2D NMR Studies of Aminoglycoside Antibiotics. Use of Relayed Coherence Transfer for ^{1}H Resonance Assignment and in Situ Structure Elucidation of Amikacin Derivatives in Reaction Mixtures†

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ABSTRACT: Phase-sensitive 2D ^{1}H/^{1}H COSY spectra can be used to identify the structures of individual pure specimens of the aminoglycoside antibiotic amikacin and its *N*-hemisuccinyl derivatives. However, even at 500 MHz the 2D chemical shift dispersion does not allow for unambiguous assignment of all cross-peaks. By use of 2D relayed coherence transfer experiments (RELAY) optimized to detect two-step ^{1}H/^{1}H scalar interactions in which one of the *J*-values is small, sufficient additional correlations can be obtained from the frequency-isolated resonances to allow facile tracing of all scalar connectivities. Complete assignments of the ^{1}H NMR spectra of amikacin, its 6′-*N*-hemisuccinamide, and a novel bis(acylate) [γ-*N*-(*p*-vinylbenzoyl)amikacin 6′-*N*-hemisuccinamide] were obtained for aqueous media. The NMR spectrum of amikacin free base was also assigned in dimethyl sulfoxide solution. The RELAY experiment can be extended to the analysis of reaction mixtures, which allows for the identification and resonance assignment of regioisomeric amikacin haptens in the mixture state. All of the *N*-monohemisuccinyl isomers of amikacin have been identified in reaction mixtures through the RELAY experiment. The relative reactivities of the amino functions of amikacin toward acylating agents were found to be 6′-N > 3-N ≳ 3″-N ≳ γ-N. However, this reactivity order is altered after the initial acylation event.

Aminoglycoside antibiotics are commonly used for counteracting Gram-negative bacterial infections. Among these agents, amikacin (II), a semisynthetic compound derived from kanamycin A (I), is particularly useful against strains resistant to other antibiotics (Tally et al., 1975). Due to the narrow therapeutic window for this drug (Follath et al., 1981), assays for monitoring serum levels are required. For most envisionable assays based on immunochemical techniques, selective haptenization is an essential first step for polyfunctional drugs such as amikacin: regioselectively placed tether points are required for immunogen formation, enzyme conjugation, and polymer immobilization techniques. The polyfunctionality of amikacin allows the attachment of multiple small molecule species, while its high density of hydrophilic groups should counteract the solubility problems encountered when the small molecules are hydrophobic species. The styrene monomer, *p*-vinylbenzoic acid, which has been attached to monoclonal antibodies for use in a polymerization-based phase-separation immunoassay (Nowinski & Hoffman, 1985; Auditore-Hargreaves et al., 1987), is such a hydrophobic species. We expected that the use of haptenized amikacin as a polyfunctional water-soluble "spacer arm" for such immunoassay studies (Houghton et al., 1986) would improve solubility and signal generation. The possibility of haptens linked at each ring amino group and at the primary amino terminus of the 1-*N*-(α-hydroxy-γ-aminobutyryl) (HAP)[1] side chain presented itself as the means both for obtaining probes for each potentially immunogenic region of amikacin and for converting amikacin into the desired "spacer arm". Thus, we set out to prepare a variety of regiospecifically N-hemisuccinylated amikacin derivatives that could then be used for subsequent tethering to antibodies or labeling with fluorophores or other readily detected moieties. For the applications that we envisioned for these materials, we required a direct synthesis from amikacin rather than the circuitous haptenization methods reported by Singh et al. (1984) that utilize protecting and blocking groups.

Recent advances in NMR methodology prompted us to choose ^{1}H NMR as the analytic tool for our studies. ^{13}C NMR has been used extensively (Nagabhushan et al., 1978; Singh et al., 1984; Everett & Tyler, 1985) for aminoglycoside antibiotics, but to our knowledge complete ^{1}H resonance assignments have not been reported for antibiotics of the deoxystreptamine class.[2] An extensive body of literature on ^{1}H

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[1] Abbreviations: 1D, one dimensional; 2D, two dimensional; NMR, nuclear magnetic resonance; HPLC, high-performance liquid chromatography; ODS, octadecylsilyl; TLC, thin-layer chromatography; HAP, α-hydroxy-γ-aminobutyryl [(1-hydroxy-3-aminopropyl)carbonyl]; SA, succinic anhydride; FT, Fourier transformation; FID, free induction decay; PD, preparatory delay; PS, phase sensitive; COSY, 2D correlated spectroscopy; NOESY, 2D nuclear Overhauser spectroscopy; DQ, double quantum; DQF, double quantum filtered; RELAY, 2D NMR experiments including a coherence relay, included in a COSY unless otherwise specified; TOCSY, total coherence transfer spectroscopy; TPPI, time proportional phase incrementation; HOHAHA, homonuclear Hartmann–Hahn spectroscopy.

[2] A complete assignment of neomycin B appeared while the manuscript was being revised (Reid & Gajjar, 1987). A nearly complete assignment for another glycoside antibiotic, erythromycin A, has appeared (Everett & Tyler, 1985).

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NMR of other complex carbohydrates (Dua et al., 1986; Vliegenthart et al., 1983), glycolipids (Dabrowski et al., 1980; Yu et al., 1986), and glycoproteins (Carver & Grey, 1981) exists; however, this does not provide correlations for assigning aminoglycoside antibiotic spectra. Furthermore, the temperature and media dependence of glycoside proton resonance chemical shifts argues against reliance on any chemical shift based assignment except those based on shifts of large magnitude ($\Delta\delta > 0.3$ ppm). Two dimensional correlated spectra should provide a basis for making definitive assignments (Bernstein & Hall, 1982; Levery et al., 1986). Of these methods, relayed coherence transfer (RELAY) (Bax & Drobny, 1985) appeared particularly attractive. In one application of the RELAY experiment to analysis of oligosaccharide ^{1}H NMR spectra (Homans et al., 1984), the authors suggested that the increased information extracted from the frequency-isolated resonances of the anomeric region would be sufficient for the study of mixtures. Our studies, reported herein, establish the utility of this approach. Resonance and structure assignments for amikacin hemisuccinamides can be achieved by relayed coherence methods without separation of the regioisomers. In this way, we were able to determine the relative reactivities of amikacin amino groups toward acylating agents and to synthesize a single regioisomer of (*p*-vinylbenzoyl)amikacin hemisuccinamide without the use of protecting groups or extensive chromatography.

Materials and Methods

Materials. All derivatization reactions employed amikacin free base (from Sigma Chemical Co., St. Louis, MO) and reagent grade chemicals using the protocols given below. NMR solvents, 99.9% D_2O, 99.96% D_2O, and 99.8% DMSO-d_6, were obtained from Aldrich Chemical Co. (Milwaukee, WI) and Cambridge Isotope Labs (Cambridge, MA).

Aminoglycoside Quantitation. Concentrations of amikacin and its derivatives were estimated by the quantitative ninhydrin assay of Sarin et al. (1981).

