Pergamon

Tetrahedron Letters 39 (1998) 13–16

TETRAHEDRON LETTERS

Exploratory Investigations into the Biosynthesis of the Antibiotic Moenomycin A

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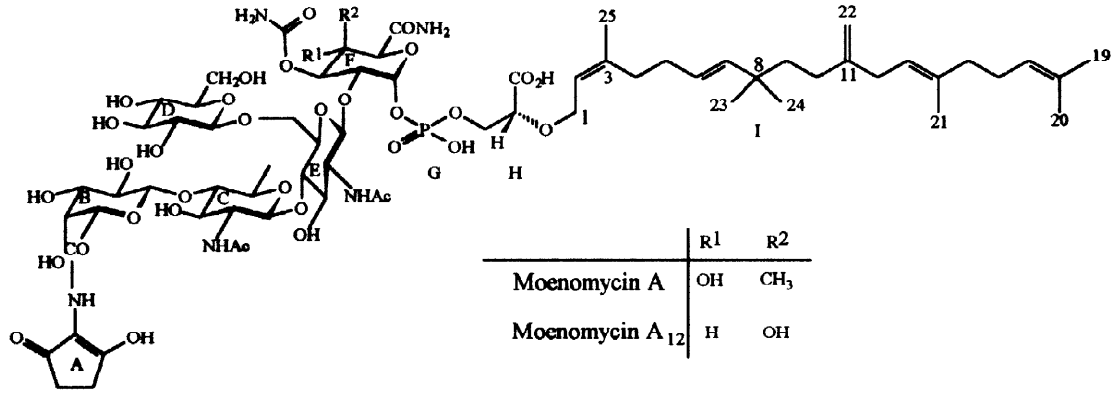
Received 16 August 1997; accepted 17 October 1997

Abstract - Feeding experiments shed light on the biosynthetic origin of the chromophore unit, the N-acetyl groups, the 4-C-methyl group of the moenuronamide unit, and the lipid part of moenomycin A.

The moenomycin antibiotics[3] are composed of an oligosaccharide part, linked via phosphoglycerate to a C_{25} lipid moiety. The other terminus is 2-aminocyclopentane-1,3-dione (the chromophore of moenomycin). Some of the moenomycin building blocks are common, others like the lipid moiety (I) and the moenuronamide unit (F) are characteristic for the moenomycin-type antibiotics. 2-Aminocyclopentane-1,3-dione is a constituent of other microbial metabolites, too.[4]

Almost nothing is known on the origin of the unusual structural elements and how the complex array of building blocks is assembled in the course of the biosynthesis. Here, we report the results of a series of feeding experiments.



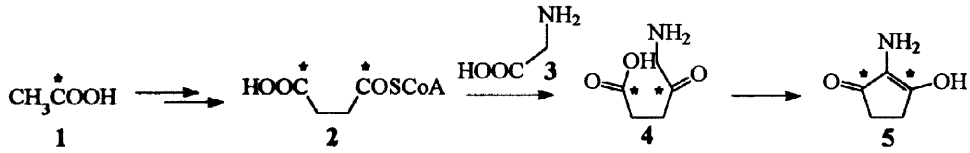
	R1	R2
Moenomycin A	OH	CH_3
Moenomycin A_{12}	H	OH

In a first set of experiments cultures of *Streptomyces ghanaensis* ATCC 14672 wilde type (DSM) were grown in Erlenmeyer flasks (medium 1[5]). The cultures were shaken for 290 h. First traces of moenomycin were detected by HPLC[6] after 100 h. At the end the maximum amount of moenomycin was in the range of 0.02 g/l.[7] Only moenomycins A and A_{12} could be detected. When the experiment was repeated using a 5 l laboratory fermenter[8] oxygen supply of the culture could be controlled and improved. Under these conditions the formation of moenomycin could be detected after 12 h. The moenomycin concentration after 130 h was much higher (roughly 0.16 g/l) than in the Erlenmeyer experiment showing the importance of sufficient oxygen supply. Again, only moenomycins A and A_{12} could be identified. We conclude from these experiments that *S. ghanaensis* ATCC 14672 wilde type under the conditions reported produces only moenomycins A and A_{12}.[9]

For the feeding experiments *Streptomyces ghanaensis* H2 (semi-producing strain from the Hoechst Marion Roussel collection) was used.[10] This strain produces all five moenomycins which are separated by reversed phase HPLC.[11] An improved isolation procedure for the moenomycins was developed including an ultrafiltration step.[12] Sodium [1-^{13}C]acetate (2.25 g/l) was administered using a pulse feeding protocol (the first dose at the end of the logarithmic phase, 45 h).[13,14] After altogether 165 h the moenomycins were isolated using

