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Supporting Information

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Evidence for the Natural Toxins from the Mushroom *Trogia venenata* as a Cause of Sudden Unexpected Death in Yunnan Province, China**

Zhong-Yu Zhou, Guo-Qing Shi, Robert Fontaine, Kun Wei, Tao Feng, Fang Wang, Gang-Qiang Wang, Yan Qu, Zheng-Hui Li, Ze-Jun Dong, Hua-Jie Zhu, Zhu-Liang Yang, Guang Zeng, and Ji-Kai Liu**

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Table 1. ^{1}H and ^{13}C NMR data of compounds 1 and 2 at 500 MHz in DMSO-d_6 and D_2O, respectively

1. Supplementary Methods

a. General

Melting point was obtained on an X-4 micro melting point apparatus. Optical rotations were measured on a Horiba SEPA-300 polarimeter. IR spectra were obtained on a Tensor 27 FT-IR spectrometer (Bruker Optics GmbH, Ettlingen, Germany) with KBr pellets. NMR spectra were recorded on Bruker AV-400 and Bruker DRX-500 spectrometer (Bruker BioSpin GmbH, Rheinstetten, Germany). Chemical shifts (*d*) were expressed in ppm with reference to the solvent signals. EIMS were measured on Finnigan-MAT 90 (Thermo Fisher Scientific Inc., Waltham, MA, United States), ESI-MS and HR-ESI-MS were recorded with an API QSTAR Pulsar 1 spectrometer (MDS Sciex, Concord, Ontario, Canada). Preparative MPLC was performed on Büchi apparatus equipped with Büchi fraction collector C-660, Büchi pump module C-605 and manager C-615. Silica gel (200-300 mesh, Qingdao Marine Chemical Inc., Qingdao, China), microcrystalline cellulose (20-160 μm, Merck, Darmstadt, Germany), Amberlite 732 cation exchange resin (Na^+ form, Sinopharm Chemical Reagent Co., Ltd., Shanghai, China), RP-18 (40-75 μm, Fuji Silysia Chemical Ltd., Aichi, Japan) and Sephadex LH-20 (Amersham Biosciences, Uppsala, Sweden) were used for column chromatography. Fractions were monitored by TLC and spots were visualized by heating silica gel plates sprayed with 0.4% ninhydrin in acetone.

All reactions were performed under nitrogen atmosphere with dry solvents under anhydrous conditions. All solvents were purified and dried by standard techniques, and distilled prior to use. Yields refer to chromatographically and spectroscopically

homogeneous materials. Reagents were used as received without further purification unless otherwise stated. The selectively protected amino acid (*N-tert*-Boc-D-aspartic acid *tert*-butyl ester) was obtained from BetaPharma (Shanghai, China) Company.

b. Animals

Healthy male and female ICR mice, weighing 18–22 g, were purchased from Experimental Animal Center of Kunming Medical College (Certificate No. SCXK2005-0008). The animals were housed on a constant 12-h light/12-dark cycle in temperature-controlled central animal facility (18–22 °C) and allowed free access to solid food and tap water. All procedures were in accordance with the Institute Ethical Committee for Experimental Use of Animals.

c. *Trogia venenata* Zhu L. Yang, sp. nov. (MycoBank: MB 561711)

Pileus 1–6 cm latus, flabeliformis vel petaloideus, pallide incarnatus, albidus vel brunneolus, subtranslucentius, glabrus, margine undulato. Stipes 0.3–2 (3.5) × 0.2–0.4 cm, solidus, subglabrus, pallide incarnatus, albidus vel brunneolus. Lamellae angustae, 0.5–1 (3) mm altae, subdistantae vel distantae, pallide incarnatae vel albidae, interdum dichotomis, decurrentibus. Caro in pileo tenuis, 0.5–1.5 mm crassa, albida vel pallide incarnata, inordora, ceraceo-mollis, in stipite cartilaginea. Basidia 4-sporigera. Sporae 6–8 (9) × 4–5 (5.5) µm, non-amyloideae. Fibulae praesentes. Ad truncos delapsos in silva, gregaria. Typus: Z.L.Yang 5256 (KUN HKAS 56679), 3 July 2009, alt. 2100 m, Jietou, Tengchong, Yunnan, China.

d. Extraction and isolation

The mushroom *Trogia venenata* was collected from Tengchong county of Yunnan Province, China, in July 2009, and identified by Prof. Zhu-Liang Yang, Kunming Institute of Botany, Chinese Academy of Sciences. The voucher specimen (KUN HKAS 56679) was deposited at the Herbarium of the Kunming Institute of Botany, CAS.

The fresh fruiting bodies of *Trogia venenata* (9.7 kg) were extracted successively with ethanol three times at room temperature. The combined extraction was concentrated to dryness *in vacuo* to afford a crude extract (60.2 g), which was suspended in water and partitioned with ethyl acetate. Animal testing confirmed that the toxic factor was present in the aqueous phase. The water extract (40.5 g) was subjected to preparative medium pressure liquid chromatography (MPLC) on a reversed-phased C_{18} column (MeOH-H_2O, 0%-100%) to yield 16 fractions. Fraction A, eluted with pure water, was further separated by Amberlite 732 cation exchange resin column chromatography (eluted from H_2O to 1.0 M NH_4OH), and fraction A3 was obtained from the 0.3 M NH_4OH eluate. Fraction A3 was further repeatedly purified by column chromatography over microcrystalline cellulose (*n*-BuOH-EtOH-HOAc-H_2O, 4:1:1:2), Sephadex LH-20 (MeOH-H_2O, 7:3), and RP-C_{18} silica gel (pure water) to yield compounds **1** (905 mg), **2** (436 mg) and **3** (44.5 mg).

2*R*-amino-4*S*-hydroxy-5-hexynoic acid (amino acid **1**): colorless prisms (aq.

MeOH); mp 205-207 °C (decompose); $[a]_D^{16}$ - 13.2º (*c* 0.28, H_2O); IR (KBr) $?_{max}$ 3424, 3216, 2102, 1650, 1472, 1406 cm^{-1}; ^{1}H-and ^{13}C-NMR data, see **Supplementary Table 1**; FABMS (neg.) *m/z* 142 $[M- H]^-$; HR-ESI-MS (positive) *m/z* 166.0478 $[M + Na]^+$ (calcd for $C_6H_9NO_3Na$, 166.0480).

2*R*-amino-5-hexynoic acid (amino acid **2**): $C_6H_9NO_2$, white amorphous powders; $[a]_D^{16}$ - 2.7º (*c* 0.22, H_2O); ^{1}H-and ^{13}C-NMR data, see **Supplementary Table 1** on line; FABMS (neg.) *m/z* 126 $[M- H]^-$.

e. Calculation of the optical rotations

Absolute configuration was determined by both matrix model and optical rotations computations using DFT methods. The calculated det(D) for (2*R*,4*S*) was - 10.89, and k_0 was obtained as 1.21. The calculated optical rotations for (2*R*,4*S*) was - 32.7º, which was close to that of experimental value (- 13.2º).

f. Total synthesis of compound 1

The enantiopure amino acid **1** was synthesized in a short and efficient manner as outlined in Scheme 1. In our synthesis, the commercially available and cheap *N*-*t*-Boc-D-aspartic acid *tert*-butyl ester **4** was chosen as the starting material in that it contains all the desired potential functionalities and corresponding backbone of the target amino acid **1**. The terminal alkynyl motif was expected to be attached to the backbone to afford the ynone **6** through an addition of ethynylmagnesium bromide to a Weinreb amide derivative **5**. Asymmetric reduction of the ynone **6,** delivering the

secondary alcohol, and then an ensuing deprotection was utilized to complete the synthesis. Our synthesis began with a regular amide formation in nearly quantitatively yield. The resulting 'Weinreb' amide was then converted to the key ynone **6** using Grignard addition of ethynylmagnesium bromide in 78% yield. Enatioselectively oxazaborolidine-catalyzed (CBS) reduction of ynone **6** using a (*S*)-CBS catalyst gave enantiopure alcohol **6** in 61% yield. Deprotection of **7** with trifluoroacetic acid yielded amino acid **1**, in which both *N*-*t*-Boc and a-(*tert*-butyl ester) groups were cleaved efficiently. The synthetic sample showed NMR spectra identical to those of authentic sample. Moreover, our synthetic **1** exhibited a rotation of -13.8 (*c* 0.16, H_2O), and was consistent with that of the natural authentic sample ($[a]_D^{16}$ -13.2, *c* 0.28, H_2O); therefore, the absolute configuration of the amino acid was established.

