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THIS IS TO CERTIFY THAT THE THESIS PREPARED UNDER MY SUPERVISION BY

Mark P. Foster

ENTITLED Isolation of a Purple Pigment from Tridemnum solidum

IS APPROVED BY ME AS FULFILLING THIS PART OF THE REQUIREMENTS FOR THE

DEGREE OF Bachelor of Science in Chemistry

Instructor in Charge

APPROVED:

HEAD OF DEPARTMENT OF Chemistry

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Isolation of a Purple Pigment from *Trididemnum solidum*

By

Mark P. Foster

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in
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I. Acknowledgement

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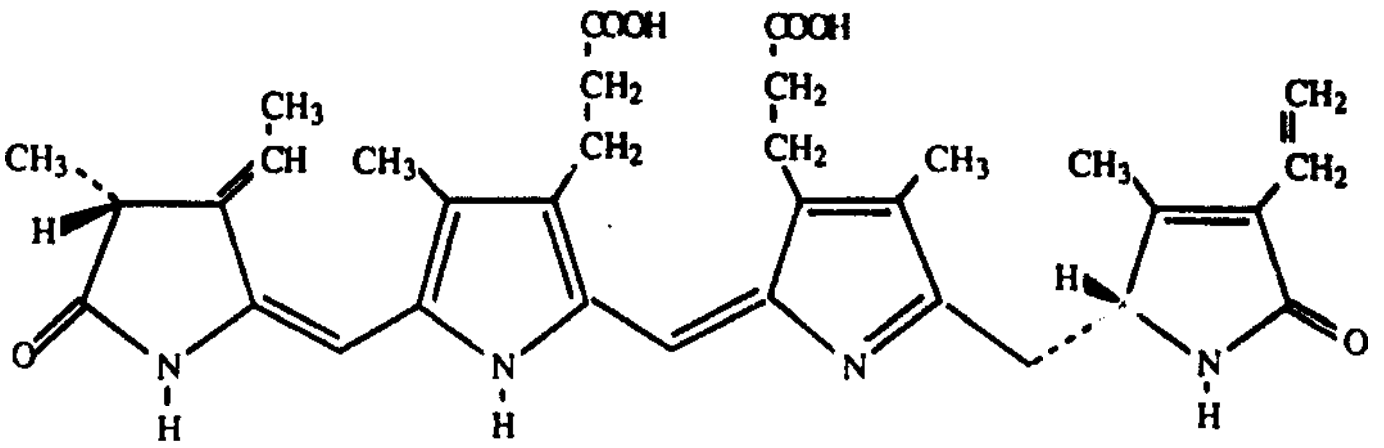
II. Introduction:

Much work has recently been carried out on the isolation and structure elucidation of pigments and other compounds from an interesting Caribbean tunicate of the family *Didemnidae*, *Trididemnum solidum*. This ascidian enjoys a symbiotic association with unicellular algae known to contain only chlorophyll a (no chlorophylls b or c) as a photosynthetic pigment.[1,2] The isolation of a novel blue-green nickel porphynoid pigment[3] and several cyclic depsipeptides (didemnins) exhibiting significant antiviral and antitumor activity[4] has encouraged further investigation into other pigments from the same organism - such as the purple pigments discussed here. The scarcity of purple pigments in lower animals also makes the isolation of these pigments of interest.

Bile pigments constitute a major class of green, red, purple, and blue pigments and are considered common in animals and plants, but not so in tunicates.[5] A purple bile pigment has, however, been isolated, along with a blue bile pigment, from a blue-green alga, specifically, in phycocyanin-rich cells of *Tolypothrix tenuis*.[6] Although initially believed to be a precursor of phycoerythrobilin (1), it was established to be an artifact formed from the biliprotein phycoerythrin (Scheme 1) by the isolation procedures used to release the covalently bound prosthetic group. The blue pigment was likewise reasoned to be an



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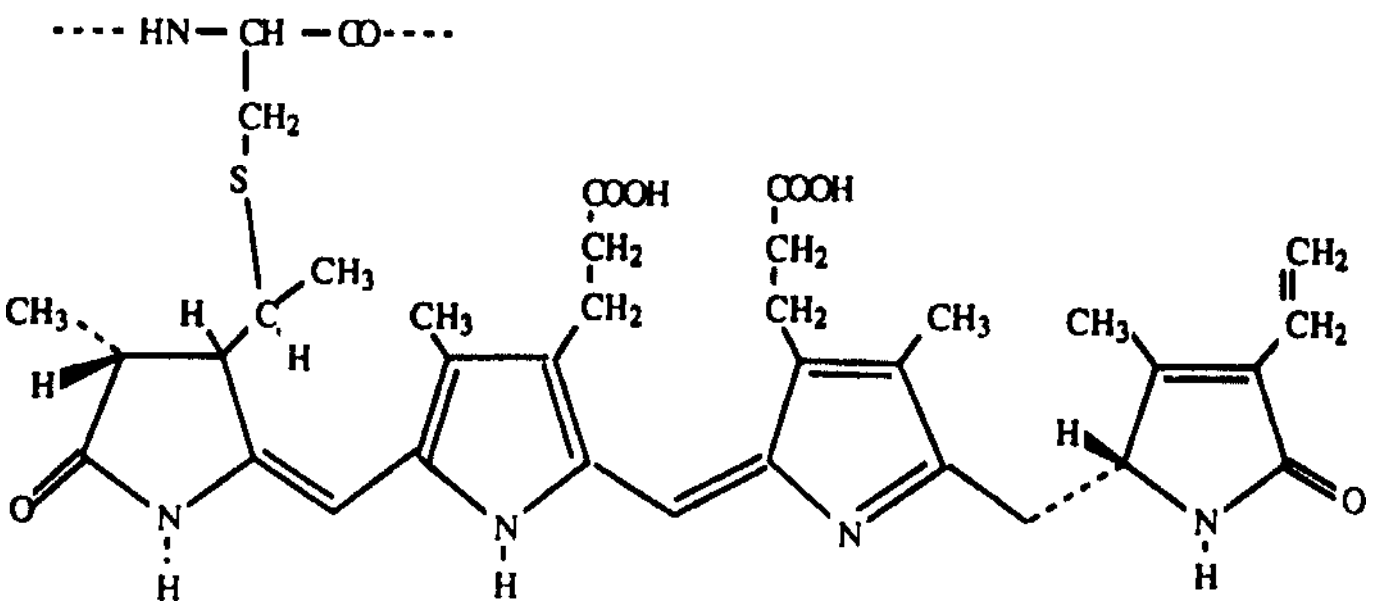
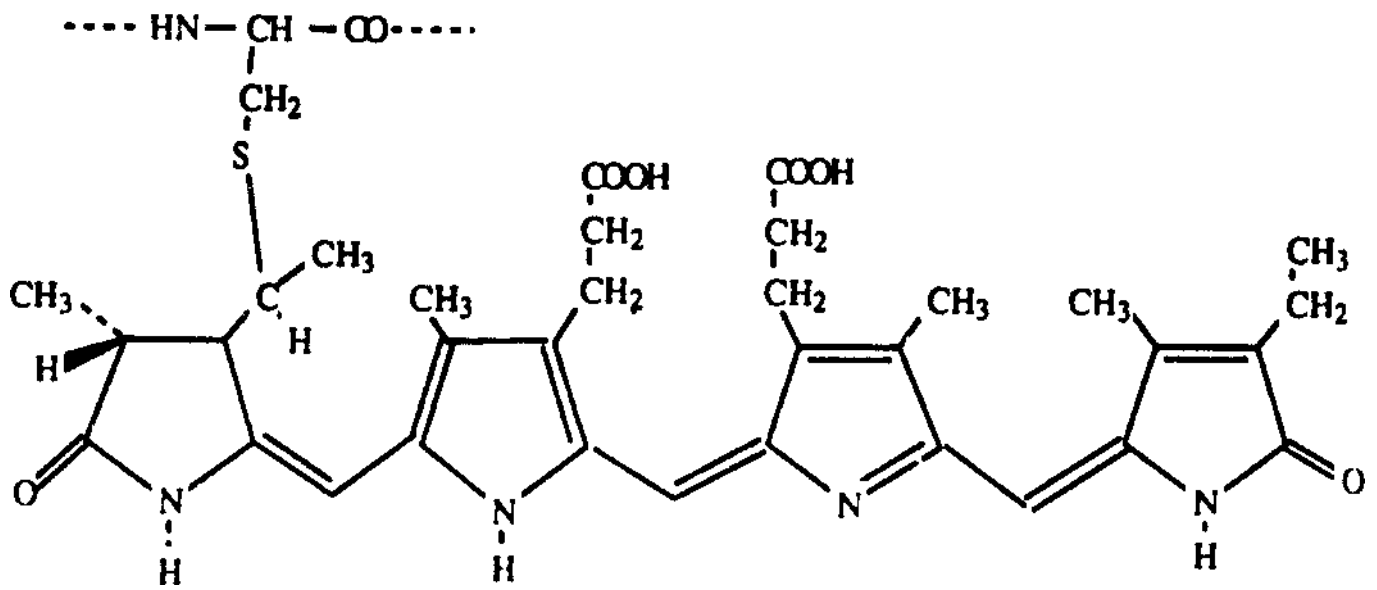


Scheme. 1 Proposed Covalent Linkage of the Prosthetic Group in Phycoerythrin

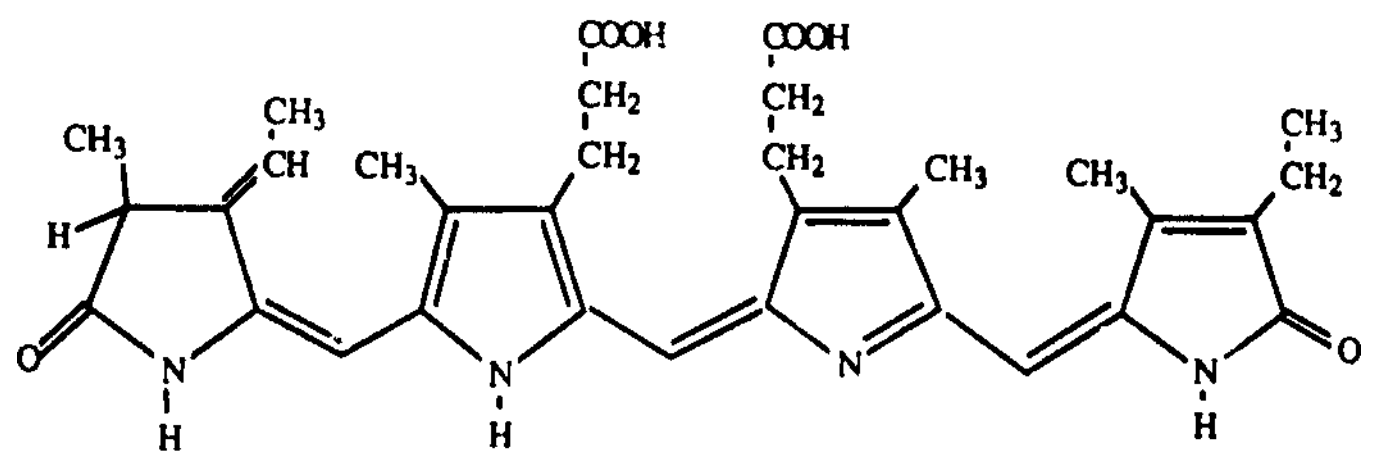
artifact from phycocyanin (Scheme 2) rather than a precursor of phycocyanobilin (2). Treatment of the purple and blue artifacts with concentrated hydrochloric acid yielded their respective phycobilins.[7]

Electronic spectra of phycobilins are characteristic, as they typically have an intense absorption peak in the visible region (ϵ = 13,500 - 92,900)[8] with a shoulder on the shorter wavelength side, plus two minor peaks in the UV region of comparable intensities (Fig. 1).[9] The lower energy peak of the two UV peaks is usually at about 350 nm for a cyanobilin and around 300 nm for an erythrobilin.[10] Phycobilins also typically demonstrate shifts toward longer wavelength when treated with hydrochloric acid (Fig. 2)[11], and their colors are solvent dependent.

NMR data, degradation studies, and chemical similarity of the "purple pigment" from *T. tenuis* to phycoerythrobilin have led to the proposal that they are isomers; the same sort of conclusion has been reached on the structure of the "blue pigment" as compared to phycocyanobilin. In fact, the similarities in degradation products, isomerizations, and NMR spectra have caused some confusion as to whether or not the respective pigments are the same as the phycobilin. Studies of electronic spectra show, however, that phycoerythrobilin and phycocyanobilin are the natural prosthetic groups of phycoerythrin and phycocyanin, respectively, and are different from the artifacts (See Fig. 3).[12,13]



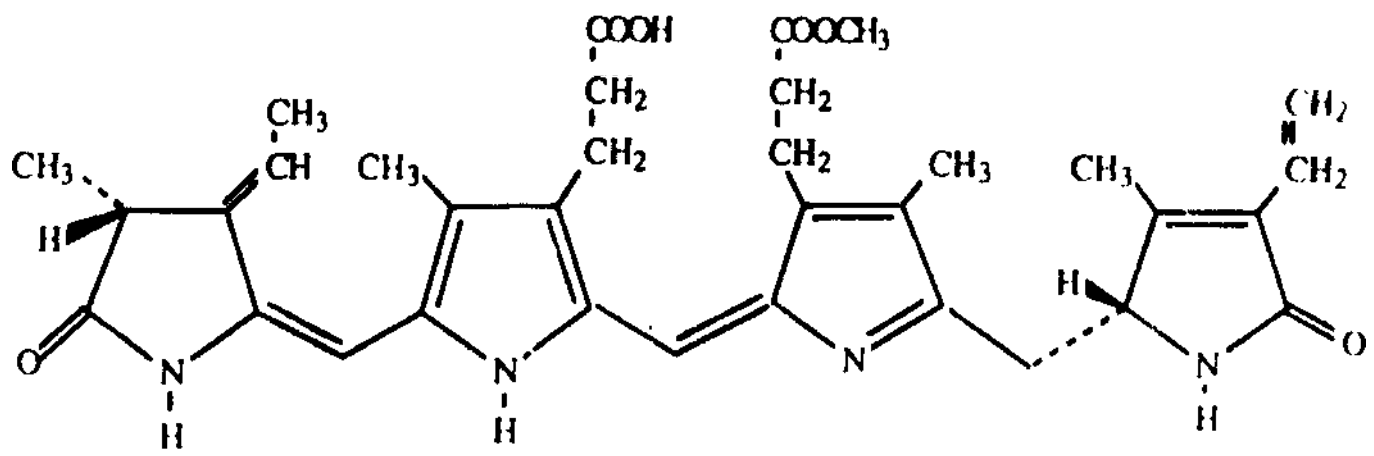
Scheme 2 Proposed Covalent Linkage of the Prosthetic Group in Phycocyanin



Aplysioviolin (3) is another interesting "violet" bile pigment that is the mono-methyl ester of phycoerythrobilin (1). Aplysioviolin is the bile pigment that provides the purple color of an excretion from certain sea hares of the genus *Aplysia*.[14,15] This is apparently a metabolite produced by the methylation of phycoerythrobilin, obtained by degradation of the red algae they feed on.

