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Conjugation of Nucleosides and Oligonucleotides by [3+2] Cycloaddition

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Received September 20, 2007

A procedure is presented for copper(I)-catalyzed [3+2] cycloaddition of nucleosides and nucleotides in near-quantitative yield. Azido−alkyne cycloaddition was applied for the preparation of a range of adenosine dimers and derivatives with versatile functionality, as well as for the smooth condensation of two oligonucleotide strands. The described technology may find valuable application in the synthesis of oligonucleotide dimers and conjugates.

Synthetic oligonucleotides have proven to be highly valuable as antisense structures for the selective silencing of specific genes.[1,2] However, clinical use of oligonucleotides is still limited to a single example, i.e., fomivirsen (Vitravene),[3] due to drawbacks regarding potency, delivery, and duration of action.[4] Second-generation involving 2′-*O*-modification, morpholino, or locked nucleic acid (LNA)-based structures[4] shows promise but a remaining hurdle involves the poor cell internalization (∼1−2%) of oligonucleotides.[5] A promising solution to the inefficient delivery of oligonucleotides lies in conjugation, e.g., with cholesterol or polycationic groups for improvement of cellular association.[6] Alternatively, conjugates with peptides or carbohydrates lead to recognition by receptors on the surface of designated cells.[7] Consequently, a large number of antisense conjugates have been prepared over the years, mostly involving 5′-conjugation due to the fact that 3′-modification is much less straightforward. The alternative conjugation via a nucleobase is more synthetically accessible but may interfere with Watson−Crick base pairing and thus hybridization. Finally, a variety of 2′-substituted AONs has been described, in particular zwitterionic derivatives, but no 2′-AON conjugates.

Apart from antisense application, oligonucleotide conjugation is also suitable for helical structure stabilization, e.g., with intercalators or minor groove binders, or covalent attachment of reporter groups, e.g., fluorescent, electrochemical, or spin labels.[8]

We here wish to report the synthesis of 2′-azide or 2′-acetylene modified adenosines as versatile building blocks for application in the mild and efficient synthesis of a variety of oligonucleotide hetero- and homoconjugates.

From the onset, a suitable technology for nucleotide conjugation appeared to be the Cu(I)-catalyzed Huisgen [3+2] cycloaddition,[9] a technique mild enough for bioconjugation of virus capsid proteins,[10] cell-surface labeling of *E. coli*,[11] and protein based profiling in living cells.[12] On the basis of acetylene−azide cycloaddition, triazole isosteres have been prepared of

