2.15 (s, 3 H), 3.71 (s, 3 H), 3.81 (dd, 1 H, J = 10, 2.5 Mz) 5.10 (d, 1 H, J = 2.5 Hz), 5.15 (m, 1 H), 5.35 (m, 1 H).

Acknowledgments. We wish to thank Drs. D. R. Diener and K. Ruetzler for collection and identification, respectively, of the biological materials. This research was supported by grants from the National Institutes of Health (AI-11969; RR-00708 to UCSD NMR Facility) and the Sea Grant Program, Department of Commerce (04-6-158-44110).

Registry No.—1, 66940-35-0; 2, 66940-36-1; 3, 66940-37-2; 4, 66940-38-3; 5, 66940-39-4; 6, 66940-40-7; 7, 66940-41-8; 8, 66940-42-9; 10, 66940-43-0; 11, 66940-44-1; 11 acetate, 66940-45-2; 12, 66984-56-3.

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Biosynthesis of the Anthracycline Antibiotics Nogalamycin and Steffimycin B

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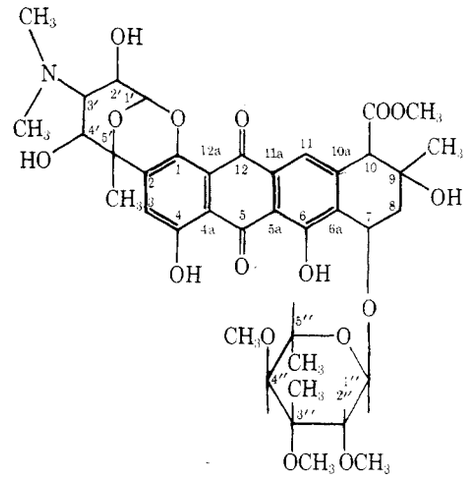
Received March 20, 1978

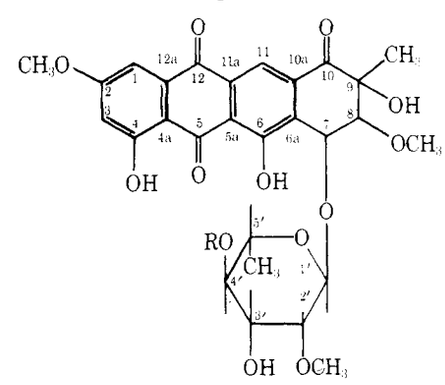
It has been shown that the aglycones of nogalamycin (1) and steffimycin B (3) arise from ten acetate units starting with the methyl groups at C-9. The neutral sugars are derived from glucose, while CH_3O and CH_3N methyl groups come from methionine.

The antibiotic nogalamycin (1) has been of interest as an antitumor agent for a number of years.[1] Some of its conversion products are even more active in this respect, and their antitumor properties are being extensively investigated.[2] Furthermore, 1 is a member of the anthracycline antibiotic family of which one member, adriamycin, is widely used in cancer chemotherapy.[3] Steffimycin (2) and steffimycin B (3) are also anthracycline antibiotics although they are only very modestly active as antitumor agents. However, the steffimycins are members of a subgroup of three anthracyclines whose structures differ markedly from the other anthracyclines. For these reasons it was felt that it would be worthwhile to investigate the biosynthesis of 1, 2, and 3 and compare their biosynthesis with those of daunomycin[4] and ϵ-pyrromycinone[5] which have already been reported. In the case of daunomycin only biosynthesis of the aglycone was established, but in the present work the biosynthesis of the sugars was also studied.

The procedure utilized to study the biosynthetic pathways of 1 and 3 was addition of ^{13}C-labeled compounds which might logically be expected to act as antibiotic precursors to fermentations of *Streptomyces nogalater*, UC-2783, and *Streptomyces elgreteus*, UC-5453, grown on minimal media. The ^{13}C-enriched 1 and 3 formed by *S. nogalater* and *S. elgreteus*, respectively, was isolated, and the positions of the ^{13}C-enriched carbon atoms established by ^{13}C NMR spectra. As a result of previous work[4,5] and current concepts of biosynthesis, it seemed very probable that both aglycones would be built completely from acetate units. For example, it has been shown[4] that the aglycone of daunomycin arises through a polyketide intermediate derived from acetate and one unit of propionate with loss of the terminal carboxyl group. Ollis and co-workers[5] have proposed a similar biosynthetic pathway for ϵ-pyrromycinone, the aglycone of rutilantin. A common biosynthetic pathway for formation of hydroxyanthraquinones by fungi is the condensation of ten acetate units.[6] Accordingly, *S. nogalater* and *S. elgreteus* fermentations in appropriate carbon-poor media were enriched with $CH_3{}^{13}COONa$ and $^{13}CH_3COONa$ to give 1 and 3 labeled with