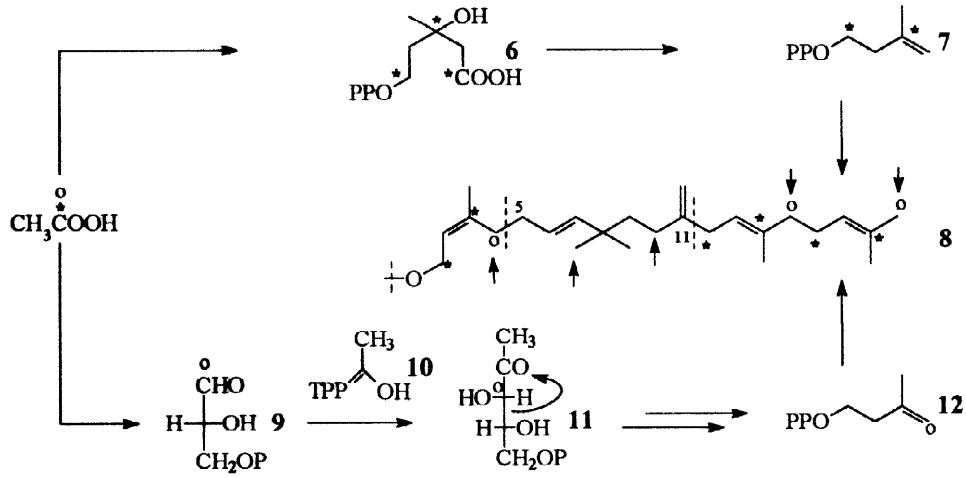
0040-4039/98/$19.00
PII: S0040-4039(97)10456-7

the new procedure. For an NMR analysis ^{13}C NMR spectra were recorded in 25:1 methanol-water. Well-resolved spectra were obtained. All signals of the lipid part could be assigned by comparison with previous results (see Table).[15] For a quantitative analysis the inverse gated decoupling ^{13}C NMR spectrum of the unlabelled moenomycin mixture was recorded in a 0.05 mol/l $Cr(acac)_3$ solution in 25:1 methanol-water. Under these conditions suppressing NOE enhancements and relaxation effects all signals gave practically the same integral. When the spectrum of labelled moenomycin was recorded under identical conditions, from the results a number of conclusions could easily be drawn: (i) acetate was incorporated into the sugar N-acetyl groups (4.7 %[16]), (ii) the chromophore part was labelled at positions 1 and 3 (1.7 %) in agreement with the labelling pattern that could be expected according to the biosynthetic pathway elucidated by Floss and coworkers.[17] Enrichment of the carbamoyl-C was also observed (2.1 %) indicating metabolic cleavage of acetic acid.

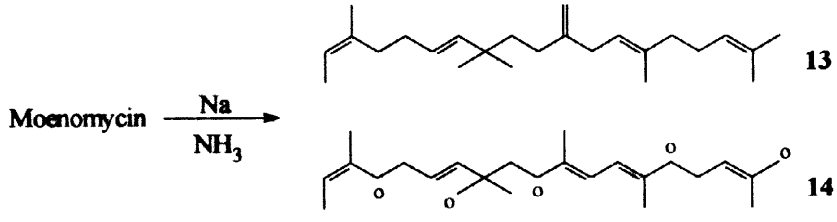


1 2 3 4 5

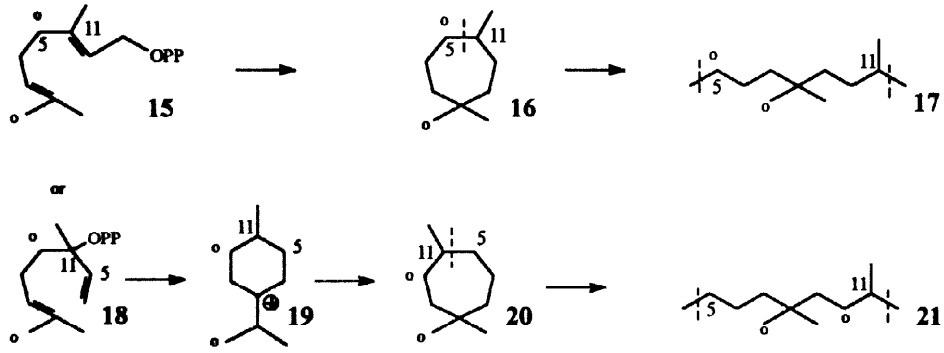
Incorporation of the labelled acetate into the lipid part was very poor. A careful analysis of the signal intensities gave the enrichments summarized in the Table (enriched positions are indicated by ↓ in formula **8**). The results show immediately that acetate is not incorporated into the moenocinol unit via the normal mevalonate pathway which would have demanded enhancements at positions indicated by * in the three isoprenoid units of formula **8**. The results are rather in agreement with a biosynthetic formation of isopentenyl diphosphate via the 1-deoxy-D-xylulose pathway.[18] The 1-labelled acetate would thus have been converted into 1-labelled glyceraldehyde-3-phosphate (**9**) via oxaloacetate. Reaction with nucleophilic thiamine diphosphate-activated acetaldehyde (**10**) would then have yielded 1-deoxy-D-xylulose (**11**) and the latter on rearrangement (via **12**) the isoprenoid carbon skeleton with the label in the positions indicated in the isoprenoid units of formula **8** by °.[19] It is probably of significance and corroborating the above arguments that incorporation (1.0 %) was also found at C-1 of the glyceric acid unit (δ = 177.3).