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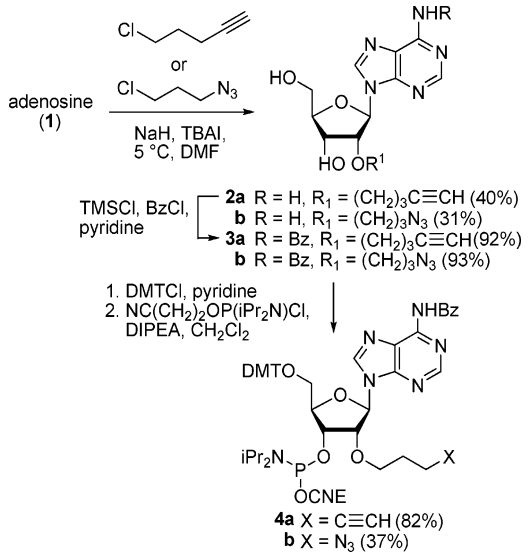
(1) (a) Zamecnik, P. C.; Stephenson, M. L. *Proc. Natl. Acad. Sci. U.S.A.* **1978**, *75*, 280−284. (b) Stephenson, M. L.; Zamecnik, P. C. *Proc. Natl. Acad. Sci. U.S.A.* **1978**, *75*, 285−288.
(2) (a) Muthiah, M. *Antisense Nucleic Acid Drug. Dev.* **2002**, *12*, 103−128. (b) Aboul-Fadl, T. *Curr. Med. Chem.* **2005**, *12*, 2193−2214.
(3) (a) Bennett, C. F. *Biochem. Pharmacol.* **1998**, *55*, 9−19. (b) Jarvis, L. M. *Chem. Eng. News* **2006**, 13−18.
(4) Corey, D. R. *Nature Chem. Biol.* **2007**, *3*, 8−11.
(5) (a) Gray, G. D.; Basu, S.; Wickstrom, E. *Biochem. Pharmacol.* **1997**, *53*, 1465−1476. (b) Stein, C. A.; Tonkinson, J. L.; Zhang. L. M.; Yakubov, L.; Gervasoni, J.; Taub, R.; Rotenberg, S. A. *Biochemistry* **1993**, *32*, 4855−4861. (c) Bennett, C. F.; Chiang, M. Y.; Chan, H.; Shoemaker, J. E.; Mirabelli, C. K. *Mol. Pharmacol.* **1992**, *41*, 1023−1033.
(6) Shi, F.; Hoekstra, D. J. *Controlled Release* **2004**, *97*, 189−209.
(7) Manoharan, M. *Antisense Nucleic Acid Drug Dev.* **2002**, *12*, 103−128.
(8) Zatepsin, T. S.; Stetsenko, D. A.; Gait, M. J.; Oretskaya, T. S. *Bioconj. Chem.* **2005**, *16*, 471−489.
(9) (a) Tornøe, C. W.; Christensen, C.; Meldal, M. J. *Org. Chem.* **2002**, *67*, 3057−3064. (b) Rostovtsev, V. V.; Green, L. G.; Fokin, V. V.; Sharpless, K. B. *Angew. Chem., Int. Ed.* **2002**, *41*, 2596−2599.
(10) Wang, Q.; Chan, T. R.; Hilgraf, R.; Fokin, V. V.; Sharpless, K. B.; Finn, M. G. *J. Am. Chem. Soc.* **2003**, *125*, 3192−3193.
(11) Link, A. J.; Tirrell, D. A. *J. Am. Chem. Soc.* **2003**, *125*, 11164−11165.
(12) Speers, A. E.; Adam, G. C.; Cravatt, B. F. *J. Am. Chem. Soc.* **2003**, *125*, 4686−4687.
(13) Bock, V. D.; Speijer, D.; Hiemstra, H.; van Maarseveen, J. H. *Org. Biomol. Chem.* **2007**, *5*, 971−975 and references cited herein.
(14) Wilkinson, B. L.; Bornaghi, L. F.; Poulsen, S. A.; Houston, T. A. *Tetrahedron* **2006**, *62*, 8115−8125.
(15) (a) Kuijpers, B. H. M.; Groothuys, S.; Keereweer, A. R.; Quaedflieg, P. J. L. M.; Blaauw, R. H.; van Delft, F. L.; Rutjes, F. P. J. T. *Org. Lett.* **2004**, *6*, 3123−3126. (b) Dondoni, A.; Giovannini, P. P.; Massi, A. *Org. Lett.* **2004**, *6*, 2929−2932. (c) Lin, H.; Walsh, C. T. *J. Am. Chem. Soc.* **2004**, *126*, 13998−14003.
(16) (a) Lazrek, H. B.; Engels, J. W.; Pfleiderer, W. *Nucleosides Nucleotides* **1998**, *17*, 1851−1856. (b) Park, S. M.; Lee, Y. S.; Kim, B. H. *Chem. Commun.* **2003**, 2912−2913. (c) Gierlich, J. G.; Burley, G. A.; Gramlich, P. M. E.; Hammond, D. M.; Carell, T. *Org. Lett.* **2006**, *8*, 3639−3642. (d) Burley, G. A.; Gierlich, J.; Mofid, M. R.; Nir, H.; Tal, S.; Eichen, Y.; Carell, T. *J. Am. Chem. Soc.* **2006**, *128*, 1398−1399. (e) Seela, F.; Sirivolu, V. R. *Chem. Biodiv.* **2006**, *3*, 509−514. (f) Kosiova, I.; Kovackova, S.; Kois, P. *Tetrahedron* **2007**, *63*, 312−320. (g) Seela, F.; Sirivolu, V. R. *Helv. Chim. Acta* **2007**, *90*, 535−552.
(17) (a) Seo, T. S.; Li, Z.; Ruparel, H.; Ju, J. *J. Org. Chem.* **2003**, *68*, 609−612. (b) Lee, L. V.; Mitchell, M. L.; Huang, S.-J.; Fokin, V. V.; Sharpless, K. B.; Wong, C.-H. *J. Am. Chem. Soc.* **2003**, *125*, 9588−9589. (c) Weller, R. L.; Rajski, S. R. *Org. Lett.* **2005**, *7*, 2141−2144. (d) Devaraj, N. K.; Miller, G. P.; Ebina, W.; Kakaradov, B.; Collman, J. P.; Kool, E. T.; Chidsey, C. E. D. *J. Am. Chem. Soc.* **2005**, *127*, 8600−8601. (e) Bouillon, C.; Meyer, A.; Vidal, S.; Jochum, A.; Chevolot, Y.; Cloarec, J.-P.; Praly, J.-P.; Vasseur, J.-J.; Morvan, F. *J. Org. Chem.* **2006**, *71*, 4700−4702. (f) Meyer, A.; Bouillon, C.; Vidal, S.; Vasseur, J.-J.; Morvan, F. *Tetrahedron Lett.* **2006**, *47*, 8867−8871. (g) Kosiova, I.; Kovackova, S.; Kois, P. *Tetrahedron* **2007**, *63*, 312−320.

