Biosynthesis of the Pulvomycin Aglycon in *Streptoverticillium Netropis*

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Introduction

Pulvomycin, **1**, is a 22-membered macrocyclic polyketide natural product which can be isolated from the fermentative culture broth of *Streptoverticillium netropis*. The structure of pulvomycin was first reported in 1957.[1] A similar isolate, named labilomycin because of its instability, was isolated in 1963,[2,3] but was subsequently shown to be identical with pulvomycin.[4] The structure originally proposed for **1** was accepted for nearly 20 years before inconsistencies between the NMR spectra of **1** and its suggested structure were noted.[5] An accurate structure for **1** was determined by Smith and coworkers using a series of NMR and MS experiments.[5] The antibiotic has attracted interest because of its mode of action. Like most macrolides **1** inhibits prokaryotic protein synthesis. **1** is unusual, however, in that it acts by blocking the formation of a ternary complex between the prokaryotic elongation factor Tu, GTP, and the aminoacyl-^{t}RNA.[6]

On close inspection, the structure of **1** would appear to deviate from the classical polyketide biosynthetic model.[7] Intriguingly, the C-42 and C-43 methyl groups are appended to positions formally derived from the carboxyl groups of the relevant extender units. Two possible biosynthetic models can be postulated to account for the structure of **1**. Either the entire aglycon backbone is synthesized as one contiguous series of thirteen malonyl-CoA and three methylmalonyl-CoA extender units elaborated on an acetyl-CoA starter unit, or the aglycon backbone is made from three separate polyketide fragments (C-13 to C-34, C-41 to C-1, and C-5 to C-12) linked so that the sense of the central (C-5 to C-12) fragment was reversed with respect to the other fragments.

To distinguish between these two models a series of basic labeling experiments has been carried out, the results of which are reported in this paper.

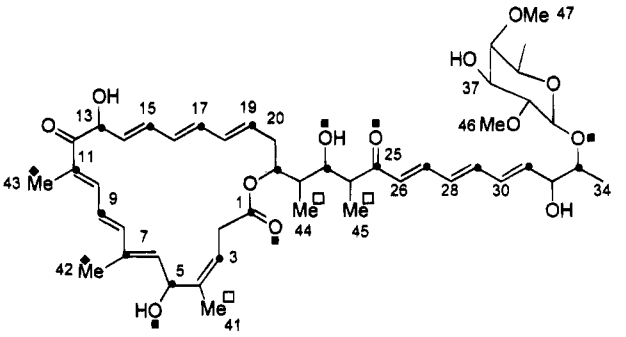

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Figure 1. Positions in the aglycon structure which become enriched with ^{13}C on feeding [1-^{13}C]acetate (●), [1,2-$^{13}C_2$]-acetate (◆) {for clarity, enrichments in the aglycon backbone have been omitted}, [*methyl*-^{13}C]methionine (□), and [1-$^{18}O_2$,^{13}C]-acetate (■).

Results

Antibiotic Production. *S. netropis* was supplied as a slant culture of an isolate obtained by Panlabs Inc. in late 1992. The culture was propagated on slants and then stored at 4 °C. Initial fermentative propagation of the culture gave the expected mycelial yields of 45–60 g L^{-1} but little detectable production of **1**. After much optimization of the culture medium composition the yield of **1** was raised to 36 mg L^{-1}. The material isolated was more than 95% pure as shown by TLC and ^{1}H NMR. The ^{1}H and ^{13}C NMR spectra were almost identical to those reported by Smith and co-workers.[5]

NMR Assignments. While Smith *et al.* had correctly assigned the majority of the ^{13}C NMR signals to carbon atoms in the aglycon structure, a number of ambiguities still remained. The ambiguities were resolved by further NMR experiments: an HMBC experiment done on unlabeled **1** and an INADEQUATE experiment done on **1** recovered from a culture to which [1,2-$^{13}C_2$]acetate had been added.

The INADEQUATE experiment gave data which allowed the assignment of C-4, C-7, and C-11. The signal at 141.6 ppm and the signal at 135.6 ppm could be assigned to C-4 and C-7, respectively, through their couplings to C-3 and C-8. The signal at 131.4 ppm could then be assigned to C-11 by elimination.

The HMBC experiment allowed the unambiguous assignment of the carbonyl carbon atoms at C-12 and C-25 to the signals at 205.9 and 200.5 ppm, respectively. In the case of C-12, a two-bond coupling to H-13 and a three-bond coupling to H-10 were observed. The carbonyl carbon, C-25, showed two-bond couplings to H-24 and H-26, and a further three-bond coupling to H-27.

