CHEMISTRY
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Supporting Information

Synthesis, Characterization, and In Vitro Evaluation of Pro2-Ile3-S-Deoxoamaninamide and Pro2-D-allo-Ile3-S-Deoxoamaninamide; Implications for Structure Activity Relationships in Amanitin Conformation and Toxicity

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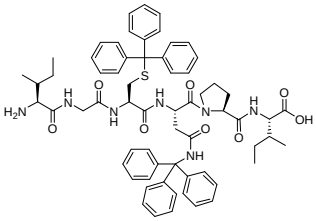
Contents

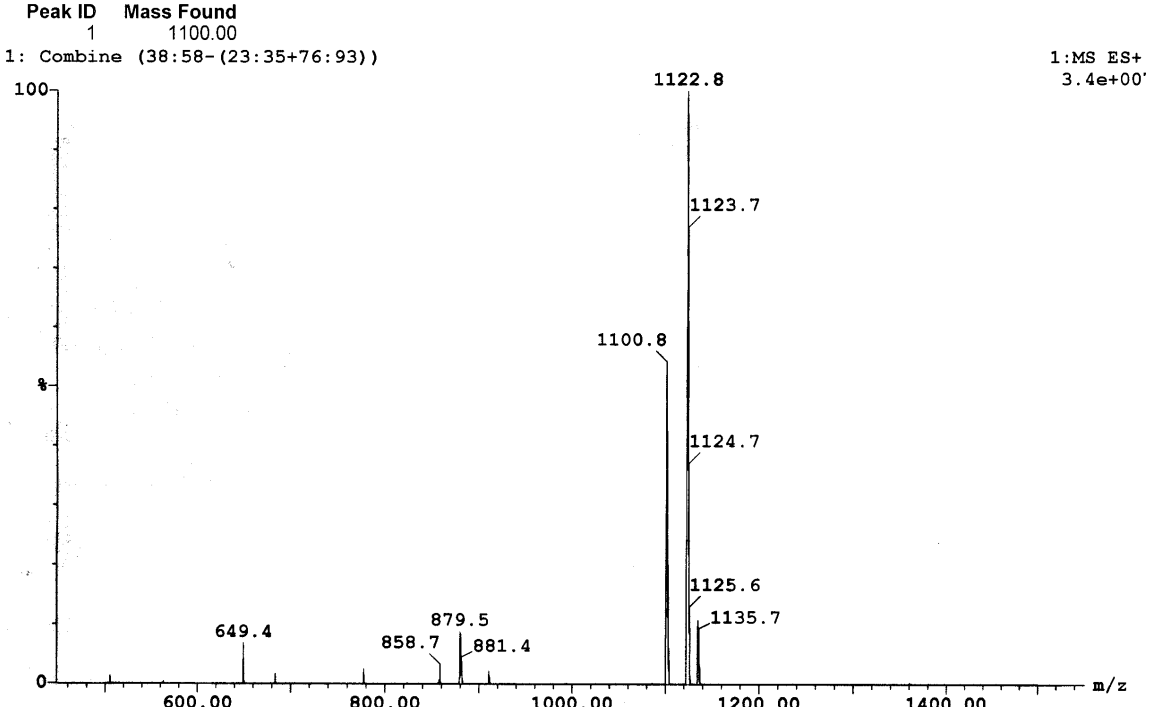
Characterisation of linear hexapeptide 2:



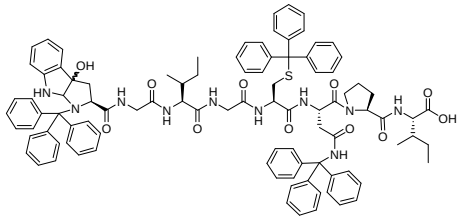
Chemical Formula: $C_{64}H_{73}N_7O_8S$
Exact Mass: 1099.5241
Molecular Weight: 1100.3715

H-Ile-Gly-Cys(Tr)-Asn(Tr)-Pro-Ile-OH

1100.8 $(M+H)^+$; 1122.8 $(M+Na)^+$



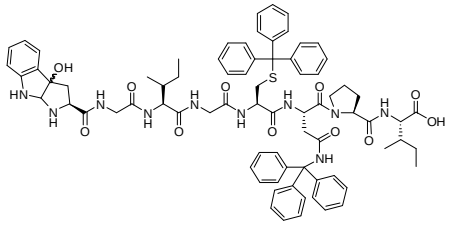
Characterisation of linear octapeptide 3:



Chemical Formula: $C_{96}H_{100}N_{10}O_{11}S$
Exact Mass: 1600.7294
Molecular Weight: 1601.9466

Tr-Hpi-Gly-Ile-Gly-Cys(Tr)-Asn(Tr)-Pro-Ile-OH

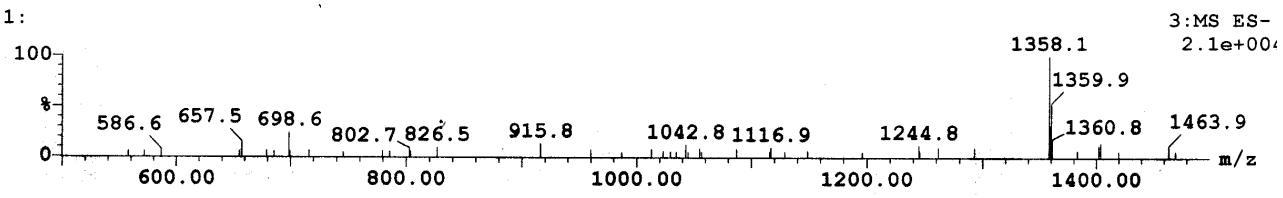
But following deprotection with HFIP structure will be:



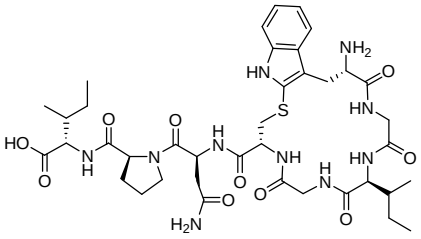
Chemical Formula: $C_{77}H_{86}N_{10}O_{11}S$
Exact Mass: 1358.6198
Molecular Weight: 1359.6321

H-Hpi-Gly-Ile-Gly-Cys(Tr)-Asn(Tr)-Pro-Ile-OH

1358.1 $(M-H)^-$

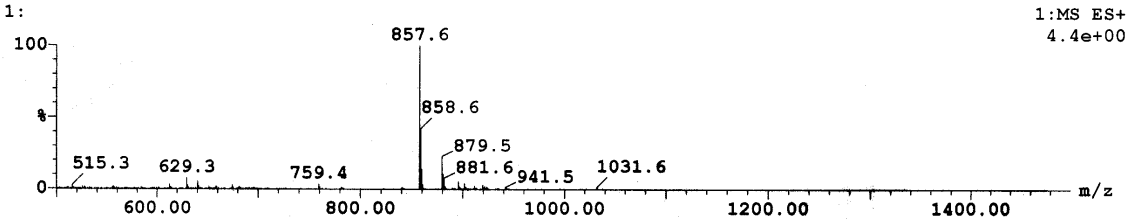


Characterisation of monocyclic octapeptide 4:

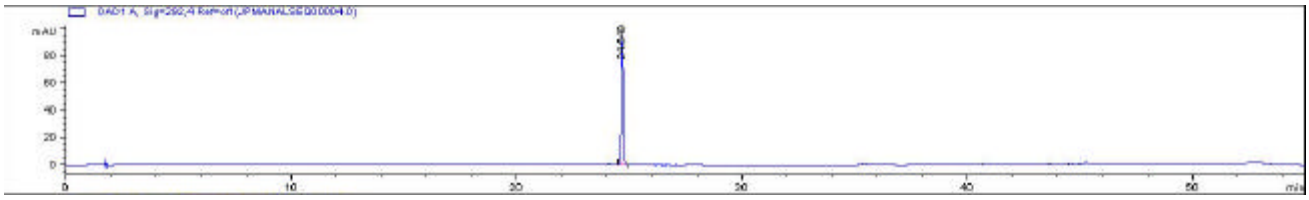


Chemical Formula: $C_{39}H_{56}N_{10}O_{10}S$
Exact Mass: 856.3902
Molecular Weight: 856.9879

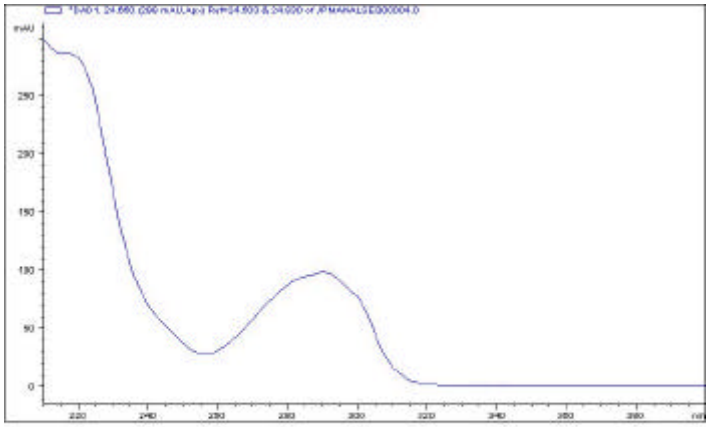
Mass Spectrometry: 857.6 $(M+H)^+$



HPLC trace following purification:

