Controlled coupling of aminoglycoside antibiotics to proteins for use in homogeneous enzyme immunoassays

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This paper is dedicated to Professor Peter Yates on the occasion of his 60th birthday

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Selective *N*-acylation of aminoglycoside antibiotics with the *N*-hydroxysuccinimide ester of methyldithioacetic acid, followed by reaction with methanethiol or dithioerythritol, gives sulfhydryl labeled antibiotics. Alternatively, the nucleophilic sulfhydryl group is incorporated into an antibiotic by treatment with *N*-acetyl-*d,l*-homocysteine thiolactone. These derivatives couple readily with proteins that have previously been modified with bromoacetylglycyl groups to provide conjugates for use in the development of homogeneous enzyme immunoassays.

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La *N*-acylation sélective d'antibiotiques aminoglycosidiques, à l'aide de l'ester *N*-hydroxysuccinimide de l'acide méthyldithioacétique, suivie d'une réaction avec du méthanethiol ou du dithioérythritol conduit à des antibiotiques portant des groupes sulfhydryles marqués. On peut aussi incorporer le groupe sulfhydryle nucléophile dans un antibiotique en le faisant réagir avec de la *N*-acétyl *d,l*-homocystéine thiolactone. Grâce à un couplage facile de ces dérivés avec des protéines qui auraient préalablement été modifiées à l'aide de groupes bromoacétylglycyles, on peut obtenir des dérivés conjugués qui peuvent être utilisés pour développer des méthodes immunologiques homogènes de détermination des enzymes.

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Aminoglycoside antibiotics, products of mold metabolites, are commonly given intravenously to patients suffering from severe infection of Gram-negative bacteria. Serious oto- and nephrotoxic side effects are often encountered during the course of therapy (1, 2). This generally happens when the concentration of an antibiotic in patient blood is kept abnormally high for an extended period of time. The optimum therapeutic window for these antibiotics, which differs somewhat from drug to drug, is narrow (3). For example, in serum the therapeutic range of the two most commonly used drugs, gentamicin and tobramycin, is between 2–8 μg/mL while that of amikacin, a semi-synthetic antibiotic, is between 5–25 μg/mL. Titration of the plasma concentration of these antibiotics is often practiced as an aid to therapy and to decrease the risk of undesirable side effects during the course of treatment.

A number of analytical methods based on chromatographic, microbiological, and immunochemical techniques have been used to monitor blood level concentration of aminoglycosides (4, 5). Homogeneous enzyme immunoassay (EMIT®)[3] (6, 7), one of the more versatile immunochemical methods, has found wide acceptance by commercial clinical laboratories for routine monitoring of drugs in biological fluid. It utilizes drug specific antibodies and drug enzyme conjugate as the key reagents. The antidrug antibodies are generally harvested from animals injected at regular intervals with immunogens (drugs coupled to proteins). The conjugates are readily prepared by reacting an activated carboxylic acid derivative of an appropriately functionalized drug with the amino groups of enzymes and carrier proteins. Since the aminoglycoside antibiotics, **1**–**6**, are polyamino compounds (Fig. 1), this coupling method is of limited application for preparation of their protein conjugates. Activation of their carboxylic acid derivatives would result in polymerization. During the course of development of EMIT assays for these antibiotics, we solved this problem by application of a novel three-stage heterobifunctional coupling method (8, 9). It involves (*a*) selective modification of an antibiotic with a nucleophilic sulfhydryl group, (*b*) labeling of the protein with electrophilic bromoacyl groups, (*c*) coupling under gentle experimental conditions.

Although a host of nucleophilic and electrophilic groups can be considered (10), the sulfhydryl and bromoacyl groups were preferred for ease of their reactivity at 0°C in aqueous buffered media.

Appropriately derivatized antibiotics (haptens)[4] are required to achieve their chemical attachment to proteins. Preparation of gentamicin, amikacin, and tobramycin haptens requires only subtle chemical modifications in such a way that most of the structural integrity of these drugs is kept intact. Only a limited chemistry of these aminoglycosides has been reported in the literature. This is presumably due to their complex structures and insolubility in most of the organic solvents. Our approach to preparing their haptens rests on modification of the antibiotic amines with a sulfhydryl group. Because of the general lability of sulfhydryl groups to aerial oxidation, the antibiotic was selectively acylated with methyldithioacetic acid. Cleavage with dithioerythritol or methanethiol gave the desired drug hapten. Alternatively, the nucleophilic suflhydryl group was generated by controlled reaction of an antibiotic with *N*-acetyl-*d,l*-homocysteine thiolactone.

Proteins were modified with a number of bromoacetylglycyl groups. Coupling with the sulfhydryl haptens afforded the desired drug immunogens and the drug enzyme conjugates (Scheme 1).

Gentamicin hapten

Perhaps the most commonly utilized antibiotic of its class, gentamicin is available as a mixture of at least three components, **1**–**3**. It is commonly resolved through liquid–liquid partition chromatography (11). This method is both tedious and time consuming. Because of the quantities of material needed,

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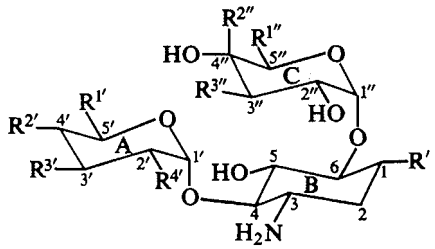
[2] Contribution no. 106 from Syva Research Department.

[3] EMIT® is a trademark for homogenous enzyme immunoassays and is held by Syva Corporation, Palo Alto, California.

[4] A hapten is a compound carrying suitable functionalities, for coupling to proteins.

FIG. 1. Chemical structures for some of the commonly used aminoglycoside antibiotics.



