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Stereochemical Assignment of Intermediates in the Rifamycin Biosynthetic Pathway by Precursor-Directed Biosynthesis

Ingo V. Hartung, Mathew A. Rude, Nathan A. Schnarr, Daniel Hunziker, and Chaitan Khosla*

Departments of Chemistry, Chemical Engineering, and Biochemistry, Stanford University, Stanford, California 94305-5025

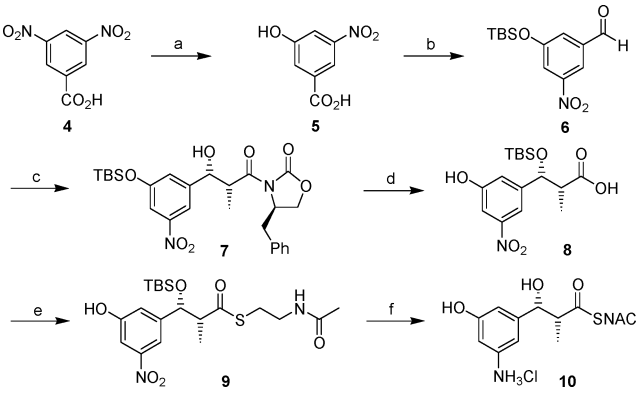
Received March 6, 2005; E-mail: khosla@stanford.edu

Natural and semisynthetic rifamycins are potent inhibitors of bacterial DNA-dependent RNA polymerase and are used for the treatment of tuberculosis and AIDS-associated mycobacterial infections.[1] The parent compound, rifamycin B **1**, is biosynthesized by the soil bacterium *Amycolatopsis mediterranei* employing the assembly line methodology of a modular polyketide synthase (PKS).[2] The first of five PKS enzymes (RifA−E) is primed with 3-amino-5-hydroxybenzoic acid (AHBA, Figure 1), which is derived from the amino-shikimate pathway. The last step of this pathway comprises the aromatization of amino-DHS catalyzed by AHBA synthase (RifK).[3] Knock-out of RifK activity delivers a mutant *A. mediterranei* strain (HGF003) that is incapable of rifamycin B production unless AHBA is supplemented. Formation of the naphthalene core of rifamycin B likely occurs after three rounds of C_2 elongation. The absolute and relative configuration of both stereocenters introduced by module 1 is obscured by the naphthalene aromatization. Premature chain release provides the shunt product P8/1-OG (or the derived lactone **2**), which was isolated from mutated *A. mediterranei* strains as well as from engineered heterologous producing organisms.[4] Feeding of 3,5-dihydroxybenzoic acid or 3-hydroxybenzoic acid instead of AHBA to the *rifK*(−) mutant of *A. mediterranei* produced analogues of P8/1-OG but no higher polyketides.[5] After a total of 10 rounds of C_2 elongation the fully assembled polyketide chain is cyclized to the macrolactam proansamycin X, which undergoes post-PKS modifications to finally yield rifamycin B **1**.

Modified rifamycins are, to date, only accessible by semisynthesis from natural rifamycins. Precursor-directed biosynthesis, in which synthetic compounds are converted into modified natural products by engineered organisms,[6] might complement semisynthetic approaches in opening access to currently inaccessible rifamycins. An understanding of specificity barriers within the rifamycin PKS constitutes one prerequisite for such an approach. The promiscuity of the loading module and module 1 of RifA has been investigated previously.[5,7] We have extended these studies now by synthesizing all four diastereomers of the biosynthetic substrate for module 2 of the rifamycin PKS in form of their *N*-acetylcysteamine (SNAC) thioester (**3**, Figure 1). Only one diastereomer was turned over in vivo into rifamycin B, thus establishing the obscured absolute and relative configuration of the native biosynthetic intermediates.

