HYDROXYLATION AND *N*-DEMETHYLATION OF CLARITHROMYCIN (6-*O*-METHYLERYTHROMYCIN A) BY *MUCOR CIRCINELLOIDES*

TAKASHI ADACHI, JOJI SASAKI and SADAFUMI OMURA

Research Center,
Taisho Pharmaceutical Co., Ltd.,
1-403 Yoshino-cho, Ohmiya-shi,
Saitama 330, Japan

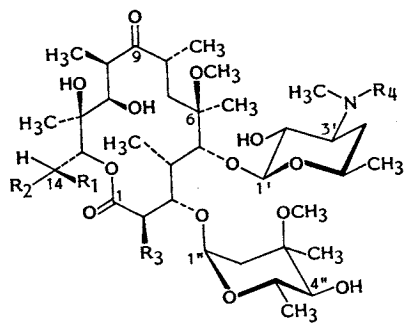
(Received for publication April 17, 1989)

In the course of studying the metabolism of clarithromycin (**1**, 6-*O*-methylerythromycin A, TE-031), (14*R*)-14-hydroxyclarithromycin (**2**) was isolated from human urine as a major and an active metabolite[1,2]. For further pharmacological and toxicological studies on this metabolite, we obtained a significant quantity of **2** by the microbial transformation of **1** using *Mucor circinelloides* f. *griseo-cyanus* IFO 4563[3]. MCALPINE *et al.* isolated 15-hydroxyclarithromycin (**3**) as a minor metabolic product of **1** using the same microorganism[4]. We have also isolated four minor metabolites of **1**;**3**, 16-hydroxyclarithromycin (**4**), *N*-demethylclarithromycin (**5**) and a 15-membered translactonization compound (**6**). Compound **6** is a ring-expanded derivative of **2** involving the 14-hydroxyl and the lactone groups. The structure determination of **6** is described elsewhere[5].

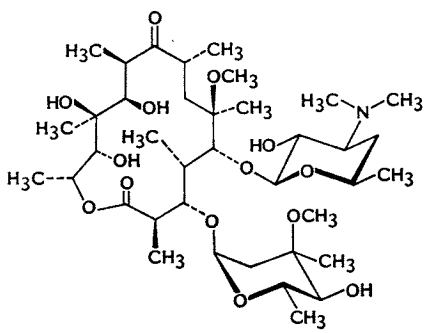
The present paper describes the isolation and the structure determination of the metabolites **4** and **5**.

Clarithromycin (**1**, 125 g) was used as the starting material for the microbial transformation studies. The fermentation conditions and extraction procedure were similar to those described in the previous paper[3].

Column chromatography (LiChroprep Si 60, E. Merck) of the crude extract (*ca.* 90 g) with $CHCl_3$ - MeOH - conc NH_4OH (20 : 1 : 0.1) afforded first **1**, and then 2.71 g of **2** (after recycling mixed fractions and crystallization from ethanol): Rf 0.58 (Silica gel 60 F_{254} plate, Art. No. 5715, E. Merck, developed two times with $CHCl_3$ - MeOH - conc NH_4OH (20 : 1 : 0.1)). There were then eluted the minor metabolic products, which were repeatedly chromatographed on silica gel columns under similar conditions. Crystallization afforded 37 mg of **3** (Rf 0.33), 111 mg of **4**



1 R_1=H R_2=R_3=R_4=CH_3
2 R_1=OH R_2=R_3=R_4=CH_3
3 R_1=H R_2=CH_2OH R_3=R_4=CH_3
4 R_1=H R_3=CH_2OH R_2=R_4=CH_3
5 R_1=R_4=H R_2=R_3=CH_3



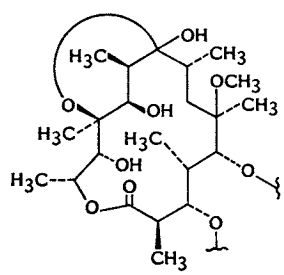


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Table 1. ^{1}H NMR chemical shifts[a] of **1**, **4** and **5**.

