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iSPAAC: isomer-free generation of a Bcl-x_L-inhibitor in living cells

Christian Lis[a], Stefan Rubner[a], Corinna Gröst[a], Ralf Hoffmann[b], Daniel Knappe[b], Thorsten Berg* [a]

Abstract: Strain-promoted azide-alkyne cycloadditions (SPAAC) have proven extremely useful for labeling of biomolecules, but typically produce isomeric mixtures. This is not appropriate for the formation of bioactive molecules in living cells. Here, we report the first use of SPAAC for the isomer-free synthesis of a bioactive molecule, both *in vitro* and inside cultured cells. We developed the symmetrical cyclooctyne SYPCO and used it for the generation of a chemically uniform triazole inhibitor of protein-protein interactions mediated by Bcl-x_L via isomer-free SPAAC (iSPAAC). Tumor cells treated with the reactants of the iSPAAC reaction contained higher concentrations of triazole, and displayed higher apoptosis levels, than cells treated with pre-synthesized triazole. We envision iSPAAC as a broadly applicable method for modulating intracellular targets with organic molecules with molecular weights prohibitively large for cellular uptake, via smaller and thus more cell-permeable components.

Strain-promoted azide-alkyne cycloadditions (SPAAC) have proved extremely useful for bioorthogonal chemistry. While the reaction between cyclooctyne and phenyl azide was first described more than half a century ago,[1] the recent application of SPAAC by Bertozzi's group[2] for the detection of cell-surface azido sugars via a biotinylated cyclooctyne has opened up a new research field. A number of strained cycloalkynes have been developed for the labeling of biomolecules with fluorophores and other reporter molecules in living cells.[3] While SPAAC obviates the need for the toxic copper ions required for copper-catalyzed azide-alkyne cycloadditions (CuAAC),[4] a significant drawback of SPAAC is the lack of a preferred relative orientation of the azide and the cycloalkyne in the transition state. SPAAC with unsymmetrical cycloalkynes generates regioisomers, typically in similar ratios[2, 5], while SPAAC with the symmetrical cycloalkyne BCN[6] generates stereoisomers (Figure 1a). While this inherent lack of regio- or stereoselectivity is typically considered to be less relevant for the labeling of biomolecules, it precludes the use of SPAAC for the intracellular synthesis of bioactive molecules. Regio- and stereoisomers of bioactive organic molecules generally have substantially different binding affinities for their biological targets, and may even display affinities for different targets altogether; therefore, the interpretation of cell-based data generated with isomeric mixtures is difficult.

We recently presented the pyrrolocyclooctyne PYRROC as the first cycloalkyne that, in the reaction with azides, forms triazoles in an isomer-free manner (Figure 1b).[7] Isomer-free strain-promoted azide-alkyne cycloaddition (iSPAAC) by pyrrolocyclooctynes is achieved by placement of the substituent R^1, which is required for functionalization, at the nitrogen atom of the pyrrole ring annulated at the 5,6-positions of the cyclooctyne. Substituents at the α-positions of the pyrrole have to be placed symmetrically. iSPAAC is anticipated to enable the intracellular generation of bioactive molecules, whose molecular weight is prohibitively large for cellular uptake, by two smaller and thus more cell-permeable building blocks (Figure 1c).[8] Intracellular assembly of bioactive molecules by iSPAAC would help to reconcile the requirement of organic modulators of protein-protein interactions for a higher molecular weight in order to target large protein-protein interfaces effectively,[9] with the superior cell permeability and/or oral bioavailability of smaller compounds.[10] Unfortunately, the sterically demanding *tert*-butyl groups of PYRROC precluded its use for creating bioactive molecules by iSPAAC. In addition, the conformational flexibility conferred by the alkyl linker on the pyrrole nitrogen of PYRROC is typically not beneficial for placement of protein-binding substituents.

