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The versatility of asymmetric aminoethyl-tetrazines in bioorthogonal chemistry

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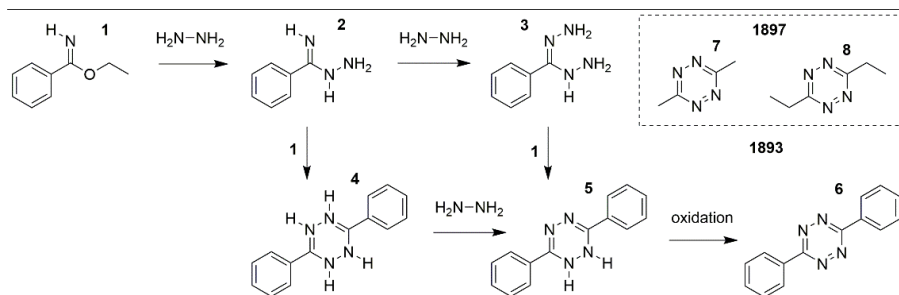
Chapter 1: A brief history of tetrazine chemistry and their application in bioorthogonal reactions

Introduction

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

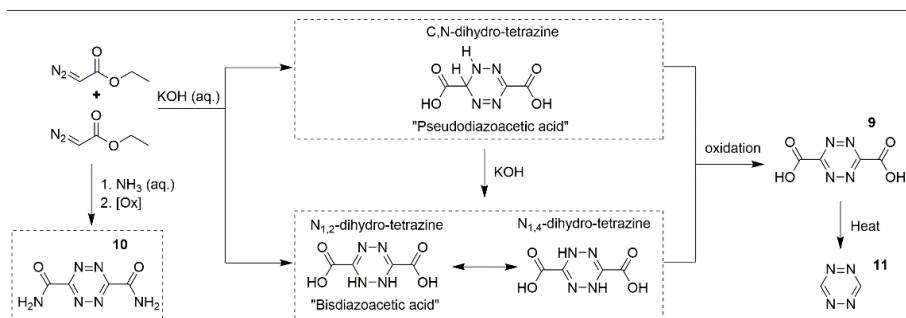
Symmetric functionalized tetrazines

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



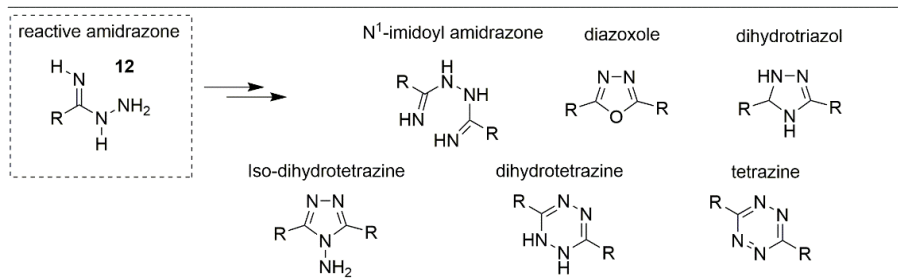
Scheme 1: Synthesis of tetrazine **6** performed by Pinner in 1893 from benzimidooxime **1**, hydrazine and aqueous potassium hydroxide.

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



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

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

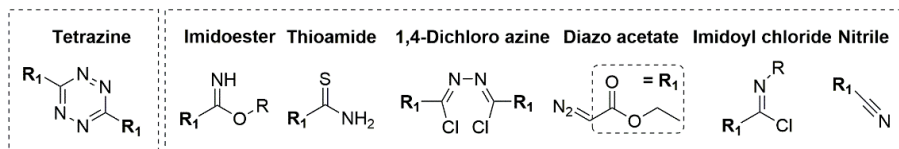


Scheme 3: Identified byproducts and unreacted intermediates that may be present in a reaction mixture during the synthesis of dihydro-1,2,4,5-tetrazines.