10.1021/jo702023s CCC: $40.75
Published on Web 12/04/2007

SCHEME 1. Adenosine 2′-*O*-Alkylation and Processing to Phosphoramidite Building Blocks 4a and 4b

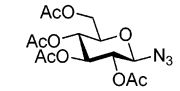
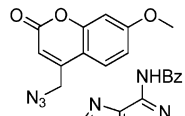
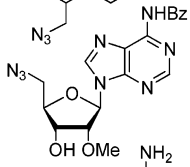
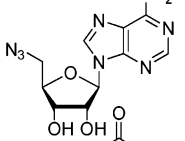
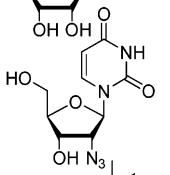
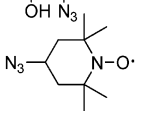


biopolymers such as proteins,[13] sugars,[14] and combinations thereof.[15] Triazole derivatives of nucleotides have also been reported, attached at the nucleobase[16] or at the 5′-position.[17] We recognized that, with a single exception,[17f] in all cases acetylene modified nucleosides were applied, forming a limiting factor in the design of the conjugation partner. Moreover, for application in antisense conjugates, nucleobase modification will in most cases be unfavorable for hybridization, whereas 5′-conjugation is clearly of interest but only of marginal improvement with respect to known protocols.

In contrast, substitution at 2′-OH, a known strategy to lock ribose in a 3′-*endo* conformation, allows the introduction of either an acetylene or an azide. Moreover, a 2′-modification can be tolerated at any ribose of the oligonucleotide backbone, including multiple modifications. Thus, we decided to prepare 2′-*O*-(3-azidopropyl) and 2′-*O*-(4-pentynyl) modified adenosine, with reduced steric interaction in comparison to the shorter 2′-*O*-(2-azidoethyl)[18] and 2′-*O*-propargyl,[19] and a potential positive effect on hybridization.[20] To avoid the common lengthy routes to 2′-modified nucleobases, direct 4-pentynylation or 3-azidopropylation of adenosine 2′-OH was investigated.[21] Gratifyingly, it was found that treatment of a solution of adenosine (**1**) in DMF with sodium hydride and an alkyl chloride at 5 °C, followed by stirring for 3 days at 55 °C, led to a mixture of 2′- and 3′-*O*-alkylated isomers (ratio ∼5:1), of which the desired 2′-*O*-isomer **2** could be readily fractionated by column chromatography and selective crystallization from ethanol.

