compounds were homogeneous on TLC (**24b**, $R_f$ 0.24; **25b**, $R_f$ 0.27; MeOH/$CHCl_3$, 5:95).

For **24b**: $^1H$ NMR ($CDCl_3$) $\delta$ 0.88 (br t, $J$ = 6.0 Hz, 3 H, $CH_2CH_3$), 1.27 (br s, 14 H, $(CH_2)_7CH_3$), 1.42 (s, 9 H, $t$-Bu), 1.59–1.97 (m, 2 H, $S(O)CH_2CH_2$), 2.77 (d of t, $J$ = 8.0 Hz, 2 H, $S(O)CH_2CH_2$), 2.89–3.21 (AB part of ABX spectrum, 7 lines, 2 H, $CHCH_2S(O)$), 3.47 (br t, $J$ = 6.0 Hz, 1 H, $CH_2OH$), 3.64–3.99 (AB part of ABX spectrum, 8 lines, 2 H, $CHCH_2OH$), 3.89–4.26 (m, 1 H, $CHCH_2OH$), 5.50 (br d, 1 H, NH). Anal. Calcd for $C_{18}H_{37}NO_4S$: C, 59.47; H, 10.26; N, 3.85. Found: C, 59.46; H, 10.34; N, 3.89.

For **25b**: $^1H$ NMR ($CDCl_3$) $\delta$ 0.88 (br t, $J$ = 6 Hz, 3 H, $CH_2CH_3$), 1.27 (s, 14 H, $(CH_2)_7CH_3$), 1.43 (s, 9 H, $t$-Bu), 1.56–2.10 (m, 2 H, $S(O)CH_2CH_2$), 2.82 (d of t, $J$ = 8 Hz, 2 H, $S(O)CH_2CH_2$), 3.02 and 3.16 (AB part of ABX spectrum, 8 lines, $J_{AX}$ = 6.0 Hz, $J_{BX}$ = 3.7 Hz, $J_{AB}$ = 13.5 Hz, 2 H, $CHCH_2S(O)$), 3.59–3.92 (AB part of ABX spectrum, 7 lines, 2 H, $CHCH_2OH$), 3.98–4.44 (m, 1 H, $CHCH_2OH$), 5.59 (br d, $J$ = 7 Hz, 1 H, NH). Anal. Calcd for $C_{18}H_{37}NO_4S \cdot {}^1/_2H_2O$: C, 58.03; H, 10.01; N, 3.76. Found: C, 57.72; H, 10.08; N, 3.73.

*N*-(*tert*-Butyloxycarbonyl)-*S*-oxo-*S*-decyl-D-cysteine methyl ester was prepared by oxidation of the corresponding sulfide **23b** (5 mmol) with sodium metaperiodate according to the procedure that has been described earlier[32] to give the desired product as a mixture of diastereomers in 99% yield: TLC $R_f$ 0.31 (eluent MeOH/$CHCl_3$, 3:97); $^1H$ NMR ($CDCl_3$) $\delta$ 0.86 (br t, $J$ = 6.0 Hz, 3 H, $CH_2CH_3$), 1.26 (br s, 14 H, $(CH_2)_7CH_3$), 1.44 (s, 9 H, $t$-Bu), 1.53–1.73 (m, 2 H, $S(O)CH_2CH_2$), 2.73 (d of t, $J$ = 7.0 Hz, 2 H, $S(O)CH_2CH_2$), 3.06–3.31 (m, 2 H, $CHCH_2S(O)$), 3.77 (s, 3 H, $CO_2CH_3$), 4.53–4.82 (m, 1 H, $CHCH_2S(O)$), 5.51–5.86 (m, 1 H, NH). Anal. Calcd for $C_{19}H_{37}NO_5S$: C, 58.28; H, 9.52; N, 3.58. Found: C, 58.56; H, 9.54; N, 3.50.

**S-Oxo-S-propyl-D-cysteinol (26a).** This compound was prepared from **24a** by treatment with trifluoroacetic acid according to the procedure that has been described before:[32] TLC $R_f$ 0.46 (eluent *sec*-BuOH/$NH_4OH$, 55:22); $^1H$ NMR ($D_2O$) $\delta$ 1.08 (t, $J$ = 7.5 Hz, 3 H, $CH_2CH_3$), 1.80 (sextet, $J$ = 8.0 Hz, 2 H, $CH_2CH_3$), 2.93 (t, $J$ = 8.0 Hz, 2 H, $S(O)CH_2CH_2$), 2.83 and 3.00 (AB part of ABX spectrum, 8 lines, $J_{AX}$ = 5.4 Hz, $J_{BX}$ = 11.7 Hz, $J_{AB}$ = 13.2 Hz, 2 H, $CHCH_2S(O)$), 3.20–3.47 (m, 1 H, $CHCH_2OH$), 3.60 and 3.66 (AB part of ABX spectrum, 8 lines, $J_{AX}$ = 9.0 Hz, $J_{BX}$ = 2.7 Hz, $J_{AB}$ = 11.4 Hz, 2 H, $CHCH_2OH$).

**S-Oxo-S-decyl-D-cysteinol (26b).** This compound was prepared from **24b** by treatment with trifluoroacetic acid according to the procedure that has been described earlier:[32] TLC $R_f$ 0.62 (eluent *sec*-BuOH/$NH_4OH$, 55:22); $^1H$ NMR ($CD_2Cl_2$) $\delta$ 0.73–0.95 (m, 3 H, $CH_2CH_3$), 1.29 (br s, 8 H, $(CH_2)_4CH_3$), 1.70 (br s, 8 H, $S(O)CH_2(CH_2)_4$), 2.58–2.93 (m, 4 H, $CHCH_2S(O)$, $S(O)CH_2CH_2$), 3.48–3.68 (m, 1 H, $CHCH_2OH$), 3.75 (s, 2 H, $CHCH_2OH$).

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**Registry No.** **2**, 77880-75-2; **3**, 77880-77-4; **4**, 77880-76-3; **5**, 61787-30-2; **6**, 60484-34-6; **7** (isomer 1), 105498-95-1; **7** (isomer 2), 105498-96-2; **8**, 88001-59-6; **9**, 91922-13-3; **10**, 105431-37-6; **11**, 105431-38-7; **12**, 105431-39-8; **13**, 105431-40-1; **14**, 105456-27-7; **15**, 58462-97-8; **18**, 88001-57-4; **19**, 105431-41-2; **20**, 105431-42-3; **21**, 105431-43-4; **22**, 91922-11-1; **22** (*N*-BOC), 91922-10-0; **23a**, 105431-44-5; **23a** (sulfoxide) (isomer 1), 105456-28-8; **23a** (sulfoxide) (isomer 2), 105431-55-8; **23b**, 105431-45-6; **23b** (sulfoxide) (isomer 1), 105431-56-9; **23b** (sulfoxide) (isomer 2), 105431-57-0; **24a**, 105431-46-7; **24b**, 105431-47-8; **25a**, 105431-48-9; **25b**, 105431-49-0; **26a**, 105431-50-3; **26b**, 105431-51-4; **27**, 28277-67-0; **28**, 57412-59-6; $(BOC)_2O$, 24424-99-5; benzyl mercaptan, 100-53-8; D-cysteine, 921-01-7; 1-bromopropane, 106-94-5; 1-bromodecane, 112-29-8; *S*-propyl-D-cysteine, 85955-34-6; *S*-decyl-D-cysteine, 105431-52-5; *S*-propyl-D-cysteine methyl ester hydrochloride, 105431-53-6; *S*-decyl-D-cysteine methyl ester hydrochloride, 105431-54-7.

# Comparison of Aminoglycoside Antibiotics with Respect to Uptake and Lethal Activity in *Escherichia coli*

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Forty-five aminoglycoside antibiotics and related compounds were compared for their ability to induce the accumulation of dihydrostreptomycin in *Escherichia coli* K12. The common aminoglycosides and a streptothricin antibiotic all induced enhanced uptake within a relatively narrow concentration range. These concentrations were lethal to the bacteria. Comparison of aminoacyl derivatives of tobramycin and apramycin, the latter synthesized utilizing transition-metal cations to selectively control the site of substitution, revealed that 1-*N*-aminoacyl modifications resulted in an increased ability to induce enhanced uptake. 2′-*N*-Aminoacyl modifications were also effective at inducing enhanced uptake, albeit without noticeable improvement over parent. The findings from this structure–activity comparison support the proposition that aminoglycosides share a common critical target (most likely the ribosome), which, when acted upon, results in both drug accumulation and killing.

