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ORIGINAL ARTICLE

Occurrence and biosynthesis of C-demethylactinomycins in actinomycin-producing *Streptomyces chrysomallus* and *Streptomyces parvulus*

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Streptomyces chrysomallus **and** ***Streptomyces parvulus*** **produce novel C-demethylactinomycins besides their normal actinomycins when fed with 3-hydroxyanthranilic acid (3-HA). The 3-HA is incorporated into pentapeptide lactone precursors in competition with the regular precursor 4-methyl-3-hydroxyanthranilic acid (4-MHA). The resultant 3-HA pentapeptide lactones can condense with each other, as well as with the continuously formed 4-MHA pentapeptide lactones giving C-demethylactinomycins lacking one or both methyl groups in their phenoxazinone chromophores. In case of C-demethylactinomyins lacking one methyl group, the condensation was shown to be regiospecific directing the 3-HA portion almost exclusively to the α-side of the phenoxazinone chromophore. As 3-HA is a weaker substrate for the 4-MHA-incorporating enzyme actinomycin synthetase I than 4-MHA, C-demethylactinomycins never exceeded 7–8% of total actinomycin formed. Surprisingly, C-demethylactinomycins (up to 0.8%) were also found in the actinomycin mixtures of unsupplemented streptomycete cultures after longer cultivation times, indicating the natural presence of 3-HA. Feeding with 3-hydroxykynurenine (3-HK) induced also formation of C-demethylactinomycins indicating that 3-HK is source of 3-HA. Analysis of tryptophan metabolites in the intracellular pools of the streptomycetes using 5-^{3}H-tryptophan as radiotracer revealed formation of 4-MHA, but not of 3-HA. This indicates that intracellular 3-HK is almost exclusively converted to 3-hydroxy-4-methylkynurenine (4-MHK), which has been identified previously as direct precursor of 4-MHA. However, small amount of 3-HK leaking out from the 4-MHA pathway can be prematurely converted to 3-HA all along the cultivation of the streptomycetes resulting in the formation of natural C-demethylactinomycins.**
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INTRODUCTION

Actinomycins are bicyclic chromopeptide lactone antibiotics produced by various streptomycetes (Figure 1a). In their structures, two pentapeptide lactone rings are attached in amide linkages to a phenoxazinone dicarboxylic acid. To date, more than 30 different natural actinomycins are known, which differ from each other by amino acid substitutions in several positions of their peptide lactone rings, whereas their phenoxazinone chromophore actinocin (2-amino-4,6-dimethyl phenoxazine-3-one-1,9-dicarboxylic acid) is always the same.[1,2] Actinocin is unique being the only phenoxazinone found in nature that contains two methyl groups at the 4- and 6-position of its phenoxazinone ring system;[3] for example, in contrast to other phenoxazones like the ommochromes or its didemethyl-homolog cinnabarinic acid.[4,5]

Biosynthetically, actinomycin and its chromophore are formed by the oxidative condensation of two 3-hydroxy-4-methylanthranilic acid (4-methyl-3-hydroxyanthranilic acid, 4-MHA) pentapeptide lactones (Figure 1a). These are nonribosomally assembled from 4-MHA and the amino acids of the peptide lactone rings by the actinomycin synthetases ACMS II and ACMS III in conjunction with the 4-MHA activating enzyme (ACMS I) and the 4-MHA carrier protein (AcmACP).[6] 4-MHA is the starter building block of the nonribosomal assembly of the 4-MHA pentapeptide lactones. In the initiation step, ACMS I activates 4-MHA as adenylate with subsequent thioesterification to the AcmACP carrier protein, which then reacts with threonine, the first amino acid of the pentapeptide chain bound as thioester to ACMS II.[7] ACMS I was intensively characterized and shown to activate *in vitro* a large number of benzene carboxylic acids

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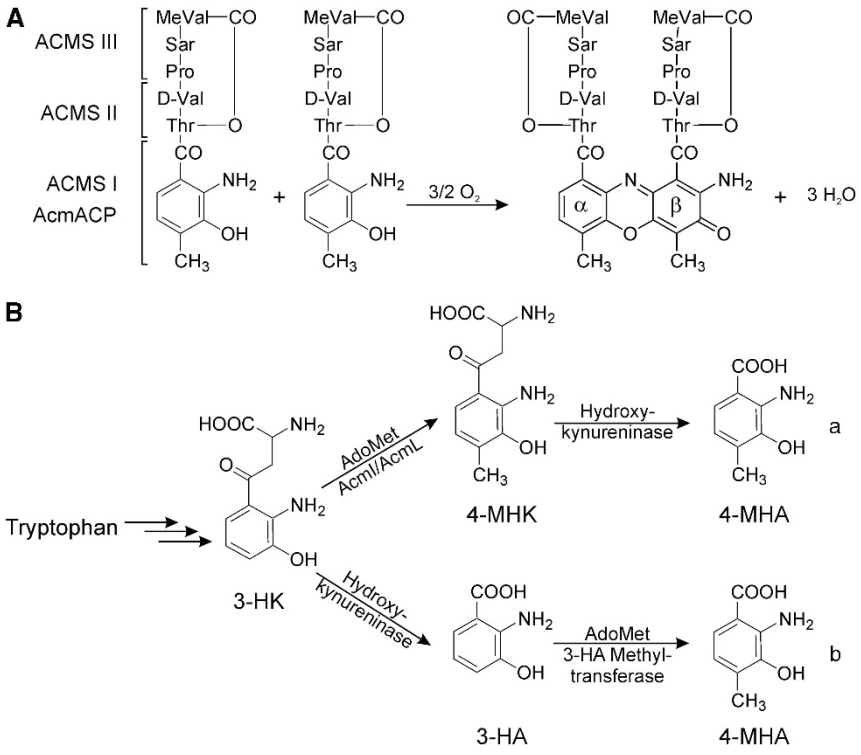

