IDENTIFICATION OF URINARY METABOLITES OF RIFAMYCIN LM 427 IN MAN

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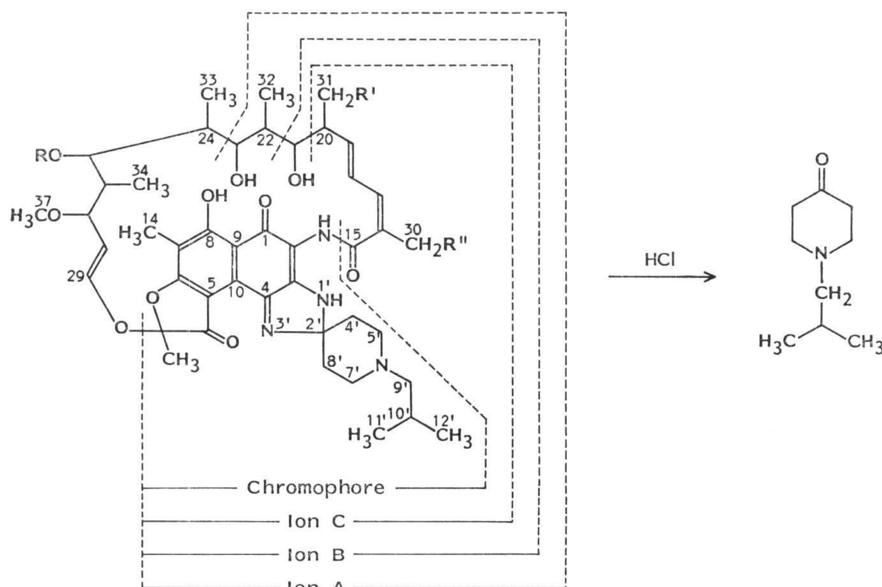
LM 427 (**1**) is a new spiropiperidylrifamycin,[1] with activity against atypical mycobacteria and many rifampicin resistant *Mycobacterium tuberculosis* strains.[2] In the present paper the spectroscopic and chemical evidence leading to the identification of three metabolites (**2**, **3** and **4**), present in human urine is described.

LM 427 was administered *per os* to six healthy volunteers as a single dose (300 mg). The urines of each subject were collected during the first 24 hours after administration and pooled (total volume 8 liters). The compounds were isolated by extraction with a 9:1 mixture of EtOAc and 1-propanol, followed by preparative chromatography on silica gel plates ($CHCl_3$ - MeOH, 9:1). According to a semiquantitative evaluation of the TLC plates, the most abundant compound **1** is equivalent to the total of metabolites **2** and **3**, which are almost in the same amount; on the contrary compound **4** is present in a smaller amount.* Acid hydrolysis of **2**, **3** and **4** (carried out at 40°C for 3 hours with 2 N HCl in acetone, followed by neutralization with $NaHCO_3$ and extraction with $CHCl_3$) affords, as in the case of **1**, the unchanged *N*-isobutyl-4-piperidone moiety, identified by GC-MS comparison with an authentic sample, thus ruling out any modification of this part of the molecule.

In the EI-mass spectrum of **2** (Table 1) the

Scheme 1.



	R	R′	R″
1	$R=\overset{36}{C}H_3\overset{35}{C}O$,	R′=H,	R″=H
2	R=H,	R′=H,	R″=H
3	$R=\overset{36}{C}H_3\overset{35}{C}O$,	R′=OH,	R″=H
4	$R=\overset{36}{C}H_3\overset{35}{C}O$,	R′=H,	R″=OH

* Experimental conditions for HPLC separation of **1**, **2** and **3**: Waters Bondapak C 18 column; mobile phase A H_2O - CH_3CN, 69:31 to pH 2 with H_3PO_4; mobile phase B CH_3CN; elution in a linear gradient from 10 to 20% B in 10 minutes, followed by a second 10 minutes linear gradient from 20 to 70% B; flow rate 2 ml/minute at room temp; detection 254 nm; retention times (minutes) 5.6 (**2**), 6.5 (**3**), 9.7 (**1**).

Table 1. Main peaks in the EI-mass spectra.*

Ion	m/z (relative intensity)			
	1	2	3	4
$[M]^{+\cdot}$	846 (12)	804 (17)	862 (8)	862 (12)
$[M - CH_3OH]^{+\cdot}$	814 (2)	772 (9)	830 (1)	830 (2)
$[M - CH_3OH - C_3H_7]^+$	771 (4)	729 (7)	787 (1)	787 (3)
Ion A	632 (1)	632 (2)	648 (1)	648 (2)
Ion B	574 (4)	574 (3)	590 (2)	590 (3)
Ion C	544 (2)	544 (2)	560 (4)	560 (5)
Chromophore	422 (4)	422 (6)	422 (2)	422 (4)
$i\ C_4H_9\text{-}\overset{+}{N}(\text{-}CH\text{=}CH_2)\text{=}CH_2$	112 (100)	112 (100)	112 (100)	112 (100)

* Data obtained at 70 eV by means of a Varian MAT 311-A spectrometer.

