SUPPLEMENTARY MATERIAL

Nigericin and grisorixin methyl ester from the Algerian soil-living *Streptomyces youssoufiensis* SF10 strain: a computational study on their epimeric structures and evaluation of glioblastoma stem cells growth inhibition

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Abstract

The present work describes the metabolites produced by a strain identified as *Streptomyces youssoufiensis*, whose secondary metabolites profile has not been studied so far. The crude ethyl acetate extract was analyzed by high performance liquid chromatography-electrospray ionization mass spectrometry (HPLC-ESIMS), leading to the detection of the ionophoric polyethers nigericin, epinigericin, abierixin and the newly isolated grisorixin methyl ester. The presence of epimeric forms of nigericin/epinigericin and grisorixin/epigrisorixin has spurred DFT computational calculations. This analysis was able to provide the relative stability of the most favored epimers, setting the basis for general structural considerations applicable to several other polyethers. Both nigericin sodium salt and grisorixin methyl ester showed to affect glioblastoma stem cells proliferation in a dose-dependent manner, with a higher activity for the more lipophilic grisorixin methyl ester (GI_{50} values of 3.85 and 3.05 µM for VIPI and COMI human glioblastoma stem cells, respectively).

Keywords: Streptomyces, HPLC-ESI/MS, Polyethers, DFT calculation, Cytotoxicity, Glioblastoma.

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Scheme S1. Chromatographic purification of polyether metabolites present in the extract of SF10 strain: abierixin, nigericin, epinigericin and grisorixin methyl ester. FC = flash chromatography; RP = reversed phase; PLC = preparative TLC; DCM = dichloromethane.

Table S2. Energy values obtained by DFT calculation at a B3LYP/6-31G(d,p) level of theory for simplified structures taken as model for the F-ring of the indicated polyether metabolite. The C-numbering resumes that reported for polyethers in Figure 1.

Experimental

1.Isolation and identification of the bacterial strain

A semi-arid soil sample (10 g) collected at Chélia Mountain (35°19′6″N, 6°38′15″E), in Khenchela (North-eastern Algeria), was diluted in 100 mL of sterile distilled water. The mixture was allowed to settle, and then serial ten-fold dilutions were prepared. An aliquot (0.1 mL) was taken from each dilution and spread evenly over the surface of Olson medium containing polymexin and nystatin (each at a 0.050 mg/mL concentration). Plates were incubated at 30°C for 14 days. Actinobacteria colonies were picked and purified by serial subcultures on yeast malt extract agar (YMEA) medium. Among the eleven actinomycetes strains isolated, SF10 strain (where the acronym SF indicates Soil Forest because Chélia Mountain is rich in forest), was selected based on its ability to inhibit the growth of pathogenic and phytopathogenic bacteria (MRSA and *Bacillus subtilis*) and fungi (*Fusariumoxysporum*). Methods and media described by the International Streptomyces Project (ISP) as well as light microscopy and scanning electron microscopy (SEM-FEG Jeol JSM-7001F) were used to assess the strain morphology. After a preliminary identification, the genomic DNA was isolated from SF10 strain according to a reported method (Pospiech and Neuman 1995). The 16S rRNA genes from pure culture was amplified using the universal primers F27 [5'AGAGTTTGATCCTGGCTCAG3'] and R1492 [5'TACGGCTACCTTGTTACGACTT3'] (Heueret al. 1997; Monciardiniet al. 2002). Sequences were assembled using Sequencher software (version 4.7) and analyzed by the Basic Local Alignment Search Tool (BLAST) on NCBI. Sequence of SF10 strain was deposited in the GenBank under accession number KU 373054.