Proton No.	Multiplicity	Chemical shifts (ppm)		
		1[2)]	**4**	**5**
2-H	(dq)	2.89	2.95[b]	2.87
3-H	(dd)	3.77	3.90	3.76
4-H	(ddq)	1.92	1.97	1.94
5-H	(d)	3.67	3.71	3.67
7-H_{eq}	(dd)	1.72	1.72	1.67
7-H_{ax}	(dd)	1.85	1.87	1.78
8-H	(ddq)	2.59	2.58	2.59
10-H	(dq)	3.00	3.06	3.00
11-H	(d)	3.76	3.74	3.77
13-H	(dd)	5.05	5.14	5.06
14-H	(ddq)	1.48	1.50	1.48
	(ddq)	1.92	1.93	1.92
15-H (14-CH_3)	(t)	0.85	0.87	0.84
16-H (2-CH_3)	(d)	1.20	—	1.21
16-H (2-CH_2)	(dd)	—	3.76~3.84	—
17-H (4-CH_3)	(d)	1.10	1.13	1.05
18-H (6-CH_3)	(s)	1.41	1.43	1.42
19-H (8-CH_3)	(d)	1.14	1.14	1.14
20-H (10-CH_3)	(d)	1.13	1.14	1.13
21-H (12-CH_3)	(s)	1.12	1.13	1.13
6-OCH_3	(s)	3.04	3.05	3.04
1′-H	(d)	4.44	4.46	4.42
2′-H	(dd)	3.19	3.22	3.13
3′-H	(ddd)	2.41	2.48	2.46
4′-H_{ax}	(ddd)	1.21	nd	nd
4′-H_{eq}	(ddd)	1.66	1.71	nd
5′-H	(ddq)	3.48	3.52	3.55
6′-H (5′-CH_3)	(d)	1.23	1.23	1.22
3′-$N(CH_3)_2$	(s)	2.28	2.32	—
3′-NCH_3	(s)	—	—	2.42
1″-H	(dd)	4.93	5.18	4.92
2″-H_{ax}	(dd)	1.59	1.63	1.58
2″-H_{eq}	(dd)	2.37	2.31	2.36
4″-H	(dd)	3.02	3.04	3.03
5″-H	(dq)	4.01	4.01	4.01
6″-H (5″-CH_3)	(d)	1.30	1.31	1.30
7″-H (3″-CH_3)	(s)	1.25	1.26	1.26
3″-OCH_3	(s)	3.33	3.31	3.32

[a] δ values in ppm from TMS, measured in $CDCl_3$ at 400 MHz; as determined from a ^{1}H-^{1}H 2D homonuclear shift correlated experiments.

[b] (ddd).

nd: Not determined because of the complexities of the spectra.

(Rf 0.41), 57 mg of **5** (Rf 0.29) and 231 mg of **6** (Rf 0.18). Compounds **3** and **6** were identical to 15-hydroxyclarithromycin and a translactonization derivative of **2**, respectively, reported in refs 4 and 5, according to mass, ^{1}H and ^{13}C NMR spectroscopy. Physico-chemical properties of **4** and **5** are as follows. **4**: MP 164~167°C and then 206~208°C (prisms, crystallized from ethanol); UV λ_{max}^{EtOH} nm (ε) 285 (49.5), 223 (471.2); $[\alpha]_D^{24}$ −72.3° (*c* 0.5, EtOH); IR ($CHCl_3$) cm^{-1} 3406, 3000~2700, 1729, 1694; fast atom bombardment (FAB)-MS *m/z* (MH^+) 764; *Anal* Calcd for $C_{38}H_{69}NO_{14}$: C 59.74, H 9.10, N 1.83. Found: C 59.47, H 9.09, N 1.90. **5**: MP 220~222°C (prisms, crystallized from ethanol); UV λ_{max}^{EtOH} nm (ε) 289 (33.6), 212 (334.1); $[\alpha]_D^{24}$ −78.3°

Table 2. ^{13}C NMR chemical shifts[a] of **1**, **4** and **5**.

Carbon No.	Chemical shifts (ppm)		
	1[2)]	**4**	**5**
C-1	175.9	174.7	175.7
C-2	45.1	51.6	45.1
C-3	78.5	76.5	78.3
C-4	39.3	38.6	39.0
C-5	80.8	81.1	81.2
C-6	78.5	78.5	78.4
C-7	39.4	39.6	39.3
C-8	45.3	45.3	45.1
C-9	221.1	221.1	220.9
C-10	37.3	37.0	37.3
C-11	69.1	69.0	69.1
C-12	74.3	74.2	74.2
C-13	76.7	77.7	76.7
C-14	21.1	20.9	21.0
C-15 (14-CH_3)	10.6	10.7	10.6
C-16 (2-CH_3)	16.0	—	16.0
C-16 (2-CH_2)	—	61.5	—
C-17 (4-CH_3)	9.1	9.1	9.6
C-18 (6-CH_3)	19.8	19.6	19.7
C-19 (8-CH_3)	18.0	18.1	18.0
C-20 (10-CH_3)	12.3	12.2	12.3
C-21 (12-CH_3)	16.0	16.0	16.0
6-OCH_3	50.7	51.0	50.6
C-1′	102.9	102.8	102.3
C-2′	71.0	71.0	75.1
C-3′	65.6	65.5	60.3
C-4′	28.6	28.5	37.4
C-5′	68.8	68.8	68.5
C-6′ (5′-CH_3)	21.5	21.4	21.2
3′-$N(CH_3)_2$	40.3	40.2	—
3′-NCH_3	—	—	33.3
C-1″	96.1	95.7	96.1
C-2″	34.9	35.2	34.9
C-3″	72.7	72.7	72.7
C-4″	78.0	77.8	77.9
C-5″	65.8	66.0	65.7
C-6″ (5″-CH_3)	18.7	18.7	18.7
C-7″ (3″-CH_3)	21.5	21.4	21.5
3″-OCH_3	49.5	49.5	49.4

[a] δ values in ppm from TMS, measured in $CDCl_3$ at 100.4 MHz; as determined from a ^1H-^{13}C 2D heteronuclear shift correlated experiments.

