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A Novel Triacylglycerol from A Cytotoxic Extract of A Red Sea Marine Fungus

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INVESTIGATION of the CH_2Cl_2 extract of a taxonomically unidentified anamorphic fungus strain MF 014, has yielded the glycerol triester, 1,3-*O*-bis-*Z*-11-eicosenoyl 2-*O*-*Z* -9, *Z* -12-nonadecadienoyl glycerol. The structure of this novel metabolite was established on the basis of extensive 1D- and 2D-NMR spectroscopic studies as well as on mass spectrometric analysis. The extract exhibited *in vitro* cytotoxic activity against human amniotic epithelial cells (FL cells) in the neutral red assay.

Keywords: Anamorphic fungi , Marine fungi, Triacylglycerol and Cytotoxic activity

Marine-derived fungi have proven to be rich sources of secondary metabolites that have both unique structures [(1-3)] and potential as pharmaceutical leads [(4)]. In particular, fungi obtained from woody substrates have yielded novel metabolites with potent antibacterial and cytotoxic activities [(5-6)].

This potential has led us to investigate the biological activity and the chemistry of the secondary metabolites contained in the dichloromethane extract of an anamorphic fungal strain MF 014 obtained from a submerged mangrove collected at the shore of the Red Sea, Safaga, Egypt.

Extract of strain MF 014 yielded the triacylglycerol, 1,3-*O*-bis-*cis*-11-eicosenoyl 2-*O*-*cis*-9,*cis*-12-nonadecadienoyl (1) that proved to be a new natural product. Isolation and structure elucidation of the compound (1) by NMR spectroscopy and by mass spectrometry are reported herein. On the other hand, the extract exhibited *in vitro* cytotoxic activity.

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Experimental

Instruments and materials

NMR spectra were measured by a Jeol ECA 500 MHz NMR spectrometer, at 500 MHz. ^{1}H chemical shifts (δ) were measured in ppm, relative to TMS and ^{13}C NMR chemical shifts to $CDCl_3$ and converted to TMS scale by adding 77.36. Typical conditions: spectral width = 8 kHz for ^{1}H and 30 kHz for ^{13}C, 64 K data points and a flip angle of 45. ESIFTMS spectra were measured on a Finnigan LTQ-FTMS (Thermo Electron, Bremen, Germany,Department of Chemistry, Humboldt-Universität zu Berlin). UV spectra were recorded on a Shimadzu UV-Visible-1601 Spectrophotometer (manufacturer, town, country). $[\alpha]_D^{25}$ were measured on a Kruess polarimeter-8001 (A. Kruess, Optronic, town, Germany). TLC analysis was carried out on Merck silica gel 60, 0.2 mm, aluminium sheets (20 x 20 cm). Preparative TLC was performed on Merck, silica gel 60, 2 mm, aluminium sheets, (20 x 20 cm). Solvent systems for TLC: *i*)- *n*-hexane: Et_2O: AcOH :MeOH (90:20:2:3,*v*/*v*); *ii*)- ether:*n*-hexane:AcOH (20:89:10, *v*/*v*) and *iii*)- $CHCl_3$:n-hexane: MeOH (14:4:1, *v*/*v*). GC analysis was performed on a DB 23 column from J & W Scientific Unc. (Folsom, CA, country) (30m x 0.25 mm i.d., d*f* 0.25 mm). The temperature program was 150-175 °C, 1.4 °C/min then 175-25°C/ min

Fungal material

The fungus was isolated by micromanipulation from the mangrove samples, collected at the shore of the Red Sea, Safaga, Egypt and plated out on sterile agar. Further clean-up was performed until a pure culture could be obtained. The standard conditions encompassed the growth in modified Hagem-medium pH 7.5 (ammonium succinate 0.5 g, KH_2PO_4 0.5 g, $MgSO_4 \times 7\ H_2O$ 0.5 g, $FeCl_3$ [1%] 0.5 ml, glucose 5.0 g, malt extract 5.0 g, aqua demineralisata ad 1000 ml (substances obtained from VWR International) in Erlenmeyer flasks of 100 ml volume, shaking at 125 rounds per minute and a natural day/night-rhythm. After 5 weeks, the culture was harvested. Media and mycelia were separated by filtration through a folded filter. Voucher specimens are deposited in the culture collection of the Institute of Pharmacy of the University Greifswald (voucher number MF014). Because of lacking sporulation an exact taxonomic determination of the fungus was not possible. It could only be determined as Anamorphic fungus. A voucher specimen is currently deposited in the culture collection of Anamorphic fungi at the Institute of Pharmacy, Ernst-Moritz-Arndt University Greifswald (MF014). Despite several attempts, the taxonomic identification of the producer has not yet been possible so far, because sporulation could not be induced under artificial conditions. Thus, it can only be referred to as an anamorphic fungus, pending further notice.