Figure 1 (**A**) Structures and formation of actinomycin D (synonymous with C_1 and IV). Shown are the 4-MHA-pentapeptide lactones (left) and actinomycin D (right). The different rings of actinomycins are indicated as α or β according to ref. 2. Aniso-actinomycins have different α and β pentapeptide lactone rings in contrast to iso-actinomycin. Actinomycins of the actinomycin C complex differ from actinomycin D (C_1) by replacement of one or both D-valine residues through D-allo-isoleucine. (**B**) Formation of 4-MHA from 3-HK in *Streptomyces chrysomallus* (**a**) and *Streptomyces antibioticus* (**b**). In (**a**) 4-MHA is derived from 4-MHK, whereas in (**b**) it is derived from 3-HA. AdoMet (S-adenosyl-L-methionine), Sar (N-methylglycine), MeVal (N-methyl-L-valine).

structurally related to 4-MHA among them 3-hydroxyanthranilic acid (3-HA), the demethyl homolog of 4-MHA.[8,9]

Genetic and biochemical studies in *Streptomyces chrysomallus* and *Streptomyces parvulus* showed that 4-MHA is derived from 3-hydroxy-4-methylkynurenine (4-MHK) by cleavage catalyzed by a hydroxykynureninase (Figure 1b).[10,11] The 4-MHK itself is obtained from 3-hydrokynurenine (3-HK), a metabolite from tryptophan, by methylation at its C-4 position. This step is catalyzed by the methyltransferases AcmI/AcmL encoded by the paralogous genes, *acmI* and *acmL*, located in the actinomycin biosynthetic gene cluster.[12] By contrast, in actinomycin-producing *Streptomyces antibioticus* 4-MHA seems to be derived directly from 3-HA. A 3-HA methyltransferase was isolated previously from *S. antibioticus* that specifically methylates 3-HA, but not 3-HK.[13,14] Like 4-MHA from 4-MHK in *S. chrysomallus*, the 3-HA in *S. antibioticus* could be derived from 3-HK. 3-HK is generated in the tryptophan degradation pathway via formyl-kynurenine and kynurenine. This pathway normally operates in eukaryotes and a number of bacteria to produce 3-HA as end product necessary as precursor in the synthesis of NAD.[15] Moreover, in eukaryotes 3-HA can have additional roles such as precursor of the phenoxazinone cinnabarinic acid, which is a pigment in various fungi[16] or in mammalian tissue, where oxidation of 3-HA to cinnabarinic acid may have a role in the protection against oxidative damage.[17]

Genomic data indicate that streptomycetes lack the ability to synthesize 3-HK in their primary metabolism. Apparently, NAD synthesis in these bacteria follows a different route without intermediacy of 3-HA.[15] Therefore, the 3-HK pathway in the actinomycin-producing streptomycetes serves only to synthesize 4-MHA. However, it seemed curious that 4-MHA biosynthesis differs in such an important step of C-methylation in *S. chrysomallus* and *S. antibioticus*. To clarify this important question, we investigated the role of 3-HA as precursor of the actinomycin chromophore in *S. chrysomallus* and *S. parvulus*.

MATERIALS AND METHODS

Chemicals and radiochemicals

DL-3-Hydroxy-kynurenine, L-leucine, L-isoleucine, L-threonine, L-valine, L-proline, L-glycine and L-methionine were purchased from Sigma (Deisenhofen, Germany). 3-HA and fluorescence silica gel $60F_{254}$ coated alumina sheets were from Merck (Darmstadt, Germany). Actinomycin D was purchased from Applichem (Darmstadt, Germany). 4-MHA was synthesized as described.[8] [^{14}C-methyl]-L-methionine (55 mCi mmol^{-1}), U-[^{14}C]-L-valine (260 mCi mmol^{-1}), U-[^{14}C]-L-threonine (192 mCi mmol^{-1}), U-[^{14}C]-L-proline (252 mCi mmol^{-1}), U-[^{14}C]-L-glycine (105 mCi mmole^{-1}), U-[^{14}C]-L-glutamic acid (238 mCi mmol^{-1}) and L-[5-^{3}H]-tryptophan (17.9 Ci mmol^{-1}) were from Amersham Bioscience (Braunschweig, Germany). All other chemicals were of the highest purity commercially available.

Strains and cultures

Strain Sc1 of *S. chrysomallus* was described previously.[18] It was derived from *S. chrysomallus* ATCC 11523. *Streptomyces parvulus* ATCC 12434 was from the American Type Culture Collection. All strains were maintained on CM agar.[18] Growth of *S. chrysomallus* and *S. parvulus* in liquid culture for producing mycelium actively synthesizing actinomycins was either for up to 2 days in liquid CM[18] or for 3 to 4 days in a glutamate−mineral salts medium.[19] All

cultures were performed with 1% maltose plus 0.1% glucose as carbon source (both sterilized separately) using 500 ml Erlenmeyer flasks containing 200 ml medium. Incubation was at 28 °C and 220 r.p.m. in a New Brunswick G 25 shaker.

