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Oxylipins from the microalgae *Chlamydomonas debaryana* and *Nannochloropsis gaditana* and their activity as TNF-α inhibitors

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ABSTRACT

The chemical study of the microalgae *Chlamydomonas debaryana* and *Nannochloropsis gaditana* has led to the isolation of oxylipins. The samples of *C. debaryana* have yielded the compounds (4*Z*,7*Z*,9*E*,11*S*,13*Z*)-11-hydroxyhexadeca-4,7,9,13-tetraenoic acid (**1**), (4*Z*,7*E*,9*E*,13*Z*)-11-hydroxyhexadeca-4,7,9,13-tetraenoic acid (**2**), (4*Z*,6*E*,10*Z*,13*Z*)-8-hydroxyhexadeca-4,6,10,13-tetraenoic acid (**3**), (4*Z*,8*E*,10*Z*,13*Z*)-7-hydroxyhexadeca-4,8,10,13-tetraenoic acid (**4**), and (5*E*,7*Z*,10*Z*,13*Z*)-4-hydroxyhexadeca-5,7,10,13-tetraenoic acid (**5**), which are derived from the fatty acid 16:4$\Delta^{4,7,10,13}$ together with the compound (5*Z*,9*Z*,11*E*,15*Z*)-13-hydroxyoctadeca-5,9,11,15-tetraenoic acid (**7**) derived from coniferonic acid (18:4$\Delta^{5,9,12,15}$). In addition, the known polyunsaturated hydroxy acids 11-HHT (**6**), (5*Z*,9*Z*,11*E*)-13-hydroxyoctadeca-5,9,11-trienoic acid (**8**), (13*S*)-HOTE (**9**), (9*E*,11*E*,15*Z*)-13-hydroxyoctadeca-9,11,15-trienoic acid (**10**), 9-HOTE (**11**), 12-HOTE (**12**), 16-HOTE (**13**) and (13*S*)-HODE (**14**) have also been obtained. The chemical study of *N. gaditana* has led to the isolation of the hydroxy acid (15*S*)-HEPE (**15**) derived from EPA (20:5$\Delta^{5,8,11,14,17}$). The structures of the isolated compounds were established by spectroscopic means. The optical activity displayed by oxylipins **1**, **2**, **6**, **7**, **9**, **10**, **14**, and **15** suggests the occurrence of LOX-mediated pathways in *C. debaryana* and *N. gaditana*. In anti-inflammatory assays, all the tested compounds inhibited the TNF-α production in LPS-stimulated THP-1 macrophages. The most active oxylipin was the C-16 hydroxy acid **1**, which at 25 μM caused a 60% decrease of the TNF-α level.

Introduction

Oxylipins are a large and structurally diverse family of lipid metabolites generated by the oxidation of polyunsaturated fatty acids (PUFAs) (Mosblech et al., 2009). Oxylipins are widespread in nature occurring in animals (Shimizu, 2009), plants, mosses, algae, bacteria, and fungi (Andreou et al., 2009). In general, oxylipins are bioactive metabolites involved in regulating developmental processes and in environmental and pathological responses.

Most plant oxylipins are derived from linoleic acid (LA, 18:2$\Delta^{9,12}$), α-linolenic acid (ALA, 18:3$\Delta^{9,12,15}$) or roughanic acid (16:3$\Delta^{7,10,13}$), whose enzymatic peroxidation is catalyzed by α-dioxygenase (α-DOX) or, mainly, by lipoxygenase (LOX) enzymes (Andreou and Feussner, 2009; Liavonchanka and Feussner, 2006). The hydroperoxides thus formed can subsequently be transformed by the action of different enzymes into an array of metabolites, including hydroxy and oxo fatty acids, divinyl ethers, volatile aldehydes, and jasmonates (Mosblech et al., 2009), which play important roles as signal molecules and defensive compounds (Blée, 2002; Howe and Schilmiller, 2002; Weber, 2002).

Red and brown macroalgae have also been source of a variety of metabolites derived from C18 and C20 PUFAs such as arachidonic acid (ARA, 20:4$\Delta^{5,8,11,14}$) and eicosapentaenoic acid (EPA, 20:5$\Delta^{5,8,11,14,17}$) *via* LOX-mediated pathways. Although studies on green seaweeds are much scarcer, oxylipin formation seems mainly based on the oxidation of C18 PUFAs (Andreou et al., 2009; Gerwick, 1994; Gerwick and Singh, 2002; Guschina and Harwood, 2006).

With respect to microalgae, initial research on some diatom species showed that the oxidation of PUFAs followed by chain cleavage leads to an array of polyunsaturated aldehydes involved in the chemical defense against predation (Cadwell, 2009; Fontana et al., 2007; Pohnert, 2005). Later, a series of hydroxy acids,

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hydroxy-epoxy acids, and oxoacids derived from the LOX-mediated oxidation of the PUFAs 16:3$\Delta^{6,9,12}$, 16:4$\Delta^{6,9,12,15}$, and EPA have also been identified in various species of diatoms (Cutignano et al., 2011; Fontana et al., 2007; Lamari et al., 2013). Only a few additional accounts of oxylipins from microalgae have been reported and they are restricted to the identification of hydroxy acids derived from LA and ALA in the chlorophycean *Dunaliella acidophila* (Pollio et al., 1988) and in the cyanobacteria species *Lygnbya majuscula* (Cardellina and Moore, 1980), *Anabaena flos-aquae f. flos-aquae* (Murakami et al., 1992), *Oscillatoria redekei* HUB 051 (Mundt et al., 2003), and *Nostoc* spp. (Lang and Feussner, 2007; Lang et al., 2008).

As a part of our project aimed to the study of bioactive compounds from microalgae grown in outdoor mass cultures, we have examined the biomass derived from cultures of the freshwater species *Chlamydomonas debaryana* (Class Chlorophyceae) and of the seawater species *Nannochloropsis gaditana* (Class Eustigmastophyceae). The genus *Chlamydomonas* comprises a high variety of microalgal species, mostly from freshwaters and often abundant in nutrient-rich environments (Klochkova et al., 2008). The biomass samples obtained from cultures of *C. debaryana* have yielded an array of monohydroxylated C16 and C18 acids (Fig. 1). The C16 derivatives include the new compounds **1**–**5** and the known related acid **6**. The C18 hydroxy acids include the new compound **7**, together with the known acids **8**–**14**. On the other hand, microalgae of the genus *Nannochloropsis* are mostly marine species that are used as food in aquaculture and are renown by their high content in EPA (Forján et al., 2011). The study of the biomass of *N. gaditana* has led to the isolation of the C20 hydroxy acid **15**. In addition, the major isolated compounds have been tested in TNF-α inhibition assays to evaluate their anti-inflammatory properties.

Results and discussion

Compound **1** was obtained as an optically active oil ($[\alpha]_D$ = +7.5 (*c* 0.08, MeOH)), whose molecular formula $C_{16}H_{24}O_3$ was determined by HRMS. The ^{13}C NMR spectrum exhibited a signal at δ_C 177.1 attributable to a carboxylic acid function, eight signals between 120 and 140 ppm due to four disubstituted double bonds, and a signal at δ_C 73.2 assigned to a hydroxy-bearing methine (Table 1). These functions accounted for the five unsaturation degrees deduced from the molecular formula and indicated that compound **1** was a monohydroxylated hexadecatetraenoic acid. The positions of the double bonds and the hydroxy group were defined from the COSY and HMBC spectra (Fig. 2). The location of a double bond at C-13,C-14 was readily inferred from the COSY coupling of the methyl group at δ_H 0.95 (t, *J* = 7.5 Hz, Me-16) with the methylene protons at δ_H 2.05 (H_2-15) which in turn were coupled with the olefinic proton at δ_H 5.46 (H-14). The olefinic carbons C-13 and C-14 showed HMBC correlations with two allylic methylene protons at δ_H 2.31/2.26 (H_2-12) that were coupled in the COSY

