ENZYMATIC SYNTHESIS OF ACTINOMYCIN D AND ANALOGUES CONTAINING *N*-METHYLALANINE FROM SYNTHETIC PENTAPEPTIDE LACTONE PRECURSORS

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The actinomycins constitute a family of chromopeptide antibiotics containing an aminophenoxazinone chromophore attached to two pentapeptide lactone units of variable amino acid content[1]. It was postulated as early as 1956 that the final step in the biosynthesis of an actinomycin involves the oxidative condensation of two molecules of a 3-hydroxy-4-methylanthraniloyl (4-MHA) peptide lactone[2] (Fig. 1). Subsequently, several total syntheses of actinomycins were reported employing chemical oxidation to achieve the same transformation[1]. Evidence for a biosynthetic role for this reaction includes the isolation of the enzyme, phenoxazinone synthase (PHS) from *Streptomyces antibioticus*, which catalyzes the oxidation of various *o*-aminophenols to the corresponding phenoxazinones[3]. Thus, with 4-MHA as substrate, actinocin (the actinomycin chromophore) is formed. Several actinocyl peptides, representing portions of the antibiotic molecule, were similarly produced from the corresponding 4-MHA peptides[4]. In addition, *in vivo* experiments have shown that 3-hydroxy-4-methylbenzoic acid is a strong inhibitor of actinomycin production by *Streptomyces chrysomallus*[5]. Novel compounds, shown to accumulate in the presence of this 4-MHA analogue, were identified as 3-hydroxy-4-methylbenzoyl pentapeptide lactones. Since these compounds lack the *o*-amino group of 4-MHA, they are unable to oxidize to actinomycins.

We now report the *in vitro* formation of actinomycin D (AMD) in mg quantities from a synthetic 4-MHA peptide lactone (**1**, R = Thr-D-Val-Pro-Sar-MeVal lactone) when incubated with a cell-free extract of *S. antibioticus*. The AMD was purified from reaction mixtures by silica gel chromatography and shown to cochromatograph with authentic AMD by TLC and HPLC. It was characterized by UV-VIS and IR spectroscopy and by ^{252}Cf plasma desorption MS (PD-MS). Difference spectra with calf thymus DNA were identical with those of authentic AMD and the antimicrobial activities (vs. *Staphylococcus aureus* and *Bacillus subtilis*) were comparable (Table 1). A similar experiment was performed using an analogue (**2**) of **1** in which *N*-methylalanine replaced *N*-methylvaline. The resulting 5,5'-MeAla AMD (**3**) was identical with the known synthetic compound[6].

Naturally occurring actinomycins are generally produced as mixtures containing both *iso* and *aniso* congeners having identical and differing peptide units, respectively[1]. The relative concentrations of these products differ from those expected from random oxidative condensations of the available precursor peptide lactones. Discrimination in favor of one of the two possible isomeric *aniso* compounds is generally observed. As a model for an enzymatic oxidation of a mixture of two different 4-MHA peptide lactones, equimolar quantities of **1** and **2**

Fig. 1. Enzymatic formation of actinomycin from two molecules of 4-MHA peptide lactone.

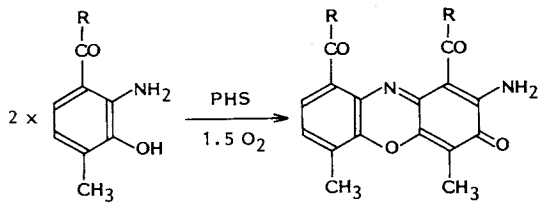

Table 1. MICs (μg/ml) of actinomycins.

Actinomycin	*S.a.*	*B.s.*
AMD, authentic	0.25	0.09
AMD, enzymatic	0.25	0.08
5,5'-MeAla AMD, synthetic	0.58	0.72
5,5'-MeAla AMD, enzymatic	0.5	0.55
5-MeAla AMD (**4**)	0.4	0.2
5-MeAla AMD (**5**)	0.45	0.2

Abbreviations: *S.a.*, *Staphylococcus aureus*; *B.s.*, *Bacillus subtilis*.

were incubated with PHS resulting in the formation of four actinomycins separable by TLC and HPLC. Two of the products were identified as AMD and **3**, while the other two, **4** and **5**, were shown to be the expected *aniso* isomers by PD-MS. The four actinomycins were produced in equal amounts, there being no discrimination in this case.

A determination of kinetic parameters with PHS and various substrates revealed apparent *Km* values for 4-MHA, 4-MHA methyl ester, **1** and **2** of 4.1, 4.5, 1.1 and 5.3×10^{-4} molar, respectively. Thus the postulated natural precursor (**1**) has the highest affinity for the enzyme of the substrates tested. This supports the postulate that the final step in the biosynthesis of an actinomycin consists of a PHS-catalyzed oxidative condensation of two molecules of 4-MHA pentapeptide lactone. This methodology may have application to the *in vitro* synthesis of actinomycin analogues.

