Production of Hydroxy Unsaturated Fatty Acids Using Crude Lipoxygenase Obtained from Infected Rice Plants

Tadahiro Kato,* Tsutomu Watanabe, Toshifumi Hirukawa, Norihiro Tomita, and Tsuneo Namai†

Department of Chemistry, Faculty of Science, Science University of Tokyo, Kagurazaka 1-3, Shinjuku-ku, Tokyo 162

†Faculty of Agriculture, Yamagata University, Wakaba-machi 1-23, Tsuruoka 997

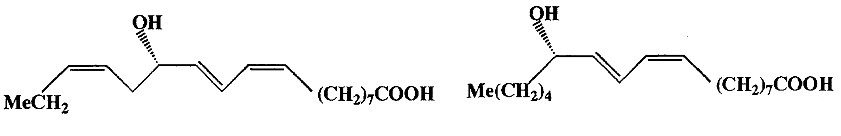
(Received December 8, 1995)

In order to explore the oxidation mode of lipoxygenase (LOX) obtained from infected rice plants, typical unsaturated fatty acids (**3**—**8**) were treated with LOX and oxygen. It was observed that ω-10 and ω-6 positions of unsaturated fatty acids were oxidized predominantly in the cases of exotic ω-6 (**4** and **5**) and ω-3 (**7** and **8**) series, respectively. In the case of endogenous fatty acids (**3** and **6**), oxidation at ω-6 position predominated. All the allylic alcohols obtained by reduction of the oxidation products with $NaBH_4$ possess *S*-configuration.

In our previous paper, we have explained that the allylic alcohols such as **1** and **2** are accumulated as defense substances to rice blast fungus in the infected rice plants.[1] We have further demonstrated that the activity of lipoxygenase (LOX) increases markedly in rice plants in response to the infection with the pathogens.[2] LOX may participate in the synthesis of the allylic alcohols (**1** and **2**) via the corresponding hydroperoxy fatty acids (Fig. 1). Since the allylic alcohols possessing the same partial structure of the fatty acids are expected to serve as chiral synthons in chemical synthesis, it is of particular interest to examine whether exotic polyunsaturated fatty acids are generally oxidized by the action of crude enzyme solution of infected rice plants. In addition, someone still must explore the easy way of selective oxidation[3] of polyunsaturated fatty acids since some of the oxygenated fatty acids have been reported to possess specific physiological activities.[4] This paper concerns the oxidation mode of crude LOX from infected rice plants toward the easily available polyunsaturated fatty acids (**3**—**8**) listed in Chart 1.

First, the oxidation mode of endogenous linoleic acid (**3**) was examined in detail. Linoleic acid was stirred at room temperature in the crude enzyme solution for 2 h and then extracted with ether. The enzyme solution was simply prepared by homogenation of fresh leaves of infected rice plants in McIlvaine buffer solution at pH 6.7. The ether extracts were treated with $NaBH_4$ to convert the hydroperoxides into the corresponding alcohols. Esterification of the reduction products with diazomethane followed by separation with silica-gel column chromatography gave, in addition to recovered methyl linoleate, crude allylic alcohols in 23% yield (45% conversion yield). HPLC analysis of the crude alcohols showed that it was a 85 : 15 mixture of two components, which were separated by preparative HPLC to isolate the major alcohol (**9**) and the minor one (**10**). Each of the alcohols was converted to the corresponding trimethylsilyl ether (R=TMS) and benzoate ($R=COC_6H_5$), respectively. The detailed H–H COSY analysis in the 400 MHz ^{1}H NMR spectra of the benzoates demonstrates the presence of α-benzoyloxy-$\beta(E)$, $\delta(Z)$-diene moiety [H_α at $\delta=5.55$ with $J_{\alpha,\beta}=7.0$ Hz, H_β at 5.68 with $J_{\beta,\gamma}=15.2$ Hz, H_γ at 6.59 with $J_{\gamma,\delta}=11.1$ Hz, H_δ at 5.96 with $J_{\delta,\varepsilon}=10.8$ Hz and H_ε at 5.46 (m)]. The presence of the conjugated diene unit was supported by UV spectra of **9** and **10**, showing the absorption maximum at 236 nm. The position of the hydroxyl group was estimated by the fragmentation pattern of the trimethylsilyl ethers. The strong fragment ions were observed at *m/z* at 173 and 311 derived from the cleavage between C13–C14 bond (*m/z* 311) and C12–C13 bond (*m/z* 173) in the mass spectrum of trimethylsilyl ether of **9** (R = TMS). The fragment ions caused by the similar bond fissions were observed in **10** (R = TMS) as shown in Fig. 2. The *S*-configuration of the asymmetric positions was determined by CD spectra of benzoates (**9** and **10**, $R=COC_6H_5$), both showing a positive Cotton effect.[5] Optical rotation of the benzoates of **9** and **10** ($R=COC_6H_5$) had +74.5° and +78.5°, respectively. Oishi and co-workers[6] have achieved the asymmetric synthesis of **9**, the benzoate of which was reported to have $[\alpha]_D$ +64.1°. Since the intermediate alcohol prepared by microbial reduction of α,γ-conjugated ynenone, was described to be over 99% ee in Oishi's synthesis, the evidence of the optical rotations suggests that the allylic alcohols obtained by the present method are over 99% ee. It should be noted that the corresponding benzoates isolated previously from the infected rice plants showed $[\alpha]_D$ +5.8° and +2.2°, respectively.[1] This fact indicates clearly that the allylic alcohols isolated from the infected rice plants are heavily racemic although the racemization mechanism in the rice plants is obscure at present.

