Drug Development

International Edition: DOI: 10.1002/anie.201610372
German Edition: DOI: 10.1002/ange.201610372

# Protein-Templated Fragment Ligations—From Molecular Recognition to Drug Discovery

*Mike Jaegle, Ee Lin Wong, Carolin Tauber, Eric Nawrotzky, Christoph Arkona, and Jörg Rademann**

**Keywords:**
dynamic covalent chemistry · fragment-based drug discovery · high-throughput screening · molecular recognition · protein-templated reactions

*Protein-templated fragment ligation is a novel concept to support drug discovery and can help to improve the efficacy of protein ligands. Protein-templated fragment ligations are chemical reactions between small molecules ("fragments") utilizing a protein's surface as a reaction vessel to catalyze the formation of a protein ligand with increased binding affinity. The approach exploits the molecular recognition of reactive small-molecule fragments by proteins both for ligand assembly and for the identification of bioactive fragment combinations. In this way, chemical synthesis and bioassay are integrated in one single step. This Review discusses the biophysical basis of reversible and irreversible fragment ligations and gives an overview of the available methods to detect protein-templated ligation products. The chemical scope and recent applications as well as future potential of the concept in drug discovery are reviewed.*

**From the Contents**



## 1. Introduction and Definition

Protein-binding, bioactive molecules, the starting points to future drugs, are in most cases identified and optimized in two clearly separated steps: first small molecules are synthesized chemically or isolated from natural sources, then their biochemical properties are investigated in bioassays (Figure 1 A). Both steps can be repeated iteratively during the optimization process. Accordingly, the classical process for lead discovery is characterized by the strict separation of the generation of chemical libraries and their screening for biological activities in a protein-based or cellular assay.

Over the last two decades, however, an alternative concept of drug discovery has emerged that aims at the integration of chemical synthesis and bioassays.[1–3] This approach exploits the molecular recognition of reactive small-molecule fragments by proteins both for assembly of the chemical ligand and for the identification of bioactive fragment combinations and is, therefore, denominated as "protein-templated fragment ligation".

Protein-templated fragment ligation is an attractive alternative to classical drug discovery for several reasons: The combination of chemical synthesis and bioassay in one step considerably confines the effort for chemical synthesis to the most active fragment combinations and, thus, is highly efficient, saving time, money, energy, and chemical resources. Only small libraries of a few hundred to a thousand reactive fragments are required to cover the relevant chemical space and to test huge numbers of potential fragment combinations or fragment ligation products. Moreover, fragment ligations enable the detection of low-affinity fragments for a spatially resolved protein binding site, since the binding of the reactive primary fragment or protein probe amplifies the binding of a secondary fragment at a precisely defined location.

In this Review we will present the current state and the future potential of protein-templated fragment ligations in drug discovery. For this purpose we propose a comprehensive definition of protein-templated fragment ligations:

Chemical reactions between two or more small molecules ("fragments") that utilize the protein's surface as a catalyst to accelerate the formation of protein ligands with increased binding affinity are defined as protein-templated fragment ligations.

This definition encompasses both reversible and irreversible ligation reactions. We consider the combined coverage of both reaction types a fitting approach, as all templated ligations share the same biophysical principles, pose the same challenges for the detection of products, and contribute excellent examples to the drug discovery process. Moreover, the definition creates a clear distinction between templated fragment ligations and other catalytic transformations exerted by proteins similar to the turn-over of enzyme substrates. Remarkably, protein-templated fragment ligation reactions following this definition are the established mode of action of several clinically admitted drugs, which suggests that the reactions can indeed proceed efficiently under physiological conditions. For example, the anti-Parkinson drug carbidopa binds to the enzyme DOPA decarboxylase and reacts with the cofactor pyridoxal phosphate to form a hydrazone as the active inhibitor (Figure 2 A).[4] Likewise, the anticonvulsive drug vigabatrin reacts in a protein-templated mode with the cofactor of GABA transaminase to form a Michael acceptor intermediate, which leads to the irreversible inhibition of the enzyme (Figure 2 B).[5] Other examples

[*] M. Jaegle, E. L. Wong, C. Tauber, E. Nawrotzky, Dr. C. Arkona, Prof. Dr. J. Rademann
Freie Universität Berlin, Medicinal Chemistry
Königin-Luise-Strasse 2 + 4, Berlin, 14195 (Germany)
E-mail: joerg.rademann@fu-berlin.de
The ORCID identification number(s) for the author(s) of this article can be found under:
https://doi.org/10.1002/anie.201610372.

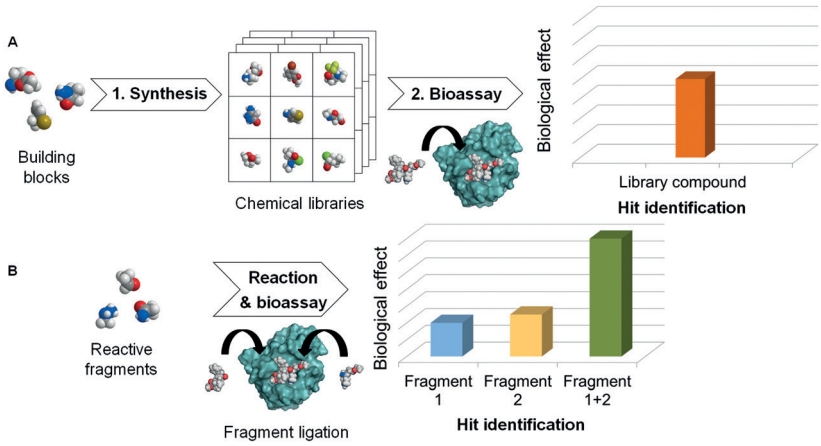



***Figure 1.*** A) In the classical approach to drug discovery, large presynthesized chemical libraries of drug-like molecules are screened in the bioassay to obtain hits. B) In templated fragment ligations, much smaller collections of reactive fragments with molecular weights of $<250$ Da are employed. The assembly of the fragments on the protein template enables the synthesis and identification of bioactive fragment combinations in one step.

of protein-templated fragment ligations in admitted drugs are found for selegiline[6] (reacts with cofactor FAD in the depression target monoamine oxidase) and for isoniazid (reacts with $NAD^+$ in *mycobacterium tuberculosis* catalase).

As fragment ligations are driven by thermodynamic interactions between the fragments and the protein, Section 2 considers the biophysical basis of protein-templated fragment ligations to highlight the conceptual potential of the method. Special emphasis will then be given to the detection of products of fragment ligation reactions, which is a major and general challenge in fragment ligation assays (Section 3). In Section 4 we will give an overview of the chemical reactions used so far in templated ligations and also discuss possible future extensions of this reaction set. Reversible reactions, which have been studied in the context of dynamic covalent chemistry[7–14] and irreversible reactions, also denominated as target-guided synthesis (TGS),[15] are treated together, as both reaction categories deliver examples of templated fragment ligations and in many cases it is difficult to categorize one reaction unambiguously. Representative recent applications of templated ligations in fragment-based drug discovery are reported in Section 5, thus demonstrating how far the method has developed to date. Finally, in Section 6 we will discuss the current state of protein-templated fragment ligations, considering the strengths of this method and the requirements for it to succeed. The complementarity of the method with classical ligand screening and fragment-based methods will be considered, thereby leading to an outlook on the relevance and further development of protein-templated fragment ligations for future drug discovery.

## 2. The Biophysical Basis of Protein-Templated Fragment Ligations

In protein-templated fragment ligations the protein serves as a catalyst for the assembly of protein ligands from protein-

*Jörg Rademann studied chemistry and biochemistry at the Universities of Constance (Germany) and Rutgers University (NJ, USA). He completed his PhD with Richard R. Schmidt at the University of Constance on the solid-phase synthesis of oligosaccharides. After postdoctoral research at Carlsberg Laboratory in Copenhagen (Denmark) with Morten Meldal and Klaus Bock, he established his own group at Eberhard-Karls-University in Tübingen, where he studied the use of polymer reagents in organic and medicinal chemistry. He became Professor of Medicinal Chemistry at Freie Universität Berlin in 2004, where his team set up one of the first academic screening facilities in Europe at the Leibniz Institute of Molecular Pharmacology (FMP). From 2010 to 2013 he was chair of Medicinal Chemistry at the University of Leipzig, and in 2013 he became full professor at Freie Universität Berlin.*
*Mike Jaegle, Ee Lin Wong, Carolin Tauber, and Eric Nawrotzky are PhD students in the group, coming from academic backgrounds in chemistry, pharmacy, and molecular biology from universities in Freiburg (M.J.), Johor Bahru, Taipei, and Seoul (E.L.W.), Berlin (C.T.), and Leipzig (E.N.). They all work on projects involving protein-templated reactions on various protein targets including proteases, phosphatases, and protein–protein interactions.*
*Christoph Arkona studied biochemistry at the Leibniz University in Hannover and joined the group as a senior scientist after finishing a PhD in plant biochemistry and many years of drug development in the biotech industry.*
*The photo shows from left to right: Mike Jaegle, Eric Nawrotzky, Jörg Rademann, Carolin Tauber, Christoph Arkona, and Ee Lin Wong.*

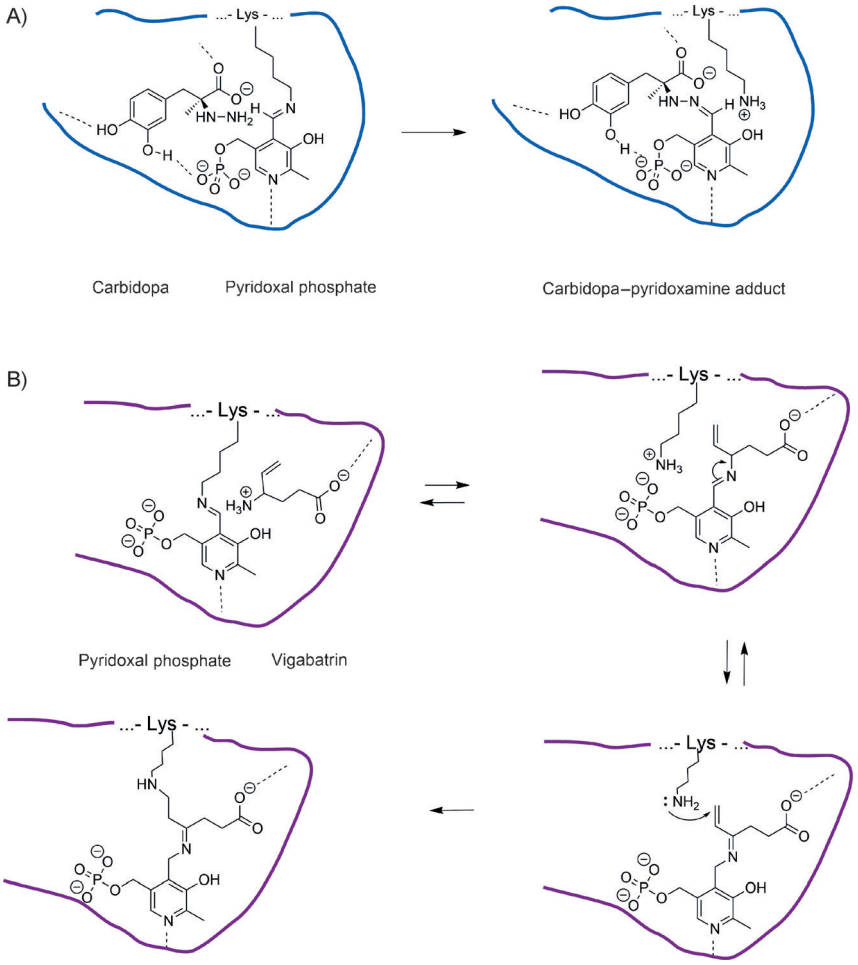



***Figure 2.*** Protein-templated fragment ligation is the mode of action of several clinically admitted drugs, including carbidopa (A) and vigabatrin (B).

binding small-molecule fragments. The molecule fragments are chemically reactive and are linked covalently to yield fragment combinations with improved binding affinities and biological activities. The process requires a chemically reactive, dynamic system that is able to adapt on the molecular level by the formation and—in the case of reversible reactions—recleavage of covalent chemical bonds and by evolving into a thermodynamically more favored state, thus furnishing optimized protein ligands. Such adaptive systems can be considered as examples of molecular learning, where the term "learning" is applied here to describe an adaptive, chemically evolving and self-optimizing system.

The additivity of free binding energies is the driving force of protein-templated fragment ligations (Figure 3).[16,17] Two fragments bind to the protein independently and without any overlapping of their free energies of binding, $\Delta G_1$ and $\Delta G_2$.[18] The linking of two fragments by a reversible or irreversible chemical reaction forms a ligation product with a free binding energy $\Delta G_{lig} = \Delta G_1 + \Delta G_2 + X$, where $X$ represents the deviation from the addition of the binding energies. Provided that the covalent linking of the two fragments is additive ($X = 0$), the obtained fragment combination product will display a binding affinity that is the product of the binding affinities of its fragments: $K_D = e^{-\Delta G/RT} = K_{D1}K_{D2}$.[19,20] For example, two fragments with $K_D$ values of 1 mM will result in a fragment ligation product of 1 μM. In fact, fragment combinations can also be strongly superadditive, as a result of the additional binding energy of the linker or entropic gain, which results in an even stronger enhancement of binding affinities.[19] Likewise, the linking of two fragments can reduce the binding energy of the ligation product below additivity if the linker contributes unfavorably to the binding.

## 3. Detection of Protein-Binding Fragments and Fragment Ligation Products

The analytical detection of protein-binding fragments constitutes a major challenge and has been a significant limitation to the development and exploitation of fragment-based methods in drug discovery. The main reason for this detection problem is the low affinity of protein-binding fragments requiring high fragment concentrations to generate and possibly saturate the detection signal. In some assays, such as fluorescence anisotropy or saturation transfer difference (STD) NMR, high protein concentrations can be used instead to saturate the binding of the small-molecule ligand. Compared to other fragment-based methods without ligation, the detection problem, however, is significantly reduced in protein-templated fragment ligations due to the higher affinity of the fragment ligation product formed. As

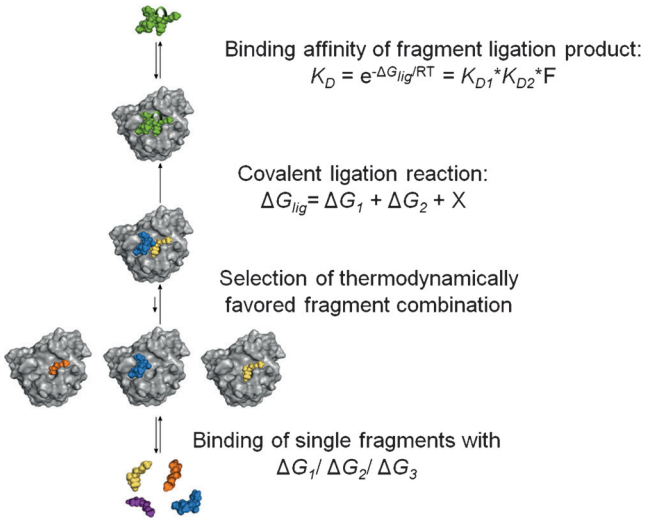



***Figure 3.*** The additivity of binding energies in fragment ligations results in an enhanced binding affinity of fragment ligation products.

a result, fragment ligation assays can, in principle, detect even low-affinity fragments that would not be identified in other fragment assays, for example, because of concentration limits. In general, fragments have to be present at concentrations that exceed their dissociation constants ($K_D$ values) by a factor of at least 10 for a strong, saturated detection signal to be measured. In contrast, in the case of a fragment ligation assay, concentrations of the starting fragment below the $K_D$ value are typically used to effect only partial inhibition, which can be saturated by the formation of the stronger binding ligation product.