Knowing that phycoerythrobilin and phycocyanin are tetrapyrroles found in blue green algae containing only chlorophyll a (4), it would not be unreasonable to suggest that their biosynthetic pathway is in some way related to the porphynoid; moreover, their structures have the related 'type-IX' orientations of their substituents.[16]

Another interesting pigment is a blue dipyrroldipyrromethene prodigiosin analog (5), reported isolated from an unclassified compound ascidian,[17] which is identical to that previously reported isolated from a mutant strain of the bacterium *Serratia marcescens*.[18]



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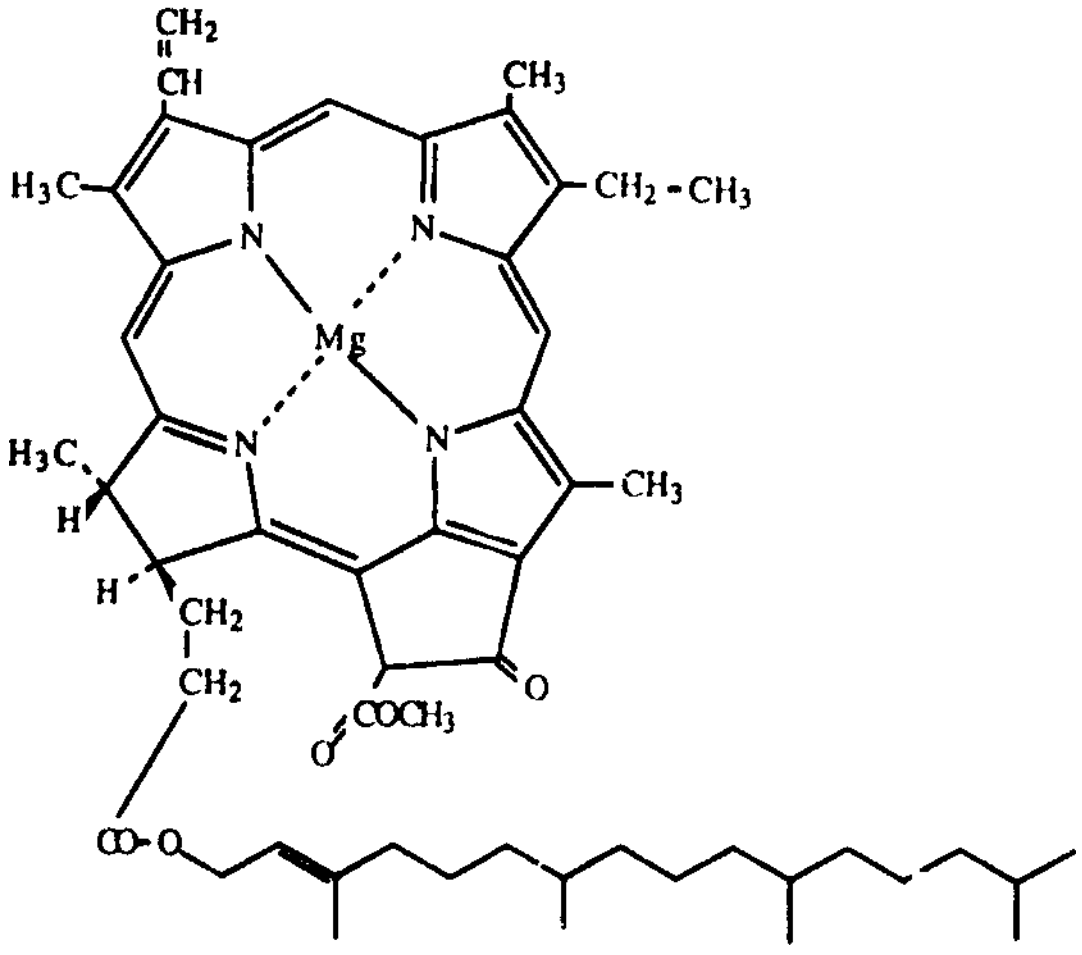

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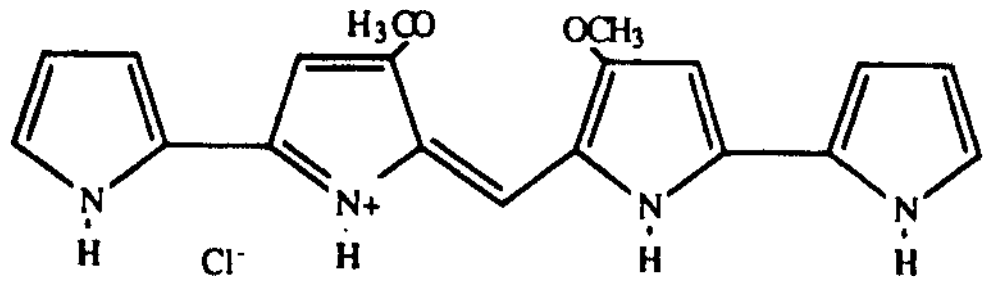

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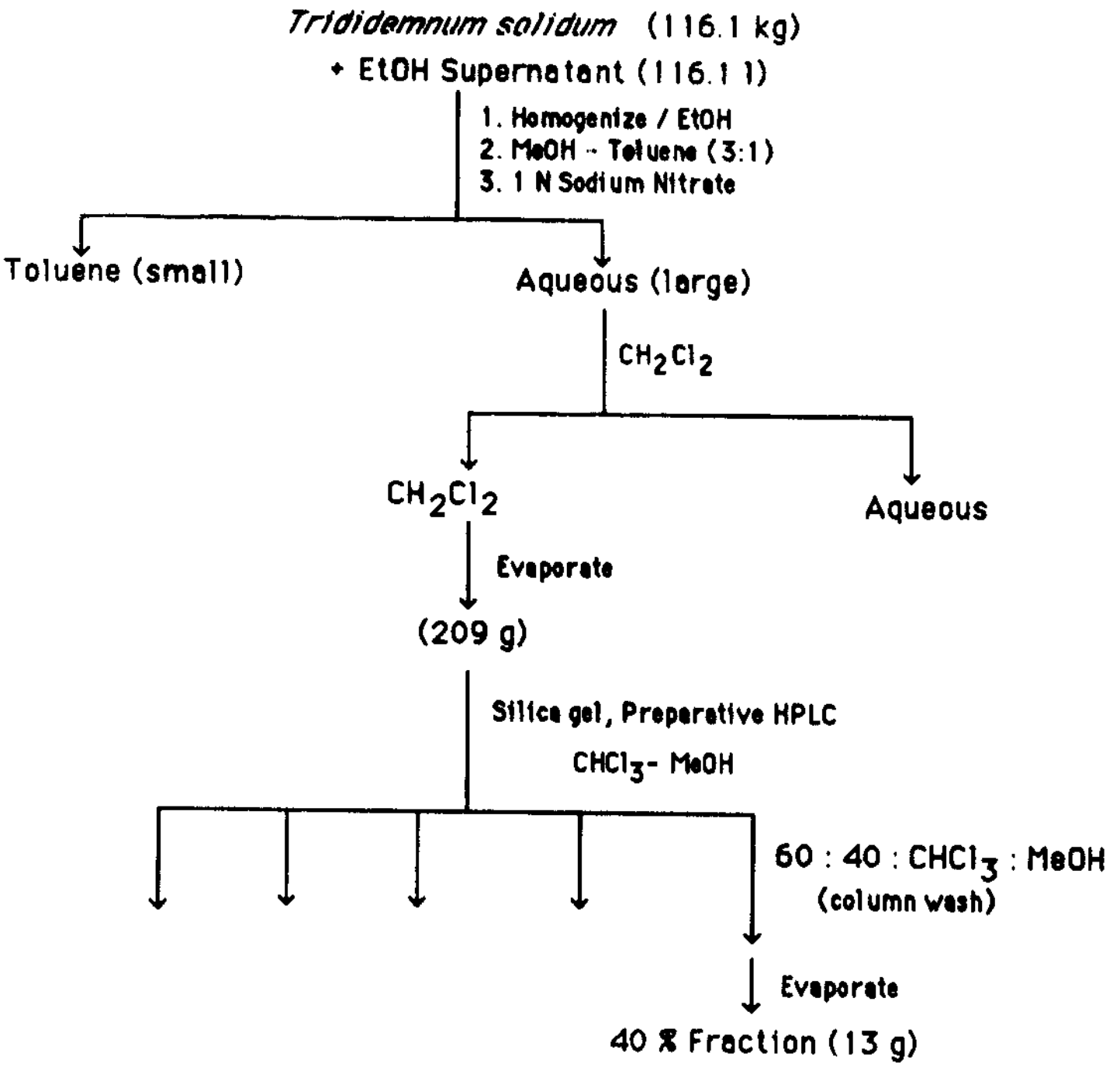
III. Discussion

a. Isolation:

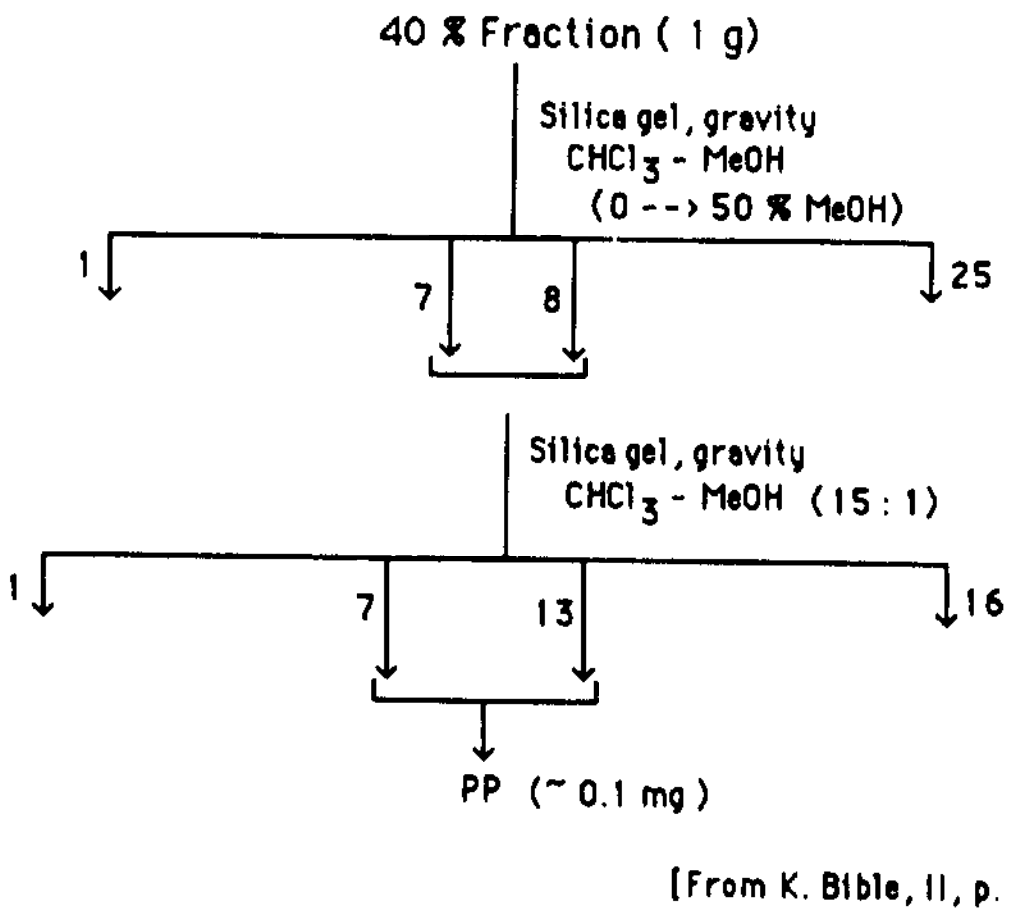
In the present study, the pigments from *Trididemnum solidum* were found to be difficult to purify (see Schemes 3 6) and yields were low (~34 parts per billion - FI82: 3.02 mg from 89.23 kg of tunicate [wet weight]). Unfortunately, reliance had to be placed upon inefficient modes of separation such as gravity liquid chromatography and preparative reversed phase TLC due to the fact that use of HPLC was unsuccessful. The use of HPLC with C_{18} and cyanopropyl columns and most eluting solvents apparently failed to elute the pigments, as no strong chromophore was detected at various wavelegnths. Use of a silica gel column in HPLC was precluded by the high amount of methanol required to elute the pigments (40%) and the destructive effect of that quantity of methanol on the column.

Solubility limits do not seem to be the cause of the elution problems as the pigments were found to be very soluble in methanol, chloroform and water, and moderately soluble in ethyl acetate (almost insoluble in hexane). The pigments showed high affinity for both normal and reversed phase TLC supports and large amounts of eluting solvents were required. Use of a methanol - chloroform eluent mixture actually increased mobility on reversed phase in comparison to chloroform alone.

