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# THE TRITERPENES FROM *STAUNTONIA HEXAPHYLLA* CALLUS TISSUES AND THEIR BIOSYNTHETIC SIGNIFICANCE

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ABSTRACT.—The sixteen oleanane-type triterpenes including five new compounds **12–16**, 24-methylenecycloartanol, stigmasterol, and campesterol have been isolated from the callus tissues of *Stauntonia hexaphylla*, and ten of these triterpenes were epimeric pairs at the 3-position. Furthermore, these compounds were arranged in a biogenetic pathway based on their degree of oxidation.

*Stauntonia hexaphylla* Decne. (Lardizabalaceae), Japanese name mube, is an evergreen woody climber that is widely distributed in thickets in lowlands and foothills in warmer regions of Japan, Korea, and China (1). A decoction of the stem and the root of the plant or the pericarp of the fruit is used as a diuretic.

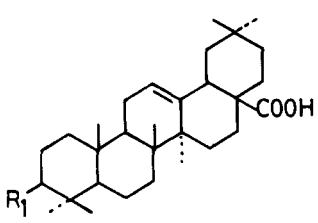
The fruits also activate the circulation and brighten the eyesight (2). From the seeds, three acidic triterpene glycosides, mubenins A, B, and C, have been reported (3). Previously, we have reported six triterpenes and four saponins representing seven new compounds from the callus tissue of the Lardizabalaceous plant *Akebia quinata* (4–6).

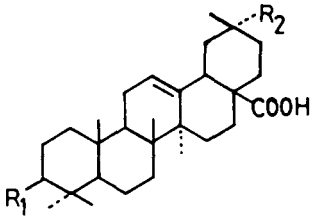
We now report the isolation and the structure elucidation of two phytosterols and sixteen triterpenes from the callus tissues of *S. hexaphylla*, five of which were new compounds from natural sources. Furthermore, the hypothetical biogenesis of these triterpenes is discussed.

## RESULTS AND DISCUSSION

Callus tissues were established from the stem of *S. hexaphylla* and cultured on Murashige and Skoog's medium (minus glycine) containing 2,4-D (1 mg/liter) with kinetin (0.1 mg/liter). The callus tissues were extracted successively with MeOH and EtOAc, and the extracts were combined. The concentrated extracts were chromatographed on a Si gel column to afford sixteen triterpenes [**1–16**], 24-methylenecycloartanol, stigmasterol, and campesterol. The structures of the compounds were identified as oleanolic acid [**3**], akebonoic acid [**7**], 3-*epi*-akebonoic acid [**8**], quinatic acid [**9**], 3β-hydroxy-29-al-olean-12-en-28-oic acid [**10**], and mesembryanthemoidigenic acid [**11**] by comparison with the spectral data reported from the callus tissues of *A. quinata* (4,5). Furthermore, β-amyrin [**1**], erythrodiol [**2**], 3-*epi*-oleanolic acid [**4**], 3-*O*-acetyloleanolic acid [**5**], oleanonic acid [**6**], and 24-methylenecycloartanol were determined by spectroscopic means. Furthermore, as minor compounds, the five new compounds **12–16** were isolated from the callus tissues of *S. hexaphylla*.

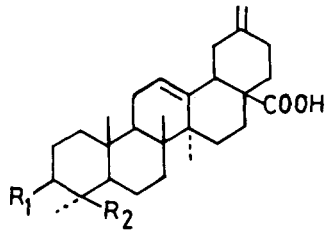
The $^1$H-nmr spectrum of compound **13** showed six tertiary methyl signals at δ 0.77–1.17 (each s), signals ascribable to the carbinolic methylene at δ 3.29 (2H, s) and the olefinic proton at δ 5.32 (1H, t, $J$ = 3.4 Hz). Furthermore, a signal attributable to an acetyl methyl group at δ 2.07 (3H, s) and a triplet-like signal to the β proton on the acetyl-bearing carbon (C-3) at δ 4.64 (1H) were shown (7). The ms spectrum of **13** showed the molecular ion peak $[M]^+$ at *m/z* 514 and exhibited the significant fragment peaks at *m/z* 264 [a], 249 [b], 233 [a − 31], 189 [b − 60], and 187 [a − 31 − 46], which could be assigned