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

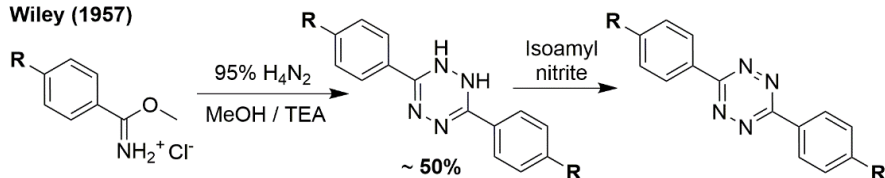
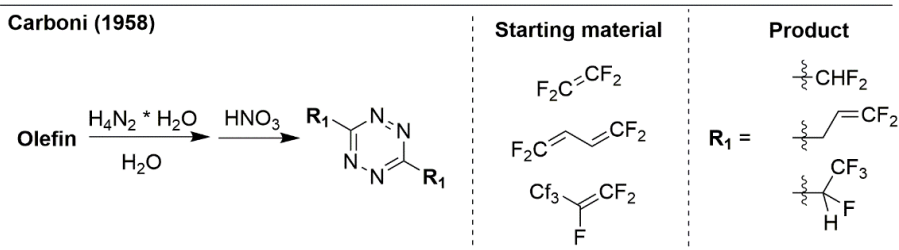
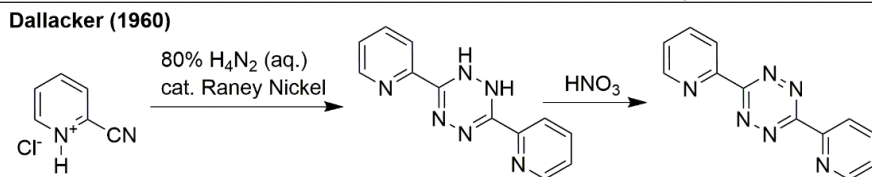
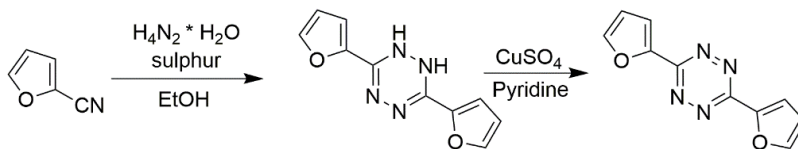
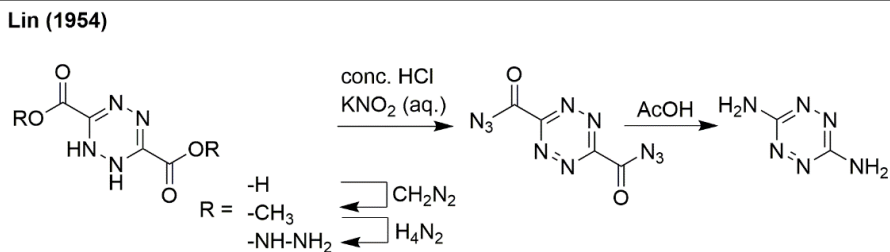
Researchers also explored the use of (dihydro)tetrazines as intermediates to functionalize into new tetrazines (**Scheme 5**). For instance, Lin^[29] was able to modify carboxy-dihydrotetrazine and produce 3,6-diamino-1,2,4,5-tetrazine via Curtius rearrangement of the azide intermediate. Marcus^[30]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

