Enzyme Inhibitors

In Situ Click Chemistry: Enzyme-Generated Inhibitors of Carbonic Anhydrase II**

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Traditional lead discovery approaches often rely on synthesizing and screening large numbers of compounds to identify the few inhibitor molecules that these libraries contain. In

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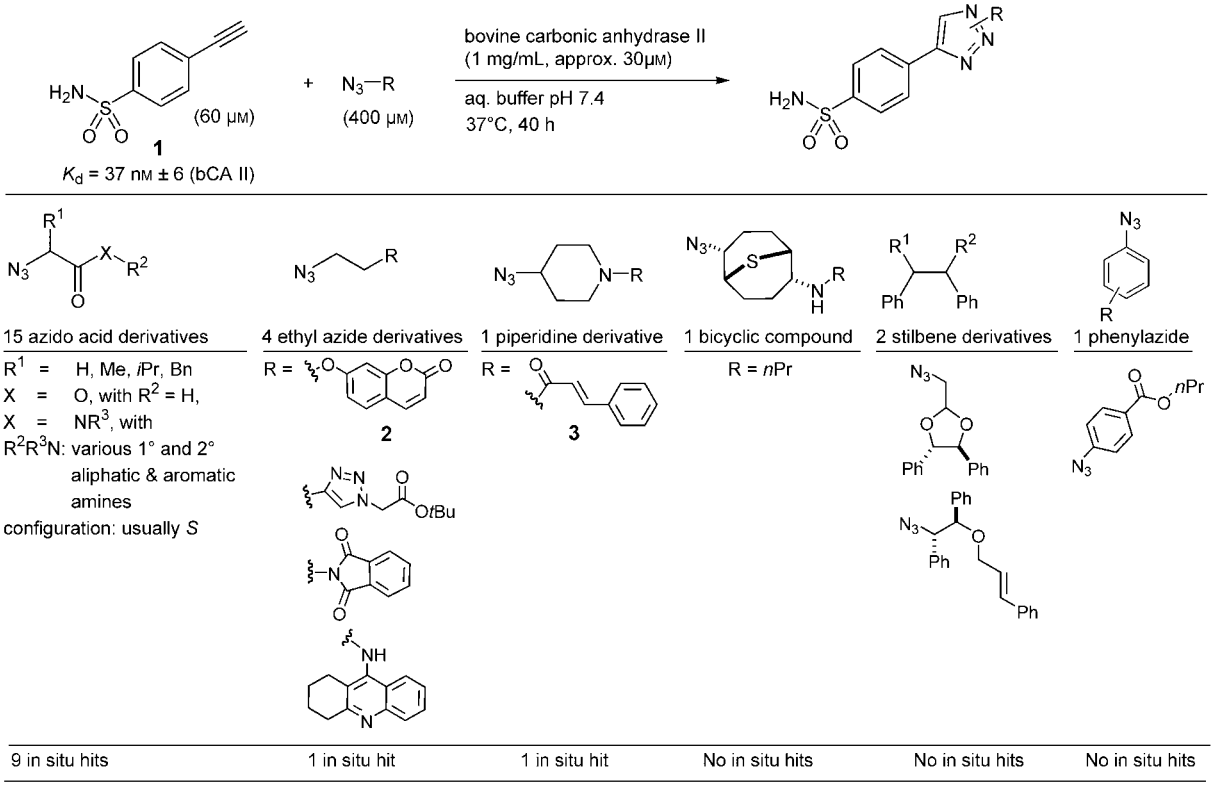
contrast, target-guided synthesis (TGS) approaches employ the target proteins themselves for assembling potent inhibitors from reactive building blocks within the confines of their binding sites. In principle, hit discovery by TGS becomes independent of the actual function of the target because it relies solely on its ability to bind reagents and hold them in close proximity until they become connected through the "arranged" chemical reaction. Since the initial report of TGS by Rideout et al. 20 years ago,[1,2] several approaches have been developed: dynamic combinatorial chemistry,[3–12] stepwise target-guided synthesis,[13,14] and kinetically controlled target-guided synthesis.[15–23] The latter approach employs irreversible reactions to assemble highly reactive reagents (strong electrophiles or nucleophiles, metathesis catalysts etc.), which can cause side reactions or even destroy the enzymic target. In contrast, the in situ click chemistry approach to TGS[15,24] employs the completely bioorthogonal [1,3]-dipolar cycloaddition reaction between azides and acetylenes which was pioneered by Rolf Huisgen.[25] This process is self-contained, hence there are no external reagents, catalysts, or byproducts that might interfere, and the reactants themselves are "invisible" in biological milieu. Most importantly, the cycloaddition reaction, which is slow at room temperature, is accelerated tremendously when azide and acetylene groups are held together in close proximity to lead to irreversible formation of triazoles.[26–28] Previously, we have employed this approach to assemble acetylcholinesterase (AChE) inhibitors with subpicomolar binding affinities from reagents that carried known active- and peripheral-site binders.[15,24]

