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Development of ^{99m}Tc-labelled the d,l diastereoisomer of HM-PAO for cerebral blood flow imaging

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AUTHOR(S)

Lun Xiao

INSTITUTE

The Institute of Atomic Energy, Radioisotope Department
Beijing, People's Republic of China

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Progress Report

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Progress Report

Development of Tc-99m-Labelled the d,l-Diastereoisomer of HM-PAO for Cerebral Blood Flow Imaging.

[a]Lan-qin Bai Jin-Jie Huang [b]Lun Xiao Li Fan Sou-zhen Bai

Hui Jing Guo-Li Li

Radioisotope Department, Institute of Atomic Energy, Beijing, China.

Summary

The d,l-diastereoisomer of hexamethyl propyleneamine oxime (HM-PAO) was selected as the preferred ligand for Tc-99m as a tracer for cerebral perfusion imaging. Further improvement of the synthesis and isolation method of HM-PAO resulted in pure d,l-HM-PAO and pure meso-HM-PAO. The neutral, lipophilic Tc-99m complexes of d, l-HM-PAO and meso-HM-PAO were formed in high yield by stannous reduction of Mo-99/Tc-99m generator eluate respectively. Two minutes following i.v. administration of Tc-99m-d,l-HM-PAO in mice, 2.24% of the injected dose appears in the brain. Little washout of the tracer is observed upto 24 hr postinjection. Two minutes following i.v. administration of Tc-99m-meso-HM-PAO in mice. 1.9% of the injected dose appears in the brain. The radioactivity of Tc-99m meso-HM-PAO declined faster than that of Tc-99m-d,l-HM-PAO did in the brain upto 24 hr postinjection.

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a —— Who is responsible for experimental method, results abtained, conclusion drawn.

b —— who is an adviser on this project.

Introduction

Following the discovery that the ^{99m}Tc complex of propyleneamine oxime (PnAO) is neutral and lipophilic (1) and demonstrates transient flow-related brain uptake in rats (2) , dogs (3) and humans (4),a large number of derivatives of PnAO were synthesized at the Amersham international laboratories (5).The aim of this work was to obtain a ligand which not only transported Tc-99m across the BBB, but allowed the radiotracer to be retained with a fixed distribution for a time sufficient to permit SPECT imaging. From that study, the ligand which combined the best overall features of high brain uptake,fixed regional distribution within the brain, and ease of radiopharmaceutical preparation was hexamethyl propyleneamine oxime (HM-PAO) . The potential of HM-PAO shown in laboratory animals (6) was confirmed (7) by the initial clinical finding.

HM-PAO exists in two diastereoisomeric forms, d, l- and meso- (Fig. 1).While the original clinical studies with HM-PAO were conducted with a mixture of these two isomeric forms, it was subsequently shown in rats (8) and humans (9) that one of the diastereoisomers, d, l-HM-PAO, provides a Tc-99m complex with superior brain uptake and retention compared with the complex from the stereoisomeric mixture.We now report on further studies which are the improvement of synthesis method of HM-PAO and the examination of the structure and chemical characteristics of d,l-HM-PAO and meso-HM-PAO.

Material and Methods

Chemistry

1. Synthesis of d, l-HM-PAO

HM-PAO was prepared in a two step process from commercially available

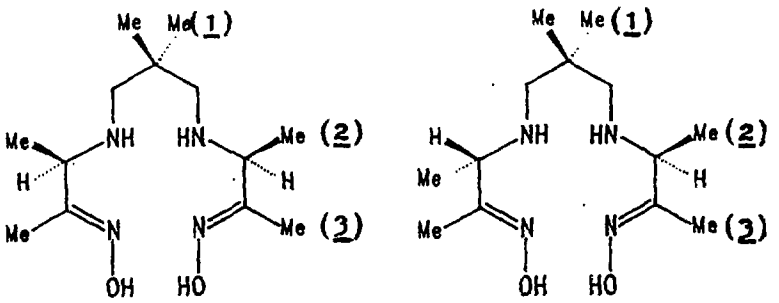

FIGURE 1
Diasteriosomers of HM-PAO.

meso-HM-PAO

d, 1-HM-PAO

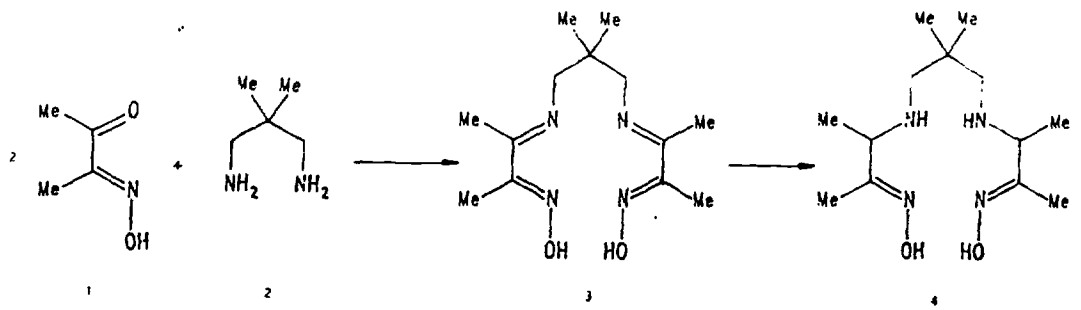

FIGURE 2
Synthesis of HM-PAO.

materials. Butanedione monoxime was condensed with 2, 2-dimethyl-1, 3-propanediamine, and the resultant bisimine was reduced with sodium borohydride Fig. 2). The ligand was purified by crystallization from ethyl acetate, and assayed for purity by ^1H NMR. IR and Mass spectroscopy, elemental analysis and melting point measurement.

(a) preparation of 4, 8-diaza-3, 6, 6, 9-tetra-methylundecane-3, 8-diene-2, 10-dione bisoxime.

2. 3-Butanedione monoxime (9. 1 g, M=101, 90.1 mMol) was dissolved in 10 ml absolute ethanol (with further purification) and the solution was stirred and heated to ~40℃. To this was added a solution of 2, 2-dimethyl-1, 3-propanediamine (5.88 ml. 49 mMol) in absolute ethanol (5ml) slowly. The solution was stirred at 52-55℃ for 30 min and then allowed to cool room temperature and stood over night. The precipitate was removed by filtration and washed with ether (with further purification). Drying under high vacuum for 2 hr gave the product: 8.5 g(69. 8%) . Recrystallization from benzene gave crystals for analysis: melting point: 120-122℃ .IR (KBr) (cm^{-1}) 1635 (C=N,C=NOH), 1390, 1370 (CH_3-C-CH_3), 923 (C=N); ^1H NMR (d_6-DMSO) δ 3.2(4H,s,CH_2N),1.9 (12H, s. MeC). 1.0 (6H, s. CMe_2) ; Anal. Calc. for $C_{13}H_{24}N_4O_2$: C. 58.2: H, 9.0: N. 20.9; Found: C. 58.7 :H. 9.5: N, 20.4.

(b) preparation of (RR.SS)-4.8-diaza-3, 6, 6, 9-tetra-methylun decane-2. 10-dione bisoxime (d,l-HM-PAO).

The above bisimine (15.1g, 56.3 mMol) was slurried in ethanol (without further purification)(122.5 ml) at 0℃. Potassium hydoxide (0.47 g,8.4 mMol)was added. Then sodium borohydride (2.76 g, 73 mMol) was added in portions over 30 min. and the mixture was stirred at 0℃ for 2 hr. Water (60.4 ml) was added and the mixture was stirred well for a further 2 hr.The ethanol was removed. The PH was adjusted to 11, and the resulting precipitate was removed by filtration. washed with a little

water and dried giving the impure HM-PAO: 8.2 g (54%) . The precipitate recrystallized from benzene and provided the diastereoisomeric mixture of HM-PAO free from major impurities: 2.9 g (35%)(m.p. 119.7-123.4℃). Fractional crystallization from ethyl acetate provided the pure d,l-diastereoisomer (m.p. 128.8-131.6℃) , and pure meso-diastereoisomer (m.p. 147-148℃). IR (KBr) 3310 cm^{-1}(OH) , 3200-3100 cm^{-1} (OH, NH) . 1380, 1370 cm^{-1} (CH_3-C-CH_3); ^1H NMR (d_6-DMSO) for d,l-HM-PAO, δ 10.25 (2H,s, OH).3.32 (2H.s,NH),3.13 (2H, q, CH), 2.13 (4H, q, CH_2), 1.65 (6H,s,CH_3)1. 07 (6H,d,CH_3). 0.79 (6H, s, CH_3); Anal. calc. for $C_{13}H_{28}N_4O_2$: C. 57.2; H, 10.4; N, 20.6; Found: C, 57.41; H, 10.51; N, 20.59%. Mass spectrum:m/e=272(M^+).

2. Characterization and determination of structures of HM-PAO diastereoisomers.

Organic compound of HM-PAO was characterized by melting point, ^1H NMR and IR and Mass spectroscopies and element analysis. In addition ^1H NMR served as a suitable analytical tool for the assessment of relative amounts of d, l and meso HM-PAO isomers in a diastereoisomeric mixture and for the determination of the purity of either one of the two diastereoisomers alone in order to effectively screen these d,l-HM-PAO fractions during separation between the d.l-and meso-HM-PAO diastereoisomers.

(a) The melting point was determined with a BÜCHI 535 melting point apparatus.

(b) The mass spectra were measured with ZAB-2F spectrometer.

(c) Nuclear magnetic resonance (NMR) spectra were recorded with AM 500 spectrometer with TMS as an internal standard.

(d) Elemental analysis was performed by Carlo-Erba 1106 elemental analyser.

(e) IR spectra were obtained with PE 580B Infrared Spectrometer.

Table 1

Assignment, Chemical Shifts (δ, ppm) and Coupling Constants (J, Hz) of d,l and meso HM-PAO Diastereoisomers

Isomer	Me^1	Me^{2*}	Me^3	CH_2N^{**}	CHMe
d,l	0.7880	1.0802,1.0668	1.6469	2.1779,2.0739	3.1294
	s	d,J = 6.71	s	ABq,J = 11.56	q,J=6.77
meso	0.7804,0.7775	1.0774,1.0640	1.6470	2.1766,2.0756	3.1423
	s	d,J = 6.71	s	ABq,J = 10.90	q,J = 6.71

Measured in DMSO at 25℃,500 MHz.For positions of functionality see Figure 1.

Abbreviations: s-singlet, d-doublet, ABq-AB quartet.

* The chemical shifts of the two lines of the Me^2 doublet are given.

