# Supplementary Information
# Imaging mass spectrometry and MS/MS molecular networking reveals chemical interactions among cuticular bacteria and pathogenic fungi associated with fungus-growing ants

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## Supplementary Methods

### *Breast cancer bioassay*

Activity against the human breast cancer cell line MCF-7 (ATCC, Manassas, VA, USA) was done as described by Higginbotham et al.[2]. In short, the cells were incubated with RPMI-1640 supplemented with gentamicin, L-glutamine, $NaHCO_3$, HEPES buffer, and FBS at 37°C. Compound was diluted in RPMI-1640 media, added to the cells, and incubated for 48 h at 37°C. After the cells were fixed with trichloroacetic acid, rinsed with water and treated with sulphorhodamine B, the mixture was allowed to react for 15–30 min at 22°C. Then the cells were rinsed with trichloroacetic acid, dried, and treated with Tris-HCl (10 mM; pH 7) for 15 min. Finally color intensity was read at 570 nm, in a color plate reader (Benchmark Bio-Rad). Adriamycin diluted in DMSO was used as positive control (normal $IC_{50}$ value 20–50 nM).

### *Plasmodium falciparum bioassay*

Activity against the causative agent of malaria was performed by culturing human erythrocytes and infecting them with *P. falciparum*, as described by Trager and Jensen, 1976[3]. Briefly, the HB3 (Chloroquine sensitive) strain of *P. falciparum* was cultured in RPMI 1640, supplemented with 10% human serum (from O+ blood) at a hematocrit of 2% human erythrocytes (O+) at 37°C in a gas mixture of 5% $CO_2$, 5% $O_2$, and 90% $N_2$. Parasites were kept in the same phase of the life cycle through synchronization in a temperature cycling incubator[4]. The parasites were initially tested with 10 µg/mL of the extract containing the compound, while the $IC_{50}$ was obtained by adding no more than 10% v/v of the compound to the well, at different concentrations. The culture was incubated further for 24 h after which PicoGreen DNA fluorescent dye (Invitrogen, USA) was added to a final concentration of 1%. After 30 min incubation the signal was read on a fluorescence plate reader[5]. Chloroquine was used as reference positive control (average $IC_{50}$: 24.3 nM).

### *Trypanosoma cruzi bioassay*

Activity against the causative agent of Chagas disease was performed using a colorimetric method, assessing Tulahuen LacZ clone C4 of *T. cruzi* parasites expressing β galactosidase (ATCC, Manassas, VA, USA)[6]. Concisely, assays were performed in duplicate on amastigotes culture in RPMI-1640 supplemented with L-glutamine, HEPES buffer, $NaHCO_3$, dilution of a penicillin-streptomycin mix (1:100) and FBS at 37°C, them exposed to different concentrations of the test compounds under an atmosphere of 5% $CO_2$/95% air[2]. Cleavage of chlorophenol red-β-D-galactoside (CPRG, Roche Applied Science) by β-Gal expressed by the parasite was measured at 570 nm to

detect color intensity, in a color plate reader (Benchmark Bio-Rad). Nifurtimox diluted in RPMI-1640 medium was used as a positive control ($IC_{50}$ 0.15–13.4 µM)[2,6,7].

***Antibiotic susceptibility test***

Assays against strains of *Candida albicans* (ATCC® 10231™), *Staphylococcus aureus* subsp. *aureus* (ATCC® 43300™) and *Bacillus subtilis* subsp. *subtilis* (ATCC® 6051™) were performed using disk diffusion test, in duplicate as described by Boned[8] with some modifications[9]. Briefly, petri dishes with Müeller-Hinton agar were inoculated with a suspension of each test organism equivalent to 0.5 McFarland solutions, followed by the application of 6 mm papers disk on the surface of the agar and then impregnated with 25 µL of the compound at a concentration of 1024, 768, 512, 384, 256 and 128 µg/mL. Petri dishes were incubated at 30ºC for 18-24 hours before inhibitions zone were measured. MIC values were determined using regression analysis of inhibition zones, considering the absorptive model of diffusion[8].

***Antifungal bioassay***

Assays against fungal strains *Escovopsis sp*. (CBR53 and CBR38), *A. fumigatus* (ATCC® 1028™) were performed in duplicate using 6 mm papers disk containing three different concentrations of the compounds (1024, 512, and 256 µg/Ml). Hyphae plugs of fungal strains were placed in the center of the petri dishes containing Müeller-Hinton agar and incubated at 30ºC, evaluation of inhibition zones were carried out at 24, 48, 72, 96 and 120 hours[10].

## Supplementary Figures

**Figure S1.** MALDI imaging mass spectrometry experiment of the interaction between the bacterium *Streptomyces* CBR53 and the fungus *Escovopsis* TZ49, showing ions from 50 m/z to 800 m/z

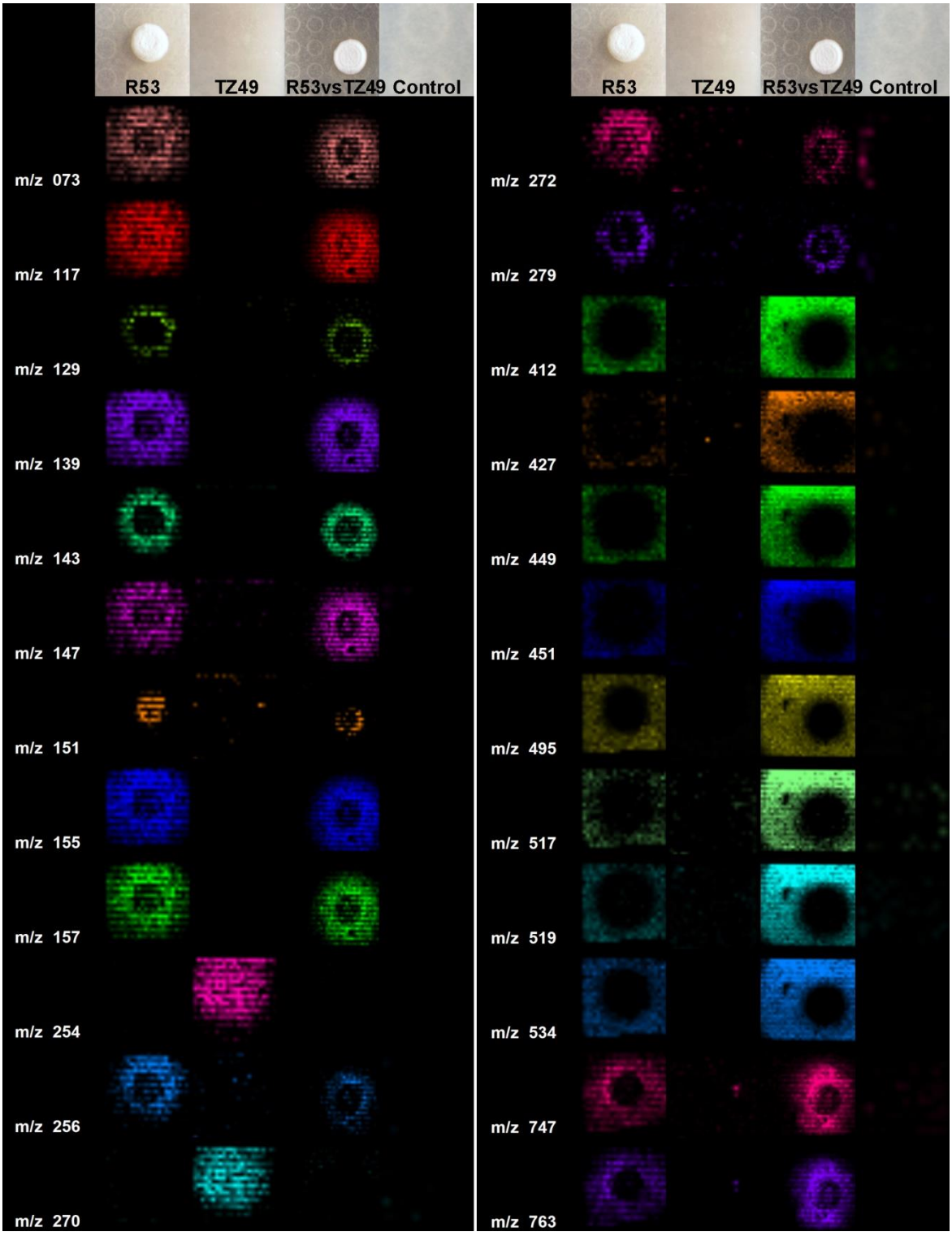


**Figure S2.** MALDI imaging mass spectrometry experiment of the interaction between the bacterium *Streptomyces* CBR53 and the fungus *Escovopsis* TZ49, showing ions from 800 m/z to 2500 m/z