Although the detection of fragment ligation products is thereby strongly facilitated compared to single fragments, it remains a challenge for several reasons. As the ligation products possess a higher binding affinity than the starting fragments to the target protein, their formation is auto-inhibitory and the amount of ligation product is limited strictly by the concentration of the protein template. As a result, fragment ligation products have to be detected against a strong background of excess nonreacted fragments.

Other challenges can arise from the reactivity of fragments in the fragment ligation assays. Although some reactive fragments such as those used in dipolar cycloaddition reactions are truly bioorthogonal, several fragment ligations rely on electrophiles that might also react with protein nucleophiles as reaction partners. Control experiments that distinguish the effect of one fragment from the effect of a fragment combination are routinely conducted to avoid interference of such reactions with the assay read-out. In addition, it is generally recommended in hit validation to use independent secondary assays to detect false-positive hit fragments.

Several classical analytical methods have been widely applied and adapted to the detection of templated fragment ligations (Figure 4). Most studies in the field have used liquid chromatography, usually in combination with mass spectrometry (LC-MS).[13,21–30] In some examples, specific methods of NMR spectroscopy were developed.[31–36] In addition, fragment ligations have been studied by X-ray crystallography.[37–42] Finally, as a powerful complement, the detection of fragment ligation products by various bioassays has been developed over recent years.[1,10,43–48]

The detection of fragment ligation products by LC-MS has become substantially easier over the last two decades because of the rapid development of this method in terms of chromatographic separation and the sensitivity of mass detectors.[13,25–28] Not only detection but also quantification and structure elucidation of ligation products is facilitated by using extracted-ion or single-ion chromatography and MS-MS techniques. Some limitations remain, however. As chromatographic separation takes some time, LC-MS detection is best suited for irreversible or quasi-irreversible ligation reactions.[15,49] In the case of reversibly formed ligation products, their chemical fixation by a chemical reaction or a pH shift can be an option. This strategy was followed, for example, in the seminal report by Huc and Lehn on "virtual chemical libraries", in which the unstable imine ligation products were converted into stable amines by chemical reduction during the ligation reaction.[21] A second limitation

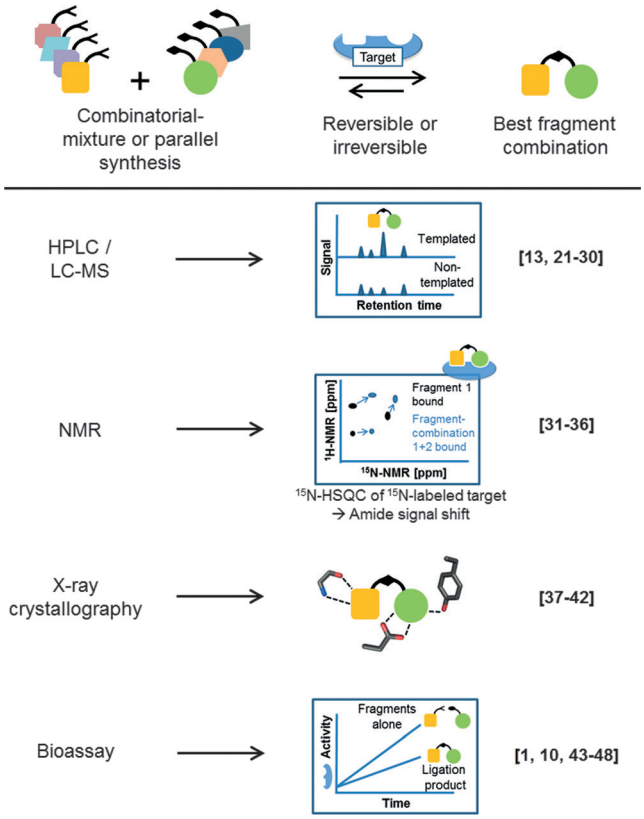



***Figure 4.*** Overview of analytical methods used for the detection of fragment ligation products.

of LC-MS detection can arise from the buffer systems used in fragment ligation reactions. Many buffer ions used in ligation assays interfere with the detection of ligation products in the mass detector, whereas buffer salts such as ammonium formiate or acetate that are highly compatible with LC-MS may interfere with the ligation reaction by adding reactive nucleophiles in strong excess. In recent years, the detection of protein-binding fragments by native protein MS has developed into a mature technology and it is very likely that this method will also be useful for the detection of fragment ligation products.[50–53]

NMR spectroscopy has the big advantage over LC that it can monitor ligation reactions directly in the assay solution. As standard NMR spectroscopy requires high (mM) concentrations of fragment ligation products in volumes of around 0.5 mL for the chemical analysis, large amounts of proteins are required.[54] Therefore, the method is only applicable to proteins that are available in large quantities and only a few experiments with low throughput can be conducted. The throughput can be enhanced if mixtures of reactive fragments are employed that undergo reversible, covalent ligation reactions.[33,55–57] These mixtures are denominated as dynamic combinatorial libraries (DCL), or dynamic covalent libraries, and can in principle form all possible combinations of the available fragment building blocks, that is, in the case of $n$ and $m$ complementary reactive building blocks, a number of $n \times m$ possible products are obtained. The protein template shifts the equilibrium in favor of the best binding and, thus, most

stabilized ligation product, which is then detected by one of the classical analytical methods.

Several NMR methods have been adapted specifically for the detection of fragment ligation products. Ramström and co-workers have investigated $^{1}$H STD NMR spectroscopy for the detection of hemithioacetals formed by a protein-templated reaction in aqueous medium (Figure 5).[58] $^{1}$H STD NMR spectroscopy is a method that exploits the selective transfer of proton magnetization from the protein to reversibly bound ligands and was used here to identify binding fragments from a DCL. For a proof of concept study, β-galactosidase was selected as the target protein that catalyzes the hydrolysis of O-β-galactosides and contains no cysteine residues in the active site. A mixture of five thiols and two aldehydes was employed, thereby resulting in the potential formation of ten hemithioacetals. It was demonstrated that 1-β-mercapto-D-galactose binds more strongly to the target enzyme than do the four other thiols and strongly enhanced the signals of both aldehydes in the STD spectrum—presumably through the formation of hemothioacetals. Surprisingly, the binding of the two sugars could not be suppressed by the addition of substrate, thus suggesting additional allosteric binding sites for these fragments. Significant inhibition of substrate hydrolysis could be confirmed for one thiol–aldehyde combination, which also suggests the formation of hemithioacetals as active inhibitors. Another application of STD NMR spectroscopy was published recently by the Hirsch research group.[36] Recently, $^{11}$B NMR spectroscopy was applied by Claridge and co-workers for the detection of fragment ligation products (see Figure 10 in Section 5).[59]

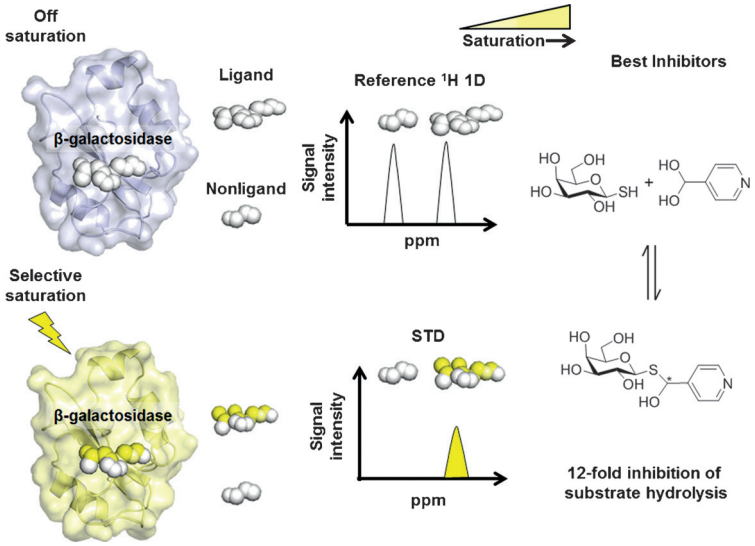



***Figure 5.*** Application of $^{1}$H STD NMR spectroscopy for detecting the protein-templated formation of hemithioacetals from a dynamic combinatorial library and enzymatic selection of the best inhibitor.[58]

In addition to ligand-based NMR spectroscopy, there have been intensive applications of protein-based NMR methods for the detection of protein-binding fragments.[60,61] Protein NMR spectroscopy uses in many cases proteins that have been produced with $^{13}$C and $^{15}$N isotopes to enhance the NMR signals. Fragment binding can then be observed, for example, in 2D HSQC experiments from perturbations of the chemical shifts. The method was introduced as "structure–activity relationships (SAR) by NMR spectroscopy" and has the big advantage of furnishing information on the binding site of fragments if the signals in the NMR spectra are assigned to the protein structure.[62] Protein-based NMR spectroscopy should also be a valuable method for the investigation of fragment ligation reactions, although we could not find applications of it so far.

X-ray crystallography has found broad application in fragment-based drug discovery, and its attractiveness comes from the detailed structural information it reveals of the fragment–protein complex.[63] This information can be extremely helpful in the design of fragment combination products. Several studies have so far used protein crystallography for the detection of fragment ligation products.[37–42]

The rapid and parallel detection of potent fragment ligation products formed in protein-templated reactions was finally realized by the introduction of bioactivity-based assays. The initial strategy was denominated as dynamic ligation screening (DLS), as reversible ligation reactions were first investigated. DLS increased the sensitivity of ligand detection considerably, and the site-directed discovery of low affinity fragments with millimolar $K_D$ values was realized (Figure 6).[10,43] Bioactivity-based detection methods require only minimal amounts of protein; usually low nanomolar concentrations of the protein are sufficient. They can be conducted using standard assay equipment such as microtiter plates and automated pipetting devices used for the handling of fragment libraries. In enzyme assays, the sensitivity is further enhanced by the catalytic activity of the

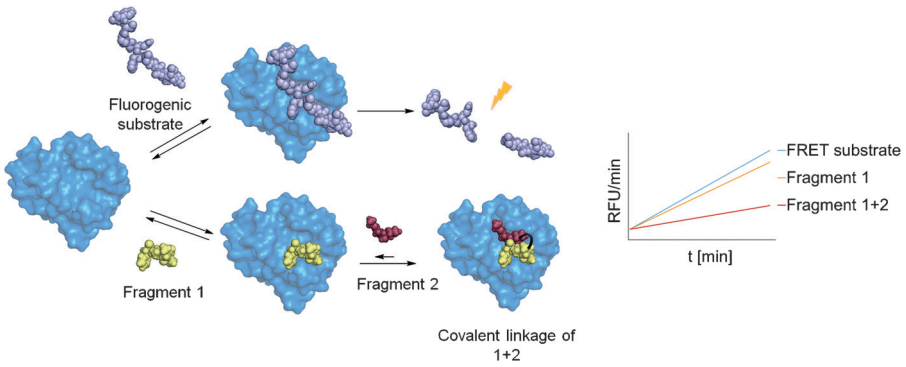



***Figure 6.*** Concept of dynamic ligation screening (DLS). A fluorogenic substrate competes with the reactive inhibitory fragment 1 for the active site of the protease. The addition of reactive fragment 2 shifts the equilibrium toward the covalent linking of two fragments on the protein surface, thereby leading to an increase in inhibition.

protein targets resulting in the rapid turnover of many of the fluorogenic or chromogenic substrate molecules. Fluorescence resonance energy transfer (FRET) has also been used for the detection of fragment combinations.[44] To reach the highest sensitivity, the primary reactive fragment has to be added to the enzyme assay at a concentration that results in 10–20% inhibition, thereby leaving a measurement window of 80–90% for detection of the enhanced inhibition after addition of the secondary fragment.

Various bioassay formats have so far been adapted to dynamic ligation screening (Figure 7). As already described, fluorogenic substrates can be used in competition assays, competing with the fragment ligation product for the enzyme's binding site. In this assay the best fragment combination results in the strongest inhibition of the enzymatic reaction (Figure 7A).

Alternatively, the substrate itself can be constructed with a reactive group that allows for fragment ligations in which the ligated fragment can increase the affinity of the substrate to the target protein. These substrate-enhancement assays (Figure 7B) enable the site-directed detection of protein-binding fragments in secondary binding sites that lead to accelerated turnover of the substrate.[45] Competitive binders can also be detected in this assay format. In the special case that the reactive, fluorogenic molecule is completely inactive without ligation of a fragment, it has been denominated as a presubstrate and is especially sensitive to the detection of chemically unstable, transient ligation products such as hemiacetals and hemithioacetals.[46] The method has been used successfully for the identification of secondary-site binding fragments of serine proteases[46] and of protein tyrosine phosphatases (PTPs),[45] where it was capable of detecting specific secondary-site binders as a starting point for the construction of PTP-specific inhibitors.