Aminoglycoside antibiotics are accumulated by an active energy-requiring transport mechanism in bacteria.[1,2] Bryan and Kwan[3] have described this transport as consisting of an initial ionic binding followed by two energy-dependent phases (EDP-I and -II). EDP-I is a slow, energized uptake phase during which the drug promotes its own uptake. The resulting rapid, linear rate of uptake is defined as EDP-II. There is considerable evidence that the induction of EDP-II requires interaction between the drug and a sensitive ribosome.[3-5]

Aminoglycoside-induced EDP-II appears to be associated with the drug-induced killing event(s). There are

(1) Hancock, R. E. W. *J. Antimicrob. Chemother.* **1981**, *8*, 429.
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0022-2623/87/1830-0333$01.50/0 

numerous examples where inability to induce EDP-II coincides with reduction or loss of lethality. For example: (i) EDP-II and killing are lost under anaerobic conditions,[6,7] (ii) EDP-II and killing are lost or reduced in certain respiratory-deficient mutants,[7,8] (iii) inactivation of aminoglycosides by plasmid-encoded modifying enzymes is accompanied by loss of EDP-II,[9] (iv) EDP-II and killing are eliminated or reduced by treatment with certain electron-transport inhibitors and uncouplers,[7,10] (v) streptomycin resistance in the ribosomally mediated rpsL mutant of *E. coli* is accompanied by loss of EDP-II,[11] and (vi) EDP-II and killing can be eliminated by treatment with subinhibitory concentrations of chloramphenicol.[12]

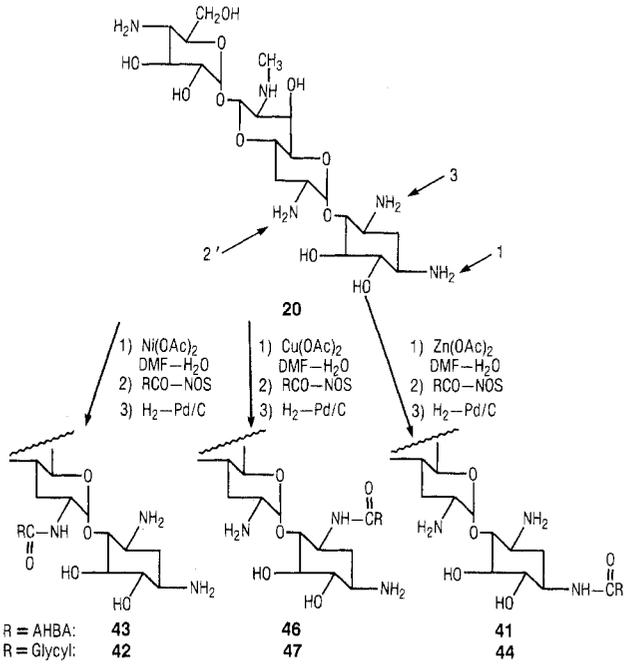
The apparent connection between onset of EDP-II and killing by aminoglycoside antibiotics raises important questions about the relationship between uptake and lethality. For example: (i) Do aminoglycosides differ in their ability to induce EDP-II? (ii) Are the concentrations needed to induce EDP-II lethal? To help answer these questions, we compared 22 common aminoglycosides with respect to their ability to promote both onset of EDP-II and lethal effects in *E. coli* K12.

Since the discovery of butirosin, amikacin, and fortimicin has emphasized the favorable effects of aminoacyl substituents on aminoglycosides, a short series of such derivatives of the structurally novel aminoglycoside apramycin was synthesized, utilizing transition-metal cations to selectively control the site of acylation. This methodology allowed the facile preparation of aminoacyl derivatives of apramycin and nebramine, which were then compared with analogous derivatives of tobramycin with respect to promotion of aminoglycoside uptake and lethality. In this manner, the effects of chemical modification of aminoglycosides on the induction of EDP-II and killing were further examined.

## Chemical Modification

A wide variety of derivatives of numerous aminoglycosides have been prepared over many years in the effort to improve the antimicrobial activity and/or decrease the toxicity of this class of antibiotics.[13] Apramycin (**20**) is a structurally unique aminoglycoside;[14] however, derivatives of this novel antibiotic have not been extensively reported. Several derivatives of apramycin (**20**) were prepared in an effort to study more closely the effects of structural modification on induction of enhanced uptake and to determine if a derivative more potent than the parent could be found. Emphasis was placed initially on aminoacyl derivatives, especially the γ-amino-α-hydroxybutyryl (AHBA) derivatives (**41**, **43**) analogous to butirosin (**1**) and amikacin (**5**). It should be noted from Table II that these latter two compounds have lower $C_{50}$ values (the concentration needed to induce half maximum rate of uptake) than their nonacylated analogues, ribostamycin (**22**) and kanamycin A (**21**), respectively.

**Scheme I.** Synthesis of *N*-Acyl Derivatives of Apramycin




The exploration of structure–activity relationships was initiated by acylation of the 1-, 3-, and 2′-amino groups of apramycin with the AHBA and glycyl substituents, the latter found in fortimicin (**19**) and sporaricin (**18**). These derivatives were most readily synthesized by the utilization of transition-metal cations to selectively control the site of acylation.[15] Although transition-metal cations have been used as temporary protecting groups in aminoglycoside chemistry by other investigators, their studies did not reveal any significant change in site of monoacylation as the metal cation was varied.[16–18] This was consistent with our results, since it was only with the aminoglycosides containing 2-deoxystreptamine monosubstituted at the 4-hydroxyl group [e.g., apramycin (**20**), nebramine (**38**)] that this major variation of reaction site with cation was observed. The 2-deoxystreptamine-containing aminoglycosides that are disubstituted (e.g., gentamicin, tobramycin) behaved as reported previously.[16–18] The reactions of apramycin (**20**) are summarized in Scheme I.

All of the reactions were performed by mixing an aqueous solution of apramycin with the appropriate transition-metal salt and then diluting the mixture with DMF. Variations in this order of mixing were generally unsuccessful, probably due to incomplete formation of the requisite complexes in the heterogeneous reaction mixtures. DMF was the organic solvent most frequently used, but it could be replaced by other polar, water-miscible organic solvents, such as $Me_2SO$, HMPA, sulfolane, formamide, tetramethylurea, *N*-methylpyrrolidinone, dioxane, THF, *t*-BuOH, acetone, or acetonitrile. Solvent composition was not critical, ranging from 2:1 DMF–water (ho-

(6) Bryan, L. E.; Kwan, S. *J. Antimicrob. Chemother.* **1981**, *8* (Suppl D), 1.
(7) Miller, M. H.; Edberg, S. C.; Mandel, L. J.; Behar, C. F.; Steigbigel, N. H. *Antimicrob. Agents Chemother.* **1980**, *18*, 722.
(8) Bryan, L. E.; Van Den Elzen, H. M. *Antimicrob. Agents Chemother.* **1977**, *12*, 163.
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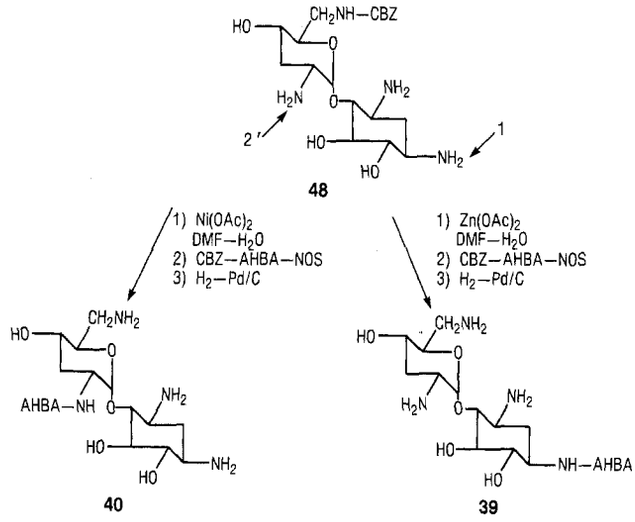
(15) Kirst, H. A.; Truedell, B. A.; Toth, J. E. *Tetrahedron Lett.* **1981**, *22*, 295.
(16) Nagabhushan, T. L.; Cooper, A. B.; Turner, W. N.; Tsai, H.; McCombie, S.; Mallams, A. K.; Rane, D.; Wright, J. J.; Reichert, P.; Boxler, D. L.; Weinstein, J. *J. Am. Chem. Soc.* **1978**, *100*, 5253.
(17) Hanessian, S.; Patil, G. *Tetrahedron Lett.* **1978**, 1031, 1035.
(18) Tsuchiya, T.; Takagi, Y.; Umezawa, S. *Tetrahedron Lett.* **1979**, 4951.

