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Communication

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Selective Irreversible Chemical Tagging of Cysteine with 3-Arylpropiolonitriles

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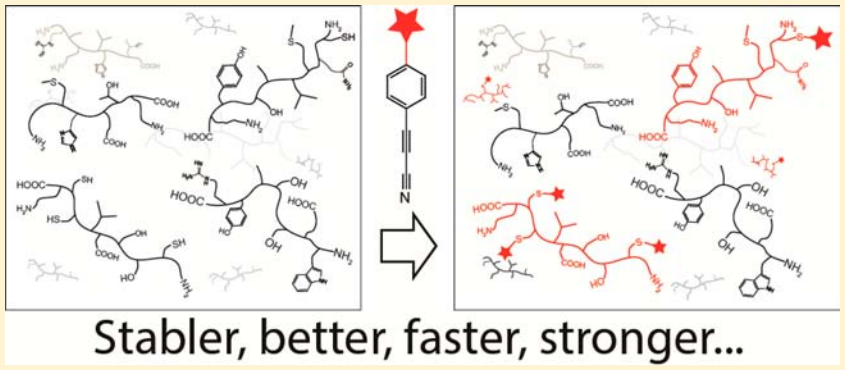
Supporting Information



ABSTRACT: Exquisite chemoselectivity for cysteine has been found for a novel class of remarkably hydrolytically stable reagents, 3-arylpropiolonitriles (APN). The efficacy of the APN-mediated tagging was benchmarked against other cysteine-selective methodologies in a model study on a series of traceable amino acid derivatives. The selectivity of the methodology was further explored on peptide mixtures obtained by trypsin digestion of lysozyme. Additionally, the superior stability of APN–cysteine conjugates in aqueous media, human plasma, and living cells makes this new thiol-click reaction a promising methodology for applications in bioconjugation.

INTRODUCTION

Cysteine is the only coded amino acid that carries a nucleophilic thiol group (-SH) which, particularly in its deprotonated thiolate form ($-S^-$), largely exceeds the reactivity of any other nucleophilic function in proteins.[1] As a result, chemospecific cysteine derivatization is by far the most widely used method for chemical tagging of proteins.[2−17] Among the vast number of chemical cysteine modification methods reported in the literature, such reagents as N-substituted maleimides, 4-vinylpyridines, and iodoacetamides are most commonly used.[2,4] All of them possess drawbacks limiting their general applicability for cysteine labeling which are mainly the presence of undesired side reactions during conjugation (in case of iodoacetamides and maleimides)[2] and the relative instability of resulting conjugates in aqueous media.[18−23] A major drawback which recently became more important with the rise of antibody–drug conjugates (ADC) is the instability of the adduct in biological media due to reversible thiol exchange and other side reactions.[24,25] Therefore, the development of more efficient site-specific cysteine labeling methodologies is an extremely rewarding target and a great challenge in protein modification. Research in this field is very active and chemical functionalities such as vinyl sulfones[26,27] and Julia–Kocienski-like sulfone derivatives[28] were recently reported as an alternative to classically used thiol labeling reagents.

Reactions of electron deficient acetylenes with thiols have been known for decades, being, however, mostly conducted in organic solvents.[29−33] Only a handful of reactions in aqueous media have been reported to date.[9,34−36] Notably, Che and co-workers described the use of arylalkynones as cleavable reagents for the modification of cysteine-containing peptides[35] and reported a FRET-based fluorescent probe for detection of cysteine and homocysteine,[36] in both cases exploiting the interchangeability of the resulting vinyl sulfide linkage adduct.

These early reports prompted us to further investigate the reactivity profile of electron deficient acetylenes toward amino acid residues in order to develop a nonexchangeable cysteine tagging methodology. Elevated stability of addition product is of crucial importance in a number of applications, such as protein conjugation and immobilization.[12,37−44]

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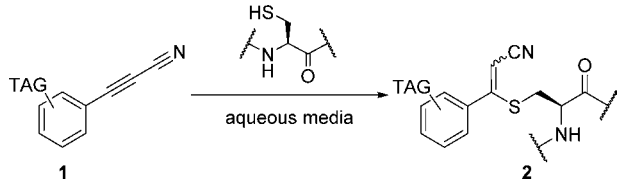
RESULTS AND DISCUSSION

Herein we report a novel class of chemoselective reagents, the 3-arylpropiolonitriles (APN, **1**), which can be used for cysteine tagging in complex aqueous media (Scheme 1). The main

Scheme 1. Cysteine Tagging Using 3-Arylpropionitriles (APN)



advantages of these new reagents are their very high selectivity for thiols, formation of stable adducts against thiol exchange and hydrolysis, and superior stability in biological environments. Application of these compounds allows for achieving quantitative alkylation of all −SH residues without cross-reactions with other functional groups and obtaining highly stable ligation products.

