The versatility of asymmetric aminoethyl-tetrazines in bioorthogonal chemistry

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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

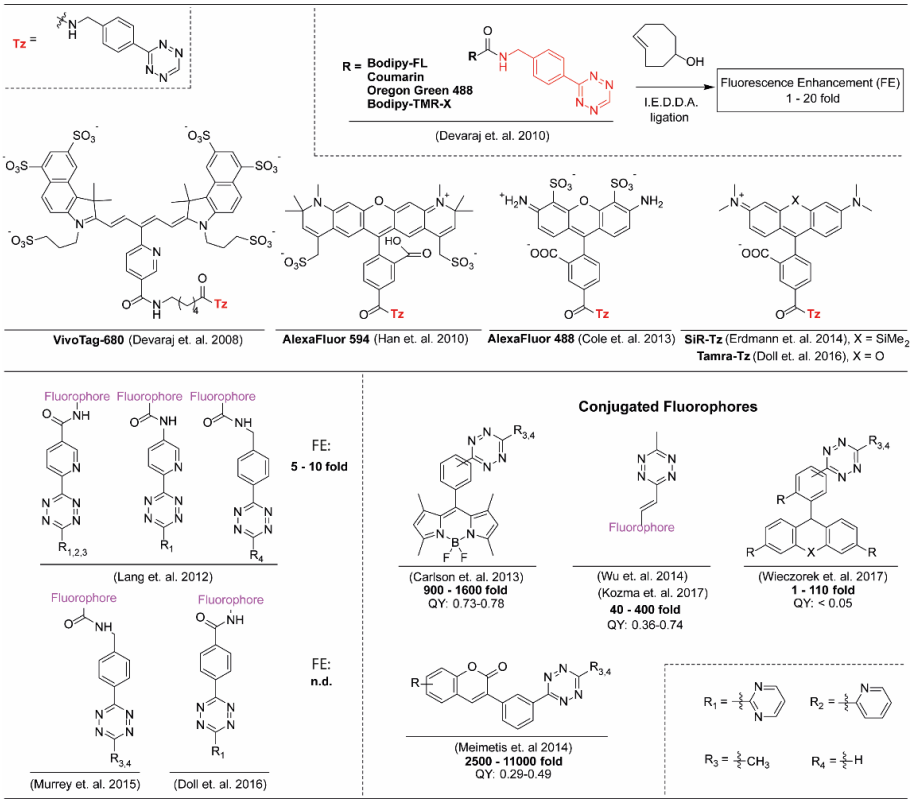


Figure 1: Examples of tetrazine-modified fluorophores described in the literature.

(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**).

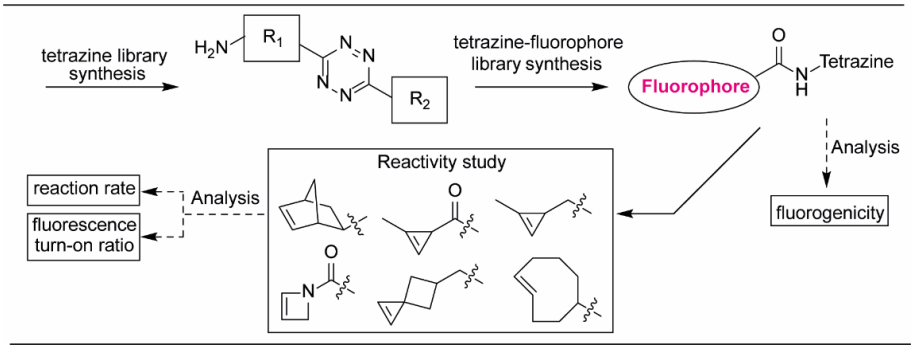
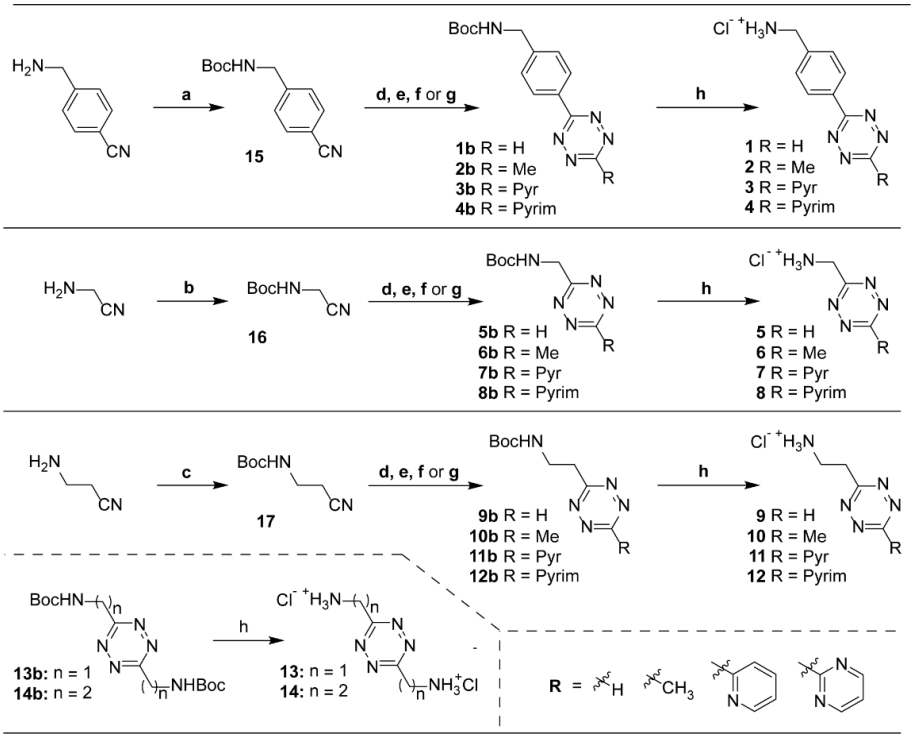


Figure 2: Flowchart regarding the aim of this chapter: Through synthesis of a focused library of amino-alkyl and amino-aryl functionalized tetrazines a tetrazine-fluorophore library can be prepared. These compounds can then be analyzed in their application in IEDDA-mediated conditional fluorescence.

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**).

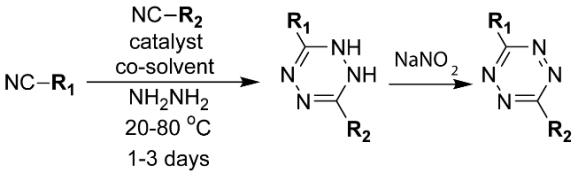


(**a**) Boc_2O, NaOH, H_2O, r.t., o.n. (**b**) Boc_2O, TEA, DCM, reflux, o.n. (**c**) Boc_2O, TEA, DCM, r.t., o.n. (**d**) N_2H_4, $Zn(OTf)_2$, formamidine acetate (**e**) N_2H_4,, $Ni(OTf)_2$, acetonitrile (**f**) N_2H_4,, $Zn(OTf)_2$, 2-cyanopyridine (**g**) N_2H_4,, $Zn(OTf)_2$, 2-cyanopyrimidine (**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



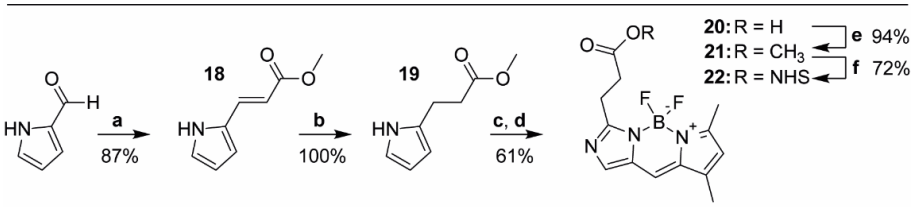
R_1	R_2	co-solvent	catalyst	container	T (°C)	time	yield	Tz
15	Form. Ac.	dioxane	$Zn(OTf)_2$	Tube	60	o.n.	34%	**1b**
16		-	ZnI_2	Tube	30	3 days	14%	**5b**
17		dioxane	$Zn(OTf)_2$	Tube	20	3 days	6%	**9b**
15	Me-CN	dioxane	$Ni(OTf)_2$	Tube	60	o.n.	16%	**2b**
16		-	$Zn(OTf)_2$	Flask	80	o.n.	31%	**6b**
17		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]