Scheme 3. Isolation of Crude Purple Pigments

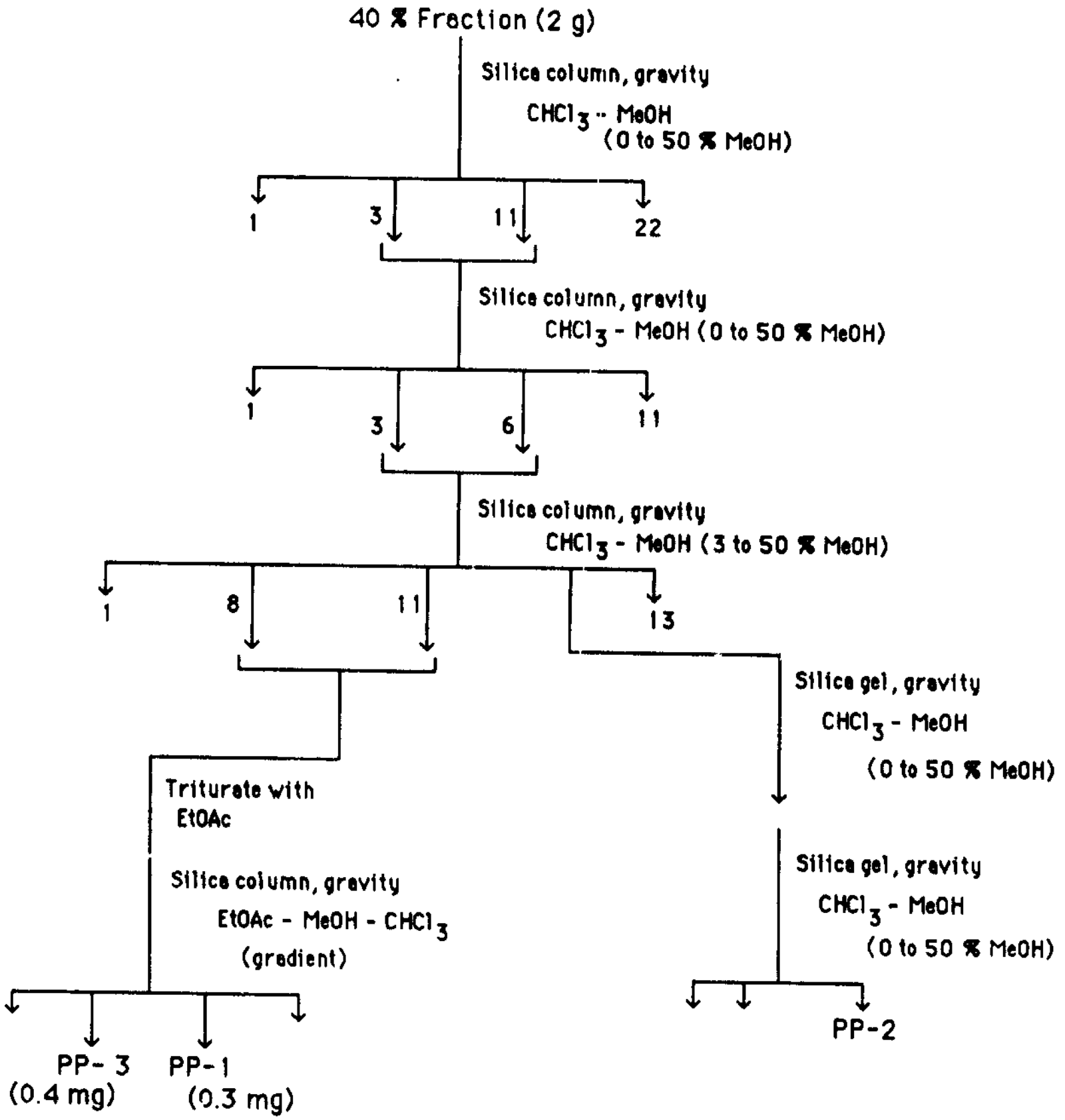


Scheme 4. Isolation of PP

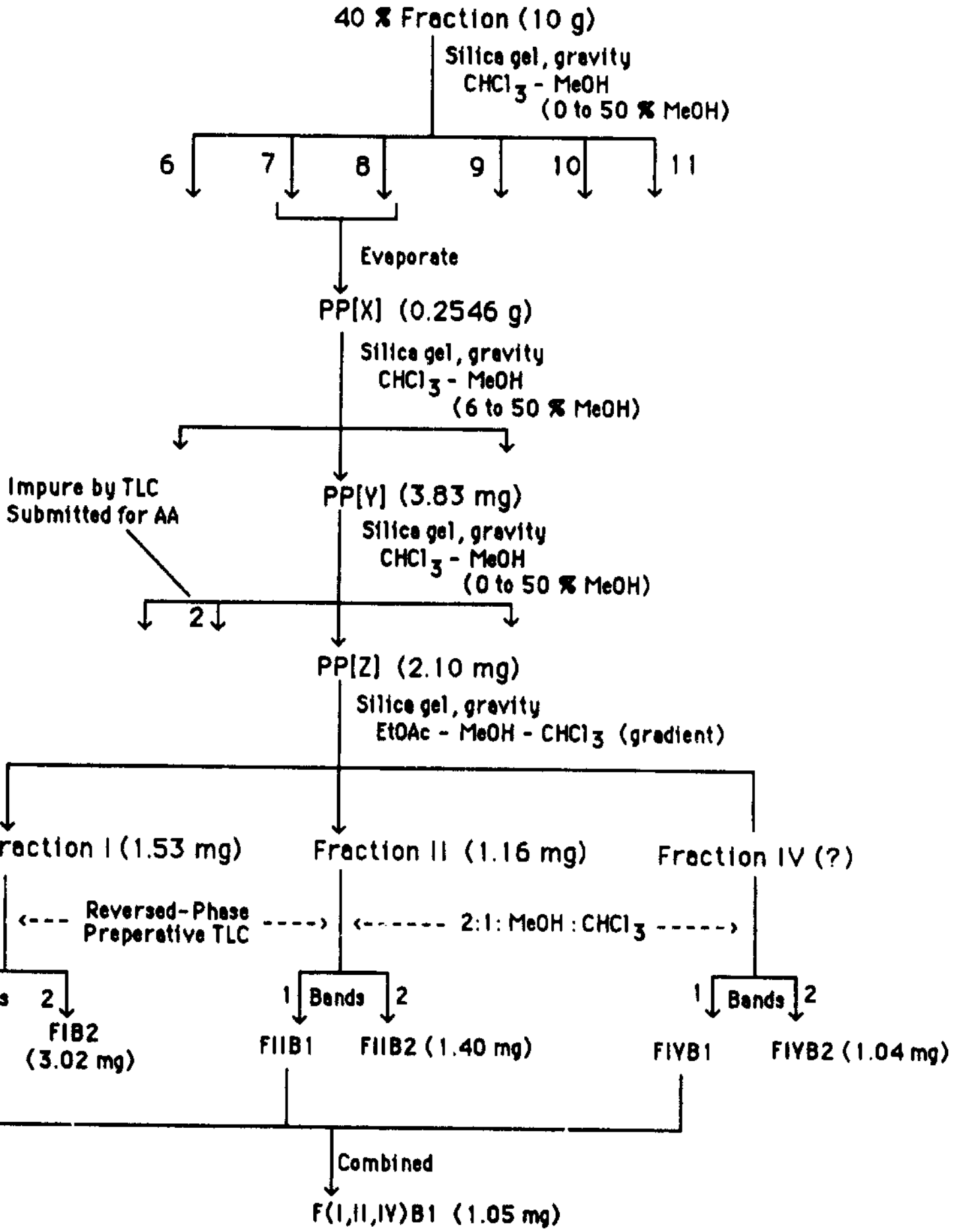


[From K. Bible, II, p. 9]

Scheme 5. Isolation of PP's 1-3



Scheme 6. Isolation of Fractions I, II & IV, Bands 1 & 2



b. Electronic Spectra

The electronic spectra of the purple pigments isolated from *T. solidum* (Figs. 4, 5) clearly resemble the analogous spectra of phycobilins extracted from marine algae (Fig. 1). The *T. solidum* pigments all contain an intense visible peak (and shorter wavelength shoulder), along with two less intense UV peaks. They are more likely to be analogs of phycoerythrobilin than phycocyanobilin, as the lower energy UV peak is closer to the wavelength expected for an erythrobilin than for a cyanobilin (~300 vs. ~350). Also, artifacts of phycoerythrobilin are generally purple, whereas artifacts of phycocyanobilin are blue.

The effect of acid and base on the absorption spectra of the *T. solidum* pigments is also helpful in their characterization. The purple pigments demonstrated a shift of 45 nm toward a longer wavelength (579 → 624 nm) upon addition of hydrochloric acid (see Table 2), a shift also characteristic of phycobilins (Fig. 2). Solvent dependence of the purple isolated pigments is also consistent with a phycobilin-type structure, as they turned bright blue in chloroform and purple in methanol. This shift toward longer wavelength corresponds to a change in the degree of conjugation of the delocalized aromatic system.

That the purple pigments from *T. solidum* shift 317 nm towards shorter wavelength upon addition of base (see Table 2) is evidence of a major disruption in the conjugation of the system at high pH. The partial reversibility of the

shift upon addition of acid suggests that it is not due to a reductive/oxidative cleavage of the tetrapyrrole chain but to protonation-deprotonation of nitrogen or oxygen.

Although the molecular weight of the *T. solidum* pigments is not known, an assumed molecular weight of 500 daltons (585 for phycoerythrobilin) yields a molar extinction coefficient of only ~1,300 for one pigment (FIB2 -- Table 1, Scheme 6) if all 2.97 mg of the sample represents the pigment. Since many bile pigments have extinction coefficients in the range of 20,000 to 80,000 (see Ref. 8), this may suggest that the actual weight of the isolated chromophore nucleus is on the order of 150 to 40 μg. This may indicate that the samples are impure and/or the chromophores are bound to molecules of high molecular weight (e.g. proteins).

c. Mass Spectra

Samples PP-1, PP-2, and PP-3 yielded various peaks by positive ion FABMS corresponding to m/z of 1350.5, 955.6, 705.5, 515.5, and 372.3 (see Table 4). These peaks were absent in the FAB^+ spectra of pigments FIB1 and FIIB2. The fact that the peaks observed in the spectra of PP's 1-3 were removed by separation techniques used to obtain the latter fractions suggests that those peaks corresponded to impurities in the samples. These latter samples did contain, however, a repetitive 44-mass unit pattern as would be expected from having polyethylene oxide as an impurity.

d. NMR

Peaks in the ^{1}H NMR and ^{13}C NMR spectra of the pigments appeared to be due to impurities in the samples (see Table 3 and Fig. 10). Aside from peaks due to chloroform at δ 7.28 ppm, water at δ 1.72 ppm, acetone at δ 2.06 ppm and silicone grease at δ 0.09 ppm, there were peaks corresponding to -CH-O-, $-CH_2-O-$, and $-CH_2-$ at δ 3.65, 3.40, and 1.13 ppm, respectively, that were coupled to each other (see Fig. 11). These peaks may be due to polyethylene oxide, concurring with the observations in FABMS (above). Peaks at δ 1.28 and 0.92 were also coupled (see Fig. 11) and may correspond to CH_3- and $-CH_2-$ from hydrocarbon wax impurities.

The ^{13}C NMR spectra of sample FIB2 likewise lacked any "real" peaks, the most outstanding belonging to $CHCl_3$ at δ 77 ppm (see Fig. 15). Minor peaks at δ 75, 71, and 17 ppm probably correspond to -CH-O-, $-CH_2-O-$, and $-CH_3$, respectively, and are probably due to the same impurities producing the peaks in the ^{1}H NMR spectra.

e. General

The mass spectral and NMR data obtained on the pigments are apparently derived from the various impurities present in the samples. Since color intensity was preserved at the same time NMR and mass peaks were lost during purification, it can be assumed that the peaks were due to impurities, or

at least to non-essential, loosely bound parts of the chromophore.

More detailed structure studies will require sufficient pure material for degradation work and NMR and mass spectral analysis. This necessitates a more efficient means of purification. HPLC using a different solid support may still be a viable purification method; countercurrent distribution may also be very effective in the purification of the pigments; ion chromatography is another possibility. Derivatization of the pigments to form more stable chromophores would potentially facilitate isolation and eliminate some of their erratic properties.

IV. Experimental

a. General:

Electronic spectra were obtained on either a Perkin Elmer 552 UV or a Perkin-Elmer λ 3 UV/VIS spectrophotometer. Mass spectral data were obtained on a ZAB SE spectrometer, and NMR spectra obtained on Varian 400 and 500 MHz FT-NMR instruments, in "100%" $CDCL_3$. For HPLC, two systems were used: Beckman 114M pumps with a Beckman 165 variable wavelength UV/VIS detector, and an Altex 110A pump using a Beckman 154 UV detector. Unless otherwise specified, chromatography refers to liquid gravity columns using silica gel (0.05 to 0.2 mm, Brinkmann) as solid support and an elution gradient comprised of chloroform-methanol. Purity assessment involved silica gel and/or C_{18} TLC on relevant fractions and observation of visual spots, fluorescent spots under long and short wavelength UV lights and development in an iodine chamber. Fractions were stored under nitrogen in the dark, below 0° C.

b. Isolation of Crude Purple Pigments

Trididemnum solidum was collected in the Caribbean, off the coast of Belize, preserved in ethanol, and shipped to Urbana. Aproximately 116.1 kg of this preserved tunicate was homogenized in 116.1 L of ethanol and ~400 L of 3:1: methanol:toluene (Scheme 3). After the homogenate was filtered, ~170.86 L of 1 N sodium nitrate solution was

added, and the material was partitioned between the smaller toluene and larger aqueous layers.

Extraction of the aqueous layer with dichloromethane and evaporation of the organic solvent yielded 209.0 g of material. This mixture was then subjected to silica gel preparative HPLC using chloroform-methanol as solvent and eluted with progressively increasing concentrations of the more polar solvent (methanol). The pigment was collected in a mixture of other pigments in a fraction eluted with 60:40: chloroform:methanol (the column wash), yielding 13 g of dark green-black material referred to as the "40% fraction". Three different chromatographic separations of the 40% fraction were performed to obtain the purple pigments.

c. Chromatographic Isolation of Purple Pigments

i. Isolation of PP (Scheme 4)

A purple pigment was obtained from 1 g of the 40% fraction by purification on a series of two silica gel liquid chromatography (gravity) columns using a chloroform-methanol eluent with progressively increasing proportions of methanol; this yielded less than 100 μg, and was designated PP.

ii. Isolation of PP's 1-3 (Scheme 5)

Similar chromatographic conditions were used with 2 g of the 40% fraction to obtain additional pigment for analysis; in this case three purple pigments were resolved. The pigment with a lower R_f (silica gel, chloroform -

methanol), was designated PP-2 and was noticeably (visually) redder than the higher R_f pigments. Purification of these latter pigments proved quite difficult as they were poorly resolved from other impurities (and each other) in the sample, and often formed a rather diffuse band on progression down the column. Application of the sample to three chloroform-methanol silica columns yielded poor results (impure), so a different solvent system was tried. An ethyl acetate - methanol - chloroform silica column separated two purple pigments and removed some impurities. The designation PP-3 was given to the pigment with the higher R_f because it exhibited peculiar chromatographic properties on the column. Rather than eluting as a band, it diffused throughout the entire column but was surprisingly resolved from the other pigments in the column upon elution. The name PP-1 was designated for the pigment that eluted in a thin band after PP-3 and other impurities (green, yellow). Pigments PP-1 and PP-3 were not distinguishable on silica gel TLC using ethyl acetate : methanol : chloroform : 5:1:3 as an eluent, nor were they distinguishable on the basis of R_f in 9:1 : chloroform : methanol. Co-spotting PP-1 and PP-3 showed that they were unresolvable in this system (SiO_2 TLC, $CHCl_3$: MeOH). The yields of PP-1,2 and 3 were each less than 1 mg; thus, their properties were not easily analyzed and their purity was suspect.

iii. Isolation of Fractions I, II and IV, Bands 1 and 2 (Scheme 6)

A third portion of the "40% fraction" (10 g) was chromatographed on a large column [8 cm x 80 cm, containing about 2.2 kg of silica gel (dry weight)], eluting with a chloroform-methanol gradient, and processed to obtain additional purple pigments. Two fractions appearing (by TLC) to contain only one (the same) purple pigment (as well as green and fluorescent impurities) were combined (PP[X]), yielding a dry mass of 0.2548 g. Adjacent fractions also contained purple pigments but they appeared to have different R_f's from PP[X] and in one case appeared to be a mixture of purple pigments of higher and lower R_f. Chromatography of PP[X] removed significant quantities of green, red, and fluorescent white and red impurities and produced 3.83 mg of combined purple pigment designated PP[Y] (plus some fluorescent red impurity). Chromatography of PP[Y] produced fraction PP[Z], which appeared pure by TLC visualization techniques and weighed 2.10 mg.

Due to the procedure employed previously by which a third purple pigment was separated using a different solvent system, PP[Z] was loaded on a silica column packed in 5:1:3 : EtOAc:MeOH:$CHCL_3$. Elution with a solvent system of the same composition caused the entire sample to spread out along the length of the column (22 cm x 1.5 cm), but it was not eluted. Eluent composition was then changed (stepwise, not gradient) to 5:1, then to 2:1: $CHCL_3$:MeOH. At this

point, the "purple streak" divided into three bands, one relatively narrow band high on the column (Fraction IV), another relatively narrow band just above the midpoint on the column (Fraction II), and a third band extending from ~1 cm below the second to the bottom of the column (Fraction I) Continuing to elute with 2:1 : $CHCL_3$:MeOH rapidly eluted all of the bands. The lower and middle bands merged as elution progressed. Fraction I (FI) weighed 1.53 mg, Fraction II (FII) 1.16 mg. The weight of Fraction IV (FIV) was not accurately measured but the quantity was noticeably much smaller than the other two. Since the total weight of the fractions eluted (I + II = 2.69 mg) is greater than the weight of the sample loaded (2.1 mg), it is reasonable to assume that the samples contain a large quantity of silica gel, probably due to the high concentration of methanol used to elute the pigments. The fractions appeared free of impurities by silica gel TLC.

Preparative reversed-phase TLC seemed effective in removing a series of fluorescent bands in addition to separating two bands of purple pigments, band 1 at R_f = 0.4 (minor) and band 2 at R_f = 0.33 (major), respectively. This separation of two bands was consistent for Fractions I, II, and IV as was the resolution of various pigmented, fluorescent, and iodine-reactive bands. Extraction of the purple pigment from C_{18} bonded phase was not 100% efficient. Chloroform was ineffective in extracting the pigments from

the C_{18} TLC material but was aided by a more polar solvent, methanol.

The first band (minor one) was combined for the three fractions as the quantity was minimal in all cases. This and the separated fractions I, II and IV were filtered through 0.45-μ Alcron LC13 disposable filters using 1:1 : $CHCl_3$:MeOH and weighed; Fraction I, Band 2 (FIB2), 3.02 mg; Fraction II, Band 2 (FIIB2), 1.40 mg; Fraction IV, Band 2 (FIVB2), 1.04 mg; combined Fractions I, II, IV, Band 1 (FI,II,IVB1), 1.05 mg. The purity of the samples is once again suspect as the combined yield is much greater than the weight of one of the earlier fractions they were derived from (PP[Z], 2.10 mg).

d. Electronic Spectra:

The electronic spectra for the pigments in methanol are given in Table 1 (and Figs. 4 and 5). Assuming the recorded weights represented pure pigment, and that the molecular weight was 500 daltons, molar extinction coefficients for FIB2 and FIIB2 were 1317 and 1767, respectively. Acid-base effects on the spectra are shown in Table II (as well as Figs. 6 and 7, and 8 and 9).

