Braunicene, a Novel Cyclic C_{32} Isoprenoid from *Botryococcus braunii*

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Abstract: A new isoprenoid hydrocarbon, braunicene (**11**), was isolated from the Berkeley strain of *Botryococcus braunii* grown in culture. The structure of the compound was determined by ^1H and ^{13}C NMR spectroscopy. Braunicene is a $C_{32}H_{54}$ member of the 1′–3 fused botryococcene family of hydrocarbons with a pentasubstituted cyclohexyl ring at the end of the 1′ branch of the molecule. The absolute configuration of the quaternary carbon at the 3 terminus was shown to be *S*, as in the parent botyrococcene, by degradation. Mechanisms for the biosynthesis of braunicene are discussed.

Botryococcus braunii is a widely distributed fresh water colonial green alga that produces and accumulates massive quantities of hydrocarbons.[1,2] Under appropriate conditions, rust-colored algal blooms form on the surfaces of lakes and ponds, and cast-up colonies sometimes leave extensive rubbery deposits on the shoreline.[3] Paleobotanical studies suggest that *B. braunii* is a primary source of hydrocarbons in many oil-rich deposits dating from the Ordovician period to the present.[4] Botyrococcane, a hydrocarbon bearing a distinctive structural signature from the organism, comprises over 1% of some crude oils from Sumatra,[5] the highest concentration of a single complex biological fossil marker found in a crude oil.

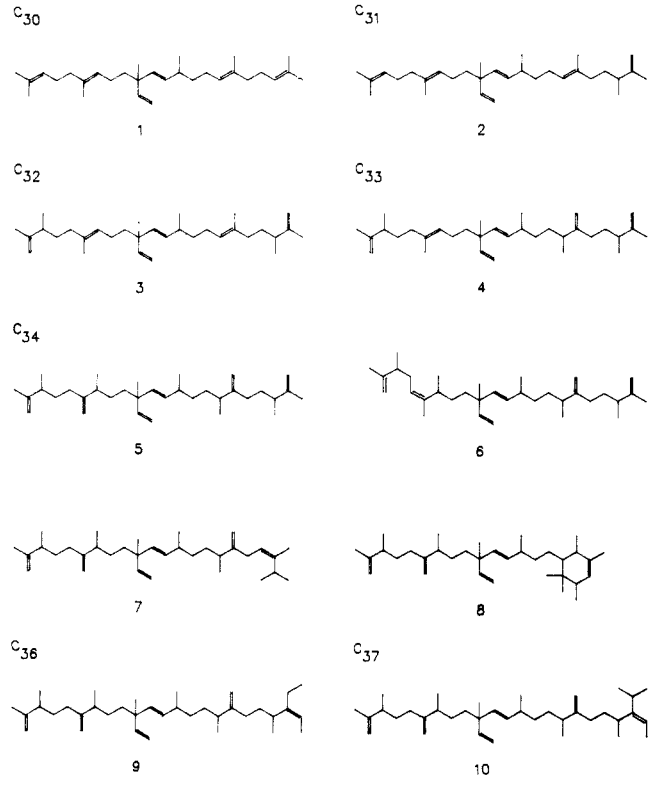
There are two forms of *B. braunii*. Both have similar morphologies, but the hydrocarbons in the oil sack that surrounds the cell are different. The L-form produces a series of linear odd-numbered C_{23}–C_{31} alkyl dienes and trienes.[6] The B form, however, synthesizes a novel family of highly branched C_{30}–C_{37} hydrocarbons, collectively called botryococcenes. Botryococcenes are isoprenoids with an unusual 1′–3 fusion of isoprene units in the center of the chain. Biosynthetic experiments suggest that a parent C_{30} botryococcene is constructed from the hydrocarbon moieties of two molecules of farnesyl diphosphate and that higher members of the family are generated by successive methylations with *S*-adenosylmethionine.[6–8] There is strong evidence from model studies for formation of 1′–3 fused isoprenoids from cyclopropylcarbinyl diphosphates by carbocationic rearrangements mechanistically related to those postulated for the conversion of presqualene diphosphate to squalene.[9–13] Recent stereochemical studies support this hypothesis.[14]

From the few detailed studies of the hydrocarbons in *B. braunii* that have been reported,[3,6,15–21] it appears that genetic and environmental factors are responsible for substantial variations in the composition of the mixtures. Although approximately 30 botryococcenes have been identified by GCMS, only the 10, whose structures are shown in Chart I, were assigned because of problems associated with resolving individual components by preparative-scale chromatographic techniques.[22] In a recent study of botryococcenes in the Berkeley isolate of *B. braunii*, Wolf and co-workers[6] discovered a new C_{32} hydrocarbon. We now describe experiments that establish the structure of the compound that we call braunicene (**11**), a botryococcenoid with a cyclohexyl moiety at the terminus of the 1 branch of the 1′-3 linkage.[32] A related cyclic structure was recently reported by Metzger et al.[21] for a C_{34} member of the family. We also report the absolute configuration of the quaternary (C10) chiral center in the molecule.

Results

The hydrocarbon fraction of *B. braunii* grown in an airlift culture under constant illumination consisted of a complex mixture of botryococcenes that constituted 28–34% of the colony dry weight. Fractionation of the oil from cultures grown to stationary

Chart I. Structures of Botryococcenes



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(1) Belcher, J. H. *Arch. Mikrobiol.* **1968**, *61*, 335–346.
(2) Wolf, F. R.; Cox, E. R. *J. Phycol.* **1981**, *17*, 395–405.
(3) Wake, L. V.; Hillen, L. W. *Biotechnology and Bioengineering*; Wiley: New York, 1980; Vol. 22, pp 1637–1656.
(4) Cane, R. F. *Trans. R. Soc. South Aust.* **1977**, *101*, 153–164.
(5) Moldowan, J. M.; Seifert, W. K. *J. Chem. Soc., Chem. Commun.* **1980**, 912–914.
(6) Wolf, F. R.; Nemethy, E. K.; Blanding, J. H.; Bassham, J. A. *Phytochemistry* **1985**, *24*, 733–737.
(7) Casadevall, E.; Metzger, P.; Puech, M. P. *Tetrahedron Lett.* **1984**, *25*, 4123–4126.
(8) Metzger, P.; David, M.; Casadevall, E. *Phytochemistry* **1987**, *26*, 129–134.
(9) Poulter, C. D.; Moesinger, S. G.; Epstein, W. W. *Tetrahedron Lett.* **1972**, 67–70.
(10) Epstein, W. W.; Poulter, C. D. *Phytochemistry* **1973**, *12*, 737–747.
(11) Poulter, C. D. *J. Agric. Food Chem.* **1974**, *22*, 167–173.
(12) Poulter, C. D.; Muscio, O. J.; Goodfellow, R. J. *Biochemistry* **1974**, *13*, 1530–1538.
(13) Poulter, C. D.; Marsh, L. L.; Hughes, J. M.; Argyle, J. C.; Satterwhite, D. M.; Goodfellow, R. J.; Moesinger, S. G. *J. Am. Chem. Soc.* **1977**, *99*, 3816–3823.

