Design, Synthesis, and Evaluation of Novel Fluoroquinolone−Aminoglycoside Hybrid Antibiotics

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A series of new hybrid structures containing fluoroquinolone (ciprofloxacin) and aminoglycoside (neomycin) antibiotics linked via 1,2,3-triazole moiety were designed and synthesized, and their antibacterial activities were determined against both Gram-negative and Gram-positive bacteria, including resistant strains. The nature of spacers in both the ciprofloxacin and neomycin parts greatly influenced the antibacterial activity. The majority of hybrids was significantly more potent than the parent neomycin and overcame most prevalent types of resistance associated with aminoglycosides. Selected hybrids inhibited bacterial protein synthesis with the potencies similar to or better than that of neomycin and were up to 32-fold more potent inhibitors than ciprofloxacin for the fluoroquinolone targets, DNA gyrase and toposiomerase IV, indicating a balanced dual mode of action. Significant delay of resistance formation was observed in both *E. coli* and *B. subtilis* to the treatment with ciprofloxacin−neomycin hybrid in comparison to that of each drug separately or their 1:1 mixture.

Introduction

The increasing emergence of antibiotic resistance in pathogenic bacteria to current antibiotics is of growing importance worldwide. Among the different classes of clinically important antibiotics that largely suffered from the resistance problem during the past few decades is the aminoglycoside class of drugs. These antibiotics have broad-spectrum of activity against both Gram-negative and Gram-positive bacteria by selectively targeting bacterial protein synthesis machinery and have been used for over 50 years. Such a prolonged clinical and veterinary use of currently available aminoglycosides has resulted in effective selection of resistance, which severely limits their usefulness.[1] The most prevalent mechanism in clinical isolates of resistant bacteria is the bacterial acquisition of aminoglycoside-modifying enzymes, which modify the antibiotics by *N*-acetyltransferase (AAC[a]), *O*-phosphotransferase (APH), and *O*-nucleotidylyltransferase (ANT) activities.[2] Among these enzymes families, aminoglycoside 3′-phosphotransferases [APH(3′)'s], of which seven isozymes are known, are widely represented. These enzymes catalyze phosphorylation at the 3′-OH of both neomycin and kanamycin classes of aminoglycosides, rendering the resulting phosphorylated products inactive.

Although most of these enzymes are typically monofunctional enzymes, the recent emergence of genes encoding bifunctional aminoglycoside-modifying enzymes is another sophistication relevant to the clinical use of aminoglycosides.[3] Among them, the bifunctional AAC(6′)/APH(2″) enzyme has been detected in *Enterococcus*,[4] *Staphylococcus*,[5] and *Streptococcus*[3] isolates, including the methicillin-resistant *Staphylococcus aureus* (MRSA),[6] and has been the most extensively investigated because of the large number of clinically important aminoglycosides that are susceptible to modification with this enzyme.[7,8]

To tackle the problem of bacterial resistance caused by enzymatic modification, many analogues of aminoglycosides have been synthesized by direct chemical modification of existing aminoglycoside drugs.[9,10] Earlier investigations in this direction have yielded several semisynthetic drugs such as amikacin, dibekacin, and arbekacin.[9,11] However, new resistance to these drugs has emerged soon after their introduction to the clinic.[1,12] Therefore, there is an urgent need for new classes of aminoglycosides that are active against known and spreading resistance mechanisms. One such strategy that has been pursued in recent years employs a combination of two different drugs in one molecule.[13] With this strategy, each drug moiety is designed to bind independently to two different biological targets and synchronously accumulate at both target sites. Such dual action drugs, or hybrid drugs, offer the possibility to overcome the current resistance and in addition to reduce the appearance of new resistant strains.[14] Several successful applications of hybrid drugs approach have been reported.[15−17] The dual action compounds, combining fluoroquinolone (enrofloxacin or norfloxacin) and cephalosporin (cefamandole) moieties with an amide linkage, were particularly potent against *Enterobacter* species.[16] Fluoroquinolone−anilinouracil hybrids linked via their secondary amino groups have also been synthesized.[17] A series of oxazolidinone−quinolone hybrid structures, which simultaneously act on two different cellular functions, DNA replication and protein synthesis, have been reported.[14,18] The best compounds of this series exhibited a balanced dual mode of action and overcame the majority of known resistance mechanisms to quinolones and linezolid in clinically relevant Gram-positive pathogens.

We previously reported that the modification of clinical aminoglycoside neomycin B (NeoB) by linking a variety of sugars at C5″-OH group via glycosidic linkage resulted in a

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[a] Abbreviations: AAC, aminoglycoside *N*-acetyltransferase; APH, aminoglycoside *O*-phosphotransferase; ANT, aminoglycoside *O*-nucleotidylyltransferase; MRSA, methicillin-resistant *Staphylococcus aureus*; NeoB, neomycin B; Cipro, ciprofloxacin; TIPSCl, triisopropylchlorosilane; PMB, *p*-methoxybenzyl; TBAF, tetra-*n*-butylammonium fluoride; CAN, cerium ammonium nitrate; DCC, *N*,*N*′-dicyclohexylcarbodiimide; TEMPO, 2,2,6,6-tetramethylpiperidine-1-oxyl; BAIB, [bis(acetoxy)iodo]benzene; HOBT, hydroxybenzotriazole; MIC, minimal inhibitory concentration; TopoIV, topoisomerase IV; IC_{50}, half-maximal inhibitory concentration.

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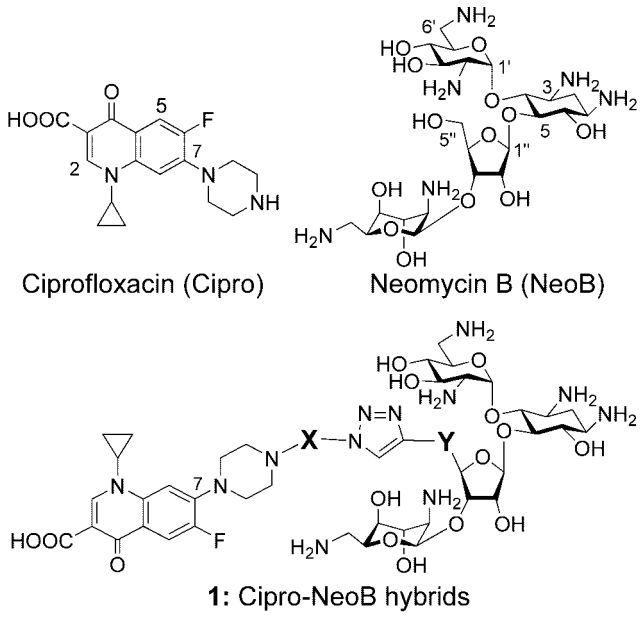


Figure 1. Structures of ciprofloxacin, neomycin B, and the designed hybrids **1**.

new class of pseudopentasaccharides that exhibited similar or better antibacterial activities to that of the parent NeoB against selected bacterial strains.[19] However, while the specificity constant values (k_{cat}/K_m) of these derivatives with the aminoglycoside resistance enzyme APH(3′)-IIIa were in general lower than that of NeoB, the best compounds exhibited these values only about 10-fold lower than that of NeoB, suggesting that several different conformations of the designed structures can bind productively the APH(3′)-IIIa and lead to the enzyme-catalyzed phosphoryl transfer process. Similar superb substrate promiscuity of APH(3′)-IIIa with other nonsugar modifications of NeoB at the C5″ position has been recently reported,[20] although several of these derivatives exhibited enhanced antibacterial activity compared to the parent NeoB. To further explore the modification of NeoB at the C5″ position to maximize the ability of new derivatives to compromise with the activity of APH(3′) and other aminoglycoside-modifying enzymes, we undertook additional synthesis of this class of compounds. Specifically, having been encouraged by the recently reported potential of hybrid antibiotics, we have prepared a new hybrid structures of NeoB and the fluoroquinolone−ciprofloxacin (Cipro) linked via 1,2,3-triazole moiety, which we called Cipro−NeoB hybrids (compounds **1**, Figure 1).

Quinolones exert their antibacterial activity by targeting bacterial DNA gyrase and topoisomerase IV (TopoIV) and inhibiting DNA replication process.[21] Specifically, they bind to complexes that form between DNA and DNA gyrase or TopoIV. The quinolone−gyrase−DNA or quinolone−TopoIV−DNA complex formation inhibits DNA replication and cell growth and is responsible for the bactericidal action of quinolones.[22] On the basis of these data, we hypothesized that the Cipro−NeoB hybrids (**1**), because of the presence of the highly positively charged NeoB, could afford favorable binding to that of Cipro to these ternary complexes by forming additional contacts to DNA and/or DNA−protein interface and as such exhibit better inhibition and improved antibacterial activity. The well-established binding of aminoglycosides to DNA,[23] along with the inhibition of various nucleic acid metabolizing enzymes by aminoglycosides,[24] supported this hypothesis.

The present work describes the synthesis and biological evaluation of the Cipro−NeoB hybrids (**1**), where the two pharmakophores are linked through different spacers: spacer X in the Cipro part and spacer Y in the NeoB part. We report that selected hybrid structures have high potency against both Gram-negative and Gram-positive bacteria including MRSA and strains harboring either the monofunctional APH(3′) enzyme or the bifunctional AAC(6′)/APH(2″) enzyme. We also show that new hybrids exhibit the dual mode of action by inhibiting both targets: bacterial protein synthesis and topoisomerase/gyrase.

Chemistry

The synthesis of the Cipro−NeoB hybrids **1** is described in Scheme 1. We separately prepared nine azido derivatives of Cipro (compounds **2a**−**i**) and three different alkyne derivatives of NeoB (compounds **3a**−**c**), which were then coupled via "click reaction"[25] to afford a library of 17 different Cipro−NeoB hybrids **1a**−**q**. The spacers X and Y were selected to vary both the length and chemical nature of the linkage between the two pharmakophores Cipro and NeoB.

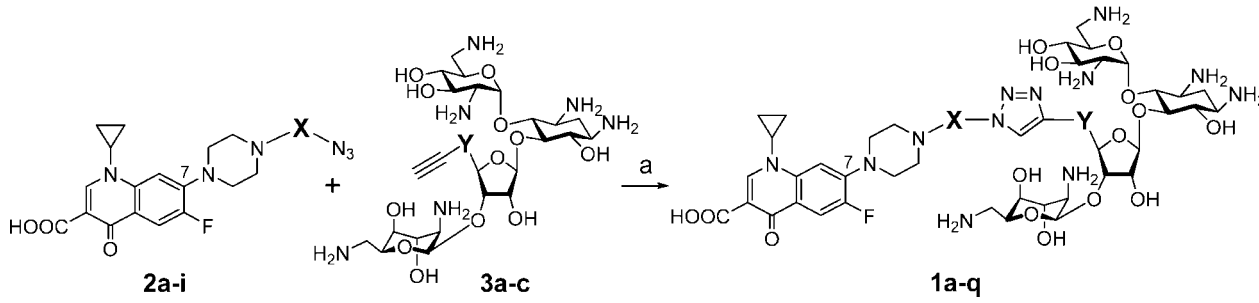
Several SAR studies on fluoroquinolones have demonstrated a high tolerance for structural variations at the 7-position of the phenyl ring (Figure 1), including alkylations at the terminal nitrogen of the piperazine moiety.[14,16−18,26,27] On the basis of this information, we choose to modify Cipro at the terminal nitrogen of the piperazine moiety with various linkers containing an azide group. The derivatives **2a**−**i** were prepared by direct coupling of the commercial Cipro with the corresponding bromoazides/chloroazides under reflux and base conditions ($NaHCO_3$, CH_3CN), and the yields of these products are summarized in Table 1. The bromoazides/chloroazides were synthesized from the corresponding dibromo or bromochloro compounds according to published procedure.[28]

The synthesis of the alkyne derivatives of NeoB (compounds **3a**−**c**) is described in Scheme 2. For the preparation of the alkyne derivative **3a**, commercial NeoB was first converted to the corresponding per-azido derivative according to published procedure,[29] followed by selective protection of the primary hydroxyl to afford the intermediate **4**. Protection of all the secondary hydroxyls with *p*-methoxybenzyl (PMB) ether and selective removal of silyl ether with TBAF gave compound **5**. Treatment of **5** with propargyl bromide under base conditions of NaH afforded the 5″-alkyne derivative **6**. Two deprotection steps, removal of PMB ether protections with CAN followed by Staudinger reaction to convert all the azides to the corresponding amines, gave the alkyne derivative **3a**.

