The versatility of asymmetric aminoethyl-tetrazines in bioorthogonal chemistry

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proefschrift

ter verkrijging van
de graad van doctor aan de Universiteit Leiden,
op gezag van rector magnificus prof.dr.ir. H. Bijl,
volgens besluit van het college voor promoties
te verdedigen op donderdag 20 februari 2025
klokke 13:00 uur

door

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geboren te Eindhoven
in 1986

Promotores:	Prof. dr. H.S. Overkleeft	
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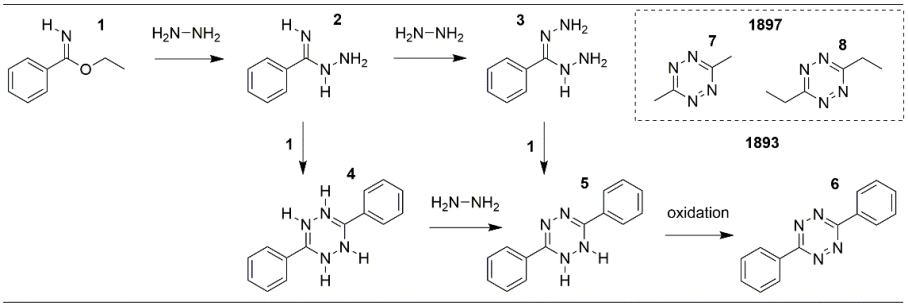
Chapter 1: A brief history of tetrazine chemistry and their application in bioorthogonal reactions

Introduction

Tetrazines have recently received considerable attention in organic chemistry and chemical biology as one of the stalwart reagents in bioorthogonal chemistry. It is one of two reaction partners present in one of the most versatile "click" reactions to date: the inverse electron demand Diels-Alder (IEDDA) reaction. Tetrazines have attracted the interest of organic chemists for over a century. Tetrazines are also the mainstay of the work described in this Thesis, including a focus on the synthesis of functionalized tetrazines for the ensuing study on their efficacy in IEDDA reactions. To set the stage, this introduction chapter provides a literature survey of tetrazine chemistry developments throughout history and their application in bioorthogonal reactions.

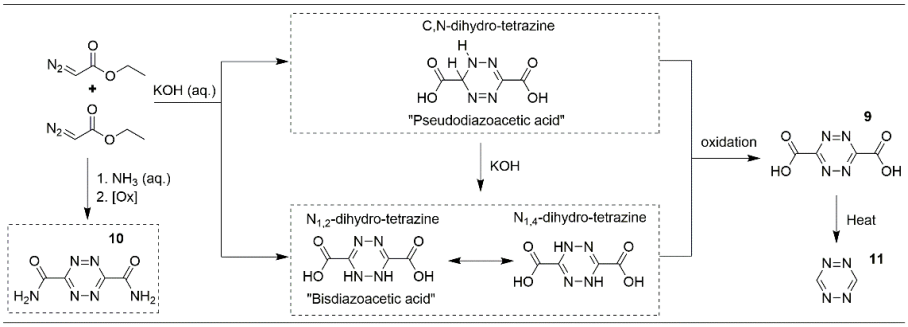
Symmetric functionalized tetrazines

One of the first documentations of tetrazine synthesis comprises the work of Pinner, as published in 1893 in the "Berichte der Deutschen Chemischen Gesellschaft" (now: European Journal of Inorganic Chemistry).[1] Here the reaction of imidoester **1** with hydrazine is described (**Scheme 1**). The formed intermediates **2** and **3** could react with an additional equivalent of imidoester **1** to form di-benzamidine **4** and a previously unknown structure **5**, which he named "dihydrotetrazine", amongst many other byproducts. This "dihydrotetrazine" would oxidize in the presence of air, or by using a variety of oxidative reagents, into structure **6** which he named "tetrazine". Pinner continued to publish several papers using the same technique to synthesize an array of 3,6-diaryl-1,2,4,5-tetrazines.[2-5] Alkyl-tetrazines **7** and **8** were also mentioned as products, but were not isolated at this time.[5b]



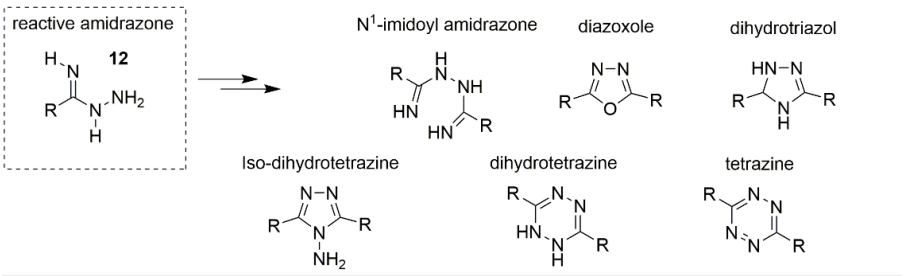
Scheme 1: Synthesis of tetrazine **6** performed by Pinner in 1893 from benzimidoester **1**, hydrazine and aqueous potassium hydroxide.

In the following decades, other scientists, at first primarily in German literature, published new methods to produce tetrazines using various starting materials, reagents, solvents and oxidation methods (**Table 1**). In the work of Junghahn[6-7] the use of thioamides is described to produce aniline-like functionalized tetrazines. Following the work of Hantsch[8] on the reactions of diazoacetate, Curtius[9-11] and Müller[12-13] described the use of diazoacetates without the use of hydrazine to form the so-called "pseudodiazoacetic acid" (1,6-dihydro) and "bisdiazoacetic acid" (1,2-dihydro) dihydro-1,2,4,5-tetrazine intermediates, which could be oxidated to produce dicarboxy-functionalized tetrazine **9** (**Scheme 2**). By addition of ammonia instead of potassium hydroxide, carboxamide-functionalized tetrazine **10** could be produced. Additionally, the discovery was made that 3,6-dicarboxy-1,2,4,5-tetrazine **9** could be heated to undergo decarboxylation resulting in the now well-known "non-functionalized" 3,6-hydro-1,2,4,5-tetrazine **11**.



Scheme 2: The use of ethyl diazoacetate in basic aqueous solutions to form dicarboxamide- and dicarboxy-functionalized tetrazines **9** and **10**, as well as non-functionalized tetrazine **11**.

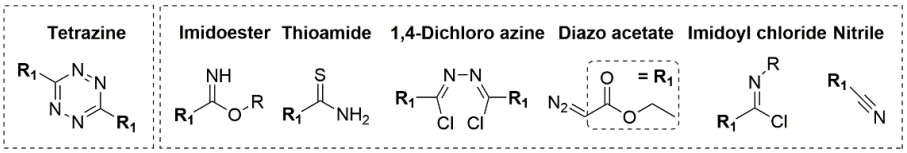
Stollé used 1,4-dichloro azines[14-15] and imidoyl chlorides[16-17] with hydrazine to synthesize a variety of tetrazines. Based on the work of Curtius[18] on reactions of nitriles with hydrazine, Hoffman[19] synthesized tetrazines **6** and **7** from benzonitrile and acetonitrile by use of hydrazine followed by oxidation. Lifschitz[20] used the technique to produce highly nitrogen rich compounds including di-tetrazyl-tetrazine. In 1921, Müller[21] elaborated on difficulties when using the starting materials mentioned before to produce tetrazines (**Scheme 3**). The formed amidrazone intermediate **12** proved to be a species that would readily react again to give, in addition to the desired product, a complex mixture of compounds. These mixtures included amidazone, dihydrotriazole, dihydrazidine, diazoxole, iso-dihydrotetrazine, various forms of dihydrotetrazines, alongside several tetrazines. Additionally, Müller describes the synthesis of various tetrazines using nitriles as starting material using anhydrous hydrazine. In 1930, Curtius[22] used anhydrous hydrazine as well to synthesize m-carboxyphenyl functionalized tetrazines.



Scheme 3: Identified byproducts and unreacted intermediates that may be present in a reaction mixture during the synthesis of dihydro-1,2,4,5-tetrazines.

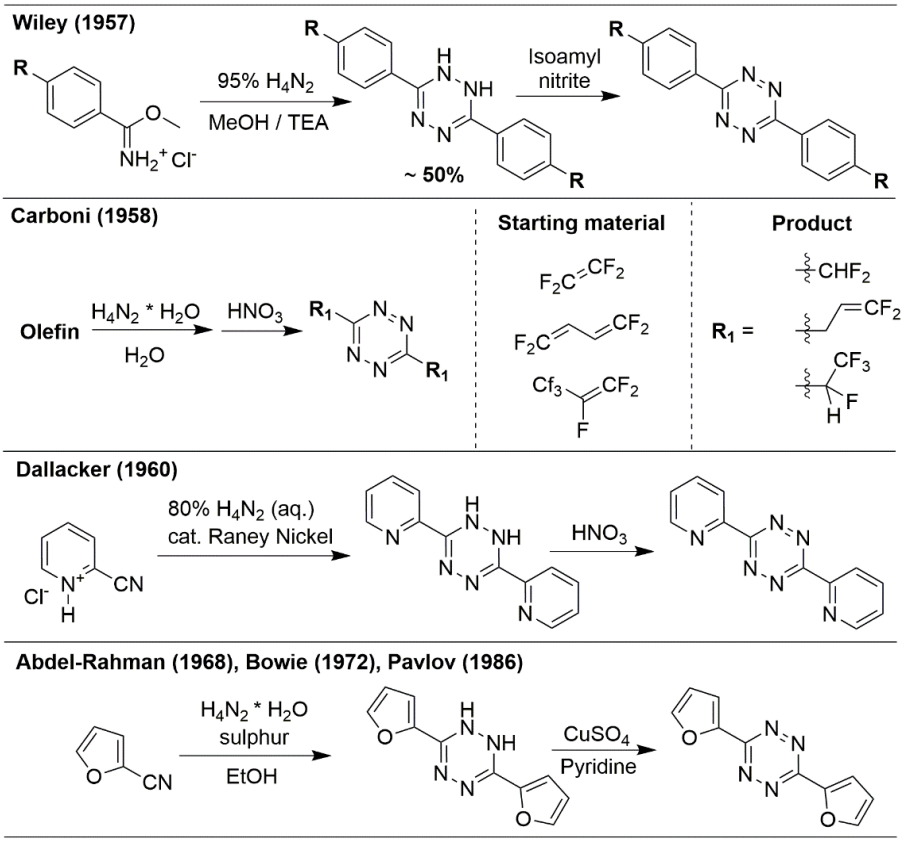
At around the 1950's the tetrazine field was further in development, but there were still discoveries on new and optimized syntheses of symmetric tetrazines (**Scheme 4**). For instance, while reproducing the synthesis of known tetrazines, Wiley[23] obtained higher yields using an anhydrous hydrazine/methanol/triethylamine solvent mixture. Carboni[24] synthesized fluoro-functionalized tetrazines from fluoro-olefins. Libman[25], and later Dallacker using ***Raney Nickel Catalyst***[26], obtained pyridyl-functionalized tetrazines from cyano-pyridine. Furthermore, the use of sulphur (in ethanol) as an additive was introduced[27] which allowed access to new tetrazines including furanyl-tetrazines.[28]

Researchers also explored the use of (dihydro)tetrazines as intermediates to functionalize into new tetrazines (**Scheme 5**). For instance, Lin[29] was able to modify carboxy-dihydrotetrazine and produce 3,6-diamino-1,2,4,5,-tetrazine via Curtius rearrangement of the azide intermediate. Marcus[30] was then able to prepare various hydrazino-functionalized tetrazines from this diamino-functionalized tetrazines.

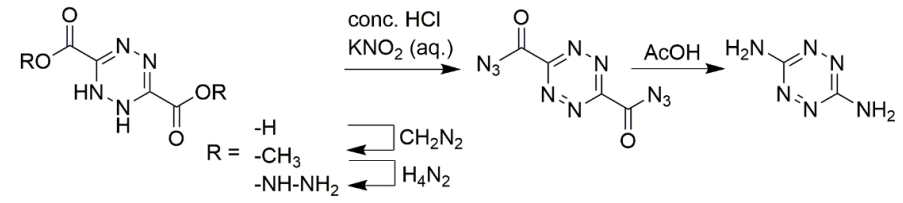


Starting Material	Product, R_1 =	Reagents	Oxidation	Author	Year
Imidoesters	phenyl	H_4N_2*H_2SO_4 KOH (aq.)	air / $FeCl_3$ / H_2O_2 / H_2CrO_4 / HNO_2	A. Pinner[1]	1893
	p-tolyl			A. Pinner[2]	1894
	Furyl			A. Pinner[3]	1895
	napthyl, benzyl			A. Pinner[4]	1897
	p-nitrophenyl			A. Pinner[5]	1897
Thioamides	phenyl, benzyl	H_4N_2 (aq.) EtOH	HNO_2 / $FeCl_3$	A. Junghahn[6]	1898
	m-anilyl, acetanilide, *p*-amino-benzyl, *p*-acetamido-benzyl			A. Junghahn[7]	1902
Diazo acetates	COOH	KOH (aq.)	HNO_2	(A. Hantzch[8]) T. Curtius[9]	(1900) 1906
	$CONH_2$	NH_3 (aq.)	$NaNO_2$		
	H	heat, H_2SO_4 (aq.)	-	T. Curtius[10]	1907
	COOH	conc. KOH (aq.)	HNO_2 / Br	T. Curtius[11]	1908
	COO-Et	(N.A.)	HNO_2	E. Müller[12]	1908
	CONH-Et	ethylamine	AcOH, KNO_2	E. Müller[13]	1909
	CONH-Me	methylamine			
	CO-Piperidyl	piperidine	HNO_2		
1,4-Dichloroazines	*p*-bromophenyl	H_4N_2*H_2O, Et_2O	air / iso-amyl nitrite	R. Stollé[14]	1906
	fluorenyl			R. Stollé[15]	1913
Imidoyl chlorides	phenyl	phenylhydrazine	air	R. Stolle[16]	1914
	phenyl	H_4N_2*H_2O, EtOH	air	M. Busch[17]	1914
Nitriles	guanidyl	H_4N_2*H_2O, EtOH	air	(T. Curtius[18]) K.A. Hofmann[19]	(1894) 1912
	phenyl				
	methyl				
	tetrazyl	H_4N_2*H_2O, EtOH	HCl (aq), $NaNO_2$	J. Lifschitz[20]	1915
	methyl	H_4N_2*H_2O, EtOH	HNO_2	E. Müller[21]	1921
	Ethyl		AcOH, $NaNO_2$		
	phenyl, *p*/*m*/*o*-tolyl	H_4N_2 (anhydrous)	AcOH, $NaNO_2$		
	m-carboxyphenyl	H_4N_2 (anhydrous)	AcOH, $NaNO_2$	T. Curtius[22]	1930
Imidoesters	phenyl, biphenyl, *m*-tolyl	H_4N_2 (95%), MeOH, TEA	iso-amyl nitrite, EtOH	R.H. Wiley[23]	1957
Fluoro olefins	fluoroalkyl	H_4N_2 (aq.)	HNO_3, AcOH	R.A. Carboni[24]	1958
Nitriles	4-pyridyl	H_4N_2*H_2O, EtOH	AcOH, $NaNO_2$	D.D. Libman[25]	1956
	4-pyridyl, 3-pyridyl, 2-pyridyl	H_4N_2*H_2O cat. Raney-Nickel	HNO_3, AcOH	F. Dallacker[26]	1960
	furanyl	H_4N_2*H_2O sulphur	$CuSO_4$, pyridine	P.A. Pavlov[28]	1986

Table 1: Early literature on the synthesis of symmetrical tetrazines.



Scheme 4: Several examples of developments into symmetrical tetrazines by use of alternative approaches.



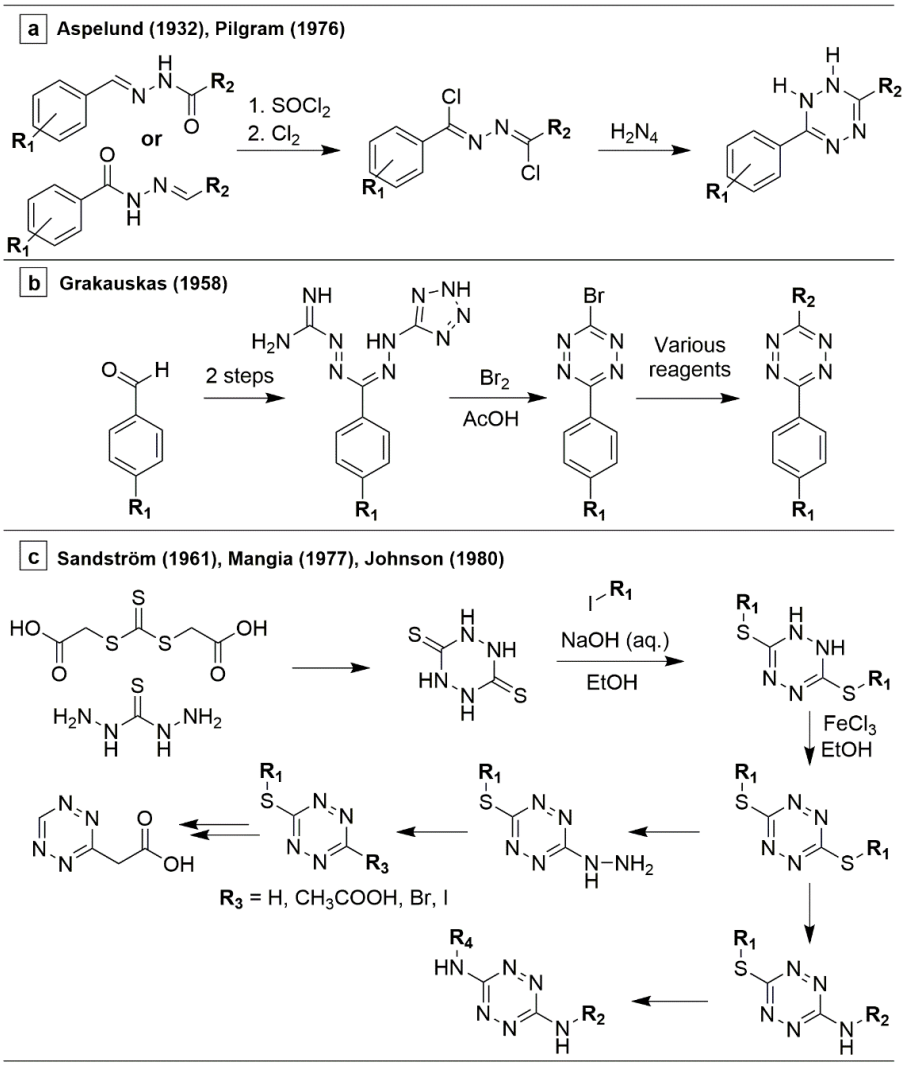
Scheme 5: An example of developments into symmetrical tetrazines by modification of existing tetrazines.

Asymmetric functionalized tetrazines

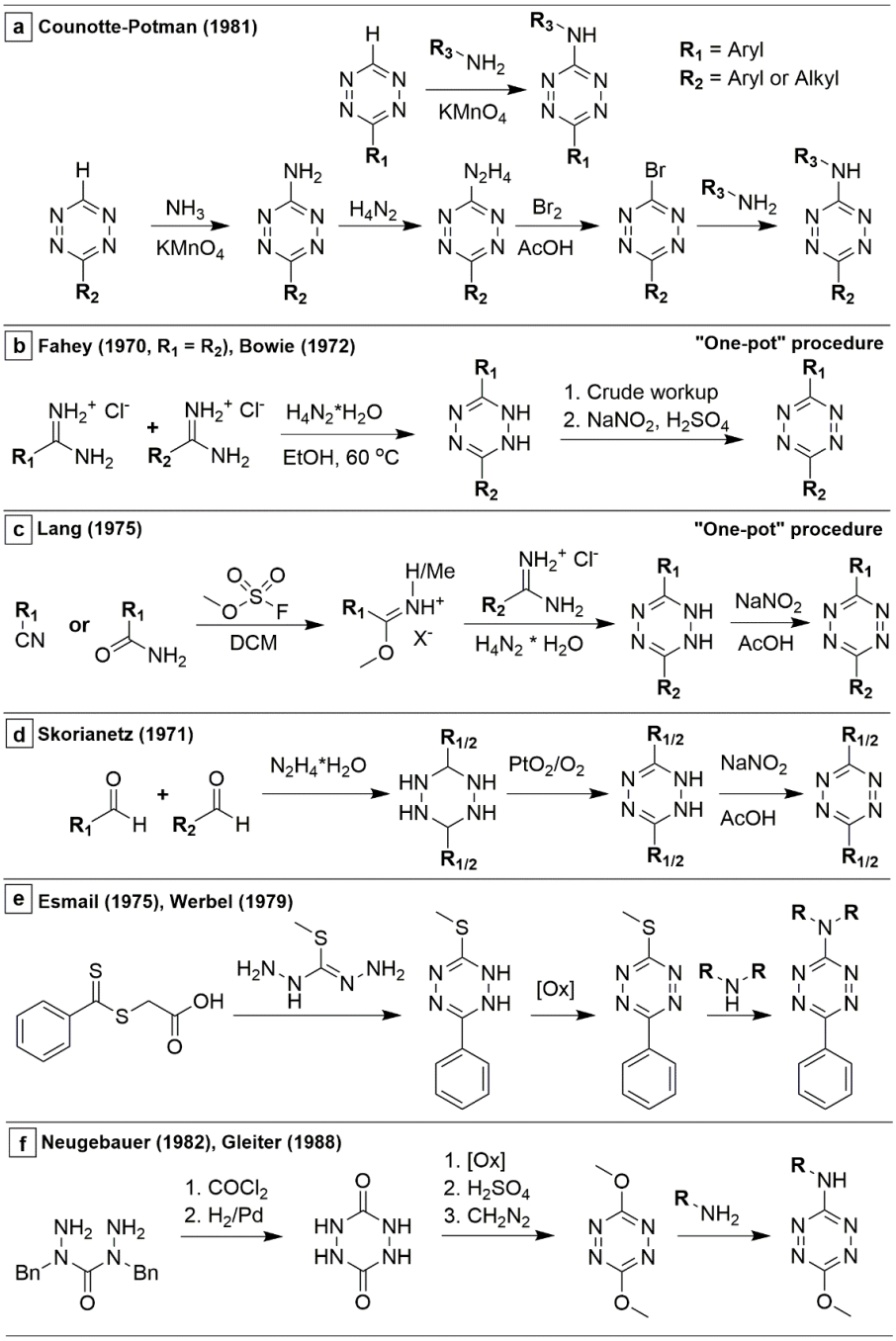
In 1930, one of the first occurrences of asymmetric synthesis of tetrazines is described by Aspelund[31-32] (**Scheme 6-a**), by using asymmetric 1,4-dichloro azines. Little other research was published until the 1950s, but from there research on finding ways to synthesize asymmetric tetrazines steadily increased as time progressed (**Table 2**). Of interest was the work of Grakauskas[33] in 1958 (**Scheme 6-b**). In his quest to synthesize aryl-tetrazoles, one set of conditions Grakauskas used yielded a red crystalline byproduct instead of the desired tetrazole. This byproduct was identified as the asymmetric 3-bromo-6-phenyl-tetrazine and was used to synthesize a unique library of 26 asymmetrically functionalized tetrazines. The method was later used as well by Ershov[34] and Werbel[35] leading to a library of asymmetric tetrazines. In 1961, Sandström[36] was able to synthesize alkylthio-tetrazines from dithio-*p*-urazine (**Scheme 6-c**). This allowed Mangia[37] to prepare several 6-hydro-, 6-carboxymethyl- and 6-halide-functionalized asymmetric 3-methylthio-1,2,4,5-tetrazines, which after further reaction, gave 3-carboxymethyl-1,2,4,5-tetrazine. The alkylthio-tetrazines also allowed Johnson[38] to prepare a multitude of asymmetric thio/amino-functionalized tetrazines. Aspelund's 1,4-dichloroazine approach was used again in multiple occasions[39], and is still used today in a slightly improved method[40].

Counotte-Potman, while exploring the mechanism of the substitution of amines and halides by hydrazine in tetrazines[41a], found an alternative way towards asymmetric hydrazino-tetrazines from asymmetric hydro-functionalized tetrazines[41b] (**Scheme 7-a**). Furthermore, a new method[41c] of alkylamination was developed to prepare not only 6-alkylamino-3-aryl-tetrazines, but also 6-alkylamino-3-alkyl-tetrazines. In 1970, Fahey[42] experimented on the use of amidinium chloride reagents and hydrazine hydrate in ethanol, which included heating to 60°C and noticed an improvement over the use of imidoesters (**Scheme 7-b**). This "one-pot" procedure only included a single work-up step where the crude dihydro-tetrazine intermediate was precipitated before oxidation. In 1972, Bowie[43] modified the procedure of Fahey to include a mixture of amidines in order to prepare asymmetrical tetrazines in yields up to 33%. Bowie also performed the synthesis of the same tetrazines by using nitrile reagents and Sulphur as an additive, however the yields were considerably lower. Lang[44] created a variation of Bowie's mixed amidine "one-pot" procedure. By using nitrile or carboxamide reagents, which were pre-activated using methylfluorosulfonate (including methanol when using nitriles), Lang prepared methyl imidoester intermediates under highly exothermic conditions (**Scheme 7-c**). To this intermediate hydrazine hydrate and an amidinium chloride reagent were added to form asymmetric dihydrotetrazines before oxidation to the tetrazine product. Around the same time Skorianetz[45] developed a different approach where

mixed aldehydes reacted in hydrazine hydrate to form a mixture of hexahydro-tetrazines, which could immediately be oxidized to dihydrotetrazines using PtO_2/O_2 (**Scheme 7-d**). Esmail[46] reacted thiobenzoylthio-acetic acid with S-methyl isothiocarbonohydrazide to synthesize dihydrotetrazines, which were oxidized using peroxide into 3-methylthio-6-phenyl tetrazine (**Scheme 7-e**). This enabled Werbel[35] to synthesize various 3-aryl-6-amino-tetrazines. Neugebauer[47a] used, instead of dithio-p-urazine, p-urazine to synthesize dimethoxy tetrazines, which could be efficiently substituted at a single position (**Scheme 7-f**).[47b]



Scheme 6: Synthesis approaches towards asymmetric tetrazines.



Scheme 7: Synthesis approaches towards asymmetric tetrazines.

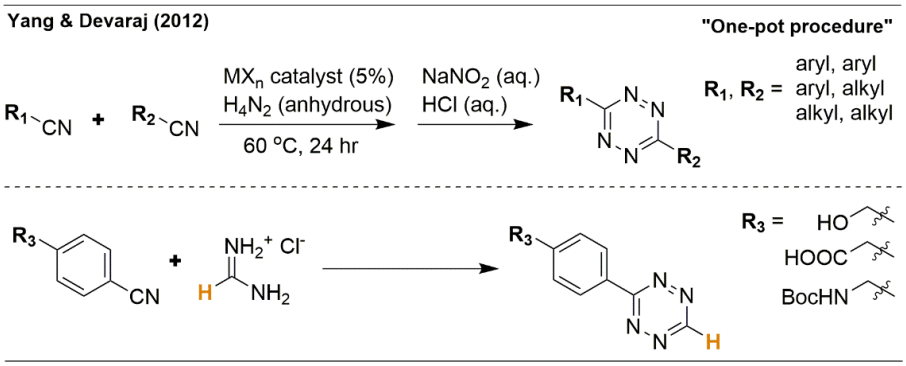
Starting Material	R_1 =	R_2 =	Reagents	Oxidation	Author	Year
1,4-Dichloro azines	phenyl	benzhydryl	$H_4N_2*H_2O$	Iso-amyl nitrite	H. Aspelund[31]	1930
	benzhydryl	benzhydryl				
	methyl	benzhydryl			H. Aspelund[32]	1932
	aryl	perfluoroalkyl	H_4N_2 (95%), EtOH	$FeCl_3$	K. Pilgram[39a]	1976
	m-nitrophenyl	*p*-CF_3-phenyl	$H_4N_2*H_2O$, MeCN	AcOH, $NaNO_2$	D.S. Liu[39b]	2012
	t-butyl / aryl	aryl	$H_4N_2*H_2O$, EtOH	AcOH, $NaNO_2$	D. Wang[40a]	2013
	2-pyridyl	COO-Et	$H_4N_2*H_2O$, EtOH	AcOH, $NaNO_2$	A. Jemas[40b]	2022
Formazans	aryl	Br	Bromine, AcOH	-	V.A. Graskausas[33]	1958
					V.A. Ershov[34]	1971
					L.M. Werbel[35]	1979
Aldehydes	methyl	ethyl, *i*-propyl, *t*-butyl	$H_4N_2*H_2O$	1. PtO_2/O_2 2. AcOH, $NaNO_2$	W. Skorianetz[45]	1971
$(CH_3S)_2$-Tetrazine	methylthio	H, NH_3, $NHNH_2$, CH_2COOH, Cl, Br	-	-	(J. Sandström)[36] A. Mangia[37]	(1961) 1977
	R_1-N	R_2-N	-	-	J.L. Johnson[38]	1980
Hydro-tetrazines	alkylamino	aryl, alkyl	-	-	A.D. Counotte-Potman[41]	1981
Amidinium Salts	aryl	aryl (R_1 = R_2)	$H_4N_2*H_2O$, EtOH	H_2SO_4, $NaNO_2$	J.L. Fahey[42]	1970
	aryl	aryl, methyl	$H_4N_2*H_2O$, MeOH	HNO_2	R.A. Bowie[43]	1972
	aryl	aryl, alkyl			S.A. Lang[44]	1975
Exotic	methylthio	phenyl	-	-	R. Esmail[46]	1975
	R_1R_2-N	aryl	-	-	L.M. Werbel[35]	1979
	R_1-N	O-Me	-	-	R. Gleiter[47b]	1988

Table 2: Early literature on the synthesis of asymmetrical tetrazines.

The use of catalysts in one pot tetrazine synthesis

The amount of research performed on the synthesis of tetrazines steadily increased into the early 21st century, however was mostly limited to the techniques described above, sometimes with small variations. This changed in 2021 when Yang[48] found that the use of soluble metal salts at increased temperature could catalyze the reaction of tetrazines from nitriles. The metal ions could coordinate to the nitrile starting material and, by acting as a Lewis acid, could activate the initial hydrazone formation, in this way kickstarting the reaction process towards dihydrotetrazines. Additional to the use of soluble metal salts, the reaction was performed in a sealed pressure tube, which prevented the formed ammonia to boil off at the 60 °C reaction temperature. It appeared that zinc^{2+} and nickel^{2+} performed very well in combination with weaker coordinating counterions, and near quantitative yields were obtained.

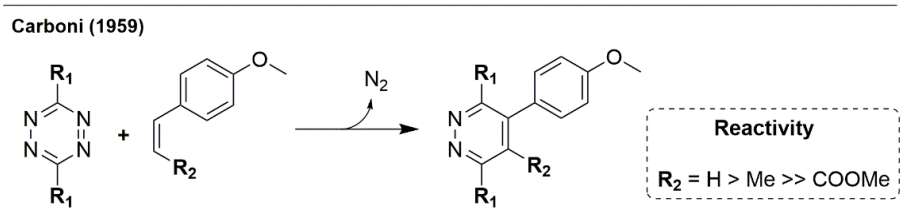
Additionally, the reaction appeared to perform better on aryl nitriles compared to alkyl nitriles. When performing the reaction on mixed nitriles ($\mathbf{R_1 \neq R_2}$) a whole variety of asymmetric tetrazines could be obtained, which were otherwise very difficult or impossible to obtain.



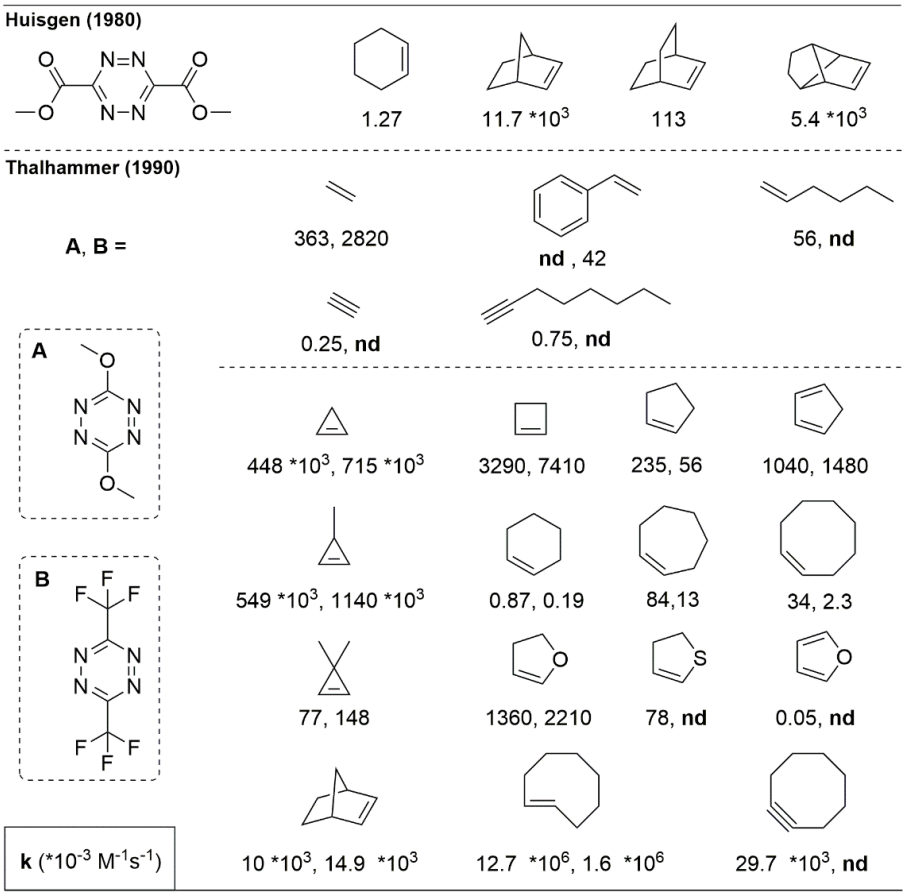
Scheme 8: Synthesis of tetrazines using soluble metal catalyzed one-pot procedure developed by Yang in 2012.

IEDDA chemistry and bioorthogonal reactions

Alongside the developments described in literature on tetrazine chemistry itself, publications on its use also continued. A very important discovery, and related to the work described in this thesis, was made in 1959 by Carboni[49] (**Scheme 9**). His fluoro-functionalized (electron poor) tetrazines were able to undergo a 1,4-addition reaction with alkenes similar to the Diels-Alder reaction. Unlike the classical Diels-Alder reaction, this required the dienophiles to have inverse electron properties. The dienophile appeared to be more reactive when functionalized with electron donating groups and less reactive when functionalized with electron withdrawing groups. With this inverse electron demand Diels Alder (IEDDA) reaction Carboni was able to prepare a multitude of pyridazines. The discovery led to other researchers exploring the properties of this IEDDA reaction as well, including the reaction's kinetic properties as shown in the work of Huisgen[50] in 1980 and Thalhammer[51] in 1990 (**Scheme 10**).

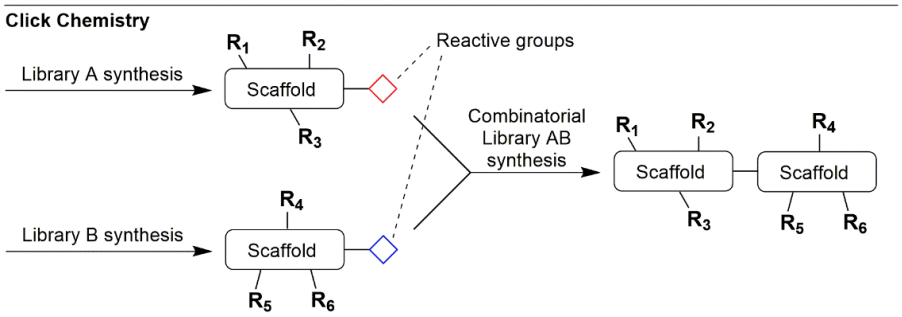


Scheme 9: IEDDA reaction as shown in Carboni's publication in 1959.



Scheme 10: A selection of results of tetrazines reacting with various alkenes in dioxane at 25°C (Huisgen) or 20 °C (Thalhammer).

At the turn of the millennium, a new concept termed "click chemistry" emerged where the goal was to design molecular building blocks functionalized to be reacted together in an easy-to-use and robust manner in an effort to prepare complex larger molecules in a modular approach that would allow the synthesis of large combinatorial libraries (**Scheme 11**).[52-53] This sparked researchers to use and improve on known chemical reactions for this purpose. One of those, the 1,3-dipolar cycloaddition between alkynes and azides[54] (**Scheme 12-a**), was improved on drastically by the discovery[55-56] that Cu^{1+} could catalyze the reaction by 10^7-fold[57] (**Scheme 12-b**) and make it regioselective. This newfound copper catalyzed azide-alkyne cycloaddition (CuAAC) reaction revolutionized the application of click chemistry as a broadly applicable and robust tool for when any molecule or structure needed to be attached to another.



Scheme 11: A representation of click chemistry: through synthesis of two libraries, each containing a click reactive group, a combinatorial library could be synthesized by combination of both libraries. Changing either one of the libraries for another library, could result in a different combinatorial library.

Around the same time, Carolyn Bertozzi was working on ligation methods in biological systems[58], and while doing so named them "bioorthogonal" reactions (**Figure 1**). The term "bioorthogonal" comprises a combination of "bio", a reaction performed in a biological environment, and "orthogonal", a term used in chemistry to define reactions and processes that occur in each other's presence without any cross interaction. The CuAAC reaction was optimized for bioorthogonal reactions[59] (**Scheme 12-c**), however the requirement of Cu^{1+} was regarded to be toxic for living organisms[60], rendering its use limited. In order to overcome this limitation, in 2004, Bertozzi used strained alkynes in the form of cyclooctynes to react with azides in a 1,3-dipolar cycloaddition reaction, without the use of copper ions, while retaining part of the reactivity increase compared to non-strained alkynes (**Scheme 12-d**).[61] The strain-promoted azide-alkyne cycloaddition (SPAAC) reaction was used on cell surface labeling of metabolically incorporated N-azido-acetylmannosamines and, while the use of copper was prevented, the efficiency was reduced compared to the CuAAC reaction. When the SPAAC reaction was compared to the "Staudinger ligation"[58, 62], a reaction based on the Staudinger reaction[63] (**Scheme 13-a**) and developed by Bertozzi as well a few years before (**Scheme 13-b**), the SPAAC reaction appeared to perform at around half the efficiency compared to the Staudinger ligation.

In 2008, in an effort to expand the tools usable for bioorthogonal click chemistry, Hildebrand[64] and Fox[65] independently applied IEDDA chemistry for this purpose (**Scheme 14-a**). The reaction, which appeared to perform in a bioorthogonal manner, in both cases used with *trans*-cyclooctene as one of its reaction partners and proved to perform in live cells at reaction rates much faster than the SPAAC, Staudinger ligation and CuAAC reactions. Together with the increased accessibility to novel tetrazines, as a result of the work of Yang[48] in 2012, researchers were motivated to

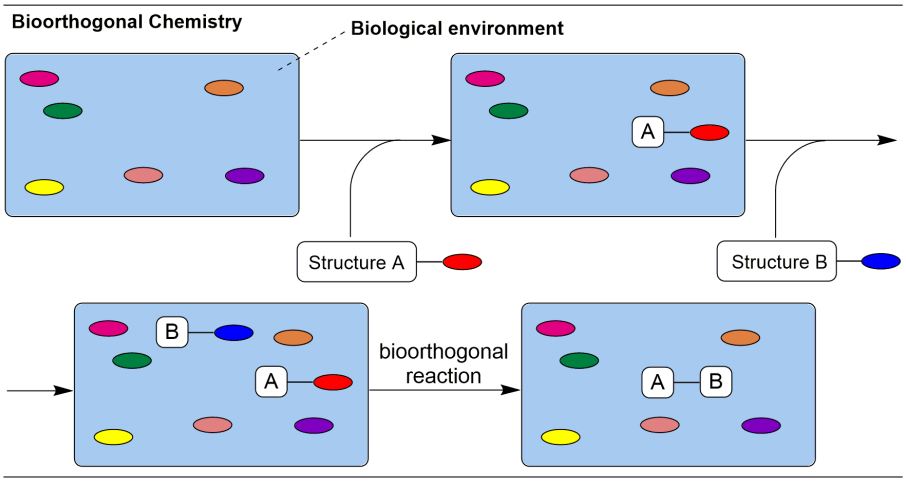
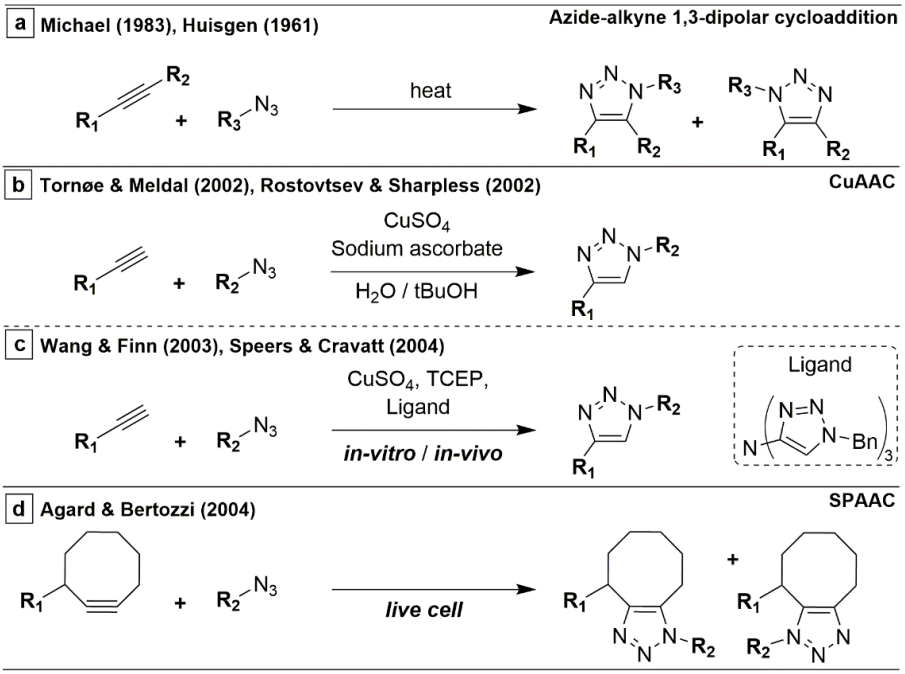
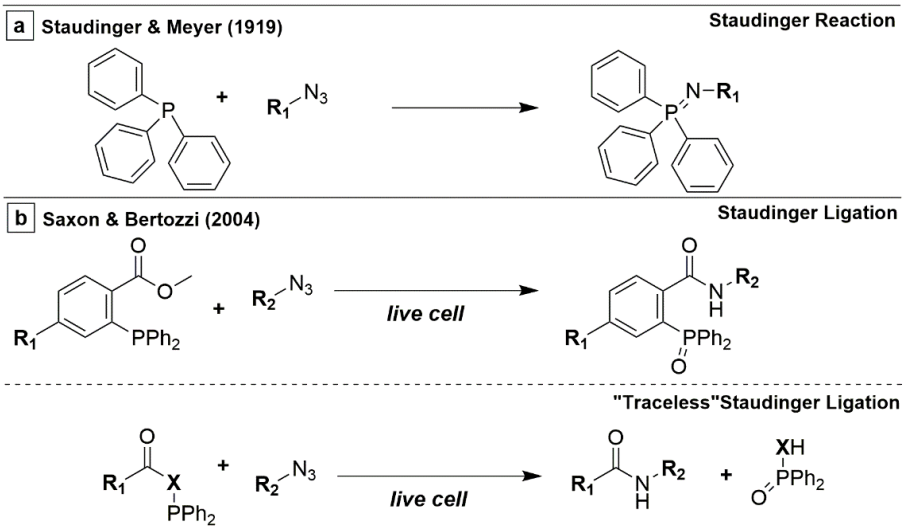


Figure 1: A representation of bioorthogonal chemistry: (1) the biological environment to which (2) structure A with a "red" bioorthogonal group is added, and then (3) structure B with a "blue" bioorthogonal group is added, after which (4) structures with "red" and "blue" bioorthogonal groups react with eachother without affecting the biological environment.

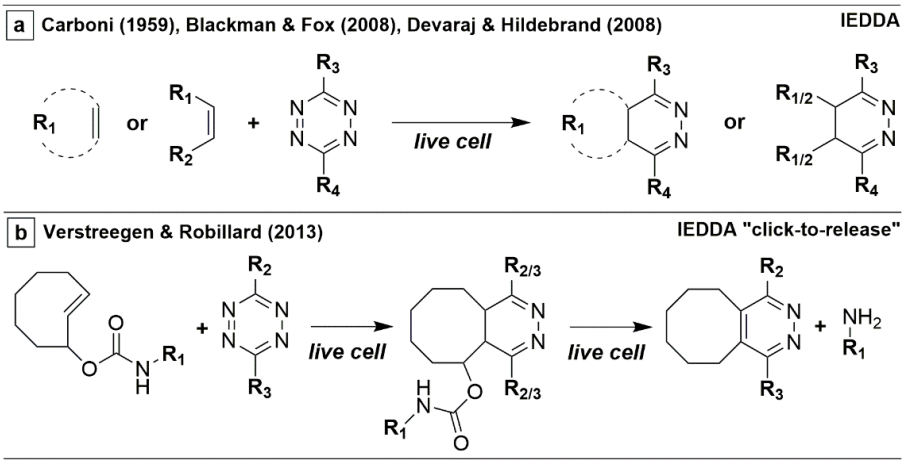


Scheme 12: Overview of the azide-alkyne 1,3-dipolar cycloaddition, CuAAC and SPAAC reactions.

synthesize new asymmetric tetrazines[66], or modify existing ones[67] for bioorthogonal conjugations. In 2013, this incentive also led to the discovery of the IEDDA-mediated "click-to-release" reaction by Robillard[68] (**Scheme 14-b**). Robillard was able to prepare *trans*-cyclooctenes with a moiety covalently linked at the allylic position. The *trans*-cyclooctene would, after reaction with the tetrazine, undergo an elimination reaction resulting in the release of the attached moiety. For this reaction type too, researchers were motivated to synthesize new tetrazines in order to improve the characteristics for the "click-to-release" reaction. [69-70]



Scheme 13: Overview of the Staudinger reaction, Staudinger ligation, and "traceless" Staudinger ligation.



Scheme 14: Overview of the IEDDA and IEDDA "click-to-release" reactions.

Aim and outline of this thesis

The core aim of this thesis is to prepare a group of asymmetric amino-alkyl functionalized tetrazines. These tetrazines are then chemically characterized and used for various bioorthogonal “click” and “click-to-release” type reaction in order to determine how they function compared to other tetrazines from the literature.

Chapter 2 describes the design and synthesis of 2-amino-1-carboxy-ethyl tetrazines in order to mimic naturally occurring amino-acids with minimal steric hindrance. The tetrazine-amino acid is incorporated in known FA-CMK (and FA-BOMK) protease inhibitors through substitution of the phenylalanine (F) amino acid and analyzed on SDS-PAGE after incubation with lysates and labeling with fluorophores by means of IEDDA reaction.

Chapter 3 describes the design and synthesis of symmetric and asymmetric 2-aminoethyl and 2-aminomethyl tetrazines. These tetrazines are then functionalized with fluorophores, chemically characterized, used in (cell surface) labeling (lipids, metabolically incorporated mannosamines, and sterculic acid) on living cells.

Chapter 4 describes the use of tetrazines-fluorophores from chapter 3 in order to perform the simultaneous labeling (dual-labeling) of metabolically incorporated mannosamines and sterculic acid. A mixture of tetrazine-fluorophores is used to perform two IEDDA reactions in a single biological system by exploiting the differences in their physical properties.

Chapter 5 describes the use of a TCO-AMC assay to characterize the tetrazines from chapter 3 on their performance during “click-to-release” reactions.

Chapter 6 describes the development and use of an assay that allows the characterization of tetrazines on how they perform in the “click-to-release” of alkyl amines. The assay uses a bifunctional-TCO functionalized with the EDANS/DABCYL fluorophore/quencher pair.

Chapter 7 summarizes the thesis and provides the design and execution of several projects as future prospects.

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Chapter 2: Incorporation of tetrazine alanine as amino acid analogue in peptidase inhibitors for activity-based protein profiling

Introduction

Activity-based protein profiling (ABPP) is a well-established methodology to identify enzymes and visualize their activities within complex biological systems.[1] ABPP makes use of irreversible enzyme inhibitors termed activity-based probes (ABPs). These ABPs in general are substrate analogues featuring an electrophilic warhead strategically mimicking and replacing an essential part of the natural substrate and are furthermore equipped with a reporter tag extending from a non-essential part of the inhibitor. ABPP works especially well for enzymes that process their substrate through a covalent enzyme-substrate intermediate, such as the serine hydrolyses and cysteine proteases for which the technology was originally developed.[2] Key to the success of ABPP is the choice of the reporter tag, which should not interfere with enzyme recognition and its subsequent irreversible interaction with the enzyme's active site. Affinity tags (Biotin and a variety of fluorophores) can be, and have been, utilized as reporter tags for ABPP on enzymes that are generous to the addition of molecular bulk to the ABP[3], whereas smaller bioorthogonal tags (azide, alkyne, strained alkene) can be employed for a so called "two-step ABPP", where reporter tags are introduced as a second step after initial binding of the ABP to the enzymes, due to the more restrictive nature of the substrate binding site.[3, 4] The bioorthogonal tags are selected on their reactivity (fast, efficient, reactive only towards their orthogonal counterpart bearing a fluorophore or affinity tag), but also on their size, which should be as small and accessible as possible. In this respect - and this is the objective of this chapter - small tetrazine bearing amino acids are designed to mimic the structural and spatial properties of phenylalanine/tyrosine amino acids and are incorporated in model methylketone peptidase inhibitors[5] to explore their capability to act as an alternative bioorthogonal tag with excellent reactivity properties.

Synthesis of peptidase inhibitors containing tetrazine alanine

In order to prepare potential peptidase inhibitors containing (methyl-)tetrazine alanine (Tza or Tzm) as an analogue for phenylalanine (Phe) or tyrosine (Tyr) at the peptidases N-terminus, a synthetic approach was designed where both the N_α-protected tetrazine alanine building block (PG-Tzx-OH) and warhead building block (H-AA-warhead) could be synthesized separately, and condensed to each other to form the target inhibitor (**Figure 1**).

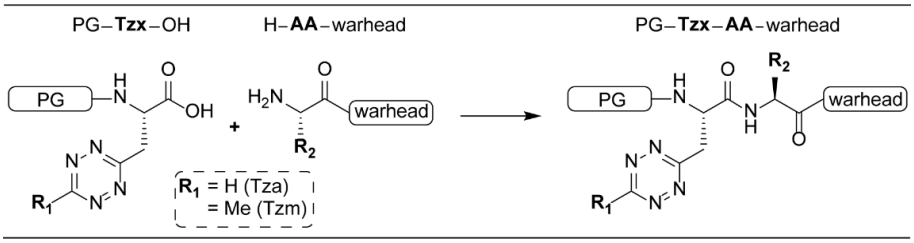
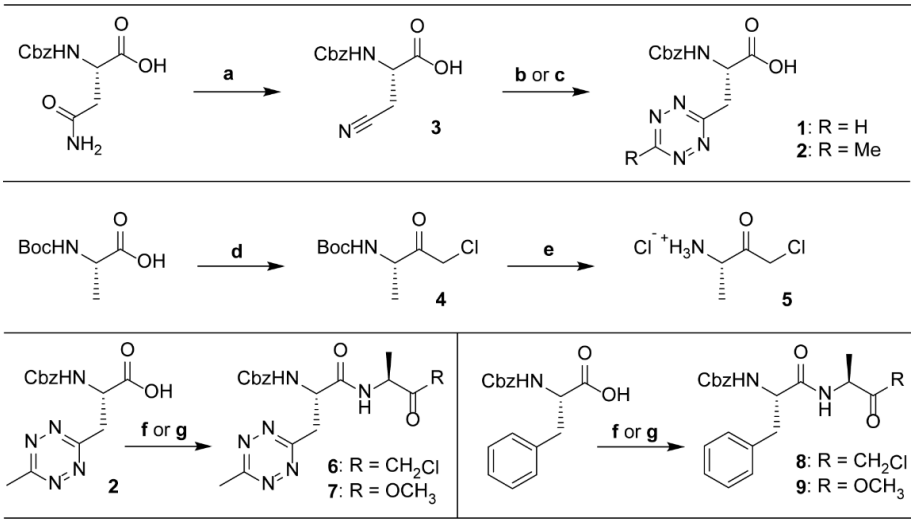


Figure 1: Synthesis approach for peptidase inhibitors containing a N-terminal protection and C-terminal warhead.

First, N_α-Cbz tetrazine alanine **1** and **2** were prepared in two steps (**Figure 2**). N_α-Cbz asparagine (Cbz-Asp-OH) was dehydrated using DCC to form N_α-Cbz cyanoalanine **3**. Compound **3** was then treated with either $Zn(OTf)_2$/formamidine or $Ni(OTf)_2$/acetonitrile in anhydrous hydrazine, after which the crude dihydrotetrazine intermediate was oxidized to yield tetrazine alanine **1** (Cbz-**Tza**-OH) or **2** (Cbz-**Tzm**-OH) respectively.[6] In parallel, N_α-Boc alanine (Boc-Ala-OH) was converted to Boc-Ala-CMK **4** using a diazomethane generation kit.[7] The N_α-Boc protective group was then removed using a HCl/Dioxane solution to obtain H-Ala-CMK **5** as a hydrochloride salt. The initial design was to incorporate Cbz-Tza-OH **1** instead of Cbz-Tzm-OH **2** in the peptidase inhibitor, however tetrazine alanine **1** was prone to degradation after synthesis. The synthesis was therefore continued using Cbz-Tzm-OH **2**. Cbz-Tzm-OH **2** and H-Ala-CMK **5** were condensed using isobutylchloroformate, obtaining the desired inhibitor Cbz-Tzm-Ala-CMK **6**. As control inhibitors Cbz-Tzm-Ala-OMe **7**, Cbz-Phe-Ala-CMK **8** and Cbz-Phe-Ala-OMe **9** were also synthesized using similar approaches. The N_α-Boc variants of each inhibitors were also prepared (**Figure 3**). The purpose of these inhibitors was to remove their N_α-Boc protective group to gain access to H-Tzm-Ala-CMK type inhibitors with a free N-terminus. N_α-Boc cyanoalanine **10** and N_α-Boc (methyl-)tetrazine alanine **11** and **12** were prepared using a synthetic strategy similar to that for compounds **6** – **9** yielding **11** (Boc-**Tza**-OH) and **12** (Boc-**Tzm**-OH). A test deprotection of the N_α-Boc protective group was successfully performed on both **11** and **12** yielded H-Tza-OH **13** and H-Tzm-OH **14** respectively in quantitative yield. This suggested the suitability of the approach for the synthesis of free N-terminus ABPs. Following the successful synthesis of **13** and

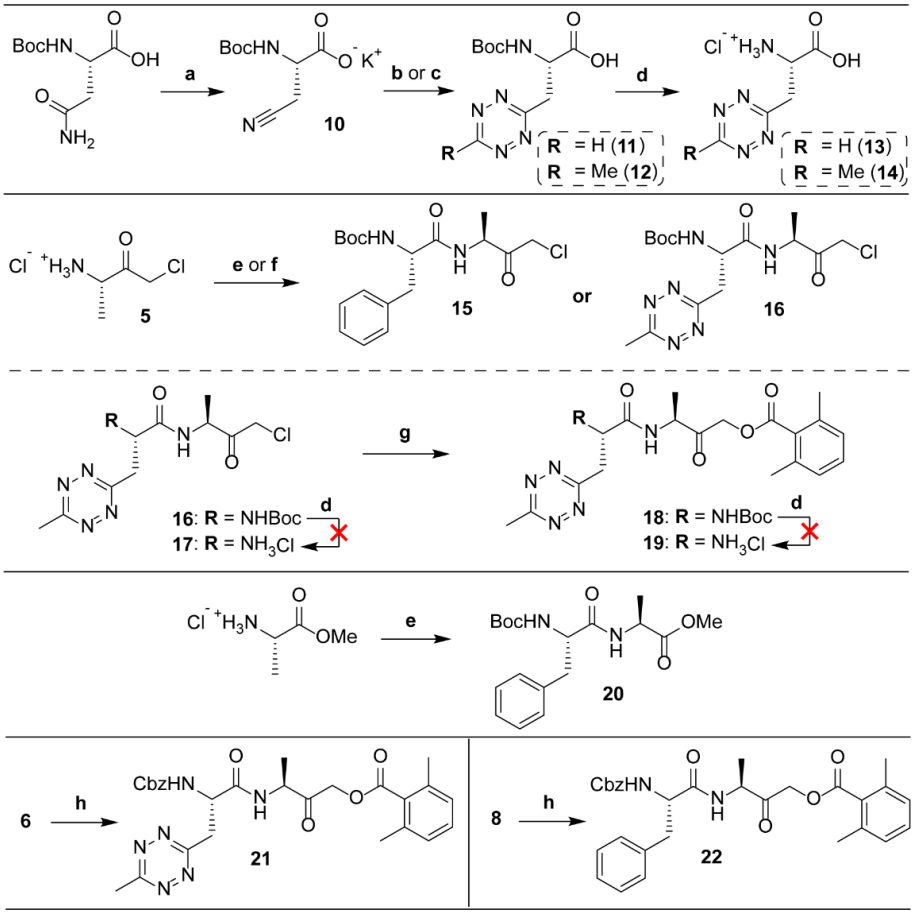
14, peptidase inhibitors Boc-Phe-Ala-CMK **15**, Boc-Tzm-Ala-CMK **16** and control inhibitor Boc-Phe-Ala-OMe **20** were also synthesized.



a) Cbz-Asp-OH, DCC, acetone/pyridine, r.t., 2 hrs, 93%. **b**) formamidine acetate, $Zn(OTf)_2$, dioxane/hydrazine, r.t., 3 days, 14%. **c**) acetonitrile, $Ni(OTf)_2$, dioxane/hydrazine, r.t., 3 days, 11%. **d**) First: Boc-Ala-OH, NMM, IBCF, THF, -25 °C to r.t., 60 min. Second: Diazomethane, Et_2O, 69%. **e**) 4M HCl/dioxane, dioxane, r.t., 2 hrs, quant. **f**) First: IBCF, TEA, THF, r.t., 45 min. Second: 5, TEA, DMF, r.t., 60 min, 21% (**6**), 47% (**8**). **g**) First: IBCF, TEA, THF, r.t., 45 min. Second: H-Ala-OMe*HCl, TEA, DMF, r.t., 50 min, 28% (**7**), 65% (**9**).

Figure 2: Synthesis of N_α-Cbz protected CMK peptidase inhibitors **6** and **8** and methyl ester control inhibitors **7** and **9**.

Next, to obtain H-Tzm-Ala-CMK **17**, compound **16** was treated with a 2M HCl/dioxane/DCM solution in an attempt to remove the N_α-Boc at the N-terminus. However, this resulted in total degradation of inhibitor **17**. The specific composition of the dipeptide was postulated to lead to undefined degradation inside the acidic environment. On the other hand, the disappearance of the distinct red color of the tetrazine, yielding a pale solid, was an indication that the tetrazine moiety was degraded under these conditions. In an attempt to solve this problem, the CMK warhead was substituted for the less reactive 2,6-dimethyl-benzyloxymethylketone (BOMK) warhead.[8a-d] Via a fairly straight forward substitution of the chloride for 2,6-dimethylbenzoate in the presence of excess potassium fluoride, Boc-Tzm-Ala-CMK **16** could be converted to the BOMK warhead functionalized inhibitor Boc-Tzm-Ala-BOMK **18**.



a) DCC, acetone/pyridine, r.t., 2 hrs, 96%. **b**) formamidine acetate, $Zn(OTf)_2$, dioxane/hydrazine, r.t., 3 days, 4%. **c**) acetonitrile, $Ni(OTf)_2$, dioxane/hydrazine, r.t., 3 days, 11%. **d**) 4M HCl/dioxane, DCM, r.t., 2 hrs, quant. **e**) First: Boc-Phe-OH, IBCF, TEA, THF, r.t., 60 min. Second: **5**, TEA, DMF, r.t., 60 min, 62%. **f**) First: **12**, IBCF, TEA, THF, r.t., 60 min. Second: **5**, TEA, DMF, r.t., 60 min, 80%. **g**) 2,6-dimethylbenzoic acid, KF, DMF, r.t., 2 hr, 86%. **h**) 2,6-dimethylbenzoic acid, KF, DMF, r.t., 1 day.

Figure 3: Synthesis N-terminal Boc/Cbz protected peptidase inhibitors containing CMK/BOMK warhead.

Unfortunately all efforts to deprotect the N_α-Boc protective group on inhibitor **18** resulted in degradation of the material as well. Because Boc-Tzm-Ala-BOMK **18** itself could potentially be an interesting inhibitor, the set was expanded to include N_α-Cbz BOMK inhibitors **21** and **22** explore the usefulness of the BOMK warhead. Inhibitors **21** and **22** were then synthesized successfully from chloromethylketone inhibitors **6** and **8** respectively.

ABPP of tetrazine-alanine modified peptidase inhibitors

To test the suitability of Cbz-Phe-Ala-CMK **8** and Cbz-Tzm-Ala-CMK **6** as ABPs for ABPP (**Figure 4**), their capacity to inhibit peptidases was first assessed in a competition study (**Figure 5**). For this study, lysates of mouse RAW 264.7 macrophages[9], and human-derived Jurkat T-cell line[10] were used and fluorescent activity based probe Cy5-DCG-04[11] was used as competitor ABP. This competitor ABP is a derivative of the naturally occurring epoxysuccinate E-64 papain-like cysteine protease inhibitor[12], and serves as a fluorescent marker for non-inhibited proteases. The competition study was performed by incubating the lysates of RAW 264.7 macrophages (**Figure 6 – assay 1**) or Jurkat T-cells (**Figure 6 – assay 2**) in a sodium acetate buffered solution (pH 5.5) with variable concentrations of inhibitor **8** ([C] = 0-500 nM, 30 min, 37 °C), followed by a second incubation with marker Cy5-DCG-04 ([C] = 1 µM, 30 min, 37 °C). The lysates were denatured by addition of mercaptoethanol and heat and Cy5-DCG-04 fluorescence was analyzed using SDS-PAGE. In control lane 1 (no inhibitor **8**), the labeling pattern of Cy5-DCG-04 shows a distinct pattern of strong and weakly visible bands, likely belonging to peptidases within the cathepsin and calpain family.[13] Increasing concentrations of Cbz-Phe-Ala-CMK **8** were able to inhibit a portion of the peptidases visualized by Cy5-DCG-04. To analyze characteristics of Cbz-Tzm-Ala-CMK **6** compared to Cbz-Phe-Ala-CMK **8** the competition study was repeated using Jurkat T-cells (**Figure 6 – assay 3 + 4**) including one of the two inhibitors ([C] = 0-10 µM, 30 min, 37 °C). The results showed near identical fluorescence patterns generated by the Cy5-DCG-04 marker.

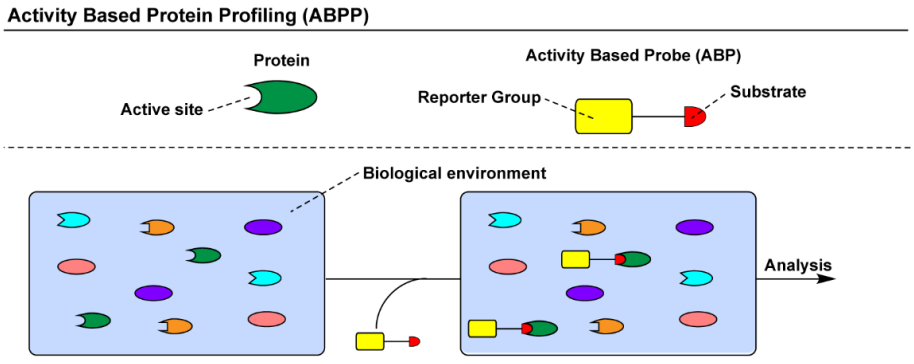


Figure 4: General representation of ABPP: The activity based probe (ABP) has a substrate that covalently binds with the active site of proteins bearing a certain type of active site. This ABP-bound protein can then be analyzed using its reporter group.

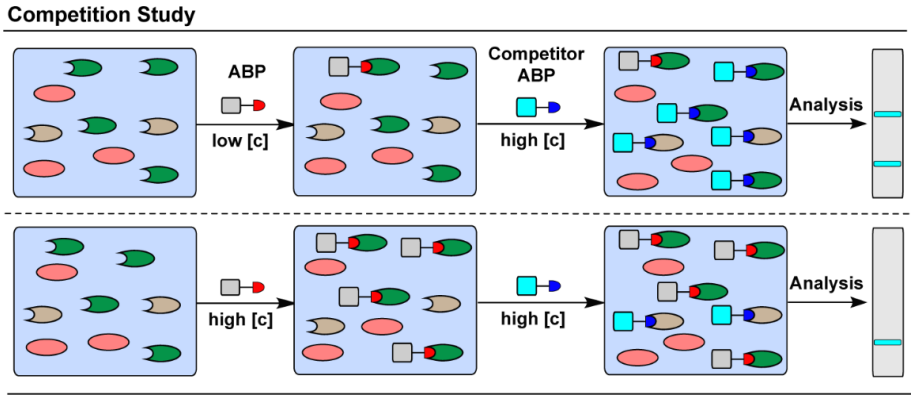


Figure 5: Competition study: The ABP's substrate binds covalently to the enzyme's active site. This is based on concentration, duration, temperature and reactivity. By varying one of the parameters, such as concentration, the amount of remaining enzyme available for a competitor probe changes and can be analyzed using the reporter groups of both probes. Probes can be introduced consecutively, or together.

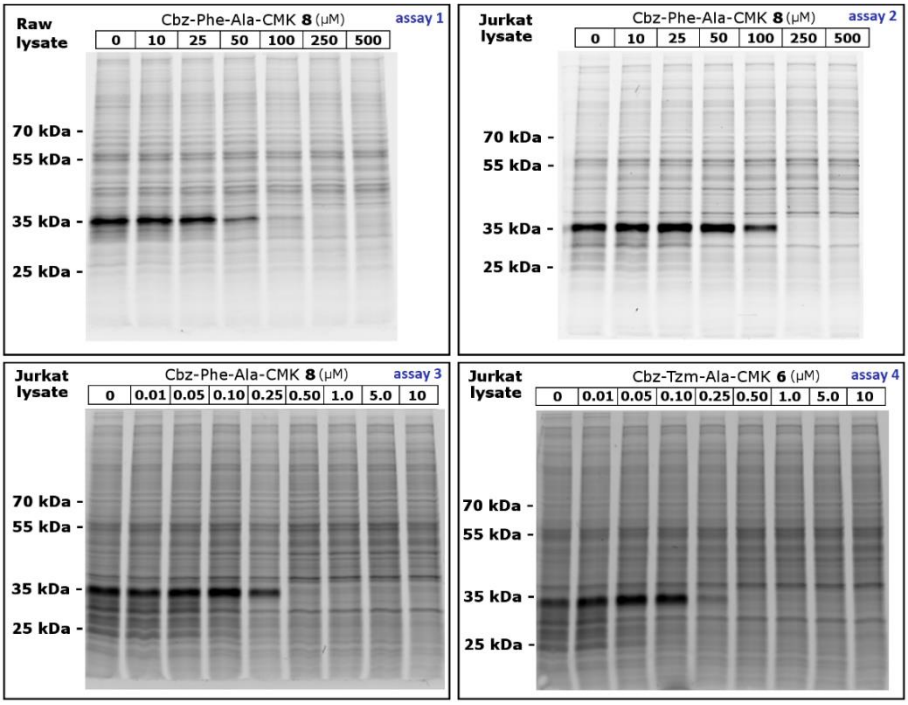


Figure 6: Lysates from RAW 264.7 macrophages (**assay 1**) or Jurkat T-cells (**assay 1-3**) were incubated with compound **8** or **6** at variable concentrations, followed by Cy5-DCG-04. Peptidase-bound Cy5-DCG-04 was visualized using SDS-PAGE fluorescence analysis (700/50 nm).

After the successful results of the competition study, a two-step ABPP experiment (**Figure 7**) was performed using Cbz-Tzm-Ala-CMK **6**, and the TCO-functionalized AF488 fluorophore. Jurkat T-cell lysates were incubated with varying concentrations of Cbz-Tzm-Ala-CMK **6** ([C] = 0-10 µM, 30 min, 37 °C), this was followed by a second incubation with the TCO-AF488 fluorophore, which attaches to the tetrazines through IEDDA reaction. Analysis was performed by denaturing the lysate followed by SDS-PAGE. The results from the SDS-PAGE analysis (**Figure 8 – assay 5**) showed many proteins labeled by inhibitor **6**. Interestingly, at low concentrations (0.1-1 µM), the expected labeling pattern (similar to figure **6**) was observed.

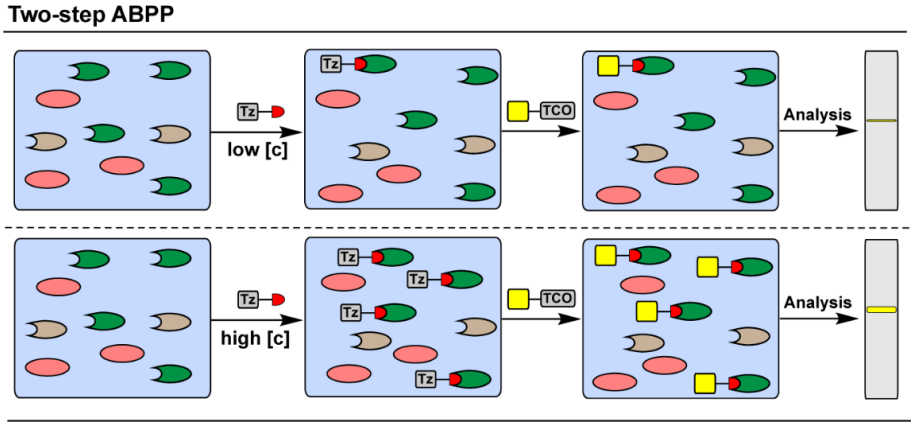


Figure 7: Two-step ABPP**:** The tetrazine-functionalized ABP binds to the enzyme's active site in varying amounts dependent on the ABP concentration. Treatment of the mixture with TCO-AF488, reacting with the tetrazine, results in AF488-labeled enzyme.

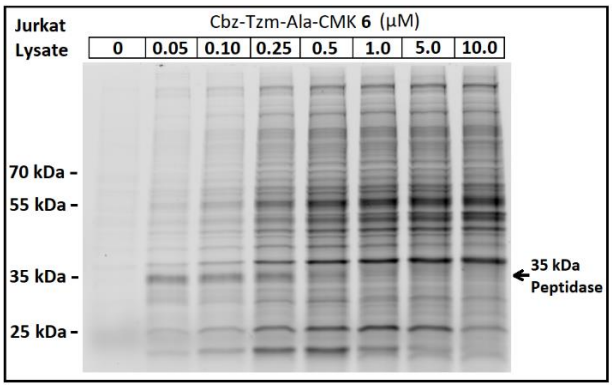


Figure 8: (**assay 5**) Jurkat T-cell lysate was incubated with **6** in a sodium acetate buffer solution at pH 5.5 at variable concentration, followed by incubation with AF488-TCO, denaturing and SDS-PAGE fluorescence analysis (532/28 nm).

At higher concentrations however, the labelling of the 35 kDa peptidase band disappeared, with the appearance of other protein bands. One possible explanation for this could be that, at high concentrations of **6**, proteases that are involved in formation of the 35kDa peptidase band are inhibited.

The next step was to determine if a two-step ABPP competition assay (**Figure 9**) was possible using Cbz-Tzm-Ala-CMK **6** and Cbz-Phe-Ala-CMK **8**, where by addition of **8** at various concentrations. First, Jurkat T-cell lysate was incubated with **8** and excess inhibitor was removed by various forms of precipitation (**Figure 10 – assay 6**), because the presence of unreacted inhibitor **8** during the incubation with AF488-TCO could influence the SDS-PAGE analysis through loss of signal, high background fluorescence or off-target labeling. From these, acetone precipitation worked best. It was then assessed whether further denaturing the proteins would influence the labelling with AF488-TCO, as steric constraints of the active site could limit accessibility of the AF488-TCO fluorophore (**Figure 10 – assay 7**). The tetrazine inhibitor was stable towards the denaturing conditions (25% SDS, 95 °C, 5 minutes), however this did not lead to improved labelling. Incubation at 20 °C for 45 minutes did lead to a change of signal. Finally, the concentration of AF488-TCO was optimized (**Figure 10 – assay 8**). Concentrations above 0.5 µM of AF488-TCO resulted in unwanted background fluorescence at the 20 – 30 kDa region, so the optimized concentration was set at 0.5 µM. With these conditions in hand, the two-step ABPP competition assay was performed to assess whether Cbz-Tzm-Ala-CMK **6** and Cbz-Phe-Ala-CMK **8** would have a specificity towards the same proteases. Lysates from Jurkat cells were incubated with varying concentrations of inhibitor **8** before incubation with inhibitor **6** (**Figure 10 – assay 9**) at 3.0 µM. Between 100-500 nM the 35 kDa peptidase signal disappeared exclusively, with other bands remaining visible. This indicated incomplete inhibition of the other proteases by **8** at the tested concentrations or a divergence in target specificity of **6** and **8**.

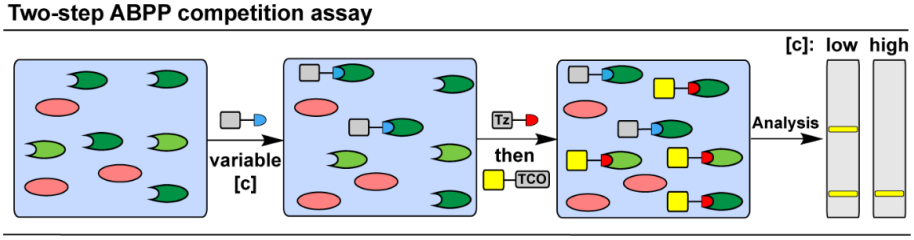


Figure 9: Two-step ABPP competition study: The ABP binds covalently to the enzyme's active site. By varying the concentration, the amount of remaining enzyme available for the tetrazine-functionalized ABP changes and can be analyzed through incubation with AF-488-TCO. Probes can be introduced consecutively, or together.

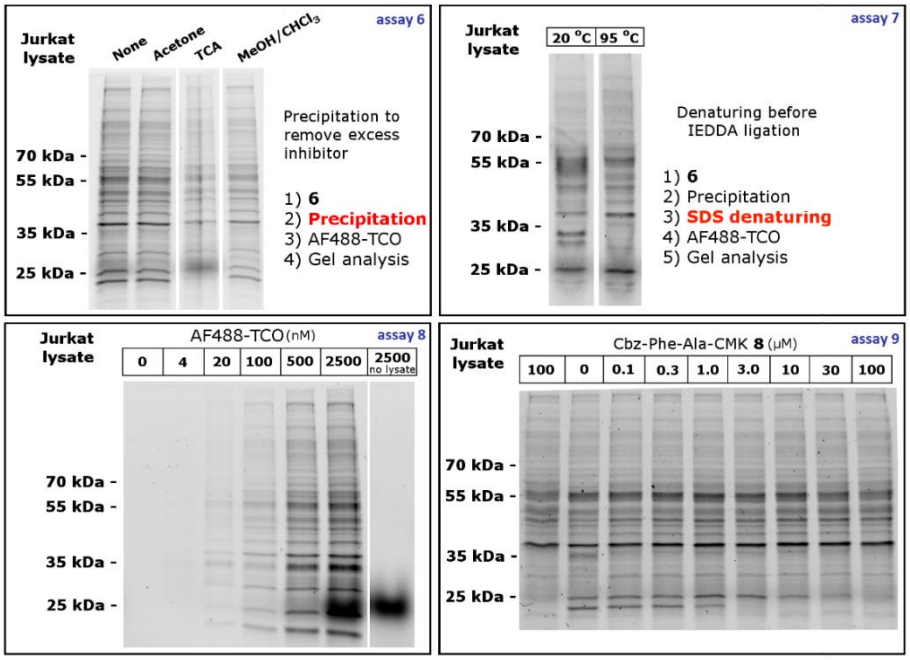


Figure 10: (**assay 6**) SDS-PAGE fluorescence analysis (532/28 nm) of acetone / trichloroacetic acid / methanol-chlorofom precipitation prior to incubation with AF488-TCO. (**assay 7**) SDS-PAGE fluorescence analysis (532/28 nm) of SDS denaturing at room temperature or 95 °C prior to incubation with AF488-TCO. (**assay 8**) SDS-PAGE fluorescence analysis (532/28 nm) of variable AF488-TCO concentrations. (**assay 9**) SDS-PAGE fluorescence analysis (532/28 nm) of variable concentrations inhibitor **8** prior to incubation with inhibitor **6**.

Conclusion

Cbz and Boc protected methyltetrazinealanine (Tzm) inhibitors **6**, **15** (CMK warhead), and **17**, **18** (BOMK warhead) were succesfully synthesized as peptidase inhibitors. A Cy5-DCG-04 competition assay used to compare methyltetrazinealanine inhibitor **6** to phenylalanine inhibitor **8** showed a high similarity in peptidases inhibited the 35kDa peptidase. Following these results the two-step ABPP labeling of peptidases and proteinases targeted by Cbz-Tzm-Ala-CMK **6** was also successful. It was shown that after incubation with **6** proteins can be precipitated with acetone and denatured using mercaptoethanol and heat before labeling with AF488-TCO, without affecting the test result. This validates the tetrazine/TCO two-step ABPP labeling technique as a versatile alternative candidate to other ABPP approaches for these proteins.

Compound synthesis

Procedure A: (Diazomethane generation) An Aldrich Mini Diazald® apparatus with Clear-Seal® joint funnel and receiver is used to add diazomethane. The "dry-ice condenser" is filled with dry ice and iso-propanol. To the "ether trap" a tube leading to an Erlenmeyer filled with a 1:1 solution of H_2O:AcOH is attached. A solution of KOH (5 g, 89 mmol) dissolved in 8 ml H_2O and 10 ml EtOH is placed in the "reaction vessel". The 100 ml Clear-Seal® joint receiving flask containing the pre-activated amino acid is attached and cooled in a dry-ice/EtOH bath. The Clear-Seal® joint separating funnel (containing 2.5 g (11.5 mmol) Diazald® dissolved in 25 mL Et_2O) is attached. On top of the separating funnel a nitrogen balloon is added to ensure a slight overpressure. To start the diazomethane generation the "reaction vessel" is heated to 65 °C and the Diazald® solution is dropwise added over 30-60 minutes. A slight reflux is observed in the reaction vessel, and a yellow condensed liquid is generated at the dry-ice condenser. After emptying the separating funnel, repeatedly, additional batches of 10 mL Et_2O are dropwise added to the reaction vessel until the condensed liquid turns nearly colorless.

Compound 1: 0.979 mmol (0.243 g) of compound **1**, 4.98 mmol (0.518 g) of formamidine acetate and 0.27 mmol (0.099 g) of $Zn(OTf)_2$ were added to a pressure tube. Then 1.5 mL of dry dioxane was added. The tube was sealed and 1.5 mL of anhydrous hydrazine was quickly injected under heavy stirring, while maintaining room temperature in a water bath. The reaction mixture was stirred for three days. The rubber seal was carefully punctured, slowly releasing the generated NH_3 gas. The reaction mixture was dissolved in 10 mL of 4M $NaNO_2$ (aq.) and 25 mL of 2M HCl (aq.) was added dropwise under heavy stirring until gas formation stops (pH = 2-3). Then 0.1M HCl (aq.) was added and the watery solution was extracted two times with EtOAc. The organic layers were combined, dried with $MgSO_4$ and concentrated using rotary evaporation. Purification was performed with silica column chromatography using an 2:2:96 AcOH/EtOAc/DCM eluent resulting in 0.040 g (0.132 mmol, 14%) of compound **1** as a thick red oil. **TLC**: Rf = 0.5, 2% AcOH in EtOAc. **^{1}H NMR** (400 MHz, Acetone) **δ** 10.42 (s, 1H), 7.52 – 7.18 (m, 5H), 6.87 (d, *J* = 8.1 Hz, 1H), 5.05 (s, 2H), 3.92 (ddd, *J* = 23.1, 15.0, 6.9 Hz, 2H). **^{13}C NMR** (101 MHz, Acetone) **δ** 172.29, 170.84, 159.22, 156.89, 137.86, 129.20, 128.68, 128.59, 66.95, 53.41, 38.33.

Compound 2: 0.979 mmol (0.243 g) of compound **1**, 4.79 mmol (0.25mL, 0.197 g) of acetonitrile and 0.26 mmol (0.094 g) of $Ni(OTf)_2$ were added to a pressure tube. Then 1.5 mL of dry dioxane was added. The tube was sealed and 1.5 mL of anhydrous hydrazine was quickly injected under heavy stirring, while maintaining room temperature in a water bath. The reaction mixture was stirred for three days. The rubber seal was carefully punctured, slowly releasing the generated NH_3 gas. The reaction mixture was dissolved in 10 mL of 4M $NaNO_2$ (aq.) and 25 mL of 2M HCl (aq.) was added dropwise under heavy stirring until gas formation stops (pH = 2-3). Then 0.1M HCl (aq.) was added and the watery solution was extracted two times with EtOAc. The organic layers were combined, dried with $MgSO_4$ and concentrated using rotary evaporation. Purification was performed with silica column chromatography using an 2:2:96 to 2:10:88 AcOH/EtOAc/DCM eluent resulting in 0.034 g (0.107 mmol, 11%) of compound **2** as a thick red oil. **TLC**: Rf = 0.5, 2% AcOH in EtOAc. **^{1}H NMR** (400 MHz, Acetone) **δ** 7.45 – 7.20 (m, 4H), 6.79 (d, *J* = 8.6 Hz, 1H), 4.98 (td, *J* = 8.4, 5.6 Hz, 1H), 4.00 – 3.70 (m, 2H), 2.96 (s, 3H). **^{13}C NMR** (101 MHz, Acetone) **δ** 172.29, 168.55, 167.82, 156.84, 137.92, 129.18, 128.64, 128.56, 66.87, 53.45, 37.69, 21.13.

Compound 3: 13.56 mmol (3.611 g) of Cbz-Asn-OH was dissolved in 35 mL of dry Acetone:Pyridine (1:1, v:v), 14.99 mmol of DCC (3.092 g) was dissolved in 35 mL of dry

Acetone and added to the solution. The reaction mixture was stirred at room temperature for 2 hours. Reaction completion was checked by TLC (Rf = 0.4, 25% MeOH in DCM). The reaction mixture was filtered and concentrated using rotary evaporation. The slurry was re-suspended in H_2O, adjusted to pH = 9 using an aqueous 1M k_2CO_3 solution, washed three times with $CHCl_3$, acidified to pH = 1 using an aqueous 1M HCl solution, extracted three times with $CHCl_3$, then dried using $MgSO_4$ and concentrated using rotary evaporation. 3.113 g (12.54 mmol, 93%) of compound **3** was obtained as a white solid. **^{1}H NMR** (400 MHz, MeOD) **δ** 7.54 – 7.11 (m, 5H), 5.10 (m, 4H), 4.62 – 4.41 (m, 1H), 2.98 (ddd, *J* = 25.1, 17.0, 6.6 Hz, 2H). **^{13}C NMR** (101 MHz, MeOD) **δ** 170.85, 156.86, 136.51, 128.16, 127.74, 127.47, 117.07, 66.60, 50.40, 20.02.