Feeding Experiments. Significant enrichments derived from [1-^{13}C]acetate were observed for 17 centers, each occurring at every alternate carbon atom in the aglycon structure (Figure 1). The degree of enrichment, as determined from an inverse-gated ^{1}H-decoupled ^{13}C NMR spectrum, ranged form 2.3 to 4.6%.

A subsequent, similar experiment with [1-^{13}C]propionate showed no significant ^{13}C enrichment in the isolated antibiotic.

A feeding experiment with [*methyl*-^{13}C]methionine was carried out to determine the biosynthetic origin of the methyl substituents of the aglycon backbone (Figure 1). Significant enrichments of 30–65% were observed for

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(1) Zief, M.; Woodside, R.; Schmitz, H. *Antibiot. Chemother.* **1957**, *7*, 384.
(2) Akita, E.; Maeda, K; Umezawa, H. *J. Antibiot., Ser. A* **1963**, *16*, 147.
(3) Akita, E.; Maeda, K; Umezawa, H. *J. Antibiot., Ser. A* **1964**, *17*, 200.
(4) Schwartz, J. L.; Tishler, M.; Arison, B. H.; Shafer, H.; Omura, S. J. *J. Antibiot.* **1976**, *29*, 236.
(5) Smith, R. J.; Williams, D. H.; Barna, J. C. J.; McDermott, I. R.; Haegele, K. D.; Piriou, F.; and Higgins, W. *J. Am. Chem. Soc.* **1985**, *107*, 2849.
(6) Wolf, H.; Assmann, D.; Fischer, E. *Proc. Natl. Acad. Sci., U.S.A.* **1978**, *75*, 5324.
(7) Masamune, S.; Bates, G. S.; Corcoran, J. W. *Angew. Chem., Int. Ed. Engl.* **1976**, *16*, 585; Hutchinson, C. R. *Acc. Chem. Res.* **1983**, *16*, 7; Simpson, J. *Nat. Prod. Rep.* **1986**, *3*, 339; Hutchinson, C. R. *Nat. Prod. Rep.* **1987**, *4*, 133; Bu'Lock, J. C. In *Comprehensive Organic Chemistry*; Barton, D.; Ollis, W. D., Eds.; Pergamon: Oxford, 1979; Vol. 5, p 927; Robinson, J. A. *Chem. Soc. Rev.* **1988**, *17*, 383.

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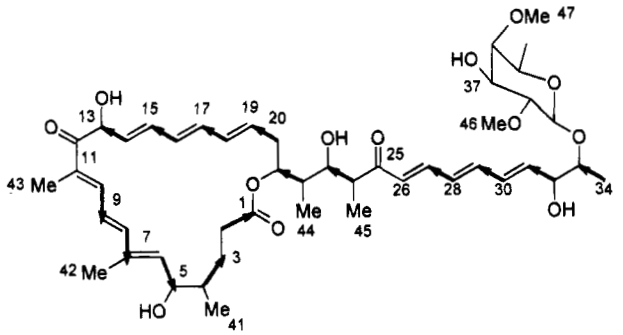

Figure 2. Positions of incorporation of intact C–C units from a feeding experiment with [1,2-$^{13}C_2$]acetate as determined by an INADEQUATE experiment on the recovered natural product. See text for a discussion of the origin of the C-11/C-12 and C-25/C-26 units.

C-41, C-44, and C-45 in the aglycon and C-46 and C-47 in the labiloside moiety. In contrast, no enrichments were observed for C-42 and C-43.

[1,2-$^{13}C_2$]Acetate was fed to producing cultures to determine if the entirety of the aglycon backbone was derived from acetate. The antibiotic isolated was analyzed by inverse-gated ^{1}H-decoupled ^{13}C NMR and by an INADEQUATE experiment. ^{13}C-Enrichments were observed for all of the aglycone backbone carbon atoms as well as C-42 and C-43 (Figure 1). Incorporation of a complete two-carbon unit was observed at fifteen of the expected seventeen acetate derived parts of the aglycone structure (Figure 2). The remaining two unaccounted for units could not be rigorously assigned in the INADEQUATE experiment because of the poor signal to noise level arising from the inherent insensitivity of the INADEQUATE experiment and the small sample size. In addition quaternary carbon centers in both of the missing units would lower their inherent detectability. Evidence for the intact incorporation of a two-carbon unit at C-25/C-26 and C-12/C-13, however, was sought in the 1D ^{13}C NMR spectrum of the isolated antibiotic. Clear doublets centered around the main peak could be seen for signals assigned to both C-12 and C-13. Similar doublets centered around the signals for C-25 and C-26 could be seen although not assigned with absolute confidence due to the complexity of the spectrum in this region. It seems likely that C-12/C-13, at least, is derived intact from acetate.

