Purification and structure elucidation of antifungal and antibacterial activities of a newly isolated *Streptomyces* sp. strain US80

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Abstract

A new actinomycete strain designated as US80 producing antimicrobial activities against Gram-positive and Gram-negative bacteria and fungi was isolated from Tunisian oasis soil. Cultural characteristic studies strongly suggested that this strain belongs to the genus *Streptomyces*. Nucleotide sequence of the 16S rRNA gene (1517 pb) of the *Streptomyces* sp. strain US80 exhibited high similarity (97-98 %) to other *Streptomyces* 16S rRNA genes. A similarity of 98 % was obtained with the 16S rRNA gene of *Streptomyces roseoflavus*, which produces the aminoglycoside antibiotic flavomycin. The study of the influence of different nutritional compounds on the production of bioactive molecules showed that the highest antimicrobial activities were obtained when glucose at 1 % (w/v) was used as the sole carbon source in the presence of magnesium. Extraction of the fermentation broth of the *Streptomyces* sp. strain US80 and various separation and purification steps led to the isolation of three pure active molecules. The chemical structure of these three compounds, namely irumamycin (**1a**), X-14952B (**1b**) and 17-hydroxy-venturicidin A (**1c**) was established on the basis on their IR, ESI-MS, ^{1}H and ^{13}C/APT NMR data and by comparison with reference data from the literature.

Keywords: Antimicrobial activities; *Streptomyces* sp. US80; Macrolides; Irumamycin; X-14952B; 17-Hydroxy-venturicidin A.

1. Introduction

The resistance of a large number of pathogenic bacteria and fungi to bioactive secondary metabolites in common use is presently an urgent focus of research, and new antifungal and antibacterial molecules are necessary to combat these pathogens. Filamentous soil bacteria belonging to the genus *Streptomyces* are rich sources of a high number of bioactive natural products with biological activity, which are extensively used as pharmaceuticals and agrochemicals. These bacteria produce about 75 % of the commercially and medically useful antibiotics **[14]**, and approximately 60 % of antibiotics developed for agricultural use were isolated from *Streptomyces* species as well **[23]**. To identify new isolated *Streptomyces* species, several techniques have been developed, including selective plating methods **[8]**, the proof of the presence of L,L-diaminopimelic acid and the absence of characteristic sugars in the cell wall **[10]** and the construction of genetic marker systems **[25]**. In addition, 16S rRNA sequence data have proved invaluable in Streptomycetes systematic, in which they have been used to identify several newly isolated Streptomycetes **[12]**.

The fungus *Fusarium oxysporum* sp. *albedinis* (Foa) **[3, 4]** caused the destruction of a high number of palms in the oases of Algeria and Morocco but not in those of Tunisia. This surprising fact could be due to the physico-chemical characteristics of the Tunisian oasis soil, and/or to the presence of some antagonistic microorganisms, which could inhibit the Foa development and dissemination. Hence, the screening of new bacterial strains having antifungal activities from the soil of Tunisia oases could be a helpful idea in this field.

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This paper describes the isolation of a new actinomycete strain, called US80, from Tunisian oasis soil producing antibacterial and antifungal activities also against Foa. The identification of this strain and the study of the influence of different nutritional compounds in the biosynthesis of the bioactive molecules are reported. The extraction, purification as well as the structure elucidation of three antimicrobial molecules from the strain US80 was addressed and their biological activity described.

2. Materials and methods

2.1. Microorganisms and plasmids

The US80 strain was isolated as a producer of potent antimicrobial activities and was used as the source of chromosomal DNA to amplify the 16S rRNA gene. *Escherichia coli* TOP10 (Invitrogen), F-*mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) ϕ80*lac*ZΔM15 Δ*lac*X74 *deo*R *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L *end*A1 *nu*pG, and *E. coli* DH5α **[5]** were used as host strains.

Bacterial strains: *E. coli* ATCC 8739; *Micrococcus luteus* LB 14110; *Bacillus subtilis* ATCC 6633 and *Staphylococcus aureus* ATCC 6538 were used as indicator microorganisms for the antibacterial activity essays.