g. Synthesis of *N*-*tert*-Boc-D-aspartic acid 1-(*tert*-butylester)-*N*-methoxy-*N*-methylamide (5)

(Benzotriazol-1-yloxy) tris(dimethylamino) phosphonium hexafluorophosphate (BOP•PF_6, 4.86 g, 11 mmol) was added to a stirred solution of *N*-*tert*-Boc-D-aspartic acid *tert*-butyl ester (**4**, 2.89 g, 10 mmol) and triethylamine (1.12 g, 11 mmol) in dichloromethane (100 mL) at ambient temperature. After 5 min of stirring *O*,*N*-dimethylhydroxylamine hydrochloride (1.15 g, 11.5 mmol) was added, followed by triethylamine (1.12 g, 11 mmol). All solid materials were dissolved in 10 min, and the stirring of the mixture was continued for 10 h at ambient temperature. The reaction was followed by TLC on silica gel with petroleum ether: EtOAc 1 : 1 as

eluent. The solution was then washed with 1 M HCl (1 x 50 mL), H_2O (1 x 50 mL), 1 M $NaHCO_3$, (2 x 50 mL), and dried (Na_2SO_4). Concentration *in vacuo* gave a yellow, oily product (4.45 g). Column chromatography (100 g of silica gel, petroleum ether : EtOAc = 6 : 4) gave the title product as a colorless oil (**5**, 3.22 g, 97%). ^{1}H NMR($CDCl_3$, 400 MHz) d: 1.43 (s, 18H, 2×(C(CH_3)$_3$), 2.86 (m, 2H, CH_2), 3.15(s, 3H, CH_3), 3.67 (s, 3H, CH_3), 4.44 [dt, 1 H, *J*(NHCH) = 8.4 Hz, CH], 5.66 (d, 1H, *J* = 8.5 Hz, NH); ^{13}C NMR ($CDCl_3$, 100 MHz) d: 27.6, 28.3, 31.9, 34.6, 50.3, 61.2, 79.5, 81.7, 155.7, 170.5, 171.6; $[a]_D^{16}$ -18.9 ^{o} (*c* 0.89, $CHCl_3$); HR-ESI-MS (positive) *m/z* 333.2024 $[M+H]^+$ (calcd for $C_{15}H_{29}N_2O_6$ 333.2025).

h. Synthesis of (*R*)-2-*tert*-butoxycarbonylamino-4-oxohex-5-ynoic acid *tert*-butyl ester (6)

Typically to a cooled, -78 ^{o}C, stirred solution of the 'Weinreb' amide (*N*-*t*-Boc-D-aspartic acid 1-(*tert*-butylester)*N*-methoxy-*N*-methylamide) (**5**, 332 mg, 1.0 mmol) in Et_2O (12 mL), ethynyl magnesium bromide (10mL of a 0.5 M solution in THF, 5 mmol) was added dropwise. The reaction was stirred for one hour before being warmed to room temperature and stirred overnight. The reaction was then poured onto a vigorously stirred mixture of Et_2O, 1 M potassium dihydrogen orthophosphate and ice, and the aqueous layer was extracted with Et_2O. The combined organic extracts were washed with 1 M potassium dihydrogen orthophosphate, saturated aqueous sodium bicarbonate solution, and brine, and then dried over Na_2SO_4 before concentrating *in vacuo* to give the crude product.

Purification by flash column chromatography (SiO_2, petroleum ether: EtOAc 8 : 1) yielded 230 mg (78%) as a colourless oil (**6**). ^{1}H NMR($CDCl_3$, 400 MHz) d: 1.42 (s, 18H, 2×($C(CH_3)_3$), 3.06 (dd, 1H, *J* = 5.0, 18.0 Hz, CH(H)), 3.26 (dd, 1H, *J* = 5.0, 18.0 Hz, CH(H)), 3.30 (s, 1H, H–C=C), 4.43–4.47 (m, 1H, CH), 5.34–5.38 (d, 1H, *J* = 8.0 Hz, NH); ^{13}C NMR ($CDCl_3$, 100 MHz) d: 27.8, 28.2, 47.6, 49.9, 79.7, 80.0, 80.8, 82.7, 155.4, 169.5, 184.3; $[a]_D^{16}$ -24.8 ^{o} (*c* 0.21, $CHCl_3$); HR-ESI-MS (positive) *m/z* 320.1466 $[M+Na]^+$ (calcd for $C_{15}H_{23}NO_5Na$ 320.1473).

i. Synthesis (2*R*,4*S*)-2-*tert*-butoxycarbonylamino-4-hydroxyhex-5-ynoic acid *tert*-butyl ester (7)

A freshly prepared solution of borane-dimethyl sulfide (4 mL, 0.5 M in toluene, 2 mmol) was added to a solution of (*R*)-2-*tert*-butoxycarbonylamino-4-oxohex-5-ynoic acid *tert*-butyl ester (**6**, 297 mg, 1 mmol, azeotropically dried with benzene) and (*S*)-B-methyl CBS catalyst (554 mg, 2 mmol) in 10 mL toluene at -40 °C. After the mixture was stirred for 15 h at -40 °C, methanol (8 mL) was added, followed by water (10 mL) and Et_2O (40 mL), and the mixture was warmed to 23 °C. Water (40 mL) and Et_2O (40 mL) were added, and the aqueous portion was extracted with Et_2O (2×100 mL). The combined organic fractions were dried ($MgSO_4$) and concentrated *in vacuo*. Purification by flash column chromatography (SiO_2, petroleum ether : EtOAc 8 : 1) yielded 178 mg (61%) as a colourless oil (**7**). ^{1}H NMR ($CDCl_3$, 400 MHz) d: 1.40 (s, 18H, 2×($C(CH_3)_3$), 1.69 (dt, 1H, *J* = 12.0 Hz, CH_2-H), 2.20 (dt, 1H, *J* = 12.0 Hz, CH_2-H), 2.40 (s, 1H, H–C=C), 4.38-4.29 (m, 1H, CHOH), 5.36 (d, 1H, *J* = 6.3 Hz,

NH); ^{13}C NMR ($CDCl_3$, 100 MHz) d: 27.9, 28.2, 42.3, 50.6, 58.3, 72.7, 82.8, 83.7, 157.0, 171.1; $[a]_D^{16}$ -23.0 ^{o} (*c* 0.29, $CHCl_3$). HR-ESI-MS (positive) *m/z* 300.1809 $[M+H]^+$ (calcd for $C_{15}H_{26}NO_5$ 300.1810).

j. Synthesis of 2*R*-amino-4*S*-hydroxy-5-hexynoic acid (1)

The (2*R*, 4*S*)-2-*tert*-butoxycarbonylamino-4-hydroxyhex-5-ynoic acid *tert*-butyl ester (**7**, 80 mg, 0.27 mmol) was treated with anhydrous trifluoroacetic acid (2 mL) at 0 ^oC. The mixture was then stirred at ambient temperature for 4 hours, and concentrated *in vacuo*. The residue was dissolved in methanol (three times, 3×2 mL) and concentrated *in vacuo*. The final residue was purified by column chromatography (100 g of microcrystalline cellulose, *n*-BuOH: EtOH: AcOH: H_2O 80: 15: 10: 20), and yielded 40 mg (99%) as a colourless crystals (**1**). ^1H NMR (CD_3OD, 400 MHz) d: 2.19-2.10 (m, 2H, CH_2), 2.88 (s, 1H, H–C=C), 3.80 (s,1H, CHN), 4.58 (s, 1H, CHOH); ^{13}C NMR (CD_3OD, 150 MHz) d: 38.7, 54.1, 60.7, 74.8, 85.1, 174.0; $[a]_D^{16}$ -13.8^o (*c* 0.16, H_2O); IR (KBr) ?$_{max}$ 3424, 3216, 2102, 1650, 1472, 1406 cm^{-1}; HR-ESI-MS (positive) *m/z* 166.0481 $[M + Na]^+$ (calcd for $C_6H_9NO_3Na$, 166.0480).

