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Preparation of 5'-Azido-5'-Deoxyguanosine and Its Efficiency for Click Chemistry

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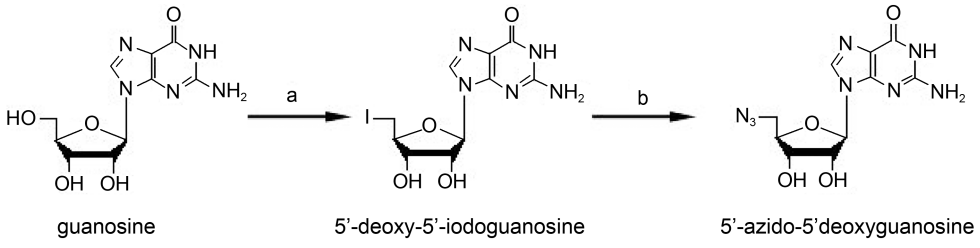
RNA plays important roles in many fundamental processes in cells, including regulation of protein biosynthesis, RNA splicing, and retroviral replication, with remarkable features.[1] In this regard, site-specific substitution and derivatization of RNA can provide powerful tools for studying RNA structure and function.[2] The modification of either the 3'- and 5'-termini or an internal position of the oligonucleotides with a primary alkylamine group is a widely used method for introducing additional functional groups to the RNA.[2,3] In particular, several 5'-modifications of RNA molecules such as sulfhydryl modification for functionalizing the 5'-terminus of RNA by a transcription or kinase reaction have been shown to have broad applications in studying RNA structures, mapping RNA-protein interactions, and *in vitro* selection of catalytic RNAs,[4] since a unique functional group incorporated into the RNA can be subsequently conjugated to the desired molecule by a selective chemical reaction.

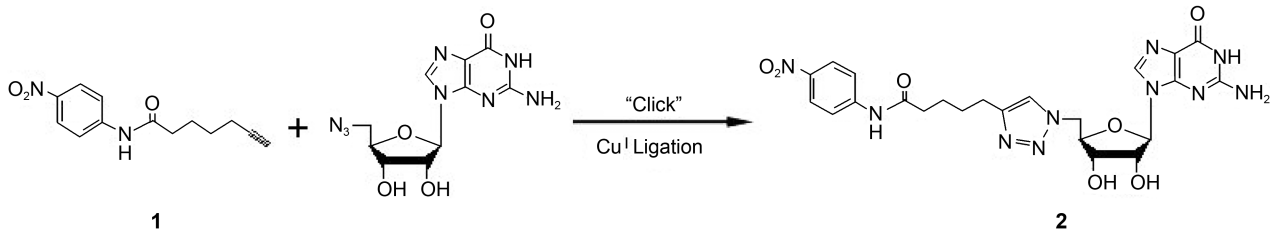
There is, however, still a need to develop coupling chemistry with high stability and yield to modify RNA and other biomolecules. Ideal coupling functional groups are required to be stable under aqueous reaction conditions, and the coupling reaction should be highly chemoselective.[5] From this point of view, Cu^{I}-catalyzed azide-alkyne cycloaddition (CuAAC or click chemistry) to form the triazole version of Huisgen's [2+3] cycloaddition family may be the best choice,[6] because this 1,3-dipolar cycloaddition chemistry is very chemoselective, only occurring between alkynyl and azido functional groups with high yield, and because the resulting 1,2,3-triazoles are stable at aqueous conditions and high temperature.

We report here a two-step synthetic method for 5'-azido-5'-deoxyguanosine by adapting literature procedures[3,7] (Scheme 1) and its efficiency for click chemistry using 6-heptynoyl *p*-nitroaniline (see Experimental Section), in consideration that the click chemistry with rapid reaction between the azide and alkyne groups to form a covalent triazole linkage without cross reacting with other functional groups has been used to great effect in bioconjugation chemistry and that RNA has an inherently much more labile ribophosphate backbone than DNA due to the existence of the 2'-hydroxy groups vicinal to the internucleotide phosphate, which has likely deterred researchers from using Cu^{I} click chemistry on RNA.

As summarized in Scheme 1, 5'-iodo-5'-deoxyguanosine (or iodinated guanosine) was synthesized as previously



Scheme 1. Synthetic scheme for 5'-azido-5'-deoxyguanosine. Reagents and conditions: (a) $P(Ph)_3$, I_2, imidazole, *N*-methyl-2-pyrrolidinone, 3 h, 71%; (b) NaN_3, DMF (dry), 80 °C, 24 h, 69%.



Scheme 2. Click chemistry between alkynyl and azido functional groups. 5'-Azido-5'-deoxyguanosine can be used for the click chemistry reaction with 6-heptynoyl *p*-nitroaniline **1**, in order to quantitatively monitor click labeling of the azide nucleoside to the alkyne dye.

described,[3] and used for reaction with sodium azide for preparation of 5'-azido-5'-deoxyguanosine. 5'-Azido-5'-deoxyguanosine was characterized by proton NMR and then quantitatively tested as a substrate for the click chemistry with 6-heptynoyl *p*-nitroaniline (Scheme 2), since the nitro functional group of 6-heptynoyl *p*-nitroaniline can be used to monitor and quantify click labeling of the azide nucleoside to the alkyne dye.

