15

Synthesis and Functionalization of Biomolecules via Click Chemistry

Christine Schilling, Nicole Jung and Stefan Bräse

15.1 Introduction

The products of the click reaction – the 1,2,3-triazoles – are a desired target class as they provide additional functionality, such as hydrogen bonding and coordination prospects. Furthermore, they possess a broad spectrum of biological properties, not only anti-HIV, anti-allergenic and antibacterial features but also fungicidal and herbicidal activity.[1] As such, the click reaction is a very attractive method in material science as well as in the development of novel biologically active compounds.

There are two possible applications of the click reaction, the first of which is to *construct* novel compounds/materials, in which new modified building blocks are added to the material/compound. The second method is to *modify* existing materials/compounds through chemical alteration.[2] One example of the latter involves the labeling of molecules *in vivo* and *in vitro* for detection and purification purposes. The click reaction also receives substantial attention in the field of polymer sciences.[4] The reaction can be applied to the immobilization/modification of, for example, carbohydrates and proteins on solid surfaces,[3,5] azido-sugars on gold to generate carbohydrate self-assembled monolayers (SAMs),[6] and to the addition of electroactive as well as bioactive recognition elements to electrode surfaces.[7]

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15.2 Labeling of Macromolecular Biomolecules

15.2.1 Fluorescent Labeling

A broad range of alternatives are known for the introduction of markers into organic molecules. Of these, the bonding of fluorescent molecules is one of the most widely used techniques. While coumarin, fluorescein and rhodamine are the preferred sources for these fluorescent markers, other less frequently used molecules can be introduced via click reaction, thus enabling the analysis of the coupled compounds via fluorescent measurements. The principle of these fluorescent labeling strategies is demonstrated below, with fluorescein as the example (Figure 15.1).

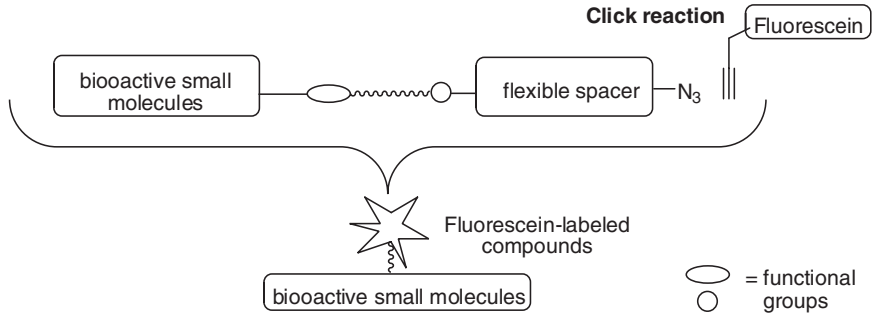
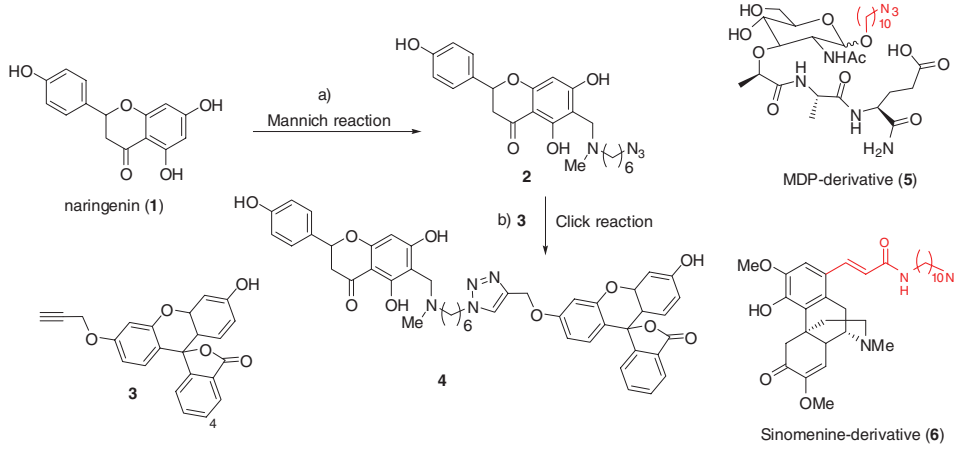


Figure 15.1 *Schematic overview of the fluorescent labeling through click chemistry.*[8]

In principle, two possible fluorescein-derivatives exist, which are useful for click chemistry. The first derivative – compound **3** (Scheme 15.1) – employed by Yao *et al.*[8] differs from the second one (not shown) used by Koberstein *et al.*,[9] Pierters *et al.*[10] and Wooley *et al.*,[11] in terms of the position of the clickable functionalization. Yao *et al.* used *O*-propargylated fluorescein derivatives, whereas the other groups performed click reactions



Scheme 15.1 *Fluorescent labeling of naringenin through click reaction: (a) formaldehyde, $MeNH(CH_2)_6N_3$, $ZnCl_2$, EtOH, 65 °C, 3 h, 87%; (b) $CuSO_4{\cdot}5H_2O$ (cat.), sodium ascorbate, fluorescein derivative 3, $^tBuOH/H_2O$, 67%.*[8]

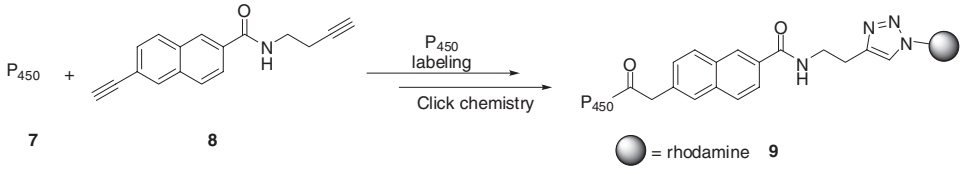
with fluorescein-azides, bearing the functionalized side chain in position 4. As natural products in their original structure contain no azides, the desired functionality has to be introduced through selective azidation of the target bioactive compound or through reaction of the latter with azide-containing molecules. The second method was performed by Yao *et al*.[8] The group chose four bioactive compounds, which were derived in order to react in click reactions and therefore bind the fluorescent label fluorescein. Fluorescein was selected from the available organic dyes because fluorescein and its derivatives are still cheaper than most of the other fluorescent molecules. Scheme 15.1 illustrates the selective attachment of an azide-bearing side chain on naringenin (**1**) via Mannich reaction of the most nucleophilic ring with formaldehyde and the azide-bearing amine.

After the introduction of the side chain, click reaction yielded the desired fluorescein-derived flavone **4**, which could then be analyzed regarding its influence on plant root nodulation. Similar reactions to those presented in Scheme 15.1 generated natural product derivatives **5** and **6**, which were both connected to azide-containing side chains and were used for click reaction with fluorescein-derivative **3** to give labeled analogs of the natural products MDP (muramyl dipeptide) and sinomenine.

Fluorescent labeling with fluorescein is also used for the labeling of proteins in the detection of cancer-linked galectin-3, as shown by Pieters *et al*.[10] Beyond this investigation, there are few publications dealing with the introduction of fluorescein into nanoparticles via click reaction[11] and the labeling of polymer materials. In the latter case, the immobilization of fluorescein-derivatives is undertaken, in order to demonstrate the reactivity of surface-bound alkynes.[9]

Rhodamine is a second compound used for the fluorescent labeling of enzymes and bioactive compounds. Taunton *et al*.[12] demonstrated that the combination of rhodamine incorporation with photo-affinity labeling could be used to identify protein targets of cyclodepsipeptides. For this reason, cyclodepsipeptides containing a propargyl functionality were reacted via photo-affinity labeling with the target proteins and subsequently 'clicked' with azide-derived rhodamine. Similar approaches have also been recognized for the tagging of probes with biotin.[13]

As shown in labeling strategies with biotin in Scheme 15.6 and Scheme 15.7 (later on in this chapter), rhodamine derivatives are used for activity-based protein profiling (ABPP). The bioorthogonal coupling reaction can be performed with an alkyne-modified rhodamine, which is covalently attached to an enzyme bearing an azide functionality.[14] The inverse case, the reaction of an azide containing rhodamine with alkynylated enzymes (Scheme 15.2), has also been established (compare to MacKinnon *et al.*,[12]). In the latter case, cytochrome P450 (**7**) was labeled with a 2-ethinylnaphthalene-derived activity-based probe **8**. After metabolization of the probe and anchoring to P450, rhodamine was introduced by click reaction to give **9**.[15]