We now report the identification of novel carbonic anhydrase (CA) inhibitors using in situ click chemistry. Carbonic anhydrases are zinc-containing enzymes that catalyze the interconversion of HCO_3^- and CO_2. They are involved in key biological processes connected with respiration and the transport of CO_2/HCO_3^-, acid secretion and pH control, bone resorption, calcification, tumorigenicity, and many others.[29,30] Systemically and topically administered carbonic anhydrase inhibitors have long been used to control the elevated intraocular pressure associated with glaucoma.[31,32] Most inhibitors are aromatic or heteroaromatic sulfonamides[30,33,34] whose anion coordinates to the Zn^{2+} ion in the active site.[35]

Inspired by the work of Whitesides and co-workers on 4-carboxybenzenesulfonamide-derived carbonic anhydrase inhibitors,[33,36–39] we selected the acetylenic benzenesulfonamide **1** as a reactive scaffold for capturing complementary azide reagents to form "divalent" CA inhibitors in situ (see Scheme 1).[40] Scaffold **1** binds to bovine carbonic anhydrase II (bCA II) with nanomolar affinity ($K_d = 37$ nM ± 6), as determined by a competition assay with 5-(dimethylamino)-1-napthalenesulfonamide (dansyl amide, DNSA).[33,41] A total of 24 azide reagents, which cover 6 different structural themes, were employed as complementary reagents for in situ click chemistry screening. α-Azido carboxamide derivatives were best represented as we intended to probe for analogues of the peptidic carboxybenzenesulfonamide-derived inhibitors reported by Whitesides.[33,36–39]

The in situ click chemistry experiments were conducted in parallel by using 96-well microtiter plates. Each well con-



Scheme 1. In situ screening protocol and reagents. Control experiments to test for false-positive results were performed under identical conditions either in the absence of enzyme or in the presence of bCA II and ethoxazolamide (200 μM; see Figure 1).

tained a mixture of bCA II (30 μM), the acetylene (60 μM), and a given azide reagent (400 μM) in phosphate buffer solution (pH 7.4).[41] The formation of the product was monitored by HPLC analysis and mass spectrometric detection through electrospray ionization and selected ion mode monitoring (LC/MS-SIM).[24] Under these conditions, over 99% of the active sites are occupied by ethynyl benzenesulfonamide (**1**), whereas the formation of triazole product from the enzyme-free background reaction remains below the LC/MS-SIM detection limit after 40 h at 37°C. In fact, the acetylene concentration can be raised to as high as 400 μM without producing detectable amounts of triazoles which demonstrates that the uncatalyzed reaction is extremely slow. Two sets of control experiments were conducted in parallel to test for false positives: Each acetylene/azide combination was incubated with mixtures of enzyme and the known active-site inhibitor ethoxazolamide (200 μM) (see Figure 1 and Table 2) and with bovine serum albumin (BSA) in place of bCA II.

Analysis of each reaction mixture by LC/MS-SIM revealed that 12 out of 24 reagent combinations had led to the formation of triazole products in the presence of the enzyme. Of these, 11 combinations did not form triazoles in the corresponding control experiments in the presence of BSA or enzyme/inhibitor, thereby validating these compounds as in situ hits and demonstrating that their formation required the enzyme active site to be accessible. Figure 1 illustrates the results for the acetylene/azide pair **1**/**4**. Most of the in situ hits are derived from α-substituted azido acid amides. The two exceptions are reagents **2** and **3**, which are azide derivatives of a coumarin and a piperidine, respectively. Conversely, 9 out of 12 azides that do not react in situ are not based on α-substituted azido acid platforms.

