Heterologous Production of 4-*O*-Demethylbarbamide, a Marine Cyanobacterial Natural Product

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Supporting Information

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Genetic manipulation, bacterial strains and culture conditions

Genetic manipulation was carried in *Escherichia coli* DH5α and plasmid Litmus 28 (New England Biolabs) by using standard protocols. The expression vector pDHS702, *E. coli–Streptomyces* shuttle vector, contains the *pik*AI promoter.[1] Cosmid pLM49 containing barbamide (**1**) biosynthetic genes was previously selected from the genomic library of *Moorea producens* strain 19L.[2] The engineered strain of *Streptomyces venezuelae* DHS2001 (a *pik* PKS gene-deleted mutant)[3] was propagated on SPA agar plates (0.1% yeast extract, 0.1% beef extract, 0.2% tryptose, 1.0% glucose, 1.5% agar, and trace amount of ferrous sulfate). Transformation of DNA into protoplasts of *S. venezuelae* was performed as described.[4] *E. coli* strains were grown at 37°C in LB (Luria-Bertani) medium with ampicillin (50 μg mL^{-1}), apramycin (25 μg mL^{-1}) and tetracycline (10 μg mL^{-1}). For production of 4-*O*-demethylbarbamide (**2**), the recombinant strain of *S. venezuelae* expressing barbamide biosynthetic genes were cultivated at 30°C for 6 days on R2YE agar supplemented with an antibiotic (50 μg mL^{-1} thiostrepton).[4]

Construction of expression vector and *S. venezuelae* mutant strain

Barbamide biosynthetic gene cluster (GenBank accession number AF516145) was cloned into pDHS702 vector.[1] Expression vector, pYJ1614 was constructed by using BAC subcloning kit (GENE BRIDGES) based on the Red/ET recombination technology with the following modifications (Table S1; Figure S2). Cosmid pLM49 and pRed/ET plasmid (Red/ET expression plasmid) were used to transform *E. coli* strain DH5α by electroporation. The transformant was selected by apramycin (selection for the cosmid pLM49) and tetracycline (selection for the pRed/ET). The plasmid pYJ1613 was designed to contain two ~600 bp DNA fragments for homologous recombination between pLM49 and pDHS702. The *Eco*RI-*Bam*HI fragment (containing the upstream and 5'-region of *barA*) and the *Bam*HI-*Nsi*I fragment (containing the downstream and 3'-region of *barK*) were amplified from cosmid pLM49 by PCR using *Taq* DNA polymerase (New England Biolabs), sequenced, and ligated into pDHS702 giving the plasmid pYJ1613. The deoxyoligonucleotide primer pairs used for PCR of the homology regions in this study are summarized in Table S2. The linear vector pYJ1613 treated by a restriction enzyme *Bam*HI was introduced by electroporation into the DH5α containing cosmid pLM49 and pRed/ET plasmid. L-arabinose was added for induction of the expression of genes mediating Red/ET and the cell was incubated at 37°C, the temperature at which all proteins necessary for the subsequent recombination was expressed, and then selected by ampicillin. The resulting plasmid pYJ1614 containing *barA-B-C-D-E-F-G-H-I-J-K* genes was transformed into *S. venezuelae* mutant DHS2001, generating YJ348.

HPLC-ESI-MS/MS analysis of barbamide and 4-*O*-demethylbarbamide

The grown cultures (1.5 L) of the mutant strain YJ348 were blended with 2 volumes of MeOH and shaken at room temperature for 3 h. The cell debris was removed by centrifugation before rotary evaporation. After adjustment of the pH of the extracts to 3 by addition of 0.02 N HCl, the extracts were immediately partitioned twice with an equal volume of EtOAc. The organic phases were evaporated to dryness, and then kept in the freezer until analyzed. The residue was reconstituted in MeOH and analyzed by HPLC-electrospray ionization mass spectrometry (HPLC-ESI-MS/MS) using a Waters/Micromass Quattro micro/MS apparatus. Separation was performed on a 150 × 3.9 mm Nova-Pak C_{18} (4.0 μm, Waters) reversed-phase column. The analytes were eluted at a flow rate of 180 μL min^{-1} with a gradient of 5 mM (w/v) ammonium acetate, 0.05% HOAc (v/v) in H_2O (A) and 80% (v/v) MeCN with the same additives concentration (B) at 20% to 70% B for 25 min, to 90% B for 20 min, maintained at 90% B for 10 min, and then to 20% B for another 10 min for column re-equilibration. The column effluent was directed to the ESI-MS, which was operated in the positive ion mode, without splitting (see Figure S3 and Figure S4).

Isolation and identification of barbamide and 4-*O*-demethylbarbamide

Approximately 104 g (dry wt) of laboratory cultured *Moorea producens* were extracted repeatedly with CH_2Cl_2/MeOH (2:1, 1L x 6) to afford 8.2548 g of crude extract. This material was fractionated by silica gel vacuum liquid chromatography using a stepwise gradient solvent system of increasing polarity (EtOAc in hexanes mixtures), starting from 100% hexanes to 100% EtOAc, followed by 25% MeOH/EtOAc and 100% MeOH. A total of nine fractions (A-I) were produced. The ^{1}H NMR-guided fractionation of fraction D (elution with 40% EtOAc/hexanes, 1.4439 g) via silica gel column chromatography (35% EtOAc in hexanes) afforded 0.9418 g of a mixture containing barbamide (**1**) as the main component according to ^{1}H NMR. LC-MS analysis of this mixture allowed detecting trace amounts of 4-*O*-demethylbarbamide (**2**; $[M+H]^+$ *m/z* 446.97) accompanying barbamide (**1**; $[M+H]^+$ *m/z* 460.94, $[M+Na]^+$ *m/z* 483.01) in the mixture (Figure S5).

The barbamide (**1**) containing fraction (4.0 mg) was subjected to chromatographic isolation. To accomplish this, HPLC was performed using an analytical reversed-phase (RP) HPLC column (250 × 4.6 mm, BDS Hypersil C_{18}, 5.0 μm, Thermo) and the sample was then eluted with the isocratic 50% (v/v) MeCN in the analytical HPLC system (Acme 9000 HPLC, YL Instrument Co., Ltd., Korea) coupled with a UV detector set to 210 nm at a flow rate of 1 mL min^{-1} over a period of 60 min. The mixture barbamide was chromatographically separated into 4-*O*-demethylbarbamide (**2**) (0.5 mg, t_R = 25.5 min) and barbamide (**1**) (2.0 mg, t_R = 48.5 min). NMR samples were prepared by dissolving 4-*O*-demethylbarbamide (**2**) in 200 μL of DMSO-d_6 (Sigma) and placing the solution in a 5 mm Shigemi advanced NMR microtube (Shigemi Inc.) matched to the solvent. The ^{1}H, ^{13}C, and 2D NMR spectra were acquired using Bruker Avance II 900 spectrometer (900 MHz) at 298K. Chemical shifts were reported in ppm using the deuterated solvent as an internal reference. The assignment of **2** was carried out by comparison with previously assigned ^{1}H, ^{13}C NMR spectra of **1** and by a combination of 1D and 2D NMR experiments. All NMR data processing was done using the Mnova (Mestrelab Research S.L.).

For further spectroscopic data analyses on 4-*O*-demethylbarbamide (**2**), 8 mg of the barbamide containing fraction of *M. producens* extract was subjected to RP-HPLC (Luna 5 µ C18, 250 × 4.6 mm, Phenomenex). The gradient elution (starting with 60% MeCN/H_2O at 1 mL min^{-1} for 20 min, then linear gradient to 100% MeCN at 1 mL min^{-1} for the next 20 min) afforded **2** (1.2 mg, t_R = 18.0 min) and **1** (5.2 mg, t_R = 28 min). The ^1H, and 2D NMR spectra of 4-*O*-demethylbarbamide (**2**) were recorded with DMSO-d_6 as an internal standard (δ_C 39.51, δ_H 2.50) on a Bruker 600 MHz spectrometer equipped with 1.7 mm MicroCryoProbe at 298K. All NMR data processing was done using the Mnova (Mestrelab Research S.L.). 1D and 2D NMR spectral data of authentic barbamide (**1**) and its natural derivative 4-*O*-demethylbarbamide (**2**) are listed in Table S3 (see also Figure S6 to S10).

