A New Substrate Specificity for Acyl Transferase Domains of the Ascomycin Polyketide Synthase in *Streptomyces hygroscopicus*

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Ascomycin (FK520) is a structurally complex macrolide with immunosuppressant activity produced by *Streptomyces hygroscopicus*. The biosynthetic origin of C12-C15 and the two methoxy groups at C13 and C15 has been unclear. It was previously shown that acetate is not incorporated into C12-C15 of the macrolactone ring. Here, the acyl transferase (AT) domain of 8 in the ascomycin polyketide synthase was replaced with heterologous ATs by double homologous recombination. When AT8 was replaced with methylmalonyl-CoA-specific AT domains, the strains produced 13-methyl-13-desmethoxyascomycin, whereas when AT8 was replaced with a malonyl-specific domain, the strains produced 13-desmethoxyascomycin. These data show that ascomycin AT8 does not use malonyl- or methylmalonyl-CoA as a substrate in its native context. Therefore, AT8 must be specific for a substrate bearing oxygen on the alpha carbon. Feeding experiments showed that [13C]glycerol is incorporated into C12-C15 of ascomycin, indicating that both modules 7 and 8 of the polyketide synthase use an extender unit that can be derived from glycerol. When AT6 of the 6-deoxyerythronolide B synthase gene was replaced with ascomycin AT8 and the engineered gene was expressed in *Streptomyces lividans* /H9251, the strain produced 6-deoxyerythronolide B and 2-demethyl-6-deoxyerythronolide B. Therefore, although neither malonyl-CoA nor methylmalonyl-CoA is a substrate for ascomycin AT8 in its native context, both are substrates in the foreign context of the 6-deoxyerythronolide B synthase. Thus, we have demonstrated a new specificity for an AT domain in the ascomycin polyketide synthase and present evidence that specificity can be affected by context.

Ascomycin (FK520) is closely related to tacrolimus (FK506), which is used to prevent xenograft rejection in human patients. Dosing of tacrolimus is difficult because metabolism varies between patients and different co-administered drugs (1–3). Because initial metabolism is due to cytochrome P450-mediated demethylation of the 13-methoxy group (4, 5), we wished to replace the 13-methoxy group with a hydrogen or methyl group and determine whether this increased metabolic stability. Because these analogues could not be obtained by current chemical methods, we sought the desired analogues of ascomycin modified at C13 (Fig. 1) using PKS1 engineering (6, 7).

The macrolactone precursor of ascomycin is biosynthesized by a large PKS complex consisting of a loading module for a shikimate-derived starter unit; 10 modules for malonyl, methylmalonyl, or other PKS extender units; and a peptide synthetase module for addition of pipocoleate (8–13). FK520 and FK506 have methoxy groups at C13 and C15, which could be derived by post-PKS hydroxylation followed by O-methylation or by direct incorporation of an extender unit with an oxygen at the alpha carbon. Feeding of [13C]acetate was reported to label C8-C9 and C20-C23 of the macrolactone ring, as expected, but not C12-C15 in either FK520 or FK506 (8). A [1-13C]erythrose feed, used to establish that the dihydroxycyclohexane starter unit is derived from shikimate, unexpectedly labeled C12 and C14, implying that the extender unit is more readily derived from erythrose than from acetate.

Sequence analysis of the ascomycin gene cluster from *Streptomyces hygroscopicus* (14) showed that it contains close homologues of the genes in the FK506 cluster (9–11) that encode the three PKS subunits, the peptide synthetase, the lysine cyclodeaminase, the C9 hydroxylase, the 31-O-methyltransferase, and the putative 9-hydroxyl oxidase. In the flanking regions of the FK520 cluster, additional genes were found with proposed roles in synthesis of precursors, including a putative methoxymalonyl-ACP precursor for the 13- and 15-methoxy groups of FK520 (14).