Chapter 1



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

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

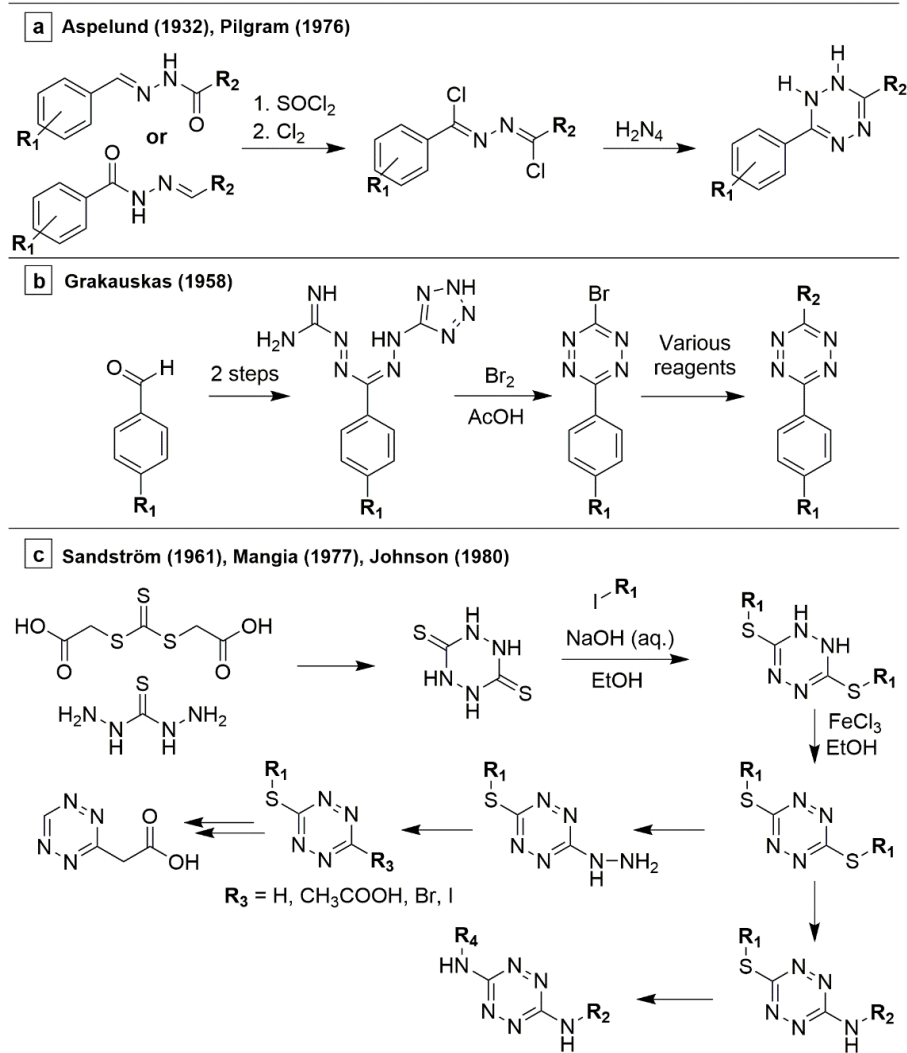
Wiley (1957)**Carboni (1958)****Dallacker (1960)****Abdel-Rahman (1968), Bowie (1972), Pavlov (1986)****Scheme 4:** Several examples of developments into symmetrical tetrazines by use of alternative approaches.**Lin (1954)****Scheme 5:** An example of developments into symmetrical tetrazines by modification of existing tetrazines.