Preparation of extracts

The mycelia were lyophilized and extracted successively, for 24 hr each with dichloromethane followed by ethanol (Carl Roth GmbH & Co, Karlsruhe, Germany) and *aqua demineralisata,* by soxhlet. Between each extraction step the biomass was dried at room temperature. The extracts were evaporated *in vacuo* and stored at - 20 °C. The CH_2Cl_2 extract yield was 198 mg of crude material.

Biological assay

The cytotoxicity of the CH_2Cl_2 extract was determined in the neutral red assay according to Al-Fatimi *et al*. with etoposid as positive control[7].

Isolation and identification of 1,3-O- Z-11-eicosenoyl 2-O- Z -9, Z -12-nonadecadienoyl glycerol (1)

Repeated preparative TLC, using solvent system (*i*) (twice) for the CH_2Cl_2 extract (117 mg) of fungal strain MF014 afforded a crude sample of compound 1. For purification, the process was repeated using solvent (*ii*) (thrice), then by using solvent (*iii*) (twice), thus yielding a pure sample (11.8) mg of 1.

1,3-O-bis- Z -11-eicosenoyl 2-O- Z -9, Z -12-nonadecadienoyl glycerol (1)

Viscous oil, R_f- values (x 100): 65 (*i*); 82 (*ii*); 89 (*iii*). HRESIMS (negative mode) *m/z* = 951.6977 $[M - H]^-$ ($C_{62}H_{111}O_6$). Alkaline hydrolysis: 1.18 mg of 1 were treated with sodium methylate (0.5 ml, 1 % in methanol (0.5 ml, 1 % w/v) for 60 min at 55 °C and then with one N methanolic HCl (0.5 ml) for 3 min. The solvent was evaporated *in vacuo*, and the reaction mixture was subsequently extracted with 50 ml of *n*-pentane. Aliquots for GC-MS analysis were directly injected into the instrument (Fig. 1); GC data: methyl nonadecadienoate: R_t: 28.874 min; methyl eicosinoate: R_t: 26.666 min. EI-MS data: methyl nonadecadienoate : *m/z*: 308 $[M]^+$, 277 $[M-31]^+$, 165, 151, 95, 81, 67, 55; methyl eicosinoate: *m/z*: 324 $[M]^+$, 292 $[M-32]^+$, 137, 109, 95, 81, 74, 55. 1H NMR of 1, (δppm): Glycerol moiety: 5.238 (triplet of triplet, *sn*-2-glycerol proton); 4.272 [(d,d, J=11.5 Hz and J=4.1 Hz) and 4.133 (d,d, J=12 Hz and J=5 Hz), *sn*-1 and *sn*-3 glycerol methylenic protons] ; Acyl moieties: 5.330 (m, olefinic protons, H-11, H-12, H-9', H-10', H-12', H-13'); 2.744 (AB system in the form of a triplet, *bis*-all CH_2 in nonadecadienoyl moiety, H2-11'); 2.319 (m, CH2 groups α to the 3 carbonyl carbons, H2-2 and H2-2'); 2.040 (m, allylic CH_2 groups number 8 and 14 in nonadecadienoyl moiety); 2.029 (m, allylic CH_2 groups, H2-8 and H2-11 in eicosenoyl moieties); 1.595 (m, CH_2 groups, two H2-3 and one H2- 3', β to the three carbonyl carbons); 1.299 and 1.279 (2 fused m, CH_2 groups, H2-4, H2-5, H2-6, H2-7, H2-8, H2- 9, H2-14, H2-15, H2-16, H2-16, H2-17 in the eicosenoyl moieties together with H2-5', H2-6', H2-7', H2-15' and H2-16' in the nonadecadienoyl moiety); 0.911 (t, terminal methyl groups, H3-20 and H3-19'). ^{13}C NMR of 1, (δ ppm): glycerol moiety: 62.149 (*sn*-C-1 & *sn*-C- 3), 68.892 (*sn*-C-2); Eicosenoyl moieties: 173.244 (Carbonyl ester C-1); 34.030 (C-2); 24.865 (C-3); 27.182(C-10); 129.824 (C-11); 129.938 (C-12); 27.207 (C-13); 31.888 (C-18); 22.659 (C-19); 14.049 (terminal methyl, C-20); Nonadecadienoyl moiety: 172.830 (Carbonyl ester C-1'); 34.009 (C-2'), 24.865 (C-3'); 27.156 (C-8'); 129.991 (C-9'); 128.074 (C-10'); 25.618 (C- 11'); 127.880 (C-12'); 130.177 (C-13'); 27.156 (C-14'); 31.510 (C-17'); 22.548 (C-18'); 14.020 (terminal methyl, C-19'); Unassigned CH_2 groups in the acyl moieties: 29.751, 29.685, 29.642, 29.605, 29.510, 29.459, 29.342, 29.326, 29.304, 29.253, 29.175, 29.154, 29.107, 29.074, 29.036, 28.996 (C-4 to C-9 and C-14 to C-17 in the eicosenoyl moieties and C-4', to C-7', C-15' and C-16' in the nonadecadienoyl moiety).

