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ANTIBACTERIAL SESQUITERPENE ARYL ESTERS FROM *ARMILLARIA MELLEA*

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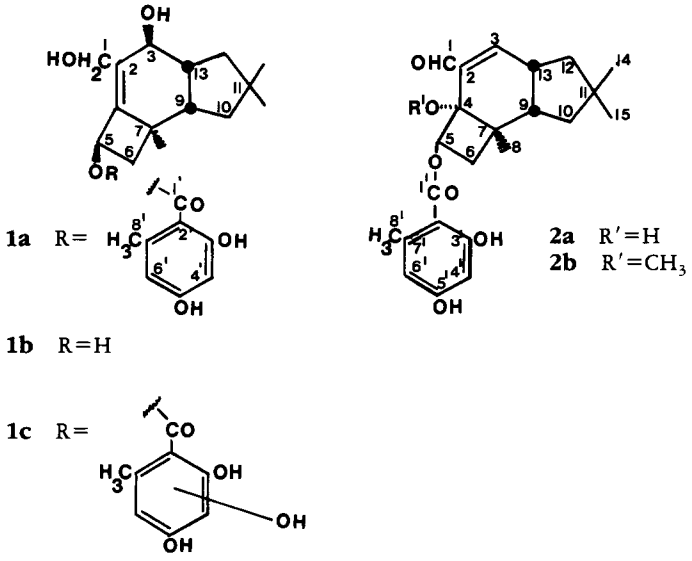
ABSTRACT.—Investigation of the mycelial extract of *Armillaria mellea* led to the isolation of the known melleolide (**2a**) and two new sesquiterpene aryl eters, 4-*O*-methylmelleolide (**2b**) and judeol (**1c**). Their structures were deduced from spectral data and that of (**2b**) confirmed by X-ray analysis. The new esters (**1c**) and (**2b**) showed strong antibacterial activity against gram-positive bacteria.

The basidiomycete *Armillaria mellea* (Vahl ex Fr) Kummer, known as the Honey or Bootlace mushroom, was reported to produce in culture compounds having antibacterial and antifungal activities (1). Our initial investigations (2) on this fungus indicated the presence of a group of aryl esters of sesquiterpenes and led to the isolation and structural elucidation of the orsellinate of armillol (**1a**), which was the first naturally occurring sesquiterpenoid orsellinate. Armillol (**1b**), as well as the metabolites of *Clitocybe illudens* (illudol and neoilludol (3-5) and those of *Fomitopsis insularis* (Δ^6-protoilludene and Δ^7-protoilluden-6-ol) (6), belong to the protoilludane family of sesquiterpenes. A second orsellinate ester, melleolide, was isolated subsequently (7) from cultures of *A. mellea*, and the structure (**2a**) was assigned, based essentially on an X-ray analysis.

Further investigation of the mycelial extract of *A. mellea* has now led to the isolation of additional sesquiterpenoid aryl esters. The extract was fractionated by Sephadex LH-20 and flash chromatography (Kieselgel). After separation of the major component, the orsellinate of armillol (**1a**), a less polar fraction was obtained, which proved to be a mixture of two compounds. Repeated chromatography of this mixture yielded the pure compounds A and B.

Compound A, mp 198-200°, had a molecular formula $C_{23}H_{28}O_6$, which was supported by cims with $|MH|^+$ at *m/z* 401 and a characteristic fragmentation ion at *m/z* 233 due to a loss of orsellinic acid from the $|MH|^+$ ion. Eims showed the base peak at *m/z* 151 corresponding to the ion $|C_6H_2(OH)_2MeCO|^+$. A 400 MHz ^{1}H-nmr spectrum revealed singlets due to an aldehyde (δ 9.42) and three aliphatic and one aromatic methyl groups (δ 0.99, 1.03, 1.32, and 2.28). Extensive decoupling experiments allowed the assignments of the other protons. A comparison of these nmr data and those reported for melleolide (**2a**) (7) suggested that the compounds were identical. An X-ray analysis of compound A established unequivocally that it has structure **2a**.