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Table I. ^1H and ^{13}C NMR Spectral Data for Branuicene

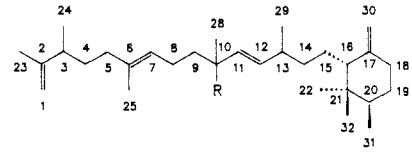
assignment	HETCOR:[a] δ (ppm) ^{13}C	^1H	COSY:[b] cross peaks	HETDJ:[a] protonation	^1H NOE's
C1	109.4	4.69	1.66, 2.12	CH_2	
C2	150.1			C	
C3	40.8	2.12	1.01, 1.35, 1.47, 1.66, 4.69	CH	
C4	33.4	1.35, 1.47	1.89, 2.11	CH_2	
C5	37.5	1.89	1.35, 1.47, 5.10	CH_2	5.10
C6	134.9			C	
C7	124.7	5.10	1.58, 1.89, 1.91	CH	1.37, 1.89
C8	23.2	1.91	1.37, 5.10	CH_2	1.58
C9	41.4	1.37	1.91	CH_2	5.10
C10	42.0			C	
C11	135.5	5.33	2.05, 5.18	CH	1.08, 1.37
C12	134.2	5.18	5.33, 2.05	CH	0.95, 1.08
C13	37.4	2.05	0.95, 0.98, 1.18, 5.18, 5.33	CH	
C14	35.9	0.98, 1.18	1.31, 1.44, 2.05	CH_2	
C15	24.1	1.31, 1.44	0.98, 1.18, 1.63	CH_2	
C16	56.7	1.63	1.31, 1.44, 4.52, 2.07 (or 2.10)	CH	4.52
C17	149.6			C	
C18	31.0	2.07, 2.10	1.21, 1.46, 1.63, 4.68	CH_2	
C19	32.1	1.21, 1.46	1.62, 2.07, 2.10	CH_2	
C20	34.8	1.62	0.78, 1.21, 1.46	CH	
C21	36.8			C	
C22	26.9	0.87	0.75	CH_3	0.75, 1.62
C23	19.0	1.66	4.69	CH_3	
C24	19.7	1.01	2.12	CH_3	
C25	15.9	1.58	1.91, 5.10	CH_3	1.91
C26	146.8	5.81	4.94, 4.96	CH	1.08
C27	111.1	4.94, 4.96	5.81	CH_2	1.08
C28	23.7	1.08		CH_3	1.37, 1.91, 4.94, 5.18, 5.35, 5.81
C29	21.0	0.95	2.05	CH_3	
C30	109.1	4.52, 4.69	1.63, 2.07, 2.09	CH_2	1.63
C31	15.8	0.78	1.62	CH_3	
C32	21.7	0.75	0.87	CH_3	0.87, 1.63

[a] Taken at 500 MHz. [b] Taken at 400 MHz.

phase by conventional chromatography on silica gel and reversed-phase HPLC gave a single pure component, braunicene (**11**). A high-resolution mass spectrum of the compound had a molecular ion at m/z 438.4225, in excellent agreement with the calculated value of 438.4228 for a $C_{32}H_{54}$ hydrocarbon. When the composition of the oil was monitored throughout the incubation, small amounts of braunicene were detected during the first few days of an incubation. However, the proportion of the hydrocarbon increased rapidly during late exponential phase and reached a maximum of approximately 25% of the total botryococcene fraction after 3–4 days into stationary phase (22–23 day old cultures).

Preliminary data indicated that braunicene was not a typical acyclic botryococcenoid. The hydrocarbon had six degrees of unsaturation, while only five double bonds were evident in ^1H and ^{13}C NMR spectra. The presence of a ring was also suggested by comparisons of the methyl regions in ^1H NMR spectra for braunicene and the linear C_{32} botryococcene 3.[20] The latter has four methyls attached to sp^2 hybrid carbons and four to sp^3 centers, while braunicene has only two methyls on sp^2 carbons, with the remaining six on sp^3 centers.

The carbon skeleton of braunicene was established from ^1H and ^{13}C NMR spectra summarized in Table I. HET2DJ[23] experiments revealed that **11** contained five C, eight CH, 11 CH_2, and eight CH_3 units. The olefinic subset consisted of three C, four



11, R = $^{26}CH{=}^{27}CH_2$

12, R = CH_2CH_2

CH, and three CH_2 units. HETCOR[24] data established connectivity between carbons and directly attached protons and revealed those methylene carbons (C4, C14, C15, C18, C19, C30) bearing nonequivalent protons. ^1H chemical shifts in complex regions were deduced from HETCOR and HOM2DJ[25] spectra. COSY[26] and NOE difference spectra were used to determine connectivity between protonated carbons.

A 400-MHz COSY spectrum of braunicene is shown in Figure 1. Starting at the end of the 3 branch of the 1′–3 linkage, COSY crosspeaks were seen from the vinyl protons at C1 to the C23 methyl (J = 0.9 and 1.3 Hz) and to the allylic proton at C3, which, in turn, was coupled to both diastereotopic methylene protons at C4 and the C24 methyl. The C5 methylene signals had crosspeaks to both C4 methylene protons and across the double bond to the vinyl proton at C7. The C7 methine was coupled to the C25 methyl (J = 1.2 Hz) and to the C8 methylene (J = 7.1 Hz), which also had crosspeaks to the C9 methylene (J = 8.5 Hz) and the C25 methyl. At this point COSY connectivity was interrupted by the quaternary center at C10.