Analytical methods

Solvent systems for thin layer chromatography were solvent system I (ethylacetate-hexane-acetic acid, 8:2.5:0.5, by vol.), solvent system II (ethylacetate-methanol-water-dimethylformamid, 100:5:5:2, by vol.), solvent system III (ethylacetate-methanol-water-dimethylformamid, 100:3:3:1, by vol.) and solvent system IV (butanol-acetic acid-water; 4:1:1, by vol.). HPLC separations of actinomycin mixtures were performed on a prepacked EnCaPharm 100-RP18 column (Molnar Institute, Berlin, Germany) isocratically with 60% (by vol.) acetonitrile-water at a flow rate of 1 ml min^{-1} using a Pharmacia LKB (Pump 2248, WWM 2141) HPLC system (GE Healthcare Life Sciences, Little Chalfont, UK) or a Waters (Pump 515, PAD 996) HPLC System. Detection wavelength was 441 nm. Actinomycin contents of cultures were determined by the extraction of culture aliquots with ethylacetate, and subsequent spectrophotometric determination at 441 nm using authentic actinomycin D as standard. Extracts containing radioactive actinomycins and related compounds were determined by counting aliquots in a Wallac 1409 liquid scintillation counter (Wallac, Turku, Finland). Radioactive compounds on TLC plates were localized by radioscanning with a RITA radioscanner (Raytest, Straubenhardt, Germany) or by autoradiography with a Kodak BioMax MR Film. The radioactive zones were scraped off the plates and extracted with methanol. After evaporation to dryness, the residue was hydrolyzed in 250 µl 6 M HCl for 20 h at 105 °C. The dried hydrolysate was dissolved in 50% ethanol, applied to silica gel plates and chromatographed in solvent system IV along with amino acid standards. Radioactive amino acids were detected by radioscanning or autoradiography. Standards were visualized by spraying with 0.1% ninhydrin in ethanol.

Short-term radioincorporation experiments and micro-preparative incubations of streptomycete mycelial suspensions

For short-term radio incorporation experiments, mycelium from a 100 ml culture of an actinomycin-producing streptomycete culture was harvested by suction-filtration on a Buechner funnel and after washing with tap water was suspended in 40 ml of tap water. 5 ml aliquots from the cell suspensions were transferred to 100 ml Erlenmeyer flasks. 3-HA, 4-MHA or 3-HK were added in the indicated concentrations together with 1 µCi of ^{14}C-labeled amino acid; that is, either threonine, valine, proline, glycine, methionine or 10 µCi ^{3}H-labeled amino acids such as tryptophan. The suspension was incubated at 30 °C and 220 r.p.m. in a New Brunswick G 25 Shaker (2.5 cm displacement) for up to 60 min. Radioactive actinomycins and other neutral compounds were extracted twice with 3 ml portions of EtOAc. After acidification to pH 2 with 1 N HCl of the incubation mixture, acidic metabolites were extracted with 3 ml EtOAc. Two hundred microlitres from the combined extracts were counted. The remainder was evaporated to dryness. The residue was dissolved in a minute amount of EtOAc, applied to silica TLC plates and separated by using solvent system II or III (for actinomycins) or solvent system I (for acidic metabolites such as 4-MHA or 3-HA).

Micro-preparative small scale incubations were performed as follows: Mycelium from cultures of a streptomycete actively synthesizing actinomycins was harvested by suction on a Buechner funnel and after washing with tap water resuspended in 40% of the original volume of tap water containing a carbon source (0.5 −1% glucose or galactose) and 1 −2 mM of each L-threonine, L-valine, L-proline, L-glycine and 100 µM L-methionine. 5 to 8 ml portions of this suspension were given in 100 ml Erlenmeyer flasks and 3-HA, 3-HK or 4-MHA were added at concentrations varying from 15 to 250 µM. Controls contained no such additive. After shaking at 220 r.p.m. and 30 °C for up to 4 h each flask was extracted with EtOAc and the extracts separated by TLC or HPLC or by a combination of both.

Mass spectrometric analysis

MALDI-time-of-flight (TOF) mass spectra were recorded using a Bruker Autoflex instrument (Bruker Daltonic GmbH, Bremen, Germany) equipped with a 337 nm nitrogen laser for desorption and ionization. Two-microlitres samples of HPLC-purified actinomycin species were mixed with the same volume of matrix medium (a saturated solution of α-cyano-4-hydroxycinnamic acid in 50% aqueous acetonitrile containing 0.1% trifluoroacetic acid), spotted on the target, air dried and measured. Spectra were recorded by positive ion detection in reflector mode. The acceleration and reflector voltages were 19 and 20 kV in pulsed ion extraction mode. A molecular gate of 350 Da improved the measurement by filtering out most matrix ions. Monoisotopic mass numbers were obtained. The structure of actinomycins was investigated by post source decay-MALDI-TOF MS. The fragment spectra of *mono*-C-demethyl- and *di*-C-demethylactinomycin were compared with that of actinomycin D as the reference.

