# Cell-Free Biosynthesis and Source of Hydroxyl Groups in (12R, 13S)-Dihydroxy-(5Z,8Z, 10E, 14Z)-Eicosatetraenoic Acid, a Novel Eicosanoid from the Marine Alga Gracilariopsis lemaneiformis

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# CELL-FREE BIOSYNTHESIS AND SOURCE OF HYDROXYL GROUPS IN (12*R*,13*S*)-DIHYDROXY-(5*Z*,8*Z*,10*E*,14*Z*)-EICOSATETRAENOIC ACID, A NOVEL EICOSANOID FROM THE MARINE ALGA *GRACILARIOPSIS LEMANEIFORMIS*

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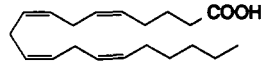
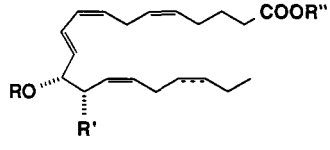
ABSTRACT.—A cell-free biosynthetic study with the marine macrophyte *Gracilariopsis lemaneiformis* showed by mass spectrometry and $^{13}C$ nmr that molecular oxygen was the source of the two hydroxyl groups in the novel eicosanoid (12*R*,13*S*)-dihydroxy-(5*Z*,8*Z*,10*E*,14*Z*)-eicosatetraenoic aicd [**3**].

*Gracilariopsis lemaneiformis* (Bory) Dawson, Acleto et Foldvik (Gracilariaceae) is a benthic red marine alga present in abundance in sandy-rock habitats along the Oregon coast (1). As a result of our survey of marine algae from along this coast for their biologically active natural products, we found this seaweed to contain several 12-lipoxygenase (12-LO) metabolites of arachidonic acid [**1**] (2,3) such as (12*S*)-hydroxyeicosatetraenoic acid (12*S*-HETE) [**2**], (12*R*,13*S*)-dihydroxy-(5*Z*,8*Z*,10*E*,14*Z*)-eicosatetraenoic acid (12*R*,13*S*-diHETE) [**3**], and (12*R*,13*S*)-dihydroxy-(5*Z*,8*Z*,10*E*,14*Z*,17*Z*)-eicosapentaenoic acid (12*R*,13*S*-diHEPE) [**7**]. The latter compound possesses inhibitory activity to canine $Na^+/K^+$ ATPase, human lymphocyte 5-lipoxygenase, and human neutrophil degranulation (4). While this is not the only Oregon marine alga to contain 12*R*,13*S*-diHETE (4,5), its ready availability and richness of 12-LO metabolites made it our first choice for further studies aimed at probing the biosynthetic mechanism by which this metabolite is formed.

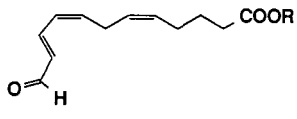
Previously, we showed that the $Me_2CO$ powder crude enzyme preparation from *G. lemaneiformis* was able to metabolize exogenously supplied arachidonic acid to the known 12-LO metabolite, 12*S*-HETE [**2**] (6). Development of this cell-free system circumvents the problem of poor cell wall penetration by exogenously supplied organic



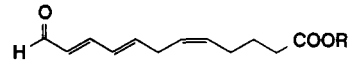

**1**




**2** R=R'=R"=H
**3** R=R"=H, R'=OH
**4** R=OH, R'=R"=H
**5** R=H, R'=OH, R"=Me
**6** R=TMSi, R'=OTMSi, R"=Me
**7** ω3, R=R"=H, R'=OH
**8** ω3, R=H, R'=OH, R"=Me
**9** R=R'=H, R"=Me




