PN-AAZ-805
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FIRST PROGRESS REPORT

PROJECT Nº 936-5542

INVESTIGATION OF THE CICATRIZANT PROPERTIES OF THE PLANT EXTRACT SANGRE DE GRADO AND ITS POSSIBLE UTILIZATION AS A THERAPEUTIC AGENT.

PRINCIPAL INVESTIGATOR: ABRAHAM J. VAISBERG, Ph.D.

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UNIVERSIDAD PERUANA CAYETANO HEREDIA

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LIMA-PERU.

PERIOD COVERED: SEPTEMBER 1st THROUGH DECEMBER 31st.

Rec'd in SCI: FEB 1 1984

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Description of Research Carried out.-

In the following pages and according to the original chronogram of activities we describe the work that has been done in our laboratory during the last 4 months in relation to the isolation purification and characterization of the alkaloids from Sangre de Grado.

Besides the work done on the chemistry of Sangre de Grado, we have been getting ready the assay systems for the testing of the biological activity and possible carcinogenic or tumor promoter activity of the alkaloid from Sangre de Grado which we think will be fully characterized in the following two months.

Results and Discussion.-

1. Isolation and Purification of the alkaloids from Sangre de Grado.

   1000 mls of the red sap Sangre de Grado extracted from Croton lechleri are treated with concentrated ammonium hidroxide bringing the pH to 11 . The resulting brownish-red viscous mass is then exhaustively extracted with chloroform. The chloroform fractions are then pooled together and reduced in vacuo at 40°C yielding 6.8693 gr of a beige solid.

   This beige solid is boiled for 10 minutes in 150 ml of methanol and the insoluble fraction containing the alkaloids is removed through a hot in vacuo filtration step. A dark orange resin remains with the hot metanol.

   Further separation is accomplished as follows: The whitish solid that resulted from the previous step is swirled in 250 mls of methanol at room temperature. This results in the settling down of a yellow solid to the bottom of the flask and the removal of a white solid -- through decantation of the white methanolic suspension.

2'

This previous step was repeated until the methanol decanted appeared clear.

Finally the yellow solid is recrystalized in Methanol-Dichloromethane (4:1) thus yielding 2.5 gr. of high melting (Melts with decomposition above 300°C) yellow cristals.

The white solid obtained from reducing the methanolic suspension is recrystalized in pure methanol yielding 230 mg of white crystals with a high melting point (Melts with decomposition above 300°C). These white crystals turn yellowish with time.

1000 ml. of sap

↓ alkaline with concentrated NH_4OH

brownish-red viscous mass

↓ extracted with $CHCl_3$

chloroform extraction

↓ reduce in vacuo at 40°C

6.8793 g of a beige solid

↓ boiled in methanol and filtered

↙ insoluble whitish solid

↘ dark orange resin in methanol

↓ swirl at room temperature methanol and decant

↙ yellow solid

↘ white methanolic suspension

recrystalize in $MeOH:CHCL_3$ 4:1

↓ 2.5g of yellow crystals

↓ reduce in vacuo at 40°C

white solid

↓ recrystalize in MeOH

230 mg of white crystals

2. Chromatografic Results.

a. Thin layer chromatography of the yellow and white solid on Silica gel sheets (Kodak 13181 Silica gel with fluorescent indicator, No. 6060) resolved one spot in each case with an Rf value of 0.63 for the yellow solid and 0.58 for the white solid using as eluent Toluene: Chloroform: Methanol (9:2:3).

Both of these spots gave positive reaction to Dragendorff's reagent according to Munier and Machenboeuf.

b. Bidimensional Development on Kieselgel HF254 (type 60) with eluent $CHCl_3$: MeOH 5:1 Also reveals only one spot for both the yellow and white solid.

c. Thin layer Reverse Phase Sheet (C-18) gave no results with the following eluents:

MeOH:	H_2O	7:3
MeOH	H_2O	4:6
Pure MeOH		
MeOH:	H_2O	8:2
MeOH:	CH_3CN	7:3
MeOH:	CH_3CN: H_2O	5:3:2

The results obtained in parts a) and b) are given only to ascertain the purity of the products obtained. However, it is necessary to seek a chromatographic system that will enable us to carry out the alkaloid separation on chromatographic columns, since we regard that such a technique will render a more efficient, reliable and reproducible method.

It is for this reason that further chromatographic systems are being carried out such as Reverse Phase C-18 thin layer chromatography sheets, which up to this time positive results have not been obtained.

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Ultraviolet (Figures 1a, 1 b) and Infrared (Figures 2a, 2b) Spectra of the yellow and white solids obtained in the previous steps show great similarity between both compounds. We still need more information from an NMR and MS Spectra in order to elucidate structure.

We have not been able to obtain an NMR Spectra of our compounds because the two NMR spectrometers available are being repaired.

We have sent samples to the University of Heidelberg in order to obtain the MS Spectra since there is no MS Spectrometer in our country.

FIGURE 1a Ultraviolet spectrum of the yellow crystals purified from Sangre de Grado, the solvent was chloroform.

FIGURE 1b Ultraviolet spectrum of the white crystals purified from Sangre de Grado. The solvent was chloroform.

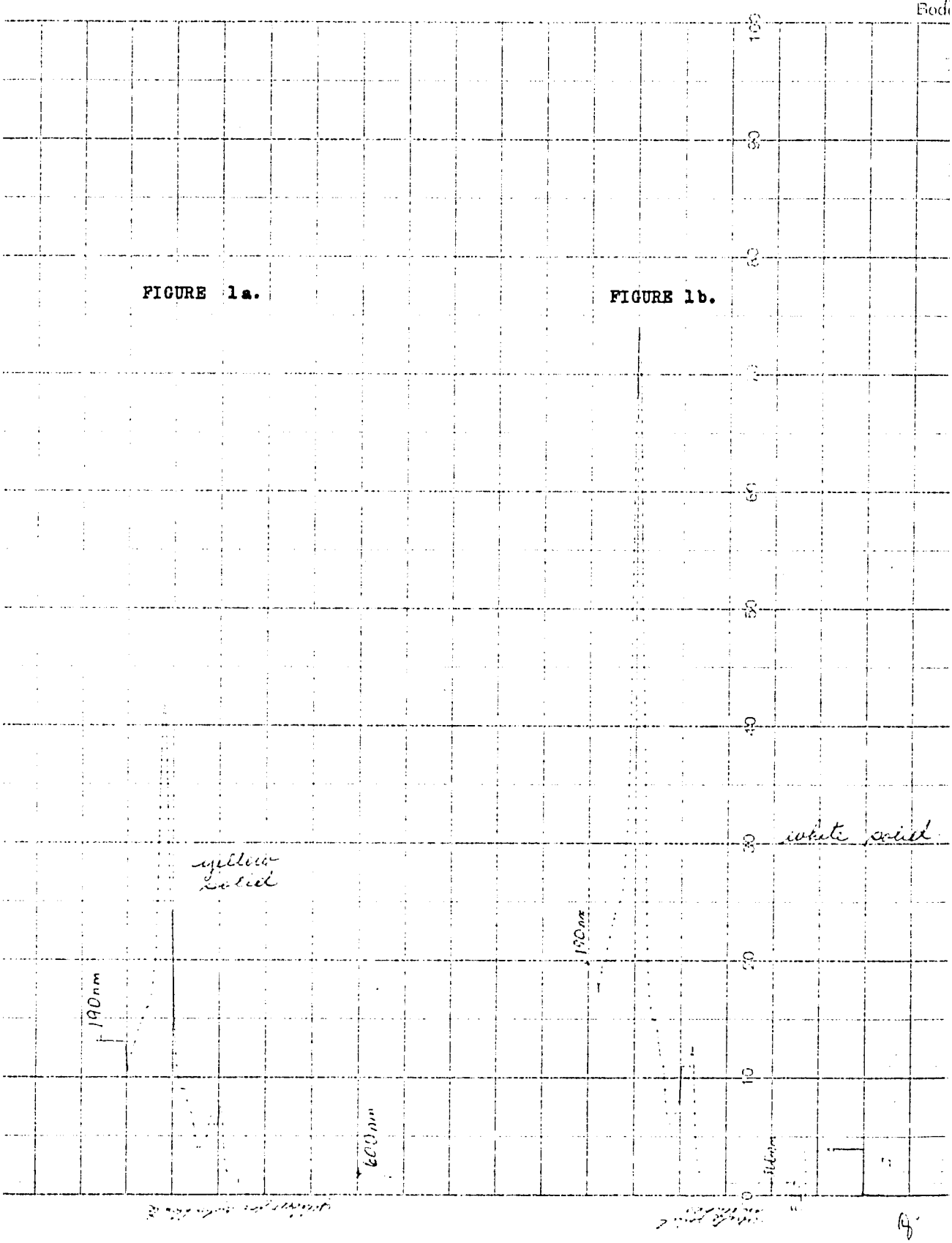

FIGURE 1a.
FIGURE 1b.
yellow solid
190nm
600nm
190nm
white solid
100
90
80
70
60
50
40
30
20
10
0

