Regiospecificity of Aminoglycoside Phosphotransferase from *Enterococci* and *Staphylococci* (APH(3′)-IIIa)†

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ABSTRACT: The broad-spectrum aminoglycoside phosphotransferase, APH(3′)-IIIa, confers resistance to several aminoglycoside antibiotics in opportunistic pathogens of the genera *Staphylococcus* and *Enterococcus*. The profile of the drug resistance phenotype suggested that the enzyme would transfer a phosphate group from ATP to the 3′-hydroxyl of aminoglycosides. In addition, resistance to the 3′-deoxyaminoglycoside antibiotic, lividomycin A, suggested possible transfer to the 5″-hydroxyl of the ribose [Trieu-Cuot, P., & Courvalin, P. (1983) *Gene 23*, 331−341]. Using purified overexpressed enzyme, we have prepared and purified the products of APH(3′)-IIIa-dependent phosphorylation of several of aminoglycoside antibiotics. Mass spectral analysis revealed that 4,6-disubstituted aminocyclitol antibiotics such as amikacin and kanamycin are monophosphorylated, while 4,5-disubstituted aminoglycosides such as butirosin A, ribostamycin, and neomycin B are both mono- and diphosphorylated by APH(3′)-IIIa. Using a series of one- and two-dimensional ^{1}H, ^{13}C, and ^{31}P NMR experiments, we have unambiguously assigned the regiospecificity of phosphoryl transfer to several antibiotics. The 4,6-disubstituted aminocyclitol antibiotics are exclusively phosphorylated at the 3′-OH hydroxyl, and the 4,5-disubstituted aminocyclitol antibiotics can be phosphorylated at both the 3′- and 5″-hydroxyls. The first phosphorylation can occur on either the 3′- or 5″-hydroxyl group of neomycin B or butirosin A. Initial phosphotransfer to the 3′-position predominates for butirosin while the 5″-OH is favored for neomycin. These results open the potential for the rational design of aminoglycoside kinase inhibitors based on functionalization of either the 6-aminohexose or the pentose rings of aminoglycoside antibiotics.

The principle mechanism of bacterial resistance to the aminoglycoside antibiotics occurs by covalent modification catalyzed by a group of resistance enzymes. These include ATP-dependent *O*-phosphotransferases (APH)[1] or *O*-adenyltransferases and acetyl CoA-dependent *N*-acetyltransferases (Davies, 1991; Umezawa & Kondo, 1982). Gram positive microorganisms of the genera *Staphylococcus* and *Enterococcus* are opportunistic pathogens that pose a significant health threat, especially in hospital settings where they are second only to *Escherichia coli* in their frequency of infection (Moellering, 1991). These bacteria have been shown to express members of all three classes of aminoglycoside-inactivating enzymes (Leclerq et al., 1992), including APH(3′)-IIIa, which has the broadest substrate range of all known 3′-phosphotransferases (Shaw et al., 1993).

Most aminoglycoside antibiotics incorporate a central six-membered aminocyclitol ring. This ring can be linked to aminosugars through C-4 and C-6, e.g., amikacin, or C-4 and C-5, e.g., butirosin (Figure 1). APH(3′)-IIIa confers resistance to a number of 3′-hydroxyl-bearing aminoglycoside antibiotics,[2] which identifies it as an APH(3′); it also confers resistance to, and will phosphorylate (McKay et al., 1994), the 3′-deoxyaminoglycoside lividomycin A. This observation has been taken as evidence for phosphate transfer to the 5″-OH (Trieu-Cuot & Courvalin, 1983). Early ^{1}H NMR decoupling experiments at an unknown field strength with lividomycin A phosphorylated by crude enzyme fractions of drug resistant *E. coli* and *Pseudomonas aeruginosa*, identified the 5″-OH as the site of phosphorylation by APH(3′)-I (Kondo et al., 1972). Chemical synthesis of 5″-phospholividomycin A and co-migration of the methanolysis products of enzymatically inactivated drug upon high-voltage paper electrophoresis also corroborated assignment of the site of phosphorylation to the primary hydroxyl of the lividomycin ribose ring (Yamamoto et al., 1972). However, the complete NMR assignment of either lividomycin or lividomycin phosphate has not been reported.

We have overexpressed and purified APH(3′)-IIIa and identified the site of phosphorylation of kanamycin A to be the 3′-OH by a combination of mass spectral and ^{1}H, ^{13}C, and ^{31}P NMR analyses (McKay et al., 1994). However, we have not assigned the site of phosphorylation of any other aminoglycoside. We anticipated that lividomycin A would be phosphorylated at position 5″ on the basis of the above literature precedent, but we had no evidence that this would

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[1] Abbreviations: APH, aminoglycoside phosphotransferase; ESI-MS, electrospray ionization mass spectrometry; FID, free induction decay; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid.

[2] Literature precedent has the 6-aminohexose ring linked to C-6 of the aminocyclitol labeled as the prime (′) ring and the 3-aminohexose or the pentose rings, respectively, linked to C-6 or C-5 of the aminocyclitol labeled as the double-prime (″) ring. To be consistent, we have also adopted this nomenclature as well and designated the rings by letters as indicated in Figure 1.

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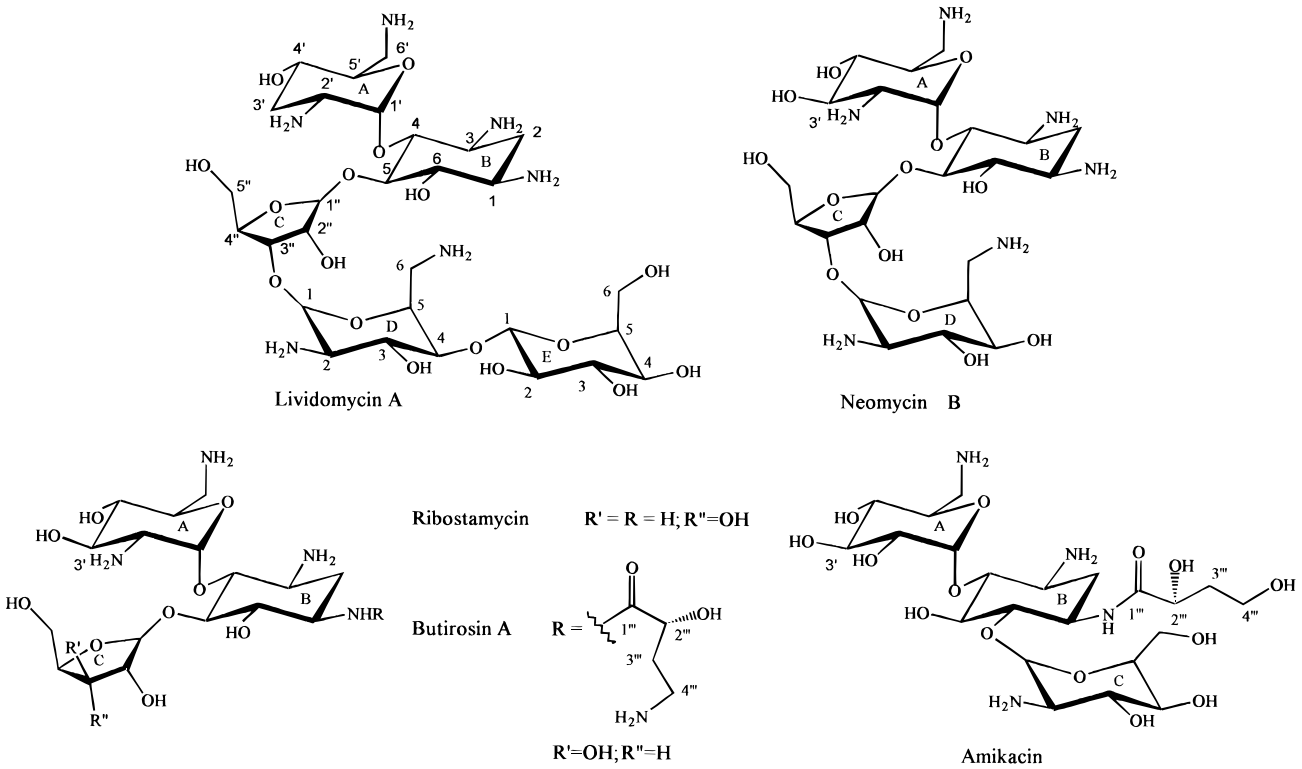

