EARLY INTERMEDIATES IN THE BIOSYNTHESIS OF ANSAMYCINS

II. ISOLATION AND IDENTIFICATION OF PROANSAMYCIN B-M1 AND PROTORIFAMYCIN I-M1

ORESTE GHISALBA, PETER TRAXLER, HERMANN FUHRER and WILHELM J. RICHTER

Pharmaceuticals Division and Central Function Research of CIBA-GEIGY Limited, Basel, Switzerland

(Received for publication March 3, 1979)

Proansamycin B-M1 and protorifamycin I-M1 were isolated as minor compounds from fermentations of the protorifamycin I producing strain *Nocardia mediterranei* F 1/24[1], identified by means of chemical and spectroscopic methods and shown to be degradation products of the hypothetical proansamycin B postulated in part I of this series of papers[1] and of protorifamycin I, respectively.

Proansamycin B-M1 and protorifamycin I-M1 (see Fig. 1) are the first products of the ansamycin group with an opened ansa chain to be reported so far. Their structure is closely related to ansamycins like protorifamycin I[1], protostreptovaricins I~V[2], damavaricins[3] and rifamycin W[4,5] but the bond between C-5 and C-11 is cleaved (for numbering see part I[1]) and the aliphatic chain is attached to the chromophore by the amide-bond at C-2 only. As in protorifamycin I and in the protostreptovaricins the hydroxyl group at C-8 is missing. WHITE *et al.*[6,7] have demonstrated by incorporation studies and ^{13}C-NMR spectroscopy that C-5 and C-11 of rifamycin S originate from one acetate unit (C-5 from C-2 and C-11 from C-1 of acetate or malonyl-CoA respectively). Identical results have been reported for rifamycin W[4] and streptovaricin D[8].

In view of these results proansamycin B-M1 and protorifamycin I-M1 are more likely to be metabolites (degradation products) of early ansamycin precursors than to be early precursors themselves.

Experiments and Results

Fermentation, Isolation and Purification of Proansamycin B-M1 and Protorifamycin I-M1

The mutant selection and the preliminary investigations with mutant strain *Nocardia mediterranei* F 1/24 have been described in the preceding paper[1].

To obtain large enough amounts of the minor compounds from *Nocardia mediterranei* F 1/24 fermentations were carried out in 30-liter fermenters with liquid complex medium 148[1] and industrial fermentation medium 151b[1] (inoculate, 5% pre-culture in liquid complex medium 148; fermentation, 8 days at 28°C; aeration, 1 liter air/liter culture/min.; pressure, 0.2 bar; stirring velocity, 700 rpm).

After fermentation the culture filtrates were acidified (pH 2.2 with sulfuric acid) and extracted with equal amounts of ethyl acetate using a LUWESTA-extractor EG 1006 (150 liters/hour). The extracts were evaporated to dryness under reduced pressure and the red-brown residues were stirred with petroleum ether. The petroleum ether insoluble residues were dried under high vacuum. From the fermentation with liquid complex medium 148, 26 g of dark brown amorphous powder was obtained. The fermentation with industrial fermentation medium 151b yielded 78 g of dark brown

amorphous powder.

Proansamycin B-M1 and protorifamycin I-M1 were isolated from these crude mixtures by repeated chromatography on silica-gel columns with chloroform and increasing amounts of methanol as eluents and then purified by preparative TLC on silica gel PF 254 plates (1.5-mm layer, Merck) with chloroform - methanol (90: 10 and 80: 20) as solvent system. Chromatographically pure proansamycin B-M1 (420 mg) and proansamycin B-M1 (60 mg) with minor impurities were obtained from the fermentation with liquid complex medium 148 (Rf = 0.55, TLC on silica gel 60 F254 plates (Merck) with chloroform - methanol (80: 20)). The fermentation with industrial fermentation medium 151b yielded only 150 mg of proansamycin B-M1. These yields show that proansamycin B-M1 is accumulated to a greater extent under the suboptimal conditions in liquid complex medium 148 than under the optimal conditions in industrial fermentation medium 151b.

