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BIOMOLECULES PRODUCED IN LIQUID-STATE FERMENTATION BY A MARINE-DERIVED FUNGUS, *Penicillium roqueforti*

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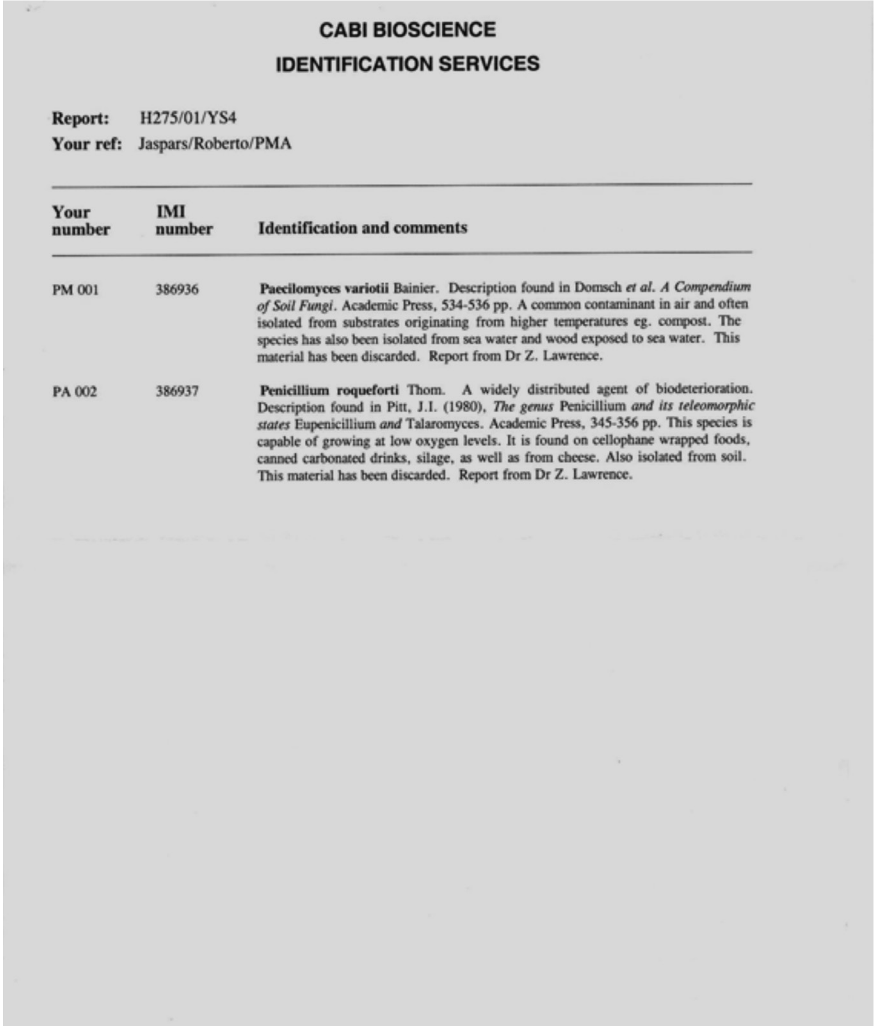
Table 1S. Optical rotation described to Ergosterol Peroxide (**27**) and 9(11)-dehydroergosterol peroxide (**28**)

Compounds mixture (EP: DHPE, %)	$[\alpha]_D^{20}$
0:100 (DHEP pure)	+80,0
Experimental *P. roqueforti* mixture	-9,7
81,5: 18,5 (Mediavilla)[48]	-12,5
84:16 (Fisch *et al.*)[47]	-14,2
100:0 (EP pure)	-32,9



CABI BIOSCIENCE
IDENTIFICATION SERVICES

Report: H275/01/YS4
Your ref: Jaspars/Roberto/PMA

Your number	IMI number	Identification and comments
PM 001	386936	**Paecilomyces variotii** Bainier. Description found in Domsch *et al. A Compendium of Soil Fungi*. Academic Press, 534-536 pp. A common contaminant in air and often isolated from substrates originating from higher temperatures eg. compost. The species has also been isolated from sea water and wood exposed to sea water. This material has been discarded. Report from Dr Z. Lawrence.
PA 002	386937	**Penicillium roqueforti** Thom. A widely distributed agent of biodeterioration. Description found in Pitt, J.I. (1980), *The genus* Penicillium *and its teleomorphic states* Eupenicillium *and* Talaromyces. Academic Press, 345-356 pp. This species is capable of growing at low oxygen levels. It is found on cellophane wrapped foods, canned carbonated drinks, silage, as well as from cheese. Also isolated from soil. This material has been discarded. Report from Dr Z. Lawrence.

Figure 1S. *Identification report of the fungal strain by CABI Bioscience, Surrey, UK*

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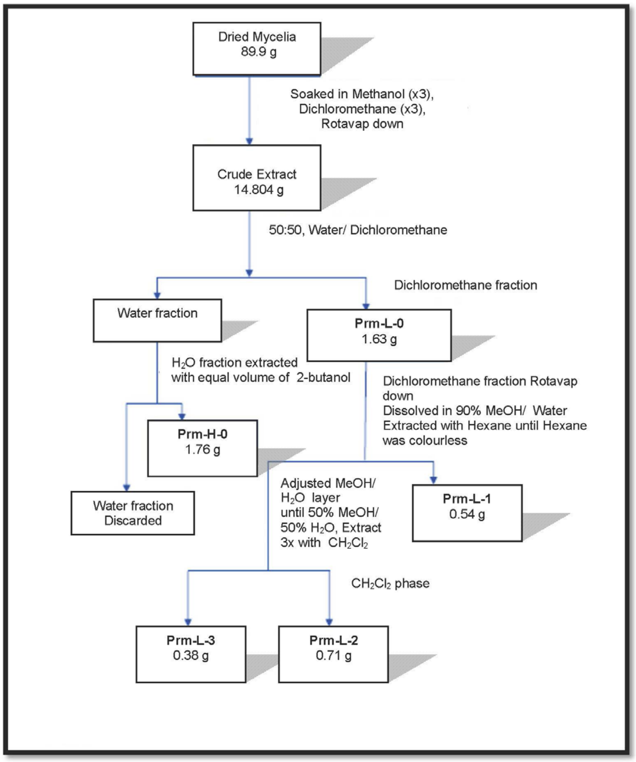


Figure 2S. *Solvent-solvent processing scheme used for partitioning of Penicillium roqueforti mycelia, adapted from Kupchan et al. (1973)*

Descriptive mycelial study

89.9 g of dried mycelium was obtained by maceration extraction in CH_2Cl_2 (x3, 24 h each one, at room temperature). After the filtration, evaporation and drying (vacuum), this gave 14.804 g of raw extract.

The ^{1}H-NMR spectrum of this mixture of substances revealed the presence of aromatic protons at δ 8.4-7.2, olefinic protons at δ 6.6-5.0, geminal to heteroatom protons at δ 4.0-3.2, and typical hydrocarbon chain protons at δ 3.0-0.8.

This raw extract was subjected to the partitioning scheme described in Figure 2S. Thus, it was dissolved in 200 ml of CH_2Cl_2 and was partitioned in a separatory decantation funnel with an additional 200 mL of H_2O. The organic phase was separated and the aqueous phase re-extracted with CH_2Cl_2 (x3). The organic phases were combined to yield 1.637 g of the crude liposoluble fraction (**Prm-L-0**).

The ^{1}H-NMR spectrum of this mixture revealed the presence of olefinic protons at δ 5.8-5.0, geminal to heteroatom protons at δ 4.0-3.0 and typical hydrocarbon chain protons at δ 3.0-0.8.

Finally, from the liposoluble fraction, three different sub-fractions were obtained: **Prm-L-1**, **Prm-L-2** and **Prm-L-3**. The aqueous phase was re-extracted with 2-butanol to give the "hidrosoluble (**Prm-H-0**) fraction".

Study of the liposoluble-1 fraction (Prm-L-1)

540 mg of a colourless oil was produced that, on TLC analysis; was seen to be a mixture of five major substances. The following volatile substances were identified by GC-MS:
2-Hexyl-1-decanol (**4**; n= 5, m= 5; Rt= 15.670; 0.083 mg)
Pentadecanoic acid, methyl ester (**6**; n= 13; Rt= 16.622; 0.61 mg)
9-Hexadecenoic acid, methyl ester, (Z)- (**8**; n= 5, m= 7; Rt= 17.175; 0.316 mg)
Hexadecanoic acid, methyl ester (**6**; n= 14; Rt= 17.302; 12.16 mg)
Heptadecanoic acid, methyl ester (**6**; n= 15; Rt= 17.942; 0.44 mg)
8,11-Octadecadienoic acid, methyl ester (**11**; n= 5, m= 6; Rt= 18.403; 8.060 mg)
Octadecanoic acid, methyl ester (**6**; n= 16; Rt= 18.568; 1.93 mg)

The product weighed a total 409.24 mg that corresponded to non-volatile material formed by components that did not volatilize at the injector temperature used, or were outside the predetermined scanning time in the method. The ^{1}H-NMR spectrum detected an AB system at δ 6.8-6.0, olefinic protons (δ 5.6-5.0), geminal to heteroatom protons (δ 4.1-3.0) and typical protons of hydrocarbon chains (δ 3.0-0.5). The ^{13}C-NMR spectrum showed carbonyl carbons (δ 178-173), olefinic carbons (δ 142-116), geminal to heteroatom carbons (δ 83-60) and saturated carbons typical of hydrocarbon chains (δ 56-11). This suggests the presence of ergosterol peroxide and unsaturated triglycerides.

It was filtered through lipophilic Sephadex LH-20 eluting with CH_2Cl_2: MeOH (1: 1), which gave three homogeneous fractions by TLC: **Prm-L-1-a** (290 mg); **Prm-L-1-b** (105 mg); and **Prm-L-1-c** (110 mg).

The **Prm-L-1-a** mixture was fractionated by semi-preparative HPLC (normal phase, Hex: EtOAc, 80: 20) to give three sub-fractions: **Prm-L-1-a-1, Prm-L-1-a-2** and **Prm-L-1-a-3**. All of these were analyzed by NMR and GC-MS as described below.

Prm-L-1-a-1

This gave 45 mg of an oil in which the following volatile substances were identified by GC-MS:
Octadecane (**1**; n= 15; Rt= 16.418; 0.2 mg)
Hexadecanoic acid, methyl ester (**6**; n= 14; Rt= 17.297; 1.891 mg)
Heptadecanoic acid, methyl ester (**6**; n= 15; Rt= 17.933; 0.12 mg)
8,11-Octadecadienoic acid, methyl ester (**11**; n= 5, m= 6; Rt= 18.391; 3.62 mg)
Heptadecanoic acid, 16-methyl-, methyl ester (**9**; n= 13; Rt= 18.558; 1.04 mg)
9-Octadecenoic (*oleic*) acid (Z)-, tetradecyl ester (**20**; n= 7; Rt= 19.817; 0.47 mg)

This gave a total of 36.039 mg of non-volatile material, presumably formed by components that did not volatilize at the injector temperature used, or that were outside the pre-determined scanning time in the method. This material was identified, by integrating the ^{1}H-NMR spectrum, as a mixture (1.0: 1.6) of ergosterol peroxide (**27**) {δ 6.55-6.14 (AB system characteristic)} and unsaturated triglyceride triolein type (**15**) {δ 5.28 (m); δ 4.34-4.01 (two characteristic dd); δ 2.79 (t); δ 2.31 (t); δ 2.04 (m); δ 1.63 (m); δ 1.29 (m); δ 0.92 (t)}.

Prm-L-1-a-2

This gave 187 mg of an oil in which the following volatile substances were identified by GC-MS:
Nonanoic acid, 9-oxo-, methyl ester (**21**; Rt= 13.687; 3.42 mg)
Tetradecanoic acid, methyl ester (**6**; n= 12; Rt= 15.898; 0.07 mg)
Pentadecanoic acid, methyl ester (**6**; n= 13; Rt= 16.598; 1.57 mg)
Hexadecanoic acid, methyl ester (**6**; n= 14; Rt= 17.270; 154.73 mg)
11-Octadecenoic acid, methyl ester (**8**; n= 5, m= 9; Rt= 18.389; 25.38 mg)

This weighed a total 1.60 mg of non-volatile components that did not volatilize at the injector temperature used or that were outside the pre-determined scanning time in the method. Using ^{1}H-NMR, this was identified as ergosterol peroxide (**27**) {(δ 6.55-6.14) characteristic dd of the AB system produced by the vinyl protons at C-6 and C-7}.

