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Structure Elucidation of the Bioactive Metabolites of ML-236B (Mevastatin) Isolated from Dog Urine

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Two active metabolites of ML-236B, 4β,6α-dihydroxy ML-236B (M3, **2**), and 3β-hydroxy ML-236B (M4, **3**), were isolated from the urine of ML-236B-treated dogs. The structures of M3 and M4 were elucidated on the basis of spectral data and confirmed by X-ray analysis.

Keywords——ML-236B metabolite; mevastatin; X-ray analysis; ^{1}H-NMR; mass spectrometry

Introduction

The fungal product ML-236B (mevastatin, **1**), isolated from cultures of *Penicillium citrinum*, has been introduced as a potent competitive inhibitor of 3-hydroxy-3-methylglutaryl (HMG)-coenzyme A (CoA) reductase[1-4] and the same compound was independently isolated from *Penicillium brevicompactum* and named compactin by Brown *et al.* at Beecham.[5]

Among the five metabolites isolated from the urine of ML-236B-treated dogs, three metabolites tentatively designated as M3, M4, and M5 retained inhibitory activity against HMG-CoA reductase, and M5 was readily identified as the acid form of ML-236B (**1a**).[6] This paper deals with the structure elucidation of M3 and M4. M3 has been identified as 4β,6α-dihydroxy ML-236B acid (**2a**) and M4 as 3β-hydroxy ML-236B acid (**3a**), based on comparisons of the spectroscopic data of ML-236B with those of metabolites. To obtain confirmation of the structure of M4, X-ray crystallographic analysis was carried out.

Results and Discussion

4β,6α-Dihydroxy ML-236B (M3)

The urine of ML-236B-treated dogs was chromatographed on an Amberlite XAD-2 column. The methanol fraction containing the crude metabolite was further purified as described in the experimental section and M3 was obtained in the acid form (**2a**). Alternatively, the metabolite was isolated after being converted to the lactone form (**2**) by incubating the crude metabolite with *p*-toluenesulfonic acid at 37 °C overnight.

The mass spectrum (MS) of **2a**, whose molecular formula was established to be $C_{23}H_{38}O_8$ by high-resolution measurement, has a molecular ion peak at *m/z* 442; the difference of 34 amu, as compared to the parent molecule ML-236B (**1**) could be explained by the addition of two hydroxyl groups. This was consistent with the introduction of five tetramethylsilane (TMS) moieties into **2a**, indicating opening of the δ-lactone ring of ML-236B to give a 3,5-dihydroxyheptanoic acid side chain, and hydroxylation at another two sites. The following

