Solid-Supported Synthesis and Click Conjugation of 4′-*C*-Alkyne Functionalized Oligodeoxyribonucleotides

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4′-*C*-[*N,N*-Di(4-pentyn-1-yl)aminomethyl]thymidine and 4′-*C*-[*N*-methyl-*N*-(4-pentyn-1-yl)aminomethyl]thymidine 3′-(2-cyanoethyl-*N,N*-diisopropyl)phosphoramidites (**1**, **2**) were synthesized, and one or two such monomers were incorporated into a 15-mer oligodeoxyribonucleotide. After chain assembly, azido-functionalized ligands, including appropriate derivatives of 1,4-phenylenedimethaneamine, mannose, paromamine, and neomycin, were conjugated to the alkynyl groups by the click chemistry on a solid support. The influence of the 4′-modifications on the melting temperature with DNA and 2′-*O*-methyl RNA targets was studied. Oligonucleotides containing one to four mannose ligands in the central part of the chain (up to two 4′-*C*-[*N,N*-di(4-pentyn-1-yl)aminomethyl]thymidine units) form equally stable duplexes with complementary 2′-OMe RNA as the corresponding unmodified DNA sequence. At high salt content, the mannose conjugation is even stabilizing. On using a DNA target, a modest destabilization occurs. All the amino group bearing conjugates stabilized the duplexes, the DNA·DNA duplexes more than the DNA·2′-*O*-methyl RNA duplexes.

INTRODUCTION

Conjugation to peptides, carbohydrates, or small molecule compounds has been extensively studied as a possible way to improve the delivery and targeting of siRNA and antisense oligonucleotides (*1*−*15*), and the synthesis of such conjugates has been recently reviewed (*16*−*21*). Both carbohydrate−carbohydrate (*22, 23*) and carbohydrate−protein interactions (*24*−*26*) are known to play a central role in cellular recognition. These interactions are multipodal; simultaneous interaction with several monosaccharide ligands is required for high-affinity binding (*27*−*30*). Accordingly, the most attention has been paid to preparation of oligonucleotide conjugates bearing a multiarmed glycocluster at either terminus of the chain (*31*−*36*). As far as glycotargeting of siRNA is concerned, another approach appears, however, possible, viz., decoration of the sense strand with sugars. A prerequisite for this approach is that the sugar ligands do not markedly destabilize the duplex. In other words, the sense strand is aimed at serving as a scaffold to which the lectin-binding sugar ligands are tethered at appropriate distances throughout the molecule. Since the carrier is simultaneously able to hybridize with the antisense strand, the latter becomes enriched at the cell surface and, hence, internalizated by receptor-mediated endocytosis.

Several examples of oligonucleotide glycoconjugates bearing the sugars at intrachain positions have been published. These cannot, however, be exploited as sense strands in siRNA, since the sugar ligands are attached either to base moieties (*37*−*41*) or to non-nucleosidic units incorporated in the sugar−phosphate backbone (*42*−*44*). Both of these conjugations markedly destabilize the duplex. In fact, the only example of conjugation that does not markedly affect the duplex stability is offered by 4′-*C*-tethered glycoconjugates obtained by subjecting a 4′-*C*-azidomethyl incorporating oligonucleotide to Cu(I) catalyzed 1,3-dipolar cycloaddition, the so-called click reaction (*45, 46*), with an alkyne-derivatized sugar (*47*). The latter approach, however, suffers from the limitation that the 4′-*C*-azidomethyl unit has to be introduced into the chain by H-phosphonate coupling, since the intramolecular Staudinger reaction prevents preparation of the phosphoramidite building block. In the present study, the corresponding alkyne-derivatized nucleosides, viz., 4′-*C*-[*N,N*-di(4-pentyn-1-yl)aminomethyl]thymidine and 4′-*C*-[*N*-methyl-*N*-(4-pentyn-1-yl)aminomethyl]thymidine monomers (**1**, **2**), have been synthesized and incorporated into oligonucleotides by phosphoramidite chemistry. The 4-pentynyl group was chosen as the alkyne spacer to provide the 4′-*C*-modified nucleoside with sufficient flexibility to ensure efficient click reaction with azides (*48*) and proper alignment of the conjugate groups upon duplex formation. A related strategy has previously been applied to solid-supported derivatization of oligonucleotides containing ω-alkynylated nucleobases (*48, 49*). Monomers **1** and **2** allowed the attachment of two or one conjugative group per modified residue, respectively (Figure 1). The ligands used for the conjugation included azido derivatives of mannose (**3**), *p*-aminomethylbenzene (**4**), and aminoglycosides paromamine (**5**) and neomycin (**6**). The effects of these conjugate groups on the melting temperature of the duplexes with cDNA and 2′-*O*-methyl RNA sequences were determined to assess the contributions of electrostatic interactions and sterical factors to the efficiency of hybrization.

EXPERIMENTAL PROCEDURES

General Remarks. The NMR spectra were recorded at 500 MHz. The mass spectra were recorded using EI or ESI ionization. The RP HPLC analyses of the oligonucleotide conjugates were performed using a Thermo ODS Hypersil C18 (250 × 4.6 mm, 5 μm) analytical column, gradient elution from 0% to 50% MeCN in 0.1 mol L^{-1} triethylammonium acetate (aq) buffer over 60 min, flow rate 1 mL min^{-1}, and detection at 260 nm.

4′-*C*-Aminomethyl-3′-*O*-(4,4′-dimethoxytrityl)thymidine (8). 4′-*C*-Azidomethyl-3′-*O*-(4,4′-dimethoxytrityl)thymidine (2.14 g, 3.6 mmol) (*47*) was dissolved in THF (24 mL). Triphenylphosphine (1.50 g, 5.7 mmol), water (64 μL), and a drop of 33% aq NH_3 (∼20 μL) was added, and the reaction was stirred overnight. The reaction mixture was evaporated to dryness and purified by silica gel chromatography (10−20% MeOH in DCM containing 0.1% triethylamine). The yield was 1.54 g (75%) as a white solid foam. ^{1}H NMR (500 MHz, CD_3CN) δ_{ppm} 7.50−7.47

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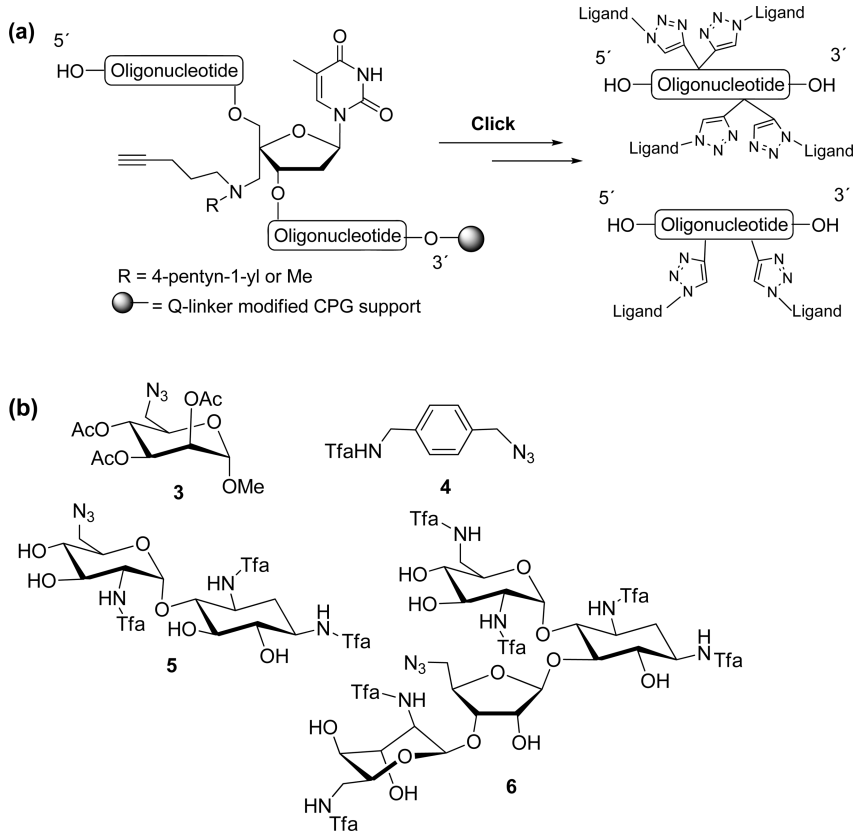


Figure 1. (a) Solid-supported conjugation of oligodeoxyribonucleotides by the click reaction. (b) Azido-functionalized ligands used in the click reaction.

(m, 2H), 7.40−7.32 (m, 6H), 7.27 (m, 1H), 7.09 (br d, 1H, J = 1.0 Hz), 6.93−6.88 (m, 4H), 6.12 (dd, 1H, J = 8.0 Hz, 4.0 Hz), 4.34 (dd, 1H, J = 7.5 Hz, 7.5 Hz), 3.79 (s, 3H), 3.78 (s, 3H), 3.60 (d, 1H, J = 12.0 Hz), 3.40 (d, 1H, J = 12.0 Hz), 3.34 (d, 1H, J = 13.5 Hz), 3.09 (d, 1H, J = 14.0 Hz), 1.98 (m, 1H), 1.78 (br d, 3H, J = 1.0 Hz), 1.32 (ddd, 1H, J = 14.5 Hz, 8.3 Hz, 4.3 Hz). ^{13}C NMR (125 MHz, CD_3CN) δ_{ppm} 163.7, 159.0, 159.0, 150.6, 145.6, 136.4, 136.1, 136.0, 130.5, 130.5, 128.2, 128.0, 127.1, 113.2, 113.2, 110.4, 87.4, 87.2, 83.6, 73.7, 64.6, 55.0, 43.5, 38.2, 11.5. ESI-MS: $[M + Na]^+$ $C_{32}H_{35}N_3NaO_7$ requires 596.2367, found 596.2376.

4′-*C*-[*N*,*N*-Di(4-pentyn-1-yl)aminomethyl]thymidine (9). Compound **8** (1.4 g, 2.4 mmol) was dissolved in MeOH (40 mL) and 4-pentynal (0.4 g, 4.9 mmol), $NaBH_3CN$ (0.46 g, 7.3 mmol), and acetic acid (140 μL) were added on an ice bath. The reaction was allowed to proceed overnight at room temperature. To remove the 4,4′-dimethoxytrityl group, 80% aqueous acetic acid was added, and after 2 h, the reaction mixture was evaporated to dryness. The residue was purified by silica gel chromatography (5−20% MeOH in DCM) yielding product **9** (0.65 g, 66%) as a powder. ^{1}H NMR (500 MHz, CD_3CN) δ_{ppm} 7.59 (d, 1H, J = 1.0 Hz), 6.28 (dd, 1H, J = 7.8 Hz, 6.3 Hz), 4.41 (dd, 1H, J = 5.0 Hz, 3.0 Hz), 3.61 (d, 1H, J = 11.5 Hz), 3.52 (d, 1H, J = 11.5 Hz), 2.92 (d, 1H, J = 14.5 Hz), 2.86 (d, 1H, J = 14.5 Hz), 2.78−2.71 (m, 2H), 2.68−2.61 (m, 2H), 2.32−2.20 (m, 6H), 2.19 (t, 2H, J = 2.8 Hz), 1.86 (br d, 3H, J = 1.0 Hz), 1.76−1.66 (m, 4H). ^{13}C NMR (125 MHz, CD_3CN) δ_{ppm} 163.9, 150.6, 136.3, 109.9, 87.3, 84.9, 84.0, 74.1, 69.0, 65.8, 56.7, 53.9, 40.2, 25.4, 15.7, 11.6. HRMS (EI): M^+ $C_{21}H_{29}N_3O_5$ requires 403.2107, found 403.2113.