RESULTS AND DISCUSSION

3-HA induces synthesis of new compounds in *S. parvulus* and *S. chrysomallus*

Washed mycelial suspensions of *S. parvulus* and S. *chrysomallus* actively synthesizing actinomycin D or actinomycin C (a mixture of actinomycins C_1, C_2 and C_3, Figure 1a) were incubated in short-term experiments (30 −60 min) with externally added 3-HA or 4-MHA in the presence of ^{14}C-threonine. Surprisingly, the determination of total EtOAc-extractable radioactivity from incubations of both strains revealed that not only 4-MHA stimulated ^{14}C-threonine incorporation into products, but also 3-HA. In both cases, the total incorporation of radiolabel into products was 10–20% higher than in the control without added 3-HA or 4-MHA. This was surprising, because in view of our recent findings that 4-MHA is derived from 4-MHK, we expected that 3-HA would have no effect on actinomycin synthesis[12], and therefore pointed to the possibility that an alternative pathway of synthesis of 4-MHA from 3-HA would exist. However, when we chromatographed the extracts on TLC plates, we saw that addition of 3-HA had not stimulated actinomycin synthesis, but instead had led to the formation of new compounds with lower R_f-value than actinomycin (Figure 2a, Supplementary Figure S1). The new 3-HA-inducible radioactive compounds were isolated from the plates and subjected to acid hydrolysis and amino acid analysis, which revealed the presence of ^{14}C-threonine. Additional radioincorporation experiments with ^{14}C-glycine, ^{14}C-valine (or ^{14}C-isoleucine) or ^{14}C-proline showed that these amino acids were also incorporated (not shown). This indicated that the new compounds had the same peptide rings as actinomycin D or actinomycin C, respectively.

In order to get sufficient quantities for analysis of the new compounds, mycelial suspensions were incubated with all non-labeled amino acids of the actinomycin peptide chain and with 4-MHA or 3-HA in long-term experiments (see Materials and methods). In a typical experiment with ca. 150 mg (wet weight) washed *S. parvulus* or *S. chrysomallus* mycelium 75–125 µg of total actinomycins were obtained in 6 h incubations. HPLC separation of an extract obtained from an incubation of *S. parvulus* shows that 3-HA had induced the appearance of a new compound represented by a peak at ca. 7 min (compound **1**), which was missing in the separation of the control extract containing only actinomycin D (ca. 9 min) (Figure 2b). The 7 min peak had a shoulder at its leading edge representing a minor product (compound **2**).

HPLC separation of a similarly obtained extract from incubations with *S. chrysomallus* containing 3-HA revealed also the formation of new compounds. These had retention times different from those of the three actinomycins C_1, C_2, and C_3 in the control incubation (Supplementary Figure S1). However, the peaks overlapped each other and were difficult to separate. Their spectral analysis indicated that they had absorption maxima between 439 and 441 nm similar to that of authentic actinomycin (441 nm). This suggested the presence

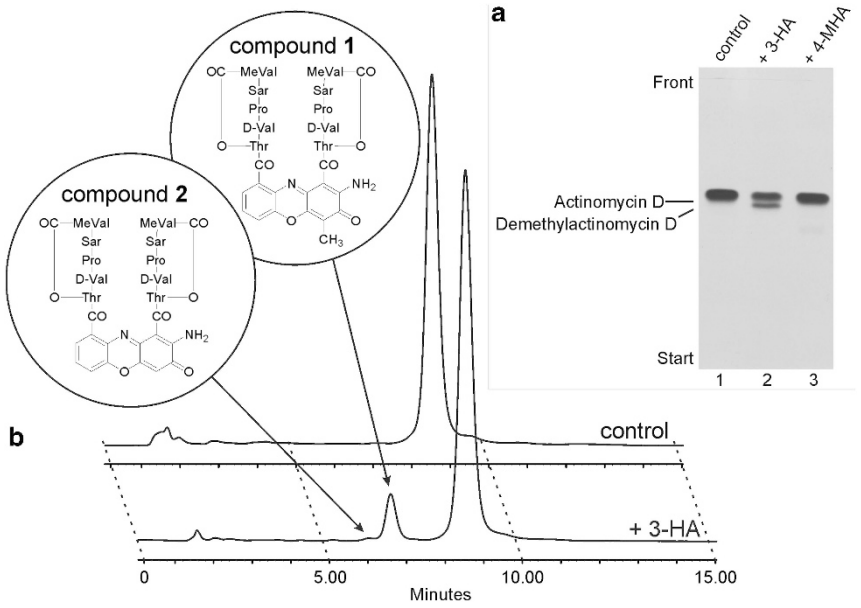

Figure 2 Formation of C-demethylactinomycins in *S. parvulus*. (**a**) TLC separations of radioactive products formed in radioincorporation experiments with ^{14}C-methionine. Lane 1: control, lane 2: with 100 μM 3-HA, lane 3: with 100 μM 4-MHA. Incubation time was 30 min. Solvent system was III. Time of exposure to x-ray film was 3 h. (**b**) HPLC separations of EtOAc extracts from micro-preparative incubations of *S. parvulus* mycelial suspension in the presence and absence of 250 μM 3-HA. Compounds 1 and 2 were identified by MS as *mono*-C-demethylactinomycin D and *di*-C-demethylactinomycin D, respectively.

of phenoxazinone chromophores in their structures similar to the actinomycin chromophore, actinocin. As these new actinomycins apparently were lacking one or both methyl groups in their chromophores, but also had different pentapeptide lactone rings differing by 14 mass units (D-valine *vs.* D-*allo*-isoleucine) from each other, the analysis of these compounds was complicated. Therefore, we concentrated on the analysis of the two compounds (**1** and **2**) from *S. parvulus*. To this end, compound **1** and compound **2** each were repurified by two further rounds of HPLC and subjected to MALDI-TOF MS.

