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Isolation, purification and structure elucidation of three new bioactive secondary metabolites from *Streptomyces lividans* AM

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Abstract: Microorganisms are a huge mine of bioactive metabolites, and actinomycetes are one of the very active groups in this area. In this article, we are concerned about the full taxonomical characterization of *Streptomyces lividans* AM, isolated from Egyptian soil. This isolate produced three new bioactive metabolites, namely: 1-Nona-decanoyl,4-oleyl disuccinate (**1**), filoboletic acid; (9*Z*,11*E*)-8,13-dihydroxy octadeca-9,11-dienoic acid (**2**), and sitosteryl-3β-D-glucoside (**3**). Extensive 1D and 2D NMR and HR-mass spectrometry were used to elucidate the structures of the three compounds. Moreover, ten known compounds were also identified. The antimicrobial activity of the producing organism and newly reported compounds (**1–3**) was investigated against a selected group of pathogenic microorganisms. A full taxonomical characterization of the strain was described as well.

Introduction

Microbial worlds are unlimited resources of bioactive secondary metabolites. Secondary metabolites are crucial players in microbial development and interactions with other organisms. This mine of highly effective metabolites is still deep and needs more and more research. One of these good resources is *Streptomyces* sp., which is confirmed as one of the most productive sources for innovation of lead drugs (Blunt *et al.*, 2003; Laatsch, 2010). Most *Streptomyces* species have the potential to synthesize several specific biologically active chemicals (Goodfellow and Haynes, 1984), which present a diversity of biological properties acting as antibiotics (Maiti *et al.*, 2020; Ibrahim *et al.*, 2019; Butler, 2004), antimycotics (Singh and Rai, 2013), antivirals (Raveh *et al.*, 2013; Wei *et al.*, 2014), anticancer (Davies-Bolorunduro *et al.*, 2019), herbicides, pesticides, anti-parasitic, enzyme inhibitors (Bo *et al.*, 2019; Olano *et al.*, 2009), and pharmacologically active agents (Ahmad *et al.*, 2017). However, many habitats of these Gram-positive bacteria are still unexplored as potential sources of new bioactive natural products (Baltz, 2007; Butler *et al.*, 2013). In this article, three new compounds were isolated from full-identified *Streptomyces lividans* AM additional to ten known compounds. This article represents another good step to discover some elements of microbial, secondary compounds.

Materials and Methods

General experimental procedure

NMR spectra: 1D (^{1}H NMR, ^{13}C NMR, DEPT) and 2D (COSY, HMQC, and HMBC) NMR spectra were quantified on Bruker Avance DRX 500 and DRX 600 MHz spectrometers employing standard pulse sequences with reference to residual solvent signals. HR-EI-MS had been calculated using the GCT Premier spectrometer. The ultraviolet-visible (UV–Vis) spectrum was measured on Spectro UV–Vis Double Beam PC8 scanning auto Cell UVD-3200, LABOMED, INC. Column chromatography has been performed on the silica gel 60 (0.040–0.063 mm,

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Merck) and Sephadex LH-20 as the stationary phases. Preparative TLC (0.5 mm thick) and analytical TLC with pre-coated Merck silica gel 60 $PF_{254+366}$. R_f values and analysis of chromatograms were performed under UV light (254 and 366 nm) and further by heating after spraying with anisaldehyde sulfuric acid reagent.

Isolation of *Streptomyces sp*. Isolate AM

The *Streptomyces isolate* AM has been isolated from a soil sample obtained from the governorate of Dakahlia, Egypt using starch nitrate medium (g/L) (Starch, 20; KNO_3, 2; K_2HPO_4, 1; $MgSO_4.7H_2O$, 0.5; NaCl, 0.5; $FeSO_4.7H_2O$, 0.01; $CaCO_3$, 3; agar, 20 in 1 L of distilled water (Waksman, 1959).

Characterization of *Streptomyces sp*. Isolate AM

Morphology and cultural properties

Substrate mycelium color, aerial mycelium colors, and diffusible pigment production were observed on ISP media as previously described (Shirling and Gottlieb, 1972). Spore chain morphology and the spore surface of the strain AM were examined after 14 days using Scanning Electron Microscope Jeol JSM-6360 LA.