4-*O*-demethylbarbamide (2): pale yellow oil; $[\alpha]^{27}_D$ -98 (*c* 0.23, MeOH); ^1H, ^{13}C and 2D NMR data, see Table S3; HRESITOFMS *m/z* $[M + Na]^+$ 469.0279 (calcd. for $C_{19}H_{21}Cl_3N_2O_2SNa$, 469.0282), see Figure S11.

Molluscicidal Assay

The molluscicidal effect of 4-*O*-demethylbarbamide (**2**) was evaluated according to a previously outlined procedure using the test organism *Biomphalaria glabrata*.[5] A stock solution of 20 mg mL^{-1} of pure compound in EtOH was prepared, of which 20 µL was taken and diluted to 24 mL with distilled H_2O. The snails were placed in wells of varying concentrations and observed after 24 h. If no heartbeat could be detected upon examination under a dissecting microscope (20× magnification), the snails were assessed as dead. For the experiment, three technical replicates were used for each data point (see Figure S12).

Detection of mRNA transcripts by RT-PCR

At day 5, total RNA was isolated from *S. venezuelae* mutant YJ348 grown on R2YE plates. Isolation of RNA was performed as described.[6] Primers (21-mers) were designed to possess a similar T_m value 60°C and generate PCR products of approximately 500 bp. The oligonucleotide primers and the target genes are summarized in Table S4. Analysis of semi-quantitative reverse transcriptase PCR (RT-PCR) was performed by using a One-Step RT-PCR kit (Qiagen) as recommended by manufacturer. A total of 8 µL (*barE, barF* and *barG*, 200 µg mL^{-1}; *barJ*, 800 µg mL^{-1}) of total RNA was used as a template for reverse transcription and PCR amplification under the following conditions : 50°C for 30 min, followed by 95°C for 15 min and then 40 to 49 cycles of 94°C for 1 min (*barE, barF* and *barG*, 40 cycles; *barJ*, 49 cycles), 55°C for 1 min, and 72°C for 1 min. Positive controls were carried out with each experimental reaction using 16S rRNA. Negative controls were carried out with *Taq* DNA polymerase (Stratagene) in the absence of reverse transcriptase to confirm that the amplified products were not derived from chromosomal DNA. After RT-PCR amplification was complete, 50 µL of the reaction products were electrophoresed onto a 1% agarose gel containing 0.5 µg mL^{-1} of ethidium bromide and photographed under UV light by using Gel Doc 2000 (Bio-Rad) (see Figure S13).

Table S1. List of plasmids used in this study

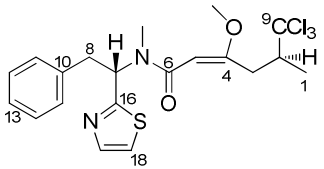
Plasmid	Description	Reference
pDHS702	*E. coli–Streptomyces* shuttle vector containing *pikAI* promoter and thiostrepton resistance marker	Xue and Sherman (2001) [1]
pYJ1613	pDHS702 derivative; homology region of *barA* (600 bp)-homology region of *barK* (600 bp)	This study
pYJ1614	pDHS702 derivative; *barA-barB-barC-barD-barE-barF-barG-barH-barI-barJ-barK*	This study

Table S2. Deoxyoligonucleotide primer pairs used for the construction of expression plasmid

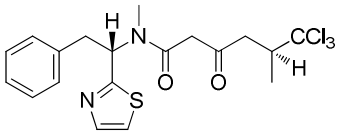
Name	Direction	Sequence (5'-3') [a]	Restriction site
barA	Forward	CAAGCAGAATTCGCCTTGGCCTTGGCCATA	*Eco*RI
	Reverse	ATCGTGGGATCCATGTACAGCGCAATTTCA	*Bam*HI
barK	Forward	GCTTTGGGATCCCTTCGAATGAATAGGAGT	*Bam*HI
	Reverse	TTTTCTATGCATTGCGGATACCAAATATCA	*Nsi*I

[a] Underlined sequence indicates restriction enzyme cleavage site.

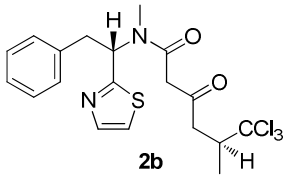
Table S3. NMR data of barbamide (**1**; DMSO-d_6; ^{1}H-NMR 900 MHz; ^{13}C-NMR 225 MHz), and four isomers of 4-*O*-demethylbarbamide (**2a** to **2d**; DMSO-d_6; ^{1}H-NMR 600 MHz; ^{13}C-NMR 150 MHz)



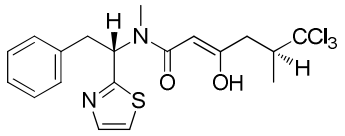
Barbamide (**1**)



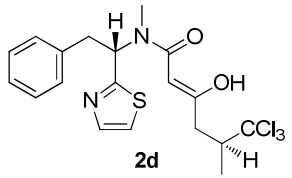
2a



2b



2c



2d

Position	Barbamide (**1**)		**2a** (63%) [a] (*trans* amide/ 4-keto)		**2b** (26%) [a] (*cis* amide/ 4-keto)		**2c** (7%) [a] (*trans* amide/ $\Delta^{4,5}$ *Z*)		**2d** (4%) [a] (*cis* amide/ $\Delta^{4,5}$ *Z*)	
	δ_C	δ_H, mult (*J* in Hz)	δ_C [b]	δ_H, mult (*J* in Hz)	δ_C [b]	δ_H, mult (*J* in Hz)	δ_C [b]	δ_H, mult (*J* in Hz)	δ_C [b]	δ_H, mult (*J* in Hz)
1	15.2	1.10 d (6.5)	16.9	1.17 d (6.8)	16.9	1.18 d (5.8)	15.6	1.19 d (6.0)	15.6	1.21 d (6.5)
2	51.9	2.84 [c]	49.5	3.05 [c]	49.5	3.01 [c]	51.3	2.93 [c]	51.5	2.93 [c]
3a	34.0	2.77 [c]	45.5	3.03 [c]	45.5	3.05 [c]	38.3	2.93 [c]	38.4	2.88 [c]
3b		3.01 dd (13.1, 12.4)		2.54 dd (18.0, 9.5)		2.49 [c]		2.16 dd (13.6, 10.5)		2.08 dd (13.9, 10.7)
4	167.1		201.7		201.7		174.4		173.4	
5a	94.0	5.37 brs	48.8	3.61 brs	48.2	3.64 d (16.4)	88.8	5.47 brs	88.2	5.83 brs
5b				3.60 brs		3.19 d (16.4)				
6	166.8		167.5		167.1		171.8		172.1	
7	54.3	6.31 dd (10.1, 5.3)	54.6	6.21 dd (10.1, 5.3)	59.8	5.59 dd (10.2, 4.8)	54.1	6.27 dd (9.9, 5.7)	58.0	6.00 dd (10.6, 4.7)
8a	35.8	3.25 dd (14.5, 5.3)	36.1	3.51 dd (14.5, 5.3)	37.0	3.57 dd (13.9, 4.8)	35.8	3.56 [c]	36.3	3.59 [c]
8b		3.55 dd (14.5, 5.6)		3.24 dd (14.5, 10.1)		3.27 dd(13.9, 10.2)		3.29 [c]		3.31 [c]
9	105.7		105.3		105.3		105.1		105.2	
10	137.7		137.5		137.1		137.4		137.0	
11	128.8	7.28 [c]	128.9	7.28 [c]	129.2	7.36 d (7.3)	- [d]	- [d]	- [d]	- [d]
12	128.1	7.28 [c]	128.9	7.28 [c]	128.3	7.32 t (7.3)	- [d]	- [d]	- [d]	- [d]
13	126.3	7.28 [c]	126.5	7.19 [c]	126.9	7.20 [c]	- [d]	- [d]	- [d]	- [d]
14	128.1	7.28 [c]	128.9	7.28 [c]	129.2	7.32 t (7.3)	- [d]	- [d]	- [d]	- [d]
15	128.8	7.28 [c]	128.9	7.28 [c]	128.3	7.36 d (7.3)	- [d]	- [d]	- [d]	- [d]
16	169.4		168.2		167.1		168.3		169.1	
17	142.0	7.78 d (3.4)	142.8	7.80 d (3.4)	142.9	7.85 d (3.4)	142.76	7.81 [c]	142.85	7.85 [c]
18	120.7	7.70 d (3.4)	121.3	7.74 d (3.4)	122.0	7.81 d (3.4)	121.34	7.73 [c]	121.95	7.79 [c]
*N*CH$_3$	30.7	2.88 s	30.3	2.81 s	28.0	2.69 s	29.9	2.85 s	27.3	2.71 s
*O*CH$_3$	55.5	3.60 s								