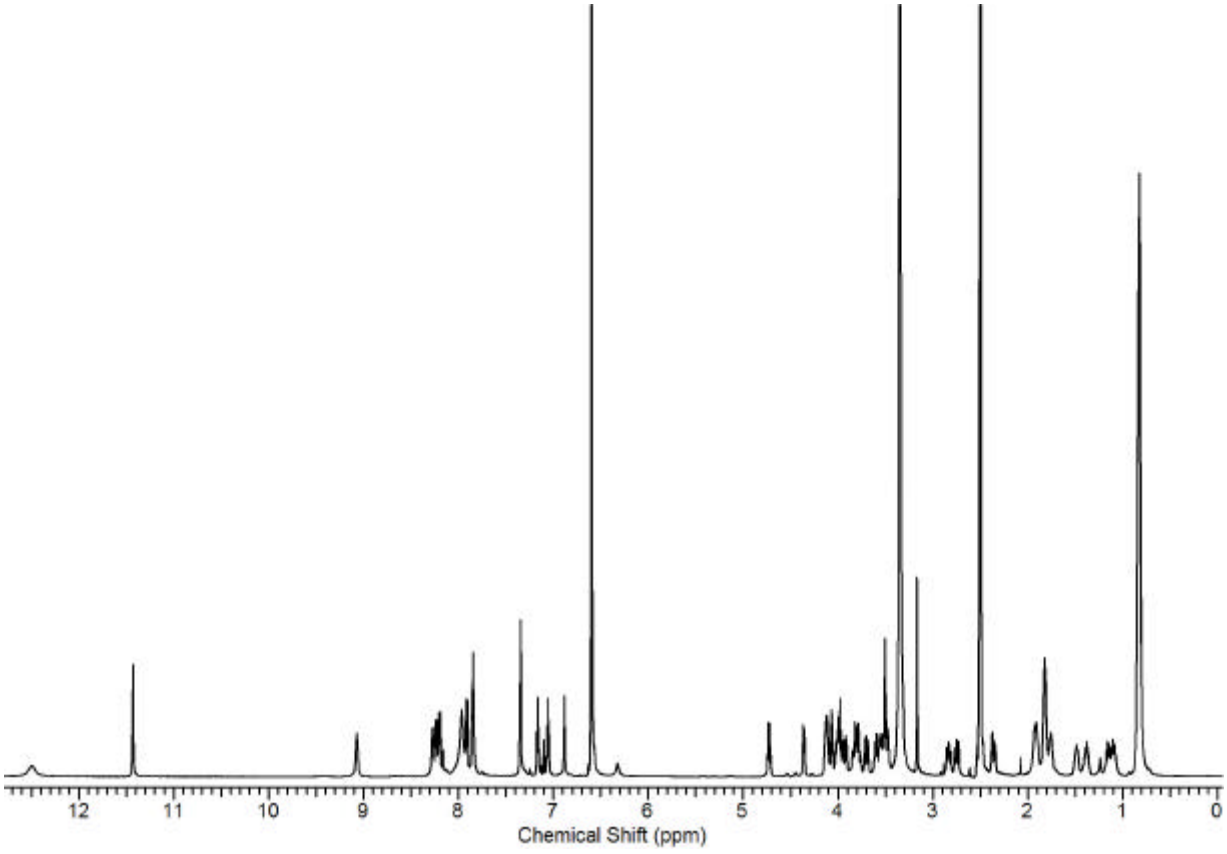


UV absorption from DAD of HPLC: $?_{max}$ = 290 nm

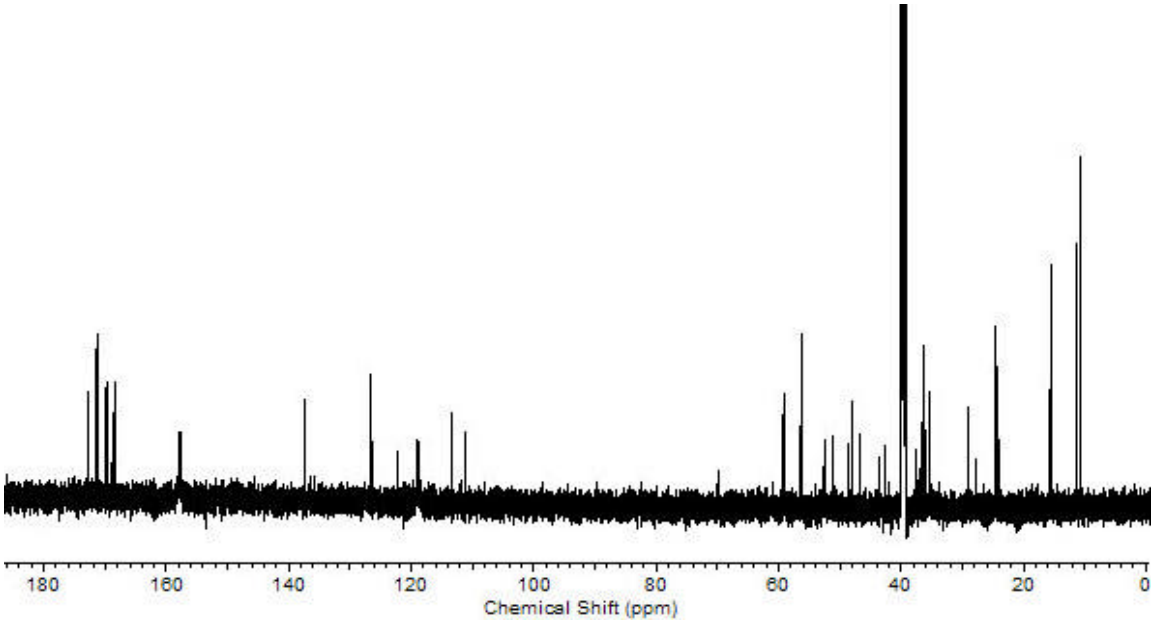


NMR spectra of monocycle octapeptide 4

1H NMR



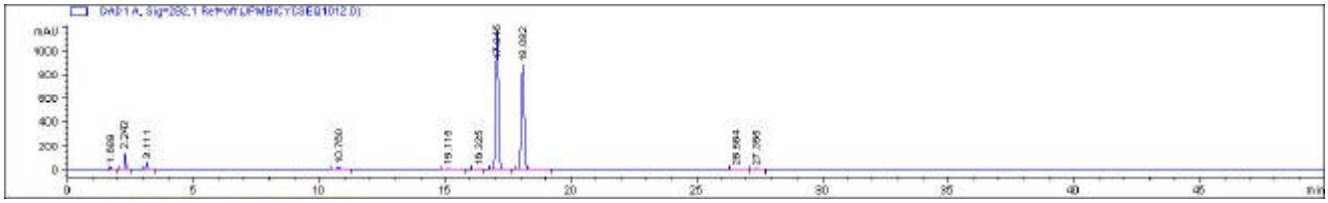
13C NMR



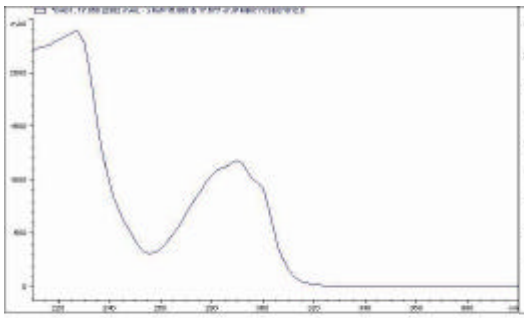
HPLC purification of bicyclic octapeptide 1:

Purification was initially with silica gel column chromatography ($CHCl_3$/MeOH/H_2O 90:13:1). One spot was obtained - rf ~ 0.5.

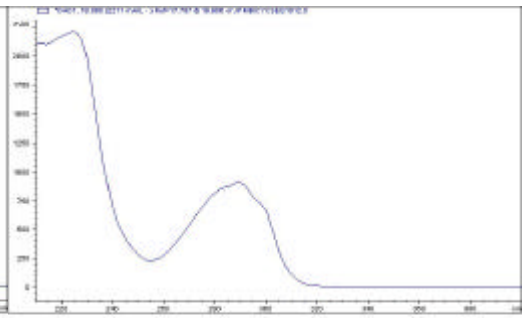
This product was injected on the HPLC (C18, H_2O/MeCN (0.1%TFA)) ?= 229 & 292 nm.



Peak 1 - compound **1a**



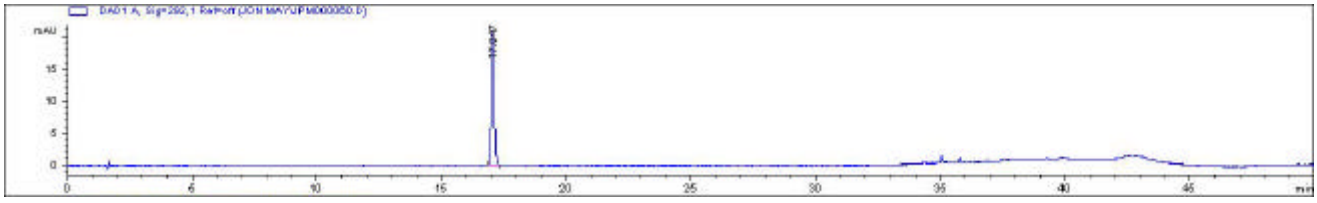
Peak 2 – compound **1b**



Both peaks show the characteristic tryptathionine UV absorption spectrum ($?_{max}$= 290 nm) and desired mass. Analytical HPLC traces showed that these compounds could be separated from each other.