Antibiotic	R^1	$R^{1'}$	$R^{2'}$	$R^{3'}$	$R^{4'}$	$R^{1''}$	$R^{2''}$	$R^{3''}$
1 Gentamicin C_1	NH_2	$CH(CH_3)NHCH_3$	H	H	NH_2	H	CH_3	$NHCH_3$
2 Gentamicin C_1a	NH_2	CH_2NH_2	H	H	NH_2	H	CH_3	$NHCH_3$
3 Gentamicin C_2	NH_2	$CHCH_3NH_2$	H	H	NH_2	H	CH_3	$NHCH_3$
4 Tobramycin	NH_2	CH_2NH_2	OH	H	NH_2	CH_2OH	H	NH_2
5 Kanamycin A	NH_2	CH_2NH_2	OH	OH	OH	CH_2OH	H	NH_2
6 Amikacin	NH $\vert$ $COCH(OH)(CH_2)_2NH_2$	CH_2NH_2	OH	OH	OH	CH_2OH	H	NH_2

Protein — [Lys-$NHCOCH_2NHCOCH_2Br$]$_n$ + Drug—$NHCO\sim SH$

↓

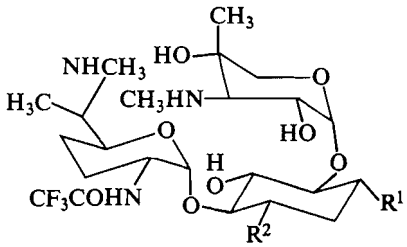
Protein—[Lys-$NHCOCH_2NHCOCH_2S\sim COHN$—Drug]$_n$

Protein = bovine serum albumin, glucose-6-phosphate dehydrogenase
Drug = Gentamicin C_1, amikacin, tobramycin

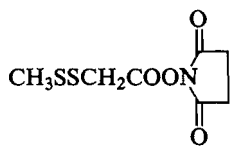
SCHEME 1. Selective coupling of aminoglycosides to proteins.

we considered this procedure to be less attractive and developed a more practical high-pressure liquid chromatographic method for separation. Gram quantities of the pure, individual components are readily available by using this technique.

Each of the antibiotic components **1**–**3** has a number of amino and hydroxyl functionalities. Since gentamicin C_1, **1**, has the least number of primary amino groups and the design of a hapten involved selective *N*-acylation, it was chosen as a starting material. The more reactive $R^{4'}$ amino group was protected by reaction with *S*-ethyl trifluorothioacetate (12). Careful acylation of the product **7** with the *N*-hydroxysuccinimide



7 $R^1 = R^2 = NH_2$
8 $R^1 = R^2 = NHCOCH_2SSCH_3$
9 $R^1 = R^2 = NHCOCH_2SH$



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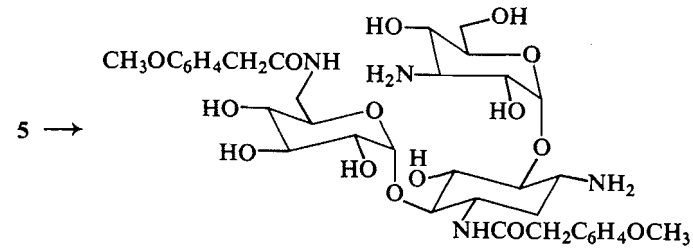
ester of methyldithioacetic acid, **10**, gave a mixture from which diacylated compound **8** was isolated in moderate yields. A small amount of a by-product, considered to be a monoacylated derivative (R^1 or R^2 = $NHCOCH_2SSCH_3$ in **8**) by nmr analysis, could also be obtained. All attempts to improve the yield of the



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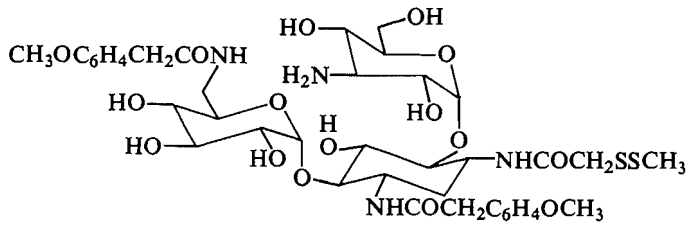

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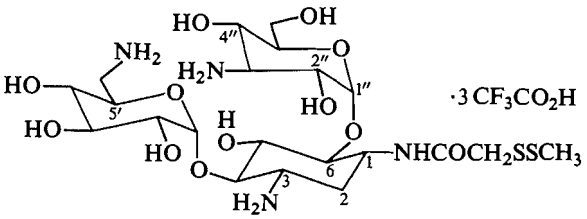

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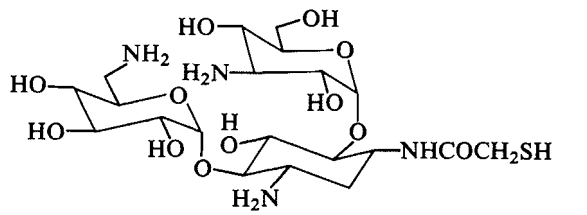

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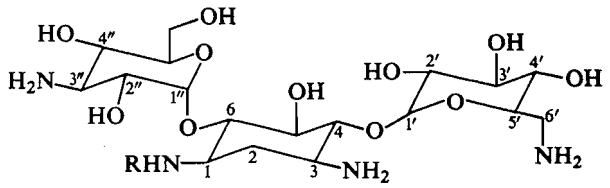
TABLE 1. Carbon-13 chemical shifts, δ values, of kanamycin A, amikacin, and amikacin pro-hapten (structurally significant carbons)



5 R = H, kanamycin A
6 R = $COCH(OH)CH_2CH_2NH_2$, amikacin
13 R = $COCH_2SSCH_3$, amikacin pro-hapten

Carbon nucleus	Kanamycin A			Amikacin			Amikacin pro-hapten		
	pH ≤4	≥11	Δ	≤4	≥11	Δ	≤4	≥11	Δ
1	50.6	51.2		49.5	50.4		49.9	50.4	
2	28.2	35.8	−7.6	30.8	35.0	−4.2	31.0	35.0	−4.0
3	48.4	49.6		48.6	49.4		48.6	49.5	
4	78.8	86.8	−8.0	79.9	87.7	−7.8	80.0	87.6	−7.6
5	73.4	74.9		73.2	75.4		73.2	75.2	
6	84.5	88.5	−4.0	81.2	81.3	0	81.7	82.0	−0.3
5′	69.4	73.0	−3.6	69.5	73.8	−4.3	69.5	73.7	−4.2
2″	68.9	72.5	−3.6	68.8	72.9	−3.9	68.7	72.7	−4.0
4″	66.3	70.0	−3.7	66.4	70.2	−3.8	66.4	70.1	−3.7

monoacylated product failed. The bismethyldithioacetyl derivative was cleaved to hapten **9** with methanethiol for *in situ* coupling to the bromoacetylglycyl modified enzyme, glucose-6-phosphate dehydrogenase (G6PDH) (8).