The choice of extender unit by a modular PKS system is determined by the acyl transferase (AT) domain of each module (6, 15–17). Comparison of methylmalonyl-CoA- and malonyl-CoA-specific AT domain sequences shows that they cluster into two groups with sequence motifs that diverge according to the specificity of the domain (15). More recently, AT domains specific for ethylmalonyl units have been identified, and they are most closely related to the methylmalonyl-specific domains (18). In addition, there is circumstantial evidence that some domains are specific for an extender unit with oxygen on the alpha carbon (19–21). Despite the availability of the *Escherichia coli* malonyl-CoA:ACP transacylase crystal structure (22) and extensive biochemical experiments with both the rat fatty acid synthase and several bacterial PKSs (23–27), the structural basis for AT domain substrate specificity has not been established.

Here we describe replacement of the ascomycin AT8 domain with AT domains specific for malonyl or methylmalonyl ex-
tender units, resulting in the production of analogues modified at C13 and showing that ascomycin AT8 is specific for an 

**EXPERIMENTAL PROCEDURES**

**Strains and Media**—The ascomycin-producing strain, *S. hygroscopicus* ATCC 14891, was plated on SY medium (10 g of soluble starch, 2 g of yeast extract, and 20 g of agar per liter) to prepare spore stocks and grown in TSBGM (tryptic soy broth supplemented with 50 mM TES buffer, pH 7, and 1% glucose) for production of ascomycin or its analogues. Growth and transformation of *Streptomyces lividans* K4-114 has been described previously (28). Use of the phage vector KC515 followed established procedures (28).

**Preparation of Phage Carrying AT Replacement Cassettes**—PKS module 8 was isolated as a 4.6-kb SpEI fragment from cosmid pKOS65C31 (14) and cloned into Litmus28 (New England Biolabs), and the orientation with a unique ScaI site proximal to the SpeI site of the polylinker was used (pKOS60-21). A synthetic linker was ligated between the SpeI and ScaI sites and then subsequently ligated between the SpAI and A/III sites to give pKOS60-29, which was used as a template to isolate regions flanking AT8 by PCR. The PCR mixtures for all reactions described herein contained (50 μM template to isolate regions flanking AT8 by PCR. The PCR mixtures for all reactions described herein contained (50 μM dNTPs, 10 μM 7-deaza-dGTP; Roche Molecular Biochemicals), 10% Me2SO, 400 nM of each primer, and 1 μM of template (either diluted plasmid DNA, undiluted genomic DNA, or high-titer phage stock), 10× Pfu buffer, 10× 2-deoxynucleotide triphosphate mix (200 μM each deoxynucleotide triphosphate except 100 μM dGTP and 100 μM 7-deaza-dGTP; Roche Molecular Biochemicals), 10% MeSO4, 400 nM of each primer, and 1 μM of cloned Pfu polymerase (Stratagene). Reactions were cycled 25–30 times at 95°C for 30 s, 60°C for 30 s, and 72°C for 2 min. To amplify the 5′-flanking region, the forward primer was 5′-CGACTCTACATGGGCAAGATCTGC-3′, and one of two reverse primers was used: 1) 5′-CCAGCTTGACGGCCGTCG-3′, which introduced an AvrII site near the 3′ end of the ketoacyl synthase domain, or 2) 5′-CGGGTACCTGGTGCGC-3′, which introduced an NheI site near the 5′ end of these PCR products were cloned as SpeI to AvrII and SpeI to NheI fragments into Litmus28 and Litmus38, respectively, to give pKOS60-37-4 and pKOS60-37-2. The 3′-flanking region was isolated using a forward primer (5′-GATGTTACACGGCTGCGAACGATCCGGGTCG-3′) that introduced an XhoI site at the AT/ dehydratase boundary plus a BglII site to facilitate the construction; the reverse primer was 5′-CGACTCTACATGGGCAAGATCTGC-3′. This PCR product was isolated as a BglII to AvrII fragment and ligated to pKOS60-37-4 cut with Acc65I and AvrII or pKOS60-37-2 cut with BglII and AvrII, giving pKOS60-39-13 and pKOS60-39-1, respectively. Heterologous AT domains were obtained by PCR using primers that introduced AvrII and XhoI or NheI and XhoI sites at the 5′ and 3′ ends, respectively. Each heterologous AT domain was cloned into pKOS60-39-13 (AvrII-XhoI) or pKOS60-39-1 (NheI-XhoI) to create a series of AT replacement cassettes. Each replacement cassette was isolated as a BglII to NsiI fragment and ligated to KC515 DNA cut with BamHI and PstI, and the ligation mixture was introduced into *S. lividans* TK24 by transfection. The resulting plaques were purified, and high-titer phage stocks were prepared as described previously (28). Recombinant phage preparations were checked by PCR using a pair of primers annealing with the dehydratase/keto reductase-flanking sequence.