Physiological properties

The ability of the isolate AM to utilize different carbon sources was determined according to Shirling and Gottlieb (1966). Lecithinase activity was determined on egg–yolk plate medium (Nitsch and Kutzner, 1969). Hydrolysis of starch (Goodfellow and Orchard, 1974) and Liquefaction of gelatin (Waksman, 1961) were also investigated. The studied isolate AM was inoculated into skimmed milk medium (Iwasaki *et al*., 1981) and incubated at 30°C, then the degree of coagulation or liquefaction was recorded after 14 days of incubation. Melanoid production was determined after 4 days of incubation by using different media such as peptone-yeast extract-iron agar, tyrosine agar, and tryptone-yeast broth medium (Shirling and Gottlieb, 1966). The tested isolate was grown on modified Bennett's agar medium that supplemented separately with adenine (1%), hypothanthine (0.4%), allantoin (1%), and uric acid (1%). The degradation was determined after 7, 14, and 21 days of incubation (Jones, 1949). The clear zone formed around the tested isolate growth was recorded as a positive result. The ability of the isolate AM to reduce nitrates to nitrites was checked after 7 and 14 days as reported by Williams *et al.* (1983). Sodium chloride tolerance of the selected isolate AM was investigated according to Tresner *et al.* (1968). The capacity of the isolate AM to produce hydrogen sulfide was detected by inserting lead acetate paper strips into the neck of the culture tube containing peptone iron agar media. The formation of hydrogen sulfide was indicated by the blackening of lead acetate strips after 7 days of incubation (Küster and Williams, 1964). The ability of the tested isolate to decompose carboxymethyl cellulose (CMC) was quantified according to Prasad *et al.* (2013) using a sterilized Hutchinson liquid medium (Crawford and McCoy, 1972) supplemented with CMC 1% (w/v).

Sequence alignment and phylogenetic analysis

The partial 16S rRNA gene sequence of strain AM was aligned with the corresponding 16S rRNA sequences of the genus *Streptomyces* retrieved from the GenBank databases by using BLAST (www.ncbi.nlm.nih.gov/blast) (Altschul *et al*., 1997) and the software package MEGA 5 (Tamura *et al*., 2007) was used for multiple alignment and phylogenetic analysis. The phylogenetic tree was created via the Neighbor-joining method (Saitou and Nei, 1987).

Fermentation, working up and isolation

The spore suspension of *S. lividans* AM has been inoculated into 100 mL of ISP2 medium and cultivated at 28°C for 3 days. Five mL of seed culture was inoculated into 1L Erlenmeyer bearing modified rice-solid medium: Commercial rice 100 mg; 150 distilled water containing yeast extract 0.4% and malt extract 1% each flask under aseptic conditions. The culture was incubated for 14 days at 37°C. The obtained culture was collected and soaked in methanol, followed by filtration. After filtration, the water/methanol fraction was concentrated in *vacuo*. The remaining water residue was mixed with ethyl acetate. The ethyl acetate phase containing the microbial organic extract was finally separated and concentrated in *vacuo* till dryness delivering the desired reddish-brown crude extract.

The crude extract (3.2 g) was fractionated by silica gel column chromatography (column: 60 cm × 3 cm) eluting with a cyclohexane-DCM-MeOH gradient to afford four fractions on the basis of the analysis of TLC. Fraction I (0.91 g) was purified by silica gel CC, eluting with a cyclohexane-DCM gradient to give colorless oil of glycerol linoleate (25 mg), colorless semi-solid of linoleic acid (500 mg), and a colorless oil of 1-nona-decanoyl,4-oleyl disuccinate (**1**, 22 mg). Fraction II (0.51 g) was divided into sub-fractions IIa (0.22 g) and IIb (0.18 g) by silica gel CC, eluting with CH_2Cl_2-CH_3OH. Purification of IIa by Sephadex LH-20 CC (CH_2Cl_2/40% MeOH) afforded filoboletic acid (**2**, 3 mg) as a colorless oil, and colorless solids from ferulic acid (**4**, 11 mg), indole-3-acetic acid methyl ester (2 mg), 4-hydroxy-phenyl acetic acid (6 mg). Purification of sub-fraction IIb by Sephadex LH-20 CC (DCM/MeOH [60:40]) resulted in 4-hydroxy-phenyl acetic acid (6 mg) as colorless solid. Fraction FIII was subjected to purification on silica gel eluted with DCM-MeOH followed by Sephadex LH-20 to afford two colorless solids of 3-(hydroxy-acetyl)-indole (3 mg), indol-3-carboxylic (2 mg). An application of the last fraction IV to Sephadex LH-20 (MeOH) led to colorless solids of *p*-hydroxybenzoic acid (3 mg), uracil (12 mg), and sitosteryl-3β-D-glucoside (**3**, 22 mg).

1-nona-decanoyl,4-oleyl disuccinate (1) $C_{41}H_{74}O_6$ (662): Colorless oil, showing similar chromatographic properties to linoleic acid; R_f = 0.55 (DCM/MeOH [95:5]). For ^1H NMR (300 MHz, $CDCl_3$) and ^{13}C NMR (125 MHz, $CDCl_3$) data, see Tab. 3. (+) ESI MS *m/z* (%): 685 ($[M+Na]^+$). (+) HR-ESI MS *m/z*: 685.53875 (calcd. 685.53774 for $C_{41}H_{74}O_6Na$).