**Scheme II.** Synthesis of *N*-Acyl Derivatives of Nebramine




mogeneous solutions) to 5:1 DMF–water (heterogeneous mixtures for $Ni^{2+}$ and $Zn^{2+}$). Selectivity for acylation at the 2′-amino group with $Ni(OAc)_2$ and at the 3-amino group with $Cu(OAc)_2$ was generally high; acylation at the 1-amino group with $Zn(OAc)_2$ was somewhat less selective since minor amounts of the 3-*N*- and 2′-*N*-acyl isomers as well as disubstituted products were usually observed. Even so, the proportion of 1-*N*-acyl product was substantially greater than that obtained in the absence of $Zn(OAc)_2$, thereby resulting in both a higher yield and more efficient isolation and purification of product. The acetate salt generally gave the best results, as found in other studies.[16] In an effort to improve the selectivity for 1-N-acylation by zinc salts, 16 other zinc salts were tried, but none gave better results than zinc acetate.

The structures of the acyl derivatives were assigned on the basis of their field desorption mass spectra and their $^{13}C$ NMR spectra in both acidic and basic solution. The $^{13}C$ NMR assignments for apramycin were made according to published data.[19,20] The $^{13}C$ NMR spectra for the *S*-γ-amino-α-hydroxybutyryl derivatives (**41**, **43**) of apramycin are given in Table I and illustrate the expected shifts for the carbon atoms at the site of substitution.

Since the transition metal cation directed acylation appeared to be generally applicable for 4-*O*-glycosyl monosubstituted derivatives of 2-deoxystreptamine, the 1- and 2′-*N*-AHBA derivatives (**39**, **40**) of the pseudodisaccharide nebramine (**38**) were also synthesized by this methodology. The reaction sequence is illustrated in Scheme II. Since the primary 6′-amino group was not complexed by the transition-metal cations and was more readily acylated than any of the secondary amino groups, it first had to be protected, e.g., as its *N*-CBZ derivative. Selective acylation then proceeded as outlined above and the desired products were cleanly obtained after the usual deprotection and isolation procedures. If the 6′-amino group was not first protected, then the appropriate di-*N*-acyl derivatives were formed having the same acyl moiety on the 6′-amino group as on the 1,3 or 2′-amino group.

## Uptake and Lethality

Accumulation of [$^3$H]dihydrostreptomycin (DHS) by *E. coli* is shown in the lowest curve in Figure 1. [$^3$H]DHS (0.5 μg/mL) was taken up at a slow but increasing rate

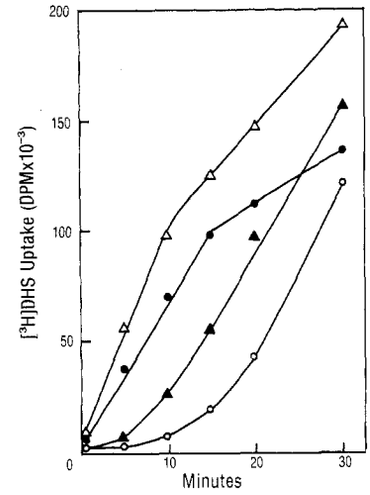



**Figure 1.** Effect of preexposure of *E. coli* K12 to gentamicin C complex on uptake of [$^3$H]DHS. Cells were preexposed to gentamicin C complex (○, 0 μg/mL; ▲, 0.1 μg/mL; △, 1.0 μg/mL; ●, 10 μg/mL) from 20 min, and uptake of [$^3$H]DHS was measured over a 30-min period as described in the Experimental Section.

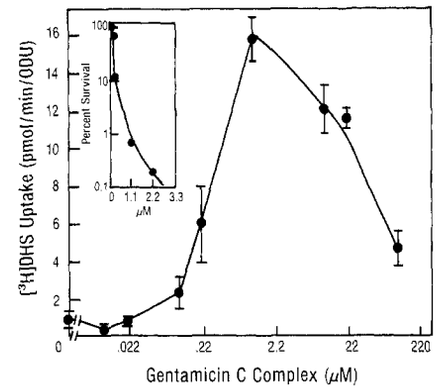



**Figure 2.** Effect of concentration of gentamicin C complex on rates of [$^3$H]DHS uptake and viability. Preexposure to aminoglycoside for 20 min and subsequent measurement of [$^3$H]DHS uptake and viability (insert) were as described in the Experimental Section. Uptake rates represent an average of three separate determinations of accumulated [$^3$H]DHS during a 5-min incubation. The bars represent the standard error of the three determinations.

(EDP-I) for 15–20 min. A fully induced, linear rate of uptake (EDP-II) was reached in approximately 20 min. However, when cells were preexposed to nonradiolabeled gentamicin for 20 min prior to addition of [$^3$H]DHS, a concentration-dependent induction of EDP-II occurred. The extent of induction was indicated by increasing rates of [$^3$H]DHS uptake. The results in Figure 1 show that preincubation concentrations up to 1.0 μg/mL of gentamicin led to increased rates of [$^3$H]DHS accumulation. The rate of accumulation was reduced when cells were preincubated with 10 μg/mL of gentamicin. Figure 2 shows more clearly the effect of gentamicin concentration on induction of enhanced [$^3$H]DHS uptake. Induction required a minimum concentration of approximately 0.11 μM and maximum uptake was seen following preexposure to 1.1 μM gentamicin. Preexposure to higher concentrations gave reduced rates of accumulation.

Dose–response curves were generated for each of the common aminoglycoside antibiotics listed in Table II. The curve for each drug was qualitatively similar to that for gentamicin (Figure 2). Preexposure to increasing concentrations of antibiotic, up to a critical concentration, resulted in increased rates of [$^3$H]DHS uptake. Beyond

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**Table I.** $^{13}C$ NMR Data[a]

| carbon | pH 11 | | | pH 2 | | |
|---|---|---|---|---|---|---|
| | apramycin (20) | 1-*N*-AHBA (41) | 2′-*N*-AHBA (43) | apramycin (20) | 1-*N*-AHBA (41) | 2′-*N*-AHBA (43) |
| 1 | 51.1 | 50.5 | 51.0 | 50.7 | 50.1 | 50.6 |
| 2 | 36.7 | 35.0 | 36.3 | 29.3 | 31.3 | 29.0 |
| 3 | 50.3 | 50.2 | 50.4 | 49.6 | 49.7 | 50.0 |
| 4 | 87.9 | 87.2 | 87.4 | 78.8 | 79.6 | 78.8 |
| 5 | 76.8 | 77.1 | 76.6 | 76.0 | 76.7 | 76.1 |
| 6 | 78.5 | 75.3 | 78.3 | 73.5 | 74.7 | 73.4 |
| 1′ | 101.6 | 101.6 | 98.5 | 96.2 | 96.3 | 97.5 |
| 2′ | 49.8 | 49.9 | 48.7 | 49.0 | 49.0 | 47.9 |
| 3′ | 32.9 | 33.0 | 29.7 | 27.8 | 27.9 | 29.0 |
| 4′ | 68.0 | 67.9 | 67.5 | 67.0 | 66.9 | 67.7 |
| 5′ | 71.0 | 71.0 | 71.1 | 70.5 | 70.4 | 70.6 |
| 6′ | 66.2 | 66.4 | 66.1 | 63.5 | 63.7 | 63.7 |
| 7′ | 62.3 | 62.4 | 62.2 | 60.4 | 60.4 | 60.4 |
| 8′ | 96.4 | 96.5 | 96.5 | 93.7 | 93.7 | 93.7 |
| $CH_3$ | 32.9 | 33.0 | 32.9 | 31.3 | 31.3 | 31.1 |
| 1″ | 95.3 | 95.3 | 95.3 | 95.5 | 95.4 | 95.3 |
| 2″ | 71.7 | 71.8 | 71.7 | 71.3 | 71.3 | 71.2 |
| 3″ | 74.1 | 74.2 | 74.2 | 70.2 | 70.2 | 70.1 |
| 4″ | 53.2 | 53.3 | 53.1 | 53.5 | 53.3 | 53.0 |
| 5″ | 73.4 | 73.6 | 73.4 | 69.1 | 69.2 | 69.0 |
| 6″ | 61.6 | 61.8 | 61.6 | 61.3 | 61.4 | 61.2 |
| AHBA | | 177.1 | 176.0 | | 176.3 | 175.5 |
| AHBA | | 70.7 | 70.5 | | 70.5 | 70.3 |
| AHBA | | 37.8 | 37.6 | | 37.9 | 37.6 |
| AHBA | | 35.6 | 33.2 | | 31.6 | 31.7 |