(*c* 0.5, EtOH); IR ($CHCl_3$) cm^{-1} 3509, 3000~2700, 1728, 1687; FAB-MS *m/z* (MH^+) 734; *Anal* Calcd for $C_{37}H_{67}NO_{13}$: C 60.55, H 9.20, N 1.91. Found: C 60.32, H 9.19, N 1.90.

The molecular formula of **4** was determined as $C_{38}H_{69}NO_{14}$ from the elemental analysis, FAB-MS and ^{13}C NMR spectra, indicating that **4** was derived from **1** by the introduction of one oxygen atom. The ^1H and ^{13}C NMR spectra of **4** were directly compared with those of **1** (Tables 1 and 2). In the ^1H NMR spectrum of **4**, the methyl signal of 16-H (2-CH_3) disappears and new signals are observed at 3.76~3.84 ppm. The resonance of 2-H in **4** appears at 2.95 ppm as a double-double-doublet, whereas the corresponding proton of **1** resonates at 2.89 ppm as a doublet of quartet. This indicates the introduction of a hydroxyl group into C-16. In

Table 3. *In vitro* antimicrobial activities of microbial transformation products.

Strain	MICs (μg/ml)				
	1	**2**	**4**	**5**	**6**
Staphylococcus aureus 209P-JC	0.10	0.20	0.78	1.56	50
S. aureus BB	0.10	0.20	0.78	1.56	50
S. aureus Smith 4	0.20	0.39	1.56	3.13	>100
S. aureus J-109	>100	>100	>100	>100	>100
S. aureus B1	>100	>100	>100	>100	>100
S. aureus C1	3.13	12.5	25	100	>100
S. epidermidis sp-al-1	0.10	0.20	0.78	1.56	100
Enterococcus faecalis ATCC 8043	0.05	0.05	0.10	0.20	12.5
Bacillus subtilis ATCC 6633	0.05	0.10	0.39	0.39	50
Micrococcus luteus ATCC 9341	0.025	0.025	0.05	0.10	3.13
Escherichia coli NIHJ JC-2	100	50	>100	>100	>100
E. coli K-12	12.5	12.5	25	>100	>100
Klebsiella pneumoniae IFO 3317	25	25	100	>100	>100
Branhamella catarrhalis NNBr-3	0.10	0.10	0.39	1.56	12.5

Inoculum size: 10^6 cfu/ml.
Medium: Sensitivity Test Agar (Eiken).

the ^{13}C NMR spectrum of **4**, the methylene signal (C-16) at 61.5 ppm is newly observed, replacing the methyl signal (C-16) at 16.0 ppm in **1**. The signal of C-2 at 51.6 ppm in **4** is shifted downfield by 6.5 ppm compared with that in **1** by the β-substituent effect. Other chemical shifts of **4** are similar to those of **1**. Erythromycin F (16-hydroxyerythromycin A) had been isolated from the mother liquors of *Streptomyces erythreus*[6]. The present spectral data of **4** are consistent with the published substituent effects of hydroxylation at C-16. The structure of **4** is therefore determined to be 16-hydroxyclarithromycin.

In the FAB-MS analysis of **5**, the protonated molecular ion peak (*m/z* 734) and the base peak (*m/z* 144) indicate the removal of the methyl group from the desosamine moiety of **1**. In the ^1H NMR spectrum of **5**, the *N*-methyl signal at 2.42 ppm integrates for only 3H. The upfield shifts of the β-carbons (C-3′ and *N*-CH_3) and the downfield shifts of the γ-carbons (C-2′ and C-4′) are observed. Compound **5** is identified as *N*-demethylclarithromycin, previously isolated from human urine as a metabolite of **1**.

The antimicrobial activities of **4** are 2- to 8-fold less than those of **1** and **2** as shown in Table 3. 16-Hydroxylation of **1** results in greater decrease of activity than does 14-hydroxylation. Compound **5** is further 2-fold less active than **4**. The translactonization derivative **6** shows only slight activity.

By the microbial transformation of clarithromycin with *Mucor circinelloides*, 14-, 15- and 16-hydroxyclarithromycins were obtained. It is interesting to note that the carbons near to the lactone group were preferentially hydroxylated by this microorganism.

In our previous studies on the metabolism of clarithromycin in humans, 14-hydroxylation and *N*-demethylation had been observed[1,2]. These reactions were also observed in the microbial transformation. The microbial transformation process represents a useful method for preparing metabolites of medicinal substances as well as for obtaining novel compounds.

Acknowledgment

We thank Mr. H. KONDOH for technical NMR assistance, and Mr. T. ONO for antibiotic susceptibility test.

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