**4** $R_1$=α-OH
**5** $R_1$=β-OAc
**6** $R_1$=$=O$

**10** $R_1$=β-OH, $R_2$=−CHO
**11** $R_1$=β-OH, $R_2$=−$CH_2OH$
**12** $R_1$=α-OH, $R_2$=−$CH_2OH$
**13** $R_1$=α-OAc, $R_2$=−$CH_2OH$
**14** $R_1$=β-OAc, $R_2$=−$CH_2OH$
**15** $R_1$=α-OAc, $R_2$=−COOH
**16** $R_1$=β-OAc, $R_2$=−COOH

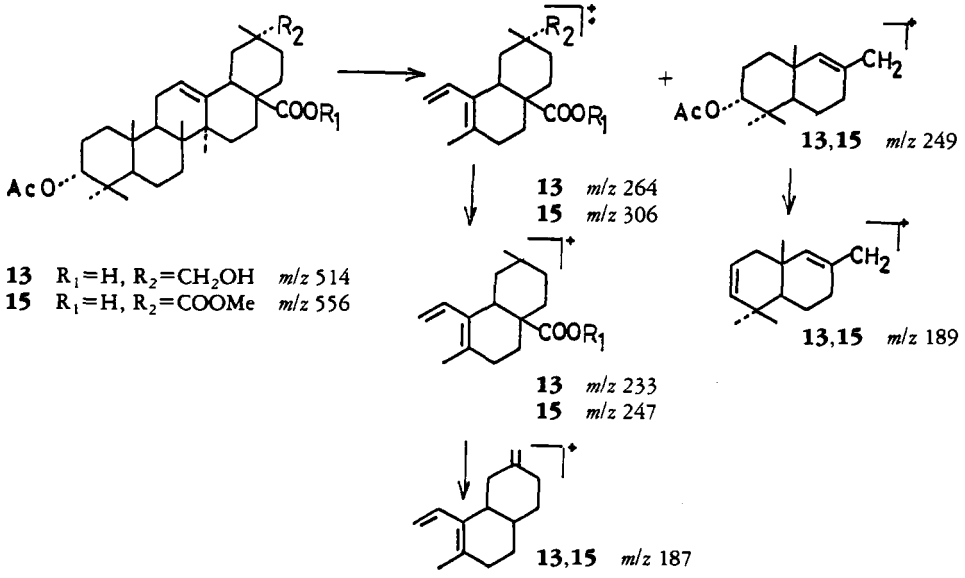


**7** $R_1$=β-OH, $R_2$=−Me
**8** $R_1$=α-OH, $R_2$=−Me
**9** $R_1$=α-OH, $R_2$=−$CH_2OH$

FIGURE 1. Triterpenes from callus tissues of *Stauntonia hexaphylla*.

to the fragments derived by retro-Diels-Alder cleavage of the β-amylin-$\Delta^{12}$ skeleton (8), and the fragments at *m/z* 264, 233, and 187 showed the same pattern as those of the C, D, and E rings of **11** (Scheme 1) (4). Furthermore, the $^{13}$C-nmr spectrum of **13** showed signals at δ 74.4 (t) and 78.2 (d) (Table 1); the latter value showed at higher field for C-3 than the δ 81.0 (d) observed for **5** with a 3β-acetyl group.

From the above-mentioned $^1$H-nmr




SCHEME 1. Fragmentation of compounds **13** and **15**.

TABLE 1. $^{13}$C-Nmr Chemical Shifts of Compounds **5**, **11**, **13**, **15**, and **16** ($CDCl_3$, δ, ppm).[a]