With the requisite adenosyl acetylene (**2a**) and azide (**2b**) at hand, Cu(I) catalyzed [3+2] cycloaddition of **2a** with benzyl azide was explored. Initial attempts on Huisgen cycloaddition catalyzed by $Cu(OAc)_2$ and sodium ascorbate in a mixture of water/^{t}BuOH or water/DMSO at room temperature or at elevated temperature led to poor conversion and significant green coloring of the solution, an effect presumably caused by complexation of copper(II) to the free adenine. A more rewarding result was obtained by stirring a stoichiometric mixture of **2a** and benzyl azide in the presence of copper wire in a 9:1 mixture of CH_3CN and H_2O at 35 °C, leading to the formation of the desired triazole in excellent yield (Table 1, entry 1). It was found that removal of the copper wire after 1 h avoided the undesired green coloring. Much to our satisfaction, brief stirring in the presence of copper wire led to successful [3+2] cycloaddition for a range of different azides, as summarized in Table 1. For example, adenosine derivative **2a** was conjugated to a fluorescent coumarin label (entry 3), three different azidonucleosides (entries 4−6), as well as a nitroxyl spin label, leading to the desired triazole products in near-quantitative yields in all cases.

TABLE 1. Copper(I)-Catalyzed [3+2] Cycloaddition of 2a with Various Azides

entry	R—N_3	time (h)	yield (%)
1	BnN_3	18	94
2		24	98
3		18	97
4		12	98
5		12	96
6		12	95
7		16	95

Similar to the acetylene modified adenosine **2a**, the 2′-*O*-azide functionalized adenosine **2b** (Table 2) underwent smooth Huisgen cycloaddition with a diverse set of reaction partners, including a propargylated coumarin (entry 3) and a fluorescence quencher (entry 5). The latter compound may be of particular interest for the application of FRET studies of antisense strand labeled with fluorescent probes.

Having access to a range of functionalized adenosine monomers, the remaining key question to be addressed concerned the applicability of the building blocks **2a** and **2b** for incorporation into an oligonucleotide strand. We were also intrigued if such a high level of efficiency in cycloaddition

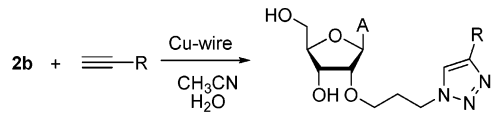
(18) Grøtli, M.; Douglas, M.; Eritja, R.; Sproat, B. S. *Tetrahedron* **1998**, *54*, 5899−5914.

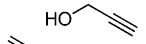
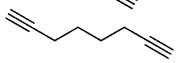
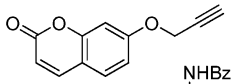
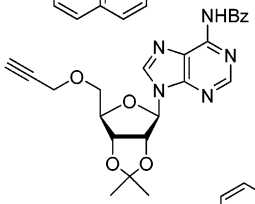
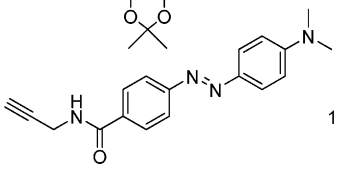
(19) Egli, M.; Minasov, G.; Tereshko, V.; Pallan, P. S.; Teplova, M.; Inamati, G. B.; Lesnik, E. A.; Owens, S. R.; Ross, B. S.; Prakash, T. P.; Manoharan, M. *Biochemistry* **2005**, *44*, 9045−9057.

(20) Cuenoud, R.; Casset, F.; Hüsken, N. F.; Wolf, R. M.; Almann, K.-H.; Martin, P.; Moser, H. E. *Angew. Chem., Int. Ed.* **1998**, *37*, 1288−1291.

