Proc. Nat. Acad. Sci. USA
Vol. 71, No. 8, pp. 3260–3264, August 1974

Ansamycin Biogenesis: Studies on a Novel Rifamycin Isolated from a Mutant Strain of *Nocardia mediterranei*

(^{13}C-enriched precursors/^{13}C magnetic resonance spectroscopy/blocked mutant)

RICHARD J. WHITE, EDOARDO MARTINELLI, AND GIANCARLO LANCINI

Research Laboratories, Gruppo Lepetit S.p.A., via Durando, 38, 20158 Milano, Italy

Communicated by Melvin Calvin, April 22, 1974

ABSTRACT **A novel ansamycin, rifamycin W, was isolated from a mutant strain of *Nocardia mediterranei*. The metabolic origin of rifamycin W was studied by ^{13}C nuclear magnetic resonance spectroscopy. Examination of the proton-decoupled pulse and Fourier transform ^{13}C spectra of rifamycin W biogenetically enriched with [*1*-^{13}C]-, [2-^{13}C]-, and [*3*-^{13}C]propionate and with [*1*-^{13}C]acetate has revealed that the alignment of *acetate* and *propionate* units corresponds to that previously proposed for rifamycin S. Washed mycelium from a rifamycin B-producing strain of *N. mediterranei* transformed rifamycin W into rifamycin B. We suggest that rifamycin W is a normal intermediate in the biosynthesis of the other rifamycins. These results, together with the structural similarity of rifamycin W to the streptovaricins, reinforce our hypothesis that a common progenitor is involved in the biogenesis of all naphthalenic ansamycins.**

The rifamycins are a family of closely related antibiotics first isolated from the fermentation medium of *Nocardia mediterranei* at the Lepetit Laboratories in 1957 (1, 2). They were the first examples of a novel class of secondary metabolites characterized by the possession of an aliphatic ansa chain bridging an aromatic chromophore, which Prelog has denominated the ansamycins (3). Members of this group so far identified can be divided into two subgroups according to the chromophore present: those containing a naphthalenic moiety [rifamycins, tolypomycins (4), and streptovaricins (5)] and those containing a benzenic moiety [maytansines (6), colubrinol (7), and geldanamycin (8)]; representative structures are shown in Scheme 1.

In a previous publication (9) describing the biosynthesis of rifamycin S, we proposed a general scheme for the biogenesis of the ansamycin carbon skeleton in which a single polyketide chain is initiated by a seven carbon amino precursor containing a six-membered ring. According to this scheme, the naphthalenic chromophore, when present, is formed by closure of a second ring including the 2nd, 3rd, and 4th carbons of the polyketide chain, e.g., C-7, C-6, and C-5 in the case of the rifamycins. The results obtained with rifamycin S (9) also implied that a common progenitor might be involved in the formation of the different naphthalenic ansamycins. This precursor would have fundamentally the same carbon skeleton as the streptovaricins, and would give rise to the rifamycins by loss of a propionate-derived methyl from the ansa chain and introduction of an ether linkage between the ansa and chromophore. This proposal has promptly received support from the results of [*1*-^{13}C]propionate incorporation into streptovaricin D (10) that indicated a biosynthetic scheme for this compound identical to that independently proposed on the basis of the results obtained for rifamycin S (9).

During our screening program of *N. mediterranei* mutants as a source of new natural rifamycins, we have isolated a morphological variant that produced a mixture of novel rifamycins. In the present communication we describe the isolation of the major component, rifamycin W, from the fermentation broth of this mutant and report its biosynthesis and relationship to the normal fermentation product, rifamycin B. Evidence for the structure of this compound will be reported elsewhere (11). The present results fully support our proposals concerning the biosynthesis of rifamycin S and are consistent with the existence of a common progenitor of the naphthalenic ansamycins.