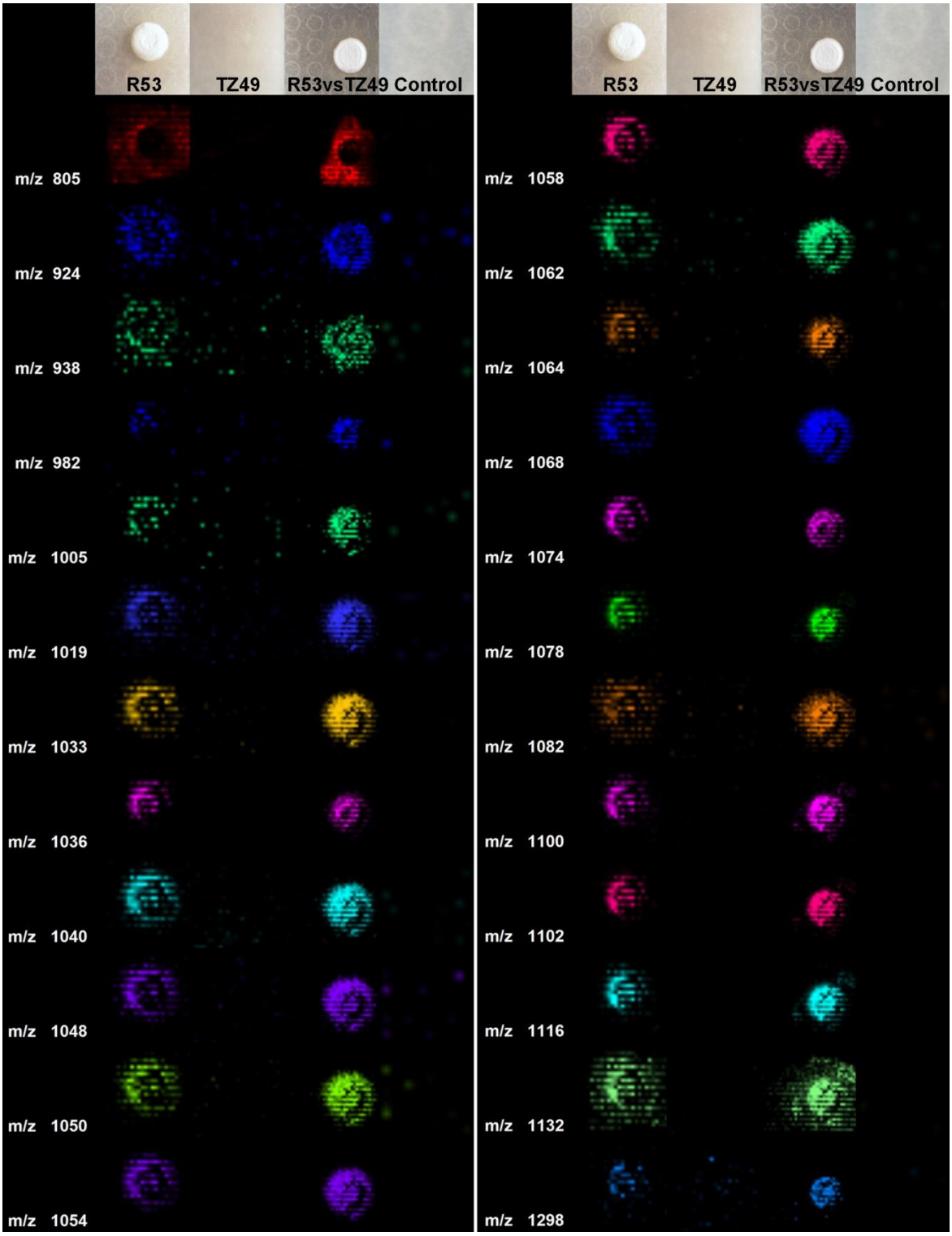


**Figure S3.** $^{1}$H-NMR spectra of elaiophylin (400 MHz, DMSO-d6)

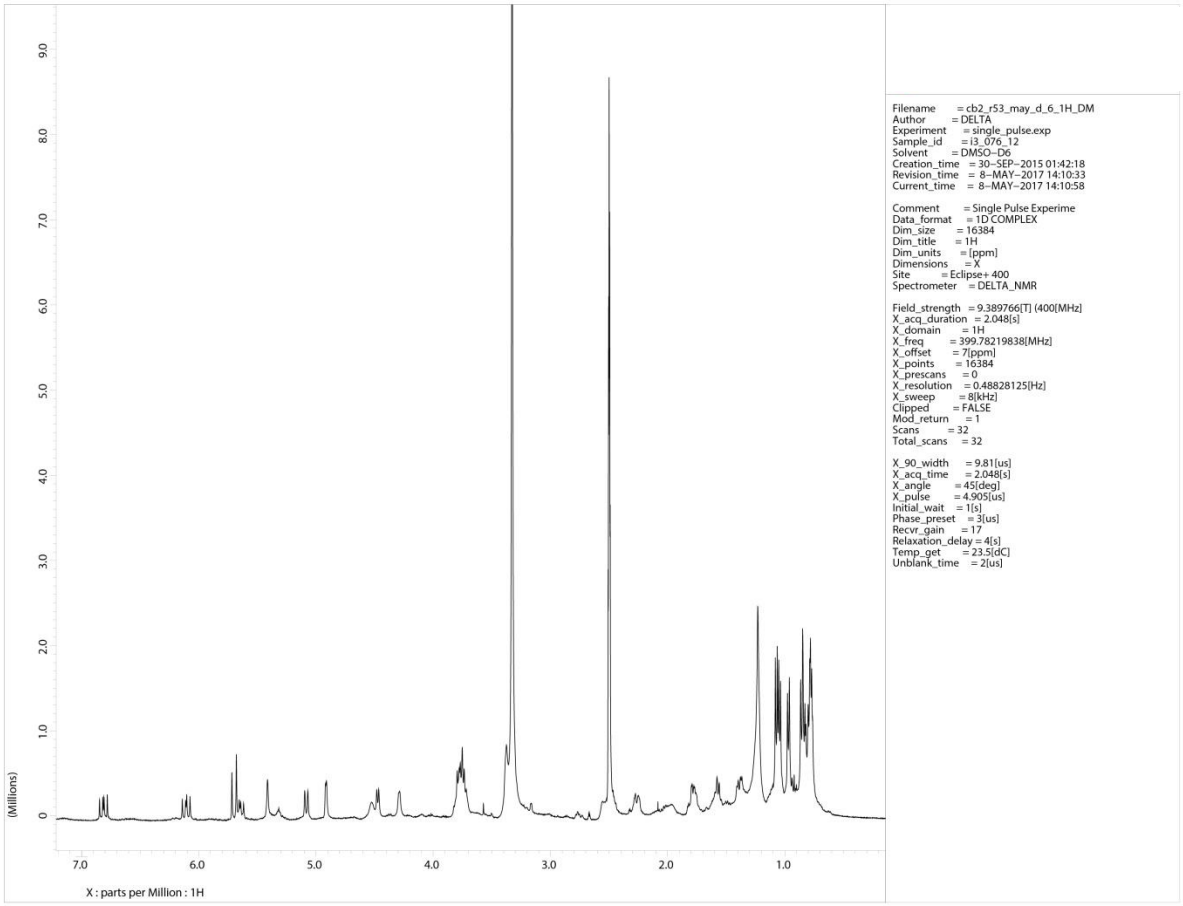


**Figure S4.** $^{13}$C-NMR spectra of elaiophylin (100 MHz, DMSO-d6)

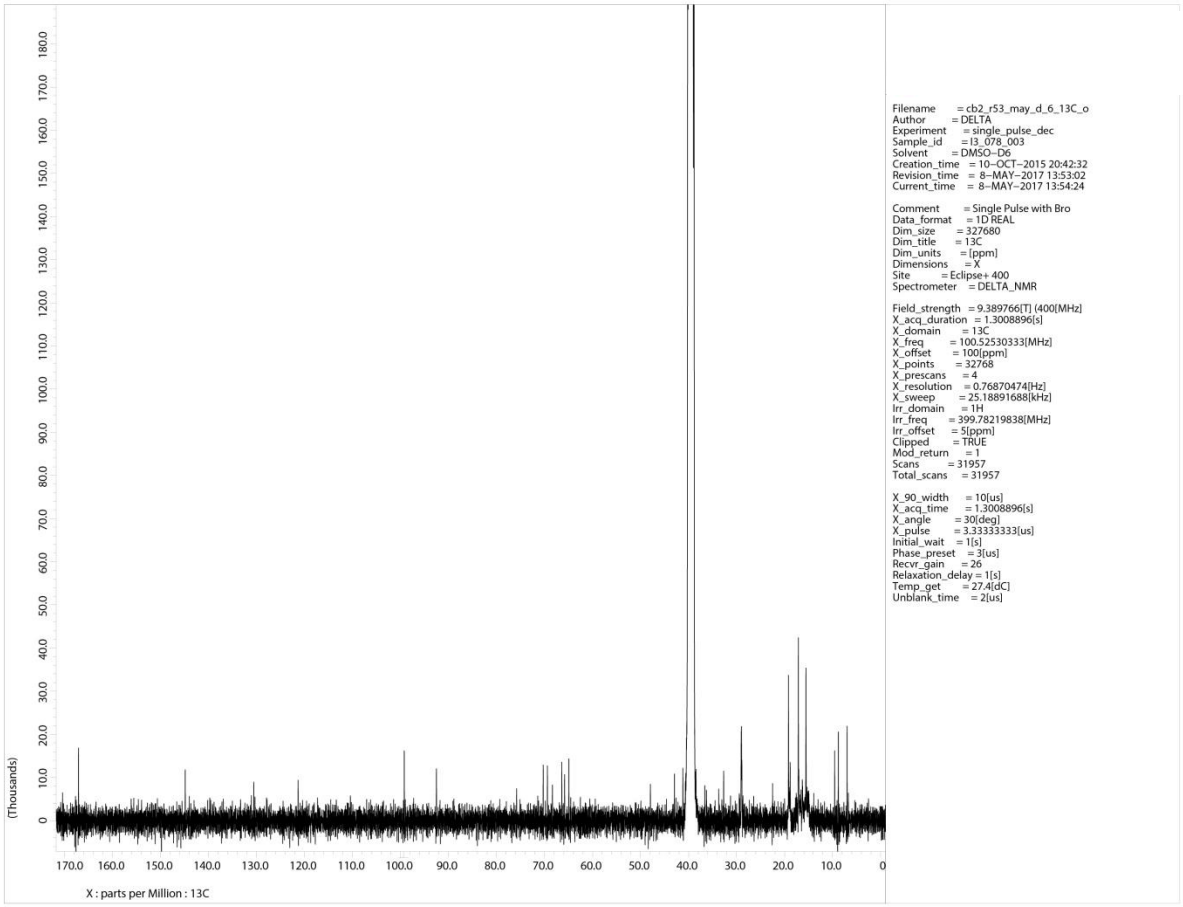


**Figure S5**. Direct MS-MS comparison of elaiophylin macrolide from the extract of *Streptomyces* CBR53 with elaiophylin-CDM (bronze spectrum) from GNPS spectral library (CCMSLIB00000479766). Picture show a