ChemComm

COMMUNICATION

View Article Online
View Journal | View Issue

Cite this: *Chem. Commun.*, 2022, **58**, 8388

Received 27th April 2022,
Accepted 22nd June 2022

DOI: 10.1039/d2cc02379b

rsc.li/chemcomm

Modular solid-phase synthesis of electrophilic cysteine-selective ethynyl-phosphonamidate peptides†

Sarah Hansen,[ab] Jan Vincent V. Arafiles,[a] Philipp Ochtrop[ab] and Christian P. R. Hackenberger *[ab]

We report an efficient method to install electrophilic cysteine-selective ethynyl-phosphonamidates on peptides during Fmoc-based solid phase peptide synthesis (SPPS). By performing Staudinger-phosphonite reactions between different solid supported azido-peptides and varying ethynylphosphonites, we obtained ethynyl-phosphonamidate containing peptidic compounds after acidic deprotection, including an electrophilic cell-penetrating peptide that showed high efficiency as an additive for cellular delivery of proteins.

Peptides have emerged as increasingly valuable modalities for both basic research and for the applied biomedical sciences,[1] underpinned by the plethora of methods available for the generation and screening of peptide libraries against challenging pharmaceutical targets.[2,3] Chemical strategies to conjugate peptides to scaffolds, polymers or biopolymers to further increase their therapeutic potential are therefore high in demand.[4] For the generation of peptide–protein or -antibody conjugates it is particularly attractive to incorporate electrophilic thiol-selective moieties into peptides,[5,6] as it allows the reaction with cysteines in proteins or antibodies, which can be obtained from pre-existing disulfides by straightforward reduction protocols or easily introduced by site-directed mutagenesis.[7,8]

Among the various approaches for cysteine-selective bioconjugation, the reaction of thiols with maleimides is very popular due to the fast reaction kinetics in aqueous buffers.[7,8] However, maleimide linkages are often reported to undergo decomposition in the presence of external thiols *via* Retro-Michael addition.[9] To provide an alternative, our laboratory has introduced electron-deficient ethynyl-phosphonamidates as a new compound class for cysteine conjugation.[10] These reagents displayed superior stability in serum when compared to maleimide linkages and are exceptionally chemoselective towards cysteine residues.[10,11]

Whereas maleimides are most often incorporated into functional modules as a pre-activated entity, *e.g.*, *via* the commercially available succinimidyl-4-(*N*-maleimidomethyl)-cyclohexane-1-carboxylate (SMCC) building block, we showed that ethynyl-phosphonamidates are accessible from azides in chemoselective Staudinger-Phosphonite Reactions (SPhR)[10,12–14] displaying high functional group tolerance under ambient conditions.[15–17] Despite the application of this protocol in the synthesis of several cysteine-reactive molecules, including peptides, we had previously refrained from implementing Staudinger reactions with P(III)-reagents in common solid phase peptide synthesis schemes due to the intrinsic lability of P(=O)–N bonds, especially under acidic deprotection conditions.[18–22]

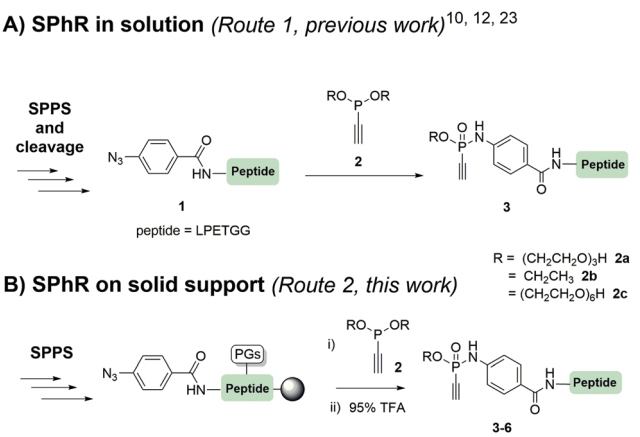
Encouraged by our recent findings that *N*-aryl substituted phosphonamidate-conjugates show excellent stability under physiological conditions and can only be cleaved quantitatively using strong acids,[10,11,23] we set out to revisit a reaction protocol to produce ethynyl-phosphonamidate-containing peptides on a solid support. (Scheme 1) Furthermore, our observation that acidic conditions allow the isolation of unsaturated phosphonothiolates[24] even in the presence of other thiol nucleophiles prompted us to probe whether thiol-containing cleavage cocktails would be tolerated in an optimized solid phase synthesis protocol to mitigate unwanted oxidation of amino acid side chains. We envisioned that an Fmoc-SPPS based methodology with its inherent advantages including easy removal of reagents as well as modularity and straight-forward adaptability of the synthesis route would drastically increase the accessibility of electrophilic ethynyl-phosphonamidate modified peptides applicable for selective thiol modification.

