Two Novel Spiramycins Obtained from Commercial Samples: Isolation and Elucidation of Structure

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(Received for publication October 19, 1995)

Spiramycin (SPM), produced by *Streptomyces ambofaciens*[1], is a mixture of sixteen-membered macrolide antibiotics[2,3]. The structure of the main component, spiramycin I is shown in Fig. 1. During HPLC of commercial spiramycin samples it was observed that besides spiramycins I, II and III, several other related products were present as shown in Fig. 2[4]. Neospiramycins (NSPM) I, II and III correspond to spiramycins I, II and III without the neutral sugar mycarose[5]. The content of unknown compounds **1** and **2** is about 0.35% and 0.25% respectively, as calculated by normalization. The more polar impurities, eluted before NSPM I, are unstable and therefore difficult to isolate.

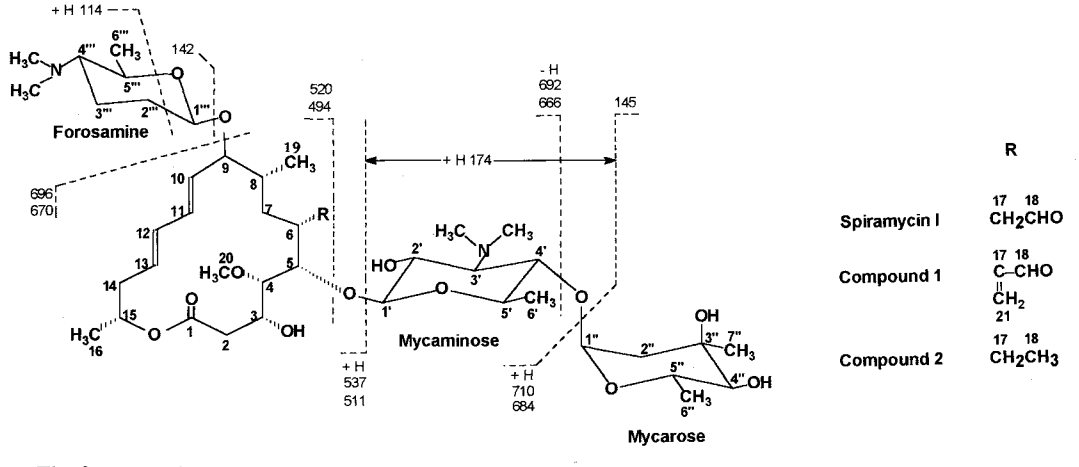
The isolation procedure of compounds **1** and **2** is outlined in Fig. 3. The samples for open column chromatography were dissolved in mobile phase CH_2Cl_2-CH_3OH-25% NH_4OH (97:3:0.2, v/v/v). The eluate from open column chromatography was monitored by TLC. The fractions of interest were combined and evaporated *in vacuo*. Further purification was achieved by semi-preparative HPLC in the same conditions as shown in Fig. 2, using a 250 mm × 9 mm i.d. column and a flow rate of 6 ml/minute.

Physico-chemical properties of compounds **1** and **2** are summarized in Table 1. Each gives a single peak in HPLC and a single spot in TLC. In the FTIR spectrum of compound **1**, the absorption band at 1674 cm^{-1} agrees with an aldehydic group conjugated with a methylene group. The absorption band at 1716 cm^{-1} points to a lactone[6]. Absorption bands at 3086 cm^{-1} and 905 cm^{-1} suggested the presence of a methylene group. In the spectrum of compound **2**, the absorption band at 1725 cm^{-1} points to a lactone group, but the absorption is weaker than that of SPM I which contains both aldehyde and lactone groups.

The structures of the two new 16-membered macrolides were derived primarily from ^{13}C NMR and Liquid SIMS (LSI-MS). Comparison of the ^{13}C NMR spectrum of **1** and **2** with that of spiramycin I clearly shows the presence of three unaltered sugar moieties, together with a methylene group in **1** and a supplementary methyl group in **2**. The chemical shifts of these compounds are shown in Table 2. The assignments are made in analogy with literature and are confirmed by DEPT or APT spectra[7~10].