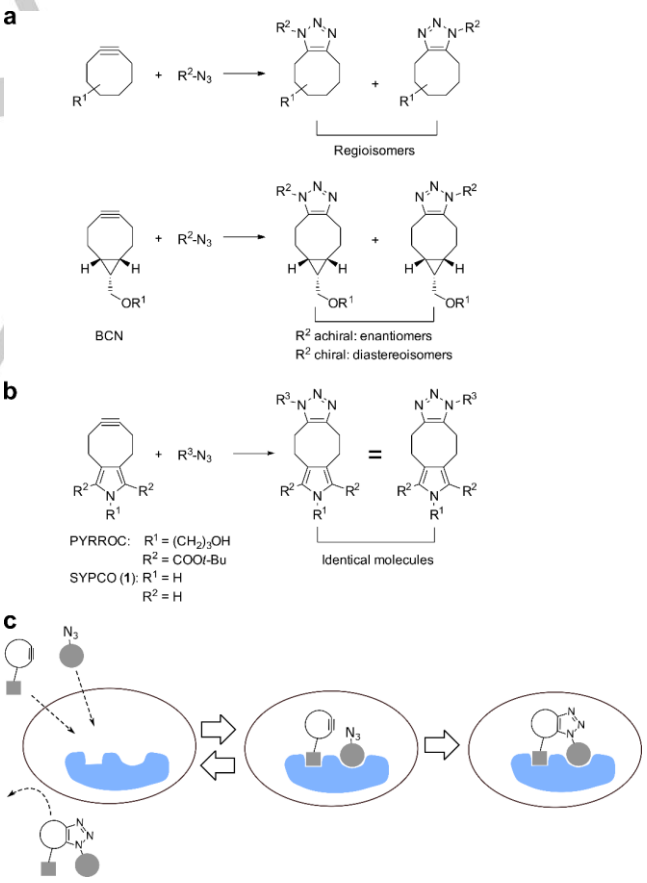

Figure 1. SPAAC between strained cycloalkynes and azides. a) SPAAC with non-symmetrical cyclooctynes generates regioisomers; SPAAC with BCN is expected to generate stereoisomers. b) Isomer-free SPAAC with symmetrical pyrrolocyclooctynes. c) Concept of intracellular formation of large bioactive ligands from two smaller fragments by iSPAAC.

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To facilitate isomer-free generation of bioactive molecules by iSPAAC, we designed and synthesized the symmetrical pyrrolocyclooctyne SYPCO (**1**, Figure 2a). SYPCO, like PYRROC,[7] does not form isomers in the reaction with azides (Figure 1b). However, unlike PYRROC, SYPCO does not display sterically demanding substituents in the α-positions of the pyrrole ring, and allows for direct placement of the functional substituent R^1 on the pyrrole nitrogen. Synthesis of SYPCO started with the pyrrolocyclooctene **2**, which can be prepared on a multigram scale as previously described.[7] Reduction of its pyrrole moiety with $NaCNBH_3$ [11] and re-installation of the *tert*-butoxycarbonyl (Boc) group afforded the 3-pyrroline **3**, which was tetrabrominated to yield **4**. Treatment with KO^{t}Bu in THF recreated the pyrrole system in the vinyl bromide **5**. A second elimination step with KO^{t}Bu in diethylether / *n*-hexane and catalytic amounts of 18-crown-6 provided SYPCO (**1**) in 5.5 % total yield over 5 synthetic steps based on **2**.

SYPCO (**1**) displayed a second-order rate constant of 0.020 ± 0.004 $M^{-1}s^{-1}$ in the reaction with benzyl azide in acetonitrile as analyzed by ^{1}H-NMR (Figure S1 in the Supporting Information), which is three-fold lower than the rate constant of PYRROC (k = 0.060 ± 0.004 $M^{-1}s^{-1}$).[7] A drop in rate constant could be advantageous in this context, increasing the likelihood that the reactants cross the cell membrane before cycloaddition takes place. Only one peak was observed for the benzylic protons of the resulting triazole, confirming the absence of regioisomers. Further support for isomer-free triazole formation in SPAAC with SYPCO (**1**) arises from its reaction with a chiral azide derived from *N-tert*-butoxycarbonyl protected L-alanine, which showed only one singlet for the *tert*-butyl group in the ^{1}H-NMR spectrum (Figure S1 in the Supporting Information). In contrast, the *tert*-butyl group of the triazole obtained by reacting the same chiral azide with BCN (Figure 1a) accounts for two singlets in the ^{1}H-NMR spectrum, consistent with the formation of diastereoisomers (Figure S1 in the Supporting Information).