Prm-L-1-a-3

This gave 53 mg of a yellow oil in which the following volatile substances were identified by GC-MS:

1-Dodecanol (**3**; n= 10; Rt= 12.437; 0.0002 mg)
1-Tridecanol (**3**; n= 11; Rt= 14.847; 0.2912 mg)
1-Hexadecanol (**3**; n= 14; Rt= 16.329; 0.3238 mg)
Triolein (**15**; Rt= 18.389; 0.26 mg)
Eicosanoic acid (**5**; n= 18; Rt= 18.962; 2.78 mg)
1-Docosanol (**3**; n= 20; Rt= 22.482; 2.28 mg)

It gave 47.065 mg of non-volatile components that did not volatilize at the injector temperature used or that were outside the pre-determined scanning time in the method. Using ^{1}H-NMR, this was identified as a mixture of unsaturated triglycerides similar to triolein (**15**) {δ 5.22 (m); δ 4.35-3.90 (two characteristic dd); δ 2.78 (t); δ 2.33 (t); δ 2.04 (m); δ 1.62 (m); δ 1.29 (m); δ 0.91 (m)}. The ^{13}C-NMR spectrum also supports the carbonyl carbon allocation at δ 173.979-173.948; olefinics at δ 130.054-127.927; geminal to oxygens at the structural sub-unit of the glycerol (δ 72.124-62.001), and the same for the hidrocarbon aliphatic chains at δ 34.286-14.163.

Via re-chromatography over silica gel with hexane- ethyl acetate (98: 2), some 2.17 mg of a homogeneous oil was obtained by TLC, the spectroscopic data of which were consistent with the structure of triolein (**15**):

I.R ($CHCl_3$) - 3029.48; 3006.99; 1738.68; 1653.08; 1232.81; 1168.45 cm^{-1}

^{1}H-NMR ($CDCl_3$) - δ 5.34 (6H, t, J= 5.6 Hz); 5.27 (1H, dd, J= 4.3 Hz; J= 5.9 Hz); 4.30 (2H, dd, J= 4.3 Hz; J= 11.9 Hz); 4.14 {2H, dd (J= 5.9 Hz; J= 11.9 Hz); 2.30 (6H, t, J= 7.5 Hz); 2.01 (12H, m); 1.61 (6H, m); 1.27 (60H, m); 0.89 (9H, t, J= 6.6 Hz)}.

MS, m/*z* (%) - 603.5567 (M$^+$-$C_{18}H_{33}O_2$; 49.16 %); 602.5514 (M$^+$-$C_{18}H_{34}O_2$; 30.03 %); 265.2749 ($C_{18}H_{33}O$; 23.14 %); 55.0413 (100 %).

^{13}C-NMR (300 MHz, $CDCl_3$) - δ 14.018; 22.606; 24.767; 27.087; 27.130; 29.022; 29.094; 29.248; 29.454; 29.623; 29.684; 31.835; 33.930; 34.092; 62.006; 68.810; 129.592; 129.892; 172.723; 173.132; 173.73.

The **Prm-L-1-b** fraction (105 mg) was a mixture of at least six substances, a fact that was revealed by analytical TLC. However, using HPLC, ten major substances were separated of which only the following volatile components were identified by GC-MS:

Hexadecanoic acid, methyl ester (**6**; n= 14; Rt= 17.288; 5.01 mg)
Octadecanoic acid, methyl ester (**6**; n= 16; Rt= 18.554; 0.67 mg)

The fractionation was carried out by semi-preparative HPLC (normal phase, Hex: EtOAc, 80: 20) to give the following sub-fractions: **Prm-L-1-b-1** and **Prm-L-1-b-2**.

Prm-L-1-b-1

This gave 78 mg of a semi-solid material which GC-MS analysis allowed to identify as the following volatile substances:

1-Hexadecene (**2**; n= 13; Rt= 14.845; 0.96 mg)
Pentadecanoic acid, methyl ester (**6**; n= 13; Rt= 16.584; 0.67 mg)
2-Pentadecanone, 6,10,14-trimethyl- (**25**; Rt= 16.711; 1.43 mg)
9-Hexadecenoic acid, methyl ester, (Z)- (**8**; n= 5, m= **7**; Rt= 17.145; 34.08 mg)
7,10,13-Eicosatrienoic acid, methyl ester (**13**; Rt= 19.542; 12.6 mg)
6,9,12,15-Docosatetraenoic acid, methyl ester (**14**; Rt= 20.205; 7.73 mg)
9,12-Eicosadienoic (*linoleic*) acid (Z,Z)-, ethyl ester (**12**; Rt= 21.454; 6.18 mg)
9,12-Octadecadienoic (*linoleic*) acid (Z,Z)-, 2,3-dihydroxypropyl ester (**18**; Rt= 22.143; 4.4 mg)
Docosanoic acid, methyl ester (**6**; n= 20; Rt= 22.244; 0.46 mg)
Tetracosanoic acid, methyl ester (**6**; n= 22; Rt= 25.823; 1.14 mg)

The total weight was 8.35 mg of non-volatile material that, as before, did not volatilize at the injector temperature used, or were outside the pre-determined scanning time for the method. A study of the integral curve of the ^{1}H-NMR spectrum of this fraction indicated that it was a mixture 2.3: 0.4 of ergosterol peroxide (**27**), and 9(11)-dehydroergosterol peroxide (**28**) {δ 6.55-6.20 (two characteristic AB systems slightly offset from one another); δ 5.43-5.10 (m, vinyl protons); δ 3.96 (m, characteristic of H geminal to hydroxyl at C-3)}; δ 2.14-0.60 (m, CH_2; and CH_3). These deductions are confirmed by the ^{13}C-NMR spectrum, wherein the following is observed: carbonyl carbons (δ 179.440), aromatics (δ 167.800), olefinics (δ 135.000-126.000); geminal to oxygen atoms (δ 82.000-65.000); and methine/ methylene /methyls (δ 55.000-12.500).

Prm-L-1-b-2

This gave 23 mg of a semi-solid compound with the following volatile substances identified by GC-MS:

Nonanoic acid, 9-oxo-, methyl ester (**21**; Rt= 13.680; 0.1500 mg)
Tetradecanoic acid, 1-(hydroxymethyl)-1,2-ethanediyl ester (**16**; Rt= 14.751; 0.0501 mg)
1-Hexadecene (**2**; n= 13; Rt= 14.842; 0.2276 mg)
Tetradecanoic acid, methyl ester (**6**; n= 12; Rt= 15.882; 0.094 mg)
Pentadecanoic acid, methyl ester (**6**; n= 13; Rt= 16.595; 0.436 mg)
Pentadecanoic acid, 14-methyl-, methyl ester (**9**, n= 11; Rt= 17.267; 14.39 mg)
Heptadecanoic acid, methyl ester (**6**; n= 15; Rt= 17.908; 0.308 mg)
11-octadecenoic acid, methyl ester (**8**; n= 5, m= 9; Rt= 18.382; 0.7964 mg)
Octadecanoic acid, methyl ester (**6**; n= 16; Rt= 18.522; 1.46 mg)

There were 5.0429 mg of non-volatile components that did not volatilize at the injector temperature used, or that were outside the pre-determined scanning time in the method. A study of the integral curve of the ^{1}H-NMR spectrum of this fraction indicated that it was a mixture 6.0: 1.0 (86%: 14%) of ergosterol peroxide (**27**), and 9(11)-dehydroergosterol peroxide (**28**) {δ 6.65-6.21 (two characteristic AB systems, slightly offset); δ 5.45-5.02 (m, vinyl protons); δ 3.97 (m, characteristic of H geminal to hydroxyl at C-3}; δ 3.68 (hydroxyl proton); δ 2.42-0.72 (m, methylenes and methyls). This fraction was subjected to semi-preparative HPLC (normal phase, hexane-ethyl acetate, 80: 20) which yielded 3 mg of pure ergosterol peroxide (**27**):

M.P.= 177- 179 ºC

IR ($CHCl_3$) - 3618.18; 3024.99; 3011.06; 2957.69; 2933.7; 2871.04; 1603.24; 1461.19;
1377.92; 1221.18; 1208.93 and 973.53 (Δ^{22}-trans) cm^{-1}.

MS, m/*z* (%): 428.329 (1.8 %); 410.042 (1.0 %); 396.113 (100 %); 363.099 (88.2 %);
303.052 (1.7 %); 271.062 (19.0 %); 253.076 (17.2 %); 217.061 (11.6 %).

^{1}H-NMR (250 MHz, $CDCl_3$) - δ 6.58-6.11 (2H, characteristic AB system, CH=CH); δ 5.17-5.00 (2H, m, C=CH); δ 3.98 (1H, m, CH-OH); δ 2.15-1.19 (20H, m, CH and CH_2); δ 1.08-0.74 (18H, CH_3).

^{13}C-NMR (250 MHz, $CDCl_3$) - δ 12.921; 17.595; 18.231; 19.691; 19.986; 20.669; 20.933; 23.433; 28.681; 29.721; 30.140; 33.090; 34.721; 36.987; 39.363; 39.767; 42.794; 44.580; 51.101; 51.707; 56.209; 66.504; 79.453; 82.046; 130.703; 132.305; 135.209; 135.411.

Fraction Prm-L-1-c

This consisted of 110 mg of an oily material which was shown by analytical TLC to be a mixture of six substances. A GC-MS analysis

identified/ quantified these volatile components:
2-Butyl-1-octanol (**4**; n= 3, m= 3; Rt= 12.528; 0.14 mg)
2-Hexyl-1-octanol (**4**; n= 3, m= 5; Rt= 13.252; 0.055 mg)
1-Dodecanol 3,7,11-trimethyl (**24**; Rt= 14.471; 0.34 mg)
2-Hexyl-1-decanol (**4**; n= 5, m= 5; Rt= 15.647; 0.131 mg)
Hexadecanoic acid, methyl ester (**6**; n= 14; Rt= 17.290; 5.78 mg)
Octadecanoic acid, methyl ester (**6**; n= 16; Rt= 18.552; 0.86 mg)
13-Docosenoic (*erucic*) acid, (Z)- (**8**; n= 7, m= 11; Rt= 19.815; 0.3 mg)

This gave 93.936 mg of non-volatile material that was fractionated by semi-preparative HPLC into two fractions: fraction **Prm-L-1-c-1** and fraction **Prm-L-1-c-2.**

Prm-L-1-c-1

This consisted of 55 mg of a white solid substance which was identified again as ergosterol peroxide (**27**) on account of its physio-chemical constants and spectroscopic data:
M.P.= 178-180 ºC (crystallized from methanol)
$[\alpha]_D^{20°C}$ ($CHCl_3$, c 1.24)= -25º
IR ($CHCl_3$) - 3618.18; 3024.99; 1603.24; 1461.19; 1377.92; 1221.18; 1208.93; 973.53
(Δ^{22}-trans) cm^{-1}.
MS, m/*z* (%): 428.917 (1.4 %); 410.921 (3.0 %); 395.069 (33.6 %); 362.991 (16.2 %);
336.997 (7.2 %); 252.956 (7.1 %); 151.972 (28.8 %); 80.981 (48.6 %); 68.991 (93.7 %); 28.105 (100 %).
HRMS, m/*z* (formula): 428.32890 ($C_{28}H_{44}O_3$); 410.31140 ($C_{28}H_{42}O_2$); 396.33612
($C_{28}H_{44}O$); 303.19601 ($C_{19}H_{27}O_3$).
^{1}H-NMR (250 MHz, $CDCl_3$) - δ 6.58-6.19 (2H, characteristic AB system, CH=CH); δ 5.23 (2H, m, C=CH); δ 4.01 (1H, m, CH-OH); δ 3.65 (1H, OH); δ 2.20-1.18 (20H, m, CH and CH_2); δ 1.08-0.75 (18H, CH_3).
^{1}H-NMR (400 MHz, $CDCl_3$) - See table 2.
^{13}C-NMR (250 MHz, $CDCl_3$) - δ 12.890; 17.595; 18.200; 19.675; 19.986; 20.653; 20.902; 23.417; 28.681; 29.721; 30.140; 33.090; 34.705; 36.972; 39.363; 39.782; 42.794; 44.580; 51.086; 51.691; 56.209; 66.504; 79.453; 82.185; 130.784; 132.337; 135.240; 135.442.
^{13}C-NMR (400 MHz, $CDCl_3$)- See table 2.

Prm-L-1-c-2

This gave 50 mg of a semi-solid substance with some minor volatile components identified by GC-MS:
1-Pentadecene (**2**; n= 12; Rt= 13.972; 0.098 mg)
1-Eicosanol (**3**; n= 18; Rt= 16.327; 0.073 mg)
9-Octadecenoic acid (Z)-, 9-octadecenyl ester, (Z)- (**19**; Rt= 19.769; 0.533 mg)

This weighed a total 48.666 mg of non-volatile material that did not volatilize at the injector temperature used or that were outside the pre-determined scanning time in the method. A study of the ^{1}H-NMR spectrum indicates that it was ergosterol peroxide again (**27**) {δ 6.57-6.17 (2H, characteristic AB system, CH=CH); δ 5.21 (2H, m, C=CH); δ 4.10 (1H, m, CH-OH); δ 2.43 (1H, OH); δ 2.20-1.16 (20H, m, CH and CH2); δ 1.09-0.72 (18H, CH3)}.

