
This is an electronic reprint of the original article.
This reprint may differ from the original in pagination and typographic detail.

Parkkari, Teija; Koskinen, Ari M.P.; Myllymäki, Mikko; Savinainen, Juha R.; Saario, Susanna M.; Castillo-Melendez, Joel A.; Laitinen, Jarmo T.; Nevalainen, Tapio; Järvinen, Tomi
a-methylated derivatives of 2-arachidonyl glycerol: Synthesis, CB1 receptor activity and enzymatic stability

Published in:
Bioorganic and Medicinal Chemistry Letters

DOI:
[10.1016/j.bmcl.2006.01.101](https://doi.org/10.1016/j.bmcl.2006.01.101)

Published: 01/01/2006

Document Version
Peer reviewed version

Please cite the original version:
Parkkari, T., Koskinen, A. M. P., Myllymäki, M., Savinainen, J. R., Saario, S. M., Castillo-Melendez, J. A., Laitinen, J. T., Nevalainen, T., & Järvinen, T. (2006). a-methylated derivatives of 2-arachidonyl glycerol: Synthesis, CB1 receptor activity and enzymatic stability. *Bioorganic and Medicinal Chemistry Letters*, (16), 2437-2440. <https://doi.org/10.1016/j.bmcl.2006.01.101>

This material is protected by copyright and other intellectual property rights, and duplication or sale of all or part of any of the repository collections is not permitted, except that material may be duplicated by you for your research use or educational purposes in electronic or print form. You must obtain permission for any other use. Electronic or print copies may not be offered, whether for sale or otherwise to anyone who is not an authorised user.

α -Methylated derivatives of 2-arachidonoyl glycerol: Synthesis, CB1 receptor activity, and enzymatic stability

Teija Parkkari,^{a,*} Mikko Myllymäki,^b Juha R. Savinainen,^c Susanna M. Saario,^a
Joel A. Castillo-Meléndez,^b Jarmo T. Laitinen,^c Tapio Nevalainen,^a
Ari M. P. Koskinen^b and Tomi Järvinen^a

^aDepartment of Pharmaceutical Chemistry, University of Kuopio, PO Box 1627, FIN-70211 Kuopio, Finland

^bHelsinki University of Technology, PO Box 6100, FIN-02015 TKK, Finland

^cDepartment of Physiology, University of Kuopio, PO Box 1627, FIN-70211 Kuopio, Finland

Abstract— α -Methylated analogues of the endogenous cannabinoid, 2-arachidonoyl glycerol (2-AG), were synthesized aiming to the improved enzymatic stability of 2-AG. In addition, the CB1 activity properties of fluoro derivatives of 2-AG were studied. The CB1 receptor activity was determined by the [³⁵S]GTP γ S binding assay, and the enzymatic stability of α -methylated analogues was determined in rat cerebellar membranes. The results indicate that even if the α -methylated 2-AG derivatives are slightly weaker CB1 receptor agonists than 2-AG, they are clearly more stable than 2-AG. In addition, the results showed that the replacement of the hydroxyl group(s) of 2-AG by fluorine does not improve the CB1 activity of 2-AG.

© 2006 Elsevier Ltd. All rights reserved.

2-Arachidonoyl glycerol (2-AG) was first reported in 1995 as a weak cannabinoid receptor agonist with similar cannabinergic character as the traditional plant-derived cannabinoids and the endogenous cannabinoid, *N*-arachidonoyl ethanol amide (AEA).^{1,2} At that time, AEA was studied extensively and it was considered as the main endogenous ligand for the cannabinoid receptors. However, later on it has been confirmed that 2-AG is a full efficacy agonist and probably the main endogenous ligand for both the CB1 and CB2 receptors.^{3–6} 2-AG is known as the most efficacious CB1 receptor ligand so far. In the [³⁵S]GTP γ S binding assays with the rat cerebellar membranes, E_{\max} value for 2-AG has been reported to be 620 ± 5 (%basal \pm SEM), while the respective figures, for example, for AEA and CP55,940, are 484 ± 7 and 510 ± 4 .⁶ In spite of the good efficacy and potency of 2-AG (Table 1), its usage as a pharmacological tool or pharmaceutical is not convenient due to its fast enzymatic degradation. The low enzymatic stability of 2-AG set a starting point for this