Identification of the 3-HA-inducible actinomycins as C-demethylactinomycins D by MALDI-TOF MS

The MALDI-TOF mass spectra of compounds **1** and **2** along with the mass spectrum of purified actinomycin D as control are shown in Figures 3a–c. The spectrum of compound **1** (Figure 3b) shows a mass peak $[M+H]^+$ at *m/z* 1242 consistent with the lack of one methyl group (-14 mass units) in the structure of actinomycin D with a molecular mass of *m/z* 1256 (Figure 3a). The spectrum of compound **2**, the minor product, exhibits a mass peak $[M+H]^+$ at *m/z* 1228 (Figure 3c), which is 28 mass units lower than that of actinomycin D. In all spectra the sodium and potassium adducts $[M+Na]^+$ and $[M+K]^+$ of these species appeared at 22 and 38 higher mass units.

The assignment of compounds **1** and **2** to the proposed structures was confirmed by fragment analysis applying post source decay-MALDI-TOF MS. The post source decay-fragment spectrum of actinomycin D is demonstrated in Supplementary Figure S2 together with a compilation of the mass data given in Supplementary Table S1 of representative fragments of actinomycin D and its demethylated species. From these data it is apparent that the b-ions of the di-, tri-, tetra- and pentapeptide fragments of the peptide lactone rings of all three compounds are the same, indicating that these molecules are identical in their peptide portions. However, fragment ions formed by elimination of portions of the peptide lactone moieties of actinomycin D and the demethyl species still containing the phenoxazinone chromophore differ in the expected manner; that is, the masses of corresponding fragments of *mono*-C-demethyl- and *di*-C-demethylactinomycin D are 14 and 28 mass units lower than those of actinomycin D (Supplementary Table S1).

These results clearly indicate that the new compounds are lacking one and two methyl groups at the phenoxazinone-chromophore, respectively (Figure 3). Fragments of the phenoxazinone chromophore itself could not be detected. *Di*-C-demethylactinomycins were as yet only known from previous chemical synthesis.[20,21] *Mono*-C-demethylactinomycins are described here for the first time. They arise by cross-condensation between 3-HA pentapeptide lactones and 4-MHA pentapeptide lactones. The mass spectrometric analysis, however, did not allow determining whether the single methyl group of *mono*-C-demethylactinomycin chromophore was located in the 4- or 6-position of the phenoxazinone ring system or was randomly distributed (see below).

3-HA does not interfere with 4-MHA synthesis in *S. parvulus*

Determination of the time course of C-demethylactinomycin formation along with actinomycin formation during long-term incubation of mycelial suspensions of *S. parvulus* with non-labeled amino acids and 3-HA is shown in Figure 4. Although the short-term radioincorporation experiment (Figure 2), represented a sudden burst of radiolabel incorporation upon addition of 3-HA in excess, during long-term incubations the amounts of C-demethylactinomycin D never exceeded 7−8% of the total actinomycin mixture (Figure 4). The formation of actinomycin remained practically unaffected by the presence of 3-HA, and the increase of total actinomycins formed in its presence was solely due to the C-demethylactinomycin formed. The main product formed from 3-HA was the *mono*-C-demethylactinomycin (compound **1**), whereas formation of the *di*-C-demethylactinomycin (compound **2**) was practically neglectable. Obviously, 3-HA was not converted to 4-MHA, otherwise it should have stimulated

