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Supporting Information on analgesic activity assay in vivo.

(a) Effect of enkephalin and enkephalin analogs on the mechanically induced pain (heat; Hot Plate method) in mice:

Albino Swiss mice weighing between 20-22 g were subjected to the hot plate test. The test substances dissolved in physiological solution were administered in varying doses by intraperitoneal (i.p.) route with 26 gauge needle. The hot plate (Socrel, model DS-37, Italy) was adjusted to the temperature of 55.0 ± 0.1 °C. The individual reaction time (licking of paws) was recorded before administering the test substances. The cut off point of reaction time was about 12 secs to avoid the thermal injury to the paw. Analgesic response was calculated as the percent increase in reaction time before and after the administration of test substances measured at intervals of 30, 60, 90 and 120 min. An initial gap of 30 min was given after each administration before starting the measurements. The ED_{50} values were calculated using the probit analysis and compared to that of Leu-enkephalin methyl ester. The results are summarised in Table 1.

Table 1. Analgesic activity assay by hot plate method.

Compound	ED_{50} (μmol/animal)	Peak analgesic activity (min)	Relative analgesic activity Leu-enk. Me ester = 1
5a	1.14	90	1.18
5b	1.48	30	0.91
6a	2.51	60	0.54
6b	2.22	90	0.61
Leu-enk. Me ester	1.35	60	1.00

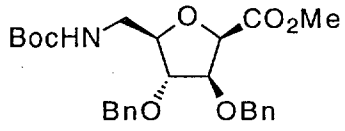
(b) Effect of enkephalin and enkephalin analogs on the mechanically induced pain (Tail clip method) in mice:

Albino Swiss mice weighing between 20-22 g were used for the experiment. The pain was induced by applying an artery clip to the base of the mouse tail. The animals which

responded by biting the clip or attempting to remove the clip within 2-3 seconds were selected a day prior to experiment. The test substances dissolved in physiological solution were administered in varying doses by i.p. route with 26 gauge needle and measurments were made after 30 min. at intervals of 30, 60, 90, 120 min. The analgesic activity was expressed as a percent of mice not showing the biting response or attempt to remove the clip. The cut off time was about 6-8 seconds to avoid mechanical injury to the mice. The ED_{50} values were calculated using the probit analysis and compared to that of Leu-enkephalin methyl ester. The results are summarised in Table 2.

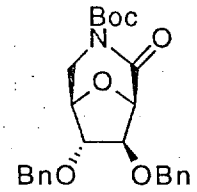
Table 2. Analgesic activity assay by tail clip method.

Compound	ED_{50} (μmol/animal)	Duration of analgesic activity (min)	Relative analgesic activity Leu-enk. Me ester = 1
5a	1.17	90	1.76
5b	1.33	90	1.55
6a	-	-	-
6b	2.45	120	0.84
Leu-enk. Me ester	2.06	90	1.00



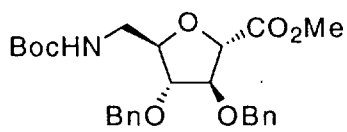
10

Selected physical data for compound **10**: R_f = 0.35 (silica gel, EtOAc:petroleum ether 1:3); $[\alpha]_D^{20}$ = 9.4 (*c* = 1.0, $CHCl_3$); ^{1}H NMR (200 MHz, $CDCl_3$): δ 7.4-7.2 (m, 10 H, aromatic), 5.3 (m, 1 H, N*H*Boc), 4.68 (d , J = 5 Hz, 1 H, H_2), 4.48 (ABq, 4 H, PhCH_2O-), 4.22 (dd, *J* = 5, 2 Hz, 1 H, H_3), 4.13 (m, 1 H, H_5), 3.88 (dd, *J* = 3, 2 Hz, 1 H, H_4), 3.72 (s, 3 H, CO_2CH_3), 3.4 (m, 2 H, H_6), 1.42 (s, 9 H, Boc); ^{13}C NMR (50 MHz, $CDCl_3$): δ 169.6, 156.1, 137.3, 137.1, 128.5, 128.4, 128, 127.8, 127.7, 83.6, 83.2, 82.7, 80, 79.1, 72.4, 72.1, 52, 42.2, 28.4; MS (LSIMS): *m/z* (%): 494 (25) [M^++Na], 472 (20) [M^++H], 372 (100) [M^++H-Boc].



