Primer Unit Specificity in Rifamycin Biosynthesis Principally Resides in the Later Stages of the Biosynthetic Pathway

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Polyketides are a structurally diverse class of natural products which are important as antibiotics, anticancer drugs, and other pharmaceutically active agents. Biosynthesis of these compounds is achieved by repetitive condensations of simple monomers, a process which closely parallels fatty acid synthesis.[1] In contrast to the fatty acid synthases, polyketide synthases (PKSs) generate a vast variety of different products by using a broad palette of primer as well as different elongation units and by varying the degree of processing after each condensation step. This processing also includes control of the stereochemical course of the reduction of the β-keto carbonyl group. Although these mechanisms could lead to the production of a vast collection of compounds, naturally occurring PKSs typically produce a single product or a small group of closely related compounds. The possibility of predictively altering PKSs in order to obtain novel analogues of known natural products has generated considerable interest. A better understanding of the molecular recognition features of different enzymes within a PKS is a crucial prerequisite for this goal.

Analogous to the extensively investigated 6-deoxyerythronolide B synthase (DEBS),[2,3] the polyketide backbone of rifamycin B (**1**), an ansamycin produced by *Amycolatopsis mediterranei*,[4] is also synthesized by a modular PKS (Figure 1).[5] In contrast to DEBS however, which uses a propionyl-CoA-derived primer unit, the rifamycin PKS is primed by 3-amino-5-hydroxybenzoic acid (AHBA, **2**). Analysis of mutant strains of *A. mediterranei* has led to the identification of a tetraketide shunt product P8/1-OG (**3**) and a macrolactam compound, protorifamycin I (**4**),[6,7] which was considered to be a precursor of rifamycin W (**5**), rifamycin S (**6**), and rifamycin B (**1**). However, subsequent studies with ^{18}O-labeled primer units suggested that 8-deoxyansamycins such as **4** are not true intermediates in rifamycin B biosynthesis.[8] More recently, molecular genetic analysis has suggested that the biosynthesis of AHBA (**2**), as well as its activation into a coenzyme A thioester, is tightly coordinated with polyketide biosynthesis in *A. mediterranei*.[5] Thus, the absence of any metabolically available AHBA analogues provides one mechanism for controlling the overall selectivity of the polyketide pathway with respect to the primer unit. We sought to determine if other enzymes in the pathway also contribute to the observed primer unit specificity.

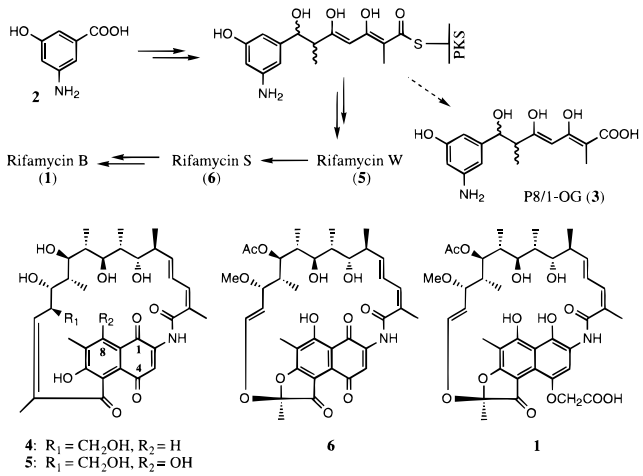

Figure 1. Rifamycin biosynthesis.

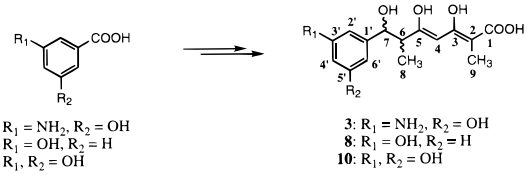

Figure 2. Precursor directed biosynthesis of P8/1-OG analogues. The tetraketide shunt product P8/1-OG (**3**) is synthesized by an earlier characterized mutant in the rifamycin pathway.[6] In this study, the feeding of precursors **7** and **9** to a strain blocked in AHBA (**2**) biosynthesis resulted in the production of **8** and **10**, respectively.