Filoboletic acid; (9 *Z*,11 *E*)-8,13-dihydroxy octadeca-9,11-dienoic acid (2) $C_{18}H_{32}O_4$ (312): Colorless semi solid, UV faint absorbing, stained as dark violet and later as grey. R_f = 0.35 (CH_2Cl_2/7% CH_3OH). The ^1H ($CDCl_3$, 300 MHz):

δ = 6.18 (m, 1H), 6.05 (m, 1H), 5.61 (m, 2H), 4.10 (m, 2H), 2.39 (brm, 2H), 2.08 (br. m, 2H), 1.65 (brm, 2H), 1.30 (br. s, 12H), 0.91 (m, 3H). **(+) ESI MS** *m/z* (%): 335 [M+Na]). **(-) ESI MS** *m/z* (%): 311 [M-H]$^-$). (+) HR-ESI MS *m/z*: 335.21942 (calcd. 335.21928 for $C_{18}H_{32}O_4Na$). (-) HR-ESI MS *m/z*: 311.22178 (calc. 311.22168 for $C_{18}H_{31}O_4$).

Sitosteryl-3 *β*-D-glucoside (3): $C_{35}H_{60}O_6$ (576): Colorless solid, UV non absorbing or fluorescence, detected by anisaldehyde/sulfuric acid as dark brown, turned later to black. R_f = 0.25 (CH_2Cl_2/12% CH_3OH). ^{1}HNMR (DMSO-d_6, 300 MHz) and ^{13}C NMR (DMSO-d_6, 125 MHz) data are listed in Tab. 4. (+) ESI MS: *m/z* (%): 599 [M+Na]$^+$; (+) HR ESI MS: *m/z* (%): 599.4267 (calcd. 599.4282 for $C_{35}H_{60}O_6Na$).

Ferulic acid; 3-(4-Hydroxy-3-methoxy-phenyl)-acrylic acid (4) $C_{10}H_{10}O_4$ (194): Yellow solid, showing UV absorbance, and stained pink-violet on spraying with anisaldehyde/sulfuric, R_f = 0.26 (DCM/MeOH, 90:10). ^{1}HNMR (300 MHz, $CDCl_3$): δ = 7.19 (d, 2.0, 1H, H-5), 7.08 (dd, 8.2, 2.0, 1H, H-9), 6.83 (d, 8.1, 1H, H-8), 6.61 (d, 15.9 Hz, 1H, H-3), 6.33 (d, 15.9 Hz, 1H, H-2), 3.90 (s, 3H, OCH_3-7). ^{13}C NMR (125 MHz, $CDCl_3$): δ = 169.5 (C_q-1), 149.9 (C_q-7), 147.9 (C_q-6), 145.5 (CH-3), 126.3 (C_q-4), 122.5 (CH-9), 115.0 (CH-8), 114.4 (CH-2), 110.2 (CH-5), 56.2 (7-OCH_3). (+) ESI MS *m/z* (%): 195 ([M+H]$^+$), 217 ([M +Na]$^+$); (-) ESI MS *m/z* (%): 195 ([M-H]$^-$).

Antimicrobial assay using agar diffusion test

Antimicrobial activity testing of the crude extract of *Streptomyces lividans* AM and the desired new compounds (**1-3**) were carried against a collection of microorganisms using the agar diffusion method. Paper-disk diffusion assay (Bayer *et al.*, 1966) with some modifications has been followed to measure the antimicrobial activity. Twenty milliliters of medium seeded with test organism were poured into 9 cm sterile Petri dishes. After solidification, the paper disks were positioned on inoculated agar plates and allowed to distribute the loaded substances at 4°C for 2 h. The plates were incubated for 24 h at 35°C. Both bacteria and yeasts were grown on nutrient agar medium (g/l): Beef extract, 3; peptone, 10; and agar, 20. The pH was adjusted to 7.2. Fungal strain was grown on potato dextrose agar medium (g/l): Potato extract, 4; Dextrose, 20; Agar No. 1, 15 (pH 6). After incubation, the zones of inhibition were measured against the test microorganisms comprising: Gram-positive bacteria; (*Bacillus cereus* ATCC6633 and *Staphylococcus aureus* ATCC6538-P), Gram-negative bacteria (*Pseudomonas areuginosa* ATCC 27853), and yeast (*Candida albicans* ATCC 10231).

Results and Discussion

Characterization of the Streptomyces isolate

Morphology and culture properties of Streptomyces lividans AM

Morphological characteristics of the isolate AM were illustrated in Tab. 1. The isolate growth on starch nitrate media, ISP 2 and ISP 4 media was excellent, good on ISP 5 and ISP 7, and weak at ISP 3 and ISP 6. No diffusible pigment was observed in all tested media. Scanning electron microscope images of the isolate AM grown on starch nitrate agar medium showed that the spore chain is spiral where the spore surface ornamentation is smooth (Fig. 1).

