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Water-soluble, stable and azide-reactive strained dialkynes for biocompatible double strain-promoted click chemistry†

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The Sondheimer dialkyne is extensively used in double strain-promoted azide–alkyne cycloadditions. This reagent suffers with poor water-solubility and rapidly decomposes in aqueous solutions. This intrinsically limits its application in biological systems, and no effective solutions are currently available. Herein, we report the development of novel highly water-soluble, stable, and azide-reactive strained dialkyne reagents. To demonstrate their extensive utility, we applied our novel dialkynes to a double strain-promoted macrocyclisation strategy to generate functionalised p53-based stapled peptides for inhibiting the oncogenic p53-MDM2 interaction. These functionalised stapled peptides bind MDM2 with low nanomolar affinity and show p53 activation in a cellular environment. Overall, our highly soluble, stable and azide-reactive dialkynes offer significant advantages over the currently used Sondheimer dialkyne, and could be utilised for numerous biological applications.

Introduction

Strain-promoted azide–alkyne cycloaddition (SPAAC) reactions are widely employed for covalent modification of biomolecules in living systems.[1] These utilise a high-energy, strained cyclooctyne molecule as the reaction driving force, thus avoiding the use of cytotoxic copper catalysts employed in copper(I)-catalysed azide–alkyne cycloadditions (CuAAC).[2–4]

A double SPAAC has been developed using Sondheimer dialkyne **1**, which contains two strained alkyne moieties both able to undergo SPAAC reactions (Fig. 1a).[5,6] The use of a double-strained dialkyne reagent enables efficient conjugation of two azide containing molecules in a metal-free environment. The Sondheimer dialkyne has been used for a range of applications, including the macrocyclisation of bis-azide functionalised peptides,[7–12] chemical modification of azido-proteins in cells,[5] post-synthetic modification of metal–organic framework

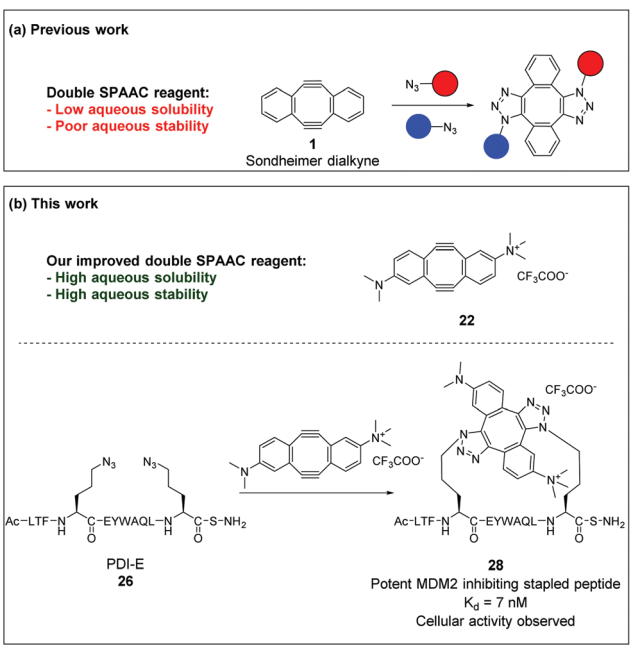

Fig. 1 (a) Previous work: development of the Sondheimer dialkyne for double strain-promoted azide–alkyne cycloaddition (SPAAC). (b) This work: development of a highly water-soluble and stable, strained dialkyne reagent and an example of its application in the generation of p53-MDM2 inhibiting stapled peptides.