APN can be obtained in two straightforward steps from iodoarenes via Sonogashira coupling with propargyl alcohol followed by tandem oxidation with MnO_2 in the presence of ammonia solution.[45] In order to compare APN with reactive probes classically used for cysteine labeling (**4**−**6**) and other electron-deficient alkynes, described by Che (**7**−**8**),[35] we conducted a model study with various potentially reactive UV-traceable amino acid derivatives (Figure 1).

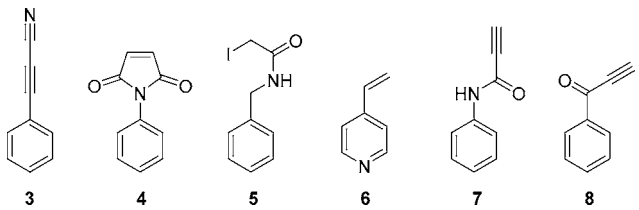


Figure 1. Set of reactive probes investigated in preliminary study.

To test aqueous stability and reactivity toward thiols, electrophilic probes **3**−**8** were reacted with a cysteine derivative at 1 mM concentration of each reagent in PBS buffer at pH 7.6 at 25 °C (see Supporting Information, Figure S7); reactions were monitored by HPLC over 1 h (Table 1).

We found that all reagents **3**−**8** gave from moderate (**5**−**7**) to very good yields (**3**, **4**, and **8**). 3-Phenylpropiolonitrile (**3**) reacted with cysteine derivative following second order kinetics

Table 1. Hydrolytic Stability and Reactivity of Reactive Probes 3−8 with a UV-Traceable Cysteine Derivative

	3	4	5	6	7	8
Degradation,[a] %	0	25	0	0	0	0.9
k_{obs}, s^{-1}	-	7×10^{-5}	-	-	-	2×10^{-6}
Conversion,[a] %	94	99	81	60	72	99
k_2, M^{-1} s^{-1}	3.1	>50	1.2	0.4	0.7[b]	>50

[a]Conversion of electrophile in 1 h was measured by calibrated UV-HPLC traces, using benzamide as an internal standard at 1 mM concentrations of reagents in PBS:DMSO (4:1) at pH 7.6 at 25 °C. [b]100 μM concentrations of reagents were used for determining the reaction constant.

of k_2 = 3.1 M^{-1} s^{-1}, which is 2.6 times higher than that exhibited by iodoacetamide (**5**, k_2 = 1.2 M^{-1} s^{-1}), about 4 times higher, compared to alkynoic amide (**7**, k_2 = 0.8 M^{-1} s^{-1}) and exceeds that of 4-vinylpyridine (**6**, k_2 = 0.4 M^{-1} s^{-1}) by the factor of 8.

The kinetics of reaction of APN **3** with cysteine derivative was slower than that of N-phenylmaleimide **4** and alkynone **8** where complete conversion was achieved in less than 30 min of reaction (k_2 was estimated to be higher than 50 M^{-1} s^{-1}).

Concerning aqueous stability of reactive probes, we found that maleimide **4** underwent 25% of hydrolysis after 1 h in PBS at 1 mM concentration (k_{obs} = 7 × 10^{-5} s^{-1}), while very little (for alkynone **8**) or no degradation (probes **3**, **5**−**7**) could be detected for the other tested electrophiles. Interestingly, even after a month at room temperature no traces of hydrolyzed product could be detected for APN **3**. It is noteworthy that maleimide sensitivity to hydrolysis may explain in part the need to add an excess of maleimide-containing probe when performing labeling of complex biological mixtures.[7]

Moreover, the addition product of the reaction between APN **3** and cysteine derivative was completely stable in a wide range of pH (from 0 to 14), in the presence of an excess of such nucleophiles as thiophenol, cysteine, thioethanol, glutathione (100−1000 equiv, 10−100 mM), as well as reducing agents such as TCEP or DTT (0.1−1 M) at pH 7.4. We then studied the possible thiol exchange by incubation of the addition product with 100 equivalents of thiophenol under slightly basic conditions (TRIS buffer, pH 9.5). HPLC monitoring showed no significant exchange even after 2 h of incubation (Supporting Information, Table S1). In comparison, adducts of model alkynoic amide **7** and alkynone **8** described by Che and collaborators,[35] underwent complete cleavage in 30 min under the same conditions.