HPLC chromatogram reveals the utility of 6-heptynoyl *p*-nitroaniline **1** constructed by the click chemistry for 5'-azido-5'-deoxyguanosine analysis. In the presence of $CuSO_4$ and copper wire, compound **1** was mixed with 5'-azido-5'-deoxyguanosine at room temperature for 24 h following the literature procedures,[8] and the reaction mixture was subjected to the HPLC experiments by adapting literature conditions.[9] As shown in Figure 1, before the click chemistry a single major peak that was assigned as 6-heptynoyl *p*-nitroaniline was monitored at 350 nm (upper chromatogram), while the lower chromatogram after the click chemistry demonstrates that 5'-azido-5'-deoxyguanosine was almost completely consumed and converted to compound **2** *via* the click chemistry. The collected HPLC fractions for compound **2** were sampled for UV absorbance measurement as illustrated in Figure 2, of which the λ_{max} is the same with that of 6-heptynoyl *p*-nitroaniline. These results indicate that 5'-azido-5'-deoxyguanosine may be used for the click chemistry for rapid labeling and ligation of RNA, as long as the "clickable" azido group is able to be synthetically or enzymatically introduced to the RNA 5'-termini, because it is known[5,6] that RNA degradation is minimized through stabilization of the Cu^{I} in aqueous buffer with acetonitrile as cosolvent and no other ligand and that the ability to use click chemistry directly to label RNA would offer a more rapid process and provide an orthogonal method to the current *N*-hydroxysuccinimide (NHS) chemistry that would enable more facile dual labeling of oligonucleotides. In this respect,

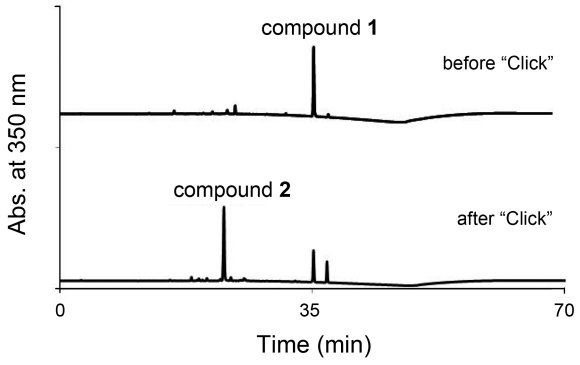


Figure 1. HPLC chromatogram before and after the click chemistry between 5'-azido-5'-deoxyguanosine and 6-heptynoyl *p*-nitroaniline **1**. In the presence of $CuSO_4$ and copper wire, 6-heptynoyl *p*-nitroaniline **1** was mixed with 5'-azido-5'-deoxyguanosine at room temperature for 24 h (see Experimental Section), and the reaction mixture was subjected to the HPLC experiments monitored simultaneously at 260 nm and 350 nm. Retention times of ~36 min for compound **1** and ~23 min for compound **2** were assigned, respectively, under the HPLC conditions employed.

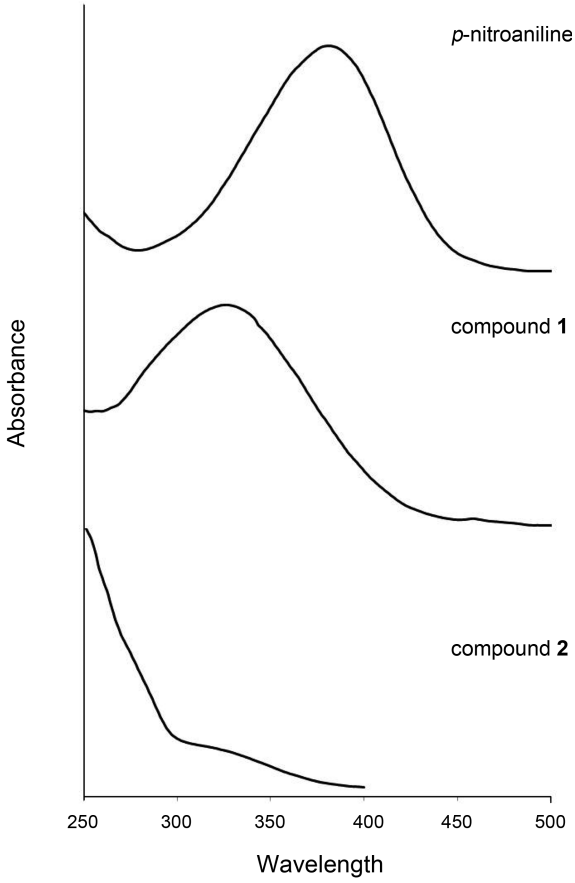


Figure 2. UV/Vis spectra for *p*-nitroaniline, 6-heptynoyl *p*-nitroaniline **1**, and compound **2**.

further studies on whether 5'-azido-5'-deoxyguanosine can be synthetically and enzymatically installed on RNA are in progress, and will be published elsewhere.