Antifungal activity was determined against *Verticillium dahliae*, *Fusarium* sp. and *Candida tropicalis* R2 CIP203.

pIJ2925 **[7]** derivative of pUC18 and pCR-Blunt vector (Invitrogen) *Col* E1 origin (pUC-derived) Kn^R were used as the cloning vectors.

pLF1 is a derivative of pCR-Blunt vector carrying a 1.5-kb fragment corresponding to the whole 16S rRNA gene of the strain US80 (this work). pLF2 and pLF3 are derivatives of pIJ2925 carrying respectively the 0.9-kb and 0.6-kb *Eco*RI-*Eco*RI DNA fragments of the 16S RNA gene of the strain US80 (this work).

2.2. Culture conditions

E. coli DH5α was grown on Luria Bertani (LB) plates supplemented with ampicillin (50 µg/ml) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (40 µg/ml) when appropriate **[19]**. Transformation of *E. coli* DH5α with pIJ2925 derivatives was carried out according to Hanahan **[5]**. Growth and transformation of TOP10 *E. coli* strain with the pCR-Blunt vector derivative were carried out according to the manufacture's instructions (Invitrogen). For the isolation of actinomycete strains, soil samples collected from different oasis regions of Tunisia, were spread on solid boiled bran barley medium **[13]**: 0.2 % yeast extract and 2 % agar were added to a supernatant of a 4 % boiled bran barley. The pH was adjusted to 7. After incubation at 30 ~ 40 °C for several days, colonies showing sporulation and filamentous aspect were picked and propagated on the same solid medium.

For determination of antibacterial activities, indicator microorganisms were grown overnight in LB medium at 30 °C for *M. luteus* LB 14110 and *B. subtilis* ATCC 6633, and at 37 °C for *E. coli* ATCC 8739 and *S. aureus* ATCC 6538, then diluted 1:100 in LB medium and incubated for 5 h under constant agitation of 200 rpm at the appropriate temperature.

For antifungal activity determination, *Candida tropicalis* R2 CIP203 was grown in YP10 medium (10 g/l yeast extract, 10 g/l peptone, 100 g/l glucose, 15 ml of 2 g/l adenine solution) at 30 °C for 24 h in an orbital incubator with shaking at 200 rpm. *Verticillium dahliae* and *Fusarium* sp. were grown in potato dextrose agar (PDA) during 7 days at 30 °C. Spores were collected in sterile distilled water and then adjusted to a spore density of approximately 10^4 spores/ml.

The strain US80 was grown in tryptic soy broth (TSB) for the preparation of genomic DNA **[6]**. Cultural characteristics of strain US80 were compared on the basis of observations made after 7, 14 and 21 days incubation on nutrient agar, Sabouraud agar and yeast malt agar media **[22]**. To investigate the influence of the medium on antimicrobial production, spores at 10^7/ml were used to inoculate 1000 ml Erlenmeyer flasks with four indents, containing 200 ml of Bennett medium (peptone 2 g/l, yeast extract 1 g/l, beef extract 1 g/l) supplemented at 1 % (w/v) with one of the five tested carbon sources (starch, fructose, glycerol, glucose and saccharose). After incubation at 30 °C for 72 h in an orbital incubator with shaking at 200 rpm, biological activities were assayed for each culture supernatant. Influence of magnesium, potassium and trace mineral oligoelements on active molecules production was also investigated. The final magnesium and potassium concentration was 2 and 1 mmol/l, respectively. For trace mineral oligoelements (40 mg $ZnCl_2$, 200 mg $FeSO_4{\cdot}7H_2O$, 6.5 mg H_3BO_3 and 13.5 mg $MoNa_2O_4{\cdot}2H_2O$ per 100 ml distilled water), 1.5 ml were added to 200 ml of growth medium.