Protorifamycin I-M1 (60 mg) was only isolated from the fermentation with industrial fermentation medium 151b (Rf 0.26, TLC on silica gel 60 F254 plates (Merck) with chloroform - methanol (80: 20).

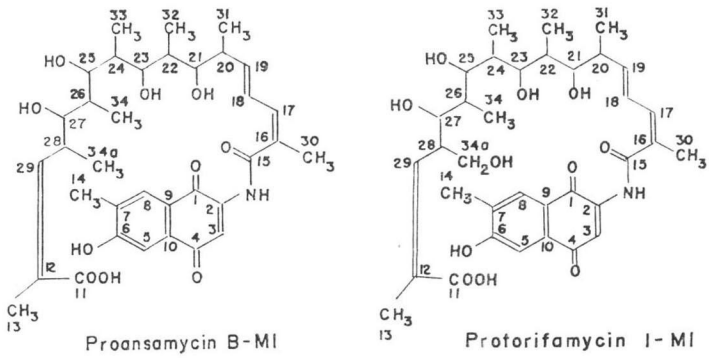
Structures of Proansamycin B-M1 and Protorifamycin I-M1

Proansamycin B-M1

Proansamycin B-M1 forms yellow needles from methanol (melting point 163~166°C, $[\alpha]_D^{20}$ (CH_3OH) $+18\pm1°$). The UV spectrum shows variation of the maxima depending on the pH; spectrum in ethanol pH 8: 315 (ε=14,000), 550 (1,300), shoulders at 262 and 370 nm; in ethanol pH 4: 212 (ε=59,000), 278 (50,000), 312 (26,400)nm, shoulder at 345 nm; in ethyl acetate: 275 (ε=30,500), 312 (17,600)nm, shoulders at 269 and 331 nm; in 0.1 N NaOH; 298 (ε=17,500), 385 (9,600)nm, shoulders at 240 and 540 nm. Potentiometric titration in 80% ethylene glycol monomethyl ether with 0.1 N trimethylammonium hydroxide gave pKa 7.88. In the FD mass spectrum $(M+H)^+$ and $(M+Na)^+$ ions were found at *m/e* 642 and *m/e* 664, respectively. After silylation the molecular ion was visible at *m/e* 1073 which indicates the presence of six exchangeable protons. From elemental analyses and from the data of ^{13}C-NMR and mass spectroscopy the molecular formula of proansamycin B-M1 was deduced to be $C_{35}H_{47}NO_{10}$. Anal.: Calc. for $C_{35}H_{47}NO_{10}\cdot 2\,CH_3OH$: C, 62.98%; H, 7.80%; N, 1.99%. Found: C, 62.10%; H, 7.80%; N, 2.45%; S, none.

The postulated structure of proansamycin B-M1 (Fig. 1) is in good accordance with the data of the 360-MHz-NMR and ^{13}C-NMR spectra (Fig. 2 and Table 1). In the ^{1}H-NMR spectrum the signals of four olefinic protons are present at very similar chemical shifts, as was found earlier in the corresponding spectra of protorifamycin I[1] and rifamycin W[1,5]. The existence of the double bond between C-12 and C-29 was further confirmed by double resonance experiments. Besides the signal of the aromatic C-3 proton at 7.51 ppm two

Fig. 1. Structures of proansamycin B-M1 and protorifamycin I-M1



further singlets of aromatic protons were found at 7.35 ppm and 7.80 ppm.

When comparing this to the ^{1}H–NMR spectrum of protorifamycin I, which lacks a C-8 hydroxyl group, the signal at 7.80 ppm can be attributed to the C-8 proton. This means that proansamycin B-M1 is also an ansamycin lacking the C-8 hydroxyl group. Because the C-14 aromatic methyl group (2.2 ppm) and the phenolic hydroxyl group at C-6 are still present in proansamycin B-M1, the additional aromatic singlet at 7.35 ppm can only be attributed to the C-5 proton, thus indicating that the ansa chain is cleaved between C-5 and C-11. On the other hand the amide proton was localized as an exchangeable singlet at 9 ppm confirming the attachment of the ansa chain to the chromophore by an amide-bond.

