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A Straightforward Preparation of Aminoglycoside–Dinucleotide and –diPNA Conjugates via Click Ligation Assisted by Microwaves

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An alternative and straightforward method to prepare aminoglycoside–dinucleotide and –diPNA conjugates is reported, which is based on copper-catalyzed Huisgen azide-alkyne cycloaddition ("click chemistry" ligation) assisted by microwave irradiation. This method permitted conjugations to be performed in aqueous solution, in very short times and with readily prepared precursors.

Introduction

Abbreviations: Asc: sodium ascorbate, Bhoc: benzhydryloxycarbonyl, Boc: *tert*-butoxycarbonyl, CPG: controlled pore glass CuAAC: copper-catalyzed Huisgen azide-alkyne cycloaddition, DIEA: *N*,*N*-diisopropylethylamine, DMF: *N*,*N*-dimethylformamide, DMSO: dimethyl sulfoxide, ESI: electrospray ionization mass spectrometry, Fmoc: 9-fluorenylmethoxycarbonyl, HOBt: *N*-hydroxybenzotriazole, HPLC: high performance liquid chromatography, MALDI-TOF: matrix assisted laser desorption ionization-time of flight mass spectrometry, MW: microwave irradiation, NMP: *N*-methylpyrrolidone, PNA: peptide nucleic acid, TBTA: tris(benzyltriazolylmethyl)amine, TFA: trifluoroacetic acid, THAP: 2,4,6-trihydroxyacetophenone, t_R: retention time.

Aminoglycoside antibiotics are known bactericidal agents. However, toxicity, target promiscuity and the appearance of resistance mechanisms have depreciated their clinical use.[1] The alarming decrease in the activity of the current antibiotic repertoire has renewed the interest for chemical analogues of aminoglycosides.[2] Efforts have recently been fuelled by the vast knowledge acquired on the structure of ribosomal RNA[3] (the main biological target of aminoglycosides) but also by potential therapies based on RNA ligands.[4] Among many other aminoglycoside derivatives, aminoglycoside–oligonucleotide conjugates[5–7] have recently been considered as specific ligands of bacterial and viral RNA due to the additional chemical recognition properties conferred by oligonucleotide strands. Furthermore, free aminoglycosides[8] and aminoglycosides as Cu^{II} complexes[9] are also known to produce the degradation of nucleic acids. Recent results suggested that if aminoglycosides are conjugated with oligonucleotides or peptide nucleic acids (PNAs), the conjugates could behave as selective artificial ribonucleases.[10–11] Based on these reports, we were interested in further studying the properties of aminoglycoside–oligonucleotide conjugates as specific RNA ligands. Here, we report on a procedure for obtaining neomycin– or paromomycin–dinucleotide and –diPNA conjugates (Scheme 1) which combines copper-catalyzed Huisgen azide-alkyne cycloaddition (CuAAC) with microwave irradiation (MW).

Results and Discussion

Aminoglycoside–oligonucleotide conjugates were known by forming thiourea,[6,12] amide,[7,13–14] and phosphate[11] linkages, or by nucleobase derivatization.[15] We reasoned that CuAAC, the best known "click chemistry" transformation,[16] could also be a useful method for producing the ligation of aminoglycosides and oligonucleotides. CuAAC, specifically a 1,3-dipolar cycloaddition of azides and alkynes which produces a triazole ring,[17] has become an outstanding method in medicinal chemistry[18] and means of building new materials.[19] Moreover, more recently it has been used for biomolecule ligation and bioconjugation.[20–21] Consequently, CuAAC has been extensively employed to synthesize both oligonucleotide[22–26] and aminoglycoside derivatives.[27–29] In connection with our objectives, during this work, the synthesis of aminoglycoside–oligonucleotide conjugates by CuAAC was reported for the first time by ligation of a 4′-*C*-(azidomethyl)thymidine-containing oligonucleotide and an alkynyl neamine derivative.[30]

Here, we describe a straightforward alternative approach to obtain aminoglycoside–nucleotide conjugates via CuAAC, by coupling azido-aminoglycosides with alkynyl dinucleotides in aqueous solution (Scheme 2). The reasons for this choice were that alkynyl oligonucleotides can be

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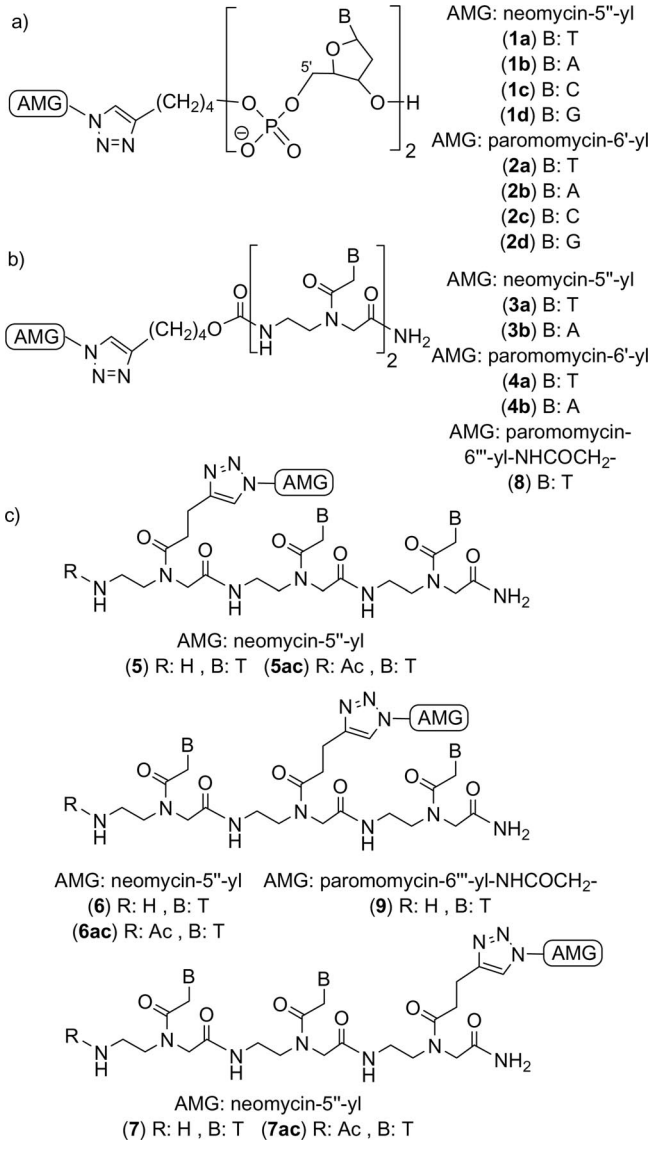
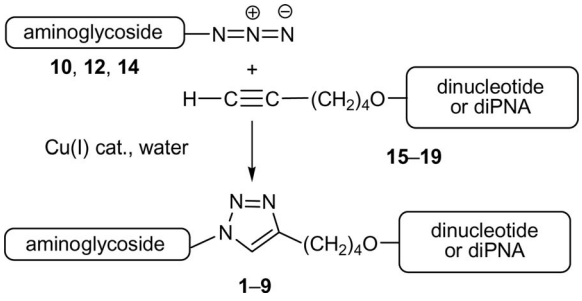