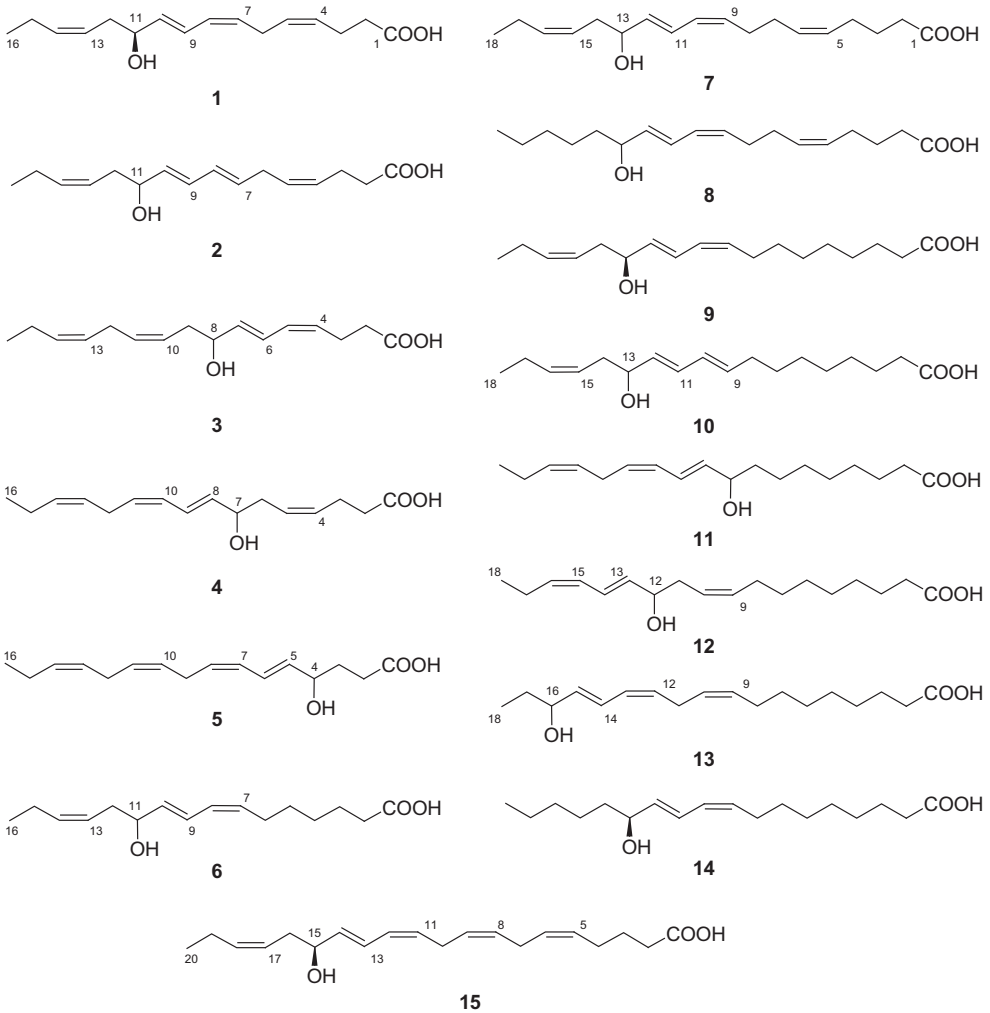

Fig. 1. Structures of the hydroxy acids **1**–**14** isolated from *Chlamydomonas debaryana* and of the hydroxy acid **15** isolated from *Nannochloropsis gaditana*.

Table 1
^{1}H and ^{13}C NMR data of compounds **1**, **2**, and **6** (CD_3OD).

Position	**1**[a]		**2**[b]		**6**[a]	
	δ_C	δ_H, m (*J* in Hz)	δ_C	δ_H, m (*J* in Hz)	δ_C	δ_H, m (*J* in Hz)
1	177.1		177.4		177.9	
2	35.1	2.32 m	35.2	2.32 m	35.1	2.26 t (7.5)
3	23.9	2.38 m	23.9	2.35 m	26.0	1.60 m
4	129.5	5.40 m	130.1	5.43 m	29.8	1.36 m
5	129.8	5.40 m	129.1	5.43 m	30.4	1.41 m
6	26.9	2.97 brdd (7.3,5.8)	31.2	2.85 dd (6.4,6.4)	28.5	2.20 dt (7.6,7.6)
7	130.7	5.36 m	133.4	5.65 dt (15.2,6.4)	132.8	5.40 dt (10.9,7.6)
8	129.3	5.97 dd (11.1,10.8)	131.3	6.04 dd (15.2,10.3)	129.4	5.96 dd (11.2,10.9)
9	126.3	6.54 dddd (15.2,11.1,1.2,1.2)	131.6	6.16 dd (15.2,10.3)	126.6	6.49 dd (15.3,11.2)
10	137.3	5.67 dd (15.2,6.5)	135.0	5.56 dd (15.2,6.4)	136.8	5.63 dd (15.3,6.8)
11	73.2	4.12 dt (6.5,6.5)	73.3	4.05 dt (6.4,6.4)	73.3	4.10 dt (6.6,6.6)
12	36.2	2.31 m	36.2	2.29 m	36.3	2.30 m
		2.26 ddd (14.5,7.4,7.4)		2.24 m		2.24 m
13	125.5	5.36 m	125.6	5.33 m	125.5	5.35 dtt (10.6,7.3,1.5)
14	134.6	5.46 dtt (10.7,7.3,1.5)	134.5	5.45 m	134.6	5.46 dtt (10.6,7.3,1.5)
15	21.7	2.05 qdd (7.5,7.5,1.3)	21.7	2.04 qdd (7.8,7.3,1.5)	21.7	2.05 qdd (7.5,7.5,0.9)
16	14.5	0.95 t (7.5)	14.5	0.95 t (7.6)	14.5	0.95 t (7.6)

[a] ^{1}H at 600 MHz and ^{13}C at 150 MHz.
[b] ^{1}H at 500 MHz and ^{13}C at 125 MHz.

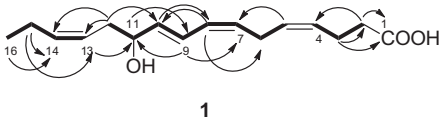

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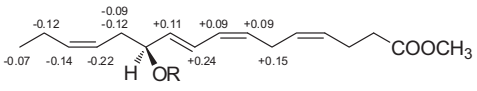
Fig. 2. Key COSY (bold) and HMBC (arrow) correlations observed for compound **1**.

spectrum with the oxymethine proton at δ_H 4.12 (H-11), thus defining the location of the hydroxy group at C-11. The sequence of COSY couplings observed from the oxymethine proton along four olefinic protons at δ_H 5.67 (H-10), 6.54 (H-9), 5.97 (H-8), and 5.36 (H-7) indicated that the hydroxy-bearing methine at C-11 was adjacent to a conjugated diene system at C-7,C-8 and C-9,C-10. The HMBC correlation of the carboxylic carbon (C-1) with the allylic methylene protons at δ_H 2.38 (H_2-3) which were also coupled in the COSY spectrum with the olefinic protons signal at δ_H 5.40 (m, H-4 and H-5) defined the location of the remaining double bond at C-4,C-5. The proton coupling constants $J_{7,8}$ = 10.8 Hz, $J_{9,10}$ = 15.2 Hz, and $J_{13,14}$ = 10.7 Hz indicated the 7*Z*,9*E*,13*Z* configuration. The *Z* geometry of the double bond at C-4,C-5 was supported by the chemical shifts of the allylic methylene carbon C-3 at δ_C 23.9 and the *bis*-allylic methylene carbon C-6 at δ_C 26.9 (Breitmaier and Voelter, 1989). The absolute configuration at C-11 was determined by NMR analysis of the derivatives **1r** and **1s** obtained by esterification of **1** with $TMSCH_2N_2$ and subsequent treatment of the methyl ester **1a** with (*R*)- and (*S*)-α-methoxy-α-phenylacetic acids (MPA), respectively. Positive chemical shift differences ($\Delta\delta = \delta_R - \delta_S$) were obtained for H-6, H-7, H-8, H-9, and H-10 while negative values were obtained for H-12, H-13, H-14, H-15, and Me-16 (Fig. 3), indicating an *S* configuration at C-11 (Seco et al., 2004). All these data led to define for compound **1** the structure (4*Z*,7*Z*,9*E*,11*S*,13*Z*)-11-hydroxyhexadeca-4,7,9,13-tetraenoic acid.



1a R= H
1r R= MPA(*R*)
1s R= MPA(*S*)

Fig. 3. $\Delta\delta$ (δ_R–δ_S) observed for the MPA esters **1r** and **1s**.

Compound **2** ($[\alpha]_D$ = +12.0 (*c* 0.10, MeOH)) possessed the molecular formula $C_{16}H_{24}O_3$ that indicated that it was an isomer of compound **1**. The ^{13}C NMR spectrum, which was closely similar to that of compound **1**, indicated that compound **2** was also a C-16 acid containing four disubstituted double bonds and a secondary hydroxy group. The analysis of the COSY and HMBC correlations established that these functions were located at the same positions than in compound **1** and therefore compound **2** had to be a stereoisomer of **1**. The difference between compounds **1** and **2** was found at the geometry of the double bond at C-7,C-8 upon observation in **2** of a coupling constant of 15.2 Hz between H-7 and H-8, that indicated the *trans* relationship between these protons. This proposal was also consistent with the upfield shift of the *bis*-allylic methylene protons H_2-6 at δ 2.85 (δ 2.97 in **1**) and the downfield shift of the C-6 resonance at δ_C 31.2 (δ_C 26.9 in **1**). These data led to propose for compound **2** the structure (4*Z*,7*E*,9*E*,13*Z*)-11-hydroxyhexadeca-4,7,9,13-tetraenoic acid. The absolute configuration at C-11 remains unassigned because the small amount of compound available precluded the preparation of the MPA esters.