Table 1. Electronic Spectra of Purple Pigments.[a]

Sample	$\lambda\lambda_{max}$(A), nm		
PP-1	584,	543(sh), 300	247
PP-2	579,	544(sh), 298,	266
PP-3	587,	545(sh), 302,	247
PP[Z]	584,	543(sh), 300,	247
FIB2	579(1.57), 550(1.22), 294(.387), 242(.417)		
FIIB2	579(.954), 550(.781), 296(.270), 244(.324)		

(a. See also Figures 4 and 5)

Table 2. Effect of Acid and Base on λ_{max}[b]

Sample	pH	$\lambda\lambda_{max}$(A), nm		
PP	neutral	582(1.8)	300(.65)	247(.8)
PP	alkaline	565(1.4)	---	---
PP[Z]	neutral	579(1.0)	---	---
PP[Z]	acidic	624(0.1)	644(.09)	---

(b. See also Figures 6, 7 and 8, 9)

e. NMR and Mass Spectral Data:

Impurities in the sample gave strong peaks which complicated interpretation of sample spectra.

1. NMR

The ^{1}H NMR spectrum (Fig. 10) of the "cleanest" samples, FIB2 and FIIB2 (identical spectra), in "100%" $CDCl_3$, contained the peaks in Table 3.

Table 3. Chemical Shifts of ^{1}H NMR Peaks[a]

δ (ppm)	Functionality
7.28	$CHCL_3$
3.65	$-\underset{|}{C}H-O-$
3.40	$-CH_2-O-$
2.06	Acetone
1.72	H_2O
1.28	$-CH_2-$
1.13	$-CH_2-$
0.92	$-CH_3$
0.09	Silicone grease

(a. See also Fig. 10)

COSY (2-D NMR, Fig. 11)) and decoupling experiments showed coupling between the peaks at δ 3.4 and 3.65, which were also coupled to the peak at δ 1.13. Peaks at δ 1.28 and 0.92 were coupled to one another. [See Figs. 10,11,12,13,14]

^{13}C NMR spectra showed no aromatic peaks, and none downfield of chloroform (77 ppm). Small peaks were observed at 75, 71, and 17 ppm (See Fig. 15).

ii. Mass Spectra

Spectra of the "dirtier" samples PP's 1-3 yielded the peaks in the FAB^+ spectra shown in Table 4.

Table 4. Peaks in FAB^+ Mass Spectra of PP's 1-3.

Sample	Fig #	m/z
PP-1	16	705.4983
		515.4125
		372.2450
PP-2	17	515.7
		705.8
PP-3	18	1350.5
		955.5788
		705.4983
		515.5
		372.3

All of these peaks disappeared in the positive ion FABMS spectra for FIB2 and FIIB2. Their spectra contained no strong peaks but showed a repetitive 44-mass unit pattern. (See Figs. 19 and 20).

f. Atomic Absorption:

One of the fractions from the column that produced Fraction PP[Z] (Fraction 2 from PP[Y] - Scheme 6) containing a purple pigment with some impurities was submitted for atomic absorption metal analysis. None of the metals tested for was detected within the limits of the instrument, which were: Ni, V, and Fe, > 0.2%, Mg, > 0.02%, Cu, >0.15%, Co, >0.3%, Cr, >0.4%, and Mn, >0.15%.

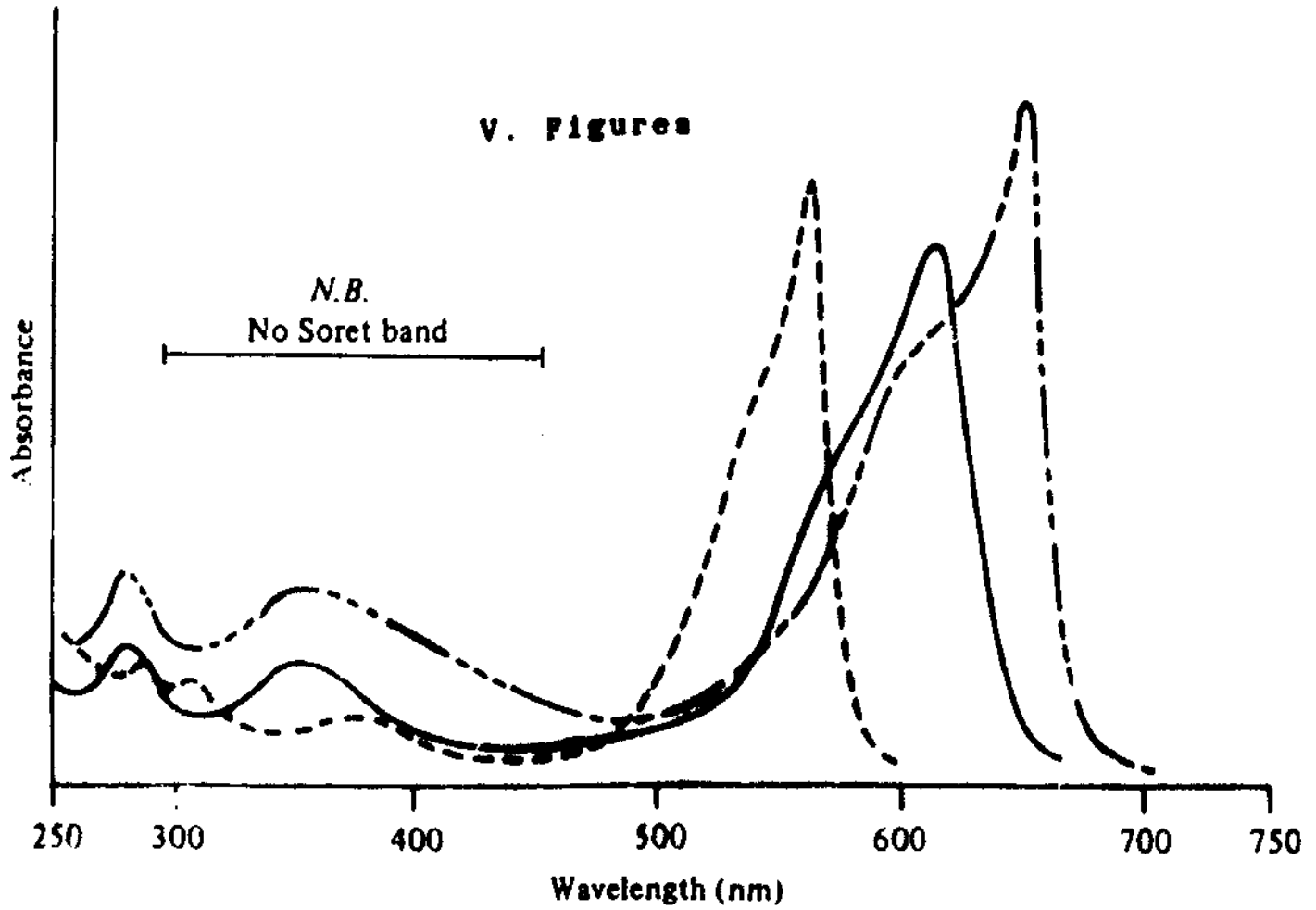


Figure 1. Typical Phycocyanin Electronic Absorption Spectra. [Britton, p. 159]

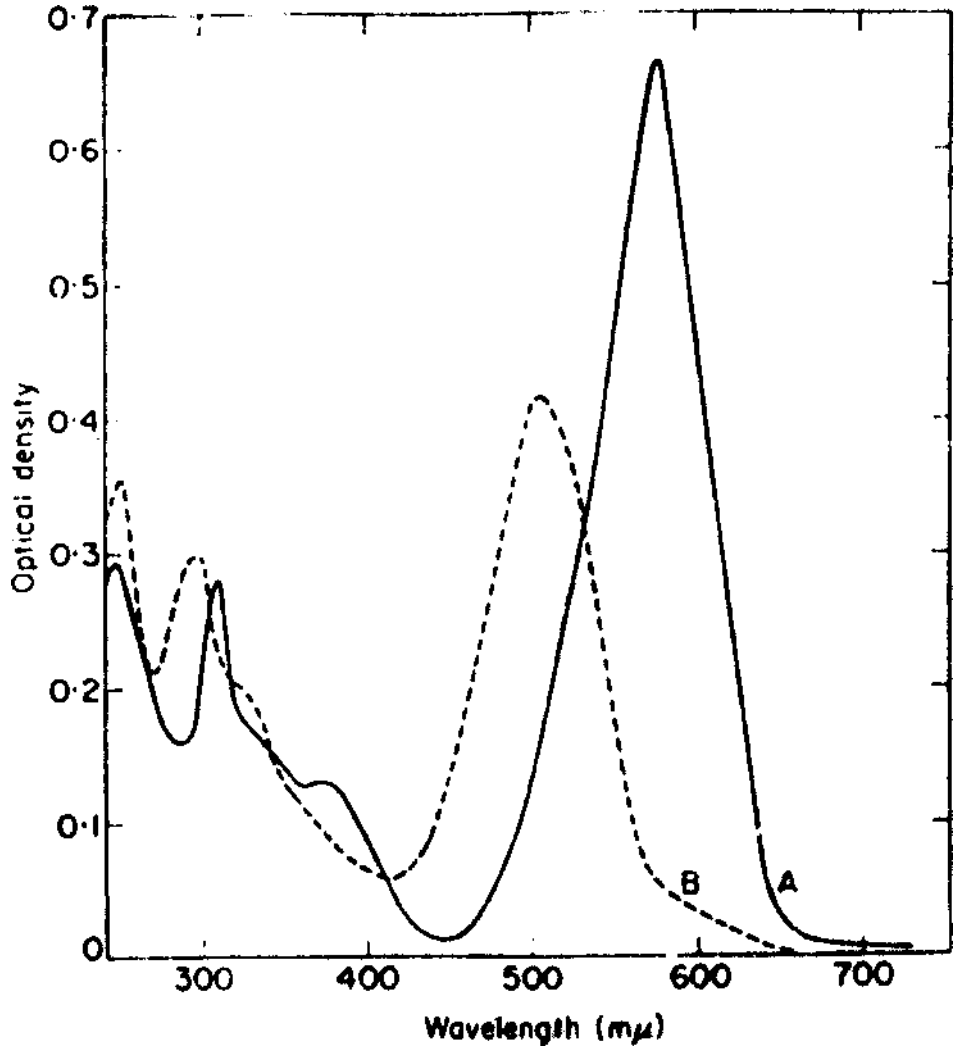


Figure 2. Absorption Spectra of Phycoerythrobilin as a hydrochloride (A) and as free base (B). [Goodwin, p. 189]

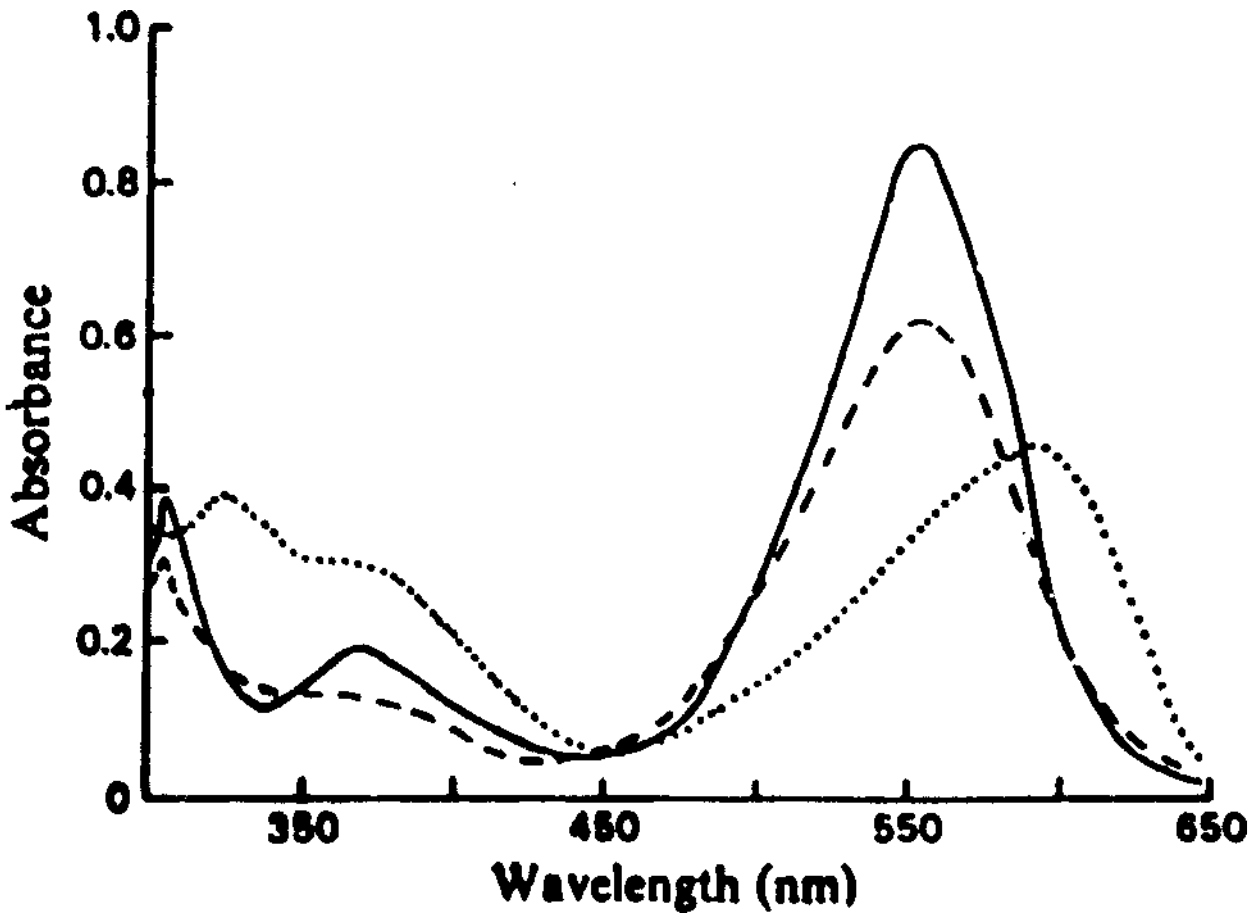


Figure 3. Absorption Spectra of Phycoerythrin (____), Phycoerythrobilin (----), "Purple Pigment" (••••). [P. O'Carra, et al. 1980, p. 305]