MATERIALS AND METHODS

Occurrence and Isolation of Rifamycin W. Mutant 126 is a morphological variant of *N. mediterranei* strain AE/1 (12) obtained by mutagenesis of spores with nitrosoguanidine (conditions as in ref. 13). Rifamycin W was produced by fermentation of mutant 126 in a complex organic medium (14) for 180 hr at 28°. Fermentation broths were filtered, adjusted to pH 2.0, and extracted with ethyl acetate. This organic extract was further purified by extraction of rifamycins into 10 mM sodium phosphate buffer (pH 7.38), which was then shaken with chloroform (discarding organic layer) and finally adjusted to pH 2.0 and back-extracted with ethyl acetate. The last stage of the purification procedure was a counter-current distribution of 150 transfers with ethyl acetate–10 mM sodium phosphate buffer, pH 6.5 (1:1) as solvent system. Rifamycin W, the major component, peaked at fraction 106. After the appropriate fractions were pooled and concentrated, it was crystallized from ethyl acetate and dried at 60°.

[^{14}C]Rifamycin W was obtained by addition of 250 μCi of sodium [*1*-^{14}C]propionate to a fermentation (0.5 μCi/ml; 10 mCi/mol) and then purification of the rifamycin W as described above.

The concentration of rifamycin W in solution was estimated from the absorbance at 540 nm in 0.1 N sodium hydroxide, taking a log ϵ of 3.73 (11). Rifamycin B was estimated by a specific differential spectrophotometric technique (15).

Preparation of ^{13}C-Enriched Rifamycin W. Essentially the same procedure was used as that previously described for the preparation of ^{13}C-labeled rifamycin S (9); [^{13}C]propionate and [^{13}C]acetate were added to fermentations twice a day to give 200 μg/ml. Total precursor added was 1.2 mg/ml in

Abbreviation: NMR, nuclear magnetic resonance.

