MUTASYNTHESIS OF NEW ANTIBIOTICS

Kenneth L. Rinehart, Jr.
University of Illinois, Urbana, Illinois 61801, USA

Abstract - Studies of the biosynthesis of aminocyclitol antibiotics, especially neomycin, have led to development of the mutasynthetic technique for the preparation of related antibiotics. A specific type of mutant strain of the microorganism which normally produces the aminocyclitol antibiotic is first prepared. The mutant sought is one unable to produce the aminocyclitol subunit and thus it can produce the normal antibiotic only in the presence of added native aminocyclitol. Alternatively, it can produce related (mutasynthetic) antibiotics in the presence of other aminocyclitols (mutasynthons). Mutasynthesis is not limited to aminocyclitol antibiotics but it has now been used to produce compounds related to essentially all of the clinically useful aminocyclitol antibiotics. Not all potential mutasynthons are accepted by the mutants and some accepted lead to antibiotics more widely altered than by the simple substitution of a natural by an unnatural aminocyclitol. Thus, mutasynthesis has considerable potential in the study of the structural requirements for bioactivity as well as in the design of new antibiotics.

The search for new antibiotics follows many trails (1,2). The classical biological approach is that of screening, in which a soil sample is examined for the microorganisms which produce antibiotics. Although somewhat random, this method has led historically to some of the greatest successes in chemotherapy through the discovery of tetracycline, cephalosporin, chloramphenicol and erythromycin, to name but a few. As screening has become more expensive and the probability of finding a new antibiotic statistically less attractive, newer approaches to the development of antibiotics have been investigated. A number of these newer procedures are at least partially chemical in nature. Total synthesis has only rarely provided useful new antibiotics due to the complexity of the structures involved, with their many asymmetric centers. However, partial synthesis has provided us with the semisynthetic penicillins and other useful chemotherapeutic agents (3), while chemical modification of older antibiotics, such as the conversion of rifamycin B to rifampicin (4) and that of lincomycin to clindamycin (5), has also proved successful. What I shall discuss here is a different approach to the preparation of new antibiotics, a procedure for which we have coined the term mutasynthesis.

The mutasynthetic technique is an outgrowth of our studies on the biosynthesis of aminocyclitol antibiotics (6) and I should like to turn first to that subject. Representatives of this class of antibiotics are characterized by the presence of an aminocyclohexanol or related subunit and include streptomycin and neomycin, which were among the first antibiotics discovered. The class has maintained a place of importance in chemotherapy through the more recent additions of kanamycin, gentamicin, spectinomycin, and ribostamycin to the list of clinically useful compounds. Clinically useful antibiotics of this class are shown in Figs. 1-3. As is evident from the figures the class can be further divided into subclasses consisting of those which contain deoxystreptamine (neomycins, ribostamycin, kanamycins, gentamicins) and those which do not. Among the latter, streptomycin (which contains streptidine, a substituted streptamine) and spectinomycin (which contains actinamine, a substituted 2-epistreptamine) are the most important.

The particular aminocyclitol antibiotic we chose to study first was neomycin, whose structure, shown in Fig. 1, we had assigned over a period of several years (7). Neomycin is produced by Streptomyces fradiae and is a potent inhibitor of gram-positive organisms, including Mycobacteria. Used initially in the treatment of tuberculosis, its nephrotoxicity and ototoxicity have more recently restricted its use to topical applications and oral administration prior to intestinal surgery. Biosynthesis of neomycin has been mainly studied by adding labeled precursors to cultures of Streptomyces fradiae growing in shake flasks, isolating the antibiotic, and locating label. Radioactive precursors were originally employed (8), but a more convincing demonstration of the nature of the precursors has been obtained using 13C labeling and 13C NMR to locate label (9).
Fig. 1. 4,5-Disubstituted deoxystreptamine antibiotics.

Fig. 2. 4,6-Disubstituted deoxystreptamine antibiotics.
Before the $^{13}$C incorporation results could be interpreted, of course, all the signals in the $^{13}$C NMR spectrum of neomycin B had to be assigned and $^{13}$C-labeled precursors had to be prepared. For the study of its $^{13}$C NMR spectrum, the antibiotic was first converted to its hexa-N-acetyl derivative, whose spectrum is shown in Fig. 4. This spectrum was then reconstructed from those of its subunits shown in Fig. 5, starting with that of the simplest, di-N-acetyl-deoxystreptamine, and proceeding through tetra-N-acetylneamine to tetra-N-acetylribostamycin which, with methyl di-N-acetylneosaminide B, provided a composite model for hexa-N-acetylneomycin B (10). Once the $^{13}$C NMR spectrum of unlabeled hexa-N-acetylneomycin B was established, it was simple to interpret the spectrum of the same compound, also shown in Fig. 4, isolated from biosynthetic experiments employing [6-$^{13}$C]glucose and [1-$^{13}$C]glucosamine. Synthesis of these precursors followed the routes shown in Fig. 6.

Fig. 5. Subunits of neomycin B employed as model compounds in assigning $^{13}$C NMR spectrum of neomycin B (10).
Fig. 4. Proton-decoupled $^{13}$C NMR spectrum of hexa-N-acetyleneomycin B: top spectrum, unlabeled; second from top, labeled by D-[6-$^{13}$C]glucose; third, labeled by D-[1-$^{13}$C]glucosamine.
Mutasynthesis of new antibiotics

Fig. 6. Syntheses of D-[6-13C]glucose (9), D-[1-13C]glucosamine (9), and D-[15N]glucosamine (41).

