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Ethynyl, 2-Propynyl, and 3-Butynyl C-Phosphonate Analogues of Nucleoside Di- and Triphosphates: Synthesis and Reactivity in CuAAC

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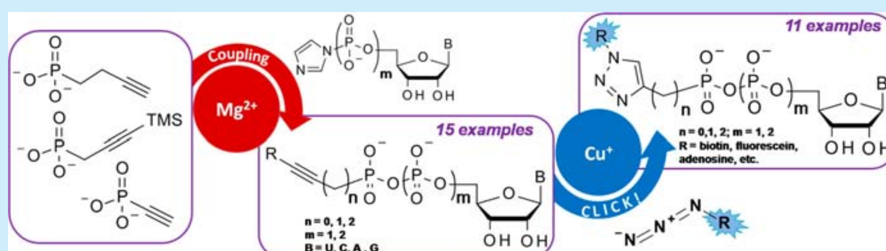
Supporting Information



ABSTRACT: The synthesis and reactivity of a novel class of clickable nucleotide analogues containing a C-phosphonate subunit that has an alkyne group at the terminal position of the oligophosphate chain are reported. The C-phosphonate subunits were prepared by simple one- or two-step procedures using commercially available reagents. Nucleotides were prepared by $MgCl_2$-catalyzed coupling reactions and then subjected to CuAAC reactions with various azide compounds to afford 5′-γ-labeled nucleoside triphosphates in excellent yields.

Labeled, synthetic nucleotide derivatives can be used as probes to investigate biological processes and as tools for biotechnology and drug discovery. Mononucleotides modified at the terminal position of the (oligo)phosphate chain are particularly useful as binding probes,[1] reporter substrates and enzymatic inhibitors,[2] donors of labeled phosphate moieties,[3] and reagents for single-molecule sequencing.[4] Among the various nucleotide modification methods, click chemistry—in particular, the copper catalyzed azide−alkyne cycloaddition (CuAAC)—has emerged as the leading approach. Despite significant advances in tagging, bioconjugation, and chemical ligation of nucleosides and (oligo)nucleotides using CuAAC,[5] only a relatively limited number of works describe the use of terminal phosphate modification to afford "clickable" nucleotides. The modifications that have been reported so far involve a formal replacement of the terminal phosphate with a phosphoester or phosphoroamidate moiety containing an azide or alkyne group (Figure 1A−C); an azide-carrying C-phosphonate ATP analogue has also been reported (Figure 1A).[6] Such modified nucleotides have already been used to examine biologically and medicinally relevant processes. For example, propargyl phosphoesters of ADP and GDP (Figure 1B) have been used to construct compound libraries and to find therapeutic inhibitors of the proteins, human fucosyltransferase, and tyrosine kinase.[2b,6c] Recently, a ferrocene-labeled ATP derivative containing a clickable alkyne moiety was developed for electrochemical detection of kinase-catalyzed protein phosphorylation.[7]

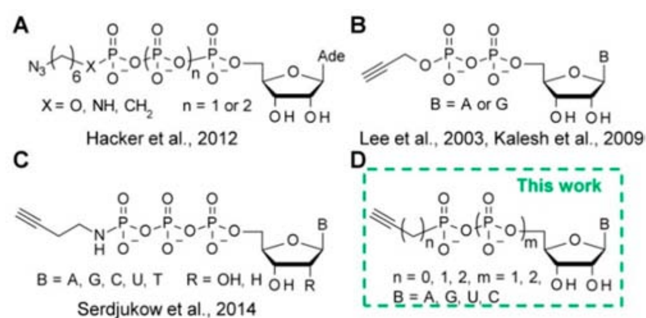


Figure 1. Nucleotide analogues modified at the terminal phosphate moiety to make them suitable for CuAAC reactions.

New methods for functionalizing terminal phosphate groups with clickable moieties are needed to increase the variety of

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structures and to tune chemoenzymatic properties of probes. In this work, we report for the first time the synthesis and properties of novel clickable nucleotide analogues containing a C-phosphonate subunit with an alkyne group.