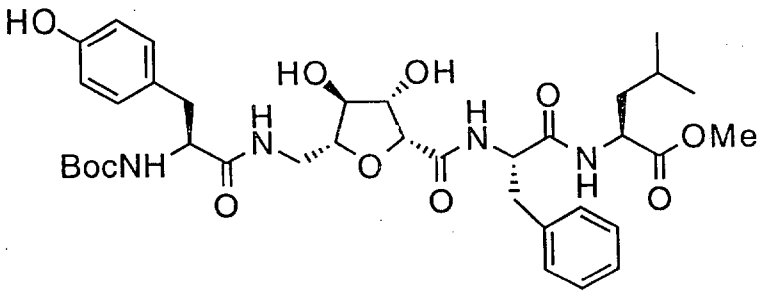
11

Selected physical data for compound **11**: R_f = 0.5 (silica gel, EtOAc:petroleum ether 1:3); $[\alpha]_D^{20}$ = -18.4 (*c* = 1.0, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.4-7.28 (m, 10 H, aromatic), 4.77 and 4.45 (ABq, 2 H, PhCH_2O-), 4.76 (d, *J* = 5 Hz, 1 H, H_2), 4.5 (ABq, 2 H, PhCH_2O-), 4.44 (d, *J* = 5 Hz, 1 H, H_3), 4.3 (dd, *J* = 5.3, 2.2 Hz, 1 H, H_5), 4.04 (d, *J* = 2.2 Hz, 1 H, H_4), 3.83 (dd, *J* = 12.8, 5.3 Hz, 1 H, H_6), 3.55 (d, *J* = 12.8 Hz, 1 H, $H_{6'}$), 1.54 (s, 9 H, Boc); ^{13}C NMR (50 MHz, $CDCl_3$): δ 166.2, 152, 137.1, 136.9, 128.5, 128.4, 128.1, 128, 127.8, 86.8, 86.3, 83.6, 79.6, 77.8, 72.9, 71.7, 48.9, 28; MS (LSIMS): *m/z* (%): 439 (10) [M^+], 384 (100) [M^++H-CH_2=C(CH_3)].



13

Selected physical data for compound **13**: R_f = 0.4 (silica gel, EtOAc:petroleum ether 1:3); $[\alpha]_D^{20}$ = 14.3 (*c* = 1.0, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.4-7.2 (m, 10 H, aromatic), 5.0 (m, 1 H, N*H*), 4.6 and 4.47 (two ABq, 4 H, PhCH_2O-), 4.65 (s, 1 H, H_2), 4.33 (s, 1 H, H_3), 4.3 (m, 1 H, H_5), 3.88 (d, *J* = 1.8 Hz, 1 H, H_4), 3.72 (s, 3 H, CO_2CH_3), 3.48 and 3.38 (two m, 2 H, $H_{6,6'}$), 1.42 (s, 9 H, Boc); MS (LSIMS): *m/z* (%): 494 (16) [M^++Na], 472 (10) [M^++H], 372 (100) [M^++H-Boc].



Boc-Tyr-Gaa-Phe-Leu-OMe (**5a**)

Selected physical data : R_f = 0.35 (silica gel, EtOAc).

^{1}H NMR (500 MHz, DMSO-d_6):

Chemical shifts (δ) in ppm

Residue	NH	CH_α	CH_β(pro-S)	CH_β(pro-R)	Others
Tyr	6.89 (d)	4.04	2.82 (dd)	2.64 (dd)	Aro_ε = 7.01 (d) Aro δ= 6.61 (d) OH = 9.15 (s) Boc = 1.29 (s)
Phe	8.41 (d)	4.60	3.36	3.06 (dd)	Aro = 7.14-7.22 (m)
Leu	7.93 (d)	4.24	1.43	1.51	CH_γ = 1.73 CH_3(pro-R) = 0.81 (d) CH_3(pro-S) = 0.77 (d) CO_2CH_3 = 3.60 (s)

Gaa: NH = 8.21 (t), H6(pro-S) = 2.92, H6(pro-R), H5, H4 = 3.76-3.84, H3 = 4.05 (t), H2 = 4.26 (d), OH (4) = 5.34 (d), OH (3) = 5.97 (d).

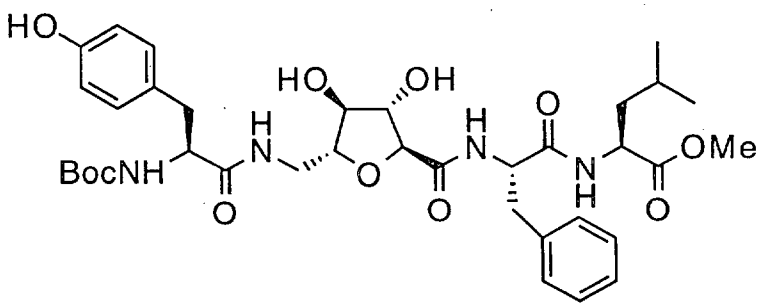
Coupling constants (J) in Hz

Residue	$J_{NH-\alpha}$	$J_{\alpha-\beta(pro-S)}$	$J_{\alpha-\beta(pro-R)}$	Others
Tyr	8.7	4.4	10.0	$J_{\beta1-\beta2}$ = 13.7 $J_{\delta-\varepsilon}$ = 8.4
Phe	9.3	3.1	11.4	$J_{\beta1-\beta2}$ = 14.0
Leu	7.3	4.6	10.4	$J_{\beta(pro-S)-\beta(pro-R)}$ = 13.6 $J_{\beta(pro-S)-\gamma}$ = 9.6 $J_{\beta(pro-R)-\gamma}$ = 4.6 $J_{\gamma-\delta(pro-S)}$ = 6.5 $J_{\gamma-\delta(pro-R)}$ = 6.5

Gaa: $J_{NH-6,6'}$ = 3.5, $J_{6-6'}$ = 11.4, J_{2-3} = 3.8, J_{H3-OH} = 3.8, J_{H4-OH} = 3.5, others not available.