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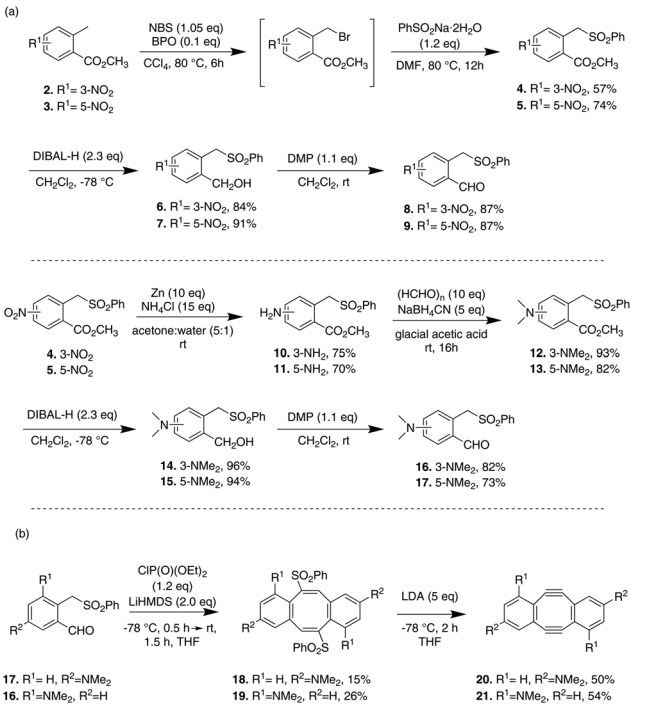
thin films,[13] tetramerisation of HIV-related peptides,[14] and for the preparation of fluorescent dyes.[15] Notably, these dialkynes are fundamentally limited in their biological application due to inherently poor water-solubility and aqueous stability ($\tau_{1/2} \sim$ 10 min at pH = 7.4).[16,17]

Following the introduction of SPAAC there has been a considerable amount of research towards the development of new water-soluble, stable, and azide-reactive strained cyclooctyne based reagents. Highly reactive strained alkyne reagents allow probing of fast biological processes and enable reactions in biological systems at low reagent concentrations. As a result, numerous derivatives of strained cyclooctyne molecules which exhibit either improved water-solubility, stability or azide-reactivity have been synthesised and applied to SPAAC *in vitro* and *in vivo*.[18–21] However, research has been less successful towards the development of water-soluble, stable and azide-reactive dialkyne reagents, despite their extensive utility.[22]

In this work, we report the synthesis of water-soluble, stable, and azide-reactive strained dialkyne reagents which address these limitations. *Meta*-trimethylammonium substituted Sondheimer dialkyne **22** exhibited high water-solubility, excellent stability and high azide-reactivity (Fig. 1b). Finally, these dialkynes were applied to a double strain-promoted macrocyclisation strategy[7] to generate functionalised p53-based stapled peptides which inhibit the oncogenic p53-MDM2 interaction (Fig. 1b).



Scheme 1 (a) Synthesis of benzyl sulfone substituted aldehyde substrates. (b) Synthesis of substituted dialkynes.

Results and discussion

Novel nitro and amine substituted dialkynes were designed to improve the physical characteristics of these reagents. The substitutions were made at the *ortho*- and *meta*-positions relative to the alkyne moiety based on Orita's synthetic route.[22,23] The *ortho*-nitro and *meta*-nitro substituted sulfones **8** and **9** were synthesised by subjecting commercially available nitro substituted arenes **2** and **3** to Wohl–Ziegler bromination, followed by nucleophilic substitution with benzenesulfinic acid sodium salt (Scheme 1a). The resulting benzyl sulfones **4** and **5** were subjected to DIBAL-H reduction followed by Dess–Martin oxidation to deliver the desired benzyl sulfone aldehyde substrates **8** and **9**. Reduction of the nitro-group in sulfones **4** and **5**, followed by reductive methylation afforded dimethyl substituted anilines **12** and **13**.[24,25] DIBAL-H reduction followed by Dess–Martin oxidation[26] gave the desired *ortho*- and *meta*-dimethylamine substituted substrates **16** and **17**.

Substrates **16** and **17** were then transformed to the respective cyclic diacetylenes through double elimination.[23] These substrates were then subjected to Wittig–Horner conditions in the presence of LiHMDS and diethyl chlorophosphate to form the cyclised intermediates **18** and **19** in 15 and 26% respective yields, which were finally treated with LDA to eliminate the two phenylsulfonyl groups, affording the desired substituted strained dialkynes **20** and **21** in moderate yields (Scheme 1b). When this protocol was applied to nitro-substituted aldehydes **8** and **9** the Wittig–Horner conditions were unsuccessful, and no product was formed.