** The chemical shifts of the AB quartet were calculated.

3. Technetium complexation

Sodium pertechnetate-Tc-99m was obtained from a 99Mo/Tc-99m generator which was prepared by the other group in our Department. A typical preparation is given for Tc-99m-d,1-HM-PAO and Tc-99m-meso-HM-PAO. 1 mg of d, 1HM-PAO or 1 mg of meso-HM-PAO was dissolved in 4.5 ml of 0.9% saline in a 10 ml glass vial under shaking by a shaking machine. 8 μg of stannous chloride dihydrate (20μl of 1.77×10^{-3} Mol/L in 0.1 N HCl) was added, and then 0.5-0.8 ml of sodium [Tc-99m] pertechnetate in 0.9% saline containing 15-30 mCi (555-1110 MBQ) was injected into the vial and eluate was less than 2 hr old. The vial was shaken for two minutes. The final concentration of d,1-HM-PAO or meso-HM-PAO was 0.2 mg/ml in the PH range 7-8. Complex radiochemical purity (RCP) was assayed by thin layer chromatography.

The influences of 6 μg, 8 μg, 20 μg and 32 μg of stannous chloride dihydrate in 0.9 % saline on the percentage of the primary lipophilic complex and the secondary hydrophilic complex were studied.

Tedchnetium-99m-d, 1-HM-PAO was also prepared from a kit containing 1 mg of d,1-HM-PAO, 8 μg stannous choride dihydrate and 4.5 mg sodium chloride in a 10 ml glass vial, freeze-dried, stoppered under nitrogen and sealed. Five milliliters of sodium pertechnetate (Tc-99m, 3-6 mCi/ml) was injected into the vial. The vial was shaken to dissolve the solid contents.

4. Analysis of Tc-99m complexes.

The percents of the primary lipophilic complex, the hydrophilic complex

species, free pertechnetate, and hydrolyzed reduced Tc-99m were measureed using three-strip method (thin layer chromatography) which was previously described by Neirinckx et al.(11).

This method involves a combination of three chromatographic systems: (a) a silica gel thin-layer chromatography strip (TLC-SG,made in Huang Yan county, Zhejiang province,China) developed with methyl ethyl ketone (MEK), (b) TLC-SG developed with 0.9% W/V aqueous sodium chloride, and (c) Whatman No. 1 paper (Gelman sciences, Inc, Ann Arbor, MI) developed with 50% V/V aqueous acetonitrile. After development, the strips dried, and the radioactivity distribution was determined using radiochromatographic scanner.

Biology

1. Biodistribuiton of Tc-99m complexes in mice.

Male albino mice (kunming strain), 18-20 g, were injected in a lateral tail vein with 2 μCi of Tc-99m-d,l-HM-PAO in 200 μl of isotonic saline solution. The RCP of all preparations was checked at all times.The purity of the Tc-99m complex was not less than 95%. The percent of the primary lipophilic complex was not less than 85%. The mice were killed by cervical dislocation and decapitation at 2, 5, 20, min, 1, 2, 4, and 24 hr after injection. At killing, a sample of blood was collected into a pre-weighed container. Ten organs were removed intact, including brain, liver, lung, stomach, kidney, bladder urine, heart, bone, spleen and blood. The radioactivity of each sample was counted, and the activity expressed as percent of injected dose per organ; the percentage of activity in blood was calculated on the basis of activity and weight of the sample and body composition data (blood represents 7 % of the mouse body weight)

Results

Chemistry

The route of synthesis of HM-PAO is shown in Figure 2. Condensation of the propanediamine (Fig 2, No. 2) with two molecular equivalents of the keto-oxime (Fig 2. No.1) provides the bisimine (Fig 2. No.3) in 69.8 % yield. Reduction of the two imine groups with sodium borohydride provides HM-PAO (Fig 2. No.4) as an almost equal mixture of the two diastereoisomers, Meso- and d, l- as demonstrated by proton NMR spectra (Fig 3).

Proton NMR results have shown spectral differences between the uncomplexed d,l-HM-PAO and meso-HM-PAO ligands(12) . Use of a "meso contaminated " d,l-HM-PAO ligand for complexation of Tc-99m will result in reduced brain concentration of the tracer complex for cerebral imaging. The importance of the purity of the d,l-HM-PAO ligand has led to the proton nuclear magnetic resonance study of these diastereoisomeric ligands whose results are given below.

Figure 4 depicts five 500 MHz ^{1}H NMR Spectra: trace (a) purified meso isomer, trace (b) purified d, l isomer, trace (c) 2 : 3 mixture of d, l: meso isomers.The peak position and assignments are given in Table 1. The different chemical environment for the geminal methyl groups (1) (Fig. 1) for the methyl group (2) (Fig. 1) attached to the asymmetric carbon and for the CH_2N-moieties result in the following features. In the case of the meso HM-PAO, the two geminal methyls are diastereotopic and therefore two distinct singlets are observed in the Fig. 4c, spectra. The doublets associated with methyl group (2) (Fig.1) are shifted in the purified d,l-HM-PAO spectrum compared to that of the meso-HM-PAO as is the AB quartet assigned to the CH_2N- moiety. The latter more significant shift is clearly depicted in the spectrum of the d,l meso HM-PAO diastereoisomeric mixture (Fig. 5).

Using a resolution enhancement procedure and integration methods of the ABq peaks (Fig. 5a, 5b, 5c) (respective d,l and meso peaks) as well as the high field peaks (Table 1) we have determined a 93%, 77%, 60%, in the d,l fractions during separation between the d,l and the meso HM-PAO diastereoisomers by repeated fraction crystallizations. In the purified d,l spectrum (Fig.5d) there can not be observed the meso impurities or unknown impurities of comparable amounts.

The freeze-dried formulation of d,l-HM-PAO allows the Tc-99m complex to be prepared simply by adding generator eluate to the vial. Thin layer chromatography permits the quantitative determination of radioactive components following complex formation . In Table 2 are listed the Rf values of the observed radioactive components on the three chromatographic systems used. Addition of Tc-99m pertechnetate to this freeze-dried formulation provides the primary lipophilic complex of d,l-HM-PAO in >90% yield immediately after complex formation. Other observed components are a less lipophilic Tc-99m complex of d,l-HM-PAO (termed the secondary complex), and very small amounts of reduced hydrolyzed technetium and pertechnetate.

Combined use of the three chromatographic systems allows the full quantitative assessment of the radiochemical composition of the mixture using the following relationships:

% reduced, hydrolyzed Tc= % activity remaining at the origin in the whatman No.1 /50% aq. acetonitrile system;

% pertechnetate = % activity at the solvent front in the TLC-SG / saline system;

% secondary complex = % activity at Rf 0-0.3 in the TLC-SG /MEK system minus % reduced hydrolyzed Tc;

% primary complex = % activity at Rf 0.3-1 in the TLC-SG / MEK system minus % pertechnetate.

Table 2

Rf Values of the Tc-99m Components TLC

Component	system 1*	2**	3***
[Tc-99m] d,l-HM-PAO primary	0.9-1.0	0	0.9-1.0
[Tc-99m] d,l-HM-PAO secondary	0	0	0.9-1.0
Tc-99m reduced, hydrolyzed	0	0	0
[Tc-99m] pertechnetate	0.9-1.0	0.9-1.0	0.9-1.0

* System 1:ITLC/SG-MEK.

** System 2:ITLC/SG-saline.

*** System 3:Whatman No. 1-50% aqueous acetonitrile.

Table 3

Effect of the Amounts of Stannous Chloride Dihydrate on the Tc-99m Complex.

$SnCl_2 \cdot 2H_2O$	Tc-99m complex No.1 (%)	Tc-99m complex No.2 (%)
6μg	91.8	4.5
8μg	93.3	1.14
10μg	87.0	1.5
20μg	79.5	1.3
32μg	66.0	5.0

The effect of the amount of stannous chloride dihydrate on the Tc-99m complex is shown in table 3. If the amount of stannous chloride dihydrate is less or more than 8 μg .the percent of the Tc-99m lipophilic chelate decreased apparantly.

Biology

The biodistribution of the primary complex of d,l-HM-PAO is shown in Table 4. The primary complex displays 2.24% (% i.d. in whole organ) brain uptake soon after injection. Retention of activity is high. with 97% of the initial activity remaining in the brain at 1 hr postinjection, and 72% at 24 hr postinjection.

Table 4

Biodistribution of the Primary Tc-99m Complex of d, l-HM-PAO in Mice

(mean± s.d of three Animals, %injected Dose in Whole Organ)

organ	Time postinjection						
	2 min	5 min	20 min	1 hr	2 hr	4 hr	24 hr
Blood	10.4±0.8	9.9±3.1	11.5±1.7	6.1±1.9	5.4±1.3	3.7±0.5	1.7±0.5
Heart	1.3±0.2	0.9±0.05	0.8±0.2	0.9±0.09	0.6±0.2	0.6±0.06	0.4±0.16
Liver	10.4±2.3	10.3±2.1	8.6±1.9	5.4±2.5	7.9±3.6	5.7±1.1	1.4±0.20
lung	4.7±1.6	2.5±0.6	2.8±1.1	2.5±0.4	2.7±0.5	1.9±0. 3	0.9±0.3
spleen	0.76±0.36	0.42±0.06	0.31±0.03	0.41±0.10	0.42±0.06	0.22±0.04	0.17±0.18
Stomach	0.81±0.27	1.40±0.41	0.75±0.22	0.62±0.33	0.49±0.24	1.25±0.13	0.25±0.12
Kidney	3.58±0.96	3.48±0.19	2.48±0.40	2.31±1.05	2.1±0.30	1.64±0.51	0.57±0.22
Bladder (Urine)	0.54±0.12	0.12±0.08	4.2±0	11.99±0	14.95±11.39	7.35±7.30	0.46±0.09
Brain	2.02±0.29	2.08±0.37	2.24±0.33	2.18±0.23	2.13±0.20	1.65±0.17	1.61±0.18
Bone	0.15±0.12	0.07±0.02	0.03±0.01	0.10±1.52	0.13±0.13	0.1±0. 06	0.18±0.16

Table 5

Biodistribution of the Primary Tc-99m complex of meso-HM-PAO in Mice (mean±s.d of Three Animals. %injected Dose in Whole Organ)

Organ	Time postinjection						
	2 min	5 min	20 min	1 hr	2 hr	4 hr	24 hr
Blood	5.59±0.99	2.53±1.36	1.88±0.33	4±1.26	1.48±0.57	1.16±0.18	0.30±0.11
Heart	0.54±0.06	0.49±0.12	0.33±0.07	0.26±0.06	0.25±0.11	0.16±0.02	0.06±0.02
Liver	10.18±1.66	12.37±1.77	16.77±3.11	11.33±0.98	7.01±2.8	6.01±1.30	2.66±0.4
Lung	1.61±0.22	1.60±0.09	1.02±0.04	1.0±0.22	0.56±0.03	0.54±0.08	0.20±0.02
Spleen	0.51±0.26	0.34±0.12	0.47±0.05	0.17±0.05	0.03±0.04	0.11±0.02	0.15±0.02
Stomach	0.48±0.31	0.76±0.22	0.71±0.28	0.81±0.09	0.56±0.14	0.41±0.11	0.15±0.07
Kidney	2.13±0.29	2.49±0.44	2.17±0.38	1.66±0.37	1.4±0.31	0.91±0.09	0.38±0.07
Bladder (urine)	0.36±0.44	0.29±0.3	4.4±0	4.13±2.65	4.48±0	8.13±6.8	0.06±0.02
Bone	0.14±0.10	0.9±0.05	0.12±0.05	0.07±0.05	0.05±0.02	0.03±0.01	0.005±0.003
Brain	1.93±0.19	1.25±0.04	0.83±0.11	0.84±0.08	0.76±0.21	0.70±0.14	0.49±0.08

Table 5 shows the biodistribution of the primary complex of meso-HM-PAO. The mouse brain uptake of radioactivity is 1.93% at 2 min postinjection. Retention of activity is lower than that of primary complex of d,l-HM-PAO, with 44% of the initial activity remaining in the brain at 1 hr postinjection, and 25% at 24 hr postinjection.