4′-*C*-[*N*,*N*-Di(4-pentyn-1-yl)aminomethyl]-5′-*O*-(4,4′-dimethoxytrityl)thymidine (12). To a solution of compound **9** (0.39 g, 1.0 mmol) in 20 mL of dry pyridine, 4,4′-dimethoxytrityl chloride (0.35 g, 1.0 mmol) was added and the mixture was stirred overnight at room temperature. The volatiles were removed under reduced pressure, and the residue was purified by silica gel chromatography (50−90% ethyl acetate in petroleum ether + 0.1% triethylamine). The yield was 0.29 g (42%) as a white solid foam. ^{1}H NMR (500 MHz, $CDCl_3$) δ_{ppm} 7.40−7.37 (m, 2H), 7.33−7.23 (m, 8H), 6.87−6.82 (m, 4H), 6.46 (dd, 1H, J = 9.0 Hz, 5.5 Hz), 4.69 (dd, 1H, J = 4.0 Hz, 4.0 Hz), 3.79 (s, 6H), 3.12 (d, 1H, J = 9.5 Hz), 3.03 (d, 1H, J = 14.5 Hz), 3.01 (d, 1H, J = 9.5 Hz), 2.91−2.83 (m, 2H), 2.80 (d, 1H, J = 14.0 Hz), 2.58−2.50 (m, 2H), 2.38 (m, 1H), 2.25−2.10 (m, 5H), 1.95 (t, 2H, J = 2.8 Hz), 1.77−1.65 (m, 4H), 1.54 (d, 3H, J = 1.0 Hz). ^{13}C NMR (125 MHz, $CDCl_3$) δ_{ppm} 163.7, 158.8, 150.3, 144.1, 135.5, 135.2, 135.0, 130.1, 128.1, 128.1, 127.3, 113.3, 110.9, 87.2, 86.1, 84.9, 83.6, 76.1, 69.0, 67.2, 58.0, 55.3, 53.9, 41.4, 25.1, 16.4, 12.0. HRMS (EI): M^+ $C_{42}H_{47}N_3O_7$ requires 705.3414, found 705.3403.

3′-*O*-[(2-Cyanoethoxy)-(*N*,*N*-diisopropylamino)phosphinyl]-4′-C-[*N*,*N*-di(4-pentyn-1-yl)aminomethyl]-5′-*O*-(4,4′-dimethoxytrityl)thymidine (1). Compound **12** (0.25 g, 0.35 mmol) was dried over P_2O_5 in a vacuum desiccator and dissolved in dry DCM (5 mL). Triethylamine (248 μL, 1.8 mmol) and 2-cyanoethyl *N*,*N*-diisopropylphosphoramidochloridite (94.5 μL, 0.4 mmol) were added to the reaction solution under nitrogen. After 1 h, the reaction was completed and the mixture was eluted through a short, dried silica gel column (50% ethyl acetate in hexane, 0.1% triethylamine). The faster-eluting diastereomer I was partly obtained as a pure compound, while diastereomer II remained contaminated by diastereomer I (see Supporting Information). Yield was 0.29 g (91%) as colorless oil. Diastereomer I: ^{1}H NMR (500 MHz, CD_3CN) δ_{ppm} 9.42 (s, 1H), 7.68 (br d, 1H, J = 1.0 Hz), 7.53−7.49 (m, 2H), 7.41−7.32 (m, 6H), 7.28 (m, 1H), 6.93−6.89 (m, 4H), 6.24 (dd, 1H, J = 6.5

Hz, 5.5 Hz), 5.01 (ddd, 1H, J = 10.5 Hz, 7.0 Hz, 7.0 Hz), 3.80 (s, 6H), 3.74−3.58 (m, 5H), 3.13 (d, 1H, J = 11.0 Hz), 2.68−2.46 (m, 8H), 2.32 (m, 2H), 2.10 (t, 2H, J = 2.8 Hz), 2.07−1.94 (m, 4H), 1.52−1.40 (m, 4H), 1.39 (br d, 3H, J = 1.0 Hz), 1.21 (d, 6H, J = 6.5 Hz), 1.20 (d, 6H, J = 6.5 Hz); ^{13}C NMR (125 MHz, CD_3CN) δ_{ppm} 163.9, 158.8, 150.5, 145.1, 136.0, 135.8, 135.7, 130.3, 130.3, 128.2, 127.9, 127.0, 118.3, 113.1, 113.1, 109.9, 88.7, 88.7, 86.6, 84.5, 83.0, 72.5, 72.4, 68.7, 64.7, 58.6, 58.5, 55.8, 55.0, 54.2, 43.1, 43.0, 38.6, 38.5, 26.5, 24.0, 24.0, 24.0, 23.9, 20.1, 20.1, 15.7, 11.2; ^{31}P NMR (200 MHz, $CDCl_3$) δ_{ppm} 148.7. Diastereomer II: ^{1}H NMR (500 MHz, CD_3CN) δ_{ppm} 9.45 (s, 1H), 7.67 (s, 1H), 7.53−7.47 (m, 2H), 7.41−7.31 (m, 6H), 7.27 (m, 1H), 6.94−6.87 (m, 4H), 6.28 (dd, 1H, J = 6.0 Hz, 6.0 Hz), 4.95 (ddd, 1H, J = 9.5 Hz, 6.8 Hz, 6.8 Hz), 3.87 (m, 1H), 3.83−3.71 (m, 7H), 3.68−3.55 (m, 3H), 3.12 (d, 1H, J = 10.5 Hz), 2.72−2.42 (m, 8H), 2.30 (m, 2H), 2.09 (t, 2H, J = 2.8 Hz), 2.06−1.93 (m, 4H), 1.51−1.38 (m, 7H), 1.18 (d, 6H, J = 6.5 Hz), 1.10 (d, 6H, J = 6.5 Hz); ^{13}C NMR (125 MHz, CD_3CN) δ_{ppm} 163.9, 158.8, 150.5, 145.1, 136.1, 135.7, 135.6, 130.3, 130.3, 128.2, 127.9, 127.0, 118.6, 113.2, 113.1, 110.1, 88.4, 88.4, 86.7, 84.4, 82.9, 73.7, 73.6, 68.7, 64.9, 58.2, 58.0, 55.8, 55.0, 54.2, 43.1, 43.1, 38.8, 26.5, 24.3, 24.2, 23.8, 23.8, 20.2, 20.1, 15.7, 11.2; ^{31}P NMR (200 MHz, CD_3CN) δ_{ppm} 148.9. ESI-MS: $[M+H]^+$ $C_{51}H_{65}N_5O_8P$ requires 906.4565, found 906.4552.

4′-*C*-[*C*-(4-Pentyn-1-yl)aminomethyl]thymidine (10). Compound **8** (0.92 g, 1.6 mmol) was dissolved in MeOH (20 mL) and 4-pentynal (0.18 g, 2.2 mmol), $NaBH_3CN$ (0.30 g, 4.8 mmol) and acetic acid (92 μL) were added on an ice bath. The reaction was allowed to proceed overnight at room temperature. To remove the 4,4′-dimethoxytrityl group, 80% aqueous acetic acid was added, and after 2 h, the reaction mixture was evaporated to dryness. The residue was purified by silica gel chromatography (5−20% MeOH in DCM), giving compound **10** in 94% yield (0.51 g) as a white powder. ^{1}H NMR (500 MHz, MeOD/CD_3Cl (1:1)) δ_{ppm} 7.61 (s, 1H), 6.38 (dd, 1H, J = 8.3 Hz, 6.3 Hz), 4.53 (dd, 1H, J = 4.0 Hz, 4.0 Hz), 3.65 (s, 2H), 3.37 (m, 1H), 3.23−3.13 (m, 3H), 2.48 (m, 1H), 2.38−2.31 (m, 3H), 2.20 (br s, 1H), 1.96 (m, 2H), 1.91 (s, 3H); ^{13}C NMR (125 MHz, MeOD/CD_3Cl (1:1)) δ_{ppm} 164.7, 151.2, 136.5, 111.4, 86.1, 85.7, 81.5, 74.1, 70.7, 65.1, 49.7, 47.8, 39.4, 24.1, 15.6, 12.0. ESI-MS: $[M+H]^+$ $C_{16}H_{24}N_3O_5$ requires 338.1711, found 338.1725.

4′-*C*-[*N*-Methyl-*N*-(4-pentyn-1-yl)aminomethyl]thymidine (11). Compound **10** (0.45 g, 1.3 mmol) was dissolved in MeOH (10 mL), and paraformaldehyde (44 mg), $NaBH_3CN$ (92 mg, 1.5 mmol), and acetic acid (76 μL) were added on an ice bath. The mixture was stirred at room temperature over the weekend, and paraformaldehyde (22 mg) and $NaBH_3CN$ (50 mg, 0.8 mmol) were added to complete the reaction. The reaction was evaporated to dryness and purified by silica gel chromatography (10−20% MeOH in DCM). The yield was 0.44 g (93%). ^{1}H NMR (500 MHz, CD_3CN) δ_{ppm} 7.48 (s, 1H), 6.40 (dd, 1H, J = 8.5 Hz, 6.0 Hz), 4.45 (dd, 1H, J = 4.5 Hz, 4.5 Hz), 3.66 (d, 1H, J = 11.5 Hz), 3.61 (d, 1H, J = 12.0 Hz), 3.41 (d, 1H, J = 14.0 Hz), 3.31 (m, 1H), 3.17 (m, 2H), 2.86 (s, 3H), 2.44−2.28 (m, 5H), 1.95−1.87 (m, 5H); ^{13}C NMR (125 MHz, CD_3CN) δ_{ppm} 163.7, 150.6, 136.1, 110.5, 85.9, 85.6, 82.5, 74.3, 70.1, 65.3, 59.0, 58.1, 43.6, 38.8, 23.2, 15.2, 11.6. ESI-MS: $[M+H]^+$ $C_{17}H_{26}N_3O_5$ requires 352.1867, found 352.1849.