Compound B, less polar than melleolide (**2a**), had mp 189-191°. The molecular formula $C_{24}H_{30}O_6$ (M^+ 414.2048) was deduced from cims, which showed the $|MH|^+$ ion peak at *m/z* 415 and the characteristic fragmentation ion at *m/z* 247 due to loss of orsellinic acid from the |MH| ion. Eims of compound B, like that of **1a** and **2a** revealed the base peak at *m/z* 151 due to fragment ion $|C_6H_2(OH)_2MeCO|^+$. As the molecular weight of compound B was found to be 14 amu higher than that of **2a**, the presence of a methoxyl instead of the hydroxyl group on the sesquiterpenoid moiety was suspected. In agreement, the cims of compound B had an ion peak at *m/z* 383 due probably to loss of MeOH from the $|MH|^+$ ion, and furthermore the 400 MHz ^{1}H-nmr spectrum



showed a signal for a methoxyl group at δ 3.22. This spectrum displayed resonances for an aldehyde group, three aliphatic and one aromatic methyl groups, and double resonance studies led to the assignment of the remaining protons. The ^{1}H-nmr spectrum of compound B, presented in Figure 1 and in the Experimental, and that of melleolide (**2a**) showed near identity of the chemical shifts of most of the protons. The structure 4-*O*-methylleolide (**2b**) for compound B was further supported by interpretation of its ^{13}C-nmr spectrum and by comparison with that of melleolide (**2a**). The former spectrum displayed an additional signal due to the methoxy group (δ_c 53.9).

X-RAY ANALYSIS OF 4-*O*-METHYLMELLEOLIDE (**2b**).—The molecular structure of **2b** has been confirmed by single crystal X-ray diffraction analysis. Compound **2b** crystallizes as tiny, well-defined plates (size 0.3×0.2×0.02 mm) from a mixture of H_2O and MeOH (over a 2 week period) to give a solvate mp 78-79°. (After resolidification, the mp raised to 180-185°.) Upon standing in air, the crystals are destroyed within a minute because of solvent loss from the packing. A wet crystal was therefore sealed in a small glass capillary and mounted on a Four-Circle automatic diffractometer, equipped with a graphite monochromator and operating with CuKα radiation.

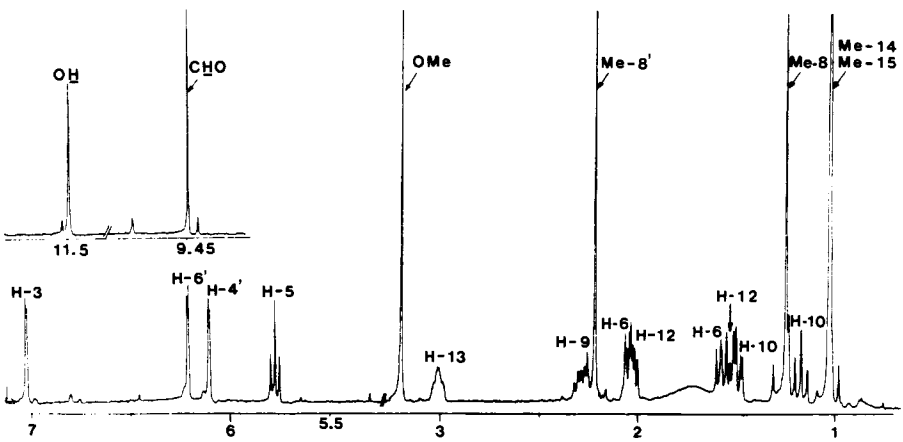


FIGURE 1. 400 MHz ^{1}H-nmr spectrum of 4-*O*-methylmelleolide (**2b**).