ABT-737 (Figure 2b) is a potent inhibitor of the protein-protein interactions mediated by Bcl-x_L and other anti-apoptotic Bcl-2 family proteins.[12] It binds to the BH3 domain of Bcl-x_L over an extended surface area and has a high molecular weight of 813 g/mol. While it is still cell-permeable in tissue culture, its low oral bioavailability has precluded its further development as an anti-tumor agent.[13] We chose ABT-737 as a model system to explore the concept of creating a large triazole inhibitor from two smaller components, i.e. a functionalized cycloalkyne and an azide, *in vitro* and in living cells (Figure 1c), and to assess whether assembly of the inhibitor by iSPAAC increases the intracellular concentration as compared to treatment of cells with pre-synthesized triazole. We chose to replace the central *N*-phenyl piperazine moiety of ABT-737 by the fused ring system created by the SPAAC reaction with SYPCO, resulting in the design of the ABT-737 mimetic **6** (Figure 2b). Functionalization of SYPCO (**1**) with the 4-chloro biphenyl group required for **6** was achieved by deprotonation of the pyrrole nitrogen of **1** and subsequent acylation with the methyl ester **7** to afford **8** (Figure 2c). The azide **9** required for the generation of **6** was synthesized from sulfonamide **9h**, which was obtained in a 8-step synthesis in a modified procedure for the acylsulfonamide part of ABT-737 (Figure 2c, Scheme S1 in the Supporting Information).[14] iSPAAC between **8** and **9** afforded the ABT-737 mimetic **6** (Figure 2b,c).

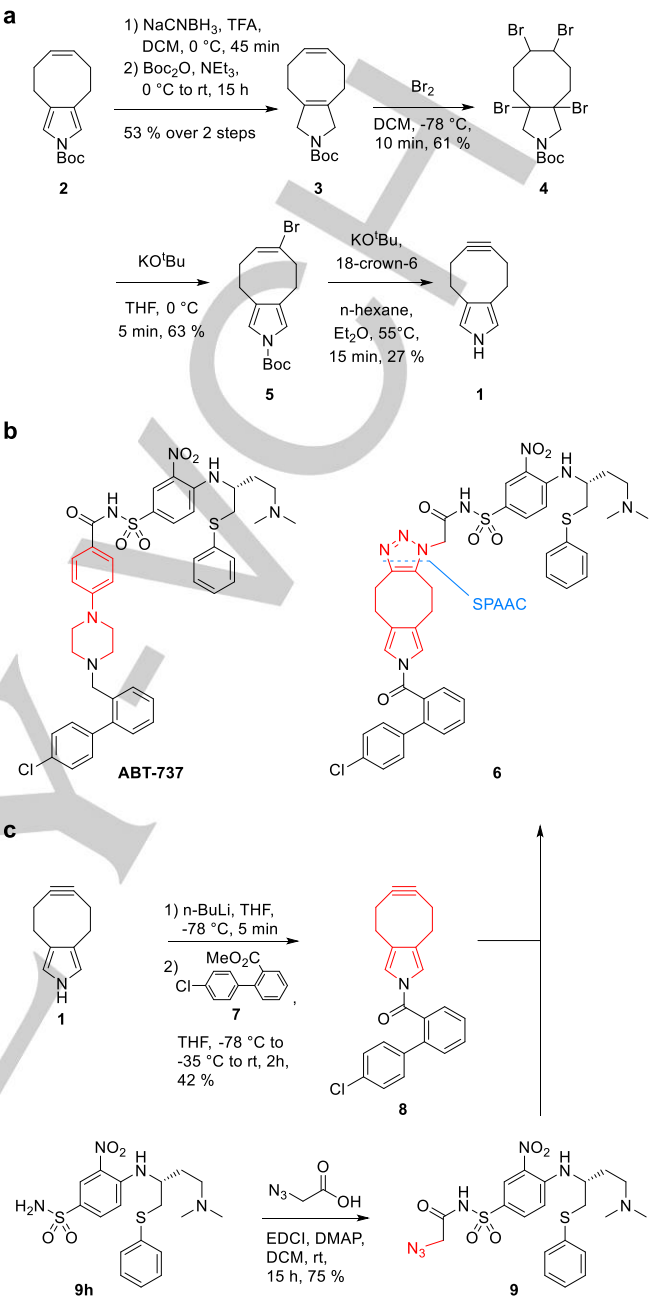

Figure 2. SYPCO facilitates the rational design of a Bcl-x_L inhibitor by iSPAAC. a) Synthesis of SYPCO (**1**). b) Structures of ABT-737 and its triazole-based mimetic **6**. c) Synthesis of the iSPAAC reactants **8** and **9**.

