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Biosynthetic Investigations of the V-Type ATPase Inhibitors Bafilomycin A_1, B_1 and Concanamycin A

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The plecomacrolides bafilomycin A_1 and B_1 (**1**, **2**) and concanamycin A (**3**), produced by different *Streptomyces* species, show a unique macrolactone structure with characteristic side chains and exhibit striking biological activities including distinct V-type ATPase inhibition. The biosynthesis of **1** and **2** has been established by feeding experiments with ^{13}C-labelled precursors. Both, bafilomycin (**1**, **2**) and concanamycin (**3**) feature an "unusual C_2 chain extension unit" of as yet unknown origin which was addressed by feeding labelled 2-hydroxy- and 2-methoxymalonyl-derivatives.

The secondary metabolites bafilomycin A_1 and B_1 (**1**, **2**)[1,2] and concanamycin A (**3**)[3,4] were discovered by biological screening methods from various *Streptomycetes* strains and belong to the plecomacrolide family due to their unusually folded side chain.[5] **1** and **2** consist of a 16-membered macrolactone ring with a C_3 spacer unit bridging a cyclic hemiacetal with different substituents. Likewise **3** consists of an 18-membered macrolactone ring with the same C_3 spacer and a related cyclic hemiacetal.

Bafilomycin A_1 and B_1 (**1**, **2**) and concanamycin A (**3**) are very potent and specific inhibitors of vacuolar (V-type) ATPase enzymes, which led to substantial interest in these metabolites.[6~9] The V-type ATPases are involved in bone resorption processes in osteoclasts, therefore such macrolides could be used in treating osteoporosis.[10~12] Additionally they display antifungal, antiparasitic and antitumor activity.[13,14] The practical use of the macrolides (**1**, **2** and **3**) is limited due to fatal toxicity, which has led to intense semi-synthetic programmes.[15~19] A second valid approach to creating novel related compounds is biosynthetic engineering.[20~22] Related macrolides could be generated by altering the normal course of biosynthetic events, either through mutasynthesis or by genetic manipulation of the biosynthetic machinery. Clearly there is a structural relationship between the bafilomycins and concanamycins, but it remains to be seen whether this is reflected in having a similar biosynthetic origin. Though concanamycin biosynthesis has been studied previously through stable isotope feeding experiments[5], similar approaches concerning bafilomycin biogenesis are missing as yet. The knowledge of how nature assembles these macrolides is necessary for running biosynthetic engineering programmes. To that end we set about investigating the biosynthesis of the bafilomycins, in the first instance through stable isotope labelled precursor feeding experiments.

Biosynthesis

The plecomacrolide structures indicate a polyketide macrolactone skeleton which is generated from precursors of the carboxylic acid pool. Both bafilomycin (**1**, **2**) and concanamycin (**3**)[5] contain the as yet unidentified, glycerol-based "unusual C_2 chain extender unit", also referred to as a "glycolate" unit.[23] Such C_2 units, all bearing an α-methoxy group of yet unidentified origin, are also found in other macrolide or macrolactam antibiotics like soraphen[24], FK 520[25], ansamitocin[23], geldanamycin[26] or leucomycin[27]. The fungistatic antibiotic zwittermicin[28] contains both a hydroxymalonate as well as an aminomalonate extender unit. Based on potentially

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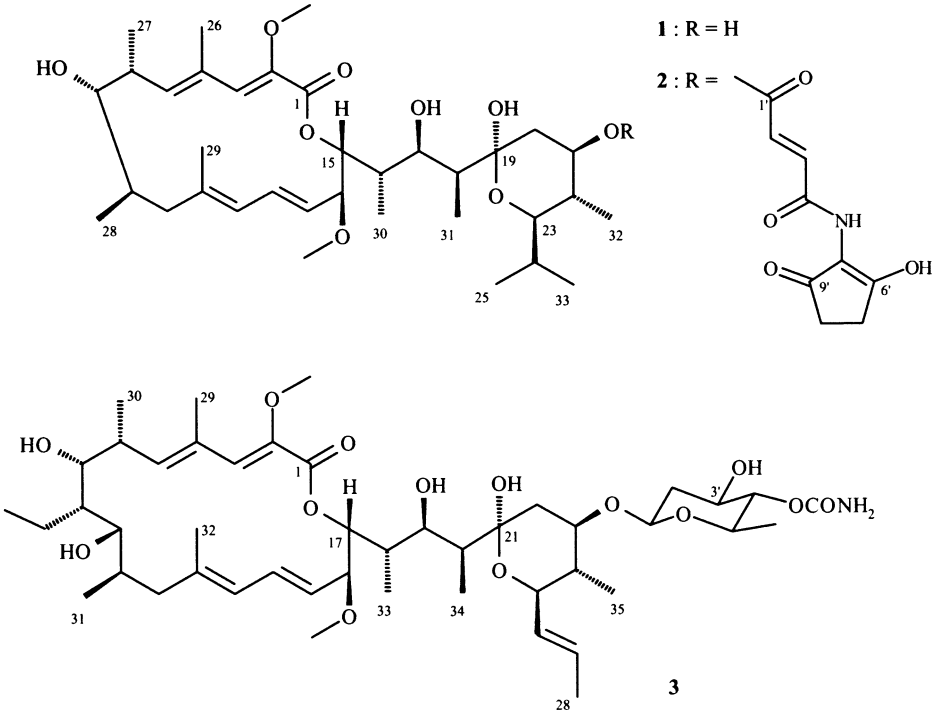
Dedicated to Prof. Dr. H. G. Floss on the occasion of his 70th birthday