4′-*C*-[*N*-Methyl-*N*-(4-pentyn-1-yl)aminomethyl]-5′-*O*-(4,4′-dimethoxytrityl)thymidine (13). To a solution of compound **11** (0.36 g, 1.0 mmol) in 15 mL of dry pyridine, 4,4′-dimethoxytrityl chloride (0.42 g, 1.2 mmol) was added, and the mixture was stirred overnight at room temperature. The reaction mixture was evaporated and purified by silica gel chromatography (5% MeOH in DCM + 0.1% triethylamine). The yield was 0.27 g (40%) as a white solid foam. ^{1}H NMR (500 MHz, CD_3CN) δ_{ppm} 7.49−7.45 (m, 2H), 7.37−7.31 (m, 7H), 7.26 (m, 1H), 6.92−6.87 (m, 4H), 6.29 (dd, 1H, J = 7.5 Hz, 6.5 Hz), 4.63 (dd, 1H, J = 5.5 Hz, 3.5 Hz), 3.79 (s, 6H), 3.22 (d, 1H, J = 10.0 Hz), 3.10 (d, 1H, J = 10.0 Hz), 2.85 (d, 1H, J = 14.0 Hz), 2.69 (d, 1H, J = 14.5 Hz), 2.56 (m, 1H), 2.45 (m, 1H), 2.30−2.20 (m, 5H), 2.16−2.10 (m, 3H), 1.62 (m, 2H), 1.54 (s, 3H); ^{13}C NMR (125 MHz, CD_3CN) δ_{ppm} 163.8, 158.8, 150.6, 144.9, 135.8, 135.7, 135.5, 130.2, 130.2, 128.1, 128.0, 127.0, 113.2, 110.1, 86.8, 86.7, 84.2, 84.1, 74.4, 68.9, 66.5, 59.9, 57.8, 55.0, 42.9, 40.4, 26.0, 15.6, 11.4. ESI-MS: $[M+Na]^+$ $C_{38}H_{43}N_3NaO_7$ requires 676.2993, found 676.2997.

3′-*O*-[(2-Cyanoethoxy)-(*N*,*N*-diisopropylamino)phosphinyl)]-4′-*C*-[*N*-methyl-*N*-(4-pentyn-1-yl)aminomethyl]-5′-*O*-(4,4′-dimethoxytrityl)thymidine (2). Compound **13** (0.27 g, 0.4 mmol) was dried over P_2O_5 in a vacuum desiccator and dissolved in dry DCM (5 mL). Triethylamine (293 μL, 2.1 mmol) and 2-cyanoethyl *N,N*-diisopropylphosphoramidochloridite (111 μL, 0.5 mmol) were added to the reaction solution under nitrogen. After 1 h, the reaction was completed and the mixture was eluted through a short, dried silica gel column (50% ethyl acetate in hexane, 0.1% triethylamine). The faster eluting diastereomer I was partly obtained as a pure compound, while diastereomer II remained contaminated by diastereomer I (see Supporting Information). Yield was 0.31 g (87%) as a white solid foam. Diastereomer I: ^{1}H NMR (500 MHz, CD_3CN) δ_{ppm} 8.89 (s, 1H), 7.54−7.49 (m, 3H), 7.40−7.32 (m, 6H), 7.28 (m, 1H), 6.92−6.88 (m, 4H), 6.24 (dd, 1H, J = 7.0 Hz, 5.0 Hz), 4.98 (ddd, 1H, J = 10.5 Hz, 7.3 Hz, 7.3 Hz), 3.80 (s, 6H), 3.75−3.59 (m, 4H), 3.42 (d, 1H, J = 10.5 Hz), 3.22 (d, 1H, J = 10.5 Hz), 2.62−2.47 (m, 6H), 2.41−2.29 (m, 2H), 2.20 (s, 3H), 2.09−2.03 (m, 3H), 1.53 (m, 2H), 1.44 (br d, 3H, J = 1.0 Hz), 1.21 (d, 6H, J = 7.5 Hz), 1.20 (d, 6H, J = 7.0 Hz); ^{13}C NMR (125 MHz, CD_3CN) δ_{ppm} 163.6, 158.8, 150.3, 145.1, 135.8, 135.8, 135.7, 130.3, 128.2, 127.9, 127.0, 118.6, 113.1, 110.0, 88.9, 88.8, 86.5, 84.4, 83.2, 72.7, 72.5, 68.6, 64.3, 58.7, 58.5, 58.0, 55.0, 43.4, 42.9, 38.5, 26.5, 24.0, 23.9, 20.1, 20.1, 15.6, 11.3; ^{31}P NMR (200 MHz, CD_3CN) δ_{ppm} 148.6. Diastereomer II: ^{1}H NMR (500 MHz, CD_3CN) δ_{ppm} 8.94 (s, 1H), 7.52−7.48 (m, 3H), 7.38−7.31 (m, 6H), 7.27 (m, 1H), 6.91−6.87 (m, 4H), 6.27 (dd, 1H, J = 7.0 Hz, 6.0 Hz), 4.90 (ddd, 1H, J = 9.5 Hz, 7.0 Hz, 6.0 Hz), 3.87 (m, 1H), 3.79 (s, 6H), 3.77−3.56 (m, 3H), 3.43 (d, 1H, J = 10.5 Hz), 3.21 (d, 1H, J = 10.0 Hz), 2.69 (t, 2H, J = 6.0 Hz), 2.62−2.47 (m, 4H), 2.42−2.26 (m, 2H), 2.20 (s, 3H), 2.09−2.02 (m, 3H), 1.52 (m, 2H), 1.45 (br d, 3H, J = 1.0 Hz), 1.19 (d, 6H, J = 6.5 Hz), 1.12 (d, 6H, J = 6.5 Hz); ^{13}C NMR (125 MHz, CD_3CN) δ_{ppm} 163.5, 158.8, 150.3, 145.1, 135.8, 135.8, 135.7, 130.3, 128.2, 127.9, 127.0, 118.6, 113.1, 110.2, 88.5, 88.4, 86.6, 84.4, 83.1, 74.0, 73.9, 68.6, 64.5, 58.8, 58.2, 58.0, 54.9, 43.4, 43.0, 38.6, 26.5, 24.2, 24.1, 23.8, 23.8, 20.2, 20.1, 15.6, 11.2; ^{31}P NMR (200 MHz, CD_3CN) δ_{ppm} 148.9. ESI-MS: $[MH]^+$ $C_{47}H_{61}N_5O_8P$ requires 854.4252, found 854.4247.

***N*-[4-(azidomethyl)benzyl]-2,2,2-trifluoroacetamide (4).** *N*-[4-(Azidomethyl)benzyl]-(4-methoxytrityl)amine (**15**) was first synthesized from *N*-(4-methoxytrityl)-1,4-phenylenedimethanamine (**14**). Compound **14** (1.00 g, 2.4 mmol) was dissolved in DCM (4 mL), and an aqueous solution of K_2CO_3 (3.7 mmol)/$CuSO_4$ (15 μmol) (7 mL) and MeOH (30 mL) were added. Triflyl azide (4.9 mmol, theoretical amount calculated from trifluoromethanesulfonyl anhydride used in the preparation) was prepared as previously reported (*50*) and added to the reaction mixture. The reaction was stirred overnight at room temperature and evaporated to dryness. The residue was dissolved in ethyl acetate and extracted with water. The organic fraction was dried with Na_2SO_4, evaporated, and purified by silica gel chromatography (10−20% ethyl acetate in petroleum ether, 0.1%

triethylamine). Compound **15** was obtained in 96% yield (1.03 g) yield. ^{1}H NMR (500 MHz, $CDCl_3$) δ_{ppm} 7.57−7.52 (m, 4H), 7.47−7.40 (m, 4H), 7.32−7.24 (m, 6H), 7.22−7.17 (m, 2H), 6.85−6.81 (m, 2H), 4.32 (s, 2H), 3.79 (s, 3H), 3.34 (s, 2H), 1.84 (br s, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ_{ppm} 158.0, 146.2, 141.4, 138.1, 129.8, 128.5, 128.4, 128.3, 127.9, 126.3, 113.2, 70.5, 55.2, 54.6, 47.6. To the solution of compound **15** (0.95 g, 2.2 mmol) in 10 mL of DCM, trifluoroacetic acid (0.34 mL, 4.4 mmol) and MeOH were added. After 1 h stirring at room temperature, the reaction mixture was evaporated. Without further purification, the detritylated product was subjected to trifluoroacetylation with methyl trifluoroacetate (1.60 g, 12.5 mmol) in a mixture of triethylamine (1.6 mL) and MeOH (9 mL). After 2.5 h, the mixture was evaporated and purified by silica gel chromatography (5−50% ethyl acetate in petroleum ether). The yield was 0.50 g (88%) as a yellowish solid foam. ^{1}H NMR (500 MHz, $CDCl_3$) δ_{ppm} 7.35−7.30 (m, 4H), 6.65 (br s, 1H), 4.54 (d, 2H, J = 6.0 Hz), 4.35 (s, 2H); ^{13}C NMR (125 MHz, $CDCl_3$) δ_{ppm} 157.2, 136.0, 135.7, 128.9, 128.5, 115.8, 54.3, 43.6. ESI-MS: $[M+Na]^+$ $C_{10}H_9F_3N_4NaO$ requires 281.0621, found 281.0602.

1,3,2′-Tri-*N*-trifluoroacetylparomamine (17). Paromamine trihydrochloride (**16**) was obtained by hydrolysis of paromomycin sulfate under acidic conditions (1 mol L^{-1} HCl aq in MeOH, reflux overnight), as described previously (*51*). To the suspension of compound **16** (2.96 g, 6.8 mmol) in a mixture of MeOH (60 mL) and triethylamine (14.4 mL), methyl trifluoroacetate (8.76 g, 68.4 mmol) was added. After a few minutes, the solution cleared for a while, but soon the product began to precipitate. The reaction was stirred for 1 h at room temperature, and the precipitated product was filtered and washed with MeOH. Compound **17** was received in 76% yield (3.20 g) as a white powder. ^{1}H NMR (500 MHz, D_2O/acetone-d_6) δ_{ppm} 5.38 (d, 1H, J = 3.5 Hz), 4.02 (m, 1H), 3.90 (dd, 1H, J = 10.5 Hz, 3.5 Hz), 3.84 (m, 1H), 3.73−3.65 (m, 4H), 3.58 (dd, 1H, J = 9.5 Hz, 9.0 Hz), 3.55−3.40 (m, 3H), 1.93 (m, 1H), 1.70 (ddd, 1H, J = 12.5 Hz, each); ^{13}C NMR (125 MHz, D_2O/acetone-d_6) δ_{ppm} 97.6, 79.4, 76.9, 73.6, 73.0, 70.5, 70.0, 60.6, 54.7, 50.1, 49.3, 31.9 (peaks referring to Tfa are not listed). ESI-MS: $[M+Na]^+$ $C_{18}H_{22}F_9N_3NaO_{10}$ requires 634.1054, found 634.1052.