Scheme 1. Molecules synthesized in the present work via CuAAC: a) aminoglycoside–dinucleotide conjugates, b) and c) aminoglycoside–diPNA conjugates.

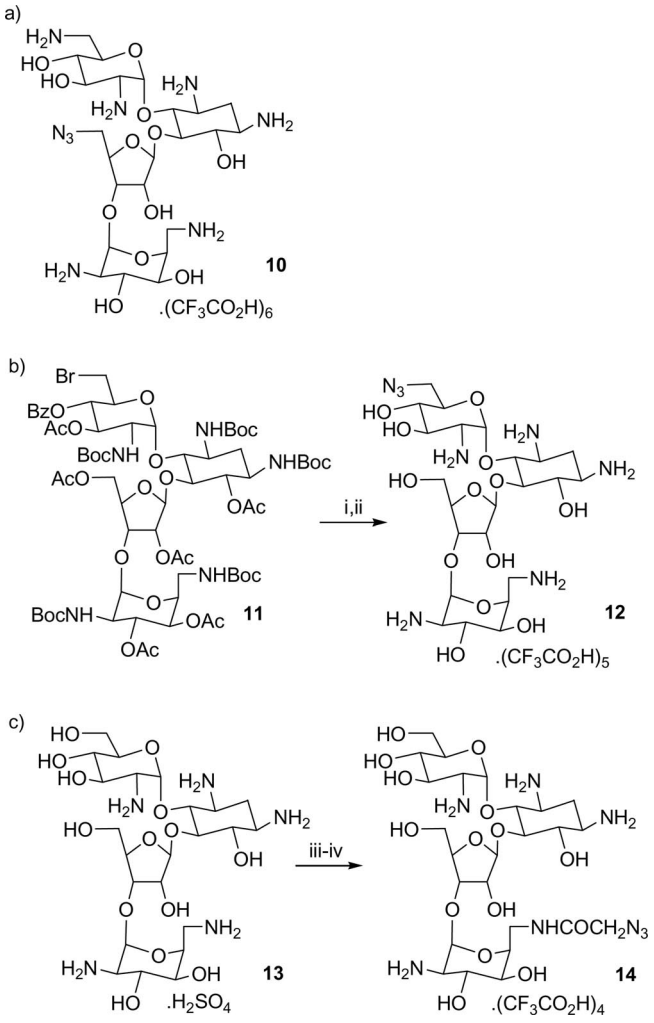
readily obtained by solid-phase synthesis,[31] and several methods are reported to yield azido-aminoglycosides.[27] Furthermore, due to the orthogonality of azides and alkyne functions, no additional protection was needed for the synthetic precursors and ligation could be performed in water, where the reagents and products are highly soluble. In comparison with alternative metal-free ligations,[32] the triazole ring produced by the copper-catalyzed reaction has shown a convenient covalent knot for conjugates acting as RNA ligands,[29] by low steric hindrance and potentially forming polar and stacking interactions. Here, this approach was tested by preparing 5′′-neomycin, 6′- or 6′′′-paromomycin conjugates of dinucleotides dTT, dCC, dAA, dGG, and also, of PNA analogues H-tt-NH_2 and H-aa-NH_2 (Scheme 1). These conjugates that combine diversed aminoglycosides, nucleobase sequences, oligonucleotide backbones and linkers were devised to explore specific interactions with RNA secondary structures and to offer new insights into the design of novel RNA ligands. Currently, we are evaluating these molecules as A-site ribosomal RNA ligands and results will be reported elsewhere.



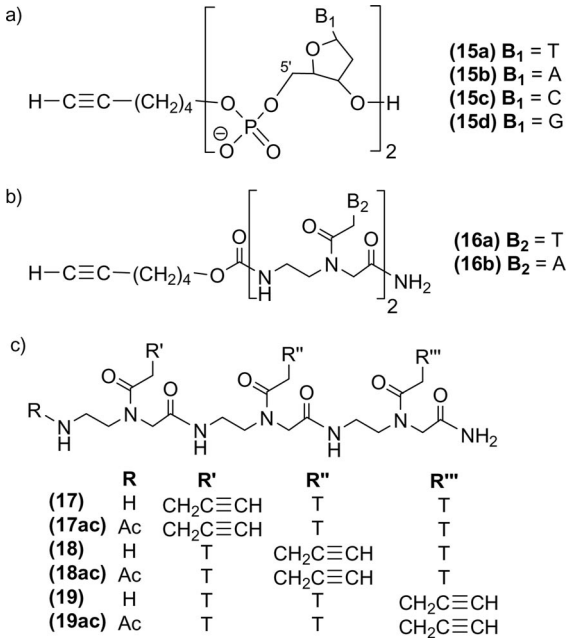
Scheme 2. Synthesis of aminoglycoside–dinucleotide and aminoglycoside–diPNA conjugates via CuAAC.