FIGURE 1: Structures of aminoglycoside antibiotics.

be the case given the availability of eight other primary and secondary hydroxyls which could also potentially be sites of phosphorylation (Figure 1). In addition, if we accepted the view that phosphorylation should occur at either the 3′- or the 5″-OH, then what would occur when the enzyme was presented with compounds such as neomycin or butirosin, which possess hydroxyls at both these positions? We have therefore explored the stoichiometry and regiospecificity of phosphoryl transfer by APH(3′)-IIIa with a number of aminoglycosides. We present definitive evidence that the sites of phosphorylation are the 3′- and 5″-hydroxyls and show that compounds that incorporate both of these groups can be diphosphorylated.

MATERIALS AND METHODS

Chemicals. Neomycin B, lividomycin A, butirosin A, amikacin, and ribostamycin were obtained from Sigma (St. Louis, MO). ATP was from Boehringer-Mannheim (Laval, Quebec) and sodium hydroxide (50% w/w) was from Fisher (Unionville, Ontario). D_2O (99.9%) was obtained from Isotec (Miamisburg, OH). APH(3′)-IIIa was purified from the overexpressing construct pETSACG1 in *E. coli* BL21 (DE3) as previously detailed (McKay et al., 1994).

Preparation and Purification of Phosphorylated Aminoglycosides. Phosphorylated aminoglycosides were prepared using purified APH(3′)-IIIa as previously described (McKay et al., 1994), except that the phosphorylations were carried out in 50 mM HEPES, pH 7.5, 10 mM $MgCl_2$ and in the presence of at least a 3-fold molar excess of ATP. The reaction was initiated by the addition of 1 mg of APH-(3′)-IIIa and was typically allowed to proceed for 45−48 h at 37 °C with shaking.

The phosphorylated derivatives were purified by batchwise application of the reaction mixture to a 50 mL slurry of AG 50W-X8 resin (Bio-Rad) (NH_4^+ form) and allowed to equilibrate for 1 h with slow stirring. The resin was then applied to a scintered glass funnel and washed with 1 L of H_2O, and phosphorylated aminoglycosides were eluted with 500 mL of 1% ammoniuim hydroxide. Fractions (50 mL) were collected, analyzed by silica gel TLC (MeOH:NH_4OH 5:2) and visualized with ninhydrin. Those fractions containing phosphorylated aminoglycoside were pooled and lyophilized.

The lyophilized product was taken up in 1 mL of H_2O and applied to a Sephadex G-10 (Pharmacia) column (2 × 81 cm), and the phosphorylated aminoglycosides were desalted at a flow rate of 1.5 mL/min. Fractions were tested for the presence of derivatized aminoglycosides by TLC analysis, and the peak fractions were pooled and applied directly to a Mono S column (HR5/5) (Pharmacia) equilibrated with H_2O. The column was treated with stepwise linear gradients of 0−300 mM ammonium hydroxide at 4 °C. The elution profiles were monitored at 210 nm, and peak fractions that were confirmed to contain phosphorylated aminoglycosides (by TLC) were pooled and lyophilized.

Further purification of butirosin A diphosphate and neomycin B diphosphate for NMR analysis was achieved by isocratic chromatography on a CSC-Spherisorb-ODS2 C-18 reverse phase column (5 μm 25 × 0.46 cm) using 8% CH_3-CN with 0.016 M heptafluorobutyric acid as mobile phase (McLaughlin & Menion, 1992). Elution of aminoglycosides was carried out at 0.5 mL/min, and compounds were detected at 220 nm. Peaks were tested for aminoglycoside presence by TLC, and positive fractions were pooled and lyophilized.

Quantitative HPLC Analysis of Aminoglycosides. Aminoglycosides were applied to a Dionex CarboPac PA1 carbohydrate analysis column (4 × 250 mm) equilibrated with 0.5 mM NaOH. After injection, the column was washed with 0.5 mM NaOH for 5 min. A linear gradient from 0.5

to 15 mM NaOH over 10 min was used to elute the non-phosphorylated aminoglycosides, and this was followed by a rapid increase to 70 mM NaOH and a linear gradient from 70 to 100 mM NaOH over 4 min to elute the HEPES buffer. An additional linear gradient from 100 to 2000 mM NaOH over 15 min resulted in the elution of phosphoryalted aminoglycosides, ATP and ADP. A post-column addition of 500 mM NaOH was used for analysis with a Dionex ED40 pulsed amperometric detector. Data were processed using the Dionex PeakNet software package, and chromatograms were subtracted from a water-only injection base line. The detector settings were essentially as previously described (Statler, 1990). The potential was set at 0.10 V for 1.50 ms whereupon integration began and continued for a further 1.50 ms. At this point, the voltage was increased to 0.60 V for 120 ms after which the voltage was stepped up to −0.80 V for 300 ms before returning to 0.10 V.

Mass Spectrometry of Phosphorylated Aminoglycosides. The mass to charge ratio of purified phosphorylated aminoglycosides were determined using electrospray ionization mass spectrometry (ESI-MS) in both positive and negative ion modes at the McMaster Regional Centre for Mass Spectrometry. The spectra were acquired with a Fison platform quadrupole spectrometer with the aminoglycosides typically being dissolved in a solution of 50:50 acetonitrile: water with either trifluoroacetic acid or ammonium hydroxide as the organic modifier to enhance ion formation.