2.3. Biological essay of antimicrobial activities

To isolate new actinomycete strains producing antimicrobial activities, we have used the solid media bioassay test against *M. luteus* LB 14110 (Gram-positive bacteria), *E. coli* ATCC 8739 (Gram-negative bacteria), *Verticillium dahliae* and *Fusarium* sp.

In solid media, after incubation of the selected strains for 7 days at the appropriate growth temperature, an agar disk was recuperated and picket in LB plates covered by 3 ml of top agar containing 50 µl of a 5 h culture of *M. luteus* LB 14110 or *E. coli* ATCC 8739 test strains, then incubated overnight at 30 °C for *M. luteus* and at 37 °C for *E. coli*. For antifungal activity determination, plates were covered by 3 ml of top agar containing 100 µl of spore suspension already prepared from *Verticillium dahliae* or *Fusarium* sp.

In liquid media, a paper disk was impregnated with 80 µl of the corresponding sample and then laid on the surface of an agar plate containing 3 ml of top agar inseeded by 40 µl of an 5 h old culture of one of the used bacteria for antibacterial tests: *M. luteus* LB 14110, *B. subtilis* ATCC 6633, *E. coli* ATCC 8739 and *S. aureus* ATCC 653850, and by 50 µl of *Candida tropicalis* R2 CIP203 culture or 100 µl of spores suspension of *Verticillium dahliae* or *Fusarium* sp. for antifungal activities. After 2 h at 4 °C, plates containing *M. luteus*, *B. subtilis*, *Candida tropicalis* R2 CIP203, *Verticillium dahliae* and *Fusarium* sp. were incubated at 30 °C and those inoculated with *E. coli* and *S. aureus* at 37 °C, all for overnight except *Verticillium dahliae* and *Fusarium* sp. for 48h. Antifungal activities against *Fusarium oxysporum* sp. *albedinis* were determined in the laboratory of microbiology of the faculty of sciences Marrakech-Morocco, as it is prohibited to manipulate this *Fusarium* sp. in Tunisia.

The antimicrobial activity of the three pure compounds was determined under the same conditions as in liquid media. The quantity used for each pure molecule was 20 µg. Plates were examined for evidence of antimicrobial activities represented by an inhibition zone of the corresponding indicator micro-organisms.

2.4. Spectroscopic measurements

NMR spectra were measured on a Varian Inova 600 (599.740 MHz) spectrometer. ESI-MS was recorded on a Quattro Triple Quadrupole Mass Spectrometer, Finnigan TSQ 7000 with nano-ESI-API-ion source. ESI-HRMS were measured on Micromass LCT mass spectrometer coupled with a HP 1100 HPLC with a Diode Array Detector. Reserpin (MW = 608) and Leucin-Enkephalin (MW = 555) were used as standards in positive and negative mode. IR spectra were recorded on a Perkin-Elmer 1600 Series FT-IR spectrometer as KBr pellets. Flash chromatography was carried out on silica gel (230-400 mesh). Thin layer chromatography (TLC) was performed on Polygram SIL G/UV_{254} (Macherey-Nagel & Co.). R_f values were measured on Polygram SIL G/UV_{254} (Macherey-Nagel & Co.). Size exclusion chromatography was done on Sephadex LH-20 (Pharmacia).

2.5. Extraction and purification of active compounds

Spores at 10^7/ml of strain US80 were used to inoculate 1000 ml Erlenmeyer flasks with four indents, containing 200 ml of Bennett medium supplemented with 1 % (w/v) of glucose and magnesium (2 mmol/l final concentration). After incubation at 30 °C for 24 h in an orbital incubator with shaking at 200 rpm, this pre-culture was used to inoculate (5 % v/v) a total volume of 15 l culture medium having the same composition as the pre-culture (200 ml in 1000 ml Erlenmeyer flasks). After three days incubation as for the pre-culture, the culture broth was filtered to separate mycelium and supernatant, which were treated separately as follows: the mycelium was lyophilized, extracted with acetone (10 %) and the solution concentrated on a Rotavapor. The supernatant was extracted twice with an equal volume of ethyl acetate and the combined organic layers were evaporated. The brown gum obtained from the combined extracts was dissolved in 100 ml methanol/cyclohexane (v/v). The antimicrobial activities were observed only in the methanolic phase, which was evaporated to dryness.