7 8 9

Scheme 15.2 *Acetylene-modified 2-ethinylnaphthalene as a derivative for conjugation via click chemistry to azide modified rhodamine.*[15]

There are several derivatives of fluorescein-type molecules that are applied in fluorescent labeling and in *in vivo* imaging. Figure 15.2 shows four compounds (**10**–**13**) linkable via alkyne-containing side chains, which were used for click reactions with azide-derived glycans. These fluorescent molecules consist of either a linear alkyne (**12**, **13**) or a difluorinated cyclooctyne (**10**, **11**). Given the ring strain of cyclooctynes in combination with the presence of the strong electron-withdrawing group, the latter enable copper-free click chemistry.[16]

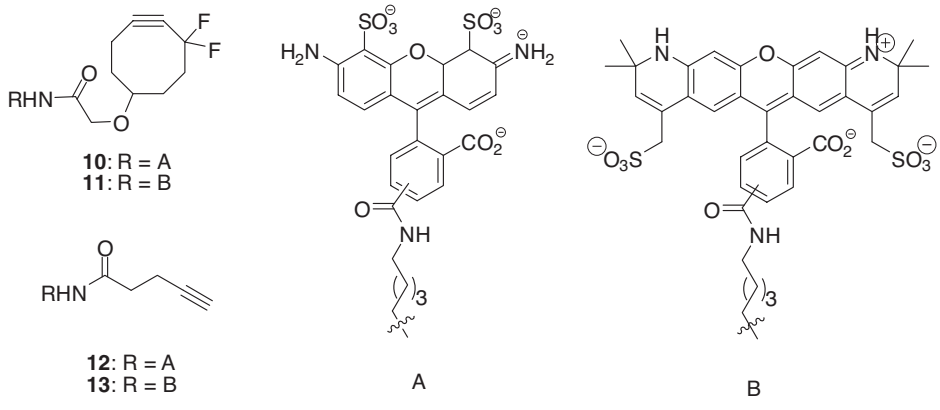
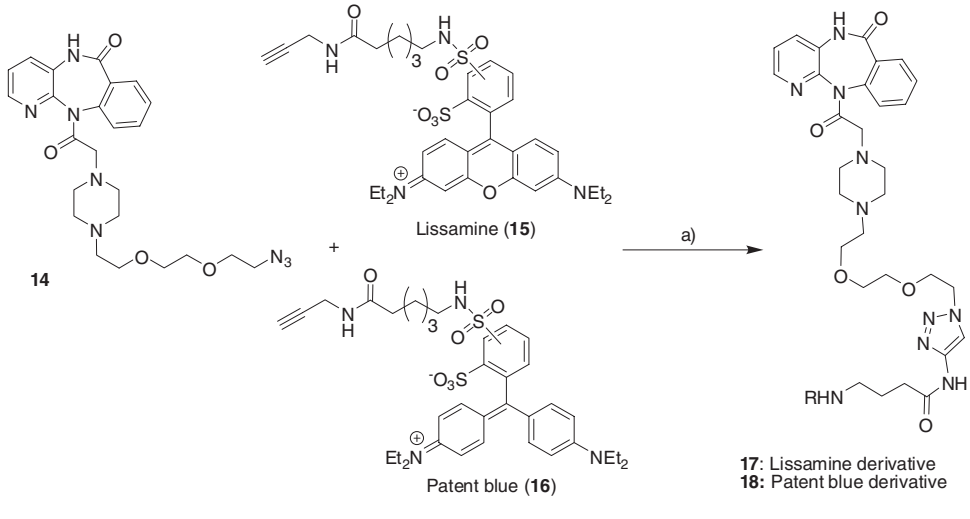


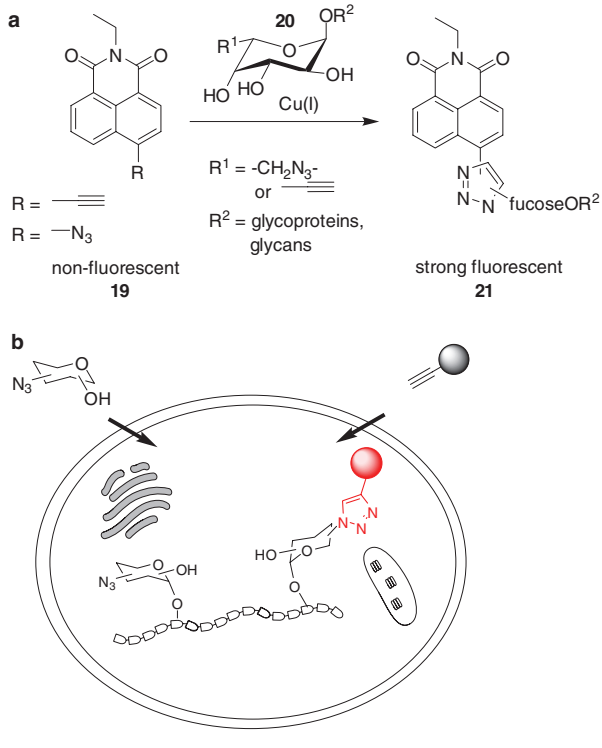
Figure 15.2 *Derivatives of Alexa Fluor 488 and Alexa Fluor 568 for copper-free click chemistry.*[16]

Rhodamine-derived molecules have also been used for the labeling of receptor ligands. Bonnet *et al*. illustrated the utility of Lissamine (**15**) (fluorescent dye) and Patent blue derivatives (**16**) (nonfluorescent dye) for the investigation of ligand interactions with the human muscarin M1 receptor.[28]



Scheme 15.3 *Labeled pirenzepine derivatives **17** and **18** obtained via click chemistry. (a) $CuSO_4$ (10% Cu) wire, MeCN/H_2O (9:1), 4 h.*[28]

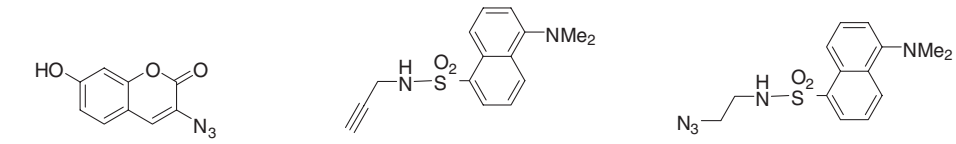
Sawa *et al*. showed that *in vivo* imaging of fucosylated glycans bearing an azide functionality is possible via glycan labeling with 1,8-naphthalimide-derivative **19**.[17] This non-fluorescent imide can be bound via click chemistry to the target 6-modified fucose analogs, thus turning into its fluorescent form (Scheme 15.4). After formation of the triazole ring, the naphthalimide-derivative **21** is strongly fluorescent and the intracellular localization of fucosylated glycoconjugates can be detected by fluorescent microscopy.



Scheme 15.4 *Glycan labeling: (a) fluorescent adduct **21** is generated through the click reaction of fucoside **20** with the probe **19**; (b) Strategy for specific fluorescent labeling of fucosylated glycans in cells.*[17]

The labeling of sugar derivatives through the attachment of biotin as well as the fluorogenic derivation by coumarin-derivatives has been presented by Wong *et al*.[18] For this reason, 3-azido-7-hydroxycoumarin (**22**) has been synthesized and connected via click reaction with alkynyl-substituted sugar analogs (Figure 15.3).

Other derivatives for fluorescent labeling are those of 5-(dimethylamino)naphthalene-1-sulfonyl-like compound **23** or **24**[19,20] which can be attached, for example, to azide-containing side chains of sugar-derivatives. In order to gain information about the K30 antigen and its mode of operation, Du *et al*.[19] demonstrated the labeling of the K30 antigen repeating unit via click chemistry of **23** with an azide containing a tetrameric sugar-derivative.



