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Cannabioxepane, a novel tetracyclic cannabinoid from hemp, *Cannabis sativa* L.

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ABSTRACT

Apart from large amounts of cannabidiol, the known stilbenoids **1**–**4** and the oxylipins **7** and **8**, a fibre cultivar of *Cannabis sativa* derived from the historical *Carmagnola* variety gave the novel spiranic stilbenoid isocannabispiradienone (**5**) and the biphenyl-type cannabinoid cannabioxepane (CBX, **6**), a tetracyclic compound characterized by an unprecedented C-5/C-8′ oxygen bridge and devoid of cannabinoid activity. Structures were established by analysis of MS and NMR data, and the biogenetic derivation of the new compounds is discussed.

1. Introduction

Hemp, *Cannabis sativa* L. (Cannabaceae), is one of the best investigated plant species from both biomedical and phytochemical points of view. Hemp was domesticated thousands of years ago, and records of its use as a medicine and/or a divinatory agent date back to 3000 B.C.[1] The relevance of this plant for humans is still enormous, since the fibre-phenotype is extensively cultivated as a source of textiles and of an edible oil, while the psychotropic phenotype is the most popular illicit recreational drug in the world. Unsurprisingly, the phytochemistry of *C. sativa* has been thoroughly investigated, and several hundred compounds, often structurally unique, have been isolated from this plant, whose phytochemical profligacy is testified by the presence of alkaloids, fatty acids and esters, terpenoids, quinones, flavonoids, stilbenoids and, above all, cannabinoids.[2] These meroterpenoids are derived from the alkylation of a C_{12} (or C_9) olivetol-type polyketide with a monoterpene unit, and represent one of the best examples of the contribution of natural products to biomedical research. Thus, the study of the psychotropic properties of Δ^9-THC has led to the discovery of the metabotropic cannabinoid receptors (CB_1 and CB_2)[3] and, subsequently, of their endogenous agonists, a group of fatty acid amides and esters exemplified by anandamide.[4] Cannabinoids and endocannabinoids have always been a hot topic for chemical and biomedical research, and the successful development of Sativex™, a combination of natural cannabinoids approved in Canada, UK and Spain for the symptomatic treatment of multiple sclerosis, will undoubtedly foster novel activities in the area. This includes the characterization of minor, previously overlooked natural cannabinoids that, given the privileged-structure status of the class, might provide new lead structures for drug discovery.

Besides the well known Δ^9-THC, about one hundred further cannabinoids have been obtained from *C. sativa*. These compounds can be sorted out into seven major structural types, whose skeletal diversity arise from different cyclizations of cannabigerol (CBG) and the subsequent rearrangement of these primary cyclization products (Fig. 1).[5] The parent compounds of these classes are cannabichromene (CBC), cannabicyclol (CBL), cannabidiol (CBD), cannabielsoin (CBE), Δ^9-THC and cannabicoumaronone (CBCON), with the most recent additions to the cannabinoid inventory falling into the CBCON,[6] CBC and CBG families.[7] Despite all these studies, structural diversity within cannabinoids is far from having been exhaustedly unravelled, as shown by the recent discovery of cannabimovone (CBM, Fig. 1).[8] This CBD analogue shows a unique resorcinyl *abeo*-menthane structure, and was isolated from an Italian fibre hemp derived from the historical cultivar *Carmagnola*.[9] Investigation of the relatively apolar fractions from an extract of this plant has now led to the isolation of five stilbenoids (**1**–**5**), including the new spirane isocannabispiradienone (**5**), and of a novel bis-oxygen bridged diphenyl-type cannabinoid, which we have named cannabioxepane (**6**, CBX).