(14) White, J. D.; Somers, T. C.; Reddy, G. N. *J. Am. Chem. Soc.* **1986**, *108*, 5352–5353.
(15) Maxwell, J. R.; Douglas, A. G.; Eglinton, G.; McCormick, A. *Phytochemistry* **1968**, *7*, 2157–2171.
(16) Cox, R. E.; Burlingame, A. L.; Wilson, D. M.; Eglinton, G.; Maxwell, J. R. *J. Chem. Soc., Chem. Commun.* **1973**, 284–285.
(17) Largeau, C.; Casadevall, E.; Berkaloff, C.; Dhamelincourt, P. *Phytochemistry* **1980**, *19*, 1043–1051.
(18) Wake, L. V.; Hillen, L. W. *Aust. J. Mar. Freshwater Res.* **1981**, *32*, 353–367.
(19) Galbraith, M. N.; Hillen, L. W.; Wake, L. V. *Phytochemistry* **1983**, *22*, 1441–1443.
(20) Metzger, P.; Casadevall, E. *Tetrahedron Lett.* **1983**, *24*, 4013–4016.
(21) Metzger, P.; Casadevall, E.; Pouet, M. J.; Pouet, Y. *Phytochemistry* **1985**, *24*, 2995–3002.
(22) See ref 21 and references therein.
(23) Bodenhausen, G.; Freeman, R.; Turner, D. L. *J. Chem. Phys.* **1976**, *65*, 839–840.
(24) Bax, A.; Freeman, R.; Morris, G. *J. Magn. Reson.* **1981**, *42*, 501–505.
(25) Freeman, R.; Hill, H. D. W. *J. Chem. Phys.* **1971**, *54*, 301–313.
(26) Bax, A.; Freeman, R.; Morris, G. *J. Magn. Reson.* **1981**, *42*, 164–168.

Table II. ^1H Spectral Data[a] for Keto Ester **13**

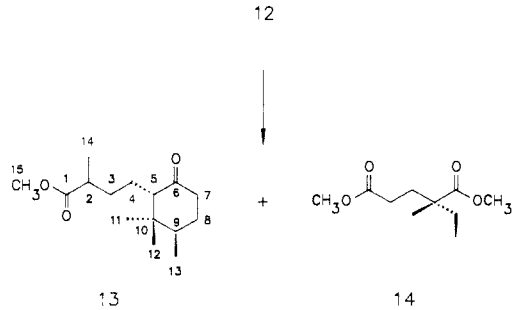
assignment (corresponding resonance in **11**)	δ (ppm)	COSY cross peaks	^1H NOE's	$J_{^1H-^1H}$
C2 (C13)	2.41	1.14, 1.33, 1.50		
C3 (C14)	1.33	1.45, 1.50, 1.65, 2.41		
C3 (C14)	1.50	1.33, 1.45, 1.65, 2.41		
C4 (C15)	1.45	1.33, 1.50, 1.65, 2.02		3.4
C4 (C15)	1.65	1.33, 1.45, 1.50, 2.02	1.33, 1.45, 1.83, 2.38, 2.41	11.1
C5eq (C16)	2.02	1.45, 1.65, 2.28	0.84, 0.90	1.3, 3.4, 11.1
C7eq (C18)	2.28	1.58, 1.90, 2.02, 2.38		1.3, 5.6, 13.9
C7ax (C18)	2.38	1.58, 1.90, 2.28	1.65, 1.83, 1.90, 2.28	6.6, 10.0, 13.9
C8eq (C19)	1.90	1.58, 1.83, 2.28, 2.38		4.2, 5.6, 6.6, 13.0
C8ax (C19)	1.58	1.83, 1.90, 2.28, 2.38		5.6, 9.3, 10.0, 13.0
C9ax (C20)	1.83	0.98, 1.58, 1.90	0.90, 0.98, 1.65, 2.38	4.2, 6.8, 9.3
C11eq (C22)	0.90		0.84, 1.83	
C12ax (C32)	0.84		0.90	
C13eq (C31)	0.98	1.83		6.8
C14 (C29)	1.14	2.41		7.0
C15	3.68			

[a] Taken at 500 MHz.

A second lengthy connectivity pattern began at C11 with crosspeaks to the C12 vinyl proton (J =15.7 Hz) and the C13 methine (J = 1.0). The signal for the C13 methine had crosspeaks to the C12 resonance (J = 7.8 Hz), the C29 methyl (J = 7.0 Hz), and the nonequivalent C14 methylene protons, both of which were also coupled to the two diastereotopic protons at C15. The C16 methine resonance coupled strongly to the C15 proton signal at 1.44 ppm (J = 11.7 Hz), weakly to the other methylene peak at 1.31 ppm (J = 3.4 Hz), and trans across the exocyclic double bond to the C30 signal at 4.52 ppm (J = 2.8 Hz), which was also coupled to the other C30 resonance at 4.68 ppm (J = 1.3 Hz). Both protons at C30 gave crosspeaks to the two C18 signals at 2.07 and 2.10 ppm. COSY crosspeaks also joined the nonequivalent methylenes at C18 and C19 and the C20 methine to signals for the C19 methylene and C31 methyl. Finally, the vinyl proton at C26 was linked to the C27 resonances at 4.94 ppm (J = 17.2 Hz) and 4.96 ppm (J = 10.6 Hz) by strong crosspeaks.

Additional connections were established from NOE measurements. The protons surrounding C10 were tied together by an extensive network of NOEs. NOEs were found between the C28 methyl and the *Z* proton at C27; the olefinic protons at C11, C12, and C26; and the methylene protons at C8 and C9. In the cyclohexyl moiety, the C22 methyl gave NOE's to the C31 and C32 methyls. The above data are sufficient to establish the basic skeleton of braunicene. In addition, NOE's between protons at C5 and C7 and between protons at C8 and C25 demonstrated that the stereochemistry of the trisubstituted double bond between C6 and C7 is *E*. The large (15.7 Hz) coupling constant between the olefinic protons at C11 and C12 showed that the stereochemistry of the disubstituted double bond was *E* as well.

The relative stereochemistry and preferred conformation of the cyclohexyl moiety in **11** could not readily be assigned because of spectral overlap. Selective reduction of the C26–C27 double bond in **11** with diimide gave the dihydro derivative **12**, which underwent ozonolysis followed by oxidation and esterification with diazomethane to afford a 1:1 mixture of keto ester **13** and diester **14**.