The ^{13}C NMR spectrum of compound **1** thus shows a signal at 195.7 ppm, which is assigned to the aldehydic carbonyl (C-18). Compared to SPM I, the upfield shift of 8 ppm points to a conjugated carbonyl, corresponding to a methylene group positioned at C-17. Indeed, in the olefinic region there are two new sp^2 hybridized carbon signals at 147.9 and 139.4 ppm, while in the substituted alkyl region one CH_2 signal (C-17) is absent. DEPT and APT-spectra further indicate a quaternary (147.9 ppm) and a methylenic carbon (139.4 ppm), which confirms the presence of a CH_2=C–CHO portion. As expected C-6 is shifted to a lower field because of the presence of a new β-substituent. In contrast, C-7 is shifted to lower

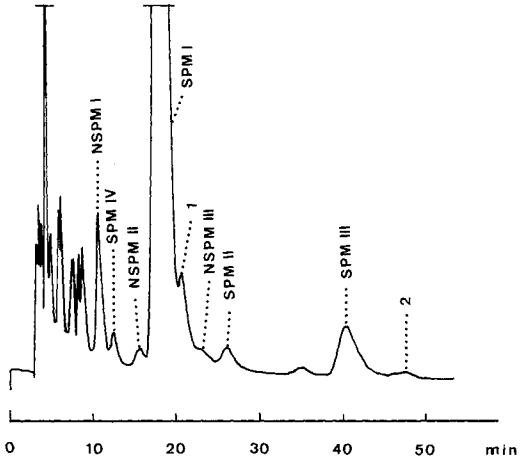
Fig. 1. Structures of spiramycin I and of compounds **1** and **2**.



The figures at the top and bottom are *m/z* values of compound **1** and **2** respectively.

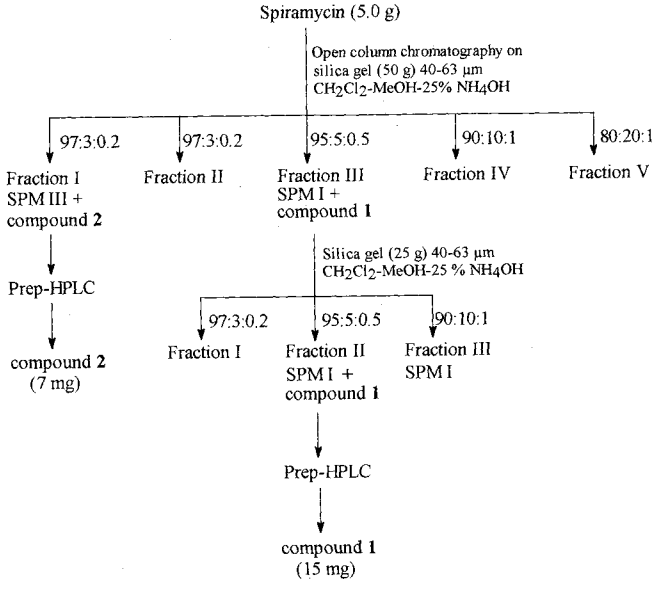
Fig. 2. Typical HPLC chromatogram of commercial spiramycin.

NSPM I: neospiramycin I; SPM IV: spiramycin IV; NSPM II: neospiramycin II; SPM I: spiramycin I; 1: compound **1**; NSPM III: neospiramycin III; SPM II: spiramycin II; SPM III: spiramycin III; 2: compound **2**.



HPLC conditions: column: poly(styrene-divinylbenzene) PLRP-S, 1000 Å, 8 μm, 250 × 4.6 mm (Polymer Laboratories, Shropshire, UK); column temperature: 60°C; mobile phase: CH_3CN - 0.2 M K_2HPO_4 pH 9.0 - water (38 : 5: up to 100, v/v/v); flow rate: 1 ml/minute; detection: UV at 232 nm; injected volume: 20 μl; concentration of sample: 1 mg/ml.

Fig. 3. Scheme for isolation and purification of compounds **1** and **2**.



field probably because of some conformational rearrangement due to the new substituent. In ^1H NMR, the aldehyde group at 9.58 ppm is at higher field than

Table 1. Physico-chemical properties of compounds **1** and **2**.

Compound	1	2
Appearance	White powder	White powder
Molecular formula	$C_{44}H_{74}O_{14}N_2$	$C_{43}H_{76}O_{13}N_2$
LSI-MS[a]: $(M+H)^+$ m/z	855	829
FTIR (KBr) cm^{-1}	3466, 3086, 2983, 2940, 2769, 1716, 1674, 1602, 1055, 998, 905	3466, 2940, 1725, 1625, 1055, 998
UV λ_{max}^{MeOH} nm	226	228
Rf[b]	0.36	0.56

[a] Samples were dissolved in thioglycerol; [b] TLC on silica gel was carried out with Et_2O - MeOH - 25% NH_4OH (90 : 10 : 1). Spots were visualized by spraying with 1 M H_2SO_4 in 95% EtOH and heating at 120°C for 10 minutes. Rf of SPM I is 0.27.

that of SPM I, as it is conjugated with the methylene group. The rest of the ^1H spectrum was not analysed and assigned in detail, since there were a lot of overlapping of the signals in this 200 MHz spectrum. In the ^{13}C NMR spectrum of compound **2**, an aldehyde signal is clearly absent but one additional methyl signal is present at 12.5 ppm. The carbon C-17 is shifted more than 20 ppm to higher field. This shows that C-18 is now a methyl group. C-6 is found at a lower field because of the larger deshielding of a β-methyl group in comparison with a β-aldehyde. There also is no aldehyde group signal in ^1H NMR.

