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Aromatic *C*-Methyltransferases with Antipodal Stereoselectivity for Structurally Diverse Phenolic Amino Acids Catalyze the Methylation Step in the Biosynthesis of the Actinomycin Chromophore[†]

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ABSTRACT: The actinomycin biosynthetic gene cluster of *Streptomyces chrysomallus* harbors two paralogous genes, *acmI* and *acmL*, encoding methyltransferases. To unveil their suspected role in the formation of 3-hydroxy-4-methyl-anthranilic acid (4-MHA), the building block of the actinomycin chromophore, each gene was expressed in *Escherichia coli*. Testing the resulting ~40 kDa His_6-tagged proteins with compounds of biogenetic relevance as substrates and *S*-adenosyl-L-methionine revealed that each exclusively methylated 3-hydroxykynurenine (3-HK) with formation of 3-hydroxy-4-methylkynurenine (4-MHK) identified by its *in vitro* conversion to 4-MHA with hydroxykynureninase. AcmI and AcmL methylate also hydroxyphenylamino propanoic acids such as *p*-tyrosine, *m*-tyrosine, or 3,4-dihydroxy-L-phenylalanine (DOPA) but at a lower rate than 3-HK. The presence of the α-amino group was necessary for substrate recognition. Phenolic acids with shorter chains such as 4-hydoxyphenyl-L-glycine (HPG), 3-hydroxybenzoic acid (3-HB), or 3-hydroxyanthranilic acid (3-HA) gave no product. Both enzymes were stereospecific for the optical configuration at α-C with unprecedented antipodal selectivity for the D-enantiomer of 3-HK and the L-enantiomer of *p*-tyrosine or *m*-tyrosine. AcmI and AcmL show sequence similarity to various *C*- and *O*-methyltransferases from bacteria. Phylogenetic analysis places them into the clade of *C*-methyltransferases comprising among others orthologues involved in 4-MHA formation of other biosynthesis systems and methyltransferases putatively involved in the *C*-methylation of tyrosine. Remarkably, computational remodelling of AcmI and AcmL structures revealed significant similarity with the 3-D structures of type 1 *O*-methyltransferases from plants such as caffeic acid *O*-methyltransferase (COMT) and other phenylpropanoid methyltransferases. The relevance of 3-HK or 3-HA methylation in the actinomycin biosynthesis pathways of different actinomycetes is discussed.

The actinomycins, bicyclic chromopeptide lactone antibiotics produced by various streptomycete strains, are formed, in the last step of their biosynthesis, by the dimerization of monocylic arylpentapeptide lactones which represent actinomycin halves. These half molecules possess the compound 3-hydroxy-4-methylanthranilic acid (4-MHA[1]) as a formal aminoterminus (Figure 1A) (*1*). 4-MHA contains an *o*-aminophenol grouping which can undergo phenol oxidation resulting in the phenoxazinone chromophore of the actinomycins (Figure 1A). The 4-MHA pentapeptide lactones are assembled from 4-MHA and the five amino acids of the peptide lactone rings in a nonribosomal mechanism (*2*, *3*). While the amino acids of the actinomycin peptide rings are derived from the cellular pool of free proteinogenic amino acids (*4*), 4-MHA is a unique compound derived from tryptophan (Figure 1B). It exclusively occurs in the actinomycete family of bacteria, where it is a building block of several secondary metabolites among them the actinomycins (*5*−*8*). More frequent in nature than 4-MHA is its homologue 3-hydroxyanthranilic acid (3-HA) which is also derived from tryptophan (Figure 1B). It is present in fungi, yeasts, higher eukaryotes, and in a limited number of bacteria as an intermediate of the biosynthesis of NAD^+ or in the catabolism of tryptophan (*9*). However, streptomycetes do not appear to use 3-HA for nicotinic acid synthesis and most probably synthesize NAD^+ via the aspartate pathway (*10*). Nevertheless, a link between 3-HA and its homologue 4-MHA was established previously by the discovery of an enzyme from actinomycin-producing *Streptomyces antibioticus* that specifically methylates 3-HA at its 4-position in an *S*-adenosyl-L-methionine (AdoMet)-dependent manner (Figure 1B) (*11*, *12*). This indicates that 3-HA could be an intermediate in 4-MHA synthesis. The actinomycin biosynthetic gene cluster from *Streptomyces chrysomallus* consists of two large inverted repeats which probably arose by gene duplication of a primordial actinomycin biosynthesic gene cluster (*13*). As a consequence, there are nine genes in duplicate, several of which are most probably involved in 4-MHA synthesis. In particular, two paralogous genes encode kynureninase-like enzymes (*acmH* and *acmK*) with substrate preference for 3-hydroxykynurenine (3-HK) resembling in some way the corresponding enzymes from fungi or mammals involved in the formation of 3-HA from 3-HK (*13*) (Figure S1 in

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[1]Abbreviations: 4-MHA, 3-hydroxy-4-methylanthranilic acid; 3-HA, 3-hydroxyanthranilic acid; 4-MHK, 3-hydroxy-4-methylkynurenine; 3-HK, 3-hydroxykynurenine; DOPA, 3,4-dihydroxy-L-phenylalanine.