2. Crude extract and metabolite profile by LC-ESI MS analysis

Pure culture of SF10 strain was used to inoculate plates of Bennett's agar medium. After 14 days incubation at 30°C, the mycelial mass together with the culture medium was cut into small pieces, subjected to sonication and macerated overnight in ethyl acetate (x2). The combined filtrates were evaporated *in vacuo* to give a yellow crude extract (1.0 g). For HPLC-ESIMS analysis, the crude extract was dissolved in methanol (1mg/mL), loaded on an Eclipse reversed-phase column (HP, Hypersil BDS-C18, 4.6x150mm, 3.5µm) and analyzed by a Hewlett–Packard HP1100 HPLC-UV Diode Array detector (DAD), coupled online to an Esquire-Bruker–Daltonics ion trap mass spectrometer. The mobile phase used was: A (MeOH: H_2O, 9:1,+ 0,1%

TFA) for 25min and B (MeOH 100%, + 0,1% TFA) from 25min to 50min, flow 1mL/min. The measurements were performed in positive and negative ion detection mode with a scan range between *m/z* 200–900.

3.General procedures for isolation and structural elucidation

Solvents were purchased from Sigma Aldrich Europe or Alfa Aesar and were used without any further purification. All evaporations were carried out at reduced pressure. Thin-layer chromatography (TLC) was carried out on Merck Kieselgel Si60 PF254 and the spots visualized at 254 nm or by carbonization after treatment with an acidic solution of cerium (IV) sulfate. Preparative TLC was performed using 20 cm×20 cm Merck Kieselgel 60 F254, 0.5 mm plates. Reversed phase RP-FC was carried out on a Merck Lichroprep RP-18 column. RP-HPLC was carried out using Luna C18, 5µm, 250X460 nm, or Eclipse C18, 3.5µm columns and 1mL/min flow. Polarimetric data were obtained with a Bellingham & Stanley Limited ADP 440 apparatus, reporting $[\alpha]_D$ in dm^{-1}deg mL g^{-1}. Infrared (IR) spectra were recorded by a FT-IR Tensor 27 Bruker spectrometer with Attenuated Transmitter Reflection (ATR) configuration, with 1 cm^{-1} resolution. NMR spectra were recorded by an Avance 400 Bruker spectrometer by using a 5 mm BBI probe; ^{1}H NMR spectra at 400 MHz and ^{13}C at 100 MHz, in $CDCl_3$ (δ_H = 7.25 ppm and δc = 77.0 ppm). Structural assignments are by Heteronuclear Single Quantum Correlation (HSQC) and Heteronuclear Multiple Bond Correlation (HMBC) experiments. ESI-MS data and tandem fragmentation (MS/MS) spectra were recorded using a Bruker Esquire LC ion trap mass spectrometer, equipped with an electrospray ion (ESI) source in positive or negative ion mode, by injection of a methanol solution of the sample into the source. MS conditions: source temp. 300°C, nebulizing gas N_2, 4 l min^{-1}, cone voltage 32 V, scan range *m/z* 100–900. Fragmentation experiments were carried out using helium to collisionally activate the selected primary ions. High-resolution ESI MS measurements for grisorixin methyl ester were obtained by direct infusion using an Orbitrap Fusion Tribrid® mass spectrometer.

4. Isolation and structural elucidation of the metabolites

The EtOAc crude extract (0.8 g) was subjected to FC on a column of silica gel, 15–25 µm (Merck), using dichloromethane/MeOH at gradient elution. Eluted fractions were collected sequentially, concentrated *in vacuo* and combined after TLC analysis. In detail, fractions 28-34,

eluted with dichloromethane/MeOH 8:2, were combined (0.10 g) and further purified on Merck Lichroprep RP-18 column, using a gradient of MeOH/H_2O, to give fr. 10 (10 mg), fr. 17-18 (36 mg) and fr. 20-23 (19 mg). Fr. 10 was subsequently purified by RP HPLC (MeOH/H_2O + 0.1% TFA 6:4) to provide abierixin (t_R=5.5 min, 1.2 mg). Fr. 17-18 were subjected to PLC (hexane/isopropanol 9:1), which allowed to recover nigericin (18.0 mg) and epinigericin (6.5 mg). Fr. 20-23 were subjected to RP HPLC (MeOH/H_2O + 0.1% TFA, 9:1 from 0 to 25 min, then MeOH + 0.1% TFA) to obtain grisorixin methyl ester (t_R= 35.3 min, 10 mg). An overview of the purification workup is reported in Supporting, Scheme S1.

