Synthesis of (25*S*)-[26-$^2H_1$]Cholesterol and ^1H N.m.r. Signal Assignments of the *pro-R* and *pro-S* Methyl Groups at C-25

Shujiro Seo,* Yohko Yoshimura, Tomohiro Satoh, Atsuko Uomori, and Ken'ichi Takeda
Shionogi Research Laboratories, Shionogi and Co. Ltd., Fukushima-ku, Osaka 553, Japan

(25*S*)-[26-$^2H_1$]Cholesterol (**11**) has been prepared from diosgenin (**1**), and the absolute configuration at C-25 of the crucial intermediate, (25*R*)-26-hydroxycholesterol (**6**), has been identified by *X*-ray crystallographic analysis. The two methyl groups at C-25 of cholesterol have been examined by ^1H n.m.r. spectroscopy and the two sets of doublet methyl signals due to 27-H and 26-H have been observed at δ_H 0.862 and 0.866 downfield from the internal reference. The former was assigned to 27-H, the *pro-R* methyl group attached at C-25, and the latter to 26-H, the *pro-S* methyl group attached at C-25. The ^{13}C n.m.r. signal assignments of the methyl groups at C-25 reported by Popjak were confirmed on the basis of the signals of the deuteriated cholesterol (**11**).

A large number of naturally occurring sterols and steroids have on their side-chain an isopropyl group; the two chemically equivalent methyl groups of this group, for cholesterol, are nonequivalent in biochemical reactions. Thus, biological oxidations distinguish between the two C-25 methyl groups of cholesterol to give (25*R*)-26-hydroxycholesterol[1,2] and (25*S*)-26-hydroxycholesterol,[1,2] and in the biosynthesis of cholesterol, *pro-R* and *pro-S* methyl groups at C-25 originate from C-2 and C-6 of mevalonate (MVA), respectively.[3] The magnetic nonequivalency of these methyl groups was also observed by ^{13}C n.m.r. spectroscopy, and the two methyl signals were assigned on the basis of their biochemical nonequivalence.[4]

In contrast, sterols which have no C-24 substituent on their side chain show, by n.m.r. spectroscopy, magnetic equivalence of their two C-25 methyl groups.[5] When the side-chain is substituted at C-24, as in campesterol or sitosterol, the two C-25 methyl groups have different chemical shifts in both their ^1H n.m.r.[6] and ^{13}C n.m.r. spectra.[7] Recently, we reported assignments of the ^{13}C methyl signals based on biosynthetic evidence.[8]

We have re-examined cholesterol carefully by ^1H Fourier transform (F.t.) n.m.r. spectroscopy at 400 MHz and observed the magnetic nonequivalence of 26-H† and 27-H;† they appeared at 0.866 and 0.862 p.p.m. This fact prompted us to prepare (25*S*)-[26-$^2H_1$]cholesterol to assign the methyl resonances based on evidence other than biochemical nonequivalence.