MS (LSIMS): *m/z* (%): 737 (34) [M^++Na], 715 (36) [M^++H], 615 (100) [M^++H-Boc];

HRMS (LSIMS) calcd. for $C_{36}H_{51}N_4O_{11}$ [M^++H]: 715.3554, found: 715.3557.



Boc-Tyr-Maa-Phe-Leu-OMe (**6a**)

Selected physical data : R_f = 0.35 (silica gel, EtOAc).

^{1}H NMR (400 MHz, DMSO-d_6):

Chemical shifts (δ) in ppm

Residue	NH	CH_α	CH_β(pro-S)	CH_β(pro-R)	Others
Tyr	6.74 (d)	4.08	2.92	2.92	Aro_ε = 7.02 Aro δ = 6.6 OH = 9.12 (s) Boc = 1.3 (s)
Phe	8.41 (d)	4.54	2.82	2.6	Aro = 7.24-7.15 (m)
Leu	7.58 (d)	4.30	1.44	1.5	CH_γ = 1.7 CH_3(pro-R) = 0.88 (d) CH_3(pro-S) = 0.82 (d) CO_2CH_3 = 3.60 (s)

Maa: NH = 8.01 (t), H6(pro-S), H6(pro-R) = 3.3, H5 = 3.84, H4 = 3.72, H3 = 3.96, H2 = 4.08, OH (4) = 5.25 (d), OH (3) = 5.55 (d).

Coupling constants (J) in Hz

Tyr: $J_{NH-\alpha}$ = 8.5, $J_{\delta-\varepsilon}$ = 8.4; **Phe:** $J_{NH-\alpha}$ = 8.1; **Leu:** $J_{NH-\alpha}$ = 9.0, $J_{\gamma-\delta(pro-S)}$ = 6.5, $J_{\gamma-\delta(pro-R)}$ = 6.5; **Maa**: $J_{NH-6,6'}$ = 5, J_{H4-OH}, J_{H3-OH} = 3.5; others not available.

MS (LSIMS): *m/z* (%): 715 (28) [M^++H], 615 (100) [M^++H-Boc].

HRMS (LSIMS) calcd. for $C_{36}H_{51}N_4O_{11}$ [M^++H]: 715.3554, found: 715.3523.