**Construction of Strains with AT8 Replaced by Heterologous ATs**—Spores of *S. hygroscopicus* (107) were suspended in Difco nutrient broth, heat-shocked at 50°C for 10 min, and mixed with an equal number of plaque-forming units of recombinant phage in Difco nutrient broth. The mixture was spread on R2YE plates (28), and after an overnight incubation at 30°C, plates were overlaid with 1.25 mg of methyltriphenylmethane suspended in 1 ml of H2O. After 7–10 days, sporulating colonies were streaked on SY agar containing 50 μg/ml thioestrepton. Colonies were picked from these plates after about 5 days and macerated in 200 μl of H2O using a microfuge pestle. Half the suspension was inoculated into a 25 × 150-mm tube with two 4-mm glass beads and 5 ml of TSBGM; the other half was inoculated into the same medium with 50 μg/ml thioestrepton. After 2–3 days, a 1.5-ml sample of each thioestrepton-containing culture was harvested for total DNA isolation, and a 0.75-ml sample was prepared for LC-MS analysis by adding an equal volume of MeCN and clarifying by centrifugation. DNA was isolated by the SDS, proteinase K, phenol method (28) and analyzed by Southern blot hybridization using the digoxigenin labeling and detection kits supplied by Roche Molecular Biochemicals. Each culture grown without thioestrepton was spread on an SY agar plate to obtain spores to screen for the second recombination event. Selected spore preparations from the nonselective propagation plates above were streaked and spread on several plates at about 100–200 colonies/plate. When colonies were sufficiently sporulated, they were replica-plated onto SY agar plates containing 50 μg/ml thioestrepton. After 4–5 days, putative these recombinants were identified and cloned on the same day, and the broth was extracted as described above for LC-MS analysis (see below). Strains producing 13-DMA or 13-MDMA were designated KOS65-170 and KOS60-135, respectively.

**Precursor Labeling Studies**—[13C2]Glycine and [13C3]glycerol were obtained from Cambridge Isotope Laboratories. [13C3]Glycerol, a mixture of ethyl [13C2]bromoaacetate (Aldrich; 98%; 13C; 1.0 g), sodium acetate (1.0 g), and anhydrous N,N-dimethylformamide (15 ml), was stirred at 80°C for 3 h. Water was added, and the mixture was extracted three times with EtO. The combined organic layers were washed twice with H2O, washed once with brine, and dried over Na2SO4. After removal of solvent, a clear oil (0.45 g) was obtained. 1H NMR (CDCl3) δ 4.59 (2H, dd, J = 149, 4.8 Hz), 4.23 (2H, qd, J = 7.2, 3.2 Hz), 2.16 (3H, s), 1.29 (3H, t, J = 7.2 Hz). The acetate was dissolved in MeOH (15 ml), 0.5 N LiOH (15 ml) was added, and the mixture was stirred overnight at room temperature. After neutralization with 2 N HCl, the mixture was extracted twice with ether. The aqueous layer was dried under high vacuum to give [13C2]lithium glycolate (508 mg; concentration and sodium acetate) and NaH (1H NRCD (D2O); δ 7.82, 1.31 (3H, t, J = 7.2 Hz), 1.45–1.60, 3.5 Hz). [13C2]NMR (D2O); δ 179.1 (d, J = 55 Hz), 60.3 (d, J = 55 Hz).