Hemisuccinylation. Amikacin free base was dissolved in distilled water to an initial concentration of 300 mg/mL (pH about 11). For most reactions the pH was adjusted to 9.95 by addition of dilute HCl. To this solution was added with stirring 2.12 M succinic anhydride/CH_3CN, in 20–60 portions over an hour, with constant pH monitoring with a glass electrode. After each addition, the initial nominal pH (uncorrected for solvent composition changes) was reestablished by the addition of 1 N aqueous NaOH. The efficiency of N-succinylation was determined from the difference in equivalents of NaOH consumed in the experiment and in a control in which no amikacin was present. Efficiency was 70–73% at pH 9.95 determined by using three 0.42 molar equivalent aliquots of succinic anhydride. Immediately before chromatography the concentration was adjusted to 200 mg/mL at pH 6.8–7.0. A carboxymethylethyl-Sephadex C-25–120 column (25–45 mL/g of amikacin derivatized) was first equilibrated with 1 M phosphate buffer (pH 7.0) and then washed with 2 volumes of 2 M aqueous LiCl followed by distilled water until the eluant refractive index matched that of the eluate. Elution was achieved with 1 volume of distilled water followed by four volumes of a linear 0–250 mM $NaHCO_3/Na_2CO_3$ buffer (pH 9.4) gradient. Fractions were monitored by TLC on 250 μm SiO_2 plates by using 11:3:3 3% NH_4OH/acetone/*n*-BuOH with ninhydrin spray detection. Observed R_f values were 0.14 for amikacin, 0.4 for monosuccinylamikacin derivatives, 0.55 for bis and higher succinylamikacin derivatives. The same sequence is observed upon elution from the Sephadex column, with the monosuccinyl compounds eluting at about 150–170 mM CO_3^-, just prior to recovered amikacin.

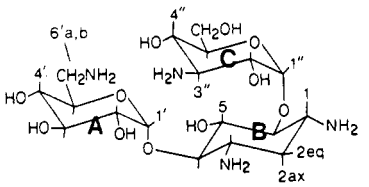
Procedure for the Large-Scale Monosuccinylation Reaction. A 10-g portion of amikacin was succinylated to the extent of 30% (assuming 72% efficiency of SA utilization) by using 30 aliquots of SA over a 1-h period with the pH maintained between 9.3 and 10.0. The reaction product was stored at 279 K for 2 months and then adjusted to a 50-mL volume at pH 6.8 for chromatography on a 5.5 × 20 cm column of carboxymethylethyl-Sephadex C-25–120 preequilibrated with 2 M LiCl. After a thorough H_2O wash [which eluted a small amount of bis(hemisuccinamide)], the products were eluted with a linear 0–250 mM CO_3^- (pH 9.4) gradient (1.6 L): fractions 110–114 were bissuccinylated, fraction 117 (148 mM CO_3^-) was ~50:50 bis- and monosuccinylated. TLC monitoring revealed traces of amikacin appearing in fraction 132 forward. Fraction 136 was judged by TLC to be about 25% amikacin. Fractions 122–133 (770 mg, 26% of theory) correspond to net monosuccinyl derivatives. Fraction numbers in this paper refer to this particular chromatography.

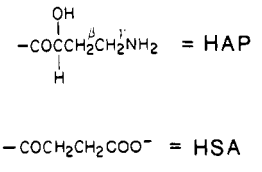
Vinylbenzoylation. Fractions 124–129 were pooled for vinylbenzoylation. A 188-mg portion was dissolved in 70 mL of 1:1 H_2O/CH_3CN and adjusted to pH 10. To this solution were added, at 1-h intervals, four portions of 96 mg of the *N*-hydroxysuccinimide ester of *p*-vinylbenzoic acid in 1.4 mL of CH_3CN, with the pH (~9.5) readjusted to 10 after each addition with 1 N aqueous NaOH. The volume was reduced to 27 mL by rotary evaporation, the pH was adjusted to 4, and the products were separated on a ODS HPLC column (1.0 × 20 cm) with a 10 mL/min flow of a 0.1% aqueous CF_3CO_2H to CH_3CN gradient. The major portion of monovinylbenzoylated material eluted as a single peak and was >90% pure by analytic HPLC and NMR.

Sample Preparation for NMR Spectroscopy. Solutions for NMR spectroscopy were prepared from lyophilized column chromatography fractions by dissolution to volume with 99.9$^+$% D_2O followed by relyophilization and then dilution to volume with 99.99$^+$% D_2O containing 20–60 mM potassium or sodium phosphate as the buffer at a pH of 6–9.6, uncorrected for D_2O activity difference at the glass electrode. Unless otherwise specified the buffer concentration was 50 mM, pH 7.4, and the total concentration of derivatized amikacin was in the 3–7-mM range. Spectra in DMSO-d_6 employed nominal 99.8% D solvent to which 2% (v/v) 99.9% D_2O had been added. The observed intensities in the spectra, however, indicate a higher protium content. Amikacin free base was dissolved directly in DMSO-d_6 (2% D_2O) or in buffered D_2O. The free amikacin concentration was 7 mM in the DMSO solution examined.

NMR Spectroscopy. All spectra were obtained at 308 ± 2 K by use of 5-mm sample tubes on a Bruker WM-500 spectrometer equipped with an Aspect 2000 computer for FID signal accumulation and storage. The Aspect 2000 was also used for Fourier transformation (FT) of all 1D spectra and control spectra for 2D experiments. Typical parameters for such 1D spectra were 4000-Hz spectral width, 12-μs acquisition pulse (13 μs = 90°), 8K or 16K channel quadrature detection (1 or 0.5 Hz/real point), with a 2-s preparatory relaxation delay. For 2D experiments, serial files of 2K complex FIDs were accumulated and stored by the Aspect 2000. For all 2D spectra, FID files were then copied onto magnetic tape and transferred to a VAX 11/780 for transformation and plotting using software developed by Dr. Dennis Hare. All chemical shifts are referenced to tetramethylsilane internal standard (DMSO solutions) or 2,2-dimethyl-2-sila-

Chart I





designation	substitutents	
I, kanamycin A	none	
II, amikacin	1 -N-HAP	
III	1 -N-HAP	6'-N-HSA
IV	1 -N-HAP	3''-N-HSA
V	1 -N-HAP	3-N-HSA
VI	1 -N-COCH(OH)CH_2CH_2NH-HSA	

pentane-5-sulfonate (D_2O solutions).

Phase sensitive detected COSY (PS-COSY) spectra were collected as two serial files with a 90° phase shift [the hypercomplex method applied to NOESY by States et al. (1982)] rather than by TPPI (Marion & Wüthrich, 1983), {[PD – $90°_{\phi 1}$ – t_1 – $90°_{\phi 2}$ – $AQ_{\psi 1}(t_2)]_{8n}$ write file 1 ... [PD – $90°_{\phi 1}$ – t_1 – $90°_{\phi 2+90}$ – $AQ_{\psi 1+90}(t_2)]_{8n}$ write file 2 ... increment $(t_1)_m$} with phase cycling $\psi 1 = \phi 1 = x\ y\ -x\ -y\ -x\ -y\ x\ y$ and $\phi 2 = x\ y\ -x\ -y\ x\ y\ -x\ -y$. The processing of hypercomplex data sets has been described (Andersen et al., 1987). Magnitude representations of PS-COSY data are obtained by using a magnitude transform for $t_1 \rightarrow \omega_1$ and retain most of the resolution enhancement associated with "dispersion zeroing" (as illustrated in supplemental Figure 2). Experimental parameters were PD = 1.2 s, with eight FIDS collected for each file at each t_1 with 400–475 t_1 increments achieved and shifted sine bell apodization functions (10–25° shift for $t_2 \rightarrow \omega_2$, 10° for $t_1 \rightarrow \omega_1$) applied. All spectra are displayed in their unsymmetrized form at a resolution of 3 Hz/real point in ω_2.