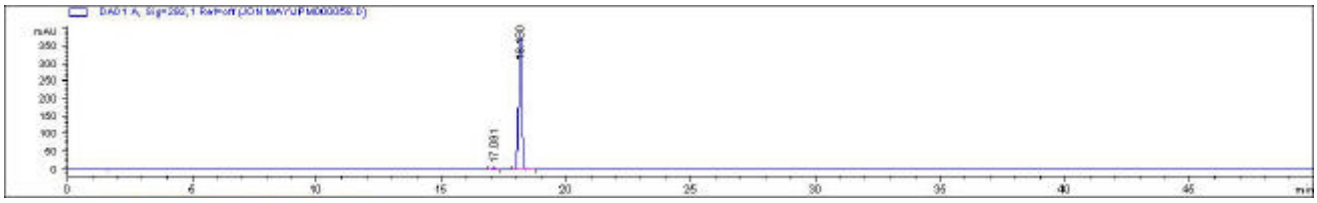
Analytical HPLC trace for bicyclic octapeptide 1a:

Analytical HPLC trace: rt = 17.95



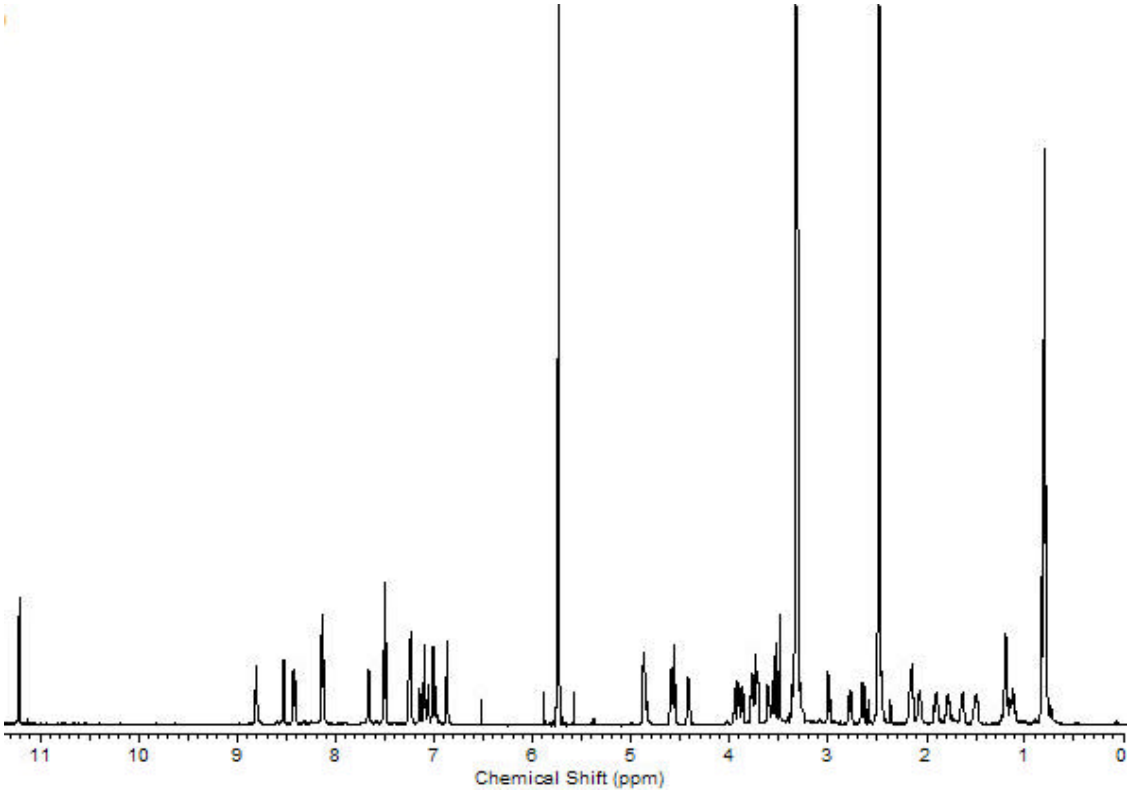
Analytical HPLC trace for bicyclic octapeptide 1b:

Analytical HPLC trace: rt = 18.13

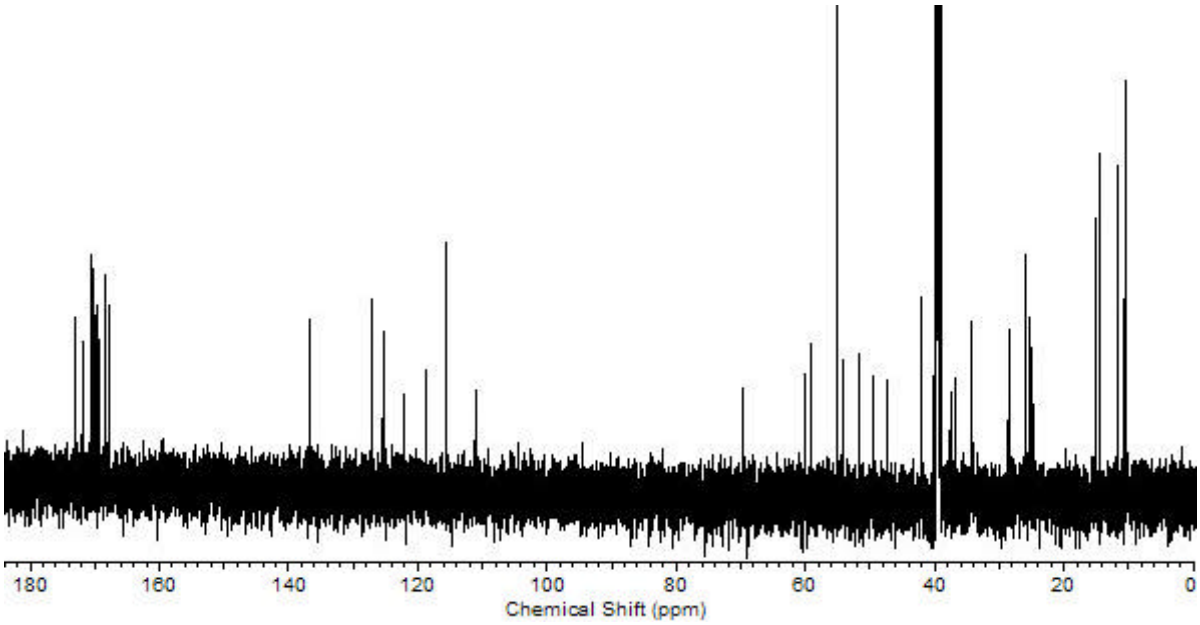


NMR spectra of 1a

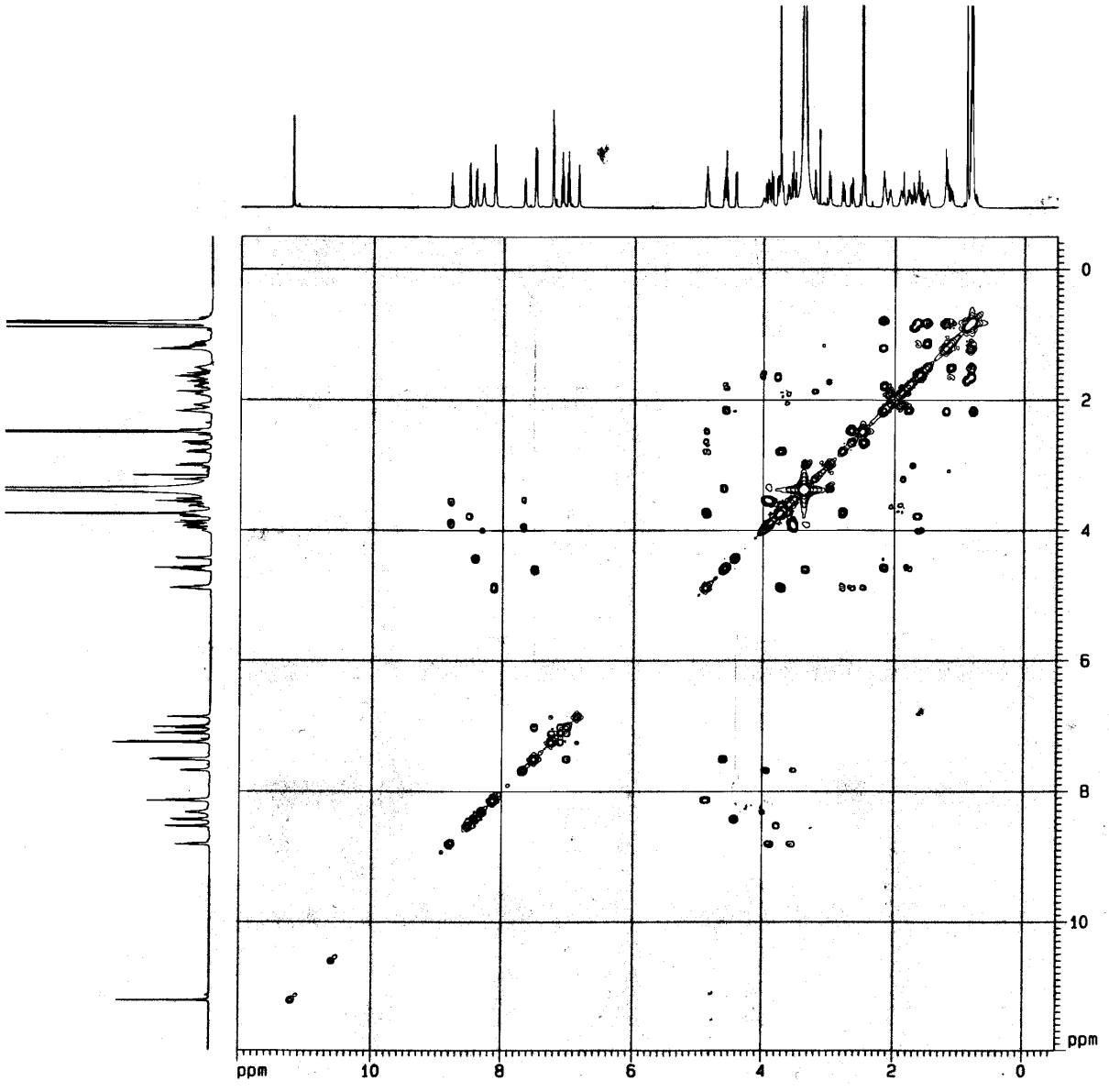
1H NMR



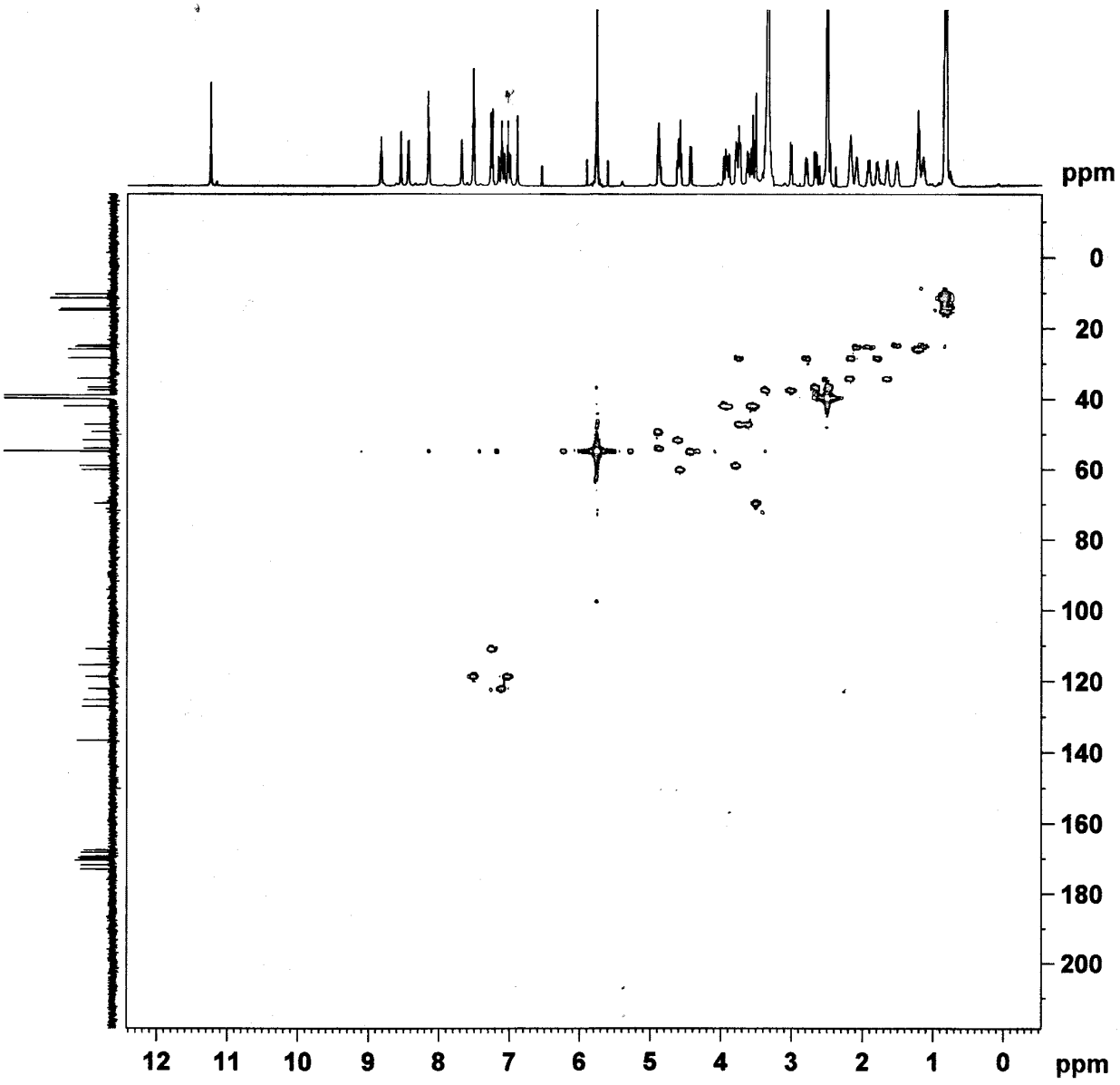
13C NMR


180
160
140
120
100
80
60
40
20
0
Chemical Shift (ppm)