These compounds were separated by HPLC. The cyclohexyl carbonyl in **13** shifted the adjacent α protons downfield and permitted us to assign all of the resonances in the cyclohexyl moiety (Figure 2). A network of COSY crosspeaks (see Table II), which encompassed all of the ^1H spins except for the C11, C12, and C15 methyl groups, extended from the protons of the C14 methyl, across the C6 carbonyl moiety, to the C13 methyl in a pattern similar to that described for the 1′ branch of braunicene. The C8 proton resonance at 1.58 ppm had large diaxial couplings to protons at 1.83 (C9) and 2.38 (C7) ppm, establishing that all three were axial. Thus, the C13 methyl and the C7 proton at 2.28 ppm were equatorial.

The long-range coupling between between the equatorial C7 resonance and the C5 proton suggested that the latter was also

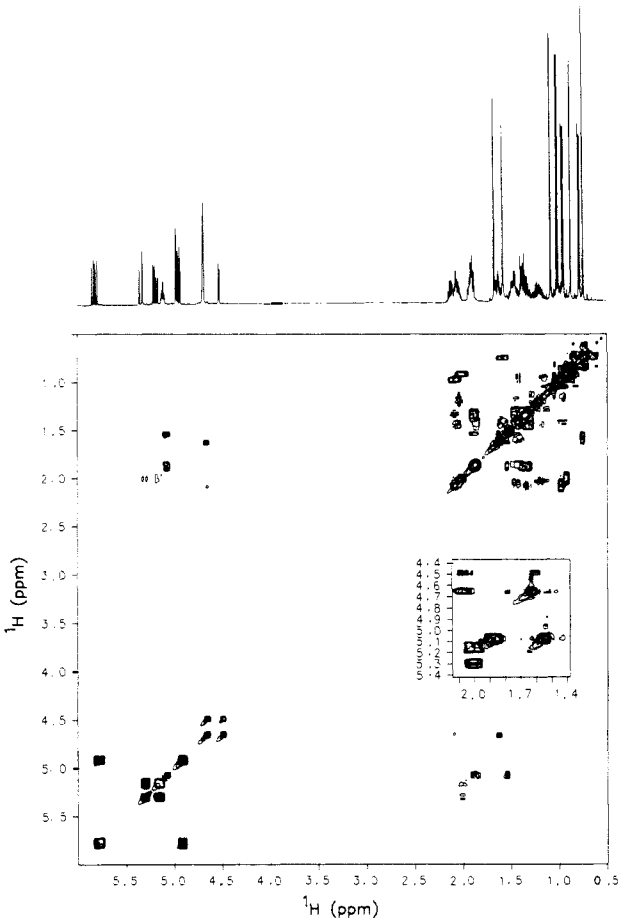


Figure 1. A 400-MHz ^1H COSY spectrum[26] of braunicene (**11**) in $CDCl_3$ at 26 °C. A one-dimensional projection is shown along the *x* axis. A total of 64 × 256 scans were collected by using a delay, D3, of 70 ms to enhance crosspeaks due to long-range couplings. The inset is an expansion at higher sensitivity of the crosspeaks between olefinic and aliphatic protons.

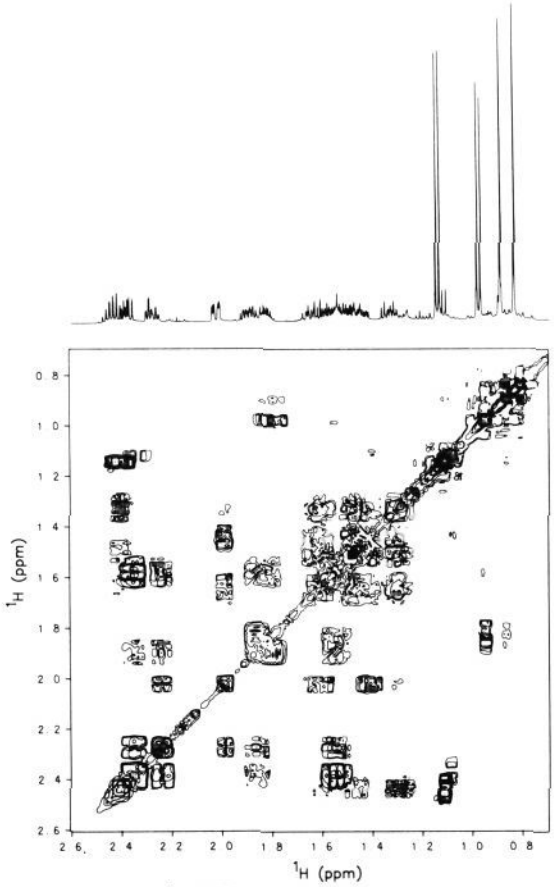
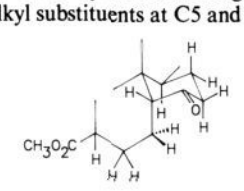


Figure 2. A 400-MHz ^{1}H COSY spectrum[26] of keto ester **13** in $CDCl_3$ at 26 °C. A one-dimensional projection is shown along the *x* axis. A total of 128 × 256 scans were collected by using a delay, D3, of 50 ms to enhance crosspeaks due to long-range couplings.

equatorial and formed a typical four-bond *W* interaction. This point was verified by NOE experiments. Of the two diastereotopic C4 protons, only the one at 1.65 ppm gave NOE's to the axial protons at C7 and C9. The large coupling constant (11.1 Hz) between the signal at 1.65 ppm and the proton at C5 and the NOE's described above indicated that the axial side chain was oriented away from the cyclohexane ring with one of the methylene hydrogens thrust under the ring near the two axial protons at C7 and C9. There was no NOE between the C5 proton and the axial C7 proton in keto ester **13**. However, upon standing in the refrigerator at 4 °C for several weeks, the keto ester partially isomerized to its C5 epimer, **15**. The major change in the ^{1}H NMR spectrum of **13** upon epimerization to **15** was a downfield shift for the C5 proton from 2.02 to 2.10 ppm (broad d, J = 9.75 Hz). The C5 resonance in **15** was no longer coupled to the equatorial C7 proton and gave NOE's to both diaxial hydrogens on the bottom face of the cyclohexane ring. These results established that the alkyl substituents at C5 and C9 in **13** were trans



13

and that the preferred conformation of the cyclohexane ring was a chair with the alkyl substituent at C5 in the axial position. Since ozonolysis of braunicene gave only trans keto ester **13**, the cyclohexyl ring in the hydrocarbon must have trans substituents as well. Comparisons between the ^{1}H NMR spectra of **11** and **13** suggest that the conformations of the cyclohexyl rings are also similar.