Double quantum filtered COSY (DQF-COSY) spectra were obtained by using a local variant of the procedures of Piantini et al. (1982) and Rance et al. (1983) with a 16-step phase cycle (quadrature detection in t_2 only) and were subjected to a direct magnitude 2D transform using 10° shifted sine bell apodization in both dimensions.

Relayed coherence transfer (RELAY) spectra were obtained by using the PD–90–t_1–90–τ/2–180–τ/2–90–t_2 pulse sequence, with the phase cycling reported by Bax and Drobny (1985). The preparatory delay was 2 s, and 16 FIDs/t_1 collected out of 300 t_1s gave sufficient signal to noise ratios and resolution. A number of relay intervals (τ = 38, 56, 80, and 110 ms) were employed. In practice, the τ = 56 ms experiment yielded substantial coherence transfer (A → X) for $J_{AM} \simeq 2$ Hz and $J_{MX} \simeq 10$ Hz, which are typical values for J_{12} and J_{23}, respectively, in the sugar rings of amikacin (see Chart I). Vicinal diaxial COSY peaks and relay peaks arising from H-2 → H-3 → H-4 coherence transfer are retained at diminished but observable intensity. Magnitude 2D transforms were carried out with the following apodization functions: $t_2 \rightarrow \omega_2$, 10–45° shifted sine bell; $t_1 \rightarrow \omega_1$, 10° shifted sine bell. The resulting 1K (real) by 0.5K (zero-filled) matrix was displayed without symmetrization as a 1K by 1K contour plot. The nominal resolution in ω_2 was 3 or 4.5 Hz/real point.

Of the RELAY experiments, only that shown for fraction 136 uses the full 32-step phase cycle which eliminates double relays and reduces the intensity of the autopeaks and direct COSY peaks (Bax & Drobny, 1985). Thus, for all other RELAY spectra, the anomeric resonance shifts reveal very minor 1,4 cross-peaks as double relays. All spectra of mixtures were obtained at pH 7.0–7.8 (uncorrected glass electrode readings) with a total amikacin concentration of 4 ± 1 mM.

2D double quantum relay (DQ-RELAY) spectra were obtained by incorporating a relay into a DQ sequence, essentially as described by Macura et al. (1984), but using a different 16-step phase cycle (Bax et al., 1980; Hoult & Richards, 1975). The DQ-RELAY spectrum shown in this paper was obtained with the transmitter placed at the low-field end of the spectrum, as the phase cycle used does not allow for quadrature detection in the ω_1 dimension.

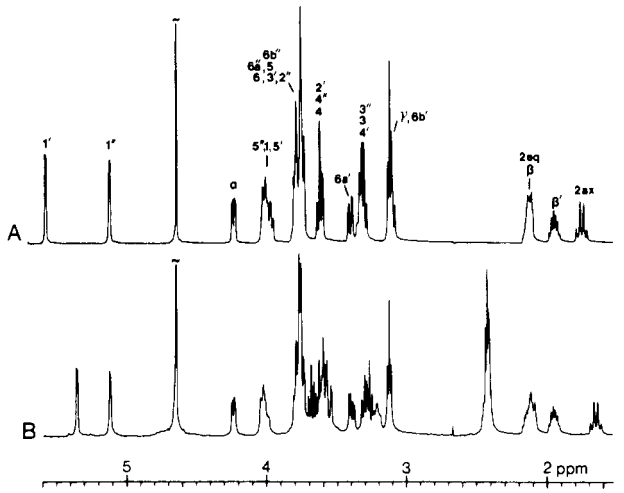


FIGURE 1: 500-MHz ^1H NMR spectra of (A) amikacin and (B) amikacin 6'-*N*-hemisuccinamide. The spectra were recorded in the same pH 7.4 D_2O buffer at a 4.5 mM concentration of amikacin.

RESULTS

Reaction of Amikacin with Limited Quantities of Succinnic Anhydride. Upon titration with succinic anhydride (SA), amikacin is rapidly hemisuccinylated in acetonitrile/water at all pH values between 8.8 and 11.2. After a 60-min reaction period at pH 9.95, differential titration versus a buffer control revealed that 69–72% of SA consumption was due to amide derivatization of amikacin when SA was present initially in 1.0–0.3 molar equivalent amounts. A preliminary NMR examination of products from reactions at SA/amikacin = 0.7–1.0 revealed complex mixtures. Based on the known reactivity of kanamycin A and tobramycin, acylation at 6'-N and then 3-N (Kawaguchi et al., 1972; Koch et al., 1974), hapten structures III and V were likely components of the mixtures. The terminal amino group of the amikacin side chain should be accessible to acylating agents, suggesting hapten VI as a possible structure (see Chart I).

Initial attempts to obtain a monohemisuccinamide utilized 0.42 molar equivalent of SA. Chromatography of a 1-g scale amikacin derivatization (at pH 9.95–9.6) on a 1.5 × 10 cm column of carboxymethylethyl-Sephadex (with Li as the counterion), eluting with a linear pH 9.4 carbonate buffer gradient, afforded 133 mg of a well-resolved monohemisuccinamide fraction that was homogeneous by TLC (38% yield on the basis of the expected succinylation efficiency). The NMR spectra of this product and the starting amikacin in phosphate-buffered D_2O appear as Figure 1. These spectra established that the product was a single regioisomer (>96%), but the carbohydrate methine region was so complex as to preclude resonance assignment. Resorting to a different solvent system did not help matters; in DMSO the spectra exhibited even worse signal overlap (see supplemental Figure 1). Even after the assignment of the amikacin spectrum (vide infra), a comparison of panels A and B of Figure 1 does not reveal the position of acylation.

Subsequent experiments did not lead to the isolation of a single pure hemisuccinimide. Figure 2 shows NMR spectra of the anomeric and HAP side-chain α-methine regions for

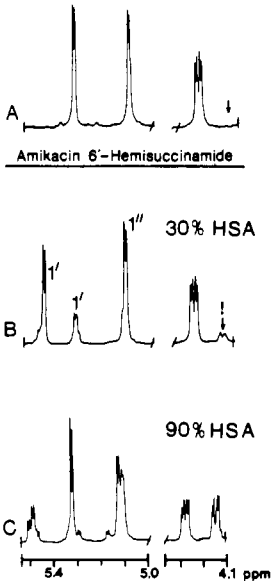


FIGURE 2: NMR spectra of the anomeric (δ 5.52–5.00) and HAP side-chain α-proton (δ 4.34–4.09) regions of succinylation reaction mixtures. The arrow indicates the position of the additional HAP signal due to (an)other regioisomer(s) which is present even at low conversion. The same regions of the 6′-*N*-hemisuccinamide spectrum are included for reference.