Table 1. Comparison of efficacy (E_{\max}) and potency (pEC_{50}) values of 2-AG and compounds **1a–b**, **2a–b**, and **3a**

Compound	CB1 activation	
	E_{\max} (%basal \pm SEM, $n = 3$)	$pEC_{50} \pm$ SEM, ($n = 3$)
2-AG	620 ± 17	6.0 ± 0.1
1a	407 ± 20	5.0 ± 0.1
1b	227 ± 19	4.8 ± 0.0
2a	168 ± 16	4.8 ± 0.4
2b	NA	NA
3a	NA	NA

NA, no detectable CB1 activation at 10^{-4} M concentration.

study where 2-AG was methylated at its α -position in order to reduce its metabolism to arachidonic acid (AA). In addition to the low stability, a formation of AA may be a drawback. AA is unwanted metabolite in several therapy targets, like in the eye, since it is the precursor of the inflammation mediators.

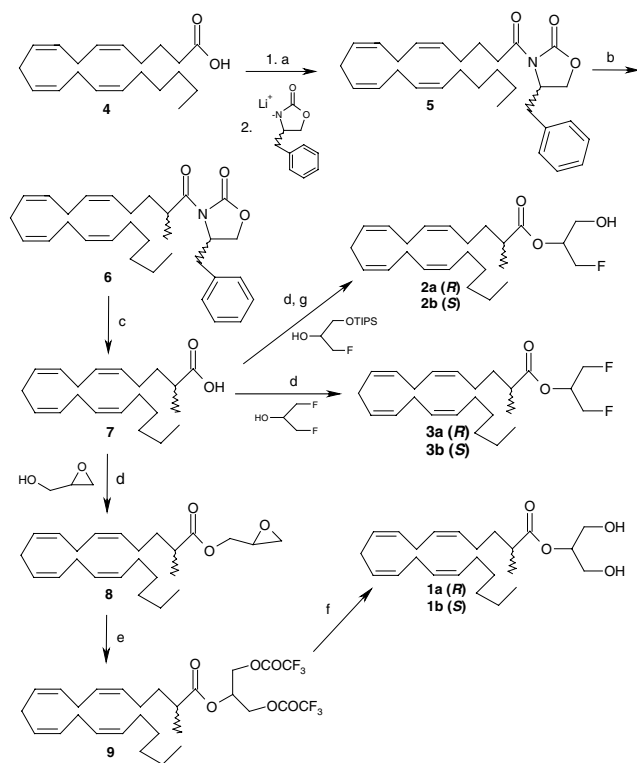
The aim of the present study was to improve the enzymatic stability of 2-AG, and thereby, to prevent formation of AA and prolong duration of action. The CB1 activity as well as the enzymatic stability were studied separately with both enantiomers. Finally, it was

Keywords: Cannabinoid; CB1 receptor; 2-AG; Enzymatic stability; Stereoselective synthesis.

determined if the activity properties of 2-AG can be improved by replacing the head hydroxyl group(s) by fluorine as it is in a case of AEA.^{7,8} The CB1 activation parameters of the synthesized compounds were determined by using the [³⁵S]GTPγS binding assay. The stability studies were conducted in rat brain homogenate and in membrane-free buffer solution.

Scheme 1 illustrates the synthesis methods for the compounds **1a–b**,^{13,14,19} **2a–b**,^{15,16,19} and **3a–b**.^{17–19} The chiral key synthon **7** was prepared using Evan's chiral auxiliary as previously described.⁹ The synthesis of endogenous 2-AG and its analogues is very challenging since its susceptibility for an isomerization and auto-oxidation. Stamatov and Stawinski developed an efficient synthetic strategy, which was utilized in the synthesis of final products **1a–b**.¹⁰ (*S*)- or (*R*)-2-methylarachidonic acid **7** was esterified with (+/–)-glycidol in the presence of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimidehydrochloride (EDC) and 4-dimethylaminopyridine (DMAP) to give the glycidyl derivatives **8**, which was then treated with TFAA giving trifluoroacetate ester **9**. This was further converted into the desired products by transesterification using pyridine and methanol in dichloromethane–hexane.