| Carbon | Compound | | | | |
|---|---|---|---|---|---|
| | **5** | **11** | **13** | **15** | **16** |
| C-1 | 38.1 | 38.5 | 33.6 | 33.6 | 38.2 |
| C-2 | 23.6 | 27.5 | 22.7 | 22.7 | 23.5 |
| C-3 | 81.0 | 79.1 | 78.2 | 77.3 | 80.9 |
| C-4 | 37.7 | 38.8 | 36.5 | 36.5 | 37.8 |
| C-5 | 55.4 | 55.3 | 50.2 | 50.2 | 55.4 |
| C-6 | 18.2 | 18.4 | 18.2 | 18.2 | |
| C-7 | 32.6 | 32.7 | 32.6 | 32.5 | |
| C-8 | 39.4 | 39.3 | 39.5 | 39.5 | |
| C-9 | 47.6 | 47.7 | 47.5 | 47.4 | |
| C-10 | 37.0 | 37.1 | 37.1 | 37.0 | |
| C-11 | 23.4 | 23.1 | 23.1 | 23.2 | |
| C-12 | 122.6 | 122.8 | 123.1 | 123.4 | |
| C-13 | 143.6 | 143.2 | 143.3 | 142.8 | |
| C-14 | 41.6 | 41.6 | 41.8 | 41.7 | |
| C-15 | 27.7 | 27.7 | 27.7 | 27.6 | |
| C-16 | 22.9 | 23.5 | 23.4 | 23.8 | |
| C-17 | 46.6 | 47.0 | 46.8 | 46.5 | |
| C-18 | 41.0 | 40.5 | 40.4 | 40.2 | |
| C-19 | 45.7 | 40.1 | 40.2 | 39.8 | |
| C-20 | 30.7 | 35.8 | 35.8 | 42.2 | |
| C-21 | 33.6 | 31.6 | 31.6 | 31.3 | |
| C-22 | 32.5 | 32.7 | 32.5 | 31.3 | |
| C-23 | 28.1 | 28.1 | 28.3 | 27.8 | 28.1 |
| C-24 | 17.2 | 15.6 | 21.9 | 21.9 | 16.9 |
| C-25 | 15.2 | 15.3 | 15.1 | 15.4 | 15.4 |
| C-26 | 16.7 | 16.9 | 17.1 | 17.0 | 16.7 |
| C-27 | 25.9 | 25.9 | 26.1 | 26.1 | 25.9 |
| C-28 | 184.0 | 178.1 | 179.2 | 178.8 | |
| C-29 | 33.1 | 74.4 | 74.4 | 177.8 | |
| C-30 | 23.6 | 19.0 | 19.0 | 19.4 | |
| Ac | 21.3 | — | 21.3 | 21.3 | |
| CO | 171.1 | — | 166.7 | 170.7 | |
| COOMe | — | 51.6 | — | 51.6 | |
| COOMe | — | — | — | 51.8 | |

[a]All signals were corroborated by DEPT techniques.

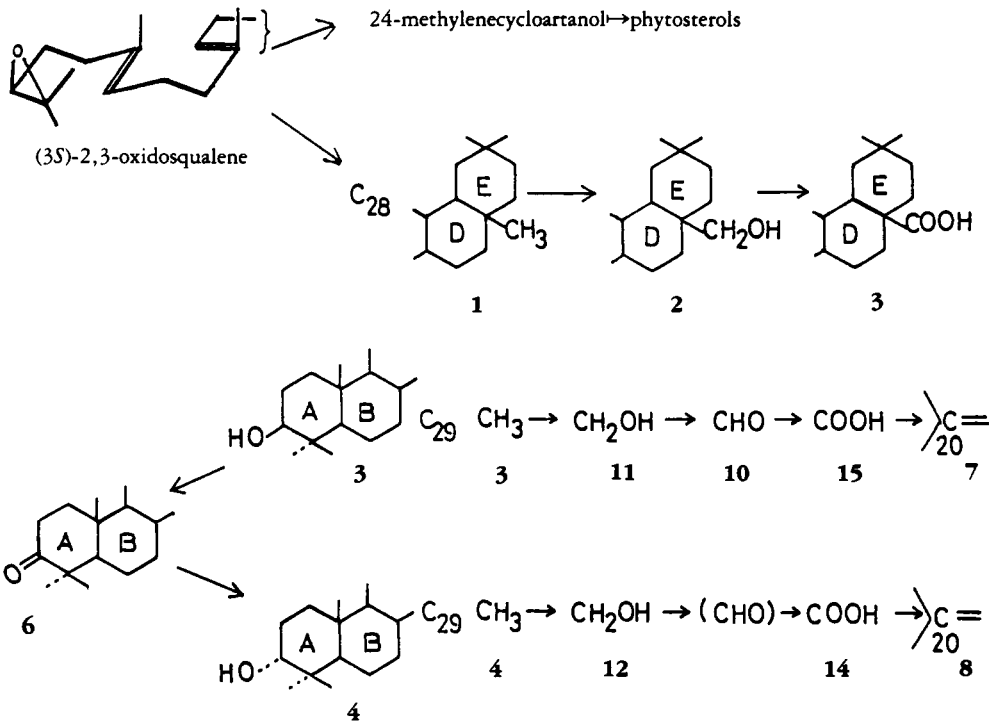
and $^{13}$C-nmr data, the configuration of the acetyl group at C-3 was confirmed as the axial (α) orientation (9, 10). Furthermore, the values of the chemical shifts for other carbons except for the A/B ring were coincident with that of mesembryanthemoidigenic acid [**11**] (Table 1). Therefore, **13** was determined as 3-*O*-acetyl-3-*epi*-mesembryanthemoidigenic acid, which is a new compound. In addition, compound **14** was determined as 3-*O*-acetylmesembryanthemoidigenic acid by the spectral data.