*Data of abierixin (**1**)*

White solid; $[\alpha]_D$ = 39.3°(c=0.001, MeOH), lit. (David et al. 1985) +45° (c=0.03, MeOH); IR: 3407, 2927, 1692, 1649, 1458, 1378, 1052 and 956 cm^{-1}. ^1H NMR and ^{13}C NMR ($CDCl_3$) spectra resulted in agreement with the reported data (Siwen et al. 2011). ESIMS(+): *m/z* 747.5 $[M+Na]^+$; ESI(+)MS/MS (747.5) *m/z* 729.4; ESIMS3(+) 729: *m/z* 685.3. ESIMS(-): *m/z* 723.5 $[M-H]^-$; ESIMS/MS(-) (723.5): *m/z* 679.3, 439.3.

*Data of nigericin (**2**)*

White solid. IR: 3446, 2930, 1715, 1266, 1127 cm^{-1}. ^1H NMR and 13C NMR ($CDCl_3$) spectra showed signals superimposable to the reported data (Gong et al. 2010). ESIMS(+): *m/z* 747.5 $[M+Na]^+$; ESI(+)MS/MS (747.5) *m/z* 729.4; $ESIMS^3$(+) 729: *m/z* 685.3. ESIMS (-): *m/z* 723.5 $[M-H]^-$; ESIMS/MS(-) (723.5): *m/z* 679.3 439.3.

*Data of epinigericin (**3**)*

White solid. IR: 3200, 2900, 1580, 1380, 1120, 1040, 950, 650 cm^{-1}. NMR data are in agreement with the reported ones (Berrada et al. 1987). ESIMS(+): *m/z* 747.5 $[M+Na]^+$; ESIMS/MS(+) (747.5) *m/z* 729.4; $ESIMS^3$(+)729: *m/z* 685.3. ESIMS(-): *m/z* 723.5 $[M-H]^-$; ESIMS/MS(-) (723.5): *m/z* 679.3, 439.3.

*Data of grisorixin methyl ester (**4**)*

White solid; $[\alpha]_D$ = +9.1(c=0.004 g/mL, MeOH); FT-IR: ν 3432 (vs), 2928 (vs), 1739 (s), 1459 (s), 1377 (m), 1220 (w), 1113 (s), 1061 (vs), 961 (s) cm^{-1}; ^1H NMR ($CDCl_3$, 400MHz) : δ= 4.19 (2H, m), 4.12 (2H, m), 3.87 (1H, brd, *J* =8.6 Hz), 3.72 (3H, s), 3.68 (1H, m), 3.58 (1 H, brd, *J* =9.8 Hz), 3.38 (3H, s), 3.36(1H, m), 2.50 (1H, m), 2.47 (1H, m), 2.10-1.42 (20H, series of m), 1.16 (3H, s), 1.02 (3H, d , *J*=7.0 Hz), 0.99-0.93 (series of d, 18H, *J*=6.9Hz), 0.92 (3H, d, *J*= 8.0

Hz), 0.86 (3H, brd, *J*=6.9 Hz), 0.84 (3H, d, *J*=6.7Hz); ^{13}C-NMR (100 MHz, $CDCl_3$): δ= 176.4, 107.6, 98.3, 86.3, 84.3, 81.7, 79.6, 78.8, 73.7, 69.4, 60.3, 58.8, 51.7, 45.7, 43.1, 40.3, 39.5, 37.2, 36.8, 35.6, 35.3, 34.3, 33.6, 32.6, 31.9, 29.6, 29.2, 27.6, 27.2, 25.9, 25.8, 24.0, 22.7, 17.7, 16.3, 16.1, 14.1, 13.0, 12.5, 11.6; Significative HSQC correlations: 3.38 ppm (s, MeO-C16) with 58.8; 3.72 ppm (s, COOMe) with 51.7 ppm and HMBC correlations: 3.38 ppm (s, MeOC-16) with 79.6 ppm (C-11), 3.72 ppm (s, COOMe) with 176.4 ppm (COO); ESI(+)MS: *m/z* 745.48526 $[M+Na]^+$, ESI(+)MS/MS (745): *m/z* 713.46007 $[M-CH_3OH+Na]^+$, HRESI(+)MS: *m/z* 745.48526±0.00500 (calcd. for $C_{41}H_{70}NaO_{10}$, 745.486120); ESI(-)MS: *m/z* 721.48169 $[M-H]^-$, HRESI(-)MS: *m/z* 721.48169 ±0.00500 (calcd. for $C_{41}H_{69}O_{10}$, 721.489622).