Interestingly, mainly Z-isomers of vinyl-sulfides were obtained during the addition of different thiols to the APN. We attributed the remarkable stability of APN−thiol conjugates to the stabilization of obtained cyanoacrylate by delocalization of the alkene's electronic density in the aromatic ring. To prove this hypothesis, we studied 3-cyclohexylpropiolonitrile, containing a cyclohexyl moiety instead of phenyl ring. Indeed, obtained cyanoacrylates were not as hydrolytically stable as aryl-substituted analogs. Moreover, the kinetics of thiol addition were 10 times slower in the case of 3-cyclohexylpropiolonitrile compared with 3-phenylpropiolonitrile **3** (Supporting Information, Figure S14).

With these promising results in hand, we decided to broaden our study and search for possible side reactivities with other nucleophilic amino acids. For this purpose we reacted electrophiles **3**−**6** with a pool of amino acid derivatives. As previously, reactions were analyzed by HPLC after 1 h of reaction. We found that all studied electrophiles appeared quite chemoselective for cysteine. However, in our rather stringent conditions (1 mM concentration of reagents) maleimide **4** showed significant conversion in the presence of glycine, serine, and histidine derivatives (Supporting Information, Table S4).

We then studied the influence of various substituents on the aryl moiety of APN on its reactivity with cysteine derivative (30 min at 25 °C, 50 μM concentration). In general, we have found an important detrimental effect when introducing substituents in the *ortho*-position. Concerning the effect of substituents in the *para*-position, we noted that electron-donating groups decreased the yield, while electron-withdrawing groups had the opposite effect. For instance, *p*-NH2-substituted APN gave a

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7% yield of cysteine adduct, while *p*-NHAc afforded 52% yield that is comparable to an unsubstituted APN, and *p*-CONHMe yielded 86% (Supporting Information, Table S1).

In order to prove the selectivity of APN in the labeling of complex peptide mixtures, we have attached the APN electrophilic tag to TMPP (tris(2,4,6,-trimethoxyphenyl)-phosphonium), a highly responsive MS tag. Once conjugated to a native peptide, TMPP modifies its chromatographic behavior by delaying retention time in reverse-phase LC and strongly enhances its signal detection.[46] As a consequence we expected to detect any nonspecific reaction of APN with a very high sensitivity.

APN-TMPP probe (**10**, Figure 2) was synthesized in four steps with overall yield of 23% (Supporting Information, Figure S1). Its labeling effectiveness was tested on a peptide mixture generated by using lysozyme as a model protein containing 6 cysteine residues. Lysozyme was reduced by TCEP (Tris(2-carboxyethyl)phosphine hydrochloride) and submitted to trypsin digestion.

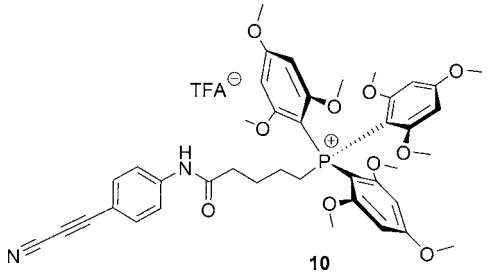

Figure 2. APN-TMPP probe.

Evaluation of APN-TMPP chemoselectivity was carried out by studying its reaction with tryptic digest at a 200:1 molar ratio of APN-TMPP (1 μM) to protein (around 10:1 to cysteine moieties) at 25 °C for 1 h. Peptide mixtures obtained with and without chemical derivatization were analyzed by LC-MS/MS.

As we expected, all detectable cysteine-containing peptides reacted with a probe and were delayed while cysteine-free peptides were unaffected (Table 3). We estimated the labeling efficiency based on the ratio between intensities of labeled and nonlabeled peptides by LC-MS. More than 98% of the detected peptides were completely tagged (Supporting Information, Figure S2). LC-MS results show clearly that cysteine-containing peptides have an increased retention time due to the addition of the hydrophobic TMPP group, whereas the retention time of all other peptides remained unchanged.