Discussion

The pure uncomplex d, l-HM-PAO and the pure uncomplex meso-HM-PAO were obtained. The ligand d,l-HM-PAO readily forms a neutral, lipophilic complex with Tc-99m from a freeze-dried kit to provide a new radiopharmaceutical for imaging cerebral perfusion.

High brain uptake of Tc-99m-d,l-HM-PAO was demonstrated in mouse, and up to 24 hr postinjection little washout occured. However, whole brain uptake of Tc-99m-meso-HM-PAO in mouse was lower, and the washout was faster than that of Tc-99m-d,l-HM-PAO up to 24 hr postinjection.

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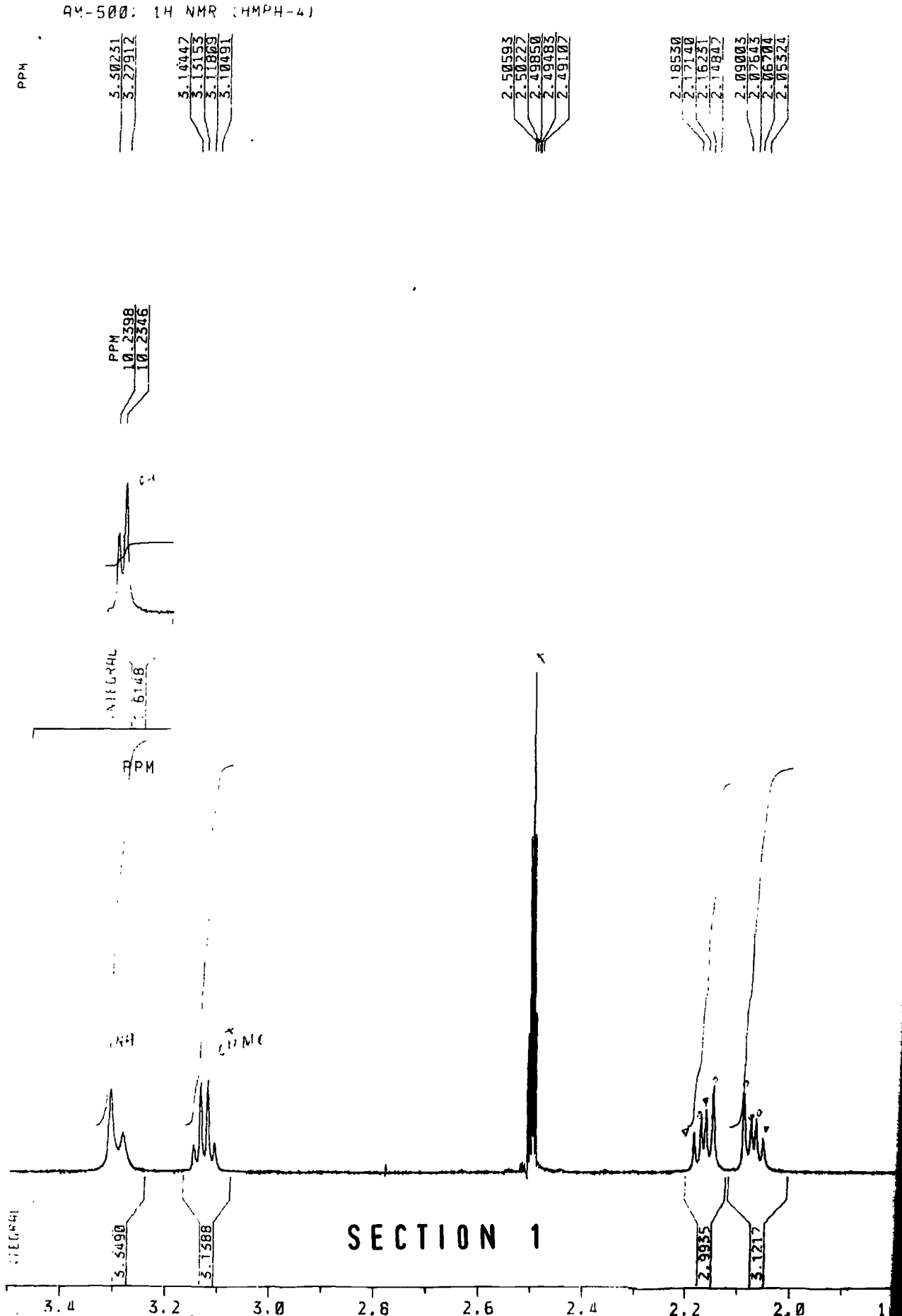
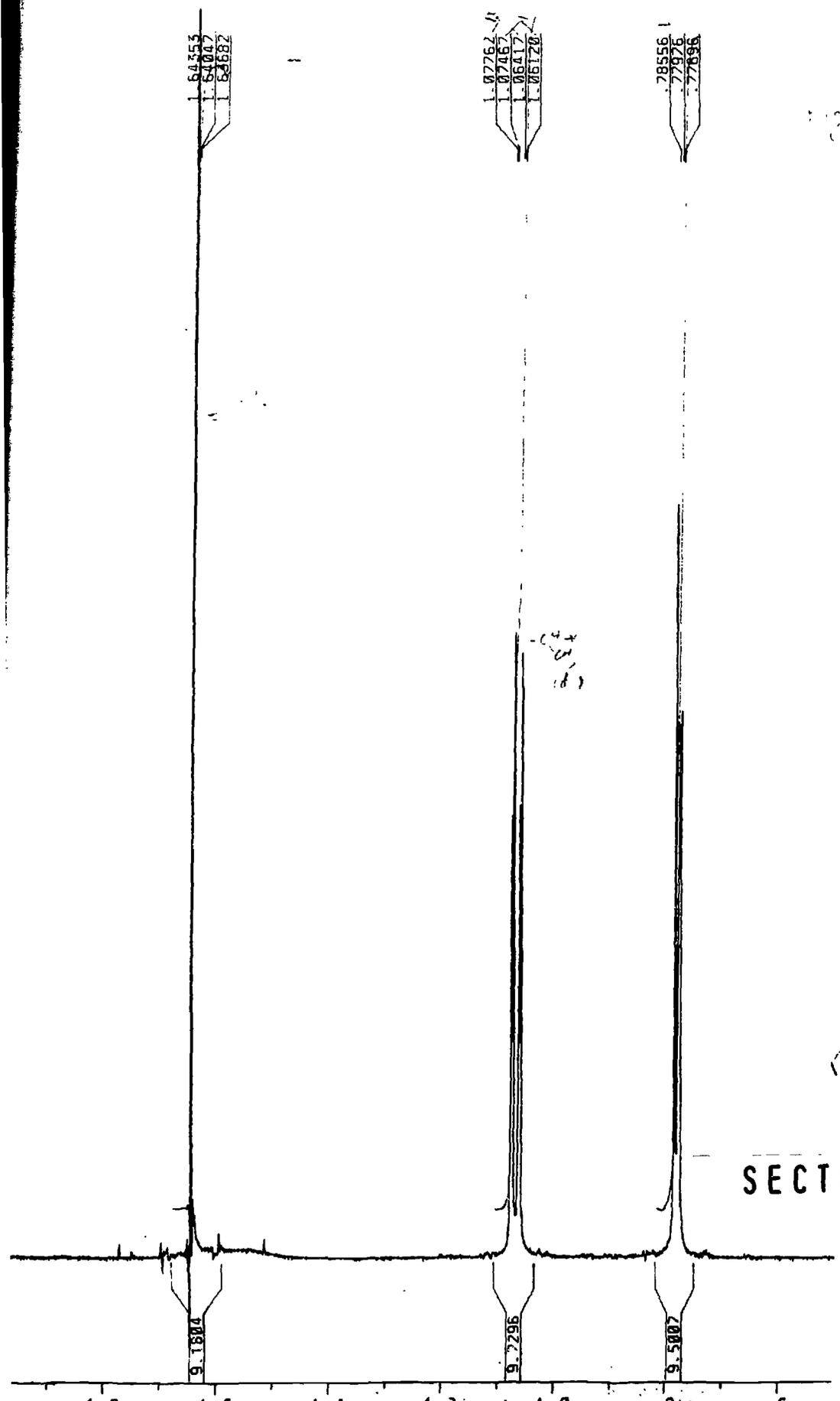


Fig. 3 500 MHz ^{1}H NMR spectra in d_6-DMSO of diastereoisomeric mixture of d,l-



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HZ/CM	44.129
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SR	7789.44

SECTION .2

of d,l- and meso- diastereoisomers. ∆ — meso isomer, o — d,l isomer.