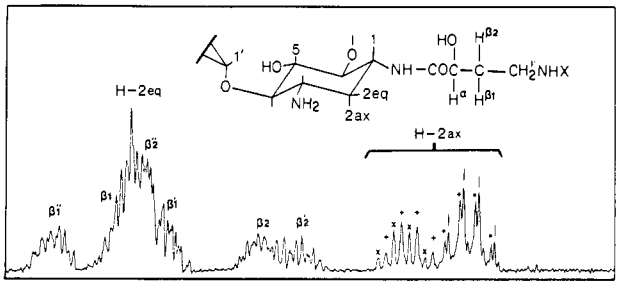


FIGURE 3: Resolution-enhanced spectrum (δ 2.20–1.20) of fraction 121, an early fraction in the elution of the net mono hemisuccinyl derivatives from a large-scale succinylation of amikacin. Resonances are labeled β (in the HAP side chain) or H-2ax or H-2eq (of deoxystreptamine). In the case of H-2ax any pure isomer should yield a sharp quartet. Symbols indicating four quartets appear above the trace.

a similarly produced reaction mixture with the same degree of net hemisuccinylation. Even at very low conversion (15%, data not shown) other regioisomers formed; these are evident in the ^{1}H NMR spectra as an upfield HAP α-methine resonance indicated by the bold arrow. At 90% hemisuccinylation more complicated mixtures were formed. Regioisomers could also be detected of the deoxystreptamine ring. The NMR trace in Figure 3 shows four distinct quartets for the 2-axial proton of this ring with two isomers each in an upfield (~1.42 ppm) and downfield (~1.53 ppm) position. Fortunately, the powerful NMR analytical methods that we could bring to bear on these mixtures allowed us to proceed without having to discern the particular features (pH control, chromatographic conditions, etc.) that had resulted in the initial isolation of what we now recognize as hapten III.

Structure Elucidation of Amikacin 6′-N-Hemisuccinamide. A direct comparison of the COSY spectra obtained for amikacin and the one pure hemisuccinamide (shown in Figure 4) eventually led to a tentative assignment of both spectra and to a secure determination of the succinylation site. The spectrum of amikacin free base in DMSO, although it exhibited more signal overlap, could be assigned in a relatively straightforward manner. This results from the fact that H-2″ and H-5″ (ring C) and H-2′ (ring A) were at unique chemical

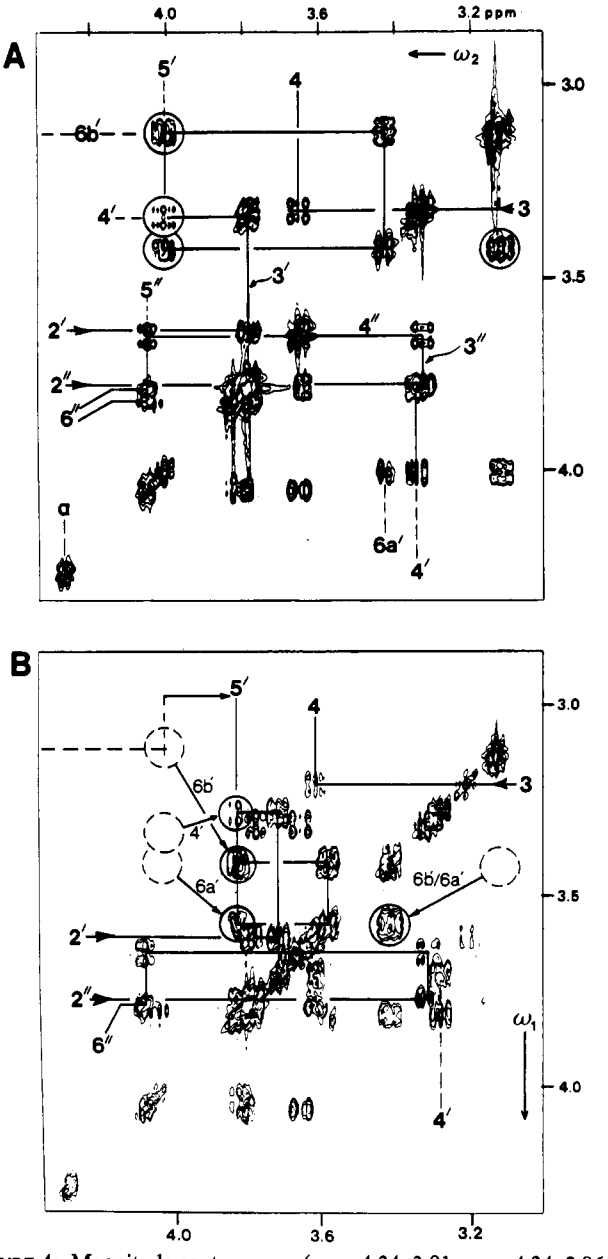


FIGURE 4: Magnitude contour map (ω_2 = 4.34–3.01, ω_1 = 4.34–2.86 ppm) comparison of the amikacin (A) and amikacin 6′-*N*-hemisuccinimide (B) PS-COSY spectra (4.5 mM, D_2O, pH 7.4). Horizonal line labels indicate the resonance assignments corresponding to the intersection at the ω_1 chemical shift axis. Vercial line labels correspond to chemical shift assignments at the ω_2 scale. The encircled cross-peaks correspond (ω_2/ω_1) to 5′/6b′, 5′/4′, 5′/6a′, and 6b′/6a′. Their displacement from panel A to panel B reveals the 6′-N-acylation-induced shifts for these four resonances of the 6-amino sugar ring. The chemical shift (ω_1) of deoxystreptamine proton H-3 (indicated as a horizontal line in the upper right of each panel) was derived from the H-3 cross-peaks with both resonances (ω_2) due to the C-2 methylene (see Figure 5 for a more complete depiction). The chemical shifts of H-2′ and H-2″ were located from the cross-peak occurring at the anomeric resonance positions (off scale).

shifts with no overlapping resonances. A direct, unambiguous connectivity trace from the more downfield anomeric resonance through an upfield C-3 methine (3-NH_2-sugar) to a downfield pair of signals due to the C-6 methylene results (supplemental Figure 1). Rings A and B could also be traced in a similar manner.[3] This analysis revealed that δ(H-1″) > δ(H-1′) in

[3] In the case of the DMSO spectrum of amikacin, the direct linear connectivity analysis became difficult at the 4-position of each ring. The three C-4 methine proton resonances are essentially frequency coincident (δ = 2.998 ± 0.006) as shown in Figure 1 of the supplementary material.

Table I: Proton Resonance Assignments for 3-Aminoglucose (Ring C) and 6-Aminoglucose (Ring A) Residues of Amikacin and Amikacin 6′-*N*-Hemisuccinamide[a]

compd, solv	ring	proton chemical shifts (ppm)						
		H-1	H-2	H-3	H-4	H-5	H-6a	H-6b
II, DMSO	A	4.908	3.281	3.402	3.000	3.562	*2.824*	*2.574*
	C	4.952	3.104	*2.793*	*2.992*	3.708	3.550	3.458
II, D_2O	A	5.613	3.644	3.785	3.356	*4.009*	*3.438*	*3.130*
	C	5.156	*3.780*	3.331	*3.656*	4.055	~3.820	~3.790
III, D_2O	A	5.386	~3.630	3.725	3.308	3.835	3.600	3.434
	C	5.145	*3.780*	*3.341*	3.670	4.069	~3.820	3.800

[a] Shifts that are particularly media and temperature sensitive are in italics; those shifts that are insensitive and can serve as internal standards are underlined.