Acquisition of NMR Spectra. Unless otherwise stated all NMR spectra were recorded on a Bruker Avance DRX-500 spectrometer. Proton spectra were recorded at 500.13 MHz using a 5 mm broadband inverse probe with triple-axis gradient capability. Spectra were obtained in 32−96 scans over spectral widths ranging from 3.600 to 4.000 kHz in 32k data points (4.551−4.096 s acquisition time). Solvent suppression was achieved by presaturation of the HDO signal during a 1.0 s relaxation delay. The temperature of the sample was maintained at 30 °C by a Bruker Eurotherm variable temperature unit. Free induction decays (FIDs) were processed using Gaussian multiplication (line broadening, −1.5 Hz; Gaussian broadening, 0.2) and were zero-filled to 64k before Fourier transformation.

^{13}C NMR spectra were recorded at 125.772 MHz over a 30.102 kHz spectral width in 32k data points (0.544 s acquisition time). Single-pulse spectra were obtained using a 5.0 μs pulse width (35° flip angle) and a 0.5 s relaxation delay. Edited spectra were acquired with the standard *J*-modulated spin sort or DEPT pulse sequences. The ^{13}C 90° pulse width was 12.7 μs, while the ^{1}H 90° pulse width through the decoupler channel was 6.4 μs. A 1.0 s relaxation delay was used. The delay time in the DEPT experiments was set to 0.003 571 s. The FIDs were processed using exponential multiplication (line broadening, 4.0 Hz) and were zero-filled to 64k before Fourier transformation.

^{31}P NMR spectra were obtained at 202.459 MHz over a 29.412 kHz spectral width in 32k data points (0.279 s acquisition time). Single-pulse spectra were recorded using a 4.5 μs pulse width (45° flip angle) and a 0.5 s relaxation delay. The FIDs were processed using exponential multiplication (line broadening, 4.0 MHz) and were zero-filled to 64k before Fourier transformation.

Proton COSY 2-D NMR spectra were in the absolute value mode using the pulse sequence 90° − t_1 − 45° − ACQ. Spectra were acquired in 8−32 scans for each of the 256 FIDs that contained 2k data points over the previously mentioned spectral widths. The ^{1}H 90° pulse width was 6.6 μs. During the 1.0 s relaxation delay the residual HDO peak was suppressed by presaturation. Zero-filling in the *F*1 dimension produced a 1k × 1k data matrix with a digital resolution in the range 3.20−4.00 Hz/point in both dimensions. During 2-D Fourier transformation a sine-bell squared window function was applied in both dimensions. The transformed data were then symmetrized.

Inverse-detected ^{1}H−^{13}C chemical shift correlation spectra were acquired in the phase-sensitive mode using the HSQC pulse sequence (Bodenhausen & Rubin, 1980). The FIDs in the *F*2 (^{1}H) dimension were obtained over the previously mentioned spectral widths, while in the *F*1 dimension the ^{13}C spectral widths ranged from 12.600 to 14.000 kHz. Each FID was acquired in 16−32 scans. The fixed delays during the pulse sequence were a 1.0 s relaxation and a 0.001 786 s delay for polarization transfer. The 90° ^{1}H pulse width was 6.6 μs, while the 90° ^{13}C pulse width in inverse mode was 11.6 μs. The data were processed using a sine-bell squared window function shifted by π/2 in both dimensions.

HMQC-TOCSY ^{1}H−^{13}C chemical shift correlation spectra were acquired in the phase-sensitive mode using the standard pulse sequence (Lerner & Bax, 1986). The FIDs in the *F*2 (^{1}H) dimension were obtained over the previously mentioned spectral widths, while in the *F*1 dimension the ^{13}C spectral widths ranged from 12.600 to 14.000 kHz. Each FID was acquired in 64 scans. The fixed delays during the pulse sequence were a 1.0 s relaxation, a 0.3 s delay between the BIRD pulse and the inverse detection pulse sequence, a 0.003 571 s delay for the BIRD pulse and polarization transfer, and a 120 ms spin lock time. The 90° ^{1}H pulse width was 6.6 μs, while the 90° ^{13}C pulse width in inverse mode was 11.6 μs. The TOCSY part of the pulse sequence used a 27.0 μs 90° spin locking pulse width along with 2500 μs trim pulses on either side of the spin lock pulses. The data were processed using a sine-bell squared window function shifted by π/2 in both dimensions.

Selective 1-D TOCSY ^{1}H NMR spectra were recorded over a 5.530 kHz spectral width in 32k data points (2.949 s acquisition time). Gaussian-shaped pulses were defined by 256 data points with the pulse being truncated at 1% of the maximum pulse amplitude. The 90° Gaussian pulse width was 43.0 μs. This pulse was followed by a 40 μs fixed delay and then by the standard TOCSY-MLEV-17 spin lock. A 1.0 s relaxation delay was used. The 90° ^{1}H spin lock pulse width was 27.0 μs. The 120 ms spin lock period was followed by a *z*-filter (Macura et al., 1981, Sorensen et al., 1984) which contained ten variable delays ranging from 4 to 18 ms. The transmitter offset was adjusted to the frequency of the ^{1}H being selectively excited. Thirty-two scans were acquired for each of the delays in the *z*-filter for a total of 320 scans. The FIDs were processed using Gaussian multiplication (line broadening, −1.5 Hz; Gaussian broadening, 0.2) and were zero-filled to 64k before Fourier transformation.

The ^{1}H−^{31}P 2-D chemical shift correlation experiments were performed on a Bruker AM-500 NMR spectrometer equipped with a 5 mm broadband inverse probe. The pulse sequence used was the heteronuclear equivalent of the COSY pulse sequence with the phase-sensitive modification (Bain, 1988). The 90° ^{31}P pulse width was 24.0 μs, and the ^{1}H 90° pulse through the decoupler channel was 6.4 μs. Spectra

were acquired in 32 scans for each of the 1024 FIDs, which contained 512 data points in *F*2. The *F*2 spectral width was 500.0 Hz. In *F*1, with the transmitter offset position at the high-frequency end of the ^{1}H spectrum, the total spectral width was 2.616 kHz. A relaxation delay of 1.0 s was used. The data were zero-filled to 4k in *F*1 and apodized with expotential multiplication in both dimensions (line broadening, 1.0 Hz). The 2-D Fourier transformation and phase correction were performed using a real Fourier transformation as would be used for processing phase-sensitive 2-D spectra acquired with time-proportional phase incrementation (Marion & Wüthrich, 1983).

Spectra for the butirosin diphosphate and neomycin monophosphate samples were acquired using a 2.5 mm triple-resonance microprobe with single-axis gradient capability. Experimental conditions were similar to those stated above with the following changes in pulse widths: ^{1}H 90° pulse width, 3.5 μs; ^{1}H 90° spin lock pulse width, 26.9 μs; and ^{13}C 90° pulse width, 8.0 μs.

The compounds used in this study were dissolved in "100%" D_2O to an average concentration of approximately 7 mg/mL. The pD of the phosphorylated derivatives was between 8 and 9, while the pD of the sulfate salts of the unreacted drugs was between 6 and 7.5. The microprobe samples contained approximately 1 mg of compound dissolved in 150 μL of D_2O. Proton chemical shifts are reported in ppm relative external tetramethylsilane using the residual HDO assigned to 4.60 ppm as internal reference. ^{13}C chemical shifts are reported relative to external dioxane in D_2O at 66.6 ppm.