k. Acute toxicity experiments

ICR Mice were maintained at standard conditions of 12 h light/darkness, humidity and temperature. They were kept in cages, fed on standard diet with free access to water throughout the period of experimentation. Each sample (**1** and **2**) was dissolved in water and orally force-fed to mice by using an intragastric needle (male/female,

18-22 g of weight). Pilot tests were conducted to determine the dose range of the compounds **1** and **2** to be administered in mice. The maximum dose producing 0% death and the minimum dose that produced 100% death were obtained. From these, 0.2 mL/kg of compounds **1** or **2** with 5 graded doses was given intragastric administration once to 5 groups of 6 mice each. The animals were observed for symptoms of toxicity and mortality within 7 days.

l. HPLC-MS/MS analysis of victim's blood

An Aglient 1200 series instrument equipped with an Agilent Zorbax XDB-C18 column (5 μm, 4.6 × 250 mm) was used for high-performance liquid chromatography (HPLC) analysis. A Bruker HCT/Esquire instrument was used for tandem mass spectrometry (MS/MS) analysis.

m. The serum creatine kinase assay in mice

Healthy adult ICR mice were randomly divided into five groups: group 1, negative control group (CON, water-treated); group 2, positive control given *p*-phenylenediamine 70 mg/kg body weight (PD); groups 3-5, three doses samples treated group given series of concentrations of compounds **1** (20, 40, and 60 mg/kg) and **2** (10, 20, and 40 mg/kg). Each sample was dissolved in water and force-fed to mice by using a catheter once a day for three days. The blood samples of mice were collected from tail vein 4 hours after the final dose, and centrifuged at 3000 rpm for 10 min in 4°C to obtain serum. The creatine kinase concentration of the serum was

determined with the mouse creatine kinase ELISA kit (Wuhan EIAab Science Co., Ltd., China).

2. Supplementary figures

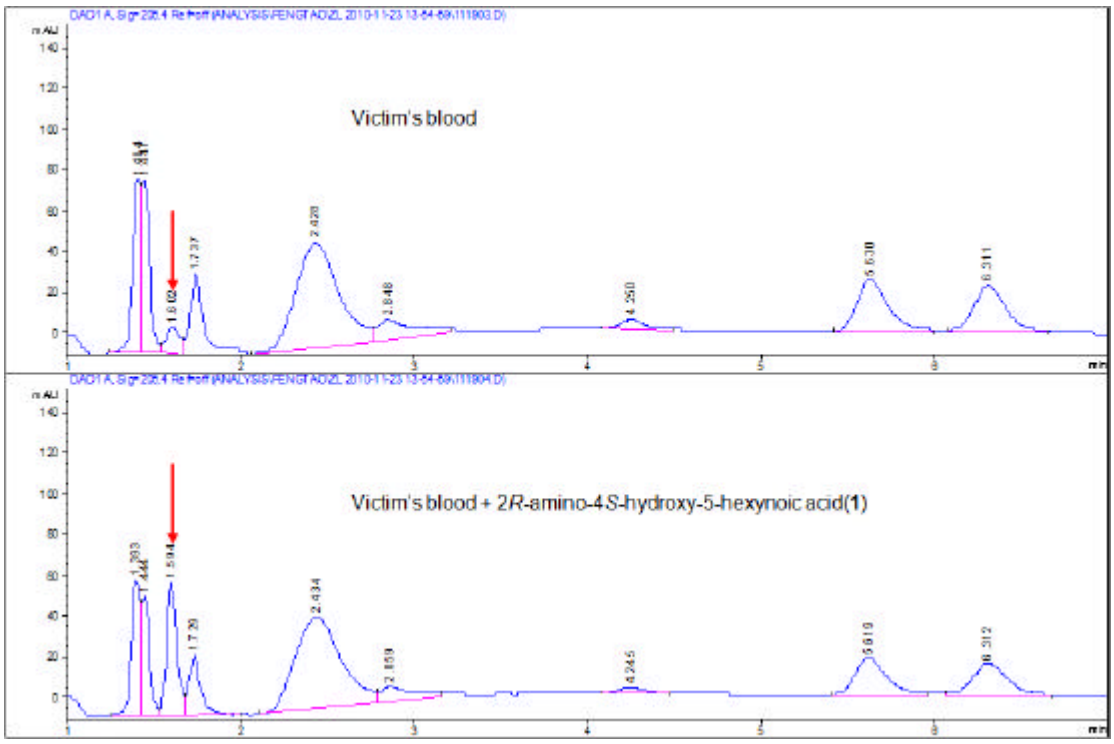

Figure 1. Upper: HPLC profile of a dead man's heart blood. Bottom: HPLC profile of a dead man's heart blood and added authentic sample (2*R*-amino-4*S*-hydroxy-5-hexynoic acid, **1**). The samples were eluted with pure water and monitored by UV absorption at 205 nm with a photodiode array detector (Agilent Zorbax XDB-C18 column, 5 μm, 4.6 × 250 mm).

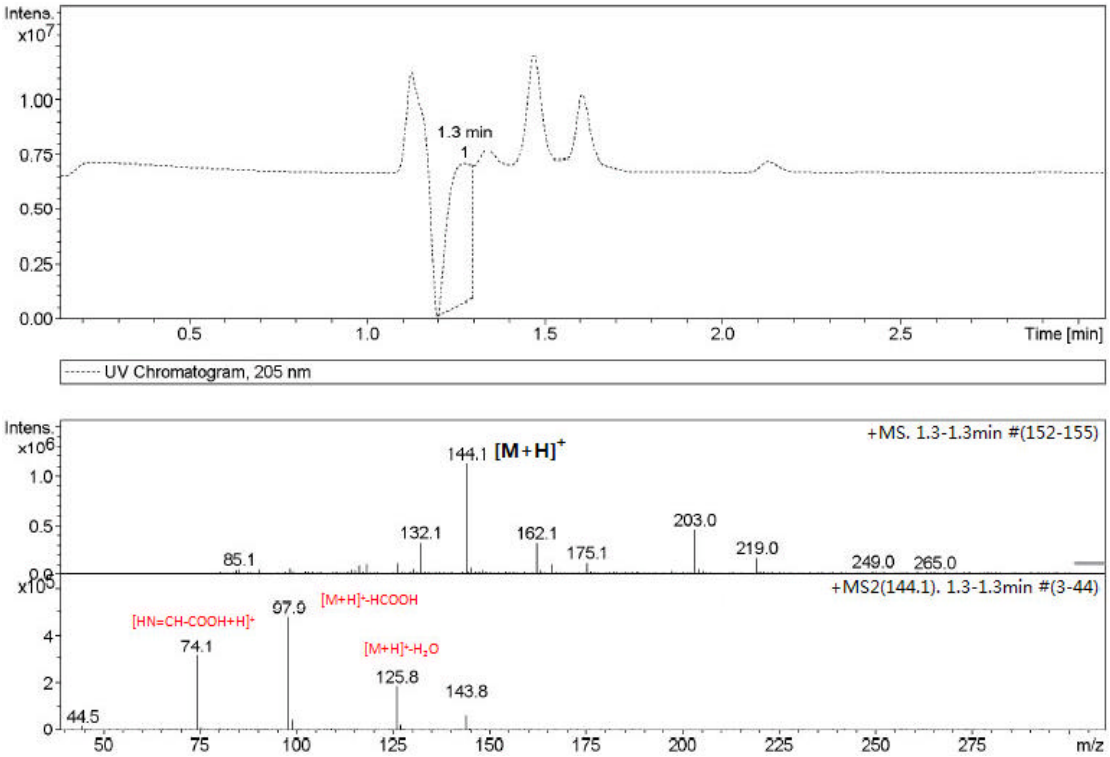

Figure 2. Upper: An ESI-MS survey scan of the dead man's heart blood during LC-MS analysis at time 1.3 min. Bottom: The corresponding ESI-MS/MS scan of precursor ion *m/z* 144.1. The samples were eluted with pure water and monitored by UV absorption at 205 nm with a photodiode array detector (Agilent Zorbax XDB-C18 column, 5 μm, 4.6 × 250 mm). The fragment ions at *m/z* 126, 98, 74 are $[M+H]^+$-H_2O, $[M+H]^+$-HCOOH, $[HN=CH-COOH+H]^+$, respectively.