[a] Spectra were obtained in $D_2O$, with dioxane as internal standard; chemical shifts are expressed in $\delta$ values (ppm downfield from tetramethylsilane).

**Table II.** Effects of Aminoglycoside Antibiotics on Induction of Enhanced [$^3$H]DHS Uptake and Killing

| aminoglycoside | no. | $C_{50}$[a] | uptake at $C_{50}$[b] | % kill at $C_{50}$ | $LD_{90}$[c] |
|---|---|---|---|---|---|
| butirosin | 1 | 0.13 | 4.0 | 88 | 0.12 |
| lividomycin A | 2 | 0.18 | 5.5 | 40 | 0.87 |
| gentamicin $C_{1a}$ | 3 | 0.20 | 4.7 | 88 | 0.22 |
| gentamicin $C_1$ | 4 | 0.21 | 5.5 | 85 | 0.25 |
| amikacin | 5 | 0.21 | 4.7 | 76 | 0.32 |
| tobramycin | 6 | 0.24 | 8.3 | 54 | 0.71 |
| gentamicin complex | 7 | 0.24 | 7.3 | 72 | 0.43 |
| gentamicin $C_2$ | 8 | 0.26 | 6.5 | 98 | 0.14 |
| kanamycin B | 9 | 0.27 | 6.8 | 31 | 1.1 |
| sisomicin | 10 | 0.31 | 7.0 | 77 | 0.49 |
| netilmicin | 11 | 0.32 | 5.8 | 86 | 0.38 |
| paromomycin | 12 | 0.32 | 3.9 | 61 | 0.78 |
| neomycin | 13 | 0.34 | 1.6 | 82 | 0.46 |
| streptomycin | 14 | 0.34 | 5.8 | 88 | 0.36 |
| dideoxykanamycin B | 15 | 0.51 | 8.1 | 74 | 0.89 |
| seldomycin 5 | 16 | 0.51 | 7.1 | 80 | 0.73 |
| mannosylparomomycin | 17 | 0.57 | 4.4 | 78 | 0.88 |
| sporaricin A | 18 | 0.59 | 5.4 | 42 | 2.6 |
| fortimicin A | 19 | 0.86 | 5.6 | 93 | 0.70 |
| apramycin | 20 | 0.87 | 4.0 | 95 | 0.67 |
| kanamycin A | 21 | 0.95 | 3.5 | 93 | 0.85 |
| ribostamycin | 22 | 1.2 | 2.6 | 99 | 0.17 |

[a] Concentration ($\mu$M) required to give half the maximum rate of enhanced [$^3$H]DHS uptake estimated from dose–response curves analogous to that shown in Figure 2. Dose–response curves were drawn with use of data averaged from triplicate experiments. [b] Estimated rate of [$^3$H]DHS uptake [pmol min$^{-1}$ (optical density unit)$^{-1}$] at the $C_{50}$. [c] Lethal dose ($\mu$M) required to kill 90% of the population.

the critical concentration, a decreased rate of uptake was observed. Although this apparent inhibitory effect at higher concentrations was observed for each compound listed in Table II, the effect was less pronounced for some aminoglycosides [e.g., kanamycin A (21), fortimicin A (19), sporaricin A (18)] than for others. A reduced rate of uptake following preexposure to high concentrations was not seen with kanamycin in the study reported by Höltje.[21]

(21) Höltje, J. V. *Antimicrob. Agents Chemother.* **1979**, *15*, 177.

**Table III.** Induction of Enhanced [$^3$H]DHS Uptake by Various Amino-Containing Compounds and Antibiotics

| compound | no. | $C_{50}$[a] | uptake at $C_{50}$[b] |
|---|---|---|---|
| spectinomycin | 23 | >10 | NA[c] |
| hygromycin B | 24 | >50 | NA |
| sorbistin A | 25 | >50 | NA |
| spermidine | 26 | >50 | NA |
| cinodine | 27 | >100 | NA |
| tetracycline | 28 | >50 | NA |
| chloramphenicol | 29 | >50 | NA |
| 2-deoxystreptamine | 30 | >50 | NA |
| *N*-methylstreptothricin F | 31 | 0.10 | 4.1 |

[a] As described in Table II except values are expressed as $\mu$g/mL, and dose–response curves were drawn with use of data from single experiments. [b] As described in Table II. [c] Not applicable.

To compare antibiotics for their ability to induce enhanced uptake (EDP-II) of [$^3$H]DHS, the concentration that induced half of the maximum measured rate ($C_{50}$) was estimated from the dose–response data for each antibiotic. This value, rather than the minimum concentration giving the maximum rate of [$^3$H]DHS uptake, was calculated due to the experimental difficulties in precisely estimating the latter value. In addition, rates of [$^3$H]DHS uptake and the lethal effects following preexposure to the $C_{50}$ were determined for each compound. Lethality was measured by using the same experimental conditions used to measure [$^3$H]DHS uptake (see Figure 2 insert). The percent kill and rate of [$^3$H]DHS uptake at the $C_{50}$ plus the $LD_{90}$ (concentration needed to kill 90% of the population) are presented together in Table II.

The compounds in Table II have been listed in order of increasing $C_{50}$ values. Considering the diversity of structures, it is remarkable that the $C_{50}$ values all fall within a relatively narrow concentration range (approximately 0.1–1 $\mu$M or 0.1–0.5 $\mu$g/mL). It is also apparent from cell-viability measurements that each drug was lethal at its $C_{50}$, although in three cases, killing was less than 50%. With the exception of only sporaricin A (18), the $LD_{90}$ values also fell within a narrow range of concentrations (0.1–1 $\mu$M). Of several compounds representing other

**Table IV.** Effects of Aminoglycoside Derivatives on Induction of Enhanced [$^3$H]DHS Uptake and Killing

| derivative | no. | $C_{50}$[a] | uptake at $C_{50}$[b] | % kill at $C_{50}$ | $LD_{90}$[c] |
|---|---|---|---|---|---|
| tobramycin | 6 | 0.24 | 8.3 | 54 | 0.71 |
| 1-*N*-AHBA[d] | 32 | 0.081 | 6.4 | 68 | 0.14 |
| 1-*N*-HACA[e] | 33 | 0.12 | 4.4 | 50 | 0.63 |
| 1-*N*-HPCA[f] | 34 | 0.12 | 4.4 | 65 | 0.31 |
| 2′-*N*-HPCA | 35 | 0.34 | 4.6 | 87 | 0.38 |
| 3″-*N*-HPCA | 36 | 4.3 | 3.2 | 92 | 4.0 |
| 6′-*N*-HPCA | 37 | ≥7.8 | ≥1.1 | ≥8 | >17 |
| nebramine | 38 | 2.0 | 2.9 | 49 | 6.7 |
| 1-*N*-AHBA | 39 | 0.56 | 6.8 | 18 | 10 |
| 2′-*N*-AHBA | 40 | 2.7 | 8.3 | 64 | 12 |
| apramycin | 20 | 0.87 | 4.0 | 95 | 0.67 |
| 1-*N*-AHBA | 41 | 0.39 | 2.4 | 53 | 1.2 |
| 2′-*N*-glycyl | 42 | 0.45 | 3.3 | 53 | 0.83 |
| 2′-*N*-AHBA | 43 | 0.84 | 2.6 | 93 | 0.72 |
| 1-*N*-glycyl | 44 | 1.1 | 2.1 | 94 | 0.84 |
| β-methyl aprosaminide[g] | 45 | 4.6 | 2.1 | 74 | 7.6 |