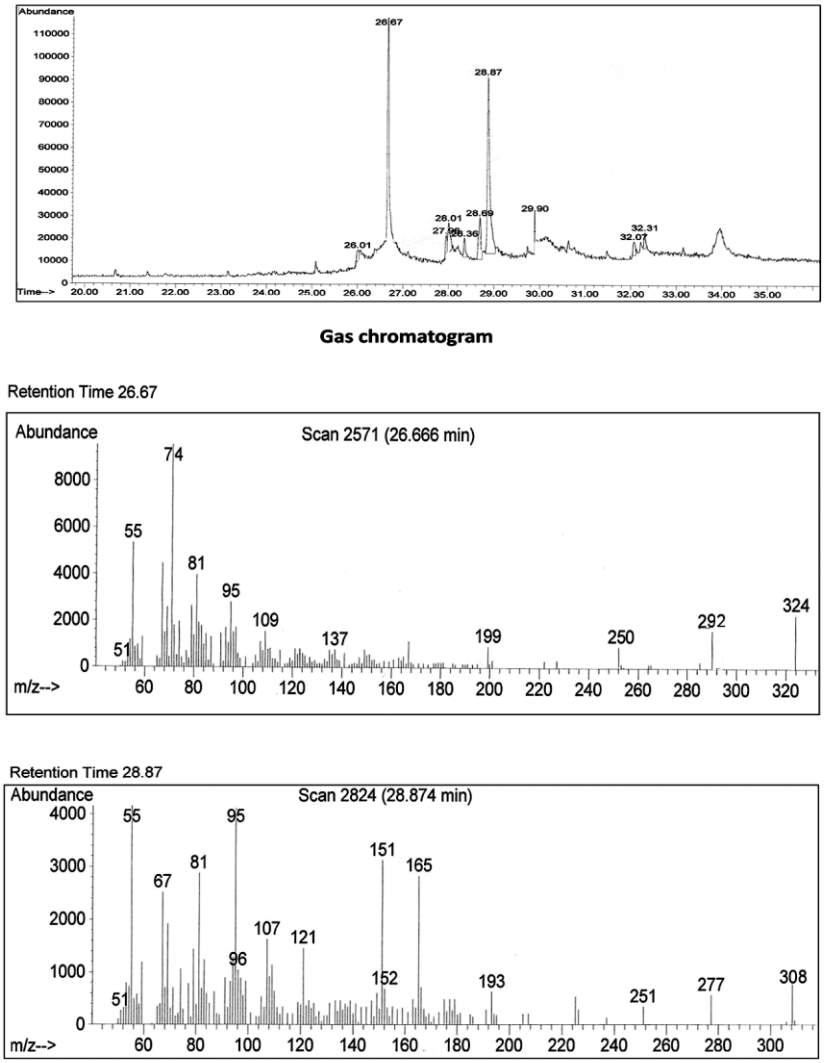
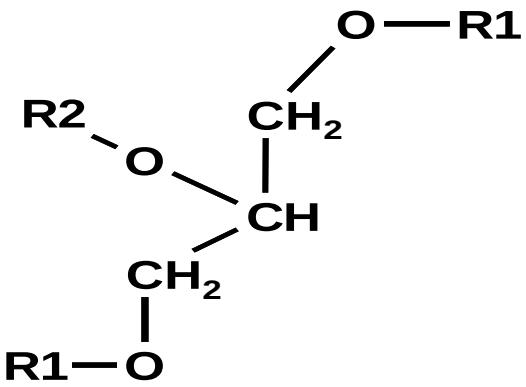
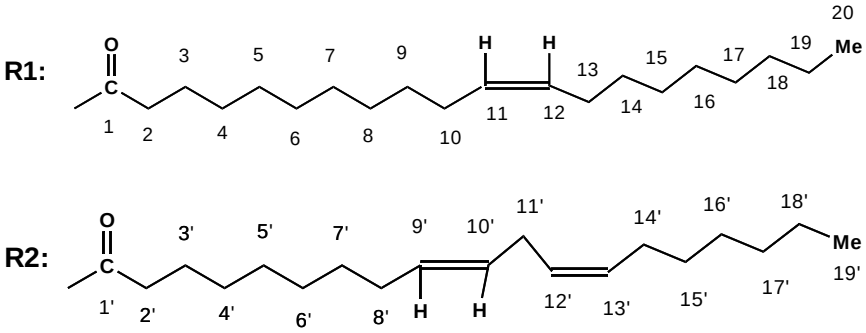


