Electronic Supplementary Material (ESI) for Molecular BioSystems

Supporting Information

Heterologous Production of Bisucaberin using a Biosynthetic Gene Cluster Cloned from a Deep Sea Metagenome

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General Experimental Procedures. FAB mass spectra were measured on a JEOL JMS-700 MStation mass spectrometer using glycerol as matrix. UV spectum was recorded on a Jasco V-530 UV/Vis spectrophotometer in MeOH. NMR spectra were recorded on a JEOL JNM−A500 NMR spectrometer at 500 MHz for ^{1}H and at 125 MHz for ^{13}C in DMSO *d*-6 + CD_3OD (1 : 1). Chemical shifts of ^{1}H and ^{13}C NMR spectra were referenced to the solvent peaks: δ_H 3.30 and δ_C 49.0 for CD_3OD.

Extraction of Metagenomic DNA from Deep Sea Sediment

Bottom sediment and benthos including marine sponges and corals were collected by dredging from the depth of approximately 150-1,000 m off Tokara Islands in East Chinese Sea (November, 2009). Samples were directly poured into flasks containing sterilized sea water, and then vigorously shaken overnight at room temperature to wash out bacteria cells from the deep sea samples. The flasks were kept for 30 min to settle large materials, and then supernatant was transferred into centrifuge bottles. After slow centrifugation (1,000 rpm for 5min) to remove high density contamination, the supernatant was re-centrifuged 5,000 rpm for 5min to afford cell pellet. Combined pellet was suspended into 20 mL of TES buffer (20 mM Tris-HCl (pH 8.0), 100 mM EDTA (pH 8.0), 50 mM NaCl, 25% sucrose), and then centrifuged at 8,000 rpm for 10 min, followed by re-suspended in 18 mL of TES buffer. A 900 μL of 10% SDS and 90 μL of proteinase K (100 mg/mL) were added in this suspension, and this as incubated at 37°C for 1 hour. After the addition of 3 mL of 5 M NaCl and 2.4 mL of 10% cetyltrimethylammonium bromide (CTAB) in 0.7 M NaCl, the cell lysate was heated at 65°C for 10 min. An equal volume of phenol-chloroform-isoamyl alcohol (25 : 24 : 1) was added to the solution. This was mixed gently, and then centrifuged at 8000 rpm for 10 min. The recovered upper layer was washed with chloroform-isoamyl alcohol (24 : 1). Followed by the crude metagenomic DNA was concentrated by precipitation from the aqueous layer with the addition of 0.6 volume of isopropanol.

Purification of Metagenomic DNA and Library Construction

Crude metagenomic DNA was size fractionated by agarose gel electrophoresis (1% low melting

point agarose gel, 30 V for 15 hours). The DNA above 23 kbp was recovered from the gel by thermostable β-agarase (Nippon Gene, Tokyo, Japan) following the protocol provided by the manufacturer. Purified DNA was blunt-ended with End-It DNA End-Repair Kit (Epicentre, Madison, WI), followed by ligated into pCC1FOS fosmid vector (Epicentre). This was subjected to packaging reaction with MaxPlax Lambda Packaging Extract (Epicentre), and then transfected into *Escherichia coli* EPI300-T1^{R} (Epicentre) and plated on the LB agar containing chloramphenicol as a selection marker following the protocol provided by the manufacturer.

Siderophore Assay

Sterilized chrome azurol S (CAS) stock solution (0.1 mM $FeCl_3$, 1.0 mM HCl, 1.0 mM CAS, 2.0 mM CTAB) was mixed with melted LB agar (1 : 9), followed by this was solidified to make CAS assay plates. Metagenomic clones or paper discs (6 mm in diameter) absorbing test samples were put on CAS assay plates, then kept overnight at 37^{o}C and room temperature respectively.

Cloning of the Bisucaberin Biosynthetic Gene Cluster

Fosmid DNA obtained from a siderophore producing clone MG110-3 was partially digested by Sau3AI. Nucleotide fragments between 5-10 kbp was recovered from the agarose electrophoresis gel, ligated into pHY300PLK plasmid vector (Takara Bio, Shiga, Japan), and transformed into NEB10β competent cell (New England Biolabs, Ipswich, MA). Siderophore producing subclone, MG116-3, which containing approximately 5.5 kbp insert DNA was subjected to nucleotide sequencing (DNA sequencing was performed by Hokkaido System Science (Sappro, Japan), Oligo DNAs were purchased from Invitrogen (Carlsbad, CA)). DNA analysis including contig construction and open reading frame detection was done by Vector NTI (Invitrogen). A homology search was performed by NCBI BLAST.