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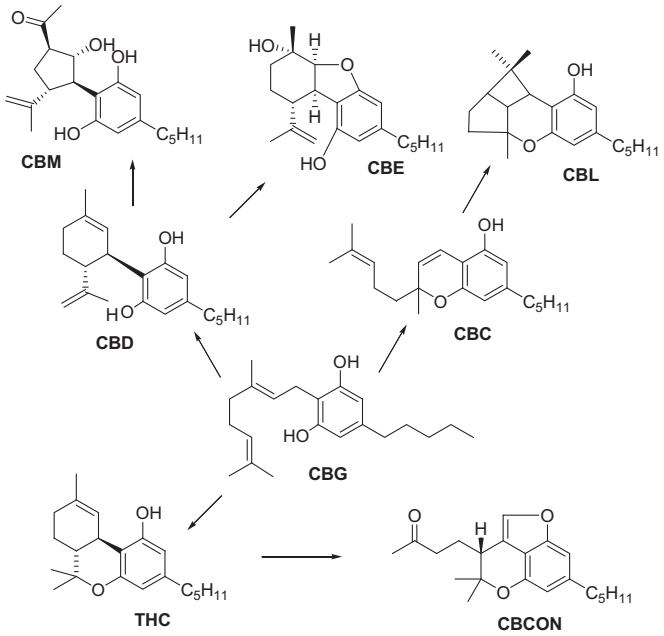

Fig. 1. The parent compounds of the cannabinoid structural classes and their demonstrated or postulated biogenetic relationships.

2. Results and discussion

Dried female flowerheads of *C. sativa* were extracted with acetone at room temperture. Removal of the solvent left a gummy residue that was partitioned between 1:1 aqueous methanol and petroleum ether. The defatted polar phase was then concentrated and extracted with CH_2Cl_2. The organic phase was dried (Na_2SO_4) and evaporated to afford a black gum, that was purified by flash chromatography on RP-18 silica gel (Biotage equipment). Repeated purification of the obtained fractions by gravity column chromatography on silica gel (petroleum ether/EtOAc mixtures) followed by HPLC led to the isolation of cannabispirane (**1**),[10] β-cannabispiranol (**2**),[11] α-cannabispiranol (**3**),[12] cannabispiradienone (**4**)[13] and two new compounds, isocannabispiradienone (**5**) and cannabioxepane (**6**) (Fig. 2).

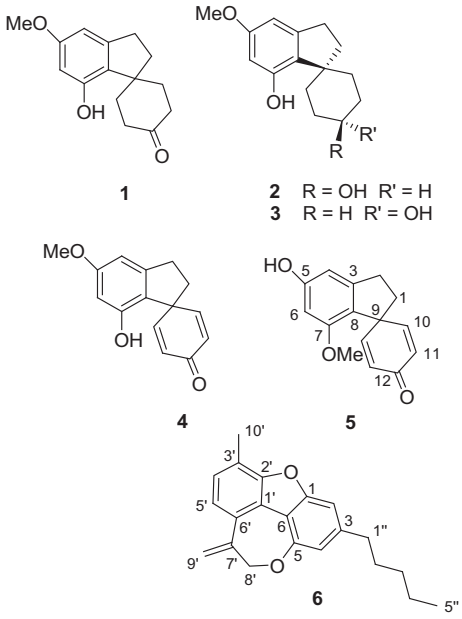

Fig. 2. The six phenolic compounds isolated from *C. sativa* L., including the new isocannabispiradienone (**5**) and cannabioxepane (**6**).

Two polyunsaturated fatty acids were also obtained, namely 12*S*-hydroxy-9*Z*,13*E*,15*Z*-octadecatrienoic acid (**7**) and 16*R*-hydroxy-9*Z*,12*Z*,14*E*-octadecatrienoic acid (**8**) (Fig. 3). The known compounds **1**–**4** were identified by comparison of their spectral data with those reported in the literature.[10–13] The fatty acids **7** and **8** have been recently obtained as methyl esters from *Swertia japonica* (Gentianaceae),[14] but had never been reported from *C. sativa*.

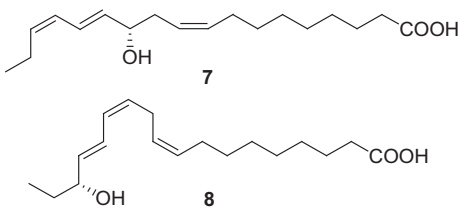

Fig. 3. The two polyunsaturated fatty acids **7** and **8** isolated from *C. sativa* L.