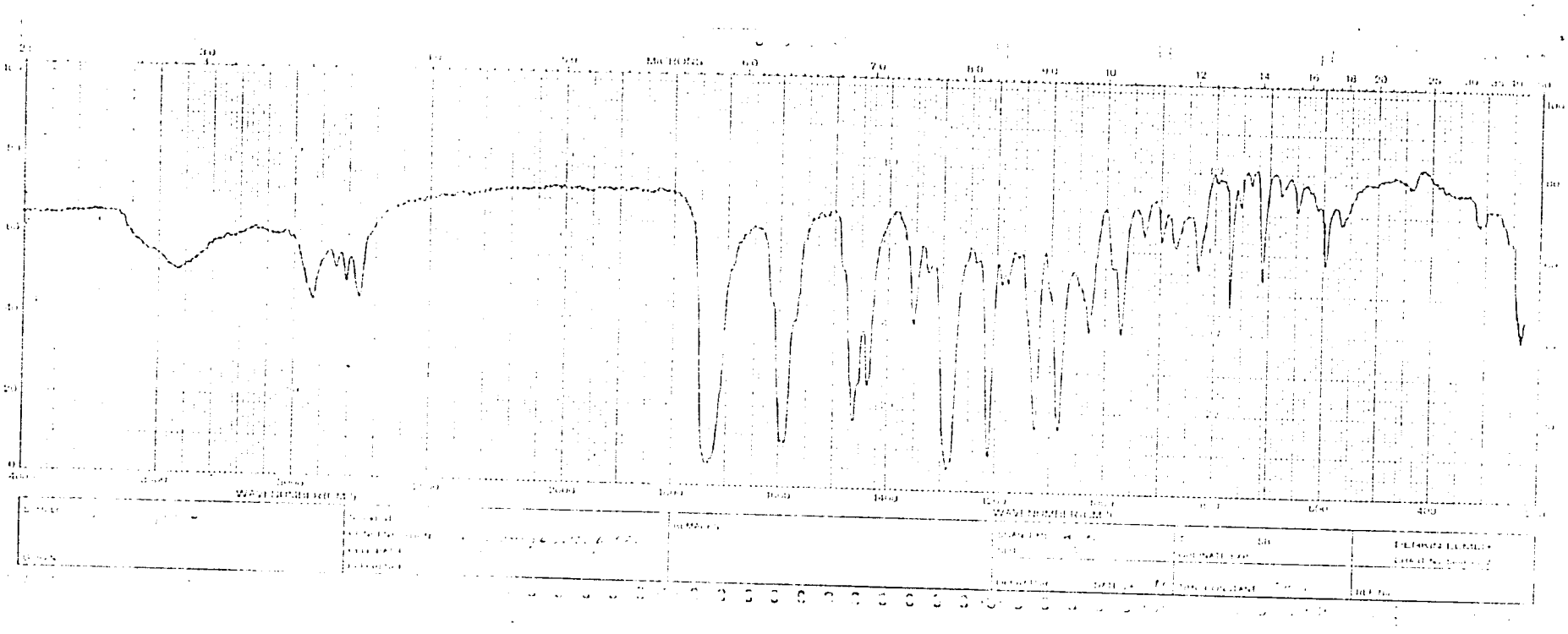

FIGURE 2a. Infrared spectrum of the yellow crystals purified from Sangre de Grado in KBr.

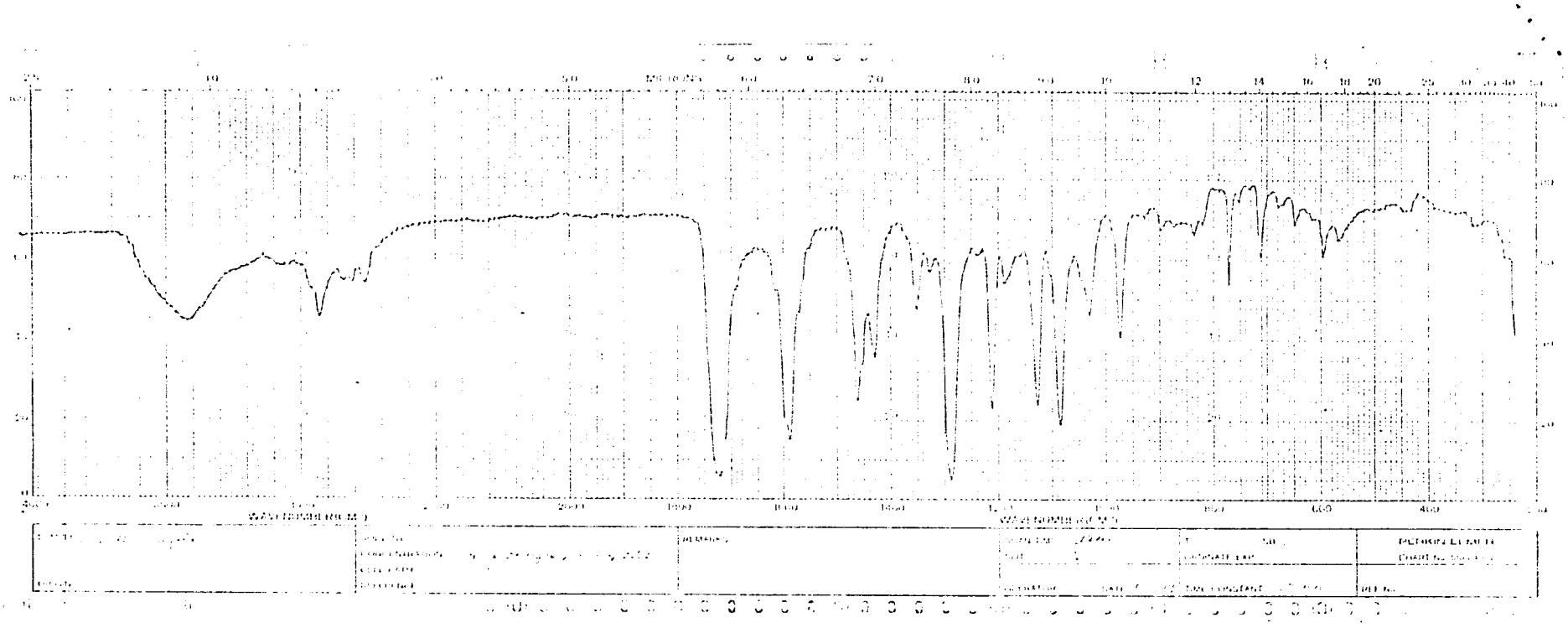

FIGURE 2b. Infrared spectrum of white crystals purified from Sangre de Grado in KBr.

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SECOND PROGRESS REPORT

PROJECT Nº 956 - 5542

INVESTIGATION OF THE CICATRIZANT PROPERTIES OF THE PLANT EXTRACT SANGRE DE GRADO AND ITS POSSIBLE UTILIZATION AS A THERAPEUTIC AGENT.

PRINCIPAL INVESTIGATOR: ABRAHAM J. VAISBERG, PH.D.

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DEPARTMENT OF MICROBIOLOGY
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PERIOD COVERED: September 1st 1983 through June 30th, 1984.

AUG 13 1984

Description of the Research Carried Out.-

In the following pages and according to the chronogram of activities we describe the work that has been done in our laboratory during the last 10 months in relation to the extraction purification, characterization and quantitation of the alkaloids from Sangre de Grado.
We will also describe the experiments on the possible carcinogenic and/or Tumor promoter activities of Sangre de Grado and its active principle.
During this reporting period two trips to the peruvian jungle have been carried out in order to collect Sangre de Grado and the botanical report is being prepared by Dr. Ramon Ferreyra from the Museum of Natural History and will be included in the next report.

Results and Discussion

I. Isolation, Purification, Characterization and Quantitation of the alkaloids from Sangre de Grado.-

1. Extraction, Purification and Quantitation.-

The extraction procedure of the alkaloids from Sangre de Grado is practicaly the same one described in the first progress report with a couple of slight modifications that are the result of successive - trials in order to obtain the best separation possible (Figure 1). This method of extraction and purification although tedious and time consuming, will be the one that we will use in the future since as we will describe in the following section all the other methods - that we have tried did not improve our separation and purification procedures.

The yield of alkaloid mixture obtained from Sangre de Grado varies depending on several factors that we will try to corroborate in future collection trips. These factors are age (size) of the tree and time of the day when the Sangre de Grado is collected. The older the tree and the earlier the collection time (6 a m) the higher the concentration of alkaloid mixture in the extract. Our yields oscilated between 1.69% and almost 0% of alkaloid mixture in Sangre de Grado. From this alkaloid mixture two different solids could be separated: A yellow cristaline solid and a white amorphous solid, being the former the more abundant in the mixture.