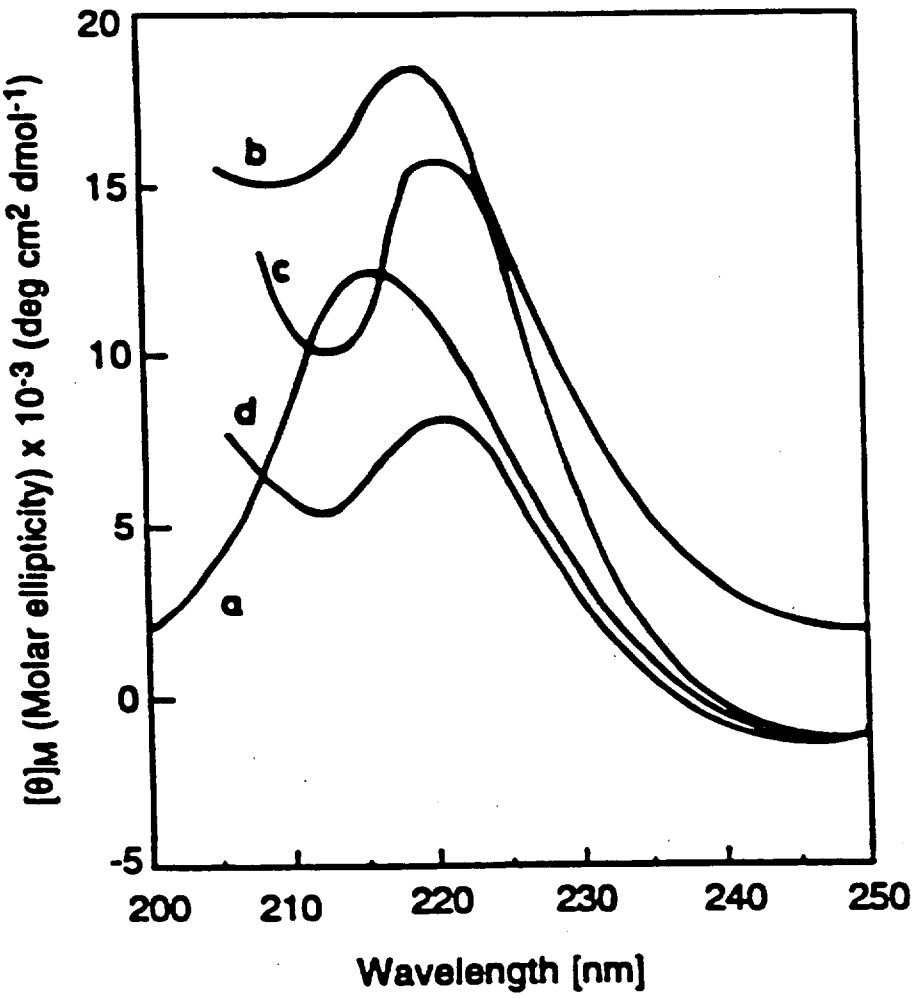
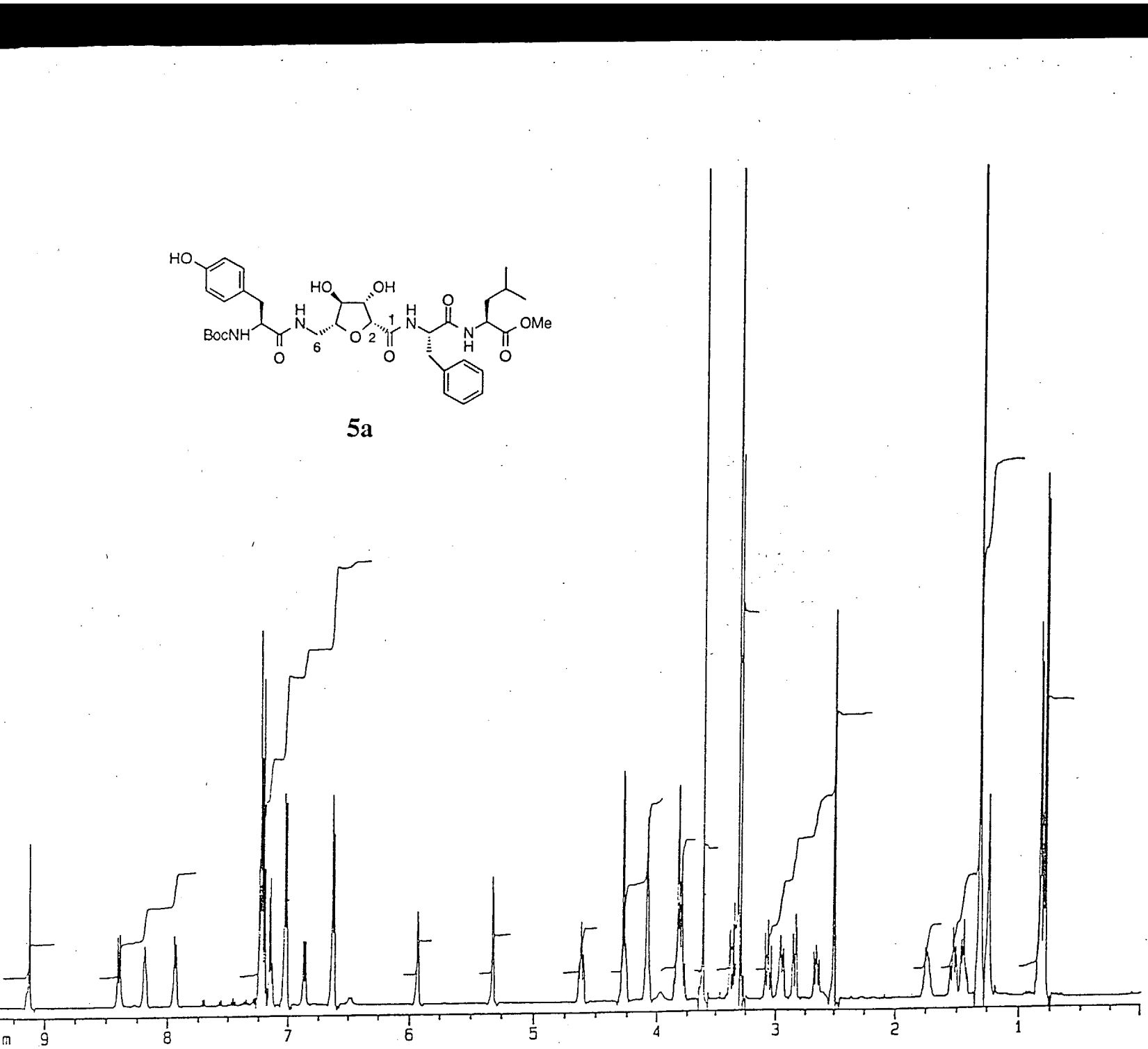