The alkyne group in our analogues is either directly attached to phosphorus via a P–C^{sp} bond or separated from the phosphorus by a one- or two-carbon atom spacer (Figure 1D), which allows for the synthesis of new, previously unavailable structural patterns. Fifteen nucleotide analogues varying in nucleobase type and phosphate chain length were prepared using an efficient, divalent metal chloride-mediated coupling reaction between imidazole activated nucleotides and C-phosphonate subunits containing an appropriate alkyne moiety (butynyl, propargyl, or ethynyl). Synthesis of the C-phosphonate starting materials was also developed. To demonstrate the utility of our nucleotides for CuAAC reactions, we prepared a set of 11 model conjugates with azide-containing biotin, fluorescent tags, or nucleoside moieties.

The first step in our study was to prepare phosphonate subunits containing an alkyne moiety. The standard method of preparing C-phosphonates containing a P–C^{sp3} bond is the use of the Michaelis–Arbuzov reaction between trialkyl phosphites and an appropriate halogen derivative.[8] The alkyl protecting groups can be subsequently removed by treatment with TMS-Br.[9] This approach appeared feasible for the synthesis of butynyl- and propargylphosphonate subunits (**1a** and **1b**, respectively); however, to enable deprotection under milder conditions, we used tris(trimethylsilyl) phosphite as a starting material for our syntheses. The triethylammonium salt of 3-butynylphosphonate (**1a**) was synthesized by a reaction of neat tris(trimethylsilyl) phosphite with 4-bromo-1-butyne (Figure 2A), followed by the removal of the silyl protecting groups

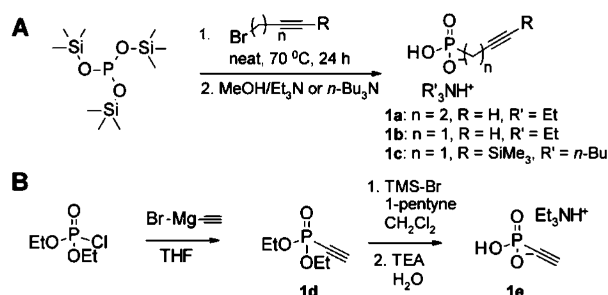

Figure 2. Synthesis of C-phosphonates containing an alkyne group.

using triethylamine in methanol. Aqueous work-up and evaporation under reduced pressure gave crude **1a**, which was at least 80% pure as judged by ^{31}P NMR (δ_P +25.70 ppm; Figure S1A, Supporting Information) with H-phosphonate (δ_P +3.59 ppm, ~ 15%) and inorganic phosphate (δ_P +0.86 ppm, ~2%) as byproducts. A test coupling reaction of this product with imidazole-activated AMP (AMP-Im, **2a**; Figure S2) in DMF in the presence of $MgCl_2$ yielded the desired phosphonate analogue **3a** within 1 h and in 74% yield as determined by reversed-phase HPLC (RP) and MS (t_R = 7.8 min, m/z = 462.1; Figure 3A) together with AMP and AppA as the only byproducts. Therefore, we assumed that further purification of crude **1a** before coupling with activated nucleotides was not required.

To obtain propargylphosphonate **1b**, we reacted propargyl bromide and tris(trimethylsilyl) phosphite under the conditions used to prepare **1a**. Unfortunately, several products were observed by ^{31}P NMR, (Figure S1B), including **1b** (34%, +10.83 ppm), allenylphosphonate **1f** (51%, +16.55 ppm), propargylester of **1b** (15%, +17.14 ppm), and inorganic phosphate (~1%, +0.85 ppm).[10]