The principle of bioactivity-based detection of fragment ligation products has also been extended from enzymatic assays to protein binding assays, thus enabling the identification of ligands of non-enzymatic protein-binding sites. Protein-binding assays are principally conducted either in homogeneous solution or heterogeneously after attachment of one binding partner to a solid phase or surface. A homogeneous protein-binding assay for protein-templated ligation reactions has been demonstrated by using fluorescence polarization (FP), also known as fluorescence anisotropy, for detection (Figure 7C,D).[47] For this assay, a reactive protein-binding peptide fragment was labeled with a fluoro-

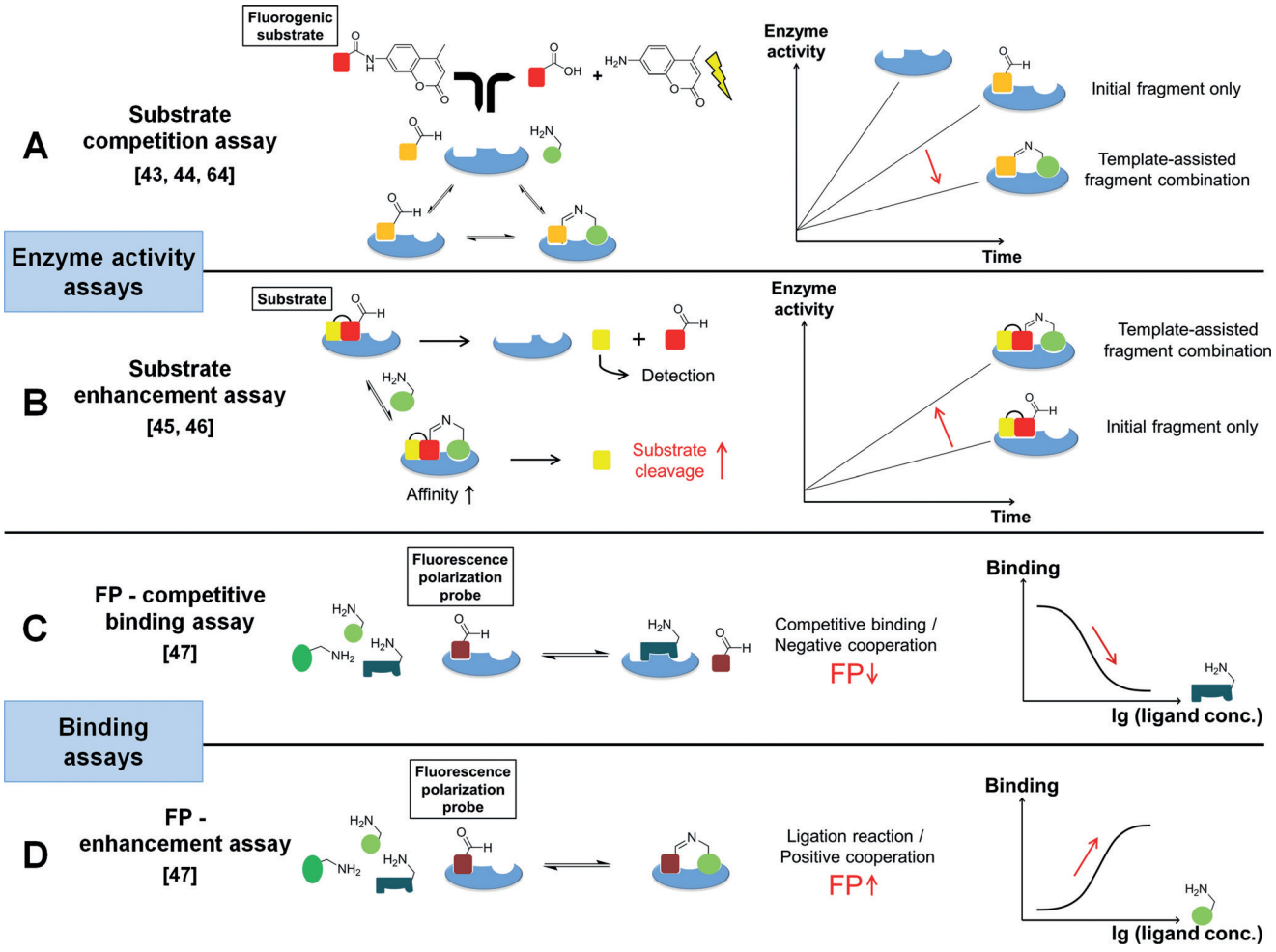



*Figure 7.* Overview and representative examples of bioactivity-based detection used for the detection of the templated formation of fragment combinations.

phore. The FP ligand (10 nM) was dissolved in a buffer containing the target protein at a concentration leading to about 50% ligand binding. Then a library of fragments was screened for those modulating the FP. The assay enabled the identification both of competing (Figure 7C) and of enhancing fragments (Figure 7D) in a single experiment, and the templating effect exerted by the protein was quantified for the best enhancing fragment combination. Reductive amination of the labeled fragment with the best enhancing fragment resulted in a picomolar inhibitor. Similar to the enzyme inhibition assays, homogeneous protein-binding assays such as FP assays can be conducted in high-throughput equipment, in small assay volumes, and at low (nM) protein concentrations, provided a ligand with sufficient affinity is available as a starting point.

Label-free, homogeneous protein-binding assays will be an attractive complement for the detection of bioactive fragment ligation products. For example, thermal shift assays are used increasingly for the detection of protein–ligand complexes.[65,66] The method monitors the defolding ("melting") of proteins at increasing temperatures by using fluorophores that show increased fluorescence when they come into contact with the hydrophobic core of unfolding proteins. Fragment binding can be observed by a significant enhancement of the protein's melting temperature because of the free binding energy of the ligand. Even more thermodynamic information about the binding of fragments is revealed by isothermal titration calorimetry and future studies will most likely use this technique—although it requires a large quantity of protein and can be conducted only at low throughput.[67–70] Heterogeneous protein-binding assays have been applied to the investigation of protein–fragment binding. These methods are label-free with respect to the fragments; however, covalent immobilization or labeling with affinity probes such as biotin are required.[71–74]

Recently, the bioactivity-based detection of fragment ligation products has been further extended from protein assays to even more complex cellular experiments.[75,76] Ohkanda and co-workers have reported the intracellular generation of the inhibitor **3** of the 14-3-3 protein by intracellular oxime ligation (Figure 8). The 14-3-3 protein is involved in protein–protein interactions (PPIs), which are especially difficult to inhibit, as interactions of large and dynamic interaction surfaces have to be disrupted by molecules that are still able to penetrate cells through cellular uptake. The authors realized that the reactive aldehyde derivative of fusicoccin A (Fc, **1**) binds in a hydrophobic cavity adjacent to the binding site of peptide **2**, which contains a hydroxylamine functionality. They were able to demonstrate the templated formation of a heterobivalent ligation product, the potent oxime inhibitor **3**, in vitro in the presence of 14-3-3 protein. Next, the authors investigated the intracellular formation of **3** in stably transformed HEK293 (Flag 14-3-3 ζ cell line). HPLC analysis showed the formation of **3** in cells treated with **1** and **2**, which led to the highest cytotoxic effect. In contrast, chemically synthesized **3** was inactive in the cells, possibly because of the large molecular size that impedes their cell-penetrating properties (Figure 8).[77] Thus, this study demonstrates that even large molecules that potentially modulate PPIs can be generated through intracellular protein-templated fragment ligation.

In summary, the considerable progress made in detection methods over the last few years, especially in bioactivity-based methods, but also in the classical analytical methods and biophysical detection strategies, has facilitated the identification of bioactive fragment ligation products in protein-binding, enzymatic, and cellular assays. This development will enable the discovery of further examples for templated fragment ligation reactions in the future and thereby contribute to the successful application of fragment ligation assays.

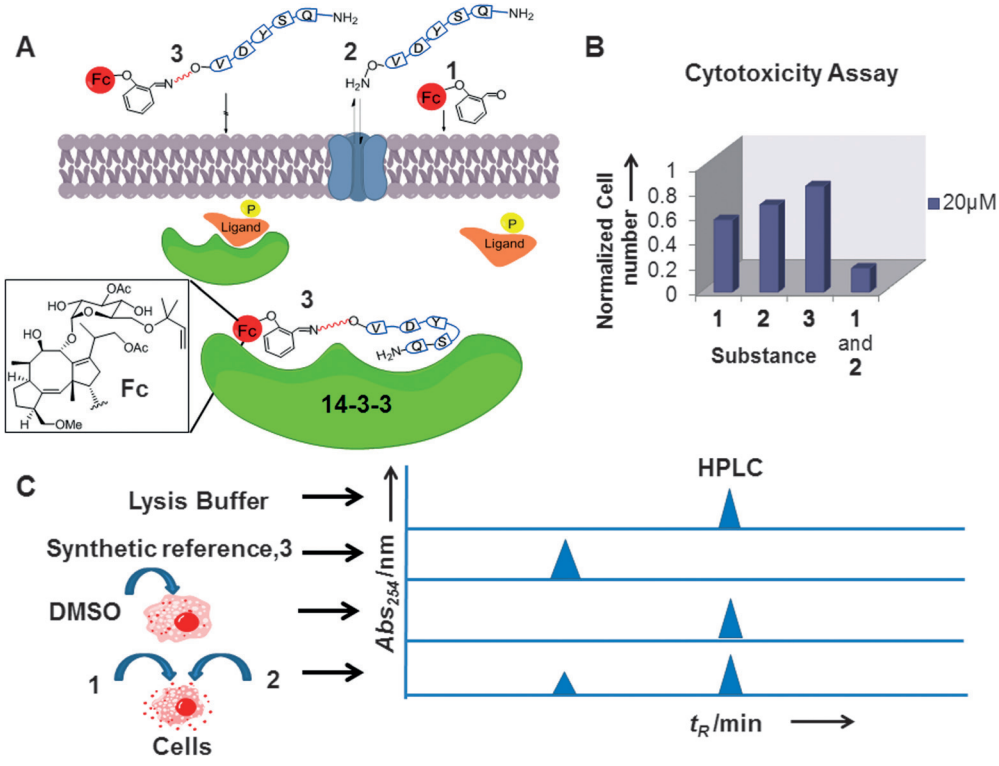



***Figure 8.*** A) Intracellular generation of the 14-3-3 inhibitor **3** through protein-templated oxime ligation. B) Cytotoxicity assay of HEK293: Flag 14-3-3 ζ cells treated individually with 20 μM of compound **1**, **2**, and **3** as well as **1** and **2** combined. C) HPLC analysis of the intracellular formation of the oxime ligation product **3**.[77]

## 4. Chemistry of Protein-Templated Fragment Ligations

Numerous reaction types have so far been employed for templated fragment ligations. Here, we will give an overview of these reaction types, focusing on the recent additions to this repertoire and giving an outlook on further extensions that can reasonably be expected. From a practical viewpoint, the degree of reversibility of the ligation reaction is an important criterium, as it affects the detection and isolation of the ligation products. Thus, reaction types used for templated fragment ligations are categorized here according to their reversibility (Table 1).

Additions of heteronucleophiles to aliphatic or aromatic aldehydes/ketones typically represent reversible ligation reactions that equilibrate rapidly in aqueous solutions as a result of low activation barriers. There are, however, marked differences with respect to reversibility and kinetics depending on the reacting nucleophile.

Hemiacetals and hemithioacetals are rapidly formed in water from aldehydes, alcohols, and thiols through templated fragment ligations, although they cannot be isolated as stable ligation products.[46,58,78,79] The formation of both hemiacetals and hemithioacetals as ligation products is impeded by competition of the heteronucleophiles with a high molar excess of water (55 M), which furnishes hydrates as alternative reaction products. Nevertheless, it could be demonstrated by using NMR spectroscopy[58] and bioactivity-based assays[46] that a protein template is able to shift the ligation equilibrium and, thus, to enable the identification of hemiacetals and hemithioacetals as ligation products.[46] In contrast to the hemiacetals, "full" acetals or dithioacetals (obtained by the addition of two alcohol or thiol nucleophiles to one carbonyl group) have not been reported as templated ligation products and cannot be expected to be formed because of the high activation barrier to the carbocation intermediates in this reaction.[80–82]

Many templated ligation reactions involve nitrogen nucleophiles that react with carbonyl electrophiles. The addition of primary amines to aldehydes furnishes hemiaminals as intermediates, which react further to form imines.[126,127] Imine formation is also a process that equilibrates rapidly and requires nonprotonated amines for reactivity.[10,43,44,46,47,83,84] Thus, the process depends on the p$K_a$ value of the amine nucleophile and the pH value of the reaction buffer. Imines are considerably more stable than hemiacetals, especially those formed from aromatic amines, and can even be isolated in some cases.

One consequence of the increased stability of imines is that the equilibration of dynamic combinatorial libraries may take significantly longer than the equilibration of hemiacetals or hemithioacetals. The stability of amine–aldehyde ligation products can be further enhanced if the aldehyde carries acidic α-hydrogen atoms, which allow the formation of enamines. Another possibility is the chemical fixation of imines by an irreversible reaction, for example, reductive amination to yield secondary amines.[86,99] The stability of the ligation products of nitrogen nucleophiles with aldehydes is further elevated in the case of hydroxylamines, hydrazines, and acyl hydrazides.[14,77,86–97,100] The formed products, oximes and (acyl) hydrazones, are stable at physiological pH values and can be isolated by standard procedures, such as column chromatography. Accordingly, the ligation reaction is equilibrated very slowly or aniline has to be added as a catalyst for the formation of the hydrazine or oxime.[86,87,89,99] Alternatively, the equilibration of acyl hydrazones can be accelerated by the addition of acid.[98]

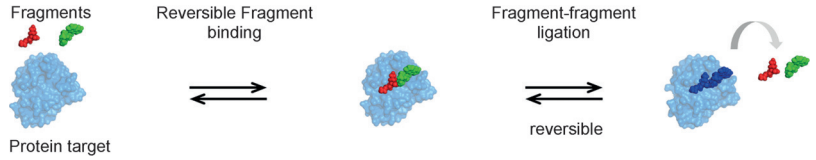
Further ligations of heteronucleophiles with aldehydes are awaiting further investigation. For example, thiols can be added to imines to furnish N,S-acetals in a ligation equilibrium that can possibly be shifted by interaction with a protein template.[43,46,112] Bioactivity data obtained from the investigation of a presubstrate have suggested that the trimeric complexes of aldehyde, amine, and thiol have a stronger affinity to the protein target, as expressed by a reduced $K_M$ value and increased turnover of the enzyme substrates.[46] Likewise, the observed superadditive binding and inhibition of peptide aldehydes and amines quantified by the FP assay can be interpreted by the formation of such thioaminal products.[43,47] Mannich bases, a special form of stable aminals, can also be expected to be suitable for protein-templated reactions, a topic which is currently under investigation in our group.