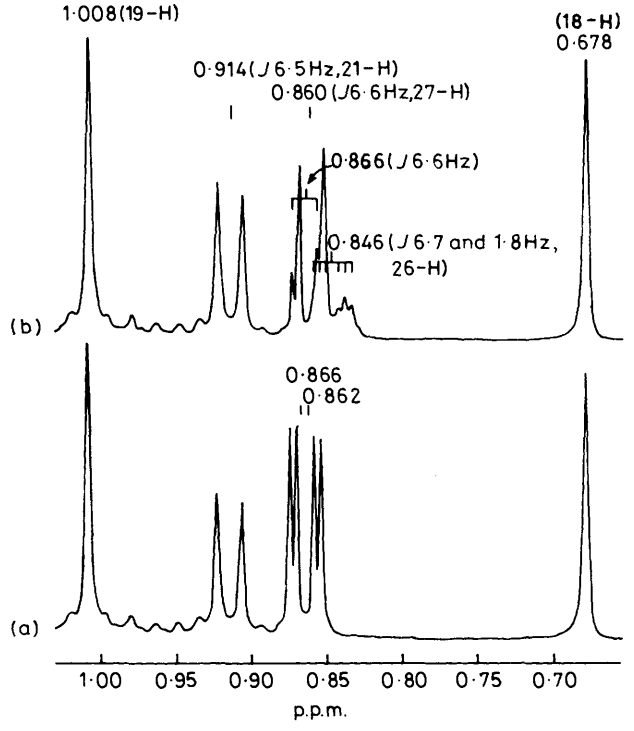


Figure. Highfield ^1H n.m.r. spectra of (a) cholesterol (**12**) and (b) (25*S*)-[26-$^2H_1$]cholesterol (**11**), were recorded on a Varian XL-400 n.m.r. spectrometer in [^2H]chloroform; spectral width, 3 800 Hz; pulse width, 5.0 μs; acquisition time, 3.950 s; accuracies of δ and *J* are ±0.0006 p.p.m. and ±0.25 Hz. A doublet of triplets signal at 0.846 p.p.m. was clearly observed by resolution enhancement and Gaussian apodization of the spectrum

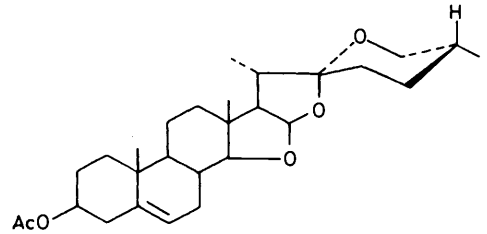
Results and Discussion

To prepare (25*S*)-26-deuteriated cholesterol (**11**), we chose diosgenin acetate (**1**) as the starting material, the configuration at C-25 of diosgenin having been established by *X*-ray analysis.[9] Clemmensen reduction of diosgenin acetate (**1**) gave (25*R*)-16β,26-dihydroxycholesterol (**2**).[10] Since the 16β-hydroxy group is sterically hindered, *p*-nitrobenzoylation of (**2**) afforded the 3,26-bis-*p*-nitrobenzoyl derivative (**3**). Huang-Minlon reduction of the 16-oxo compound (**5**), which was obtained by Jones' oxidation of (**3**) followed by deprotection, afforded 26-hydroxycholesterol (**6**).

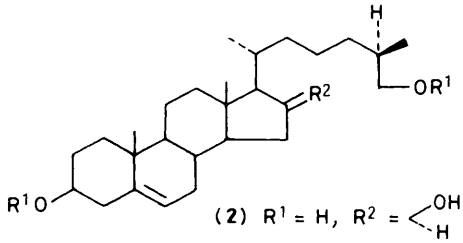
Since the configuration at C-25 of spirostanols can be reversed by acid treatment,[11] *X*-ray diffraction was used to confirm the C-25 configuration of (25*R*)-26-hydroxycholesterol. In the Clemmensen reduction, we treated diosgenin in hydrochloric acid; the optical rotation of the 26-hydroxycholesterol obtained, $[\alpha]_D^{24.0}$ −40.6°, was in poor agreement with the literature value, $[\alpha]_D$ −33.5°.[12]

To remove the 26-hydroxy group, (25*R*)-26-hydroxycholesterol (**6**) was converted into the 26-trityl ether (**7**), which was acetylated to (**8**). Acid treatment of (**8**) gave 3-*O*-acetyl-26-hydroxycholesterol (**9**), which was converted into the 26-tosylate (**10**). On treatment with lithium aluminium deuteride,

† Although Popjak proposed the *pro-R* and *pro-S* methyl groups at C-25 of cholesterol (**12**) be numbered C-26 and C-27, respectively, we use C-26 for the *pro-S* methyl group at C-25 and C-27 for the *pro-R* methyl group at C-25 of cholesterol (**12**) in accordance with the numbering of (**11**)



(1)