The molecular formula $C_{16}H_{24}O_3$ determined for compounds **3**, **4**, and **5** indicated that they also were isomers of compound **1**. The ^{13}C NMR spectrum of each compound contained a signal attributable to a carboxylic acid function, eight signals due to olefinic carbons and one signal due to a secondary hydroxy group (Table 2). Furthermore, similar to compound **1** the ^{1}H NMR spectra of **3**–**5** exhibited between 4.0 and 6.6 ppm a set of signals diagnostic of a moiety featuring the hydroxy group adjacent to a *E*,*Z* conjugated diene. This assignment was confirmed by the COSY spectra that showed, for each compound, a spin system consisting of the oxymethine proton and four consecutive olefinic protons. In addition, the three compounds exhibited an HMBC correlation between the terminal methyl group and an olefinic carbon, indicating the presence of a double bond at the n-3 position (Δ^{13}). These data suggested that compounds **3**–**5** were, as compound **1**, hydroxy acids derived from the oxidation of the fatty acid 16:4$\Delta^{4,7,10,13}$ at different positions of the chain.

In compound **3** the conjugated diene was deduced to be located at $\Delta^{4Z,6E}$ and the hydroxy group at C-8. Key data were the HMBC correlations of the methylene protons H_2-3 (δ_H 2.47) with two olefinic carbons at δ_C 131.0 (C-4) and 130.1 (C-5), the correlation of this latter carbon with the *trans*-olefinic proton at δ_H 5.67 (dd, *J* = 15.2 and 6.4 Hz, H-7), and the COSY coupling of this proton with the oxymethine proton at δ_H 4.13 (H-8). The presence of a double

Table 2
^{1}H and ^{13}C NMR data of compounds **3–5** (CD_3OD).[a]

Position	**3**		**4**		**5**	
	δ_C	δ_H, m (*J* in Hz)	δ_C	δ_H, m (*J* in Hz)	δ_C	δ_H, m (*J* in Hz)
1	177.7		178.5		179.1	
2	35.5	2.33 t (7.3)	35.9	2.30 t (6.9)	32.3	2.33 t (7.6)
3	24.5	2.47 dt (7.3,7.3)	24.5	2.33 m	33.9	1.80 m
4	131.0	5.40 m	131.4	5.45 m	72.6	4.15 dt (6.8,6.8)
5	130.1	5.99 dd (10.8,10.8)	127.3	5.45 m	137.3	5.66 dd (15.2,6.6)
6	126.3	6.53 dddd (15.2,10.8,1.5,1.0)	36.3	2.33 m	126.5	6.57 dddd (15.2,10.8,1.5,1.0)
7	137.4	5.67 dd (15.2,6.4)	73.1	4.13 dt (6.9,6.4)	129.3	5.99 dd (11.3,11.3)
8	73.1	4.13 dt (6.4,6.4)	137.2	5.67 dd (15.2,6.4)	130.8	5.36 m
9	36.3	2.33 m	126.4	6.54 dddd (15.2,10.8,1.5,1.0)	26.9	2.97 dd (7.3,5.9)
10	126.3	5.40 m	129.1	5.97 dd (10.8,10.3)	128.5	5.36 m
11	131.2	5.43 m	131.0	5.35 m	129.8	5.36 m
12	26.6	2.79 dd (6.1,6.1)	26.8	2.93 dd (7.8,6.4)	26.4	2.82 dd (6.4,5.9)
13	128.2	5.28 m	127.8	5.30 m	128.1	5.30 m
14	132.8	5.37 m	133.1	5.40 m	132.9	5.38 m
15	21.5	2.07 m	21.5	2.09 m	21.5	2.08 qdd (7.8,7.8,1.5)
16	14.6	0.96 t (7.3)	14.6	0.97 t (7.6)	14.6	0.96 t (7.6)

[a] ^{1}H at 500 MHz and ^{13}C at 125 MHz.

bond at C-10,C-11 was proposed from the additional COSY coupling of the oxymethine proton H-8 with an allylic methylene at δ_H 2.33 (H_2-9) that was correlated in the HMBC with the olefinic carbons at δ_C 126.3 (C-10) and 131.2 (C-11). The 10*Z*,13*Z* geometry was supported by the chemical shift of the *bis*-allylic methylene carbon C-12 at δ_C 26.6 (Breitmaier and Voelter, 1989). These data led to propose for compound **3** the structure (4*Z*,6*E*,10*Z*,13*Z*)-8-hydroxyhexadeca-4,6,10,13-tetraenoic acid.

In compound **4** the methylene protons adjacent to the carboxy group (H_2-2, δ_H 2.30) exhibited an HMBC correlation with the olefinic carbon at δ_C 131.4 (C-4) indicating the presence of a double bond at C-4,C-5. Following this, the HMBC correlation of the olefinic carbon C-5 (δ_C 127.3) with the oxymethine proton at δ_H 4.13 (H-7) defined the position of the hydroxy group at C-7. Consequently, the accompanying conjugated diene system had to be at $\Delta^{8E,10Z}$. The chemical shifts of the allylic methylene carbons C-3 (δ_C 24.5) and C-12 (δ_C 26.8) were consistent with the 4*Z*,13*Z* configuration. Therefore, the structure (4*Z*,8*E*,10*Z*,13*Z*)-7-hydroxyhexadeca-4,8,10,13-tetraenoic acid was defined for compound **4**.

In compound **5** the hydroxy group was located at C-4 on the basis of the COSY correlation between the oxymethine proton at δ_H 4.15 (H-4) and the methylene protons at δ_H 1.80 (m, H_2-3) which in turn were correlated in the HMBC with the carboxylic carbon (δ_C 179.1, C-1). Therefore, the two conjugated double bonds were placed at $\Delta^{5E,7Z}$. The ^{1}H NMR spectrum showed the signals of two *bis*-allylic methylenes at δ_H 2.97 (H_2-9) and 2.82 (H_2-12). These data indicated that the molecule featured three methylene-interrupted double bonds. Therefore, after the double bond at C-7,C-8 another two double bonds had to be located at C-10,C-11 and C-13,C-14. This proposal was confirmed upon observation of the HMBC correlations of the methylene protons at δ_H 2.97 (H_2-9) with carbons at δ_C 129.3 (C-7), 130.8 (C-8), 128.5 (C-10) and 129.8 (C-11) and those of the protons at δ_H 2.82 (H_2-12) with C-10,C-11, and the olefinic carbons at δ_C 128.1 (C-13) and 132.9 (C-14), this latter one also exhibiting a correlation with the terminal methyl group. The chemical shifts of the *bis*-allylic methylene carbons C-9 (δ_C 26.9) and C-12 (δ_C 26.4) indicated the 10*Z*,13*Z* geometry of the double bonds. All these data led to propose for compound **5** the structure (5*E*,7*Z*,10*Z*,13*Z*)-4-hydroxyhexadeca-5,7,10,13-tetraenoic acid.

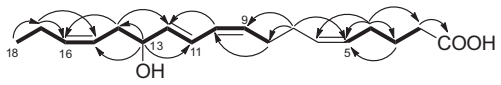
Compound **6** was obtained as an optically active oil ($[\alpha]_D$ = +6.7 (*c* 0.06, MeOH)) whose molecular formula $C_{16}H_{26}O_3$ was obtained by HRMS. The NMR spectra of **6** were similar to those of compound **1** although only six signals due to olefinic methines were observed in the ^{13}C NMR spectrum (Table 1). The analysis of the COSY and HMBC correlations defined for compound **6** a structure identical to that of compound **1** from the terminal methyl group up to C-7. The remaining signals of the spectra of **6** were due to a sequence of five methylenes that connected C-7 to the carboxylic group. Therefore compound **6** was identified as (7*Z*,9*E*,13*Z*)-11-hydroxyhexadeca-7,9,13-trienoic acid (11-HHT). This compound has been previously identified as an oxylipin formed in plants (Montillet et al., 2004; Polkowska-Kowalczyk et al., 2008), used in bioactivity studies (Prost et al., 2005), and prepared for MS analysis (Montillet et al., 2004). However, to the best of our knowledge, this is the first description of the NMR spectroscopic data of 11-HHT (**6**).