Two other alkyne derivatives of NeoB, compounds **3b**,**c**, contain a terminal alkyne group connected to the NeoB moiety at 5″-position via an amide linkage (Scheme 2b). For the assembly of these derivatives, the readily available 5″-alcohol **7**[30] was converted to the corresponding 5″-acid **8** according to the published procedure.[31] The resulting acid was then coupled with the commercially available alkyne−amines in the presence of DCC to afford the corresponding amides **9a**,**b** in reasonable yields. Finally, removal of all the ester protections ($MeNH_2$, MeOH) and Staudinger reaction yielded the alkyne derivatives **3b**,**c** in excellent isolated yields.

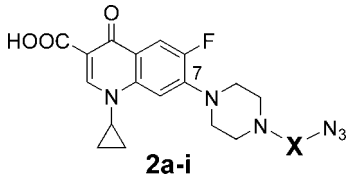
The key coupling reaction between NeoB-alkyne derivatives (compounds **3a**−**c**) and Cipro-azide derivatives (compounds **2a**−**i**) was performed under microwave irradiation (∼40 s) in the presence of organic base (7% Et_3N in water) and the Cu(I) catalyst to ensure the production of a single (anti) stereoisomer at the triazole moiety.[25,32] The reaction proceeded almost

Scheme 1. Strategy for the Assembly of Cipro−NeoB Hybrids **1a**−**q**[a]



[a] (a) $[(CH_3CN)_4Cu]PF_6$, 7% Et_3N in water, microwave irradiation 40 s.

Table 1. Chemical Yields and Preparation of Ciprofloxacin−Azido Derivatives **2a**−**i**



compd	azido linker coupled with Cipro[a]	X	yield (%)
2a	(Cl)Br-$(CH_2)_2$-N_3	-$(CH_2)_2$-	75
2b	(Cl)Br-$(CH_2)_3$-N_3	-$(CH_2)_3$-	70
2c	(Cl)Br-$(CH_2)_4$-N_3	-$(CH_2)_4$-	80
2d	(Cl)Br-$(CH_2)_5$-N_3	-$(CH_2)_5$-	65
2e	Br-$(CH_2)_6$-N_3	-$(CH_2)_6$-	57
2f	Br-$CH_2CH(OH)CH_2$-N_3	-$CH_2CH(OH)CH_2$-	31
2g	Br-$(CH_2)_2$-O-$(CH_2)_2$-N_3	-$(CH_2)_2$-O-$(CH_2)_2$-	75
2h	Br-CH_2-*m*C_6H_4-CH_2-N_3	-CH_2-*m*C_6H_4-CH_2-	44
2i	Br-CH_2-*p*C_6H_4-CH_2-N_3	-CH_2-*p*C_6H_4-CH_2-	30

[a] The azido linkers were prepared from the corresponding commercially available dibromo or bromochloro compounds according to a published procedure.[28]

quantitatively in the presence of 1.2 equiv of Cipro-azide derivative and 1 equiv of NeoB-alkyne. The unreacted Cipro-azide could then be easily separated from the hybrid product by passing the reaction mixture through a short column of Amberlite CG-50 (H^+ form) resin. As test cases indicated that the protocol provided excellent yields of highly pure product, the three NeoB-alkyne derivatives **3a**−**c** were coupled with selected Cipro-azide derivatives **2a**−**i** to produce 17 hybrid products **1a**−**q** (Table 2); all compounds were synthesized at a minimum of a 0.05 mmol scale to provide approximately 50 mg of each product (as a free base form). The structures of **1a**−**q** were confirmed by a combination of various 1D and 2D NMR techniques, including 2D $^1H-^{13}C$ HMQC and HMBC, 2D COSY, and 1D selective TOCSY experiments, along with mass spectral analysis.

Results and Discussion

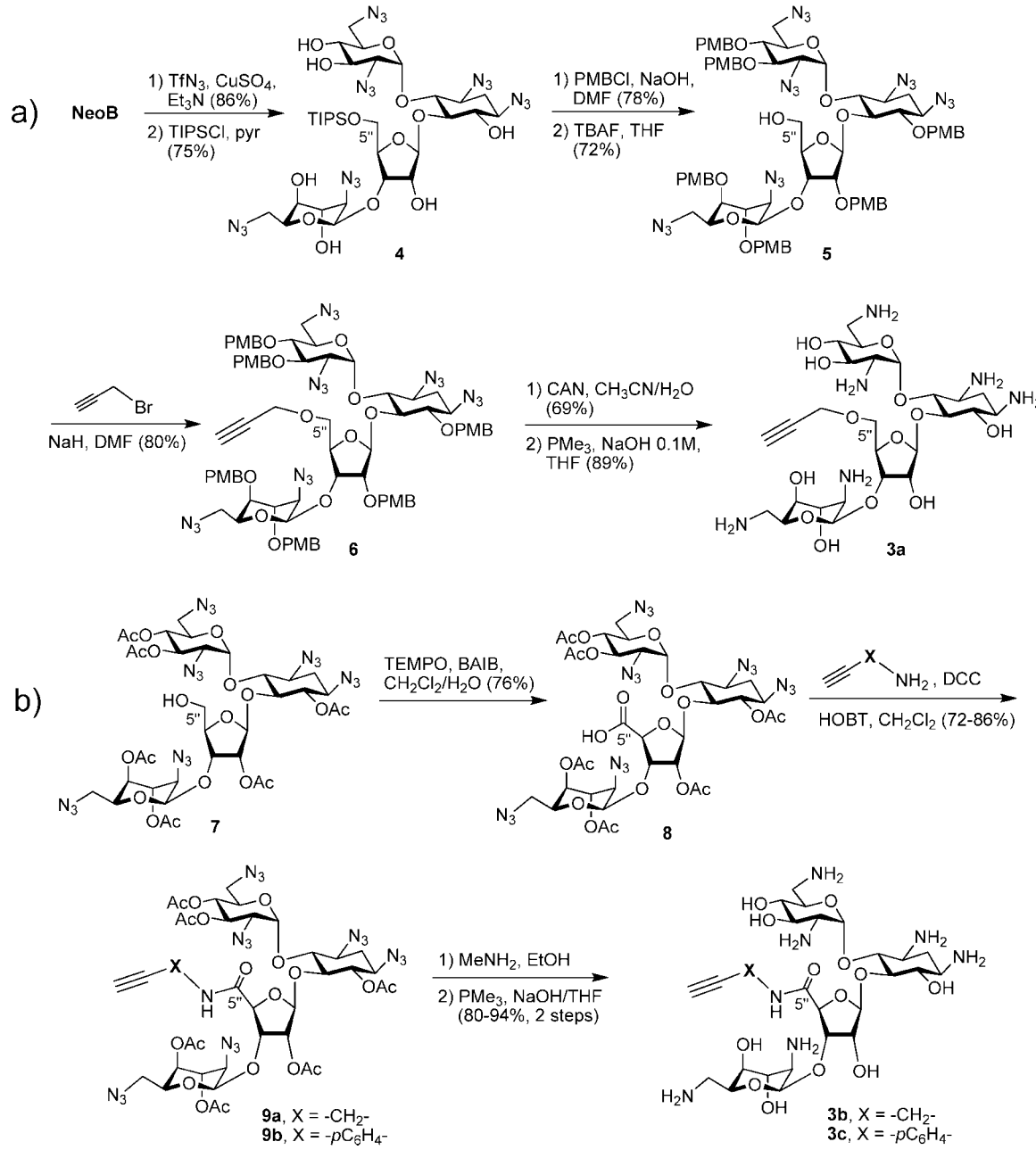
The Cipro−NeoB hybrids **1a**−**q** were tested for in vitro antibacterial activity against a panel of susceptible and resistant bacterial strains using Cipro and NeoB as controls (Table 2). Data for selected Gram-negative and Gram-positive strains are reported as minimal inhibitory concentration (MIC) values. Resistant strains included *Escherichia coli* AG100A and AG100B (Gram-negative) and methicillin-resistant *S. aureus* (MRSA) (ATCC 43300, Gram-positive). *E. coli* AG100A and AG100B are aminoglycosides-resistant laboratory strains that harbor Kan^r transposon Tn903.[33] MRSA (ATCC 43300) is one of the leading causes of bacterial infections and exerts high level of resistance to aminoglycosides.[34]

As can be deduced from the MIC data, all of the synthesized hybrids exhibited significant antibacterial activity. This activity was especially improved in comparison to that of NeoB, while the activity of all the hybrids was lower than that of Cipro. The most prominent improvement was observed against all *E. coli* strains including aminoglycosides-resistant strains *E. coli* AG100A and *E. coli* AG100B. On average, the hybrids showed 2−8 and 2−16 times better activity than NeoB against *E. coli* R477-100 and *E. coli* 25922, respectively, and this was much higher against the resistant *E. coli* AG100A and *E. coli* AG100B strains with **1i** and **1q** as the most active derivatives. Compound **1i** was 128-fold more potent than NeoB against *E. coli* AG100B and *E. coli* AG100A; compound **1q** was 32-fold better than NeoB against *E. coli* AG100B and 253-fold better against *E. coli* AG100A. Unlike the activity against Gram-negative *E. coli*, most of the hybrids were less active than the parent NeoB against the Gram-positive *Bacillus subtilis*, susceptible to aminoglycosides. However, all the hybrids displayed significantly better potency against the aminoglycosides-resistant Gram-positive MRSA with the activities of 8- to 128-fold better than that of NeoB. The hybrid compounds **1i** and **1q** retained activity similar to that of NeoB in *B. subtilis* and displayed the most prominent activity against the MRSA.

As to the SAR among the 17 hybrid structures, it appears that the length of the linear aliphatic chain at position X (**1a**−**e** and **1j**−**l**) has no particular influence on antibacterial activity, as the variation in antibacterial potency against individual strains is very little. This is valid when comparing between **1a**−**e** or between **1j**−**l** of the same sets with respect to the Y spacer or between **1a**−**e** and **1j**−**l** of different sets. Hybrids consisting of the linear aliphatic chain incorporating alcohol functionality (**1f** and **1m**) show better activity than those incorporating ether functionality (**1g** and **1n**) at position X. Among the derivatives containing an aromatic linker at position X (**1h**,**i** and **1o**,**p**), the derivative **1i** that contains para-substituted benzene ring at both X and Y positions displayed the most prominent activity against all bacterial strains tested. Surprisingly, the only hybrid that displayed a spectrum of activity similar to that of **1i** was the hybrid **1q** that contains the shortest linkers at both X and Y positions. Nevertheless, the observation that the majority of hybrids are more active than NeoB against Gram-negative bacteria (*E. coli*) while retaining moderate activities against the susceptible Gram-positive bacteria (*B. subtilis*) prompted us to further investigate the activity of hybrids against *E. coli* strains harboring particular aminoglycosides resistant plasmids.

For this purpose, we tested selected hybrids against five isogenic *E. coli* strains (Table 3). *E. coli* (pSF815) and *E. coli* (pET9d) are laboratory resistant strains derived by transforma-

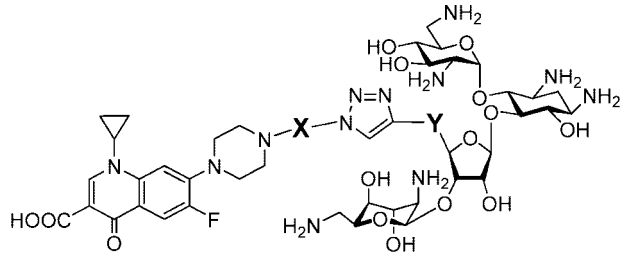
Scheme 2. Synthesis of NeoB 5″-Alkyne Derivatives



tion of *E. coli* XL1 blue (background strain) with the pSF815 and pET9d plasmids, respectively. The pSF815 encodes for the bifunctional AAC(6′)/APH(2″) resistance enzyme, which catalyzes acetylation of the amino group at 6′-NH_2 and phosphorylation at the 2″-OH. The pET9d encodes for the APH(3′)-Ia resistance enzyme, which catalyzes phosphorylation at the 3′-OH of both neomycin and kanamycin families of aminoglycosides. The last two isogenic strains used were *E. coli* BL21 (background strain) and *E. coli* (pETSACG1). The latter was derived by transformation of *E. coli* BL21 with the pETSACG1 plasmid that encodes for the APH(3′)-IIIa resistance enzyme. These three enzymes are among the most prevalent modes of resistance found in aminoglycosides resistance strains.[5,34,35,2] Since the aminoglycoside resistance of the engineered strains, *E. coli* (pSF815), *E. coli* (pET9d), and *E. coli* (pETSACG1), is mediated only because of the presence of the respective cloned resistance enzyme, comparison of the MIC values against each pair of the resistant and background strains eliminates other effects that could affect the activity of the tested compound, like penetration or solubility. Consequently, a poorer substrate for the resistance enzyme should have a low ratio between the MIC values of the resistant and nonresistant strains, as demonstrated in several earlier studies.[19,20,36]