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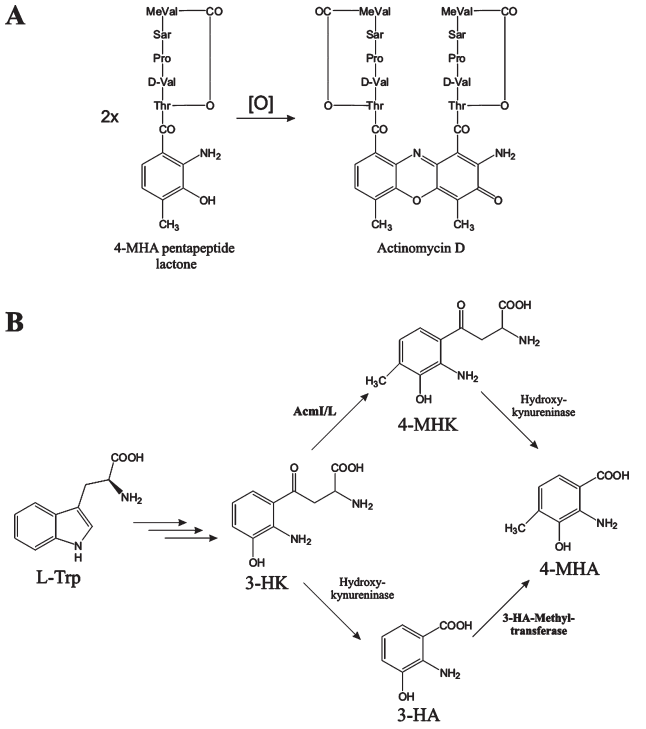

FIGURE 1: Actinomycin D structure and possible pathways of 4-MHA formation in actinomycin-producing streptomycetes. (A) Structure of actinomycin and 4-MHA pentapeptide lactone (Sar = *N*-methylglycine, MeVal = *N*-methyl-L-valine). (B) Alternative pathways for the methylation step during conversion of L-tryptophan to 3-hydroxy-4-methyl-anthranilic acid 4-MHA) (3-HK = 3-hydroxykynurenine; 3-HA = 3-hydroxyanthranilic acid; 4-MHK = 3-hydroxy-4-methylkynurenine).

the Supporting Information). Interestingly, downstream of each of these two hydroxykynureninase genes a gene, *acmI* or *acmL*, respectively, is located (Figure S1 in the Supporting Information) each encoding a protein with similarity to various *C*- and *O*-methyltransferases from bacteria and plants. These two methyltransferase genes would be candidates to be the *S. chrysomallus* orthologues of the above-mentioned 3-HA 4-methyltransferase from *S. antibioticus* (*12*). On the other hand, it could not be ruled out that the methylation step is earlier than at 3-HA because previous *in vivo* feeding of *S. antibioticus* cells with chemically synthesized intermediates had suggested some evidence that methylation would take place at the stage of 3-HK and not 3-HA because both 3-hydroxy-4-methylkynurenine (4-MHK) and 4-MHA diluted incorporation of labeled tryptophan into the chromophore of the antibiotic in contrast to 3-HA (*14*). To clarify this issue, we set out to express both *acmI* and *acmL* in *E. coli* and test their gene products for their substrate specificity. It will be shown here that both AcmI and AcmL methylate 3-HK rather than 3-HA indicating an alternative step of methyl group introduction into the benzene ring of 4-MHA in 4-MHA-producing actinomycetes. A detailed characterization of these two novel methyltransferases will also be presented.

MATERIAL AND METHODS

Strains and Cultures. *Streptomyces chrysomallus* strains Sc1 (wild type) and Sc-white (actinomycin nonproducing) and their cultivations were described previously (*13*). For the preparation of protein extracts from *S. chrysomallus* strains, mycelium grown in the glutamate-mineral salts medium (*15*) supplemented with 1% maltose using 500-mL Erlenmeyer flasks containing 200 mL of medium was used exclusively (*13*). The harvest of cells was after 48 h of growth. *E. coli* strains were DH5α (*16*) and M15 (Qiagen). They were grown as described in ref *17*.

DNA Manipulation. All DNA manipulations were according to ref *17*. PCR amplification of *acmI* and *acmL* from chromosomal DNA of *S. chrysomallus* strain Sc1 was by using primer pairs AcmI_Forw (5′-GGA**GGTACC**ATGGCAGACG-TCCGCCCCTTC-3′) AcmI_Rev (5′-GGAG**AAGCTT**TCACT-TGACCGCGCTGATCAC-3′), AcmL_Forw (5′-GA**GGTAC-C**ATGCCTCACGCGTCCCCGCTCA-3′), and AcmL_Rev (5′-GCT**AAGCTT**TCACTTGACCGCGCTGATCAC-3′), each derived from the 5′- and 3′-ends of *acmI* and *acmL*, respectively (Genbank HM038106). Primers carried *Kpn*I (5′) and *Hind*III (3′) sites at their ends. Conditions for PCR were (1) 2 min 95 °C; (2) 30 cycles consisting each of 1 min 95 °C, 30 s 60 °C, 1 min 72 °C; (3) 10 min 72 °C. The resulting fragments were TOPO-cloned into pCR4-TOPO (Invitrogen) and subsequently cloned as *Kpn*I-*Hind*III fragments into *Kpn*I/*Hind*III-cleaved plasmid pQE30 (Qiagen). Correctness of the gene sequence was established by DNA sequencing. The resultant plasmids were pAcmI-pQE30 or pAcmL-pQE30 allowing expression of cloned genes as aminoterminal His_6-fusion proteins in *E. coli* strain M15. To prepare a non-His_6 version of AcmL, the corresponding *Kpn*I and *Hind*III fragment from pAcml-pQE30 was ligated to *Kpn*I/*Hind*III-cleaved pQE50 (Qiagen). The resultant plasmid was pAcmL-pQE50.