Study of the liposoluble fraction-2 (Prm-L-2)

710 mg were obtained for this material, semi-solid in appearance. By analysis with TLC, five major substances were identified. The ^{1}H-NMR spectrum revealed signs of aromatic protons (δ 8.4-6.91), olefinic (δ 5.54-5.22), geminal to heteroatom (δ 4.51-3.49) and hydrocarbon saturated chains (δ 2.84 to 0.75). The ^{13}C-NMR spectrum gave carbonyl carbons (δ 173.529), aromatics (δ 146.419-142.103), olefinics (δ 132.305-127.880), geminal to heteroatom (δ 76.642-54.439) and typical aliphatic hydrocarbon chains (δ 42.825-14.117).

These data indicate the presence of an aromatic hydrocarbon mixture, endoperoxides and polyunsaturated triglycerides. This fraction was filtered through Sephadex (lipophilic LH-20), resulting in the following subfractions: **Prm-L-2-a**, **Prm-L-2-b**, **Prm-L-2-c** and **Prm-L-2-d**.

Prm-L-2-a

This gave 35 mg of an oily material in which only one volatile component present at the trace level was identified by GC-MS:
13-Docosenoic (*erucic*) acid, (Z)- (**8**; n= 7, m= 11; Rt= 19.816; 0.034 mg)

It gave 34.918 mg of non-volatile material that did not volatilize at the injector temperature used or that was outside the pre-determined scanning time in the method. A study of the ^{1}H-NMR spectrum (δ 1.92-0.73) indicated that it was a mixture of high molecular weight alkanes.

Prm-L-2-b

This gave 355 mg of an oil with the following volatile trace substances detected by GC-MS:
2-Butyl-1-octanol (**4**; n= 3, m= 3; Rt= 12.527; 0.0287 mg)
Tridecanoic acid, 13-formyl-, ethyl ester (**22**; Rt= 18.352; 0.0988 mg)
9,12-Octadecadienoic acid (Z,Z)-, 2-hydroxy-1-(hydroxymethyl) ethyl ester (**17**; Rt= 18.811; 0.0637 mg)

By analytical HPLC (normal phase, Hexane: AcOEt, 1: 1) two major peaks were observed; they were separated by semi-preparative HPLC under the same conditions, resulting in the following fractions: **Prm-L-2-b-1** and **Prm-L-2-b-2**.

Prm-L-2-b-1

This gave 270 mg of a white solid which crystallized from methanol to give crystals with m.p.= 171-176 °C and $[\alpha]_D^{20}$= -9.7 ($CHCl_3$, c 1.2). In its IR spectrum, hydroxyl groups are to be observed at 3400.00 cm^{-1}. By the rest of the spectroscopic data (mainly the integral data of the ^{1}H-NMR spectrum at 400 MHz), in the area of the two AB systems that are resolved in the range at δ 6.55-6.15, this was identified as a mixture 79: 21 of the ergosterol peroxide (**27**) and 9(11)-dehydroergosterol peroxide (**28**). This deduction is confirmed again by the ^{13}C-NMR spectrum at 250 MHz which shows carbons in the major component (**27**) at δ 12.875; 17.579; 18.185; 19.660; 19.970; 20.638; 20.902; 23.402; 28.665; 29.686; 30.094; 33.075; 34.690; 36.972; 39.332; 39.767; 42.779; 44.564; 51.070; 51.691; 56.194; 66.488; 79.453; 82.185; 130.768; 132.321; 135.224; 135.426 and some of the minor at δ 125.000; 130.784; 130.970; 132.337; 135.240; 135.442; and the mass spectrum by high resolution where peaks can be seen at m/*z* 428.32761 (4.7 %; calculated for $C_{28}H_{44}O_3$ 428.32905); 426.31196 (1.5 %; calculated for $C_{28}H_{42}O_3$ 426.31340); 410.3170 (10.7 %); 408.30139 (5.4 %); 396.3778 (32.2 %); 394.32213 (20.3 %); 303.19458 (5.9 %); 301.17893 (5.9 %).

Prm-L-2-b-2

This gave 30 mg of an oily material with the following substances identified by GC-MS analysis:
Tetradecane (**1**; n= 11; Rt= 13.235; 0.0058 mg)
Heptadecane (**1**; n= 14; Rt= 15.642; 0.0240 mg)
9-Hexadecenoic acid, tetradecyl ester, (Z)- (**20**; n= 5; Rt= 16.501; 0.1400 mg)

It gave 29.94 mg of non-volatile material that did not volatilize at the injector temperature used, or that were outside the pre-determined scanning time in the method. By ^{1}H-NMR sprectroscopy, this was revealed to be a mixture of steroidal unsaturated waxes {δ 5.56-5.03 ($\mathrm{C{=}C\underline{H}}$); 4.81-4.57 ($\mathrm{COOC\underline{H}}$); 2.84-2.64 ($\mathrm{C{=}C\text{-}C\underline{H}_2\text{-}C{=}C}$); 2.15-1.40 ($\mathrm{C\underline{H}_2COO}$, $\mathrm{C\underline{H}}$ and $\mathrm{C\underline{H}_2}$ of steroid skeletons); 1.38-1.11 ($\mathrm{C\underline{H}_2}$, intense, long hydrocarbon chains); 1.04-0.72 ($\mathrm{C\underline{H}_3}$, intense, angular methyl steroid skeletons)}. The attempts to separate these substances were unsuccessful.

Prm-L-2-c

This gave 109 mg of a semi-solid material with the following volatile substances as identified by GC-MS analysis:

1-Eicosanol (**3**; n= 18; Rt= 16.358; 0.0093 mg)

This means that this fraction contains 107.1924 mg of non-volatile components that did not volatilize at the temperature used for the injector or that exceeded the pre-determined scanning time in the method. Two subfractions were obtained by normal phase semi-preparative HPLC: **Prm-L-2-c-1** and **Prm-L-2-c-2**.

Prm-L-2-c-1

This gave 10 mg of an oily material, the ^{1}H-NMR spectrum of which revealed signals indicative of waxes structurally similar to those identified by GC-MS in other fractions {δ 5.57-5.26 ($\mathrm{C{=}C\underline{H}}$); 4.81-4.54 ($\mathrm{COOC\underline{H}}$); 2.83-2.69 ($\mathrm{C{=}C\text{-}C\underline{H}_2\text{-}C{=}C}$); 2.21-1.48 ($\mathrm{C\underline{H}_2CO}$, $\mathrm{C{=}C\text{-}C\underline{H}_2}$); 1.43-1.19 ($\mathrm{C\underline{H}_2}$, intense, long hydrocarbon chains); 1.01-0.74 (terminal $\mathrm{C\underline{H}_3}$)}.

Prm-L-2-c-2

This gave 83 mg of a semi-solid material with only one volatile substance identified by GC-MS analysis:

1-Eicosanol (**3**; n= 18; Rt= 16.357; 1.66 mg)

This gave 82.961 mg of non-volatile material that did not volatilize at the injector temperature used or that were outside the pre-determined scanning time in the method. This component was identified by the ^{1}H-NMR spectrum as ergosterol peroxide (**27**) {δ 6.57-6.17 (2H, characteristic AB system, $\mathrm{C\underline{H}{=}C\underline{H}}$); 5.21 (2H, m, $\mathrm{C{=}C\underline{H}}$); 4.10 (1H, m, CH-OH); 2.17-1.10 (m, $\mathrm{C\underline{H}}$ and $\mathrm{C\underline{H}_2}$ of the steroid skeleton); 1.09-0.72 (angulars $\mathrm{C\underline{H}_3}$)}.

Prm-L-2-d

This gave 105 mg of a viscous oil which revealed the following volatile components by GC-MS analysis:

Benzaldehyde, 4-hydroxy (**26**; Rt= 13.454; 0.1 mg)
1-Eicosanol (**3**; n= 18, Rt= 16.352; 0.062 mg)
Pentadecanoic acid, methyl ester (**6**; n= 13; Rt= 16.622; 0.036 mg)
Hexadecanoic acid, methyl ester (**6**; n= 14; Rt= 17.290; 1.97 mg)
9-Octadecenamide (**10**; Rt= 18.366; 0.85 mg)
8,11-Octadecadienoic acid, methyl ester (**11**; n= 5, m= 6; Rt= 18.389; 1.0 mg)
Octadecanoic acid, methyl ester (**6**; n= 16; Rt= 18.556; 0.54 mg)
Tricosane (**1**; n= 20; Rt= 19.753; 0.53 mg)
9-Octadecenoic (*oleic*) acid (Z)-, tetradecyl ester (**20**; n= 7; Rt= 19.817; 0.86 mg)

This revealed a total of 98.99 mg of non-volatile material,the components of which did not volatilize at the injector temperature used or that were outside the pre-determined scanning time in the method. By ^{1}H-NMR spectrum analysis, a mixture made up of phenol aldehydes was determined {δ 12.29; 12.10; 9.88; 7.91-7.50; 7.15- 6.67} together with waxes. This was purified by low pressure CC (silica gel, hexane- ethyl acetate, 90: 10) that yielded the fraction **Prm-L-2-d-1** (25 mg), proved to be homogeneous by TLC. This was crystallized from methanol to give colorless prisms of 4-hydroxy-benzaldehyde (**26**):

M.P. - 118-119 ºC (Methanol).
I.R ($\mathrm{CHCl_3}$) - 3590.81; 3338.71; 3002.84; 1687.99; 1604.06; 1586.85; 1511.54; 1442.68; 1274.83; 1223.19; 1156.48; 1102.69; 859.3; 838.01 cm^{-1}.
^{1}H-NMR - See table 3.
^{13}C-NMR - See table 3.
MS, m/*z* (%): 106.0794 (M$^+$; 2.5 %); 105.0747 (M$^+$-H; 16.7 %); 79.0599 (52.61 %);
78.0516 (M$^+$-CO; 25.43 %); 77.0444 (M$^+$-H -CO; 72.4 %).

Study of the liposoluble fraction-2 (Prm-L-3)

This gave 380 mg of a viscous oil which ^{1}H-NMR spectrum ($\mathrm{DOCD_3}$) showed to be one or more polyhydroxy compounds {δ 5.16 (1H, s wide; 4.05-3.35 (6H, m)}. This material was purified by reverse-phase semi-preparative HPLC, resulting in the fraction **Prm-L-3-1** (310 mg). This was also analyzed by GC-MS, allowing us to identify the following volatile substance:

1-Eicosanol (**3**; n= 18; Rt= 16.357; 1.66 mg)

There were 305.45 mg of non-volatile material, the components of which did not volatilize at the injector temperature used or were outside the pre-determined scanning time in the method. By ^{1}H-NMR spectrum analysis this seemed to be made up of one or more mono-saccharides in the form of α-pyranose {δ 5.10 (1H, d, J= 3.6 Hz, $\mathrm{C\underline{H}}$ anomeric); 3.83-3.21(9H, m, $\mathrm{C\underline{H}{+}C\underline{H}_2}$ geminals to OH groups)}; the ^{13}C-NMR spectrum ($\mathrm{DOCD_3}$) also confirmed that deduction (δ 94.948 for the anomeric carbon and 74.442-54.716 for the other carbons).

Through an analysis of this fraction by HPLC with a Shodex OH Pak SB806 HQ column thermostated at 30 °C (water 0.05% $\mathrm{NaN_3}$ as eluent at a flow of 1.0 ml/ min and a refractive index detector), and after filtration through a Sep-Pak $\mathrm{C_{18}}$ of Water cartridge, this was found to be made up of two components, a minor component (30.63%) with a retention time (Rt= 11.481 min) matching that of the D-(-)mannitol {a mixed injection of the **Prm-L-3-1** fraction with an authentic sample of D-(-)-mannitol had the same chromatogram with the peak of the minor substance (Rt= 11.481) increased in intensity (57.38%)}, and another major substance (69.11%) with a retention time (Rt= 12.711) which did not coincide with authentic samples of commercial monosaccharides as D-(+)-Glucose (Rt= 11.481) or D-(+)-mannose (Rt= 11.188). A more detailed study of this gluco-pyranose will be published shortly.