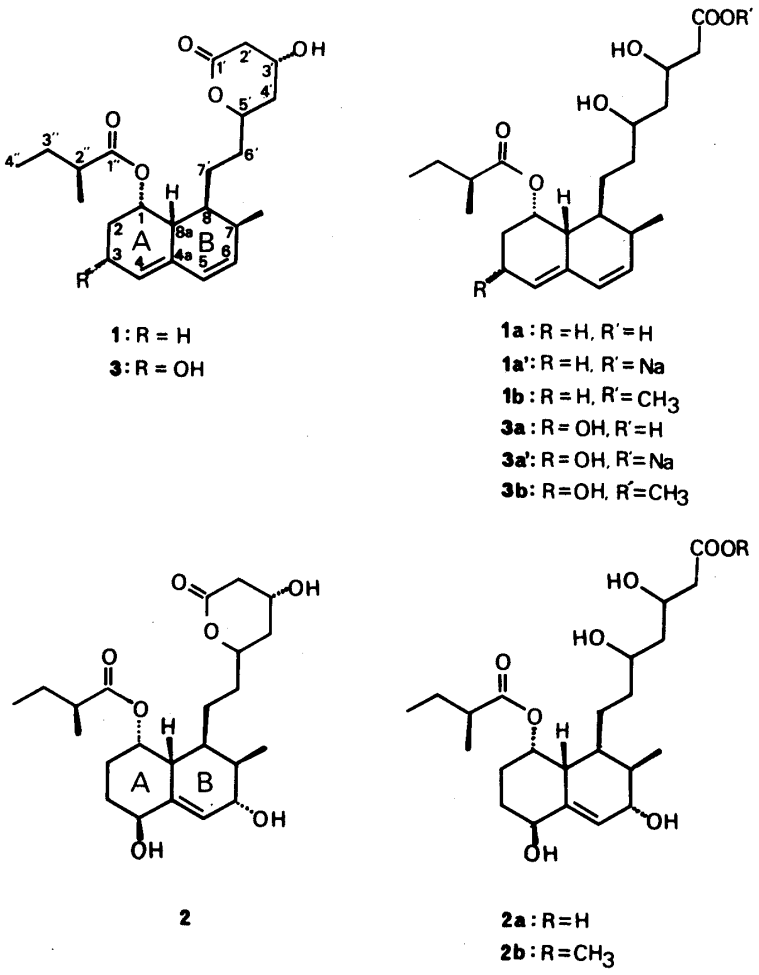

Fig. 1

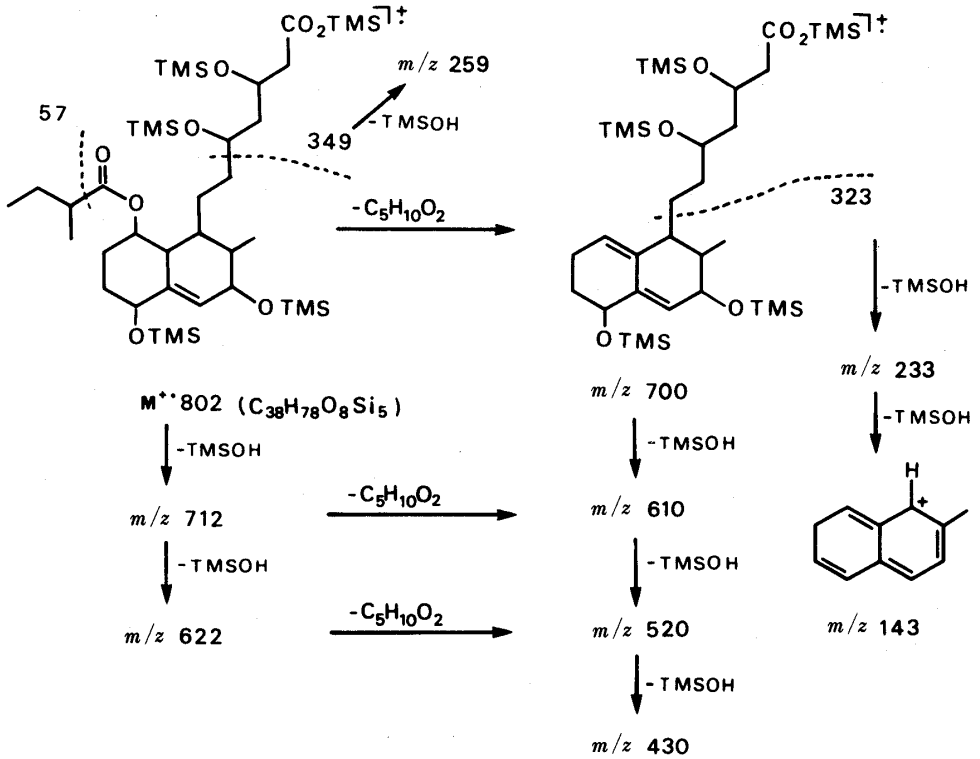

Fig. 2. Mass Spectral Fragmentation Modes of the TMS Derivative of **2a**

TABLE I. ^{1}H-NMR Data for **1a′**, **1**, **2a**, **2**, **3a′** and **3b**

	1a′[a]	**1**[b]	**2a**[a]	**2**[a]	**3a′**[a]	**3b**[b]
H_1	5.30 (br)	5.33 (t, 4.0)	5.26 (br)	5.28 (br)	5.36 (ddd, 4.5, 3, 1.5)	5.42 (ddd, 4.5, 3, 1.5)
H_{2a}	—	1.6—1.7 (m)	—	2.08 (tdd, 14.0, 4.5, 2.3)	1.1—1.7 (m)	1.1—1.7 (m)
H_{2b}	—	1.6—1.7 (m)	—	1.7—1.8 (m)	2.49 (dddd, 13.0, 6.5, 4.5, 1.7)	2.58 (dddd, 14.0, 6.5, 4.5, 1.5)
H_{3a}	—	2.1—2.4	—	1.7—1.8	—	—
H_{3b}	—	2.1—2.4 (m)	—	1.83 (tdd, 14.0, 4.0, 3.3)	4.30 (dddd, 10.0, 6.5, 3, 2)	4.40 (ddt, 10.0, 6.5, 2.5)
H_4	5.51 (br)	5.56 (dd, 6.0, 4.0)	4.21 (br)	4.21 (t, 2.5)	5.50 (dd, 3.0, 2.0)	5.56 (dd, 3.2, 1.5)
H_5	5.95 (d, 10.0)	5.98 (d, 9.3)	5.77 (dd, 5.0, 2.0)	5.77 (dd, 5.0, 2.0)	5.98 (d, 9.8)	5.99 (d, 9.8)
H_6	5.74 (dd, 10.0, 6.0)	5.74 (dd, 9.3, 6.0)	3.84 (dd, 5.0, 4.5)	3.84 (dd, 5.0, 3.4)	5.89 (dd, 9.3, 5.9)	5.90 (dd, 9.8, 5.6)
H_7	—	2.38 (m)	1.86 (m)	1.92 (m)	2.3—2.4 (m)	2.3—2.4 (m)
H_{8a}	—	2.14 (d, 9.0)	2.33 (m)	2.35 (d, 7.4)	2.3—2.4 (m)	2.3—2.4 (m)
7-Me	0.90 (d, 7.0)	0.90 (d, 7.5)	0.84 (d, 7.0)	0.84 (d, 6.8)	0.91 (d, 7.3)	0.89 (d, 6.5)
$H_{2'a}$	—	2.62 (ddd, 17.7, 4.0, 1.6)	2.43	2.53 (ddd, 17.7, 3.5, 1.6)	2.26 (dd, 15.5, 7.8)	2.49 (d, 6.4)
$H_{2'b}$	—	2.73 (dd, 17.5, 4.9)	2.43	2.72 (dd, 17.7, 4.6)	2.34 (dd, 15.5, 4.8)	2.49 (d, 6.4)
$H_{3'}$	4.07 (tt, 8.0, 6.0)	4.36 (quin, 4.0)	4.09 (m)	4.25 (quin, 4.3)	4.07 (m)	4.25 (tt, 7.5, 6.0)
$H_{5'}$	3.70 (m)	4.63 (m)	3.74 (m)	4.63 (m)	3.69 (m)	3.78 (m)
$H_{2''}$	2.27 (sex, 7.0)	2.37 (sex, 6.9)	2.33 (sex, 7.0)	2.39 (sex, 7.0)	2.35 (sex, 7.0)	2.34 (sex, 7.0)
2″-Me	1.12 (d, 7.0)	1.12 (d, 8.0)	1.12 (d, 7.0)	1.12 (d, 7.8)	1.11 (d, 7.3)	1.11 (d, 6.8)
3″-Me	0.91 (t, 7.0)	0.89 (t, 7.5)	0.89 (t, 7.0)	0.89 (t, 7.3)	0.91 (t, 7.5)	0.88 (t, 7.5)