Docking of **6** into the co-crystal structure of the Bcl-x_L/ABT-737 complex[15] with AutoDock Vina[16] indicated a very similar binding mode to that of ABT-737 (Figure 3a). **6** was found to inhibit the interaction between Bcl-x_L and the Bak BH3 domain with an IC_{50} of 37.7 ± 3.5 µM as analyzed in a competitive binding assay based on fluorescence polarization (Figure 3b). The azide **9** and, especially, the cycloalkyne **8**, displayed significantly weaker effects (Figure 3b). In order to investigate whether iSPAAC between **8** and **9** is templated by Bcl-x_L, we analyzed the reaction rate in the absence or presence of Bcl-x_L by reversed phase (RP)-HPLC. The second-order rate constant at room

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temperature in the absence of protein was determined as 1.14 ± 0.19 M^{-1} s^{-1} (Figure 3c, Figure S2 in the Supporting Information), which represents a 50 to 60-fold rate acceleration as compared to the reaction of **1** with benzyl azide in acetonitrile (k = 0.020 ± 0.004 $M^{-1}s^{-1}$, Figure S1 in the Supporting Information). Previous reports of rate accelerations in SPAAC caused by shifting from a purely organic solvent to a mixture of organic solvent and water have involved up to 28-fold increases,[3b, 5b, 5c, 17] while rate accelerations exceeding 100-fold have been predicted for changing from an entirely organic solvent to an entirely aqueous environment.[17] Our previous work also revealed a dramatically accelerated conversion of an identical pair of SPAAC reactants by changing the solvent from acetonitrile to an entirely aqueous buffer.[7] Nevertheless, it is also conceivable that the higher rate of reaction between **8** and **9**, as compared to the reaction between **1** and benzyl azide, is partially caused by the electronic differences between the reagents.[18] As expected, triazole **6** eluted as a single peak in the HPLC (Figure S2 in the Supporting Information), consistent with its isomer-free formation from **8** and the chiral azide **9**.

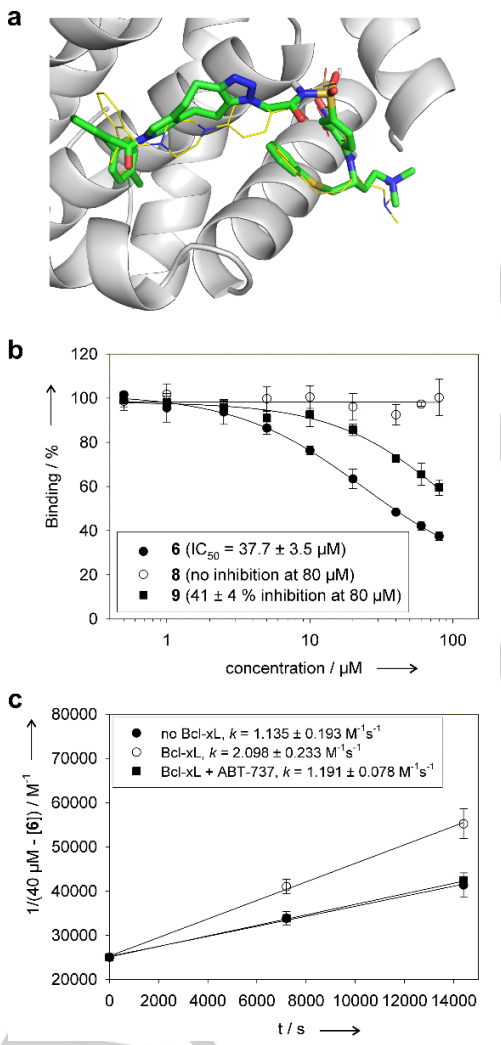

Figure 3. a) Docking of **6** into the ABT-737-binding pocket of Bcl-x_L. The position of ABT-737 (yellow) in the co-crystal structure (PBD ID 2YXJ)[15] is shown for comparison. The figure was created with PyMol.[19] b) Effect of **6**, **8**, and **9** on binding of a fluorescent-labeled Bak-derived peptide to Bcl-x_L analyzed by fluorescence polarization (n = 3). c) RP-HPLC analysis of the reaction between **8** and **9** in the absence of Bcl-x_L (n=3), in the presence of Bcl-x_L (n=4), and in the presence of both Bcl-x_L and ABT-737 (n = 3).