In the ^{13}C–NMR spectrum (see Table 1) most of the signals of the 35 C-atoms could be assigned by direct comparison with the ^{13}C–NMR spectrum of protorifamycin I and with the aid of the off-resonance spectrum. The two signals at 169.3 ppm and 167.5 ppm can be attributed to the amide-C-15 and to the carboxyl-C-11. The signal of the primary alcoholic carbon C-34a earlier found at 64.3 ppm in protorifamycin I has moved upfield and is now present at 10.2 ppm, among the signals of other methyl groups, indicating that C-34a is present in proansamycin B-M1 as a methyl group. For the rest of the C-atoms of the ansa chain there were no significant differences in the chemical shifts compared with protorifamycin I. According to the off-resonance spectrum proansamycin B-M1 has seven doublets in the range between 110 ppm and 150 ppm, which correspond to the four olefinic carbon atoms C-17, C-18, C-19 and C-29 and to the three aromatic carbon atoms bearing a proton, namely C-3, C-5 and C-8. Due to the proton in posi-

Table 1. ^{13}C–NMR data of protorifamycin I, proansamycin B-M1 and protorifamycin I-M1 (δ values in ppm).

Carbon	Protorifamycin I (in CD_3OD)	Proansamycin B-M1 (in DMSO-d_6)	Protorifamycin I-M1 (in CD_3OD)
C(1)	180.2	178.9	180.5
C(2)	140.8*	140.3	141.8
C(3)	118.2	114.9*	116.7*
C(4)	187.1	184.9	187.3
C(5)	131.3	110.9*	112.2*
C(6)	159.4	162.1	163.6
C(7)	133.0	130.2*	132.2*
C(8)	131.6	129.6	131.1
C(9)	124.0	121.4	123.2
C(10)	128.5	126.9	129.7
C(11)	200.2	167.5	168
C(12)	141.7*	132.0*	133.7*
C(15)	172.0	169.3	169.7
C(16)	131.9	124.4	129.1
C(17)	135.1	138.2	139.0
C(18)	126.1	124.4	127.7
C(19)	141.5*	146.4 146.9	146.0 146.9
C(29)	141.5*		
C(21)	71.0		
C(23)	74.4	69.9 72.5	72.3 74.8
C(25)	68.8	73.5 76.8	75.4 75.9
C(27)	78.8		
C(20)	39.0		37.6 38.4
C(22)	34.1	35.0 36.1	39.3
C(24)	37.7	37.1 39.5	
C(26)	43.8	40.3	42.6
C(28)	49.3		44.2
C(13)	18.0		
C(14)	17.0		
C(30)	20.2	9.0 10.2	9.6 10.1
C(31)	11.1*	10.2 10.5	13.1 16.4
C(32)	11.5*	16.7 16.8	17.0 17.5
C(33)	8.8	20.1	20.6
C(34)	12.5*		
C(34a)	64.3	10.2	60.3

* tentative assignment

} no precise assignment possible

Fig. 2. 360-MHz-NMR spectra of proansamycin B-M1: A) spectrum in DMSO-d_6 and B) spectrum in DMSO-d_6 after proton-exchange with D_2O.