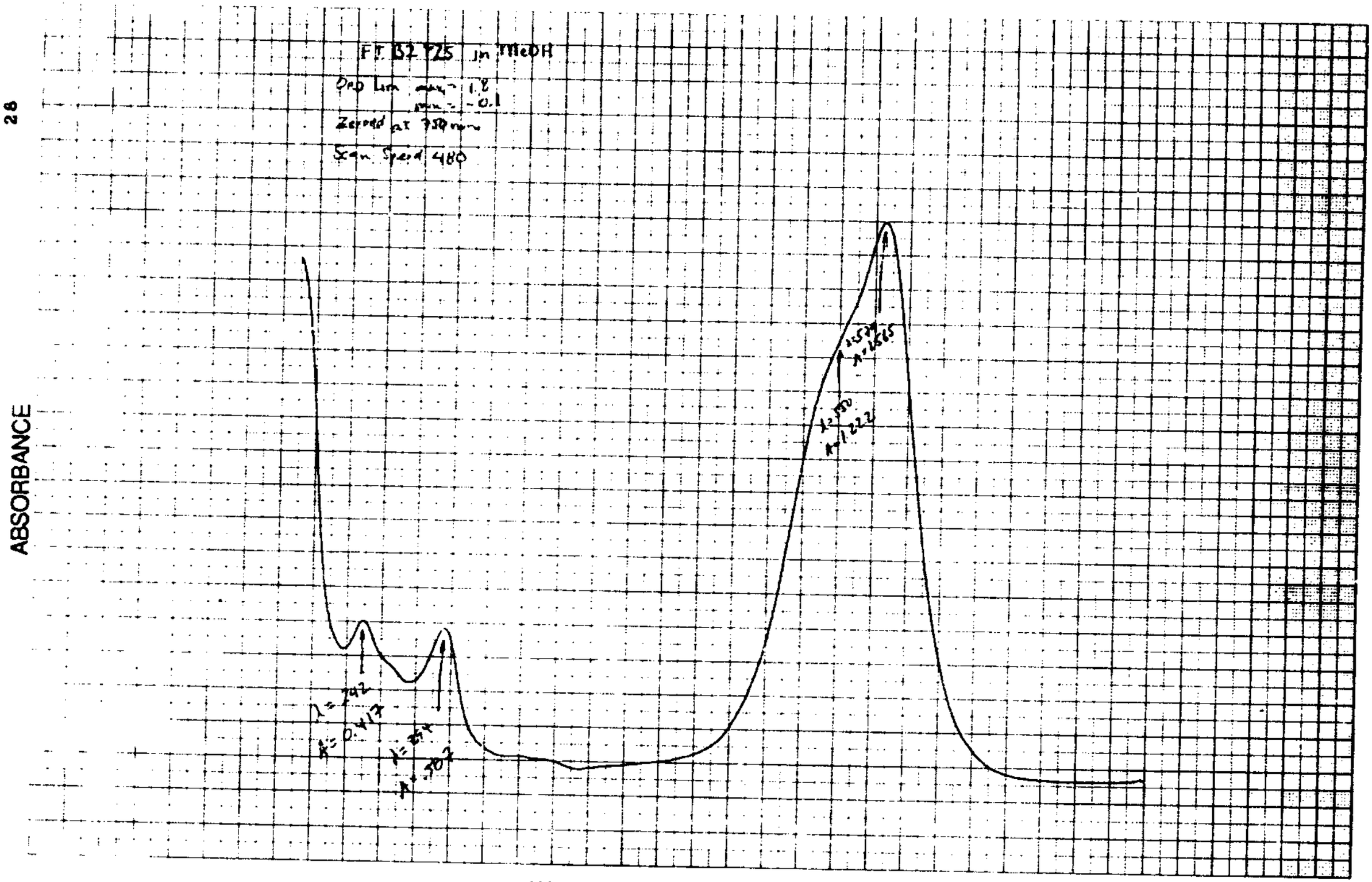


Figure 4. Electronic Spectrum of FIB2

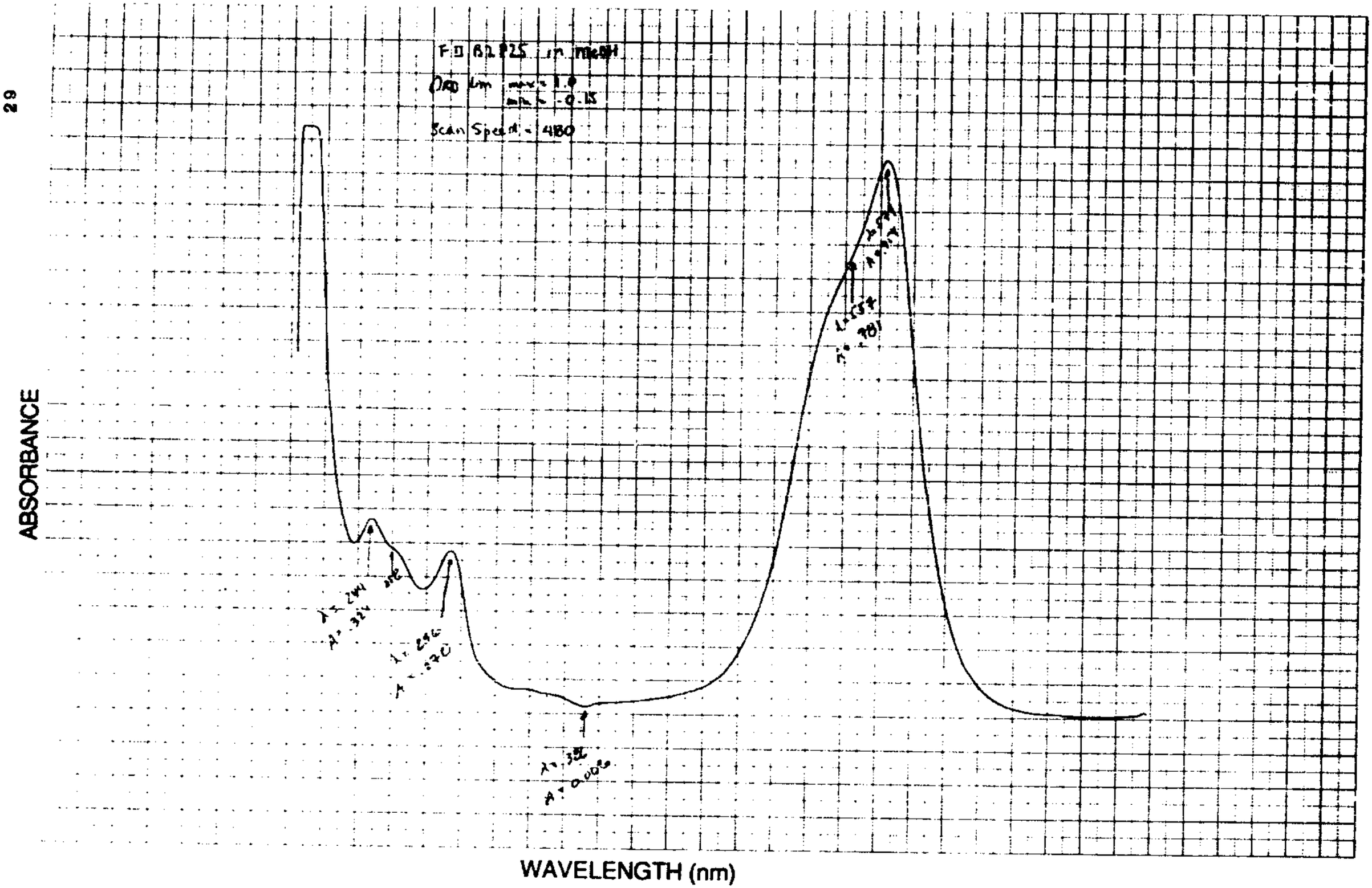


Figure 5. Electronic Spectrum of FIIB2

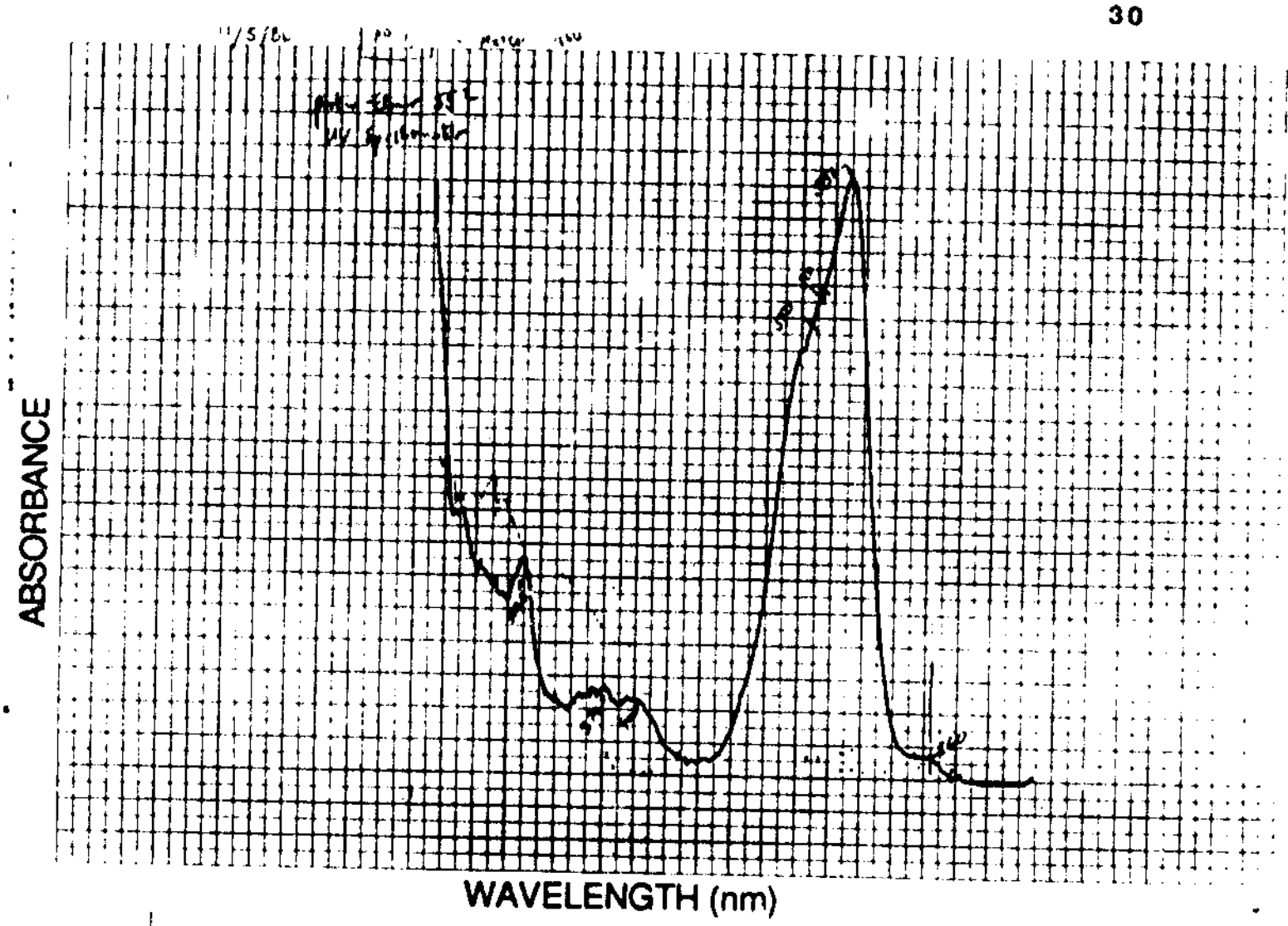


Figure 6. Electronic Spectrum of PP

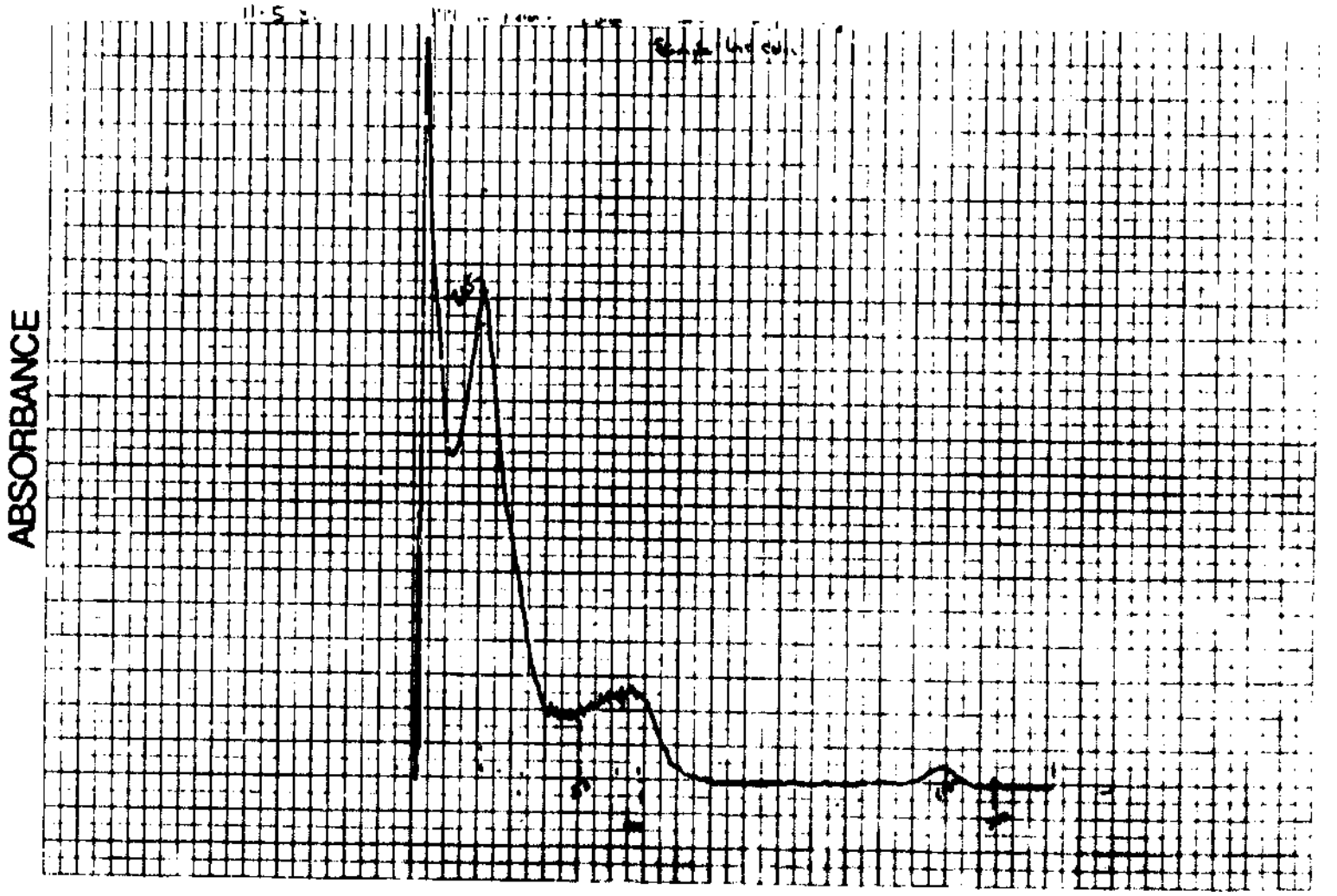


Figure 7. Electronic Spectrum of PP + Bee

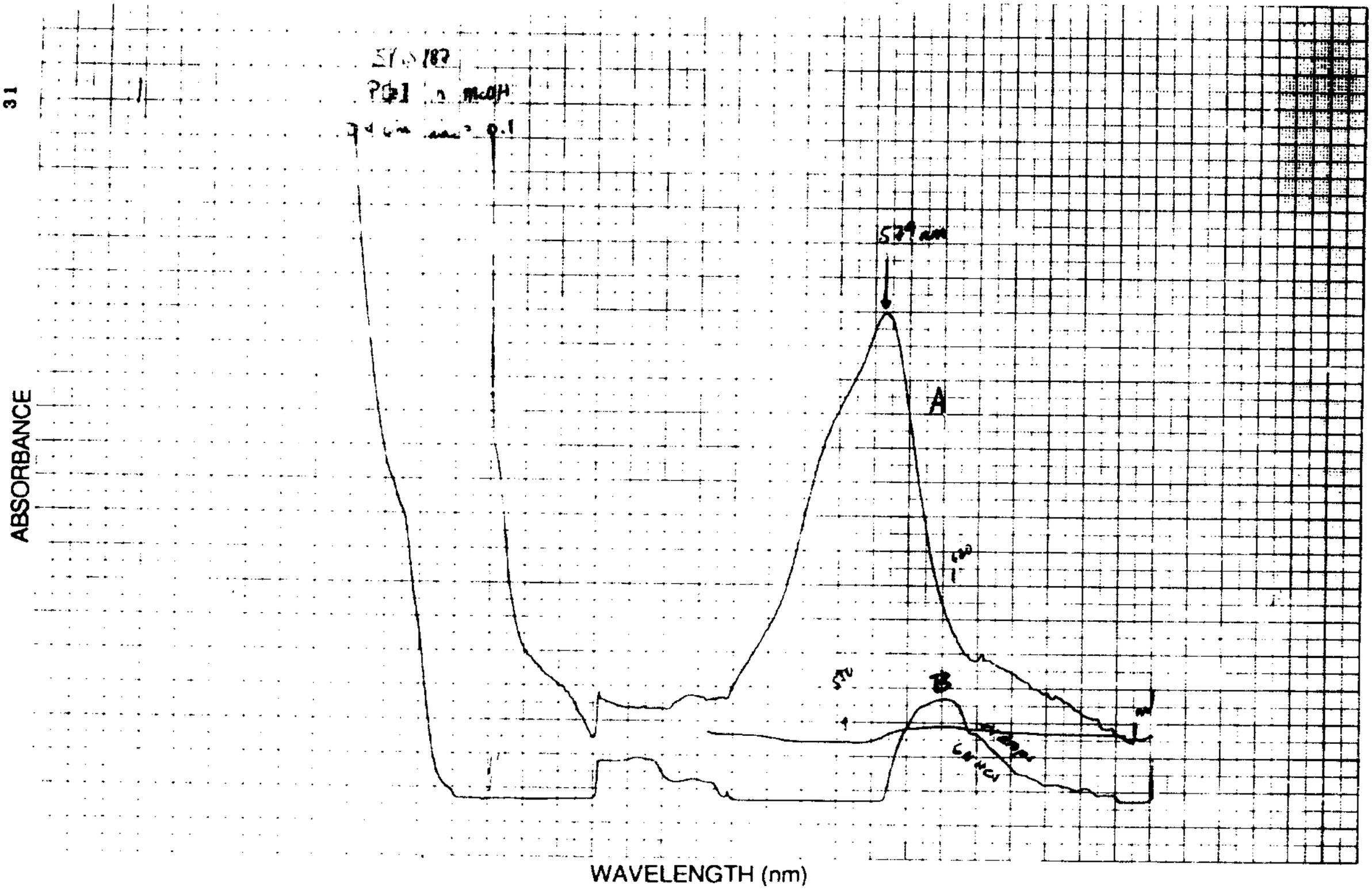


Figure 8. Electronic Spectrum of PP[Z] (A), + 4 drops 6 N HCL (B)

5/15

P ~ 1 mg

~ >8 drops 6N HCl

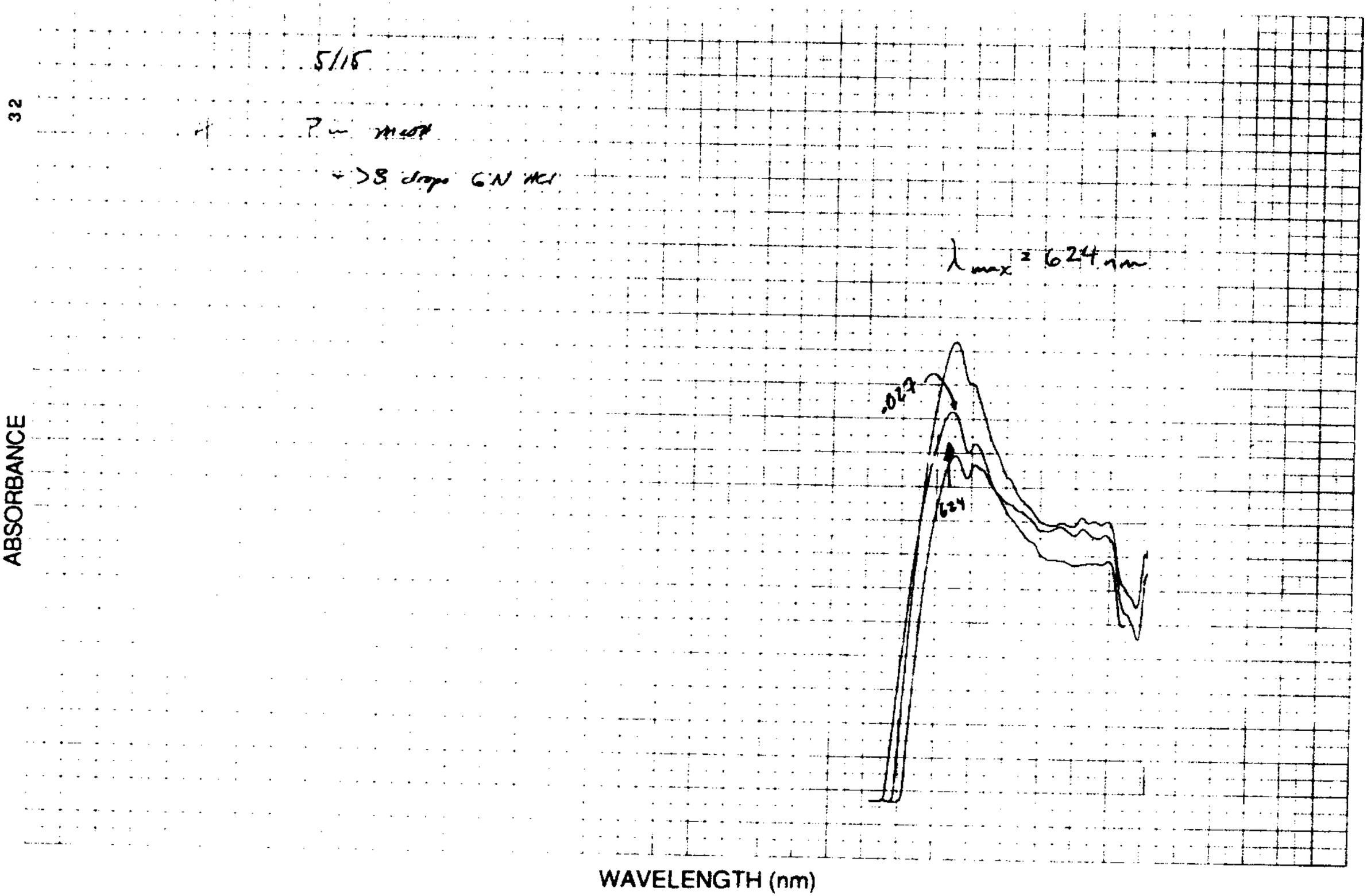


Figure 9. Electronic Spectrum of PP[Z] + Excess 6 N HCl

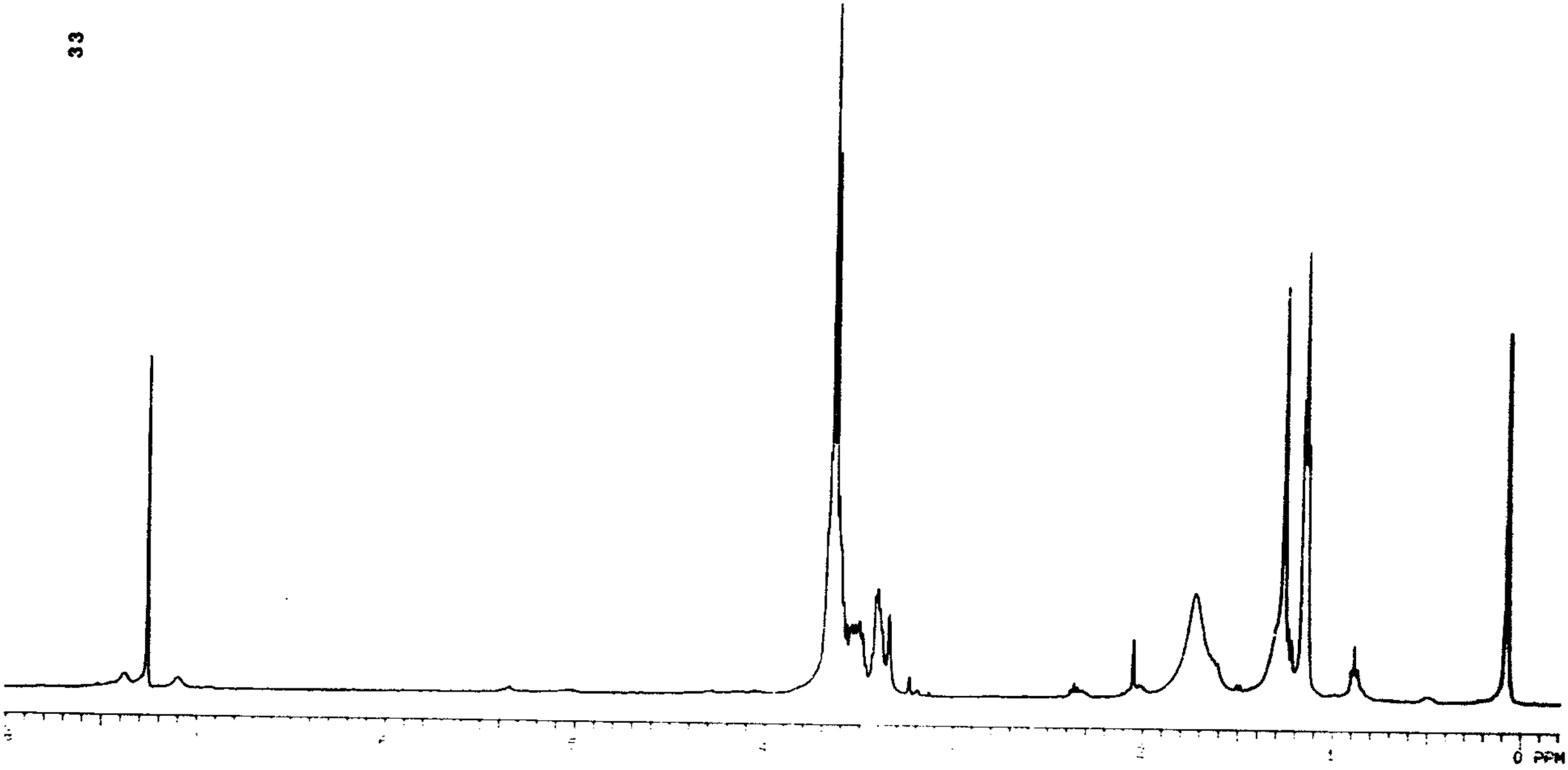


Figure 10. ^{1}H NMR, FIB2.

F2B2 IN 100% CDCL3

EXPS PULSE SEQUENCE: COSY
DATE 94-04-07
SOLVENT CDCL3
FILE H74708

ACQUISITION		DEC. & VT	
TN	1.700	DN	1.700
SW	3635.0	DO	0
AT	0.141	DM	NNN
NP	[illegible]4	DLP	20
[illegible]		[illegible]MO	N
	3		
D	[illegible].500	PROCESSING	
D2	0	RE	0.009
TO	-200	FN	2048
NT	96	AF	0.035
CT	0	MATH	F
PW90	39.3	FN2	2048
D3	0	RE2	0.009
SW2	3635.0	AF2	0.035
NI	512		
BS	16	DISPLAY	
SS	2	SP	-120.8
IL	Y	WP	3635.0
IN	N	VS	90
DP	N	SP2	-120.8
HS	NN	WP2	3635.0
ALOCK	N	SC	15
SPIN	0	WC	215
GAIN	20.0	IS	92
		RFL	120.0
		RFP	0
		TH	5
		SC2	0
		WC2	370
		IHS	1.000
		RFL2	120.0
		RFP2	0
AI IC AV			

Figure 11. COSY (2-D ^{1}H NMR), FIIB2

F1B2 IN 100% CDCL3

EXP3 PULSE SEQUENCE: STD1H