Study of the hidrosoluble fraction (Prm-H-0)

The fraction which was obtained directly from the resultant aqueous phase by separating the liposoluble element from the crude extract, was re-extracted (equal volume, x1) with 2-butanol. The evaporation of the solvent gave 1761 mg of a viscous oil (**Prm-H-0**) which was shown to be “highly polar” by thin layer chromatography in its normal phase. In the ^{1}H-NMR spectrum of this crude fraction, geminal to heteroatom protons were observed (δ 3.83-3.21) together with the typical unsaturated hydrocarbon chains (δ 5.32 and 2.31-0.79). The ^{13}C-NMR spectrum shows olefinic carbons at δ 130.240-127.865; geminal to heteroatoms at δ 77.590-54.517 and typical aliphatic hydrocarbon chains (δ 31.507-9.598). It follows, therefore, that there is probably presence of a mixture of sugars with impurities from the previous lipid fractions. Indeed, by GC-MS, the following volatile organic components were detected:

Geranyl isovalerate (**23**; Rt= 14.005; 10.28 mg)
9-Octadecenoic (*oleic*) acid (Z)-, tetradecyl ester (**20**; n= 7; Rt= 19.818; 4.98 mg)
Pentacosane (**1**; n= 22; Rt= 21.820; 2.86 mg)

Two subfractions were separated by semi-preparative reverse phase HPLC: **Prm-H-0-1** and **Prm-H-0-2**.

Prm-H-0-1

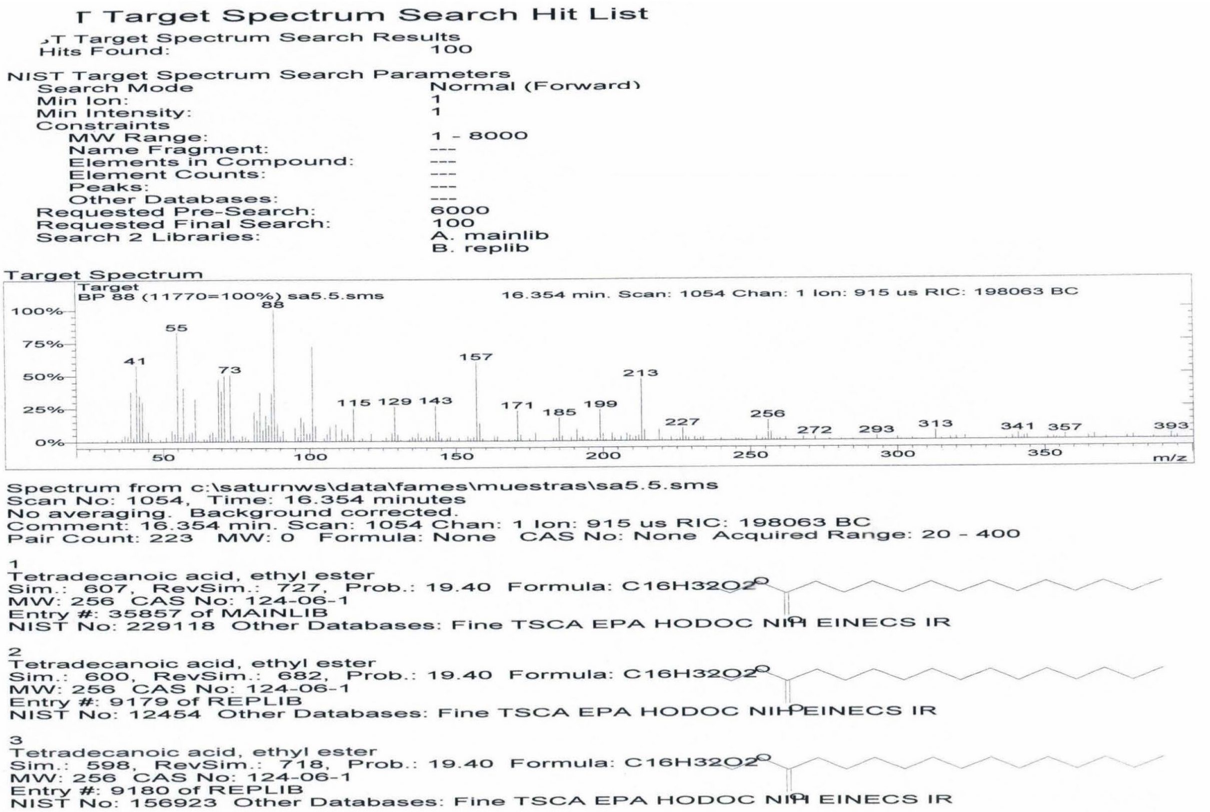
This gave 8.5 mg of a viscous oil. By GC-MS, the following volatile compounds were detected:
Dodecane (**1**; n= 9; Rt= 12.075; 0.00032 mg)
2-Butyl-1-octanol (**4**; n= 3, m= 3; Rt= 12.517; 0.00053 mg)
Tetradecanoic acid (**5**; n= 12; Rt= 14.766; 0.00131 mg)
9-Octadecenamide (**10**; Rt= 18.352; 0.00475 mg)
9-Octadecenoic acid (Z)-, 9-octadecenyl ester, (Z)- (**19**; Rt= 19.796; 0.00401 mg)

This gave 8.49 mg of non-volatile material, the components of which did not volatilize at the injector temperature used or that were outside the pre-determined scanning time for the method. By analysis of the ^{1}H-NMR spectrum, this appeared to be made of phospho- and glycolipids that remained unidentified.

Prm-H-0-2

This gave 1670 mg of a semi-solid material with the following volatile components detected by GC-MS:
2-Butyl-1-octanol (**4**; n= 3, m= 3; Rt= 12.528; 0.22 mg)
2-Hexyl-1-decanol (**4**; n= 5, m= 5; Rt= 15.649; 0.16 mg)
Pentadecanoic acid (**5**; n= 13; Rt= 15.906; 0.22 mg)
Pentadecanoic acid, 14-methyl-, methyl ester (**9**, n= 11; Rt= 17.279; 0.61 mg)
13-Docosenoic (*erucic*) acid, (Z)- (**8**; n= 7, m= 11; Rt= 19.810; 0.59 mg)

This gave a total of 1664.93 mg of non-volatile material that did not volatilize at the injector temperature used or that were outside the pre-determined scanning time for the method.



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Target Spectrum
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Sim.:  607,  RevSim.:  727,  Prob.: 19.40  Formula: C16H32O2
MW: 256  CAS No: 124-06-1
Entry #: 35857 of MAINLIB
NIST No: 229118  Other Databases: Fine TSCA EPA HODOC NIH EINECS IR

2
Tetradecanoic acid, ethyl ester
Sim.:  600,  RevSim.:  682,  Prob.: 19.40  Formula: C16H32O2
MW: 256  CAS No: 124-06-1
Entry #: 9179 of REPLIB
NIST No: 12454  Other Databases: Fine TSCA EPA HODOC NIH EINECS IR

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Tetradecanoic acid, ethyl ester
Sim.:  598,  RevSim.:  718,  Prob.: 19.40  Formula: C16H32O2
MW: 256  CAS No: 124-06-1
Entry #: 9180 of REPLIB
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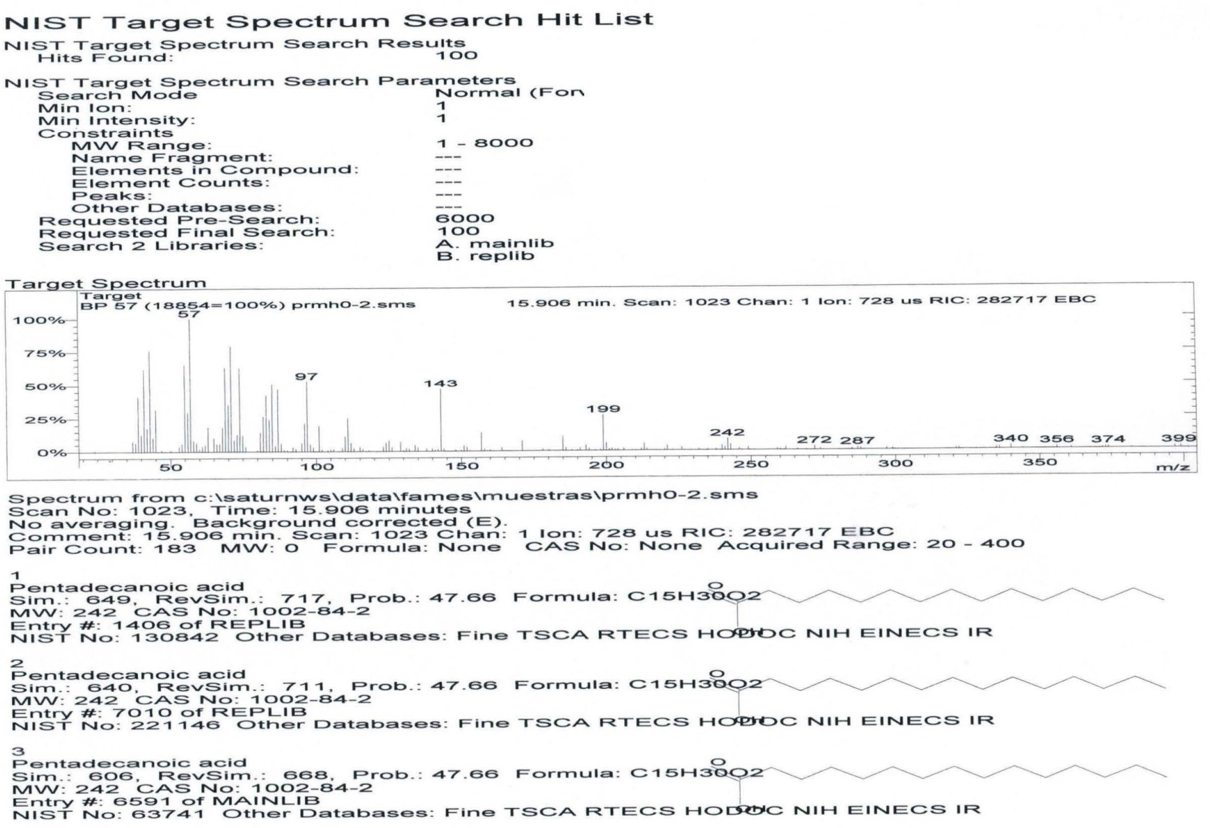
Figure 3S. *GC-MS fingerprint of the tetradecanoic acid ethyl ester (**5**, n= 12)*



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Pentadecanoic acid
Sim.:  649,  RevSim.:  717,  Prob.: 47.66  Formula: C15H30O2
MW: 242  CAS No: 1002-84-2
Entry #: 1406 of REPLIB
NIST No: 130842  Other Databases: Fine TSCA RTECS HODOC NIH EINECS IR

2
Pentadecanoic acid
Sim.:  640,  RevSim.:  711,  Prob.: 47.66  Formula: C15H30O2
MW: 242  CAS No: 1002-84-2
Entry #: 7010 of REPLIB
NIST No: 221146  Other Databases: Fine TSCA RTECS HODOC NIH EINECS IR

3
Pentadecanoic acid
Sim.:  606,  RevSim.:  668,  Prob.: 47.66  Formula: C15H30O2
MW: 242  CAS No: 1002-84-2
Entry #: 6591 of MAINLIB
NIST No: 63741  Other Databases: Fine TSCA RTECS HODOC NIH EINECS IR
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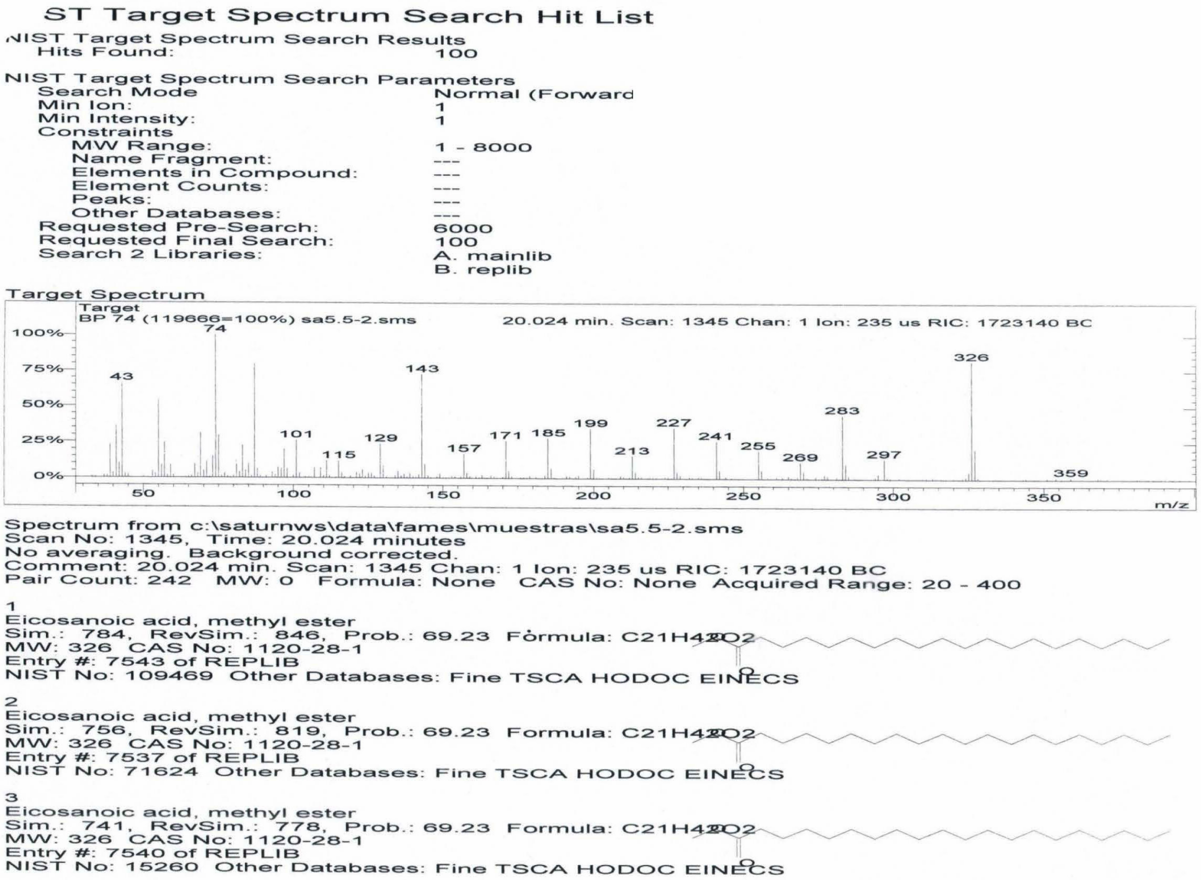
Figure 4S. *GC-MS fingerprint of the pentadecanoic acid (**5**, n= 13)*