The system is triclinic, space group P1, Z=2 corresponding to ≃ 70 non-hydrogen atoms in the asymmetric unit (two molecules and two to four molecules of solvent). The cell parameters are: a=16.252 (**4**); b=9.588 (**3**); c=9.315 (**3**) Å; α=117.1 (**2**)°; β=85.16 (**8**)°; and γ=102.0 (**1**)°. Of a total of 3450 scanned intensities, only 2151 with $I \geq 2\sigma$ (I) were considered as observed. The structure has been solved by direct methods (8) with some difficulties, which are inherent to the space group. The correct structure has been found in the E-map corresponding to the highest figure of merit only after lowering the number of Σ2 relationships developed in the multisolution by increasing the threshold of their consistencies. A fragment of 25 atoms with plausible bond distances and angles was the starting point of Fourier recycling procedures to give the complete molecular structure. The isotropic refinements converged to a $R=\Sigma \| F_o| - | F_c| | / \Sigma |F_o| = 12\%$. At this stage, an absorption correction according to the method described by Walker *et al.* (9) was applied. Using anisotropic thermal factors and theoretically positioned hydrogens, the final R was 8.1% with two well defined molecules of MeOH and a third disordered one. The view given in Figure 2 shows only one of the two molecules of the asymmetric unit.

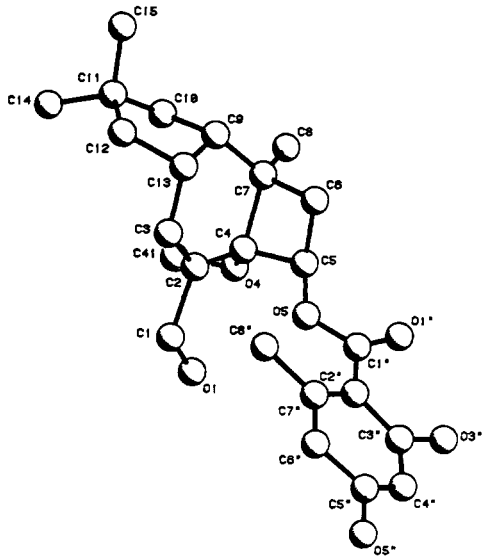


FIGURE 2. Molecular structure of 4-*O*-methylmelleolide (**2b**).

The two molecules of the asymmetric unit (Figure 3) are not equivalent. Whereas the cyclobutane rings adopt similar folded conformations [puckering angle around C(6)-C(5)=20.5° and 17.6° in molecules 1 and 2, respectively], their cyclopentane ring conformations are quite different: In molecule 1, the pseudo-rotation phase (10) is ρ=5.8° with a maximum puckering angle θ_m=37° indicating a twist conformation whereas in molecule 2, these parameters are ρ=18.4° and θ_m=42.2°, which are relevant of a pure envelope conformation.

The aldehyde function is found highly agitated in molecule 1 so that the corresponding bond distances and angles are poorly defined. A (F_o-F_c) Fourier map calculated at the end of the refinements shows many spurious peaks (<0.7 e $Å^{-3}$) around the CHO group of molecule 1; they have not been analyzed. The three molecules of MeOH located in the packing are involved in a rather limited H-bond network (Figure 3).

The atomic coordinates as well as the recalculated mean isotropic thermal factors are given in Table 1. The bond lengths, valency angles and coordinates of the calculated hydrogen atoms are deposited as a supplementary material.