The α-methylated monofluoro derivatives of 2-AG, **2a** (*R*) and **2b** (*S*), and the difluoro derivatives **3a** (*R*) and **3b** (*S*) were prepared by coupling (*S*)- or (*R*)-2-methylarachidonic acid **7** with (*rac*)-1-fluoro-3-(tri-2-propyl)-



Scheme 1. Reagents and conditions: (a) Pivaloyl chloride, Et₃N, THF, 84–97%; (b) 1—NHMDS, THF, –78 °C, 2—MeI, 60–82%; (c) LiOOH, THF, 65–83%; (d) EDC or DCC, DMAP, CH₂Cl₂, 60–93%; (e) TFAA, CH₂Cl₂, 96%; (f) pyridine, MeOH, CH₂Cl₂/hexane, quantitative yield; (g) TBAF, THF, 89%.

siloxo-propan-2-ol and 1,3-difluoro-2-propanol using EDC/DMAP. Finally, the TIPS-protective groups were removed by an excess of tetrabutylammonium fluoride trihydrate (TBAF).

The [³⁵S]GTPγS membrane binding studies were performed in an optimized condition, where the enzymatic degradation of endocannabinoids has been minimized.⁶ Maximal agonist responses (*E*_{max}, %basal) and potencies (*pEC*₅₀) were determined from dose–response curves. The CB1-dependent activity was confirmed by antagonizing half-maximal responses with the CB1-selective antagonist AM251 (10^{–6} M). The results are presented as means ± SEM of at least three independent experiments, performed in duplicate. Data analysis was calculated as non-linear regressions by GraphPad Prism 4.0.

The enzymatic stability studies were carried out in rat cerebellar membranes prepared as previously described.¹¹ Preincubations (80 μL, 30 min at 25 °C) contained 10 μg membrane protein, 44 mM Tris–HCl (pH 7.4), 0.9 mM EDTA, 0.5% BSA, and 1.25% (v/v) DMSO as a solvent for drugs. The incubations (90 min at 25 °C) were initiated by adding 40 μL of preincubated membrane cocktail, giving a final volume of 400 μL. The final volume contained 5 μg membrane protein, 52 mM Tris–HCl (pH 7.4), 1.0 mM EDTA, 95 mM NaCl, 4.8 mM MgCl₂, 0.5% BSA, and 50 μM of the drug (2-AG, **1a–b**). At time-points of 0 and 90 min, 100 μL samples were removed from incubation, acetonitrile (200 μL) was added to stop the enzymatic reaction, and pH of the samples was simultaneously decreased with phosphoric acid (added to acetonitrile) to 3.0, in order to stabilize the drugs against a possible post-incubation chemical acyl migration reaction. Samples were centrifuged at 23,700g for 4 min at RT prior to HPLC analysis²⁰ of the supernatant. The susceptibility of 2-AG and **1a–b** for the enzymatic degradation was finally determined based on the formation of AA or α-methyl-AA during the 90 min incubation period. The results are presented as means ± SEM of three independent experiments, performed in duplicate.

The CB1 receptor activation data are presented in the Table 1. Among the series, only the compounds **1a–b** showed appreciable dose-dependent CB1 receptor activity. The (*R*) enantiomer (**1a**) gave significantly better CB1 activity than the (*S*) enantiomer (**1b**) which is quite interesting finding since it has been reported that stereochemistry at the α-position is not that significant when affinity for the CB1 receptor is measured.⁹ Weak potency and efficacy values were achieved for the monofluoro derivative **2a**, however, with the (*S*) enantiomer no detectable CB1 activation at 10^{–4} M concentration was observed. The replacement of the both hydroxyl groups with fluorine (**3a**) led to a loss of the CB1 activity. Since the *R* enantiomer was proven to be inactive, the activity of the (*S*) form (**3b**) was not determined.