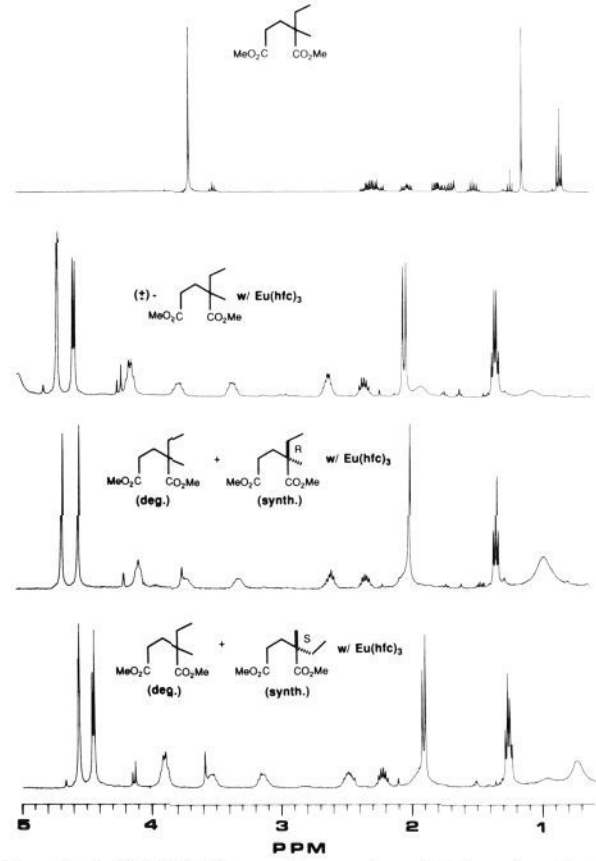


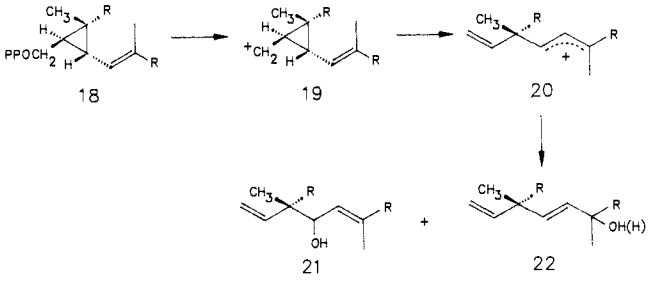
Figure 3. A 400-MHz ^{1}H spectral comparison of **15** from degradation of braunicene with synthetic *R* and *S* diesters in the presence of the chiral shift reagent Eu(hfc)$_3$.

The absolute configuration at C10 of braunicene was established in a manner similar to that described for botryococcene.[14] As previously described, diester **14** was obtained in the same sequence of reactions that gave keto ester **13**. This material was then compared with authentic samples of the *R* and *S* enantiomers of **14**. The assignment of absolute stereochemistry to C_{34} botryococcene previously made use of the (2*R*,5*S*)- and (2*S*,5*S*)-2,5-dimethyl-2-ethyl-5-hydroxypentanoic acid lactone (**16** and **17**,

16 17

respectively)[14] whose configurations were rigorously established by their derivation from propylene oxide, together with an X-ray crystallographic analysis of the dicyclohexylamine salt of the opened hydroxy acid. The latter determination allowed a relative correlation of the quaternary center in botryococcene with the absolute configuration at C5. Treatment of **16** or **17** with sodium hypobromite effected concomitant saponification, oxidation of the secondary alcohol, and haloform reaction of the resulting methyl ketone to yield a dicarboxylic acid, which, upon exposure to diazomethane, gave the corresponding dimethyl ester. With use of *tris[3-[[(heptafluoropropyl)hydroxy]methylene]-(+)-camphorato]*europium(III) (Eu(hfc)$_3$) as a chiral shift reagent, it was first determined that clear separation of major signals could be seen in the ^{1}H NMR spectrum of racemic diester. Mixtures (1:1) of **14** from degradation of braunicene with synthesized *R* and *S* diesters were then dosed with increasing concentrations of Eu(hfc)$_3$, and the ^{1}H NMR spectra were measured. As seen from Figure 3, the diester from degradation is clearly identical with the substance of *R* configuration, thereby specifying C10 of braunicene as *S*.