**10** R=H
**11** R=Me




**12** R=H
**13** R=Me

substrates, a traditional obstacle encountered in biosynthetic experiments with marine macrophytes (7). Of the eicosanoids produced by *G. lemaneiformis*, 12*R*,13*S*-diHETE [**3**] was the one of principal biosynthetic interest because of its unique oxidation at C-13, an unusual position for oxidation in eicosanoids. The conceivable mechanisms for introduction of this oxygen atom (Figure 1) involve initial 12-lipoxygenation to yield 12-hydroperoxyeicosatetraenoic acid (12-HPETE) [**4**] followed by (a) trans-12*R*,13*R*-epoxide formation and attack by $H_2O$ with inversion at C-13; (b) inter- or intramolecular rearrangement of the distal hydroperoxy oxygen of 12-HPETE (10,11), although involving a novel mechanism of oxidation at C-13; or (c) independent molecular oxygen oxidation of C-13 by a separate catalytic activity (i.e., cytochrome P-450). Employing this cell-free preparation from *G. lemaneiformis*, we have effectively probed this mechanistic feature of 12*R*,13*S*-diHETE biosynthesis. This is the first time that such a detailed feature of lipid biosynthesis has been examined in a marine macrophyte.

## RESULTS AND DISCUSSION

Incubation of arachidonic acid [**1**] with *G. lemaneiformis* $Me_2CO$ powder led to the production of an assortment of uv-absorbing and acid-charring products by tlc analysis. Following methylation of this mixture, five oxidized products could be separated by repetitive normal phase hplc using various solvent mixtures. These methyl ester derivatives were each characterized by high field nmr and in most cases, low resolution gc-eims of the corresponding TMSi ethers for compounds **5**, **8**, and **9** and ir spectroscopy as well. Compound **2** was shown by these techniques to be identical to 12-HETE (<1% yield), the product we had earlier characterized from these incubation experiments (6). Derivatives **5** and **8** were deduced by $^1$H-$^1$H COSY as methyl *erythro*-12,13-dihydroxy-(5*Z*,8*Z*,10*E*,14*Z*)-eicosatetraenoate (methyl 12,13-diHETE, 8% of recovered lipids) and methyl *erythro*-12,13-dihydroxy-(5*Z*,8*Z*,10*E*,14*Z*,17*Z*)-eicosapentaenoate (methyl 12,13-diHEPE, 8% of recovered lipids) respectively, by comparison to $^1$H-nmr data we had for other derivatives of these same dihydroxyeicosanoids (4). This was confirmed by lreims of each of these in which major ions for the expected fragments from C-12–C-

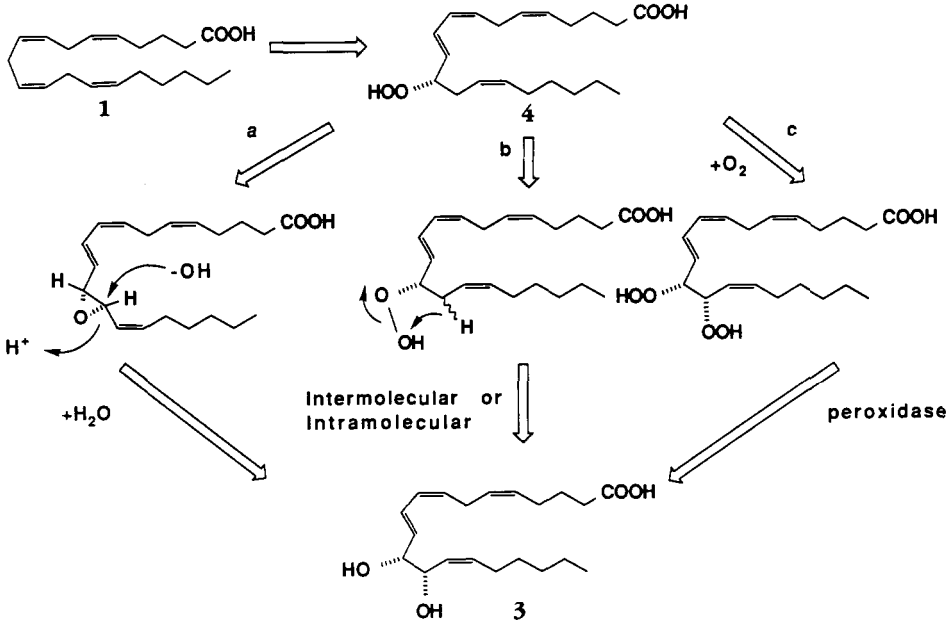



FIGURE 1. Potential biosynthetic alternatives leading to the formation of 12*R*,13*S*-diHETE [**3**].