DATE 04-24-87
SOLVENT CDCL3
FILE TEST

ACQUISITION		DEC. & VT	
TN	1.700	DN	1.700
SW	10000.0	DO	ARRAY1
AT	3.200	DN	NNN
NP	64000	DLP	20
PW	33.3	HOMO	N
P1	0		
D1	0	PROCESSING	
D2	0	SE	0.318
TO	500	LB	1.000
NT	1.00E 7	MATH	F
CT	144		
PW90	33.3	DISPLAY	
BS	16	SP	251.5
SS	0	WP	759.3
IL	N	VS	264
IN	N	SC	0
DP	Y	WC	400
HS	NN	IS	2673
ALOCK	N	RFL	5509.2
GAIN	20.0	RFP	2903.3
		TH	26
		INS	1.000

DC

DO ARRAY

INDEX	VALUE	INDEX	VALUE
1	0		

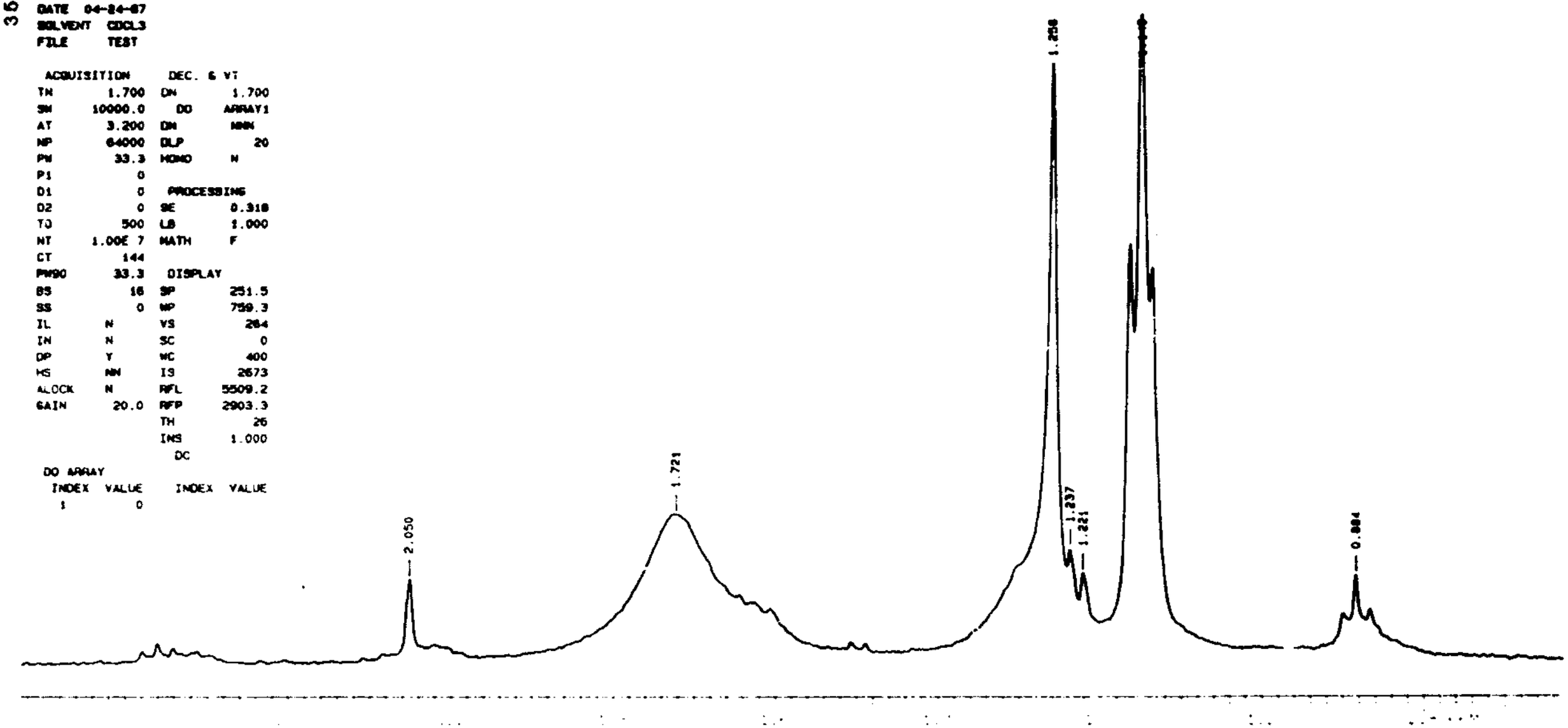


Figure 12. FIB2 0.8 - 2.4 ppm

```
F1B2 IN 100% CDCL3

EXP3  PULSE SEQUENCE: STD1H
DATE  04-24-87
SOLVENT  CDCL3
FILE     TEST

 ACQUISITION     DEC. & VT
TN       1.700  DN       1.700
SW   10000.0    DO    ARRAY1
AT       3.200  DN       YYY
NP       64000  DLP        14
PW        33.3  HOMO      Y
P1           0
D1           0   PROCESSING
D2           0  SB       0.318
TD         500  LB       1.000
NT         128  MATH     F
CT          65
PW90      33.3   DISPLAY
BS          16  SP     -202.9
SS           0  WP     3485.5
IL       N      VS        330
IN       N      SC          0
DP       Y      WC        400
HS       NN     IS       2873
ALOCK    N      RFL    9509.2
GAIN      20.0  RFP    2903.3
                TH         26
                INS     1.000
                  DO

DO ARRAY
 INDEX  VALUE    INDEX  VALUE
   1   -430.2
```