singlet fragment at 729.3878 Da, which correspond to the fragmentation of the symmetrical groups bonded to the macrolactone ring of elaiophylin.

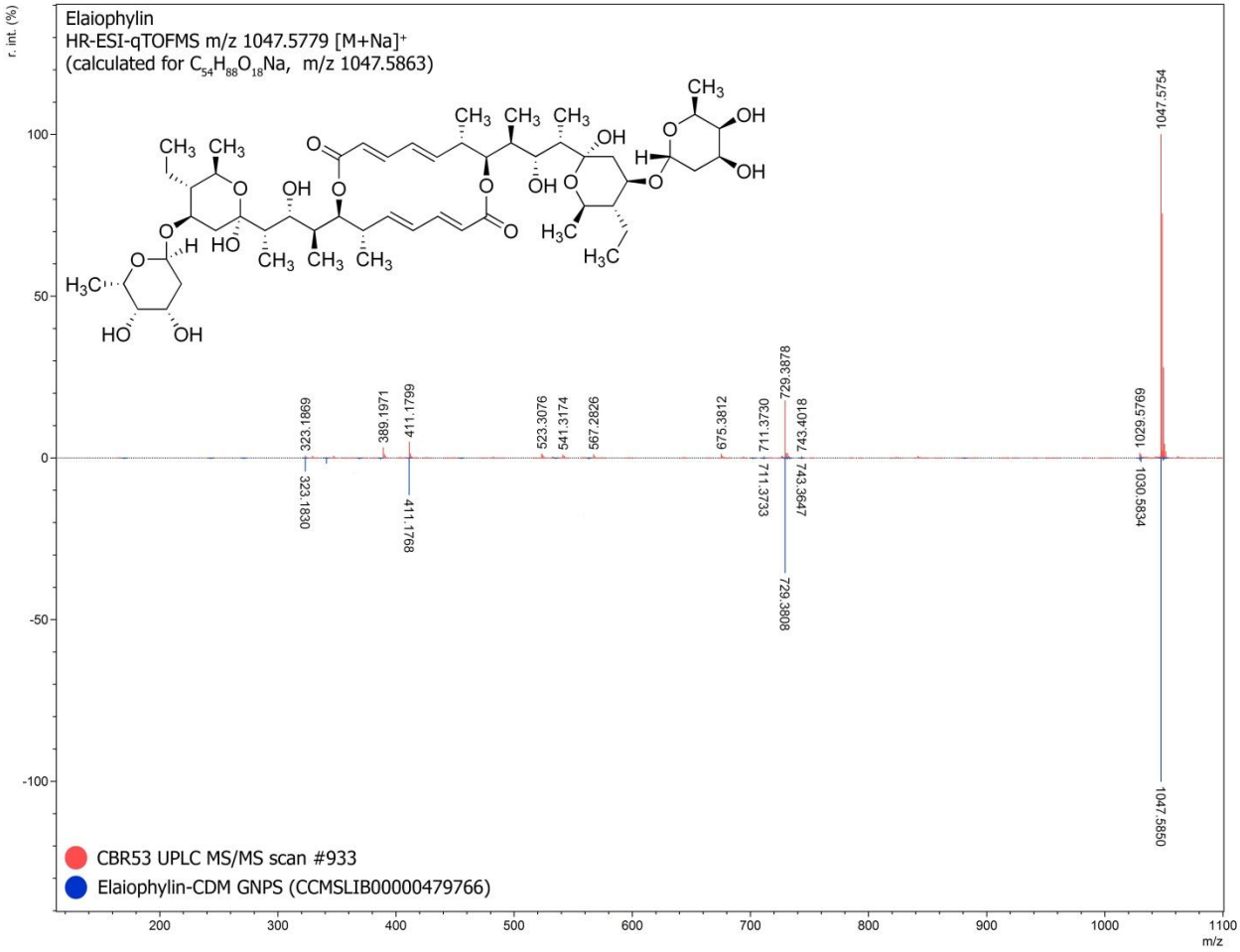


**Figure S*6*.** Efomycin G from the extract of *Streptomyces* CBR53. The spectrum show three mayor fragment ions: 411.1819 m/z which correspond to the aglycone; and the ions 715.3734, 729.3860 m/z, which indicate a cleavage of the nonsymmetrical groups between carbons 9-10. and 9''-10''.