a) Measured in CD_3OD. *b*) Measured in $CDCl_3$. Chemical shifts are given relative to internal TMS as a reference. Coupling patterns and coupling constants (Hz) are shown in parentheses. Abbreviations: s, singlet; d, doublet; dd, double doublet; ddd, double double doublet; dddd, double double double doublet; t, triplet; tdd, triple double doublet; ddt, double double triplet; tt, triple triplet; m, multiplet; sex, sextet; quin, quintet; br, broad.

peaks suggest the presence of a 3,5-dihydroxyheptanoic acid side chain and 2-methylbutanoyl group, as depicted in Fig. 2: *m/z* 802 ($M^{+\cdot}$), 787 ($M-CH_3$), 712 (M−TMSOH), 700 ($M-C_5H_{10}O_2$), 610 ($M-C_5H_{10}O_2-$TMSOH), 520 ($M-C_5H_{10}O_2-2\cdot$TMSOH), 430 ($M-C_5H_{10}O_2-3\cdot$TMSOH), 259 ($C_{11}H_{23}O_3Si_2$), and 57 (C_4H_9).

The above observations were supported by comparison of the proton nuclear magnetic resonance (^{1}H-NMR) signals of M3 with those of ML-236B. The methyl triplet at δ 0.89 ($J=$ 7 Hz), methyl doublet at δ 1.12 ($J=7$ Hz), and methine sextet at δ 2.33, which is coupled to the latter methyl doublet, are coincident with the signals of **1a′** assigned to 3″-Me, 2″-Me, and

$H_{2'}$, within $\Delta\delta$ 0.05 ppm (Table I). The two key signals of the heptanoic acid side chain which appear at δ 4.07 ($H_{3'}$) and 3.74 ($H_{5'}$) in **1a'** were also observed at δ 4.09 and 3.74 in **2a**, while these signals were shifted to δ 4.25 and 4.63, respectively, in **2**. A similar spectral change was observed when ML-236B was converted from the acid form (**1a**) to the lactone form (**1**). The lactonization is also confirmed by the large geminal coupling constant (17.7 Hz) between $H_{2'a}$ and $H_{2'b}$, characteristic of a position adjacent to a carbonyl group. This is consistent with two hydroxyl groups being introduced on a decalin moiety.

The disappearance of the typical ultraviolet (UV) absorption of a transoid conjugated diene system in **2** and **2a** indicates that the hydroxylation was accompanied by reduction of the diene system.

In the ^{1}H-NMR spectrum of **2** (and **2a**), the two oxygen-bearing methine protons appearing at δ 4.21 and 3.84 could be considered as the sites of hydroxylation. These signals could be assigned on the basis of extensive decoupling experiments combined with nuclear Overhauser effect (NOE) measurements.