Amikacin hapten

A relatively new member in its class for treatment of Gram-negative bacteria, amikacin is particularly useful for patients having strains resistant to all other aminoglycosides (13). Hapten **14** was designed so that most of the structural topology of the drug would remain intact and exposed after coupling to proteins. Such a conjugate should produce drug specific antibodies.

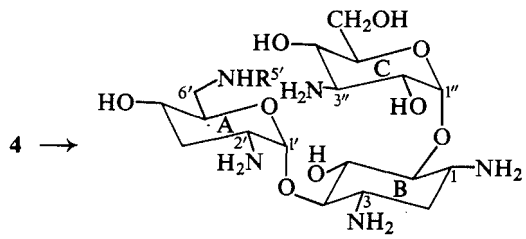
Our synthetic approach for amikacin hapten **14** followed that of the parent drug from kanamycin A (14, 15). The order of reactivity for acylation of amino groups in kanamycin A, **5**, has been determined as C-6′ > C-1 > C-3 > C-3″, which makes its direct transformation to **14** by reaction with **10** less attractive. A more involved route, utilizing the protection of amino groups before acylation, was therefore followed. The C-1 and C-3″ amino groups of **5** were first protected by reacting it with nickel acetate in dimethyl sulfoxide. Treatment of the nickel complex with *p*-methoxybenzyl-5-(4,6-dimethylpyrimidine-2-yl)thiocarbonate, followed by removal of the metal with hydrogen sulfide, gave 3,6′-di-*N*-*p*-methoxybenzyloxycarbonyl kanamycin A, **11**, identical in all respects to an authentic sample (16). Based on the findings of Hanessian and Patel (17) that the vicinal C-3″ amino and C-4″ hydroxyl groups selectively complex with the Group II transition metals such as Cu^{2+} in protic media, **11** was stirred with Ni^{2+} acetate in a 1:1 mixture of tetrahydrofuran and water. The resulting nickel complex, which now has a free C-1 amino group, was then condensed with the activated methyldithioacetic acid **10**. Removal of the metal with Chelex-100® resin gave **12**, which after cleavage of the *p*-methoxybenzyloxycarbonyl protecting groups with trifluoroacetic acid afforded the desired pro-hapten **13** in an overall yield of 15% from kanamycin A. The hapten **14** was readily obtained by stirring it with dithioerythritol under an inert atmosphere for *in situ* coupling to modified proteins. The pure hapten could not be isolated, presumably due to its sensitivity to aerial oxidation.

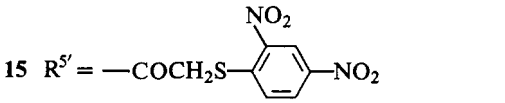
The chemical structure **13** of the amikacin pro-hapten is in accord with its elemental analysis and spectral data. The exact site of acylation was determined from the pH-dependent ^{13}C nmr chemical shift of its carbon nuclei β to amino groups (Table 1). A change in pH from 4 to 11 caused 2,5′, 2″, and 4″ carbon nuclei to experience downfield shifts of ~4 ppm, similar to the ones observed for amikacin (18). As expected, the C-6 nucleus showed insignificant change and the shift of approximately 4 ppm in the C-2 nucleus, one half the value of the same carbon atom in kanamycin A (7.6 ppm shift), is in accord with acylation only at the C-1 amino group.

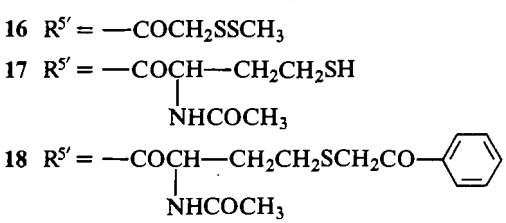
Tobramycin hapten

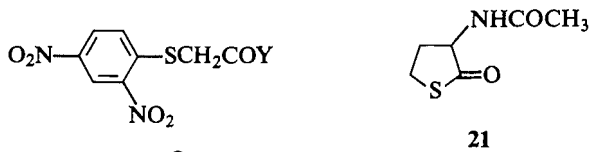
Our strategy to prepare its hapten directly from the parent drug by selective acylation of the C-6 amino group of **4** relied heavily on the relative basicities of the five amino groups of tobramycin, which by pH-dependent nmr studies have been reported in the order C-6′ > C-3 > (C-2′, C-3″) > C-1 (19). Such a hapten would furthermore provide the maximum exposure of the antibiotic ring C after attachment to proteins, the part of structures at which tobramycin differs substantially from gentamicins **1**–**3**. More selective anti-tobramycin antibodies are therefore expected.

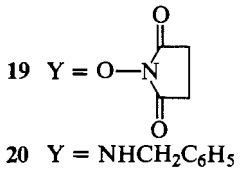
Reaction of tobramycin (**4**) with the *N*-hydroxysuccinimide ester of 2,4-dinitrophenylthioglycolic acid afforded monoacylated product **15**. Analysis of its ultraviolet spectrum at 331 nm and comparison with the benzamide derivative **20** of 2,4-dinitrophenylthioglycolic acid as a model compound clearly showed that only one 2,4-dinitrophenyl moiety had been incorporated into a molecule of **4**. That the monoacylation had indeed taken place only at the C-6 amino group was in accord with the mass spectral fragmentation of the derivative **15**. As expected, appropriate fragments attributed to the cleavage of

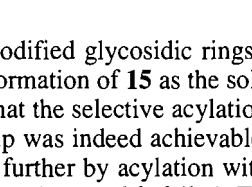












unmodified glycosidic rings B and C were observed (20).