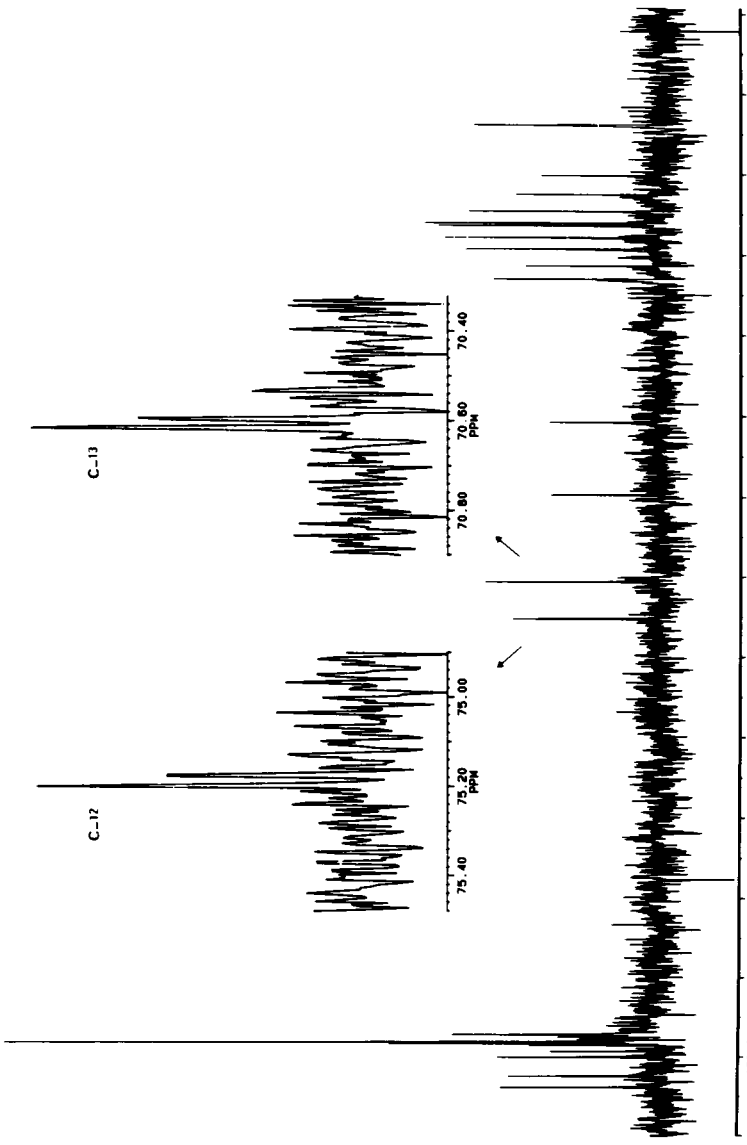



FIGURE 2. DEPT-45 $^{13}C$-nmr spectrum of methyl 12*R*,13*S*-diHETE (1.0 mg of **5** in ca. 0.4 ml $C_6D_6$, 75 MHz, 297°, 10,168 scans) showing that both C-12 and C-13 possess $^{16}O$-$^{13}C$ and $^{18}O$-$^{13}C$ signals (shifted 0.02 ppm in each case).

13 cleavage were observed at *m/z* 295 and 199 for **6** and *m/z* 295 and 197 for the TMSi ether derivative of **8**. The structures of two aldehyde-containing products, **10** and **12**, isolated again as methyl ester derivatives (**11**, 4% of recovered lipids; **13**, 2% of recovered lipids), were determined by comparisons to published and reference $^1$H-nmr spectra (8,9).