Fig. 1. Structural formulae of the plecomacrolides bafilomycin A_1 (**1**) and B_1 (**2**) and concanamycin A (**3**).



corresponding gene functions hydroxymalonate or methoxymalonate have been proposed as intermediates.[29)]

Feeding sodium [1-^{13}C]acetate led to strong signal enhancement of C-11 and C-19 and moderate enhancement of C-3, C-5, C-7, C-9, C-15, C-17 and C-21 in **1** and **2** (Table 1). Feeding of [1,2-$^{13}C_2$]acetate thus revealed the direction of bafilomycin biogenesis by strong spin-spin coupling of C-11/C-12 and C-19/C-20 (Table 2) as depicted in Fig. 2. The results indicate two chain extensions with acetate and low enrichments for the methylmalonyl-CoA derived carbons due to acetate catabolism. C-1′, C-4′ and C-6′, C-9′ are additionally enriched in bafilomycin B_1 (**2**) indicating two 'tail-to-tail' linked acetate units. They reveal succinate as the biosynthetic precursor for the fumaric acid unit (C-1′∼C-4′) and the C_5N unit (C-5′∼C-9′). This corresponds with the known biogenetic pathway of its precursor 5-aminolevulinic acid, which is built up from succinyl-CoA and glycine by a 5-aminolevulinate synthase.[30,31)]

Expected signal enhancement in the ^{13}C NMR data from [3-^{13}C]propionate feeding reflects high specific incorporation for C-26 to C-32 in both compounds and therefore gave evidence that the seven methyl groups derive from methylmalonyl-CoA as chain extension unit ruling out methionine. [1-^{13}C]Isobutyrate as a feasible building block resulted in the exclusive signal enhancement of C-23 of **1** and **2** confirming the notion that isobutyrate is the starter unit of the polyketide chain during bafilomycin biogenesis.

Administration of [U-$^{13}C_3$]glycerol led to intact specific incorporation of C-1/C-2 and C-13/C-14 (Table 2) for the "unusual C_2 extender units", which confirms the hypothesis that these C_2 units arise from the carbohydrate metabolism. C-11/C-12 and C-19/C-20 also showed a significant labelling pattern (due to scrambling from glycerol into malonyl-CoA) while C-24/C-33 represents a coupling pair of carbon atoms from the incorporation of glycerol into the isobutyrate unit *via* pyruvate. The two methoxy groups were labelled after feeding of [methyl-^{13}C]methionine as expected.

According to known chain extension mechanisms 2-hydroxymalonyl- and 2-methoxymalonyl-thioesters are plausible precursors. ^{13}C labelled 2-hydroxy- (**4**) and 2-methoxymalonyl-*N*-acetylcysteamine (SNAC) thioester (**5**) were synthesized by FLOSS *et al.*[32)] as cell-permeable CoA-analogues. To prove the origin of the "unusual C_2 units" **4** and **5** were each administered to the growing cultures of the producer strains of **1**, **2** and **3**. The ^{13}C-NMR data showed no isotope enrichment for the respective

Table 1. Specific incorporation of sodium[1-^{13}C]acetate, sodium[3-^{13}C]propionate, sodium[1-^{13}C]isobutyrate and [methyl-^{13}C]methionine into **1** and **2**.

