2-Demethylmonensins A and B

Novel Minor Congeners of Monensins from *Streptomyces cinnamonensis*

Stanislav Pospíšil*, Petr Sedmera and Vladimír Havlíček

Institute of Microbiology, Academy of Sciences of the Czech Republic, Víděňská 1083, 142 20 Prague 4, Czech Republic

(Received for publication March 12, 1996)

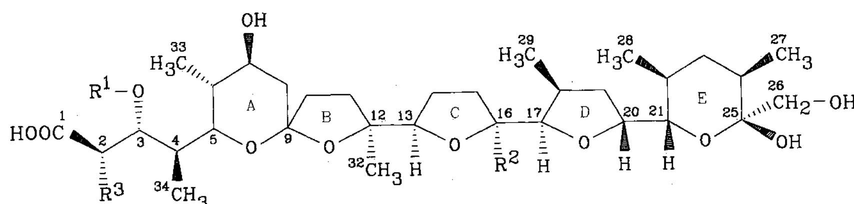
In the course of the study of metabolites produced by various mutant strains of *Streptomyces cinnamonensis*, we have found that besides monensins[1)] A (**1**) and B (**2**) and traces of 3-*O*-demethylmonensins[2)] A (**3**) and B (**4**), some of the mutants excrete small amount of new substances exhibiting standard color reaction with vanillin reagent[3)]. They were isolated and structures determined by FAB-MS, ^{1}H and ^{13}C NMR spectroscopy as 2-demethylmonensins A (**5**) and B (**6**).

The strain *Streptomyces cinnamonensis* TR-26 (Collection of IM Prague) was cultivated under submerged conditions on soybean meal medium[4)] for 6 days at 34°C on an orbital shaker (162 rpm). At the end of the fermentation, the broth was made alkaline by 0.1 M NaOH to pH 8.5. Cells were separated by centrifugation. Monensins were reextracted from the methanolic extract of the mycelium into $CHCl_3$. The concentrated substances were dried over anhydrous Na_2SO_4, solvent was removed *in vacuo*, and the residue was subjected to column chromatography (silica gel; heptane - ethyl acetate - methanol 5 : 4 : 1, v/v/v). Fractions containing new compounds were further chromatographed by preparative TLC on silica gel[5)]; Rf values in the system heptane - ethyl acetate - methanol (5 : 4 : 1, v/v/v; silica gel plate) were: **1**-Na 0.44, **2**-Na 0.38, **5**-0.29, **6**-0.33. The last purification step involved Sephadex LH-20 chromatography using methanol as a mobile phase and crystallisation from diethyl ether - H_2O. Melting points: **5** 80～83°C, **6** 73～76°C.

Both compounds **5** and **6** were isolated as free acids. The absence of stoichiometric sodium was confirmed by atomic absorption spectroscopy using the Na salt of **1** as a standard. The commonly used procedure for salt preparation (shaking with saturated $NaHCO_3$ solution) failed in this case.

First-order positive-ion FAB mass spectrum of (**5**) revealed the sodiated molecule at *m/z* 679 (sodium was trapped from matrix), *i.e.* at a mass of 14 daltons lower, than that of the sodium salt of monensin A (**1**). The first fragmentation step of the $[M+Na]^+$ ion involves loss of carboxyl and the side chain attached to the ring A. The daughter ion spectrum of this ion upon collisional activation is completely identical to that of molecular ion of 3-*O*-demethylmonensin A (**3**). Therefore, the possible sites of demethylation are C-2 or C-3. Unfortunately, our efficient negative ion fast atom bombardment tandem mass spectrometry protocol[6)] is applicable to sodium salts only and cannot be used for **5** and **6**.

The distribution of ^{13}C chemical shifts in **5**—eight methyls, ten methylenes (one of OCH_2 type), twelve methines (seven OCH), and five quaternary carbons (one C=O, 2× –OCO–, 2× –C–O–) closely resembles that of monensin A. However, one methyl is missing and there is one CH_2 more. According to ^{1}H NMR, the molecule of **5** contains one aliphatic methoxyl, one primary, five secondary, and one tertiary methyl. Thus, the missing methyl belongs to the secondary type. COSY spectrum reveals spin system $CH_2CH(O-)CH(CH_3)CH(O-)$-$CH(CH_3)CH(O-)CH_2$, $-CH_2-CH_2$, $-OCHCH_2CH_2$, $-OCHCH(CH_3)CH_2CH(O-)CH$ $(O-)CH(CH_3)CH_2$-$CH(CH_3)-$ neatly fitting the structure of **1** having a CH_2 group at position 2 instead of $-CH(CH_3)$ one. Except the side chain atoms, both ^{1}H and ^{13}C NMR parameters of corresponding protons or carbons in pairs 1～5 and 2～6 are very close. The difference in C-1 chemical shifts (Table 3) is striking but not without precedences; such values were observed with laidlomycin[7)] (176.46 ppm),