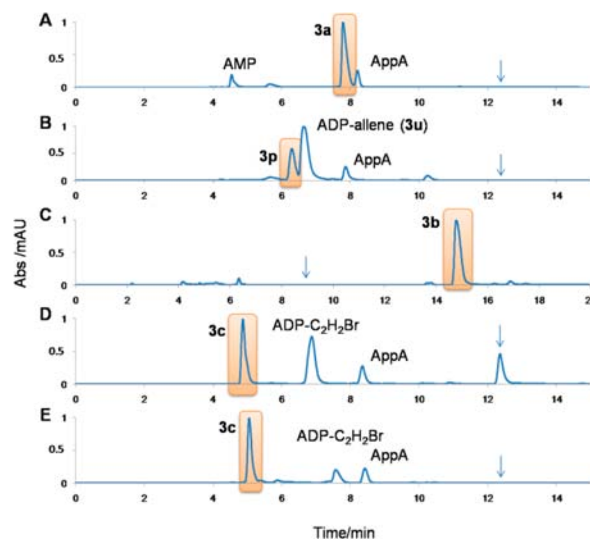

Figure 3. Representative RP HPLC profiles from pilot coupling reactions of adenosine 5′-phosphorimidazolide (**2a**) with crude subunits **1a** (A), **1b** (B), **1c** (C), and **1e** prepared in the absence (D) or presence (E) of 1-pentyne. Note the different time scale and different HPLC gradient for panel C (see the Supporting Information for details).

Consequently, when crude **1b** was coupled with **2a**, two major products were detected by HPLC, the desired C-phosphonate analogue of ADP (**3p**, t_R = 6.30 min; 23%) and an isomeric allene analogue (**3u**, t_R = 6.64 min; 58%) (Figure 3B and Figure S3). The isomers were isolated on preparative scale and identified using ^{1}H and ^{31}P NMR as shown in Figures S3 and S4. To prevent allene formation, we replaced propargyl bromide with 3-bromo-1-(trimethylsilyl)-1-propyne in the reaction with tris(trimethylsilyl) phosphite. This resulted in almost quantitative formation of the desired product. After removal of the two labile *O*-TMS groups and simple work-up, the tributylammonium salt of 3-(trimethylsilyl)-propargylphosphonate **1c** was isolated in 90% yield without any byproducts detectable by ^{31}P NMR (Figure S1C). When compound **1c** was subjected to coupling with AMP-Im, the desired ADP analogue (**3b**) was formed as the sole product in less than 1 h (Figure 3C). We decided to keep the TMS group on the propargyl moiety for coupling with imidazole-activated nucleotides since the presence of lipophilic TMS group improved the chromatographic properties of the nucleotides and the removal of TMS at the stage of nucleotide could be conveniently monitored by HPLC.

To prepare C-phosphonates with an alkyne group directly attached to phosphorus via a P–C^{sp} bond (**1e**), ethynylmagnesium bromide was reacted with diethyl chlorophosphate followed by alkyl group removal.[11] Reaction of diethyl chlorophosphate with 1 equiv of ethynylmagnesium bromide in THF afforded the expected ethynylphosphonate diethyl ester as the sole product (as evidenced by TLC and MS), which was isolated in 38% yield after silica gel purification.

After removal of alkyl groups using TMS-Br, two products were identified using ^{31}P NMR and MS: the desired phosphonate **1d** (δ_P −10.83 ppm, m/z 105.0, 44%) and a

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byproduct (δ_P +8.38 ppm, m/z 184.5 and 186.7, 56%), which we identified as a product of HBr addition to the triple bond (Figure S1D).[11] Consequently, the formation of two products with distinct retention times was also observed by RP HPLC when crude **1e** was subjected to coupling with AMP-Im (ADP-C_2H, **3c**, t_R = 4.87 min, 32% and ADP-C_2H_2Br, t_R = 6.88 min, 39%; Figure 3D). To minimize the amount of **1d** undergoing conversion into unwanted bromoalkene, an excess of 1-pentyne was added to the reaction mixture as a "scavenger". According to ^{31}P NMR (Figure S1E), using 10 equiv of 1-pentyne increased the amount of desired product from 44% to 76%. The coupling reaction of the resulting crude C-phosphonate **1d** with AMP-Im yielded the desired product in 70% yield (Figure 3E). The relatively large difference in retention times between the desired product and byproducts (ADP-C_2H, t_R = 5.05 min, and ADP-C_2H_2Br, t_R = 6.73 min, respectively), indicated that the two could be easily separated using RP HPLC.