Compound 4: 5.28 mmol (0.998 g) of Boc-Ala-OH was dissolved in 25 mL of dry THF, cooled to -25 °C, and 6.82 mmol (0.75 ml, 0.69 g) of N-methylmorpholine (NMM) and 6.55 mmol (0.85 mL, 0.90 g) isobutyl chloroformate (IBCF) were added to the solution. The reaction mixture was stirred for 1 hour while warming to room temperature. Then, using an Aldrich Mini Diazald® apparatus 8.3 mmol diazomethane in Et_2O was added (**procedure A**). The reaction was quenched by dropwise addition of 10 ml of conc. HCl (aq) in AcOH (1:1, v:v) and then warmed to room temperature. Et_2O and sat. K_2CO_3 (aq.) were added before extracting the mixture and partitioning the layers. The organic layer was dried using Na_2SO_4 and concentrated using rotary evaporation. Purification was performed with silica column chromatography using an 5-20% EtOAc in pentane eluent resulting in 0.764 g (3.45 mmol, 69%) of compound **4** as a colorless oil. **TLC**: Rf = 0.3, 20% EtOAc in pentane. **^{1}H NMR** (400 MHz, $CDCl_3$) **δ** δ 5.18 (s, 1H), 4.47 (p, *J* = 7.2 Hz, 1H), 4.27 (d, *J* = 3.7 Hz, 2H), 1.40 (s, 9H), 1.33 (d, *J* = 7.2 Hz, 3H). **^{13}C NMR** (101 MHz, $CDCl_3$) **δ** 202.17, 155.32, 80.38, 77.48, 77.16, 76.84, 53.24, 46.35, 28.35, 17.42.

Compound 5: 1.8 mmol (0.40 g) of compound **4** was dissolved in 3.5 ml dry dioxane and 2.4 ml (9.6 mmol) 4M HCl in dioxane was dropwise over 1 minute to the solution. The reaction mixture was stirred for 2 hours before it was concentrated using rotary evaporation obtaining compound **5** in quantitative yield as a paly white solid. **^{1}H NMR** (400 MHz, DMSO) **δ** 8.31 (br, 3H), 4.80 (dd, *J* = 65.4, 17.2 Hz, 2H), 4.26 (q, *J* = 6.9 Hz, 1H), 1.41 (d, *J* = 7.3 Hz, 3H). **HR-MS**: [$C_4H_8ClNO+H^+$] found 122.0367, calculated 122.0294.

Compound 6: 0.11 mmol (34 mg) of compound **2** was dissolved in 1.6 ml dry THF, 0.11 mmol (14.6 mg, 200 µl of a 0.5 M solution in dry THF) IBCF and 0.10 mmol (10.9 mg, 200 µl of a 0.5 M solution in dry THF) TEA were added and the reaction mixture was stirred for 45 minutes at room temperature. An 1.8 ml dry DMF solution containing 0.11 mmol (17 mg) compound **5** and 0.13 mmol (13 mg, 17.9 µL) TEA was dropwise added to the reaction mixture and stirred for an additional 60 minutes at room temperature. Then, 50 ml EtOAc was added, and the organic layer was washed with 5% citric acid (aq.), water and brine, dried with Na_2SO_4 and concentrated using rotary evaporation. Purification was performed with silica column chromatography using an 20-40% EtOAc in pentane eluent resulting in 9.6 mg (0.023 mmol, 21%) of compound **6** as pink solid **TLC**: Rf = 0.5, 50% EtOAc in pentane. **^{1}H NMR** (400 MHz, $CDCl_3$) **δ** 7.41 – 7.28 (m, 5H), 7.06 (d, *J* = 6.9 Hz, 1H), 6.03 (d, *J* = 8.6 Hz, 1H), 5.21 – 5.03 (m, 2H), 4.96 (q, *J* = 6.1 Hz, 1H), 4.72 (p, *J* = 7.1 Hz, 1H), 4.20 (s, 2H), 3.79 (qd, *J* = 16.0, 6.0 Hz, 2H), 3.04 (s, 3H), 1.35 (d, *J* = 7.2 Hz, 3H). **^{13}C NMR** (126 MHz, $CDCl_3$) **δ** 200.99, 170.04, 168.03, 166.90, 156.20, 135.88, 128.75, 128.55, 128.34, 67.69, 52.85, 52.39, 46.18, 36.91, 21.32, 17.17. **HRMS:** [$C_{18}H_{21}ClN_6O_4+H]^+$: found 421.1390, calculated 421.1313.

Compound 7: 25 µmol (7.9 mg) of compound **2** was dissolved in 300 µl dry THF, 25 µmol (3.4 mg, 100 µl of a 0.25 M solution in dry THF) IBCF and 25 µmol (2.5 mg, 100 µl of a 0.25 M

solution in dry THF) TEA were added and the reaction mixture was stirred for 45 minutes at room temperature. An 1.3 ml dry DMF solution containing 25 µmol (3.5 mg) alanine methyl ester hydrochloride and 30 µmol (3.0 mg, 4.1 µL) TEA was dropwise added to the reaction mixture and stirred for an additional 60 minutes at room temperature. Then, EtOAc was added, and the organic layer was washed with 5% citric acid (aq.), water and brine, dried with Na_2SO_4 and concentrated using rotary evaporation. Purification was performed with silica column chromatography using an 20-40% EtOAc in pentane eluent resulting in 2.8 mg (7 µmol, 28%) of compound **7** as pink solid **TLC**: Rf = 0.4, 50% EtOAc in pentane. **^{1}H NMR** (400 MHz, $CDCl_3$) **δ** 7.44 – 7.28 (m, 5H), 6.88 (d, *J* = 7.3 Hz, 1H), 6.03 (d, *J* = 8.6 Hz, 1H), 5.15 – 5.03 (m, 2H), 4.95 (q, *J* = 6.7, 6.1 Hz, 1H), 4.50 (p, *J* = 7.3 Hz, 1H), 3.91 – 3.72 (m, 2H), 3.71 (s, 3H), 3.04 (s, 3H), 1.36 (d, *J* = 7.3 Hz, 3H). **^{13}C NMR** (126 MHz, $CDCl_3$) **δ** 172.90, 169.59, 167.93, 167.08, 156.12, 135.96, 128.73, 128.49, 128.32, 67.58, 52.73, 48.43, 37.16, 21.32, 18.27. **HRMS:** $[C_{18}H_{22}N_6O_5+H]^+$: found 403.1726, calculated 403.1652.

Compound 8: 0.10 mmol (30 mg) Cbz-Phe-OH was dissolved in 1.6 ml dry THF, 0.10 mmol (13.7 mg, 200 µl of a 0.5 M solution in dry THF) IBCF and 0.10 mmol (10.1 mg, 200 µl of a 0.5 M solution in dry THF) TEA were added and the reaction mixture was stirred for 1 hour at room temperature. An 1.8 ml dry DMF solution containing 0.10 mmol (16 mg) compound **5** and 0.12 mmol (12 mg, 16.7 µL) TEA was dropwise added to the reaction mixture and stirred for an additional 30 min at room temperature. Then, 50 ml EtOAc was added, and the organic layer was washed with 5% citric acid (aq.), 5% $NaHCO_3$ (aq.), water and brine, dried with Na_2SO_4 and concentrated using rotary evaporation. Purification was performed with silica column chromatography using an 20-30% EtOAc in pentane eluent resulting in 19.1 mg (0.047 mmol, 47%) of compound **8** as white solid **TLC**: Rf = 0.6, 50% EtOAc in pentane. **^{1}H NMR** (400 MHz, $CDCl_3$) **δ** 7.43 – 7.23 (m, 8H), 7.19 (d, *J* = 6.8 Hz, 2H), 6.61 (d, *J* = 5.5 Hz, 1H), 5.45 (d, *J* = 7.3 Hz, 1H), 5.08 (s, 2H), 4.68 (p, *J* = 7.1 Hz, 1H), 4.46 (d, *J* = 7.1 Hz, 1H), 4.09 (s, 2H), 3.20 – 2.97 (m, 2H), 1.29 (d, *J* = 7.1 Hz, 3H). **^{13}C NMR** (101 MHz, $CDCl_3$) **δ** 200.81, 170.96, 156.10, 136.08, 129.39, 128.93, 128.71, 128.43, 128.18, 127.41, 77.48, 77.16, 76.84, 67.37, 56.26, 52.11, 46.14, 38.50, 17.11. **HRMS**: $[C_{21}H_{23}ClN_2O_4+H]^+$: Found 403.1419, calculated 403.1346.

Compound 9: 0.10 mmol (30 mg) of Cbz-Phe-OH was dissolved in 1.6 mL dry THF, 0.10 mmol (13.7 mg, 200 µl of a 0.5 M solution in dry THF) IBCF and 0.10 mmol (10.1 mg, 200 µL of a 0.5 M solution in dry THF) TEA were added and the reaction mixture was stirred for 1 hour at room temperature. A 1.8 mL dry DMF solution containing 0.10 mmol (14 mg) alanine methyl ester hydrochloride and 0.12 mmol (12 mg, 16.7 µL) TEA was dropwise added to the reaction mixture and stirred for an additional 30 min at room temperature. Then, EtOAc was added, and the organic layer was washed with 5% citric acid (aq.), 5% $NaHCO_3$ (aq.), water and brine, dried with Na_2SO_4 and concentrated using rotary evaporation. Purification was performed with silica column chromatography using an 20-30% EtOAc in pentane eluent resulting in 24.8 mg (0.065 mmol, 65%) of compound **9** as white solid **TLC**: Rf = 0.5, 50% EtOAc in pentane **^{1}H NMR** (400 MHz, $CDCl_3$) **δ** 7.40 – 7.12 (m, 11H), 6.44 (d, *J* = 7.2 Hz, 1H), 5.40 (d, *J* = 8.1 Hz, 1H), 5.08 (s, 2H), 4.48 (dp, *J* = 14.2, 7.2 Hz, 2H), 3.70 (s, 3H), 3.19 – 2.99 (m, 2H), 1.32 (d, *J* = 7.2 Hz, 3H). **^{13}C NMR** (101 MHz, $CDCl_3$) **δ** 172.93, 170.51, 156.02, 136.31, 136.23, 129.47, 128.78, 128.65, 128.32, 128.15, 127.17, 67.18, 56.10, 52.60, 48.25, 38.62, 18.38. **HRMS:** $[C_{21}H_{24}N_2O_5+H]^+$: found 385.1762, calculated 385.1685.

Compound 10: 19.27 mmol (4.470 g) of Boc-Asn-OH was dissolved in 50 mL of dry Acetone:Pyridine (1:1, v:v), 22.12 mmol of DCC (4.565 g) was dissolved in 40 mL of dry acetone and added to the solution. The reaction mixture was stirred at room temperature for 2 hours.

Reaction completion was checked by TLC (Rf = 0.5, 30% MeOH in DCM). The reaction mixture was filtered and concentrated using rotary evaporation. The slurry was redissolved in 10 mL (10 mmol) 1M k_2CO_3 solution, washed three times with $CHCl_3$ and concentrated using rotary evaporation. 4.680 g (18.57 mmol, 96%) of compound **10** was obtained as a white solid. **^{1}H NMR** (400 MHz, DMSO) **δ** 6.25 (d, *J* = 5.4 Hz, 1H), 3.65 (q, *J* = 5.2 Hz, 1H), 2.96 – 2.67 (m, 2H), 1.39 (s, 9H). **^{13}C NMR** (101 MHz, DMSO) **δ** 169.90, 154.91, 119.22, 78.02, 51.60, 28.16, 21.59.

Compound 11: 0.976 mmol (0.246 g) of compound **10**, 10.08 mmol (1.050 g) of formamidine acetate and 0.26 mmol (0.092 g) of $Zn(OTf)_2$ were added to a pressure tube. Then 1.5 mL of dry dioxane was added. The tube was sealed and 1.5 mL of anhydrous hydrazine was quickly injected under heavy stirring, while maintaining room temperature in a water bath. The reaction mixture was stirred for three days. The rubber seal was carefully punctured, slowly releasing the generated NH_3 gas. The reaction mixture was dissolved in 10 mL of 4M $NaNO_2$ (aq.) and 25 mL of 2M HCl (aq.) was added dropwise under heavy stirring until gas formation stops (pH = 2-3). Then 1M HCl (aq.) was added and the watery solution was extracted two times with EtOAc. The organic layers were combined, dried with $MgSO_4$ and concentrated using rotary evaporation. Purification was performed with silica column chromatography using an 2:2:96 AcOH/EtOAc/DCM eluent resulting in 11 mg (0.041 mmol, 4.2%) of compound **11** as a thick red oil. **TLC**: Rf = 0.5, 2% AcOH in EtOAc. **^{1}H NMR** (400 MHz, $CDCl_3$) **δ** 10.25 (s, 1H), 8.41 (s, 1H), 5.63 (d, *J* = 7.9 Hz, 1H), 4.97 (m, 1H), 3.92 (ddd, *J* = 22.4, 15.5, 5.7 Hz, 2H), 1.38 (s, 9H). **^{13}C NMR** (101 MHz, $CDCl_3$) **δ** 174.60, 169.67, 158.27, 155.52, 81.00, 51.79, 37.91, 28.33.

Compound 12: 9.05 mmol (2.28 g) of compound **10**, 155 mmol (5.0 mL, 6.36 g) of acetonitrile and 2.40 mmol (0.872 g) of $Ni(OTf)_2$ were added to a pressure tube. The tube was sealed and 16 mL of anhydrous hydrazine was quickly injected under heavy stirring, while maintaining room temperature in a water bath. The reaction mixture was stirred for three days. The rubber seal was carefully punctured, slowly releasing the generated NH_3 gas. The reaction mixture was dissolved in 400 mL of DCM/AcOH (1:1, v:v) While stirring, the reaction mixture was added dropwise. Then 10 g solid $NaNO_2$ was added portion wise over 30 minutes. The mixture was concentrated using rotary evaporation, re-dissolved in EtOAc, washed with aqueous 2M HCl (check for pH = 1) and brine, dried using $MgSO_4$ and concentrated using rotary evaporation. Purification was performed with silica column chromatography using an 2:2:96 to 2:10:88 AcOH/EtOAc/DCM eluent resulting in 0.279 g (0.985 mmol, 11%) of compound **12** as a thick red oil. **TLC**: Rf = 0.5, 2% AcOH in EtOAc. **^{1}H NMR** (400 MHz, $CDCl_3$) **δ** 8.20 (s, 1H), 5.63 (d, *J* = 7.6 Hz, 1H), 4.93 (d, *J* = 4.9 Hz, 1H), 3.85 (ddd, *J* = 22.1, 15.3, 5.3 Hz, 2H), 3.04 (s, 3H), 1.37 (s, 9H). **^{13}C NMR** (101 MHz, $CDCl_3$) **δ** 174.66, 167.90, 166.60, 155.52, 80.82, 51.86, 37.26, 28.32, 21.22.

Compound 13: Compound **11** was dissolved in DCM, cooled to 0 °C, and a 4M HCl in dioxane solution was added dropwise over 1 minute. The solution was stirred for 4 hours forming a suspension. The suspension was concentrated using rotary evaporation resulting in compound **13** in quantitative yield.

Compound 14: 0.071 mmol (20 mg) of compound **12** was dissolved in 0.4 mL DCM, cooled to 0 °C, and 0.4 mL of a 4M HCl in dioxane solution was added dropwise over 1 minute. The solution was stirred for 4 hours forming a suspension. The suspension was concentrated using rotary evaporation resulting in compound **14** in quantitative

yield. **^{1}H NMR** (400 MHz, D_2O) **δ** 4.68 – 4.62 (m, 1H), 3.92 (d, *J* = 6.2 Hz, 2H), 2.95 (s, 3H). **^{13}C NMR** (101 MHz, D_2O) **δ** 170.86, 168.27, 165.42, 50.98, 34.11, 20.18.

Compound 15: 0.40 mmol (106 mg) of Boc-Phe-OH was dissolved in 7 mL dry THF, 1.08 mmol (147 mg, 139 µL) IBCF and 0.49 mmol (50 mg, 69 µL) TEA were added and the reaction mixture was stirred for 1 hour at room temperature. An 7 mL dry DMF solution containing 0.49 mmol (78 mg) compound **5** and 1.2 mmol (122 mg, 168 µL) TEA was dropwise added to the reaction mixture and stirred overnight at room temperature. Then, 140 mL EtOAc was added, and the organic layer was washed with 5% citric acid (aq.), 5% $NaHCO_3$ (aq.), water and brine, dried with Na_2SO_4 and concentrated using rotary evaporation. Purification was performed with silica column chromatography using an 20-50% EtOAc in pentane eluent resulting in 91 mg (0.247 mmol, 62%) of compound **15** as white solid **TLC**: Rf = 0.5, 20% EtOAc in pentane. **^{1}H NMR** (400 MHz, $CDCl_3$) **δ** 7.36 – 7.16 (m, 5H), 7.05 (s, 1H), 5.34 (d, *J* = 6.9 Hz, 1H), 4.66 (m, 1H), 4.44 (m, 1H), 4.08 (s, 2H), 3.05 (s, 2H), 1.41 (s, 9H), 1.30 (d, *J* = 7.1 Hz, 3H). **^{13}C NMR** (101 MHz, $CDCl_3$) **δ** 200.88, 171.64, 155.63, 136.41, 129.35, 128.74, 127.17, 80.42, 55.78, 52.12, 46.28, 38.37, 28.32, 16.84.

Compound 16: 0.106 mmol (30 mg) of compound **12** was dissolved in 3 mL dry THF, cooled to 0 °C, 0.116 mmol (16 mg, 15 µL) IBCF and 0.115 mmol (12 mg, 16 µL) TEA were added and the reaction mixture was stirred for 30 minutes. A 2 mL dry THF solution containing 0.165 mmol (26 mg) compound **5** and 0.208 mmol (21 mg, 29 µL) TEA was dropwise added to the reaction mixture and stirred for 1 hour while warming to room temperature. Then, EtOAc was added, and the organic layer was washed water, brine, dried with Na_2SO_4 and concentrated using rotary evaporation. Purification was performed with silica column chromatography using an 25-50% EtOAc in pentane eluent resulting in 33 mg (0.085 mmol, 80%) of compound **16** as a red solid. **TLC**: Rf = 0.5, 50% EtOAc in pentane. **^{1}H NMR** (400 MHz, $CDCl_3$) **δ** 7.22 (d, *J* = 6.4 Hz, 1H), 5.81 (m, 1H), 4.85 (m, 1H), 4.79 – 4.67 (m, 1H), 4.23 (m, 2H), 3.80 – 3.71 (m, 2H), 3.03 (s, 3H), 1.41 – 1.35 (m, 12H). **^{13}C NMR** (101 MHz, $CDCl_3$) **δ** 201.05, 170.41, 167.89, 167.03, 79.23, 52.51, 52.33, 46.27, 46.20, 36.94, 28.31, 21.25, 17.09.

Compound 18: 49 µmol (19 mg) of compound **16** was dissolved in 1 mL of dry DMF and 153 µmol (23 mg) of 2,6-dimethylbenzoic acid was added before adding 517 µmol (30 mg) of solid KF and stirring the solution for 2 hours at room temperature. Reaction completion was checked by TLC (Rf = 0.55, 50% EtOAc in pentane). Then, 10 mL EtOAc was added, and the organic layer was washed two times with 10 mL 5% $NaHCO_3$ (aq.), 10 mL brine, dried with $MgSO_4$ and concentrated using rotary evaporation. Purification was performed with silica column chromatography using an 25-50% EtOAc in pentane eluent resulting in 21 mg (42 µmol, 86%) of compound **18** as a red solid. **^{1}H NMR** (500 MHz, $CDCl_3$) **δ** 7.20 (t, *J* = 7.6 Hz, 1H), 7.12 (s, 1H), 7.04 (d, *J* = 7.6 Hz, 2H), 5.74 (d, *J* = 7.4 Hz, 1H), 5.00 (m, 2H), 4.87 (dd, *J* = 13.7, 6.1 Hz, 1H), 4.67 (p, *J* = 7.0 Hz, 1H), 3.78 (d, *J* = 6.2 Hz, 2H), 3.03 (s, 3H), 2.37 (s, 6H), 1.43 (m, 12H). **^{13}C NMR** (126 MHz, $CDCl_3$) **δ** 201.69, 170.35, 169.08, 167.90, 167.12, 135.79, 132.45, 129.91, 127.82, 66.23, 52.49, 51.93, 51.84, 36.83, 28.35, 21.26, 20.02, 17.16.

Compound 20: 1.0 mmol (0.265 g) Boc-Phe-OH was dissolved in 4 ml of dry DMF, 1.2 mmol (0.167 g) alanine methyl ester hydrochloride, 1.2 mmol (0.162 g) HOBt and 1.3 mmol (0.131 g, 180 µL) TEA were added and stirred for 4 hours at room temperature. 100 mL of DCM was added and the organic layer was washed with sat. $NaHCO_3$ (aq.), 1M HCl (aq.), water and brine, dried using $MgSO_4$, filtered and concentrated using rotary evaporation. Purification was performed with silica column chromatography using an 30-50% EtOAc in pentane eluent resulting in 300 mg (0.856 mmol, 86%) of compound **20** as a white solid. **^{1}H NMR** (400 MHz,

$CDCl_3$) **δ** 7.34 – 7.10 (m, 5H), 6.44 (d, *J* = 7.1 Hz, 1H), 5.01 (s, 1H), 4.52 (p, *J* = 7.0 Hz, 1H), 4.36 (d, *J* = 5.9 Hz, 1H), 3.71 (s, 3H), 3.07 (t, *J* = 6.1 Hz, 2H), 1.40 (s, 9H), 1.34 (d, *J* = 7.2 Hz, 3H). **^{13}C NMR** (101 MHz, $CDCl_3$) **δ** 172.97, 170.83, 136.60, 129.50, 128.77, 127.09, 55.68, 52.59, 48.23, 38.47, 28.37, 18.53.

Compound 21: 3.85 µmol (1.62 mg) of compound **6** was dissolved in 70 µL of dry DMF and 13 µmol (2 mg) of 2,6-dimethylbenzoic acid was added before adding 34 µmol (2 mg) of solid KF and stirring the solution for 1 day at room temperature. Reaction completion was checked by TLC (Rf = 0.6, 50% EtOAc in pentane). The reaction mixture was diluted with 1 mL EtOAc, washed with 1 mL sat. $NaHCO_3$ (aq.) and 1mL brine. The organic layer was dried using $MgSO_4$ and concentrated under rotary evaporation. Purification was performed with silica column chromatography using an 50:50 EtOAc/pentane eluent resulting in compound **21** as a red solid. **^{1}H NMR** (400 MHz, Chloroform) **δ** 7.39 – 7.29 (m, 5H), 7.24 – 7.18 (m, 1H), 7.05 (d, *J* = 7.6 Hz, 2H), 7.00 (s, 1H), 5.99 (d, *J* = 8.6 Hz, 1H), 5.20 – 4.86 (m, 5H), 4.65 (p, *J* = 6.8 Hz, 1H), 3.91 – 3.70 (m, 2H), 3.03 (s, 3H), 2.38 (s, 6H), 1.40 (d, *J* = 7.2 Hz, 3H).

Compound 22: 4.0 µmol (1.7 mg) of compound **8** was dissolved in 70 µL of dry DMF and 13 µmol (2 mg) of 2,6-dimethylbenzoic acid was added before adding 34 µmol (2 mg) of solid KF and stirring the solution for 1 day at room temperature. Reaction completion was checked by TLC (Rf = 0.4, 40% EtOAc in pentane). The reaction mixture was diluted with 1 mL EtOAc, washed with 1 mL sat. $NaHCO_3$ (aq.) and 1mL brine. The organic layer was dried using $MgSO_4$ and concentrated under rotary evaporation. Purification was performed with silica column chromatography using an 20:80 to 40:60 EtOAc/pentane eluent resulting in compound **22** as a red solid. **^{1}H NMR** (400 MHz, $CDCl_3$) **δ** 7.46 – 7.19 (m, 11H), 7.10 (d, *J* = 7.7 Hz, 2H), 6.46 (d, *J* = 6.3 Hz, 1H), 5.30 (d, *J* = 7.2 Hz, 1H), 5.14 (s, 2H), 4.92 (dd, *J* = 39.8, 16.9 Hz, 2H), 4.69 (p, *J* = 7.1 Hz, 1H), 4.48 (d, *J* = 6.8 Hz, 1H), 4.16 (q, *J* = 7.1 Hz, 2H), 3.14 (ddd, *J* = 33.8, 13.8, 6.8 Hz, 1H), 2.43 (s, 6H), 1.40 (d, *J* = 7.1 Hz, 3H).

Biological Protocols

Cell Culturing

Jurkat T-cells were cultured in RPMI-1640 with 10% FBS, penicillin (100 U/mL), streptomycin (0.10 mg/mL) and Gibco GlutaMAX (2 mM). Cells were kept at a cell density between $1*10^5$ and $1*10^6$ cells/mL in T-25/T-75/T-150 flasks. Culture conditions were 95% air and 5% CO_2 at 37°C.

RAW 264.7 macrophages were cultured in DMEM with 10% FBS, penicillin (100 U/mL), streptomycin (0.10 mg/mL) and Gibco GlutaMAX (2 mM). Culture cell density was kept between $1*10^6$ and $2*10^6$ cells/mL in 10 cm Petri dishes. Culture conditions were 95% air and 5% CO_2 at 37°C.

Cell Harvesting

Cell harvest: Cells were collected (13 million / tube) and centrifuged at 300 RCF. The medium was decanted and the tubes were cooled to -20 °C before flash freezing in liquid N_2 for storage at -80 °C op to 3 months.

Cell Lysis

Lysis Buffer: A 50 mM NaOAc (aq.) solution was titrated to pH = 5.5 via dropwise addition of 1M NaOH (aq.) and supplemented with DTT (4 mM), $MgCl_2$ (10 mM) and 0.2% Triton-X 100, before diluting with water (1:1, v:v) and re-titrating to pH 5.5.

Cell Lysis: Frozen harvested cells were transferred to ice (-20 °C) and kept for 30 minutes to defrost. Excess liquid was removed before mixing the suspension with 25 µL lysis buffer for cell lysis (1 hour, -20 °C). The suspension was flash frozen three times (1 min liq. N_2 freeze, 7-10 min -20 °C thaw), centrifuged (30 min, 4°C, 30.000 RCF) and the lysate supernatant was collected to ice (-20 °C).

Bradford assay: Lysate supernatant (1 µL) was diluted with H_2O (39 µL), from which 1 µL was transferred to a 96 well plate containing 100 µL Bradford solution (1 part Bradford reagent, 4 parts H_2O) and measured at 595 nm absorption wavelength on a BioRAD iMark™ microplate reader. Lysates were measured triplo against a freshly prepared BSA in H_2O calibration line (2.0 mg/mL – 0.0625 mg/mL range).

Stock lysate: Lysate supernatant was diluted to a protein concentration of 1.8 µg/µL using 50 mM NaOAc (aq.) at -20 °C, separated into Eppendorf tubes (100 µL stocks, -20 °C) and flash frozen in liquid N_2 for storage at -80 °C op to 2 weeks.

Protein Precipitation

Acetone precipitation: Eppendorf tubes containing lysate in NaOAc buffered water were diluted with acetone (4x volume), cooled to -20 °C for 30 minutes, and centrifuged at 4 °C for 10 minutes before removing the supernatant. The remaining pellet (poorly visible) was redissolved in the original amount of NaOAc buffered water.

MeOH/$CHCl_3$ precipitation: Eppendorf tubes containing lysate in NaOAc buffered water were diluted with methanol (4x volume), chloroform (1x volume) and water (3x volume) respectively, vortexed, and centrifuged. The top and bottom layers were removed while keeping the precipitated protein at the interface. The protein was redissolved in the original amount of NaOAc buffered water.

SDS-PAGE

SDS-PAGE Protocol: 10 lane SDS-PAGE plates were prepared using: **(1)** Add 3 mL running gel (36% of H_2O, 25% of Buffer A, 33.5% of Acrylamide 30% (29:1) solution, 5% Glycerol, 0.5% APS, and 0.05% TEMED), followed by 60 µL iPrOH and 30 minutes polymerization. **(2)** Fill with stacking gel (60% H_2O, 24% Buffer B, 14.4% Acrylamide 30% (29:1) solution, 1.2% APS, 0.12% TEMED) before 30 minutes polymerization. Samples were denatured using 4 µL β-mercaptoethanol and heating to 95 °C for 5 minutes prior to loading. Samples were run at 90 volt for 15 minutes, followed by 150 volt for 80-90 minutes against a PAGE-ruler calibration ladder. Gel analysis was performed on a BioRAD ChemiDOC™ MP imaging system: Cy3-channel (602/50 nm) for PAGE-ruler lines (25kDa / 70 kDa), Cy5-channel (700/50 nm) for Cy5-DCG-04 labelled proteins and AF488-channel (532/28 nm) for AF488 labelled proteins. To verify protein concentration a Coomassie staining was performed. The Coomassie staining was performed on a BioRAD ChemiDOC™ MP imaging system: Coomassie (590/110 nm).

Biological Experimental

Assay 1 + 2: Stock RAW / Jurkat cell lysate (100 µL) was defrosted on ice (-20 °C) for 1 hour, and divided over 10 Eppendorf tubes (10 µL each, -20 °C). Per Eppendorf tube, 1.0 µL Cbz-Phe-Ala-CMK **8** (11x concentration, 1% DMSO) was added to reach the desired concentration, before incubating for 30 minutes at 37 °C. Then, per Eppendorf tube, 1.2 µL of Cy5-DCG-04 (12 µM, 6.5% DMSO) was added to reach 1 µM concentration, before incubating for 30 minutes at 37 °C. Analysis of the samples was performed via SDS-PAGE analysis using the standard protocol.

Assay 1

			Lane Content							
			Ruler	Samples						
Incubation	Content	volume (end [C])	0	1	2	3	4	5	6	7
-	RAW Lysate (1.8 µg/µL)	µL	-	10	10	10	10	10	10	10
1 (30 min)	Cbz-Phe-Ala-CMK **8**	µL (range)	-	- (0)	1 (0.01)	1 (0.025)	1 (0.05)	1 (0.10)	1 (0.25)	1 (0.50)
2 (30 min)	Cy5-DCG-04	µL (20 µM)	-	1	1	1	1	1	1	1

Assay 2

			Lane Content							
			Ruler	Samples						
Incubation	Content	volume (end [C])	0	1	2	3	4	5	6	7
-	Jurkat Lysate (1.8 µg/µL)	µL	-	10	10	10	10	10	10	10
1 (30 min)	Cbz-Phe-Ala-CMK **8**	µL (range)	-	- (0)	1 (0.01)	1 (0.025)	1 (0.05)	1 (0.10)	1 (0.25)	1 (0.50)
2 (30 min)	Cy5-DCG-04	µL (20 µM)	-	1	1	1	1	1	1	1

Assay 3 + 4: Stock Jurkat cell lysate (100 µL) was defrosted on ice (-20 °C) for 1 hour, and divided over 10 Eppendorf tubes (10 µL each, -20 °C). Per Eppendorf tube, 1.0 µL Cbz-Phe-Ala-CMK **8** / Cbz-Tzm-Ala-CMK **6** (11x concentration, 1% DMSO) was added to reach the desired concentration, before incubating for 30 minutes at 37 °C. Then, per Eppendorf tube, 1.2 µL of Cy5-DCG-04 (12 µM, 6.5% DMSO) was added to reach 1 µM concentration, before incubating for 30 minutes at 37 °C. Analysis of the samples was performed via SDS-PAGE analysis using the standard protocol.

Assay 3

			Lane Content									
			Ruler	Samples								
Incubation	Content	volume (end [C])	0	1	2	3	4	5	6	7	8	9
-	Jurkat Lysate (1.8 µg/µL)	µL	-	10	10	10	10	10	10	10	10	10
1 (30 min)	Cbz-Phe-Ala-CMK **8**	µL (range)	-	- (0)	1 (0.01)	1 (0.05)	1 (0.10)	1 (0.25)	1 (0.50)	1 (1.0)	1 (5.0)	1 (10.0)
2 (30 min)	Cy5-DCG-04	µL (20 µM)	-	1	1	1	1	1	1	1	1	1

Assay 4

			Lane Content									
			Ruler	Samples								
Incubation	Content	volume (end [C])	0	1	2	3	4	5	6	7	8	9
-	Jurkat Lysate (1.8 µg/µL)	µL	-	10	10	10	10	10	10	10	10	10
1 (30 min)	Cbz-Tzm-Ala-CMK **6**	µL (range)	-	- (0)	1 (0.01)	1 (0.05)	1 (0.10)	1 (0.25)	1 (0.50)	1 (1.0)	1 (5.0)	1 (10.0)
2 (30 min)	Cy5-DCG-04	µL (20 µM)	-	1	1	1	1	1	1	1	1	1

Assay 5: Stock Jurkat cell lysate (100 µL) was defrosted on ice (-20 °C) for 1 hour, and divided over 10 Eppendorf tubes (10 µL each, -20 °C). Per Eppendorf tube, 1.0 µL Cbz-Tzm-Ala-CMK **6** (11x concentration, 1% DMSO) was added to reach the desired concentration, before incubating for 30 minutes at 37 °C. Then, per Eppendorf tube, 1 µL of AF488-TCO (6 µM, 10% DMSO) was added to reach 0.5 µM concentration, before incubating for 30 minutes at 37 °C. Then, per Eppendorf tube, 1.3 µL of Cy5-DCG-04 (13 µM, 6.5% DMSO) was added to reach 1

µM concentration, before incubating for 30 minutes at 37 °C. Analysis of the samples was performed via SDS-PAGE analysis using the standard protocol.

Assay 5			Lane Content									
			Ruler	Samples								
Incubation	Content	volume (end [C])	0	1	2	3	4	5	6	7	8	9
-	Jurkat Lysate (1.8 µg/µL)	µL	-	10	10	10	10	10	10	10	10	10
1 (30 min)	Cbz-Tzm-Ala-CMK **6**	µL (range)	-	1 (0)	1 (0.01)	1 (0.05)	1 (0.10)	1 (0.25)	1 (0.50)	1 (1.0)	1 (5.0)	1 (10.0)
	Acetone precipitation											
2 (30 min)	AF488-TCO	µL (500 nM)	-	1	1	1	1	1	1	1	1	1
3 (30 min)	Cy5-DCG-04	µL (20 µM)	-	1	1	1	1	1	1	1	1	1

Assay 6: Stock Jurkat cell lysate (100 µL) was defrosted on ice (-20 °C) for 1 hour, and divided over 6 Eppendorf tubes (10 µL each, -20 °C). Per Eppendorf tube, 1.0 µL Cbz-Tzm-Ala-CMK **6** (5.5 µM, 1% DMSO) was added to reach 0.5 µM concentration, before incubating for 30 minutes at 37 °C. Then, the protein was precipitated using the acetone (lane 1+2), TCA (lane 3+4), or methanol/chloroform (lane 5+6) precipitation protocol. Then, per Eppendorf tube, 1 µL of AF488-TCO (6 µM, 10% DMSO) was added to reach 0.5 µM concentration, before incubating for 30 minutes at 37 °C. Analysis of the samples was performed via SDS-PAGE analysis using the standard protocol.

Assay 6			Lane Content						
			Ruler	Samples					
Incubation	Content	volume (end [C])	0	1	2	3	4	5	6
-	Jurkat Lysate (1.8 µg/µL)	µL	-	10	10	10	10	10	10
1 (30 min)	Cbz-Tzm-Ala-CMK 6	µL (500 nM)	-	1	1	1	1	1	1
	Precipitation			Acetone		TCA		$MeOH/CHCl_3$	
2 (30 min)	AF488-TCO	µL (500 nM)	-	1	1	1	1	1	1

Assay 7: Stock Jurkat cell lysate (100 µL) was defrosted on ice (-20 °C) for 1 hour, and divided over 2 Eppendorf tubes (10 µL each, -20 °C). Per Eppendorf tube, 1.0 µL Cbz-Tzm-Ala-CMK **6** (33 µM, 1% DMSO) was added to reach 3.0 µM concentration, before incubating for 30 minutes at 37 °C. Then, the protein was precipitated using the acetone precipitation protocol. Then, the lysates were denatured using an SDS solution at 20 or 95 °C for 45 minutes. Then, per Eppendorf tube, 1 µL of AF488-TCO (6 µM, 10% DMSO) was added to reach 0.5 µM concentration, before incubating for 30 minutes at 37 °C. Analysis of the samples was performed via SDS-PAGE analysis using the standard protocol excluding denaturing step.

Assay 8: Stock Jurkat cell lysate (100 µL) was defrosted on ice (-20 °C) for 1 hour, and divided over 10 Eppendorf tubes (10 µL each, -20 °C). Per Eppendorf tube, 1.0 µL Cbz-Tzm-Ala-CMK **6** (5.5 µM, 1% DMSO) was added to reach 0.5 µM concentration, before incubating for 30 minutes at 37 °C. Then, the protein was precipitated using the acetone precipitation protocol. Then, per Eppendorf tube, 1 µL of AF488-TCO (12x concentration, 10% DMSO) was added to reach the desired concentration, before incubating for 30 minutes at 37 °C. Analysis of the samples was performed via SDS-PAGE analysis using the standard protocol.

Assay 8			Lane Content									
			Ruler	Samples								
Incubation	Content	volume (end [C])	0	1	2	3	4	5	6	7	8	9
-	Jurkat Lysate (1.8 µg/µL)	µL	-	10	10	10	10	10	10	10	10	10
1 (30 min)	Cbz-Tzm-Ala-CMK **6**	µL (500 nM)	-	1	1	1	1	1	1	1	0	1
	Acetone precipitation											
3 (30 min)	AF488-TCO	µL (range)	-	1 (2500)	1 (500)	1 (100)	1 (20)	1 (4)	1 (1)	1 (0)	1 (2500)	1 (2500)

Assay 9: Stock Jurkat cell lysate (100 µL) was defrosted on ice (-20 °C) for 1 hour, and divided over 10 Eppendorf tubes (9 µL each, -20 °C). Per Eppendorf tube, 1.0 µL Cbz-Phe-Ala-CMK **8** (10x concentration, 1% DMSO) was added to reach the desired concentration, before incubating for 30 minutes at 37 °C. Then, per Eppendorf tube, 1.0 µL Cbz-Tzm-Ala-CMK **6** (33 µM, 1% DMSO) was added to reach 3.0 µM concentration, before incubating for 30 minutes at 37 °C. Then, the protein was precipitated using the acetone precipitation protocol. Then, per Eppendorf tube, 1 µL of AF488-TCO (12x concentration, 10% DMSO) was added to reach the desired concentration, before incubating for 30 minutes at 37 °C. Analysis of the samples was performed via SDS-PAGE analysis using the standard protocol.

Assay 9			Lane Content									
			Ruler	Samples								
Incubation	Content	volume (end [C])	0	1	2	3	4	5	6	7	8	9
-	Jurkat Lysate (1.8 µg/µL)	µL	-	10	10	10	10	10	10	10	10	10
1 (30 min)	Cbz-Phe-Ala-CMK **8**	µL (range µM)	-	1 (100)	1 (0)	1 (0.1)	1 (0.3)	1 (1.0)	1 (3.0)	1 (10.0)	1 (30.0)	1 (100.0)
2 (30 min)	Cbz-Tzm-Ala-CMK **6**	µL (3.0 µM)	-	1	1	1	1	1	1	1	1	1
	Acetone precipitation											
3 (30 min)	AF488-TCO	µL (500 nM)	-	1	1	1	1	1	1	1	1	1

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Chapter 3: Synthesis of amino-functionalized tetrazines and their use in cellular fluorescence microscopy imaging

Introduction

The inverse electron demand Diels-Alder (IEDDA) reaction has received considerable attention in the past decade as a very effective bioorthogonal reaction to connect tetrazines and strained alkenes (**Figure 1**). Thanks to its high efficiency and almost complete selectivity, IEDDA has been applied in many processes. Initial studies utilized a single tetrazine handle connected to numerous well-known fluorophores (for instance, bodipy, coumarin, cyanine, xanthone dyes) providing fluorogenic bioorthogonal reagents for reaction with strained alkenes on antibodies[1], quantum dots[2], lipids[3], cell surface glycans[4-6] and anticancer drugs.[7] Parallel to these studies, alternative tetrazine structures were developed for FRET-based fluorogenic imaging[8-9], as well as TBET-based fluorogenic imaging by conjugation of the tetrazine to the fluorophore itself. [10-14] Downsides to these approaches are the price

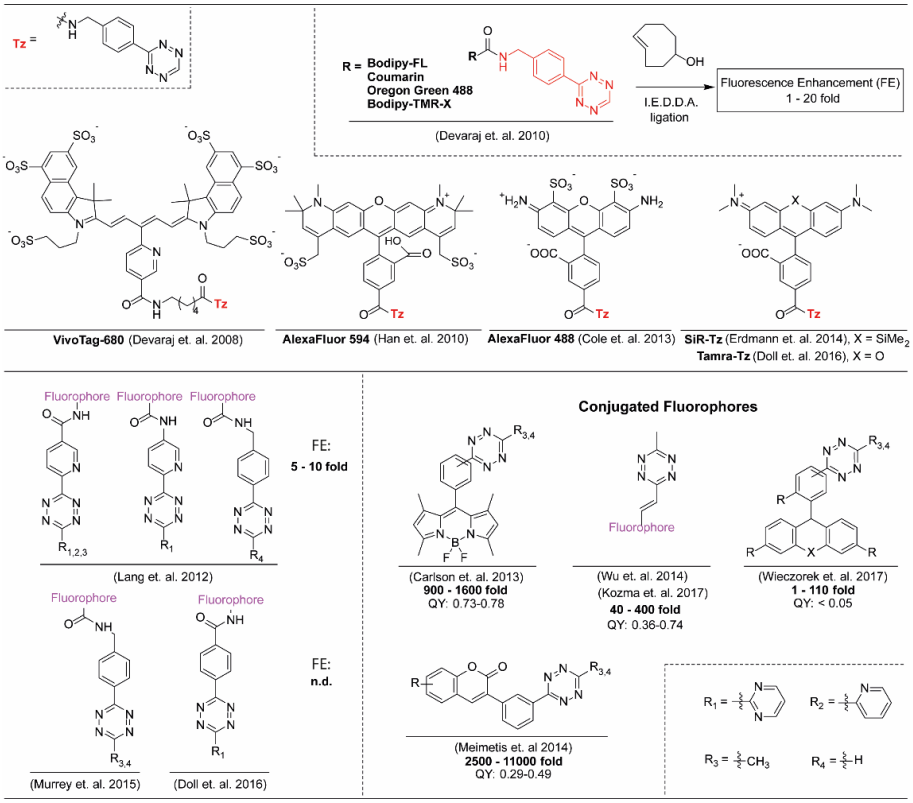


Figure 1: Examples of tetrazine-modified fluorophores described in the literature.

(commercial fluorophores), difficulty of synthesis (conjugated fluorophores), reduced quantum yields (xanthone fluorophores), and poor water solubility (non-sulfated fluorophores). When looking at the tetrazine handles, only a few attempts have been made to optimize their structure for fluorogenicity, quantum yield and water solubility. Many of the widely accessible fluorophores (coumarin, Bodipy-FL, Bodipy-TMR, Cy5) are cheap, easily synthesized and have high quantum yields, and so most gain in IEDDA turn-on fluorescence of these tetrazine-modified fluorophores, when reacting with strained alkenes, appears in optimization of the properties of the tetrazine moieties. In this chapter, a series of tetrazine handles are evaluated on their IEDDA reactivity towards a wide variety of strained alkenes (**Figure 2**).

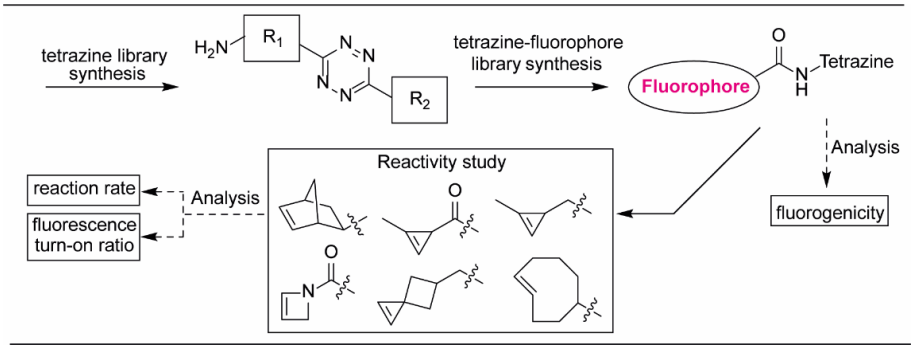
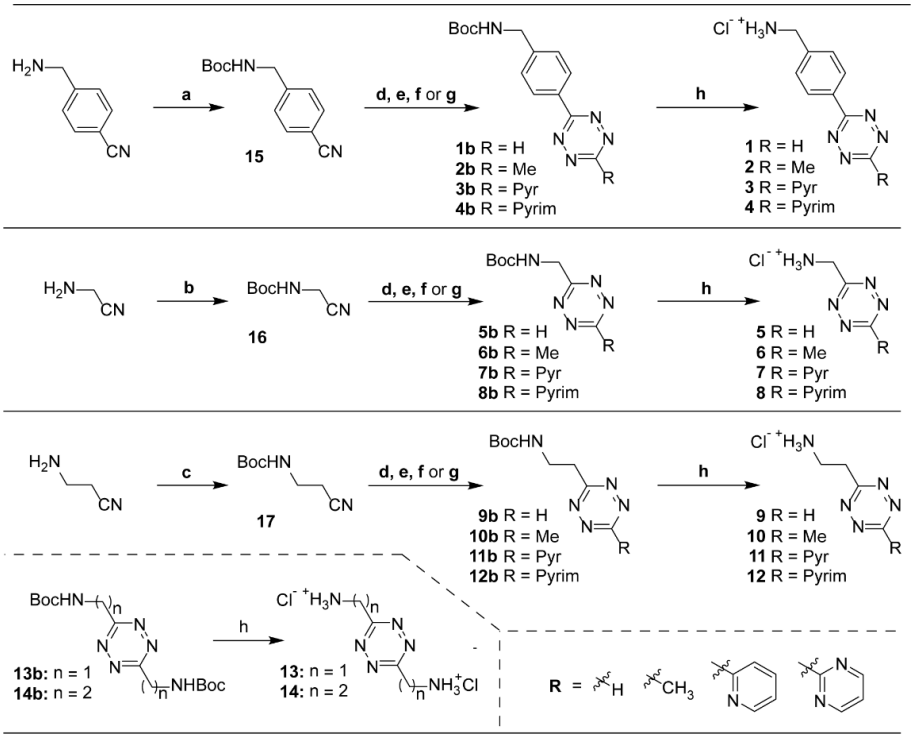


Figure 2: Flowchart regarding the aim of this chapter: Through synthesis of a focused library of amino-alkyl and amino-aryl functionalized tetrazines a tetrazine-fluorophore library can be prepared. These compounds can then be analyzed in their application in IEDDA-mediated conditional fluorescence.

Results & Discussion

As the first step, a library of amino functionalized tetrazines was synthesized (**1**-**14**, **Scheme 1**). Readily accessible N-Boc-protected aminonitriles **15**-**17** were prepared in good (**17**) to quantitative yields (**15**, **16**) by treatment of the corresponding primary amine precursors with di-*tert*-butyl dicarbonate in the presence of an appropriate base. Compounds **15**-**17** were subsequently converted into N-Boc protected aminoalkyl tetrazines **1b-14b** by Lewis acid catalyzed condensation of the nitriles with hydrazine followed by oxidation with sodium nitrite under acidic conditions.[15, 8] Optimization of this two-step synthetic protocol was required to ensure successful synthesis of each tetrazine (**Table 1**). At the condensation stage five parameters were varied (co-solvent, catalyst, reaction vessel, temperature and reaction time) to obtain optimal conditions tailored for each tetrazine. The synthesis of **1b**, **6b**, **9b**, **10b**, and **14b** required dioxane as a cosolvent to ensure efficient formation of the dihydrotetrazine intermediate. Following results in the literature $Zn(OTf)_2$, $Ni(OTf)_2$ and ZnI_2 were studied as catalysts in dihydrotetrazine

formation.[15a] For most reactions $Zn(OTf)_2$ proved the optimal catalyst, however $Ni(OTf)_2$ worked well with in reactions having acetonitrile as the nitrile component (**6b**, **10b**).

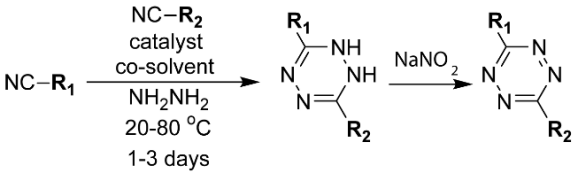


(**a**) Boc_2O, NaOH, H_2O, r.t., o.n. (**b**) Boc_2O, TEA, DCM, reflux, o.n. (**c**) Boc_2O, TEA, DCM, r.t., o.n. (**d**) N_2H_4, $Zn(OTf)_2$, formamidine acetate (**e**) N_2H_4,, $Ni(OTf)_2$, acetonitrile (**f**) N_2H_4,, $Zn(OTf)_2$, 2-cyanopyridine (**g**) N_2H_4,, $Zn(OTf)_2$, 2-cyanopyrimidine (**h**) 4M HCl/Dioxane:DCM (1:1, v:v).

Scheme 1: Synthesis of amino functionalized tetrazine library **1**-**12**.

The syntheses of compounds **2b**-**4b**, **7b**, **8b**, and **11b** were performed in a round-bottom flask under inert atmosphere ("Flask") while the other reactions were executed in a closed pressure resistant test-tube ("Tube"). A closed set up prevents the loss of ammonia that is formed as a side product in the condensation stage and apparently has a beneficial effect on the solubility of components present during this stage. However, consistent improvements of the yields were not observed for all tetrazines when executing the condensation stage in a closed vessel. The reaction temperature and time were adjusted simultaneously, thus the condensations that required lower temperatures (20-30 °C) were left to react 3 days while those at higher temperatures (60-80 °C) were reacted overnight. [15, 8] Oxidation of the formed dihydropyridazine intermediates was facilitated by transferring the reaction mixture

to a 1:1 solution of AcOH and DCM followed by addition of solid $NaNO_2$. [8] For the oxidation of the dihydropyridazine intermediates towards tetrazines **1b**, **3b** and **10b** the reaction mixture was transferred to an aqueous $NaNO_2$ solution followed by addition of an aqueous HCl solution following the procedure described in the



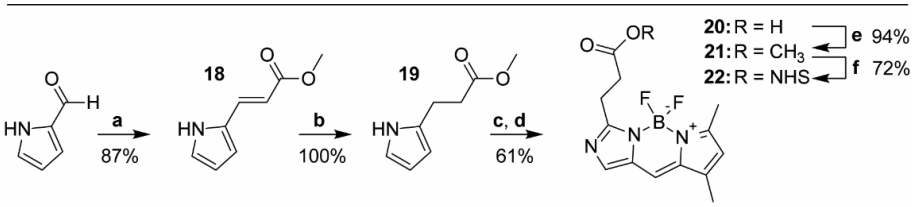
R_1	R_2	co-solvent	catalyst	container	T (°C)	time	yield	Tz
15	Form. Ac.	dioxane	$Zn(OTf)_2$	Tube	60	o.n.	34%	**1b**
16		-	ZnI_2	Tube	30	3 days	14%	**5b**
17		dioxane	$Zn(OTf)_2$	Tube	20	3 days	6%	**9b**
15	Me-CN	dioxane	$Ni(OTf)_2$	Tube	60	o.n.	16%	**2b**
16		-	$Zn(OTf)_2$	Flask	80	o.n.	31%	**6b**
17		dioxane	$Ni(OTf)_2$	Tube	60	o.n.	23%	**10b**
15	**Pyr-CN**	-	$Zn(OTf)_2$	Flask	60	o.n.	13%	**3b**
16		-	$Zn(OTf)_2$	Flask	80	o.n.	53%	**7b**
17		-	$Zn(OTf)_2$	Flask	60	o.n.	49%	**11b**
15	Pyrim-CN	-	$Zn(OTf)_2$	Tube	60	o.n.	27%	**4b**
16		-	$Zn(OTf)_2$	Flask	80	o.n.	7%	**8b**
17		-	$Zn(OTf)_2$	Flask	60	o.n.	16%	**12b**
16	Form. Ac.	-	ZnI_2	Tube	30	3 days	18%	**16b**
17	Pyr-CN	dioxane	$Zn(OTf)_2$	Tube	60	o.n.	20%	**17b**

Table 1: General procedure for the synthesis of N-Boc protected tetrazines **1b-14b**. Variable parameters are used to synthesize tetrazines **1b-14b**, which include co-solvent, catalyst, contained, temperature, and time.

literature to oxidize dihydroterazines of various nature.[15a] N-Boc-protected symmetric aminoalkyl tetrazines **13b** and **14b** were formed as side products in the synthesis of tetrazines **5b** and **11b** respectively. The N-Boc protective group could be readily removed in all cases under anhydrous acidic conditions (4M HCl in dioxane) without decomposition of the tetrazine core yielding the target aminoalkyl tetrazines **1**-**14** in quantitative yields.

Following the synthesis of tetrazine handle library **1-12**, the tetrazines were characterized on their fluorogenicity, as well as their reactivity and fluorescence turn-on ratio when reacted with strained alkenes. As shown in literature, tetrazines have an optimal effect when they are FRET-quenching green fluorescent dyes due to their absorbance around 500-550 nm. [7] For this reason, the well accessible Bodipy-

FL was chosen to be attached to the tetrazine. Following literature procedures, succinimide ester **22** of Bodipy-FL could be synthesized in five steps starting from pyrrole-2-carboxaldehyde (Scheme 2).[16] The 2-formylimidazole starting material was converted by a HWE-reaction to form methylpropenoate **18**, its alkene was then



(**a**) pyrrole-2-carboxaldehyde, methyl (triphenylphosphoranylidene) acetate, DCE, 50 °C, o.n. (**b**) 10% Pd/C, H_2, MeOH, 2 hours. (**c**) 3,5-dimethylpyrrole-2-carboxaldehyde, $POCl_3$, DCM, 0 °C to r.t., 3 hours. (**d**) BF_3*Et_2O, TEA, DCM, 0 °C to r.t., o.n. (**e**) 2.25 M HCl in H_2O:THF, o.n. (**f**) NHS, DIC, DMF, o.n.

Scheme 2: Synthesis of Bodipy-FL NHS ester **22**.

reduced with palladium on carbon to give methylpropanoate **19**. Then, $POCl_3$ mediated condensation of **19** with 1,3-dimethylpyrrole-2-carboxaldehyde, followed by complexation with boron trifluoride diethyl etherate produced methyl ester **20**. Hydrolysis of methyl ester **20** using an acidic aqueous solution formed carboxylic acid **21**, which was finally condensed with N-hydroxy-succinimide yielding target Bodipy-FL succinimide ester **22** on a 1.3-gram scale, with a 36% overall yield. Following this synthesis, tetrazines **1**-**12** were reacted with Bodipy-FL succinimide ester **22** to give tetrazine fluorophores **23**-**34** (**Figure 3**). Aminomethylphenyl tetrazines **1**-**4** could be attached with relative ease to **22** forming Bodipy tetrazines **23**-**26**, however the presence of TEA in the reaction caused degradation of the tetrazines leading to suboptimal yields. During the syntheses of Bodipy-FL tetrazines **27**-**30**, the very poor stability of aminomethyl tetrazines **5-8** towards bases was visible, leading to very low yields. In the case of tetrazine **8**, immediate degradation of the tetrazine (formation of N_2 gas) upon addition of a base (TEA, pyridine, or $NaHCO_3$) was observed making the synthesis of Bodipy-FL tetrazine **30** impossible using this method.

To explain this behavior, a mechanism can be proposed based on the acidity of the benzylic proton next to the conjugated system of the tetrazine. This proton is easily removed in the presence of electron withdrawing groups, and in the case of tetrazine **8** is removed first due to the strong electron withdrawing effect of both the pyrimidyl tetrazine moiety and the cationic ammonium species. Even in neutral aqueous conditions, degradation of these tetrazines were observed within hours. Fortunately though, aminoethyl tetrazines **9**-**12** did not show this liability towards bases in solution, and remained intact up several days when exposed to solutions containing TEA. Apparently, thanks to the longer ethyl group the contribution of the cationic

ammonium species towards the acidity of the benzylic proton is reduced. Therefore, aminoethyl tetrazines **9**-**12** could be used to synthesize Bodipy-FL tetrazines **31**-**34** in acceptable yields. The proposed base-lability also explains the work-around required for tetrazines **1**-**4** and amino functionalized bipyrimidyl tetrazines in literature.[8]

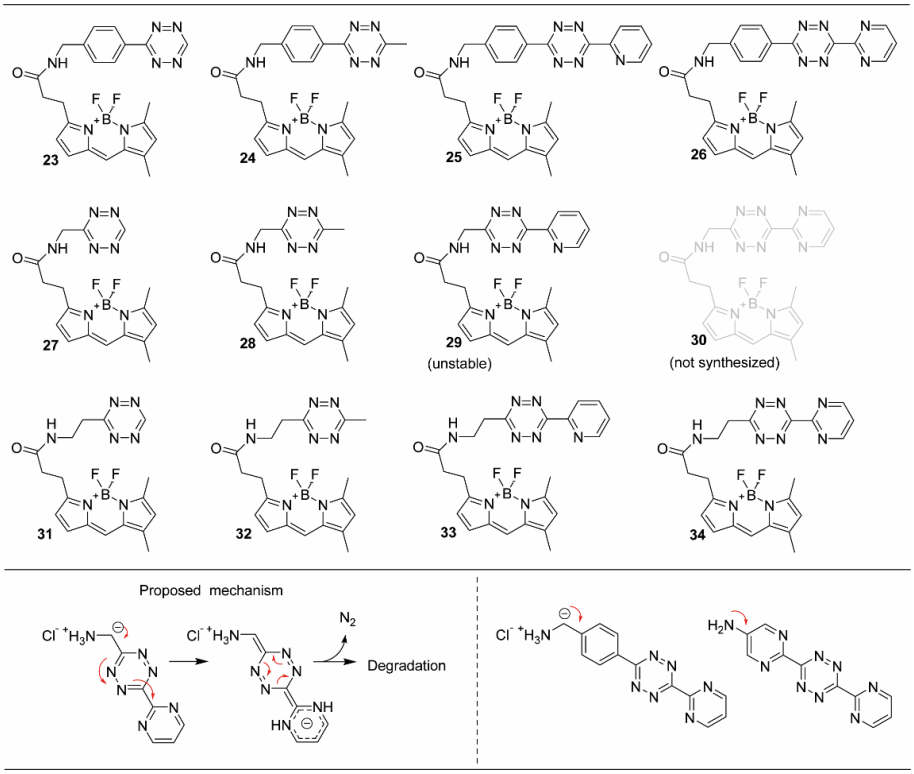
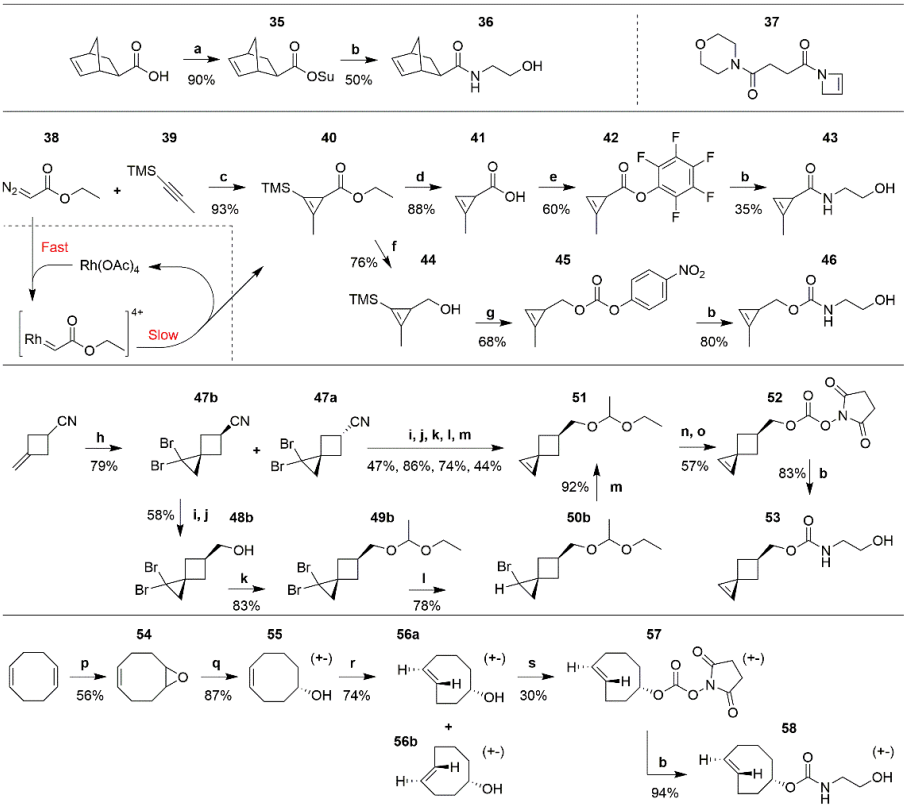


Figure 3: Synthesized library of tetrazine functionalized Bodipy-FL **23-34**.

With Bodipy-FL tetrazine **23**-**34** in hand the last synthetic step comprised creating water soluble alkenes (**Scheme 3**). Adapted from literature procedures, the synthesis of norbornene **36** starting from exo-5-norbornene carboxylic acid in 3 steps[17], acyl-cyclopropene **43** and alkyl-cyclopropene **46** starting from diazoacetate **38** and propyne **39** both in 4 steps[4, 18], spirohexene **53** starting from 3-methylene-1-cyano-cyclobutane in 7 steps[19] (*cis route:* 6%, 18% in literature, *trans route:* 16%, 9% in literature), and transcyclooctene **58** starting from 1-5-cyclooctadiene (via cis/trans photoisomerization) in 5 steps[20] (*photo-isomerization:* 74%, 77% in literature) proved straightforward with yields similar to the followed procedures. A few noteworthy improvements were performed. Rhodium catalyzed synthesis (**Table 2**) of cyclopropenes, of which the general method was likely first described in 1978[21], was first used to prepare cyclopropenes **40** (72%), **41** (75%) and **44** (82%) in 1981.[18]

In more recent literature, in some occurrences the synthesis of cyclopropene **40** was either low yielding[22], or poorly described and documented. [4, 23]. And because the original work by Zefirov and co. workers[18] is only accessible in printed paper, the work was often not read or cited, leading to Thomas and co. workers[24] claiming unprecedented results (71%) and sustainability (5 mol% iPrCuCl catalyst).



(**a**) NHS, DCC, THF, r.t., 3 hours. (**b**) 2-amino ethanol, TEA, DCM, r.t., 2 hours. (**c**) $Rh(OAc)_4$, r.t., o.n. (**d**) KOH, $MeOH/H_2O$, r.t., o.n. (**e**) Pentafluorophenol, DMAP, EDC*HCl, r.t., o.n. (**f**) DiBAl-H, Et_2O/THF, 0 °C, 30 min. (**g**) CsF, 18-crown-6, THF, r.t., 2.5 hours, then paranitrophenol chloroformate, pyridine, DCM, r.t., o.n. (**h**) CTAB, $CHBr_3$, NaOH (aq.), r.t., o.n. (**i**) DiBAl-H, toluene, -78 °C to r.t., 2 hours. (**j**) $NaBH_4$, EtOH, 0 °C, 1 hour. (**k**) vinyl ethyl ether, $TsOH*H_2O$, Et_2O, 0 °C, 1 hour. (l) $(iPrO)_4Ti$, EtMgBr, Et_2O, 30 min, r.t. (**m**) KOtBu, DMSO, 0 °C to r.t., 5 hours. (**n**) 1.5 M HCl in H_2O/THF, 0 °C, 30 min. (**o**) DSC, TEA, ACN, 0 °C to r.t., o.n. (p) mCPBA, $CHCl_3$, 0 °C to r.t., o.n. (**q**) $LiAlH_4$, THF, 0 °C to r.t., o.n. (r) flow irradiation (254 nm), $AgNO_3$ impregnated silica column, methyl benzoate/Et_2O/heptane, 20 hours. (s) DSC, TEA, ACN, r.t., 3 days.

Scheme 3: Synthesis of solubilized alkenes as reactive handles.

The reaction could be optimized by lowering the addition speed of diazoacetate **38** relative to the amount of $Rh(OAc)_4$ catalyst, and so reducing the amount of propyne **39** necessary. Previous procedures required a substantial excess of propyne **39** to prevent the *in situ* formed rhodium carbene intermediate from reacting with remaining **38**. The speed of addition of **38** must not exceed the maximum turnover frequency (TOF) of the catalyst. This can be done by either increasing the amount of catalyst,[25] or, for a more sustainable result, by lowering the addition speed of **38** (this work), resulting in high reaction yields (93% for both). By doing so **38** is immediately consumed by the available catalyst, preventing formation of unwanted byproduct diethyl fumarate, which is formed through reaction of the intermediate rhodium carbene with remaining **38**. The advantage of lowering the addition speed of **38** is to utilize the high turnover number, which reached (TON = 490, 0.19% catalyst) compared to Qian and co-workers (TON = 19, 5.0% catalyst). This allowed a large reduction of catalyst at the cost of a significantly longer reaction time. Finally, it appears that adding DCM as a co-solvent lowers the catalyst's TOF threefold compared to propyne **39** as the sole solvent, without any benefit.

	Reagent **38**	$Rh(OAc)_4$	Propyn **36**					
	mmol	mmol (%)	mmol (eq.)	Solvent	Time	Yield	**TON***	**TOF**
Zefirov et. al. 1981	50	0.156 (0.31)	142 (2.8)	-	5 h	72%	230	46
Pallerla et. al. 2005	4.5	0.09 (2.0)	8.9 (2.0)	DCM	2 h	42%	21	11
Patterson et. al. 2012	no experimental reported					70%		
Elling et. al. 2016	no experimental reported					71%		
Qian et. al. 2019	10	0.50 (5.0)	22 (2.2)	DCM**	2 h	93%	19	9
This work	30	0.11 (0.37)	102 (3.4)	-	7 h	90%	246	35
	58	0.11 (**0.19**)	102 (**1.8**)	-	15 h	**93%**	**490**	**33**

TON = Turnover number (mol/mol catalyst) * If reaction duration = catalyst lifetime
TOF = Turnover frequency (mol/mol catalyst per hour) ** = not properly reported

Table 2: Optimizations from literature and this work for synthesis of compound **40** from **38** and **39**.

During the synthesis of spirohexene **53** (**Scheme 3**) a few issues were encountered that needed to be resolved. Debromination of compound **49** into compound **50** was originally performed with near equimolar EtMgBr (1.1 eq.) over 6 hour and NMR tracking (70% trans, 60% cis), but this procedure proved poorly reproducible (possibly due to reagent quality) and labor intensive. However, using a small excess of EtMgBr (1.6 eq.) full conversion of the material could be achieved within 1 hour at higher yields (*route 1:* 78% trans product, *route 2:* 87% cis product). According to the literature, synthesis of compound **51** required cooling the DMSO solvent to 0 °C, before addition of KOtBu (98% yield). This is likely a documentation error, because when reproducing their work at 0 °C the reaction mixture solidified and was therefore poorly reproducible (44% yield). At room temperature the reaction proceeded without any problems (92% yield). Finally, the two-step synthesis of compound **52** from compound **51** proceeded through a spirohexene alcohol

intermediate that is volatile (<300 mbar), and unstable at -20 °C. This crude intermediate was therefore immediately used after synthesis, resulting in the desired succinimide product at yields comparable to those reported (57% compared to 63% over 2 steps). [19]

With all the reagents in hand, the kinetic properties of the tetrazines when reacted with the different alkenes was determined. At first acyl- and alkyl-cyclopropenes **43** and **46** were analyzed by reacting them with fluorogenic bodipy tetrazine **23** and measuring the fluorescence emergence over time (**Figure 4**). At the used alkene concentrations (400 µM), which are high when compared to concentrations expected in cellular assays, acyl-cyclopropene **43** showed no detectible reactivity (non-determinable fluorescence emergence) with respect to alkyl-cyclopropene **46**, which showed a clearly visible fluorescence increase within the measured timeframe. Norbornene **36** also showed no detectible reactivity (data not shown). As a result, norbornene **36** and acyl-cyclopropene **43** were not used in further analysis.

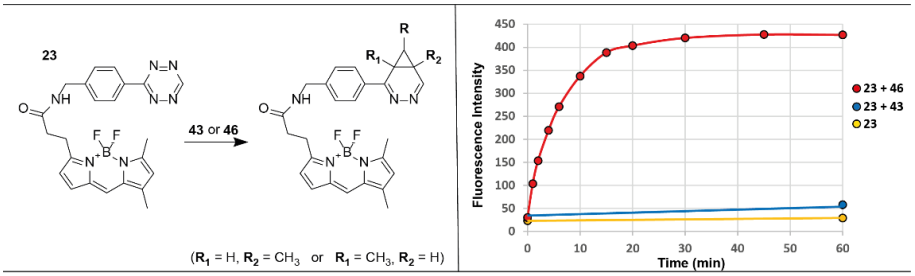


Figure 4: Fluorescence of Bodipy-FL tetrazine **23** (4.0 µM) during reaction with acyl-cyclopropene **43** (400 µM) and alkyl-cyclopropene **46** (400 µM) in phosphate buffer (0.2M, pH 7.4).

Following this initial analysis, fluorogenic bodipy tetrazines **23**-**28** and **31**-**34** were analyzed next by reacting them with either alkyl-cyclopropene **46** (**Figure 5**), aziridine **37** (**Figure 6**), or *trans*-cyclooctene **58** (**Figure 7**) in a phosphate buffered solution. Some interesting results were obtained from this experiment. All bodipy tetrazines appeared to react a bit slower with azetine **37** (k-values between 0.017 – 0.68 $M^{-1} s^{-1}$) compared to alkyl-cyclopropene **46** (k-values between 0.052 – 1.86 $M^{-1} s^{-1}$). As expected, *trans*-cyclooctene **58** (k-values between 0.8 $*10^6$ – 4.0 $*10^6$ $M^{-1} s^{-1}$) reacted extremely fast with the bodipy tetrazines and the concentrations in the solution had to be lowered by 1,000-fold for the fluorophore, and 10,000-fold for the TCO to be able to track the reaction (**Figure 8**, top). Interestingly, when looking at the fluorescence emerging from the reaction of bodipy tetrazines **23**, **25**, **26**, **31**, **33** and **34** with *trans*-cyclooctene **58**, an unclear effect was observed where the fluorescence would be quenched shortly after, possibly due to rearrangement or oxidative effects within the reaction mixture possibly forming an aromatic diazine

moiety with fluorogenic properties (**Figure 8**, bottom). Some reactions even showed an increase of fluorescence after this effect, possibly due to the reaction of this hypothetical diazine moiety with remaining excess TCO in the solution at a slower rate. These effects were not observed for reactions performed with azetine **37**, and slightly observed for some reaction performed with alkylcyclopropene **46**.

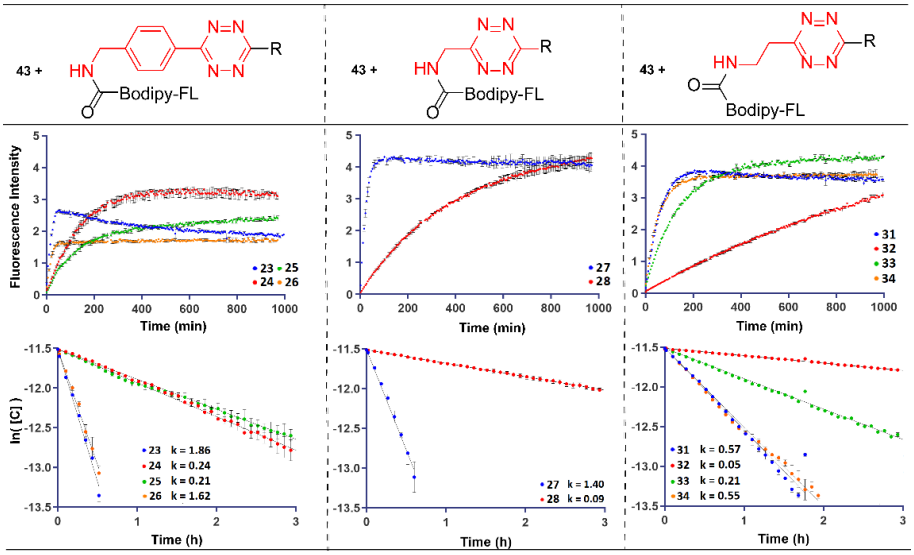


Figure 5: Fluorescence emergence of Bodipy-FL tetrazines **23**-**28** and **31**-**34** (10.0 µM) upon reaction with alkyl-cyclopropene **46** (500 µM) in PBS (pH 7.4). Reaction rate constants were determined by linear approximation of the measurements until the concentration of non-reacted Bodipy-FL tetrazine reached [C] ≤ 1.37 µM (= $e^{-13.5}$ µM).

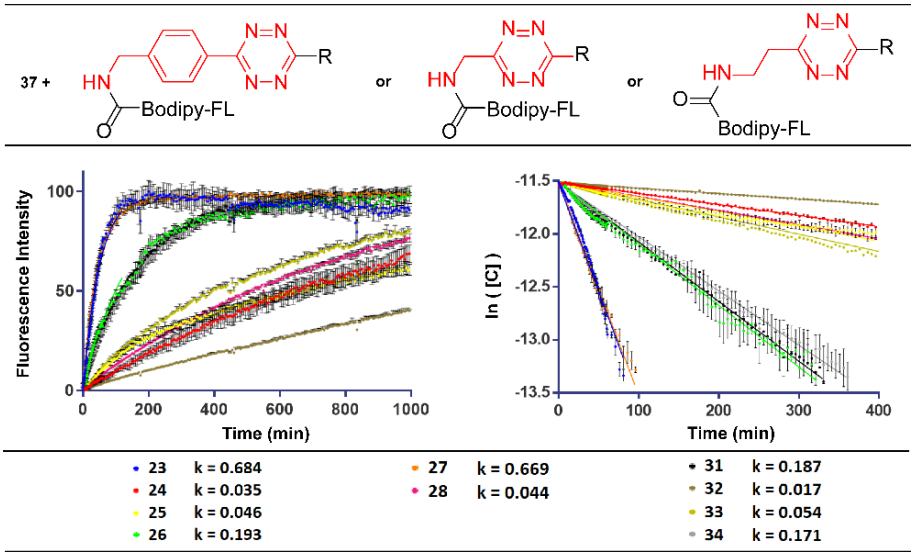


Figure 6: Fluorescence of Bodipy-FL tetrazines **23**-**28** and **31**-**34** (10.0 µM) upon reaction with azetine **37** (500 µM) in PBS (pH 7.4). Reaction rate constants were determined by linear approximation of the measurements until the concentration of non-reacted Bodipy-FL tetrazine reached [C] ≤ 1.37 µM (= $e^{-13.5}$ µM).

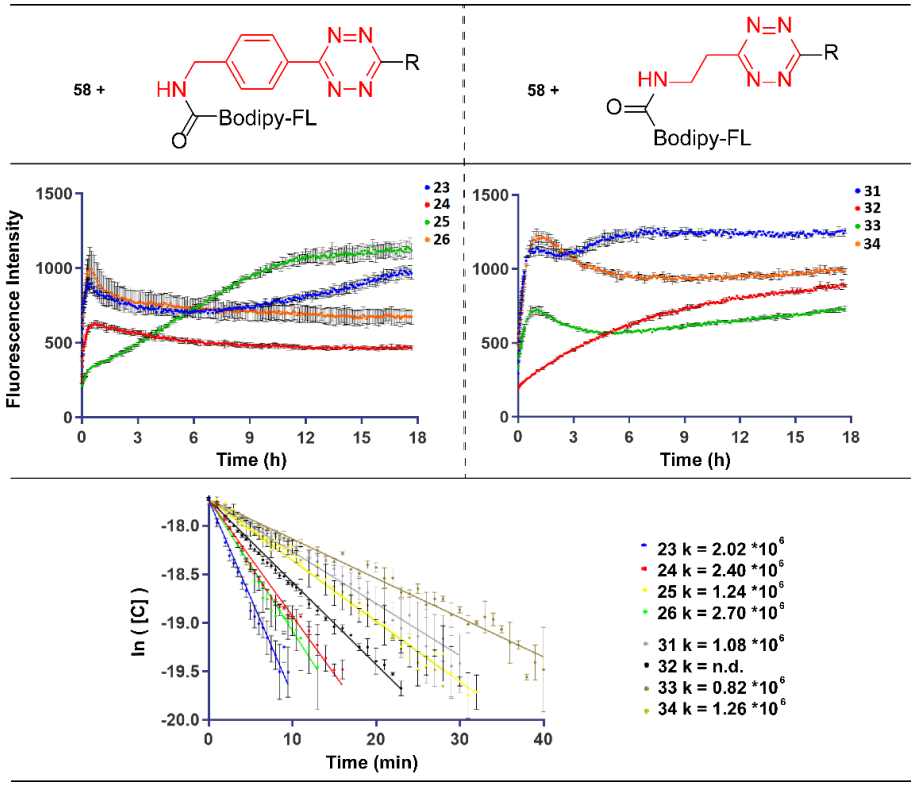


Figure 7: Fluorescence of Bodipy-FL tetrazines **23**-**26** and **31**-**34** (10 nM) upon reaction with transcyclooctene **58** (50 nM) in PBS (pH 7.4). Reaction rate constants were determined by linear approximation of the measurements until the estimated concentration of non-reacted Bodipy-FL tetrazine **24** reached [C] ≤ 2.06 nM (= e^{-20} µM).

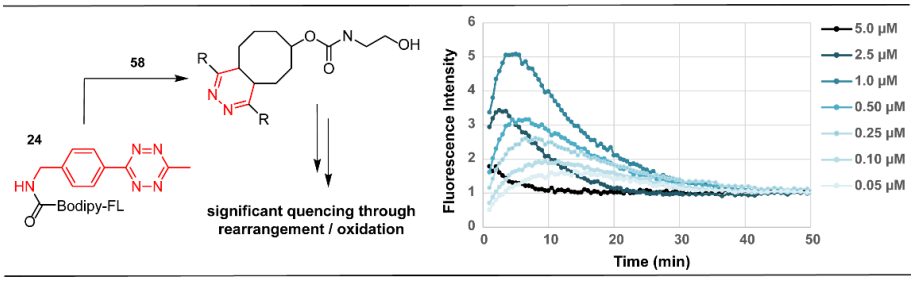


Figure 8: Fluorescence of Bodipy-FL tetrazine **24** (10 nM) upon reaction with transcyclooctene **58** (5.0 – 0.05 µM) in PBS (pH 7.4) to determine the fluorescence increase and decrease over time (normalized at t = 56 minutes).

Having established their propensity to react with strained alkynes and produce IEDDA-dependent fluorescence, selected bodipy tetrazines were evaluated in live-cell imaging of various human cell lines (HELA, U2OS, HEK) in model studies taken from the literature. These model studies comprised 1) the visualization of: alkene modified DOPE phospholipids[3a] after uptake and incorporation in cellular membranes; 2) the visualization of engineered sialylated glycans at the cell surface[5, 26-27] that are the result of metabolic uptake and processing of alkene modified N-acetyl mannosamines (ManNAc); and **3**) imaging the naturally occurring plant lipid sterculic acid (8-(2-octyl-I-cyclopropenyl)octanoic acid)[28], after its internalization by cells (**Figure 9**). For this purpose, strained-alkene-modified variations of the three metabolites (DOPE, ManNAc and sterculic acid) were required. Sterculic acid, known for its potential to inhibit the enzyme stearoyl-CoA desaturase (SCD)[29] located at the endoplasmatic reticulum, is commonly obtained from the *Sterculia foetida* plant seeds. It is commercially available and contains a strained alkene of its own and therefore does not require any modification. DOPE lipids **59**, **60** and **61** were each synthesized as TEA salts in one step by reacting DOPE with **45**, **52** or **57**, followed by silica column chromatography using TEA neutralized silica in combination with 0.25% TEA containing eluent. The use of TEA appeared necessary as the products proved unstable when isolated as a free acid instead of the TEA salt. An attempt was made to prepare an azetine modified DOPE lipid from azetine **37**, however even though the product could be formed, it appeared to be unstable under the purification methods used. N-(acylcyclopropene) mannosamine **63** was synthesized according to literature using a 4-step procedure.[5] An attempt was made to synthesize N-(alkyl-cyclopropene) mannosamine **62** from intermediate **A3** (see experimental), however no reaction occurred. After addition of DMAP migration of the 3'-O-acetyl to the neighboring amine occurred and N-acetyl-3'-O-(alkyl-cyclopropene) mannosamine **A4** (see experimental) was obtained. In the end, N-(alkyl-cyclopropene) mannosamine **62** was synthesized according to the literature procedures involving functionalization of the amine with alkylcyclopropene **52** before acetylation of the hydroxyl groups.[26-27]

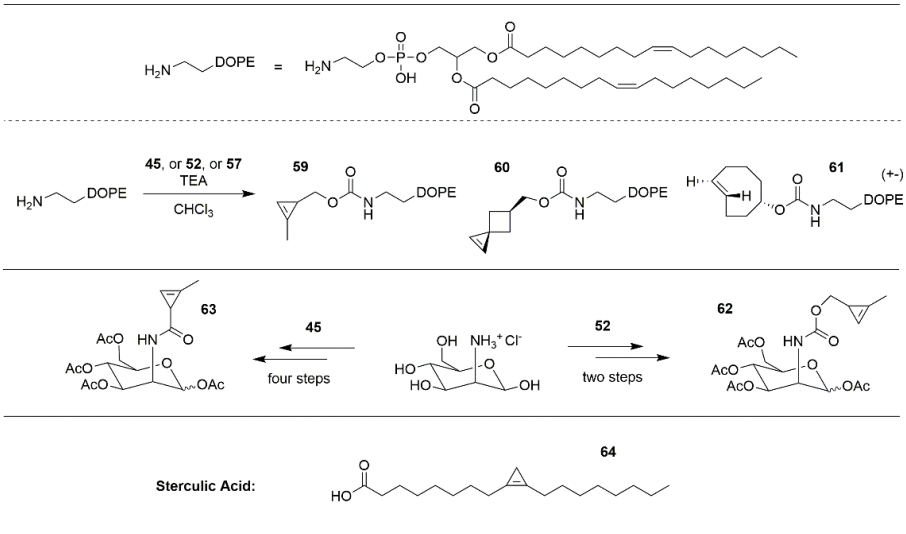


Figure 9: **Top:** Synthesis of DOPE phospholipids **59**, **60**, and **61**, and N-alkene modified mannosamines **62** and **63**. **Bottom**: Naturally occurring sterculic acid **64**.