The crude methanolic extract (1.87 g) was separated on Sephadex LH 20 (MeOH/50% $CHCl_3$) into three fractions by tracing their colour reactions with anisaldehyde/sulphuric acid. Re-chromatography of fraction 1 on silica gel delivered 21.5 mg of irumamycin (**1a**) as a white powder with R_f = 0.39 in CH_2Cl_2/MeOH (95:5) on TLC with anisaldehyde/sulphuric acid giving a greenish, later on black spot. Repeated chromatography of fraction 2 on Sephadex LH 20 yielded 12.2 mg X-14952B (**1b**) as a white amorphous solid with R_f = 0.21 in CH_2Cl_2/MeOH (95:5). From fraction 3, in the same way as for **1b**, 27.7 mg 17-hydroxy-venturicidin A (**1c**) were obtained. **1c** and **1b** were giving similar colour reactions as **1a**.

17-Hydroxyventuricidin A (**1c**). White amorphous powder, R_f= 0.18 ($CHCl_3$/MeOH 95:5). - IR (KBr): ν = 3449, 2969, 2938, 1710, 1617, 1384, 1339, 1231, 1077, 974 cm^{-1}. - (+)-ESI MS: *m/z* (%) = 1553 ($[2M+Na]^+$, 40), 788 ($[M+Na]^+$, 100); (-)-ESI MS: *m/z* = 764 ($[M-H]^-$); (+)-HRESI MS *m/z* = 788.4555 (calcd. 788.45557 for $C_{41}H_{67}NO_{12}Na^+$), 783.50007 (calcd. 783.50013 for $C_{41}H_{67}NO_{12}$ + NH_4^+). - ^{13}C and ^1H NMR (Tables 1 and 2).

2.6. DNA isolation and manipulation

Total DNA preparation from strain US80 was carried out according to Hopwood et al. **[6]**. Small-scale plasmid preparations from *E. coli* were performed as described by Sambrook et al. **[19]**. Digestion with restriction endonucleases, separation of DNA fragments by agarose gels electrophoresis, dephosphorylation with alkaline calf intestinal phosphatase, ligation of DNA fragments and transformation of *E. coli* were done according to Sambrook et al. **[19]**.

Nucleotide sequence of the 16S rRNA gene of strain US80 was determined on both strands using the dideoxy chain-termination method **[20]**. The nucleotide sequence of the whole 16S rRNA gene (1517 pb) of strain US80 has been deposited in the GenBank (EMBL) under the accession number AJ639841.

2.7. PCR amplification of the 16S rRNA gene of strain US80

PCR amplification of the 16S rRNA gene of strain US80 was performed using two primers 5'-AGAGTTTGATCCTGGCTCAG-3' and 5'-AAGGAGGTGATCCAGCCGCA-3' as described by Edwards et al. **[2]**. Approximately 300 ng genomic template DNA was used with 150 pmol of each primer per 50 µl reaction volume. To improve the denaturation of the DNA, 5 % (v/v) DMSO was added to the reaction mixture. Amplification was performed in an automated thermocycler (Perkin Elmer) using 1U Pfu DNA polymerase (Stratagene) and the recommended buffer system according to the following amplification profile: 94 °C (3 min) followed by 45 cycles of denaturation at 94 °C (30 s), annealing at 60 °C (1 min) and extension at 72 °C (3 min). The PCR reaction mix was analysed by agarose gel electrophoresis and the DNA of the expected size was purified and then cloned into pCR-Blunt vector yielding pLF1**.**

Table 1. Comparison of ^{13}C NMR chemical shifts ($CDCl_3$, δ values) of X-14952B(**1b**), 17-hydroxy-venturicidin A (**1c**) and irumanolide II (**1d**)