The $^{1}$H nmr of compound **12** showed the six tertiary methyl signals at δ 0.78–1.15 (each s), the signals ascribable to the carbinolic methylene at δ 3.29 (2H), and a proton attributable to the 3β-proton at δ 3.41 (1H, br s). The ms spectrum of **12** showed $[M]^+$ at *m/z* 472 and 264, 233, 207, 201, 190, 189, and 187, which showed a similar fragmentation pattern with **11** (Scheme 1) (4). Also, the $^{13}$C-nmr spectrum of the A/B ring of **12** was coincident with that of **8** (4). Moreover, the values of the other chemical shifts except for A/B ring were coincident with that of **13**. Thus, **12**

was determined as 3-*epi*-mesembryanthemoidigenic acid. The $^1$H-nmr spectrum of compounds **15** and **16** showed two singlets attributable to acetoxyl methyl groups at δ 2.04 (3H, s) and 2.06 (3H, s), and two signal groups were exhibited as triplet-like at δ 4.63 (1H, t, $J$ = 2.6 Hz) and as a multiplet at δ 4.48 (1H), which were attributable to the α and β protons at C-3 on the acetoxyl-bearing carbon, respectively, that were deduced as a mixture of α and β configuration at C-3 (7). Furthermore, **15** and **16** afforded dimethyl esters on treatment with $CH_2N_2$, and its $^1$H-nmr spectrum exhibited the signals of four methyl esters in the region of δ 3.63–3.67. The $^{13}$C-nmr spectrum (Table 1) of the mixture showed two signals attributable to C-3 with the α and β configuration of the hydroxyl group at δ 77.3 (d) and δ 80.9 (d), which were moreover presumed to be a mixture of the equatorial and the axial signals bearing acetoxyl at C-3. Moreover, each signal was coincident with those of the A/B ring of **5** and **13** (Table 1). From the ms spectrum, the molecular ion peak $[M]^+$ was shown at *m/z* 556; significant fragment peaks were also exhibited at *m/z* 306 [a], 249 [b], 247 [a − 59], 189 [b − 60], and 187 [a − 59 − 60] arising from retro-Diels-Alder cleavage. The occurrence of fragment ions at *m/z* 306, 247, and 187 indicated that two methyl esters were located on the D/E rings of **15** and **16** (Scheme 1). Therefore, compounds **15** and **16** were presumed to be a mixture of 3-*O*-acetylserragenic acid [**16**] and 3-*O*-acetyl-3-*epi*-serragenic acid [**15**], respectively. The ratio of compounds **15** and **16** was approximately 3:2. It is very interesting from a biosynthetic point of view to produce so many triterpene compounds of different degrees of oxidation in a biosynthetic sequence and so many pairs of 3-epimers from one kind of the plant cell culture. Furthermore, plant tissue culture may provide a new source for these natural products.

SPECULATIVE BIOGENESIS OF 30-NOROLEANANE TYPE TRITERPENES IN CALLUS TISSUES OF *S. HEXAPHYLLA*.—The sixteen triterpenoids isolated from callus tissues of *S. hexaphylla* have the oleanane type skeleton, and the 30-noroleanane type triterpenes are rare compounds from natural sources except for norarjunolic acid (11) and eupteleogenin (12). It is interesting from the biosynthetic point of view that the following hypothetical biogenetic sequence for **7** can be presumed theoretically based on the co-occurrence of these constituents of different degrees of oxidation at C-29. They are at first derived stepwise by oxidation of β-amyrin [**1**] which is biosynthesized from (3*S*)-2,3-oxidosqualene (Scheme 2) (13), and the further biosynthetic transformation of **1** proceeds through erythrodiol [**2**] by hydroxylation and then to oleanolic acid [**3**] by oxidation at C-28 as shown in Scheme 2 (14,15). On the other hand, hydroxylation of **3** at C-29 will produce mesembryanthemoidigenic acid [**11**], and further oxidation of **11** at C-29 proceeds to aldehyde **10** and with successive oxidation to serragenic acid [**16**]. Finally decarboxylation of **16** may occur at C-29 to produce akebonoic acid [**7**] (30-noroleanolic aicd). On the other hand, the co-occurrence of 3α- and 3β-hydroxyl-triterpenes raises the question of the biosynthetic origin of 3α-hydroxyl. It has previously been reported that [$^{14}C_6$]-maslinic acid biosynthesized by the callus tissues of *Isodon japonica* from [2 − $^{14}$C] mevalonate was converted into 3-*epi*-maslinic acid via the 3-ketone (16,17). Because of the co-existence of oleanonic acid [**6**] in callus tissues of *S. hexaphylla*, 3-*epi*-oleanolic acid [**4**] may be formed from oleanolic acid [**3**] via the same sequences as has been shown for 3-*epi*-maslinic acid.

