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Synthesis of a combinatorial compound library based on the double click reaction

Master's thesis

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"I declare that this thesis is my original author's work, which has been composed solely by myself (under the guidance of my consultant). All the literature and other resources from which I drew information are cited in the list of used literature and are quoted in the paper. The work has not been used to get another or the same title. "

Hradec Králové, August 2022 .....

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Table of Contents

1. List of Abbreviations

AChE	acetylcholinesterase
ACN	acetonitrile
AD	Alzheimer's disease
AS-MS	affinity selection-mass spectrometry
BSA	bovine serum albumin
BuChE	butyrylcholinesterase
ChEs	cholinesterases
CuAAC	copper-catalyzed azide–alkyne cycloaddition
DCM	dichloromethane
DD-CoA	2-*trans*-dodecenoyl-coenzyme A
DIPEA	*N,N*-diisopropylethylamine
DMF	dimethylformamide
DMSO	dimethylsulfoxide
DTNB	5,5'-dithio-bis-(2-nitrobenzoic acid)
EDTA	ethylenediaminetetraacetic acid
EtOAc	ethyl acetate
EtOH	ethanol
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HR-MS	high-resolution mass spectrometry
HTS	high-throughput screening
LC	liquid chromatography
LC-MS	liquid chromatography-mass spectrometry

MOE	Molecular Operating Environment
NMR	nuclear magnetic resonance
OBOC	one-bead-one-compound
PBP	penicillin-binding protein
PEP	phosphoenolpyruvate
PIPES	piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)
Ro5	Lipinski's rule of 5
S_N2	nucleophilic substitution type 2
SPAAC	strain-promoted azide–alkyne cycloaddition
TB	tuberculosis
TCEP	tris(2-carboxyethyl)phosphine
TEA	triethylamine
THF	tetrahydrofuran
TLC	thin layer chromatography
TsOH	*p*-toluenesulfonic acid
UNAG	UDP-*N*-acetylglucosamine
UV	ultraviolet
VS	virtual screening

2. Abstract (English)

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Faculty of Pharmacy in Hradec Králové

Department of Pharmaceutical Chemistry and Pharmaceutical Analysis

Author: Mária Krutáková

Supervisor: Assoc. Prof. PharmDr. Jan Zitko, Ph.D.

Title: Synthesis of a combinatorial compound library based on the double click reaction

Click chemistry is a powerful tool in drug discovery. It is very efficient in creating compound libraries through the combinatorial methodology. The copper(I)-catalyzed 1,2,3-triazole-forming reaction between azides and terminal alkynes has become the gold standard of click chemistry due to high reaction efficiency, mild reaction conditions, chemo- and regioselectivities. The molecules with triazole moiety display a broad spectrum of favorable properties and have been used in the development of antibacterial, antiviral, anti-inflammatory, anticancer, and anti-tubercular agents. This work focused on preparing a compound library using double-click reactions. Firstly, we synthesized several compounds with two alkyne groups ("alkyne cores") and a diverse group of structurally simple azides. In the next step, one equivalent of alkyne core was reacted with two equivalents of all prepared azides. The reaction between each alkyne core and ten azides yielded 100 compounds. The prepared libraries of compounds were assayed on several validated pharmacological targets, including human butyryl- and acetylcholinesterase, penicillin-binding protein 1b, InhA, and MurA. Inhibition of butyrylcholinesterase and InhA was observed, thus clearly demonstrating the validity of the initial idea. As a continuation of this work, various methods will be used to identify compounds causing inhibition from the compound mixtures.

Keywords: azide–alkyne cycloaddition; click chemistry; combinatorial chemistry; compound library

3. Abstract (Slovak)

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Katedra farmaceutickej chémie a farmaceutickej analýzy

Autor: Mária Krutáková

Vedúci diplomovej práce: doc. PharmDr. Jan Zitko, Ph.D.

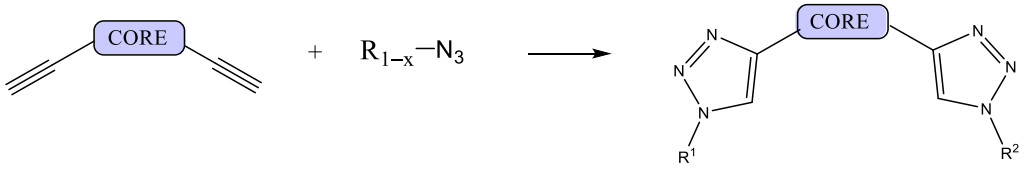
Názov: Syntéza kombinatoriálnej knižnice zlúčenín pomocou dvojitej click reakcie

Click chémia je významným nástrojom pri objavovaní liečiv. Je veľmi užitočná pri vytváraní knižníc zlúčenín prostredníctvom kombinatoriálnych metód. Meďou(I) katalyzovaná 1,2,3-triazolotvorná reakcia medzi azidmi a terminálnymi alkínmi sa stala zlatým štandardom click chémie vďaka vysokej účinnosti reakcie, miernym reakčným podmienkam, chemo- a regioselektivite. Zlúčeniny, obsahujúce triazol vo svojej štruktúre, vykazujú široké spektrum výhodných vlastností a môžu byť uplatnené ako potenciálne antibakteriálne, antivírové, protizápalové, protinádorové látky alebo ako antituberkulotiká. Táto práca bola zameraná na prípravu knižnice zlúčenín s využitím dvojitej click reakcie. Najskôr sme nasyntetizovali niekoľko zlúčenín s dvoma alkínovými skupinami („alkínové jadrá“) a rôznorodú skupinu azidov s jednoduchou štruktúrou. V nasledujúcom kroku reagoval jeden ekvivalent alkínového jadra s dvoma ekvivalentmi všetkých pripravených azidov. Reakcia medzi každým alkínovým jadrom a desiatimi azidmi mala výťažnosť 100 zlúčenín. Pripravené knižnice zlúčenín sa testovali na niekoľkých overených farmakologických cieľoch, vrátane ľudskej butyryl- a acetylcholínesterázy, penicilín viažucich proteínov 1b, InhA a MurA.
U butyrylcholínesterázy a InhA bola pozorovaná inhibícia, čím bola jasne preukázaná správnosť pôvodnej myšlienky. V nadväznosti na túto prácu budú použité rôzne metódy pre identifikáciu jednotlivých zlúčenín spôsobujúcich inhibíciu.

Kľúčové slová: azid–alkín cykloadícia; click chémia; kombinatoriálna chémia; knižnica zlúčenín

4. Aim of work

This work aimed to synthesize a double click compound library using copper-catalyzed azide–alkyne cycloaddition (CuAAC), also known as click reaction (Scheme 1). If we take, for instance, one dialkyne core and 30 different azides, the reaction gives, in theory, 30^2 = 900 various compounds. If we use 30 cores and 30 azides, we can theoretically produce 27 000 compounds. Therefore, a large number of compounds can be formed in a relatively short time under simple reaction conditions.



Scheme 1: Idea for double click compound library, X = number of azides.

The click reaction itself was preceded by the preparation of alkyne and azide intermediates. The intention was to design dialkynes with different physicochemical properties, positions on the core ring, and distances between both alkyne moieties. Some examples of the proposed cores are displayed in Figure 1.

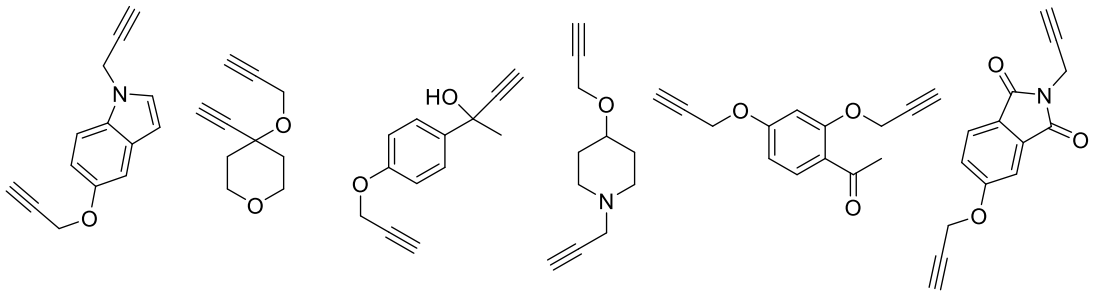


Figure 1: The example of proposed dialkyne cores.

The proposed azides were designed as simple structures (aromatic/aliphatic) with diverse physicochemical properties (lipophilic/hydrophilic, acidic/basic/neutral). Some examples of the proposed cores are displayed in Figure 2.

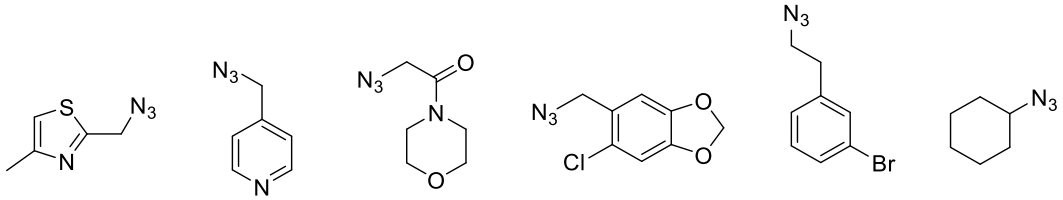


Figure 2: The example of proposed azides.

The biological activity of prepared compound libraries was tested on several enzymes. Individual compounds responsible for enzyme inhibition will be examined in the future when more complex compound libraries will be prepared.

5. Theoretical part

5.1 Introduction

Drug discovery can be characterized as the long and demanding process through which potential therapeutic agents are identified. The drug discovery campaigns aim to recognize new molecules that may be valuable in treating diseases that do not have definitively beneficial therapies and are absolutely or potentially life-threatening.[1] The process of discovering, testing, and approving a new drug has changed over the last century. Drug discovery has evolved from the isolation of active ingredients from traditional remedies and natural sources to a multidisciplinary procedure that involves the cooperation of biologists, pharmacologists, and chemists.[2] Medicinal chemistry and *in vitro* screening have been accelerated by incorporating automation and developing technologies such as recombinant DNA and transfection technology. High-throughput screening (HTS), parallel synthesis, automation technologies, and combinatorial chemistry have facilitated the synthesis and biological evaluation of many potentially valuable compounds.[3] The rational design of ligands is still the "gold standard" in pharmaceutical chemistry, especially when the target is structurally defined.[2] Current drug discovery relies on screening vast chemical libraries against various intracellular and extracellular targets to find novel therapeutic agents with the desired mode of action. To aid the process and build a screening library, click chemistry can be used. Thus, the drug discovery process can be notably accelerated by utilizing a few practical and reliable reactions. Click chemistry simplifies compound synthesis, providing faster lead discovery and optimization, making it one of the most powerful drug discovery tools.[4]

5.2 Combinatorial chemistry in the drug discovery

Combinatorial chemistry represents a broad spectrum of techniques that can rapidly generate a massive number of structurally diverse compounds. These extensive, organized collections of compounds, called libraries, can be assembled from pure compounds, compound mixtures, or virtual structures created by computational methods. The prepared chemical libraries can be subsequently screened to identify the desired interaction with a biological target. Afterward, the interaction can be specified directly

(position-addressable libraries), or through decoding (with chemical or genetic methods).[5] Compared to traditional pharmaceutical research, the undeniable advantage of combinatorial chemistry is that we can produce hundreds, thousands, or even millions of compounds in a shorter time, with lower costs and higher efficiency (Figure 3).[6] In the beginning, most of the combinatorial chemistry methods were introduced in the field of peptide chemistry. Since the therapeutic use of peptides is minor, mainly due to poor oral bioavailability and metabolic instability, the focus of combinatorial chemistry has gradually shifted to peptide analogs and, lastly, to small organic molecules.[7] Numerous combinatorial library techniques can be applied in this area involving split and mix, parallel chemistry, solution phase, and solid phase synthesis.[8] Parallel synthesis can be accomplished robotically or manually and carried out in solution or on a solid support. The structural diversity of created libraries is usually smaller. On the other side, the one-bead-one-compound (OBOC) libraries prepared by a split-mix strategy result in a greater diversity of bead-bound library compounds. However, the positive bead isolated from screening has to be decoded using a physical or chemical barcode.[5] DNA-encoded chemical libraries are sets of molecules individually linked to DNA tags that serve as identification barcodes.[9] Other types of libraries are biological such as yeast display, phage-display, and polysome-display peptide libraries.[5]

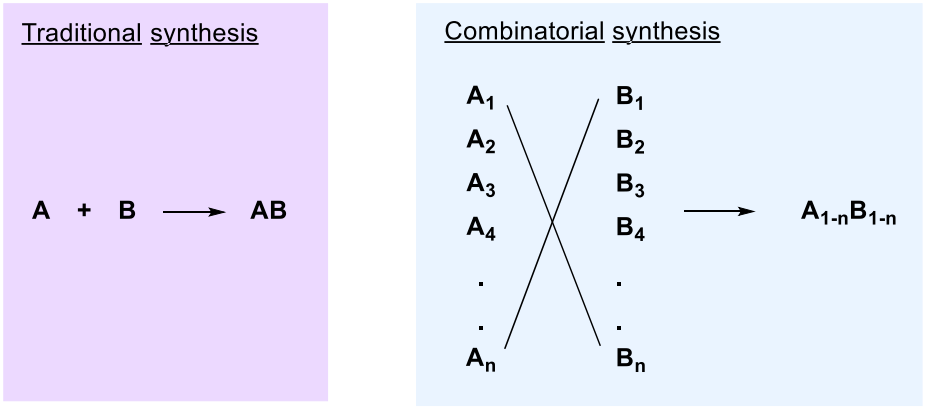


Figure 3: Comparison of traditional and combinatorial syntheses. Adapted and modified from Terrett et al.[6]

5.2.1 Screening of combinatorial libraries

Screening of combinatorial libraries can be divided into physical screening and virtual screening (VS). VS is an *in silico* technique that uses computational methods to predict or simulate how a compound interacts with a target protein.[5] The aim of using virtual methods is to speed up the discovery process, reduce the number of candidates to be tested experimentally and rationalize their selection.[10] Due to the enormous potential of VS methodologies, various structure-based methods (molecular docking, structure-based pharmacophores, and *de novo* design) and ligand-based methods (ligand-based pharmacophore screening, machine learning approaches, and quantitative structure-activity relationships) have been developed.[11] Physical screening approaches like HTS can investigate the activity of hundreds of thousands of compounds per day, providing real results. However, these methods are much more expensive and slower compared to VS. The choice of screening techniques largely depends on the nature of the combinatorial libraries. The most frequent assay to screen a library is to determine the binding of the library compounds to the biomolecular target using enzymatic and cell-based assays.[5] Of note, one of the HTS techniques is affinity selection-mass spectrometry (AS-MS).

5.2.2 Affinity selection-mass spectrometry

AS-MS enables rapid screening of huge collections of compounds to identify ligands for the specific biological target. In AS-MS, the targeted protein does not need to be labeled, and compounds can be tested as mixtures and do not have to be tagged as well because each compound is identified by its unique mass. AS-MS may enable the discovery of ligands that act through allosteric binding and other mechanisms. Furthermore, AS-MS allows the identification of molecular targets of compounds with an unknown mechanism. On the other hand, this method does not give information regarding the target binding site. The general workflow involves incubating a mixture of molecules with a target biomolecule in solution (Figure 4a).[12] The number of compounds used in the mixture usually falls in the range of 400–3 000, but the screening of 20 000 compounds at once was also reported.[13] Molecules that do not bind to the biomolecule are separated, leaving the binder-target complexes in the solution. Washing, (ultra)filtration or size-exclusion chromatography is used to eliminate unbound compounds from binder-target complexes

(Figure 4b). Subsequently, complexes are denatured (Figure 4c), allowing the identification of ligands by applying liquid chromatography (LC) combined with high-resolution mass spectrometry (HR-MS, Figure 4d). In the incubation and binding stage, the target is commonly present in the molar excess compared to the ligands, thus avoiding competition that could preclude the detection of ligands with lower affinity for the target. The sensitivity of modern mass spectrometers means that only µg quantities of ligands and targets are required to test a huge compound library. Moreover, the screening is provided with non-encoded libraries of pooled compounds; therefore, both time and costs are saved compared to HTS techniques that assay compounds individually.[12,14]

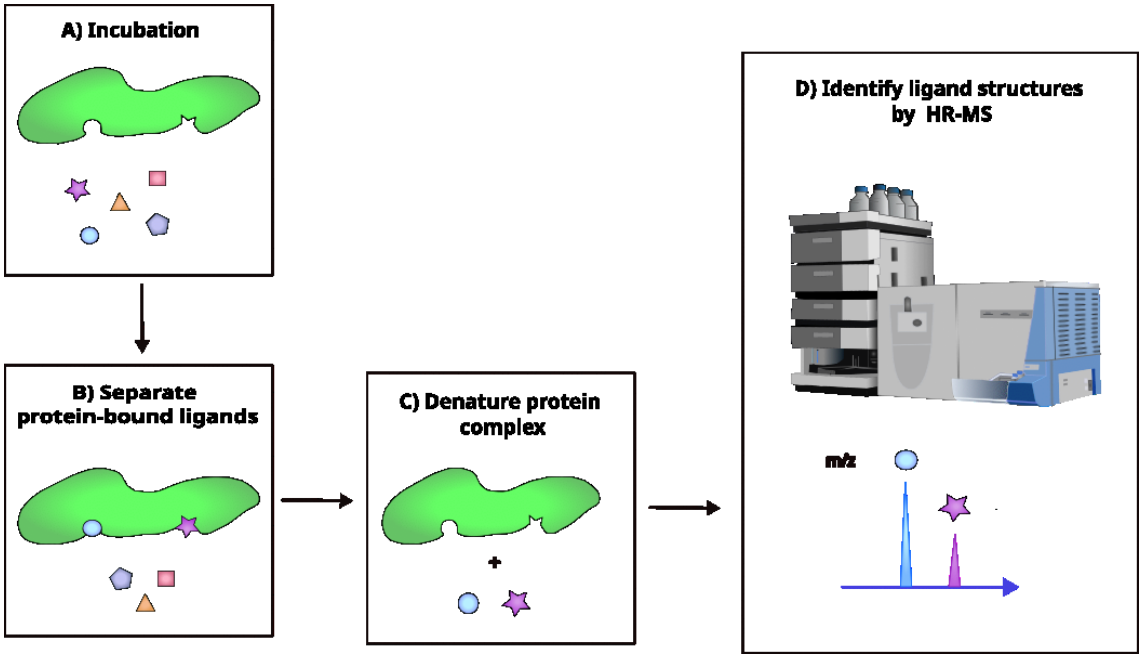


Figure 4: General workflow of AS-MS screening for protein-ligand identification. Adapted and modified from Prudent et al.[12]

5.3 Click chemistry

Examination of molecules produced by nature (polysaccharides, nucleic acid, and proteins) shows that the carbon-heteroatom bond is preferred over the carbon-carbon bond. From monomers, relatively simple building blocks are created by large oligomers or polymers through the carbon-heteroatom links (C-X-C). This strategy can be described as a version of combinatorial chemistry in a "nature's way" with impressive diversity and modularity. Following nature, K. B. Sharpless and his co-workers in 2001 presented a

new synthetic approach, click chemistry.[15] The majority of click chemistry reactions involve forming carbon-heteroatom bonds, mainly nitrogen, oxygen, and sulfur. Click chemistry reactions have been described as "modular, wide in scope, give very high yields, generate only inoffensive byproducts that can be removed by nonchromatographic methods, and be stereospecific (but not necessarily enantioselective)."[16] The reactions have simple reaction conditions, using easily available reagents and starting materials, green solvents, water, or even no solvents. Click chemistry has diverse applications in several research areas, such as polymer and material sciences, bioconjugation, and organic synthesis. Moreover, click chemistry reactions are a meaningful part of drug discovery; they are very efficient in creating compound libraries through combinatorial methodologies. Compound libraries obtained this way could contribute to the discovery of bioactive molecules and therapeutic agents.[17]

5.3.1 Types of click reactions

Click reactions can be broadly classified into four categories (Figure 5).

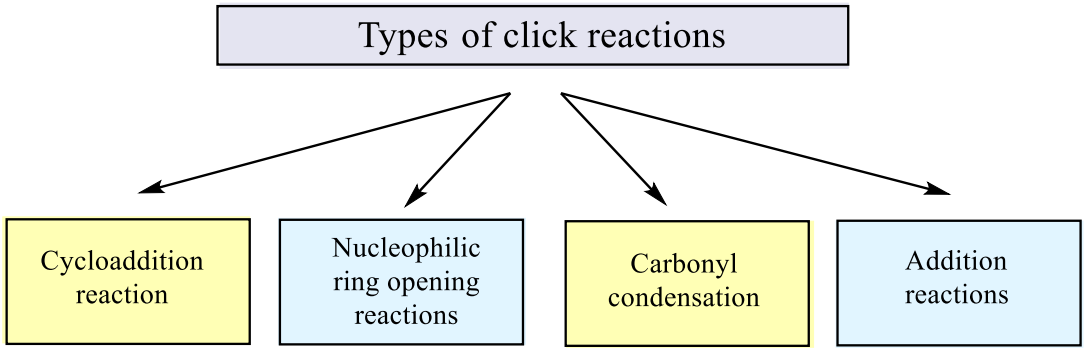


Figure 5: Types of click reactions.