The synthesis of *syn*-AHBA diketide **10** is shown in Scheme 1. 3,5-Dinitrobenzoic acid **4** was converted into phenol **5** applying the Rickards' procedure.[8] $NaBH_4-I_2$ reduction,[9] MnO_2 oxidation, and TBS protection provided carbaldehyde **6** as a common precursor for all four diastereomers. *syn*-Evans aldol conditions[10] employing the (4*R*)-benzyloxazolidinone delivered aldol **7**. TBS protection and LiOOH-mediated cleavage of the auxiliary (accompanied by cleavage of the phenolic TBS group) was followed by formation of the *N*-acetylcysteamine (SNAC) thioester **9**. Finally, HF-mediated

Scheme 1. Representative Synthesis of AHBA Diketide **10**[a]



[a] Reaction conditions: (a) 1. LiOMe, HMPA, 85 °C, 20 h, 86%; 2. AcOH, HBr, reflux, 20 h, 95%. (b) 1. $NaBH_4$, I_2, THF, room temperature (rt), 6 h, 79%; 2. MnO_2, CH_2Cl_2/acetone (10:1), rt, 24 h, 75%; 3. TBSCl, imid., DMF, rt, 85−90%. (c) (4*R*)-*N*-Propionyl-4-benzyloxazolidinone (1.1 equiv), Bu_2BOTf (1.27 equiv), DIPEA (1.43 equiv), Et_2O, 97%, d.r. > 25:1. (d) 1. TBSOTf, 2,6-lutidine, CH_2Cl_2, 0 °C to rt, 97%; 2. $LiOH\cdot H_2O$, H_2O_2, THF, 0 °C → rt, 91%. (e) 1. CDI (2.1 equiv), DMF, rt, 3 h; 2. HSNAC, DMAP, DMF, rt, 84%. (f) 1. HF (30%), CH_3CN, 0 °C to rt, 94%; 2. H_2, Pd/C, EtOH/HCl (1 N) 10:1, rt, 99%.

TBS cleavage and hydrogenation in the presence of HCl gave rise to ammonium hydrochloride **10**.

The enantiomeric syn-diketide **11** (Scheme 2) was accessible in analogous fashion from carbaldehyde **8** using the opposite enantiomer of the oxazolidinone. anti-Diketides **12** and **13** were prepared applying Heathcock's *anti*-aldol conditions.[11]

The hydrochlorides of all four diastereomers **10**−**13** were transformed into free anilines prior to administration to *A. mediterranei* strain HGF003 cultures by adjustment of substrate solutions to pH 7.2. Fermentation in the presence of AHBA was used as positive control. Analysis of the crude extracts by HPLC and LC−MS using commercially available rifamycin B as reference showed that only anti-diketide **12** was transformed into rifamycin B, while substrates **10**, **11**, and **13** did not deliver any detectable amount of **1**. The turnover efficiency of the natural diketide analogue **12** into **1** is at least 100 times lower than that of AHBA, possibly reflecting a fundamental difference in the loading mechanism of SNAC substrate **12** compared to that of AHBA. To further underscore this finding, in vitro labeling and digestion experiments with RifA module 2 (RM2) were undertaken. A stand-alone protein construct of RM2 was incubated with substrates **10**−**13** and digested with trypsin, and the resulting fragments were analyzed by LC−MS. Substrate **12** was the only diketide to covalently label the KS domain of RM2, indicating the KS domain of RM2 provides a selectivity barrier to diketide incorporation.

Earlier studies have established that PKS modules possess intrinsic selectivity barriers discriminating between naturally and