5. Computational details on the study of epimerization

Starting structures were generated by molecular mechanics minimization, using PC Model (Serena Software, version 7.00). Quantum chemical calculations were performed on a Pentium IV/3.6 GHz personal computer using the Gaussian 03W revision E.01 package program set (Frisch et al. 2004). Restricted density functional theory (DFT) was used for geometry optimization invoking gradient geometry optimization. The basis set of choice was 6–31 G (d,p) for all the atoms. The gradient-corrected DFT with the three-parameter hybrid functional (B3) (Becke 1993) for the exchange part and the Lee–Yang–Parr (LYP) correlation function (Lee et al. 1988) were utilized. The simulations in the presence of water were achieved using the polarizable continuum model (PCM). The optimized structural parameters were employed in the vibrational energy calculations at the DFT levels to characterize all stationary points as minima. For each optimized structure, no imaginary wave number modes were obtained, proving that a local minimum on the potential energy surface was actually found.

6. Cytotoxicity evaluation

Human glioblastoma stem cells (COMI and VIPI) were cultured in DMEMF12 and Neurobasal media (ratio 1:1, pH 7.4), supplemented with B27 (100X), 2 mmol/L Glutamax, 100 U/mL penicillin G, 20 ng/mL EGF, 10 ng/mL FGF2 and 2 µg/mL heparin. GSCs were grown in adherence on laminin in a 5% CO_2 environment at 37°C.

In the *in vitro* assay 4000 cells/well for COMI and 3500 cells/well for VIPI were seeded in 96-well plate on laminin in adherence and incubated at 37 °C, 5% CO_2, for 24 h. The cells were then

treated with nigericin sodium salt or grisorixin methyl ester at desired concentrations. Following drug addition, the plates were incubated for 48 h at 37 °C, 5% CO_2. Hoechst 33342 (Thermo Fisher Scientific) and propidium iodide (PI, Sigma Aldrich) were then added to a final concentration of 1μg/mL and incubated for 20 min at room temperature in the darkness. The plates were read using Operetta High Content Imaging System (Perkin Elmer). All the experiments were performed in technical quadruplicates and in biological triplicates. The number of live cells for each treatment was estimated by subtracting PI positive cells from the total number of cells counted using Hoechst. The percentage of viable cells was calculated as normalized to control treatment (100% viability). Dose-response curves were plotted and growth inhibition 50 (GI_{50}) values calculated for nigericin sodium salt and grisorixin methyl ester. The final GI_{50} value for each drug was calculated as mean of the three biological replicates (standard deviation reported).

7. References

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Figure S1. Spore chain morphology of SF10 *Streptomyces* observed by scanning electron microscopy (SEM) analysis.