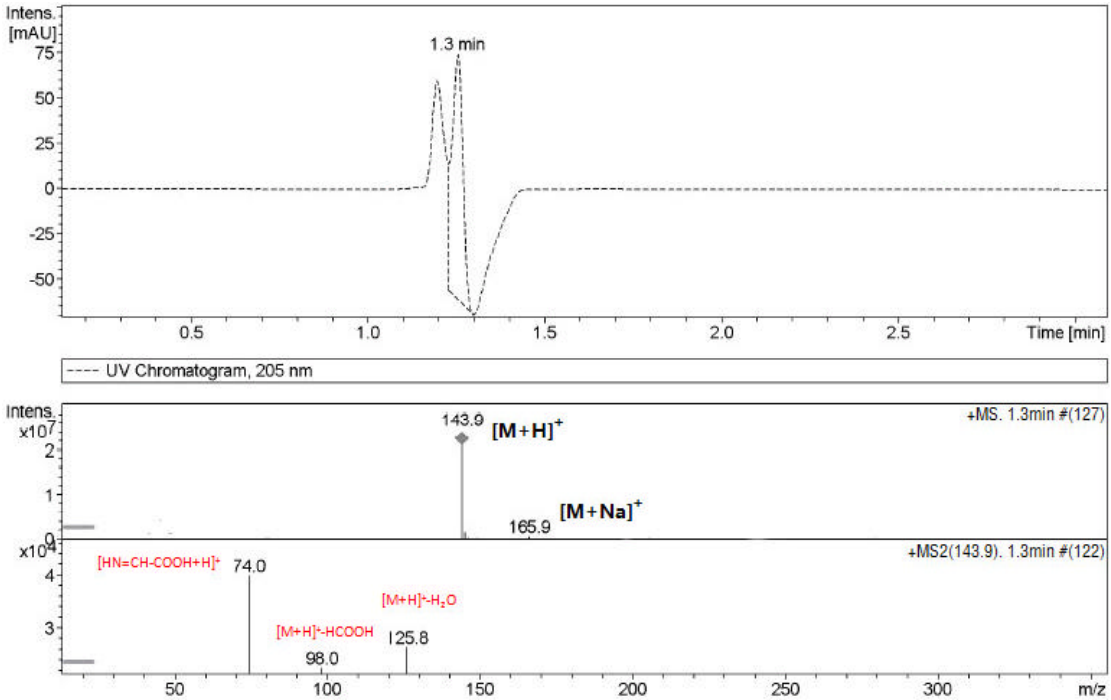

Figure 3. Upper: An ESI-MS survey scan of 2*R*-amino-4*S*-hydroxy-5-hexynoic acid (**1**) during LC-MS analysis at time 1.3 min. Bottom: The corresponding ESI-MS/MS scan of precursor ion *m/z* 143.9. The sample was eluted with pure water and monitored by UV absorption at 205 nm with a photodiode array detector (Agilent Zorbax XDB-C18 column, 5 μm, 4.6 × 250 mm). The fragment ions at *m/z* 126, 98, 74 are $[M+H]^+$-H_2O, $[M+H]^+$-HCOOH, $[HN=CH-COOH+H]^+$, respectively.