The data in Table 3 illustrate that the MIC values for NeoB are >64-, 16-, and 8-fold higher for the resistant strains than the respective nonresistant strains. As expected, the activity of NeoB against the resistant strain harboring bifunctional AAC(6′)/APH(2″) resistance is significantly lower than those harboring monofunctional APH(3′)-Ia or APH(3′)-IIIa resistance. All the tested hybrids were less effective than Cipro but displayed significant to excellent activities against resistant strains with the potencies far greater than that of the parent NeoB. The hybrid structures also displayed similar (*E. coli* XL1 blue) or better (*E. coli* BL21) antibacterial efficiency compared to NeoB against susceptible strains. Subsequently, the MIC ratio for each tested hybrid (calculated by dividing the MIC value against resistant

Table 2. Chemical Yields and MICs of the Hybrids **1a**−**q** against Selected Bacterial Strains[a]



1a-q

				MIC (μg/mL)[b]					
compd	X	Y	yield (%)	*E.coli* R477-100	*E.coli* ATCC 25922	*E.coli*[c] AG100B	*E.coli*[c] AG100A	*B. subtilis* ATCC 6633	MRSA ATCC 43300
Cipro				0.02	0.02	0.05	<0.005	0.02	0.20
NeoB				24	48	384	96	1.5	384
1a	-$(CH_2)_2$-	-C_6H_4-NHCO-	63	12	6	24	0.75	6	48
1b	-$(CH_2)_3$-	-C_6H_4-NHCO-	90	6	3	12	1.5	6	48
1c	-$(CH_2)_4$-	-C_6H_4-NHCO-	92	12	6	24	1.5	6	24
1d	-$(CH_2)_5$-	-C_6H_4-NHCO-	94	12	6	24	1.5	12	48
1e	-$(CH_2)_6$-	-C_6H_4-NHCO-	96	6	6	12	1.5	12	48
1f	-$CH_2CH(OH)CH_2$-	-C_6H_4-NHCO-	92	6	3	6	0.75	6	24
1g	-$(CH_2)_2$-O-$(CH_2)_2$-	-C_6H_4-NHCO-	95	24	12	12	1.5	12	24
1h	-CH_2-*m*C_6H_4-CH_2-	-C_6H_4-NHCO-	95	6	12	12	1.5	6	12
1i	**-CH_2-*p*C_6H_4-CH_2-**	**-C_6H_4-NHCO-**	**91**	**1.5**	**3**	**3**	**0.75**	**1.5**	**3**
1j	-$(CH_2)_2$-	-CH_2-NHCO-	97	12	6	12	1.5	3	24
1k	-$(CH_2)_3$-	-CH_2-NHCO-	93	12	6	12	1.5	6	48
1l	-$(CH_2)_5$-	-CH_2-NHCO-	96	24	6	24	1.5	12	48
1m	-$CH_2CH(OH)CH_2$-	-CH_2-NHCO-	85	6	3	12	0.75	3	12
1n	-$(CH_2)_2$-O-$(CH_2)_2$-	-CH_2-NHCO-	90	12	6	12	1.5	12	24
1o	-CH_2-*m*C_6H_4-CH_2-	-CH_2-NHCO-	93	6	6	12	0.75	3	6
1p	-CH_2-*p*C_6H_4-CH_2-	-CH_2-NHCO-	96	6	6	12	1.5	3	3
1q	**-$(CH_2)_2$-**	**-CH_2-O-CH_2-**	**97**	**3**	**3**	**12**	**0.38**	**0.75**	**6**

[a] The boldface rows in the table highlight the most potent compounds. [b] The MIC values represent the results obtained in parallel experiments with two different starting concentrations of the tested compound (384 and 1.5 μg/mL). [c] Kanamycin resistant *Escherichia coli* strains expressing APH(3′)-I aminoglycoside resistance enzyme.[33]

Table 3. Antibacterial Activities of Selected Hybrids against *E. coli* XL1 Blue and *E. coli* BL21 (Background Strains) and Their Engineered Variants

	MIC (μg/mL)			MIC (μg/mL)		MIC (μg/mL)		
compd	XL1 blue	XL1 blue/pSF815, expressed enzyme AAC(6′)-APH(2″)	MIC ratio[a]	XL1 blue/pET9d, expressed enzyme APH(3′)-Ia	MIC ratio[a]	BL21	BL21/pETSACG1, expressed enzyme APH(3′)-IIIa	MIC ratio[a]
Cipro	0.10	0.38	3.8	0.10	1	<0.005	<0.005	1
NeoB	6	>384	>64	96	16	6	48	8
1i	3	24	8	3	1	0.4	0.4	1
1m	6	48	8	12	2	0.2	0.2	1
1o	6	24	4	6	1	0.4	0.4	1
1p	6	6	1	6	1	0.4	0.4	1
1q	3	12	4	12	4	0.2	0.2	1

[a] The MIC ratios were calculated by dividing the MIC value against resistant strain by that against the respective background strain.

strain to the MIC value against susceptible strain) was significantly lower than that calculated for the NeoB. Most importantly, this MIC ratio was 1 for the majority of cases: for all the hybrids against *E. coli* (pETSACG1); for the hybrids **1i**, **1p**, and **1o** against *E. coli* (pET9d); and for the **1p** against *E. coli* (pSF815). The observed identical MICs of the hybrids against different isogenic pairs of bacteria indicate that the reason for the observed sensitivity of the *E. coli* harboring AAC(6′)/APH(2″) (in the case of **1p**), APH(3′)-Ia (in the cases of **1i**, **1p**, and **1o**), or APH(3′)-IIIa (in all the tested hybrids) is the inferior activity of these enzymes toward particular hybrids rather than reduced permeability of the hybrid structures.

To further substantiate this observation, a detailed kinetic analysis of the purified APH(3′)-IIIa enzyme with NeoB along with the selected hybrid **1m** (that displayed the MIC ratio of 1 against pETSACG1) was carried out according to the previously reported procedure.[19] The measured K_m (μM), k_{cat} (s^{-1}), and k_{cat}/K_m ($M^{-1}\cdot s^{-1}$) values were 5.7 ± 0.7, 2.4 ± 0.1, and 42 × 10^{-4} for NeoB and 86.8 ± 8.9, 2.5 ± 0.1, and 2.9 × 10^{-4} for the hybrid **1m**. The kinetic constants measured for NeoB are similar to previously reported values.[19,37] The observed data indicate that the hybrid **1m** is a poorer substrate of APH(3′)-IIIa than NeoB. The observed decrease in specificity for **1m** is caused primarily by its poor ability to saturate the enzyme, as judged from its elevated K_m (87 μM) compared to that of the NeoB (K_m = 6 μM). The observed kinetic data with **1m** are consistent with the antibacterial data (Table 3). In fact, comparison of its K_m value (87 μM) with the MIC of 5.5 μM (0.2 μg/mL) against *E. coli* (pETSACG1) suggests that the bacteria are killed at far lower concentration before the enzyme's full activity is reached.

From the data in Table 3 it was interesting to find out that the MIC ratio for Cipro in the case of the isogenic *E. coli* XL1 blue (pSF815)/*E. coli* XL1 blue strains was 3.8, indicating a modification of this important clinical antibiotic by the aminoglycoside resistant AAC(6′)/APH(2″) enzyme. Since two

Table 4. Activity of Selected Hybrids as Inhibitors of DNA Gyrase, TopoIV, and Bacterial Protein Synthesis

	IC_{50} (μM)		
compd	DNA gyrase[a]	TopoIV[b]	protein synthesis[c]
Cipro	1.3 ± 0.1	10.8 ± 0.3	inactive
NeoB	inactive	inactive	10.5 ± 0.1
1f	0.073 ± 0.005	0.58 ± 0.04	2.2 ± 0.6
1i	0.085 ± 0.003	0.55 ± 0.06	16.7 ± 4.4
1q	0.041 ± 0.009	7.90 ± 0.25	18.1 ± 4.9

[a] Supercoiling assay with *E. coli* DNA gyrase. The IC_{50} was defined as the drug concentration that reduced the enzymatic activity observed with drug-free controls by 50%. See Experimental Section for assay details. [b] Relaxation assay with *E. coli* TopoIV. The IC_{50} was defined as the drug concentration that reduced the enzymatic activity observed with drug-free controls by 50%. See Experimental Section for assay details. [c] In vitro transcription/translation assay with *E. coli* S30 extract system. See Experimental Section for assay details.

other strains harboring APH(3′) activity displayed MIC ratios of 1, we anticipated that Cipro may undergo N-acetylation at the terminal nitrogen of its piperazine moiety by AAC(6′)/APH(2″). This suggestion is supported by recent reports demonstrating that some common aminoglycoside acetyltransferases (AACs), including AAC(6′)s, are capable of performing N-acetylation of flouroquinolones having a free amino group at 7-position.[38,39] In addition, a close inspection of the data in Table 2 reveals that Cipro displayed particularly reduced activity against *S. aureus* (MRSA): MIC of 0.2 in comparison to MIC values of 0.05 to <0.005 against other tested strains. Since the bifunctional AAC(6′)/APH(2″) enzyme is the most frequently encountered aminoglycoside-modifying enzyme in staphylococci, including MRSA,[6] the observed reduced activity of Cipro against *S. aureus* (MRSA) may be due to the modification of Cipro by AAC(6′)-APH(2″). To further investigate these observations, purification of the bifunctional AAC(6′)/APH(2″) and the detailed kinetic analysis of Cipro with the homogeneous enzyme, along with the structural characterization of the enzymatic product, are currently underway.

To investigate the possibility of a dual mode of action, we measured for the hybrids **1f**, **1i**, and **1q** both the inhibition of protein synthesis in an in vitro transcription/translation assay and the inhibition of the enzymes that are targeted by the quinolones, DNA gyrase, and TopoIV (Table 4 and Figure 2). The observed data show that the hybrid compounds inhibited bacterial protein synthesis with potencies similar to or better than that of NeoB, confirming their strong aminoglycoside character and the observed antibacterial activity. On the basis of the observed reduced antibacterial activity of all the hybrids in comparison to that of Cipro (Tables 2 and 3), we expected that the hybrids should be weaker DNA gyrase and TopoIV inhibitors than Cipro. However, the hybrids **1f**, **1i**, and **1q** displayed far greater activities than Cipro in both the DNA gyrase and TopoIV assays, indicating the dual mode of action of these molecules. The measured IC_{50} values for **1f**, **1i**, and **1q** were 18-, 15-, and 32-fold superior to that of Cipro in DNA gyrase assay and 19-, 20-, and 1.4-fold superior to Cipro in TopoIV assay. It is of note that the IC_{50} values determined for Cipro for the inhibition of DNA gyrase and TopoIV are very similar to those previously reported.[18,40] These data clearly confirm our design principle of the Cipro−NeoB hybrids and their desired dual mode of action. The observed difference between antibacterial performance and targets inhibition can be best explained by the reduced cell penetration of the hybrid structures in comparison to Cipro. Both the higher molecular weight and the higher charge of the hybrids compared with those of Cipro might contribute to their reduced cell penetration. Nevertheless, the observed superior activity of the selected hybrids (like **1i** and **1q**) compared to that of the parent NeoB against a variety of Gram-negative and Gram-positive bacteria including resistant strains along with their established dual mode of action warrants further investigation of these compounds.

Finally, one advantage of hybrid drugs is their potential to slow the emergence of resistance.[13,17] The underlying hypothesis is that treatments that inhibit multiple targets might delay and decrease the pathogen's ability to accumulate simultaneous mutations that affect the multiple targets.[41] To assess the potential of emergence of antibacterial resistance to Cipro−NeoB hybrids, we used a procedure of selective pressure in which both *E. coli* ATCC 35218 and *B. subtilis* ATCC 6633 were exposed to subinhibitory ($^1/_2$ MIC) concentrations of Cipro, NeoB, Cipro/NeoB mixture (1:1 molar ratio), and hybrid **1i** during 15 successive subcultures (Figure 3). We note that the MIC values of the Cipro/NeoB mixture (1:1 molar ratio) against the bacterial strains mentioned in Table 2 were very similar to that of Cipro (on the weight basis of the composition of Cipro in the mixture; data not shown). Therefore, in these experiments we were very interested, in addition to Cipro and NeoB, to also include the mixture Cipro + NeoB. As can be seen from the data in Figure 3, the relative MIC values of Cipro, NeoB, and Cipro + NeoB mixture increased by 75-, 4-, and 20-fold against *E. coli* and by 37.5-, 8-, and 7.6-fold against *B. subtilis* while that of the hybrid **1i** remained unchanged against both *E. coli* and *B. subtilis*. Similar emergence of resistance under same experimental conditions has been reported for Cipro[42] and aminoglycosides.[43] To our knowledge, the ability of hybrid drugs to delay the emergence of resistance development was not demonstrated for the previously reported hybrid structures.[13−18] As such, the observed delay of resistance development to the hybrid **1i**, compared to that of Cipro, NeoB, and the mixture Cipro + NeoB in both the Gram-negative (*E. coli*) and Gram-positive (*B. subtilis*) bacteria, is very encouraging.