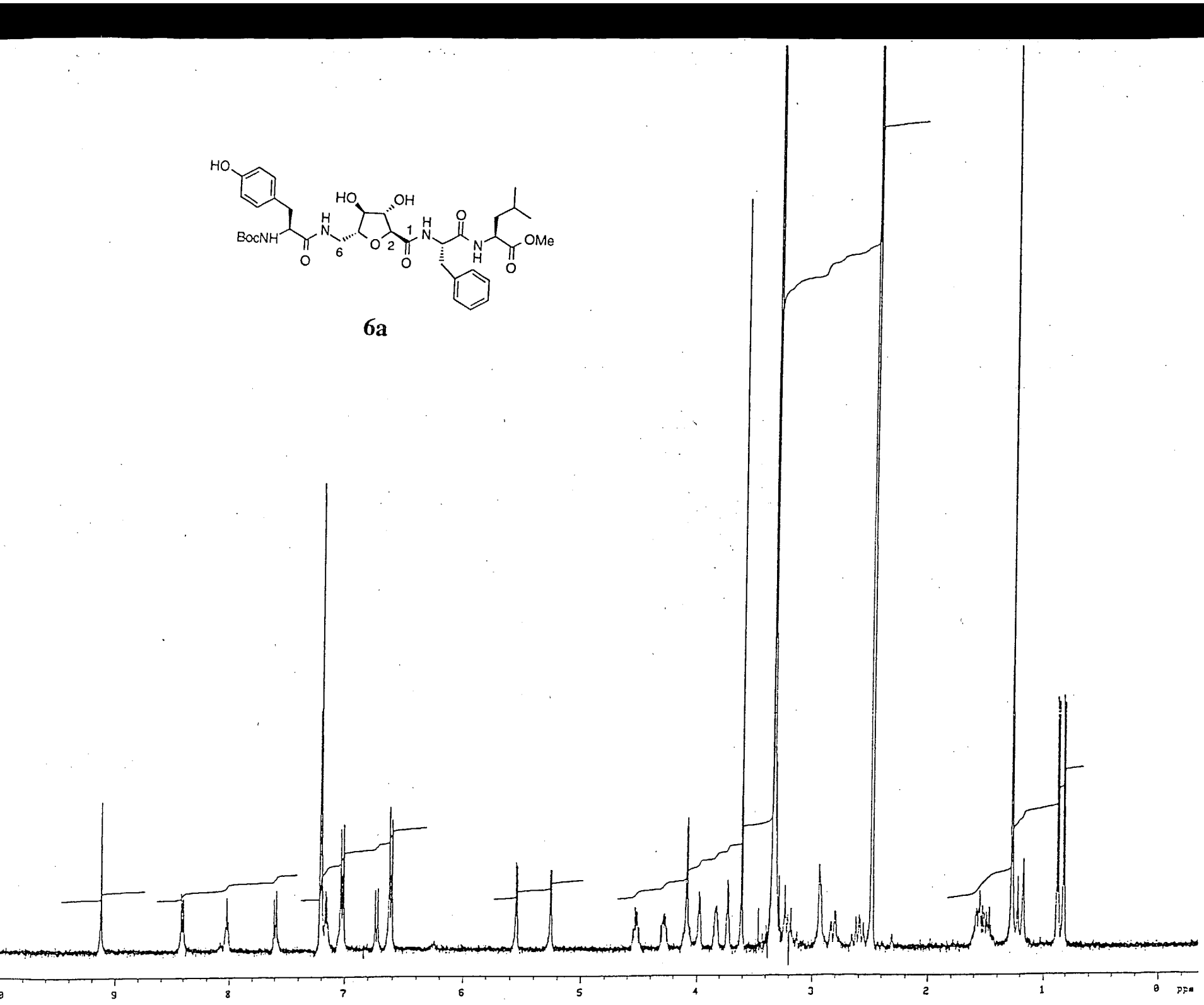