As aminoglycoside precursors, azides **10**, **12** and **14** were prepared from neomycin and paromomycin, respectively (Scheme 3), in order to explore derivatization of the aminoglycoside backbone in three different rings and with a different number of amine groups. The synthesis of 5′′-azido-5′′-deoxyneomycin (**10**) was carried out essentially as described from neomycin (four steps, 37%).[27] Paromomycin derivative **12** (Scheme 3, b) was prepared by sodium azide substitution on hexa-*O*-acetyl-4′-*O*-benzoyl-6′-bromopentakis(*N*-*tert*-butoxycarbonyl)-6′-deoxyparomomycin[33] and subsequent deprotection with aq. conc. ammonia and trifluoroacetic acid, resulting in a global 60% yield. Derivative **14** that contained an azide group at the idose ring was prepared (Scheme 3, c), by selective monoacylation of 6′′′ primary amine of paromomycin with succinimidyl azidoacetate. Product was temporally protected with Boc groups to allow purification by chromatography, and deprotected by treatment with trifluoroacetic acid. It was obtained in 28% yield from paromomycin.

The corresponding *O*-alkynyl dinucleotides (**15a**–**15d**, Scheme 4, a) were solid-phase synthesized by the standard phosphoramidite method, and the hexyn-5-yl appendage was incorporated via 5′-phosphorylation.[22,31] Alkynyl dinucleotides were finally deprotected and cleaved from the resin by treatment with aq. conc. ammonia, and as they were sufficiently pure (>80% after HPLC analysis), they were used without further purification in the following CuAAC step. Our results below confirm that intermediate purification steps can be minimized, without significantly compromising the scope of the ligation reaction. The corresponding diPNAs **16**–**19** (Scheme 4 parts b–c) were assembled by solid-phase synthesis on a Sieber resin, by use of standard Fmoc/Bhoc methodology. The hexynyl appendage of diPNAs **16a**–**16b** was incorporated after the removal of the *N*-terminal Fmoc group, by reaction with 5-hexyn-1-yl *p*-nitrophenyl carbonate at room temperature for 2 h. To synthesize diPNAs **17**–**19** and **17ac**–**19ac**, the alkynyl appendage was introduced via N^{α}-(2-Fmoc-aminoethyl)-N^{α}-(3-pentynoyl)glycine[34] under the same conditions as the nucleobase monomers. At the end of the synthesis, the *N*-terminal Fmoc group was first removed, in order to pro-



Scheme 3. Azido-aminoglycosides **10**–**14**. a) 5′′-Azido-5′′-deoxyneomycin (**10**). b) Synthesis of 6′-azido-6′-deoxyparomomycin (**12**). c) Synthesis of 6′′′-*N*-azidoacetylparomomycin (**14**). Reagents and conditions: i) NaN_3, DMF, 80 °C, 4 h, 75%; ii) NH_3 in CH_3OH, 60 °C, 4 h + 40% TFA in CH_2Cl_2, 2 h, 80%; iii) *N*-succinimidyl azidoacetate, $NaHCO_3$ 10%, water/DMF + Boc_2O; iv) 40% TFA in CH_2Cl_2, 2 h, 28% (from paromomycin).



Scheme 4. Dinucleotide and diPNA precursors: a) 5′-*O*-hexynyl dinucleotides (**15a**–**15d**). b) *N*-hexynyloxycarbonyl diPNAs (**16a**, **16b**). c) diPNAs (**17**–**18** and **17ac**–**18ac**) containing N^{α}-(2-Fmoc-aminoethyl)-N^{α}-(3-pentynoyl)glycine.

duce the free amine derivatives **17**–**19** or subsequently acetylated to obtain **17ac**–**19ac**. Modified PNA **17**–**19** were devised to evaluate the effect of i) the ligation position of the aminoglycoside to the polyamide backbone and ii) the presence of a free amine or an acetamide group at the *N*-terminus in the affinity properties of conjugates. Alkynyl diPNAs were finally cleaved from the resin and deprotected by treatment with TFA/*m*-cresol (19:1) for 10 min at room temperature. Just as with the dinucleotide derivatives, no further purification of diPNAs was neeeded before performing the ligation step.

Our ligation approach was first assayed in the preparation of neomycin-TT conjugate (**1a**). Among the plethora of methods known to generate the Cu^{I} catalyst,[35] we opted for the in situ reduction of $CuSO_4$ with sodium ascorbate. The results of our assays are summarized in Table 1 (see HPLC profiles in Supporting Information). In every assay, 0.1 μmol of hexynyl oligonucleotide **15a** reacted with an excess of 5′′-azido-5′′-deoxyneomycin **10** (1.67 equiv.). To avoid the formation of potentially degradative Cu^{II}-aminoglycoside species,[9a] Cu^{I} was first obtained from the reaction of $CuSO_4$ and sodium ascorbate (Asc), followed by the addition of the dinucleotide and the aminoglycoside. After some preliminary experiments, only moderate cycloaddition was produced (Table 1, assay 1.3) by heating a solution containing 0.24 mM dinucleotide, 0.40 mM azido-aminoglycoside, Cu^{I} catalyst generated from 0.05 mM $CuSO_4$ and 1.2 mM ascorbate, and 0.05 mM of tris(benzyltriazolylmethyl)amine[36] (TBTA) to 70 °C for 24 h. Previously, when the reaction was tested in the absence of TBTA, no significant conjugation was observed after 24 h at 70 °C (assay 1.1). Thus, TBTA, which acted here as Cu^{I} chelator, was a determinant factor to enhance conjugation and limit the degradation of the oligonucleotide, in accordance with other previous reports.[24a,37] Copper concentration should also be conveniently optimized. Without TBTA, the increase of copper concentration from 0.05 mM to 0.1 mM was found to be counterproductive, as the conjugate was produced in low amounts and accompanied of several degraded products (assay 1.2). MW was also assayed, as it was recently reported to be compatible with the synthesis of oligonucleotide derivatives.[23a,24,25] Notably, MW produced an extraordinary acceleration of the reaction and improved the efficiency of conjugation, but only if TBTA was present (assay 1.3). In the absence of chelator, by MW irradiating at 80 °C, very low conversion and degradation were observed after 1 h, even if Cu^{I} concentration was increased (assay 1.4). When 0.07 mM TBTA was present, the irradiation produced a significant increase of conversion; up to