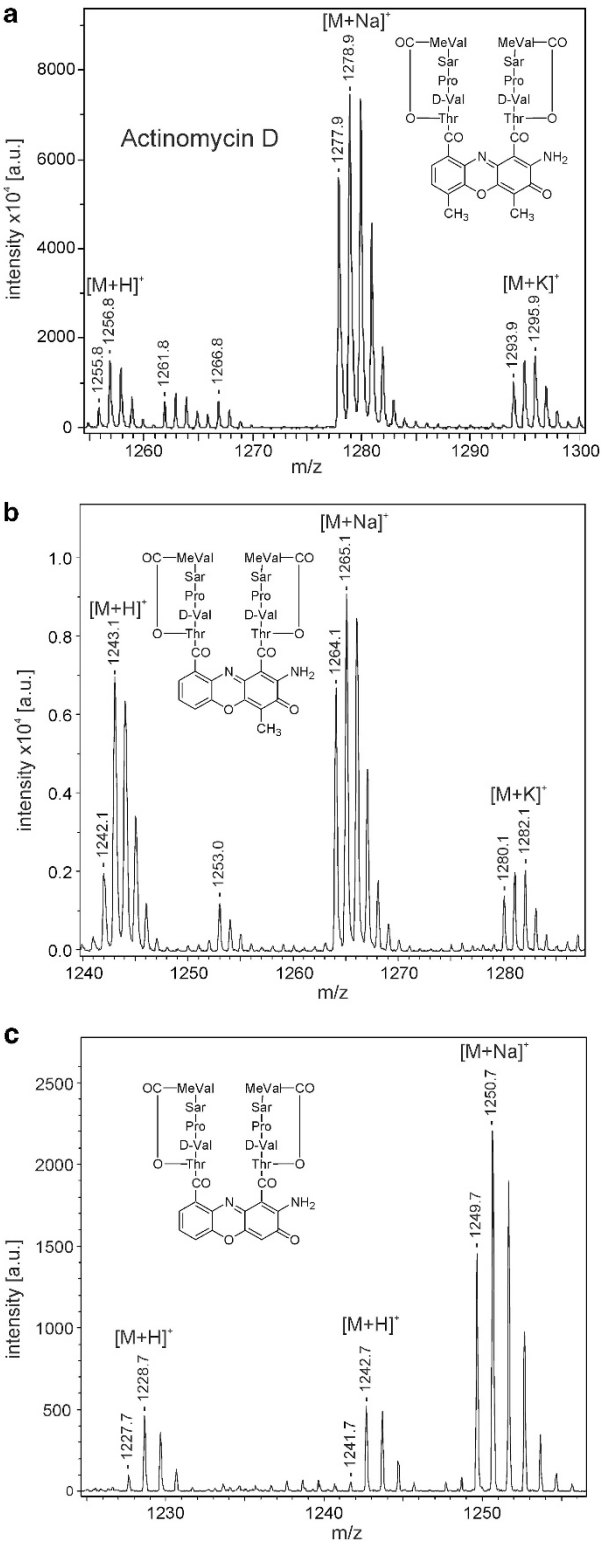

Figure 3 MALDI-TOF mass spectra of actinomycin D and related C-demethylactinomycins. (**a**) Spectrum of actinomycin D. (**b**) Spectrum of *mono*-C-demethylactinomycin D (compound 1), (**c**) spectrum of *di*-C-demethylactinomycin D (compound 2). The second $[M+H]^+$ at 1242.7 is due to the presence of *mono*-C-demethylactinomycin D traces in the *di*-C-demethylactinomycin D sample.

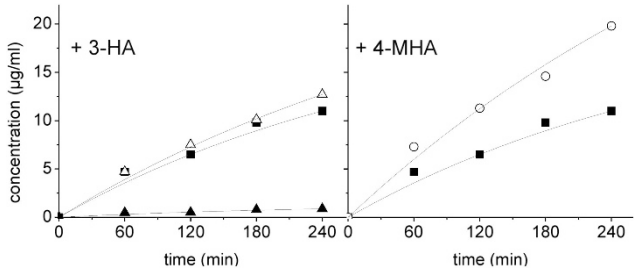

Figure 4 Time course of C-demethylactinomycin and actinomycin formation in *S. parvulus* washed mycelial suspensions. Left: Formation of *mono*-C-demethylactinomycin D (▲) and actinomycin D (△) in the presence of 100 µM 3-HA; formation of actinomycin (■) in control culture without 3-HA. Right: Formation of actinomycin in the presence of 4-MHA (○) and formation of actinomycin in control culture without 4-MHA (■). Conditions (see Materials and methods).

actinomycin synthesis as did 4-MHA, which drastically enhanced actinomycin synthesis (Figure 4).

The data also confirm that 4-MHA, which is present at only trace level in actinomycin-producing streptomycetes[9,22] is a much better substrate for incorporation into the aroyl pentapeptide lactone precursor than 3-HA even if the latter is present in excess. In fact, previous analysis of the 4-MHA-activating enzyme ACMS I from *S. chrysomallus* showed much higher catalytic efficiency for 4-MHA as substrate ($k_{cat}/K_m = 0.47\,min^{-1}\,\mu M^{-1}$) than for 3-HA ($k_{cat}/K_m = 0.018\,min^{-1}\,\mu M^{-1}$) in the acyl-adenylate formation reaction.[9]

Demethylactinomycins are present in natural actinomycin mixtures from streptomycetes

We analyzed actinomycin mixtures from *S. parvulus* cultures of different ages to see, whether they contained C-demethylactinomycins formed in natural conditions. Indeed, product mixtures from 6 and more days old cultures contained a minor compound with the same λ_{max} (441 nm) as actinomycin and with the same retention time in the HPLC (ca. 7 min) as the above-described 3-HA-inducible *mono*-C-demethylactinomycin D. MALDI-TOF MS revealed that it was *mono*-C-demethylactinomycin D (not shown). Its amount in the cultures never exceeded more than 0.5–0.8% of the total actinomycin mixtures (after 6 days of cultivation), whereas the *di*-C-demethylactinomycin made up only up to 0.02% of the actinomycin mixtures. These findings not only showed that C-demethylactinomycins are formed in natural conditions, but that 3-HA must be naturally occurring in *S. parvulus*. Inspection of the natural actinomycin mixtures of *S. chrysomallus* cultures revealed small amounts of C-demethylactinomycins too (not shown).

3-HA in actinomycin-producing *S. parvulus* is derived from 3-HK

In eukaryotes and in those bacteria, where 3-HA is a precursor of nicotinic acid, 3-HA was shown to be derived from tryptophan in the 3-HK pathway.[23] However, analysis of various bacterial genomes strongly suggested that the genus *Streptomyces* lacks the 3-HK pathway, and nicotinic acid is most probably produced by an alternative pathway.[15] Therefore, we asked for the origin of the 3-HA responsible for the formation of C-demethylactinomycins occurring in natural actinomycin mixtures. As the actinomycin biosynthetic gene cluster of *S. chrysomallus*[6] contains genes encoding enzymes such as tryptophan dioxygenase, kynurenine

formamidase and hydroxykynureninase involved in 4-MHA synthesis it seemed reasonable to assume that the 4-MHA pathway catalyzed formation of 3-HA as a by-product. We showed previously that hydroxykynureninase is present in actinomycin-producing cells of *S. chrysomallus* at high activity and *in vitro* can convert not only 4-MHK to 4-MHA, but also 3-HK to 3-HA.[6,24] To test whether 3-HK was converted to 3-HA *in vivo* and thus could induce C-demethylactinomycin C formation, 3-HK was incubated in short-term experiments with mycelial suspensions of *S. chrysomallus* containing ^{14}C-threonine. In fact, TLC analysis of the radioactive actinomycin mixtures clearly revealed that 3-HK in 20−250 μM concentrations induced formation of C-demethylactinomycin C along with significant reduction of actinomycin C formation. The most likely explanation for this was that the 3-HK became converted to 3-HA to induce synthesis of C-demethylactinomycin. The data in Figure 5 confirm this showing that addition of 3-HK led to more C-demethylactinomycin than when the same amount of 3-HA was added. Most probably, the added 3-HK penetrated the cells better than 3-HA and thus, a higher intracellular 3-HA concentration was reached than with addition of 3-HA. The same effect of 3-HK to induce formation of C-demethylactinomycin D with simultaneous reduction of actinomycin production was also observed in short-term incubation of *S. parvulus* mycelium (not shown).