Asymmetric functionalized tetrazines

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

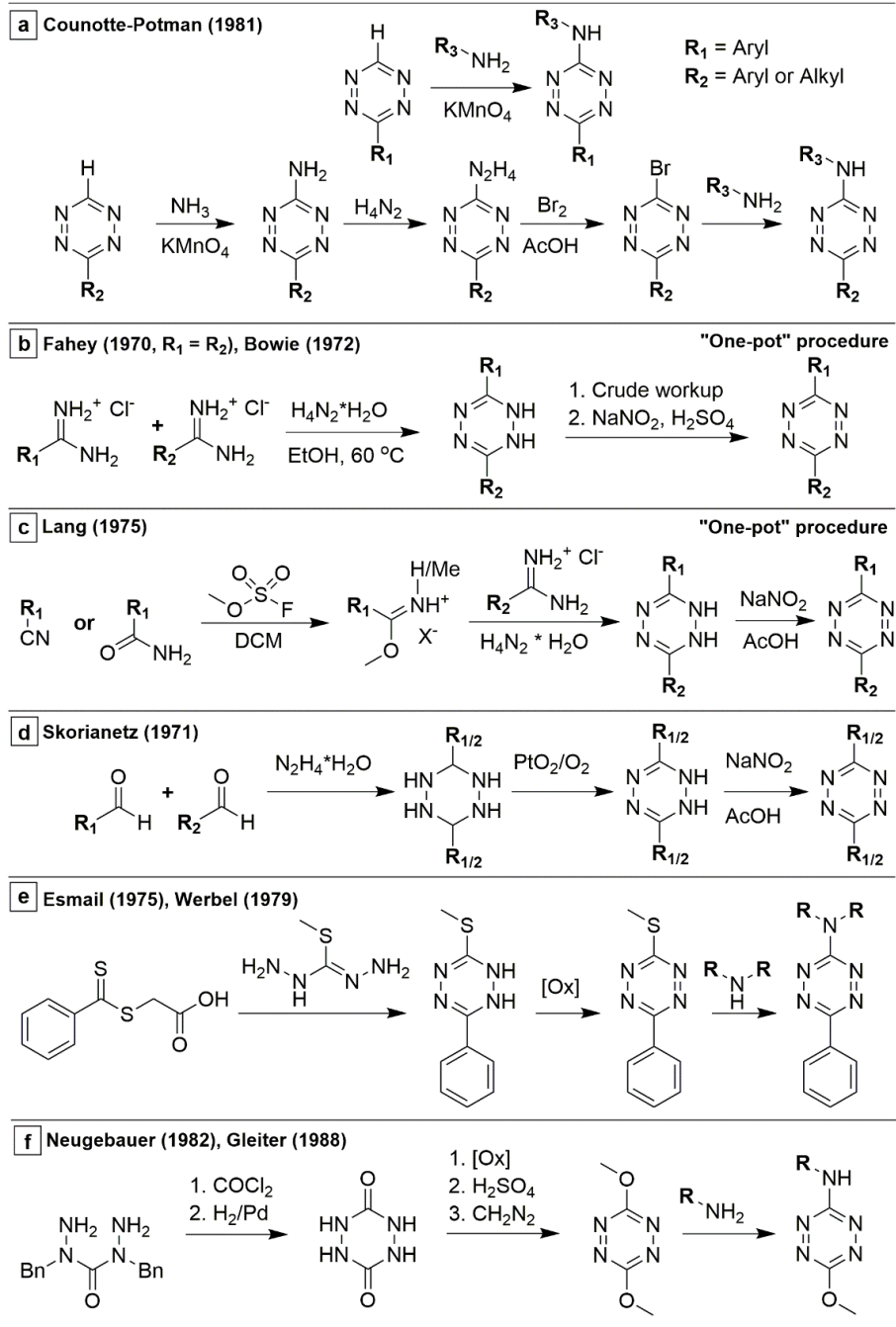
Counotte-Potman, while exploring the mechanism of the substitution of amines and halides by hydrazine in tetrazines^[41a], found an alternative way towards asymmetric hydrazino-tetrazines from asymmetric hydro-functionalized tetrazines^[41b] (**Scheme 7-a**). Furthermore, a new method^[41c] of alkylation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

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



Scheme 6: Synthesis approaches towards asymmetric tetrazines.

Chapter 1



Scheme 7: Synthesis approaches towards asymmetric tetrazines.

Starting Material	R ₁ =	R ₂ =	Reagents	Oxidation	Author	Year
1,4-Dichloro azines	phenyl	benzhydryl	H ₄ N ₂ *H ₂ O	Iso-amyl nitrite	H. Aspelund ³¹	1930
	benzhydryl	benzhydryl			H. Aspelund ³²	1932
	methyl	benzhydryl				
	aryl	perfluoroalkyl	H ₄ N ₂ (95%), EtOH	FeCl ₃	K. Pilgram ^{39a}	1976
	<i>m</i> -nitrophenyl	<i>p</i> -CF ₃ -phenyl	H ₄ N ₂ *H ₂ O, MeCN	AcOH, NaNO ₂	D.S. Liu ^{39b}	2012
	<i>t</i> -butyl / aryl	aryl	H ₄ N ₂ *H ₂ O, EtOH	AcOH, NaNO ₂	D. Wang ^{40a}	2013
	2-pyridyl	COO-Et	H ₄ N ₂ *H ₂ O, EtOH	AcOH, NaNO ₂	A. Jemas ^{40b}	2022
Formazans	aryl	Br	Bromine, AcOH	-	V.A. Graskausas ³³	1958
					V.A. Ershov ³⁴	1971
					L.M. Werbel ³⁵	1979
Aldehydes	methyl	ethyl, <i>i</i> -propyl, <i>t</i> -butyl	H ₄ N ₂ *H ₂ O	1. PtO ₂ /O ₂ 2. AcOH, NaNO ₂	W. Skorianetz ⁴⁵	1971
(CH ₃ S) ₂ -Tetrazine	methylthio	H, NH ₃ , NNNH ₂ , CH ₂ COOH, Cl, Br	-	-	(J. Sandström) ³⁶ A. Mangia ³⁷	(1961) 1977
	R ₁ -N	R ₂ -N	-	-	J.L. Johnson ³⁸	1980
Hydro-tetrazines	alkylamino	aryl, alkyl	-	-	A.D. Counotte-Potman ⁴¹	1981
Amidinium Salts	aryl	aryl (R ₁ = R ₂)	H ₄ N ₂ *H ₂ O, EtOH	H ₂ SO ₄ , NaNO ₂	J.L. Fahey ^[42]	1970
	aryl	aryl, methyl	H ₄ N ₂ *H ₂ O, MeOH	HNO ₂	R.A. Bowie ^[43]	1972
	aryl	aryl, alkyl			S.A. Lang ^[44]	1975
Exotic	methylthio	phenyl	-	-	R. Esmail ⁴⁶	1975
	R ₁ R ₂ -N	aryl	-	-	L.M. Werbel ³⁵	1979
	R ₁ -N	O-Me	-	-	R. Gleiter ^{47b}	1988