5.3.1.1 Cycloadditions

These primarily refer to 1,3-dipolar cycloaddition and hetero-Diels–Alder cycloadditions. CuAAC is the most popular reaction in drug discovery due to its biocompatibility, specificity, and favorable physicochemical properties of triazoles, products of the reaction (Figure 6a).[18] Hetero-Diels–Alder reaction involves at least one heteroatom and can be defined as a [4+2] cycloaddition between an electron-rich diene and electron-poor dienophile to form various heterocycles (Figure 6b). Some attractive features of hetero-

Diels–Alder are high yields and minimal side reactions; in addition, the presence of a metal catalyst is not required.[19] The strain-promoted alkyne-azide cycloaddition (SPAAC) is a biorthogonal, copper-free reaction in which various cyclooctyne derivates react with azide-bearing molecules (Figure 6c). Under mild conditions, a stable triazole product is formed simply by mixing and stirring.[20]

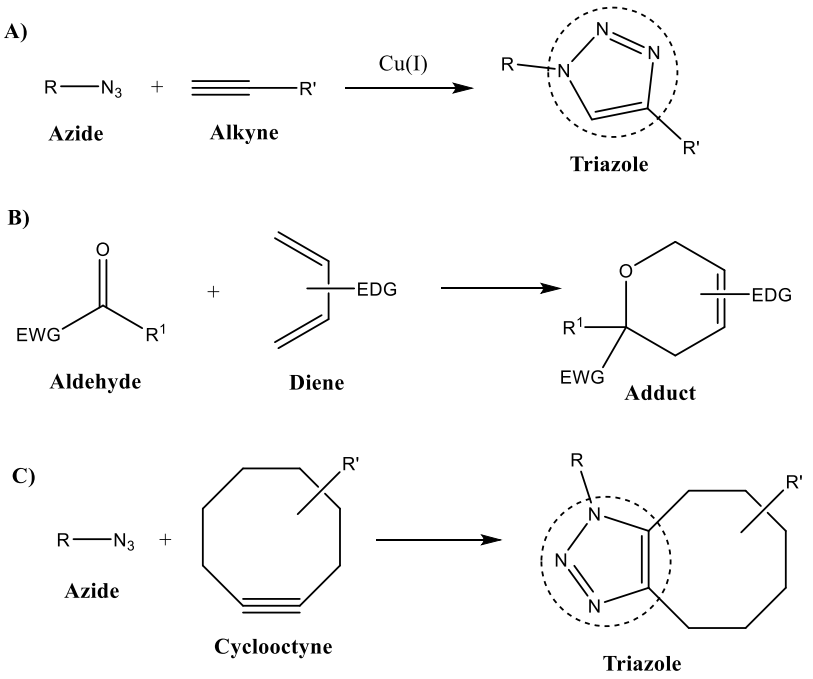


Figure 6: (a) Copper-catalyzed azide–alkyne cycloaddition. (b) An Example of a standard hetero Diels–Alder reaction, **EWG** = electron-withdrawing group, **EDG** = electron-donating group. (c) Strain-promoted azide–alkyne cycloaddition. Adapted and modified from Kaur et al.[18]

5.3.1.2 Nucleophilic ring-opening reactions

These reactions are realized by the nucleophilic opening of strained heterocyclic electrophiles such as epoxides, aziridines, cyclic sulfamides, cyclic sulfates, and cyclosulfonium ions. The first two mentioned, epoxy derivates and aziridines, are the most commonly used substrates for nucleophilic attack (Figure 7). Epoxides (aziridines) contain a three-membered ring with high tension. Nucleophile attacks the electrophilic C of the C-O (C-N) bond, causing it to break, resulting in ring-opening and relieving the ring strain. The reaction is often performed in alcohol/water solvent or without solvent and leads to the formation of diverse bioactive compounds.[21]

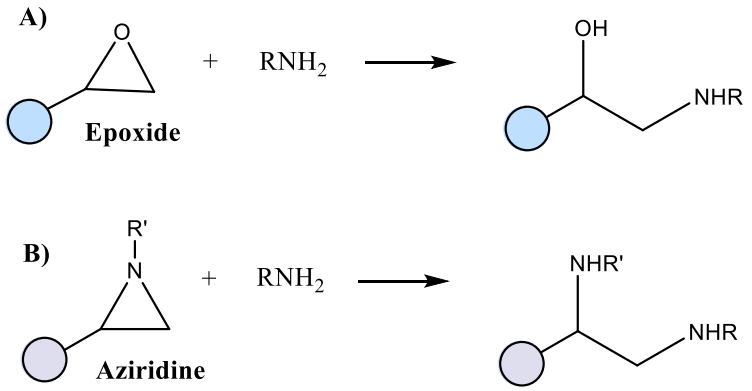


Figure 7: An example of nucleophilic ring-opening reactions for (a) epoxide derivates, (b) aziridines. Adapted and modified from Kaur et al.[18]

5.3.1.3 Carbonyl condensation

Carbonyl condensation includes carbonyl reactions of non-aldol types, such as the formation of ureas, thioureas, hydrazones, oximes, amides, aromatic heterocycles, etc. Carbonyl chemistry of aldol type is not considered click chemistry because it has a longer reaction time and gives byproducts.[22] Examples of a carbonyl reaction for hydrazones and oximes are displayed in Figure 8.

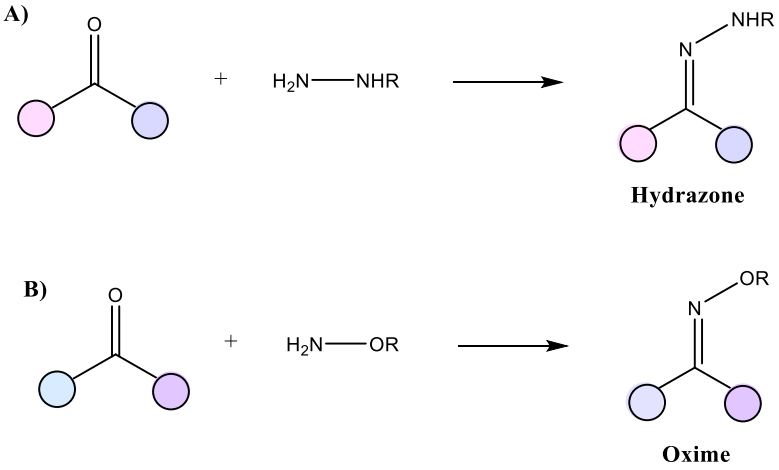


Figure 8: An example of non-aldol type carbonyl reactions (a) hydrazones, (b) oximes. Adapted and modified from Kaur et al.[18]

5.3.1.4 Addition reactions

Reactions include additions to carbon-carbon multiple bonds, such as thiol-ene and thiol-yne.[18] Thiol-ene/ -yne additions are coupling reactions between alkene/alkyne to form a carbon-sulfur bond. The product of the thiol-ene click reaction is an alkyl sulfide,

and the reaction can proceed through two mechanisms: free-radical addition and Michael addition (Figure 9a,b).[23] The thiol-yne reaction is typically facilitated by a radical initiator or a photoinitiator (Figure 9c) and proceeds via a radical-mediated mechanism, resulting in alkenyl sulfide products.[24] Thiol-ene/yne click reactions have found their application in polymer and material sciences.[21]

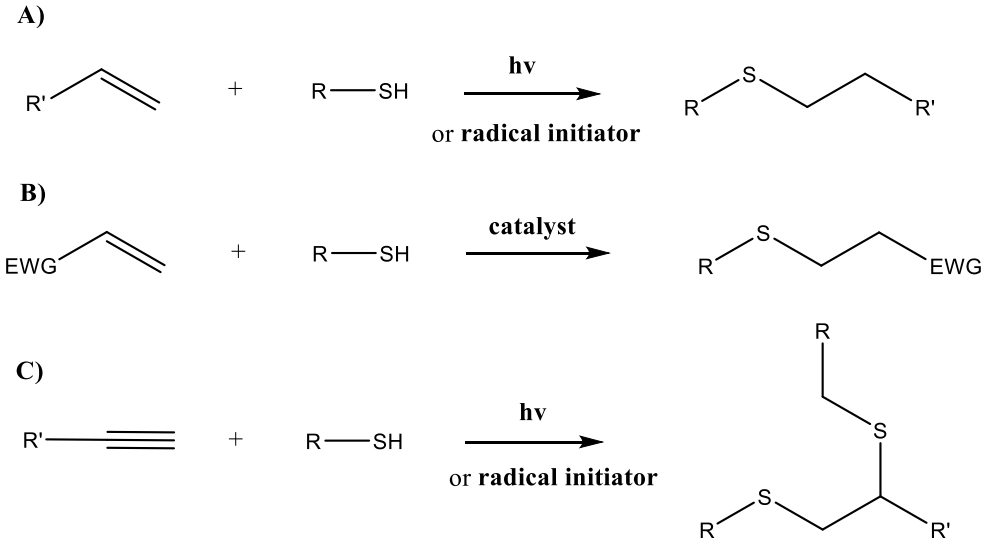
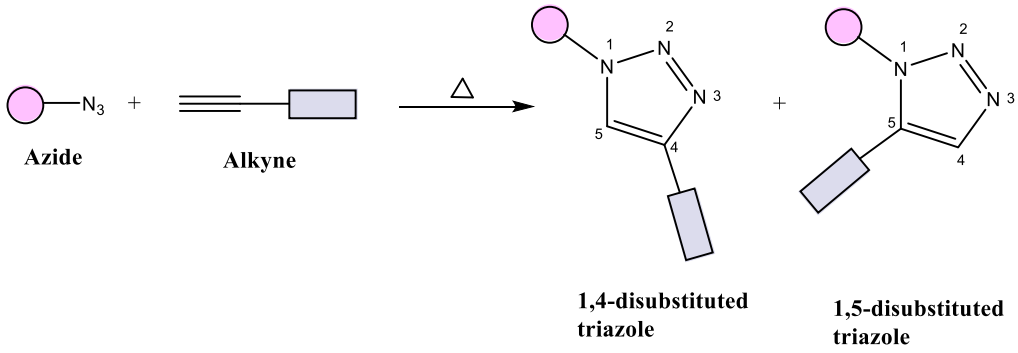


Figure 9: (a) Thiol-ene reaction; (b) Thiol-Michael addition; **EWG** = electron withdrawing group; (c) Thiol-yne reaction. Adapted and modified from Sharma et al.[25]

5.3.2 Azide–alkyne Huisgen cycloaddition

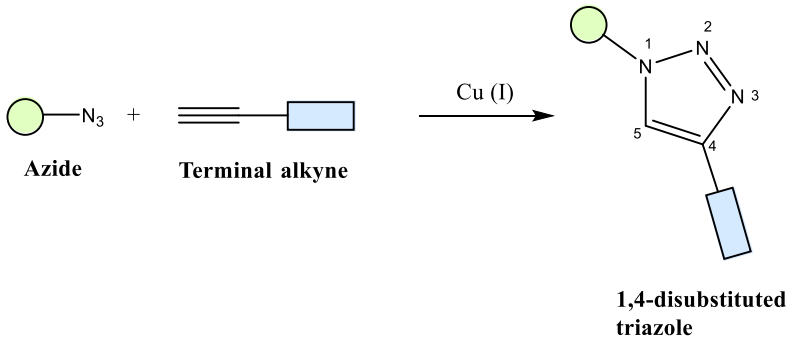
Huisgen 1,3-dipolar azide–alkyne cycloaddition belongs to a large class of pericyclic reactions between 1,3-dipole (azide) and a dipolarophile (alkyne). It transforms terminal alkynes and organic azides to the corresponding disubstituted 1,2,3-triazoles. Azides fulfill the condition of 1,3-dipole because they have delocalized electrons and charge separation over three atoms.[26] Unfortunately, the classic Huisgen 1,3-cycloaddition proceeds slowly without a catalyst and usually requires high temperatures. Furthermore, the reaction is not regioselective and produces an equimolar mixture of 1,4- and 1,5-disubstituted triazoles, which is not favorable when developing compound libraries (Scheme 2).[18]



Scheme 2: Thermal Huisgen 1,3-dipolar cycloaddition between azide and alkyne.

5.3.3 Copper-catalyzed azide–alkyne cycloaddition

In the presence of the catalytic amount of copper(I), there is an enormous acceleration of reaction rate, thus eliminating the need for a high temperature to perform the reaction. Additionally, a dramatic improvement of the regioselectivity is observed; only 1,4-disubstituted-1,2,3-triazole is formed (Scheme 3).[27] Simple reaction conditions, high yields, easy workup, regioselectivity, chemospecificity, and fast reaction kinetics made this reaction the model example of click chemistry. Despite all the advantages this reaction offers, some crucial limitations exist, such as the biocompatibility of 1,2,3-triazoles, copper toxicity and its *in vivo* safety. Some azides are explosive, which can represent a problem, especially in a large-scale synthesis in pharmaceutical research.[18]



Scheme 3: Copper-catalyzed azide–alkyne cycloaddition.

5.3.3.1 Copper catalyst

The active catalytic copper is in oxidation state +1, although precatalyst mixtures can contain copper(0), copper(I) species, or copper(II) salts. The copper(II) salts (commonly $CuSO_4$) are used together with reducing agents (usually sodium ascorbate). An alternative

reducing agent, tris(2-carboxyethyl)phosphine (TCEP), is particularly suitable for application in biological systems because it also protects cysteine residues from oxidative coupling.[28,29] The advantages of reducing copper(II) salts include compatibility with oxygen and water, no need for a base in the reaction, and yields are usually very high, without the presence of byproducts. Another option is the direct addition of copper(I) salts, for example, copper(I) iodide, copper(I) bromide, or copper(I) acetylide into the reaction mixture.[18] The copper needs to be stabilized against moisture and efficiently solubilized to avoid the disproportionation to copper(0) and copper(II). The method must be performed under an inert atmosphere, or the presence of amine bases such as triethylamine (TEA) or *N,N*-diisopropylethylamine (DIPEA) is required for the catalyst to work.[29,30] Most of the reactions with copper halide catalysis proceed smoothly at room temperature; however, there are many reports about using heating or applying microwave irradiation.[31] An alternative way to obtain the copper(I) catalyst is by *in situ* oxidation of elemental copper(0).[18] This route requires a longer reaction time (12–24 hours), but it usually produces very pure triazole with low levels of copper contamination. The reaction can be sped up by microwave irradiation at elevated temperature.[30,32]

5.3.3.2 Solvents

CuAAC is compatible with a large variety of solvents. Solvents for CuAAC (and for click chemistry generally) should fulfill the following criteria: availability, ability to dissolve a wide range of compounds, low cost, and chemical inertness toward the reactants and products formed in the reaction. The classic examples of organic solvents employed in CuAAC are dichloromethane (DCM), toluene, chloroform (non-coordinating solvents), tetrahydrofuran (THF), pyridine, dioxane (weakly coordinating solvents), dimethylformamide (DMF), dimethylsulfoxide (DMSO), acetone, ethanol (EtOH), methanol (polar solvents), and water alone or aqueous solvents including mixtures of water with alcohol or acetone.[18,28] An alternative way is to replace traditional organic solvents with biorenewable and biodegradable green solvents such as water, glycerol, 2-methyl-THF, γ-valerolactone, lactic acid, or deep eutectic solvents. Deep eutectic solvents combine two or three chemical substances, which form a new eutectic mixture by creating a tridimensional hydrogen-bond network. An example may be a mixture of quaternary ammonium salt in combination with hydrogen bond donors like polyols (ethylene glycol, glycerol), urea, choline chloride, biorenewable organic acids.[33]

5.3.3.3 Mechanism of reaction

Fokin, Sharpless, and co-workers proposed an early mononuclear mechanism that started with copper(I) acetylide formation. Coordination of azide to copper(I) acetylide results in a complex of all three components. The complex is subsequently transformed into six-membered copper(III) metallacycle. Ring contraction follows with a reduction of copper(III) to copper(I) to give copper triazolide. In the last phase, triazolide has to gain a proton from an alkyne. The exchange of proton between triazolide and alkyne can facilitate using the base. The early mechanism can be considered a good starting point for further studies of reaction mechanisms. One-step mechanism from neutral copper(I) acetylide was excluded after the results of an extensive density functional theory study in 2005. The formation of a six-membered metallacycle requires high activation energy. Subsequent computational and experimental studies suggested that adding another copper atom could overcome the activation barrier of metallacycle formation. That led to a change of the mechanistic view from mononuclear to dinuclear, which is a currently excepted mechanism. The dinuclear copper catalysis model involves two copper centers, and the reaction is initiated by forming π,σ-bis(copper) acetylide (Figure 10a). Two copper atoms participate in the reaction, one as a purely bound ligand and the other creating weak π complexation. The acetylide, which enlists in both π and σ bonding with copper, reacts with azide to generate azide/alkyne/copper(I) ternary complex (Figure 10b). Six membered copper(III) metallacycle formation occurs (Figure 10c), followed by the creation of copper(I) triazolide (Figure 10d), which deprotonates an alkyne to finish a cycle, and the final product 1,4-disubstituted 1,2,3-triazole (Figure 10e) is generated. Both π,σ-bis(copper) acetylide and copper(I) triazolide have been isolated and characterized with X-ray crystallography, which gave credibility to the dinuclear catalysis model.[34,35]

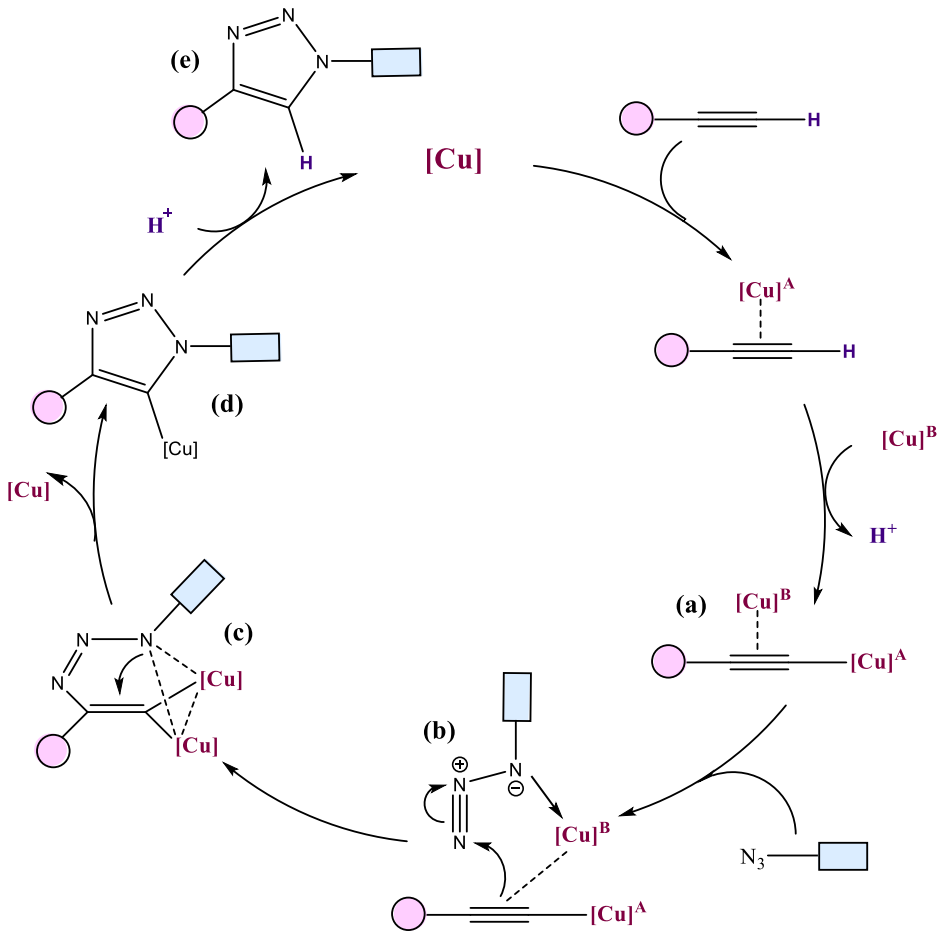


Figure 10: Dinuclear mechanism of CuAAC. (a) π,σ-bis(copper)acetylide, (b) azide/alkyne/copper(I) ternary complex, (c) copper(III) metallacycle, (d) copper(I) triazolide, (e) 1,4-disubstituted 1,2,3-triazole. Adapted and modified from Worrell et al.[36]

5.3.4 Ruthenium-catalyzed azide–alkyne cycloaddition (RuAAC)

Another catalyst applied in click chemistry is ruthenium. The most effective are ruthenium(II) complexes containing a [Cp*RuCl] unit in an aprotic solvent. Similar to CuAAC, a relatively large variety of solvents can be used for the reaction; the most common are benzene, toluene, and THF. Organic azides react with a broad range of terminal alkynes, selectively producing 1,5-disubstituted 1,2,3-triazoles (Figure 11a). The use of ruthenium catalyst allows coupling of the internal alkynes with azides resulting in the formation of 1,4,5-trisubstituted 1,2,3-triazoles (Figure 11b). RuAAC has found application in the field of medicinal and supramolecular chemistry, polymer synthesis, and organocatalysis.[37, 38]

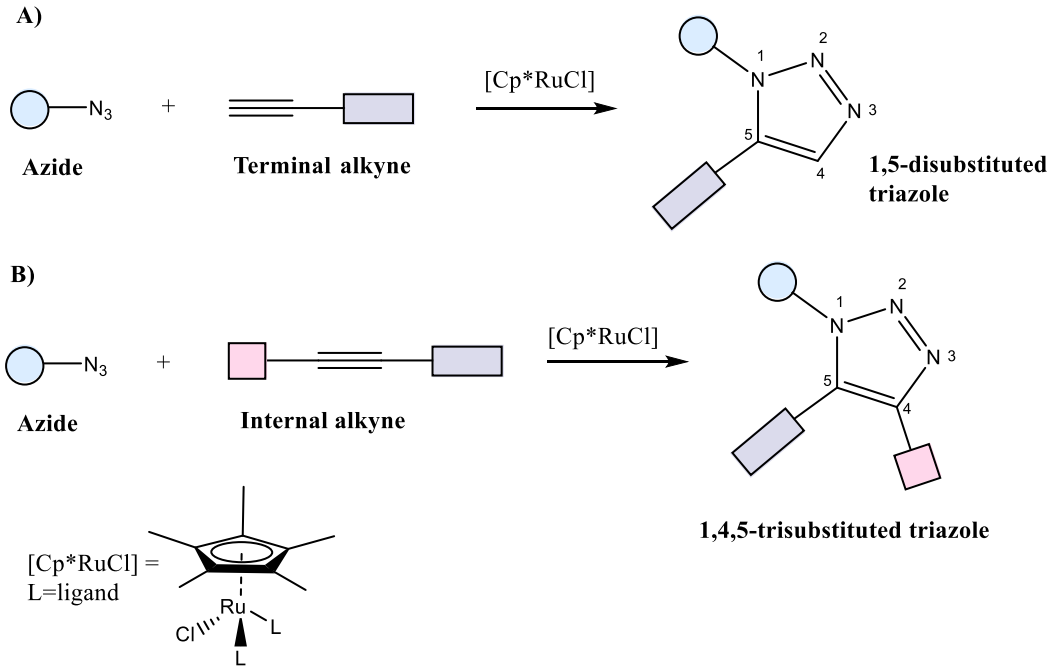


Figure 11: Ruthenium-catalyzed azide–alkyne cycloaddition with (a) terminal alkyne, (b) internal alkyne. Adapted and modified from Kaur et al.[18]

5.3.5 Application of click chemistry in the drug discovery

Click chemistry has proven to be an effective tool for generating stable 1,2,3-triazole-containing molecules. It has attracted interest in almost all aspects of drug discovery, such as lead discovery through combinatorial methodology, proteomics and DNA research with bioconjugation reactions, and target-templated *in vitro* chemistry. 1,2,3-Triazoles are unsaturated, aromatic, π electron-deficient, possess a strong dipole moment (5.2–5.6 Debye), and are capable of interaction with the biological targets, via dipole-dipole interaction or hydrogen bonding. They are stable to both metabolic and chemical degradation and inert to hydrolytic, reducing, and oxidizing conditions, even at high temperatures.[17] 1,4-Substituted 1,2,3-triazole has multiple roles in bioactive molecules: as a basic pharmacophore element or as a molecular scaffold, it regulates other pharmacophore elements to maintain an active conformation; as a connecting group, it links conjugated molecules or probes.[39] The triazole-based molecules showed a broad spectrum of favorable biological activities and were used in the development of anti-inflammatory, antibacterial, anti-tubercular, antiviral, and anticancer therapeutic agents (Figure 12a).[18] The approved drugs are displayed in Figure 12b. The application of click

chemistry in drug discovery will be discussed in the following sections via the selected examples.