Figure 1. CD spectra (in TFE) of **5a** (a), **5b** (b), **6a** (c), and **6b** (d).



H NMR spectrum of **5a** (500 MHz, DMSO-d_6)



6a

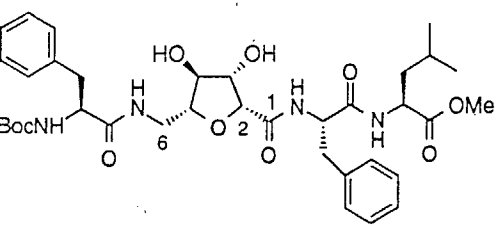
^{1}H NMR spectrum of 6a (400 MHz, DMSO-d_6)

STANDARD 1H OBSERVE

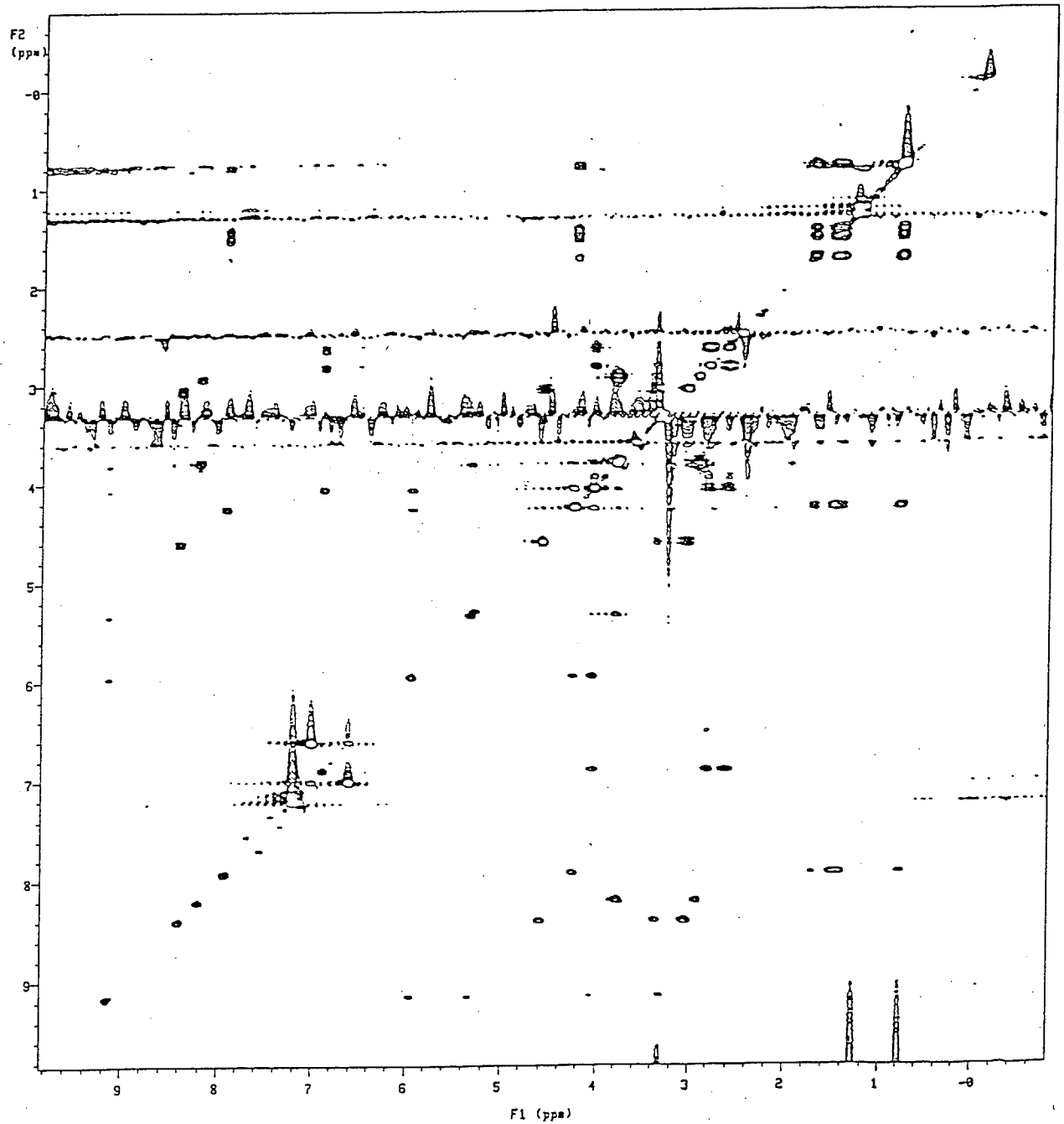
exp3 pulse sequence: tocsy

SAMPLE		DEC. & VT		ACQUISITION ARRAYS	
date	Dec 10 97	dn	H1	array	phase
solvent	DMSO	dof	-1095.3	arraydim	256
file	exp	dm	nnn		
ACQUISITION		dmm	c	1	phase
sfrq	399.952	dmf	200	1	1
tn	H1	homo	n	2	2
at	0.241	PROCESSING			
np	2048	gf	0.111		
sw	4253.5	gfs	not used		
wb	2400	wtfile			
ss	8	proc	ft		
ds	8	fn	2048		
spw	45	math	f		
sw	37.0				
dlvl	57	werr			
d1	9.3	wexp			
d2	undefined	wbs			
d1	1.500	wnt			
gresat	0	2D PROCESSING			
gzlvl	0.080	gf1	0.028		
gzlvlm	0.0020	gfs1	not used		
tof	-620.1	wtfile1			
ct	8	proc1	ft		
ct	8	fn1	1024		
lock	n	DISPLAY			
gain	15	sp	-318.9		
FLAGS		wp	4253.5		
il	n	vs	600		
in	n	sc	0		
dp	y	wc	155		
hs	n	hzmm	27.44		
wspul	n	is	500.00		
2D ACQUISITION		rfl	1314.8		
sw1	4253.5	rfp	995.9		
fn1	128	th	3		
phase	arrayed	ins	1.000		
2D DISPLAY		ai cdc ph			
sp1	-318.9				
wp1	4253.5				
lvl2	0				
tm2	155				
rfl1	1314.8				
rfp1	995.9				