Compound **7** was obtained as an optically active compound ($[\alpha]_D$ = +7.1 (*c* 0.07, MeOH)) whose molecular formula $C_{18}H_{28}O_3$ was determined by HRMS. The ^{13}C NMR spectrum (Table 3) exhibited signals due to a carboxylic acid function (δ_C 179.0), four disubstituted double bonds (δ_C 136.9, 134.6, 132.3, 130.8, 130.5, 129.6, 126.6, and 125.5), a hydroxy-bearing methine (δ_C 73.3/δ_H 4.11), and a terminal methyl group (δ_C 14.5/δ_H 0.95), indicating that compound **7** was a monohydroxylated octadecatetraenoic acid. The location of a double bond at C-15,C-16 was readily inferred from the COSY coupling of the methyl group at δ_H 0.95 (t, *J* = 7.5 Hz, Me-18) with the methylene protons at δ_H 2.05 (H_2-17) which in turn were coupled with the olefinic proton at δ_H 5.46 (H-16) (Fig. 4). The olefinic carbons C-15 and C-16 showed HMBC correlations with two allylic methylene protons δ_H 2.30/2.23 (H_2-14) that showed COSY coupling with the oxymethine proton at δ_H 4.11 (H-13), thus defining the location of the hydroxy group at C-13. The sequence of COSY couplings observed from the oxymethine proton at δ_H 4.11 (H-13) along four consecutive olefinic protons at δ_H 5.64 (H-12), 6.50 (H-11), 5.97 (H-10) and 5.42 (H-9), indicated the presence of two conjugated double bonds at C-9,C-10 and C-11,C-12. The 9*Z*,11*E*,15*Z* geometry of the double bonds was assigned on the basis of the proton coupling constants $J_{9,10}$ = 10.5 Hz, $J_{11,12}$ = 15.3 Hz, and $J_{15,16}$ = 11.1 Hz. The remaining double bond of the molecule was located at C-5,C-6 from the HMBC correlation of the olefinic carbon at δ_C 130.5 (C-5) with the methylene protons at δ_H 1.64 which were identified as H_2-3 from their HMBC correlation with the carboxylic carbon (C-1). The *Z* geometry of the double bond at C-5,C-6 was deduced from the chemical shifts of the allylic methylene carbons C-4 and C-7 at δ_C 27.7 and 28.3, respectively (Breitmaier and Voelter, 1989). The absolute configuration at C-13 could not be investigated due to the paucity of compound for further studies. All these data and the remaining COSY and

Table 3
^{1}H and ^{13}C NMR data of compounds **7**, **8**, and **15** (CD_3OD).

Position	**7**[a]		**8**[b]		**15**[a]	
	δ_C	δ_H, m (*J* in Hz)	δ_C	δ_H, m (*J* in Hz)	δ_C	δ_H, m (*J* in Hz)
1	179.0		178.9		177.6	
2	35.3	2.25 t (7.5)	35.4	2.25 t (7.3)	34.4	2.29 t (7.3)
3	26.4	1.64 tt (7.5,7.5)	26.5	1.64 tt (7.3,7.3)	26.0	1.66 tt (7.3,7.3)
4	27.7	2.09 dt (7.5,7.2)	27.7	2.08 dt (7.3,7.3)	27.6	2.13 m
5	130.5	5.40 m	130.5	5.40 m	130.1	5.37 m
6	130.8	5.40 m	130.8	5.40 m	129.8	5.37 m
7	28.3	2.12 dt (7.8,7.2)	28.3	2.12 dt (6.8,6.8)	26.5	2.83 brdd (5.4,5.4)
8	28.8	2.23 m	28.8	2.24 m	128.7	5.37 m
9	132.3	5.42 dt (10.5,7.5)	132.2	5.42 m	129.6	5.37 m
10	129.6	5.97 dd (11.1,10.8)	129.7	5.98 dd (11.2,10.8)	27.0	2.96 m
11	126.6	6.50 dd (15.3,11.1)	126.5	6.49 dd (15.2,11.2)	130.7	5.37 m
12	136.9	5.64 dd (15.3,6.7)	137.5	5.62 dd (15.2,6.8)	129.3	5.97 dd (10.9,10.9)
13	73.3	4.11 dt (6.5,6.5)	73.4	4.07 dt (6.8,6.8)	126.4	6.55 dd (15.2,10.7)
14	36.2	2.30 m 2.23 m	38.4	1.52 m 1.46 m	137.3	5.67 dd (15.2,6.5)
15	125.5	5.35 m	26.3	1.38 m 1.33 m	73.2	4.12 dt (6.5,6.5)
16	134.6	5.46 qtt (11.1,7.2,1.4)	33.0	1.33 m	36.3	2.31 m 2.25 m
17	21.7	2.05 qd (7.5,7.5)	23.7	1.33 m	125.5	5.37 m
18	14.5	0.95 t (7.5)	14.4	0.90 t (6.9)	134.6	5.46 dtt (10.9,7.3,1.5)
19					21.7	2.05 qd (7.5,7.5)
20					14.5	0.95 t (7.6)

[a] ^{1}H at 600 MHz and ^{13}C at 150 MHz.
[b] ^{1}H at 500 MHz and ^{13}C at 125 MHz.



7

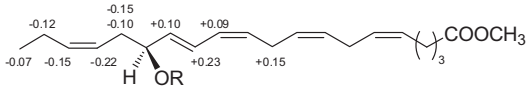
Fig. 4. Key COSY (bold) and HMBC (arrow) correlations observed for compound **7**.

HMBC correlations defined the structure (5*Z*,9*Z*,11*E*,15*Z*)-13-hydroxyoctadeca-5,9,11,15-tetraenoic acid for compound **7**.

The HRMS and NMR data of compound **8** led to define the structure (5*Z*,9*Z*,11*E*)-13-hydroxyoctadeca-5,9,11-trienoic acid. The only previous account on this compound was found in a Russian-written paper that deals with the enzyme-catalyzed oxidation of pinolenic acid ($18{:}3\Delta^{5,9,12}$) (Kuklev et al., 1993). The HRMS, NMR, and optical rotation data of compound **9** allowed to identify this compound as (9*Z*,11*E*,13*S*,15*Z*)-13-hydroxyoctadeca-9,11,15-trienoic acid [(13*S*)-HOTE] (Yadav et al., 1992; Zheng et al., 2009). Compound **10** was identified as (9*E*,11*E*,15*Z*)-13-hydroxyoctadeca-9,11,15-trienoic acid, recently described by Zheng et al. (2009). Although these authors proposed a 13*R* configuration for the compound ($[\alpha]_D$ = +4.3, *c* 1.0, MeOH), it is worth noting that this assignment was not supported by any data nor study of the absolute configuration. Therefore, although compound **10** ($[\alpha]_D$ = +4.0, *c* 0.1, MeOH) obtained from the microalga *C. debaryana* is identical to the acid described by Zheng et al. (2009), in the absence of further data we present the structure **10** with the absolute configuration unassigned. Compounds **11**, **12**, and **13** were obtained as optically inactive compounds that were identified as (10*E*,12*Z*,15*Z*)-9-hydroxyoctadeca-10,12,15-trienoic acid (9-HOTE) (McLean et al., 1996), (9*Z*,13*E*,15*Z*)-12-hydroxyoctadeca-9,13,15-trienoic acid (12-HOTE) (Pagani et al., 2011), and (9*Z*,12*Z*,14*E*)-16-hydroxyoctadeca-9,12,14-trienoic acid (16-HOTE) (Dong et al., 2000), respectively. Compound **14** was identified, on the basis of its optical rotation, MS, and NMR data as (9*Z*,11*E*,13*S*)-13-hydroxyoctadeca-9,11-dienoic acid [(13*S*)-HODE] (Dong et al., 2000; Yadav et al., 1992).

Compound **15** was obtained as an optically active oil ($[\alpha]_D$ = +4.9 (*c* 0.11, MeOH)) whose molecular formula $C_{20}H_{30}O_3$ was established by HRMS. The analysis of the NMR data led to conclude that compound **15** was (5*Z*,8*Z*,11*Z*,13*E*,17*Z*)-15-hydroxyeicosa-5,8,11,13,17-pentaenoic acid (15-HEPE). This acid has been cited in a variety of pharmacological studies (*eg.* Vang and Ziboh, 2005) and it has also been identified as an oxylipin of diatoms (Cutignano et al., 2011; d'Ippolito et al., 2009). However, to the best of our knowledge, this is the first description of the NMR data of 15-HEPE (**15**). Further, the optical activity of any of the enantiomers of 15-HEPE has not been found reported in literature. Therefore, the absolute configuration of **15** was determined by NMR analysis of the diastereomeric esters **15r** and **15s**. Positive chemical shift differences ($\Delta\delta = \delta_R - \delta_S$) were obtained for H-10, H-12, H-13 and H-14 while negative values were obtained for H-16, H-17, H-18, H-19, and Me-20 (Fig. 5), in agreement with an *S* configuration at C-15 (Seco et al., 2004).