DNA Sequence of Bisucaberin Biosynthetic Gene Cluster

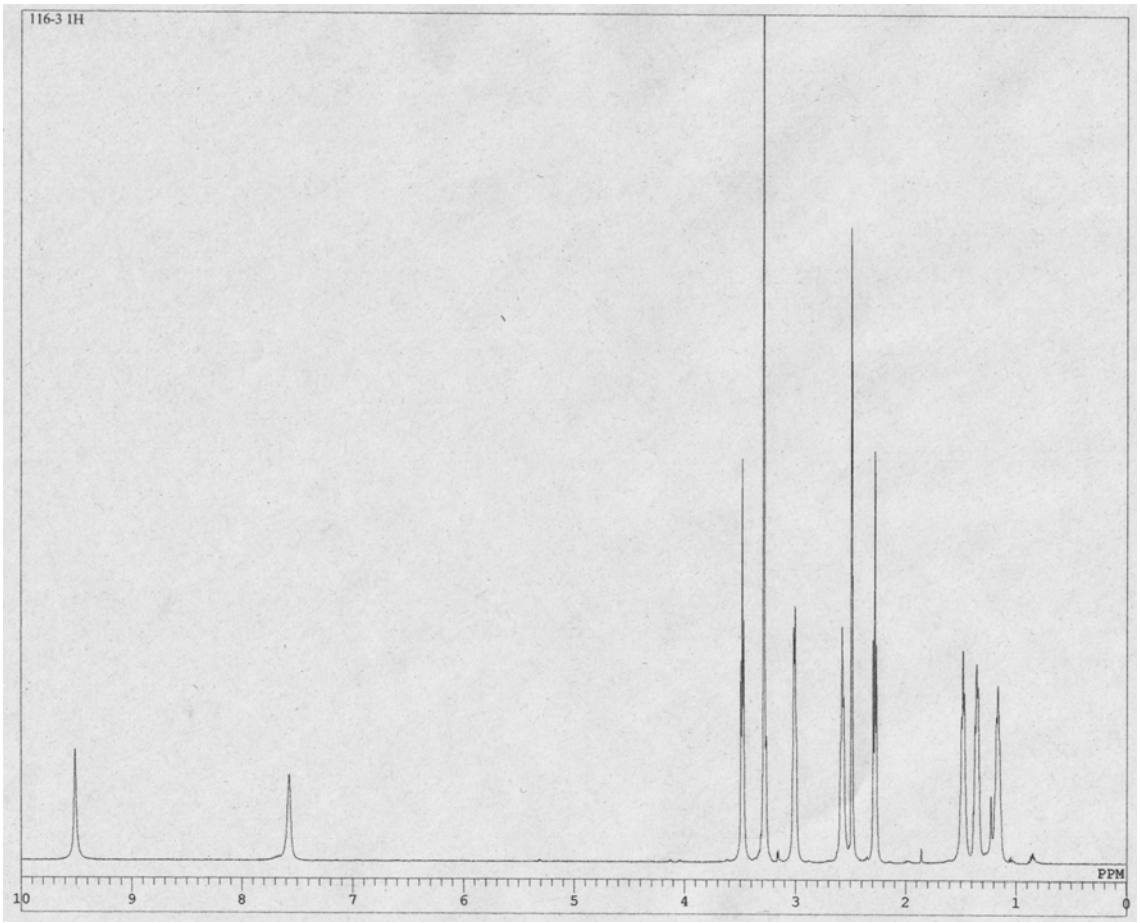
DNA Data Bank of Japan (DDBJ); AB643578, bisucaberin biosynthetic gene cluster from marine environmental sample.