A second portion of the *G. lemaneiformis* $Me_2CO$ powder was used for the production of $^{18}$O-labeled 12*R*,13*S*-diHETE [**3**]. The different potential origins ($H_2O$ versus $O_2$) for oxygen at C-12 and C-13 were distinguished by forming metabolite **3** in the presence of a 50% $^{16}O/^{18}O$ gas mixture. Direct tlc analysis of the products of this incubation in an acidified solvent [EtOAc-hexane-HOAc 64:35:1)] demonstrated the existence of several oxidized arachidonic acid metabolites. The crude methylated extract was repetitively chromatographed by normal phase hplc to yield 1.0 mg of pure methyl 12*R*,13*S*-diHETE [**5**]. The 300 MHz $^1$H nmr, $^{13}$C nmr, and low resolution gc-eims (of the corresponding bis-TMSi either) were fully descriptive of methyl 12,13-diHETE when compared with data from our earlier work in this structural class (4). A small positive rotation for this biosynthetically produced sample of compound **5** confirmed its enzymatic origin and 12*R*,13*S* absolute stereochemistry as previously found for the natural product **3** (2,12). That this rotation was less than that measured for authentic samples of **5** indicated either partial racemization or, more likely, a less accurate measurement given the small sample size.

A DEPT-45 $^{13}$C nmr spectrum of oxygen-isotope-labelled **5** displayed signals for all of the protonated carbons (13). Of primary interest were the C-12 and C-13 signals resonating at δ 75.20 and 70.62 (4), respectively, which, following Gaussian resolution enhancement, showed two $^{18}$O-isotope-shifted carbon signals at δ 75.18 and 70.60 (Figure 2). The magnitude of the chemical shift dispersion between these $^{16}$O-$^{13}$C and $^{18}$O-$^{13}$C signals (0.02 ppm) is in good agreement with literature values for carbons with singly bonded oxygen atoms (14). The presence of $^{18}$O at both sites necessarily excludes the involvement of $H_2O$ in these oxidations of C-12 or C-13. The biosynthetic origin of these oxygen atoms in $^{18}$O-labeled **5** was confirmed by mass spectrometric analysis (lr gc-eims) of the bis-TMSi ether **6**. Ions observed at *m/z* 494 $[M]^+$ (0.18%), 496 $[M+2]^+$ (0.30%), and 498 $[M+4]^+$ (0.25%) clearly demonstrated that the C-12 and C-13 oxygen atoms both originated from molecular oxygen.

From these experiments, it is clear that molecular oxygen is used by *G. lemaneiformis* to oxidize both C-12 and C-13 enzymatically, beginning with an arachidonic acid precursor. The oxidation at C-12 is most likely mediated by a 12-lipoxygenase enzyme, while that at C-13 results either from separate oxidation at C-13 or from rearrangement of the proposed C-12 hydroperoxide intermediate (Figure 1, b or c). Further investigations probing the nature of these unique algal lipoxygenase and oxidase enzyme systems are currently under way (15).

## EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Ir spectra were recorded on a Nicolet 5 DXB FT 15 spectrophotometer. Nmr spectra were recorded on a Bruker ACP 300 nmr spectrometer, and all chemical shifts are reported relative to an internal TMS standard. Mass spectra were obtained on a Finnigan 4023 mass spectrometer. Hplc was performed with Waters M-6000 and M-45 pumps, U6K injectors, and a Waters lambda-Max 480 lc spectrophotometer. Tlc used Merck aluminum-backed tlc sheets (Si gel 60 $F_{254}$). All solvents were distilled from glass before use.

COLLECTION OF *G. LEMANEIFORMIS* AND FORMATION OF $Me_2CO$ POWDER.—*G. lemaneiformis* was collected and an $Me_2CO$ powder produced as previously detailed (6).