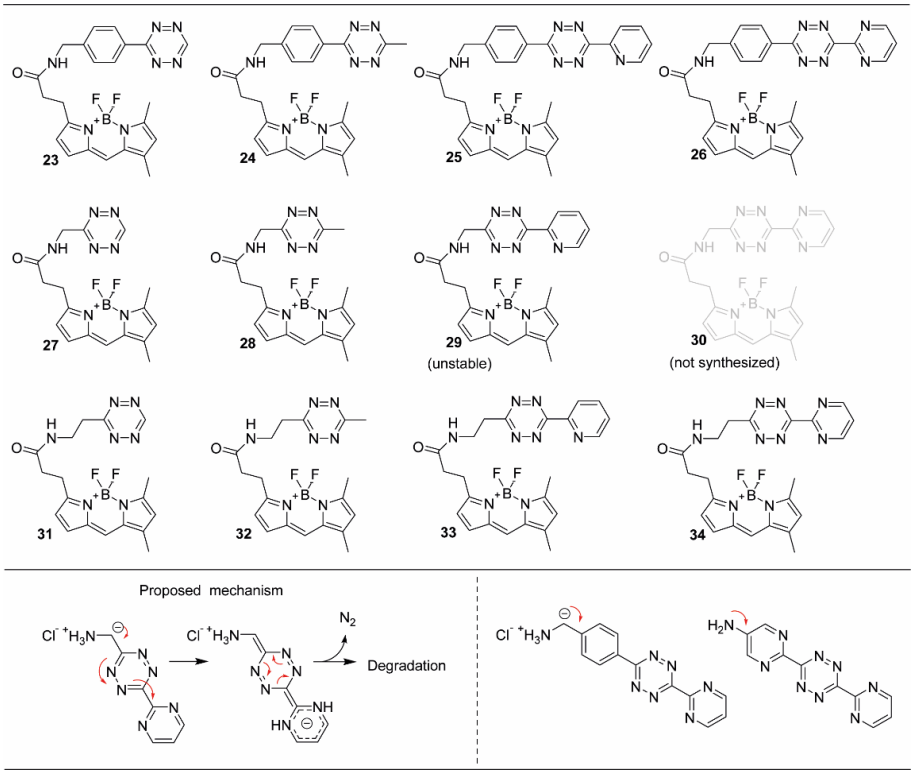
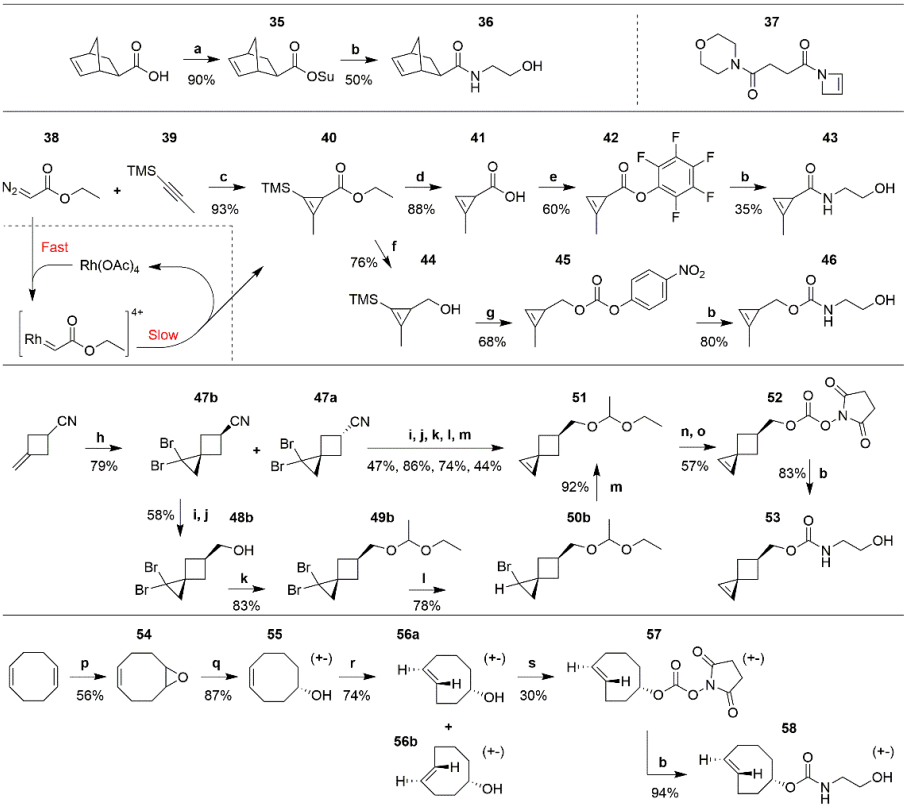


Figure 3: Synthesized library of tetrazine functionalized Bodipy-FL **23-34**.

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.

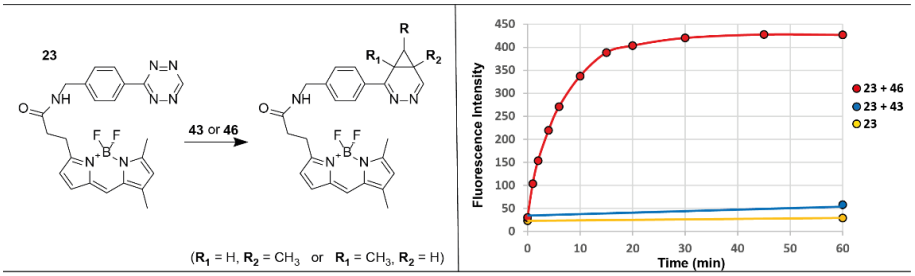


Figure 4: Fluorescence of Bodipy-FL tetrazine **23** (4.0 µM) during reaction with acyl-cyclopropene **43** (400 µM) and alkyl-cyclopropene **46** (400 µM) in phosphate buffer (0.2M, pH 7.4).

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**.

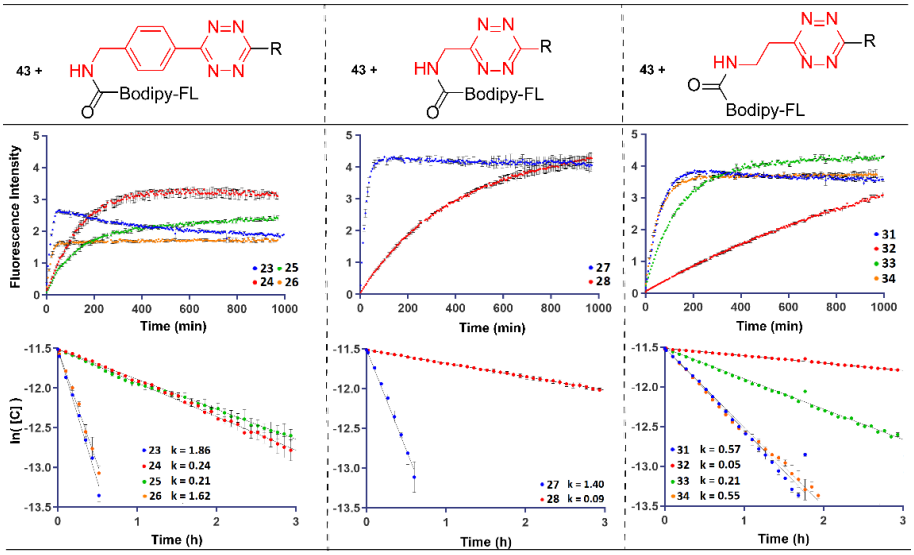


Figure 5: Fluorescence emergence of Bodipy-FL tetrazines **23**-**28** and **31**-**34** (10.0 µM) upon reaction with alkyl-cyclopropene **46** (500 µM) in PBS (pH 7.4). Reaction rate constants were determined by linear approximation of the measurements until the concentration of non-reacted Bodipy-FL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

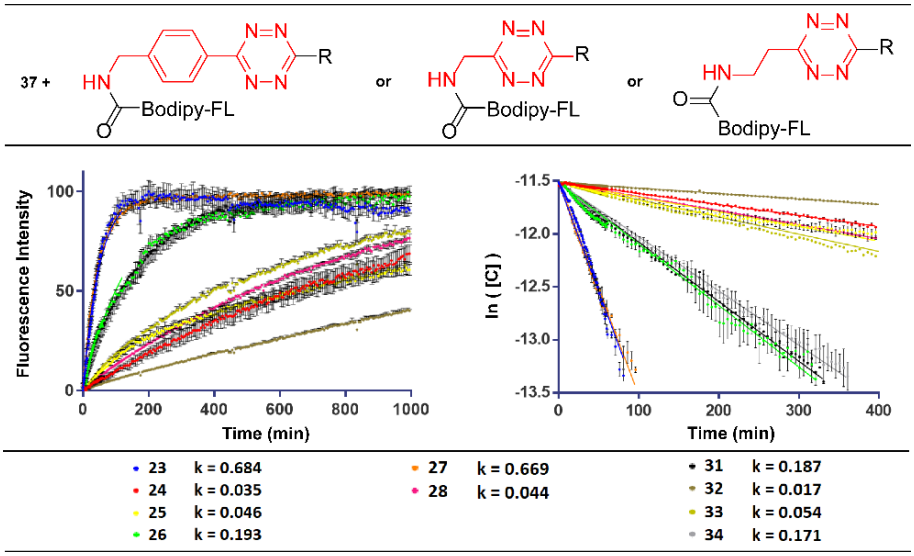


Figure 6: Fluorescence of Bodipy-FL tetrazines **23**-**28** and **31**-**34** (10.0 µM) upon reaction with azetine **37** (500 µM) in PBS (pH 7.4). Reaction rate constants were determined by linear approximation of the measurements until the concentration of non-reacted Bodipy-FL tetrazine reached [C]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

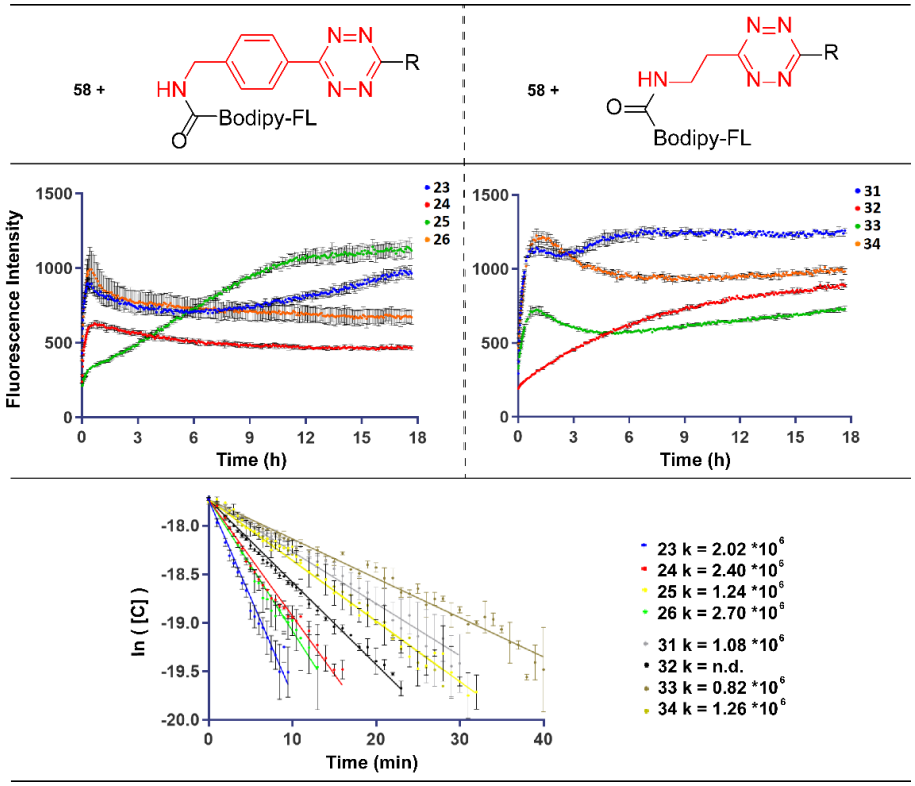


Figure 7: Fluorescence of Bodipy-FL tetrazines **23**-**26** and **31**-**34** (10 nM) upon reaction with transcyclooctene **58** (50 nM) in PBS (pH 7.4). Reaction rate constants were determined by linear approximation of the measurements until the estimated concentration of non-reacted Bodipy-FL tetrazine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