C No.	1b	1c	1d	C No.	1b	1c	1d
1	173.4 s	173.4 s	173.8 s	18-CH_3	5.5 q	5.5 q	5.5 q
2	43.3 t	43.2 t	43.6 t	19	81.9 d	81.8 d	82.2 d
3	94.0 s	93.9 s	94.3 s	20	33.4 d	33.5 d	32.8 d
4	35.1 t	35.0 t	35.2 t	20-CH_3	15.8 q	15.8 q	16.0 q
5	116.8 d	116.7 d	117.2 d	21	37.0 t	35.9 t	36.2 t
6	134.0 s	132.7 s	133.2 s	22	32.5 d	32.4 d	32.8 d
6-CH_3	19.1 q	19.1 q	19.2 q	22-CH_3	12.6 q	14.0 q	12.3 q
7	80.0 d	79.9 d	80.3 d	23	76.7 d	77.6 d	77.7 d
8	137.7 s	134.7 s	135.0 s	24	55.1 d	48.3 d	48.5 d
8- CH_3	10.7 q	10.6 q	10.8 q	24-CH_2CH_3	22.6 t	-	-
9	129.3 d	129.3 d	129.9 d	24-CH_2CH_3	11.7 q	14.1 q	14.4 q
10	27.0 t	27.0 t	27.3 t	25	217.2 s	216.8 s	217.3 s
11	26.0 t	25.9 t	26.6 t	26	37.9 t	35.9 t	37.2 t
12	35.0 t	34.5 t	37.4 t	27	7.2 q	7.4 q	7.6 q
13	82.2 d	82.2 d	75.1 d	1’	98.2 d	98.1 d	-
14	134.3 d	134.3 d	135.7 d	2’	37.0 t	36.8 t	-
15	134.0 d	133.9 d	135.2 d	3’	75.2 d	74.8 d	-
16	42.0 d	42.0 d	42.2 d	3’$OCONH_2$	157.5 s	157.5 s	-
16-CH_3	17.1 q	17.1q	17.4 q	4’	74.9 d	75.0 d	-
17	77.7 d	77.7 d	77.9 d	5’	71.9 d	71.9 d	-
18	34.5 d	34.5 d	34.8 d	5’-CH_3	17.8 q	17.7 q	-

Table 2. ^{1}H NMR chemical shifts ($CDCl_3$, δ values) of 17-hydroxy-venturicidin A (**1c**)

H No.	^{1}H NMR	H No.	^{1}H NMR
1	-	18-CH_3	0.92
2	2.40~2.60	19	4.86 d
3	-	20	1.67
4	2.10~2.30	20-CH_3	0.83
5	5.52	21	1.10, 1.42
6	-	22	1.58
6-CH_3	1.48	22-CH_3	0.82
7	4.45	23	3.54
8-CH_3	1.40	24	2.68
9	5.45	24-CH_3	1.02
10	1.84~2.10	25	-
11	1.20~1.50	26	2.50
12	1.50~1.60	27	1.26
13	3.93	1’	4.60
14	5.60	2’	1.62, 2.24
15	5.30	3’	4.66
16	2.40	4’	3.16
16-CH_3	1.08	5’	3.20
17	3.18	5’-CH_3	1.31
18	1.98		

3. Results and discussion

3.1. Isolation and identification of strain US80

A new actinomycete strain called US80 isolated from Tunisian oasis soil produced antimicrobial activities against Gram-positive and Gram-negative bacteria and fungi (Fig. 1). Permissive temperature ranges for growth of the strain US80 were 25 to 37 °C with an optimum at 30 °C. According to the cultural characteristics (Table 3), strain US80 grew well and the colonies were detachable. The colours of the vegetative and aerial mycelia were yellowish and white-brown, respectively. The spore chains were greyish-yellow. The comparison of these obtained cultural characteristics with those of the known actinomycete species described in Bergey’s Manual of Systematic Bacteriology **[11]**, strongly suggested that strain US80 belongs to the genus *Streptomyces*. Total nucleotide sequence of 1517 bp (accession n° AJ639841), of the 16S rRNA gene of the *Streptomyces* sp. US80 was determined in both strands. The alignment of this sequence through matching with reported 16S rRNA gene sequences in the gene bank shows high similarity (97-98 %) to *Streptomyces* 16S rRNA genes. Highest similarity (98 %) was obtained with the 16S rRNA gene of *Streptomyces roseoflavus,* which produces the aminoglycoside antibiotic, flavomycin **[1]**.