Thus, it was found that the oxidation of endogenous linoleic acid (**3**) occurs at both ω-6 and ω-10 positions, the former position being oxidized predominantly. The rest of exotic polyunsaturated fatty acids (**4**—**8**) including en-



1 **2**

Fig. 1. Allylic alcohols from infected rice plants.

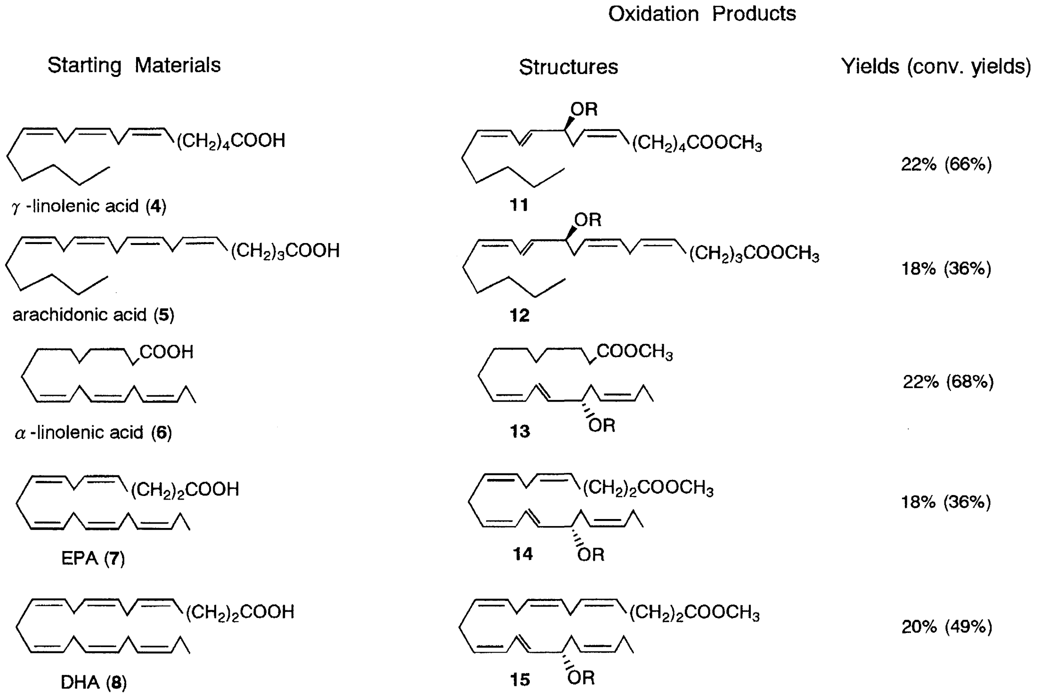


Chart 1. Major oxidation products of polyunsaturated fatty acids.