Enzyme Assay. The enzymatic activity of APH(3′)-IIIa was monitored by coupling ADP release upon aminoglycoside phosphorylation to NADH oxidation through pyruvate kinase and lactate dehydrogenase in the presence of excess phosphoenol pyruvate as previously described (McKay et al., 1994).

RESULTS

Analysis of Progress Curves. Examination of the progress of aminoglycoside phosphorylation catalyzed by APH(3′)-IIIa using the lactate dehydrogenase/pyruvate kinase coupled assay system at saturating ATP revealed some intriguing patterns. The 4,6-disubstituted deoxystreptamine aminoglycosides such as kanamycin and amikacin (not shown) as well as the 3-deoxy-4,5-disubstituted drug lividomycin A displayed a linear decrease in absorbance at 340 nm followed by a horizonal plateau after all of the drug was titrated by the enzyme (Figure 2). The plateau for these drugs occurred at concentrations of NADH which were equimolar with the added aminoglycoside. Surprisingly, other 4,5-disubstituted aminoglycosides including neomycin B and ribostamycin displayed nonlinear progress curves with distinct inflection points at or around the expected point where we would predict that the substrate would be titrated to completion (Figure 2). Butirosin A did not give biphasic kinetics over the observed time period (60 min). These results suggested that multiple phosphorylation of some 4,5-disubstituted aminoglycosides was occurring. On the basis of these results we expected that monophosphoryl aminoglycoside dissociated from the enzyme before further modification occurred. We predicted that if the monophosphoryl derivative did not dissociate and was tightly bound, we would first see a burst

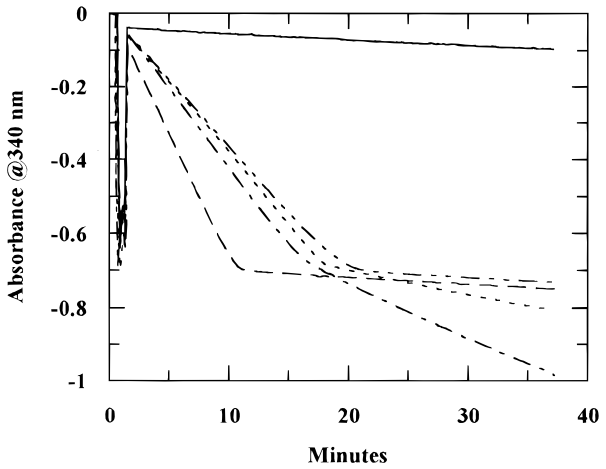

FIGURE 2: APH(3′)-IIIa-dependent ADP release progress curves. ADP formation was monitored continuously by coupling to pyruvate kinase/actate dehydrogenase as described in Materials and Methods. Curves are shown for no added aminoglycoside (—), lividomycin A (- - -), butirosin A (-···-), ribostamycin (···), and neomycin B (-·-). Aminoglycosides were added to 100 μM.

Table 1: ESI-MS of Aminoglycosides Phosphorylated by APH(3′)-IIIa

antibiotic	*m*/*z* (Da/e)	stoichiometry of phosphorylation
4,6-disubstituted aminoglycosides		
kanamycin A[a]	565.3	mono
amikacin	666.3	mono
4,5-disubstituted aminoglycosides		
lividomycin A	842.4	mono
neomycin B	695.2	mono
	774.5	di
butirosin A	636.1	mono
	714.1	di
ribostamycin	535.3	mono
	615.2	di

[a] From McKay et al. (1994).

equivalent to the enzyme concentration followed by a progress curve with little or no inflection point. Alternatively, if only some of the monophosphoryl derivative did not dissociate, we would predict that the inflection point would not occur at 1 equiv of aminoglycoside and may not in fact be readily apparent. However, since the inflection point occurred after titration of 1 equiv of drug, we expected that monophosphoryl aminoglycoside was dissociating from the enzyme as predicted by the kinetic mechanism (McKay & Wright, 1995), and this was further supported by HPLC evidence (vide infra).

Stoichiometry of Phosphoryl Transfer by ESI-MS. Aminoglycosides phosphorylated by APH(3′)-IIIa were purified by a combination of low- and high-resolution ion exchange techniques and analyzed by ESI-MS in both the positive and negative ion modes. Compounds with mass to charge ratios consistent with monophosphorylation were found for all aminoglycosides tested (Table 1). In addition, incubation of 4,5-disubstituted aminoglycosides which possessed both 3′- and 5″-hydroxyl groups (neomycin B, butirosin A, and ribostamycin) with APH(3′)-IIIa also produced compounds with mass to charge ratios consistent with diphosphorylation. These compounds were produced in varying amounts depending on the drug and could readily be purified from the incubation mixtures, although with neomycin B, the

Table 2: ^{1}H Chemical Shifts (ppm) of Lividomycin and Lividomycin Phosphate in D_2O

ring	proton	lividomycin	lividomycin phosphate
A	1	5.448	5.216
	2	3.532	3.500
	3a[a]	1.849	1.717
	3e[b]	2.150	2.149
	4	3.613	3.604
	5	3.694	3.657
	6	3.750	3.728
	6′	3.615	3.602
B	1	3.208	2.792
	2a[a]	1.708	1.202
	2e[b]	2.316	1.948
	3	3.329	2.852
	4	3.836	3.397
	5	3.737	3.600
	6	3.604	3.311
C	1	5.224	5.224
	2	4.296	4.077
	3	4.444	4.391
	4	4.062	4.098
	5	3.761	3.847
	5′	3.630	3.810
D	1	5.172	4.894
	2	3.484	2.930
	3	4.304	3.611
	4	3.745	4.089
	5	4.319	4.167
	6	3.351	3.251
	6′	3.193	3.069
E	1	4.965	4.938
	2	3.876	3.828
	3	3.680	3.683
	4	3.547	3.518
	5	3.398	3.417
	6	3.766	3.764
	6′	3.615	3.614

[a] Axial protons. [b] Equatorial protons.

reaction had to be quenched after 1.5 h to isolate the monophosphoryl derivative.

Determination of the Regiospecificity of Phosphorylation by NMR. We have assigned the ^{1}H and ^{13}C spectra for both phosphorylated and unphosphorylated aminoglycosides. These assignments permit unambiguous determination of the sites of phosphorylation to be at the 3′-OH for amikacin, neomycin B, and butirosin A and at the 5″-OH for lividomycin A, neomycin B, and butirosin A.

The site of phosphorylation of this series of aminoglycosides was established by the application of a variety of one- and two-dimensional NMR techniques. These methods were used to initially determine the complete assignment of the ^{1}H and ^{13}C NMR spectra of the non-phosphorylated compounds. Spectra of the phosphorylated aminoglycosides were then assigned by comparison with the data on the non-phosphorylated carbohydrates, and the position of the phosphate group was confirmed by NMR techniques such as selective ^{31}P spin decoupling or the application of the heteronuclear equivalent of the COSY experiment (Bain, 1988; Maudsley & Ernst, 1977). This pulse sequence produces a correlation between the phosphorus and the neighboring protons with which it is spin coupled. Additional evidence for locating the phosphate group was derived from the observation of ^{1}H−^{13}P and ^{31}P−^{13}C spin coupling.