Physiological and chemotaxonomic characteristics of Streptomyces lividans AM

The physiological and chemotypic characteristics of the isolate AM were illustrated in Tab. 2, where no melanoid pigment was formed in peptone-yeast extract iron agar or tyrosine agar. Isolate AM utilizes D-Glucose, L-arabinose, D-xylose, i-inositol, D-mannitol, D-fructose, rhamnose, sucrose, and raffinose for growth, but growth on sucrose or raffinose may be less than on the other carbon sources. It degrades adenine, uric acid, allantoin, hypoxanthine, and cellulose. Coagulation and peptonization of milk were positive, whereas lecithinase activity was negative (Fig. 2). Nitrate hydrolysis and H_2S production were positive.

Molecular identification using 16S rRNA

The obtained 479 base pair sequence was analyzed using nucleotides BLAST, where it was compared with other rRNA genes that have been sequenced so far. A phylogenetic tree (Fig. 3) based on 16S rRNA gene sequences of members of the genus *Streptomyces* was constructed according to the neighbor-joining method using MEGA 5. The sequence analysis showed a close relationship to *Streptomyces lividans* strain TNAU1 (HQ897160.1) with a maximum identity of 97%. Moreover, the nucleotide sequence (479 base pairs) was deposited in the GenBank sequence database as *Streptomyces lividans* strain AM, and the accession number MF623053 was obtained.

Fermentation and structure elucidation

The *Streptomyces lividans* AM was fermented on rice-solid medium and the strain organic extract displayed potent activity versus *Pseudomonas aeruginosa* (30 mm), *Candida albicans* (25 mm), *Bacillus cereus* (22 mm), and moderate activity against *Staphylococcus aureus* (12 mm). In the chemical screening through TLC analysis, several bands of wide polarity were detected: (a) a group of non-UV-absorbing compounds, detected as intensive violet-blue bands by heating after spraying with anisaldehyde/sulfuric acid reagent; (b) UV absorbing bands, some of them showed pink, brown, violet, and/or orange colorization with anisaldehyde/sulfuric acid; (c) while the remaining UV-absorbing zones showed no color staining with the same reagent. Separation using a different series of chromatographic skills, namely silica gel and Sephadex LH-20 CC, afforded 1-nona-decanoyl, 4-oleyl disuccinate (**1**), filoboletic acid; (9*Z*,11*E*)-8,13-dihydroxy octadeca-9,11-dienoic acid (**2**), and sitosteryl-3*β*-D-glucoside (**3**). Further ten known compounds were afforded: ferulic acid (**4**) (Al-Refa, 2008; Hamed *et al.*, 2017), glycerol monolinoleate (Naureen *et al.*, 2015), linoleic acid (Mahmoud, 2005), indol-3-acetic acid methyl ester, 4-hydroxy-phenyl acetic acid (Mahmoud, 2005), 2-hydroxy-phenyl acetic acid (Mahmoud, 2005), 3-(hydroxy-acetyl)-indole (Mahmoud, 2005), indol-3-carboxylic (Mahmoud, 2005; Shaaban *et al.*, 2002), p-hydroxybenzoic acid (Mahmoud, 2005), and uracil (Mahmoud, 2005).

TABLE 1

Phenotypic characterization of the isolate AM

Medium	Color of			Growth
	Aerial mycelium	Substrate mycelium	Diffusible mycelium	
1- Starch nitrate agar	Gray	Off-white	No pigment	Excellent
2- Yeast extract-malt extract agar (ISP 2)	Gray	Colorless	No pigment	Excellent
3- Oat meal agar (ISP 3)	Gray	Cream	No pigment	Weak
4- Inorganic salt -starch agar (ISP 4)	White	Light gray	No pigment	Excellent
5- Glycerol asparagine agar (ISP 5)	White	Light brown	No pigment	Good
6- Peptone yeast extract iron agar (ISP 6)	Weak growth	Weak growth	No pigment	Weak
7- Tyrosine agar (ISP 7)	Gray	Dark Brown	No pigment	Good