[a] Values in parentheses refer to the percentage of the mixture that this form represents. [b] Derived from HSQC and HMBC data. [c] Multiplicity of the peak is obscured. [d] The chemical shift of the peak was not able to be determined due to overlap.

Table S4. Primers for RT-PCR

Primer	**Sequence (5'-3')**	**Description**
barE_f	GCAGAAACGGTATTACGTCCA	Forward primer for *barE*
barE_r	CGCACTAAAGAAAATCGCAAG	Reverse primer for *barE*
barF_f	TTTTCGTAGCAGCGGAAATTA	Forward primer for *barF*
barF_r	GACAGAAATTGTTGCCGAAAA	Reverse primer for *barF*
barG_f	TCTGTATCTGGAAAGCCGAGA	Forward primer for *barG*
barG_r	AACGTGCTAAATCCCCTGTTT	Reverse primer for *barG*
barJ_f	ACCCACTTTCACCTCTGGACT	Forward primer for *barJ*
barJ_r	TATGGGGCATACCCTGTGATA	Reverse primer for *barJ*

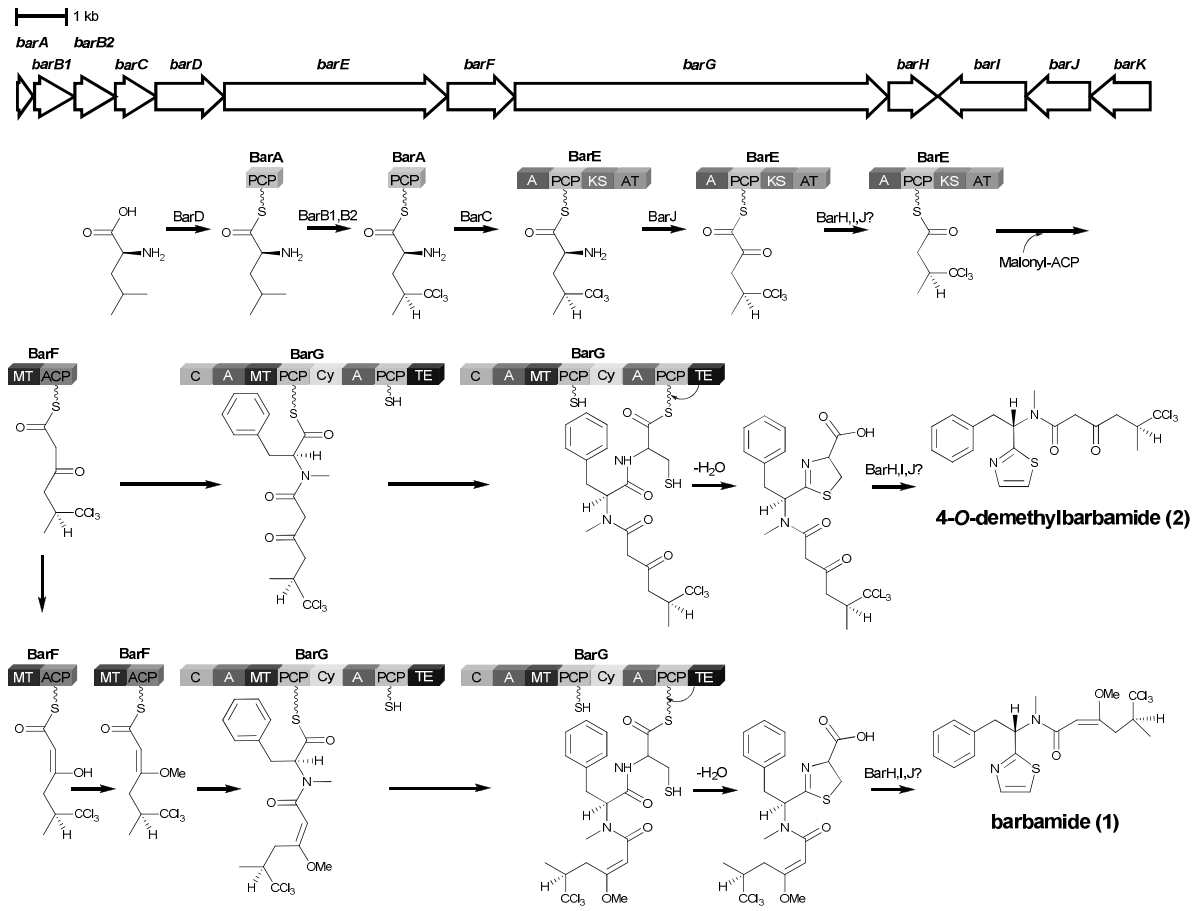


Figure S1. Organization of the barbamide gene cluster and the proposed biosynthetic pathways for 4-*O*-demethylbarbamide (**2**) and barbamide (**1**). L-leucine is activated through the BarD adenylation domain and loaded onto the BarA peptidyl carrier protein (PCP). The pro-*R* C-5 methyl group of leucine is then trichlorinated via the tandem action of two nonheme Fe^{II} halogenases, BarB1 and BarB2,[7] and transferred to the PCP domain of the NRPS/PKS (nonribosomal peptide synthetase/polyketide synthase) hybrid BarE probably by the function of BarC thioesterase (TE) II. The trichloroleucyl moiety linked to the PCP domain of BarE presumably undergoes oxidative deamination by the putative aminooxidase BarJ followed by a one-carbon truncation. The putative amidohydrolase and decarboxylase BarH, BarJ, and a protein of unknown function (BarI) could be involved in catalysing this one-carbon truncation. The resulting trichloroisovaleryl group is subsequently extended by a malonyl unit, and the BarF methyltransferase (MT) domain is presumed to catalyse formation of the unusual *E*-enol methyl ether. Next, the chain is extended by phenylalanine, which is *N*-methylated by the MT of BarG, and then further extended by condensation with cysteine that is cyclized to a thiazoline ring system. The TE domain in module BarG and the three proteins BarH, BarI, and BarJ may be involved in the final decarboxylation to produce the thiazole ring, thus completing the biosynthesis of **1**.