The procedures used to analyze the NMR spectra of the aminoglycosides can be illustrated by the results for lividomycin and its phosphate analog. The assignment of the ^{1}H NMR spectra began by identifying the various methylene proton signals by using the standard inversion−recovery pulse sequence. The shorter relaxation times of the methylene protons resulted in a partial resolution of the A, D, and E ring C-6 protons, the C ring C-5 protons, and the A and B ring C-3 and C-2 methylene protons, respectively. The COSY-45 pulse sequence was then used to determine the connectivity of the remaining protons. The anomeric protons were readily identified as the doublets between 4.8 and 5.5 ppm, and their COSY correlations with neighboring C-2 protons were observed. For example, the anomeric doublet at 5.448 ppm was correlated with the C-2 proton multiplet at 3.532 ppm. This multiplet was in turn connected with the methylene protons at 1.849 and 2.150 ppm. These correlations thus identified the anomeric C-2 and C-3 protons of ring A (Table 2). The COSY spectrum permitted the partial assignment of the other carbohydrate rings but the overlap of signals between 3.0 and 4.0 ppm made this analysis difficult.

The ^{13}C NMR spectrum of lividomycin was then examined using editing techniques such as DEPT. The anomeric carbons were also deshielded relative to the other carbohydrate carbons. In particular the anomeric carbon of ring C followed the tendency for a higher frequency chemical shift (110.2 ppm) for the ribofuranose structure (Kalinowski et al., 1988). The methylene carbons with oxygen substituents were located near 60 ppm, while the methylene carbon corresponding to the E ring C-6 was assigned to 40.5 ppm. The assignment of the remaining carbons and protons relied on the application of ^{1}H−^{13}C chemical shift correlation techniques such as the HSQC (Bodenhausen & Rubin, 1980) and HMQC-TOCSY (Figure 3) (Lerner & Bax, 1986) pulse sequences. The HSQC 2-D spectrum provided the one-bond ^{1}H−^{13}C correlations. For example, the anomeric carbon for ring C observed at 110.2 ppm was connected to the proton at 5.224 ppm. The connectivities observed for the lowest frequency methylene carbons at 29.0 and 29.8 ppm allowed their assignment to C-2 of ring B and C-3 of ring A, respectively.

One of the more informative techniques was the hybrid pulse sequence HMQC-TOCSY (Figure 3). This method provides a one-bond correlation between a carbon and its directly bonded proton while the TOCSY part of the pulse sequence transfers this magnetization to the rest of the proton spin system. A carbon will then display correlations to all the other protons of that particular spin system and therefore allow the resolution of all the protons of a specific carbohydrate ring. For example, the methine carbon at 81.0 ppm corresponding to C-4 of ring C was connected not only to the directly bonded proton at 4.062 ppm but also showed TOCSY correlations to all of the other ring C protons at 5.225 (H-1), 4.296 (H-2), 4.444 (H-3), 3.761 (H-5), and 3.630 (H-5′) ppm. A similar analysis could be performed for the other carbohydrate ring systems and the data are summarized in Tables 2−4.

The analysis of the NMR spectra of the phosphorylated lividomycin followed similar procedures. However, in order to resolve some severely overlapped proton multiplets near 3.65 ppm, the 1-D equivalent of the TOCSY pulse sequence incorporating selective excitation was used (Kessler et al.,

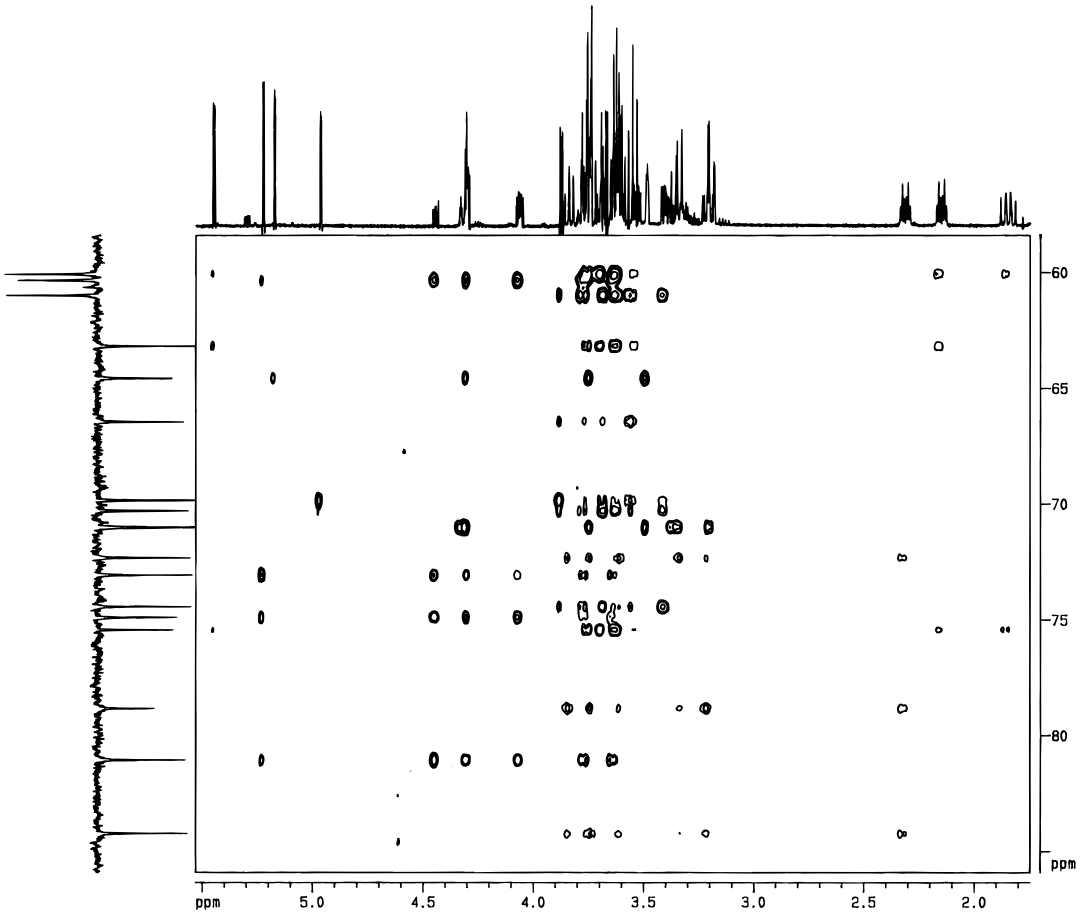

FIGURE 3: Portion of the HMQC-TOCSY of lividomycin A. The spectrum was acquired at 30 °C on a Bruker Avance DRX-500 spectrometer as described in Materials and Methods.