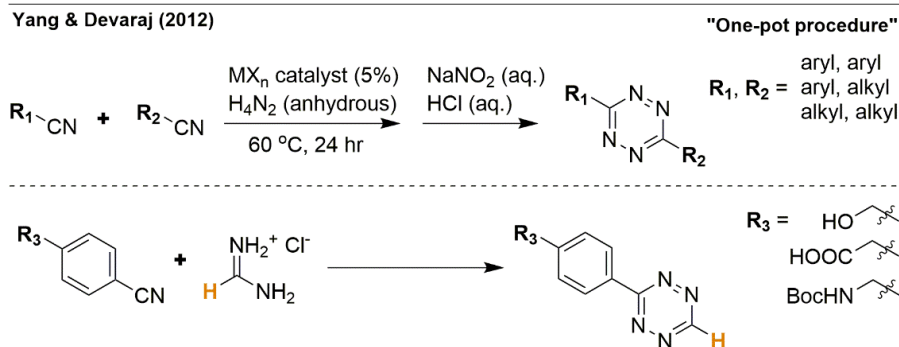
Table 2: Early literature on the synthesis of asymmetrical tetrazines.

The use of catalysts in one pot tetrazine synthesis

The amount of research performed on the synthesis of tetrazines steadily increased into the early 21st century, however was mostly limited to the techniques described above, sometimes with small variations. This changed in 2021 when Yang^[48] found that the use of soluble metal salts at increased temperature could catalyze the reaction of tetrazines from nitriles. The metal ions could coordinate to the nitrile starting material and, by acting as a Lewis acid, could activate the initial hydrazone formation, in this way kickstarting the reaction process towards dihydrotetrazines. Additional to the use of soluble metal salts, the reaction was performed in a sealed pressure tube, which prevented the formed ammonia to boil off at the 60 °C reaction temperature. It appeared that zinc²⁺ and nickel²⁺ performed very well in combination with weaker coordinating counterions, and near quantitative yields were obtained.

Chapter 1

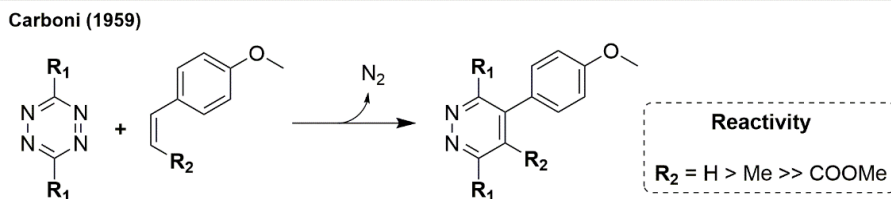
Additionally, the reaction appeared to perform better on aryl nitriles compared to alkyl nitriles. When performing the reaction on mixed nitriles ($R_1 \neq R_2$) a whole variety of asymmetric tetrazines could be obtained, which were otherwise very difficult or impossible to obtain.