Isocannabispiradienone (**5**) has the molecular formula $C_{15}H_{14}O_3$ (by HR-ESIMS), indicative of nine unsaturation degrees. ^{1}H NMR spectrum of **5** ($CDCl_3$) was reminiscent of that reported for **4**, being characterized by two coupled 2H doublets at δ_H 6.86 and 6.26 (J=10.0 Hz), two coupled 2H triplets at δ_H 3.06 and 2.25 (J=7.8 Hz), and two *m*-coupled aromatic protons at δ_H 6.19 and 6.36 (J=2.1 Hz). A methyl singlet resonating at δ_H 3.61 and an exchangeable 1H singlet at δ_H 4.73 completed the signals of the ^{1}H NMR spectrum of **5**. All the proton signals were associated with those of the directly attached carbon atoms with the help of the 2D NMR HSQC spectrum. The presence of a cross-conjugated cyclohexadienone moiety was then deduced from the following experimental evidences: (i) the ^{13}C NMR spectrum of **5** showed only 12 signals, as a result of the presence of symmetrical cyclohexadienone sub-structure (and the accidental isochronicity of two aromatic carbons, see below); (ii) the presence of a doubly conjugated ketone (C-12) was suggested by the ^{13}C NMR resonance at δ_C 186.9 and by the IR (KBr) peak at ν_{max} 1650 cm^{-1}; (iii) the HMBC cross-peaks of H-10/H-14 with C-12, (C-14/C-10) and with the unprotonated C-9 (δ_C 52.6). The latter carbon atom was subsequently identified as the spiro-carbon connecting the dienone ring with a five-membered ring, as indicated by the HMBC cross-peaks H-10/C-1, H-10/C-8 (δ_C 121.4), H-1/C-8, H-1/C-9 and H-1/C-3 (δ_C 147.2). The structural framework of isocannabispiradienone (**5**) is completed by a dioxygenated phenyl ring condensed with the five-membered ring. The signal at δ_H 6.36 showed HMBC cross-peaks with C-2, C-3, C-8, with the oxygenated C-5 (δ_C 157.6) and with the second aromatic methine carbon (C-6), and therefore it can be confidently assigned to H-4. Accordingly, the signal at δ_H 6.19, showing HMBC correlations with both the oxygenated carbons (C-5 and C-7) as well as with C-4 and C-8, can be assigned to H-6.

Unfortunately, the location of the methyl group could not be unambiguously determined through the HMBC spectrum since the carbon resonances of C-5 and C-7 were coincident. However, inspection of the ROESY spectrum allowed a straightforward solution to this problem; the OH signal at δ_H 4.73 showed cross-peaks with both H-4 and H-6, while the methoxy singlet at δ_H 3.61 showed cross-peak only with H-6, in agreement with its location at C-7, thus completely defining the chemical structure of **5**.

A derivation of isocannabispiradienone (**5**) from cannabispiradienone (**4**) by methyl swapping between the two phenolic hydroxyl seems unlikely. More plausibly, **4** and **5** might derive from the regiochemically divergent oxidative coupling of the same dihydrostilbene precursor (Fig. 4). Just like cannabispiradienone (**4**) is the alleged precursor of cannabispirane and cannabispiranol,[13] so isocannabispiradienone might be the precursor of isocannabispirane.[15] Therefore, the existence of isocannabispiranols in cannabis extracts could be foreseen.

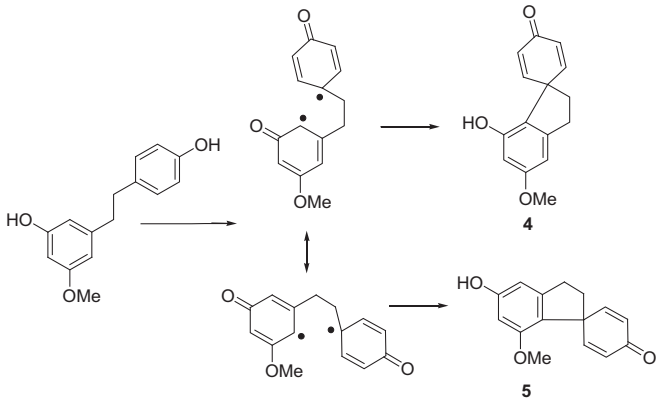

Fig. 4. Postulated biogenetic origin of isocannabispiradienone (**5**).