Analytical Methods. Solvent systems for thin layer chromatography of 4-MHA were solvent system I (ethylacetate−methanol−water, 20:1:1, by volume), II (hexane−ethylacetate−acetic acid, 8:2.5:0.5, by volume), and III (chloroform−acetic acid, 9:1, by volume); for kynurenine and hydroxykynurenine solvent systems IV (*n*-butanol−acetic acid−water, 4:1:1, by volume), V (*n*-propanol−acetic acid−water, 7:3:2, by volume), and VI (*i*-propanol−dibutylether−acetic acid−water, 4:3:3:2, by volume); and for chiral chromatography solvent systems VII (methanol−water 1:7, by volume) and VIII (acetone−methanol−water, 4:2:2, by volume). Separation of formyl-L-kynurenine, L-kynurenine, DL-kynurenine, 3-hydroxy-DL-kynurenine, anthranilic acid, 3-hydroxyanthranilic acid, and 3-hydroxy-4-methylanthranilic acid by HPLC was on a Eurospher 100 C18 (5 μm) column (250 mm × 4.6 mm) (Knauer AG, Berlin, Germany) with a water−acetonitrile gradient (containing 0.1% trifluoroacetic acid) from 0 to 60% acetonitrile in 15 min at a flow rate of 1 mL min^{-1}. Detection wavelengths were at 340 nm (3-hydroxyanthranilic acid, 4-methyl-3-hydroxyanthranilic acid), 320 nm (kynurenine), or 368 nm (3-hydroxy-DL-kynurenine).

Enzyme Assays. In the case of radioactive assay, His_6-tagged AcmI or AcmL (purified according to the protocols given in the Supporting Information) were incubated with 1 μCi [^{14}C]-methyl-adenosyl-L-methionine and 1 mM nonlabeled 3-HK or other substrates in a total volume of 100 μL at 30 °C for up to 1 h.. The amounts of protein ranged between 50 and 500 μg. In the case of tyrosine methylation, the reaction time was up to 12 h. The buffer was buffer B. After the end of incubations, the reaction mixtures were brought to pH 2 and applied to small Dowex-50 (H^+-form) columns (half-filled Pasteur pipettes) and washed with 0.5 M HOAc, and the products eluted with 2 M NH_3. After freeze-dying, the products were taken up in 50 μL of ethanol−water (7:3, by volume), applied to silica gel thin layer chromatography (TLC) plates, and chromatographed with solvent system IV or V. TLCs were analyzed by radioscanning (RITA, Raytest, Straubenhardt, Germany) or by autoradiography using X-ray

film Biomax MR (Kodak). Occasionally, in the assays with 3-HK, 4-MHK formation was monitored via 4-MHA formation in a coupled assay with hydroxykynureninase (50 μL of enzyme extract containing 5−10 mg mL^{-1} protein). Usually, the hydroxykynureninase was added at the end of incubation which was continued for another 30 min at 30 °C after which 4-MHK was completely converted to 4-MHA. After addition of 2 mL of water, the pH was brought to pH 2 and the reaction mixture was extracted with two portions of 2 mL of EtOAc. The combined extracts were evaporated to dryness, the residue was dissolved in a minute amount of EtOAc, applied to the silica TLC plate, and chromatographed in solvent systems II, III, or VI. Detection of radioactive 4-MHA was by radioscanning or by autoradiography. Assays to demonstrate 4-MHA formation from 3-HA catalyzed by AcmI or AcmL (at 100−200 μg mL^{-1} protein concentration), or enzyme extracts from *S. chrysomallus* strains (concentration of 5−10 mg mL^{-1} protein), contained 1 mM 3-HA and 1 μCi [^{14}C-methyl]-*S*-adenosyl-L-methionine and 500−5000 μg of protein at 30 °C in a total volume of 100 μL. The buffer was buffer B.

In the case of nonlabeled substrates, AcmI or AcmL (50−500 μg of protein) were incubated with AdoMet (50−1000 μM), 3-HK (100−1500 μM), or tyrosine (1000 μM) in total volumes of 100 μL at different temperatures and for different times. The buffer was buffer B. At the end of the incubations, 10 μL of 50% trichloroacetic acid (TCA) was added and the resulting precipitate was removed by centrifugation at 14 000 rpm (Eppendorf centrifuge 5417C) at room temperature. The supernatant was applied to a HPLC column and separated as above. 3-HK had a elution time of 12 min and the methylation product 4-MHK had one of 14 min (Figure S2 in the Supporting Information). Peaks were localized by monitoring at the indicated wavelength (see above), and quantitation was performed by referring to a calibration curve established by chromatography of various known amounts of reference material on the same HPLC column.

*Preparation of 3-Hydroxy-*D*- and* L*-Kynurenine (3-*D*-HK and 3-*L*-HK) by Chiral TLC of Racemic 3-HK.* Separation of racemic 3-hydroxykynurenine into its enantiomers was performed by chiral TLC using solvent system VII. The bands representing the L- or D-enantiomer were scraped off the plates and extracted each with 2 mL of 50% ethanol, 1 mM DTE. Rechromatography of each band on HPLC showed that both had the same retention time as racemic 3-HK (12 min). Treatment of the material contained in the band of higher R_f-value (R_f = 0.32) with L-amino acid oxidase (Sigma) revealed that it represented the L-enantiomer usually contaminated with a little rest of D-enantiomer as shown by its conversion to xanthurenic acid (16 min) (Figure S3A in the Supporting Information). By contrast, treatment of the material contained in the band of lower R_f-value (R_f = 0.27) with L-amino acid oxidase left the compound unchanged except for a little rest of L-enantiomer present in the sample. This indicated that the band of higher R_f-value represents L-3-HK and the band of lower R_f-value D-3-HK. D- and L-*m*-tyrosine were prepared from racemic *m*-tyrosine essentially by the same procedure except that for chiral TLC solvent system VIII was used (R_f = 0.76; R_f = 0.79, respectively).

Sequence Analysis and Data Handling. Sequence analysis was done with PC/Gene (Intelligenetics, Mountain View) and BlastP (National Center for Biotechnology Information). Multiple alignments were performed with ClustalW2 (*18*) and edited with GeneDoc (*19*); tree analysis was with clustalW and edited with Drawtree (http://www.phylodiversity.net) or by using the phylogeny.fr web service ((*20*), http://www.phylogeny.fr/). Structure modeling was with the aid of programs such as Phyre (*21*) or I-tasser (*22*). Molecular graphics images were produced using the UCSF Chimera package from the Resource for Biocomputing, Visualization and Informatics at University of California at San Francisco (http://www.cgl.ucsf.edu/chimera).