COSY NMR of 1a

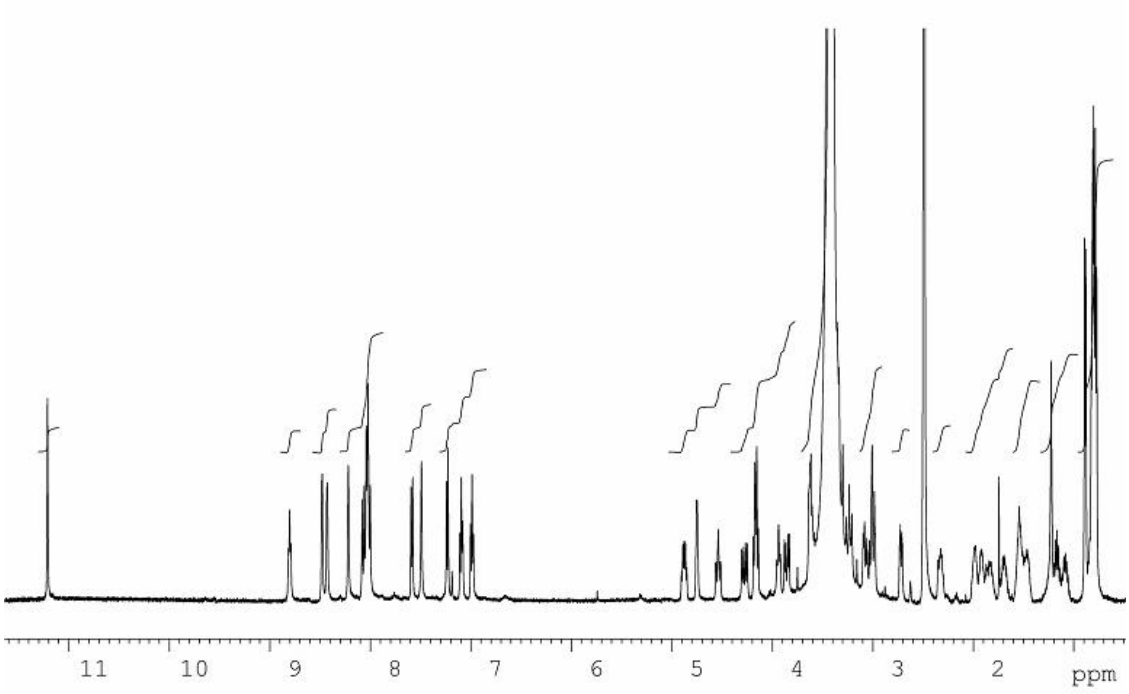


HMQC NMR of 1a

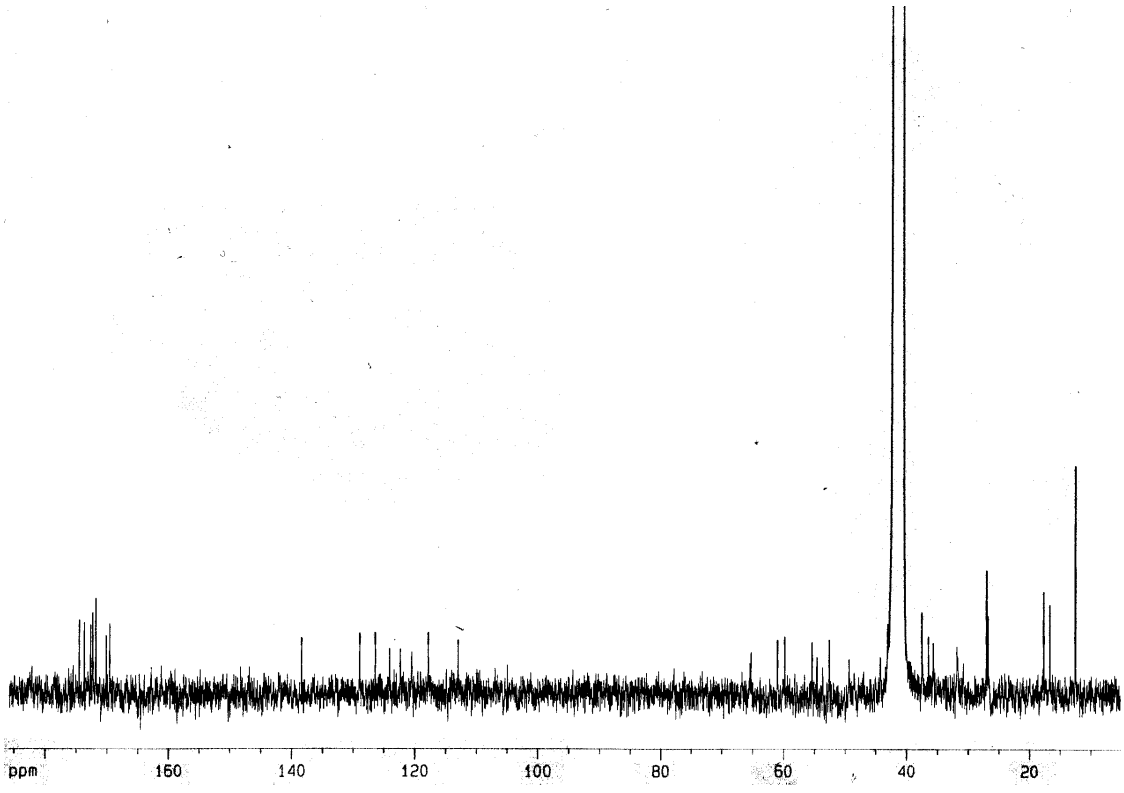

ppm
0
20
40
60
80
100
120
140
160
180
200
12
11
10
9
8
7
6
5
4
3
2
1
0
ppm

NMR spectra of 1b

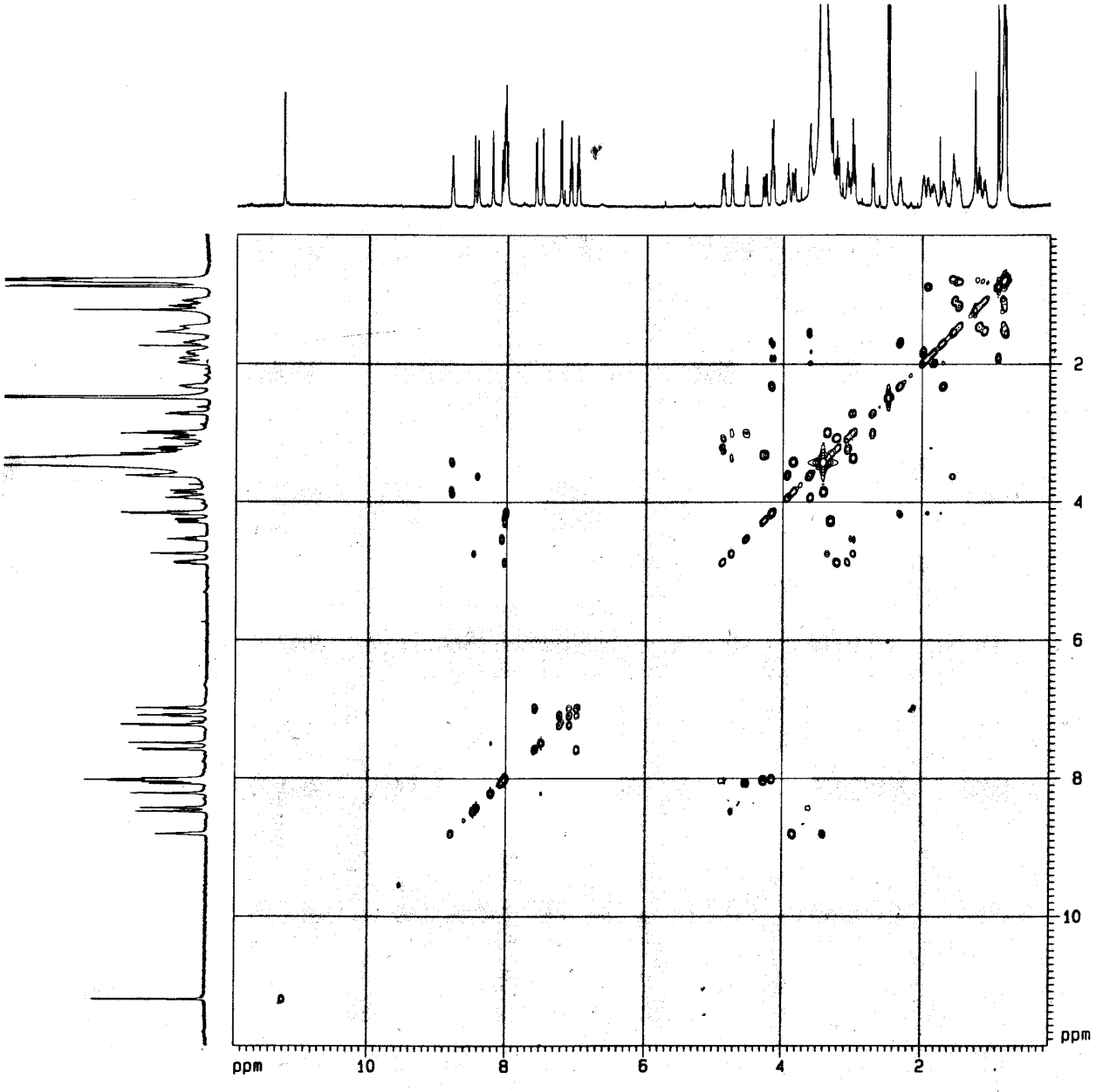
1H NMR



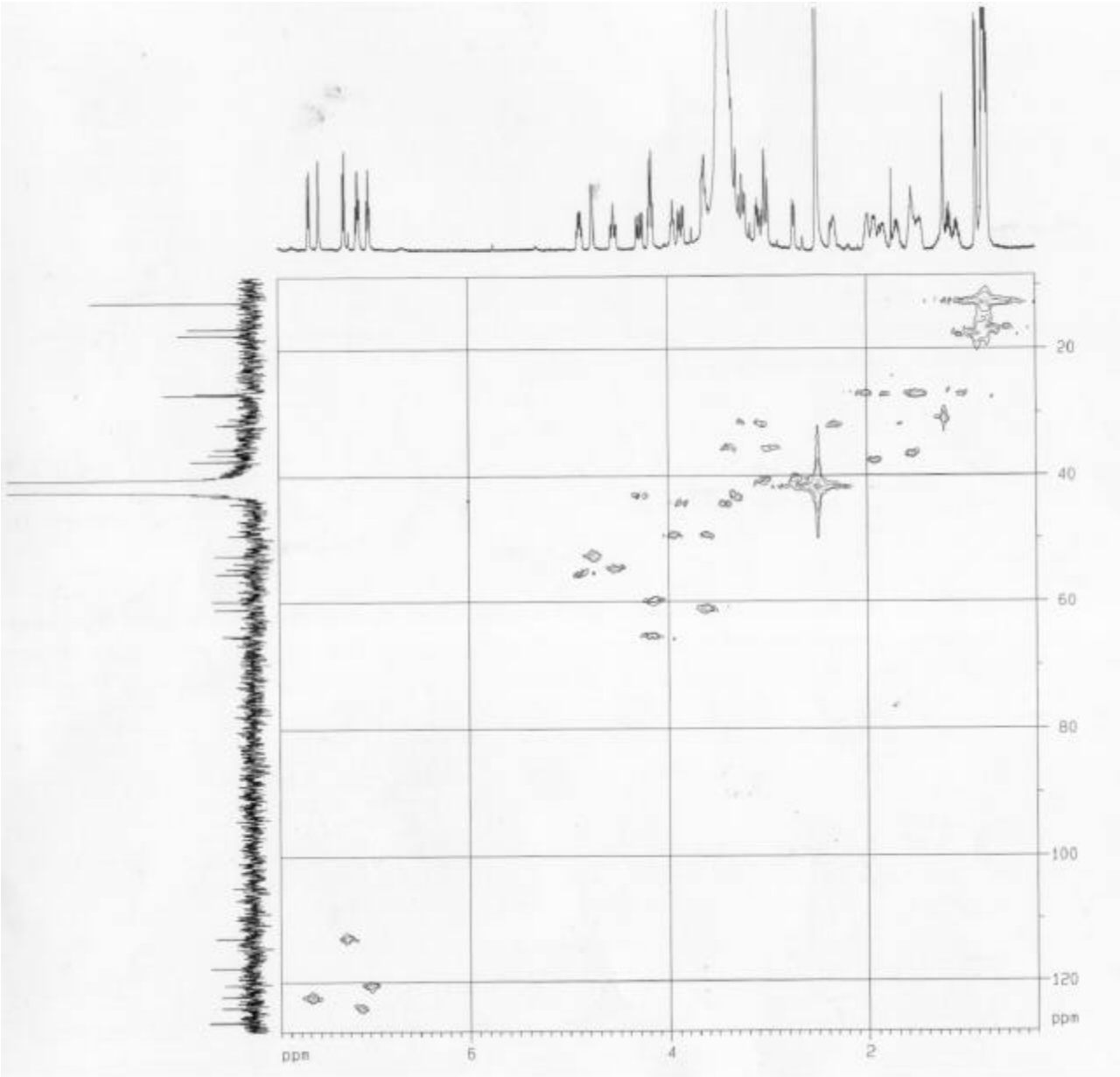
13C NMR



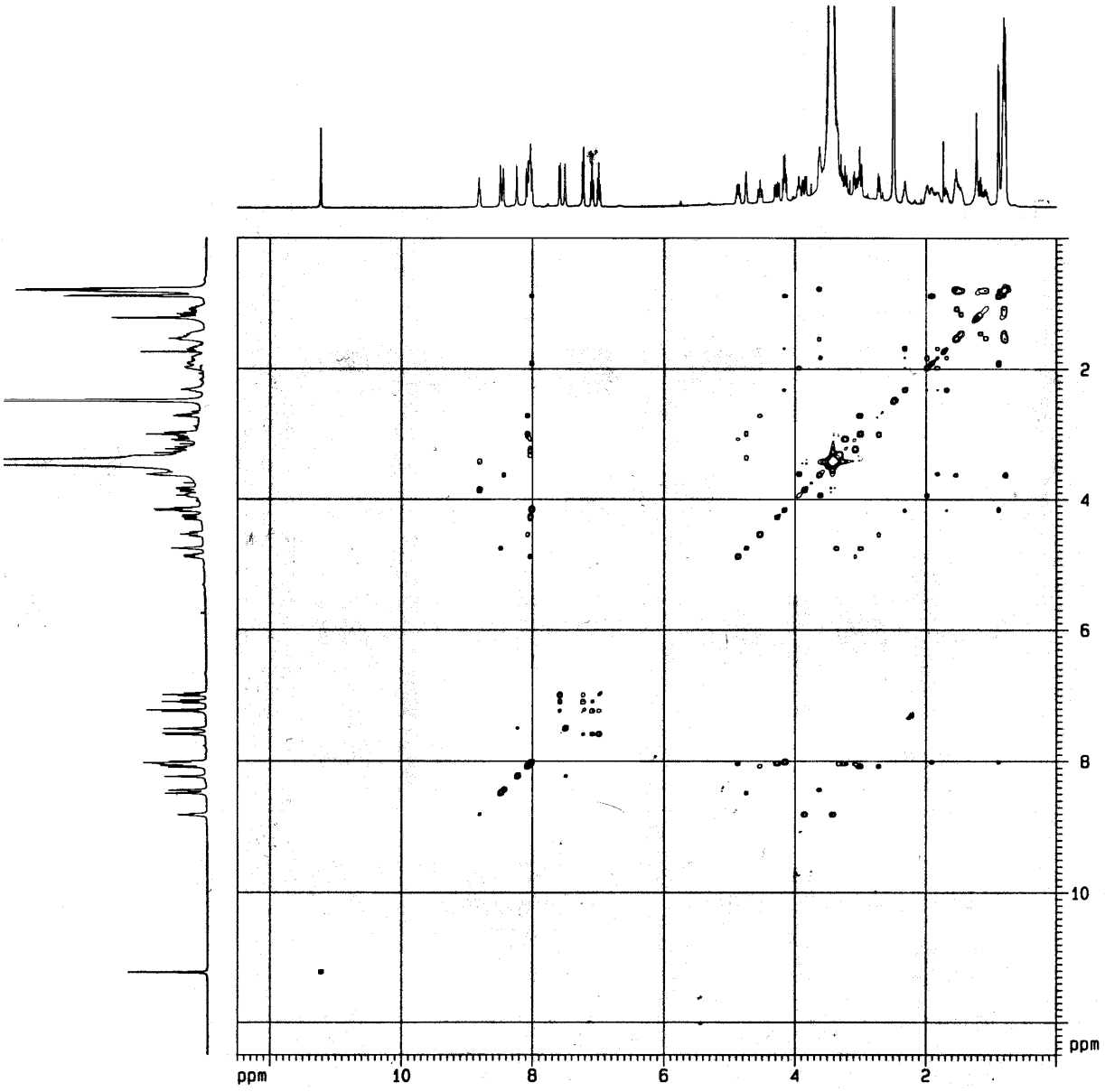
COSY NMR of 1b



HMQC NMR of 1b

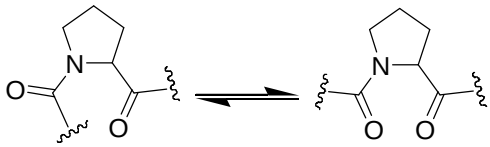


TOCSY NMR of 1b



Study of isomers of 1: 1) cis/trans isomerization study.

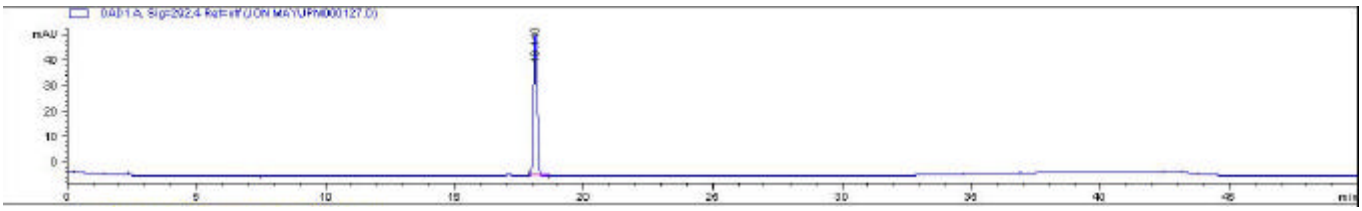
In order to rule out the possibility of cis-trans isomerization about the proline secondary amide bond.



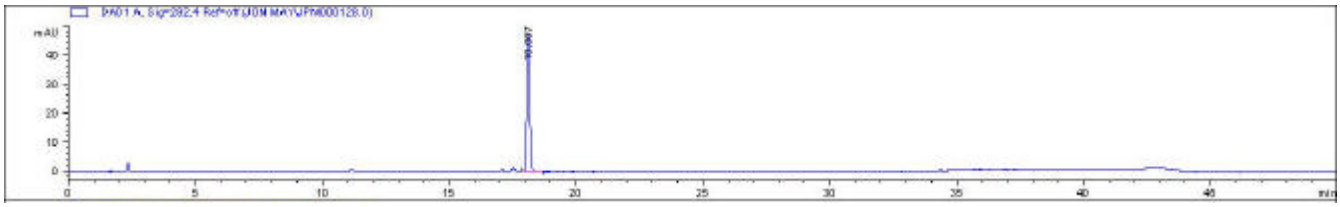
cis trans