HR-ESIMS established the molecular formula of cannabioxepane (CBX, **6**) as $C_{21}H_{22}O_2$, implying eleven degrees of unsaturation. The ^{1}H NMR spectrum of **6** ($CDCl_3$, Table 1) showed a pair of coupled doublets at δ 7.34 and 7.16, four singlets between δ 7.10 and 5.40, a methylene broad singlet at δ 4.85, a deshielded methyl singlet at δ 2.56 and, finally, five well resolved multiplets between δ 2.70 and 0.80, which, on the basis of a 2D COSY spectrum, were combined into a linear pentyl chain (see Fig. 5). The 2D NMR HSQC spectrum of **6** made it possible to associate all the proton signals with those of the directly linked carbons, revealing that the singlets at δ_H 5.88 and 5.44 are actually linked at the same carbon at δ_C 118.6 and that the singlet at δ_H 4.85 must be an oxymethylene group (δ_C 75.1). The ^{13}C NMR spectrum of **6** ($CDCl_3$, Table 1) showed also the signals of nine unprotonated carbon atoms, all resonating in the sp^2 region of the spectrum, between δ_C 120 and 158. The above data were strongly suggestive of the presence of two phenyl rings and of an additional carbon-carbon double bond. Therefore, to account for the nine unsaturations implied by the molecular formula of CBX, two additional rings were required. A series of key $^{2,3}J_{H,C}$ correlations evidenced by the HMBC spectrum (Fig. 5) were critical to assemble the skeletal fragments and to draw the structure of CBX, allowing also the complete assignment of the proton and carbon resonances as reported in Table 1.

In particular, the three correlations exhibited by H_3-10′ with the oxygenated C-2′ (δ_C 153.7), with C-3′ and with the methine carbon C-4′ (δ_C 127.4), along with the correlations of H-5′ with C-1′, C-3′ and C-6′ allowed the identification of a tetrasubstituted phenyl ring bearing a methyl group and an oxygen atom on adjacent positions. The unprotonated sp^2 carbon C-7′ was attached at C-6′ on the basis of the HMBC cross-peaks H-5′/C-7′, H_2-9′/C-6′, H_2-9′/C-7′, H_2-8′/C-6′, H_2-8′/C-7′ and, these data, together with the HMBC cross-peak H_2-9′/C-8′ allowed also to establish the linkage of both the sp^2 methylene carbon (C-9′) and the oxygenated sp^3 methylene (C-8′) at C-7′. The second phenyl ring was also tetrasubstituted, and its linkage with the pentyl chain at C-3 was indicated by the HMBC cross-peaks of H_2-1″ with C-3 and with the methine carbons C-2 and C-4. The pattern of HMBC cross-peaks shown by H-2 and H-4 suggested that both C-1 (δ_C 157.5) and C-5 (δ_C 155.2) are oxygenated carbons, and the key HMBC cross-peak H_2-8′/C-5 indicated the presence of an oxygen bridge to connect C-8′ and C-5. Finally, the direct linkage of C-6 with C-1′ (defining a seven-membered ring) and the presence of a second oxygen bridge to connect C-1 with C-2′ (defining a furan ring), completed the dioxygenated tetracyclic structure of cannabioxepane (**6**), and allowed complete rationalization of the NMR and MS data, which was in perfect agreement with biogenetic arguments.

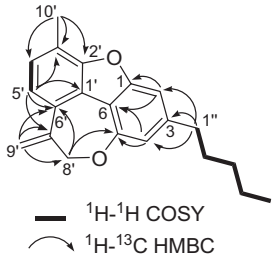

Fig. 5. COSY and $^{2,3}J$ H→C HMBC correlations of cannabioxepane (**6**).