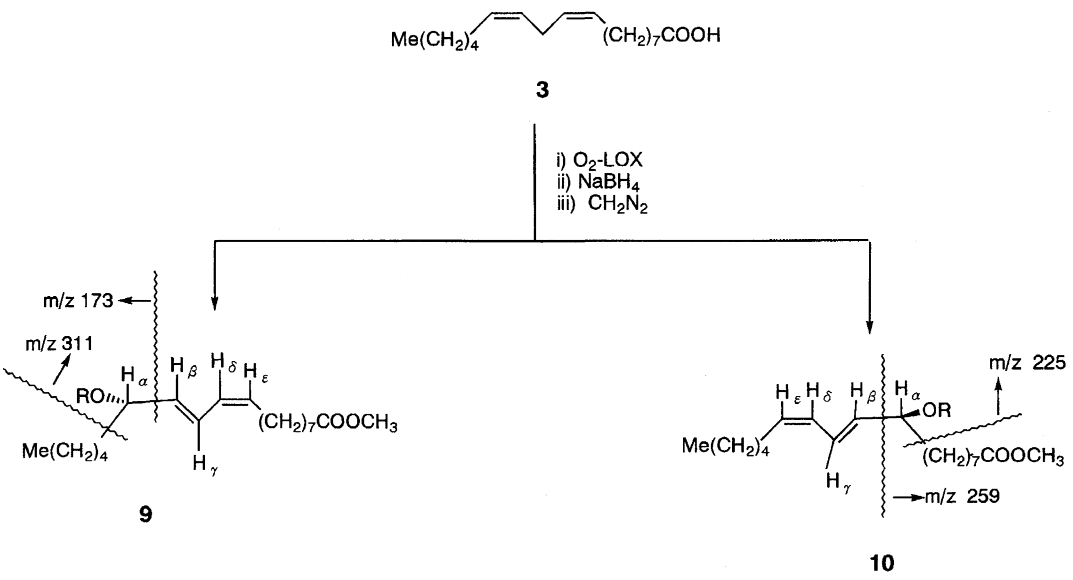


Fig. 2. Oxidation mode of linoleic acid (**3**) with LOX.

dogenous α-linolenic acid (**6**) were treated with LOX under the same conditions and the structures of the resultant allylic alcohols were elucidated by the same methods as described in the case of linoleic acid. The results are summarized in Chart 1. The CD spectra of the benzoates indicate that all the allylic alcohols derived from hydroperoxides by reduction with $NaBH_4$ have *S*-configuration. Although exact enantiomeric excess still remains undetermined, the optical rotations of the benzoates indicate that all the allylic alcohols were formed with high enantiomeric excess. It was observed that oxidation occurred at ω-10 position with more than 90% regioselectivity in the cases of ω-6 type fatty acids such as γ-linolenic (**4**) and arachidonic (**5**) acids. On the other hand, ω-6 position is oxidized with high selectivity in the series of ω-3 type polyunsaturated fatty acids such as eicosapentaenoic acid (**7**) (EPA) and docosahexaenoic acid (**8**) (DHA).[7] It should be noted that oxidation at ω-6 position predominates in the case of endogenous fatty acids (**3** and **6**).

Since the infected rice plants can be stored in refrigerator at $-30\ ^\circ\text{C}$ for a long time, the present finding provides a method for preparation of various types of allylic alcohols possessing *S*-configuration from easily available polyunsaturated fatty acids.

Experimental

Column chromatographic purifications were carried out on kieselgel 60, Art 7734 (70—230 mesh) or 5% silver nitrate impregnated kieselgel 60, Art 7734 using solvent as indicated. Unless otherwise stated, all the spectroscopic data were determined on pure samples obtained by column chromatography or preparative HPLC, checking the purity by TLC or HPLC analyses. The IR spectra were determined on a Hitachi 270-30 spectrometer. The NMR spectra were recorded with a Brucker AM 400 spectrometer with tetramethylsilane as an internal standard. The mass spectra, CD spectra, and optical rotations were measured with a Hitachi M-80 mass spectrometer, JASCO J-500A spectropolarimeter, and Perkin–Elmer polarimeter, respectively. The CD spectra were measured in EtOH and expressed with λ_{ext}.

Purification of Polyunsaturated Fatty Acids. To an ether (20 cm^3) solution of each of the commercially available fatty acids (300 mg) was added an excess of diazomethane in ether solution. The mixture was kept at room temperature for 10 min. The volatile materials were removed and crude oily ester was purified with 5% $AgNO_3$ impregnated silica-gel (15 g) column chromatography (hexane : AcOEt=20 : 1). After the purified methyl ester (610 mg) in methanolic 2 M KOH (10 cm^3) ($M=\text{mol dm}^{-3}$) was stirred at room temperature for 6 h, the methanol was removed in vacuo at 30 °C. The residue was diluted with 20 cm^3 each of water and ether and the mixture was shaken vigorously. The resultant emulsified mixture was frozen at -30 °C overnight to isolate the ether phase from the frozen water. After the frozen mixture was gently warmed to room temperature, the ether phase was separated from the aqueous phase. 0.1 M HCl was added to the aqueous phase and the mixture was extracted with ether. The ether solution was washed with brine and dried over $MgSO_4$. Evaporation of volatile materials afforded free acid (540 mg).