(21) (a) Yano, J.; Kan, L. S.; Ts'O, P. O. P. *Biochim. Biophys. Acta* **1980**, *629*, 178−183. (b) Wagner, E.; Oberhauser, B.; Halzner, A.; Brunar, H.; Issakides, G.; Schaffner, G.; Cotten, M.; Knollmüller, M.; Noe, C. R. *Nucl. Acids Res.* **1991**, *19*, 5965−5971.

TABLE 2. Copper(I)-Catalyzed [3+2] Cycloaddition of 2b with Various Acetylenes



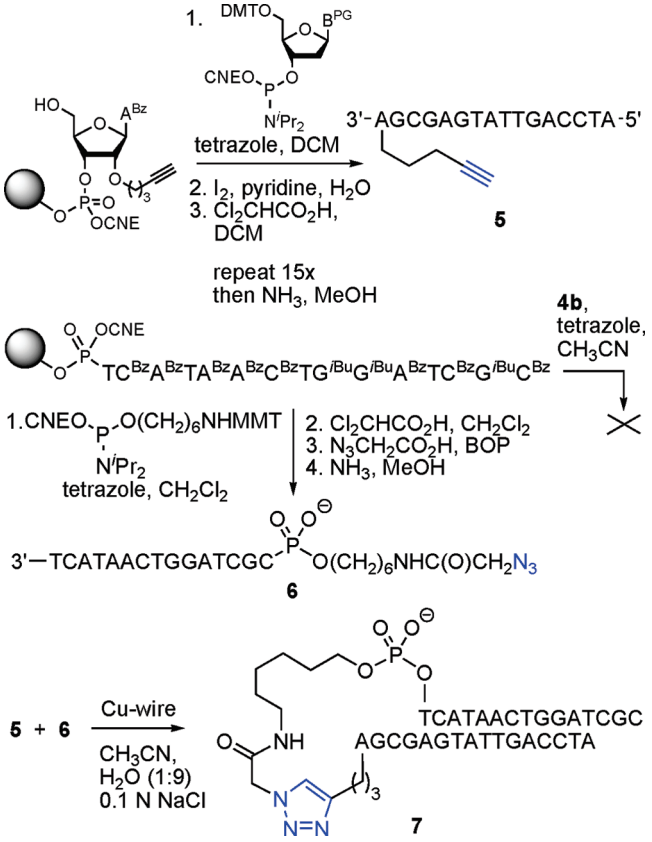
entry	—R	time (h)	yield (%)
1		2	97
2		16	90
3		5	98
4		12	92
5		16	98

would be obtained in the conjugation of two oligonucleotide strands. RNA chimeras find use, for example, in the selective delivery of siRNA to prostate cancer cells,[22] but so far such chimeras have not been prepared via azide−acetylene condensation. Some Cu(I)-catalyzed cycloadditions of acetylene charged nucleotides and azides are known, but require excess azide[17a] or microwave conditions.[17e,f] Thus, to prepare the requisite oligonucleotides, the 2′-modified nucleosides **2a** and **2b** were converted into the corresponding 5′-*O*-DMT-3′-*O*-phosphoramidite derivatives **4a** and **4b** with use of standard protocols. It was noticed that, whereas the conversion of **3a** into **4a** proceeded without incidence, the high instability of **4b** necessitated rapid synthesis (40 min), workup, purification on silica gel (5 min), and concentration. At this stage, the suitability of phosphoramidites **4a**/**b** for either 3′- or 5′-incorporation into an oligonucleotide (DNA) strand was investigated by automated solid-phase synthesis on universal CPG (Scheme 2). Gratifyingly, attachment of the acetylene-modified phosphoramidite **4a** to the resin, as well as repetitive coupling with standard 2′-deoxyribonucleoside phosphoramidites, proceeded smoothly to give the 16-mer chain 3′-rA[$(CH_2)_3$(C≡CH)d(GCGAGTATTGACCTA)-5′ (**5**) in excellent yield and purity after cleavage from the resin. The azido-containing phosphoramidite **4b**, on the other hand, failed to incorporate into the nucleotide 3′-(dTCATAACTGGATCGC)-rA[$(CH_2)_3N_3$]-5′, giving solely the truncated 15-mer. Apparently, the azido function negatively influenced the coupling behavior of the 3′-*O*-phosphoramidite, presumably due to iminophosphorane formation by Staudinger reaction, as reported for 2-azidoadenosine.[23] Indeed, simply dissolving **4b** in acetonitrile led to spontaneous degradation, as judged by ^{31}P NMR, with