One of the oxygen-bearing protons at δ 3.84 was found to be coupled to the olefinic proton at δ 5.77 and the methine multiplet at δ 1.92 with J values of 5.0 and 3.4 Hz, respectively. As irradiation of the methyl doublet at δ 0.84 (7-Me) changed the multiplet at δ 1.92 to a triplet, the signals at δ 1.92, 3.84, 5.77 were assigned to H_7, H_6 and H_5, respectively. Thus, one of the hydroxyl groups could be located at C(6). An allylic coupling between H_5 and H_{8a} allowed the assignment of the double bond between C(4a) and C(5).

The configuration of the hydroxyl group at C(6) could be assigned as α from NOE observed at H_6 on irradiation of 7-Me (10%).[7b]

Based on the assignment of the methine signal at δ 5.28 to H_1, the following assignments could be established. The H_1 signal (δ 5.28) is coupled to nonequivalent methylene signals at δ 2.08 and *ca.* 1.7—1.8, and H_{2a} at δ 2.08 is coupled to one of the other nonequivalent methylene signals at δ 1.83 (H_{3b}) with J = 14 Hz. This large coupling constant requires an antiperiplanar arrangement between H_{2a} and H_{3b}, and relatively small coupling constants between H_1 and vicinal protons, H_{8a}, H_{2a} and H_{2b} suggest H_1 is in gauche relation to them. This indicates that ring A takes a chair conformation. The H_{3b} signal is also coupled to the oxygen-bearing proton at δ 4.21 with J = 3.3 Hz, leading to the assignment of another

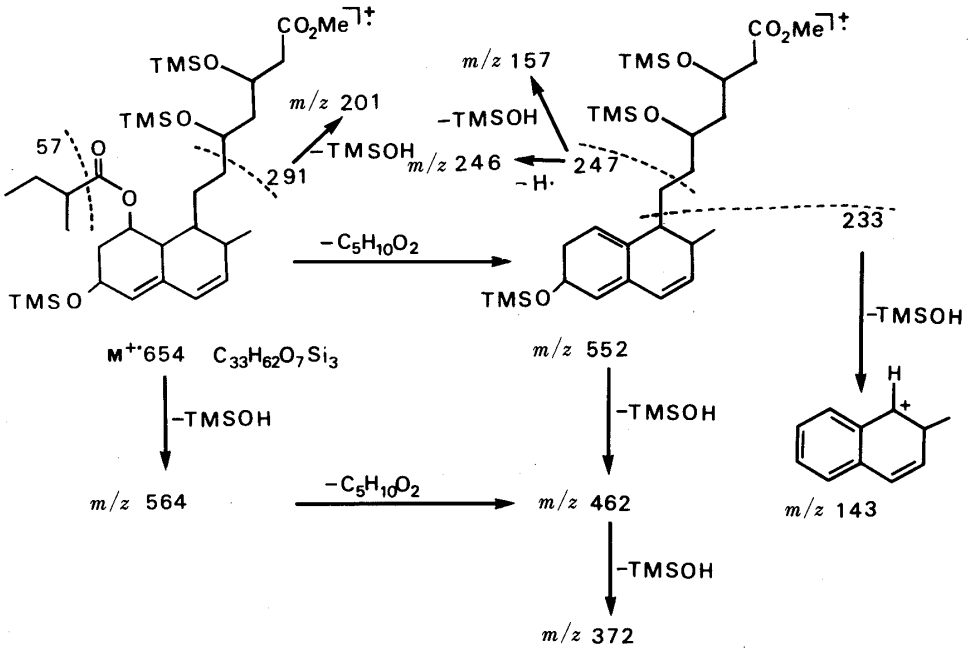

Fig. 3. Mass Spectral Fragmentation Modes of the TMS Derivative of **3b**

hydroxyl group at C(4). This assignment could be supported by the observation of a large NOE at H_5 (18%) on irradiation of H_4 (δ 4.21). H_4 is in a gauche relation to H_{3a} and H_{3b}, because the H_4 signal appears as a broad triplet with small coupling constants (J=2.5 Hz). Taking into account the chair conformation of ring A, this leads to H_4 being in an equatorial position. The hydroxyl group at C(4), therefore, could be assigned as β-axial.

Based on the absolute configuration of ML-236B,[7a] these new chiral centers of M3 have been established as 4S and 6S.[7b]

3β-Hydroxy ML-236B (M4)

Dog urine was applied to an Amberlite XAD-2 column, and eluted with 50% aqueous acetone. The crude metabolite was then extracted with ethyl acetate and the pH was adjusted to 3 with trifluoroacetic acid. The metabolite was further purified after methylation with diazomethane. The sodium salt of M4 was obtained by hydrolysis of the methyl ester with 0.1 N NaOH.

The highest ion peak of trimethylsilylated **3b** was m/z 654, while that of the deuterotrimethylsilylated **3b** was m/z 681. This difference of 27 amu can be explained by the incorporation of three TMS groups into **3b**. Thus, the molecular formula of trimethylsilylated **3b** was determined as $C_{33}H_{62}O_7Si_3$, and the metabolite could be considered as monohydroxylated ML-236B. The fragmentation mode indicated the presence of a 3,5-dihydroxyheptanoic acid side chain and a 2-methylbutanoic acid group (Fig. 3).