Experimental

Large-scale Enzymatic Synthesis of AMD

3-Hydroxy-4-methyl-2-nitrobenzoyl-Thr-D-Val-Pro-Sar-MeVal lactone[6] (26 mg) in MeOH (8 ml) containing 0.4 N HCl-dioxane (100 μl) was hydrogenated over 10% Pd-C for 2 hours. After filtration and evaporation, the residual 4-MHA peptide lactone hydrochloride (15 mg) was dissolved in 0.5 M acetate buffer (pH 5.2, 10 ml). This was incubated with 100 μl of a cell-free extract of *S. antibioticus* in the same buffer (20 ml) at 37°C for 3 hours. After 4 extractions with EtOAc the extracts were washed with aqueous NaCl, dried (Na_2SO_4) and evaporated. The residue was chromatographed on a column of silica gel (Merck, grade 60, 230~400 mesh) with EtOAc-EtOH (19:1) and then on acid-washed alumina[7] with EtOAc. After evaporation *in vacuo* AMD was obtained as a red solid (11 mg). TLC on silica gel with EtOAc-EtOH (9:1) gave a single red spot alone or mixed with authentic AMD (Rf 0.63). HPLC[8,9] on Novapak C_{18} (5 μm) with 55% acetonitrile gave a single peak (Rt 8.7 minutes) alone or mixed with authentic AMD. UV λ_{max}^{MeOH} nm (ε) 240 (33,000), 445 (25,000). IR ν_{max} ($CHCl_3$) cm^{-1} 3011, 2966, 1750, 1689, 1664, 1649, 1632, 1583, 1514, 1489, 1406 (identical with AMD). PD-MS: *m/z* 1,256.4 (M+H) and 1,278.7 (M+Na), indicating M=1,255; $C_{62}H_{86}N_{12}O_{16}$ requires M=1,255.4. A difference spectrum[10] with calf thymus DNA was identical with that of authentic AMD, with a minimum at 425 nm and a maximum at 475 nm.

Large-scale Enzymatic Synthesis of a Mixture Containing AMD and Analogues having *N*-Methylalanine in Place of *N*-Methylvaline

3-Hydroxy-4-methyl-2-nitrobenzoyl-Thr-D-Val-Pro-Sar-MeAla lactone[6] (6.3 mg, 10 μmol) and 3-hydroxy-4-methyl-2-nitrobenzoyl-Thr-D-Val-Pro-Sar-MeVal lactone (6.6 mg, 10 μmol) were each hydrogenated in MeOH (2 ml) containing 0.4 N HCl-dioxane (25 μl) over 10% Pd-C for 2 hours. After filtration and evaporation, each residual 4-MHA peptide lactone was dissolved in 0.5 M acetate buffer (pH 5.2, 2 ml). Aliquots (1 ml) of each solution were mixed and a cell-free extract (20 μl) of *S. antibioticus* diluted with the buffer (4 ml) was added. At timed intervals during incubation at 37°C, aliquots (1 ml) were removed and twice extracted with EtOAc; the extracts were adjusted to 25 ml for OD readings at 445 nm. These indicated that at the following times (minutes) the completeness of reaction was as follows (%): 5 (17), 10 (26), 20 (40), 30 (47) and 60 (64). HPLC of the extracts as above gave peaks with Rt=0.75, 3.0, 3.5 and 8.7 minutes; the latter corresponded with AMD. The peak at 0.75 minute corresponded with samples of 5,5'-MeAla AMD produced both by chemical synthesis[6] and by incubation of **2** (2 μmol) with the cell-free extract under the same conditions. The intermediate peaks **4** and **5** were identified as the isomeric 5-MeAla AMD's by PD-MS: **4**, *m/z* 1,228.5 (M+H) and 1,250.6 (M+Na); **5**, *m/z* 1,228.4 and 1,250.0; both indicate that M=1,227 ($C_{60}H_{82}N_{12}O_{16}$ requires M=1,227.3). The 4 peaks were equal in area and the various timed fractions did not vary in this respect.

Conditions for the Assay of Phenoxazinone Synthase

The enzymatic assay as described previously[3] is based on the increase in OD at 443 nm due to formation of actinomycin. Incubations were performed in a recording Gilford spectrophotometer at 37°C. The reaction mixture contained 2 μmol of substrate, 250 μmol of acetate buffer (pH 5.3), enzyme and water to a total volume of 3 ml. Incubations were carried out for 10 minutes, and reaction rates were measured during 5~10 minutes.

Antimicrobial Activities (MICs) of Actinomycins

S. aureus or *B. subtilis* was inoculated into 50 ml of Trypticase soy broth (TSB; BRL, Baltimore) and incubated overnight at 37°C. Each culture (0.1 ml) was then inoculated into fresh TSB medium (50 ml) and reincubated for 8 hours at which time 0.1 ml of

the rapidly growing culture was mixed with an additional 50 ml of TSB medium. The diluted cultures (0.1 ml) were employed for determinations of the MICs in 2 ml of TSB medium.

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