62% after 30 min and up to 72% after 1 h (assays 1.5–1.6). Finally, optimal conversion into **1a** was observed if irradiation was conducted at 100 °C for 1 h (assay 1.7). Most probably, in the presence of a chelating agent as TBTA that stabilizes Cu^{I} and prevents complexation with aminoglycosides, MW experiments benefited from the high heating rate of water, the selective activation of the polar intermediates involved in CuAAC, and that reactions can be carried out at higher temperatures than by conventional heating.

Table 1. Assays[a] of synthesis of 5′′-neomycin-5′dTT (**1a**).

	15a [mM]	**10** [mM]	Cu^{II} [mM]	Asc [mM]	TBTA [mM]	*T* [°C][b]	Time [h]	Conv. [%][c]
1.1	0.24	0.40	0.05	1.2	–	70	24	n.d.
1.2	0.24	0.40	0.1	2.4	–	70	52	n.d.
1.3	0.24	0.40	0.05	1.2	0.05	70	24	moderate
1.4	0.24	0.40	0.1	2.4	–	80 MW	1	n.d.
1.5	0.34	0.60	0.07	1.2	0.07	80 MW	0.5	51
1.6	0.34	0.60	0.07	1.2	0.07	80 MW	1	61
1.7	0.34	0.60	0.07	1.2	0.07	100 MW	1	73

[a] Assays were performed with 0.1 μmol dinucleotide **15a** (the HPLC profiles are shown in ESI). [b] MW means microwaves irradiation. [c] Conversion of dinucleotide **15a** into conjugate according to the HPLC analyses.

Based on the conditions of assay 1.5, which efficiently rendered **1a**, we continued by synthesizing the other conjugates in Scheme 1. The results are summarized in Table 2. The temperature should be optimized to maximize conversion: the optimal temperature was found to be 100 °C for TT conjugates (**1a**, **2a**), and 80 °C for other dinucleotide conjugates (**1b**–**1d**, **2b**–**2d**). Notably, we managed to increase the scale from 0.1 μmol to 0.5–1.0 μmol without significant differences in conversion or conjugate purity. The same procedure was also successfully applied for the preparation of PNA conjugates **3a**–**3b** and **4a**–**4b** although reactions were heated to 120 °C and extended to 1.5 h to maximize conversion. Probably, conjugations were slower than in dinucleotide derivatives due to the absence of favourable electrostatic interactions between aminoglycosides and diPNAs. Conversion into conjugates was mostly higher than 70% according to the HPLC analyses, except for conjugates **5**–**7** and **5ac**–**7ac**, which were obtained in lower yields. However, low amounts of fragmented products were inevitably observed, probably caused by heating at the high temperature needed to maximize conjugations. At room temperature, conjugates shown to be stable for extended times (1–2 d) in the presence of copper and ascorbate reagents. All the conjugates were conveniently purified by reversed-phase liquid chromatography and identified by mass spectrometry. Regardless of high conversions, the presence of impurities chromatographically close to our desired products made difficult their purification, and it was probably the reason for some of the low-to-moderate yields shown in Table 2. Notably, TT and AA conjugates were generally obtained with better yields than their CC and GG counterparts. No significant differences in yields were observed between dinucleotide and diPNA conjugates of the same sequence. With respect to conjugates synthesized from diPNAs containing N^{α}-aminoethyl-N^{α}-(3-pentynoyl)glycine and 5′′-azido-5′′-deoxyneomycin (**10**), we observed that free amine PNAs **17**–**19** produced conjugates with lower conversion and lower purity than their acetylated counterparts **17ac**–**19ac**. Although we could not ascertain the exact reasons for that, it clearly related to the presence of a *N*-terminal amine, with independence of the ligation position of aminoglycoside. Their lower conversions could be related to unfavourable electrostatic between both protonated PNA and aminoglycoside in the course of the ligation reaction. No significant improvements could be obtained by extending reaction times nor increasing temperatures. Conjugates **8** and **9** were synthesized by ligation of 6′′′-*N*-azidoacetylparomomycin **14** and diPNAs **16a** and **18ac**. Here, ligations required longer times of microwave irradiation (2 h) than those carried out with the other two aminoglycosides.

Table 2. Synthesis[a] of conjugates **1**–**4**.

Aminoglycoside + dinucleotide/diPNA	Conjugate	*T* [°C][b]	Time [h]	Crude yield[c] [%]	Isolated yield[d] [%]
10 + **15a**	**1a**	100 (MW)	1.0	80	27
10 + **15b**	**1b**	80 (MW)	1.0	55	28
10 + **15c**	**1c**	80 (MW)	1.0	43	14
10 + **15d**	**1d**	80 (MW)	1.0	63	11
10 + **16a**	**3a**	120 (MW)	1.5	68	21
10 + **16b**	**3b**	120 (MW)	1.5	65	19
12 + **15a**	**2a**	100 (MW)	1.0	56	33
12 + **15b**	**2b**	80 (MW)	1.0	70	9
12 + **15c**	**2c**	80 (MW)	1.0	60	30
12 + **15d**	**2d**	80 (MW)	1.0	77	5
12 + **16a**	**4a**	120 (MW)	1.5	76	25
12 + **16b**	**4b**	120 (MW)	1.5	65	20
10 + **17**	**5**	120 (MW)	1.5	n.d.[e]	15
10 + **17ac**	**5ac**	120 (MW)	1.5	36	22
10 + **18**	**6**	120 (MW)	1.5	n.d.[e]	13
10 + **18ac**	**6ac**	120 (MW)	1.5	55	38
10 + **19**	**7**	120 (MW)	1.5	81	18
10 + **19ac**	**7ac**	120 (MW)	1.5	64	40
14 + **16a**	**8**	120 (MW)	2.0	62	20
14 + **18ac**	**9**	120 (MW)	2.0	55	17

[a] Experiments were performed at 0.5–1 μmol scale. [b] MW means microwaves irradiation. [c] Percentages of conjugates in synthesis crudes according to HPLC analysis. [d] Isolated yield after purification. [e] Not determined because of overlapping of chromatographic bands.