To further improve their physical properties, dimethylamine substituted dialkynes **20** and **21** were treated with methyl trifluoromethanesulfonate in dichloromethane to give trimethylammonium substituted dialkynes **22** and **24** (see ESI 2.1† for details).[28] The dialkynes **22** and **24** were further methylated in acetonitrile to deliver bis-ammonium dialkynes **23** and **25**. Gratifyingly, all of the trimethylammonium substituted dialkynes **22–25** were found to be readily soluble in water thereby overcoming this limitation. Water-solubility of *meta*-substituted bis-ammonium dialkyne **23** was determined experimentally by measuring its octanol–water partition coefficient using the shake-flask method,[29] which demonstrated **23** ($\log P = -1.50$) to be more soluble than the uncharged dialkyne **20** ($\log P = 1.11$), which was consistent with predicted solubility trends (Fig. 2).

Next, the aqueous stability of these highly water-soluble trimethylammonium substituted dialkynes **22** and **23** was investigated. Dialkynes **22** and **23** were subjected to aqueous buffer conditions (1 mM in PBS, pH 7.4, r.t.) and decomposition was monitored by UV spectroscopy (see ESI 2.3† for details). We observed no decomposition of **22** nor **23** even after 7 days. Under the same conditions, Sondheimer dialkyne **1** is known to have a decomposition half-life of 10 ± 2 minutes.[16] Moreover, **23** when stored neat at r.t. did not show any decomposition after two weeks, whereas the Sondheimer dialkyne completely decomposes within two days.[16] We believe that these dialkynes will exhibit similar stability to thiols as Sondheimer dialkyne.

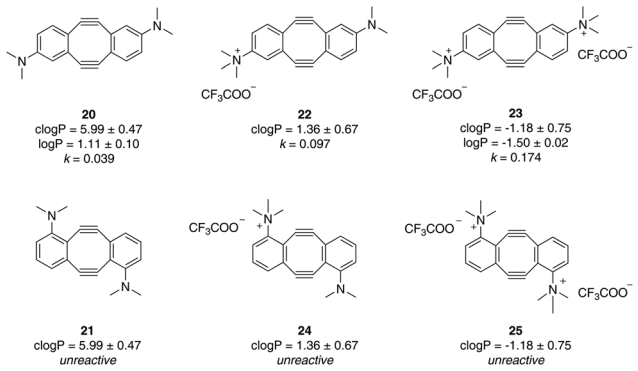
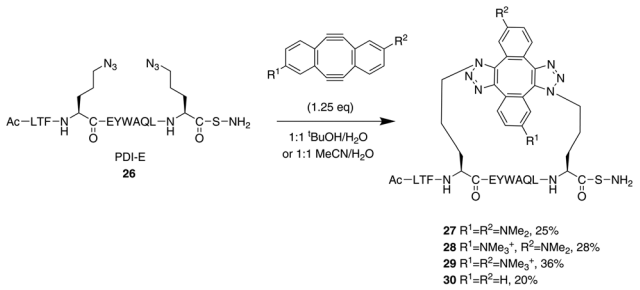


Fig. 2 Predicted (clog *P*) water-solubility values of substituted dialkynes **20–25**.[27] Experimentally determined (log *P*) values and second-order rate constants (*k*, in M^{-1} s^{-1}) indicated below.



Scheme 2 Application of substituted Sondheimer dialkynes in double strain-promoted macrocyclisation strategy to generate functionalised stapled peptides for p53-MDM2 inhibition. The yields depicted are the isolated yields of the major fraction of the stapled peptides obtained after HPLC purification. The major isomer of functionalised stapled peptides separated by HPLC purification were tested for biological activity.