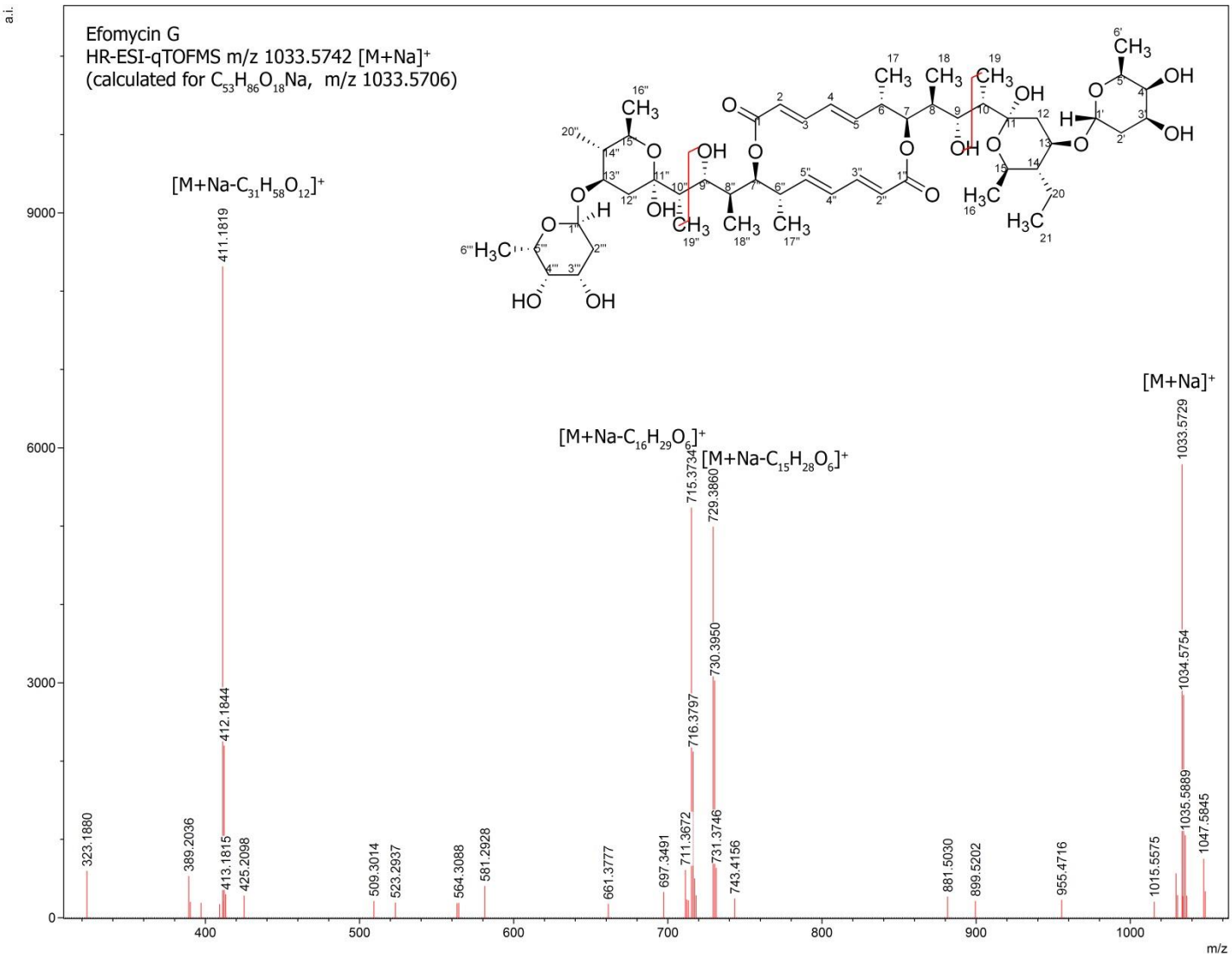


**Figure S7.** Efomycin A from the extract of *Streptomyces* CBR53. The spectrum show three mayor fragment ions: 411.1800 m/z which correspond to the aglycone; and the ions 743.3699, 729.3763 m/z, which indicate a cleavage of the nonsymmetrical groups between carbon 9-10 and 9''-10''.

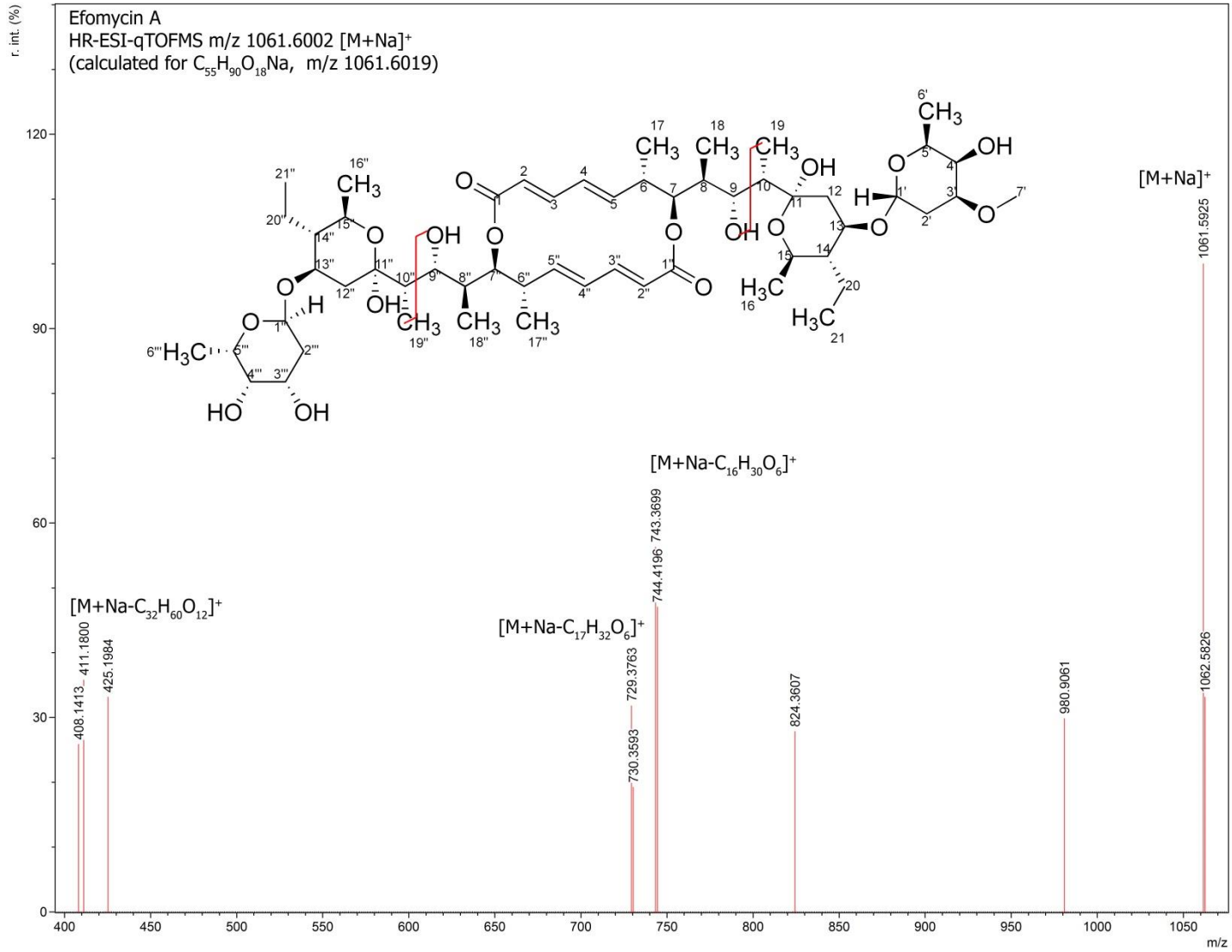


**Figure S8.** MS-MS comparison of unknown nodes 1065.6007, 1079.6139 and 1093.6222 from the extract of *Streptomyces* CBR53, with elaiophylin-CDM bronze spectrum from GNPS spectral library (CCMSLIB00000479766). Spectra show chemical shifts of 18, 32 and 46 Dalton related to elaiophylin.