Trapping intracellular 4-MHA and 3-HA in *S. parvulus* using 5-^{3}H-tryptophan as radiotracer

The temporary reduction of actinomycin synthesis during the short-term experiments with externally added 3-HA or 3-HK let raise the question for the fate of the intracellular 4-MHA: Did its synthesis still continue or was it suppressed by 3-HA at one of its biosynthesis steps? If 3-HA would compete with 4-MHA, 4-MHA should accumulate to some degree in the cell. To test this, we performed short-term radioincorporation experiments with mycelial suspensions of *S. parvulus* with 5-^{3}H-tryptophan in the presence 3-HA or 3-HK. In fact, in these incubations appreciable formation of radioactive 4-MHA was observed in the presence of 3-HA and even more 4-MHA in the presence of 3-HK, whereas in the control (without 3-HA or 3-HK) 4-MHA was nearly undetectable (Figure 6a lanes 3, 4, 5). These findings show that during actinomycin formation in normal conditions no 4-MHA accumulates, because it is immediately incorporated into actinomycin during its formation. In presence of 3-HA (or 3-HK), which competes with 4-MHA for incorporation into the antibiotic, 4-MHA accumulates, which shows, however, that 3-HA does not inhibit its formation. This conclusion is also supported by the observation, that the increase of intensities of the 4-MHA bands in Figure 6a shows inverse correlation with the reduced intensities of the bands of radiolabeled actinomycins when 3-HA or 3-HK were present or in other words that the total amounts of 4-MHA formed in the presence or absence of 3-HA or 3-HK are practically equal and that 3-HA neither stimulates nor inhibits 4-MHA synthesis.

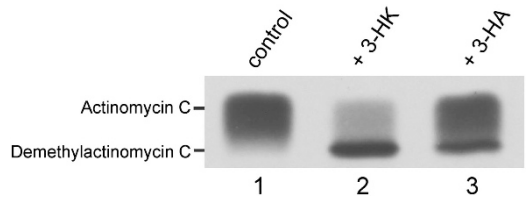

Figure 5 3-HK stimulates demethyl-actinomycin formation in *S. chrysomallus*. TLC separations of radioactive products formed in radioincorporation experiments with ^{14}C-threonine. Lane 1: control, lane 2: with 100 μM 3-HK, lane 3: with 100 μM 3-HA. Incubation time was 30 min. Solvent system was III. Time of exposure to x-ray film was 6 h.

Remarkably, in all of these experiments radioactive 3-HA was never detected neither in the controls nor when 3-HA (3-HK) was externally added. Apparently, in natural conditions (without added 3-HK) the conversion of intracellular 3-HK to 4-MHK catalyzed by AcmI and AcmL in *S. chrysomallus* (or *S. parvulus*) is so rapid that 3-HA cannot be formed from 3-HK. This also implicates, that the intracellular 3-HK level in *S. chrysomallus* (or *S. parvulus*) is strictly regulated to ensure that 3-HK is converted quantitatively to 4-MHA. Only when the 3-HK level in the cells is raised by external addition of 3-HK enhanced 3-HA synthesis is observed leading to detectable formation of C-demethylactinomycins. These data strongly suggest that 3-HA is no regular intermediate in 4-MHA biosynthesis, and instead that 3-HA is only a minute by-product of the 4-MHA pathway originating from premature cleavage of small amounts of 3-HK leaking out from the 4-MHA biosynthetic pathway.

Regiospecificity of 3-HA pentapeptide lactone condensation

The analysis of the labeling pattern of actinomycin and C-demethylactinomycin during the incubation with ^{14}C-threonine in the presence of 3-HA (Figure 6a) showed that both actinomycin and C-demethylactinomycin were labeled in their peptide lactone ring with ^{14}C-threonine. This proved that both types of actinomycins were formed. Surprisingly, however, in the case of 5-^{3}H-tryptophan we saw only labeling of the actinomycin bands, but not of C-demethylactinomycin. This was comprehensible for *di*-C-demethylactinomycin with the chromophore formed from two non-labeled 3-HA residues. For the main compound *mono*-C-demethylactinomycin with a chromophore formed from one 4-MHA and one 3-HA residue the lack of label could only be explained when the tritium label in the 5-position of the 4-MHA portion in the chromophore was lost. This can only happen when the radiolabeled 4-MHA portion carrying tritium in the 5-position is directed to the β-side of the phenoxazinone ring system and the 3-HA portion to the α-side (Figure 6b). Otherwise, when 4-MHA was on the α-side, tritium label had to be retained (Figure 6b). This results clearly show regiospecific incorporation of 3-HA pentapeptide lactones to the α-side of the *mono*-C-demethylactinomycins. The enzymatic basis of regiospecificity of the phenoxazinone formation reaction is unclear, because *S. chrysomallus* and *S. parvulus* are lacking phenoxazinone synthase or peroxidase activity present in *S. antibioticus* or *S. griseus*, respectively, which catalyze the oxidative condensation of *o*-aminophenols to the corresponding phenoxazinones.[25,26] It can therefore not be excluded, that the actinomycins of *S. parvulus* and *S. chrysomallus* are formed by spontaneous nonenzymatic condensation of 4-MHA pentapeptide lactones like in the chemical synthesis of the actinomycin chromophore described earlier.[27] Interestingly, in case of actinomycin C_2, which carries one peptide lactone ring with D-valine and one containing D-*allo*-isoleucine, the D-*allo*-isoleucine containing peptide lactone ring is almost exclusively directed to the β-side[2,28], which suggests preference of the β-side for the more hydrophobic aroyl pentapeptide lactone. More chemical work will be necessary to study the mechanism of mixed-type phenoxazinone formation.