INCUBATION OF *G. LEMANEIFORMIS* $Me_2CO$ POWDER WITH ARACHIDONIC ACID [**1**] AND ISOLATION OF METHYL ESTER DERIVATIVES **5**, **8**, **9**, **11**, AND **13**.—Incubation of 50 mg arachidonic acid with 27.8 g *G. lemaneiformis* $Me_2CO$ powder under conditions essentially as previously reported (6) gave 19

mg of extractable products which yielded derivatives **5** (1.6 mg), **8** (1.6 mg), **9** (ca. 0.1 mg), **11** (0.3 mg), and **13** (0.8 mg) following methylation and separation by repetitive normal phase hplc [(1) 35% EtOAc/hexane, 4.6 mm × 250 mm, 5 μ Nucleosil; (2) 30% EtOAc/hexane, 2 × 4.1 mm × 250 mm, 10 μ Versapak]. Additionally, approximately 10 mg of unmetabolized arachidonic acid was present as methyl arachidonate.

*Methyl (12R,13S)-dihydroxy-(5Z,8Z,10E,14Z)-eicosatetraenoate* [**5**].—Derivative **5** was isolated as a colorless oil: ir ($CHCl_3$) 3415, 2929, 2739, 1437, 1220, 1157, 1025, 998 $cm^{-1}$; $^1H$ nmr (300 MHz, $C_6D_6$) δ 6.77 (1H, dddd, $J$ = 15.3, 11.1, 1.0, 1.0 Hz, H-10), 6.03 (1H, bdd, $J$ = 11.1, 11.1 Hz, H-9), 5.77 (1H, dd, $J$ = 15.3, 6.3 Hz, H-11), 5.55 (1H, m, H-14), 5.46 (1H, m, H-15), 5.44 (1H, m, H-8), 5.39 (1H, m, H-6), 5.24 (1H, dtt, $J$ = 10.8, 6.3, 1.6 Hz, H-5), 4.45 (1H, ddd, $J$ = 7.6, 4.0, 3.8 Hz, H-13), 4.19 (1H, m, H-12), 3.33 (3H, s, OMe), 2.86 (2H, dddd, $J$ = 6.7, 6.7, 1.8, 1.8 Hz, $H_2$-7), 2.21 (1H, bd, $J$ = 4.0 Hz, H-12′), 2.06 (2H, t, $J$ = 7.4, $H_2$-2), 1.95 (2H, dt, $J$ = 7.3, 7.3 Hz, $H_2$-16), 1.92 (2H, dt, $J$ = 7.4, 7.4 Hz, $H_2$-4), 1.73 (1H, bd, $J$ = 4.0 Hz, H′-13), 1.57 (2H, tt, $J$ = 7.4, 7.4 Hz, $H_2$-3), 1.18 (2H, m, $H_2$-17), 1.18 (2H, m, $H_2$-19), 0.86 (3H, t, $J$ = 6.9 Hz, $H_3$-20); lr gc-eims (TMSi ether, trimethylimidazole, 70 eV) obs. $m/z$ $[M]^+$ 494 (0.2%), $[M-C_4H_7O_2]^+$ 407 (0.9%), $[M-TMSiOH]^+$ 404 (3.8%), $[M-2\ TMSiOH]^+$ 314 (1.0%), $[M-C_8H_{14}OTMSi]^+$ 295 (18.4%), $[M-C_{13}H_{18}O_2TMSiOH]^+$ 199 (7.1%), $[TMSi]^+$ 73 (100%).