These spectra demonstrated that C-6 of glucose labels C-6 of neosamines B and C (units B and C, respectively, in Fig. 5) and C-5 of ribose (unit R in Fig. 5) as well as C-2 of deoxystreptamine (unit D in Fig. 5), that C-1 of glucosamine labels C-1 of neosamines B and C, C-1 of ribose and C-1 of deoxystreptamine (Fig. 7). These experiments also demonstrated that glucosamine is the more direct precursor of the neosamines, but glucose is the more direct precursor of deoxystreptamine and ribose. The latter result was confirmed by an experiment employing [15N]glucosamine, which demonstrated that isotope was incorporated into the neosamines but not into deoxystreptamine. Thus, the biosynthetic labeling pathway shown in Fig. 8 was established for the neomycin subunits.

More important from the standpoint of the development of the mutasynthetic technique was the demonstration of steps beyond the subunits. Of the four C5 and C6 units only D-ribose and deoxystreptamine could be demonstrated to be incorporated directly into neomycin; neosamine C (and presumably neosamine B) was not. These incorporation studies were carried out (Fig. 9) by administering radioactive precursors [1-14C]deoxystreptamine, [1-14C]ribose (11) and [1-14C]neosamine C (12) to S. fradiae and locating the label in the appropriate subunit of neomycin.

The demonstration that deoxystreptamine was incorporated as a unit led us to adopt a different approach, the mutant approach, in our biosynthetic studies. Specifically, a mutant of Streptomyces fradiae was sought which could not biosynthesize deoxystreptamine, hence could not biosynthesize neomycin. The experiments were carried out by treating a growing culture of S. fradiae with a mutagen (normally nitrosoguanidine) to kill nearly all of the cells, in the hopes that the few survivors had mutated. This was easily tested by replicate plating—growing the survivors in media both with and without added deoxystreptamine. Such a mutant (a D" mutant) of S. fradiae was found after many attempts (13). In addition to confirming the direct place of deoxystreptamine on the biosynthetic pathway the mutant allowed the testing of other potential neomycin precursors containing deoxystreptamine—neamine and ribostamycin (14), ribosyl-deoxystreptamine and neobiosaminy(1B)
Fig. 7. Biosynthetic labeling of neomycin B by D-[1-14C]glucose (8), D-[6-13C]glucose (6) and D-[O-15C]glucosamine (9).

Fig. 8. Proposed biosynthetic pathways from 1- and 6-labeled glucose and glucosamine to labeled neomycin B subunits.
Most importantly, the existence of this D' mutant allowed us to test the concept of mutasynthesis. The mutant of *S. fradiae* which could produce neomycin only in the presence of added deoxystreptamine was next tested for whether aminocyclitols closely related to deoxystreptamine would be accepted by the mutant as replacements for deoxystreptamine and used to produce new antibiotics related to neomycin. This is the technique for producing new antibiotics which we now call mutasynthesis. Correspondingly, any antibiotics produced by the procedure are referred to as mutasynthetic.

Note a: The latter two compounds were provided by Professor S. Hanessian, University of Montreal.
antibiotics, while the surrogate aminocyclitols added to the fermentation broth are referred to as mutasynthons.

In a successful mutasynthesis experiment at least four distinct steps are required: preparation of the mutasynthon, incorporation of the mutasynthon, isolation and characterization of the mutasynthetic antibiotic, and biological evaluation of the mutasynthetic antibiotic. We shall turn first to the preparation of the mutasynthons.

In initial studies with the Streptomyces fradiae mutant (17), a total of 30 aminocyclitols and substituted aminocyclitols were tested as mutasynthons and others have been tested in later studies (18). These compounds are shown in Figs. 11-13. Several were readily prepared by hydrolysis of other antibiotics, such as streptidine and streptamine (from

\[
\begin{array}{cccc}
R & R' & R'' & R''' \\
\text{NHAc} & \text{NHAc} & \text{H} & \text{H} \\
\text{NHAc} & \text{NHAc} & \text{H} & \text{OH} \\
\text{NHAc} & \text{NHAc} & \text{OH} & \text{H} \\
\text{NHCONH}_2 & \text{NHCONH}_2 & \text{H} & \text{OH} \\
\text{NHCH}_3 & \text{NHCH}_3 & \text{H} & \text{H} \\
\text{NHCH}_3 & \text{NHCH}_3 & \text{OH} & \text{H} \\
\text{N(CH}_2)_3 & \text{N(CH}_2)_3 & \text{H} & \text{H} \\
\text{N(CH}_2)_3 & \text{N(CH}_2)_3 & \text{OH} & \text{H} \\
\text{N(CH}_2)_3 & \text{N(CH}_2)_3 & \text{H} & \text{OH} \\
\text{N(CH}_2)_3 & \text{N(CH}_2)_3 & \text{H} & \text{H} \\
\text{NH}_2 & \text{NH}_2 & \text{H} & \text{Cl} \\
\text{NH}_2 & \text{NH}_2 & \text{H} & \text{NH}_2 \\
\text{OH} & \text{OH} & \text{H} & \text{H} \\
\text{OH} & \text{OH} & \text{H} & \text{OH} \\
\text{OCONH}_2 & \text{NH(=NH)NH}_2 & \text{H} & \text{OH} \\
\text{NH} & \text{NH} & \text{H} & \text{OH} \\
\text{OH} & \text{OH} & \text{H} & \text{OH} \\
et
\end{array}
\]