TOCSY spectrum (DMSO-d_6) of **5a**



5a



TANDARD 1H OBSERVE

xp3 pulse sequence: tocsy

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ate	Dec 10 97	dn	H1	array	phase
olvent	DMSO	dof	-1095.3	arraydim	256
ile	exp	dm	nnn		
ACQUISITION		dmm	c	1	phase
frq	399.952	dmf	200	1	1
	H1	homo	n	2	2
	8.241	PROCESSING			
	2048	gf	0.111		
	4253.5	gfs	not used		
	2400	utfile			
	8	proc	ft		
	8	fn	2048		
ur	45	math	f		
	37.0				
lvl	57	werr			
	9.3	wexp			
	undefined	wbs			
	1.500	wnt			
esat	0	2D PROCESSING			
x	0.080	gf1	0.028		
ia	0.0020	gfs1	not used		
f	-620.1	utfile1			
	8	proc1	ft		
	8	fn1	1024		
ock	n	DISPLAY			
in	15	sp	2026.2		
FLAGS		wp	1679.8		
	n	vs	700		
	n	sc	0		
	y	wc	155		
	n	hzmm	27.44		
pul	n	is	500.00		
2D ACQUISITION		rf1	1314.8		
1	4253.5	rfp	995.9		
	128	th	3		
ase	arrayed	ins	1.000		
2D DISPLAY		ai cdc ph			
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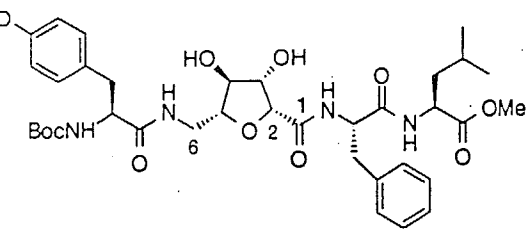
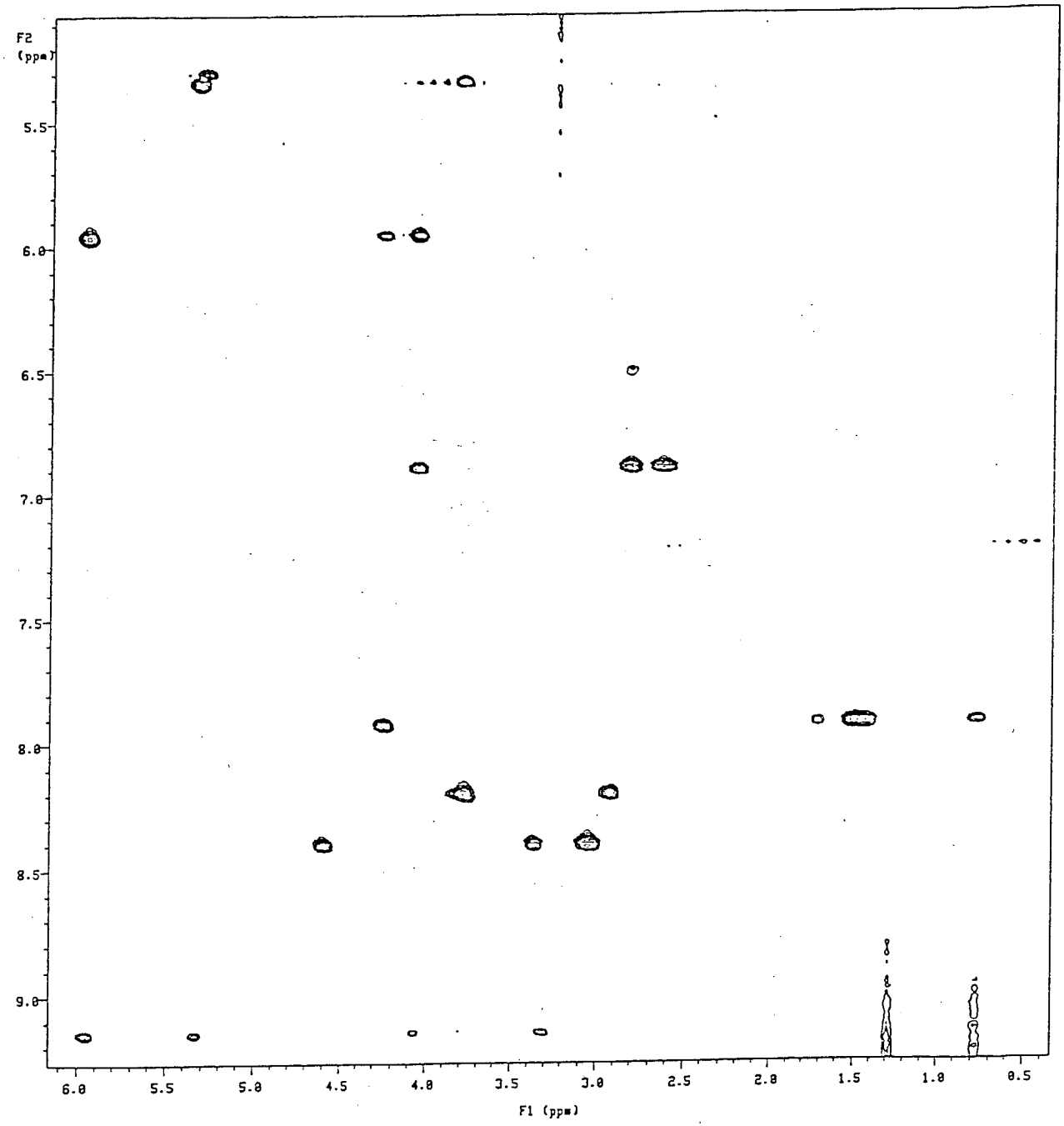
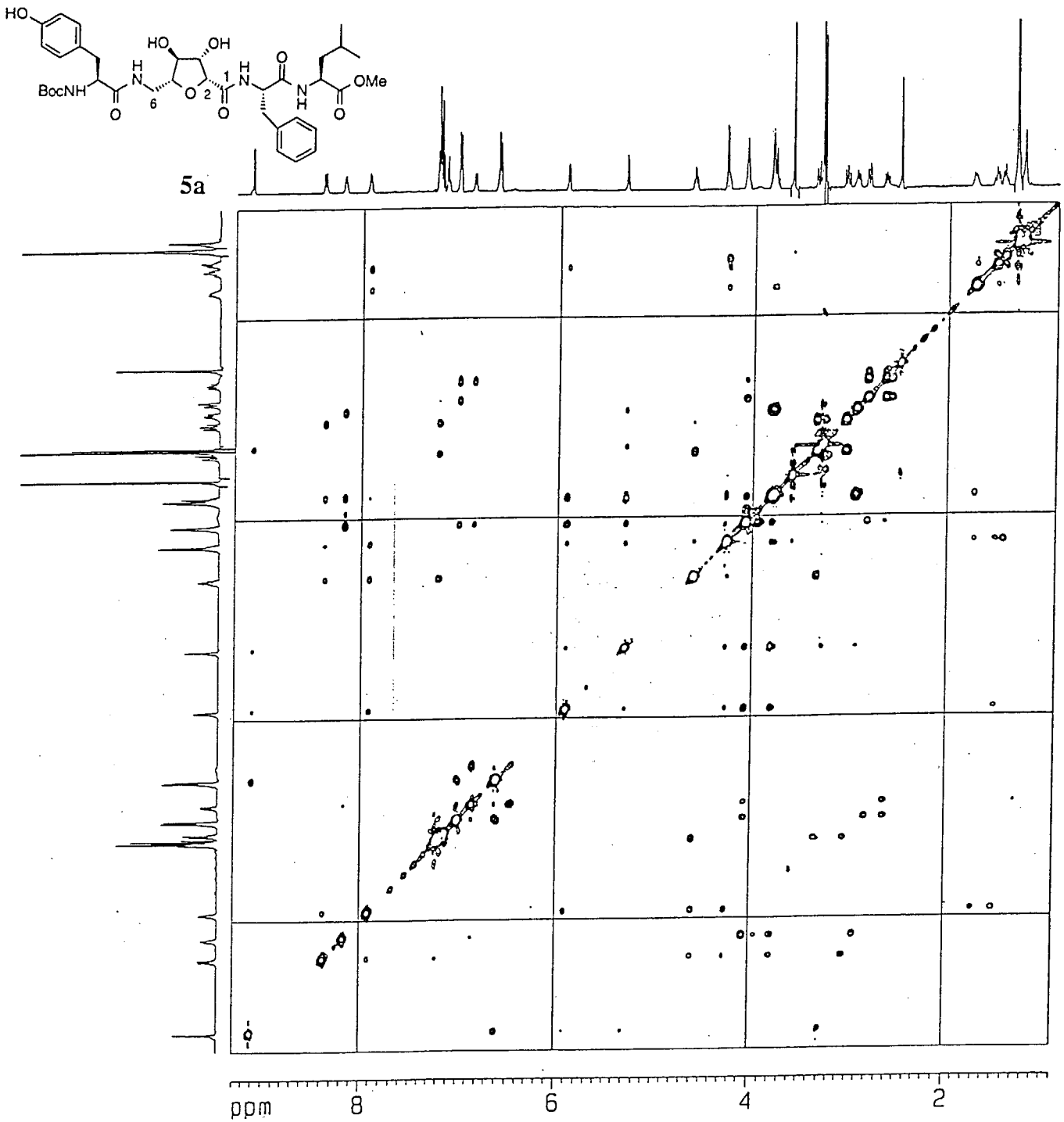
1	2322.4				
2	0				
2	155				
11	1314.8				
p1	995.9				