Thin layer chromatography on Silica gel 60 F-254 (Merck Nº 5539) of

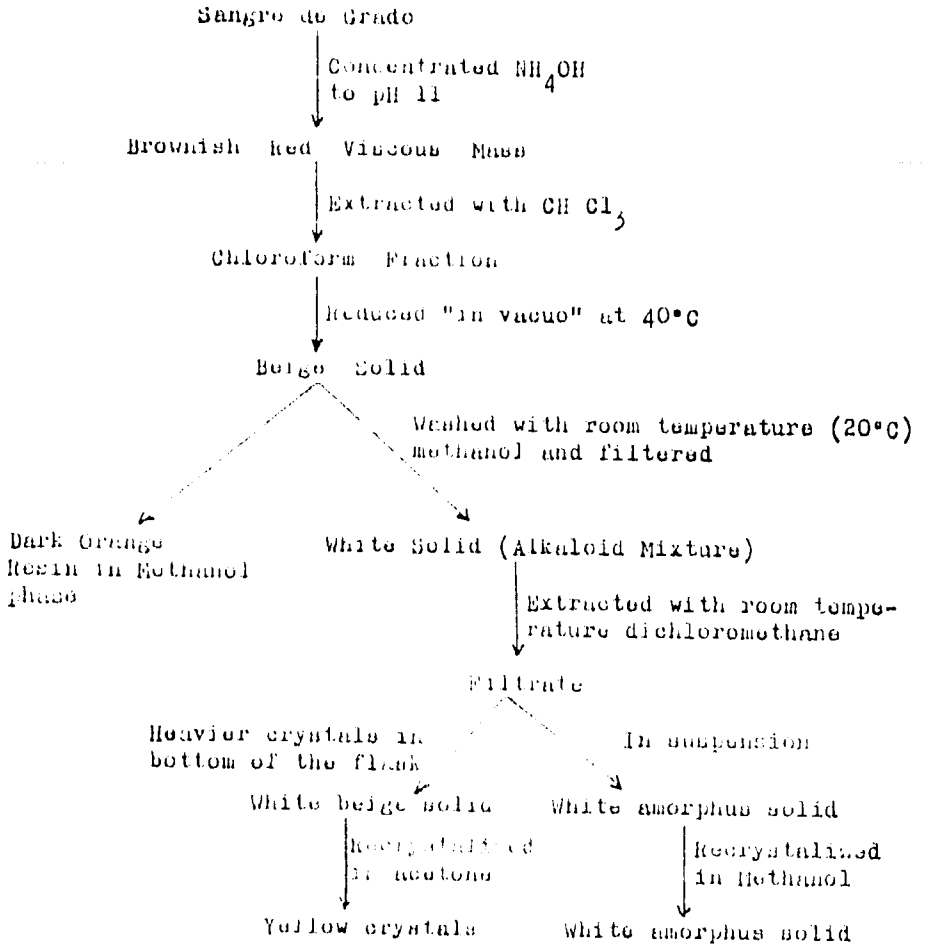

FIGURE 1.- Extraction and Purification Procedure of the alkaloid from Sangre de Grado.

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the two solids and the resin [illegible] Dichloromethane : Methanol (5:1) gave the following [illegible]

- The resin gave three spots [illegible] 0.30 and 0.13 respectively and which fluoresced when [illegible] long wave UV light (366 nm).
- The yellow crystals gave [illegible] spot with an rf of 0.485, which fluoresced when exposed to [illegible] UV light (254 nm) and gave a positive reaction with the [illegible] reagent according to Munier and Machenboeuf.
- The white amorphus solid [illegible] spots which fluoresced when exposed to short wave UV light [illegible] from this spots the ones with an rf of 0.[illegible] and [illegible] the other three with rfs of 0.28, 0.19 and 0.05 [illegible] in trace amounts.
- From all this spots [illegible] rf of 0.4[illegible] gave a positive reaction with the [illegible] according to Munier and Machenboeuf.

2. Separation and [illegible] and Medium Pressure Liquid Chromatography.

Trying to [illegible] column and medium [illegible] results [illegible]

a. [illegible]

[illegible]

[illegible]

[illegible]

[illegible]

Eluent

[illegible]

[illegible]

[illegible]

[illegible] ml NH_4OH

[illegible] ml NH_4OH

[illegible] ml [illegible]

[illegible] the eluent [illegible]

[illegible] acidic with [illegible]

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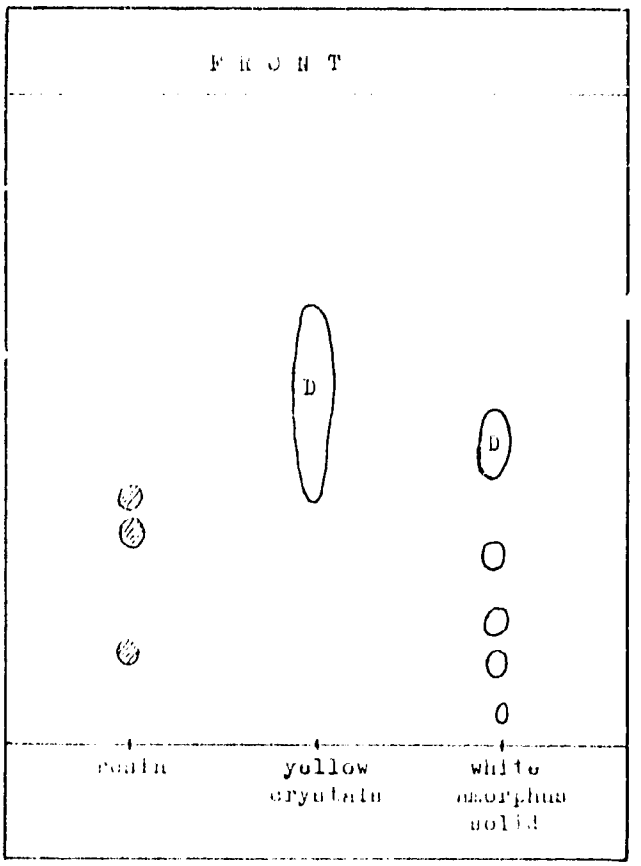

D - Dragendorff positive

Figure 2.- Thin layer chromatography of the alkaloid and resin obtained during the extraction of Sangre de Drago

TLC was performed on Silica Gel 60 F 254 using as eluent a mixture of dichloromethane, Methanol (9:1).

The resin spots fluoresced only when excited with long wave UV light of 366 nm (◍) and all the other spots with short wav. UV light of 254 nm (○).

rfs for the resin spots were: 0.36, 0.30 and 0.15 respectively

rf for the yellow crystal was 0.485

rfs for the white amorphus solid were: 0.45, 0.38, 0.19, 0.12 and 0.05 respectively.

Best Available Document

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acid since on TLC this would favor separation. Later on the eluent was made basic in order to help remove alkaloid substances from the stationary phase. If no basic fraction is used, the alkaloid content tends to remain impregnated on the stationary phase.

62 fractions were collected the volume of each of this fraction oscilated between 10-60 ml. Each fractions was then chromatographed on Silica gel 60 F 254 thin layer plates using dichloromethane: Methanol (3:1) made acid as eluent.

The results are shown in Figure 3: the first 50 fractions gave one spot with equal rf values of approximately 0.48. This result indicate poor separation and thus regards the technique as inefficient.

b. Medium Pressure Liquid Chromatography.-

A medium pressure column was used with the following characteristics:

Silica gel 60 as stationary phase.

The dimension of the stationary phase was 25 x 2 cm.

The eluent used in this system was dichloromethane: Methanol (3:1)

The detector used was UV 254 nm lamp.

Sample: 4 mgr of the alkaloid mixture.

The graph shown in Figure 4 was obtained showing no separation. The graph gives the times at which the substances are expelled and detected with the UV light of 254 nm. At 60 seconds one single peach was obtained showing that everything is expelled with no separation. The column was run for an additional 4 hours to make sure that nothing remained in the column.

c. Reverse Phase Chromatography.-

Reverse phase C-8 was attempted to seek for a system that could be used on Medium Pressure Column with Reverse C-8 Stationary Phase. The following systems were used unsuccesfully.