Scheme I. A Mechanism for Formation of 1′–3 Terpenes



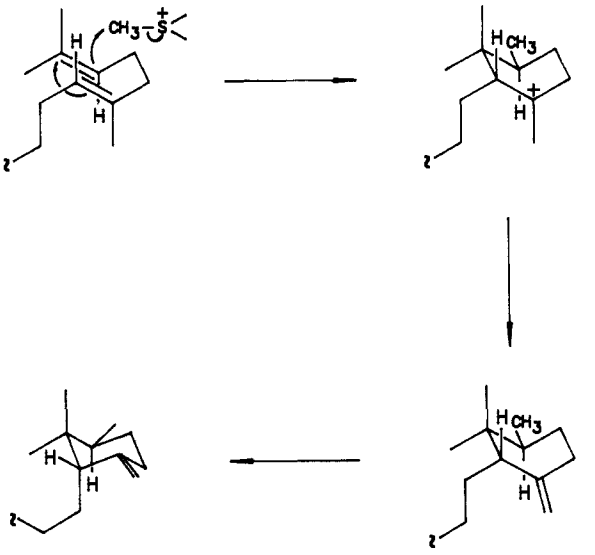
Discussion

Braunicene (**11**) is a new C_{32} member of the botryococcene family. Typical of all botryococcenoids, the carbon skeleton of **11** is constructed from two C_{15} farnesyl moieties joined by a 1′–3 fusion, and the molecule has an *E*-disubstituted double bond in the 1′ branch of the chain. In addition, braunicene contains a unique pentasubstituted cyclohexane ring at the terminus of the 1′ branch. Several years ago, as the result of model studies on the mechanism of the conversion of presqualene diphosphate to squalene, Poulter and co-workers[13] proposed that isoprenoids with 1′–3 linkages are formed by rearrangement of cyclopropylcarbinyl precursors. They discovered that artemisia alcohol (**21**, R = CH_3) and yomogi alcohol (**22**, R = CH_3), monoterpenes with 1′–3 linkages, were the principal products of solvolysis of a variety of chrysanthemyl derivatives (**18**, R = CH_3) in water[9] and were formed by hydration of allylic cation (**20**, R = CH_3), following rupture of a cyclopropane bond in cyclopropylcarbinyl cation **19**, as illustrated in Scheme I.[13] A similar mechanism was proposed for the biosynthesis of artemisia and yomogi alcohols. They also postulated that the 1′–3 linkage in botryococcenes was formed by a similar rearrangement of a cyclopropylcarbinyl precursor, in this case the C_{30} cation (R = $C_{11}H_{19}$) generated from presqualene diphosphate, in a reaction terminated by transfer of hydride from NADH or NADPH to the allylic cation.[13] White and co-workers[14] recently discovered that C_{34} botryococcene **5** has a 10*S*,13*R* configuration. The absolute configuration at C10 is consistent with biosynthesis of botryococcenes from the natural (1*R*,2*R*,3*R*) enantiomer of presqualene diphosphate. Furthermore, the 13*R* center is presumably formed by delivery of hydride to the *si* face of allylic cation (**20**, R = $C_{11}H_{19}$). A comparison of the biosynthesis of botryococcene and squalene from presqualene diphosphate indicates that the relative positions of the C_{30} substrate and the nicotinamide cofactor are similar in the enzyme–substrate complexes of squalene synthetase and botryococcene synthetase. Since *B. braunii* produces squalene, the botryococcene enzyme may well have evolved from squalene synthetase. We have now found that braunicene also has the 10*S* configuration.

Pulse-chase experiments by Wolf and co-workers[6] and feeding studies by Metzger et al.[8] provide strong evidence for formation of higher botryococcenes from **1** by successive electrophilic methyl transfers from *S*-adenosyl methionine to carbon–carbon double bonds in the isoprenoid chains. A similar mechanism is presumably responsible for cyclization. As illustrated in Scheme II, electrophilic methylation at C20 of a C_{30} or C_{31} precursor could generate a cyclohexyl cation, which, upon elimination of a hydrogen from the methyl at C30, would yield braunicene. The trans juxtaposition of the substituents at C16 and C20 probably arises from a sterically favored *si–si* methylation–cyclization from a "boat" conformation.

Our experiments do not permit us to determine which of the two methylations in braunicene occurs first. Given the rich variety of patterns seen in this family of hydrocarbons, it is quite possible that methylases in the botryococcene pathway have broad substrate specificities and that there are multiple routes to polymethylated derivatives. There is also a report of methylation of squalene by *B. braunii*,[20] which may result from an adventitious methyl addition by a botryococcene methylase. The only other cyclic botryococcene reported to date is a C_{34} hydrocarbon bearing a pentamethylated cyclohexenyl moiety at the end of the 1′ branch.

Scheme II. A Mechanism for Cyclizations Initiated by *S*-Adenosyl Methionine



Metzger and co-workers[21] proposed a methyl-initiated cationic cyclization similar to that shown in Scheme II, followed by elimination to produce an endocyclic trisubstituted double bond, which was then further methylated to account for the structure. It is worth noting that biosynthesis of braunicene peaks late in the growth cycle of the organism when the culture changes color from deep green to bright brown-orange as carotenoids accumulate. It will be interesting to see if the activities for methyl-initiated cyclization in the botryococcene pathway and proton-initiated cyclization in carotenogenesis are expressed in a coordinate manner.

Iris produces methylated triterpenoids derived from squalene,[27-29] some of which contain cyclohexyl rings presumably formed during methylation by *S*-adenosyl methionine of isoprenoid chains by reactions similar to those proposed for biosynthesis of braunicene. These compounds are thought to be the precursors of the fragrant violet-scented irones found in the plant. Both cis and trans isomers, α- and γ-irone respectively, are found in the essential oil of iris. Presumably the trans stereochemistry in γ-irone is generated by cyclization of the isoprenoid chain in the "boat" conformation shown in Scheme II and the cis stereochemistry in α-irone by cyclization of the "chair" conformer. The Berkeley isolate of *B. braunii* contains several unidentified botryococcenes, and it will be of interest to see if cis braunicenes are also produced by the alga.

Experimental Section

NMR spectra were recorded on Varian XL-300, XL-400, or VXR-500 spectrometers and were referenced to internal tetramethylsilane. COSY spectra were obtained according to the protocol of Bax and co-workers[26] with a delay, D3, between the second pulse and the acquisition period to enhance weak long-range interactions. Homonuclear two-dimensional *J*-correlated spectra (HOM2DJ) were obtained by the pulse sequence reported by Freeman and Hill,[25] heteronuclear two-dimensional *J* spectra (HET2DJ) by the procedure of Bodenhansen and co-workers,[23] and heteronuclear chemical shift correlated spectra (HETCOR) by the method of Bax et al.[24] Mass spectra were recorded on a VG Micromass 7070E mass spectrometer. A DB-5 fused-silica capillary column (30 m × 0.25 mm) was used for all GCMS work. Rotations were measured with a Perkin-Elmer Model 241 MC polarimeter.

Cultures. Air lift cultures were grown according to the protocol of Wolf et al.[6] Standard growth media contained the following components (milligrams/liter of deionized water): $Ca(NO_3)_2 \cdot 4H_2O$ (100), NH_4Cl (26.5), $MgSO_4 \cdot 7H_2O$ (25), K_2HPO_4 (10), H_3BO_3 (0.6), Na_2EDTA (7.7),

(27) Marner, F. J.; Krick, W.; Gellrich, B.; Jaenicke, L.; Winter, W. *J. Org. Chem.* **1982**, *47*, 2531–2536.
(28) Krick, W.; Marner, F. J.; Jaenicke, L. *Z. Naturforsch., C: Biosci.* **1983**, *38C*, 179–184.
(29) Krick, W.; Marner, F. J.; Jaenicke, L. *Z. Naturforsch., C: Biosci.* **1983**, *38C*, 689–692.

$ZnCl_2$ (0.624), $CuCl_2 \cdot 2H_2O$ (0.268), $NaMoO_4 \cdot 2H_2O$ (0.252), $CoCl_2 \cdot 6H_2O$ (0.420), $FeSO_4 \cdot 7H_2O$ (2.5), $MnCl_2 \cdot 4H_2O$ (0.360), MES (1950). pH was adjusted to 6.5 with 3 N potassium hydroxide. Cultures were started by innoculation of 800 mL of sterile media in a 1-L culture flask with 50 mL of a stationary-phase culture (20–22 days old). The contents of the flask were aerated with 1% CO_2-enriched air introduced through a gas dispersion tube. Temperature was maintained at 25 °C, and the flask was illuminated continuously with cool-white fluorescent light. Cultures followed a typical exponential growth curve and reached stationary phase after 16–17 days.