[methyl-13C2]Methoxyacetic acid:ethyl diazoacetate (1.8 ml) was added dropwise to a stirred solution of Rh2(OAc)2 (10 mg) in [13C]methanol (isoe; 99 atom% 13C; 1.0 g). After 4 h, the mixture was diluted with 10 ml of hexanes and filtered through a 1 cm plug of silica gel. The silica was washed with 3.1 hexanes/ether, and the eluates were combined and concentrated to give 1.35 g of ethyl [methyl-13C2]methoxyacetate as a colorless liquid. 1H NMR (CDCl3); δ 4.28 (2H, q, J = 7.2 Hz), 4.03 (2H, d, J醋酸 = 4.8 Hz), 3.45 (3H, d, J醋酸 = 142 Hz), 1.31 (3H, t, J = 7.2 Hz). The ester was distilled in 2 ml of MeOH and treated with 10 ml of 6 N NaOH for 12 h. The mixture was extracted three times with CH2Cl2, and then the aqueous phase was acidified to below pH 2 using concentrated HCl and diluted with 100 ml of saturated aqueous NaCl before continuous extraction with ether for 24 h. The ether extract was dried over MgSO4, filtered, and evaporated to yield 1.5 g of an oil. Bulk-to-bulb distillation under reduced pressure yielded clean [methyl-13C2]methoxyacetic acid (0.70 g). 1H NMR (CDCl3); δ 4.09 (2H, d, J醋酸 = 4.8 Hz), 3.48 (3H, d, J醋酸 = 142 Hz).

[methyl-13C2]Methoxymalonic acid:5-diazo-Meldrum’s acid (2 g), [13C]ethanol (2 g), Rh2(OAc)2 (250 mg), and tolune (25 ml) were mixed in an Ace pressure tube. The capped tube was heated at 140°C for 2 h, the reaction was filtered, and the filtrate was evaporated. The residue was dissolved in H2O and extracted three times with EtOAc. The combined organic layers were dried over MgSO4, filtered, and evaporated to yield an oil (1.35 g), which was identified as a mixture of dimethyldimethoxymalate and monoethylmethoxymalate. The oil...
was dissolved in MeOH (5 ml), 6 N NaOH (10 ml) was added, and the mixture was left overnight at room temperature. After cooling on ice, the mixture was acidified to pH < 1 with HCl and filtered. The filtrate was dried under high vacuum to yield a yellow solid (1 g). To remove the inorganic salt, the solid was extracted three times with acetone; the acetone extracts were evaporated to give methoxymalonyl-ACP as a yellow solid (0.65 g). $^1$H NMR (acetone-d$_6$): $\delta$ 4.47 (1H, d, $J = 4$ Hz), 3.46 (3H, d, $J = 143$ Hz). $^{13}$C NMR (acetone-d$_6$): $\delta$ 167.0, 79.9, 57.5 (enriched).

Cultures grown in TSBGM were harvested at 24 h, washed twice, and resuspended in 100 mM MES, pH 6.0, 1% glucose to the original culture volume. The resting cell suspension was shaken in baffled flasks at 30 °C and 175 rpm. The $^{13}$C-labeled precursors were added in three equal portions at 24, 36, and 48 h to obtain 0.5 g/l total 13C activity in the final concentration. Resting cell cultures were harvested at 56 h by adding half the volume of the culture of MeOH. The resulting broth/extract was clarified by centrifugation and loaded at 25 ml/min onto a column of Diaion HP-20ss pre-equilibrated in MeOH-H$_2$O (2:1). The column volume was 5% the volume of the broth/extract. The column was eluted at 8 ml/min with MeOH-H$_2$O at ratios of 1:2 (2 column volumes), 1:1 (4 column volumes), 7:3 (4 column volumes), and 9:1 (4 column volumes). The 9:1 eluent was concentrated to give crude ascomycin, which was chromatographed on Bond-Elut ODS solid phase extraction cartridges by eluting with MeOH-H$_2$O (9:1). This material was suitable for NMR analysis.