*Epimer

\[
\begin{array}{cccc}
R & R' & R'' & R''' \\
\text{H} & \text{I} & \text{H} & \text{OH} \\
\text{OH} & \text{OH} & \text{H} & \text{OH} \\
\text{OH} & \text{H} & \text{OH} & \text{NH}_2 \\
\text{OH} & \text{H} & \text{OH} & \text{OH}^* \\
\text{H} & \text{H} & \text{OCH}_3 & \text{OCH}_3 \\
et
\end{array}
\]

*Epimer

Fig. 11. Unsuccessful attempts to incorporate mutasynthons into neomycin or neamine analogs (17).
Mutasynthesis of new antibiotics

Fig. 12. Preparation of mutasynthetic antibiotics related to neomycin (13,19).

Fig. 13. Additional mutasynthons giving bioactive neomycin or neamine analogs (15,16).

streptomycin), bluensidine (from bluensomycin), actinamine (from spectinomycin), and hyosamine (from hygromycin B), or hydrolysis of modified antibiotics, such as 6-O-methyldeoxystreptamine from N-acetyl-0-methylneomycin and 5,6-di-O-methyldeoxystreptamine from N-acetyl-0-methylneamine. Other aminocyclitols, such as 2,5- and 2,6-dideoxystreptamine (or their polyacetyl derivatives) were obtained from other laboratories (Note b), while still others, e.g., 2-epi-, 6-epi, and 2,6-diepistreptamine, were synthesized in our laboratory by routes involving cyclization of nitrohexosamines (20).

The second step involved in mutasynthesis of a new antibiotic is the one least under the control of the chemist, since the organism either accepts the mutasynthon or it does not. A standard fermentation of the mutant strain is carried out except that the mutasynthon, the aminocyclitol, is added to the fermentation medium. If, after completion of the fermentation, the broth inhibits growth of an appropriate test organism, a new antibiotic may be

Note b: Especially that of Professor T. Suami at Keio University.
presumed to have been formed, although it must first be isolated and characterized. With the original mutant strain of Streptomyces fradiae, only two aminocyclitols served as successful mutasynthons--streptamine and 2-epistreptamine (Fig. 12). However, additional mutants of S. fradiae (Note c) have been tested as well, and some of these mutants gave new antibiotics with mono-N-methyldeoxystreptamine, streptidine, 2,5- and 2,6-dideoxystreptamines, and 2-bromo- and 6-bromostreptamines in addition to streptamine and 2-epistreptamine (Figs. 12, 13). 2,6-Dideoxystreptamine has also recently been employed by Gero, Akhtar, et al., to give 6-deoxynemycin from a mutant of S. fradiae (19).

Isolation and purification of the mutasynthetic antibiotics follow standard procedures for neomycin, mainly ion exchange and ion exclusion chromatography. The mutasynthetic antibiotic produced by addition of streptamine was termed hybrimycin A and that from 2-epistreptamine hybrimycin B, the name hybrimycin being applied since their structures (Fig. 12) were in a sense "hybrids" of neomycin and streptomyacin or neomycin and spectinomycin. Paper chromatography demonstrated that hybrimycins A and B consisted of two components each (hybrimycins A1 and A2, B1 and B2) which appeared from Rf values and rotational differences to be related to one another as neomycin B is related to neomycin C (13).

More conclusive evidence has been provided by mass spectrometry. In earlier characterization it was necessary to derivatize the highly polar antibiotics to obtain their mass spectra. Those chosen, the N-acetyl-0-trimsyl and the N,O-trimsyl derivatives, have quite high molecular weights (Fig. 14), but they are sufficiently volatile for direct probe electron impact mass spectrometry and the pertrimyl derivatives will even pass through a gas chromatograph for combination with mass spectrometry (21). Characteristic fragment ions are also observed in their mass spectra (Fig. 14). More recently we have characterized mutasynthetic antibiotics by field desorption mass spectrometry (22). This new technique, which ionizes molecules in the solid state on a specially prepared emitter wire is ideally suited for non-polar molecules of the aminocyclitol class. Mass marking, formerly a problem with field desorption mass spectrometry, is now carried out with hexakis-(multifluoroalkoxyl)-sym-triphosphazenes (23). Under conditions of low emitter current molecular ions are almost the only ions seen, while with higher emitter current fragment ions are more prominent (Fig. 15). Both the electron impact mass spectral studies with the derivatives and the field desorption studies with under-derivatized hybrimycin B2 demonstrate that the central aminocyclitol ring contains an extra hydroxyl group in hybrimycin B2.

A still more powerful technique for the characterization of mutasynthetic antibiotics is 13C NMR spectroscopy. The 13C NMR spectrum of neomycin displays

---

Note c: Obtained from Dr. G. Kimura, Tokyo Tanabe Co.
Fig. 15. Field desorption mass spectra of neomycin B, of subunits of neomycin B, and of hybrimycin B2, whose structure is shown in Fig. 12. Note the shift of all peaks of neomycin B by 16 amu in the spectrum of hybrimycin B2, due to its extra oxygen atom.