The present results are in perfect agreement with two feeding experiments performed many years ago.[20] Labelled moenomycin (feeding experiments with sodium [1-^{13}C]acetate) was at that time cleaved reductively with sodium in liquid ammonia to produce the reduction products **13** (31% yield) and **14** (47% yield). The



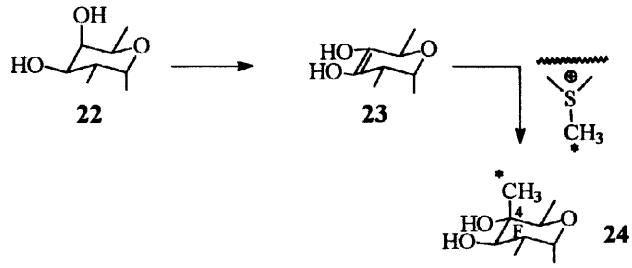
spectrum of labelled **14** was analyzed. The enrichments of two experiments are summarized in columns 4 and 5 of the Table. As far as the non-isoprenoid C_{10} middle part of moenocinol is concerned, many years ago we speculated that this unit could be formed by anti-Markovnikov cyclization of geranyl diphosphate to give **16** and opening of the bond between C-5 and C-11 (moenomycin numbering). This would lead from **12** via **15/18** and **16** to **17**. The present results appear to rule out this mechanism. However, cyclization of geranyl diphosphate (or rather linalyl diphosphate) in the Markovnikov sense (→ **19**) followed by rearrangement (conceivably via a carane intermediate) to give **20** and cleavage of the C-5 - C-11 bond would lead to the labelling pattern found in the experiment (compare **21** and **8**).



In a final set of experiments (*S. ghanaensis* H2, gyratory shaker, medium 1) the formation of moenomycin was studied in the presence of methionine. Methionine consumption and moenomycin production were followed by HPLC. After considerable experimentation methionin was administered in a single dose after 48 h (1 g/l). HPLC indicated that it was consumed after 120 h. The moenomycin concentration in the liquid medium started to increase after all methionin had been consumed reaching finally twice the value of cultures without added methionine. When (S)-[$^{13}CH_3$]methionine was administered the isolated moenomycin (mixture containing mainly moenomycin A) showed one enriched carbon signal, that of the 4-methyl group in unit F (20 %). We conclude from this experiment that the branching methyl group in unit F of moenomycin is introduced via a sequence consisting of (i) oxidation, (ii) dienolate methylation, and (iii) reduction (**22**→**23**→**24**). Such a sequence was postulated for related cases many years ago.[21]

Table: Feeding experiments with [1-^{13}C]-CH_3COONa

		% enrichment		
		moenomycin		14
1	n.d.	n.d.	0.3	0.0
2	123.0	0.1	0.5	0.0
3	141.8	0.0	0.3	0.0
4	33.3	0.6	1.2	1.1
5	32.6	0.4	0.5	0.3
6	126.8	0.2	0.2	0.0
7	141.4	0.0	0.4	0.0
8	36.3	0.1	0.5	0.2
9	42.7	0.0	0.4	0.0
10	32.2	0.9	1.2	1.1
11	151.0	0.0	0.0	0.0
12	35.8	0.0	0.3	0.0
13	123.4	0.4	0.4	0.0
14	137.3	0.0	0.3	0.0
15	40.7	0.7	1.4	1.1
16	27.6	0.0	0.7	0.5
17	125.3	0.4	0.3	0.0
18	132.2	0.2	0.3	0.0
19	25.9	0.8	1.1	1.0
20	17.8	0.3	0.3	0.0
21	16.2	0.0	0.4	0.0
22	109.3	0.4	0.4	0.0
23	27.8	0.8	1.1	1.1
24	27.8	0.0	0.0	0.0
25	24.0	0.0	0.4	0.2



Acknowledgements - We thank Dr.Marina Vogel for technical advice and Prof.P.Kleber (Leipzig) and Prof.M.H.Zenk (München) for helpful comments. The group at Leipzig acknowledges generous support by the

Deutsche Forschungsgemeinschaft (We 595/24-1 and Innovationskolleg „Chemisches Signal und biologische Antwort"), the Fonds der Chemischen Industrie, and Hoechst Marion Roussel (Frankfurt and Romainville).