Fig. 1. GC and mass spectra of the hydrolysis products of compound 1.



Compound 1



Results and Discussion

Biological assay

In the course of our biological screening assays we found that all the extracts of MF 0014 did not show antibacterial activity. The following strains were used as test organisms: *Staphylococcus aureus* (ATCC 6538), *Bacillus subtilis* (ATCC 6051), *Escherichia coli* (ATCC 11229), *Pseudomonas aeruginosa* (ATCC 22853) and as multiresistant strains: *Staphylococcus aureus* (Northern Germany Epidemic Strain, NGES), *Staphylococcus epidermidis* (LHI, No. 847) and *Staphylococcus haemolyticus* (LHI, No. 535). Ampicillin and gentamicin were used as positive control. Instead, the CH_2Cl_2 extract displayed cytotoxic activity against human amniotic epithelial cells (FL cells) in the neutral red assay. The recorded IC_{50} (0.01) of the extract was found equal to that of the etoposid control. Low yield of the isolated pure compound 1 prevented the biological evaluation of which. This distinguishable effect of the extract might be attributed to the existing combination of constituents or it could be due to specific compound (s) present in it.

Isolation and structure elucidation

Compound (1) was obtained pure by repeated preparative TLC on silica gel of the CH_2Cl_2 extract. Conventional and spectral analysis mainly by NMR spectroscopy and by mass spectrometry indicated that the compound is a novel natural product. Compound (1), is a viscous oil which appeared as a discrete dull brown spot on silica gel TLC under UV light (λ = 254 nm). The spot was further visualized by spraying with 10 % (*w*/*v*) cupric sulphate in 8 % (*w*/*v*) phosphoric acid, followed by heating at 180 °C for 60 min[8], possessed R_f- values = 0.65 in solvent system *n*-hexane : ether : AcOH : MeOH (90:20:2:3,*v*/*v*) [9]. Complete alkaline hydrolysis of compound 1 was achieved in sodium methylate solution (1 %, *w*/*v*, 60 min, 55 °C), followed by treatment of the reaction mixture with anhydrous 1 N HCl in methanol (3 min., 55 °C) [10]. The fatty acid methyl esters formed were extracted with *n*-pentane and directly injected into the GC/MS for comparision, (Fig. 1). Furthermore, TLC analysis of authentic samples was performed in system XYZ, thus confirming the presence of only methyl *Z*-11-eicosenoate (*Rt*: 28.87 min; mass ions at *m/z*: 324 $[M]^+$, 292 $[M-32]^+$), and methyl *Z*-9,*Z*-12-nonadecadienoate (*Rt*: 26.67min; mass ions at *m/z*: 308 $[M]^+$, 177 $[M-32]^+$). Comparative TLC of the residue, in MeOH left after *n*-pentane extraction indicated the absence of 1 and the presence of glycerol instead [11]. Compound 1 exhibited the molecular formula $C_{62}H_{111}O_6$ as proven by negative HRESIMS $[M-H]^-$: 951.6977. The spectrum revealed in addition, ions at *m/z* 57.5105, 641.5101 and 348.4502, corresponding to (M –eicosenoyl, – H)$^-$; (M – eicosenoate – H)$^-$ and (M – eicosenoyl – eicosenoate – H)$^-$, respectively. The molecular weight determined and the fragmentation pattern of were in consistent with a di-eicosenoyl, monononadecadienoyl glycerol structure. Interpretation of ^{1}H, ^{13}C, DEPT, COSY, HSQC and HMBC NMR spectroscopic data (see Experimental) of 1 indicated the presence of a methylene proton resonance at δ 2.744 ppm (AB system in the form of a triplet, H2-11') in the ^{1}H spectrum which was found correlated in the HSQC spectrum to the methylenic (DEPT) carbon located at δ 25.618 ppm. Both values are typical for a *bis*-all CH_2, -11') group in between two olefinic bonds [12-13], a conjugated system that ensures the presence of the previously identified conjugated nonadecadienoic acid in the molecule of 1. The *Z*-configuration of the protons of both olefinic bonds in this system was concluded from the δ values of the corresponding carbons at 129.98 (C-9'), 128.075 (C-10'), 127.880 (C-12') and 130.177 (C-13') [14]. It should be noted, however, that although the carbon signals intensities are not an accurate measure for the number of carbon nuclei producing these signals, yet the recorded intensities of the carbon signals of the nonadecadienoyl moiety in the ^{13}C NMR spectrum of 1 were found to be of approximately of half value when compared with those of the carbon signals of the eicosenoyl moieties.