3-azido-7-hydroxycoumarin (**22**)

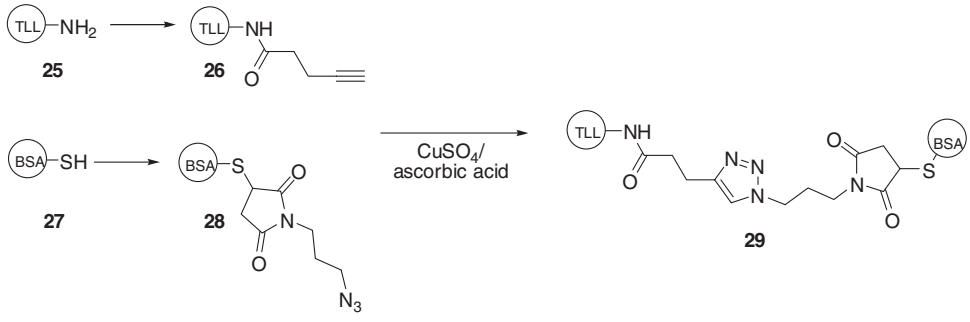
5-(dimethylamino)-*N*-(2-propynyl)-1-naphthalene-sulfonamide (**23**)

N-(2-azidoethyl)-5-(dimethylamino) naphthalene-1-sulfonamide (**24**)

Figure 15.3 *Derivatives for fluorogenic labeling via click chemistry.*[18–20]

15.2.2 Labeling of Bovine Serum Albumin

Nolte *et al*. developed a method of synthesizing enzyme dimers via click reaction.[21] These dimers consist of one specific enzyme in combination with bovine serum albumin (BSA), which forms a so-called 'protein foot' for the immobilization of the whole surface construct (Scheme 15.5). The BSA-labeled enzyme can be further anchored onto surfaces used for single enzyme studies. The synthesis of the acetylene moiety **26** was built up through amide formation on the enzyme – *Thermomyces lanuginosa* lipase (TLL) – via the addition of pentynecarboxylic acid under peptide coupling conditions. Compound **26** was connected with an azide-functionalized BSA component **28** to give the triazole target compound **29**.



Scheme 15.5 *BSA-labeling of enzymes: synthesis of TLL–BSA dimer* ***29****.*[21]

Thermomyces lanuginosa lipase has been the target of additional enzyme studies, with the click reaction as the key step. In another approach by Nolte *et al*., TLL was successfully immobilized on gold nanoparticles through the formation of triazoles as the connecting group.[22] These functional hybrids of lipase and gold nanoparticles were created through the use of azide-functionalized gold nanoparticles that can be prepared via the treatment of citrate-stabilized gold hydrosols with aqueous solutions of an azide-containing thiol-linker. After functionalization of the gold surface, the addition of acetylene-modified enzymes enabled their immobilization via triazole linkage. The connection of BSA to macromolecules is an established method, even in the synthesis of biohybrid amphiphiles.[23] Via click chemistry, Rutjes *et al*. connected terminal azide-functionalized polystyrene and alkyne-functionalized BSA derivatives to produce giant amphiphiles.

15.2.3 Biotin-labeling of Biomolecules: ABPP

Click chemistry is an often-used tool in order to label molecules with biotin, both *in vivo* and *in vitro*. Biotin is a popular label because of its affinity to avidin and streptavidin. Therefore, bioconjugations with biotin (and other markers) are of great interest in reference to ABPP. This technique enables the binding of enzymes to a mechanism-based inhibitor that is bound to a fluorophore, an affinity tag or a bioorthogonal chemical reporter. The bioorthogonal chemical reporter can be reacted with different probes, such as biotin-derivatives, through the use of click reactions. The use of this reaction in order to convert bioorthogonal reporters *in vivo* into easily detectable derivatives facilitates the covalent binding of azide- or alkyne-containing molecules to enzymes. This method is very useful because the often bulky and noncell permeable tags can be formed afterwards *in vitro*. The biotin-labeled proteins are then purified and analyzed (via bonding to avidin or strepatavidin) and can be applied for the investigation of the proteins expressed in cancer proteomes. A schematic presentation of the ABPP-mechanism is illustrated in Scheme 15.6.

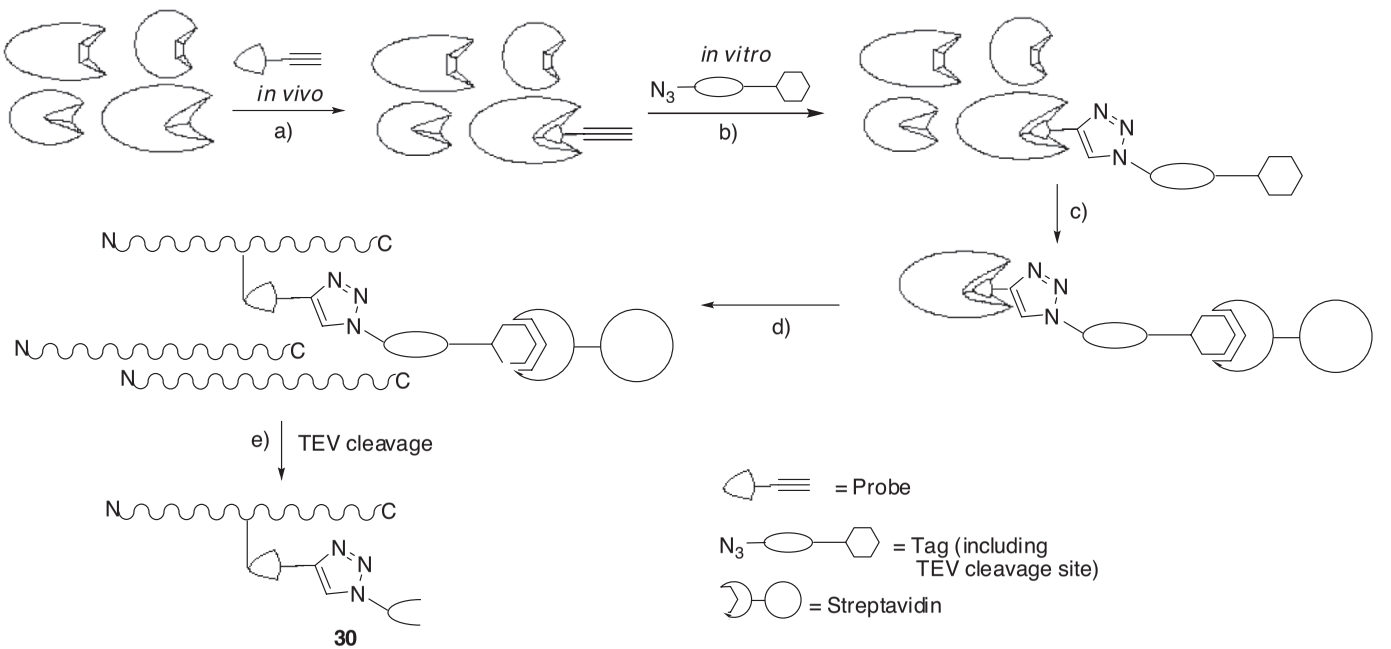
In the first step, active enzymes of the proteome can be labeled *in vivo* with alkyne or azide-containing probes (a, Scheme 15.6) that are converted into triazoles through the *in vitro* addition of the counterpart (b). Subsequently, the labeled proteins have to be enriched by binding them to streptavidin (c). Cravatt *et al.*[24] expanded the ABPP concept through combination with tandem orthogonal proteolysis (d), in order to ascertain the parallel characterization of the probe-labeled proteins and the sites of probe modification. Afterwards, proteolysis was performed by on-bead trypsin-digestion. The supernatant is removed after the digestion and the probe-labeled peptides can be cleaved from the beads through incubation with *tobacco etch virus protease* (TEV) to give conjugates **30**. The peptides – eluted after the trypsin and TEV digestion – can be analyzed separately (Scheme 15.6).

One recent example of biotinylation experiments can be illustrated through the proteome analysis of *Pseudomonas aeruginosa* NagZ.[25] Vocadlo *et al.* incubated the investigated proteome with 2AA5FGF (**32**), which was then anchored on the activated enzymes **31** via the formation of an ester bond with the nucleophile of the β-glucosaminidase. Thereafter, ligation with the biotin-containing reporter group was achieved through Staudinger reaction (not shown) or click reaction. The target enzymes **35** were purified and identified via immobilized streptavidin stationary phase (Scheme 15.7).