Therefore, it is also presumed that 3-*epi*-oleanolic acid [**4**] is converted to 3-*epi*-akebonic acid [**8**] via the intermediates of the 3-epi derivatives **12** and **14** in the same sequences as shown in the




SCHEME 2. Postulated biosynthetic sequence of 30-nor-oleanane triterpenes and phytosterols in *Stauntonia hexaphylla* callus tissues.

pathway of akebonic acid [7]. Thus, a hypothetical biochemical pathway for 30-noroleanane triterpenes is proposed as shown in Scheme 2. It is very interesting from a biosynthetic point of view to produce a number of biogenetic triterpenoid intermediates stepwise from one plant cell culture such as *S. hexaphylla*. The feeding experiments of the hypothetical biogenetic pathway are now in progress. The use of the callus cultures of higher plants may provide a good method for biosynthetic studies. Studies with the intact plant material would not have allowed such complete determination of the biogenetic sequences.

## EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Melting points were determined on a Yanagimoto micro melting point apparatus and were uncorrected. The ir and optical rotation were recorded on JASCO A-302 and JASCO DIP-4, respectively. The $^1$H-nmr spectra were measured at 400 MHz and the $^{13}$C-nmr spectra were measured at 100.6 MHz on a Brucker AM-400, at room temperature with $CDCl_3$ and pyridine-$d_5$ solution and TMS as internal standard. The multiplicities for the $^{13}$C-nmr spectra were determined by DEPT spectra of 45°, 90°, and 135°. Mass spectra (70 eV) were taken with a direct probe (Hitachi-80M).

PLANT MATERIAL.—*S. hexaphylla* Decne was collected in August 1982 at the Medicinal Plant Garden of this college.

DERIVATION AND CULTURE OF CALLUS TISSUES.—The callus tissues from the stalk were established in February 1983. Murashige and Skoog's medium (minus glycine) (M&S) containing 2,4-D (1 mg/liter, 3 mg/liter) and kinetin (0.1 mg/liter) as plant growth regulators were used for induction of callus tissues. The callus tissues were subcultured every 5–6 weeks onto fresh M&S containing 2,4-D (1 mg/liter) and kinetin (0.1 mg/liter) at 26 ± 1° in the dark.

EXTRACTION AND ISOLATION.—The fresh callus tissues (820 g, fresh wt) were extracted with cold MeOH and EtOAc in a Waring blender. The solution of extract was combined and concentrated under reduced pressure to yield the extract, which was partitioned between $CHCl_3$

and $H_2O$ to obtain the organic-solvent-soluble fraction. The $CHCl_3$ solution was chromatographed over a column of Si gel (Merck 9385), and elution with $CHCl_3$ with increasing proportions of MeOH afforded crude triterpene mixtures. The mixtures were purified repeatedly by rechromatography over a Si gel column [hplc, CIG column system (Kusano Scientific, Tokyo) with Iatrobeads as the stationary phase (60 Si gel, IATRON, Tokyo)] using the combination of hexane/EtOAc/MeCN, which afforded compounds **1–16**, 24-methylenecycloartanol, stigmasterol, and campesterol.

*3*-epi-*Mesembryanthemoidigenic acid* [**12**].—$^1$H-nmr ($CDCl_3$) δ 0.78 (3H, s), 0.84 (3H, s), 0.92 (3H, s), 0.95 (3H, s), 0.96 (3H, s), 1.15 (3H, s), 3.29 (2H, s), 3.41 (1H, bs), 5.31 (1H, t); eims *m/z* (rel. int.) $[M]^+$ 472 (24), 264 (77), 233 (100), 207 (32), 201 (56), 190 (14), 189 (10), 187 (17).