We found that the coupling reactions of **1a–e** with imidazolide-activated nucleotides occurred efficiently in DMF in the presence of a 4–8-fold excess of $MgCl_2$.[12] To demonstrate the generality of our method, we synthesized a series of 15 ribonucleotides (**3a–o**, Figure 4) differing in

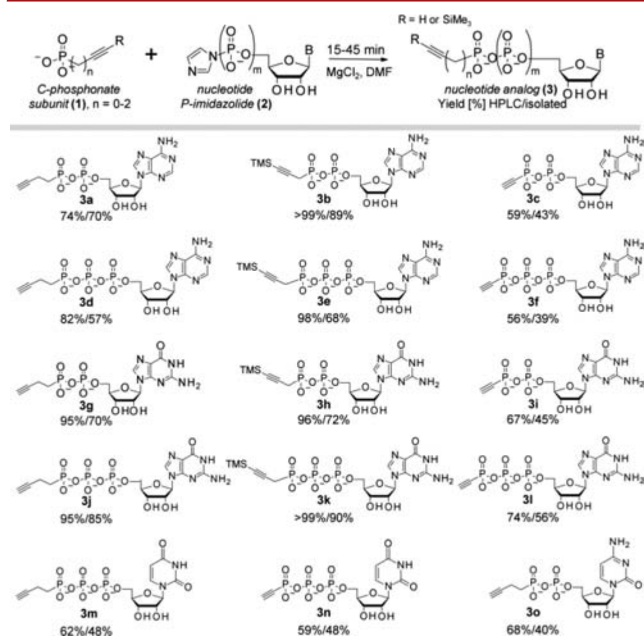

Figure 4. Synthesis of C-phosphonate nucleotide analogues **3a–o** containing an alkyne moiety.

phosphate chain length (diphosphate and triphosphate) and nucleobase type (A, G, C, U) starting from the corresponding nucleotide imidazolide derivatives (**2a–f**, Figure S2).[13] The coupling reactions gave good to excellent yields 56–99%, usually within less than 1 h (Figure 4). The products were isolated by ion-exchange chromatography (if necessary additional purification was by RP HPLC), and their structures and purities were confirmed by HRMS, ^1H NMR, and ^{31}P NMR (Supporting Information).

Couplings with compound **1c** produced nucleotides carrying a TMS-propargylphosphonate moiety, and thus, the TMS group had to be removed before subsequent CuAAC reactions. The TMS removal could be performed under mild conditions (aqueous ammonia or TBAF)[14] either on the ion-exchange purified product or at the stage of unpurified product in the post-coupling reaction mixture (Table 1). In the former case, lyophilization of the aqueous phase afforded products of high

Table 1. Synthesis of Deprotected Nucleotides 3p–t

entry	starting material	reaction conditions[a]	product	HPLC yield (%)
1	**3b**	A	**3p** (m = 1, B = A)	100
2	**3b**	B	**3p** (m = 1, B = A)	100
3	**3b**[b]	B	**3p** (m = 1, B = A)	73[c]
4	**3e**	A	**3r** (m = 2, B = A)	100
5	**3e**	B	**3r** (m = 2, B = A)	100
6	**3h**	A	**3s** (m = 1, B = G)	100
7	**3k**	A	**3t** (m = 2, B = G)	100

[a]Conditions A: 1.1 equiv of 1 M TBAF in THF, ACN, rt, 12 h. Conditions B: 10% $NH_{3(aq)}$, rt, 2 h. [b]Crude product in post-reaction mixture. [c]Overall HPLC yield for two-step procedure: coupling and subsequent deprotection.

purity, by HPLC and NMR, that were suitable for further CuAAC reactions without additional purification. The compounds showed high stability in aqueous solutions at various pH values (Figures S5–7) and upon storage as solid samples, which makes them more stable than the corresponding phosphoramidate analogues (acid-sensitive) and at least as stable as phosphoester analogues.[6a]

To verify the suitability of the compounds for CuAAC reactions, a selection of them were reacted with azides varying in size, hydrophobicity, and linker length between the azido group and the rest of molecule (**4a–g**, Figure S8), including dansyl (**4a**), fluorescein (**4b** and **4f**), biotin (**4c**), 5′-azido-5′-deoxyadenosine (**4d**), and pyrene (**4e** and **4g**). All reactions proceeded rapidly under standard CuAAC conditions[15] to produce fluorescently labeled nucleotides (**5a–g**), biotinylated nucleotides (**5j,k**), or novel dinucleoside triphosphate mimics (**5h,i**) in high yields (Figure 5). Notably, product **5d**, which is derived from two sterically demanding compounds, was also efficiently formed under standard conditions, thus indicating that the close proximity of the oligophosphate moiety does not impair reactivity of the alkyne. In fact, in a simple competition experiment between **3d** and **3f** we found that ethynyl analogues were more reactive in CuAAC than the corresponding 3-butynyl analogues (Figure S9), which suggests that they are suitable for the preparation of complex and sterically crowded nucleotide derivatives.