Methanol: H_2O (4:6)

Methanol: H_2O (8:2)

Methanol pure

Methanol: CH_3CN (7:3)

Methanol: CH_3CN: H_2O (5:32)

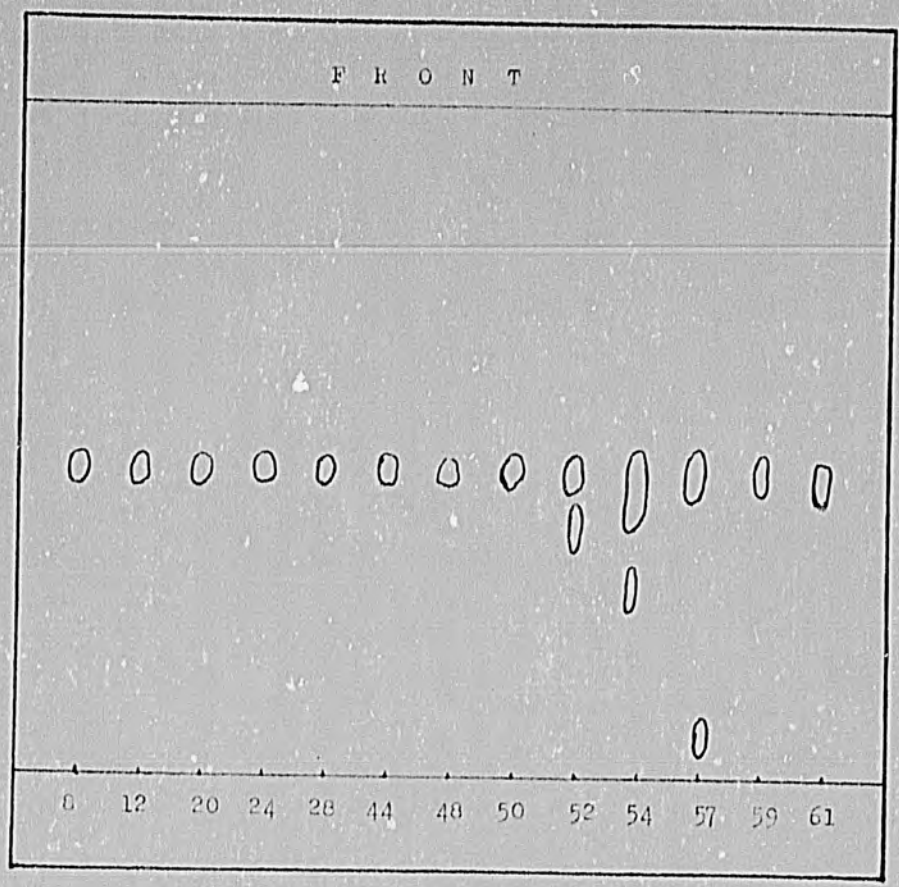

FIGURE 3.- Thin layer chromatography of representative fractions eluted from the dry column. The system was Silica gel 60 F254 Merck and the eluent was dichloromethane: Methanol (3:1) made acid with acetic acid.

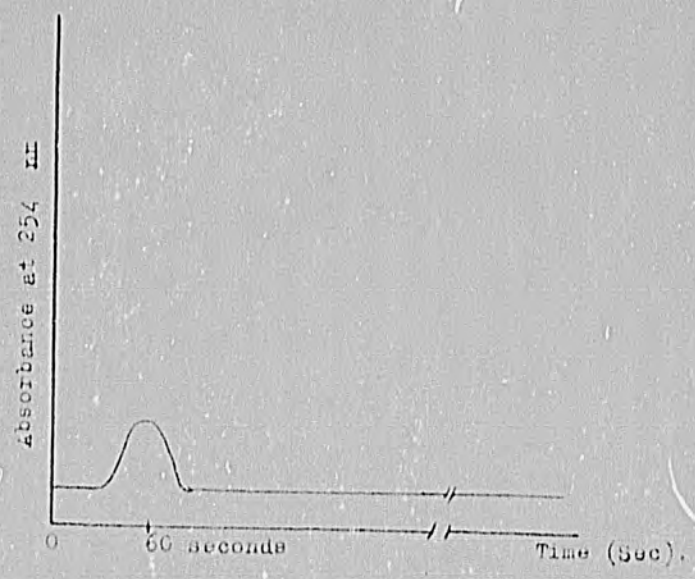

FIGURE 4.- Elution pattern of the crude alkaloid mixture from Sangre de Grado from a Silica gel 60 Medium Pressure Column of the following characteristics:
Stationary phase Silica gel 60
Dimension: 25 x 2 cm.
Detector: UV 254 nm lamp
Eluent: Dichloromethane: Methanol (3:1).
Column was run up to 4 hours.

Since in all cases the sample wouldn't run, no attempt was made to run a Medium Pressure Column with reverse C-8 Stationary Phase.

3. Characterization of the alkaloids from Sangre de Grado.

The information we have accumulated on the yellow alkaloid crystals obtained during the purification procedures of Sangre de Grado is in agreement with Taspine being the identity of these crystals.

The ultra violet spectrum (Figure 5 A) of the hydrochloride of the yellow crystals is identical to the UV spectrum (Figure 5B) of a reference sample provided by Dr. Norman R. Farnsworth from the College of Pharmacy, University of Illinois.

The yellow alkaloid crystals showed an UV spectrum with absorption maxima similar to the ones reported previously by G. Persinos, R. Blomster, D. Blake and N. Farnsworth (J. Pharmaceutical Sciences - (1979) 68: 124-126) and Shamma and Moniot (Chem Commun (1971), 1965).

The mass spectra performed by Dr. Bernhard Tauscher at the Organic - Chemistry Institute of the University of Heidelberg showed a molecular weight of 369 which is in agreement with the molecular weight reported for Taspine (Persinos et al (1979) J. Pharm. Sci. 68: 124 - 126).

An NMR spectrum of the yellow alkaloid crystals from Sangre de Grado shown in Figure 7 in agreement with that of Taspine reported in the literature (M. Shamma and J. L. Moniot Chem. Commun (1971, 1065).

The ultraviolet spectrum of hydrochloride of white amorphus solid from Sangre de Grado (Figure 6) was also identical to the reference sample of Taspine-HCl provided by Dr. Farnsworth.

No NMR spectrum of this white amorphus solid has been obtained yet because of its low solubility in the necessary volume for the determination.

The NMR Spectrometer used was a Bruker WP 80-FT.

4. Preparation of Taspine Hydrochloride.-

HCl gas was passed through a chloroform solution of Taspine (yellow alkaloid crystals) until no further precipitation was observed. The alkaloid hydrochloride was then removed by filtration and washed with chloroform several times until a white solid was obtained which was then dryed in vacuo at 70 C.

An identical procedure was followed for obtaining the hydrochloride of the white amorphus solid.

FIGURE 5.- Ultraviolet Spectra of the hydrochloride of the yellow crystals from Sangre de Grado (5A) and of the reference sample of Taspine hydrochloride provided by Dr. -- Norman R. Farnsworth (5 B) Both spectra were run in H_2O.

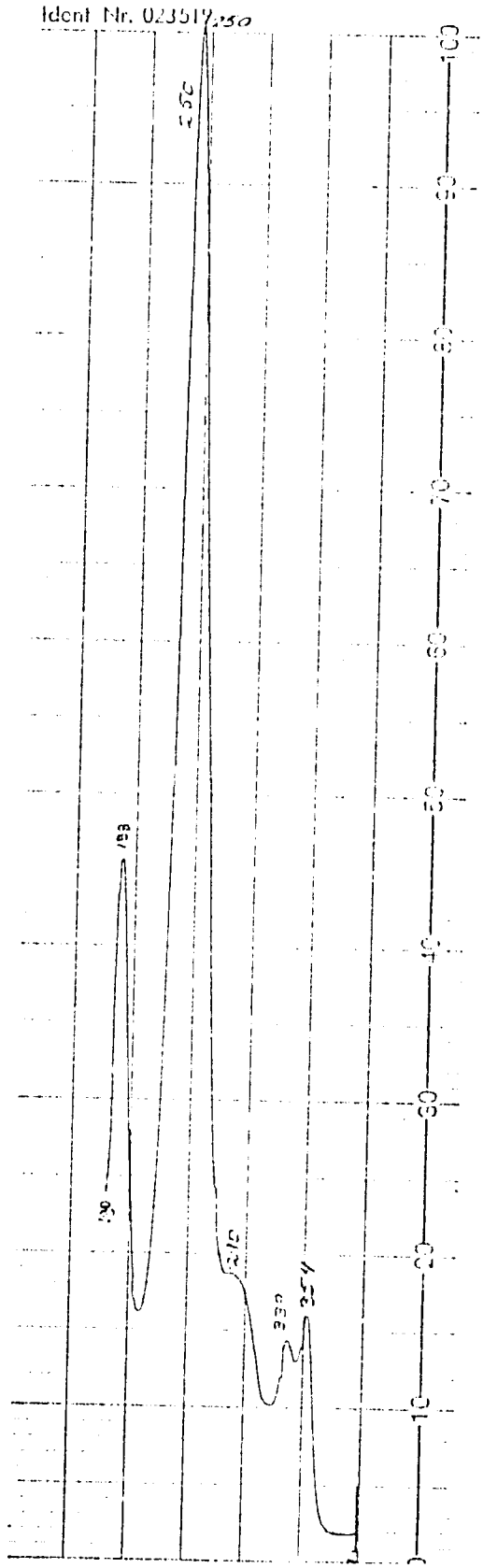
Ident Nr. 023519
250
250
100
90
80
70
60
50
40
30
20
10
0
190
193
210
330
354