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                                            B. replib
Target Spectrum
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Eicosanoic acid, methyl ester
Sim.:  784,  RevSim.:  846,  Prob.: 69.23  Formula: C21H42O2
MW: 326  CAS No: 1120-28-1
Entry #: 7543 of REPLIB
NIST No: 109469  Other Databases: Fine TSCA HODOC EINECS

2
Eicosanoic acid, methyl ester
Sim.:  756,  RevSim.:  819,  Prob.: 69.23  Formula: C21H42O2
MW: 326  CAS No: 1120-28-1
Entry #: 7537 of REPLIB
NIST No: 71624  Other Databases: Fine TSCA HODOC EINECS

3
Eicosanoic acid, methyl ester
Sim.:  741,  RevSim.:  778,  Prob.: 69.23  Formula: C21H42O2
MW: 326  CAS No: 1120-28-1
Entry #: 7540 of REPLIB
NIST No: 15260  Other Databases: Fine TSCA HODOC EINECS
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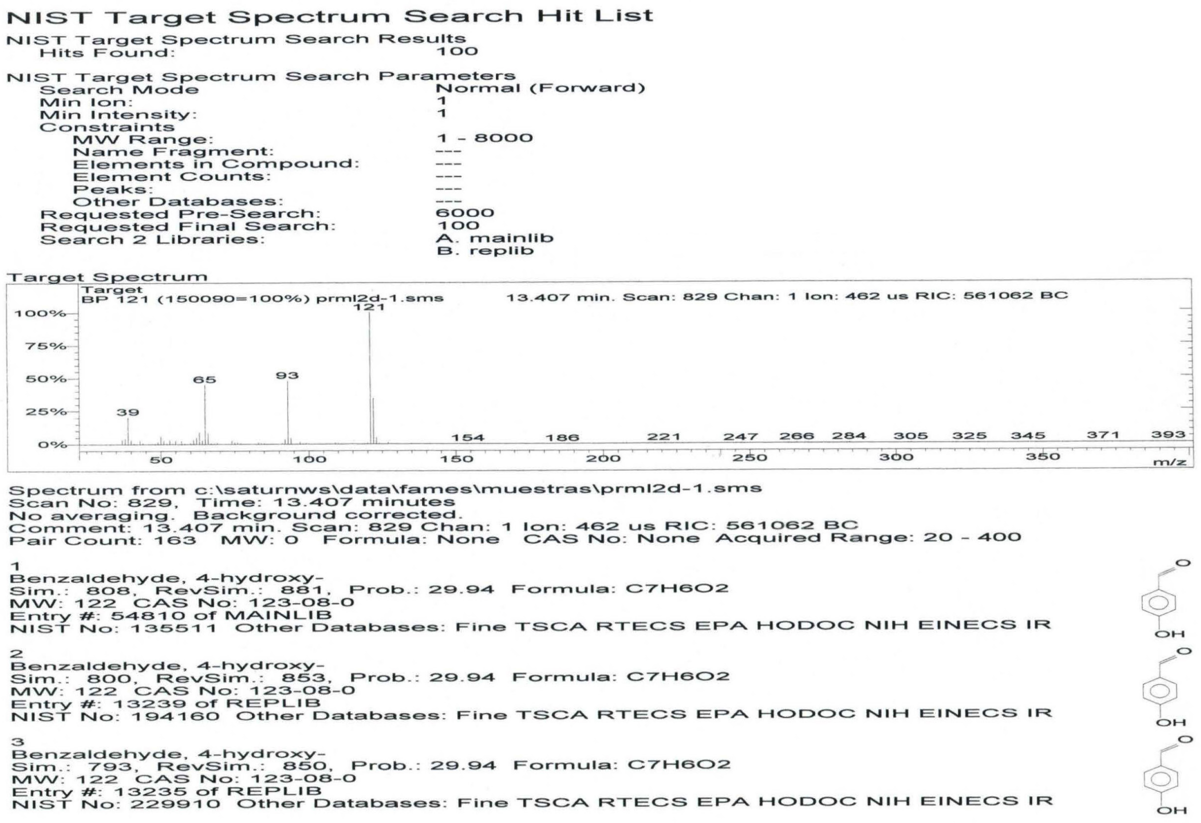
Figure 5S. *GC-MS fingerprint of the eicosanoic acid methyl ester (**5**, n= 18)*



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                                         B. replib
Target Spectrum
    Target
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Pair Count: 163   MW: 0   Formula: None   CAS No: None  Acquired Range: 20 - 400

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Benzaldehyde, 4-hydroxy-
Sim.:  808,  RevSim.:  881,  Prob.: 29.94  Formula: C7H6O2
MW: 122  CAS No: 123-08-0
Entry #: 54810 of MAINLIB
NIST No: 135511  Other Databases: Fine TSCA RTECS EPA HODOC NIH EINECS IR

2
Benzaldehyde, 4-hydroxy-
Sim.:  800,  RevSim.:  853,  Prob.: 29.94  Formula: C7H6O2
MW: 122  CAS No: 123-08-0
Entry #: 13239 of REPLIB
NIST No: 194160  Other Databases: Fine TSCA RTECS EPA HODOC NIH EINECS IR

3
Benzaldehyde, 4-hydroxy-
Sim.:  793,  RevSim.:  850,  Prob.: 29.94  Formula: C7H6O2
MW: 122  CAS No: 123-08-0
Entry #: 13235 of REPLIB
NIST No: 229910  Other Databases: Fine TSCA RTECS EPA HODOC NIH EINECS IR
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***Figure 6S.** GC-MS fingerprint of the 4-hydroxibelzaldehyde (**26**)*