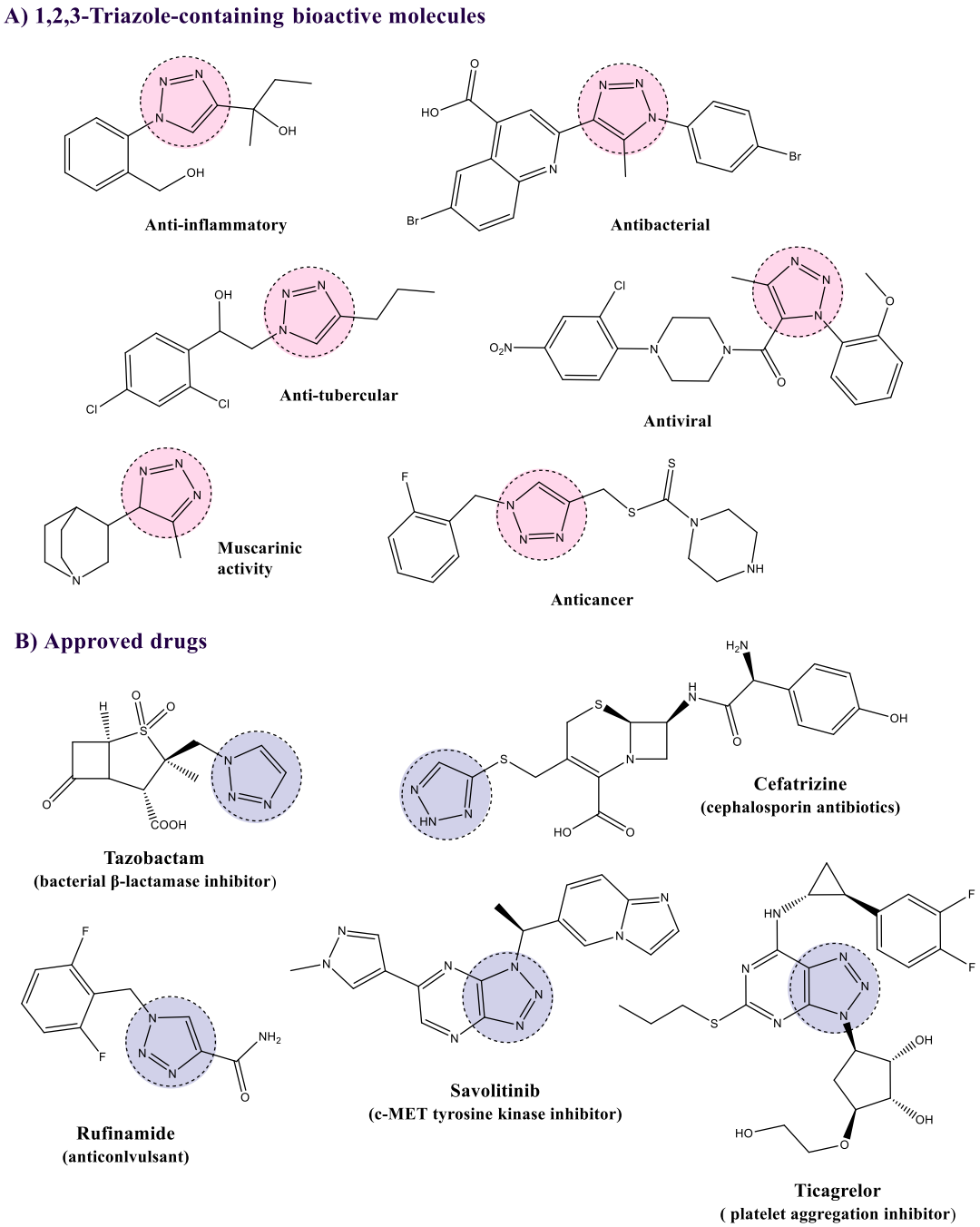


Figure 12: (a) Some examples of 1,2,3-triazole-containing bioactive molecules. (b) Examples of approved drugs containing the 1,2,3-triazole moiety, c-MET= mesenchymal-epithelial transition factor. Adapted and modified from Dheer et al.[40]

5.3.5.1 The 1,2,3-triazole ring as a bioisostere in medicinal chemistry

The necessity to overcome drug resistance, search for safer alternatives, or attempts to improve the pharmacokinetic profile, necessitates for a constant optimization process. Applying a bioisosteric replacement is a widely used technique in the modification of active molecules. Structural features and marked stability of 1,2,3-triazole enable it to exchange different functional groups, justifying its wide use as a bioisostere for synthesizing novel active molecules. 1,2,3-Triazoles are suitable replacements of the aromatic ring, particularly heteroaromatic rings such as imidazole, pyrazole, oxazole, but also 1,2,4-triazole. 1,2,3-triazoles can be also used as isosteres of carboxylic acid or esters, although these are less typical replacements. The 1,2,3-triazole ring is used as an ester isostere to reduce their susceptibility to enzymatic degradation *in vivo*. On the other hand, amide bond replacement is more frequent.[41] Amides and 1,4-disubstituted 1,2,3-triazoles have a similar dipole moment, distances between substituents, and H-bond acceptor capacity (Figure 13). The 1,2,3-triazole ring has two lone pairs of electrons, due to which it can act as a weak hydrogen-bond acceptor. Furthermore, the intense dipole moment of the 1,2,3-triazole ring polarizes the C (5) proton so it can behave as a hydrogen-bond donor similar to amidic NH.[17] The 1,4-disubstituted 1,2,3-triazole unit is bioisosteric with a peptide bond, but it is extremely stable to hydrolysis. The ability to obtain proteolysis-stable isosteres has resulted in the application of 1,2,3-triazoles in the peptidomimetics field.[39]

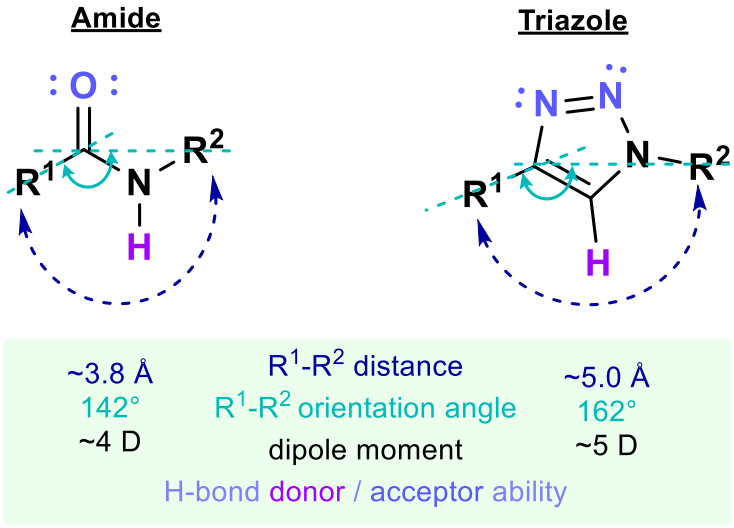


Figure 13: The comparison of amide and 1,2,3-triazole moiety. Adapted and modified from Doiron et al.[42]

5.3.5.2 The 1,2,3-triazole ring as a linker

The 1,2,3-triazole moiety, which is very stable under physiological conditions, is also a favorable linker. 1,2,3-Triazole linker has been used to produce homodimer and heterodimer drugs. A series of bivalent dopamine D2 receptor ligands, polyphenols-based molecules linked via 1,2,3-triazole with monocyclic β-lactams, and chloroquinoline core conjoined with β-lactam through 1,2,3-triazole linkage can be mentioned as an example.[17] The 1,2,3-triazole linker has been used to generate inhibitors interacting with two different binding sites of the same enzyme. This strategy was applied to synthesize protein tyrosine phosphatase 1B inhibitors and 1,2,3-triazole-based arginine-glycine-aspartate (RGD) mimetics.[17] Additionally, 1,2,3-triazole is widely used as a linker unit of targeting conjugates or probes, as a linker unit of drug carriers, and as a linker to generate conjugated ligands.[39]

5.3.5.3 1,2,3-Triazole-based molecules as antibacterial agents

Antibiotics are undoubtedly indispensable and life-saving weapons against numerous bacterial infections. In the last century, research has produced many new antibiotics; however, since the 1990s, the number of new antimicrobial agents has been declining sharply, and at the same time, antimicrobial resistance has been developing. A multidrug-resistant bacteria, resistant to at least three different classes of antibiotics, have become common, mainly in hospital environments. There is a risk that we may return to a pre-antibiotic era when even milder bacterial infections could become lethal.[43] In recent years, click chemistry has been used to synthesize novel antibacterial agents containing 1,2,3-triazole structural motifs. One example is 1,2,3-triazoles-based chalcone compounds. The activity of dehydroacetic acid-chalcone-1,2,3-triazoles hybrids (Figure 14a) screened against bacterial strains (*Escherichia coli* and *Bacillus subtilis*) and fungal strains (*Candida albicans* and *Aspergillus niger)* proved the antimicrobial potential. The antibacterial activity can be increased with terminal bromo and methoxy groups on the benzene ring. In the presence of the nitro group, better antifungal activity was observed.[44] Another class of compounds containing Schiff base linked to 1,2,3-triazoles with terminal silatrane group was synthesized via click silylation reaction. The structure in Figure 14b showed the best activity against *Staphylococcus aureus* and *Staphylococcus epidermidis.* In a different experiment, metronidazole-triazole hybrids were synthesized (Figure 14c), examined against methicillin-resistant *Staphylococcus aureus,* and compared to the

reference drug oxacillin.[45] Among other potential antibacterial therapeutics, a series of novel aryl-substituted-1,2,3-triazoles linked to a carbohydrate unit (Figure 14d) prepared by a standard click reaction of aryl azides with sugars were described. The activity was evaluated against *Staphylococcus aureus, Pseudomonas aeruginosa*, *Candida albicans,* and *Aspergillus niger* and revealed a potent activity of compound d compared to other tested compounds.[46] A library of new 1,2,3-triazole-thiazole hybrids was designed (Figure 14e), synthesized by a multi-component reaction approach, and screened for their antibacterial potential against seven bacterial strains. Most of the tested compounds exhibited promising activity with a minimum inhibitory concentration in a range of 2.8 to 15.7µM.[47]

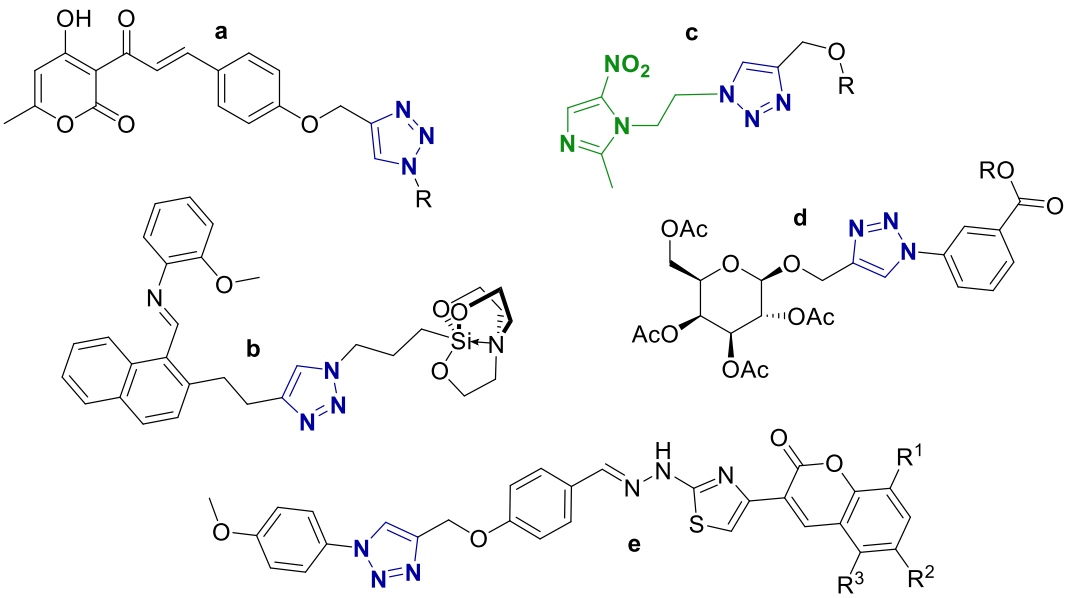


Figure 14: Some examples of 1,2,3-triazole-containing molecules possessing antibacterial activity. The triazole ring is highlighted in blue, and metronidazole in green.

5.3.5.4 1,2,3-Triazole-based molecules with activity against Alzheimer's disease

Alzheimer's disease (AD) is a multifactorial neurodegenerative disorder, the leading cause of dementia in the elderly. The prevalence of AD is rising rapidly among the aging population; the most recent data indicate that, by 2050, the prevalence of dementia will be doubled in Europe and tripled worldwide.[48] Typical characteristics of AD are progressive memory loss and functional impairment. A person with dementia is no longer entirely independent; thus, the disease impacts not only the individual but also his family and society. Despite the seriousness of the disease, current treatments cannot achieve a

satisfactory therapeutic effect or stop disease progression.[49] Through the click chemistry approach, the class of tacrine-1,2,3-triazole hybrids was prepared (Figure 15a) and these compounds exhibited a good inhibition activity toward cholinesterases (ChEs).[45,50] A series of 1,2,3-triazole-supported chromenone carboxamides (Figure 15b) was synthesized and compounds proved to be promising inhibitors of ChEs. Compound with 3,4-dimethyl substitution on the aromatic ring exhibited the best acetylcholinesterase (AChE) activity.[51] Other potential triazole-based molecules revealing the anti-Alzheimer's activity include the tacrine-coumarin hybrids linked to the 1,2,3-triazole unit (Figure 15c) [52], multifunctional iminochromene-*2H*-carboxamide derivates containing aminomethylene triazole ring (Figure 15d), 1,2,3-triazole linked acridone derivates (Figure 15e), and tryptamine triazole hybrids (Figure 15f).[53]

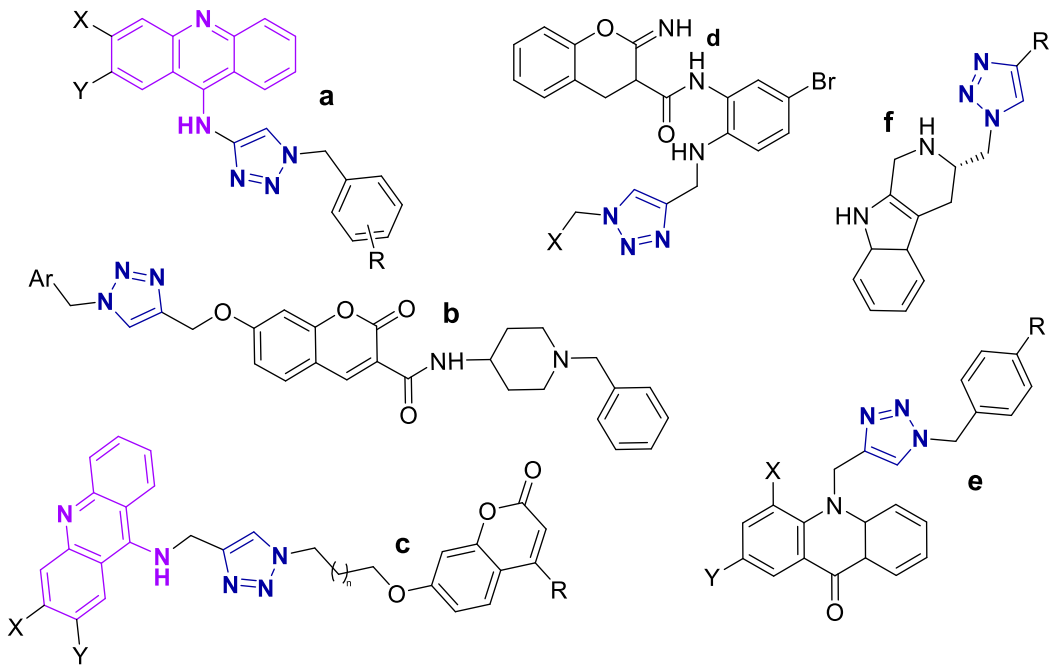


Figure 15: Some examples of 1,2,3-triazole-containing molecules possessing anti-Alzheimer's activity. The triazole ring is highlighted in blue and tacrine in violet.

5.3.5.5 1,2,3-Triazole-based molecules with anti-tubercular activity

Tuberculosis (TB), caused by a pathogenic group of organisms, *Mycobacterium tuberculosis* complex, is one of the leading causes of death worldwide. Although drug-sensitive TB is curable, the risk is mainly multi-drug resistance TB when the strain is resistant to both isoniazid and rifampicin. Despite enormous efforts to identify novel anti-tubercular agents, only a few candidates have entered clinical trials in recent

decades.[54] Molecules containing 1,2,3-triazole moiety may show promising anti-TB activities. Similar to isoniazid, they inhibit bacterial growth by blocking mycolic acid synthesis. In the following section, triazole-containing structures as anti-TB agents will be mentioned.[55] A small library of benzotiazinone-based 1,2,3-triazoles (Figure 16a) was developed via the click chemistry method. The compounds with 4-fluoro and 4-chloro substitution on benzene ring were demonstrated to be the most efficient against *Mycobacterium tuberculosis* and *Mycobacterium bovis*.[56] A series of *N*-substituted-phenyl-1,2,3-triazole-4-carbaldehydes (Figure 16b) was prepared by the reaction of aromatic amine hydrochlorides with diazomalonalaldehyde. Click chemistry has also been used to develop a class of quinolone-coupled 1,2,3-triazoles and the most potent was the quinolone-linked triazole sugar hybrid (Figure 16c).[40] Propylene-1,2,3-triazoles fused with isatin-moxifloxacin hybrids were synthesized, and activity against *Mycobacterium tuberculosis* was measured. The most active compound (Figure 16d) displayed similar MIC values as isoniazid.[57] Other examples of 1,2,3-triazoles with anti-tubercular activity include hybridized triazole skeletons with some pharmacologically active structures, such as coumarin (Figure 16e) and isoniazid (Figure 16f).[54]

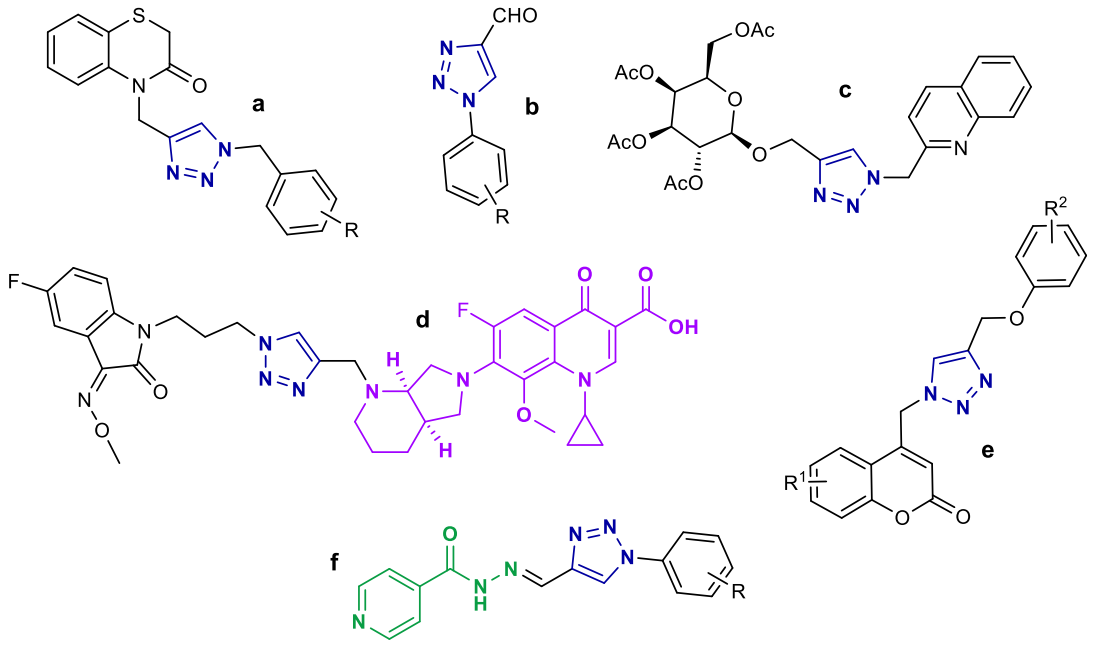


Figure 16: Some examples of 1,2,3-triazole-containing molecules possessing anti-tubercular activity. The triazole ring is highlighted in blue, moxifloxacin in violet and isoniazid in green.

6. Experimental part

6.1 Materials and methods

All experiments were performed at the Department of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Ljubljana. Reagents used in the experimental part were purchased from different commercial vendors: Sigma-Aldrich (Schnelldorf, Germany), Fluka (Buchs, Switzerland), Apollo scientific (Bredbury, United Kingdom), Acros organics (Geel, Belgium). Solvents were obtained from Merck (Darmstadt, Germany) and Carlo Erba reagents (Val-de-Reuil, France). Chemicals and solvents were used directly, without additional purification.

Thin-layer chromatography (TLC) was used as a reaction-monitoring tool and for column chromatography fraction differentiation. TLC detection was performed on pre-coated silica 60 F_{254} aluminium sheets manufactured by Merck (Darmstadt, Germany) with visualization under ultraviolet (UV) light at 254 nm or with TLC visualization reagents (ninhydrin, phosphomolybdic acid).

Products were separated and purified by column chromatography. Silica gel 60 with a particle size of 0.040–0.063 mm from Merck (Darmstadt, Germany) was used as the stationary phase. Mobile phases were prepared from several solvents and are indicated next to each synthetic procedure.

Nuclear magnetic resonance (NMR) spectra were recorded on BRUKER AVANCE III 400 spectrometer. Chemical shifts are given in ppm relative to standard tetramethylsilane.

HRMS spectrum was recorded on Thermo Scientific Q Exactive Plus mass spectrometer (Thermo Fisher Scientific) in positive electrospray ionization mode.

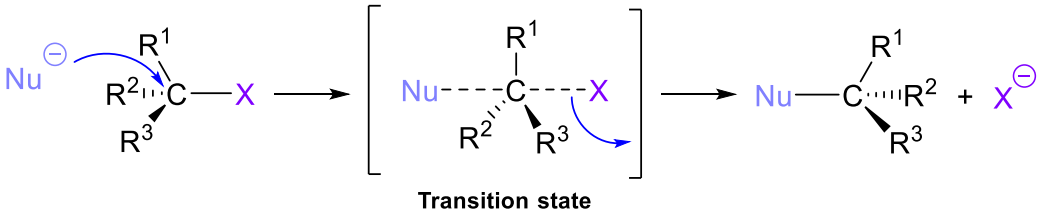
The melting points were determined by observation on a microscope Cambridge Instruments with heating table Reichert-Jung and thermometer Testoterm GmbH & Co. Reported melting points are uncorrected.