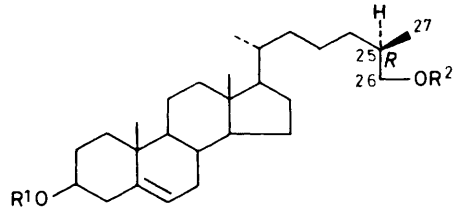


(2) $R^1 = H$, $R^2 = \langle^{OH}_{H}$

(3) $R^1 = p\text{-}O_2NC_6H_4CO$, $R^2 = \langle^{OH}_{H}$

(4) $R^1 = p\text{-}O_2NC_6H_4CO$, $R^2 = O$

(5) $R^1 = H$, $R^2 = O$



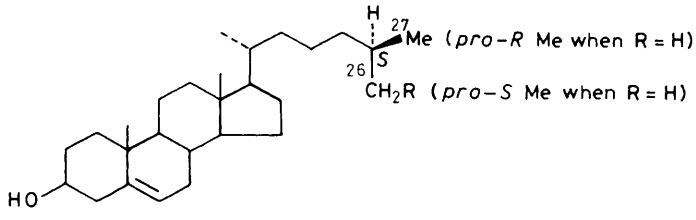
(6) $R^1 = R^2 = H$

(7) $R^1 = H$, $R^2 = CPh_3$

(8) $R^1 = Ac$, $R^2 = CPh_3$

(9) $R^1 = Ac$, $R^2 = H$

(10) $R^1 = Ac$, $R^2 = p\text{-}MeC_6H_4SO_2$



(11) R = ^2H

(12) R = H

compound (**10**) afforded (25*S*)-[26-$^2H_1$]cholesterol (**11**), the configuration at C-25 of which should be the same as that of (25*R*)-26-hydroxycholesterol (**6**). Mass spectrometry of (**11**) showed that 94% of the cholesterol had a single deuterium atom at C-26 (M^+, *m/z* 387).

As shown in the Figure (a), the ^1H n.m.r. spectrum of cholesterol (**12**) examined by 400 MHz F.t. n.m.r. spectroscopy at high accuracy (±0.006 p.p.m.) showed two doublets at 0.862 (*J* 6.6 Hz) and 0.866 p.p.m. (*J* 6.6 Hz) downfield from the internal reference corresponding to the isopropyl methyl groups in the side chain. In the ^1H spectrum [see Figure (b)] of (25*S*)-[26-$^2H_1$]cholesterol (**11**), the signal at 0.866 p.p.m. had shifted upfield owing to the deuterium isotope shift [13] (−0.020 p.p.m.) and appeared as a doublet of triplets at 0.846 p.p.m. ($^3J_{H,H}$ 6.7 and $^2J_{H,D}$ 1.8 Hz). Consequently, this signal was assigned to 26-H. A further doublet signal at 0.862 p.p.m. shifted upfield and appeared at 0.860 p.p.m. ($^3J_{H,H}$ 6.6 Hz) was assigned to 27-H. The weak doublet signal at 0.866 p.p.m. ($^3J_{H,H}$ 6.6 Hz) [see Figure (b)] was due to the unlabelled methyl (26-H) signal generated from the hydrogen atom in 99% deuteriated lithium aluminium hydride. In the ^1H complete decoupled ^{13}C n.m.r. spectrum of (25*S*)-[26-$^2H_1$]cholesterol (**11**), C-27 appeared at 22.5 p.p.m. and C-26 originally at 22.7 p.p.m. shifted upfield by 0.2 p.p.m. owing to the deuterium isotope shift [13] and appeared at 22.5 p.p.m. as a triplet, $^1J_{C,D}$ 18 Hz. This conclusion agrees with the assignments made by Popjak based on biosynthetic findings.[4]

Experimental

M.p.s were taken on a hot plate and are uncorrected. ^1H N.m.r. spectra were recorded on a Varian EM-390 spectrometer in [^2H]chloroform unless otherwise stated. ^{13}C F.t. n.m.r. spectra were recorded on a Varian XL-100-12A spectrometer operating at 25.16 MHz at 30 °C using a 10-mm spinning spherical tube. F.t. n.m.r. measurement conditions were as follows: spectral width, 4 131 Hz; pulse flipping angle, 40°; acquisition time, 0.992 s; number of data points, 8 192. Chemical shifts are given in δ (p.p.m.) downfield from internal tetramethylsilane. The accuracies of δ_H and δ_C are ±0.01 and ±0.04 p.p.m., respectively. Optical rotations were determined on a Hitachi Perkin-Elmer 141 instrument and mass spectra were recorded on a RMV-8GN spectrometer.