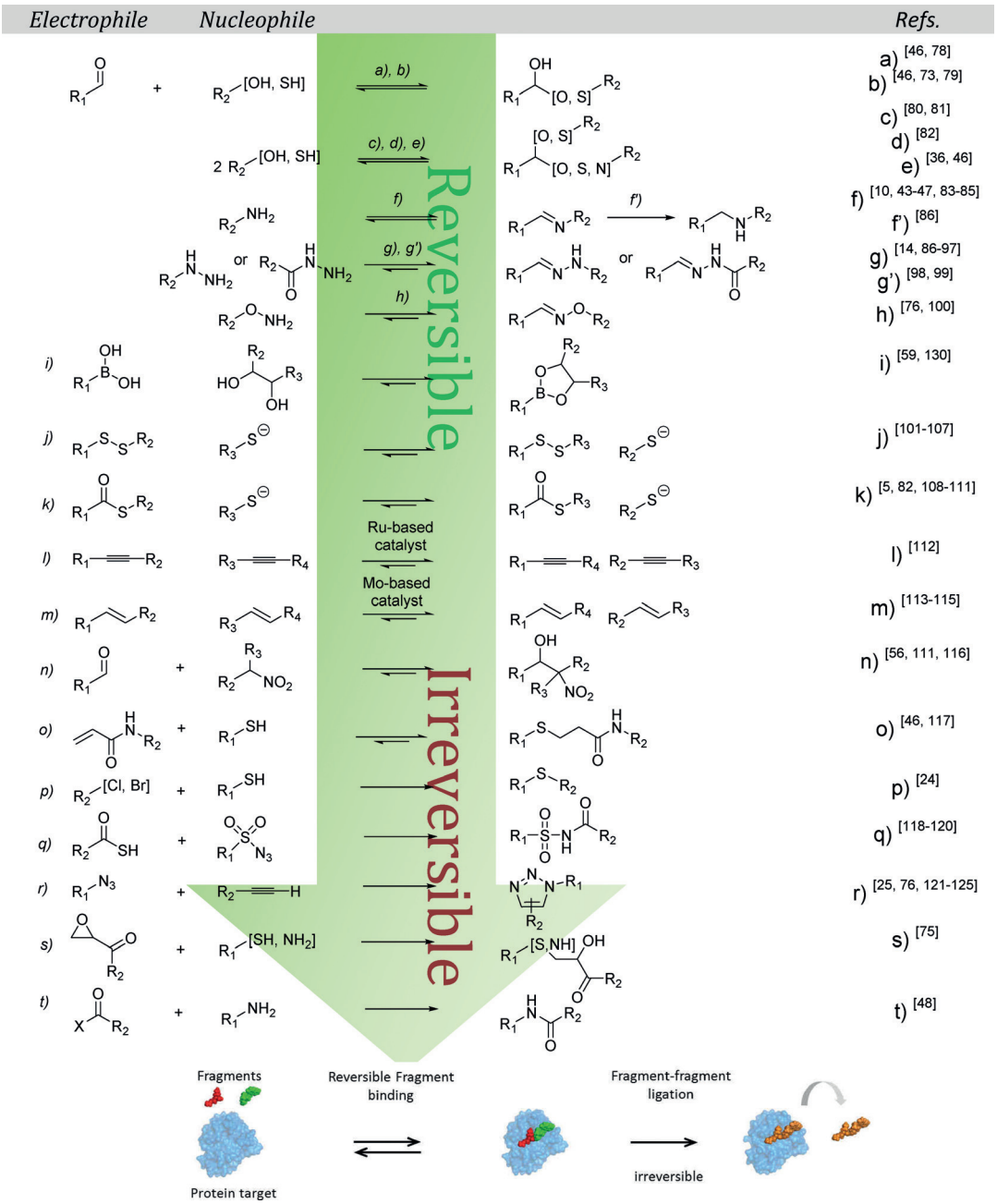
Further templated fragment ligations involve the addition of heteronucleophiles to C electrophiles other than aldehydes. Thio-Michael additions have been reported in several cases, and the products can usually be isolated, even though the reaction can be reversible if the product favors retro-addition through β-elimination.[46,117] Alkylations are usually truly irreversible ligations; in most cases thiolates are employed as nucleophiles for highest reactivity, and aliphatic halogenides, epoxides, or sulfonates have been used as electrophiles.[118–120] These studies suggest that alternative C electrophiles should be useful in protein-templated reactions. For example, nucleophilic substitutions at electron-poor aryl moieties could be an interesting extension of the reaction repertoire.

Boric acid and boronic acids have been used as alternative electrophiles. The use of diols as bisnucleophiles furnishes boronate esters as ligation products with limited stability.[59,125] Other popular examples of reversible reactions that furnish stable products that can be isolated are disulfide formation and disulfide exchange reactions through the use of thiolates as the reactive nucleophiles under slightly basic and nonreducing conditions.[101–107] A similar exchange reaction of thiolates has been reported for the thioester exchange reaction.[33,55,82,108–110] C–C bond-forming reactions are of special interest in templated ligations, as they can extend the choice and diversity of the accessible ligations products considerably. Few examples have been reported to date. Ramström and co-workers have described a reversible templated Henry-aldol addition of nitroalkanes to aldehydes, in which the formed, best-bound secondary alcohol was preferably acylated by the lipase as the protein target in the assay.[56] Although no further reports on templated aldol reactions have yet been published, this reaction type should be highly suitable for fragment ligation chemistry considering the detailed literature on aldol reactions in water and under

**Table 1:** Fragment ligation reactions employed in FBDD.




a) Hemiacetals, b) hemithioacetals, c) acetals. d) thioacetals, e) N,S-acetals, f) imines, g) hydrazones, g') acylhydrazones, h) oximes, i) boronates, j) disulfides, k) thioesters, l) alkene metathesis, m) alkyne metathesis, n) nitroaldols o) alkylation, p) amidation between sulfonylazides and thioacids, q) 1,3-dipolar cycloaddition, r) addition, s) ring opening, t) amidation between amines and active esters.

mild reaction conditions.[56,111,116,128] Likewise, additions of cyanide anions and isocyanides have been reported in water, which suggests that Passerini and Ugi reactions might be highly suitable for protein-templated reactions. The same applies to the C–C bond formations by alkene and alkyne metathesis reactions, which have so far been demonstrated as ligation reactions in water.[112–115]

Classical examples of irreversible templated reactions have been reported for dipolar cycloaddition reactions, including azide–alkyne ligations that furnish 1,4-disubstituted

1,2,3-triazoles, and sulfonylazide–thioacid ligations that provide sulfonylamides through a cyclic intermediate.[24,25,76,118–124] It is clear that numerous other ligations based on cycloaddition reactions may also succeed. Besides cycloaddition reactions, however, irreversible ligations have not yet been investigated much and future extensions are necessary to provide ligation products that cover a larger part of the biologically relevant chemical space. In general, chemoselective reactions in water are required for a successful ligation. Ideally, the reaction should provide a linker between the reacting fragments that supports binding or at least does not interfere with it. For example, the structural analysis of the world drug index (a collection of molecules with reported bioactivity) revealed that amide linkages are privileged linkers in bioactive compounds.[129] Thus, a protein-templated amidation reaction can be considered an especially useful extension of the fragment ligation repertoire. Very recently the first background-free protein-templated amidation reactions were discovered.[48] Other reaction types that could considerably extend the opportunities of fragment ligations are reactions that are able to connect fragments without a linker remaining in the product, such as cross-coupling reactions or reactions forming heterocycles as the connection between two fragments instead of a classical linker moiety.

## 5. Practical Examples and Applications of Fragment Ligations

Having defined fragment ligation reactions, explained their biophysical background, useful detection strategies, and the underlying chemical transformations, we are now going to focus on the application of the concept for the discovery and optimization of protein ligands. We will start with reversible ligation reactions and then proceed to the irreversible ones.

Many of the first applications of reversible, templated fragment ligations were reported for dynamic combinatorial libraries (DCLs). As a recent representative example of this line of research, we highlight the studies by Hirsch and co-workers.[36,98] The authors employed endothiapepsin as a target and model enzyme for other aspartic acid proteases. Nine hydrazides (**4**–**12**) and one bisaldehyde were used to form a dynamic library derived from two fragment hits[36] that bind to adjacent binding pockets of endothiapepsin (Figure 9).[98] To ease analysis, two sublibraries (DCL-1 and DCL-2) were formed that consisted of four and five hydrazides. Reversed-phase HPLC and LC-MS were employed to analyze the templated formation of the bisacylhydrazone ligation products in the presence of endothiapepsin. Out of the potential 78 different bisacylhydrazones and 12 monoacylhydrazones, only 6 possible fragment combinations showed significant amplification of the HPLC signal in the presence of the endothiapepsin template. Two of the found combinations, **13** and **16**, were synthesized and tested in a fluorescence-based biochemical assay. Both were reported to be active, and the best inhibitor, **13**, showed a 240-fold increase in potency compared to the starting fragments, thus illustrating a successful example of a templated three-fragment ligation reaction. The authors demonstrated in this study that, unlike other fragment optimization methods such as fragment growing or merging, templated fragment ligation is especially sensitive for discovering combined fragments with superadditivity, where their ligand efficiencies were not only maintained but improved. In their approach, the challenge lay in preserving the binding mode of the fragments and finding a well-fitting linker that provides additional interactions to the target. Despite the positive results, the study also demonstrates the limitations of DCL, namely the tedious analysis of complex combinatorial mixtures and the limitation to few dynamic reactions that deliver non-druglike products such as acylhydrazones.

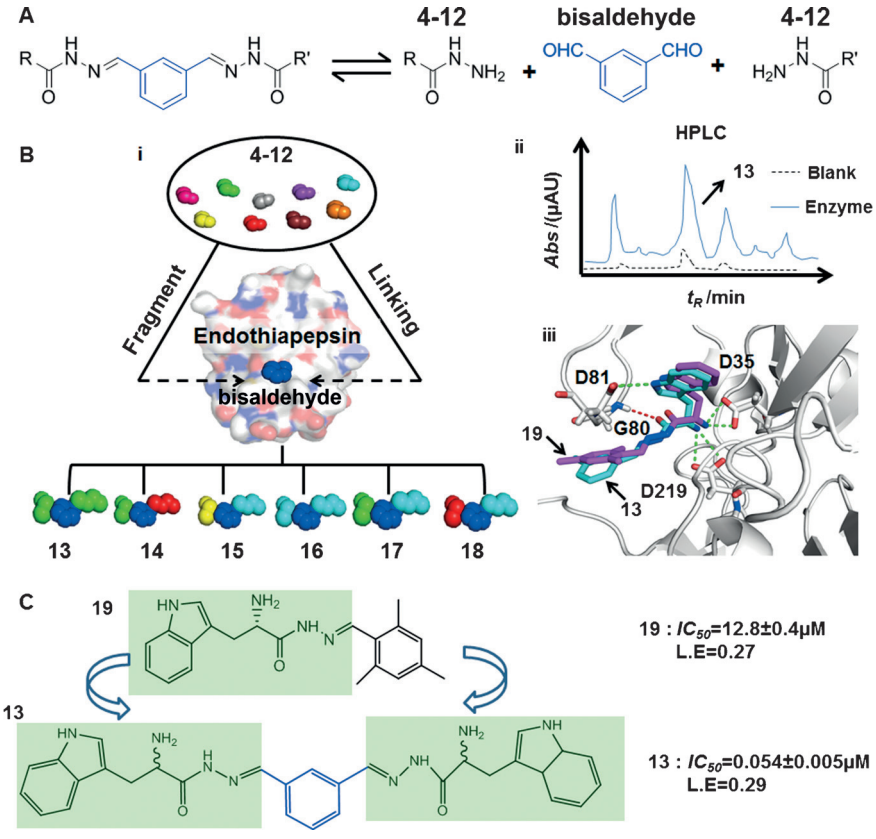



***Figure 9.*** Protein-templated fragment ligations using a dynamic combinatorial library.[98] A) Isophthalaldehyde **3** and nine hydrazides, **4**–**12** form up to 81 bisacylhydrazones. B i) The model protein endothiapepsin is equilibrated with the DCL for the formation of potential inhibitors. B ii) HPLC analysis shows that the formation of the bisacylhydrazone **13** was amplified in the presence of the enzyme. B iii) Binding modes of **19** (purple) and **13** (cyan) were determined by X-ray crystallography (PDB IDs: 4KUP and 5HCT respectively). C) The best compound **13** exhibits a 240-fold improvement in potency and higher ligand efficiency compared to the starting fragment **19**.

The same limitations are displayed in the study by Claridge and co-workers who applied $^{11}B$ NMR spectroscopy for the detection of serine protease inhibitors from a DCL.[59] For a proof-of-concept study, α-chymotrypsin (αCT) was recruited as a model enzyme, and boronic acids were ligated with added sugar molecules to form improved enzyme inhibitors. The formation of ternary complexes of enzyme, boronic acid, and sugar diol was monitored by $^{11}B$ NMR and $^{1}H$-WaterLOGSY (water-ligand observed by gradient spectroscopy). These findings encouraged the groups of Schofield and Claridge to apply native mass spectrometry to detect hits from a DCL. Reversibly formed boronate esters were identified as inhibitors of prolyl hydroxylase domain isoform 2 (PHD2), an enzyme of the 2-oxoglutarate (2-OG) oxygenase family (Figure 10).[130] PHD2 is an $Fe^{II}$-containing 2-OG oxygenase which helps to regulate human hypoxic response, thus making it an excellent drug target for the treatment of anemia and ischemia-related diseases.

In the experiments, a heterocyclic iron-binding boronic acid fragment was incubated with several pools of diol ligands and subsequently analyzed by native MS. The best-binding fragment combinations were identified by the shift in protein mass and converted into stable, boron-free inhibitors by using Suzuki cross-coupling reactions, which resulted in inhibitors of the enzyme being active down to the nanomolar range. The results of the fragment ligation assays were further validated in another study by using a $^{1}H$ NMR based method.[131]

The tethering approach is a classical example of reversible templated fragment ligations using disulfide exchange reactions.[132] This method exploits the reversible disulfide exchange reaction between a natural or an engineered cysteine residue on the protein surface and a disulfide-containing small-molecule fragment in solution. Further developments by the Erlanson group using so-called "extenders" made this approach even more applicable for fragment combination reactions.[133] In 2008 they reported an inhibitor screening that targeted the aurora kinase (Figure 11).[134] The first step was to introduce a cysteine residue near the ATP binding site. Next, an extender was synthesized containing two disulfide-containing residues and a diaminopyridine group, which is known to bind to the purine binding site. One disulfide residue was able to react and exchange with the introduced cysteine residue, the other one was able to bind a secondary disulfide fragment. If this secondary fragment was able to interact with the adaptive region of the protein adjacent to the extender, a thermodynamically stabilized disulfide bond was formed. This stabilized complex was subsequently detected by MS measurements through modification of the protein's mass. In their experiments, a library containing roughly 4500 disulfide-containing fragments was screened in pools of 10 compounds, which resulted in the identification of several fragment combinations. A few modifications of the corresponding fragments led to the formation of a stable inhibitor with affinity in the micromolar range. A similar approach has been successfully applied to another kinase, 3-phosphoinositide-dependent protein kinase-1 (PDK1), by attaching the extender through an irreversible alkylation reaction.[135]

Dynamic ligation screening was introduced in 2008 to overcome the detection limits of combinatorial methods such as DCL. The first bioactivity-based detection of dynamically formed fragment ligation products has been demonstrated for

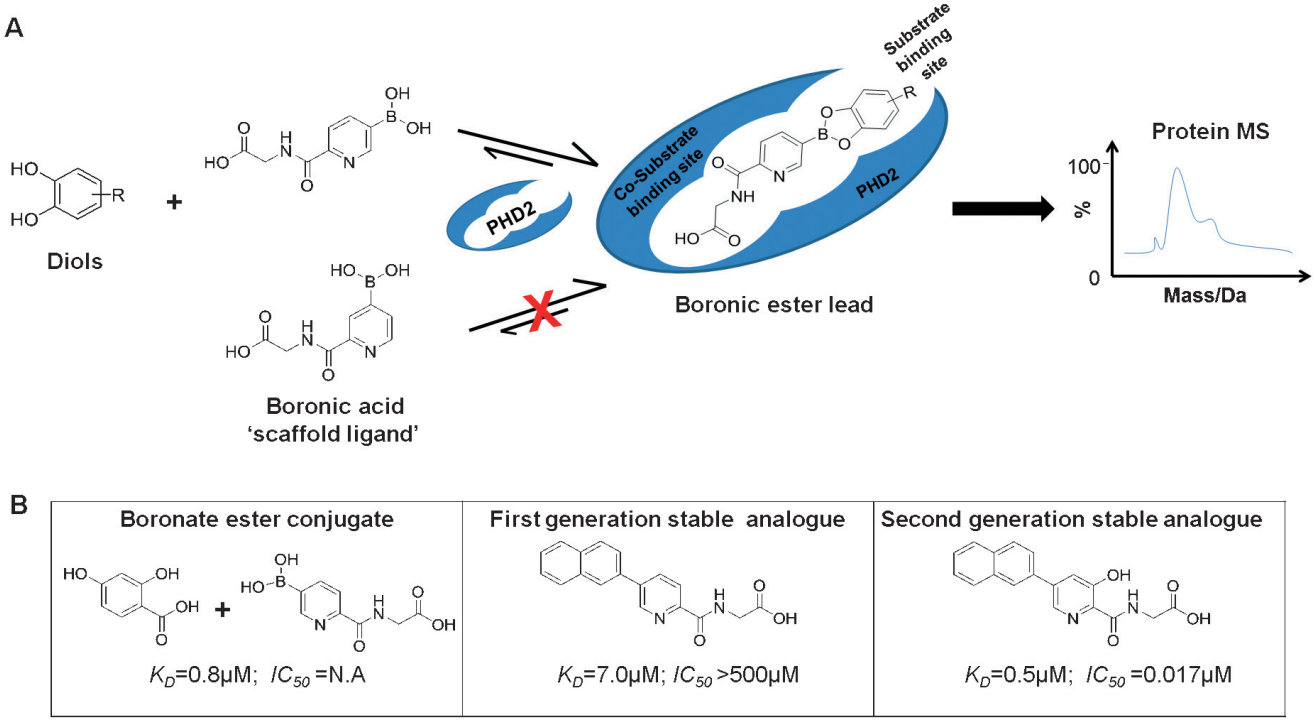



***Figure 10.*** A) Schematic representation of the generation of boronate acid/ester leads using protein-directed dynamic combinatorial libraries containing diols and boronic acids.[130] B) The identification of potent boronate ester conjugates by using a boronic acid "scaffold ligand" leads to the discovery of first and second generation stable analogues that inhibit PHD2 in the nanomolar range.