A pure sample of **1a** was taken and diluted in water. An aliquot was taken and run on the HPLC. The rest of the solution was heated to 95ºC for 5hr. Following heating another aliquot was taken and run on the HPLC. In both cases the retention time was identical.

Before heating:



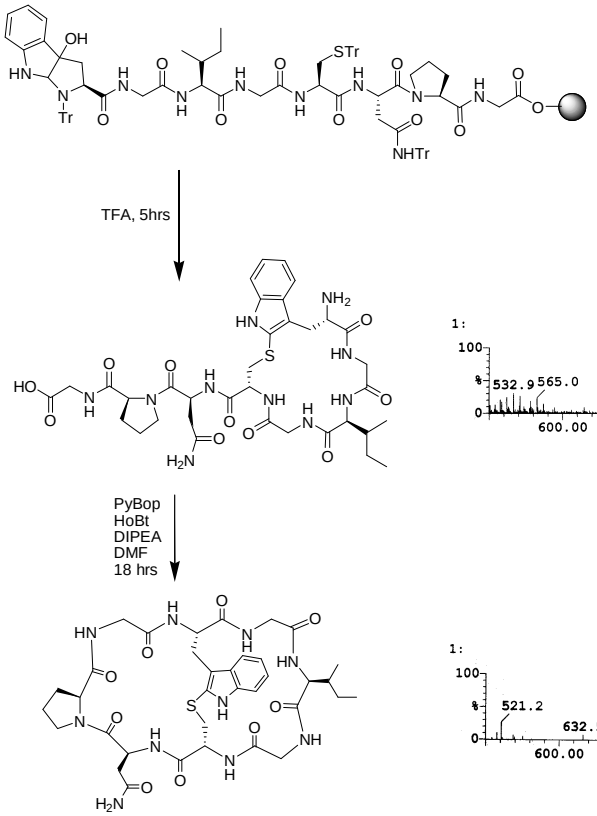
After heating:



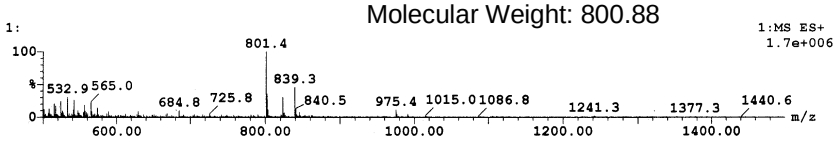
An identical experiment was performed on a sample of **1b** with the same result.

Study of isomers of 1: 2) synthesis of Pro^2-Gly^3-*S*-deoxo amaninamide (**5**)

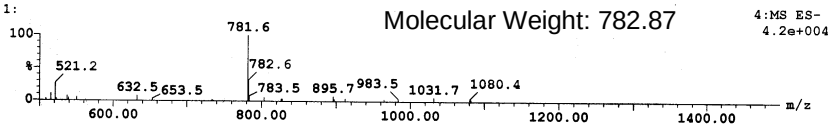
An analogous peptide was synthesized with Gly at the C-terminus (**5**). This can not racemize at the C-terminus during macrocyclization, hence if we get just one desired tryptathionine product it is good evidence that we are witnessing an epimerization and not formation of atropisomers.