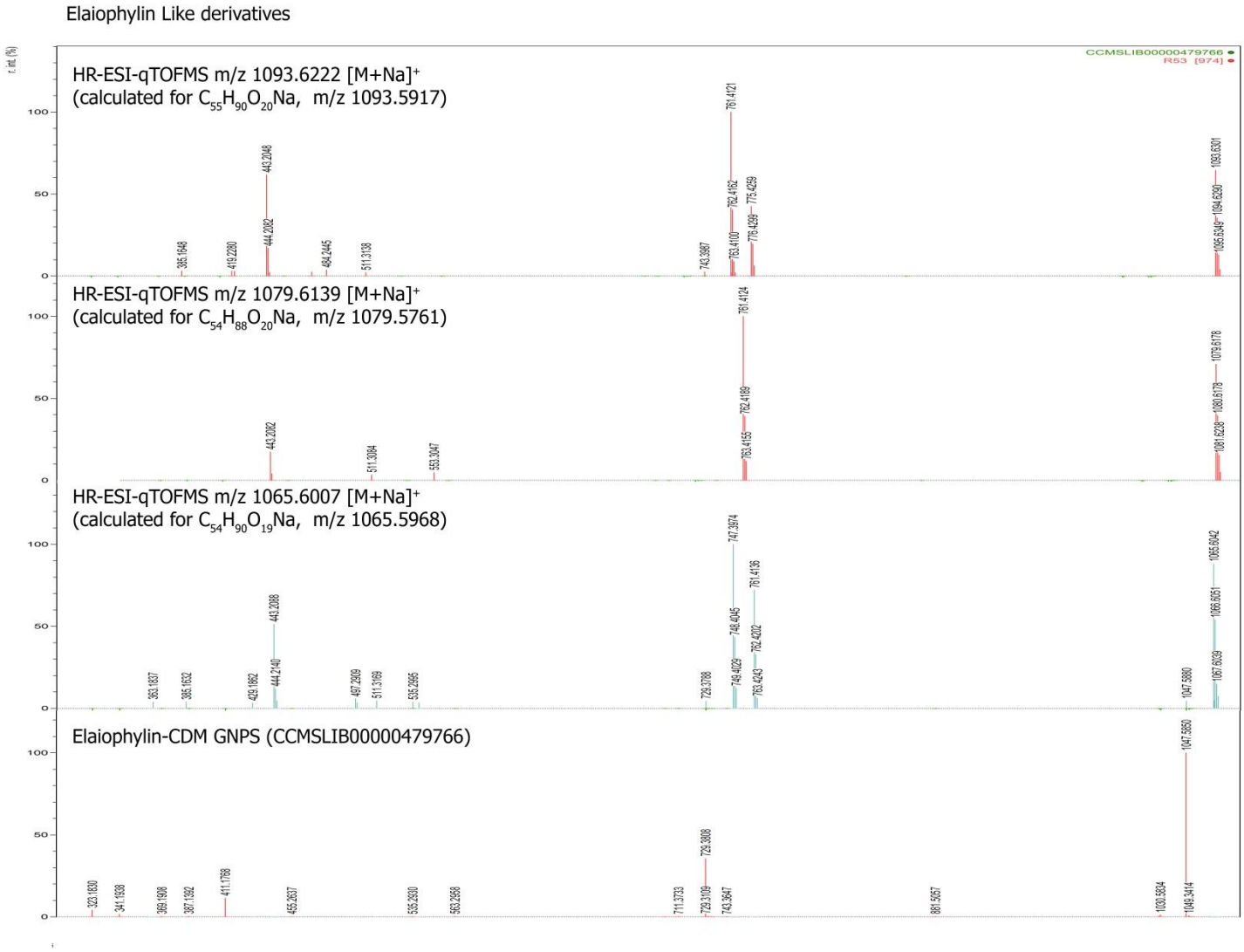


**Figure S9.** Direct MS-MS comparison of shearinine D from the extract of *Escovopsis* TZ49 with silver spectrum of Shearinine D_130028 from GNPS spectral library (CCMSLIB00000478461)

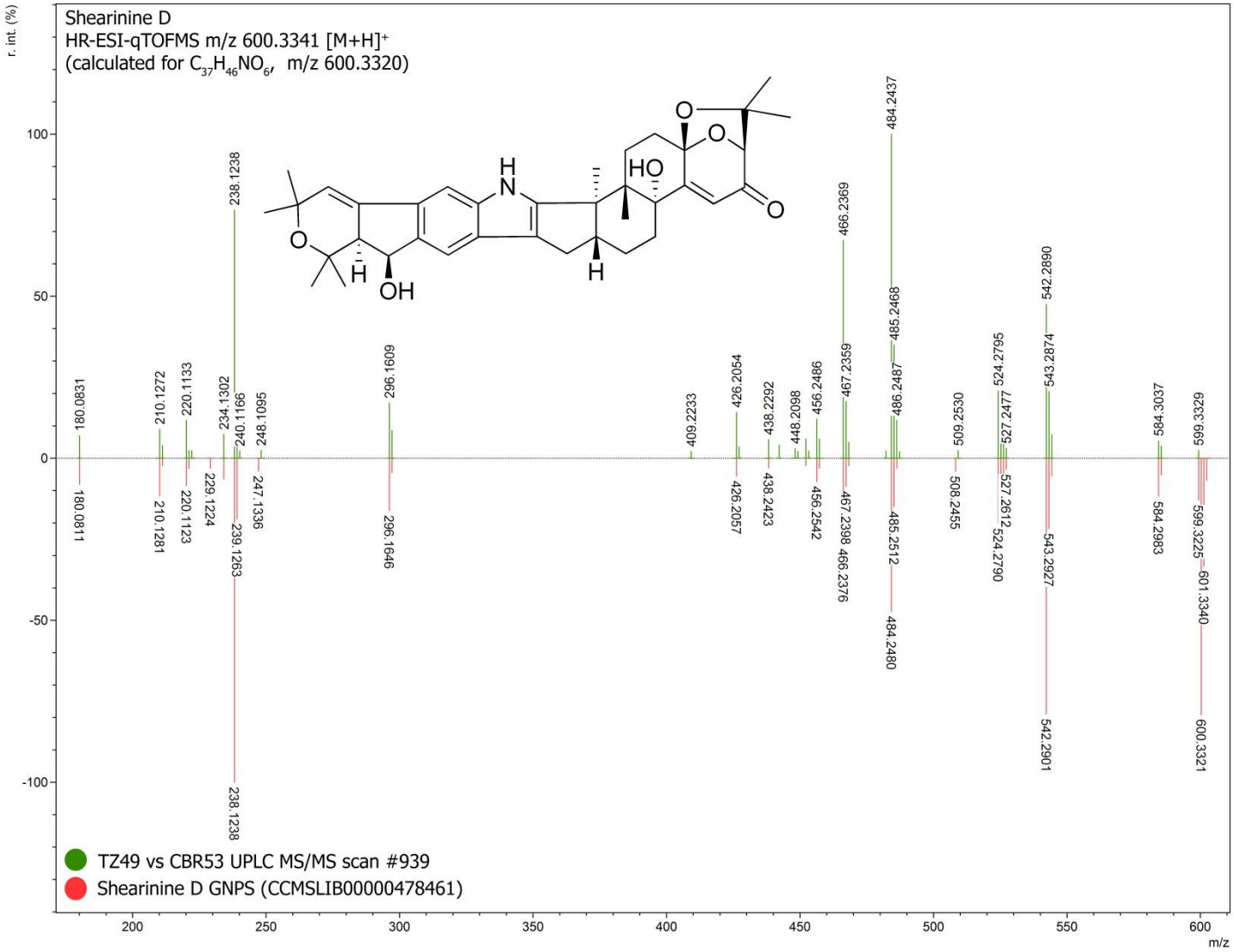


**Figure S10.** Direct MS-MS comparison of shearinine F from the extract of *Escovopsis* TZ49 with silver spectrum of Shearinine F_120146 from GNPS spectral library (CCMSLIB00000478066)