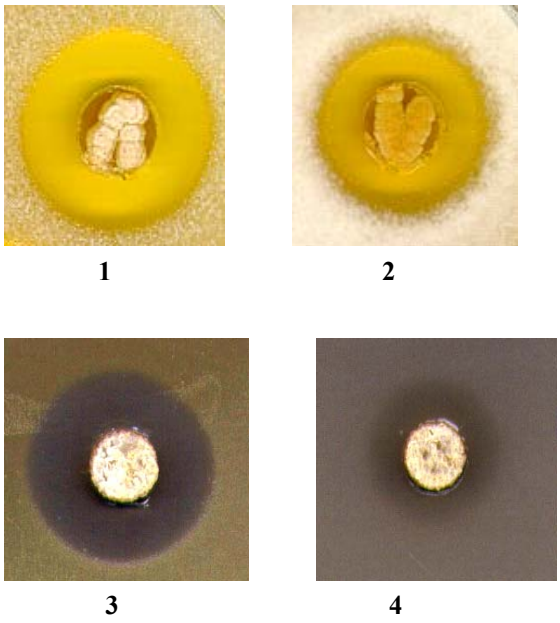

Fig. 1. Antimicrobial activities in solid media of strain US80 against the four used indicator microorganisms. *Fusarium* sp. (1); *Verticillium dahliae* (2); *M. luteus* LB 14110 (3) and *E. coli* ATCC 8739 (4).

Table 3. Cultural characteristics of strain US80

Medium	Growth	Vegetative mycelia	Aerial mycelia	Spores
Nutrient Agar	well, spreading	abundant, yellowish	detachable, white-brown	moderate, white-yellow
Sabouraud Agar	well, partitioned	abundant, yellowish	detachable, white-yellow	abundant, yellowish
Yeast malt Agar	moderate, elevated	moderate, brown	moderate, white-brown	moderate, greyish

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Flavomycin is a broad-spectrum antibiotic effective against both Gram positive and Gram negative bacteria. It interferes with protein synthesis in sensitive bacterial cells such as species of *Proteus* and *Staphylococcus*. It is mainly used topically in the treatment of skin and mucous membrane infections, wounds and burns.

3.2. Optimization of the culture conditions and quantification of the antimicrobial activities in liquid media

Four carbohydrates (starch, fructose, glucose, saccharose) and glycerol were tested as sole carbon source at 1 % (w/v) in the Bennett liquid medium. Maximal activity was obtained when glucose was used as carbon source. To further optimize the culture conditions, trace mineral oligoelements, potassium and magnesium salts were tested using glucose as carbon source. The results showed that antimicrobial activities against the used indicator microorganisms were observed under all three conditions; however, the combination of glucose and magnesium produced the best results. In that case, we obtained antimicrobial activities against all tested microorganisms including the *Fusarium oxysporum* sp. *albedinis* (data not shown).

3.3. Purification and structure elucidation of the active compounds

The bulk fermentation was performed on a 15 l scale of *Streptomyces*. sp. strain US80 on Bennett medium supplemented with 1 % (w/v) of glucose and magnesium at 2 mmol/l (final concentration) for three days. Both mycelium and filtrate were extracted with acetone and ethyl acetate, respectively. Repeated chromatography of the combined extracts delivered three compounds, which were identified as irumamycin (**1a**), X-14952B (**1b**), and 17-hydroxy-venturicidin A (**1c**) by ^{1}H, ^{13}C NMR and mass spectra and comparison with published data (Fig. 2).