6′-*O*-(4,4′-Dimethoxytrityl)-1,3,2′-tri-*N*-trifluoroacetylparomamine (18). Compound **17** (1.96 g, 3.2 mmol) was dried over P_2O_5 in a vacuum and dissolved in dry pyridine (70 mL). 4,4′-Dimethoxytrityl chloride (1.09 g, 3.2 mmol) was added and the mixture was stirred overnight at room temperature. After evaporation, the residue was partitioned between ethyl acetate and saturated aq $NaHCO_3$. The organic fraction was dried with Na_2SO_4, evaporated, and purified by silica gel chromatography (10% MeOH in DCM + 0.1% triethylamine) yielding product **18** (2.00 g, 68%) as a purplish foam. ^{1}H NMR (500 MHz, CD_3CN) δ_{ppm} 8.07 (br d, 1H, J = 7.0 Hz), 7.54 (br d, 2H, J = 8.5 Hz), 7.52−7.49 (m, 2H), 7.39−7.32 (m, 6H), 7.26 (m, 1H), 6.92−6.88 (m, 4H), 5.30 (d, 1H, J = 3.0 Hz), 4.05−3.92 (m, 2H), 3.89−3.75 (m, 10H), 3.70−3.61 (m, 3H), 3.53 (dd, 1H, J = 9.5 Hz, 9.0 Hz), 3.49 (m, 1H), 3.42−3.34 (m, 2H), 3.27 (dd, 1H, J = 10.0 Hz, 3.5 Hz), 3.23 (dd, 1H, J = 10.0 Hz, 2.5 Hz), 2.01 (ddd, 1H, J = 13.0 Hz, 4.5 Hz, 4.5 Hz), 1.71 (ddd, 1H, J = 13.0 Hz, each); ^{13}C NMR (125 MHz, CD_3CN) δ_{ppm} 158.6, 145.3, 136.2, 136.2, 130.2, 128.2, 127.8, 126.7, 113.0, 98.4, 85.6, 80.1, 76.5, 73.7, 72.0, 71.5, 70.3, 62.0, 54.9, 54.8, 50.1, 49.6, 31.5 (peaks referring to Tfa are not listed). ESI-MS: $[M+Na]^+$ $C_{39}H_{40}F_9N_3NaO_{12}$ requires 936.2360, found 936.2349.

6′-*O*-(4,4′-Dimethoxytrityl)-5,6,3′,4′-tetra-*O*-acetyl-1,3,2′-tri-*N*-trifluoroacetylparomamine (19). Compound **18** (1.68 g, 1.8 mmol) was dissolved in dry pyridine and acetic anhydride (1.4 mL, 14.8 mmol), and a catalytic amount of 4-dimethylaminopyridine was added. The mixture was heated at 60 °C for 3 h and evaporated to dryness. The residue was dissolved in ethyl acetate and extracted with saturated aq $NaHCO_3$. The organic fraction was dried with Na_2SO_4, evaporated, and purified by silica gel chromatography (30% ethyl acetate in DCM + 0.1% triethylamine). The product (**19**, 1.96 g) was isolated as white foam in 98% yield.^{1}H NMR (500 MHz, CD_3CN) δ_{ppm} 7.72 (d, 1H, J = 9.0 Hz), 7.56 (d, 1H, J = 9.0 Hz), 7.52−7.48 (m, 2H), 7.39−7.30 (m, 7H), 7.27 (m, 1H), 6.95−6.90 (m, 4H), 5.42 (dd, 1H, J = 10.0 Hz, 9.5 Hz), 5.36 (d, 1H, J = 3.5 Hz), 5.31 (dd, 1H, J = 10.0 Hz, 9.5 Hz), 5.08 (dd, 1H, J = 10.0 Hz, 10.0 Hz), 5.06 (dd, 1H, J = 10.5 Hz, 10.0 Hz), 4.43 (ddd, 1H, J = 10.0 Hz, 9.5 Hz, 3.8 Hz), 4.29 (m, 1H), 4.20 (m, 1H), 4.07 (dd, 1H, J = 10.0 Hz, 10.0 Hz), 3.87 (m, 1H), 3.81 (s, 3H), 3.81 (s, 3H), 3.47 (dd, 1H, J = 10.5 Hz, 1.5 Hz), 2.83 (dd, 1H, J = 10.5 Hz, 2.5 Hz), 2.10 (ddd, 1H, J = 13.0 Hz, 4.5 Hz, 4.5 Hz), 2.02−1.94 (m, 7H), 1.92 (s, 3H), 1.71 (s, 3H); ^{13}C NMR (125 MHz, CD_3CN) δ_{ppm} 170.3, 170.2, 169.4, 168.7, 158.7, 158.7, 144.9, 136.1, 135.6, 130.1, 130.0, 128.1, 127.9, 126.9, 113.1, 113.0, 96.1, 85.6, 75.1, 74.6, 72.9, 70.9, 69.4, 67.4, 60.2, 55.0, 52.0, 48.6, 48.1, 31.1, 20.0, 19.8, 19.8, 19.7 (peaks referring to Tfa are not listed). ESI-MS: $[M+Na]^+$ $C_{47}H_{48}F_9N_3NaO_{16}$ requires 1104.2783, found 1104.2695.

5,6,3′,4′-Tetra-*O*-acetyl-1,3,2′-tri-*N*-trifluoroacetylparomamine (20). A solution of 3% dichloroacetic acid in DCM (30 mL) was added to compound **19** (1.90 g, 1.8 mmol) and MeOH was added. The reaction was stirred at room temperature for 4 h and evaporated to dryness. The residue was dissolved in ethyl acetate and extracted with saturated aq $NaHCO_3$. The organic fraction was dried with Na_2SO_4, evaporated, and purified by silica gel chromatography (70% ethyl acetate in petroleum ether). The product (**20**, 1.27 g) was isolated in 92% yield. ^{1}H NMR (500 MHz, CD_3CN) δ_{ppm} 7.84 (d, 1H, J = 9.0 Hz), 7.58 (d, 1H, J = 8.5 Hz), 7.30 (d, 1H, J = 9.0 Hz), 5.31 (dd, 1H, J = 9.5 Hz, 9.5 Hz), 5.27 (d, 1H, J = 4.0 Hz), 5.14 (dd, 1H, J = 10.5 Hz, 9.5 Hz), 5.07 (dd, 1H, J = 10.0 Hz, 9.5 Hz), 5.03 (dd, 1H, J = 10.5 Hz, 10.5 Hz), 4.38−4.17 (m, 3H), 4.09 (dd, 1H, J = 10.0 Hz, 9.5 Hz), 3.81 (ddd, 1H, J = 10.0 Hz, 4.0 Hz, 2.0 Hz), 3.66 (ddd, 1H, J = 12.5 Hz, 6.5 Hz, 2.0 Hz), 3.53 (ddd, 1H, J = 12.5 Hz, 6.0 Hz, 4.0 Hz), 2.96 (t, 1H, J = 6.3 Hz), 2.13 (ddd, 1H, J = 13.5 Hz, 4.5 Hz, 4.5 Hz), 2.04−1.96 (m, 4H), 1.96 (s, 3H), 1.95 (s, 6H); ^{13}C NMR (125 MHz, CD_3CN) δ_{ppm} 170.5, 170.2, 169.8, 169.4, 96.1, 75.5, 74.5, 72.9, 70.5, 70.3, 67.6, 59.7, 52.1, 48.7, 48.0, 31.2, 19.9, 19.8, 19.8, 19.7 (peaks referring to Tfa are not listed). ESI-MS: $[M+Na]^+$ $C_{26}H_{30}F_9N_3NaO_{14}$ requires 802.1476, found 802.1474.

6′-Azido-6′-deoxy-5,6,3′,4′-tetra-*O*-acetyl-1,3,2′-tri-*N*-trifluoroacetylparomamine (21). Compound **20** (0.73 g, 0.9 mmol) was dried over P_2O_5 in a vacuum and dissolved in dry DCM (6 mL). 10% DBU (1,8-diazabicyclo[5.4.0]undec-7-ene, 1.4 mL, 0.9 mmol) solution in MeCN and trifluoromethanesulfonyl anhydride (0.19 mL, 1.1 mmol) were added on an ice bath. After 30 min, the ice bath was removed, and the reaction was stirred at room temperature for 1.5 h. The reaction mixture was partitioned between ethyl acetate and water. The organic phase was separated, dried with Na_2SO_4, and evaporated to dryness. The residue was purified by silica gel chromatography (50% ethyl acetate in hexane) yielding triflate product (0.46 g, 53%), which was directly subjected to azidation step. To a solution of triflate product (0.45 g, 0.5 mmol) in dry DMF (3 mL), sodium azide (0.16 g, 2.5 mmol) was added and the mixture was stirred overnight at room temperature. The reaction mixture was partitioned between diethyl ether and water. The organic phase was separated, dried with Na_2SO_4, and evaporated to dryness. The residue was purified by silica gel chromatography (50% ethyl acetate in petroleum ether) yielding product **21** (0.39 g, 98%). ^{1}H NMR (500 MHz, CD_3CN) δ_{ppm} 7.83 (d, 1H. J = 9.0 Hz), 7.58 (d, 1H, J = 8.0 Hz), 7.31 (d, 1H, J = 8.5

Hz), 5.32 (dd, 1H, J = 10.0 Hz, 9.5 Hz), 5.28 (d, 1H, J = 4.0 Hz), 5.15−5.07 (m, 2H), 5.04 (dd, 1H, J = 10.5 Hz, 10.5 Hz), 4.40−4.27 (m, 2H), 4.22 (m, 1H), 4.06 (dd, 1H, J = 10.0 Hz, 9.5 Hz), 3.92 (m, 1H), 3.56 (dd, 1H, J = 13.5 Hz, 2.5 Hz), 3.36 (dd, 1H, J = 13.5 Hz, 4.3 Hz), 2.13 (ddd, 1H, J = 13.0 Hz, 4.8 Hz, 4.8 Hz), 2.04 (ddd, 1H, J = 13.0 Hz each), 1.99 (s, 3H), 1.96 (s, 3H), 1.95 (s, 3H), 1.94 (s, 3H); ^{13}C NMR (125 MHz, CD_3CN) δ_{ppm} 170.4, 170.2, 169.4, 96.0, 75.8, 74.5, 72.9, 70.1, 69.3, 68.1, 52.0, 50.1, 48.7, 48.0, 31.1, 19.9, 19.8, 19.7, 19.7 (peaks referring to Tfa are not listed). ESI-MS: $[M+Na]^+$ $C_{26}H_{29}F_9N_6NaO_{13}$ requires 827.1541, found 827.1496.

6′-Azido-6′-deoxy-1,3,2′-tri-*N*-trifluoroacetylparomamine (5). To a solution of compound **21** (0.40 g, 0.5 mmol) in dry MeOH (12 mL), sodium methoxide (160 μL of 0.6 mol L^{-1} solution in MeOH) was added. After 1 h, the mixture was neutralized with a strong cation-exchange resin (Dowex 50WX8−200, H^+-form) and filtered. The solution was evaporated, yielding pure product **5** (0.28 g, 88%) as a solid powder. ^{1}H NMR (500 MHz, CD_3CN + drop of CD_3OD) δ_{ppm} 8.54 (d, 1H. J = 9.0 Hz), 8.15 (d, 1H, J = 9.0 Hz), 8.01 (d, 1H, J = 8.0 Hz), 5.21 (d, 1H, J = 3.5 Hz), 4.02 (m, 1H), 3.95 (m, 1H), 3.84 (m, 1H), 3.69 (ddd, 1H, J = 10.0 Hz, 3.0 Hz, 3.0 Hz), 3.63−3.56 (m, 2H), 3.54 (dd, 1H, J = 13.5 Hz, 2.5 Hz), 3.50 (dd, 1H, J = 9.5 Hz, 9.0 Hz), 3.47−3.41 (m, 2H), 3.34 (dd, 1H, J = 10.0 Hz, 9.5 Hz), 2.00 (ddd, 1H, J = 13.0 Hz, 4.3 Hz, 4.3 Hz), 1.68 (ddd, 1H, J = 13.0 Hz each); ^{13}C NMR (125 MHz, CD_3CN + drop of CD_3OD) δ_{ppm} 98.6, 81.2, 76.2, 73.7, 72.0, 71.0, 70.3, 54.7, 50.5, 50.1, 49.5, 31.6 (peaks referring to Tfa are not listed). ESI-MS: $[M+Na]^+$ $C_{18}H_{21}F_9N_6NaO_9$ requires 659.1119, found 659.1122.