FIGURE 5 A

21

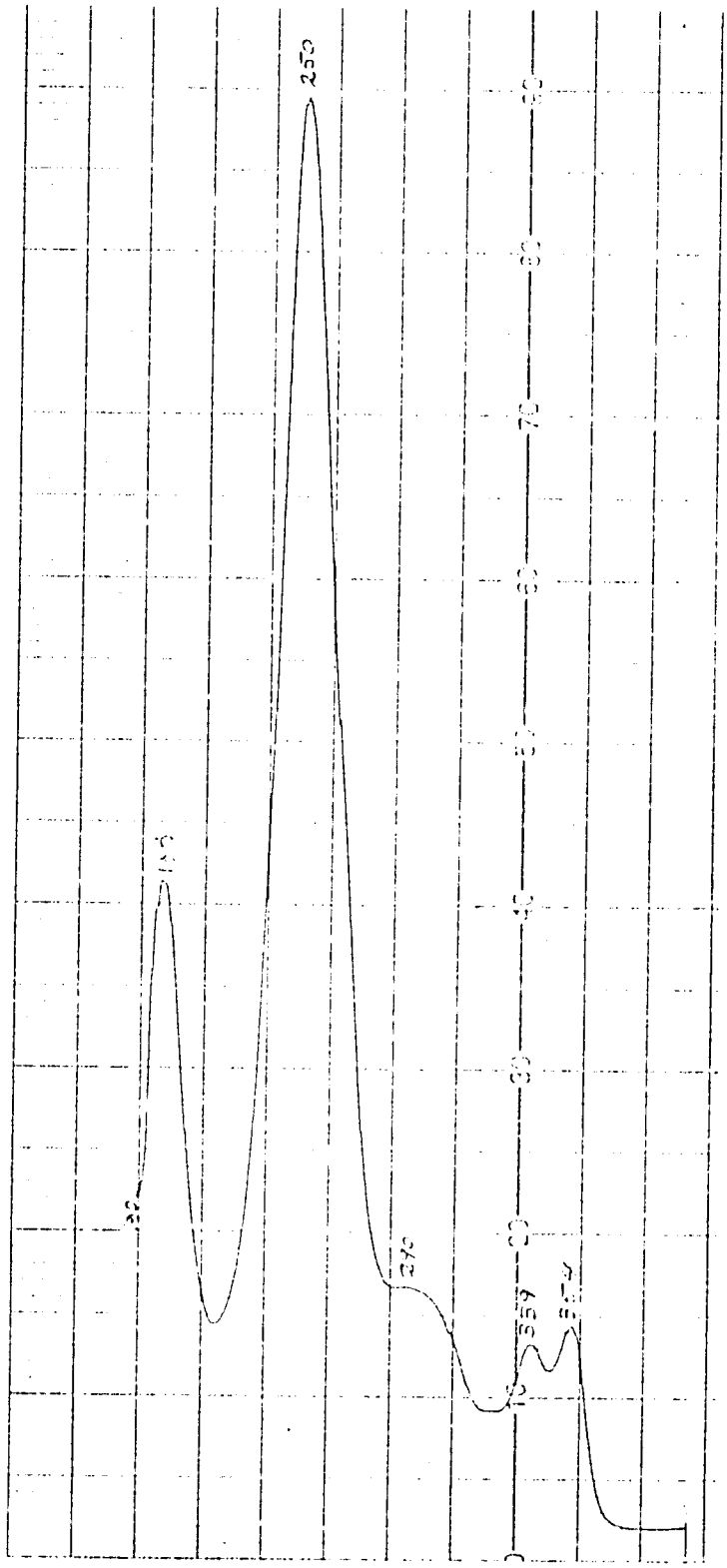

FIGURE 5 B

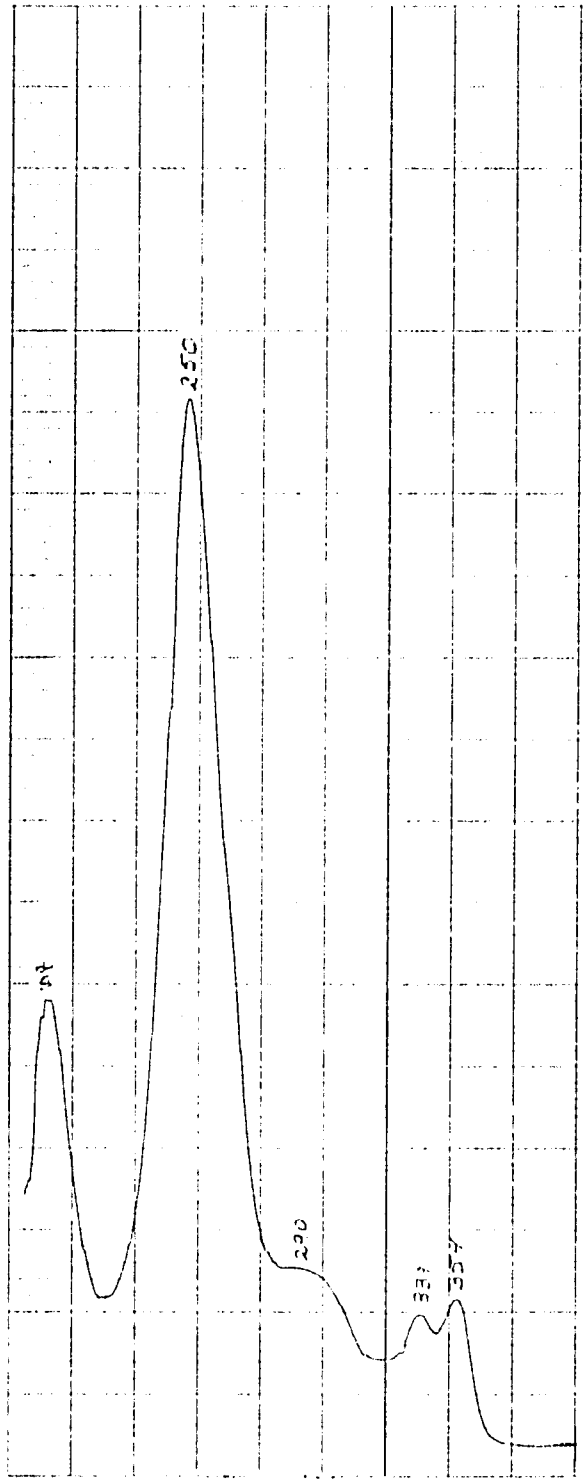

FIGURE 6 UV Spectrum of the Hydrochloride of the White Amorphus Solid from Sangre de Grado in H_2O.

FIGURE 7.- NMR Spectrum of the yellow crystals obtained during the purification procedure of Sangre de Grado.

·S1· 10·000 30
·PP

ADDRESS	FREQUENCY	PPM	INTEGRAL	INTENSITY
680	658.955	5.236	560.160	282.945
720	650.161	6.127	1116.064	338.416
1001	588.356	7.356	1501.762	239.840
1030	581.978	7.276	3490.550	1353.136
1041	579.559	7.244	1014.918	197.986
1067	573.640	7.173	2171.536	310.165
2089	349.055	4.363	295.536	39.328
2132	328.600	4.107	13312.512	2437.376
2274	308.365	3.854	346.608	44.400
2364	288.569	3.607	1200.976	32.278
2400	280.651	3.508	1614.528	106.034
2417	276.912	3.461	992.656	95.042
2441	271.633	3.395	1161.728	125.221
2686	217.747	2.721	1488.704	134.421
2709	212.688	2.658	815.024	91.792
2722	209.828	2.622	2110.656	146.176
2745	202.570	2.530	921.280	74.992
2810	189.373	2.367	12766.720	2450.238
2907	169.138	2.114	318.992	44.640
3022	143.845	1.798	3746.992	416.224
3661	3.299	0.041	428.768	61.392
3676	0.000	0.000	3621.792	1551.104
3691 -	3.299 -	0.041	64.784	49.154
3763 -	20.235 -	0.252	99.056	31.776

II. Studies on the mutagenic, [illegible] and tumor promoter potentialities of Sangre de Grado and Taspine-HCl.

1. Effect of Taspine. HCl on the frequency of sister chromatid exchanges.

Sister chromatide exchanges are easily visualized in metaphase chromosomes are considered sensitive indicators of chromosome damage and have been used as indicators of mutagenesis (Latt S.A. (1973) Proc. Natl. Acad. Sci. USA 70: 3395 - 3399).