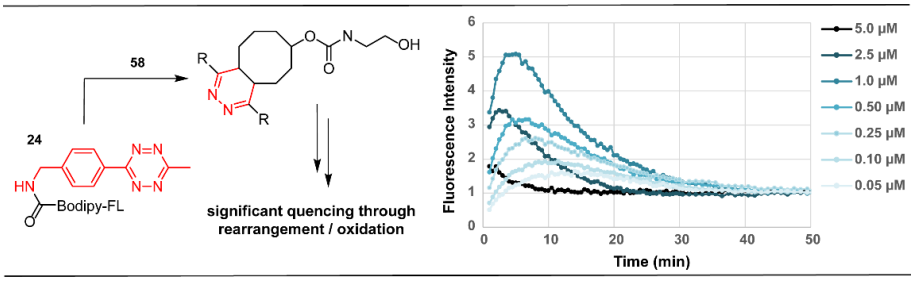


Figure 8: Fluorescence of Bodipy-FL tetrazine **24** (10 nM) upon reaction with transcyclooctene **58** (5.0 – 0.05 µM) in PBS (pH 7.4) to determine the fluorescence increase and decrease over time (normalized at t = 56 minutes).

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]

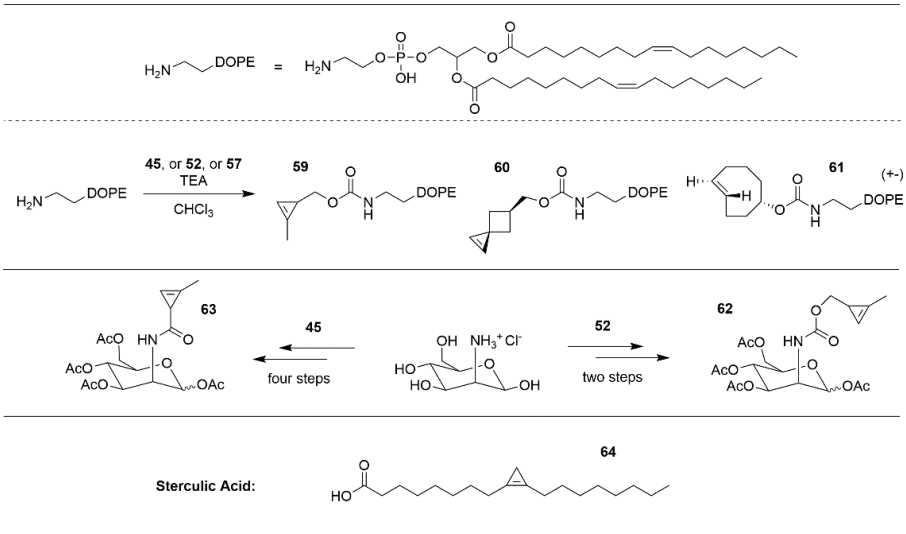


Figure 9: **Top:** Synthesis of DOPE phospholipids **59**, **60**, and **61**, and N-alkene modified mannosamines **62** and **63**. **Bottom**: Naturally occurring sterculic acid **64**.

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.

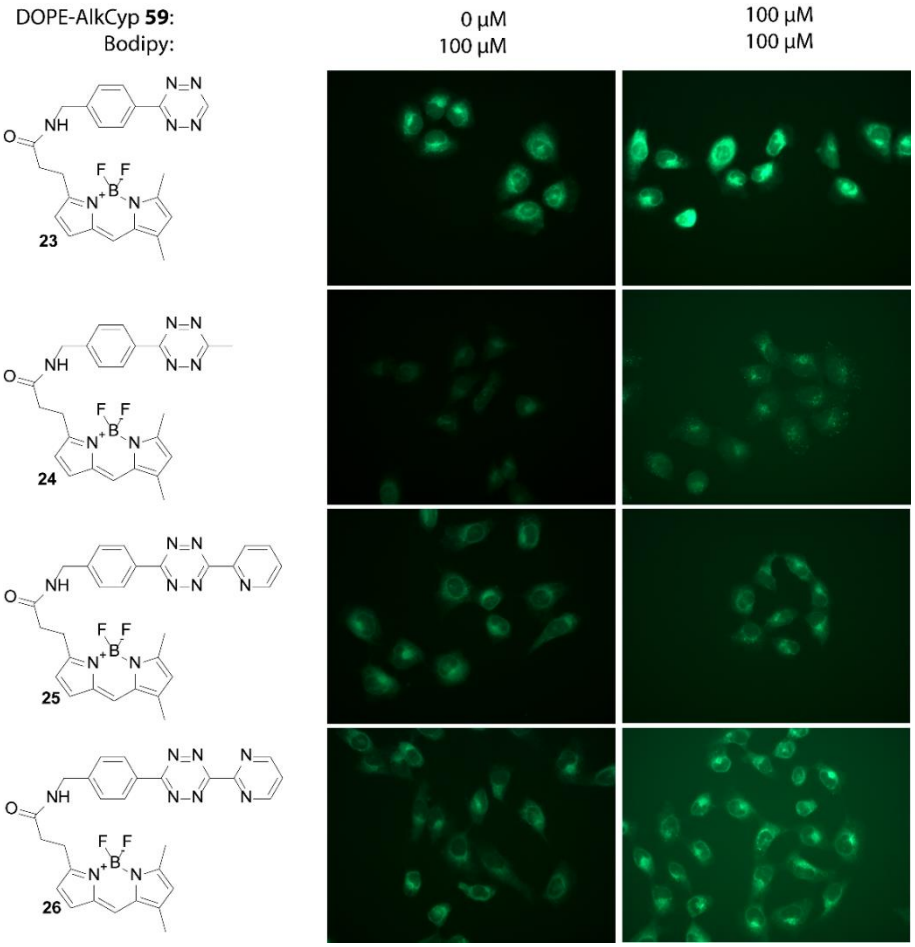


Figure 10: Live-cell fluorescence imaging of U2OS cells after Incubation without (left) and with (right) alkyl-cyclopropene DOPE lipid **59** for 1 hour, followed by incubation with (top to bottom) Bodipy-FL tetrazines **23**, **24**, **25**, or **26** for 1 hour.

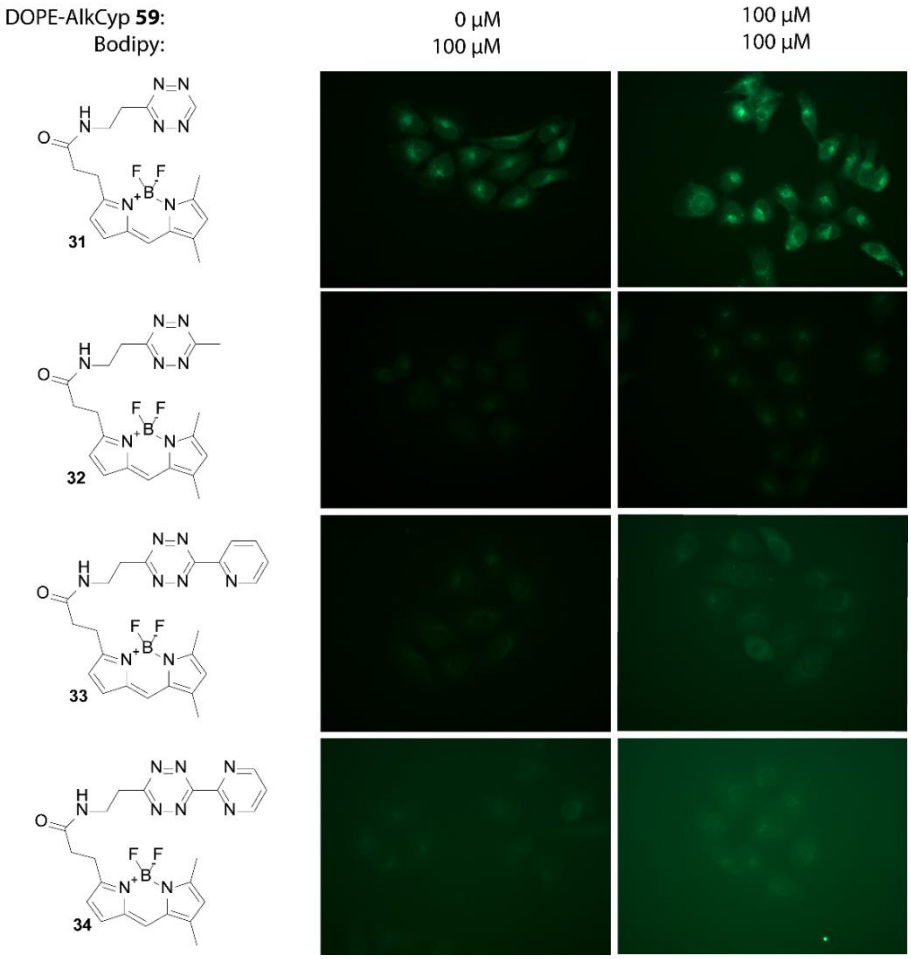


Figure 11: Live-cell fluorescence imaging of U2OS cells after Incubation without (left) and with (right) alkyl-cyclopropene DOPE lipid **59** for 1 hour, followed by incubation with (top to bottom) Bodipy-FL tetrazines **31**, **32**, **33**, or **34** for 1 hour.