Formation of **15** as the sole monoacylated product established that the selective acylation of tobramycin at the C-6′ amino group was indeed achievable. Our attempt to extend this reaction further by acylation with **10**, with the object of preparing the pro-hapten **16**, failed. The product had an amide carbonyl infrared absorption at 1650 cm^{-1} and showed complete absence of an SCH_3 signal in it proton nmr spectrum. We rationalize these observations as due to the presumed instability of the disulfide group in the product to the experimental conditions.

Amines are reported to open *N*-acetyl-*d,l*-homocysteine thiolactone, **21**, quantitatively (21). Our attention was therefore turned to using this thiolactone as a potential synthon for introduction of a mercapto group into tobramycin. Indeed, when aqueous tobramycin, under high dilution, was stirred under argon, with 0.5 mol-equivalents of the thiolactone **21**, a new compound **17** was obtained as the sole product by tlc analysis. That it is the monoacylated derivative **17** was proven by reaction, without further purification, with ω-bromoacetophenone and by comparing the spectral data of the product **18** with 2-hydroxyethylmercaptophenone as a model compound.

The tobramycin hapten **17**, prepared *in situ*, was used directly for coupling to bromoacetylglycyl modified G6PDH and bovine serum albumin.

Modification of proteins for coupling to aminoglycosides

Development of an EMIT® assay for an aminoglycoside antibiotic requires drug specific antibodies and active drug enzyme conjugates. The immunogens needed for raising the antibodies to antibiotics are often prepared by coupling an aminoglycoside with a carrier protein in the presence of 1-ethyl-3(3-dimethylaminopropyl)carbodiimide (EDAC). Such a method would lead to random coupling of antibiotics, resulting in an heterogeneous mixture of immunogens. This not only leads to a lot-to-lot variation for preparation of immunogens but, in our experience, elicits poor quality (low titer) antisera. As an attempt to solve this problem, the antibiotic immunogens were prepared by using a more controlled heterobifunctional coupling procedure, shown in Scheme 1. For example, the amikacin and tobramycin immunogens were readily prepared by coupling at pH ~ 8 bovine serum albumin, modified with a number of bromoacetylglycyl groups, with the sulfhydryl labeled antibiotic haptens **14** and **17**. Selective reaction of the modified proteins with the sulfhydryl groups of antibiotics is expected because of more than hundred-fold greater reactivity of thiols as compared to amino groups with bromoacyl groups (22). This is in agreement with the observation that only a single product **18** is formed upon reacting **17** with ω-bromoacetophenone under similar conditions. Use of radiolabeled drugs showed that 8–12 haptens could be readily incorporated into proteins using this procedure. Superior titer antibodies were obtained from sheep injected with these immunogens.

Labeling of the enzyme, glucose-6-phosphate dehydrogenase, with aminoglycosides using the direct EDAC coupling procedure turned out to be a more serious problem. The method afforded enzyme conjugates which were generally found inactive, presumably due to extensive polymerization, and therefore could not be used in EMIT® assays. Extension of the protocol similar to the one used for the preparation of antibiotic immunogens afforded useable enzyme conjugates. The procedure has been described previously in detail for preparation of the G6PDH–gentamicin conjugates (8). The method has now been extended for preparation of the G6PDH conjugates of other aminoglycosides, including tobramycin, netilmicin, and amikacin. These conjugates are found useful for development of their EMIT® assays (23, 24).

A brief mention of the method for preparation of the enzyme–amikacin conjugate for use in development of its EMIT® assay is provided as an example. The enzyme, G6PDH, was labeled with a number of bromoacetyl groups by reaction in a buffer at pH ~6, with bromoacetylglycyl-*N*-hydroxysuccinimide ester. The modified enzyme was then treated at 0°C, under an inert atmosphere, with the freshly prepared amikacin hapten **14** in a buffered medium at pH 8. The amikacin–G6PDH conjugate, with a residual activity of ~25% of the native enzyme, was readily inhibited by anti-amikacin antibodies. The conjugate was used to develop its EMIT® assay in the range of 2.5–50 μg/mL. The assay has the usual convenient protocol like other EMIT® assays and provides patient sample quantitation in less than a minute (6, 7).

Experimental

Melting points are uncorrected and were determined with a Thomas–Hoover capillary apparatus. The ir spectra were measured on a Perkin–Elmer 297 spectrometer and the proton nmr spectra were recorded on a 90 MHz Varian EM-390 instrument using tetramethylsilane as an internal standard. The ^{13}C nmr were taken on a Bruker WM-300 with dioxane as an internal standard and uv spectra were recorded on a Cary-15 instrument. Enzyme activity of drug–enzyme conjugates was measured on a Gilford Stasar III spectrometer. Analytical tlc analyses were performed on Analtech silica gel GF and E Merck silanized silica gel 60 plates, using 5% ammonium molybdate – sulfuric acid or 0.1% ninhydrin in isopropanol as sprays for viewing of the developed plates. Preparative tlc work was carried using Analtech 1000-μm thick silica G plates.

Separation of gentamicin isomers 1–3

Ammonia gas was bubbled through a stirring suspension of gentamicin sulfate (60 g) in 750 mL of methanol. After 3 h, the slurry was filtered and the filtrate was evaporated to give 35 g of gentamicin-free base as a white foam. The tlc analysis ($CHCl_3/CH_3OH/NH_4OH$, 10:10:3) showed two major and one minor spots.

Two silica cartridges (Waters preparative 500), in series, were equilibrated with the lower phase of a 2:1:1 mixture of chloroform, isopropanol, and 14% aqueous ammonium hydroxide. A solution of gentamicin-free base (10 g) in methylene chloride (total volume, 15 mL) was loaded onto the first column. Elution with the same mixture of solvents at 300 mL/min afforded fractions which after evaporation gave tlc pure gentamicin C_1 (**1**, 3.39 g). The elution solvent was then made more basic by changing it to the lower layer of a 2:2:1 mixture of chloroform, isopropanol, and concentrated ammonium hydroxide. The fractions were continuously monitored by analytical tlc. Pooling of the like fractions followed by evaporation afforded the remaining two components, namely gentamicin C_2 (**3**, 1.5 g) and gentamicin C_{1a} (**2**, 2.1 g).