A feeding experiment with [1-^{13}C,$^{18}O_2$]acetate was done to determine the origin of some of the oxygen atoms on the aglycon backbone. Incorporation of intact ^{18}O–^{13}C units was indicated by observation of ^{18}O satellites in the ^{13}C signals for C-1, C-5, C-23, C-25, and C-33 (Figure 1). A possible satellite upfield of the C-21 resonance could not be ascribed to an ^{18}O isotope induced chemical shift change with absolute confidence because of the presence of other signals in this part of the spectrum.

Discussion

From the data obtained in the feeding studies, the following observations can be made about the biosynthesis of the pulvomycin aglycon by *S. netropis*.

1. The aglycon backbone is derived entirely from acetate. The backbone is probably synthesized by the iterative Claisen condensation of 16 malonyl-CoA extender units onto an acetyl-CoA starter unit. The chemistry involved in this process will be similar to that

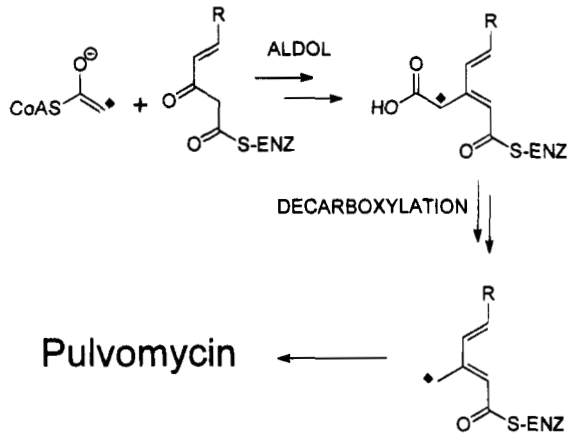

Figure 3. Biosynthetic origin of the aglycon backbone methyl substitution in pulvomycin. Methyl substituents at C-1 of a backbone acetate unit come from the C-2 position of another acetate. ◆ represents a ^{13}C-label.

catalyzed by fatty acid synthase and other polyketide synthase complexes.[7]

2. The methyl substituents on the aglycon backbone come from either methionine (C-41, C-44, and C-45) or the C-2 position of acetate (C-42 and C-43) but not from propionate. C-42 and C-43 are derived from the C-2 position of acetate as these positions were enriched when [1,2-$^{13}C_2$]acetate was fed to the fermentative culture but not when [1-^{13}C]acetate was used. The origin of the methyl group depends on where it is attached to the aglycon backbone. Methyl groups attached to carbons in the backbone arising from C-2 of acetate come from methionine. The methionine-derived methyl groups would be introduced most probably by *S*-adenosylmethionine-dependent methylation of an intermediate enolate species. Methyl groups attached to carbons in the backbone arising from C-1 of acetate come from C-2 of another acetate unit. The acetate-derived methyl groups would most probably arise from an aldol condensation-decarboxylation process (Figure 3). The use of the C-2 of acetate in this way and the mixed origin of the methyl substituents are somewhat unusual although the attachment of the C-2 position of an acetate, itself not in the polyketide backbone, to the C-1 position of another acetate unit within a polyketide backbone has been observed for rhizoxin[8] and myxovirescin.[9]

3. At five sites (C-1, C-5, C-23, C-25, and C-33) a ^{13}C–^{18}O bond is incorporated intact from [1-^{13}C,$^{18}O_2$]acetate into the aglycon structure. Only one of the carboxyl oxygen atoms at C-1 is ^{18}O labeled, as would be expected: [1-^{13}C,$^{18}O_2$]acetate at some point would be activated as a CoASH ester. The satellite peak is a clear singlet with a larger chemical shift difference from the main peak than observed with the other satellite peaks. This would suggest that the carbonyl oxygen was labeled rather than the ester oxygen and that no scrambling of the label between the carbonyl oxygen and the ester oxygen had occurred. This would imply that a carboxylic acid at C-1 was not involved in the biosynthesis and that a thioester was the substrate for the lactonization process. The remaining three oxygenation sites, at C-12, C-13, and C-32, could arise from O_2 by oxidative pro-

(8) Kobayashi, H.; Iwasaki, S.; Yamada, E.; Okuda, S. *J. Chem. Soc., Chem. Commun.* **1986**, 1702.
(9) Trowitzch, W.; Gerth, K.; Wray, V.; Höfle, G. *J. Chem. Soc., Chem. Commun.* **1983**, 1174.

cesses. For example, the oxygen at C-32 could arise from P_{450}-dependent oxidation of this allylic position. A scheme involving P_{450}-dependent epoxidation of a precursor olefin, followed by hydrolytic opening of the epoxide to set up the C-32–C-33 diol, is unlikely as the oxygen atom at C-33 has been shown to derive from acetate. The oxygenation at C-12 and C-13 could, however, quite likely arise from such an epoxidation followed by hydrolytic opening of the epoxide and regioselective oxidation. We could not unequivocally establish the origin of the oxygen atom attached to C-21 in these experiments because of the complexity of the ^{13}C NMR spectrum in the region of the C-21 signal.