1



2, R = H
3, R = CH_3

^{13}C. Isolation of the products was carried out, and ^{13}C NMR spectra were obtained to establish the positions of the carbon atoms enriched with ^{13}C. Similar procedures were used, but

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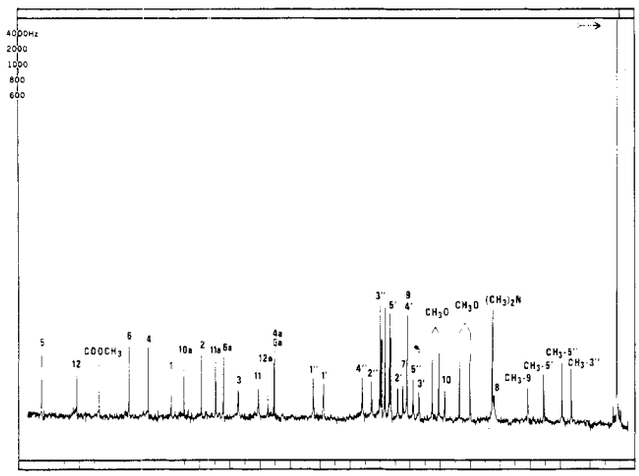

Figure 1. ^{13}C NMR spectrum ($CDCl_3$) of nogalamycin at natural abundance ^{13}C concentration.

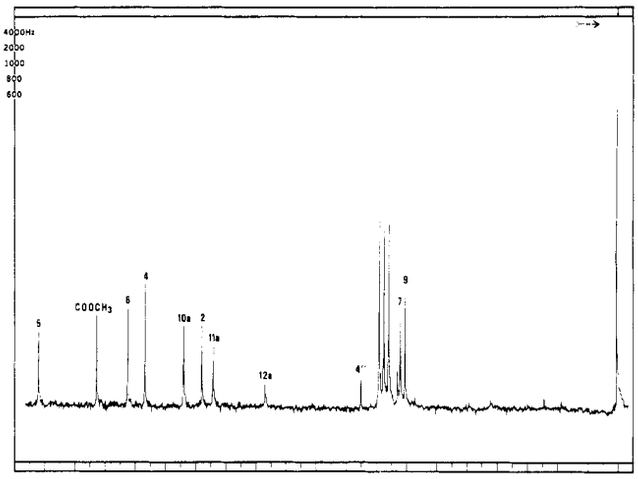

Figure 3. ^{13}C NMR spectrum ($CDCl_3$) of nogalamycin from cultures supplemented with $CH_3{}^{13}COONa$.

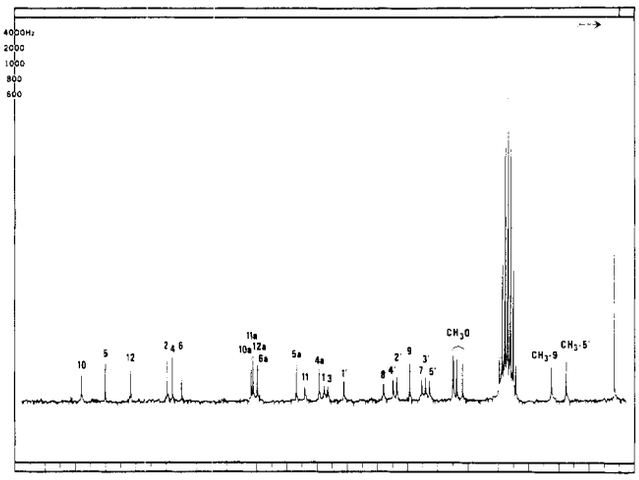

Figure 2. ^{13}C NMR spectrum (Me_2SO-d_6) of steffimycin B at natural abundance ^{13}C concentration.