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

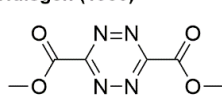
IEDDA chemistry and bioorthogonal reactions

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



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

Huisgen (1980)



1.27

 11.7×10^3 

113

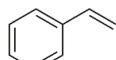
 5.4×10^3

Thalhammer (1990)

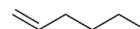
A, B =



363, 2820



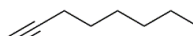
nd, 42



56, nd

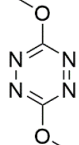


0.25, nd



0.75, nd

A

 448×10^3 , 715×10^3 

3290, 7410



235, 56



1040, 1480

 549×10^3 , 1140×10^3 

0.87, 0.19

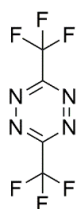


84, 13



34, 2.3

B



77, 148



1360, 2210



78, nd

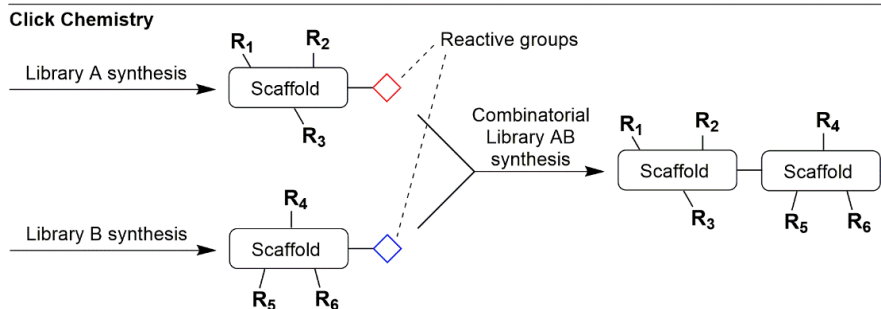


0.05, nd

 12.7×10^6 , 1.6×10^6  29.7×10^3 , nd k ($\times 10^{-3} \text{ M}^{-1} \text{ s}^{-1}$) 10×10^3 , 14.9×10^3

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

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



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

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

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

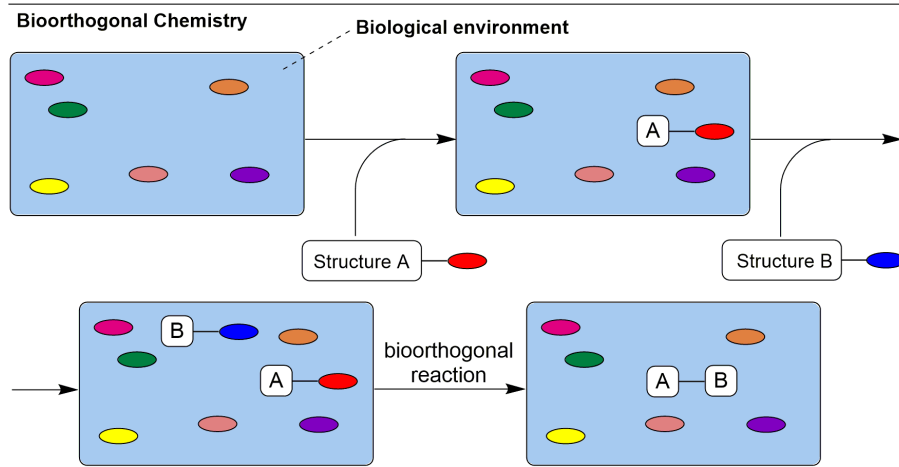
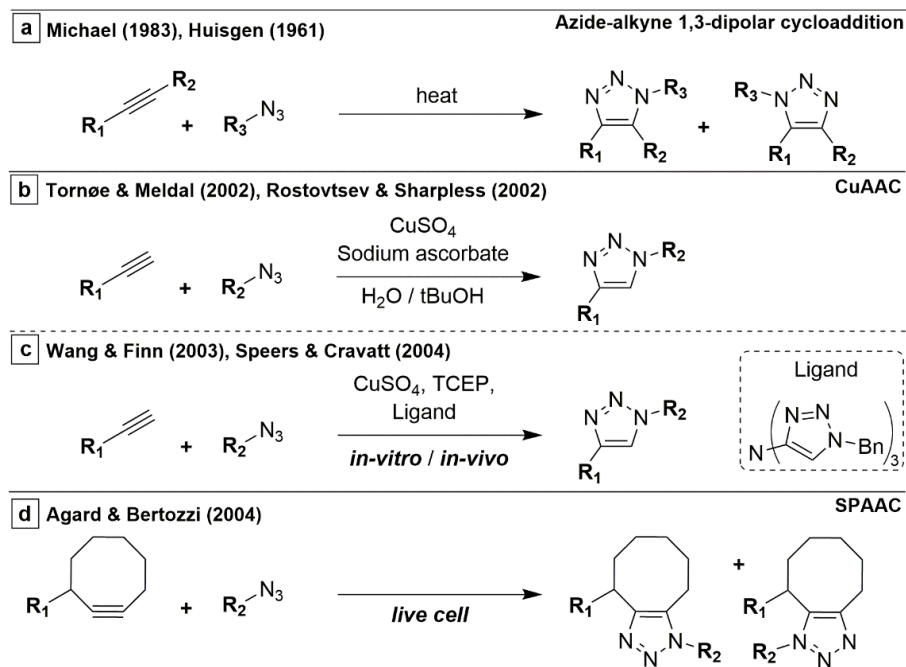