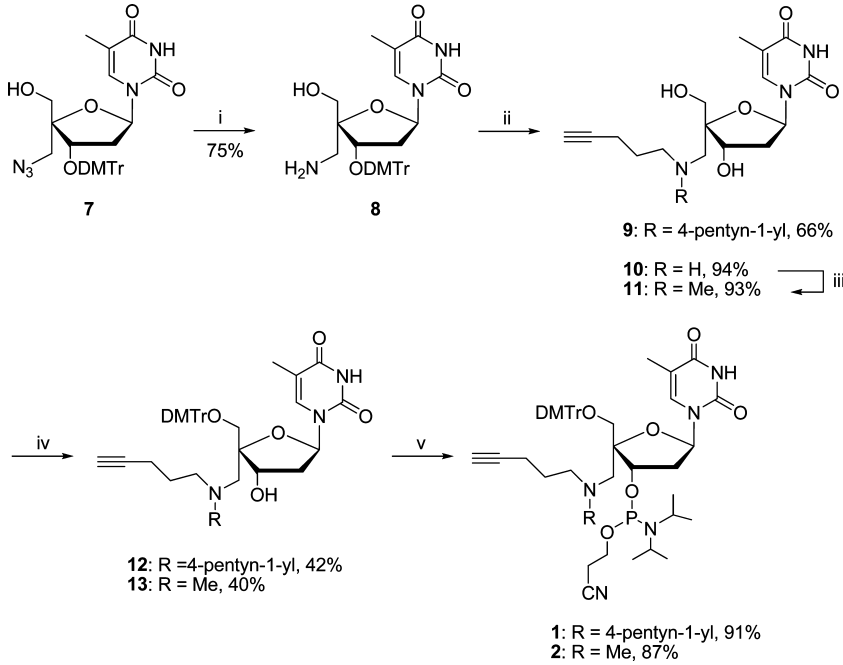
5″-*O*-(4,4′-Dimethoxytrityl)-1,3,2′,6′,2‴,6‴-hexa-*N*-trifluoroacetylneomycin (23). Triethylamine (3.6 mL, 26 mmol) and methyl trifluoroacetate (2.6 mL, 26 mmol) were added to a mixture of neomycin trisulfate (**22**, 2.0 g, 2.2 mmol) and MeOH (10 mL). The mixture was stirred overnight at room temperature and evaporated to dryness. Saturated $NaHCO_3$ was added, and the crude product was extracted with ethyl acetate. The organic layers were combined, dried over Na_2SO_4, filtered, and evaporated to dryness. The residue was further dried by coevaporation with dry pyridine and dissolved in dry pyridine, and then 4,4′-dimetoxytrityl chloride (0.80 g, 2.4 mmol) was added to the mixture. After overnight reaction, saturated $NaHCO_3$ was added to the mixture and the product was extracted with ethyl acetate. The organic layers were combined, dried over anhydrous Na_2SO_4, filtered, and evaporated to dryness. The residue was purified by silica gel chromatography (10% MeOH, 1% triethylamine in DCM) to yield 2.35 g (73%) of the product (**23**) as white foam.^{1}H NMR (500 MHz, CD_3OD) δ_{ppm} 7.46 (m, 2H), 7.36−7.30 (m, 6H), 7.21 (m, 1H), 6.90 (m, 4H), 5.78 (d, 1H, J = 3.3 Hz), 5.16 (d, 1H, J = 4.6 Hz), 5.09 (d, 1H, J = 1.5 Hz), 4.29 (dd, 1H, J = 4.8 Hz, both), 4.21−4.20 (m, 2H), 4.14 (m, 1H), 4.11 (dd, 1H, J = 6.7 Hz, both), 4.01 (m, 1H), 3.97 (m, 1H), 3.92 (m, 1H), 3.87 (dd, 1H, J = 9.9 and 8.8 Hz), 3.80 (s, 6H), 3.75−3.63 (m, 5H), 3.60 (dd, 1H, J = 13.5 and 7.4 Hz), 3.56−3.51 (m, 2H), 3.35−3.31 (m, 3H), 3.19 (dd, 1H, J = 10.5 and 3.1 Hz), 2.00 (ddd, 1H, J = 12.9 Hz, 4.2 and 4.2 Hz), 1.77 (ddd, 1H, J = 12.7 Hz, each); ^{13}C NMR (125 MHz, CD_3OD) δ_{ppm} 157.93, 157.85, 145.0, 136.0, 135.6, 130.2, 129.9, 128.0, 127.4, 126.3, 112.7, 109.0, 97.6, 96.3, 87.4, 86.3, 81.9, 76.9, 75.6, 74.8, 73.0, 72.1, 71.5, 70.3, 69.8, 69.2, 67.1, 62.5, 54.3, 53.5, 51.3, 49.6, 49.1, 40.4, 39.6, 31.5 (peaks referring to Tfa are not listed). ESI-MS: $[M+Na]^+$ $C_{56}H_{58}F_{18}N_6NaO_{21}$ requires 1515.3260, found 1515.3239.

5″-Azido-5″-deoxy-6,3′,4′,2″,3‴,4‴-hexa-*O*-acetyl-1,3,2′,6′,2‴,6‴-hexa-*N*-trifluoroacetylneomycin (25). Compound **23** (4.60 g, 3.1 mmol) was dried over P_2O_5 in vacuum and dissolved in dry pyridine (10 mL). Acetic anhydride (3.5 mL, 37.0 mmol) and a catalytic amount of 4-dimethylaminopyridine were added, and the reaction was stirred overnight at room temperature. After evaporation, the residue was partitioned between ethyl acetate and saturated aq $NaHCO_3$. The organic phase was separated, dried with Na_2SO_4, and evaporated to dryness. The residue was dissolved in 3% dichloroacetic acid in DCM (40 mL) and MeOH was added. After stirring the reaction 3 h at room temperature, the mixture was evaporated and extracted with ethyl acetate and saturated aq $NaHCO_3$. The organic phase was dried with Na_2SO_4, evaporated to dryness, and purified by silica gel chromatography (5−10% MeOH in DCM) to yield 3.90 g (87%) of the product **24** as a white foam. ^{1}H NMR (500 MHz, $CDCl_3$ + drop of CD_3OD) δ_{ppm} 5.84 (d, 1H, J = 2.0 Hz), 5.17 (d, 1H, J = 2.5 Hz), 5.14 (dd, 1H, J = 9.5 Hz, 9.5 Hz), 4.97 (dd, 1H, J = 3.0 Hz, 3.0 Hz), 4.91 (m, 1H), 4.89−4.81 (m, 2H), 4.80 (d, 1H, J = 1.5 Hz), 4.76 (dd, 1H, J = 5.8 Hz, 2.3 Hz), 4.37−4.24 (m, 2H), 4.17−4.05 (m, 4H), 3.99 (m, 1H), 3.95 (dd, 1H, J = 9.5 Hz, 8.5 Hz), 3.78 (dd, 1H, J = 13.0 Hz, 2.5 Hz), 3.74−3.64 (m, 3H), 3.63−3.53 (m, 2H), 3.32 (dd, 1H, J = 14.3 Hz, 5.8 Hz), 3.23 (dd, 1H, J = 14.0 Hz, 9.5 Hz), 2.18 (s, 3H), 2.15 (s, 3H), 2.10 (s, 3H), 2.08−2.02 (m, 7H), 1.99 (s, 3H), 1.81 (ddd, 1H, J = 13.0 Hz each); ^{13}C NMR (125 MHz, $CDCl_3$ + drop of CD_3OD) δ_{ppm} 171.0, 170.8, 170.5, 170.3, 168.7, 168.5, 107.4, 97.4, 95.0, 82.8, 80.9, 75.1, 75.0, 74.9, 73.3, 72.3, 69.9, 69.5, 68.2, 66.8, 66.2, 59.0, 51.7, 48.6, 48.1, 47.4, 40.1, 39.9, 31.3, 20.5, 20.2, 20.2, 20.2 (peaks referring to Tfa are not listed).

Compound **24** (1.07 g, 0.7 mmol) was dried over P_2O_5 in vacuum and dissolved in dry DCM (5 mL). 10% DBU (1.1 mL, 0.7 mmol) in MeCN and trifluoromethanesulfonyl anhydride (0.15 mL, 0.9 mmol) were added on an ice bath. After 30 min, the ice bath was removed and the reaction was stirred at room temperature for 2 h. The reaction mixture was partitioned between ethyl acetate and water. The organic phase was separated, dried with Na_2SO_4, and evaporated to dryness. The residue was purified by silica gel chromatography (50−80% ethyl acetate in hexane) yielding the triflate product (0.59 g, 50%) as reddish foam. The crude product was subjected as such to azidation. Accordingly, to a solution of the triflate product (0.59 g, 0.4 mmol) in dry DMF (3.5 mL), sodium azide (0.13 g, 2.0 mmol) was added, and the mixture was stirred overnight at room temperature. The reaction mixture was partitioned between diethyl ether and water. The organic phase was collected, dried with Na_2SO_4, and evaporated to dryness. The residue was purified by silica gel chromatography (50−70% ethyl acetate in hexane) yielding **25** (0.38 g, 69%). ^{1}H NMR (500 MHz, CD_3CN) δ_{ppm} 7.87 (d, 1H, J = 9.5 Hz), 7.59 (br t, 1H), 7.54 (d, 1H, J = 8.5 Hz), 7.50−7.42 (m, 2H), 7.23 (d, 1H, J = 9.5 Hz), 5.86 (d, 1H, J = 3.5 Hz), 5.24−5.17 (m, 2H), 4.99 (d, 1H, J = 3.0 Hz), 4.98−4.84 (m, 5H), 4.44−4.34 (m, 2H), 4.28 (m, 1H), 4.22 (m, 1H), 4.13−4.04 (m, 3H), 4.01 (ddd, 1H, J = 10.5 Hz, 3.5 Hz, 3.5 Hz), 3.89−3.81 (m, 2H), 3.69 (dd, 1H, J = 13.5 Hz, 3.0 Hz), 3.62 (ddd, 1H, J = 14.5 Hz, 5.0 Hz, 3.0 Hz), 3.56−3.41 (m, 4H), 2.12 (s, 3H), 2.10 (s, 3H), 2.08−2.04 (m, 4H), 2.03 (s, 3H), 2.02 (s, 3H), 2.00 (s, 3H), 1.96 (m, 1H); ^{13}C NMR (125 MHz, CD_3CN) δ_{ppm} 170.4, 170.3, 170.2, 170.0, 169.2, 168.7, 107.8, 96.9, 95.5, 82.7, 79.5, 76.5, 74.8, 74.7, 74.6, 71.3, 69.9, 68.4, 68.1, 67.6, 65.5, 51.7, 50.5, 48.8, 48.5, 47.6, 39.5, 38.9, 30.8, 20.1, 20.1, 19.9, 19.8, 19.8, 19.7 (peaks referring to Tfa are not listed). ESI-MS: $[M+Na]^+$ $C_{47}H_{51}F_{18}N_9NaO_{24}$ requires 1490.2652, found 1490.2649.