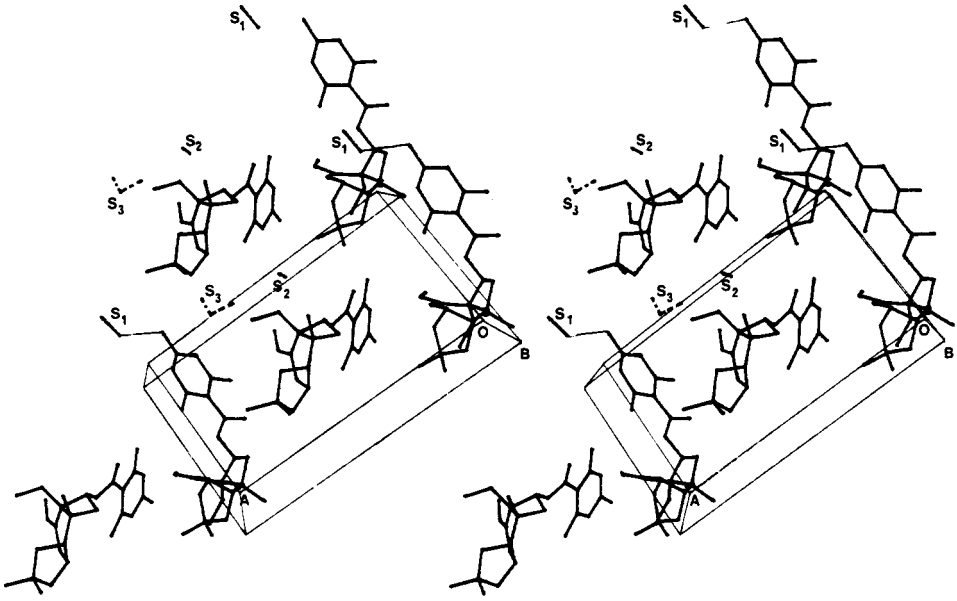


FIGURE 3. Stereo diagram of the packing of (**2b**) showing the content of three different cells. The molecules of MeOH are labelled S_1 to S_3. The carbon atom of S_3 is statistically distributed between two different positions.

Analysis of fractions more polar than melleolide (**2a**) and its 4-*O*-methyl ether (**2b**) indicated the presence of a complex mixture of compounds. An amorphous minor product (chromatographically homogeneous), for which the name judeol is proposed, was isolated. The cims of judeol showed a $|MH|^+$ ion at *m/z* 419 corresponding to the molecular formula $C_{23}H_{30}O_7$ and displayed a fragmentation ion at *m/z* 217 due to loss of a mole of hydroxyorsellinic acid and of a mole of H_2O from the $|MH|^+$ ion. The eims registered a peak at *m/z* 216 and one of high intensity at *m/z* 184 $|C_6H(OH)_3MeCOOH|^+$. The 400 MHz ^{1}H-nmr spectrum of judeol contained signals due to three aliphatic and one aromatic methyl groups (0.97, 1.06, 1.09, and 2.67), and the remaining protons were assigned as follows: δ_H 1.16, 1.35, 1.43, and 1.79 (4H, 4×dd, J=12.0, 9.0, 7.0 Hz, H-10 and H-12), 1.95 and 2.61 (2H, 2×dd, J=8.0 and 12.0 Hz, H-6), 2.32-2.42 (2H, m, H-9 and H-13), 4.19 [1H, dd, J=8.0 and 2.0 Hz, H-3 (C*H*OH)], 4.29 and 4.35 [2H, 2×d, J=13.0 Hz, H-1(-CH_2OH)], 5.94 [1H, br t, J=6.0 Hz, H-5 (CH-OOC-)], 6.46 (1H, s, H-4′ or H-6′). Thus, this spectrum reveals a striking resemblance to that of armillol ester (**1a** (2), except in the aromatic region. Judeol (**1c**) contains only one aromatic proton, and since its molecular formula differs from that of (**1a**) by one oxygen, it is likely that (**1c**) possesses an additional hydroxyl substituent in the orsellinic acid moiety. Judeol on methanolysis gave armillol (**1b**) (tlc analysis), but the paucity of material prevented further analysis of this natural product and the exact assignments of the positions of the three phenolic hydroxyl groups.

The bioassays were carried out using conventional antibiotic discs. 4-*O*-Methylmelleolide (**2b**) and judeol (**1c**) showed strong antibacterial activity against gram-positive bacteria, such as *Bacillus subtilis* 5262 (ATCC 6633) and *Staphylococcus aureus* 209 P (ATCC 53156) (minimum value 5.6γ and 8.7γ per disc, respectively). The compounds did not inhibit the growth of gram-negative bacteria (*Pseudomonas aeruginosa, Escherichia coli*).