The results of the stability studies are presented in Table 2. In the membrane-free buffer, the ester bond of 2-AG was stable, even though acyl migration from 2-AG into

Table 2. Relative (% \pm SEM) concentrations of 2-AG (1(3)-AG), and **1a–b** (α -Me-1-AG) and their degradation products (AA or α -Me-AA) in the rat cerebellar membranes after 90 min incubation

Compound:	2-AG		1a		1b	
	0	90	0	90	0	90
2-AG/ α -Me-2-AG	86 \pm 3	5 \pm 1	84 \pm 1	23 \pm 1	82 \pm 2	24 \pm 1
1-AG / α -Me-1-AG	14 \pm 3	18 \pm 4	14 \pm 1	46 \pm 1	17 \pm 2	50 \pm 2
AA / α -Me-AA	0 \pm 0	77 \pm 4	3 \pm 0	31 \pm 2	1 \pm 0	27 \pm 3

1(3)-AG can be observed. However, in the rat cerebellar membrane incubations about 77% of 2-AG was degraded into AA due to the enzymatic activity present in the studied tissue. The acyl migration is not likely to have an impact on the metabolism since it has been reported that the rates of the enzymatic degradation for 2-AG and 1-AG are approximately the same.¹²

Compounds **1a** and **1b** behaved similarly in the membrane-free buffer solution as 2-AG; the α -methylation did not prevent the acyl migration. Nevertheless, the hypothesis that α -methylation of 2-AG could reduce the metabolism was proven to be correct since only about 30% of **1a** and **1b** were degraded into corresponding α -methyl-arachidonic acids (α -Me-AA) in the rat cerebellum membranes after 90 min incubation. The stereochemistry of the compound does not have a significant role in metabolism.

In conclusion, the results indicate that even if the stereochemistry of an α -position of 2-AG does not play a role in a ligand affinity for the CB1 receptor, it has a significant role in a G-protein activation. The potency and efficacy values of the α -methylated 2-AG derivatives are slightly weaker compared to 2-AG, however, it is noteworthy that derivatives are clearly more stable than 2-AG, and therefore, it can be expected that their duration of action in a target tissue is longer. Finally, the results showed that the replacement of the hydroxyl group(s) of 2-AG by fluorine does not improve the activity of 2-AG as it is in the case of AEA.

Acknowledgments

The authors are grateful to Mrs Tiina Koivunen and Mrs Helly Rissanen for their skilful technical help. The study was supported by a grant from the National Technology Agency of Finland and Academy of Finland (Grant No. 107300).