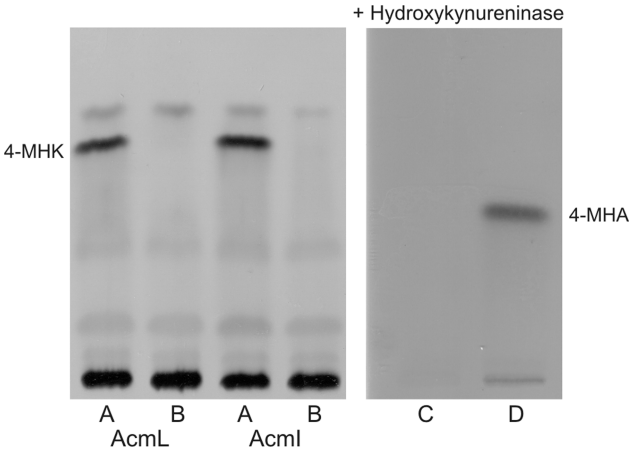

FIGURE 2: 3-Hydroxykynurenine methyltransferase activity of AcmI and AcmL. (Left) Autoradiograph of TLC separations of reaction mixtures of *E. coli* cell extracts containing His$_6$AcmL or His$_6$AcmI with ^{14}C-methyl-AdoMet in the presence (A) and absence (B) of 3-HK (solvent system IV). (Right) Conversion of the putative 4-MHK into 4-MHA by hydroxykynureninase. His$_6$AcmL was incubated with ^{14}C-methyl-AdoMet in the absence (C) and presence (D) of 3-HK and subsequent postincubation with hydroxykynureninase. The mixtures were extracted with EtOAC under acidic conditions, and the residues after evaporation were chromatographed on a TLC plate with solvent system II. The time of exposure to the X-ray film was 2 days.

RESULTS AND DISCUSSION

Heterologous Expression of acmI and acmL in E. coli and Search for the Substrate of Their Gene Products. Both *acmI* and *acmL* were PCR-amplified from chromosomal DNA of strain *S. chrysomallus* Sc1 and cloned in-frame into expression vector pQE30. The resultant plasmids were AcmI-pQE30 and AcmL-pQE30. SDS-PAGE analysis showed that cell extracts of IPTG-induced *E. coli* M15 strains harboring the plasmids each contained an abundant 40 kDa protein band apparently representing His$_6$AcmI and His$_6$AcmL. At growth temperatures lower than 25 °C, both proteins were soluble. Incubations of the cell extracts with [^{14}C-methyl]-AdoMet and different biogenetically relevant intermediates of tryptophan catabolism such as formyl-L-kynurenine, DL-kynurenine, 3-hydroxy-DL-kynurenine (3-HK), anthranilic acid, or 3-hydroxyanthranilic acid (3-HA) surprisingly revealed formation of a product only in the presence of 3-HK. In different TLC systems this product always had a slightly higher R_f-value than 3-HK from which it was argued that it might be 3-hydroxy-4-methylkynurenine (4-MHK) (Figure 2).

Structural Evidence of Enzymatically Formed 3-Hydroxy-4-methyl-kynurenine. Hydroxykynureninase from actinomycin-producing *S. chrysomallus* was shown to be capable to convert 3-HK to 3-HA *in vitro* (*13*). From this it was expected that it could convert the putative 4-MHK to 4-MHA (see Figure 1). In fact, when hydroxykynureninase was added to the His$_6$AcmI- or His$_6$AcmL-containing incubations with 3-HK and ^{14}C-AdoMet and incubation was resumed further, the final product was radioactive 4-MHA as confirmed by thin-layer chromatographic

comparisons with authentic 4-MHA (Figure 2). Henceforward, the assays were performed with nonlabeled AdoMet and 3-HK. Separation of these reaction mixtures by RP-HPLC revealed a product eluting at ~14 min (Figure S2 in the Supporting Information), i.e., with ~2 min higher retention time than 3-HK, which could be enzymatically converted to 4-MHA whereas the educt 3-HK was converted to 3-HA. Mass spectrometric analysis of the material contained in the peak eluting at 14 min revealed a mass peak [MH^+] of 239 in accordance with the calculated molecular mass of 238 Da of 4-MHK.

Purification and Properties of His_6AcmI and His_6AcmL. His_6AcmI and His_6AcmL were purified to homogeneity (Figure S4 in the Supporting Information). Their calculated molecular masses are 39.74 and 39.67 kDa, respectively, which fit the sizes of the denatured proteins of 40.1 ± 0.2 kDa as estimated from SDS-PAGE (Figure S4 in the Supporting Information). Gel filtration on Superdex Tm75 and Superdex Tm200 columns revealed native M_rs of 73 000 ± 1800, which suggests that both enzymes are dimers. At 30 and 37 °C, 4-MHK formation (in the presence of 1 mM of each 3-HK and AdoMet) proceeded linearly with time for 3 h for both enzymes. After this period, the reaction slowed due to interference with the nonenzymatic oxidative conversion of 3-HK to the corresponding phenoxazinone. In the case of tyrosine methylation, linearity was observed for at least 8 h in the same conditions (see below). The temperature optimum measured in 30 min incubations at different temperatures was 37 °C for His_6AcmI and 42 °C for His_6AcmL. Sulfhydryl-protecting agent such as dithioerythritol (generally used at 4 mM concentration) significantly protected both enzymes against loss of specific activity in incubations as well as in storage conditions in agreement with the presence of two and four cysteines in the sequences of AcmI and AcmL, respectively. However, iodoacetamide or dibromopropanone had no significant effect on the activity of these enzymes compared with controls without these inhibitors. This makes a role of thiol groups in their reaction mechanism unlikely. The pH range of enzyme activities was from pH 6.3 to 8.3. In phosphate-containing buffer, their activity declined drastically above ~pH 7.2 indicating that the HPO_4^{2-} ion most probably inhibited the enzyme. The fact that both enzymes do not methylate DL-kynurenine indicates the requirement of the phenolic OH-group for activity as has been noted for the *C*-methyltransferases CouO and NovO which both catalyze *in ortho* methylation of phenolic intermediates in the biosynthesis of the antibiotics novobiocin and coumermycin, respectively (*23*). The most likely formation of a phenolate ionic group may activate the benzene ring *in ortho* facilitating attack to the methyl group of AdoMet (*23*). Both AcmI and AcmL did not show dependence on earth alkali metal ions in contrast to type 2 *O*-methyltransferases which *O*-methylate biphenolic substrates such as catechol-*O*-methyltransferases in the presence of Mg^{2+} only (*24*). Enzymes were stable when kept at −80 °C in the presence of 20% glycerol with no loss of activity after at least 3 months.