To evaluate the tolerance of the PKS toward priming by alternative aromatic acids, a mutant of *A. mediterranei* that is incapable of AHBA biosynthesis was fed with exogenous AHBA (**2**),[9] as well as the related compounds 3-hydroxybenzoic acid (**7**) and 3,5-dihydroxybenzoic acid (**9**) (Figure 2). The construction of this mutant strain (HGF003), in which the AHBA synthase has been inactivated, has been described elsewhere.[9] The choice of these analogues was based on their ready availability, as well as earlier reports which showed that addition of these analogues to ansamycin fermentations resulted in a reduction of ansamycin titer levels.[10]

AHBA (**2**) and its analogues **7** and **8** were fed to both the wild-type and blocked mutant strains of *A. mediterranei*, and the ethyl acetate extracts of the fermentation media were investigated by HPLC and TLC analysis. Notably, feeding of 1.0 g/L of the natural primer unit[11] **2** to the mutant strain led to the production of **1**,[9] as shown by comparison to a commercially available standard. The yield of rifamycin B (**1**) relative to the amount of

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AHBA (**2**) added was 3.4%. No other major polyketide product was detected in the fermentation broth, and there was not any significant production of PKS related compounds by the mutant strain when the starter unit was omitted. However, addition of the primer unit analogue 3-hydroxybenzoic acid (**7**) at 1.0 g/L to the fermentation medium resulted in the production of a major new compound which was isolated by silica gel column chromatography.[12] The yield of this polyketide relative to starting material was 3.6%. Comparison of the NMR spectroscopic data with the reported values for P8/1-OG (**3**) (present as the free acid) revealed a striking similarity of the chain portion of the compound whereas the signals for the aromatic part proved to be different. On this basis, as well as mass spectrometric and isotope labeling data, it was therefore concluded that the new compound posessed the structure of the corresponding P8/1-OG analogue **8**.[13,14] Similarly, upon administration of the starter unit analogue 3,5-dihydroxybenzoic acid (**9**) (1.0 g/L), the tetraketide **10** bearing two aromatic hydroxy groups was found at 6.2% yield in the fermentation medium of the mutant strain.[15]

The isolation of the tetraketides **8** and **10** may be considered as a further example of the powerful concept of using blocked mutants for the synthesis of unnatural PKS products by supplying unnatural or synthetic compounds able to prime the PKS system.[16,17] Furthermore, our results indicate that, whereas the enzymes involved in the early stages of the rifamycin polyketide pathway (including those for activating the free acid into a CoA thioester, transferring the primer unit onto the first module, and elaborating it into a tetraketide) have relatively broad specificity toward alternative primer units, the acitvity of one or more enzyme(s) in the later stage of rifamycin biosynthesis is crucially dependent upon the presence of the aromatic amino group.

The exact enzyme(s) which present a barrier to polyketide chain elongation and elaboration remain unknown. On one hand, it is possible that the enzymes of the fourth module of the rifamycin PKS show absolute discrimination against substrates lacking the aromatic amino group. On the other hand, even though the biosynthetic block may actually occur at a later stage in the pathway (including possibly chain release via macrolactam formation), the tetraketide is preferentially released due to the presence of the relatively labile β,δ-diketo carbonyl system (or its enol forms). Further mutasynthetic studies[18] using more advanced intermediates may shed light on this unexpected molecular recognition feature of the rifamycin PKS.

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Supporting Information Available: Tables of ^{13}C and ^{1}H NMR data (2 pages). See any current masthead page for ordering and Internet access instructions.

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(12) Strains were grown in Petri dishes containing 40 mL of YMG-medium (4.0 g of yeast extract, 10.0 g of malt extract, 4.0 g of glucose, 1.5% agar, pH was adjusted to 7.3 prior to sterilization). For polyketide production, the plates were incubated for 8−10 days. After 3 days, a sterile solution of the starter unit in DMSO/water 9:1 (pH adjusted to 7.2) was overlaid on the bacteria. Following growth, the solid medium was homogenized and extracted three times with ethyl acetate containing 1% acetic acid. After drying and filtration, the solvent was removed by rotary evaporation and the residue was subjected to column chromatography using ethyl acetate/hexanes 4:1 (containing 1% acetic acid) as an eluant. In all cases, a titer of 80−130 mg/L of tetraketide was observed.