Compound **1a** was obtained as white solid which on TLC gave slowly a black colour after spraying with anisaldehyde/sulphuric acid, which is typical for macrolides. The ^{1}H NMR spectrum of **1a** was rich in aliphatic proton signals with additional four olefinic multiplets: In addition to other signals, a triplet at δ 0.84, five doublets at δ 1.18, 0.94, 0.80, 0.78 and 0.70 and three singlets at δ 1.38, 1.30 and 1.24 of altogether nine methyl groups were observed. The ^{13}C/APT NMR spectra displayed 41 signals for nine methyl, eight methylene and thirteen methine groups, of which nine were bearing oxygen. In the low field region, signals of two acetal groups (δ 98.1, 93.8), six olefinic, an amide (δ 157.6), a lactone or carboxylic acid (δ 173.1), and a ketone (δ 211.0) carbon atom appeared. Two signals in the acetal region indicated the presence of sugar residues. The ESI mass spectrum exhibited a molecular weight of *m/z* 763, and high resolution delivered the molecular formula $C_{41}H_{65}NO_{12}$. A substructure search in AntiBase **[9]** supported by the NMR data and the molecular weight led to irumamycin (**1a**), which was previously isolated from *Streptomyces subflavus* subsp. *irumaensis* subsp. nov. AM-3603 by Ômura et al. **[15]** and is reported as the first antifungal drug active against the phytopathogens *Piricularia oryzae, Sclerotinia cinerea* and *Botrytis cinerea* **[16]**. Irumamycin (**1a**) has found use in agriculture **[15]**.

Compound **1b** was obtained as an amorphous solid, which showed similarities in TLC, ^{1}H and ^{13}C NMR spectra, indicating also structural similarities with irumamycin (**1a**). The proton NMR spectrum of **1b** indicated 9 methyl groups as well; the methyl singlet at δ 1.24 in **1a** was, however, replaced by an additional methyl triplet at δ 0.84 in **1b**. The ^{13}C NMR spectrum delivered 42 signals. The chemical shift of the sugar residue and the lactone part of the aglycone moiety in **1b** were nearly identical to those of **1a**. The carbon signals of the epoxy ring in **1a** at δ 66.1 (C_q) and 64.1 (CH) were replaced by methine signals at δ 77.0 and 55.2 in **1b**. The spectra indicated the presence of the same 20-membered lactone ring in **1b** as in **1a**, and the changes must have taken place in the side chain. ESI MS delivered a molecular weight, which is *Δm* 16 higher than that of **1a**. A substructure search in AntiBase suggested the identity with X-14952B (**1b**)**,** which was confirmed by comparison with the published data **[17]**.

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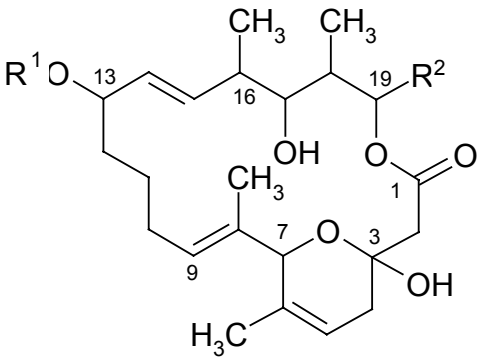


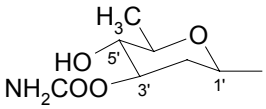
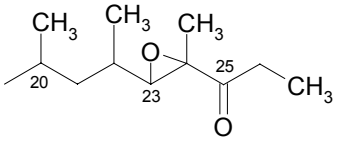
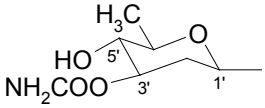
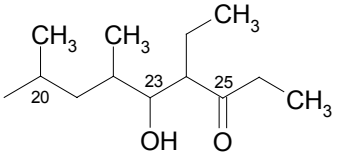
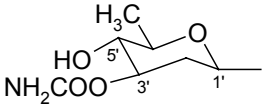
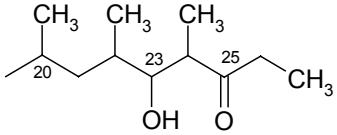
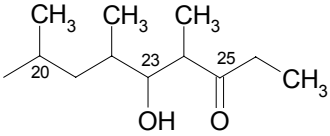
	R^1	R^2
Irumamycin (**1a**):		
X-14952 B (**1b**):		
17-Hydroxy-venturicidin A (**1c**):		
Irumanolide (**1d**)	H	