	R^1	R^2	R^3
Monensin A (**1**)	$\overset{36}{CH_3}$	$\overset{31}{CH_3}\overset{30}{CH_2}$	$\overset{35}{CH_3}$
Monensin B (**2**)	$\overset{36}{CH_3}$	$\overset{31}{CH_3}$	$\overset{35}{CH_3}$
3-*O*-Demethylmonensin A (**3**)	H	CH_3CH_2	CH_3
3-*O*-Demethylmonensin B (**4**)	H	CH_3	CH_3
2-Demethylmonensin A (**5**)	CH_3	CH_3CH_2	H
2-Demethylmonensin B (**6**)	CH_3	CH_3	H

Table 1. Selected proton signals (ppm, 400 MHz, $CDCl_3$) of **1**, **5**, and **6**.

Proton	**1**[17]	**5**	**6**
2	2.616 dq	2.611 dd	2.563 dd
		2.457 dd	2.445 dd
3	3.216 dd	3.703 ddd	3.869 ddd
4	2.183 m	2.208 m	2.168 m
5	4.070 dd	4.013 dd	3.929 dd
6	2.167 ddq	1.992 ddq	2.069 ddq
7	3.860 dd	3.856 dd	3.716 dd
8	1.980 dd	1.980 dd	1.952 dd
	1.700 dd	1.729 dd	1.725 dd
13	3.450 dd	3.465 dd	3.521 dd
14	1.690 m	1.714 m	1.664 m
	1.570 m	1.665 m	1.618 m
15	1.900 m	1.892 m	2.021 m
		1.709 m	1.850 m
17	4.090 d	4.033 d	3.943 d
18	2.333 m	2.234 m	2.237 m
19	2.200 m	2.161 m	2.209 m
	1.380 m	1.383 m	1.411 m
20	4.333 ddd	4.327 ddd	4.336 ddd
21	3.940 dd	3.935 dd	3.939 dd
22	1.270 m	1.270 m	1.272 m
24	1.500 m	1.498 m	1.492 m
26	3.678 d	3.663 d	3.763 d
	3.496 d	3.502 d	3.485 d
27	0.877 d	0.875 d	0.874 d
28	0.856 d	0.856 d	0.840 d
29	0.927 d	0.978 d	0.909 d
30	1.550 dq	1.556 q	—
	1.370 dq		—
31	0.948 t	0.948 t	1.176 s
32	1.496 s	1.481 s	1.447 s
33	0.890 d	0.886 d	0.899 d
34	1.099 d	1.069 d	1.064 d
35	3.367 s	3.348 s	3.343 s
36	1.259 d	—	—

Table 2. Selected proton-proton coupling constants [Hz] in **1**, **5**, and **6**.

i, j	**1**[17]	**5**	**6**
2d, 2u	—	13.2	16.0
2d, 3	10.2	9.9	9.4
2u, 3	—	4.8	3.1
2, 36	6.7	—	—
3, 4	2.0	2.3	2.3
4, 5	11.5	11.4	10.2
4, 34	6.8	6.9	6.8
5, 6	1.9	2.3	2.8
6, 7	≈2	2.6	2.4
6, 33	7.1	7.2	7.2
7, 8d	3.5	3.0	3.3
7, 8u	>2	2.7	2.7
8d, 8u	14.5	n.d.	n.d
13, 14d	n.d.	5.1	4.9
13, 14u	n.d.	10.4	10.5
17, 18	4.0	4.0	3.8
18, 29	6.8	7.0	6.9
19d, 20	5.8	5.8	5.9
19u, 20	10.5	10.6	10.5
20, 21	2.6	2.9	2.7
21, 22	10.6	10.4	10.3
22, 28	6.6	6.5	6.4
24, 27	6.3	6.3	6.2
26d, 26u	11.3	11.1	11.7
30d, 30u	14.5	n.o.	—
30, 31	7.4	7.3	—

d Downfield, u upfield (for magnetically nonequivalent methylene protons); n.o. not observed; n.d. not determined.