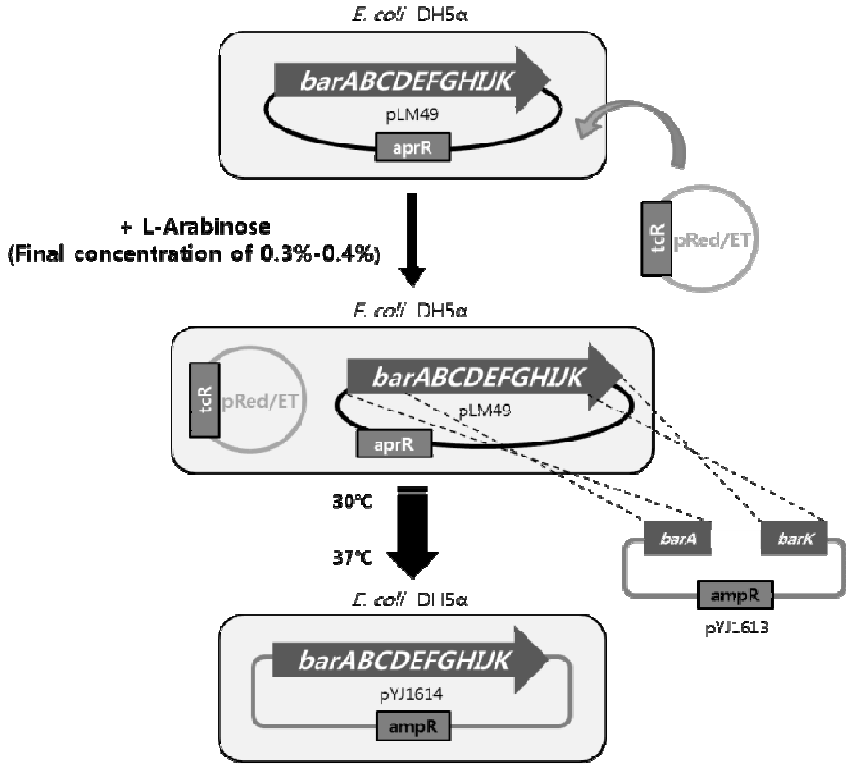


***Figure S2*.** Experimental outline for the construction of pYJ1614.

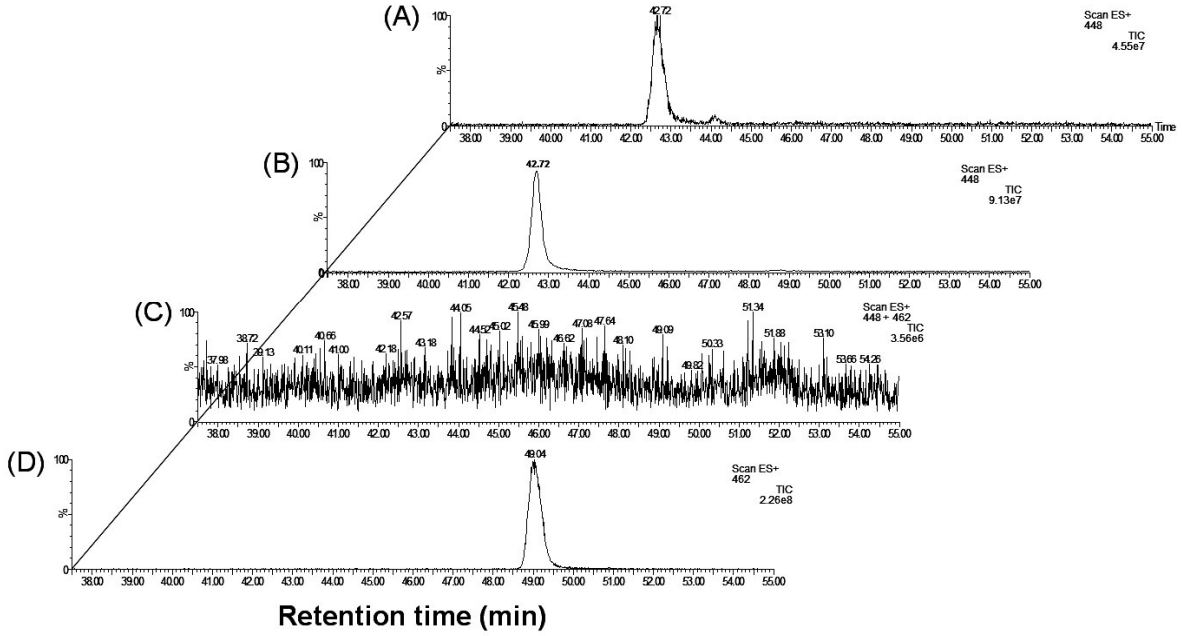


Figure S3. Selected ion chromatograms at *m/z* 448 and 462 for the $[M+H]^+$ of 4-*O*-demethylbarbamide (**2**) and barbamide (**1**), respectively, for the (A) organic extract obtained from the culture of recombinant strain *S. venezuelae* YJ348, (B) extract from the culture of *S. venezuelae* YJ348 spiked with authentic 4-*O*-demethylbarbamide, (C) extracts from the cultures of recombinant strains of *S. venezuelae* DHS2001 harboring an empty vector as control, and (D) authentic barbamide (**1**).

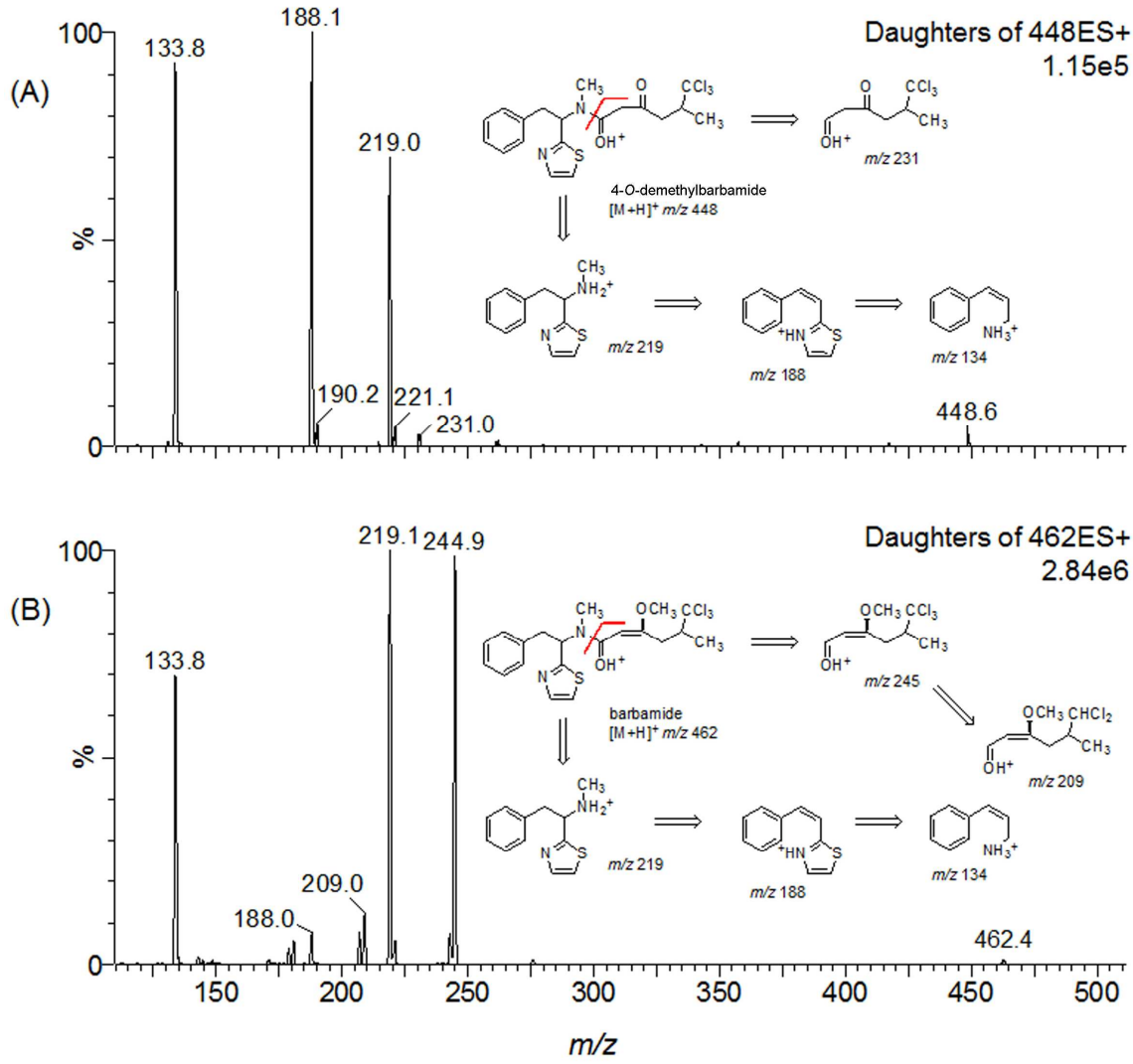


Figure S4. ESI-MS/MS spectra and suggested fragmentation patterns of (A) 4-*O*-demethylbarbamide (**2**) obtained from the culture of *S. venezuelae* YJ348 and (B) authentic barbamide (**1**).