The above conclusions were supported by the close similarity between the ^{1}H-NMR spectra of the sodium salts of **3a** and **1a** with the exception of an oxygen-bearing methine signal appearing at δ 4.30 (at δ 4.40 for **3b**). This observation strongly suggests that the hydroxylation occurred on a decalin moiety, while the UV absorption spectrum with λ_{max} at 230, 237 and 245 nm (in methanol) indicated the retention of the transoid diene system.

The site of hydroxylation was readily assigned on the basis of extensive decoupling experiments on **3b** (Table I). An oxymethine proton assigned to H_1 at δ 5.42 is coupled to H_{2a} (δ *ca.* 1.60), H_{2b} (δ 2.58), and H_{8a} (δ *ca.* 2.35) with J=1.5, 4.5, and 3 Hz, respectively. These small coupling constants indicate that H_1 is in a gauche relation with H_{2a}, H_{2b} and H_{8a}. The allylic oxymethine proton at δ 4.40 was assigned to H_3, because it is coupled to H_{2a}, H_{2b} and H_4 (δ 5.56) with J=10, 6.5, and 2—3 Hz, respectively. The *trans* and *cis* relation of H_3 with H_{2a} and H_{2b} was assigned from their coupling constants. The small coupling constant between

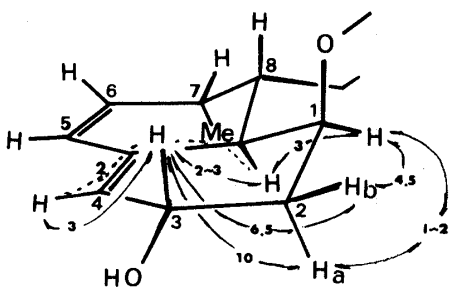

Fig. 4. Proton Spin–Spin Coupling Constants for the Bis-dehydrodecalin Ring of **3b** and Its Conformation

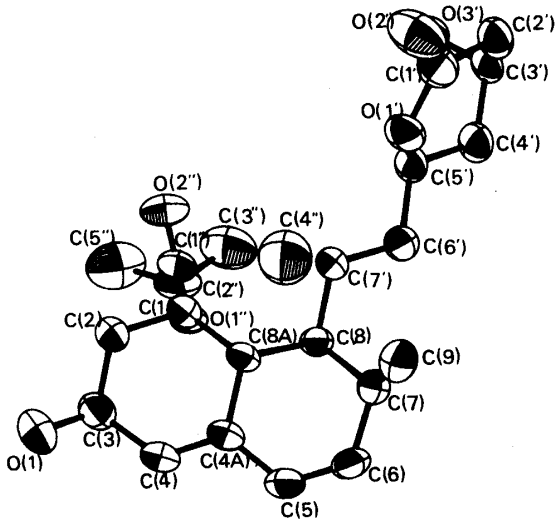

Fig. 5. An ORTEP Drawing of **3**

TABLE II. Final Coordinates ($\times 10^4$) and Equivalent Thermal Parameters for Non-hydrogen Atoms of **3**, with e.s.d.'s in Parenthese

	x	y	z	B_{eq}
C(1)	7848 (6)	4596 (2)	8261 (6)	3.4 (0.2)
C(2)	9134 (6)	4945 (2)	8590 (6)	3.8 (0.2)
C(3)	9395 (6)	5410 (2)	7573 (7)	4.3 (0.2)
C(4)	8021 (6)	5665 (2)	7124 (6)	3.7 (0.2)
C(4A)	6712 (6)	5460 (2)	7348 (5)	3.3 (0.2)
C(5)	5421 (6)	5743 (2)	6896 (6)	4.1 (0.2)
C(6)	4118 (6)	5552 (2)	7114 (6)	4.3 (0.2)
C(7)	3812 (6)	5009 (2)	7804 (6)	3.9 (0.2)
C(8)	5129 (6)	4629 (2)	7711 (6)	3.2 (0.2)
C(8A)	6480 (6)	4941 (2)	8182 (5)	3.0 (0.1)
C(9)	3270 (7)	5110 (2)	9288 (6)	4.7 (0.2)
C(1′)	3077 (7)	3083 (2)	11447 (7)	4.7 (0.2)
C(2′)	2044 (7)	2642 (2)	11117 (7)	4.6 (0.2)
C(3′)	2203 (6)	2406 (2)	9696 (7)	4.0 (0.2)
C(4′)	2299 (6)	2866 (2)	8678 (7)	4.5 (0.2)
C(5′)	3566 (6)	3226 (2)	8991 (6)	3.9 (0.2)
C(6′)	3615 (6)	3762 (2)	8190 (6)	4.2 (0.2)
C(7′)	4945 (6)	4102 (2)	8534 (6)	3.7 (0.2)
C(1″)	8561 (7)	3818 (2)	6889 (6)	4.2 (0.2)
C(2″)	8842 (8)	3614 (2)	5442 (6)	5.3 (0.2)
C(3″)	7911 (11)	3113 (3)	5131 (9)	9.3 (0.3)
C(4″)	6526 (0)	3194 (0)	5240 (0)	11.0 (0.0)
C(5″)	10408 (11)	3457 (3)	5307 (9)	9.5 (0.3)
O(1)	10259 (5)	5797 (2)	8348 (5)	6.8 (0.2)
O(1′)	3636 (4)	3387 (1)	10425 (4)	4.5 (0.1)
O(2′)	3456 (6)	3191 (2)	12585 (5)	6.8 (0.2)
O(3′)	3504 (5)	2091 (1)	9586 (4)	4.9 (0.1)
O(1″)	8063 (4)	4332 (1)	6904 (4)	3.6 (0.1)
O(2″)	8806 (5)	3557 (1)	7913 (4)	5.6 (0.1)