In conclusion, we showed that in actinomycin-producing *S. parvulus* and *S. chrysomallus* 3-HA does not stimulate incorporation of radiolabeled amino acids into actinomycins. Instead, hitherto

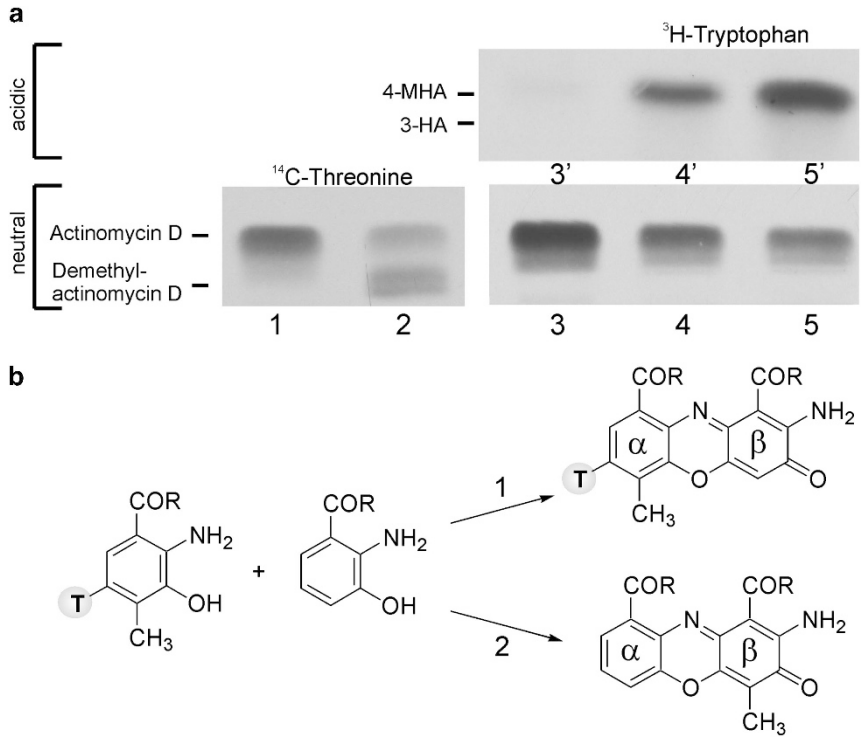

Figure 6 (**a**) Intracellular 4-MHA in *S. parvulus* in response to externally added 3-HA and 3-HK. TLC separations of neutral and acidic extracts from radioincorporation experiments of *S. parvulus* mycelium with 5-^{3}H-tryptophan in the presence of external 3-HA (lanes 4, 4′), 3-HK (lanes 5, 5′) and without addition (lanes 3, 3′). Upper panel: formation of radioactive 4-MHA in response to added 3-HA or 3-HK; lower panel: formation of actinomycin D in the same incubations. TLC separations of control incubations with ^{14}C-threonine show that formation of *mono*-C-demethylactinomycin D had indeed occurred in the presence of 3-HA (lane 2). Lane 1 (without 3-HA). Solvent system for acidic extracts was I and for neutral extracts II. (**b**) Two possibilities for regioselectivity in the phenoxazinone condensation reaction between non-labeled 3-HA- and 5-labeled 4-MHA-pentapeptide lactones. In 1 the tritium label in the product is retained, whereas in 2 it is lost. The pentapeptide lactone rings are symbolized by R.

unrecognized compounds, the C-demethylactinomycins, are formed, which possess phenoxazinone moieties, in which one or both 4-MHA residues are replaced by 3-HA. This is due to the competition of 3-HA with 4-MHA for incorporation into actinomycin pentapeptide lactone precursors.[8,9] Importantly, 3-HA does not affect 4-MHA synthesis, and therefore 4-MHA pentapeptide lactones are continued to be formed in cultures thus allowing further synthesis of actinomycins and only low amounts of *mono*-C- and *di*-C-demethylactinomycins. Interestingly, we could show that C-demethylactinomycins occur in traces as natural metabolites in actinomycin mixtures from older streptomycete cultures. This indicates that small quantities of 3-HA are formed during cultivation of these streptomycetes. However, since externally added 3-HA does not stimulate actinomycin synthesis and 3-HA was never detectable during short term radioincorporation experiments (in contrast to 4-MHA) we infer that the 3-HA responsible for C-demethylactinomycin formation in natural conditions is a by-product of 4-MHA synthesis derived from premature cleavage of 3-HK by hydroxykynureninase rather than a precursor of 4-MHA itself. Genomic data indicate that the genus *Streptomyces* lacks the gene encoding the enzyme kynurenine monoxygenase in primary metabolism.[15] Apparently, 3-HK synthesis and hence 3-HA formation is restricted to those streptomycetes which produce actinomycins or other 4-MHA-containing secondary metabolites.[6,15,29]

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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