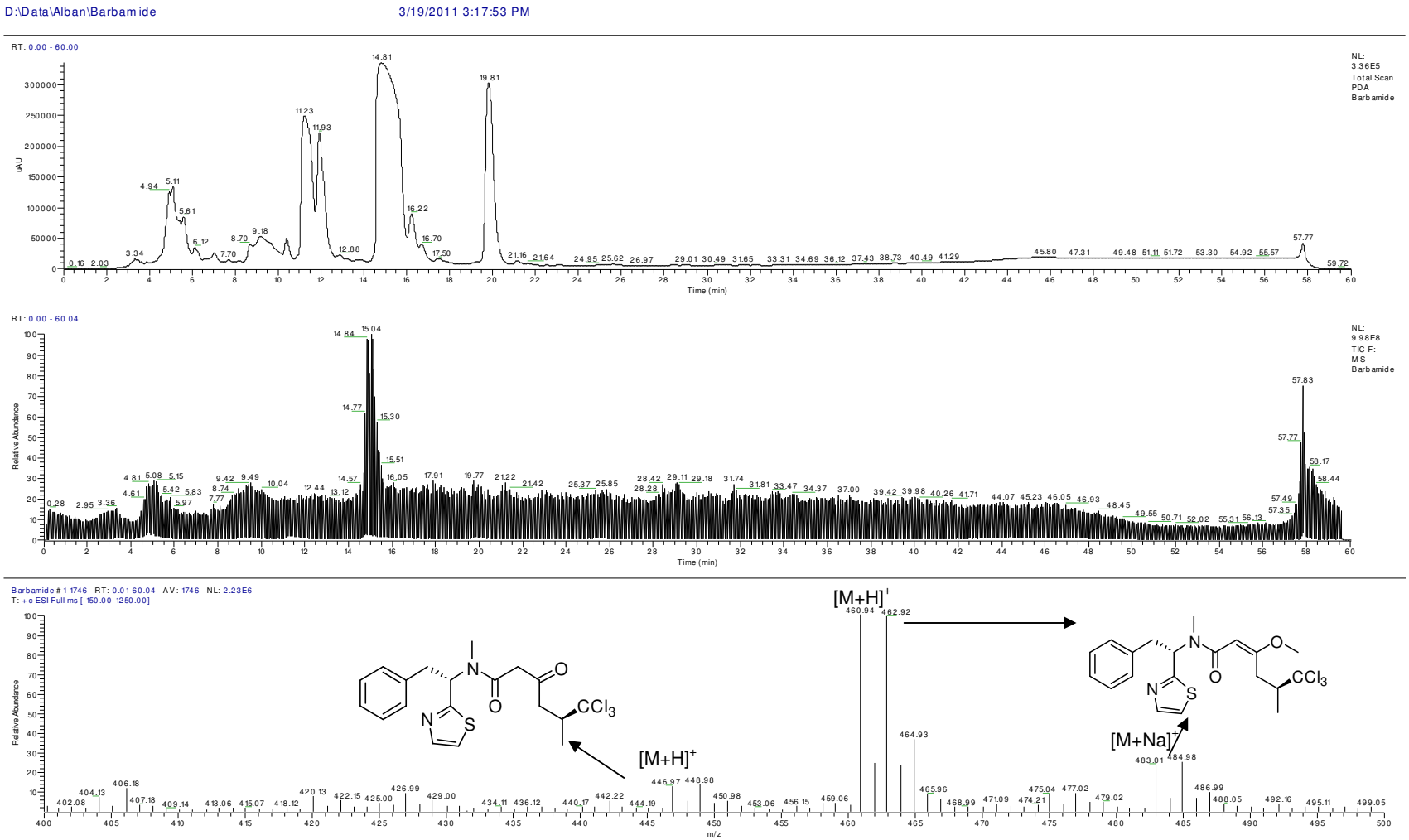


Figure S5. LC-MS chromatogram of the extract of *M. producens* containing barbamide (**1**) and 4-*O*-demethylbarbamide (**2**) (Finnigan LCQ Advantage Max ESI LCMS, linear gradient 60-100% MeCN/H_2O, 40 min): (top) PDA detection, (middle) total ion chromatogram, (bottom) accumulated mass spectra of the extract from 0 min to 60 min.

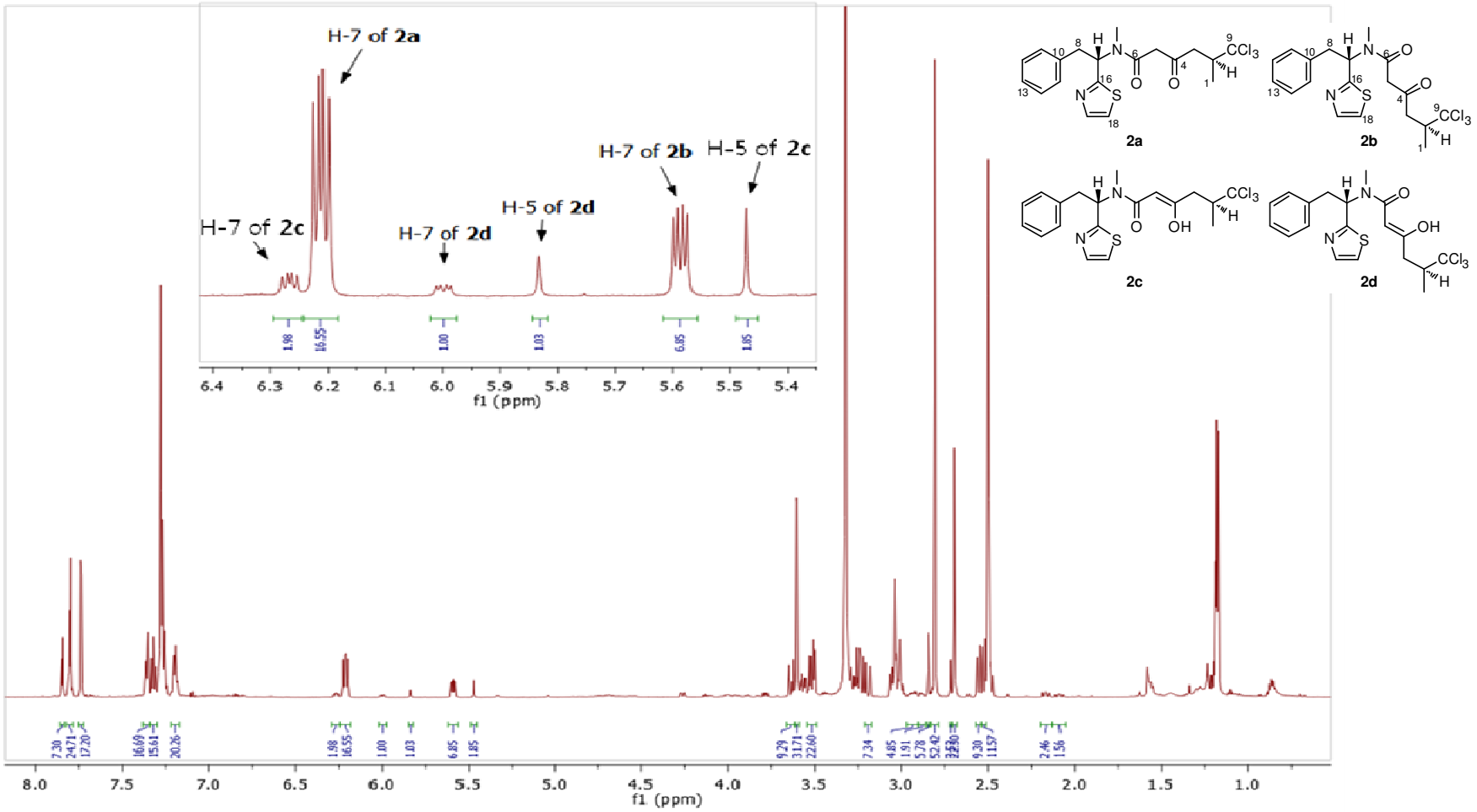


Figure S6. ^{1}H NMR spectrum of 4-*O*-demethylbarbamide (**2**) (600 MHz, DMSO-*d*6).