Table 2. ^{1}H NMR chemical shifts, multiplicity and coupling constants.[a]

Proton	δ (ppm) Multiplicity[a]					
	1		2		3	
NH	8.23	s	8.26	s	8.01	s
NH-1′	9.03	br s	9.72	br s	8.74	br s
OH-8	14.40	s	—[c]		14.66	s
H-13	1.73	s	1.72	s	1.76	s
H-14	2.31	s	2.28	s	2.30	s
H-17	5.9~6.4	m	5.8~6.4	m	6.20	J=2, 11 Hz dd
H-18	5.9~6.4	m	5.8~6.4	m	6.37	J=11, 16 Hz dd
H-19	5.9~6.4	m	5.8~6.4	m	5.97	J=16 Hz d
H-20	2.2~2.4	m	2.38	m	2.2~2.3	m
H-21	3.65	m	3.70	J=9 Hz d	3.78	s
OH-21	(3.35)[b]	s	(2.95)[b]	s	(3.16)[b]	s
H-22	1.7~2.0	m	1.7~2.1	m	1.8~2.0	m
H-23	2.8~3.0	m	3.30	m	2.8~3.0	m
OH-23	(3.60)[b]	s	(4.15)[b]	s	(3.79)[b]	s
H-24	1.42	m	1.7~2.1	m	1.4~1.7	m
H-25	4.72	J=11, 2 Hz dd	3.54	J=8, 10 Hz dd	4.88	J=10, 2 Hz dd
OH-25	—	—	2.87	J=8 Hz d	—	—
H-26	1.7~2.0	m	1.7~2.1	m	1.4~1.7	m
H-27	3.25	m	3.35	m	3.34	m
H-28	5.09	J=8, 13 Hz dd	5.18	J=10, 12 Hz dd	4.99	J=6, 12 Hz dd
H-29	6.10	J=13 Hz d	5.8~6.4	m	6.06	J=1, 12 Hz dd
H-30	2.04	s	2.03	s	2.07	s
H-31	0.82	J=7 Hz d	0.83	J=7 Hz d	2.8~3.0	m
H-32	1.02	J=7 Hz d	1.07	J=7 Hz d	1.17	J=7 Hz d
H-33	0.58	J=7 Hz d	0.54	J=7 Hz d	0.57	J=7 Hz d
H-34	−0.17	J=7 Hz d	−0.15	J=7 Hz d	−0.13	J=7 Hz d
H-36	1.99	s	—	—	2.04	s
H-37	3.06	s	3.16	s	3.05	s
H-4′, H-8′	1.6~2.0	m	1.7~2.1	m	1.4~1.7	m
H-5′, H-7′	2.65, 2.90	m	2.64, 2.92	m	1.85, 2.60	m
H-9′	2.2~2.4	m	2.2~2.3	m	2.2~2.3	m
H-10′	1.7~2.0	m	1.7~2.1	m	1.8~2.0	m
H-11′, H-12′	0.94	J=6.5 Hz d	0.93	J=6.5 Hz d	0.92	J=6.5 Hz d

[a] 200 MHz, $CDCl_3$ solutions, s=singlet; br s=broad singlet; d=doublet; dd=doublet of doublets; m=multiplet.
[b] Values can be interchanged.
[c] Not detected.

Table 3. ^{13}C NMR chemical shift assignments.[a]

Carbon atom	δ (ppm)			Carbon atom	δ (ppm)		
	1	2	3		1	2	3
1	182.1	181.4	181.5	24	38.1	38.5	37.7
2	114.6	114.9	113.9	25	73.6	71.2	74.0
3	142.7	142.0	142.3	26	38.6	40.0	39.0
4	155.6	155.3	153.3	27	81.2	85.7	79.0
5	112.6	112.0	111.9	28	116.1	113.8	116.5
6	168.4	168.1	168.0	29	145.1	147.8	147.5
7	109.8	110.1	109.2	30	20.5	20.3	20.0
8	171.9	171.1	171.6	31	17.4	17.0	74.7
9	107.9	108.4	107.1	32	11.9	12.5	14.0
10	125.7	125.4	[c]	33	9.1	8.9	9.5
11	191.8	191.3	191.4	34	11.4	11.4	8.7
12	105.6	105.1	105.0	35	171.9	—	172.0
13	22.0	22.8	21.3	36	20.5	—	20.1
14	7.7	7.7	7.4	37	56.5	55.5	56.6
15	168.7	168.6	168.4	2′	95.0	94.9	94.2
16	132.0	132.4	131.1	4′	(36.0)[b]	(35.8)[b]	(35.4)[b]
17	132.7	133.0	133.4	5′	51.7	[51.6][b]	51.3
18	124.8	123.8	123.2	8′	(36.7)[b]	(36.5)[b]	(36.1)[b]
19	141.2	141.2	143.4	7′	51.7	[51.8][b]	51.3
20	39.1	39.4	[d]	9′	66.6	66.5	66.0
21	73.2	72.2	73.7	10′	26.2	26.1	25.6
22	33.7	33.5	33.5	11′	20.9	20.9	20.6
23	77.4	76.7	78.4	12′	20.9	20.9	20.6