(22) (a) McNamara, J. O.; Andrechek, E. R.; Wang, Y.; Viles, K. D.; Rempel, R. E.; Gilboa, E.; Sullenger, B. A.; Giangrande, P. H. *Nat. Biotechnol.* **2006**, *24*, 1005−1015. (b) Chu, T. C.; Twu, K. Y.; Ellington, A. D.; Levy, M. *Nucleic Acids Res.* **2006**, *34*, e73.

(23) Wada, T.; Mochizuki, A.; Higashiya, S.; Tsuruoka, H.; Kawahar, S.; Ishikawa, M.; Sekine, M. *Tetrahedron Lett.* **2001**, *42*, 9215−9219.

SCHEME 2. Solid-Phase Synthesis of 2′-*O*-Modified Oligonucleotides 5 and 6 and [3+2] Cycloaddition



near-complete disappearance of starting material after two weeks. Consequently, an alternative approach was conceptualized involving the introduction of azido functionality after oligonucleotide assembly. To this end, an *N*-MMT protected phosphoramidite was attached at the 5′-terminus of the 15-mer oligonucleotide, followed by deprotection and azidoacetylation to give, after base cleavage, compound **6**.

Finally, [3+2] cycloaddition of fragments **5** and **6** was attempted. First of all, a template-directed cycloaddition of the two complementary sequences was attempted.[24] It was found that no reaction took place in the absence of copper, but treatment of the two strands **5** and **6** in an CH_3CN/H_2O mixture (1:9) with copper wire led to rapid disappearance of both nucleotides and the formation of a single new compound as judged by HPLC. The resulting product was unambiguously identified as the triazole-linked conjugate **7** by MALDI-TOF mass spectrometry.

In conclusion, a fast and practical procedure for the 2′-*O*-alkylation of adenosine was developed for introduction of azido or acetylene functionality, which could be converted into a range of conjugates including other nucleosides, spin-labels, and fluorescent probes. Conversion of the acetylene containing compound **4a** into the corresponding phosphoramidite proceeded smoothly, but not for azide **4b**, necessitating azide introduction at a late stage. Conjugation of the oligonucleotides by 1,3-dipolar cycloaddition readily occurred in the presence of copper wire,

(24) (a) Poulin-Kerstien, A. T.; Dervan, P. B. *J. Am. Chem. Soc.* **2003**, *125*, 15811−15821. (b) Kumar, R.; El-Sagheer, A.; Tumpane, J.; Lincoln, P.; Wilhelmsson, L. M.; Brown, T. *J. Am. Chem. Soc.* **2007**, *129*, 6859−6864.

thus paving the way for the synthesis of unique, spacer-linked oligonucleotide chimeras.

Experimental Section

Synthesis of 2′-*O*-(1-Pentyn-5-yl)adenosine (2a). Adenosine (5 g, 18.72 mmol) was dissolved in hot anhydrous DMF (250 mL) under an inert atmosphere. The solution was cooled to 5 °C, and NaH (1 g, 25 mmol, 60% dispersion in mineral oil) was subsequently added, followed by the addition of TBAI (1.5 g, 4.06 mmol) and 5-chloro-1-pentyne (2.5 mL, 23.84 mmol). The reaction mixture was allowed to stir for 3 days at 55 °C. Evaporation of the solvent under reduced pressure resulted in a suspension, which was absorbed on silica gel. Flash chromatography (CH_2Cl_2/MeOH, 95:5) gave a mixture of 2′-*O*- and 3′-*O*-alkynylated products. Selective crystallization from anhydrous ethanol provided the title compound 1 (2.5 g, 40%) as colorless needles. ^{1}H NMR (300 MHz, DMSO) δ 8.36 (s, 1H), 8.12 (s, 1H), 7.33 (br s, 2H), 6.00 (d, J = 6 Hz, 1H), 5.39 (m, 1H), 5.17 (d, J = 5.4 Hz, 1H), 4.46 (m, 1H), 4.29 (m, 1H), 3.97 (m, 1H), 3.64−3.32 (m, 4H), 2.64 (m, 1H), 2.08 (m, 2H), 1.58 (m, 2H); ^{13}C NMR (75 MHz, DMSO) δ 156.1, 152.5, 149.0, 139.7, 119.3, 86.3, 86.0, 83.9, 81.0, 71.1, 69.0, 68.3, 61.4, 28.1, 14.3; HRMS calcd for $C_{15}H_{19}N_5O_4$ (M + Na^+) 356.1335, found 356.1308.