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4 For leading references, see Cho, H.; Beale, J.M.; Graff, C.; Mocek, U.; Nakagawa, A.; Ōmura, S.; **Floss, H.G.** *J. Am. Chem. Soc.* **1993**, *115*, 12296-12304.

5 Medium 1: cornsteep liquor (6.6 g), soybean meal (25.6 g), $CaCO_3$ (3.3 g), soybean oil (23.4 g), $(NH_4)_2SO_4$ (11.7 g), corn starch (26.3 g), amylase bac. (1.5 mg), $CoSO_4$ (0.94 mg), KH_2PO_4 (93.8 mg), Genapol®(20 per cent in water, 25.0 g), deion. water (1.0 l), pH 7. Preculture medium: cornsteep liquor (0.4 g), soybean meal (3.0 g), $CaCO_3$ (0.5 g), soybean oil (0.4 g), glucose (2.6 g), KH_2PO_4 (0.1 g), deion. water (0.1 l), pH 7. All fermentations were performed at 37°C.

6 Analytical HPLC: 250 mm x 4 mm SEPSIL 100-5C_{18}; elution with 63:37 of solution A and acetonitrile. Solution A: Sodium heptanesulfonate (3 g), K_2HPO_4*3 H_2O (26.2 g), KH_2PO_4 (0.6 g) and water, final volume: 1 l; pH 8.0.

7 The moenomycin concentrations given in the text refer to the liquid medium.

8 Type L 1523 (Bioengineering, Wald, Switzerland), media and conditions as described in ref.[5]

9 See however, Subramaniam-Niehaus, B.; Schneider, T.; Metzger, J.W.; Wohlleben, W. *Z. Naturforsch.* **1997**, *52c*, 217-226.

10 Gyratory shaker, medium 1[5] or medium 2. For the feeding experiments with NaOAc medium 2 was used: cornsteep liquor (28.5 g), soybean meal (34.1 g), $CaCO_3$ (4.3 g), $(NH_4)_2SO_4$ (3.0 g), starch (32.9 g), amylase bac. (1.5 mg), $MgSO_4$ (0.23 g), $FeSO_4$ (0.2 mg), $ZnSO_4$ (0.8 mg), $CuSO_4$ (2.9 mg), $CoSO_4$ (1.0 mg), glucose (6.3 g), KH_2PO_4 (0.12 g), Genapol®(20 per cent in H_2O, 25.0 g), deion. H_2O (1.0 l), pH 6.5.

11 See, Donnerstag, A.; Marzian, S.; Müller, D.; Welzel, P.; Böttger, D.; Stärk, A.; Fehlhaber, H.-W.; Markus, A.; van Heijenoort, Y.; van Heijenoort, J. *Tetrahedron* **1995**, *51*, 1931-1940.

12 Cells were separated from the medium by centrifugation. The supernatant after solvent evaporation was stirred with 80:20 methanol-water. Cell disintegration was achieved by sonication in methanol. The filtrates from both fractions were combined and after solvent evaporation the residue was partitioned between butanol and water. The aqueous phase was purified by ultrafiltration (Amicon YM 3000 (cutoff 3000 Da)). Medium-pressure LC (RP_{18}, solvent described above, ref.[6], however, using ½ of the salt concentrations) followed by desalting (ultrafiltration) yielded the pure mixture of moenomycins.

13 A total of 15 additions of [1-^{13}C]acetate in intervals of 8 h.

14 In test experiments consumption of acetate was followed with the test kit of Boehringer (Mannheim).

15 See M_A (9) in Kempin, U.; Hennig, L.; Welzel, P.; Marzian, S.; Müller, D.; Fehlhaber, H.-W.; Markus, A.; van Heijenoort, Y.; van Heijenoort, J. *Tetrahedron* **1995**, *51*, 8471-8482. The chemical shifts of C-19^I and C-20^I have to be reversed.

16 Enrichments were calculated according to Scott. The integrals were referenced to C-2^A.
Scott, A.I.; Townsend, C.A.; Okada, K.; Kajiwara, M.; Cushley, R.J.; Whitman, P.J.; *J. Am. Chem. Soc.* **1974**, *96*, 8069-8080.

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19 See also Seto, H.; Watanabe, H.; Furihata, K. *Tetrahedron Lett.* **1996**, *37*, 7979-7982.

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