(25R)-16β,26-*Dihydroxycholesterol* (**2**).—To diosgenin acetate (**1**) (1 g), m.p. 202—206 °C $[\alpha]_D^{24.0}$ −119.9° ($CHCl_3$, *c* 1.001), was added zinc amalgam [freshly prepared from $HgCl_2$ (3 g) and zinc powder (30 g) in 1.3% hydrochloric acid] in 90% ethanol (100 ml). Concentrated hydrochloric acid (30 ml) was added over 1.5 h to the above mixture heated under reflux; the heating was then continued for a further 1.5 h. The mixture was cooled, the inorganic material filtered off, and the filtrate concentrated under reduced pressure to 50 ml; it was then extracted with chloroform, and the extract washed with water, dried (Na_2SO_4), and evaporated. Chromatography of the residue on silica gel eluted with hexane–ethyl acetate–chloroform (3:4:3) gave (25*R*)-16β,26-dihydroxycholesterol (**2**) (367 mg), m.p. 182—183 °C, δ_H ($CDCl_3$ + CD_3OD 1 drop) 0.87 (3 H, s, 18-H), 0.87 (3 H, d, *J* 6.6 Hz, 27-H), 0.95 (3 H, d, *J* 6.6 Hz, 21-H), 1.00 (3 H, s, 19-H), 3.37 (2 H, d, *J* 6 Hz, 26-H), 3.4 (1 H, m, 3-H), 4.28 (1 H, m, 16-H), and 5.31 (1 H, d, *J* 5 Hz, 6-H).

(25R)-3β,25-O-*Bis*-p-*nitrobenzoyl*-16β,26-*dihydroxycholesterol* (**3**).—To (25*R*)-16β,26-dihydroxycholesterol (**2**) (180 mg) in ether (30 ml) and pyridine (2 ml) was added *p*-nitrobenzoyl chloride (500 mg) and the mixture was left for 3 h at room temperature. Methanol was then added to destroy the excess of chloride. After work-up, the ethyl acetate extract was purified by silica gel chromatography, eluting with hexane–

ethyl acetate–chloroform (8:1:2), to give (25*R*)-3β,26-*O*-bis-*p*-nitrobenzoyl-16β,26-dihydroxycholesterol (**3**) (120 mg), m.p. 206—208 °C, δ_H ($CDCl_3$ + CD_3OD 1 drop) 0.90 (3 H, s, 18-H), 0.97 (3 H, d, *J* 6.5 Hz, 27-H), 1.03 (3 H, d, *J* 6.5 Hz, 21-H), 1.08 (3 H, s, 19-H), 4.3 (3 H, m, 16-H and 26-H), 4.87 (1 H, m, 3-H), 5.43 (1 H, d, *J* 6 Hz, 6-H), and 8.25 and 8.30 (4 H, ABq, *J* 8 Hz, ArH).

(25R)-3β,26-O-*Bis*-p-*nitrobenzoyl*-16-*oxo*-26-*hydroxycholesterol* (**4**).—Jones' reagent (0.5 ml) was added to a stirred solution of compound (**3**) (100 mg) in acetone (15 ml) with ice cooling and stirring was then continued for 1.5 h. After work-up, the ether extract was purified by silica gel chromatography with hexane–ethyl acetate–chloroform (4:2:1) as eluant to give (25*R*)-3β,26-*O*-bis-*p*-nitrobenzoyl-16-oxo-26-hydroxycholesterol (**4**) (97 mg), m.p. 155—157 °C (Found: C, 68.85; H, 6.9; N, 3.9. $C_{41}H_{50}N_2O_9$ requires C, 68.88; H, 7.05; N, 3.92%); δ_H($CDCl_3$) 0.85 (3 H, s, 18-H), 1.00 (3 H, d, *J* 6.0 Hz, 27-H), 1.05 (3 H, d, *J* 6.6 Hz, 21-H), 1.12 (3 H, s, 19-H), 4.18 and 4.27 (2 H, AB part of ABX, *J* 11, 6, and 6 Hz, 26-H), 4.93 (1 H, m, 3-H), 5.45 (1 H, d, *J* 5 Hz, 6-H), and 8.25 and 8.30 (4 H, ABq, *J* 8 Hz, ArH).