The cell line that we used for this experiment was the V79/AP4 obtained by Ford and Yerganian in 1958 and were lung fibroblasts derived from a chinise hamster embryo Cricetulus griseus. This cell line has 22 chromosomes and was obtained from the Laboratorio de Mutagenesi e Defferenziamento. Pisa, Italy.

1.1 Evaluation of Citotoxicity of Taspine-HCl.

Our first task was to determine the non-citotoxic concentration of Taspine-HCl to be utilized and for this we used two criteria.

a. Celular destruction and/or dettachment of cells from the plates.

For this purpose 24 hours before the test 3×10^5 V79/AP4 cells were inoculated into 60 mm tissue culture petri -- dishes and grown in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum and 0.5 mg/ml of gentamicin and incubated in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. 24 hours later Taspine-HCl was - added to the media at different concentrations and plates were incubated for another 24 hours.

The results showed that concentrations of 1.76, 0.88 and [illegible] Taspine HCl were toxic for the cells, while concentrations of 0.44 and 0.088 µg/ml of Taspine-HCl -- were innocuous for the cells.

b. Simultaneous determination of the integrity of the cell membrane and the activity of intracelular enzymes.-

This method is a modification and combination of the methods of Rotman and Papermaster (Proc.Natl. Acad. Sci.USA

55: 134-141, 1966) and Edidin (J. Immunol. 104: 1306, - 1970).

This technique makes use of two dyes:

- The first one a non polar non fluorescing compound fluorescein diacetate that could easily traverse the cell membrane. Ones inside the cell, inespecific estearases liberate the fluorescein which when exposed to UV light fluoresces with a green color.
- The second one is a polar fluorescent dye, etidium bromide which upon excitation with UV light fluoresces red. This dye could only enter the cells when the membrane is damaged.

The concentration of Fluorescein diacetate and etidium bromide used were 2 µgr/ml and 4 µgr/ml respectively. When cells are treated with the two dyes and observed under UV light in a fluorescent microscope, healthy cells will show a bright green fluorescence, unhealthy cells will show a light green fluorescence and damaged and - dead cells will show a red fluorescence

This method was also used in order to evaluate the citotoxic concentration of Taspine HCl and the results were in agreement with the ones described above, that is concentrations of Taspine HCl between 1.76 and 0.44 µg/ml were citotoxic while concentration of 0.264 µg/ml and lower were not citotoxic and will be the ones utilized in the experiment that follows.

1.2 Evaluation of the effect of Taspine HCl on Sister Chromatid Exchange.-

For this test, V79/AP4 cells that were growing exponentially were seeded on 60 mm tissue culture petri dishes (3×10^5 - cell per plate) using Dulbecco's modified Eagles's medium suplemented with 5% Fetal bovine serum and 0.5 mg/ml of gentamicin and incubated in a humidified atmosphere of 5% CO_2 and 95% air at 37C.

Twenty four hours later two sets of plates received Taspine HCl to a final concentration of 0.264 µg/ml and 0.088 µg/ml respectively. A third set of plates did not receive any Taspine

HCl and remained as controls. Simultaneously with the treatment the three sets of plates received 5' Bromodeoxiuridine to a final concentration of 3 µgr/ml of medium. Plates where then incubated for and additional 22 hours in a humidified atmosphere of 5% CO_2 95% air at 37C (about two replicative rounds).
At the end of the 22 hours of incubation, Colcemid was added to the plates to a final concentration of 0.05 µg/ml and plates were incubated for an additional two hours. After this last incubation cells were fixed in ethanol: acetic acid (3:1) and stained with the Fluorochrome plus Giemsa technique of Perry and Wolff (Nature 251, 156-158, 1974) and Latt (Proc. Natl. Acad. Sci USA 70, 3395-3399, 1973).
The results are shown in Table 1 and two prototypes of the metaphases observed are shown in Figure 8. What we observe is that Taspine HCl at non citotoxic concentrations increases the frequency of sister chromatid exchanges.
We are in the process of repeating this experiment for the third time and are including groups of cells that have been treated with the crude Sangre de Grado.

2. Studies on the carcinogenic and/or tumor promoter potentializer of Sangre de Grado and Taspine HCl.

We have already started our experiments on the carcinogenic and/or tumor promoter potentializer of Sangre de Grado using two stage mouse skin carcinogenesis system.
Female Swiss mice of 3 months of age were shaved with surgical clippers 2 days before treatment and those in the resting phase of the hair cycle were used. Mice are housed 10 per cage and food and water are available ad libitum. There are 30 animals per each experimental group.

- The carcinogenic compound used is 7-12 Dimethylbenz (a) anthracene (DMBA). The carcinogenic single dose is 100 n moles and the initiator single dose is 10 n moles.
- The tumor promoter compound used is 12-O- Tetradecanoyl phorbol 13-acetate (TPA) and the concentration was 2 µg in 0.1ml of acetone.

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TABLE 1

Effect of Taspine HCl on the frequency of Sister Chromatid Exchange (SCE).

Treatment	SCE/Metaphase
Control	7.22 ± 1.86
Taspine HCl 0.088 µg/ml	8.14 ± 1.99
Taspine HCl 0.264 µgr/ml	11.40 ± 2.82

3×10^5 V79/AP4 cells were inoculated into 60 mm Tissue Culture dishes in Dulbecco's modified Eagle's medium suplemented with 5% Fetal bovine serum and 0.5 mg/ml of gentamicin and incubated in a humidified atmosphere of 5% CO_2, 95% air at 37°C for 24 hours. Then different concentrations of Taspine HCl or medium were added to the corresponding plates simultaneously with 5' Bromodeoxiuridine at a final concentration of 3 µg/ml of medium. 22 hours later colcemid to a final concentration of 0.05 µg/ml of medium was added to the plates and incubated for 2 hours. Then cells were fixed and stained.

This data is the result of two separate experiments and a total of 57 metaphases were read in each of the treatment groups.

Significance of the differences were calculated according to the student t test. The difference between the control and the Taspine at 0.088 µg/ml group ($p < 0.05$) and between the control and the Taspine HCl 0.264 µg/ml group ($p < 0.01$) were significant.

Figure 3.- Metaphases from control and Tespine HCl. Treated cells for the determination of sister chromatide exchanges. Seven exchanges are visualized in the metaphase from the control cell (3A) and 15 exchanges in the Tespine HCl treated cell (3B).

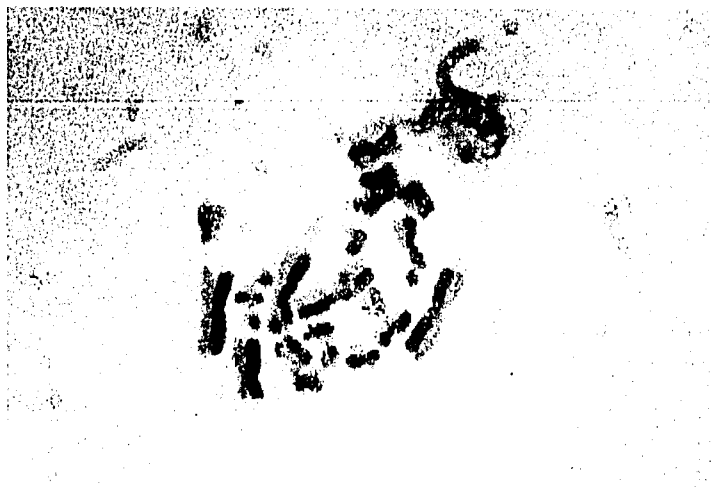

FIGURE 8 A

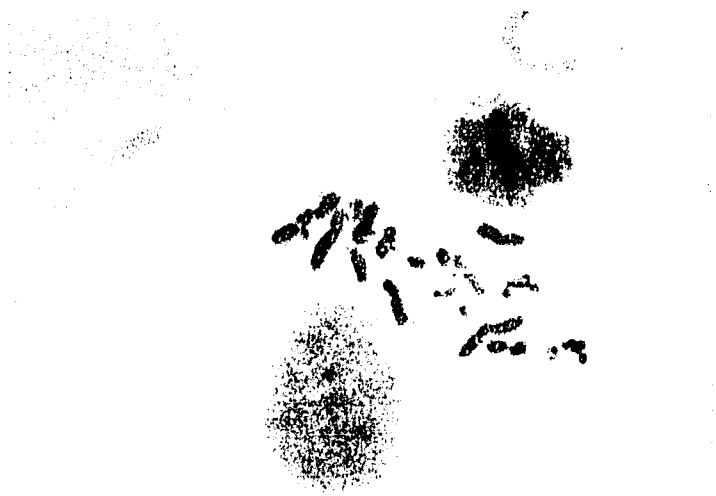

FIGURE 8 B

The groups we have already started are as follows:

Group I: 0.1 ml acetone twice weekly

Group II: 100 n moles of DMBA in 0.1 ml acetone

Group III: 10 n moles of DMBA in 0.1 ml acetone

Group IV: 10 n moles of DMBA in 0.1 ml acetone first week
2 γg TPA in 0.1 ml of acetone twice weekly - thereafter.