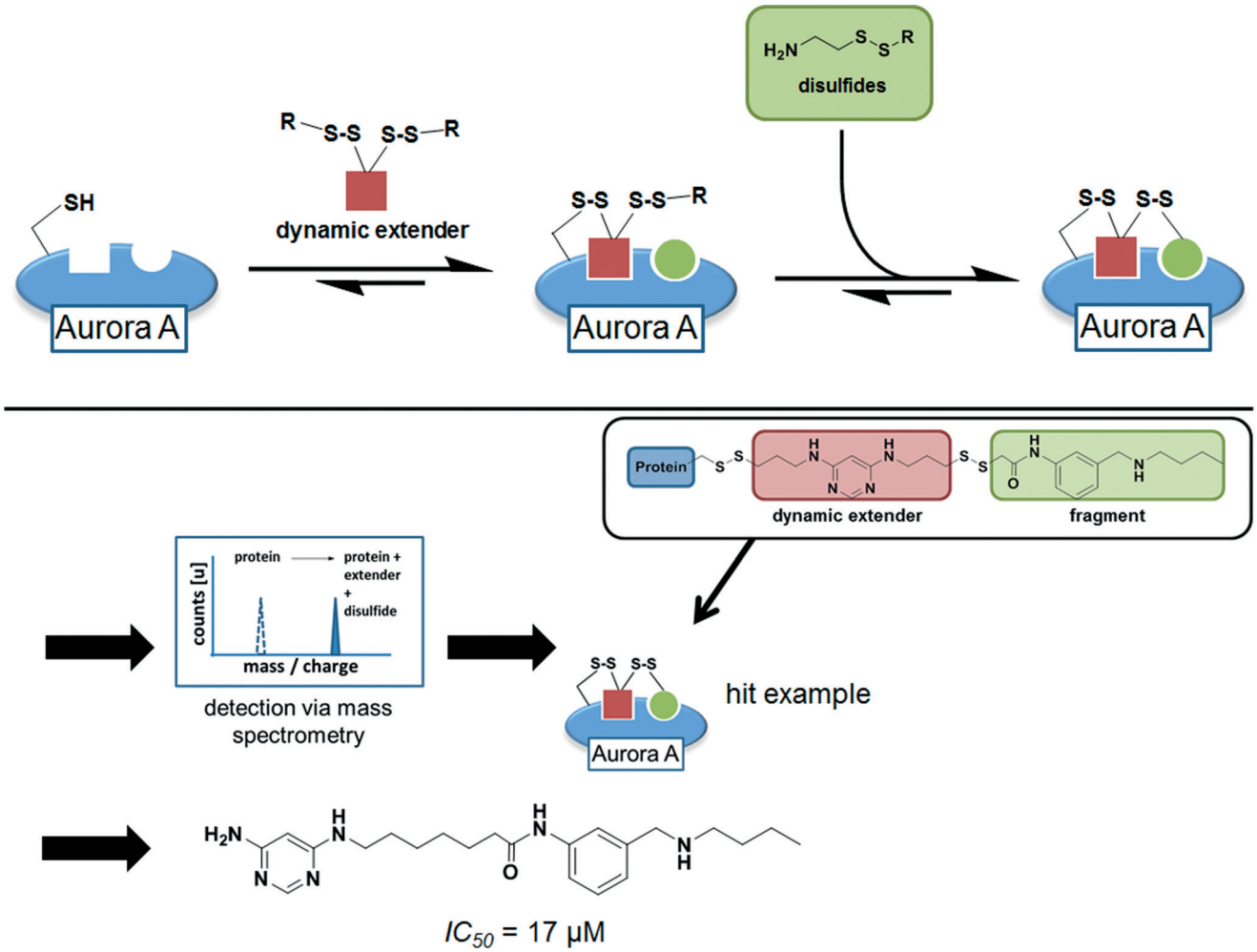



***Figure 11.*** A tethering approach using a dynamic extender has been applied to screen for fragments binding to Aurora kinase.[134]

the SARS coronavirus main protease (SARS-CoV $M^{pro}$, Figure 12).[43] SARS-CoV $M^{pro}$ is a virus-encoded cysteine protease essential for the replication of the virus inside the infected host cells. Inhibitors targeting $M^{pro}$ are, therefore, relevant as potential antivirals.[136,137] Since proteases possess defined binding pockets for the side chains of peptides, it was possible to develop a peptide aldehyde inhibitor that positions an electrophilic aldehyde precisely at the active site of the protease. Dynamic ligation screening of a fragment library of 234 diverse nucleophiles revealed several fragment hits that targeted the S1′ pocket of the enzyme with $K_I$ values in the millimolar range. The best fragment hit was converted into the corresponding aldehyde fragment and assayed again for enhanced inhibition against an amine library, thereby providing a secondary hit fragment for the adjacent S1 pocket of the enzyme's binding site. Only two iterations of dynamic ligation screening starting from a peptide inhibitor resulted in the selection of two millimolar-active small-molecule fragments, and the covalent combination of these fragments by reductive amination revealed an entirely nonpeptidic inhibitor of SARS-CoV $M^{pro}$ with a $K_I$ value of 2.9 μM.

Further examples of bioactivity-based dynamic ligation assays are summarized in Table 2. Caspase 3 is a protein that plays an essential role in the execution phase of cell apoptosis and, therefore, a potential drug target in traumatic brain injury and amyotrophic lateral sclerosis as well as Alzheimer's and Parkinson's disease. The protein was tested with a nanomolar fluorophore-labeled peptidyl ketoaldehyde inhibitor and a total of 7397 fragments, including 4019 nucleophilic and primary amines in 384-well microtiter plates, by applying the FP binding assay described above. We observed no change for most of the fragments tested. 78 fragments led to lower FP signals (negative cooperativity) and 176 fragments evoked a significantly stronger FP signal (positive cooperativity) than the control. 21 fragments were confirmed as competitive inhibitors of caspase-3 with $K_I < 10$ μM. The best cooperatively binding fragment **B** was linked covalently to the starting ligand **A** by reductive amination, thereby resulting in a potent inhibitor of caspase-3 with a $K_I$ value of 80 pM.

Dynamic ligation assays were further implemented for secondary-site screening of four closely related protein tyrosine phosphatases (PTPs): human PTP1B, PTPN7, PTPN12 (= SHP2), and mycobacterial MPTPA.[45] In general, the development of specific PTP inhibitors is considered to be a major challenge that raises doubts on the druggability of this physiologically relevant class of enzymes. By using 4-formyl-

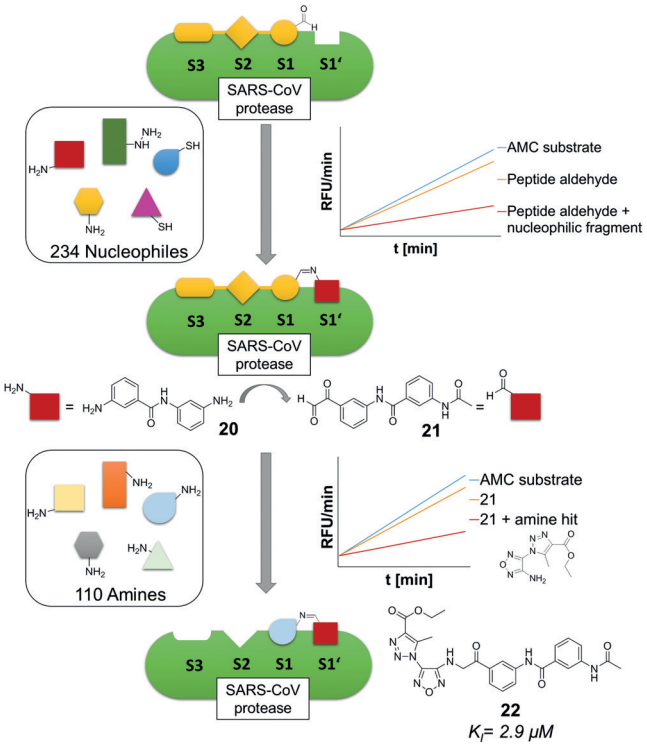



***Figure 12.*** Development of a fragment-based, nonpeptidic SARS-CoV main protease inhibitor starting from a peptide aldehyde.[43] Dynamic ligation screening of a library of nucleophiles yielded the amine **20**, which binds to the S1′ subpocket of the protease. Amine **20** was converted into the electrophile **21**, which furnished a secondary hit fragment in the next iteration. Reductive amination of fragment **21** and the best amine hit from the secondary screening yielded inhibitor **22**.

phenyl phosphate as an electrophilic PTP substrate, specific secondary-site binding fragments could be identified for each of the PTPs from a library of only 110 primary amines. The specific secondary site binders were detected using a "dynamic substrate enhancement" assay (see Figure 7B): active fragments form a ligation product with the PTP substrate which results in a higher stability of the enzyme–substrate complex and a lowered $K_M$ value. This leads to an amplified release of phosphate ions, which was determined in a Malachite green assay. Replacement of the phenyl phosphate substrate by a noncleavable phosphotyrosine mimetic and covalent linking to the selected MPTPA-specific amine fragment furnished inhibitors of protein tyrosine phosphatase A (MPTPA) from *Mycobacterium tuberculosis*, with no activity for the other three PTPs. This result suggests that second-site targeting of PTPs enables the development of selective PTP inhibitors.

Dynamic ligation screening has been further extended toward aspartic proteases, this time by employing a fluorescence resonance energy transfer (FRET) assay.[44] A peptide aldehyde was used as a directing probe to identify the active-site binding fragments of the aspartic protease β-secretase (BACE-1). This enzyme is known to be the main culprit in the aggregation of amyloid-β-peptides, which is a major pathological hallmark of Alzheimer's Disease (AD). Thus, considerable efforts have been made to discover BACE-1 inhibitors for potential therapeutic treatment of AD. Instead of reversible hemithioacetal formation, the aldehyde hydrate was formed to bind the catalytic aspartic acid dyad through formation of a hydrogen bond. Dynamic ligation of the peptide aldehyde with an amine nucleophile reversibly yielded an imine product. The peptide aldehyde, which serves as a chemically reactive inhibitor (CRI), revealed 3-(3-aminophenyl)-2*H*-chromen-2-one to be a competitive BACE1 inhibitor. The identified 3-(aminophenyl)coumarin fragment was used as a starting point for hit optimization and a low micromolar ($K_I = 3.7$ μM) BACE1 inhibitor was developed. Another application of bioactivity-based detection has been reported recently for the aspartic protease endothiapepsin.[64]

In the most recent application, reversible protein-templated fragment ligation has been extended toward the discovery of irreversible inhibitors of enteroviral proteases (Figure 13).[85] Epoxyaldehyde **23**, a modified partial structure of the known inhibitor of cysteine proteases E-64 and a weak irreversible inhibitor of the 3C protease of Coxsackie 3B virus, was tested with a library of 850 primary amine fragments. 5-Aminopyrazolone **24** was discovered as a fragment hit that led to a superadditive inhibition of the protease and covalent modification of the enzyme by addition of both fragments. Iterative optimization of ligation products of **23** and **24** finally led to sub-micromolar, broad-spectrum inhibitors of enteroviral proteases, with 300- to 500-fold acceleration of the protein deactivation rate and no significant cross-reactivity with nonviral proteases.

Early studies in the area of irreversible protein-templated fragment ligations were conducted by the Sharpless group and denominated as kinetic target-guided synthesis. They used dipolar cycloadditions of azides and alkynes for in situ click reactions templated by the protein acetylcholinesterase (AChE), which is a target in the treatment of Alzheimer's disease.[30] In a follow-up study from 2005, the method was again applied to the same target, with the goal of identifying and synthesizing a fragment combination that occupied both the active center and the peripheral binding site (Figure 14).[123]

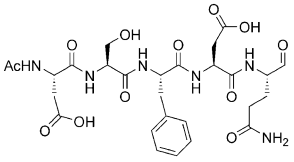
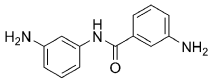
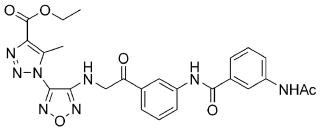
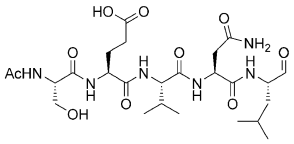
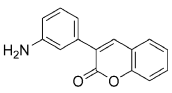
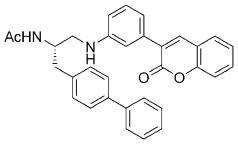
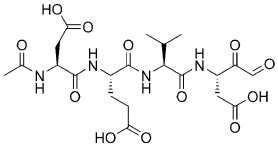
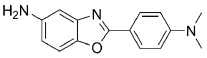
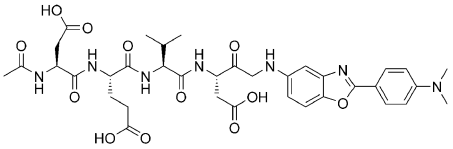
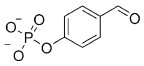
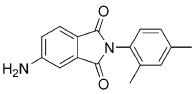
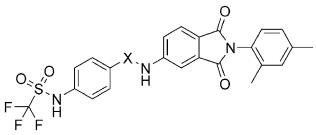
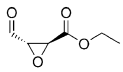
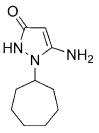
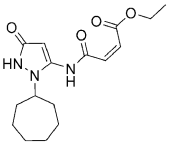
As a starting point, the established AChE inhibitor tacrine was used, which binds to the active center of the enzyme. Tacrine was modified by introducing an azide functionality. A compound collection of 23 terminal acetylenes was composed of peripheral site binding fragments and tested to detect the formation of the 1,2,3-triazole by dipolar cycloaddition. The first LC-MS results confirmed the protein's templating effect during the ligation reaction of the bound fragments. Only in the presence of the enzyme were 1,5-disubstituted (*syn*) triazoles formed, with only little or no background reaction.