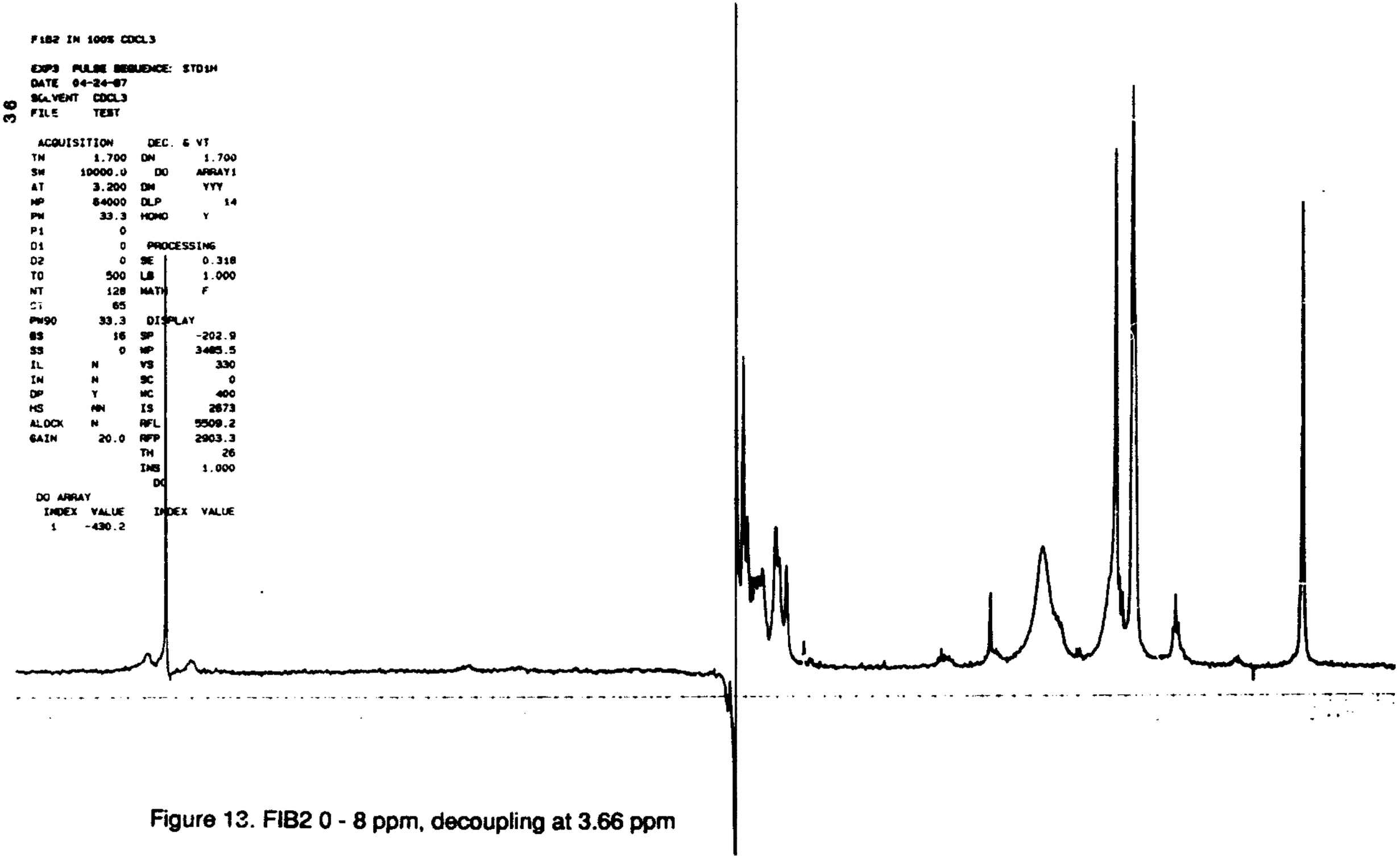


Figure 13. FIB2 0 - 8 ppm, decoupling at 3.66 ppm

FIB2 IN 100% CDCL3

EXPS PULSE SEQUENCE: STD1H
DATE 04-04-07
SOLVENT CDCL3
FILE TEST

ACQUISITION		DEC. & VT	
TN	1.700	ON	1.700
SW	10000.0	DO	ARRAY1
AT	3.200	ON	YYY
NP	64000	DLP	14
PW	33.3	HOMO	Y
P1	0		
D1	0	PROCESSING	
D2	0	SE	0.318
TO	500	LB	1.000
NT	128	MATH	F
CT	85		
PW90	33.3	DISPLAY	
BS	16	SP	251.5
SS	0	WP	759.3
IL	N	VS	383
IN	N	SC	0
DP	Y	WC	400
HB	NN	IS	2673
ALOCK	N	RFL	9509.2
GAIN	20.0	RFP	2803.3
		TH	26
		INS	1.000

DC

DO ARRAY

INDEX	VALUE	INDEX	VALUE
1	-430.2		

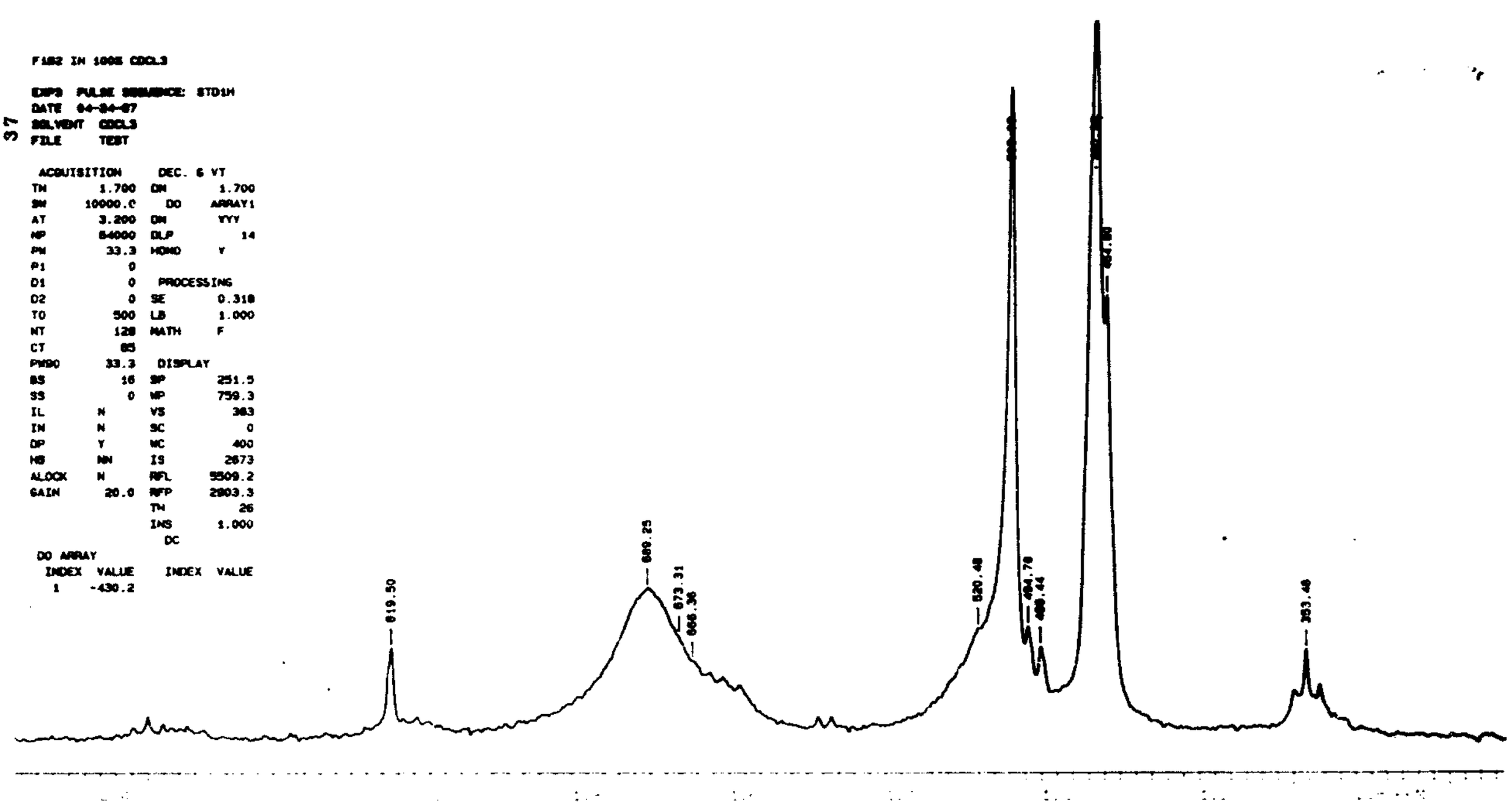


Figure 14. FIB2 0.8 - 2.4 ppm, decoupled at 3.66 ppm

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```
BALAZS RDA9 1349-9-6
SC-28026 DEVILS ADVOCATE
75.7 MG/DMSO C13 APTC
W70013 EH

EXP2  PULSE SEQUENCE: APT
DATE  04-24-97
SOLVENT  CDCL3
FILE     APTC

 ACQUISITION     DEC. & VT
TN       13.000  ON        1.000
SW     34865.8   OO            0
AT       1.830   ON          YNY
NP       128000  DNM         B
PW         14.5  DNP        8400
P1         28.0  DNP        40.0
D1        0.500  HOMO        N
D2      7.00E-3
TD        2000.0  PROCESSING
NT      1.00E 8   EM        0.318
CT         18404  LB        1.000
PW90       14.5  MATH        F
D3      1.00E-2
DLP            0  DISPLAY
FB         18300  SP      -3887.5
BS            64  WP      34865.8
SS             0  VS         1418
IL             N  SC            0
IN             N  WC          250
DP             Y  IS           50
ALOCK          N  RFL      3887.5
GAIN        28.0  RFP            0
                  TM           35
                  DMM       1.000
                    AI
```

Figure 15. ^{13}C NMR FIB2 -20 -> 250 ppm

38