Position	Chemical shift (ppm)		Specific incorporation							
			[1-^{13}C] Acetate		[3-^{13}C] Propionate		[1-^{13}C]Iso-butyrate		[Methyl-^{13}C] methionine	
	1	**2**	**1**[a]	**2**[a]	**1**[b]	**2**[c]	**1**[a]	**2**[a]	**1**[b]	**2**[c]
1	167.6	167.7	0.58	0.30	-0.34	-0.22	0.20	0.92	-0.09	-0.05
2	141.4	141.6	0.08	0.08	-0.34	-0.24	0.07	0.78	-0.55	0.15
2-OCH_3	60.1	60.3	0.23	0.14	-0.20	-0.10	-0.02	-0.23	**56.98**	**51.13**
3	133.9	134.0	**5.93**	**3.18**	0.29	0.19	0.51	0.92	-0.29	-0.17
4	133.1	133.3	0.22	0.07	0.50	0.43	0.35	0.89	-0.12	0.55
5	143.5	143.6	**6.12**	**4.18**	0.45	0.28	0.68	0.11	0.06	-0.87
6	37.0	37.2	0.12	-0.05	0.32	0.24	-0.05	0.41	-0.03	0.45
7	81.1	81.3	**5.34**	**3.10**	0.05	0.09	0.28	0.20	-0.13	0.51
8	41.4	40.6	0.37	-0.07	0.83	0.12	-0.42	0.47	0.11	0.26
9	41.5	41.7	**5.45**	**3.04**	0.49	0.31	0.71	0.97	-0.04	0.76
10	143.6	143.7	-0.01	-0.91	0.60	0.27	0.14	0.91	0.17	0.09
11	125.3	125.5	**16.35**	**7.97**	-0.03	0.14	0.82	0.58	-0.18	0.04
12	133.5	133.6	0.03	-0.15	0.03	0.02	0.31	0.49	0.22	0.03
13	127.1	127.3	0.59	0.17	-0.29	-0.22	0.16	-0.16	-0.08	-0.13
14	82.6	82.8	0	0	-0.28	-0.22	0	0	-0.11	0.38
14-OCH_3	55.7	55.8	0.43	0.24	-0.23	-0.09	0.38	0.22	**57.45**	**40.77**
15	77.0	77.2	**6.12**	**3.28**	0.54	0.29	0.54	0.03	-0.38	0.25
16	37.5	37.7	0.08	-0.02	0.41	0.27	-0.11	0.56	-0.04	0.07
17	71.0	71.1	**6.14**	**2.77**	0.15	0.08	-0.12	0.25	0.18	0.35
18	42.3	42.4	0.06	-0.05	0.46	0.24	-0.34	0.20	0.03	0.27
19	99.3	99.3	**16.24**	**9.97**	0.01	0.31	0.16	0.96	0	0.12
20	43.8	40.4	0.04	-0.08	-0.02	0	-0.53	0.68	0.18	0
21	70.9	75.8	**5.35**	**2.78**	0.28	0.37	0.30	-0.34	0.07	-0.58
22	40.4	38.5	0.12	0.05	0.20	0.56	-0.07	0.16	0.10	0.04
23	76.1	76.1	0.34	0.14	0.02	0.01	**62.25**	**35.31**	-0.04	-0.02
24	28.2	28.4	0.20	0.04	0	0.03	-0.57	0.19	0.00	0.57
25	21.3	21.3	0.38	0.10	0.28	0.18	0.13	0.39	0.13	0.49
26	14.0	14.1	0.35	0.18	**9.57**	**10.12**	0.30	0.39	0.16	0.94
27	17.3	17.4	0.33	0.10	**8.69**	**8.50**	0.26	0.33	0.17	0.73
28	21.7	21.8	0.36	0.15	**9.59**	**9.14**	0.25	0.40	0.11	0.76
29	20.1	20.3	0.20	0.12	**9.97**	**8.61**	0.22	0.38	0.20	0.95
30	9.8	10.0	0.14	0.17	**9.33**	**9.90**	0.14	0.49	0.06	0.30
31	7.1	7.2	0.27	0.24	**8.31**	**10.25**	-0.01	0.53	0.12	0.56
32	12.1	12.4	0.41	0.11	**10.58**	**9.77**	0.24	0.61	0.17	-0.05
33	14.3	14.4	0.29	0.13	0.33	0.14	-0.07	0.40	0.12	0.05
1'	-	164.8	-	**6.62**	-	0.83	-	1.71	-	-0.50
2'	-	133.4	-	0.13	-	**2.30**	-	1.07	-	-0.59
3'	-	133.8	-	0.14	-	**2.38**	-	0.46	-	-0.48
4'	-	164.2	-	**5.82**	-	0.67	-	0.85	-	-0.68
5'	-	115.2	-	0.14	-	-0.09	-	0.84	-	-0.60
6'	-	175.6	-	*	-	-	-	-	-	-
7'	-	26.2	-	-	-	-	-	-	-	-
8'	-	32.6	-	-	-	-	-	-	-	-
9'	-	198.1	-	*	-	-	-	-	-	-