DMSO. Unfortunately, hemisuccinylation products could not be analyzed in this medium. Initial attempts to assign the D_2O spectra by analogy were confounded by the chemical shift reversal of the anomeric resonances upon solvent change.

The aqueous media spectra (Figure 4) had to be analyzed without reference to the assignments established in organic media. In each case the scalar pathway analysis began with (1) the recognition that the lowest frequency quartet (J = 12.7 Hz) must correspond to the axial proton at C-2 of the deoxystreptamine (ring B) and (2) the observation that the 4.2 ppm resonance couples only to protons of chemical shift less than 2.2 ppm and thus must be the α-proton of the HAP side chain attached at the deoxystreptamine 1-amino group. The side-chain β resonances so located in turn revealed the γ-CH_2NH_2 group and thus set the stage for identifying the 6′-CH_2NH_2 unit of ring A. The two downfield resonances, $\delta > 4.9$, could be safely assigned to anomeric protons. However, specific assignments to rings A and C required a secure correlation to the C-3 methines—the 3-amino sugar (ring C) would then be distinguished by the upfield location of H-3. One resonance assignment distinguishing between rings A and C was clear in the D_2O COSY of amikacin: the CH_2NH_2 group of ring A, revealed by a large upfield geminal cross-peak, led to a secure assignment of the H-5′ resonance. With these assignments in hand, comparison with the COSY of the pure hemisuccinamide (Figure 4) revealed downfield shifts ($\Delta\delta$ = 0.30 and 0.15 ppm) for the 6′-methylene signal upon hemisuccinylation—proof that the HSA moiety is conjugated to ring A. Other nearby hydrogens (H-5′ and H-4′) and some at more remote sites are shifted upfield upon 6′-N-acylation. The shifts associated with 6′-N-hemisuccinylation are shown in the lower panel of Figure 4.

Attempts to assign the resonances in magnitude COSY spectra failed. Phase-sensitive COSY spectra were collected for amikacin and the pure hemisuccinamide, and these spectra provided distinct advantages over routine magnitude COSY spectra—a narrower diagonal and cross-peak multiplet structure. The latter served to distinguish sugar ring 5/4 and 5/6 cross-peaks from those due to 2/3 and 3/4 interactions. Double quantum filtered (DQF) COSYs were also collected. Up to this point in the resonance assignments, the H-2 → H-3 correlations for rings A and C, and thus also the specific assignment of the anomeric hydrogens, had to be viewed as only tentative since they depended, in each case, on a choice between alternate cross-peak alignments that were at or beyond the nominal resolution of these 2D experiments; H-2′ and H-2″ are each nearly shift coincident with several other ring methine signals. The connectivity traces shown in Figure 4 are consistent with the small chemical shift differences (<5 Hz) seen in the correlated spectra collected on three different days with imperfect temperature control. The assignments were not fully confirmed until RELAY experiments were performed (vide infra).

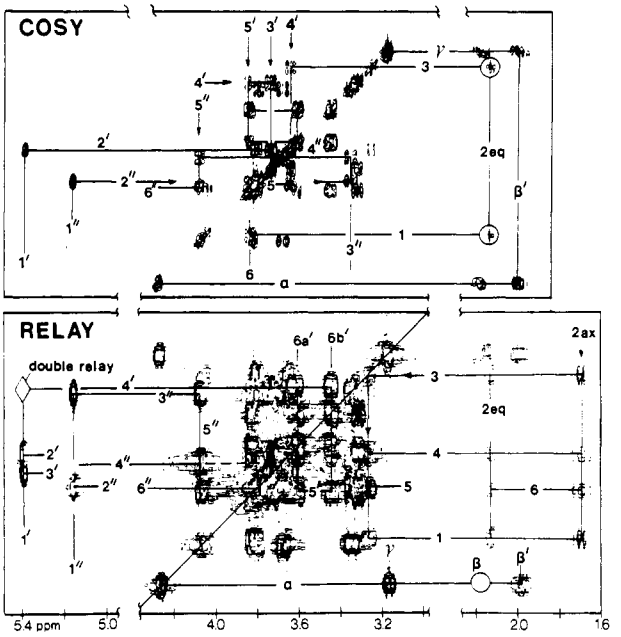


FIGURE 5: Comparison of the PS-COSY (magnitude representation) and RELAY experiments (τ = 56 ms, PD = 2.0 s) recorded for the same solution of hapten III. For this particular RELAY the $t_2 \rightarrow \omega_2$ transform was performed after a 45° shifted sine bell apodization in order to retain the significant direct COSY peaks. The long-range 1′/4′ cross-peak seen in the anomeric region is not due to a nonzero long-range coupling, J(1′,3′) or J(2′,4′), but rather is a double relay due to residual 90° pulse character for the 180° (chemical shift refocusing) pulse. This can result when the full 32-step phase cycle is not employed.

The complete connectivity traces for the deoxystreptamine rings are not shown in Figure 4. The near equivalence of chemical shifts of the hydrogens at C-5 and C-6 placed the cross-peak too close to the diagonal. Neither DQF-COSY spectra nor PS-COSY spectra of the hemisuccinyl derivative provided sufficiently enhanced resolution near the diagonal to provide for secure assignments. Figure 2 of the supplementary material traces the deoxystreptamine connectivities (derived from assignments obtained in the RELAY experiment, Figure 5) through this diagonal region of the DQF- and PS-COSY spectra and illustrates the expected advantage of these techniques relative to routine magnitude COSY spectra.

Relayed coherence transfer (Wagner, 1983; Eich et al., 1982) provides a means for reading additional chemical shift assignments not only from the anomeric proton resonances but also from the resonances due to the side-chain α-proton and the C-2 methylene and H-3 of the deoxystreptamine. The thus facilitated assignment process is illustrated for the 6′-*N*-hemisuccinamide in Figure 5. Similar studies of amikacin in DMSO-d_6 also gave unambiguous assignments. Resonance assignments in D_2O and DMSO appear in Tables I and II. The top panel of Figure 5 shows the scalar connectivity analysis

Table II: Proton Resonance Assignments for Deoxystreptamine Residue (Ring B) and HAP Side Chain of Amikacin and Amikacin 6′-*N*-Hemisuccinamide[a]

	proton chemical shifts (ppm)											
	ring B							HAP				
compd, solv	H-1	H-2ax	H-2eq	H-3	H-4	H-5	H-6	H-α	H-β	H-β′	H-γ	H-γ′
II, DMSO	~3.660	1.175	1.893	*2.634*	3.004	3.428	~3.620	3.390	1.543	1.748	*2.665*	*2.680*
II, D_2O	4.040	*1.788*	*2.156*	*3.360*	*3.656*	3.786	3.824	4.268	1.981	2.168	*3.156*	*3.156*
III, D_2O	~4.050	*1.660*	2.130	*3.250*	3.640	~3.790	~3.820	4.272	1.987	2.175	3.164	3.164

[a] Shifts that are particularly media and temperature sensitive are in italics; those shifts that are insensitive and can serve as internal standards are underlined as in Table I.