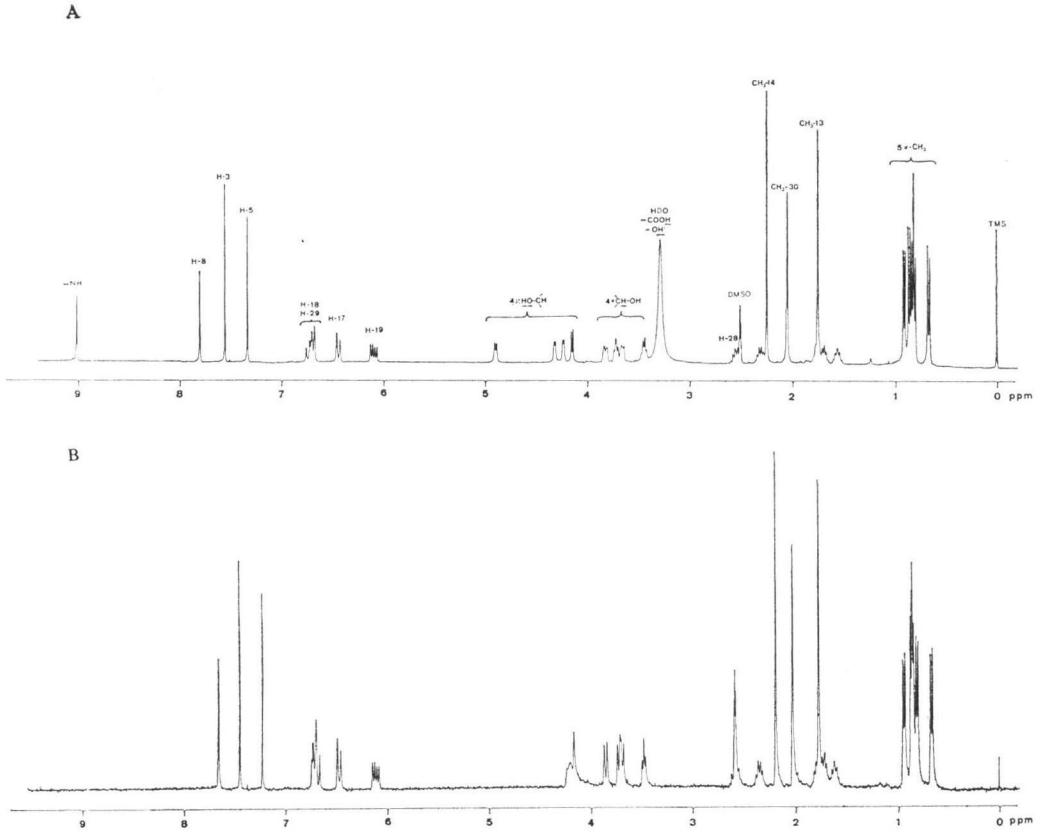
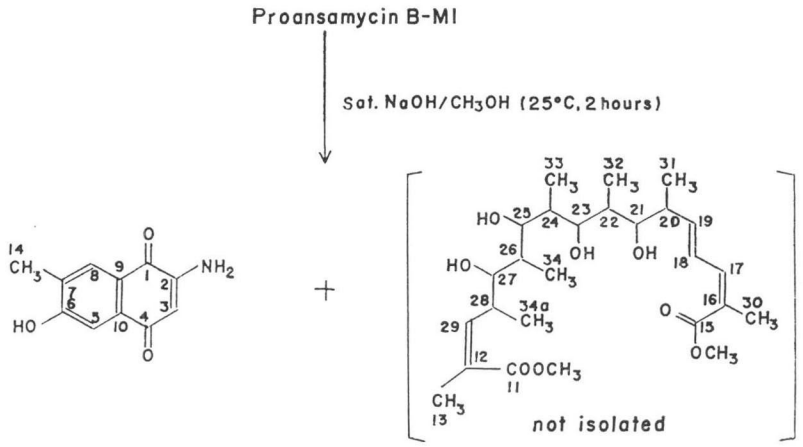


Fig. 3. Hydrolysis of proansamycin B-M1.



tion C-5 instead of the attached ansa chain as in protorifamycin I, shift differences for some aromatic carbons were found in the ^{13}C-NMR spectrum. The most drastic one was for C-5 itself, which shifted from 131.1 ppm in protorifamycin I to 110.9 ppm in proansamycin B-M1. The shift differences

for most of the other aromatic carbon atoms are within 4 ppm of those of protorifamycin I.