Compounds **1**–**14** isolated from *C. debaryana* and **15** from *N. gaditana* are polyunsaturated hydroxy acids whose formation can be explained through the oxidation either enzymatic or chemical of the corresponding fatty acids (Table 4). The optical activity exhibited by compounds **1**, **2**, **6**, **7**, **9**, **10**, **14**, and **15** strongly indicates that these metabolites are enzymatically formed, likely through the action of LOXes. The first step in LOX-mediated lipid peroxidation is a stereoselective hydrogen removal from a *bis*-allylic methylene to yield an intermediate fatty acid radical, whose reaction with oxygen can lead to hydroperoxide formation at [+2] or [−2] position (Andreou and Feussner, 2009; Liavonchanka and Feussner, 2006). It is well known that in plants LOX enzymes mediate the specific insertion of molecular oxygen into either the



15a R= H
15r R= MPA(*R*)
15s R= MPA(*S*)

Fig. 5. $\Delta\delta$ ($\delta_R - \delta_S$) observed for the MPA esters **15r** and **15s**.

Table 4
Fatty acid composition (% of total fatty acids ± SD, $n = 3$) of the samples of *C. debaryana* and *N. gaditana*.

Fatty acids	*C. debaryana*		*N. gaditana*
	sample 0902-12	sample-1702-12	
C14	2.46 ± 0.01	2.71 ± 0.09	7.11 ± 0.26
C16	11.45 ± 0.02	11.44 ± 0.02	25.02 ± 0.63
C16Δ^{7}	1.88 ± 0.00	1.80 ± 0.00	0.00
C16Δ^{9}	0.33 ± 0.00	0.38 ± 0.00	32.97 ± 1.06
C16$\Delta^{7,10}$	1.13 ± 0.01	1.22±0.00	0.00
C16$\Delta^{4,7,10}$	0.43 ± 0.00	0.45 ± 0.00	0.00
C16$\Delta^{7,10,13}$	1.78 ± 0.00	2.14 ± 0.01	0.00
C16$\Delta^{4,7,10,13}$	21.00 ± 0.01	20.61 ± 0.09	0.00
C18	1.46 ± 0.00	1.45 ± 0.01	0.18 ± 0.01
C18Δ^{9}	0.96 ± 0.00	0.78 ± 0.00	4.07 ± 0.04
C18Δ^{11}	3.70 ± 0.00	3.55 ± 0.00	0.00
C18$\Delta^{9,12}$	6.33 ± 0.00	6.02 ± 0.01	2.72 ± 0.10
C18$\Delta^{5,9,12}$	3.25 ± 0.01	3.90 ± 0.01	0.00
C18$\Delta^{9,12,15}$	40.70 ± 0.02	40.50 ± 0.07	0.00
C18$\Delta^{5,9,12,15}$	3.14 ± 0.00	3.07 ± 0.02	0.00
C20$\Delta^{5,8,11,14}$	0.00	0.00	2.88 ± 0.15
C20$\Delta^{5,8,11,14,17}$	0.00	0.00	25.05 ± 1.83

position 9 (9-LOX) or 13 (13-LOX) of the C18 fatty acids ALA and LA to yield the corresponding 9- and 13-hydroperoxy derivatives, in most cases with *S* configuration (Andreou et al., 2009). Analogous pathways for fatty acid oxidation have been proposed to explain the production of oxylipins in some green macrophytic algae (Andreou et al., 2009; Tsai et al., 2008; Gerwick, 1994). Similar to plants and green algae, oxylipins **9** (13*S*-HOTE) and **14** (13*S*-HODE) could arise in the microalga *C. debaryana* through the 13*S*-LOX-mediated oxidation of ALA and LA, respectively, to yield the corresponding (13*S*)-hydroperoxides, whose subsequent reduction would lead to the hydroxy fatty acids. In this context it is worth noting that it has been sequenced a LOX from the related species *Clamydomonas reinhardtii*, showing about 35% homology to plant and moss LOXes. (Andreou et al., 2009). On the other hand, studies on plants containing high amounts of roughanic acid (16:3$\Delta^{7,10,13}$), the so-called "16:3 plants", have indicated that 9- and 13-LOXes that oxidize C18 PUFAs also mediate the oxidation of the 16:3$\Delta^{7,10,13}$ acid to yield the 7- and 11-hydroperoxides, respectively (Montillet et al., 2004; Polkowska-Kowalczyk et al., 2008). Thus, in the microalga *C. debaryana* the 11-hydroxy acid **6** can be proposed to arise from the action of a 13*S*-LOX on roughanic acid. We have not found data regarding to oxylipins derived from the tetraenoic acids 16:4$\Delta^{4,7,10,13}$ and 18:4$\Delta^{5,9,12,15}$ (coniferonic acid), but it seems likely that these acids in *C. debaryana* have also experienced a 13-LOX mediated oxidation to yield the hydroxy acids **1** and **7**, respectively. The hydroxy acids **2** and **10**, which are geometric isomers of **1** and **9**, respectively, could also derive from the oxidation of the acid 16:4$\Delta^{4,7,10,13}$ and ALA with concomitant *cis* to *trans* double bond isomerisation (Fukushige et al., 2005). The major oxylipins isolated from *C. debaryana* were compounds **1** and **9**, a result consistent with the prominence of the fatty acids 16:4$\Delta^{4,7,10,13}$ and ALA (*ca.* 20% and 40% of total fatty acids, respectively) in the examined biomass (Table 4).

On the other hand, the optically inactive hydroxy acids **3**–**5**, **8**, and **11**–**13** would arise from the ROS-mediated oxidation of the corresponding C16 and C18 fatty acids. The coexistence of metabolites arisen from enzymatic and chemical oxidation of PUFAs has often been recorded, since physiological and environmental conditions may affect the intensity and nature of lipid peroxidation (Berger et al., 2001; Montillet et al., 2004; Polkowska-Kowalczyk et al., 2008).The possibility of compounds **3**–**5**, **8**, and **11**–**13** be artefacts formed during the purification steps may also be considered. Nonetheless, other extracts obtained from this and other species of microalgae that have been subjected to the same isolation procedures were devoid of this class of hydroxy acids.

The results herein described for *C. debaryana*, together with a first report of the hydroxy acids (12*R*)-HOTE and (9*S*)-HOTE in the green microalga *D. acidophila* (Pollio et al., 1988), represent the only accounts of the isolation of oxylipins from green microalgae.

During our research we have studied two batches of *C. debaryana* cultured at different dates. Differences in the total content of oxylipins per dry weight and in their relative amounts were observed among the two biomass samples (See Experimental). Since these polyenoic hydroxy acids proved to be rather unstable, chemical differences among the two biomass samples could partly be due to different extent of degradation of compounds during the purification steps. Nonetheless, it is well known that the production of oxylipins in plants, seaweeds, and microalgae is induced by a variety of biotic and abiotic factors (Blée, 2002; Cutignano et al., 2011; Potin, 2008). Thus, it can also be considered that differences in the profile of oxylipins among samples of *C. debaryana* can be due to culture, harvesting, or environmental conditions, a topic that is currently the focus of new studies in our laboratories.

The occurrence of oxylipin **15** (15*S*-HEPE) in the biomass sample of *N. gaditana* strongly suggests that this microalga features a 15-LOX and represents the first account of oxylipins from microalgae of the class Eustigmatophyceae. The oxidation of EPA (20:5$\Delta^{5,8,11,14,17}$) by different LOXes has been previously proposed to explain the biosynthesis of a variety of metabolites obtained from red and brown algae (Gerwick, 1994; Guschina and Harwood, 2006). More recently, the 15-LOX mediated oxidation of EPA to yield the 15-hydroperoxide and then (15*S*)-HEPE has also been described in diatoms (d'Ippolito et al., 2009; Lamari et al., 2013).

From the pharmacological point of view, some monohydroxylated compounds derived from the oxidation of PUFAs are known to be ligands of peroxisome proliferator-activated receptors (PPARs) (Itoh et al., 2008; Willson et al., 2000). These are nuclear transcription factors that play a central role in regulating the storage and catabolism of dietary fatty acids, being also involved in pathological processes such as inflammation, atherosclerosis, and cancer (Willson et al., 2000). In this regard, (13*S*)-HODE (**14**) has been shown to induce apoptosis in colorectal cancer cells through binding and down-regulating PPAR-δ (Shureiqi et al., 2003). The EPA-derived hydroxy acid (15*S*)-HEPE (**15**) together with (15*S*)-HETrE, the analogous derivative of dihomo-γ-linolenic acid (20:3$\Delta^{8,11,14}$), have shown to inhibit the growth and the production of ARA-derived metabolites in human prostatic cancer cells with a possible mechanism consisting on binding to and activating PPAR-γ (Vang and Ziboh, 2005). 13-HODE (**14**) has also been described to play a role in modulating cutaneous hyperproliferation and in the suppression of the activity of the epidermal protein kinase C-β (Ziboh et al., 2000). On the other hand, monohydroxy acids derived from LA, ALA, and EPA have shown activity in assays aimed to detect anti-inflammatory activity. Thus, 13-HOTE (**9**) has been described to suppress the IL-1β induced expression of matrix metalloproteinases (MMPs), enzymes that degrade the cartilage-specific extracellular matrix (Schulze-Tanzil et al., 2002); compounds 16-HOTE (**13**), 13-HODE (**14**), and the methyl ester of 9-HOTE (**11**) suppress the PMA-induced inflammation on mouse ears (Dong et al., 2000), and 15-HEPE (**15**) inhibits the production of the proinflammatory leukotriene B_4 in RBL-1 cells (Ziboh et al., 2000).