Fatty Acid Suspension in Tris-HCl Buffer. The purified DHA (540 mg) was added to distilled water (48 cm^3) containing 2 drops of Tween 80 and the mixture was stirred vigorously with a magnetic stirrer. The mixture was diluted with 48 cm^3 of Tris-HCl buffer solution (pH 7.6) and stirred again for 30 min. The resultant suspension was kept at -30 °C before usage. The Tris-HCl buffer suspension of the rest of the fatty acids was prepared similarly.

Crude LOX Solution of Infected Rice Plants. Fresh leaves (100 g) of infected rice plants (cultivar, Aichi Asahi) were cut to about 10 mm length and then frozen by liquid nitrogen and ground with a pestle and mortar. The powdered leaves were transferred into 2 dm^3 of MacIlvaine buffer (pH 6.7) and homogenized with Polytron Kinematica. During the homogenation procedure, the mixture was kept at 5 °C by chilling with ice water. The homogenate was filtered by four layers of gauze, and the filtrate was used for the oxidation of fatty acids.

Oxidation of Fatty Acids with Crude LOX Solution. Tris-HCl buffer suspension (108 cm^3) of linoleic acid (600 mg) was added to the LOX solution prepared from 100 g of infected rice leaves and the mixture was stirred at 23 °C for 3 h. 5 M HCl solution (10 cm^3) and ether (850 cm^3) were added and the stirring was continued for 30 min. The resultant emulsified mixture was frozen at -30 °C overnight to isolate the ether phase. The ether extraction was repeated twice and the combined ether solutions were washed with brine and dried ($MgSO_4$). The ether was removed and residue was dissolved into a mixture of MeOH (100 cm^3) and borate buffer (100 cm^3, pH 9.0). $NaBH_4$ (200 mg) was added to the stirred MeOH–borate buffer solution under ice cooling and stirring was continued for 30 min. After acidification with aq HCl, the mixture was extracted with ether, and the combined ether solutions were washed with brine, dried ($MgSO_4$), and finally diazomethane in ether was added to the solution after removal of $MgSO_4$. The volatile materials were removed and the residue was chromatographed on silica-gel (75 g) with hexane–AcOEt 10 : 1, and then 5 : 1, and finally with AcOEt. From 10 : 1 and 5 : 1 eluants (each 500 cm^3) were obtained recovered methyl linoleate (300 mg) and hydroxylinoleate (150 mg), respectively. The AcOEt eluant contained highly oxygenated linoleate (295 mg). High-performance liquid chromatography [column; microporasil. solvent; hexane–EtOH 300 : 1] shows that it is a 85 : 10 mixture of **9** and **10** (R=H). The mixture was separated by preparative HPLC [column, ERC-0155 (i.d. 30 mm × o.d. 250 mm), solvent; hexane–EtOH 200 : 1].

Trimethylsilylation of Methyl Ester of Hydroxy Fatty Acids. Methyl 13-Hydroxylinoleate. After a mixture of 13-hydroxylinoleate (**9**) (4.7 mg), triethylamine (6.5 μl), trimethylsilyl chloride (4 μl), and 4-dimethylaminopyridine (DMAP) (1 mg) in dichloromethane (1 cm^3) was kept at room temperature overnight, the reaction mixture was diluted with CH_2Cl_2, washed with aq ammonium chloride solution and then brine. The CH_2Cl_2 solution was dried over Na_2SO_4 and the volatile materials were removed in vacuo. The residue was chromatographed on silica-gel (5 g) eluting with benzene to provide the trimethylsilyl ether (6.0 mg). The TMS ethers of the rest of the methyl ester of hydroxy fatty acids were similarly prepared and purified.