References and notes

- Mechoulam, R.; Ben-Shabat, S.; Hanus, L.; Ligumsky, M.; Kaminski, N. E.; Schatz, A. R.; Gopher, A.; Almog, S.; Martin, B. R.; Compton, D. R.; Pertwee, R. G.; Griffin, G.; Bayewitch, M.; Barg, J.; Vogel, Z. *Biochem. Pharmacol.* **1995**, *50*, 83.
- Sugiura, T.; Kondo, S.; Sukagawa, A.; Nakane, S.; Shinoda, A.; Itoh, K.; Yamashita, A.; Waku, K. *Biochem. Biophys. Res. Commun.* **1995**, *215*, 89.
- Sugiura, T.; Kodaka, T.; Nakane, S.; Miyashita, T.; Kondo, S.; Suhara, Y.; Takayama, H.; Waku, K.; Seki, C.; Baba, N.; Ishima, Y. *J. Biol. Chem.* **1999**, *274*, 2794.
- Sugiura, T.; Kondo, S.; Kishimoto, S.; Miyashita, T.; Nakane, S.; Kodaka, T.; Suhara, Y.; Takayama, H.; Waku, K. *J. Biol. Chem.* **2000**, *275*, 605.
- Gonsiorek, W.; Lunn, C.; Fan, X.; Narula, S.; Lundell, D.; Hipkin, R. W. *Mol. Pharmacol.* **2000**, *57*, 1045.
- Savinainen, J. R.; Saario, S. M.; Niemi, R.; Jarvinen, T.; Laitinen, J. T. *Br. J. Pharmacol.* **2003**, *140*, 1451.
- Adams, I. B.; Ryan, W.; Singer, M.; Razdan, R. K.; Compton, D. R.; Martin, B. R. *Life Sci.* **1995**, *56*, 2041.
- Lin, S.; Khanolkar, A. D.; Fan, P.; Goutopoulos, A.; Qin, C.; Papahadjis, D.; Makriyannis, A. *J. Med. Chem.* **1998**, *41*, 5353.
- Goutopoulos, A.; Fan, P.; Khanolkar, A. D.; Xie, X.-Q.; Lin, S.; Makriyannis, A. *Bioorg. Med. Chem.* **2001**, *9*, 1673.
- Stamatov, S. D.; Stawinski, J. *Tetrahedron Lett.* **2002**, *43*, 1759.
- Savinainen, J. R.; Jarvinen, T.; Laine, K.; Laitinen, J. T. *Br. J. Pharmacol.* **2001**, *134*, 664.
- Saario, S. M.; Savinainen, J. R.; Laitinen, J. T.; Jarvinen, T.; Niemi, R. *Biochem. Pharmacol.* **2004**, *67*, 1381.