Conclusions

To summarize, we have reported a straightforward procedure for the synthesis of aminoglycoside–dinucleotide conjugates derived from neomycin and paromomycin, for the first time by a combination of the Huisgen alkyne–azide cycloaddition and microwaves irradiation. Notably, ligations can be performed in aqueous solution, in very short times and from readily prepared precursors. In spite of what could be expected from the known degradative properties

of aminoglycosides and their copper complexes, ligations were straightforwardly achieved by Cu^I catalysis in the presence of a Cu^I chelator such as TBTA under an inert atmosphere. The same method could also be applied to the preparation of diPNA conjugates, just by extending reaction times. The aminoglycoside conjugates that were prepared in this work are currently being tested as selective effectors of ribosomal RNA and the results will be reported elsewhere.

Experimental Section

General: Unless otherwise indicated, all chemicals were purchased from commercial suppliers (reagent grade) and used without purification. Dry CH_3CN was obtained by distillation over CaH_2 and storage over CaH_2 lumps. CH_2Cl_2 was neutralized and dried by passing through basic Al_2O_3 and storage over CaH_2. Nitrogen was bubbled through DMF in order to remove volatile contaminants, then the DMF was dried by storage over CaH_2. NMR spectra were recorded with a Varian Mercury 400 MHz. MALDI-TOF spectra of oligonucleotides were recorded in a Perseptive Biosystems Voyager DE-RP instrument, by using 2,4,6-trihydroxyacetophenone (THAP) as a matrix.

6′-Azido-6′-deoxyparomomycin (12): Aminoglycoside **11**[33] (300 mg, 0.195 mmol) and sodium azide (26 mg, 0.39 mmol) were dissolved in 12 mL of anhydrous DMF, and the mixture was heated at 80 °C for 4 h. The solvent was removed by evaporation and co-evaporation with CH_3CN, to obtain a solid residue. The residue was redissolved in AcOEt and it was washed twice with water, dried on $MgSO_4$ and the solvent was evaporated. Hexa-*O*-acetyl-6′-azido-4′-*O*-benzoyl-pentakis(*N*-*tert*-butoxycarbonyl)-6′-deoxyparomomycin (**I**) was obtained as a yellowish solid. No further purification was needed according to TLC and NMR analyses; yield 215 mg, 75%. TLC (CH_2Cl_2/CH_3OH, 95:5) R_f = 0.42; m.p. 168–173 °C. ^1H NMR ([D_6]DMSO, 400 MHz); only most relevant signals: δ = 7.84 (d, J = 7.7 Hz, 2 H, NHBoc), 7.65 (t, J = 7.3 Hz, 1 H, NHBoc), 7.50 (t, J = 7.7 Hz, 2 H, NHBoc), 6.87 (d, J = 8.4 Hz, 1 H, NHBoc), 6.82 (d, J = 9.5 Hz, 1 H, NHBoc), 6.20 (d, J = 9.5 Hz, 1 H, NHBoc), 5.54 (d, J = 3.4 Hz, 1 H), 5.40 (d, J = 9.8 Hz, 1 H), 5.54 (br. s, 1 H), 5.39 (d, J = 9.6 Hz, 1 H), 5.19 (br. s, 1 H), 5.13 (t, J = 9.2 Hz, 1 H), 5.05 (t, J = 9.2 Hz, 1 H), 4.82 (br. s, 1 H), 4.57 (m, 2 H), (m, 45 H, CH_3 *t*Bu) ppm. IR (KBr): $\tilde{\nu}$ = 3379, 2978, 2933, 2107, 1747 cm^{-1}. MALDI-TOF MS (positive mode) *m*/*z*: 1519.0 [M + Na]$^+$ (calcd. for $C_{67}H_{100}N_8O_{30}Na^+$, 1519.6), 1534.0 [M + K]$^+$ (calc for $C_{67}H_{100}N_8O_{30}K^+$, 1535.6). ESI HRMS (positive mode) *m*/*z*: 1497.6572 [M + H]$^+$ (calcd. for $C_{67}H_{101}N_8O_{30}^+$, 1497.6618; differential –3.1 ppm), 1519.6396 [M + Na]$^+$ (calc for $C_{67}H_{100}N_8O_{30}Na^+$, 1519.6437; differential –2.7 ppm).