At the outset of our studies, we synthesized a model peptidic ethynyl-phosphonamidate based on our previously published in-solution approach (Scheme 1).[10] Thus, we first synthesized

[a] *Leibniz-Forschungsinstitut für Molekulare Pharmakologie (FMP), Chemical Biology Department, Robert-Rössle-Str. 10, 13125 Berlin, Germany. E-mail: hackenbe@fmp-berlin.de*

[b] *Humboldt Universität zu Berlin, Department of Chemistry, Brook Taylor Str. 2, 12489 Berlin, Germany*

† Electronic supplementary information (ESI) available. See DOI: https://doi.org/10.1039/d2cc02379b



Scheme 1 General principle for the synthesis of peptidic ethynyl-phosphonamidates in solution or on solid support.

azido-peptide **1** *via* standard Fmoc-SPPS on a Rink amide resin from peptide LPETGG and commercial 4-azido benzoic acid.

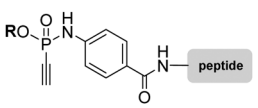
Azido-peptide **1** was isolated in 40% yield. In parallel, ethynyl-phosphonite **2a** (R = $(CH_2CH_2O)_3H$) was synthesized based on a published procedure from commercial bis-(diisopropylamino)-chlorophosphine and ethynyl-magnesium bromide.[12] Following our published procedure,[23] azido-peptide **1** was then solubilized in dry DMF and reacted with ethynyl-phosphonite **2a** overnight at room temperature. The corresponding ethynyl-phosphonamidate **3a** was isolated in 29% yield *via* HPLC, resulting in an overall yield of 12%, including the peptide synthesis (Table 1). We observed significant formation of an aryl amine-containing peptide under these conditions, which could result from phosphonimidate or -amidate hydrolysis as known from Staudinger reductions of azides.[13]

Next, we aimed to investigate if we could obtain ethynyl-phosphonamidate **3a** by a Staudinger-phosphonite reaction on solid support starting from a Rink-amide resin. (Scheme 1) Immobilized azido-peptide **1** was reacted with 1, 3, or 5 equivalents of ethynyl-phosphonite **2a** in DMF at room temperature overnight. (Fig. 1a) Next, a test cleavage with 95% trifluoroacetic acid (TFA), 2.5% triisopropylsilane (TIS), and 2.5% deionized water (cocktail **I**) was performed for 30 minutes at room temperature. Subsequent UPLC-ESI-MS analysis showed that the desired product was indeed formed, and that full conversion of the azide was achieved with 5 equivalents of ethynyl-phosphonite **2a**, thereby supporting our hypothesis that ethynyl-phosphonamidate peptides like **3a** are sufficiently stable against standard harsh cleavage conditions and can indeed be obtained *via* SPPS schemes.