Sequence Analysis of AcmI and AcmL. BLASTP analysis of AcmI and AcmL sequences revealed high similarity to a large number of proteins annotated as *C*- or *O*-methyltransferases. There were three proteins with outstanding similarity (54% identity) to AcmI and AcmL. SfmM2 is a postulated *C*-methyltransferase encoded by the saframycin biosynthesis gene *sfmM2* from *Streptomyces lavendulae* (*25*). *SfmM2* in conjunction with the genes *sfmM3* and *sfmD* was shown to be involved in synthesis of 3-hydroxy-5-methyl-4-*O*-methyl-tyrosine (3h5m*O*meTyr)

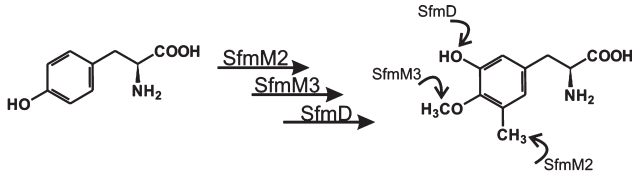

FIGURE 3: Reaction scheme of transformation of tyrosine into 3-hydroxy-5-methyl-4-*O*-methyl-tyrosine (3h5m*O*meTyr), from ref *25*; for details see text.

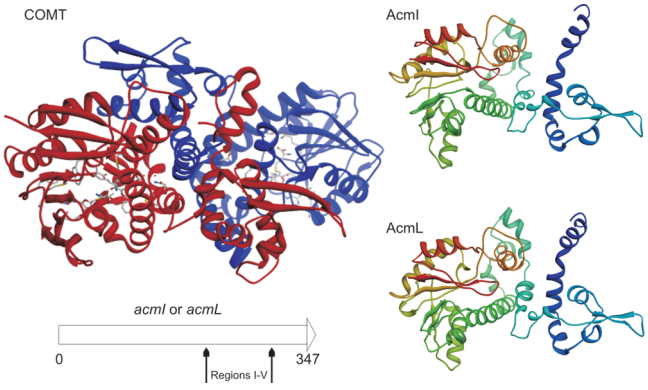

FIGURE 4: Computed 3-D structures of AcmI and AcmL. Structures were calculated with aid of Phyre (*21*) and Chimera (see Materials and Methods) based on the structure of caffeic acid *O*-methyltransferase (COMT) from *Medicago sativa* (*27*) (top). Indicated are the AdoMet-binding sites and the ferulic acid substrate binding sites of the dimer (from ref *27*). The indicated conserved motifs of the AdoMet binding site are I (LDVGGxG), II (GINFDLPHV), III (EHVGGDMF), IV (NGKVI), and V (GGKERT) all taken from ref *28*.

from tyrosine (Figure 3). 3h5m*O*meTyr is a building block of saframycin and also of the closely related antibiotic safracin from *Pseudomonas fluorescens* (*25*). *SfmM2* was assigned to encode introduction of the methyl group in C5 of 3h5m*O*meTyr and *sfmM3* was assigned to encode the *O*-methylation of the tyrosine phenol group. The temporal order of these methylation steps was not elucidated (*25*). The next hit was an *O*-methyltransferase (ZP_05534762) from *Streptomyces viridochromogenes* with unknown function whereas the third was SibL encoded by the gene *sibL* of the sibiromycin gene cluster from *Streptosporangium sibiricum* (*7*). Sibiromycin, a diazepine antibiotic, contains 4-MHA as a building block in its structure which suggests that SibL is the orthologue of AcmI and AcmL in the biosynthesis of 4-MHA in *S. sibiricum* because it is the only methyltransferase gene in that gene cluster.

Structure prediction programs such as Phyre (*21*) or I-tasser (*22*) revealed high structural similarity of AcmI and AcmL to type 1 plant *O*-methyltransferases (*26*) such as caffeic acid *O*-methyltransferase (COMT) from *Medicago sativa* (*27*). The structural remodelling of AcmI and AcmL based on the COMT structure suggests that like COMT AcmI and AcmL are dimers thus indirectly confirming the determinations of their native M_rs mentioned above. AcmI and AcmL are highly similar to COMT in their AdoMet binding regions displaying the same five signature sequences which, importantly, are located in the carboxyterminal portion of the proteins (*28*, *29*). This is the case for all type 1 *O*-methyltransferases, e.g., chalcone *O*-methyltransferase and isoflavone *O*-methyltransferase IOMT (Figure 4) (*29*). Moreover, the *N*-methyltransferase PhzM from *Pseudomonas aeruginosa* (*30*) from its structure also belonging to type 1