Software ChemDraw professional 20.0 (PerkinElmer, Massachusetts, USA) was used for drawing structural formulas of compounds, reaction schemes, figures, and calculations of molecular weights.

The virtual libraries generated by click reactions 16 were enumerated using Molecular Operating Environment (MOE), 2022.02 Chemical Computing Group ULC, Montreal, QC, Canada. The molecular descriptors of individual compounds were calculated by the same software. Upon creation of libraries derived from cores IS-MK4, IS-MK7 and IS-MK8 (Click6, Click3 and Click4), the possible chirality of the core was disregarded.

6.2 Azides synthesis and characterization

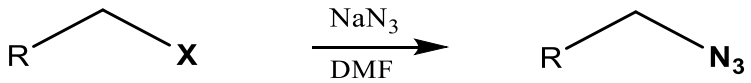
We used the most general method for azide preparation, i.e. nucleophilic substitution type 2 (S_N2) of alkyl halides (or methanesulfonate) using sodium azide (Scheme 4). The azide ion is an excellent nucleophile and will readily participate in S_N2 reactions. Dimethylformamide (DMF) as a polar aprotic solvent increases the reaction rate. Due to its high polarity, it dissolves many salts, and tends to solvate metal cations, but not nucleophile anions. Consequently, unsolvated anions are more efficient nucleophiles, and reactions are faster.[58]



Scheme 4: General mechanism of S_N2 reactions, X = leaving group, Nu = nucleophile.

6.2.1 The general procedure for the azides preparation

To a solution of starting material (1 equiv.) in DMF (1.5 mL per 1 mmol), 1.5 equiv. of NaN_3 was added (Scheme 5). The reaction was stirred at room temperature overnight. The mixture was extracted with ethyl acetate (EtOAc, 50 mL), washed three times with water (80 mL) and brine (80 mL). The organic layer was dried over anhydrous Na_2SO_4 and filtered. The solvent was removed under reduced pressure, and the purity of the product was checked by TLC. If more than one spot was on the TLC plate, the compound was purified by column chromatography.

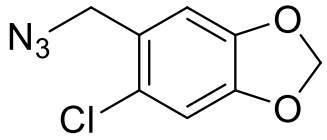


Scheme 5: General procedure of azides preparation, R = cycloalkane, aryl, heterocycle, X = - Cl, -Br, $-OSO_2CH_3$.

6.2.2 Characterization of prepared azides:

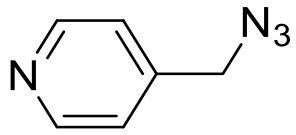
5-(Azidomethyl)-6-chlorobenzo[*d*][1,3]dioxole

Azide **IS-MK-AZ1** was synthesized from 5-chloro-6-(chloromethyl)benzo[*d*][1,3]dioxole (2 g, 9.46 mmol) according to the general procedure described previously.

Label	IS-MK-AZ1	CAS number: **1421616-94-5**
Molecular weight	211.61 g/mol	
Chemical formula	$C_8H_6ClN_3O_2$	
Appearance	Colorless crystals	
Melting point	30.5–31.7 ˚C	
Theoretical yield	2 g	
Actual yield	1.74 g (87 %)	
Retention factor	0.53 (Hex:EtOAc = 4:1)	
^{1}H NMR (400 MHz, $CDCl_3$)	δ 6.88 (s, 1H), 6.85 (s, 1H), 6.01 (s, 2H), 4.39 (s, 2H).	
The compound is described by Pandley et al.[59]		

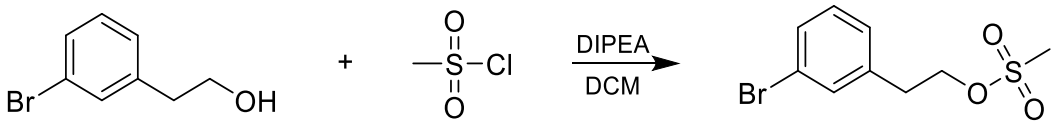
4-(Azidomethyl)pyridine

Azide **IS-MK-AZ2** was synthesized from 4-(bromomethyl)pyridine hydrobromide (1 g, 3.83 mmol) according to the general procedure described previously.

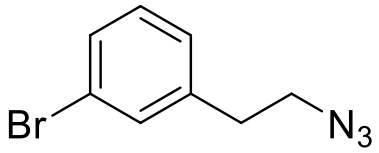
Label	IS-MK-AZ2	CAS number: **864528-34-7**
Molecular weight	134.14 g/mol	
Chemical formula	$C_6H_6N_4$	
Appearance	Yellow liquid	
Theoretical yield	0.51 g	
Actual yield	0.24 g (47 %)	
Retention factor	0.22 (Hex:EtOAc = 2:1)	
^{1}H NMR (400 MHz, $CDCl_3$)	δ 8.65–8.59 (m, 2H), 7.27–7.23 (d, J = 6.0 Hz, 2H), 4.41 (s, 2H).	
The compound is described by Piccinno et al.[60]		

1-(2-Azidoethyl)-3-bromobenzene

2-(3-Bromophenyl)ethan-1-ol (4 g, 20 mmol) was dissolved in DCM (25 mL) and cooled to 0 ˚C. After adding DIPEA (5.1 mL, 30 mmol) and methanesulfonyl chloride (2.32 mL, 30 mmol), the reaction was stirred at room temperature for 3 hours (Scheme 6). TLC indicated complete conversion of starting material. DCM was evaporated, and the mixture was extracted with EtOAc, washed with water (2×100 mL) and brine (1×100 mL). The organic layer was dried over anhydrous Na_2SO_4 and filtered. The evaporation of the solvent gave an oily product. The second step (azide formation) was realized according to the general procedure. The crude product was purified by column chromatography (mobile phase: Hex:EtOAc = 5:1).

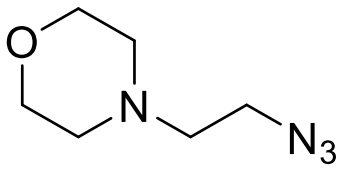


Scheme 6: The first step of synthesis IS-MK-AZ3.

Label	IS-MK-AZ3	CAS number: **198633-81-7**
Molecular weight	226.08 g/mol	
Chemical formula	$C_8H_8BrN_3$	
Appearance	Yellow liquid	
Theoretical yield	4.63 g	
Actual yield	2.25 g (49 %)	
Retention factor	0.67 (Hex:EtOAc = 4:1)	
^{1}H NMR (400 MHz, $CDCl_3$)	δ 7.42–7.36 (m, 2H), 7.23–7.12 (m, 2H), 3.51 (t, *J* = 7.1 Hz, 2H), 2.86 (t, *J* = 7.1 Hz, 2H).	
The compound is described by Meng et al.[61]		

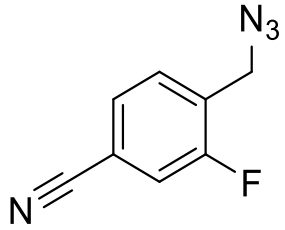
4-(2-Azidoethyl)morpholine

Azide **IS-MK-AZ4** was synthesized from 4-(2-chloroethyl)morpholine hydrochloride (2 g, 10.75 mmol) according to the general procedure described previously.

Label	IS-MK-AZ4	CAS number: **660395-39-1**
Molecular weight	156.19 g/mol	
Chemical formula	$C_6H_{12}N_4O$	
Appearance	Orange liquid	
Theoretical yield	1.68 g	
Actual yield	1.21 g (72 %)	
Retention factor	0.43 (Hex:EtOAc = 1:2)	
^{1}H NMR (400 MHz, $CDCl_3$)	δ 3.79–3.69 (m, 4H), 3.35 (t, *J* = 5.9 Hz, 2H), 2.62–2.57 (m, 2H), 2.54–2.48 (m, 4H).	
The compound is described by Mengji et al.[62]		

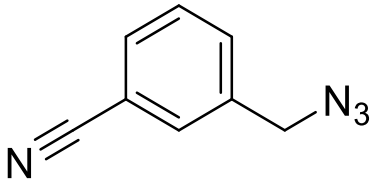
4-(Azidomethyl)-3-fluorobenzonitrile

Azide **IS-MK-AZ5** was synthesized from 4-(bromomethyl)-3-fluorobenzonitrile (2 g, 9.34 mmol) according to the general procedure described previously.

Label	IS-MK-AZ5	CAS number: **368426-24-8**
Molecular weight	176.15 g/mol	
Chemical formula	$C_8H_5FN_4$	
Appearance	Colorless solid	
Melting point	25.9–27.5 °C	
Theoretical yield	1.65 g	
Actual yield	1.24 g (75 %)	
Retention factor	0.48 (Hex:EtOAc = 4:1)	
^{1}H NMR (400 MHz, $CDCl_3$)	δ 7.56–7.48 (m, 2H), 7.44–7.38 (m, 1H), 4.51 (s, 2H).	
The compound is described by Araldi and Semple.[63]		

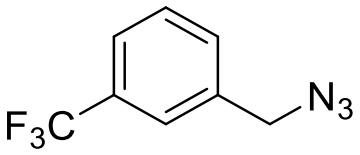
3-(Azidomethyl)benzonitrile

Azide **IS-MK-AZ6** was synthesized from 3-(bromomethyl)benzonitrile (1.42 g, 10.2 mmol) according to the general procedure described previously.

Label	IS-MK-AZ6	CAS number: **328552-90-5**
Molecular weight	176.15 g/mol	
Chemical formula	$C_8H_6N_4$	
Appearance	Yellow liquid	
Theoretical yield	1.15 g	
Actual yield	0.86 g (74.8 %)	
Retention factor	0.54 (Hex:EtOAc = 4:1)	
^{1}H NMR (400 MHz, $CDCl_3$)	δ 7.67–7.61 (m, 1H), 7.57 (dt, *J* = 7.8, 1.5 Hz, 1H), 7.54–7.48 (m, 1H), 4.43 (s, 1H).	
The compound is described by Chen et al.[64]		

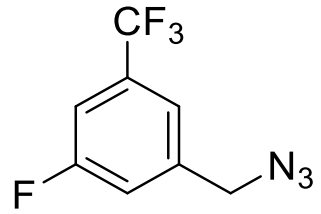
1-(Azidomethyl)-3-(trifluoromethyl)benzene

Azide **IS-MK-AZ7** was synthesized from 1-(chloromethyl)-3-(trifluoromethyl)benzene (1 g, 5.14 mmol) according to the general procedure described previously.

Label	IS-MK-AZ7	CAS number: **620533-90-6**
Molecular weight	201.15 g/mol	
Chemical formula	$C_8H_6F_3N_3$	
Appearance	Pale yellow liquid	
Theoretical yield	1.03 g	
Actual yield	0.65 g (63 %)	
Retention factor	0.59 (Hex:EtOAc = 4:1)	
^{1}H NMR (400 MHz, $CDCl_3$)	δ 7.67–7.56 (m, 2H), 7.55–7.48 (m, 2H), 4.44 (s, 2H).	
The compound is described by Yang et al.[65]		

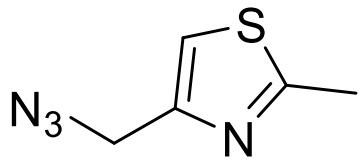
1-(Azidomethyl)-3-fluoro-5-(trifluoromethyl)benzene

Azide **IS-MK-AZ8** was synthesized from 1-(bromomethyl)-3-fluoro-5-(trifluoromethyl)benzene (1.1 g, 3.89 mmol) according to the general procedure described previously.

Label	IS-MK-AZ8	CAS number: **620533-87-1**
Molecular weight	219.14 g/mol	
Chemical formula	$C_8H_5F_4N_3$	
Appearance	Pale yellow liquid	
Theoretical yield	0.95 g	
Actual yield	0.57 g (60 %)	
Retention factor	0.64 (Hex:EtOAc = 4:1)	
^{1}H NMR (400 MHz, $CDCl_3$)	δ 7.38 (apps, 1H), 7.34–7.28 (m, 1H), 7.27–7.22 (m, 1H), 4.45 (s, 2H).	
The compound is described by Meng et al.[61]		

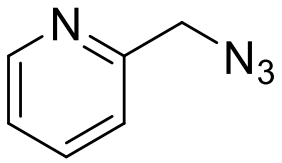
4-(Azidomethyl)-2-methylthiazole

Azide **IS-MK-AZ9** was synthesized from 4-(chloromethyl)-2-methylthiazole hydrochloride (1.5 g, 8.13 mmol) according to the general procedure described previously.

Label	IS-MK-AZ9	CAS number: **864262-96-4**
Molecular weight	154.19 g/mol	
Chemical formula	$C_5H_6N_4S$	
Appearance	Brown-orange liquid	
Theoretical yield	1.25 g	
Actual yield	0.652g (52 %)	
Retention factor	0.49 (Hex:EtOAc = 4:1)	
^{1}H NMR (400 MHz, $CDCl_3$)	δ 7.08–7.06 (m, 1H), 4.44 (s, 2H), 2.72 (s, 3H).	
The compound is described by Allen et al.[66]		

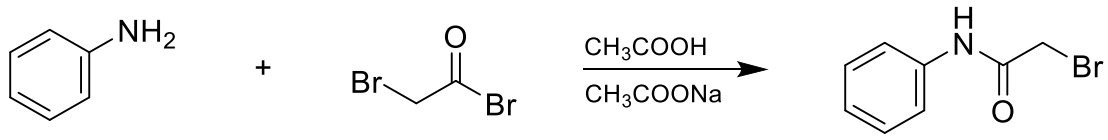
2-(Azidomethyl)pyridine

Azide **IS-MK-AZ10** was synthesized from 2-(bromomethyl)pyridine hydrobromide (2 g, 7.91 mmol) according to the general procedure described previously.

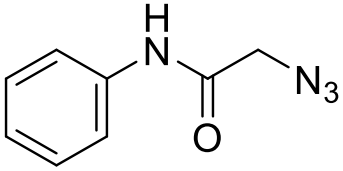
Label	IS-MK-AZ10	CAS number: **609770-35-6**
Molecular weight	134.14 g/mol	
Chemical formula	$C_6H_6N_4$	
Appearance	Yellow liquid	
Theoretical yield	1.36 g	
Actual yield	0.43 g (31.6 %)	
Retention factor	0.61 (Hex:EtOAc = 1:2)	
^{1}H NMR (400 MHz, $CDCl_3$)	δ 8.65–8.56 (m, 1H), 7.73 (td, *J* = 7.7, 1.7 Hz, 1H), 7.38–7.32 (m, 1H), 7.28–7.23 (m, 1H), 4.50 (s, 2H).	
The compound is described by Mamidyala et al.[67]		

2-Azido-*N*-phenylacetamide

Aniline (2.31 g, 24.8 mmol) and acetic acid (19.86 mL, 347.2 mmol) were added into a 100 mL round bottom flask. The reaction was cooled down in the water bath (10–15 ˚C) and after adding bromoacetyl bromide (2.7 mL, 31 mmol), the reaction mixture was stirred for 30 minutes in the water bath. To this solution sodium acetate (6.1 g, 74.4 mmol) dissolved in water (20 mL) was added (Scheme 7). The reaction was carried out at the room temperature overnight and then filtered on the Büchner funnel. Pure compound was transferred into a round bottom flask, and the general procedure for azide formation followed. The final product was purified by column chromatography (mobile phase: Hex:EtOAc = 2:1).

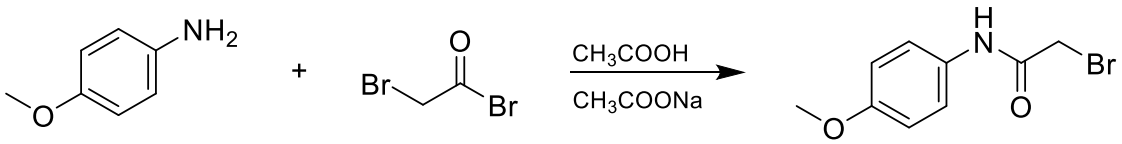


Scheme 7: The first step of synthesis IS-MK-AZ11.

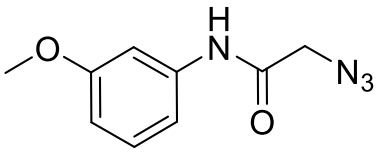
Label	IS-MK-AZ11	CAS number: **10258-71-6**
Molecular weight	176.18 g/mol	
Chemical formula	$C_8H_8N_4O$	
Appearance	Colorless solid	
Melting point	80.7–81.5 °C (82–83 °C according to the literature [68])	
Theoretical yield	3.77 g	
Actual yield	1.794 g (47.6 %)	
Retention factor	0.43 (Hex:EtOAc = 2:1)	
^{1}H NMR (400 MHz, DMSO-*d*$_6$)	δ 10.15 (s, 1H), 7.64–7.57 (m, 2H), 7.38–7.28 (m, 2H), 7.13–7.03 (m, 1H), 4.05 (s, 2H).	
^{13}C NMR (101 MHz, DMSO-*d*$_6$))	δ 166.36, 159.60, 139.64, 129.67, 111.59, 109.13, 105.15, 54.99, 51.37.	
The compound is described by Tiew et al.[69]		

2-Azido-*N*-(3-methoxyphenyl)acetamide

Into a 100 mL round bottom flask 3-methoxyaniline (3.05 g, 24.8 mmol) and acetic acid (19.86 mL, 347.2 mmol) were added. The reaction was cooled down in the water bath (10–15 ˚C) and after adding bromoacetyl bromide (2.7 mL, 31 mmol), the reaction mixture was stirred for 30 minutes in the water bath. To this solution sodium acetate (6.1 g, 74.4 mmol) dissolved in water (20 mL) was added (Scheme 8). The reaction was carried out at room temperature overnight and then filtered on the Büchner funnel. Pure compound was transferred into a round bottom flask, and the general procedure for azide formation followed. The final product was purified by column chromatography (mobile phase: Hex:EtOAc = 2:1).



Scheme 8: The first step of synthesis IS-MK-AZ12.

Label	IS-MK-AZ12	CAS number: **1160748-24-2**
Molecular weight	206.21 g/mol	
Chemical formula	$C_9H_{10}N_4O_2$	
Appearance	Colorless crystals	
Melting point	59.9–60.7 °C	
Theoretical yield	6.14 g	
Actual yield	1.54 g (25 %)	
Retention factor	0.34 (Hex:EtOAc = 2:1)	
^{1}H NMR (400 MHz, DMSO-d_6))	δ 10.14 (s, 1H), 7.31 (t, *J* = 2.1 Hz, 1H), 7.23 (t, *J* = 8.1 Hz, 1H), 7.14 (dd, *J* = 8.1, 0.8 Hz, 1H), 6.67 (ddd, *J* = 8.1, 2.1, 0.8 Hz, 1H), 4.04 (s, 2H), 3.74 (s, 3H).	
^{13}C NMR (101 MHz, DMSO-d_6))	δ 166.36, 159.60, 139.64, 129.67, 111.59, 109.13, 105.15, 54.99, 51.37.	
The compound is described by Phatak et al.[70]		

6.3 Alkyne core synthesis and characterization

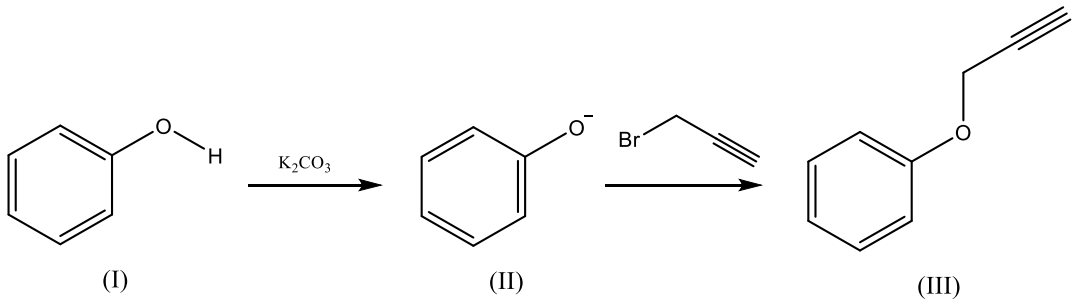
Alkyne cores were designed to encompass different orientations and distances between alkyne moieties. All molecules were purified using classical purification methods such as extraction and column chromatography.

6.3.1 Alkylation with propargyl bromide

The nucleophilic substitution of alkyl halides with the phenolate anion or alkoxides leads to the formation of (unsymmetric) ethers. Generally, even weak bases are able to form the phenolate anion. Potassium carbonate, as a mild base was used to deprotonate phenol derivates. However, a stronger base is needed to prepare the alkoxide ion. As a strong base, sodium hydride can deprotonate weak Brønsted acids. NaH reacts intensely with water; therefore, our reactions with NaH were carried out in an argon atmosphere.

6.3.1.1 Method A: *O*-alkylation of phenol derivates

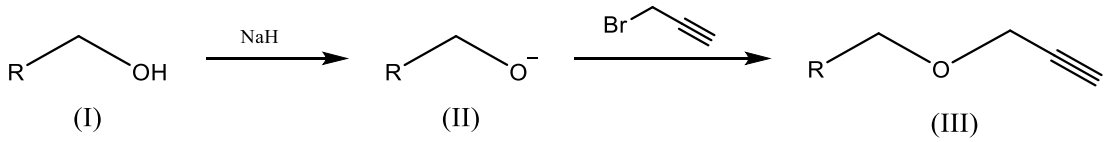
To a solution of starting material (1 equiv.) in acetonitrile (ACN), K_2CO_3 (3 equiv.) and propargyl bromide (2 equiv.) were added. The mixture was heated at 60 °C for 24 hours (Scheme 9). The conversion of the reactant to the product was checked by TLC. ACN was evaporated and the mixture was then extracted from EtOAc. The organic layer was washed with 1 M HCl, water, and brine. The organic layer was dried over anhydrous $NaSO_4$ and concentrated under reduced pressure. The residue was purified by column chromatography to obtain the final or intermediate product.



Scheme 9: *O*-alkylation of phenols. The phenolic hydroxy group (I) is exposed to basic conditions, phenolate anion is formed (II), which reacts with propargyl bromide to yield *O*-propargylated product (III).

6.3.1.2 Method B: *O*-alkylation of alcohols

One equiv. of starting material was added to the round bottom flask filled with argon, and 30 mL of anhydrous THF was added with a syringe. The reaction mixture was cooled down in the ice bath and then carefully two equiv. of NaH (60 % dispersion in mineral oil) was added. After 15 minutes, propargyl bromide (2 equiv.) was added (Scheme 10). The reaction was carried out under the argon atmosphere for three days. Saturated aqueous NH_4Cl was then used to quench the excess of NaH. THF was evaporated and the mixture extracted with EtOAc. The organic phase was washed with 1 M HCl (2 × 60 mL) and brine (1 × 60 mL), followed by drying over anhydrous Na_2SO_4, filtration, and evaporation of the solvent. The residue was purified by column chromatography.