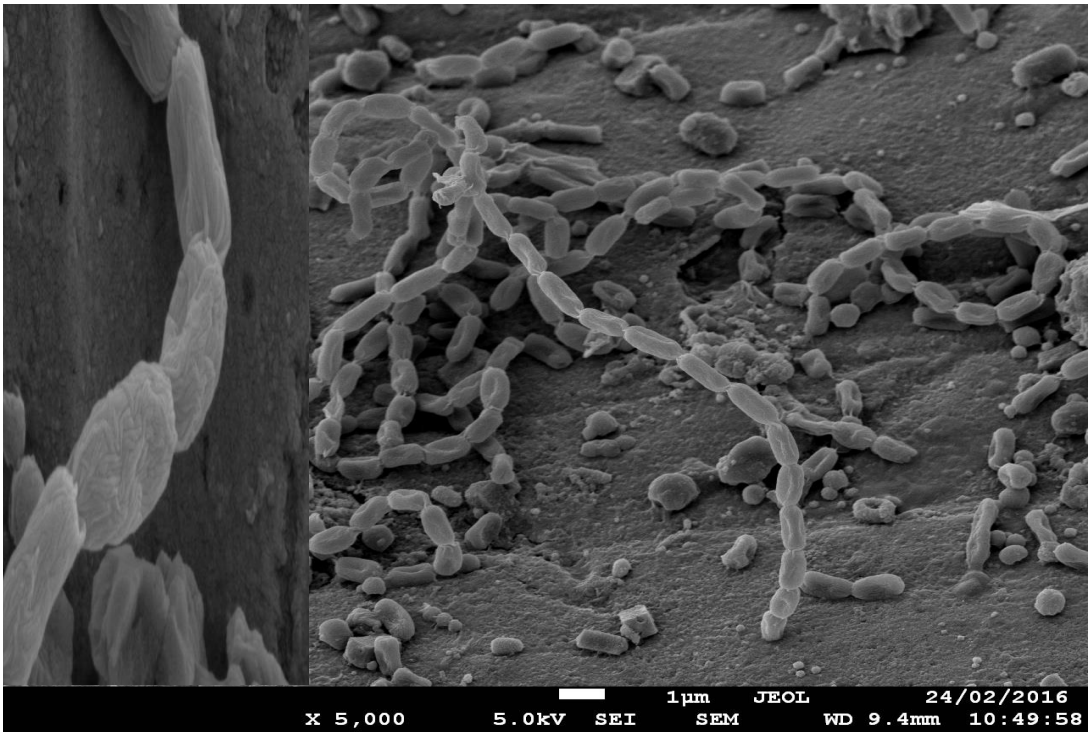
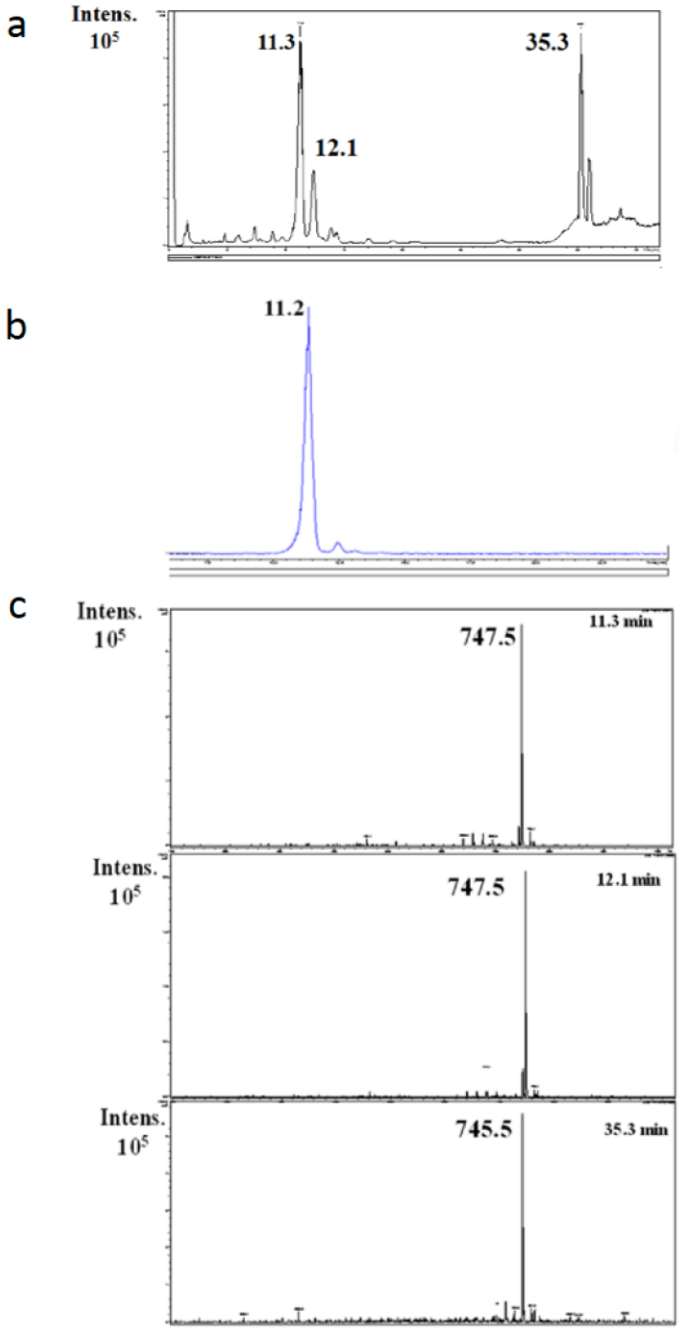