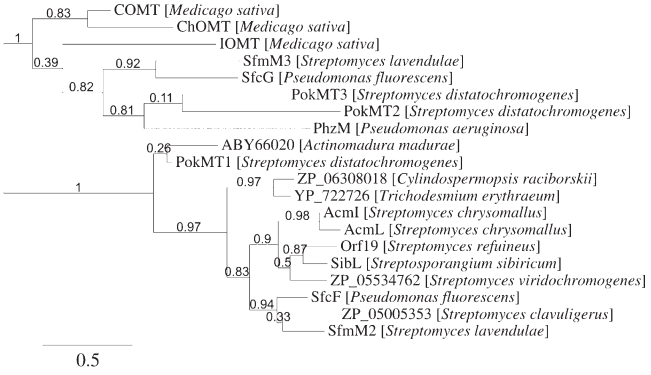

FIGURE 5: Phylogenetic tree of various *O*- and *C*-methyltransferases with similarity to AcmI and AcmL. Upper clade: *O*-methyltransferases type I and related methyltransferases. Lower clade: *C*-methyltransferases. Accession Numbers not included are COMT P28002, ChOMT P93324, IOMT O24529, SfmM3 ABI22145.1, SfmM2 ABI22137, SfcG AAL33762, PokMT3 ACN64847, PokMT2 ACN64843, PhzM PAO1]NP_252898, PokMT1 ACN64833, AcmI ADG27364, AcmL ADG27351, Orf19 ABW71850, SibL ACN39735, SfcF AAL33761. Both ZP_05005353 and ZP_05534762 are annotated as *O*-methyltransferases.

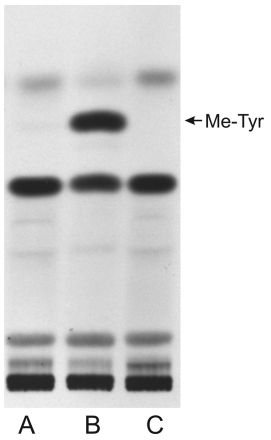

FIGURE 6: Methylation of L-tyrosine catalyzed by AcmL. TLC separations of reaction mixtures containing 50 μg of AcmL, 1 μCi ^{14}C-AdoMet, 1 mM L- or D-tyrosine. (A) with D-tyrosine; (B) with L-tyrosine; (C) without substrate. Incubation was for 8 h as described in the Materials and Methods. The time of exposure to the X-ray film was 3 days.

O-methyltransferases has striking similarity to AcmI and AcmL. Less similarity to the type 1 *O*-methyltransferases is seen in the aminoterminal region of AcmI and AcmL which covers the substrate binding regions in COMT. A sequence comparison of this region contained in the different *C*-methyltransferases along with AcmI and AcmL revealed as yet no relevant clues of which residues in AcmI or AcmL would play a role in 3-HK binding. These analyses also showed that the *C*-methyltransferases CouO and NovO (*23*) from coumermycin and novobiocin-producing streptomycetes are not related to AcmI or AcmL. Both CouO and NovO have their AdoMet binding site in the aminoterminal portion of their sequences and also are shorter than AcmI and AcmL and therefore do not align with them significantly.

Phylogenetic Analysis of AcmI and AcmL. A phylogenetic tree calculated from similar sequences selected from a BLASTP search using AcmI and AcmL as a query revealed an *O*-methyltransferase clade, among them COMT, and a *C*-methyltransferase clade, among them AcmI and AcmL (Figure 5). The aforementioned SibL and also Orf19, a methyltransferase gene located in the anthramycin biosynthetic gene cluster from *Streptomyces refuineus* (*5*), which like actinomycin or sibiromycin contains 4-MHA as building block, are located in a sister clade near to AcmI and AcmL as to be expected for typical orthologues catalyzing the same reaction. In another sister clade are located SfmM2 and its orthologue SfcF, which are presumed to catalyze the *C*-methylation of either tyrosine or of *O*-methyl-L-tyrosine in the synthesis of the above-mentioned 3h5m*O*meTyr (*25*). Similarly, the methyltransferase ZP_05534762 from *S. viridochromogenes* is located in the same subclade as SibL and Orf19 leaving little doubt that it is a *C*-methyltransferase rather than a *O*-methyltransferase as originally annotated in Genbank. In addition to COMT, the clade of *O*-methyltranferases contains other type 1 *O*-methyltransferases from plants like COMT, ChOMT, or IOMT (*27*, *31*) and other experimentally proven *O*-methyltransferases such as PokMT2 and PokMT3 from *Streptomyces diastatochromogenes* involved in the *O*-methylation of polyketomycin precursor (*32*) or the methyltransferase SfmM3 and its orthologue SfcG both assigned to *O*-methylate tyrosine in the formation of 3h5m*O*meTyr during the biosyntheses of saframycin or safracin, respectively (*25*). Their location in the clade of *O*-methyltransferases supports their postulated roles as *O*-methyltransferases and the validity of the phylogenetic analysis shown here.

AcmI and AcmL Have Tyrosine Methyltransferase Activity. In view of their similarity to SfmM2 and SfcG of saframycin and safracin biosynthesis systems, respectively, AcmI and AcmL enzymes were tested whether they could methylate tyrosine and other substrates with structural similarity to tyrosine or 3-HK. Figure 6 shows that AcmI (like AcmL) can methylate tyrosine giving a product that is different from *O*-methyl-tyrosine. *O*-Methyl-tyrosine itself or L-phenylalanine were not methylated apparently because they lack a phenolic hydroxyl group which appears of crucial importance for activity as in the case of 3-HK (see above). Measurements of the time course of tyrosine methylation along with that of 3-HK revealed a ~5-fold lower activity in the case of tyrosine (Figure S6 in the Supporting Information). Longer incubation of AcmL with unlabeled tyrosine and AdoMet afforded formation of an amount of product sufficient for mass spectrometric analysis which revealed a [MH^+] of 213 consistent with the calculated mass of putative 3-methyl-tyrosine of 212 Da. Interestingly, AcmI (and AcmL) also methylate *m*-tyrosine to the same extent as tyrosine whereas 3,4-dihydroxy-L-phenylalanine (DOPA) was methylated in minute amounts. By contrast, compounds with shorter or no aliphatic side chains such as 4-hydroxyphenyl-L-glycine, 3-hydroxy-benzoic acid, 4-hydroxybenzoic acid, or 3-HA gave no product. Compounds such as caffeic acid, 3-(4-hydroxyphenyl)-propionic acid, 3-hydroxy-acetophenone, and 2-(3,4-dihydroxyphenyl)ethylamine (dopamine) also gave no response to the enzymes indicating that the α-amino acid grouping in the side chain is necessary for substrate recognition. The finding that AcmI and AcmL methylate tyrosine but not *O*-methyl-tyrosine suggests that in saframycin or safracin synthesis, *C*-methylation of tyrosine by SfmM2 is prior to its *O*-methylation by SfmM3. This detail was not addressed in the previous studies of 3h5m*O*-meTyr synthesis (*25*).