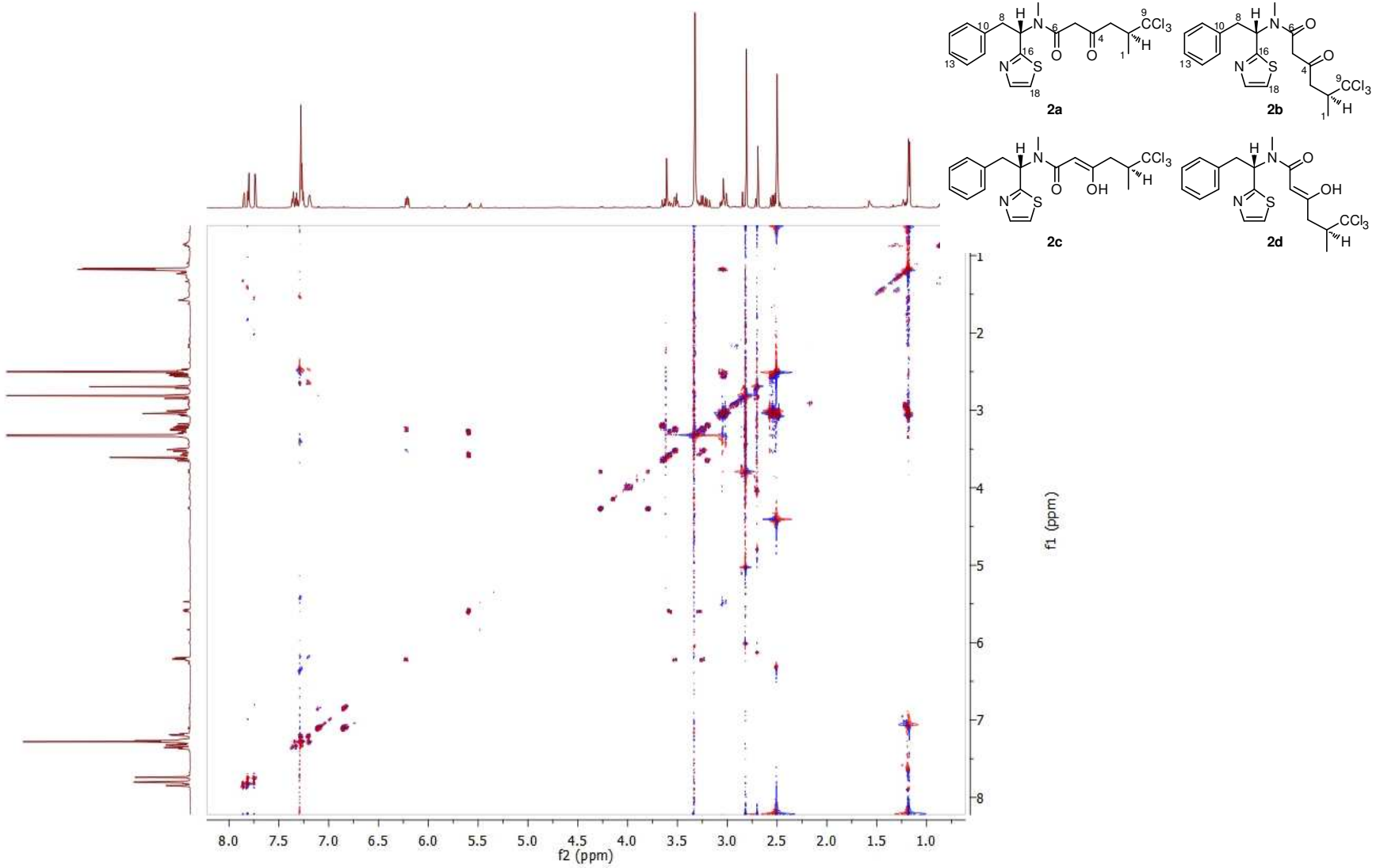


Figure S7. DQF-COSY spectra of 4-*O*-demethylbarbamide (**2**) (600 MHz, DMSO-*d*6).