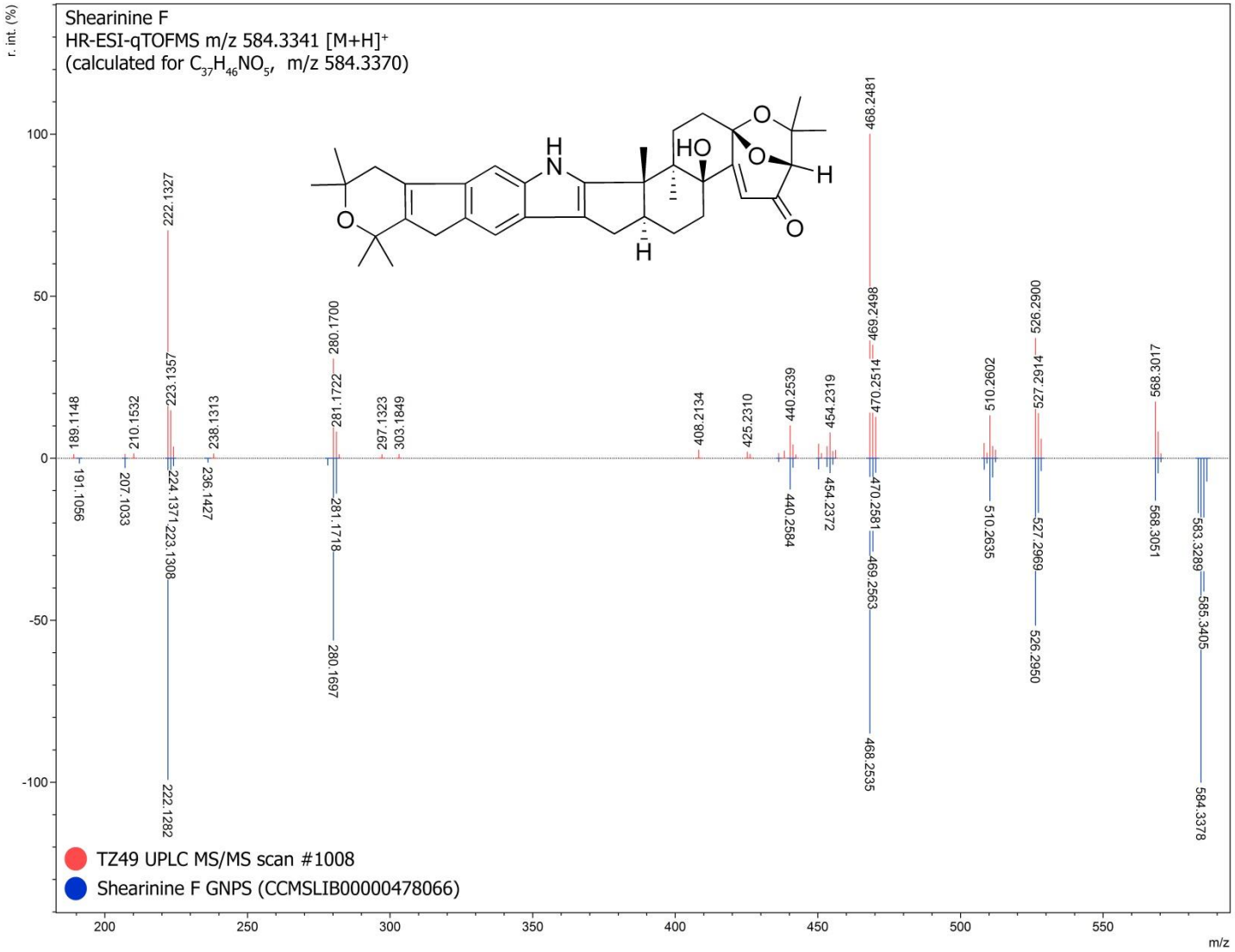


**Figure S11.** MALDI imaging mass spectrometry experiment of the interaction between the bacterium *Streptomyces* CBR38 and the fungus *Escovopsis* ACRO424, showing ions from 50 m/z to 2500 m/z

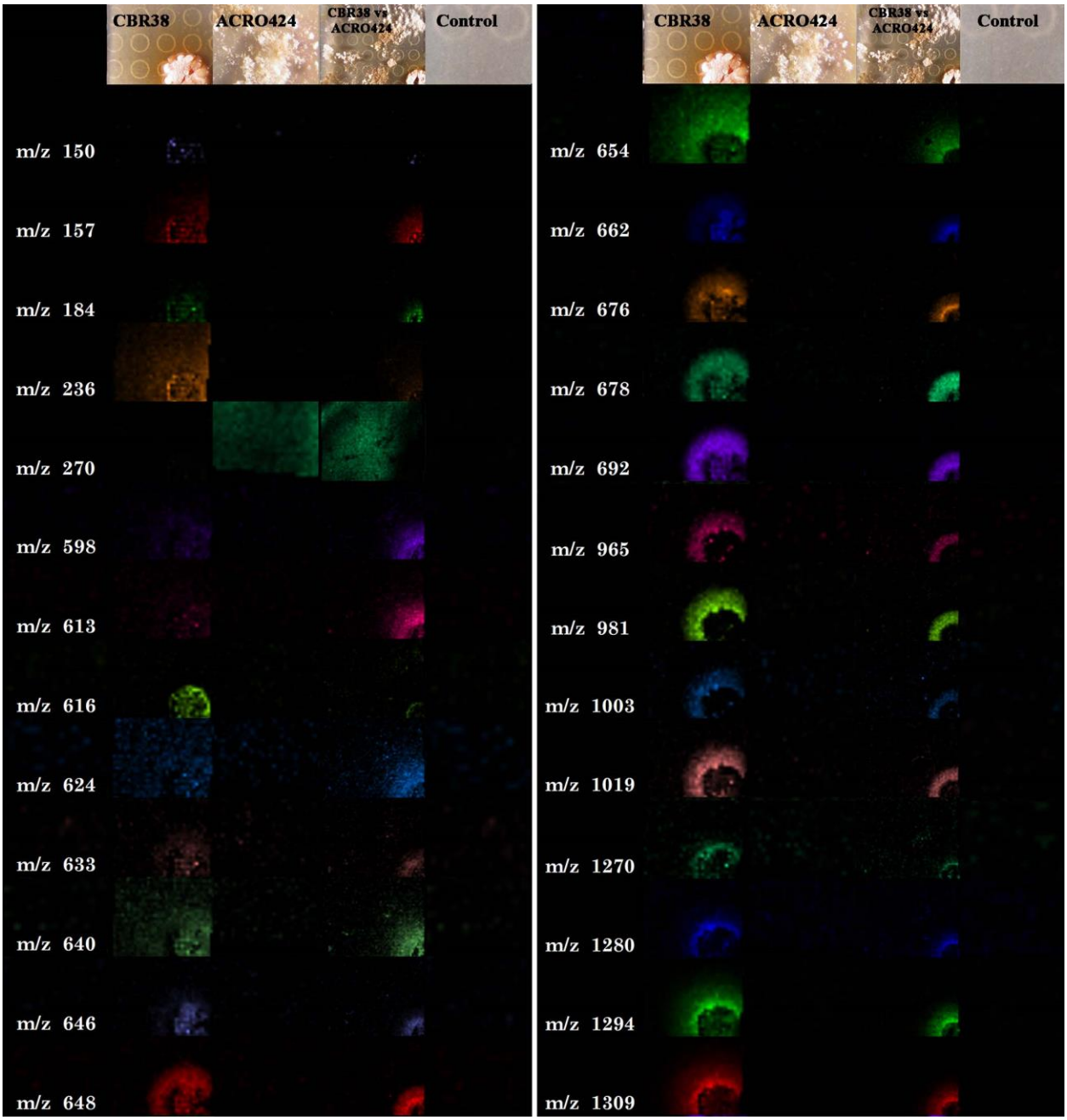


**Figure S12.** Direct MS-MS comparison of actinomycin D from the extract of *Streptomyces* CBR38 with actinomycin D gold spectrum from GNPS spectral library (CCMSLIB00000006871)