Experimental Section

Strains. Two slant cultures of *S. netropis* reisolated in late 1992 were obtained from Panlabs and stored at 4 °C.

Slant Propagation. A sterile solution of glycerol (10 mL, 10% v/v) was introduced to a slant culture and the mycelia scraped off into suspension. The mycelia were further dispersed by vortexing. The mycelial suspension so obtained was recovered and stored in 0.5 mL aliquots at −78 °C. Ten 20 mL slants comprising Difco yeast extract (0.4% w/v), Aldrich malt extract (1% w/v), dextrose (anhydrous, 0.4% w/v), and Difco Agar (2% w/v) in water, adjusted to pH 7.3 with KOH solution, were inoculated with 0.5 mL samples of the mycelial suspension. The slants were grown on at 28 °C for 16 days and then stored at 4 °C.

Production Cultures. The mycelial suspensions of *Streptoverticillium netropis* were propagated in a two-stage fermentation. Initially an aliquot of the mycelial suspension (0.5 mL) was used to inoculate a vegetative culture consisting of Difco tryptic soy broth (3% w/v) in tap water (100 mL). The vegetative cultures were grown in 500 mL nonbaffled Erlenmeyer flasks for 24 h at 28 °C and shaken at 280 rpm (*ca.* 7 cm "throw") in the dark. Aliquots of the vegetative culture (10% v/v) were used to inoculate 33 100 mL fermentative cultures which were then grown in 500 mL nonbaffled Erlenmeyer flasks. The fermentative medium consisted of Promosoy 100 (0.75% w/v),[10,11] dextrose (1.5% w/v), NaCl (0.5% w/v), KH_2PO_4 (0.03% w/v), $CaCO_3$ (0.1% w/v), $FeSO_4{\cdot}7H_20$ (0.0033% w/v), and $ZnSO_4{\cdot}7H_2O$ (0.0033% w/v) in tap water at pH 6.5. The fermentative cultures were shaken at 280 rpm for 36 h in the dark at 28 °C before the cells were harvested.

Typical Extraction Procedure. All operations were carried out under diffuse light; extended operations were carried out at 4 °C. The cells from 2 L of fermentative broth were harvested at 36 h after inoculation by centrifugation (4000 g, 15 min) to give 45–60 g of wet mycelial cake. The mycelial cake was stirred vigorously with CH_2Cl_2–methanol (250 mL; 3:1 v/v) in the dark at 4 °C for 3 h. The extract emulsion was separated by centrifugation (4000 g, 5 min) into three layers: a colorless, clear, mainly aqueous upper layer; a layer of mycelial debris; and a clear, fluorescent green organic phase. The organic phase was recovered, dried over anhydrous Na_2SO_4, and filtered, and the solvent was removed by evaporation *in vacuo* to give a yellow oil. The mycelia and upper phase were combined and extracted a second time. The organic extracts were combined and then fractionated by column chromatography on silica gel, eluting with ethyl acetate–methanol (97:3) to give **1** as a pale yellow, semicrystalline solid in yields between 5 and 50 mg.

NMR Studies. ^1H spectra and ^{13}C spectra were recorded at 300.1 and 75.47 MHz, respectively, on a Bruker AF300 NMR spectrophotometer at 298 K. Samples of **1** were dissolved in 0.5 mL of methyl-d_3 alcohol-d (99.8 atom % ^2H). ^1H spectra were recorded both before and after extended ^{13}C NMR experiments to show that no degradation of the antibiotic had occurred. The INADEQUATE spectrum was run assuming an average $J_{^{13}C-^{13}C}$ coupling of 55 Hz.

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(10) Incubations with the following soy bean meals were attempted, each at a concentration of 1% w/v: Sigma type I (6.6 mgL^{-1} of **1**), Sigma type II (13.0), Promosoy-100 (16.4), Schoenenberger (13.3), Iowa meal (5.8), BBL Trypticase Soy Broth (5.8), and Difco Trypticase Soy Broth (4.3).

(11) The following yields of **1** were obtained at different concentrations of Promosoy-100 in the fermentative cultures: 0.5% w/v (32 mgL^{-1} of **1**), 0.75% (36), 1.0% (31), 1.25% (18), and 1.50% (<10).