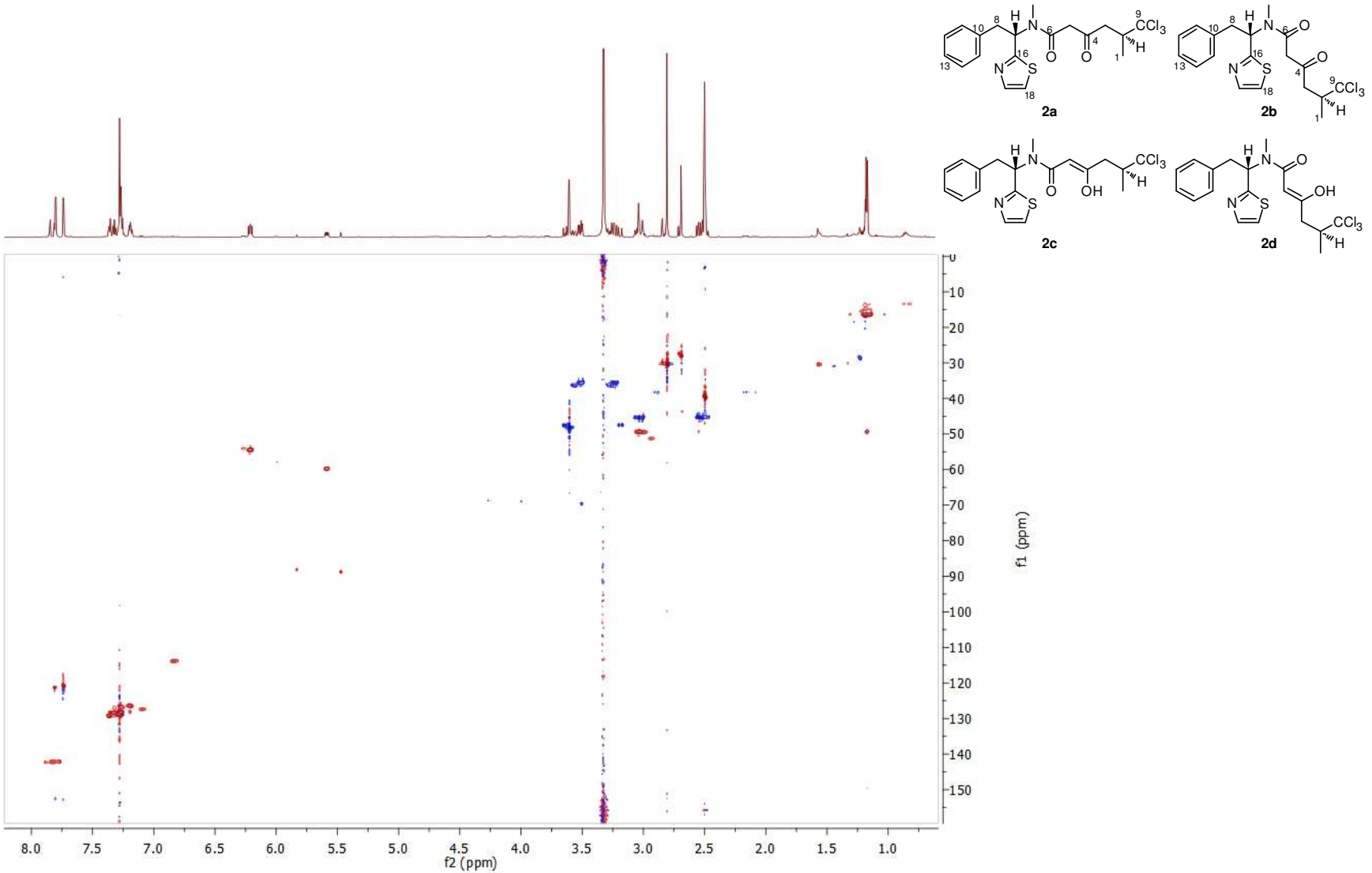


Figure S8. HSQC spectra of 4-*O*-demethylbarbamide (**2**) (600 MHz, DMSO-*d*6).