- 2-((*R*)-2-Methyl-arachidonoyl) glycerol (**1a**). ¹H NMR (CDCl₃): δ 5.44–5.31 (m, 8H), 4.92 (qn, $J = 5.0$ Hz, 1H), 3.82 (d, $J = 5.0$ Hz, 4H), 2.85–2.80 (m, 6H), 2.53 (st, $J = 6.8$ Hz, 1H), 2.13–2.03 (m, 4H), 1.82–1.75 (m, 1H), 1.54–1.46 (m, 1H), 1.39–1.25 (m, 6H), 1.19 (d, $J = 7.0$ Hz, 3H), 0.89 (t, $J = 7.0$ Hz, 3H). ¹³C NMR (CDCl₃): δ 176.9, 130.5, 129.0, 128.7, 128.6, 128.3, 128.1, 127.8, 127.5, 75.0, 62.4, 39.1, 33.5, 31.5, 29.3, 27.2, 25.6 (3C), 24.8, 22.5, 17.0, 14.0. $[\alpha]^{20} +15^\circ$ ($c = 0.19$; CH₂Cl₂). Elemental analysis: calculated for C₂₄H₄₀O₄*1/3 H₂O: C 72.32%, H 10.28%; found: C 72.50%, H 10.30%.
- 2-((*S*)-2-Methyl-arachidonoyl) glycerol (**1b**). ¹H NMR (CDCl₃): δ 5.44–5.31 (m, 8H), 4.92 (qn, $J = 5.0$ Hz, 1H), 3.83–3.82 (m, 4H), 2.85–2.80 (m, 6H), 2.57–2.50 (m, 1H), 2.13–2.03 (m, 4H), 1.82–1.75 (m, 1H), 1.55–1.47 (m, 1H), 1.39–1.25 (m, 6H), 1.19 (d, $J = 7.0$ Hz, 3H), 0.89 (t, $J = 7.0$ Hz, 3H). ¹³C NMR (CDCl₃): δ 176.9, 130.5, 128.9, 128.8, 128.6, 128.3, 128.1, 127.8, 127.5, 75.0, 62.5, 39.1, 33.5, 31.5, 29.3, 27.2, 25.6 (3C), 24.8, 22.5, 17.1, 14.0. $[\alpha]^{20} -11^\circ$ ($c = 0.37$; CH₂Cl₂). Elemental analysis: calculated for C₂₄H₄₀O₄: C 70.21%; H 10.31%; found: C 70.54%, H 10.02%.
- 2-((*R*)-2-Methyl-arachidonoyl)-(*rac*)(1-fluoro,3-hydroxy)-propyl ester (**2a**). ¹H NMR (CDCl₃): δ 5.43–5.31 (m, 8H), 5.08 (dq, $J = 4.6$ Hz, $J_{H-F} = 20.9$, 1H), 4.58 (dd, $J = 4.5$ Hz, $J_{H-F} = 47.1$ Hz, 2H), 3.82 (br s, 2H), 2.85–2.80 (m, 6H), 2.54 (st, $J = 7.0$ Hz, 1H), 2.11 (q, $J = 7.0$ Hz, 2H), 2.06 (q, $J = 6.9$ Hz, 2H), 1.82–1.74 (m, 1H), 1.54–1.47 (m, 1H), 1.39–1.25 (m, 6H), 1.19 (dd, $J = 1.3$ Hz, 7 Hz, 3H), 0.89 (t, $J = 7.0$ Hz, 3H). ¹³C NMR (CDCl₃): δ 176.3, 130.5, 128.9, 128.8, 128.6, 128.3, 128.1, 127.8, 127.5, 81.4 (d, $J_{C-F} = 172.3$ Hz), 72.8 (d, $J_{C-F} = 19.7$ Hz), 60.9 (d, $J_{C-F} = 7.0$ Hz), 39.0, 33.5, 31.5, 29.3, 27.2, 25.6 (3C), 24.8, 22.5, 17.0, 14.0. $[\alpha]^{20} -15^\circ$ ($c = 0.36$; CH₂Cl₂). GC-MS(EI) M = 394.