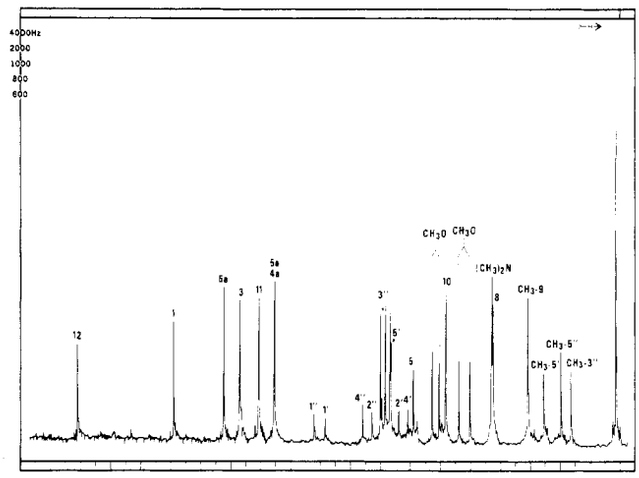

Figure 4. ^{13}C NMR spectrum ($CDCl_3$) of nogalamycin from cultures supplemented with $^{13}CH_3COONa$.

adding $^{13}CH_3$-labeled methionine and uniformly ^{13}C-labeled glucose, to study incorporation of one-carbon units and sugar biosynthesis.

Before discussing biosynthesis, it is necessary to reassign some of the ^{13}C chemical shifts previously published for 1.[7] The ^{13}C NMR spectrum of 1 was originally taken in $CDCl_3$ and C-7 and C-4′ were assigned chemical shifts of δ 69.7 and 71.1, respectively. The spectra of several of the analogues were taken in DMF-d_7, and in the spectrum of the analogue which has nogalose replaced by methoxyl, the chemical shift assigned to C-7 was δ 72.5. This discrepancy was somewhat surprising. A ^{13}C NMR spectrum of 1 has now been taken in DMF-d_7, and the resulting differences in chemical shifts of some of the carbon atoms makes it necessary to reverse the C-7 and C-4′ assignments. In the DMF-d_7 spectrum, the peaks for C-2′, C-3′, and C-5′ have moved downfield by δ 0.6 while the peak originally assigned to C-4′ has moved downfield by δ 2.2. The peak movements are much more consistent if the chemical shift originally assigned to C-7 is assigned as C-4′ which would then have moved downfield by δ 0.6 in DMF-d_7. Also, the new value agrees much better with that for C-7 in the analogue mentioned above. In the DMF-d_7 spectrum, the peaks for C-8, C-9, and C-10 have undergone very substantial changes from the values obtained for them in $CDCl_3$ with C-8 and C-9 peaks moving upfield by δ 1.3 and 1.1, respectively, while the C-10 peak moved downfield by δ 1.2. The change of δ 2.2 downfield for the reassigned value of C-7 would fit in with these reasonably large changes which probably are a result of conformation differences in the two solvents perhaps arising from DMF-d_7 binding of the C-9 hydroxyl group. In order to investigate the chemical shifts assigned to C-4a and C-12a, about which there was some doubt, a gated decoupling spectrum was run in DMF-d_7. In such a spectrum, C-4a should show coupling with the proton at C-3, but C-12a should be a singlet as no protons are in appropriate positions for coupling. The spectrum showed a well-defined singlet at δ 116.9 establishing that this value should be assigned to C-12a as the δ 116.1 peak in the $CDCl_3$ spectrum moved to δ 116.9 in DMF-d_7. The peaks at δ 115.0 and 114.7 showed so much splitting that the exact coupling was not established. However, these results make necessary the assignment of peaks at δ 116.1 and 114.1 in the $CDCl_3$ spectrum of 1 to C-12a and C-4a, respectively.

The chemical shifts in the ^{13}C NMR spectrum of 1 have thus been completely assigned,[7] and the spectrum is shown in Figure 1. The spectra derived from 1 enriched with ^{13}C by addition of $CH_3{}^{13}COONa$ and $^{13}CH_3COONa$ are shown in Figures 3 and 4, respectively. In both cases a polyketide intermediate derived totally from acetate should give ten strong peaks assignable to alternate carbon atoms in the aglycone. The addition of $CH_3{}^{13}COONa$ should have enriched carbons starting at C-9, and $^{13}CH_3COONa$ should enrich the carbon atoms starting with CH_3 at C-9. The spectrum (Figure 3) derived from 1 enriched with $CH_3{}^{13}COONa$ has chemical shifts of δ 191.2 (C-5), 171.9 ($COOCH_3$), 161.7 (C-6), 155.9 (C-4), 143.2 (C-10a), 137.2 (C-2), and 133.4 (C-11a) which are so strong relative to the signals for adjacent carbon atoms that