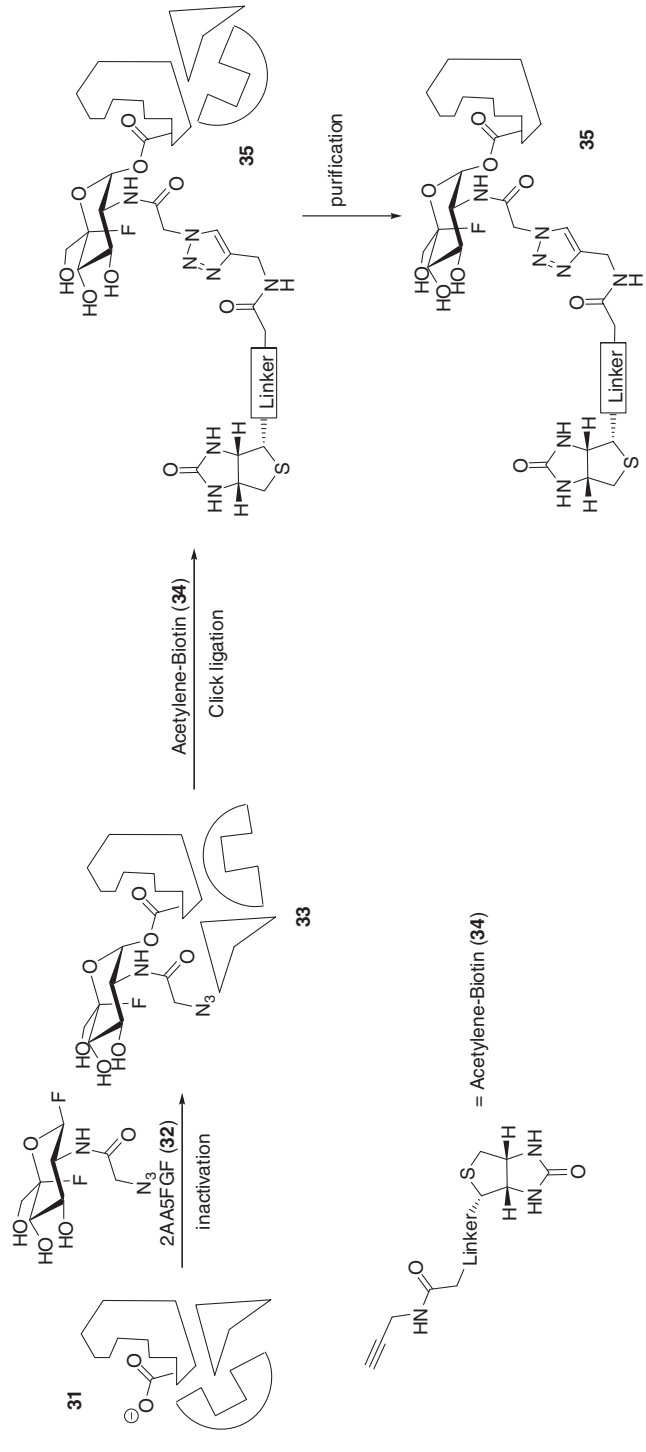
The essential azido-compound **32** has been synthesized via a five-step protocol including two fluorination steps (using DAST and $AgBF_4$) and the transformation of an phthalimido-protected amine into the corresponding azide through deprotection with N_2H_4, acylation and substitution with NaN_3.

A similar approach for the biotin-labeling of glucosamines has been recently published by Gurcel *et al.*[26] Given its importance for the activity of many nuclear and cytoplasmic proteins, the group investigated dynamic glycosylation. The bioorthogonal click reaction has been used to connect biotinylated acetylenes or azides with *N*-acetylglucosamine (GlcNAc) analogs that contain azides or alkynes. Because of biotinylation and subsequent affinity purification on streptavidin beads, thirty-two *O*-GlcNAc-azido-tagged proteins were identified.

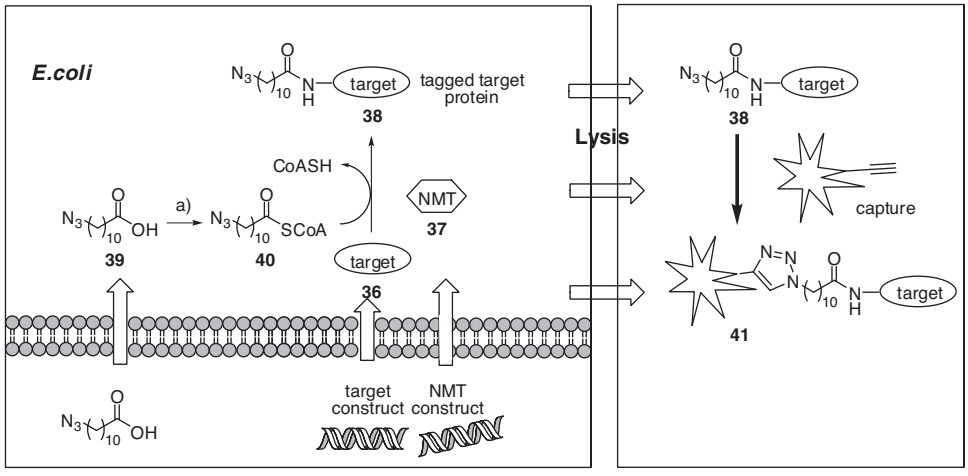
Furthermore, biotin-labeling was used by Tate *et al.*[27] in a specific manner: the group used *N*-myristoyl transferase for the introduction of 'clickable' components into target enzymes



Scheme 15.6 *ABPP in combination with tandem orthogonal proteolysis strategy: (a) in vivo labeling; (b) in vitro click reaction; (c) streptavidin addition; (d) trypsin digestion; (e) TEV cleavage.*[24]



Scheme 15.7 *Activity-based detection and purification strategy using 2-azidoacetamido-2-deoxy-5-fluoro-β-*D*-glucopyranosyl fluoride (2AA5FGF).*[25]



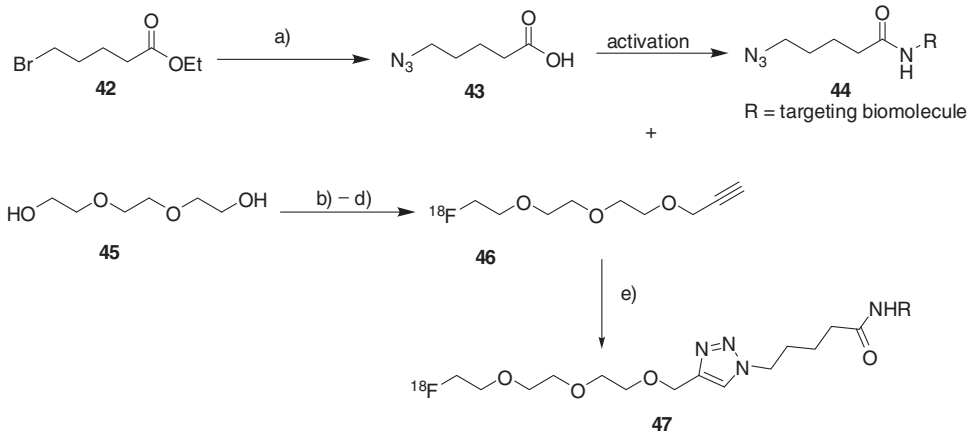
Scheme 15.8 *E. coli co-expression system applied to azide and alkyne tagging of a protein* **36** *in vivo. (a) CoASH, acetyl-CoA synthetase.*[27]

(Scheme 15.8). In their search for methodologies for posttranscriptional labeling concepts, Tate *et al*. found that the use of *N*-myristoyl transferase facilitates the *in vitro* and *in vivo* site-specific generation of *N*-terminal azide-tagged recombinant proteins. These tagged proteins can be 'clicked' after cell lysis with the corresponding acetylenes to produce biotin-labeled triazole linker-containing proteins. The entire procedure is briefly described in Scheme 15.8. The required myristic acid analogs **39** were synthesized starting from bromine precursors that could be transferred into the azide-containing compound via substitution with sodium azide. Incubation of the target enzyme **36** with the functionalized myristic acid derivatives **40** in combination with *N*-myristoyl transferase **37** yielded tagged target proteins **38** in *E. coli*.