[a] As described in Table II except dose–response curves were drawn with use of data from single experiments. [b] As described in Table II. [c] As described in Table II. [d] γ-Amino-α-hydroxybutyryl. [e] 3-Hydroxyazetidinyl-3-carbonyl (see ref 27). [f] 3-Hydroxypyrrolidinyl-3-carbonyl (see ref 27). [g] See ref 14.

aminocyclitol, aminosugar, or polyamine structures, only *N*-methylstreptothricin F (**31**) induced enhanced [$^3$H]DHS uptake in *E. coli* (Table III). Two non-aminoglycoside inhibitors of ribosome function [tetracycline (**28**) and chloramphenicol (**29**)] were ineffective as inducers.

The aminoglycoside derivatives in Table IV are representative of modifications of different amino groups on or near the 2-deoxystreptamine moiety of tobramycin and apramycin. The data from Table II for tobramycin (**6**) and apramycin (**20**) have been included for comparison purposes. Unlike the results in Table II, the range of $C_{50}$ values was noticeably wider for these compounds, and although each derivative demonstrated lethal activity at the concentration corresponding to the $C_{50}$, the $LD_{90}$ values for some of these drugs were much higher than those shown in Table II.

The dose–response curves for most of the derivatives were very similar to that shown in Figure 2. No maximum enhanced rate of uptake was seen for 6′-N-HPCA-tobramycin (**37**, the 3-hydroxypyrrolidinyl-3-carbonyl derivative) so the $C_{50}$ given for it is a minimum estimate. This derivative also had very poor lethal activity with less than 20% kill at 17μM.

## Discussion

Forty-five compounds were evaluated in this study as inducers of enhanced [$^3$H]DHS uptake. Since initial rates of uptake of [$^3$H]DHS were measured immediately following preexposure to the test compound, the extent of induction could be estimated by the rate at which [$^3$H]DHS was accumulated. It should be pointed out that the absolute rates of [$^3$H]DHS uptake in these experiments were very much dependent on time of preexposure as well as concentrations of preexposure drug and [$^3$H]DHS. Therefore, standard conditions were selected so that effects of [$^3$H]DHS on its own uptake were minimized. Since the time of preexposure was held constant, $C_{50}$ values for aminoglycosides and derivatives reflect the ability of these drugs to induce EDP-II under the specific experimental conditions employed.

Each of the aminoglycosides listed in Table II induced enhanced uptake of [$^3$H]DHS in *E. coli* K12. This extends an earlier report[21] wherein kanamycin and streptomycin were shown to induce enhanced uptake of [$^3$H]DHS in *E. coli*. The aminoglycosides in Table II contain either streptamine, 2-deoxystreptamine, or fortamine as the aminocyclitol moiety. However, not every antibiotic containing an aminocyclitol enhances uptake of [$^3$H]DHS, as exemplified by spectinomycin and hygromycin B (Table III), which contain actinamine and hyosamine, respectively. Spectinomycin was previously reported to be a noninducer.[22] Sorbistin A, in which the aminocyclitol ring has been replaced by an acyclic moiety, was a noninducer. Furthermore, 2-deoxystreptamine alone and the polyamine compounds spermidine and cinodine were noninducers. These results demonstrate that a polyamino structure is not sufficient and that the stereochemical arrangement of the several amino groups in the aminoglycosides is critical for induction of EDP-II. Surprisingly, the non-aminocyclitol antibiotic *N*-methylstreptothricin F[23] was an effective inducer with a $C_{50}$ of 0.1 μg/ml (0.19 μM). *N*-Methylstreptothricin F is the first example of a compound lacking the conventional aminocyclitol moieties but which nevertheless enhances the uptake of [$^3$H]DHS similar to conventional aminoglycosides. This phenomenon has not previously been recognized or even expected.

Although not an inducer, hygromycin B is accumulated at an enhanced rate when *E. coli* is preexposed to tobramycin.[24] Thus, uptake via EDP-II is not limited to those compounds that induce. Other studies have shown that polyamines such as spermidine[22] and the basic amino acid arginine[3] are transported at enhanced rates following induction of EDP-II. This is consistent with the notion that EDP-II is dependent on a perturbation of the ribosome,[1] but once induced, uptake of a wider variety of amino-containing compounds is effected.

Quantitatively, there was little difference among the commonly recognized aminoglycosides with respect to ability to induce EDP-II (Table II compounds). The estimated rates of uptake occurring at the $C_{50}$ also were similar for each of the compounds. Considerably more variation was seen among the derivatives of tobramycin and apramycin, which clearly emphasizes that the relative lack of variation in Table II is not simply fortuitous. The $C_{50}$ value for each of the 1-*N*-acyl derivatives (**32–34**) of tobramycin was noticeably lower than that for tobramycin (**6**) itself (see Table IV). The 1-*N*-AHBA derivatives of nebramine (**39**) and apramycin (**41**) showed a similar pattern. This is consistent with the trend seen with butirosin (**1**) and amikacin (**5**) wherein these drugs showed an improved facility to induce enhanced uptake compared with their unacylated analogues (**22**, **21**) (see Table II). Some variation in the 1-*N*-acyl group is possible, since the AHBA (**32**), HACA (**33**, 3-hydroxyazetidinyl-3-carbonyl), and HPCA (**34**) derivatives of tobramycin all had lower $C_{50}$ values. Just as interesting was the effect of acylation on the 2′-amino group; in our limited series, these derivatives (**35**, **40**, **42**, **43**) were as effective as parent antibiotic in penetrating and killing. In contrast, modifications distant from the 2-deoxystreptamine moiety (**36**, **37**, **45**) were generally deleterious.

In spite of the diversity, the $C_{50}$ values for most of the derivatives in Table IV appear to be lethal concentrations. As indicated by the $LD_{90}$ figures, higher $C_{50}$ values seem to be accompanied by reduced lethal activity. Thus, induction of enhanced uptake appears to correlate with killing. Although circumstances have been reported where

(22) Höltje, J. V. *Eur. J. Biochem.* **1978**, *86*, 345.
(23) Hunt, A. H.; Hamill, R. L.; DeBoer, J. R.; Presti, E. A. *J. Antibiot.* **1985**, *38*, 987.
(24) Rao, R. N.; Allen, N. E.; Hobbs, J. N., Jr.; Alborn, W. E., Jr.; Kirst, H. A.; Paschal, J. W. *Antimicrob. Agents Chemother.* **1983**, *24*, 689.

this correlation is absent (e.g., the stimulation of streptomycin uptake by puromycin[5]), the data are consistent with the hypothesis that there is a common, critical target for aminoglycoside antibiotics (most likely the ribosome) and that the critical concentration needed to act on or at this target results in both enhanced drug uptake and killing.[1]

Nearly one-half of the compounds listed in Table II were included by Benveniste and Davies[25] in their study of structure–activity relationships among aminoglycoside antibiotics. Those authors concluded that deoxystreptamine-containing aminoglycosides had widely differing abilities to inhibit poly(U)-directed polyphenylalanine synthesis on isolated *E. coli* K-12 ribosomes. However, the variation was seen at 10 and 50 μg/mL; few differences were seen at 0.1 or 1.0 μg/mL. In the present study, induction of half-maximum EDP-II required concentrations less than 1.0 μg/mL (Table II). Assuming no limitations in ribosome accessibility in the strain of *E. coli* K12 used in the present study, these low concentrations are lethal and are likely affecting ribosome function. However, as Benveniste and Davies showed,[25] few differences between aminoglycosides could be detected at these concentrations. Again, the data suggest a common critical target for these drugs.