Table S1. Morphological and physiological characteristics of *Streptomyces* sp. SF10

Morphological characteristics		**Degradation activities**	
Color of spore mass	Yellow	Starch	-
Carbon source utilization		Casein	-
D-glucose	+++	Gelatin	++
Lactose	+	Adenine	-
Cellulose	-	Tween	-
Arabinose	+	Tyrosine	-
D-xylose	+	Pectin	-
Saccharose	-	Cellulose	-
D-fructose	++	**Growth in the presence of inhibitory compounds**	
D-maltose	++	NaCl 4%	+++
D-galactose	+	7%	±
D-ribose	+++	10%	-
Sorbitol	-	13%	-
Inositol	±	phenol	-
Mannitol	+++	Crytal violet	-
		Sensibility to antibiotics [a)]	
Nitrogen utilization		(µg/mL) mm	
Asparagine	+	Amikamycin (30)	22
Methionine	-	Spiramycin (100)	0
Aspartic acid	+++	Erythromycin (15)	0
Cysteine	-	Emipeneme (10)	24
Tyrosine	+++	Nalidixique acid (30)	0
Arginine	-	Cefuroxime (30)	0
Proline	++	Streptomycin (10)	0
Glycine	+		
Tryptophan	-		

a) Diameter of inhibition (mm) at the concentration indicated in brackets

Figure S2. LC-MS analysis (RP18, MeOH/H_2O + 0.1% TFA gradient elution) of the ethyl acetate extract from SF10 strain. (a) Extracted-ion chromatogram. (b) The peak at 11.3 min associated to nigericin by comparison with a standard sample. (c) MS signals of $[M+Na]^+$ ions corresponding to the indicated retention times.



Scheme S1. Chromatographic purification of polyethermetabolitespresent in the extract of SF10 strain: abierixin, nigericin, epinigericin and grisorixinmethyl ester. FC = flash chromatography; RP = reversed phase; PLC = preparative TLC; DCM = dichloromethane.