Production and Isolation of Bisucaberin

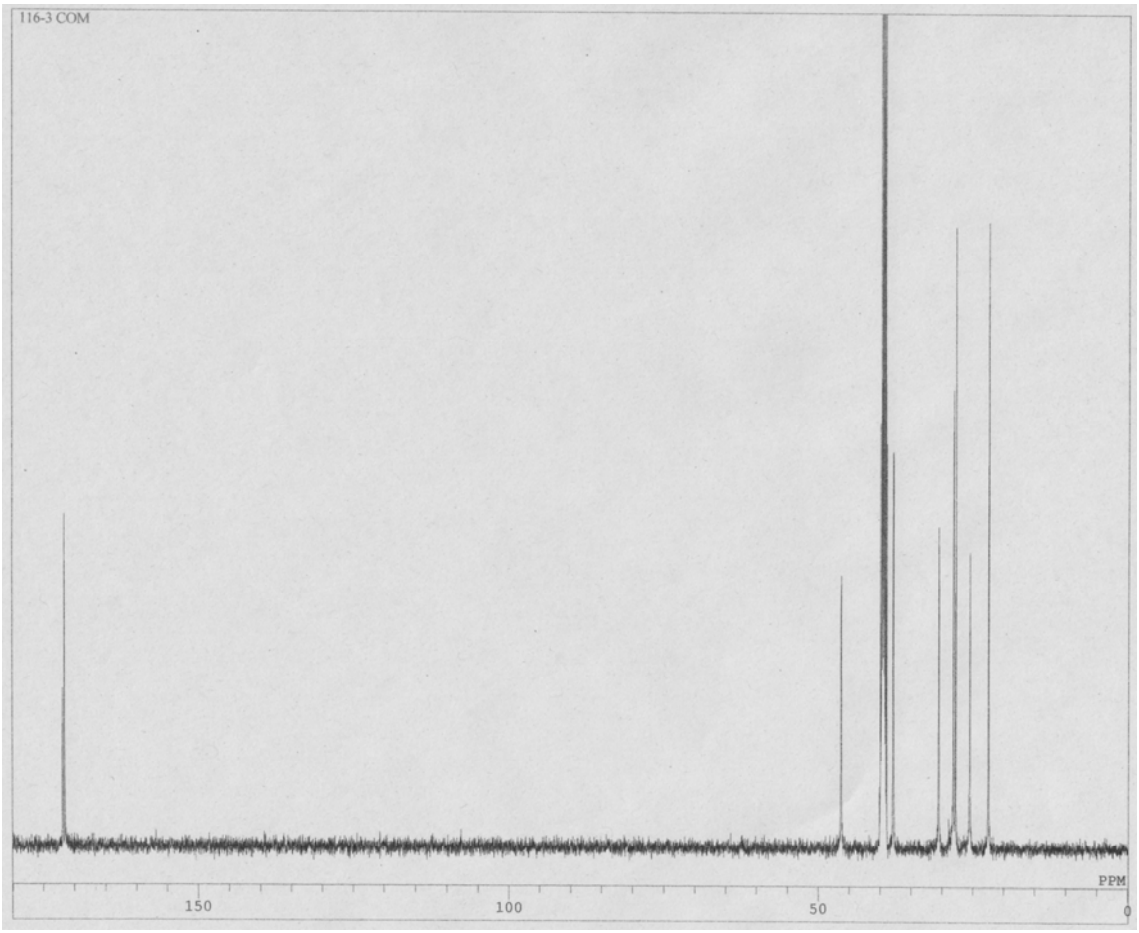
Bisucaberin producing clone, MG116-3, was cultured in LB medium supplemented with chloramphenicol (30 μg/mL) at 30^{o}C for 3 days under shaken. The centrifuge clarified conditioned medium was subjected to the solid phase extraction with C18 resin, and then eluted with MeOH. The concentrated extract was fractionated by C18 column chromatography with stepwise gradient system from pure water to methanol. Siderophore activity was found in the 30% and 60% methanol fractions.

They were combined and subsequently chromatographed by gel filtration with Sephadex G-10 (with water) followed by Sephadex LH-20 (with MeOH). Active fraction obtained after two steps of gel filtration was separated by reversed phase HPLC using C30 column (Develosil C30) to afford nearly pure material. Active compound was isolated by recrystallization from water to form white powder as a single isolated compound (100.7 mg from 12 L culture broth).