(25R)-26-*Hydroxy*-16-*oxocholesterol* (**5**).—Compound (**4**) (520 mg) was refluxed for 2 h in 1% potassium hydroxide in methanol (30 ml). After work-up, the chloroform extract was purified by silica gel chromatography with hexane–ethyl acetate (1:1) as eluant to give (25*R*)-26-hydroxy-16-oxocholesterol (**5**) (288 mg), m.p. 170 °C (Found: C, 77.45; H, 10.7. $C_{27}H_{44}O_3$ requires C, 77.83; H, 10.65%); δ_H($CDCl_3$) 0.82 (3 H, s, 18-H), 0.89 (3 H, d, *J* 6.5 Hz, 27-H), 0.97 (3 H, d, *J* 6.5 Hz, 21-H), 1.02 (3 H, s, 19-H), 3.37 and 3.46 (2 H, AB part of ABX, *J* 11, 6 and 6 Hz, 26-H), 3.63 (1 H, m, 3-H), and 5.32 (1 H, d, *J* 5 Hz, 6-H).

(25R)-26-*Hydroxycholesterol* (**6**).—Compound (**5**) (100 mg) was heated for 1.5 h at 130 °C with hydrazine hydrochloride (180 mg) and 80% hydrazine hydrate (950 mg) in triethyleneglycol (5.2 ml). Potassium hydroxide (450 mg) was added to the mixture which was then heated at 210 °C for 3.5 h with distillation of the water. The mixture was cooled, diluted with water, and the product extracted with chloroform. The crude product was purified by silica gel chromatography with n-hexane–ethyl acetate (1:1) as eluant to afford (25*R*)-26-hydroxycholesterol (**6**) (94 mg), m.p. 169—170 °C (from chloroform) and 176 °C (from methanol) (Found: C, 80.3; H, 11.7. $C_{27}H_{46}O_2$ requires C, 80.54; H, 11.52%; $[\alpha]_D^{24.0}$ −40.6° (*c* 1.277, $CHCl_3$), δ_H($CDCl_3$) 0.67 (3 H, s, 18-H), 0.90 (3 H, d, *J* 6 Hz, 27-H), 0.96 (3 H, d, *J* 6 Hz, 21-H), 1.00 (3 H, s, 19-H), 3.40 and 3.50 (2 H, AB part of ABX, *J* 11, 6, and 6 Hz, 26-H), 3.5 (1 H, m, 3-H), 5.33 (1 H, d, *J* 5 Hz, 6-H); δ_C* ($CDCl_3$ + CD_3OD 1 drop) 11.88 (C-18), 16.53 (C-27), 18.70 (C-21), 19.41 (C-19), 21.14 (C-11), 23.50 (C-23), 24.33 (C-15), 28.29 (C-16), 31.51 (C-2), 31.96 (C-7 and C-8), 33.62 (C-24), 35.81 (C-20 and C-25), 36.23 (C-22), 36.57 (C-10), 37.32 (C-1), 39.86 (C-12), 42.18 (C-4), 42.38 (C-13), 50.22 (C-9), 56.22 (C-17), 56.84 (C-14), 68.38 (C-26), 71.68 (C-3), 121.66 (C-6), and 140.89 (C-5). A crystal obtained from chloroform when subjected to *X*-ray diffraction measurement was shown to be identical with a crystal of (25*R*)-26-hydroxycholesterol.[14]