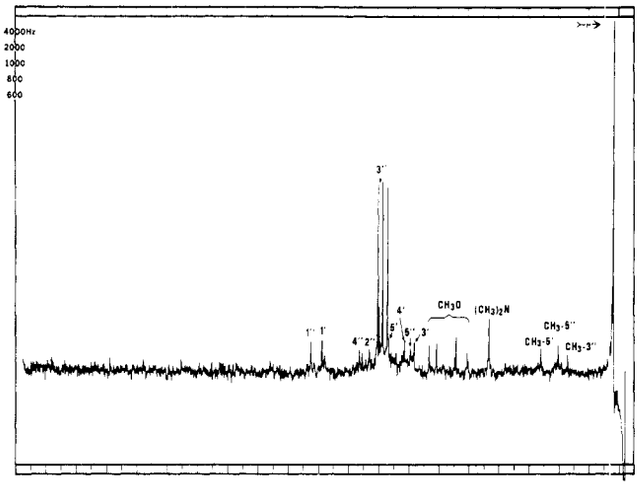

Figure 5. ^{13}C NMR spectrum ($CDCl_3$) of nogalamycin from cultures supplemented with uniformly ^{13}C-labeled D-glucose.

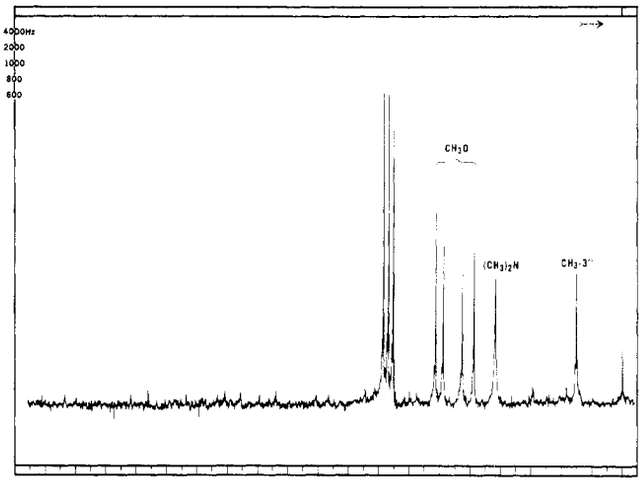

Figure 6. ^{13}C NMR spectrum ($CDCl_3$) of nogalamycin from cultures supplemented with $^{13}CH_3$-labeled methionine.

it is quite clear that these carbon atoms are enriched in ^{13}C as expected if the aglycone were derived from ten acetate units. The strong peaks at δ 69.9 and 71.5 must arise from C-9 and C-7, respectively. The expected tenth peak, which after the above reassignment would be at δ 116.1, would arise from C-12a if the polyacetate biogenesis view is correct. There is a peak at this position albeit one not nearly so strong as the others. However, in view of the absence of other aromatic carbon peaks in this area, it must arise from an enriched C-12a carbon atom. In such case, the labeled carbon atoms would be exactly as expected for polyacetate biosynthesis of the aglycone portion of **1**. In addition, weakly enriched peaks arising from some of the carbon atoms in the two sugars can be observed, but this would have no bearing on the biosynthesis of aglycone. The spectrum (Figure 4) of material derived from $^{13}CH_3COONa$ enrichment is somewhat more complex. Only six peaks in the carbonyl and aromatic region are quite obviously strongly enriched in ^{13}C with respect to other aromatic and carbonyl peaks, and polyketide biosynthesis would require seven. The peak with a chemical shift of δ 114.1 is slightly higher than other enriched carbon atom peaks so it seems probable that it represents C-4a and C-5a after reassignment of C-4a. Such a situation would be that expected from polyacetate biosynthesis. The methyl carbon at C-9 would be expected to be enriched, and this is the case as is obvious from comparing methyl peak heights in Figure 1 with those in Figure 4. In the spectrum of **1** the ratio of the height of the C-9 CH_3 peak to the height of the C-5′ CH_3 peak is 0.66 while in the spectrum in Figure 4 it is 2.13 showing quite clearly that the CH_3 carbon at C-9 is enriched. The same argument applied to the peaks arising from C-8 and C-10 established that they have been substantially enriched. Thus, the expected ten-carbon atoms have been shown to arise from C-2 of acetate, and the consistency of $CH_3{}^{13}COONa$ and $^{13}CH_3COONa$ is perfect with the reassignment of C-4a, C-12a, C-7, and C-4′. However, a number of peaks arising from sugar carbon atoms and various methyl groups are high enough to indicate ^{13}C enrichment of these carbon atoms. Such incorporations are known to occur.