Beyond these enzyme labeling methods with biotin, several biologically active molecules have also been labeled. One example is the tagging of enzyme ligands to characterize ligand–receptor interactions. This concept has been extended to ligands that were mentioned in the first chapter of this article, in reference to labeling with fluorescent markers.[28]

15.2.4 Fluorine Labeling

^{18}F-labeling has been used extensively in the observation of peptidic structures. The peptides under investigation can be labeled with ^{18}F-containing substances via click reaction when either ^{18}F-containing azides or alkynes are prepared. Scheme 15.9 illustrates the synthesis of an ^{18}F-labeled alkynyl-chain that has been incorporated into peptides through triazole formation of compounds **44** and **46**.[29] The synthesis has been attained via the integration of ^{18}F into the alkyne-bearing component, as demonstrated by Wuest *et al*. The aforementioned group synthesized ^{18}F-containing alkynylated aryls that have been used for the labeling of neurotensin by click reaction.[30]



Scheme 15.9 *Radiosynthesis of ^{18}F-PEG-alkyne intermediate* ***46*** *and click reaction with azide* ***44****. (a) NaN_3 then H^+ or OH^-; (b) propargyl bromide, NaH, THF, r.t. 18 h; (c) TsCl, NEt_3, MeCN, r.t., 16 h; (d) K_2CO_3, KF, $K^+[^{18}F]F^-$, MeCN, 90 °C, 40 min; (e) Cu^{2+}, ascorbate, THF, r.t., 24 h.*[29]

While the formation of the ^{18}F-labeled building block **46** for click reaction can be completed in a three-step synthetic protocol starting with triethyleneglycol (**45**), there are other methods to prepare ^{18}F-modified alkynes via the direct addition of ^{18}F-sources ($[^{18}F]KF$) to tosylated alkynyl alcohols.[31]

^{18}F-bearing azide-derivatives for click reaction with alkynes can be synthesized following a procedure established by Glaser and Arstad.[32] This approach is favorable because of the substantial number of readily available alkynes that can be used for the click reaction. The synthesis of the fluorinated compound **48** was achieved via nucleophilic fluorination of 2-azidoethyl-4-toluenesulfonate using Kryptofix 222 $K^+[^{18}F]F^-$ (Figure 15.4).

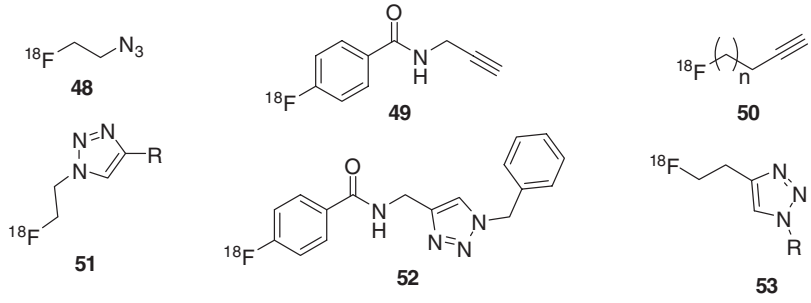


Figure 15.4 *^{18}F-containing azides and alkynes for click chemistry.*[30–32]

15.3 Syntheses of Natural Products and Derivatives

To this day, sophisticated knowledge of molecular biology and the advances in biomedical research have increased the number of known targets for therapeutic intervention. Thus,

the interest in natural compounds with high bioactive potential for novel pharmacological screenings has risen exponentially. The rapid and well-known regioselective 1,2,3-triazole synthesis can be used to generate an enormous chemical database with potential bioactivity for screening purposes.[33,34]

Glycopeptides, for example, constitute an important class of natural products, most of which are involved in biochemical transformations. Today, the synthesis of different glycopeptidomimetics[35,36] and analogs as well as the synthesis of new macrolide antibiotics,[37] enzyme inhibitors,[38,39] steroids[40] and natural nucleosides[41] remains a great challenge.

Specific problems, such as the instability in *in vivo* applications, the synthesis of glycosidic-linked saccharides and peptides as well as antibiotic resistance, have led to an alternative synthesis in the case of novel peptidomimetics and macrolides, e.g. C-linked analogs or the indirect linkage via a 1,2,3-triazole ring.

In one approach, the peptide chain was replaced by either a carbamate derivative **54** (*in vitro* activity against HL-60 human leukemia, HT-29 human colon carcinoma and antineoplatic activities) or by 2(1*H*)pyrazinones **55** via 1,2,3-triazole in a microwave-enhanced regioselective cycloaddition reaction, thus unveiling a new class of interesting analogs of glycopeptidomimetics (Figure 15.5).[35]

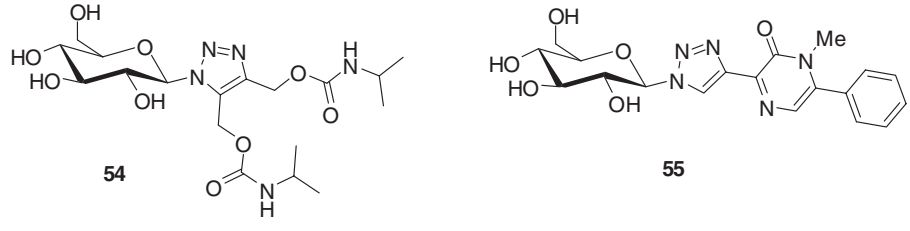
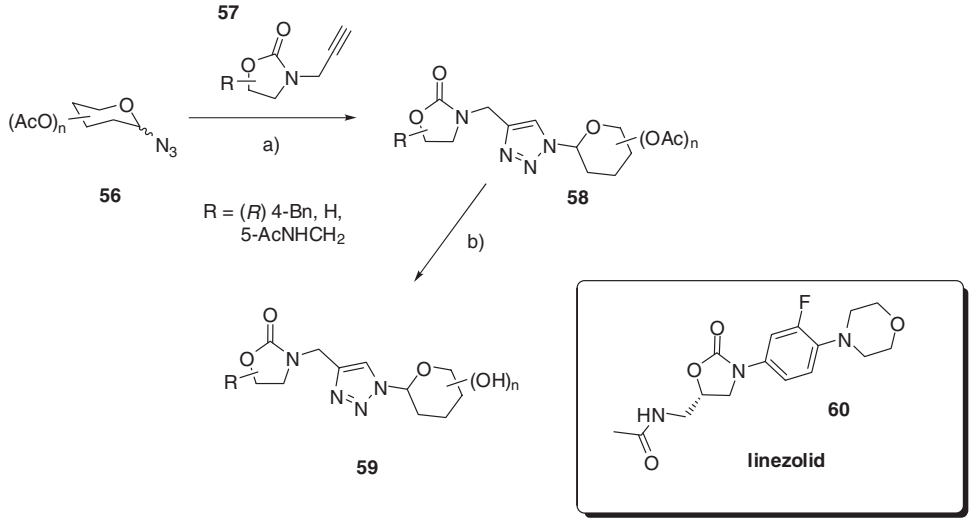


Figure 15.5 *Glycopeptidomimetics: carbamate derivative **54** and 2(1*H*)pyrazinone **55**.*[35]

Through its mechanism of action, the antibacterial drug linezolid (**60**), which is an oxazolidinone derivative, has prompted further investigation in terms of chemical modification and structure–activity relationship (SAR) studies. Chang and his coworkers[42] designed a variety of new molecules containing an oxazolidinone, a triazole ring as mimic of the benzene ring, and a pyranose component as a surrogate of the morpholine-moiety of linezolid (Scheme 15.10).

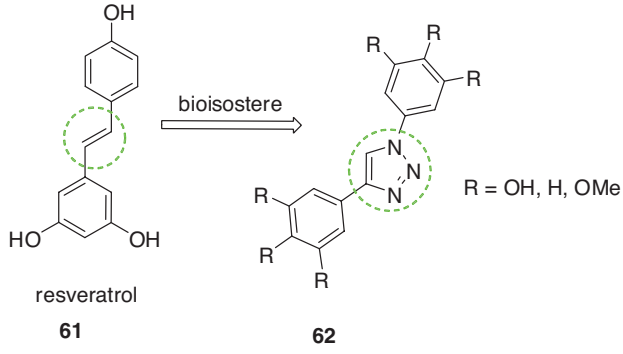
SAR studies have unveiled an essential *N*-aryl group directly linked to the oxazolidinone, which was mimicked through triazole-formation (*N*-aryl-type linkage). The compounds were tested for inhibition of various strains of bacteria and fungi but surprisingly no inhibitory activity was observed. In the field of macrolide antibiotics, Omura *et al*. designed several 8,9-anhydroerythromycin A 6,9-hemiketal analogs with anti-MRSA (multiresistent *Staphylococcus aureus*) and -VRE (vancomycin-resistant enterococci) activity using click chemistry.[37] Besides many carbohydrate and nucleotide conjugates with biological activity, natural products based on a simple organic molecule such as resveratrol **61** are also well known. Highly concentrated in wine, resveratrol plays a crucial role in the cardiovascular system.