3-O-*Acetyl*-26-*hydroxy*-26-O-*tritylcholesterol* (**8**).—Triphenylmethyl chloride (58 mg) was added to 26-hydroxycholesterol (**6**) (40 mg) in pyridine (0.5 ml) and the mixture heated at 80 °C for 2 h. When no starting material was detectable by t.l.c., acetic anhydride (0.3 ml) was added slowly to the mixture which was then heated at the same temperature for 2 h. After cooling, methanol was added slowly to destroy the excess of reagent, and then the reaction mixture was diluted with water. The ethyl acetate extract was purified by t.l.c. [silica, hexane–ethyl acetate–chloroform (8:1:1)] to give 3-*O*-acetyl-26-hydroxy-26-*O*-tritylcholesterol (**8**) (49.8 mg), *m/z* 626 (M^+ − AcOH); δ_H($CDCl_3$) 0.67 (3 H, s, 18-H), 0.93 (6 H, d, *J* 6 Hz, 21-H and 27-H), 1.01 (3 H, s, 19-H), 2.00 (3 H, s, Ac), 2.84 and 2.90 (2 H, AB part of ABX, *J* 9, 6, and 6 Hz, 26-H), 4.62 (1 H, m, 3-H), 5.35 (1 H, d, *J* 5 Hz, 6-H), and 7.2—7.5 (15 H, m, ArH).

3-O-*Acetyl*-26-*hydroxycholesterol* (**9**).—Trityl acetate (**8**) (40 mg) in ethanol (5 ml) with concentrated hydrochloric acid (5 drops) was stirred for 2 h at room temperature after which the reaction mixture was diluted with water. The mixture was extracted with ethyl acetate and the extract purified by silica gel chromatography with hexane–ethyl acetate–chloroform (4:1:1) as eluant to afford 3-*O*-acetyl-26-hydroxycholesterol (**9**) (26 mg) (Found: C, 78.25; H, 10.3. $C_{29}H_{48}O_3$ requires C, 78.32; H, 10.88%); δ_H($CDCl_3$) 0.67 (3 H, s, 18-H), 0.91 (6 H, d, *J* 6.5 Hz, 21-H and 27-H), 1.01 (3 H, s, 19-H), 2.01 (3 H, s, Ac), 3.40 and 3.49 (2 H, AB part of ABq, *J* 11, 6, and 6 Hz, 26-H), 4.60 (1 H, m, 3-H), and 5.36 (1 H, d, *J* 5 Hz, 6-H).

3-O-*Acetyl*-26-*hydroxy*-26-O-*tosylcholesterol* (**10**).—Toluene-*p*-sulphonic acid (40 mg) was added to the 26-hydroxy compound (**9**) (26 mg) in pyridine (1 ml) and the mixture was left for 6 h at room temperature. After work-up, the ether extract was purified by t.l.c. [silica, hexane–ethyl acetate (3:1)] to give 3-*O*-acetyl-26-*O*-tosyl-26-hydroxycholesterol (**10**) (20 mg).

(25S)-[26-$^2H_1$]*Cholesterol* (**11**).—The tosylate (**10**) (20 mg) was added to lithium aluminium deuteride (20 mg, Merck 99 ^2H atom%) in ether (2 ml) and stirred for 2 h at room temperature. The excess of reagent was destroyed with 5% aqueous sodium hydrogencarbonate and the inorganic material was then filtered off. The filtrate was evaporated to dryness and the residue was purified by t.l.c., with hexane–ethyl acetate–chloroform (4:1:1) as eluant, to give (25*S*)-[26-$^2H_1$]cholesterol (**11**) (14 mg), *m/z* 387 (M^+, 100%) and 386 (6%); $[\alpha]_D^{24.0}$ −42.3° (*c* 1.249 $CHCl_3$); δ_H (±0.0006 p.p.m. in $CDCl_3$ at 400 MHz) 0.678 (3 H, s, 18-H), 0.846 (2 H, dt, *J* 6.7 and 1.8 Hz, 26-H), 0.860 (3 H, d, *J* 6.6 Hz, 27-H), 0.914 (3 H, d, *J* 6.5 Hz, 21-H), 1.008 (3 H, s, 19-H), 3.53 (1 H, m, 3-H), and 5.355 (1 H, d, *J* 5.2 Hz, 6-H); δ_C($CDCl_3$) 11.88 (C-18), 18.74 (C-21), 19.41 (C-19), 21.11 (C-11), 22.50 (t, *J* 19 Hz, C-26), 22.54 (C-27), 23.87 (C-23), 24.31 (C-15), 27.95 (C-25), 28.24 (C-16), 31.71 (C-2), 31.94 (C-7 and C-8), 35.81 (C-20), 36.22 (C-22), 36.53 (C-10), 37.30 (C-1), 39.53 (C-24), 39.82 (C-12), 42.35 (C-4 and C-13), 50.20 (C-9), 56.22 (C-17), 56.81 (C-14), 71.81 (C-3), 121.72 (C-6), and 140.80 (C-5).