Fig. 2. The structures of compounds **1a** ~ **1d**

Compound **1c** showed similar chromatographic and spectroscopic properties as **1a** and **1b**. In the proton NMR spectrum, the major difference was the absence of the triplet at δ 0.84 in **1b**, which was replaced by a doublet at δ 1.02 **1c**. The molecular weight was deduced to be 765 Dalton from the ESI mass spectrum with a *pseudo*-molecular ion at *m/z* 788 $[M+ Na]^+$, and the molecular formula was determined to be $C_{41}H_{67}NO_{12}$ by high resolution. The ^{13}C NMR spectrum showed all 41 carbon signals. Comparison of the ^{13}C NMR data of **1c** with those of **1a** and **1b** suggested that all compounds possessed the same lactone structure and sugar residue, and that the differences were localized in the side chain. Among the carbon atoms of the side chain, the signal at δ 22.6 of the methylene group at C-24 (24-CH_2CH_3) was missing, so that a methyl group must be attached directly to C-24. The structure **1c** was finally confirmed by comparison of the ^{13}C NMR data with those of irumanolide II (**1d**) **[18]** (Table 1) which is the aglycon of **1c**. Compound **1c** is identical with YP-02259L-C **[21]**, which had been described previously, however, without a full spectroscopic characterisation.

The antimicrobial activities of the three pure compounds (irumamycin, X-14952B and 17-hydroxyventuricidin A) are shown in Table 4. All the three pure compounds inhibited the growth of the two tested filamentous fungi (*Verticillium dahliae* and *Fusarium* sp.) and of *Candida tropicalis* R2 CIP203. The highest antifungal activity was obtained with irumamycin (**1a**). The three compounds exhibited an inhibitory activity only against Gram-positive bacteria and molecules **1b** and **1c** showed a greater activity than **1a**.

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Test organism	Diameter of inhibition zones (mm)		
	1a	1b	1c
M. luteus LB 14110	21	31	32
B. subtilis ATCC 6633	22	25	28
S. aureus ATCC 6538	20	22	22
E. coli ATCC 8739	0	0	0
Verticillium dahliae	22	17	18
Fusarium sp.	20	15	14
Candida tropicalis R2 CIP203	20	13	12

To our knowledge, US80 is the only described strain, which produces all these three active molecules at once. In addition, and according to the results of the antimicrobial activities of the studied three pure compounds and those obtained in liquid media (inhibition of both Gram-positive and Gram-negative bacteria), we can consider that US80 strain produces not only the three active molecules described in this work but also at least one other active compound. In spite of the high similarity between the nucleotide sequence of the 16S rRNA genes of the US80 strain and the flavomycin producer *Streptomyces roseoflavus*, there are several reasons to consider that the US80 strain is different to the irumamycin producer *Streptomyces subflavus* subsp. *nirumaensis* subsp. nov. AM-3603 already described by Ômura et al. (1982). Firstly, identity of two *Streptomyces* strains requires also 100% identity of their 16S rRNA nucleotide sequence. Secondly, the *Streptomyces roseoflavus* has been described as flavomycin (aminoglycoside antibiotic) producer whereas the US80 strain did not afford the latter but synthesizes at least four active metabolites including three different macrolides.
All these data suggest strongly that the three strains evoked in this work (US80 strain, S. *subflavus* subsp. *nirumaensis* subsp. nov. AM-3603 and *Streptomyces roseoflavus*) are different

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