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Melting points were determined on a Kofler melting

TABLE 1. Positional Parameters and Calculated Mean Isotropic U Factors ($\times 10^4$)

	X	Y	Z	U
Molecule 1:				
C1	5913(9)	4056(18)	6337(24)	124(30)
C2	5628(8)	5304(16)	5871(17)	80(21)
C3	5979(9)	5324(16)	4469(18)	73(21)
C4	4983(8)	6075(17)	6728(17)	112(23)
C5	4092(9)	5172(17)	6303(19)	108(24)
C6	4003(9)	5938(18)	5285(22)	100(28)
C7	4732(8)	7301(14)	6146(14)	65(18)
C8	4424(9)	8723(16)	7551(17)	88(22)
C9	5393(8)	7764(16)	5073(16)	88(21)
C10	6137(10)	9098(19)	5997(21)	124(29)
C11	6813(9)	8981(17)	4711(16)	78(22)
C12	6657(12)	7303(23)	3482(23)	136(35)
C13	5816(10)	6435(18)	3876(18)	75(24)
C14	7712(11)	9402(28)	5400(24)	154(39)
C15	6694(15)	10055(22)	3976(24)	240(42)
C41	5931(11)	7660(22)	9145(18)	94(29)
O1	5623(8)	3741(17)	7330(17)	140(23)
O4	5104(6)	6763(11)	8496(11)	94(15)
O5	4054(5)	3454(10)	5528(11)	71(13)
C1′	3328(9)	2536(20)	5540(18)	89(25)
C2′	3350(9)	777(18)	4519(19)	72(22)
C3′	2704(10)	−236(20)	4880(20)	94(27)
C4′	2648(10)	−1913(21)	4084(21)	121(28)
C5′	3165(11)	−2474(18)	2888(21)	65(27)
C6′	3848(10)	−1514(18)	2462(18)	88(25)
C7′	3907(10)	155(19)	3260(20)	93(27)
C8′	4603(10)	1147(19)	2688(21)	119(28)
O1′	2772(7)	3045(14)	6335(17)	145(23)
O3′	2118(7)	240(13)	6017(13)	123(19)
O5′	3156(7)	−4137(13)	1969(13)	103(19)
Molecule 2:				
C1′	1238(9)	3842(14)	3920(14)	43(18)
C2′	826(8)	2321(15)	2723(14)	46(17)
C3′	1040(8)	1031(15)	2715(14)	59(17)
C4′	150(8)	2206(14)	1606(15)	58(19)
C5′	−696(8)	2322(18)	2338(15)	84(21)
C6′	−990(8)	546(16)	1624(15)	68(20)
C7′	−243(8)	414(15)	419(15)	65(19)
C8′	−566(9)	238(17)	−1197(16)	74(23)
C9′	233(8)	−874(14)	113(14)	55(17)
C10′	1003(8)	−998(16)	−1056(15)	90(20)
C11′	1714(8)	−1570(16)	−513(14)	69(19)
C12′	1320(10)	−1728(17)	1025(16)	108(24)
C13′	657(9)	−606(14)	1678(15)	79(20)
C14′	1910(12)	−3173(20)	−1766(21)	114(29)
C15′	2528(9)	−304(18)	−82(18)	77(24)
C41′	1238(9)	3416(19)	252(19)	96(25)
O1′	1073(6)	5175(11)	4174(11)	75(15)
O4′	407(5)	3341(10)	948(9)	68(12)
O5′	−682(5)	3154(10)	4116(10)	83(13)
C1″	−1287(7)	3915(15)	4888(15)	70(19)
C2″	−1290(8)	4514(15)	6640(15)	56(18)
C3″	−1793(7)	5619(16)	7572(15)	65(19)
C4″	−1857(10)	6225(17)	9259(17)	75(22)
C5″	−1328(8)	5847(15)	10033(16)	78(20)
C6″	−838(8)	4725(14)	9205(13)	65(18)
C7″	−751(8)	4137(14)	7553(16)	68(19)
C8″	−195(9)	2899(18)	6675(18)	91(23)
O1″	−1834(6)	4129(13)	4210(17)	121(17)
O3″	−2279(6)	6140(11)	6858(11)	96(15)
O5″	−1388(6)	6483(12)	11663(10)	91(15)
Solvent:				
MEO1	9893(6)	6350(12)	13113(12)	74(2)
MEC1	9950(10)	7640(19)	14769(20)	82(4)
MEO2	4347(8)	4746(16)	10059(15)	110(4)
MEC2	4260(14)	2955(26)	9509(26)	126(7)
MEO3	7068(19)	2546(34)	10361(35)	203(10)
MEC3[a]	7000(23)	2028(44)	11450(46)	84(10)
MEC3[a]	6230(42)	2751(76)	10097(79)	109(20)