The LSI-MS spectrum of compound **1** shows a $(M+H)^+$ ion at m/z 855. The spectrum also shows a $(M+Na)^+$ ion at m/z 877, $(M+K)^+$ ion at m/z 893 and $(M+H+thioglycerol)^+$ ion at m/z 963. The LSI-MS spectrum of compound **2** shows a $(M+H)^+$ ion at m/z 829, a $(M+Na)^+$ ion at m/z 851, $(M+K)^+$ ion at m/z 867 and $(M+H+thioglycerol)^+$ ion at m/z 937. As expected, the molecular weight of both compounds is confirmed by the presence in their EI-MS spectra of a molecular ion at m/z 854 and m/z 828 respectively. As for spiramycin I[11,12], most electron impact fragmentations involve cleavage of the glycosidic bonds yielding ions containing a terminal sugar unit as well as ions containing the aglycone, Fig. 1. Abundant sugar ions also occur in the LSI-MS spectra. Further decomposition of the primary sugar ions leads to the intense low-mass ions[13]. The m/z values of diagnostically important ions and their relative intensity are reported in Table 3. Comparison with spiramycin I which has molecular weight 842, shows that compound **1** has a mass of 12 units more and compound **2** has a mass of 14 units less than spiramycin I. So mass spectrometry confirms NMR results: compound **1** is deduced to be 17-methylenespiramycin and compound **2** is also deduced to be 18-deoxy-18-dihydrospiramycin.

Table 2. ^{13}C NMR chemical shift assignments for compounds **1** and **2** in acetone-d_6[a].

Carbon	SPM I	Compound **1**	Compound **2**	Carbon	SPM I	Compound **1**	Compound **2**
Aglycone moiety				Mycaminose moiety			
1	174.0	174.1	174.4	1′	104.6	104.7	104.1
2	38.8	39.1	38.8	2′	72.2	71.2	71.7
3	69.2	67.5	69.3	3′	69.5	68.9	69.6
4	86.1	85.9	86.5	4′	75.9	76.7	76.5
5	79.6	80.2	79.7	5′	73.5	71.9	72.9
6	31.2	34.3	38.0	6′	19.3	19.4	19.4
7	31.0	35.3	31.7	3′-$N(CH_3)_2$	42.4	41.1	42.2
8	31.9	32.2	32.6	Mycarose moiety			
9	78.4	79.2	78.6	1″	97.2	96.2	95.4
10	129.7	129.4	129.8	2″	41.8	41.5	41.5
11	134.8	135.1	135.0	3″	69.8	70.5	70.8
12	133.7	133.7	133.8	4″	77.2	78.0	76.8
13	131.5	131.8	131.6	5″	66.5	66.4	67.9
14	42.0	42.1	42.1	6″	18.8	18.7	18.6
15	69.7	69.7	70.2	7″	25.8	26.4	26.5
16	20.4	20.5	20.4	Forosamine moiety			
17	43.5	147.9	20.8	1‴	100.0	99.8	99.9
18	203.5	195.7	12.5	2‴	32.1	31.3	32.2
19	15.6	15.4	15.9	3‴	18.6	19.9	18.6
20	61.7	61.9	61.7	4‴	65.9	66.4	66.2
21	—	139.4	—	5‴	74.0	73.2	73.1
				6‴	19.3	19.6	19.6
				4‴-$N(CH_3)_2$	40.9	41.0	41.3

[a] All assignments are made in analogy with references 7, 8, 9, 10 and are confirmed by APT- or DEPT- spectra. TMS was used as internal standard.

Table 3. Diagnostically important ions in the mass spectra of compounds **1** and **2**[a].

Compound **1**				Compound **2**			
LSI-MS		EI-MS		LSI-MS		EI-MS	
m/z	%[b]	*m/z*	%	*m/z*	%	*m/z*	%
963	3.1	854	2.6	937	2.9	828	4.0
893	0.4	710	31.0	867	4.2	684	20.1
877	1.7	696	0.9	851	5.0	670	1.0
855	3.3	692	2.2	829	36.8	666	1.5
174	69.5	649	2.9	174	71.3	623	5.9
145	31.7	537	2.9	145	48.3	511	3.2
142	55.1	520	2.0	142	65.2	494	2.5
114	45.8	174	42.3	114	41.3	174	39.2
101	100	142	45.2	101	100	142	40.6
		114	54.9			114	42.8
		87	100			87	92.2
		71	77.2			71	100

[a] Positive ion LSI-MS spectra were obtained on a KRATOS Concept ^1H double-focusing mass spectrometer. The compounds were dissolved in thioglycerol matrix. The EI-MS spectra were recorded on a KRATOS MS-50 double-focusing mass spectrometer at 70 eV and 200°C.