N-*Hydroxysuccinimide ester of methyldithioacetic acid (10)*

Into a 50-mL round-bottom flask was placed 1.2 g (8.86 mmol) of methyldithioacetic acid (8), 35 mL of dichloromethane, and 1.09 g (9.54 mmol) of *N*-hydroxysuccinimide (crystallized from ethyl acetate). The solution was cooled in an ice bath, to which was then added 1.96 g (9.54 mmol) of freshly distilled *N,N'*-dicyclohexylcarbodiimide. The reaction mixture was stirred, and after 4 h the solution was filtered through a sintered glass funnel. Precipitated urea was washed with dichloromethane. The light brown filtrate was concentrated under vacuum at ambient temperature. The residue was dissolved in 10 mL of methylene chloride and applied to the top of a 2.5 × 43 cm dry glass column packed with 110 g of silanized silica gel (70–230 mesh, E Merck reagent). Elution with a mixture of hexane/methylene chloride (1:1) afforded **10** as a white solid (1.2 g, 60%). Recrystallization from methylene chloride/hexane afforded crystals, mp 79–81°C; ir (KBr): 1760, 1790, 1830 cm^{-1}; nmr ($CDCl_3$) δ: 2.5 (s, 3H, SCH_3), 2.85 (s, 4H), and 3.74 (s, 2H, CH_2); ms, *m/e*: 235 (M^+), 120, 93. *Anal.* calcd. for $C_7H_9NO_4S_2$: C 35.73, H 3.85, N 5.95, S 27.25; found: C 36.22, H 4.02, N 6.18, S 27.25.

*Preparation of 3-*N*-methyldithioacetyl-2'-*N*-trifluoroacetyl gentamicin C_1 (8)*

Into a 250-mL 3-necked round-bottom flask, equipped with an argon inlet, rubber septum, and magnetic stirrer, was placed 2'-*N*-trifluoroacetyl gentamicin C_1 (**7**, 1.5 g, 2.6 mmol), prepared from gentamicin C_1 according to the literature procedure (11), in 80 mL of dry tetrahydrofuran. To this was added dropwise over 2 h the activated ester **10** of methyldithioacetic acid (560 mg, 2.8 mmol) (8) in 15 mL dimethylformamide. The mixture was allowed to stir overnight at room temperature and the solvent was removed under reduced pressure at 40°C. Careful chromatography of the crude product on a column of silica gel using chloroform/methanol (2:1) followed by chloroform/methanol/17% NH_4OH (2:1:1) yielded two products.

The minor product, R_f 0.3 ($CHCl_3/CH_3OH$/17%NH_4OH, 2:1:1), considered to be a monoacylated derivative, exhibited the following spectral data: ir (KBr): 3300 (NH_2,OH), 1720 (CF_3CO), and 1660 (amide CO) cm^{-1}; ^1H nmr ($CDCl_3$) δ: 1.1 (d, $CHCH_3$ J = 6 Hz), 1.2 (s, CH_3), 2.4 (s, $NHCH_3$), 2.5 (s, S—CH_3), 2.7 (s, $NHCH_3$), 3.4 (s, CH_2SS). The major product, identified as **8**, had its R_f 0.6 and showed bands in its ir spectrum identical to the monoacylated derivative mentioned above. It showed signals in its nmr ($CDCl_3$) spectrum at δ: 1.1 (d, J = 6 Hz, $CHCH_3$), 1.26 (s, $C(OH)CH_3$), 3.40 (s, SCH_2CO). *Anal.* calcd. for $C_{29}H_{50}N_5O_{10}F_3S_4$: C 42.80, H 6.15, N 8.61, F 7.01, S 15.72; found: C 42.47, H 6.17, F 6.90, N 8.32, S 15.98.

*3,6'-Di-*N*-p-methoxybenzyloxycarbonyl kanamycin A (11)*

A solution of 1 *N* sodium hydroxide in deionized water was passed through a column (35 cm × 4 cm) packed with 350 mL of Bio-Rad Ag1X8 resin (20–50 mesh, chloride form) until the eluent showed a negative test for chloride ions. An aqueous solution of kanamycin A sulfate (23 g, Sigma Company) in 2 L of deionized water was then passed through the column at 8 mL/min and the solution collected. The column was further washed with 3 L of deionized water. The total eluent was concentrated under vacuum at 45°C to approximately 400 mL. Methanol (400 mL) was added to the concentrate, which was kept stirring and maintained at about 45°C. A white amorphous precipitate of the free kanamycin A (**5**), mp >245°C (dec.), was formed and collected by filtration. Treatment of the filtrate with an additional 400 mL of methanol afforded a second crop of **5**.

A solution of the kanamycin free base **5** (4.6 g, 9.5 mmol) and nickelous acetate (15 g, 60 mmol) in anhydrous DMSO was stirred in a round-bottomed flask for 30 min. A deep green solution was formed, to which was added dropwise, with stirring, *p*-methoxybenzyl-*S*-(4,6-dimethylpyrimidine-2-yl)thiocarbonate (7.22 g, 23.7 mmol) in 60 mL of DMSO. After stirring overnight the green solution was placed in a 2-L Erlenmeyer flask and treated with a liter of diethylether. The mixture was swirled vigorously, allowed to settle, and the ethereal phase decanted. This was repeated three more times, when a green semisolid material was obtained. The semisolid residue was dissolved in methanol (300 mL) and filtered. Hydrogen sulfide was slowly bubbled through the filtrate for 30 min and the reaction set aside with stirring for 24 h.