*Methyl (12R,13S-dihydroxy-(5Z,8Z,10E,14Z,17Z)-eicosapentaenoate* [**8**].—Derivative **8** was isolated as a colorless oil: ir ($CHCl_3$) 3442, 2964, 1738, 1492, 989 $cm^{-1}$; $^1H$ nmr (300 MHz, $C_6D_6$) δ 6.76 ($^1H$, dddd, $J$ = 15.3, 11.1, 1.2, 1.2 Hz, H-10), 6.02 (1H, bdd, $J$ = 11.1, 11.1 Hz, H-9), 5.74 (1H, dd, $J$ = 15.3, 6.2 Hz, H-11), 5.23–5.55 (7H, m, H-5, -6, -8, -14, -15, -17, -18), 4.44 (1H, m, H-13), 4.17 (1H, m, H-12), 3.33 (3H, s, OMe), 2.85 (2H, bdd, $J$ = 6.6, 6.6 Hz, $H_2$-7), 2.16 (1H, m, H-12′), 2.77 (2H, m, $H_2$-16), 2.06 (2H, t, $J$ = 7.4, $H_2$-2), 1.95 (2H, dt, $J$ = 7.4, 7.4 Hz, $H_2$-4), 1.93 (2H, dq, $J$ = 7.5, 7.5 Hz, $H_2$-19), 1.71 (1H, m, H′-13), 1.54 (2H, tt, $J$ = 7.4, 7.4 Hz, $H_2$-3), 0.88 (3H, t, $J$ = 7.5 Hz, $H_3$-20); lr gc-eims (TMSi ether, trimethylimidazole, 70 eV) obs. $m/z$ $[M]^+$ 492 (1.4%), $[M-C_4H_7O_2]^+$ 405 (1.4%), $[M-TMSiOH]^+$ 402 (1.3%), $[M-2\ TMSiOH]^+$ 312 (1.8%), $[M-C_8H_{12}OTMSi]^+$ 295 (13.1%), $[M-C_{13}H_{18}O_2TMSiOH]^+$ 197 (7.1%), $[TMSi]^+$ 73 (100%).

*Methyl 12-oxo-(5Z,8Z,10E)-dodecatrienoate* [**11**] *and methyl 12-oxo-(5Z,8E,10E)-dodecatrienoate* [**13**].—Derivative **11** was isolated as a colorless oil and showed, for corresponding signals, $^1H$-nmr bands highly comparable to those reported for the parent acid (8): $^1H$ nmr (300 MHz, $CDCl_3$) 9.60 (1H, d, $J$ = 8.0 Hz, H-12), 7.48 (1H, dd, $J$ = 15.1, 11.1 Hz, H-10), 6.28 (1H, dd, $J$ = 11.1, 10.2 Hz, H-9), 6.17 (1H, dd, $J$ = 15.1, 8.0 Hz, H-11), 5.94 (1H, dt, $J$ = 10.2, 7.0 Hz, H-8), 5.4 (2H, m, H-5, -6), 3.68 (3H, s, OMe), 3.09 (2H, bdd, $J$ = 7.0, 7.0 Hz, $H_2$-7), 2.34 (2H, t, $J$ = 7.3, $H_2$-2), 2.14 (2H, dt, $J$ = 7.3, 7.3 Hz, $H_2$-4), 1.73 (2H, tt, $J$ = 7.3, 7.3 Hz, $H_2$-3). Derivative **13** was also isolated as a colorless oil and had a $^1H$-nmr spectrum identical to that of an authentic standard (9).