[b] %: relative intensity.

The antibacterial activity of compound **1** and **2** was compared with that of the chemical reference substance of the European Pharmacopoeia by the agar diffusion test[14] using *Bacillus subtilis*. The potency of compound **1** and **2** was respectively 3 and 10 times less than that of the reference.

Acknowledgments

The National Fund for Scientific Research (Belgium) is acknowledged for financial support. The authors thank I. QUINTENS for technical and secretarial assistance. K. GOOSSENS and R. DE BOER, Department of Chemistry, Faculty of Sciences,

are acknowledged for FTIR and EI-MS measurements, respectively.

References

1) PINNERT-SINDICO, S.; L. NINET, J. PREUD HOMME & C. COSAN: A new antibiotic-spiramycin. Antibiot. Ann. 1954/1955: 724~727, 1955
2) KUEHNE, M. E. & B. W. BENSON: The structures of the spiramycins and magnamycin. J. Am. Chem. Soc. 87: 4660~4662, 1965
3) OMURA, S.; A. NAKAGAWA, M. OTANI, T. HATA, H. OGURA & K. FURUHATA: Structure of the spiramycins (foromacidines) and their relationship with the leucomycins and carbomycins (magnamycins). J. Am. Chem. Soc. 91: 3401~3404, 1969
4) LIU, L.; E. ROETS & J. HOOGMARTENS: Liquid chromatography of spiramycin on poly(styrene-divinylbenzene). J. Chromatogr., in preparation
5) TAKAHIRA, H.: The studies on spiramycin derivatives. Jpn. J. Antibiotics 23: 424~428, 1970
6) WANG, Y.; L. JIN, W. JIN, X. ZHANG, Y. ZENG, X. XU & J. YAO: Cloning of midecamycin 4″-acyltransferase and its expression in spiramycin producing strains. Chinese J. Biotechnology 8(1): 1~14, 1992
7) SANO, H.; H. TANAKA, K. YAMASHITA, R. OKACHI & S. OMURA: Chemical modification of spiramycins. V. Synthesis and antibacterial activity of 3′- or 4‴-de-N-methylspiramycin I and their N-substituted derivatives. J. Antibiotics 38: 186~196, 1985
8) KONDO, A.; T. NAKATANI, Y. KUZUYA, H. SUZUKI, H. SANO, K. YAMAGUCHI, K. SHIRAHATA, K. TAKAHASHI, K. KITA, Y. NISHIIE & S. MIZUNO: Studies on acetylspiramycin 1. Separation and chemical structures of acetylspiramycin components. Jpn. J. Antibiotics 43: 1152~1163, 1990
9) RAMU, K.; S. SHRINGARPURE, S. COOPERWOOD, J. M. BEALE & J. S. WILLIAMSON: ^{1}H-NMR and ^{13}C-NMR spectral assignments of spiramycins I and III. Pharmaceutical Research 11(3): 458~465, 1994
10) ALAM, P.; J. BARBER, R. J. BRENNAN, K. KENNEDY & M. H. H. TEHRANI: ^{1}H and ^{13}C NMR spectra of spiramycin I in organic and aqueous solutions. Magnetic Resonance in Chemistry 33: 228~235, 1995
11) SHIDA, Y.; L. J. DETERDING, K. O'HARA, M. KONO & K. B. TOMER: Macrolide antibiotics structure determination by Fast Atom Bombardment/Tandem Mass Spectrometry. Tetrahedron 49: 9221~9234, 1993
12) SANO, H.; T. SUNAZUKA, H. TANAKA, K. YAMASHITA, R. OKACHI & S. OMURA: Chemical modification of spiramycins. IV. Synthesis and *in vitro* and *in vivo* activities of 3″,4″-diacylates and 3,3″,4″-triacylates of spiramycin. J. Antibiotics 37: 760~772, 1984
13) GIOIA, B. & A. VIGEVANI: Stereochemical effects in the electron impact mass spectra of some 2,6-dideoxy-3-C-methylhexopyranoses. Organic Mass Spectrometry 11: 71~78, 1976
14) European Pharmacopoeia, Maisonneuve, Sainte Ruffine, France, 2nd edn., monograph 293,1984