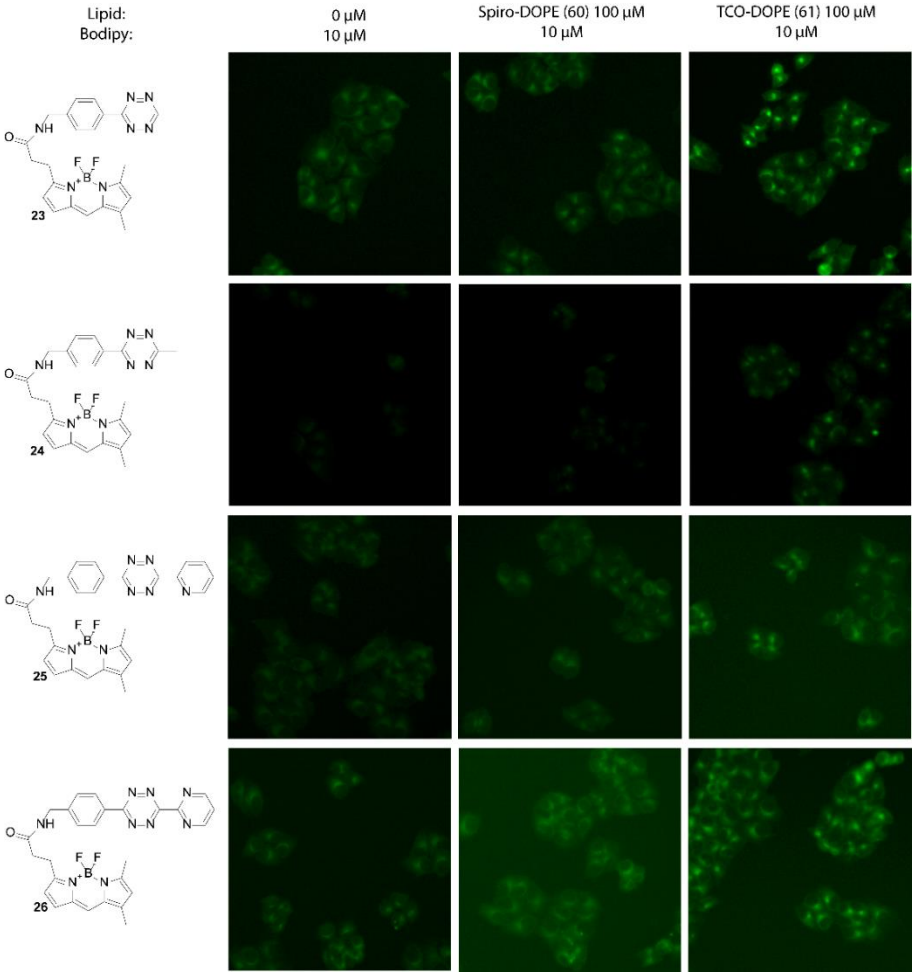


Figure 12: Live-cell fluorescence imaging of HeLa cells after Incubation without (left) lipid, with (middle) spirohexene DOPE lipid **60**, or with (right) *trans*-cyclooctene **61** for 1 hour, followed by incubation with (top to bottom) Bodipy-FL tetrazines **23**, **24**, **25**, or **26** for 1 hour.

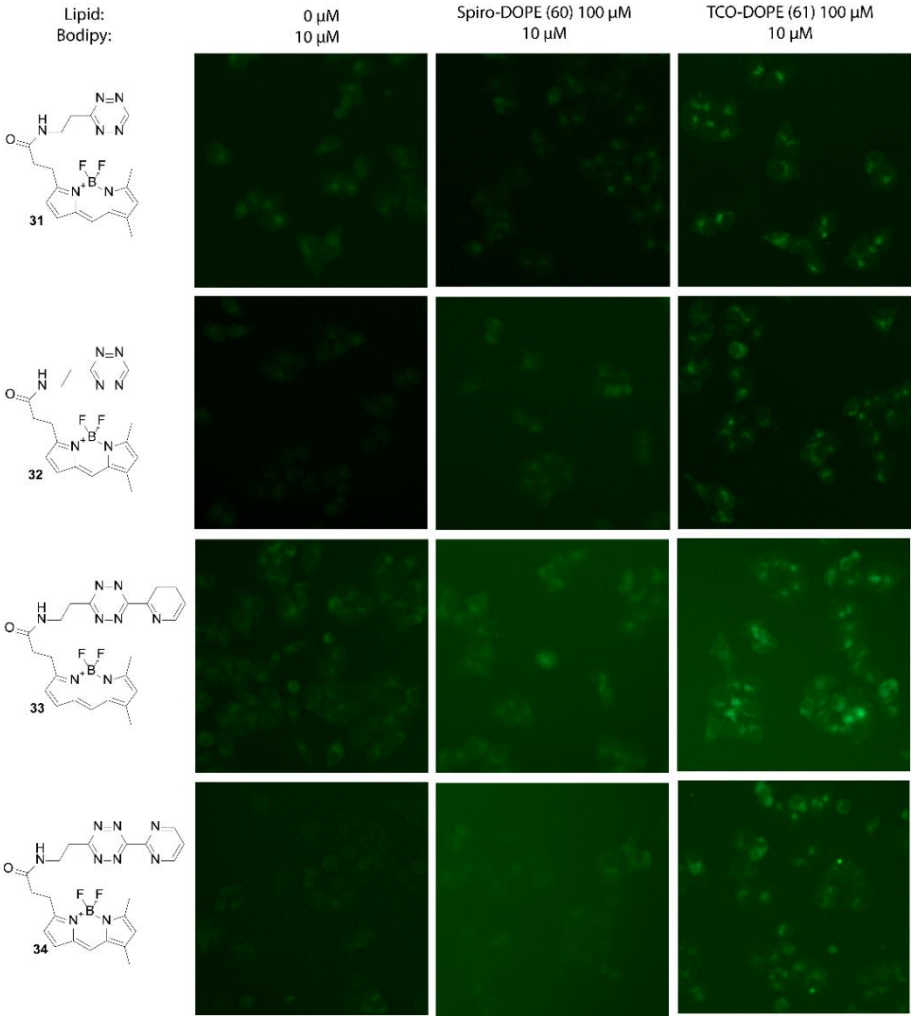


Figure 13: Live-cell fluorescence imaging of HeLa cells after Incubation without (left) lipid, with (middle) spirohexene DOPE lipid **60**, or with (right) *trans*-cyclooctene **61** for 1 hour, followed by incubation with (top to bottom) Bodipy-FL tetrazines **31**, **32**, **33**, or **34** for 1 hour.

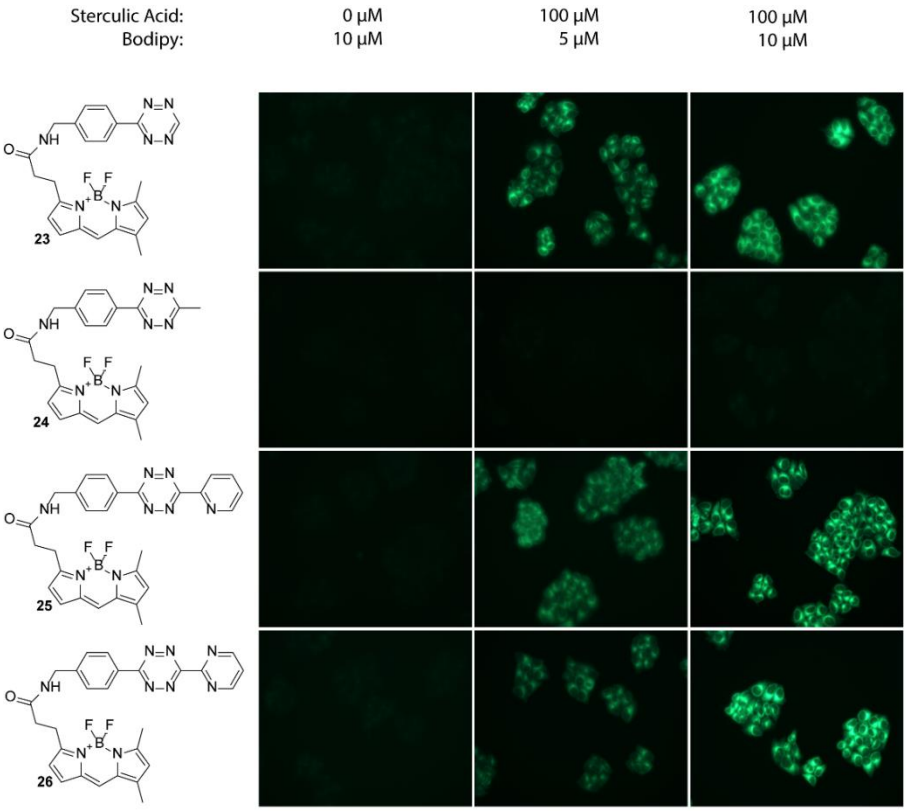


Figure 14: Live-cell fluorescence imaging of U2OS cells after Incubation without (left) lipid, with (middle and right) sterculic acid for 1 hour, followed by incubation with (top to bottom) Bodipy-FL tetrazines **23**, **24**, **25**, or **26** at variable concentrations for 1 hour.