**Expression of DEBS with AT6 Replaced by Ascomycin AT8**—The Streptomyces expression vector pKAO127 containing the SCP2* replicon and the DEBS genes expressed via the actI promoter and actI-ORF4 transcription activator were described previously (29). Constructs with SpeI and PstI sites engineered at the eryAT6 boundaries have also been described previously (6). The ascomycin AT8 was isolated by PCR with primers that introduced a SpeI site at the 5’ boundary and an NsiI site at the 3’ boundary. The PCR product was cloned into pUCmT28 to give PKOSS8-178, which was checked by sequencing, and subsequently inserted between the engineered SpeI and PstI sites flanking DEBS AT6. This was assembled into the DEBS expression construct pKSOSS8-187. The construct was introduced into S. lividans K4-114 by standard protoplast transformation (28), and the transformants were cultured in R5 medium for production of the 6-DEB analogue.

**LC-MS Analysis**—Samples were analyzed by on-line extraction by LC-MS using a system comprised of a 10 port, 2 position switching valve/injector, Beckman System Gold HPLC, an Alltech evaporative light scattering detector, and a PE SCIEX API100 LC-mass spectrometry—LC-MS using a system comprised of a 10 port, 2 position switching valve/injector, Beckman System Gold HPLC, an Alltech evaporative light scattering detector, and a PE SCIEX API100 LC-mass spectrometry equipped with an atmospheric pressure chemical ionization source. For ascomycin analogue analyses, whole cultures were extracted by adding 1 volume of MeCN and clarified by centrifugation. Sample (250 μl) was loaded onto an Upchurch 4.3/10 mm ODS guard column that was pre-equilibrated with 15% MeCN. A linear gradient from 15% MeCN to 100% MeCN-H$_2$O at ratios of 1:2 (2 column volumes), 1:1 (4 column volumes), 7:3 (4 column volumes), and 9:1 (4 column volumes). The 9:1 eluent was concentrated to give crude ascomycin, which was chromatographed over Bond-Elut ODS solid phase extraction cartridges by eluting with MeOH-H$_2$O (9:1). This material was suitable for NMR analysis.

**RESULTS**

**Replacement of AT8 in the Ascomycin PKS**—We previously hypothesized that AT8 of the ascomycin PKS selects the unusual precursor methoxymalonyl-ACP leading to direct incorporation of the methoxy group at C13 (14). To explore this, ascomycin AT8 was replaced with heterologous ATs of known specificity by double homologous recombination using the phage vector KC515 as shown in Fig. 2. Between 10 and 100 thiostrpton-resistant colonies were obtained after infection with recombinant phages, whereas KC515 alone gave no thiostrpton-resistant colonies. Ten isolates each of lysogens from the rapamycin AT3 or erythromycin AT2 construct were analyzed in detail. None produced detectable ascomycin or related products, consistent with insertion of the phage into a gene essential for ascomycin production. Southern blot hybridization experiments (using XhoI or Acc65I digestion of genomic DNA) showed that of the erythromycin AT2 lysogens, seven arose by recombination at the ketoacyl synthase 8 sequence, and three arose by recombination at the dehydratase 8/keto reductase 8 sequence, whereas of the rapamycin AT3 lysogens, eight recombined at the dehydratase 8/keto reductase 8 sequence, one recombined at the ketoacyl synthase 8, and one apparently recombined at the dehydratase 1/keto reductase 1 sequence, which is 98% identical over 1 kb with keto reductase 8/dehydratase 8. Three lysogens from the malonyl-specific rapamycin AT12 construct also did not produce FK520, and the expected first crossover event was verified using PCR.