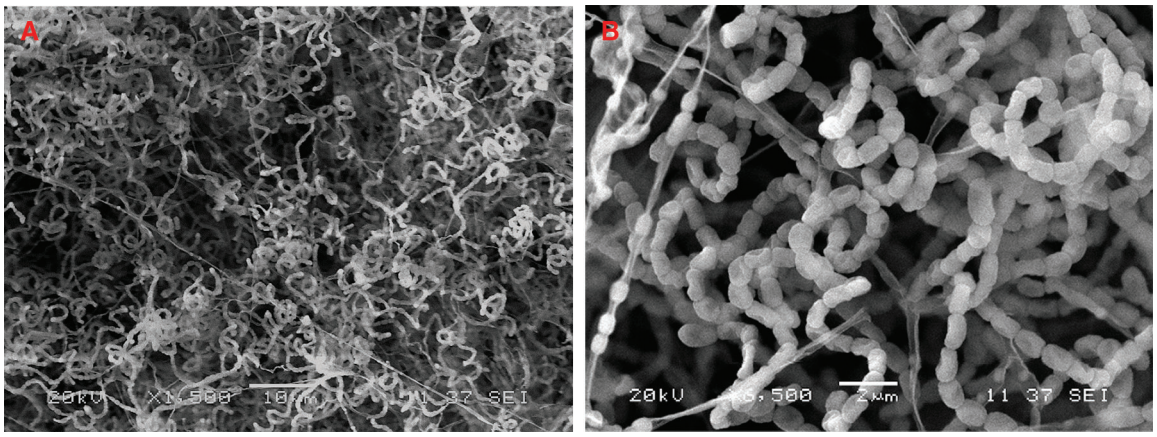

FIGURE 1. Scanning electron micrograph of the isolate AM grown on starch nitrate agar; (A) spore chain and (B) spore surface ornamentation.

TABLE 2

Major phenotypic characteristics of the isolate AM

Characteristics	Results
Gram stain	+
Growth temperature range	20°C–40°C
Growth pH range	5–9
NaCl tolerance	Up to 3%
Degradation of	
Adenine	+
Uric acid	+
Hypoxanthine	+
Allantoin	+
Starch hydrolysis	+
Gelatin liquefaction	–
Coagulation of milk	+
Peptonization of milk	+
Cellulose decomposition	+
Nitrate reduction	+
Lecithinase activity	–
H_2S production	+
Melanoid pigment production	
Peptone yeast iron agar	–
Tyrosine agar	–
Tryptone yeast broth	–
Utilization of different carbon source	
D- Glucose	+
Arabinose	+
D- Xylose	+
D- Fructose	+
Galactose	+
Mannose	+
Ribose	+
Rhamnose	+
lactose	+
Sucrose	±
Raffinose	±

TABLE 3

^{13}C (125 MHz) and ^{1}H NMR (500 MHz) data of 1-nona-decanoyl, 4-oleyl disuccinate (1) in $CDCl_3$

Nr	δ_C	δ_H (*J* * [Hz$^{\#}$])	Nr	δ_C	δ_H (*J* *[Hz$^{\#}$])
1	173.1		3'	27.5	2.79 (t, 6.7)
2	47.2	3.62 (t, 6.5)	4'	173.2	
3	25.7	1.34 (m)	1''	173.6	
4	23.3	1.39–1.34 (m**)	2''	38.8	3.19 (t, 6.5)
5	28.8	1.39–1.34 (m)	3''	28.4	1.53 (m)
6	29.3	1.39–1.34 (m)	4''	23.2	1.39 (m)
7	29.4	1.39–1.34 (m)	5''	29.2	1.39–1.34 (m)
8	29.3	1.39–1.34 (m)	6''	29.1	1.39–1.34 (m)
9	29.23	1.39–1.34 (m)	7''	29.3	1.39–1.34 (m)
10	29.24	1.39–1.34 (m)	8''	26.7	2.08 (m)
11	29.22	1.39–1.34 (m)	9''	127.8	5.71 (m)
12	29.25	1.39–1.34 (m)	10''	129.9	5.71 (m)
13	29.24	1.39–1.34 (m)	11''	26.7	2.08 (m)
14	29.23	1.39–1.34 (m)	12''	29.2	1.39–1.34 (m)
15	29.22	1.39–1.34 (m)	13''	29.1	1.39–1.34 (m)
16	29.20	1.39–1.34 (m)	14''	29.1	1.39–1.34 (m)
17	31.4	1.39–1.34 (m)	15''	29.3	1.39–1.34 (m)
18	22.1	1.39–1.34 (m)	16''	31.4	1.39–1.34 (m)
19	13.05	0.90 (t, 6.8)	17''	22.1	1.34 (m)
1'	173.6		18''	13.05	0.90 (t, 6.8)
2'	30.2	2.48 (t, 6.7)			

Note: *J = coupling constant, $^{\#}$Hz = Hertz, **m = multiplet.