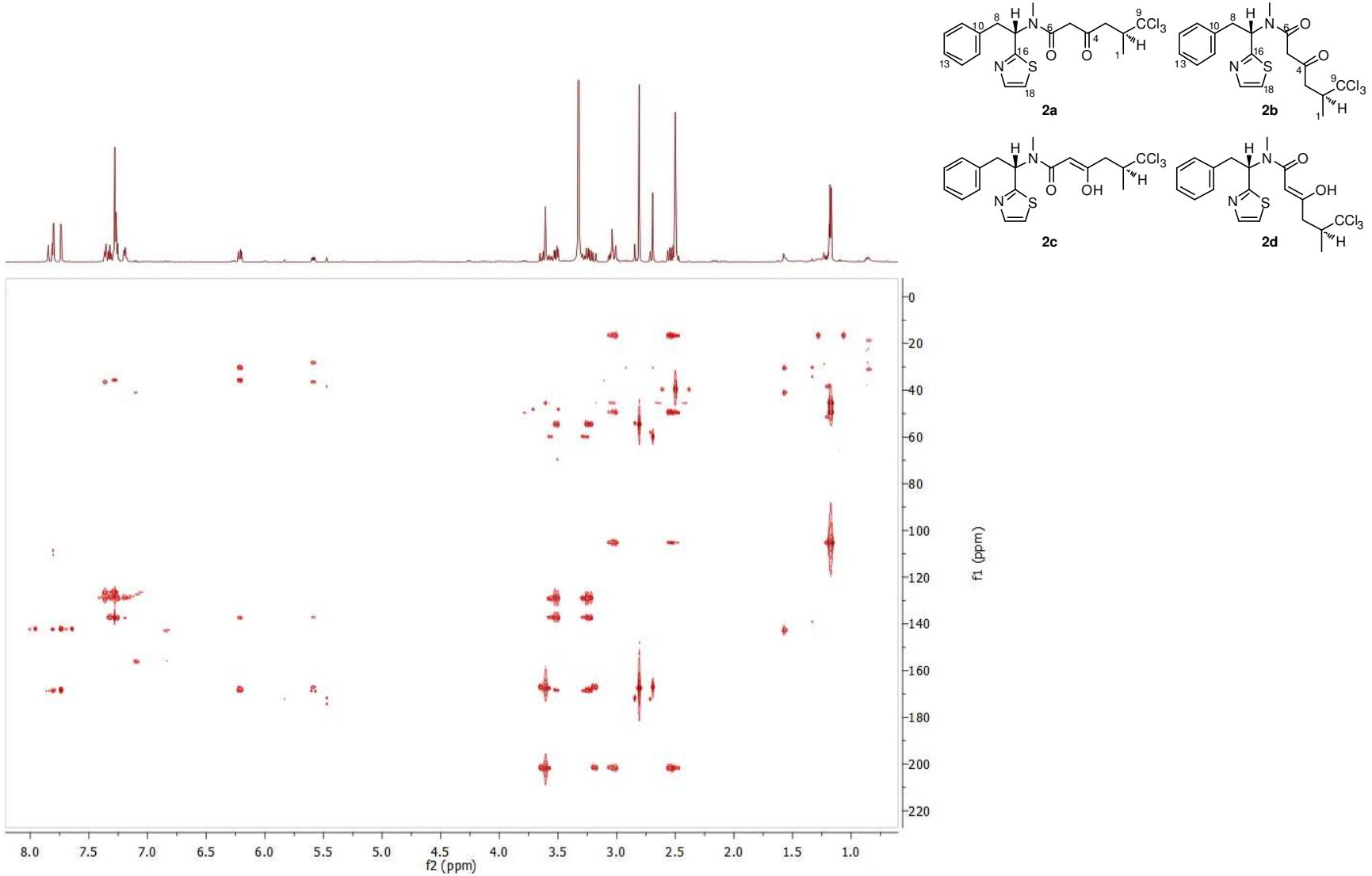


***Figure S9*.** HMBC spectra of 4-*O*-demethylbarbamide (**2**) (600 MHz, DMSO-*d*6).

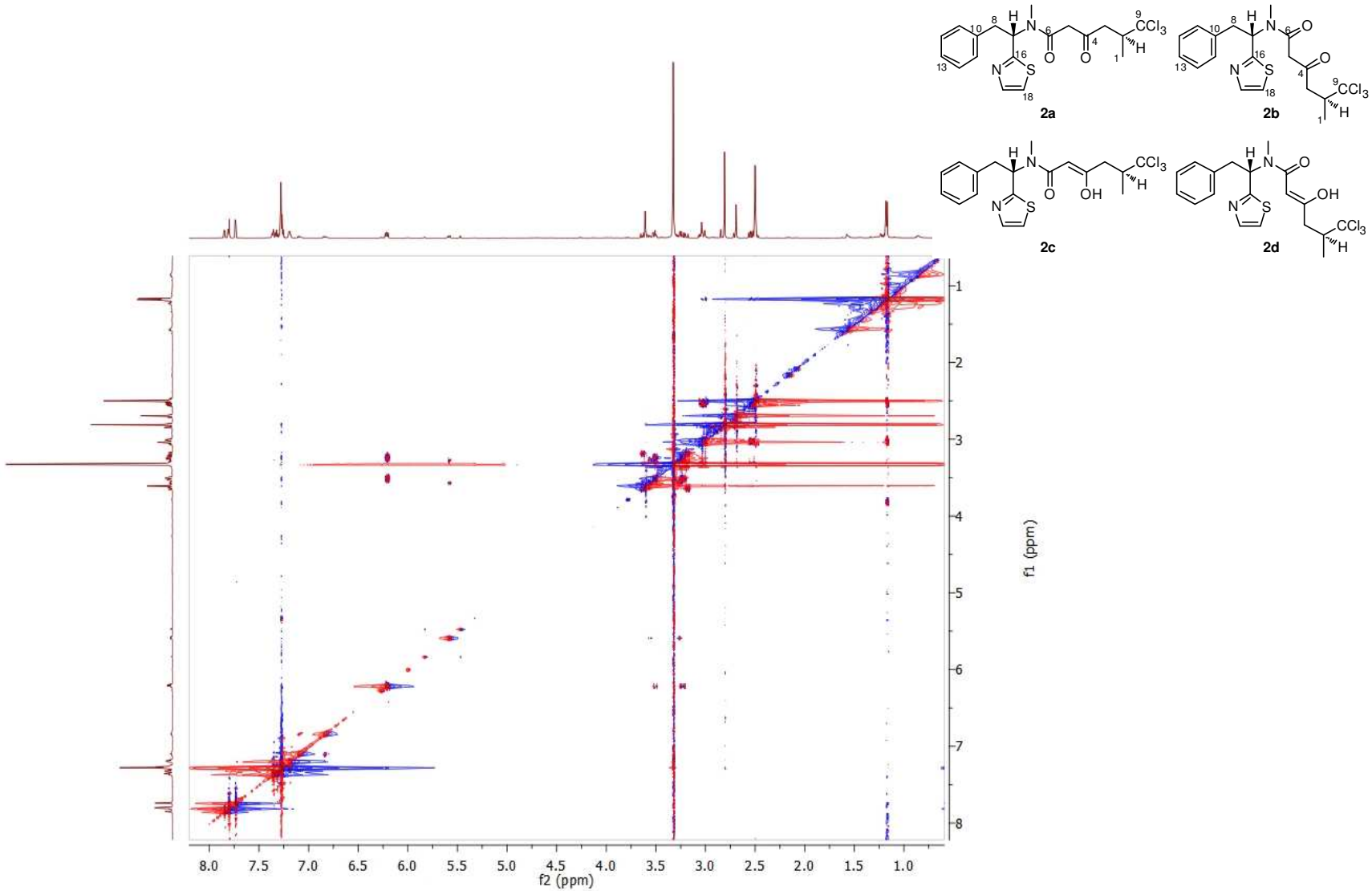


Figure S10. NOESY spectra of 4-*O*-demethylbarbamide (**2**) (600 MHz, DMSO-*d*6).

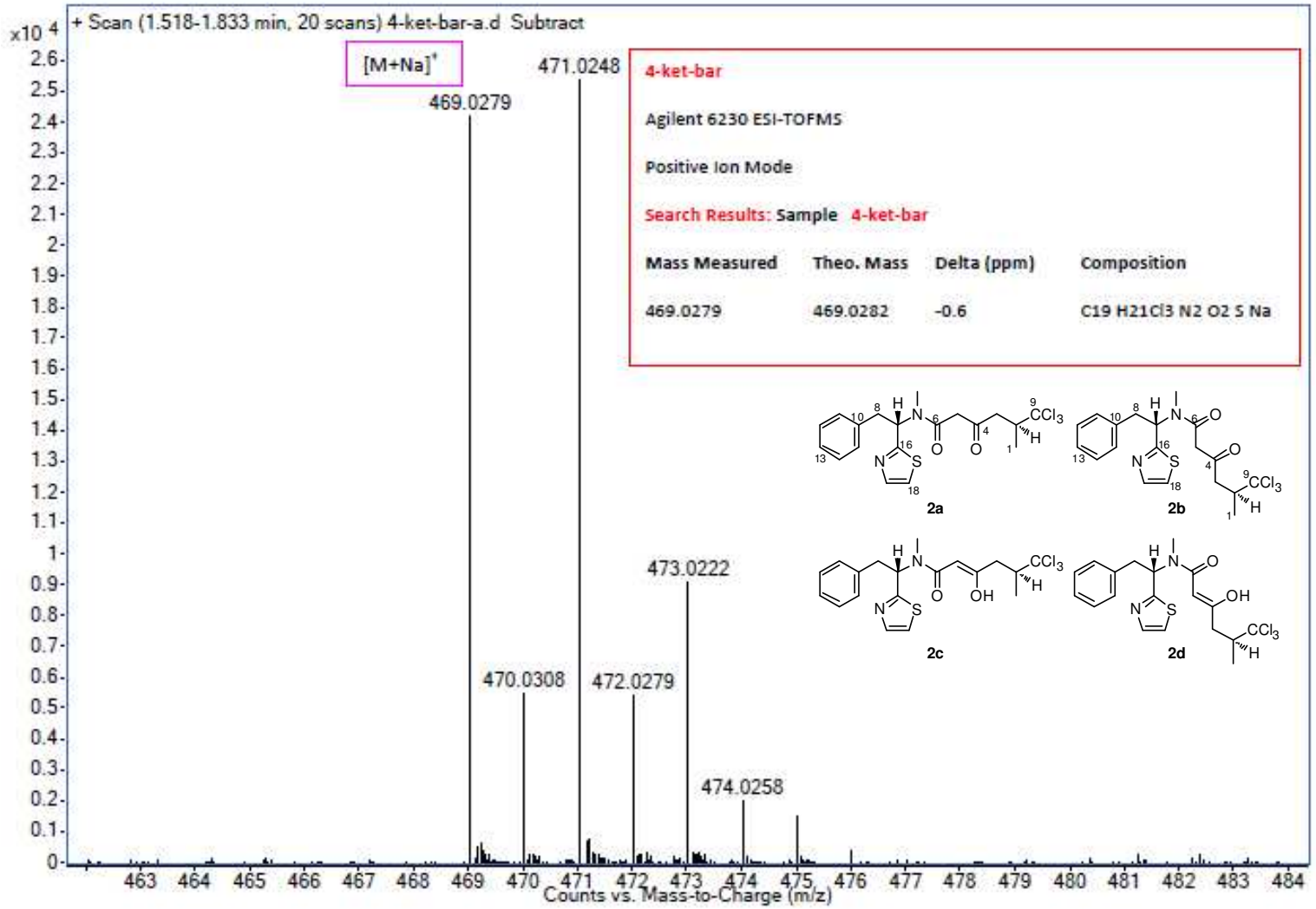


Figure S11. HR-ESI-TOF-MS spectra of 4-*O*-demethylbarbamide (**2**).

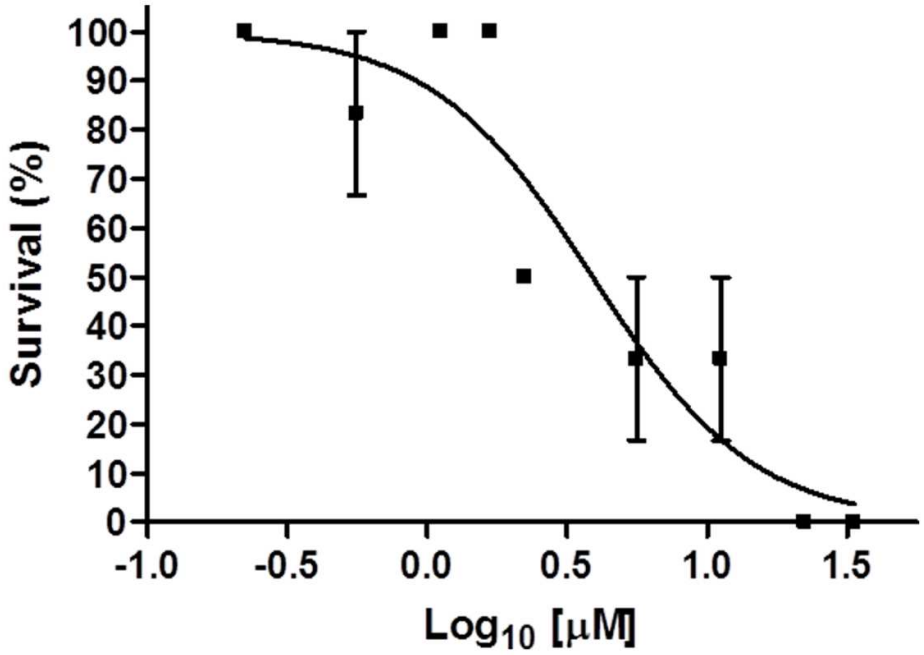


Figure S12. Molluscicidal activity of 4-*O*-demethylbarbamide (**2**).

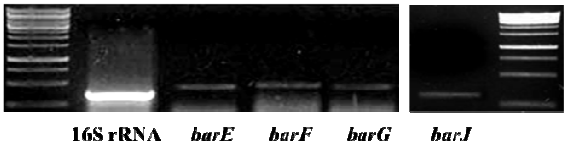


Figure S13. Gene expression analyses by RT-PCR applied to the mRNAs isolated from *S. venezuelae* YJ348 strain.

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