A remaining challenge is to develop the targets responsible for each individual effect, such as the impact on the lipid metabolism and platelet function of resveratrol, because



Scheme 15.10 *Synthesis of different linezolid mimics* ***59*** *using click chemistry. (a) Cu(OAc)$_2$, sodium ascorbate, MeOH–THF–H$_2$O, sonication; (b) NaOMe, MeOH.*[42]

a high micromolar concentration of the natural product is necessary to exert its effects.[33] Genazzani and his coworkers observed some triazole analogs whose bioactivities are in fact comparable to a resveratrol-like action (Scheme 15.11).



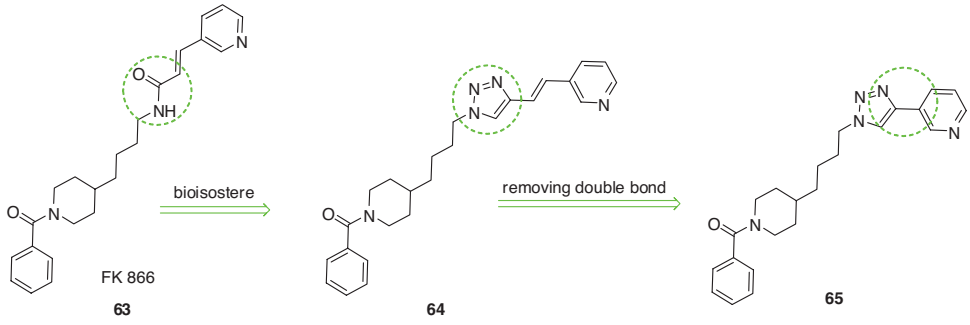
Scheme 15.11 *Resveratrol* ***61*** *and its triazole-modified analogs* ***62****.*[33]

Other such examples are the natural products steganacin and podophyllotoxin, both of which inhibit the assembly of tubulin into microtubules and possess cytotoxic activity against several cancer cell lines.[43] Therefore, a lactone group was replaced with 1,5-disubstituted 1,2,3-triazoles, synthesized via well-known ruthenium-catalyzed click chemistry.

In medical chemistry, one of the great challenges is to create novel, effective chemotherapeutic agents with specificity for cancer cells, combined with low systemic toxicity.

Moreover, the need for antitumor drugs continually increases. Novel targets of these drugs are enzymes of NAD (nicotinamide adenine dinucleotide) biosynthesis and recycling pathways, given that tumor cells have a higher turnover rate than healthy cells. Thus, Tron and his coworkers[38] developed bioactive compounds useful for medical chemistry, such as isosteric triazole analogs of FK866, which blocks nicotinamide phosphoribosyltranferase (NMPRTase).

The therapeutic potential of analogs has led to novel syntheses of compounds via click chemistry, in which the amide bond is replaced by a triazole ring, acting as a bioisostere (Scheme 15.12). The required azide was synthesized starting from commercial available 4-piperidine butyric acid hydrochloride, which was reduced to the corresponding alcohol using lithium aluminum hydride, *N*-benzoylated and then converted into an azide with DPPA (diphenylphosphorylazide and sodium azide).

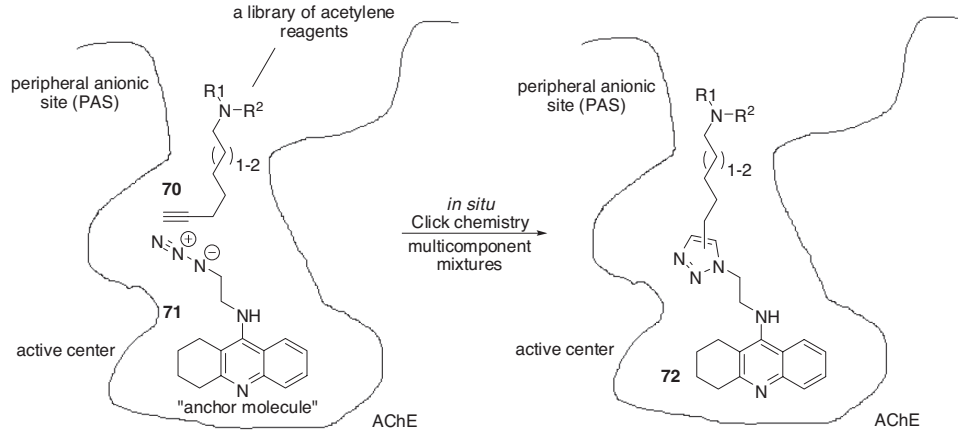


Scheme 15.12 *1,4-Disubstituted triazole analogs of FK866.*[38]

The novel triazole analogs were then tested in a cell viability assay (neuroblastoma cell line, SH-SY5Y) and the intracellular NAD levels were measured with a cycling assay after incubation with compounds **64** and **65** for 24 h.[38] Surprisingly, analog **64** – the closest analog to FKK866 – displayed an IC_{50} value of 3.0 ± 0.2 μM, higher than the analog **65**, which lacks the olefinic moiety (shortened distance between the triazole and the pyridine ring). Jiang *et al*. reported another example of an enzyme inhibitor against H5N1, an avian influenza virus (AIV), synthesized via click chemistry.[44]

15.4 Enzymes and Click Chemistry

The formation of an enzyme inhibitor through chemical reaction of two components bound to the enzyme can be effectively performed if the coupling reaction commonly takes place with high yields. Given this and the aforementioned attributes of the click reaction, the click reaction is the reaction of choice for the development of enzyme inhibitors. Enzyme ligands with known affinity for the active side of enzymes were chosen to bind to two distinct, neighboring binding sides and were connected via a linker that is able to perform click reactions. Given the enzyme-enforced proximity of the two reaction partners, the formation of these triazole-connected twofold ligands should be catalyzed by the enzyme. Through this strategy, the enzyme is used to catalyze the formation of its own inhibitor.



Scheme 15.13 In situ *click chemistry screening for AChE inhibitors with novel peripheral site ligands.*[47]

The most famous example for the exploration of these enzyme inhibitory ligands is the search for acetylcholinesterase inhibitors (AChE). Sharpless and Kolb's respective groups investigated tacrine and phenylphenanthridinium derivatives,[45,46] in terms of their function as inhibitors of *Electrophorus electricus* and mouse AChE. The target acetylcholinesterase was chosen because of its role in the central and peripheral nervous system and its importance in the investigation of acetylcholine in neurotransmission. Two sites of AChE were chosen for the binding of the acetylene and the azide component: the catalytic site of the enzyme at the bottom of a 20 Å deep narrow gorge and another peripheral binding site near the protein surface (Scheme 15.13). The potentially potent inhibitors of AChE were identified through the incubation of several in preliminary experiments selected ligands **66-69** (Figure 15.6, all containing azide as well as alkyne functionalities) and the target enzyme for six days at room temperature (series of 49 binary mixtures incubated). From the theoretically feasible combinations of products, only one was observed. It was shown that, while the enzyme-catalyzed reaction with the triazoles was merely selective, in particular with respect to the formation of the *syn*-isomer, the antitriazole was not detected.