Harvest and Extraction. Two 800-mL 23 day old cultures were filtered through 5-μm Nitex cloth, and the orange-brown mass was washed three times with 50 mL of 0.9% saline. The residue was lyophilized for 24 h to yield 3.6 g of dry cells. The material was stirred with 40 mL of 1:1 (v/v) hexanes–acetone for 3 h under nitrogen. Solvent was removed, and the extraction was repeated. A final extraction was performed with 1:1 (v/v) chloroform–methanol. The extracts were combined and filtered. Solvent was removed at reduced pressure to yield a deeply colored oil. The residue was loaded on a 2.5 cm × 16 cm column of silica gel (Baker, 60–200 mesh) and eluted with 4 column volumes of dry hexanes. Solvent was removed at reduced pressure to yield 1.08 g of a colorless oil.

Purification of Braunicene (11). The oil (1.00 g) was loaded on a 2.5 cm × 60 cm silica gel column (Baker, 200–400 mesh, activated by heating at 150 °C for 48 h prior to chromatography) and eluted by gravity with 3 L of dry hexanes. Fractions were monitored by gas chromatography on a DB-5 fused silica capillary column (30 m × 0.25 mm, 245 °C) and were combined into four groups on the basis of GC profiles. The first group (249 mg of a colorless oil) was further purified by HPLC on a 5 μm ODS-Hypersil semi-preparative column (30 cm × 7.8 mm) with elution by acetonitrile by repeated injection of 5–8 mg of material. Fractions containing the last major peak to elute were combined, and solvent was removed to yield 149 mg (15% of the starting sample) of a colorless oil: $[\alpha]^{25}_D$ −29° ($CHCl_3$, c 3.15); IR (neat) 3069, 2964, 2957, 2933, 2926, 2869, 1646, 1453, 1387, 1372, 999, 976, 910, 888, and 737 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) δ 5.81 (1 H, dd, J = 17.2 and 10.6 Hz), 5.33 (1 H, dd, J = 15.7 and 1.0 Hz), 5.18 (1 H, dd, J = 15.7 and 7.8 Hz), 5.10 (1 H, tm, J = 7.1 Hz), 4.96 (1 H, dd, J = 10.6 and 1.5 Hz), 4.94 (1 H, dd, J = 17.2 and 1.5 Hz), 4.69 (3 H, m), 4.52 (1 H, dd, J = 2.8 and 1.3 Hz), 2.12 (1 H, m), 2.10 (1 H, m), 2.07 (1 H, m), 2.05 (1 H, m), 1.91 (2 H, dt, J = 7.1 and 8.5 Hz), 1.89 (2 H, t, J = 7.9 Hz), 1.66 (3 H, dd, J = 1.3 and 0.9 Hz), 1.63 (1 H, ddd, J = 11.7, 3.4, 2.8 Hz), 1.62 (1 H, m), 1.58 (3 H, d, J = 1.2 Hz), 1.47 (1 H, m), 1.46 (1 H, m), 1.44 (1 H, m), 1.37 (2 H, t, J = 8.5 Hz), 1.35 (1 H, m), 1.31 (1 H, m), 1.21 (1 H, m), 1.18 (1 H, m), 1.08 (3 H, s), 1.01 (3 H, d, J = 6.8 Hz), 0.98 (1 H, m), 0.95 (3 H, d, J = 7.0 Hz), 0.87 (3 H, s), 0.78 (3 H, d, J = 6.5 Hz), 0.75 (3 H, s); HRMS, m/z 438.4225 ($C_{32}H_{54}$, 438.4228); GCMS, m/z (17 eV, intensity) 69 (31), 95 (100), 109 (66), 123 (80), 137 (28), 149 (22), 191 (23), 205 (20), 217 (16), 231 (12), 273 (9), 355 (3), 368 (5), 423 (5), 438 (M^+, 22).

Dihydrobraunicene (12). A solution of 48 mg (0.11 mmol) of braunicene in 15 mL of 1:4 (v/v) 2-propanol–ethanol was cooled to 0 °C. Four drops of a 1 mM aqueous solution of cupric acetate followed by addition of 0.5 mL of a solution of hydrogen peroxide in ethanol (1.8 mL of 30% H_2O_2 in 4.0 mL of ethanol) and 32 μL of hydrazine at 10-min intervals. After 12 additions, starting material was consumed as determined by gas chromatography (DB-5, 260 °C). Excess hydrogen peroxide was decomposed with 5 mL of saturated sodium thiosulfate solution. The resulting mixture was added to brine and extracted three times with 20-mL portions of hexanes. The extracts were washed with brine and dried over magnesium sulfate. Solvent was removed at reduced pressure, and the residue was purified by HPLC on a 5 μm ODS-Hypersil column (30 cm × 7.8 mm) with elution by methanol to yield 28 mg (58%) of a colorless oil: $[\alpha]^{25}_D$ −27.5° ($CHCl_3$, c 1.09); IR (neat) 3068, 2962, 2932, 2824, 1649, 1644, 1461, 1453, 1327, 976, 887 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 5.19 (1 H, d, J = 15.8 Hz), 5.11 (1 H, tm, J = 7.1 Hz), 5.10 (1 H, dd, J = 15.8 and 7.6 Hz), 4.69 (3 H, m), 4.52 (1 H, dd, J = 2.5 and 1.1 Hz), 2.00–2.15 (4 H, m), 1.90 (2 H, dt), 1.88 (2 H, t), 1.67 (3 H, dd, J = 1.3 and 0.9 Hz), 1.63 (1 H, m), 1.62 (1 H, m), 1.58 (3 H, d, J = 1.2 Hz), 1.50–1.40 (3 H, m), 1.39–1.15 (9 H, m), 1.02 (3 H, d, J = 6.9 Hz), 0.98 (1 H, m), 0.95 (3 H, d, J = 6.7 Hz), 0.92 (3 H, s), 0.87 (3 H, s), 0.78 (3 H, d, J = 6.8 Hz), 0.78 (3 H, t, J = 7.5 Hz), 0.75 (3 H, s); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 8.6, 15.9, 16.0, 19.1, 19.8, 21.4, 21.8, 22.8, 23.0, 24.2, 27.0, 31.1, 32.2, 33.5, 33.8, 34.8, 36.1, 36.9, 37.6, 37.7, 38.6, 40.9, 41.3, 56.8, 109.0, 109.3, 124.9, 133.9, 134.6, 136.6, 149.6, 150.1; HRMS, m/z 440.4340 ($C_{32}H_{56}$, 440.4385); GCMS, m/z (17 eV) 69, 83, 95 (base), 109, 123, 137, 151, 163, 177, 191, 205, 219, 245, 276, 302, 341, 370, 411, 440 (M^+).

