Biosynthetic Origins of the Oxygen Atoms in the Ansamycin Antibiotics Rifamycin B, O, and S

Margaret G. Anderson, Dominic Monypenny, Rodney W. Rickards,* and Jennifer M. Rothschild

Research School of Chemistry, Australian National University, G.P.O. Box 4, Canberra, A.C.T. 2601, Australia

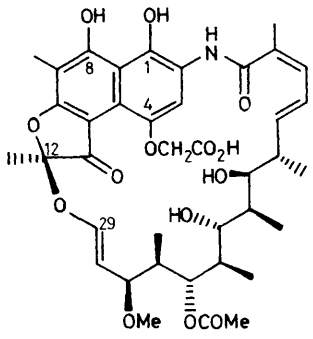
Cultures of *Nocardia mediterranea* utilise atmospheric oxygen to form the C-1 oxygen function and C-29 vinyl ether group of the ansamycin antibiotics rifamycin B, O, and S, while the C-8 phenolic hydroxy group and C-8 carbon atom are both derived from the carboxy group of 3-amino-5-hydroxybenzoic acid; these results exclude 8-deoxyansamycins as possible biosynthetic intermediates.

Rifamycins B(**1**), O(**2**), S(**3**), and SV (the quinol corresponding to S) are the most important antibiotics of the ansamycin group.[1,2] From these related fermentation products of *Nocardia mediterranea* is prepared the clinically valuable semisynthetic antibacterial agent rifampicin. The biosynthesis of the molecular skeleton of these naphthalenoid ansamycins, and of their benzenoid analogues, stems from 3-amino-5-hydroxybenzoic acid [AHB, (**4**)].[3] This natural aromatic amino acid[4] initiates formation of a polyketide *ansa* chain, which is extended primarily by propionate and acetate units.[2] Oxidative cyclisation between C-6 of the AHB unit and the polyketide, either before or after lactam formation, closes the second ring of the naphthalenoid nuclei.[5] Secondary structural modifications occurring in both the polyketide chain and the nucleus give rise to the known variants of the basic rifamycin carbon skeleton.[1,2] The terminal stages in rifamycin biosynthesis have been considered[2] to proceed from the hypothetical proansamycin B (**5**)[6] *via* sequential hydroxylation at C-34a and C-8 to protorifamycin I (**6**)[6] and rifamycin W (**7**),[7] oxidative loss of C-34a and insertion of an ether link into the *ansa* ring then leading to rifamycin S (**3**).[8] Modification of the quinonoid ring, after reduction to the quinol SV, then yields rifamycins B (**1**) and O (**2**).[9]

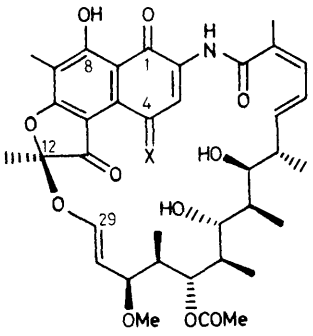
We present evidence for the origins of the biogenetically significant oxygen atoms at C-1, C-8, and C-29 in rifamycins B, O, and S,[10] which negates these accepted terminal stages of rifamycin biosynthesis.

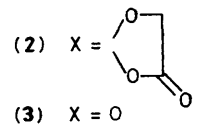
N. mediterranea (ATCC strain 21789) was cultured on a rotary shaker for 74 h. Fermentation was then continued in a closed system under an artificial atmosphere consisting of N_2 (80%) and $^{18}O_2$ (20%, 98 atom % ^{18}O) for a further 22 h. Since the extracted rifamycin B (**1**) does not give useful mass or ^{13}C n.m.r. spectra,† it was oxidised directly with manganese

† Rifamycin B (**1**) is too involatile for electron impact (EI) or chemical ionisation (CI) mass spectrometry, while ^{13}C n.m.r. spectral signals from the aromatic nucleus are broadened by the presence of naphthyloxy radicals and are not observable.