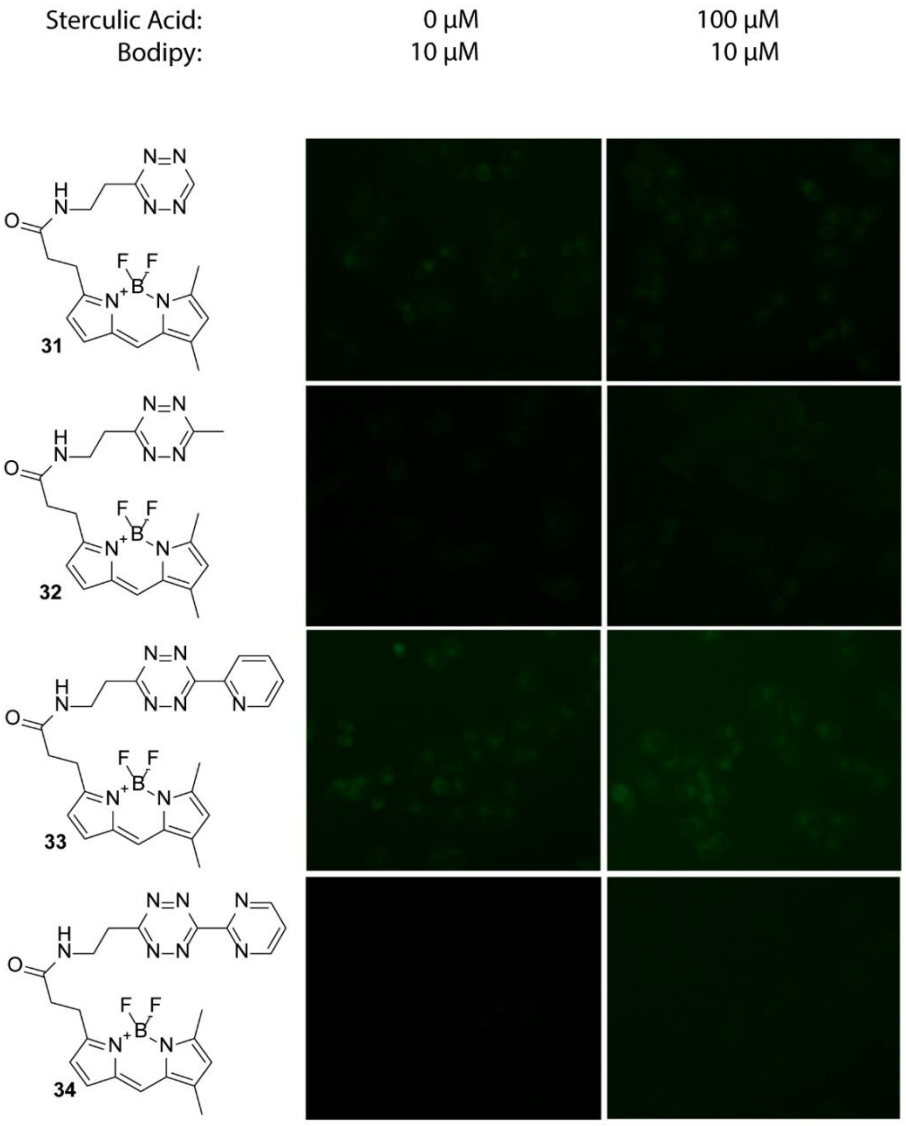


Figure 15: Live-cell fluorescence imaging of U2OS cells after Incubation without (left) lipid, with (right) sterculic acid for 1 hour, followed by incubation with (top to bottom) Bodipy-FL tetrazines **31**, **32**, **33**, or **34** for 1 hour.

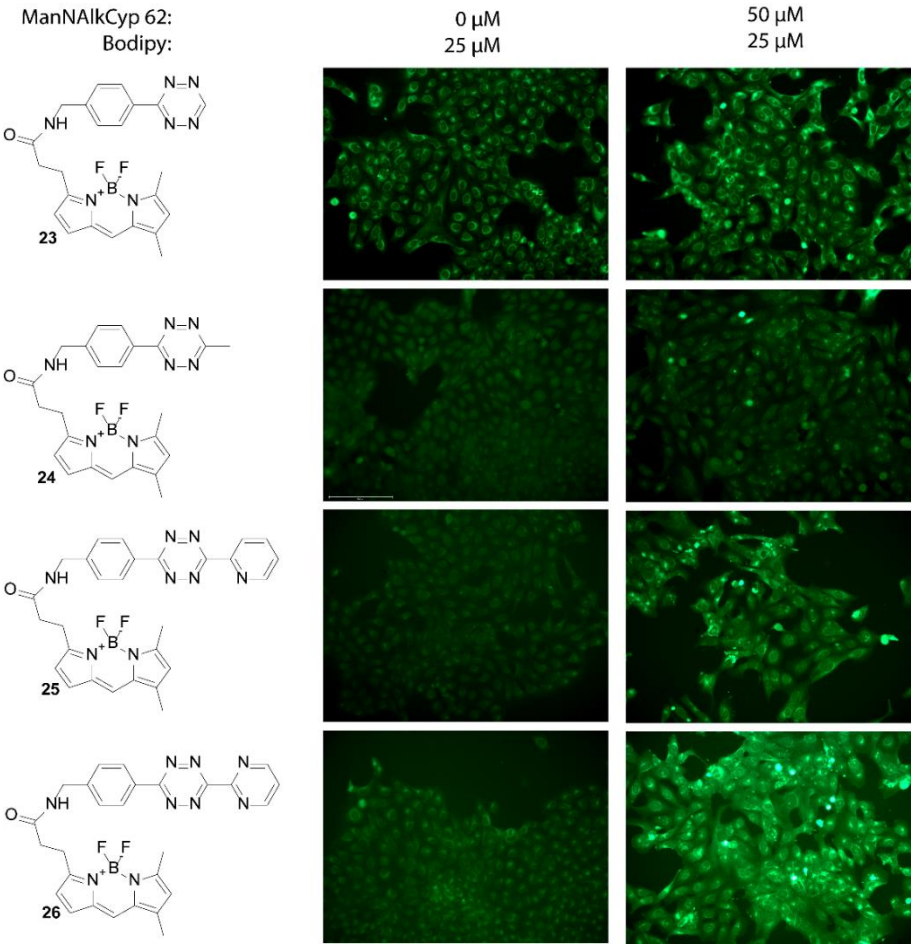


Figure 16: Live-cell fluorescence imaging of U2OS cells after Incubation without (left) lipid, with (right) N-(alkyl-cyclopropene) mannosamine **62** for 3 days, followed by incubation with (top to bottom) Bodipy-FL tetrazines **23**, **24**, **25**, or **26** for 1 hour.

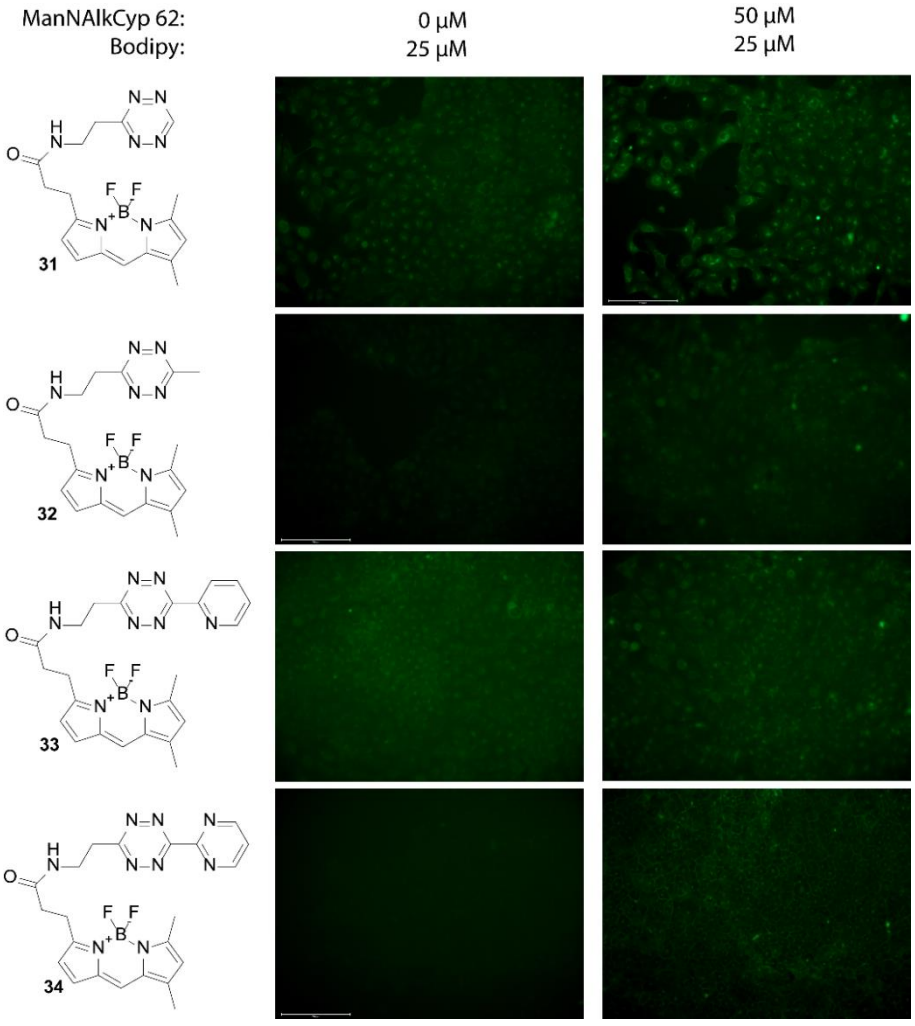


Figure 17: Live-cell fluorescence imaging of U2OS cells after Incubation without (left) lipid, with (right) N-(alkyl-cyclopropene) mannosamine **62** for 3 days, followed by incubation with (top to bottom) Bodipy-FL tetrazines **31**, **32**, **33**, or **34** for 1 hour.

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.