Cannabioxepane (**6**, CBX) is characterized by an unprecedented tetracyclic skeleton including, in addition to two phenyl rings, a furan and an oxygenated seven-membered ring. This skeleton bears some similarity to some cannabinoids from the CBE family (Fig. 1), and particularly with the aromatized analogue named cannabifuran,[16] however, it is noteworthy that no member of the CBE family shows a fourth ring. On the other hand, in the structure of Δ^9-THC and its derivatives (of their alleged CBG precursors) the oxygen atom at C-5 is involved in the formation of a six-membered ring with the unprotonated carbon of the isopropyl side chain (C-7′). CBX is the first cannabinoid to show a linkage between the oxygen atom at C-5 and C-8′, thus giving rise to the unprecedented seven-membered ring. From a biosynthetic standpoint, CBX could be derived from the 7-*endo-tet* opening of the 7′-8′ epoxide of a CBE-like precursor, followed by dehydration (Fig. 6). While cyclization of CBD-epoxides strongly favours the formation of pyrane derivatives via a 6-*exo-tet* process, it was noticed that closure of a furan ring between C-2′ and the 1-hydroxyl could steer cyclization towards the formation of an alternative 7-*endo-tet* process, with formation of an oxepane rather than a pyran ring.[17] It seems therefore logical to assume that dehydrocannabifuran epoxide **9**, a yet unreported cannabinoid, is the ultimate precursor of CBX.

CBX was evaluated for its affinity to CB_1, CB_2 and $TRPA_1$ receptors but no significant activity was detected. This result is in perfect agreement with the marked reduction of CB affinity caused by aromatization of the terpene-derived six-membered ring and etherification of the phenolic 1-hydroxyl, both detrimental for activity.[18]

In conclusion, phytochemical analysis of a fibre cultivar of *C. sativa* afforded, in addition to the novel spiranic stilbenoid isocannabispiradienone (**5**), the biphenyl-type cannabinoid cannabioxepane (CBX, **6**), a tetracyclic compound characterized by an unprecedented C-5/C-8′ oxygen bridge and devoid of cannabinoid activity.

Table 1
^{1}H (500 MHz) and ^{13}C (125 MHz) NMR Data of Cannabioxepane (**6**) in $CDCl_3$

Pos.	δ_C, mult.	δ_H, mult., *J* in Hz
1	157.5, C	
2	104.4, CH	7.01, s
3	144.3, C	
4	110.5, CH	6.72, s
5	155.2, C	
6	112.5, C	
1′	125.5, C	
2′	153.7, C	
3′	120.7, C	
4′	127.4, CH	7.16, d, 6.2
5′	118.4, CH	7.34, d, 6.2
6′	131.2, C	
7′	142.8, C	
8′	75.1, CH_2	4.85, br s
9′a	118.6, CH_2	5.88, br s
9′b		5.44, br s
10′	15.1, CH_3	2.56, s
1″	36.6, CH_2	2.70, t, 7.6
2″	31.4, CH_2	1.66, m
3″	31.2, CH_2	1.32, m
4″	22.7, CH_2	1.30, m
5″	14.3, CH_3	0.88, t, 7.0

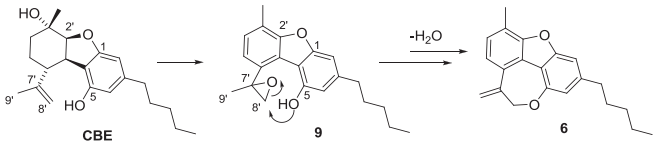

Fig. 6. Possible biogenesis of CBX from CBD via the epoxide **9**.

3. Experimental

3.1. General

Optical rotations ($CHCl_3$) were measured at 589 nm on a P2000 Jasco polarimeter using a 10 cm microcell. UV spectra were measured on a Thermo Scientific (mod. Nanodrop2000c) instrument. ^1H (500 MHz) and ^{13}C (125 MHz) NMR spectra were measured on a Varian INOVA spectrometer. Chemical shifts were referenced to the residual solvent signal ($CDCl_3$: δ_H 7.26, δ_C 77.0). Homonuclear ^1H connectivities were determined by the COSY experiment. One-bond heteronuclear $^1H-^{13}C$ connectivities were determined with the HSQC experiment. Two- and three-bond $^1H-^{13}C$ connectivities were determined by gradient-HMBC experiments optimized for a $^{2,3}J$ of 9 Hz. Through-space ^1H connectivities were evidenced by using a ROESY experiment with a mixing time of 500 ms. Low- and high-resolution ESI-MS spectra were performed on LTQ Orbitrap XL (Thermo Scientific) mass spectrometer. Medium pressure liquid chromatography was performed on a Büchi apparatus using a silica gel (230–400 mesh) column; HPLC were achieved on a Knauer apparatus equipped with a refractive index detector and analytical LUNA (Phenomenex) SI60 (250×4 mm) columns.