Chemical modification of aminoglycoside antibiotics has traditionally concentrated on derivatization of the 1-amino group on the 2-deoxystreptamine moiety, leading to semisynthetic antibiotics such as amikacin and netilmicin. Our study has demonstrated that modification of the 2′-amino group of either tobramycin or apramycin also yields antibiotics that effectively penetrate and kill bacteria. Consequently, this suggests that chemical modification of the 2′-amino group of aminoglycosides in novel ways may represent a fertile new approach to the synthesis of aminoglycoside derivatives and that a more thorough investigation of this direction may lead to a potentially useful series of new antibiotics.

## Experimantal Section

**Bacteria and Growth Conditions.** *E. coli* K12 (ATCC 25868) was used in all experiments. Cells were grown in a tryptone-peptone broth (TPB) medium[26] and incubated at 37 °C with shaking.

**Assay of Enhanced Rates of Dihydrostreptomycin (DHS) Uptake.** Cells grown overnight were used to inoculate 50 mL of fresh broth to a starting $OD_{600}$ (optical density, 600 nm) of approximately 0.05. Incubation was continued until the $OD_{600}$ reached 0.20. Aliquots (10 mL) of this culture were pipetted into 25-mL Erlenmeyer flasks to which the appropriate antibiotic was added. Incubation was continued for 20 min at 37 °C. Following incubation, cells were collected by centrifugation, (10 min, 10000 rpm, 23 °C), pellets were resuspended in an equal volume of TPB, and 2.0-mL aliquots were dispensed in 25-mL Erlenmeyer flasks. [$^3$H]DHS (1.0 μCi/mL, 3.8 Ci/mmol, or 0.38 μg; Amersham) was added to each flask, and cultures were incubated for 5.0 min at 37 °C. At the end of this time, 0.5-mL samples were withdrawn and pipetted directly onto 0.45-μm millipore filters that had been presoaked with 0.1 M LiCl.[22] Each filter was washed three times with 0.1 M LiCl, placed into a scintillation vial, and dried. Radioactivity was measured with use of a toluene-based scintillation fluid.

**Viability Measurements.** Cells were preexposed to antibiotics by using the same procedure described above. Following the 20-min preexposure, cell pellets were resuspended to an $OD_{600}$ of 0.10–0.15 in TPB. Serial dilutions were made in the same broth and plated onto tryptone–peptone agar. Colonies were counted after overnight incubation at 37 °C.

**Antibiotics.** All antibiotic stock solutions were freshly prepared on the day of use. Concentrations of drugs listed in Table II were adjusted for potency, which ranged from 590 to 1000 μg/mg. $C_{50}$ and $LD_{90}$ values (see Tables II–IV) were expressed as concentration of free base.

Antibiotics were kindly provided by the following sources: Bristol (amikacin), Meiji Seika (kanamycin B, dideoxykanamycin B, ribostamycin), Schering (gentamicin $C_1$, $C_{1a}$, $C_2$, sisomicin, netilmicin), Parke-Davis (butirosin, paromomycin), Shionogi (1-*N*-AHBA-, 1-*N*-HACA-,[27] 1-*N*-HPCA-,[27] 2′-*N*-HPCA-, 3″-*N*-HPCA-, and 6′-*N*-HPCA-tobramycin), Abbott (spectinomycin, fortimicin A, seldomycin 5), Kowa (sporaricin A, lividomycin A, mannosylparomomycin), Bristol-Banyu (sorbistin A), Lederle (cinodine). Kanamycin A, gentamicin complex, streptomycin, and neomycin were purchased from Sigma Chemical Co. *N*-Cbz-γ-amino-α-hydroxybutyric acid was kindly provided by Bristol Laboratories.

**Physical–Chemical Methodology.** Nuclear magnetic resonance (NMR) spectra were recorded on a Varian Associates FT-80 or JEOL JNM-PS-100 spectrometer. Field-desorption mass spectra were taken on a Varian MAT 731 spectrometer using carbon dendrite emitters. Melting points were obtained with a Mel-Temp apparatus and are uncorrected. Thin-layer chromatography was carried out with E. Merck silica gel plates, using ninhydrin reagent for visualization.

**Preparation of $N^1$-[(*S*)-γ-Amino-α-hydroxybutyryl]-apramycin (41).** A solution of apramycin (3.07 g, 5.7 mmol) in water (40 mL) was treated with zinc(II) acetate dihydrate (5.0 g, 22.8 mmol), and this mixture was diluted with dimethylformamide (200 mL). The resultant white opaque mixture was stirred for 1 h and treated with *N*-[(*S*)-γ-[(benzyloxycarbonyl)-amino]-α-hydroxybutyryloxy]succinimide (2.86 g, 8.2 mmol), slowly yielding a homogeneous solution. After the mixture had stirred overnight at ambient temperature, the solvent was evaporated under reduced pressure. The resultant residue was dissolved in a 10:10:1 mixture of dichloromethane/methanol/concentrated ammonium hydroxide and loaded on a silica gel column (Grace 950, 500 g) preeluted with the same solvent mixture. The column was eluted stepwise with two different mixtures of dichloromethane/methanol/ammonium hydroxide (10:10:1, then 3:3:1). Fractions containing the desired product were found by thin-layer chromatography, combined, concentrated, and lyophilized, yielding a glassy residue (1.7 g) containing $N^1$-[(*S*)-γ-[(benzyloxycarbonyl)amino]-α-hydroxybutyryl]apramycin. This residue was dissolved in a solution of 25% aqueous dioxane (50 mL) and glacial acetic acid (1 mL), treated with 5% palladium on carbon (700 mg), and hydrogenated at 4 atm for 24 h at room temperature. The palladium catalyst was removed by filtration and the filtrate was evaporated to dryness. The resultant residue was loaded on a column of 115 mL of Bio-Rex 70 (ammonium hydroxide cycle, pH 9.5), and the column was eluted with a linear gradient of 0.01 N ammonium hydroxide (2 L) and 0.4 N ammonium hydroxide (2 L). The fractions collected were analyzed by thin-layer chromatography and by their antimicrobial activity against the apramycin-resistant *E. coli* X563. The two fractions active against the above bacterium were combined and lyophilized to yield $N^1$-[(*S*)-γ-amino-α-hydroxybutyryl]apramycin (**41**) (200 mg).

**Preparation of $N^1$-Glycylapramycin (44).** Apramycin (2.16 g, 4 mmol) was dissolved in water (40 mL) and treated with zinc(II) acetate dihydrate (3.5 g, 16 mmol). The resulting white mixture was diluted with dimethylformamide (200 mL) and treated with *N*-[[*N*-(benzyloxycarbonyl)glycyl]oxy]succinimide (1.84 g, 6 mmol). After the solution had been stirred overnight at room temperature, the solvent was evaporated under reduced pressure. The residue thus obtained was dissolved in 0.1 N hydrochloric acid (250 mL) and was treated for 1 h with a slow stream of gaseous hydrogen sulfide, yielding a white precipitate, which was stirred overnight and then filtered. The filtrate was evaporated to dryness, and the residue was dissolved in 1 N sodium hydroxide (50 mL solution, pH 9.4). The solution was loaded on a column of 300 mL of Bio-Rex 70 (ammonium hydroxide cycle, pH 10, preeluted with 0.01 N ammonium hydroxide). After loading, the column was eluted with 400 mL of 0.01 N ammonium hydroxide,

(25) Benveniste, R.; Davies, J. *Antimicrob. Agents Chemother.* **1973**, *4*, 402.
(26) Allen, N. E.; Alborn, W. E., Jr.; Hobbs, J. N., Jr.; Kirst, H. A. *Antimicrob. Agents Chemother.* **1982**, *22*, 824.
(27) Igarashi, K *Jpn. J. Antibiot.* **1979**, *32*, S-187.

followed by a linear gradient of 0.01 N ammonium hydroxide (2 L) and 0.05 N ammonium hydroxide (2 L). $N^1$-[*N*-(Benzyloxycarbonyl)glycyl]apramycin was readily eluted, giving 0.61 g (21%) of relatively pure product. Later fractions contained smaller amounts of the 2′-N-substituted and 3-N-substituted isomers.