The mixture was filtered through a bed of Celite and washed with methanol. To the concentrated filtrate (~300 mL) was added an excess of ethyl acetate and the pH adjusted to 9.0 with ammonium hydroxide. The crude aminoglycoside product **11** separated out as an oily solid. The semisolid material was redissolved in a minimum amount of methanol and reprecipitated with a 1:1 mixture of acetonitrile and ethyl acetate. The solid was filtered, washed with ethyl acetate, and dried under vacuum over P_2O_5. The title compound **11** was obtained as a white solid (5.1 g, 65%), mp 220–235°C (dec.); ir (KBr): 3395 (br), 1700, 1620 cm^{-1}. Its R_f value on a silica tlc plate (CH_3OH/NH_4OH, 9.5:05) and infrared spectrum were identical to those of the authentic sample (16). *Anal.* calcd. for $C_{36}H_{52}N_4O_{17}$: C 53.19, H 6.44, N 6.89; found: C 52.99, H 6.29, N 6.90.

*1-*N*-Methyldithioacetyl-3,6'-di-*N*-p-methoxybenzyloxycarbonyl kanamycin A (12)*

A mixture of **11** (2.5 g, 3 mmol) and nickelous acetate (4 g, 16 mmol) in 100 mL of 1:1 tetrahydrofuran–water was stirred for 1 h. A deep green solution was formed. To the stirring green solution was added dropwise a solution of the activated ester **10** of methyldithioacetic acid (1 g, 4.5 mmol) in tetrahydrofuran (30 mL). The reaction mixture was stirred for 16 h and treated with an excess of Bio-Rad Chelex-100 resin. The mixture was stirred overnight and filtered. The resin was washed with a mixture of tetrahydrofuran–water (1:1) until the filtrate was free of aminoglycoside by tlc analysis. The colorless filtrate was concentrated under vacuum at 20°C, when a white suspension was formed. The mixture was centrifuged. The supernatant was removed and made alkaline to pH 9.5 with ammonium hydroxide. A white precipitate was formed and removed by centrifugation. The precipitate was washed twice with deionized water and once with methanol by repeatedly suspending and centrifuging it in these liquids. The white solid was lyophilized, affording **12** as a tlc pure white powder (1.4 g, 50%), mp 200–205°C (dec.). Its proton nmr spectrum (DMF-d_7) showed signals at δ: 2.46 (s, 3H, S—CH_3), 3.2–4.2 (m), 3.8 (s, OCH_3) 5.0 (br s, 4H, —O—CH_2 Ar), and AA'XX' doublet of a doublet centered at 7.1 (aromatics, 8H) and ir absorption bands (KBr) at 3350 (br), 1700, 1645 cm^{-1}. *Anal.* calcd. for $C_{39}H_{56}N_4O_{18}S_2$: C 50.20, H 6.05, N 6.00, S 6.87; found: C 50.50, H 6.12, N 5.90, S 6.89.

*Amikacin pro-hapten, 1-*N*-methyldithioacetyl kanamycin A, (13)*

Into a dry two-necked flask was placed 1.5 g (1.50 mmol) of **12** (previously dried at 60°C/0.1 Torr over P_2O_5 (1 Torr = 133.3 Pa) and a stream of argon was passed over it. The contents of the flask were cooled in an ice bath and 25 mL ice-cooled trifluoroacetic acid (distilled from P_2O_5) was added with swirling. After 5 min the acid, from the deep purple colored mixture, was removed at room temperature under vacuum (0.1 Torr). The residual solid material was pulverized

and washed with diethyl ether. This was repeated twice with ether and twice with dichloromethane, leaving **13** as an off-white solid. Chromatography on a 250-g silanized silica gel RP-2 column using a gradient of 0–20% of methanol in methylene chloride afforded the pure hapten as a white solid (670 mg, 45%), mp > 185°C (dec.). It showed signals in its ^{1}H nmr spectrum (CD_3OD) at δ: 2.46 (s, 3H, S—CH_3), 5.15 (br s, 1H, —O*CH*O—), 5.5 (br s, 1H, O*CH*O), along with multiplets for other protons between δ 2.8–4.4. *Anal.* calcd. for $C_{21}H_{40}N_4O_{12}S_2 \cdot 3CF_3CO_2H \cdot H_2O$: C 33.60, H 4.69, N 5.80, F 17.70, S 6.64; found: C 33.25, H 4.66, N 5.78, F 17.40, S 6.53.

The ^{13}C nmr spectrum of **13** (D_2O–dioxane) exhibited its carbon signals at the following chemical shifts: for pH ≤4.0, δ: 49.94 (C-1), 30.98 (C-2), 48.60 (C-3), 79.98 (C-4), 73.15 (C-5), 81.70 (C-6), 96.33 (C-1′), 72.86 (C-2′), 71.59 (C-3′ and C-4′), 69.54 (C-5′), 41.09 (C-6′), 98.99 (C-1″), 68.73 (C-2″), 55.98 (C-3″), 66.39 (C-4″), 73.15 (C-5″), 60.57 (C-6″), 22.76 (S—CH_3), 41.65 (S—CH_2), and 173.15 (C=O); for pH ≥11.0, δ: 50.39 (C-1), 35.02 (C-2), 49.51 (C-3), 87.62 (C-4), 75.23 (C-5), 82.02 (C-6), 99.51 (C-1′), 72.69 (C-2′), 71.82 (C-3′ and C-4′), 73.73 (C-5′), 42.27 (C-6′), 100.26 (C-1″), 72.69 (C-2″), 54.42 (C-3″), 70.29 (C-4″), 73.25 (C-5″), 61.0 (C-6″), 42.27 (S—CH_2), and 179.0 (C=O).