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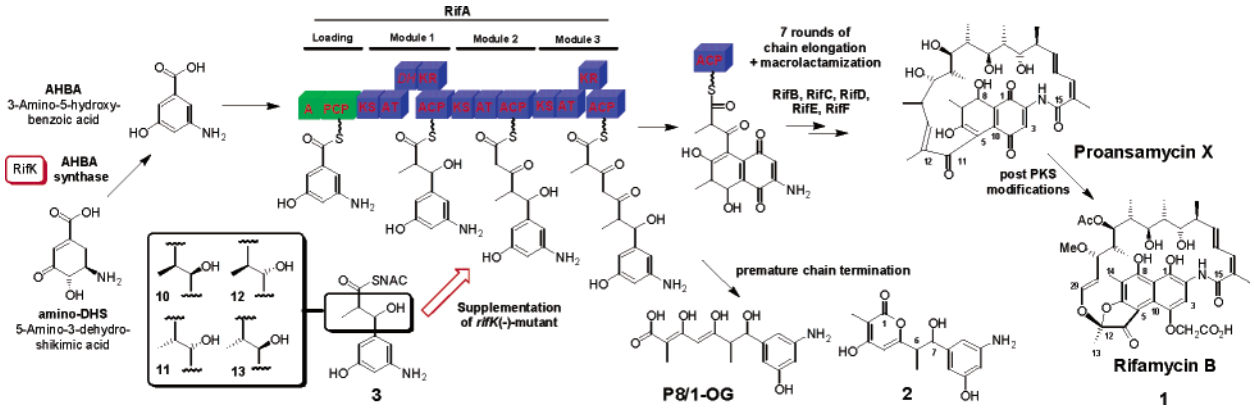
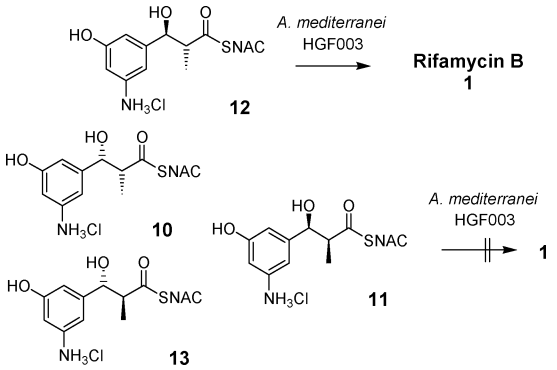


Figure 1. Biosynthesis of rifamycin B. RifA is primed with AHBA, derived from amino-DHS by action of AHBA synthase (RifK). The loading module consists of an adenylation domain (A) for AHBA activation and a peptidyl carrier protein (PCP). Subsequently, 10 cycles of C_2 elongation are carried out by RifA−RifE. Each cycle plus reductive modifications is catalyzed by one module composed of a β-keto synthase domain (KS), an acyl transferase domain (AT), an acyl carrier protein (ACP), and optionally a keto reductase domain (KR) or a dehydratase domain (DH, inactive in module 1). Premature chain release after module 3 of RifA gives rise to the shunt product P8/1-OG (or the derived lactone **2**). Macrolactamization of the fully assembled polyketide chain is catalyzed by amide synthetase RifF leading to proansamycin X, which undergoes post-PKS modifications finally yielding rifamycin B.

Scheme 2 In Vivo Transformations



nonnaturally configured substrates.[12] Therefore, substrate **12** likely contains the natural configuration of intermediates in the rifamycin PKS pathway. Additionally, the keto reductase domain of module 1 can be expected to be D-selective based on sequence analysis,[13] thus establishing that, for example, C7 in lactone **2** is S-configured (as in **11** and **12**). The P8/1-OG-derived lactone **2** shows a large ^{1}H NMR coupling constant (>9 Hz) for this benzylic proton.[4a] Computational minimization of conformational energies for the syn- and anti-diastereomers of lactone **2** led to a dihedral angle of 173.86° for the *anti*-**2**, which is in agreement with the observed coupling constant (Karplus calculation: 9.1 Hz; measured: 9.0 Hz in d_6-DMSO). This additional evidence underscores that anti-diketide **12** contains the natural configuration of biosynthetic intermediates in the rifamycin PKS pathway.

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Supporting Information Available: Synthetic procedures, protein expression, and purification conditions, in vitro labeling procedure, characterization data, LC−MS traces, KR sequence alignment, and modeling results. This material is available free of charge via the Internet at http://pubs.acs.org.

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