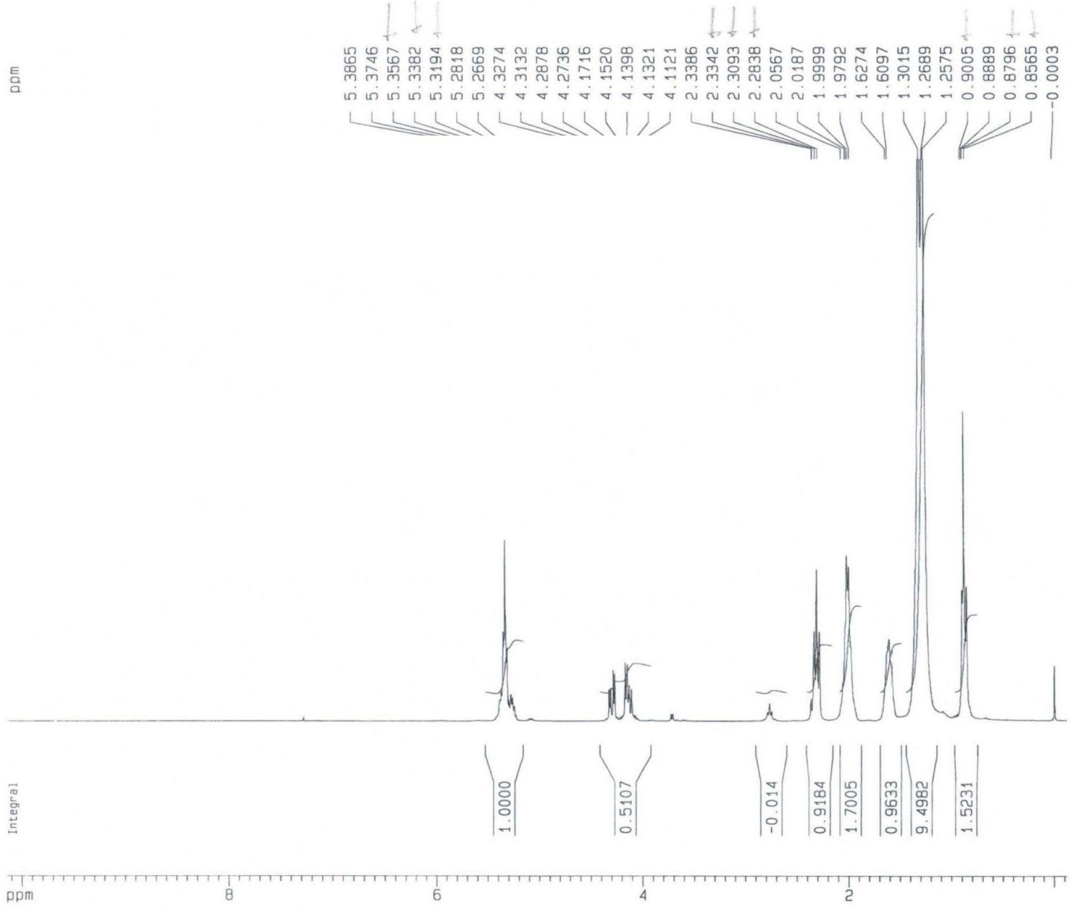

***Figure 7S.** ^{1}H-NMR spectrum (CDCl$_3$, 300 MHz) of the Triolein (**15**)*

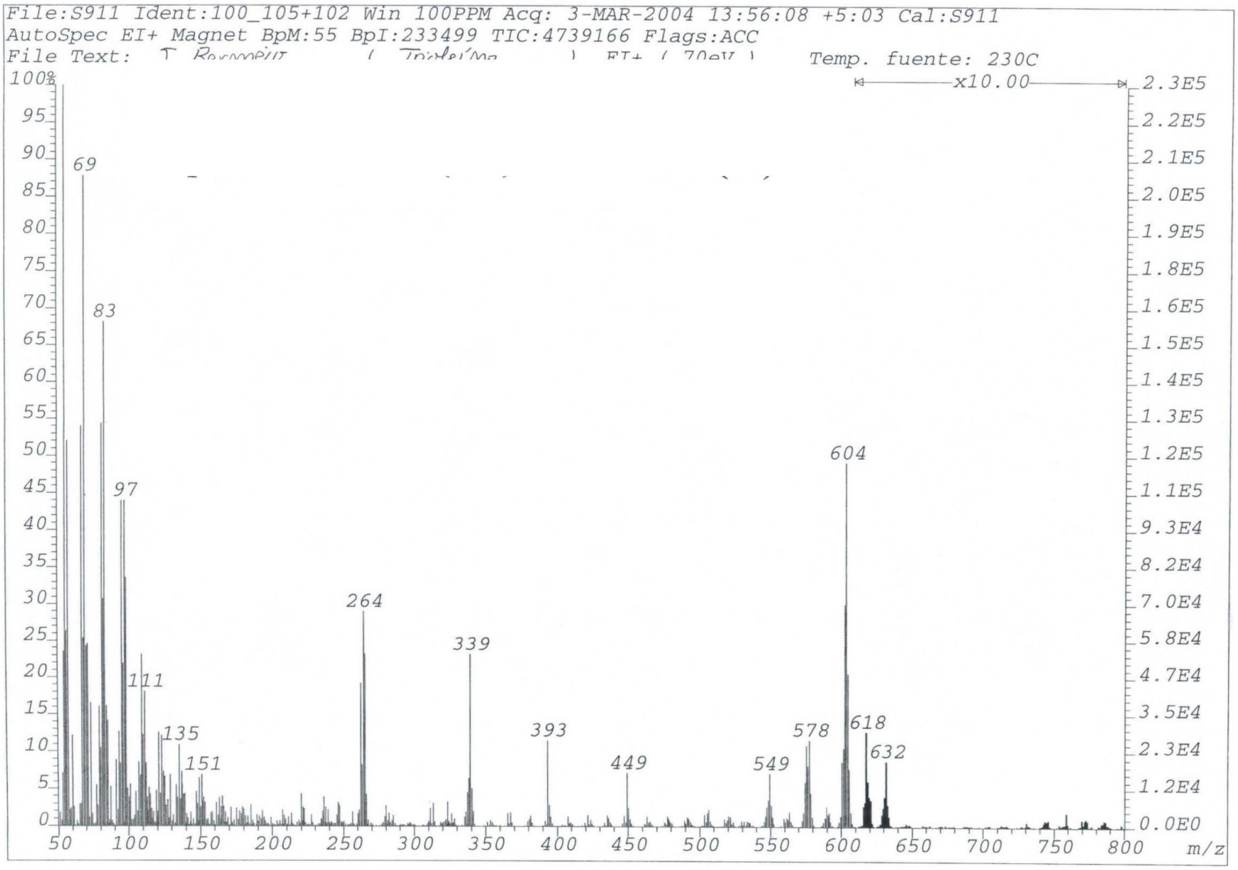

Figure 8S. *MS spectrum of the Triolein (**15**)*

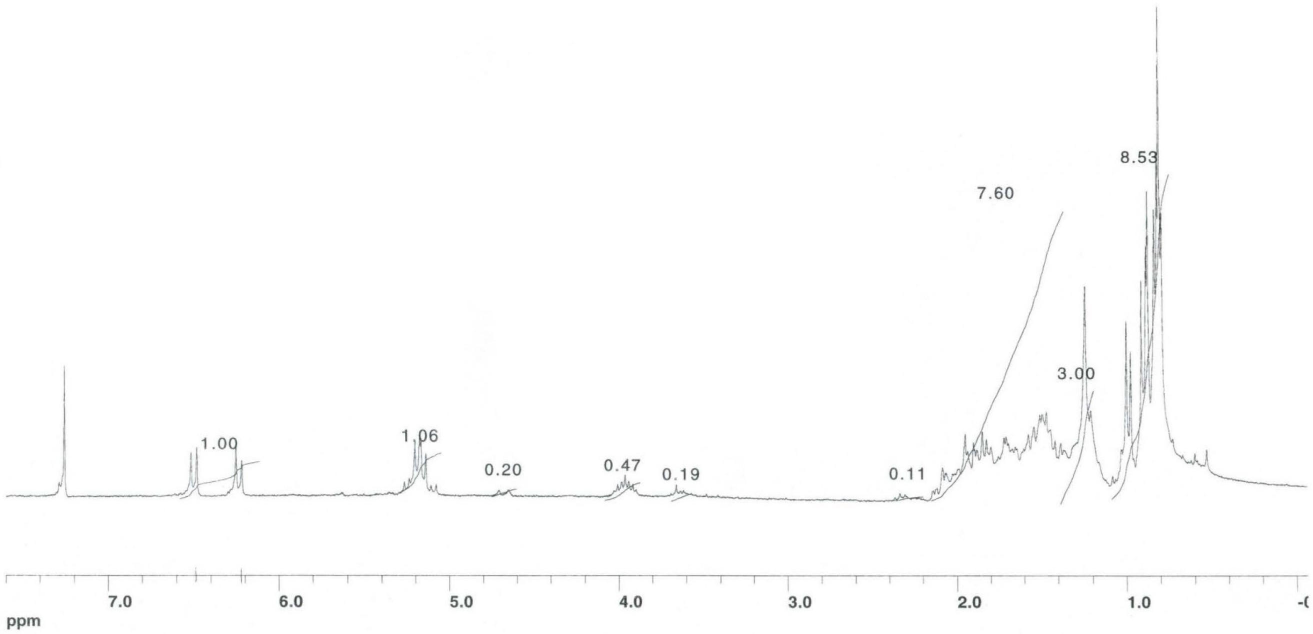

Figure 9S. *^{1}H-NMR spectrum (CDCl$_3$, 250 MHz) of the Ergosterol Peroxide (**27**)*

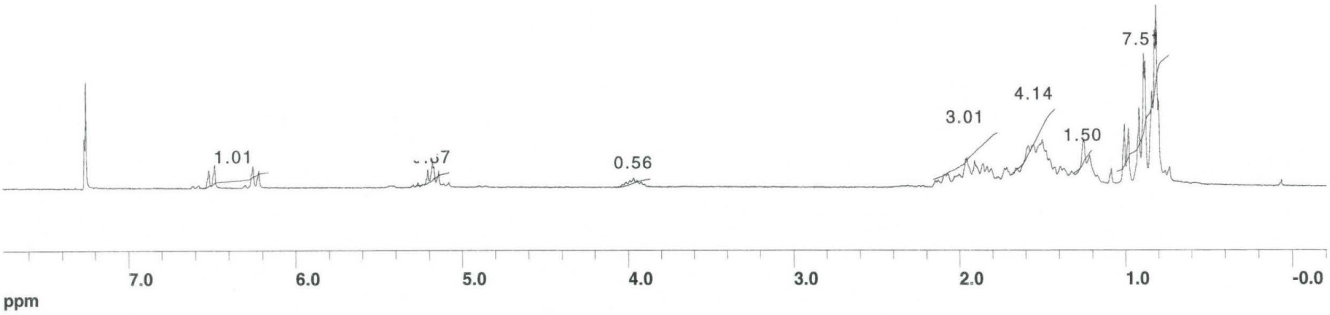

Figure 10S. *^{1}H-NMR spectrum ($CDCl_3$, 250 MHz) of the ergosterol peroxide (**27**) and its derivative, 9(11)-dehydroergosterol peroxide (**28**)*

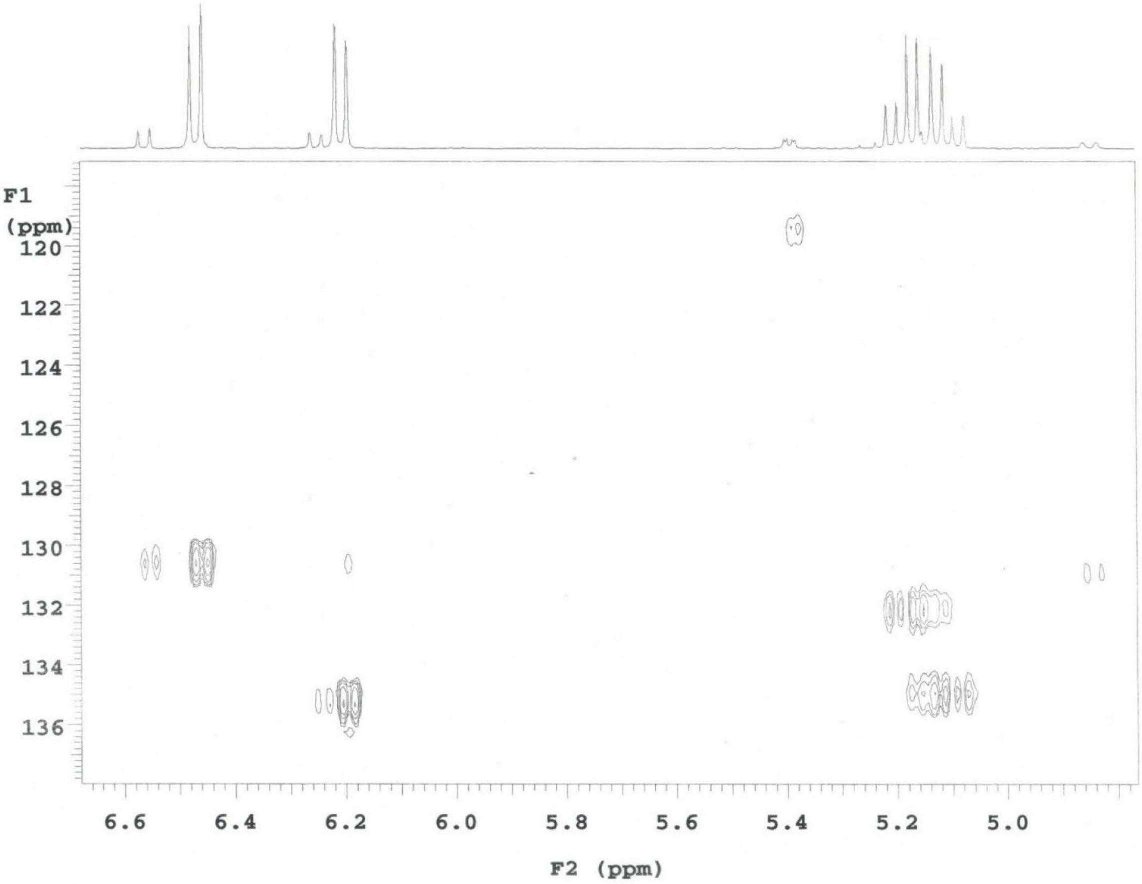

Figure 11S. *HSQC spectrum ($CDCl_3$, 400 MHz) of the ergosterol peroxide (**27**) and its derivative, 9(11)-dehydroergosterol peroxide (**28**)*

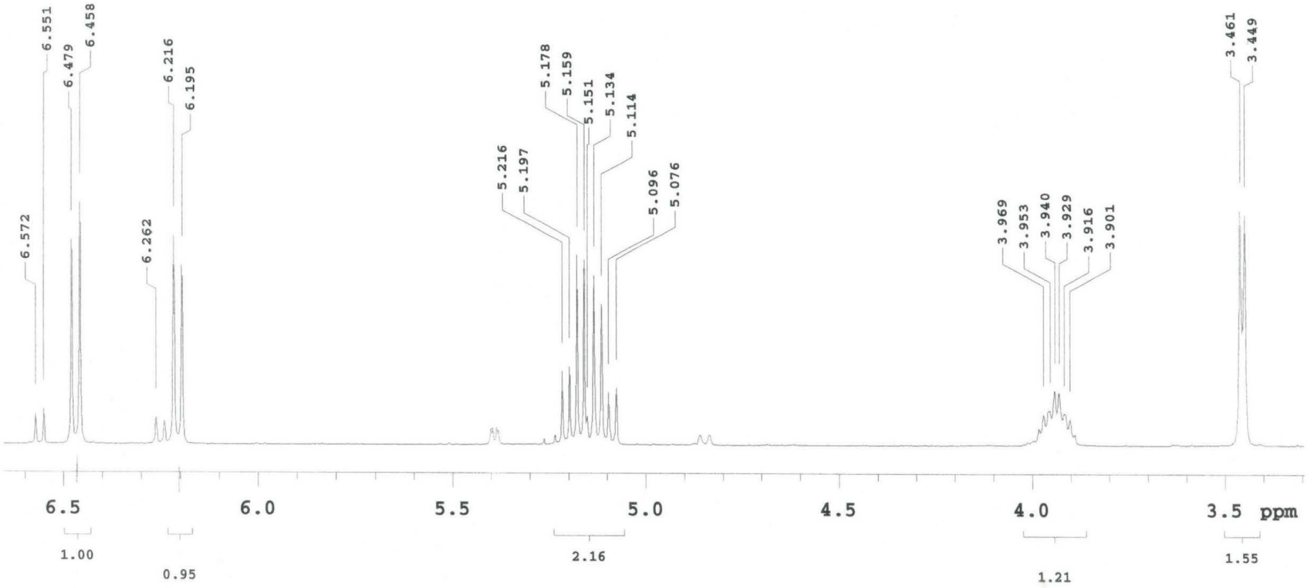

Figure 12S. *^{1}H-NMR spectrum (CDCl$_3$, 400 MHz) of the ergosterol peroxide (**27**) and its derivative, 9(11)-dehydroergosterol peroxide (**28**)*