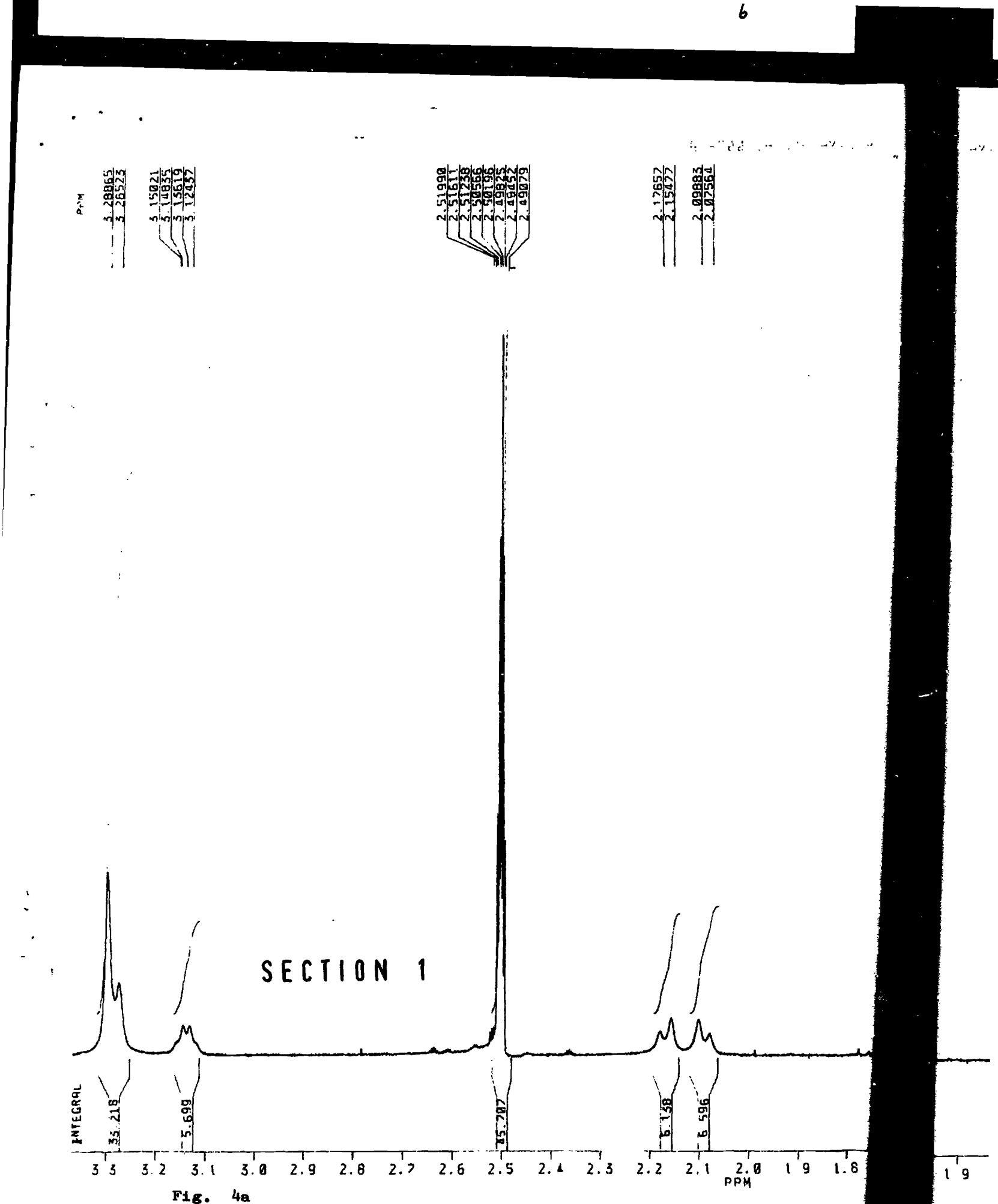
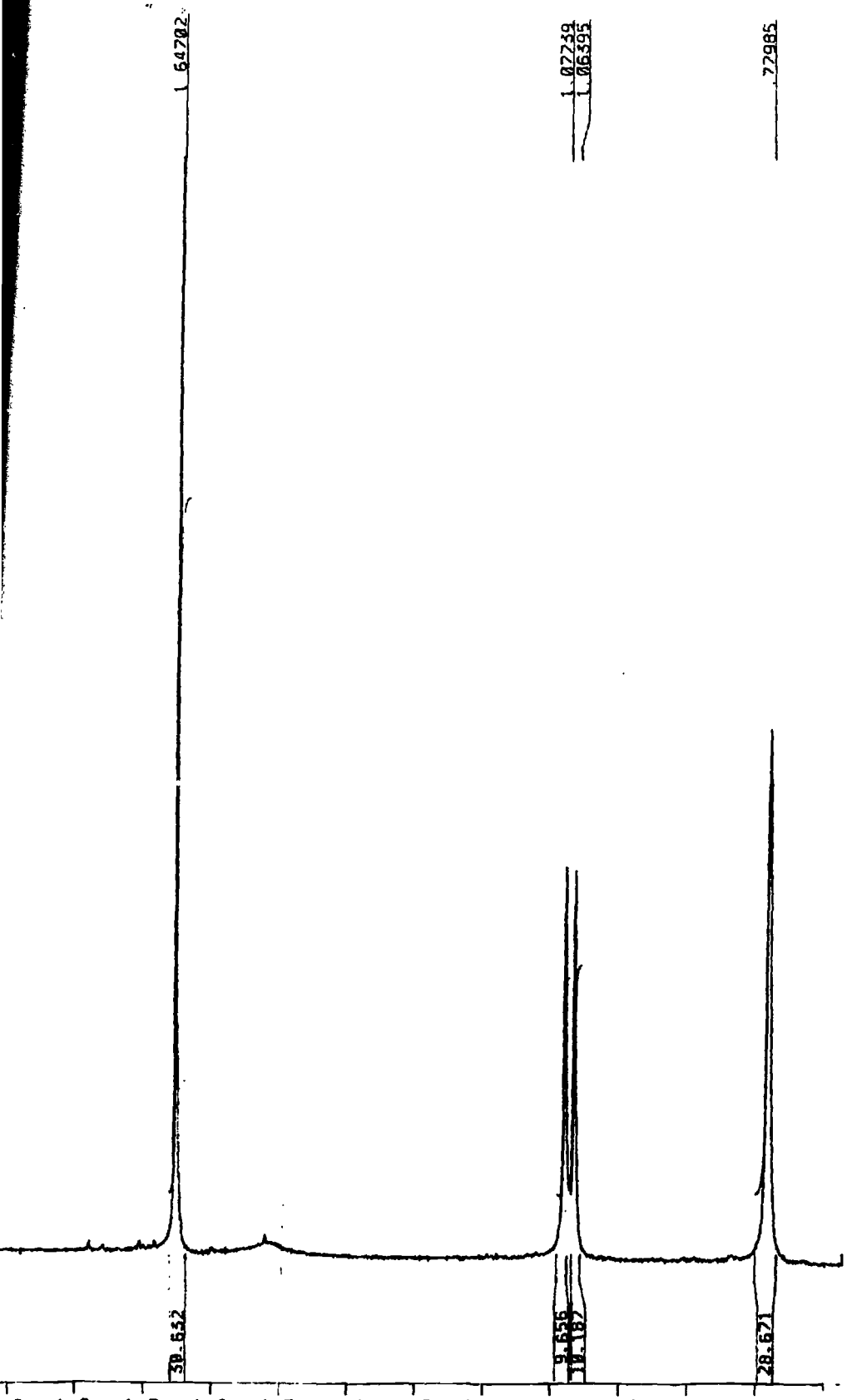


Fig. 4a

500 MHz ^{1}H NMR spectra in d_6-DMSO of purified meso isomer.



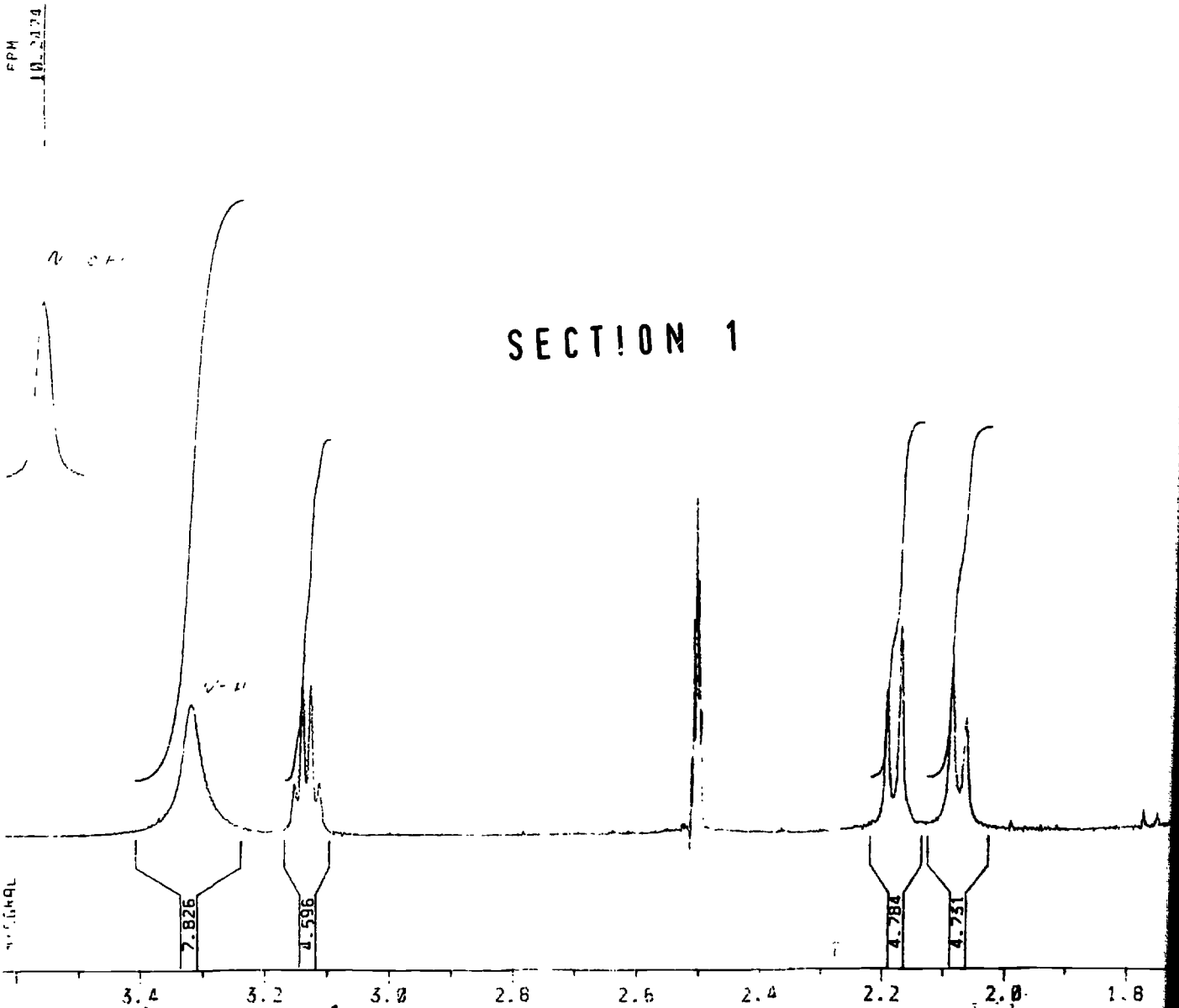
BRUKER

HM4:9H.001
DATE 5-7-89

SF	500.138
SY	60.0
O1	8577.082
SI	32768
TD	32768
SW	1984.127
HZ/PT	.121
PW	10.5
RD	0.0
AQ	8.258
RG	61
NS	96
TE	243
FW	2500
O2	9472.650
OP	30L PO
LB	-.300
GB	.300
CX	34.00
CY	30.00
F1	3.367P
F2	.670P
HZ/CM	39.661
PPM/CM	.079
SR	7789.68