Aminoglycoside **I** (200 mg, 0.134 mmol) was dissolved in 15 mL of NH_3 in CH_3OH (aprox. 7 N), the solution was transferred into a screw-thread vial and heated at 60 °C for 9 h. The solvent was evaporated to obtain a solid residue, which was subsequently treated with 20 mL of 40% TFA in CH_2Cl_2 for 2 h at room temperature. Then, the solution was evaporated to near dryness, and a solid was precipitated by addition of Et_2O. Centrifugation and Et_2O washings rendered aminoglycoside **12** as a brownish solid, pure enough not to be further purified; yield 68 mg, 80%. TLC (CH_2Cl_2/MeOH, 8:2): baseline;[38] m.p. 172–177 °C. ^1H NMR (D_2O, 400 MHz):[40] δ = 5.78 (d, J = 4.4 Hz, 1 H, H$^{1'}$), 5.26 (d, J = 2.4 Hz, 1 H, H$^{1''}$), 5.15 (d, J = 1.6 Hz, 1 H, H$^{1'''}$), 4.38 (dd, J = 6.5, 4.8 Hz, 1 H, H$^{3''}$), 4.25 (dd, J = 4.8, 2.3 Hz, 1 H, H$^{2''}$), 4.17 (m, 1 H, H$^{3'}$), 4.09 (t, J = 3.1 Hz, 1 H, H$^{3'''}$), 4.07 (m, 1 H, H$^{4''}$), 3.89 (dd, J = 9.9, 9.2 Hz, 1 H, H^4 or H^6), 3.75–3.81 (m, 3 H, H^5, H$^{5'}$, H$^{5a''}$), 3.65–3.71 (m, 3 H, H$^{4'''}$, H$^{6a'}$, H$^{6b'}$), 3.62 (dd, J = 12.4, 5 Hz, 1 H, H$^{5b''}$), 3.55 (dd, J = 10.5, 9.2 Hz, 1 H, H^4 or H^6), 3.38–3.50 (m, 4 H, H$^{6'}$, H$^{2'''}$, H$^{6b'''}$, H$^{5'''}$); 3.31 (dd, J = 10.7, 4.0 Hz, 1 H, H$^{2'}$), 3.18–3.29 (m, 3 H, H$^{4'}$, H^1, H^3), 2.36 (dt, J = 4, 12.4 Hz, 1 H, H^{2a}), 1.74 (q, J = 12.4 Hz, 1 H, H^{2b}) ppm. ^{13}C NMR (D_2O, 100 MHz): δ = 118.9, 115.0, 110.2, 95.6, 95.4, 84.5, 81.5, 81.3, 76.7, 76.5, 75.2, 73.5, 72.6, 72.5, 70.3, 70.2, 69.8, 68.8, 67.7, 67.4, 60.2, 53.8, 50.9, 50.8, 49.8, 49.5, 48.8, 40.5, 30.6, 28.1, 25.1, 21.3 ppm. IR (KBr): $\tilde{\nu}$ = 2926, 2116, 1676 cm^{-1}. MALDI-TOF MS (positive mode) *m*/*z*: 641.6 [M + H]$^+$ (calcd. for $C_{23}H_{45}N_8O_{13}^+$, 641.3), 663.7 [M + Na]$^+$ (calcd. for $C_{23}H_{44}N_8O_{13}Na^+$, 663.3), 679.7 [M + K]$^+$ (calcd. for $C_{23}H_{44}N_8O_{13}K^+$, 679.3). ESI HRMS (positive mode) *m*/*z*: 641.3115 [M + H]$^+$ (calcd. for $C_{23}H_{45}N_8O_{13}^+$, 641.3100; differential 2.2 ppm), 663.2929 [M + Na]$^+$ (calcd. for $C_{23}H_{44}N_8O_{13}Na^+$, 663.2920; differential 1.3 ppm), 321.1598 [M + H]$^{2+}$ (calcd. for $C_{23}H_{46}N_8O_{13}^{2+}$, 321.1586; differential 3.5 ppm).

6′′′-*N*-Azidoacetylparomomycin (14): *N*-Succinimidyl azidoacetate was prepared by reaction of azidoacetic acid[41] (540 mg, 5.4 mmol), *N*-hydroxysuccinimide (402 mg, 5.4 mmol) and dicyclohexylcarbodiimide (720 mg, 5.4 mmol) in anhydrous DMF (10 mL) for 1 h at 5 °C. The mixture was filtered through glass wool and added drop by drop, in three portions, to a solution of paromomycin. H_2SO_4 (1.25 g, 1.8 mmol) in 0.1 M $NaHCO_3$/DMF (4:3, 50 mL), and left to react for 18 h. Dioxane was added (200 mL), and the pH was adjusted to 10 by addition of $NaHCO_3$. Boc_2O was added (2.4 g, 10.8 mmol), and the mixture heated at 60 °C. Two additional portions of Boc_2O (1.3 g, 5.4 mmol) were added after 12 h and 25 h, respectively. After 3 d of reaction, the solvent was evaporated to near dryness, and redissolved in ethyl acetate and water. The organic layer was separated, washed twice with water, dried on Na_2SO_4 and evaported to dryness. Product was purified by medium pressure chromatography (MPLC), by employing a 20–90% linear gradient of 0.045% TFA/water and 0.045% TFA/CH_3CN. The fractions corresponding to 6′′′-*N*-azidoacetyl-tetrakis(*N*-*tert*-butoxycarbonyl)paromomycin (**II**) were pooled, evaporated and freeze-dried, to yield a white solid. TLC (CH_2Cl_2/MeOH, 9:1): 0.58. ^1H NMR ([D_6]DMSO, 400 MHz); only most relevant signals:[39] δ = 8.06 (t, J = 3.6 Hz, 1 H, N*H*COCH$_2$N$_3$), 6.80 (d, J = 8 Hz, 1 H, NHBoc), 6.64 (d, J = 6 Hz, 1 H, NHBoc), 6.19 (d, J = 9.6 Hz, 1 H, NHBoc), 5.86 (d, J = 10 Hz, 1 H, NHBoc), 1.37 (br. s, 36 H, CH_3 *t*Bu) ppm. MALDI-TOF MS (positive mode) *m*/*z*: 1121.7 [M + Na]$^+$ (calcd. for $C_{45}H_{78}N_8O_{23}Na^+$, 1122.1), 1137.7 [M + K]$^+$ (calc for $C_{45}H_{78}N_8O_{23}K^+$, 1138.2).