Identifiable resonances for all 23 carbon atoms, as we saw in Fig. 4. By comparing the $^{13}$C NMR spectrum of hybrimycin B1 with that of neomycin C (Fig. 16) one can see immediately that the methylene carbon (C-2, at 29.6 ppm) of deoxystreptamine has been lost; it is replaced by a new carbinol absorption, located at 70.0 ppm, while the only other carbons at slightly different chemical shifts in the two antibiotics are those near C-2 of deoxystreptamine, i.e., C-1, C-3, C-4, and C-6. Thus, hybrimycin B1 is 2-hydroxyneomycin C (Fig. 12). These techniques have combined to establish hybrimycins A1 and A2 as 2-hydroxyneomycins B and C and hybrimycins B1 and B2 as 2-epi-p-hydroxyneomycins B and C. Additional confirmation of the structural assignments derives from hydrolysis of hybrimycins A1 and A2 to hybrimycin A3, a neamine analog and similar hydrolytic results with hybrimycins B1 and B2 to give hybrimycin B3. The structure assigned to 6-deoxyneomycin (Fig. 12) by Gero, et al. (19), was based on hydrolytic experiments, which gave 6-deoxyneamine (which in turn gave 2,6-dideoxystreptamine) and methyl neobiosaminides B and C.

2,5-Dideoxystreptamine cannot, of course, give a neomycin analog, since it lacks the requisite 5-hydroxyl group for attachment to ribose. Thus, the mutasynthetic antibiotic produced is an analog of neamine instead, i.e., 5-deoxyneamine. The latter compound has recently been prepared synthetically by Suami, et al. (24), and it is of some interest to compare the two routes, as shown in Fig. 17. The synthetic route starts with neamine (thus, ultimately, with neomycin) and gives 5-deoxyneamine in 9% yield after 9 steps (24). The mutasynthetic route starts with 1,4-cyclohexadiene and requires 5 steps to produce 2,5-dideoxystreptamine in 24% yield (25), which is then converted mutasynthetically in one step to 5-deoxyneamine. The mutasynthetic procedure is clearly simpler, though the overall yield depends on the percentage incorporation of 2,5-dideoxystreptamine into 5-deoxyneamine.
Fig. 16. $^{13}$C NMR spectra of neomycin B and the related mutasynthetic antibiotic hybrimycin B1, whose structure is shown in Fig. 12.

Fig. 17. Synthetic and mutasynthetic routes to 5-deoxyneamine. The overall yield in the purely synthetic route (top line) was 9% (24), while the overall yield to 2,5-dideoxy streptamine (bottom line) was 24% (25).
In addition to the work described above with Streptomyces fradiae, mutasynthetic studies have also been carried out with a number of other microorganisms which produce aminocyclitol antibiotics. Paromomycin (Fig. 1) is produced as a minor component of crude neomycin; thus, it is not surprising that Streptomyces rimosus forma paromomycinus, which produces paromomycin, can be induced to mutate to a non-producing strain which cannot form deoxystreptamine. The mutant strain of S. rimosus has thus far only been shown to accept two mutasynthons: streptamine, which produces the mutasynthetic antibiotics hybrimycins C1 and C2 (14), and 2,6-dideoxystreptamine, which produces 6-deoxyparamomycins I and II (19). Structures of these antibiotics (Fig. 18) have been assigned by methods precisely analogous to those described above for the neomycin analogs.

Since ribostamycin is actually a part of neomycin (Fig. 1), one might a priori expect the mutasynthetic technique to be successful with Streptomyces ribosidificus, which produces ribostamycin. This has been confirmed by Kojima and Satoh in the Meiji Seika laboratories (26). The deoxystreptamine-lacking mutant of S. ribosidificus was similar to those described above in its acceptance of deoxystreptamine-related mutasynthons (Fig. 19) but was unusual in that it accepted neamine as well as deoxystreptamine, and gave a mutasynthetic antibiotic (3',4'-dideoxyribostamycin) from 3',4'-dideoxyneamine (26). This argues that the biosynthesis of ribostamycin proceeds from deoxystreptamine to neamine to ribostamycin, which probably has significance for the biosynthesis of neomycin as well. The structures of the mutasynthetic ribostamycin analogs were established by mass spectrometric comparison to that of ribostamycin. The mutasynthetic route to 3',4'-dideoxyribostamycin (which starts with neamine) (27) can be compared to the purely synthetic route, which starts with ribostamycin (Fig. 20) (28).

A D mutant has also been reported recently from Bacillus circulans, which normally produces butirosin, a ribostamycin derivative (29). Unlike the S. ribosidificus mutant, it did not form the parent antibiotic (butirosin) in the presence of neamine (or of ribostamycin, or of the L-N-L-(γ-amino-α-hydroxybutyryl) derivatives of deoxystreptamine or neamine (Fig. 21). The D mutant of B. circulans has been employed to prepare 2-hydroxybutirosin from streptamine and 5-deoxybutirosamine from 2,5-dideoxystreptamine. The structures of the mutasynthetic antibiotics were assigned by hydrolysis to subunits and by mass spectra of the antibiotics' N-acetyl O-methyl derivatives, which are of lower molecular weight than the O-trimsyl analogs.