Group V: 10 n moles of DMBA in 0.1 ml acetone first week
0.1 ml of 50% aqueous suspension of Sangre de Grado twice weekly thereafter.

Group VI 0.1 ml of 50% aqueous suspension of Sangre de Grado twice weekly.

In a couple of weeks we will start with the groups of mice that will be treated with Taspine-HCl.

XN-AAZ-805-B

3. N-22

THIRD PROGRESS REPORT
PROJECT No. 936-5542

INVESTIGATION OF THE CICATRIZANT PROPERTIES OF THE PLANT EXTRACT SANGRE DE GRADO AND ITS POSSIBLE UTILIZATION AS A THERAPEUTIC AGENT.

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PERIOD COVERED: SEPTEMBER 1st 1983 THROUGH
DECEMBER 31st 1984.

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DESCRIPTION OF THE RESEARCH CARRIED OUT.-

In the following pages and according to the chronogram of activities we describe the work that has been done in our laboratory during the last 16 months. This report includes the previous findings on the extraction, purification, characterization and quantitation of the alkaloids from Sangre de Grado as well as new findings on the extraction and purification of the alkaloids present in the leaves of Croton lechleri.
We will also describe the previous as well as the new experiments on the possible carcinogenic and/or tumor promoter activities of Sangre de Grado and its active principle.
During this reporting period one trip to the peruvian jungle has been carried out in order to collect Sangre de Grado, and the botanical report is being prepared by Dr. Ramon Ferreyra from the Museum of Natural History. This trip, was to the Iquitos area where we were able to locate a privately owned land that had trees of what appeared to be the species we are working with and which will allow as to do long term studies on the same plants without having to worry about the plants been tored down.

RESULTS AND DISCUSSION.

1. ISOLOATION, PURIFICATION, CHARACTERIZATION AND QUANTITATION OF THE ALKALOIDS FROM SANGRE DE GRADO AND FROM THE LEAVES OF THE Croton lechleri.

1. EXTRACTION, PURIFICATION AND QUANTITATION OF THE ALKALOID FROM SANGRE DE GRADO.-

Collection of Sangre de Grado: A trip to the peruvian jungle for the collection of Sangre de Grado was organized in December of 1984. This trip was to Iquitos, where we have located a privately owned land that has 41 trees which apparently belong to the species we are working with. We were able to obtain the botanical sample from seven of these trees and we also extracted Sangre de Grado at different times of the day from seven different trees. In Table 1 we show that the amount of Sangre de Grado obtained varies from tree to tree but there is no difference between the different extraction times. For each of this Sangre de Grados we determined the alkaloid content and found that the values oscilated between 3.4 and 1.5 mg/ml. The total yield was found to be considerably higher in the sample taken from 6 to 7 a.m. The botanical samples are being analized in order determine the species, which on the preliminary inspection appeared to be Croton lechleri.
We are planing to make several trips to the same location this year and extract Sangre de Grado from the same trees as well as from others in order to obtain information on the optimun collection season.

The extraction procedure of the alkaloids from Sangre de Grado is practicaly the same one described in the first progress report with a couple of slight modifications that are the result of succesive trials in order to obtain the best separation possible (Figure 1).
This method of extraction and purification although tedious and time consuming, is the one being used now since as we will describe in the following section al the other methods that we have tried did not improve our separation and purification procedures.

This extraction procedure yields an alkaloid mixture from which two different solids could be separated: A yellow cristaline solid and a white amorphous solid, being the former the more abundant in the mixture.
Thin layer chromatography on Silica gel 60 F-254 (Merck No. 5539) of the two solids and the resin using as eluent Dichloromethane:Methanol (3:1) gave the following results (Figure 2):

- The resin gave three spots with rfs of 0.36, 0.30 and 0.13 respectively and which fluoresced when exposed to long wave UV light (366 nm).

- The yellow crystals gave only one large spot with an rf of 0.485, which fluoresced when exposed to short wave UV light (254 nm) and gave positive reaction with the Dragendorf reagent according to Munier and Machenboeuf.

- The white amorphus solid gave several spots which fluoresced when exposed to short wave UV light. From these spots the ones with an rf of 0.439 and 0.12 where intense while the other three with rfs of 0.28, 0.19 and 0.05 respectively where in trace amounts. From all these spots only the one with an rf of 0.439 gave a positive reaction with the Dragendorf reagent according to Munier and Machenboeuf.

2. SEPARATION AND PURIFICATION USING DRY COLUMN AND MEDIUM PRESSURE LIQUID CHROMATOGRAPHY.

Trying to seek better chromatographic means of separation, Dry Column an Medium Pressure Liquid Chromatography were used obtaining the results that we describe bellow.

a. Dry column chromatography

The system used was:

Stationary phase: Silica Woelm TSC UV 254 III activity.
Dimension of the stationary phase 43 x 2.5 cm.
Sample: 330 mg of crude alkaloid mixture.

Eluent:

1100 ml of CH_2Cl_2: MeOH (3:1) made acid with 10 ml acetic acid.
160 ml of CH_2Cl_2 : MeoH (5:3) made acid with 2 ml acetic acid.
180 ml of CH_2Cl_2 : MeOH (1:1) made acid with 2 ml acetic acid.
500 ml of CH_2Cl_2 : MeOH (85:15) made basic with 5 ml NH_4OH 28%
170 ml of CH_2Cl_2 : MeOH (10:7) made basic with 2 ml NH_4OH 28%
200 ml of CH_2Cl_2 : MeOH (1:1) made basic with 2 ml NH_4OH 28%.

The column was eluted increasing the polarity of the eluent to favor separation. The eluent at first was made acidic with acetic acid since on TLC this would favor separation. Later on the eluent was made basic in order to help remove alkaloid substances from the stationary phase. If no basic fraction is used, the alkaloid content tends to remain impregnated on the stationary phase.
62 fracions were collected, the volume of each of these fractions oscilated between 10-60 ml. Each fractions was then chromatographed on silica gel 60 F 254 thin layer plates using dichloromethane: Methanol (3:1) made acid as eluent.
The results are shown in Figure 3: the first 50 fractions gave one spot with equal rf values of approximately 0.48. This result indicates poor separation and thus regards the technique as inefficient.

b. Medium Pressure Liquid Chromatography
A medium pressure column was used with the following characteristics:
Silica gel 60 as stationary phase.
The dimension of the stationary phase was 25 x 2 cm.
The eluent used in this system was dichloromethane: Methanol (3:1).
The detector used was UV 254 nm lamp.
Sample: 4 mgr of the alkaloid mixture.
The graph shown in Figure 4 was obtained showing no separation.
The graph gives the times at which the substances are expelled and detected with the UV light of 254 nm. At 60 seconds one single peack was obtained showing that everything is expelled with no separation. The column was run for an additional 4 hours to make sure that nothing remained in the column.

c. Reverse Phase Chromatography
Reverse phase C-8 was attempted to seek for a system that could be used on Medium Pressure Column with Reverse C-8 Stationary Phase.

The following systems were used unsuccessfully.

Methanol: H_2O (4:6)
Methanol: H_2O (8:2)
Methanol pure
Methanol: CH_3CN (7:3)
Methanol: CH_3CN: H_2O (5:3:2)
Since in all cases the sample wouldn't run no attempt was made to run a Medium Pressure Column with reverse C-8 Stationary Phase.

3. Characterization of the alkaloids from Sangre de Grado.

The information we have accumulated on the yellow alkaloid crystals obtained during the purification procedures of Sangre de Grado is in agreement with Taspine being the identity of these crystals.
The ultraviolet spectrum (Figure 5 A) of the hydrochloride of the yellow crystals is identical to the UV spectrum (Figure 5B) of a reference sample provided by Dr. Norman R. Farnsworth from the College of Pharmacy, University of Illinois.
The yellow alkaloid crystals showed an UV spectrum with absorption maxima similar to the ones reported previously by G. Persinos, R. Blomster, D. Blake and N. Farnsworth (J. Pharmaceutical Sciences (1979) 68: 124-126) and Shamma and Moniot (Chem. Commun (1971), 1965).
The mass spectra performed by Dr. Bernhard Tauscher at the OrganicChemistry Institute of the University of Heidelberg showed a molecular weight of 369 which is in agreement with the molecular weight reported for Taspine (Persinos et al (1979) J. Pharm. Sci. 68: 124-126).
An NMR spectrum of the yellow alkaloid crystals from Sangre de Grado shown in Figure 7 is in agreement with that of Taspine reported in the literature (M. Shamma and J.L.Moniot Chem. Commun (1971, 1065).
The ultraviolet spectrum of the hydrochloride of the white amorphus solid from Sangre de Grado (Figure 6) was also identical to the reference sample of Taspine-HCL provided by Dr.Farnsworth.
No NMR spectrum of this white amorphus solid has been obtained yet because of its low solubility in the necessary volume for the determination.
The NMR Spectrometer used was a Bruker WP 80-FT.