2,4-Dinitrophenylthioglycolic acid N-*hydroxy succinimide ester (**19**)*

To a mixture of 2,4-dinitrophenylthioglycolic acid (0.774 g, 3 mmol) and *N*-hydroxysuccinimide (0.345 g, 3 mmol) in 25 mL of freshly distilled THF at 0°C under nitrogen was added *N,N′*-dicyclohexylcarbodiimide (DCC) (0.927 g, 4.5 mmol). The mixture was stirred. After 4 h, a white precipitate was formed and was filtered. The solvent was removed from the filtrate under reduced pressure and the solid residue was triturated with 10% dichloromethane in hexanes. The solid which remained undissolved was collected by filtration. Recrystallization of the solid from ethyl acetate – DMF gave **19** as a light yellow solid (0.854 g, 80% yield), mp 198–199°C; ir (KBr): 1820, 1775, 1740 cm^{-1}; ^{1}H nmr (DMSO-d_6) δ: 8.88 (d, J = 1.5 Hz, 1H), 8.47 (q, J_1 = 5.0, J_2 = 1.5, 1H), 7.90 (d, J = 5.0 Hz, 1H), 4.85 (s, 2H), 2.83 (s, 4H). *Anal.* calcd. for $C_{12}H_9N_3O_8S$: S 40.51, H 2.55, N 11.58, S 9.17; found: C 40.56, H 2.53, N 11.83, S 9.00.

*Reaction of tobramycin with 2,4-dinitrophenylthioglycolic acid NHS ester (**19**)*

To a stirring solution of tobramycin (**4**, 0.92 g, 2 mmol) in 200 mL of water at 0°C, pH 10.76, was added very slowly a solution of 2,4-dinitrophenylthioglycolic acid NHS ester **18** (0.365 g, 1 mmol) in 25 mL of anhydrous DMF. The pH of the solution was maintained at 10.5–10.9 throughout the reaction (10% sodium hydroxide). After addition, the mixture was stirred at 0°C for 2 h and filtered. The filtrate was washed several times with ethyl acetate. The aqueous layer was evaporated to dryness under reduced pressure to give a solid residue. The solid residue was chromatographed on preparative silica gel plates, using chloroform/methanol/ammonium hydroxide (v/v 1:2:1) for elution, to give **15** as an orange solid (0.2 g, 28.3%), mp 210°C (dec.); ir (KBr): 3700–2400, 1645 cm^{-1}; uv (H_2O): 331 nm (ε 6532); EI ms, *m/e* (relative abundance): 163(8), 203(2), 306(1.5), 323(3), 351(2), 407(2.5). *Anal.* calcd. for $C_{26}H_{41}N_7O_{14}S$: S 4.53; found: S 4.74.

*Preparation of benzamide derivative **20***

To a solution of 2,4-dinitrophenylthioglycolic acid NHS ester **18** (0.19 g, 0.54 mmol) in 5 mL of anhydrous DMF at 0°C under nitrogen, was added dropwise a solution of benzylamine (0.06 g, 0.54 mmol). After addition, the mixture was stirred at 0°C for 3 h. The solvent was removed under reduced pressure to give an orange solid residue. Preparative tlc on silica plates using chloroform/methanol (95:5) gave **20** (0.16 g, 85%), which upon recrystallization from methanol gave deep yellow needles, mp 148–151°C; ir (KBr): 3300, 1640 cm^{-1}; uv (50% THF in water): 335 (ε 9000); nmr (DMSO-d_6) δ: 8.78 (d, 1H), 8.35 (q, 1H), 7.80 (d, 1H), 7.20 (s, 5H), 4.28 (d, 2H), 4.05 (s, 2H); *Anal.* calcd. for $C_{15}H_{13}N_3O_5S$: C 51.60, H 3.80, N 12.00, S 9.32; found: C 51.72, H 3.80, N 12.69, S 9.20.

*Reaction of tobramycin with activated methyldithioacetic ester (**10**)*

To a stirring solution of tobramycin (0.723 g, 1.55 mmol) in 20 mL of dimethylformamide, 20 mL of water, and 10 drops of triethylamine at 0°C and under nitrogen was added very slowly a solution of methyldithioacetic acid NHS ester (**10**, 0.364 g, 1.55 mmol) in 4 mL of anhydrous DMF. After addition, the mixture was stirred at 0°C for 2 days. The solvent was removed under reduced pressure to give a white residue. The solid residue was chromatographed on silica plates, elution with methanol/acetic acid/water (3:1:1) or methanol/chloroform/ammonium hydroxide (2:1:1) gave a solid product which showed in its ir spectrum an amide band at 1650 cm^{-1}. Its ^{1}H nmr spectrum, however, showed the absence of an S—CH_3 signal.

Reaction of tobramycin with N-*acetyl*-d,l-*homocysteine thiolactone (**21**)*

A steady stream of dry nitrogen was bubbled through a solution of tobramycin (0.934 g, 2 mmol) in 75 mL of water at pH 10.12 for 30 min. To this stirring solution was added slowly (15 drop/s) a solution of the homocysteine thiolactone **20** (0.159 g, 1 mmol, Sigma Chemicals) in 5 mL of oxygen-free tetrahydrofuran. The pH of the mixture was maintained at 10.12–10.25 throughout the addition with 10% sodium hydroxide. After addition, the mixture was stirred for 1 h. The pH of the solution was adjusted to 7 with 5% hydrochloric acid. The solution was washed carefully with oxygen-free ethyl acetate (4 × 30 mL) and the aqueous layer was collected. The pH of the solution was adjusted to 10.12 with 10% sodium hydroxide. Lyophilization of the solution gave a white solid (ca. 800 mg). The analytical silica gel tlc showed a single new product (R_f 0.39) and the unreacted starting tobramycin (R_f 0.10) using methanol/chloroform/ammonium hydroxide (2:1:1) as the solvent system.

The mixture obtained by the addition of tobramycin to *N*-acetylhomocysteine thiolactone (**4** + **17**, 700 mg) was dissolved in 25 mL of oxygen-free water. The pH of the solution was adjusted to 7.5 with 5% hydrochloric acid. To this solution was added slowly a solution of ω-bromoacetophenone (0.1 g, 0.5 mmol) in 5 mL of THF at 0°C under nitrogen. The pH of the reaction mixture was maintained at 7.1–7.5 with 10% sodium hydroxide solution. After addition, the mixture was stirred for 2 h and filtered. The filtrate was washed with ethyl acetate and lyophilized to give a white solid residue. The solid was chromatographed on silica tlc plates; elution with chloroform/methanol/ammonium hydroxide (1:2:1) gave **18** as a white solid (60 mg); ir (KBr): 3700–2400, 1640 cm^{-1}; uv (H_2O): 250 nm (ε 10 496). The 2-hydroxyethylmercaptoacetophenone, prepared in 91% yield by condensing bromoacetophenone with 2-mercaptoethanol, had its ultraviolet maxima in ethanol at 242 nm (ε 10 014).