[a] 50 MHz, C_6D_6 solutions.
[b] Values in parentheses and brackets can be interchanged.
[c] Overlap by solvent.
[d] Probably overlapped by C-37 signal.

molecular ion is observed at m/z 804; the fragmentation pattern is the same as previously described,[3] in particular the fragments corresponding to the chromophore and those shown as ions A, B and C (Scheme 1) are unchanged with respect to **1**, indicating that the modification is confined to the "ansa" region C-24 to C-29. In the 200 MHz ^1H NMR spectrum of **2** (Table 2) the signal of CH_3-36 (δ: 1.99 ppm in **1**) is missing and H-25 shifts upfield from 4.72 (value observed for **1**) to 3.54 ppm. The coupling (J=8 Hz) with the OH signal at 2.87 ppm is clearly observable. The ^1H chemical shift values for **1**, also reported in Table 2, are completely consistent with the structure and in agreement with the reported values for rifamycin S.[4] The signals of C-35 and C-36 (171.9 and 20.5 ppm respectively, in **1**) are missing from the ^{13}C NMR spectrum of **2** (Table 3) and the C-25 peak (73.6 ppm in **1**) is observed at 71.2 ppm, whereas all other signals are found as expected and in agreement with literature values[5] and known substituent effects. The data allow one to assign to **2** the structure of the 25-*O*-deacetyl derivative of LM 427; the same deacylation process was reported for rifampicin.[6] The assignment has been confirmed by direct comparison with an authentic sample prepared[1] from 25-*O*-deacetylrifamycin S.[7]

As far as compound **3** is concerned, its EI-mass spectrum shows the molecular ion at m/z 862, *i.e.*, the molecular weight is 16 mass units higher than that of the parent compound. The same increase of 16 mass units is observed for ions A, B and C, while the chromophore ion is unchanged (Table 1). The above data indicate that an oxygen atom has been introduced in the "ansa" segment C-15 to C-20. In the ^1H NMR spectrum (Table 2) the CH_3-31 doublet (0.82 ppm for **1**) is missing whereas an additional signal corresponding to a CH_2OH group is found in the range 2.8~3.0 ppm, badly overlapped by the H-23 multiplet. The presence of the additional CH_2OH group causes noticeable conforma-

tional changes in that region of ansa chain. Coupling constants different from the reported values[4] are observed for H-19 (d, $J_{19,18}=16$ Hz) and H-21, which appears (both in $CDCl_3$ and in C_6D_6) as a broad singlet at 3.78 ppm. For this reason it has not been possible to detect a sizeable correlation peak between CH_2OH-31 and H-21 in a 2D homo-correlation experiment carried out on a C_6D_6 solution of **3**. Similar conformational effects were not observed for 3,31-dihydroxyrifamycin S in $CDCl_3$ solution.[8] In the ^{13}C NMR spectrum, the C-31 signal (17.4 ppm in **1**) is missing, and an additional peak appears in a region appropriate for a CH_2OH group (74.7 ppm). On the basis of the above data, the structure of the 31-hydroxyl derivative of LM 427 is assigned to compound **3**.

A third, minor metabolite (**4**) was also isolated; its EI-mass spectrum shows the molecular ion at *m/z* 862, indicating that also in this case an oxygen atom has been introduced in the molecule. The fragmentation pattern shows that the chromophore is unchanged; therefore the additional oxygen again must be located in the "ansa" portion. Due to the minimal amount of **4** it has not been possible to perform a complete NMR analysis, however, the ^1H NMR spectrum ($CDCl_3$) showed the absence of the olefinic C-30 methyl singlet and the presence of all the other "ansa" methyl signals. Therefore, we suggest for **4** the structure of the 30-hydroxyl derivative of **1**. These results indicate that the hydroxylation pattern in humans parallels to some extent the microbial oxidation at the C-30 and C-31 methyl groups.[8,10]

The biological activity of **2** is quite similar to that of LM 427, whereas metabolites **3** and **4** show less activity.[9]

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