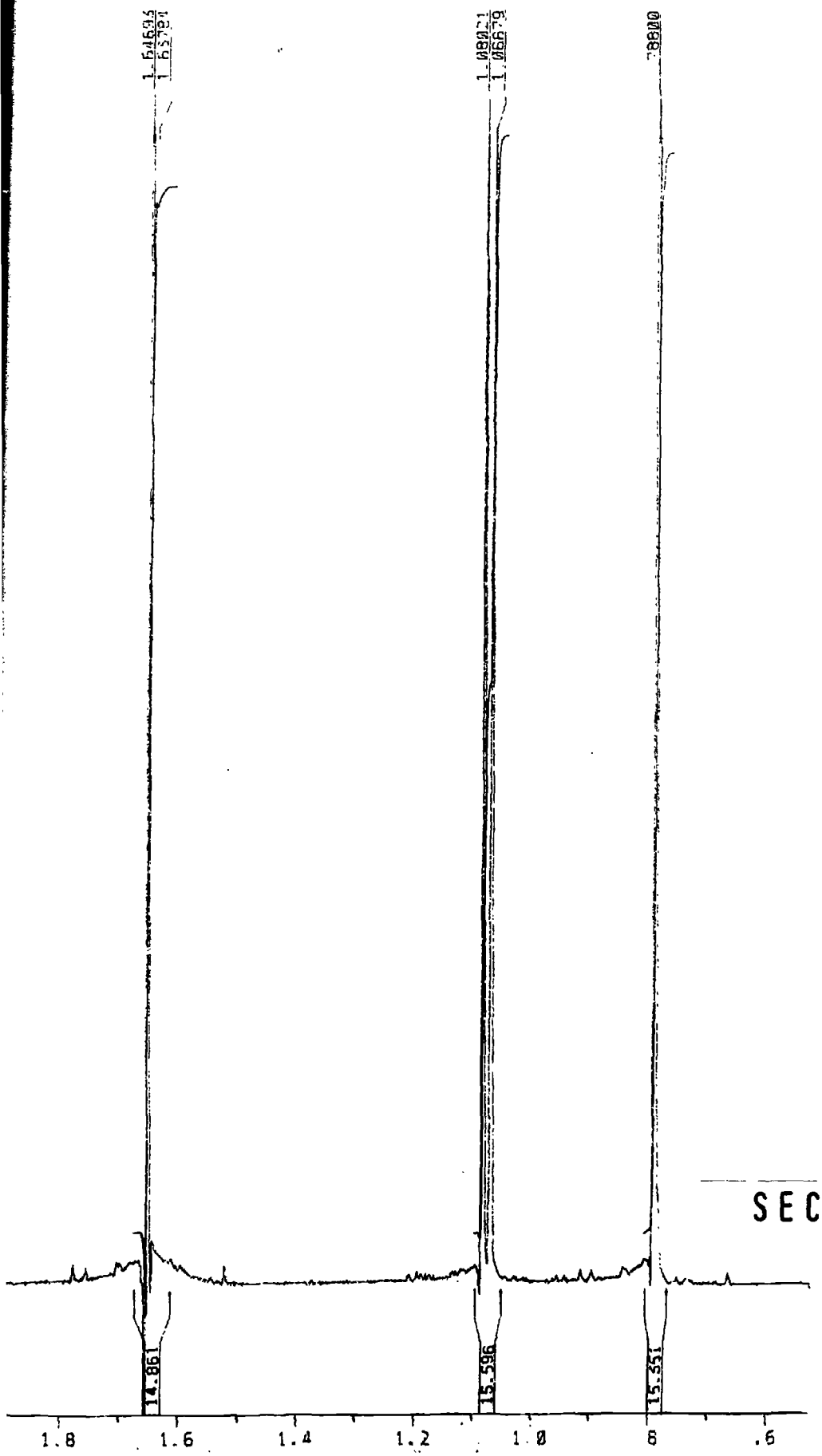
Meso—HMP 30

SECTION 2



SECTION 1

Fig. 4b 500 MHz ^{1}H NMR spectra in d_6-DMSO of purified d,l isomer.



BRUKER

XS1H.001
DATE 31-5-89

SF 500.138
SY 60.0
D1 10328.516
SI 32768
TD 32768
SW 6024.096
HZ/PT .368

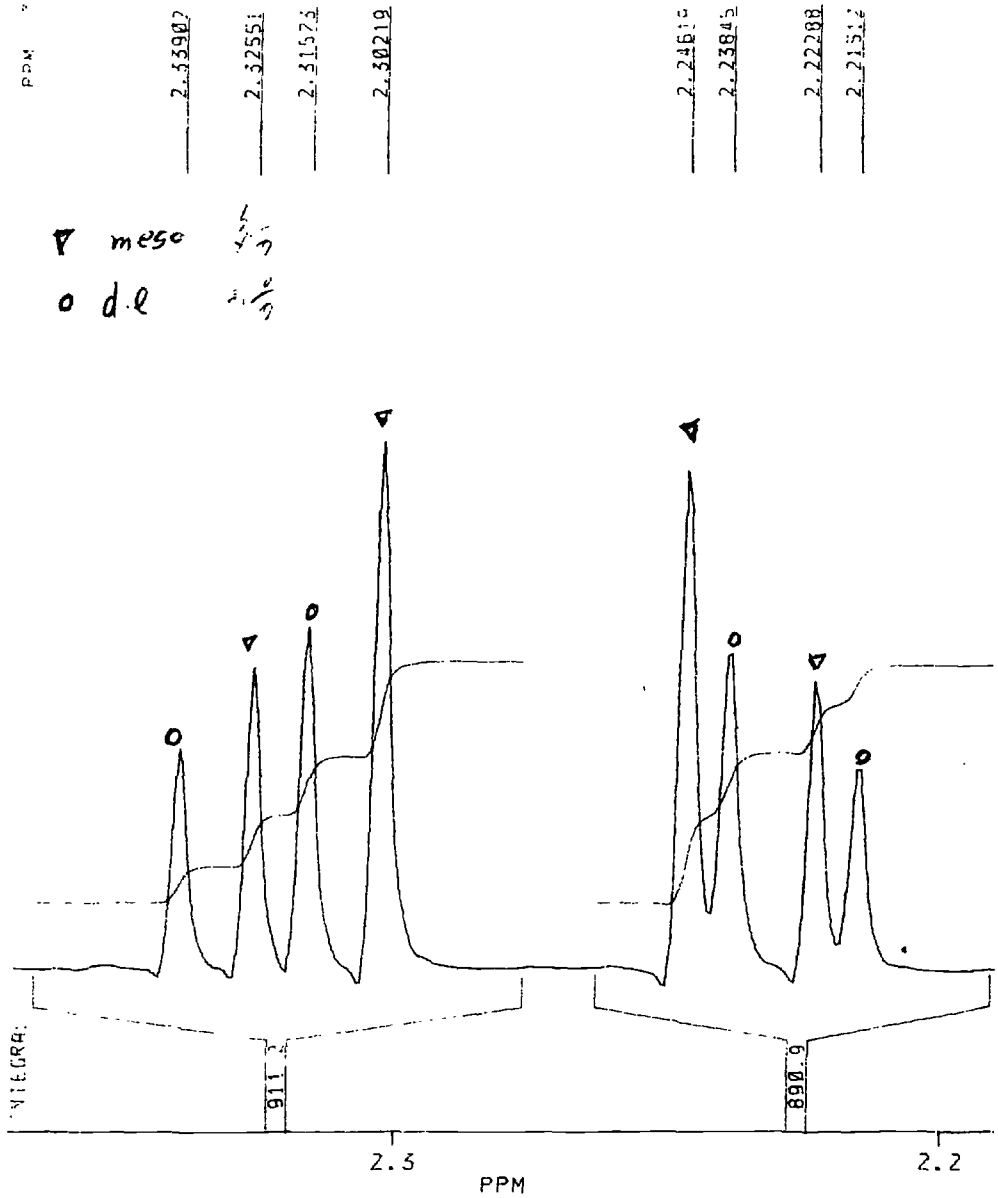
PW 11.9
RG 0.0
AQ 2.722
RG 61
NS 24
TE 243