Bromoacetylglycine N-*hydroxysuccinimide ester*

To a solution of bromoacetylglycine (463 mg, 2.37 mmol) and *N*-hydroxysuccinimide (273 mg, 2.37 mmol) in 15 mL of anhydrous tetrahydrofuran at 0°C, under nitrogen, was added *N,N′*-dicyclohexylcarbodiimide (DCC) (732 mg, 50% excess). The resulting solution was stirred at 0°C for 4 h, when a white precipitate had formed. The precipitate was removed by filtration and the filtrate evaporated to dryness under vacuum. The solid residue was triturated with a 1:4 mixture of dichloromethane in hexanes. The solid was collected by filtration, and recrystallization from dichloromethane – ethyl acetate – hexanes gave the title compound as ten colored crystals (400 mg, 58%), mp 122–123°C; ir (KBr); 3270, 1830, 1780, 1650 cm^{-1}; ^{1}H nmr ($CDCl_3$ – DMSO-d_6) δ: 2.88 (s, 4H), 3.90 (s, 2H), 4.55 (d, 2H), 8.50 (m, 1H). *Anal.* calcd. for $C_8H_9NO_5Br$: C 32.76, H 3.07, N 9.56, Br 27.30; found: C 32.62, H 2.99, N 9.42, Br 27.53.

Labeling of bromoacetylglycine–NHS ester to bovine serum albumin (BSA)

To a solution of BSA (644 mg, 0.01 mmol) in 32 mL of 0.1 *M* borate buffer, pH 8.44, at 0°C was added dropwise a solution of bromoacetylglycine–NHS ester (293 mg, 1 mmol) in 5 mL of dry DMF. All solutions were saturated with argon before use. The addition took 20 min. The conjugation was closely monitored by pH meter. While the initial pH of the protein solution was 8.44, it dropped

to around 8.0 and was maintained to ±0.5 pH units with 0.1 *N* sodium hydroxide or 0.1 *N* hydrochloric acid. The pH, after additions of the NHS ester, was adjusted to 8.04; the solution was allowed to stir in an ice bath for 40 min, after which time the pH had changed to 7.15. This pH was again adjusted to 6.8 with the acid. The bromoacetylglycyl labeled BSA conjugate, about 50 mL, was purified by passing through a 100-ml G-50 Sephadex column using deionized water as eluent. Appropriate fractions were pooled and used either immediately or the following day (stored in cold room).

Conjugation of amikacin hapten ***14*** *to BSA. Formation of an immunogen*

The amikacin pro-hapten **13** (44 mg) was dissolved in 2 mL of methanol and taken into a 10-ml round-bottomed (or a pear shaped) flask. A slow stream of argon was bubbled through the solution for 30 min. To this stirring solution under argon was now added dithioerythritol (180 mg) in degassed methanol (6 mL), along with a drop of triethylamine. Progress of the reaction was continuously monitored by using silica tlc plates. After completion of the reaction (ca. 2 h), the solution was dried by evaporation and the residue stored under nitrogen.

The bromoacetylglycyl-labeled BSA solution (ca. 10 mL) prepared from 60 mg of BSA was degassed by bubbling a slow stream of argon. The pH of the solution was maintained around 7.3 using 0.2 *M* phosphate buffer. To this solution at 0°C was now added dropwise the cleaved amikacin hapten **14** in 0.5 mL of DMF (degassed with argon). The reaction mixture was stirred overnight in the cold room. The BSA–amikacin conjugate was dialyzed in a Spectropor membrane tubing (mol. wt. cutoff 6000–8000), dialyzed against 4 L of deionized water, and adjusted to pH 8.0 with ammonium hydroxide. The dialyzing medium was changed three times after every 3 h. The residual conjugate in the membrane tube was centrifuged at ~10,000 rpm (to remove any suspended material) and the liquid was lyophilized to give 56 mg of the conjugate.

*Conjugation of tobramycin-*N*-acetyl-*d,l*-homocysteine thiolactone (17) with bromoacetylglycine-labeled G6PDH*

To a solution of bromoacetylglycine-labeled G6PDH (3.3 mL, 0.0245 μmol of G6PDH) prepared as described elsewhere (8), at 0°C, pH 7.7 (adjusted with 10% NaOH), was added in 5-μL portions a solution of **17** (36.8 mg, only 25% of which is assumed to be the desired mercaptotobramycin) in 0.5 mL of 0.01 *M*, pH 6.8 phosphate buffer. The pH of the reaction solution was maintained at around 7.5–7.7 throughout the reaction with 5% hydrochloric acid. The reaction was constantly followed by checking the enzyme activity on a Gilford spectrophotometer. The reaction was stopped when a total of 240 μL of the tobramycin sulfhydryl derivative **17** had been added. No loss of the enzyme activity was noticed throughout the reaction. The solution of tobramycin–G6PDH conjugate was stored at 0°C for 5 days and chromatographically purified by a Sephadex G-50 column (eluting with 0.01 *M*, pH 6.8 phosphate buffer) for use in an EMIT® assay.

Conjugation of amikacin hapten ***14*** *to glucose-6-phosphate dehydrogenase*

The amikacin hapten **14**, freshly prepared by cleaving the disulfide linkage of **13** as described above, was coupled to bromoacetylglycine-labeled G6PDH by a procedure identical to the one described for the preparation of gentamicin–G6PDH conjugates (8). The amikacin–G6PDH conjugate, with 25–30% of the native enzyme activity, was ~75% inhibitable by addition of anti-amikacin antibodies. Most of the inhibited activity was reversible by addition of the drug.

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