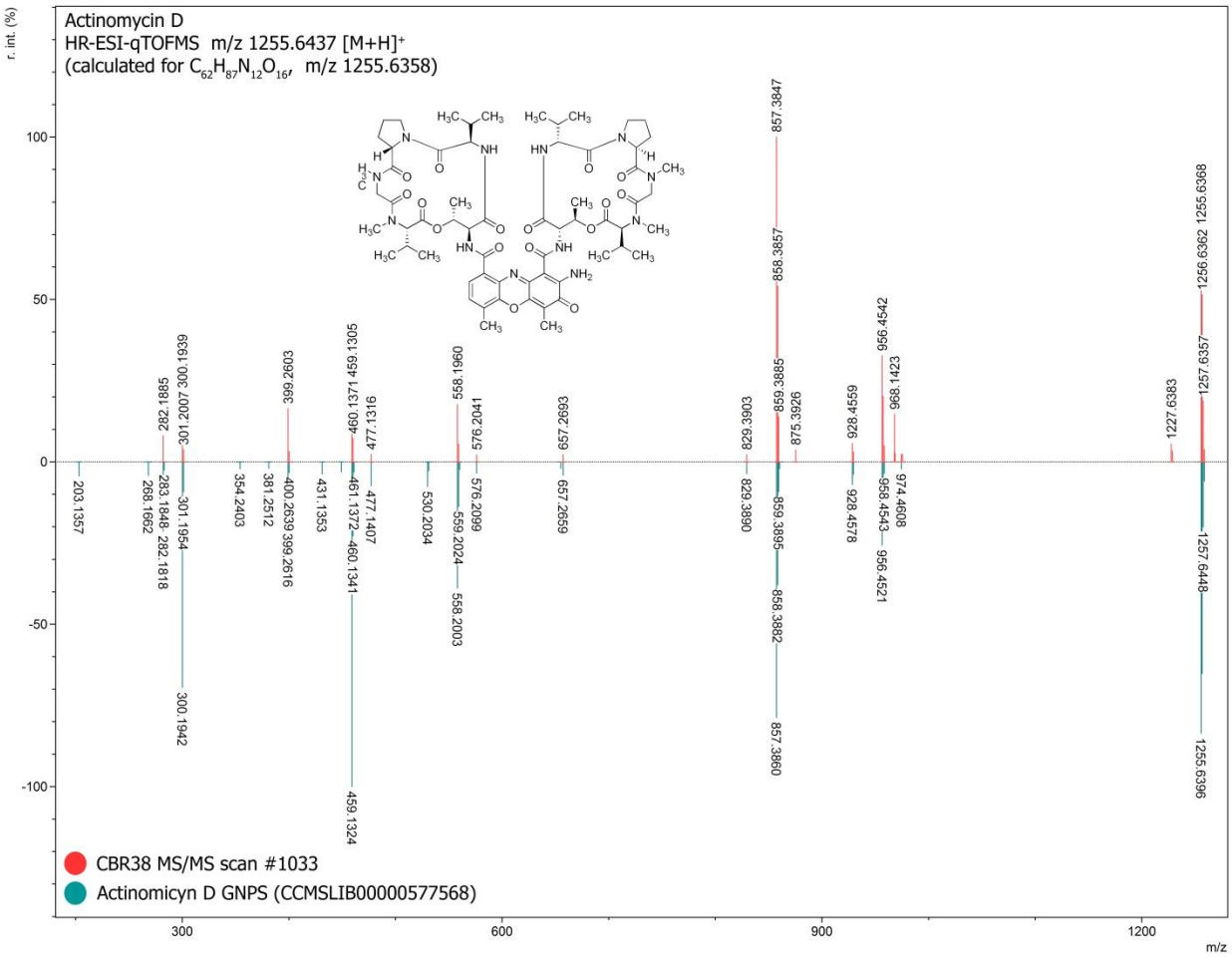


**Figure S13.** Direct MS-MS comparison of actinomycin X2 from the extract of *Streptomyces* CBR38 with actinomycin X2 gold spectrum from GNPS spectral library (CCMSLIB00000577607)

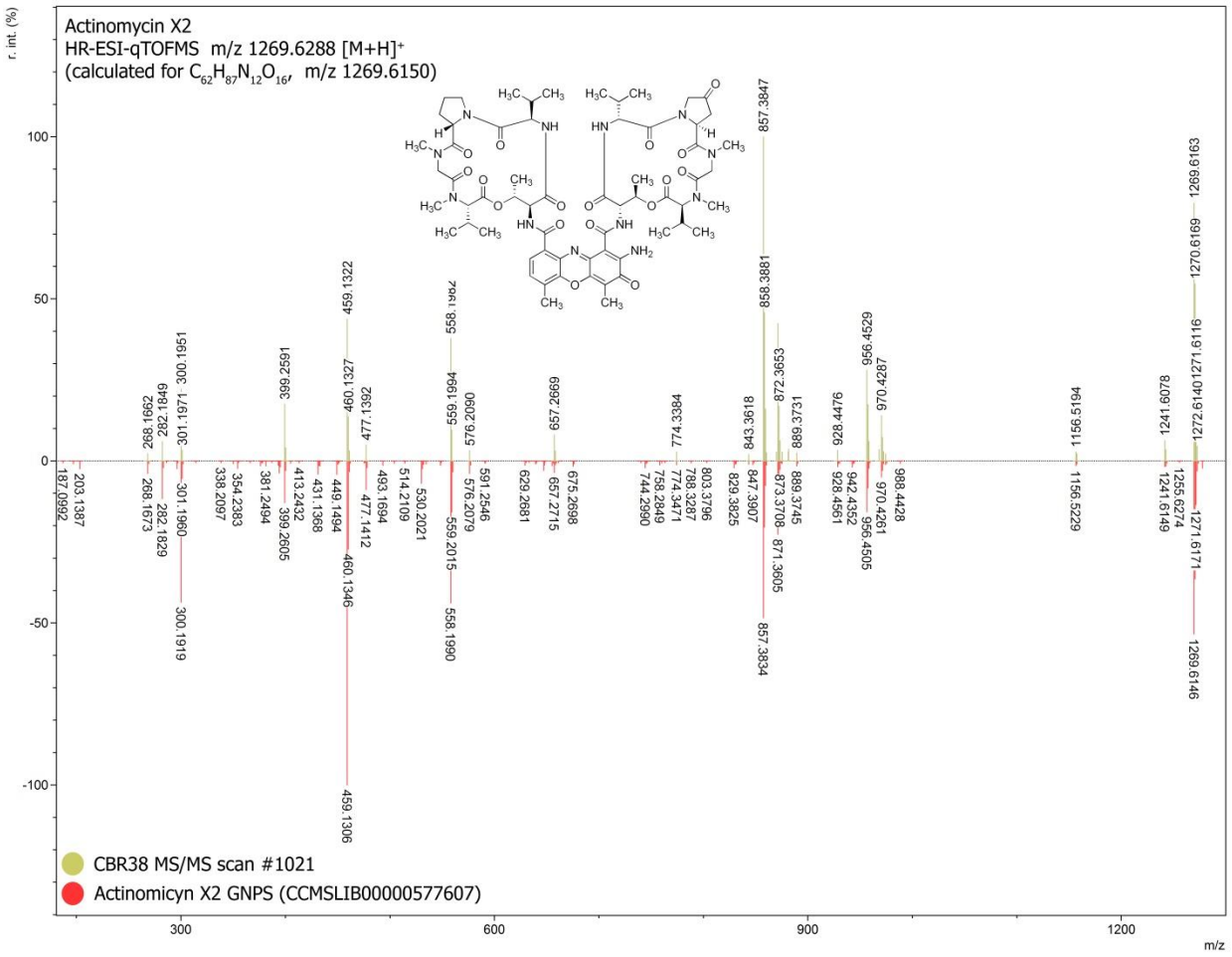
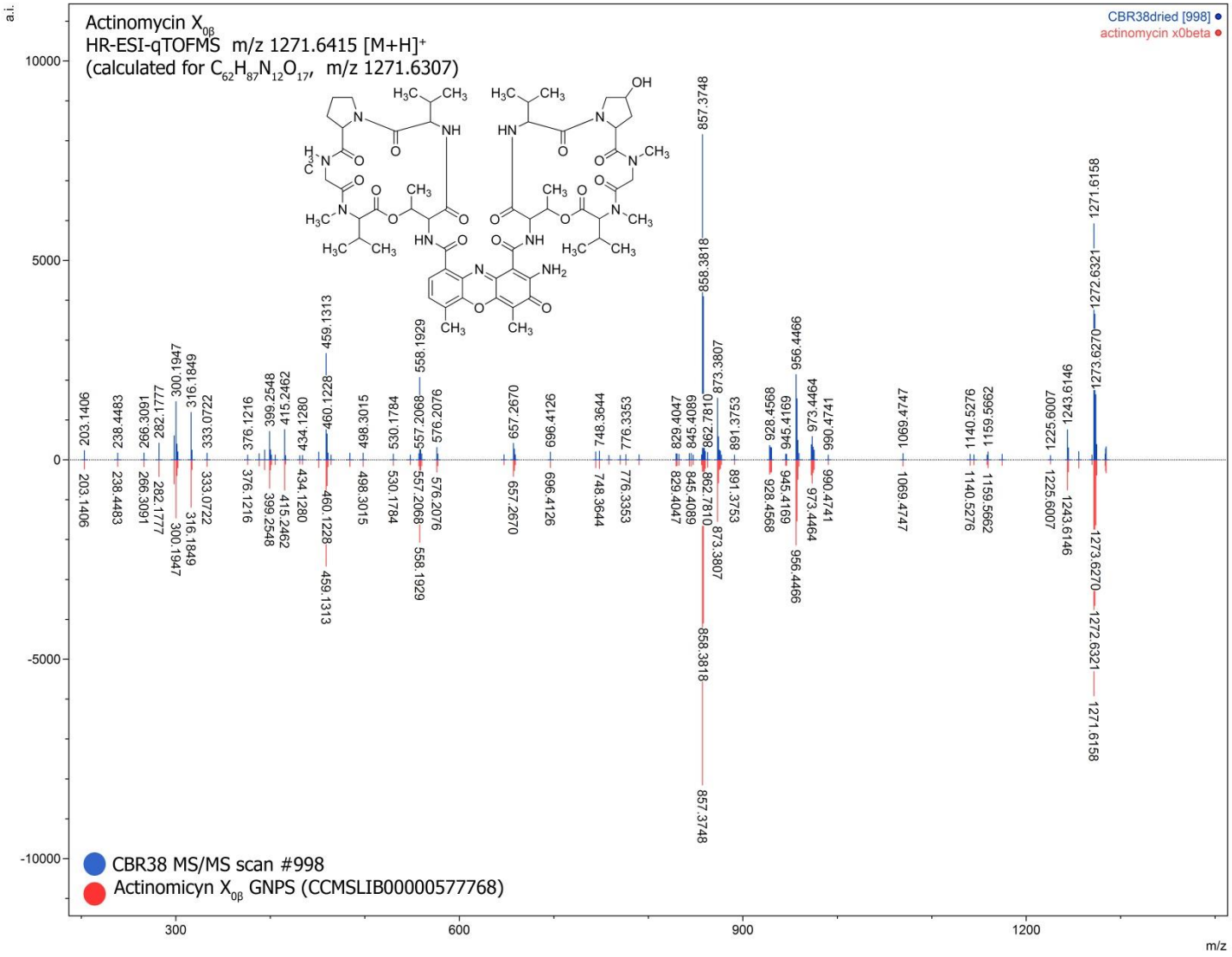
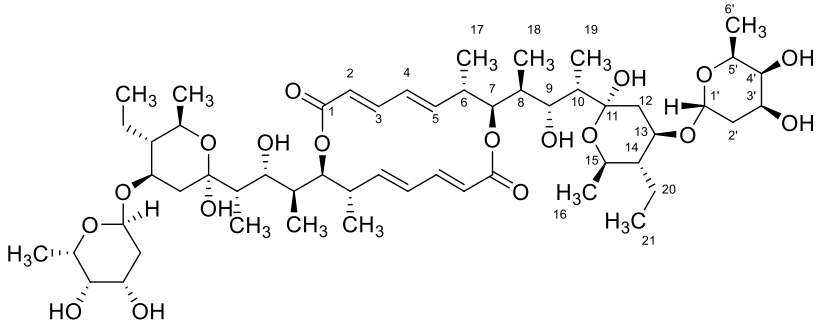