3α-O-*Acetylmesembryanthemoidigenic acid* [**13**].—Colorless, amorphous, $[\alpha]^{18}D$ + 106.7° (*c* = 0.03, $CHCl_3$); ir ν max ($CHCl_3$) 1720 $cm^{-1}$; $^1$H-nmr ($CDCl_3$) δ 0.77 (3H, s), 0.85 (3H, s), 0.88 (3H, s), 0.93 (3H, s), 0.97 (3H, s), 1.17 (3H, s), 2.07 (3H, s), 2.87 (1H, dd, *J* = 4, 8 Hz), 3.29 (2H, s), 4.63 (1H, bs), 5.32 (1H, t); eims *m/z* (rel. int.) $[M]^+$ 514 (1), 454 (5), 264 (70), 249 (7), 233 (100), 201 (55), 190 (35), 189 (24), 187 (15).

*3*-O-*Acetylserratagenic acid* [**15,16**].—Mp 290° >.

*3*-O-*Acetyl-3*-epi-*serratagenic acid* [**16**].—$^1$H-nmr ($CDCl_3$) δ 0.73 (3H, s), 0.84 (3H, s), 0.88 (3H, s), 0.92 (3H, s), 1.19 (3H, s), 1.26 (3H, s), 2.06 (3H, s), 2.90 (1H, dd), 3.64 (3H, s), 3.67 (3H, s), 4.63 (1H, t, *J* = 2.6 Hz), 5.33 (1H, t, *J* = 2.6 Hz).

*3*-O-*Acetylserratagenic acid* [**15**].—$^1$H-nmr ($CDCl_3$) δ 0.72 (3H, s), 0.85 (3H, s), 0.86 (3H, s), 0.92 (3H, s), 1.12 (3H, s), 2.04 (3H, s), 2.90 (1H, dd), 3.63 (3H, s), 3.67 (3H, s), 4.48 (1H, m), 5.32 (1H, t, *J* = 2.6 Hz); eims *m/z* (rel. int.) $[M]^+$ 556 (2.5), 525 (1), 496 (11), 481 (3.5), 306 (55), 293 (12), 249 (6), 247 (48), 246 (13), 233 (10), 215 (13), 203 (5), 190 (38), 189 (18), 188 (19), 187 (100), 173 (20).

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## LITERATURE CITED

1. J. Ohwi, in: "Flora of Japan." Ed. by F.G. Meyer and E.H. Waker, Smithsonian Institution, Washington, DC, 1984, p. 461.
2. L.M. Perry, "Medicinal Plants of East and Southeast Asia: Attributed Properties and Uses," MIT Press, Cambridge, MA, 1980, pp. 195–196.
3. T. Takemoto and K. Kametani, *Ann. Chem.*, **685**, 237 (1965).
4. A. Ikuta and H. Itokawa, *Phytochemistry*, **25**, 1625 (1986).
5. A. Ikuta and H. Itokawa, *Phytochemistry*, **27**, 3809 (1988).
6. A. Ikuta and H. Itokawa, *Phytochemistry*, in press (1989).
7. N.S. Bhacca and D.H. Williams, "Application of NMR Spectroscopy in Organic Chemistry," Holden-Day, San Francisco, 1964, p. 78.
8. H. Budzikiewicz, J.M. Wilson, and C. Djerassi, *J. Am. Chem. Soc.*, **85**, 3688 (1963).
9. R.J. Abraham and P. Loftus, "Proton and Carbon-13 NMR Spectroscopy: An Integrated Approach." Wiley, New York, 1978.
10. J. Asakawa, R. Kasai, K. Yamasaki, and O. Tanaka, *Tetrahedron*, **33**, 1935 (1977).
11. R. Higuchi and T. Kawasaki, *Chem. Pharm. Bull.*, **20**, 2143 (1972).
12. T. Murata, S. Imai, M. Imanishi, M. Goto, and K. Morita, *Tetrahedron Lett.*, 3215 (1965).
13. D.H.R. Barton, T.R. Jarman, K.G. Watson, and D.A. Widdowson, *J. Chem. Soc., Chem. Commun.*, 861 (1974).
14. H.W. Kircher, *Phytochemistry*, **19**, 2707 (1980).
15. W.D. Nes, M. Benson, and E. Heftomann, *Phytochemistry*, **20**, 2299 (1981).
16. Y. Tomita and S. Seo, *J. Chem. Soc., Chem. Commun.*, 707 (1973).
17. S. Seo, T. Tomita, and K. Tori, *J. Am. Chem. Soc.*, **103**, 2075 (1981).

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