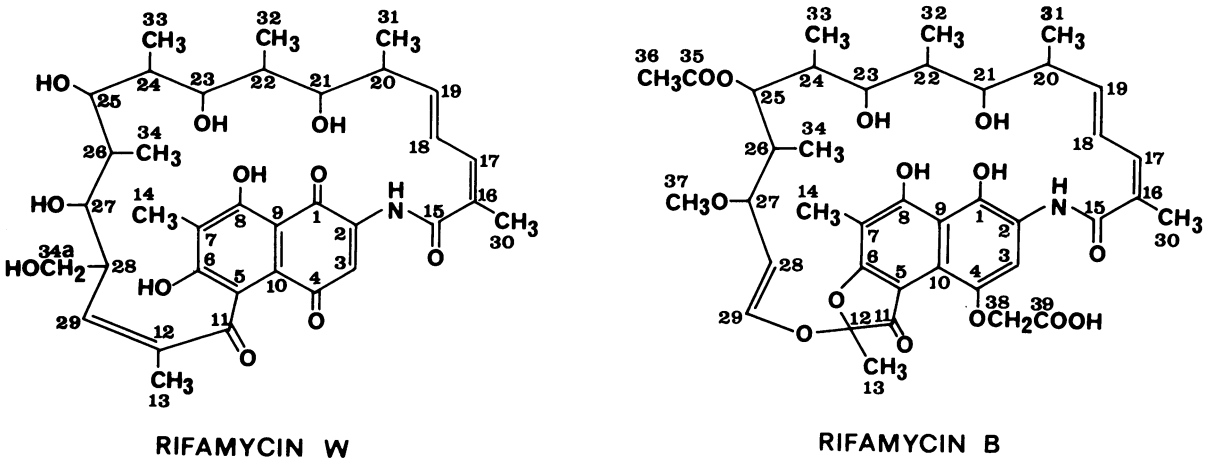
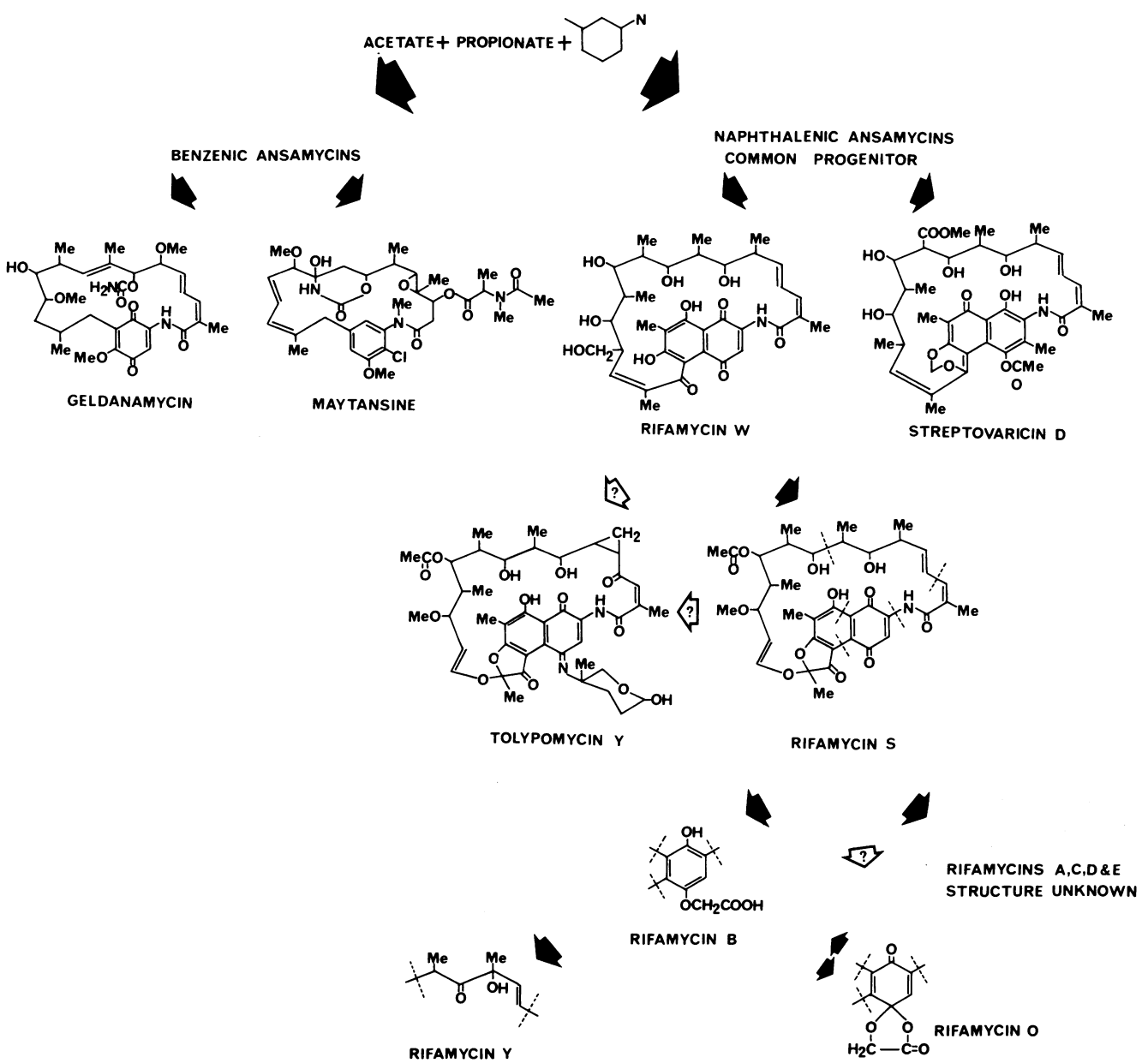


FIG. 1. Structural formulas of rifamycins W and B.



SCHEME 1. Proposed biogenetic interrelationships for the ansamycins with special reference to the rifamycins.