Having confirmed the reaction with single fragment pairs, the authors turned their attention to reactions containing mixtures of up to 10 acetylene compounds. Indeed the protein template induced the formation of highly potent fragment combinations with dissociation constants in the low picomolar or even femtomolar range.

Azide–alkyne ligations have also been applied to other protein targets.[76,121–125,138] For example, the formation of a known nanomolar inhibitor of HIV protease containing an *anti*-substituted 1,2,3-triazole was accelerated in the presence

***Table 2:*** Applications of protein-templated fragment ligation based on dynamic ligation screening.[a]

| Protein | Starting ligand | Hit fragment | Fragment combination | Ref. |
|---|---|---|---|---|
| SARS-CoV Mpro |  |  |  | [43] |
| | 11 μM | >1 mM | 2.9 μM | |
| BACE-1 |  |  |  | [44] |
| | 20.7 μM | 146 μM | 3.7 μM | |
| Caspase-3 |  |  |  | [47] |
| | 25 nM | 120 μM | 0.08 nM | |
| MPTPA |  |  |  | [45] |
| | $K_M$ = 515 μM | >500 μM | X(C=O): 13 μM<br>X($CH_2$): 11 μM | |
| CB3-3CV- protease |  |  |  | [85] |
| | $k_{inact}/K_I$ = 2.36 $M^{-1}s^{-1}$ | >1 mM | $k_{inact}/K_I$ = 606 $M^{-1}s^{-1}$ | |

[a] $K_I$ values are given, unless noted otherwise.

of the enzyme.[138] In this case, the reaction was considerably slower than that reported for the pico-/femtomolar AChE inhibitors, and this time a clear background reaction that furnished the *syn*-triazole was observed. Protein-templated azide–alkyne "click" reactions have also been successfully applied to the Abl tyrosine kinase.[121] The Fukase research group has demonstrated the templated synthesis of functional mimetics of the Grb2-SH2 domain which inhibited the growth of cancer cells in vitro.[76] To obtain the best inhibitor through the templated reaction of azide and alkyne fragments it is crucial to exclude even tiny amounts of copper ions from the reaction. The groups of Miyata and Finn reported the in situ synthesis of an inhibitor of the histone deacetylase 8 (HDAC-8) from an alkyne fragment bearing a hydroxamate and an azide fragment.[122] Surprisingly, the formed ligation product was not the best inhibitor by far and contained a 1,4-disubstituted *anti*-triazole instead of the more active *syn*-triazol. A detailed scrutiny of these results revealed that the reaction contained traces of $Cu^I$ ions, which were bound by the metal-binding site of HDAC-8 and were sufficient to catalyze the reaction to the less-favored cycloaddition product.

In addition to azide–alkyne ligations, other dipolar cycloadditions have also been investigated. Reactions of thioacids with sulfonylazide fragments, which provide acylsulfonamides via a cyclic intermediate, constitute especially successful examples and have been denominated as sulfo-click reactions. In 2011, the Manetsch group demonstrated protein-templated, irreversible ligation reactions with a particularly challenging target: the PPI domain Bcl-$X_L$.[120] The interaction of Bcl-$X_L$ with the BH3 peptide is a crucial step in the regulation of apoptosis (programmed cell death). Inhibitors of this interaction might be potent anticancer agents. In a screening for new modulators, the Bcl-$X_L$ protein was used as a template for the protein-templated sulfo-click reaction of a sulfonylazide and a thioacid fragment (Figure 15). It could be shown that formation of the micromolar Bcl-$X_L$ inhibitor proceeded in a protein-templated reaction; less inhibitor was

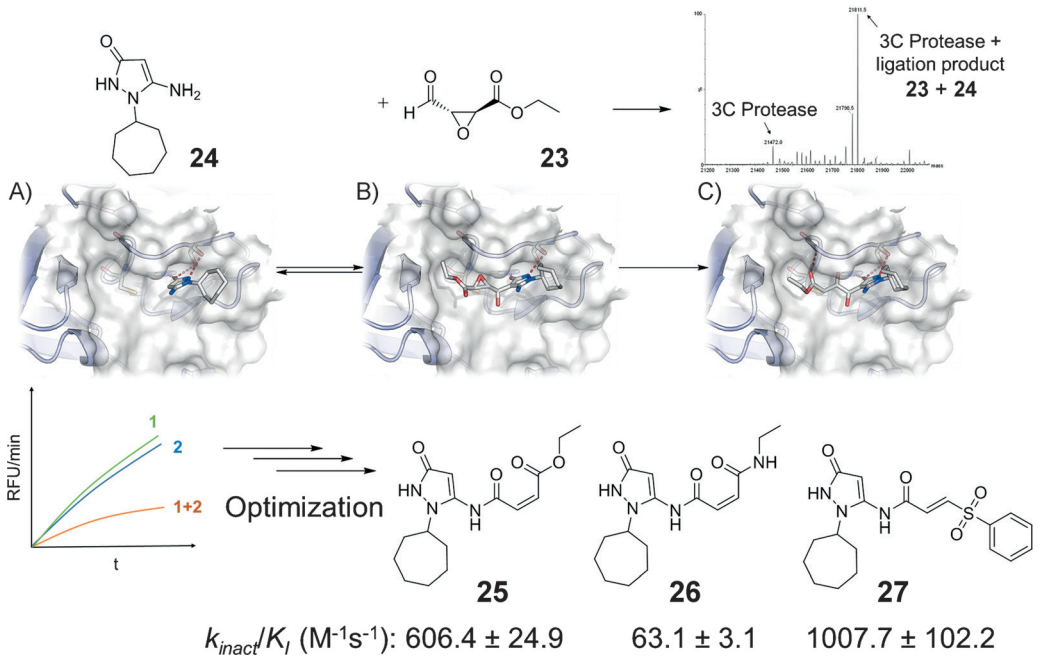



***Figure 13.*** Discovery of irreversible inhibitors of Coxsackie virus B3 3C protease by using reversible protein-templated fragment ligations.[85] Nucleophilic amine fragment **24** binds to the S1 pocket of the enzyme (A) to reversibly form a fragment ligation product with the biselectrophilic warhead **23** (B). Next the epoxide is opened by attack of the cysteine side chain in the active site, as detected by LC-MS (C). The ligation product displays a superadditive inhibitory effect in the FRET assay and is optimized to potent broadband inhibitors of enteroviral and rhinoviral 3C proteases.

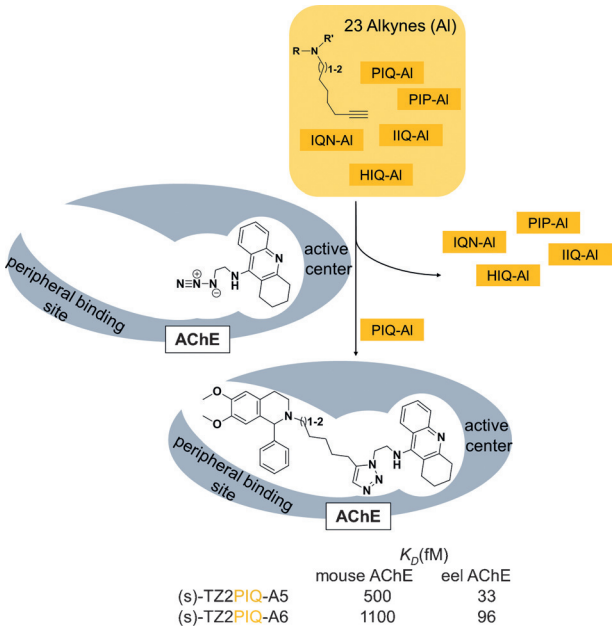



***Figure 14.*** Development of AChE inhibitors by protein-templated fragment ligation reactions.[123] The AChE inhibitor tacrine was modified with an azide functionality and acts as an anchoring molecule in the active site of the protein. Mixtures of various acetylenes were added and the templated reaction furnished two highly potent *syn*-1,2,3-triazoles.

formed without the protein or on blocking the binding site by the BH3 peptide, while addition of an inactive mutated BH3 peptide had no effect.

Protein-templated, irreversible fragment ligations were recently established for the formation of amide bonds (amidation reactions), one of the most relevant fragment linkages in bioactive compounds and clinically admitted drugs (Figure 16).[47] A collection of active esters **28**–**40** covering a broad range of chemical reactivity was incubated with 4-aminomethylbenzamidine (**41**) and protein factor Xa, a drug target from the blood coagulation cascade, to discover conditions for protein-templated amidation reactions. Two active ester fragments, phenyl ester **39** and trifluoroethyl ester **40,** displayed a clear protein-templated amidation of **41** in the substrate competition assay and in LC-MS, and afforded the nanomolar inhibitor **42** from two weakly binding fragments with millimolar affinities. Extracted-ion chromatography with a QTOF detector was used to quantify the progress of the protein-templated formation of inhibitor **42**. Interestingly, the reaction of the trifluoroethyl ester **40** proceeded without detectable background reaction and was autoinhibited at a saturation concentration of 10 nM of the free inhibitor **42**. The inhibitor displayed a remarkable superadditive enhancement of its free binding energy (the $K_I$ value was 29 nM instead of 3 μM for the additive case), which was proven to result from the relatively decreased entropy of binding because of fragment linking. The protein–inhibitor complex was crystallized and the obtained high-resolution structure allowed the authors to rationalize the templated amidation reaction in steric and mechanistic detail.

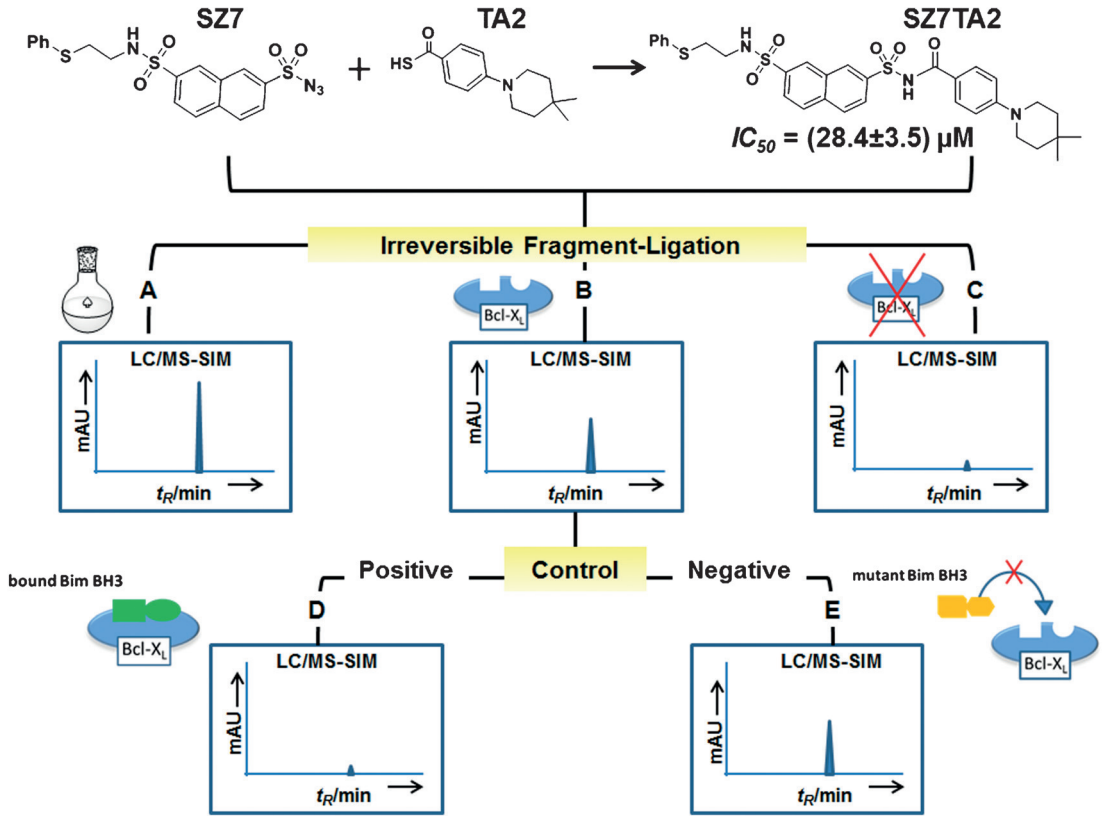



*Figure 15.* Templated formation of sulfonamide inhibitor SZ7TA2 of the protein Bcl-$X_L$ from fragments sulfonylazide SZ7 and thioacid TA2.[119] A) Reference compound obtained by chemical synthesis. B) Templated reaction: fragments incubated in the presence of target protein; Bcl-$X_L$ serves as a template for the formation of SZ7TA2. C) Background reaction: fragments incubated without Bcl-$X_L$ lead only to small amounts of the product. D) Blocking of the Bcl-$X_L$ binding site by the Bim-BH3 peptide suppresses the product formation. E) Mutated Bim-BH3 has only a low affinity towards Bcl-$X_L$, thereby leading to no inhibition of the templated fragment ligation reaction.

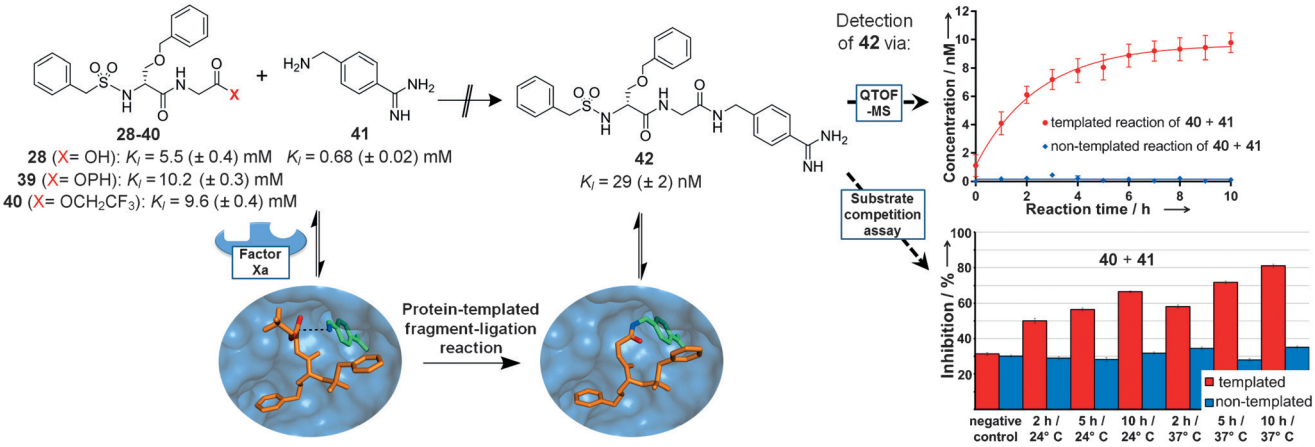



*Figure 16.* Protein-templated amidation of active esters **39**/**40** with 4-aminomethylbenzamidine (**41**) furnished the nanomolar inhibitor of the protein factor Xa.[47] The protein-templated, background-free reaction was demonstrated in a bioactivity-based assay and the autoinhibitory kinetics of inhibitor formation was proven by QTOF-MS. The crystal structure of the protein–inhibitor complex enabled the pathway of the templated reaction to be modeled.