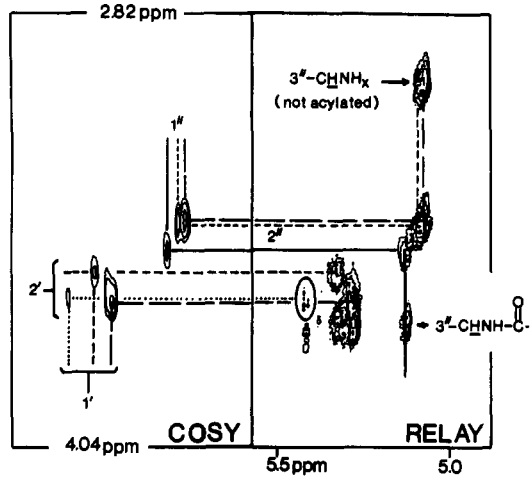


FIGURE 6: Contour map presentations of 2D spectra of a mixture of amikacin monohemisuccinamides. The horizontal scale is ω_2 in all cases. Vertical line labels thus correspond to chemical shift assignments at the ω_2 scale. A comparison of the anomeric region (δ_2 = 5.56–4.98) COSY and RELAY contour maps for the hemisuccinamide mixture in fraction 121.

proceeding from the resonances due to H-1′, -1″, -2eq, and -2ax. Note that the potential confusion in assigning the H-3′/H-2′ and H-3″/H-2″ cross-peaks due to the presence of other cross-peaks along the $\omega_1 = \delta(2')$ and $\delta(2'')$ lines, which could have led to a misassignment of the resonances due to the 3-amino and 6-amino sugar rings, was clarified by the RELAY spectrum (lower panel). In the RELAY spectrum, the 3-amino versus 3-hydroxy sugar distinction is immediately evident in cross-peaks involving the anomeric protons. The crowded central region (δ 3.50–3.95) becomes more difficult to interpret, but all chemical shift assignments can be made from frequency-isolated resonances in the upfield and downfield portions of the spectra.

In Situ Assignment of Structures in the Mixture State. Mixtures of regioisomers with identical net succinylation proved to be virtually inseparable by routine ion-exchange or adsorption chromatography. COSY spectra were not capable of resolving the numerous overlapping cross-peaks observed in reaction mixtures and chromatographic fractions. (Figure 3 in the supplementary material illustrates the changes in the downfield regions in sequential fractions spanning the net monosuccinylated eluates from an attempted chromatographic purification.) The only spectral regions that contained any frequency-resolved signals due to individual isomers were the downfield anomeric portion and the extreme upfield region (as seen in Figure 3, vide supra).

The RELAY experiment gave numerous additional chemical shift assignments from these few resolved signals. In the case of the anomeric region (see Figures 6 and 7) for each resolved resonance, it was immediately evident whether it was due to H-1′ or H-1″ and whether the 3″-position had been acylated. In instances in which the full phase cycle was not used, a double relay frequently gave an assignment of the C-4 methine as well. Even in the mixture state, it is possible to assign resonances and structures to the different regioisomers present in the mixture. In some instances cross-peaks could have been assigned to either direct ($J_{i,i+1}$) or relayed coherences, and in a few instances a comparison with a COSY spectrum was useful for distinguishing these possibilities (as illustrated in Figure 6). Certain chemical shift values appear

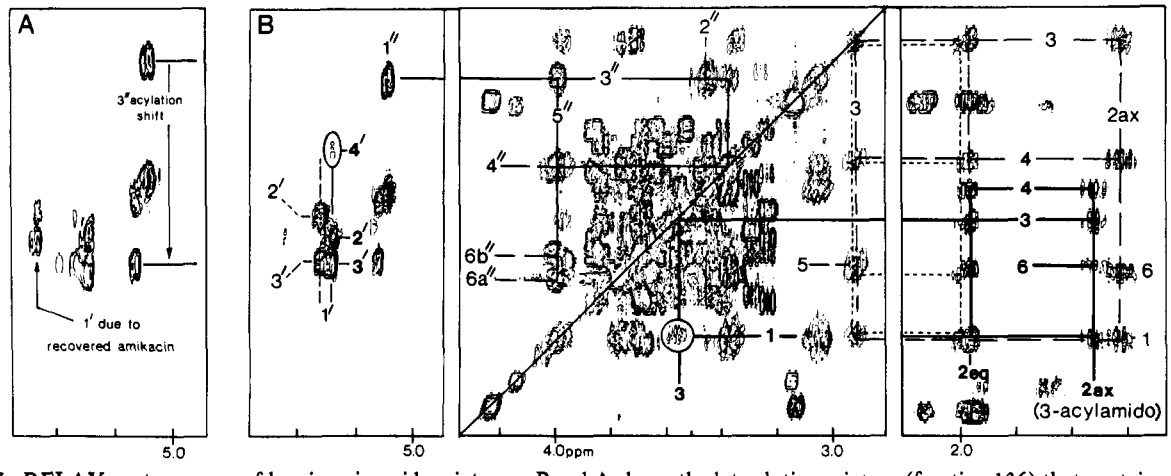


FIGURE 7: RELAY contour maps of hemisuccinamide mixtures. Panel A shows the late eluting mixture (fraction 136) that contains recovered amikacin and illustrates (1) the observed 3″-N-acylation shift and (2) the appearance of RELAY experiments obtained with a full 32-step phase cycle at each t_1 value. Even at lower cutoff levels, no trace of the 1′ → 4′ double relay seen in panel B is found in this experiment. Panel B shows portions of the RELAY spectrum of fraction 127. The ω_2 scale is discontinuous; ω_1 spans 4.36–2.81 ppm. The low-frequency H-1′ shows a double relay, encircled. Further correlations based on the location of the H-1″ relay to H-3″ are shown to the left of the diagonal. Evidence for three distinguishable types of deoxystreptamine rings in fraction 127 appears to the right of the diagonal. The normal (non-3-N-acylated) rings are shown in dashed and broken lines. The 3-*N*-hemisuccinamide correlation is shown in bold lines and position designations; the H-3/H-1 RELAY peak is encircled.

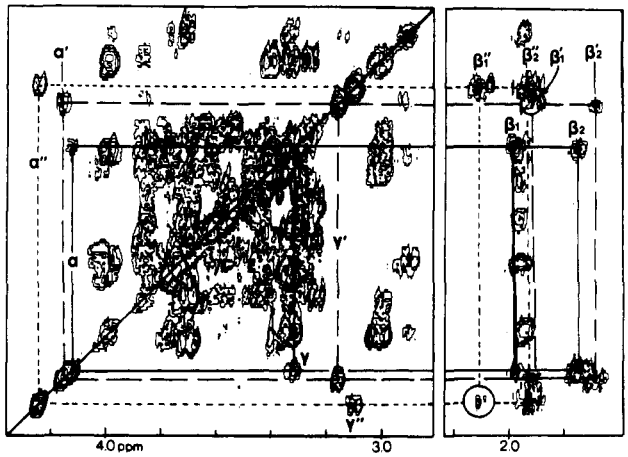


FIGURE 8: Evidence for hemisuccinylation of the γ-NH_2 group of the HAP side chain. Fraction 121 shows three distinct α/γ RELAY peaks and each full side-chain connectivity can be traced. The γ-N-acylated species is shown with solid-line correlations. The set designated by a single prime is attributed to a deoxystreptamine 3-N-acylated species (see Figure 7, panel B). The 6′-N- and 3″-N-acylated species appear together at a shift unaltered relative to amikacin.

to be quite sensitive to the buffer concentration, Li ion content, and other factors associated with specific column chromatography conditions, and thus it is not possible to identify previously encountered isomers by chemical shift correspondences. The lack of alignment of the H-3″ relay peaks from the upfield H-1″ resonances is an instance of this type. Due to the variable chemical shift values, the RELAY map of each fraction had to be analyzed independently. In order to confirm the presence of amikacin or its 6′-*N*-hemisuccinamide in the mixtures, fraction 127 was spiked with pure 6′-*N*-hemisuccinamide and fraction 136 with amikacin for additional 1D spectral determinations.