With lipids **59**-**61**, mannosamine **62**-**63** and sterculic acid **64** in hand, fluorescence live-cell imaging was performed. In the first instance, HeLa and U2OS cells were incubated with lipids **59** (**Figure 10**), **60** and **61** (**Figure 11**) respectively, after which one of each of the bodipy tetrazines **23**-**26** and **31**-**34** were added. Unfortunately, control and sample cells gave minor to no difference in fluorescence, with an increase in signal for the more reactive spirohexene **60** as well as *trans*-cyclooctene **61**. While for bodipy tetrazine **21** a fluorescence image could be produced that proved highly similar to that described in the literature[3a] by tweaking the contrast, the images obtained (**Figure 10**, **11**) show only marginal fluorescence, indicating that the lipids and/or fluorogenic tetrazines are poorly taken up by the cells, or that the bioorthogonal molecules localize in different cellular compartments where they do not meet and react. From both control and sample cells the internalized bodipy tetrazines appeared to be localized around/inside the endoplasmic reticulum, whereas potential subcellular localization of the lipids in different compartments could not be established. Next, and in a similar approach, U2OS cells were incubated with sterculic acid **64** (**Figure 12**), after which one of each of the bodipy tetrazines **23**-**26** and **31**-**34** were added. In steep contrast to the DOPE lipid experiments, sterculic acid showed a very strong fluorescence increase around the endoplasmic reticulum when bodipy tetrazines **23**, **25** or **26** were added, while giving nearly no visible fluorescence increase for bodipy tetrazines **24**, or **31**-**34**. Though no final conclusions can be drawn at this stage, the difference in IEDDA-induced fluorescence may be caused by the difference in substituents on the tetrazine moiety in bodipy tetrazines **23**, **25** and **26**. Due to the poor results of **24** there is no clear indication

that this effect is caused by the additional phenyl ring specifically, and the results from the kinetic analysis on alkyl-cyclopropene **47** indicates that a difference in reactivity is likely not the cause either. Finally, U2OS cells were incubated with mannosamine **62** (**Figure 13**, **14**) for several days, to allow metabolic incorporation into sialylated glycans at the cell surface, after which one of each of the bodipy tetrazines **23**-**26**, and **31**-**34** were added. Similar to the DOPE experiments, cell imaging showed only minor to no difference in fluorescence when comparing the control and sample cells. When fluorescence was observed, this was located internally, instead of at the cell surface. A likely reason for this is the glycans at the cell surface are poorly accessible by hydrophobic molecules as they are surrounded by many other hydrophilic glycans forming a so-called dense hydrophilic coating (glycocalyx) around the cell surface. [30]

In conclusion, this chapter presents a concise and general route of synthesis of a focused library of differently substituted tetrazines and their ligation to Bodipy-FL fluorophores, yielding a set of fluorogenic tetrazines for IEDDA-mediated conditional fluorescence in complex biological samples. Matching results in the literature, the synthesis and purification of the modified tetrazines can be hampered by base lability which in some, but not all, cases can be circumvented by adapting synthesis strategies, purification protocols and if needed the design of the tetrazine. The presented kinetics and fluorescence emergence following IEDDA of these fluorogenic tetrazines with a select group of strained alkenes (for which also synthesis protocols are presented) provide the beginning of the assembly of a panel of bioorthogonal IEDDA reagent pair for selecting the optimal combination depending on the biochemical or cell biological experiment at hand. In a series of three individual cell labeling and imaging experiments, one on DOPE phospholipids, one on cell surface glycan engineering and one on imaging of the naturally-occurring strained alkene-containing sterculic acid, the value of the reagent set is described. As is evidenced, for instance, one cannot discard a single fluorogenic tetrazine for bioorthogonal cell imaging based on one strained alkene-modified metabolite: whereas bodipy tetrazines **23**, **25** and **26** (as indeed all fluorogenic compounds) proved unable to identify strained alkene DOPE molecules inside cells, they proved very effective in localization, in cells, of externally added sterculic acid. More cell imaging experiments are needed to disclose the full potential of the set of reagents here, but results such as they have been obtained so far indicate that tuning tetrazine moieties holds promise for IEDDA-mediated cell imaging and possibly also for other chemical biology studies relying on the timely activating of a (bio)molecule by means of the versatile bioorthogonal reaction pair: strained alkenes and tetrazines.

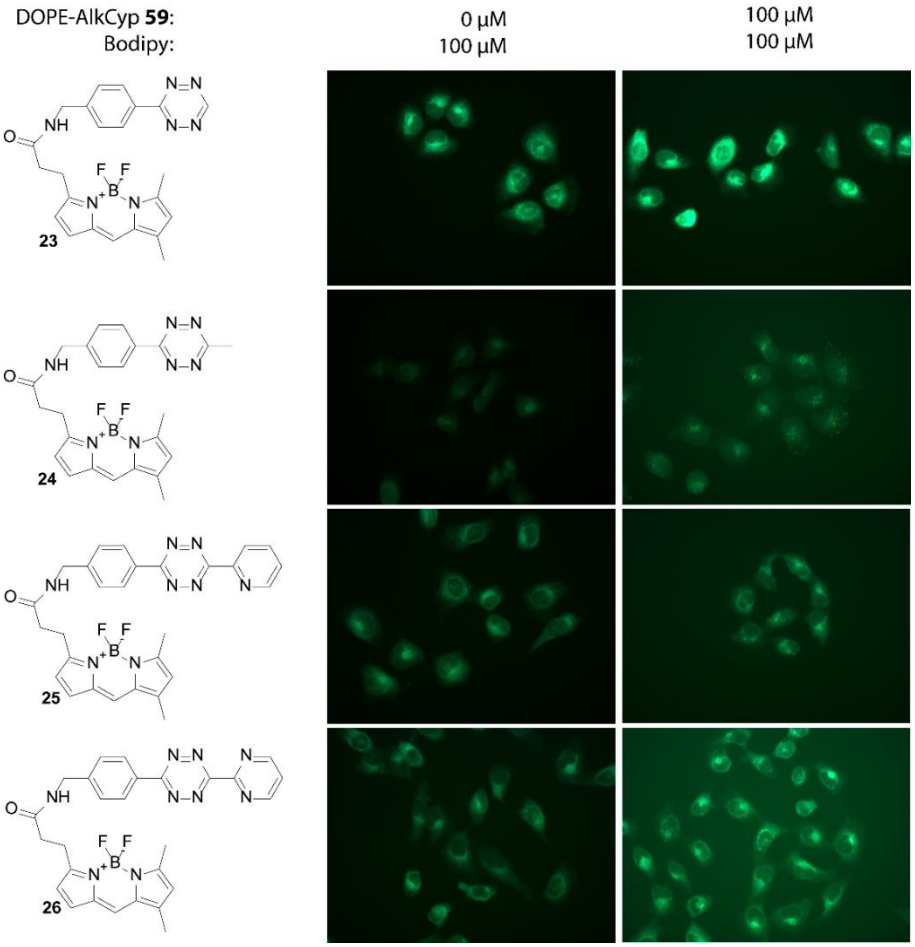


Figure 10: Live-cell fluorescence imaging of U2OS cells after Incubation without (left) and with (right) alkyl-cyclopropene DOPE lipid **59** for 1 hour, followed by incubation with (top to bottom) Bodipy-FL tetrazines **23**, **24**, **25**, or **26** for 1 hour.

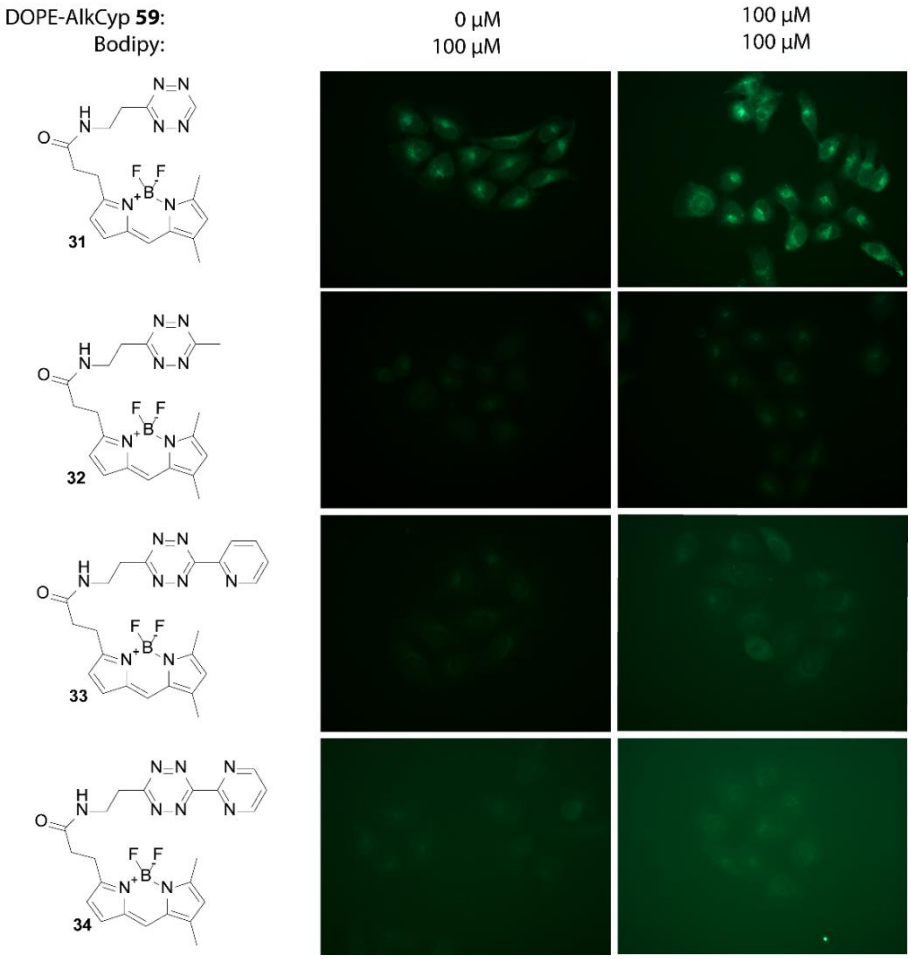


Figure 11: Live-cell fluorescence imaging of U2OS cells after Incubation without (left) and with (right) alkyl-cyclopropene DOPE lipid **59** for 1 hour, followed by incubation with (top to bottom) Bodipy-FL tetrazines **31**, **32**, **33**, or **34** for 1 hour.

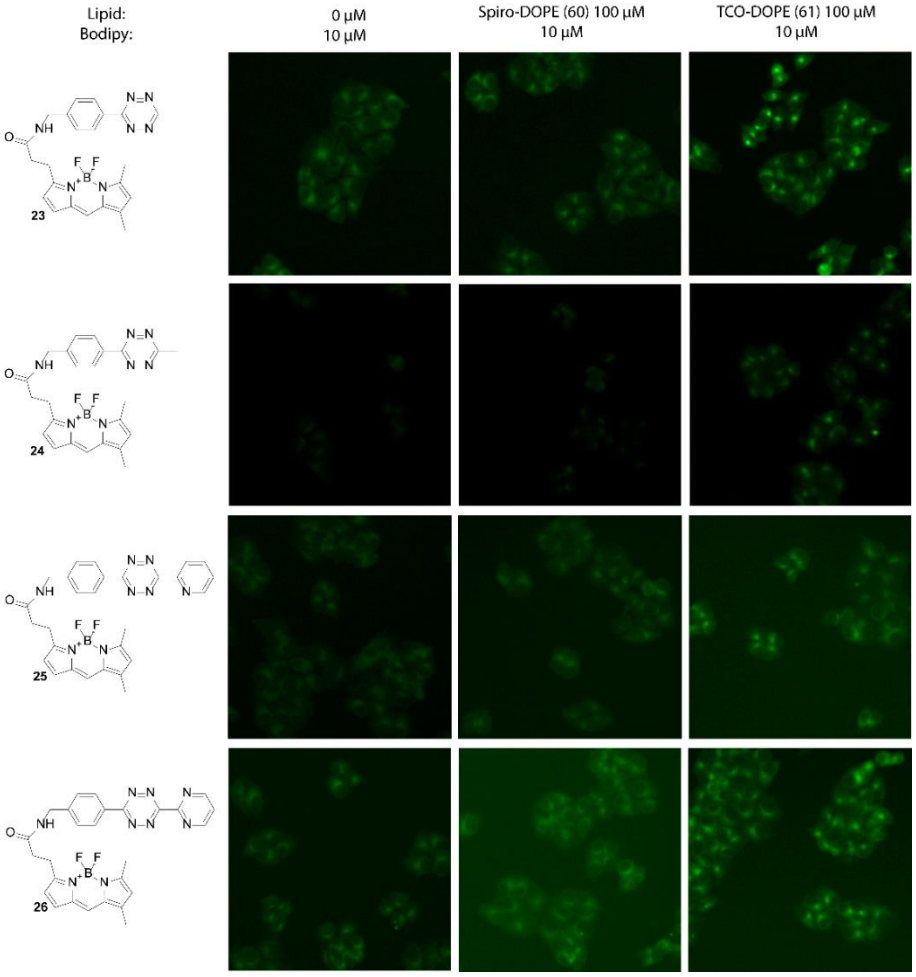


Figure 12: Live-cell fluorescence imaging of HeLa cells after Incubation without (left) lipid, with (middle) spirohexene DOPE lipid **60**, or with (right) *trans*-cyclooctene **61** for 1 hour, followed by incubation with (top to bottom) Bodipy-FL tetrazines **23**, **24**, **25**, or **26** for 1 hour.

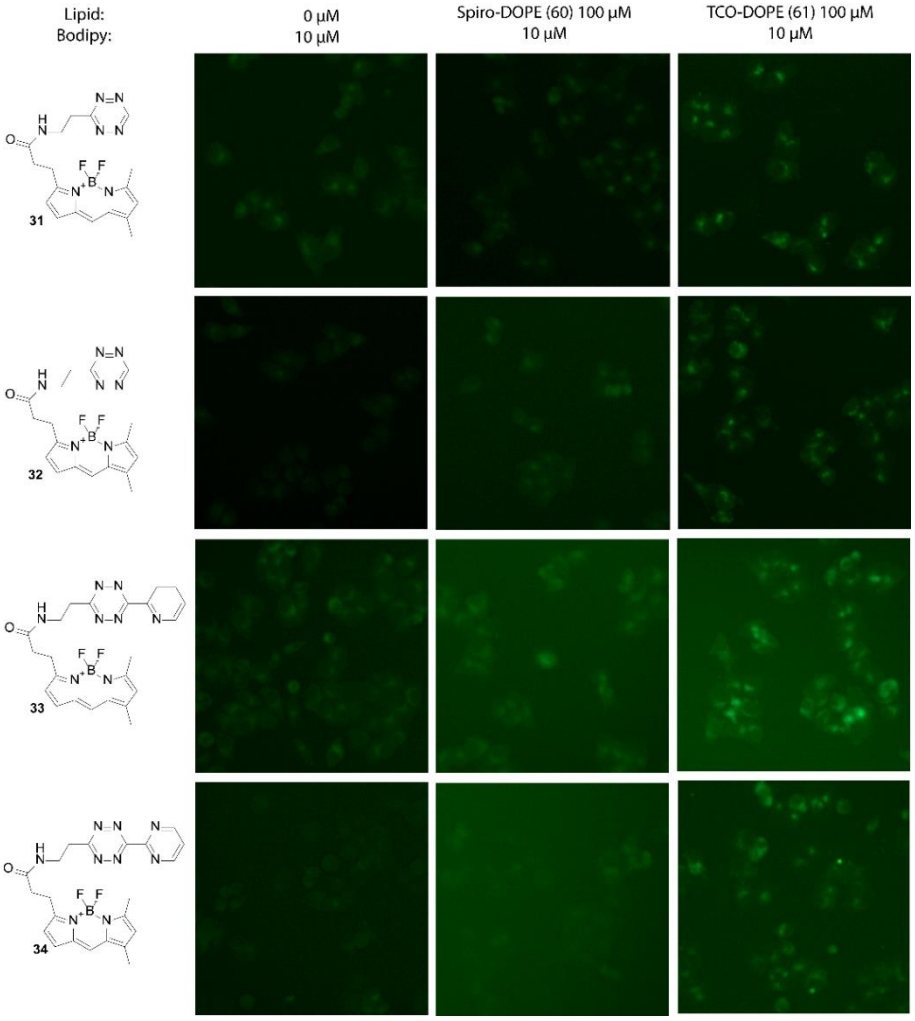


Figure 13: Live-cell fluorescence imaging of HeLa cells after Incubation without (left) lipid, with (middle) spirohexene DOPE lipid **60**, or with (right) *trans*-cyclooctene **61** for 1 hour, followed by incubation with (top to bottom) Bodipy-FL tetrazines **31**, **32**, **33**, or **34** for 1 hour.

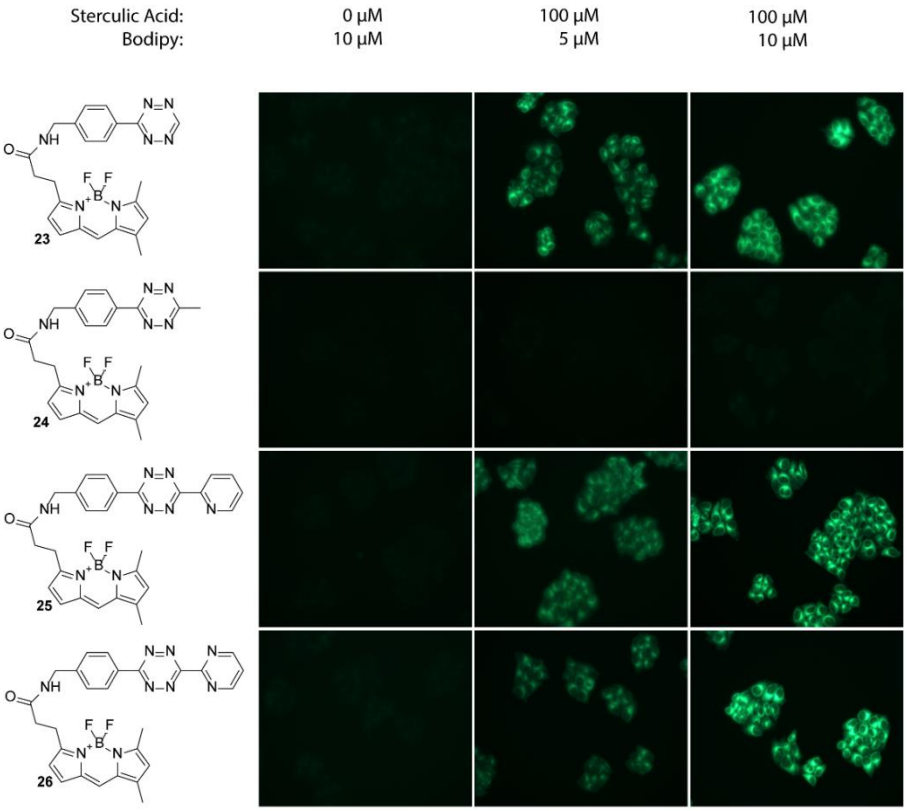


Figure 14: Live-cell fluorescence imaging of U2OS cells after Incubation without (left) lipid, with (middle and right) sterculic acid for 1 hour, followed by incubation with (top to bottom) Bodipy-FL tetrazines **23**, **24**, **25**, or **26** at variable concentrations for 1 hour.

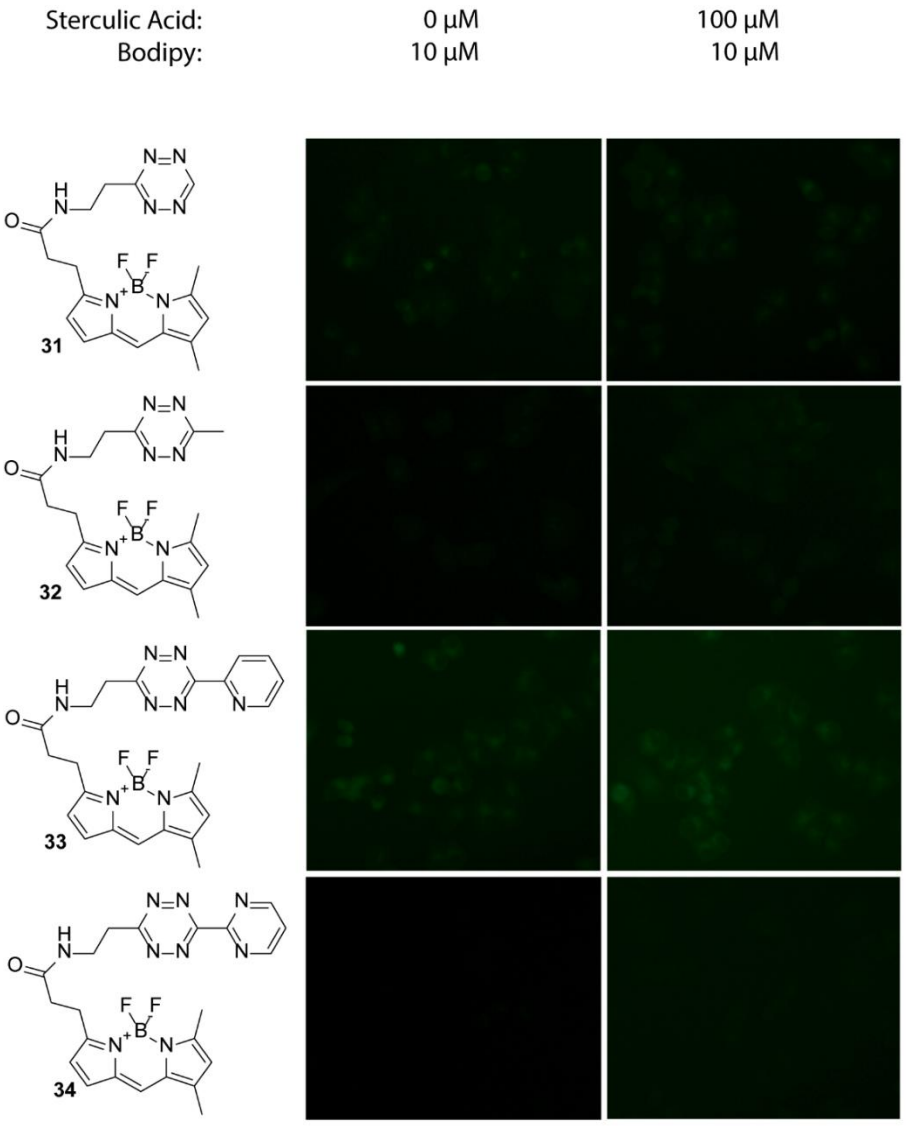


Figure 15: Live-cell fluorescence imaging of U2OS cells after Incubation without (left) lipid, with (right) sterculic acid for 1 hour, followed by incubation with (top to bottom) Bodipy-FL tetrazines **31**, **32**, **33**, or **34** for 1 hour.

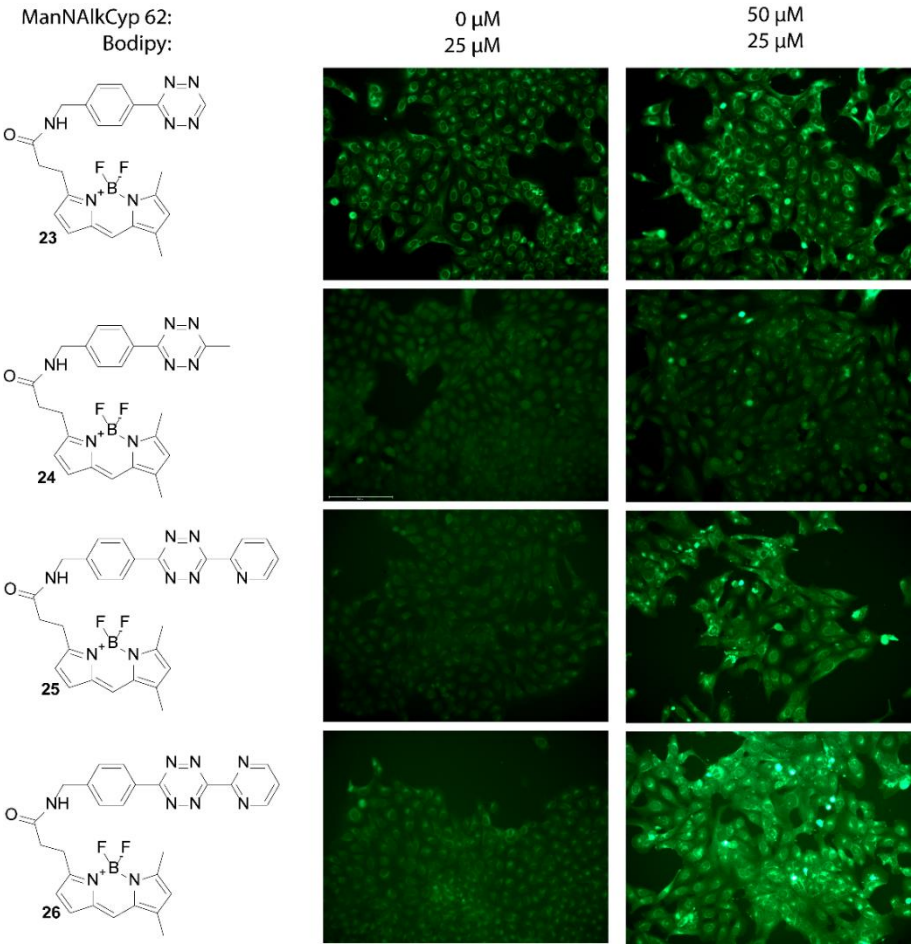


Figure 16: Live-cell fluorescence imaging of U2OS cells after Incubation without (left) lipid, with (right) N-(alkyl-cyclopropene) mannosamine **62** for 3 days, followed by incubation with (top to bottom) Bodipy-FL tetrazines **23**, **24**, **25**, or **26** for 1 hour.

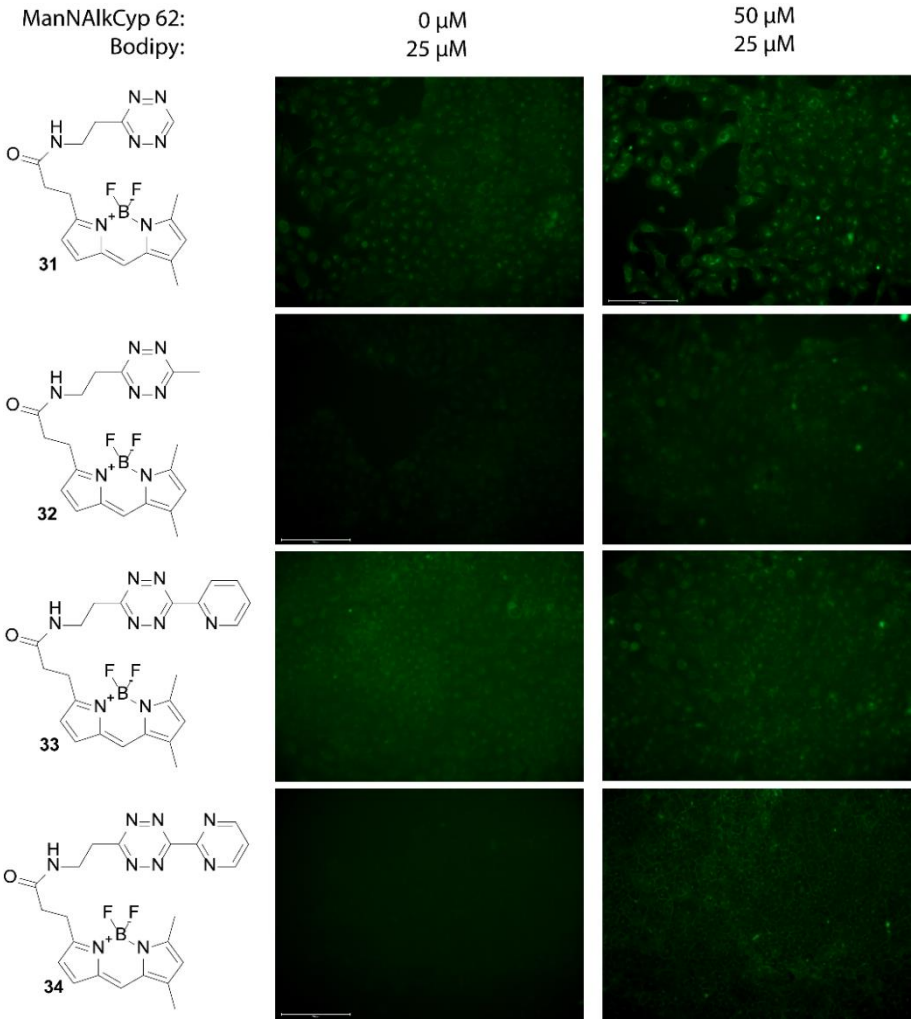


Figure 17: Live-cell fluorescence imaging of U2OS cells after Incubation without (left) lipid, with (right) N-(alkyl-cyclopropene) mannosamine **62** for 3 days, followed by incubation with (top to bottom) Bodipy-FL tetrazines **31**, **32**, **33**, or **34** for 1 hour.

General Procedures

Preparation of anhydrous hydrazine. 6.00 mol (300 mL) of hydrazine monohydrate was added to 3.6 mol (200 g) of KOH pellets. The mixture refluxed for 3 hours (>140 °C) and 5.5 mol (175 mL) hydrazine was collected via distillation (>140 °C). This hydrazine was added to 75 mmol (30 g) of NaOH pellets. The mixture refluxed for 1 hour (135 °C) and 3.7 mol (120 mL) of dry hydrazine was collected via distillation.

Procedure A (**flask**). First 1 eq. of R_1-CN nitrile reagent (**15**, **16** or **17**), 5 eq. of R_2-CN (formamidine acetate, acetonitrile, 2-cyano pyridine or 2-cyano pyrimidine) and 0.25 eq. of catalyst (zinc triflate, zinc iodide, or nickel triflate) were added to a flask under inert nitrogen atmosphere. Then (if used) dry dioxane (1.6 mL/mmol) was added. Anhydrous hydrazine (50 eq., 1.6 mL/mmol) was added dropwise under heavy stirring, while maintaining room temperature in a water bath. After 5 minutes (if required) the temperature was slowly adjusted to the desired value. After the desired time a dihydrotetrazine containing reaction mixture was obtained.

Procedure B (**pressure tube**). First 1 eq. of R_1-CN nitrile reagent (**15**, **16** or **17**), 5 eq. of R_2-CN (formamidine acetate, acetonitrile, 2-cyano pyridine or 2-cyano pyrimidine) and 0.25 eq. of catalyst (zinc triflate, zinc iodide, or nickel triflate) were added to a pressure tube. Then (if used) dry dioxane (1.6 mL/mmol) was added. The tube was sealed and anhydrous hydrazine (50eq., 1.6 mL/mmol) was quickly injected under heavy stirring, while maintaining room temperature in a water bath. After 5 minutes (if required) the temperature was slowly adjusted to the desired value. After the desired time, the reaction was cooled to room temperature and the rubber seal was carefully punctured, slowly releasing the generated NH_3 gas. In this way a dihydrotetrazine containing reaction mixture was obtained.

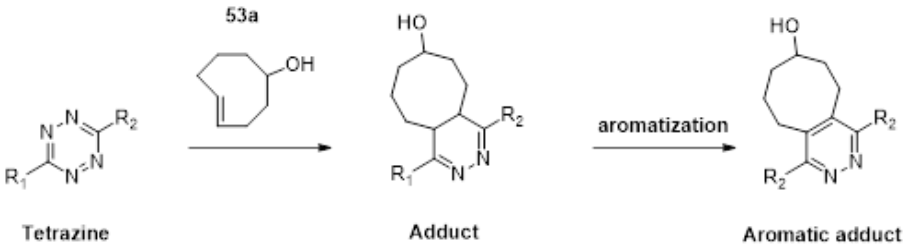
Procedure C (**oxidation**). A mixture of DCM/AcOH (1:1, v:v, 20 mL/mmol) was prepared. While stirring, the dihydrotetrazine containing reaction mixture was added dropwise. Solid $NaNO_2$ (20 eq., 1.5 g/mmol) was added portion wise over 30 minutes. The mixture was concentrated using rotary evaporation, re-dissolved in EtOAc, washed with H_2O ($NaHCO_3$ (aq.) or HCl (aq.)), dried over $MgSO_4$ and concentrated using rotary evaporation. The crude product was purified by column chromatography.

Procedure D (**oxidation**). The dihydrotetrazine containing reaction mixture was dissolved in 4M $NaNO_2$ (aq.) (40 eq., 10 mL/mmol) and 2M HCl (aq.) (60 eq., 30 mL/mmol) was added dropwise under heavy stirring until gas formation stops (pH = 2-3). Then 0.1M HCl (aq.) (50 mL/mmol) was added and the watery solution was extracted multiple times with EtOAc (50 mL/mmol). The organic layers were combined, dried over $MgSO_4$ and concentrated using rotary evaporation. The crude product was purified by column chromatography.

Procedure E (**N-Boc deprotection**). The N-Boc protected tetrazine was dissolved in dry DCM (1 mL/30 mg) and a 4M HCl in dioxane solution (1 mL / 30 mg) was added dropwise over 1 minute while stirring at room temperature. The reaction mixture was stirred at room temperature for 2 hours. The resulting suspension was centrifuged and the colorless supernatant was removed. The colorful precipitate was washed 2 times via re-suspension in 10 mL of dry dioxane, centrifugation, and partitioning from the colorless supernatant. The precipitate was re-suspended in 5 mL of dry dioxane, transferred to a flask and concentrated

using rotary evaporation resulting in quantitative N-Boc deprotected product as an ammonium chloride salt.

HRMS analysis of analytically unstable tetrazines. Tetrazines that could not be identified by HRMS were reacted with 4-OH-TCO at 1mM concentration to form a non-eliminating adduct for HRMS.



Procedure: For each tetrazine 160 µl DMSO was placed in a small vial and 20 µl of 10mM tetrazine solution was added, followed by 20 µl of 10mM TCO **53a**. Near-instantaneous discoloration was observed, where after the vials were stored at -20 °C until HRMS analysis was performed. Due to aromatizing effects of the product tetrazines were identified by **adduct mass** (m/z) or **aromatic adduct mass** (m/z -2).

Kinetics

Fluorometric IEDDA reactivity analysis: The reactivity alkenes **36**, **43** and **46** were analyzed by incubation of 2.4 µL of a 10 mM bodipy-tetrazine **23** in DMSO solution with 6 mL of 0.2M phosphate buffered water (pH = 9.4), to form a 4.0 µM solution. The resulting solution was divided into two quartz cuvettes, 3 mL each, one as a control sample (A), and one as a test sample (B). 2.4 µL of a 500 mM solution of alkene **36**, **43** or **46** was added to test sample (B), and mixed thoroughly. Both control sample (A) and test sample (B) the fluorescence intensity (λ_{ex} = 504 nm at 3 nm width, λ_{em} range = 475-550 nm at 3 nm width, increment = 3 nm, scanning speed = slow, sensitivity = low) was determined on a Shimadsu fluorometer.

Tecan IEDDA reactivity analysis for azetine 37 and alkyl-cyclopropene 46: The reactivity of alkyl-cyclopropene **46** and azetine **37** compared to bodipy tetrazines **23**-**26**, and **31**-**34** was analyzed using the following method: 10 mM bodipy-tetrazine DMSO stocks were warmed to room temperature and used to prepare fresh 20 µM bodipy tetrazine PBS stock solutions (1% DMSO). 500 mM alkyl-cyclopropene **46** or azetine **37** DMSO stock was warmed to room temperature and used to prepare fresh 1.0 mM alkyl-cyclopropene **46** or azetine **37** PBS stock solution (1% DMSO). Using a black Greiner 96-well plate, for each bodipy tetrazine, in a single column, two control wells (A) were filled with 200 µL PBS each, two fluorophore control wells (B) were filled with 100 µL PBS each, and two sample wells (C) were filled with 100 µL of 100 nM alkyl-cyclopropene **46** or azetine **37** PBS stock solution each. Using a multichannel pipet, to every column of 6 samples, 100 µL of PBS (A), or 100 µL of 20 µM bodipy tetrazine PBS stock solution (B, C) was mixed thoroughly. For both control samples (A, B) and test sample (C) the fluorescence intensity (λ_{ex} = 491 nm at 5 nm bandwidth, λ_{em} = 525 nm at 5 nm bandwidth, Gain = 100, Flashes = Mode 1 [400Hz] 50) was determined on a Tecan microplate reader at various kinetic intervals.

Tecan IEDDA reactivity analysis for various concentrations of *trans*-cyclooctene 58: The reactivity of trans-cyclooctene **58** at various concentrations compared to bodipy tetrazine **24** was analyzed using the following method: 10 mM bodipy-tetrazine **24** DMSO stock was warmed to room temperature and used to prepare fresh 20 nM bodipy tetrazine **24** PBS stock solution (1% DMSO). 500 µM TCO **58** DMSO stock was warmed to room temperature and used to prepare fresh 10, 5, 2, 1, 0.5, 0.2, 0.1 µM TCO **58** PBS stock solutions (10% - 0.1% DMSO). Using a black Greiner 96-well plate, for each TCO concentration, in a single column, two control wells (A) were filled with 200 µL PBS each, two fluorophore control wells (B) were filled with 100 µL PBS each, and two sample wells (C) were filled with 100 µL of desired concentration TCO **58** PBS stock solution each. Using a multichannel pipet, to every column of 6 samples, 100 µL of PBS (A), or 100 µL of 20 nM bodipy tetrazine **24** PBS stock solution (B, C) was mixed thoroughly. For both control samples (A, B) and test sample (C) the fluorescence intensity (λ_{ex} = 491 nm at 10 nm bandwidth, λ_{em} = 525 nm at 10 nm bandwidth, Gain = 100, Flashes = Mode 1 [400Hz] 50) was determined on a Tecan microplate reader at various kinetic intervals.

Tecan IEDDA reactivity analysis 3 for *trans*-cyclooctene 58 on bodipy tetrazines: The reactivity trans-cyclooctene **58** compared to bodipy tetrazines **23**-**26**, and **31**-**34** was analyzed using the following method: 10 mM bodipy-tetrazine DMSO stocks were warmed to room

temperature and used to prepare fresh 20 nM bodipy tetrazine PBS stock solutions (1% DMSO). 500 µM TCO **58** DMSO stock was warmed to room temperature and used to prepare fresh 100 nM TCO **58** PBS stock solution (0.02% DMSO). Using a black Greiner 96-well plate, for each bodipy tetrazine, **in a single column**, two control wells (A) were filled with 200 µL PBS each, two fluorophore control wells (B) were filled with 100 µL PBS each, and two sample wells (C) were filled with 100 µL of 100 nM TCO **58** PBS stock solution each. Using a multichannel pipet, to **every row of 5 samples**, 100 µL of PBS (rows A), or 100 µL of 20 nM bodipy tetrazine PBS stock solution (rows B, rows C) was mixed thoroughly. For both control samples (A, B) and test sample (C) the fluorescence intensity (λ_{ex} = 491 nm at 10 nm bandwidth, λ_{em} = 525 nm at 10 nm bandwidth, Gain = 100, Flashes = Mode 1 [400Hz] 50) was determined on a Tecan microplate reader at various kinetic intervals.

Cell Culturing procedures

Full medium preparation: A Dulbecco's Modified Eagle Medium (DMEM) 500 mL bottle was warmed to 37 °C. 50 mL of medium was replaced with 50 mL of Newborn Calf Serum (NCS) (warmed to 37 °C), 1 mL of pennicilin (200 IU/mL) + strepomycin (200 µg/mL) and 5 mL of GlutaMAX^{tm} were added and the bottle was mixed before use. Between uses the medium was stored at 4 °C (up to 1 month), and prior to use re-warmed to 37 °C. For phenylred-free full medium, a 500 mL bottle of phenylred-free DMEM was used during the preparation.

Trypsin solution preparation: To a 50 mL tube, 20 mL of PBS (15mM KH_2PO_4, 15mM Na_2HPO_4, 150mM NaCl, pH 7.4), 25mL of PBS/EDTA (0.4 mg/mL EDTA) and 5 mL of 10X trypsin (2.5 mg/mL) were warmed to 37 °C, added together and mixed thoroughly. Between uses the trypsin solution was stored at 4 °C (up to 1 month), and prior to use re-warmed to 37 °C.

Cell counting: 5 µL of Trypan Blue stain was gently mixed with 5 µL of suspended non-adherent cells. The mixed solution was loaded into a disposable cell counting plate and used to determine the concentration of cells present, as well as the fraction of live cells.

Hela / U2OS cell seeding: Hela / U2OS cells were seeded twice a week, at variable 3-4 day intervals. First, the cell culturing flask was removed from the cell culturing incubator, and the cells were inspected using light-microscopy to spot any abmormalities. The old medium was removed, 2.0 mL of trypsin solution was added and the cells were allowed to detatch from the flasks surface for several minutes. 8.0 mL of full medium was added to the flask and the contents were gently homogenized using a serological pipet. Cells were counted, and 100.000 cells were seeded to a new flask after filling the flask with a calculated amount of full medium to obtain a total of 10 mL. The new flask was then returned to the cell culturing incubator.

Human Bone Osteosarcoma Epithelial Cells (U2OS) fluorescence microscopy using DOPE lipids 59, 60 and 61: U2OS cells were cultured using full medium (phenylred-free) and during seeding a fraction of the homogenized cell solution was diluted and homogenized with full medium (phenylred-free) to 50.000 cells / 3 mL. This solution was then devided onto 6 well plates, (10 cm^2) 3 mL per well, moved to the cell culturing incubator and allowed to adhere and grow overnight. The next day, the old medium was removed, and 1 mL of full medium (phenylred-free) was added to each well. For the control wells 2 µL of DMSO was added and gently homogenized, and for the sample wells 2 µL of 50 mM alkyl-cyclopropene **59**, was added and gently homogenized, resulting in the final concentrations of 0 µM and 100 µM alkyl-cyclopropene **59** for the control and sample wells respectively. Cells were incubated for 1 hour, the medium was removed and the cells were gently washed with pre-warmed PBS. Then, 1 mL of full medium (phenylred-free) was added to each well. For the control wells 1 µL of DMSO was added and gently homogenized, and for each sample well the 1 µL of the 10 mM desired bodipy tetrazine (**23**-**26**, **31**-**34**) was added and gently homogenized, resulting in the final concentrations of 0 µM and 10 µM bodipy tetrazine (**23**-**26**, **31**-**34**) for the control and sample wells respectively. Then, to each well 1.5 µL of 100 µM Hoechst solution was added and gently homogenized, resulting in a final concentration of 150 nM. Cells were incubated for 1 hour, the medium was removed and the cells were gently washed twice with pre-warmed PBS. Then, 1 mL of PBS was added to each well. And the plate was analysed using an EVOS fluorescence microscope at 60x amplification, using DAPI and Trans channel filters to verify

cell healthiness, before imaging using the GFP channel filter. Raw images obtained were adjusted using ImageJ software v1.52n (contrast adjusted from 0-255 to 5-100, 400x400 pixel image selection).

Human Cervical Cancer Cells (HeLa) fluorescence microscopy using spirohexene 60 or *trans*-cyclooctene 61: Hela cells were cultured using full medium (phenylred-free) and during seeding a fraction of the homogenized cell solution was diluted and homogenized with full medium (phenylred-free) to 50.000 cells / 3 mL. This solution was then devided onto 6 well plates, (10 cm^2) 3 mL per well, moved to the cell culturing incubator and allowed to adhere and grow overnight. The next day, the old medium was removed, and 1 mL of full medium (phenylred-free) was added to each well. For the control wells 2 µL of DMSO was added and gently homogenized, and for the sample wells 2 µL of 50 mM spirohexene **60** or *trans*-cyclooctene **61** was added and gently homogenized, resulting in the final concentrations of 0 µM and 100 µM spirohexene **60** or *trans*-cyclooctene **61** for the control and sample wells respectively. Cells were incubated for 1 hour, the medium was removed and the cells were gently washed with pre-warmed PBS. Then, 1 mL of full medium (phenylred-free) was added to each well. For the control wells 1 µL of DMSO was added and gently homogenized, and for each sample well the 1 µL of the 10 mM desired bodipy tetrazine (**23**-**26**, **31**-**34**) was added and gently homogenized, resulting in the final concentrations of 0 µM and 10 µM bodipy tetrazine (**23**-**26**, **31**-**34**) for the control and sample wells respectively. Cells were incubated for 1 hour, the medium was removed and the cells were gently washed twice with pre-warmed PBS. Then, 1 mL of PBS was added to each well. The plate was analysed using an EVOS fluorescence microscope at 60x amplification, Trans channel filters to verify cell location, before imaging using the GFP channel filter. Raw images obtained were adjusted using ImageJ software v1.52n (contrast adjusted from 0-255 to 20-100, 400x400 pixel image selection).

Human Bone Osteosarcoma Epithelial Cells (U2OS) fluorescence microscopy using sterculic acid: U2OS cells were cultured using full medium prepared with phenylred-free DMEM, and during seeding a fraction of the homogenized cell solution was diluted and homogenized with phenylred-free DMEM to 25.000 cells / 1 mL. This solution was then devided onto 8 well rectangular plates, (1 cm^2) 200 µL per well, moved to the cell culturing incubator and allowed to adhere and grow overnight. The next day, the old medium was removed, and 200 µL of full medium (phenylred-free) was added to each control and sample well containing freshly premixed 0 µM or 100 µM sterculic acid (0.5% DMSO) respectively. Cells were incubated for 1 hour, the medium was removed and the cells were gently washed with pre-warmed PBS. Then, 200 µL of full medium (phenylred-free) was added to each control and sample well containing freshly premixed 0 µM, 5 µM or 10 µM bodipy tetrazine (**23**-**26**, **31**-**34**) (1% DMSO) respectively. Cells were incubated for 1 hour, the medium was removed and the cells were gently washed twice with pre-warmed PBS and 200 µL of PBS was added to each well. The plate was analysed using an EVOS fluorescence microscope at 40x amplification, Trans channel filters to verify cell location, before imaging using the GFP channel filter. Raw images obtained were adjusted using ImageJ software v1.52n (contrast adjusted from 0-255 to 20-200 for **31**-**34**, 400x400 pixel image selection).

Human Bone Osteosarcoma Epithelial Cells (U2OS) fluorescence microscopy using mannosamine 62: U2OS cells were cultured using full medium prepared with phenylred-free

DMEM, and during seeding a fraction of the homogenized cell solution was diluted and homogenized with phenylred-free DMEM to 25.000 cells / 1 mL. This solution was then devided onto 8 well rectangular plates, (1 cm^2) 200 µL per well, moved to the cell culturing incubator and allowed to adhere and grow overnight. The next day, the old medium was removed, and 200 µL of full medium (phenylred-free) was added to each control and sample well containing freshly premixed 0 µM or 50 µM mannosamine **62** (0.2% DMSO) respectively. Cells were incubated for 2 days, the medium was removed and the cells were gently washed with pre-warmed PBS. Then, 200 µL of full medium (phenylred-free) was added to each control and sample well containing freshly premixed 0 µM or 10 µM bodipy tetrazine (**23**-**26**, **31**-**34**) (1% DMSO) respectively. Cells were incubated for 1 hour, the medium was removed and the cells were gently washed twice with pre-warmed PBS and 200 µL of PBS was added to each well. The plate was analysed using an EVOS fluorescence microscope at 20x amplification, Trans channel filters to verify cell location, before imaging using the GFP channel filter. Raw images obtained were adjusted using ImageJ software v1.52n (contrast adjusted from 0-255 to 30-150, 400x400 pixel image selection).

Compound synthesis

Compound 1b: Synthesis was performed in a closed pressure tube (**procedure B**) at 60 °C overnight. 1.08 mmol (0.250 g) of compound **15**, 9.91 mmol (1.032 g) of formamidine acetate, 0.534 mmol (0.194 g) $ZnOTf_2$, 1 mL of dioxane and 50 mmol (1,6 mL, 1,6 g) of anhydrous hydrazine were used. Oxidation (**procedure C**) was performed using 80 mL of a DCM/AcOH (1:1, v:v) mixture and 21.0 mmol (1.45 g) solid $NaNO_2$. Purification was performed with silica column chromatography using an 1% to 5% EtOAc in DCM eluent resulting in 0.106 g (0.369 mmol, 34.2%) of compound **1b** as a pink solid. **TLC**: Rf = 0.6, 5% EtOAc in DCM. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 10.15 (s, 1H, Tz, CH), 8.48 (d, *J* = 8.2 Hz, 2H, phenyl, CH), 7.44 (d, *J* = 8.2 Hz, 2H, phenyl, CH), 5.28 (s, 1H, NH), 4.38 (d, *J* = 5.7 Hz, 2H, CH_2), 1.43 (s, 9H, Boc, CH_3). ^{13}C NMR (101 MHz, $CDCl_3$) δ: 166.21, 157.76, 156.04, 144.80, 130.46, 128.49, 128.07, 79.77, 44.31, 28.42. HRMS (m/z): $[C_{14}H_{17}N_5O_2 + Na]^+$ calculated 310.1274, found 310.1284.

Compound 1: N-Boc deprotection of compound **1b** was performed using 4M HCl in dioxane according to **procedure E** obtaining compound **1** in quantitative yield as a pink solid. ^{1}H NMR (400 MHz, DMSO) δ: 10.63 (s, 1H, Tz, CH), 8.75 (s, 3H, NH_3Cl), 8.51 (d, *J* = 8.2 Hz, 2H, phenyl, CH), 7.81 (d, *J* = 8.2 Hz, 2H, phenyl, CH), 4.16 (d, *J* = 4.2 Hz, 2H, CH_2). ^{13}C NMR (101 MHz, DMSO) δ: 165.27, 158.22, 138.95, 131.82, 129.93, 127.86, 41.79. HRMS (m/z): $[C_9H_9N_5 + H]^+$ calculated 188.0931, found 188.0937.

Compound 2b: Synthesis was performed in a flask (**procedure A**) under nitrogen atmosphere at 80 °C overnight. 0.987 mmol (0.229 g) of compound **15**, 4.8 mmol (0.25 mL, 0.20 g) of acetonitrile, 0.23 mmol (0.084 g) $ZnOTf_2$ and 50 mmol (1.6 mL, 1.6 g) of anhydrous hydrazine. Oxidation (**procedure C**) was performed using 20 mL of DCM/AcOH (1:1, v:v) and 12 mmol (0.8 g) solid $NaNO_2$. Purification was performed with silica column chromatography using an 2% to 4% EtOAc in DCM eluent resulting in 0.091 g (0.30 mmol, 30.6%) of compound **2b** as a pink solid. **TLC**: Rf = 0.5, 5% EtOAc in DCM. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.48 (d, *J* = 8.2 Hz, 2H, phenyl, CH), 7.45 (d, *J* = 8.2 Hz, 2H, phenyl, CH), 5.19 (s, 1H, NH), 4.39 (d, *J* = 5.6 Hz, 2H, CH_2), 3.05 (s, 3H, Tz, CH_3), 1.45 (s, 9H, Boc, CH_3). ^{13}C NMR (101 MHz, $CDCl_3$) δ: 167.23, 163.90, 156.06, 144.09, 130.73, 128.17, 128.06, 79.79, 44.38, 28.46, 21.20. HRMS (m/z): $[C_{15}H_{19}N_5O_2 + Na]^+$ calculated 324.1431, found 324.1440.

Compound 2: N-Boc deprotection of compound **2b** was performed using 4M HCl in dioxane according to **procedure E** obtaining compound **2** in quantitative yield as a purple solid. ^{1}H NMR (400 MHz, DMSO) δ: 8.68 (s, 3H, NH_3Cl), 8.49 (d, *J* = 8.3 Hz, 2H, phenyl, CH), 7.79 (d, *J* = 8.4 Hz, 2H, phenyl, CH), 4.16 (d, *J* = 5.6 Hz, 2H, CH_2), 3.41 (s, HOD), 3.01 (s, 3H, Tz, CH_3), 2.50 (m, DMSO). ^{13}C NMR (101 MHz, DMSO) δ: 167.28, 163.04, 138.50, 131.87, 129.91, 127.53, 41.82, 40.15, 39.94, 39.73, 39.52, 39.31, 39.10, 38.89 (DMSO-d6), 20.91. HRMS (m/z): $[C_{10}H_{11}N_5 + H]^+$ calculated 202.1087, found 202.1090.

Compound 3b: Synthesis was performed in a flask (**procedure A**) under nitrogen atmosphere at 80 °C overnight. 1.03 mmol (0.238 g) of compound **15**, 5.2 mmol (0.50 mL, 5.4 g) of 2-pyridinecarbonitrile, 0.24 mmol (0.086 g) $ZnOTf_2$ and 50 mmol (1.6 mL, 1.6 g) of anhydrous hydrazine were used. Oxidation (**procedure C**) was performed using 20 mL of DCM/AcOH (1:1, v:v) and 22 mmol (1.5 g) solid $NaNO_2$. Purification was performed with silica column chromatography using an 2% to 30% EtOAc in DCM eluent resulting in 0.200 g (0.549 mmol, 53.3%) of compound **3b** as a purple solid and 3,6-di-2-pyridyl-1,2,4,5-tetrazine as a purple

solid byproduct. **TLC**: Compound **3b** Rf = 0.7, 3,6-di-2-pyridyl-1,2,4,5-tetrazine Rf = 0.2, 100% EtOAc. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.92 (d, *J* = 4.5 Hz, 1H, pyr, CH), 8.63 (d, *J* = 7.9 Hz, 1H, pyr, CH), 8.58 (d, *J* = 8.2 Hz, 2H, phenyl, CH), 7.95 (ddd, *J* = 7.8, 7.8, 1.6 Hz, 1H, pyr, CH), 7.52 (dd, *J* = 7.5, 4.8 Hz, 1H, pyr, CH), 7.47 (d, *J* = 8.2 Hz, 2H, phenyl, CH), 5.25 (s, 1H, NH), 4.41 (d, *J* = 5.6 Hz, 2H, CH_2), 1.44 (s, 9H, Boc, CH_3). ^{13}C NMR (101 MHz, $CDCl_3$) δ: 164.17, 163.38, 156.08, 150.95, 150.28, 144.73, 137.51, 130.44, 128.67, 128.12, 126.38, 123.93, 79.80, 44.40, 28.46. HRMS (m/z): $[C_{19}H_{20}N_6O_2 + H]^+$ calculated 365.1721, found 365.1725.

Compound 3: N-Boc deprotection of compound **3b** was performed using 4M HCl in dioxane according to **procedure E** obtaining compound **3** in quantitative yield as a pink solid. ^{1}H NMR (400 MHz, DMSO) δ: 8.94 (d, *J* = 4.1 Hz, 1H, pyr, CH), 8.78 (s, 3H, NH_3Cl), 8.62 (d, *J* = 7.9 Hz, 1H, pyr, CH), 8.58 (d, *J* = 8.3 Hz, 2H, phenyl, CH), 8.20 (ddd, *J* = 7.8, 7.8, 1.7 Hz, 1H, pyr, CH), 7.84 (d, *J* = 8.4 Hz, 2H, phenyl, CH), 7.77 (ddd, *J* = 7.6, 4.8, 1.0 Hz, 1H, pyr, CH), 4.18 (d, *J* = 5.7 Hz, 2H, CH_2). ^{13}C NMR (101 MHz, DMSO) δ: 163.33, 163.01, 150.28, 149.75, 139.08, 138.51, 131.63, 130.04, 128.03, 126.86, 124.23, 41.85. HRMS (m/z): $[C_{14}H_{12}N_6 + H]^+$ calculated 265.1196, found 265.1208.

Compound 4b: Synthesis was performed in a flask (**procedure A**) under nitrogen atmosphere at 80 °C overnight. 0.991 mmol (0.230 g) of compound **15**, 4.85 mmol (0.510 g) of 2-pyrimidinecarbonitrile, 0.26 mmol (0.095 g) $ZnOTf_2$ and 50 mmol (1.6 mL, 1.6 g) of anhydrous hydrazine were used. Oxidation (**procedure C**) was performed using 20 mL of DCM/AcOH (1:1, v:v) and 22 mmol (1.5 g) solid $NaNO_2$. Purification was performed with silica column chromatography using an 2% to 30% EtOAc in DCM eluent resulting in 0.026 g (0.071 mmol, 7.2%) of compound **4b** as a pink solid. **TLC**: Rf = 0.4, 100% EtOAc. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 9.12 (d, *J* = 4.4 Hz, 2H, pyrim, CH), 8.67 (d, *J* = 8.0 Hz, 2H, phenyl, CH), 7.58 (t, *J* = 4.3 Hz, 1H, pyrim, CH), 7.52 (d, *J* = 7.7 Hz, 2H, phenyl, CH), 5.11 (s, 1H, NH), 4.45 (d, *J* = 4.5 Hz, 2H, CH_2), 1.47 (s, 9H, Boc, CH_3). ^{13}C NMR (101 MHz, $CDCl_3$) δ: 164.43, 163.15, 159.65, 158.51, 156.08, 145.15, 130.32, 129.20, 128.25, 122.59, 79.96, 44.49, 28.51. HRMS (m/z): $[C_{18}H_{19}N_7O_2 + H]^+$ calculated 366.1673, found 366.1678.

Compound 4: N-Boc deprotection of compound **4b** was performed using 4M HCl in dioxane according to **procedure E** obtaining compound **4** in quantitative yield as a red solid. ^{1}H NMR (400 MHz, DMSO) δ: 9.20 (d, *J* = 4.9 Hz, 2H, pyrim, CH), 8.77 – 8.46 (m, 5H, phenyl, CH | NH_3Cl), 7.94 – 7.75 (m, 3H, phenyl, CH | pyrim, CH), 4.20 (q, *J* = 5.8 Hz, 2H, CH_2). ^{13}C NMR (101 MHz, DMSO) δ: 163.34, 163.00, 159.11, 158.56, 139.18, 131.56, 129.98, 128.34, 123.04, 41.88. HRMS (m/z): $[C_{13}H_{11}N_7 + H]^+$ calculated 266.1149, found 266.1160.

Compound 5b: Synthesis was performed in a closed pressure tube (**procedure B**) at 30 °C for 3 days. 1.02 mmol (0.160 g) of compound **16**, 5.09 mmol (0.530 g) of formamidine acetate, 0.31 mmol (0.099 g) ZnI_2 and 50 mmol (1.6 mL, 1.6 g) of anhydrous hydrazine were used. Oxidation (**procedure C**) was performed using 20 mL of DCM/AcOH (1:1, v:v) and 22 mmol (1.5 g) solid $NaNO_2$. Purification was performed with silica column chromatography using an 3% to 10% EtOAc in DCM eluent resulting in 0.030 g (0.14 mmol, 13.9%) of compound **5b** as a pink solid and 0.032 g (0.094 mmol, 18.4%) of compound **16b** as a pink solid byproduct. **TLC**: Compound **5b** Rf = 0.6, compound **16b** Rf = 0.3, 20% EtOAc in DCM. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 10.27 (s, 1H, Tz, CH), 5.58 (s, 1H, NH), 5.01 (d, *J* = 5.0 Hz, 2H, CH_2), 1.45 (s, 9H, Boc, CH_3). ^{13}C NMR (126 MHz, $CDCl_3$) δ: 169.54, 158.83, 155.90, 80.66, 43.92, 28.43, 0.13. HRMS (m/z): aromatic adduct $[C_{17}H_{27}N_3O_3 + H]^+$ calculated 308.1969, found 308.1972.

Compound 5: N-Boc deprotection of compound **5b** was performed using 4M HCl in dioxane according to **procedure E** obtaining compound **5** in quantitative yield as an orange solid. ^{1}H NMR (400 MHz, DMSO) δ: 10.76 (s, 1H, Tz, H), 9.09 (s, 3H, NH_3Cl), 4.75 (s, 2H, CH_2). ^{13}C NMR (101 MHz, DMSO) δ: 166.37, 159.12, 41.25. HRMS (m/z): aromatic adduct $[C_{11}H_{18}N_3O_1 + H]^+$ calculated 208.1444, found 208.1450.

Compound 6b: Synthesis was performed in a closed pressure tube (**procedure B**) at 60 °C overnight. 1.03 mmol (0.161 g) of compound **16**, 4.8 mmol (0.25 mL, 0.20 g) of acetonitrile, 0.26 mmol (0.092 g) $NiOTf_2$, 1.5 mL of dioxane and 47 mmol (1.5 mL, 1.5 g) of anhydrous hydrazine were used. Oxidation (**procedure D**) was performed using 40 mmol (10 mL) of 4M $NaNO_2$ (aq.) and 60 mmol (30 mL) 2M HCl (aq.). Purification was performed with silica column chromatography using an 10% to 20% EtOAc in pentane eluent resulting in 0.053 g (0.24 mmol, 22.7%) of compound **6b** as a bright pink solid. **TLC**: Rf = 0.25, 25% EtOAc in pentane. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 5.67 (s, 1H, NH), 4.92 (d, *J* = 5.6 Hz, 2H, CH_2), 3.04 (s, 3H, Tz, CH_3), 1.42 (s, 9H, Boc, CH_3). ^{13}C NMR (101 MHz, $CDCl_3$) δ: 168.42, 166.54, 155.91, 80.38, 43.47, 28.37, 21.24. HRMS (m/z): $[C_9H_{15}N_5O_2 + Na]^+$ calculated 248.1118, found 248.1123.

Compound 6: N-Boc deprotection of compound **6b** was performed using 4M HCl in dioxane according to **procedure E** obtaining compound **6** in quantitative yield as a pink solid. ^{1}H NMR (400 MHz, DMSO) δ: 9.05 (s, 3H, NH_3Cl), 4.70 (s, 2H, CH_2), 3.03 (s, 3H, Tz, CH_3). ^{13}C NMR (101 MHz, DMSO) δ: 168.48, 163.64, 40.85, 20.96. HRMS (m/z): $[C_4H_7N_5 + H]^+$ calculated 126.0775, found 126.0775.

Compound 7b: Synthesis was performed in a flask (**procedure A**) under nitrogen atmosphere at 60 °C overnight. 0.986 mmol (0.154 g) of compound **16**, 7.3 mmol (0.70 mL, 7.7 g) of 2-pyridinecarbonitrile, 0.26 mmol (0.094 g) $ZnOTf_2$ and 47 mmol (1.5 mL, 1.5 g) of anhydrous hydrazine were used. Oxidation (**procedure C**) was performed using 20 mL of DCM/AcOH (1:1, v:v) and 22 mmol (1.5 g) solid $NaNO_2$. Purification was performed with silica column chromatography using an 10% to 50% EtOAc in pentane eluent resulting in 0.139 g (0.482 mmol, 48.9%) of compound **7b** as a red solid and 0.288 g (1.22 mmol) of compound **19** as a red solid byproduct. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.94 (d, *J* = 4.0 Hz, 1H, pyr, CH), 8.63 (d, *J* = 7.8 Hz, 1H, pyr, CH), 7.97 (t, *J* = 7.4 Hz, 1H, pyr, CH), 7.55 (dd, *J* = 6.9, 5.0 Hz, 1H, pyr, CH), 5.76 (s, 1H, NH), 5.05 (d, *J* = 5.2 Hz, 2H, CH_2), 1.44 (s, 9H, Boc, CH_3). ^{13}C NMR (101 MHz, $CDCl_3$) δ: 167.44, 164.46, 151.04, 150.08, 137.63, 126.66, 124.27, 80.51, 43.74, 28.43. HRMS (m/z): $[C_{13}H_{16}N_6O_2 + H]^+$ calculated 289.1408, found 289.1418.

Compound 7: N-Boc deprotection of compound **7b** was performed using 4M HCl in dioxane according to **procedure E** obtaining compound **7** in quantitative yield as an orange solid. ^{1}H NMR (400 MHz, DMSO) δ: 9.14 (s, 3H, NH_3Cl), 8.94 (d, *J* = 4.2 Hz, 1H, pyr, CH), 8.59 (d, *J* = 7.9 Hz, 1H, pyr, CH), 8.18 (ddd, *J* = 7.8, 7.8, 1.6 Hz, 1H, pyr, CH), 7.76 (dd, *J* = 6.8, 4.8 Hz, 1H, pyr, CH), 4.81 (q, *J* = 5.6 Hz, 2H). ^{13}C NMR (101 MHz, DMSO) δ: 164.36, 164.10, 150.69, 149.60, 138.26, 127.05, 124.43, 41.10. HRMS (m/z): $[C_8H_8N_6 + H]^+$ calculated 189.0883, found 189.0889.

Compound 8b: Synthesis was performed in a flask (**procedure A**) under nitrogen atmosphere at 60 °C overnight. 1.07 mmol (0.167 g) of compound **16**, 5.09 mmol (0.535 g) of 2-pyrimidinecarbonitrile, 0.26 mmol (0.095 g) $ZnOTf_2$ and 50 mmol (1.6 mL, 1.6 g) of anhydrous hydrazine were used. Oxidation (**procedure C**) was performed using 20 mL of DCM/AcOH (1:1, v:v) and 22 mmol (1.5 g) solid $NaNO_2$. Purification was performed with silica column

chromatography using an 10% to 60% EtOAc in DCM eluent resulting in 0.049 g (0.17 mmol, 15.9%) of compound **8b** as a red solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 9.13 (d, *J* = 4.9 Hz, 2H, pyrim, CH), 7.60 (t, *J* = 4.9 Hz, 1H, pyrim, CH), 5.66 (s, 1H, NH), 5.12 (d, *J* = 5.6 Hz, 2H, CH_2), 1.46 (s, 9H, Boc, CH_3). ^{13}C NMR (126 MHz, $CDCl_3$) δ: 167.96, 164.14, 159.47, 158.60, 155.97, 122.81, 80.67, 43.89, 28.46. HRMS (m/z): $[C_{12}H_{15}N_7O_2 + Na]^+$ calculated 312.1180, found 312.1180.

Compound 8: N-Boc deprotection of compound **8b** was performed using 4M HCl in dioxane according to **procedure E** obtaining compound **8** in quantitative yield as a pale orange solid. The compound proved too unstable for NMR analysis.

Compound 9b: Synthesis was performed in a closed pressure tube (**procedure B**) at 20 °C for 3 days. 2.04 mmol (0.348 g) of compound **17**, 10.11 mmol (1.053 g) of formamidine acetate, 0.48 mmol (0.173 g) $ZnOTf_2$, 3.0 mL of dioxane and 101 mmol (3.2 mL, 3.2 g) of anhydrous hydrazine were used. Oxidation (**procedure D**) was performed using 80 mmol (20 mL) of 4M $NaNO_2$ (aq.) and 120 mmol (60 mL) 2M HCl (aq.). Purification was performed with silica column chromatography using an 5% EtOAc in DCM eluent resulting in 0.026 g (0.12 mmol, 5.9%) of compound **9b** as a red solid. **TLC**: Rf = 0.6, 5% EtOAc in DCM. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 10.23 (s, 1H, Tz, CH), 5.01 (s, 1H, NH), 3.76 (dt, *J* = 6.3, 6.2 Hz, 2H, CH_2), 3.59 – 3.54 (t, *J* = 6.2 Hz, 2H, CH_2), 1.38 (s, 9H, Boc, CH_3). ^{13}C NMR (126 MHz, $CDCl_3$) δ: 171.24, 158.24, 155.89, 79.81, 38.41, 36.23, 28.45. HRMS (m/z): aromatic adduct $[C_{17}H_{27}N_3O_3 + H]^+$ calculated 322.2125, found 322.2130.

Compound 9: N-Boc deprotection of compound **9b** was performed using 4M HCl in dioxane according to **procedure E** obtaining compound **9** in quantitative yield as a bright pink solid. ^{1}H NMR (500 MHz, DMSO) δ: 10.61 (s, 1H, Tz, H), 8.30 (s, 3H, NH_3Cl), 3.67 (t, *J* = 7.1 Hz, 2H, CH_2), 3.47 – 3.38 (m, 2H, CH_2). ^{13}C NMR (126 MHz, DMSO) δ: 169.16, 158.28, 36.50, 32.54. HRMS (m/z): aromatic adduct $[C_{12}H_{19}N_3O + H]^+$ calculated 222.1601, found 222.1606.

Compound 10b: Synthesis was performed in a closed pressure tube (**procedure B**) at 60 °C overnight. 1.65 mmol (0.280 g) of compound **17**, 19 mmol (1.0 mL, 0.79 g) of acetonitrile, 0.34 mmol (0.120 g) $NiOTf_2$, 2.4 mL of dioxane and 76 mmol (2.4 mL, 2.4 g) of anhydrous hydrazine were used. Oxidation (**procedure C**) was performed using 40 mL of DCM/AcOH (1:1, v:v) and 22 mmol (1.5 g) of solid $NaNO_2$. Purification was performed with silica column chromatography using an 3% to 10% EtOAc in DCM eluent resulting in 0.062 g (0.26 mmol, 15.8%) of compound **10b** as a red solid. **TLC**: Rf = 0.5, 20% EtOAc in DCM. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 5.13 (s, 1H, NH), 3.68 (dt, *J* = 6.3, 6.2 Hz, 2H, CH_2), 3.46 (t, *J* = 6.2 Hz, 2H, CH_2), 2.99 (s, 3H, Tz, CH_3), 1.33 (s, 9H, Boc, CH_3). ^{13}C NMR (101 MHz, $CDCl_3$) δ: 168.03, 167.59, 155.83, 79.51, 38.41, 35.44, 28.35, 21.14. HRMS (m/z): aromatic adduct $[C_{16}H_{29}N_3O_3 + H]^+$ calculated 336.2282, found 336.2287.

Compound 10: N-Boc deprotection of compound **10b** was performed using 4M HCl in dioxane according to **procedure E** obtaining compound **10** in quantitative yield as a bright pink solid. ^{1}H NMR (500 MHz, DMSO) δ: 8.26 (s, 3H, NH_3Cl), 3.61 (t, *J* = 7.1 Hz, 2H, CH_2), 3.42 – 3.34 (m, 2H, CH_2), 2.97 (s, 3H, Tz, CH_3). ^{13}C NMR (126 MHz, DMSO) δ: 167.27, 166.29, 36.69, 31.94, 20.74. HRMS (m/z): $[C_5H_9N_5 + H]^+$ calculated 140.0931, found 140.0931.

Compound 11b: Synthesis was performed in a flask (**procedure A**) under nitrogen atmosphere at 60 °C overnight. 0.940 mmol (0.160 g) of compound **17**, 5.2 mmol (0.50 mL, 0.54 g) of 2-

pyridinecarbonitrile, 0.25 mmol (0.090 g) $ZnOTf_2$ and 47 mmol (1.5 mL, 1.5 g) of anhydrous hydrazine were used. Oxidation (**procedure D**) was performed using 80 mmol (20 mL) of 4M $NaNO_2$ (aq.) and 120 mmol (60 mL) 2M HCl (aq.). Purification was performed with silica column chromatography using an 10% to 60% EtOAc in pentane eluent resulting in 0.038 g (0.13 mmol, 13.4%) of compound **11b** as a pink oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.91 (dd, *J* = 3.9, 0.8 Hz, 1H, pyr, CH), 8.60 (d, *J* = 7.9 Hz, 1H, pyr, CH), 7.95 (ddd, *J* = 7.8, 7.8, 1.7 Hz, 1H, pyr, CH), 7.53 (ddd, *J* = 7.6, 4.8, 1.0 Hz, 1H, pyr, CH), 5.19 (s, 1H, NH), 3.78 (dt, *J* = 6.2, 6.1 Hz, 2H, CH_2), 3.61 (t, *J* = 6.1 Hz, 2H, CH_2), 1.34 (s, 9H, Boc, CH_3). ^{13}C NMR (101 MHz, $CDCl_3$) δ: 169.00, 163.84, 155.88, 150.97, 150.21, 137.54, 126.50, 124.02, 79.61, 38.41, 35.62, 28.39. HRMS (m/z): adduct $[C_{22}H_{32}N_4O_3 + H]^+$ calculated 401.2547, found 401.2554.

Compound 11: N-Boc deprotection of compound **11b** was performed using 4M HCl in dioxane according to **procedure E** obtaining compound **11** in quantitative yield as a pink solid. ^{1}H NMR (400 MHz, DMSO) δ: 8.92 (ddd, *J* = 4.7, 1.6, 0.8 Hz, 1H, pyr, CH), 8.55 (d, *J* = 7.9 Hz, 1H, pyr, CH), 8.33 (s, 3H, NH_3Cl), 8.17 (ddd, *J* = 7.8, 7.8, 1.7 Hz, 1H, pyr, CH), 7.74 (ddd, *J* = 7.6, 4.8, 1.1 Hz, 1H, pyr, CH), 5.70 (s, HOD), 3.74 (t, *J* = 7.0 Hz, 2H, CH_2), 3.46 (dd, *J* = 12.5, 6.4 Hz, 2H, CH_2). ^{13}C NMR (101 MHz, DMSO) δ: 167.20, 163.40, 150.52, 149.98, 138.25, 126.78, 124.11, 36.79, 32.27. HRMS (m/z): $[C_9H_{10}N_6 + H]^+$ calculated 203.1040, found 203.1048.

Compound 12b: Synthesis was performed in a closed pressure tube (**procedure B**) at 60 °C overnight. 5.87 mmol (0.999 g) of compound **17**, 60.11 mmol (6.317 g) of 2-pyrimidinecarbonitrile, 0.81 mmol (0.296 g) $ZnOTf_2$ and 315 mmol (10 mL, 10 g) of anhydrous hydrazine were used. Oxidation (**procedure C**) was performed using 150 mL of DCM/AcOH (1:1, v:v) and 145 mmol (10 g) solid $NaNO_2$. Purification was performed with silica column chromatography (3 times) using an 50% to 100% EtOAc in pentane eluent resulting in 0.474 g (1.56 mmol, 26.6%) of compound **12b** as a pink solid. **TLC**: Rf = 0.4, 100% EtOAc. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 9.12 (d, *J* = 4.9 Hz, 2H, pyrim, CH), 7.59 (t, *J* = 4.9 Hz, 1H, pyrim, CH), 5.13 (s, 1H, NH), 3.82 (dt, *J* = 6.2, 6.1 Hz, 2H, CH_2), 3.68 (t, *J* = 6.1 Hz, 2H, CH_2), 1.37 (s, 9H, Boc, CH_3). ^{13}C NMR (101 MHz, $CDCl_3$) δ: 169.48, 163.36, 159.40, 158.44, 155.84, 122.64, 79.56, 38.37, 35.66, 28.34. HRMS (m/z): adduct $[C_{21}H_{31}N_5O_3 + H]^+$ calculated 402.2500, found 402.2498.

Compound 12. N-Boc deprotection of compound **12b** was performed using 4M HCl in dioxane according to **procedure E** obtaining compound **12** in quantitative yield as a pink solid. ^{1}H NMR (600 MHz, DMSO) δ: 9.19 (d, *J* = 4.9 Hz, 2H, pyrim, CH), 8.24 (s, 3H, NH_3Cl), 7.84 (t, *J* = 4.9 Hz, 1H, pyrim, CH), 3.76 (t, *J* = 7.0 Hz, 2H, CH_2), 3.53 – 3.45 (m, 2H, CH_2). ^{13}C NMR (151 MHz, DMSO) δ: 167.49, 163.29, 159.15, 158.57, 123.04, 66.35, 36.72, 32.36. HRMS (m/z): $[C_8H_9N_7 + H]^+$ calculated 204.0992, found 204.0999.

Compound 13b: *For synthesis details, see **Compound 5b**.* ^{1}H NMR (400 MHz, $CDCl_3$) δ: 5.68 (s, 2H, NH), 4.95 (d, *J* = 5.6 Hz, 4H, CH_2), 1.43 (s, 18H, Boc, CH_3). ^{13}C NMR (101 MHz, $CDCl_3$) δ: 167.71, 155.93, 80.50, 43.57, 28.40. HRMS (m/z): $[C_{14}H_{24}N_6O_4 + Na]^+$ calculated 363.1752, found 363.1759.

Compound 13: N-Boc deprotection of compound **13b** was performed using 4M HCl in dioxane according to **procedure E** obtaining compound **13** in quantitative yield as a bright pink solid. ^{1}H NMR (400 MHz, DMSO) δ: 9.16 (s, 6H, NH_3Cl), 4.82 (s,4H, CH_2). ^{13}C NMR (101 MHz, DMSO) δ: 165.09, 40.94. HRMS (m/z): $[C_4H_8N_6 + H]^+$ calculated 141.0883, found 141.0880.

Compound 14b: (*Alternative synthesis for **Compound 11b**)* Synthesis was performed in a closed pressure tube (**procedure B**) at 60 °C overnight. 2.02 mmol (0.344 g) of compound **17**, 12 mmol (1.0 ml, 1.1 g) of 2-pyridinecarbonitrile, 0.48 mmol (0.173 g) $ZnOTf_2$, 3.0 mL of dioxane and 101 mmol (3.2 mL, 3.2 g) of anhydrous hydrazine were used. Oxidation (**procedure D**) was performed using 80 mmol (20 mL) of 4M $NaNO_2$ (aq.) and 120 mmol (60 mL) 2M HCl (aq.). Purification was performed with silica column chromatography using an 10% to 60% EtOAc in pentane eluent resulting in 0.034 g (0.11 mmol, 5.4%) of compound **11b** as a pink oil and 0.074 g (0.20 mmol, 19.8%) of compound **14b** as a red solid byproduct. **TLC**: Compound **11b** Rf = 0.2, compound **14b** Rf = 0.5, 50% EtOAc in pentane. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 5.12 (s, 2H, NH), 3.71 (dd, *J* = 12.3, 6.2 Hz, 4H, CH_2), 3.49 (t, *J* = 6.1 Hz, 4H, CH_2), 1.35 (s, 18H, Boc, CH_3). ^{13}C NMR (101 MHz, $CDCl_3$) δ: 168.48, 155.90, 79.58, 38.50, 35.61, 28.42. HRMS (m/z): $[C_{16}H_{28}N_6O_4 + Na]^+$ calculated 391.2065, found 391.2070.

Compound 14: N-Boc deprotection of compound **14b** was performed using 4M HCl in dioxane according to **procedure E** obtaining compound **14** in quantitative yield as a pink solid. ^{1}H NMR (400 MHz, DMSO) δ: 8.31 (s, 6H, NH_3Cl), 3.66 (t, *J* = 6.9 Hz, 4H, CH_2), 3.47 – 3.31 (m, 4H, CH_2). ^{13}C NMR (101 MHz, DMSO) δ: 166.89, 37.00, 32.11. HRMS (m/z): $[C_6H_{12}N_6 + H]^+$ calculated 169.1197, found 169.1199.

Compound 15[7]: 55.0 mmol (12.0 g) of Boc_2O was dissolved in 100 mL water, 150 mmol (6 g) of NaOH and 49.8 mmol (8.43 g) of 4-(aminomethyl) benzonitrile hydrochloride were added respectively and the solution was stirred overnight. The precipitate was isolated by centrifugation and dried using rotary evaporation, yielding compound **15** in quantitative yield as a white solid. ^{1}H NMR (400 MHz, DMSO) δ: 7.77 (d, *J* = 7.5 Hz, 2H, phenyl, CH), 7.52 (t, *J* = 5.7 Hz, 1H, NH), 7.42 (d, *J* = 7.6 Hz, 2H, phenyl, CH), 4.21 (d, *J* = 5.3 Hz, 2H, CH_2), 1.39 (s, 9H, Boc, CH_3). ^{13}C NMR (101 MHz, DMSO) δ: 155.85, 146.12, 132.23, 127.71, 118.92, 109.50, 78.10, 43.20, 28.20.

Compound 16[8]: 19.41 mmol (2.992 g) of aminoacetonitrile bisulfate was dissolved in 50 mL of dry DCM, 79 mmol of TEA (11 mL, 8.0 g) and 39.35 mmol of Boc_2O (8.589 g) were added respectively and the suspension was refluxed overnight. Reaction completion was checked by TLC (Rf = 0.6, 25% EtOAc in pentane). The organic layer was washed two times with water, two times with brine, dried over $MgSO_4$ and concentrated using rotary evaporation. 2.018 g (12.92 mmol, 66.6%) of compound **16** was obtained as an orange oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 5.58 (s, 1H, NH), 3.99 (d, *J* = 5.8 Hz, 2H, CH_2), 1.39 (s, 9H, Boc, CH_3). ^{13}C NMR (101 MHz, $CDCl_3$) δ: 155.25, 116.79, 81.04, 29.06, 28.15.

Compound 17[8]. 50.1 mmol (3.51 g) of 3-amino propionitrile was dissolved in 100 mL of dry DCM, 60 mmol of Boc_2O (13 g) was added and the solution was stirred overnight at room temperature. Reaction completion was checked by TLC (Rf = 0.4, 25% EtOAc in pentane). The organic layer was washed with water, brine, dried over $MgSO_4$ and concentrated using rotary evaporation. 8.30 g (48.8 mmol, 97.4%) of compound **17** was obtained as a clear solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 5.25 (s, 1H, NH), 3.33 (q, *J* = 6.3 Hz, 2H, CH_2), 2.54 (t, *J* = 6.3 Hz, 2H, CH_2), 1.38 (s, 9H, Boc, CH_3). ^{13}C NMR (101 MHz, $CDCl_3$) δ: 155.67, 118.33, 79.99, 36.73, 28.25, 18.83. HRMS (m/z): $[C_8H_{14}N_2O_2 + Na]^+$ calculated 193.0947, found 193.0954.

Compound 18: 21.9 mmol (2.145 g) of pyrrole-2-carboxaldehyde was dissolved in 50 mL of dry 1,2-dichloroethane, 4.47 mmol (1.493 g) of methyl (triphenylphosphoranylidene)acetate was added, and the clear yellow reaction mixture was stirred overnight at 50 °C turning clear

red. Reaction completion was checked by TLC (Rf = 0.60, 25% EtOAc in pentane). The reaction mixture was concentrated using rotary evaporation and purified using silica column chromatography resulting in 2.984 g (19.68 mmol, 87.2%) of compound **18** as a pale-yellow solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 9.32 (s, 1H), 7.60 (d, *J* = 15.9 Hz, 1H), 6.93 (d, *J* = 1.1 Hz, 1H), 6.57 (s, 1H), 6.28 (d, *J* = 3.4 Hz, 1H), 6.10 (d, *J* = 15.9 Hz, 1H), 3.78 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ: 168.68, 134.94, 128.48, 122.81, 114.61, 110.95, 110.62, 51.68.

Compound 19: 18.8 mmol (2.838 g) of compound **18** was dissolved in 100 mL of dry MeOH, the solution was degassed through sonication under N_2 gas flow, 0.21 mmol Pd/C (223 mg, 10 wt. % loading) was added and H_2 gas bubbled through the reaction mixture (using a balloon and needle) for 2 hours at room temperature. The reaction mixture was filtered over a thin layer of celite, and concentrated using rotary evaporation, resulting in 2.860 g (18.68 mmol, 99.5%) of compound **19** as a yellow oil (stable at -20 ^{0}C as a pale brown solid). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.55 (s, 1H), 6.68 (td, *J* = 2.6, 1.6 Hz, 1H), 6.11 (dd, *J* = 5.8, 2.8 Hz, 1H), 5.93 (dt, *J* = 3.2, 2.5 Hz, 1H), 3.71 (s, 3H), 2.93 (t, *J* = 6.8 Hz, 2H), 2.66 (t, *J* = 6.8 Hz, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ: 174.66, 131.05, 116.93, 108.11, 105.59, 51.94, 34.44,

Compound 20: 8.96 mmol (1.372 g) of compound **19** was dissolved in 45 mL of dry DCM, the solution was cooled to 0 °C and 10.0 mmol (1.237 g) of 3,5-dimethylpyrrole-2-carboxaldehyde was added. Then, 10.1 mmol (0.95 mL, 1.56 g) of phosphoryl chloride was added dropwise to the solution stirring for 3 hours while warming to room temperature. Reaction completion was checked by TLC (Rf = 0.6, 25% EtOAc in pentane, on neutral TLC plate dipped in 10% TEA in EtOAc and dried prior to use). Then, the reaction mixture cooled to 0 °C, and 39.5 mmol (5.5 mL, 4.0 g) TEA was dropwise added to neutralize the reaction mixture, followed by dropwise addition of 35.6 mmol (4.4 mL, 5.1 g) of boron trifluoride diethyl etherate and stirring overnight while warming to room temperature. Reaction completion was checked by TLC (Rf = 0.5, 25% EtOAc in pentane). The reaction mixture was filtered, diluted to 2 L with DCM, washed with 2 L $NaHCO_3$, dried twice over $NaSO_4$, filtered, and concentrated using rotary evaporation. Purification was performed with silica column chromatography using an 10 to 20% EtOAc in pentane eluent resulting in 1.675 g (5.47 mmol, 61.0%) of compound **20** as a black solid with a green/red glow). ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.05 (s, 1H), 6.85 (d, 1H), 6.24 (d, 1H), 6.08 (s, 1H), 3.68 (s, 3H), 3.28 (t, 2H), 2.76 (t, 2H), 2.54 (s, 3H), 2.21 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 173.01, 160.48, 156.98, 144.00, 135.28, 133.36, 128.11, 123.92, 120.48, 116.65, 51.78, 33.30, 24.03, 14.99, 11.31.