1986, 1991). This pulse sequence used a Gaussian-shaped pulse to selectively excite a single proton, and this was followed by a short fixed delay and the rest of the standard TOCSY pulse sequence. The data were then acquired as a 1-D spectrum. Where this technique proved to be useful was in the resolution of the E ring protons, in particular H-3. Proton 3 near 3.6 ppm was completely hidden by overlap with a number of other multiplets. Selective excitation of E H-2 at 3.828 ppm with the 1-D TOCSY pulse produced a completely resolved spectrum of the ring E protons. The E H-3 was located at 3.683 ppm and appeared as a doublet of doublets with $^3J_{2,3} = 3.3$ and $^3J_{3,4} = 9.6$ Hz. This technique facilitated the resolution of the other ^{1}H spin systems in this aminoglycoside.

The location of the phosphate was initially confirmed by examining the ^{13}C spectrum for coupling to ^{31}P (Tables 4 and 5). This coupling was initially observed on the methine carbon at 81.1 ppm ($^3J_{C,P} = 8.1$ Hz) and the methylene carbon at 62.5 ppm ($^2J_{C,P} = 4.5$ Hz). These were assigned to C-4 and C-5, respectively, of ring C. The couplings observed on these carbon signals were consistent with two- and three-bond ^{13}C−^{31}P couplings (Kalinowski et al., 1988). Additional evidence for the location of the phosphate at C-5 of ring C was the ^{1}H−^{31}P correlation observed by using the heteronuclear equivalent of the COSY pulse sequence. The method had been modified for phase-sensitive detection (Bain, 1988). This pulse sequence will detect all one-bond and long-range heteronuclear coupling interactions and does not require any prior knowledge of these coupling constants since there are no delay times that are determined by the magnitude of these couplings. The method, however, has very low sensitivity. The correlation observed between the phosphorous and the methylene protons at 3.847 and 3.810 ppm confirms phosphorylation occurring at the 5″-position of ring C. The assignment of these methylene protons was confirmed by the previously mentioned techniques. Similar methods were used to assign amikacin and amikacin phosphate (Table 6; complete assignment tables can be found in the supporting information).

The positions of the phosphate groups in the diphosphorylated aminoglycosides neomycin (Tables 7) and butirosin (Tables 8) were determined by the observation of ^{31}P couplings in the ^{1}H and ^{13}C NMR spectra. However, the differentiation of the two signals in the ^{31}P spectra was achieved by recording a ^{1}H NMR spectrum with selective decoupling at each phosphorous resonance. For example, in the case of neomycin irradiation of the phosphorous signal at 2.176 ppm removed the ^{31}P coupling on the ^{1}H multiplet at 4.364 ppm which had been assigned to the A ring H-3. A similar experiment confirmed the assignment of the 0.821 ppm ^{31}P signal to the phosphate group on position 5 of ring C.

HPLC Analysis of the Phosphorylation of Neomycin B. The progress of the phosphorylation of neomycin B was monitored by anion exchange HPLC with a pulsed amperometric detector to visualize the UV-transparent aminoglycosides. The disappearance of neomycin B (retention time, 10 min) was followed by the rise in a peak corresponding to monophosphoryl neomycin (25 min), and this compound was then converted to the diphospho derivative (28 min)

Table 3: $^{1}H-^{1}H$ Coupling Constants (Hz) of Lividomycin and Lividomycin Phosphate in D_2O

	ring proton		lividomycin	lividomycin phosphate
$^{3}J_{H,H}$	A	1,2	3.5	3.8
		2,3a[a]	11.2	11.4
		2,3e[b]	4.5	4.4
		4,3a	9.7	10.3
		4,3e	4.4	4.4
		4,5	7.0	9.5
		5,6	2.9	2.0
		5,6′	7.8	5.6
	B	1,2a	12.6	12.3
		1,2e	4.3	4.3
		1,6	10.5	9.7
		3,2a	12.6	12.3
		3,2e	4.3	4.3
		3,4	9.8	9.3
		4,5	9.2	9.3
		5,6	9.2	9.7
	C	1,2	2.4	4.1
		2,3	4.8	5.7
		3,4	6.8	5.7
		4,5	3.0	2.7
		4,5′	4.4	4.0
	D	1,2	1.9	1.9
		2,3	2.9	2.9
		3,4	2.9	3.2
		4,5	2.4	2.4
		5,6	10.4	10.1
		5,6′	2.2	2.6
	E	1,2	1.9	1.8
		2,3	3.3	3.3
		3,4	9.5	9.6
		4,5	6.7	9.8
		5,6	2.2	2.2
		5,6′	5.5	6.5
$^{2}J_{H,H}$	A	3a,3e	−12.7	−11.4
		6,6′	−11.2	−12.1
	B	2a,2e	−12.6	−12.7
	C	5,5′	−12.2	−11.9
	D	6,6′	−13.2	−13.3
	E	6,6′	−12.1	−12.3
$^{4}J_{H,H}$	D	2,4		1.1

[a] Axial protons. [b] Equatorial protons.

Table 4: ^{13}C Chemical Shifts (ppm) of Lividomycin and Lividomcin Phosphate in D_2O

ring carbon		lividomycin	lividomycin phosphate
A	1	94.4	95.9
	2	48.0	48.4
	3	29.8	30.7
	4	63.2	63.4
	5	75.4	73.9
	6	60.1	60.5
B	1	50.0	50.3
	2	29.0	34.1
	3	49.1	49.9
	4	78.8	84.3
	5	84.2	84.7
	6	72.3	76.0
C	1	110.2	109.6
	2	73.1	73.6
	3	74.9	75.5
	4	81.0	81.1
	5	60.3	62.5
D	1	95.4	99.3
	2	50.9	52.2
	3	64.6	66.8
	4	71.0	72.3
	5	71.0	71.7
	6	40.5	40.5
E	1	98.6	98.3
	2	69.8	70.0
	3	70.3	70.3
	4	66.4	66.6
	5	74.4	74.2
	6	61.0	61.1

Table 5: ^{31}P Chemical Shifts and $^{31}P-^{1}H$ and $^{31}P-^{13}C$ Coupling Constants in Lividomycin Phosphate in D_2O

	ring phosphorus		chemical shift (ppm)	coupling constant (Hz)
	C		4.468	
$^{3}J_{P,H}$	C	P,5		4.4
		P,5′		5.9
$^{2}J_{P,C}$	C	P,C-5		4.5
$^{3}J_{P,C}$	C	P,C-4		8.1

Table 6: ^{31}P Chemical Shifts and $^{31}P-^{1}H$ and $^{31}P-^{13}C$ Coupling Constants in Amikacin Phosphate in D_2O[a]

	ring phosphorus		chemical shift (ppm)	coupling constant (Hz)
	A		4.929	
$^{3}J_{H,P}$	A	H-3,P		7.0
$^{2}J_{C,P}$	A	C-3,P		4.8
$^{3}J_{C,P}$	A	C-2,P		3.2

[a] Complete assignments of amikacin and amikacin phosphate can be found in the supporting information.