Aminoglycoside **II** was treated with 20 mL of 40% TFA in CH_2Cl_2 for 2 h at room temperature. The solution was evaporated to near dryness, and a solid was precipitated by addition of Et_2O. Centrifugation and Et_2O washings rendered aminoglycoside **14** as a white solid, pure enough not to be further purified. Yield (from paromomycin): 350 mg, 28%. TLC (CH_2Cl_2/MeOH, 8:2): baseline;[38] m.p. 141–142 °C. ^1H NMR (D_2O, 400 MHz):[40] δ = 5.81 (d, J = 4 Hz, 1 H, H$^{1'}$), 5.40 (d, J = 2.4 Hz, 1 H, H$^{1''}$), 5.22 (d, J = 1.6 Hz, 1 H, H$^{1'''}$), 4.44 (t, J = 6.40 Hz, 1 H, H$^{3''}$), 4.37 (dd, J = 3.8, 2 Hz, 1 H, H$^{2''}$), 4.27 (t, J = 3.1 Hz, 1 H, H$^{3'''}$), 4.22 (m, 1 H, H$^{4''}$), 4.15 (m, 1 H, H$^{3'}$), 4.08 (s, 2 H, CH_2N_3), 3.89–4.03 (m, 7 H, H^5, H$^{5a''}$, H$^{5'''}$, H^{6a}, H$^{6b'}$, H^4 or H^6), 3.68–3.76 (m, 5 H, H$^{4'}$, H$^{4'''}$, H^4 or H^6, H$^{5b''}$, H$^{6a'''}$), 3.57 (s, 1 H, H$^{2'''}$), 3.44–3.52 (m, 4 H, H$^{2'}$, H^1 or H^3, H$^{5'}$, H$^{6b'''}$), 3.31–3.40 (m, 2 H, H^1 or H^3), 2.49 (dt, J = 4, 12.4 Hz, 1 H, H^{2a}), 1.84 (q, J = 12.8 Hz, 1 H, H^{2b}) ppm. ^{13}C NMR (D_2O, 100 MHz): δ = 170.8, 110.1, 96.2, 95.7, 84.7, 81.6, 75.7, 73.9, 73.7, 72.7, 72.7, 69.4, 69.0, 67.9, 66.5, 63.7, 60.5, 54.0, 52.1, 51.1, 50.0,

49.0, 39.8 ppm. MALDI-TOF MS (positive mode) *m*/*z*: 699.6 [M + H]$^+$ (calcd. for $C_{25}H_{47}N_8O_{15}^+$, 699.7), 721.6 [M + Na]$^+$ (calcd. for $C_{25}H_{46}N_8O_{15}Na^+$, 721.7), 737.5 [M + K]$^+$ (calc for $C_{25}H_{46}N_8O_{15}K^+$, 737.8). ESI HRMS (positive mode) *m*/*z*: 699.3156 [M + H]$^+$ (calcd. for $C_{25}H_{47}N_8O_{15}^+$, 699.3155; differential –0.1 ppm).

5′-*O*-Hexynyl Dinucleotides (15a–15d): Dinucleotides were assembled on a CPG resin using standard phosphite triester methodology (ABI 381 synthesizer). The phosphoramidite derivatives of 2′-deoxynucleosides (A^{Bz}, C^{Bz}, G^{iBu} and T), the corresponding nucleoside-controlled pore glass supports and reagents and solvents for solid-phase oligonucleotide synthesis were from Glen Research. *O*-Hexynyl phosphoramidite was prepared by reaction of 5-hexyn-1-ol and 2-cyanoethoxy-bis(diisopropylamino)phosphane, and incorporated to the 5′-end of oligonucleotides by extending the coupling time to 15 min. Oligonucleotides were deprotected and cleaved from the resin by treatment with aq. conc. ammonia at 60 °C for 6 h, except for **15a**, which was treated for 2 h at room temperature. As HPLC analyses showed purities >75%, products were readily used in the following ligation step. Products were quantified by UV absorption at 260 nm by considering the experimental ε_{260} values of the corresponding dinucleotides. The following Table 3 summarizes the characterization data of the dinucleotides that were synthesized.

Table 3. Characterization data of 5′-*O*-hexynyl dinucleotides.

Dinucleotides	MALDI-TOF[a] [M – H]$^-$ *m*/*z*	HPLC[b] t_R [min]	ε_{260}
15a	704.2 (705.2)	16.5	16800
15b	723.1 (723.2)	13.3	27400
15c	674.3 (675.2)	12.2	14600
15d	754.2 (755.2)	11.7	21600

[a] MALDI-TOF spectra were obtained in a Perseptive Biosystems Voyager DE-RP instrument (negative and reflector mode) by using THAP/ammonium citrate matrix. The corresponding calculated monoisotopic masses are shown in parenthesis. [b] Oligonucleotides were analyzed by reversed-phase HPLC, by using a linear gradient of 0 to 30% B in 20 min, where solvent A was 0.05 M triethylammonium acetate and solvent B was CH_3CN.

Alkynyl diPNAs (16a–16b, 17–19 and 17ac–19ac): diPNAs (40 μmol) were manually assembled on a commercially available Fmoc-XAL-polyethyleneglycol-polystyrene resin (Novabiochem) following standard procedures. Fmoc/Bhoc monomers were purchased from Applied Biosystems, with the exception of N^{α}-(2-Fmoc-aminoethyl)-N^{α}-(3-pentynoyl)glycine which was prepared essentially as described.[34] To prepare *N*-hexynyloxycarbonyl diPNAs **16a**–**16b**, after the removal of the Fmoc group (20% piperidine in DMF), the resin was treated with 10 equiv. of 5-hexyn-1-yl *p*-nitrophenyl carbonate [freshly prepared by reaction of 5-hexyn-1-ol with bis(*p*-nitrophenyl) carbonate in anhydrous CH_2Cl_2 and DIEA, quantitative yield] and 10 equiv. of DIEA, in NMP at room temperature for 2 h. With respect to diPNAs containing N^{α}-(2-aminoethyl)-N^{α}-(3-pentynoyl)glycine, the Fmoc group was removed to produce free *N*-terminal amino diPNAs **17**–**19** or after the Fmoc removal, the resin was subsequently acetylated by treatment with Ac_2O/DIEA in DMF in the case of diPNAs **17ac**–**19ac**. To produce cleavage and deprotection, the corresponding resin was washed and treated with TFA/*m*-cresol, 19:1 for 10 min at room temperature, solvents were removed by evaporation and coevaporation with anhydrous ether. The resulting residue was resuspended with aq. 0.1% TFA and filtered to separate the resin beads. The aqueous solution that contained the diPNA was washed with AcOEt to remove *m*-cresol and freeze-dried. Crude diPNAs could be readily used in the following ligation step. Products were quantified by UV absorption at 260 nm, considering the calculated values of ε_{260} (ε_{260} = 17200 M^{-1} cm^{-1} for dithymine PNAs and ε_{260} = 30800 M^{-1} cm^{-1} for **16b**). Aliquots were purified by HPLC for characterization purposes. Table 4 summarizes the characterization data for diPNAs.