Compound 21: 5.32 mmol (1.627 g) of compound **20** was dissolved in 50 mL of THF, 50 mL of 4.5M HCl (aq.) was added and the reaction mixture was stirred overnight at room temperature. Reaction completion was checked using TLC (Rf = 0.5, 100% EtOAc). The reaction mixture was quenched with 250 mL of sat. $NaHCO_3$ (aq.) to pH = 7, 200 mL of 10% citric acid (aq.) was added and the reaction mixture was extracted twice with 400 mL EtOAc. The organic layers were combined, dried over $NaSO_4$ and concentrated using rotary evaporation. Purification was performed with silica column chromatography (very tall column) using an 100% EtOAc eluent resulting in 1.452 g (4.97 mmol, 93.5%) of compound **21** as a red solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.40 (s, 1H), 6.99 (d, 1H), 6.33 (d, 1H), 6.20 (s, 1H), 3.20 (t, 2H), 2.71 (t, 2H), 2.51 (s, 3H), 2.26 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ: 174.93, 128.26, 124.46, 120.03, 116.22, 32.69, 23.64, 13.52, 9.86.

Compound 22 (**A-092)**: 4.73 mmol (1.382 g) of compound **21** was dissolved in 50 mL of dry DMF, 10.1 mmol (1.158 g) of N-hydroxy-succinimide and 10.3 mmol (1.6 mL, 1.30 g) of

diisopropylcarbodiimide were added respectively, and the reaction mixture was stirred overnight at room temperature. Reaction completion was checked using TLC (Rf = 0.4, 50% EtOAc in pentane). The reaction mixture was concentrated using rotary evaporation, taken up in 30 mL of DCM/EtOAc/pentane (10:2:3, v:v:v) and purified using silica column chromatography using an 40 to 50% EtOAc in pentane eluent resulting in 1.334 g (3.43 mmol, 72.4%) of compound **22** as a bright orange/red solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.09 (s, 1H), 6.87 (d, *J* = 3.9 Hz, 1H), 6.32 (d, *J* = 4.0 Hz, 1H), 6.11 (s, 1H), 3.37 (t, *J* = 7.4 Hz, 2H), 3.07 (t, *J* = 7.4 Hz, 2H), 2.81 (d, *J* = 1.9 Hz, 4H), 2.55 (s, 3H), 2.23 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 169.29, 168.02, 161.42, 154.82, 144.67, 135.72, 133.45, 128.08, 124.25, 120.96, 116.92, 30.49, 25.77, 23.54, 15.20, 11.51.

General procedure for synthesis of compounds 23-34: Two solutions are prepared. Solution **A**: 1 equivalent of compound **22** is dissolved in dry DMF (0.1M). Solution **B**: 2-4 equivalents of tetrazine **1-12** are dissolved in dry DMF (0.1M) forming a suspension, 2 equivalents of TEA are added to this suspension, resulting in a red suspension or red clear solution. Then, solution **B** is added to solution **A** and the reaction mixture is stirred for 5 to 60 minutes at room temperature. The reaction mixture is dropped in (10x volume) of 0.1M HCl (aq.) and extracted with (10x volume) EtOAc. The organic layer is washed with brine, dried using $MgSO_4$ and concentrated using rotary evaporation. Purification is performed using a gradient of EtOAc in DCM resulting in compounds **23-34**.

Compound 23: Following *General procedure for synthesis of compounds 23-34*, using 15 µmol (6 mg) of compound **22**, resulting in 7.6 µmol (3.5 mg) of compound **23** as a red solid. Duration: 60 min. Column: 30% EtOAc in DCM. TLC: Rf = 0.5, 30% EtOAc in DCM. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 10.21 (s, 1H), 8.48 (d, *J* = 8.4 Hz, 2H), 7.33 (d, *J* = 8.4 Hz, 2H), 7.11 (s, 1H), 6.90 (d, *J* = 4.0 Hz, 1H), 6.32 (d, *J* = 4.0 Hz, 1H), 6.27 (s, 1H), 4.51 (d, *J* = 5.9 Hz, 2H), 3.32 (t, *J* = 7.3 Hz, 2H), 2.78 (t, *J* = 7.3 Hz, 2H), 2.53 (s, 3H), 2.23 (s, 3H).

Compound 24: Following *General procedure for synthesis of compounds 23-34*, using 21 µmol (8 mg) of compound **22**, resulting in 13 µmol (6 mg, 60%) of compound **24** as a red solid. Duration: 30 min. Column: 10 to 30% EtOAc in DCM. TLC: Rf = 0.2, 10% EtOAc in DCM. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 8.44 (d, *J* = 8.4 Hz, 2H), 7.32 (d, *J* = 8.3 Hz, 2H), 7.09 (s, 1H), 6.88 (d, *J* = 4.0 Hz, 1H), 6.30 (d, *J* = 4.0 Hz, 1H), 6.27 (s, 1H), 6.07 (s, 1H), 4.49 (d, *J* = 5.9 Hz, 2H), 3.31 (t, *J* = 7.3 Hz, 2H), 3.09 (s, 3H), 2.76 (t, *J* = 7.3 Hz, 2H), 2.52 (s, 3H), 2.23 (s, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ: 171.80, 167.32, 164.07, 160.68, 156.98, 144.28, 143.46, 135.39, 133.55, 130.78, 128.43, 128.36, 128.22, 123.99, 120.65, 117.74, 43.29, 36.11, 24.99, 21.28, 15.07, 11.44.

Compound 25: Following *General procedure for synthesis of compounds 23-34*, using 21 µmol (8 mg) of compound **22**, resulting in 17 µmol (9 mg, 80%) of compound **25** as a red solid. Duration: 10 min. Column: 15 to 45% EtOAc in DCM. TLC: Rf = 0.4, 50% EtOAc in DCM. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 8.97 (d, *J* = 4.0 Hz, 1H), 8.69 (d, *J* = 7.9 Hz, 1H), 8.54 (q, 2H), 8.01 (td, *J* = 7.8, 1.8 Hz, 1H), 7.57 (ddd, *J* = 7.6, 4.7, 1.1 Hz, 1H), 7.35 (d, *J* = 8.5 Hz, 2H), 7.10 (s, 1H), 6.89 (d, *J* = 4.0 Hz, 1H), 6.33 (s, 1H), 6.31 (d, *J* = 4.0 Hz, 1H), 6.06 (s, 1H), 4.51 (d, *J* = 5.9 Hz, 2H), 3.32 (t, *J* = 7.3 Hz, 2H), 2.78 (t, *J* = 7.3 Hz, 2H), 2.52 (s, 3H), 2.23 (s, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ: 171.80, 164.35, 163.54, 160.70, 156.93, 151.06, 150.48, 144.07, 137.61, 135.40, 133.55, 130.51, 128.72, 128.55, 128.36, 126.46, 124.04, 123.98, 120.67, 117.76, 43.32, 36.08, 24.97, 15.07, 11.47.

Compound 26: Following *General procedure for synthesis of compounds 23-34*, using 21 µmol (8 mg) of compound **22**, resulting in 7.4 µmol (4 mg, 35%) of compound **26** as a red solid. **Duration:** 10 min. Column: 30 to 100% EtOAc in DCM. TLC: Rf = 0.3, 75% EtOAc in DCM. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 9.14 (d, *J* = 4.9 Hz, 2H), 8.58 (d, *J* = 8.4 Hz, 2H), 7.59 (t, *J* = 4.9 Hz, 1H), 7.35 (d, *J* = 8.4 Hz, 2H), 7.10 (s, 1H), 6.89 (d, *J* = 4.0 Hz, 1H), 6.31 (d, *J* = 4.0 Hz, 2H), 6.06 (s, 1H), 4.52 (d, *J* = 5.9 Hz, 2H), 3.32 (t, *J* = 7.3 Hz, 2H), 2.78 (t, *J* = 7.3 Hz, 2H), 2.52 (s, 3H), 2.23 (s, 3H).

Compound 27: Following *General procedure for synthesis of compounds 23-34*, using 21 µmol (8 mg) of compound **22**, resulting in 2.6 µmol (1 mg, 12%) of compound **27** as a red solid. Duration: 5 min. Column: 25% EtOAc in DCM. TLC: Rf = 0.5, 50% EtOAc in DCM. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 10.23 (s, 1H), 7.09 (s, 1H), 6.88 (d, *J* = 4.0 Hz, 1H), 6.69 (s, 1H), 6.28 (d, *J* = 4.0 Hz, 1H), 6.13 (s, 1H), 5.09 (d, *J* = 5.7 Hz, 2H), 3.32 (t, *J* = 7.5 Hz, 2H), 2.80 (t, *J* = 7.5 Hz, 2H), 2.56 (s, 3H), 2.26 (s, 3H).

Compound 28: Following *General procedure for synthesis of compounds 23-34*, using 21 µmol (8 mg) of compound **22**, resulting in 21 µmol (8.5 mg, 100%) of compound **28** as a red solid. Duration: 15 min. Column: 20 to 40% EtOAc in DCM. TLC: Rf = 0.5, 50% EtOAc in DCM. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 7.08 (s, 1H), 6.87 (d, *J* = 3.9 Hz, 1H), 6.69 (s, 1H), 6.28 (d, *J* = 4.0 Hz, 1H), 6.12 (s, 1H), 5.03 (d, *J* = 5.6 Hz, 2H), 3.31 (t, *J* = 7.5 Hz, 2H), 3.05 (s, 3H), 2.77 (t, *J* = 7.5 Hz, 2H), 2.55 (s, 3H), 2.25 (s, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ: 172.31, 168.42, 166.19, 160.54, 157.10, 144.09, 135.32, 133.51, 128.35, 123.99, 120.62, 117.53, 42.43, 35.86, 24.79, 21.30, 15.10, 11.47.

Compound 29: Following *General procedure for synthesis of compounds 23-34*, using 21 µmol (8 mg) of compound **22**, resulting in 2.5 µmol (1 mg, 10%) of compound **29** as a red solid. Duration: 20 min. Column: 50 to 100% EtOAc in DCM. TLC: Rf = 0.2, 50% EtOAc in DCM. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.96 (d, *J* = 3.8 Hz, 1H), 8.64 (d, *J* = 7.9 Hz, 1H), 8.00 (t, *J* = 7.8 Hz, 1H), 7.58 (dd, *J* = 7.6, 4.7 Hz, 1H), 7.08 (s, 1H), 6.86 (d, *J* = 3.9 Hz, 1H), 6.72 (s, 1H), 6.29 (d, *J* = 3.9 Hz, 1H), 6.11 (s, 1H), 5.15 (d, *J* = 5.6 Hz, 2H), 3.33 (t, *J* = 7.4 Hz, 2H), 2.81 (t, *J* = 7.5 Hz, 2H), 2.56 (s, 3H), 2.24 (s, 3H).

Compound 31: Following *General procedure for synthesis of compounds 23-34*, using 26 µmol (10 mg) of compound **22**, resulting in 23 µmol (9 mg, 87%) of compound **31** as a red solid. **Duration:** 7 min. Column: 10 to 30% EtOAc in DCM. TLC: Rf = 0.5, 100% EtOAc. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 10.10 (s, 1H), 7.03 (s, 1H), 6.83 (d, *J* = 3.9 Hz, 1H), 6.24 (d, *J* = 4.0 Hz, 2H), 6.13 (s, 1H), 3.85 (dd, *J* = 12.3, 6.2 Hz, 2H), 3.47 (t, *J* = 6.2 Hz, 2H), 3.19 (t, *J* = 7.3 Hz, 2H), 2.61 (t, *J* = 7.4 Hz, 2H), 2.54 (s, 3H), 2.27 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ: 171.97, 170.91, 160.44, 157.97, 156.85, 144.02, 135.08, 133.23, 128.20, 123.81, 120.52, 117.57, 36.56, 35.77, 35.32, 24.78, 14.97, 11.36.

Compound 32: Following *General procedure for synthesis of compounds 23-34*, using 26 µmol (10 mg) of compound **22**, resulting in 19 µmol (8 mg, 74%) of compound **32** as a red solid. Duration: 10 min. Column: 10 to 30% EtOAc in DCM. TLC: Rf = 0.4, 100% EtOAc. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.03 (s, 1H), 6.83 (d, *J* = 4.0 Hz, 1H), 6.25 (d, *J* = 4.0 Hz, 1H), 6.22 (s, 1H), 6.12 (s, 1H), 3.83 (q, *J* = 6.2 Hz, 2H), 3.42 (t, *J* = 6.2 Hz, 2H), 3.20 (t, *J* = 7.4 Hz, 2H), 2.99 (s, 3H), 2.60 (t, *J* = 7.4 Hz, 2H), 2.54 (s, 3H), 2.26 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ: 172.06, 167.99, 167.67, 160.47, 157.14, 144.03, 133.42, 128.31, 123.88, 120.58, 117.74, 36.77, 35.96, 34.77, 24.98, 21.23, 15.08, 11.49.

Compound 33: Following *General procedure for synthesis of compounds 23-34*, using 26 µmol (10 mg) of compound **22**, resulting in 15 µmol (7 mg, 57%) of compound **33** as a red solid. Duration: 10 min. Column: 20 to 70% EtOAc in DCM. TLC: Rf = 0.2, 100% EtOAc. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.94 (ddd, *J* = 4.7, 1.6, 0.8 Hz, 1H), 8.58 (d, *J* = 7.9 Hz, 1H), 7.97 (td, *J* = 7.8, 1.8 Hz, 1H), 7.56 (ddd, *J* = 7.6, 4.8, 1.1 Hz, 1H), 6.93 (s, 1H), 6.78 (d, *J* = 4.0 Hz, 1H), 6.35 (s, 1H), 6.23 (d, *J* = 4.0 Hz, 1H), 5.99 (s, 1H), 3.90 (q, *J* = 6.1 Hz, 2H), 3.53 (t, *J* = 6.2 Hz, 2H), 3.21 (t, *J* = 7.3 Hz, 2H), 2.63 (t, *J* = 7.3 Hz, 2H), 2.49 (s, 3H), 2.13 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ: 172.14, 168.89, 163.77, 160.49, 156.93, 150.97, 150.34, 144.02, 137.55, 135.10, 133.33, 128.23, 126.54, 124.08, 123.79, 120.51, 117.74, 77.48, 77.16, 76.84, 36.58, 35.88, 34.98, 25.00, 15.05, 11.38.

Compound 34: Following *General procedure for synthesis of compounds 23-34*, using 15 µmol (6 mg) of compound **22**, resulting in 8.4 µmol (4 mg, 56%) of compound **34** as a red solid. Duration: 15 min. Column: 15 to 50% Acetone in DCM. TLC: Rf = 0.5, 50% Acetone in DCM. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 9.11 (d, *J* = 4.9 Hz, 2H), 7.58 (t, *J* = 4.9 Hz, 1H), 7.00 (s, 1H), 6.82 (d, *J* = 3.9 Hz, 1H), 6.30 (s, 1H), 6.25 (d, *J* = 3.9 Hz, 1H), 6.02 (s, 1H), 3.91 (q, *J* = 6.1 Hz, 2H), 3.60 (t, *J* = 6.2 Hz, 2H), 3.21 (t, *J* = 7.3 Hz, 2H), 2.62 (t, *J* = 7.4 Hz, 2H), 2.51 (s, 3H), 2.18 (s, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ: 172.16, 169.44, 163.47, 160.52, 159.66, 158.51, 157.07, 143.97, 135.18, 133.41, 128.29, 123.88, 122.69, 120.48, 117.74, 36.80, 35.93, 35.21, 24.96, 15.08, 11.43.

Compound 35: 3.59 mmol (0.496 g) of exo-carboxynorbornene was dissolved in 5 mL of dry THF, 4.37 mmol (0.504 g) of N-hydroxysuccinimide (HOSu) and 4.37 mmol (0.901 g) dicyclohexylcarbodiimide (DCC) were added to the solution. The reaction mixture was stirred for 3 hours. 5 mL of EtOAc was added to the reaction before filtering the solution over a pad of celite. The filtrate was concentrated using rotary evaporation. Purification was performed with silica column chromatography using an 20 to 40% EA in pentane eluent resulting in 0.764 g (3.25 mmol, 89.7%) of compound **35** as a white solid. TLC: Rf = 0.45, 20% EA in pentane. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 6.18 (dd, *J* = 5.6, 3.0 Hz, 1H), 6.12 (dd, *J* = 5.6, 3.1 Hz, 1H), 3.24 (s, 1H), 2.97 (s, 1H), 2.48 (ddd, *J* = 9.0, 4.5, 1.2 Hz, 1H), 2.05 – 1.95 (m, 1H), 1.50 (ddd, *J* = 11.5, 7.1, 2.7 Hz, 2H), 1.45 – 1.38 (m, 1H). ^{13}C NMR (101 MHz, $CDCl_3$) δ: 171.70, 169.44, 138.59, 135.32, 47.17, 46.44, 41.82, 40.33, 31.01, 25.66.

Compound 36: 0.40 mmol (94 mg) of compound **35** was dissolved in 2 mL of dry DCM, 0.48 mmol (67 µL, 49 mg) of TEA and 0.48 mmol (29 µL, 29 mg) of 2-amino ethanol were added respectively and the reaction mixture was stirred for 1 hour at room temperature. Reaction completion was checked by TLC (Rf = 0.3, 100% EA). The reaction mixture was filtered and concentrated. Upon concentrating the mixture, degradation of the product was observed. Purification was performed with silica column chromatography using an 1 to 2.5% MeOH in DCM eluent resulting in 36 mg (0.20 mmol, 50.0%) of compound **36** as a colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 6.44 (s, 1H), 6.18 – 6.00 (m, 2H), 3.68 (s, 2H), 3.39 (s, 2H), 2.90 (s, 2H), 2.03 (d, *J* = 4.5 Hz, 1H), 1.86 (d, *J* = 11.1 Hz, 1H), 1.66 (d, *J* = 8.1 Hz, 1H), 1.32 (d, *J* = 8.5 Hz, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ: 138.31, 136.06, 62.27, 47.28, 46.41, 44.66, 42.60, 41.64, 30.67.

Compound 40 (Method A): 0.11 mmol (50 mg) of $Rh(OAc)_4$ was dissolved in 102 mmol (14.4 mL, 10.0 g) of propyne **39**, and 29.9 mmol (3.6 mL, 3.41 g) of diazoacetate **38** (87% wt. in DCM) was added dropwise over 7 hours using a syringe pump (0.54 mL/h) while stirring at room temperature overnight. The reaction mixture was diluted with 3.6 mL pentane and filtered over celite. The filtrate was distilled at 110 °C to remove the pentane, followed by distillation at 150 °C to recover 6.36 g of propyne **39** containing a small amount of pentane.

The remaining liquid consisted of a mixture of 5.4 g (27 mmol, 90%) of compound **40** and 1.35 g of propyne **39** as a yellow oil, and was used without further purification. **(Method B):** 0.11 mmol (50 mg) of $Rh(OAc)_4$ was dissolved in 102 mmol (14.4 mL, 10.0 g) of propyne **39**, and 58 mmol (7.0 mL, 6.63 g) of diazoacetate **38** (87% wt. in DCM) was added dropwise over 15 hours using a syringe pump (0.48 mL/h) while stirring at room temperature overnight. The reaction mixture was diluted with 7.0 mL pentane and filtered over celite. The filtrate was distilled at 110 °C to remove the pentane, followed by distillation at 150 °C to recover propyne **39**. The remaining liquid consisted of a mixture of 10.3 g (52 mmol, 93%) of compound **40** and 3.4 g of propyne **39** as a brown oil, and was used without further purification. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 4.18 – 4.03 (m, 1H), 2.20 (s, 1H), 1.98 (s, 0H), 1.24 (t, *J* = 7.1 Hz, 1H), 0.20 (s, 4H). ^{13}C NMR (101 MHz, $CDCl_3$) δ: 176.23, 122.67, 104.21, 59.53, 21.35, 14.93, 11.98, -1.46.

Compound 41: 25 mmol (5.0 g) of compound **40** (6.69g of a 75% wt. mixture) was dissolved in 60 mL of MeOH, 97.5 mmol of KOH (65 mL of an 1.5M aqueous solution) was added and the reaction mixture was stirred overnight at room temperature. Reaction completion was checked by TLC (Rf = 0.5, 100% EA). Methanol was removed using rotary evaporation (40 °C, 50 mbar) and the remaining aqueous solution was acidified using 100 mL of a 1M HCl (aq.) solution. The solution was extracted 4 times with 100 mL EtOAc and the organic layers were combined, dried using $MgSO_4$, filtered and concentrated using rotary evaporation. Purification was performed with silica column chromatography using an 20% EtOAc in pentane eluent resulting in 2.16 g (22.0 mmol, 87.9%) of compound **41** as a pale-yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 11.56 (s, 1H), 6.28 (s, 1H), 2.10 (d, *J* = 1.1 Hz, 3H), 2.03 (d, *J* = 1.4 Hz, 1H). ^{13}C NMR (101 MHz, $CDCl_3$) δ: 183.25, 111.08, 94.12, 25.80, 19.78, 10.26.

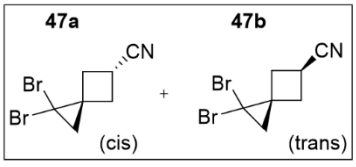
Compound 42: 15.1 mmol (1.483 g) of compound **41** was dissolved in 70 mL DCM, 22.5 mmol (4.15 g) of pentafluoro phenol, 20.7 mmol (3.96 g) of EDC*HCl and 1.7 mmol (0.21 g) of DMAP were added respectively and the reaction mixture was stirred overnight at room temperature. Reaction completion was checked by TLC (Rf = 0.80, 10% Et_2O in pentane). 100 mL DCM was added to the solution, before washing it with 1M HCl (aq.), sat. $NaHCO_3$ (aq.) and brine. The organic layer was dried using $MgSO_4$, filtered and concentrated using rotary evaporation. Purification was performed with silica column chromatography using an 2 to 5% Et_2O in pentane eluent resulting in 2.39 g (9.04 mmol, 59.8%) of compound **42** as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 6.51 – 6.36 (m, 1H), 2.40 (d, *J* = 1.5 Hz, 1H), 2.25 (d, *J* = 1.2 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ: 172.15, 142.63, 142.55, 140.55, 140.02, 139.22, 138.05, 136.72, 111.25, 93.98, 19.52, 10.33.

Compound 43: 0.12 mmol (32 mg) of compound **32** was dissolved in 1 mL of dry DCM, 0.12 mmol (17 µL, 12 mg) of TEA and 0.23 mmol (14 µL, 14 mg) of 2-amino ethanol were added respectively and the reaction mixture was stirred for 2 hours at room temperature. Reaction completion was checked by TLC (Rf = 0.5, 10% MeOH in EtOAc). The reaction mixture was quenched by adding 5 mL 0.1M HCl (aq.). An attempt to extract the product using EtOAc failed, so 100 mL EtOAc was added and $MgSO_4$ was used to dry the biphasic solution, before filtering and concentrating using rotary evaporation. Purification was performed with silica column chromatography using an 0 to 10% MeOH in EtOAc eluent resulting in 6 mg (0.043 mmol, 35%) of compound **43** as a colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 6.51 – 6.34 (m, 1H), 6.02 (s, 1H), 3.73 – 3.62 (m, 2H), 3.42 (dd, *J* = 10.0, 5.5 Hz, 2H), 2.17 (d, *J* = 1.2 Hz, 3H), 2.03 (d, *J* = 1.5 Hz, 1H). ^{13}C NMR (101 MHz, $CDCl_3$) δ: 177.90, 113.79, 96.05, 63.00, 42.88, 22.43, 10.77.

Compound 44: 27.3 mmol (5.42 g) of compound **40** was dissolved in 50 mL of dry Et_2O, the reaction mixture was cooled to 0 °C and 70 mmol (70 mL) of 1.0 M diisobutylaluminium hydride in THF was added dropwise over 1 minute. The reaction mixture was stirred for 30 minutes. The reaction was quenched by adding a solution of 400 mmol (20 g) of Rochelle salt in 150 mL H_2O, extracted with 100 mL of Et_2O, dried using $MgSO_4$, filtered and concentrated using rotary evaporation. Purification was performed with silica column chromatography using an 10 to 30% Et_2O in pentane eluent resulting in 3.264 g (20.9 mmol, 76.5%) of compound **44** as a colorless oil. TLC: Rf = 0.4, 30% Et_2O in pentane. ^1H NMR (400 MHz, $CDCl_3$) δ: 3.48 (d, *J* = 4.6 Hz, 2H), 2.21 (s, 3H), 1.56 (t, *J* = 4.6 Hz, 1H), 0.16 (s, 9H). ^{13}C NMR (101 MHz, $CDCl_3$) δ: 135.63, 111.41, 69.44, 22.32, 13.60, -0.98.

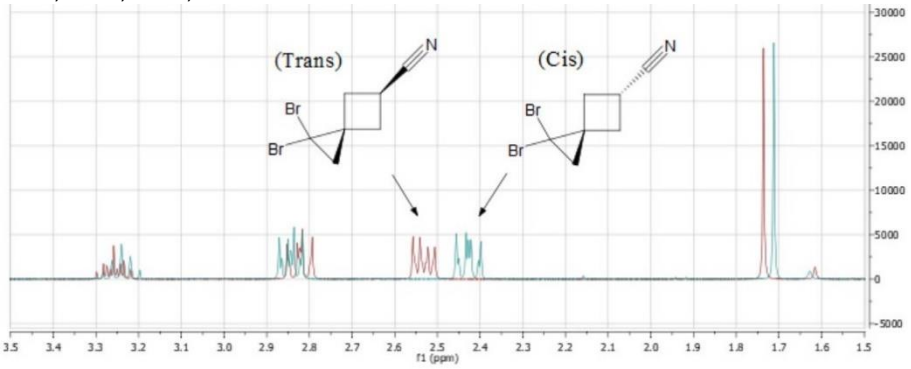
Compound 45: 1.1 mmol (0.172 g) of compound **44** was dissolved in 5 mL of dry THF, 1.2 mmol (0.320 g) of 18-crown-6 and 1.15 mmol (0.175 g) of cesium fluoride powder were added respectively. The mixture was stirred for 2.5 h at room temperature. Reaction completion was checked by TLC (Rf = 0.2, 30% Et_2O). Next, the mixture was diluted with 16 mL of dry DCM and 9.9 mmol (0.8 mL, 0.78 g) of pyridine and 2.2 mmol (0.445 g) of 4-nitrophenyl chloroformate were added respectively. The mixture was stirred overnight at room temperature. Reaction completion was checked by TLC (Rf= 0.5, 10% Et_2O in pentane). The reaction mixture was concentrated using rotary evaporation, dissolved in Et_2O, washed with sat. 10% $NaHCO_3$ (aq.), dried over $MgSO_4$, filtered and concentrated using rotary evaporation. Purification was performed with silica column chromatography using an 5 to 20% Et_2O in pentane eluent resulting in 0.185 g (0.74 mmol, 67.5 %) of compound **45** as a pale yellow oil. TLC: Rf = 0.5, 10% Et_2O in pentane. ^1H NMR (400 MHz, $CDCl_3$) δ: 8.31 – 8.23 (m, 2H), 7.43 – 7.34 (m, 2H), 7.260, 6.61 (t, J = 1.6 Hz, 1H), 4.23 – 4.10 (m, 2H), 2.17 (d, 3H, J = 1.2 Hz), 1.77 (td, 1H, J = 5.3, 1.5 Hz). ^{13}C NMR (101 MHz, $CDCl_3$) δ: 155.87, 152.79, 145.38, 125.40, 121.9, 120.29, 101.80, 77.55, 16.74, 11.79.

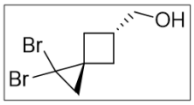
Compound 46: 0.80 mmol (250 mg) of compound **45** was dissolved in 8.0 mL of dry DCM, 0.80 mmol (0.11 mL, 0.080 g) of TEA and 1.6 mmol (0.10 mL, 0.10 g) of 2-aminoethanol were added respectively. The reaction mixture was stirred for 3 hours at room temperature. Reaction completion was checked by TLC (Rf = 0.4, 100% EtOAc). The reaction mixture was directly purified with silica column chromatography using an 25 to 100% EtOAc in pentane eluent resulting in 109 mg (0.64 mmol, 80.0%) of compound **46** as a colorless oil. TLC: Rf = 0.3, 30% Et_2O in pentane. ^1H NMR (400 MHz, $CDCl_3$) δ: 6.52 (t, J = 1.4 Hz, 1H), 5.48 (t, J = 5.9 Hz, 1H), 3.87 (d, J = 4.8 Hz, 2H), 3.64 (t, J = 5.2 Hz, 2H), 3.50 (s, 1H), 3.27 (q, J = 5.4 Hz, 2H), 2.08 (d, J = 1.2 Hz, 3H), 1.58 (d, J = 5.3 Hz, 1H). ^{13}C NMR (101 MHz, $CDCl_3$) δ: 157.82, 120.66, 102.19, 72.63, 62.08, 43.44, 17.17, 11.67.



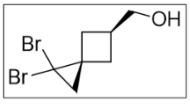
Compound 47: 5 mmol (0.50 mL, 0.47 g) of 3-methylene-2-cyano-cyclobutane was dissolved in 10 mmol (0.9 mL, 2.53 g) of bromoform, 0.05 mmol (18 mg) of CTAB was added, followed by dropwise addition of 2.5 g NaOH dissolved in 2.5 mL H_2O over 1 minute. The biphasic solution was stirred overnight resulting in a black suspension. The reaction was poured in 100 mL of H_2O and extracted twice with 50 mL DCM. The organic layers were combined, washed with H_2O twice, washed with brine, dried using $MgSO_4$, filtered and concentrated using rotary evaporation. Purification was performed with silica column chromatography using an 0-3% Et_2O in pentane eluent resulting in 0.524 g (1.98 mmol, 40.4 %) of compound **47b** as a white solid, followed by an 3-10% Et_2O

in pentane eluent resulting in 0.507 g (1.91 mmol, 39.0%) of compound **47a** as a white solid. Compound **47a**: TLC: Rf = 0.10, 10% Et_2O in pentane. ^1H NMR (400 MHz, $CDCl_3$) δ: 3.32 – 3.12 (m, 1H), 2.93 – 2.73 (m, 2H), 2.47 – 2.31 (m, 2H), 1.71 (s, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ: 121.41, 34.23, 33.55, 31.89, 31.60, 14.96. Compound **47b**: TLC: Rf = 0.15, 10% Et_2O in pentane. ^1H NMR (400 MHz, $CDCl_3$) δ: 3.26 (tt, *J* = 9.6, 6.4 Hz, 1H), 2.89 – 2.76 (m, 2H), 2.53 (dd, *J* = 14.1, 6.4 Hz, 2H), 1.74 (s, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ: ^{13}C NMR (101 MHz, $CDCl_3$) δ 122.08, 33.93, 33.23, 31.96, 16.38.



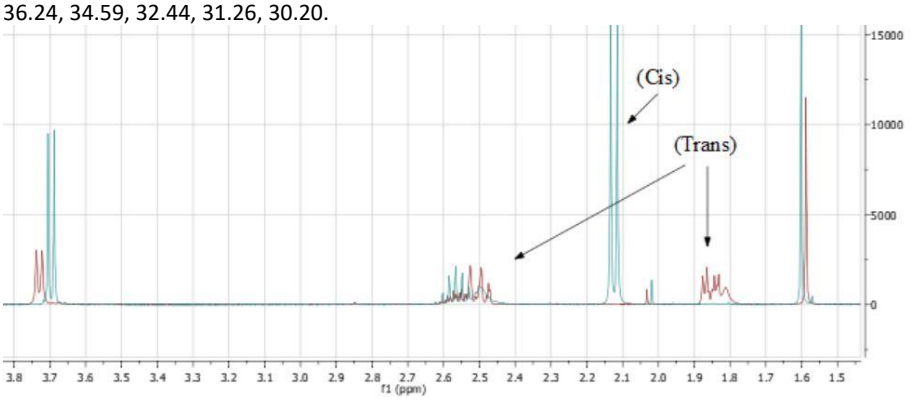


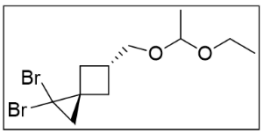
Compound 48a: 1.57 mmol (0.415 g) of compound **47a** was dissolved in 0.3 mL dry toluene, cooled down to -78 °C, and 3.1 mmol (3.1 mL of a 1M solution in toluene) of DiBAl-H was added dropwise before stirring the suspension for 2 hours while warming to room temperature. Reaction completion was checked by TLC (Rf = 0.7, 25% EtOAc in pentane). The clear reaction mixture was quenched via dropwise addition to 3 mL of a 1M HCl (aq.) solution. The aqueous solution was extracted three times with DCM. The organic layers were combined, washed with H_2O and brine, dried using $MgSO_4$, filtered and concentrated using rotary evaporation. The concentrate was taken up in 0.6 mL dry EtOH, cooled to 0 °C, 2.4 mmol (91 mg) of $NaBH_4$ was added portion wise, and the reaction mixture was stirred for 1 hour. Reaction completion was checked by TLC (Rf = 0.4, 25% EtOAc in pentane). The reaction mixture was quenched via dropwise addition of 3 mL of 1M HCl (aq.). The aqueous solution was extracted two times with DCM. The organic layers were combined, washed with H_2O and brine, dried using $MgSO_4$, filtered and concentrated using rotary evaporation. Purification was performed using silica column chromatography using an 10-30% EtOAc in pentane eluent, resulting in 0.200 g (0.74 mmol, 47.2%) of compound **48a** as a colorless liquid. ^1H NMR (400 MHz, $CDCl_3$) δ: 3.70 (d, *J* = 6.9 Hz, 2H), 2.62 – 2.52 (m, 1H), 2.50 (s, 1H), 2.12 (d, *J* = 7.5 Hz, 4H), 1.60 (s, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ: 66.43, 36.23, 34.14, 32.26, 31.46, 29.53.



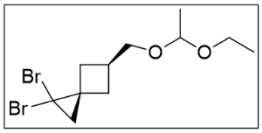
Compound 48b: 15.9 mmol (4.201 g) of compound **47b** was dissolved in 8 mL dry toluene, cooled down to -78 °C, and 32 mmol (32 mL of a 1M solution in toluene) of DiBAl-H was added dropwise before stirring the clear solution for 2 hours while warming to room temperature. Reaction completion was checked by TLC (Rf = 0.4, 15% Et_2O in pentane). The clear reaction mixture was quenched by adding 32 mL of a 1M HCl (aq.) solution. The aqueous solution was extracted three times with DCM. The organic layers were combined, washed with H_2O and brine, dried using $MgSO_4$, filtered and concentrated using rotary evaporation. The concentrate was taken up in 8 mL dry EtOH, cooled to 0 °C, 24 mmol (0.91 g) of $NaBH_4$ was added portion

wise, and the reaction mixture was stirred for 1 hour. Reaction completion was checked by TLC (Rf = 0.1, 15% Et_2O in pentane). The reaction mixture was quenched via dropwise addition of 40 mL of 1M HCl (aq.). The aqueous solution was extracted two times with DCM. The organic layers were combined, washed with H_2O and brine, dried using $MgSO_4$, filtered and concentrated using rotary evaporation. Purification was performed using silica column chromatography using an 5-20% EtOAc in pentane eluent, resulting in 2.490 g (9.22 mmol, 58.0%) of compound **48b** as a colorless liquid. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 3.73 (d, *J* = 6.4 Hz, 2H), 2.62 – 2.45 (m, 3H), 1.90 – 1.75 (m, 3H), 1.59 (s, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ: 66.77, 36.24, 34.59, 32.44, 31.26, 30.20.



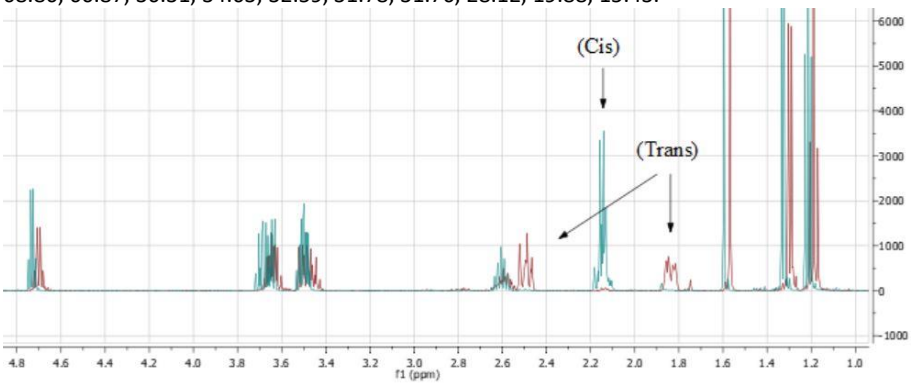


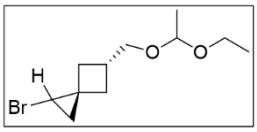
Compound 49a: 0.74 mmol (0.200 g) of compound **48a** was dissolved in 4 mL of dry Et_2O, 0.05 mmol (10 mg) of TsOH monohydrate was added, and the reaction mixture was cooled to 0 °C before dropwise addition of 6 mmol (0.6 mL, 0.43 g) of vinyl ethyl ether over 1 minute. The reaction mixture was stirred for 1 hour, filtered while cold over basic Al_2O_3 (half a Pasteur pipet in volume), and concentrated using rotary evaporation. Purification was performed with silica column chromatography using an 5% Et_2O in pentane eluent, resulting in 0.219 g (0.43 mmol, 86.5%) of compound **49a** (mixture of enantiomers) as colorless oil. TLC: Rf = 0.7, 10% Et_2O in pentane. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 4.71 (q, *J* = 5.4 Hz, 1H), 3.70 – 3.59 (m, 2H), 3.51 – 3.43 (m, 2H), 2.64 – 2.51 (m, 1H), 2.17 – 2.06 (m, 4H), 1.57 (d, *J* = 2.4 Hz, 2H), 1.30 (d, *J* = 5.4 Hz, 3H), 1.19 (t, *J* = 7.1 Hz, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ: 99.69, 77.41, 77.16, 76.91, 68.60, 60.90, 36.34, 34.04, 32.46, 31.98, 31.93, 27.69, 19.89, 15.42.



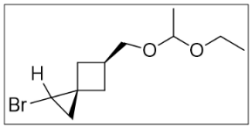
Compound 49b: 8.90 mmol (2.402 g) of compound **48b** was dissolved in 40 mL of dry Et_2O, 0.46 mmol (87 mg) of TsOH monohydrate was added, and the reaction mixture was cooled to 0 °C before dropwise addition of 54.6 mmol (5.25 mL, 3.94 g) of vinyl ethyl ether over 5 minutes. The reaction mixture was stirred for 1 hour, filtered while cold over 4.5 g of basic Al_2O_3 (0.5 g / mmol) using a filter syringe, and concentrated using rotary evaporation. Purification was performed with silica column chromatography using an 2-5% Et_2O in pentane eluent, resulting in 2.533 g (7.41 mmol, 83.2%) of compound **49b** (mixture of enantiomers) as colorless oil. **TLC**: Rf = 0.3, 4% Et_2O in pentane. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 4.70 (q, *J* = 5.4 Hz, 1H), 3.69 – 3.58 (m, 2H), 3.53 – 3.40 (m, 2H), 2.65 – 2.53 (m, 1H), 2.53 – 2.43 (m, 2H), 1.89 – 1.78 (m, 2H), 1.57 (s, 2H), 1.30 (d, *J* =

5.4 Hz, 3H), 1.19 (t, *J* = 7.1 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ: 99.66, 77.48, 77.16, 76.84, 68.86, 60.87, 36.31, 34.63, 32.59, 31.78, 31.76, 28.12, 19.88, 15.43.



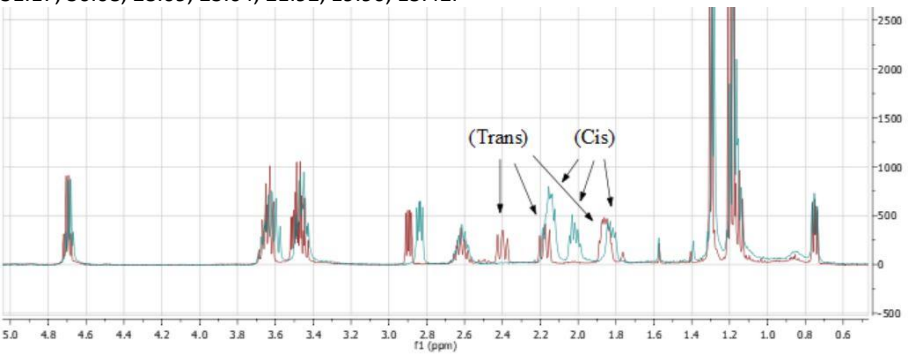


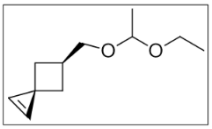
Compound 50a: 0.585 mmol (0.200 g) of compound **49a** was dissolved in 1.5 mL of dry Et_2O, 0.037 mmol (11 µL, 10.5 mg) of $(iPrO)_4Ti$ was added, followed by dropwise addition of 1 mL (1.0 mmol) a freshly prepared 1M EtMgBr in Et_2O solution. Initial formation of the $Et_2(iPrO)_2Ti$ catalyst was observed by decoloring of the formed yellow solution, where after the reaction started to turn brown indicating progress of the debromination reaction. The reaction was stirred for 30 minutes and quenched by dropwise addition to 3 mL of a sat. NH_4Cl (aq.) solution, which was extracted twice with Et_2O. The organic layers were combined, washed twice with H_2O, dried using $MgSO_4$, filtered and concentrated under reduced pressure. NMR analysis showed 66% conversion, so the procedure was repeated with 0.5 mmol EtMgBr, resulting in full conversion of the starting material. 114 mg (0.433 mmol, 74.0%) of compound **50a** was obtained (inseparable mixture of diastereo- and enantiomers) as a pale-yellow oil. TLC: Rf = 0.50, 5% Et_2O in pentane. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 4.69 (q, *J* = 5.3 Hz, 1H), 3.71 – 3.55 (m, 2H), 3.53 – 3.38 (m, 2H), 2.84 (dd, *J* = 7.7, 4.3 Hz, 1H), 2.62 (dt, *J* = 13.5, 6.9 Hz, 1H), 2.21 – 2.08 (m, 2H), 2.08 – 1.95 (m, 1H), 1.82 (dd, *J* = 11.6, 5.4 Hz, 1H), 1.29 (d, *J* = 4.9 Hz, 3H), 1.23 – 1.06 (m, 4H), 0.75 (dd, *J* = 6.6, 4.4 Hz, 1H). ^{13}C NMR (101 MHz, $CDCl_3$) δ: 99.65, 69.18, 69.08, 60.96, 60.80, 34.03, 32.01, 31.97, 31.24, 31.18, 29.72, 28.13, 23.08, 21.56, 21.54, 19.90, 19.88, 15.39.



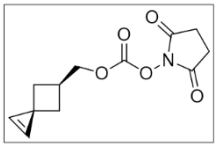
Compound 50b: 7.16 mmol (2.448 g) of compound **49b** was dissolved in 15 mL of dry Et_2O, 0.36 mmol (110 µL, 105 mg) of $(iPrO)_4Ti$ was added, followed by dropwise addition of 9.3 mmol EtMgBr (3.1 mL of a 3M solution in Et_2O). Initial formation of the $Et_2(iPrO)_2Ti$ catalyst was observed by decoloring of the formed yellow solution, where after the reaction started to turn brown indicating progress of the debromination reaction. The reaction was stirred for 30 minutes and quenched by dropwise addition to 20 mL of a sat. NH_4Cl (aq.) solution, which was extracted twice with Et_2O. The organic layers were combined, washed with H_2O, washed with brine, dried using $MgSO_4$, filtered and concentrated under reduced pressure. NMR analysis showed 40% conversion, so the procedure was repeated with 3.1 mmol EtMgBr, resulting in full conversion of the starting material. 1.472 g (5.59 mmol, 78.2%) of compound **50b** was obtained (inseparable mixture of diastereo- and enantiomers) as a pale-yellow oil. TLC: Rf = 0.50, 5% Et_2O in pentane. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 4.70 (q, *J* = 5.4 Hz, 1H), 3.70 – 3.59 (m, 2H), 3.53 – 3.41 (m, 2H), 2.90 (dd,

J = 7.7, 4.3 Hz, 1H), 2.68 – 2.56 (m, 1H), 2.40 (dd, *J* = 11.2, 9.7 Hz, 1H), 2.22 – 2.13 (m, 1H), 1.92 – 1.80 (m, 2H), 1.30 (d, *J* = 5.4 Hz, 3H), 1.19 (t, *J* = 7.1 Hz, 3H), 1.17 – 1.11 (m, 1H), 0.75 (dd, *J* = 6.7, 4.4 Hz, 1H). ^{13}C NMR (101 MHz, $CDCl_3$) δ: 99.63, 69.36, 60.89, 60.86, 32.18, 32.15, 31.22, 31.17, 30.08, 28.09, 23.04, 21.92, 19.90, 15.42.



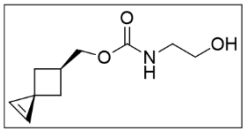


Compound 51 (**Method A**): 0.433 mmol (114 mg) of compound **50a** was dissolved in 2 mL of DMSO, cooled to 0 °C (according to literature procedure) forming a solid, and 1mL of DMSO containing 0.93 mmol (104 mg) of KOtBu was added dropwise on top of the solid surface. The reaction mixture was allowed to "stir" for 5 hours while warming up to room temperature. The reaction mixture was quenched by dropwise addition to 20 mL sat. NH_4Cl (aq.), and extracted twice with Et_2O. The organic layers were combined, washed with brine, dried using $MgSO_4$, and concentrated using rotary evaporation. Purification was performed with silica column chromatography using an 5% Et_2O in pentane eluent resulting in 35 mg (0.19 mmol, 44%) of compound **51** (mixture of enantiomers) as a colorless oil. (**Method B**): 5.32 mmol (1.40 g) of compound **50b** was dissolved in 20 mL of DMSO, and 8.5 mL of DMSO containing 8.5 mmol (0.95 g) of KOtBu was added dropwise to the solution over 20 minutes. The reaction mixture was allowed to stir for 1 hours. The reaction mixture was quenched by dropwise addition to 200 mL sat. NH_4Cl (aq.), and extracted twice with Et_2O. The organic layers were combined, washed twice with H2O, once with brine, dried using $MgSO_4$, and concentrated using rotary evaporation. Purification was performed with silica column chromatography using an 2-4% Et_2O in pentane eluent resulting in 0.895 g (4.91 mmol, 92.3%) of compound **51** (mixture of enantiomers) as a colorless oil. TLC: Rf = 0.55, 5% Et_2O in pentane. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.40 (dd, *J* = 14.4, 1.2 Hz, 2H), 4.72 (q, *J* = 5.4 Hz, 1H), 3.73 – 3.56 (m, 2H), 3.55 – 3.40 (m, 2H), 2.48 – 2.33 (m, 1H), 2.31 – 2.16 (m, 2H), 1.83 (ddd, *J* = 11.0, 8.0, 3.2 Hz, 2H), 1.31 (d, *J* = 5.4 Hz, 3H), 1.21 (t, *J* = 7.1 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ: 122.03, 121.84, 99.61, 70.23, 60.81, 38.92, 38.88, 27.88, 21.46, 19.98, 15.44.

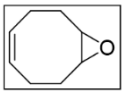


Compound 52: 3.84 mmol (0.700 g) of compound **51** was dissolved in 8 mL of acetonitrile, cooled to 0 °C, 8 mL of 3M HCl aq. was added dropwise, and the reaction mixture was stirred for 30 minutes. Reaction completion was checked by TLC (Rf = 0.2, 20% EtOAc in pentane). The reaction mixture was extracted with Et_2O, washed with brine, dried using $MgSO_4$, filtered and concentrated using rotary evaporation (40 °C, >500 mbar), resulting in the unstable and volatile crude intermediate. The crude intermediate was taken up in 8 mL of dry acetonitrile, cooled to 0 °C and 7.9 mmol (1.1 mL, 0.80 g) of TEA followed by portion wise addition of 8.0 mmol (2.05 g) N,N'-disuccinimidyl carbonate were

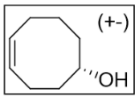
added over 30 minutes. The clear solution was allowed to stir overnight at room temperature forming a suspension. The suspension was concentrated using rotary evaporation, taken up in a minimal amount of 1:2:2 mixture of Et_2O:pentane:chloroform for silica column chromatography. Purification was performed using an 5-20% Et_2O in pentane eluent resulting in 0.541 g (2.15 mmol, 56.6%) of compound **52** as a white solid. TLC: Rf = 0.9, 50% EtOAc in DCM. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.39 – 7.35 (s, 1H), 7.23 – 7.05 (m, 1H), 4.35 (d, *J* = 7.4 Hz, 2H), 2.72 (s, 4H), 2.62 – 2.49 (m, 1H), 2.30 (s, 1H), 2.29 – 2.19 (m, 2H), 1.89 (ddd, *J* = 12.4, 5.8, 1.1 Hz, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ: 168.87, 151.61, 128.89, 128.09, 125.17, 121.18, 120.91, 76.84, 75.33, 37.80, 26.49, 25.29, 20.77.



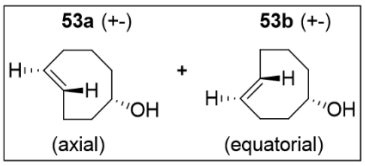
Compound 53: 0.20 mmol (50 mg) of compound **52** was dissolved in 1 mL of dry DCM, 0.20 mmol (28 µL, 20 mg) of TEA and 0.39 mmol (24 µL, 24 mg) 2-amino ethanol were added and the reaction mixture was stirred for 30 minutes at room temperature. The reaction mixture was directly used for purification. Purification was performed using an 20-60% EtOAc in DCM eluent resulting in 33 mg (0.167 mmol, 83.4%) of compound **53** as a colorless oil. TLC: Rf = 0.5, 50% EtOAc in DCM. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.46 – 7.31 (m, 2H), 5.33 (s, 1H), 4.11 (d, *J* = 7.4 Hz, 2H), 3.68 (t, *J* = 5.0 Hz, 2H), 3.31 (d, *J* = 3.2 Hz, 2H), 2.86 (s, 1H), 2.52 – 2.36 (m, 1H), 2.27 – 2.11 (m, 2H), 1.85 (dd, *J* = 12.5, 5.4 Hz, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ: ^{13}C NMR (101 MHz, $CDCl_3$) δ 157.88, 121.76, 121.48, 69.72, 62.30, 43.51, 38.35, 27.09, 21.21.



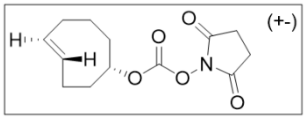
Compound 54: 0.68 mol (117 g, 1.0 L of a 0.68M solution in chloroform) of *meta*-chloroperbenzoic acid was slowly added to 0.55 mol (59.1 g, 61.4 mL) of cyclooctadiene, using a dropping funnel over 2 hours at 0°C, and the reaction mixture was stirred overnight at room temperature. Reaction completion was checked by TLC (Rf = 0.5, 10% Et_2O in pentane). The reaction mixture concentrated using rotary evaporation and purified with silica column chromatography using an 5-10% Et_2O in pentane eluent resulting in 39.3 g (0.316 mol, 56%) of compound **54** with solvent impurities (20% wt, Et_2O/pentane). **TLC**: Rf = 0.5, 10% Et_2O in pentane. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 5.62 – 5.44 (m, 2H), 3.06 – 2.93 (m, 2H), 2.52 – 2.31 (m, 2H), 2.18 – 2.05 (m, 2H), 2.00 (m, 4H). ^{13}C NMR (101 MHz, $CDCl_3$) δ: 128.88, 56.74, 28.16, 23.73.



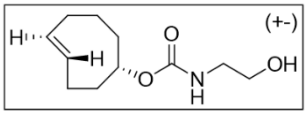
Compound 55: 316 mmol (39.3 g) of compound **54** was dissolved in 200 mL of dry THF. To this solution 248 mmol (62 mL) 4M $LiAlH_4$ in THF was added dropwise at 0°C and the reaction mixture was stirred overnight at room temperature. The reaction mixture was quenched by adding 200 mL H_2O and the resulting suspension was filtered. The filtrate was extracted with Et_2O, the organic layer was washed with brine, dried with $MgSO_4$ and concentrated using rotary evaporation. Purification was performed with silica column chromatography using pure pentane followed by 25-75% Et_2O in pentane eluents resulting in 34.8 g (276 mmol, 87.3%) of compound **55** as a colorless oil. TLC: Rf = 0.5, 50% Et_2O in pentane. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 5.75 – 5.52 (m, 2H), 3.79 (td, *J* = 8.9, 4.1 Hz, 1H), 2.36 – 2.04 (m, 4H), 2.00 – 1.78 (m, 2H), 1.75 – 1.45 (m, 4H). ^{13}C NMR (101 MHz, $CDCl_3$) δ: 130.26, 129.68, 72.87, 37.83, 36.40, 25.77, 25.00, 22.88.



Compound 56: 7.9 mmol (1.0 g) of compound **55** was irradiated (λ = 254 nm) for 20 hours in presence of 23.8 mmol methyl benzoate (3.24 g, 2.97 mL, 3 eq.) in a quartz flask containing 500 mL of 10% Et_2O in heptane. During irradiation the reaction mixture was circulated at a flow rate of 40 mL/min through a column (40 g size) filled with 8 cm of dry silica followed by 17 g of 10% $AgNO_3$ impregnated silica. After 20 hours a sample was taken to confirm total consumption of compound **55** using methyl benzoate as a reference. The irradiation was turned off and the column was flushed and rinsed by circulating 500 mL fresh 10% Et_2O in heptane, followed by circulation with air for 2 hours to try the column. The silica was transferred to an Erlenmeyer flask and 250 mL of 28% NH_4OH (aq.), followed by 250 mL of DCM were added, while stirring for 1 hour. The suspension is filtered, the layers were separated, the organic layer was dried with $MgSO_4$, filtrated and concentrated using rotary evaporation. The crude mixture containing axial and equatorial isomers was purified using silica column chromatography using an 1-8% EtOAc in pentane eluent, resulting in 2.31 mmol (291 mg, 29%) of compound **56a** (axial isomer, enantiomeric mixture) and 3.55 mmol (448 mg, 45%) of compound **56b** (equatorial isomer, enantiomeric mixture). Compound **56a**: TLC: Rf = 0.30, 25% EtOAc in pentane. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.26 (s, 0H), 5.62 – 5.43 (m, 2H), 4.07 – 3.93 (m, 1H), 2.42 – 1.99 (m, 5H), 1.91 – 1.53 (m, 5H). Compound **56b**: TLC: Rf = 0.60, 25% EtOAc in pentane. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.26 ($CDCl_3$) 5.60 – 5.25 (m, 2H), 3.44 – 3.30 (m, 1H), 2.25 (dddt, *J* = 25.4, 11.6, 5.7, 3.3 Hz, 3H), 1.88 (dqd, *J* = 13.9, 6.2, 5.0, 2.6 Hz, 4H), 1.71 – 1.44 (m, 3H).

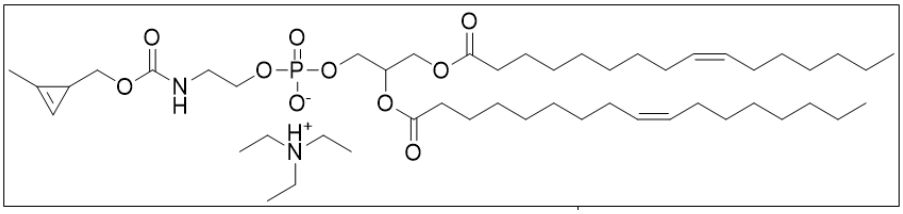


Compound 57: 0.634 mmol (80 mg) of **56a** was dissolved in 5 mL of dry acetonitrile, 2.2 mmol (0.24 g, 0.3 mL) TEA, followed by slow addition of 1.56 mmol (400 mg) N,N'-disuccinimidyl carbonate and the reaction mixture was stirred for three days. The reaction mixture was suspended in Et_2O, washed with 0.1M HCl (aq.), washed with brine, dried with $MgSO_4$ and concentrated using rotary evaporation. Purification was performed using silica column chromatography using an 10-20% EtOAc in pentane eluent, resulting in 50 mg (0.187 mmol, 29.5%) of compound **57** as clear oil. TLC: Rf = 0.7, 100% EtOAc. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.26 ($CDCl_3$), 5.73 – 5.45 (m, 2H), 4.95 (ddt, *J* = 10.5, 5.5, 1.3 Hz, 1H), 2.81 (d, *J* = 5.3 Hz, 5H), 2.51 – 2.25 (m, 4H), 2.24 – 2.09 (m, 2H), 1.85 (m, 2H), 1.79 – 1.57 (m, 3H), 1.42 – 1.26 (m, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ: 168.92, 151.02, 135.67, 131.31, 78.67, 77.48 - 76.84 ($CDCl_3$), 40.54, 34.13, 32.17, 29.66, 27.65, 25.55.

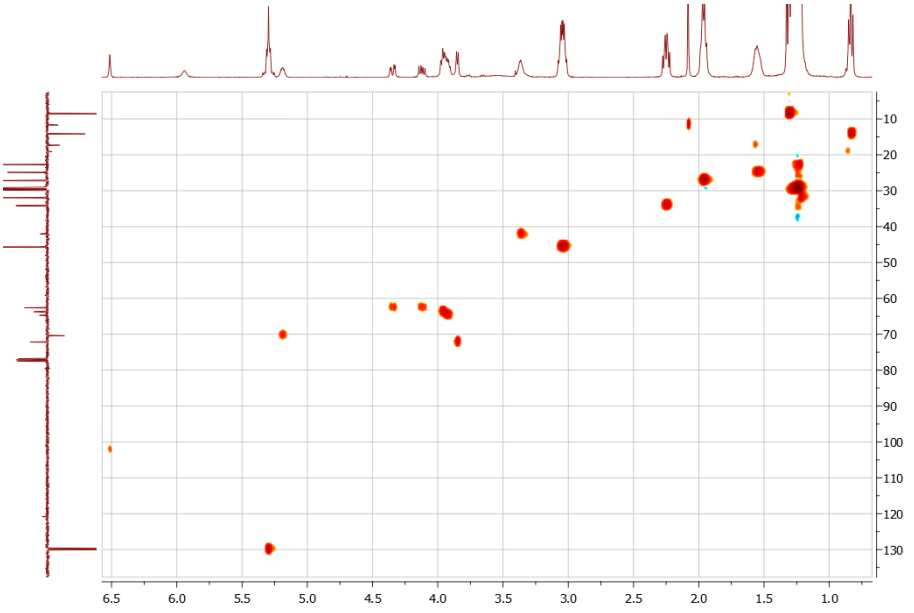


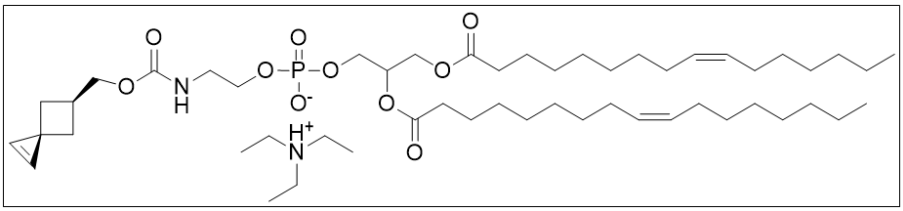
Compound 58: 0.10 mmol (30 mg) of compound **57** was dissolved in 1 mL of dry DCM, 0.10 mmol (14 µL, 10 mg) of TEA and 0.20 mmol (12 µL, 12 mg) of 2-amino ethanol were added respectively and the reaction mixture was stirred for 30 minutes at room temperature. Reaction completion was checked by TLC (Rf = 0.5, 50% EtOAc in DCM) and the reaction appeared to be at 50% completion. No progress was observed over 3 hours, so 0.10 mmol (6 µL, 6 mg) of 2-amino ethanol was added and the reaction mixture was stirred for another 3 hours. The reaction appeared to be complete according to TLC analysis. The reaction mixture was directly used for purification. Purification was performed using an 50% EtOAc in DCM eluent resulting in 20 mg (0.094 mmol, 94%) of compound **58** as a colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 5.58 – 5.47 (m, 2H), 5.23 (s, 1H), 4.90 (d, *J* = 5.3 Hz, 1H), 3.76 – 3.64 (m, 2H), 3.36 (s, 2H), 2.50 (s, 1H), 2.36 – 2.19 (m, 4H), 2.16 – 2.03 (m, 1H), 1.82 (ddd, *J* = 19.9, 11.7, 5.2 Hz, 2H), 1.73 – 1.41 (m, 2H), 1.29 – 1.13 (m, 1H).

^{13}C NMR (101 MHz, $CDCl_3$) δ: 135.45, 131.86, 70.61, 62.60, 43.57, 41.16, 34.41, 32.75, 30.08, 28.10.

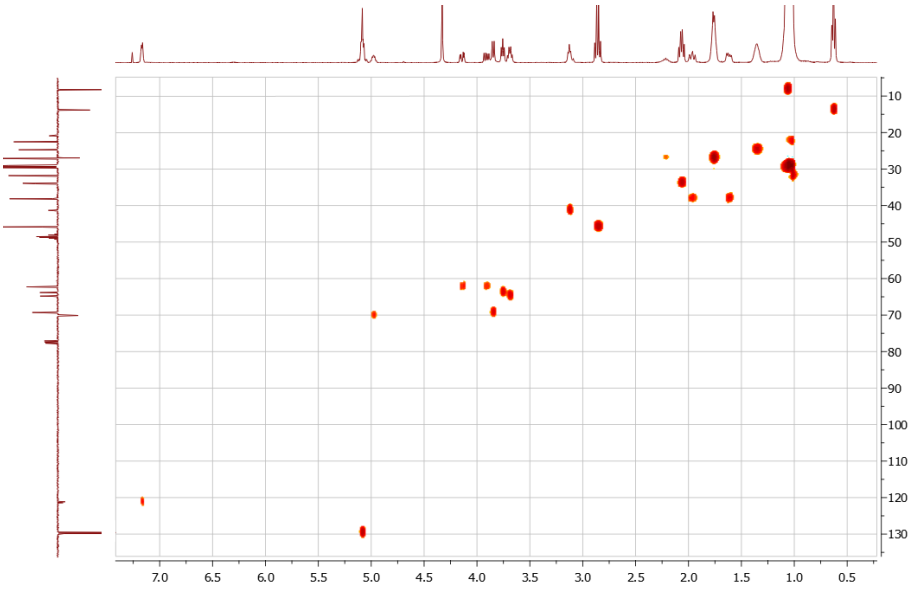


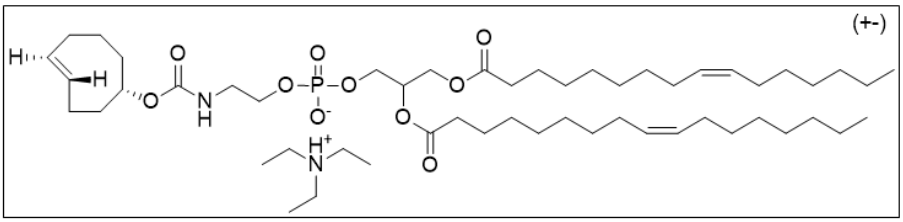
Compound 59: Compound **45** was dissolved dry chloroform, TEA and DOPE were added respectively and the reaction mixture was stirred overnight at room temperature. Reaction completion was checked by TLC (20% MeOH in $CHCl_3$). The reaction mixture was directly used for purification. Purification was performed using TEA neutralized silica and a 0-4% MeOH with 0.25% TEA in $CHCl_3$ eluent resulting in compound **59** as a colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 12.14 (s, 1H), 6.51 (s, 1H), 5.94 (s, 1H), 5.36 – 5.24 (m, 4H), 5.23 – 5.13 (m, 1H), 4.35 (dd, J = 12.0, 3.3 Hz, 1H), 4.12 (dd, J = 12.0, 6.5 Hz, 1H), 4.00 – 3.88 (m, 4H), 3.85 (d, J = 5.1 Hz, 2H), 3.42 – 3.28 (m, J = 2.6 Hz, 2H), 3.12 – 2.96 (m, 6H, TEA), 2.25 (dd, J = 13.3, 7.4 Hz, 4H), 2.08 (d, J = 0.9 Hz, 3H), 2.03 – 1.91 (m, 8H), 1.56 (d, J = 5.0 Hz, 5H), 1.41 – 1.09 (m, 49H, DOPE, TEA), 0.84 (t, J = 6.8 Hz, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ: 173.47, 173.08, 157.12, 130.03, 129.76, 120.83, 72.20, 70.42, 70.34, 64.72, 63.75, 62.63, 45.68, 42.05, 34.28, 34.10, 31.95, 29.81, 29.79, 29.57, 29.36, 29.30, 29.28, 29.20, 29.16, 27.26, 27.23, 24.93, 24.89, 22.73, 19.13, 17.33, 14.17, 11.72, 8.61. HSQC:



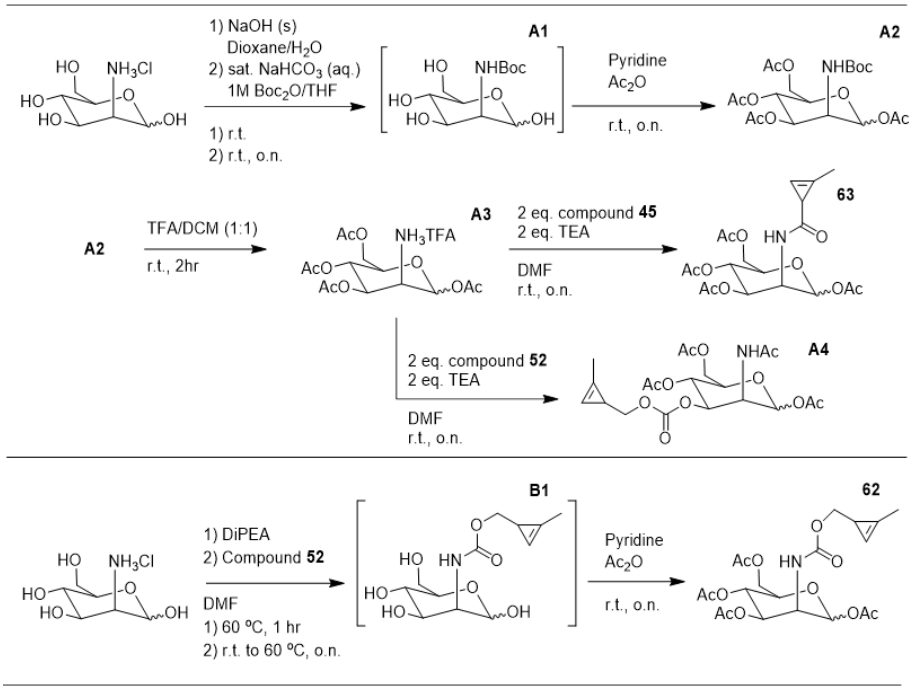


Compound 60: 0.18 mmol (46 mg) of compound **52** was dissolved in 2.0 mL of dry chloroform, 0.39 mmol (52 µL, 40 mg) of TEA and 0.171 mmol (127 mg) of DOPE were added respectively and the reaction mixture was stirred overnight at room temperature. Reaction completion was checked by TLC (Rf = 0.5, 20% MeOH in $CHCl_3$). The reaction mixture was directly used for purification. Purification was performed using TEA neutralized silica and a 0-4% MeOH with 0.25% TEA in $CHCl_3$ eluent resulting in 140 mg (0.149 mmol, 87%) of compound **60** as a colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$:MeOD) δ: 7.17 (d, J = 4.0 Hz, 2H), 5.15 – 5.02 (m, 4H), 5.01 – 4.93 (m, 1H), 4.14 (dd, J = 12.0, 3.4 Hz, 1H), 3.91 (dd, J = 12.0, 6.6 Hz, 1H), 3.85 (d, J = 7.4 Hz, 2H), 3.76 (t, J = 5.8 Hz, 2H), 3.69 (dd, J = 12.4, 5.4 Hz, 2H), 3.13 (t, J = 4.9 Hz, 2H), 2.86 (q, J = 7.3 Hz, 6H, TEA), 2.27 – 2.15 (m, 1H), 2.07 (dd, J = 13.2, 7.4 Hz, 4H), 1.96 (dd, J = 11.5, 9.5 Hz, 2H), 1.76 (d, J = 5.7 Hz, 8H), 1.62 (dd, J = 12.3, 5.5 Hz, 2H), 1.36 (d, J = 3.4 Hz, 4H), 1.14 – 0.95 (m, 49H, DOPE, TEA), 0.63 (t, J = 6.8 Hz, 6H). ^{13}C NMR (101 MHz, $CDCl_3$:MeOD) δ: 173.40, 172.98, 157.36, 129.60, 129.30, 121.19, 120.92, 69.90, 69.82, 69.05, 64.62, 64.56, 63.60, 63.54, 62.04, 48.91, 48.70, 48.49, 48.28, 48.06, 47.85, 47.64, 45.64, 41.14, 41.07, 37.91, 33.81, 33.66, 31.56, 29.39, 29.37, 29.16, 28.97, 28.94, 28.88, 28.86, 28.77, 28.74, 28.72, 26.83, 26.80, 26.72, 24.52, 24.48, 22.31, 20.66, 13.59, 8.01. HSQC:





Compound 61: 26 µmol (7 mg) of compound **57** was dissolved in 0.2 mL of dry chloroform, 26 µmol (3.6 µL, 2.6 mg) of TEA and 17.4 µmol (13 mg) of DOPE were added respectively and the reaction mixture was stirred overnight at room temperature. Reaction completion was checked by TLC (Rf = 0.5, 15% MeOH in $CHCl_3$). The reaction mixture was directly used for purification. Purification was performed using TEA neutralized silica and a 0-4% MeOH with 0.25% TEA in $CHCl_3$ eluent resulting in 15 mg (15.1 µmol, 87%) of compound **61** as a colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$:MeOD) δ: 5.52 – 5.25 (m, 2H), 5.12 (dt, J = 5.7, 3.5 Hz, 4H), 5.02 (td, J = 8.7, 5.3 Hz, 1H), 4.64 (dd, J = 10.2, 5.1 Hz, 1H), 4.18 (dd, J = 12.0, 3.4 Hz, 1H), 3.95 (dd, J = 12.0, 6.7 Hz, 1H), 3.82 – 3.74 (m, J = 13.1, 7.4 Hz, 2H), 3.74 – 3.64 (m, 2H), 3.20 – 3.14 (m, 2H), 2.90 (q, J = 7.3 Hz, 6H, TEA), 2.17 – 1.99 (m, 8H), 1.90 – 1.84 (m, 1H), 1.84 – 1.75 (m, 8H), 1.68 – 1.61 (m, 1H), 1.61 – 1.52 (m, 1H), 1.46 (dd, J = 13.6, 4.6 Hz, 1H), 1.43 – 1.36 (m, 4H), 1.36 – 1.28 (m, 1H), 1.15 – 1.02 (m, 50H). ^{13}C NMR (101 MHz, $CDCl_3$:MeOD) δ: 173.59, 173.16, 156.74, 135.12, 131.32, 129.71, 129.42, 129.41, 70.04, 70.00, 64.54, 63.53, 62.24, 48.91, 48.74, 48.57, 48.40, 48.23, 48.06, 47.89, 45.85, 41.29, 41.23, 40.77, 34.02, 33.94, 33.77, 32.44, 31.64, 29.58, 29.48, 29.46, 29.45, 29.24, 29.05, 29.03, 28.97, 28.94, 28.87, 28.86, 28.84, 28.82, 27.63, 26.92, 26.89, 25.26, 25.04, 24.62, 24.58, 22.39, 22.01, 13.66, 8.14. HSQC:



Compound A2: 4.92 mmol (1.062 g) of D-mannosamine hydrochloride was added to 25 mL of a dioxane:H_2O mixture (4:1, v:v), 5.21 mmol (0.21 g) of NaOH was added and the reaction mixture was stirred at room temperature until a clear solution was obtained. Then, 4.6 mmol (4 mL) of a saturated $NaHCO_3$ (aq) solution and 5 mmol (5 mL) of a freshly prepared solution of Boc_2O/THF were added and the reaction mixture was stirred overnight at room temperature. Reaction completion was checked by TLC (Rf = 0.8, 30% MeOH in CH_2Cl_2). The reaction mixture was concentrated using rotary evaporation to an oily suspension, and dried via co-evaporation with dioxane. The crude mixture was suspended in MeOH (instead of CH_2Cl_2, according to literature) and filtered. The solution was again concentrated using rotary evaporation to form, resuspended in methanol and the solvent was decanted into a new container and concentrated using rotary evaporation to form intermediate **A1** as a foamy solid. Without further purification Intermediate **A1** was dissolved in 10 mL of dry pyridine, 26 mmol (2.5 mL) of Ac_2O was added and the reaction mixture was stirred overnight at room temperature. Reaction completion was checked by TLC (Rf = 0.9, 100% EtOAc). 50 mL of EtOAc was added to the reaction mixture. The reaction mixture was washed with 1.0M HCl (aq.), washed with brine, dried using $MgSO_4$, and concentrated using rotary evaporation. Purification was performed with silica column chromatography using a 20%-40% EtOAc in Pentane eluent resulting in 1.259 g (2.814 mmol, 57%) of compound **A2** (α:β mixture, 3:1) as a white foam. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 6.00 (s, 1H, α), 5.79 (s, 1H, β), 5.30 – 5.02 (m, 2H), 5.02 – 4.90 (m, 1H), 4.38 (dd, *J* = 9.2, 3.2 Hz, 1H, β), 4.26 (dd, *J* = 9.2, 2.9 Hz, 1H, α), 4.19 (m, 1H), 4.08 – 3.98 (m, 1H), 3.98 – 3.92 (m, 1H, α), 3.73 (m, 1H, β), 2.23 – 1.81 (m, 12H), 1.39 (s, 9H). ^{13}C NMR (101 MHz, $CDCl_3$) δ: 170.66, 170.10, 169.77, 168.56, 168.25, 155.83, 155.17, 92.06, 90.86, 80.36, 80.06, 73.26, 71.44, 70.11, 69.25, 65.54, 65.44, 62.16, 62.02, 50.67, 50.41, 28.21, 20.87, 20.82, 20.73, 20.67.

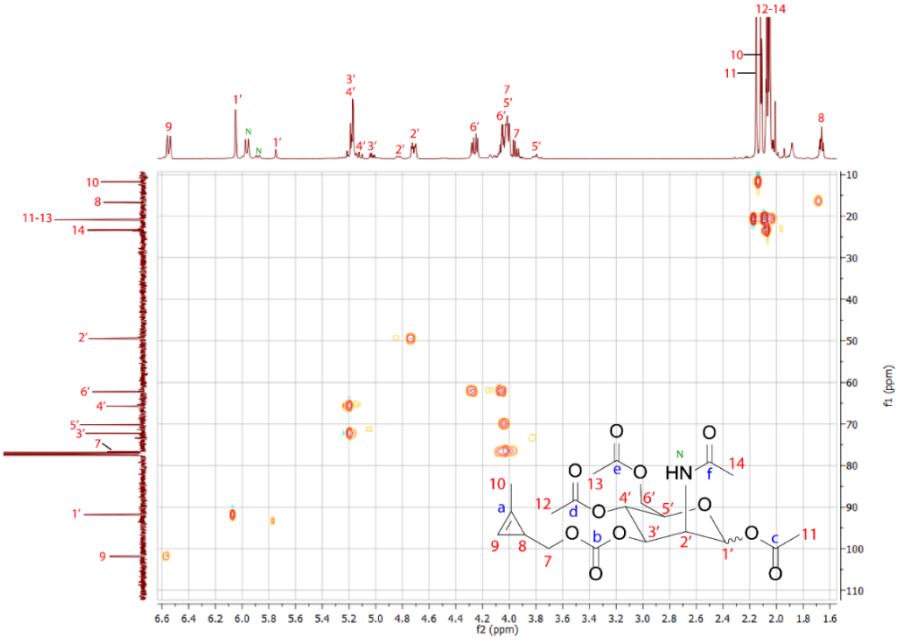
Compound A3: 186 µmol (86 mg) of compound **A2** was dissolved in 2 mL of TFA:DCM mixture (1:1, v:v) and the reaction mixture was stirred for 2 hours at room temperature. Reaction completion was checked by TLC (two spots, Rf = 0.3, 100% EtOAc). The reaction mixture was concentrated using rotary evaporation, and co-evaporated twice with MeOH resulting in Compound **A4** (α:β mixture, 3:1) in quantitative yield. ^{1}H NMR (400 MHz, MeOD) δ: 6.28 (d, *J* = 1.6 Hz, 1H, α), 6.10 (d, *J* = 1.8 Hz, 1H, β), 5.53 – 5.46 (m, 1H), 5.37 (t, *J* = 9.9 Hz, 1H, α), 5.25 (t, *J* = 9.9 Hz, 1H, β), 4.32 (m, 2H), 4.22 (ddd, *J* = 9.6, 5.7, 2.6 Hz, 1H, α), 4.11 (m, 1H,), 4.03 (ddd, *J* = 9.4, 5.9, 2.4 Hz, 1H, β), 4.02 – 3.97 (m, 1H), 3.93 (dd, *J* = 4.8, 1.8 Hz, 1H), 2.19 (s, 3H), 2.16 (s, 1H), 2.11 (d, *J* = 2.0 Hz, 4H), 2.07 – 2.02 (m, 9H). ^{13}C NMR (101 MHz, MeOD) δ: 172.32, 171.22, 170.92, 169.48, 90.75, 90.46, 74.54, 71.66, 70.28, 68.77, 66.39, 66.27, 63.27, 52.98, 52.23, 20.58, 20.49.

Compound 63: 50 µmol (23 mg) of compound **A3** was dissolved in 0.5 mL dry DMF, 109 µmol (29 mg) of compound **42** and 100 µmol (14 µL) of TEA were added and the reaction mixture was stirred overnight at room temperature. Reaction completion was checked by TLC (Rf = 0.5, 100% EtOAc). 5 mL of EtOAc was added to the reaction mixture and the resulting solution was washed with 0.1M HCl (aq.), washed with brine, dried using $MgSO_4$, and concentrated using rotary evaporation. Purification was performed with silica column chromatography using a 50%-100% EtOAc in Pentane eluent resulting in 5.0 mg (11.7 µmol, 23%) of compound **63** as a colorless solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 6.46 (d, *J* = 9.1 Hz, 1H), 6.00 (dd, *J* = 4.6, 1.7 Hz, 1H), 5.62 (dd, *J* = 15.3, 9.6 Hz, 1H), 5.30 td, , *J* = 10.0, 4.4 Hz, 1H), 5.15 (td, *J* = 10.1, 3.2 Hz, 1H), 4.67 (ddd, *J* = 9.5, 6.0, 1.8 Hz, 1H), 4.25 (ddd, *J* = 12.1, 7.6, 4.6 Hz, 1H), 4.10 – 3.99 (m, 2H), 2.23 (d, *J* = 1.0 Hz, 1.5H), 2.19 (d, *J* = 1.0 Hz, 1.5H), 2.17 (s, 3H), 2.11 (s, 3H), 2.08 – 2.03 (m, *J* = 3.6 Hz, 4H), 1.99 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ: 176.16, 170.63, 170.22, 169.82, 168.38, 114.64, 113.64, 95.95, 95.46, 92.01, 70.21, 69.24, 69.06, 65.58, 65.33, 62.20, 62.04, 49.19, 49.14, 22.50, 21.02, 20.90, 20.86, 20.83, 20.79, 10.79, 10.64.

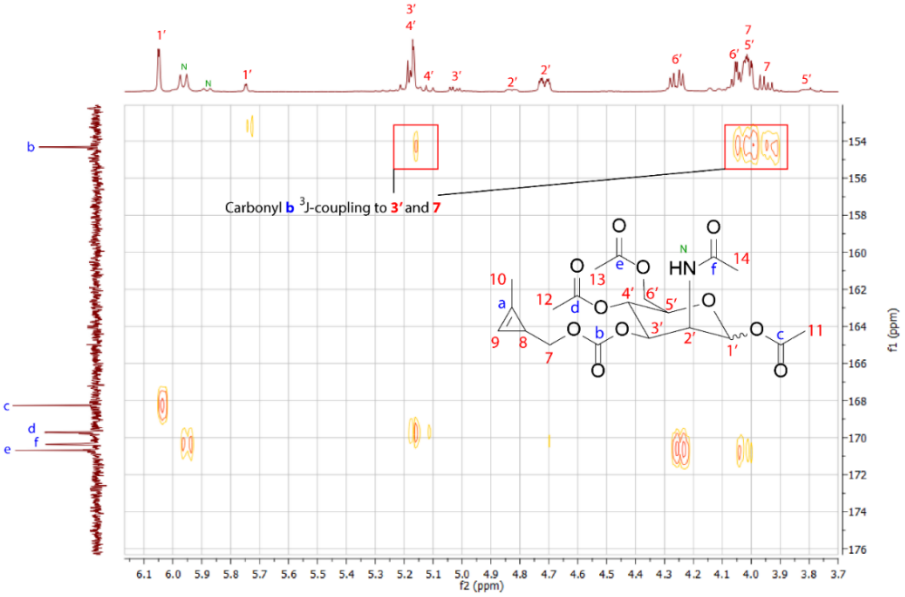
Compound 62: 0.20 mmol (43 mg) of D-mannosamine hydrochloride was added to 1 mL of a dry DMF, 0.80 mmol (139 µL) of DiPEA was added and the reaction mixture was stirred at 60 °C until a clear solution was obtained. Then, the mixture was allowed to cool to room temperature, 0.40 mmol (100 mg) of compound **45** was added and the reaction mixture was stirred overnight at room temperature, then overnight at 60 °C, and finally three days at room temperature. Reaction completion was checked by TLC (Rf = 0.7, 20% MeOH in CH_2Cl_2). The reaction mixture containing compound **B1** was concentrated using rotary evaporation. This crude mixture was dissolved in 2 mL of Pyridine, 5 mmol (0.5 mL) of Ac_2O was added and the reaction mixture was stirred overnight at room temperature. Reaction completion was checked by TLC (Rf = 0.6, 50% EtOAc in pentane). The reaction mixture was concentrated using rotary evaporation. 5 mL of DCM was added to the reaction mixture and the resulting solution was washed with twice with 10% $KHSO_4$ (aq.), washed with brine, dried using $MgSO_4$, and concentrated using rotary evaporation. Purification was performed with silica column chromatography using a 10%-70% EtOAc in pentane eluent resulting in 14 mg (31 µmol, 16%) of compound **62** as a colorless solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 6.59 (d, *J* = 6.0 Hz, 1H), 6.09 (s, 1H, α), 5.85 (s, 1H, β), 5.31 (dd, *J* = 10.1, 4.2 Hz, 1H), 5.21 (t, *J* = 10.1 Hz, 1H), 5.02 (d, *J* = 9.4 Hz, 1H), 4.50 – 4.43 (m, 1H, β), 4.34 (dd, *J* = 8.9, 3.4 Hz, 1H, α), 4.26 (dd, *J* = 12.3, 4.2 Hz, 1H), 4.07 – 3.91 (m, 4H), 2.18 (s, 3H), 2.15 (s, 3H), 2.11 (s, 3H), 2.06 (s, 3H), 2.02 (s, 3H), 1.67 (t, *J* = 4.5 Hz, 1H). ^{13}C NMR (126 MHz, $CDCl_3$) δ: 171.32, 170.77, 170.25, 169.75, 168.32, 156.39,

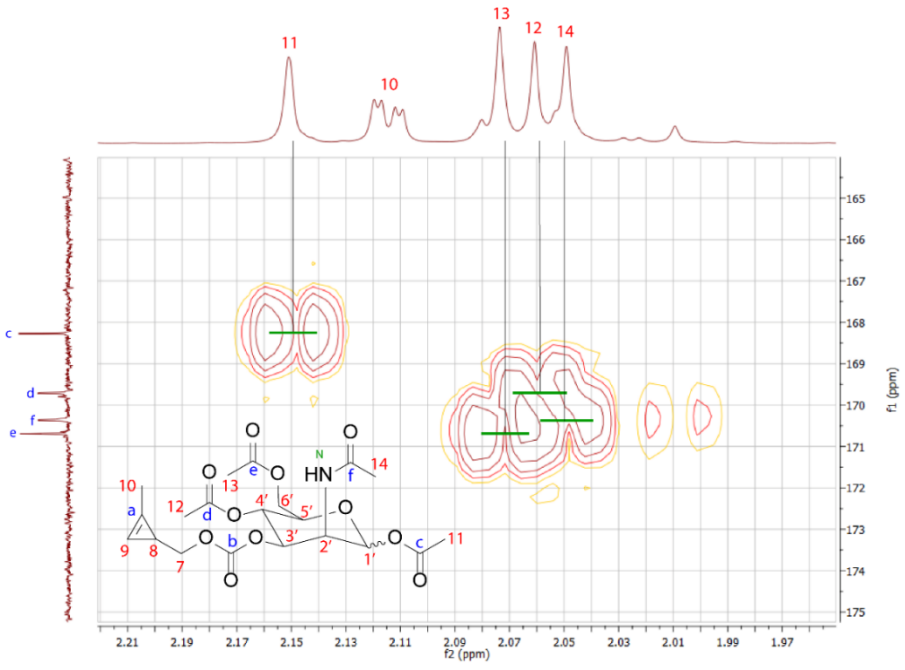
102.21, 92.09, 73.51, 70.30, 69.29, 65.44, 62.06, 60.55, 51.20, 21.21, 21.05, 20.92, 20.80, 17.17, 11.82.