This observation further supported the assumption that compound 1 is a symmetrical *sn*-1,3-di-eicosenoyl *sn*-2-nonadecadienoyl glycerol. Direct correlations observable in the HSQC and HMBC spectra proved this hypothesis, whereby a $^3(J)$ correlation recognised in the HMBC spectrum was found correlating the *sn*-2-glycerol proton signal at δ 5.238 (triplet of triplet) to the less intense carbonyl carbon signal located at δ 172.832, C-1'. Another cross peak was found correlating the remaining intense carbonyl carbon signal at δ 173.244 (C-1) with the equivalent *sn*-1 and *sn*-3 glycerol methylenic protons at δ 4.272 (d,d, J=11.5

Hz and J=4.1 Hz) and at δ 4.133 (d,d, J=12 Hz and J=5 Hz). Furthermore, in the ^{13}C NMR spectrum, theassigned carbon resonances belonging to the two equivalent *Z*-11-eicosenoyl moieties possessed closely similar chemical shifts to those reported for *sn*-1,*sn*-3-bis-eicosenoyl glycerol[15]. It should be noted, however, that no unambiguous assignments of the carbon resonances of the CH_2 groups number, 4 up to 9 and 14 to 17 in the eicosenoyl moieties and number 4', 5', 6', 7', 15' and 16' in the nonadecadienoyl moiety were possible on the basis of NMR. The data given above finally confirmed the structure of compound 1 to be 1,3-*O*-bis-*Z*-11-eicosenoyl 2-*O*-*Z*-9,*Z*-12- nonadecadienoyl glycerol, a new triacyl glycerol which has not been reported before as a natural product.

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ثلاثى اسيل جليسيرول جديد من خلاصة مضادة للسرطان مشتقة من فطر بحرى من البحر الاحمر

محمود نوار ، سحر حسين ، أماني هاشم ، هبه بركات، جودرون ميرنيتس*، بيأته سيبروس*، ميتشانيل لينشيد و أولريكا ليندكويست*****
قسم كيمياء العقاقير وتصنيف النباتات - المركز القومى للبحوث - مصر ،*مركز الكائنات البحرية - جرايفزفالد ، *جامعة هومبلدت- برلين و*** معهد الصيدلة- جامعة جريفزفالد- ألمانيا .

أدى فحص سلالة ديوتيروميسيت MF 014 الى فصل ١،٣-أ-مماثل-Z-١١-آيكوزينويل ٢-أ-Z-٩،Z -١٢-نونا ديكاداي-اينويل جليسيرول. و تمت البرهنة على صحة التركيب بدراسة اطياف الرنين النووى الغناطيسى ذات البعد الواحد وذات البعدين واطياف الكتلة. بالاضافة الى ذلك فقد وجد ان الخلاصة موضوع الدراسة قد اظهرت قدرتها كمضاد للسرطان .