(13) Spectroscopic data for **8** (refer to Figure 2 for numbering): ^{1}H NMR (400 MHz, acetone-d_6) δ 7.15 (t, J = 7.84 Hz, H5′), 6.88 (t, br, 1H, J = 2.07 Hz, H2′), 6.84 (d, br, 1H, J = 7.46 Hz, H4′ or H6′), 6.75 (dd, br, 1H, J = 8.00, 2.44 Hz, H4′ or H6′), 6.09 (s, 1H, H4), 4.70 (d, 1H, J = 8.61 Hz, H7), 2.81 (qd, 1H, J = 8.54, 7.02 Hz), 1.87 (s, 3H, $C9H_3$), 0.95 (d, 3H, J = 7.05 Hz, $C8H_3$), OH not observed; ^{13}C NMR (100 MHz, acetone-d_6): δ 166.07 (C1), 165.29 (C3), 165.24 (C5), 158.14 (C3′), 145.61 (C1′), 129.86 (C4′), 118.86 (C6′), 115.22/114.53 (C2′/C5′), 101.04 (C4), 98.56 (C2), 76.18 (C7), 47.10 (C6), 15.43 (C8), 8.61 (C9). MS (ESI^+) 277 [$(M-H_2O)H^+$]; MS (ESI^-) 275 [$((M-H_2O-H)^-$]; R_f = 0.41 (ethyl acetate/hexanes 4:1, containing 1% acetic acid). The polyketide origin of the tetraketide **8** was proved with two feeding experiments using uniformly labeled $^{13}C_3$-propionate and $^{13}C_2$-acetate at a concentration of 100 and 300 mg/L, respectively. Propionate feeding gave rise to the expected labeling of carbon centers C1, C2, and C9 (first propionate unit, 1.0% specific incorporation rate) as well as C5, C6, and C8 (second propionate unit, 0.9% specific incorporation, compound **8a**) whereas incubation with labeled acetate lead to enrichment at C3 and C4 (0.8% specific incorporation, compound **8b**). On the basis of the $^{13}C-^{13}C$−coupling constants, the connectivities in the chain were assigned (J_{CC} (Hz)) for **8a**: C1−C2 = 78.4, C2−C9 = 47.5, C5−C6 = 51.3, C6−C8 = 35.0. For **8b**: C3−C4 = 58.0. Assignments of the aromatic carbon centers, where possible, are based on chemical shift estimation.

(14) Mass spectrometric analysis of **8** did not reveal the correct molecular ion by various techniques; however, a unit mass of 276 corresponding to a dehydrated fragment was observable with both ESI^+ and ESI^-. Compound **8** was treated with diazomethane to yield the corresponding monomethyl ester derivative **11** in excellent yield. Even then, the molecular ion of the methylated compound was not observable, but the expected mass of 290 corresponding to the dehydrated fragment was found with FAB^+ as well as ESI^+ and ESI^-. Spectroscopic data for the methyl derivative **11**: ^{1}H NMR (400 MHz, acetone-d_6) δ 8.33 (s, br, 1H, C3′-OH), 7.17 (t, 1H, J = 7.63 Hz), 6.90 (s, br, 1H, H2′), 6.86 (d, br, 1H, J = 7.63 Hz, H4′ or H6′), 6.76 (dd, br, 1H, J = 8.03, 2.47 Hz, H4′ or H6′), 6.43 (s, 1H, H4), 4.72 (dd, 1H, J = 8.85, 3.96 Hz, H7), 4.52 (d, 1H, J = 4.03 Hz, C7−OH), 3.94 (s, 3H, OCH_3), 2.91−2.84 (m, 1H) covered with OH/H_2O., 1.82 (s, 3H, $C9H_3$), 0.98 (d, 3H, J = 7.02 Hz, $C8H_3$); ^{13}C NMR (100 MHz, acetone-d_6) δ 166.69, 166.56, 165.25 (C1, C3, and C5), 158.08 (C3′), 145.76 (C1′), 129.79 (C4′), 118.79 (C6′), 115.16/114.46 (C2′/C5′), 100.32 (C4), 95.98 (C2), 76.27 (C7), 56.76 (OCH_3), 47.62 (C6), 15.57 (C8), 8.57 (C9); MS (ESI^+) 291 ($(MH)^+$); MS (ESI^-) 289 ($(M-H)^-$); HRMS (FAB^+, NBA/NaI) calcd for $(C_{16}H_{20}O_6 - H_2O)H^+$ 291.1232, found 291.1244; R_f = 0.50 (ethyl acetate/hexanes 4:1).

(15) Spectroscopic data for **10**: ^{1}H NMR (400 MHz, acetone-d_6) δ 6.38 (d, 2H, J = 2.13 Hz, H2′ and H6′), 6.27 (t, 1H, J = 2.13 Hz, H4′), 6.10 (s, 1H, H4), 4.61 (d, 1H, J = 8.68, H7), 2.78 (qd, 1H, J = 8.48, 7.09 Hz, H6), 1.86 (s, 3H, $C9H_3$), 0.97 (d, 3H, J = 7.05 Hz, $C8H_3$), OH not observed; ^{13}C NMR (100 MHz, acetone-d_6) δ 166.38, 165.47, 165.41 (C1/C3/C5), 159.14 (C3′/C5′), 146.42 (C1′), 106.14 (C2′/C6′), 102.57, 101.10 (C4′ and C4), 98.58 (C2), 76.31 (C7), 46.95 (C6), 15.49 (C8), 8.57 (C9); R_f = 0.50 (ethyl acetate containing 1% acetic acid).

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