Compound A4: (In an attempt to prepare compound **62**), 0.78 mmol of compound **A3** was dissolved in 4.0 mL dry DMF, 0.94 mmol (0.23 g) of compound **45** and 3.9 mmol (0.54 mL) of TEA were added and the reaction mixture was stirred overnight at room temperature. No reaction was observed. A catalytic amount of DMAP was added and the reaction was stirred overnight again. Reaction completion was checked by TLC (Rf = 0.6, 100% EtOAc). 50 mL of EtOAc was added to the reaction mixture and the resulting solution was washed with sat. $NaHCO_3$ (aq.), 0.1M HCl (aq.), washed with brine, dried using $MgSO_4$, and concentrated using rotary evaporation. Purification was performed twice with silica column chromatography, loading the crude mixture in a minimal amount of DCM, and using a 50%-100% EtOAc in Pentane eluent resulting in 36 mg (quantitative yield) of compound **A4** as a mixture of diastereomers. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 6.55 (d, *J* = 8.7 Hz, 1H), 6.05 (d, *J* = 1.7 Hz, 1H), 5.96 (d, *J* = 8.9 Hz, 1H), 5.22 – 5.12 (m, 2H), 4.71 (ddd, *J* = 8.9, 4.0, 1.9 Hz, 1H), 4.30 – 4.20 (m, 1H), 4.08 – 3.88 (m, 4H), 2.15 (s, 3H), 2.11 (dd, *J* = 3.1, 1.1 Hz, 3H), 2.07 (s, 3H), 2.06 (s, 3H), 2.05 (s, 3H), 1.66 (tt, *J* = 5.5, 1.5 Hz, 1H). ^{13}C NMR (101 MHz, $CDCl_3$) δ: 170.69, 170.37, 170.36, 169.72, 169.70, 168.27, 154.33, 120.41, 120.15, 101.96, 101.75, 91.81, 72.25, 70.14, 65.69, 62.24, 49.47, 23.38, 20.97, 20.85, 20.77, 16.73, 16.72, 11.72, 11.70. HSQC:

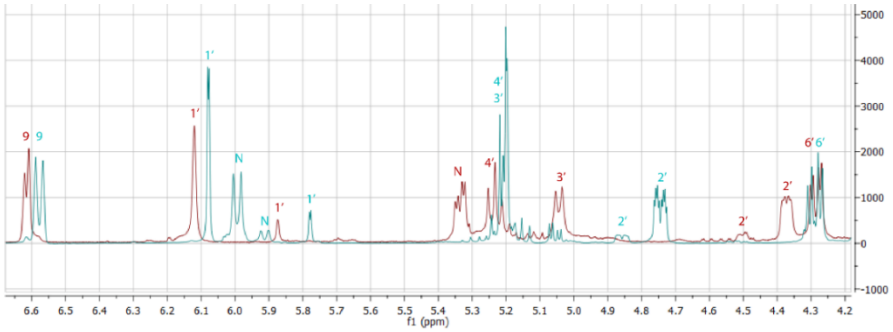


Compound A4 (HMBC)





Red: Compound **62**. **Blue**: Compound **A4** (downfield 2', downfield 3', downfield N)



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Chapter 4: Simultaneous dual-labeling of live cells using hydrophilic and hydrophobic tetrazine fluorophores

Introduction

In the previous chapter it was shown not to be possible to visualize N-akylcyclopropene mannosamine (ManNAlkCyp **62**, Chapter 3), which was metabolically incorporated in the hydrophilic glycocalyx (hydrophilic glycan rich region on the outside of the cell membrane), using tetrazine-functionalized fluorophores (**23**-**26**, **31**-**34**, Chapter 3). It was postulated that the compounds were not hydrophilic enough when compared to tetrazine-functionalized fluorophores successfully used by others in similar experiments.[1] To overcome this limitation, and not resort to difficult-to-synthesize zwitterionic fluorophores (e.g. disulfo-cyanine dyes or Alexa Fluor dyes), tetrazine-functionalized fluorophores were designed using Boc-Tzm-OH **3** as a starting point. The approach was somewhat inspired by the success of Calfluors[2], with hydrophilic moieties not attached to the fluorophore directly (**Figure 1**). In the first part of this chapter the synthesis of these molecules is described, followed by the successful labeling and fluorescence imaging of metabolically incorporated N-alkylcyclopropene mannosamine (**62**, Chapter 3) on live cells.

Dual-labeling is used to track biomolecules in tandem.[3] Previous studies generally describe the use of multiple techniques to label two biomolecules consecutively. Such approaches may suffer from a number of limitations, such as differences in the concentrations of the biomolecules to be studied, and differences in reaction kinetics of the labeling chemistries employed. In the second part of this chapter, the dual-labeling strategy is employed to simultaneously track both sterculic acid (Compound **64**, Chapter 3) and N-alkylcyclopropene mannosamine (**62**, Chapter 3) using a mixture of tetrazine-functionalized fluorophores comprising of a relative hydrophobic Bodipy-FL (Bodipy-FL **25**, Chapter 3) and relative hydrophilic Bodipy-TMR **7** or Cy5 **8**. Using a fluorophore mixture instead of consecutive labeling allows the number of steps to be reduced increasing the ease of working with sensitive live cells. As is shown here, application of a combination of tetrazines allows low concentrations, gives fast reaction times and therefore enables to swiftly perform dual-labeling of two biomolecules at once.

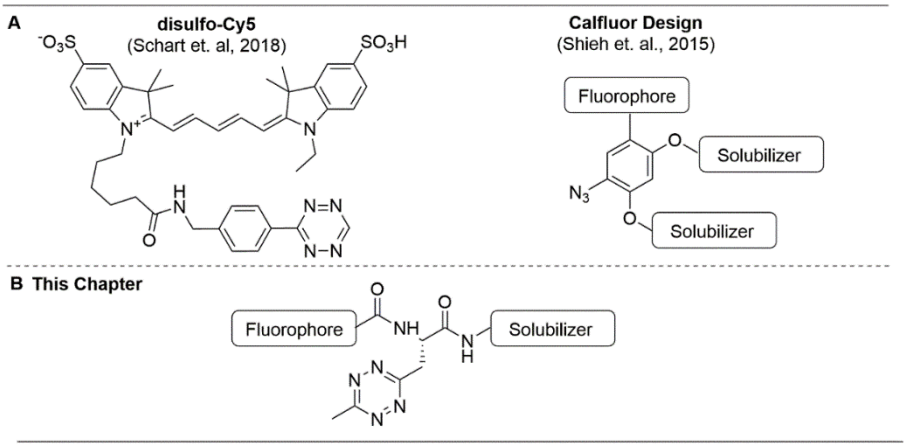


Figure 1: A) Example fluorophores used in literature to label metabolically incorporated N-functionalized mannosamines. B) General design of hydrophilic tetrazine-functionalized fluorophores in this chapter.

Synthesis of hydrophilic tetrazine-functionalized fluorophores

Zwitterionic linker **2** was synthesized in three steps from N,N-dimethyl-1,3-diaminopropane by Boc protection, subsequent reaction with 1,3-propanesultone and a final Boc deprotection under acidic anhydrous conditions (**Figure 2**). Linker **2** was then attached to Boc-Tzm-OH **3**, the synthesis of which is described in Chapter 3. Compound **3** was activated using DCC and NHS and linker **2** was attached at the C-terminal position. The Boc protective group was then removed after which three fluorophores were attached individually to give compounds **6**-**8**.

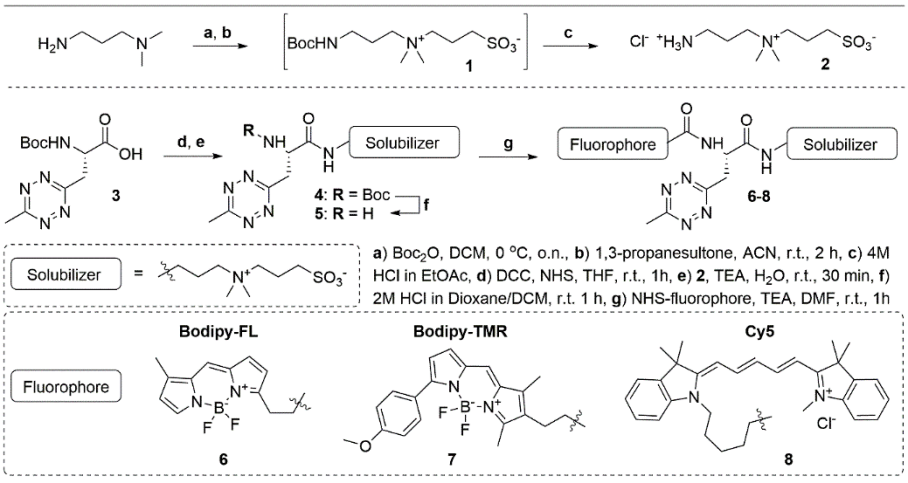


Figure 2: Synthesis of hydrophilic tetrazine-functionalized fluorophores **6**-**8**.

Regiospecific dual-labeling using hydrophilic and hydrophobic fluorophores

Following the synthesis of the hydrophilic tetrazine-functionalized fluorophores above, U2OS cells were incubated with N-alkylcyclopropene mannosamine (**62**, Chapter 3) via similar protocol to the tests performed in Chapter 3. After 2 days, the medium was refreshed, and the fluorophores were introduced for 2 hours before fluorescence imaging (**Figure 3**). The images clearly indicate the labelling of the cell membranes glycocalyx. A clear contrast to the results obtained using tetrazine-functionalized fluorophores (**23**-**26**, **31**-**34**, Chapter 3) from the previous chapter, where these parts of the cell were not visualizable.

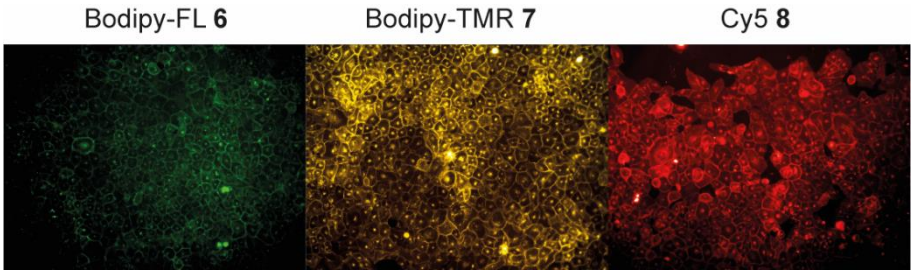


Figure 3: Fluorescence imaging of U2OS cells treated with N-alkylcyclopropene mannosamine (**62**, Chapter 3) and then with Bodipy-FL **6** (left), Bodipy-TMR **7** (middle) and Cy5 **8** (right).

To determine whether each fluorophore was capable of only labeling its respective target selectively purely based on location, instead of the type of bio-orthogonal chemistry used, a dual-labelling was performed . It was already shown that the hydrophobic tetrazine-functionalized Bodipy-FL (**25**, Chapter 3) was not capable of labeling metabolically incorporated mannosamines, due to the inability to reach inside the glycocalyx. It was still unclear whether the hydrophilic tetrazine-functionalized Bodipy-TMR **7** would in turn be unable to cross the cell membrane to reach the inside of the cell; or at least be unable to reach the hydrophobic niches in which sterculic acid had accumulated. HEK cells were therefore incubated with N-alkylcyclopropene mannosamine (**62**, Chapter 3) for 2 days, followed by a one-hour incubation with sterculic acid (**Figure 4**). The cells were washed and a mixture of Bodipy-FL (**25**, Chapter 3) and Bodipy-TMR **7** were added simultaneously, incubated for 2 hours, washed and imaged at the appropriate excitation/emission channels. Bodipy-FL (**25**, Chapter 3), which was postulated to react only in the lipophilic regions of the cell, showed an intracellular distribution. Conversely, the signal from Bodipy-TMR **7** was restricted to the cell membrane. Merged images (**Figure 4**) of the fluorescence from Bodipy FL (**25**, Chapter 3) and Bodipy-TMR **7**, depicted in green and red respectively, indeed show limited overlap (yellow pixels).[4-7] which could also be the result of the poor axial resolution of the widefield fluorescence

microscope used in these experiments. To better confirm the location-dependent labelling the experiment was repeated using confocal microscopy, where the axial resolution is < 1 micron (**Figure 5**). The single Z-resolved images indeed show no overlap between the two signals, supporting the hypothesis that Bodipy-FL tetrazine and **7** can be used for regiospecific labelling with the same chemistry.

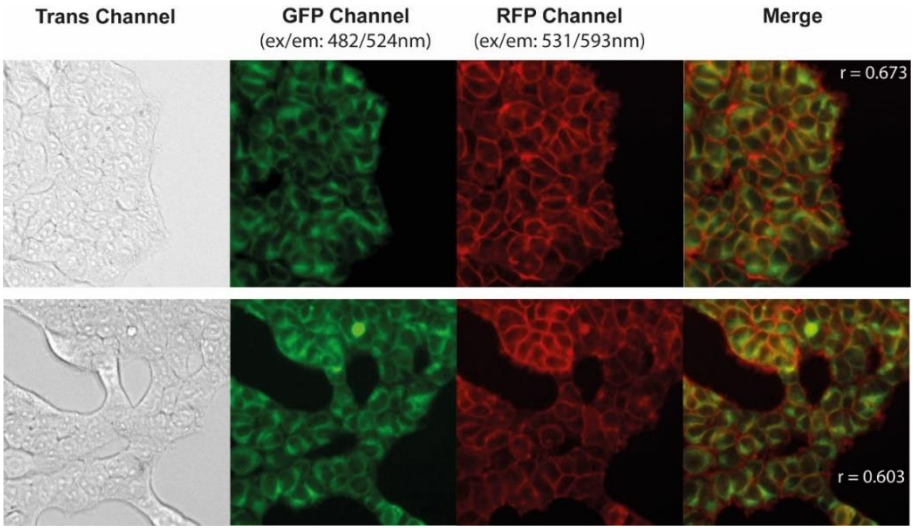


Figure 4: Fluorescence imaging of HEK cells, consecutively incubated with (**62**, Chapter 3) and (**64**, Chapter 3), before simultaneous incubation with Bodipy-FL (**25**, chapter 3) (**GREEN**) and Bodipy-TMR **7** (**RED**). Merged images of both fluorescence channels are included with their respective Pearson's coefficient (**r**).[4-7]

Conclusion

Hydrophilic tetrazine-functionalized fluorophores **6**-**8** were successfully synthesized by attachment of a hydrophilic zwitterionic moiety at the N-terminus, and hporophores to the C-terminus of Boc-Tzm-OH **3**. These fluorophores were capable to enter the glycocalyx coating of the surface of humane cell lines and label metabolically incorporated N-akylcyclopropene mannosamine. Furthermore Bodipy-TMR **7** was used in parallel with Bodipy-FL (**25**, Chapter 3) for the simultaneous regiospecific labeling of strained alkenes, and subsequent fluorescence imaging using confocal-microscopy showing minimal co-localization. These results, to my knowledge, offer the first example regiospecific labelling using parallel identical chemical reactions.

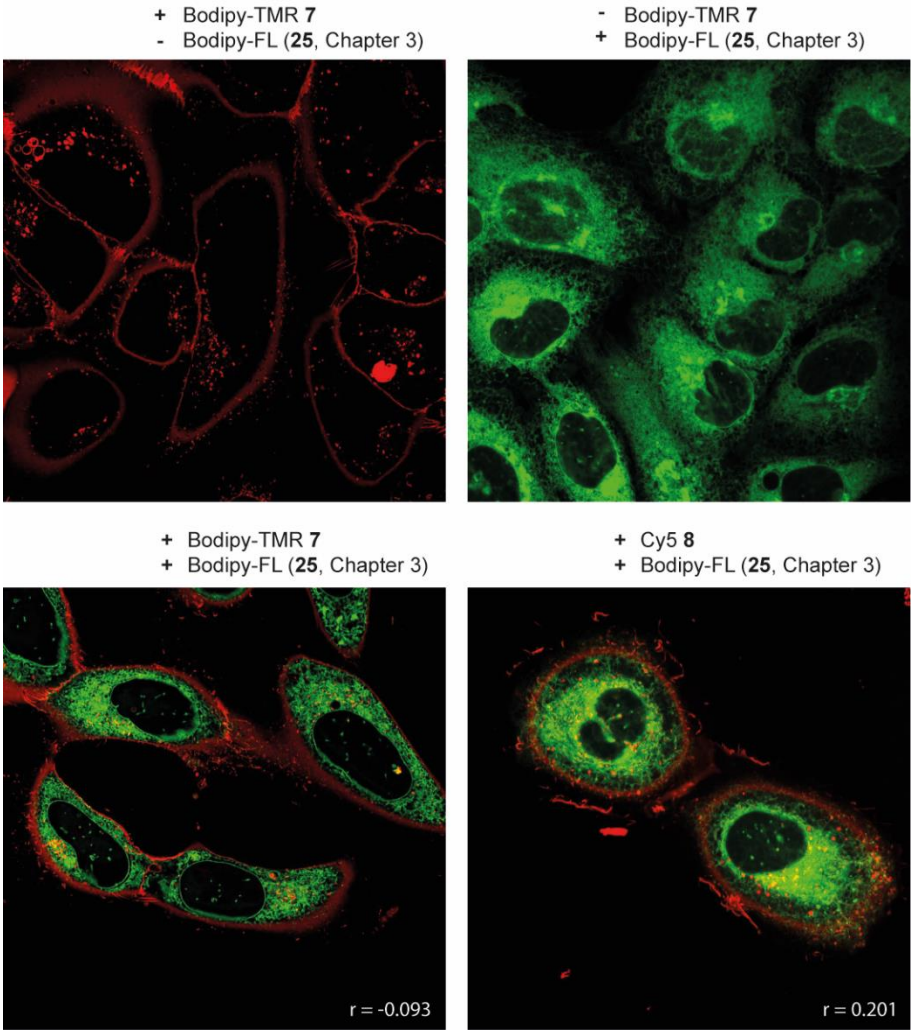


Figure 5: Confocal fluorescence imaging of HEK cells, consecutively incubated with N-alkylcyclopropene mannosamine (**62**, Chapter 3) and sterculic acid (**64**, Chapter 3), before simultaneous incubation with Bodipy-FL (**25**, Chapter 3) (GREEN) and Bodipy-TMR **7** or Cy5 **8** (RED). Merged images of both fluorescence channels are included with their respective Pearson's coefficient (**r**).[4-7]

Cell culturing and imaging procedures

HEK / U2OS cell seeding: HEK / U2OS cells were seeded 2 times a week, at variable 3-4 day intervals. First, the cell culturing flask was removed from the cell culturing incubator, and the cells were inspected using light-microscopy to spot any abmormalities. The old medium was removed, 2.0 mL of trypsin solution was added and the cells were allowed to detatch from the flasks surface for several minutes. 8.0 mL of full medium was added to the flask and the contents were gently homogenized using a serological pipet. Cells were counted, and 100.000 cells were seeded to a new flask after filling the flask with a calculated amount of full medium to obtain a total of 10 mL. The new flask was then returned to the cell culturing incubator.

Human Bone Osteosarcoma Epithelial cells (U2OS) fluorescence microscopy using mannosamine (62, Chapter 3): U2OS cells were cultured using full medium prepared with phenylred-free DMEM, and during seeding a fraction of the homogenized cell solution was diluted and homogenized with phenylred-free DMEM to 25.000 cells / 1 mL. This solution was then devided onto 8 well rectangular plates, (1 cm^2) 200 µL per well, moved to the cell culturing incubator and allowed to adhere and grow overnight. The next day, the old medium was removed, and to each sample well 200 µL of freshly premixed full medium (phenylred-free) containing 100 µM mannosamine **62** (0.2% DMSO). Cells were incubated for 2 days, the medium was removed and the cells were gently washed with pre-warmed medium. Then, 200 µL of full medium (phenylred-free) was added to each well containing freshly premixed 25 µM of tetrazine fluorophore (**6**-**8**) (0.25% DMSO). Cells were incubated for 1 hour, the medium was removed and the cells were gently washed twice with pre-warmed PBS and 200 µL of PBS was added to each well. The plate was analysed using an EVOS fluorescence microscope at 20x amplification, Trans channel filters to verify cell location, before imaging using the GFP/RFP/Cy5 channel filter. Raw images obtained were adjusted using ImageJ software v1.52n (contrast adjusted for **6**: 0-255 to 70-100, **7**: 0-255 to 40-200, **8**: 0-255 to 50-150).

Human Embryonic Kidney cells (HEK) fluorescence microscopy using mannosamine (62, Chapter 3) and sterculic acid (64, Chapter 3): HEK cells were cultured using full medium prepared with phenylred-free DMEM, and during seeding a fraction of the homogenized cell solution was diluted and homogenized with phenylred-free DMEM to 50.000 cells / 1 mL. This solution was then devided onto 8 well rectangular plates, (1 cm^2) 200 µL per well, moved to the cell culturing incubator and allowed to adhere and grow overnight. The next day, the old medium was removed*, and to each sample well 200 µL of freshly premixed full medium (phenylred-free) containing 100 µM mannosamine **62** (0.2% DMSO). Cells were incubated for 2 days, the medium was removed* and the cells were gently washed* with pre-warmed medium. Then, to each sample well 200 µL of freshly premixed full medium (phenylred-free) containing 50 µM sterculic acid **64** (0.5% DMSO). Cells were incubated for 1 hour, the medium was removed* and the cells were gently washed* twice with pre-warmed medium. Then, 200 µL of full medium (phenylred-free) (0.30% DMSO) was added to each well containing freshly premixed 25 µM of tetrazine fluorophore (**7**) and 5 µM of tetrazine fluorophore (**25**, Chapter 3). Cells were incubated for 2 hours, the medium was removed* and the cells were gently washed* twice with pre-warmed medium and 200 µL of pre-warmed medium was added to each well. Plates were analysed using (**A**) An EVOS fluorescence microscope at 20x amplification, Trans channel filters to verify cell location, before imaging using the GFP and

RFP channel filters. Raw images obtained were adjusted using ImageJ software v1.52n (contrast adjusted for Trans-channel: 0-255 to 80-225, GFP-channel: 0-255 to 30-100, RFP-channel: 0-255 to 30-100, 400x400 pixel image selection), or (**B**) Confocal microscope. Raw slices from image sets obtained were selected using ImageJ software v1.52n without adjusting contrast or pixel image selection.

* When removing or old medium or washing cells in this procedure, only around 80% was removed to prevent agitation of the sensitive HEK cells

Pearson's coefficient calculation: The Pearson's coefficient (**r**)[5, 6] was determined in ImageJ using JACoP plugin[4] and applying the Costes[7] automatic thresholds.

Compound Synthesis

Compound 2 (MJ456): 11 mmol (2.4 g) of Boc_2O was dissolved in 10 mL of DCM, 10 mmol (1.26 mL) of N,N-Dimethyl-1,3-diaminopropane was dissolved in 10 mL of DCM, both solutions were cooled 0 °C, added together and stirred overnight at 0 °C. The crude reaction mixture was concentrated using rotary evaporation and used without further purification. Then, 1.45 g (est. 7.5 mmol) of Boc-protected intermediate was dissolved in 10 mL of dry acetonitrile, 10 mmol (1.2 g) of 1,3-propanesultone was added and the reaction mixture was stirred for 2 hours at room temperature. The reaction mixture was concentrated using rotary evaporation and directly purified using silica column chromatography resulting in intermediate **1**. Intermediate **1** was treated with dry 4M HCl in EtOAc and concentrated using rotary evaporation resulting in compound **2**. ^{1}H NMR (101 MHz, D_2O) δ: 3.56 – 3.50 (m, 2H), 3.50 – 3.44 (m, 2H), 3.16 (s, 6H), 3.14 – 3.07 (m, 2H), 3.03 – 2.97 (m, 2H), 2.30 – 2.15 (m, 4H). ^{13}C NMR (101 MHz, D_2O) δ: 62.37, 60.52, 50.78, 47.07, 36.23, 20.49, 18.09.

Compound 4 (AS295, AS284, A268): 0.21 mmol (60 mg) of Boc-Tzm-OH **3** was dissolved in 3 mL dry THF, 0.34 mmol (39 mg) of N-hydroxysuccinimide and 0.28 mmol (58 mg) of N,N'-dicyclohexylcarbodiimide were added and the reaction mixture was stirred for 1 hour at room temperature. Reaction completion was checked by TLC (NHS-intermediate: Rf = 0.8, 1:1 EtOAc/DCM. Boc-Tzm-OH: Rf = 0.4, 1:1 EtOAc/DCM). The reaction mixture was filtered through a pad of celite. Then, 0.38 mmol (100 mg) of solubilizer, 0.38 mmol (53 µL) of TEA and 1.5 mL H_2O were added to the solution and the reaction mixture was stirred for 30 minutes at room temperature. Reaction completion was checked by TLC (Product: Rf = 0.1, 10% H_2O in acetonitrile. NHS-intermediate: Rf = 0.8, 10% H_2O in acetonitrile. Boc-Tzm-OH: Rf = 0.4, 10% H_2O in acetonitrile.) The reaction mixture was concentrated using rotary evaporation and directly purified using silica column chromatography using an 5-13% H_2O in acetonitrile eluent, resulting in 89 mg (0.18 mmol, 86%) of compound **4** as a pink solid. ^{1}H NMR (400 MHz, DMSO) δ: 8.17 (t, *J* = 5.7 Hz, 1H), 7.16 (d, *J* = 8.5 Hz, 1H), 4.50 (td, *J* = 8.5, 5.7 Hz, 1H), 3.48 – 3.37 (m, 3H), 3.25 (dd, *J* = 11.1, 5.6 Hz, 2H), 3.20 – 3.11 (m, 2H), 3.00 (s, 6H), 2.94 (s, 3H), 2.47 (t, *J* = 6.9 Hz, 2H), 2.07 – 1.90 (m, 2H), 1.83 (dd, *J* = 10.8, 5.5 Hz, 2H), 1.29 (s, 9H).^{13}C NMR (101 MHz, DMSO) δ 170.24, 167.00, 166.95, 155.11, 78.54, 62.50, 61.18, 53.29, 50.08, 47.63, 37.20, 35.77, 28.08, 22.32, 20.70, 18.88. ESI-MS (m/z): $[C_{19}H_{35}N_7O_6S+H]^+$ calculated 490.24, found 490.08.

Compound 5 (AS296, AS293, A268): 0.17 mmol (81 mg) of compound **2** was dissolved in 3 mL dry DCM, 2 mL of 4M HCl in dioxane was added and the reaction mixture was stirred for 1 hour at room temperature. The reaction mixture was concentrated using rotary evaporation resulting in 71 mg (0.17 mmol, quant.) of compound **3** as a pink solid. ^{1}H NMR (400 MHz,

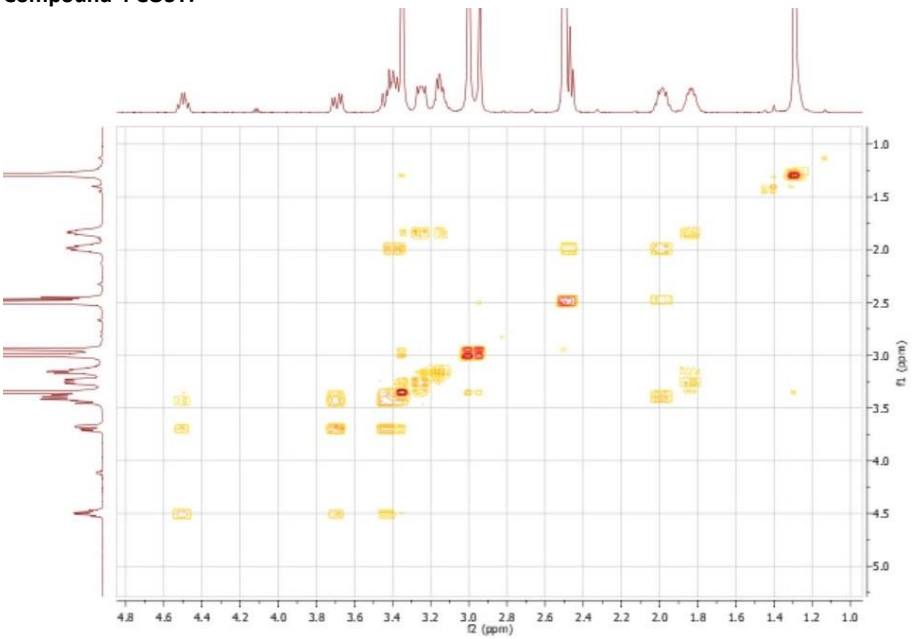
MeOD) δ: 8.72 (t, *J* = 5.9 Hz, 1H), 4.58 (t, *J* = 5.8 Hz, 1H), 3.93 (d, *J* = 5.8 Hz, 1H), 3.74 (d, *J* = 5.0 Hz, 1H), 3.62 – 3.52 (m, 2H), 3.51 – 3.40 (m, 2H), 3.19 (s, 3H), 3.17 (s, 3H), 3.16 – 3.12 (m, 2H), 3.05 (s, 3H), 2.87 (t, *J* = 7.0 Hz, 2H), 2.27 – 2.14 (m, 2H), 2.05 (dd, *J* = 15.4, 11.1 Hz, 2H). ^{13}C NMR (101 MHz, MeOD) δ: 160.19, 159.54, 157.11, 54.25, 53.48, 43.45, 42.37, 40.37, 40.16, 39.95, 39.73, 39.52, 39.31, 39.09, 38.88, 27.98, 27.50, 14.35, 11.91, 10.55. ESI-MS (m/z): $[C_{14}H_{27}N_7O_4S+H]^+$ calculated 390.19, found 390.25.

Compound 6 Bodipy-FL-Tzm-Sol (A270): 26 µmol (10 mg) of Bodipy-FL NHS ester, 026 µmol (10 mg) of compound **5** and 0.21 mmol (30 µL) of TEA were dissolved in 1 mL dry DMF and the reaction mixture was stirred for 1 hour at room temperature. Reaction completion was checked by TLC (Rf = 0.3, 20% H_2O in acetonitrile). The reaction mixture was concentrated using rotary evaporation and directly purified using silica column chromatography using an 2-10% H_2O in acetonitrile eluent, resulting in 1 mg (1.5 µmol, 5.7%) Compound **6** as an orange solid. ^{1}H NMR (400 MHz, MeOD) δ 7.43 (s, 1H), 7.00 (d, *J* = 3.8 Hz, 1H), 6.33 (d, *J* = 3.9 Hz, 1H), 6.23 (s, 1H), 4.96 – 4.91 (m, 2H), 3.79 – 3.67 (m, 2H), 3.56 – 3.47 (m, 2H), 3.43 – 3.34 (m,2H), 3.23 – 3.15 (m, 2H), 3.11 (s, 3H), 3.09 (s, 3H), 2.99 (s, 3H), 2.86 (td, *J* = 6.6, 2.3 Hz, 2H), 2.69 (t, *J* = 7.6 Hz, 2H), 2.51 (s, 3H), 2.29 (s, 3H), 2.22 (dd, *J* = 14.9, 8.0 Hz, 2H), 2.00 (dd, *J* = 15.6, 10.5 Hz, 2H).

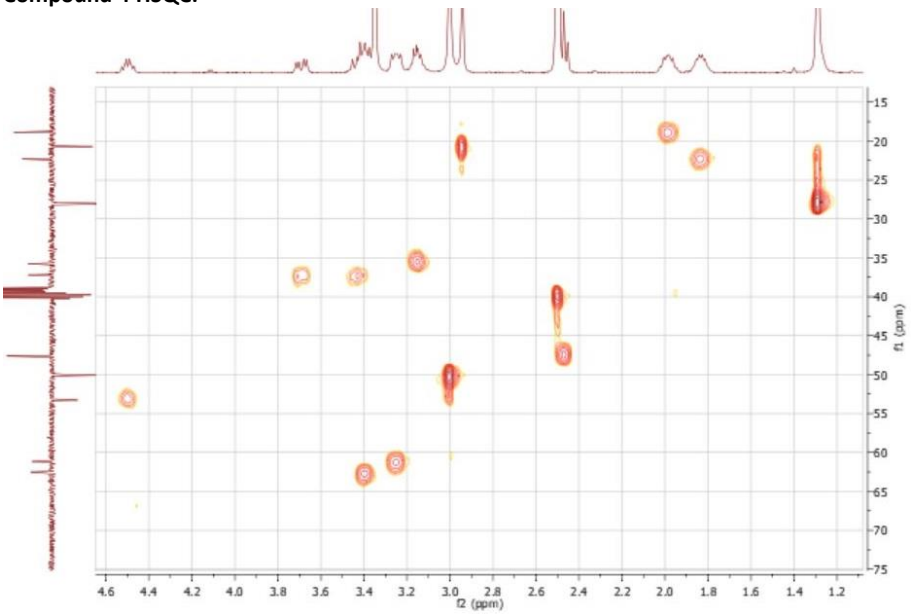
Compound 7 Bodipy-TMR-Tzm-Sol (A302): 40 µmol (20 mg) of Bodipy-TMR NHS ester, 38 µmol (15 mg) of compound **5** and 72 µmol (10 µL) of TEA were dissolved in 1 mL dry DMF and the reaction mixture was stirred for 1 hour at room temperature. Reaction completion was checked by TLC (Rf = 0.4, 20% H_2O in acetonitrile). The reaction mixture was concentrated using rotary evaporation and directly purified using silica column chromatography using an 5-20% H_2O in acetonitrile eluent, resulting in 8 mg (10 µmol, 26%) Compound **7**.

Compound 8 Cy5-Tzm-Sol (A294): 50 µmol (30 mg) of Cy5 NHS ester, 77 µmol (30 mg) of compound **5** and 160 µmol (22 µL) of TEA were dissolved in 1 mL dry DMF and the reaction mixture was stirred for 1 hour at room temperature. Reaction completion was checked by TLC (Rf = 0.5, 25% H_2O in acetonitrile). The reaction mixture was concentrated using rotary evaporation and purified using HPLC resulting in Compound **8** as a blue solid. ESI-MS (m/z): $[C_{46}H_{64}N_9O_5S]^+$ calculated 854.47, found 854.40.

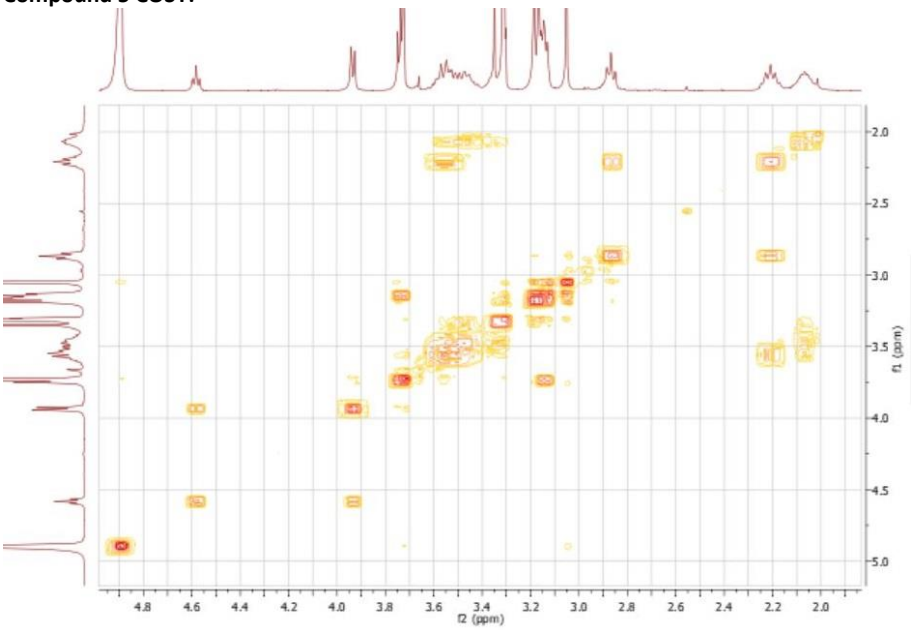
Compound 4 COSY:



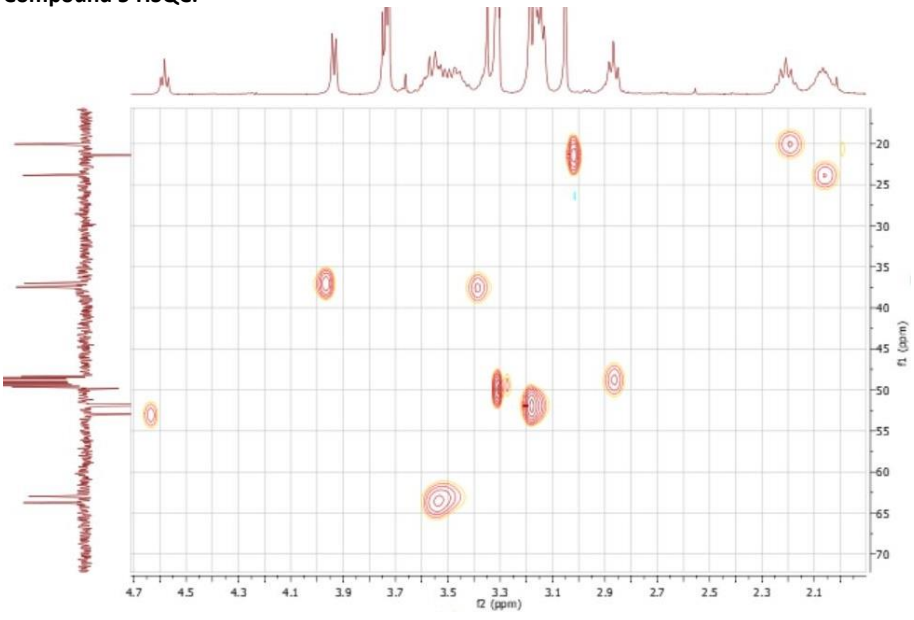
Compound 4 HSQC:



Compound 5 COSY:



Compound 5 HSQC:



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Chapter 5: Fast and pH-independent elimination of *trans*-cyclooctene using aminoethyl functionalized tetrazines

Introduction

Inverse electron demand Diels-Alder (IEDDA)-pyridazine elimination tandem reactions, in which the allylic substituent on *trans*-cyclooctene is eliminated following reaction with tetrazines, are gaining interest as a versatile bioorthogonal process. One potential shortcoming of the currently used reactions is their propensity to run faster and more efficient at increasing acidity of the reaction medium. This feature, caused by the nature of the tetrazines used in these contemporary studies, dictates that at biologically relevant pH (ranging broadly from 4 to 7) IEDDA-based elimination/biological activation processes may proceed at a suboptimal speed. In this chapter, aminoethyl substituted tetrazines are presented as the first pH-independent reagents showing invariably fast elimination kinetics at all biologically relevant pH's.

Bioorthogonal chemistry – the execution of selective chemical conversions within a biological sample – has provided a wealth of information on a wide variety of biological processes.[1] Initial work included the controlled ligation reactions within biological systems. Examples of such bioorthogonal ligations include copper-catalyzed Huisgen cycloadditions[2], Staudinger ligations[3], inverse electron demand Diels-Alder (IEDDA) reactions[4], amongst other chemistries[5]. Bioorthogonal ligations have found wide application in adding a reporter group after a biochemical/biological process or pathway has run to completion. In this instance, one of the bioorthogonal reaction partners, ideally a modified substrate containing a metbolically inert and small moiety (alkyne, azide, cyclopropene, respective towards the above mentioned bioorthogonal ligation reactions), is engineered within the molecule of interest that is processed by the biological system of interest. Such entity, when compared to the final reporter moiety (a fluorophore, biotin or any other type of tag), has a much better chance to proceed through the physiological process or pathway similar to the unmodified substrate. The ligation itself should proceed with high speed and efficiency (also in terms of side reactions which should be minimal) to maximize sensitivity in detection and minimize false positives (for instance due to side reactions).

Bioorthogonal reactions have also been used to unmask functional groups in living systems.[6] A molecule of interest is rendered biologically inert by introducing a chemical functionality masking the natural functional group. The molecule can then be re-activated by removing the introduced masking chemical group using the bio-oorthogonal reaction partner. In bioorthogonal ligation, and perhaps even more so in bioorthogonal elimination, reaction speed and selectivity are essential.

The IEDDA-pyridazine elimination reaction[7] – a "click-to-release" reaction by which an allylic substituent on *trans*-cyclooctene (2-TCO, axial (E)-cyclooct-2-en-1-ol) is eliminated upon rearrangement of the pyridazine intermediate – has proven particularly favorable in this regard. Depening on the nature of the chosen IEDDA reaction pair (diene, and dienophile), it has excellent biocompatibility[9] and low toxicity[10]. *In vivo* applications include chemical control of drug release[11], control over T-cell activation[8], release of drugs from hydrogels[12], as well as the control over kinase activity in mice[10]. The use of this reaction for the localised release of a chemotherapeutic is now even pursued in human clinical trials.[15]

Mechanistic insight into the IEDDA click-to-release reaction has provided a pathway towards its improvement (**Figure 1**).[13] Chen and co-workers, for example, discovered that asymmetric tetrazines carrying both an electron-donating and electron-withdrawing substituent showed a significant improvement in the elimination rates compared to their symmetric counterparts, leading to improved results of their most prominent tetrazine **1** over 3,6-dimethyl tetrazine **2**[13a]. Weissleder and co-workers used carboxy-functionalized tetrazine **3** and observed elimination rates much faster than tetrazine **2**.[13b] It was also discovered that, for tetrazines containing simple alkyl substituents, elimination rates are very sensitive to the pH of the reaction medium: while release is completed within an hour at acidic conditions (tetrazine **2**, pH 5.0), it takes several hours under physiological conditions (tetrazine **2**, pH 7.0)[13b], as the elimination step is subject to general acid catalysis. What was lacking at the onset of these studies were tetrazines that maintain fast elimination kinetics over the whole biologically relevant pH range (pH 3.5 – pH 7.5). It was postulated that tetrazines **4**, **5**, **6**, **7** and **17** could serve as pH-independent eliminating tetrazines. The amine on the aminoethyl substituent, as cationic ammonium functionality at and below physiological pH, would function as general acid during the elimination step, potentially improving both release rate and efficiency.

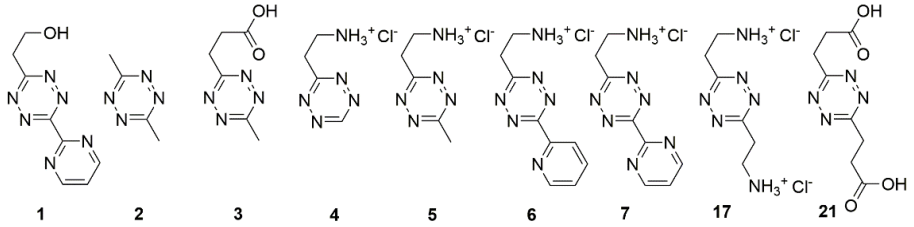
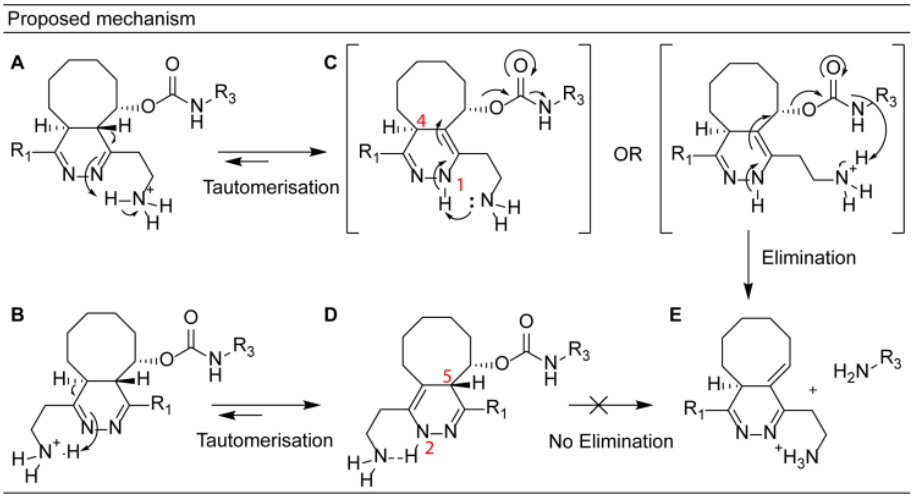


Figure 1. Known tetrazines (**1**, **2**, **3** and **21**) and aminoethyl substituted tetrazines (**4**, **5**, **6**, **7** and **17**) synthesized in chapter 3.

In this chapter the elimination behavior of the family of aminoethyl tetrazines synthesized in **chapter 3** are explored. It is shown that, through intramolecular proton delivery, an increase in the elimination rate by 18-fold compared to the fastest reported literature tetrazines **1** and **3** could be attained. Furthermore, this intramolecular proton source renders the reaction pH independent with minimal change in the reaction rates from pH 3 to pH 7.4.

Design

Based on the functional groups present on the synthesized tetrazines from chapter 3, a selection was made focusing on aminoethyl functioalized tetrazines. Similar to tetrazines having a carboxylate functionality,[13b] an aminoethyl could possibly catalyze both the 4,5- to 1,4-tautomerization and subsequent elimination process (**Scheme 1**). The [4+2] IEDDA cycloaddition of an assymetric tetrazine (with respect to the substitutents at C2 and C5) with a 2-TCO can proceed with the aminoethyl functionality ending up at either the eliminating end (**A**, "head-to-head"), or the non-eliminating end (**B**, "head-to-tail") of the adduct. In the "head-to-head" adduct a tautomerisation of the initial 4,5-tautomer may be promoted leading to either the eliminating (**C**) 1,4-tautomer or the non-eliminating (**D**) 2,5-tautomer. Elimination (**E**) may then be driven through the proximity of the intramolecular catalytic amine (acting as a base) near both the N-1 of the dihydropyridazine core and carbamate linkage.



Scheme 1. Proposed mechanism for the tautomerisation and subsequent elimination via intramolecular catalysis by aminoethyl functionality.

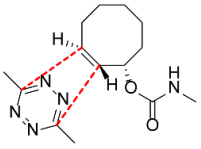
To investigate the behavior of tetrazines **4**-**7** during addition and subsequequent elimination density functional theory (DFT) calculations were performed on the

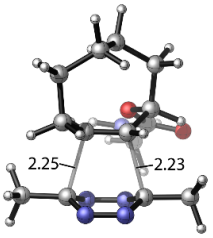
transition states of these tetrazines (**Figure 1** and **2**)[14]. The transition states of the cycloaddition step for tetrazines **2** and **4-7** was evaluated computationally in reaction with a model alpha-carbamate modified *trans*-cylooctene (**Figure 1a-b**). The results suggest that the IEDDA reaction path towards the "head-to-head" adduct is favoured over the reaction path that leads to the "head-to-tail" adduct for all calculated aminoethyl functionalized tetrazines. In the "head-to-head" transition states the cationic ammonium functionality indeed interacts with the carbamate linkage resulting in an energetically favorable approach. For example, the "head-to-head" transition state (TS-1, **Figure 1a**) for tetrazine **5** proved to be 2.5 kcal/mol lower than the "head-to-tail" transition state (TS-2, **Figure 1a**). The thermodynamic preferences for the other aminoethyl tetrazines **4**, **6**, **7** appeared to be even greater at 4.4 - 4.6 kcal/mol (**Figure 1a-b**).

After establishing the theoretically favored geometries of the transition state in the cycloaddition step, the lowest energy geometries of the formed adducts were investigated (**Figure 2**). From these calculations it follows that tautomerization from initial 4-5-dihydropyridazine towards eliminating 1,4-dihydropyridazine appears energetically favorable and shows prominent interaction between the aminoethyl functionality and the N-1 of the dihydropyridazine core in both neutral (**Figure 2A**) and cationic (**Figure 2B**) state. The hypothetical proton transfer through a six-membered-cyclic-transition-state is geometrically feasible in the kinetically favored "head-to-head" adduct and likely to facilitate both tautomerization and elimination (**Scheme 1**). The design consideration described above, supported by the calculations, indicated that the tetrazines **4-7** would show fast and pH independent kinetics for the "click-to-release" reaction.

Tetrazine 2

TS

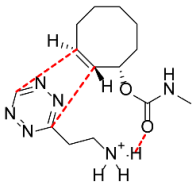


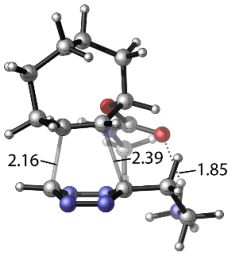


$\Delta G^{\ddagger}_{aq}$ (kcal/mol)	20.6
ν_i (cm^{-1})	-408.0i

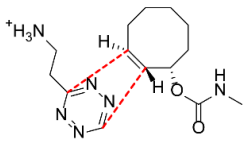
Tetrazine 4

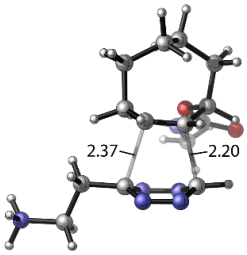
TS1 - "Head-to-Head"





TS2 - "Head-to-Tail"

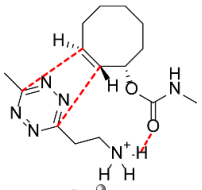


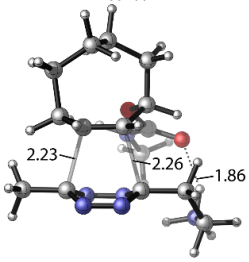


14.5	$\Delta G^{\ddagger}_{aq}$ (kcal/mol)	18.9
-368.8i	ν_i (cm^{-1})	-337.3i

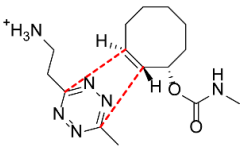
Tetrazine 5

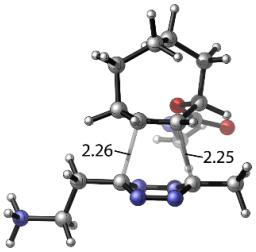
TS1 - "Head-to-Head"





TS2 - "Head-to-Tail"





17.8	$\Delta G^{\ddagger}_{aq}$ (kcal/mol)	21.3
-403.5i	ν_i (cm^{-1})	-387.8i

Figure 1a: Transition states for "head-to-head" (**TS1**) and "head-to-tail" (**TS2**) reactions of tetrazines **2, 4** and **5** with model axial (E)-cyclooct-2-ene. Distances of interest (forming C-C bonds, interactions) are labelled in Å. Reported transition states were used for initial approximations.[14] All structures were optimized with Gaussian 09 using the ωB97XD long-range corrected hybrid functional and 6-31+G(d) as basis set. Optimization was done in combination with a polarizable continuum model (PCM) using water as solvent parameter.

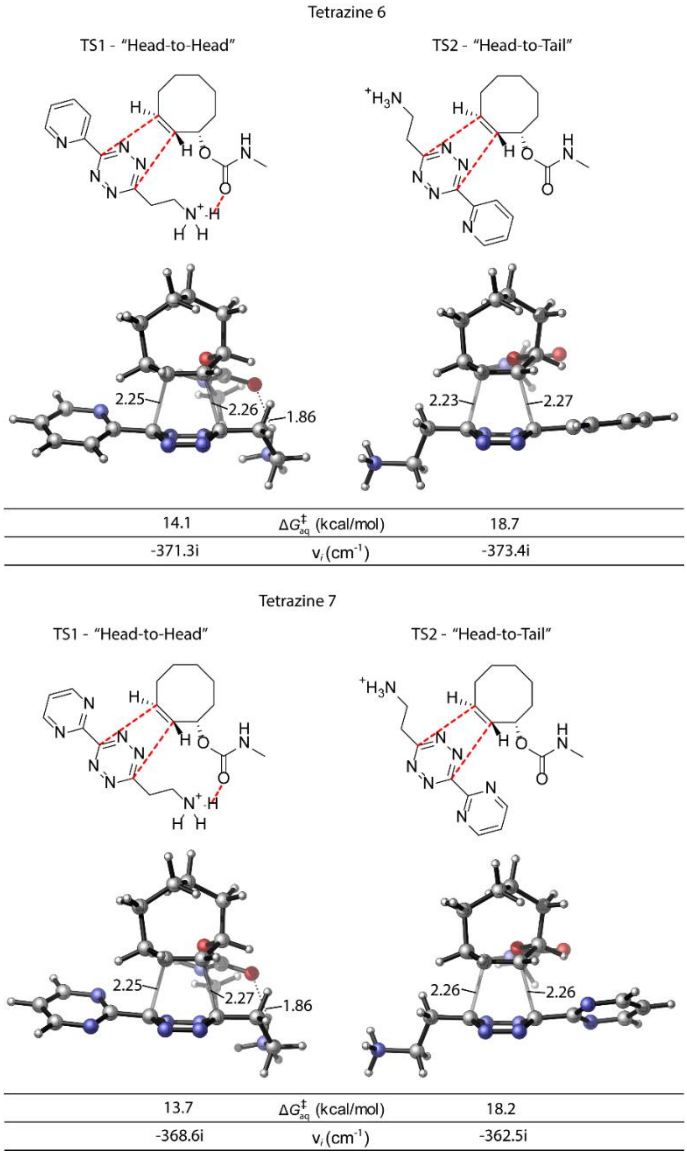


Figure 1b: Transition states for "head-to-head" (**TS1**) and "head-to-tail" (**TS2**) reactions of tetrazines **6** and **7** with model axial (E)-cyclooct-2-ene. Distances of interest (forming C-C bonds, interactions) are labelled in Å. Reported transition states were used for initial approximations.[14] All structures were optimized with Gaussian 09 using the ωB97XD long-range corrected hybrid functional and 6-31+G(d) as basis set. Optimization was done in combination with a polarizable continuum model (PCM) using water as solvent parameter.

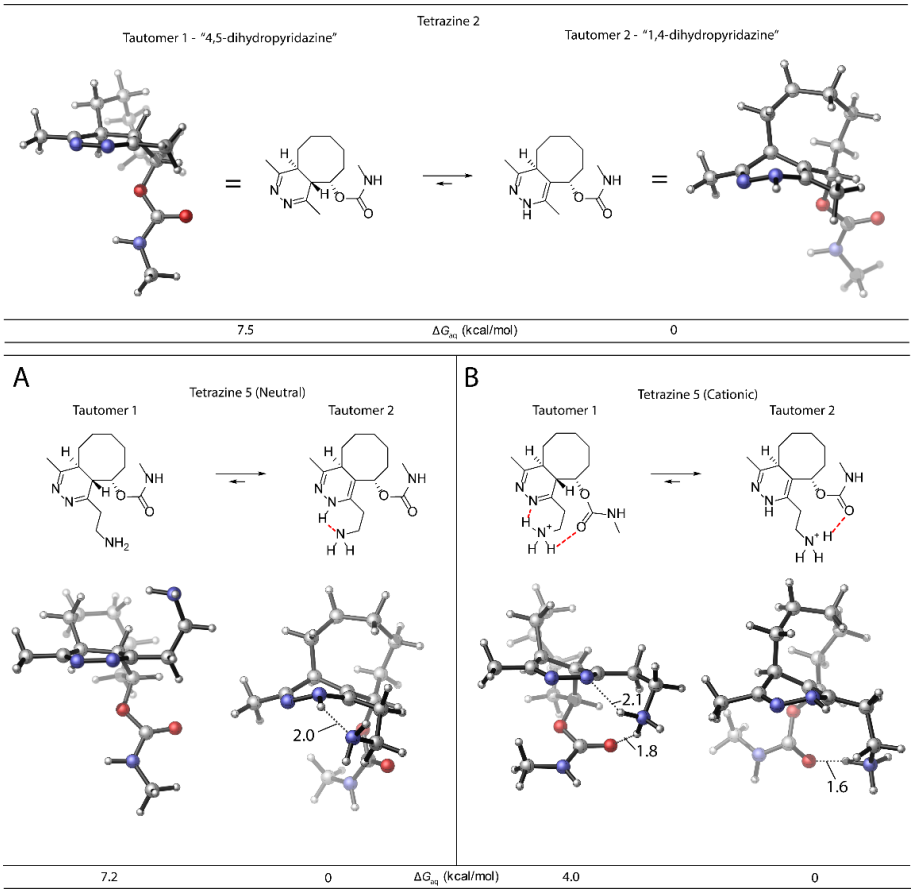


Figure 2. Lowest energy geometries of dihydropyridazine adducts. Proton transfer is geometrically feasible (red lines). **A**) "Neutral" Initial 4,5-tautomer (left) and thermodynamically favorable 1,4-tautomer (right) **B**) "Cationic" Initial 4,5-tautomer (left) and thermodynamically favorable 1,4-tautomer (right). Conformer distributions were generated with Spartan 10 program using molecular mechanics with MMFF94 as force field, for the formed initial 4,5-tautomer and subsequent 1,4-tautomer after cycloaddition of tetrazines **2** and **5** with model axial (E)-cyclooct-2-en. All generated structures were further optimized in the gas-phase with Gaussian 09 using the ωB97XD functional with 6-311G(d,p) basis set and optimized with PCM using water as solvent parameter.

Determination of elimination kinetics

To probe the importance of the intramolecular proton delivery by the aminoethyl functionality the efficacy of tetrazines **4**-**7** and **17** (**Figure 3B**), in a IEDDA-click to release reaction with trans-cyclooctene **X**, was compared to the efficacy of tetrazines from literature, known to be either of the "non-releasing" (**23**, **24**) or "releasing" (**1**, **2**, **25**) type (**Figure 3A**). Comparative studies with N-Boc protected tetrazines **4b**-**16b** (**Figure 3B-C**) and tetrazines **8**-**16** (**Figure 3D**), in which the amino group is expected to be unable to provide intramolecular assistance for geometrical reasons, were conducted as well.

To assess the elimination properties of all tetrazines a direct fluorescence-based assay[13a] that allows monitoring the reaction in real time was chosen (**Figure 4A**). This method was preferred over LC-MS based analysis[13a][13b] as it allows a rapid measurement of several tetrazines simultaneously, acquisition of more data (at the initial stage of the reaction) and is not affected by possible "pseudo-release" artefacts caused by LC-MS analysis.[13b] Tetrazine elimination properties were assessed through reaction with fluorogenic 2-TCO-modified 7-amino-4-methylcoumarin **28** (2-TCO-AMC)[13a] and tracking the product AMC **27** by measuring its characteristic fluorescence at 450 nm (**Figure 4B**).

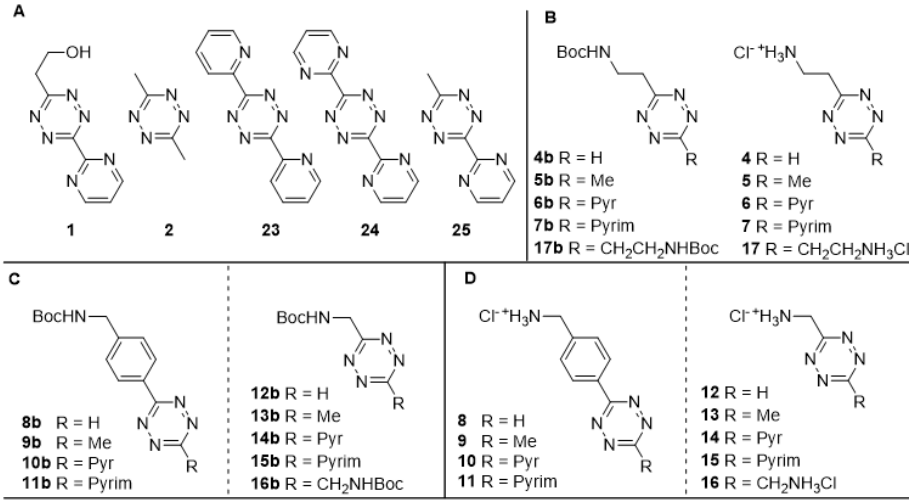


Figure 3. A) Tetrazines **1**, **2**, **23**-**25** used as literature references. B) Aminoethyl tetrazines **4**-**7** and **17**, and N-Boc protected aminoethyl tetrazines **4b**-**7b** and **17b**. C) N-Boc protected tetrazines **8b**-**16b**. D) tetrazines **8**-**16**.

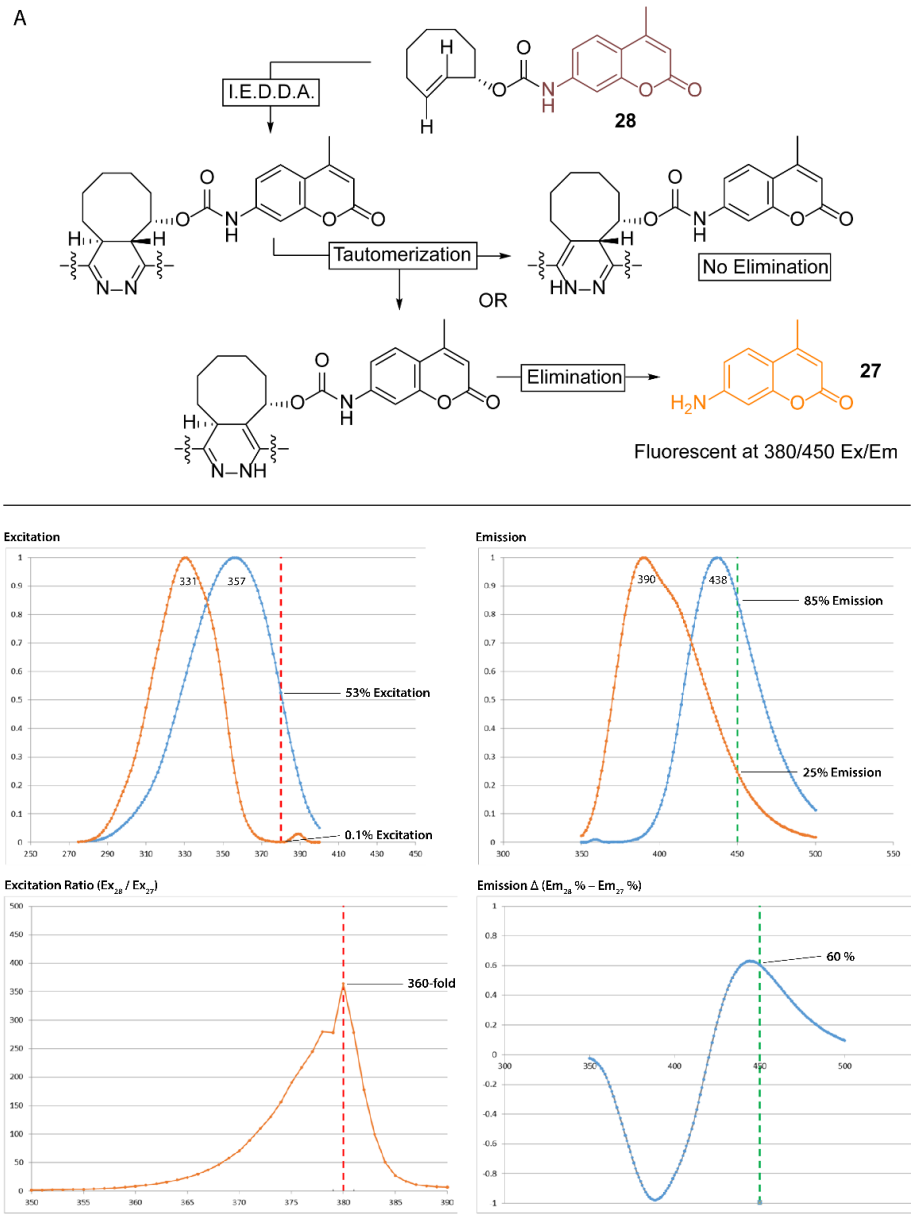
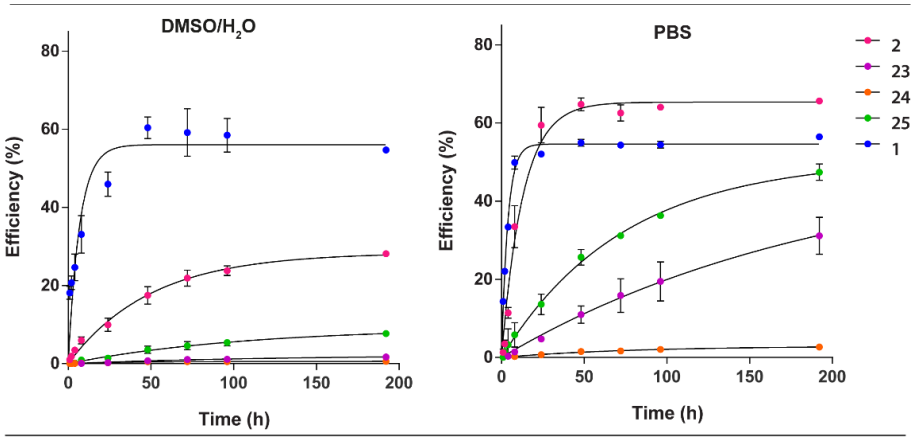
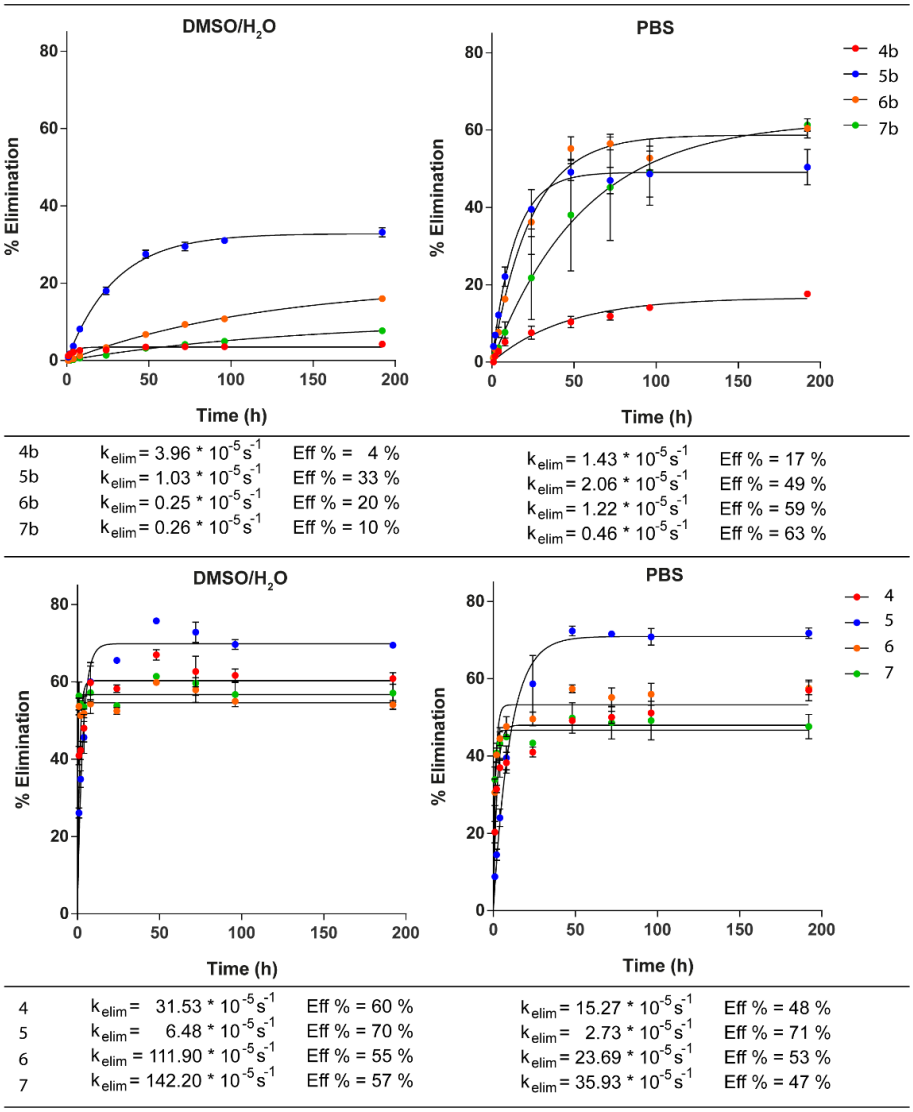


Figure 4 A) Fluorogenic assay: 2-TCO-AMC **28** is used as a measurement tool to track the release of fluorescent AMC **27** upon reaction with tetrazines. B) Normalized excitation (left) and emission (right) spectra of AMC **27** (blue) and 2-TCO-AMC **28** (orange). Chosen excitation wavelength (380nm, excitation ratio (Ex_{27} / Ex_{28}) = 360-fold) is visualized by the vertical red line. Chosen emission wavelength (450nm, Emission Δ (Em_{27} % – Em_{28} %) = 60%) is visualized by the vertical green line.



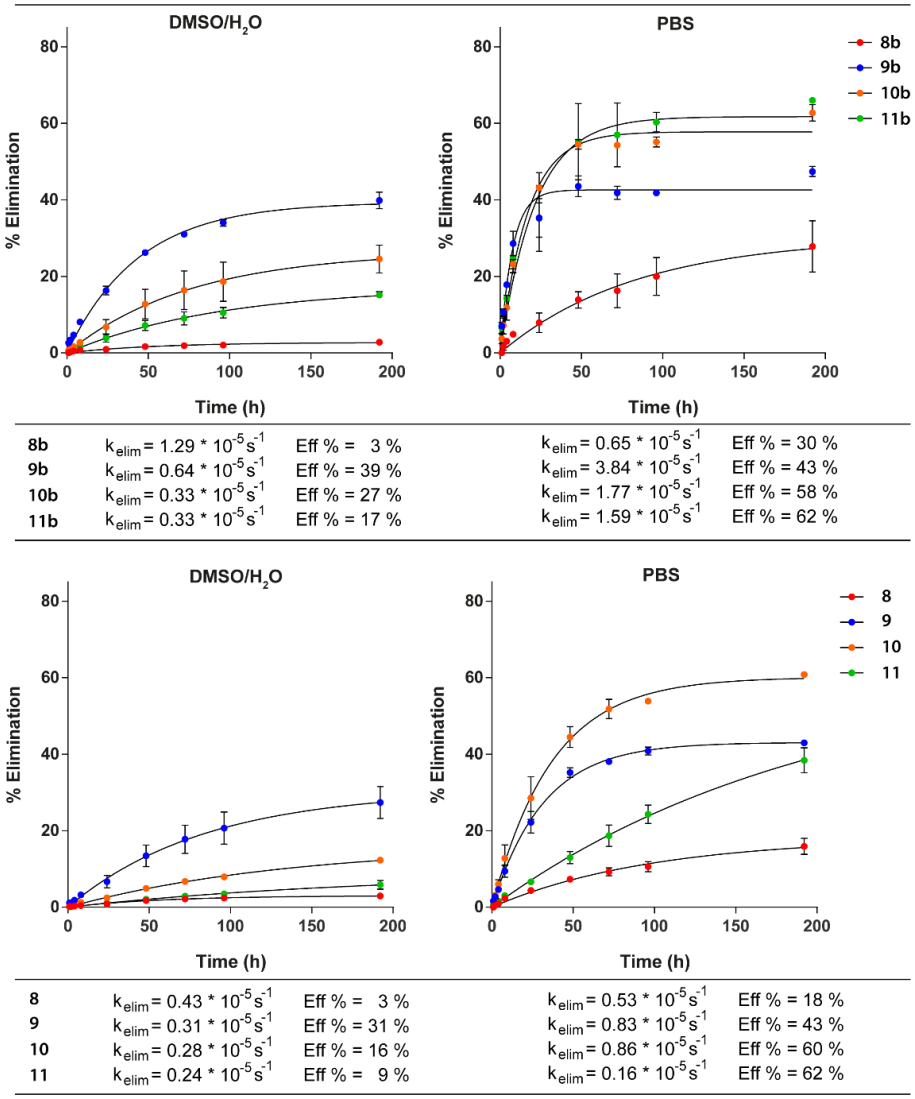
	DMSO/H_2O		PBS	
2	k_{elim} = 0.79 * 10^{-5} s^{-1}	Eff % = 28 %	k_{elim} = 2.91 * 10^{-5} s^{-1}	Eff % = 65 %
23	k_{elim} = 0.36 * 10^{-5} s^{-1}	Eff % = 2 %	k_{elim} = 0.15 * 10^{-5} s^{-1}	Eff % = 51 %
24	k_{elim} = 1.01 * 10^{-5} s^{-1}	Eff % = 1 %	k_{elim} = 0.62 * 10^{-5} s^{-1}	Eff % = 3 %
25	k_{elim} = 0.41 * 10^{-5} s^{-1}	Eff % = 9 %	k_{elim} = 0.47 * 10^{-5} s^{-1}	Eff % = 51 %
1	k_{elim} = 2.07 * 10^{-5} s^{-1}	Eff % = 56 %	k_{elim} = 8.86 * 10^{-5} s^{-1}	Eff % = 55 %

Figure 5: Corrected normalized elimination efficiency over time of tetrazines **1, 2, 23, 24** and **25** in DMSO/H_2O (1:1, v:v) and PBS (0.25% DMSO). Maximum elimination efficiencies (Eff %) were determined by first-order exponential decay approximations (black lines).



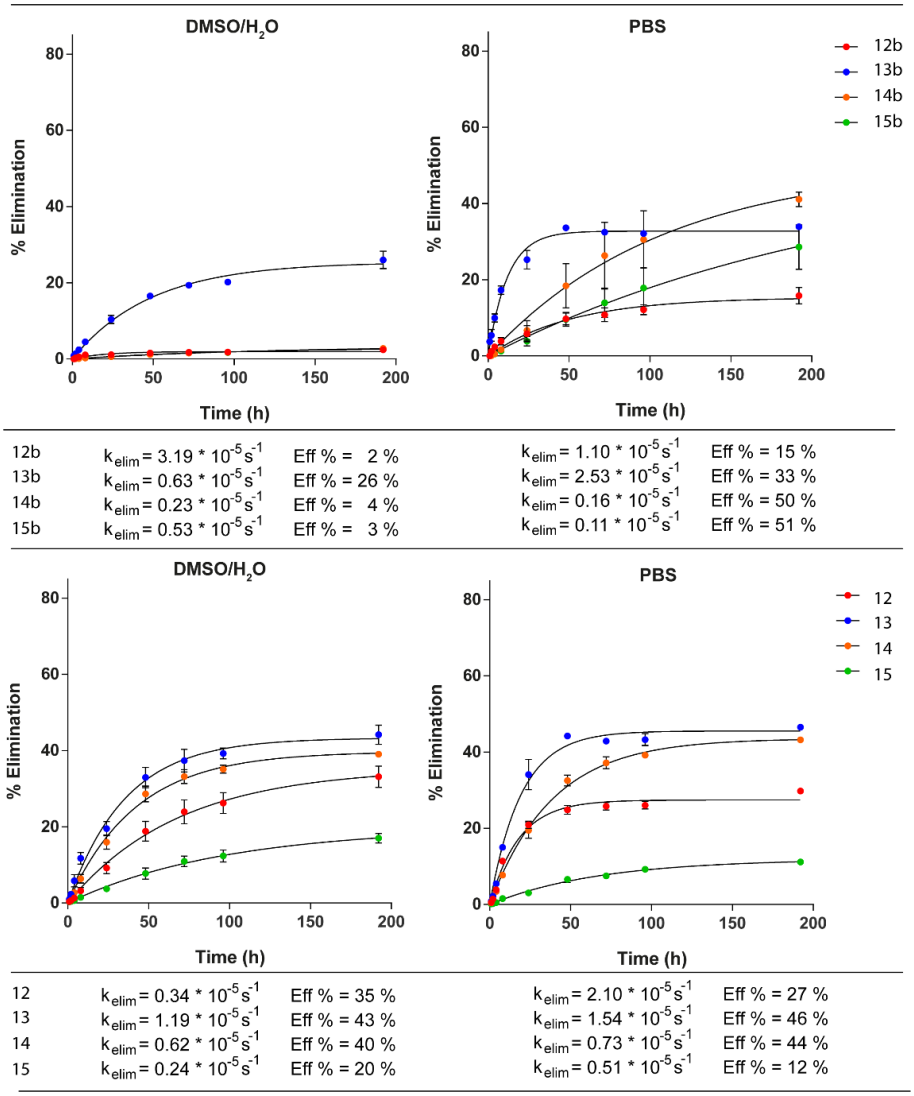
	DMSO/H_2O		PBS	
4b	k_{elim} = 3.96 * 10^{-5} s^{-1}	Eff % = 4 %	k_{elim} = 1.43 * 10^{-5} s^{-1}	Eff % = 17 %
5b	k_{elim} = 1.03 * 10^{-5} s^{-1}	Eff % = 33 %	k_{elim} = 2.06 * 10^{-5} s^{-1}	Eff % = 49 %
6b	k_{elim} = 0.25 * 10^{-5} s^{-1}	Eff % = 20 %	k_{elim} = 1.22 * 10^{-5} s^{-1}	Eff % = 59 %
7b	k_{elim} = 0.26 * 10^{-5} s^{-1}	Eff % = 10 %	k_{elim} = 0.46 * 10^{-5} s^{-1}	Eff % = 63 %
4	k_{elim} = 31.53 * 10^{-5} s^{-1}	Eff % = 60 %	k_{elim} = 15.27 * 10^{-5} s^{-1}	Eff % = 48 %
5	k_{elim} = 6.48 * 10^{-5} s^{-1}	Eff % = 70 %	k_{elim} = 2.73 * 10^{-5} s^{-1}	Eff % = 71 %
6	k_{elim} = 111.90 * 10^{-5} s^{-1}	Eff % = 55 %	k_{elim} = 23.69 * 10^{-5} s^{-1}	Eff % = 53 %
7	k_{elim} = 142.20 * 10^{-5} s^{-1}	Eff % = 57 %	k_{elim} = 35.93 * 10^{-5} s^{-1}	Eff % = 47 %

Figure 6a: Corrected normalized elimination efficiency over time of tetrazines **4b**-**7b** and **4**-**7** in DMSO/H_2O (1:1, v:v) and PBS (0.25% DMSO). Maximum elimination efficiencies (Eff %) were determined by first-order exponential decay approximations (black lines).



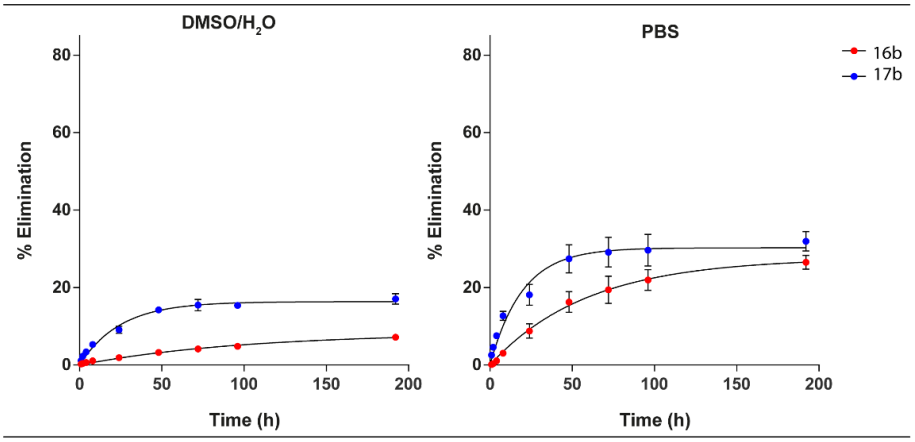
	DMSO/H_2O		PBS	
8b	k_{elim} = 1.29 * 10^{-5} s^{-1}	Eff % = 3 %	k_{elim} = 0.65 * 10^{-5} s^{-1}	Eff % = 30 %
9b	k_{elim} = 0.64 * 10^{-5} s^{-1}	Eff % = 39 %	k_{elim} = 3.84 * 10^{-5} s^{-1}	Eff % = 43 %
10b	k_{elim} = 0.33 * 10^{-5} s^{-1}	Eff % = 27 %	k_{elim} = 1.77 * 10^{-5} s^{-1}	Eff % = 58 %
11b	k_{elim} = 0.33 * 10^{-5} s^{-1}	Eff % = 17 %	k_{elim} = 1.59 * 10^{-5} s^{-1}	Eff % = 62 %
8	k_{elim} = 0.43 * 10^{-5} s^{-1}	Eff % = 3 %	k_{elim} = 0.53 * 10^{-5} s^{-1}	Eff % = 18 %
9	k_{elim} = 0.31 * 10^{-5} s^{-1}	Eff % = 31 %	k_{elim} = 0.83 * 10^{-5} s^{-1}	Eff % = 43 %
10	k_{elim} = 0.28 * 10^{-5} s^{-1}	Eff % = 16 %	k_{elim} = 0.86 * 10^{-5} s^{-1}	Eff % = 60 %
11	k_{elim} = 0.24 * 10^{-5} s^{-1}	Eff % = 9 %	k_{elim} = 0.16 * 10^{-5} s^{-1}	Eff % = 62 %

Figure 6b: Corrected normalized elimination efficiency over time of tetrazines **9b**-**11b** and **9**-**11** in DMSO/H_2O (1:1, v:v) and PBS (0.25% DMSO). Maximum elimination efficiencies (Eff %) were determined by first-order exponential decay approximations (black lines).