We also investigated the azide-reactivity of these amine substituted dialkynes **20–25** in a SPAAC, with benzyl azide as a model substrate in methanol. We studied the reaction kinetics by monitoring the consumption of dialkynes **20–25** using UV spectroscopy and measured their second-order rate constants (see ESI 2.10† for details). The second-order rate constants determined experimentally are depicted in Fig. 2. We observed that the substitution of a single dimethylamine substituent at the *meta*-position in **20** with a charged trimethylammonium substituent (**22**, 0.097 M^{-1} s^{-1}) led to a 2.5-fold increase in the rate when compared to **20**. Installation of another trimethylammonium substituent at *meta*-position in **22** (**23**, 0.174 M^{-1} s^{-1}) pleasingly led to a further 1.8-fold increase in the rate over dialkyne **22**. However, presence of a dimethylamine or trimethylammonium substituent at the *ortho*-position (**21**, **24**, and **25**) led to a significant decrease in the rate of reaction, making them essentially unreactive towards benzyl azide at rt (see ESI Fig. S2.10†). Overall, trimethylammonium substituted **22** and **23** were found to be the most reactive dialkynes. We believe that the high azide-reactivity of **22** and **23** stems from the electronic effect of the charged trimethylammonium substituent which potentially lowers the transition state barrier required for SPAAC with azide, as has been reported previously with BARAC in the presence of a fluoro substituent.[30] The poor reactivity of *ortho*-substituted **21**, **24**, and **25** is attributed to the steric effects of bulky dimethylamine/trimethylammonium substituent.

With substituted Sondheimer dialkynes **20–25** in hand, we investigated their application in stapling phage display peptide PDI-E **26** (Ac-LTF**X**EYWAQL**X**S-NH_2, **X** = Orn(N_3)) for targeting the oncogenic p53-MDM2 interaction.[31,32] The p53 protein is a transcription factor and tumour suppressor. Its activity is inhibited by interaction with the E3 ligase MDM2, which is overexpressed in many cancers and targets p53 for proteasome-mediated degradation.[33] The stapling of peptide **26** with Sondheimer dialkyne **1** has been previously shown by our group to be a potent inhibitor of the p53-MDM2 interaction.[7] Stapling reactions between peptide **26** and dialkynes **20**, **22**, and **23** successfully gave the stapled peptides **27–29** in 25–36% yields within 24 h (Scheme 2).[34] *ortho*-Substituted dialkynes **21**, **24**, and **25** were found to be unreactive under the reaction conditions.

Functionalised stapled peptides **27–29** were then tested in a competitive fluorescence polarisation (FP)[35,36] assay for MDM2 binding affinity (see ESI 2.8† for details). Stapled peptide **28** which contained one trimethylammonium substituent, was found to exhibit the highest binding affinity (7.0 ± 3.9 nM). Installation of a second trimethylammonium group on the dialkyne caused a slight reduction in the binding affinity (**29**, 17.0 ± 6.2 nM).

A crystal structure of MDM2 complexed with *meta*-trimethylammonium functionalised peptide **28** was obtained to help determine whether the charged trimethylammonium group interacts with the protein (17-108, E69A/K70A;[37] Fig. 3). Previous studies have shown that the staple moiety of the peptide can interact with the protein.[7,38–41] The MDM2-bound stapled peptide **28** was shown to be in an α-helical conformation as expected, with the binding triad (F3, W7, L10) in the correct orientation to engage the respective binding hotspots on MDM2 (Fig. 3: Top view).[7] Two MDM2-bound peptide molecules were observed in one asymmetric unit, with the staple moiety shifted away from the MDM2 surface due to the charged trimethylammonium group (see ESI Fig. S2.7.1.3.1a

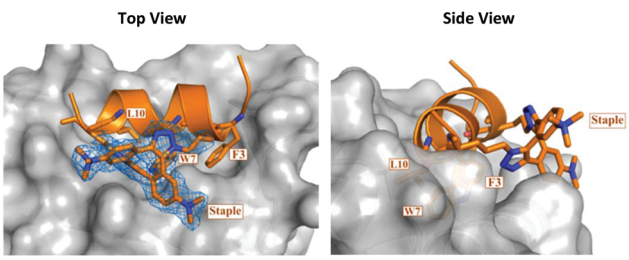


Fig. 3 Crystal structure of **28** bound to MDM2 at 2.0 Å resolution (PDB ID: 6H22). Top and side views depicting the bound stapled peptide **28** in α-helical conformation and the staple as *anti* regioisomer (only the staple and side chains of the three binding residues are shown for clarity). The 2Fo-Fc electron density map is contoured at 1σ.