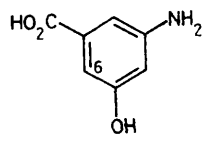


(1)

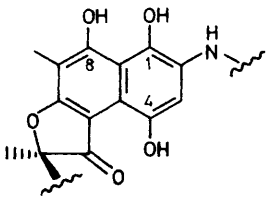


(2) X = 

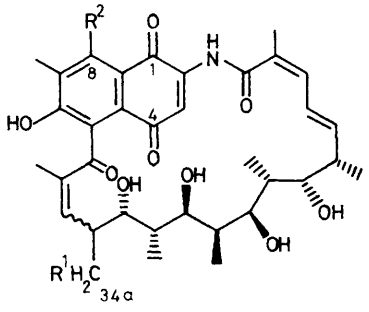
(3) X = O



(4)



(8)



(5) R^1 = H, R^2 = H

(6) R^1 = OH, R^2 = H

(7) R^1 = OH, R^2 = OH

dioxide[11] and purified as rifamycin O(**2**). Negative ion chemical ionisation (NICI) mass spectrometry of this rifamycin O showed intense fragment ions at *m/z* 709 and 713, with associated ^{13}C isotope peaks in similar intensity ratios, arising by $C^{16}O_2$ loss from weak molecular ions of the compositions $C_{39}H_{47}N^{16}O_{14}$ and $C_{39}H_{47}N^{16}O_{12}{}^{18}O_2$. The intensities of these two fragment ions are in the ratio 3 : 1, reflecting the amounts of rifamycin B (**1**) formed under the normal and $^{18}O_2$ atmospheres. In particular, there is no evidence for the presence of other rifamycin O species (with $M - CO_2$ ions at *m/z* 711 or 715) incorporating either one or three ^{18}O atoms.

The duplicity of signals in the ^1H and ^{13}C n.m.r. spectra of this semisynthetic rifamycin O (**2**) indicated it to be a mixture of two diastereoisomers resulting from creation of the spiro centre at C-4.[12] Resolution enhancement of the ^{13}C n.m.r. spectrum (75 MHz, $CDCl_3$) showed upfield ^{18}O-induced shifts ($\Delta\delta$ 27 and 50 p.p.b.) associated with the resonances due to C-1 (δ 182.64, 182.58) and C-29 (δ 145.92, 145.33) only.

Hydrolysis[11] of rifamycin O (**2**) to rifamycin S (**3**) removed the source of diastereoisomerism and the duplicity of the n.m.r. signals. Resolution enhancement then showed upfield ^{18}O-isotope shifts ($\Delta\delta$ 37 and 26 p.p.b.) for C-1 (δ 184.58) and C-12 (δ 108.24) only. NICI mass spectrometry of the quinone S (**3**) was complicated by partial reduction in the spectrometer to the quinol form, rifamycin SV, resulting in multiple isotope patterns in the parent ion region. In addition to molecular ions at *m/z* 695 and 697 with natural isotopic abundance, however, both forms showed second molecular ion sets carrying two ^{18}O atoms at *m/z* 699 and 701. The quinone S fragmented by loss of the *ansa* chain to give ions of structure (**8**)[13] at *m/z* 273 and 275, the latter containing the single ^{18}O atom present in the nucleus at C-1.

These results obtained from fermentation of *N. mediterranea* under successive atmospheres of $^{16}O_2$ and $^{18}O_2$ establish that only the C-1 oxygen function and the C-29 vinyl ether group of rifamycins B(**1**), O(**2**), and S(**3**) are introduced oxidatively. These two sites are labelled to the same extent by the ^{18}O isotope, and singly labelled species are not detectable. Consequently, at the time the atmosphere is changed and some three-quarters of the ultimate rifamycin B is already present, there is no appreciable pool of any intermediate in which only one of these oxidation processes has yet occurred.