$B_{eq} = 4/3 \cdot \sum_i \sum_j \beta_{ij} \boldsymbol{a}_i \cdot \boldsymbol{a}_j$.

H_3 and H_4 indicated that these atoms are at a maximum inclination. Allylic long-range coupling between H_4 and H_{8a} ($J = 1.5$ Hz), and homoallylic long-range coupling between H_3 and H_{8a} ($J = 2.5$ Hz) indicate that one of the conjugated double bonds should be located between C(4) and C(4a). Thus, the other double bond should be located between C(5) and C(6), which is consistent with the coupling pattern of H_7 (δ *ca.* 2.36), H_6 (δ 5.90) and H_5 (δ 5.99).

The coupling pattern described above allowed us to assign the conformation of the decalin ring system as depicted in Fig. 4; H_1 and H_{2b} are in quasi-equatorial and H_{2a}, H_3 and H_{8a} are in quasi-axial positions. Thus, the newly introduced hydroxyl group at C(3) is assigned quasi-equatorial (β-)orientation. A "W" type long-range coupling between H_4 and H_{2b} suggests that the A ring has a half-chair conformation.

To confirm the structure indicated by the above spectroscopic study, X-ray analysis of the lactone form of M4 (**3**), obtained by microbial conversion of ML-236B,[8] was carried out. The structure was solved by the direct method with MULTAN-78. All the non-hydrogen atoms except one were automatically detected on an *E*-map in the first trial. The missing atom was found by a successive Fourier synthesis. The hydrogen atoms were all discernible on a difference Fourier map after several cycles of isotropic refinement. In the course of anisotropic refinement for all non-hydrogen atoms, temperature factors of the terminal butyrate group

TABLE III. Bond Lengths and e.s.d.'s for Non-hydrogen Atoms of **3** (Å)

C(1)–C(2)	1.506 (8)	C(1′)–O(1′)	1.347 (7)
C(1)–C(8A)	1.532 (7)	C(1′)–O(2′)	1.193 (8)
C(1)–O(1″)	1.483 (6)	C(2′)–C(3′)	1.505 (9)
C(2)–C(3)	1.525 (8)	C(3′)–C(4′)	1.500 (8)
C(3)–C(4)	1.492 (8)	C(3′)–O(3′)	1.442 (7)
C(3)–O(1)	1.453 (7)	C(4′)–C(5′)	1.504 (8)
C(4)–C(4A)	1.339 (8)	C(5′)–C(6′)	1.525 (8)
C(4A)–C(5)	1.458 (8)	C(5′)–O(1′)	1.452 (7)
C(4A)–C(8A)	1.519 (7)	C(6′)–C(7′)	1.529 (8)
C(5)–C(6)	1.320 (8)	C(1″)–C(2″)	1.516 (8)
C(6)–C(7)	1.514 (8)	C(1″)–O(1″)	1.339 (6)
C(7)–C(8)	1.543 (8)	C(1″)–O(2″)	1.204 (7)
C(7)–C(9)	1.550 (8)	C(2″)–C(3″)	1.529 (10)
C(8)–C(8A)	1.544 (8)	C(2″)–C(5″)	1.517 (12)
C(8)–C(7′)	1.525 (7)	C(3″)–C(4″)	1.313 (10)
C(1′)–C(2′)	1.480 (8)		

TABLE IV. Bond Angles and e.s.d.'s for Non-hydrogen Atoms of **3** (°)