In conclusion, we reported the synthesis, stability, and reactivity of a novel class of clickable nucleotide analogues bearing a C-phosphonate subunit containing an alkyne group. A series of 15 nucleotides was prepared using an efficient, magnesium chloride mediated coupling of imidazole-activated nucleotides with C-phosphonate subunits **1a**, **1c**, and **1e**. The C-phosphonate subunits were prepared by simple one- or two-step procedures from commercially available reagents. The compounds show high stability and react efficiently in a CuAAC reaction under standard conditions. Due to the presence of a C-phosphonate moiety, the compounds and their conjugates may possess unique structural and chemical properties beneficial for biological applications. As such, the reported analogues may serve as structural frameworks for the

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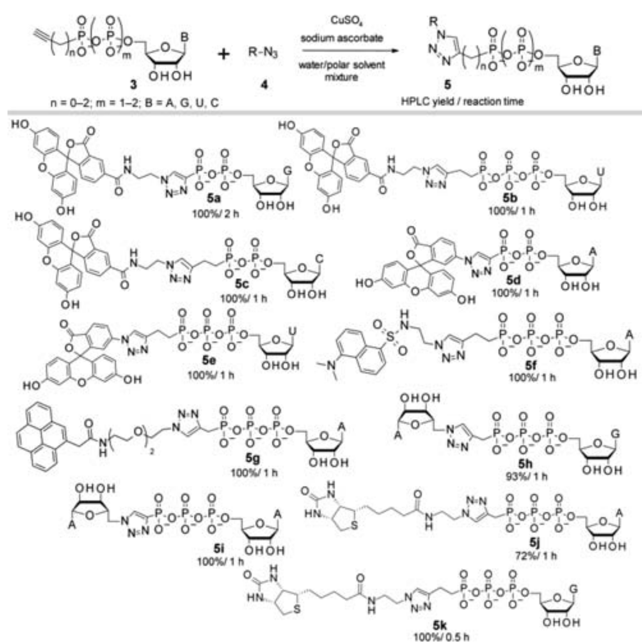

Figure 5. Synthesis of nucleotide conjugates from nucleotide C-phosphonate analogues using CuAAC.

development of novel nucleotide-based fluorescent probes, affinity purification ligands, or enzymatic inhibitors with exceptional chemical and enzymatic stability. The applications of the reported nucleotide analogues and their derivatives as RNA polymerase substrates, kinase substrates, and dinucleoside polyphosphate mimics are under investigation.

■ ASSOCIATED CONTENT

Supporting Information

Figures S1−S9, detailed experimental procedures, HPLC profiles of reaction mixtures and purified compounds, and NMR and HRMS spectra. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.5b01346.

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) George Cisar, E. A.; Nguyen, N.; Rosen, H. *J. Am. Chem. Soc.* **2013**, *135*, 4676.

(2) (a) Hardt, N.; Hacker, S. M.; Marx, A. *Org. Biomol. Chem.* **2013**, *11*, 8298. (b) Kalesh, K. A.; Liu, K.; Yao, S. Q. *Org. Biomol. Chem.* **2009**, *7*, 5129. (c) Hacker, S. M.; Pagliarini, D.; Tischer, T.; Hardt, N.; Schneider, D.; Mex, M.; Mayer, T. U.; Scheffner, M.; Marx, A. *Angew. Chem., Int. Ed.* **2013**, *52*, 11916.

(3) (a) Suwal, S.; Senevirathne, C.; Garre, S.; Pflum, M. K. H. *Bioconjugate Chem.* **2012**, *23*, 2386. (b) Martić, S.; Rains, M. K.; Freeman, D.; Kraatz, H.-B. *Bioconjugate Chem.* **2011**, *22*, 1663.