5″-Azido-5″-deoxy-1,3,2′,6′,2‴,6‴-hexa-*N*-trifluoroacetylneomycin (6). To a solution of compound **25** (0.43 g, 0.3 mmol) in dry MeOH (7 mL), sodium methoxide (200 μL of 0.6 mol L^{-1} solution in MeOH) was added. After 6 h, the mixture was neutralized with a strong cation-exchange resin (Dowex 50WX8−200, H^+-form) and filtered. The solution was evapo-

Scheme 1[a]



[a] Reagents: (i) PPh_3, H_2O, NH_3 (aq), THF; (ii) 4-pentynal (1 or 2 equiv), $NaBH_3CN$, AcOH, MeOH; (iii) paraformaldehyde, $NaBH_3CN$, AcOH, MeOH; (iv) DMTrCl, pyridine; (v) 2-cyanoethyl *N,N*-diisopropylphosphoramidochloridite, Et_3N, DCM.

rated yielding pure product **6** (0.34 g, 95%) as a solid powder. ^{1}H NMR (500 MHz, CD_3OD) δ_{ppm} 9.35 (d, 1H, J = 9.0 Hz), 9.27 (t, 1H, J = 5.5 Hz), 9.23 (d, 1H, J = 8.0 Hz), 8.82 (t, 1H, J = 5.5 Hz), 8.73 (d, 1H, J = 9.5 Hz), 8.33 (d, 1H, J = 9.5 Hz), 5.73 (d, 1H, J = 3.0 Hz), 5.12−5.08 (m, 2H), 4.23−4.08 (m, 5H), 4.05 (ddd, 1H, J = 10.0 Hz, 10.0 Hz, 4.0 Hz), 3.97−3.87 (m, 3H), 3.80−3.51 (m, 11H), 3.42 (dd, 1H, J = 13.3 Hz, 3.3 Hz), 3.23 (dd, 1H, J = 9.5 Hz, 9.0 Hz), 1.99 (ddd, 1H, J = 13.0 Hz, 4.3 Hz, 4.3 Hz), 1.73 (ddd, 1H, J = 12.5 Hz each); ^{13}C NMR (125 MHz, CD_3OD) δ_{ppm} 110.3, 97.6, 96.5, 87.6, 79.9, 76.2, 74.6, 73.6, 72.0, 71.7, 70.3, 70.2, 69.3, 67.7, 53.7, 51.3, 51.2, 49.8, 49.0, 40.4, 40.0, 31.5 (peaks referring to Tfa are not listed). ESI-MS: $[M+Na]^+$ $C_{35}H_{39}F_{18}N_9NaO_{18}$ requires 1238.2018, found 1238.2027.

Synthesis of Oligodeoxyribonucleotides. The modified oligodeoxyribonucleotides were synthesized on an Applied Biosystems 392 DNA synthesizer in a 1.0 μmol scale using commercial 500 Å CPG-Q-adenosine support (Q = hydroquinone-*O*,*O*′-diacetate). For phosphoramidites **1** and **2** (0.13 mol L^{-1} solution in dry MeCN), a prolonged coupling time of 10 min was used. Otherwise, standard DNA protocol was applied. The oligonucleotides were released from the support with concentrated ammonia (33% aqueous NH_3) at 55 °C overnight. For the click reactions, the support-bound material **26**−**29** were treated for 5−8 min with 10% DBU in MeCN (1 mL, dried over molecular sieves), after which the support was sequentially washed with MeCN, DMF, MeOH, water, and MeOH.

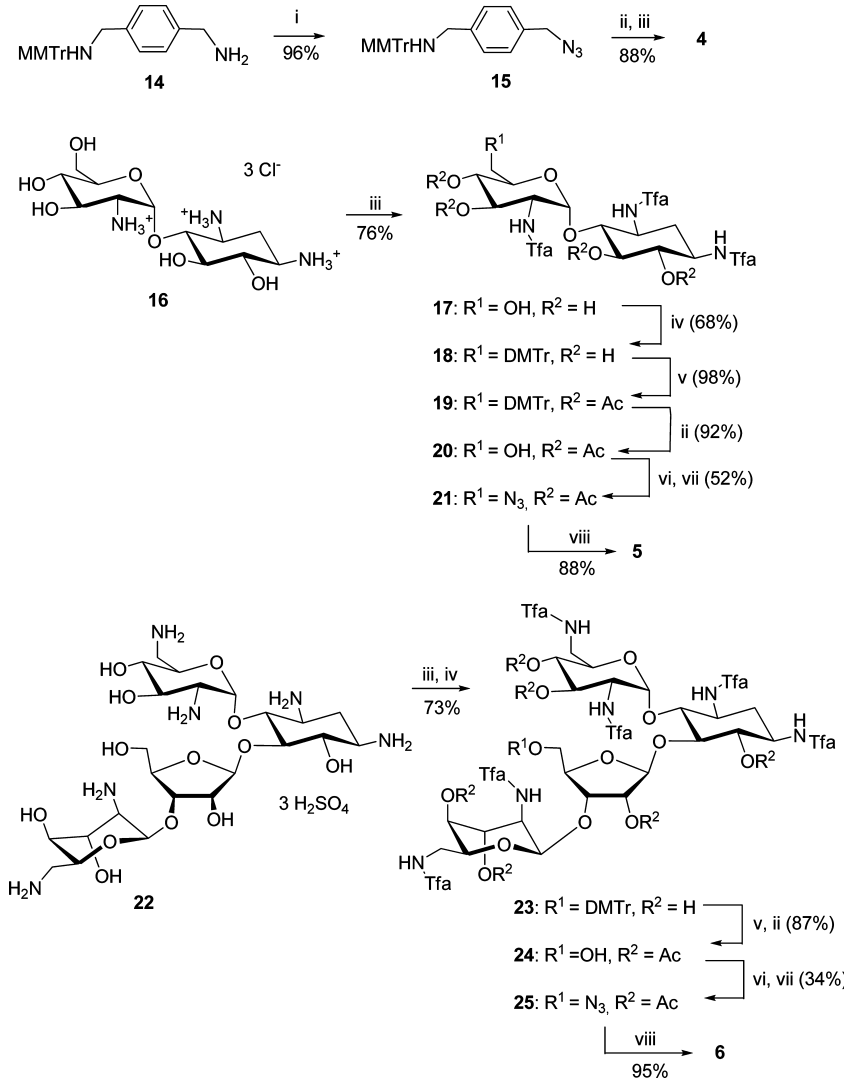
Click Conjugation. Azide-functionalized ligands, **3**−**6**, were conjugated to the alkynyl groups of oligonucleotides **26**−**29**. The solutions of ligands (50 mmol L^{-1}) in MeOH were added onto the support-bound oligonucleotides as follows: 15 equiv for **27**, 30 equiv for **26** and **29**, and 40 equiv for **28**. After that, $CuSO_4$ and tris[(1-benzyl-1,2,3-triazol-4-yl)methyl]amine (TBTA-ligand) were added as a 25 mmol L^{-1} solution in a 12:3:1 mixture of water, DMSO, and 2-butanol. The amounts added were as follows: 7.5 equiv for **27**, 15 equiv for **26** and **29**, and 20 equiv for **28**. Finally, sodium ascorbate (15, 30, and 40 equiv for **27**, **26**, **29**, and **28**, respectively) as 0.1 mol L^{-1} aqueous solution was added. The mixture was shaken at room temperature for 5 h. The support was collected by filtration and washed first with water, DMF, and MeOH. To remove the copper salt and TBTA ligand as quantitatively as possible, the support was then treated for 10 min with aq EDTA solution (0.1 mol L^{-1}), washed with water, and then for another 10 min with aqueous solution of neomycin trisulfate (0.1 mol L^{-1}). Finally, the support was washed with water, DMF, DCM, MeOH, and MeCN and subjected to standard ammonolytic release. After evaporation, the oligonucleotide conjugate was purified by RP HPLC.

Melting Temperature Studies. The melting curves (absorbance versus temperature) were measured at 260 nm on a Perkin-Elmer Lambda 35 UV−vis spectrometer equipped with a multiple cell holder and a Peltier temperature-controller. The temperature was changed at a rate of 0.5 °C/min (from 20 to 90 °C). The measurements were performed in 10 mmol L^{-1} potassium phosphate buffer (pH 7) containing 0.1 mol L^{-1} or 1.0 mol L^{-1} NaCl. The oligonucleotide conjugates and their cDNA or 2′-*O*-methyl RNA targets were used at a concentration of 2 μmol L^{-1}. T_m values were determined as the maximum of the first derivate of the melting curve.

RESULTS AND DISCUSSION

Synthesis of 4′-*C*-[*N*,*N*-di(4-pentyn-1-yl)aminomethyl]thymidine and 4′-*C*-[*N*-methyl-*N*-(4-pentyn-1-yl)aminomethyl]thymidine 3′-(2-Cyanoethyl-*N*,*N*-diisopropylphosphoramidites (1, 2). 4′-*C*-Azidomethyl-3′-*O*-(4,4′-dimethoxytrityl)-thymidine (**7**) was synthesized in good yield (75%) from 4′-*C*-hydroxymethylthymidine as described previously (*47*). Because the azido group can be easily converted to amino group by Staudinger reaction, it allows further functionalization. Accordingly, 4′-*C*-aminomethyl-3′-*O*-(4,4′-dimethoxytrityl)thymidine (**8**) was obtained by the reaction of compound **7** with triphenylphosphine and water (Scheme 1). Compound **8** was then subjected to reductive alkylation in the presence of $NaBH_3CN$ under acidic conditions. On using 2 equiv of 4-pentynal (*52*), 4′-*C*-[*N*,*N*-di(4-pentyn-1-yl)aminomethyl]thymidine (**9**) was ob-

Scheme 2[a]



[a] Reagents: (i) TfN_3, K_2CO_3, $CuSO_4$, H_2O, MeOH, DCM; (ii) DCA, DCM, MeOH; (iii) TfaOMe, Et_3N, MeOH; (iv) DMTrCl, pyridine; (v) Ac_2O, DMAP, pyridine; (vi) Tf_2O, 10% DBU in MeCN, DCM; (vii) NaN_3, DMF; (viii) NaOMe, MeOH.

tained. 4′-*C*-[*N*-Methyl-*N*-(4-pentyn-1-yl)aminomethyl]thymidine (**11**) was, in turn, prepared by two consecutive alkylation steps. Reaction with 4-pentynal gave 4′-*C*-[*N*-(4-pentyn-1-yl)aminomethyl]thymidine (**10**), which was then *N*-methylated with paraformaldehyde. After the reductive alkylations, the 3′-*O*-4,4′-dimethoxytrityl group was removed with 80% acetic acid. Compounds **9** and **11** were then converted to 5′-*O*-(4,4′-dimethoxytrityl) ethers (**12**, **13**) and phosphitylated to phosphoramidites **1** and **2**.