In the present study, the polyunsaturated hydroxy acids **1**, **9**, **11**, and **14**, which are the major oxylipins obtained from *C. debaryana*, together with **15** obtained from *N. gaditana*, were tested for their activity as inhibitors of the tumour necrosis factor α (TNF-α), a potent proinflammatory cytokine mainly produced by monocytes and macrophages in immunologic and inflammatory responses (Iqbal et al., 2013). For compounds **6** and **13** there were not enough

amounts of pure samples and an HPLC fraction composed by **6** and **13** (1:1) was evaluated. Assays were performed on the human THP-1 macrophages using lipopolysaccharide (LPS) as the triggering factor to stimulate the TNF-α production. In order to rule out cytotoxic effects, the compounds were assayed at a maximum concentration of 100 μM which do not affect THP-1 cell viability in the SRB assay. To test the effects of the oxylipins on the TNF-α production, THP-1 macrophages were pretreated with each oxylipin, then stimulated with LPS and finally analyzed to quantify TNF-α (Fig. 6). During the incubation time of 24 h, control THP-1 macrophages produced 16.77 ng/mL of TNF-α. After stimulation with LPS (1 μg/mL) the TNF-α production increased about tenfold, up to 153.17 ng/mL. The treatment of cells with oxylipins **1**, **9**, **11**, **14**, or **15** at 100 μM strongly inhibited the TNF-α production by 87%, 86%, 85%, 98%, and 90%, respectively, upon comparison with LPS-stimulated untreated THP-1 cells. The mixture of **6** and **13** was rather less active and only 45% of inhibition was observed. It is noticeable the activity exhibited by compound **14**, which caused an almost total inhibition of the TNF-α production. At the concentration of 50 μM compounds **1** and **11** again exhibited high activity, causing 78% and 72% decrease of the TNF-α level, respectively. Compounds **9** and **14** were also significantly active at 50 μM, inhibiting the production of TNF-α by 54% and 52%, respectively. When compounds were tested at 25 μM, the new oxylipin **1** proved to be the most potent TNF-α inhibitor, causing 60% decrease of the TNF-α level upon comparison with LPS-stimulated untreated THP-1 cells. At the same concentration of 25 μM, compound **11** inhibited the production of TNF-α by 44% and compounds **9**, **14**, and **15** exhibited a weaker inhibitory effect, less than 25%.

In conclusion, we have found that the cultured biomass of the green microalga *C. debaryana* contains an array of oxylipins, including new monohydroxy acids derived from the highly unsaturated $16{:}4\Delta^{4,7,10,13}$ and $18{:}4\Delta^{5,9,12,15}$ fatty acids. The occurrence of optically active oxylipins suggests the existence of LOX-mediated pathways in *C. debaryana* that oxidatively transform C16 and C18 PUFAs. The obtention of the EPA-derived metabolite (15*S*)-HEPE (**15**) from the biomass of *N. gaditana* indicates that this microalga features a 15-LOX and represents the first account of oxylipins in microalgae of the Eustigmatophyceae class. Bioactivity assays have shown the significant activity of the tested hydroxy acids as TNF-α inhibitors, in particular of the new oxylipin **1**, and evidence the potential of microalgal biomass to contain, in addition to the well known long chain PUFAs, other minor bioactive lipid metabolites.

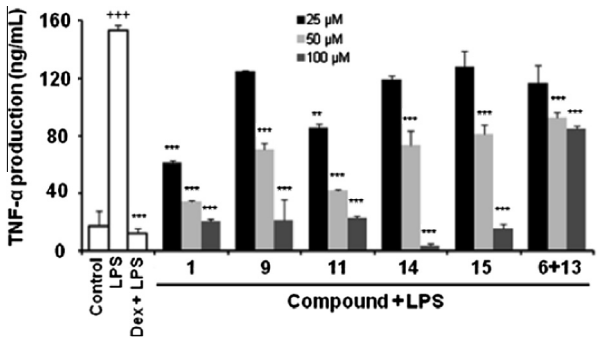


Fig. 6. Effect of oxylipins **1**, **9**, **11**, **14**, **15**, and **6** + **13** on LPS-induced TNF-α production by THP-1 macrophages. Cells were incubated with compounds (25, 50, and 100 μM) for 1 h and then stimulated for 24 h with LPS (1 μg/mL). TNF-α was quantified in supernatants using ELISA assay. Dexamethasone (Dex) was used as positive reference compound at concentration of 1 μM. Data are means ±SEM from six independent experiments. Statistical analysis was performed by ANOVA followed by Tukey test ($n = 6$; $^{+++}p < 0.001$ vs control; $^{***}p < 0.001$ vs LPS; $^{**}p < 0.01$ vs LPS).

Experimental section

General experimental procedures

Optical rotations were measured on a Perkin-Elmer 241 polarimeter. IR spectra were recorded on a Perkin-Elmer FT-IR System Spectrum BX spectrophotometer. UV/Vis analysis were performed on a Helios γ Unicam spectrophotometer. ^{1}H and ^{13}C NMR spectra were recorded on Varian INOVA 600 or Agilent 500 spectrometers using $CDCl_3$ or CD_3OD as solvents. Chemical shifts were referenced using the corresponding solvent signals [δ_H 7.26 and δ_C 77.0 for $CDCl_3$, and δ_H 3.30 and δ_C 49.0 for CD_3OD]. COSY, HSQC, and HMBC experiments were performed using standard Varian pulse sequences. High resolution mass spectra (HRMS) were obtained on a Waters SYNAPT-2G spectrometer. GC/MS analyses were performed on a QUATTRO Micro GC instrument. Column chromatography was carried out on Merck Silica gel 60 (70–230 mesh). HPLC separations were performed on a LaChrom-Hitachi apparatus using LiChrospher Si-60 (Merck, 250 × 10 mm, 10 μm) and Luna Si (2) (Phenomenex, 250 × 4.6 mm, 5 μm) columns (flow rates 3 mL/min and 1 mL/min, respectively, volume of injection 150 μL), and monitored by a differential refractometer RI-71 or an UV detector L-7400 (Merck) set at $\lambda = 254$ nm. PMA, dexamethasone, and LPS were purchased from Sigma.

Biological material

The species used in the present study were *C. debaryana* (freshwater species, strain ITC-09; Phylum Chlorophyta, Class Chlorophyceae, Order Volvocales, Family Chlamydomonaceae) and *N. gaditana* (seawater species, strain ITC-Nano-01; Phylum Heterokontophyta, Class Eustigmatophyceae, Order Eustigmatales, Family Eustigmataceae). Stocks of both strains are maintained at the Microalgae Collection of the Instituto Tecnológico de Canarias (Gran Canaria, Spain). Maintenance of stocks, scale-up of inocula and outdoor production of biomass were performed using the minimal medium (Sueoka, 1959) for *C. debaryana* and f/2 medium (Guillard, 1975) for *N. gaditana*. Scale-up of the inocula was performed indoor using 10 L polycarbonate containers (Carboys, Nalgene) at 12:12 h light–dark cycle, 20 °C, and aeration with 3% CO_2. Outdoor production was done in batch mode. For *C. debaryana* cultures, 225 L of minimal medium were prepared using previously chlorinated freshwater. Similarly, 225 L of *f*/2 medium were prepared for *N. gaditana* cultures, using previously chlorinated seawater. Every batch was inoculated with 10% (v/v) of inoculum. Cultures were performed during February 2012 using a 3 m^2 conventional paddle-wheel driven raceway pond with 15 cm culture depth, placed in a greenhouse at Instituto Tecnológico de Canarias (Playa de Pozo Izquierdo, Gran Canaria, Spain). Ponds were supplied with pure CO_2 to maintain the culture pH between 7 and 7.5. Cell growth was daily monitored by optical density measurements at 750 nm using a UV/Vis spectrophotometer. Cultures were harvested in the late stationary phase, when maximal biomass was achieved, using a Westfalia industrial centrifuge. After centrifugation, the microalgal biomass was immediately frozen at −20 °C until lyophilized to dryness.