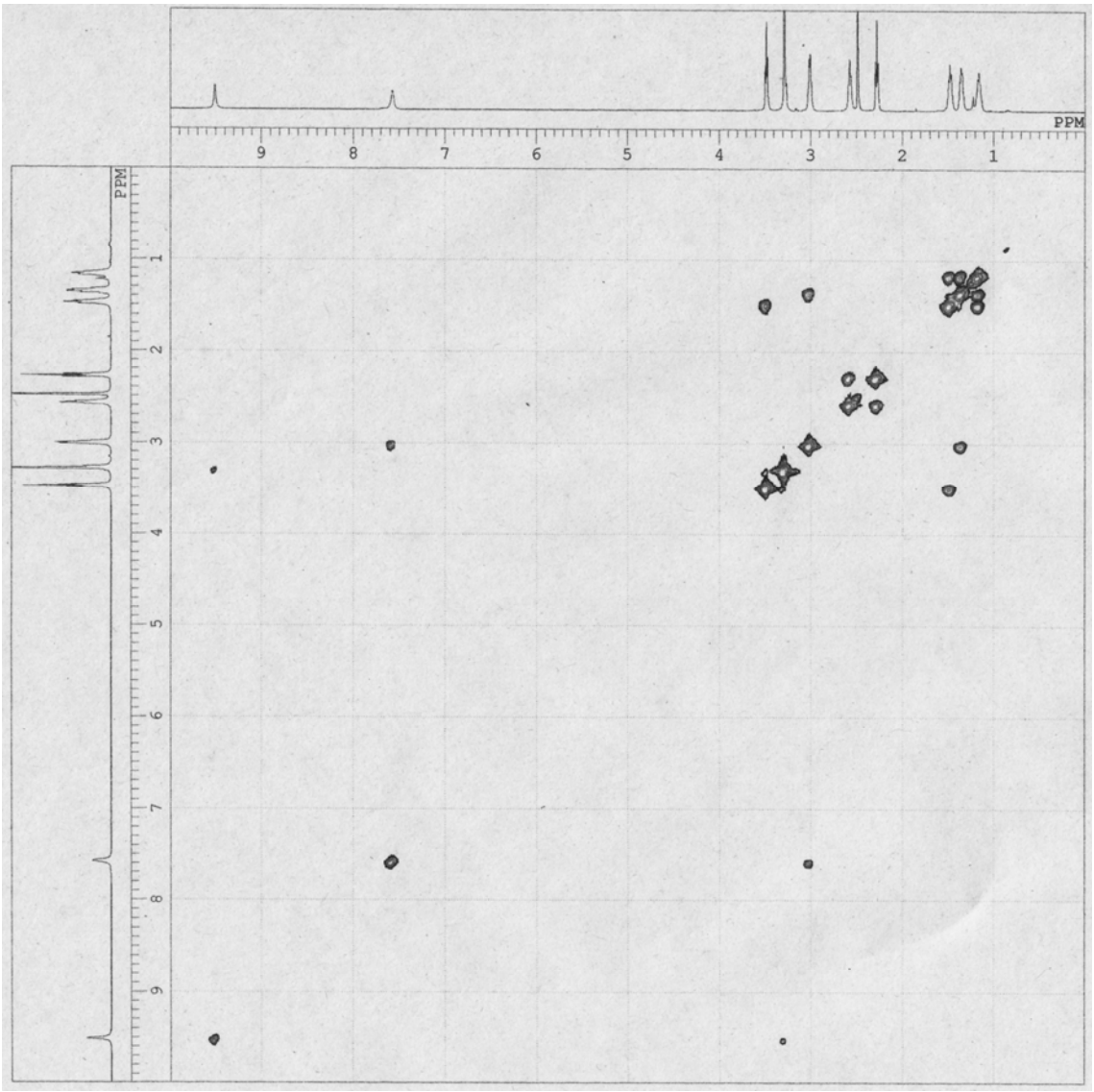
Bisucaberin (1): white powde; UV (MeOH) No λ_{max} above 220 nm; ^{1}H and ^{13}C NMR, see **Table 1**; HR-FABMS (glycerol) *m/z* 399.2235 (calcd for $C_{18}H_{31}N_4O_6$, -0.8 mmu).