Synthesis of Ligands 3−6. The azido-functionalized ligands (**4**−**6**) used in the click conjugation were synthesized as outlined in Scheme 2. The azidation of previously synthesized (*47*) *N*-(4-methoxytrityl)-1,4-phenylenedimethanamine (**14**) was carried out by the Cu^{2+}-catalyzed diazo transfer described in the literature (*50*). The acid-labile trityl protection of the product (**15**) was changed to a base-labile trifluoroacetyl protection, removable by the conventional ammonolysis used for the release and deprotection of support-bound oligonucleotides.

Paromamine trihydrochloride (**16**) was obtained by acidic hydrolysis of commercially available paromomycin sulfate (*51*). After trifluoroacetylation of the amino groups, the primary 6′-hydroxyl group was protected as a 4,4′-dimethoxytrityl ether (**18**) and the remaining hydroxyl functions were acetylated. The 6′-position was then detritylated, sulfonated, and subjected to azidation. Since the azidation gave poor yields using mesyl esters as a starting material, conversion of the 6′-hydroxyl group to a triflate was attempted. The conventional treatment with triflic anhydride in the presence of pyridine, however, yielded a 6′-pyridinium salt instead of the desired product. Replacement of pyridine by less nucleophilic DBU then provided the desired triflate, which may be azidated to product **21** in 52% overall yield. This was finally deacetylated by methoxide ion-catalyzed transesterification in methanol to obtain product **5**, applicable to postsynthetic conjugation of the alkynylated oligonucleotides. 5″-Azido-1,3,2′,6′,2‴,6‴-hexa-*N*-trifluoroacetylneomycin (**6**) was synthesized starting from neomycin trisulfate (**22**) according to the same protocol as described for the paromamine derivative **5** above. The mannose-derived ligand, methyl 2,3,4-tri-*O*-acetyl-6-azido-6-deoxy-α-D-mannopyranoside (**3**), was prepared as previously reported (*53*).

Oligonucleotide Synthesis and Click Conjugation. 4′-*C*-[*N,N*-Di(4-pentyn-1-yl)aminomethyl]thymidine and 4′-*C*-[*N*-methyl-*N*-(4-pentyn-1-yl)aminomethyl]thymidine monomers **1** and **2** were incorporated into 15-mer oligodeoxyribonucleotides **26**−**29** using standard phosphoramidite coupling chemistry except for a prolonged 10 min coupling time. The coupling yields were 97%. Interestingly, we noticed that these modified oligonucleotides did not withstand solid-supported click con-

Scheme 3. Synthesis of the Conjugated Oligodeoxyribonucleotides Containing One 4′-*C*-Modified Monomer

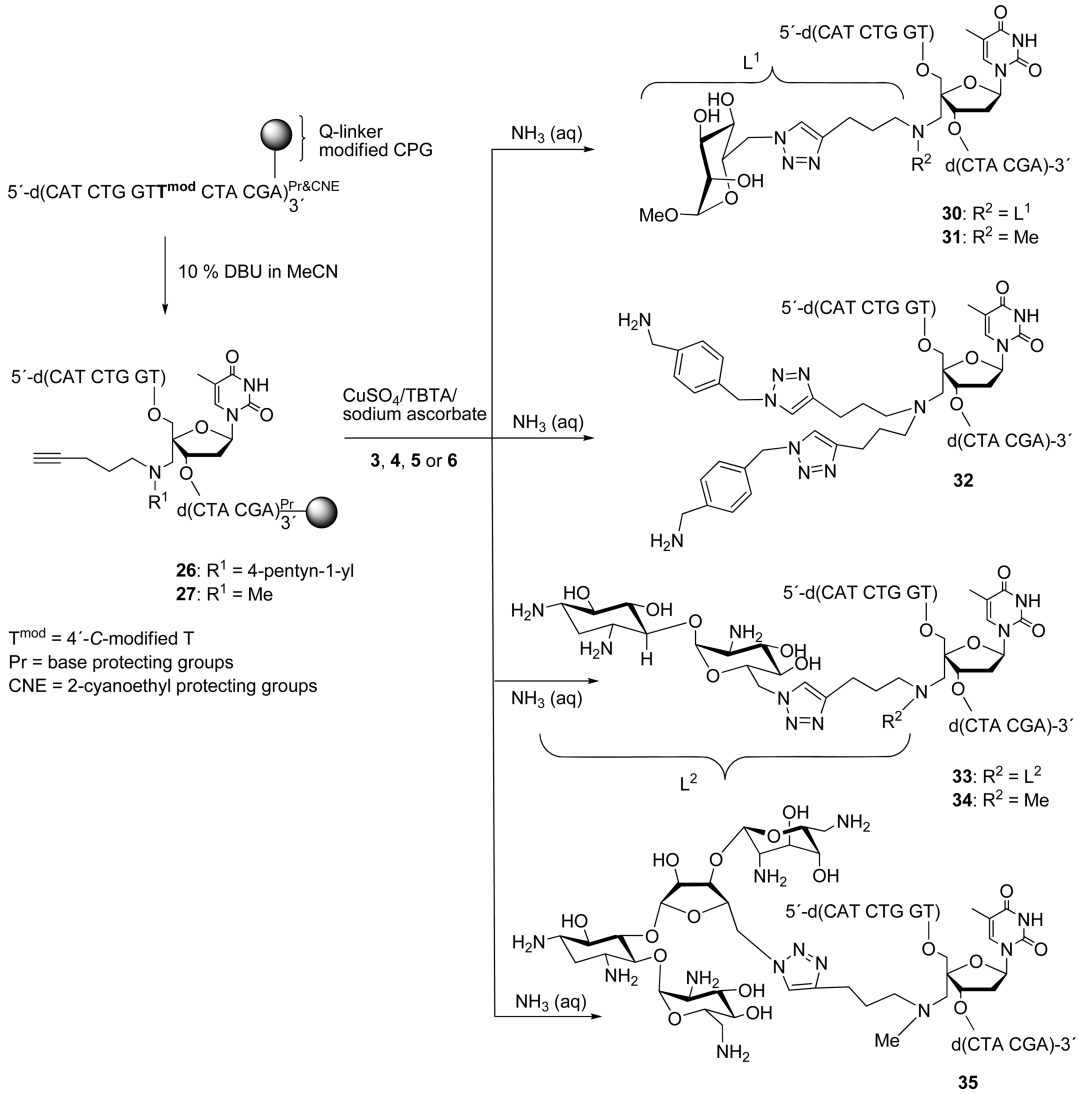


Table 1. Melting Experiments of the Oligonucleotide Conjugates and Their Complementary DNA or 2′-*O*-Methyl-RNA Sequences[a]

	compl. DNA T_m/°C and (Δ T_m/°C)		compl. 2′-*O*-methyl RNA T_m/°C and (Δ T_m/°C)	
	0.1 mol L^{-1} NaCl	1.0 mol L^{-1} NaCl	0.1 mol L^{-1} NaCl	1.0 mol L^{-1} NaCl
5′-CAT CTG GTT CTA CGA	52.8	61.9	54.1	65.0
5′-CAT CTG GT**B**CTA CGA (**26**)	54.5 (+1.7)	61.3 (−0.6)	54.7 (+0.6)	65.1 (+0.1)
5′-CAT C**B**G GTT C**B**A CGA (**28**)	52.5 (−0.3)	59.7 (−2.2)	53.0 (−1.1)	65.4 (+0.4)
5′-CAT CTG GT**D** CTA CGA (**27**)	54.6 (+1.8)	62.5 (+0.6)	55.7 (+1.6)	66.4 (+1.4)
5′-CAT C**D**G GTT C**D**A CGA (**29**)	53.5 (+0.7)	61.6 (−0.3)	52.4 (−1.7)	64.7 (−0.3)
5′-CAT CTG GT**E** CTA CGA (**30**)	53.8 (+1.0)	62.1 (+0.2)	54.3 (+0.2)	65.5 (+0.5)
5′- CAT C**E**G GTT C**E**A CGA (**36**)	51.3 (−1.5)	59.6 (−2.3)	53.8 (−0.3)	66.1 (+1.1)
5′-CAT CTG GT**F** CTA CGA (**31**)	54.6 (+1.8)	62.2 (+0.3)	54.6 (+0.5)	65.3 (+0.3)
5′-CAT CTG GT**H** CTA CGA (**32**)	57.9 (+5.1)	63.7 (+1.8)	54.2 (+0.1)	64.3 (−0.7)
5′-CAT C**H**G GTT C**H**A CGA (**37**)	62.0 (+9.2)	66.6 (+4.7)	56.7 (+2.6)	66.5 (+1.5)
5′- CAT C**I**G GTT C**I**A CGA (**38**)	61.0 (+8.2)	66.5 (+4.6)	57.3 (+3.2)	67.1 (+2.1)
5′- CAT CTG GT**J** CTA CGA (**33**)	57.9 (+5.1)	62.2 (+0.3)	53.2 (−0.9)	64.8(−0.2)
5′-CAT CTG GT**K** CTA CGA (**34**)	57.8 (+5.0)	63.5 (+1.6)	54.1 (±0.0)	65.1 (+0.1)
5′- CAT C**K**G GTT C**K**A CGA (**39**)	58.2 (+5.4)	62.8 (+0.9)	55.1 (+1.0)	65.2 (+0.2)
5′-CAT CTG GT**L** CTA CGA (**35**)	56.1 (+3.3)	62.1 (+0.2)	52.5 (−1.6)	64.2 (−0.8)

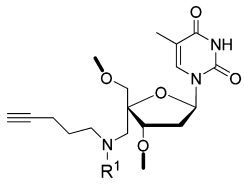
[a] In 10 mmol L^{-1} potassium phosphate buffer (pH 7) containing 0.1 mol L^{-1} or 1.0 mol L^{-1} NaCl. The oligonucleotide conjugates and their cDNA or 2′-*O*-methyl RNA targets were used a concentration of 2 μmol L^{-1}.

jugation as long as their phosphate groups were 2-cyanoethyl protected, but a chain cleavage took place leaving the modified monomer attached to the 5′-terminus of the support-bound oligonucleotide. By contrast, the 4-pentyn-1-yl functionalized oligonucleotides that had not been subjected to the conditions of click reaction could be isolated in intact form by conventional ammonolysis. To carry out the click reaction on a solid support, the 2-cyanoethyl groups had to be removed prior to the click reaction, which was achieved by treatment with 10% DBU in MeCN (5−8 min). Since the

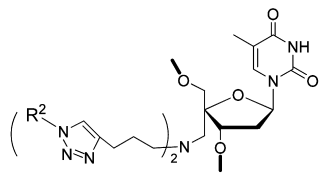
succinyl linker is not fully compatible with the DBU treatment, but undergoes a premature cleavage via succinimide formation (*54*), all the conjugate syntheses were carried out on a Q-linker loaded CPG support (*55*). After exposure of the internucleosidic linkages as phosphodiesters, the azido-functionalized ligands **3**−**6** were attached by the click reaction ($CuSO_4$/TBTA/sodium ascorbate, 5 h, r.t) and the conjugates obtained were released from the support and deprotected by ammonolysis (Scheme 3). The crude products (**30**−**39**; see Table 1) were purified by RP-HPLC, and their identity was verified by ESI-MS. Figure 2 shows representative examples of the HPLC traces of the crude oligonucleotides **35**, **36**, and **39** and the homogeneous products after purification. In each case, the desired conjugate was clearly a main product.