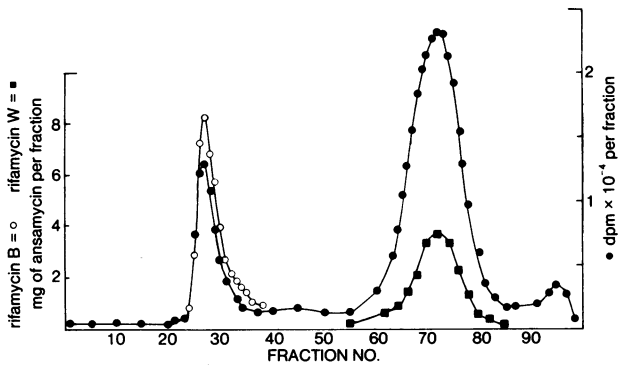


FIG. 2. Transformation of [^{14}C]rifamycin W into [^{14}C]rifamycin B by washed mycelium. Washed mycelium (1.45 g dry weight) from a rifamycin B-producing strain of *N. mediterranei* was resuspended in 100 ml of 10 mM sodium phosphate buffer pH 6.5 containing 0.15% (w/v) sodium diethylbarbiturate; 50 mg of [^{14}C]rifamycin W (6500 dpm/mg) was added. The suspension was incubated at 28° for 24 hr; [^{14}C]rifamycins were recovered by extraction with ethyl acetate at pH 2.0. Unlabeled carrier rifamycin B (50 mg) was added to the organic extract (which already contained 17 mg of [^{14}C]rifamycin B), and the mixture was subjected to counter-current distribution in ethyl acetate–10 mM sodium phosphate buffer pH 6.0 (1:1) as solvent system. Fractions were analyzed for radioactivity by scintillation counting and for rifamycins B and W by spectrophotometric methods.

each case. All the precursors used were 90% enriched in ^{13}C except for [*3*-^{13}C]propionate, which was 60% enriched. [^{13}C]Acetate and [^{13}C]propionate were obtained from Merck Sharp and Dohme Ltd., Montreal, Canada.

RESULTS

Occurrence and Physicochemical Properties of Rifamycin W. Fermentation of the parent strain *N. mediterranei* in medium containing sodium diethyl barbiturate (16) results in an almost exclusive synthesis of rifamycin B. Under the same conditions mutant 126 produced a mixture of novel ansamycins and no rifamycin B at all. The major component of this complex has been isolated as a pure crystalline solid and has been assigned (11) the formula $C_{35}H_{45}O_{11}N$ (calculated: C = 64.11, H = 6.92, O = 26.84, N = 2.14; found: C = 63.64, H = 6.81, O = 26.76, N = 2.13). The UV and visible absorption spectrum was very similar to that of rifamycin S (17), having maxima in 0.1 M NaOH at 540 nm (log = 3.73), 350 nm (log ϵ = 4.09), and 239 nm (log ϵ = 4.56), with shoulders at 285 nm and 255 nm. Spectrophotometric studies and potentiometric titration revealed the presence of two ionizable functions on the chromophore, both acidic, and pK_a values of 4.9 and 11.1. The infrared spectrum lacked the bands of the acetoxyl and the dihydrofuranone carbonyls, characteristic of other rifamycins.

The proposed structural formulation of rifamycin W is shown in Fig. 1, where rifamycin B, the normal fermentation product, is included for comparison. Detailed physicochemical data and evidence for the structure is presented elsewhere (11).

Transformation of Rifamycin W into Rifamycin B. In two respects rifamycin W resembles the streptovaricins rather than rifamycin B: it lacks the ether linkage between C-29 and C-12 and it has an extra carbon on C-28 (see Scheme 1). Thus, it was important to establish whether rifamycin W was an intermediate in the biosynthesis of the other rifamycins or a shunt metabolite. In the light of previous observations on the capacity of washed mycelium to transform exogenous rifamycins (18), we have added ^{14}C-labeled rifamycin W (500 μg/ml; 6500 dpm/mg) to mycelium of a rifamycin B-producing strain of *N. mediterranei*. Under the conditions used, the endogenous synthesis of rifamycin B was very low: a control flask produced 4 μg/ml in 24 hr while the flask containing [^{14}C]rifamycin W synthesized 89 μg/ml. Total rifamycins were extracted and, after addition of unlabeled carrier rifamycin B, analyzed by Craig counter-current distribution (Fig. 2). The rifamycin B synthesized was labeled, as shown by the coincidence of the peaks for radioactivity and absorption.