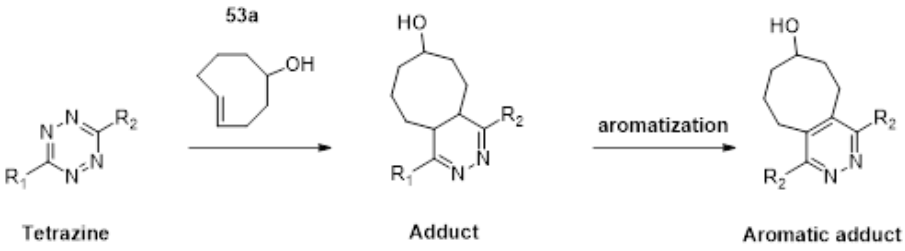
Procedure C (**oxidation**). A mixture of DCM/AcOH (1:1, v:v, 20 mL/mmol) was prepared. While stirring, the dihydrotetrazine containing reaction mixture was added dropwise. Solid $NaNO_2$ (20 eq., 1.5 g/mmol) was added portion wise over 30 minutes. The mixture was concentrated using rotary evaporation, 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.

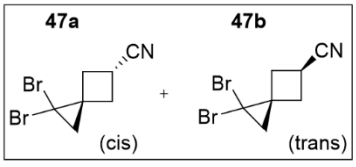
Compound 42: 15.1 mmol (1.483 g) of compound **41** was dissolved in 70 mL DCM, 22.5 mmol (4.15 g) of pentafluoro phenol, 20.7 mmol (3.96 g) of EDC*HCl and 1.7 mmol (0.21 g) of DMAP were added respectively and the reaction mixture was stirred overnight at room temperature. Reaction completion was checked 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.

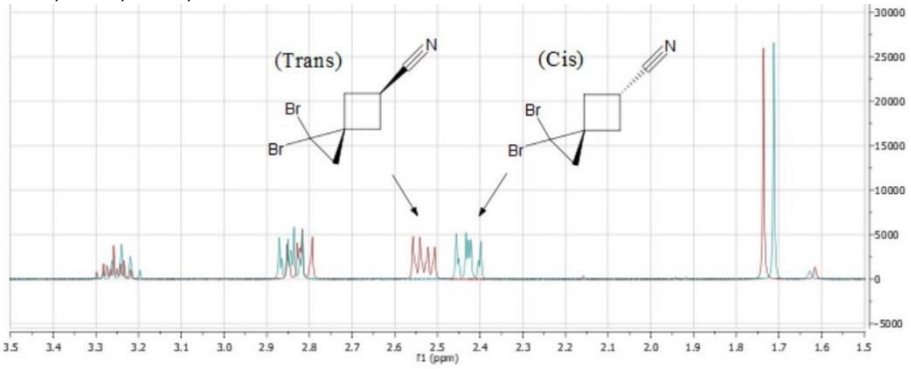
Compound 45: 1.1 mmol (0.172 g) of compound **44** was dissolved in 5 mL of dry THF, 1.2 mmol (0.320 g) of 18-crown-6 and 1.15 mmol (0.175 g) of cesium fluoride powder were added respectively. The mixture was stirred for 2.5 h at room temperature. Reaction completion was checked by TLC (Rf = 0.2, 30% 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.

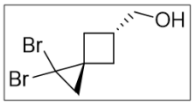
Compound 46: 0.80 mmol (250 mg) of compound **45** was dissolved in 8.0 mL of dry DCM, 0.80 mmol (0.11 mL, 0.080 g) of TEA and 1.6 mmol (0.10 mL, 0.10 g) of 2-aminoethanol were added respectively. The reaction mixture was stirred for 3 hours at room temperature. Reaction completion was checked by TLC (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.



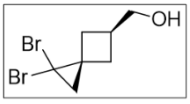
Compound 47: 5 mmol (0.50 mL, 0.47 g) of 3-methylene-2-cyano-cyclobutane was dissolved in 10 mmol (0.9 mL, 2.53 g) of bromoform, 0.05 mmol (18 mg) of CTAB was added, followed by dropwise addition of 2.5 g NaOH dissolved in 2.5 mL H_2O over 1 minute. The biphasic solution was stirred overnight resulting 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.



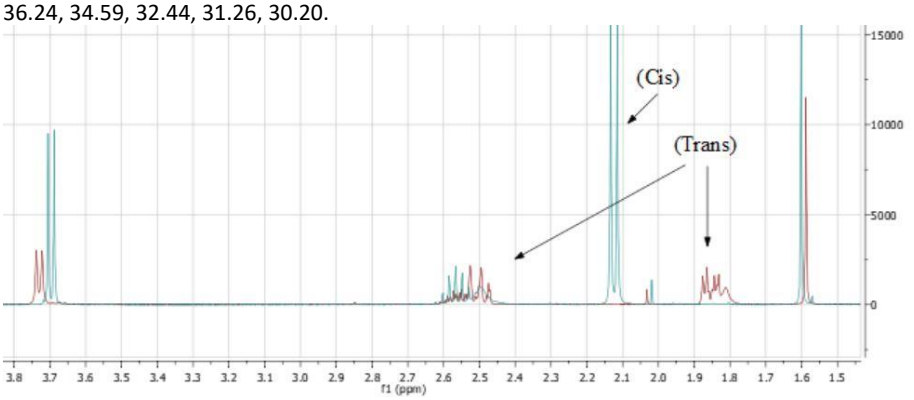


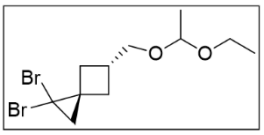
Compound 48a: 1.57 mmol (0.415 g) of compound **47a** was dissolved in 0.3 mL dry toluene, cooled down to -78 °C, and 3.1 mmol (3.1 mL of a 1M solution in toluene) of DiBAl-H was added dropwise before stirring the suspension for 2 hours while warming to room temperature. Reaction completion was checked 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.



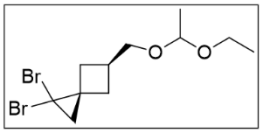
Compound 48b: 15.9 mmol (4.201 g) of compound **47b** was dissolved in 8 mL dry toluene, cooled down to -78 °C, and 32 mmol (32 mL of a 1M solution in toluene) of DiBAl-H was added dropwise before stirring the clear solution for 2 hours while warming to room temperature. Reaction completion was checked 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.



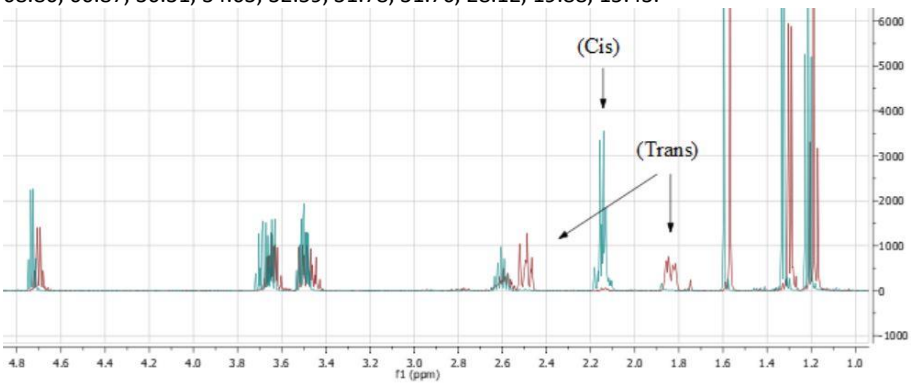


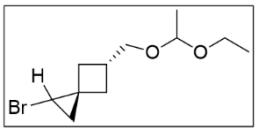
Compound 49a: 0.74 mmol (0.200 g) of compound **48a** was dissolved in 4 mL of dry Et_2O, 0.05 mmol (10 mg) of TsOH monohydrate was added, and the reaction mixture was cooled to 0 °C before dropwise addition of 6 mmol (0.6 mL, 0.43 g) of vinyl ethyl ether over 1 minute. The reaction mixture was stirred 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.



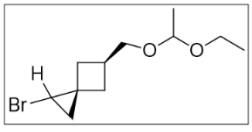
Compound 49b: 8.90 mmol (2.402 g) of compound **48b** was dissolved in 40 mL of dry Et_2O, 0.46 mmol (87 mg) of TsOH monohydrate was added, and the reaction mixture was cooled to 0 °C before dropwise addition of 54.6 mmol (5.25 mL, 3.94 g) of vinyl ethyl ether over 5 minutes. The reaction mixture was 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.



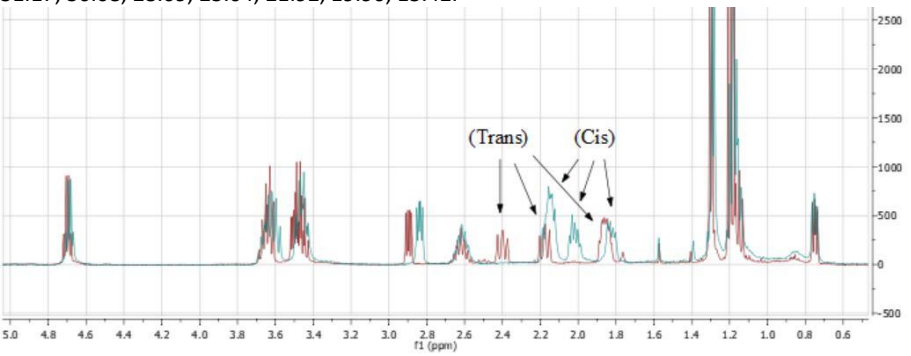


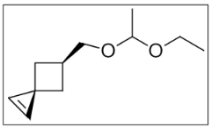
Compound 50a: 0.585 mmol (0.200 g) of compound **49a** was dissolved in 1.5 mL of dry Et_2O, 0.037 mmol (11 µL, 10.5 mg) of $(iPrO)_4Ti$ was added, followed by dropwise addition of 1 mL (1.0 mmol) a freshly prepared 1M EtMgBr in Et_2O solution. Initial formation of the $Et_2(iPrO)_2Ti$ catalyst was observed 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.