Due to the introduction of a permanent positive charge given by the tag, ionization efficiency of modified peptides in ESI-MS was increased. Enhancement of the peptide signal ranged from 2-fold to roughly 8-fold. It is noteworthy that we were not able to detect any APN-TMPP labeled noncysteine containing peptides.

This clearly illustrated the high selectivity of the APN toward bio-thiols. In order to evaluate the other required feature of an ideal bioconjugation agent, we decided to conduct a side-by-side stability comparison of APN and maleimide conjugates. To monitor bond breakage at low concentrations and in biological environments we synthesized two FRET-based probes **P1** and **P2**, containing APN- and maleimide-thioether scaffold, respectively (Figure 3A). In these probes, cleavage of any bond between fluorescent dye (5′-TAMRA) and quencher (BHQ-2) will result in the appearance of the fluorescence signal. Since these two probes differ only by their core APN− or maleimide−thiol linkage, we assume that the appearance of fluorescence will account for instability of the scaffold. Moreover, because of very similar structure of two probes, we assumed their cellular uptake to be comparable.

To test stability in human plasma, we incubated both probes (1 μM) at 37 °C, while monitoring the appearance of fluorescence at 580 nm over 15 h (Figure 3B). We detected that incubation of **P1** showed only 1.5 times increase of fluorescence, while **P2** gave a much stronger increase (4-fold) due to degradation in biological medium in accordance with previously described plasma-instability of maleimide conjugates.[24]

The biolabilities of the probes were then studied in living cells (BNL CL.2). To provide quantitative and spatial information about probe hydrolysis, fluorescence activated-cells were imaged by flow cytometry and confocal microscopy. First, cells were loaded with the FRET-based probes (1 μM, 2 h), incubated for different times and fluorescence activation was recorded by flow cytometry (Figure 3C). The results indicated higher stability of APN probe **P1** vs maleimide probe **P2** over the given time. After one day of incubation, the maleimide probe exhibited a 4-fold higher level of fluorescence than the APN probe.

For confocal imaging, cells were loaded with both probes (5 μM, 90 min) and nuclei were stained with Hoechst 33258 (5 μg/mL, 30 min). Resulting images are shown in Figure 3 (probe **P1** − image D; probe **P2** − image E). Compared to **P1**,

Table 3. LC-MS Analyses of Tryptic Digest of Lysozyme before and after Reaction with APN-TMPP (10)

	before tagging		after tagging			
peptide sequence[a]	*m/z* (charge state)	RT (min)	*m/z* (charge state)	RT (min)	ΔRT (min)	number of tags
^{24}**C**ELAAAMK^{31}	418.70 (+2)	13.9	545.89 (+3)	24.2	10.3	1
^{24}**C**ELAAAMoxK^{31}	426.70 (+2)	12.6	551.23 (+3)	23.7	11.1	1
^{40}GYSLGNWV**C**AAK^{51}	634.81 (+2)	19.8	689.96 (+3)	25.3	5.5	1
^{80}WW**C**NDGR^{86}	468.69 (+2)	16.5	579.22 (+3)	25.7	9.2	1
^{92}NL**C**NIP**C**SALLSSDITASVN**C**AK^{114}	779.71 (+2)	23.7	949.20 (+5)	30.3	6.6	3
^{52}FESNFNTQATNR^{63}	714.83 (+2)	13.5	714.83 (+2)	13.5	0	0
^{135}GTDVQAWIR^{143}	523.27 (+2)	17.3	523.27 (+2)	17.3	0	0
^{64}NTDGSTDYGILQINSR^{79}	585.28 (+2)	18.2	585.28 (+2)	18.2	0	0
^{116}IVSDGNGMNAWVAWR^{130}	559.27 (+2)	20.9	559.27 (+2)	20.9	0	0

[a]Cysteine residues are in bold.