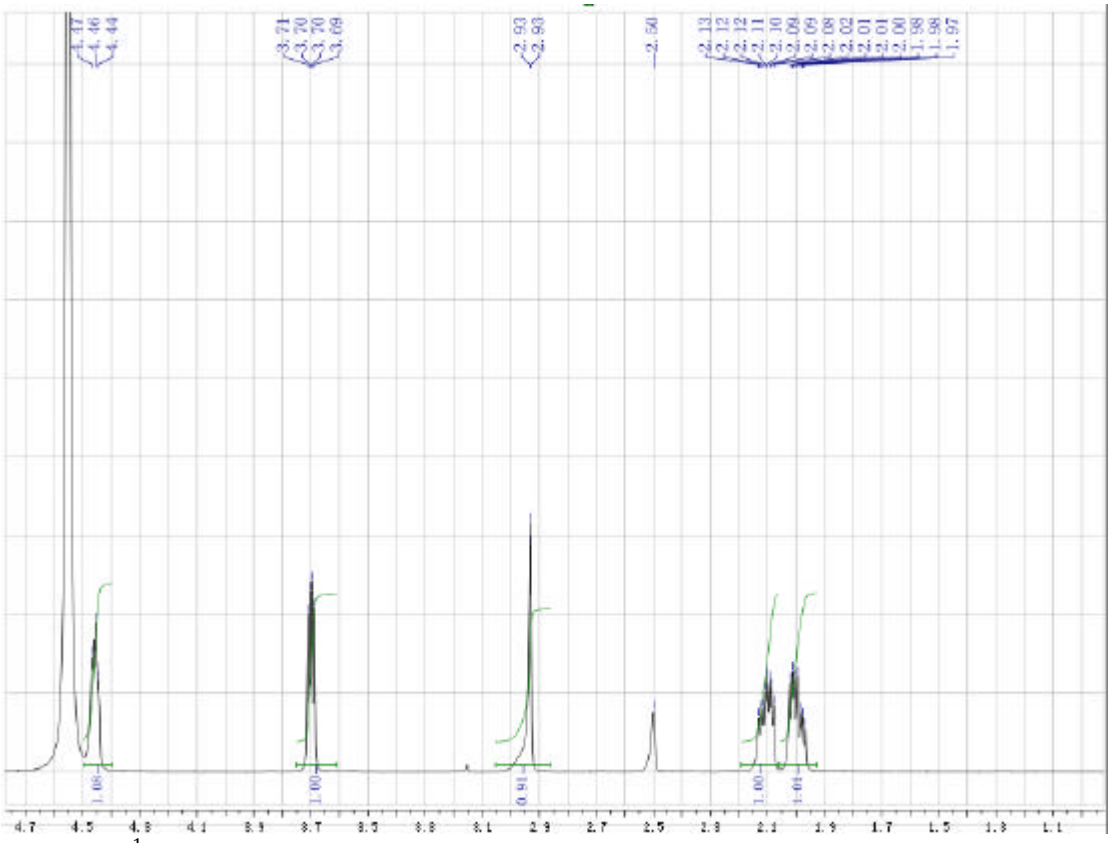

Figure 4. ^{1}H NMR spectrum of **1** (natural) in D_2O

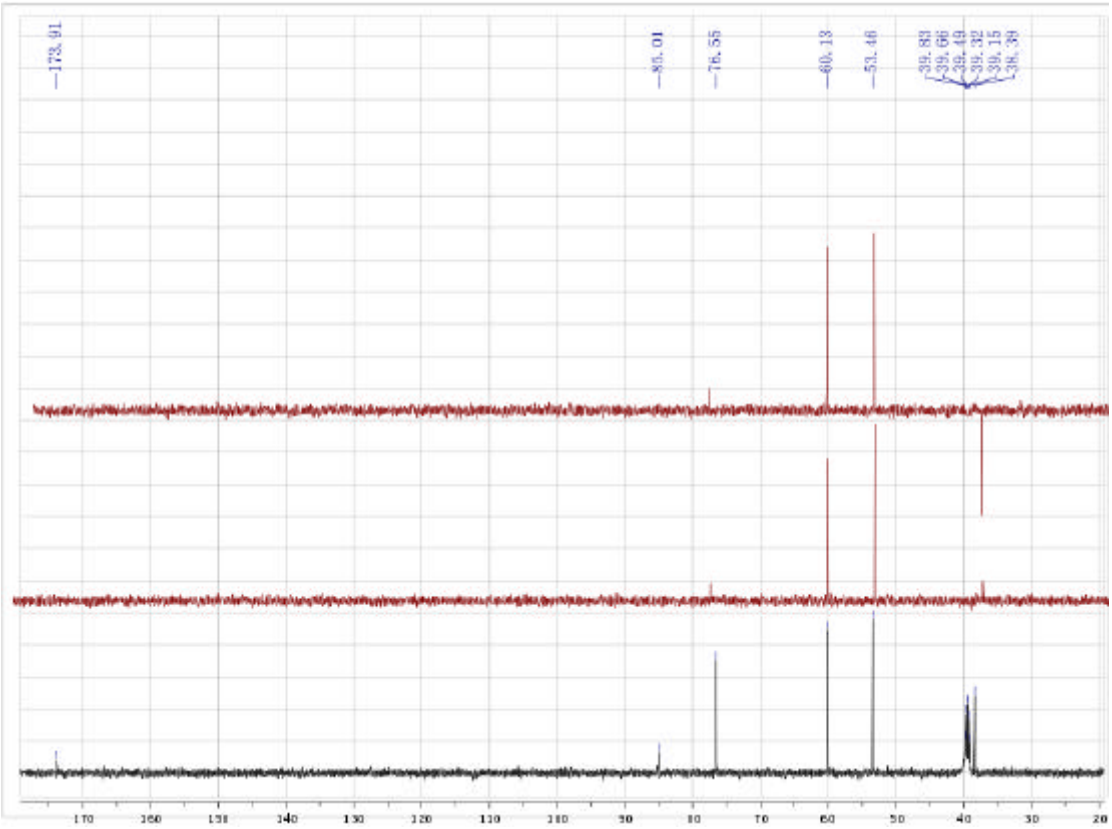

Figure 5. ^{13}C NMR spectrum of **1** (natural) in D_2O

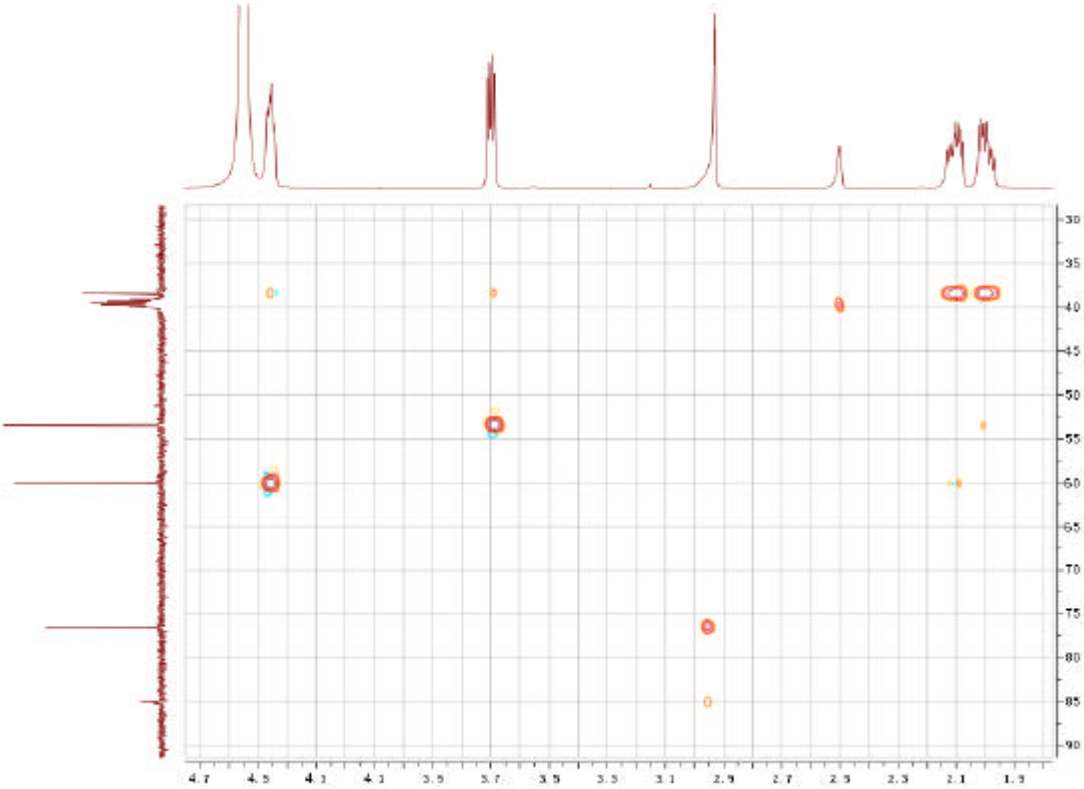

Figure 6. HSQC spectrum of **1** (natural) in D_2O

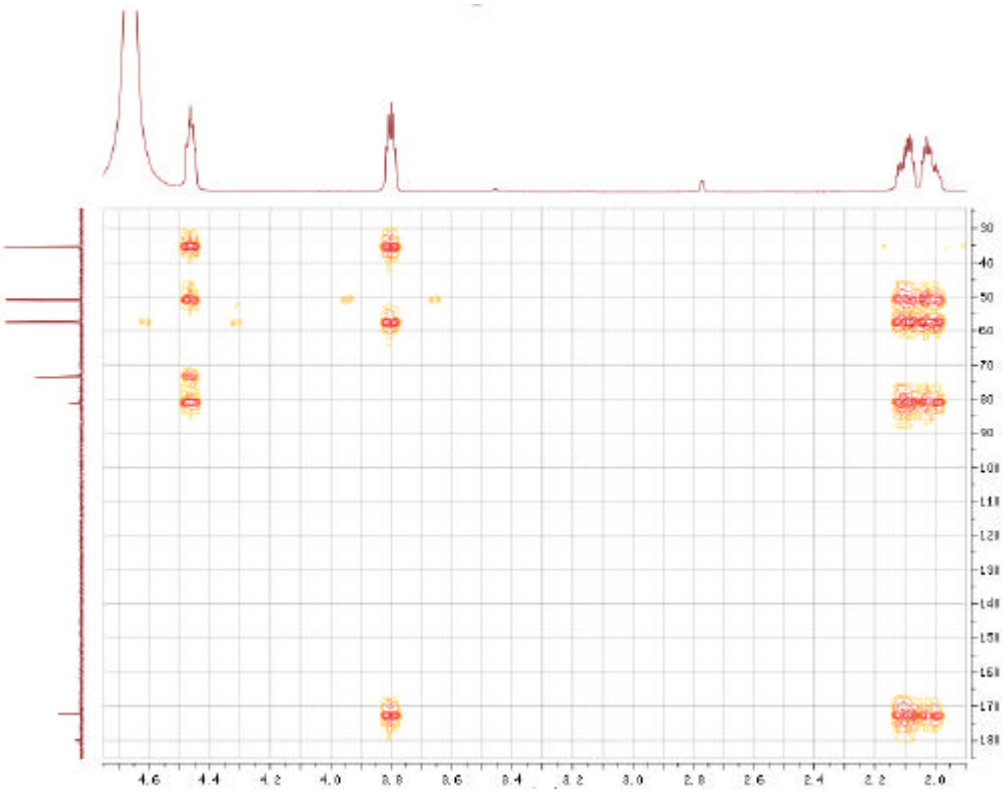