[a]Occupancy factors are 70% and 30%, respectively.

point apparatus and are uncorrected. The uv spectra were measured on a Bausch and Lomb spectrometer model 505. Electron-impact mass spectra (ei) were taken on MS 50-AEI and VG 70-70 spectrometers and chemical ionization mass spectra (ci) using isobutane as reactant gas were recorded on a modified (11) MS-9 spectrometer. The 400 MHz ^{1}H-nmr and 100.61 ^{13}C-nmr spectra were recorded with a Bruker WM 400 in $CDCl_3$ solution.

CULTURE CONDITIONS.—*A. mellea* (CBS 111.29) was initiated on malt agar for 14 days. A good distribution of mycelium and rhizomorph was obtained. Roux flasks (20×250 ml) each containing Difco potato-dextrose broth (PDB) (150 ml) were inoculated with *A. mellea* and incubated at 25° for 23 days.

ISOLATION OF MELLEOLIDE (**2a**), 4-*O*-METHYLMELLEOLIDE (**2b**), AND JUDEOL (**1c**).—The mycelium was extracted with MeOH. The methanolic extract was diluted with H_2O and extracted with Et_2O to give a brown oil (10 g), which was chromatographed on a Sephadex LH-20 column to give twenty-five fractions.

Fraction 16 (2.09 g) was further purified by flash chromatography [Kieselgel PF_{254}; eluant: *n*-hexane-Me_2CO (3:1)] and afforded the orsellinate of armillol (**1a**) (1.4 g). Fractions 6-8 (580 mg) were re-chromatographed on Kieselgel 60H [eluant: *n*-hexane-Me_2CO (5:1)]. A sub-fraction (210 mg) gave a white solid which was recrystallized from CCl_4 mp 180-183°. Tlc analysis [solvent systems: C_6H_6-Me_2CO (4:1), C_6H_6-EtOH (7:1), and $CHCl_3$-MeOH-H_2O (7:1:1, lower layer)] showed the solid to be a mixture. Rechromatography of this mixture on Kieselgel [eluant: C_6H_6-Me_2CO (20:1)] afforded two fractions. Purification of these fractions on columns of Kieselgel 60H (1 atm) afforded the compounds A and B. Fraction 12 (103 mg) was a complex mixture as shown by tlc analysis [solvent system: *n*-hexane-Me_2CO (3:1)]. Chromatography of the mixture on a Kieselgel column (60H, 1 atm) and elution with *n*-hexane-Me_2CO (5:1) afforded judeol (**1c**) and other amorphous materials that were not investigated further. Judeol (**1c**) was purified by preparative tlc [solvent: *n*-hexane-EtOAc (1:3)] and subsequent chromatography on Kieselgel (60H) [eluant: $CHCl_3$-Me_2CO (9:1)] gave a chromatographically pure compound.

MELLEOLIDE (**2a**) (COMPOUND A).—Recrystallization of compound A from C_6M_6 gave prisms mp 196-198° [lit(7) mp 198-200°]; cims $|MH|^+$ at *m/z* 401.