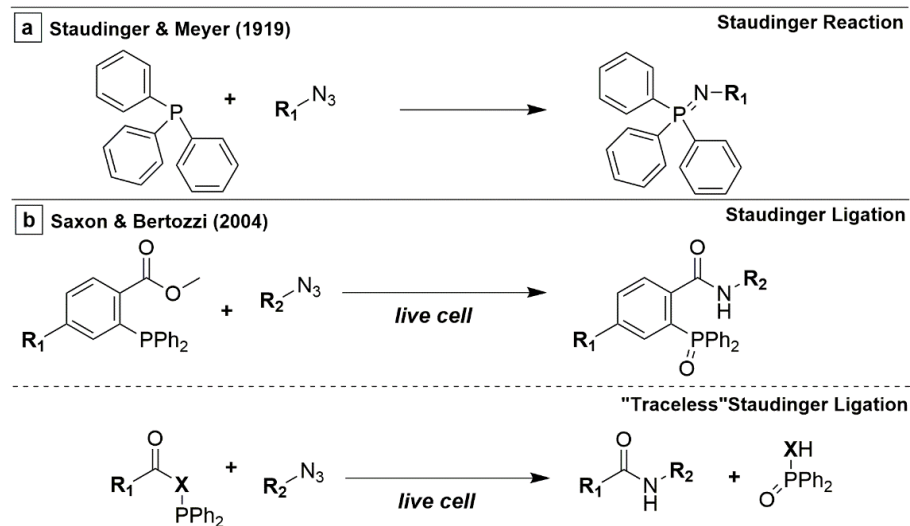
Figure 1: A representation of bioorthogonal chemistry: (1) the biological environment to which (2) structure A with a “red” bioorthogonal group is added, and then (3) structure B with a “blue” bioorthogonal group is added, after which (4) structures with “red” and “blue” bioorthogonal groups react with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



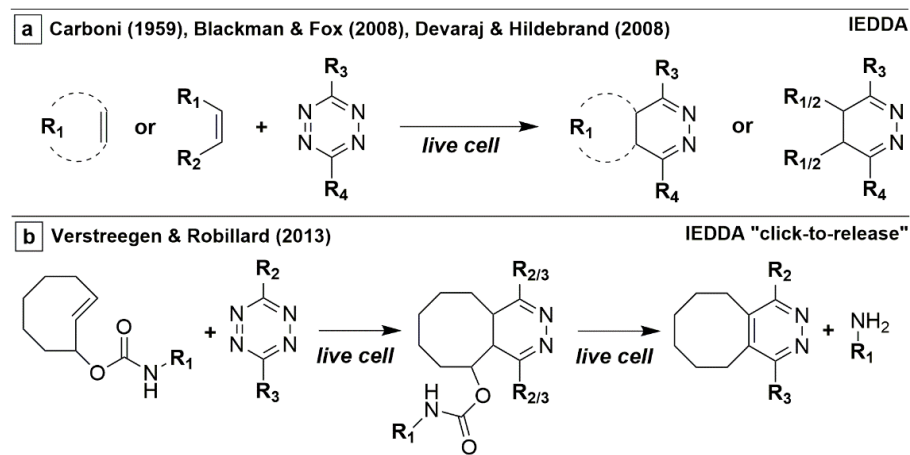
Scheme 12: Overview of the azide-alkyne 1,3-dipolar cycloaddition, CuAAC and SPAAC reactions.

Chapter 1

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



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



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

Aim and outline of this thesis

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

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

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

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

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

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

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

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