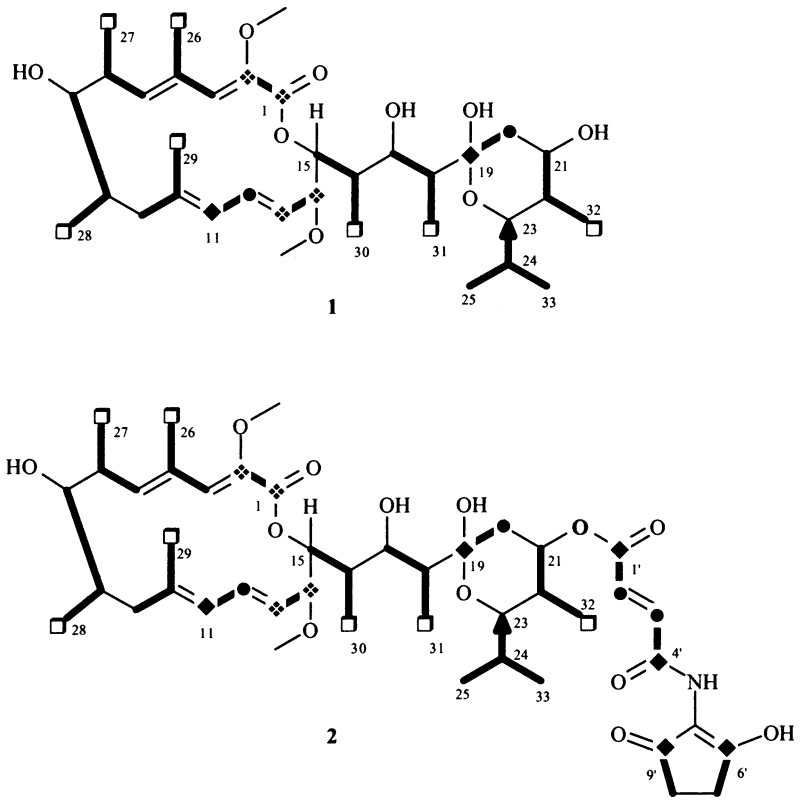
[a] Relative enrichments were normalized to the peak intensity of the C-14 signal

[b] Relative enrichments were normalized to the peak intensity of the C-24 signal

[c] Relative enrichments were normalized to the peak intensity of the C-20 signal

* signals for C-6'~C-9' show strong line broadening due to keto-enol tautomerism, whereas the signal integrals of C-6' and C-9' clearly indicate enrichment.

Fig. 2. ^{13}C enrichment for **1** and **2** after feeding of different precursors.



purified plecomacrolides bafilomycin (2 mg of **1** and 5 mg of **2**) and concanamycin (39 mg of **3**).

Discussion

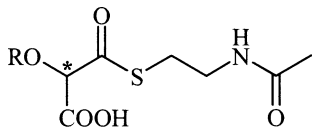
The results confirm the assembly of the macrolactone ring of bafilomycin A_1 (**1**) and B_1 (**2**) from an isobutyrate starter unit and eleven additional chain extension steps which involves seven propionate units, two acetate units and the two unusual C_2 extender units, probably on a type I modular polyketide synthase. The unusual tetrahydropyran ring of the bafilomycin plecomacrolides represents the start of the polyketide chain. As shown for asukamycin[30] and reductiomycin[31] the feeding experiments reveal that the characteristic side chain of **2** is constructed from succinate and 5-aminolevulinic acid and then probably attached to C-21 of the macrolactone ring of **1** in post PKS steps to form **2**. The "unusual C_2 extender units" in the macrolactone ring, which do not arise from acetate derive from the triose pool of the carbohydrate metabolism, but the potential precursors, the 2-hydroxy- and 2-methoxymalonate analogues **4** and **5**, were not incorporated. This observation corresponds with studies on ansamitocin[32,33] and FK520[34], which imply that the C_2 unit is biosynthesized on an acyl carrier protein, that presumably is used to load the ACP domain of the extension module. SNAC esters have been used *in vivo* and *in vitro* as mimics for coenzyme A thioesters[35], and are either incorporated in biosynthesis directly or after transthioesterification onto coenzyme A. Exogenous hydroxy- and methoxymalonyl-SNAC esters may be not able to intercept with the mechanism of C_2 unit biosynthesis and transfer due to the high level of protein-protein recognition which is required by such processes.