Incorporation of ^{13}C-Enriched Precursors. The proton-decoupled natural abundance ^{13}C nuclear magnetic resonance (NMR) spectrum of rifamycin W in dimethyl sulfoxide is shown in Fig. 3A; assignment of the resonances will be discussed in a separate publication (11). Also given in Fig. 3 are the ^{13}C NMR spectra of rifamycin W obtained from fermentations to which [*1*-^{13}C]propionate, [*2*-^{13}C]propionate [*3*-^{13}C]propionate, and [*1*-^{13}C]acetate had been added. All the spectra from the [^{13}C]propionate-supplemented fermentations show eight enriched carbons. In the case of [*1*-^{13}C]-acetate, a large number of peak heights are enhanced, but comparison with the other spectra shows that the majority of such carbons derive from propionate, and the enrichments seen are the result of an indirect incorporation of acetate by way of propionate (or its metabolic equivalent), as previously noted for rifamycin S (9). If one disregards the positions enriched by both precursors, only two are left that specifically derive from [*1*-^{13}C]acetate, C-11 and C-17. Thus, there are eight *propionate* units and two *acetate* units in rifamycin W.

DISCUSSION

Studies on the incorporation of ^{13}C-enriched precursors suggested that a common scheme of biogenesis is operative for the rifamycins (9) and streptovaricins (10) in which a seven-carbon amino compound containing a six-membered ring initiates a single polyketide chain composed of two *acetate* and eight *propionate* units.* The naphthalenic chromophore is formed by closure of a second ring including the first *propionate* and *acetate* unit of the polyketide chain. Failure to form a second ring would result in the formation of a benzenic ansamycin such as geldanamycin. Although the proposed biogenetic scheme presents few surprises for the streptovaricins, it implies that the rifamycins derive from a precursor that must subsequently lose a propionate-derived methyl group from carbon-28 and have an oxygen inserted between two carbons of the same propionate unit (a most unusual metabolic event). The isolation of rifamycin W possessing all the essential features of this hypothetical progenitor supports our proposal.

Examination of the ^{13}C NMR spectra of rifamycin W biogenetically enriched with [^{13}C]acetate and [^{13}C]propionate demonstrates the metabolic capacity of *N. mediterranei* to synthesize the rifamycin carbon skeleton from a seven-carbon amino moiety of unknown origin and a single polyketide

* Throughout this article the terms *acetate* and *propionate* are used to denote the chemical entity participating in the biological reaction.