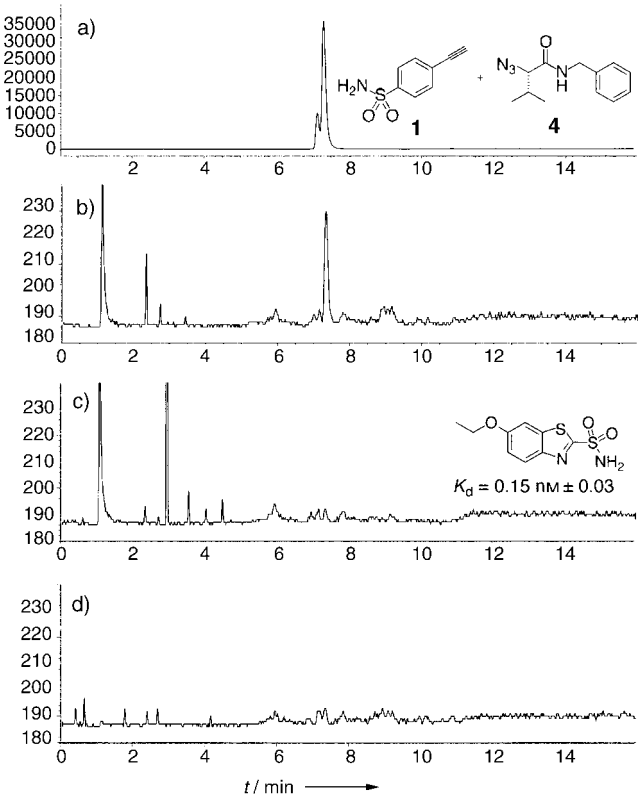


Figure 1. Results of in situ click chemistry between acetylene **1** and azide **4**, monitored by HPLC. a) Thermal reaction (85°C, 6 d): triazole products between 7.1 and 7.3 min; b) Reaction with bCA II (1 mg mL^{-1}; 37°C, 40 h): product at 7.3 min; c) bCA II (1 mg mL^{-1}), ethoxazolamide (200 μM; 37°C, 40 h); d) Bovine serum albumin (1 mg mL^{-1}) instead of bCA II (37°C, 40 h).

A comparison of the in situ reaction products with pure 1,4-disubstituted ("*anti*") triazoles, which were prepared by the Cu^{I}-catalyzed reaction of the precursor azides and acetylenes,[42,43] and *syn*/*anti*-triazoles, which were prepared by thermal cycloaddition, by LC/MS-SIM reveals the enzyme reaction to be highly *anti*-selective, in contrast to the thermal cycloaddition (see Supporting Information). The great *anti* selectivity further supports the notion that the formation of the product is enzyme controlled.

For the determination of inhibition constants at 25°C, all 24 *anti*-triazoles were synthesized from their respective acetylene/azide precursors under the copper-catalyzed reaction conditions recently developed in the laboratories of Sharpless/Fokin and Meldal.[42,43] The dissociation constants for these compounds were measured with a fluorescence-based assay,[39,44] which uses DNSA as a reporting ligand that is displaced by the test compound.[41]

Without exception, the in situ hit compounds display significantly higher binding affinities for bCA II (K_d = 0.2–7.1 nM) than the acetylene scaffold **1** (K_d = 37 nM ± 6) or the azide reagents (high micromolar affinities and above) from which they are derived (Table 1 and Table 2). These results demonstrate that the azide/acetylene in situ click chemistry can be used to produce enzyme inhibitors in which only one of the components binds well to the target. The dissociation constants of the triazoles prepared in situ compare favorably with those of the drugs acetazolamide (K_d = 6.4 nM) and ethoxazolamide (K_d = 0.15 nM; see Table 2). Of the six azide chemotypes in this initial screen (see Scheme 1), α-azido acid derived triazolyl benzenesulfonamides gave the most potent inhibitors with subnanomolar dissociation constants (Table 1). The amide residue can vary over a wide range without leaving the subnanomolar regime which suggests that the azido acid residues, and not the amide substituents, are responsible for high activity. From the reagents that were not derived from azido acids (Table 2), only the hydrophobic stilbene derivative **7** gave a product with low nanomolar activity. The other structural types were much less active: the runners-up were the coumarin and piperidine derivatives **8** and **9**, which displayed a 5 to 7.5 times higher affinity than the starting acetylene **1**.

Interestingly, six out of ten compounds that were not formed by the enzyme were also highly active with dissociation constants of less than 7.5 nM. These results demonstrate that compounds that are formed in situ are very likely to be potent inhibitors—in this case without exception—whereas the reverse is not true which leads to "false negatives" from the perspective of in situ screening. We do not regard this as a drawback because the in situ hits invariably display high binding affinities, that is, there are no "false positives". The best in situ hits (Table 1) have a 185-times higher binding

Table 1: Summary of in situ click chemistry experiments and dissociation constants of azido acid derived triazolyl benzenesulfonamides with K_d values of less than 7.5 nM.