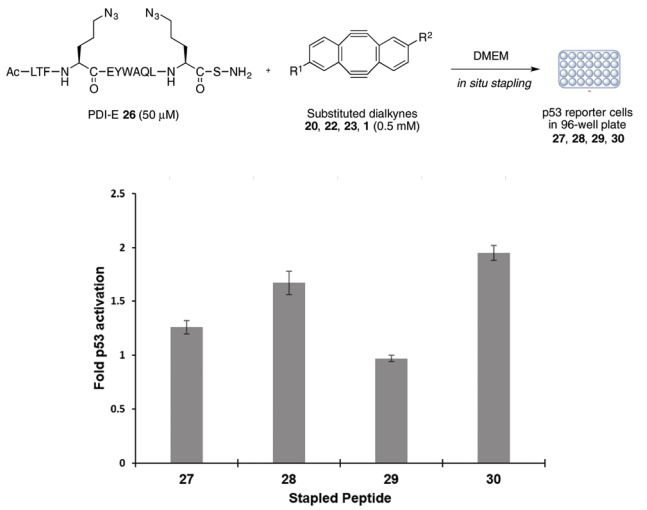


Fig. 4 p53 activation in a cellular reporter assay for *in situ* stapling of peptide **26** (50 μM) with substituted dialkynes **20**, **22**, **23** and dialkyne **1** (0.5 mM) in parallel, giving stapled peptides **27**, **28**, **29**, and **30** respectively (performed as technical triplicate). Data reported as fold activation over 1% DMSO. DMEM-Dulbecco's Modified Eagle's Medium.

and S2.7.1.3.1b†). The bis(triazolyl) staple of the bound peptide **28** exists as the *anti* regioisomer with the charged trimethylammonium group pointing away from MDM2 surface (Fig. 3: Side view).

Next, we tested the ability of functionalised stapled peptides **27–29** to inhibit the p53-MDM2 interaction in an established p53 reporter cell-based assay.[36] We treated p53 reporter cells with linear peptide **26** and dialkynes **1**, **20**, **22**, and **23** in a 96-well format (Fig. 4). *In situ* parallel stapling with multiple substituted dialkynes provides a faster way of screening resultant stapled peptides directly in the cellular medium, without performing individual stapling reactions and purifications for each dialkyne variant.[7,42] p53 activation values are reported as fold activation over the activation value obtained when cells were treated with 1% DMSO. This provides an indirect comparative measure of the cellular p53 levels where higher values are indicative of increased cellular activity. We also tested the corresponding purified stapled peptides **27–29** in this assay which confirmed the potencies (see ESI 2.9† for details). Peptide **29** gave a negligible response in this assay, most likely due to cell permeability issues with the doubly charged dialkyne. Peptide **28** was able to permeate the cell and gave 1.7-fold activation of p53. We feel that the advantages in usability of our water-soluble, stable and azide-reactive dialkynes make these reagents a substantial improvement over the Sondheimer dialkyne.

Conclusions

In conclusion, substituted strained dialkyne reagents have been synthesised for biocompatible double strain-promoted click chemistry. These highly azide-reactive novel dialkyne reagents were shown to be both water-soluble and stable under aqueous conditions. To demonstrate their extensive utility, we applied these dialkynes to an *in situ* double-strain promoted macrocyclisation strategy to generate functionalised stapled peptide inhibitors of the oncogenic p53-MDM2 interaction. These stapled peptides were all shown to bind MDM2 with low nanomolar affinity by FP assay. Furthermore, the structure of our MDM2-bound functionalised stapled peptide **28** was elucidated through X-ray crystallography, which confirmed the α-helical conformation of the peptidic backbone, and indicated the staple moiety was shifted away from the MDM2 surface. Peptide **28** was found to permeate the cell and gave comparable biological data to the unsubstituted dialkyne. These water-soluble, stable and azide-reactive strained dialkyne reagents could be successfully applied to numerous therapeutically relevant protein–protein interactions and conjugation applications.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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42 Gradual product formation over 18 h and losses due to the formation of syn/anti byproducts in an in situ stapling assay could result in slight variation in the cellular response levels observed.