Conclusions

A series of hybrid compounds containing a covalently linked fluoroquinolone (Cipro) and aminoglycoside (NeoB) with potent antibacterial activity and dual mode of action has been discovered. The nature of the spacers in fluroquinolone and aminoglycoside parts greatly influenced the antibacterial activity. The hybrids were significantly more potent than the parent NeoB, especially against Gram-negative bacteria and Gram-positive MRSA, and overcame most prevalent types of resistance associated with aminoglycosides. The hybrids inhibited bacterial protein synthesis with potencies similar to or better than that of NeoB and were up to 32-fold more potent inhibitors than Cipro for the fluoroquinolone targets, DNA gyrase, and TopoIV. One case study also demonstrated a significant delay of resistance formation in both Gram-negative (*E. coli*) and Gram-positive (*B. subtilis*) bacteria to the treatment with Cipro−NeoB hybrid in comparison to that of each drug (Cipro and NeoB) separately or their 1:1 mixture.

Experimental Section

General Methods. ^{1}H NMR spectra (including DEPT, 2D COSY, 2D TOCSY, 1D TOCSY, HMQC, HMBC) were routinely recorded on a Bruker Avance 500 spectrometer, and chemical shifts reported (in ppm) are relative to internal Me_4Si ($\delta = 0.0$) with $CDCl_3$ as the solvent and to HOD ($\delta = 4.63$) with D_2O as the solvent. ^{13}C NMR spectra were recorded on a Bruker Avance 500 spectrometer at 125.8 MHz, and the chemical shifts were reported (in ppm) relative to the residual solvent signal for $CDCl_3$ ($\delta = 77.00$) or to external sodium 2,2-dimethyl-

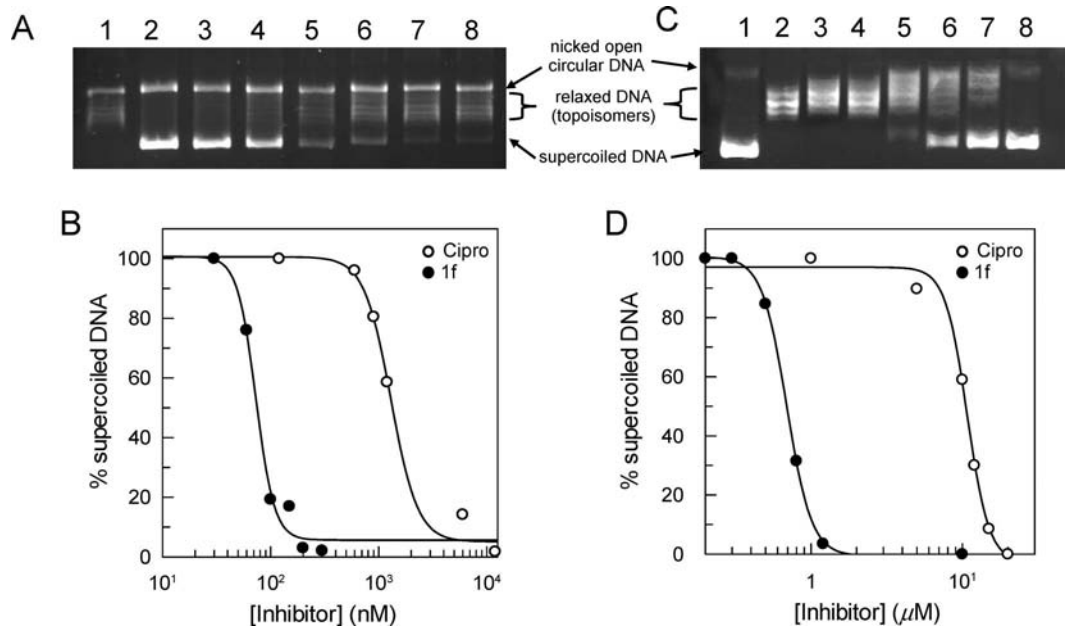


Figure 2. Representative comparative data for the inhibition of DNA gyrase (A and B) and TopoIV (C and D) with Cipro and compound **1f**. (A) A 1% agarose gel shows the inhibitory activity of **1f** against DNA gyrase: lane 1, relaxed DNA; lane 2, supercoiling reaction by DNA gyrase without presence of inhibitor; lanes 3−8, same as lane 1 but in the presence of 30, 60, 100, 150, 200, and 300 nM inhibitor **1f**. (B) Semilogarithmic plot of in vitro DNA gyrase supercoiling reaction inhibition, measured for Cipro and **1f**. (C) A 1% agarose gel shows the inhibitory activity of **1f** against TopoIV: lane 1, supercoiled DNA; lane 2, relaxation reaction by TopoIV without the presence of inhibitor; lanes 3−8, same as lane 1 but in the presence of 0.2, 0.3, 0.5, 0.8, 1.2, and 10 μM **1f**. (D) Semilogarithmic plot of TopoIV inhibition, measured for Cipro and **1f**. The percentages of the supercoiled DNA were calculated from the electrophoresis images by using ImageJ Launcher program (Rasband, W. Bethesda, MD) and plotted as functions of drug concentration. Each data point represents the average of two to three independent experimental results.

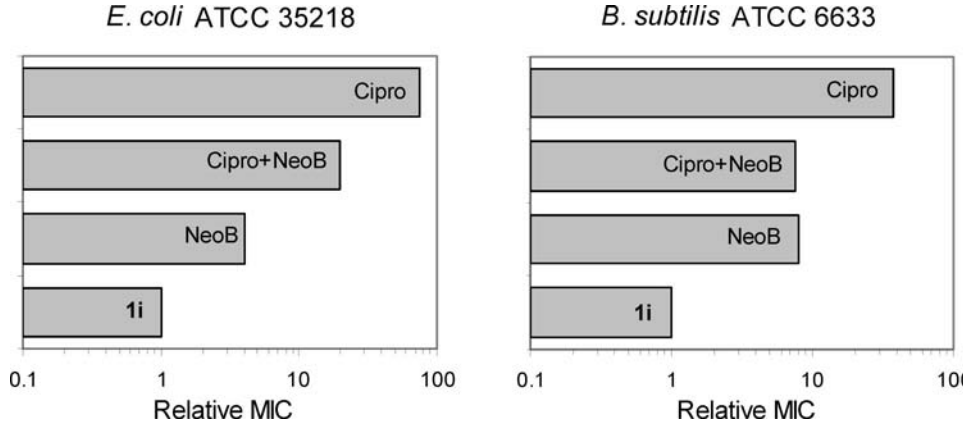


Figure 3. Comparative study on the emergence of resistance in *E. coli* and *B. subtilis* after 15 serial passages in the presence of Cipro, NeoB, Cipro + NeoB mixture (1:1 molar ratio), and hybrid structure **1i**. Relative MIC is the normalized ratio of MIC obtained for a given subculture to MIC obtained upon first exposure.

2-silapentane sulfonate ($\delta = 0.0$) for D_2O as the solvent. Mass spectra were obtained either on a Bruker Daltonix Apex 3 mass spectrometer under electron spray ionization (ESI) or by a TSQ-70B mass spectrometer (Finnigan Mat). Reactions were monitored by TLC on silica gel 60 F_{254} (0.25 mm, Merck), and spots were visualized by charring with a yellow solution containing $(NH_4)Mo_7O_{24}\cdot 4H_2O$ (120 g) and $(NH_4)_2Ce(NO_3)_6$ (5 g) in 10% H_2SO_4 (800 mL). Flash column chromatography was performed on silica gel 60 (70−230 mesh). All reactions were carried out under an argon atmosphere with anhydrous solvents, unless otherwise noted. IR spectra ($CHCl_3$) were recorded on a Bruker vector 22 spectrophotometer, and only significant peaks were identified. Microwave assisted reactions were carried out in domestic microwave oven, Sauter SG251. Analytical HPLC was performed on Hitachi LC system equipped with autosampler, by using Superspher 100 RP-18 column and a detection wavelength of 271 nm. All chemicals, unless otherwise stated, were obtained from commercial sources. 1-Bromo-2-chloroethane, 1-bromo-3-chloropropane, 1-bromo-4-chlorobutane, 1-bromo-5-chloropentane, 1,6-dibromohexane, 1,3-dibromo-2-propanol, 2-bromoethyl ether, α,α′-dibromo-*m*-xylene, and α,α′-dibromo-*p*-xylene as well as 4-ethynylaniline and propargylamine were obtained from Sigma-Aldrich Israel. Compound **7** was prepared as previously reported.[30] Purity of the hybrids **1a−q** were determined by using HPLC analysis which indicated >95% purity of each product (see Supporting Information).

General Procedure for Preparation of 2a−i. A mixture of ciprofloxacin (1 mmol) and the azido linker (5 mmol) in acetonitrile (15 mL) was refluxed in the presence of powdered $NaHCO_3$ (1 mmol) for 12−24 h. When TLC ($MeOH/CH_2Cl_2$, 1:9) indicated completion of the reaction (2−14 h), the mixture was filtered and washed with excess $MeOH/CH_2Cl_2$ (1:1) and the combined filtrates were evaporated to dryness. The residue was purified by column chromatography (silica gel, $MeOH/CH_2Cl_2$, 1:10) to yield the product usually as a slightly yellow solid.

7-(4-(2-Azidoethyl)piperazin-1-yl)-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic Acid (2a). ^{1}H NMR (500 MHz, $CDCl_3$) δ_H 1.14−1.15 (d, J = 3.0 Hz, 2H, cyclopropane), 1.34−1.35 (d, J = 7.0 Hz, 2H, cyclopropane), 2.65−2.67 (t, J = 6.0 Hz, 2H, NCH_2), 2.69−2.71 (t, J = 5.0 Hz, 4H, piperazine), 3.31−3.33 (t, J = 5.0 Hz, 4H, piperazine), 3.34−3.36 (t, J = 6.0 Hz, 2H, CH_2N_3), 3.51−3.53 (m, 1H, cyclopropane), 7.29−7.30 (d, J = 7.0 Hz, 1H, C_8−H), 7.78−7.80 (d, J = 14.0 Hz, 1H, C_5−H), 8.61 (s, 1H, C_2−H). ^{13}C NMR (125 MHz, $CDCl_3$) δ_C 9.9 (CH_2 of cyclopropane), 10.0 (CH_2 of cyclopropane), 31.5 (CH of cyclopropane), 49.8 (CH_2N_3), 51.4, 54.5, 58.9 (NCH_2), 106.7, 109.6, 113.7, 121.2, 140.8, 147.6, 149.1, 154.4, 156.4, 168.8, 178.7. IR ($CHCl_3$, cm^{-1}): 2120 (N_3), 1730 (CO). MALDI TOF MS calculated for $C_{19}H_{21}FN_6O_3Na$ ([M + Na]$^+$) *m*/*e* 423.4; measured *m*/*e* 423.2.

7-(4-(3-Azidopropyl)piperazin-1-yl)-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic Acid (2b). ^{1}H NMR (500 MHz, $CDCl_3$) δ_H 1.19 (m, 2H, cyclopropane), 1.38−1.39 (d, J = 6.0 Hz, 2H, cyclopropane), 1.79−1.84 (m, 2H, CH_2 of linker), 2.52−2.54 (t, J = 7.0 Hz, 2H, NCH_2), 2.68 (m, 4H, piperazine), 3.36 (m, 4H, piperazine), 3.38−3.40 (t, J = 7.0 Hz, 2H, CH_2N_3), 3.54−3.55 (m, 1H, cyclopropane), 7.34−7.35 (d, J = 7.0 Hz, 1H, C_8−H), 7.91−7.93 (d, J = 7.0 Hz, 1H, C_5−H), 8.70 (s, 1H, C_2−H). ^{13}C NMR (125 MHz, $CDCl_3$) δ_C 10.0 (CH_2 of cyclopropane), 28.0 (CH_2 of linker), 37.1 (CH of cyclopropane), 51.2, 51.5 (CH_2N_3), 54.5, 56.8 (NCH_2), 106.6, 109.7, 113.9, 114.1, 121.4, 140.9, 147.6, 149.1, 154.4, 156.4, 168.8, 178.8. IR ($CHCl_3$, cm^{-1}): 2100 (N_3), 1722 (CO). MALDI TOF MS calculated for $C_{20}H_{23}FN_6O_3Na$ ([M + Na]$^+$) *m*/*e* 437.4; measured *m*/*e* 437.4.