TABLE 4

^{13}C (125 MHz) and ^{1}H NMR (300 MHz) data of sitosteryl-3-β-D-glucoside (3) in DMSO-d_6

Nr	δ_C	δ_H (*J* [Hz])	Nr	δ_C	δ_H (*J* [Hz])
1	36.8	1.79 (m), 0.99 (m)	19	19.0	0.96 (s)
2	29.2	1.83 (m), 1.48 (m)	20	35.4	1.34 (m)
3	76.9	3.46 (m)	21	18.5	0.90 (d, 5.6 Hz)
4	42.8	2.36 (ddd, 13.4, 4.7, 2.1)	22	33.3	1.32 (m), 1.01 (m)
5	140.2		23	25.7	1.16 (m)
6	120.9	5.32 (bd)	24	45.0	0.92 (m)
7	38.3	2.34 (ddd, 13.4, 4.7, 2.1), 1.91 (td, 12.3, 11.5, 2.7)	25	28.7	1.64 (m)
8	31.4	1.41 (m)	26	19.6	0.82 (d, 7.2)
9	49.5	0.89	27	18.9	0.80 (d, 8.1)
10	36.1		28	22.6	1.26 (m), 1.19 (m)
11	20.6	1.49 (m), 1.41 (m)	29	11.7	0.82 (d, 8.1)
12	39.0	1.96 (m), 1.15 (m)	1'	100.7	4.22 (d, 7.8)
13	41.1		2'	73.3	2.91 (t, 8.4)
14	55.9	1.39 (m)	3'	76.7	3.13 (m)
15	23.7	1.54 (m)	4'	70.0	3.04 (m)
16	27.6	1.81 (m), 1.24 (m)	5'	76.6	3.07 (m)
17	56.1	0.99 (m)	6'	61.0	3.64 (m), 3.43 (m)
18	11.6	0.65 (s)			

FIGURE 2. Negative lecithinase activity of *Streptomyces lividans* AM.

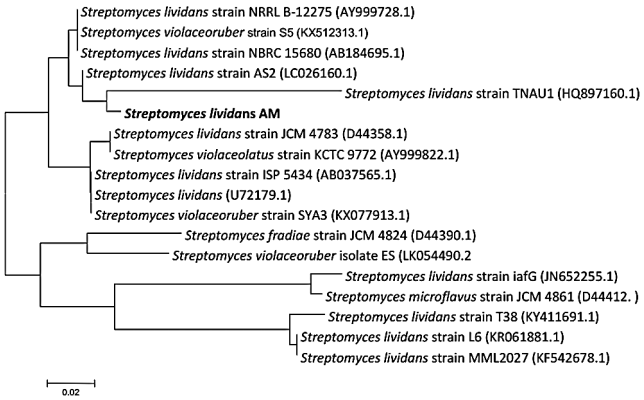

FIGURE 3. Neighbor-joining tree based on 16S rRNA gene sequences, showing the phylogenetic relationship between strain AM and related species of the genus *Streptomyces*. GenBank sequence accession numbers are indicated in parentheses after the strain name.

1-Nona-decanoyl-,4-oleyl disuccinate

Compound **1** was obtained as a colorless oil with middle polarity, exhibiting a violet staining on spraying with anisaldehyde/sulfuric acid. The molecular weight of **1** was established by ESIMS as 662 Daltons with a corresponding molecular formula of $C_{41}H_{74}O_6$, concluding the existence of five double bond equivalents (DBE). The ^{1}H NMR spectrum exhibited a multiplet signal of 2H at δ 5.71 being for olefinic proton signals, in addition to two triplet signals being for two methylene groups at δ 3.62 and 3.19, which could be attached to sp^2 systems, e.g., carbonyl of acid, ester or amide groups. Additional two triplet signals for further methylene groups were observed at δ 2.79 and 2.48 having a similar resonating pattern to those of the pervious triplet methylene groups. However, the lower chemical shifts of the last methylene groups establish their direct adjacent to sp^2 systems, having a direct attachment (i.e., ethanediyl group) owing to the shown H-H COSY correlation between H_2-2'(δ 2.79) and H_2-3'(δ 2.48). Further, multiple signals were observed at δ 1.64 and 1.53 being for additional methylene groups. Finally, the spectrum exhibited a broad signal in the region of 1.39–1.34, being for multi methylene groups, terminated by triplet methyl groups (δ 0.90).

According to the ^{13}C and HMQC spectral data (Tab. 3), compound **3** displayed forty-one carbon signals, classified into: four carbonyls appeared in the region of δ 173.6–173.2, two olefinic CH carbons at δ 129.9 and 127.8. The remaining carbons were visible in the region of δ 47.2–13.0, representing 35 sp^3 carbon signals. Based on the HMBC and H-H COSY correlations (Fig. 4, Tab. 3), the two methylene groups at δ = 2.79 and 2.48 were confirmed to be flanked by two carbonyls of carboxylic acid esters visible at δ 173.2 and 173.6, deducing the existence of a succinate system. The lengths of two fatty acid chains were deduced, representing nonadecanoeate and oleate chains, one of them is bearing an olefinic double bond, attached to both sides of the succinate ester as the low chemical shifts of their esterified carbonyls (~173).