Next, we investigated the stability of the P(═O)–N bond under TFA cleavage conditions over time. We performed test cleavages in cocktail **I** for 30, 60, 120, and 240 minutes and analyzed the change in the ratio between the desired ethynyl-phosphonamidate (green bar) and the corresponding amine (red bar) by UPLC-ESI-MS analysis and relative peak area integration. (Fig. 1b) Notably, even after two hours of exposure to 95% TFA, only 18% of P(═O)–N bond hydrolysis with respect to **3a** was observed. Generally, we observed that the overall yield depended on the cleavage time. To achieve full cleavage of the peptide from the resin with minimal amounts of P(═O)–N bond hydrolysis, we chose a cleavage time of 1 hour in 95% TFA as our optimized protocol. Under these conditions, ethynyl-phosphonamidate **3a** was isolated in 52% yield after HPLC purification. Subsequently, ethynyl-phosphonamidates **3b** and **3c** were synthesized on resin from ethynyl-phosphonites **2b** (R = CH_2CH_3) and **2c** (R = $(CH_2CH_2O)_6H$. (Table 1) Peptides **3b** and **3c** were isolated in 24% and 26% yields under the same conditions except that the global cleavage was conducted for 45 minutes rather than one hour. To investigate if our method applies to peptides with more delicate residues such as Arg, Met, or Tyr, we prepared ethynyl-phosphonamidate **4**, **5**, and **6** from model peptides TITSYR, KPQQFM, and R10. These sequences contained several sensitive amino acids next to the *N*-terminal ethynyl-phosphonamidate and served as suitable substrates to demonstrate the scope of our method. After their preparation on Rink amide resin, ethynyl-phosphonamidates **4**, **5**, and **6** were cleaved in cocktail **I** for 1 hour at room temperature and isolated in 31%, 23%, and 14% yield respectively. (Table 1) These results suggest that electrophilic peptidic ethynyl-phosphonamidates are satisfactorily stable against P(═O)–N bond hydrolysis.

Next, we wanted to investigate if thiol-containing cleavage cocktails were tolerated and if they could mitigate methionine oxidation. To this end, we prepared ethynyl-phosphonamidate peptide **4** following our on-resin protocol and performed cleavages with TFA-cocktails with and without ethanedithiol (EDT) for 1 hour. (Fig. 1c) The ratio between the ethynyl-phosphonamidate (orange bar) and the corresponding amine (blue bar) was not significantly affected by the choice of cleavage cocktail while methionine oxidation (yellow bar) was greatly reduced from 16% to 2% when EDT was used in cleavage cocktail **II** (94% TFA, 2.5% EDT, 2.5% H_2O, 1% TIS). We observed that a small amount of thiol-addition product with respect to ethynyl-phosphonamidate peptide **4** (11%, Fig. 1c) was formed, which could further be lowered to 6% by extracting the cleavage solution with hexane. These results confirm that thiol-containing cleavage cocktail **II** is indeed well tolerated during cleavage of an electrophilic ethynyl-phosphonamidate-containing peptide, and methionine oxidation could be significantly reduced.

Table 1 Synthesized peptidic ethynyl-phosphonamidates with isolated yields. For experimental conditions see the ESI



Compound	Peptide sequence	R	Yield [%]	Route
3a	LPETGG	$(CH_2CH_2O)_3H$	12	1
3a	LPETGG	$(CH_2CH_2O)_3H$	52	2
3b	LPETGG	CH_2CH_3	24	2
3c	LPETGG	$(CH_2CH_2O)_6H$	26	2
4	KPQQFM	$(CH_2CH_2O)_3H$	31	2
5	TITSYR	$(CH_2CH_2O)_3H$	23	2
6	R10	$(CH_2CH_2O)_3H$	14	2