Figure 7. HMBC spectrum of **1** (natural) in D_2O

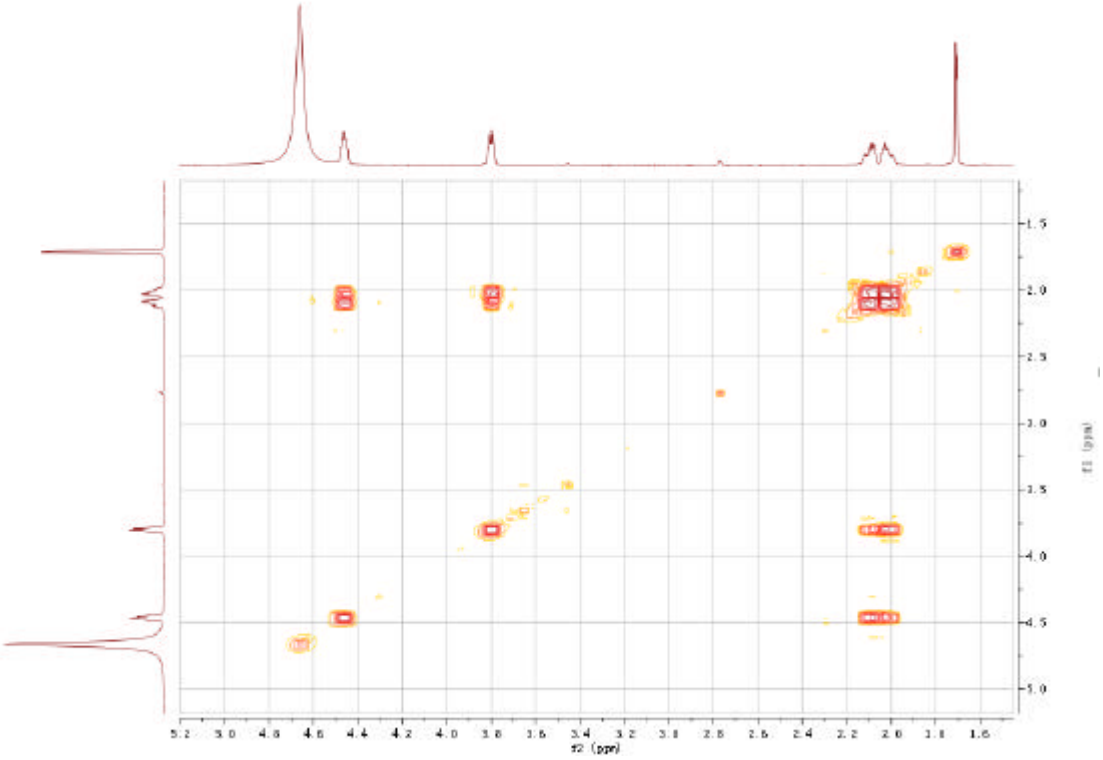

Figure 8. ^{1}H-^{1}H COSY spectrum of **1** (natural) in D_2O

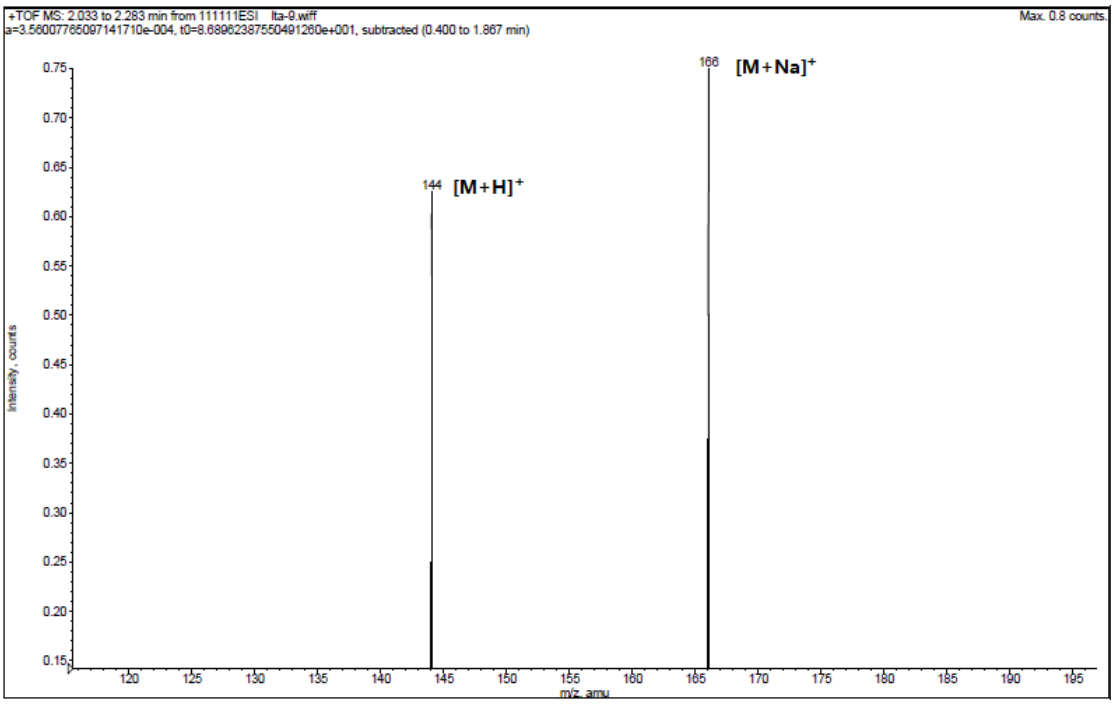

Figure 9. ESI-MS of **1** (natural)

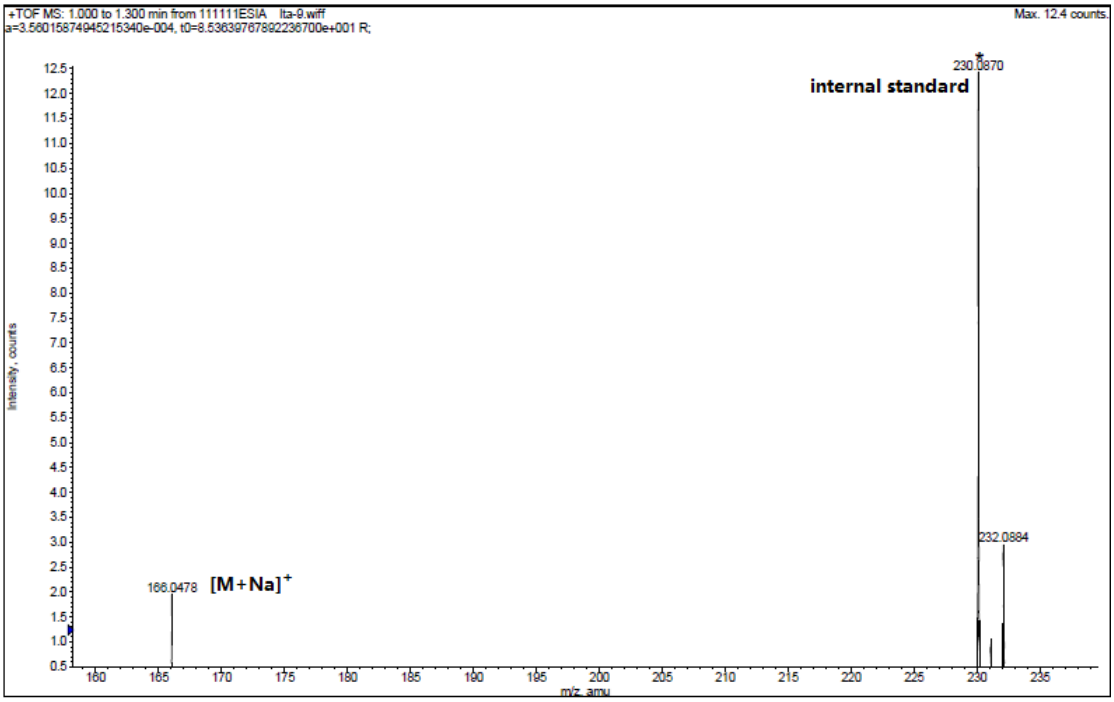

Figure 10. Positive HR-ESI-MS of **1** (natural)