We shall now shift from the 4,5- to the 4,6-disubstituted deoxystreptamine antibiotics (Fig. 2). Mutants of S. kanamyceticus which cannot synthesize deoxystreptamine have been obtained both in our laboratory (14) and those of Meiji Seika (Kojima and Satoh) (26). Pseudodisaccharides such as neamine, kanosaminyl-deoxystreptamine, or 6-amino-6-deoxyglucosyl-deoxystreptamine are not incorporated. Similar results with regard to potential mutasynthon specificity were obtained by both groups. 2-Epistreptamine was incorporated in both laboratories and N-methyldeoxystreptamine by Kojima and Satoh. Structures of the two mutasynthetic antibiotics from 2-epistreptamine and 1-N-methyl-deoxystreptamine (Fig. 22) were, surprisingly, not the simple analogs resulting from one-to-one replacement of deoxystreptamine by the mutasynthon. In addition, the 6-aminoglucose moiety of kanamycin was replaced in both mutasynthetic antibiotics by glucose itself. The S. kanamyceticus mutant example provides an important caveat for mutasynthetic antibiotic research—that the structures of the antibiotics cannot be assumed but must be proved.
Gentamicin is produced by species of the Micromonospora rather than the Streptomyces genus. Nevertheless, the biosynthesis of deoxystreptamine can be presumed to be the same for both genera and the mutasynthetic approach could be anticipated to be successful. This has been shown to be the case by Testa, et al., who prepared a mutant of *M. iny Genesis* which could not form deoxystreptamine (31). This organism normally produces sisomycin, an unsaturated analog of gentamicin C13, and the D' mutant was able to convert the mutasynthons streptamine, 2-epistreptamine, and 2,5-dideoxy-streptamine to mutasynthetic antibiotics (Fig. 23), called mutamicins. More mutamicins were formed than expected and, although the structures of some of the mutasynthetic antibiotics formed were those expected, involving simple replacement of deoxystreptamine by the mutasynthon, the structures of others involved more extensive changes. Of particular interest was the formation of a 5-deoxygentamicin A analog, since gentamicin A has recently been shown to be a biosynthetic precursor of sisomicin with this same D' mutant (32). Methods used to assign the structures of the mutamicins have not yet been described. The unexpected number of the mutamicins illustrates again the potential pitfall of assuming the structures of the mutasynthetic antibiotics in advance and argues strongly for careful characterization of the products.

Fig. 19. Preparation of mutasynthetic antibiotics related to ribostamycin (29).

Fig. 20. Comparison of mutasynthetic (26,27) and synthetic (28) routes to 3',4'-dideoxyribostamycin.
Fig. 21. Preparation of mutasynthetic antibiotics related to butirosin (29).

Fig. 22. Preparation of mutasynthetic antibiotics related to kanamycin (26). A 6'-hydroxyl replaces the 6'-amino group of kanamycin A (Fig. 2).
Fig. 23. Preparation of mutasynthetic antibiotics related to sisomicin (31). Mutamicin 2a is an analog of gentamicin A. The 3″-methylamino group is replaced by acetamino and amino groups in mutamicins 1a and 1b, respectively.

As noted at the beginning of this discussion of mutasynthetic antibiotics, there are a number of aminocyclitol antibiotics which contain aminocyclitols other than deoxystreptamine. The most important of these are the streptomycins, but before turning to mutasynthesis of antibiotics related to streptomycin, we must first discuss the biosynthesis of streptomycin itself, since it differs somewhat from that of neomycin and has been even more extensively studied (33,34). Investigations with $^{14}$C- and $^{13}$C-labeled glucoses have shown that glucose provides all the carbon atoms of streptomycin except the N-methyl carbon of N-methyl-L-glucosamine (which is derived from methionine) and the guanido carbons of streptidine (which are derived by transfer from arginine). For the most part the glucose skeleton is incorporated unrearranged (Fig. 24), though C-3 of glucose becomes the 3′-formyl carbon of...
Mutasynthesis of new antibiotics

streptose. Our own attention was primarily directed toward the streptidine ring, and $^{13}$C NMR spectroscopy was able to show that C-6 of glucose becomes C-6 of streptidine, as well as C-6' of N-methyl-L-glucosamine and C-5' of streptose (35). The $^{13}$C NMR spectrum of unla-
beled streptomycin, shown in Fig. 25, together with the spectrum of streptomycin labeled by D-[6-$^{13}$C]glucose, was assigned by consideration of the model compounds streptidine, methyl dihydrostreptobiosaminide, N-methyl-L-glucosamine, dibenzyl a-L-dihydrostreptoside (Fig. 26) and dihydrostreptomycin, and the variations in the $^{13}$C NMR spectrum of dihydrostreptomycin engendered by changes in pH (Fig. 27) (36).

![Proton-decoupled $^{13}$C NMR spectra of streptomycin](image)

The differences between the biosyntheses of deoxystreptamine and streptidine are shown first by the difference in labeling of the aminocyclitol by C-1 of glucose, which labels C-1 of deoxystreptamine but C-5 of streptidine, and by C-6 of glucose, which labels C-2 of deoxy-
streptamine but C-6 of streptidine. Moreover, myo-inositol is not incorporated into deoxy-
streptamine (12) but is incorporated well into streptidine (33). Finally, while deoxy-
streptamine is incorporated well into neomycin, streptamine has been reported not to be incorporated specifically into streptomycin (37). All of these considerations are well accounted for by the biosynthetic scheme proposed for streptidine by Walker (Fig. 28) (38).