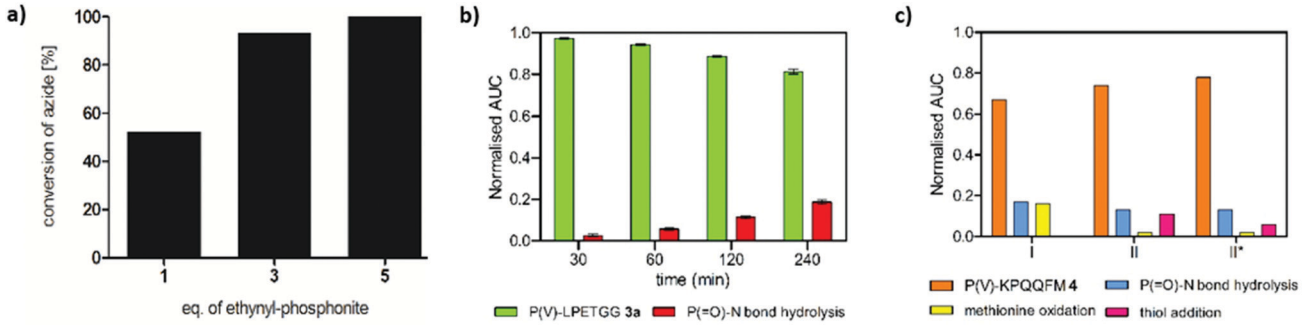


Fig. 1 (a) Conversion of azido-peptide **1** with respect to the equivalents of ethynyl-phosphonite **2a** used during on-resin synthesis of **3a**. (b) Ratio of **3a** (green bar) to the corresponding amine (red bar) at several time points during test cleavage in 95% TFA, 2.5% TIS and 2.5% H_2O. The ratios were determined by UPLC-ESI-MS analysis and relative peak area integration. (c) Ratio of ethynyl-phosphonamidate **4** (orange bar) to P(═O)–N bond hydrolysis (blue bar), methionine oxidation (yellow bar), and thiol-addition (pink bar) after test cleavage in cocktail **I** (95% TFA, 2.5% TIS, 2.5% H_2O) or cocktail **II** (94% TFA, 2.5% EDT, 2.5% H_2O, 1% TIS) for 1 hour. Ratios were determined by UPLC-ESI-MS and relative peak area integration. *After the test cleavage, the suspension was diluted with MeCN/H_2O (1 : 1) and extracted twice with hexane to remove EDT.

Finally, we evaluated whether the on-resin synthesized ethynyl-phosphonamidate-functionalized cell-penetrating peptide (CPP) **6** was applicable as a thiol-reactive additive for cellular delivery of proteins. Cell-penetrating peptides are short (less than 20 amino acids), usually highly cationic oligo-amino acids that are able to mediate translocation of proteins into cells.[25–27] However, when conjugated to a protein-of-interest, high concentrations (~50 μM) of conjugate are needed to achieve direct membrane transduction and cytosolic delivery.[28–30] To improve the efficiencies of cellular uptake of these conjugates, our lab has recently introduced CPP-additives for cell-surface anchoring, which significantly improve the direct transduction of CPP–protein conjugates.[31] A key aspect for the design of CPP-additives is an *N*-terminal thiol-reactive moiety, *e.g.* a reactive disulfide or a maleimide.[31] This moiety forms covalent linkages with cell-surface thiols that enabled CPP-additives to tether to the cell membrane and form nucleation zones where CPP–protein conjugates could readily enter. (Fig. 2a, ESI,† Fig. S1) Using the above on-resin synthesis protocol, we synthesized a linear decaarginine peptide (R10) bearing an *N*-terminal ethynyl-phosphonamidate **6** and confirmed its utility as a CPP-additive. We incubated HeLa-CCL2 cells with a protein conjugate composed of an mCherry fused to a nuclear localization signal (NLS) and a linear R10 (NLS-mCherry-R10) in the presence or absence of CPP-additives. If direct transduction through the membrane is achieved, we expect an accumulation of NLS-mCherry-R10 signal in the nucleolus, as reported previously.[31,32] Confocal microscopy images show that NLS-mCherry-R10 treatment without CPP-additives resulted in ''dot-like'' signals – indicating endocytic uptake and endosomal localization. (Fig. 2b) In contrast, applying NLS-mCherry-R10 together with **6** or the positive control maleimide-R10 **7** both resulted in nucleolar staining with NLS-mCherry-R10 (yellow arrows). Since CPP-additives require the cell-surface thiol conjugation and tethering to be functional,[31] we can infer that the on-resin synthesized **6** remains reactive and allows cell-surface labelling. Moreso, this observation also suggests that ethynyl-phosphonamidates, and other phosphorus-based thiol-reactive species, may serve as alternative thiol-reactive moieties for future designs of CPP-additives.

In summary, we present a straight-forward methodology to access electrophilic ethynyl-phosphonamidates *via* Fmoc-SPPS, which can be synthesized from aryl-azide containing peptides and ethynyl phosphonites. Despite applying highly acidic deprotection conditions, we could obtain the desired ethynyl-phosphonamidate peptides in good overall yields with only small amounts of P(═O)–N bond hydrolysis byproducts. Finally, our protocol enabled the synthesis of a CPP-additive containing a P(V)-electrophile for cell-surface attachment, which enabled the intracellular transport of a fluorescent protein, which further demonstrated the utility of these peptide-based electrophiles.