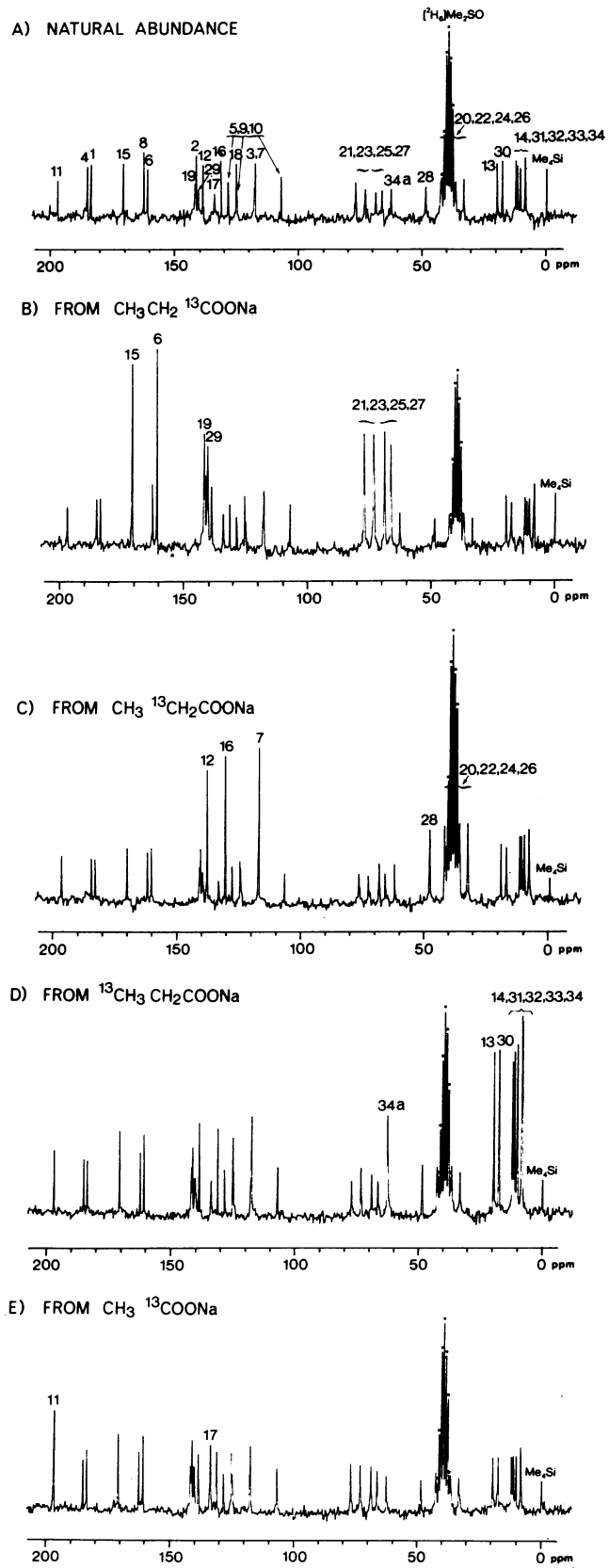


FIG. 3. Proton-decoupled ^{13}C Fourier transform NMR spectra of rifamycin W in hexadeutero dimethyl sulfoxide ($[^2H_6]Me_2SO$) recorded at 25.15 MHz. The *horizontal scale* is in parts per million downfield from dissolved tetramethyl silane (Me_4Si). 2000 Scans were made with a repetition time of 0.5 sec. *A*, Natural abundance; *B*, biogenetically enriched with [*1*-^{13}C]propionate; *C*, biogenetically enriched with [*2*-^{13}C]propionate; *D*, biogenetically enriched with [*3*-^{13}C]propionate; *E*, biogenetically enriched with [*1*-^{13}C]acetate. The *numbered carbon atoms* on the spectra of rifamycin W deriving from [^{13}C]propionate and [^{13}C]acetate are those considered as specifically enriched in ^{13}C by the precursor in question.

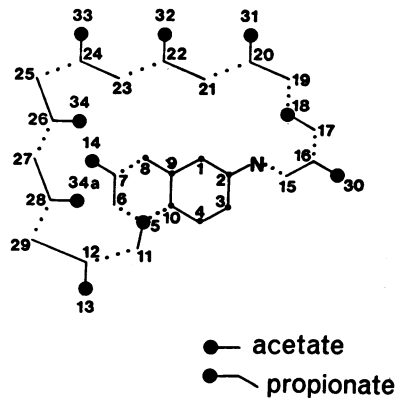


FIG. 4. Alignment of *acetate* and *propionate* units in rifamycin W.

chain comprised of eight *propionate* and two *acetate* units (see Fig. 4) aligned in the same way as previously proposed for rifamycin S (9). The results obtained with ^{13}C-enriched precursors further illustrate the usefulness of this technique in studying the biosynthesis of complex molecules. The traditional approach using radioactive precursors was impracticable in the present case as a consequence of difficulty in obtaining suitable fragments of the labeled molecule by chemical degradation.