In the presence of Bcl-x_L, the rate constant for formation of **6** (k = 2.10 ± 0.23 M^{-1} s^{-1}, Figure 3c, Figure S2 in the Supporting Information) was significantly higher than in the absence of Bcl-x_L [p = 0.0044 (t-test, two-tailed, unpaired)]. To exclude the possibility that the increased rate constant in the presence of protein was caused by non-specific effects, we also analyzed the rate in the presence of both Bcl-x_L and ABT-737, which occupies the Bcl-x_L BH3 domain.[15] Under these conditions, the rate constant was virtually the same as in the absence of protein (k = 1.19 ± 0.08 $M^{-1}s^{-1}$, Figure 3c, Figure S2 in the Supporting Information). These data indicate that iSPAAC between **8** and **9** is templated by the BH3 domain of Bcl-x_L, an effect which is expected to support formation of **6** by intracellular iSPAAC (Figure 1c). Although the observed template effect is weak, it opens up the possibility of creating more active derivatives of **6** by kinetic target-guided synthesis.[20] iSPAAC between **8** and **9** at the physiological temperature of 37 °C using 40 µM or 80 µM of reactants in the absence of Bcl-x_L provided the triazole **6** in yields of 64 ± 9 % and 84 ± 5 % respectively after 24 h (Figure S2 in the Supporting Information).

Inhibition of Bcl-x_L by ABT-737 has been reported to induce apoptosis in K562 human leukemia cells.[21] Similarly, K562 cells treated with pre-synthesized **6** at concentrations of 40 and 80 µM resulted in dose-dependent induction of apoptosis (Figure 4a, Figure S3 in the Supporting Information). Treatment of K562 cells with an equimolar mixture of cycloalkyne **8** and azide **9** (at 40 µM and 80 µM each) for 24 h resulted in a higher induction of apoptosis than treatment of cells with pre-synthesized **6** at the same concentration. Cells treated with the mixture of **8** and **9** at 40 µM showed the same degree of apoptosis as cells treated with pre-synthesized **6** at 80 µM. Either **8** or **9** alone did not significantly increase the apoptotic rate, excluding the possibility that the induction of apoptosis by the mixture of **8** and **9** was caused by the individual iSPAAC components. While further biological studies would be required to validate the Bcl-x_L-dependency of the induction of apoptosis in leukemia cells by **6**, the data are consistent with the notion that, at the same molarity, the concentration of **6** inside K562 cells is higher in cells treated with a mixture of **8** and **9** than in cells treated with pre-synthesized triazole **6**.

To investigate this possibility further, K562 cells were treated with either the mixture of **8** and **9**, or with pre-synthesized **6** (all compounds at 80 µM) for 12 h. This shorter incubation period was chosen in order to reduce the degree of membrane instability and apoptotic cell death induced by **6**, which would hamper data analysis. Subsequently, cells were lysed, and the intracellular content of **6** was analyzed via RP-HPLC. Lysates of cells treated with the mixture of **8** and **9** contained 2.2-fold higher concentrations of **6** than lysates of cells treated with pre-synthesized **6,** in parallel experiments carried out at the same time under otherwise identical conditions (p = 0.004, Fig. 4b, c). The chemical identity of **6** in lysates from cells treated with **8** and **9** was confirmed by RP-HPLC coupled mass spectrometric (MS) analysis (Fig. S4 in the Supporting Information). The more than

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two-fold higher concentrations of **6** found in cells treated with the reactants **8** and **9**, compared to cells treated with pre-synthesized **6**, indicate that over half of the molecules of **6** in lysates of cells treated with the mixture of **8** and **9** must be formed by intracellular iSPAAC. In the hypothetical scenario where **8** and **9** undergo iSPAAC exclusively in the tissue culture medium, with a 100 % yield, the maximum possible intracellular concentration of **6** would be equal to that observed in cells treated with pre-synthesized **6**. The results of the cell-based assays can only be explained by the reactants **8** and **9** having a superior cell permeability as compared to **6**, with a major proportion of iSPAAC occuring inside the cell.