With the differences between the biosyntheses of streptidine and deoxystreptamine in mind, it is clear why streptamine should not be incorporated into streptomycin: it never appears on the biosynthetic pathway of Fig. 28. On the other hand, streptidine itself does appear on the pathway, at least as its 6-phosphate, which is hydrolyzed in a perhaps reversible reaction. Curiously, no one appears to have tried to incorporate labeled streptidine into streptomycin, though streptidine was shown early to label all three C6 subunits of strepto-
mycin (37). Shier early made brief attempts to obtain a mutant of S. griseus lacking the ability to form streptidine (an $S^-$ mutant) but was unsuccessful (39). More recently,
Fig. 26. Subunits of dihydrostreptomycin employed as model compounds in assigning $^{13}$C NMR spectrum of dihydrostreptomycin (36).

Fig. 27. Carbon signals of streptomycin shifting with changes in pH (36). The location of the urethan group of bluensomycin (Fig. 3) can be assigned from the changes in chemical shift of the streptidine carbons (36).
Mutasynthesis of new antibiotics

Nagaoka and Demain have been successful in obtaining such an S" mutant and have also been successful in employing the mutasynthetic approach by adding the mutasynthon 2-deoxystreptidine to a growing culture of the mutant to produce an antibiotic different from streptomycin (Fig. 29) (40). Thus far, the mutasynthetic antibiotic has not been isolated or characterized, so it is unclear whether it represents a simple replacement of streptidine by deoxystreptidine or a more extensively modified antibiotic.

The final example of mutasynthesis involves Streptomyces spectabilis, which produces the aminocyclitol antibiotic spectinomycin (as well as the unrelated ansamycin antibiotic streptovaricin, but that is a separate subject). As noted above, spectinomycin (Fig. 3) contains the aminocyclitol actinamine (N,N'-dimethyl-2-epistreptamine) and actinamine's biosynthesis might resemble that of deoxystreptamine or that of streptidine. The biosynthesis of spectinomycin has been investigated by our own group (41) and those of Mitscher and Martin (42) and by Slechta and Coats (43) and, in fact, there are elements of both the involved in actinamine biosynthesis.

The labeling pattern from D-[6-13C]glucose resembles that of streptidine rather than that of deoxystreptamine (Fig. 30) (41). The 13C NMR spectra of unlabeled spectinomycin and of the antibiotic from the [6-13C]glucose feeding are shown in Fig. 31. Carbon atoms were assigned by comparison with actinamine and by shifts of carbons with varying pH. myo-Inositol is incorporated into actinamine, as it is into streptidine (42). On the other hand, 2-epistreptamine (didemethylactinamine) is also incorporated into actinamine (43), whereas streptamine is not incorporated into streptidine. A mutant of S. spectabilis which cannot synthesize actinamine (an A" mutant) was employed in the last experiment and in separate experiments tritium-labeled actinamine, 2-epistreptamine + [methyl-14C]methionine and N-methyl-2-epistreptamine all gave labeled spectinomycin, identified by spots which were both radioactive and antibacterial (Fig. 32).

The same A" mutant was also used in mutasynthetic experiments employing streptamine in the presence of [methyl-14C]methionine or [3H]N,N'-dimethylstreptamine, which both gave radioactive spots near those for spectinomycin. However, there was no antibacterial activity.
Fig. 30. Biosynthetic labeling of spectinomycin by D-[6-13C]glucose (41).

Fig. 31. Proton-decoupled 13C NMR spectra of spectinomycin (top spectrum, unlabeled; bottom, labeled by D-[6-13C]glucose). Labeled carbons are C-6 (66.9 ppm), C-6' (20.5 ppm), and the two N-methyl carbons (31.6, 32.0 ppm).
Mutasythesis of new antibiotics

Fig. 32. Labeling of spectinomycin by radioactive actinamine and methionine, employing an actinamine-requiring mutant of *Streptomyces spectabilis* (43).

Associated with the material from these experiments (Fig. 33). The mutasynthetic product was not isolated, but its most likely structure is 2-epispectinomycin. However, it would have to be biologically inactive, a rather surprising result. 2-Epispectinomycin has recently been synthesized (44) by chemical modification of spectinomycin (Fig. 34) and the compound is indeed devoid of antibacterial activity (Figs. 31, 32). While this does not prove the structure of the mutasynthetic product from the *S. spectabilis* mutant, it lends credibility to a likely 2-epispectinomycin structure. Since the usual rapid assay for incorporation of a mutasynth has been a test for antimicrobial activity,
we have long recognized the possibility of incorporation to give a bioinactive product. However, the recently demonstrated inactivity of 2-epispectinomycin lends additional impetus to a reinvestigation of many of the aminocyclitols which did not give new antibiotics.

We have now described all of the mutasynthetic aminocyclitol antibiotics thus far reported and can summarize them in Table 1.