Isolation of oxylipins

Microalgal dry biomass was extracted with acetone-MeOH (1:1, 75 mL/g dry wt.) at room temperature. After filtration, the solvent was evaporated under reduced pressure (bath temp. 35 °C). Two samples of lyophilized biomass of *C. debaryana*, ITC09-0902-12 (72 g) and ITC09-1702-12 (106 g), were extracted to yield 15.0 g and 19.0 g of extract, respectively. Each extract was chromatographed on a silica gel column (25 × 7 cm and 30 × 7 cm,

respectively) using solvents of increasing polarities starting with the combinations of hexanes-Et_2O (9:1, 1:1, 3:7, v/v, 1.5 L each), Et_2O 100% (3.0 L), some combinations of $CHCl_3$–MeOH (9:1 and 8:2, v/v, 1.5 L each), and finally MeOH 100% (1.5 L). The fractions eluted with hexanes-Et_2O (3:7, v/v), Et_2O 100%, and $CHCl_3$–MeOH (9:1, 8:2, v/v) were dried and resuspended in MeOH–H_2O (9:1, v/v, 50 mg/mL), transferred onto RP-18 cartridges preconditioned with MeOH-H_2O (9:1, v/v, 1 mL) and eluted with 10 mL of the same solvent. After evaporation of the solvent, each mixture was repeatedly separated by normal phase HPLC using $CHCl_3$–MeOH (99.8:0.2, v/v) or hexanes-propan-2-ol-AcOH (97:3:0.1, v/v/v) as the mobile phase until pure compounds were obtained. The extract of ITC09-0902-12 yielded dry wt. of compounds **1** (1.6×10^{-2}% dry wt.), **3** (1.1×10^{-3}% dry wt.), **4** (1.3×10^{-3}% dry wt.), **5** (2.1×10^{-3}% dry wt.), **9** (9.5×10^{-3}% dry wt.), **11** (8.1×10^{-3}% dry wt.), **12** (2.4×10^{-3}% dry wt.), **13** (8.6×10^{-3}% dry wt.), and **14** (4.2×10^{-4}% dry wt.). The extract of ITC09-1702-12 yielded compounds **1** (4.8×10^{-3}% dry wt.), **2** (3.1×10^{-3}% dry wt.), **6** (1.1×10^{-3}% dry wt.), **7** (2.0×10^{-3}% dry wt.), **8** (6.6×10^{-4}% dry wt.), **9** (1.7×10^{-2}% dry wt.), **10** (4.7×10^{-4}% dry wt.), **11** (2.6×10^{-3}% dry wt.), **12** (1.5×10^{-3}% dry wt.), **13** (1.5×10^{-3}% dry wt.), and **14** (3.5×10^{-3}% dry wt.). Extraction of the lyophilized biomass of *N. gaditana* (25 g) as described above yielded 7.0 g of extract, that was chromatographed on a silica gel column (28 × 3.5 cm) using solvents of increasing polarities starting with the combinations of hexanes-Et_2O (9:1, 1:1, 3:7, v/v, 0.5 L each), Et_2O 100% (0.2 L), some combinations of $CHCl_3$–MeOH (9:1 and 8:2, v/v, 0.2 and 0.4 L, respectively), and finally MeOH 100% (0.3 L). The fractions eluted with hexanes-Et_2O (3:7, v/v), Et_2O 100%, and $CHCl_3$–MeOH (9:1, 8:2, v/v) were separated as described above to yield compound **15** (6.0×10^{-2}% dry wt.).

Characterization of compounds

*(4Z,7Z,9E,11S,13Z)-11-hydroxyhexadeca-4,7,9,13-tetraenoic acid (**1**)*

Colorless oil; $[\alpha]_D$ = +7.5 (*c* 0.08, MeOH); IR (film) ν_{max} 3452, 1711, 1591, 1411 cm^{-1}; ^1H NMR (CD_3OD, 600 MHz) see Table 1; ^{13}C NMR (CD_3OD, 150 MHz) see Table 1; HRESIMS(−) *m/z* 263.1647 $[M-H]^-$ (calcd for $C_{16}H_{23}O_3$, 263.1647).

*(4Z,7E,9E,13Z)-11-hydroxyhexadeca-4,7,9,13-tetraenoic acid (**2**)*

Colorless oil; $[\alpha]_D$ = +12.0 (*c* 0.10, MeOH); ^1H NMR (CD_3OD, 500 MHz) see Table 1; ^{13}C NMR (CD_3OD, 125 MHz) see Table 1; HRESIMS(−) *m/z* 263.1646 $[M-H]^-$ (calcd for $C_{16}H_{23}O_3$, 263.1647).

*(4Z,6E,10Z,13Z)-8-hydroxyhexadeca-4,6,10,13-tetraenoic acid (**3**)*

Colorless oil; ^1H NMR (CD_3OD, 500 MHz) see Table 2; ^{13}C NMR (CD_3OD, 125 MHz) see Table 2; HRESIMS(−) *m/z* 263.1644 $[M-H]^-$ (calcd for $C_{16}H_{23}O_3$, 263.1647).

*(4Z,8E,10Z,13Z)-7-hydroxyhexadeca-4,8,10,13-tetraenoic acid (**4**)*

Colorless oil; ^1H NMR (CD_3OD, 500 MHz) see Table 2; ^{13}C NMR (CD_3OD, 125 MHz) see Table 2; HRESIMS(−) *m/z* 263.1649 $[M-H]^-$ (calcd for $C_{16}H_{23}O_3$, 263.1647).

*(5E,7Z,10Z,13Z)-4-hydroxyhexadeca-5,7,10,13-tetraenoic acid (**5**)*

Colorless oil; ^1H NMR (CD_3OD, 500 MHz) see Table 2; ^{13}C NMR (CD_3OD, 125 MHz) see Table 2; HRESIMS(−) *m/z* 263.1649 $[M-H]^-$ (calcd for $C_{16}H_{23}O_3$, 263.1647).

*(7Z,9E,13Z)-11-hydroxyhexadeca-7,9,13-trienoic acid (11-HHT) (**6**)*

Colorless oil; $[\alpha]_D$ = +6.7 (*c* 0.06, MeOH); ^1H NMR (CD_3OD, 600 MHz) see Table 1; ^{13}C NMR (CD_3OD, 150 MHz) see Table 1; HRESIMS(−) *m/z* 265.1809 $[M-H]^-$ (calcd for $C_{16}H_{25}O_3$, 265.1804).

*(5Z,9Z,11E,15Z)-13-hydroxyoctadeca-5,9,11,15-tetraenoic acid (**7**)*

Colorless oil; $[\alpha]_D$ = +7.1 (*c* 0.07, MeOH); IR (film) ν_{max} 3416, 1700, 1580, 1406; ^1H NMR (CD_3OD, 600 MHz) see Table 3; ^{13}C NMR (CD_3OD, 150 MHz) see Table 3; HRESIMS(−) *m/z* 291.1956 $[M-H]^-$ (calcd for $C_{18}H_{27}O_3$, 291.1960).

*(5Z,9Z,11E)-13-hydroxyoctadeca-5,9,11-trienoic acid (**8**)*

Colorless oil; ^1H NMR (CD_3OD, 500 MHz) see Table 3; ^{13}C NMR (CD_3OD, 125 MHz) see Table 3; HRESIMS(−) *m/z* 293.2115 $[M-H]^-$ (calcd for $C_{18}H_{29}O_3$, 293.2117).

*(5Z,8Z,11Z,13E,15S,17Z)-15-hydroxyeicosa-5,8,11,13,17-pentaenoic acid (15S-HEPE) (**15**)*

Colorless oil; $[\alpha]_D$ = +4.9 (*c* 0.11, MeOH); IR (film) ν_{max} 3417, 1700, 1595, 1432 cm^{-1}; ^1H NMR (CD_3OD, 600 MHz) see Table 3; ^{13}C NMR (CD_3OD, 150 MHz) see Table 3; HRESIMS(−) *m/z* 317.2123 $[M-H]^-$ (calcd for $C_{20}H_{29}O_3$, 317.2117).