$N^1$-[*N*-(Benzyloxycarbonyl)glycyl]apramycin (0.51 g, 0.70 mmol) was dissolved in a mixture of ethanol (100 mL), water (16 mL), and 1 N hydrochloric acid (4.2 mL) and hydrogenated over 5% palladium on charcoal (2 g) at 60 psi overnight at room temperature. The catalyst was filtered and the filtrate was evaporated under reduced pressure. The residue thus obtained was dissolved in water, the pH of the solution was adjusted to 8 with 1 N sodium hydroxide, and the resulting mixture was loaded on a column of 100 mL of Bio-Rex 70 (ammonium hydroxide, pH 10, preeluted with 0.01 N ammonium hydroxide). After loading, a linear gradient was begun with 0.01 N ammonium hydroxide (1 L) and 0.1 N ammonium hydroxide (1 L). The fractions containing product, found by thin-layer chromatographic analysis, were combined and lyophilized to yield 258 mg (62%) of $N^1$-glycylapramycin (**44**).

**Preparation of $N^{6'}$-(Benzyloxycarbonyl)nebramine (48).** Nebramine (15.3 g, 50 mmol) was dissolved in water (400 mL) and tetrahydrofuran (150 mL). A solution of (benzyloxycarbonyl)imidazole (11.6 g, 57 mmol) in tetrahydrofuran (300 mL) was added dropwise over a 90-min period, and the reaction mixture was stirred overnight at room temperature. Tetrahydrofuran was evaporated under reduced pressure, and the resulting aqueous solution was lyophilized.

The crude product thus obtained was dissolved in water (100 mL) and loaded on a column of 1500 mL of Bio-Rex 70 (ammonium hydroxide cycle, pH 9.7). The column was eluted stepwise with 0.005 N ammonium hydroxide (1.5 L), 0.015 N ammonium hydroxide (2 L), 0.03 N ammonium hydroxide (5 L), and 0.05 N ammonium hydroxide (10 L). $N^{6'}$-(Benzyloxycarbonyl)nebramine was eluted with 0.05 N ammonium hydroxide; fractions were analyzed by thin-layer chromatography and appropriate fractions were combined and lyophilized to give 9.7 g (44%) of product.

**Preparation of $N^1$-[(*S*)-γ-Amino-α-hydroxybutyryl]nebramine (39).** $N^{6'}$-(Benzyloxycarbonyl)nebramine (1.32 g, 3 mmol) was dissolved in water (20 mL) and treated with zinc(II) acetate dihydrate (2.63 g, 12 mmol). The mixture was diluted with dimethylformamide (100 mL), treated with *N*-[[(*S*)-γ-[(benzyloxycarbonyl)amino]-α-hydroxybutyryl]oxy]succinimide (1.58 g, 4.5 mmol) and stirred overnight at room temperature. Solvent was evaporated under reduced pressure; the resulting residue was dissolved in methanol and reevaporated to remove all dimethylformamide. The residue was dissolved in 30:10:1 dichloromethane/methanol/ammonium hydroxide and loaded on a column of 140 g of Grace 950 silica gel packed in the same solvent. The column was eluted with 30:10:1 dichloromethane/methanol/ammonium hydroxide. Appropriate fractions were combined and evaporated under reduced pressure. The resulting residue was dissolved in 1:1 dioxane/water, filtered, and lyophilized to give 1.29 g (64%) of crude $N^{6'}$-(benzyloxycarbonyl)-$N^1$-[(*S*)-γ-[(benzyloxycarbonyl)amino]-α-hydroxybutyryl]nebramine.

$N^{6'}$-(Benzyloxycarbonyl)-$N^1$-[(*S*)-γ-[(benzyloxycarbonyl)amino]-α-hydroxybutyryl]nebramine (1.0 g, 1.6 mmol) was dissolved in a mixture of dioxane (25 mL), water (25 mL), and glacial acetic acid (1 mL). Palladium on charcoal (5%, 280 mg) was added, and the mixture was hydrogenated at 42 psi overnight at room temperature. The catalyst was filtered and washed with hot water, and the filtrate was evaporated under reduced pressure. The residual oil was dissolved in water (20 mL) and loaded on a column of 70 mL of Bio-Rex 70 (ammonium hydroxide cycle, pH 10). The column was eluted with a gradient of 2 L of 0.005 N ammonium hydroxide and 3 L of 0.5 N ammonium hydroxide. Fractions were analyzed by thin-layer chromatography, and the appropriate fractions were combined and lyophilized to yield 283 mg (43%) of $N^1$-[(*S*)-γ-amino-α-hydroxybutyryl]nebramine (**39**).

**Preparation of $N^{2'}$-[(*S*)-γ-Amino-α-hydroxybutyryl]apramycin (43).** Apramycin (1.08 g, 2.0 mmol) and nickel(II) acetate tetrahydrate (1.99 g, 8 mmol) were dissolved in water (20 mL), and this solution was diluted with dimethylformamide (100 mL). The resultant opaque, blue-green mixture was stirred for 1 h at ambient temperature and then treated with *N*-[[(*S*)-γ-[(benzyloxycarbonyl)amino]-α-hydroxybutyryl]oxy]succimide (1.05 g, 3 mmol); a homogeneous green solution formed after about 30 min. After the solution had stirred overnight at ambient temperature, the solvent was evaporated under reduced pressure. The resultant residue was dissolved in a 30:15:2.5 mixture of dichloromethane/methanol/concentrated ammonium hydroxide and was loaded onto a silica gel column (Grace 950, 200 g) preeluted with the same solvent mixture. The column was successively eluted with three different mixtures of dichloromethane/methanol/concentrated ammonium hydroxide (30:15:2.5, 6:6:1, 3:3:1). The fractions containing the desired product were combined, the solvent was evaporated, and the resultant colorless glassy residue was dissolved in water and lyophilized to give $N^{2'}$-[(*S*)-γ-[(benzyloxycarbonyl)amino]-α-hydroxybutyryl]apramycin (0.60 g, 39% yield).

$N^{2'}$-[(*S*)-γ-[(Benzyloxycarbonyl)amino]-α-hydroxybutyryl]-apramycin (550 mg) was dissolved in a solution of water (30 mL), dioxane (10 mL), and glacial acetic acid (1 mL). Palladium on carbon (5%, 135 mg) was added and the mixture was hydrogenated at 45 psi for 24 h at room temperature. The palladium catalyst was removed by filtration and the filtrate was evaporated to dryness. The residual glassy product was dissolved in water and loaded onto a column of Bio-Rex 70 (290 mL, ammonium hydroxide cycle, pH 10.0). This column was eluted with a linear gradient of 0.005 N ammonium hydroxide (1 L) and 0.5 N ammonium hydroxide (1 L) followed by continued elution with 0.5 N ammonium hydroxide. The fractions containing the desired product were combined, concentrated, and lyophilized to yield $N^{2'}$-[(*S*)-γ-amino-α-hydroxybutyryl]apramycin (**43**) (383 mg, 84% yield).

**Preparation of $N^{2'}$-Glycylapramycin (42).** Apramycin (2.16 g, 4 mmol) and nickel(II) acetate tetrahydrate (4.0 g, 15 mmol) were dissolved in water (40 mL), and the resultant solution was diluted with dimethylformamide (200 mL). To this mixture was added *N*-[[*N*-(benzyloxycarbonyl)glycyl]oxy]succinimide (1.84 g, 6 mmol). After the mixture had stirred overnight at ambient temperature, the solvent was evaporated under reduced pressure. The residue was dissolved in 0.1 N hydrochloric acid (250 mL) and a slow stream of gaseous hydrogen sulfide was bubbled into the mixture for 1 h. After the mixture was stirred overnight, the precipitate was filtered and the filtrate was evaporated to dryness under reduced pressure. The resultant residue was suspended in 1 N sodium hydroxide (35 mL) and loaded on a column of 300 mL of Bio-Rex 70 (ammonium hydroxide cycle, pH 10, preeluted with 0.01 N ammonium hydroxide). The column was eluted first with 0.01 N ammonium hydroxide (400 mL) and then with a linear gradient of 0.01 N ammonium hydroxide (2 L) and 0.05 N ammonium hydroxide (2 L). Fractions containing the desired product were located by thin-layer chromatography, combined, and lyophilized to yield $N^{2'}$-[*N*-(benzyloxycarbonyl)glycyl]apramycin (1.35 g, 46% yield).