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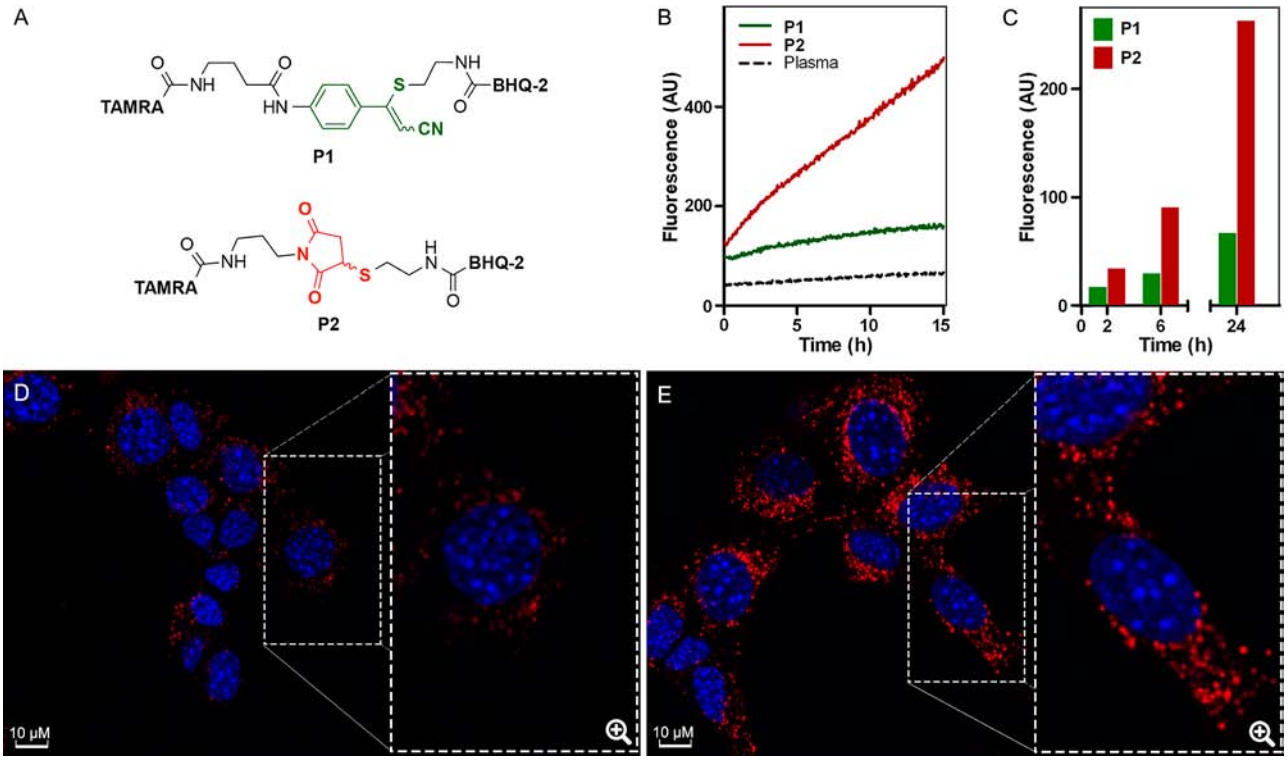

Figure 3. Structures of FRET-based probes **P1** and **P2** (A). Side-by-side comparison of the instability of probes **P1** and **P2** (1 μM) in human plasma at 37 °C (B) and in BNL CL.2 cells at different time points (C). Higher fluorescence level corresponds to higher level of degradation. In vitro imaging of BNL CL.2 cells with probe **P1** (D) and probe **P2** (E). Viable cell imaging was carried out by first staining with the different probes (5 μM, 90 min) followed by staining with Hoechst 33258 (5 μg/mL, 30 min). Confocal fluorescence microscopy imaging of viable cells was performed at a magnification of 630×.

maleimide−thiol conjugate **P2** exhibited higher fluorescent signal with a vesicular distribution, probably corresponding to endosomal uptake of the probe. The results are congruent with flow cytometry measurements and confirmed higher stability of APN− than maleimide−thiol conjugates.

CONCLUSION

As demonstrated above, we have elaborated a novel class of electron deficient acetylenes, APN, for highly chemoselective cysteine modification in aqueous medium. This methodology allowed us to surpass the main drawbacks of existing strategies, notably side reactions with other nucleophilic amino acid residues and instability of educt and addition products. These results open an interesting prospect in the field of antibody−drug conjugates and biologics, where high selectivity and biostability are key features. It can be anticipated that new heterobifunctional reagents for cross-coupling will be developed by combining APN with other reactive hooks from bioconjugation toolbox.

ASSOCIATED CONTENT

Supporting Information

General experimental procedures, materials, and instrumentation. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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