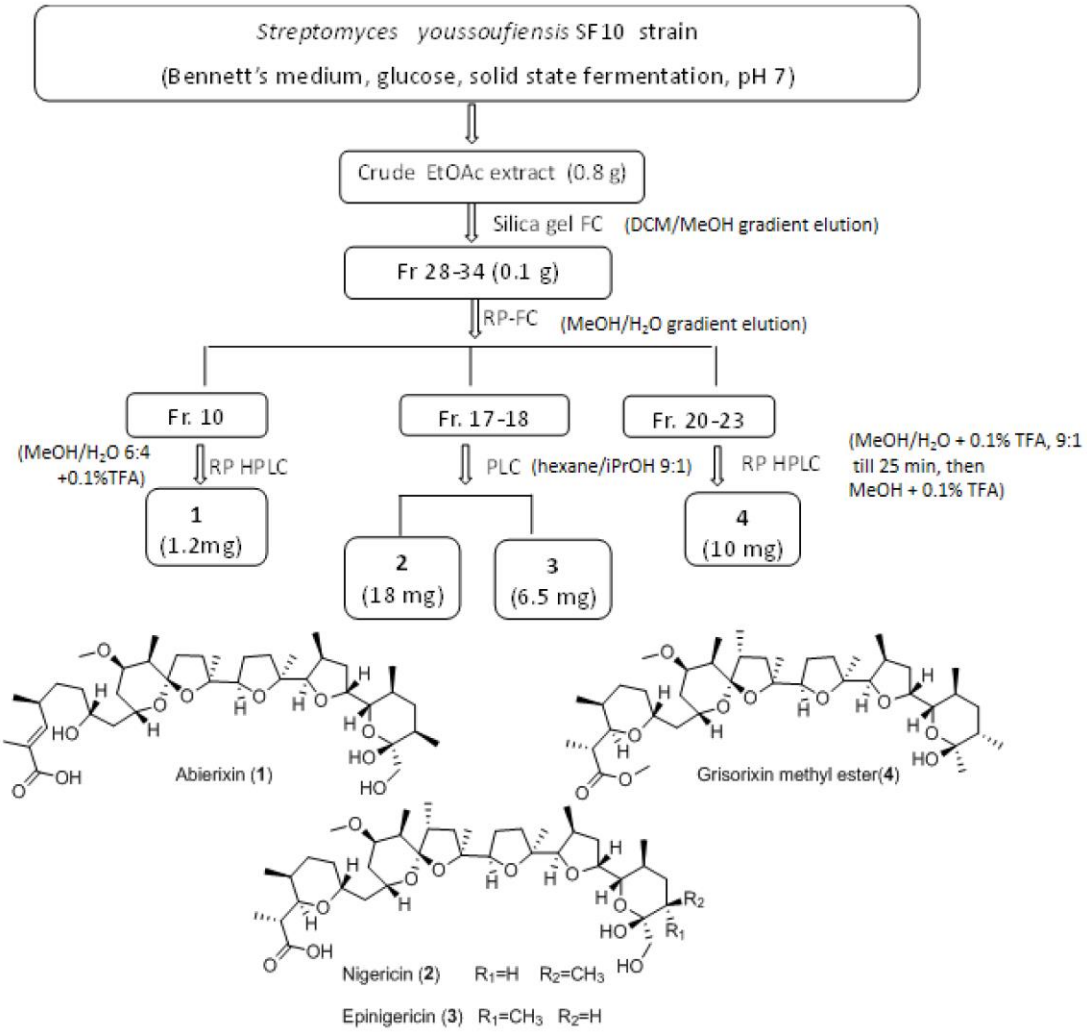
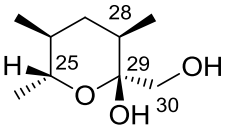
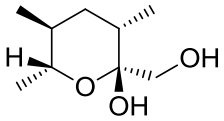
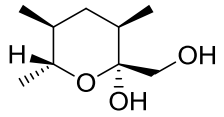
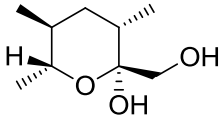
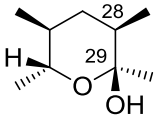
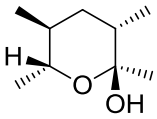
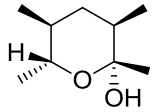
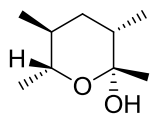


Table S2. Energy values obtained by DFT calculation at a B3LYP/6-31G(d,p) level of theory for simplified structures taken as model for the F-ring of the indicated polyether metabolite. The C-numbering resumes that reported for polyethers in Figure 1.

		In vacuo		In H_2O	
Nigericin-like metabolite	Structural Moiety	Energy (a.u.)	ΔE related to nigericin ($kJmol^{-1}$)	Energy (a.u.)	ΔE related to nigericin ($kJmol^{-1}$)
Nigericin (**2**)		-579.49456813	-	-579.50211165	-
Epinigericin (**3**)		-579.49472552	-0.4129	-579.50310163	-2.5992
C-29 epimer of nigericin		-579.49332270	+ 3.2719	-579.50051262	+4.1983
C-29 epimer of epinigericin		-579.49247169	+5.4877	-579.49975007	+6.2003
Grisorixin-like metabolite	Structural Moiety	Energy (a.u.)	ΔE related to grisorixin ($kJmol^{-1}$)	Energy (a.u.)	ΔE related to grisorixin ($kJmol^{-1}$)
Grisorixin		-504.28877454	-	-504.29328817	-
Epigrisorixin		-504.28631943	+9.9357	-504.29093337	+6.1825
C-29 epimer of grisorixin		-504.28317969	+14.6900	-504.28881966	+11.7321
C-29 epimer of epigrisorixin		-504.28136691	+19.4472	-504.28673415	+4.1127