Key features of the strategy for resonance assignment in mixtures are illustrated in Figures 6–8. For example, it was clear that the highest frequency H-1″ anomeric resonance was due to a 3″-N-acylated species. The 3″-acylation shift ($\Delta\delta$ = +0.71 ppm) is shown in panel A of Figure 7. In the RELAY spectra, three distinct 1″ resonances can be recognized in both fractions 121 and 136. The ring C (3-aminoglucose) connectivity trace for the major isomer in fraction 127 is shown in the upper left-hand portion of panel B of Figure 7. A similar analysis of two 6-aminoglucose rings appears in Figure 4 of the supplementary material. By use of RELAY maps obtained with a 56-ms relay interval, connectivity traces proceed by two step correlations (1′ → 3′ → 5′, 2′ → 4′ → 6′) since these give the more intense cross-peaks.

Three distinguishable deoxystreptamine moieties are also evident; the lower right portion of panel B (Figure 7) traces these. The unexpected 3-*N*-hemisuccinyl derivative was readily recognized by a 0.65 ppm acylation shift. The evidence for acylation of the terminal amino group of the HAP side chain appears in Figure 8. Fraction 121 shows three distinct α/γ RELAY peaks. The most upfield α-resonance shows a two-step coupling to the downfield γ-methylene at 3.325 ppm and is thus identified as the γ-*N*-hemisuccinamide. The set of scalar connected resonances indicated by double primes apparently corresponds to both the 6′-N- and 3″-N-succinylated species.

The contribution of the different regioisomers in the various fractions was estimated by comparing the intensities of diagnostic cross-peaks through the full set of eluted column fractions. The γ-N-derivatized species (hapten VI) was the minor product and was concentrated in the early fractions.

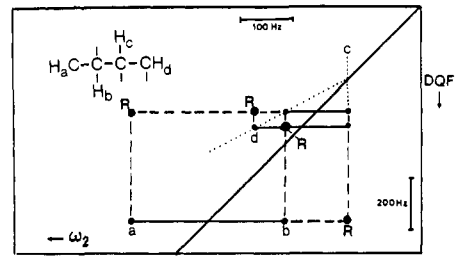


FIGURE 9: Diagramatic DQ spectrum: scalar coupled resonances appear on the same horizontal line at their respective δ_2 values equally displaced about the DQ diagonal. In order to place the DQ diagonal at 45° (and accurately present the resolution in the two dimensions) the DQ frequency axis scale (ω_1) is 2 Hz per hertz in ω_2. Inclusion of the RELAY sequence places additional signals (R) for hydrogens coupled to either member of the DQ coherence on the same line.

The net degree of 3″-N-succinylation was judged from the intensity of the upfield versus downfield H-1″/H-3″ cross-peaks. Hapten III (6′-*N*-succinylamikacin) was a major contributor in all fractions, but no single set of cross-peaks assays specifically for this regioisomer. Hapten III never constituted in excess of 50% of the material present. The 3-N-acylated species appears to be the next most prevalent form (shown by a single prime in Figure 8 and in panel B of Figure 7).

Synthesis of a Copolymerizable Spacer Arm. A molecule with a polymerizable unit distal to the tether point was one of our specific goals. The *p*-vinylbenzoyl function had previously been shown to have the desired copolymerizing characteristics (Auditore-Hargreaves et al., 1987). The monohemisuccinyl fraction (excluding the majority of the early eluting γ-*N*-hemisuccinamide) was therefore treated with an excess of the *N*-hydroxysuccinimide ester of *p*-vinylbenzoic acid at pH 10–9.5. Reverse-phase LC separated the product mixture on the basis of its vinylbenzoyl content. The bis-vinylbenzoylation fractions that were produced in this reaction were, as anticipated, not single pure substances; they could, however, be used in trial studies of antibody tethering methods. The monovinylbenzoyl fraction was judged homogeneous from its 1D ^{1}H NMR (see Figure 10, top portion). A RELAY experiment (data not shown) quickly revealed that no acyl functions were present at N-1, N-3, or N-3″ and provided a nearly complete resonance assignment. The remaining questionable assignments were due to incomplete cross-peak resolution.

The last ambiguities in the resonance assignment of the monovinylbenzoyl compound were resolved by a 2D double quantum RELAY experiment. The DQ-RELAY yields a distinctly different map format that often places RELAY peaks, which may overlap in a COSY format, at different DQ frequencies. The pulse sequence for the DQ-RELAY experiment was modeled after that used by Macura et al. (1984). The general features of DQ spectra are shown in Figure 9. In the present case, the effect of the added relay sequence can be illustrated by the remote connectivities to the γ-CH_2 and α-methine observed at the double quantum frequency corresponding to the HAP side-chain $\beta1/\beta2$ coherence (Figure 10). Continuing in this manner, a complete and unambiguous assignment was derived by using the two data sets. As part of the assignment process, we needed to ascertain which acyl function was at N-6′ and which on the side-chain γ-terminus; since the monovinylbenzoyl fraction was obtained in only modest yield, we could not assume that the hemisuccinylation site in the vinylbenzoylated product was that which predominated in the starting mixture. Pertinent shift values (in comparable aqueous buffer) for the 6′- and γ-succinyl deriv-

Table III: Proton Resonance Assignments for Amikacin Derivatives in Buffered D_2O

	proton chemical shifts (ppm)										
	3-aminoglucose (ring A)				deoxystreptamine (ring B)			HAP			
compd	H-3′	H-4′	H-6a′	H-6b′	H-1	H-2ax	H-6	H-α	H-β1	H-β2	H-γ
III	3.72	3.30	3.58	3.41	4.03	1.51	3.82	4.25	2.15	1.97	3.14
VI	3.74		3.15	2.92	4.01	1.45	3.70	4.12	2.02	1.79	3.33
VII	3.62	3.25	3.54	3.36	3.98	1.70	3.76	4.19	2.09	1.89	3.49