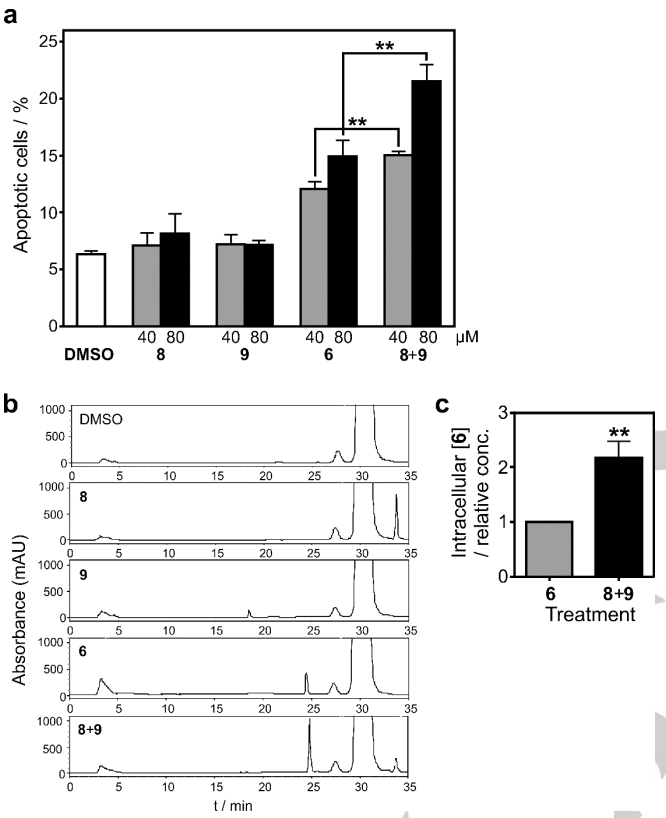

Figure 4. a) Rate of apoptosis in K562 cells treated with the individual SPAAC reactants **8** or **9**, the ABT-737 mimetic **6**, or a mixture of **8** and **9** (n = 3). Error bars represent standard deviations (SD). ** p < 0.01 (t-test, two-tailed, paired). b) HPLC analysis of lysates from cells treated with either **8**, **9**, **6**, or a mixture of **8** and **9** at 80 μM of each component. c) Relative concentrations of **6** in lysates from cells treated with either pre-synthesized **6** (80 μM) or a mixture of the iSPAAC reactants **8** and **9** (80 μM each) in parallel experiments (n = 4). Error bars represent standard deviations (SD). **p < 0.01 (t-test, two-tailed, paired).

In summary, we present iSPAAC as a method by which to form large bioactive molecules from two smaller components in cells in an isomer-free manner. We demonstrate the application of this method by using the symmetrical cycloalkyne SYPCO (**1**) to generate a rationally designed inhibitor of protein-protein interactions mediated by Bcl-x_L, both *in vitro* and in living cells, via isomer-free SPAAC. To our knowledge, our data represent the first isomer-free generation of a bioactive molecule by SPAAC.

The first application of SPAAC to living systems by the Bertozzi group[2] triggered a large number of research studies in bioorthogonal chemistry, leading to today's vastly expanded possibilities to label biomolecules in living cells. The isomer-free variant iSPAAC, using symmetrical strained cycloalkynes such as SYPCO, could similarly become widely used for the intracellular generation of bioactive molecules with higher molecular weights, via smaller and thus more cell-permeable components. Potential applications of this methodology include the isomer-free, intracellular synthesis of large bioactive molecules such as protein-protein interaction inhibitors, protein dimerizers,[22], bivalent ligands that target two separate domains within the same protein,[23] and PROTACS consisting of both a ligand of E3 ubiquitin ligases and of the protein targeted for degradation.[24] Since the concept of iSPAAC doubles most of the cut-off parameters defined by the Lipinski rules for orally bioavailable drugs, it may enable fundamentally new avenues in the development of drugs against challenging molecular targets.[10] Future challenges will involve the development of iSPAAC reagents with optimized steric demands, electronic properties, and inherent reactivities for the respective applications.

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Keywords: apoptosis • bioorthogonal chemistry • cyclooctynes • inhibitors • protein-protein interactions

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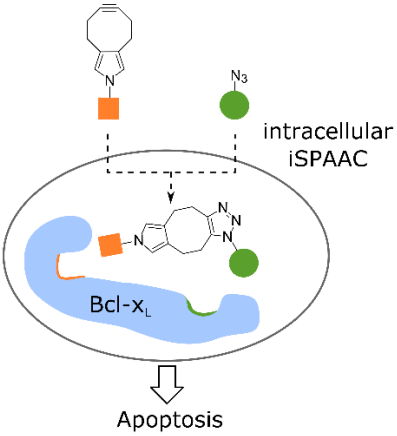
COMMUNICATION

Isomer-free zone: The formation of isomers in strain-promoted azide-alkyne cycloadditions (SPAAC) has precluded their use for the formation of bioactive molecules in living cells. Here, we present the first isomer-free generation of a bioactive molecule by SPAAC. The cycloalkyne SYPCO facilitated the intracellular synthesis of a rationally designed inhibitor of protein-protein interactions mediated by Bcl-x_L via isomer-free SPAAC (iSPAAC).



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