16. 2-((*S*)-2-Methyl-arachidonoyl)-(*rac*)(1-fluoro,3-hydroxy)-propyl ester (**2b**). ^1H NMR (CDCl_3): δ 5.43–5.31 (m, 8H), 5.08 (dq, $J = 4.6$ Hz, $J_{\text{H-F}} = 20.9$, 1H), 4.58 (dd, $J = 4.5$ Hz and $J_{\text{H-F}} = 47.1$ Hz, 2H), 3.82 (br s, 2H), 2.85–2.80 (m, 6H), 2.54 (st, $J = 7.0$ Hz, 1H), 2.11 (q, $J = 7.0$ Hz, 2H), 2.06 (q, $J = 6.9$ Hz, 2H), 1.82–1.74 (m, 1H), 1.54–1.47 (m, 1H), 1.39–1.25 (m, 6 H), 1.19 (d, $J = 7.0$ Hz, 3H), 0.89 (t, $J = 6.9$ Hz, 3H). ^{13}C NMR (CDCl_3): δ 176.3, 130.5, 128.9, 128.8, 128.6, 128.3, 128.1, 127.9, 127.5, 81.4 (d, $J_{\text{C-F}} = 172.4$ Hz), 72.8 (d, $J_{\text{C-F}} = 19.8$ Hz), 61.0 (d, $J_{\text{C-F}} = 6.9$ Hz), 39.0, 33.5, 31.5, 29.3, 27.2, 25.6 (3 C), 24.8, 22.6, 17.0, 14.0. $[\alpha]^{20} +15^\circ$ ($c = 0.13$; CH_2Cl_2). GC-MS(EI): M = 394.
17. 1,3-Difluoro-2-propyl-((*R*)-2-methyl)-arachidonate (**3a**). ^1H NMR (CDCl_3): δ 5.43–5.31 (m, 8H), 5.23 (tq, $J = 4.6$ Hz, $J_{\text{H-F}} = 19.8$ Hz, 1H), 4.58 (dd, $J = 4.6$ Hz, $J_{\text{H-F}} = 46.9$ Hz, 4H), 2.85–2.80 (m, 6H), 2.55 (st, $J = 7.0$ Hz, 1H), 2.13–2.03 (m, 4H), 1.82–1.75 (m, 1H), 1.54–1.47 (m, 1H), 1.39–1.26 (m, 6 H), 1.20 (d, $J = 7.0$ Hz, 3H), 0.89 (t, $J = 6.9$ Hz, 3H). ^{13}C NMR (CDCl_3) δ :175.7, 130.5, 128.9, 128.8, 128.6, 128.3, 128.1, 127.9, 127.5, 80.3 (ddd, $J_{\text{C-F}} = 1.4, 6.9$ and 173 Hz), 70.1 (t, $J_{\text{C-F}} = 20.8$ Hz), 39.0, 33.4, 29.3, 27.2, 25.6 (3C), 24.8, 22.6, 16.9, 14.0.
18. 1,3-Difluoro-2-propyl-((*S*)-2-methyl)-arachidonate (**3b**). ^1H NMR (CDCl_3): δ 5.43–5.31 (m, 8H), 5.23 (tq, $J = 4.6$ Hz, $J_{\text{H-F}} = 19.7$ Hz, 1H), 4.58 (dd, $J = 4.8$ Hz, $J_{\text{H-F}} = 46.9$ Hz, 4H), 2.85–2.80 (m, 6H), 2.55 (sext, $J = 7.0$ Hz, 1H), 2.13–2.03 (m, 4H), 1.82–1.75 (m, 1H), 1.54–1.47 (m, 1H), 1.39–1.26 (m, 6 H), 1.20 (d, $J = 7.0$ Hz, 3H), 0.89 (t, $J = 6.9$ Hz, 3H). ^{13}C NMR (CDCl_3): δ 175.6, 130.5, 128.9, 128.8, 128.6, 128.3, 128.1, 127.8, 127.5, 80.3 (ddd, $J_{\text{C-F}} = 1.4, 6.9, 173$ Hz), 70.1 (t, $J_{\text{C-F}} = 20.8$ Hz), 38.9, 33.4, 31.5, 29.3, 27.2, 25.6 (3C), 24.8, 22.5, 17.0, 14.0. $[\alpha]^{20} +15^\circ$ ($c = 1.0$; CH_2Cl_2). GC-MS(EI): M = 396.
19. ^1H NMR and ^{13}C NMR were recorded on a Bruker Avance 500 spectrometer operating on 500.1 and 125.8 MHz, respectively. CDCl_3 was used as a solvent, and tetramethylsilane (TMS) was used as an internal standard. The spectra were processed from the recorded FID files with MestRe-C software (version 2.3a, Departamento Química Orgánica, Universidad de Santiago de Compostela, Spain). Chemical shifts (δ) are reported in parts per million (ppm) downfield from TMS. Following abbreviations are used: s, singlet; br s, broad singlet; d, doublet; t, triplet; dd, doublet of doublets; ddd, doublet of doublets; dq, doublet of quintets; tq, triplet of quintets; qn, quintet; m, multiplet; st, sextet. Coupling constants are reported in Hz and letter J indicates 3J if not otherwise noted. ESI-MS spectra were acquired using a LCQ ion trap mass spectrometer equipped with an electrospray ionization source (Finnigan MAT, San Jose, CA, USA). Gas chromatography mass spectrum was obtained on a HP6890 GC mass spectrometer with electron-ionization detector. The free hydroxyl groups of the sample (0.1 mg/ml) in methanol were coated with silicon groups. Elemental analyses for C, H, and N were performed on a ThermoQuest CE Instruments EA1110-CHNS-O elemental analyser (ThermoQuest, Italy).
20. The analytical HPLC system consisted of a Merck Hitachi (Hitachi Ltd) L-7100 pump, D-7000 interface module, L-7455 diode-array detector (190–800 nm, set at 211 nm), and L-7250 programmable autosampler. The separations were performed with Zorbax SB-C18 endcapped reversed-phase precolumn (4.6 mm \times 12.5 mm, 5 μm) and column (4.6 mm \times 150 mm, 5 μm) (Agilent). The injection volume was 50 μL . A mobile phase mixture of 28% phosphate buffer (30 mM, pH 3.0) in acetonitrile at a flow rate of 2.0 mL min^{-1} was used. Retention times were 5.6 min for 2-AG, 6.1 min for 1-AG, 7.4 min for α -methyl-2-AG, 7.9 min for α -methyl-1-AG, 10.0 for AA, and 13.2 min for α -methyl-AA. The relative concentrations of the analytes were estimated on the basis of corresponding peak areas.