The origin of the C-8 phenolic hydroxy group was then examined using labelled AHB (**4**). [*Carboxy*-^{13}C,^{14}C,$^{18}O_2$]-AHB (**4**) hydrochloride was prepared by exchange of [*carboxy*-^{13}C,^{14}C]-AHB[14] (90 atom % excess ^{13}C) with $H_2{}^{18}O$ (97 atom % ^{18}O) catalysed by hydrogen chloride, affording radioactive material with an effective isotopic composition‡ in the carboxy group for spectroscopic purposes of $^{13}C^{18}O_2$: $^{13}C^{16}O^{18}O$: $^{12}C^{18}O_2$ of 75 : 15 : 8. This amino acid hydrochloride was pulse fed to a shaken cell suspension of *N. mediterranea*. The rifamycin B (**1**) obtained on harvest was converted into rifamycins O(**2**) and S(**3**) as before. Radioassay of the crystallised rifamycin S indicated 10% incorporation of the labelled AHB (**4**), diluted only 1 : 6 by endogenous substrate. Mass spectrometry of the rifamycin O(**2**) showed intense ions at *m/z* 709 and 712 arising by $C^{16}O_2$ loss from natural and $^{13}C^{18}O$-labelled rifamycin O species. The relative intensities of these ions confirm the 1 : 6 dilution of the labelled AHB during biosynthesis. Mass spectrometry of the rifamycin S (**3**) showed, as expected, *M* and *M* + 3 ions for both the quinone (*m/z* 695 and 698) and quinol (*m/z* 697 and 700) oxidation states, together with the quinone fragment (**8**)[13] at *m/z* 273 and 276. ^{13}C n.m.r. spectroscopy (75 MHz, $CDCl_3$) of the rifamycin O(**2**) diastereoisomers showed enhancement only of the C-8 resonance (δ 167.82) to five times natural abundance, but failed to detect any splitting of this signal due to isomers or isotopes. The derived rifamycin S (**3**) showed similar enhancement of the C-8 resonance, which was then resolvable into a minor natural component (δ 166.84) and a major ^{18}O-carrying component shifted ($\Delta\delta$ 14 p.p.b.) to higher field.

This specific incorporation of [*carboxy*-^{13}C,^{14}C,$^{18}O_2$]-AHB (**4**) into rifamycin B(**1**) by *N. mediterranea* establishes that the carboxy carbon of the amino acid is converted *with retention of an attached oxygen atom* into the C-8 phenolic functionality of rifamycins B(**1**), O(**2**), and S(**3**). Little, if any exchange of the attached oxygen with water in the culture medium occurs during this process, suggesting that enzyme-free intermediates

‡ The ratio at equilibrium follows from the isotope content of the reactants, and was confirmed by mass spectrometry.

which would be susceptible to such exchange (such as polyketides or 3-amino-5-hydroxybenzaldehyde[15]) are not involved.

It is clear from the isotopic data described here that 8-deoxyansamycins, such as protorifamycin I(**6**) and its hypothetical precursor proansamycin B(**5**),[6] are not precursors of the 8-hydroxyansamycins rifamycin B(**1**), O(**2**), S(**3**), and W(**7**). The different C-8 oxidation levels instead reflect two parallel biochemical pathways from AHB (**4**). A similar relationship probably holds for the protostreptovaricins and the 8-hydroxylated streptovaricins and damavaricins.[2,16] The status of the reported[6] 'partial transformation' of protorifamycin I(**6**) into rifamycin W(**7**), a proven precursor[8] of rifamycin S (**3**), by hydroxylation at C-8 is now uncertain. It may reflect a minor shunt pathway in the strains of *N. mediterranea* employed, or alternatively the recorded result[6] may be in error.

Received, 24th October 1988; Com. 8/04226H

References

1 For reviews see: M. Brufani, *Top. Antibiot. Chem.*, 1977, **1**, 91; W. Wehrli, *Top. Curr. Chem.*, 1977, **72**, 21; K. L. Rinehart, Jr., and L. S. Shield, *Fortschr. Chem. Org. Naturst.*, 1976, **33**, 231.