Based on the presented molecular weight, corresponding molecular formula, and intensive study of the NMR data, this directed to structure **1** as 1-nona-decanoyl,4-oleyl disuccinate. It was not possible to fix the position of the olefinic double bond position as the high overlapping of the NMR signals in the structure. However, based on the biosynthetic pathways and the produced closely linoleic acid and glycerol monolinoleate by the same strain, the location of the olefinic bond at 9-position is the most plausible for structure **1**. A search in the different databases (AntiBase, DNP and Scifinder) confirmed the natural novelty of **1**. Only six structurally related compounds to **1** were reported in the literature: 1,4-didodecanoyl succinate;1,4-di-9-octadecanoyl succinate; 1,2,3-tricosanoyl-2-hydroxy-succinate; 1,4-di-hexadecanoly-2,3-bis(3-carbony-1-oxopropoxy) succinate (Scifinder). Esters of fatty acids, namely (*R*) and (*S*)-Glycerol-monolinoleate, were reported to exhibit inhibitory activities with IC_{50} values of 45.0 and 52.0 μM, respectively, against lipoprotein-associated phospholipase A2 [Lp-PLA2]; the latter is a specific marker of vascular inflammation associated with atherosclerosis (Lee *et al.*, 2005).

Filoboletic acid

As further middle polar colorless oil, compound **3** was isolated, exhibiting faint UV absorbance during TLC, which was detected as dark violet on spraying with anisaldehyde/sulfuric acid. The molecular weight of **3** was established to be 312 Dalton based on the exhibited molecular ion peaks shown at *m/z* 335 ($[M+Na]^+$) and 311 ($[M-H]^-$) in the ESI positive and negative modes, respectively. The corresponding molecular formula of **2** was deduced as $C_{18}H_{32}O_4$ according to the HRSI MS as well.

The ^{1}H NMR spectrum exhibited three multiple signals with integration of 4H located in the olefinic region at δ 6.18 (1H), 6.05 (1H), and 5.61 (2H). At δ 4.10, multiple signals with integration of 2H being for two hydroxy methines were visible. Two strong broad multiple signals were observed at δ 2.39, 2.08 corresponding to two 2H methylene protons attached mostly to sp^2 carbons, being of the carboxylic acid and olefinic carbons, respectively. Three additional broad signals were observed at δ 1.65, 1.30, and 0.91, characteristics most likely of a long chain of methylene carbons ended by a terminal methyl group at δ 0.88. Based on these data and according to the search in AntiBase, filoboletic acid; (9*Z*,11*E*)-8,13-dihydroxy octadeca-9,11-dienoic acid was confirmed as the sole matching compound, which we report herein for the first time as a new bacterial

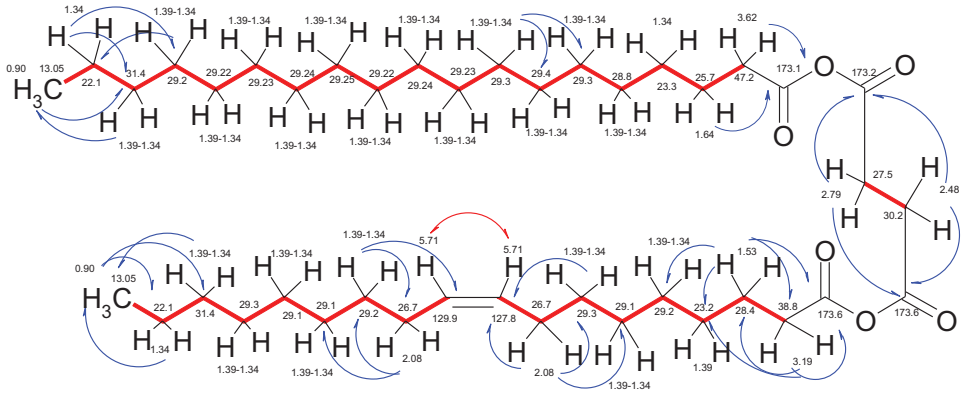

FIGURE 4. H-H COSY and HMBC connectivities of 1-nona-decanoyl-, 4-oleyl disuccinate (1).

metabolite. Filoboletic acid was previously reported as an antiviral compound produced by a fungus belonging to the genus *Filoboletus* (Simon *et al.*, 1994). However, it is reported herein to first time from bacterial strains, and particularly from *Streptomyces* sp.

Sitosteryl-3β-D-glucoside

As polar colorless solid, compound **3** was obtained from fraction IV after purification with silica gel column followed by Sephadex LH-20. It showed no UV activity during TLC. However, it was detected as a dark brown when spraying with anisaldehyde/sulfuric acid. The molecular weight of **3** was established as 576 Dalton according to ESI MS, and the corresponding molecular formula was determined as $C_{35}H_{60}O_6$ according to HRESI MS, containing 4 DBE. We report herein the full assignment of the structure (Fig. 5, Tab. 4) as it has not completely been assigned before failing 2D NMR assigning in several articles (Scifinder).