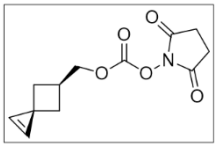
Compound 50b: 7.16 mmol (2.448 g) of compound **49b** was dissolved in 15 mL of dry Et_2O, 0.36 mmol (110 µL, 105 mg) of $(iPrO)_4Ti$ was added, followed by dropwise addition of 9.3 mmol EtMgBr (3.1 mL of a 3M solution in Et_2O). Initial formation of the $Et_2(iPrO)_2Ti$ catalyst was observed by decoloring 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.



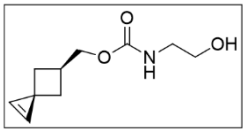


Compound 51 (**Method A**): 0.433 mmol (114 mg) of compound **50a** was dissolved in 2 mL of DMSO, cooled to 0 °C (according to literature procedure) forming a solid, and 1mL of DMSO containing 0.93 mmol (104 mg) of KOtBu was added dropwise on top of the solid surface. The reaction mixture was allowed 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.

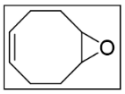


Compound 52: 3.84 mmol (0.700 g) of compound **51** was dissolved in 8 mL of acetonitrile, cooled to 0 °C, 8 mL of 3M HCl aq. was added dropwise, and the reaction mixture was stirred for 30 minutes. Reaction completion was checked by TLC (Rf = 0.2, 20% EtOAc in pentane). The reaction mixture was extracted 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

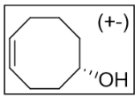
added over 30 minutes. The clear solution was allowed to stir overnight at room temperature forming a suspension. The suspension was concentrated using rotary evaporation, taken up in a minimal amount of 1:2:2 mixture of Et_2O:pentane:chloroform for silica column chromatography. Purification was performed 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.



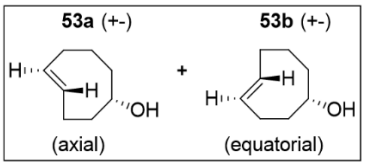
Compound 53: 0.20 mmol (50 mg) of compound **52** was dissolved in 1 mL of dry DCM, 0.20 mmol (28 µL, 20 mg) of TEA and 0.39 mmol (24 µL, 24 mg) 2-amino ethanol were added and the reaction mixture was stirred for 30 minutes at room temperature. The reaction mixture was directly used for purification. 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.



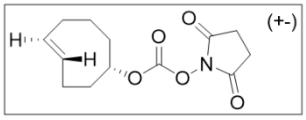
Compound 54: 0.68 mol (117 g, 1.0 L of a 0.68M solution in chloroform) of *meta*-chloroperbenzoic acid was slowly added to 0.55 mol (59.1 g, 61.4 mL) of cyclooctadiene, using a dropping funnel over 2 hours at 0°C, and the reaction mixture was stirred overnight at room temperature. Reaction completion 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.



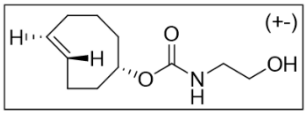
Compound 55: 316 mmol (39.3 g) of compound **54** was dissolved in 200 mL of dry THF. To this solution 248 mmol (62 mL) 4M $LiAlH_4$ in THF was added dropwise at 0°C and the reaction mixture was stirred overnight at room temperature. The reaction mixture was quenched by adding 200 mL H_2O and the resulting 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).

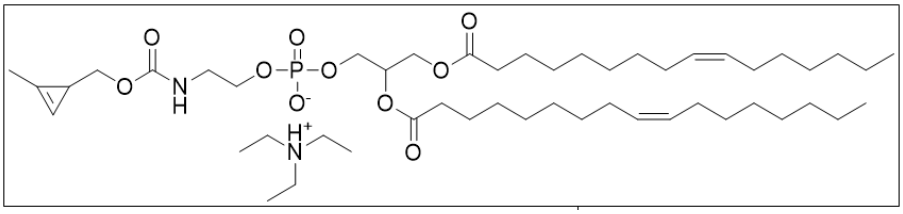


Compound 57: 0.634 mmol (80 mg) of **56a** was dissolved in 5 mL of dry acetonitrile, 2.2 mmol (0.24 g, 0.3 mL) TEA, followed by slow addition of 1.56 mmol (400 mg) N,N'-disuccinimidyl carbonate and the reaction mixture was stirred for three days. The reaction mixture was suspended in Et_2O, washed with 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.

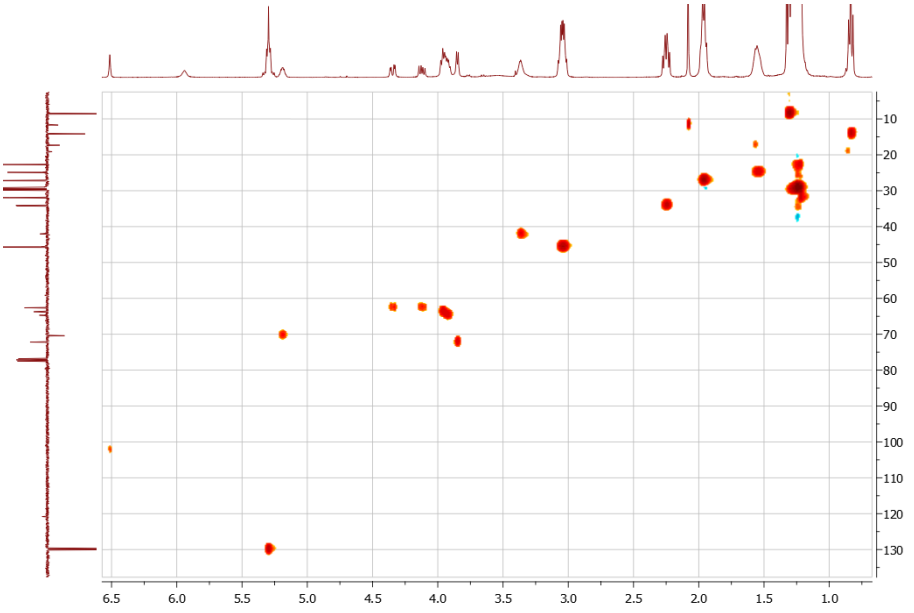


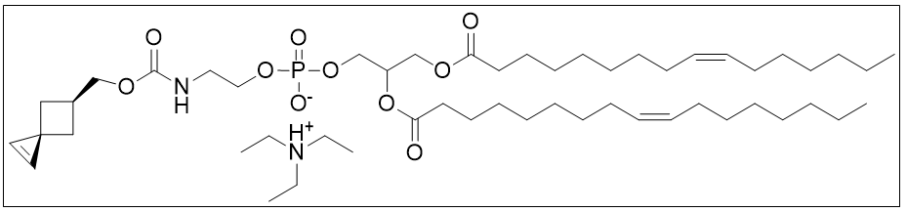
Compound 58: 0.10 mmol (30 mg) of compound **57** was dissolved in 1 mL of dry DCM, 0.10 mmol (14 µL, 10 mg) of TEA and 0.20 mmol (12 µL, 12 mg) of 2-amino ethanol were added respectively and the reaction mixture was stirred for 30 minutes at room temperature. Reaction completion was checked by TLC (Rf = 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.

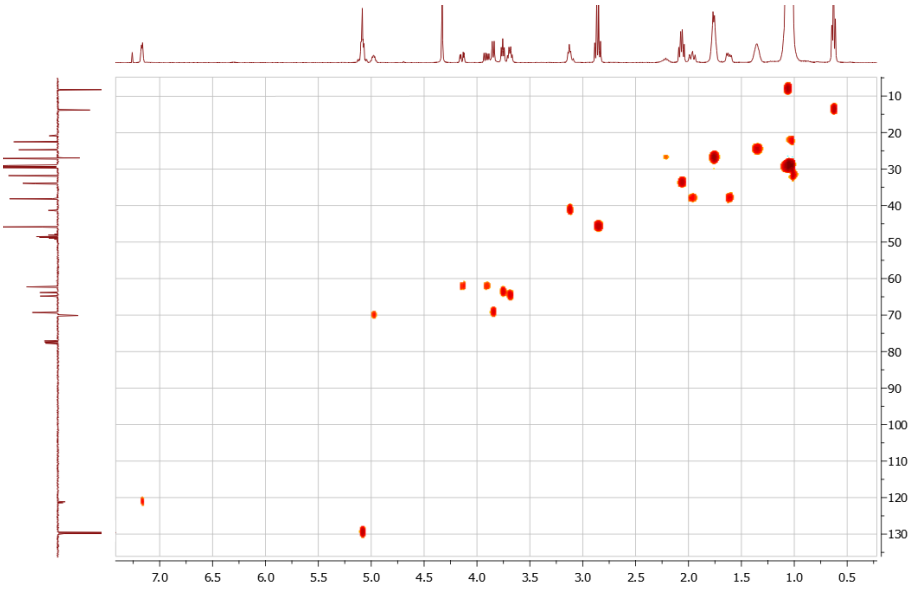


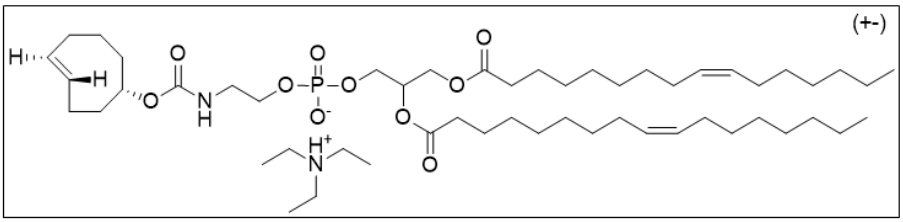
Compound 59: Compound **45** was dissolved dry chloroform, TEA and DOPE were added respectively and the reaction mixture was stirred overnight at room temperature. Reaction completion was checked by TLC (20% MeOH in $CHCl_3$). The reaction mixture was directly used for purification. Purification was 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:



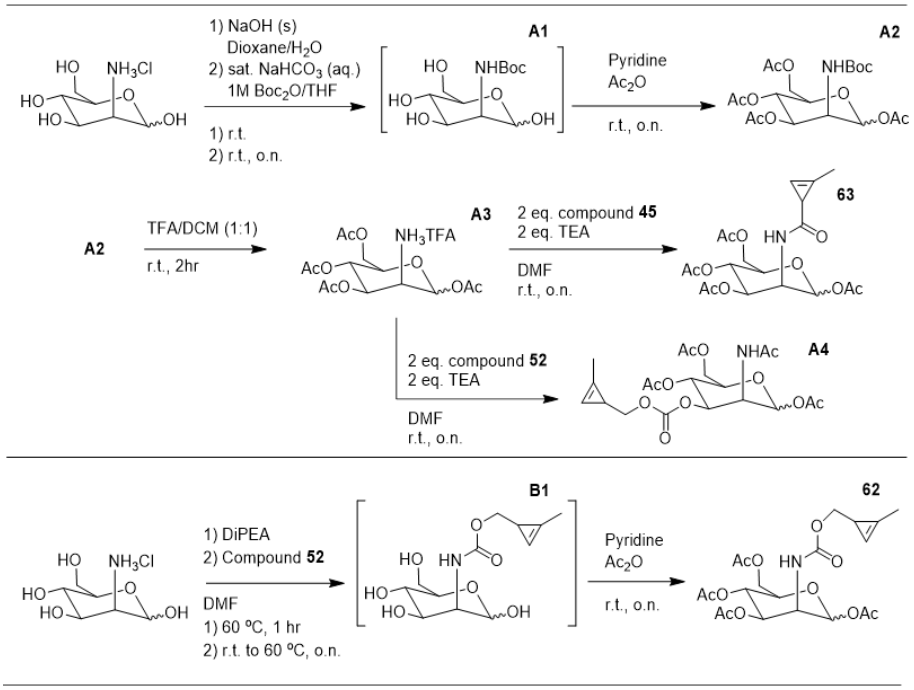


Compound 60: 0.18 mmol (46 mg) of compound **52** was dissolved in 2.0 mL of dry chloroform, 0.39 mmol (52 µL, 40 mg) of TEA and 0.171 mmol (127 mg) of DOPE were added respectively and the reaction mixture was stirred overnight at room temperature. Reaction completion was checked by TLC (Rf = 0.5, 20% 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.

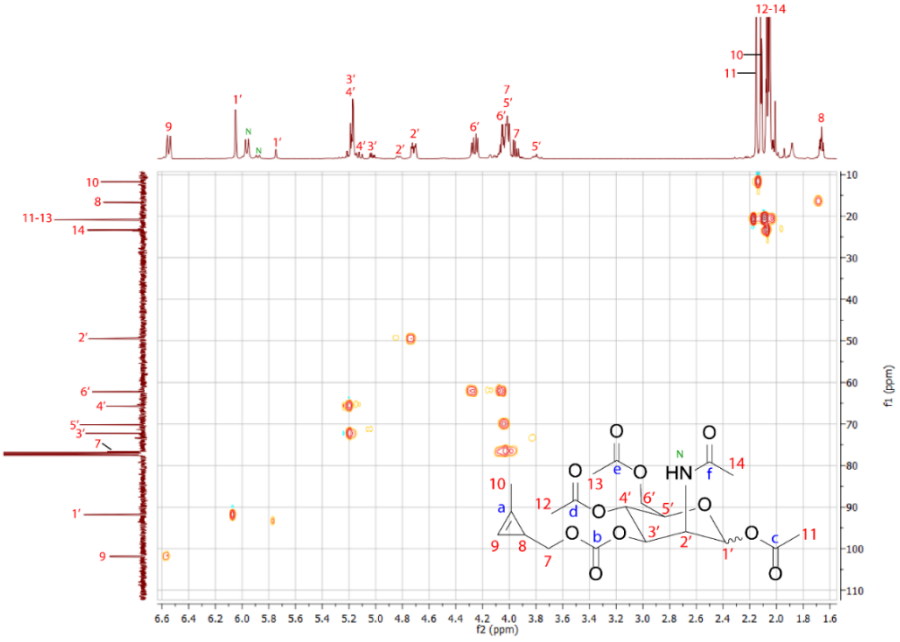
Compound A3: 186 µmol (86 mg) of compound **A2** was dissolved in 2 mL of TFA:DCM mixture (1:1, v:v) and the reaction mixture was stirred for 2 hours at room temperature. Reaction completion was checked by TLC (two spots, Rf = 0.3, 100% EtOAc). The reaction mixture was concentrated using rotary evaporation, 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.

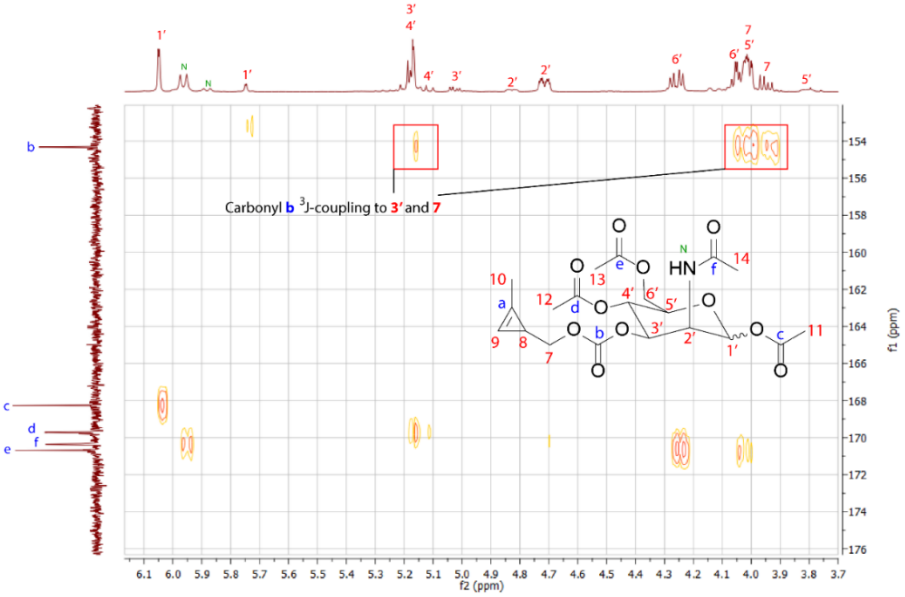
Compound 62: 0.20 mmol (43 mg) of D-mannosamine hydrochloride was added to 1 mL of a dry DMF, 0.80 mmol (139 µL) of DiPEA was added and the reaction mixture was stirred at 60 °C until a clear solution was obtained. Then, the mixture was allowed to cool to room temperature, 0.40 mmol (100 mg) of compound **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,

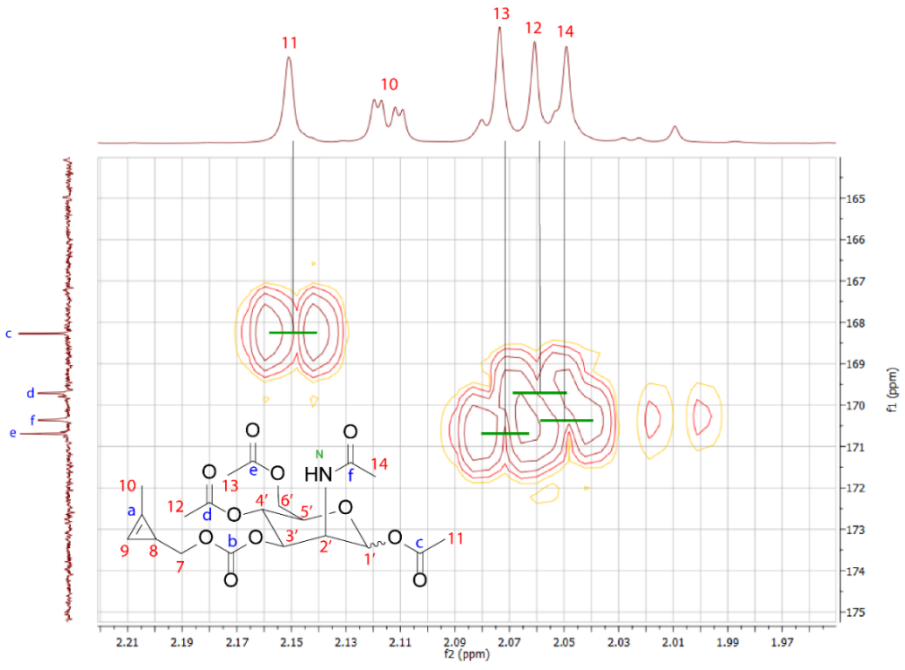
102.21, 92.09, 73.51, 70.30, 69.29, 65.44, 62.06, 60.55, 51.20, 21.21, 21.05, 20.92, 20.80, 17.17, 11.82.

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:

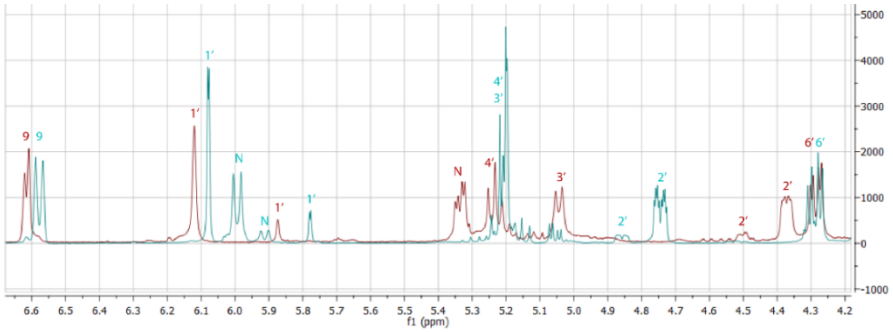


Compound A4 (HMBC)





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



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