In summary, we have developed an efficient two-step synthesis of 5'-azido-5'-deoxyguanosine from guanosine and showed that 5'-azido-5'-deoxyguanosine can be used for the click chemistry. Further improvement for the methodology will allow reduced reaction time by attaching an electron-withdrawing functional group at the end of the triple bond. It is expected that the optimized click chemistry will have potential applications in bioconjugation fields such as RNA covalent attachment on a chip, chemoselective protein modification, and immunoassays.

Experimental Section

Unless otherwise noted, reagents were obtained from commercial suppliers and were used without further purification. ^{1}H NMR spectra were carried out on Bruker 400 MHz spectrometer and TMS was used as an internal reference for ^{1}H. UV absorbance was measured using Agilent 8453 UV-Visible spectrophotometer. All experiments were performed in duplicate.

5'-Deoxy-5'-iodoguanosine was synthesized as previously described.[3]

Synthesis of 5'-Azido-5'-deoxyguanosine. By adapting

literature procedures,[7] a mixture of 5'-deoxy-5'-iodoguanosine (74 mg, 0.19 mmol) and sodium azide (25 mg, 0.38 mmol) in dry DMF (0.5 mL) was stirred at 80 °C under argon for 20 h. After cooling to room temperature, the solvent was removed under reduced pressure and the residue was stirred in water (1 mL) for 30 min. The resulting solid was collected by filtration and then washed successively with water (0.5 mL), cold ethanol (0.3 mL) and diethyl ether (0.2 mL) before drying *in vacuo* to give 5'-azido-5'-deoxyguanosine as a colorless solid (69%). ^{1}H NMR (400 MHz, DMSO-d_6): 3.52 (dd, J = 13.2, 3.6 Hz, 1H), 3.67 (dd, J = 13.2, 7.2 Hz, 1H), 3.99 (m, 1H), 4.06 (m, 1H), 4.58 (m, 1H), 5.2-5.7 (br, exchanged with D_2O, 2H), 5.72 (d, J = 5.6 Hz, 1H), 6.50 (br, exchanged with D_2O, 2H), 7.90 (s, 1H) and 10.3-10.9 (br, exchanged with D_2O, 1H).

Synthesis of 1. 6-Heptynoic acid (10 mmol) and *p*-nitroaniline (10 mmol) were dissolved in dry pyridine (30 mL). The clear yellowish solution was cooled to −15 °C and phosphorus oxychloride (11 mmol) was added dropwise with vigorous stirring. During the addition the reaction mixture turned deep red and became turbid in the course of 15 min. The color of the suspension slowly changed to orange, the reaction being complete in approximately 30 min (monitored by TLC). The reaction mixture was then quenched with crushed ice and water (100 mL) and the nitroanilide was extracted with ethyl acetate. The combined organic layers were washed successively with saturated $NaHCO_3$ and NaCl solutions. The residue was coevaporated successively with toluene, ethyl acetate and MeOH to remove residual pyridine. To remove unreacted *p*-nitroaniline, the crude reaction product was suspended in diethyl ether, filtered, and subsequently recrystallized. ^{1}H NMR (400 MHz, DMSO-d_6): 1.48-1.51 (m, 2H), 1.67-1.71 (m, 2H), 2.18-2.22 (m, 2H), 2.38-2.42 (m, 2H), 2.76 (s, 1H), 7.8 (d, 2H), 8.2 (d, 2H), 10.5 (s, 1H).

Click Chemistry. By adapting literature procedures,[8] 6-heptynoyl *p*-nitroaniline **1** (20.4 mg, 83 mmol), 5'-azido-5'-deoxyguanosine (12.9 mg, 42 μmol), $CuSO_4$ (0.4 mM, 4.2 μmol), and copper wire (20 mg, 0.315 mmol) were stirred at room temperature in solvent mixture of EtOH:H_2O:*t*-BuOH (2:3:5) until all 5'-azido-5'-deoxyguanosine was consumed (~24 h). The reaction was monitored by TLC (5:2:2, isopropanol:NH_4OH:H_2O). The copper wire was removed and the solvent mixture was evaporated *in vacuo*. The crude reaction product was resuspended in acetonitrile and loaded onto a 250-mm × 4.6-mm Hypersil ODS column (Thermo Electron Corporation, Waltham,MA) for HPLC experiments according to the literature.[9] Briefly, solvents A and B were 50 mM phosphate buffer (pH 7.0) and 70% acetonitrile, respectively. An isocratic elution of 0% B for 5 min was followed by the gradient elution from 0% B to 100% B in 40 min. A flow rate of 1.0 mL/min was used, resulting in retention times of ~36 min for compound **1** and ~23 min for compound **2**, respectively, as shown in Figure 1. Products were monitored simultaneously at 260 nm and 350 nm. Collected HPLC fractions for compounds **1** and **2** were sampled for UV absorbance measurement.

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