7-(4-(4-Azidobutyl)piperazin-1-yl)-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic Acid (2c). ^{1}H NMR (500 MHz, $CDCl_3$) δ_H 1.17 (m, 2H, cyclopropane), 1.36−1.37 (d, J = 6.0 Hz, 2H, cyclopropane), 1.62−1.65 (m, 4H, CH_2 of linker), 2.44−2.46 (t, J = 7.0 Hz, 2H, NCH_2), 2.65 (m, 4H, piperazine), 3.31−3.34 (m, 4H, piperazine; 2H, CH_2N_3), 3.54−3.55 (m, 1H, cyclopropane), 7.31−7.33 (d, J = 7.0 Hz, 1H, C_8−H), 7.82−7.85 (d, J = 7.0 Hz, 1H, C_5−H), 8.64 (s, 1H, C_2−H). ^{13}C NMR (125 MHz, $CDCl_3$) δ_C 10.0 (CH_2 of cyclopropane), 25.7 (CH_2 of linker), 28.6 (CH_2 of linker), 37.1 (CH of cyclopropane), 51.2, 51.5, 53.1 (CH_2N_3), 54.5, 59.5 (NCH_2), 106.6, 109.6, 113.7, 113.9, 121.2, 140.8, 147.6, 149.1, 154.4, 156.4, 168.7, 178.7. IR ($CHCl_3$, cm^{-1}): 2100 (N_3), 1718 (CO). MALDI TOF MS calculated for $C_{21}H_{25}FN_6O_3$ ([M + H]$^+$) *m*/*e* 429.5; measured *m*/*e* 429.4.

7-(4-(5-Azidopentyl)piperazin-1-yl)-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic Acid (2d). ^{1}H NMR (500 MHz, $CDCl_3$) δ_H 1.20 (m, 2H, cyclopropane), 1.38−1.40 (d, J = 7.0 Hz, 2H, cyclopropane), 1.42−1.47 (m, 2H, CH_2 of linker), 1.55−1.61 (m, 2H, CH_2 of linker), 1.62−1.68 (m, 2H, CH_2 of linker), 2.44−2.47 (t, J = 7.0 Hz, 2H, NCH_2), 2.68 (m, 4H, piperazine), 3.28−3.31 (t, J = 6.0 Hz, 2H, CH_2N_3), 3.36 (m, 4H, piperazine), 3.54−3.56 (m, 1H, cyclopropane), 7.35−7.36 (d, J = 7.0 Hz, 1H, C_8−H), 7.96−7.99 (d, J = 13.0 Hz, 1H, C_5−H), 8.75 (s, 1H, C_2−H). ^{13}C NMR (125 MHz, $CDCl_3$) δ_C 10.0 (CH_2 of cyclopropane), 26.4 (CH_2 of linker), 28.1 (CH_2 of linker), 30.5(CH_2 of linker), 37.1 (CH of cyclopropane), 51.6, 53.2 (CH_2N_3), 54.6, 60.0 (NCH_2), 106.5, 109.9, 114.1, 114.2, 121.5, 140.9, 147.7, 154.5, 156.5, 168.9, 178.9. IR ($CHCl_3$, cm^{-1}): 2110 (N_3), 1723 (CO). MALDI TOF MS calculated for $C_{22}H_{27}FN_6O_3$ ([M + H]$^+$) *m*/*e* 443.5; measured *m*/*e* 443.3.

7-(4-(6-Azidohexyl)piperazin-1-yl)-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic Acid (2e). ^{1}H NMR (500 MHz, $CDCl_3$) δ_H 1.18−1.19 (m, 2H, cyclopropane), 1.36−1.43 (m, 2H, cyclopropane; 4H, CH_2 of linker), 1.54−1.63 (m, 4H, CH_2 of linker), 2.42−2.45 (t, J = 7.5 Hz, 2H, NCH_2), 2.68 (m, 4H, piperazine), 3.26−3.28 (t, J = 6.5 Hz, 2H, CH_2N_3), 3.35−3.37 (m, 4H, piperazine), 3.53−3.57 (m, 1H, cyclopropane), 7.33−7.35 (d, J = 7.0 Hz, 1H, C_8−H), 7.90−7.93 (d, J = 13.5 Hz, 1H, C_5−H), 8.70 (s, 1H, C_2−H). ^{13}C NMR (125 MHz, $CDCl_3$) δ_C 10.0 (CH_2 cyclopropane), 28.4 (CH_2 of linker), 28.8 (CH_2 of linker), 30.6 (CH_2 of linker), 37.1 (CH of cyclopropane), 51.5, 53.2 (CH_2N_3), 54.6, 60.1 (NCH_2), 106.5, 109.8, 113.9, 114.1, 121.4, 140.9, 147.7, 149.1, 154.4, 156.4, 168.8, 178.8. IR ($CHCl_3$, cm^{-1}): 2110 (N_3), 1726 (CO). MALDI TOF MS calculated for $C_{23}H_{29}FN_6O_3$ ([M + H]$^+$) *m*/*e* 457.3; measured *m*/*e* 457.5.

7-(4-(3-Azido-2-hydroxypropyl)piperazin-1-yl)-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic Acid (2f). ^{1}H NMR (500 MHz, $CDCl_3$) δ_H 1.20−1.21 (d, J = 4.0 Hz, 2H, cyclopropane), 1.39−1.41 (d, J = 7.0 Hz, 2H, cyclopropane), 2.46−2.49 (dd, J = 3.0, 12.0 Hz, 1H, NCH_2), 2.57−2.62 (dd, J = 10.0, 12.0 Hz, 1H, NCH_2), 2.67−2.71 (m, 2H, piperazine), 2.87−2.92 (m, 2H, piperazine), 3.24−3.28 (dd, J_1 = 6.0 Hz, J_2=13.0 Hz, 1H, CH_2N_3), 3.36−3.39 (dd, J_1 = 6.0 Hz, J_2=13.0 Hz, 4H, piperazine), 3.44−3.47 (dd, J_1 = 4.0 Hz, J_2 = 9.0 Hz, 1H, CH_2N_3), 3.54−3.58 (m, 1H, cyclopropane), 3.94−3.98 (m, 1H, C*H*−OH), 7.34−7.36 (d, J = 7.0 Hz, 1H, C_8−H), 7.91−7.94 (d, J = 13.0 Hz, 1H, C_5−H), 8.70 (s, 1H, C_2−H). ^{13}C NMR (125 MHz, $CDCl_3$) δ_C 10.0 (CH_2 of cyclopropane), 37.2 (CH of cyclopropane), 51.6, 54.7, 56.1, 62.4, 68.0, 106.7, 109.8, 114.0, 114.2, 121.5, 140.8, 147.5, 149.2, 154.4, 156.4, 168.8, 178.8. IR ($CHCl_3$): 2110 (N_3), 1723 (CO). MALDI TOF MS calculated for $C_{20}H_{23}FN_6O_4$ ([M + H]$^+$) *m*/*e* 431.4; measured *m*/*e* 431.0.

7-(4-(2-(2-Azidoethoxy)ethyl)piperazin-1-yl)-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic Acid (2g). ^{1}H NMR (500 MHz, $CDCl_3$) δ_H 1.17−1.20 (m, 2H, cyclopropane), 1.36−1.40 (m, 2H, cyclopropane), 2.72−2.74 (t, J = 5.0 Hz, 2H, NCH_2), 2.78−2.79 (m, 4H, piperazine), 3.37−3.41 (m, 4H, piperazine; 2H, CH_2N_3), 3.53−3.57 (m, 1H, cyclopropane), 3.66−3.68 (t, J = 4.0 Hz, 2H, CH_2 of linker), 3.69−3.71 (t, J = 4.0 Hz, 2H, CH_2 of linker), 7.34−7.35 (d, J = 8.0 Hz, 1H, C_8−H), 7.92−7.95 (d, J = 13.0 Hz, 1H, C_5−H), 8.71 (s, 1H, C_2−H). ^{13}C NMR (125 MHz, $CDCl_3$) δ_C 10.0 (CH_2 of cyclopropane), 37.1 (CH of cyclopropane), 51.4, 52.5 (CH_2N_3), 55.0, 59.5 (NCH_2), 70.9 (CH_2 of linker), 71.7 (CH_2 of linker), 106.6, 109.8, 114.0, 114.1, 121.4, 140.9, 147.7, 149.1, 154.4, 156.4, 168.8, 178.8. IR ($CHCl_3$, cm^{-1}): 2110 (N_3), 1722 (CO). MALDI TOF MS calculated for $C_{21}H_{25}FN_6O_4$ ([M + H]$^+$) *m*/*e* 445.3; measured *m*/*e* 445.5.

7-(4-(3-(Azidomethyl)benzyl)piperazin-1-yl)-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic Acid (2h). ^{1}H NMR (500 MHz, $CDCl_3$) δ_H 1.16−1.19 (m, 2H, cyclopropane), 1.34−1.38 (m, 2H, cyclopropane), 2.67−2.69 (t, J = 4.5 Hz, 4H, piperazine), 3.35−3.37 (t, J = 4.5 Hz, 4H, piperazine), 3.51−3.55 (m, 1H, cyclopropane), 3.62 (s, 2H, NCH_2), 4.35 (s, 2H, CH_2N_3), 7.22−7.24 (m, 1H, aromatic), 7.30−7.37 (m, 4H, aromatic, C_8−H), 7.86−7.89 (d, J = 13.0 Hz, 1H, C_5−H), 8.67 (s, 1H, C_2−H). ^{13}C NMR (125 MHz, $CDCl_3$) δ_C 10.0 (CH_2 of cyclopropane), 31.4 (CH of cyclopropane), 51.5, 54.5, 56.5 (CH_2N_3), 64.4 (NCH_2), 106.6, 109.7, 113.8, 114.0, 121.2, 129.0, 130.7, 130.9, 137.3, 140.3, 140.9, 154.4, 156.4, 168.8, 178.7. MALDI TOF MS calculated for $C_{25}H_{25}FN_6O_3$ ([M + H]$^+$) *m*/*e* 477.2; measured *m*/*e* 477.5.

7-(4-(4-(Azidomethyl)benzyl)piperazin-1-yl)-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic Acid (2i). ^{1}H NMR (500 MHz, $CDCl_3$) δ_H 1.17−1.20 (m, 2H, cyclopropane), 1.35−1.39 (m, 2H, cyclopropane), 2.68−2.69 (t, J = 5.0 Hz, 4H, piperazine), 3.35−3.37 (t, J = 5 Hz, 4H, piperazine), 3.52−3.54 (m, 1H, cyclopropane), 3.61 (s, 2H, NCH_2), 4.34 (s, 2H, CH_2N_3), 7.29−7.31 (d, J = 7.5 Hz, 2H, aromatic), 7.33−7.34 (d, J = 7.0 Hz, 1H, C_8−H), 7.38−7.39 (d, J = 7.5 Hz, 2H, aromatic), 7.91−7.94 (d, J = 13.0 Hz, 1H, C_5−H), 8.70 (s, 1H, C_2−H). ^{13}C NMR (125 MHz, $CDCl_3$) δ_C 8.1 (CH_2 of cyclopropane), 35.2 (CH of cyclopropane), 49.7, 52.6 (CH_2N_3), 54.5, 62.4 (NCH_2), 104.7, 107.9, 112.1, 112.3, 119.5, 128.2, 129.5, 134.4, 137.9, 139.0, 145.9, 147.3, 152.6, 154.6, 167.0, 177.0. MALDI TOF MS calculated for $C_{25}H_{25}FN_6O_3$ ([M + H]$^+$) *m*/*e* 477.1; measured *m*/*e* 477.5.