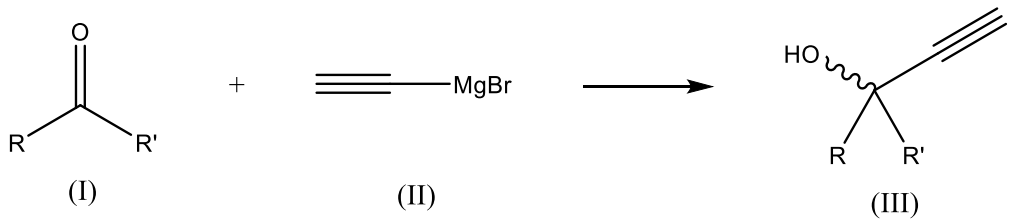


Scheme 10: *O*-alkylation of alcohols (Williamson ether synthesis). Strong base NaH deprotonates alcohol (I), alkoxide is formed (II), which reacts with propargyl bromide to yield unsymmetric ether (III).

6.3.2 Method C: Alkylation with the Grignard reagent

The Grignard reagents are organomagnesium halides with highly polar magnesium-carbon bonds. The carbon atom has a partial negative charge and reacts as a nucleophile with electrophilic centers. The Grignard reagent reacts easily with water to form an alkane.[71] This is why conditions for the reaction must be anhydrous and the reagent must be kept away from water.

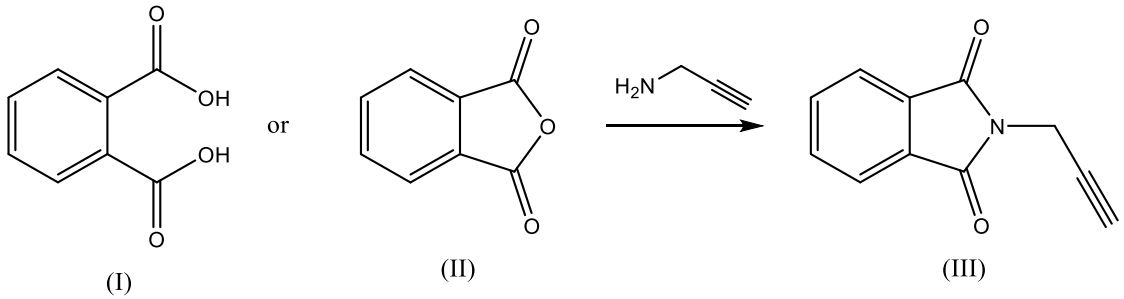
Starting material (1 equiv.) was dissolved under inert conditions in dry THF (20 mL). The reaction mixture was cooled to -70 °C (the mixture of liquid nitrogen and EtOAc). Ethynylmagnesium bromide (2 equiv.) was then added drop-wise with a syringe to the reaction mixture (Scheme 11). The reaction was carried out at room temperature for 48 hours. Then, the reaction was quenched with saturated aqueous NH_4Cl (4 mL) and the organic solvent was evaporated. The aqueous residue was extracted with EtOAc, washed with 1 M HCl (1 × 75 mL) and brine (1 × 75 mL). The organic layer was dried over anhydrous $NaSO_4$, filtered, and concentrated on a rotary evaporator. The crude product was purified by column chromatography.



Scheme 11: Grignard reagent ethynylmagnesium bromide (II) reacts with ketones (I) to form tertiary alcohol (III).

6.3.3 Method D: The preparation of *N*-substituted phthalimides

N-substituted phthalimides were synthesized by the reaction of propargylamine with phthalic anhydride/phthalic acid. *p*-Toluenesulfonic acid (TsOH), a strong organic acid, was used as a catalyst. To a solution of starting material (1 equiv.) in toluene (30 mL), TsOH (0.1 equiv.) was added and the reaction heated to 120 °C. After 10 minutes, propargylamine (1 equiv.) was added and the resulting mixture stirred under reflux for 24 hours (Scheme 12). Toluene was evaporated and the mixture extracted with EtOAc. The organic phase was washed with water (3 × 70 mL), followed by drying over anhydrous Na_2SO_4, filtration, and evaporation of the solvent. The crude product was purified by column chromatography.

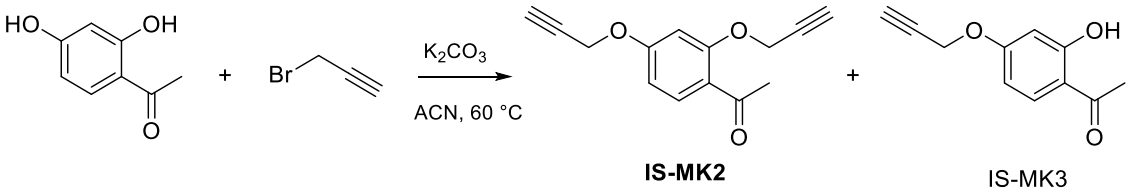


Scheme 12: Synthesis of phthalimide derivates (III), condensation of phthalic acid (I), or anhydride (II) with propargylamine.

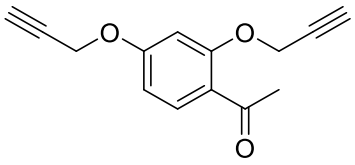
6.3.4 Description of prepared dialkyne (trialkyne) cores

1-(2,4-Bis(prop-2-yn-1-yloxy)phenyl)ethan-1-one

Dialkyne core **IS-MK2** was synthesized from 1-(2,4-dihydroxyphenyl)ethan-1-one (2 g, 13.15 mmol) according to Method A described previously (Scheme 13). The solid residue was purified by column chromatography (mobile phase: Hex:EtOAc = 4:1). The byproduct of this reaction was monoalkylated IS-MK3 (0.56 g).

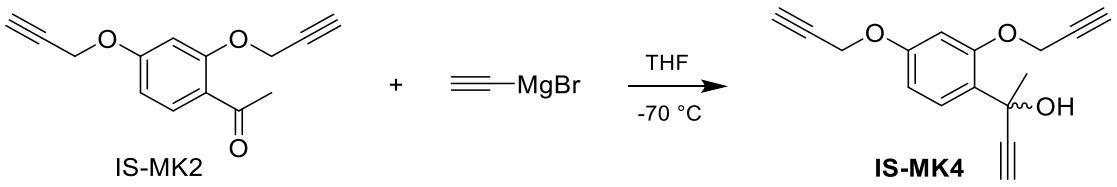


Scheme 13: Synthesis of dialkyne core IS-MK2.

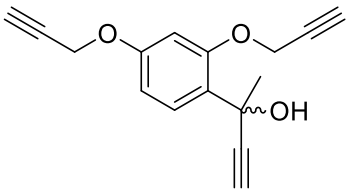
Label	IS-MK2	CAS number: **22287-70-3**
Molecular weight	228.24 g	
Chemical formula	$C_{14}H_{12}O_3$	
Appearance	Colorless solid	
Melting point	75–77 °C (78 °C according to the literature [72])	
Theoretical yield	3 g	
Actual yield	2.34 g (78 %)	
Retention factor	0.28 (Hex:EtOAc = 4:1)	
^{1}H NMR (400 MHz, $CDCl_3$)	δ 7.87–7.81 (m, 1H), 6.68–6.63 (m, 2H), 4.79 (d, *J* = 2.4 Hz, 2H), 4.75 (d, *J* = 2.4 Hz, 2H), 2.61 (s, 1H), 2.57 (t, *J* = 2.4 Hz, 1H), 2.57 (t, *J* = 2.4 Hz, 1H).	
^{13}C NMR (101 MHz, $CDCl_3$)	δ 197.40, 161.95, 158.67, 132.52, 122.26, 106.88, 100.52, 77.75, 77.66, 76.44, 76.32, 56.23, 55.94, 31.90.	
The compound is described by Ortalli et al.[73]		

2-(2,4-Bis(prop-2-yn-1-yloxy)phenyl)but-3-yn-2-ol

Trialkyne core **IS-MK4** was synthesized from **IS-MK2** (1 g, 4.58 mmol) according to Method C described previously (Scheme 14). The solid residue was purified by column chromatography (mobile phase: Hex:EtOAc = 4:1).

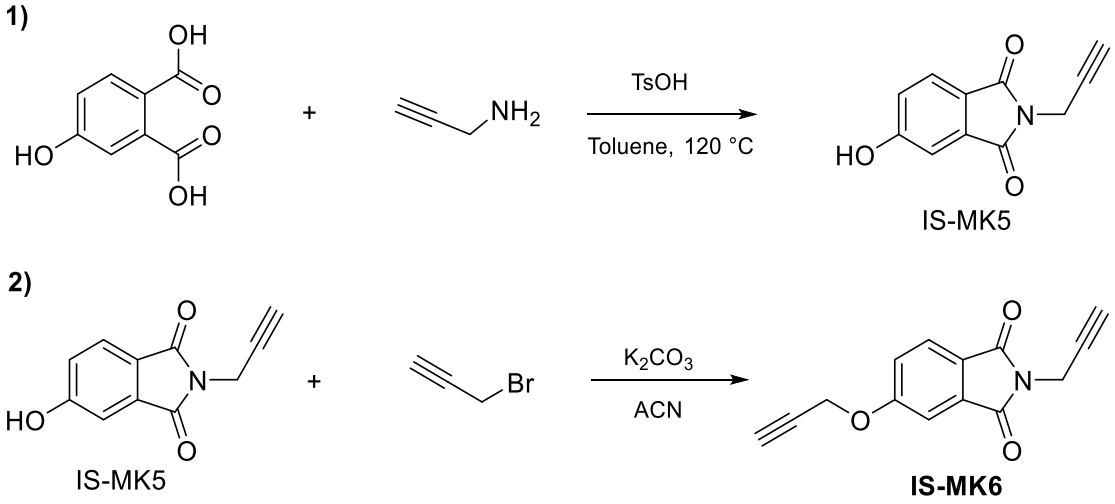


Scheme 14: Synthesis of trialkyne core IS-MK4 from IS-MK2.

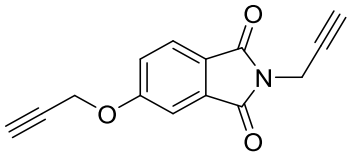
Label	IS-MK4	
Molecular weight	254.29 g/mol	
Chemical formula	$C_{16}H_{14}O_3$	
Appearance	Brown-orange liquid	
Theoretical yield	1.11 g	
Actual yield	0.13 g (11.7 %)	
Retention factor	0.60 (Hex:EtOAc = 2:1)	
^{1}H NMR (400 MHz, $CDCl_3$)	δ 7.49 (d, *J* = 8.6 Hz, 1H), 6.73 (d, *J* = 2.4 Hz, 1H), 6.60 (dd, *J* = 8.6, 2.4 Hz, 1H), 4.83–4.78 (m, 2H), 4.70 (d, *J* = 2.4 Hz, 2H), 4.06 (s, 1H), 2.57 (t, *J* = 2.4 Hz, 1H), 2.56 (s, 1H), 2.54 (t, *J* = 2.4 Hz, 1H), 1.89 (s, 3H).	
^{13}C NMR (101 MHz, CDCl3)	δ 158.27, 155.91, 127.19, 126.17, 106.18, 101.98, 87.36, 78.31, 77.78, 76.55, 75.96, 71.54, 68.28, 56.53, 55.98, 29.74	

2-(Prop-2-yn-1-yl)-5-(prop-2-yn-1-yloxy)isoindoline-1,3-dione

Dialkyne core **IS-MK6** was synthesized from 4-hydroxyphthalic acid (1 g, 5.49 mmol), which converts to 4-hydroxyphthalic anhydride after heating. The first step of the synthesis was realized according to Method D and gave us a monoalkylated IS-MK5 compound. 100 mg of IS-MK5 was taken away for the different click reactions; with the rest, we continued to the second step according to Method A (Scheme 15). The solid residue was purified by column chromatography (mobile phase: Hex:EtOAc = 6:1→2:1).

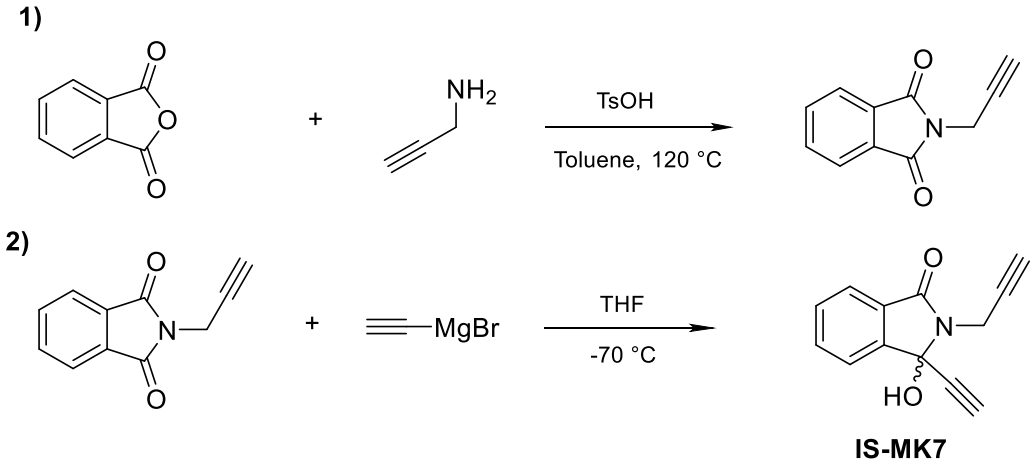


Scheme 15: The first and the second step of synthesis of IS-MK6.

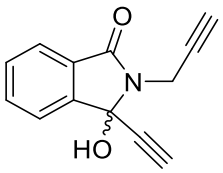
Label	IS-MK6	
Molecular weight	239.23 g/mol	
Chemical formula	$C_{14}H_9NO_3$	
Appearance	White solid	
Melting point	109.0–110.6 °C	
Theoretical yield	1.19 g	
Actual yield	0.76 g (63.8 %)	
Retention factor	0.50 (Hex:EtOAc = 2:1)	
^{1}H NMR (400 MHz, $CDCl_3$)	δ 7.81 (d, *J* = 8.3 Hz, 1H), 7.45 (d, *J* = 2.2 Hz, 1H), 7.27 (dd, *J* = 8.3, 2.2 Hz, 1H), 4.82 (d, *J* = 2.4 Hz, 2H), 4.44 (d, *J* = 2.5 Hz, 2H), 2.59 (t, *J* = 2.4 Hz, 1H), 2.23 (t, *J* = 2.5 Hz, 1H).	
^{13}C NMR (101 MHz, $CDCl_3$)	δ 166.65, 166.57, 162.53, 134.42, 125.33, 124.61, 120.87, 109.38, 77.33, 77.09, 77.07, 71.54, 56.46, 27.01.	

3-Ethynyl-3-hydroxy-2-(prop-2-yn-1-yl)isoindolin-1-one

Dialkyne core **IS-MK7** was synthesized from phthalic anhydride (2.07 g, 13.98 mmol). The first step of the synthesis was realized according to Method D, and the second step according to Method C (Scheme 16). The solid residue was purified by column chromatography (mobile phase: Hex:EtOAc = 2:1).

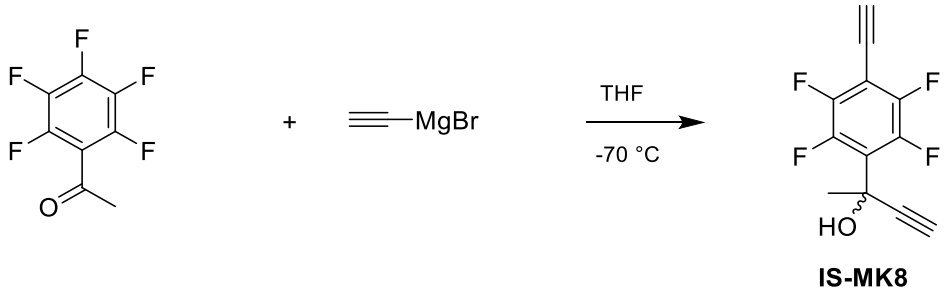


Scheme 16: The first and the second step of synthesis IS-MK7.

Label	IS-MK7	
Molecular weight	211.22 g/mol	
Chemical formula	$C_{13}H_9NO_2$	
Appearance	Colorless solid	
Melting point	160.9–163.2 °C	
Theoretical yield	2.11 g	
Actual yield	0.22 g (10.4 %)	
Retention factor	0.19 (Hex:EtOAc = 2:1)	
^{1}H NMR (400 MHz, $CDCl_3$)	δ 7.83–7.79 (m, 1H), 7.76–7.72 (m, 1H), 7.66 (td, *J* = 7.5, 1.1 Hz, 1H), 7.56 (td, *J* = 7.5, 1.1 Hz, 1H), 4.68 (dd, *J* = 17.8, 2.5 Hz, 1H), 4.23 (dd, *J* = 17.8, 2.5 Hz, 1H), 3.45 (s, 1H), 2.74 (s, 1H), 2.28 (t, *J* = 2.5 Hz, 1H).	
^{13}C NMR (101 MHz, DMSO-d_6)	δ 165.06, 146.34, 133.25, 130.13, 129.11, 122.81, 122.73, 81.80, 80.36, 79.68, 76.08, 72.92, 27.64.	

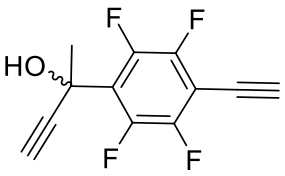
2-(4-Ethynyl-2,3,5,6-tetrafluorophenyl)but-3-yn-2-ol

Dialkyne core **IS-MK8** was synthesized from 1-(perfluorophenyl)ethan-1-one (2 g, 9.52 mmol) according to the previously described Method C (Scheme 17). The liquid residue was purified by column chromatography (mobile phase: Hex:EtOAc = 6:1).



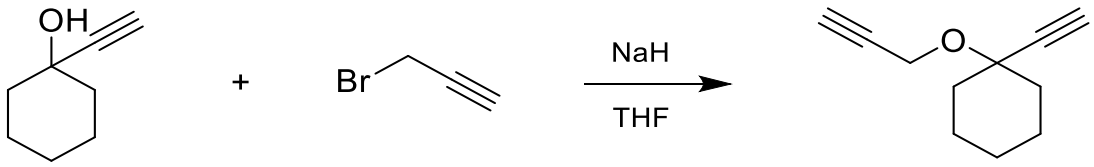
IS-MK8

Scheme 17: Synthesis of dialkyne core IS-MK8.

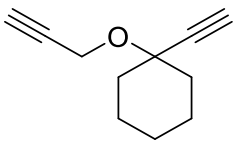
Label	IS-MK8	
Molecular weight	242.17 g/mol	
Chemical formula	$C_{12}H_6F_4O$	
Appearance	Orange liquid	
Theoretical yield	2.3 g	
Actual yield	0.36 g (15.7 %)	
Retention factor	0.43 (Hex:EtOAc = 4:1)	
^{1}H NMR (400 MHz, $CDCl_3$)	δ 4.32 (s, 1H), 3.93 (d, J = 18.6 Hz, 1H), 3.42 (d, J = 18.6 Hz, 1H), 1.75 (s, 3H).	
^{13}C NMR	not available	

1-Ethynyl-1-(prop-2-yn-1-yloxy)cyclohexane

Dialkyne core **IS-MK11** was synthesized from 1-ethynyl-1-cyclohexanol (2.54 g, 20.45 mmol) according to previously described Method B (Scheme 18). The liquid residue was purified by column chromatography (mobile phase: Hex:EtOAc = 6:1). For the visualization of the TLC plate, the phosphomolybdic acid stain was used.



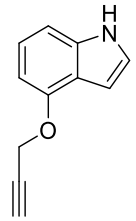
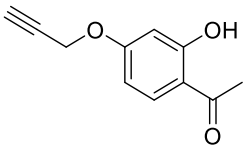
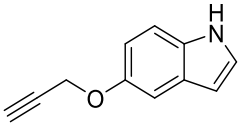
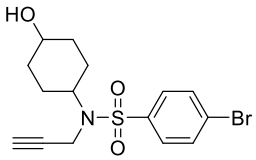
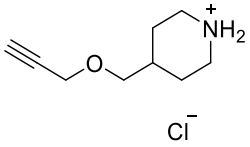
Scheme 18: Synthesis of dialkyne core IS-MK11.

Label	IS-MK11	
Molecular weight	228.24 g	
Chemical formula	$C_{11}H_{14}O$	
Appearance	Yellow liquid	
Theoretical yield	3.27 g	
Actual yield	2.32 g (71 %)	
Retention factor	0.73 (Hex:EtOAc = 4:1)	
^{1}H NMR (400 MHz, $CDCl_3$)	δ 4.31 (d, *J* = 2.5 Hz, 2H), 2.52 (s, 1H), 2.42 (t, *J* = 2.5 Hz, 1H), 2.00–1.88 (m, 2H), 1.77–1.45 (m, 8H).	
^{13}C NMR (101 MHz, CDCl3)	δ 84.24, 81.06, 74.98, 74.87, 73.60, 51.81, 37.18, 25.35, 22.83.	

6.3.5 Monoalkylated compounds

Table 1 contains the list of intermediates with only one alkyne functional group. Compound IS-MK3 was observed as a byproduct of the synthesis of IS-MK2 (see page 44). The other monoalkynes listed in Table 1 were intended as intermediates for the introduction of the second alkyne moiety, but the second-step alkylation efforts failed for these compounds. The monoalkynes mentioned in Table 1 were not further used in this work, and as intermediates, they were not fully characterized. These alkynes can be used in other click reactions in the future.

Table 1: Alkynes with one alkyne moieties; alkynes were prepared according to general methods described previously.