Acknowledgements

We thank Drs. H. Ishii and Y. Terui of these laboratories for their encouragement.

References

1 O. Berseus, *Acta Chem. Scand.*, 1965, **19**, 325.
2 S. Seo, A. Uomori, Y. Yoshimura, and K. Tori, *J. Chem. Soc., Perkin Trans. 1*, 1984, 869; references therein.
3 M. G. Kienle, R. K. Varma, L. J. Mulheirn, B. Yagen, and E. Caspi, *J. Am. Chem. Soc.*, 1973, **95**, 1996.

* A small amount of the (25*S*)-isomer of (**6**) was detected in the ^{13}C n.m.r. spectrum of the mother liquor of (**6**). The two isomers differ only by the different chemical shifts of three carbons, C-24, C-26, and C-27. The Δδ value of the corresponding carbons are so small [δ_C 33.62, 68.38, and 16.53 for the (25*R*)-isomer and δ_C 33.74, 68.28, and 16.74 for the (25*S*)-isomer] that identification of one of the isomers would be difficult by the n.m.r. method

4 G. Popják, J. Edmond, F. K. L. Anet, and N. R. Easton, Jr., *J. Am. Chem. Soc.*, 1977, **99**, 931.
5 T. A. Wittstruck, J. K. Sliwowski, and E. Caspi, *J. Chem. Soc., Perkin Trans. 1*, 1977, 1403.
6 W. C. M. C. Kokke, J. N. Shoolery, W. Fenical, and C. Djerassi, *J. Org. Chem.*, 1984, **49**, 3742; J. H. Adler, *Phytochemistry*, 1983, **22**, 607.
7 J. L. C. Wright, A. G. McInnes, S. Shimizu, D. G. Smith, J. A. Walter, D. Idler, and W. Khalil, *Can. J. Chem.*, 1978, **56**, 1898; N. Koizumi, Y. Fujimoto, T. Takeshita, and N. Ikekawa, *Chem. Pharm. Bull.*, 1979, **27**, 38.
8 S. Seo, A. Uomori, Y. Yoshimura, and K. Takeda, *J. Am. Chem. Soc.*, 1983, **105**, 6343; *J. Chem. Soc., Chem. Commun.*, 1984, 1174; A. Uomori, S. Seo, Y. Yoshimura, and K. Takeda, *ibid.*, 1984, 1176.
9 E. A. O'Donnell and M. F. C. Ladd, *Chem. Ind. (London)*, 1963, 1984.
10 R. E. Marker, R. B. Wagner, P. R. Ulshafer, E. L. Wittbecker, D. P. J. Goldsmith, and C. H. Ruof, *J. Am. Chem. Soc.*, 1947, **69**, 2167.
11 R. E. Marker and E. Rohrmann, *J. Am. Chem. Soc.*, 1939, **61**, 846.
12 E. Caspi, M. G. Kienle, R. K. Varma, and L. J. Mulheirn, *J. Am. Chem. Soc.*, 1970, **92**, 2161; R. K. Varma, M. Koreeda, B. Yagen, K. Nakanishi, and E. Caspi, *J. Org. Chem.*, 1975, **40**, 3680.
13 P. E. Hansen, *Annu. Rep. NMR Spectrosc.*, 1983, **15**, 105.
14 A. Kirfel, G. Will, R. Brennecke, and R. Tschesche, *Acta Crystallogr., Sect. B*, 1977, **33**, 895.

Received 20*th May* 1985; *Paper* 5/841