1,3,2′,6′,2‴,6‴-Hexaazido-5″-triisopropylsilyloxyneomycin (4). Commercial NeoB was converted to the corresponding perazido derivative according to the published procedure.[29] Hexaazido-NeoB (5.10 g, 6.62 mmol) was dissolved in pyridine (30 mL), and 4-DMAP (cat.) was added. The mixture was stirred at room temperature. After 15 min, triisopropylsilylchloride (TIPSCl) (1.91 g, 9.93 mmol) was added, and TLC (EtOAc, 100%) indicated completion after 3 h. The mixture was diluted with EtOAc and washed with brine, H_2SO_4 (2%), $NaHCO_3$ (sat.), and brine. Organic layers were combined, dried over $MgSO_4$, evaporated and the

residue was purified by flash chromatography (silica gel, EtOAc/hexane) to yield the silyl ether **4** as a white powder (4.60 g, 75% yield). ^{1}H NMR (500 MHz, $CDCl_3$) δ_H 0.85−0.98 (m, 15H, TIPS); ring I δ_H 3.14−3.28 (m, 3H, H-2, H-4, H-6), 3.33−3.51 (m, 1H, H-6′), 3.63−3.74 (m, 1H, H-3), 3.91−3.95 (m, 1H, H-5), 5.65−5.66 (d, J = 3.5 Hz, 1H, H-1); ring II δ_H 1.19−1.24 (ddd, $J_1 = J_2 = J_3$ = 12.5 Hz, 1H, H-2ax), 2.01−2.06 (dt, J = 4.0, 12.5 Hz, 1H, H-2eq), 3.14−3.28 (m, 2H, H-1, H-3), 3.33−3.51 (m, 3H, H-4, H-5, H-6); ring III δ_H 3.33−3.51 (m, 1H, H-5′), 3.63−3.74 (m, 1H, H-5), 4.03−4.05 (m, 2H, H-2, H-4), 4.22−4.23 (dd, J = 4.0, 4.5 Hz, 1H, H-3), 5.10−5.11 (d, J = 4.5 Hz, 1H, H-1); ring IV δ_H 2.91−2.94 (dd, J = 4.0, 10.5 Hz, 1H, H-6), 3.14−3.28 (m, 2H, H-4, H-6), 3.63−3.74 (m, 1H, H-2), 3.78−3.80 (t, J = 3.5 Hz, 1H, H-3), 3.81−3.83 (m, 1H, H-5), 4.96 (d, J = 1.0 Hz, 1H, H-1). ^{13}C NMR (125 MHz, $CDCl_3$) δ_C 11.5, 17.4, 31.6 (C-2), 50.8 (C-6′), 51.1 (C-6‴), 59.2, 59.4, 60.4, 63.2, 63.3, 68.4, 68.7, 70.5, 70.9, 71.1, 73.6, 74.3, 75.0, 75.3, 76.3, 83.1, 85.1, 96.3 (C-1‴), 98.6 (C-1′), 107.2 (C-1″). MALDI TOF MS calcd for $C_{32}H_{54}N_{18}O_{13}SiNa$ ([M + Na]$^+$) *m*/*e* 949.6; measured *m*/*e* 949.4.

1,3,2′,6′,2‴,6‴-Hexaazido-6,3′,4′,2″,3‴,4‴-hexa(4-methoxybenzyloxy)-5″-triisopropylsilyloxyneomycin (5). Compound **4** (3.0 g, 3.24 mmol) was dissolved in anhydrous DMF (20 mL), and after the mixture was stirred at 0 °C for 10 min 4-methoxybenzyl chloride (4.6 g, 29.2 mmol) and NaH (0.17 g, 7.29 mmol) were added. The mixture was allowed to warm to room temperature, and after 4 h TLC (EtOAc, 100%) indicated completion. The mixture was diluted with EtOAc and extensively washed with brine. The combined organic layer was dried over $MgSO_4$, evaporated to dryness, and used in the next step without further purification.

The crude from the previous step was dissolved in dry THF (10 mL), cooled at 0 °C, and 1 M solution of tetrabutyl ammonium fluoride in THF (3.74 mL, 3.74 mmol) was added. The reaction progress was monitored by TLC (EtOAc/hexane, 2:3), which indicated completion after 3 h. The mixture was diluted with EtOAc (300 mL) and washed with brine. The combined organic layer was dried over $MgSO_4$, evaporated, and purified by flash chromatography (silica gel, EtOAc/hexane) to yield **5** (3.5 g, 72% for two steps). ^{1}H NMR (500 MHz, $CDCl_3$) δ_H 3.74 (s, 3H, CH_3O), 3.78 (s, 3H, CH_3O), 3.80 (s, 6H, CH_3O), 3.82 (s, 6H, CH_3O), 4.30−4.41 (m, 2H, CH_2 of PMB), 4.50−4.58 (m, 4H, CH_2 of PMB), 4.64−4.67 (m, 4H, CH_2 of PMB), 4.78−4.87 (m, 2H, CH_2 of PMB), 6.75−6.76 (m, 2H, aromatic), 6.86−6.90 (m, 10H, aromatic), 7.13−7.35 (m, 12H, aromatic); ring I δ_H 3.12−3.15 (dd, J = 4.0, 10.0 Hz, 1H, H-2), 3.29−3.36 (m, 1H, H-6), 3.40−3.51 (m, 2H, H-4, H-6′), 4.03−4.07 (dd, J = 9.0, 11.0 Hz, 1H, H-3), 4.21−4.23 (m, 1H, H-5), 5.88−5.89 (d, J = 4.0 Hz, 1H, H-1); ring II δ_H 1.41−1.49 (ddd, $J_1 = J_2 = J_3$ = 12.5 Hz, 1H, H-2ax), 2.26−2.35 (dt, J = 4.0, 12.5 Hz, 1H, H-2eq), 3.29−3.36 (m, 1H, H-1), 3.40−3.51 (m, 2H, H-3, H-5), 3.62−3.66 (t, J = 10.0 Hz, 1H, H-4), 3.90−3.94 (t, J = 9.0 Hz, 1H, H-6); ring III δ_H 3.02−3.04 (dd, J = 6.0, 9.0 Hz, 1H, H-5), 3.67−3.77 (m, 2H, H-2, H-5′), 4.11−4.12 (m, 1H, H-4), 4.33−4.35 (dd, J = 4.0, 6.0 Hz, 1H, H-3), 5.68−5.69 (d, J = 5.0 Hz, 1H, H-1); ring IV δ_H 2.97−3.01 (dd, J = 4.0, 12.0 Hz, 1H, H-6), 3.12 (m, 1H, H-4), 3.40−3.51 (m, 1H, H-2), 3.67−3.77 (m, 3H, H-3, H-5, H-6′), 4.97 (d, J = 2.0 Hz, 1H, H-1). ^{13}C NMR (125 MHz, $CDCl_3$) δ_C 32.3 (C-2), 51.1 (C-6′), 55.2 (C-6″), 57.4, 59.6, 60.4, 61.9, 62.6, 71.0, 71.3, 72.0, 72.5, 72.9, 74.2, 74.6, 74.9, 75.7, 78.2, 78.6, 80.8, 81.9, 83.0, 83.9, 97.1 (C-1‴), 98.9 (C-1′), 105.4 (C-1″). MALDI TOF MS calcd for $C_{71}H_{82}N_{18}O_{19}Na$ ([M + Na]$^+$) *m*/*e* 1513.7; measured *m*/*e* 1513.5.

1,3,2′,6′,2‴,6‴-Hexaazido-6,3′,4′,2″,3‴,4‴-hexa(4-methoxybenzyloxy)-5″-(prop-2-ynyloxy)neomycin (6). Compound **5** (0.73 g, 0.49 mmol) was dissolved in dry DMF (15 mL) and was stirred at 0 °C for 10 min, followed by the addition of tetrabutylammonium iodide (0.54 g, 1.47 mmol), propargyl bromide (0.35 g, 2.97 mmol), and NaH (0.070 g, 2.94 mmol). The reaction progress was monitored by TLC (EtOAc/hexane, 4:5), which indicated completion after 4 h. The mixture was diluted with EtOAc (200 mL) and washed with brine. The combined organic layer was dried over $MgSO_4$, evaporated, and purified by flash chromatography (silica gel, EtOAc/hexane) to yield compound **6** (0.60 g, 80%). ^{1}H NMR (500 MHz, $CDCl_3$) δ_H 2.44−2.45 (t, J = 2.5 Hz, 1H, CH of triple bond), 3.73 (s, 3H, CH_3O), 3.78 (s, 3H, CH_3O), 3.80 (s, 3H, CH_3O), 3.81 (s, 3H, CH_3O), 3.82 (s, 6H, CH_3O), 4.13−4.14 (t, J = 2.5 Hz, 2H, CH_2 of linker), 4.39−4.43 (m, 4H, CH_2 of PMB), 4.49−4.66 (m, 4H, CH_2 of PMB), 4.77−4.89 (m, 4H, CH_2 of PMB), 6.72−6.74 (m, 2H, aromatic), 6.84−6.89 (m, 10H, aromatic), 7.12−7.31 (m, 12H, aromatic); ring I δ_H 3.28−3.32 (m, 1H, H-6), 3.35−3.37 (dd, J = 3.5, 10.5 Hz, 1H, H-2), 3.41−3.51 (m, 1H, H-4), 3.56−3.58 (dd, J = 3.0, 10.0 Hz, 1H, H-6′), 4.02−4.06 (dd, J = 9.5, 10.0 Hz, 1H, H-3), 4.24−4.27 (m, 1H, H-5), 6.08−6.09 (d, J = 3.5 Hz, 1H, H-1); ring II δ_H 1.39−1.46 (ddd, $J_1 = J_2 = J_3$ = 12.5 Hz, 1H, H-2ax), 2.22−2.27 (dt, J = 4.0, 12.5 Hz, 1H, H-2eq), 3.28−3.32 (m, 1H, H-1), 3.41−3.51 (m, 2H, H-3, H-5), 3.63−3.67 (t, J = 9.0 Hz, 1H, H-4), 3.89−3.92 (t, J = 9.0 Hz, 1H, H-6); ring III δ_H 3.41−3.51 (m, 1H, H-5), 3.67−3.72 (m, 2H, H-2, H-5′), 4.16−4.18 (m, 1H, H-4), 4.24−4.27 (m, 1H, H-3), 5.61−5.62 (d, J = 5.5 Hz, 1H, H-1); ring IV δ_H 2.86−2.91 (dd, J = 4.0, 13.0 Hz, 1H, H-6), 3.09 (m, 1H, H-2), 3.41−3.51 (m, 1H, H-4), 3.67−3.73 (m, 3H, H-3, H-5, H-6′), 4.95 (d, J = 1.5 Hz, 1H, H-1). ^{13}C NMR (125 MHz, $CDCl_3$) δ_C 30.3, 32.4 (C-2), 51.2 (C-6′), 55.2 (C-6″), 57.4, 58.5, 59.8, 60.4, 63.2, 69.5, 70.9, 71.0, 71.2, 71.9, 72.4, 73.0, 74.2, 74.3, 74.5, 75.0, 75.2, 78.1, 79.5, 79.7, 81.6, 82.1, 83.8, 96.2 (C-1‴), 98.2 (C-1′), 107.1 (C-1″), 113.7, 113.8, 114.0, 129.4, 129.5 L, 129.8, 130.0, 159.1, 159.2, 159.5. MALDI TOF MS calcd for $C_{74}H_{84}N_{18}O_{19}Na$ ([M + Na]$^+$) *m*/*e* 1551.8; measured *m*/*e* 1551.4.

5″-(Prop-2-ynyloxy)neomycin (3a). Compound **6** (0.43 g, 0.28 mmol) was dissolved in acetonitrile (5 mL), and after the mixture was stirred at −4 °C for 10 min, cerium(IV) ammonium nitrate (CAN) (1.0 g, 1.82 mmol) in 0.5 mL of water was added. The reaction progress was monitored by TLC (EtOAc/hexane, 4:5, and EtOAc, 100%). After 3 h, the reaction mixture was diluted with EtOAc (100 mL) and washed with brine. The combined organic layer was dried over $MgSO_4$, evaporated to dryness, and used in the next step without further purification.