4-*O*-METHYLMELLEOLIDE (**2b**) (COMPOUND B).—Recrystallization of compound B from *n*-hexane-EtOAc gave prisms mp 189-191°; $[\alpha]^{21}D$ +71° (c, 0.31 $CHCl_3$), ms M^+ at *m/z* 414.2148 (calcd 414.2042) uv λ max (MeOH) nm (ε) 215 (32,000) 262 (16,800) 299 (6405); 400 MHz ^{1}H-nmr ($CDCl_3$) δ 1.03 (s, CH_3-14), 1.05 (s, CH_3-15), 1.19 (t, J=13.0 Hz, H-10b), 1.26 (s, CH_3-8), 1.48 (dd, J=14.0, 4.0 Hz, H-10a), 1.54 (dd, J=14.0, 6.0 Hz, H-12b), 1.58 (dd, J=11.0, 9.0 Hz, H-6b), 2.03 (dd, J=14.0, 6.0 Hz, H-12a), 2.06 (dd, J=9.0, 11.0 Hz, H-6a), 2.23 (s, CH_3-8′), 2.3 (ddd, J=6.0, 13.0, 10.0 Hz, H-9), 3.05 (m, J=9.5, 9.5, 4.0, 2.3 Hz, H-13), 3.22 (s, OCH_3), 5.77 (t, J=9.0 Hz, H-5), 6.11 (d, J=2.5 Hz, H-4′), 6.21 (d, J=2.5 Hz, H-6′), 7.02 (d, J=2.5 Hz, H-3), 9.45 (s, H-1); ^{13}C-nmr ($CDCl_3$) δ 192.5s (C-1), 170.6s (C-1′), 165.3s (C-5′), 161.8s (C-3′), 156.2d (C-3), 143.5s (C-7′), 133.9s (C-2), 111.7d (C-6′), 104.6s (C-2′), 101.2d (C-4′), 80.6s (C-4), 74.6d (C-5), 53.9q (OMe), 46.9t (C-6), 43.3t (C-10), 43.3s (C-7), *39.0d (C-9), *38.9d (C-13), 38.6s (C-11), 34.0t (C-12), 31.2q 30.0q 24.4q 21.3q (Me-8, Me-8′, Me-14, Me-15) (* signals may be reversed).

JUDEOL (**1c**).—This compound was an amorphous solid that resisted crystallization. The analysis on Kieselgel indicated that it is less polar than armillol ester (**1a**) in the solvent systems: C_6H_6-Me_2CO (5:2), $CHCl_3$-EtOAc (4:1), *n*-hexane-EtOAc (1:3) and more polar than **1a** in the system $CHCl_3$-MeOH-H_2O (7:1:1, lower layer).

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NOMINATIONS FOR THE AMERICAN SOCIETY OF PHARMACOGNOSY RESEARCH AWARD

The American Society of Pharmacognosy announces the establishment of a new research award to recognize members of the Society for their outstanding contribution to research in the field of natural products.

The American Society of Pharmacognosy Research Award will be presented every third year at the Annual Meeting of the Society with a special honorarium of $2,500 and travel expenses sufficient to attend the meeting. It is anticipated that the first award will be presented at the Annual Meeting to be held in North Carolina in 1985. The recipient is required to present the keynote lecture, which will be published in the *Journal of Natural Products* as a special feature.

A nomination may be made by any member and must include a curriculum vitae of the nominee, a list of publications, and a statement emphasizing the nominee's accomplishments and their significance. Attachment of representative reprints may be helpful. Honorary Members of the Society would normally be excluded from consideration, because their achievements have been acknowledged already by the Society.

Nominations will be reviewed by the Research Award Committee consisting of Yuzuru Shimizu, Chairman, University of Rhode Island; Laurence H. Hurley, University of Texas; and John M. Cassady, Purdue University.

Nominations and inquiries should be received no later than 30 April 1985, and be directed to:

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