After growth in the absence of selection, thio$^8$ colonies appeared at a frequency of about 0.3%, half of which produced ascomycin and had therefore reverted to wild-type. With the exception of one thio$^8$ recombinant that produced no ascomycin-related compound, the remaining thio$^8$ recombinants produced a compound with an LC retention time and atomic mass consistent with either 13-DMA (for the rapamycin AT12 replacement) or 13-MDMA (for the rapamycin AT3 and erythromycin AT2 replacements). The overall statistics indicated little bias for recombination via one flanking sequence over the other. After growing selected strains in laboratory-scale stirred-tank fermenters and purifying the ascomycin analogues, structures were verified by mass spectrometry and NMR analyses. For the rapamycin AT3 replacement strain, only 13-MDMA was produced, and the relative stereochemistry of the 13-methyl group was the same as that of the 13-methoxy group of ascomycin. Thus, the α-methyl epimerization activity that occurs in rapamycin module 3 does not appear to reside on the AT domain, consistent with previous work (23). The 13-

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2 J. Carney, R. Arslanian, E. Woo, and G. Ashley, manuscript in preparation.
New Substrate Specificity in the Ascomycin PKS

**TABLE I**

| Strain          | Replacement | Ascomycin-related products detected (approximate titer) |
|-----------------|-------------|--------------------------------------------------------|
| ATCC14891       | Wild-type   | Ascomycin (50 mg/liter), 21-methyl congener (trace)    |
| KOS60-135       | Rapamycin AT3 | 13-MDMA (2 mg/liter)                                   |
| KOS454-170      | Rapamycin AT12 | 13-DMA (2 mg/liter), ascomycin (0.1 mg/liter)        |

**TABLE II**

| Carbon | δC (ppm) | 1,2,3,4-13C | [13C2]Glycolate | [13C]-MeO-acetate | [13C]-MeO-malonate | [13C2]Glycine | [13C3]Glycerol |
|--------|----------|-------------|----------------|------------------|-------------------|--------------|---------------|
| 12     | 32.6     | 36          | n.d.a          | n.d.c            | n.d.b             | d            | 2′           |
| 13     | 73.6     | 36          | n.d.a          | n.d.c            | n.d.b             | d            | 1.5′         |
| 14     | 72.8     | 44          | n.d.a          | n.d.c            | n.d.b             | d            | 2′           |
| 15     | 73.5     | 44          | n.d.a          | n.d.c            | n.d.b             | d            | 2′           |

a No detectable coupled signals, n.d., not detected; d, detected.
b Weak coupled signals, but detectable.
c Intensity of 13C satellites in relation to the intensity of the central peak in the 13C NMR spectrum.
d Intensity of 13C satellites in relation to the intensity of the central peak in the 13C NMR spectrum.

6-DEB 2-desmethyl-6-DEB not seen

**FIG. 3.** Structure of 6-DEB and potential analogues. *S. lividans* expressing DEBS produces predominantly 6-DEB. *S. lividans* expressing DEBS with AT6 replaced by ascomycin AT8 produces 6-DEB and 2-demethyl-6-DEB. No 2-methoxy-2-demethyl-6-DEB was detected. The portion of the molecules arising from incorporation by DEBS module 6 is boxed.

DMA and 13-MDMA analogues were produced at levels about 5% of parental ascomycin titers (50 mg/liter), which is better than most PKS engineering results (6). For the rapamycin AT12 replacement strain, 13-DMA was the predominant product, but a small amount (~5% of the total) of ascomycin was also observed, even after two rounds of clonal isolation. A summary of the products identified in cultures of the engineered strains is presented in Table I.

**Incorporation of 13C-labeled Precursors into Ascomycin—** It was reported previously that [1,2,3,4-13C]acetate can be incorporated into the macroalactone ring carbons C8, C9, and C20-C23 but not C12-C15 in either ascomycin or FK506 (8). To explore the origin of C12-C15 further, the incorporation of potential 13C-labeled precursors ([13C2]glycolate, [13C]-MeO-acetate, [13C]-MeO-malonate, [13C2]glycine, and [13C2]glycerol) was evaluated by 13C NMR experiments (Table II). No significant enrichment of the O-methyl carbon atoms was seen if either [13C]-methylacetate or [13C]-methylmalonate was fed. In addition, no detectable coupled signals at C12-C15 were seen after feeding glycylate. Glycylate gave weak coupled signals at C12-C15 and additional weak signals for the O-methyl carbons (at C13, C15, and C31), presumably because of metabolism via the one-carbon pool. On the other hand, glycylate feeding gave unequivocal signals resulting from 1,4-C labels for C12-C13 and C14-C15, indicating intact incorporation of two-carbon units at these positions (Table II).