The crude product from the previous step was dissolved in THF (7 mL), 0.1 M NaOH (1.5 mL) and stirred at 60 °C for 10 min, after which PMe_3 (1 M solution in THF, 4.0 mL, 4.0 mmol) was added. The reaction progress was monitored by TLC (CH_2Cl_2/MeOH/H_2O/$MeNH_2$ (33% solution in EtOH), 10:15:6:15), which indicated completion after 3.5 h. The reaction mixture was purified by flash chromatography on a short column of silica gel, and the column was washed as follows: THF, EtOH, MeOH, and finally with $MeNH_2$ (33% solution in EtOH). The fractions containing the product were combined and evaporated to dryness, redissolved in water, and evaporated again to afford the product **3a** as a free amine (162.5 mg, 89% for two steps). This product was then dissolved in water, the pH was adjusted to 7.5 with 0.01 M H_2SO_4, and the mixture was lyophilized to give the sulfate salt of **3a** (220 mg) as a white foamy solid. ^{1}H NMR (500 MHz, D_2O, pH 3.0) δ_H 2.96 (m, 1H, CH of triple bond), 4.23−4.28 (m, 2H, CH_2 of linker); ring I δ_H 3.27−3.51 (m, 4H, H-2, H-4, H-6, H-6′), 3.82−3.93 (m, 2H, H-3, H-5), 6.07−6.08 (d, J = 4.0 Hz, 1H, H-1); ring II δ_H 1.96−2.03 (ddd, $J_1 = J_2 = J_3$ = 12.5 Hz, 1H, H-2ax), 2.37−2.41 (dt, J = 4.0, 12.5 Hz, 1H, H-2eq), 3.27−3.51 (m, 2H, H-1, H-3), 3.64−3.69 (m, 1H, H-5), 4.00−4.04 (t, J = 9.5 Hz, 1H, H-6), 4.14−4.17 (t, J = 9.5 Hz, 1H, H-4); ring III δ_H 3.64−3.69 (m, 1H, H-5), 3.82−3.93 (m, 1H, H-5′), 4.23−4.28 (m, 1H, H-4), 4.40 (dd, J = 2.0, 4.0 Hz, 1H, H-2), 4.47−4.49 (dd, J = 4.0, 7.0 Hz, 1H, H-3), 5.36 (s, 1H, H-1); ring IV δ_H 3.08−3.13 (dd, J = 8.5, 13.5 Hz, 1H, H-6), 3.27−3.51 (m, 2H, H-2, H-6′), 3.75 (m, 1H, H-4), 4.14−4.17 (m, 1H, H-3), 4.23−4.28 (m, 1H, H-5), 5.22 (s, 1H, H-1). ^{13}C NMR (125 MHz, D_2O) δ_C 29.6 (C-2), 42.2 (C-6′), 42.5 (C-6‴), 50.2, 51.6, 52.6, 55.3, 60.3, 68.9, 69.4, 69.6, 71.3, 72.1, 73.2, 74.1, 74.9, 76.2, 77.2, 78.9, 81.1(CH of triple bond), 81.6, 87.1, 96.4 (C-1‴), 96.9 (C-1′), 112.5 (C-1″). MALDI TOF MS calcd for $C_{26}H_{49}N_6O_{13}$ ([M + H]$^+$) *m*/*e* 653.3; measured *m*/*e* 653.3.

1,3,2′,6′,2‴,6‴-Hexaazido-6,3′,4′,2″,3‴,4‴-hexaacetoxyneomycin-4″-carboxylic Acid (8). Compound **7**[30] (0.43 g, 0.42 mmol) was dissolved in CH_2Cl_2 (30 mL) and cooled to 5 °C. Then water (2.5 mL), TEMPO (0.013 g, 0.08 mmol), and BAIB (0.34 g, 1.06

mmol) were added. The reaction mixture was stirred at 5 °C for 40 min and then allowed slowly to warm to room temperature. The reaction progress was monitored by TLC with two solvent systems (EtOAc/hexane, 1:1, and MeOH/$CHCl_3$, 1:9), which indicated completion after 4.5 h. The mixture was cooled to 0 °C, diluted with EtOAc, quenched with $Na_2S_2O_3$, and washed with brine. The combined organic layer was dried over $MgSO_4$, evaporated, and purified by flash chromatography (silica gel, MeOH/$CHCl_3$) to yield **8** (320 mg, 76%). ^{1}H NMR (500 MHz, $CDCl_3$) δ_H 2.04 (s, 3H, OAc), 2.06 (s, 3H, OAc), 2.12 (s, 3H, OAc), 2.13 (s, 3H, OAc), 2.15 (s, 3H, OAc), 2.16 (s, 3H, OAc); ring I δ_H 3.27−3.44 (m, 1H, H-2), 3.50−3.57 (m, 2H, H-6, H-6′), 4.41−4.43 (m, 1H, H-5), 5.00 (t, J = 9.5 Hz, 1H, H-4), 5.43 (t, J = 10.5 Hz, 1H, H-3), 6.12 (s, 1H, H-1); ring II δ_H 1.63 (ddd, $J_1 = J_2 = J_3$ = 12.5 Hz, 1H, H-2ax), 2.37 (dt, J = 3.5, 12.5 Hz, 1H, H-2eq), 3.27−3.44 (m, 2H, H-1, H-3), 3.73 (t, J = 9.0 Hz, 1H, H-5), 3.96 (t, J = 9.0 Hz, 1H, H-4), 4.93−4.97 (m, 1H, H-6); ring III δ_H 4.71 (m, 1H, H-3), 4.81 (t, J = 5.0 Hz, 1H, H-2), 4.87 (d, J = 3.0 Hz, 1H, H-4), 5.54 (d, J = 5.0 Hz, 1H, H-1); ring IV δ_H 3.27−3.44 (m, 3H, H-2, H-6, H-6′), 4.05−4.07 (m, 1H, H-5), 4.71 (m, 1H, H-4), 4.95 (s, 1H, H-1), 5.05 (t, J = 2.5 Hz, 1H, H-3). ^{13}C NMR (125 MHz, $CDCl_3$) δ_C 20.4, 20.7, 20.8, 21.0, 31.2 (C-2), 50.5 (C-6‴), 50.9 (C-6′), 57.2, 58.0, 59.0, 60.7, 65.7, 68.6, 69.1, 69.3, 69.8, 73.0, 74.6, 75.2, 76.0, 79.3, 81.8, 96.5 (C-1′), 100.3 (C-1‴), 106.3 (C-1″), 168.6, 169.7, 169.8, 170.1, 170.2. MALDI TOF MS calcd for $C_{35}H_{44}N_{18}O_{20}K$ ([M + K]$^+$) *m*/*e* 1075.3; measured *m*/*e* 1075.4.

1,3,2′,6′,2‴,6‴-Hexaazido-6,3′,4′,2″,3‴,4‴-hexaacetoxy-4″-(prop-2-ynylcarbamoyl)neomycin (9a). Compound **8** (2.68 g, 2.59 mmol) was dissolved in CH_2Cl_2 (35 mL). Then DCC (0.53 g, 2.57 mmol) and HOBT (0.25 g, 1.85 mmol) were added at 0 °C. The reaction mixture was stirred at 0 °C for 1 h and allowed slowly to warm to room temperature. Then propargylamine (0.43 g, 7.81 mmol) was added. Progress of the reaction was monitored by TLC with two solvent systems (EtOAc/hexane, 1:1, and MeOH/$CHCl_3$, 1:9), which indicated completion after 4 h. The mixture was diluted with EtOAc and washed with brine. The combined organic layer was dried over $MgSO_4$, evaporated, and purified by flash chromatography (silica gel, EtOAc/hexane) to yield **9a** (2.0 g, 72%). ^{1}H NMR (500 MHz, $CDCl_3$) δ_H 2.07 (s, 3H, OAc), 2.10 (s, 3H, OAc), 2.13 (s, 6H, OAc), 2.17 (s, 3H, OAc), 2.19 (s, 3H, OAc), 2.25−2.26 (t, J = 2.5 Hz, 1H, CH of triple bond), 3.91−3.95 (m, 1H, CH_2 of linker), 4.14−4.17 (m, 1H, CH_2 of linker), 7.41−7.44 (t, J = 6.0 Hz, 1H, NH); ring I δ_H 3.23−3.26 (dd, J = 3.0, 10.0 Hz, 1H, H-2), 3.31−3.44 (m, 2H, H-6, H-6′), 4.46−4.49 (m, 1H, H-5), 4.99−5.09 (m, 1H, H-4), 5.50−5.54 (dd, J = 9.0, 11.0 Hz, 1H, H-3), 5.97−5.98 (d, J = 4.0 Hz, 1H, H-1); ring II δ_H 1.62−1.70 (ddd, $J_1 = J_2 = J_3$ = 12.5 Hz, 1H, H-2ax), 2.39−2.43 (dt, J = 4.0, 12.5 Hz, 1H, H-2eq), 3.31−3.44 (m, 1H, H-3), 3.54−3.58 (m, 1H, H-1), 3.74−3.78 (t, J = 9.0 Hz, 1H, H-4), 3.99−4.02 (t, J = 9.0 Hz, 1H, H-5), 4.99−5.09 (m, 1H, H-6); ring III δ_H 4.57−4.60 (t, J = 6.0 Hz, 1H, H-2), 4.65−4.66 (dd, J = 3.5, 6.0 Hz, 1H, H-3), 4.82 (d, J = 4.0 Hz, 1H, H-4), 5.59−5.61 (d, J = 6.0 Hz, 1H, H-1); ring IV δ_H 3.31−3.44 (m, 2H, H-2, H-6), 3.54−3.58 (m, 1H, H-6′), 4.09−4.13 (m, 1H, H-5), 4.71−4.72 (t, J = 2.0 Hz, 1H, H-4), 4.99−5.09 (m, 2H, H-1, H-3). ^{13}C NMR (125 MHz, $CDCl_3$) δ_C 22.1, 22.5, 22.7, 22.8, 30.6 (CH_2 of linker), 33.2 (C-2), 52.4 (C-6′), 52.7 (C-6‴), 59.2, 59.9, 60.9, 62.0, 67.4, 70.6, 71.0, 71.1, 71.2, 73.5, 74.8, 75.8, 76.9, 77.7, 80.4 (CH of triple bond), 80.9, 83.1, 83.3, 99.0 (C-1‴), 102.3 (C-1′), 106.7 (C-1″), 170.1, 170.4, 171.4, 171.8, 171.9. MALDI TOF MS calcd for $C_{38}H_{47}N_{19}O_{19}Na$ ([M + Na]$^+$) *m*/*e* 1096.3; measured *m*/*e* 1096.3.

4″-(Prop-2-ynylcarbamoyl)neomycin (3b). Compound **9a** (2.4 g, 2.27 mmol) was dissolved in 33% solution of $MeNH_2$ in EtOH (40 mL), and the mixture was stirred at room temperature for 30 h. The reagent and the solvent were removed by evaporation, and the residue was dissolved in THF (50 mL), NaOH 0.1 M (3 mL) and stirred at 60 °C for 10 min, after which PMe_3 (1 M solution in THF, 21.9 mL, 21.9 mmol) was added. Propagation of the reaction was monitored by TLC [CH_2Cl_2/MeOH/H_2O/$MeNH_2$ (33% solution in EtOH), 10:15:6:15], which indicated completion after 3.5 h. The reaction mixture was purified by flash chromatography on a short column of silica gel, and the column was washed as follows: THF, EtOH, MeOH, and finally $MeNH_2$ (33% solution in EtOH). The fractions containing the product were evaporated under vacuum, redissolved in water, and evaporated again to afford the product **3b** as a free amine (1.39 g, 92%). This product was then dissolved in water, the pH was adjusted to 7.5 with 0.01 M H_2SO_4, and the mixture was lyophilized to give the sulfate salt of **3b** (1.88 g) as a white foamy solid. ^{1}H NMR (500 MHz, D_2O, pH 3.17) δ_H 2.59−2.60 (t, J = 2.5 Hz, 1H, CH of triple bond), 3.86−4.02 (m, 2H, CH_2 of linker); ring I δ_H 3.20−3.24 (dd, J = 3.5, 13.5 Hz, 1H, H-6), 3.25−3.37 (m, 3H, H-2, H-4, H-6′), 3.86−4.02 (m, 2H, H-3, H-5), 6.03−6.04 (d, J = 4.0 Hz, 1H, H-1); ring II δ_H 1.92−1.98 (ddd, $J_1 = J_2 = J_3$ = 12.5 Hz, 1H, H-2ax), 2.37−2.41 (dt, J = 4.0, 12.5 Hz, 1H, H-2eq), 3.25−3.37 (m, 1H, H-1), 3.45−3.50 (m, 1H, H-3), 3.67−3.71 (m, 1H, H-5), 3.86−4.05 (m, 1H, H-6), 4.15−4.19 (t, J = 9.5 Hz, 1H, H-4); ring III δ_H 4.42−4.43 (dd, J = 2.0, 4.5 Hz, 1H, H-3), 4.47−4.48 (d, J = 7.5 Hz, 1H, H-4), 4.60−4.62 (dd, J = 4.5, 7.5 Hz, 1H, H-2), 5.44 (s, 1H, H-1); ring IV δ_H 3.13−3.17 (dd, J = 8.0, 13.5 Hz, 1H, H-6), 3.38−3.41 (dd, J = 3.0, 13.5 Hz, 1H, H-6′), 3.53 (m, 1H, H-2), 3.67−3.71 (m, 1H, H-4), 4.15−4.19 (m, 1H, H-3), 4.20−4.23 (m, 1H, H-5), 5.20 (s, 1H, H-1). ^{13}C NMR (125 MHz, D_2O) δ_C 29.6 (C-2), 30.7, 42.1 (C-6′), 42.2 (C-6‴), 50.2, 51.8, 52.5, 55.4, 68.7, 69.3, 69.5, 71.4, 72.5, 72.9, 74.0, 74.7, 76.4, 79.4, 80.9, 81.2 (CH of triple bond), 86.4, 96.5 (C-1‴), 97.0 (C-1′), 112.3 (C-1″), 173.0 (CO). MALDI TOF MS calcd for $C_{26}H_{47}N_7O_{13}K$ ([M + K]$^+$) *m*/*e* 704.2; measured *m*/*e* 704.3.