Table 4. Characterization data of alkynyl diPNAs.

diPNA	MALDI-TOF[a] [M + H]$^+$ *m*/*z*	HPLC[b] t_R [min]
16a	674.1 (674.3)	13.7[b]
16b	693.5 (691.3)	10.5[c]
17	730.4 (730.7)	10.2[b]
17ac	772.7 (772.8)	11.8[b]
18	730.9 (730.7)	10.3[b]
18ac	772.9 (772.8)	11.8[b]
19	730.8 (730.7)	10.3[b]
19ac	772.8 (772.8)	11.5[b]

[a] PNAs were characterized by MALDI-TOF mass spectrometry (positive and reflector mode) in a Perseptive Biosystems Voyager DE-RP instrument, by using THAP matrix. The corresponding monoisotopic masses are shown in parenthesis. [b] Analysis conditions: Linear gradient from 0 to 100% B in 30 min, where solvent A was 0.045% TFA/water and solvent B was 0.036% TFA/CH_3CN. [c] Analysis conditions: linear gradient from 10 to 30% B in 30 min, where solvent A was 0.045% TFA/water and solvent B was 0.036% TFA/CH_3CN.

Aminoglycoside–Dinucleotide and Aminoglycoside–diPNA Conjugates: In a Biotage microwave vial (a 0.5 mL vial for the 0.1 μmol scale or a 5 mL vial for the 0.5–1 μmol scale), 1:1 volumes of 0.48 mM $CuSO_4 \cdot 5H_2O$ (0.2 equiv.) and 12 mM sodium ascorbate

Table 5. Characterization data of aminoglycoside conjugates.

Conjugates	MALDI-TOF[a] [M + H]$^+$ *m*/*z*	HPLC t_R [min]
1a	1347.1 (1346.5)	8.6[b]
1b	1364.8 (1364.5)	8.2[b]
1c	1316.7 (1316.5)	6.8[b]
1d	1397.3 (1396.5)	7.3[b]
3a	1314.7 (1313.6)	12.5[c]
3b	1333.3 (1331.6)	12.9[c]
2a	1348.1 (1347.5)	10.0[b]
2b	1365.4 (1365.5)	9.3[b]
2c	1318.1 (1317.5)	6.4[b]
2d	1397.7 (1397.5)	8.5[b]
4a	1315.5 (1314.6)	14.3[c]
4b	1331.5 (1330.6)	13.5[c]
5	1370.3 (1370.4)	14.3[c]
5ac	1411.8 (1413.4)	17.3[c]
6	1370.3 (1370.4)	14.7[c]
6ac	1411.9 (1413.4)	16.4[c]
7	1370.4 (1370.4)	14.0[c]
7ac	1412.6 (1413.4)	15.9[c]
8	1374.7 (1372.6)	20.9[c]
9	1471.1 (1471.7)	17.9[c]

[a] MALDI-TOF spectra were obtained in a Perseptive Biosystems Voyager DE-RP instrument (reflector mode) by using THAP/ammonium citrate for the analysis of oligonucleotide conjugates, or THAP matrices for the analysis of PNA conjugates. The corresponding calculated monoisotopic masses are shown in parenthesis. [b] Analysis conditions: linear gradient of 0 to 30% B in 20 min, where solvent A was 0.05 M triethylammonium acetate and solvent B was CH_3CN. [c] Analysis conditions: linear gradient from 0 to 20% B in 30 min, where solvent A was 0.045% TFA/water and solvent B was 0.036% TFA/CH_3CN.

(5 equiv.) were reacted under argon for 5 min to produce the Cu^{I} catalyst. Then, 0.48 mM TBTA in water/MeOH (0.2 equiv.), 1 mM hexynyl dinucleotides or diPNAs (1 equiv.) and 2 mM azido-aminoglycosides (2 equiv.) in water were added. The vial was purged with argon, sealed and irradiated (Biotage Initiator™) in the conditions mentioned in the main text (see Table 1 and Table 2) and by selecting the following additional parameters: constant temperature, non pre-stirring, high absorption level and fixed hold time. During the reaction, the irradiation power was 25–40 W and the pressure increased up to 2–3 bar. The resulting crude was freeze-dried and purified by either HPLC or medium pressure chromatography, by employing linear gradients of 0.05 M triethylammonium acetate (pH 7.0) and CH_3CN, or 0.045% TFA/water and 0.036% TFA/CH_3CN. Conjugates were quantified by UV absorption at 260 nm, by considering the corresponding ε_{260} values of the unmodified dinucleotides and diPNAs, and characterized by mass spectrometry. Table 5 summarizes the characterization data of the conjugates described in this study.

Supporting Information (see also the footnote on the first page of this article): HPLC profiles of the experiments of Table 1, NMR spectra of aminoglycosides **12** and **14**, and HPLC and mass spectra analyses of dinucleotides, diPNAs and conjugates.

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[39] We could not identify the signal of CH_2N_3 protons in the spectrum of **II**, because of extensive signal overlapping in the region in which it was expected ($\delta \approx 3.5$–4.0 ppm). However, CH_2N_3 signal was readily identified as a singlet at $\delta = 4.08$ ppm in the spectrum of aminoglycoside **14**, obtained by deprotection of **II**.
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