Stereospecificity of AcmI and AcmL. To test the stereospecificity of AcmI and AcmL, both enzymes were tested with D-tyrosine. As expected it was not methylated in contrast to L-tyrosine (Figure 6). We then tested 3-hydroxy-L-kynurenine (3-L-HK) and 3-hydroxy-D-kynurenine (3-D-HK) each prepared

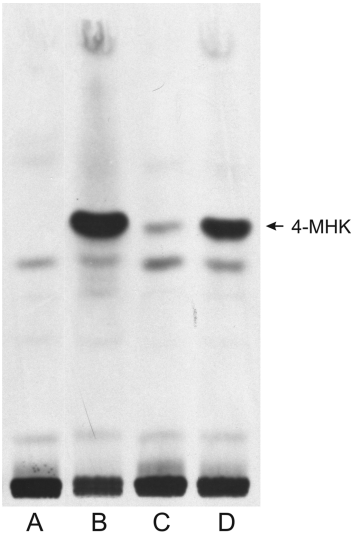

FIGURE 7: Stereospecificity of AcmI for D-3-HK. Shown are TLC separations of reaction mixtures (total volumes 100 μL) containing 160 μg of His_6AcmI, 1 μCi ^{14}C-AdoMet and the following enantiomeric forms of 3-HK: (B) 1 mM racemic 3-HK, (C) 3-L-HK isolated from a chiral TLC separation of 200 nmol of racemic 3-HK, (D) 3-D-HK isolated from the same plate, and (A) control with water instead of 3-HK. Note that because of minor overlap of the bands of the enantiomers on the TLC plate, the 3-L-HK still contains a little amount of 3-D-HK as revealed by the faint band in lane C (see also Figure S3 in the Supporting Information). Time of incubation was for 30 min at 37 °C. Solvent system IV was used. The time of exposure to the X-ray film was 3 days. The bands other than those of products are due to decomposition of ^{14}C-Adomet.

by chiral thin-layer chromatographic separation of the commercially available racemic 3-HK. Their optical configuration had been assessed by digestion with L-amino acid oxidase which converted the L-enantiomer of 3-HK to xanthurenic acid, whereas the D-enantiomer remained unchanged (see Materials and Methods and Figure S3 in the Supporting Information). Strikingly, both enzymes exclusively methylated the D-enantiomer but not the L-enantiomer (Figure 7) thus displaying total antipodal stereoselectivity for tyrosine and 3-HK. To address the role of the position of the phenolic OH group in the chiral selectivity of AcmI and AcmL, D-*m*-tyrosine and L-*m*-tyrosine were tested also. Likewise in the case of *p*-tyrosine, both enzymes exclusively methylated the L-enantiomer of *m*-tyrosine, which excludes that the 3- or 4-position of the hydroxy group would determine the stereospecificity as one could speculate when considering that 3-D-HK has a 3-hydroxy and L-tyrosine has a 4-hydroxy group. Therefore, the longer chain length of 3-HK and/or possibly the presence of the keto group in the aliphatic chain have to be drawn into consideration as major determinants specifying stereoselectivity of AcmI and AcmL toward 3-HK. Unfortunately, appropriate model compounds were not available to test them as substrates. Therefore we have to await the results of future functional and structural studies of these unique enzymes which may open a new interesting avenue in stereoenzymology. To our knowledge, exclusive antipodal stereospecificity for structurally different substrates in the same enzyme is without precedent among methyltransferases and more generally may be a very rare phenomenon in enzymology.

Kinetic Characterization of AcmI and AcmL. Determinations of kinetic constants of His_6AcmI and His_6AcmL for 3-D-HK were hampered by the fact that the separation procedure of racemic 3-D-HK and its susceptibility against oxidation yielded

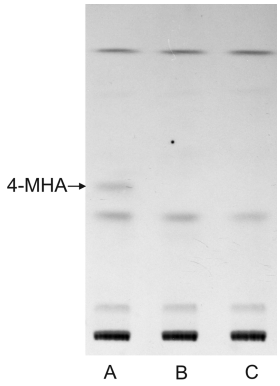

FIGURE 8: 3-HK methylating activity in *Streptomyces chrysomallus* strain Sc1. TLC separations of reaction mixtures containing desalted cell extract from *S. chrysomallus* strain Sc1 incubated with 1 μCi ^{14}C-AdoMet, 1 mM 3-HK (A) or 1 mM 3-HA (B) or water (C). The same experiments with mutant *S. chrysomallus* strain Sc-white gave no products (Figure S7 in the Supporting Information). The time of exposure to the X-ray film was 3 days.