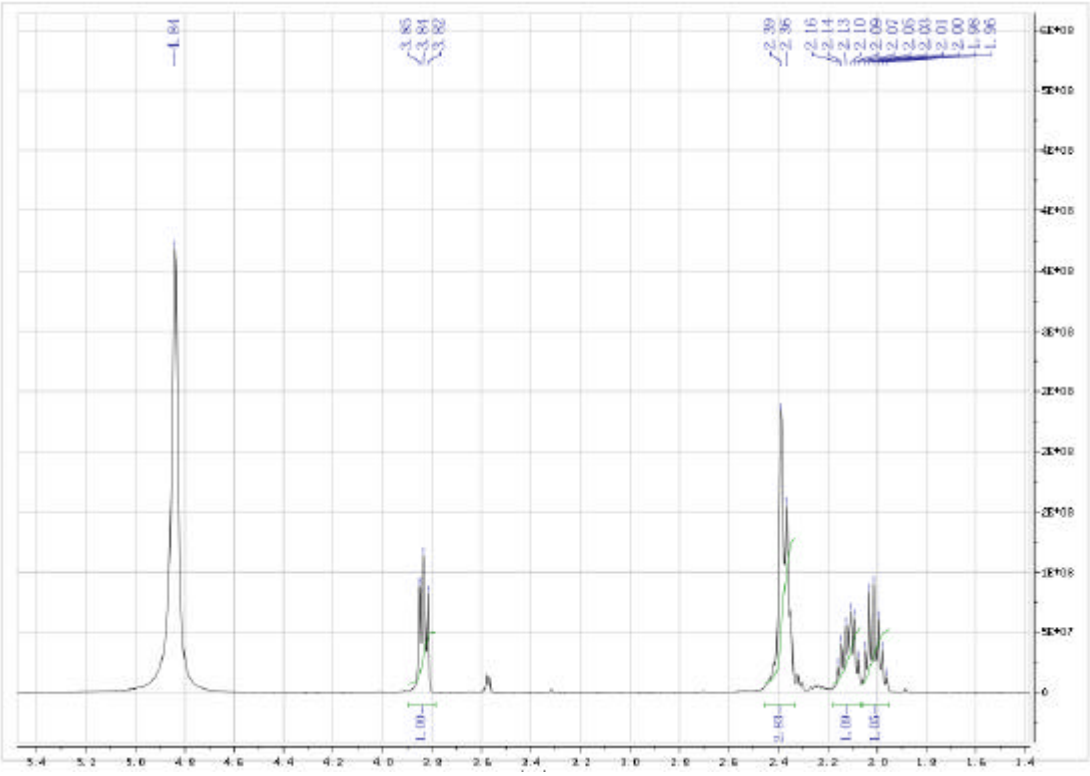

Figure 11. ^{1}H NMR spectrum of **2** (natural) in D_2O

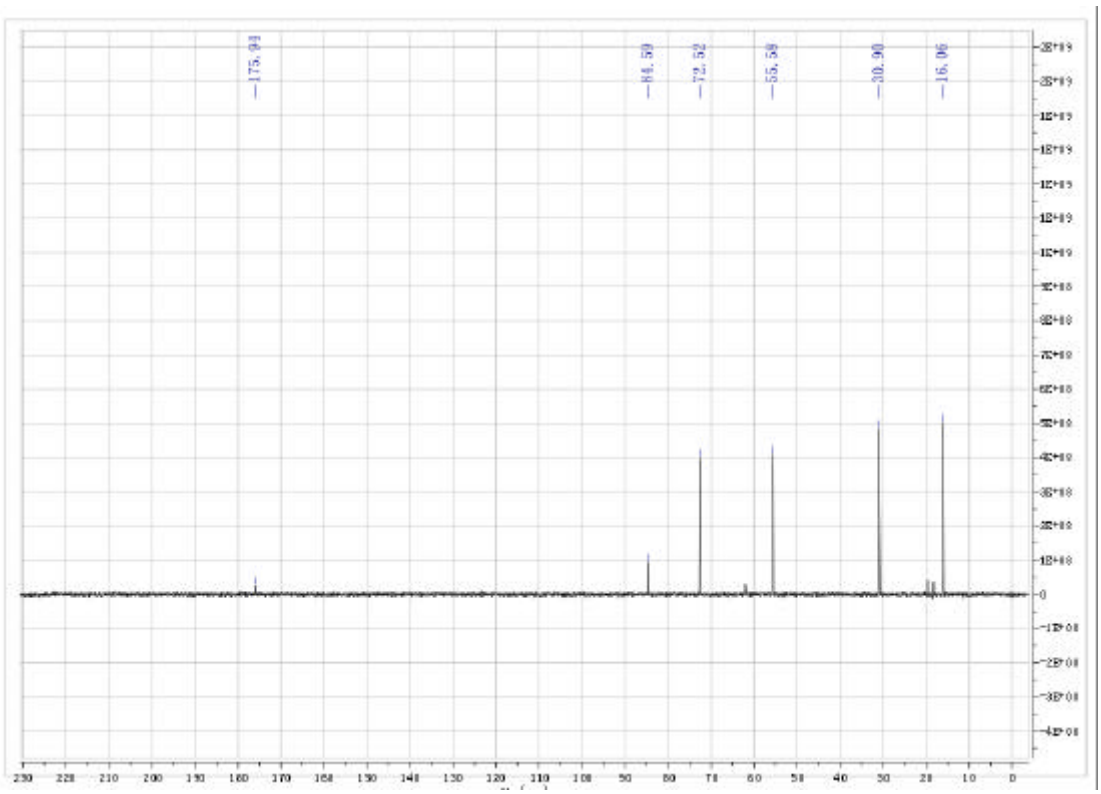

Figure 12. ^{13}C NMR spectrum of **2** (natural) in D_2O

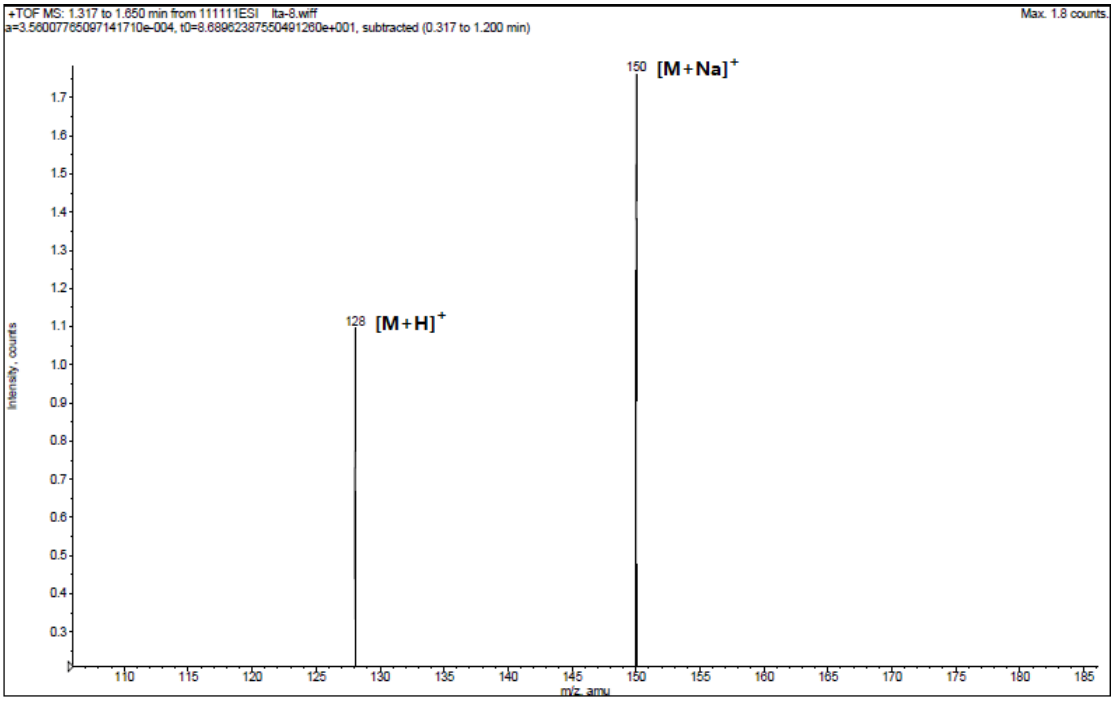

Figure 13. ESI-MS of **2** (natural)