The demonstration that *N. mediterranei* can convert this ansamycin into rifamcyin B proves that the mycelium is capable of carrying out the necessary modifications, i.e., removal of methyl group and introduction of oxygen to transform the common progenitor into rifamycin S. We suggest that rifamycin W is a normal intermediate in the formation of the other rifamycins, but cannot for the moment exclude that it is a shunt metabolite readily convertible into an intermediate. The fact that the extra carbon (C-34a) is in the form of a hydroxymethyl group implicates the common route of methyl oxidation by way of primary alcohol, aldehyde, and carboxyl groups with loss as carbon dioxide. Clearly, several mechanisms are possible for the introduction of an oxygen between C-29 and C-12; for example, epoxidation of the double bond C-29, C-12 and subsequent scission of the carbon–carbon bond, or a modified Baeyer–Villiger oxidation may be involved.

Acetylation and methylation of the hydroxyls at C-25 and C-27 must be late events in biosynthesis, as judged by the unsubstituted ansa chain of rifamycin W, in agreement with the prior isolation of mutant blocked in methylation that accumulated 27-desmethyl rifamycin SV; 27-desmethyl, 25-desacetyl rifamycin SV; and 27-desmethyl rifamycin B (19).

Prelog *et al.* (20) have recently pointed out a striking similarity in the stereochemistry of the aliphatic chain of ansamycins and the macrolide model proposed by Celmer (21). One can safely predict that C-28, which bears the extra carbon (C34a), has the same chirality as that established for the analogous carbon of the streptovaricins, but verification of this must await x-ray crystallographic studies.

In Scheme 1 we have attempted to summarize some possible biosynthetic interrelationships between the various ansamycins with special reference to the rifamycins. No indication is given as to the number of steps involved between the various intermediates. A unique sequence of reactions is probably not the normal case in secondary metabolism owing to the comparative lack of substrate specificity; consequently, the order of modifications shown must be taken with due caution (22). The scheme serves only to outline in general terms the metabolic interrelationships and a possible sequence of events during ansamycin biogenesis. Very little is known about the biosynthesis of benzenic ansamycins, and although the general scheme of biogenesis, i.e., a seven-carbon precursor initiating a single polyketide chain, seems applicable, there are no grounds for suggesting a common progenitor of the type proposed for the naphthalenic ansamycins. In addition, it should be remembered that two of the examples so far known are plant products while all the other ansamycins have been isolated from the Actinomycetes. The intriguing possibility that geldanamycin also derives from the same polyketide chain, giving rise to the naphthalenic ansamycins by a degradative process during which six carbons of the chain are lost, must await detailed studies on its biosynthesis; the beginning and end of its ansa chain are analogous to those present in the rifamycins.† The point at which tolypomycin and rifamycin biosynthesis diverge is uncertain, but a report that rifamycin B and tolypomycin Y are cosynthesized by *Streptomyces tolypophorus* (23) lends support to the idea that rifamycin S is an intermediate in both cases. Evidence for the relationship between rifamycins S, B, O, and Y has been discussed (14, 18). More recent results have indicated that rifamycin S is also the precursor of the rifamycin complex (our unpublished work), providing a further demonstration of the central role played by this rifamcyin in the formation of the other members of the family.

The isolation of rifamycin W, the "missing link" in naphthalenic ansamycin biogenesis, provides an important illustration as to the relevance of studies on the biosynthesis of antibiotics as a rational means of predicting and under standing the type of molecules that can be isolated from blocked mutants.

† **Note added in proof.** On the basis of the results of [*1*-^{13}C]-propionate incorporation, Rinehart's group has now proposed a biosynthetic pathway for geldanamycin (conforming to Scheme 1) in which the *ansa* chain is derived from four *propionates* and three *acetates* [Johnson, R. D., Haber, A. & Rinehart, K. L. (1974) *J. Amer. Chem. Soc.* **96**, 3316–3317], thus excluding the possibility that this ansamycin derives from the naphthalenic progenitor.

We thank Dr. P. Beynon of JEOL Ltd. (U.K.) for recording the ^{13}C-NMR spectra, G. Sartori for expert technical assistance, and Prof. P. Sensi and Prof. G. G. Gallo for advice and encouragement.