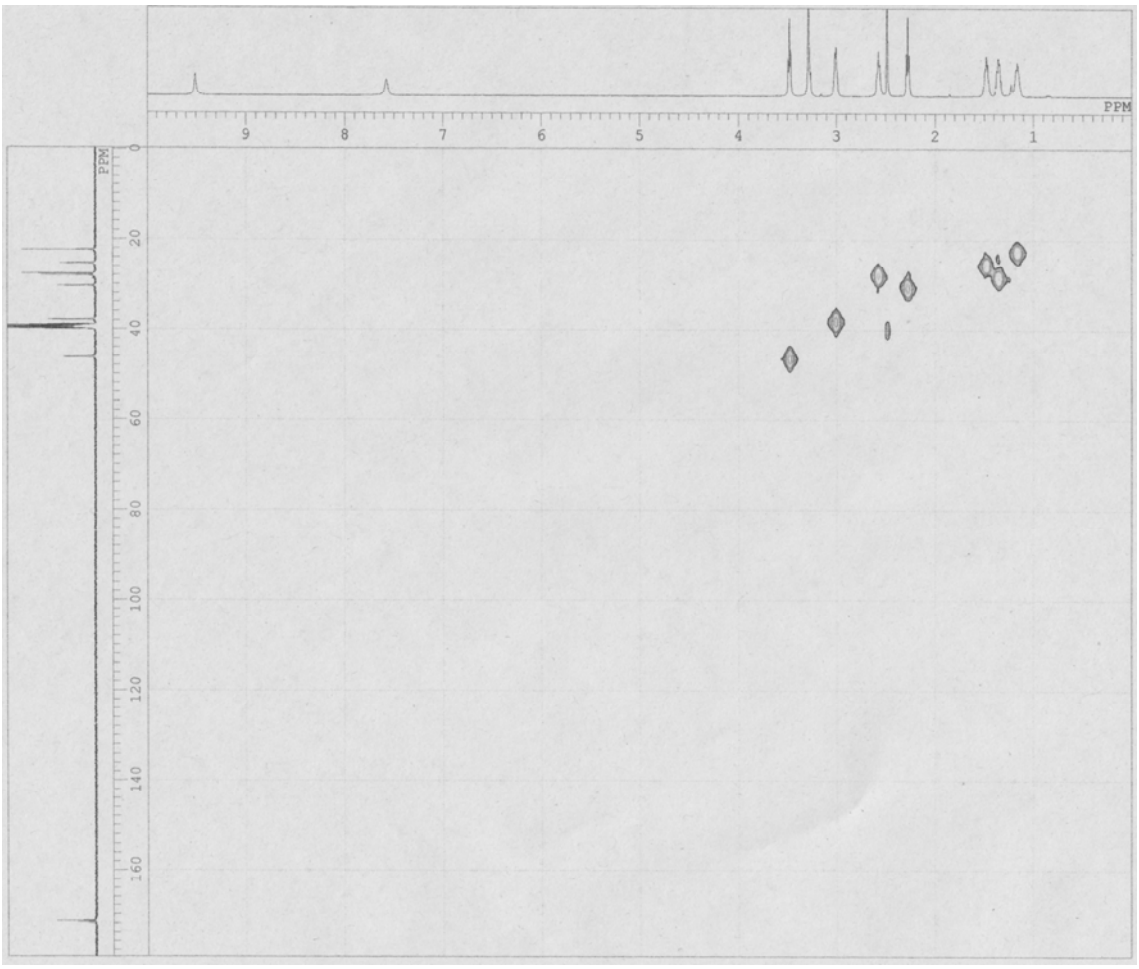
^{1}H NMR spectrum of bisucaberin (**1**) in CD_3OD + DMSO *d*-6 (1:1).