Esterification with Benzoyl Chloride. A mixture of 13-hydroxylinoleate (18.2 mg), triethylamine (0.5 cm^3), benzoyl chloride (27.2 mg), and DMAP (2 mg) in CH_2Cl_2 (1 cm^3) was stirred at room temperature overnight and then diluted with CH_2Cl_2 (4 cm^3). The CH_2Cl_2 solution was washed with aq ammonium chloride solution and then with brine. The solution was dried over $MgSO_4$ and the volatile materials were removed in vacuo. The residue was chromatographed on silica-gel (5 g) eluting first with hexane–benzene (1 : 1) and then with benzene to obtain benzoate (20 mg). The benzoate derivatives of the rest of methyl esters of the hydroxy fatty acids were prepared and purified by a similar procedure. Methyl 9-benzoyloxylinoleate (**10**; $R=COC_6H_5$) λ_{ext} nm ($\Delta\varepsilon$) 222 (-32.4) and 237 ($+54.0$). $[\alpha]_D^{22}$ +78.5° ($CHCl_3$; $c=3.0$). ^{13}C NMR ($CDCl_3$) $\delta=13.99$ (q), 22.52 (t), 24.90 (t) ×2, 27.73 (t), 29.05 (t) ×2, 29.44 (t), 29.68 (t), 31.58 (t), 34.07 (t), 34.65 (t), 51.37 (q), 75.40 (d), 127.60 (d), 128.08 (d), 128.28 (d) ×2, 129.54 (d) ×2, 130.81 (s), 130.96 (d), 132.71 (d), 133.69 (d), 165.85 (s), and 174.19 (s). Methyl 13-benzoyloxylinoleate (**9**; $R=COC_6H_5$) λ_{ext} nm ($\Delta\varepsilon$) 221 (-32.7) and 238 ($+54.0$). $[\alpha]_D^{22}$ +74.5° ($CHCl_3$; $c=4.5$). ^{13}C NMR ($CDCl_3$) $\delta=13.99$ (q), 22.52 (t), 24.90 (t) ×2, 27.73 (t), 29.05 (t) ×2, 29.44 (t), 29.68 (t), 31.58 (t), 34.07 (t), 34.65 (t), 51.37 (q), 75.35 (d), 127.60 (d), 128.08 (d), 128.28 (d) ×2, 129.54 (d) ×2, 130.81 (s), 130.96 (d), 132.71 (d), 133.64 (d), 165.85 (s), and 174.19 (s).

The rest of the fatty acids were similarly treated with crude LOX solution prepared from infected rice leaves and the reaction mixture was esterified with diazomethane followed by silica-gel column chromatography to give the following results.

γ-Linolenic Acid (4). Treatment of γ-linolenic acid (**4**) (421 mg) with crude LOX solution furnished recovered material (298 mg), 9-hydroxy-γ-linolenate (100 mg) and highly oxygenated derivative (195 mg) as their methyl ester. The crude 9-hydroxy γ-linolenate is up to 95% purity and could be purified by HPLC. Methyl 9-benzoyloxy-γ-linolenate (**11**; R=COC_6H_5) λ_{ext} nm ($\Delta\varepsilon$) 222 (-32.2) and 239 ($+49.4$). $[\alpha]_D^{22}$ ($CHCl_3$; $c = 1.30$) $+75.23°$. ^{13}C NMR ($CDCl_3$) $\delta = 13.99$ (q), 22.53 (t), 24.56 (t), 24.90 (t), 26.19 (t), 26.87 (t), 29.05 (t), 31.63 (t), 33.99 (t), 34.69 (t), 51.43 (q), 75.28 (d), 127.52 (d), 127.67 (d), 127.72 (d), 128.33 (d) ×2, 129.60 (d) ×2, 130.19 (d), 130.80 (s), 131.34 (d), 131.64 (d), 132.80 (d), 165.90 (s), and 174.08 (s). EIMS (**11**; R=TMS) Found: 380 (M, $C_{22}H_{40}O_3Si$), 291 ($C_{19}H_{31}O_2$), and 225 (base peak, $C_{13}H_{25}OSi$).

Arachidonic Acid (5). Arachidonic acid (**5**) (400 mg) was oxidized with crude LOX solution to give recovered arachidonate (211 mg), 11-hydroxyarachidonate (77 mg) and highly oxygenated arachidonate (245 mg) as their methyl ester. The crude 11-hydroxyarachidonate is up to 91% purity and could be purified by HPLC. Methyl 11-benzoyloxyarachidonate (**12**; R=COC_6H_5) λ_{ext} nm ($\Delta\varepsilon$) 222 (-32.5) and 240 ($+84.0$). $[\alpha]_D^{22}$ ($CHCl_3$; $c = 1.98$) $+77.52°$. ^{13}C NMR ($CDCl_3$) $\delta = 13.40$ (q), 22.52 (t), 24.81 (t), 24.89 (t), 25.64 (t), 26.18 (t), 26.58 (t), 31.63 (t), 33.46 (t), 34.68 (t), 51.44 (q), 75.28 (d), 127.49 (d), 127.65 (d), 127.88 (d), 128.33 (d) ×2, 128.72 (d), 128.74 (d), 129.09 (d), 129.59 (d) ×2, 130.79 (s), 131.11 (d), 131.75 (d), 132.79 (d), 165.90 (s), and 174.03 (s). CIMS (**12**; R=TMS) Found: 407 (M+H, $C_{24}H_{43}O_3Si$), 317 ($C_{21}H_{33}O_2$), and 225 ($C_{13}H_{25}OSi$).