1. Sensi, P., Greco, A. M. & Ballotta, R. (1959) in *Antibiotics Annual 1959–1960* (Antibiotics, Inc., New York), pp. 262–270.
2. Thiemann, J. E., Zucco, G. & Pelizza, G. (1969) *Arch. Mikrobiol.* **67**, 147–155.
3. Prelog, V. & Oppolzer, W. (1973) *Helv. Chim. Acta* **56**, 2279–2287.
4. Shibata, M., Hasegawa, T. & Higashide, E. (1971) *J. Antibiot.* **24**, 810–816.
5. Siminoff, P., Smith, R. M., Sokolski, W. T. & Savage, G. M. (1957) *Amer. Rev. Tuberc. Pulm. Dis.* **75**, 576–583.
6. Kupchan, S. M., Komoda, Y., Court, W. A., Thomas, G. J., Smith, R. M., Karim, A., Gilmore, C. J., Haltiwagner, R. C. & Bryan, R. F. (1972) *J. Amer. Chem. Soc.* **94**, 1354–1356.
7. Wani, M. C., Taylor, H. L. & Wall, M. E. (1973) *J. Chem. Soc. Chem. Commun.* 390.
8. De Boer, C., Meulman, P. A., Wnuk, R. J. & Peterson, D. H. (1970) *J. Antibiot.* **23**, 442–447.
9. White, R. J., Martinelli, E., Gallo, G. G., Lancini, G. & Beynon, P. (1973) *Nature* **243**, 273–277.
10. Milavetz, B., Kakinuma, K., Rinehart, K. L., Rolls, J. P. & Haak, W. J. (1973) *J. Amer. Chem. Soc.* **95**, 5793–5795.
11. Martinelli, E., Gallo, G. G., Antonini, P. & White, R. J. (1974) *Tetrahedron*, in press.
12. Esposito, A., Licciardello, G., Murthy, Y. K. S., Sacerdoti, S. A. & Sparapani, P. (1972) in *Abstracts of Fourth International Fermentation Symposium, Kyoto, Japan*, p. 217.
13. Lancini, G. & Hengeller, C. (1969) *J. Antibiot.* **22**, 637–638.
14. Lancini, G., Gallo, G. G., Sartori, G. & Sensi, P. (1969) *J. Antibiot.* **22**, 369–377.
15. Pasqualucci, C. R., Vigevani, A., Radaelli, P. & Gallo, G. G. (1970) *J. Pharm. Sci.* **59**, 685–687.
16. Lancini, G. C. & White, R. J. (1973) *Proc. Biochem.* **8**, 14–16.
17. Gallo, G. G., Pasqualucci, C. R. & Radaelli, P. (1963) *Il Farmaco* (ed. Prat.) **18**, 78–84.
18. Lancini, G. C. & Sensi, P. (1967) *Proceedings Vth International Congress of Chemotherapy*, eds. Spitzy, K. H. & Haschek, H. (Verlag der Wiener Medizinishen Akademie-Wien), Vol. 1, pp. 41–47.
19. Lancini, G. C., Hengeller, C. & Sensi, P. (1970) *Progress in Antimicrobial and Anticancer Chemotherapy* (University of Tokyo Press), Vol. 2, pp. 1166–1173.
20. Brufani, M., Kluepfel, D., Lancini, G. C. Leitich, J., Mesentsev, A. S., Prelog, V., Schmook, F. P. & Sensi, P. (1973) *Helv. Chim. Acta* **56**, 2315–2323.
21. Celmer, W. D. (1965) in *Biogenesis of Antibiotic Substances*, eds. Vanek, Z. & Hostalek, Z. (Czechoslovak. Acad. Sciences, Prague), pp. 99–129.
22. Bu' Lock, J. D. & Powell, A. J. (1965) *Experientia* **21**, 55–56.
23. Kishi, T., Yamana, H., Muroi, M., Harada, S., Asai, M., Hasegawa, T. & Mizuno, K. (1972) *J. Antibiot.* **25**, 11–15.