**TABLE 1. Summary of mutant organisms, mutasynthons and mutasynthetic antibiotics**

<table>
<thead>
<tr>
<th>Mutant (Ref.)</th>
<th>Normal Antibiotic</th>
<th>Mutasynthon</th>
<th>Mutasynthetic Antibiotic</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. fradiae (D) (13, 16, 19)</td>
<td>Neomycin</td>
<td>Streptamine</td>
<td>2-Hydroxyneomycins B, C (hybrimycins A1, A2)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2,6-Dideoxystreptamine</td>
<td>6-Deoxyneomycins B, C</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6-O-Methyldeoxystreptamine</td>
<td>Not isolated</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2-Methyldeoxystreptamine</td>
<td>Not isolated</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2,6-Bromo-2-deoxystreptamine</td>
<td>Not isolated</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2,6-Dideoxystreptamine</td>
<td>Not isolated</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Streptidine</td>
<td>Not isolated</td>
</tr>
<tr>
<td>S. rimosus (O') (14, 19)</td>
<td>Paromomycin</td>
<td>Streptamine</td>
<td>2-Hydroxyparomycins I, II (hybrimycins C1, C2)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2,6-Dideoxystreptamine</td>
<td>6-Deoxyneomycins I, II</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2,5-Dideoxystreptamine</td>
<td>Not isolated</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6-O-Methyldeoxystreptamine</td>
<td>Not isolated</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1-N-Methyldeoxystreptamine</td>
<td>Not isolated</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2-Bromo-2-deoxystreptamine</td>
<td>Not isolated</td>
</tr>
<tr>
<td>S. ribosidificus (O') (26)</td>
<td>Ribostamycin</td>
<td>Streptamine</td>
<td>2-Hydroxyribostamycin</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Epistreptamine</td>
<td>6-Deoxyribostamycin</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1-N-Methyldeoxystreptamine</td>
<td>1-N-Methylribostamycin</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3',4'-Dideoxystreptamine</td>
<td>3',4'-Dideoxyribostamycin</td>
</tr>
<tr>
<td>B. circulans (O') (30)</td>
<td>Butirosin</td>
<td>Streptamine</td>
<td>2-Hydroxybutoxirins</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2,5-Dideoxystreptamine</td>
<td>5-Deoxybutoxirins</td>
</tr>
<tr>
<td>S. kanamyceticus (O') (26)</td>
<td>Kanamycin</td>
<td>Streptamine</td>
<td>2-Hydroxy-6'-deaminoo-2-epihydroxykanamycin A</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6'-Hydroxy-6'-deaminoo-1-N-methylkanamycin A</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>6'-Hydroxy-6'-deaminoo-1-N-methylkanamycin A</td>
<td></td>
</tr>
<tr>
<td>M. inydes (O') (31)</td>
<td>Sisomicin</td>
<td>Streptamine</td>
<td>2-Hydroxysisomicin (mutacin 1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3'-N,N-Dimethyl-3'-N-acetyl-2-hydroxsisomicin (mutacin 1a)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>3'-N,N-Dimethyl-2-hydroxsisomicin (mutacin 1b)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>5-Deoxysisomicin (mutacin 2)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>5-Deoxyribostamycin A (mutacin 2a)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>2-Epihydroxsisomicin (mutacin 4)</td>
<td></td>
</tr>
<tr>
<td>S. spectabilis (A') (43)</td>
<td>Spectomycin</td>
<td>Streptamine</td>
<td>Not isolated (bioinactive)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>N,N'-Dimethylstreptamine</td>
<td>Not isolated (bioinactive)</td>
</tr>
<tr>
<td>S. griseus (C') (40)</td>
<td>Streptomycin</td>
<td>Deoxystreptidine</td>
<td>Not isolated (streptomycin A)</td>
</tr>
</tbody>
</table>

Four steps in a mutasynthesis study were listed earlier—1) preparation and 2) incorporation of the mutasynthons, and 3) isolation and characterization and 4) evaluation of the mutasynthetic antibiotics. The last step has not been discussed until now, but it represents, in a sense, the "bottom line" of a mutasynthetic investigation. In order to be developed the new antibiotics must have advantages over those previously available. Very little definitive information has thus far appeared, perhaps because yields of the mutasynthetic antibiotics are usually not very high and evaluation of any new drug is time-consuming. The most abundant information is that available for the hybrimycins (see below) but the fragmentary information on others is summarized in Table 2.

The hybrimycins have been compared with several other aminocyclitol antibiotics for their activity against twelve microorganisms (46). The order of bioactivity observed was gentamicin > hybrimycin A > neomycin ≥ kanamycin B > hybrimycin B > ribostamycin. A similar order was observed against *Mycobacterium tuberculosis*. In a more extensive comparison against 56 microorganisms hybrimycin A and neomycin were of similar activity; again, hybrimycin B was less active. The mode of action of the hybrimycins is the same as that of their parent antibiotics, the neomycins, which cause misreading of the ribosomal codon (47).

Since the antibacterial activities of the hybrimycins are at best about as high as those of the corresponding neomycins their principal clinical interest would appear to lie in their potentiality for reduced toxicity. Results along these lines have only very recently become available and they are quite encouraging. The acute toxicities (intravenous and subcuta-neous) of both hybrimycins A and B are somewhat less than those of neomycin. More importantly, the nephrotoxicity and hepatotoxicity of hybrimycin A are much lower (ca. one-half) than those of neomycin and hybrimycin B (46).

Though these initial biological evaluations are less than spectacular, the future of the mutasynthetic approach appears quite promising. Presumably some day a superior mutasynthetic antibiotic will be prepared. Moreover, the technique can be employed for other purposes—the study of structure-activity relationships, the investigation of enzyme
TABLE 2. Relative antibacterial activities of mutasynthetic antibiotics and the corresponding antibiotics of the wild strains