BIOSYNTHETIC FORMATION OF $^{18}O$-LABELED 12,13-DIHETE [**3**].—A second portion of *G. lemaneiformis* $Me_2CO$ powder [17.1 g from 157 g tissue (6)], first confirmed to be active by incubating a small portion (0.3 gm) with arachidonic acid followed by tlc analysis, was used for the production of $^{18}O$-labeled 12,13-diHETE by $^{18}O_2$. Ms analysis of this gas mixture (Cambridge Isotope) showed it to be of random speciation (23.6% $^{16}O_2$, 48.4% $^{16}O^{18}O$, 28% $^{18}O_2$), although originally sold as "Oxygen-18 $O_2$; $^{18}O$, 50%." The incubation was conducted in a closed system containing the $Me_2CO$ powder stirring in 200 ml of 0.1 M phosphate buffer (pH 7.4) containing 0.1% UCON (Union Carbide Corporation) as an anti-foaming agent. The head space (ca. 50 ml) and buffer medium were repetitively gas-stripped with $N_2$ alternating with $H_2O$-aspirated vacuum. The system was charged twice with the $^{16}O/^{18}O$ gas mixture, and 100 mg of 99% pure arachidonic acid (Sigma) dissolved in 1 ml EtOH was added. After 25 min at room temperature, the incubation was terminated by acidification to pH 4.0 (10% HCl). Organic extraction with $CHCl_3$ (700 ml) was assisted by the addition of ca. 200 ml of saturated brine. Following evaporation of the $CHCl_3$, a dark green oil (137.1 mg) was recovered. Direct tlc analysis of this material in an acidified solvent [EtOAc-hexane-HOAc (64:35:1)] demonstrated the presence of several oxidized arachidonic acid metabolites. The crude extract was methylated (ethereal $CH_2N_2$) and repetitively chromatographed by normal phase hplc [(a) 40% EtOAc/hexane, 10 mm × 50 cm, RSIL, 10μ; (b) 25% EtOAc/hexane, 2 × 3.9 mm × 25 cm, μ-Porasil, 10 μ] to yield 1.0 mg of pure methyl 12*R*,13*S*-diHETE [**5**]: $[\alpha]_D$ +10.4° ($c$ = 0.03, $Me_2CO$) [for authentic **5** $[\alpha]_D$ +84.2° ($c$ = 0.71, $Me_2CO$)]; $^1H$ nmr as reported above for unlabeled **5**; $^{13}C$ DEPT nmr (75 MHz, $C_6D_6$) δ 134.02 (C-15), 132.60 (C-8), 130.19 (C-10), 129.47 (C-5), 128.30 (C-6), tentative, obscured by solvent), 127.97 (C-9), 127.64 (C-11), 127.33 (C-14), 75.20 ($^{16}OC$-12), 75.18 ($^{18}OC$-12), 70.62 ($^{16}OC$-13), 70.60 ($^{18}OC$-13), 51.07 ($OCH_3$), 33.33 (C-2), 31.71 (C-18), 29.61 (C-17), 28.20 (C-16), 26.73 (C-7), 26.44 (C-4), 24.97 (C-3), 22.87 (C-19), 14.23 (C-20); lr gc-eims of bis-TMSi ether **6** (trimethylsilylimidazole) $m/z$ $[M+4]^+$ 498 (0.25%), $[M+2]^+$ 496 (0.30%), $[M]^+$ 494 (0.18%), $[M+4-TMSiOH]^+$ 408 (0.25%), $[M-C_4H_7O_2]^+$ 407

(3.8%), $[M+2-TMSiOH]^+$ 406 (4.4%), $[M-TMSiOH]^+$ 404 (3.8%), $[M-2TMSiOH]^+$ 314 (1.0%), $[C_9H_{15}{}^{18}O_2(TMSi)_2]^+$ 305 (7.9%), $[C_9H_{15}{}^{16}O^{18}O(TMSi)_2]^+$ 303 (11.3%), $[C_9H_{15}{}^{16}O_2(TMSi)_2]^+$ 301 (6.8%), $[C_{13}H_{18}{}^{16}O_2{}^{18}OTMSi]^+$ 297 (20%), $[C_{13}H_{18}O_3TMSi]^+$ 295 (18.4%), 266 (4.2%), 265 (4.6%), 263 (4.2%), $[C_9H_{15}{}^{18}OTMSi]^+$ 213 (6.4%), $[C_9H_{15}OTMSi]^+$ 211 (7.0%), $[C_{13}H_{17}O_2]^+$ 205 (8.1%), $[C_{18}H_{14}{}^{18}OTMSi]^+$ 201 (5.9%), $[C_8H_{14}OTMSi]^+$ 199 (7.1%), $[C_{12}H_{17}O_2]^+$ 193 (5.0%), $[C_{12}H_{13}O]^+$ 173 (9.2%), 161 (5.5%), 155 (4.4%), 149 (5.2%), 147 (7.1%), 145 (5.4%), 131 (15.6%), 129 (8.0%), 119 (7.2%), 105 (7.7%), $[C_7H_{13}]^+$ 97 (1.4%), 91 (12.3%), 81 (5.4%), 79 (6.6%), $[C_3H_5O_2]^+$ 73 (100%), 69 (17.5%), 67 (7.2%), 59 (7.3%), 55 (12.2%).

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