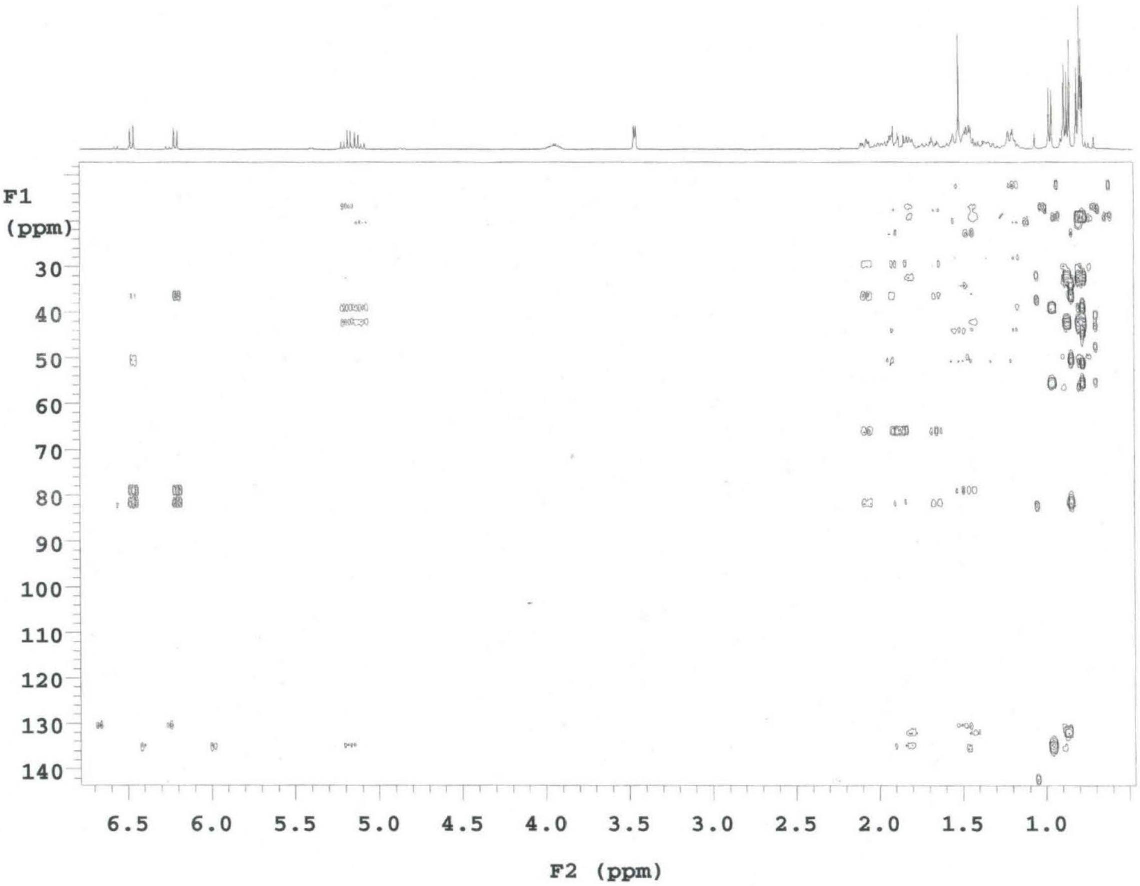

Figure 13S. *HMBC spectrum (CDCl$_3$, 400 MHz) of the ergosterol peroxide (**27**) and its derivative, 9(11)-dehydroergosterol peroxide (**28**)*

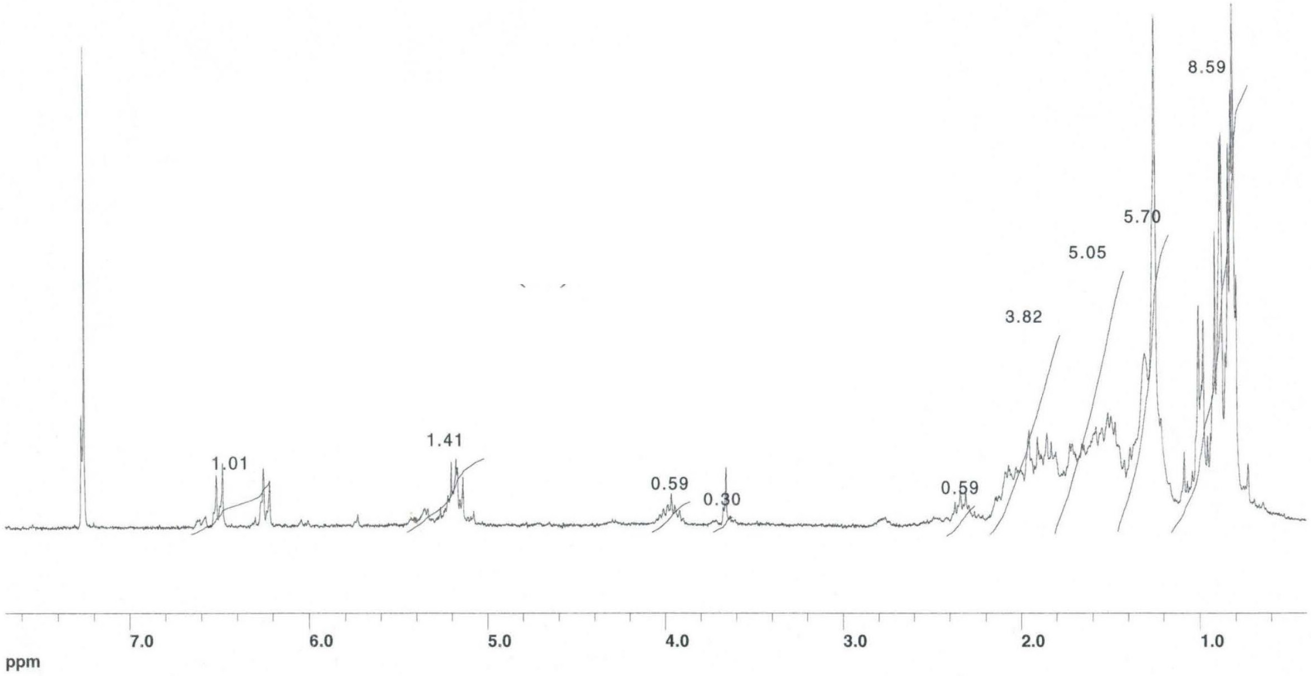

Figure 14S. *^{1}H-NMR spectrum ($CDCl_3$, 250 MHz) of the ergosterol peroxide (**27**) and its derivative, 9(11)-dehydroergosterol peroxide (**28**)*

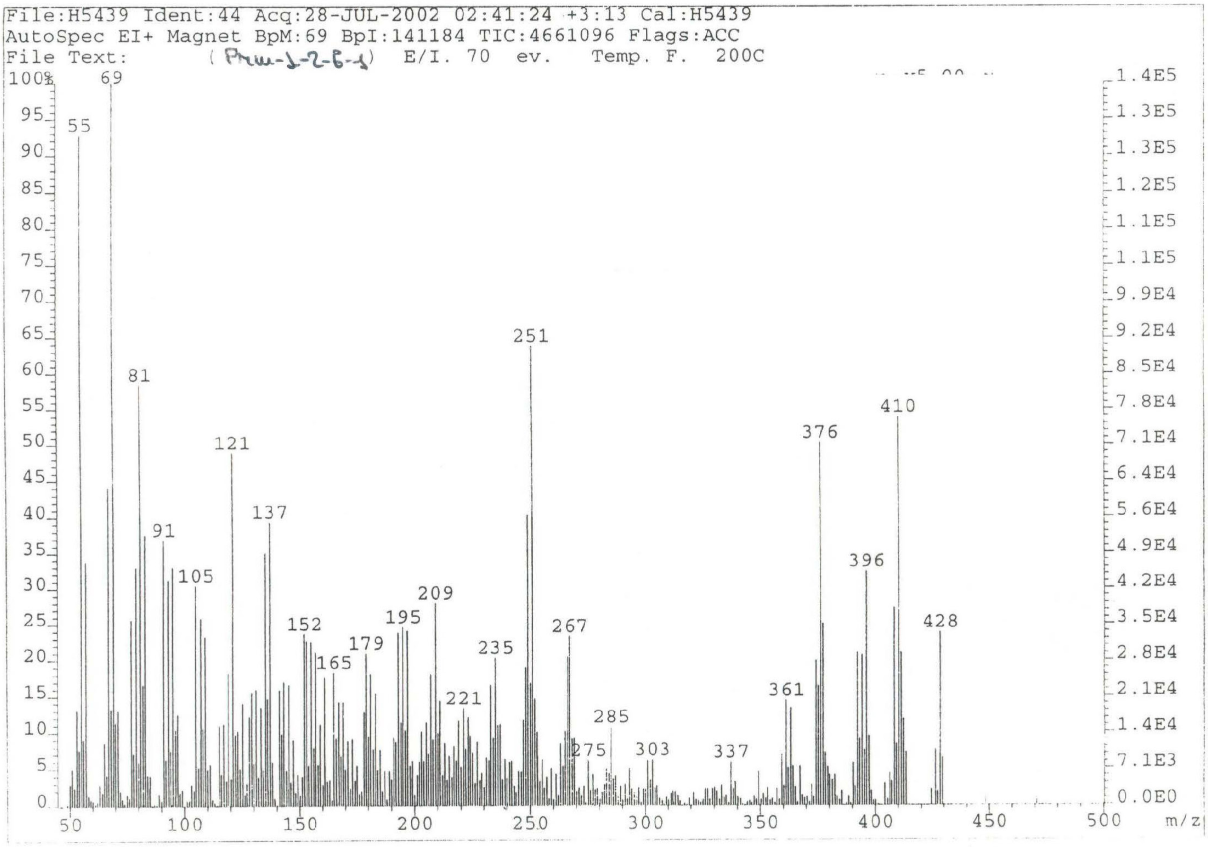

Figure 15S. *Mass spectrum (MS) of the ergosterol peroxide (**27**) and its derivative, 9(11)-dehydroergosterol peroxide (**28**)*

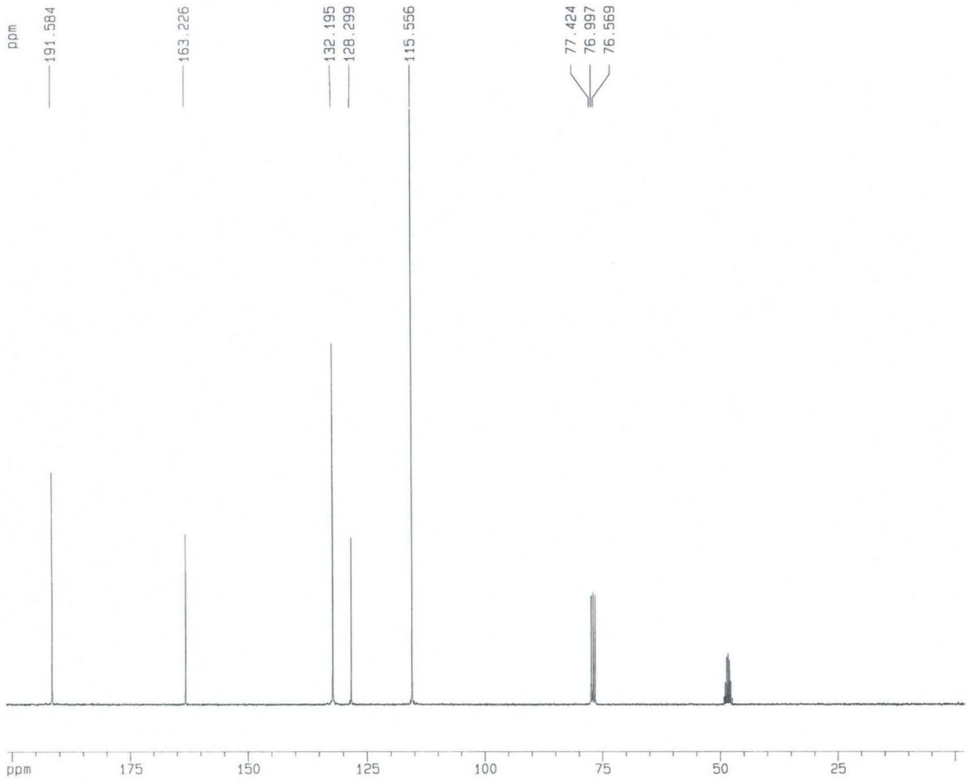

Figure 16S. *^{13}C-NMR spectrum (CD$_3$OD, 300 MHz) of the 4-hydroxybenzaldehyde (**26**)*