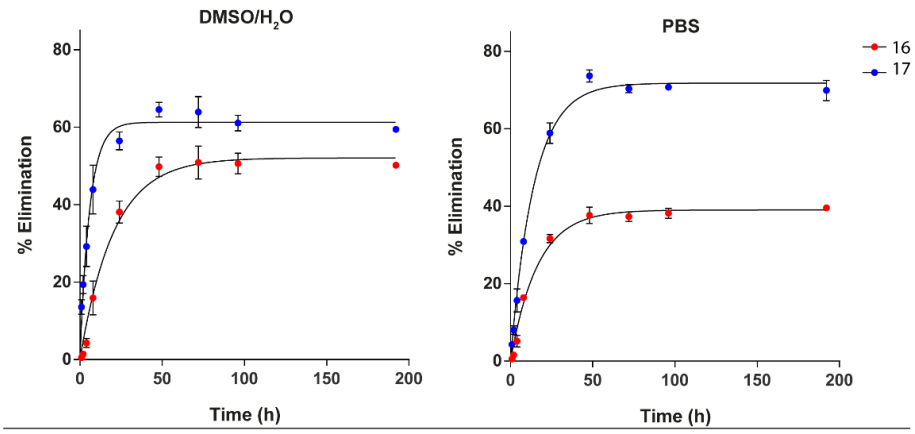


	DMSO/H_2O		PBS	
12b	k_{elim} = 3.19 * 10^{-5} s^{-1}	Eff % = 2 %	k_{elim} = 1.10 * 10^{-5} s^{-1}	Eff % = 15 %
13b	k_{elim} = 0.63 * 10^{-5} s^{-1}	Eff % = 26 %	k_{elim} = 2.53 * 10^{-5} s^{-1}	Eff % = 33 %
14b	k_{elim} = 0.23 * 10^{-5} s^{-1}	Eff % = 4 %	k_{elim} = 0.16 * 10^{-5} s^{-1}	Eff % = 50 %
15b	k_{elim} = 0.53 * 10^{-5} s^{-1}	Eff % = 3 %	k_{elim} = 0.11 * 10^{-5} s^{-1}	Eff % = 51 %
12	k_{elim} = 0.34 * 10^{-5} s^{-1}	Eff % = 35 %	k_{elim} = 2.10 * 10^{-5} s^{-1}	Eff % = 27 %
13	k_{elim} = 1.19 * 10^{-5} s^{-1}	Eff % = 43 %	k_{elim} = 1.54 * 10^{-5} s^{-1}	Eff % = 46 %
14	k_{elim} = 0.62 * 10^{-5} s^{-1}	Eff % = 40 %	k_{elim} = 0.73 * 10^{-5} s^{-1}	Eff % = 44 %
15	k_{elim} = 0.24 * 10^{-5} s^{-1}	Eff % = 20 %	k_{elim} = 0.51 * 10^{-5} s^{-1}	Eff % = 12 %

Figure 6c: Corrected normalized elimination efficiency over time of tetrazines **12b**-**15b** and **12**-**15** in DMSO/H_2O (1:1, v:v) and PBS (0.25% DMSO). Maximum elimination efficiencies (Eff %) were determined by first-order exponential decay approximations (black lines).



16b	k_{elim} = 0.42 * 10^{-5} s^{-1}	Eff % = 8 %	k_{elim} = 0.45 * 10^{-5} s^{-1}	Eff % = 28 %
17b	k_{elim} = 1.23 * 10^{-5} s^{-1}	Eff % = 16 %	k_{elim} = 1.80 * 10^{-5} s^{-1}	Eff % = 30 %



16	k_{elim} = 1.48 * 10^{-5} s^{-1}	Eff % = 52 %	k_{elim} = 3.16 * 10^{-5} s^{-1}	Eff % = 39 %
17	k_{elim} = 4.31 * 10^{-5} s^{-1}	Eff % = 61 %	k_{elim} = 2.01 * 10^{-5} s^{-1}	Eff % = 72 %

Figure 6d: Corrected normalized elimination efficiency over time of tetrazines **16b**-**17b** and **16**-**17** in DMSO/H_2O (1:1, v:v) and PBS (0.25% DMSO). Maximum elimination efficiencies (Eff %) were determined by first-order exponential decay approximations (black lines).

The elimination efficiency (Eff %) and rate (k_{elim}) of the tetrazine library was determined by exposing 2-TCO-AMC **28** (5 µM) to 4 equivalents (20 µM) of a tetrazine in PBS (pH 7.4) at 37 °C for 8 days (**Figures 6**, **7**). The solvent and concentrations were chosen to be either comparable to previous literature (DMSO/H2O) or relevant to experiments conducted in biological systems (PBS). Initially we tested the literature tetrazines **1**, **2**, **23**, **24** and **25** in both DMSO/H_2O (1:1, v:v) and PBS (0.25% DMSO) (**Figure 5**, top panels), followed by tetrazines **4b-7b**, **4-7** (Figure 6a), tetrazines **8b-11b**, **8-11** (**Figure 6b**), tetrazines **12b-15b**, **12-15** (**Figure 6c**) and tetrazines **16b-17b**, **16-17** (**Figure 6d**) under the same conditions. At various time points, the fluorescence intensity relative to 2-TCO-AMC **28** (0%) and AMC **27** (100%) was quantified from which the efficiency could be determined (Figures 5, 6, Eff% values). The results were then normalized and compared to each other. Linear approximations (black lines) were analysed, from which the elimination rate constants were extracted. Elimination rate constants (**Figure 5**, **6**, k_{elim} values) were determined for each tetrazine following first order rate equations:

$$\ln([\text{Tetrazine}]_t) = -k_{elim} * t - \ln([\text{Tetrazine}]_{t=0})$$

The obtained elimination properties of the tetrazine library in PBS were vastly different compared to DMSO/H_2O. Tetrazine **2**, for example, was both faster and more efficient in PBS. As a result, the further focus was on the experiments conducted in PBS.

The values for all tetrazines were plotted as dots in an XY-scatterplot as k_{elim} (logarithmic scale) vs Eff% (linear scale) (**Figure 7**, XY-scatterplot). Three classes of tetrazines were most conspicuous: **14b**, **15b**, **23**, **11** caused slow elimination rates ($k_{elim} < 0.20 * 10^{-5}$ s^{-1}; **Figure 7B**). **11b**, **2**, **5**, **17** all displayed intermediate elimination rates (k_{elim} between 1.5 and 3.0 * 10^{-5} s^{-1}; **Figure 7C**). Tetrazines **1**, **4**, **6**, **7** formed a third, most interesting, class of tetrazines defined by their very fast release kinetics (**Figure 7D**). While reference tetrazine **1** is fast (k_{elim} = 8.86 * 10^{-5} s^{-1}), it is still significantly slower compared to **4**, **6**, and **7** (k_{elim} = 15.3, 23.7, 35.9 10^{-5} s^{-1} respectively). Release rates for tetrazines **4**, **6** and **7** were too fast to be accurately determined by the initial assay and have therefore been displayed as the rate they are at minimum. It is noteworthy that the tetrazines with the protected amino group (**14b**, **15b**) and the tetrazines with a suboptimal positioned amino group (**11**) showed slow elimination rates (**Figure 7B**). This behavior proved to be general across the whole test set (**Figures 5**, **6**) and underscores the importance of the intramolecular proton delivery for the rate of the “click-to-release” process.

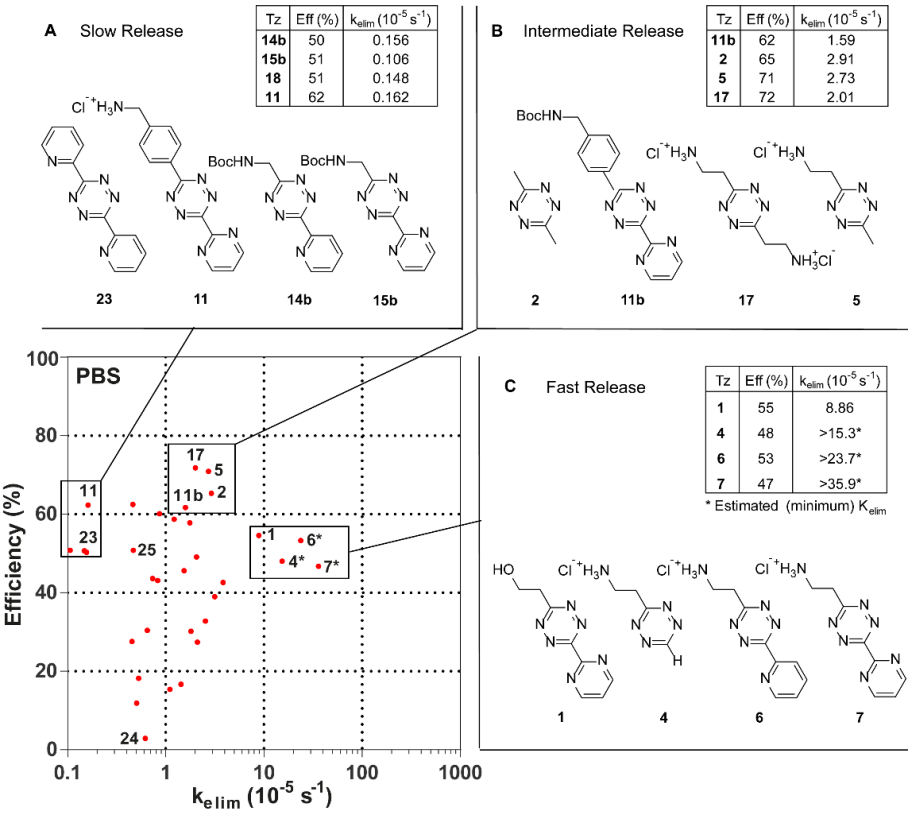


Figure 7: A) XY-scatterplot of all data obtained in PBS as solvent during the fluorescence-based assay. Each result (tetrazines **1**, **2**, **23**-**25**, **4b**-**17b**, **4**-**17**) is depicted as a dot based on their elimination efficiency and rate. B) Tetrazines **14b**, **15b**, **23** and **11** with slow release properties k_{elim} < 0.2 * 10^{-5} s^{-1}. C) Tetrazines **11b, 2**, **5** and **17** with intermediate release properties: Eff% > 60%. D) Tetrazines **1, 4**, **6** and **7** with fast release properties: k_{elim} > 8 * 10^{-5} s^{-1}.

pH dependency of elimination rates

Following these results we set out to determine whether the fast releasing aminoethyl functionalized tetrazines retained these properties over a wide pH-range. To this end, tetrazines **1**-**3**, **21**, **4b**-**7b** and **4**-**7** were exposed (**Figures 8**, **9**) to phosphate buffered solutions at various pH (0.2M [PO_4^{2-}], 10% DMSO, pH = 3.0 (red lines), 6.0 (green lines), and 7.4 (blue lines)) and the fluorescence intensity relative to 2-TCO-AMC **28** (0%) and AMC **27** (100%) was quantified at corresponding phosphate buffered solutions. At pH 3.0 (**Figure 10A**) all tetrazines showed elimination rates between 30 * 10^{-5} s^{-1} and 500 * 10^{-5} s^{-1}, indicating that the availability of protons in solution enhances the elimination rate. Lowering the proton concentration by raising pH to 6.0 or 7.4 resulted in large decreases in the elimination rates observed for most tetrazines (**Figure 10B**, **10C**). At pH 7.4 tetrazines **1**-**3**, **21**, **6b**, and **7b** showed much slower elimination rates (5-15 * 10^{-5} s^{-1}, **Figure 10C**, **10D**). Aminoethyl tetrazines **6** (k_{elim} = 241 * 10^{-5} s^{-1}) and **7** (k_{elim} = 120 * 10^{-5} s^{-1}) on the other hand showed a minimal reduction in rate at increased solvent pH (**Figure 10D**), resulting in a rate 18-27-fold faster at pH 7.4. Substituting amino (**6**, **7**) for alcohol (**1**) or carbamate (**6b, 7b**) functionality reintroduced pH-dependency in the elimination process (**Figure 11**). A carboxy functionality, as seen in tetrazine **3**, provided an overall moderate elimination rate and moderate pH-dependency (**Figure 11**).

Substitution of carboxy (**3**) for amino (**5**) functionality improved both properties. Furthermore, the fastest tetrazine **6** outperforms tetrazine **3** by 18-fold in terms of elimination rate. Finally, when comparing tetrazines **4-7**, the data also shows that the elimination rate is positively affected by electron-withdrawing substituents (pyridine/pyrimidine) explaining the differences between the tetrazines, in line with previous findings.[13b]

These results strongly support our hypothesis that the elimination process is dependent on proton availability and can be catalyzed at biologically relevant pH (3-7.4) through careful placement of cationic ammonium functionality acting as an intramolecular catalyst.

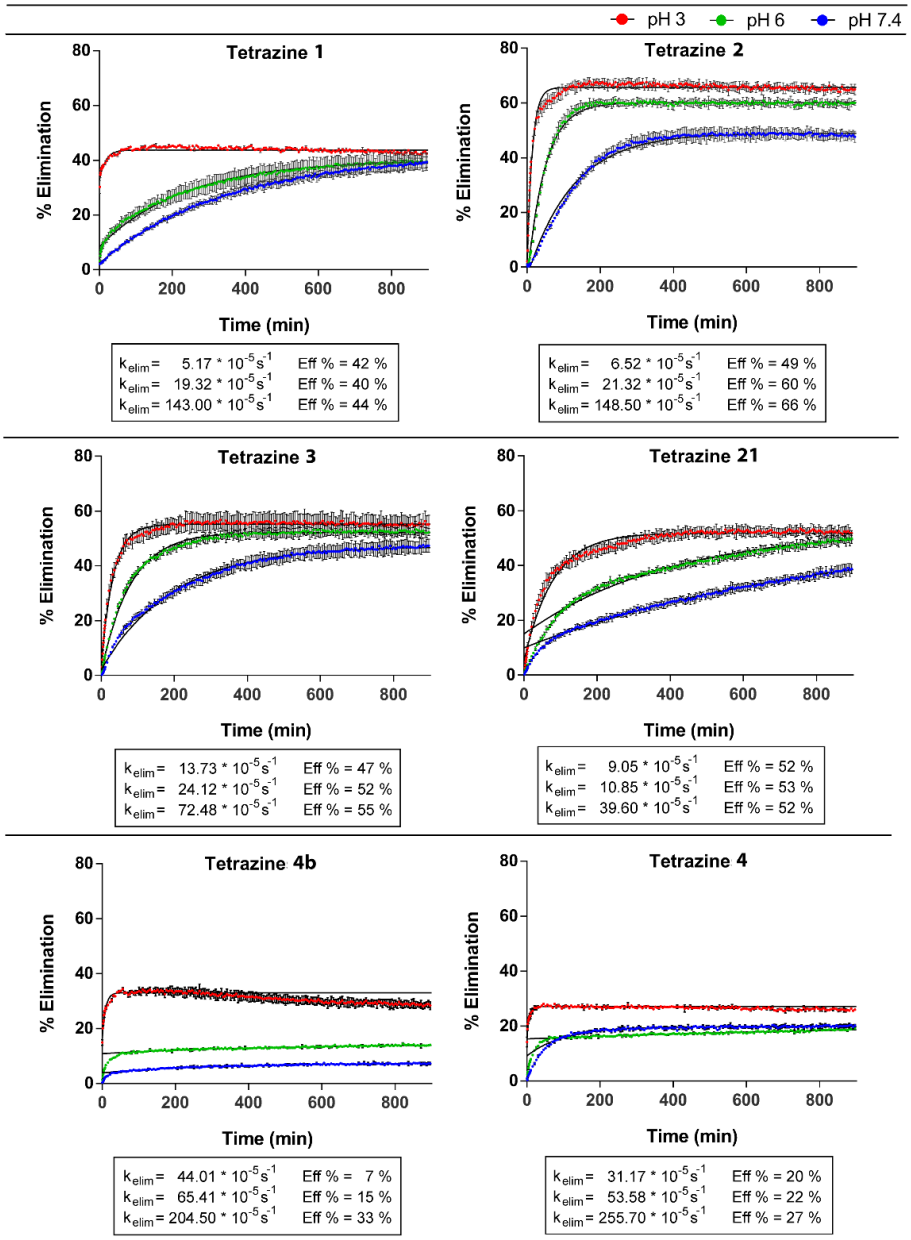


Figure 8: Corrected normalized elimination efficiency over time of tetrazines **1**, **2**, **3**, **21, 4b** and **4** in PBS at pH 3 (red), pH 6 (green) and pH 7.4 (blue). Maximum elimination efficiencies (Eff %) were determined by first-order exponential decay approximations (black lines).

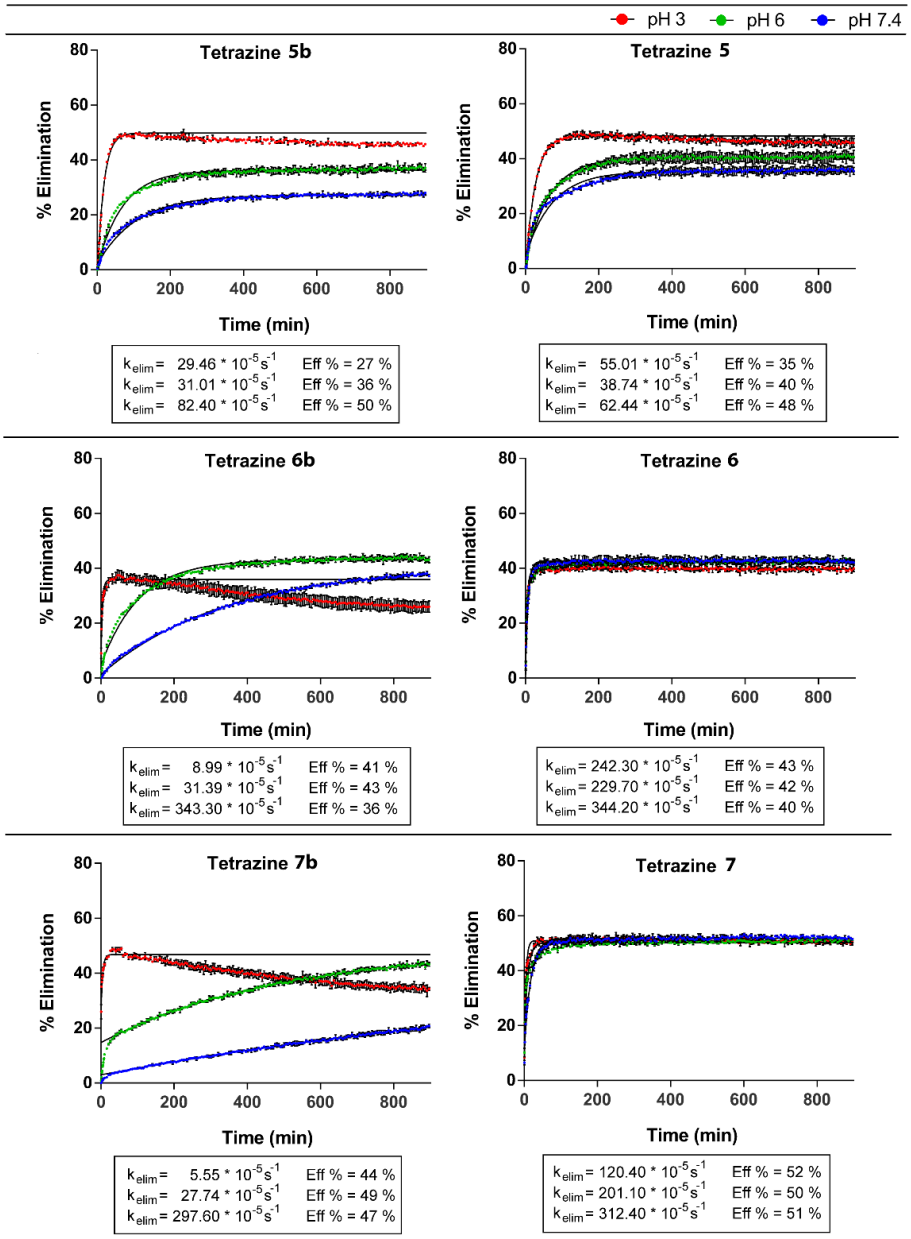


Figure 9: Corrected normalized elimination efficiency over time of tetrazines **5b**-**7b** and **5**-**7** in PBS at pH 3 (red), pH 6 (green) and pH 7.4 (blue). Maximum elimination efficiencies (Eff %) were determined by first-order exponential decay approximations (black lines).

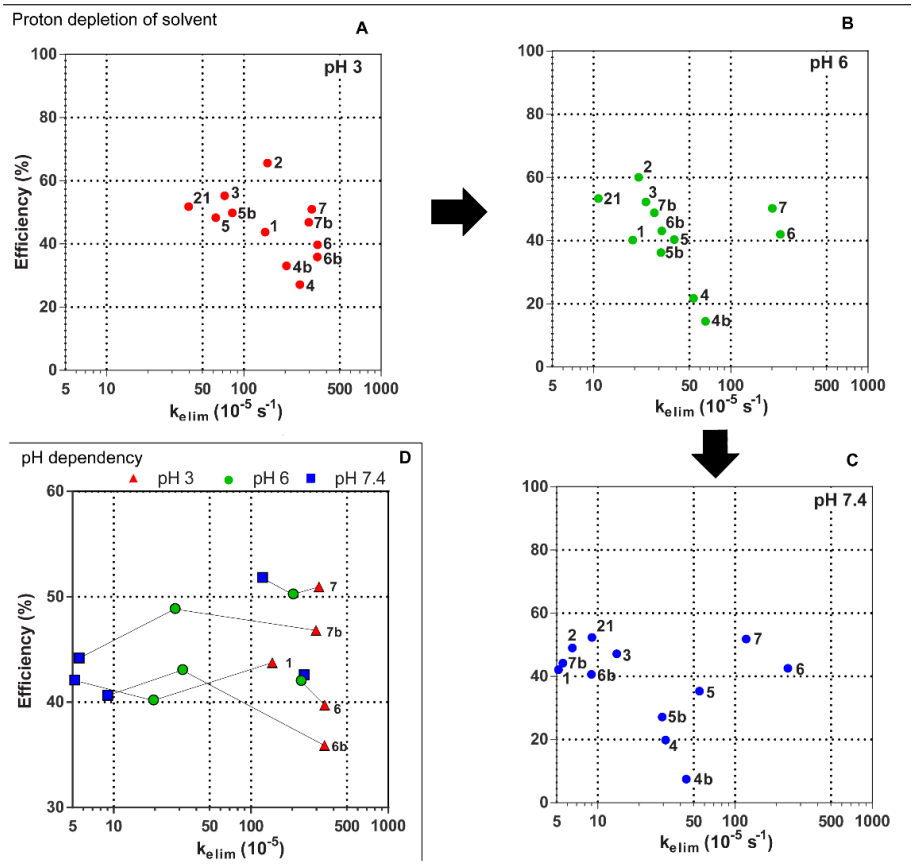


Figure 10. Proton depletion of solvent. A) Scatter plot of tetrazines at pH = 3. B) Scatter plot of tetrazines at pH = 6. C) Scatter plot of tetrazines at pH = 7.4. D) Scatter plot of pH dependent tetrazines (**6**, **7**) and pH independent tetrazines (**6b**, **7b**, **1**).

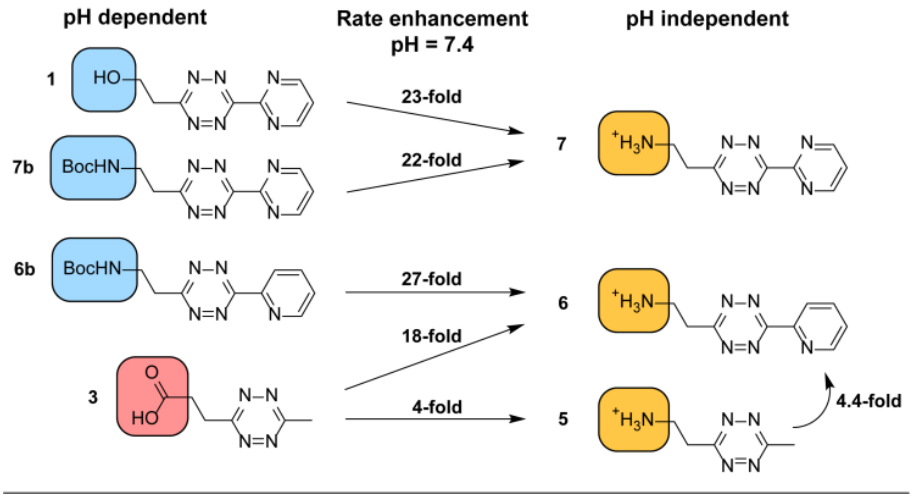


Figure 11. A) Rate enhancement of pH independent functional groups depicted in orange over pH dependent functional groups depicted in blue and red.

LC-MS analysis

In order to gain additional insight in the course of the "click-to-release" reaction with the newly developed aminoalkyl tetrazines and to study the intermediates and possible side products we also assessed the elimination rates of the key compounds **5 - 7** using reported LC-MS based approach that uses an ammonium formate buffered water-acetonitrile eluent (2.5mM $NH_4^+HCOO^-$, pH = 8.4) and taking the first analysis point at t = 5 min.[13b] Known tetrazines **1**-**3** were analyzed by this method for comparison. The elimination rates of tetrazines **2** and **3** showed results corresponding to data reported by Weissleder and coworkers[13b], where tetrazine **3** gives 48% release at t = 5 min, going up to 72% after 16 hours (Figure 12). The explanation offered by the authors invokes rapidly releasing "head-to-head" adduct and slowly releasing "head-to-tail" adduct which are formed in approximately equal amounts.[13b] The data showed much faster initial release than measured in the fluorescence assay, and this is likely due to additional "pseudo-release" or concentration effects during the LC-MS analysis. In turn, aminoethyl tetrazines **5**, **6** and **7**, showed 78%, 80% and 70% initial release respectively at t = 5 minutes with only a small further increase in release over time (Figure 12). This would correspond to the preferential formation of the rapidly releasing "head-to-head" adduct as predicted by our calculations.

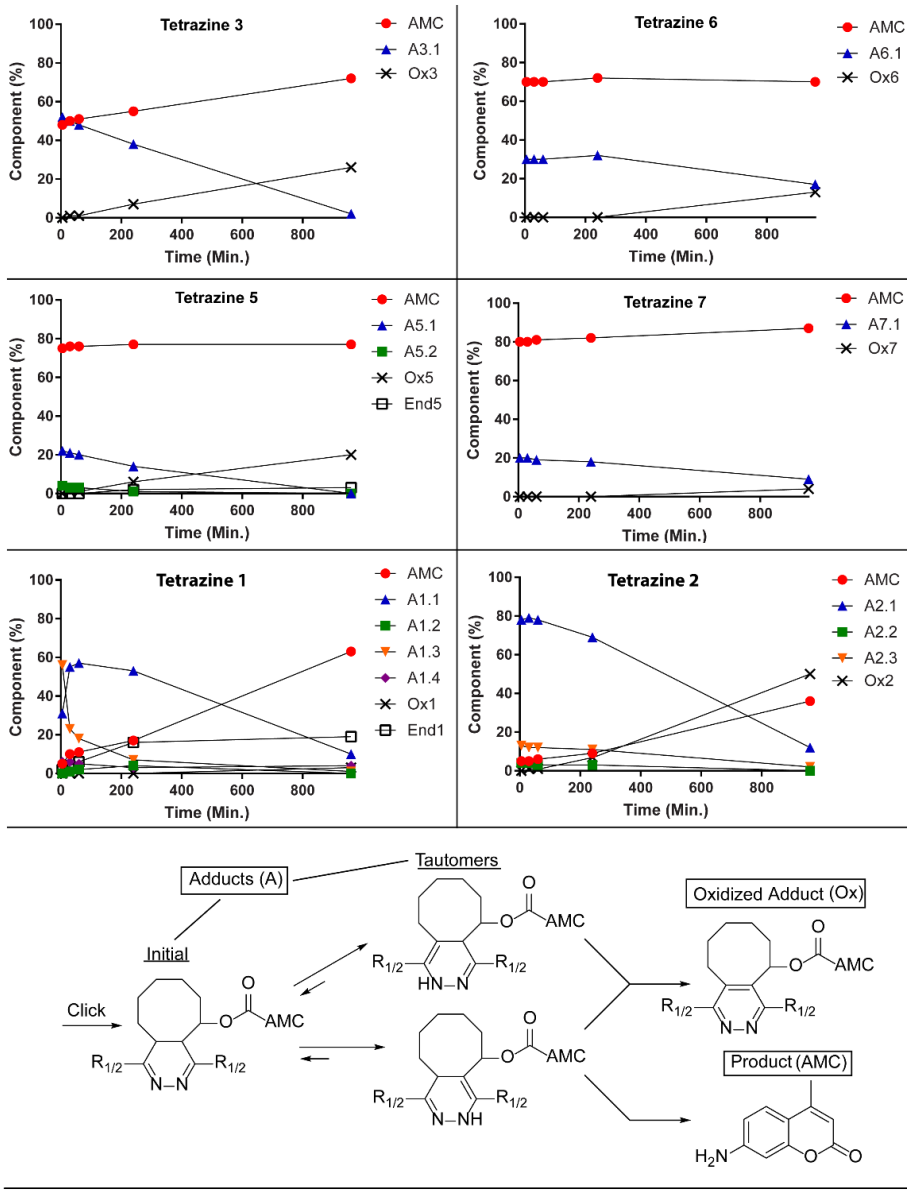


Figure 12. A) Time dependent LC-MS analysis of 2-TCO-AMC **28** with tetrazines **1**, **2**, **3**, **5**, **6** and **7** in PBS. **B)** Aminomethyl coumarin containing molecules: product "AMC", adduct "A", oxidized adduct "Ox" and dead-end adduct "End".

It is noteworthy that although multitude of different intermediates are observed, including putative “head-to-tail” and ”head-to-head” adducts, in the reaction of 2-TCO-AMC **28** with comparatively slow eliminating tetrazines **1** and **2** (Figure 12), as well as with tetrazines **3, 5, 6** and **7** (Figure 12), this does not translate into consistent formation of dead-end adduct (End) as reported by others[13b]. Furthermore the amount of oxidized adduct (Ox) is reduced for tetrazines **1**, **6**-**7** compared to tetrazines **2**, **3**, and **5**. The results of the LC-MS analysis of the “click-to-release” reaction of 2-TCO-AMC **28** with tetrazines **1** and **2** are similar to what is published by Robillard and co-workers in terms of observed intermediates and side products (Figure 12).[13c] To summarize this part, the results obtained from the LC-MS analysis of the key tetrazine derivatives demonstrate that the method shows reproducible results for the known tetrazines **2** and **3** and that the aminoethyl tetrazines **5**-**7** do perform as designed in the “click-to-release” IEDDA reaction.

Conclusions

The full kinetic profiling of a focused tetrazine library clearly shows that the presence of a properly placed cationic ammonium functionality acting as an intramolecular proton donor gives the tetrazine mediated “click-to-release” elimination a pH independent character. Asymmetric tetrazines that contain both this aminoethyl substituent and an electron-withdrawing substituent on the tetrazine core show unprecedented release rates combined with nearly complete pH independence over the whole biologically relevant pH range.

Computational Procedures

Geometry optimisation: IEDDA adduct structures

A conformer distribution search option included in the Spartan 10 program[17], with the use of MM with MMFF94 as force field, was used as starting point for the geometry optimization. All generated structures were further optimized with Gaussian 09[18] using the ωB97XD long-range corrected hybrid functional[19] and 6-311G(d,p) as basis set. Optimization was done in gas-phase and subsequently corrections for solvent effects were done by the use of a polarizable continuum model using water as solvent parameter. The electronic energies ΔE_{gas} were computed by dispersion-corrected DFT given by Equation (1), in which ΔE_{DFT} is the KS-DFT SCF energy and ΔE_{disp} is the standard atom pair-wise London dispersion energy.

$$\Delta E_{gas} = \Delta E_{DFT} + \Delta E_{disp} \quad (1)$$

The free Gibbs energy of the computed conformations was calculated using Equation (2) in which ΔE_{gas} is the gas-phase energy (electronic energy), ΔG^{T}_{RRHO} (T= 298.15 K and pressure= 1 atm.) is the sum of corrections from the electronic energy to free Gibbs energy in the rigid-rotor-harmonic-oscillator approximation (RRHO) also including zero-point-vibrational energy, and ΔG^{T}_{solv} is their corresponding free solvation Gibbs energy. Visualization of relevant structures was done with CYLview[20]. All denoted distances are expressed in ångström (Å).

$$\Delta G^{T}_{aq} = \Delta E_{gas} + \Delta G^{T}_{gas,RRHO} + \Delta G^{T}_{solv} \quad (2)$$

$$= \Delta G^{T}_{gas} + \Delta G^{T}_{solv}$$

Geometry optimization: IEDDA transition state structures

Initial guesses for the transition states were based on the work of Fox and co-workers and Houk and co-workers[21]. All generated structures were further optimized with Gaussian 09[18] using the ωB97XD long-range corrected hybrid functional[19] and 6-31+G(d) as basis set. Optimization was done in combination with a polarizable continuum model using water as solvent parameter.

General Synthetic Procedures

Preparation of anhydrous hydrazine 6.00 mol (300 mL) of hydrazine monohydrate was added to 3.6 mol (200 g) of KOH pellets, refluxed for 3 hours (>140 °C) and 5.5 mol (175 mL) hydrazine was collected via distillation (>140 °C). Then 175 mL of hydrazine was added to 75 mmol (30 g) NaOH pellets, refluxed for 1 hour (135 °C) and 3.7 mol (120 mL) of dry hydrazine was collected via distillation.

Procedure A (flask) First 1 eq. of R_1-CN nitrile reagent (**18**, **19** or **20**), 5 eq. of R_2-CN (formamidine acetate, acetonitrile, 2-cyano pyridine or 2-cyano pyrimidine) and 0.25 eq. of catalyst (zinc triflate, zinc iodide, or nickel triflate) were added to a flask under inert nitrogen atmosphere. Then (if used) dry dioxane (1.6 mL/mmol) was added. Anhydrous hydrazine (50 eq., 1.6 mL/mmol) was added dropwise under heavy stirring, while maintaining room temperature in a water bath. After 5 minutes (if required) the temperature was slowly

adjusted to the desired value. After the desired time a dihydrotetrazine containing reaction mixture was obtained.

Procedure B (pressure tube) First 1 eq. of R_1-CN nitrile reagent (**18**, **19** or **20**), 5 eq. of R_2-CN (formamidine acetate, acetonitrile, 2-cyano pyridine or 2-cyano pyrimidine) and 0.25 eq. of catalyst (zinc triflate, zinc iodide, or nickel triflate) were added to a pressure tube. Then (if used) dry dioxane (1.6 mL/mmol) was added. The tube was sealed and anhydrous hydrazine (50eq., 1.6 mL/mmol) was quickly injected under heavy stirring, while maintaining room temperature in a water bath. After 5 minutes (if required) the temperature was slowly adjusted to the desired value. After the desired time, the reaction was cooled to room temperature and the rubber seal was carefully punctured, slowly releasing the generated NH_3 gas. A dihydrotetrazine containing reaction mixture was obtained.

Procedure C (oxidation) A mixture of DCM/AcOH (1:1, v:v, 20 mL/mmol) was prepared. While stirring, the dihydrotetrazine containing reaction mixture was added dropwise. Solid $NaNO_2$ (20 eq., 1.5 g/mmol) was added portion wise over 30 minutes. The mixture was concentrated using rotary evaporation, re-dissolved in EtOAc, washed with H_2O ($NaHCO_3$ (aq.) or HCl (aq.)), dried using $MgSO_4$ and concentrated using rotary evaporation. The crude product was purified by column chromatography.

Procedure D (oxidation) The dihydrotetrazine containing reaction mixture was dissolved in 4M $NaNO_2$ (aq.) (40 eq., 10 mL/mmol) and 2M HCl (aq.) (60 eq., 30 mL/mmol) was added dropwise under heavy stirring until gas formation stops (pH = 2-3). Then 0.1M HCl (aq.) (50 mL/mmol) was added and the watery solution was extracted multiple times with EtOAc (50 mL/mmol). The organic layers were combined, dried with $MgSO_4$ and concentrated using rotary evaporation. The crude product was purified by column chromatography.

Procedure E (N-Boc deprotection) The N-Boc protected tetrazine was dissolved in dry DCM (1 mL/30 mg) and a 4M HCl in dioxane solution (1 mL / 30 mg) was added dropwise over 1 minute while stirring at room temperature. The reaction mixture was stirred at room temperature for 2 hours resulting in a suspension. The suspension was centrifuged and the colorless supernatant was removed. The colorful precipitate was washed 2 times via re-suspension in 10 mL of dry dioxane, centrifugation, and partitioning from the colorless supernatant. The precipitate was re-suspended in 5 mL of dry dioxane, transferred to a flask and concentrated using rotary evaporation resulting in quantitative N-Boc deprotected product as an ammonium chloride salt.

General Analytic Procedures

Fluorescence profile Excitation and Emission profiles of AMC **27** and 2-TCO-AMC **28** were determined in DMSO/H_2O and phosphate buffered water at 5 µM concentrations. Excitation wavelength was optimized towards highest ratio (Ex_{25} % / Ex_{24} %). Emission wavelength was optimized to maximize the fluorescence difference Δ % (Em_{25} % – Em_{24} %).

Fluorescence background analysis Background fluorescence of tetrazines was determined in PBS. 5 µl of a 10 mM solution of tetrazine (**4b**-**17b**, **4**-**17**, **1**, **2**, **23**-**25**, **3**, **21**) in DMSO was added

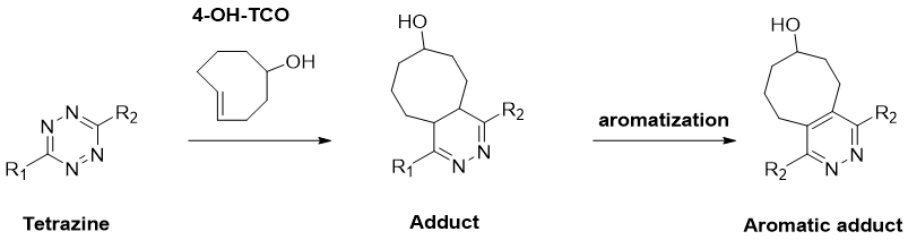
to 195µl PBS. The fluorescence intensity (λ_{ex} = 380 nm, λ_{em} = 450 nm) was determined comparing with **28** (0% elimination) and **27** (100% elimination) in matching solvent.

Elimination efficiency test A The elimination efficiency of tetrazines was determined by incubation of the various tetrazines in the desired solvent (DMSO/H_2O or PBS) at 37 °C over a time period of 8 days. 1.5 µl of a 10 mM solution of 2-TCO-AMC **28** (or AMC **27** as a control) in DMSO was dissolved in 3 mL solvent, followed by 6 µl of a 10 mM solution of tetrazine (**4b**-**17b**, **4**-**17**, **1**, **2**, **23**-**25**; or no tetrazine as a control) in DMSO. The resulting solution was then incubated at 37 °C. At time points 1, 2, 4, 8, 24, 48, 96 and 192 hours 200 µL aliquots were taken and the fluorescence intensity (λ_{ex} = 380 nm, λ_{em} = 450 nm) was determined. The test was performed twice on all tetrazines. Percentage of elimination was determined comparing with **28** (0% elimination) and **27** (100% elimination) in matching solvent.

Elimination efficiency test B The elimination efficiency of tetrazines was determined by incubation in phosphate buffer (0.2M at pH 7.4, 6 or 3) at 25 °C over a time period of 16 hours. 10 µl of a 100 µM solution of 2-TCO-AMC **28** (or AMC **27** as a control) in DMSO was dissolved in 180 µl, followed by addition of 10 µl of a 100 µM solution of tetrazine (**4b**-**7b**, **4**-**7**, **1**, **2**, **23**-**25**, **3**, **21**; or no tetrazine as a control) in DMSO. The fluorescence intensity (λ_{ex} = 380 nm, λ_{em} = 450 nm) was determined over a period of 16 hours at 1 to 5 minute intervals. The test was performed twice on all tetrazines. Percentage of elimination was determined comparing with **28** (0% elimination) and **27** (100% elimination) in matching pH solvent due to pH dependent variable fluorescence of **27**.

Elimination efficiency test C The elimination efficiency of tetrazines was determined by incubation in PBS over a time period of 1 day. 1 µl of of a 10 mM solution of 2-TCO-AMC **28** in DMSO, 10 µl of a 10 mM solution of tetrazine (**1**, **2**, **3**, **5**, **6** or **7**) in DMSO and 99 µl of PBS (pH = 7.4) were incubated in an LCMS vial and measured by C18 column LC-MS analysis using a freshly made 2.5 mM ammonium formate buffer solution (pH = 8.4 titrated using conc. NH_4OH (aq.)) and acetonitrile as eluent components. 5 µl of the solution was injected at t = 5, 30, 60, 240, 960 minutes. Intermediates and products formed were identified by mass analysis and aminomethyl coumarin bearing compounds were quantified by UV analysis.

HRMS analysis of analytically unstable tetrazines Tetrazines that could not be identified by HRMS were reacted with 4-OH-TCO at 1mM concentration to form a non-eliminating adduct for HRMS.



Procedure: For each tetrazine 160 µl DMSO was placed in a small vial and 20 µl of 10mM tetrazine solution was added, followed by 20 µl of 10mM 4-OH-TCO. Near-instantaneous

discoloration was observed, where after the vials were stored at -20 °C until HRMS analysis was performed. Due to aromatizing effects of the product tetrazines were identified by **adduct mass** (m/z) or **aromatic adduct mass** (m/z -2).

Compound Synthesis

Compound 3: Synthesis was performed in a closed pressure tube (**procedure B**) at 60 °C overnight. 1.0 mmol (0.099 g) of compound **22**, 10 mmol (0.26 ml, 0.21 g) of acetonitrile, 0.25 mmol (0.090 g) nickel triflate, 1.5 mL of dioxane and 47 mmol (1.5 mL, 1.5 g) of anhydrous hydrazine were used. Oxidation (**procedure C**) was performed using 40 mL of DCM/AcOH (1:1, v:v) and 22 mmol (1.5 g) of solid $NaNO_2$. Purification was performed with silica column chromatography using an 2:20:78 AcOH:EtOAc:DCM eluent resulting in 0.008 g (0.047 mmol, 4.7%) of compound **3** as a pink solid. ^{1}H NMR (500 MHz, MeOD) δ 3.56 (t, *J* = 7.0 Hz, 2H, CH_2), 3.03 (t, *J* = 7.0 Hz, 2H, CH_2), 2.98 (s, 3H, CH_3). ^{13}C NMR (126 MHz, MeOD) δ 176.03, 170.17, 168.98, 31.64, 30.79, 21.12. HRMS (m/z): adduct $[C_{14}H_{22}N_2O_3 + H]^+$ calculated 267.1703, found 267.1704.

Compound 16b: *For synthesis details, see **Compound 12b**.* ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.68 (s, 2H, NH), 4.95 (d, *J* = 5.6 Hz, 4H, CH_2), 1.43 (s, 18H, Boc, CH_3). ^{13}C NMR (101 MHz, $CDCl_3$) δ 167.71, 155.93, 80.50, 43.57, 28.40. HRMS (m/z): $[C_{14}H_{24}N_6O_4 + Na]^+$ calculated 363.1752, found 363.1759.

Compound 16: N-Boc deprotection of compound **16b** was performed using 4M HCl in dioxane according to **procedure E** obtaining compound **16** in quantitative yield as a bright pink solid. ^{1}H NMR (400 MHz, DMSO) δ 9.16 (s, 6H, NH_3Cl), 4.82 (s,4H, CH_2). ^{13}C NMR (101 MHz, DMSO) δ 165.09, 40.94. HRMS (m/z): $[C_4H_8N_6 + H]^+$ calculated 141.0883, found 141.0880.

Compound 17b: (*Alternative synthesis for **Compound 6b**)* Synthesis was performed in a closed pressure tube (**procedure B**) at 60 °C overnight. 2.02 mmol (0.344 g) of compound **18**, 12 mmol (1.0 ml, 1.1 g) of 2-pyridinecarbonitrile, 0.48 mmol (0.173 g) zinc triflate, 3.0 mL of dioxane and 101 mmol (3.2 mL, 3.2 g) of anhydrous hydrazine were used. Oxidation (**procedure D**) was performed using 80 mmol (20 mL) of 4M $NaNO_2$ (aq.) and 120 mmol (60 mL) 2M HCl (aq.). Purification was performed with silica column chromatography using an 10% to 60% EtOAc/Pentane eluent resulting in 0.034 g (0.11 mmol, 5.4%) of compound **6b** as a pink oil and 0.074 g (0.20 mmol, 19.8%) of compound **17b** as a red solid byproduct. **TLC**: Compound **6b** Rf = 0.2, compound **17b** Rf = 0.5, 50% EtOAc in pentane. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.12 (s, 2H, NH), 3.71 (dd, *J* = 12.3, 6.2 Hz, 4H, CH_2), 3.49 (t, *J* = 6.1 Hz, 4H, CH_2), 1.35 (s, 18H, Boc, CH_3). ^{13}C NMR (101 MHz, $CDCl_3$) δ 168.48, 155.90, 79.58, 38.50, 35.61, 28.42. HRMS (m/z): $[C_{16}H_{28}N_6O_4 + Na]^+$ calculated 391.2065, found 391.2070.

Compound 17: N-Boc deprotection of compound **17b** was performed using 4M HCl in dioxane according to **procedure E** obtaining compound **17** in quantitative yield as a pink solid. ^{1}H NMR (400 MHz, DMSO) δ 8.31 (s, 6H, NH_3Cl), 3.66 (t, *J* = 6.9 Hz, 4H, CH_2), 3.47 – 3.31 (m, 4H, CH_2). ^{13}C NMR (101 MHz, DMSO) δ 166.89, 37.00, 32.11. HRMS (m/z): $[C_6H_{12}N_6 + H]^+$ calculated 169.1197, found 169.1199.

Compound 21: Synthesis was performed in a closed pressure tube (**procedure B**) at 30 °C for 3 days. 7.87 mmol (0.780 g) of compound **22**, 0.25 mmol (0.086 g) zinc triflate, 1.5 mL of dioxane and 47 mmol (1.5 mL, 1.5 g) of anhydrous hydrazine were used. Oxidation (**procedure C**) was performed using 40 mL of DCM/AcOH (1:1, v:v) and 22 mmol (1.5 g) $NaNO_2$ (s). Purification was performed with silica column chromatography using an AcOH:EtOAc:DCM eluent resulting in 0.210 g (0.928 mmol, 23.6%) of compound **21** as a pink solid. ^{1}H NMR (400 MHz, DMSO) δ 3.47 (t, *J* = 6.8 Hz, 4H, CH_2), 2.90 (t, *J* = 6.8 Hz, 4H, CH_2). ^{13}C NMR (101 MHz, DMSO) δ 173.43, 168.73, 30.56, 29.40. HRMS (m/z): adduct $[C_{16}H_{24}N_2O_5 + H]^+$ calculated 325.1758, found 325.1767.

Compound 22: Following literature procedure[22], 300 mmol (44.14 g) of D-glutamic acid was dissolved in a solution of 300 mL 2M NaOH (aq.) (600 mmol) and portionwise over 30 minutes 200 mmol (48 g) of trichloroisocyanuric acid (TCCA) was added while stirring at room temperature in a water bath. The reaction was very exothermic and heavy foaming occurred. The reaction mixture was stirred for an additional 30 minutes, where after 50 mL of con. HCl (aq.) (600 mmol) was added. The reaction mixture was extracted with EA, dried using $MgSO_4$ and concentrated under rotary evaporation resulting in 22.72 g (229.3 mmol, 76.4%) of compound **22** as a pale yellowish solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 11.13 (s, 1H, COOH), 2.72 (t, *J* = 6.7 Hz, 2H, CH_2), 2.62 (t, *J* = 6.8 Hz, 2H, CH_2). ^{13}C NMR (101 MHz, $CDCl_3$) δ 175.69, 118.46, 29.56, 12.62.

Compound 23: *For synthesis details, see **Compound 14b**.* ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.93 (d, *J* = 4.2 Hz, 2H, pyr, CH), 8.70 (d, *J* = 7.9 Hz, 2H, pyr, CH), 7.97 (ddd, *J* = 7.8, 7.8, 1.6 Hz, 2H, pyr, CH), 7.54 (ddd, *J* = 7.5, 4.7, 0.7 Hz, 2H, pyr, CH). ^{13}C NMR (101 MHz, $CDCl_3$) δ 163.86, 151.04, 150.03, 137.55, 126.66, 124.55. HRMS (m/z): $[C_{12}H_8N_6 + H]^+$ calculated 237.0884, found 237.0892.

Compound 28: 0.21 mmol (37 mg) of aminomethylcoumarin **27**, 0.71 mmol (75 mg) of solid Na_2CO_3 and 0.14 mmol (42 mg) of triphosgene were dissolved in 4 mL DCM at 0 °C and stirred for 4 hours while warming to room temperature. The reaction mixture was reduced by rotary evaporation and redissolved in 2 mL THF. 0.15 mmol (19 mg) of 2-hydroxy-*trans*cyclooctene (2-OH-TCO, **26**) and 0.60 mmol (84 µl, 61 mg) of TEA were added and the reaction mixture was stirred overnight at room temperature. The reaction mixture was reduced under rotary evaporation, purified with silica column chromatography using an 10% to 40% EtOAc/Pentane eluent resulting in 2.3 mg (7.0 µmol, 4.7%) of compound **28** as a pale solid. ^{1}H NMR (850 MHz, $CDCl_3$) δ 7.53 (d, *J* = 8.6 Hz, 1H), 7.45 (s, 1H), 7.40 (d, *J* = 1.9 Hz, 1H), 6.96 (s, 1H), 6.19 (d, *J* = 0.8 Hz, 1H), 5.91 – 5.86 (m, 1H), 5.58 (dd, *J* = 16.5, 2.2 Hz, 1H), 5.46 (s, 1H), 2.51 – 2.47 (m, 1H), 2.41 (d, *J* = 0.8 Hz, 3H), 2.14 (dd, *J* = 15.0, 4.2 Hz, 1H), 2.08 – 2.02 (m, 1H), 2.02 – 1.98 (m, 1H), 1.93 – 1.87 (m, 1H), 1.80 – 1.74 (m, 1H), 1.73 – 1.68 (m, 1H), 1.54 – 1.47 (m, 1H), 1.14 – 1.09 (m, 1H), 0.86 – 0.81 (m, 1H). ^{13}C NMR (214 MHz, $CDCl_3$) δ 161.27, 154.57, 152.39, 152.29, 141.62, 132.51, 130.70, 125.54, 115.60, 114.42, 113.26, 105.88, 75.15, 40.75, 36.13, 36.08, 29.16, 24.32, 18.76. HRMS (m/z): $[C_{19}H_{21}NO_4 + H]^+$ calculated 328.1543, found 328.1551.

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Chapter 6: Fluorogenic Bifunctional *Trans*-Cyclooctenes as Efficient Tools for Investigating Click to Release Kinetics

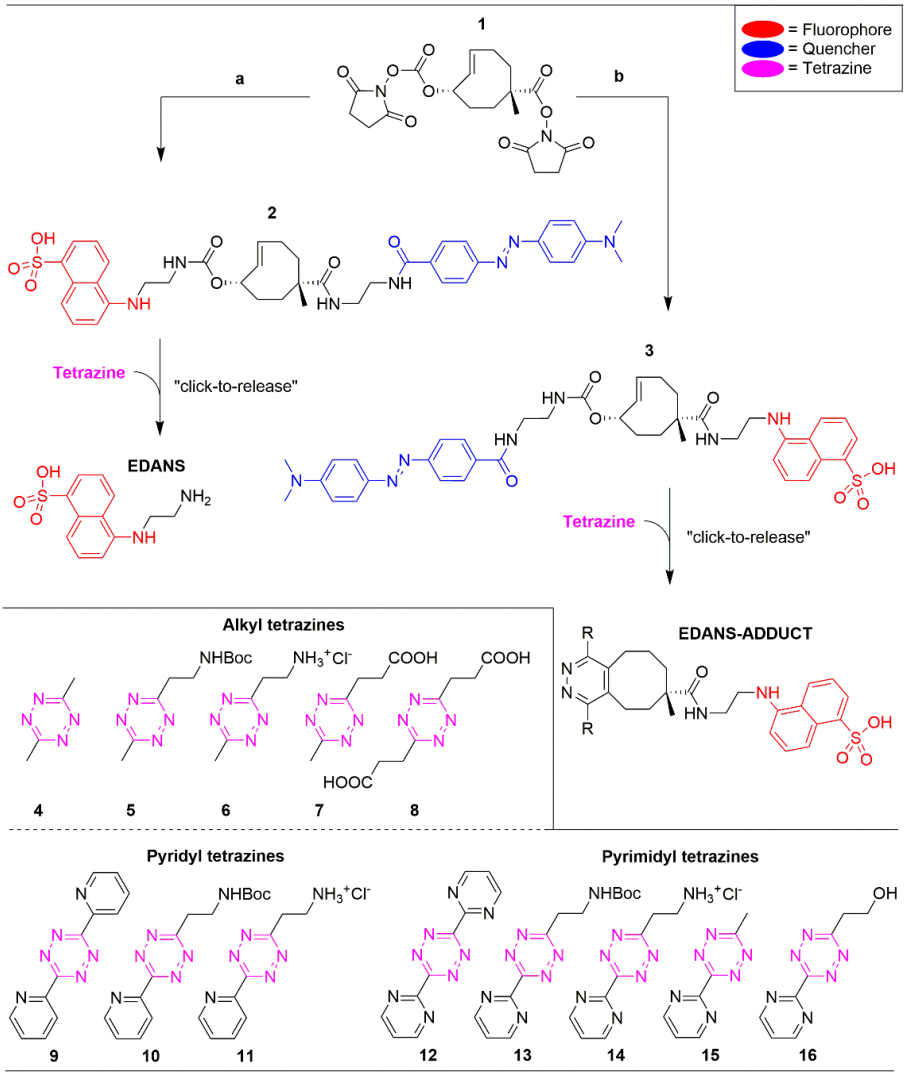
Introduction

The inverse electron demand Diels-Alder (IEDDA)-pyridazine elimination tandem reaction can be used to release allylic substituents from *trans*-cyclooctene (TCO) following a reaction with tetrazines (**chapter 5**). While the fluorogenic reporter TCO-coumarin (compound **28**, **chapter 5**) is a very useful tool to identify effective tetrazines for this elimination reaction, the results may not be an accurate fit for the actual release rates of (non-conjugated) aliphatic primary amines (often the target of TCO-caging in biological experiments). No reliable method has been reported to directly measure the true kinetic parameters of the cycloaddition and release steps in the click-to-release reaction of tetrazines with TCO-caged aliphatic primary amines. To date, by using indirect LC-MS analysis[1-2] (at non-physiological pH or non-physiological eluent), or by releasing non-aliphatic functional groups[1, 3], kinetic parameters have been obtained. Whilst being informative, these results may not translate well to the true kinetic parameters for click-to-release reactions of tetrazines with TCO-caged aliphatic primary amines in biological environments.

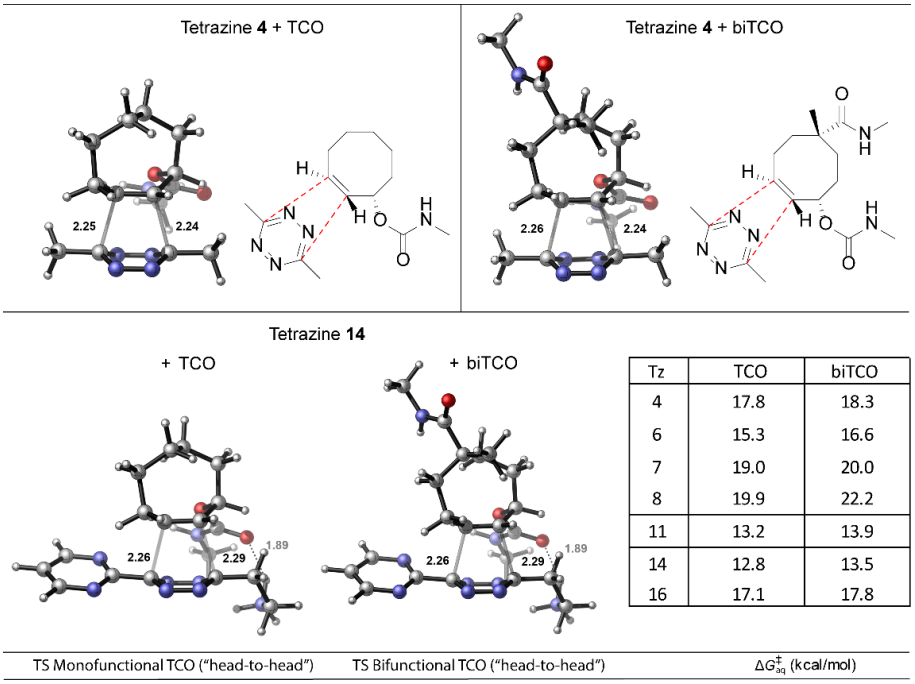
In this chapter, to circumvent limitations caused by indirect, non-physiological, or non-aliphatic conditions, fluorogenic probes **2** and **3** were designed using bifunctional *trans*-cyclooctene[4] (biTCO) and a FRET-pair based on the fluorophore EDANS and quencher DABCYL (**Scheme 1**). The resulting probes allow the direct approximation of IEDDA (K_{IEDDA}) and release ($K_{release}$) rate constants of TCO-caged primary aliphatic amines in a 96-well plate reader format, thereby representing the first tool to study click-to-release rates for TCO-caged primary aliphatic amines.

Design

The first aim of the research described in this chapter was to calculate the kinetic properties of tetrazines reacting with a bifunctional TCOs (as opposed to monofunctional TCOs). For this potent tetrazines from literature were identified, such as dimethyl tetrazine **4**, carboxyethyl modified tetrazines **7** and **8**, aminoethyl modified tetrazines **6**, **11** and **14** and hydroxyethyl modified tetrazine **16**. The ground state and the transition state (TS) energies were calculated for both reactions (**Figure 1**). The DFT calculations showed that, for these tetrazines, the IEDDA reaction is slower when reacting with bifunctional TCO compared to monofunctional TCO. The relative reactivity differences between individual tetrazines were similar for both TCOs and depended on the functional groups attached to the tetrazine core. Compared to tetrazine **4**, carboxyethyl functionality (tetrazines **7**-**8**) increased the TS energy, aminoethyl functionality (tetrazines **6**, **11**, **14**) lowered the TS energy due to



Scheme 1: (**Top**) Functionalization of bifunctional TCO **1** into probes **2** and **3** accompanied with a structure of the fluorescent end-product after reaction with tetrazine and subequent release. (**Bottom**) Previously synthesized tetrazines used in this chapter.



Tz	TCO	biTCO
4	17.8	18.3
6	15.3	16.6
7	19.0	20.0
8	19.9	22.2
11	13.2	13.9
14	12.8	13.5
16	17.1	17.8

Figure 1: "Head-to-head" transition state energies of tetrazines **2**, **6**, **7**, **8**, **11**, **14** and **16** with model mono- or bi-functional axial (E)-cyclooct-2-ene. Distances of interest (forming C-C bonds, interactions) are labelled in Å. Reported transition states were used for initial approximations.[8-9] All generated structures were further optimized with Gaussian using the M06-2X hybrid functional[6] and 6-31+G(d) as basis set. Optimization was done in combination with a polarizable continuum model using water as solvent parameter.

its beneficial interaction with the carbonyl at the TCO's allylic carbamate. The presence of electron withdrawing pyridyl and pyrimidyl functionalities also lowered the TS energy.

Determination of elimination kinetics

Starting from bifunctional TCO **1**, the activated carbonate could be functionalized first, followed by functionalizing the activated ester, to make EDANS releasing fluorogenic probe **2** and DABCYL releasing fluorogenic probe **3**. Next, both constructs were tested in a phosphate buffered solution (0.2M PO_4^{2-} solution in 10% DMSO) to determine the release reaction kinetics in the presence of commonly used tetrazines from literature (alkyl tetrazines **4-8**, pyridyl tetrazines **9-11**, and pyrimidyl tetrazines **12-16**). Initially both probes **2** and **3** were analyzed to determine the elimination behavior of the tetrazines when reacting with bifunctional TCOs. Each of the tetrazines were tested on both probes and for each probe two concentrations were used, either 400 µM (**Figure 2**) or 100 µM (**Figure 3**). The fluorescence emergence

was analyzed until t = 240 minutes, by which time the change in fluorescence had become minimal. To determine the elimination efficiency and reaction rates involved during the reaction, decay trendlines were fitted to the data using ***GraphPad Prism***. Unlike the fluorescence signals observed in **chapter 5**, where the release of coumarin from monofunctional TCO was emerging from a single "eliminating" adduct following a "one-phase exponential decay" trend accompanied with a single reaction rate, the fluorescence signal from the release of alkyl amines from both probe **2** and **3** did not fit a "one-phase exponential decay" trend, with the exception of tetrazine **4**. Alternatively a "two-phase exponential decay" trendline appeared to be

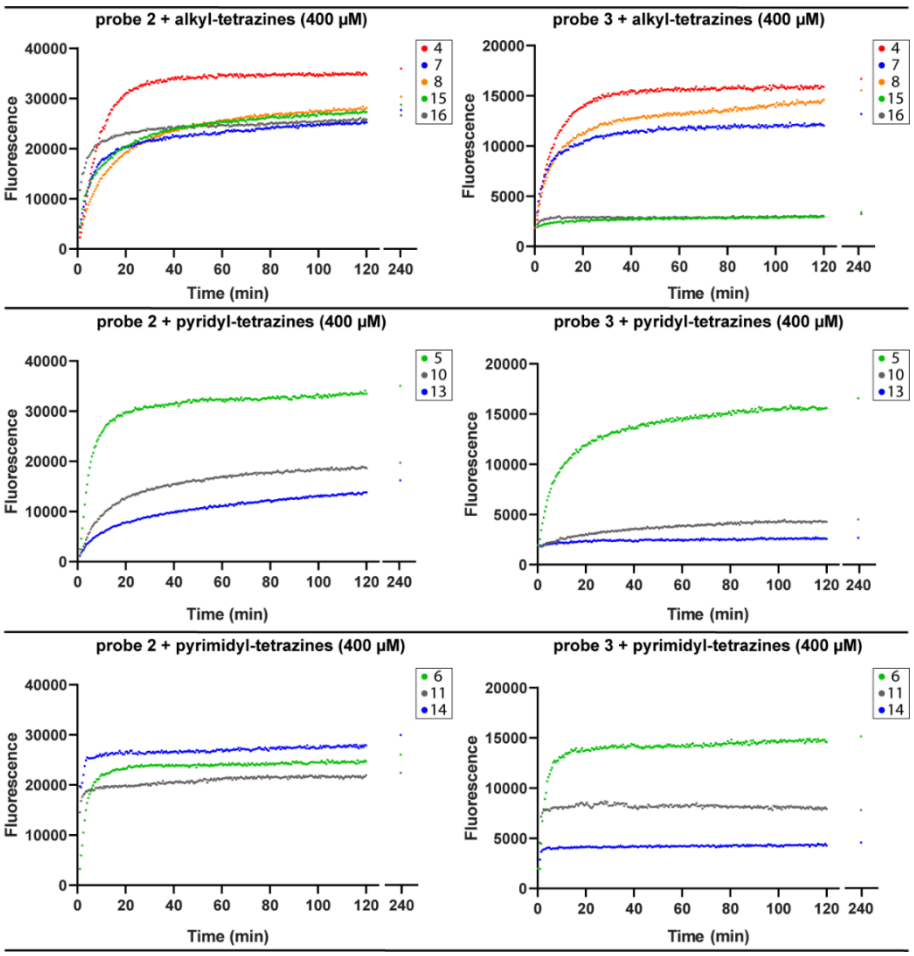


Figure 2: Raw data of the fluorescence emergence when reacting either EDANS releasing probe **2** (left, 10 µM) or DABCYL releasing probe **3** (right, 10 µM) with tetrazines **4** – **8**, **10** – **11** and **13** – **16** (400 µM) measured over a time period of 120 minutes, followed by an additional measurement at 240 minutes.

a near-perfect fit for the fluorescence signals obtained. This suggested that is was likely that when using probe **2** or **3**, having a bifunctional TCO attached to an alkyl amine, at least two measurable eliminating processes existed during the reaction with the tested tetrazines. Similar to what was shown in literature[2], this could be the result from multiple reversible tautomeric intermediates (one non-eliminating and one eliminating) formed from a single IEDDA adduct (**Scheme 2**). When comparing the fluorescence signals obtained from probe **2** and **3** it appeared that the intensity was significantly lower for DABCYL-releasing probe **3** (**Figure 4**).

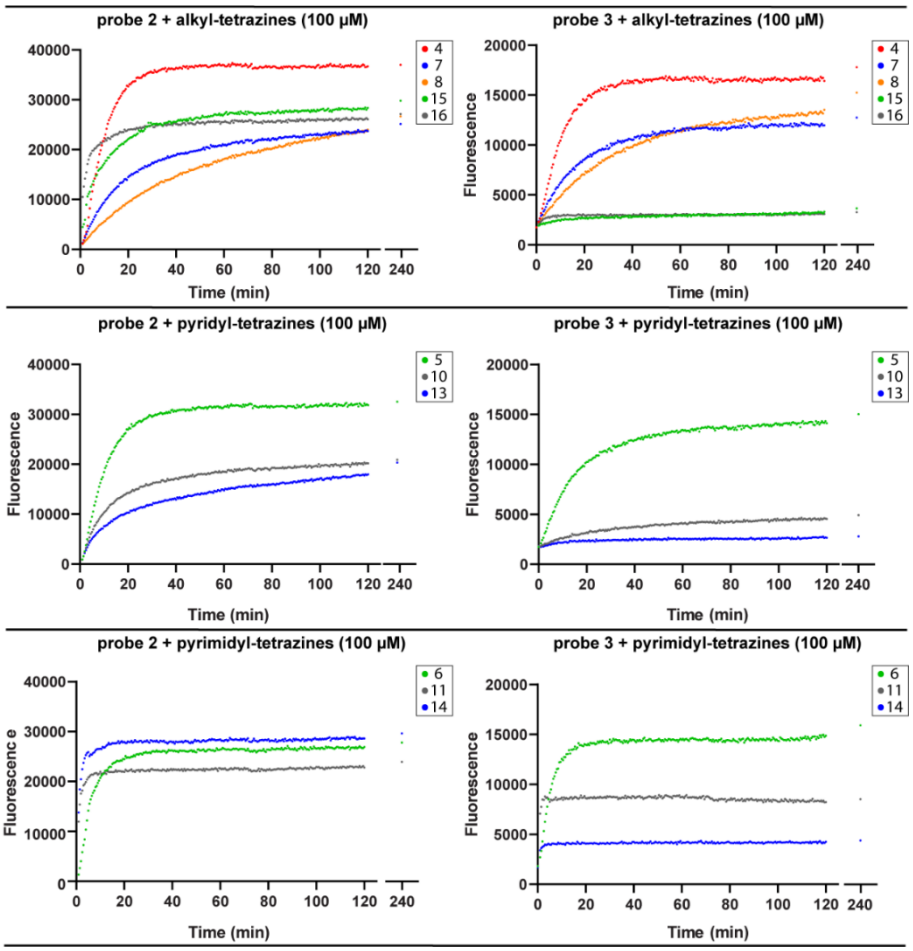
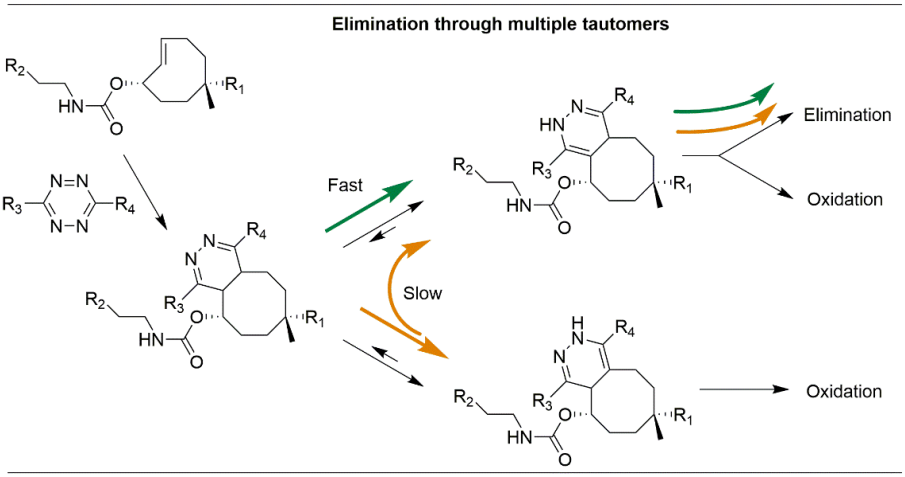


Figure 3: Raw data of the fluorescence emergence when reacting either EDANS releasing probe **2** (left, 10 µM) or DABCYL releasing probe **3** (right, 10 µM) with tetrazines **4** – **8**, **10** – **11** and **13** – **16** (100 µM) measured over a time period of 120 minutes, followed by an additional measurement at 240 minutes.

The likely reason for this difference is residual FRET-quenching occurring from the formed pyridazine moiety in close proximity to the attached EDANS, and appeared to be stronger when pyridyl (65% – 77%) or pyrimidyl (84% – 88%) functionalized compared to solely alkyl (46% – 56%) functionalized (**Scheme 3**). For the most accurate analysis and interpretation of the experiments, data obtained from using probe **2** was used to eliminate any undesired influences from FRET-quenching.



Scheme 2: Proposed mechanism of the "click-to-release" elimination reaction.

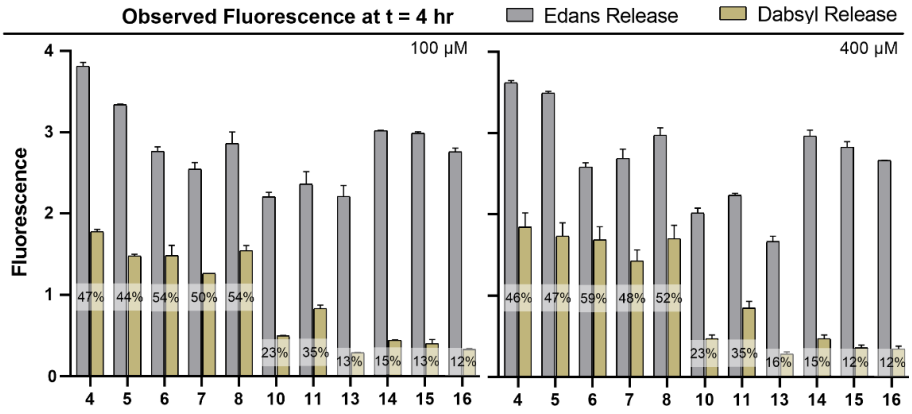
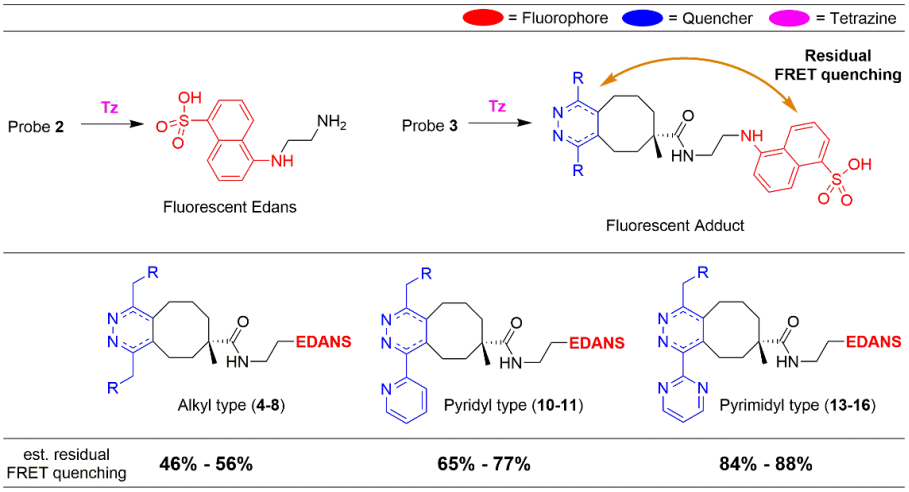


Figure 4: Differences in total amount of fluorescence after 4 hours when using probe **2** (grey) or **3** (yellow).



Scheme 3: FRET-quenching from remaining (non-)aromatic pyridazine structures present in close proximity to EDANS when using DABCYL-releasing fluorogenic probe **3**.

After the data was processed (**Figure 5**), for each tetrazine and probe concentration, it provided two reaction rate constants, "k_{fast}" and "k_{slow}", each accompanied with a fractional value describing to what extend they contribute to the total observed fluorescence. These fractional values together represent the elimination efficiency of the entire reaction after 240 minutes normalized to tetrazine **4** which was used as a reference. When looking at the data a whole variety of properties were obtained, where for a select group of tetrazines the tetrazine-specific faster elimination path (k_{fast}) was very dominant. This was observed for hydroxyethyl and aminoethyl tetrazines **6**, **11**, **14** and **16**, indicating a favorable "head-to-head" approach originating from electron-withdrawing substituents, as well as the beneficial intramolecular catalytic effect from the ammonium functionality, as shown in chapter 5, steering towards the eliminating tautomer. Interestingly, for pyridyl and pyrimidyl tetrazines, where the ammonium functionality is absent (tetrazine **15**), or Boc-protected (tetrazines **10** and **13**) the reaction rate constant from the fast elimination path (k_{fast}) is not only reduced by several folds, but the slower elimination path (k_{slow}) appeared to be the favored one as well. Asymmetric carboxylate functionalized tetrazine **8** showed a limited preference for the faster elimination path (k_{fast}) and symmetric carboxylate functionalized tetrazine **9** showed no clearly defined preference for either elimination path.

Tetrazine	[Tz] = 400 µM: Rate constant (K_{obs})	[Tz] = 400 µM: Fraction	[Tz] = 400 µM: Total*	[Tz] = 100 µM: Rate constant (K_{obs})	[Tz] = 100 µM: Fraction	[Tz] = 100 µM: Total*
4	K_{obs} = 20.2 *10^{-4}	**96.5%**	**96.5%**	K_{obs} = 18.9 *10^{-4}	**100%**	**100%**
5	K_{fast} = 43.8 *10^{-4}	74.3%	93.9%	K_{fast} = 17.3 *10^{-4}	83.8%	88.5%
	K_{slow} = 5.7 *10^{-4}	19.6%		**n.d.**	4.7%	
6	K_{fast} = 45.5 *10^{-4}	64.3%	69.8%	K_{fast} = 32.7 *10^{-4}	64.4%	75.5%
	K_{slow} = 1.6 *10^{-4}	5.5%		K_{slow} = 6.1 *10^{-4}	11.1%	
7	K_{fast} = 36.4 *10^{-4}	50.5%	74.6%	K_{fast} = 14.4 *10^{-4}	36.2%	67.7%
	K_{slow} = 3.0 *10^{-4}	24.1%		K_{slow} = 2.9 *10^{-4}	31.5%	
8	K_{fast} = 25.2 *10^{-4}	36.1%	81.5%	K_{fast} = 6.2 *10^{-4}	37.5%	81.0%
	K_{slow} = 4.9 *10^{-4}	45.4%		**n.d.**	33.5%	
10	K_{fast} = 24.4 *10^{-4}	25.9%	61.3%	K_{fast} = 21.9 *10^{-4}	31.3%	55,9%
	K_{slow} = 4.1 *10^{-4}	25.4%		K_{slow} = 4.2 *10^{-4}	24.6%	
11	K_{fast} = 113.7 *10^{-4}	51.4%	59.7%	K_{fast} = 100.2 *10^{-4}	58.1%	63.9%
	K_{slow} = 2.3 *10^{-4}	8.3%		K_{slow} = 2.5 *10^{-4}	5.8%	
13	K_{fast} = 24.5 *10^{-4}	15.6%	41.2%	K_{fast} = 21.6 *10^{-4}	21.1%	56.2%
	K_{slow} = 2.2 *10^{-4}	25.6%		K_{slow} = 2.3 *10^{-4}	35.1%	
14	K_{fast} = 111.0 *10^{-4}	71.7%	81.6%	K_{fast} = 113.3 *10^{-4}	66.0%	79.5%
	K_{slow} = 46.4 *10^{-4}	9.9%		K_{slow} = 14.9 *10^{-4}	13.5%	
15	K_{fast} = 59.0 *10^{-4}	34.1%	77.1%	K_{fast} = 57.7 *10^{-4}	28.7%	79.8%
	K_{slow} = 6.5 *10^{-4}	43.0%		K_{slow} = 8.9 *10^{-4}	51.1%	
16	K_{fast} = 76.4 *10^{-4}	54.1%	72.4%	K_{fast} = 82.2 *10^{-4}	50.4%	73.9%
	K_{slow} = 6.8 *10^{-4}	18.3%		K_{slow} = 9.9 *10^{-4}	23.5%	

*** = Relative to Tetrazine 4**

Figure 5: Two phase decay data obtained from ***GraphPad Prism*** when reacting probe **2** with tetrazines at 400 µM (left) and 100 µM (right) concentrations with a fast decay component (K_{fast}) and a slow decay component (K_{slow}).

Kinetic modeling

Because it remained unknown whether the observed reaction rate constants (k_{fast}, k_{slow}), originating from the slow or fast reaction pathway, were limited by the IEDDA ("k_{IEDDA}") or the elimination ("$k_{release}$") step, a kinetic model of the reaction was built in ***Coach 7***[10] to generate data that would represent a fluorescence signal if the theoretical model and its parameters were true.

To limit the complexity of the model, a reaction was modeled (**Figure 6**) where tetrazine ("Tz", 100 µM or 400 µM) and probe ("TCO", 10 µM) react with each other at reaction rates ("IEDDA_rate", k_{IEDDA} = variable) to form a single eliminating intermediate ("Adduct"), according to the following formula:

$$\mathbf{R_{IEDDA} = k_{IEDDA} * [Tz] * [TCO]}$$

This "Adduct" subsequently would eliminate with a specified elimination rate constant ("Elimination_rate", $k_{release}$ = 20 $*10^{-4}$ s^{-1}) to form released "Product". According to the following formula:

$$\mathbf{R_{release} = k_{release} * [Adduct]}$$

As a result from the kinetic modeling a broad list of data points could be obtained on the changing concentration of "Tz", "TCO", "Adduct" and "Product" (**Figure 7**) during the reaction. From these the "Product" data points would represent a simulated fluorescent signal. The simulated fluorescence signal, similar to an experimentally observed fluorescence signal, could be used to generate a decay trendline using ***GraphPad Prism*** to obtain reaction rate constants ("k_{obs}"). The resulting reaction rate constant "k_{obs}" that ***GraphPad Prism*** calculates when feeding ***Coach 7*** data sets would give an understanding to the relationship between the observed "k_{obs}" (calculated by ***GraphPad Prism***) and the actual rate constants "k_{IEDDA}" and "$k_{release}$" of such a reaction (**Figure 8**).

Based on the results obtained from the kinetic modeling, a ratio between the observed rate constants could be calculated using the following formula:

$$\mathbf{Ratio} = \frac{\mathbf{k_{obs,\,high\,[Tz]}}}{\mathbf{k_{obs,\,low\,[Tz]}}}$$

It appears that when the pseudo first order IEDDA rate constant (k_{IEDDA} * [Tz]) is much larger than the first order elimination rate constant ($k_{release}$), as shown for parameters A, the ratio will remain largely unchanged:

$$\frac{\mathbf{k_{obs,\,high\,[Tz]}}}{\mathbf{k_{obs,\,low\,[Tz]}}} \approx \frac{\mathbf{k_{release}}}{\mathbf{k_{release}}} \approx \mathbf{1}$$

In this situation the rate limiting step is the elimination step, which is independent of the tetrazine concentration. Here the observed rate (k_{obs}) can be used as an approximation of the elimination rate ($k_{release}$).

When the pseudo first order IEDDA rate constant (k_{IEDDA} * [Tz]) is much smaller than the first order elimination rate constant ($k_{release}$), as shown for parameters E, the ratio will be the same as the ratio between both tetrazine concentrations:

$$\frac{k_{obs,\ high\ [Tz]}}{k_{obs,\ low\ [Tz]}} \approx \frac{k_{IEDDA}*[Tz]_{high}}{k_{IEDDA}*[Tz]_{low}} \approx \frac{[Tz]_{high}}{[Tz]_{low}} \approx 4$$

In this situation the rate limiting step is the IEDDA step, which is dependent on the tetrazine concentration. Here, the observed rate (k_{obs}) can be used as an approximation of the pseudo first order IEDDA rate constant (k_{IEDDA} *[Tz]).

Finally, when the pseudo first order IEDDA rate constant (k_{IEDDA} * [Tz]) is similar to the first order elimination rate constant ($k_{release}$), as shown for parameters B, C and D, the ratio will be in between the two values. In this situation both the IEDDA step as well as the elimination step are contributing as a rate limiting step. Here, the observed rate (k_{obs}) can be used to approximate either the rates with a somewhat limited accuracy.

Analysis of experimental results

With the modeling data finished, the experimental data could be analyzed to obtain a ratio for each of the reactions k_{obs}, k_{fast}, or k_{slow}, whenever possible (**Figure 9**). From these ratio values the reaction would be considered limited by IEDDA rate when the ratio was > 3.06, limited by release rate when the ratio was < 1.66, or limited by both when the ratio was inbetween 1.66 and 3.06.

Then for the IEDDA rate limited reactions the rate constant ("k_{IEDDA}") was determined through the following formula using the lower (100 µM) concentration value, as it would provide a better approximation:

$$k_{obs,\ low\ [Tz]} / [Tz]_{low} \approx k_{IEDDA}\ (M^{-1}\ s^{-1})$$

For the release rate limited reactions the rate constant ("$k_{release}$") was determined through the following formula using the lower (400 µM) concentration value, as it would provide a better approximation:

$$k_{obs,\ high\ [Tz]} \approx k_{release}\ (s^{-1})$$

And finally for the in between reaction, both formulas were used to give a rough estimation (> 60%) of each of the rate constants.

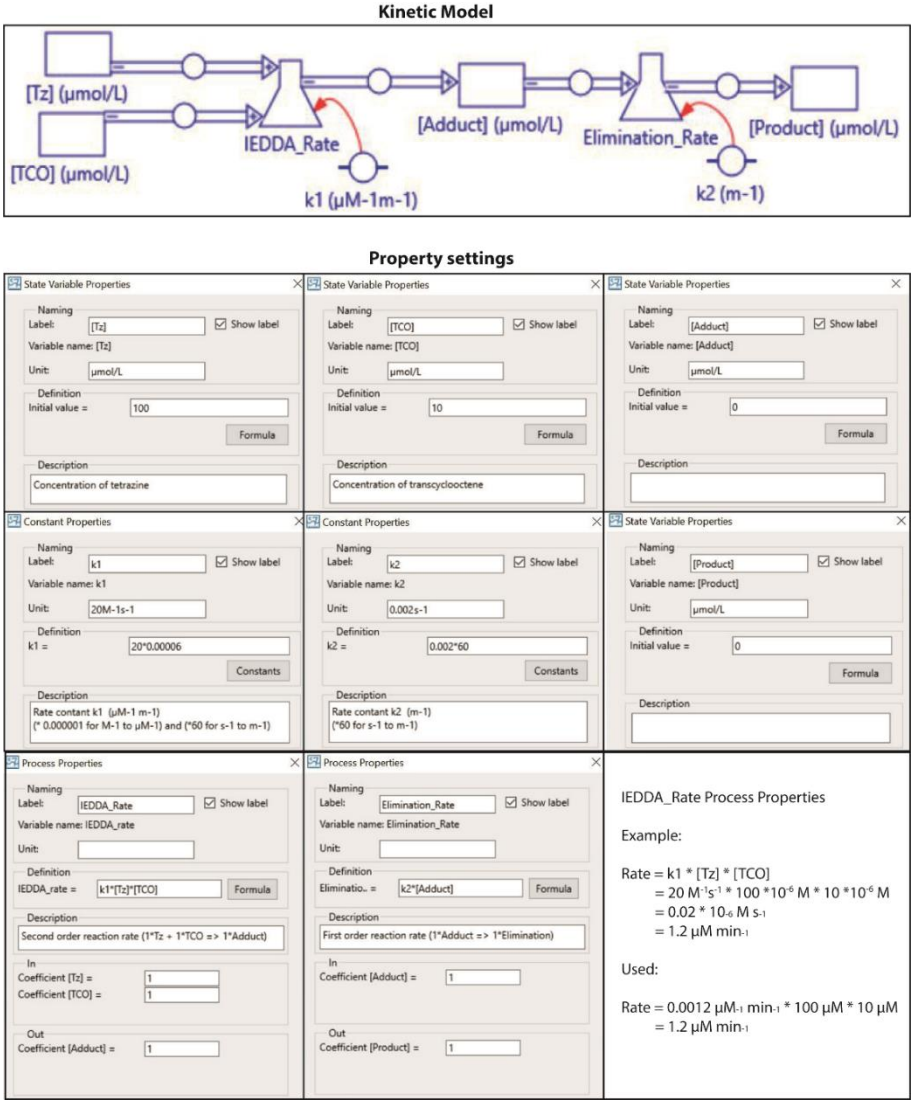


Figure 6: One phase decay click-to-release reaction model using ***Coach 7***.