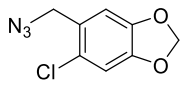
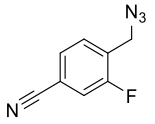
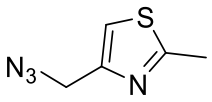
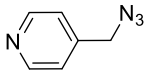
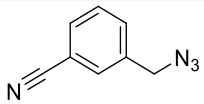
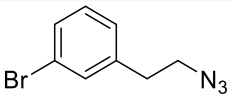
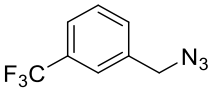
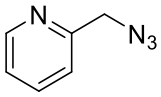
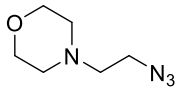
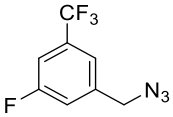
Label	Structure	Starting material	The procedure used for the preparation	Appearance Melting point Yield
IS-MK1		1*H*-indol-4-ol (2.02 g, 15.17 mmol)	Method A	colorless viscous liquid 27.9–46.2 °C 69%
IS-MK3	 CAS number: **67091-10-5**	1-(2,4-dihydroxyphenyl)ethan-1-one (2.00 g,13.15 mmol)	Method A	colorless crystals 57.5–60.0 ˚C 28%
IS-MK9	 CAS number: **153969-91-6**	1*H*-indol-5-ol (1.28 g, 9.61 mmol)	Method A	colorless crystals 51.4–53 °C 58%
IS-MK10		4-bromo-*N*-(4-hydroxycyclohexyl) benzenesulfonamide (14.51 g, 43.41 mmol)	Method A (*N*-alkylation)	white solid 134.5–136.9 °C 65%
IS-MK12	 CAS number: **1185100-09-7**	*tert*-butyl 4-((prop-2-yn-1-yloxy)methyl)piperidine-1-carboxylate (1.68 g, 7.8 mmol)	Method B *	colorless viscous liquid 30.2–53.8 °C 63%

* after alkylation, the *tert*-butyloxycarbonyl protecting group was removed using acetyl chloride

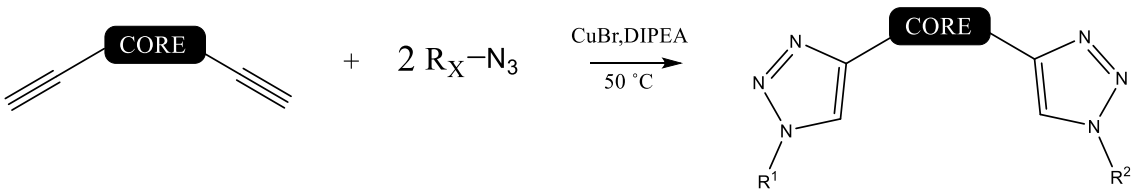
6.4 Double click reaction

Compound libraries were prepared by reacting one dialkyne core (1 equiv.) with 10 different azides (2 equiv.). For our click reactions, azides IS-MK-AZ1 to IS-MK-AZ10 were used (Table 2).

Table 2: Azides used for click reactions.

IS-MK-AZ1 	**IS-MK-AZ5** 	**IS-MK-AZ9** 
IS-MK-AZ2 	**IS-MK-AZ6** 	
IS-MK-AZ3 	**IS-MK-AZ7** 	**IS-MK-AZ10** 
IS-MK-AZ4 	**IS-MK-AZ8** 	

Firstly, the synthetic procedure was optimized by reacting one alkyne (IS-MK3) with only one azide (IS-MK-AZ1). The final conditions were: a reaction temperature of 50 ˚C, DIPEA as a base, and copper bromide (CuBr) as a catalyst (Scheme 19). Subsequently, the more complex compound libraries were prepared by reacting one equivalent of alkyne core with two equivalents of 10 prepared azides in the optimized click reaction conditions. Theoretically, the reaction between each alkyne core and 10 azides yielded 100 compounds.



Scheme 19: General procedure for the double click reaction. R_x-N_3 = 10 different azides (from IS-MK-AZ1 to IS-MK-AZ10).

6.4.1 The general procedure for click reactions

A small amount of alkyne and azide was sufficient to carry out the click reaction. Alkyne (1 equiv.) was dissolved in EtOH (1 mL) in a 10 mL round bottom flask. Afterward, 10 different azides were prepared as 0.1 M solutions in EtOH. Around 10 mg of each azide was weighed and refilled with a calculated volume of ethanol. The same volume of each azide solution was pipetted into the flask with an alkyne core (V_{azide}). To the reaction mixture, was added of DIPEA (10 µL) and CuBr (3 mg). The reaction was carried out at 50 ˚C overnight, followed by evaporation of EtOH. The mixture was extracted with EtOAc, washed with 5% EDTA (2 × 15 mL) and brine (1 × 15 mL). The organic layer was dried over anhydrous Na_2SO_4 and filtered. The solvent was removed under reduced pressure.

$$n(azide) = \frac{n(alkyne) \times Eq}{N}$$

$\boldsymbol{n_{(alkyne)}}$ the molar amount of alkyne that was used for the click reaction

Eq equivalent, the amount of azide that reacts with one mole of alkyne, for double click = 2, for triple click = 3

N number of azides; for both triple and double click, we used ten azides

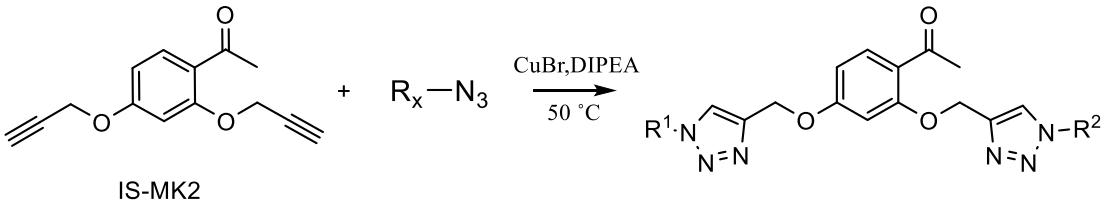
$$V(azide) = \frac{n(azide)}{c(azide)}$$

$\boldsymbol{V_{(azide)}}$ volume of each azide, that was pipetted to the reaction mixture

$\boldsymbol{c_{(azide)}}$ concentration of each azide, c of azide was always 0.1 M

$\boldsymbol{n_{(azide)}}$ the molar amount of azide

6.4.2 Click reaction number 1



Scheme 20: Synthesis of Click mixture 1, R_x-N_3 = 10 different azides (from IS-MK-AZ1 to IS-MK-AZ10).

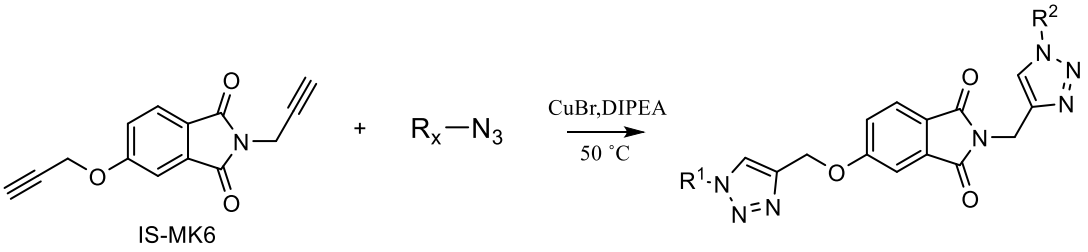
Alkyne core **IS-MK2** (20.6 mg, 0.090 mmol) was added to the round bottom flask. Around 10 mg of each azide was weighed one by one and dissolved in EtOH to get a 0.1 M concentration (Table 3). Subsequently, 180 µL of each azide solution was added to the reaction mixture flask and the general procedure for click reactions followed. Scheme 20 shows the synthesis of Click mixture 1.

Table 3: Preparation of fresh 0.1M solutions of azides for click reaction 1.

Azide	m (mg)	M (g/mol)	V_{EtOH} (mL)
IS-MK-AZ1	12.2	211.61	0.577
IS-MK-AZ2	11.1	134.14	0.827
IS-MK-AZ3	11.3	226.08	0.500
IS-MK-AZ4	12.4	156.19	0.794
IS-MK-AZ5	11.1	176.15	0.630
IS-MK-AZ6	10.0	158.16	0.632
IS-MK-AZ7	10.5	201.15	0.522
IS-MK-AZ8	10.4	219.14	0.475
IS-MK-AZ9	13.0	154.19	0.843
IS-MK-AZ10	10.5	134.14	0.783

m = weight of azide, V_{EtOH} = volume of EtOH to get 0.1 M concentration of azide

6.4.3 Click reaction number 2



Scheme 21: Synthesis of Click mixture 2, R_x-N_3 = 10 different azides (from IS-MK-AZ1 to IS-MK-AZ10).

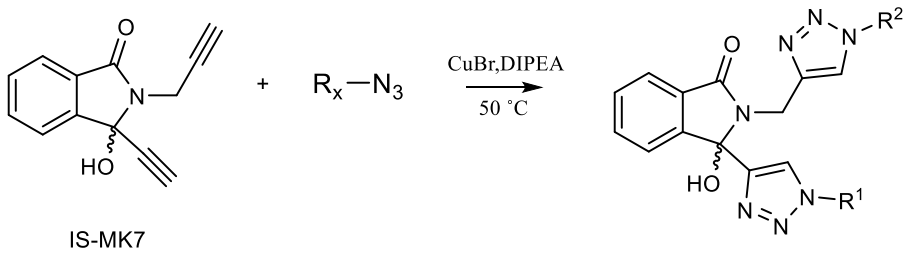
Alkyne core **IS-MK6** (20.7 mg, 0.087 mmol) was added to the round bottom flask. Around 1 mg of each azide was weighed one by one and dissolved in EtOH to get a 0.1 M concentration (Table 4). Subsequently, 173 µL of each azide solution was added to the reaction mixture flask and the general procedure for click reactions followed. Scheme 21 shows the synthesis of Click mixture 2.

Table 4: Preparation of fresh 0.1M solutions of azides for click reaction 2.

Azide	m (mg)	M (g/mol)	V_{EtOH} (mL)
IS-MK-AZ1	12.2	211.61	0.577
IS-MK-AZ2	11.1	134.14	0.827
IS-MK-AZ3	11.3	226.08	0.500
IS-MK-AZ4	12.4	156.19	0.794
IS-MK-AZ5	11.1	176.15	0.630
IS-MK-AZ6	10.0	158.16	0.632
IS-MK-AZ7	10.5	201.15	0.522
IS-MK-AZ8	10.4	219.14	0.475
IS-MK-AZ9	13.0	154.19	0.843
IS-MK-AZ10	10.5	134.14	0.783

m = weight of azide, V_{EtOH} = volume of EtOH to get 0.1 M concentration of azide

6.4.4 Click reaction number 3



Scheme 22: Synthesis of Click mixture 3, R_x-N_3 = 10 different azides (from IS-MK-AZ1 to IS-MK-AZ10).

Alkyne core **IS-MK7** (18.47 mg, 0.087 mmol) was added to the round bottom flask. Around 10 mg of each azide was weighed one by one and dissolved in EtOH to get a 0.1 M concentration (Table 5). Subsequently, 175 µL of each azide solution was added to the reaction mixture flask and the general procedure for click reactions followed. Scheme 22 shows the synthesis of Click mixture 3.

Table 5: Preparation of fresh 0.1M solutions of azides for click reaction 3.

Azide	m (mg)	M (g/mol)	V_{EtOH} (mL)
IS-MK-AZ1	13.8	211.61	0.654
IS-MK-AZ2	11.1	134.14	0.827
IS-MK-AZ3	14.5	226.08	0.640
IS-MK-AZ4	12.4	156.19	0.794
IS-MK-AZ5	11.1	176.15	0.630
IS-MK-AZ6	10.0	158.16	0.632
IS-MK-AZ7	12.9	201.15	0.643
IS-MK-AZ8	13.8	219.14	0.630
IS-MK-AZ9	13.0	154.19	0.843
IS-MK-AZ10	10.5	134.14	0.783

m = weight of azide, V_{EtOH} = volume of EtOH to get 0.1 M concentration of azide

Click mixture number 3 was analyzed with LC-HRMS in order to confirm that the desired compounds are indeed in the mixture. Because more than 100 compounds were in the mixture, we checked if a small subset of possible products were present. Namely, compounds with an amino group were deliberately selected for the analysis since they ionize very well in positive mode and thus give strong signals in MS. Also, the two signals on the chromatogram indicate two regioisomers. The mass match confirmed the presence of individual compounds in the mixture. LC-HRMS chromatogram and measured compounds are displayed in Figure 17.

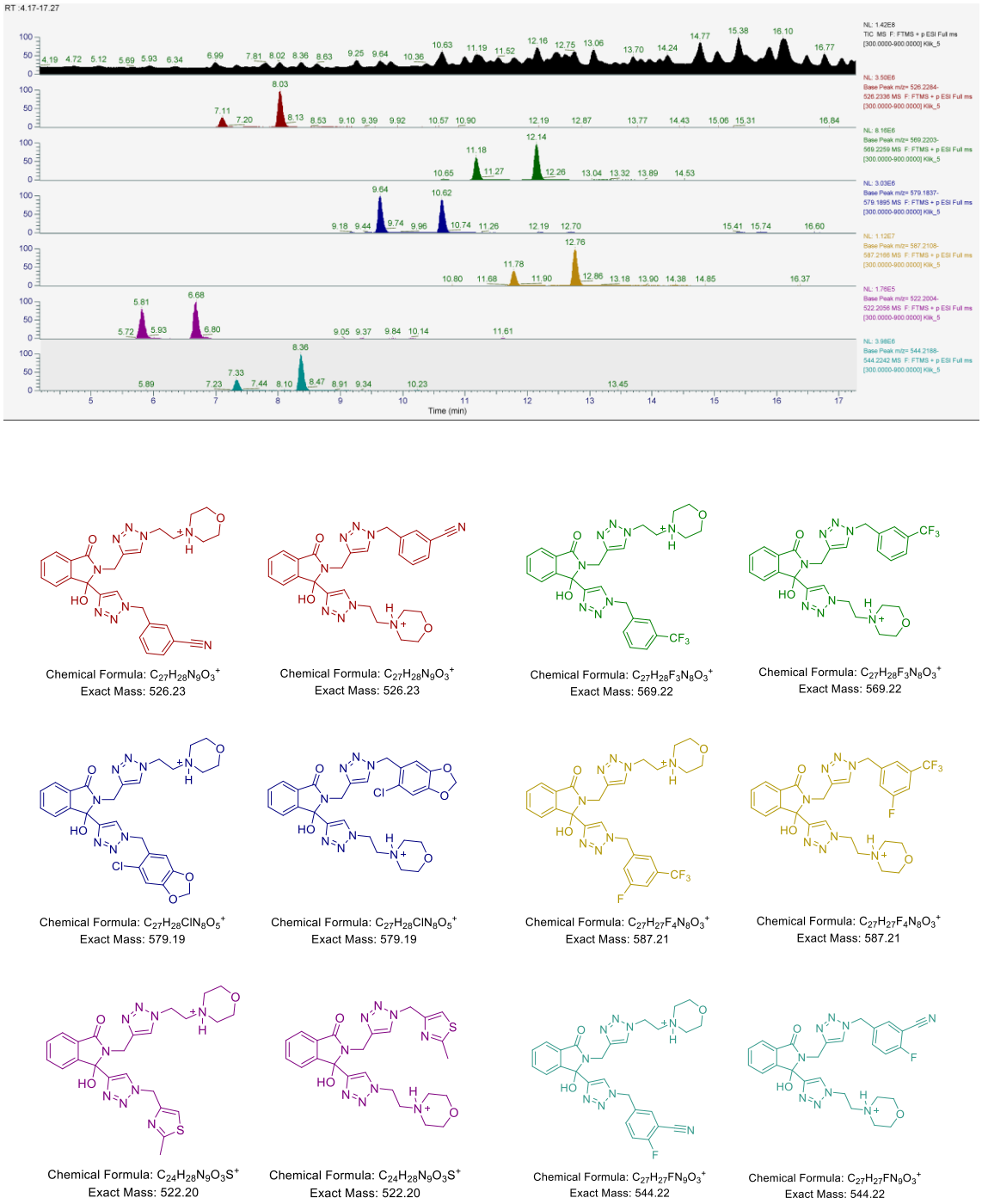
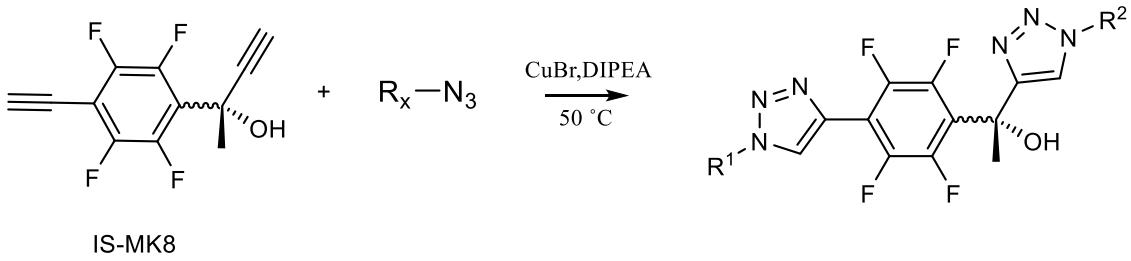


Figure 17: LC-HRMS chromatograms of Click mixture 3; the upper chromatogram represents total ion chromatogram (TIC), lower chromatograms present extracted ion chromatograms for structures below. Under the chromatogram are the structures of measured compounds with exact mass. Two peaks on the chromatogram indicate two regioisomers; the color of the regioisomers matches the color of the peaks on the chromatogram.

6.4.5 Click reaction number 4



Scheme 23: Synthesis of Click mixture 4, R_x-N_3 = 10 different azides (from IS-MK-AZ1 to IS-MK-AZ10).

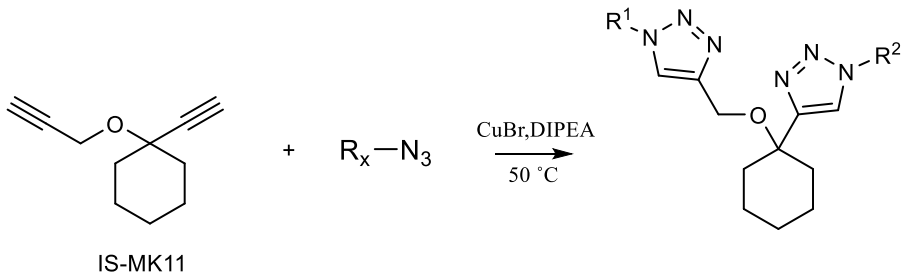
Alkyne core **IS-MK8** (18.57 mg, 0.077 mmol) was added to the round bottom flask. A small amount of azide was weighed one by one and dissolved in EtOH to get a 0.1 M concentration (Table 6). Subsequently, 153 µL of each azide solution was added to the reaction mixture flask and the general procedure for click reactions followed. Scheme 23 shows the synthesis of Click mixture 4.

Table 6: Preparation of fresh 0.1M solutions of azides for click reaction 4.

Azide	m (mg)	M (g/mol)	V_{EtOH} (mL)
IS-MK-AZ1	12.2	211.61	0.577
IS-MK-AZ2	12.7	134.14	0.947
IS-MK-AZ3	11.3	226.08	0.500
IS-MK-AZ4	13.2	156.19	0.848
IS-MK-AZ5	14.7	176.15	0.832
IS-MK-AZ6	13.1	158.16	0.830
IS-MK-AZ7	10.5	201.15	0.522
IS-MK-AZ8	10.4	219.14	0.475
IS-MK-AZ9	13.0	154.19	0.844
IS-MK-AZ10	12.5	134.14	0.933

m = weight of azide, V_{EtOH} = volume of EtOH to get 0.1 M concentration of azide

6.4.6 Click reaction number 5



Scheme 24: Synthesis of Click mixture 5, R_x-N_3 = 10 different azides (from IS-MK-AZ1 to IS-MK-AZ10).

Alkyne core **IS-MK11** (27.77 mg, 0.171 mmol) was added to the round bottom flask. A small amount of azide was weighed one by one and dissolved in EtOH to get a 0.1 M concentration (Table 7). Subsequently, 342 µL of each azide solution was added to the reaction mixture flask and the general procedure for click reactions followed. Scheme 24 shows the synthesis of Click mixture 5.

Table 7: Preparation of fresh 0.1M solutions of azides for click reaction 5.

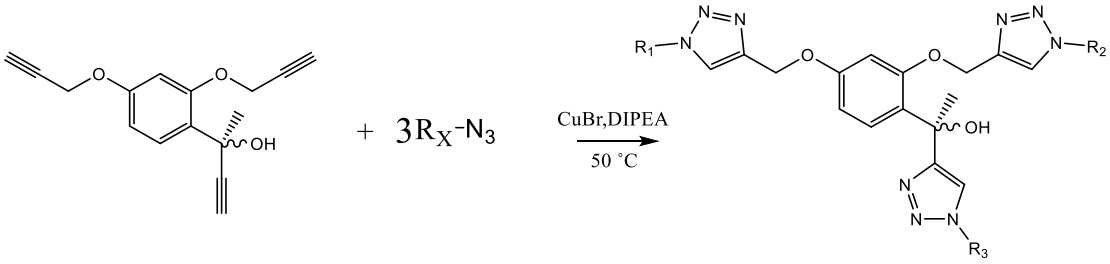
Azides	m (mg)	M (g/mol)	V_{EtOH} (mL)
IS-MK-AZ1	13.9	211.61	0.655
IS-MK-AZ2	12.7	134.14	0.947
IS-MK-AZ3	15.3	226.08	0.675
IS-MK-AZ4	13.2	156.19	0.848
IS-MK-AZ5	14.7	176.15	0.832
IS-MK-AZ6	13.1	158.16	0.830
IS-MK-AZ7	13.2	201.15	0.654
IS-MK-AZ8	16.5	219.14	0.751
IS-MK-AZ9	13.0	154.19	0.844
IS-MK-AZ10	12.5	134.14	0.933

m = weight of azide, V_{EtOH} = volume of EtOH to get 0.1 M concentration of azide

6.5 Triple click reaction

We were able to prepare the core **IS-MK4** with three alkyne functional groups. We let the core react with three equiv. of 10 different azides (Table 2). Theoretically, this reaction yielded 1 000 compounds. The reaction procedure was the same as for the double click described above.

6.5.1 Click reaction number 6



Scheme 25: Synthesis of Click mixture 6, R_x-N_3 = 10 different azides (from IS-MK-AZ1 to IS-MK-AZ10).

Alkyne core **IS-MK4** (16.9 mg, 0.066 mmol) was added to the round bottom flask. Around 15 mg of each azide was weighed one by one and dissolved in EtOH to get a 0.1 M concentration (Table 8). Subsequently, 199 µL of each azide solution was added to the reaction mixture flask and the general procedure for click reactions followed. Scheme 25 shows the synthesis of Click mixture 6.

Table 8: Preparation of fresh 0.1M solutions of azides for click reaction 6.