## 6. Discussion and Outlook: Present Status and Future of Protein-Templated Fragment Ligations

Protein-templated fragment ligations have been established as an alternative and complementary route to access bioactive protein ligands over recent years. Significant progress has been realized on several fronts. Improved and specialized analytical and bioanalytical methods have contributed both to a broad implementation and a profound understanding of the method. The chemical scope of ligation

reactions has been continually extended, and further extensions to reactions that deliver templated fragment ligation products can be expected. After reviewing the biophysical background, the underlying chemistry, and actual applications of this method, we will now consider its strengths and limitations and finally give an outlook on the future possibilities and developments of this technique as part of the drug discovery process.

The biggest advantage of protein-templated fragment ligations compared to other fragment-based methods is that it enables the site-directed, spatially resolved identification of fragments that bind to a precisely defined protein pocket. Other fragment-based methods, for example X-ray crystallography and some of the NMR-based methods, also deliver structural information on ligand binding, however, they cannot be used to screen specifically for one binding site. Site-directed fragment detection is realized by the structure and by the defined binding of the reactive starting fragment. The second major advantage of protein-templated fragment ligation assays is their sensitivity caused by the (super-) additive binding enhancement of ligated fragments. As a result, fragment ligation assays can identify fragments that are not detectable with other fragment-based discovery methods or only at considerably higher fragment or protein concentrations. Thus, the method may provide protein ligands with alternative structures and with improved ligand efficiencies. As a third advantage, protein-templated fragment ligations have practical and economic benefits, mainly arising from the integration of the synthesis of fragment combinations and the detection of bioactivity in one step. As a result, the effort and the resources required for the chemical synthesis of bioactive ligands are reduced considerably, as only bioactive fragment combinations need to be resynthesized for further structural and functional validation. Whereas classical high-throughput screening uses large libraries of drug-like molecules to cover the chemical space, fragment ligation screening needs only small libraries of reactive fragments to sample the chemical space of all potential fragment combinations.

One general requirement of fragment ligation assays is the need for a reactive starting fragment. This could be problematic if no suitable ligand of a target protein is known. In such cases, complementary methods such as classical screening or structure-based design are indispensable to provide starting points for fragment ligation.

Another major requirement of protein-templated ligation assays is the availability of ligation reactions that cover the relevant chemical space and are compatible with the conditions of the protein assay. Although, as shown in Sections 4 and 5, the number of chemical reactions that have been adapted to fragment ligation screening is constantly growing, continuous research efforts will be needed to make more of the privileged drug-like fragment linkages accessible in ligation assays. In particular, C–C bond-forming reactions, formations of heterocycles, and direct connections between cyclic fragments, which are often constructed through cross-coupling reactions, need further investigation.

The critical evaluation shows that protein-templated fragment ligations can currently be considered as a broadly applicable method that can, and should, contribute to the modern drug discovery process by complementing the other established and successful methods when its specific virtues are required: spatially resolved and site-directed screening and high sensitivity for the identification of novel fragments as well as the further chemical development of known protein ligands.

Future research will show if the specific advantages of the method will be able to provide clinical candidates with improved properties. The ultimate significance of protein-templated fragment ligations will depend on to what extent they will contribute to fulfill the promise of fragment-based drug discovery: Find better drugs with high potency, specificity, and fewer off-target related side effects. Most likely, protein-templated fragment ligations will contribute to this goal in close collaboration with other fragment-based and classical lead discovery approaches. Beyond the practical success of the method in the drug discovery process, protein-templated ligation reactions are also potent and attractive research and training tools that teach us how partial structures of a molecule can interact additively to create high-affinity protein ligands. Through this, the method helps us to understand how molecular recognition makes the molecules of life work and how molecular evolution can proceed.

## Conflict of interest

The authors declare no conflict of interest.

**How to cite:** *Angew. Chem. Int. Ed.* **2017**, *56*, 7358–7378
*Angew. Chem.* **2017**, *129*, 7464–7485

[1] M. Jaegle, E. Nawrotzky, E. L. Wong, C. Arkona, J. Rademann in *Fragment-based Drug Discovery Lessons and Outlook* (Eds.: D. A. Erlanson, W. Jahnke), Wiley-VCH, Weinheim, **2016**, pp. 293–326.
[2] J. Rademann, G. Jung, *Science* **2000**, *287*, 1947–1948.
[3] O. Ramström, T. Bunyapaiboonsri, S. Lohmann, J.-M. Lehn, *Biochim. Biophys. Acta Gen. Subj.* **2002**, *1572*, 178–186.
[4] V. Das Gupta, A. Gupta, *J. Pharm. Sci.* **1980**, *69*, 1145–1148.
[5] P. Storici, D. De Biase, F. Bossa, S. Bruno, A. Mozzarelli, C. Peneff, R. B. Silverman, T. Schirmer, *J. Biol. Chem.* **2004**, *279*, 363–373.
[6] R. Borštnar, M. Repič, M. Kržan, J. Mavri, R. Vianello, *Eur. J. Org. Chem.* **2011**, 6419–6433.
[7] P. T. Corbett, J. Leclaire, L. Vial, K. R. West, J.-L. Wietor, J. K. M. Sanders, S. Otto, *Chem. Rev.* **2006**, *106*, 3652–3711.
[8] J.-M. Lehn, *Chem. Soc. Rev.* **2007**, *36*, 151–160.
[9] S. Ladame, *Org. Biomol. Chem.* **2008**, *6*, 219–226.
[10] M. F. Schmidt, J. Rademann, *Trends Biotechnol.* **2009**, *27*, 512–521.
[11] Y. Jin, C. Yu, R. J. Denman, W. Zhang, *Chem. Soc. Rev.* **2013**, *42*, 6634–6654.
[12] M. Mondal, A. K. H. Hirsch, *Chem. Soc. Rev.* **2015**, *44*, 2455–2488.
[13] A. Herrmann, *Chem. Soc. Rev.* **2014**, *43*, 1899–1933.
[14] S. Ulrich, P. Dumy, *Chem. Commun.* **2014**, *50*, 5810–5825.
[15] D. Bosc, J. Jakhlal, B. Deprez, R. Deprez-Poulain, *Future Med. Chem.* **2016**, *8*, 381–404.
[16] G. G. Ferenczy, G. M. Keserű, *J. Chem. Inf. Model.* **2012**, *52*, 1039–1045.

[17] G. G. Ferenczy, G. M. Keserű, *MedChemComm* **2016**, *7*, 332 – 337.
[18] C. W. Murray, M. L. Verdonk, *J. Comput.-Aided Mol. Des.* **2002**, *16*, 741 – 753.
[19] W. P. Jencks, *Proc. Natl. Acad. Sci. USA* **1981**, *78*, 4046 – 4050.
[20] V. Borsi, V. Calderone, M. Fragai, C. Luchinat, N. Sarti, *J. Med. Chem.* **2010**, *53*, 4285 – 4289.
[21] I. Huc, J.-M. Lehn, *Proc. Natl. Acad. Sci. USA* **1997**, *94*, 2106 – 2110.
[22] K. C. Nicolaou, R. Hughes, S. Y. Cho, N. Winssinger, C. Smethurst, H. Labischinski, R. Endermann, *Angew. Chem. Int. Ed.* **2000**, *39*, 3823 – 3828; *Angew. Chem.* **2000**, *112*, 3981 – 3986.
[23] O. Ramström, J.-M. Lehn, *ChemBioChem* **2000**, *1*, 41 – 48.
[24] R. Nguyen, I. Huc, *Angew. Chem. Int. Ed.* **2001**, *40*, 1774 – 1776; *Angew. Chem.* **2001**, *113*, 1824 – 1826.
[25] K. B. Sharpless, R. Manetsch, *Expert Opin. Drug Discovery* **2006**, *1*, 525 – 538.
[26] X. Hu, R. Manetsch, *Chem. Soc. Rev.* **2010**, *39*, 1316 – 1324.
[27] A. J. Clipson, V. T. Bhat, I. McNae, A. M. Caniard, D. J. Campopiano, M. F. Greaney, *Chem. Eur. J.* **2012**, *18*, 10562 – 10570.
[28] R. Huang, I. Leung, *Molecules* **2016**, *21*, 910.
[29] M. Hochgürtel, H. Kroth, D. Piecha, M. W. Hofmann, C. Nicolau, S. Krause, O. Schaaf, G. Sonnenmoser, A. V. Eliseev, *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 3382 – 3387.
[30] W. G. Lewis, L. G. Green, F. Grynszpan, Z. Radić, P. R. Carlier, P. Taylor, M. G. Finn, K. B. Sharpless, *Angew. Chem. Int. Ed.* **2002**, *41*, 1053 – 1057; *Angew. Chem.* **2002**, *114*, 1095 – 1099.
[31] Y. Kubota, S. Sakamoto, K. Yamaguchi, M. Fujita, *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 4854 – 4856.
[32] W. Jahnke, A. Flörsheimer, M. J. J. Blommers, C. G. Paris, J. Heim, C. M. Nalin, L. B. Perez, *Curr. Top. Med. Chem.* **2003**, *3*, 69 – 80.
[33] R. Larsson, Z. Pei, O. Ramström, *Angew. Chem. Int. Ed.* **2004**, *43*, 3716 – 3718; *Angew. Chem.* **2004**, *116*, 3802 – 3804.
[34] C. A. Lepre, J. M. Moore, J. W. Peng, *Chem. Rev.* **2004**, *104*, 3641 – 3676.
[35] M. Angelin, R. Larsson, P. Vongvilai, O. Ramström, *J. Chem. Educ.* **2010**, *87*, 1248 – 1251.
[36] M. Mondal, N. Radeva, H. Köster, A. Park, C. Potamitis, M. Zervou, G. Klebe, A. K. H. Hirsch, *Angew. Chem. Int. Ed.* **2014**, *53*, 3259 – 3263; *Angew. Chem.* **2014**, *126*, 3324 – 3328.
[37] Y. Bourne, K. B. Sharpless, P. Taylor, P. Marchot, *J. Am. Chem. Soc.* **2016**, *138*, 1611 – 1621.
[38] T. Hirose, N. Maita, H. Gouda, J. Koseki, T. Yamamoto, A. Sugawara, H. Nakano, S. Hirono, K. Shiomi, T. Watanabe, H. Taniguchi, K. B. Sharpless, S. Omura, T. Sunazuka, *Proc. Natl. Acad. Sci. USA* **2013**, *110*, 15892 – 15897.
[39] M. Gelin, G. Poncet-Montange, L. Assairi, L. Morellato, V. Huteau, L. Dugué, O. Dussurget, S. Pochet, G. Labesse, *Structure* **2012**, *20*, 1107 – 1117.
[40] J. Yamane, N. Ohyabu, M. Yao, H. Takemoto, I. Tanaka, *J. Appl. Crystallogr.* **2010**, *43*, 1329 – 1337.
[41] M. S. Congreve, D. J. Davis, L. Devine, C. Granata, P. G. Wyatt, H. Jhoti, *Angew. Chem. Int. Ed.* **2003**, *42*, 4479 – 4482; *Angew. Chem.* **2003**, *115*, 4617 – 4620.
[42] R. E. Babine, M. J. Rynkiewicz, L. Jin, S. S. Abdel-Meguid, *Lett. Drug Des. Discovery* **2004**, *1*, 173 – 177.
[43] M. F. Schmidt, A. Isidro-Llobet, M. Lisurek, A. El-Dahshan, J. Tan, R. Hilgenfeld, J. Rademann, *Angew. Chem. Int. Ed.* **2008**, *47*, 3275 – 3278; *Angew. Chem.* **2008**, *120*, 3319 – 3323.
[44] M. I. Fernández-Bachiller, A. Horatscheck, M. Lisurek, J. Rademann, *ChemMedChem* **2013**, *8*, 1041 – 1056.
[45] M. F. Schmidt, M. R. Groves, J. Rademann, *ChemBioChem* **2011**, *12*, 2640 – 2646.
[46] E. Burda, J. Rademann, *Nat. Commun.* **2014**, *5*, 5170.
[47] M. F. Schmidt, A. El-Dahshan, S. Keller, J. Rademann, *Angew. Chem. Int. Ed.* **2009**, *48*, 6346 – 6349; *Angew. Chem.* **2009**, *121*, 6464 – 6467.
[48] M. Jaegle, T. Steinmetzer, J. Rademann, *Angew. Chem. Int. Ed.* **2017**, *56*, 3718—3722; *Angew. Chem.* **2017**, *129*, 3772—3776.
[49] E. Oueis, C. Sabot, P.-Y. Renard, *Chem. Commun.* **2015**, *51*, 12158 – 12169.
[50] A. J. R. Heck, *Nat. Methods* **2008**, *5*, 927 – 933.
[51] S.-A. Poulsen, *Aust. J. Chem.* **2013**, *66*, 1495 – 1501.
[52] E. B. Erba, C. Petosa, *Protein Sci.* **2015**, *24*, 1176 – 1192.
[53] L. Pedro, R. J. Quinn, *Molecules* **2016**, *21*, 984.
[54] J. Klages, M. Coles, H. Kessler, *Analyst* **2007**, *132*, 692 – 705.
[55] R. Larsson, O. Ramström, *Eur. J. Org. Chem.* **2006**, 285 – 291.
[56] P. Vongvilai, M. Angelin, R. Larsson, O. Ramström, *Angew. Chem. Int. Ed.* **2007**, *46*, 948 – 950; *Angew. Chem.* **2007**, *119*, 966 – 968.
[57] S. M. Turega, C. Lorenz, J. W. Sadownik, D. Philp, *Chem. Commun.* **2008**, 4076 – 4078.
[58] R. Caraballo, H. Dong, J. P. Ribeiro, J. Jiménez-Barbero, O. Ramström, *Angew. Chem. Int. Ed.* **2010**, *49*, 589 – 593; *Angew. Chem.* **2010**, *122*, 599 – 603.
[59] I. K. H. Leung, T. Brown, Jr, C. J. Schofield, T. D. W. Claridge, *MedChemComm* **2011**, *2*, 390.
[60] L. Fielding, *Curr. Top. Med. Chem.* **2003**, *3*, 39 – 53.
[61] M. Pellecchia, I. Bertini, D. Cowburn, C. Dalvit, E. Giralt, W. Jahnke, T. L. James, S. W. Homans, H. Kessler, C. Luchinat, B. Meyer, H. Oschkinat, J. Peng, H. Schwalbe, G. Siegal, *Nat. Rev. Drug Discovery* **2008**, *7*, 738 – 745.
[62] S. B. Shuker, P. J. Hajduk, R. P. Meadows, S. W. Fesik, *Science* **1996**, *274*, 1531.
[63] Z. Chilingaryan, Z. Yin, A. J. Oakley, *Int. J. Mol. Sci.* **2012**, *13*, 12857 – 12879.
[64] M. Mondal, D. E. Groothuis, A. K. H. Hirsch, *MedChemComm* **2015**, *6*, 1267 – 1271.
[65] R. Zhang, F. Monsma, *Curr. Opin. Drug Discov. Devel.* **2010**, *13*, 389 – 402.
[66] M. K. Grøftehauge, N. R. Hajizadeh, M. J. Swann, E. Pohl, *Acta Crystallogr. Sect. D* **2015**, *71*, 36 – 44.
[67] R. Perozzo, G. Folkers, L. Scapozza, *J. Recept. Signal Transduction* **2004**, *24*, 1 – 52.
[68] G. A. Holdgate, *Expert Opin. Drug Discovery* **2007**, *2*, 1103 – 1114.
[69] J. B. Chaires, *Annu. Rev. Biophys.* **2008**, *37*, 135 – 151.
[70] N. C. Garbett, J. B. Chaires, *Expert Opin. Drug Discovery* **2012**, *7*, 299 – 314.
[71] T. S. Olsson, M. A. Williams, W. R. Pitt, J. E. Ladbury, *J. Mol. Biol.* **2008**, *384*, 1002 – 1017.
[72] C. Bergsdorf, J. Ottl, *Expert Opin. Drug Discovery* **2010**, *5*, 1095 – 1107.
[73] I. Navratilova, A. L. Hopkins, *ACS Med. Chem. Lett.* **2010**, *1*, 44 – 48.
[74] T. Rogez-Florent, L. Duhamel, L. Goossens, P. Six, A. S. Drucbert, P. Depreux, P. M. Danzé, D. Landy, J. F. Goossens, C. Foulon, *J. Mol. Recognit.* **2014**, *27*, 46 – 56.
[75] T. Maki, A. Kawamura, N. Kato, J. Ohkanda, *Mol. BioSyst.* **2013**, *9*, 940 – 943.
[76] K. Tanaka, S. Shirotsuki, T. Iwata, C. Kageyama, T. Tahara, S. Nozaki, E. R. O. Siwu, S. Tamura, S. Douke, N. Murakami, H. Onoe, Y. Watanabe, K. Fukase, *ACS Chem. Biol.* **2012**, *7*, 637 – 645.
[77] P. Parvatkar, N. Kato, M. Uesugi, S.-i. Sato, J. Ohkanda, *J. Am. Chem. Soc.* **2015**, *137*, 15624 – 15627.
[78] L. You, J. S. Berman, E. V. Anslyn, *Nat. Chem.* **2011**, *3*, 943 – 948.
[79] R. Caraballo, M. Sakulsombat, O. Ramström, *ChemBioChem* **2010**, *11*, 1600 – 1606.