**Figure S14.** Direct MS-MS comparison of ion 1271.6415 with actinomycin $X_{0\beta}$ (CCMSLIB00000577768), result show twenty five shared peak (25) and less than 10 ppm of error for the calculated molecular formula.



***Supplementary Table***

**Table S1. Comparison of** $^{13}C$ and $^{1}H$ NMR spectra of compound 1 (400 MHz, DMSO-d6, δ in ppm relative to solvent) and elaiophylin data from literature[1] (360 MHz, DMSO-d6, δ in ppm relative to internal TMS); Carbon numbers represent the lower and upper half portion of the molecule.



| Carbon | Compound 1, $\delta_C$ | Elaiophylin, $\delta_C$ | Compound 1, $\delta_H$ (m, J in Hz) | Elaiophylin $\delta_H$ (m, J in Hz) |
|---|---|---|---|---|
| 1 | 167.0966 | 167.22 | - | -- |
| 2 | 121.3408 | 121.33 | 5.70 (d, *J*=15.14) | 5.66 (d, *J*=15) |
| 3 | 144.8549 | 144.86 | 6.82 (dd, *J*=15.38, 10.99) | 6.80 (dd, *J*=15, 11) |
| 4 | 130.5782 | 130.65 | 6.11 (dd, *J*=15.14, 11.23) | 6.08 (dd, *J*=15, 11) |
| 5 | 144.8549 | 144.86 | 5.64 (m) | 5.61 (dd, *J*=16, 10) |
| 6 | 41.1816 | 41.26 | 2.50 (m) | 2.50 (m) |
| 7 | 75.8113 | 75.9 | 5.09 (d, *J*=10.25) | 5.06 (d, J=11) |
| 8 | 36.2769 | 36.31 | 1.80 (m) | 1.82 (m) |
| 9 | 69.4062 | 69.53 | 3.76 (m) | 3,70 (m) |
| 10 | 42.9404 | 42.82 | 1.59 (m) | 1.6 (m) |
| 11 | 99.2291 | 99.19 | -- | -- |
| 12 | 36.6180 | 36.98 | 2.27 (m) | 2.27 (m) |
| 13 | 66.4224 | 66.42 | 3.76 (m) | 3,70 (m) |
| 14 | 47.9491 | 48.05 | 1.08 (d, *J*=6.35) | 1.1 (m) |
| 15 | 65.8198 | 65.93 | 3.76 (m) | 3,70 (m) |
| 16* | 19.1800 | 19.14 | 1.05 (d, *J*=5.86) | 1.07 (m) |
| 17* | 15.5370 | 15.49 | 0.97 (d, *J*=6.84) | 1.01 (d, *J*=6) |
| 18 | 8.7940 | 8.81 | 0.81 (m) | 0.83 (d, *J*=6) |
| 19 | 6.9848 | 6.90 | 0.81 (m) | 0.83 (d, *J*=6) |
| 20 | 19.1800 | 19.14 | 1.38 (d, *J*=4.88)<br>1.61 (m) | 1.41 (m)<br>1.60 (m) |
| 21 | 9.5663 | 9.47 | 0.81 (m) | 0.83 (d, *J*=6) |
| 1' | 92.5197 | 92.68 | 4.92 (d, *J*=3.42) | 4.90 (d, *J*=4Hz) |
| 2' | 32.6890 | 32.7 | 1.40 (m);<br>1.80 (m) | 1.41(m)<br>1.82 (m) |
| 3' | 64.9420 | 65.04 | 3.76 (m) | 3,70 (m) |
| 4' | 70.2872 | 70.39 | 3.38 (br. s.) | 3.36 (br.s.) |
| 5' | 68.3556 | 68.56 | 3.76 (m) | 3,70 (m) |
| 6' | 17.1215 | 17.10 | 1.07 (d, *J*=6.35) | 1.07 (d, *J*=6) |

* Carbon numeration corrected established by COSY correlations and literature[11,12]

***Supplementary References***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