Chemical Formula: $C_{35}H_{48}N_{10}O_{10}S$
Exact Mass: 800.33
Molecular Weight: 800.88

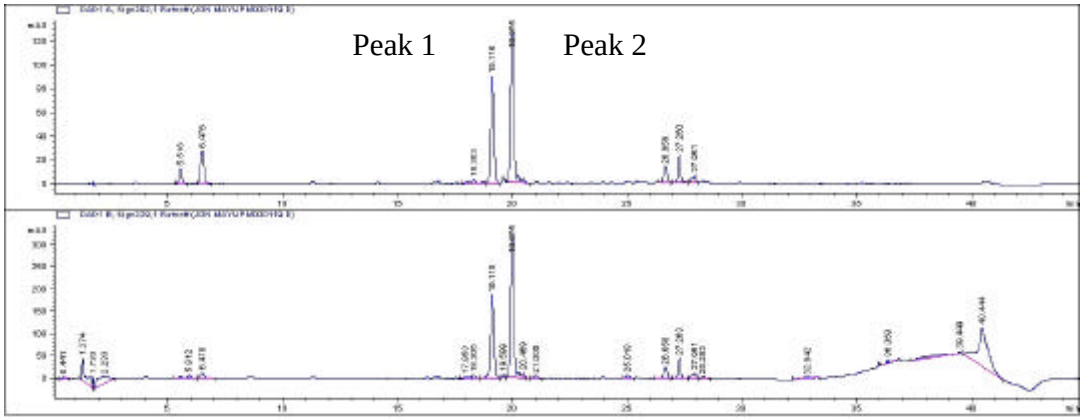


Chemical Formula: $C_{35}H_{46}N_{10}O_9S$
Exact Mass: 782.32
Molecular Weight: 782.87

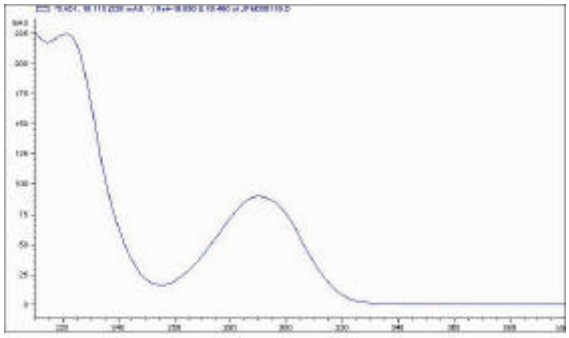


HPLC purification of 5

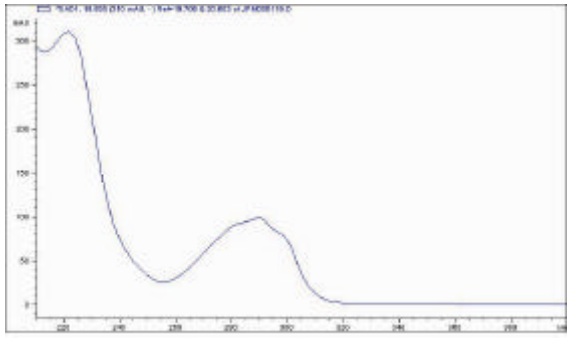
2 major peaks:



Peak 1: rt = 19.12 min; $?_{max}$ = 290 nm.



Peak 2: rt = 19.98 min; $?_{max}$ = 290 nm

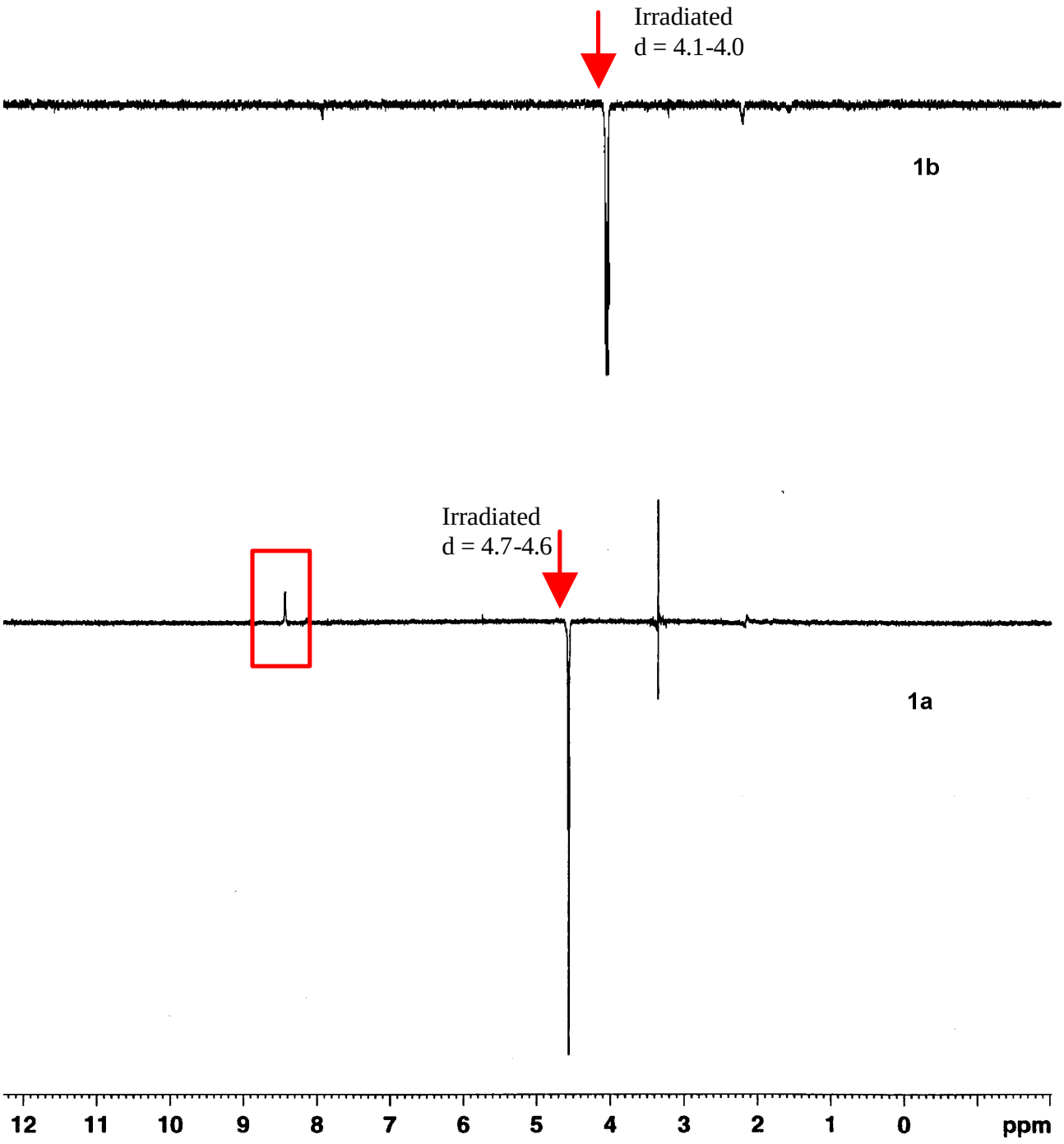


Only one peak with desired tryptathionine UV (peak 2). This is good evidence for an epimerization at the C-terminus during the final coupling step, because it appears only one tryptathionine containing product is formed with glycine as the C-terminal residue during macrocyclization.

NB. Peak 1 corresponds to a by-product introduced after the synthesis which can be easily avoided. Although this peak has a ?max at 290, the shape of the spectrum is not typical of a tryptathionine.

NMR study of NOEs for bicyclic peptides 1a and 1b

A strong NOE is observed between the Ca of i+2 (Pro^2) and the NH of i+3 (Ile^3) for the ßII-turn (**1a**), but not for the ßI-turn (**1b**). In both cases the Ca of Pro^2 was irradiated and the NH of Ile^3 was observed for NOE.



Crystallography experimental for compound 1a

Experimental

Data Collection

A colourless prism crystal of $C_{39}H_{54}N_{10}O_9S.[3.5MeOH]$ having approximate dimensions of 0.05 x 0.12 x 0.25 mm was mounted on a glass fiber. All measurements were made on a Bruker X8 APEX II diffractometer with graphite monochromated Mo-Kα radiation.

The data were collected at a temperature of -100.0 ± 0.1°C to a maximum 2θ value of 45.0°. Data were collected in a series of ϕ and ω scans in 0.50° oscillations with 60.0 second exposures. The crystal-to-detector distance was 36.00 mm.

Data Reduction

Of the 45256 reflections that were collected, 12958 were unique (R_{int} = 0.205); equivalent reflections were merged. Data were collected and integrated using the Bruker SAINT[1] software package. The linear absorption coefficient, μ, for Mo-Kα radiation is 1.33 cm^{-1}. Data were corrected for absorption effects using the multi-scan technique (SADABS[2]), with minimum and maximum transmission coefficients of 0.485 and 0.993, respectively. The data were corrected for Lorentz and polarization effects.