**Expression of DEBS with AT6 Replaced by Ascomycin AT8—** Replacement of DEBS AT6 by the malonyl-specific rapamycin AT2 and expression in *S. lividans* was previously shown to give production of 2-demethyl-6-DEB instead of 6-DEB (6). To further study the ascomycin AT8 domain, the same fusion junctions engineered in this previous construct were used to replace DEBS AT6 with ascomycin AT8. LC-MS analysis of *S. lividans* cultures expressing this construct revealed that 6-DEB and 2-demethyl-6-DEB (Fig. 3) were produced at ~7 and 3 mg/liter, respectively, or 10–20% of the level of 6-DEB produced by *S. lividans* cultures expressing the wild-type DEBS genes. No 2-methoxy-2-demethyl-6-DEB was observed. Thus, ascomycin AT8 in this foreign PKS context, expressed in a strain that does not contain the methoxymalonyl precursor, selects either malonyl-CoA or methylnalonyl-CoA as alternative substrate relatively efficiently.

**DISCUSSION**

The results of AT8 replacement in the ascomycin PKS have proven that module 8 uses a precursor other than malonyl-CoA or methylnalonyl-CoA. Moreover, we have shown that C12-C15 arise by sequential incorporation of an extender unit that can be derived from glycylate, consistent with our previous proposal, based on gene homologies, that the direct precursor comes from the glycolytic pathway (14). Recent results have confirmed that replacement of AT7 with ATs specific for malonyl, methylnalonyl, or ethylmalonyl extenders gave the predicted 15-desmethoxy analogue. Two possible reasons glycylate was not incorporated are that it was not taken up by the cells or that one of the activities needed to convert it to glycylate (e.g. tartronate semialdehyde synthase) was absent from *S. hygroscopicus* under the growth conditions used.

Our results are consistent with the proposal that methylnalonyl-ACP is the substrate used by modules 7 and 8 (14). The substrate is more likely to be a methoxymalonyl thioester rather than a hydroxymalonyl thioester for the following reasons. First, aside from fkbM, the assigned 31-O-methyltransferase gene, fkbG is the only other methyltransferase gene found in the cluster (14). It is unlikely that FkbG methylates both the 13- and 15-hydroxyl groups of a post-PKS intermediate because methyltransferases are known to have very tight substrate specificity. Second, the sequence of FkbG is most similar to two methyltransferases encoded in clusters for macrolides with a methoxy group at an α carbon and is more similar to plant caffeoyl-CoA methyltransferases than to any of the enzymes known to methylate a post-PKS macrolide intermediate (14). This is consistent with FkbG methylating a precursor to the extender unit before its incorporation into the

3 A. Schirmer, W. P. Revill, and L. Katz, unpublished data.
polyketide chain. Finally, if hydroxymalonyl units were incorporated at these positions, the polyketide would rearrange to form the hemiketal isomers known to be favored following demethylation of these methoxy groups by mammalian liver CYP3A4 (4, 5), and this could interfere with subsequent biosynthetic steps. Our previous proposal that the substrate is methoxymalonyl-ACP instead of methoxymalonyl-CoA was based on the presence of an unusual ACP gene (fkbB) in the set of five genes believed to encode the synthesis of this extender unit (14). If this hypothesis is correct, methoxymalonyl-specific AT domains may recognize features of the unusual ACP as an additional way to maintain tight substrate specificity.

The choice of precursor by a given AT domain is governed by its relative selectivity for the available substrates and the effective concentrations of those substrates. Clearly, there is an adequate supply of both malonyl-CoA and methylmalonyl-CoA in \textit{S. hygroscopicus} during ascomycin production because these precursors are incorporated by other modules of the ascomycin PKS. Despite this, AT8 in its native ascomycin PKS context may recognize features of the unusual ACP as an additional way to maintain tight substrate specificity.


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