(4) (a) Eid, J.; Fehr, A.; Gray, J.; Luong, K.; Lyle, J.; Otto, G.; Peluso, P.; Rank, D.; Baybayan, P.; Bettman, B.; et al. *Science* **2009**, *323*, 133. (b) Kumar, S.; Tao, C.; Chien, M.; Hellner, B.; Balijepalli, A.; Robertson, J. W. F.; Li, Z.; Russo, J. J.; Reiner, J. E.; Kasianowicz, J. J.; Ju, J. *Sci. Rep.* **2012**, *2*, 684. (c) Sims, P. A.; Greenleaf, W. J.; Duan, H.; Xie, X. S. *Nat. Methods* **2011**, *8*, 575.

(5) (a) Liang, L.; Astruc, D. *Coord. Chem. Rev.* **2011**, *255*, 2933. (b) El-Sagheer, A. H.; Brown, T. *Chem. Soc. Rev.* **2010**, *39*, 1388.

(6) (a) Hacker, S. M.; Mex, M.; Marx, A. *J. Org. Chem.* **2012**, *77*, 10450. (b) Serdjukow, S.; Kink, F.; Steigenberger, B.; Tomas-Gamasa, M.; Carell, T. *Chem. Commun.* **2014**, *50*, 1861. (c) Lee, L. V.; Mitchell, M. L.; Huang, S.-J.; Fokin, V. V.; Sharpless, K. B.; Wong, C.-H. *J. Am. Chem. Soc.* **2003**, *125*, 9588.

(7) Wang, N.; She, Z.; Lin, Y.-C.; Martić, S.; Mann, D. J.; Kraatz, H.-B. *Chem.—Eur. J.* **2015**, *21*, 4988.

(8) Bhattacharya, A. K.; Thyagarajan, G. *Chem. Rev.* **1981**, *81*, 415.

(9) McKenna, C. E.; Higa, M. T.; Cheung, N. H.; McKenna, M.-C. *Tetrahedron Lett.* **1977**, *18*, 155.

(10) Similar procedures starting with trialkyl phosphites were described previously and also yielded a mixture of various products; see ref 8 and: Lesnikowski, Z. J.; Schinazi, R. F. *J. Org. Chem.* **1993**, *58*, 6531.

(11) A similar observation was reported previously: McAllister, T. E.; Webb, M. E. *Org. Biomol. Chem.* **2012**, *10*, 4043.

(12) The procedure was adapted from previous syntheses of terminal phosphate-modified nucleotides; as examples, see: (a) Kadokura, M.; Wada, T.; Urashima, C.; Sekine, M. *Tetrahedron Lett.* **1997**, *38*, 8359. (b) Dabrowski-Tumanski, P.; Kowalska, J.; Jemielity, J. *Eur. J. Org. Chem.* **2013**, 2147. DMF (ca 2 mL per 1 mmol of compound **2**) was added to a flask with dry compound **1a**, **1c**, or **1e** (2.5−3.5 equiv), and the mixture was stirred at rt until complete dissolution of the C-phosphonate. Then, a P-imidazolide of the 5′-nucleotide (**2**, 1 equiv) along with $MgCl_2$ (4−8 equiv) were added. The mixture was stirred at rt for 1 h. The reaction was stopped by dilution with 10 volumes of water. The product was purified by ion-exchange chromatography on DEAE Sephadex A-25..

(13) The imidazolide derivatives were prepared using the standard Mukaiyama−Hashimoto protocol: Mukaiyama, T.; Hashimoto, M. *Bull. Chem. Soc. Jpn.* **1971**, *44*, 2284.

(14) Kielkowski, P.; Macíčková-Cahová, H.; Pohl, R.; Hocek, M. *Angew. Chem.* **2011**, *123*, 8886.

(15) An aqueous solution of analogue **3** (1 equiv, ∼25 mM) was mixed with a solution of azide **4** (1−1.5 equiv, ∼25 mM) in DMF or H_2O/*t*-BuOH or DMSO. Then, freshly prepared aqueous solutions of $CuSO_4{\cdot}H_2O$ (0.05−0.3 equiv) and sodium ascorbate (0.1−0.6 equiv) were added, and the mixture was stirred at rt for 0.5−2 h. The reaction was quenched by addition of Na_2EDTA (0.05−3.0 equiv depending on the amount of $CuSO_4$), and the product was purified using RP HPLC.

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