FW 7600
D2 9422.652
DE 30L PD

LB -2.002
GB .200
CX 35.00
CY 60.00
F1 3.622
F2 .520
HZ/CM 44.311
PPM/CM .089
SR 7789.57

SECTION .2

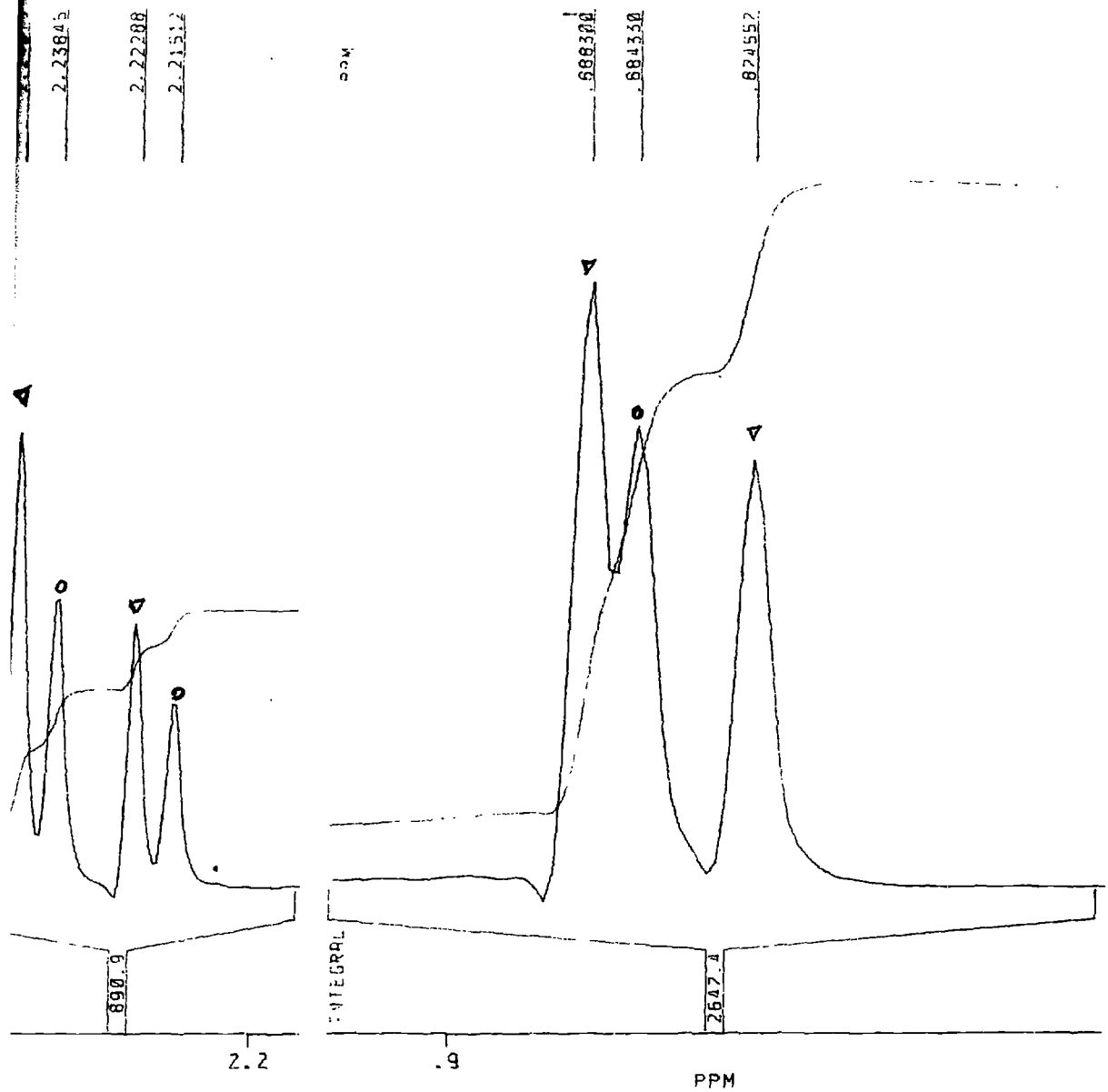
d, L-HMPA



SECTION 1

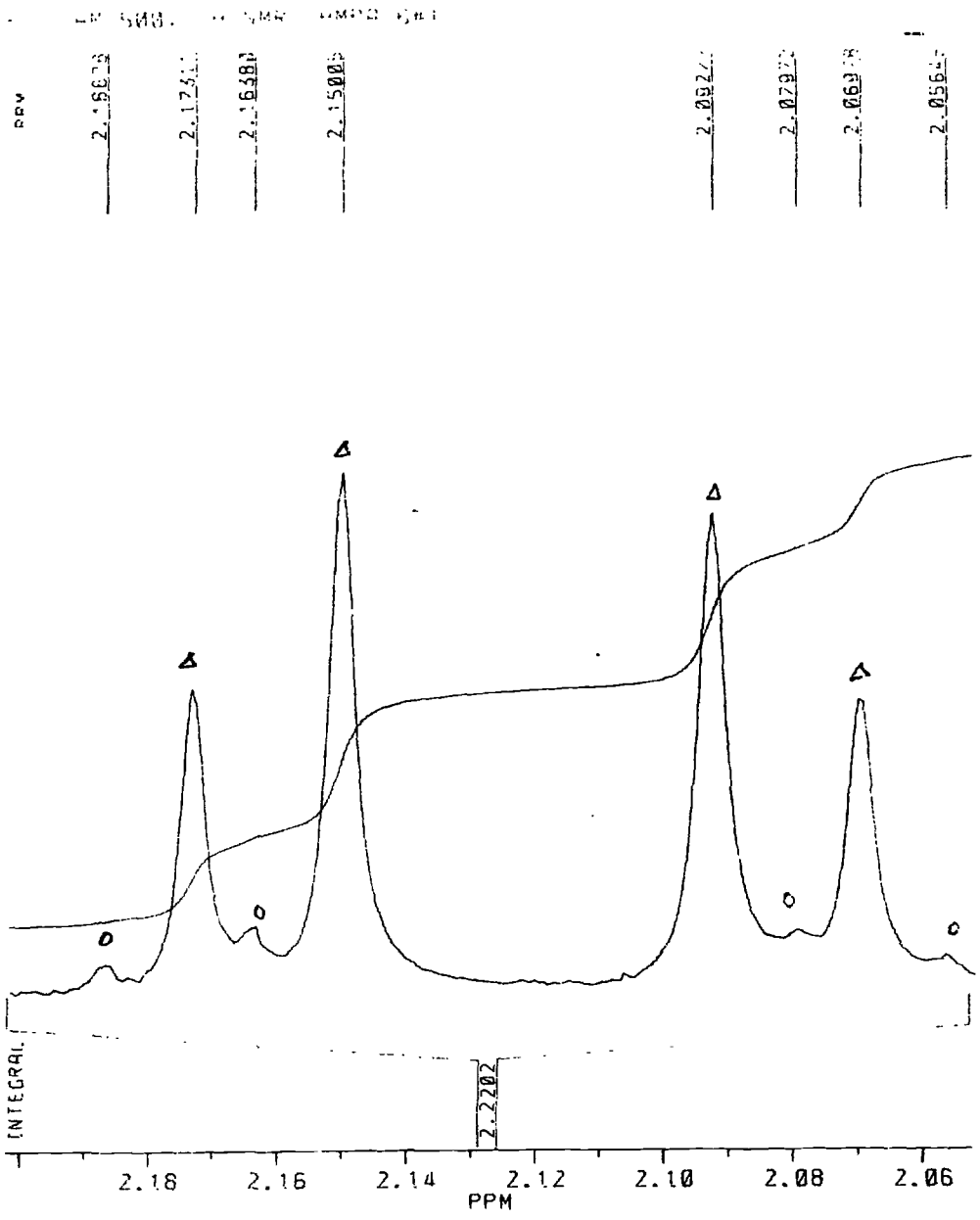
Fig. 4c

Expansion of the AB quartet region assigned to CH_2N- moiety and the two singlets region of two geminal methyls in the resolution enhanced 500 MHz ^1H NMR spectra in CD_3OD.



› CH_2N- moiety and
› in the resolution

SECTION .2

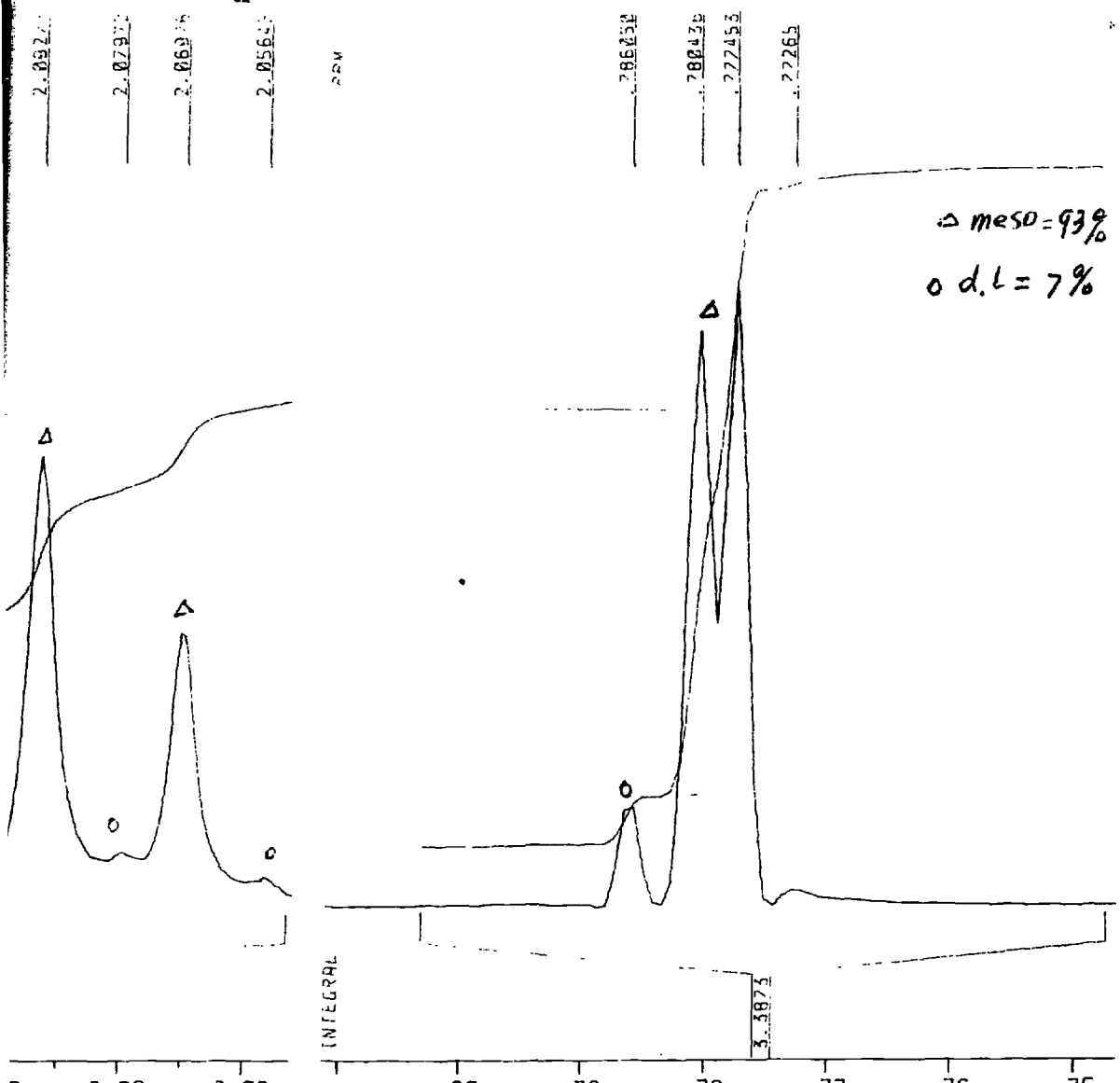


SECTION 1

Fig. 5a

Expansion of the AB quartet region assigned to CH_2N- moiety and ...sig... the high field region in the resolution enhanced 500 MHz ^1H NMR ...on spectra in d_6-DMSO of 93% meso isomer.