25-methoxymonensin A (178.96 ppm) and 25-methoxy-26-deoxymonensin B[8] (178.00 ppm). It confirms the existence of a free carboxyl group in **5** and **6**. Similarity of proton-proton coupling constants involving side chain and A-ring protons between **1** and **5** or **6** (Table 2) allows to reject the non-closed form[9].

Compound **6** gave a $[M+Na]^+$ ion at m/z 665.3 in positive-ion FAB mass spectrum. Its ^{13}C NMR spectrum, closely resembling that of monensin B (**2**) contains signals of 34 carbons (Table 3). Indeed, there are two tertiary methyls in the ^1H NMR spectrum (Table 1) in addition to all common features discussed above. Therefore, **6** is the lower homologue of **5**, *i.e.*, 2-demethylmonensin B.

According to diffusion method and comparing with the corresponding monensins **1** and **2**, the 2-demethyl derivatives had approx. 200 times lower activities against *Bacillus subtilis*.

From the biogenetic point of view, monensins are classified as oligoketides. The basic skeleton is assembled by sequential coupling of precursors derived from acetate, propionate and (in the case of monensin A) butyrate[10,11]. During the biosynthesis of the 2-demethylmonensins, the last biosynthetic unit—propionate—is replaced by acetate. By feeding of precursors to cultural medium of *Streptomyces cinnamonensis* it is possible to influence the ratio of monensins A and B produced. *E.g.* valine[12], butyrate and isobutyrate yielded higher proportion of monensin A, propionate caused shift of the biosynthesis in favour of monensin B[13]. On the other hand, acetate at low concentrations (0.05 to 0.2%, w/v) did not significantly influence the total production of monensins A and B or their proportion though the butyrate unit of monensin A was found to be produced also by a condensation of two acetate subunits[11,14]. High concentration of acetate completely inhibited the production of antibiotic. However, in no case a higher proportion of 2-demethylmonensins was observed; *e.g.*, with *S. cinnamonensis* TR-26 these compounds amounted 1~2% of the total monensin production only.

Addition of 2-demethylmonensins A and B to the known monensins A~D[1] completes the number of this family members to six. The structural diversity among these compounds is due to the use of different building blocks during their biosynthesis. The question whether there is one polyketide synthase or more of them remains open[15,16].

Table 3. Carbon signals (ppm, 100 MHz, $CDCl_3$) of **1**, **2**, **5**, and **6**.

Carbon	**1**[17]	**5**	**2**[17]	**6**
1	181.40 s	177.88 s	181.33 s	172.94 s
2	45.12 d	39.08 t	45.63 d	39.05 t
3	83.16 d	77.46 d	83.03 d	77.21 d
4	37.58 d	39.15 d	37.47 d	39.05 d
5	68.46 d	68.09 d	68.31 d	67.22 d
6	34.92 d	35.24 d	34.91 d	35.46 d
7	70.61 d	70.38 d	70.55 d	70.77 d
8	33.61 t	33.19 t	33.58 t	33.60 t
9	107.15 s	106.96 s	106.96 s	107.80 s
10	39.34 t	39.15 s	39.34 t	38.49 t
11	33.35 t	33.44 t	33.22 t	32.48 t
12	85.37 s	85.14 s	85.32 s	84.36 s
13	82.64 d	82.48 d	81.59 d	82.48 d
14	27.31 t	27.28 t	27.42 t	27.41 t
15	29.97 t	29.91 t	30.40 t	29.69 t
16	86.02 s	85.82 s	83.91 s	85.44 s
17	85.04 d	84.89 d	86.47 d	86.39 s
18	34.46 d	34.33 d	34.60 d	34.39 d
19	33.35 t	33.20 t	33.05 t	32.31 t
20	76.53 d	76.40 d	76.65 d	76.78 d
21	74.58 d	74.53 d	74.61 d	74.14 d
22	31.92 d	31.79 d	31.85 d	32.73 d
23	35.76 t	35.62 t	35.76 t	35.74 t
24	36.61 d	36.44 d	36.51 d	37.37 d
25	98.44 s	98.24 s	98.27 s	97.13 s
26	65.02 t	64.85 t	64.91 t	65.47 t
27	16.12 q	16.06 q	16.05 q	15.23 q
28	16.77 q	16.74 q	16.76 q	16.29 q
29	14.63 q	14.61 q	14.14 q	14.11 q
30	30.69 t	30.64 t	—	—
31	8.26 q	8.19 q	23.42 q	24.49 q
32	27.50 q	27.48 q	27.65 q	27.58 q
33	10.53 q	10.62 q	10.51 q	10.53 q
34	11.05 q	10.62 q	11.01 q	10.26 q
35	16.77 q	—	16.88 q	—
36	58.00 q	56.52 q	57.91 q	56.70 q

Acknowledgment

This work was supported by the Grant Agency of the Czech Republic (Grant No. 310/95/1053).