Addition of uniformly ^{13}C-labeled glucose to an *S. nogalater* fermentation was for the purpose of determining whether or not the two sugars of **1** were derived from glucose. It is known that the amino sugar has the glucose configuration,[7] although not whether it is D or L, suggesting a strong possibility of direct origin from glucose. Nogalose, the neutral sugar, has the L-rhamnose configuration, and it has been shown that many microorganisms can convert D-glucose to L-rhamnose.[8] The ^{13}C NMR spectrum in Figure 5 is that obtained from material enriched in ^{13}C by inclusion of uniformly labeled glucose in the fermentation. Carbon atoms in methyl groups and in C-1 to C-6 of nogalose were substantially enriched although not nearly at the level obtained in the aglycone arising from labeled acetate. A comparison of Figures 1 and 5 with respect to peak heights of peaks from aliphatic carbon atoms relative to those of aromatic carbons shows that peaks at δ 100.8 (C-1″), 84.3 (C-4″), 81.3 (C-2″), 78.7 (C-3″), 67.7 (C-5″), and 18.3 (CH_3 at C-5″) arise from enriched carbon atoms establishing that glucose is directly converted to nogalose. The results were not as clear with respect to the amino sugar. Peaks at δ 97.0 (C-1′), 75.1 (C-5′), 69.6 (C-4′), 66.4 (C-3′), and 24.0 (CH_3 at C-5′) have heights comparable to those from nogalose and must also arise from enriched carbon atoms, strongly suggesting that the amino sugar is derived from glucose. However, no enriched peak for C-2′ was seen. All of the methyl groups show evidence of ^{13}C enrichment indicating degradation of glucose to one-carbon fragments which were ultimately used for methylation.

The addition of $^{13}CH_3$-labeled methionine to *S. nogalater* fermentations gave **1** whose ^{13}C NMR spectrum (Figure 6) showed substantial enrichment in the four CH_3O groups (δ 61.4, 58.9, 52.6, and 48.8), in the methyl groups attached to nitrogen (δ 41.6), and in one CH_3C group, the one at C-3″ in nogalose. Relative peak heights in Figures 1 and 6 quite clearly establish the enriched carbon atoms. These results confirm that methyl groups on heteroatoms arise from methionine, and that at some stage in the conversion of D-glucose to nogalose a transfer of methyl from methionine to C-3″ of nogalose occurs. Figure 11 shows the origin of the various carbon atoms.

Investigation of the biosynthesis of **3** was carried out in the same fashion except using the organism *S. elgreteus*. Because of the nearly identical structures of **2** and **3** it was assumed that results obtained for **3** would hold for **2**. Since yields of **3** were better than those of **2**, the formation of **3** was studied. Incorporation of $CH_3{}^{13}COONa$ into **3** gave material whose ^{13}C NMR spectrum (Figure 7) indicated substantial enrichment of nine-carbon atoms with ^{13}C while there was slight enrichment of CH_3O groups and sugar carbon atoms. The enrichment of the nine most enriched carbon atoms calculated on the basis of relative peak heights was at least 20-fold. These highly enriched carbon atoms were C-5, C-2, C-4, C-6, C-10a, C-11a, C-12a, C-9, and C-7. The latter carbon atom was apparently misassigned at δ 70.2[9] originally and it should have been δ 71.6 with C-3′ at δ 70.2 (Figure 2). This pattern of enrichment would be appropriate for formation of steffimycinone (the aglycone of **2** and **3**) from ten acetate units with the