1,3,2′,6′,2‴,6‴-Hexaazido-6,3′,4′,2″,3‴,4‴-hexaacetoxy-4″-(4-ethynylphenylcarbamoyl)neomycin (9b). The titled compound was prepared as was described for the preparation of compound **9a** with the following quantities: compound **8** (1.67 g, 1.61 mmol), DCC (0.33 g, 1.61 mmol), HOBT (0.22 g, 1.61 mmol), 4-ethynylaniline (0.57 g, 4.8 mmol), DCM (20 mL). Yield: 1.57 g (86%). ^{1}H NMR (300 MHz, $CDCl_3$) δ_H 2.00 (s, 3H, OAc), 2.02 (s, 3H, OAc), 2.09 (s, 3H, OAc), 2.10 (s, 3H, OAc), 2.11 (s, 3H, OAc), 2.15 (s, 3H, OAc), 3.00 (s, 1H, CH of triple bond), 7.37−7.40 (m, 2H, aromatic), 7.47−7.50 (m, 2H, aromatic), 8.60 (s, 1H, NH); ring I δ_H 3.09−3.18 (m, 2H, H-2, H-6), 3.25−3.26 (m, 1H, H-6′), 4.38−4.44 (m, 1H, H-5), 5.03−5.04 (m, 1H, H-4), 5.39−5.45 (dd, J = 9.0, 10.5 Hz, 1H, H-3), 5.92−5.93 (d, J = 4.0 Hz, 1H, H-1); ring II δ_H 1.91−1.94 (ddd, $J_1 = J_2 = J_3$ = 12.5 Hz, 1H, H-2ax), 2.34−2.38 (dt, J = 4.0, 12.5 Hz, 1H, H-2eq), 3.31−3.53 (m, 2H, H-1, H-3), 3.66−3.72 (t, J = 9.0 Hz, 1H, H-4), 3.94−4.00 (t, J = 9.0 Hz, 1H, H-5), 4.91−4.97 (t, J = 9.0 Hz, 1H, H-6); ring III δ_H 4.64−4.75 (m, 2H, H-2, H-3), 4.78−4.80 (d, J = 4.2 Hz, 1H, H-4), 5.53−5.55 (d, J = 5.0 Hz, 1H, H-1); ring IV δ_H 3.25−3.26 (m, 1H, H-2), 3.31−3.54 (m, 2H, H-6, H-6′), 4.04−4.08 (m, 1H, H-5), 4.64−4.75 (m, 2H, H-3, H-4), 5.04 (s, 1H, H-1). ^{13}C NMR (125 MHz, $CDCl_3$) δ_C 22.1, 22.5, 22.7, 22.8, 33.0 (C-2), 51.0, 52.2 (C-6′), 52.7 (C-6″), 59.1, 59.8, 60.7, 62.2, 67.4, 70.4, 71.0, 71.1, 71.3, 74.9, 75.9, 76.9, 78.2, 78.9, 79.9, 82.7, 83.8, 85.0 (CH of triple bond), 98.9 (C-1′), 101.8 (C-1‴), 107.3 (C-1″), 120.1, 121.7, 134.7, 139.6, 168.5, 170.4, 171.4, 171.5, 171.8, 171.9. MALDI TOF MS calcd for $C_{43}H_{49}N_{19}O_{19}Na$ ([M + Na]$^+$) *m*/*e* 1158.3; measured *m*/*e* 1158.2.

4″-(4-Ethynylphenylcarbamoyl)neomycin (3c). The titled compound was prepared as was described for the preparation of **3b** with the following quantities: compound **9b** (1.13 g, 1.00 mmol), $MeNH_2$ (70 mL), Me_3P (1 M solution in THF, 8.86 mL, 8.86 mmol), NaOH (0.1 M, 2 mL), THF (20 mL). Yield: 0.58 g (80%). ^{1}H NMR (500 MHz, D_2O, pH 3.39) δ_H 3.42 (s, 1H, CH of triple bond), 7.48−7.52 (m, 4H, aromatic); ring I δ_H 3.02−3.07 (dd, J = 8.5, 13.5 Hz, 1H, H-6), 3.18−3.22 (dd, $J_1 = J_2$ = 9.0 Hz, 1H, H-4), 3.28−3.32 (m, 1H, H-2), 3.37−3.40 (dd, J = 3.0, 13.5 Hz, 1H, H-6′), 3.85−3.88 (m, 1H, H-5), 3.96−3.99 (m, 1H, H-3), 6.04−6.05 (d, J = 4.0 Hz, 1H, H-1); ring II δ_H 1.94−1.98 (ddd, $J_1 = J_2 = J_3$ = 12.5 Hz, 1H, H-2ax), 2.37−2.41 (dt, J = 4.0, 12.5 Hz, 1H, H-2eq), 3.28−3.32 (m, 1H, H-1), 3.47−3.50 (m, 1H, H-3), 3.67−3.71 (t, J = 10.0 Hz, 1H, H-6), 3.96−3.99 (m, 1H, H-5), 4.16−4.20 (t, J = 9.5 Hz, 1H, H-4); ring III δ_H 4.48−4.49 (dd, J = 2.0, 4.0 Hz, 1H, H-2), 4.64−4.68 (m, 2H, H-3, H-4), 5.47 (s,

1H, H-1); ring IV δ_H 2.70−2.74 (dd, J = 8.0, 13.5 Hz, 1H, H-6), 2.94−2.97 (dd, J = 3.0, 13.5 Hz, 1H, H-6′), 3.55 (m, 1H, H-2), 3.65 (m, 1H, H-4), 4.11−4.15 (m, 2H, H-3, H-5), 5.21 (s, 1H, H-1). ^{13}C NMR (125 MHz, D_2O) δ_C 29.6 (C-2), 41.9 (C-6′), 42.3 (C-6″), 50.2, 51.8, 52.5, 55.5, 68.6, 69.1, 69.5, 71.3, 72.4, 73.1, 74.1, 75.4, 76.4, 80.2, 80.3, 81.3 (CH of triple bond), 85.0, 86.3, 96.9 (C-1′, C-1‴), 112.4 (C-1″), 120.4, 122.7, 135.0, 138.8, 172.0 (CO). MALDI TOF MS calcd for $C_{31}H_{49}N_7O_{13}K$ ($[M + K]^+$) *m/e* 766.3; measured *m/e* 766.3.

General Procedure for the Preparation of Hybrid Structures (1a−q). A solution of compound **2** (0.06 mmol), compound **3** (0.05 mmol), $[(CH_3CN)_4Cu]PF_6$ (0.025 mmol) in 7% solution of Et_3N in water (5 mL) was placed in a glass vial (25 mL). The vial was closed with a stopper and heated in a domestic microwave oven for 40 s at maximum power. Propagation of the reaction could be monitored by TLC [CH_2Cl_2/MeOH/H_2O/$MeNH_2$ (33% solution in EtOH), 10:15:6:15]. After completion, the mixture was purified on a short column of Amberlite CG-50 (H^+-form). The column was sequentially washed by MeOH, MeOH/$MeNH_2$ (33% solution in EtOH) 95:5, MeOH/$MeNH_2$ (33% solution in EtOH) 9:1, and MeOH/$MeNH_2$ (33% solution in EtOH) 4:1. Fractions containing the product were combined, evaporated, redissolved in water, and evaporated again to afford the free amine form of the product. The product was dissolved in water, the pH was adjusted to 3.2 with TFA (0.01 M), and the mixture was lyophilized to afford the TFA salt of the final product, usually as a white foamy solid. Chemical yields of the resulting hybrids **1a−q** are given in Table 2, and their complete analytical data are given in Supporting Information.

Antibacterial Activity. The MIC values were determined using the double-microdilution method according to the National Committee for Clinical Laboratory Standards (NCCLS)[44] with starting concentration of 384 and 1.5 μg/mL of the tested compounds. All the experiments were performed in duplicate, and analogous results were obtained in two to four different experiments.

Resistance studies were performed in parallel with *E. coli* ATCC 35218 and *B. subtilis* ATCC 6633 strains in the presence of Cipro, NeoB, Cipro/NeoB mixture (1:1 molar ratio), and the hybrid **1i**. After the initial MIC experiments, MICs were determined once in 2 days for 15 passages as follows: for each compound tested, bacteria from the one-half MIC well were diluted 100-fold (50 μL of the bacterial growth in a total of 5 mL of LB medium) and were grown overnight at 37 °C. The OD_{600} of the bacteria was diluted to yield 5×10^5 cells/mL in LB (according to a calibration curve) and used again for MIC determination in the subsequent generation. In parallel, MIC evolution during these subcultures was compared concomitantly with each new generation, using bacteria harvested from control wells (wells cultured without antimicrobial agent from the previous generation). The relative MIC was calculated for each experiment from the ratio of MIC obtained for a given subculture to that obtained for first-time exposure.

Biochemical Studies. The plasmid pETSACG1 carrying the APH(3′)-IIIa gene (GenBank Accession No. V01547) was generously provided by Prof. A. Berghuis, McGill University. The plasmid pSF815 carrying the AAC(6′)-APH(2″) gene was generously provided by Prof. S. Mobashery, University of Notre Dame. The plasmid pET9d carrying the APH(3′)-Ia gene was obtained from New England Biolabs. Purification and kinetic analysis of APH(3′)-IIIa were performed according to a previously described procedure.[19]

Protein translation inhibition by the different compounds was quantified in a coupled transcription/translation assay by using *E. coli* S30 extracts for circular DNA with the pBEST*luc* plasmid (Promega), according to the manufacturer's protocol. Translation reactions (25 μL) that contained variable concentrations of the tested compound were incubated at 37 °C for 60 min, cooled on ice for 5 min, and diluted with a dilution reagent (tris-phosphate buffer (25 mM, pH 7.8), DTT (2 mM), 1,2-diaminocyclohexanetetraacetate (2 mM), glycerol (10%), Triton X-100 (1%), and BSA (1 mg/mL)) into 96-well plates. The luminescence was measured immediately after the addition of the luciferase assay reagent (50 μL; Promega), and the light emission was recorded with a Victor3 plate reader (Perkin-Elmer). The concentration of half-maximal inhibition (IC_{50}) was obtained from concentration−response curves fitted to the data of at least two independent experiments by using Grafit 5 software (Leatherbarrow, R. J. Erithacus Software Ltd.: Horley, U.K., 2001).

DNA supercoiling activity was assayed with relaxed pBR322 DNA as a substrate (TopoGEN, Inc.) according to the manufacturer's protocol. The standard reaction mixture (20 μL) contained 35 mM Tris-Cl, pH 7.5, 24 mM KCl, 4 mM $MgCl_2$, 2 mM dithiothreitol, 1.8 mM spermidine, 1 mM ATP, 6.5% glycerol, 0.1 mg/mL BSA, 12.5 ng/μL relaxed pBR322, and DNA gyrase protein. The reaction mixture was incubated at 37 °C for 1 h and then was terminated by addition of a loading dye and chloroform/isoamyl alcohol (24:1) mixture. After a brief vortex, the blue aqua phase was analyzed by electrophoresis in 1% agarose. One unit of supercoiling activity was defined as the amount of DNA gyrase required to supercoil 0.5 μg of plasmid in 1 h. The IC_{50} was defined as the drug concentration that reduced the enzymatic activity observed with drug-free controls by 50%.

DNA relaxation activity was assayed with supercoiled pBR322 DNA as a substrate (Inspiralis Ltd.) according to the manufacturer's protocol. The standard reaction mixture (20 μL) contained 40 mM HEPES-KOH, pH 7.6, 100 mM potassium glutamate, 10 mM $Mg(OAc)_2$, 10 mM dithiothreitol, 1 mM ATP, 50 μg/mL albumin, 5 ng/μL relaxed pBR322, and TopoIV protein. The reaction mixture was incubated at 37 °C for 30 min and then was terminated by addition of 2 μL of 0.5 M EDTA, 3.5 μL of loading dye, and 20 μL of chloroform/isoamyl alcohol (24:1) mixture. After a brief vortex, the blue aqua phase was analyzed by electrophoresis in 1% agarose. One unit of relaxation activity was defined as the amount of TopoIV required to relax 0.1 μg of plasmid in 30 min. The IC_{50} was defined as the drug concentration that reduced the enzymatic activity observed with drug-free controls by 50%.

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Supporting Information Available: Analytical data, ^{1}H and ^{13}C NMR spectra, and HPLC chromatograms of purity determination for all the hybrid structures (**1a−q**). This material is available free of charge via the Internet at http://pubs.acs.org.

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