In our previous study on oligonucleotides containing the 4′-*C*-azidomethylthymidine monomer, we were not able to achieve homogeneous products by the solid-supported click conjugation in case the ligand contained amino groups in their structure, but capillary electrophoresis revealed the presence of two peaks with the same molecular mass. When the reactions were performed in solution, this problem was not encountered. With 4′-*C*-[*N,N*-di(4-pentyn-1-yl)aminomethyl]thymidine and 4′-*C*-[*N*-methyl-*N*-(4-pentyn-1-yl)aminomethyl]thymidine monomers (**1**, **2**) used in the present study, capillary electrophoretic, HPLC, and ESI-MS data all indicated the presence of only one pure conjugate for each oligonucleotide. Accordingly, all conjugations could now be conveniently made on a solid support without complications.

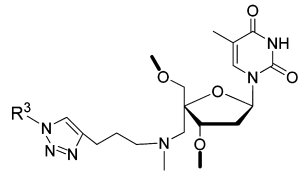
Melting Temperature Studies. Melting temperatures (T_m) for the duplexes of oligonucleotides **26**−**29** and their conjugates **30**−**39** with the cDNA and 2′-*O*-methyl RNA sequences were measured at pH 7 at the ionic strength of 0.1 and 1.0 mol L^{-1} (Table 1).



B: R^1 = 4-pentyn-1-yl
D: R^1 = Me



E: R^2 = mannose (deprotected **3**)
H: R^2 = *p*-aminomethylbenzene (deprotected **4**)
J: R^2 = paromamine (deprotected **5**)



F: R^3 = mannose (deprotected **3**)
I: R^3 = *p*-aminomethylbenzene (deprotected **4**)
K: R^3 = paromamine (deprotected **5**)
L: R^3 = neomycin (deprotected **6**)

When one 4′-*C*-modified monomer, **B** (**26**) or **D** (**27**), was incorporated into the DNA strand, the stability of the duplex increased with both DNA and 2′-*O*-Me RNA. When two such monomers were incorporated in the sequence (**28**, **29**), a slight destabilization took place.

With mannose-conjugated oligonucleotides containing a single modified monomer, either **E** (**30**) or **F** (**31**), the T_m of the DNA-hybrid was again increased, but the presence of two modified units (**36**) is destabilizing. By contrast, on using a 2′-*O*-methyl RNA target, the conjugate containing two **E** monomers and, hence, four mannose residues hybridized almost as well as the corresponding unmodified 15-mer oligodeoxyribonucleotide. At high salt concentration, the hybridization was even slightly more efficient compared to the unmodified DNA sequence.

All conjugates bearing amino groups (**32**−**35**, **37**−**39**) markedly stabilized the duplex with DNA target. With the *p*-aminomethylbenzene conjugates, the DNA-hybrids incorporating one or two **H** (**32**, **37**) or **I** (**38**) monomers were stabilized by 4−5 °C/modification. Toward 2′-*O*-methyl RNA, the stabilization was less prominent. The stabilization was more marked at low ionic strength, but it still existed at high ionic strength. Undoubtedly, the major part of the stabilization results from reduced electrostatic repulsion between negatively charged sugar−phosphate backbones and positively charged amino groups present at neutral pH. This influence plays a more important role at low ionic strength.

When one paromamine derived monomer, **J** (**33**) or **K** (**34**) was incorporated into the strand, the DNA-hybrid was stabilized by 5 °C, but the second monomer (**39**) did not result in any extra stabilization. With 2′-*O*-Me RNA target, two monomers **K** were needed to achieve even a modest increase in T_m (ΔT_m = +1.0 °C). Compared to the paromamine monomers, the neomycin group (monomer **L** in **35**) was less stabilizing than the paromamine group (monomer **K** in **34**) toward DNA, and toward 2′-*O*-Me-RNA, the effect is even destabilizing. Similarly to the *p*-aminomethylphenyl ligands, the duplex stabilizing effect of the aminoglycosides ligands is more marked at low ionic strength.

The hybridization properties of the 4′-modified oligonucleotides are encouraging. Evidently the 4′-position, facing the minor groove upon hybridization, can accommodate even rather large molecule clusters without appreciable destabilization of the duplex. The 4′-*C*-[*N,N*-di(4-pentyn-1-yl)aminomethyl]thymidine monomer (**1**) allows attachment of two ligands per residue, which may well be useful when applying the glycotargeting concept. A short segment of the duplex may be decorated with several sugar units. The results received with the mannose conjugated oligonucleotide, **36**, lend some support to the feasibility of sugar-coated oligonucleotides as carriers of RNA-type oligonucleotides. On the other hand, 4′-*C*-[*N*-methyl-*N*-(4-pentyn-1-yl)aminomethyl]thymidine monomer (**2**) can be preferable for attachment of bulky ligands such as neomycin derivatives to oligonucleotides. Among the amino group-bearing ligands, the most remarkable hybridization enhancement was obtained with *p*-aminomethylphenyl conjugates (**32**, **37**, **38**). Sterically more demanding aminoglycoside ligands (**33**−**35**, **39**) stabilized the DNA·DNA duplex less, but still notably. All amino group-bearing conjugates preferred DNA over 2′-OMe RNA. Compared to the results of the present paper, the previously observed duplex stabilizations achieved with some conjugates of the 4′-*C*-azidomethylthymidine incorporating oligonucleotides are modest (*47*). This implies that the alkyl chain of the 4′-*C*-(4-pentyn-1-ylaminomethyl)thymidine monomers (**1**, **2**) allows a more flexible alignment of the triazole ring and conjugate groups upon hybridization. The tertiary amino group, which probably is protonated under physiological conditions, further stabilizes the duplex formation by diminishing the electrostatic repulsion between the complementary chains, as evidenced by the fact that the melting temperatures observed for the oligonucleotides containing 4′-*C*-(alkynylaminomethyl)thymidine units (**26**−**29**) are higher than those observed for their 4′-*C*-azidomethylthymidine counterparts.

CONCLUSION

4′-*C*-[*N,N*-Di(4-pentyn-1-yl)aminomethyl]thymidine and 4′-*C*-[*N*-methyl-*N*-(4-pentyn-1-yl)aminomethyl]thymidine 3′-phos-

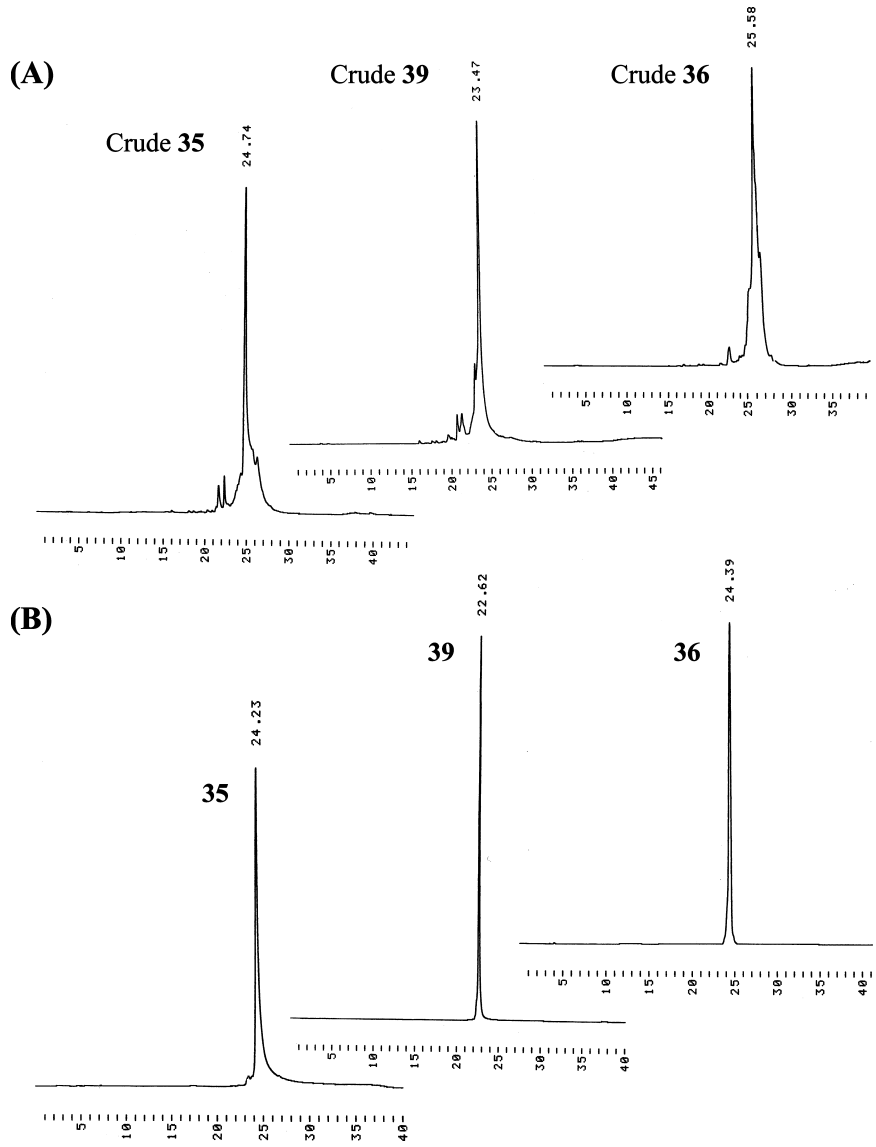


Figure 2. RP HPLC traces of the crude (A) and purified (B) oligonucleotide conjugates **35**, **36**, and **39**.

phoramidites (**1**, **2**) have been synthesized as a continuation of the previously reported preparation of 4′-*C*-azidomethylthymidine 3′-hydrogenphosphonate. Together, these building blocks enable the exploitation of the click conjugation on both ways, i.e., to have azido function present on either the oligonucleotide or ligand side. Nucleoside **1** bearing two 4-pentyn-1-yl groups is designed for high-density functionalization of modified oligonucleotides, whereas nucleoside **2** allows one-armed conjugations. Azido-derivatized ligands may be efficiently conjugated to the alkynyl groups of these monomers by the click chemistry on a solid support. However, the 2-cyanoethyl protections must be removed from the phosphates prior to the click conjugation, since the 4′-modifications used here under the click reaction conditions cause cleavage of the 5′-linked phosphate triester bond. Melting temperature studies reveal that oligonucleotides containing one to four mannose ligands in the central part of the chain (i.e., one or two modified nucleoside residue) form equally stable duplexes with complementary 2′-OMe RNA as the corresponding unmodified DNA sequence. At high salt content, the mannose conjugation is even stabilizing. The results with the space-demanding aminoglycoside ligands further demonstrate the utility of 4′-modified nucleosides **1** and **2** in glycoconjugations. All amino group-bearing conjugates stabilized the DNA·DNA duplex significantly and the DNA·2′-*O*-methyl RNA duplex notably.

ACKNOWLEDGMENT

Financial support from the Academy of Finland is gratefully acknowledged.

Supporting Information Available: NMR spectral data for **1**, **2**, **4**−**6**, **12**, and **13**. ESI-MS and RP HPLC data for oligonucleotides **26**−**39**. In addition, examples of melting temperature curves for **26**−**39** are presented. This material is available free of charge via the Internet at http://pubs.acs.org.

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