*Synthesis of the MPA esters **1r**, **1s**, **15r**, **15s***

The acid (**1** or **15**) was dissolved in MeOH (1 mL) and treated with a solution of $TMSCH_2N_2$ 2.0 M in Et_2O (100 µL). After stirring the mixture at room temp. for 30 min, the solvent was evaporated. Treatment of **1** (2.8 mg, 0.011 mmol) and **15** (7.1 mg, 0.022 mmol) as described above yielded the methyl esters **1a** and **15a**, respectively, with quantitative yield in both cases. Each methyl ester was dissolved in CH_2Cl_2 (0.5 mL) and treated with CH_2Cl_2 solutions (0.5 mL each) of *N,N′*-dicyclohexylcarbodiimide (tenfold excess over the starting compound), *N,N*-dimethylaminopyridine (fivefold excess) and the MPA acid (fivefold excess). The mixture was stirred overnight at room temp. and then purified by preparative TLC (hexanes-EtOAc 85:15, v/v) to obtain the MPA ester. Treatment of **1a** (1.6 mg, 5.8×10^{-3} mmol) and **15a** (5.4 mg, 1.6×10^{-2} mmol) with (*R*)-MPA as described above yielded compounds **1r** and **15r**, respectively. Treatment of **1a** (1.2 mg, 4.3×10^{-3} mmol) and **15a** (1.7 mg, 5.1×10^{-3} mmol) with (*S*)-MPA as described above yielded compounds **1s** and **15s**, respectively.

*Compound **1r**:* ^1H NMR ($CDCl_3$, 600 MHz) (selected data, assignments aided by a COSY experiment) δ 6.51 (1H, dd, *J* = 15.3 and 11.2 Hz, H-9), 5.94 (1H, dd, *J* = 11.4 and 11.2 Hz, H-8), 5.61 (1H, dd, *J* = 15.3 and 7.3 Hz, H-10), 5.41 (1H, m, H-7), 5.38 (1H, m, H-11), 5.34 (1H, m, H-14), 5.03 (1H, m, H-13), 2.91 (2H, *br* dd, *J* = 6.2 and 5.9 Hz, H_2-6), 2.32 (1H, m, H-12a), 2.26 (1H, m, H-12b), 1.91 (2H, qd, *J* = 7.6 and 7.6 Hz, H_2-15), 0.88 (3H, t, *J* = 7.5 Hz, Me-16).

*Compound **1s**:* ^1H NMR ($CDCl_3$, 600 MHz) (selected data, assignments aided by a COSY experiment) δ 6.27 (1H, dd, *J* = 15.2 and 10.9 Hz, H-9), 5.85 (1H, dd, *J* = 11.2 and 10.9 Hz, H-8), 5.50 (1H, dd, *J* = 15.2 and 6.5 Hz, H-10), 5.48 (1H, m, H-14), 5.40 (1H, m, H-11), 5.32 (1H, m, H-7), 5.25 (1H, m, H-13), 2.76 (2H, *br* dd, *J* = 7.3 and 7.0 Hz, H_2-6), 2.44 (1H, m, H-12a), 2.35 (1H, m, H-12b), 2.03 (2H, m, H_2-15), 0.95 (3H, t, *J* = 7.6 Hz, Me-16).

*Compound **15r**:* ^1H NMR ($CDCl_3$, 600 MHz) (selected data, assignments aided by a COSY experiment) δ 6.51 (1H, dd, *J* = 15.1 and 11.0 Hz, H-13), 5.94 (1H, dd, *J* = 11.0 and 10.7 Hz, H-12), 5.61 (1H, dd, *J* = 15.1 and 7.3 Hz, H-14), 5.34 (1H, m, H-15), 5.33 (1H, m, H-18), 5.03 (1H, dddd, *J* = 14.2, 7.2, 3.2, and 1.6 Hz, H-17), 2.91 (2H, dd, *J* = 7.3 and 7.3 Hz, H_2-10), 2.29 (1H, m, H-16a), 2.26 (1H, ddd, *J* = 14.5, 7.3, and 7.3 Hz, H-16b), 1.91 (2H, m, H_2-19), 0.88 (3H, t, *J* = 7.4 Hz, Me-20).

Compound ***15s****:* ^{1}H NMR ($CDCl_3$, 600 MHz) (selected data, assignments aided by a COSY experiment) δ 6.28 (1H, dddd, J = 15.2, 11.1, 1.3, and 1.0 Hz, H-13), 5.85 (1H, dd, J = 11.1 and 10.8 Hz, H-12), 5.51 (1H, dd, J = 15.2 and 6.7 Hz, H-14), 5.48 (1H, m, H-18), 5.40 (1H, m, H-15), 5.25 (1H, m, H-17), 2.76 (2H, m, H_2-10), 2.44 (1H, ddd, J = 14.6, 6.9, and 6.9 Hz, H-16a), 2.36 (1H, ddd, J = 13.4, 6.6, and 6.6 Hz, H-16b), 2.03 (2H, qdd, J = 7.6, 7.6, and 1.3 Hz, H_2-19), 0.95 (3H, t J = 7.4 Hz, Me-20).

Fatty acid analysis

An aliquot (15–30 mg) of the acetone-MeOH extract of each microalga was dissolved in 1 mL of MeOH–HCl (10:1, v/v). 100 μL of a solution of heptadecanoic acid in hexanes (50 mg in 5 mL) were added as internal standard and the mixture was refluxed for 1 h. After cooling, the reaction mixture was extracted with hexanes (3 × 3 mL). The organic layers were collected, rinsed with brine (3 mL) and dried over $MgSO_4$. After filtration and evaporation of the solvent under reduced pressure, the residue was purified on a small silica gel column eluting with hexanes-Et_2O (95:5, v/v). The FAME mixture was dissolved in CH_2Cl_2 and subjected to CG/MS analysis in a QUATTRO Micro GC (Waters) instrument fitted with an Supelcowax10 column (250 μm × 30 m, 0.25 μm film) with He as carrier gas (1 mL/min), operating at 70 eV. The column temperature was elevated from 50 to 220 °C (4 °C/min) and held at 220 °C for 20 min. Fatty acids were identified by comparison of retention time and mass spectral data with FAME standards and a NIST library and, when necessary, by analysis of pyrrolidine derivatives as described in literature (Kajikawa et al., 2006).

Anti-inflammatory assays

Cell culture

The THP-1 human monocytic leukemia cell line was obtained from the American Type Culture Collection (TIB-202, ATCC, USA) and cultured in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin in a humidified atmosphere containing 5% CO_2 at 37 °C.

Cell proliferation assay

Viability of THP-1 cells upon exposure to oxylipins was determined by the sulforhodamine B (SRB) assay (Skehan et al., 1990). Briefly, for differentiation into macrophages the cultured THP-1 cells in growth medium (1×10^5 cells/mL) were spiked with PMA (DMSO-dissolved, 0.8 mM) for a final concentration of 0.2 μM, transferred into 96-well plates (100 μL/well), and incubated in a humidified atmosphere of 5% CO_2 at 37 °C for three days. After that, the medium was removed, cells were washed with phosphate saline buffer (PBS, 4 °C), and then incubated for 48 h with oxylipin solutions (100 μL/well, final concentrations of 6.25, 12.5, 25, 50, and 100 μM) that were prepared by dilution of stock solutions (10 mM in DMSO) with the appropriate amounts of fresh medium. Controls were incubated in fresh medium containing DMSO 1%, v/v, which did not affect cell viability. After 48 h the cells were fixed with 50 μL of trichloroacetic acid (TCA, 50% v/v) and processed as described in the literature.

Determination of TNF-α production

Differentiation into macrophages was achieved by incubating for 3 days THP-1 cells (3×10^5 cells/mL) with PMA (final concentration of 0.2 μM) in 24-well plates (500 μL/well), as described above. After that, the medium was removed, the cells were washed with PBS (4 °C), and then incubated for 1 h with oxylipin solutions (500 μL/well, final concentration of 25, 50, and 100 μM) that were prepared by dilution of stock solutions (10 mM in DMSO) with the appropriate amounts of fresh medium. At the highest concentration of oxylipin used in the assay the concentration of DMSO was 1%, v/v. At this dose DMSO did not affect cell viability or TNF-α production. Positive controls were incubated with a dexamethasone solution (500 μL/well, final concentration of 1 μM) that was prepared by dilution of a stock solution (20 mM in DMSO) with fresh medium. Control groups were incubated with growth medium (500 μL/well) containing DMSO 1%, v/v. The inflammatory response was induced by addition of lipopolysaccharide (LPS, final concentration of 1 μg/mL). The stock LPS solution (5 mg/mL) was prepared in DMSO and then diluted with fresh medium for a final volume of 5 μL/well. An unestimulated control containing DMSO 1%, v/v, but without LPS, was also assayed. After 24 h incubation, supernatant fluids were collected and stored at −80 °C until TNF-α measurements. Commercial enzyme-linked immunosorbent assay (ELISA) kits (Diaclone GEN-PROBE) were used to quantify TNF-α according to the manufacturer's protocol. Samples were diluted by 1:100 prior to reading the absorbance at 450 nm with a microplate reader. To calculate the concentration of TNF-α, a standard curve (r^2 = 0.99) was constructed using serial dilutions of cytokine standards (range 25–1600 pg/mL) provided with the kit.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phytochem.2014.03.011.

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