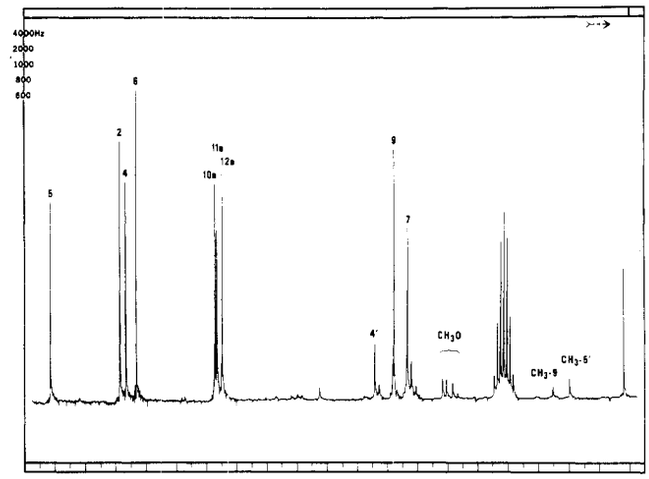

Figure 7. ^{13}C NMR spectrum (Me_2SO-d_6) of steffimycin B from cultures supplemented with $CH_3{}^{13}COONa$.

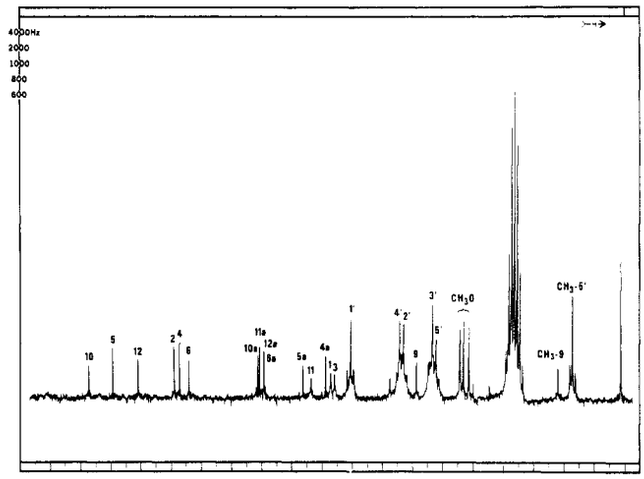

Figure 9. ^{13}C NMR spectrum (Me_2SO-d_6) of steffimycin B from cultures supplemented with uniformly ^{13}C-labeled D-glucose.

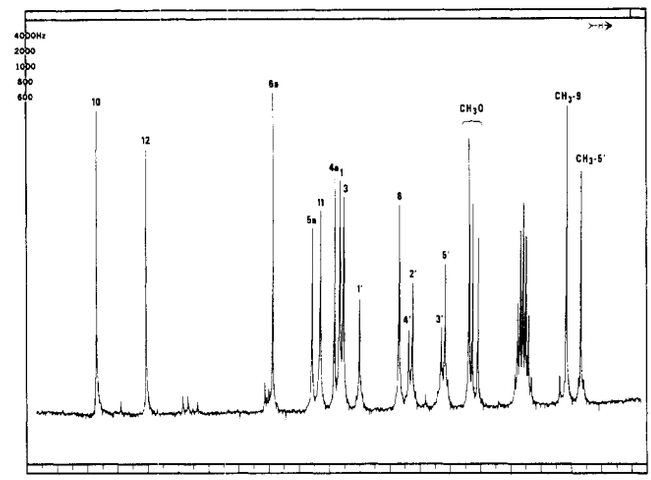

Figure 8. ^{13}C NMR spectrum (Me_2SO-d_6) of steffimycin B from cultures supplemented with $^{13}CH_3COONa$.

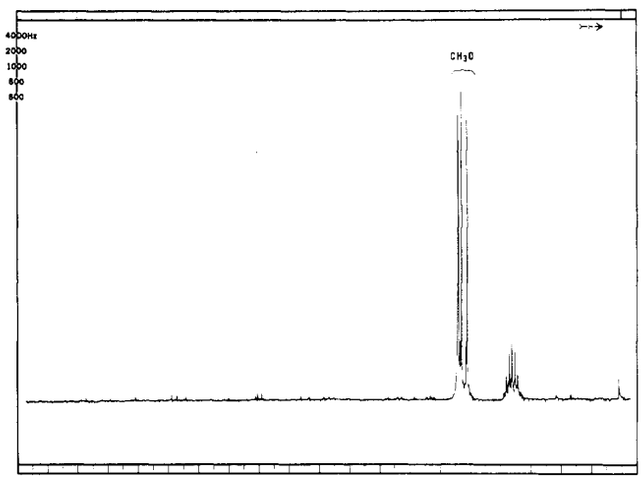

Figure 10. ^{13}C NMR spectrum (Me_2SO-d_6) of steffimycin B from cultures supplemented with $^{13}CH_3$-labeled methionine.