(Figure 4A). Integration of the peaks demonstrates the intermediacy of neomycin monophosphate (Figure 4B). These results clearly show that the second phosphorylation event occurs *after* the monophosphoryl derivative has dissociated from the enzyme, consistent with our determination of an ordered kinetic mechanism for APH(3′)-IIIa in which ATP binds first followed by the aminoglycoside (McKay & Wright, 1995).

Processivity of Phosphorylation. ^{31}P NMR of the monophosphoryl derivatives of neomycin B and butirosin A revealed two peaks corresponding to 3′- and 5″-phosphate compounds. Careful integration of these resonances revealed that there was not an equal chance of phosphorylation at either site but that there was a distinct preference for phosphorylation at the 3′-OH (70%) for butirosin A and at the 5″-OH (77%) for neomycin B. We could not achieve separation of these monophosphoryl compounds by cation exchange or reverse phase HPLC.

DISCUSSION

The phosphorylation of aminoglycosides by specific kinases is an important mechanism which some bacteria use to overcome the toxic properties of these drugs. The modified aminoglycosides no longer have lethal affinity for the target ribosome, and thus the organism can proliferate in solutions containing these antibacterial drugs. The prevalence of genes encoding these antibiotic-inactivating enzymes in pathogens found in hospital settings is a major concern. The widespread dissemination of these genes in *Enterococci* and *Staphylococci*, two classes of organisms which are frequently pathogenic in the clinics, and for which aminoglycosides are first-line antibiotics, is particularly troublesome.

Table 7: ^{31}P Chemical Shifts and ^{1}H−^{31}P and ^{13}C−^{31}P Coupling Constants of Neomycin Diphosphate in D_2O[a]

	ring phosphorus		chemical shift (ppm)	coupling constant (Hz)
	A	3	2.176	
	C	5	0.821	
$^{3}J_{H,P}$	A	H-3,P		8.5
	C	H-5,P		3.7
		H-5′,P		7.1
$^{2}J_{C,P}$	A	C-3,P		*b*
	C	C-5,P		3.5
$^{3}J_{C,P}$	A	C-2,P		*b*
		C-4,P		*b*
	C	C-4,P		8.0

[a] Complete assignment of neomycin diphosphate can be found in the supporting information. [b] Couplings not resolved.

Table 8: ^{31}P Chemical Shifts and ^{31}P−^{1}H and ^{31}P−^{13}C Coupling Constants of Butirosin A (Diphosphate$_4$)$_2$ in D_2O[a]

	ring phosphorus		chemical shift (ppm)	coupling constant (Hz)
	A	3	4.743	
	C	5	4.505	
$^{3}J_{H,P}$	A	H-3,P		8.1[b]
	C	H-5,P		6.6
		H-5′,P		6.2
$^{2}J_{C,P}$	A	C-3,P		4.5
	C	C-5,P		5.2
$^{3}J_{C,P}$	A	C-2,P		*c*
		C-4,P		*c*
	C	C-4,P		7.5

[a] Complete assignment of butirosin and butirosin diphosphate can be found in the supporting information. [b] Estimated coupling constant owing to broad linewidth. [c] Couplings not resolved.

APH(3′)-IIIa is a 31 kDa enzyme, widely distributed in *Enterococci* and *Staphylococci*, which phosphorylates a broad spectrum of aminoglycoside antibiotics (Leclerq et al., 1992). We have previously shown that APH(3′)-IIIa exclusively phosphorylates kanamycin A at the 3′-OH of the 6-amino-glucose ring (McKay et al., 1994). This was definitive evidence that the enzyme was highly regiospecific. Surprisingly, lividomycin A, a *3′-deoxyaminoglycoside*, was also phosphorylated efficiently [K_m 31 μM, k_{cat} 3.9 s^{-1} (McKay et al., 1994)]. Resistance to lividomycin had been interpreted as evidence for phosphorylation at the 5″-hydroxyl (Trieu-Cuot & Courvalin, 1983) based on early work with an APH(3′) containing crude extract from *Pseudomonas aeruginosa* (Kondo et al., 1972). Given the lack of concrete evidence for regiospecific phosphoryl transfer to lividomycin A with our broad-spectrum enzyme and the fact that the other aminoglycoside hydroxyl groups should, in principle, be as chemically reactive as either the 3′- or 5″-hydroxyls of kanamycin or lividomycin A, we sought unequivocal evidence for the site of phosphoryl transfer to several aminoglycoside antibiotics. This was made possible by our ability to readily obtain highly purified APH(3′)-IIIa using our overproducing strain.

The judicious use of several homo- and heteronuclear NMR experiments coupled with confirmation of the stoichiometry of aminoglycoside phosphorylation by ESI-MS permitted the unambiguous determination of the regiospecificity of phosphoryl transfer of amikacin to be at the 3′-OH and for lividomycin A to be at the 5″-OH. Complete NMR assignment of butirosin A diphosphate and neomycin B diphosphate unequivocally demonstrated that aminoglycosides which possess both 3′- and 5″-hydroxyls were phosphorylated at both sites. The initial phosphorylation event for these drugs appears to be random, with either the 3′- or the 5″-OH first being modified, although initial phosphorylation at the 3′-OH is preferred for butirosin A and at the 5″-OH for neomycin C by roughly 70−75%. This may indicate a subtle positioning effect which would bring the preferred hydroxyl either closer to the γ-phosphate than the other group or provide a better geometric alignment for direct in-line attack of the nucleophilic hydroxyl.[3] This first phosphoryl transfer is then followed by a slower second modification to give the diphosphoryl derivative. Neomycin and ribostamycin were readily diphosphorylated, whereas the second phosphorylation of butirosin was much slower, demonstrating the important structural role played by the (*S*)-4-amino-2-hydroxybutyryl group in butirosin.

One can readily build models in which the 3′- and the 5″-hydroxyls are within close proximity to each other, a fact which was previously noted for the diphosphorylation of ribostamycin by APH(3′)-I (Kondo et al., 1972). We have recently determined the structure of butirosin bound to a Cr•-ATP•APH(3′)-IIIa complex by paramagnetic NMR relaxation techniques (Cox et al., 1996). This structure also shows the close proximity of the 3′- and 5″-hydroxyls to each other and to the γ-phosphorus of ATP. If all 4,5-disubstituted aminoglycosides bind to the enzyme in a similar fashion, then we would expect that, upon phosphorylation, the drug would have to undergo a conformational change before the second phosphoryl transfer could occur to avoid bringing in the bulky, negatively charged phosphate into the ATP-loaded active site. This may reflect a significant change in structure for the monophosphoryl derivative of butirosin A. Unlike most aminoglycosides which demonstrate a dynamic solution structure, butirosin A shows a preferred structure in which rings A and C are in a stacked arrangement (Cox & Serpersu, 1995). It is not known what effect phosphorylation at the 3′- and 5″-positions would have on this structure, but it may show significant alteration, resulting in at least two possibilities. First, phosphorylation could result in an altered solution structure which is not readily recognized by the enzyme. Alternatively, phosphorylation may not have a significant direct effect but may "freeze" the structure in the A/B stacked conformation. Therefore, additional phosphorylation at C 5″-(or 3′-) may be sterically *and* electronically hindered due to approach of the butirosin-bound phosphate to the γ-phosphate of ATP. Regardless, it is evident that it is the (*S*)-4-amino-2-hydroxybutyryl group which dictates this effect as ribostamycin is readily diphosphorylated.