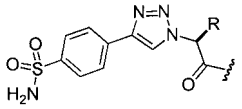
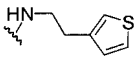
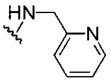
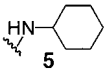
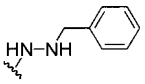
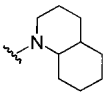
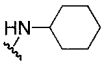
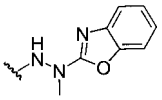
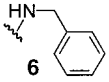
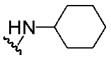
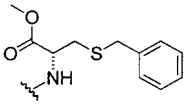
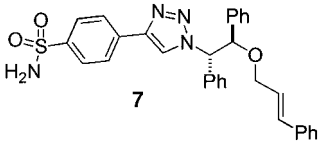
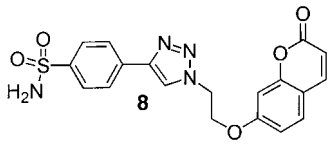
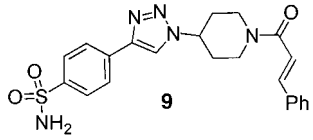
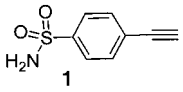
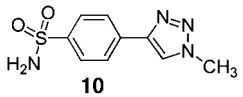
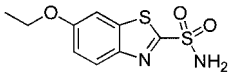
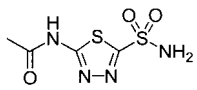
	R	In situ hit	K_d [nM]
			high affinity
	*i*Pr	Yes	0.2–0.5
	Bn	Yes	0.2–0.4
	*i*Pr	Yes	0.4–0.5
 **5**	Bn	Yes	0.5–0.9
	Bn	No	0.5 ± 0.1
	Bn	No	0.5 ± 0.1
	*i*Pr	Yes	0.8 ± 0.2
	Bn	Yes	0.9 ± 0.1
	H	No	1.1–1.4
 **6**	*i*Pr	Yes	1.9–2.3
	*i*Pr	Yes	2.4 ± 0.4
	*i*Pr	No	2.6 ± 0.2
	H	No	7.0 ± 0.5
			low affinity

Table 2: Summary of in situ click chemistry experiments and dissociation constants.

Reagent	In situ hit	K_d [nM]
 **7**	No	1.0–1.5
 **8**	Yes	4.9 ± 0.7
 **9**	Yes	7.1 ± 0.4
 **1**	N/A	37 ± 6
 **10**	N/A	38 ± 10
 ethoxazolamide	N/A	0.15 ± 0.03
 acetazolamide	N/A	6.4 ± 0.9

affinity to bCA II than the scaffold **1**, and the worst in situ hits are still more than five times as potent.

Even though the inhibitors were formed by bovine CA II, they also strongly inhibit the human enzyme (hCA II). Compounds **5** and **6** have dissociation constants of 1.2 nM (± 0.3) and 0.9 nM (± 0.2), respectively; that is, their potency is comparable to acetazolamide ($K_d = 0.9$ nM ± 0.05).[45]

Interested to what extent the binding affinities of the in situ products are influenced by the newly-formed triazole moiety, for example, by changing the pK_a value of the sulfonamide, we prepared *N*-methyltriazole **10** (Table 2). Its dissociation constant ($K_d = 38$ nM ± 10) is almost identical to that of the parent acetylene **1** which demonstrates that the triazole does not affect the binding affinity significantly and suggests that the increased affinity of the hit compounds formed in situ is largely provided by favorable binding interactions with substituents that are more remote on the azide component. Furthermore, a lack of direct triazole–protein interactions would indicate that the [3+2] cycloaddition reaction in this system is more dependent on entropic stabilization of the transition state, in contrast to the AChE system, in which the presence of strong protein–triazole attractive interactions suggests that the rate enhancement is partially due to enthalpic stabilization effects.[16] All in all, the triazole-containing linker appears to be suboptimal in the bCA II system as the observed dissociation constants are much larger than the theoretical optimum of approximately 50 pM, which is calculated based on the known affinities of scaffold **1** ($K_d = 37$ nM) and the azide reagents (high micromolar to low millimolar) and assumes the complete absence of positive or negative linker effects.[46] We are currently optimizing the linker with the aid of in situ click chemistry and investigating the ligand/protein interactions on a molecular level by X-ray crystallography.

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[45] The fluorometric assay was performed as described in the Supporting Information for bovine CA II. The dissociation constant K_d of DNSA and human CA II was determined to be 0.43 μM by titration of hCA II with DNSA.
[46] If it is assumed that the linker, that is, the triazole, does not participate in any way in binding, the free energy of binding for the divalent inhibitor can be calculated as the sum of the free energies of the individual components which in turn provides approximate values for the corresponding dissociation constant (see Ref. [39]).