References

1) Gorman, M.; J. W. Chamberlin & R. L. Hamill: Monensin, a new biologically active compound. V. Compounds related to monensins. Antimicrob. Agents Chemother. 1967: 363~368, 1968
2) Pospíšil, S.; P. Sedmera, J. Vokoun, Z. Vaněk & M. Buděšínský: 3-*O*-Demethylmonensins A and B produced by *S. cinnamonensis*. J. Antibiotics 40: 555~557, 1987
3) Haney, Jr. M. E. & M. M. Hoehn: Monensin, a new biologically active compound. Discovery and isolation. Antimicrob. Agents Chemother. 1967: 349~352, 1968
4) Pospíšil, S.: Resistance of *Streptomyces cinnamonensis* to butyrate and isobutyrate: production and properties of a new anti-isobutyrate (AIB) factor. J. Gen. Microbiol. 137: 2141~2146, 1991
5) Pospíšil, S.; P. Sedmera, V. Krumphanzl & Z. Vaněk: Biosynthesis of monensin A and B. The role of isoleucine. Folia Microbiol. 31: 8~14, 1986
6) Havlíček, V.; M. Ryska & S. Pospíšil: Negative ion fast atom bombardment tandem mass spectrometry of sodium salts of monensins and related compounds. J. Mass Spectrom. 30: 1089~1094, 1995
7) Clark, R. D.; G. L. Hedden, A. F. Kluge, M. L. Maddox, H. R. Spires & P. F. Long: Enhancement of the activity of the antibiotic laidlomycin by acylation and the ^{13}C NMR spectra of laidlomycin and its esters. J. Antibiotics 35: 1527~1537, 1982
8) Pospíšil, S.; P. Sedmera, V. Havlíček & J. Tax: Production of 26-deoxymonensins A and B by *Streptomyces cinnamonensis* in the presence of metyrapone. Appl. Environ. Microbiol. 60: 1561~1564, 1994
9) Rodios, N. A. & M. J. O. Anteunis: Non-closed forms of monensin and nigericin free acid in protic solvents. Bull. Soc.Chim. Belg. 89: 537~550, 1980
10) Day, L. E.; J. W. Chamberlin, E. Z. Gordee, S. Chen, R. L. Hamill, T. Ness, R. E. Weeks & R. Stroshane: Biosynthesis of monensin. Antimicrob. Agents Chemother. 4: 410~414, 1974
11) Pospíšil, S.; P. Sedmera, M. Havránek, V. Krumphanzl & Z. Vaněk: Biosynthesis of monensin A and B. J. Antibiotics 36: 617~ 619, 1983
12) Pospíšil, S.; E. Cimburková, K. Stajner, J. Tax, V. Krumphanzl & Z. Vaněk: Regulation of biosynthesis of monensins on chemically defined medium. Folia Microbiol. 27: 275 ~277, 1982
13) Pospíšil, S.; E. Cimburková, V. Krumphanzl & Z. Vaněk: Effect of precursors on biosynthesis of monensin A and B. Folia Microbiol. 30: 30~33, 1985
14) Sood, G. R.; D. M. Ashworth, A. A. Ajaz & J. A. Robinson: Biosynthesis of the polyether antibiotic monensin A. Results from the incorporations of labelled acetate and propionate as a probe of the carbon chain assembly processes. J. Chem. Soc. Perkin Trans. I 1988: 3183~3193, 1988
15) Hopwood, D. A. & C. Khosla: Genes for polyketide secondary metabolic pathways in microorganisms and plants. *In* Secondary metabolites: Their function and evolution. Ciba Foundation Symposium 171, pp. 88~112, Wiley, Chichester, 1992
16) Birch, A. W. & J. A. Robinson: Polyetheres. *In* Genetics and Biochemistry of Antibiotic Production. *Ed.* L. C. Vining & C. Stuttard, pp. 443~476, Butterworth-Heinemann, London, 1995
17) Anteunis, M. J. O. & N. A. Rodios: Solution conformation of monensin free acid, a typical representative of polyetherin antibiotics. Bioorg. Chem. 7: 47~55, 1978