Azides	m (mg)	M (g/mol)	V_{EtOH} (mL)
IS-MK-AZ1	13.9	211.61	0.655
IS-MK-AZ2	12.7	134.14	0.947
IS-MK-AZ3	15.3	226.08	0.675
IS-MK-AZ4	13.2	156.19	0.848
IS-MK-AZ5	14.7	176.15	0.832
IS-MK-AZ6	13.1	158.16	0.830
IS-MK-AZ7	13.2	201.15	0.654
IS-MK-AZ8	16.5	219.14	0.751
IS-MK-AZ9	13.0	154.19	0.844
IS-MK-AZ10	12.5	134.14	0.933

m = weight of azide, V_{EtOH} = volume of EtOH to get 0.1 M concentration of azide

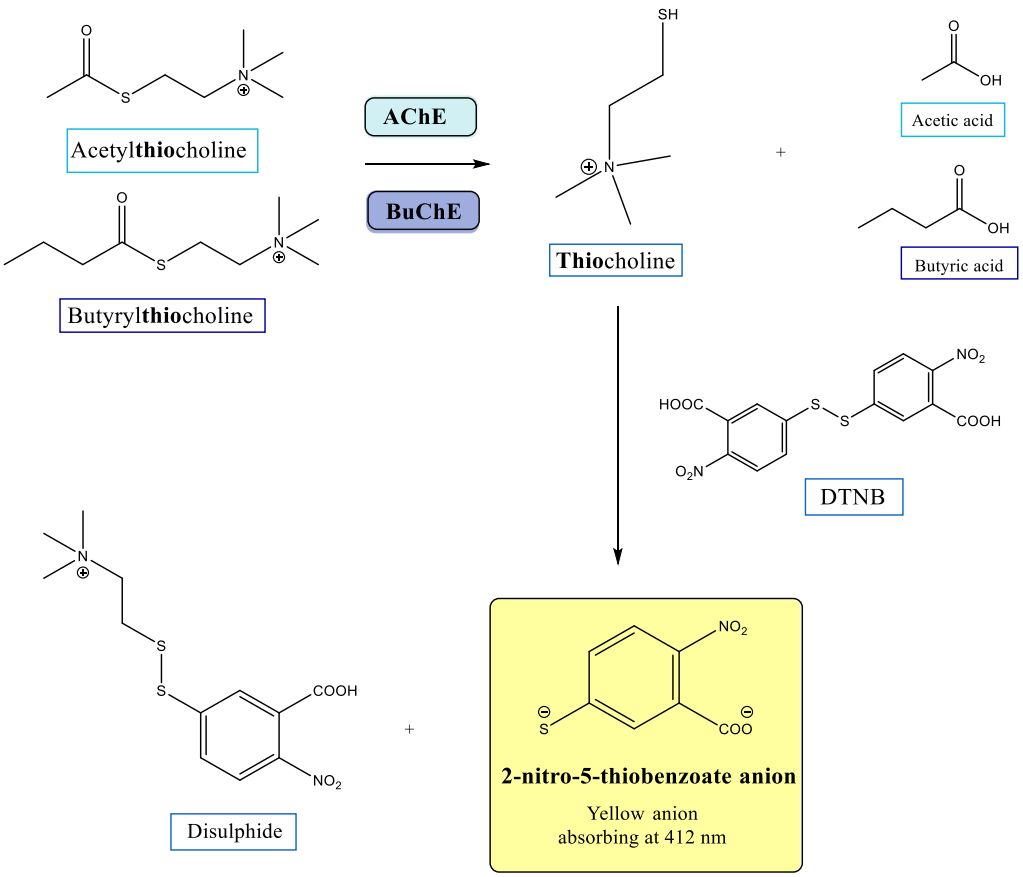
6.6 Biological assays

The prepared libraries were assayed on validated and pharmacologically relevant enzymes. In the initial stages, we focused on human AChE and butyrylcholinesterase (BuChE) with click mixtures number 1, 2 and 3. After that, all six libraries were assayed on mycobacterial 2-*trans*-enoyl-acyl carrier protein reductase (InhA), UDP-*N*-acetylglucosamine enolpyruvyl transferase (MurA), and penicillin-binding protein 1b (PBP1b).

6.6.1 hBuChE, hAChE assay

AChE and BuChE terminate cholinergic signaling by hydrolyzing acetylcholine. AChE, a specific esterase, performs 80% of hydrolytic activity in the brain, being attached to the postsynaptic membrane.[74] BuChE is pseudocholinesterase found in the central and peripheral nervous system, most tissues, and the liver.[75] BuChE plays a supportive role in the brain and accounts for about 10% of cholinesterase activity in the temporal cortex.[76] Excessive hydrolysis of acetylcholine and butyrylcholine is one of the causes of neurodegenerative diseases, such as AD. Therefore, AChE and BuChE inhibition have been documented as critical targets for the effective management of AD.[77]

The inhibitory potencies of the compounds against the ChEs were determined using the method of Ellman (Scheme 26). This method uses an alternative substrate, acetylthiocholine, instead of acetylcholine. Acetylthiocholine is enzymatically hydrolyzed to thiocholine, which reacts with 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB, Ellman's reagent). Ellman's reagent is used for the quantification of thiol groups. The product of this reaction, 5-thio-2-nitrobenzoic acid, has a yellow color with maximum absorption at 412 nm.[78]



Scheme 26: Chemical mechanism of Ellman's method. Adapted and modified from Ali-Shtayeh et al.[79]

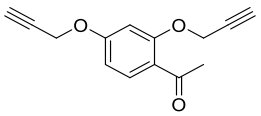
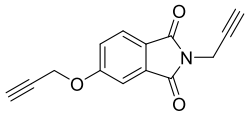
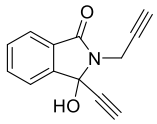
6.6.1.1 Description of the assay

Briefly, compound stock solutions in DMSO were incubated with Ellman's reagent and the ChEs (final concentrations: 370 µM Ellman's reagent, approximately 1 nM or 50 pM hBuChE or hAChE) in 0.1 M phosphate buffer pH 8.0 for 5 min at 20 °C. The enzymes were provided by Xavier Brazzolotto (Département de Toxicologie et Risques Chimiques, Institut de Recherche Biomédicale des Armées, 91223, Brétigny sur Orge, France). The reactions were started by the addition of the substrate (final concentration, 500 µM butyrylthiocholine iodide or acetylthiocholine iodide for hBuChE and hAChE, respectively). The final content of DMSO was always 1%. The increase in absorbance at 412 nm was monitored for 1 min using a 96-well microplate reader (Synergy H4, BioTek Instruments, VT, USA). The initial velocities in the presence (v_i) and absence (v_o) of the test compounds were calculated. The inhibitory potencies were expressed as the residual activities, according to RA [%] = $v_i/v_o \times 100\%$.[80]

6.6.1.2 Evaluation of the assay

The results of the hAChE and hBuChe assays are displayed in Table 9. Click mixture number 3 shows the best potential in the inhibition of BuChe. AChE is quite sensitive (assay conditions, presence of aggregates, lipophilic compounds), and denaturation of the enzyme due to precipitate might have occurred, thus leading to false-positive results. Precipitation was evident at 1 µM concentration of the tested compound. Although the precipitate was not clear at 100 nM concentration, we consider the inhibition nonspecific and the result false positive.

Table 9: Results of the hAChE and hBuChe assays, hAChE = human acetylcholinesterase, hBuChE = human butyrylcholinesterase. RA–the residual activity of the enzyme [%] in the presence of 1 µM and 100 nM compound concentration is expressed as the mean ± standard deviation of one independent experiment performed in triplicate.

Compounds	**Core alkyne**	**hAChE**	**hBuChE**
		RA (c = 1µM)** **RA (c = 100 nM**)**	**RA (c = 1µM**)** **RA (c = 100 nM**)**
Click mixture 1		22.8 ± 2.1%* 56.2 ± 4.6%	79.4 ± 1.6% 93.0 ± 0.9%
Click mixture 2		12.9 ± 2.2%* 52.7 ± 2.7%	83.4 ± 2.0% 93.8 ± 0.7%
Click mixture 3		34.7 ± 1.6%* 63.3 ± 6.7%	**46.1 ± 0.9%** **80.9 ± 0.7%**

* compounds precipitated (OD_{600} at 1 µM > 0.1 µM)
**concentration of one compound in the mixture

6.6.2 PBP1b, InhA, MurA assay

Click mixtures were prepared as stock solutions (Table 10). Around 2 mg (Click5 = 4.15 mg) of click mixture was weighed and diluted in 500 µL of DMSO. We also prepared the stock solution of azides in case the inhibition of the enzyme was caused by the azides alone.

Table 10: The stock solution concentration and the final concentration after dilution (100×).

compound library	m (mg)	M (g/mol)	V_{DMSO} (µL)	number of compounds in the mixture	the stock solution concentration		the final concentration (dilution 100×)	
					c_{mix} (mM)	c (µM)	c_{mix} (µM)	c (µM)
Click1	2.1	600	500	100	7	70	70	0.70
Click2	2.7	600	500	100	9	90	90	0.90
Click3	1.99	600	500	100	6.6	66	66	0.66
Click4	2.39	600	500	100	8	80	80	0.80
Click5	4.15	600	500	100	13.8	138	138	1.38
Click6	1.8	750	500	1000	4.8	4.8	48	0.048

m = mass of compound library, M = approximate molar mass of one compound, V = volume of DMSO, c_{mix} = concentration of compound mixture, c = concentration of one compound in a mixture

6.6.2.1 PBP1b assay

PBPs play a key role in the final stages of peptidoglycan biosynthesis. PBPs catalyze the cross-linking between glycan chains (transpeptidation) and the polymerization of glycan strands (transglycosylation). Bacteria carry multiple PBPs divided into classes A, B, or C. PBP1a and PBP1b are the main transpeptidases and transglycosylases (bifunctional) and their removal leads to the death of bacteria.[81]

PBP1b (from *Streptococcus pneumoniae*) was incubated with stock solutions of click mixtures (2 µL) in 10 mM sodium phosphate buffer (pH 7.0), containing 70 mM D-alanine and 0.01 mg/mL bovine serum albumin (BSA) for 60 min at 25 ˚C. The final volume in a well was 200 µL. Residual activities were determined from the initial rates of the hydrolysis of a thioester 2-(2-benzamidopropanoylthio) acetic acid used as reporter substrate at a concentration of 5 mM. After preincubation, the initial rate of thioester hydrolysis was measured in the presence of 1 mM DTNB by monitoring the increase in absorbance at 412 nm. The assay was carried out in triplicates. Aztreonam was used as a positive control.[82]

6.6.2.2 InhA enzymatic assay

InhA, the enoyl-ACP reductase, participates in the biosynthesis of mycolic acid, an essential lipid component of the mycobacterial cell envelope. The enzyme is the target of the first-line anti-tubercular drug isoniazid and for the second-line drug ethionamide as

well. The resistance of isoniazid and other antituberculosis drugs is increasing, so it is crucial to develop novel InhA inhibitors. InhA is considered a promising target for discovering new agents against active tuberculosis.[83]

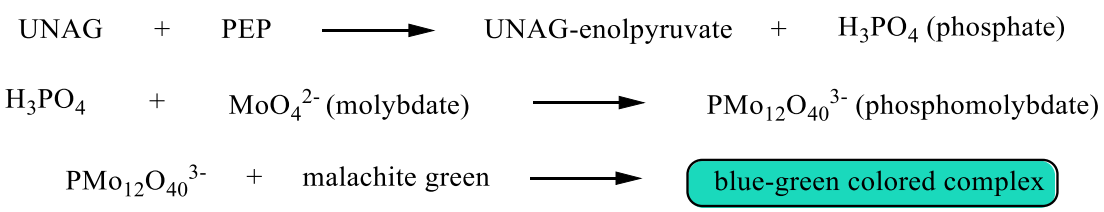
The enzymatic activity of InhA was measured spectrophotometrically by monitoring the oxidation of NADH to NAD^+ at 340 nm. Stock solutions of click mixtures (~ 0.7-1.4 μM) were prepared in 500 μL DMSO (Table 10). DMSO (200 μL) was pipetted into row A on the well plate (negative control), and triclosan (2 μL) was used as a positive control. Stock solutions (2 μL) were pipetted into rows B-H in triplicates. Reactions were initiated by the addition of InhA (50 nM) to a solution containing the inhibitor, the substrate 2-*trans*-dodecenoyl-coenzyme A (DD-CoA), NADH in piperazine-*N,N*′-bis(2-ethanesulfonic acid) (PIPES, 30nM) buffer, pH 6.8. Kinetic assays were performed at 25 °C for 10 min. The inhibitory activity of the mixtures of compounds was expressed as the percentage of residual activities.[84]

6.6.2.3 MurA assay

MurA enzyme participates in the biosynthesis of the bacterial cell wall. MurA catalyzes the first committed step in the biosynthesis of peptidoglycan, the essential cellular component. Antibiotic fosfomycin acts as an inhibitor of the MurA enzyme by forming a covalent adduct with Cys155.[85]

The inhibition of MurA was monitored with the colorimetric malachite green method. The MurA enzyme catalyzes the reaction between UDP-*N*-acetylglucosamine (UNAG) and phosphoenolpyruvate (PEP), which produces phosphate and UNAG-enolpyruvate. Phosphate forms with molybdate phosphomolybdate, which then binds to malachite green and gives a color complex (Scheme 27). Compound libraries were prepared as a stock solution (Table 10). MurA enzyme (from *Escherichia coli*) was pre-incubated with stock solutions and the substrate UNAG for 30 min at 37 °C. The reaction mixture with a final volume of 50 μL contained: 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer, 0.005% Triton X-114, 200 μM UNAG, 100 μM PEP, purified MurA (diluted in 50 mM HEPES, pH 7.8), and 0.5 μL of stock solution of tested mixtures dissolved in DMSO). The reaction was started by adding molybdate, and the mixture was incubated at 37 °C for 15 minutes. After incubation, the reaction was terminated by

adding 100 µL of BIOMOL® Green reagent (determination of free phosphate), and the absorbance was measured at 650 nm after 5 min.[86]

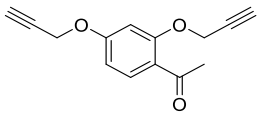
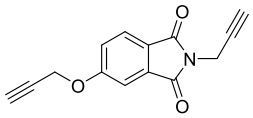
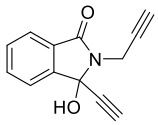
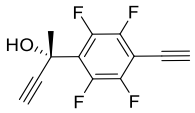
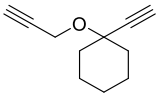
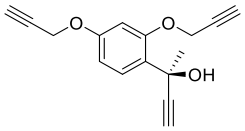


Scheme 27: The formation of a color complex in the MurA enzymatic assay.

6.6.2.4 Evaluation of the assays

The results of the PBP1b, InhA, MurA assays are displayed in Table 11. The first enzyme assay showed no hits on PBP1b. However, potent inhibition was observed in the assay with InhA. Click mixture number 1 (RA = 47.2 %) was the most promising among those tested. Inhibition was also observed in Click mixture 5 (RA = 59.8%) and Click mixture 6 (RA=55.7%). Interestingly, the mixture of azides showed inhibitory activity of MurA.

Table 11: The results of the PBP1b, InhA, MurA assays, RA = the residual activity of the enzyme [%].

Compound libraries	**Alkyne core**	RA% **PBP1b** *S. pneumoniae*	RA% **InhA**	RA% **MurA** *E.coli*
Click mixture 1		94.9	**47.2**	68.4
Click mixture 2		90.2	-10.9*	82.8
Click mixture 3		96.7	97.7	57.6
Click mixture 4		96.1	65.4	55.9
Click mixture 5		94.7	59.8	9.3
Click mixture 6		101.9	55.7	53.4
Azides mixture	———	96.2	97.9	**32.8**

*high absorbance

6.7 Enumeration of virtual libraries and assessment of druglikeness of individual compounds

Virtual libraries of compounds produced by click reactions 1–6 were generated in MOE with the intention of comparing the molecular properties of individual compounds. We focused mainly on molecular weight, number of hydrogen bond donors/acceptors, number of rotatable bonds, and log *P*. It was evaluated whether individual compounds met Lipinski's rule of 5 (Ro5) criteria and could be described as druglike. The compound is druglike if the number of Ro5 violations is less than or equal to one. Most compounds were relatively large molecules with a molecular weight greater than 500 g/mol, lipophilic with library-averaged log P ranging from 4.1 to 6.92. The library-averaged number of rotatable bonds was greater than 10 for click1 and click6, and for click2 was equal to 10. Due to polarization, the triazole also acts as a donor (with a C-H bond). However, this non-standard hydrogen bond donor is not recognized by MOE algorithm and is not included in the evaluation of compliance to Ro5. In general, all theoretically prepared compounds had a low number of hydrogen bond donors, significantly below the Ro5 limit. The number of hydrogen bond acceptors was higher than 10 for click1, click2, click3, and click6. A total of 1396 compounds did not meet the conditions for the druglike. On the other hand, 104 compounds fulfill the criteria and can be considered druglike. Statistics and histograms of individual descriptors were evaluated; see Tables 12–17 and Figures 18–21 below. The whole library of theoretically prepared compounds with calculated values of the descriptors is located as an attachment to this work in Appendix A (semi-colon separated CSV format). The explanation of individual descriptors present in the database can be found below.

The explanation of individual descriptors:

weight	Molecular weight (including implicit hydrogens) in atomic mass units with atomic weights taken from [CRC 1994].
b_rotN	Number of rotatable single bonds. Conjugated single bonds are not included (e.g. ester and peptide bonds).
lip_acc	The number of O and N atoms.
lip_don	The number of OH and NH atoms.
SlogP	Log of the octanol/water partition coefficient. This property is an atomic contribution model that calculates logP from the given structure; i.e. the correct protonation state (washed structures). Results may vary from the logP(o/w) descriptor.
logS	Log of the aqueous solubility (mol/L). This property is calculated from an atom contribution linear atom type model with r2 = 0.90.
TPSA	Polar surface area (Å2) calculated using group contributions to approximate the polar surface area from connection table information only.
lip_violation	The number of violations of Lipinski's Rule of Five.
lip_druglike	One if and only if lip_violation < 2 otherwise zero.
mutagenic	Indicator of the presence of potentially toxic groups. A non-zero value indicates that the molecule contains a mutagenic group.
rsynthA	Value in [0,1] indicating the synthetic reasonableness, or feasibility, of the chemical structure. A value of 0 means it is unlikely that the molecule can be synthesized while a value of 1 means that it is likely that the molecule can be synthesized. The value reflects the fraction of heavy atoms in the molecule that can be traced back to starting materials fragments resulting from retrosynthetic disconnection rules.

Table 12: Molecular weight statistics.

Molecular Weight	Click1	Click2	Click3	Click4	Click5	Click6
Mean	582.44	593.42	565.41	596.36	516.42	785.57
Standard deviation	47.03	47.03	47.03	47.03	47.03	57.34
Mean ± SD	582.44 ± 47.03	593.42 ± 47.03	565.41 ± 47.03	596.36 ± 47.03	516.42 ± 47.03	785.57 ± 57.34
Minimum	496.53	507.51	479.50	510.45	430.52	656.71
Maximum	680.40	691.38	663.37	694.33	614.39	932.52

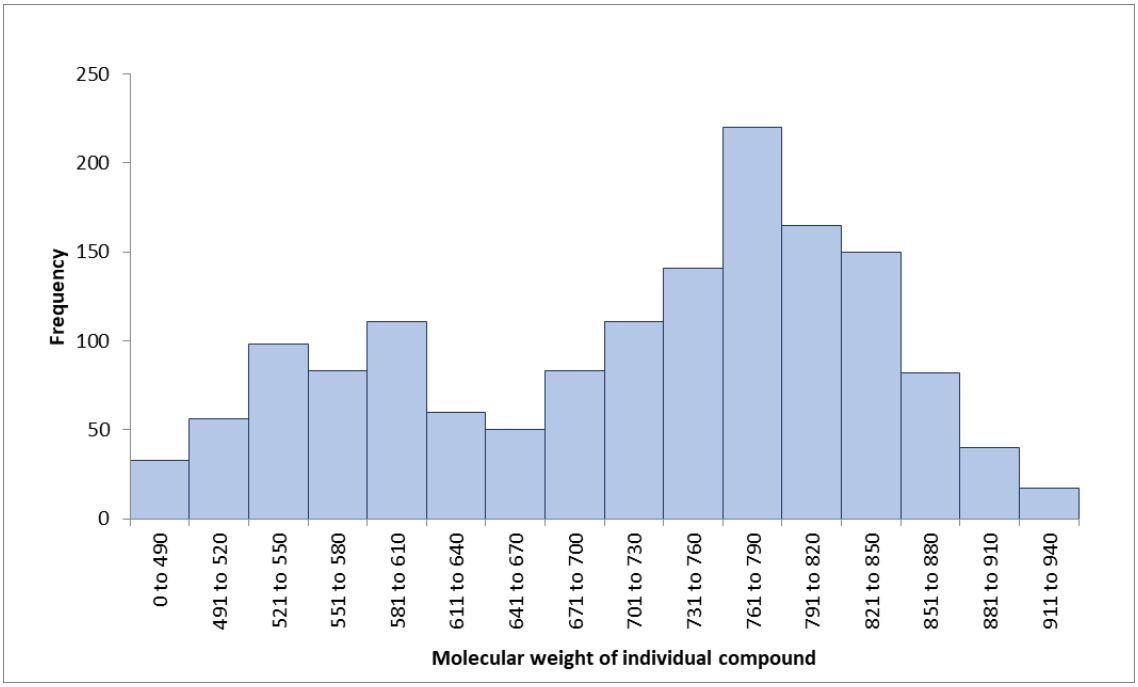


Figure 18: Histogram of molecular weight of the whole library (1500 compounds).

Table 13: Log *P* statistics.

Log *P*	Click1	Click2	Click3	Click4	Click5	Click6
Mean	5.56	4.58	4.10	5.46	5.63	6.92
Standard deviation	1.32	1.32	1.32	1.32	1.32	1.61
Mean ± SD	5.56 ± 1.32	4.58 ± 1.32	4.1 ± 1.32	5.46 ± 1.32	5.63 ± 1.32	6.92 ± 1.61
Minimum	1.96	0.97	0.49	1.86	2.02	1.52
Maximum	8.33	7.35	6.87	8.23	8.39	11.07

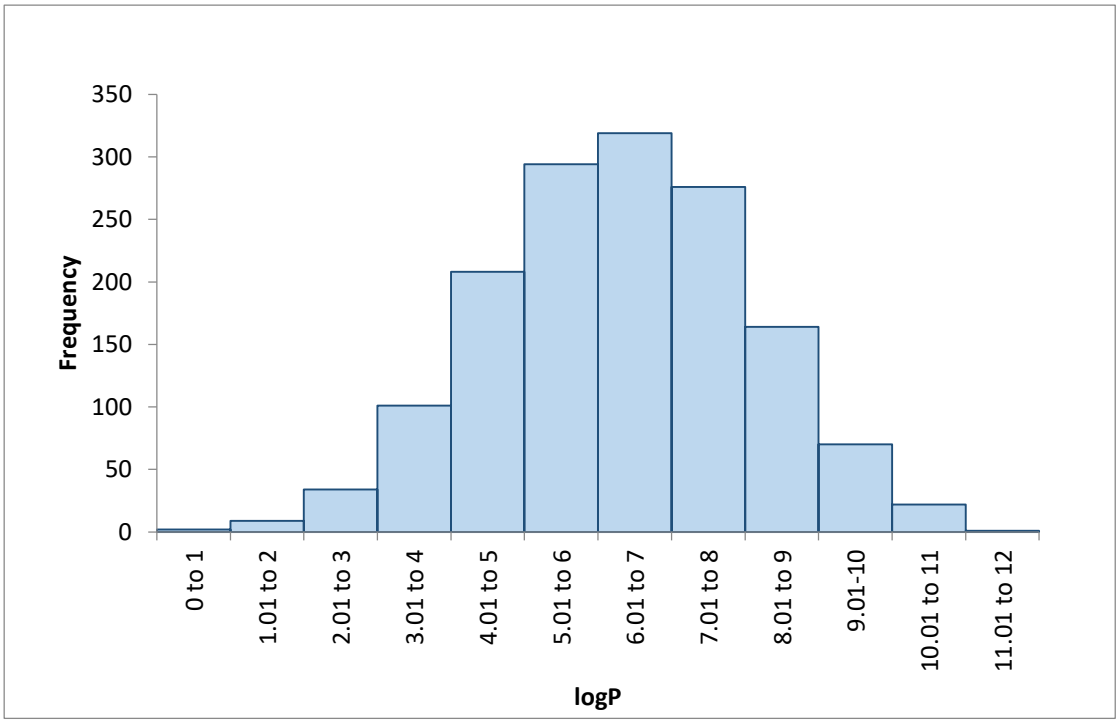


Figure 19: Histogram of log *P* of the whole library (1500 compounds).