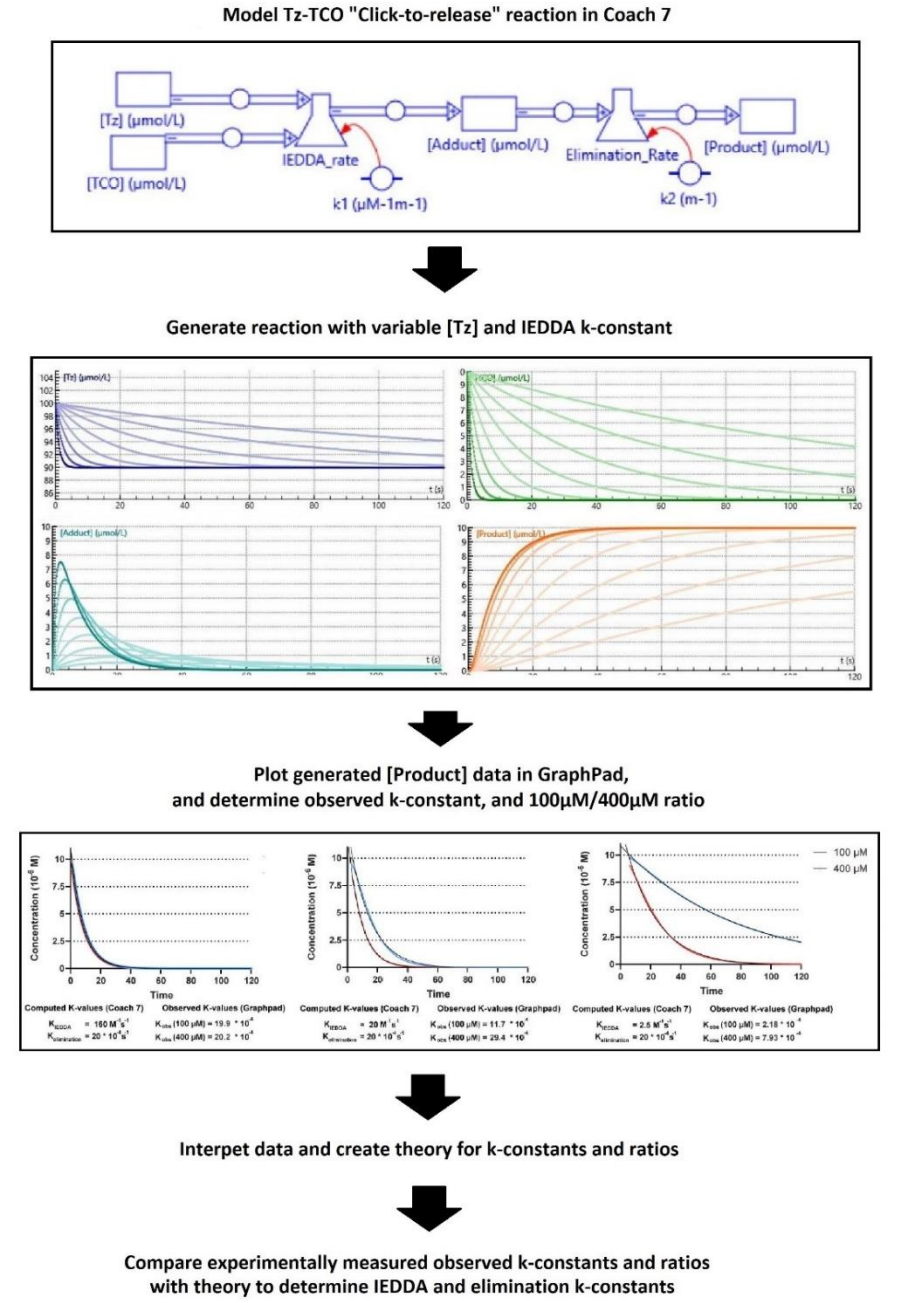
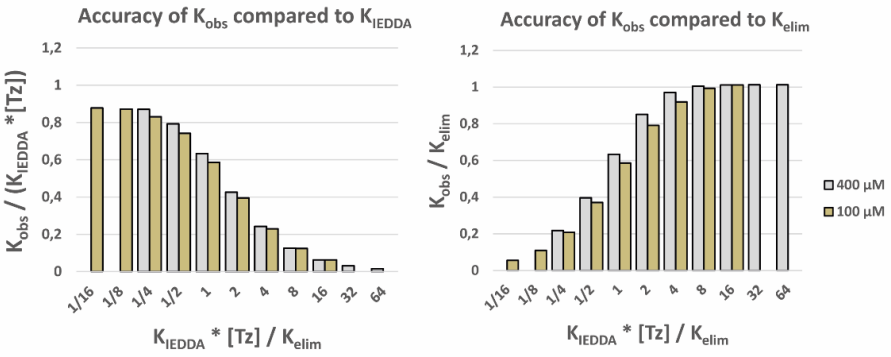


Figure 7: Data generation pathway into understanding the relationship between observed reaction rate "K_{obs}" calculated by *GraphPad Prism* and model reaction "K_{IEDDA}" and elimination "$K_{release}$" rates.

Data interpretation

	Input					Output	
	[TCO]	[Tz]	$K_{release}$	K_{IEDDA}	K_{IEDDA} * [Tz]	K_{obs}	Ratio
	µM		* 10^{-4} s^{-1}	$M^{-1}s^{-1}$	* 10^{-4} s^{-1}	* 10^{-4} s^{-1}	
A	10	100	20	160	160	19.9	1.02
		400			720	20.2	
B	10	100	20	20	20	11.7	1.66
		400			80	19.4	
C	10	100	20	10	10	7.4	2.29
		400			40	17.0	
D	10	100	20	5	5	4.2	3.06
		400			20	12.7	
E	10	100	20	1.25	1.25	1.1	3.96
		400			5.0	4.4	

$$\text{Ratio} = \frac{K_{obs}([Tz] = 400\ \mu M)}{K_{obs}([Tz] = 100\ \mu M)}$$



Kobs provides an approximation of KIEDDA when KIEDDA * [Tz] is smaller than Krelease

Kobs provides an approximation of Krelease when KIEDDA * [Tz] is larger than Krelease

Observation	Result	Interpetation
Ratio ≈ 1	k_{obs} is independent of [Tz]	$K_{obs} \approx K_{release}$
Ratio ≈ $[Tz]^{high}$ / $[Tz]^{low}$	k_{obs} is dependent of [Tz]	$K_{obs} \approx K_{IEDDA}$ * [Tz]

Figure 8: Top: Modeling parameters **A**, **B**, **C**, **D** and **E** used in ***Coach 7*** to generate simulated fluorescence signal (**input**) and the observed reaction rate constant K_{obs} obtained in ***GraphPad Prism*** by approximation of the 1st order reaction rate using the simulated fluorescence readout (**output**). **Middle:** A graphical view of how the K_{obs} generated by ***Graphpad Prism*** compares to K_{IEDDA} (left graph) or $K_{release}$ (right graph), and how accurate (closer to 1 is more accurate) this value is. **Bottom:** An explanation of what the observed reaction rate constant K_{obs} approximates to depending on the change fluorescent readout observed when adjusting the tetrazine concentration [Tz] by 4-fold within a single modeling parameter.

				Rate limiting rate constant		Lit. K_{IEDDA}
Tetrazine	Rate constant	Ratio	Rate limiting step	K_{IEDDA} (100 µM, M^{-1} S^{-}	$K_{release}$ (400 µM, s^{-1})	K_{IEDDA} (M^{-1} S^{-1})
4	K_{obs}	1.1	**release**	-	20.2 * 10^{-4}	48.7
5	K_{fast}	2.5	**both**	(est.) 17.3	(est.) 43.8 *10^{-4}	-
	K_{slow}	n.d.	n.d.	-	-	-
6	K_{fast}	1.39	**release**	-	45.5 * 10^{-4}	-
	K_{slow}	n.d.	n.d.	-	-	-
7	K_{fast}	2.5	**both**	(est.) 14.4	(est.) 36.4 *10^{-4}	17.5
	K_{slow}	1.0	**release**	-	3.0 * 10^{-4}	-
8	K_{fast}	4.1	**IEDDA**	6.2	-	6.7
	K_{slow}	n.d.	n.d.	-	-	-
10	K_{fast}	1.1	**release**	-	24.4 * 10^{-4}	-
	K_{slow}	1.0	**release**	-	4.1 * 10^{-4}	-
11	K_{fast}	1.1	**release**	-	113.7 *10^{-4}	-
	K_{slow}	1.5	**release**	-	2.3 * 10^{-4}	-
13	K_{fast}	1.1	**release**	-	24.5 * 10^{-4}	-
	K_{slow}	0.9	**release**	-	2.2 * 10^{-4}	-
14	K_{fast}	1.0	**release**	-	111.0 *10^{-4}	-
	K_{slow}	n.d.	n.d.	-	-	-
15	K_{fast}	1.0	**release**	-	59.0 * 10^{-4}	-
	K_{slow}	0.7	**release**	-	6.5 * 10^{-4}	-
16	K_{fast}	0.9	**release**	-	76.4 *10^{-4}	-
	K_{slow}	0.7	**release**	-	6.8 * 10^{-4}	-

Figure 9: Interpretation of the two phase decay data obtained from ***GraphPad Prism*** when reacting probe **2** with tetrazines at 400 µM and 100 µM concentrations with a fast decay component (K_{fast}) and a slow decay component (K_{slow}). Rate limiting steps comparison of the ratio between the two components, including respective assigned rate limiting rate constant. Known rate constants from literature were added for comparison.

Approximation of K_{IEDDA}

With all the rate constants determined, most of the tetrazines only one of the two rate constants was determined. To be able to find the other rate constant the difference between the (pseudo) first order rate constants k_{IEDDA} * [Tz] and $K_{release}$ must be adjusted towards where the desired reaction step is limiting the observed reaction rate, as shown in the middle graphs of **Figure 8**. The most feasible way to

achieve this is through adjusting the tetrazine concentration. By increasing the tetrazine concentration above the concentrations previously used it may be possible to approximate $K_{release}$ rate constants for tetrazines limited by the IEDDA reaction step, but unfortunately this was not possible in the setup used. On the other side it was possible to approximate the k_{IEDDA} rate constants for tetrazines **4**, **6**, **11** and **14** by lowering the tetrazine concentration to 5-10 µM (**Figure 10**). Unfortunately the concentration of probe **2** also had to be lowered to 100 nM, and as a result lowering the tetrazine concentrations even further resulted in a too slow reaction to yield accurate data.

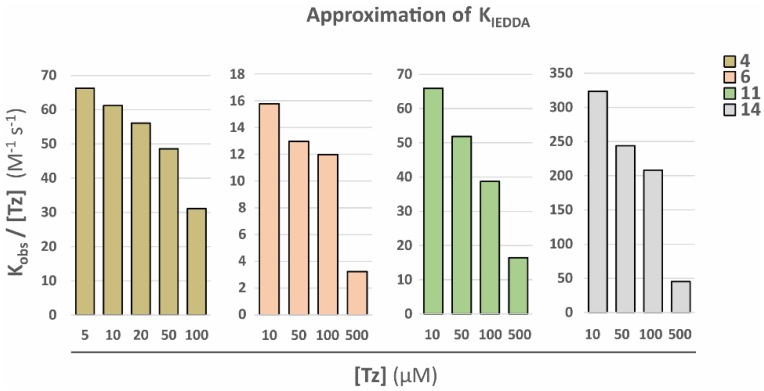


Figure 10: Approximation of K_{IEDDA}, by lowering the tetrazine concentration.

Conclusion

To conclude the chapter, probes **2** and **3** were synthesized successfully and probe **3** appeared to perform poorly due to FRET-quenching by the diazine moiety withing the product. Probe **2** performed well and the fluorescence data obtained allowed the identification of rate constants for multiple eliminating pathways within a single reaction. These rate constants could then, together with obtained knowledge from theoretically modeled data, be used to approximate the IEDDA reaction rate constant (K_{IEDDA}) or multiple eliminating rate constants (k_{fast}, k_{slow}) within a single reaction. Finally it was shown that for reactions where the eliminating reaction step was the rate limiting step, the IEDDA reaction rate constant could be approximated by lowering both the probe and tetrazine concentrations.

Computational Chemistry

Geometry optimization: structures

A conformer distribution search option included in the Spartan 10 program[5] with the use of MM with MMFF94 as force field, was used as starting point for the geometry optimization. All generated structures were further optimized with Gaussian 09 using the M06-2X hybrid functional[6] and 6-31+G(d) as basis set. Optimization was done in gas-phase and subsequently corrections for solvent effects were done by the use of a polarizable continuum model using water as solvent parameter. The denoted free Gibbs energy was calculated using Equation (1), in which ΔE_{gas} is the gas-phase energy (electronic energy), $\Delta G^{T}_{gas,QH}$ (T = 293.15 K and pressure = 1 atm.) is the sum of corrections from the electronic energy to the free Gibbs energy in the quasi-harmonic oscillator approximation, including zero-point-vibrational energy, and ΔG_{solv} is their corresponding free solvation Gibbs energy. The $\Delta G^{T}_{gas,QH}$ were computed using the quasi-harmonic approximation in the gas phase according to the work of Truhlar - the quasi-harmonic approximation is the same as the harmonic oscillator approximation except that vibrational frequencies lower than 100 cm^{-1} were raised to 100 cm^{-1} as a way to correct for the breakdown of the harmonic oscillator model for the free energies of low-frequency vibrational modes.[7] Visualization of relevant structures was done with CYLview. All denoted distances are expressed in ångström (Å).

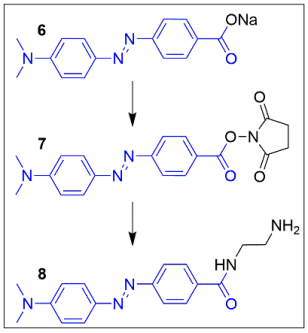
$$\Delta G^{T}_{aq} = \Delta E_{gas} + \Delta G^{T}_{gas,QH} + \Delta G_{solv} \quad (1)$$

$$= \Delta G^{T}_{gas} + \Delta G_{solv}$$

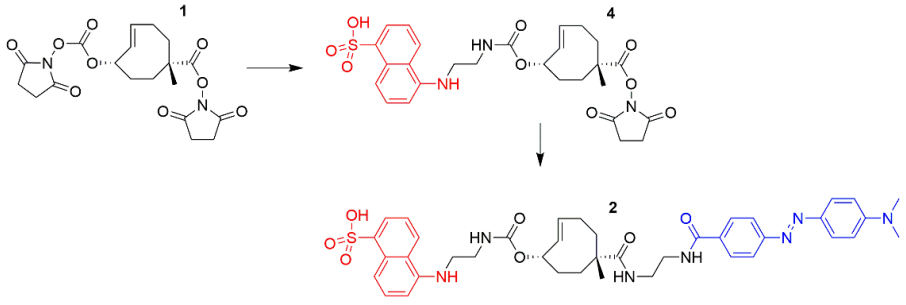
Geometry optimization: IEDDA transition state structures

Initial guesses for the transition states were based on the work of Fox and co-workers[8] and Houk and co-workers.[9] All generated structures were further optimized with Gaussian using the M06-2X hybrid functional and 6-31+G(d) as basis set. Optimization was done in combination with a polarizable continuum model using water as solvent parameter.

Compound Synthesis

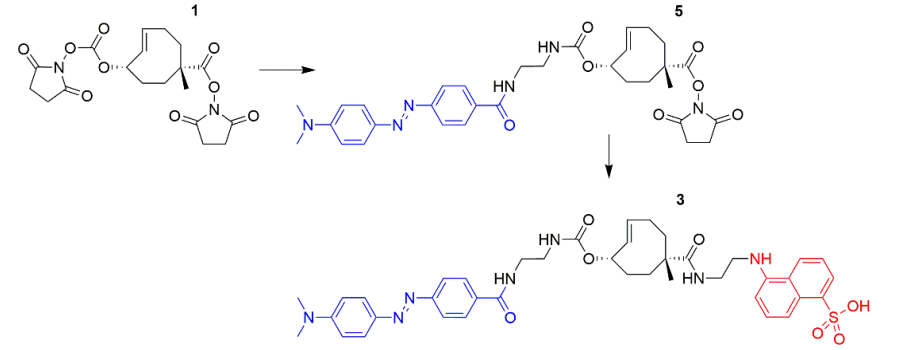


Compound 8: 0.50 mmol (145 mg) of commercially available DABCYL sodium carboxylate **6** (0.145 g, 0.50 mmol) was dissolved in 8.8 mL dry DMF. Then 0.66 mmol (76.5 mg) of N-hydroxy succinimide and 1.00 mmol (193 mg) of EDC*HCl were added and the reaction mixture was stirred overnight at room temperature. The resulting mixture was concentrated using rotary evaporation and purified with silica column chromatography over silica gel using a 0-2% MeOH in DCM eluent resulting in 169 mg (0.46 mmol, 96%) of intermediate **7**. Intermediate **7** was then dissolved in 11 mL of dry DMF, 60 mmol (4 mL) of ethylenediamine and 0.57 mmol (100 µL) of TEA were added and the reaction mixture was stirred overnight at room temperature. The resulting mixture was concentrated using rotary evaporation and purified with silica column chromatography over silica gel using a 10-20% MeOH in DCM eluent resulting in 134 mg (0.43 mmol, 93%) of compound **8**. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.30 – 8.23 (m, 2H), 8.10 (s, 2H), 8.03 (d, 2H), 6.89 (d, 2H), 3.22 (s, 6H), 3.00 – 2.90 (m, 4H). HRMS (m/z): $[C_{17}H_{21}N_5O + H]^+$ calculated 312.1819, found 312.1817.



Compound 2: 57 µmol (15 mg) of commercially available 1,5-EDANS and 47 µmol (20 mg) of compound **1** were added to a 2 mL Eppendorf tube. Then, 1 mL of dry DMF and 5 equivalents of TEA were added and the Eppendorf tube was shaken overnight in the dark (wrapped in aluminum foil). The resulting mixture was concentrated using rotary evaporation and purified with silica column chromatography using a MeOH in DCM eluent resulting in 21 mg (37 µmol, 79%) of intermediate **4.** Then, 10 µmol (4.9 mg) of intermediate **4** and 11 µmol (3.4 mg) of compound **8** were added to a 2 mL Eppendorf tube. Then, 1 mL of dry DMF and 5 equivalents of TEA were added and the Eppendorf tube was shaken overnight in the dark (wrapped in aluminum foil). The resulting mixture was concentrated using rotary evaporation and purified with silica column chromatography using a MeOH in DCM eluent resulting in 4.8 mg (6.2 µmol, 62%) of compound **2** as a red solid. ***Intermediate 4***: ^{1}H NMR (500 MHz, MeOD) δ: 8.14 (m), 7.45 – 7.32 (m), 6.74 – 6.61 (m), 5.92 (m), 5.70 (m), 3.82 (m), 3.64 – 3.56 (m), 3.53 (m), 3.49 (m), 3.38 (m), 3.09 (m), 2.82 – 2.76 (m), 2.52 (m), 2.30 – 2.22 (m), 1.28 (m). ^{13}C NMR (126 MHz,

MeOD) δ: 175.89, 175.36, 171.89, 145.48, 145.43, 141.75, 133.25, 132.10, 131.39, 130.95, 128.58, 128.52, 126.68, 125.62, 125.58, 125.50, 125.41, 123.58, 116.26, 115.40, 105.06, 73.48, 56.81, 55.77, 45.63, 45.29, 44.73, 43.76, 40.76, 39.71, 39.65, 36.63, 31.65, 31.55, 31.28, 28.80, 26.48, 26.34, 26.21, 18.47, 13.16. ***Compound 2***: ^{1}H NMR (500 MHz, MeOD) δ: 8.00 – 7.80 (m), 6.74 (m), 5.88 (m), 5.58 (m), 5.14 (m), 5.09 (m), 3.66 (s), 3.61 (m), 3.47 (m), 3.09 (s), 2.95 (s), 2.87 (s), 2.80 (m), 2.57 (m), 2.24 (m), 2.05 (m), 1.89 (m), 1.24 (m), 1.12 (m). HRMS (m/z): $[C_{40}H_{47}N_7O_7S + H]^+$ calculated 770.3330, found 770.3320.



Compound 3: 29 µmol (9.0 mg) of compound **8** and 30 µmol (13 mg) of compound **1** were added to a 2 mL Eppendorf tube. Then, 1 mL of dry DMF and 5 equivalents of TEA were added and the Eppendorf tube was shaken overnight in the dark (wrapped in aluminum foil). The resulting mixture was concentrated using rotary evaporation and purified with silica column chromatography using a MeOH in DCM eluent resulting in 21 mg (28 µmol, 96%) of intermediate **5**. Then, 5.2 µmol (3.2 mg) of intermediate **5** and 5.5 µmol (1.6 mg) of commercially available 1,5-EDANS were added to a 2 mL Eppendorf tube. Then, 1 mL of dry DMF and 5 equivalents of TEA were added and the Eppendorf tube was shaken overnight in the dark (wrapped in aluminum foil). The resulting mixture was concentrated using rotary evaporation and purified with silica column chromatography using a MeOH in DCM eluent resulting in 3.6 mg (4.6 µmol, 88%) of compound **3**. ***Intermediate 5***: ^{1}H NMR (500 MHz, $CDCL_3$) δ: 8.01 (s), 7.95 – 7.86 (m), 7.85 (d, *J* = 8.6 Hz), 7.73 (dd, *J* = 8.9, 0.7 Hz), 7.48 (dd, *J* = 1.9, 0.8 Hz), 7.22 (dd, *J* = 8.9, 1.8 Hz), 6.76 (d, *J* = 9.1 Hz), 5.92 – 5.82 (m), 5.65 – 5.55 (m), 5.18 (s), 3.75 – 3.60 (m), 3.50 (m), 3.25 – 3.00 (m), 2.96 (s), 2.88 (s), 2.81 (m), 2.27 (m), 2.04 (m), 1.91 (m), 1.40 – 1.34 (m), 1.33 – 1.22 (m). ^{13}C NMR (126 MHz, $CDCl_3$) δ: 174.4, 167.8, 157.1, 155.2, 152.9, 143.7, 141.6, 134.1, 132.1, 131.7, 131.3, 128.6, 128.1, 125.7, 125.5, 122.3, 119.7, 111.6, 110.1, 72.5, 67.2, 54.2, 46.3, 44.5, 44.4, 42.5, 41.6, 40.8, 40.4, 36.6, 35.9, 31.6, 30.6, 30.5, 29.8, 25.7, 18.7, 18.0, 17.6, 12.2, 8.8. ***Compound 3***: ^{1}H NMR (500 MHz, MeOD) δ: 8.13 (m), 7.92 (m), 7.85 (m), 7.37 (m), 6.79 (m), 6.66 (m), 5.89 (m), 5.65 (m), 3.60 – 3.35 (m), 3.20 – 3.00 (m), 2.19 (m), 2.07 (m), 1.90 (m), 1.71 (m), 1.61 (m), 1.47 (m), 1.29 (m), 1.11 (m), 0.89 (m). HRMS (m/z): $[C_{40}H_{47}N_7O_7S + H]^+$ calculated 770.3330, found 770.3321.

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Chapter 7: Summary and future prospects

This thesis describes the synthesis, analysis of various tetrazine bearing compounds and use in bioorthogonal chemistry. In **chapter 1** a literature survey of tetrazine chemistry developments throughout history is provided. Additionally an introduction to the emergence of click chemistry, bioorthogonal reactions, and IEDDA chemistry is provided. These developments are the foundations on which the work described in this thesis is built upon.

Chapter 2 reports the synthesis of tetrazine-modified alanine building blocks as functionalized amino acids to substitute natural amino acids in activity-based probes (ABP's) for bioorthogonal activity-based protein profiling (ABPP) of cysteine, serine and threonine peptidases.[1][2] One building block, Cbz-Tzm-OH (compound **2**, **chapter 2**), proved to be readily available following the developed route of synthesis, and could be incorporated in small oligopeptide chloromethylketones (CMK)[3, 4] and benzyloxymethylketones (BOMK)[5, 6] providing structures closely related to known peptidase inhibitors. CMK inhibitor **1** (**Figure 1**) could be readily prepared and was successfully used bioorthogonal ABPP. Unfortunately, the CMK warhead was too reactive and resulted the modification of numerous unspecified proteins when applied to cell lysates. BOMK inhibitor **2** (**Figure 1**) was also readily prepared, however, provided poor protein modification.

As a **future prospect**, it would be interesting to prepare ABPs containing a fluoromethylketone (FMK) warhead[7], such as inhibitor **3** (**Figure 1**). It should be possible to synthesize this inhibitor using intermediates from this thesis as described in literature[8], and may result in an ABP featuring a more reactive warhead compared to compound **2** and a less reactive warhead compared to compound **1**[1, 3].

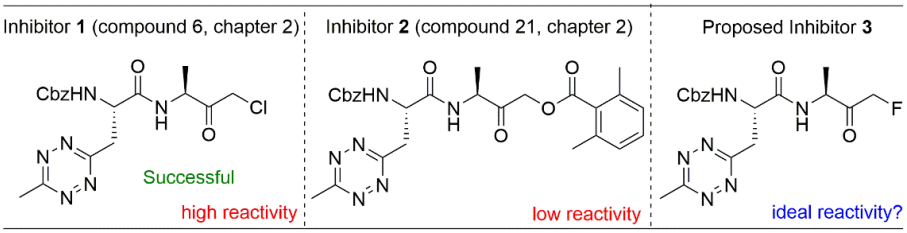
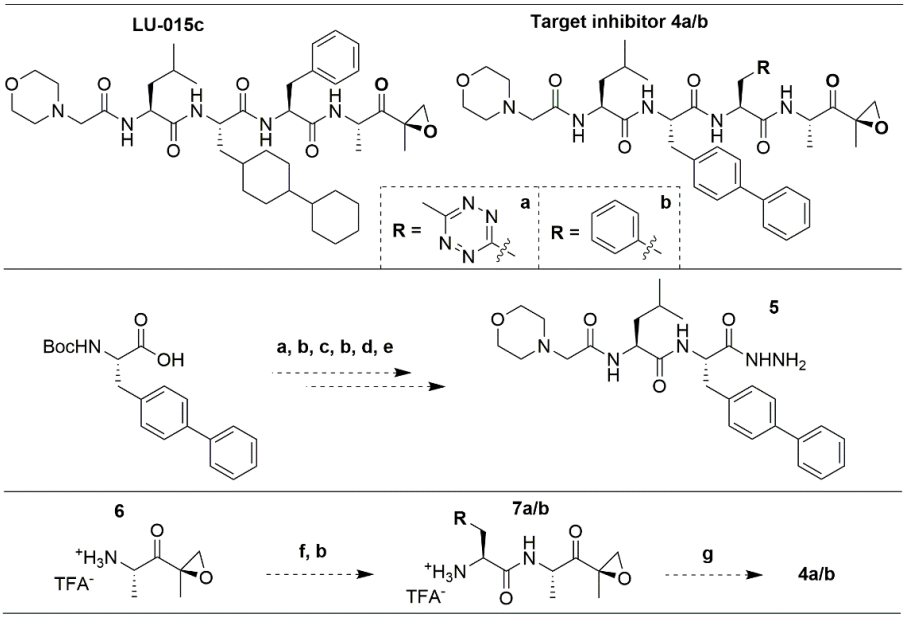


Figure 1: Structures of synthesized CMK inhibitor **1**, synthesized BOMK inhibitor **2**, and proposed FMK inhibitor **3**.

Chapter 2 also presents the synthesis of Boc-Tzm-OH (compound **12**, **chapter 2**) as an building block towards ABPs. However, after attachment of a warhead on its C-terminus, any effort to deprotect the Boc-group was unsuccessful.

As a **future prospect**, Boc-Tzm-OH could be used in the design of alternative ABPs. The non-canonical amino acid may be incorporated through replacement of the phenyl amino acid in the human proteasome and immunoproteasome B5c and B5i selective inhibitor **LU-015c**[9a] (**Figure 2**). Initial target inhibitors can be synthesized using commercially available Boc-Bip-OH instead of Boc-BiCha-OH, because this amino acid change has been shown to have only little influence on similarly used constructs[9b]. Compounds **5** and **6** may be synthesized according to literature procedures [9a, 9c]. Condensation of compound **6** with Boc-Tzm-OH or Boc-Phe-OH using HCTU, following Boc-deprotection should obtain compounds **5a/b**, which should be condensed with compound **5** to obtain target peptide epoxyketones **4a/b**.



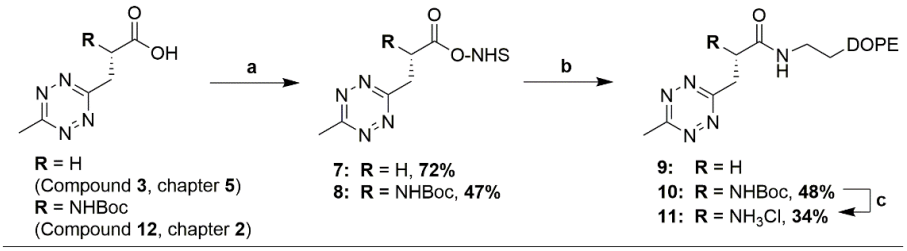
a) MeI, $KHCO_3$, DMF, **b**) TFA, DCM, **c**) Boc-Ile-OH, HCTU, DiPEA, DCM, **d**) 4-morpholineacetic acid, HCTU, DiPEA, DCM, **e**) hydrazine, MeOH, **f**) Boc-Phe-OH or Boc-Tzm-OH, HCTU, DiPEA, DCM, **g**) compound **5**, tBuONO, 4M HCl in dioxane, DMF/DCM.

Figure 2: Proposed 9-step synthesis of peptide epoxyketones **4a** (control) and **4b** (tetrazine).

Chapter 3 describes the synthesis of a library of functionalized tetrazines, as well as the synthesis and optimization of a variety of reactive alkenes. The work includes kinetic studies to determine the reactivity of the library of tetrazines towards the synthesized variety of alkenes. Additionally, these tetrazines were attached to Bodipy-FL to obtain fluorophore-tetrazine tags for bioorthogonal live cell fluorescence microscopy of alkene-functionalized molcecules present in the cell. Functionalized DOPE-lipids[10], mannosamines[11] and the naturally occurring strained

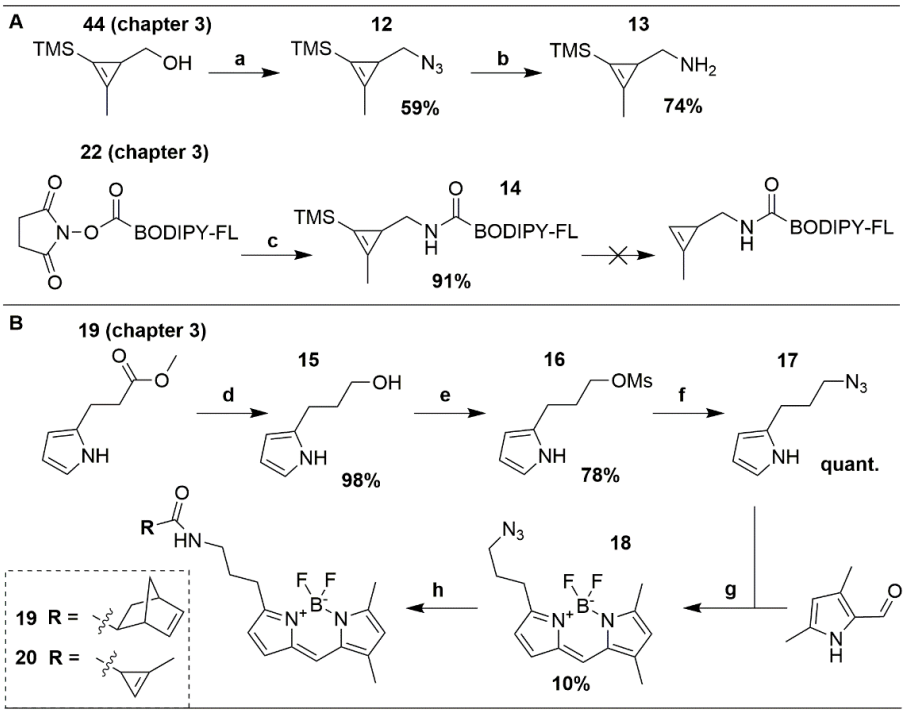
alkene-bearing sterculic acid[12] were incubated in cells and subsequently incubated with the tetrazine fluorophores. This resulted in weak fluorescence for the DOPE-lipids, as well as the mannosamines. Surprisingly, cells incubated with sterculic acid gave a strong fluorescence signal for a specific set of tetrazines. Tetrazines functionalized with the amino-methyl-phenyl linker gave a very strong fluorescence signal, while no fluorescence was observed for tetrazines functionalized with an aminoethyl linker.

As a **future prospect**, for the fluorescent imaging of alkene-bearing DOPE-lipids, tetrazine-functionalized modified DOPE-lipids **8**, **9** and **10** can be synthesized (**Figure 3**) starting from either Boc-Tzm-OH or 4-carboxyethyl-methyltetrazine (compound **3**, **chapter 5**). These functionalized lipids may be used for fluorescent imaging using commercially available or synthesized alkene-modified fluorophores **18** and **19** (**Figure 4**).



a) N-hydroxy succinimide, DCC, THF, **b**) DOPE-C_2H_4-NH_2, TEA, $CHCl_3$, **c**) 2M HCl dioxane/DCM.

Figure 3: Synthesis of tetrazine functionalized DOPE-lipids **8**, **9**, and **10**.



a) DPPA, DBU, THF, **b**) PPh_3, THF/H_2O, **c**) **12**, TEA, DMF, **d**) $LiAlH_4$, Et_2O, **e**) MsCl, TEA, DCM, **f**) NaN_3, DMF, **g**) $POCl_3$, DCM, then BF_3*OEt_2, TEA, **h**) PPh_3, THF/H_2O, then compound **35 (chapter 3)** or compound **42 (chapter 3)**, DMF.

Figure 4: (**A**) Attempted synthesis of alkene-functionalized Bodipy-FL. (**B**) Successful synthesis of alkene-functionalized Bodipy-FL **18** and **19**.

Chapter 4 describes the synthesis of highly water-soluble tetrazine fluorophores. These fluorophores were able to access the dense hydrophilic glycan coating (glycocalyx) around the cell surface and by doing so label the metabolically incorporated alkene-functionalized mannosamine (compound **62**, **Chapter 3**). By using sterculic acid and Bodipy-FL tetrazine (compound **25**, **Chapter 3**) as a second bioorthogonal pair, live cells could be incubated and labeled simultaneously to achieve multicomponent labeling ("dual-labeling") of alkene-bearing biomolecules. This was achieved by using only bioorthogonal IEDDA chemistry without any significant cross reactivity.

Building on the success of these highly water-soluble tetrazine fluorophores, as a **future prospect**, tetrazine-alkyne handles **21-26** (**Figure 5**) were synthesized. These molecules may be used to change the functionality of azide-functionalized biomolecules to alkene-functionalized biomolecules, and used in biological experiments together with the tetrazine fluorophores.

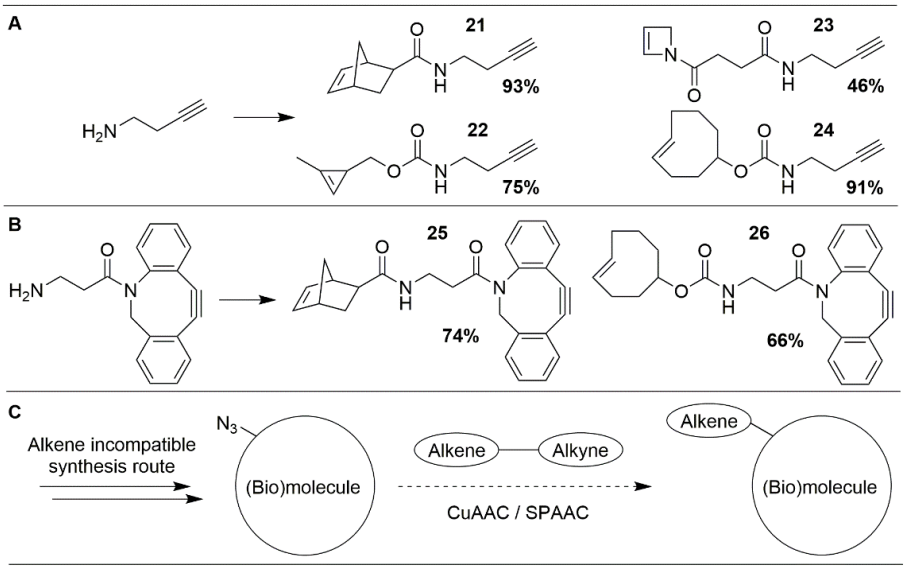
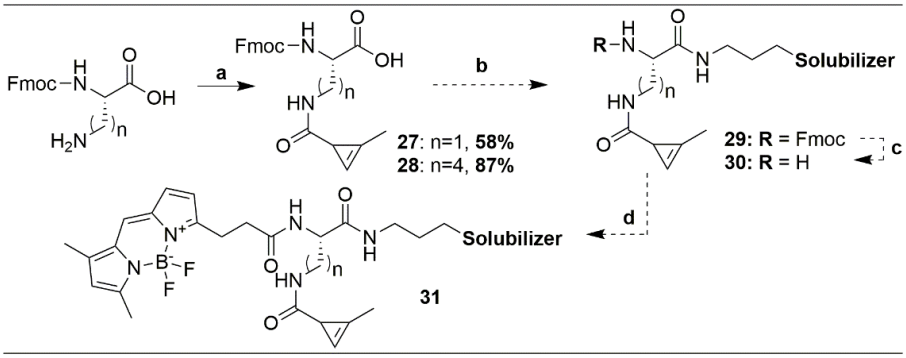


Figure 5: (**A**) Successful synthesis of propargyl-based alkene-alkynes. (**B**) Successful synthesis of DIBAC-based alkene-alkynes. (**C**) Proposed CuAAC or SPAAC mediated conversion of azide-functionalized biomolecules into alkene-functionalized biomolecules.

As another **future prospect**, it may be possible to apply the synthesis route of these fluorophores for the preparation of highly water-soluble alkene-functionalized fluorophores such as Bodipy-FL **31** (**Figure 6**).



a) Compound **42 (chapter 3)**, TEA, DMF, **b**) compound **2 (chapter 4)**, **c**) 20% Piperidine in DMF, **d**) compound **22 (chapter 3)**, TEA, DMF.

Figure 6: Synthesis of acyl-cyclopropene functionalized amino acids **27** and **28**, and proposed synthesis towards highly soluble Bodipy-FL-alkenes **31**.

As yet another **future prospect**, the highly water-soluble fluorophores may be used for the labeling of molecules less-abundant at the cells surface, such as alkene- and tetrazine-functionalized peptide epitopes, which were successfully synthesized (**Figure 7**). Through standard solid phase peptide synthesis Fmoc-SIINFEKL-OH **32** was prepared. **32** was then used to synthesize **33-36**, which may be used for MHC-I mediated presentation at the cell surface of APCs[13, 14]. Additionally using tetrazine-epitopes in combination with highly water-soluble alkene-functionalized fluorophores may also be a viable approach.

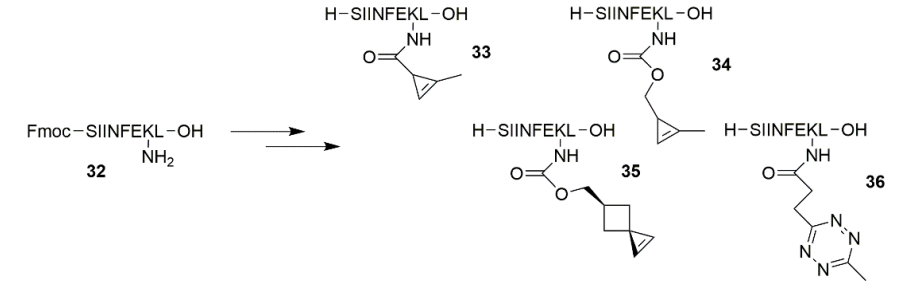
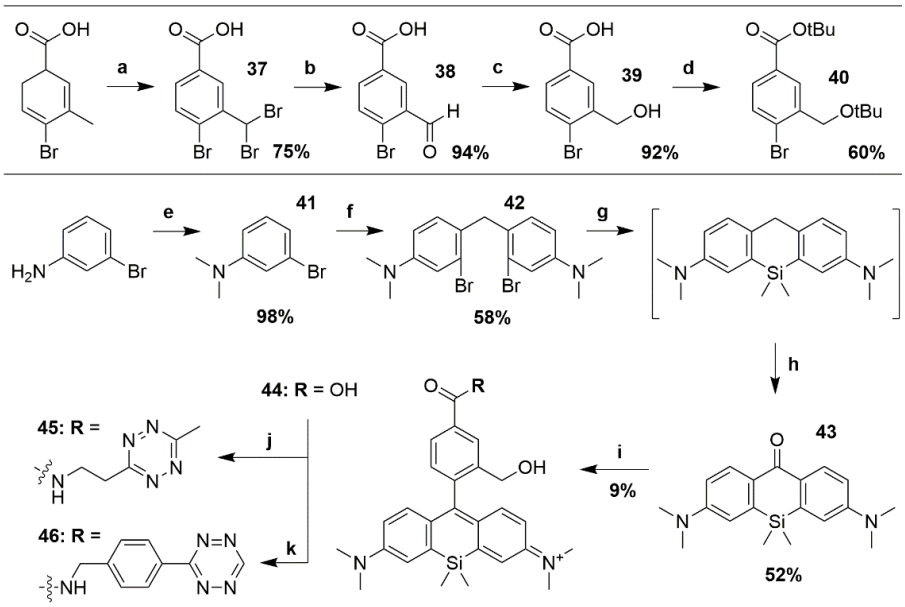


Figure 7: Successful synthesis of alkene-functionalized and tetrazine-functionalized "SIINFEKL" epitopes at the lysine position.

As a final **future prospect** for this chapter, to increase the resolution of fluorescence imaging, super-resolution may be applied by modifying the spontaneously blinking fluorophore "HMSiR" [15] at its carboxyl position, which was successfully synthesized (**Figure 8**). The molecule may then be used to label and quantify cell-surface labeled molecules[16, 17].



a) NBS, AIBN, DCE, **b**) Na_2CO_3(aq.), **c**) $NaBH_4$, THF, **d**) $MgSO_4$, H_2SO_4, tBuOH, DCM, **e**) formamide, H_2SO_4, THF then $NaBH_4$, **f**) formamide, AcOH **g**) Sec-BuLi, $SiMe_2Cl_2$, THF, **h**) $KMnO_4$, Acetone, **i**) **40**, THF then BuLi, then **42**, workup then TFA, **j**) EDC*HCl, N-hydroxy succinimide, DMF, then compound 10 chapter 3 **j**) EDC*HCl, N-hydroxy succinimide, DMF, then compound **10 (chapter 3)**, **k**) EDC*HCl, N-hydroxy succinimide, DMF, then compound **1 (chapter 3)**.

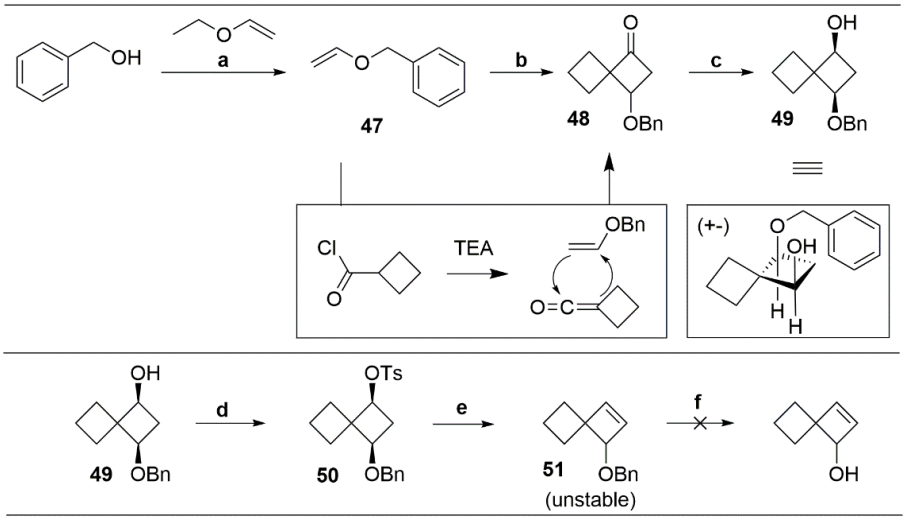
Figure 8: Successful synthesis of tetrazine-functionalized naturally blinking "HMSiR" fluorophores.

In **Chapter 5** the library of tetrazines from chapter 3 was analyzed on their capability (with respect to IEDDA rate, elimination rate and elimination efficiency) to perform a IEDDA-pyridazine elimination tandem reaction[18]. The library, consisting tetrazines functionalized with free amines or N-Boc protected amines with various spacers (methyl, ethyl, or methyl-phenyl) was analyzed on their reactivity and elimination speed with AMC-coumarin, a previously employed method from the literature[19]. The results were compared a set of literature tetrazines[19] including carboxy-functionalized tetrazines[20]. The data showed that amino-ethyl functionalized tetrazines, in particular tetrazines **6** (chapter 5) and **7** (chapter 5), showed unprecedented elimination rates, combined with respectable elimination efficiency, which was only achievable with other tetrazines by lowering the pH to non-physiological levels[20]. Unlike these other tetrazines, due to their amino-ethyl functionality, tetrazines **6** (chapter 5) and **7** (chapter 5) were not negatively affected by pH changes, or the lack of an acidic environment. This makes them highly likely an excellent choice when a high elimination rate is required.

In **Chapter 6** a tool was designed and synthesized from bifunctional trans-cyclooctane and the EDANS/DABCYL quencher pair to be able to correctly determine the reaction and elimination rates of tetrazines when releasing alkylic amines. This tool replaces the previously used AMC-coumarin method as it contained an aniline instead of a primary amine. With the help of computational modelling of the multi-step reaction in *Coach 7* and analysis of the results using *Graphpad PRiSM*, the tool was used to determine specific properties of individual tetrazines on their reaction rate and elimination rate. Additionally multiple simultaneous processes were identified and quantified and with that the rate-limiting steps could be determined.

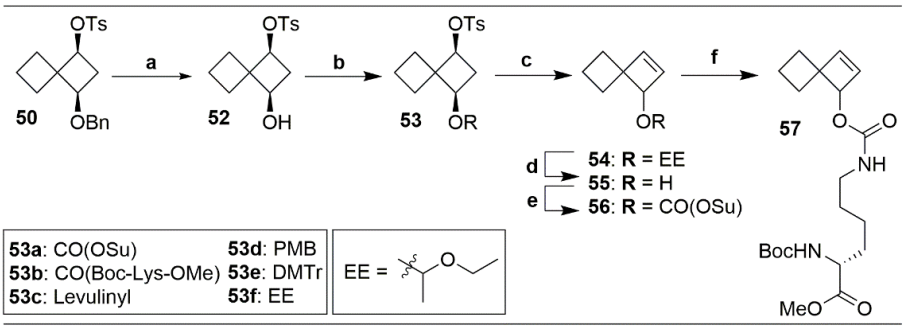
A **future prospect** to the work described here, would be the design, synthesis, and characterization of new strained alkenes that may have desirable properties for future research. One attempt was made here in the synthesis of a strained alkene termed "spiroheptene". First (**Figure 9**), benzyl vinyl ether **47** was prepared via mercury(II) trifluoroacetate catalysed vinylation of benzyl alcohol.[21] Spiroheptane **48** was synthesized via a [2+2]-cycloaddition of the *in situ* generated ketene[22] and vinyl ether **47,** which was reduced to obtain spiroheptane **48** as an enantiomeric mixture of cis-positioned (hydroxy and benzyloxy) functional groups. To form the spiroheptene structure through elimination of the hydroxyl moiety, this moiety was mesylated, tosylated or triflated, to be followed by their elimination though the use of KOtBu. Neither triflate nor mesylate were obtained in useful quantities, while tosylated spiroheptane **50** was obtained in good yield (79%) and was successfully eliminated to form spiroheptene **51**.[23] Storage of spiroheptene **51** at -20°C for several months led to the degradation of the compound, indicating a limited use for these types of molecules. Removal of the benzyl group to obtain 3-hydroxy-spiroheptene by chromium chloride and lithium iodide appeared to be unsuccessful.[24] The approach was discontinued as hydrogenation was not an option in the presence of the already formed spiroheptene functionality. As an alternative approach (**Figure 10**) spiroheptane **50** was hydrogenized to remove the benzyl group, resulting in spiroheptane **52**. The free hydroxyl was then functionalized with a variety of groups, which were successful for some (**53a**, **53b**, **53c**, **53f**) and failed for the others (**53d**, **53e**). Using **53f**, previously shown to be useful in the synthesis of spirohexene, the tosyl group was eliminated to form spiroheptene **54**. Further attempts on the elimination reaction on **53a** and **53b** appeared to be unsuccessful and returned the initial material, increasing the amount of KOtBu equivalents resulted in the degradation of the material. Compound **53c** degraded using the initial elimination procedure. Spiroheptene **54** was deprotected under acidic conditions to obtain spiroheptene **55**, which was directly used to prepare spiroheptene **56**

containing a succinimide ester. Finally spiroheptene **56** was successfully reacted with Boc-Lys-OMe resulting in spiroheptene **57**.



a) $Hg(TFA)_2$, 0°C to r.t., 2h, 75%; **b)** cyclobutanecarbonyl chloride, TEA, ACN, 90°C, 3h, 66%; **c)** $NaBH_4$, MeOH, 0°C to r.t., 3h, 69%. **d)** TsCl, pyridine, r.t., 3h, 79%; **e)** KOtBu, dry DMSO, r.t., 3h, 24% **f)** $CrCl_2$, LiI, H_2O, EtOAc, 65°C, o.n., 0%.

Figure 9: Synthesis of spiroheptene intermediates **50** and **51**.



a) Pd/C, H_2, MeOH, r.t., 3h, 88%; **b)** **53a**: $CO(OSu)_2$, DIPEA, dry ACN, r.t., o.n., 62%; **53b:** Compound **53a**, Boc-Lys-OMe acetate salt, TEA, dry DCM, rt, 4 h, 48%; for **53c**: levulinic acid, DCC, DMAP, DCM, 0° to rt, 3h, 76%; **53d**: NaH, dry DMF, 0 °C, 15 min, then PMB-Cl, 0°C, 15 min, 0%; **53e**: DMTr-Cl, pyridine, rt, o.n., 0%; **53f**: ethyl vinyl ether, PPTS, DCM, 0°C, 1h, 86%; **c)** KOtBu, dry DMSO, rt, 3h, 88%. **d)** 2M HCl (aq.), ACN, 0°, 1h; **e)** $CO(OSu)_2$, DIPEA, dry ACN, rt, overnight, 49%, **f)** Boc-Lys-OMe acetate salt, TEA, dry DCM, rt, 45 min, 32%.

Figure 10: Synthesis of target spiroheptene **56** as activated ester and spiroheptene modified lysine **57**.

Compound Synthesis

Compound 7: 0.125 mmol (21 mg) of compound **3 (chapter 5)** was dissolved in 1.0 mL of THF, 0.150 mmol (31 mg) of DCC and 0.156 mmol (18 mg) of N-hydroxy succinimide were added and the reaction mixture was stirred for 3 hours at room temperature. Reaction completion was checked by TLC (Rf = 0.6, 50% EtOAc in DCM). The reaction mixture was filtered over a pad of celite (in a glass pipette) and concentrated using rotary evaporation. Purification was performed twice by silica column chromatography using an 2.5%-20% EtOAc in DCM eluent followed by silica column chromatography using an EtOAc in pentane eluent resulting in 24 mg (0.091 mmol, 72.4%) of compound **7** as a pink solid**. ^{1}H NMR (400 MHz, $CDCl_3$) δ:** 3.74 (t, *J* = 7.2 Hz, 2H), 3.38 (t, *J* = 7.2 Hz, 2H), 3.05 (s, 3H), 2.81 (s, 4H). **^{13}C NMR (101 MHz, $CDCl_3$) δ**: 168.96, 168.07, 167.57, 167.38, 29.27, 28.06, 25.66, 21.26.

Compound 8: 0.198 mmol (56 mg) of compound **12 (chapter 2)** was dissolved in 2.0 mL of THF, 0.242 mmol (50 mg) of DCC and 0.243 mmol (28 mg) of N-hydroxy succinimide were added and the reaction mixture was stirred for 2 hours at room temperature. Reaction completion was checked by TLC (Rf = 0.6, 50% EtOAc in DCM). The reaction mixture was filtered over a pad of celite (in a glass pipette) and concentrated using rotary evaporation. Purification was performed twice by silica column chromatography using an 20%-60% EtOAc in pentane eluent followed by silica column chromatography using an 5%-20% EtOAc in DCM eluent, but failed to separate the starting material from the product. The fractions containing product were collected and concentrated using rotary evaporation. The residue was redissolved in EtOAc extracted with sat. $NaHCO_3$ (aq.), dried using $MgSO_4$, filtered and concentrated using rotary evaporation resulting in 35 mg (0.092 mmol, 46.5%) of compound **8** as a pink solid**. ^{1}H NMR (400 MHz, $CDCl_3$) δ**: 5.87 (d, *J* = 9.1 Hz, 1H), 5.34 (dt, *J* = 9.2, 5.4 Hz, 1H), 3.95 (d, *J* = 5.2 Hz, 2H), 3.05 (s, 4H), 2.77 (s, 5H), 1.39 (s, 11H). **^{13}C NMR (101 MHz, $CDCl_3$) δ**: 168.45, 168.18, 166.83, 166.06, 154.86, 80.98, 50.17, 37.43, 28.28, 25.60, 21.29.

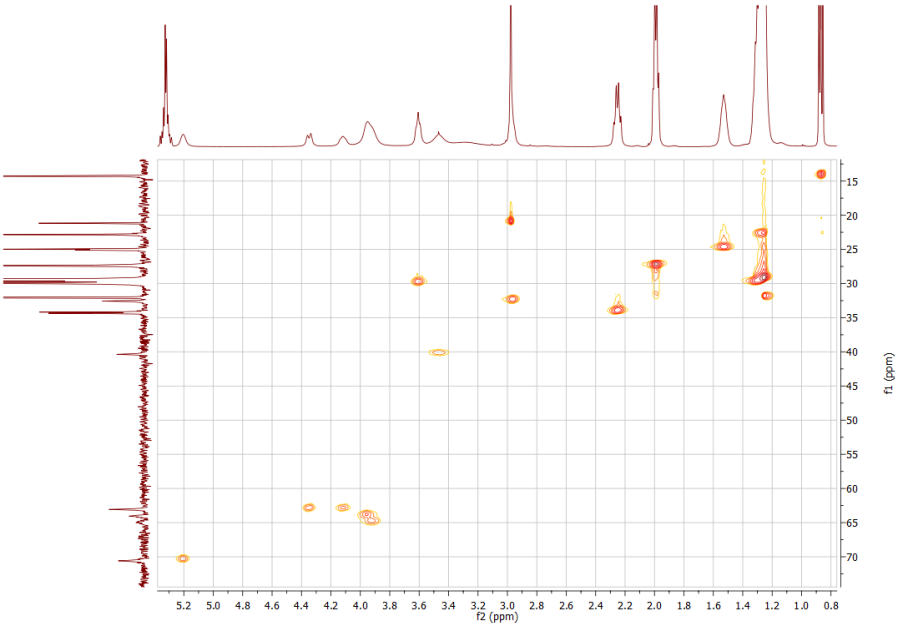
Compound 9: 0.075 mmol (20 mg) of compound **7** was dissolved in 1 mL of $CHCl_3$, 0.050 mmol (0.37 mg) of DOPE and 0072 mmol (10 µL, 7.2 mg) of TEA were added respectively and the reaction mixture was stirred for 30 minutes. 2 mL of $CHCl_3$ was added, the solution was washed with 0.1M HCl (aq.) and the organic layer was directly used for purification. Purification was performed by silica column chromatography using an 2%-5% MeOH in DCM eluent resulting in compound **9**. **^{1}H NMR (500 MHz, $CDCl_3$) δ**: 7.52 (br, 1H), 5.39 – 5.25 (m, 4H), 5.20 (s, 1H), 4.35 (d, *J* = 11.1 Hz, 1H), 4.12 (s, 1H), 3.95 (br, 4H), 3.61 (s, 2H), 3.47 (s, 2H), 3.29 (br, 1H), 2.98 (s, 5H), 2.25 (dd, *J* = 15.6, 7.9 Hz, 4H), 2.04 – 1.92 (m, 8H), 1.53 (s, 4H), 1.38 – 1.15 (m, 36H), 0.87 (t, *J* = 6.9 Hz, 6H). **^{13}C NMR (126 MHz, $CDCl_3$) δ**: 173.87, 173.63, 169.14, 167.47, 130.14, 129.76, 87.18, 70.60, 64.99, 64.04, 63.07, 40.36, 34.35, 34.18, 32.57, 32.05, 29.92, 29.69, 29.48, 29.35, 29.30, 27.38, 25.07, 24.97, 22.83, 21.17, 14.26.

Compound 10: 0.078 mmol (30 mg) of compound **8** was dissolved in 1 mL of $CHCl_3$, 0.050 mmol (0.37 mg) of DOPE and 0072 mmol (10 µL, 7.2 mg) of TEA were added respectively and the reaction mixture was stirred for 30 minutes. 5 mL of $CHCl_3$ was added, the solution was washed with 5 mL 1M HCl (aq.) and the organic layer was directly used for purification. Purification was performed by silica column chromatography using an 2%-5% MeOH in DCM eluent containing 2% AcOH resulting in 32 mg (0.036 mmol, 48%) of compound **10**. **^{1}H NMR**

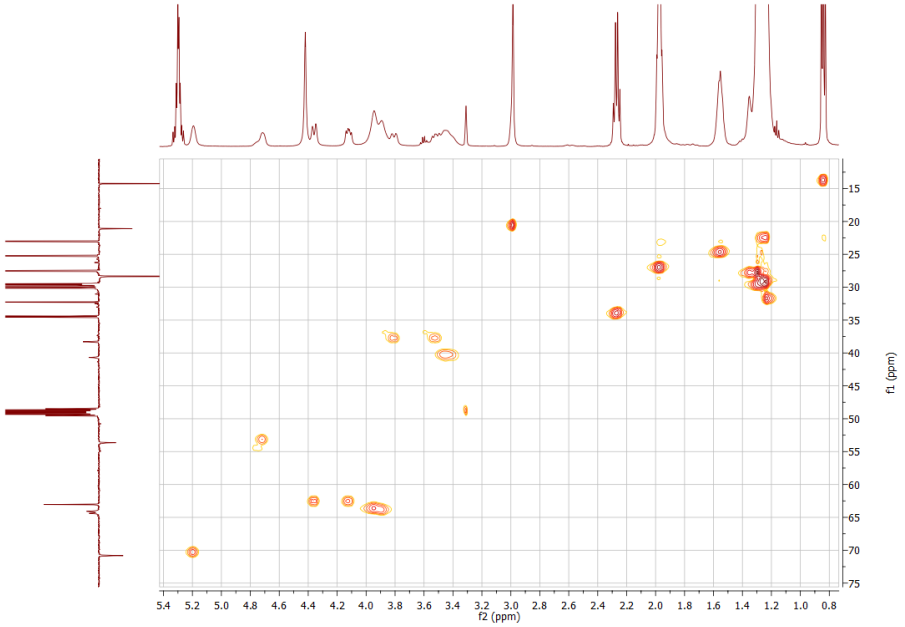
(500 MHz, MeOD) δ: 5.36 – 5.22 (m, 4H), 5.19 (s, 1H), 4.72 (s, 1H), 4.36 (d, *J* = 11.3 Hz, 1H), 4.12 (dd, *J* = 11.5, 7.0 Hz, 1H), 3.94 (s, 2H), 3.89 (s, 2H), 3.81 (d, *J* = 13.0 Hz, 1H), 3.65 – 3.35 (m, 3H), 2.99 (s, 3H), 2.27 (q, *J* = 7.6 Hz, 4H), 1.97 (dd, *J* = 11.5, 6.0 Hz, 8H), 1.56 (d, *J* = 5.1 Hz, 4H), 1.41 – 1.10 (m, 45H), 0.84 (t, *J* = 6.9 Hz, 6H). **^{13}C NMR (126 MHz, MeOD) δ**: 174.24, 173.90, 171.77, 167.89, 167.34, 156.10, 130.33, 129.99, 129.97, 80.60, 70.82, 64.39, 64.08, 63.05, 53.65, 40.69, 38.30, 34.54, 34.39, 32.25, 30.09, 29.85, 29.65, 29.64, 29.62, 29.58, 29.51, 29.49, 29.47, 29.45, 28.34, 27.53, 27.51, 25.25, 25.19, 22.99, 21.09, 14.25.

Compound 11: 10 µmol (10 mg) of compound **10** was dissolved in 1 mL of DCM and 1mL of 4M HCl in dioxane and stirred for 2 hours at room temperature. The reaction mixture was concentrated using rotary evaporation and purified using silica column chromatography using an 1%-20% MeOH in $CHCl_3$ eluent resulting in 3 mg (3,4 µmol, 34%) of compound **11**. **^{1}H NMR (400 MHz, MeOD) δ**: 5.37 – 5.23 (m, 4H), 5.19 (s, 1H), 4.67 (s, 1H), 4.35 (d, *J* = 11.7 Hz, 1H), 4.16 – 4.06 (m, 1H), 3.96 (s, 5H), 3.77 – 3.53 (m, 2H), 3.31 (s, 1H), 3.03 (s, 3H), 2.28 (dd, *J* = 14.2, 6.9 Hz, 4H), 1.97 (d, *J* = 5.2 Hz, 8H), 1.55 (s, 4H), 1.25 (d, *J* = 14.8 Hz, 36H), 0.84 (t, *J* = 6.8 Hz, 6H). **^{13}C NMR (101 MHz, MeOD) δ:** 174.31, 173.98, 168.67, 165.77, 130.35, 129.98, 129.96, 70.66, 64.64, 64.45, 62.92, 51.80, 43.08, 43.00, 40.75, 35.89, 34.55, 34.39, 32.24, 30.08, 29.85, 29.65, 29.63, 29.60, 29.57, 29.50, 29.48, 29.43, 27.52, 27.49, 25.25, 25.19, 22.99, 21.21, 14.25.

HSQC ($CDCl_3$) (compound 9):



HSQC (MeOD) (compound 10):



Compound 12: 0.79 mmol (0.323 g) of compound **44 (Chapter 3)** was dissolved in 2 mL of dry THF and cooled to 0 °C. 1.0 mmol (0.15 mL) of DBU and 1.0 mmol (0.22 mL) of DPPA were added and the reaction mixture was stirred overnight while warming to room temperature. Reaction completion was checked by TLC (Rf = 0.4, 100% pentane). Air was blown through the reaction mixture to evaporate the THF, forming a suspension. Then, 2 mL of a 1:1 (v:v) Pentane:DCM mixture was added to the suspension. The liquid fraction was then separated from the solid and directly purified with silica column chromatography using an 0-2% Et_2O in pentane eluent. Product fractions were collected and carefully concentrated using rotary evaporation (30 °C, 200 mbar) and carefully co-evaporated twice using $CHCl_3$ (30 °C, 100 mbar) resulting in 83 mg (0.47 mmol, 59%) of compound **12** as a yellow oil (unstable at room temperature). **^{1}H NMR (300 MHz, $CDCl_3$) δ**: 3.075 (m, 2H), 2.214 (s, 3H), 1.568 (t, 1H), 0.168 (s, 9H). **^{13}C NMR (75 MHz, $CDCl_3$) δ:** 134.94, 112.53, 59.98, 18.80, 13.85, -1.18.

Compound 13: 1.22 mmol (0.222 g) of compound **12** was dissolved in 2.4 mL of a 5:1 (v:v) THF:H_2O mixture, 1.6 mmol (0.418 g) of PPh_3 was added and the reaction mixture was stirred overnight at room temperature. Reaction completion was checked by TLC (Rf = 0.2, 10% MeOH in DCM). 5 mL of 1M HCl (aq.) was added and the THF was removed using rotary evaporation, and additional 5 mL of 1M HCl (aq.) was added, 10 mL of Et_2O was added and the mixture was stirred vigorously for 10 minutes to dissolve any remaining solids. The layers were separated and the water layer was neutralized to pH = 9 and extracted using DCM. The organic layer was dried using $MgSO_4$, filtered, concentrated using rotary evaporation, co-evaporated twice using $ChCl_3$. The resulting product, 0.14 g (0.90 mmol, 73.6%) of compound **13** as a yellow oil, was used without further purification. **^{1}H NMR (300 MHz, $CDCl_3$) δ**: 2.588 (m, 2H),

2.251 (s, 3H), 1.435 (t, 1H), 1.5-1.4 (br, 1H), 0.145 (s, 9H). **^{13}C NMR (75 MHz, $CDCl_3$) δ:** 136.90, 112.64, 48.81, 23.55, 13.67, -0.87.

Compound 14: 0.11 mmol (0.044 g) of compound **22 (chapter 3)** was dissolved in 1.5 mL dry DMF, 0.32 mmol (0.050 g) of compound 1**2** and 0.43 mmol (60 µL) of TEA were added respectively and the reaction mixture was stirred for 30 minutes at room temperature. Reaction completion was checked by TLC (Rf = 0.4, 10% EtOAc in DCM). 15 mL of EtOAc was added and the reaction mixture was washed with 0.1M HCl (aq.), washed with sat. $NAHCO_3$ (aq.), washed with brine, dried using $MgSO_4$, filtered and concentrated using rotary evaporation. Purification was performed with silica column chromatography using an 10-20% EtOAc in DCM eluent resulting in 43 mg (0.10 mmol, 91%) of compound **14** as a red solid. **^{1}H NMR (400 MHz, $CDCl_3$) δ**: 7.07 (s, 1H), 6.86 (d, *J* = 3.9 Hz, 1H), 6.28 (d, *J* = 4.0 Hz, 1H), 6.10 (s, 1H), 5.55 (s, 1H), 3.26 (t, *J* = 7.6 Hz, 2H), 3.10 (ddt, *J* = 66.2, 13.5, 5.1 Hz, 2H), 2.60 (t, *J* = 7.6 Hz, 2H), 2.55 (s, 3H), 2.24 (s, 3H), 2.13 (s, 3H), 1.37 (t, *J* = 4.7 Hz, 1H), 0.12 (s, 8H). **^{13}C NMR (101 MHz, $CDCl_3$) δ:** 71.39, 160.10, 157.85, 143.82, 135.98, 135.14, 133.51, 128.44, 123.86, 120.42, 117.67, 111.81, 77.48, 77.16, 76.84, 46.43, 36.43, 25.24, 19.24, 15.03, 13.21, 11.42, -1.02.

Compound 15: 2.49 mmol (0.381 g) of compound **19 (Chapter 3)** was dissolved in 20 mL of Et_2O and cooled to 0 °C. 4.0 mmol (2 mL of a 2M solution in THF) of $LiAlH_4$ was added dropwise to the solution and the reaction mixture was stirred overnight while warming to room temperature. Reaction completion was checked by TLC (Rf = 0.15, 10% EtOAc in pentane). The reaction was quenched by dropwise addition of 5 mL of a 1M NaOH (aq.) solution over 5 minutes while stirring vigorously. The organic layer was separated from the water layer, 5 mL of Et_2O was added to the water layer while stirring for 5 minutes, before separating the organic layer. The organic layers were combined, dried using Na_2SO_4, filtered and concentrated using rotary evaporation. The resulting product, 0.306 g (2.44 mmol, 98%) of compound **15** as a pale oil, was used without further purification.

Compound 16: 2.0 mmol (0.252 g) of compound **15** was dissolved in 10 mL of dry DCM and cooled to 0 °C. 3.9 mmol (0.55 mL) of TEA, and 2.6 mmol (0.20 mL) of methanesulfonyl chloride were added and the reaction mixture was stirred for 1 hour. Reaction completion was checked by TLC (Rf = 0.8, 75% EtOAc in pentane). 50 mL of DCM was added to the reaction mixture and the solution was washed with 1M HCl (aq.), washed with sat. $NaHCO_3$ (aq.), dried using $MgSO_4$, filtered and concentrated using rotary evaporation. The resulting product, 1.55 mmol (0.314 g, 77.5%) of compound **16**, was used without further purification.

Compound 17: 0.144 mmol (0.292 g) of compound **16** was dissolved in 8 mL dry DMF, 4.61 mmol (0.30 g) of NaN_3 was added and the reaction mixture was tired overnight at 70 °C. Reaction completion was checked by TLC (Rf = 0.85, 50% EtOAc in pentane). 20 mL of EtOAc was added to the reaction mixture and the solution was washed with 20 mL of water. The water layer was extracted twice using 10 mL EtOAc, all organic layers were combined and the resulting solution was washed multiple times using brine until the organic layer became clear. The organic layer was then dried using $MgSO_4$, filtered and concentrated using rotary evaporation. The resulting product, (quantitative) compound **17**, was used without further purification. **1H NMR (400 MHz, CDCl3) δ:** 6.69 (dd, J = 4.0, 2.5 Hz, 1H), 6.16 (dd, J = 5.7, 2.8

Hz, 1H), 5.96 (s, 1H), 3.34 (t, J = 6.7 Hz, 2H), 2.72 (t, J = 7.4 Hz, 2H), 1.98 – 1.86 (m, 2H). **13C NMR (101 MHz, CDCl3) δ**: 130.77, 116.66, 108.54, 105.58, 50.75, 28.96, 24.73.

Compound 18: 1.9 mmol (0.27 g) of compound **17** was dissolved in dry DCM and cooled to 0 °C, 2.1 mmol (0.260 g) of 3,5-dimethyl-1H-pyrrole-2-carboxaldehyde and 2.1 mmol (0.20 mL) of $POCl_3$ were added respectively and the reaction mixture was stirred for 6 hours while warming to room temperature. The reaction mixture was again cooled to 0 °C and 8.6 mmol (1.2 mL) of TEA was dropwise added to the solution, followed by dropwise addition of 7.6 mmol (0.94 mL) of BF_3 etherate and the reaction mixture was stirred overnight. Reaction completion was checked by TLC (Rf = 0.8, 25% EtOAc in pentane). The reaction mixture was poured into 400 mL EOAc, washed with 1M HCl (aq.), washed with sat. $NAHCO_3$ (Aq.), washed with brine, dried using $MGSO_4$, filtered and concentrated using rotary evaporation. Purification was performed with silica column chromatography using an 5-10% EtOAc in pentane eluent resulting in 55 mg (0.181 mmol, 9.5%) of compound **18** as a black solid. **1H NMR (400 MHz, CDCl3) δ:** 7.07 (s, 1H), 6.89 (d, J = 3.9 Hz, 1H), 6.26 (d, J = 3.9 Hz, 1H), 6.10 (s, 1H), 3.38 (t, J = 7.0 Hz, 2H), 3.04 (t, J = 7.7 Hz, 2H), 2.55 (s, 3H), 2.23 (s, 3H), 2.10 – 1.93 (m, 2H). **13C NMR (101 MHz, CDCl3) δ**: 160.25, 157.78, 143.85, 135.20, 133.33, 128.26, 123.82, 120.47, 116.72, 51.03, 28.22, 25.88, 15.01, 11.36.

Compound 19: 30 µmol (10 mg) of compound **18** was dissolved in 1 mL of THF, 100 µL of H_2O and 60 µmol (38 mg, 1.6 mmol/g) of polymer-bound PPh_3 were added and the reaction mixture was stirred for two days at room temperature. Reaction completion was checked by TLC (Rf = 0.2, 10% TEA in EtOAc). 6 mL of 0.1M HCl (aq.) was added and the water layer was washed three times with Et_2O. To the water layer 2 mL of sat. $NaHCO_3$ (aq.) was added and extracted three times with EtOAc. The organic layers were combined, dried using $MgSO_4$, filtered and concentrated using rotary evaporation. The obtained crude bodipy-amine intermediate was directly used by dissolving it in 1 mL of DMF, 110 µmol (27 mg) of compound **35 (chapter 3)** was added and the reaction mixture was stirred for 1 hour. Reaction completion was checked by TLC (Rf = 0.9, 100% EtOAc). 5 mL of 0.1M HCL (aq.) was added and the watery solution was extracted twice with EtOAc. The organic layers were combined, washed with brine and concentrated using rotary evaporation. Purification was performed with silica column chromatography using an 25-35% EtOAc in pentane eluent resulting in 1 mg (2.5 µmol, 8%) of compound **19**. **^{1}H NMR (400 MHz, $CDCl_3$) δ:** 7.08 (s, 1H), 6.92 (d, *J* = 4.1 Hz, 1H), 6.31 (d, *J* = 4.1 Hz, 1H), 6.12 (t, *J* = 4.2 Hz, 2H), 6.09 – 5.99 (m, 2H), 3.39 – 3.22 (m, 2H), 3.02 (t, *J* = 7.3 Hz, 2H), 2.93 (s, 1H), 2.89 (s, 1H), 2.56 (s, 3H), 2.26 (s, 3H), 1.95 (dd, *J* = 15.5, 9.2 Hz, 3H), 1.91 – 1.85 (m, 1H), 1.68 (d, *J* = 8.3 Hz, 2H), 1.36 – 1.20 (m, 4H).

Compound 20: 30 µmol (10 mg) of compound **18** was dissolved in 1 mL of THF, 200 µL of H_2O and 40 µmol (10 mg) of PPh_3 were added and the reaction mixture was stirred overnight at room temperature. Reaction completion was checked by TLC (Rf = 0.2, 10% TEA in EtOAc). 10 mL of 1.2M HCl (aq.) was added and the water layer was washed two times with Et_2O. To the water layer 50 mL of sat. $NaHCO_3$ (aq.) was added and extracted once with DCM and two times with EtOAc. The organic layers were combined, dried using $MgSO_4$, filtered and concentrated using rotary evaporation. The obtained crude bodipy-amine intermediate was directly used by dissolving it in 0.5 mL of DMF, 60 µmol (15 mg) of compound **42 (chapter 3)** was added and the reaction mixture was stirred overnight at room temperature. Reaction completion

was checked by TLC (Rf = 0.6, 100% EtOAc). 5 mL of 0.1M HCL (aq.) was added and the watery solution was extracted twice with EtOAc. The organic layers were combined, washed with brine and concentrated using rotary evaporation. Purification was performed with silica column chromatography using an 25-50% EtOAc in pentane eluent resulting in 5.0 mg (14 µmol, 47%) of compound **20** as a red solid. The NMR showed a non-removable PPh_3 impurity, and HPLC purification was required to remove this impurity. **^{1}H NMR (400 MHz, $CDCl_3$) δ:** 7.09 (s, 1H), 6.92 (d, *J* = 4.0 Hz, 1H), 6.40 (dt, *J* = 2.6, 1.2 Hz, 1H), 6.30 (d, *J* = 4.1 Hz, 1H), 6.12 (s, 1H), 5.99 (s, 1H), 3.37 – 3.22 (m, 2H), 2.98 (t, *J* = 7.3 Hz, 2H), 2.57 (s, 3H), 2.26 (s, 3H), 2.16 (d, *J* = 1.2 Hz, 3H), 2.00 (d, *J* = 1.6 Hz, 1H), 1.97 – 1.87 (m, 2H).

Compound 21: 0.40 mmol (94 mg) of compound **35 (chapter 3)** was dissolved in 2 mL of DCM, 0.80 mmol (51 µL) of propargylamine and 0.80 mmol (111 µL) of TEA were added respectively and the reaction mixture was stirred for 1 hour at room temperature. Reaction completion was checked by TLC (Rf = 0.75 in 10% EtOAc/DCM). The crude mixture was directly used for purification by silica column chromatography using an EtOAc in DCM eluent, resulting in 65 mg (0.37 mmol, 93%) of compound **21** as a white solid. **^{1}H NMR (400 MHz, $CDCl_3$) δ:** 6.27 (s, 1H), 6.10 (dd, *J* = 5.6, 2.9 Hz, 1H), 6.05 (dd, *J* = 5.6, 3.0 Hz, 1H), 4.01 (dd, *J* = 5.3, 2.6 Hz, 2H), 2.90 (d, *J* = 1.3 Hz, 1H), 2.87 (s, 1H), 2.19 (t, *J* = 2.6 Hz, 1H), 2.06 – 1.98 (m, 1H), 1.92 – 1.81 (m, 1H), 1.67 (d, *J* = 8.2 Hz, 1H), 1.35 – 1.23 (m, 2H). **^{13}C NMR (101 MHz, $CDCl_3$) δ:** 175.58, 138.30, 135.98, 79.94, 77.48, 71.37, 47.12, 46.36, 44.35, 41.58, 30.49, 29.28.

Compound 22: 0.47 mmol (116 mg) of compound **45 (chapter 3)** was dissolved in 2.5 mL of DCM, 1.0 mmol (64 µL) of propargylamine and 1.0 mmol (140 µL) of TEA were added respectively and the reaction mixture was stirred overnight at room temperature. Reaction completion was checked by TLC (Rf = 0.30 in 10% EtOAc/Pentane). The crude mixture was directly used for purification by silica column chromatography using an 5%-10% EtOAc in DCM eluent resulting in 58 mg (0.35 mmol, 75%) of compound **22** as a colorless oil. **^{1}H NMR (400 MHz, $CDCl_3$) δ:** 6.53 (s, 1H), 5.02 (s, 1H), 4.05 – 3.81 (m, 4H), 2.22 (t, *J* = 2.5 Hz, 1H), 2.10 (d, *J* = 0.9 Hz, 3H), 1.61 (t, *J* = 4.5 Hz, 1H). **^{13}C NMR (101 MHz, $CDCl_3$) δ:** 156.49, 120.70, 102.12, 80.09, 72.87, 71.45, 30.82, 17.20, 11.72.

Compound 23: 0.18 mmol (50 mg) of *p*-nitrophenyl 2-azetine-succinate was dissolved in 2 mL of DCM, 0.91 mmol (58 µL) of propargylamine and 0.90 mmol (125 µL) of TEA were added respectively and the reaction mixture was stirred overnight at room temperature. Reaction completion was checked by TLC (Rf = 0.25 in 100% EtOAc). The crude mixture was directly used for purification by silica column chromatography using an 50%-100% EtOAc in pentane eluent, followed by silica column chromatography using an 75%-100% EtOAc in DCM eluent resulting in 16 mg (0.083 mmol, 46%) of compound **23** as a white solid. **^{1}H NMR (400 MHz, $CDCl_3$) δ**: 6.77 (d, *J* = 78.1 Hz, 1H), 6.65 (s, 1H), 5.72 (d, *J* = 12.1 Hz, 1H), 4.51 (d, *J* = 57.1 Hz, 2H), 4.04 – 3.98 (m, 2H), 2.68 – 2.57 (m, 2H), 2.56 (s, 2H), 2.20 (t, *J* = 2.5 Hz, 1H). **^{13}C NMR (101 MHz, $CDCl_3$) δ**: 171.75, 166.12, 137.34, 136.99, 114.15, 114.06, 79.74, 71.44, 58.87, 56.83, 30.91, 30.81, 29.29, 27.41, 26.45.

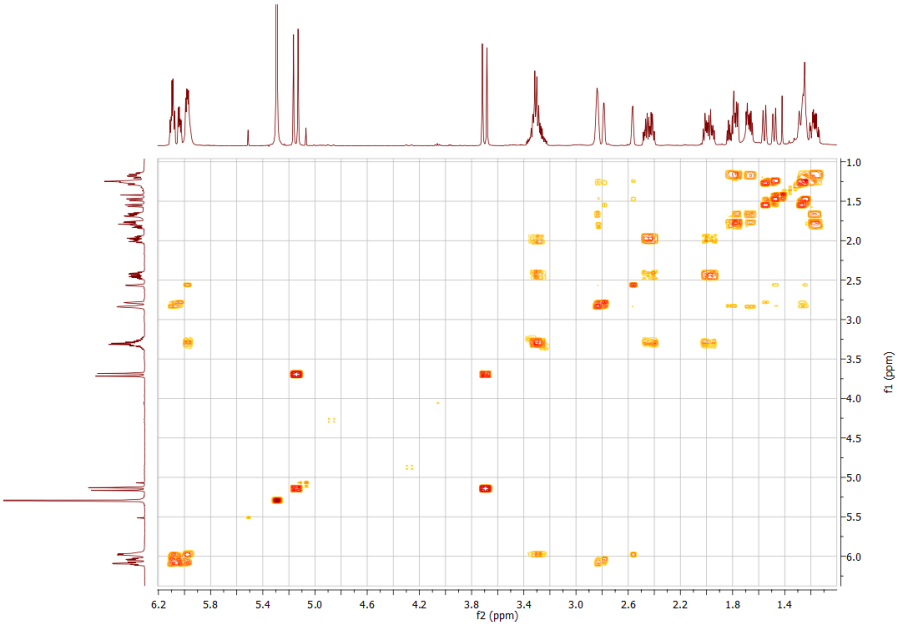
Compound 24: 0.069 mmol (20 mg) of compound **57a (chapter 3)** was dissolved in 0.5 mL of DCM, 0.34 mmol (22 µL) of propargylamine and 0.34 mmol (48 µL) of TEA were added respectively and the reaction mixture was stirred overnight at room temperature. Reaction

completion was checked by TLC (Rf = 0.60 in 20% EtOAc/Pentane). 20 mL of DCM was added and the reaction mixture was washed twice with sat. $NaHCO_3$ (aq.), washed twice with 10% $KHSO_4$ (aq.), dried with $MgSO_4$, filtered and concentrated using rotary evaporation. Purification was performed by silica column chromatography using an 5%-10% EtOAc in pentane eluent resulting in 13 mg (0.63 mmol, 91%) of compound **24** as a colorless oil. **^{1}H NMR (400 MHz, $CDCl_3$) δ**: 5.63 – 5.47 (m, 2H), 5.01 – 4.86 (m, 2H), 3.99 (dd, *J* = 5.7, 2.4 Hz, 2H), 2.39 – 2.21 (m, 5H), 2.21 – 2.01 (m, 2H), 1.91 – 1.73 (m, 2H), 1.73 – 1.60 (m, 1H), 1.60 – 1.43 (m, 1H), 1.28 – 1.14 (m, 1H). **^{13}C NMR (101 MHz, $CDCl_3$) δ**: 155.75, 135.49, 131.86, 80.00, 71.70, 70.95, 41.16, 34.41, 32.69, 30.93, 30.08, 28.13.