More support for the proposed structure was obtained from some derivatives of proansamycin B-M1 and especially from the hydrolysis of the amide-bond. Hydrogenation gave an uptake of 2.8 equivalents of hydrogen. In the ^{1}H-NMR spectrum of the hexahydro-derivative the signals of all the olefinic protons as well as of the two vinylic methyl groups have disappeared and shifted upfield. Treatment of proansamycin B-M1 with diazomethane gave the monomethyl ester derivative as the main product. After silylation it showed a molecular ion at *m/e* 1,089 (six TMS-groups) in the mass spectrum corresponding to a molecular weight of 655. In the ^{1}H-NMR spectrum an additional methyl group at 3.65 ppm was present. On the other hand, by methylation with methyl iodide and silver oxide as a catalyst, a dimethyl derivative was obtained. In the mass spectrum of the silylated product a molecular ion was detected at *m/e* 957 (four TMS-groups), corresponding to a molecular weight of 669, while in the ^{1}H-NMR spectrum two additional singlets of methyl groups at 3.65 ppm and 3.96 ppm were found.

The final proof for the postulated structure was achieved by a basic hydrolysis of the amide-bond to the chromophore and the ansa part (see Fig. 3). After chromatography on preparative TLC plates the chromophore was obtained as deep red crystals with the expected molecular ion at *m/e* 203 ($C_{11}H_9NO_3$) in the mass spectrum. The ^{1}H-NMR spectrum fully agreed with the structure of the chromophore.

In addition to the singlet for the aromatic methyl group at 2.18 ppm, three other singlets at 5.66 ppm, 7.26 ppm and 7.68 ppm can be attributed to the aromatic protons at C-3, C-5 and C-8. The chemical shifts of the C-5 and C-8 protons were very similar to what was found earlier in the ^{1}H-NMR spectrum of proansamycin B-M1, while the C-3 proton was shifted upfield due to the amino group in the C-2 position (7.06 ppm, exchanged by D_2O). The ansa chain on the other hand could not be isolated in high purity but the mass spectrum of an enriched fraction showed some typical fragmentations. With these findings the postulated structure of proansamycin B-M1 seems to be well established.

Protorifamycin I-M1

Protorifamycin I-M1 differs in its structure from proansamycin B-M1 only by the hydroxymethyl group at C-28 instead of a methyl group. In the FD mass spectrum M^+ was found at *m/e* 657 ($C_{35}H_{47}NO_{11}$). In the ^{1}H-NMR spectrum of protorifamycin I-M1, again the signals of three aromatic protons were found at 7.22 ppm, 7.57 ppm and 7.71 ppm, corresponding to H-5, H-3 and H-8. In the ^{13}C-NMR spectrum (see Table 1) the signals of only seven methyl groups were found between 9 and 21 ppm instead of eight in proansamycin B-M1. The signal of C-34a has moved downfield and was found at 60.3 ppm (compared to 64.3 ppm in protorifamycin I) indicating that C-34a is a hydroxymethyl group in protorifamycin I-M1. These results confirm the postulated structure of protorifamycin I-M1.

Biological Activity

Proansamycin B-M1 shows no activity against Gram-positive bacteria, Gram-negative bacteria and *Candida albicans* (MIC > 128 μg/ml). The exception was very weak activity against *Neisseria meningitidis* (MIC 8 μg/ml). The biological activity of protorifamycin I-M1 was not tested (lack of material) but from structural comparisons one can predict that protorifamycin I-M1 must be an inactive product, too.

Transformation Studies

Transformation assays were carried out according to the procedure described in the preceding paper[1] with washed mycelium of *Nocardia mediterranei* strains N813 (rifamycin B producer) and F 1/24 (protorifamycin I producer). No significant transformation of proansamycin B-M1 to rifamycin B or rifamycin W (strain N813) or protorifamycin I (strain F 1/24) could be detected after 80 hours of incubation. Nevertheless, part of the proansamycin B-M1 disappeared during the incubation due to biological instability. These findings indicate that proansamycin B-M1 is not a precursor of protorifamycin I and the subsequent rifamycins. It must therefore be a degradation product of an early precursor of the rifamycins (proansamycin B).