The above investigations contribute to the elucidation of the general chain extension principle of the non-acetate, "unusual C_2 unit" which extends the large variety of

Table 2. ^{13}C-^{13}C Coupling constants of **1** and **2** after feeding of sodium[1,2-$^{13}C_2$]acetate and [U-$^{13}C_3$]glycerol (*J* in Hz).

Position	[$^{13}C_2$]Acetate		[U-^{13}C]Glycerol	
	1	**2**	**1**	**2**
1			d (84 Hz)	d (83 Hz)
2			d (84 Hz)	d (83 Hz)
11	d (57 Hz)	d (57 Hz)	d (57 Hz)	d (56 Hz)
12	d (57 Hz)	d (57 Hz)	d (57 Hz)	d (56 Hz)
13			d (50 Hz)	d (50 Hz)
14			d (50 Hz)	d (50 Hz)
19	d (44 Hz)	d (44 Hz)	d (44 Hz)	d (44 Hz)
20	d (44 Hz)	d (44 Hz)	d (44 Hz)	d (44 Hz)
24			d (36 Hz)	d (36 Hz)
33			d (36 Hz)	d (36 Hz)
1'		d (75 Hz)		
2'		d (75 Hz)		
3'		d (65 Hz)		
4'		d (65 Hz)		
6'		Broad singlet		
9'		Broad singlet		

Fig. 3. The presumed precursors 2-hydroxy- (**4**) and 2-methoxy-*N*-acetylcysteamine (SNAC) thioester (**5**), asterisks indicate position of ^{13}C labelling.



4 : R = H
5 : R = CH_3

polyketide structures but hitherto did not attract high interest. These results create a platform for the production of new bafilomycin A_1 and B_1 (**1**, **2**) and concanamycin A (**3**) derivatives through biosynthetic engineering, potentially with advanced biological characteristics. This is subject of our ongoing investigations.

Experimental

General

All ^{13}C NMR spectra of **1** and **2** were measured in CD_2Cl_2 on a Varian Inova 600 spectrometer with the solvent as internal standard. ^{13}C chemical shifts are listed in Table 1. For concanamycin A (**3**) see ref. 5.

Labelled Compounds

^{13}C labelled compounds were >98% ^{13}C atom purity. Feeding studies with strain Gö 14F were performed using the sodium salts of 9.1 mM [1-^{13}C]acetate, 6.0 mM [1,2-$^{13}C_2$]acetate, 3.6 mM [3-^{13}C]propionate and 3.2 mM [1-^{13}C]isobutyrate, 5.3 mM [U-$^{13}C_3$]glycerol, 1.9 mM 2-hydroxy-[1-^{13}C]malonyl-*N*-acetylcysteamine and 0.9 mM 2-methoxy-[1-^{13}C]malonyl-*N*-acetylcysteamine[32]. For feeding studies with strain Gö 22/15 0.8 mM 2-hydroxy- and 2-methoxy-[1-^{13}C]malonyl-*N*-acetylcysteamine were administered.

Incorporation of Isotope-labelled Compounds

For feeding experiments strain *Streptomyces* sp. Gö 14F was fermentated in two 1 liter Erlenmeyer flasks, each

containing 250 ml medium E[36]. The labelled precursors were fed to the growing culture continuously from the 32th to 56th hour of incubation. Strain *Streptomyces* sp. Gö 22/15 was cultivated in a 2-liter fermentor (Biostat B, Braun) with 600 ml medium (oat bran 20 g, in 1 liter tap water, pH 7.8 prior to sterilization). Fermentations were inoculated with 10 vol-% of a 48-hour preculture and stirred at 250 rpm using an aeration of 3.3 vvm and the temperature controlled to 28°C. The ^{13}C labelled SNAC esters were fed to the cultures continuously from the 34th to 48th hour of incubation.

Fermentation and Isolation

Streptomyces sp. (strain 14F) produces both bafilomycins A_1 (**1**) and B_1 (**2**) in yields of 4 mg/liter and 16 mg/liter respectively. Production started after 20 hours and reached its maximum after 70 hours. Following feeding experiments **1** and **2** were isolated by extracting the supernatant with ethyl acetate and by flash chromatography on silica gel with cyclohexane/acetone 3 : 2. Final purification was achieved by gel permeation chromatography on Sephadex LH-20 with dichloromethane as eluent. Following feeding studies with the concanamycin (**3**) producing strain *Streptomyces* sp. Gö 22/15 efficient isolation of **3** was achieved by silica gel flash chromatography of the acetone cell extract with a gradient (chloroform/methanol from 95 : 5 to 90 : 10) to yield 45 mg/liter of **3** in high purity.[37]

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