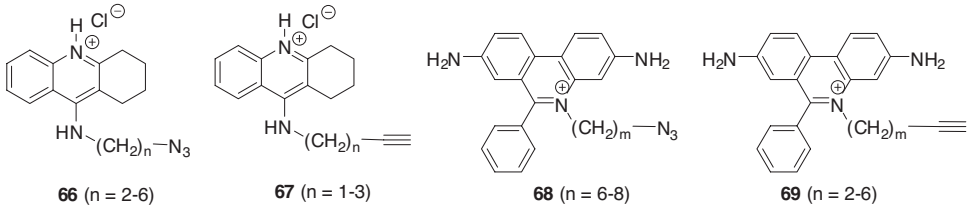
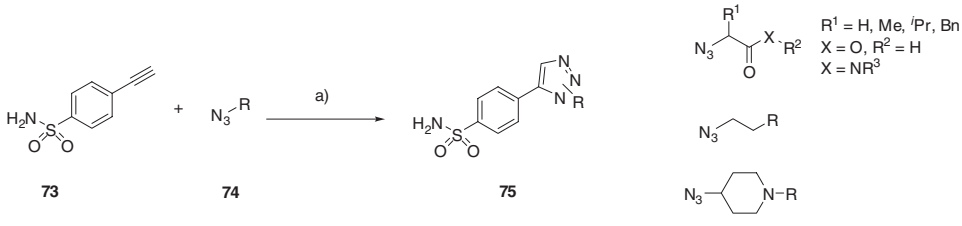


Figure 15.6 *Azide and acetylene building blocks for the formation of 49 binary mixtures and their incubation with AChE.*[45,46]

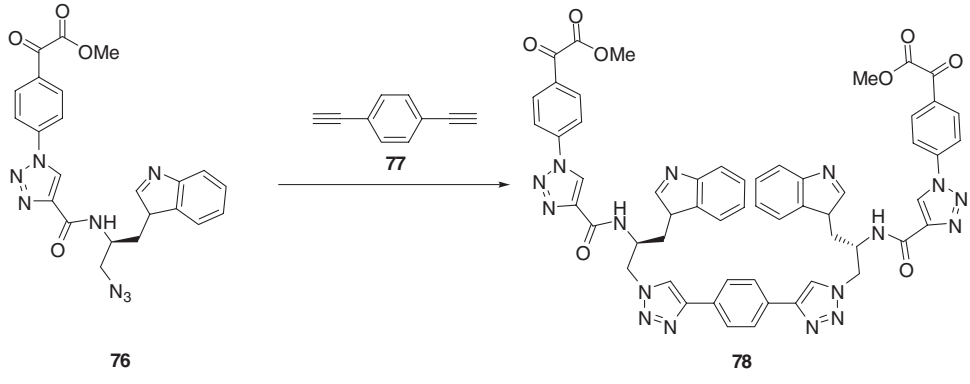
X-ray studies have proven the interaction of the bivalent ligand with protein binding sites. It has been shown that the tacrine moiety was incorporated into the active center of the enzyme and that the phenylphenanthridinium group is located on the peripheral site, both connected via a triazole linker generated by click reaction. In subsequent experiments, the phenylphenanthridinium group was replaced by phenyltetrahydroisoquinoline building blocks (**70**), which yielded inhibitors with an up-to-three-times enhanced activity in combination with the previously used tacrine moiety (**71**) (Scheme 15.13). As a well-known ligand for the active center of AChE, the tacrine group was used combined with a two-carbon azide linker, given that preceding experiments had proven this to be the optimal distance (see formation of **72**). It is possible to perform the experiments with up to 10 acetylene-containing compounds at one time, allowing the enzyme to choose between different ligands and to select the most suitable one for the click reaction. In these competing experiments, out of 10 structurally different acetylenes, only two were able to react with the enzyme-ligated azide. Interestingly, the enzyme only showed selectivity for the formation of the *syn*-triazole products, but there was no preference exhibited for the formation of one specific enantiomer. Both the *R*- and the *S*-enantiomers are used for enzyme-located click reactions.[47]

Another example of target-guided synthesis (TGS) has been illustrated by Kolb *et al*., with the synthesis of enzyme-generated inhibitors of carbonic anhydrase (CA) II.[48] Yet again, the click reaction was selected because of its bioorthogonal character in the ligation of the target-bound functionalized ligands. Compound **73** was chosen as acetylene moiety since most inhibitors of CA are aromatic or heteroaromatic sulfonamides that are able to coordinate to the Zn^{2+}-ion at the active site of the enzyme. Acetylenic benzenesulfonamide (**73**) was incubated for 40 h with bovine carbonic anhydrase II and the corresponding azide-containing counterpart **74**. Subsequently, triazole formation was observed (Scheme 15.14). Altogether, the group was able to demonstrate the formation of twelve triazoles **75**, among them triazoles from piperidine azides, bicyclic azides, stilbene azides, phenyl and ethyl azides.



Scheme 15.14 *In situ screening by click reaction in the presence of carbonic anhydrase II. (a) Bovine carbonic anhydrase II (1 mg/mL, approx. 30 μm), aqueous buffer pH 7.4, 37 °C, 40 h.*[48]

Yao *et al*.[49] investigated inhibitors of protein tyrosine phosphatase (PTP). Inspired by Zhang *et al*.'s discovery of a second binding site on PTP,[50] and based on results of researchers at Abbott, who found a cell-permeable bidentate PTP inhibitor, the group synthesized a library of potential PTP ligands with azide and alkyne functionality and thereafter combined the building blocks to 66 bidentate ligands. Subsequent *in situ* enzymatic screenings revealed a potential PTP1B inhibitor with an IC_{50}-value of 4.7 μM.



76 78

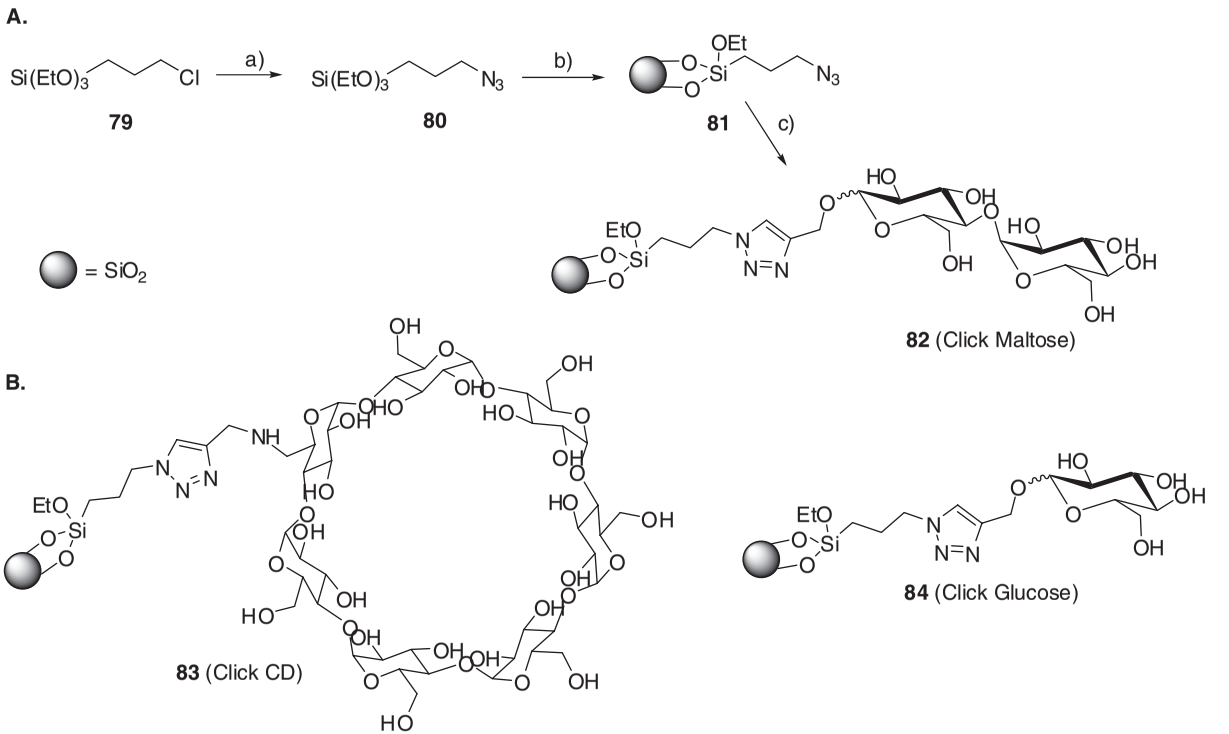
Scheme 15.15 *Structure of the best bis(α-ketocarboxylic acid) inhibitor of protein tyrosine phosphatase.*[52]