Δ — meso isomer, o — d,l isomer,



SECTION .2

...signed to CH_2N- moiety and
...on enhanced 500 MHz ^1H NMR

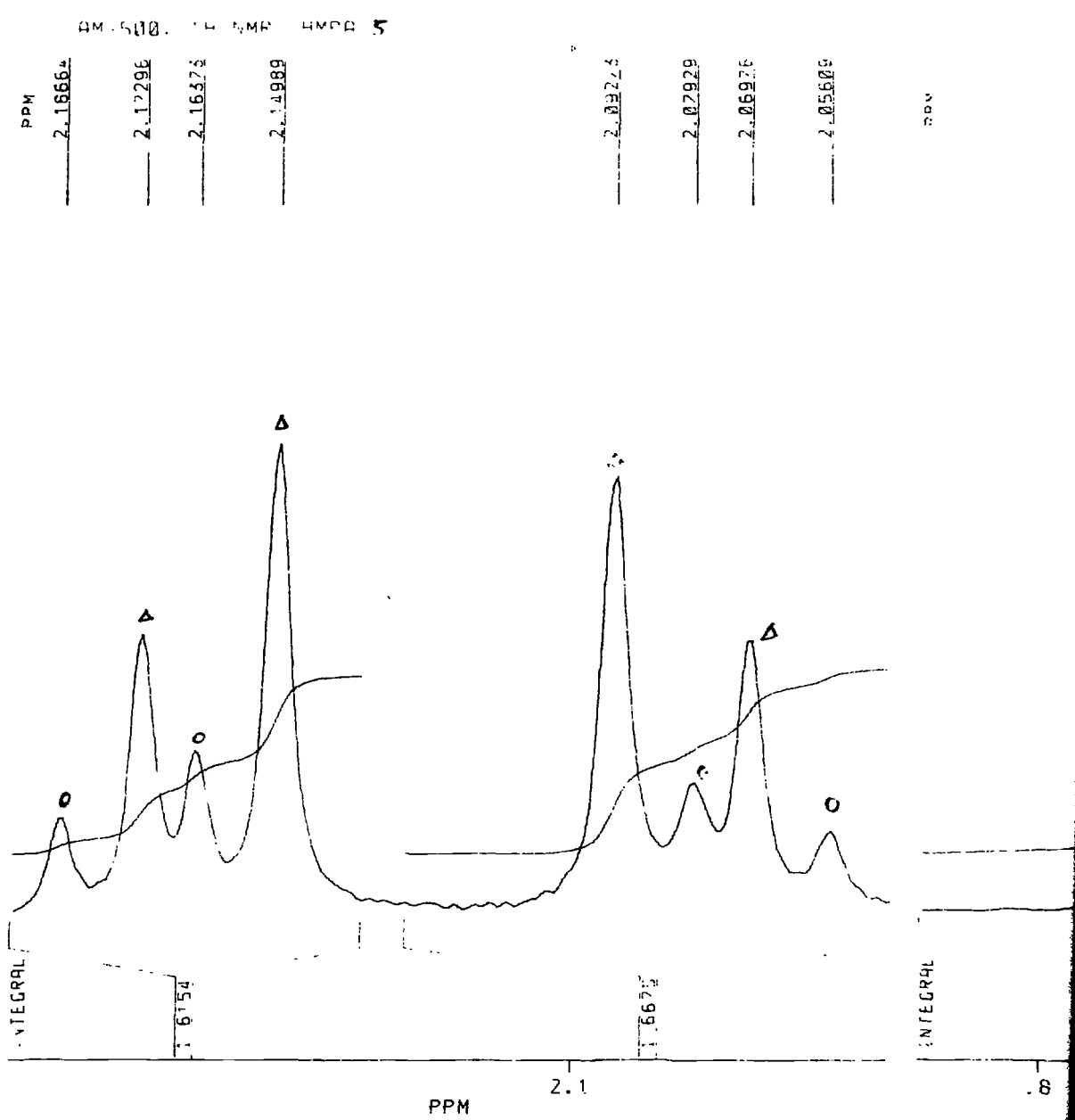
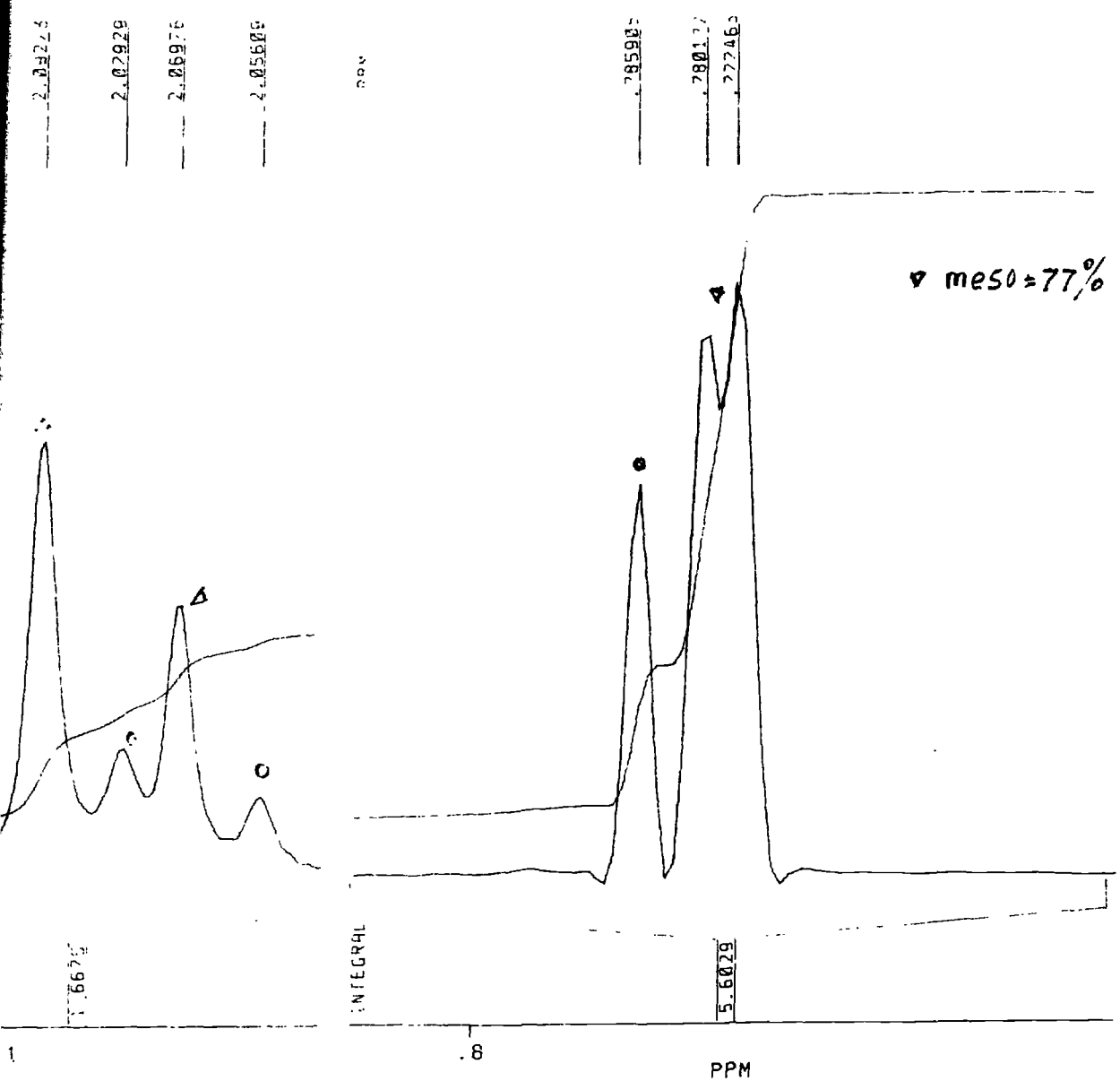


Fig. 5b

Expansion of the AB quartet region assigned to CH_2N- moiety and the high field region in the resolution enhanced 500 MHz ^1H NMR spectra in d_6-DMSO of 77% meso isomer.

Δ — meso isomer, o — d,l isomer.



SECTION .2

ıed to CH_2N- moiety and
ınhanced 500 MHz ^1H NMR

SECTION 1

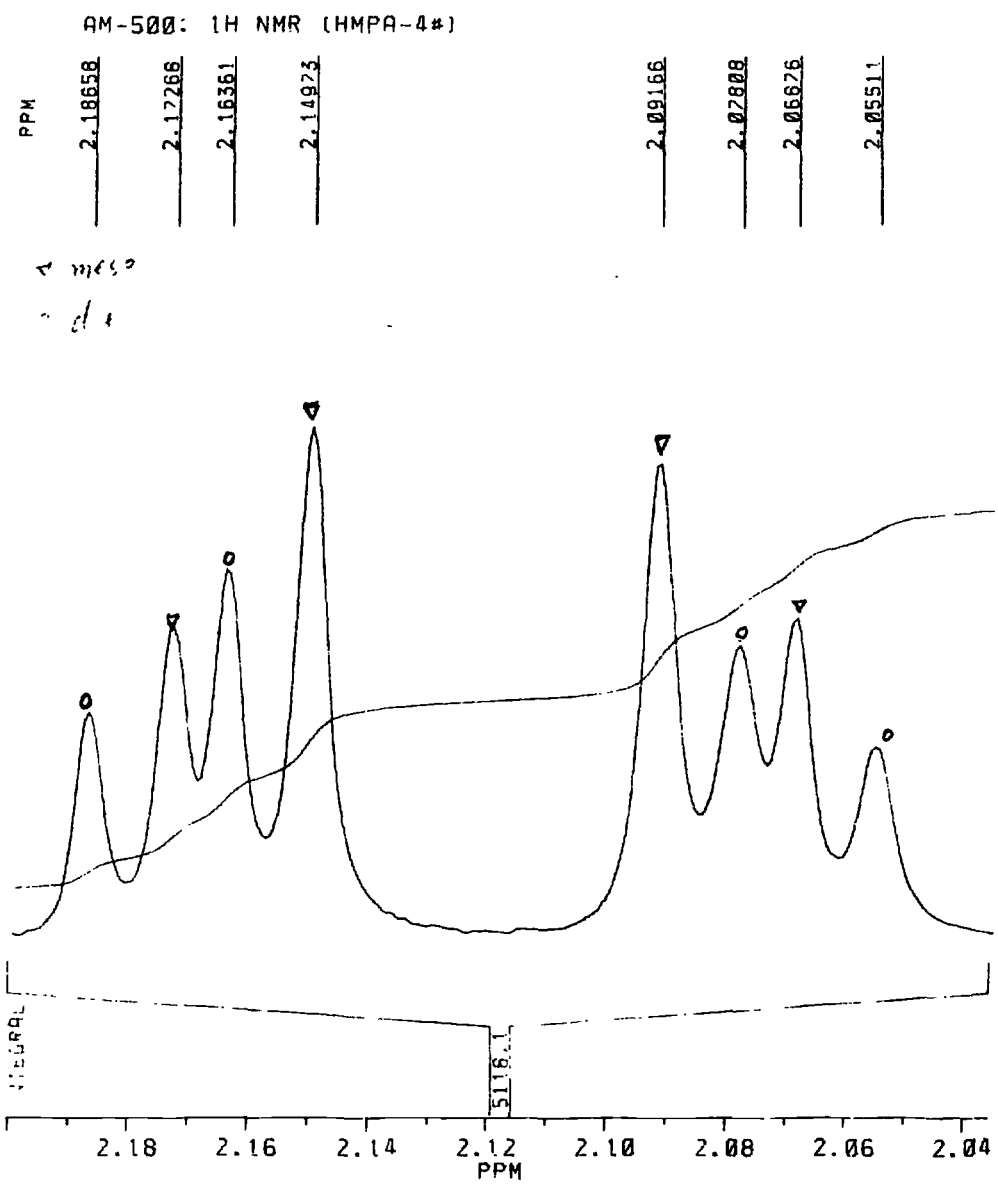
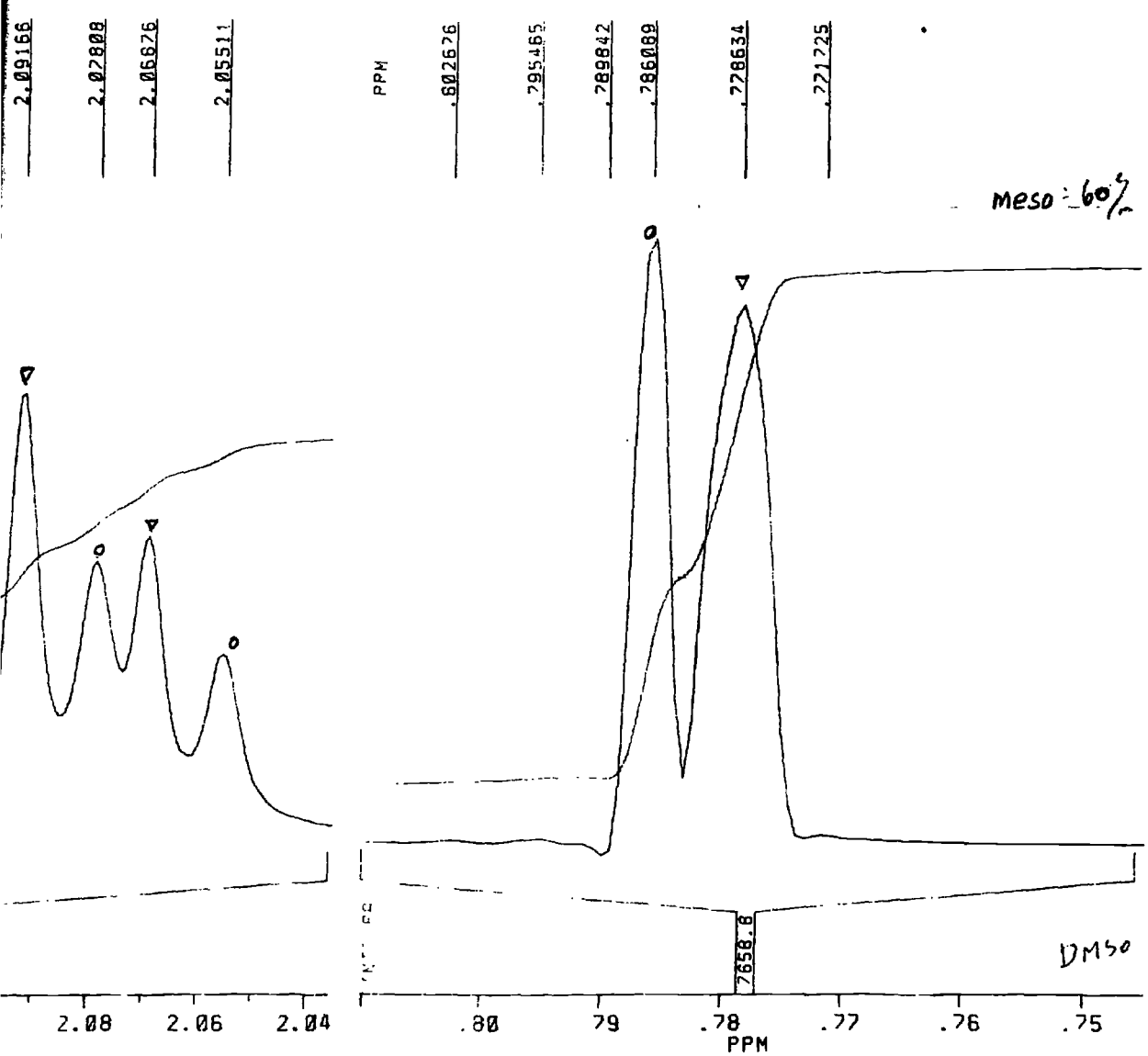