only limited amounts of 3-D-HK. Moreover, 3-HK is poorly soluble above 700 μM in water which restricts its testing over a broad substrate concentration range (*33*). *V* vs *S*-plots using racemic 3-HK as the substrate revealed K_ms for His_6AcmI and His_6AcmL of 0.61 and 0.60 mM, respectively, with both values corrected for the D-enantiomer (Figure S5 in the Supporting Information). The corresponding k_{cat}s were 0.11 min^{-1} for both enzymes. In these conditions, it cannot be excluded that 3-L-HK may inhibit the methylation of 3-D-HK in a competitive manner. Even so, these values show that AcmI and AcmL are very slow, e.g., when compared to the *C*-methyltransferase CouO from coumermycin-producing *Streptomyces spheroides* (k_{cat} = 2.2 min^{-1}). Also, in terms of catalytic efficiency, CouO greatly exceeds AcmI and AcmL in view of its low K_m of 52 μM for its substrate desmethyl-monamide (*23*). To exclude the possibility that the His_6tag present in His_6AcmI or His_6AcmL interfered with the enzymes' activities, AcmL (without His_6tag) was tested in the same conditions as His_6AcmL. However, AcmL had the same specific activity as His_6AcmL. In view of the low k_{cat}/K_m of AcmI and AcmL, it may be speculated that both enzymes in cellular conditions may be assembled in a multicomponent complex with other enzymes of the 4-MHA biosynthesis pathway to ensure high local concentrations of 3-HK in the reaction. On the other hand, protein−protein interactions with partner enzymes of the 4-MHA pathway may cooperatively activate AcmI or AcmL as reported in the case of the structurally similar pyocyanine *N*-methyltransferase PhzM from *Pseudomonas aeruginosa* (*30*) which methylates the substrate phenazine-1-carboxylic acid (PCA). Heterologously expressed PhzM is null active in the absence of its partner enzyme PhzS, a flavine-dependent oxygenase, but becomes active at an appreciable rate in its presence. PhzS replaces the carboxy group of PCA by a hydroxy group and itself is only catalytically active in a transient physical interaction with PhzM. In analogy, kynurenine hydroxylase could be a similar partner for AcmI or AcmL.

Presence of 3-HK Methylating Activity in Protein Fraction from S. chrysomallus Actively Synthesizing Actinomycin. Incubation of extracts of broken cells prepared from mycelium of *S. chrysomallus* strain Sc1 actively synthesizing actinomycin with ^{14}C-AdoMet and DL-3-HK revealed the formation of radioactive 4-MHA (Figure 8). No radioactive 4-MHK was detectable because the hydroxykynureninase present in the

extract converted it to 4-MHA. Moreover, these protein fractions did not catalyze the methylation of 3-HA to 4-MHA. Apparently, *S. chrysomallus* is lacking the orthologue of the 3-HA 4-methyltransferase from actinomycin-producing *S. antibioticus* (*11*, *12*) and instead uses AcmI and AcmL for the synthesis of 4-MHA via 4-MHK in conjunction with the hydroxykynureninases (*13*). In agreement with this, protein extracts from mutant strain *S. chrysomallus* Sc-white (*13*) in which both *acmI* and *acmL* are inactivated by gene disruption and in which both hydroxykynureninase genes are deleted showed no conversion of 3-HK to 4-MHA (Figure S7 in the Supporting Information) or of 3-HK to 4-MHK. Addition of recombinant His_6AcmL to these extracts of strain Sc-white resulted indeed in formation of 4-MHK from 3-HK but no formation of 4-MHA from 4-MHK was seen due to the absence of hydroxykynureninase. These results unambiguously prove that in the *S. chrysomallus* cellular background AcmI and/or AcmL are present and that they are the only 3-HK methylating enzyme species. In addition, they show that the hydroxykynureninases AcmH and AcmK are responsible for conversion of 4-MHK to 4-MHA.

In summary, the data presented show the presence of two highly similar C-methyltransferases, AcmI and AcmL, in actinomycin-producing *S. chrysomallus* which catalyze the methylation step in the formation of 4-MHA, the immediate precursor of the actinomycin chromophore, actinocin. Instead of methylating 3-hydroxyanthranilic acid (3-HA), as initially expected, they methylate the unusual substrate 3-hydroxy-D-kynurenine (3-HK) which is an interesting finding *per se* because 3-HK, an intermediate in tryptophan metabolism in eukaryotes and a number of bacteria, normally has the L-configuration. This implies that in the course of formation of 3-HK from cellular L-tryptophan, a racemization step must occur either acting on tryptophan itself or one of its metabolites. This finding evokes reminiscence of earlier results obtained by feeding experiments of actinomycin-producing streptomycetes which revealed that ^{14}C-D-tryptophan-(benzene ring-^{14}C) was equally well incorporated into the actinomycin chromophore as ^{14}C-L-tryptophan-(benzene ring-^{14}C) (*34*). These authors postulated the presence of a racemase that would convert D-tryptophan or one of its metabolites into the L-enantiomer (*34*). Although in the light of the data shown here, the reverse is true and their findings indicated early that stereochemistry plays a substantial role in the formation of 4-MHA from tryptophan. Attempts to isolate a tryptophan racemase from that actinomycin-producing streptomycete were unsuccessful (*34*); however, with knowledge of the stereoselectivity of AcmI and AcmL and of genes involved in 4-MHA biosynthesis, a solution of this issue may be achieved in the future.

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SUPPORTING INFORMATION AVAILABLE

Sources of materials and a detailed description of purification of His6AcmI, His6AcmL, and AcmL and figures (Figure S1−S7) referring to location of *acmI* and *acmL* in the actinomycin biosynthetic gene cluster (Figure S1); analysis of reaction products (Figure S2); optical configuration of 3-D-HK and 3-L-HK (Figure S3); purity of methyltransferases (Figure S4); kinetic analysis of AcmI and AcmL (Figure S5); time course of tyrosine methylation (Figure S6); and absence of AcmI and/or AcmL from *S. chrysomallus* mutant strain Sc-white (Figure S7). This material is available free of charge via the Internet at http://pubs.acs.org.

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