3.2. Collection, extraction and isolation

C. sativa derived from a greenhouse cultivation at CRA-CIN, Rovigo (Italy), where a voucher specimen is kept, and was collected in November 2008. The isolation and manipulation of all cannabinoids was done in accordance with their legal status (Authorization SP/101 of the Ministero della Salute, Rome, Italy).

Dried flowerheads of *C. sativa* (500 g) were heated at 120 °C for 2.5 h in a ventilated oven, to decarboxylate pre-cannabinoids. After cooling to room temperature, the plant material was extracted with acetone (2×10 L). Removal of the solvent left a gummy residue that was partitioned between 1:1 aqueous methanol (1 L) and petroleum ether (1 L). The defatted polar phase was concentrated and extracted with CH_2Cl_2. The organic phase was dried (Na_2SO_4) and evaporated to afford a black gum (10 g), that was purified by flash chromatography on RP-18 silica gel (Biotage equipment, 250 mL column, linear gradient, from MeOH/H_2O 55:45 to 90:10). Overall, five fractions (A1–A5) were collected. Fraction A2 was further fractionated by gravity column chromatography on silica gel, using acidified (0.5% HOAc) petroleum ether/EtOAc mixtures to obtain 20 subfractions (B1–B20), which were subsequently purified by HPLC. HPLC purification of fraction B2 (eluent *n*-hexane/EtOAc 85:15) yielded cannabispirane (**1**, 15.0 mg, 30 ppm on dried plant material). HPLC purification of fraction B3 (eluent *n*-hexane/EtOAc 8:2) yielded cannabispiradienone (**4**, 5.0 mg, 11 ppm on dried plant material). HPLC purification of fraction B4 (eluent *n*-hexane/EtOAc 75:25) yielded 12-hydroxy-3,9,15-octadecatrienoic acid (**7**, 6.0 mg, 12 ppm on dried plant material), isocannabispiradienone (**5**, 2.8 mg, 5 ppm on dried plant material), β-cannabispiranol (**2**, 22.0 mg, 48 ppm on dried plant material) and α-cannabispiranol (**3**, 15.0 mg, 30 ppm on dried plant material). HPLC purification of fraction B5 (eluent *n*-hexane/EtOAc 75:25) yielded 16-hydroxy-9*Z*,12*Z*,14*E*-octadecatrienoic acid (**8**, 4.0 mg, 9 ppm on dried plant material), The less polar fraction A4 was subjected to repeated column chromatographies on silica gel (petroleum ether/EtOAc mixtures) to obtain a crude fraction, which was further purified by HPLC (eluent *n*-hexane/EtOAc 95:5) to yield cannabioxepane (**6**, 3.5 mg, 7 ppm on dried plant material).

*3.2.1. Isocannabispiradienone (**5**).* Colourless amorphous solid, mp 175 °C (decomp.); IR (KBr): ν_{max} 3100, 1650 cm^{-1}. UV (MeOH): λ_{max} (ε) 215 (28,000), 240 (23,300), 277 (3000), 287 (3000) nm. ^1H NMR ($CDCl_3$, 500 MHz): δ 6.86 (H-10/H-14), 2H, d, *J*=10.0 Hz), 6.36 (H-4, 1H, d, *J*2.1 Hz), 6.26 (H-11/H-13), 2H, d, *J*10.0 Hz), 6.19 (H-6, 1H, d, *J* 2.1 Hz), 4.73 (5-OH, 1H, s), 3.61 (7-OMe, 3H, s), 3.06 (H_2-2, 2H, t, *J* 7.8 Hz), 2.25 (H_2-1, 2H, t, *J* 7.8 Hz). ^{13}C NMR ($CDCl_3$, 125 MHz): δ 186.9 (C-12), 158.9 (C-11/C-13), 157.6 (C-5, C-7), 153.1 (C-10/C-14), 147.2 (C-3), 121.4 (C-8), 103.8 (C-4), 97.5 (C-6), 55.9 (7-OMe), 52.6 (C-9), 38.1 (C-1), 31.6 (C-2). ESI-MS (positive ions) *m*/*z* 265 $[M+Na]^+$; HREIMS *m*/*z* 265.0844 $[M+Na]^+$ ($C_{15}H_{14}NaO_3$ requires 265.0841).