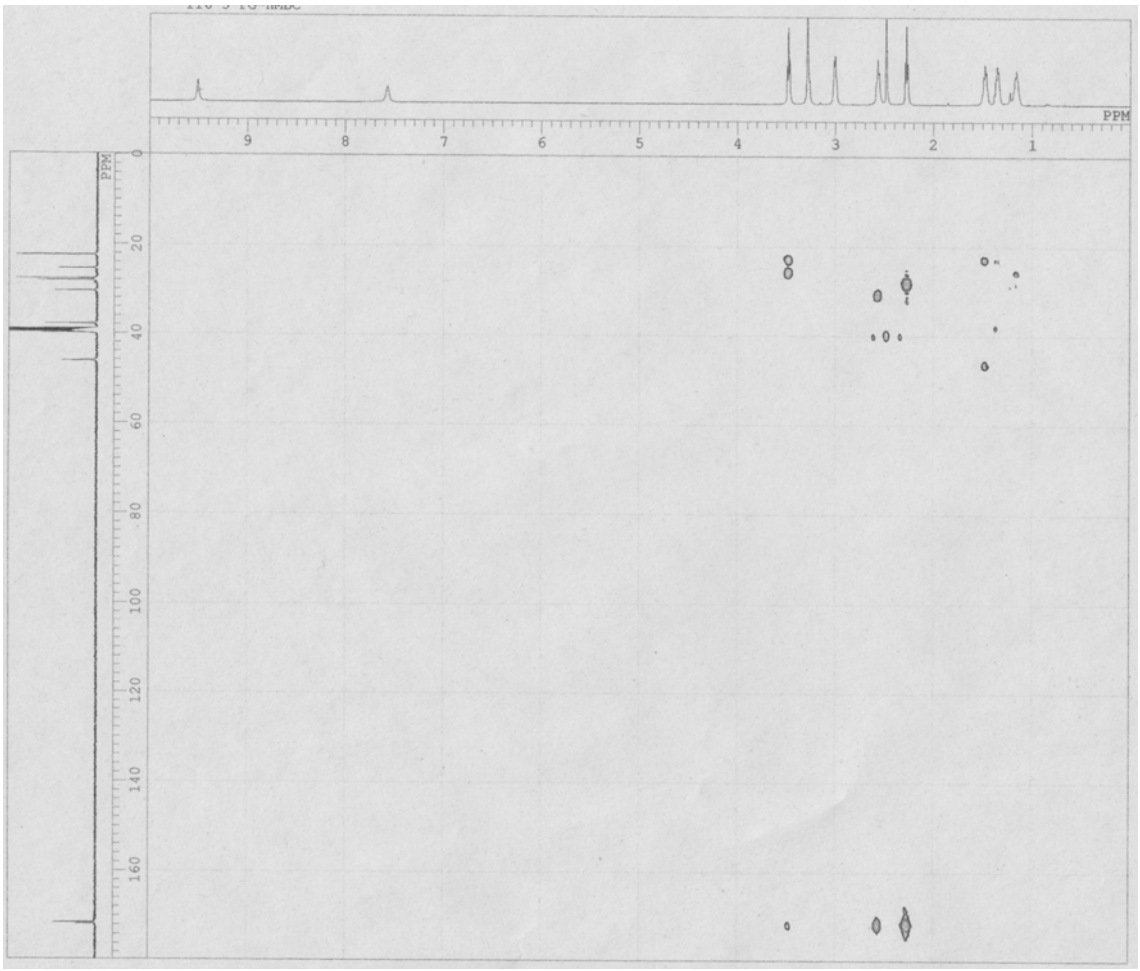
^{13}C NMR spectrum of bisucaberin (**1**) in CD_3OD + DMSO *d*-6 (1:1).



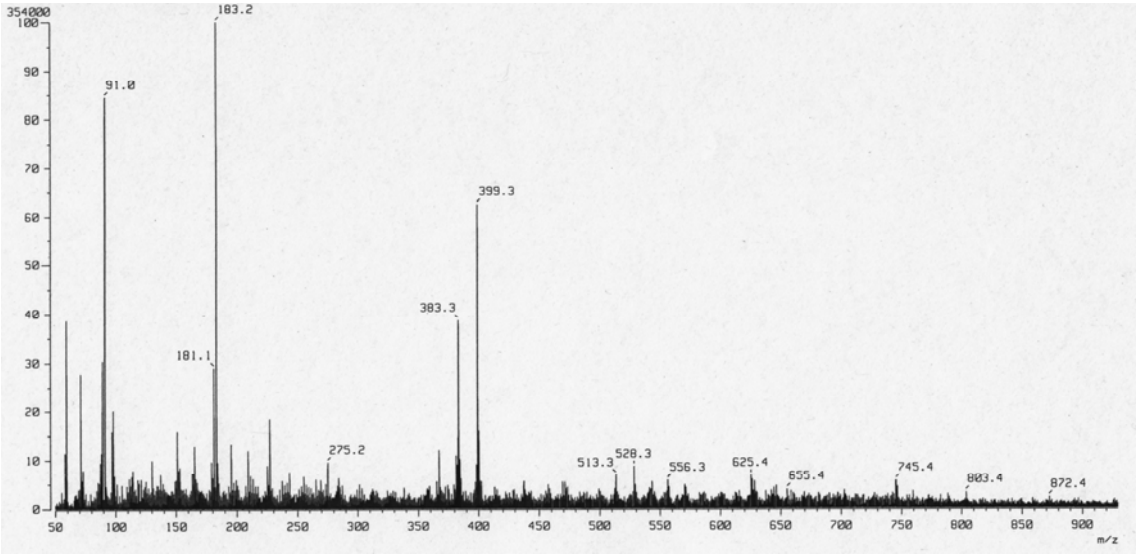
COSY spectrum of bisucaberin (**1**) in CD_3OD + DMSO *d*-6 (1:1).



HMQC spectrum of bisucaberin (**1**) in CD_3OD + DMSO *d*-6 (1:1).



HMBC spectrum of bisucaberin (**1**) in CD_3OD + DMSO *d*-6 (1:1).



Negative FABMS spectrum of bisucaberin (**1**) (matrix: glycerol)