initial acetate giving C-9 and its attached CH_3, and an eventual loss of the carboxyl carbon in the terminal acetate unit as in daunomycin. If such were the case, enrichment with $^{13}CH_3COONa$ should lead to ten highly enriched carbon atoms in steffimycinone, and this is what happens. In the spectrum (Figure 8) obtained from material isolated from an *S. elgreteus* fermentation to which $^{13}CH_3COONa$ had been added, eight aromatic and carbonyl carbon atoms gave peaks which were five- to ten-fold larger than the peaks for the other aromatic carbon atoms. Furthermore, these were the peaks expected from polyacetate synthesis being those arising from C-10, C-12, C-6a, C-5a, C-11, C-4a, C-1, and C-3. However, it appeared that the acetate methyl group had also been incorporated into the sugar and CH_3O carbon atoms making it somewhat more difficult to clearly establish that carbon at C-8 and CH_3 at C-9 were, as would be expected from acetate biosynthesis, greatly enriched with ^{13}C. The height of the C-8 peak is about the same as that of the clearly enriched carbon atoms and about 1.8 times as high as the C-2′ peak whereas in the spectrum of **3** (Figure 2) the height of the C-8 peak is lower than the C-2′ peak height. This change in relative height strongly suggests ^{13}C enrichment at C-8. The C-9 CH_3 group also gives a very strong peak, but it is not in an absolute sense a great deal stronger than is the one for methyl at C-5′. In the spectrum of **3**, the C-9 CH_3 peak is the weaker one whereas in the enriched material it is stronger, and it is obviously many-fold higher than peaks due to C-7 or C-9. Furthermore, such a carbon atom would not be expected to arise from a conversion of ^{13}C methyl or acetate to a one-carbon fragment as could carbon atoms in the sugar. Thus, the patterns seen in the spectra reproduced in Figures 7 and 8 are consistent with the view that steffimycinone arises from ten acetate units starting at the C-9 CH_3 with loss of the terminal carboxyl.

A fermentation of *S. elgreteus* to which ^{13}C uniformly labeled D-glucose had been added gave a sample of **3** whose ^{13}C NMR spectrum is shown in Figure 9. The height of peaks derived from the six C-1′ to C-6′ carbon atoms of the sugar relative to the aromatic carbon peak heights in Figure 9 and in the spectrum of **3** (Figure 2) are such that they demonstrate ^{13}C enrichment of these carbon atoms two- to fivefold. In this case, the glucose must be incorporated intact into the 2,4-di-*O*-methyl-L-rhamnose moiety. The only other carbon atoms enriched were those of the CH_3O groups again suggesting breakdown of glucose to one-carbon units which are used for methylation.

The results of addition of $^{13}CH_3$-labeled methionine to an *S. elgreteus* fermentation were quite clear cut. Figure 10 shows the spectrum of **3** derived from such a fermentation. The carbon atoms of the CH_3O groups were so highly enriched that the peaks arising from them are virtually the only ones visible, and it is clearly shown that methylation on oxygen occurs by direct transfer of methyl groups from methionine with no other methylation by methionine.

Figure 12 shows the origin of the various carbon atoms.

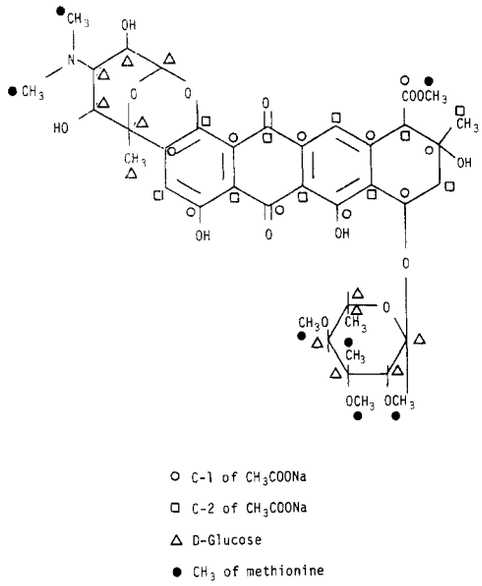

Figure 11. Origin of various carbon atoms in nogalamycin.