C(2)–C(1)–C(8A)	111.4 (2)	C(2′)–C(1′)–O(1′)	119.5 (6)
C(2)–C(1)–O(1″)	109.1 (4)	C(2′)–C(1′)–O(2′)	123.8 (6)
C(8A)–C(1)–O(1″)	107.8 (4)	O(1′)–C(1′)–O(2′)	116.7 (6)
C(1)–C(2)–C(3)	114.2 (5)	C(1′)–C(2′)–C(3′)	114.4 (5)
C(2)–C(3)–O(1)	103.6 (4)	C(2′)–C(3′)–O(3′)	110.8 (5)
C(2)–C(3)–C(4)	111.3 (4)	C(2′)–C(3′)–C(4′)	109.1 (2)
C(4)–C(3)–O(1)	110.9 (2)	C(4′)–C(3′)–O(3′)	107.4 (5)
C(3)–C(4)–C(4A)	125.5 (4)	C(3′)–C(4′)–C(5′)	110.4 (5)
C(4)–C(4A)–C(5)	121.9 (3)	C(4′)–C(5′)–O(1′)	112.8 (5)
C(4)–C(4A)–C(8A)	121.9 (5)	C(4′)–C(5′)–C(6′)	114.9 (5)
C(5)–C(4A)–C(8A)	116.0 (5)	C(6′)–C(5′)–O(1′)	104.9 (2)
C(4A)–C(5)–C(6)	123.2 (4)	C(5′)–C(6′)–C(7′)	112.1 (4)
C(5)–C(6)–C(7)	123.7 (5)	C(8)–C(7′)–C(6′)	115.6 (4)
C(6)–C(7)–C(8)	110.5 (4)	C(2″)–C(1″)–O(1″)	112.3 (5)
C(6)–C(7)–C(9)	109.6 (2)	C(2″)–C(1″)–O(2″)	124.2 (4)
C(8)–C(7)–C(9)	114.2 (4)	O(1″)–C(1″)–O(2″)	123.5 (7)
C(7)–C(8)–C(7′)	112.7 (4)	C(1″)–C(2″)–C(5″)	109.3 (5)
C(7)–C(8)–C(8A)	109.7 (2)	C(1″)–C(2″)–C(3″)	110.4 (5)
C(8A)–C(8)–C(7′)	110.7 (4)	C(3″)–C(2″)–C(5″)	109.2 (3)
C(1)–C(8A)–C(8)	115.1 (2)	C(2″)–C(3″)–C(4″)	115.2 (4)
C(4A)–C(8A)–C(8)	111.7 (4)	C(1′)–O(1′)–C(5′)	122.9 (3)
C(1)–C(8A)–C(4A)	111.5 (4)	C(1)–O(1″)–C(1″)	117.6 (3)

became rather large due to the thermal motion or disordering of these atoms. All attempts to obtain more accurate positions for these atoms failed. Thus the terminal methyl group of the 2-methyl butanoyl substituent was fixed at the final anisotropic refinement cycles and a discrepancy factor, R, of 0.064 was reached with a weighting scheme of $w=1/\sigma^2(F_o)$.

An ORTEP drawing of **3** is shown in Fig. 5, where the newly emerged chiral center at C(3) was assigned as S based on the absolute configuration of **1a**. Bond lengths and bond angles are within the normal ranges, except for C(4″)–C(3″), 1.313 Å, of the butyrate moiety, because of the factors discussed above. The atomic coordinates, bond lengths and angles are given in Tables II, III, and IV.

Both six-membered rings of the bis-dehydrodecalin ring take half-chair conformations. C(1) and C(2) are 0.44 (3) and −0.23 (3) Å from the least-squares plane through C(3), C(4),

C(4a) and C(8a), while C(8), C(8a) are 0.57 (3) and −0.14 (3) Å from the least-squares plane through C(4a), C(5), C(6), and C(7). The δ-lactone ring has an envelope conformation with a flap at C(4′).

Experimental

Administration of ML-236B——Male adult beagle dogs, weighing about 10 kg, were used. Three beagles were orally given 200 mg/kg of ML-236B and urine was collected over 24 h.

Isolation of 4β,6α-Dihydroxy ML-236B in Acid Form (2a)——The dog urine (1.5 l) was applied to an Amberlite XAD-2 column (*ca.* 2 l of resin). The methanol eluate was evaporated and the residue was dissolved in 10 ml of water. This solution was applied to a Sephadex G-10 column (70 cm × 2.5 cm diameter) and eluted with water. The fraction containing the crude metabolite was lyophilized, dissolved in 5 ml of water and rechromatographed on another Sephadex G-10 column (100 cm × 1 cm diameter) with water as the eluant. For further purification, the eluate was lyophilized, and the product was dissolved in 2 ml of 25% aqueous methanol, then applied to a Lobar column RP-8 (Merck). The fraction eluted with 25% aqueous methanol was applied to a silica gel thin layer chromatography (TLC) plate (Art. 5715, Merck) using a solvent system of benzene–acetone–acetic acid (50 : 50 : 3). The band containing the metabolite was scraped from the plate, and extracted with methanol. This extract was chromatographed on a Sephadex G-10 column (40 cm × 1 cm diameter) with water as the eluant and the metabolite was obtained by lyophilization in the acid form (*ca.* 1 mg, amorphous powder).