The same procedure with 3-azido-1-chloropropane instead of 5-chloro-1-pentyne provided compound **2b**. ^{1}H NMR (300 MHz, DMSO) δ 8.36 (s, 1H), 8.12 (s, 1H), 7.32 (br s, 2H), 6.00 (d, J = 6 Hz, 1H), 5.38 (m, 1H), 5.20 (d, J = 5.4 Hz, 1H), 4.46 (m, 1H), 4.29 (m, 1H), 3.97 (m, 1H), 3.64−3.43 (m, 4H), 3.27 (m, 2H), 1.67 (m, 2H); ^{13}C NMR (75 MHz, DMSO) δ 156.1, 152.5, 149.0, 139.6, 119.3, 86.3, 86.0, 81.0, 68.0, 66.7, 61.4, 47.6, 28.5; HRMS calcd for $C_{13}H_{18}N_8O_4$ (M + Na^+) 351.1529, found 351.1525.

General Procedure for Copper(I)-Catalyzed Azido−Alkyne Cycloaddition (CuAAC). 2′-*O*-Pentynyladenosine **2a** (67 mg, 0.2 mmol) and 1 equiv of an azido compound were dissolved in a mixture of acetonitrile (2 mL) and water (0.2 mL). The reaction mixture was stirred at 35 °C in the presence of a Cu-wired stirring bar. After 1 h, the stirring bar was removed and stirring was prolonged until all starting material was consumed. Concentration in vacuo and purification by flash chromatography (CH_2Cl_2/MeOH, 95:5) furnished the triazole-functionalized nucleosides. The same procedure was applied to azidopropyladenosine **2b** by stirring with an acetylene reaction partner.

Analytical Data for the CuAAC-Adduct of 2a and 5′-Azido-5′-deoxyadenosine (entry 5 of Table 1). ^{1}H NMR (300 MHz DMSO) δ 8.37, 8.17, 8.13.(s,1H), 8.11 (s, 1H), 7.60 (s, 1H), 7.32 (s, 2H), 5.98 (d, J = 6.0 Hz, 2H), 5.97 (d, J = 5.1 Hz), 5.79−5.29 (br s, 4H), 4.66−4.65 (m , 3H), 4.46 (t, J = 7.2 Hz, 1H), 4.31 (br s, 1H), 4.21 (br s, 2H), 3.97 (d, J = 3 Hz), 3.67−3.64 (m, 3H), 1.72−1.61 (m, 2H); ^{13}C NMR (75 MHz DMSO) δ 156.1, 156.0, 152.7, 152.5, 149.2, 149.0, 146.2, 139.8, 122.6, 119.3, 119.2, 87.7, 86.3, 86.0, 82.3, 80.8, 72.6, 70.9, 68.9, 68.7, 61.5, 60.6, 51.2, 28.9, 21,23; HRMS calcd for $C_{25}H_{31}N_{13}O_7$ (M + Na^+) 648.2367, found 648.2326.

Acknowledgment. The European Commission is kindly acknowledged for financial support (FSG-V-RNA).

Supporting Information Available: General procedures, synthesis, and characterizations of all new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

JO702023S