5a

Expansion of the TOCSY spectrum (DMSO-d_6) of **5a**





ROESY spectrum of (DMSO-d_6) **5a**

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EXPNO	2
PROCNO	1
F2 - Acquisition Parameters	
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Time	15.04
INSTRUM	drx500
PROBHD	5 mm BBI Z-G
PULPROG	roesyct
TD	1024
SOLVENT	~~CDCl3~~ DMSO
NS	16
DS	16
SWH	5165.289 Hz
FIDRES	5.044228 Hz
AQ	0.0991732 sec
RG	128
DW	96.800 usec
DE	6.00 usec
TE	300.0 K
d0	0.0000030 sec
d12	0.0000200 sec
D1	2.00000000 sec
PL1	-1.00 dB
P1	8.50 usec
PL11	21.00 dB
P15	300000.00 usec
DE	6.00 usec
SF01	500.1323040 MHz
NUC1	1H
IN0	0.00009680 sec
F1 - Acquisition parameters	
NDO	2
TO	512
SF01	500.1324 MHz
FIDRES	10.088455 Hz
SW	10.328 ppm
F2 - Processing parameters	
SI	1024
SF	500.1300135 MHz
WDW	QSINE
SSB	2
LB	0.00 Hz
GB	0
PC	1.00
F1 - Processing parameters	
SI	1024
MC2	TPPI
SF	500.1300870 MHz
WDW	QSINE
SSB	2
LB	0.00 Hz
GB	0
2D NMR plot parameters	
CX2	15.00 cm
CX1	15.00 cm
F2PL0	9.300 ppm
F2L0	4651.21 Hz
F2PHI	0.900 ppm
F2HI	450.12 Hz
F1PL0	9.300 ppm
F1L0	4651.21 Hz
F1PHI	0.900 ppm
F1HI	450.12 Hz
F2PPMCM	0.56000 ppm/cm
F2HZCM	280.07281 Hz/cm
F1PPMCM	0.55000 ppm/cm
F1HZCM	280.07288 Hz/cm