Ozonolysis of 12. A solution of 41 mg (0.094 mmol) of dihydrobraunicene in 7.0 mL of 5:1:1 (v/v/v) methylene chloride–ethyl acetate–methanol was cooled to −78 °C. Ozone was passed through the solution until a blue color persisted. An additional 2 mL of cold methanol was added, and after 10 min excess ozone was removed by gently bubbling nitrogen through the solution. Solvent was removed at reduced pressure. The residue was dissolved in 10 mL of acetone at 0 °C, and 1.4 mL of Jones reagent[30] (1.4 M, 1.9 mmol) were added. The resulting solution was allowed to stir for 10 min at 0 °C and then quenched by dropwise addition of 2-propanol until the orange-brown mixture turned green. The mixture was filtered through Celite, and the filter bed was washed with 20 mL of methylene chloride. Solvent was removed at reduced pressure. The residue was dissolved in 10 mL of ether and treated with excess diazomethane (~10 mmol) for 2 h at room temperature. Excess diazomethane was removed with a gentle stream of nitrogen. Solvent was removed at reduced pressure, and the residue was passed through a plug of silica gel (Baker, 200–400 mesh) with elution by 2:5 (v/v) ethyl acetate–hexanes. Solvent was removed at reduced pressure, and the residue was purified by HPLC on a 5 μm Porisil column (30 cm × 3.9 mm) with elution by 1:5 (v/v) ethyl acetate–hexane to yield 10.4 mg (39%) of **13** as a colorless oil: $[\alpha]^{25}_D$ −20.5° ($CHCl_3$, c 0.30); IR (neat) 2968, 2878, 1735, 1708, 1460, 1433, 1375, 1198, 1163 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) δ 3.68 (3 H, s), 2.41 (1 H, m), 2.38 (1 H, ddd, J = 13.9, 10.0, and 6.6 Hz), 2.28 (1 H, dtd, J = 13.9, 5.6, and 1.3 Hz), 2.02 (1 H, ddd, J = 11.1, 3.4, and 1.3 Hz), 1.90 (1 H, dddd, J = 13.0, 6.6, 5.6, and 4.2 Hz), 1.83 (1 H, dqd, J = 9.3, 6.8, and 4.20 Hz), 1.65 (1 H, dm, J = 11.1 Hz), 1.58 (1 H, dddd, J = 13.0, 10.0, 9.3, and 5.6 Hz), 1.50 (1 H, m), 1.45 (1 H, m), 1.33 (1 H, m), 1.14 (3 H, d, J = 7.0 Hz), 0.98 (3 H, d, J = 6.8 Hz), 0.89 (3 H, s), 0.84 (3 H, s); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 14.9, 16.9, 23.0, 23.5, 25.7, 30.7, 32.2, 35.8, 37.9, 39.5, 40.2, 51.3, 60.8, 176.9, 214.7; GCMS, m/z (17 eV) 44, 55, 59, 69, 83, 101, 111, 116, 129, 143, 144, 171. Further elution gave 6.0 mg (34%) of **14**, $[\alpha]^{25}_D$ +8.5° ($CHCl_3$, c 0.3), identical with (*R*)-(+)-**14** prepared as described below.

(*R*)-Dimethyl 2-Ethyl-2-methylglutarate (14). To a stirred solution of 385 mg (9.60 mmol) of sodium hydroxide in 2.5 mL of water and 3.5 mL of dioxane at 0 °C was added 165 μL (511 mg, 3.20 mmol) of bromine dropwise. After 10 min, a solution of 50.0 mg (0.320 mmol) of (2*R*,5*S*)-2,5-dimethyl-2-ethyl-5-hydroxypentanoic acid lactone (**16**) in 1.5 mL of dioxane was added, and the solution was maintained at 0 °C for 15 h. The mixture was warmed to room temperature, quenched with 10% aqueous sodium sulfite, and heated to 100 °C for 25 min. The cooled solution was acidified to pH 2 with 3 M hydrochloric acid and extracted with four portions of ethyl acetate. Concentration of the dried solution (sodium sulfate) gave a diacid, which was dissolved in ether and treated with excess diazomethane for 10 h. Chromatography of the concentrate on 13 g of silica gel 60 with elution by ethyl acetate–hexanes (1:5, v/v) gave 41.4 mg (64%) of (*R*)-(+)-**14** as a colorless oil: $[\alpha]^{23}_D$ +7.5° ($CHCl_3$, c 2.07) (lit.[31] $[\alpha]^{25}_D$ +9.8°). Similar treatment of 33.0 mg of **17** gave 27.5 mg (64%) of (*S*)-(−)-**14**: $[\alpha]^{23}_D$ −7.3 ($CHCl_3$, c 1.38); IR (neat) 2972, 1730, 1242, 1175 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 3.67 (6 H, s), 2.28 (2 H, m), 2.00 (1 H, m), 1.73 (2 H, m), 1.48 (1 H, m), 1.12 (3 H, s), 0.83 (3 H, t, J = 7.5 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 177.12, 173.91, 51.69, 51.64, 45.72, 33.40, 31.89, 29.75, 20.66, 8.87; MS, m/z 202 (M^+), 171, 143, 129, 116, 111, 83, 69, 55.

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(30) Bowers, A.; Halsall, T. G.; Jones, E. R. H.; Lemin, A. J. *J. Chem. Soc.* **1953**, 2548–2560.

(31) Rao, P.; Bhalla, V. K.; Nayak, U. R.; Dev, S. *Tetrahedron* **1973**, *29*, 1127.

(32) **Note Added in Proof.** A structure for braunicene was recently reported by Murakami et al. (Murakami, M.; Nakano, H.; Yamaguchi, K.; Konosu, S.; Nakayama, O.; Matsumoto, Y.; Iwamoto, H. *Phytochemistry* **1971**, *27*, 455–457).