*3.2.2. Cannabioxepane (**6**).* Colourless amorphous solid, mp 75–76 °C; IR (KBr): ν_{max} 3065, 2950, 1599, 1459, 1183 cm^{-1}. ^1H and ^{13}C NMR ($CDCl_3$) see Table 1. ESI-MS (positive ions) *m*/*z* 329 $[M+Na]^+$; HREIMS *m*/*z* 329.1522 $[M+Na]^+$ ($C_{21}H_{22}NaO_2$ requires 329.1517).

*3.2.3. 12S-Hydroxy-9Z,13E,15Z-octadecatrienoic acid (**7**)[14].* Colourless amorphous solid, mp 50–51 °C; $[\alpha]_{D^{20}}$ +2 (*c* 0.05, $CHCl_3$); CD (MeOH): λ_{max} 243 nm ($\Delta\varepsilon$=+0.3); ^1H NMR ($CDCl_3$, 500 MHz): δ 6.52 (H-14, 1H, dd, *J* 15.5, 11.0 Hz), 5.97 (H-15, 1H, t, *J* 11.0 Hz), 5.69 (H-13, 1H, dd, *J* 15.5, 6.0 Hz), 5.54 (H-9, 1H, dt, *J* 10.7, 7.2 Hz), 5.42 (H-16, 1H, dt, *J* 11.0, 7.2 Hz), 5.36 (H-10, 1H, dt, *J* 10.7, 7.2 Hz), 4.21 (H-12, 1H, m), 2.33 (H_2-11, 2H, t, *J* 7.2 Hz), 2.30 (H_2-2, 2H, t, *J* 7.2 Hz), 2.18 (H_2-17, 2H, q, *J* 7.2 Hz), 2.09 (H_2-8, 2H, q, *J* 7.2 Hz), 1.35–1.30 (H_2-7, H_2-6, H_2-5, H_2-4, 8H, m), 1.60 (H_2-3, 2H, q, *J* 7.2 Hz), 1.01 (H_3-18, 3H, t, *J* 7.2 Hz). ESI-MS (positive ions) *m*/*z* 317 $[M+Na]^+$; HREIMS *m*/*z* 317.2100 $[M+Na]^+$ ($C_{18}H_{30}NaO_3$ requires 317.2093).

*3.2.4. 16R-Hydroxy-9Z,12Z,14E-octadecatrienoic acid (**8**)[14].* Colourless amorphous solid, mp 49–50 °C; CD (MeOH): λ_{max} 229 nm ($\Delta\varepsilon$=−0.8); ^1H NMR ($CDCl_3$, 500 MHz): δ 6.52 (H-14, 1H, dd, *J* 15.0, 11.0 Hz), 6.00 (H-13, 1H, t, *J* 11.0 Hz), 5.69 (H-15, 1H, dd, *J* 15.5, 7.0 Hz), 5.45 (H-12, 1H, dd, *J* 11.0, 7.0 Hz), 5.40 (H-9, 1H, dd, *J* 11.0, 7.0 Hz), 5.35 (H-10, 1H, dt, *J* 11.0, 7.0 Hz), 4.09 (H-16, 1H, m), 2.93 (H_2-11, 2H, t, *J* 7.5 Hz), 2.31 (H_2-2, 2H, t, *J* 7.2 Hz), 2.06 (H_2-8, 2H, q, *J* 7.2 Hz), 1.60 (H_2-3, H_2-17, 4H, m), 1.35–1.30 (H_2-7, H_2-6, H_2-5, H_2-4, 8H, m), 0.88 (H_3-18, 3H, t, *J* 7.2 Hz). ESI-MS (positive ions) *m*/*z* 317 $[M+Na]^+$; HREIMS *m*/*z* 317.2090 $[M+Na]^+$ ($C_{18}H_{30}NaO_3$ requires 317.2093).

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Supplementary data

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.tet.2011.03.062.

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