Table 14: Number of rotatable bonds statistics.

Number of rotatable bonds	Click1	Click2	Click3	Click4	Click5	Click6
Mean	12.2	10.2	8.2	8.2	9.2	15.8
Standard deviation	0.7	0.7	0.7	0.7	0.7	0.85
Mean ± SD	12.2 ± 0.7	10.2 ± 0.7	8.2 ± 0.7	8.2 ± 0.7	9.2 ± 0.7	15.8 ± 0.85
Minimum	11	9	7	7	8	14
Maximum	13	11	9	9	10	17

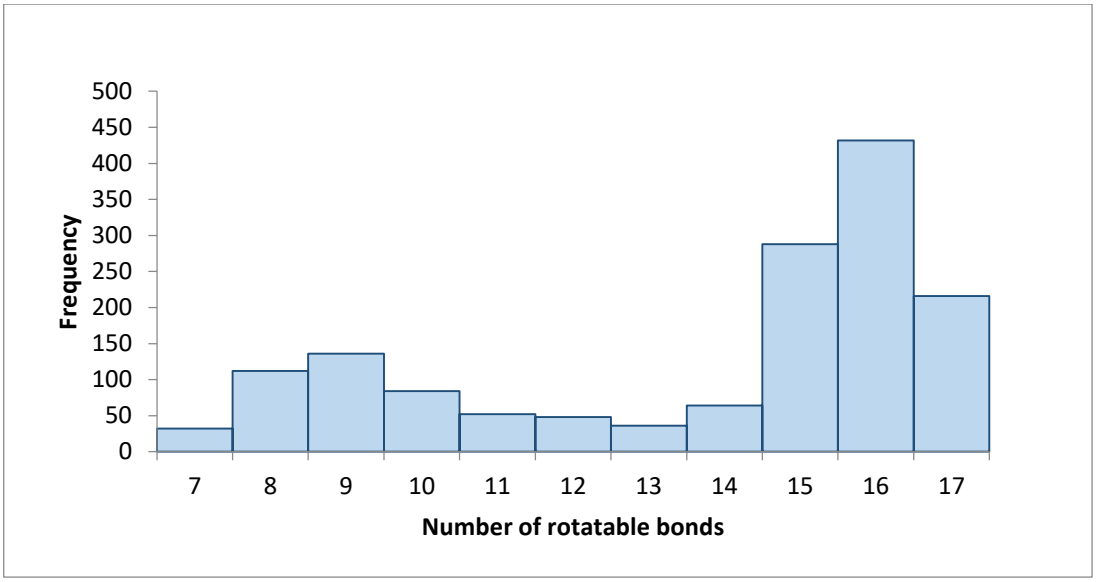


Figure 20: Histogram of the number of rotatable bonds of the whole library (1500 compounds).

Table 15: Number of hydrogen bonds acceptors statistics.

HBA	Click1	Click2	Click3	Click4	Click5	Click6
Mean	10.8	11.8	10.8	8.8	8.8	14.7
Standard deviation	0.99	0.99	0.99	0.99	0.99	1.21
Mean ± SD	10.8 ± 0.99	11.8 ± 0.99	10.8 ± 0.99	8.8 ± 0.99	8.8 ± 0.99	14.7 ± 1.21
Minimum	9	10	9	7	7	12
Maximum	13	14	13	11	11	18

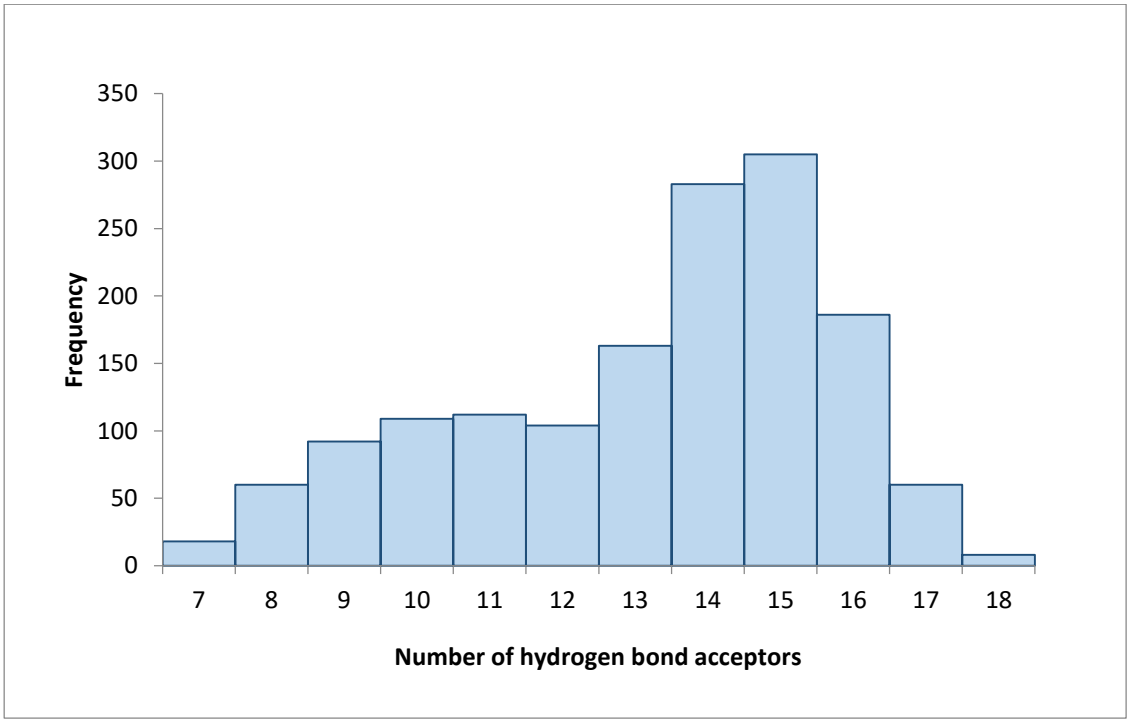


Figure 21: Histogram of the number of hydrogen bond acceptors of the whole library (1500 compounds).

Table 16: Number of hydrogen bonds donor statistics.*

HBD	Click1	Click2	Click3	Click4	Click5	Click6
Mean	0	0	1	1	0	0
Standard deviation	0	0	0	0	0	0
Mean ± SD	0	0	1	1	0	0
Minimum	0	0	1	1	0	0
Maximum	0	0	1	1	0	0

*hydrogen bond donors of triazole are omitted

Table 17: Druglikeness* statistics.

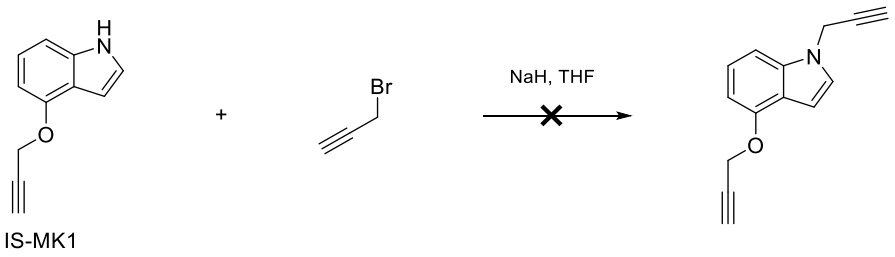
Druglikeness	Click1	Click2	Click3	Click4	Click5	Click6
Number of druglike compounds	4	0	26	33	41	0
Number of non druglike compounds	96	100	74	67	59	1000

* the compound is druglike if the number of Ro5 violations is less than or equal to one

7. Discussion

In this thesis, we aimed to synthesize compound libraries with the help of click chemistry. Experimental work was divided into three parts: the preparation of intermediates (alkynes and azides), the subsequent realization of the click reaction itself, and the biological screening on several enzymes.

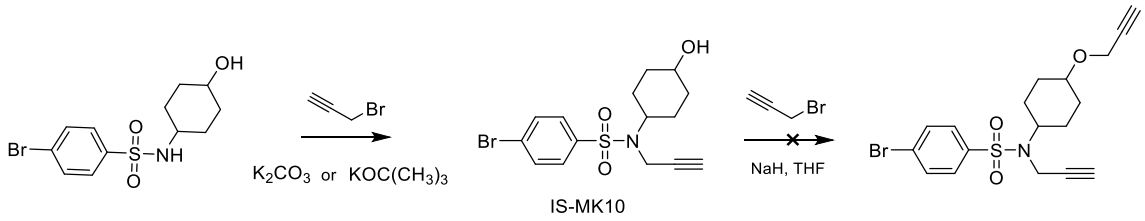
When designing the cores, we paid attention to the different spatial orientations of the alkyne groups in order to incorporate structural diversity to compound libraries. Method A (the alkylation of phenols with propargyl bromide) was the method for IS-MK1, IS-MK2, IS-MK3, IS-MK9, and IS-MK6 preparation. The yield of Method A was good and it ranged around 75%. The dialkyne core IS-MK2 was made with a one-step synthesis, whereby we also obtained alkyne IS-MK3 as a byproduct. The first step of preparing IS-MK1 and IS-MK 9 was successfully carried out with Method A. However, the second, *N*-alkylation of indol (with propargyl bromide, NaH used as a base) failed in the case of IS-MK1 (Scheme 28). We repeated the reaction twice and in both cases, the NMR spectrum contained a mixture of aromatic compounds. For this reason, we decided not to *N*-alkylate IS-MK9.



Scheme 28: Unsuccessful second alkylation of IS-MK1.

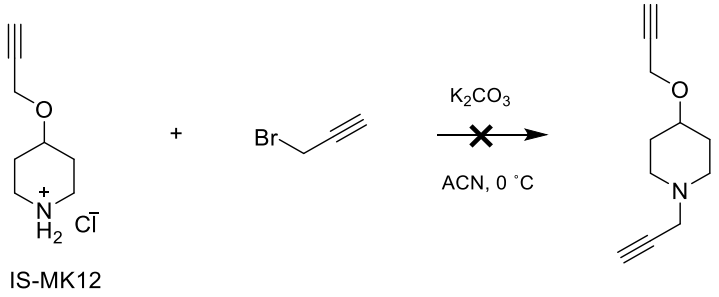
Method A was applied for the synthesis of IS-MK10, but with the difference that we alkylated the nitrogen of the sulfonamide group, not phenol. We started the reaction with a relatively large amount of 4-bromobenzenesulfonyl chloride (11 g, 43.41 mmol) and let it react with primary amine (4-aminocyclohexan-1-ol). The resulting sulfonamide was split into two halves. For one half, potassium carbonate was used as a base, whereas potassium *tert*-butoxide was used for the other (Scheme 29). Both reactions led to the formation of IS-MK10, but the product of the reaction with potassium carbonate was more pure. We started the second alkylation according to Method B, but without success

(Scheme 29) as NMR analysis did not show the desired product. Nonetheless, a monoalkylated IS-MK10 can be used for different reactions or click chemistry.



Scheme 29: First alkylation and unsuccessful second alkylation of IS-MK10.

On the other hand, Method B worked well for preparing the dialkyne core IS-MK11 with a decent yield of 71%. Alkyne IS-MK12 was also synthesized by Method B, but the reaction yield was relatively low (29%), so the reaction had to be repeated to obtain an adequate quantity for the next step. Unfortunately, *N*-alkylation of piperidine was unsuccessful (Scheme 30) as a mixture of different compounds was visible on TLC, which we were not able to separate and analyze.

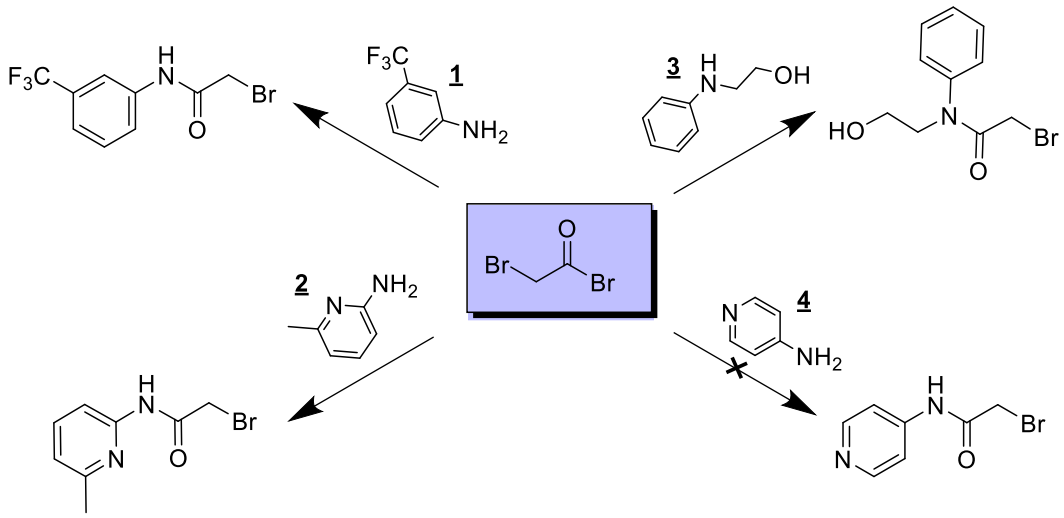


Scheme 30: Unsuccessful second alkylation of IS-MK12.

Alkylation with Grignard reagent (Method C) was applied for dialkyne cores IS-MK4, IS-MK7, and IS-MK8. The actual yields were lower in comparison to Method A. Moreover, the yields of intermediates (alkynes and azides) were not essential for us as milligram quantities were sufficient for subsequent click chemistry. Reactions were carried out at -70 °C. In the last procedure (Method D), phthalic anhydride or phthalic acid reacted with a primary amine to form *N*-substituted phthalimides. The first step of dialkyne core IS-MK7 was carried out this way. Phthalic acid creates anhydride when it is heated up, and after the reaction with propargyl amine, we received alkyne IS-MK5.

The second reagents needed for click reactions were azides. Generally, the azido compounds were obtained by halide (bromine, chloride) or mesyl group displacement. The source of azide in our reactions was sodium azide, and the reactions proceeded

according to the S_N2 mechanism. Usually, azides were purified only with extraction, without further chromatographic purification. In this way, we prepared 10 azides. To bring some diversity into azides, we attempted to synthesize some of them from anilides/aminopyridine; specifically, we chose 3-(trifluoromethyl)aniline (**1**), 6-methylpyridin-2-amine (**2**), 2- (phenylamino)ethan-1-ol (**3**), pyridin-4-amine (**4**). We used bromoacetyl bromide as a source of bromine and TEA as a base (Scheme 31). Compounds **1**, **2**, and **3** were converted to corresponding α-bromoamides, which reacted with sodium azide in the next step. Unfortunately, TLC showed us a mixture of compounds; for this reason, the final purification was not accomplished.



Scheme 31: Synthesis of α-bromoamides for the following azide preparation. The procedure failed for compound **4**. Reagents and conditions: TEA, DCM, laboratory temperature, 12h.

Our further efforts were inspired by the first step of lidocaine synthesis; instead of 2,6-dimethylaniline, we tried the reaction with 3-(trifluoromethyl)aniline and 6- methylpyridin-2-amine. For these two compounds, reactions did not work, so we tried one last time with aniline and 3-methoxyaniline. Aniline (3-methoxyaniline) was treated with bromoacetyl bromide in the presence of acetic acid and its conjugate base sodium acetate. We were successful this time and the reaction yielded α-bromoamides, which were reacted with sodium azide in the next stage. The products had to be purified additionally. As we did not need a large quantity of these azides, we decided to purify them by crystallization from methanol, which did not work according to our expectations. Therefore, we finally purified them by column chromatography and obtained desired azides IS-MK-AZ-11 and IS-MK-AZ12.

Double click reactions were realized with previously prepared dialkyne core and 10 azides. The double click reaction itself was preceded by finding the appropriate reaction conditions. For our first attempt, we used alkyne IS-MK3 and only one azide IS-MK-AZ1. CuBr was used as a catalyst, DIPEA as a base, and the reaction was carried out at room temperature overnight. According to TLC, the reaction was not completed, so we heated the reaction mixture to 50 ˚C and stirred it overnight. Heating proved beneficial as the reaction proceeded to completion. We applied the same conditions for double click, in which one alkyne core (IS-MK2) was reacted with three different azides. After one day, the reaction was extracted with ethyl acetate and washed with 5% ETDA. EDTA, the chelating agent, binds the copper, and we observed blue colored water layer (Figure 22).

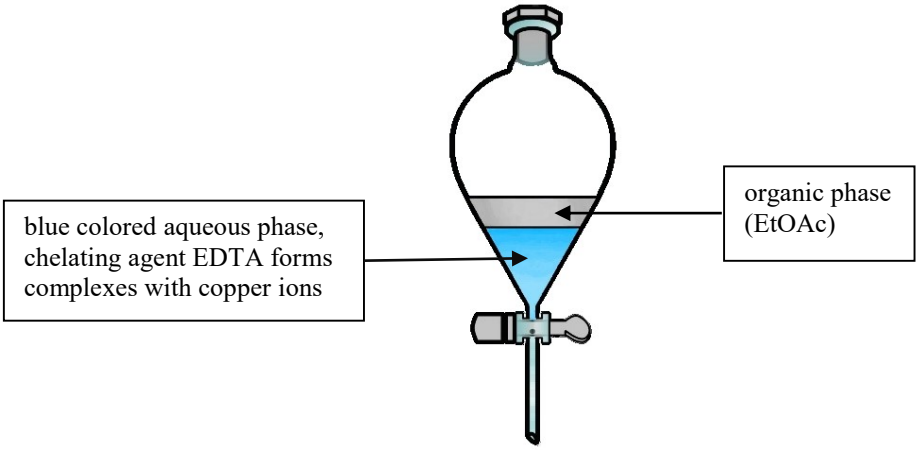


Figure 22: Extraction of click mixture with EDTA.

Afterward, more complex compound libraries were prepared by reacting one alkyne core with 10 different azides in previously optimized click reaction conditions. To illustrate one example of the complexity of our work on the one hand and the usefulness of methodology on the other, one should bear in mind that since alkynes were not symmetrical, the reaction between each alkyne core and ten azides can yield 100 compounds (triple click 1000 compounds).

The prepared compound libraries were assayed on pharmacologically relevant enzymes (involved in Alzheimer's disease and bacterial targets). The inhibitory potencies were expressed as residual activities (%). The first enzymatic essay on BuChE and AChE was measured at 1 μM and 100 nM concentrations of compounds. The best potential in the inhibition of BuChE was exhibited by Click mixture number 3 with residual activity of 41.6 % (c = 1 μM). In the AChE assay, there was precipitation of the compounds, leading

to denaturation of the enzyme and a false-positive result. Precipitation was evident at 1 μM concentration. Although the precipitate was not clear at 100 nM concentration, we also considered that the observed inhibition was nonspecific. All six libraries were assayed on PBP1b, MurA, and InhA enzymes. No inhibition was observed in PBP1b assay, whereas potent inhibition was detected in the InhA assay. The best inhibitory potential was demonstrated for Click mixture 1; nonetheless, the activity was indicated also for click mixtures 5 and 6. On a microtiter plate, the mixture of azides was pipetted to demonstrate if azides themselves showed inhibition. Interestingly, this occurred in MurA enzyme assays, where only azides showed inhibition. To summarize, the results of biological testing demonstrate the potential of this idea. It establishes the background for the generation of bigger libraries and screening on a larger scale of targets.

Click reactions, specifically triple click, have the potential to produce a large number of compounds with high diversity. On the other hand, in our setup, the compounds synthesized by click reactions have difficulty meeting Ro5, as we could see from the assessment of druglikeness (chapter 6.7). Compounds were quite bulky; the molecular weight of most of them exceeded 500 g/mol. This problem is difficult to overcome because the presence of multiple triazole rings increases molecular weight. The log *P* of prepared compounds was relatively high, which can be solved by using more hydrophilic azides for the click reactions. For most compounds, the number of hydrogen bond acceptors was greater than 10, so Ro5 was violated again. The number of rotatable bonds, another descriptor often used in the measurement of druglikeness, should be lower than 10, according to Veber et al.[87] Our compounds were quite flexible, and compounds from the triple click reaction contained 16 rotatable bonds on average.

8. Conclusion and future outlook

In this work, we designed and synthesized 12 azides and 6 alkyne cores. After both groups of compounds were prepared, we let each of the 6 alkyne cores react with 10 different azides. Since alkynes were not symmetric, the reaction between each dialkyne and 10 azides yielded, in theory, 100 compounds. We prepared one core with three alkyne moieties; and the click reaction with this core theoretically gave us 1 000 compounds.

As a result, the combinatorial chemistry approach applied in this thesis led, in theory, to 1 500 compounds in total. Clearly, double (triple) click reactions have the potential to generate a large number of compounds. On the other hand, the resulting compounds have a relatively high molecular weight, a higher number of rotatable bonds, and hydrogen bond acceptors, which can be a barrier for these compounds to become a lead structure, at least for drugs intended for peroral administration.

In conclusion, inhibition observed in the assays with BuChE and InhA demonstrates the validity of the initial idea. As a continuation of this work, various methods will be used to identify compounds causing inhibition from click mixtures. The plan is as follows: the compound mixture will be incubated with the target protein. Hopefully, some compounds will bind strongly to the protein. The mixture (target + compounds) will be filtered to discard unbound compounds. The denaturing solution will be added so that the target protein gets denaturized and releases the bound compounds. The latter are going to be filtered away and identified by LC-MS. Future plans include the generation of bigger and more complex libraries and screening them on targets with affinity selection mass spectrometry.

The disclosed library (Appendix A) can be used as an input for future computational chemistry studies.

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