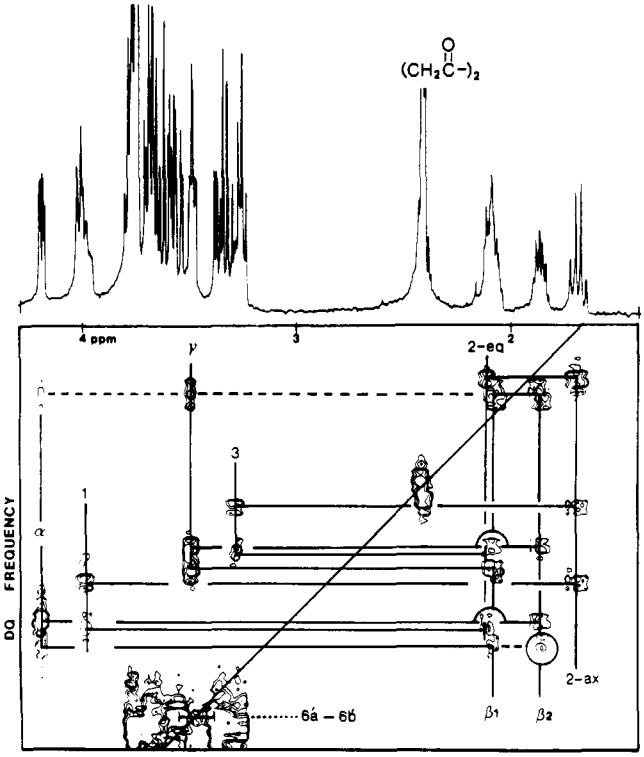


FIGURE 10: (Top) Glycosidic portion (less the anomeric region) of the NMR spectrum of γ-*N*-(*p*-vinylbenzoyl)amikacin *N*-6′-hemisuccinamide. (Bottom) Corresponding sections of the DQ RELAY spectrum: direct connectivities, equally spaced about the DQ diagonal, are shown as solid lines; remote connectivities are encircled or connected by a dashed line.

atives and the mixed bis(acylate) are given in Table III. Clearly the vinylbenzoyl function is on the side-chain pendant on ring B. The γ-CH_2 is unusually deshielded, while certain ring B resonances are shielded, presumably due to the anisotropic effect of the succinate π-system. The ring A resonances are essentially unaltered from their shifts in the 6′-*N*-monohemisuccinyl derivative.

Discussion

In this study, we gained insights into the reactivity of amikacin toward acylating agents. A detailed analysis of the monohemisuccinamide distribution observed suggests an unexpected order of reactivity 6′-N > 3-N ≳ 3″-N ≳ γ-N. Subsequent acylation regiochemistry is dramatically influenced by the initial site of attack. 6′-N-Acylation retards reaction at 3″-N, while γ-N acylation seems to block both 3-N and 3″-N reactions. These effects combined to direct vinylbenzoylation to the γ-N amino group of amikacin 6′-*N*-hemisuccinamide even though the γ-N amino is initially among the least reactive amino groups present. These conclusions concerning initial site reactivity, however, must be confirmed by further studies with more careful pH control. For example, we observed that treatment of amikacin with 0.42 equivalent of SA with the pH maintained between 11 and 10 (rather than between 10 and 9.5) gave predominantly a regioisomer (presumably γ-N- or 3-N-acylated) displaying an upfield HAP α-methine resonance. In another experiment, isomer mixtures previously free of this upfield HAP α-methine signal display it upon standing in pH 10 buffer. This suggests that 3-N-acylation may result from a subsequent transacylation (6′-N → 3-N or, less likely, 3″-N → 3-N). Two-dimensional RELAY experiments should be useful tools for studying these processes.

The relative reactivities of the amikacin amino groups toward acylating agents allowed us to prepare γ-*N*-(vinylbenzoyl)amikacin 6′-*N*-hemisuccinamide without resort to blocking strategies. This material and a variety of bis(vinylbenzoyl)amikacin hemisuccinamides have proven to be tetherable to antibodies, as we had hoped (Priest and Nelson, unpublished results). Our purpose was to attach a readily copolymerizable monomer to a monoclonal antibody in such a fashion that the monomer is readily accessible to the polymerization reactants in aqueous media and thus provide a novel means for signal isolation in immunoassays (Houghton et al., 1986; Nowinski & Hoffman, 1985). These amikacin derivatives should further improve the efficiency of signal incorporation into the antibody/antigen complex and thus increase the speed and sensitivity of this novel immunoassay method.

However, the most important feature of this study is a demonstration of the power of 2D coherence relay spectroscopies as tools for structure elucidation and analysis of mixtures of complex carbohydrates. The methods used in this study, unlike the recently introduced isotropic mixing experiment variously known as TOCSY, long-range relay, or HOHAHA [Braunschweiler & Ernst, 1983; Klevit & Drobny, 1986; Davis & Bax, 1985; for an application to an oligosaccharide see Summers et al. (1986)], can be implemented on any modern NMR instrument. Another limitation of the isotropic mixing experiment is that, at the resolution normally achieved in this experiment, one cannot rely on cross-peak multiplicities to help assign the cross-peaks, and thus one cannot tell how many coherence transfers are represented by a given cross-peak (Weber et al., 1987). The abundance of cross-peaks in an isotropic mixing spectrum, and the uncertainty of the origin of these cross-peaks, would render the isotropic mixing spectrum of a mixture of complex carbohydrates difficult, if not impossible, to interpret. In contrast, the RELAY experiment, with its predominance of one-step coherence relays, is well suited for the study of carbohydrate mixtures.

Supplementary Material Available

Four figures and their extensive legends showing the spectrum and assignment for amikacin in DMSO, the deoxystreptamine assignment in aqueous media by use of different 2D correlated methods, and 1D and 2D plots for hemisuccinylation mixtures derived from amikacin (4 pages). Ordering information is given on any current masthead page.

Registry No. Amikacin, 37517-28-5; amikacin 6′-*N*-hemisuccinamide, 113353-32-5; γ-*N*-(*p*-vinylbenzoyl)amikacin 6′-*N*-hemisuccinamide, 113353-33-6.

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On the Reaction of Ferric Heme Proteins with Nitrite and Sulfite†

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ABSTRACT: Optical and EPR spectroscopy of ferric heme proteins of the porphyrin, oxyporphyrin, and isobacteriochlorin classes has indicated that nitrite reacts with these proteins at the heme iron. Sulfite has been conclusively proven to react only with proteins containing the isobacteriochlorin macrocycle. Quantitative EPR spectroscopy of these nitrite and sulfite adducts showed that most contained a substantial quantity of undetectable heme. It is suggested that protein-induced autoreduction of nitrite (but not sulfite) and a strained and/or uniaxial *g*-tensor are the principal ways by which the silent state is produced.

The iron isobacteriochlorin siroheme (Scott et al., 1978) is the prosthetic group for many of the enzymes involved with the reduction of NO_2^- to NH_3 and of SO_3^{2-} to H_2S. Insofar as the binding of the substrate is concerned, this cofactor requirement appears to be much more stringent for sulfite than for nitrite; i.e., with the possible exception of the cytochrome d_1 of a pair of bacterial dissimilatory nitrite reductases (Sawhney & Nicholas, 1978; Kuronen & Ellfolk, 1972) sulfite has been proven to form a stable ferric complex only with proteins containing the isobacteriochlorin macrocycle. In contrast, oxidized forms of sperm whale myoglobin (Smith & Williams, 1969), human hemoglobin A (Gibson et al., 1969), *Pseudomonas putida* cytochrome P-450 (Sono & Dawson, 1982), *Chaldariomyces fumago* chloroperoxidase (Sono et al., 1986), *Pseudomonas aeruginosa* cytochrome cd_1 (Muhoberac & Wharton, 1983), and *Escherichia coli* cytochrome c_{552}

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