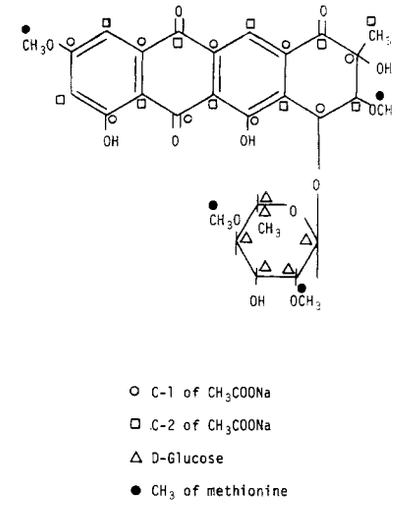

Figure 12. Origin of various carbon atoms in steffimycin B.

Experimental Section

Fermentation. *S. nogalater and S. elgreteus* were stored and maintained on sterile soils in the culture collection of The Upjohn Company and were cultured in seed media as described by Arcamone and co-workers.[10] Following 48 h of aerobic incubation of the seed stage at 28 °C, the cultures were used as inocula (5%) for an inorganic salts medium termed P.A.S.[11] enriched with 0.1% yeast extract and the indicated carbon source. Individual fermentations were carried out in 100-mL volumes in 500-mL wide-mouthed Erlenmeyer flasks shaken at 250 rpm. In all cases, the ^{13}C-enriched carbon sources were either incorporated into P.A.S. before inoculation or were added to cultures 36 h after inoculation. When $CH_3{}^{13}COONa$ or $^{13}CH_3COONa$ (90%, Stohler Isotope Chemicals) was used as a ^{13}C source, they were incorporated into sterile media at a concentration of 1 g/L of P.A.S. In these experiments the P.A.S. medium was additionally supplemented with 0.1% yeast extract and 0.5% unenriched CH_3COONa. Under these conditions, *S. nogalater* and *S. elgreteus* were cultured aerobically at 28 °C for 72–96 h. In the experiments using ^{13}C-labeled D-glucose (>50% uniformly labeled, Merck Sharp & Dohme) and $^{13}CH_3$-labeled methionine (90%, Merck Sharp & Dohme), the ^{13}C-labeled material was injected at 36 h postinoculation as a sterile aqueous solution. In both cases the ^{13}C-carbon sources were added to final concentrations of 60 mg/L of P.A.S. medium enriched with 0.1% yeast extract and 0.5% unenriched CH_3COONa. All fermentations were incubated aerobically at 28 °C for 72 h following isotope addition.

Isolation. (a) Nogalamycin (1). The isolation of 1 was carried out by a previously unpublished procedure developed by Meyer and Hofstetter.[12] A 4- to 4.5-L fermentation was adjusted to pH 2 with concentrated HCl and filtered using filter aid. The filter cake was washed with 1/10 v/v of water, and the filtrate was extracted with three 1/4 v/v of *n*-BuOH. The combined extracts were evaporated to dryness under reduced pressure, and the residue was dissolved in 100 mL of H_2O. The aqueous solution was adjusted to pH 7 with 1 N NaOH and extracted with three 40-mL portions of CH_2Cl_2. The combined extracts were evaporated to dryness under reduced pressure, and the residue was chromatographed on 20 g of silica gel using CH_2Cl_2–CH_3OH (9:1). The fractions containing pure 1 were combined on the basis of TLC in $CHCl_3$–CH_3OH–H_2O (78:20:2) and evaporated to give 30–60 mg of pure 1.

(b) Steffimycin B (3). This was isolated by the procedure of Brodasky and Reusser[13] except for the chromatographic purification. This was done by a combination of preparative TLC ($CHCl_3$–CH_3OH; 95:5) and silica gel chromatography (CH_2Cl_2–CH_3OH; 99:1). The yield from 3- to 3.5-L fermentations was 90–140 mg identified by TLC using $CHCl_3$–CH_3OH (95:5).

Acknowledgment. This work was supported in part by Contract N01-CM-43753 from the Division of Cancer Treatment, National Cancer Institute, National Institutes of Health, Education, and Welfare. Appreciation is extended to Ms. Alma Dietz and to Mrs. Grace Li for providing the inocula used in these studies.

Registry No.—1, 1404-15-5; 3, 54526-94-2.

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(12) We wish to especially thank Dr. Heinz Meyer and Mr. J. R. Hofstetter for allowing us to report this isolation procedure.
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