The authors thank Dr Jacobo Gómez González and Christian Stieger for helpful scientific discussions and Kristin Kemnitz-Hassain and Ines Kretzschmar for their excellent technical assistance. This work was supported by grants from the Deutsche Forschungsgemeinschaft (DFG, SPP1623 HA 4468/10-1 and RTG2473 ''Bioactive Peptides'', Projectnummer 392923329), the Leibniz Society (SAW-2018-FMP-4-P5label, T18/2017) and the Einstein Foundation Berlin (Leibniz-Humboldt Professorship). J. V. V. A. is funded by the Alexander von Humboldt Research Fellowship for postdoctoral researchers. Fig. 2a was created with BioRender.com.

Conflicts of interest

There are no conflicts to declare.

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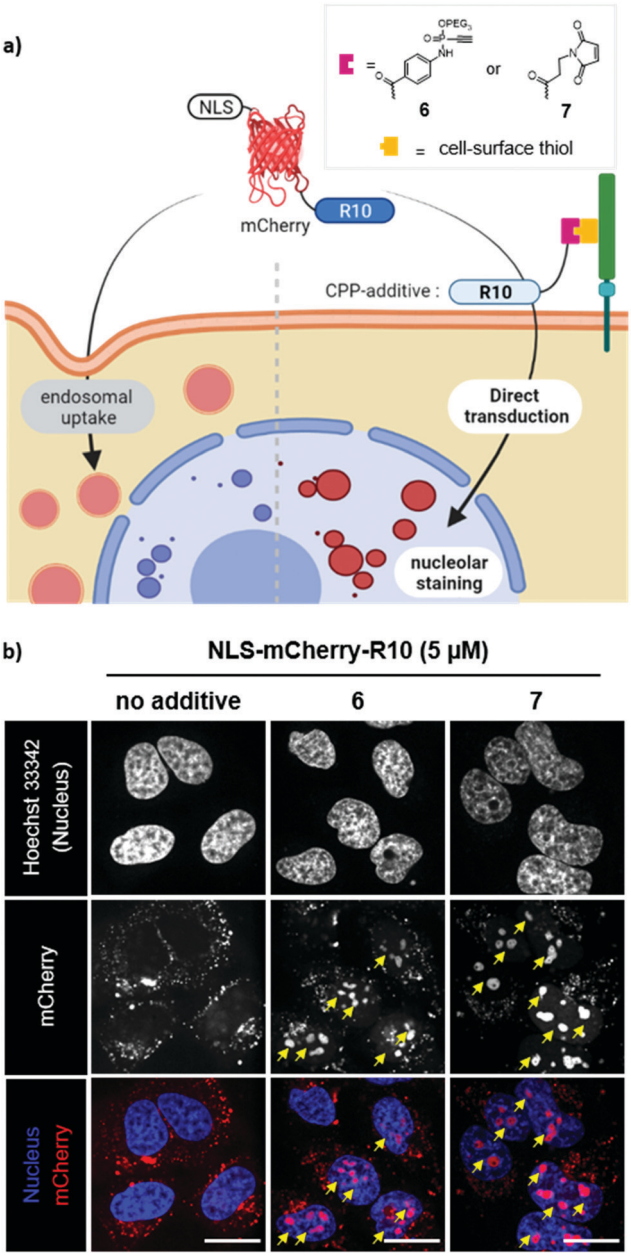

Fig. 2 Cellular uptake of NLS-mCherry-R10 facilitated by on-resin synthesized CPP-additives. (a) General concept of CPP-additive strategy. (b) Confocal microscopy images of HeLa-CCL2 cells treated with 5 µM NLS-mCherry-R_{10} and 10 µM maleimide-R10 **7** or P^{V}-R_{10} **6** for 1 hour. Yellow arrows indicate nucleoli having nucleolar staining. Scale bars = 20 µm.

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