<table>
<thead>
<tr>
<th>Mutasynthetic Antibiotic</th>
<th>Normal Antibiotic</th>
<th>Ratio</th>
<th>Remarks (Ref.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-Hydroxyneomycin &lt; Neomycin</td>
<td>0.17-1.3</td>
<td>(13)</td>
<td></td>
</tr>
<tr>
<td>2-Hydroxyneamine &lt; Neamine</td>
<td>0.06-0.25</td>
<td>(45)</td>
<td></td>
</tr>
<tr>
<td>2-Epihydroxyneomycin &lt; Neomycin</td>
<td>0.04-0.67</td>
<td>(13)</td>
<td></td>
</tr>
<tr>
<td>2-Epihydroxyneamine &lt; Neamine</td>
<td>0.02-0.25</td>
<td>(45)</td>
<td></td>
</tr>
<tr>
<td>6-Deoxyneomycin B &lt; Neomycin B</td>
<td>-------</td>
<td>&quot;Very similar&quot; except vs. E. coli, P. mirabilis, S. aureus, and S. typhimurium (19)</td>
<td></td>
</tr>
<tr>
<td>6-Deoxyneomycin C &lt; Neomycin C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-Hydroxyparomomycin I &lt; Paromomycin I</td>
<td>0.33-0.67</td>
<td>(14)</td>
<td></td>
</tr>
<tr>
<td>2-Hydroxyparomomycin II &lt; Paromomycin II</td>
<td>0.10-0.67</td>
<td>(14)</td>
<td></td>
</tr>
<tr>
<td>2-Hydroxyparomamine &lt; Paromamine</td>
<td>0.02-1.0</td>
<td>(14)</td>
<td></td>
</tr>
<tr>
<td>6-Deoxyparomomycin I &lt; Paromomycin I</td>
<td>0.25</td>
<td>(19)</td>
<td></td>
</tr>
<tr>
<td>6-Deoxyparomomycin II &lt; Paromomycin II</td>
<td>0.25</td>
<td>(19)</td>
<td></td>
</tr>
<tr>
<td>2-Hydroxyribostamycin &lt; Ribostamycin</td>
<td>0.10</td>
<td>(26)</td>
<td></td>
</tr>
<tr>
<td>2-Epihydroxyribostamycin &lt; Ribostamycin</td>
<td>&lt;0.10</td>
<td>(26)</td>
<td></td>
</tr>
<tr>
<td>1-N-Methylribostamycin &lt; Ribostamycin</td>
<td>0.25</td>
<td>(26)</td>
<td></td>
</tr>
<tr>
<td>3',4'-Dideoxyribostamycin &lt; Ribostamycin</td>
<td>0.25-2.0</td>
<td>Active against kanamycin- and ribostamycin-resistant P. aeruginosa and E. coli (26)</td>
<td></td>
</tr>
<tr>
<td>2-Hydroxybutirosin &lt; Butirosine</td>
<td>0.1-1.0</td>
<td>(30)</td>
<td></td>
</tr>
<tr>
<td>5-Deoxybutirosamine &lt; Butirosamine</td>
<td>0.5-8.0</td>
<td>(30)</td>
<td></td>
</tr>
<tr>
<td>6'-Hydroxy-6'-deaminone-2'-epihydroxykanamycin A &lt; Kanamycin A</td>
<td>-------</td>
<td>Weak bioactivity (26)</td>
<td></td>
</tr>
<tr>
<td>6'-Hydroxy-6'-deaminone-1-N-methylkanamycin A &lt; Kanamycin A</td>
<td>-------</td>
<td>Weak bioactivity (26)</td>
<td></td>
</tr>
<tr>
<td>2-Hydroxisomicin &lt; Sisomicin</td>
<td>0.1-1.0</td>
<td>Active against gentamicin-sisomicin-tobramycin adenylating K. pneumoniae and E. coli (31)</td>
<td></td>
</tr>
<tr>
<td>5-De oxy sisomicin &lt; Sisomicin</td>
<td>0.1-1.0</td>
<td>Active against gentamicin-sisomicin acetylating P. aeruginosa (31)</td>
<td></td>
</tr>
</tbody>
</table>

In conclusion, I should like to express my appreciation to my colleagues, past and present, who have contributed to the development of the ideas and techniques involved in mutasynthesis. I have already noted the paramount contribution of Dr. W. L. Shier. Biosynthetic studies on neomycin were carried out by Drs. J. L. Foght, R. F. Schimbor, and F. C. Falkner, on streptomycin by Dr. M. H. G. Munro, on spectinomycin by Dr. R. M. Stroshane, with contributions to the required 13C NMR interpretation by Dr. S. T. Truitt, to the synthesis of labeled compounds by Drs. S. Ogawa and M. Taniguchi, and to field desorption mass spectrometry by Mr. K. L. Olson. I thank also Dr. G. Kimura, Tokyo Tanabe, Dr. J. P. Rolls, The Upjohn Co., and Dr. T. H. Stoudt, Merck and Co., for fruitful collaborations, Mrs. L. S. Shield, Mrs. J. Zvilius and Miss. M. Vanko for assistance with the preparation of this manuscript, and the National Institute of Allergy and Infectious Diseases for their generous support of our efforts for many years.

Notes added in proof:
Very recent studies with a deoxystreptamine-lacking mutant of Micromonospora purpurea have demonstrated that streptamine and 2,5-dideoxystreptamine are incorporated by this mutant into 2-hydroxygentamicins and 5-deoxygentamicins, respectively, and that addition of 2,4,6/3,5-pentahydroxycyclohexanone (myo-inosose, scyllo-inosose) gives 2-hydroxygentamicins while 2,4/5-tetrahydroxycyclohexanone (a deoxyinosose) gives the gentamicins complex itself.

REFERENCES
52. S. J. Daum, D. Ross, and W. A. Goss, J. Antibiot., in press.