The tolerance of an additional bulky and negatively charged group at or near the active site of APH(3′)-IIIa is surprising. Although the enzyme does phosphorylate a broad range of drugs, these all share similar structures and are cationic. The determination of the exact geometry of active

[3] All of our current evidence for the chemical mechanism of phosphoryl transfer, including recent positional isotope exchange data (P. Thompson and G. Wright, manuscript in preparation), supports direct attack as opposed to a double-displacement mechanism.

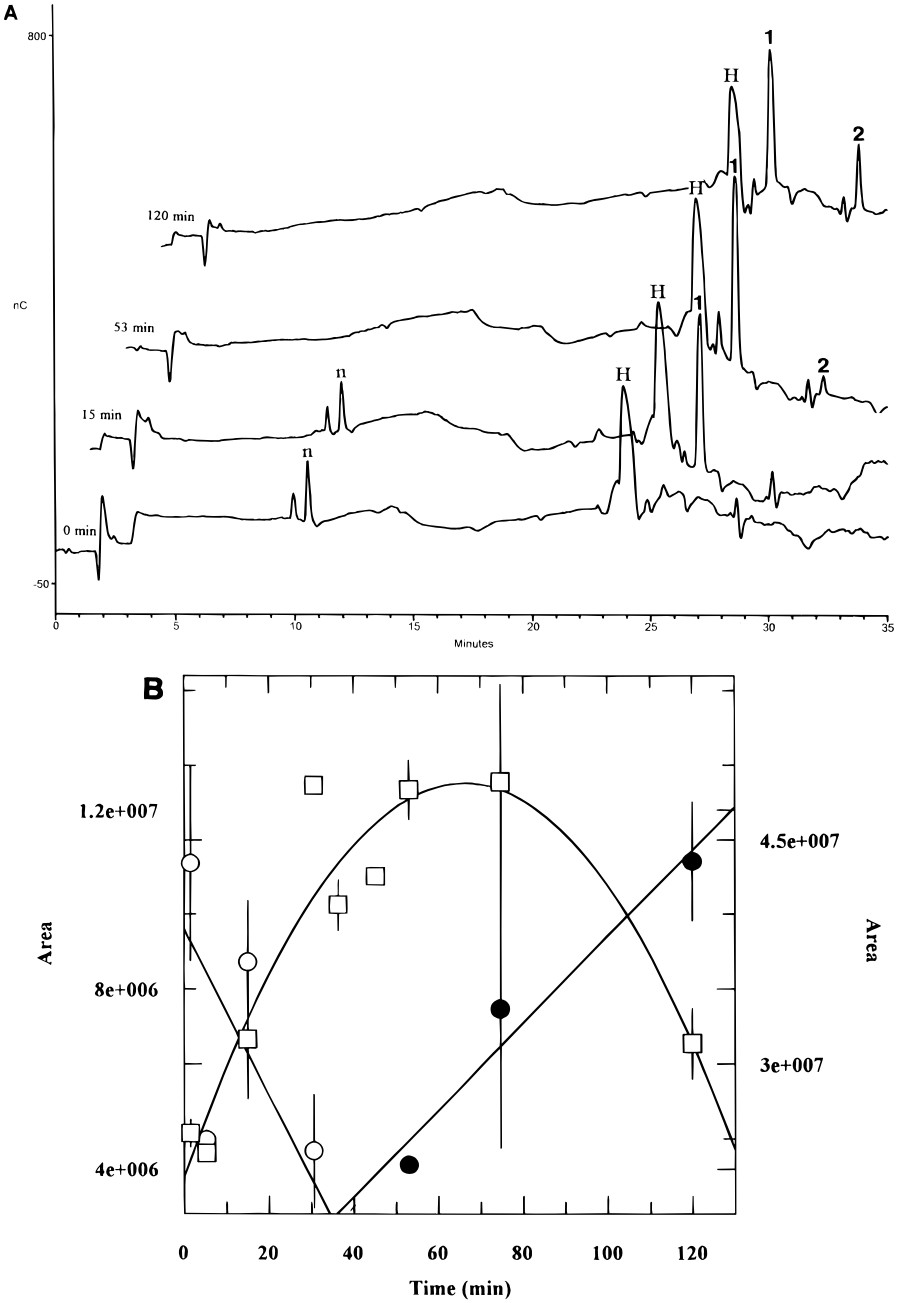

FIGURE 4: HPLC analysis of neomycin B phosphorylation by APH(3′)-IIIa. Reaction mixtures consisted of 150 μM neomycin B, 250 μM ATP, 10 μM $MgCl_2$, 40 μM KCl, 50 μM HEPES, pH 7.5. The solution was preincubated for 10 min at 37 °C, and the reaction was initiated by the addition of 0.5 nmol of APH(3′)-IIIa. At appropriate times, 500 μL was removed and quenched by freezing in liquid nitrogen. For HPLC analysis, samples were heated at 65 °C for 10 min, centrifuged at 1000 rpm, directly injected onto a Dionex CarboPac PA1 column, and analyzed as described in Materials and Methods. (A) HPLC profiles of neomycin *B*(*n*) phosphorylation over time. Initial rise of neomycin monophosphate (1) is followed by conversion to neomycin diphosphate (2). Peak marked by H is HEPES. Samples were incubated for 0, 15, 53, and 120 min. (B) Graphical analysis of conversion of neomycin B to neomycin diphosphate. Experiments were performed in duplicate and the area under the curve plotted as a function of incubation time. (○) Neomycin B (left scale); (□) neomycin monophosphate (right scale); (●) neomycin diphosphate (left scale).

site residues and substrates (currently in progress by X-ray diffraction studies) will be helpful in elucidating the structural mechanism of phosphate transfer to monophosphorylated aminoglycosides.

Implications of this Study. Roestamadji et al. (1995) have recently reported an elegant synthesis and study of mechanism-based inactivators of APH(3′)-I and APH(3′)-II. These compounds place a nitro group at position 2′ that readily eliminates inorganic phosphate upon phosphorylation at the 3′-OH, revealing an electrophilic nitroalkene that can undergo nucleophilic attack at the enzyme active site. Our demonstration of the regiospecificity of APH(3′)-IIIa opens the possibility of the synthesis of similar compounds modified at the pentose ring of 4,5-disubstituted aminoglycosides. This may be important for ease of synthesis or availability of precursors and thus expands the potential spectrum for such inhibitors.

SUPPORTING INFORMATION AVAILABLE

Nine tables of NMR assignments for aminoglycosides and their phosphorylated derivatives (9 pages). Ordering information is given on any current masthead page.

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