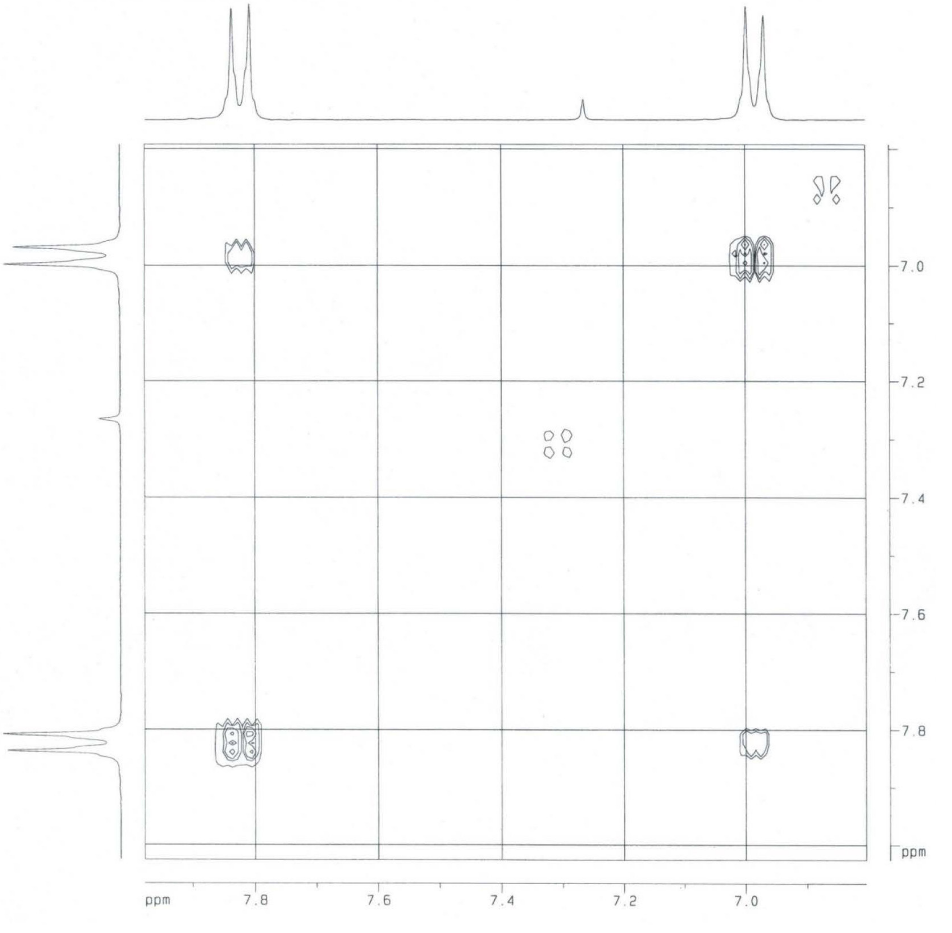

Figure 17S. *TOCSY spectrum (CD$_3$OD, 300 MHz) of the 4-hydroxybenzaldehyde (**26**)*

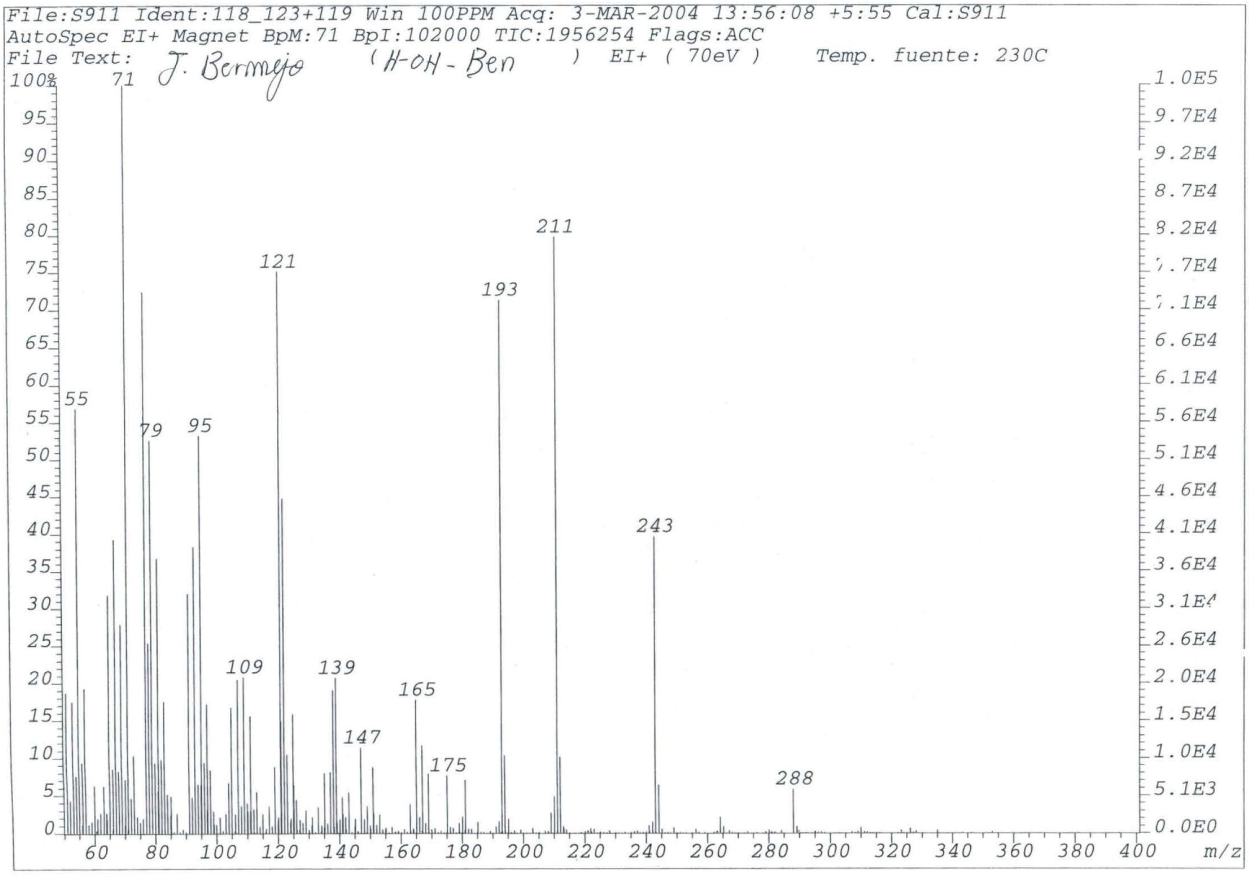

Figure 18S. *Mass spectrum (MS) of the 4-hydroxybenzaldehyde (**26**)*

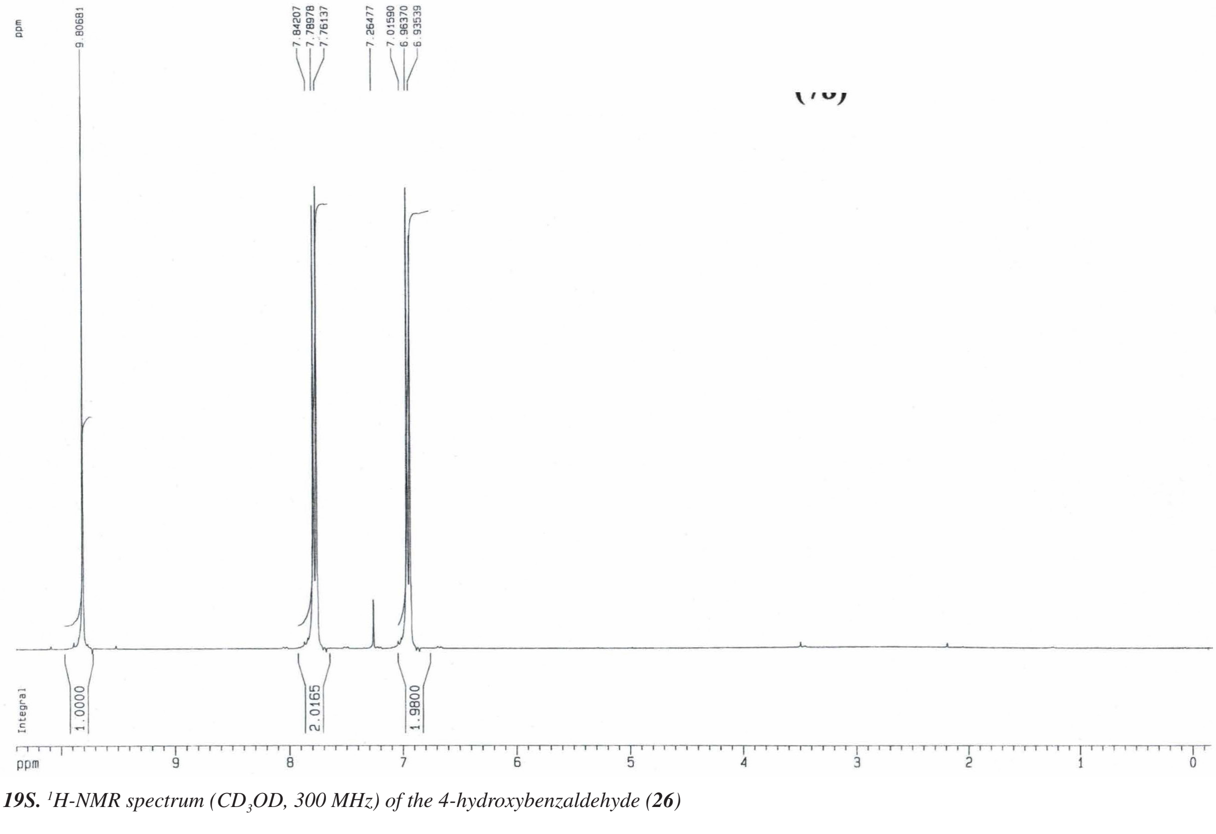

Figure 19S. *^{1}H-NMR spectrum (CD$_3$OD, 300 MHz) of the 4-hydroxybenzaldehyde (**26**)*

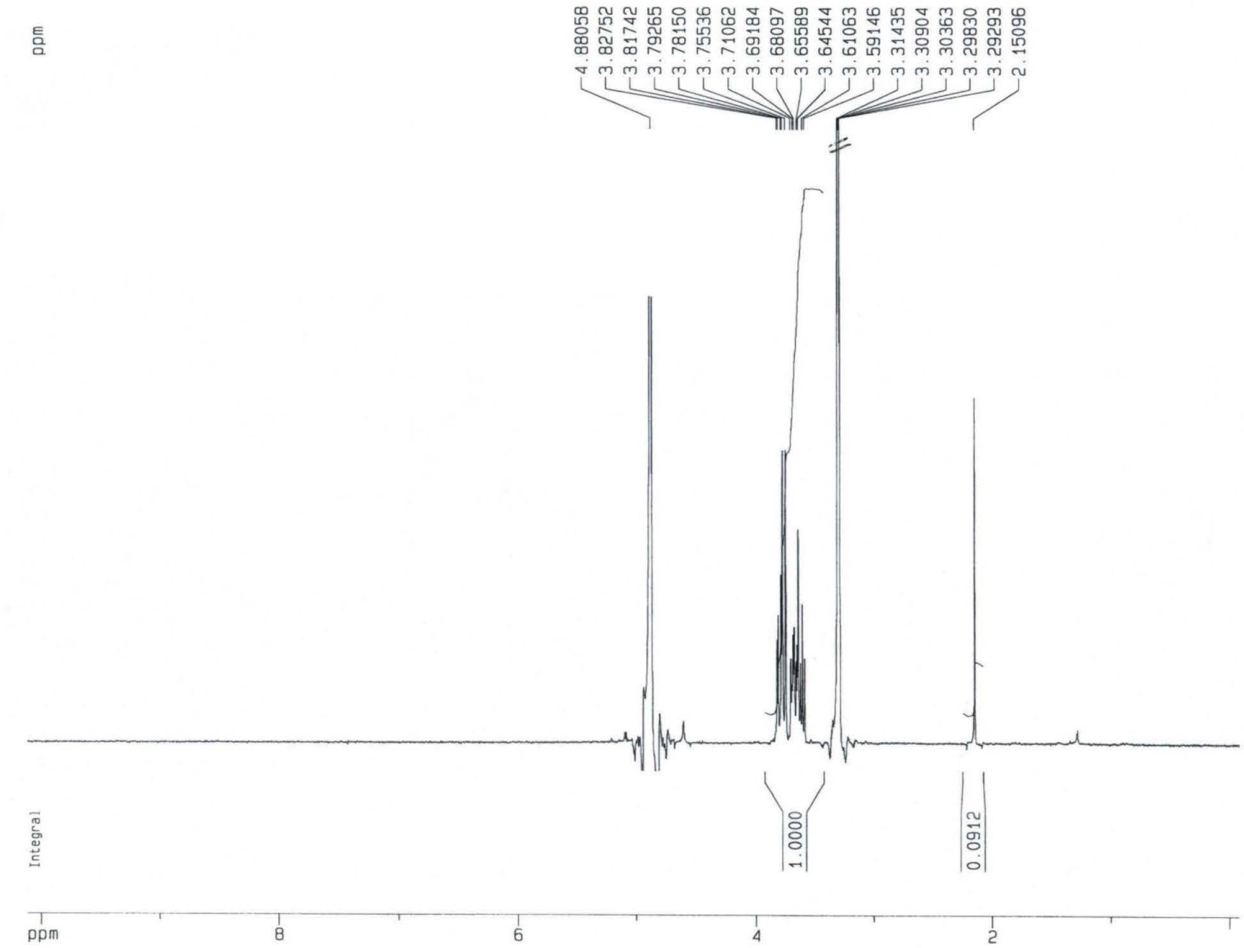

Figure 20S. *^{1}H-NMR spectrum (CD_3OD, 300 MHz) of the D-mannitol (**29**)*