Isolation of 4β,6α-Dihydroxy ML-236B (2)——After adjusting the pH of the urine (0.5 l) to 3 with HCl, the metabolite was extracted with ethyl acetate (1.5 l). The concentrated organic fraction (200 ml) was incubated for 15 h at 37 °C with *p*-toluenesulfonic acid (10 mg) to lactonize the metabolite. After being washed with 2% sodium bicarbonate, the concentrated solution was applied to a silica gel column C-200 (20 cm × 1 cm diameter) and eluted with a mixture of benzene and acetone (3 : 1). The eluate was rechromatographed on a Lobar column, RP-8 size A (Merck), with 23% aqueous acetonitrile as the eluant, and lyophilized to give M3 in the lactone form (8 mg). Amorphous powder, IR ν_{max}^{KBr} cm^{-1}: 3400, 1720, 1710.

Isolation of 3β-Hydroxy ML-236B Methylester (3b)——Dog urine (3 l) was applied to an Amberlite XAD-2 column (0.5 l of resin). The pH of the 50% aqueous acetone fraction (500 ml) was adjusted to 3 with trifluoroacetic acid, and the crude metabolite was extracted with ethyl acetate (1 l). This organic fraction containing the metabolite was washed with saturated NaCl, treated with CH_2N_2 in ethyl acetate at 0 °C and evaporated *in vacuo*. The residue was dissolved in 55% aqueous methanol and applied to a Lobar column, RP-8 size B (Merck). The eluate with 60% aqueous methanol was further rechromatographed on a μ-Bondapak C_{18} (Waters) column using 65% aqueous methanol as the eluant to give **3b** (4 mg). Amorphous powder, IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3400, 2950, 1730, 1600. UV λ_{max}^{EtOH} nm: 230, 237, 246.

Hydrolysis of 3b——The methylester of M4 (2 mg) was hydrolyzed with 0.1 N NaOH. After neutralization with 0.1 N HCl, the reaction mixture was applied to an Amberlite XAD-2 column (5 ml of resin) and eluted with 50% aqueous acetone. M4 was obtained as the sodium salt (0.78 mg). Amorphous powder, IR ν_{max}^{KBr} cm^{-1}: 3400, 2900, 1725, 1580. UV λ_{max}^{MeOH} nm: 230, 237, 245.

IR, UV——IR spectra were measured with a JASCO A-302 spectrometer and UV spectra with a Cary 17D spectrometer.

MS——Electron impact (70 eV) mass spectra were obtained using a JEOL JMS-D300 mass spectrometer. Trimethylsilyl derivatives were prepared by treating the target compound with at least 50-fold excess of a 20 : 50 : 10 : 1 mixture of BSTFA: acetonitrile : pyridine : TMCS at 50 °C for 5 min. The methyl ester derivative was prepared by treating the target compounds with an excess of diazomethane in diethylether at ambient temperature.

N,O-Bis-(trimethylsilyl)trifluoloacetamide plus 1% trimethylsilylchlorosilane (BSTFA/TMCS) and *N,O*-bis-perdeuterotrimethylsilylacetamide (BSA-d_{18}) were purchased from Pierce Chemical Co. (Rockford, Illinois, U.S.A.).

NMR——^{1}H-NMR spectra were recorded in the field-swept mode on a Varian HA-100 spectrometer using an internal homonuclear-locked mode and/or in the Fourier-transform mode on JEOL FX-200 and FX-400 spectrometers using a deuterium-locked mode. The NOE measurements were carried out in the difference spectra mode. Chloroform-*d* and methanol-d_4 were purchased from Merck Sharp and Dohme Ltd.

X-Ray Analaysis——A colorless plate crystal of 3β-hydroxy ML-236B (**3**), mp 138—142 °C, $[\alpha]_D^{22} = +194.0°$ ($c = 0.51$, MeOH), was used for the following analysis. The physicochemical properties of **3** have been reported in the literature.[8]

Crystallographic parameters and intensity data were recorded on a Rigaku four-circle diffractometer (AFC-5/RU-200). Cell constants were; $a = 9.338$ (1), $b = 24.399$ (2), $c = 9.735$ (1) Å, $D_m = 1.22$ g/cm^3, $D_c = 1.216$ g/cm^3, $Z = 4$. The space group belongs to $P2_12_12_1$, orthorhombic. All intensity measurements were made in the w–2θ scan mode up to $2\theta = 128°$ with monochromated Cu-K_α radiation. The reflections with intensities greater than $3\sigma(I_0)$ (1804 reflections) were used for the structure analysis after correction for Lorentz-polarization effects (but not for absorption).

References and Notes

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