Fig. 5c

Expansion of the AB quartet region assigned to CH_2N- moiety and the high field region in the resolution enhanced 500 MHz ^1H NMR spectra in d_6-DMSO of 60% meso isomer.

Δ — meso isomer. o — d,l isomer.



signed to CH_2N- moiety and
on enhanced 500 MHz ^1H NMR
.

SECTION .2

Fig. 3 500 MHz ^{1}H NMR spectra in d_6-DMSO of diastereoisomeric mixture of d,l-

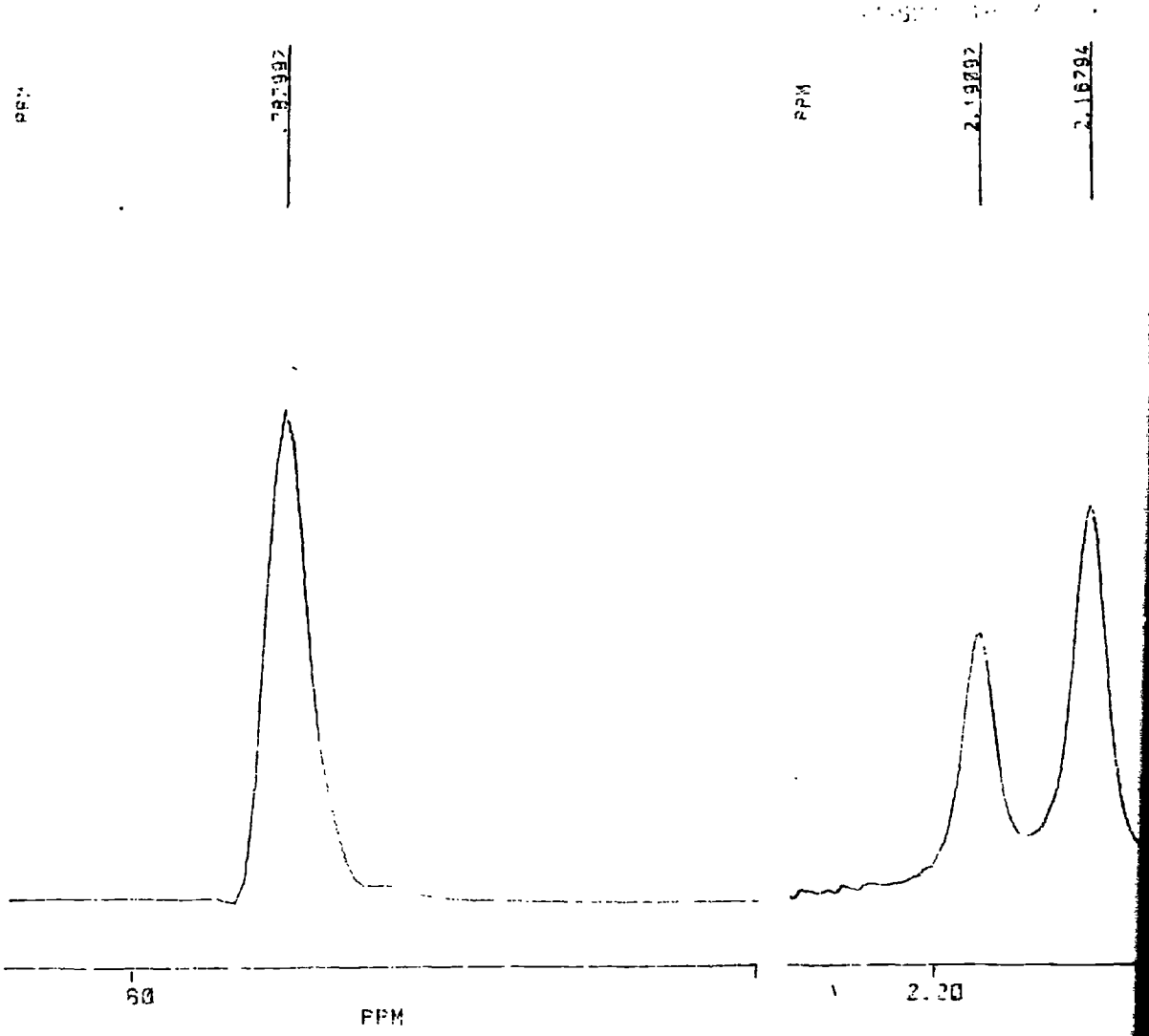
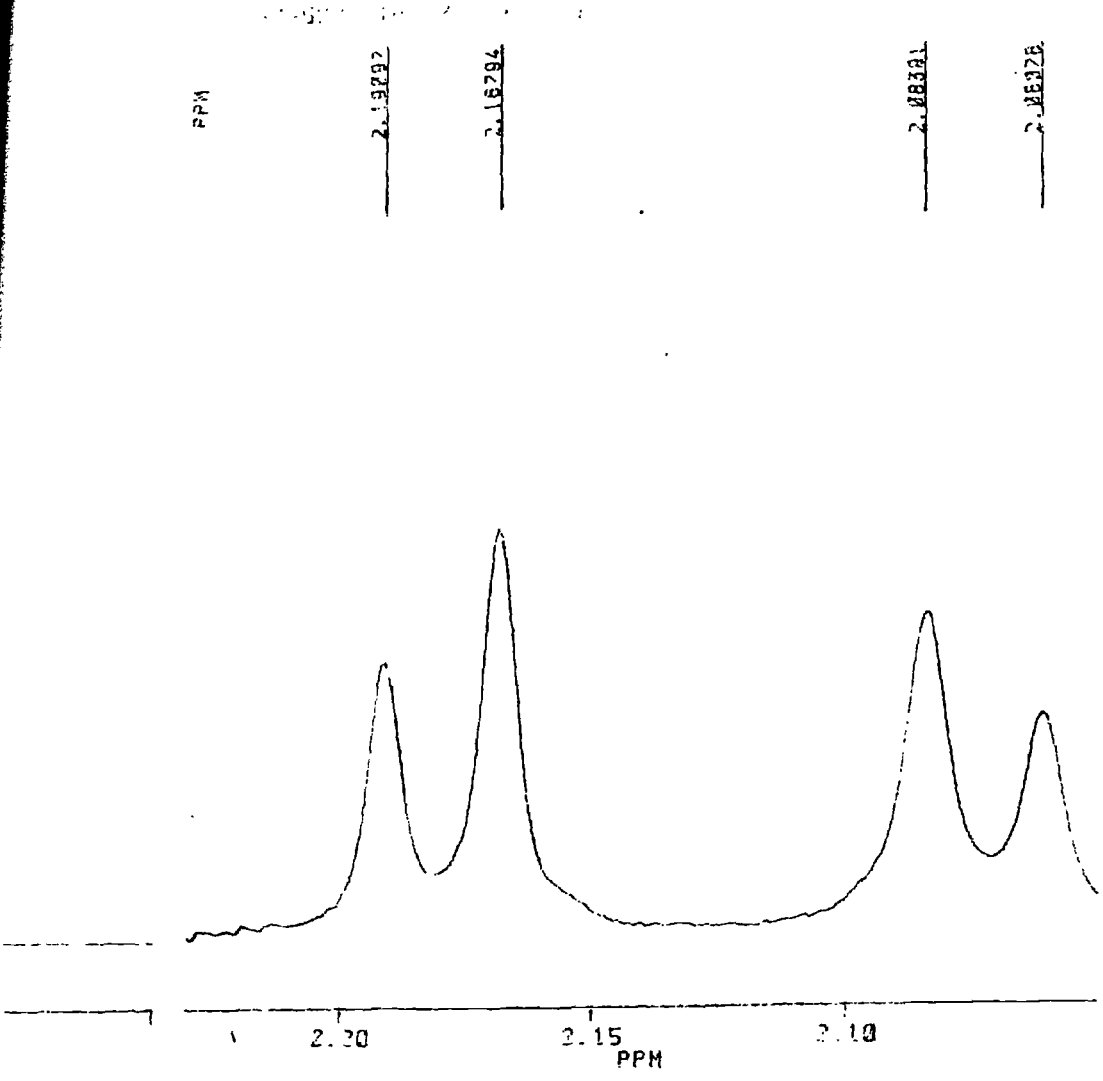


Fig. 5d

Expansion of the AB quartet region assigned to CH_2N- moiety and the high field region in the resolution enhanced 500 MHz ^{1}H NMR spectra in d_6-DMSO of the purified d,l-HM-PAO ligand.

SECTION 1

of d,l- and meso- diastereoisomers. Δ — meso isomer, o — d,l isomer.



- moiety and
0 MHz ^{1}H NMR
d.

SECTION .2