```
BALAZS RDAS 1348-8-6
SC-38026 DEVILS ADVOCATE
75.7 MR/DMSO C13 APTC
M70010 EH

EXP2  PULSE SEQUENCE: APT
DATE  04-24-87
SOLVENT  CDCL3
FILE     APTC

 ACQUISITION     DEC. & VT
TN       13.000  DN        1.000
SW     34888.8   DO            0
AT       1.838   DM          YNY
NP       128000  DMM         S
PW         14.5  DMF         8400
P1         28.0  DMP         40.0
D1        0.500  HOMO        N
D2      7.00E-3
TO       8888.8   PROCESSING
NT      1.00E 8  SI        8.318
CT         18484  LB        1.000
PWSO       14.5  MATH        F
D3      1.00E-3
DLP            0   DISPLAY
FB         18300  SP      -2887.5
SS            64  WP      34888.8
SB             0  VS         1418
IL             N  SC            0
IN             N  WC          328
DP             Y  IS           80
ALOCK          N  RFL       2887.5
GAIN        28.0  RFP            0
                  TH           28
                  INS       1.000
                    AI
```

Figure 15. ^{13}C NMR FIB2 -20 -> 250 ppm

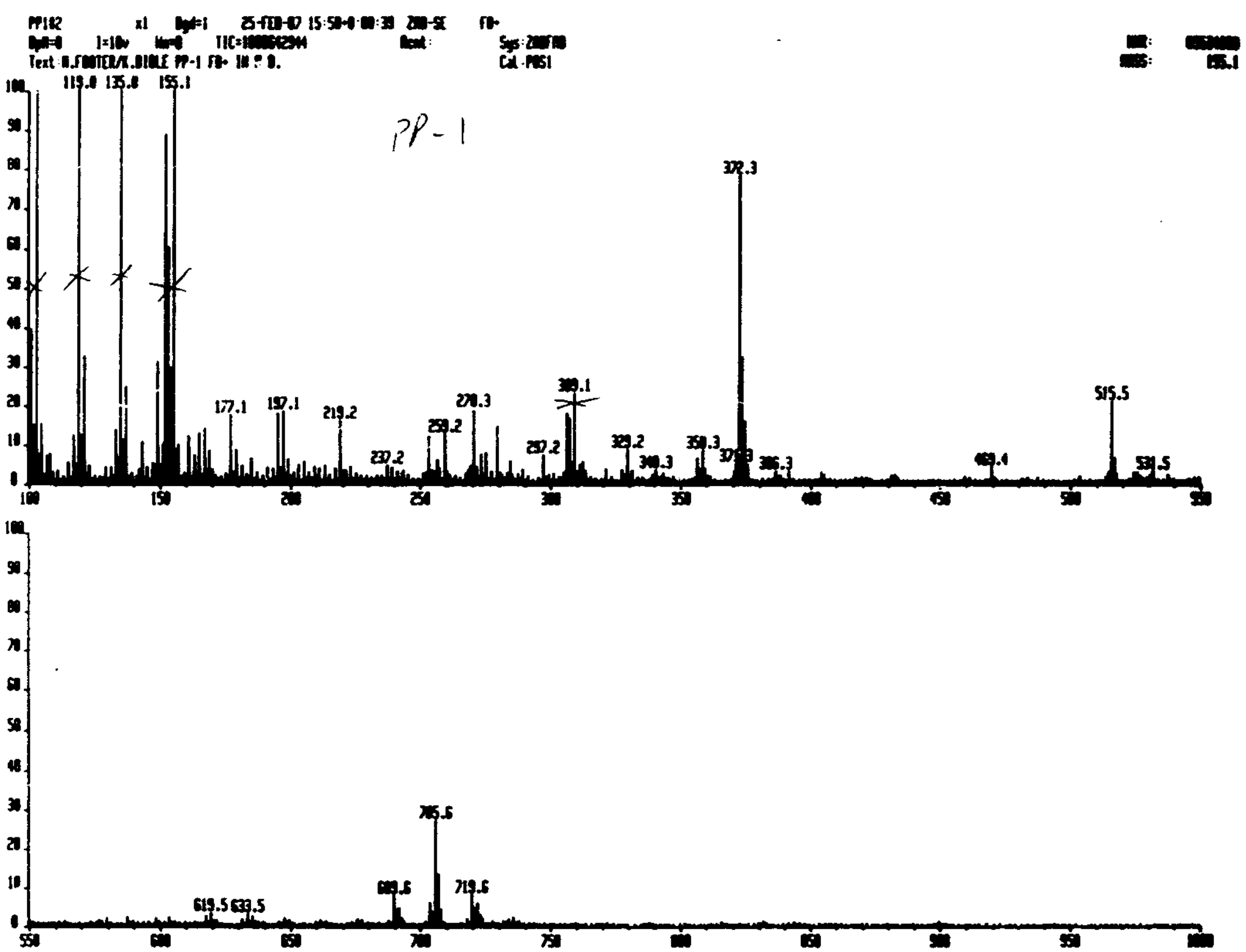

Figure 16. FAB$^+$ Spectrum of PP-1

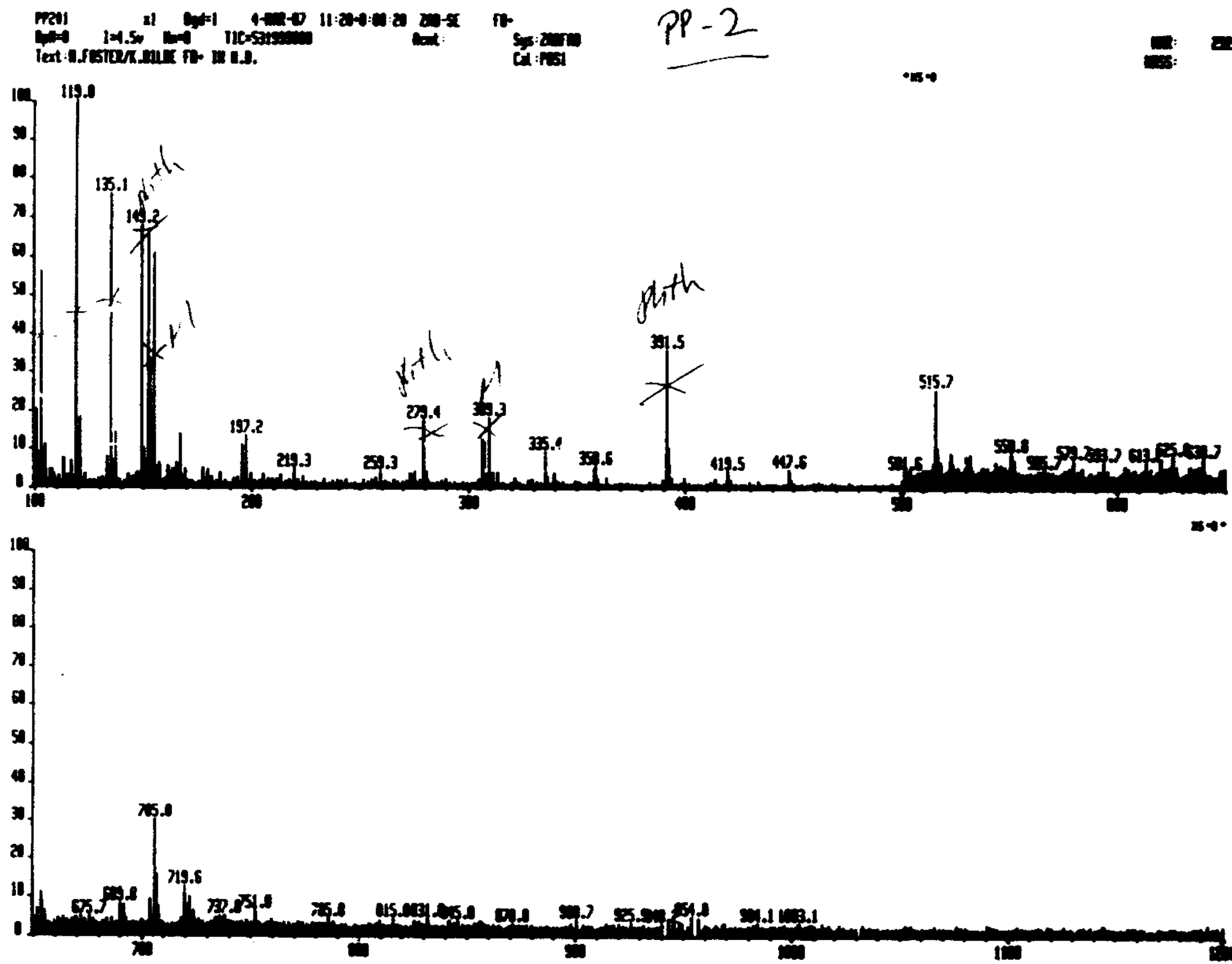

Figure 17. FAB^{+} Spectrum of PP-2

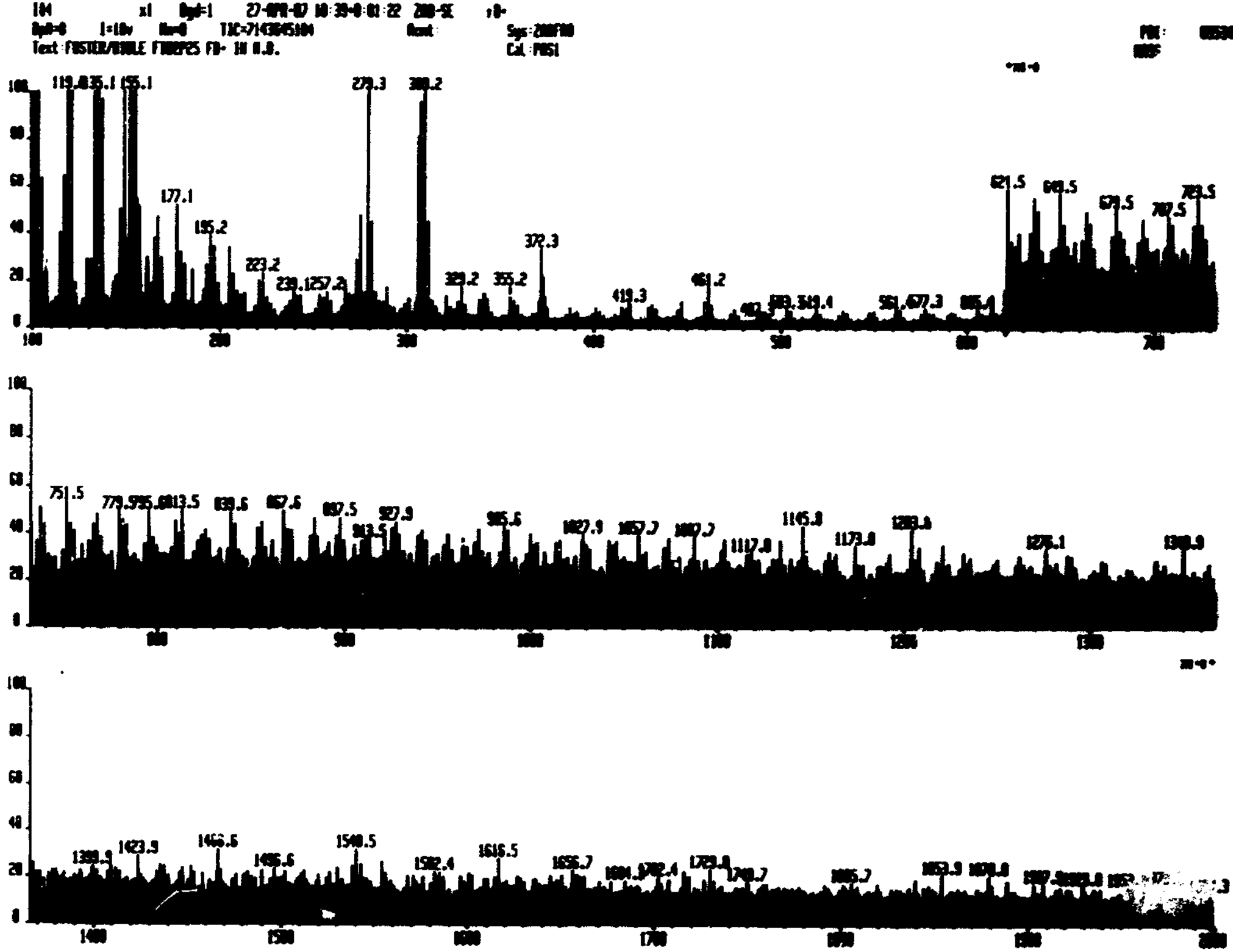

Figure 19. FAB$^+$ Spectrum of FIB2

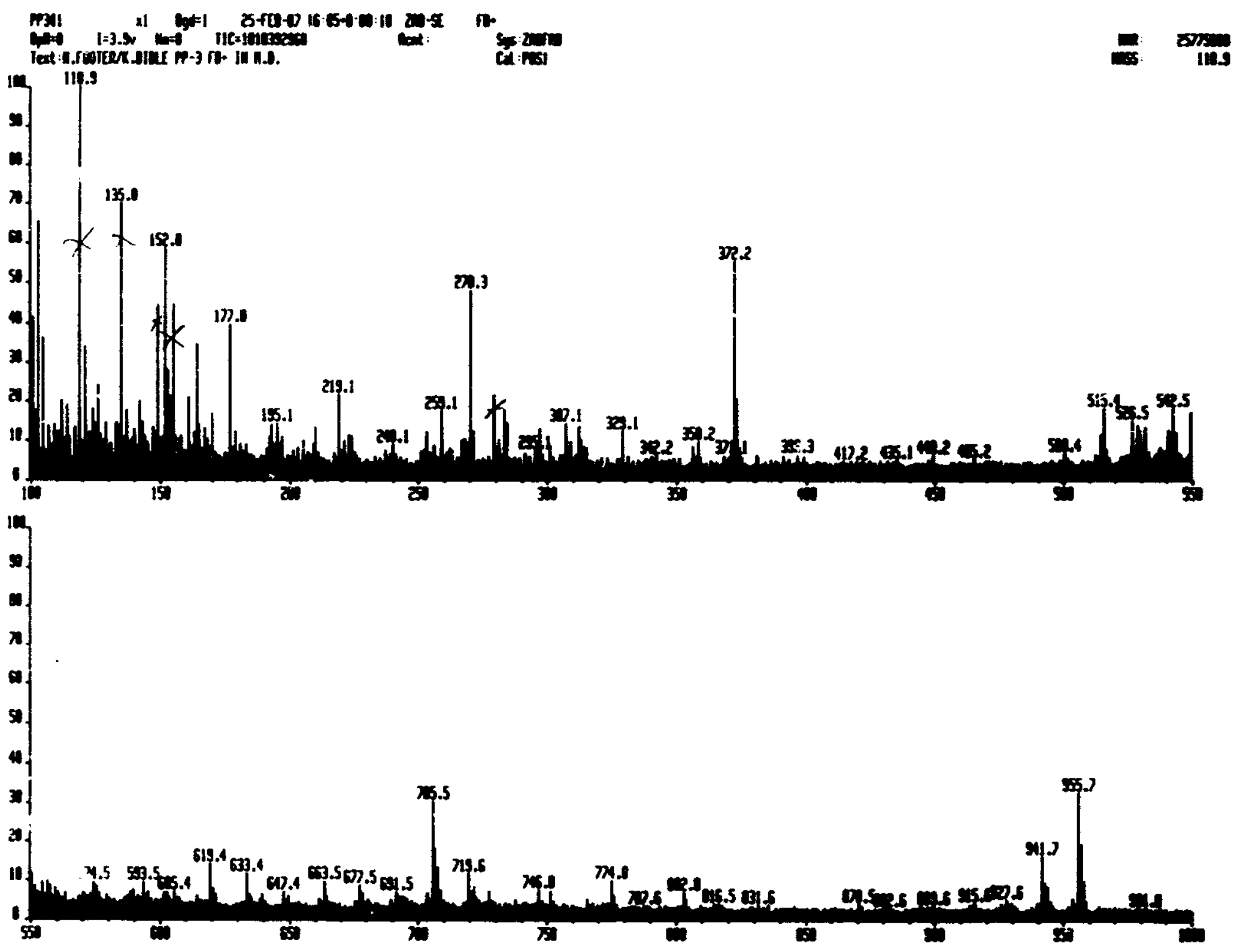

Figure 18. FAB^{+} Spectrum of PP-3

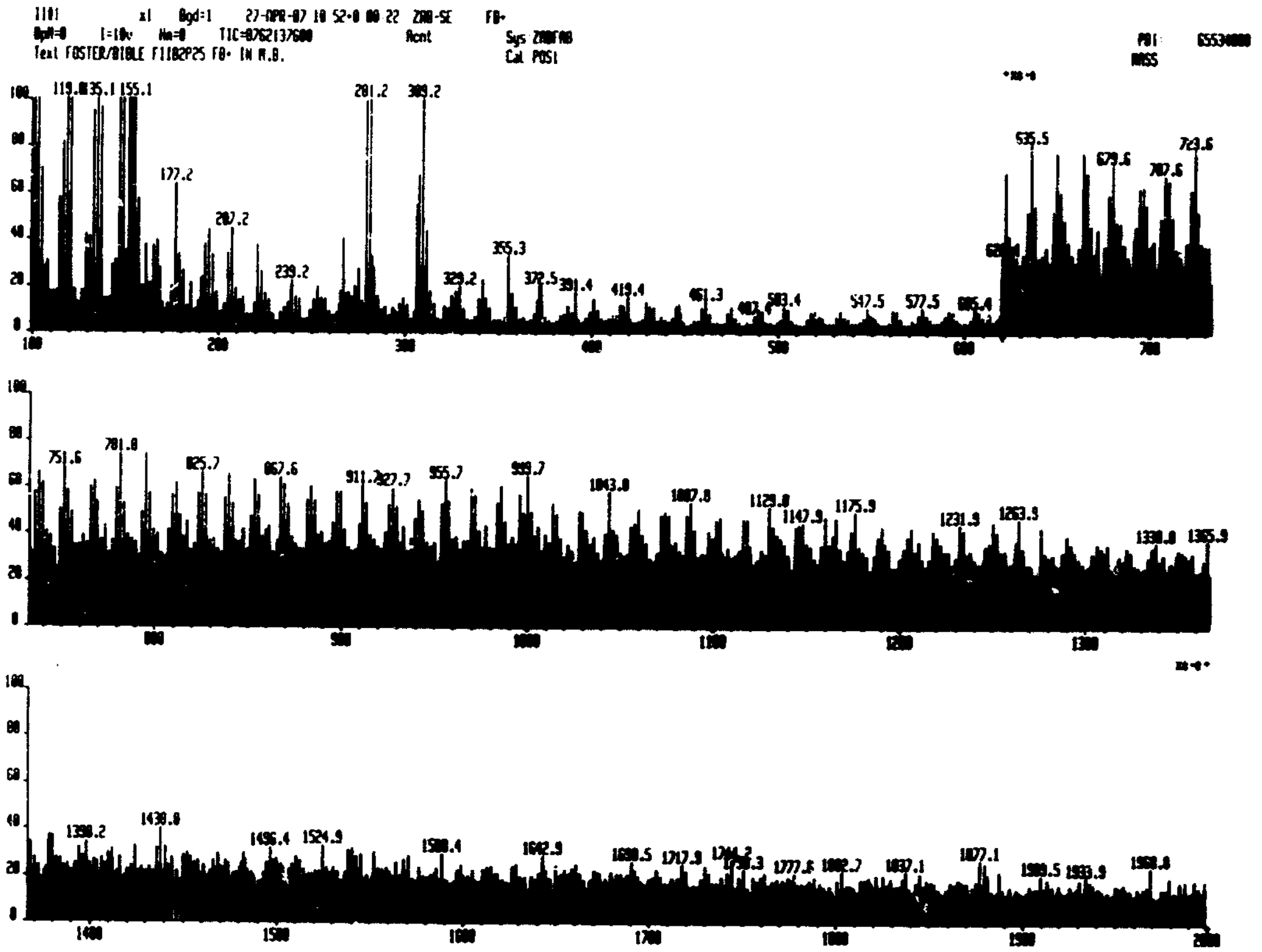

Figure 20. FAB$^+$ Spectrum of FIIB2

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