2 For biosynthetic reviews see: O. Ghisalba, *Chimia*, 1985, **39**, 79; O. Ghisalba, J. A. L. Auden, T. Schupp, and J. Nüesch, in 'Biotechnology of Industrial Antibiotics,' ed. E. J. Vandamme, Marcel Dekker, New York, 1984, p. 281; G. Lancini, in 'Biochemistry and Genetic Regulation of Commercially Important Antibiotics,' ed. L. C. Vining, Addison-Wesley, London, 1983, p. 231; G. Lancini and M. Grandi, in 'Antibiotics. Vol. IV. Biosynthesis,' ed. J. W. Corcoran, Springer-Verlag, Berlin, 1981, p. 12.

3 J. J. Kibby, I. A. McDonald, and R. W. Rickards, *J. Chem. Soc., Chem. Commun.*, 1980, 768; O. Ghisalba, H. Fuhrer, W. J. Richter, and S. Moss, *J. Antibiot.*, 1981, **34**, 58; O. Ghisalba and J. Nüesch, *ibid.*, 1981, **34**, 64; K. Hatano, S-I. Akiyama, M. Asai, and R. W. Rickards, *ibid.*, 1982, **35**, 1415; K. L. Rinehart, Jr., M. Potgieter, W. Jin, C. J. Pearce, D. A. Wright, J. L. C. Wright, J. A. Walter, and A. G. McInnes, in 'Trends in Antibiotic Research. Genetics, Biosynthesis, Actions, and New Substances,' ed. H. Umezawa, A. L. Demain, T. Hata, and C. R. Hutchinson, Japan Antibiotics Research Association, Tokyo, 1982, p. 171; T. S. Wu, J. Duncan, S. W. Tsao, C. J. Chang, P. J. Keller, and H. G. Floss, *J. Nat. Prod.*, 1987, **50**, 108.

4 J. J. Kibby and R. W. Rickards, *J. Antibiot.*, 1981, **34**, 605; Z. Jin, C. Liu, P. Chen, S. Li, X. Lu, Z. Wei, S. Jui, and C. Wang, *Weishengwu Xuebao*, 1984, **24**, 210 (*Chem. Abstr.*, 1984, **101**, 226551w).

5 R. W. Rickards, Proceedings of the Fifth Asian Symposium on Medicinal Plants and Spices, Seoul, 1984, p. 615.

6 O. Ghisalba, P. Traxler, and J. Nüesch, *J. Antibiot.*, 1978, **31**, 1124.

7 E. Martinelli, G. G. Gallo, P. Antonini, and R. J. White, *Tetrahedron*, 1974, **30**, 3087.

8 R. J. White, E. Martinelli, and G. Lancini, *Proc. Natl. Acad. Sci. USA*, 1974, **71**, 3260.

9 O. Ghisalba, R. Roos, T. Schupp, and J. Nüesch, *J. Antibiot.*, 1982, **35**, 74.

10 R. W. Rickards, presented in part at the Second International Symposium on Progress in Natural Product Chemistry, Nottingham, July, 1986, and at the First Princess Chulabhorn Science Congress, International Congress on Natural Products, Bangkok, December, 1987.

11 B. L. Seong and M. H. Han, *Chem. Lett.*, 1982, 627.

12 M. G. Anderson, R. W. Rickards, and J. M. Rothschild, unpublished work.

13 L. F. Zerilli, M. Landi, N. Rimorini, and G. G. Gallo, *Ann. Chim. (Rome)*, 1974, **64**, 199.

14 A. J. Herlt, J. J. Kibby, and R. W. Rickards, *Aust. J. Chem.*, 1981, **34**, 1319.

15 R. W. Rickards and V. Rukachaisirikul, *Aust. J. Chem.*, 1987, **40**, 1011.

16 P. V. Deshmukh, K. Kakinuma, J. J. Ameel, and K. L. Rinehart, Jr., *J. Am. Chem. Soc.*, 1976, **98**, 870.