Seto *et al*. extended the search for PTP inhibitors and generated two sequential libraries of PTP inhibitors. In the first step, 4-azidobenzoylformate was reacted with fifty-six mono- and diynes. The resulting esters were hydrolyzed and tested against Yersinia PTP and PTP1B. Four selected examples were further investigated and one of them (the precursor of compound **76**) was derived to give the second-generation PTP inhibitors (Scheme 15.15). An alcohol functionality was converted into an azide group via the Mitsunobu reaction with ZnN_3 and subsequently into a triazole group through the addition of the same fifty-six mono- and diynes from the first generation inhibitors. Click reaction of the first-generation inhibitor-derivative **76** with 1,4-diethynylbenzene (**77**) produced the precursor **78,** which was transferred into the target compound via hydrolysis of the ester functionalities. The bis(α-ketocarboxylic acid) inhibitor **78** was found to have an IC_{50}-value of 550 nM against Yersinia PTP (IC_{50} = 710 nM against TCPTP).[51,52]

Beyond the search for phosphatase inhibitors, other enzymes have been under investigation concerning their inhibition abilities. Examples include the exploration of phosphonate inhibitors for the regulation of serine hydrolases,[53] the search for Grb2 SH2 domain-binding macrocycles[54] and the investigation of glycoconjugate benzene sulfonamides as carbonic anhydrase inhibitors.[55]

15.5 Synthesis of Glycosylated Molecular Architectures

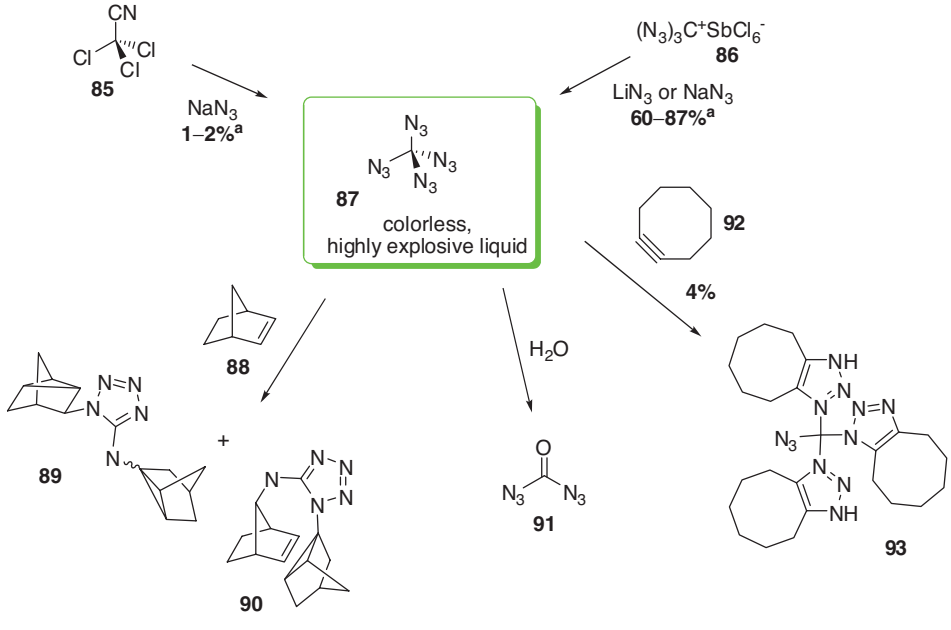
As the separation of highly polar compounds by HPLC remains a great challenge, an alternative method with a polar stationary phase and an aqueous mobile phase (hydrophilic interaction liquid chromatography, HILIC) has been developed. A variety of separation materials such as underivatized silica, amido silica, poly(succinimide)-bonded silica, polyhydroxy silica with different retention characteristics and separation selectivity have been described in the literature. Moreover, glucose, maltose and β-CD (Scheme 15.16) with several polar groups and unique structures have been immobilized via click reaction, leading to novel complex separation materials.[56]



Scheme 15.16 *(A) Synthesis of the model molecule click Maltose (**82**). (a) NaN_3, DMF, KI, 90–100 °C; (b) silica beads, DMF, 100–110 °C; (c) 1-O-propargylmaltose, MeOH–H_2O (1:1), 5 mol% $CuSO_4$, 15 mol% sodium ascorbate, r.t.; (B) click-CD (**83**) and click-glucose (**84**) as separation materials.*[56]

15.6 Synthesis of Nitrogen-rich Compounds: Polyazides and Triazoles

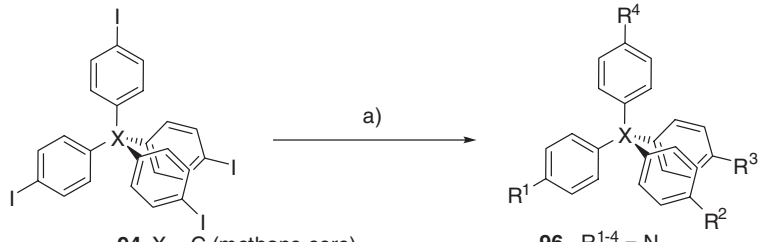
In recent years, the study of polyazides and their highly applicable energetic materials has experienced a renaissance. Numerous homoleptic azides [$M(N_3)_n$], derivable salts[57] as well as organic azides have been prepared.[58,59] A major challenge, the isolation of tetraazidomethane, which was expected to be a highly explosive compound, was overcome by Banert and coworkers in 2007 (Scheme 15.17). The lower-substituted derivative, the triazidomethane, was obtained via simple nucleophilic substitution from bromoform (CBr_4). However, all attempts to generate the homoleptic azide from halides, as well as a variety of precursors bearing donor or acceptor substituents, through different azide reagents were unsuccessful.[58]



Scheme 15.17 *Attempts to prepare the tetraazidomethane (**87**) and further reactions with this compound. The yield was determined by the integration of the ^{13}C NMR signal.*[58]

The only sign of formation was generated through the treatment of $C(CN)Cl_3$ (**85**) with sodium azide in MeCN. Subsequent approaches involved the reaction of the salt **86** with dry lithium or sodium azide, leading to significantly higher yields. Against all expectations, antimonate salts are expensive and explosive starting materials, which are therefore unsuitable for preparative purposes. The workup is less problematic and more reproducible for the first-mentioned procedure.

Special safety precautions have to be followed, because azides are highly energetic and potentially explosive materials, particularly those with low molecular weights. Pure tetraazidomethane (**87**) is extremely dangerous and can explode at any time without a recognizable cause. According to convention, the ratio of nitrogen to carbon (oxygen)



Scheme 15.18 *Ullmann-type coupling reaction leading to polyazides* ***96–100****. (a)* NaN_3*, ligand (diamine), Cu(I), sodium ascorbate, DMSO–*H_2O*, 100 °C, 48 h.*[59]

should be maximum 1:3 for a neat isolation of the yielded azide. Compounds with lower ratios should be handled with extreme caution.

Sodium azide is very toxic, similar to sodium cyanide. The use of additional metals as well as halogenated solvents such as dichloromethane in the presence of the sodium azide must be avoided.[60]

Nowadays, aryl azides are increasingly used in organic synthesis, due to the versatile transformations of the azide functional group. Thus, additional polyazides were synthesized, as previously reported by Bräse *et al*. (Scheme 15.18).[58] For the investigation of novel nanomaterials and compounds for polymer science and polymer processes as well as material and macromolecular sciences, rigid tetrafunctionalized molecules are promising compounds. The tetrasubstituted polyazides were prepared via Ullmann-type coupling reactions.

Furthermore, 1,4-disubstituted 1,2,3-triazoles, easily accessible from polyazides **96–100**, have found application in drug discovery, bioconjugation, surface modification and material development as multifunctional ligands as well as in polymer science. Additional examples, such as hyperbranched poly(1,2,3-triazoles), reported by Tang *et al*., were inclined to self-oligomerize and failed to yield soluble polymers using Cu(I)-catalyzed click polymerization.[61]

15.7 Conclusions

The benefits of the click reaction, namely high yields and biocompatibility, enable the very efficient application of this cycloaddition reaction to the synthesis of molecular architectures based on biomolecules, the synthesis of labeled biomolecules and their general application in living systems. In this article, the structure of derived markers, biomolecules, natural products and biologically active compounds containing either an azide or an alkyne functionality for click reactions is described. It has been illustrated that the potential of the click reaction lies not exclusively in its application as a labeling method, but that it can also

be used to build up novel biologically active compounds. These compounds can be created according to known active targets, but can also be identified via direct formation of triazole derivatives in the active site of enzymes through incubation with several theoretically clickable precursors.

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