Structure Solution and Refinement

The structure was solved by direct methods[3]. The molecule crystallizes with two molecules in the asymmetric unit, in addition to seven methanol molecules. There is mild disorder in the proline rings of each molecule, which in each case was modeled in two orientations with the disordered atoms

refined with isotropic thermal parameters. All other non-hydrogen atoms were refined anisotropically, while all hydrogen atoms were placed in calculated positions and not refined. The final cycle of full-matrix least-squares refinement[4] on F^2 was based on 12958 reflections and 1202 variable parameters and converged (largest parameter shift was 0.00 times its esd) with unweighted and weighted agreement factors of:

$$R1 = \Sigma \, ||Fo| - |Fc|| \, / \, \Sigma \, |Fo| = 0.215$$

$$wR2 = [\, \Sigma \, (\, w \, (Fo^2 - Fc^2)^2 \,) / \, \Sigma \, w(Fo^2)^2]^{1/2} = 0.211$$

The standard deviation of an observation of unit weight[5] was 1.1 The weighting scheme was based on counting statistics. The maximum and minimum peaks on the final difference Fourier map corresponded to 0.47 and –0.34 $e^-/Å^3$, respectively.

Neutral atom scattering factors were taken from Cromer and Waber[6]. Anomalous dispersion effects were included in Fcalc[7]; the values for $\Delta f'$ and $\Delta f''$ were those of Creagh and McAuley[8]. The values for the mass attenuation coefficients are those of Creagh and Hubbell[9]. All refinements were performed using the SHELXTL[10] crystallographic software package of Bruker-AXS.

References

(1) SAINT. Version 7.03A. Bruker AXS Inc., Madison, Wisconsin, USA. (1997-2003).

(2) SADABS. Bruker Nonius area detector scaling and absorption correction - V2.10, Bruker AXS Inc., Madison, Wisconsin, USA (2003).

(3) SIR2002 - Burla M.C., Camalli M., Carrazzini, B., Cascarano G.L., Giacovazzo C., Polidori G.,Spagna R. (2003) J. Appl. Cryst. 36, 1103.

(4) Least Squares function minimized:

$$\Sigma w(F_o^2-F_c^2)^2$$

(5) Standard deviation of an observation of unit weight:

$$[\Sigma w(F_o^2-F_c^2)^2/(N_o-N_v)]^{1/2}$$

where: N_o = number of observations

N_v = number of variables

(6) Cromer, D. T. & Waber, J. T.; "International Tables for X-ray Crystallography", Vol. IV, The Kynoch Press, Birmingham, England, Table 2.2 A (1974).

(7) Ibers, J. A. & Hamilton, W. C.; Acta Crystallogr., 17, 781 (1964).

(8) Creagh, D. C. & McAuley, W.J .; "International Tables for Crystallography", Vol C, (A.J.C. Wilson, ed.), Kluwer Academic Publishers, Boston, Table 4.2.6.8, pages 219-222 (1992).

(9) Creagh, D. C. & Hubbell, J.H..; "International Tables for Crystallography", Vol C, (A.J.C. Wilson, ed.), Kluwer Academic Publishers, Boston, Table 4.2.4.3, pages 200-206 (1992).

(10) SHELXTL Version 5.1. Bruker AXS Inc., Madision, Wisconsin, USA. (1997).

EXPERIMENTAL DETAILS

A. Crystal Data

Empirical Formula	$C_{42.5}H_{67.5}N_{10}O_{12.5}S$
Formula Weight	950.63
Crystal Color, Habit	colourless, prism
Crystal Dimensions	0.05 X 0.12 X 0.25 mm
Crystal System	orthorhombic
Lattice Type	primitive
Lattice Parameters	a = 15.7181(16) Å b = 25.125(4) Å c = 25.337(4) Å α = 90.0 ^{o} β = 90.0 ^{o} γ = 90.0 ^{o} V = 10006(2) Å^{3}
Space Group	*P* $2_12_12_1$ (#19)
Z value	8
D_{calc}	1.262 g/cm^{3}
F_{000}	4068.00
μ(MoKα)	1.33 cm^{-1}

B. Intensity Measurements

Diffractometer	Bruker X8 APEX II
Radiation	MoKα (λ = 0.71073 Å) graphite monochromated
Data Images	669 exposures @ 60.0 seconds
Detector Position	36.00 mm
$2\theta_{max}$	45.0^{o}
No. of Reflections Measured	Total: 45256
Corrections	Unique: 12958 (R_{int} = 0.205) Absorption (T_{min} = 0.485, T_{max}= 0.993) Lorentz-polarization

C. Structure Solution and Refinement

Structure Solution	Direct Methods (SIR2002)
Refinement	Full-matrix least-squares on F^2
Function Minimized	Σ w (Fo^2 - Fc^2)^2
Least Squares Weights	w=1/($\sigma^2(Fo^2)$+(0.0964P) ^{2}+ 0.0000P)
Anomalous Dispersion	All non-hydrogen atoms
No. Observations (I>0.00σ(I))	12958
No. Variables	1202
Reflection/Parameter Ratio	10.78
Residuals (refined on F^2, all data): R1; wR2	0.215; 0.211
Goodness of Fit Indicator	1.01
No. Observations (I>2.00σ(I))	6237
Residuals (refined on F): R1; wR2	0.074; 0.156
Max Shift/Error in Final Cycle	0.00
Maximum peak in Final Diff. Map	0.47 e^-/Å^3
Minimum peak in Final Diff. Map	-0.34 e^-/Å^3

Bioactivity data

Full 96 well plate, including control lanes.

Lanes 1 2 3 4 5 6

Rows
A Control (medium)

B Control (HepG2)

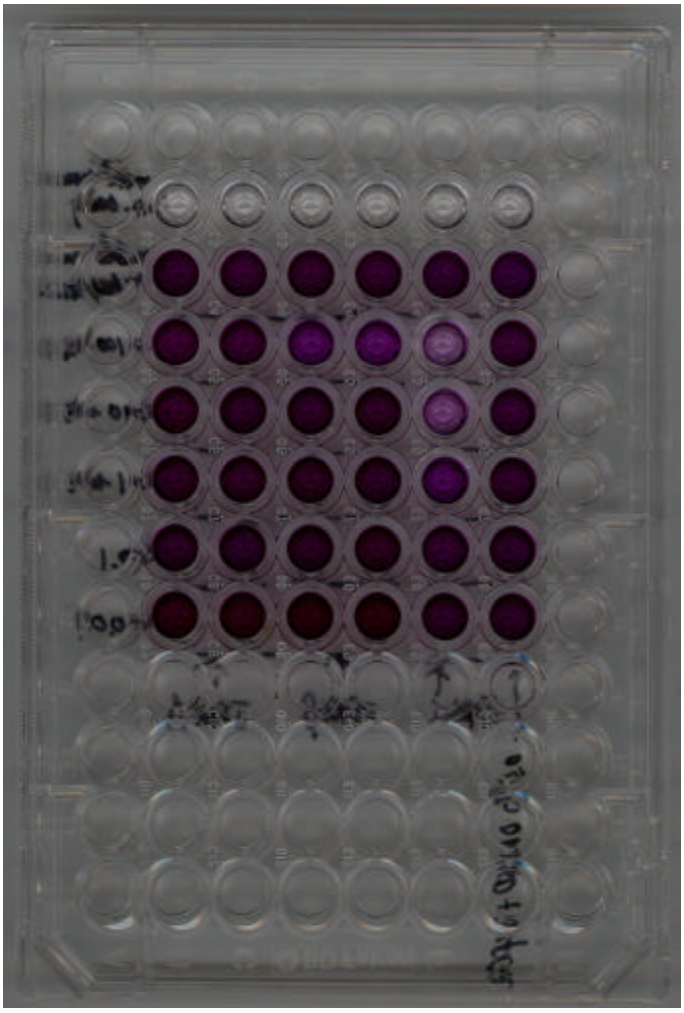
C 100 µM

D 10 µM

E 1 µM

F 0.1 µM

G 0.01 µM



Rows A and B contain no peptides; A is a control with just medium (i.e. a blank) and B is a positive control with medium and HepG2 cells. Rows C-G contain peptides at the given concentration in the media, where lanes 1 and 2 contain compound **1a**, lanes 3 and 4 contain compound **1b**, lane 5 contains a-amanitin. Lane 6 contains DMSO only.

Raw data from MTT assay

OD reading at 570 nm										
								mean	net OD	% alive cel
medium only (Blank)		0.0503	0.0477	0.0492	0.0487	0.049	0.0484	0.048883	0	
medium + hepG2		1.5811	1.4224	1.5074	1.5189	1.5851	1.5604	1.529217	1.480333	
0.5% DMSO + hepG2		1.3623	1.4875	1.3672	1.6473	1.6255		1.49796	1.449077	100
100 µM	alpha	0.2084						0.2084	0.159517	11.00816
10 µM	alpha	0.2791						0.2791	0.230217	15.88713
1 µM	alpha	0.833						0.833	0.784117	54.11147
0.1 µM	alpha	1.3074						1.3074	1.258517	86.84956
0.01 µM	alpha	1.5395						1.5395	1.490617	102.8667
100 µM	1a	1.1351	1.1729					1.154	1.105117	76.26351
10 µM	1a	1.2822	1.5688					1.4255	1.376617	94.99957
1 µM	1a	1.3793	1.3316					1.35545	1.306567	90.16546
0.1 µM	1a	1.5093	1.4463					1.4778	1.428917	98.60877
0.01 µM	1a	1.4979	1.4283					1.4631	1.414217	97.59433
100 µM	1b	0.7091	0.735					0.72205	0.673167	46.45487
10 µM	1b	1.4694	1.3938					1.4316	1.382717	95.42053
1 µM	1b	1.4684	1.3555					1.41195	1.363067	94.0645
0.1 µM	1b	1.4646	1.5271					1.49585	1.446967	99.85439
0.01 µM	1b	1.5259	1.4876					1.50675	1.457867	100.6066