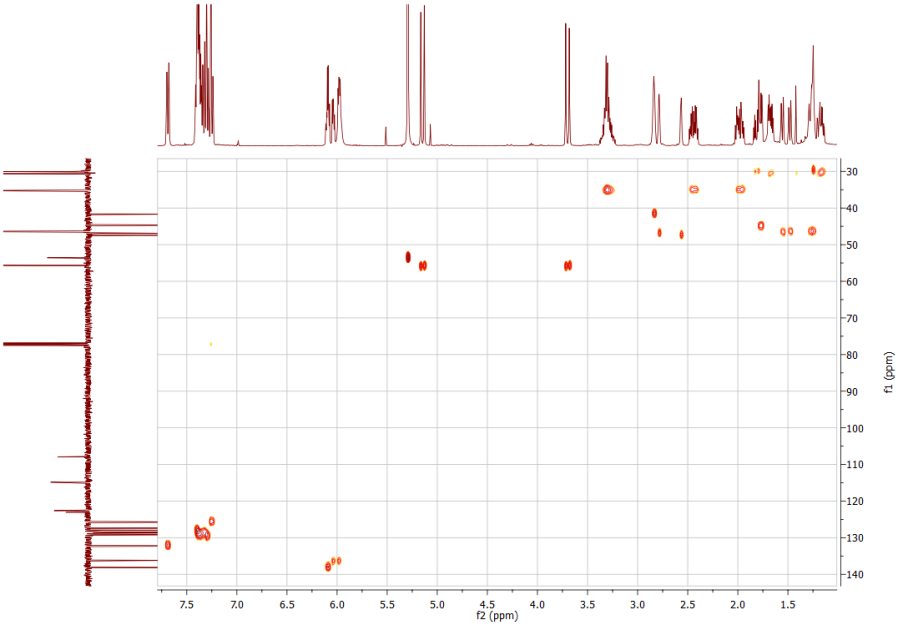
Compound 25: 38 µmol (9 mg) of compound **35 (chapter 3)** was dissolved in 0.2 mL of DCM, 40 µmol (11 mg) of DBCO-amine and 43 µmol (6 µL) of TEA were added respectively and the reaction mixture was stirred for 1 hour at room temperature. Reaction completion was checked by TLC (Rf = 0.4 in 20% EtOAc/DCM). The crude mixture was directly used for purification by silica column chromatography using an 10%-20% EtOAc in DCM eluent, resulting in 11 mg (28 µmol, 74%) of compound **25** as a colorless oil. **^{1}H NMR (400 MHz, $CDCl_3$) δ:** 7.69 (d, *J* = 7.6 Hz, 1H), 7.44 – 7.20 (m, 7H), 6.11 – 6.05 (m, 1H), 6.05 – 5.93 (m, 2H), 5.15 (d, *J* = 13.9 Hz, 1H), 3.70 (d, *J* = 13.9 Hz, 1H), 3.41 – 3.20 (m, 2H), 2.84 (s, 1H), 2.68 (dd, *J* = 88.0, 1.4 Hz, 1H), 2.50 – 2.36 (m, 1H), 2.05 – 1.90 (m, 1H), 1.78 (ddd, *J* = 5.4, 4.6, 2.2 Hz, 1H), 1.73 – 1.61 (m, 1H), 1.52 (dd, *J* = 30.1, 8.4 Hz, 1H), 1.28 – 1.23 (m, 1H), 1.22 – 1.13 (m, 1H). **^{13}C NMR (101 MHz, $CDCl_3$) δ:** 175.45, 175.28, 172.49, 172.43, 151.18, 151.13, 148.11, 138.16, 138.14, 136.19, 136.16, 132.21, 129.15, 128.71, 128.69, 128.55, 128.45, 127.96, 127.40, 125.79, 125.69, 123.10, 123.07, 122.62, 114.85, 107.92, 107.90, 55.63, 53.57, 47.43, 46.88, 46.49, 46.31, 44.74, 44.61, 41.66, 41.65, 35.31, 35.20, 35.10, 30.61, 30.05.

Compound 26: 41 µmol (12 mg) of compound **57a (chapter 3)** was dissolved in 0.2 mL of DCM, 40 µmol (11 mg) of DBCO-amine and 100 µmol (14 µL) of TEA were added respectively and the reaction mixture was stirred overnight at room temperature. Reaction completion was checked by TLC (Rf = 0.55 in 50% EtOAc/pentane). The crude mixture was directly used for purification by silica column chromatography using an 20%-40% EtOAc in pentane eluent, followed by silica column chromatography using an 20%-5% EtOAc in DCM eluent resulting in 12 mg (27 µmol, 66%) of compound **26** as a colorless oil. **^{1}H NMR (400 MHz, $CDCl_3$) δ**: 7.70 (d, *J* = 7.5 Hz, 1H), 7.44 – 7.19 (m, 7H), 5.57 – 5.38 (m, 2H), 5.16 (d, *J* = 14.0 Hz, 1H), 5.12 – 5.08 (m, 1H), 4.85 – 4.76 (m, 1H), 3.71 (d, *J* = 13.9 Hz, 1H), 3.36 – 3.11 (m, 2H), 2.49 (m, 1H), 2.32 – 2.13 (m, 4H), 2.13 – 1.89 (m, 2H), 1.88 – 1.66 (m, 2H), 1.59 (m, 1H), 1.56 – 1.21 (m, 1H), 1.21 – 1.05 (m, 1H). **^{13}C NMR (101 MHz, $CDCl_3$) δ**: 172.23, 156.15, 151.23, 148.12, 135.65, 135.53, 132.12, 132.08, 131.78, 131.62, 129.24, 129.19, 128.64, 128.52, 128.51, 128.00, 127.35, 127.32, 125.85, 125.80, 122.96, 122.79, 115.03, 107.74, 70.09, 69.97, 55.71, 55.68, 41.20, 41.17, 36.95, 36.79, 35.58, 35.38, 34.52, 34.45, 32.78, 30.06, 28.05.

COSY (compound 25):



HSQC (compound 25):



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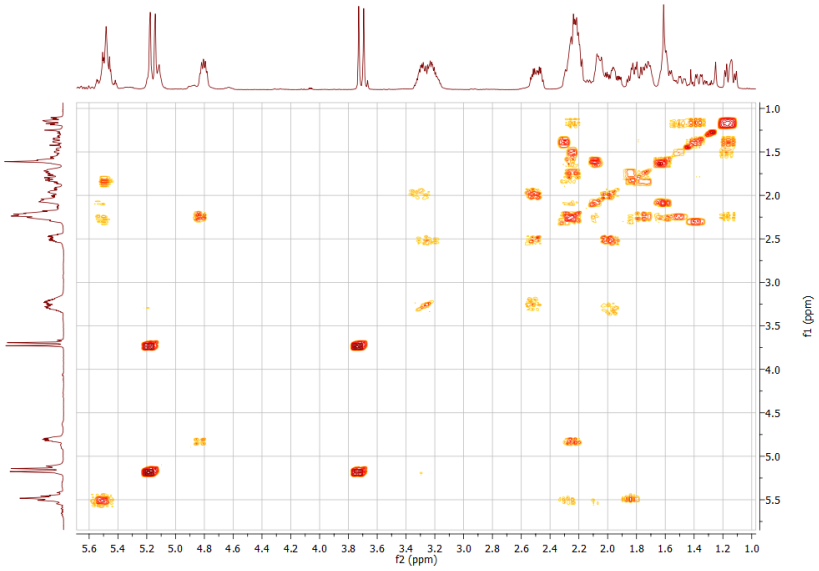
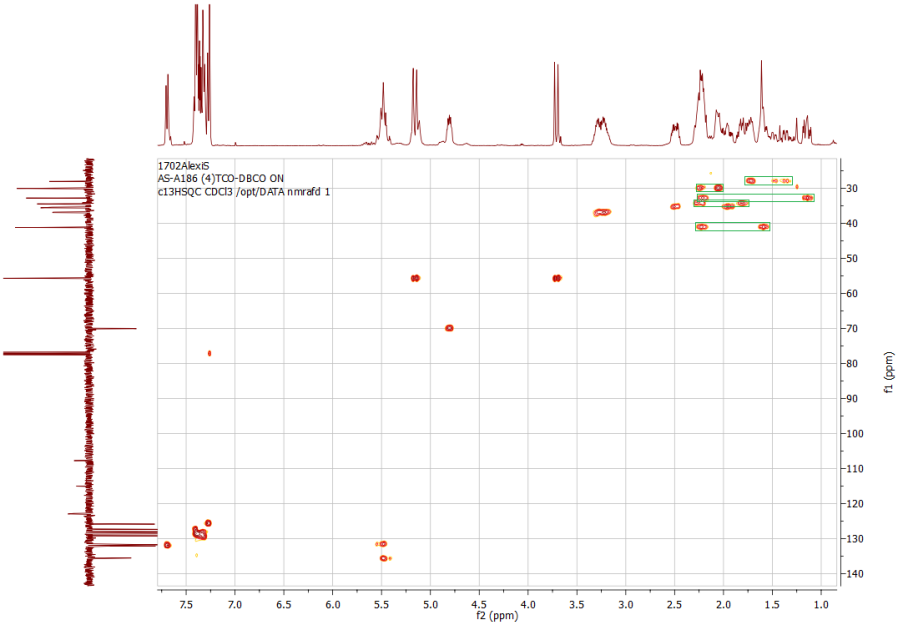


HSQC (compound 26):



Compound 27: 0.24 mmol (63 mg) of compound **42** was dissolved in 0.5 mL dimethylformamide, 0.36 mmol (130 mg) Fmoc-Dap-OH hydrochloride, 0.36 mmol (63 µL) DiPEA were added and the reaction mixture was stirred overnight at room temperature. Reaction completion was checked by TLC tracking the consumption of starting material (Rf = 0.8, 10% Et_2O in pentane) and the formation of product (Rf = 0.1, 100% EtOAc + 0.5% AcOH). 10 mL of 0.1M HCl (aq.) and 2 mL brine were added to the reaction mixture and the resulting solution was extracted twice with 10 mL EtOAc. The organic layers were combined, dried using $MgSO_4$, filtered and concentrated using rotary evaporation. Purification was performed with silica column chromatography using an 1-5% MeOH in dichloromethane containing 1% AcOH eluent resulting in 0.57 mg (0.14 mmol, 58%) of compound **27** as a colorless oil. **TLC**: Rf = 0.2, 100% EtOAc + 0.5% AcOH). **^{1}H NMR (400 MHz, $CDCl_3$) δ**: 9.42 (s, 1H), 7.73 (d, *J* = 7.4 Hz, 2H), 7.58 (t, *J* = 7.6 Hz, 2H), 7.37 (t, *J* = 7.3 Hz, 2H), 7.31 – 7.21 (m, 2H), 6.57 (d, *J* = 5.1 Hz, 1H), 6.43 – 6.22 (m, 2H), 4.54 – 4.28 (m, 3H), 4.18 (t, *J* = 6.9 Hz, 1H), 3.81 – 3.56 (m, 2H), 2.07 (d, *J* = 5.9 Hz, 3H), 2.01 (s, 1H). **^{13}C NMR (101 MHz, $CDCl_3$) δ:** 178.99, 172.50, 156.66, 143.89, 143.69, 141.34, 141.31, 127.85, 127.23, 125.27, 120.07, 113.23, 67.47, 67.43, 55.05, 54.86, 47.11, 41.89, 22.18, 10.55, 10.52.

Compound 28: 0.20 mmol (52 mg) of compound **42** was dissolved in 1 mL dimethylformamide, 0.30 mmol (110 mg) Fmoc-Lys-OH was added and the reaction mixture was stirred overnight at room temperature. Reaction completion was checked by TLC tracking the consumption of starting material (Rf = 0.8, 10% Et_2O in pentane) and the formation of product (Rf = 0.1, 100% EtOAc + 0.5% AcOH). 10 mL of 0.1M HCl (aq.) and 4 mL brine were added to the reaction mixture and the resulting solution was extracted with 10 mL EtOAc. The organic layer was dried using $MgSO_4$, filtered and concentrated using rotary evaporation. Purification was performed with silica column chromatography using an 1-5% MeOH in dichloromethane containing 1% AcOH eluent resulting in 0.78 mg (0.174 mmol, 87%) of compound **28** as a colorless oil. **TLC**: Rf = 0.2, 100% EtOAc + 0.5% AcOH). **^{1}H NMR (400 MHz, $CDCl_3$) δ:** 9.28 (s, 1H), 7.73 (d, *J* = 7.5 Hz, 2H), 7.62 – 7.55 (m, 2H), 7.37 (t, *J* = 7.4 Hz, 2H), 7.31 – 7.24 (m, 2H), 6.35 (s, 1H), 5.96 (t, *J* = 8.8 Hz, 1H), 5.91 (d, *J* = 2.8 Hz, 1H), 4.39 (dd, *J* = 12.9, 7.3 Hz, 1H), 4.33 (d, *J* = 7.2 Hz, 2H), 4.17 (t, *J* = 7.1 Hz, 1H), 3.24 (pd, *J* = 13.4, 6.7 Hz, 2H), 2.09 (s, 3H), 1.99 (s, 1H), 1.95 – 1.71 (m, 2H), 1.50 (dd, *J* = 13.3, 6.5 Hz, 2H), 1.46 – 1.31 (m, 2H**). ^{13}C NMR (101 MHz, $CDCl_3$) δ:** 177.72, 177.68, 174.72, 156.38, 144.00, 143.81, 141.31, 127.76, 127.16, 125.28, 125.25, 120.01, 113.55, 113.48, 67.08, 53.75, 47.19, 39.32, 39.24, 32.02, 29.19, 22.31, 22.24, 10.65.

Compound 32 (A): 0.25 mmol (1.25 g) of 0.2 mmol/g Fmoc-Leucine preloaded Tentagel S and 1 mmol of Fmoc-Lys(Boc)-OH, Fmoc-Glu(tBu)-OH, Fmoc-Phe-OH, Fmoc-Asn(Trt)-OH, Fmoc-Ile-OH (2x), Fmoc-Ser(tBu)-OH were used in solid phase peptide synthesis to prepare Fmoc-SIINFEKL-OH **32** as a colorless solid. LC-MS analysis: (Alltima C_{18} analytical column, linear gradient in 12.5 minutes; H_2O: 80 → 0% ; ACN: 10 → 90% ; aq. 0.5% TFA: 10%) **LC:** Rt: 6.34 min **MS:** $[C_{60}H_{84}N_{10}O_{15}]^+$: found 1185.6, calculated 1185.6.

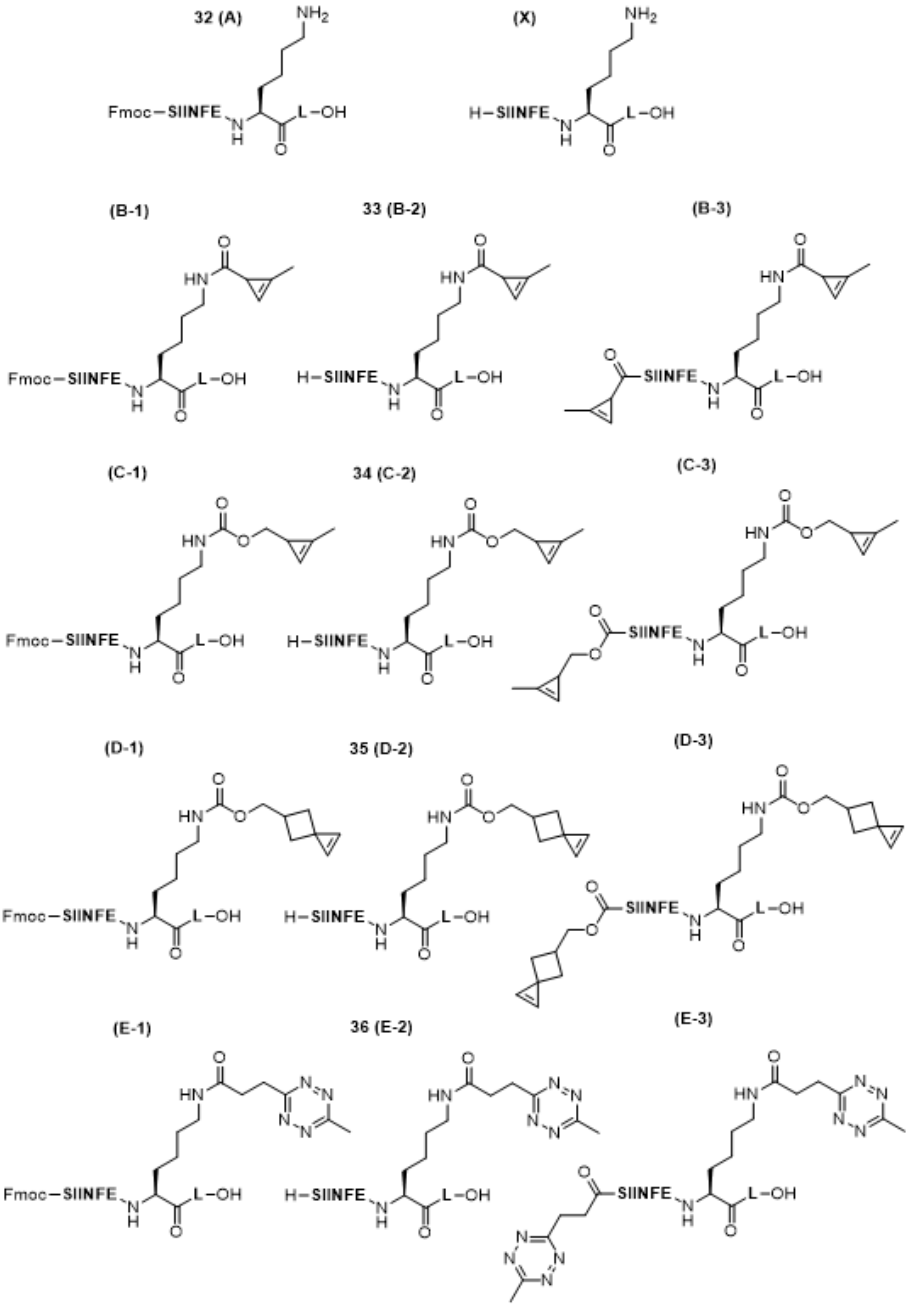
Compound 33 (B-2): **(Step 1)** 23 µmol (27 mg) of compound **32** was added to 3 mL DMF, 0.47 mmol (44 µL) N-methyl morpholine and 98 µmol (26 mg) of compound **42 (chapter 3)** were added to the solution and the reaction mixture was stirred for three days at room temperature. Reaction completion was checked by LC-MS tracking the consumption of starting compound

32. **(Step 2)** 0.75 mL of piperidine was added to form a 20% piperidine in DMF solution and the reaction mixture was stirred for 2 hours at room temperature. Reaction completion was checked by LC-MS tracking the consumption of intermediate **B-1**. The solution was dropwise titrated into an 25 mL of Et_2O, cooled to -20 °C for 30 minutes and centrifuged to form a crude pellet containing the product **33** (**B-2**), as well as double functionalized byproduct **B-3**, and unknown likely-oxidized byproduct **B-1^{ox}**. The crude product was then purified using C_{18}-reverse phase HPLC to obtain compound **B-2** as a white powder. LC-MS analysis: (Alltima C_{18} analytical column, linear gradient in 12.5 minutes; H_2O: 80 → 0% ; ACN: 10 → 90% ; aq. 0.5% TFA: 10%) **LC:** Rt: 5.2 min (**32**), 5.9 min (**B-3**), 6.8 min (**B-1^{ox}**), 7.5 min (**B-1**), **MS:** $[C_{65}H_{89}N_{10}O_{16}]^+$: found 1265.4, calculated 1265.6 (**B-1**), $[C_{50}H_{79}N_{10}O_{14}]^+$: found 1043.4, calculated 1043.6 (**32**), $[C_{55}H_{83}N_{10}O_{15}]^+$: found 1123.4, calculated 1123.6 (**B-3**) , $[C_{65}H_{89}N_{10}O_{17}]^+$: found 1281.3, calculated 1281.6 (**B-1^{ox}**)

Compound 34 (C-2): **(Step 1)** 31 µmol (35 mg) of compound **32** was added to 2 mL DMF, 236 µmol (26 µL) N-methyl morpholine and 120 µmol (30 mg) of compound **45 (chapter 3)** were added to the solution and the reaction mixture was stirred for five days at room temperature. Reaction completion was checked by LC-MS tracking the consumption of starting compound **32**. **(Step 2)** 0.5 mL of piperidine was added to form a 20% piperidine in DMF solution and the reaction mixture was stirred for 2 hours at room temperature. Reaction completion was checked by LC-MS tracking the consumption of intermediate **C-1**. The solution was dropwise titrated into an 25 mL of Et_2O, cooled to -20 °C for 30 minutes and centrifuged to form a crude pellet containing the product **34**, as well as double functionalized byproduct **C-3**, unprotected peptide **X**, and unknown likely-oxidized byproducts **32^{ox}** and **C-1^{ox}**. The crude product was then purified using C_{18}-reverse phase HPLC to obtain compound **34** as a white powder. LC-MS analysis: (Alltima C_{18} analytical column, linear gradient in 12.5 minutes; H_2O: 80 → 0% ; ACN: 10 → 90% ; aq. 0.5% TFA: 10%) **LC:** Rt: 4.7 min (**X**), 5.6 min (**34**), 7.0 min (**C-3**), 7.3 min (**C-1^{ox}**), 7.9 min (**C-1**), **MS:** $[C_{45}H_{75}N_{10}O_{13}]^+$: found 963.5, calculated 963.6 (**X**), $[C_{60}H_{85}N_{10}O_{15}]^+$: found 1185.6, calculated 1185.6 (**32**), $[C_{60}H_{85}N_{10}O_{16}]^+$: found 1201.5, calculated 1201.6 (**32^{ox}**), $[C_{66}H_{91}N_{10}O_{17}]^+$: found 1295.3, calculated 1295.7 (**C-1**), $[C_{66}H_{91}N_{10}O_{18}]^+$: found 1311.3, calculated 1311.7 (**C-1^{ox}**), $[C_{51}H_{81}N_{10}O_{15}]^+$: found 1073.5, calculated 1073.6 (**34**), $[C_{57}H_{87}N_{10}O_{17}]^+$: found 1183.3, calculated 1183.6 (**C-3**).

Compound 35 (D-2): **(Step 1)** 37 µmol (44 mg) of compound **32** was added to 1 mL DMF, 199 µmol (22 µL) N-methyl morpholine and 80 µmol (20 mg) of compound **52 (chapter 3)** were added to the solution and the reaction mixture was stirred for three days at room temperature. Reaction completion was checked by LC-MS tracking the consumption of starting compound **32**. **(Step 2)** 0.25 mL of piperidine was added to form a 20% piperidine in DMF solution and the reaction mixture was stirred for 2 hours at room temperature. Reaction completion was checked by LC-MS tracking the consumption of intermediate **D-1**. The solution was dropwise titrated into 25 mL of Et_2O, cooled to -20 °C for 30 minutes and centrifuged to form a crude pellet containing the product **35**, as well as double functionalized byproduct **D-3**. The crude product was then purified using C_{18}-reverse phase HPLC to obtain compound **35** as a white powder. LC-MS analysis: (Alltima C_{18} analytical column, linear gradient in 12.5 minutes; H_2O: 80 → 0% ; ACN: 10 → 90% ; aq. 0.5% TFA: 10%) **LC:** Rt: 6.0 min (**35**), 7.6 min (**D-3**), 7.4 min (**D-1**), **MS:** $[C_{68}H_{93}N_{10}O_{17}]^+$: found 1322.0, calculated 1321.7 (**D-1**), $[C_{53}H_{83}N_{10}O_{15}]^+$: found 1099.5, calculated 1099.6 (**35**), $[C_{61}H_{91}N_{10}O_{17}]^+$: found 1235.4, calculated 1235.7 (**D-3**).

Compound 36 (E-2): **(Step 1)** 31 µmol (35 mg) of compound **32** was added to 3 mL DMF, 146 µmol (16 µL) N-methyl morpholine and 41 µmol (11 mg) of compound **3 (chapter 5)** were added to the solution and the reaction mixture was stirred for three days at room temperature. Reaction completion was checked by LC-MS tracking the consumption of starting compound **32**. **(Step 2)** 0.75 mL of piperidine was added to form a 20% piperidine in DMF solution and the reaction mixture was stirred overnight at room temperature. Reaction completion was checked by LC-MS tracking the consumption of intermediate **E-1**. The solution was dropwise titrated into an 25 mL of Et_2O, cooled to -20 °C for 30 minutes and centrifuged to form a crude pellet containing the product **36**, as well as double functionalized byproduct **E-3**, and unknown likely-oxidized byproduct **E-1^{ox}**. The crude product was then purified using C_{18}-reverse phase HPLC to obtain compound **36** as a powder. LC-MS analysis: (Alltima C_{18} analytical column, linear gradient in 12.5 minutes; H_2O: 80 → 0% ; ACN: 10 → 90% ; aq. 0.5% TFA: 10%) **LC:** Rt: 4.4 min (**36**), 5.0 min (**E-3**), 5.9 min (**E-1^{ox}**), 6.6 min (**E-1**), **MS:** $[C_{66}H_{91}N_{14}O_{16}]^+$: found 1335.9, calculated 1335.7 (**E-1**), $[C_{66}H_{91}N_{14}O_{17}]^+$: found 1351.8, calculated 1351.6 (**E-1^{ox}**), $[C_{51}H_{81}N_{14}O_{14}]^+$: found 1113.5, calculated 1113.6 (**36**), $[C_{57}H_{87}N_{18}O_{15}]^+$: found 1263.7, calculated 1263.7 (**E-3**).


32 (A)
(X)
(B-1)
33 (B-2)
(B-3)
(C-1)
34 (C-2)
(C-3)
(D-1)
35 (D-2)
(D-3)
(E-1)
36 (E-2)
(E-3)
Fmoc–SIINFE
H–SIINFE
L–OH

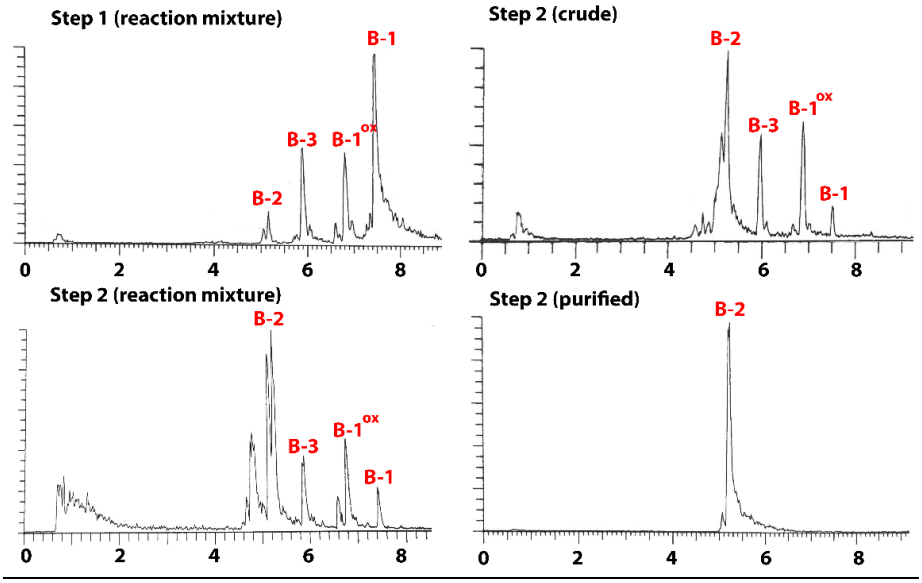


Figure: LCMS trace (m/z) of the two-step reaction and between Fmoc-SIINFEKL-OH and acyl-cyclopropene **42 (chapter 3)**.

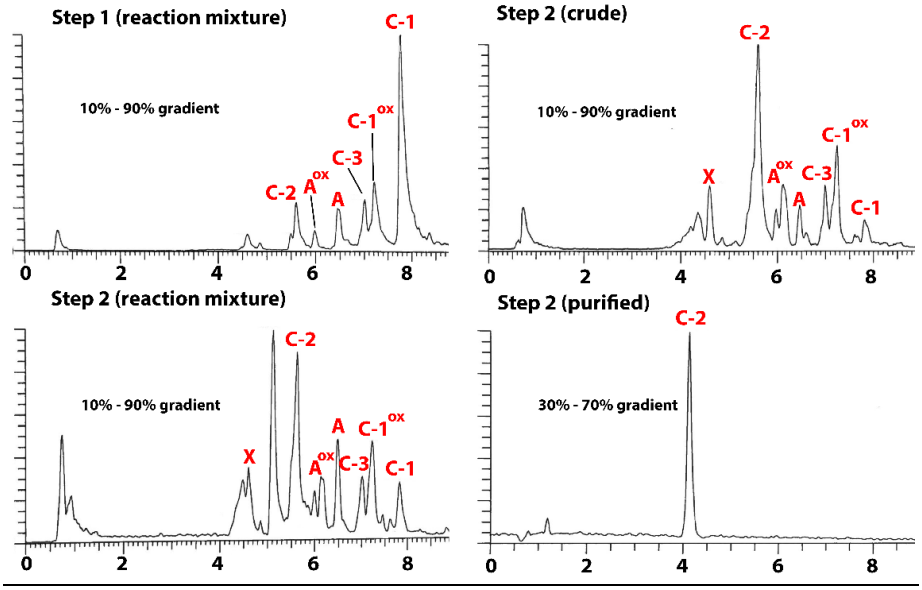


Figure: LCMS trace (m/z) of the two-step reaction between Fmoc-SIINFEKL-OH and alkyl-cyclopropene **45 (chapter 3)**.

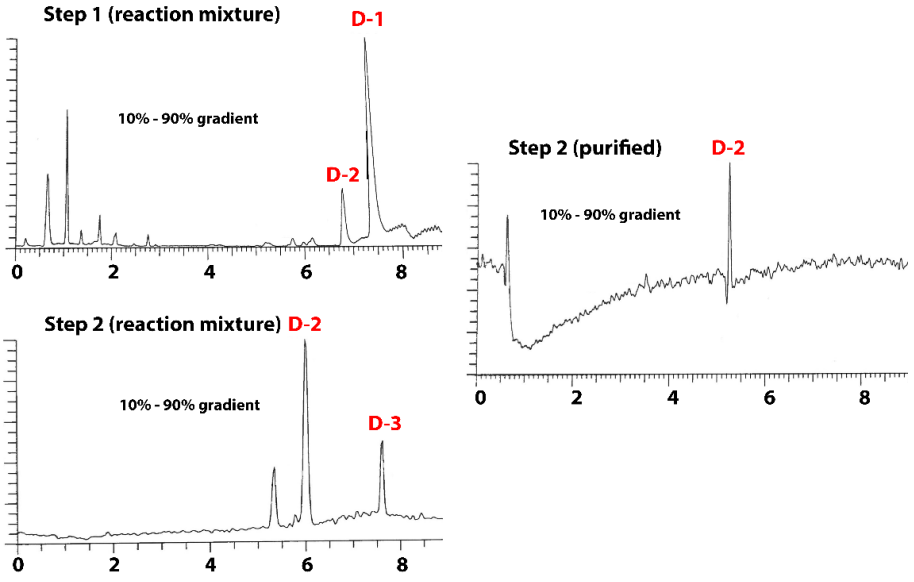


Figure: LCMS trace (m/z) of the two-step reaction between Fmoc-SIINFEKL-OH and spirohexene **52 (chapter 3)**.

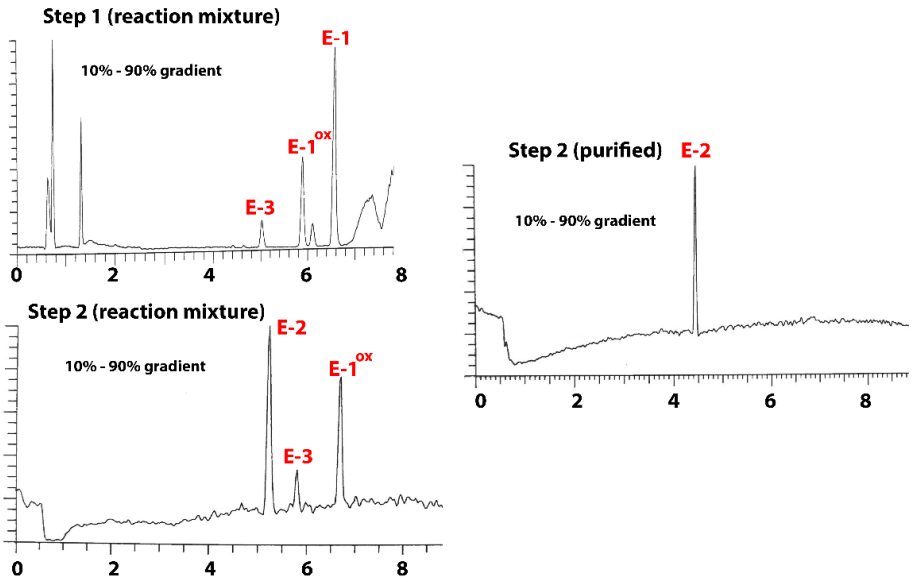


Figure: LCMS trace (m/z) of the two-step reaction between Fmoc-SIINFEKL-OH and carboxyethyl-tetrazine **3 (chapter 5)**.

Compound 37: 5.0 mmol (1.08 g) of 4-bromo-2-methylbenzoic acid was suspended in 20 mL of dry DCE, 11 mmol (2.0 g) of NBS and 0.11 mmol (18 mg) of AIBN were added and the reaction mixture was stirred overnight at 95 °C. The reaction mixture was cooled to room temperature and formed a suspension. Reaction completion was checked by TLC (Rf = 0.5, 2% AcOH in 1:1 EtOAc/DCM). 50 mL of sat. $NaHCO_3$ (aq.) and 50 mL of H_2O were added to the mixture and it was washed using 100 mL of DCM. The water layer was acidified using 75 mL of 1M HCl (aq.) and extracted using 100 mL of EtOAc. The organic layer was then washed multiple times with 0.1M HCl (aq.) until all remaining succinimide was washed away (visible by TLC at RF = 0.3, 2% AcOH in 1:1 EtOAc/DCM). The organic layer was dried using NaSO4, filtered and concentrated using rotary evaporation. Reaction completion was checked by NMR (^{1}H NMR (400 MHz, MeOD)), which could distinguish between mono-brominated intermediate (**δ**: 4.740 (CH_2Br)) and di-brominated product (**δ**: 7.313 ($CHBr_2$)). Because only 62% of the material was di-brominated, the reaction and purification steps were repeated using 5.0 mmol of NBS resulting in 1.40 g (3.76 mmol, 75.2%) of compound **37** as a slightly yellowish solid. **^{1}H NMR (300 MHz, DMSO) δ**: 8.470 (s, 1H), 7.806 (s, 2H), 7.383 (s, 1H). **^{13}C NMR (75 MHz, DMSO) δ:** 165.92, 140.25, 133.87, 131.93, 131.33, 131.10, 40.62.

Compound 38: 3.13 mmol (1.17 g) of compound **37** was suspended in 20 mL of 10% wt. $NaCO_3$ (aq.) and the reaction mixture was warmed to 70 °C for 4 hours. Reaction completion was checked by TLC (Rf = 0.6, 10% MeOH in EtOAc). While warm, the reaction mixture was filtered ,100 mL of H_2O was added, and the watery solution was washed with 100 mL EtOAc. The water layer was acidified to pH = 1 using 50 mL of 1M HCl (aq.) and extracted with 100 mL EtOAc. The organic layer was dried using $MgSO_4$, filtered and concentrated using rotary evaporation resulting in 0.675 g (2.95 mmol, 94.2%) of compound **38** as an off-white solid. **^{1}H NMR (400 MHz, DMSO) δ:** 13.50 (s, 1H), 10.22 (d, J = 0.5 Hz, 1H), 8.29 (d, J = 2.2 Hz, 1H), 8.06 (dd, J = 8.3, 1.8 Hz, 1H), 7.92 (d, J = 8.3 Hz, 1H). **^{13}C NMR (101 MHz, DMSO) δ**: 191.10, 165.89, 135.59, 134.69, 133.36, 130.94, 130.58, 130.14.

Compound 39: 2.69 mmol (0.615 g) of compound **38** was dissolved in 10 mL of THF, cooled to 0 °C, 4.15 mmol (0.157 g) of $NaBH_4$ was added and the reaction mixture was stirred for 2 hours. Reaction completion was checked by TLC (Rf = 0.8, 10% MeOH in EtOAc). The reaction mixture was poured into a mixture of 50 mL of EtOAc and 50 mL 1M HCl (aq.), while vigorously stirring. The organic layer was collected, dried using $MgSO_4$, filtered and concentrated using rotary evaporation resulting in 0.573 g (248 mmol, 92.4%) of compound **39** as a white solid. **^{1}H NMR (300 MHz, DMSO) δ**: 8.109 (s, 1H), 7.8-7.6 (m, 2H), 5.605 (br, 1H), 4.607 (s, 1H). **^{13}C NMR (75 MHz, DMSO) δ:** 166.91, 141.64, 132.39, 130.14, 129.21, 128.65, 125.69, 62.37.

Compound 40: 8.3 mmol (1.0 g) of anhydrous $MgSO_4$ was suspended in 10 mL dry DCM and stirred for 15 minutes. Then 0.10 mL of H_2SO_4 was added and the mixture was stirred for 15 minutes. To the solution 0.498 mmol (0.115 g) of compound **39** and 1.0 mL of tBuOH were added, the vessel was sealed air-tight and the reaction mixture was stirred for 4 days at room temperature. The formed white suspension was quenched by pouring it into 50 mL sat. $NaHCO_3$ (aq.) while vigorously stirring. To obtain the crude product, and tert-butyl ester intermediate, the watery solution was extracted with 100 mL of EtOAc. To recover the remaining starting-material and tert-butyl ether intermediate, the water layer was acidified using 1M HCl (aq.) and extracted with EtOAc. The crude product fraction was dried using

$MgSO_4$, filtered and concentrated using evaporation. Purification was performed by silica column chromatography using an 2%-12% EtOAc in pentane eluent resulting in 0.102 g (0.297 mmol, 59.6%) of compound **40** (TLC Rf = 0.6 in 2% EtOAc in pentane)**.** The tert-butyl ester intermediate (TLC Rf = 0.1 in 2% EtOAc in pentane) was also collected and combined with crude starting material and tert-butyl ether intermediate to be re-used. **^{1}H NMR (400 MHz, $CDCl_3$) δ:** 8.14 (s, 1H), 7.71 (dd, *J* = 8.2, 1.9 Hz, 1H), 7.54 (d, *J* = 8.3 Hz, 1H), 4.50 (s, 3H), 1.58 (s, 9H), 1.32 (s, 9H). **^{13}C NMR (101 MHz, $CDCl_3$) δ**: 165.37, 139.44, 132.31, 131.38, 130.08, 129.30, 127.24, 81.38, 74.10, 63.50, 28.28, 27.75.

Compound 41: 300 mmol (25 mL) formaldehyde was dissolved in 250 mL THF, cooled to 0 °C and 60 mL of H_2SO_4 was added to the solution. Then, dropwise over 10 minutes, 100 mmol (11 mL, 17.2 g) of 3-bromo aniline was added and the reaction mixture was stirred for an additional 10 minutes. Reaction completion was checked by TLC (Intermediate, Rf = 0.8, 25% EtOAc in pentane). 400 mmol (15 g) of $NaBH_4$ was portion wise added over a period of 30 minutes, then the reaction mixture was stirred for 1 hour while warming to room temperature. Reaction completion was checked by TLC (Rf = 0.9, 25% EtOAc in pentane). The reaction was quenched by addition of 400 mL sat. $NaHCO_3$ (aq.), THF present in the solution was removed using rotary evaporation, and the remaining watery solution was extracted twice with 500 mL $CHCl_3$. The organic layers were combined, washed with sat. $NaHCO_3$ (aq.) dried using $MgSO_4$, filtered and concentrated using rotary evaporation. Purification was performed by silica column chromatography using an 0%-4% EtOAc in pentane eluent resulting in 19.52 g (98.42 mmol, 98.4%) of compound **41** as a pale oil**. ^{1}H NMR (500 MHz, $CDCl_3$) δ**: 7.08 (t, *J* = 8.0 Hz, 1H), 6.86 – 6.80 (m, 2H), 6.63 (dd, *J* = 8.6, 2.3 Hz, 1H), 2.95 (s, 6H).

Compound 42: 88.85 mmol (17.77 g) of compound **41** was dissolved in 170 mL AcOH and 44 mmol (1.332 g, 3.6 mL of a 37% wt. solution) formaldehyde was added dropwise over 10 minutes. The reaction mixture was stirred at 80 °C for 1.5 hours, cooled to room temperature, and the reaction mixture was concentrated using rotary evaporation. The residue was suspended in 300 mL of sat. $NaHCO_3$ (aq.) and extracted three times with EtOAc (500, 100, 100 mL). The organic layers were combined, dried using $MgSO_4$ and concentrated using rotary evaporation. Purification was performed by silica column chromatography using an 2%-10% EtOAc in pentane eluent resulting in 12.84 g (31.16 mmol, 70.1%) of compound **42** as a white solid. **^{1}H NMR (500 MHz, $CDCl_3$) δ:** 6.99 (d, *J* = 2.1 Hz, 2H), 6.90 (d, *J* = 8.5 Hz, 2H), 6.62 (dd, *J* = 8.5, 2.1 Hz, 2H), 4.06 (s, 2H), 2.95 (s, 12H). **^{1}H NMR (300 MHz, $CDCl_3$) δ:** 6.97 (d, 2H), 6.88 (d, 2H), 6.61 (dd, 2H), 4.03 (s, 2H), 2.93 (s, 12H). **^{13}C NMR (75 MHz, $CDCl_3$) δ**: 150.09, 130.85, 127.12, 125.69, 116.30, 111.9340.62, 39.96.

Compound 43: 1.45 mmol (0.597 g) of compound **42** was co-evaporated in dry dioxane, dissolved in 58 mL dry THF and cooled to -78 °C. 3.1 mL *sec*-butyl lithium was added dropwise to the solution over 10 minutes and the reaction mixture was stirred for another 30 minutes. 2.6 mmol (0.32 mL) of di-chloro-di-methyl silane was added and the reaction mixture was stirred for 2 hours while warming to room temperature. The resulting dark-green solution was quenched by the addition of 2 mL 1M HCl (aq.), resulting in the formation of a black precipitate, which dissolved after the addition of 50 mL H_2O. THF present in the solution was removed using rotary evaporation, and the remaining watery solution was adjusted to pH = 10 by adding 100 mL of sat. $NaHCO_3$ (aq.). The resulting dark blue solution was extracted twice with

100 mL of EtOAc, obtaining a colorless water layer and yellow organic layers. Formation of the intermediate was checked by TLC (TEA neutralized TLC plate, Rf = 0.8, 10% EtOAc in pentane). The organic layer was dried using Na_2SO_4, filtered and evaporated using rotary evaporation, resulting in a dark green oil containing crude intermediate. The crude intermediate was directly used by dissolving it in 10 mL dry acetone, and cooling to -15°C using an iced salt bath. 600 mg of $KMnO_4$ was added in portions of 100 mg over a time period of 45 minutes to the solution and the reaction mixture was stirred for another 75 minutes. The reaction mixture was then diluted with dry acetone, filtered using filter paper, filtered using a pad of celite and concentrated using rotary evaporation into a black slurry. Purification was performed by silica column chromatography using 10%-40% EtOAc in pentane eluent resulting in 0.247 g (0.762 mmol, 52.6%) of compound **43** as a yellow (slightly green) solid. **^{1}H NMR (400 MHz, $CDCl_3$) δ**: 8.40 (d, *J* = 9.0 Hz, 1H), 6.84 (dd, *J* = 9.0, 2.8 Hz, 1H), 6.79 (d, *J* = 2.8 Hz, 1H), 3.09 (s, 6H), 0.47 (s, 2H). **^{13}C NMR (101 MHz, $CDCl_3$) δ:** 185.39, 151.55, 140.59, 131.70, 129.75, 114.35, 113.24, 40.13, -0.88.

Compound 44: 134 µmol (46 mg) of compound **40** was co-evaporated with dry dioxane, dissolved in 2.0 mL of dry THF and cooled to -78 °C. 170 µmol (0.10 mL of a 1.7M solution in pentane) tert-butyl lithium was cooled to -78 °C and added dropwise over 5 minutes. The reaction mixture was stirred for 15 minutes at -78 °C. Then, 32 µmol (10.5 mg) of compound **43** was co-evaporated with dry dioxane, dissolved in 1.0 mL of dry THF, cooled to -78 °C and dropwise added to the reaction mixture over 1 minute. The reaction mixture was allowed to stir for 2 hours while warming to room temperature. The reaction was quenched by the slow addition of 0.5 mL of 1M HCl (aq.), neutralized using 10 mL of sat. $NaHCO_3$ (aq.), and the watery solution was extracted using 10 mL of EtOAc. The organic layer was concentrated using rotary evaporation resulting a residue containing the tert-butylated crude intermediate. The residue was dissolved in 1 mL of TFA and stirred for 2 days at room temperature. The resulting suspension was filtered using a syringe filter, concentrated using rotary evaporation and purified using C_{18}-reverse phase HPLC to obtain 1.3 mg (2.8 µmol, 8.8%) of compound **44** including a small amount of impurities. **LC-MS analysis:** (Alltima C_{18} analytical column, linear gradient in 12.5 minutes; H_2O: 80 → 0% ; ACN: 10 → 90% ; aq. 0.5% TFA: 10%) **LC:** Rt: 5.18 min **MS:** $[C_{27}H_{31}N_2O_3Si]^+$: found 459.40, calculated 459.21.

Compound 44 (repurification method): Crude and semi-purified material from several reaction were combined to a single flask. Purification was performed by silica column chromatography using an 0.5%-2% H_2O in ACN eluent containing 1% AcOH resulting in 4 mg (8.7 µmol) of compound **44** as a colorless oil/solid**. ^{1}H NMR (600 MHz, MeOD) δ**: 8.02 (s, 1H), 7.85 (d, *J* = 7.8 Hz, 1H), 7.01 (d, *J* = 8.9 Hz, 2H), 6.98 (d, *J* = 2.8 Hz, 2H), 6.88 (d, *J* = 8.0 Hz, 1H), 6.71 (dd, *J* = 8.9, 2.9 Hz, 2H), 5.40 (s, 2H), 2.93 (s, 11H), 0.59 (s, 3H), 0.49 (s, 3H). **^{13}C NMR (151 MHz, MeOD) δ:** 170.24, 153.59, 150.54, 139.70, 139.38, 135.51, 132.12, 130.32, 129.99, 124.60, 124.04, 117.57, 115.70, 94.17, 73.71, 40.82, 0.24, -0.30.

Compound 45: In an Eppendorf tube 8.7 µmol (4 mg) of compound **44** was dissolved in 40 µL of DMF. Then, 16 µmol (2 mg) of N-hydroxy succinimide and 16 µmol (3 mg) of EDC hydrochloride salt were added and the reaction mixture was thoroughly mixed using a micropipette and left to react for one hour at room temperature. No reaction was observed, so over the course of several hours 16 µmol of TEA, 16 µmol of EDC hydrochloride salt and 16

μmol of N-hydroxy succinimide were added and the reaction mixture was left to react overnight at room temperature. Reaction completion was checked by TLC tracking the consumption of starting material (Rf = 0.4, 10% MeOH in DCM) and the formation of succinimide ester intermediate (Rf = 0.9, 10% MeOH in DCM).

To the reaction mixture 29 μmol (4 mg) of compound **10 (chapter 3)** and 25 μmol (3.5 μL of TEA were added and the reaction mixture was thoroughly mixed using a micropipette and left to react for two hours at room temperature. Reaction completion was checked by TLC tracking the consumption of succinimide ester intermediate (Rf = 0.60, 75% EtOAc in pentane) and the formation of product (Rf = 0.40, 75% EtOAc in pentane). 2 ml of EtOAc was added to the solution and the organic mixture was washed twice with 0.5 mL sat. $NaHCO_3$ (aq.) and concentrated using rotary evaporation. Purification was performed by silica column chromatography using an 0.5%-2% H_2O in ACN eluent resulting in compound **45** as a colorless oil/solid**.**

Compound 46: In an Eppendorf tube 2 μmol (1 mg) of compound **44** was dissolved in 10 μL of DMF. Then, 4 μmol (10 μL of a 0.4M solution in DMF) of TEA, 8 μmol (10 μL of a 0.8M solution in DMF) of N-hydroxy succinimide, 1.5 mg of EDC hydrochloride salt and an additional 10 μL of DMF were added and the reaction mixture was thoroughly mixed using a micropipette and left to react for two hours at room temperature. Reaction completion was checked by TLC tracking the consumption of starting material (Rf = 0.4, 10% MeOH in DCM) and the formation of succinimide ester intermediate (Rf = 0.9, 10% MeOH in DCM). LC-MS analysis: (Alltima C_{18} analytical column, linear gradient in 12.5 minutes; H_2O: 80 → 0% ; ACN: 10 → 90% ; aq. 0.5% TFA: 10%) **LC:** Rt: 5.77 **MS:** $[C_{31}H_{34}N_3O_5Si]^+$: found 556.3, calculated 556.2.

To the reaction mixture 4 μmol (1 mg) of compound **1 (chapter 3)** and 8 μmol (20 μL of a 0.4M solution in DMF) of TEA were added and the reaction mixture was thoroughly mixed using a micropipette and left to react for one hour at room temperature. Reaction completion was checked by TLC tracking the consumption of succinimide ester intermediate (Rf = 0.60, 75% EtOAc in pentane) and the formation of product (Rf = 0.65, 75% EtOAc in pentane). 0.5 mL of sat. $NaHCO_3$ (aq.) was added to the solution and it was extracted with 0.5 mL of EtOAc. The organic layer was washed twice with 0.5 mL sat. $NaHCO_3$ (aq.) and concentrated using rotary evaporation. The crude product was then purified using C_{18}-reverse phase HPLC to obtain compound **46.** LC-MS analysis: (Alltima C_{18} analytical column, linear gradient in 12.5 minutes; H_2O: 80 → 0% ; ACN: 10 → 90% ; aq. 0.5% TFA: 10%) **LC:** Rt: 6.01 **MS:** $[C_{36}H_{38}N_7O_2Si]^+$: found 628.2, calculated 628.3.

Benzyl vinyl ether 47: 202 mmol (21 mL) of benzyl alcohol was dissolved in 998 mmol (96 mL) vinyl ethyl ether and cooled down to 0 °C. 20 mmol (9 mg) $Hg(TFA)_2$ was added and the reaction mixture stirred for 2 hours while warming to room temperature. Reaction completion was checked by TLC (Rf = 0.4 in 100% Pentane). Et_2O was added to the reaction mixture and the organic layer was washed four times with $NaHCO_3$, dried with $MgSO_4$, filtered and concentrated using rotary evaporation. Silica chromatography was performed 0%-4% Et_2O in pentane eluent resulting in 150 mmol (20.2 g, 75.2%) of the benzyl vinyl ether **47** as a colorless oil. **^{1}H NMR (400 MHz, $CDCl_3$) δ:** 7.51 – 7.33 (m, 5H), 6.65 (dd, J = 14.3, 6.8 Hz, 1H), 4.82 (s,

2H), 4.38 (dd, J = 14.3, 2.1 Hz, 1H), 4.16 (dd, J = 6.8, 2.2 Hz, 1H). **^{13}C NMR (101 MHz, $CDCl_3$) δ:** 151.73, 136.96, 128.59, 128.00, 127.64, 127.53, 87.41, 77.48, 77.16, 76.84, 70.11.

Spiroheptane 48: 19.5 mmol (2.2 mL, 2.30 g) cyclobutanecarbonyl chloride and 35.3 mmol (4.73 g) benzyl vinyl ether **47** were dissolved in 18 mL dry ACN. 21.58 mmol (3 ml) TEA was added and the mixture was refluxed (90 °C) for 3 hours. Reaction completion was checked by TLC (Rf = 0.35 in 5% EtOAc/Pentane). 32 mL ACN was added and the reaction mixture was filtered, concentrated using rotary evaporation, redissolved in Et_2O, washed with 1M HCl (aq.), washed with brine, dried with $MgSO_4$, filtered, and concentrated using rotary evaporation. Purification was performed by silica column chromatography using an 1%-4% EtOAc in pentane eluent resulting in 11.6 mmol (2.50 g, 65.6%) of the spiroheptane **48** as a colorless oil. **^{1}H NMR (400 MHz, $CDCl_3$) δ:** 7.45 – 7.20 (m, 5H), 4.78 – 4.42 (m, 2H), 4.06 (dd, J = 6.7, 5.5 Hz, 1H), 3.06 (dd, J = 17.6, 6.8 Hz, 1H), 2.92 (dd, J = 17.5, 5.5 Hz, 1H), 2.58 (dtd, J = 11.0, 8.9, 8.4, 1.9 Hz, 1H), 2.43 (t, J = 8.0 Hz, 2H), 2.37 – 2.28 (m, 1H), 2.24 – 2.15 (m, 1H), 2.11 – 1.93 (m, 3H), 1.93 – 1.82 (m, 1H). **^{13}C NMR (101 MHz, $CDCl_3$) δ:** 210.79, 137.87, 128.61, 127.97, 127.72, 77.48, 77.16, 76.84, 71.89, 71.46, 66.64, 50.35, 27.78, 24.45, 16.66.

Spiroheptane 49: 3.72 mmol (805 mg) of spiroheptane **48** was dissolved in 17 mL dry MeOH and cooled to 0°C. 11.3 mmol (429 mg) of $NaBH_4$ was added and the reaction mixture was stirred for 3 hours while warming to room temperature. Reaction completion was checked by TLC (Rf = 0.4 in 25 % EtOAc/Pentane). The reaction was quenched by the slow addition of 85 mL water, and extracted twice with EtOAc. The organic layers were combined, washed with water, washed with brine, dried with $MgSO_4$, filtered and concentrated using rotary evaporation. Purification was performed by silica column chromatography using an 10%-25% EtOAc in pentane eluent resulting in 2.55 mmol (556 mg, 68.9%) of the spiroheptane **49** as a colorless oil. **^{1}H NMR (400 MHz, $CDCl_3$) δ:** 7.44 – 7.11 (m, 5H), 4.63 – 4.43 (m, 2H), 3.60 (t, J = 7.7 Hz, 1H), 3.41 (dd, J = 8.1, 6.6 Hz, 1H), 2.51 (dt, J = 11.3, 6.8 Hz, 1H), 2.39 – 2.23 (m, 1H), 2.17 (ddd, J = 11.6, 8.9, 6.2 Hz, 1H), 2.04 (t, J = 7.5 Hz, 2H), 1.98 – 1.78 (m, 2H), 1.53 (dt, J = 11.1, 8.2 Hz, 1H). **^{13}C NMR (101 MHz, $CDCl_3$) δ:** 138.57, 128.48, 127.68, 77.48, 77.16, 76.84, 72.19, 71.07, 66.74, 54.11, 36.72, 30.50, 19.18, 17.39.

Spiroheptane 50: 3.02 mmol (659 mg) of spiroheptane **49** was co-evaporated with dioxane and dissolved in 3 mL dry pyridine. 6.02 mmol (1.15 g) of *p*-toluenesulfonyl chloride was added and the reaction mixture was stirred for 3 hours at room temperature. Reaction completion was checked by TLC (Rf = 0.75 in 25% EtOAc/Pentane). 80 mL of 0.1M HCl (aq.) was added to the reaction mixture and extracted twice with Et_2O. The organic layers were combined, washed twice with 0.1M HCl (aq.), washed with water, washed with brine, dried with $MgSO_4$, filtered and concentrated using rotary evaporation. Purification was performed by silica column chromatography using an 1%-40% Et_2O in pentane eluent resulting in 2.38 mmol (886 mg, 78.9%) of the spiroheptane **50** as a white crystalline solid. **^{1}H NMR (400 MHz, $CDCl_3$) δ:** 7.80 (d, J = 8.4 Hz, 2H), 7.52 – 6.68 (m, 6H), 4.50 (d, J = 10.2 Hz, 2H), 4.19 (dd, J = 8.4, 7.0 Hz, 1H), 3.38 (dd, J = 8.3, 6.5 Hz, 1H), 2.45 (s, 3H), 2.44 – 2.18 (m, 3H), 2.05 – 1.89 (m, 2H), 1.89 – 1.60 (m, 3H). **^{13}C NMR (101 MHz, $CDCl_3$) δ:** 144.87, 138.13, 134.08, 129.92, 128.52, 127.93, 127.85, 127.68, 74.02, 71.77, 71.31, 53.83, 34.49, 29.95, 21.76, 20.31, 17.02.

Compound 51: 0.752 mmol (280 mg) of spiroheptane **50** was co-evaporated with dioxane and dissolved in 10 mL dry DMSO. 1.13 mmol (127 mg) KOtBu was pre-suspended in 5 mL dry DMSO and added to the solution. The reaction mixture was stirred for 5 hours at room temperature. Reaction completion was checked by TLC (Rf = 0.9 in 5% EtOAc/Pentane). 50 mL 1M HCl and Et_2O were added to the reaction mixture. The organic layer was washed twice with 1M HCl (aq.), washed three times with water, washed once with brine, dried with $MgSO_4$, filtered and concentrated using rotary evaporation. Purification was performed by silica column chromatography using an 0%-5% Et_2O in pentane eluent resulting in 0.18 mmol (37 mg, 24.3%) of the spiroheptene **51** as a colorless oil. **^{1}H NMR (400 MHz, $CDCl_3$) δ:** 7.59 – 7.13 (m, 5H), 6.34 (dd, J = 2.8, 1.2 Hz, 1H), 6.12 (dd, J = 2.8, 0.6 Hz, 1H), 4.88 – 4.46 (m, 2H), 4.22 (t, J = 0.8 Hz, 1H), 2.58 – 2.34 (m, 1H), 2.13 (dddd, J = 12.7, 9.0, 5.1, 2.1 Hz, 3H), 1.97 – 1.79 (m, 2H). **^{13}C NMR (101 MHz, $CDCl_3$) δ:** 145.36, 135.47, 128.50, 127.78, 127.65, 83.91, 77.48, 77.16, 76.84, 71.11, 30.19, 28.03, 16.56.

Compound 52: 0.268 mmol (100 mg) of spiroheptane **50** was dissolved in 8 mL MeOH and degassed using sonication under a nitrogen atmosphere for 30 min. 10 mg Pd/C was added to the solution, the mixture was flushed with hydrogen and stirred overnight at room temperature under a hydrogen atmosphere. Reaction completion was checked by TLC (Rf = 0.8 in 25% EtOAc/Pentane). The reaction mixture was concentrated using rotary evaporation resulting in 0.24 mmol (67 mg, 88%) of the spiroheptane **52** as colorless oil. **^{1}H NMR (400 MHz, $CDCl_3$) δ:** 7.77 (d, J = 8.3 Hz, 2H), 7.38 – 7.29 (m, 2H), 4.15 (dd, J = 8.2, 6.9 Hz, 1H), 3.60 (dd, J = 8.3, 6.8 Hz, 1H), 2.41 (d, J = 14.4 Hz, 4H), 2.31 (s, 1H), 2.19 (dd, J = 8.3, 7.1 Hz, 2H), 1.95 – 1.83 (m, 2H), 1.80 – 1.60 (m, 3H). **^{13}C NMR (101 MHz, $CDCl_3$) δ:** 144.99, 133.80, 129.95, 127.87, 77.48, 77.16, 76.84, 73.80, 66.01, 54.57, 36.72, 29.50, 21.73, 19.82, 16.91.

Compound 53a: 0.12 mmol (34 mg) of spiroheptane **52** was co-evaporated with dioxane and dissolved in 1 mL dry ACN. 0.26 mmol (67 mg) of N-N'-disuccinimidyl carbonate and 0.18 mmol (30 µl, 22 mg) DIPEA were added and the reaction mixture was stirred overnight at room temperature. Reaction completion was checked by TLC (Rf = 0.15 in 25% EtOAc/Pentane). The mixture was concentrated using rotary evaporation, re-dissolved in Et_2O, washed three times with 0.1 M HCl (aq.), washed once with sat. $NaHCO_3$ (aq.), washed once with brine, dried with $MgSO_4$, filtered and concentrated using rotary evaporation. Purification was performed by silica column chromatography using an 25%-60% EtOAc in pentane eluent resulting in 0.073 mmol (31 mg, 61.9%) of the spiroheptane **53a** as a colorless oil. **^{1}H NMR (400 MHz, $CDCl_3$) δ:** 7.80 (d, J = 8.0 Hz, 2H), 7.35 (d, J = 8.0 Hz, 2H), 4.50 (t, J = 7.5 Hz, 1H), 4.29 (t, J = 7.5 Hz, 1H), 2.83 (s, 4H), 2.57 (dt, J = 12.2, 7.0 Hz, 1H), 2.45 (s, 3H), 2.41 – 2.30 (m, 1H), 2.25 (dd, J = 11.4, 5.3 Hz, 1H), 2.16 – 1.93 (m, 3H), 1.79 (td, J = 9.1, 6.5 Hz, 2H). **^{13}C NMR (101 MHz, $CDCl_3$) δ:** 168.64, 151.07, 145.29, 133.61, 130.10, 127.94, 77.48, 77.16, 76.84, 74.11, 72.79, 53.71, 33.67, 29.58, 25.65, 25.56, 21.80, 21.02, 16.69.

Compound 53b: 0.14 mmol (60 mg) of spiroheptane **53a** was dissolved in 4.3 mL dry DCM. 0.17 mmol (45 mg) Boc-Lys-OMe acetate salt and 0.32 mmol (44 µl, 32 mg) TEA were added and the reaction mixture was stirred for 4 hours at room temperature. Reaction completion was checked by TLC (Rf = 0.75 in 50% EtOAc/Pentane). 10 mL of 0.1M HCl was added to the reaction mixture and extracted with DCM. The organic layer was washed twice with 0.1M HCl (aq.), washed once with brine, dried with $MgSO_4$, filtered and concentrated using rotary

evaporation. Purification was performed by silica column chromatography using an 10%-50% EtOAc in pentane eluent resulting in 0.069 mmol (39 mg, 48.2%) of the spiroheptane **53b** as a colorless oil. **^{1}H NMR (500 MHz, $CDCl_3$) δ:** 7.81 – 7.76 (m, 2H), 7.34 (d, J = 8.1 Hz, 2H), 5.07 (d, J = 8.8 Hz, 1H), 4.88 (s, 1H), 4.39 (td, J = 7.7, 4.8 Hz, 1H), 4.32 – 4.13 (m, 2H), 3.71 (s, 3H), 3.21 – 3.08 (m, 2H), 2.44 (s, 4H), 2.25 (ddt, J = 12.4, 9.0, 4.5 Hz, 1H), 2.15 – 2.05 (m, 2H), 1.94 – 1.85 (m, 1H), 1.84 – 1.68 (m, 4H), 1.66 – 1.57 (m, 1H), 1.56 – 1.46 (m, 2H), 1.42 (d, J = 1.4 Hz, 9H), 1.40 – 1.28 (m, 2H). **^{13}C NMR (126 MHz, $CDCl_3$) δ:** 173.32, 155.96, 145.02, 133.84, 129.99, 127.96, 127.87, 80.05, 77.41, 77.16, 76.91, 74.05, 67.47, 53.77, 53.75, 53.21, 52.41, 40.72, 34.09, 32.56, 29.61, 29.35, 28.42, 22.51, 21.77, 20.87, 16.84.

Compound 53c: 0.354 mmol (100 mg) of spiroheptane **52** was dissolved in 1.8 mL DCM and cooled to 0°C. 0.37 mmol (77 mg) of N,N'-Dicyclohexylcarbodiimide (DCC), 0.086 mmol (11 mg) of DMAP and 0.60 mmol (62 µL, 70 mg) levulinic acid were added and the reaction mixture stirred for 3 hours while warming to room temperature. Reaction completion was checked by TLC (Rf = 0.7 in 50% EtOAc/Pentane). The reaction mixtutre was washed with 30 sat. $NaHCO_3$ and the water layer was extracted with DCM and EtOAc. The organic layers were combined, dried with $MgSO_4$, filtered and concentrated using rotary evaporation. Purification was performed by silica column chromatography using an 10%-40% EtOAc in pentane eluent resulting in 0.270 mmol (103 mg, 76.3%) of the spiroheptane **53c** as a colorless oil. **^{1}H NMR (400 MHz, $CDCl_3$) δ:** 7.81 – 7.71 (m, 2H), 7.40 – 7.27 (m, 2H), 4.41 (dd, J = 8.3, 7.0 Hz, 1H), 4.23 (dd, J = 8.2, 7.0 Hz, 1H), 2.72 (td, J = 6.4, 1.6 Hz, 2H), 2.60 – 2.52 (m, 2H), 2.49 – 2.39 (m, 4H), 2.30 – 2.20 (m, 1H), 2.14 (s, 3H), 2.12 – 2.01 (m, 2H), 1.86 (ddt, J = 16.5, 12.2, 8.0 Hz, 2H), 1.70 (q, J = 7.7 Hz, 2H). **^{13}C NMR (101 MHz, $CDCl_3$) δ:** 206.45, 172.31, 145.02, 133.69, 129.95, 127.84, 127.80, 77.48, 77.16, 76.84, 73.85, 67.66, 53.58, 37.80, 33.70, 29.83, 29.57, 27.69, 21.69, 20.89, 16.67.

Compound 53f: 1.12 mmol (316 mg) of spiroheptane **52** was dissolved in DCM and cooled to 0°C. 0.571 mmol (144 mg) pyridinium *p*-toluenesulfonate (PPTS) was added, 6.71 mmol (642 µL, 484 mg) of vinyl ethyl ether was added dropwise over 1 min and the reaction mixture was stirred at 0°C for 1 hour. Reaction completion was checked by TLC (Rf = 0.85 in 25% EtOAc/Pentane). The reaction mixture was washed three times with sat. $NaHCO_3$, washed once with brine, dried with $MgSO_4$, filtered and concentrated using rotary evaporation. This resulted in 0.958 mmol (339 mg, 85.6%) of the spiroheptane **53f** as a colorless oil. **^{1}H NMR (400 MHz, $CDCl_3$) δ:** 8.01 – 7.62 (m, 2H), 7.34 (d, J = 8.0 Hz, 2H), 4.69 (dt, J = 31.8, 5.3 Hz, 1H), 4.19 (ddd, J = 8.6, 6.9, 1.5 Hz, 1H), 3.76 – 3.27 (m, 3H), 2.47 – 2.42 (m, 3H), 2.40 – 2.18 (m, 2H), 1.94 (dtd, J = 15.7, 8.4, 7.9, 4.0 Hz, 2H), 1.85 – 1.68 (m, 3H), 1.27 (dd, J = 5.3, 3.3 Hz, 3H), 1.16 (td, J = 7.0, 2.7 Hz, 3H). **^{13}C NMR (101 MHz, $CDCl_3$) δ:** 144.87, 134.11, 129.94, 127.96, 99.35, 98.77, 77.48, 77.16, 76.84, 74.28, 74.24, 67.21, 66.85, 61.33, 60.94, 54.12, 36.08, 35.23, 29.92, 29.46, 21.78, 20.55, 20.44, 17.03, 16.93, 15.33.

Compound 54: 0.958 mmol (339 mg) spiroheptane **53f** was co-evaporated with dioxane and dissolved in 19 mL dry DMSO. 1.43 mmol (160 mg) KOtBu was added and the reaction mixture was stirred for 3 hours at room temperature. Reaction completion was checked by TLC (Rf =0.8 in 5% EtOAc/Pentane). 100 mL of sat. NH_4Cl (as.) was added to the reaction mixture and extracted twice with Et_2O. The organic layers were combined, washed twice with sat. NH_4Cl (aq.), washed five times with water, washed once with brine, dried with $MgSO_4$, filtered and

concentrated using rotary evaporation. This resulted in 0.844 mmol (154 mg, 88.1%) of spiroheptene **54** as a pale yellow oil. **^{1}H NMR (500 MHz, $CDCl_3$) δ:** 6.31 (ddd, J = 15.2, 2.8, 1.1 Hz, 1H), 6.08 (ddd, J = 12.5, 2.8, 0.6 Hz, 1H), 4.96 – 4.63 (m, 1H), 4.34 (dt, J = 13.5, 0.8 Hz, 1H), 3.94 – 3.02 (m, 2H), 2.41 – 2.24 (m, 1H), 2.17 – 1.96 (m, 3H), 1.88 – 1.71 (m, 2H), 1.37 (dd, J = 9.0, 5.3 Hz, 3H), 1.22 (t, J = 7.1 Hz, 3H). **^{13}C NMR (126 MHz, $CDCl_3$) δ:** 145.26, 145.11, 136.34, 135.83, 99.38, 98.81, 79.83, 78.87, 77.41, 77.16, 76.91, 61.39, 60.51, 56.90, 56.75, 30.36, 30.07, 28.06, 27.98, 20.81, 20.80, 16.41, 16.17, 15.49, 15.48.

Compound 56: 0.685 mmol (125 mg) of spiroheptene **54** was dissolved in 1.4 mL ACN and cooled to 0°C. 1.4 mL 2M HCl (aq.) was added and the reaction mixture stirred for 35 min at 0°C. Reaction completion was checked by TLC (Rf = 0.25 in 5% EtOAc/Pentane). 50 mL of sat. $NaHCO_3$ was added and extracted twice with Et_2O. The organic layers were combined, washed twice with sat. $NaHCO_3$ (aq.), washed once with brine, dried with $MgSO_4$, filtered and concentrated using rotary evaporation. This resulted in 0.65 mmol (72 mg) of the spiroheptene **55** intermediate as a colorless oil. Spiroheptene **55** was dissolved in 5.5 mL dry ACN, 1.37 mmol (239 µl, 177 mg) DIPEA and 1.37 mmol (351 mg) N-N'-disuccinimidyl carbonate were added, and the reaction mixture was stirred overnight at room temperature. Reaction completion was checked by TLC (Rf = 0.25 in 10% EtOAc/Pentane). The mixture was concentrated using rotary evaporation, re-dissolved in Et_2O, washed three times with sat. $NaHCO_3$ (aq.), washed three times with 0.1 M HCl (aq.), washed once with brine, dried with $MgSO_4$, filtered and concentrated using rotary evaporation. Purification was performed by silica column chromatography using an 10%-100% Et_2O in pentane eluent, resulting in 0.34 mmol (85 mg, 49%, over two steps) of spiroheptene **56** as a colorless oil. **^{1}H NMR (400 MHz, $CDCl_3$) δ:** 6.49 (dd, J = 2.8, 1.5 Hz, 1H), 6.06 (d, J = 2.7 Hz, 1H), 5.17 (d, J = 1.5 Hz, 1H), 2.83 (s, 4H), 2.36 – 2.18 (m, 2H), 2.18 – 2.04 (m, 2H), 1.94 – 1.71 (m, 2H). **^{13}C NMR (101 MHz, $CDCl_3$) δ:** 168.85, 151.25, 148.10, 132.24, 83.79, 77.48, 77.16, 76.84, 56.27, 29.78, 28.12, 25.58, 16.03.

Compound 57: 0.080 mmol (20 mg) of spiroheptene **56** was dissolved in 2.5 mL dry DCM, 0.100 mmol (26 mg) Boc-Lys-OMe acetate salt and 0.180 mmol (25 µL) TEA were added and the reaction mixture was stirred for 45 minutes at room temperature. Reaction completion was checked by TLC (Rf = 0.8 in 5% MeOH/DCM). The reaction was quenched with 50 mL sat. NH_4Cl (aq.) and extracted twice with DCM. The organic layers were combined, washed twice with sat. NH_4Cl (aq.), washed three times with sat. $NaHCO_3$ (aq.), washed with brine, dried with $MgSO_4$, filtered and concentrated using rotary evaporation. Silica chromatography was performed using 0.25%-1% MeOH in DCM eluent, resulting in 0.025 mmol (10 mg, 31.5%) of the spiroheptene **57** as a colorless oil. **^{1}H NMR (500 MHz, $CDCl_3$) δ:** 6.38 (dd, J = 2.8, 1.4 Hz, 1H), 6.04 (d, J = 2.7 Hz, 1H), 5.12 (d, J = 4.1 Hz, 1H), 5.06 (d, J = 10.4 Hz, 1H), 4.80 (s, 1H), 4.29 (d, J = 7.7 Hz, 1H), 3.73 (s, 3H), 3.20 (q, J = 6.7 Hz, 2H), 2.33 – 2.22 (m, 1H), 2.18 – 2.09 (m, 1H), 2.11 – 1.98 (m, 2H), 1.93 – 1.72 (m, 3H), 1.65 (s, 1H), 1.53 (dt, J = 13.2, 5.9 Hz, 2H), 1.43 (s, 9H), 1.38 (d, J = 8.9 Hz, 2H). **^{13}C NMR (126 MHz, $CDCl_3$) δ**: 173.41, 156.45, 146.55, 133.97, 80.09, 78.41, 77.42, 77.17, 76.91, 56.53, 53.33, 52.44, 40.69, 40.61, 32.56, 32.48, 30.00, 29.61, 28.47, 28.15, 22.54, 16.19.

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Nederlandse samenvatting

Dit proefschrift beschrijft de synthese, analyse van verschillende tetrazine-houdende verbindingen en het gebruik in de bioorthogonale chemie. In **hoofdstuk 1** wordt een literatuuroverzicht gegeven van de ontwikkelingen op het gebied van de tetrazine chemie door de geschiedenis heen. Daarnaast wordt een inleiding gegeven tot de opkomst van click chemie, bioorthogonale reacties en IEDDA chemie. Deze ontwikkelingen vormen de basis waarop het in dit proefschrift beschreven werk is gebouwd.

Hoofdstuk 2 beschrijft de synthese van tetrazine-gemodificeerde alanine bouwstenen als gefunctionaliseerde aminozuren ter vervanging van natuurlijke aminozuren in activity-based probes (ABP's) voor bioorthogonale activity-based protein profiling (ABPP) van cysteïne-, serine- en threonine-peptidasen.[1][2] Eén bouwsteen, Cbz-Tzm-OH (verbinding **2**, hoofdstuk 2), bleek gemakkelijk te verkrijgen via de ontwikkelde syntheseroute en kon worden opgenomen in kleine oligopeptide chloormethylketonen (CMK)[3, 4] en benzyloxymethylketonen (BOMK)[5, 6] om zo structuren te leveren die nauw verwant zijn aan bekende peptidase remmers. CMK-remmer **1** (**Figuur 1**) kon gemakkelijk worden bereid en werd met succes in bioorthogonaal ABPP gebruikt. Helaas was de CMK-groep te reactief en resulteerde deze in de modificatie van talrijke niet-gespecificeerde eiwitten wanneer deze op cel-lysaten werd toegepast. BOMK-remmer **2** (**Figuur 1**) werd ook gemakkelijk bereid, maar leverde een slechte eiwitmodificatie op. **Hoofdstuk 2** beschrijft ook de synthese van Boc-Tzm-OH (verbinding **12**, hoofdstuk 2) als bouwsteen voor ABPs. Na bevestiging van een kernkop aan het C-uiteinde waren alle pogingen om de Boc-groep te verwijderen echter niet succesvol.

Hoofdstuk 3 beschrijft de synthese van een bibliotheek van gefunctionaliseerde tetrazines, evenals de synthese en optimalisatie van verschillende reactieve alkenen. Het werk omvat kinetische studies om de reactiviteit van de bibliotheek van tetrazines ten opzichte van de verschillende gesynthetiseerde alkenen te bepalen. Bovendien werden deze tetrazines aan Bodipy-FL verbonden, waardoor tetrazine-fluorofoor tags werden verkregen voor bioorthogonale levende-cel fluorescentie microscopie van alkeen-gefunctionaliseerde moleculen in de cell. De DOPE-lipiden[10], mannosaminen[11] en het in de natuur voorkomende sterculzuur[12] werden in cellen geïncubeerd en vervolgens geïncubeerd met de tetrazine-fluoroforen. Dit resulteerde in een zwak fluorescentiesignaal voor beide de DOPE-lipiden en de mannosaminen. Verrassend genoeg gaven cellen die geïncubeerd waren met stercuulzuur een sterk fluorescentiesignaal voor een specifieke reeks tetrazinens. Tetrazines gefunctionaliseerd met de amino-methyl-phenyl linker gaven een zeer sterk fluorescentiesignaal, terwijl er nagenoeg geen fluorescentie werd waargenomen voor tetrazines gefunctionaliseerd met een aminoethyl linker.

Hoofdstuk 4 beschrijft de synthese van goed wateroplosbare tetrazine fluoroforen. Deze fluoroforen waren in staat toegang te krijgen tot de dichte hydrofiele glycaancoating (glycocalyx) rond het celoppervlak en zo de metabolisch opgenomen alkeen-gefunctionaliseerde mannosamine (verbinding **62**, hoofdstuk 3) te labelen. Door stercuulzuur en Bodipy-FL-tetrazine (verbinding **25**, hoofdstuk 3) te gebruiken als een tweede bioorthogonale paar, konden levende cellen worden geïncubeerd en simultaan gelabeld worden om zo multicomponent labeling (“dual labeling”) van alkeen-houdende biomoleculen

te bereiken. Dit werd bereikt door alleen het gebruik van bioorthogonale IEDDA chemie zonder enige significante kruisreactiviteit.

In Hoofdstuk 5 werd de bibliotheek van tetrazines uit hoofdstuk 3 geanalyseerd op hun vermogen (met betrekking tot IEDDA-snelheid, eliminatiesnelheid en eliminatie-efficiëntie) om een IEDDA-pyridazine-eliminatietandemreactie uit te voeren[18]. De bibliotheek, bestaande uit tetrazines gefunctionaliseerd met vrije aminen en N-Boc-beschermde aminen met verschillende spacers (methyl, ethyl of methyl-fenyl), werd geanalyseerd op hun reactiviteit en eliminatiesnelheid met AMC-coumarine, een eerder gebruikte methode uit de literatuur[19]. De resultaten werden vergeleken met een reeks tetrazines uit de literatuur[19], waaronder carboxyl-gefunctionaliseerde tetrazines[20]. De data toonde aan dat aminoethyl-gefunctionaliseerde tetrazines, in het bijzonder tetrazines **6** (hoofdstuk 5) en **7** (hoofdstuk 5), ongekende eliminatiesnelheden vertoonden, gecombineerd met een respectabele eliminatie-efficiëntie. Deze snelheden waren voorheen alleen haalbaar met andere tetrazines door de pH te verlagen tot niet-fysiologische niveaus[20]. In tegenstelling tot andere tetrazines werden tetrazines **6** (hoofdstuk 5) en **7** (hoofdstuk 5) vanwege hun aminoethyl-functionaliteit niet negatief beïnvloed door pH-veranderingen of het ontbreken van een zure omgeving. Hierdoor zijn ze hoogst waarschijnlijk een uitstekende keuze wanneer een hoge eliminatiesnelheid vereist is.

In Hoofdstuk 6 werd een hulpmiddel ontworpen en gesynthetiseerd uit bifunctioneel trans-cycloocteen en het EDANS/DABCYL-quencher-paar om de reactie- en eliminatiesnelheden van tetrazines bij het vrijkomen van alkylaminen correct te kunnen bepalen. Dit hulpmiddel is een uitstekende vervanging voor de eerder gebruikte AMC-coumarine methode omdat deze een aniline bevatte in plaats van een primair amine. Met behulp van computationele modellering van de meerstapsreactie in *Coach 7* en analyse van de resultaten met behulp van *Graphpad PRiSM*, kon de tool gebruikt worden om specifieke eigenschappen van individuele tetrazines te bepalen wat betreft hun reactiesnelheid en eliminatiesnelheid. Bovendien werden meerdere gelijktijdige processen geïdentificeerd en gekwantificeerd en kon daarmee de snelheidsbeperkende stappen worden bepaald.

List of Publications

Fluorogenic Bifunctional trans-Cyclooctenes as Efficient Tools for Investigating Click-to-Release Kinetics
A. J. C. Sarris, M. A. R. de Geus, E. Maurits, T. Hansen, M. S. Kloet, K. Kamphorst, W. ten Hoeve, M. S. Robillard, A. Pannwitz, S. A. Bonnet, J. D. C. Codée, D. V. Filippov, H. S. Overkleeft, S. I. van Kasteren
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Fast and pH-Independent Elimination of *trans*-Cyclooctene by Using Aminoethyl-Functionalized Tetrazines
A. J. C. Sarris, T. Hansen, M. A. R. de Geus, E. Maurits, W. Doelman, H. S. Overkleeft, J. D. C. Codée, D. V. Filippov, S. I. van Kasteren
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Curriculum Vitae

Alexi Johannes Christaki Sarris was born on the 29th of July 1986 in Eindhoven. During his pre-university education (VWO) developed an strong interest in chemistry and biology. After following an introductory course on organic chemistry at the University of Leiden, he found his passion and went on to study the bachelor chemistry program "Molecular Science and Technology", a cooperation of fundamental (in)organic chemistry (University of Leiden) and Chemical Technology (University of Delft). After the first year a focus on the University of Leiden program was made, including a minor of in-depth chemistry courses. In the final year, a chemistry research internship was conducted at the Bio-organic Synthesis Department (Biosyn), part of the Leiden Institute of Chemistry (LIC). While working on "*Synthesis, analysis and reaction of caged lanthanide NMR probes*", his supervisor Wei-Min Lui provided great guidance and was an inspiration for him to continue in the research focused organic chemistry field. In 2012 he obtained his Bachelor's degree.

He went on to study the chemistry master program with a specialization in research at the University of Leiden. This included a major focus on all aspects of organic chemistry, supplemented with virology, biology, catalysis and electrochemistry courses. After an extensive master research internship at Biosyn on "*Solid Phase Synthesis of Coxsackievirus B3W VPg-Nucleopeptides*" under the guidance of Geoffroy P.P. Gential, in 2015, he obtained his Master's degree with *cum laude* distinction (GPA 8.6).

He was then offered a PhD position at Biosyn (Prof. Dr. Hermen S. Overkleeft) within the group of Prof. Dr. Sander I. van Kasteren, where "click"-type bioorthogonal chemistry was employed to explore the interface between chemistry and immunology. He was also participating in the multidisciplinary educational programme of the institute of chemical immunology (ICI), and followed a wide variety of courses within the ICI-programme on relevant scientific subjects. During his PhD, he worked on various projects focused on tetrazine-alkene mediated IEDDA chemistry and its application in biology and supervised multiple students. Most of these projects are included in this thesis, and parts of the work were presented (as poster) on various conferences including CHAINS.

He was employed at the University of Leiden until 2020, and continued his career outside of academia within the field of chemistry and biology, including biotech research and regulatory work.

Acknowledgements

The final part of the thesis comes with the acknowledgements to everyone contributing to the work described in this thesis. I would like to thank Sander for all his support and advice during my PhD, allowing me to find my path in the wonderful world of tetrazines and “click”-chemistry, and always being supportive to the projects I was working on. Without his knowledge and guidance this thesis would not contain the work I am so proud of. I would also like to thank Hermen providing insights and experience whenever I needed a clear direction in my projects. I would like to thank all the people working at Biosyn, the professors, assistant-professors, technicians and other employees were always happy to provide their time and expertise when it was required and keeping the entire department running. A special mention goes to Dima, besides being the person I would go to whenever I had any theoretical or practical question about the chemistry I was performing during my PhD, he has been a great chemistry teacher throughout my education and a positive influence in my path to becoming a professional chemist.

Over the years I’ve had the pleasure to work alongside many PhD students, post-docs, and intern students. Special mentions go to Michel, Jerre, Thomas and Shimrit, Ward, Daniël and Anouk as the people that I’ve got to know really well and consider my friends, everyone at the DM-Labs who were my daily colleagues and comrades during my PhD, everyone within the Sander-group, and also everyone within the Biosyn-group. Last but not least I am very thankful to the students I worked with Luuk, Nancy and Dyone, who showed amazing skill and perseverance in their projects and were, besides contributing to this thesis, a true enjoyment to supervise and spend my time with. Looking back to this, I am truly happy to have met each and all of you, and I am certain that in between all the fun, conversations and cups of coffee, these interactions have provided me the knowledge and strength to succeed and overcome all the difficulties in the path towards my goals.

Finally, I would like to thank my friends and family. My father and my brothers have always been very supportive throughout my PhD and after. And, most importantly, my wife Ruth and my children Elias and Hannah. Deciding to become a parent, twice, during my PhD was a very challenging and the most rewarding decision in my life. Ruth, you were a tremendous help to me for all my struggles. During periods of long and energy draining days at the lab, but also the time after leaving the University lab. With my most precious experiments successful and the book mapped out in my head, the only thing left was to write the thesis. Unfortunately things usually don’t go as we plan them. I went For two years I was stuck working in terrible and toxic work environments, leaving me struggling with emotions I couldn’t imagine. During this time, you and our children have been my joy, my beacons of hope and have kept me going even when everything felt too much. With the thesis now finally written, this chapter in my life comes to an end and hopefully, surely, will lead to a bright and exciting new one.