The above $N^{2'}$-[*N*-(benzyloxycarbonyl)glycyl]apramycin was dissolved in a mixture of ethanol (100 mL), water (10 mL), and 1 N hydroxhloric acid (10 mL). Palladium on charcoal (5%, 3 g) was added and this mixture was hydrogenated at 60 psi at ambient temperature overnight. The palladium catalyst was removed by filtration and the filtrate was placed in the refrigerator overnight. The desired crystalline $N^{2'}$-glycylapramycin (**42**) pentahydrochloride (1.01 g, 78% yield) precipitated from solution and was collected by filtration, washed with ethanol, and air-dried.

**$N^{2'}$-[(*S*)-γ-Amino-α-hydroxybutyryl]nebramine (40).** $N^{6'}$-(Benzyloxycarbonyl)nebramine (880 mg, 2 mmol) and nickel(II) acetate tetrahydrate (1.99 g, 8 mmol) were dissolved in water (20 mL), and this solution was diluted with dimethylformamide (100 mL). To the resultant mixture was added *N*-[[(*S*)-γ-[(benzyloxycarbonyl)amino]-α-hydroxybutyryl]oxy]succinimide (1.05 g, 3 mmol). After the mixture had stirred overnight at room temperature, the solvent was evaporated under reduced pressure, the residue was dissolved in methanol, and the solvent was again evaporated to remove traces of dimethylformamide. The resultant residue was dissolved in a 30:10:1 mixture of dichloromethane/methanol/ammonium hydroxide and was loaded on a silica gel column (Grace 950, 90 g) preeluted with the same solvent mixture. The desired product was eluted from the column with the 30:10:1 mixture of dichloromethane/methanol/ammonium hydroxide as eluent. The fractions containing the desired product

were combined, and the solvent was evaporated. The residue was dissolved in a 1:1 mixture of water-dioxane, filtered, and lyophilized to give $N^{6'}$-(benzyloxycarbonyl)-$N^{2'}$-[(*S*)-γ-[(benzyloxycarbonyl)amino]-α-hydroxybutyryl]nebramine (1.06 g, 79% yield).

$N^{6'}$-(Benzyloxycarbonyl)-$N^{2'}$-[(*S*)-γ-[(benzyloxycarbonyl)-amino]-α-hydroxybutyryl]nebramine (975 mg) was dissolved in a mixture of dioxane (20 mL), water (20 mL), and glacial acetic acid (1 mL). Palladium on charcoal (5%, 270 mg) was added and the mixture was hydrogenated at 40 psi overnight at ambient temperature. The palladium catalyst was removed by filtration and the filtrate was concentrated under reduced pressure and then loaded onto a column of 200 mL of Bio-Rex 70 (ammonium hydroxide cycle, pH 8.6). After first eluting with a gradient of 0.005 N ammonium hydroxide (2 L) and 0.4 N ammonium hydroxide (3 L), the desired product was eluted with 0.5 N ammonium hydroxide. The fractions containing the desired product were combined and lyophilized to yield $N^{2'}$-[(*S*)-γ-amino-α-hydroxybutyryl]nebramine (**40**) (397 mg, 70% yield).

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# Synthesis and Antitumor Activity of Fluorine-Substituted 4-Amino-2(1*H*)-pyridinones and Their Nucleosides. 3-Deazacytosines

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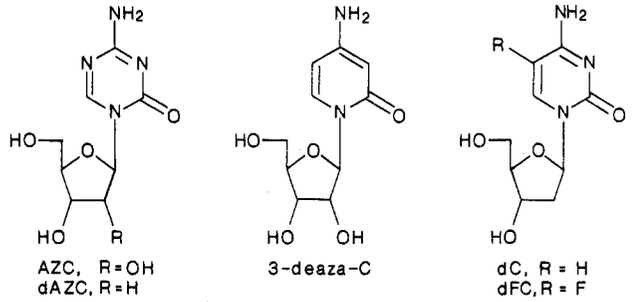
Novel fluorine-substituted deaza analogues of 5-azacytidine (AZC) and 5-aza-2′-deoxycytidine (dAZC) (3-deazacytosines) have been synthesized and tested for antitumor activity. Thus, 4-amino-3,5-difluoro-1-β-D-ribofuranosyl-2(1*H*)-pyridinone (**16**), 4-amino-3-fluoro-1-β-D-ribofuranosyl-2(1*H*)-pyridinone (**17**), 4-amino-5-fluoro-1-β-D-ribofuranosyl-2(1*H*)-pyridinone (**18**), 4-amino-1-(2-deoxy-β-D-*erythro*-pentofuranosyl)-3,5-difluoro-2(1*H*)-pyridinone (**25**), 4-amino-1-(2-deoxy-β-D-*erythro*-pentofuranosyl)-3-fluoro-2(1*H*)-pyridinone (**26**), 4-amino-1-(2-deoxy-α-D-*erythro*-pentofuranosyl)-3,5-difluoro-2(1*H*)-pyridinone (**27**), and 4-amino-1-(2-deoxy-α-D-*erythro*-pentofuranosyl)-3-fluoro-2(1*H*)-pyridinone (**28**) were prepared by standard glycosylation procedures. Requisite heterocycle 4-amino-3,5-difluoro-2(1*H*)-pyridinone (**6**) was prepared in five steps from pentafluoropyridine (**1**). Other requisite fluoro heterocycles, 4-amino-3-fluoro-2(1*H*)-pyridinone (**7**) and 4-amino-5-fluoro-2(1*H*)-pyridinone (**8**), were obtained from a bis-defluorination of 4-amino-3,5,6-trifluoro-2(1*H*)-pyridinone (**3**) with hydrazine. Acetylation of **17** provided 4-amino-3-fluoro-1-(2,3,5-tri-*O*-acetyl-β-D-ribofuranosyl)-2(1*H*)-pyridinone (**29**). Structure proof of target nucleosides and heterocyclic compounds was provided by X-ray diffraction, $^{19}F$ and $^{1}H$ NMR, and UV. The $ID_{50}$ values of fluorine-substituted 3-deazacytosines and 3-deazacytidines were greater than $1 \times 10^{-5}$ M in L1210 lymphoid leukemia cells in culture. Nucleoside **17** and its tri- and tetraacetates were the most active compounds with $ID_{50}$ values of $1.07 \times 10^{-5}$, $1.23 \times 10^{-5}$, and $1.25 \times 10^{-5}$ M, respectively. The target nucleosides and intermediate heterocycles were inactive against P388 and L1210 lymphocytic leukemia in mice, except nucleoside **17** (NSC-378066) and its triacetate **29** (NSC-382021). Nucleoside **17** exhibited confirmed DN2 activity (% T/C 169–230) at five dose levels (25–300 mg/kg). Prodrug **29** exhibited similarly confirmed L1210 in vivo activity.

5-Azacytidine[1] (AZC) and 5-aza-2′-deoxycytidine (dAZC) are toxic to cells in culture[2,3] and are effective antileukemic agents in humans.[4,5] Furthermore, the properties of cytotoxicity and anticancer activity may not be related,[6] and thus they may be noncytotoxic anticancer agents.[7] At low, sublethal concentrations, the major biochemical effect of AZC and dAZC is rapid inhibition of DNA methylation, caused by irreversible inactivation of DNA-cytosine methyltransferase.[8] At these concentrations, AZC and dAZC induce a variety of undifferentiated cells to differentiate, presumably by inhibition of DNA methylation.[8,9] It is noteworthy that the activity of dAZC against L1210 leukemia in mice correlates well with its inhibition of DNA

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(1) Abbreviations used are as follows: AZC, 5-azacytidine; dAZC, 2′-deoxy-5-azacytidine; 3-deaza-C, 3-deazacytidine; dC, 2′-deoxycytidine; *ara*-C, 1-β-D-arabinofuranosylcytosine; dFC, 2′-deoxy-5-fluorocytidine; FC, 5-fluorocytidine; DAU, 3-deazauridine; NCI, National Cancer Institute.

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