It is worthy to mention that this is the first time to report *β*-sitosteryl D-glucoside from microorganisms. In contrast *β*-sitosteryl D-glucoside has been reported commonly from many diverse plant sources, e.g., the juice of Florida Valencia oranges (*Citrus sinensis*) (Ma and Schaffer, 1953), walnut *(Juglans regia*) (Jurd, 1956), *Rhus trichocarpa* (Yasue and Kato, 1957), *H. longituba* (Takemoto and Kusano, 1966), *Zizyphus spina-christi* (Aynehchi and Kiumehr, 1974; Aynehchi and Mahoodian, 1973), *Euphorbia tinctoria* (Aynehchi and Kiumehr, 1974). *Oryza sativa* (rice) hulls (Chung *et al.*, 2005), represent one of the important sources of *β*-sitosteryl D-glucoside, and pre-germinated brown rice bran (Usuki *et al.*, 2008). Therefore, it might be that *β*-sitosteryl D-glucoside has been isolated from the rice medium during the extraction process. However, an application of the same cultivating medium for the growth of fungal strains, followed by the same extracting procedure did not afford the same metabolite. This experiment was done several times confirming the capability of *Streptomyces* sp. rather than fungal strains to release the desired metabolite owing to their containing of rather different enzymes and genomes than those present in fungi, which have the efficiency to release/bio-transform such metabolite from the main producing media (rice media). In accordance, *Streptomyces* sp. have either high capability to release rather different enzymes with high efficiency to excrete compound **3**, rather than those present in fungal strains or this compound is originally isolated from the rice medium, although this compound was not reported before from the rice bran before. Biologically, the use of sitosteryl *β*-D-glucoside as an emulsifier or preservative in food and feed was reported to exhibit no risk (Weber, 1988). Biologically, *β*-Sitosterol-3-*O*-*β*-d-glucopyranoside is selectively inhibited the activity of mammalian DNA polymerase λ (pol λ) *in vitro* (Mizushina *et al.*, 2006).

Biologically, the organic extract of the bacterial strain exhibited potent activity against the Gram-negative bacteria *Pseudomonas aeruginosa* ATCC 27853 (30 mm), the yeast *Candida albicans* ATCC 10231 (25 mm), and the Gram-positive bacteria *Bacillus cereus* ATCC6633 (22 mm), while showed moderate activity against *Staphylococcus aureus* ATCC6538-P (12 mm). However, the newly reported

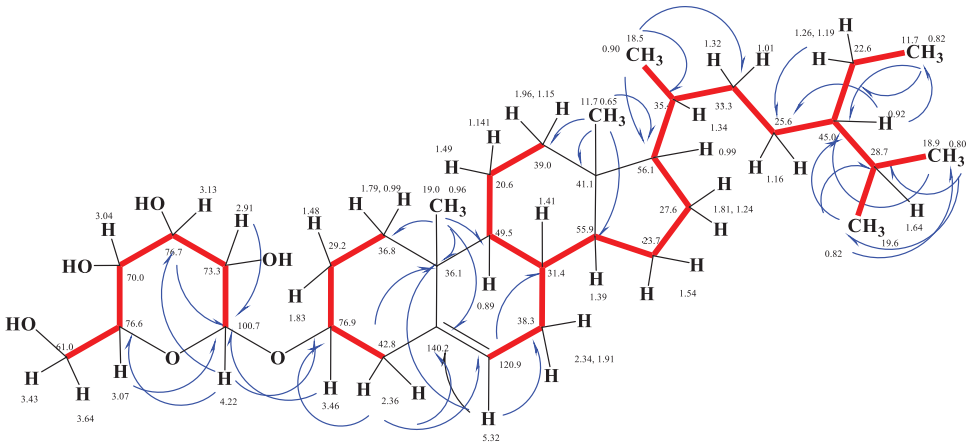

FIGURE 5. H-H COSY (–) and selected HMBC (H→C) correlations of sitosteryl-3*β*-D-glucoside (3).

compounds (**1–3**) displayed no antimicrobial activity against the referred set of pathogenic microorganisms.

Conclusion

Streptomyces lividans AM, which was isolated from an Egyptian soil sample, could produce three newly bioactive metabolites, namely 1-nona-decanoyl,4-oleyl disuccinate, filoboletic acid, (9Z,11E)-8,13-dihydroxy octadeca-9,11-dienoic acid, and sitosteryl-3β-D-glucoside. Further work needs to be done to evaluate the antiviral, antitumor, antifungal, antiparasitic, and many else of these secondary metabolites.

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