α-Linolenic Acid (6). Oxidation of **6** (1.02 g) afforded recovered α-linolenate (730 mg), hydroxylinolenate (243 mg), and highly oxygenated linolenate (195 mg) as their methyl ester. The hydroxylinolenates are a 87 : 10 mixture of 13-hydroxy- and 9-hydroxylinolenates, respectively.[8] The mixture was purified by HPLC. Methyl 9-benzoyloxylinolenate. λ_{ext} nm ($\Delta\varepsilon$) 222 (-32.0) and 238 ($+53.8$); $[\alpha]_D^{22}$ ($CHCl_3$; $c = 2.9$) $+78.0°$. Methyl 13-benzoyloxylinolenate (**13**; R=COC_6H_5) λ_{ext} nm ($\Delta\varepsilon$) 223 (-30.9) and 238 ($+52.2$); $[\alpha]_D^{22}$ ($CHCl_3$; $c = 3.2$) $+76.9°$.

Eicosapentaenoic Acid (EPA) (7). EPA (**7**) (400 mg) was oxidized to give recovered EPA (211 mg), 11-hydroxy-EPA (77 mg), and others (245 mg). The crude 11-hydroxy-EPA is up to 93% purity and could be purified by HPLC. Methyl 11-benzoyloxy-EPA (**14**; R=COC_6H_5) λ_{ext} nm ($\Delta\varepsilon$) 223 (-37.1) and 241 ($+57.3$); $[\alpha]_D^{22}$ ($CHCl_3$; $c = 2.08$) $+77.4°$. ^{13}C NMR ($CDCl_3$) $\delta = 14.12$ (q), 20.75 (t), 24.76 (t), 25.61 (t), 26.16 (t), 26.54 (t), 32.53 (t), 33.43 (t), 51.50 (q), 74.71 (d), 122.89 (d), 127.41 (d), 127.76 (d), 127.77 (d), 128.32 (d) ×2, 128.67 (d), 128.73 (d), 129.07 (d), 129.59 (d) ×2, 130.57 (s), 131.08 (d), 131.29 (d), 132.86 (d), 134.84 (d), 165.80 (s), and 174.08 (s). CIMS (**14**; R=TMS) Found: 405 (M+H, $C_{24}H_{41}O_3Si$), 335 ($C_{19}H_{31}O_3Si$), and 171 ($C_9H_{19}OSi$).

Docosahexaenoic Acid (DHA) (8). DHA (**8**) (450 mg) was oxidized with LOX solution to give recovered DHA (274 mg), 17-hydroxy-DHA (100 mg) and others (190 mg) as their methyl ester. The crude 17-hydroxy-DHA is up to 95% purity. Methyl 17-benzoyloxy-DHA (**15**; R=COC_6H_5) λ_{ext} nm ($\Delta\varepsilon$) 222 (-37.1) and 240 ($+52.8$). $[\alpha]_D^{22}$ ($CHCl_3$; $c = 1.37$) $+72.4°$. ^{13}C NMR ($CDCl_3$) $\delta = 14.12$ (q), 20.75 (t), 22.79 (t), 25.57 (t), 25.63 (t), 26.16 (t), 32.53 (t), 34.01 (t), 51.58 (q), 74.70 (d), 122.89 (d), 127.51 (d), 127.76 (d), 127.77 (d), 127.90 (d), 128.07 (d), 128.19 (d), 128.31 (d) ×2, 128.64 (d), 129.28 (d), 129.59 (d) ×2, 130.56 (s), 131.08 (d), 131.26 (d), 132.85 (d), 134.83 (d), 165.80 (s), and 174.05 (s). CIMS (**15**; R=TMS) Found: 431 (M+H, $C_{26}H_{43}O_3Si$), 361 ($C_{21}H_{33}O_3Si$), 341 ($C_{23}H_{33}O_2$), and 171 ($C_9H_{19}OSi$).

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