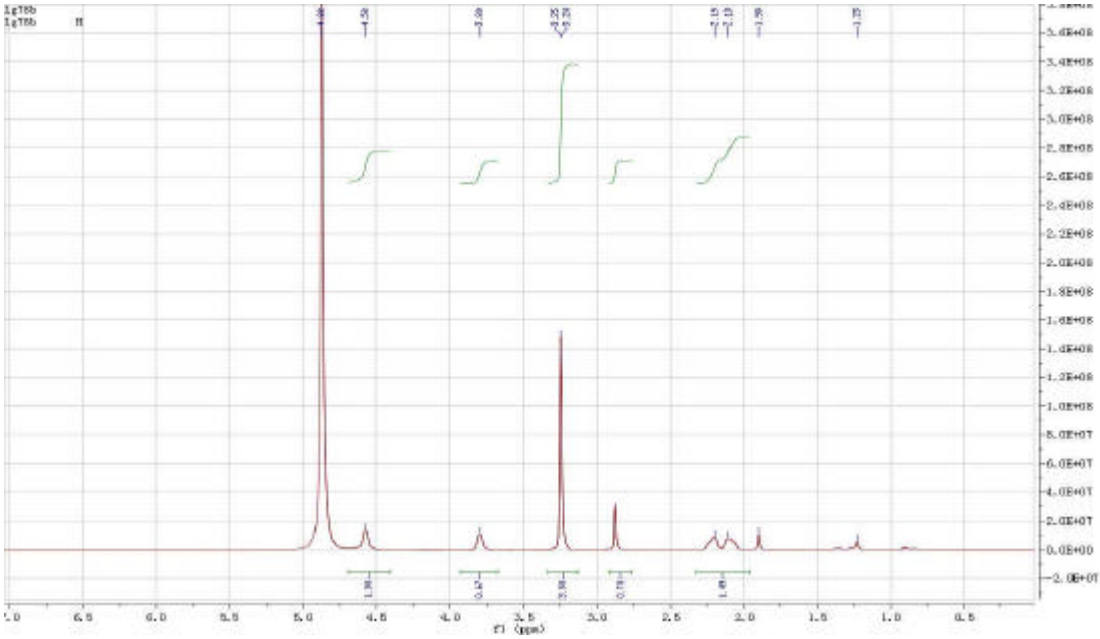

Figure 14. ^{1}H NMR spectrum of **1** (synthetic) in D_2O

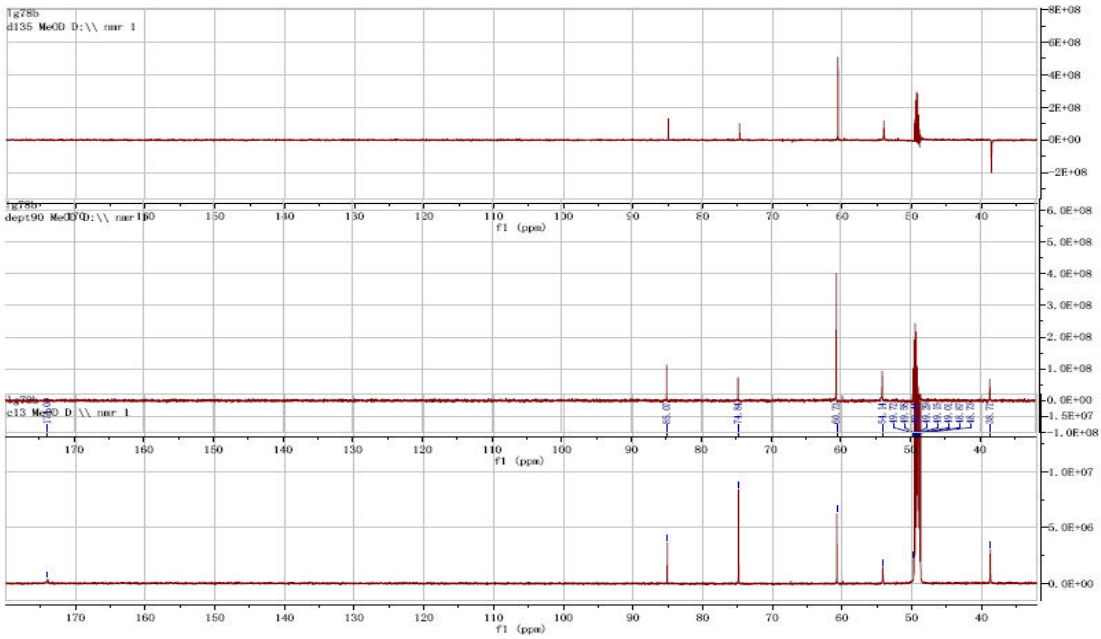

Figure 15. ^{13}C NMR spectrum of **1** (synthetic) in D_2O

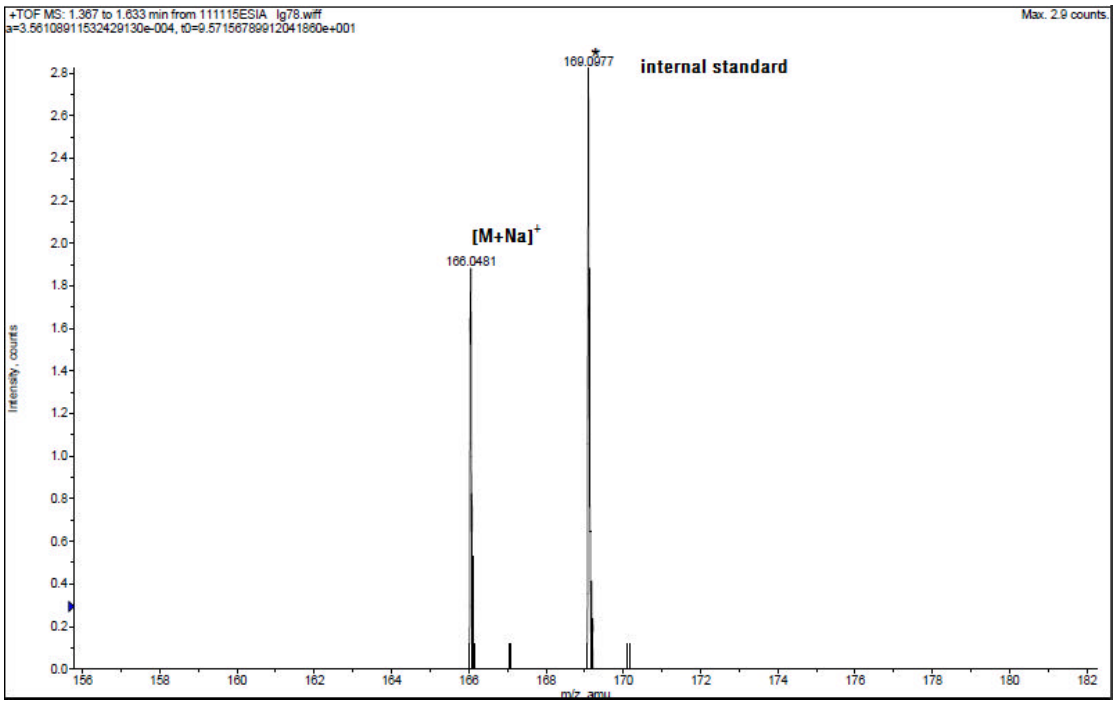

Figure 16. Positive HR-ESI-MS of **1** (synthetic)

3. Supplementary Table

Table 1. ^{1}H and ^{13}C NMR data of compounds 1 and 2 at 500 MHz in DMSO-*d*$_6$ and D_2O, respectively

No.	**1**		**2**	
	d_C	d_H	d_C	d_H
1	173.9 s		175.9 s	
2	53.5 d	3.70 (dd, 7.3, 4.3)	55.6 d	3.84 (dd, 7.0, 5.7)
3	38.4 t	2.10 (ddd, 14.6, 7.9, 4.3)	30.9 t	2.11 (m)
		2.00 (ddd, 14.6, 7.3, 4.9)		2.00 (m)
4	60.1 d	4.46 (dd, 7.9, 4.9)	16.1 t	2.38 (m)
5	85.0 s		84.6 s	
6	76.6 d	2.93 (d, 1.8)	72.5 d	2.40 (s)