Discussion

As the transformation studies indicated, proansamycin B-M1 is not a precursor itself but a metabolite of an early precursor of protorifamycin I and of the subsequent rifamycins. Proansamycin B-M1 must be a degradation product of the hypothetical proansamycin B postulated in the preceding paper[1] for the following chemical reasons:

The only difference from the hypothetical proansamycin B is the cleavage of the bond between C-5 and C-11.

From biosynthetic studies it is known that C-5 and C-11 of the rifamycins (and streptovaricins) originate from the same acetate unit. A biosynthesis of the closed ansa-chain-chromophore system (proansamycin B) by a bond formation between C-5 and C-11 is thus excluded.

The existence of proansamycin B-M1 is thus an indirect argument for the existence of proansamycin B and a support for our biosynthetic hypothesis on the structures of possible common progenitors for the naphthalenic ansamycins presented in the preceding paper[1]. Proansamycin B itself still remains to be isolated.

In an analogous way, protorifamycin I-M1 must be interpreted as a degradation product of protorifamycin I. The cleavage of the bond between C-5 and C-11 seems to be a non-specific (enzymatic) reaction for ansamycins with a protorifamycin I-type chromophore.

Acknowledgement

The authors thank J.A.L. AUDEN and W. VOSER for fermentation and extraction, P. HUG for 360-MHz-NMR spectra and J.A.L. AUDEN for reviewing the manuscript.

References

1) GHISALBA, O.; P. TRAXLER & J. NÜESCH: Early intermediates in the biosynthesis of ansamycins. I. Isolation and identification of protorifamycin I. J. Antibiotics 31: 1124~1131, 1978
2) DESHMUKH, P. V.; K. KAKINUMA, J. J. AMEEL, K. L. RINEHART, Jr., P. F. WILEY & L. H. LI: Protostreptovaricins I~V. J. Am. Chem. Soc. 98: 870~872, 1976
3) RINEHART, K. L., Jr.; F. J. ANTOSZ, P. V. DESHMUKH, K. KAKINUMA, P. K. MARTIN, B. I. MILAVETZ, K. SASAKI, T. R. WITTY, L. H. LI & F. REUSSER: Identification and preparation of damavaricins, biologically active precursors of streptovaricins. J. Antibiotics 29: 201~203, 1976
4) WHITE, R. J.; E. MARTINELLI & G. LANCINI: Ansamycin biogenesis: Studies on a novel rifamycin isolated from a mutant strain of *Nocardia mediterranei*. Proc. Nat. Acad. Sci. 71: 3260~3264, 1974
5) MARTINELLI, E.; G. G. GALLO, P. ANTONINI & R. J. WHITE: Structure of rifamycin W: A novel ansamycin from a mutant of *Nocardia mediterranei*. Tetrahedron 30: 3087~3091, 1974
6) WHITE, R. J.; E. MARTINELLI, G. G. GALLO, G. LANCINI & P. BEYNON: Rifamycin biosynthesis studied with ^{13}C enriched precursors and carbon magnetic resonance. Nature 243: 273~277, 1973
7) MARTINELLI, E.; R. J. WHITE, G. G. GALLO & P. BEYNON: Carbon-13 NMR spectrum of rifamycin S: A re-examination of the assignments with special reference to their biogenetic implication. Tetrahedron Letters 1974: 1367~1368, 1974
8) MILAVETZ B.; K. KAKINUMA, K. L. RINEHART, Jr., J. P. ROLLS & W. J. HAAK: Carbon-13 magnetic resonance spectroscopy and the biosynthesis of streptovaricin. J. Am. Chem. Soc. 95: 5793~5795, 1973