[80] D. Berkovich-Berger, N. G. Lemcoff, *Chem. Commun.* **2008**, 1686 – 1688.
[81] J. A. Berrocal, R. Cacciapaglia, S. Di Stefano, *Org. Biomol. Chem.* **2011**, *9*, 8190 – 8194.
[82] L. R. Sutton, W. A. Donaubauer, F. Hampel, A. Hirsch, *Chem. Commun.* **2004**, 1758 – 1759.
[83] C. Godoy-Alcántar, A. K. Yatsimirsky, J.-M. Lehn, *J. Phys. Org. Chem.* **2005**, *18*, 979 – 985.
[84] A. Herrmann, *Org. Biomol. Chem.* **2009**, *7*, 3195 – 3204.
[85] D. Becker, Z. Kaczmarska, C. Arkona, R. Schulz, C. Tauber, G. Wolber, R. Hilgenfeld, M. Coll, J. Rademann, *Nat. Commun.* **2016**, *7*, 12761.
[86] A. Dirksen, S. Dirksen, T. M. Hackeng, P. E. Dawson, *J. Am. Chem. Soc.* **2006**, *128*, 15602 – 15603.
[87] D. Larsen, M. Pittelkow, S. Karmakar, E. T. Kool, *Org. Lett.* **2015**, *17*, 274 – 277.
[88] J. B. Blanco-Canosa, I. L. Medintz, D. Farrell, H. Mattoussi, P. E. Dawson, *J. Am. Chem. Soc.* **2010**, *132*, 10027 – 10033.
[89] D. E. Prasuhn, J. B. Blanco-Canosa, G. J. Vora, J. B. Delehanty, K. Susumu, B. C. Mei, P. E. Dawson, I. L. Medintz, *ACS Nano* **2010**, *4*, 267 – 278.
[90] W. R. Algar, D. E. Prasuhn, M. H. Stewart, T. L. Jennings, J. B. Blanco-Canosa, P. E. Dawson, I. L. Medintz, *Bioconjugate Chem.* **2011**, *22*, 825 – 858.
[91] S. Ulrich, D. Boturyn, A. Marra, O. Renaudet, P. Dumy, *Chem. Eur. J.* **2014**, *20*, 34 – 41.
[92] E. M. Sletten, C. R. Bertozzi, *Angew. Chem. Int. Ed.* **2009**, *48*, 6974 – 6998; *Angew. Chem.* **2009**, *121*, 7108 – 7133.
[93] J. Kalia, R. T. Raines, *Angew. Chem. Int. Ed.* **2008**, *47*, 7523 – 7526; *Angew. Chem.* **2008**, *120*, 7633 – 7636.
[94] M. G. Simpson, M. Pittelkow, S. P. Watson, J. K. M. Sanders, *Org. Biomol. Chem.* **2010**, *8*, 1181 – 1187.
[95] M. E. Belowich, J. F. Stoddart, *Chem. Soc. Rev.* **2012**, *41*, 2003 – 2024.
[96] M. Sindelar, K. T. Wanner, *ChemMedChem* **2012**, *7*, 1678 – 1690.
[97] M. Sindelar, T. A. Lutz, M. Petrera, K. T. Wanner, *J. Med. Chem.* **2013**, *56*, 1323 – 1340.
[98] M. Mondal, N. Radeva, H. Fanlo-Virgós, S. Otto, G. Klebe, A. K. H. Hirsch, *Angew. Chem. Int. Ed.* **2016**, *55*, 9422 – 9426; *Angew. Chem.* **2016**, *128*, 9569 – 9574.
[99] V. T. Bhat, A. M. Caniard, T. Luksch, R. Brenk, D. J. Campopiano, M. F. Greaney, *Nat. Chem.* **2010**, *2*, 490 – 497.
[100] F. T. Kern, K. T. Wanner, *ChemMedChem* **2015**, *10*, 396 – 410.
[101] S. P. Black, J. K. M. Sanders, A. R. Stefankiewicz, *Chem. Soc. Rev.* **2014**, *43*, 1861 – 1872.
[102] J. Leclaire, L. Vial, S. Otto, J. K. M. Sanders, *Chem. Commun.* **2005**, 1959 – 1961.
[103] B. Rasmussen, A. Sørensen, H. Gotfredsen, M. Pittelkow, *Chem. Commun.* **2014**, *50*, 3716 – 3718.
[104] Z. Rodriguez-Docampo, S. Otto, *Chem. Commun.* **2008**, 5301 – 5303.
[105] N. Zhu, F. Zhang, G. Liu, *J. Comb. Chem.* **2010**, *12*, 531 – 540.
[106] B. Brisig, J. K. M. Sanders, S. Otto, *Angew. Chem. Int. Ed.* **2003**, *42*, 1270 – 1273; *Angew. Chem.* **2003**, *115*, 1308 – 1311.
[107] J. Atcher, I. Alfonso, *RSC Adv.* **2013**, *3*, 25605 – 25608.
[108] M. Angelin, R. Larsson, P. Vongvilai, O. Ramström, *J. Chem. Educ.* **2010**, *87*, 1248 – 1251.
[109] S. Ghosh, L. A. Ingerman, A. G. Frye, S. J. Lee, M. R. Gagné, M. L. Waters, *Org. Lett.* **2010**, *12*, 1860 – 1863.
[110] B. Shi, M. F. Greaney, *Chem. Commun.* **2005**, 886 – 888.
[111] M. Angelin, A. Fischer, O. Ramström, *J. Org. Chem.* **2008**, *73*, 3593 – 3595.
[112] W. Zhang, J. S. Moore, *J. Am. Chem. Soc.* **2005**, *127*, 11863 – 11870.
[113] L. Hunter, G. C. Condie, M. M. Harding, *Tetrahedron Lett.* **2010**, *51*, 5064 – 5067.
[114] P. Edwards, *Drug Discovery Today* **2007**, *12*, 497 – 498.
[115] S.-A. Poulsen, L. F. Bornaghi, *Bioorg. Med. Chem.* **2006**, *14*, 3275 – 3284.
[116] S. R. Beeren, J. K. M. Sanders in *Dynamic Combinatorial Chemistry* (Eds.: J. N. H. Reek, S. Otto), Wiley-VCH, Weinheim, **2010**, pp. 1 – 22.
[117] E. Oueis, F. Nachon, C. Sabot, P.-Y. Renard, *Chem. Commun.* **2014**, *50*, 2043 – 2045.
[118] D. T. S. Rijkers, R. Merkx, C.-B. Yim, A. J. Brouwer, R. M. J. Liskamp, *J. Pept. Sci.* **2010**, *16*, 1 – 5.
[119] X. Hu, J. Sun, H.-G. Wang, R. Manetsch, *J. Am. Chem. Soc.* **2008**, *130*, 13820 – 13821.
[120] S. S. Kulkarni, X. Hu, K. Doi, H.-G. Wang, R. Manetsch, *ACS Chem. Biol.* **2011**, *6*, 724 – 732.
[121] C. Peruzzotti, S. Borrelli, M. Ventura, R. Pantano, G. Fumagalli, M. S. Christodoulou, D. Monticelli, M. Luzzani, A. L. Fallacara, C. Tintori, M. Botta, D. Passarella, *ACS Med. Chem. Lett.* **2013**, *4*, 274 – 277.
[122] T. Suzuki, Y. Ota, Y. Kasuya, M. Mutsuga, Y. Kawamura, H. Tsumoto, H. Nakagawa, M. G. Finn, N. Miyata, *Angew. Chem. Int. Ed.* **2010**, *49*, 6817 – 6820; *Angew. Chem.* **2010**, *122*, 6969 – 6972.
[123] A. Krasiński, Z. Radić, R. Manetsch, J. Raushel, P. Taylor, K. B. Sharpless, H. C. Kolb, *J. Am. Chem. Soc.* **2005**, *127*, 6686 – 6692.
[124] H. C. Kolb, K. B. Sharpless, *Drug Discovery Today* **2003**, *8*, 1128 – 1137.
[125] M. Mondal, M. Y. Unver, A. Pal, M. Bakker, S. P. Berrier, A. K. H. Hirsch, *Chem. Eur. J.* **2016**, *22*, 14826 – 14830.
[126] O. Š. Miljanić, *Nat. Chem.* **2011**, *3*, 909 – 910.
[127] L. You, S. R. Long, V. M. Lynch, E. V. Anslyn, *Chem. Eur. J.* **2011**, *17*, 11017 – 11023.
[128] M. Angelin, R. Larsson, P. Vongvilai, M. Sakulsombat, O. Ramström in *Dynamic Combinatorial Chemistry in Drug Discovery, Bioorganic Chemistry, and Materials Science* (Ed.: B. L. Miller), Wiley, Hoboken, **2010**, pp. 169 – 200.
[129] M. Lisurek, B. Rupp, J. Wichard, M. Neuenschwander, J. P. von Kries, R. Frank, J. Rademann, R. Kühne, *Mol. Diversity* **2010**, *14*, 401 – 408.
[130] M. Demetriades, I. K. H. Leung, R. Chowdhury, M. C. Chan, M. A. McDonough, K. K. Yeoh, Y.-M. Tian, T. D. W. Claridge, P. J. Ratcliffe, E. C. Y. Woon, C. J. Schofield, *Angew. Chem. Int. Ed.* **2012**, *51*, 6672 – 6675; *Angew. Chem.* **2012**, *124*, 6776 – 6779.
[131] I. K. H. Leung, M. Demetriades, A. P. Hardy, C. Lejeune, T. J. Smart, A. Szöllössi, A. Kawamura, C. J. Schofield, T. D. W. Claridge, *J. Med. Chem.* **2013**, *56*, 547 – 555.
[132] D. A. Erlanson, A. C. Braisted, D. R. Raphael, M. Randal, R. M. Stroud, E. M. Gordon, J. A. Wells, *Proc. Natl. Acad. Sci. USA* **2000**, *97*, 9367 – 9372.
[133] D. A. Erlanson, J. W. Lam, C. Wiesmann, T. N. Luong, R. L. Simmons, W. L. DeLano, I. C. Choong, M. T. Burdett, W. M. Flanagan, D. Lee, E. M. Gordon, T. O'Brien, *Nat. Biotechnol.* **2003**, *21*, 308 – 314.
[134] M. T. Cancilla, M. M. He, N. Viswanathan, R. L. Simmons, M. Taylor, A. D. Fung, K. Cao, D. A. Erlanson, *Bioorg. Med. Chem. Lett.* **2008**, *18*, 3978 – 3981.
[135] D. A. Erlanson, J. W. Arndt, M. T. Cancilla, K. Cao, R. A. Elling, N. English, J. Friedman, S. K. Hansen, C. Hession, I. Joseph, G. Kumaravel, W.-C. Lee, K. E. Lind, R. S. McDowell, K. Miatkowski, C. Nguyen, T. B. Nguyen, S. Park, N. Pathan, D. M. Penny, M. J. Romanowski, D. Scott, L. Silvian, R. L. Simmons, B. T. Tangonan, W. Yang, L. Sun, *Bioorg. Med. Chem. Lett.* **2011**, *21*, 3078 – 3083.
[136] K. Fan, P. Wei, Q. Feng, S. Chen, C. Huang, L. Ma, B. Lai, J. Pei, Y. Liu, J. Chen, L. Lai, *J. Biol. Chem.* **2004**, *279*, 1637 – 1642.
[137] H. Yang, M. Yang, Y. Ding, Y. Liu, Z. Lou, Z. Zhou, L. Sun, L. Mo, S. Ye, H. Pang, G. F. Gao, K. Anand, M. Bartlam, R.

Hilgenfeld, Z. Rao, *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 13190 – 13195.
[138] M. Whiting, J. Muldoon, Y.-C. Lin, S. M. Silverman, W. Lindstrom, A. J. Olson, H. C. Kolb, M. G. Finn, K. B. Sharpless, J. H. Elder, V. V. Fokin, *Angew. Chem. Int. Ed.* **2006**, *45*, 1435 – 1439; *Angew. Chem.* **2006**, *118*, 1463 – 1467.

Manuscript received: October 23, 2016
Revised manuscript received: January 10, 2017
Accepted manuscript online: January 24, 2017
Version of record online: May 31, 2017