4. Preparation of Taspine Hydrochloride.-

HCL gas was passed through a chloroform solution of Taspine (yellow alkaloid crystal) until no further precipitation was observed. The alkaloid hydrochloride was then removed by filtration and washed with chloroform several times until a white solid was obtained which was then dryed in vacuo at 70 C.
An identical procedure was folloed for obtaining the

hydrochloride of the white amorphus solid.

5. EXTRACTION AND PURIFICATION OF THE ALKALOIDS FROM THE LEAVES OF THE Croton lechleri.

30 kg of dryed leaves were grinded and extracted four times with four liters of 95% ethanol. The ethanolic extracts were then pooled together and reduced in vacuo at 40C. The solid obtained was then resuspended in 4% tartaric acid and extracted with ethyl acetate. The tartaric acid phase containing the alkaloids was taken then to pH 9 using concentrated amonium hydroxide and extracted exhaustively with chloroform until no further alkaloids could be extracted. The chloroform fractions were then pooled together and reduced in vacuo at 40C yielding 0.8 g of a greenish solid (Figure 8). Thin layer chromatography of the chloroform extract on silica gel HF 254 using as eluent Chloroform:Methanol (9:1) resolved eleven spots (Figure 9). Three of these spots: Spot A with an rf of 0.63, spot B with an rf of 0.51, and spot C with an rf of 0.29 gave a positive reaction to the Dragendorf reagent according to Munier an Machenboeuf.
The extract was separated by preparative thin layer chromatography on silica gel HF 254 using as eluent a mixture of Chloroform:Methanol (9:1) from which we separated the zones that corresponded to alkaloids A, B and C.
Alkaloid B was the most abundant and was the only one that we obtained in crystaline form. This alkaloid was further purified on preparative silica gel HF 254 thin layer chromatography plates using as eluent the system Dichloromethane:Methanol (1:1). The purified alkaloid B was then recrystalized in 75% ethanol and 30 mg of white crystals were obtained.

The crystaline compound obtained showed a single spot positive to the Dragendorff reagent and with an rf of 0.51 on silica gel HF 254 TLC using Chloroform:Methanol (9:1) as eluent. This compund had a melting point of 205C with decomposition. The UV spectrum of the compound shown in Figure 10 exhibits a maximal absorption at 212 nm in ethanol. The Infrared Spectrum shown in Figure 11 reveals the presence of the following groups: N-CH3, N-H, aromatic C=C, O-CH3, aromatic C-H and C-O-C. The NMR spectrum of the alkaloid shown in Figure 12 reveals the presence of one N-methyl group (singlet at δ 2.449 ppm, 3 protons), two methoxy groups (singlets at δ 3.758 ppm and δ 3.893 ppm, 3 protons each), two aromatic protons (doublet at δ 6.28 ppm) and six additional protons as evidenced by singlets at 1.644 ppm (2 protons), δ 3.103 ppm (1 proton), δ 7.544 ppm 1 proton) respectively, and doublets at δ 6.71 ppm (2 protons).
A sample of the alkaloid B will be send to the USA shortly in order to obtain the mass spectrum.

II. CELL CULTURE STUDIES ON THE MECHANISM OF ACTION AND TOXICITY OF TASPINE HCl.

Human foreskin fibroblasts were used in these studies and they were obtained as follows: human foreskins were collected under sterile conditions and transported to our laboratory within two hours in Mimimal Essential Medium Eagles with Hanks salts (MEM-H) containing 5% newborn calf serum, 2.5 ug/ml of fungizone and 50 ug/ml of gentamycin sulfate. The tissue was weighed, minced and washed twice with phosphate buffered saline without Ca^{++} and Mg^{++} containing 0.02% ethylene-diaminetetraacetic acid (PBS-EDTA). After the last wash the tissue was incubated for 30 minutes at 37C in PBS-EDTA containing 0.1% tripsin (T-EDTA, 10 ml per gram of tissue), then allowed to sediment and the supernatant was discarded. This tripsinization procedure was repeated once more and then the tissue fragments were placed over the surface of several tissue culture flasks, a small drop of growing medium was added to each piece (MEM-H containing 10% fetal bovine serum, 10 mM Hepes and 50 ug/ml of gentamycin), and then the flask was inverted and more medium was added to prevent the pieces from drying. Once the fragments have attached (about 16 hours) the bottle was turned carefully so as the medium now bathed the fragments. Fibroblasts outgrowing from the tissue fragments were later on collected. These fibroblast were then frozen in liquid nitrogen and constitute our stock of cells for the experiments.

1. TOXICOLOGICAL STUDIES OF TASPINE-HCl ON HUMAN FORESKIN FIBROBLASTS.

It was important for us to find first the maximum dose at which we could use taspine-HCl in our fibroblast cultures. For this purpose two experiments were performed:

The first one was carried out in order to measure the effect of taspine-HCl on recently trypsinized and plated cells, since some of our experiments will make use of this type of design. Our findings are shown in Table 2, and what we observe is that we should use concentrations bellow 250 ng/ml of taspine-HCl, since at that and higher concentrations the percentage of dead cells increases dramatically.

The second experiment was carried out in order to measure the effect of taspine-HCl on subconfluent cells. The result of this experiment is shown in Table 3, and what we observe is that concentrations of 200 ng/ml and lower could be used safely since the percentage of dead cells was very low at these concentrations. Another interesting observation was the increase in cell numbers with respect to the control that we see at concentrations bellow 200 ng/ml at the 60 h. measurements. We are in the process of repeating this experiment in order to reconfirm the observation.

III. STUDIES ON THE MUTAGENIC, CARCINOGENIC AND TUMOR PROMOTER POTENTIALITIES OF SANGRE DE GRADO AND TASPINE-HCl.

1. STUDIES ON THE MUTAGENIC EFFECT OF SANGRE DE GRADO AND/OR TASPINE-HCl ON BACTERIA.

For this purpose we will use the method of Ames (Ames B.N., MacCann J., Yamasaki E. Mutation Research 31:347-364, 1975). This method utilizes several specially constructed mutants of Salmonella typhymurium selected for their sensitivity and specificity in being reverted from a histidine requirement back to prototrophy, by a wide variety of mutagens. It is also interesting to point out that since since it is known how the original mutations originated (frameshift mutation, base substitutions,etc) the method provides the mechanism by which the mutagen is causing the revertion.

The strains that we used were: TA 102, TA 100, TA 98, TA 97, and TA 1538. Strains TA 102 and TA 97 replaced strains TA 1535 and TA 1537 that were considered in the original protocol.
The method we used is as follows: To 2ml of molten top agar at 45 C, 0.1 ml of an overnight culture of the bacterial tester strain, 0.1 ml of the compound to be tested and 0.1 ml of the S-9 mix when required (rat liver homogenate, microsomal fraction) were added, mixed rapidly and poured on minimal glucose agar plates containing trace amounts of histidine. The mixing, pouring and distribution took less than 20 seconds, and the plates were left to harden for several minutes then inverted and cultured at 37 C. Two days later the colonies on the plates were counted.
The compounds tested were Sangre de Grado and taspine-HCl. Taspine-HCl was disolved in water and sterilized by filtration through millipore membranes (0.45um). Sangre de Grado was sterilized by making first a 50% suspension in ethyl alcohol and from this stock we prepared the required concentrations by dilution in sterile water.
The results of our experiments could be observed in Tables 4 and 5.

In Table 4, we show the results obtained when we tested different concentrations of both Sangre de Grado and taspine-HCl with the tester strains in the absence of S-9 mix. These concentrations,were found previously to be none toxic to the tester strains except, for the two highest concentrations of taspine-HCl on strain TA97. What we observe, is that Sangre de Grado was weakly mutagenic at concentrations of 0.22% for tester strains TA97 and TA102, as evidenced by a doubling of the number of revertants with respect to the spontaneous reversion observed. Taspine-HCl was not mutagenic at the concentrations tested.

In Table 5 we show the results obtained by testing different concentrations of Sangre de Grado and Taspine-HCl with the

tester strains in the presence of S-9 mix. What we observe is that neither Sangre de Grado nor taspine-HCl were mutagenic for the tester strains under these conditions. A positive control was run with cyclophosphamide (1.24 mg/ml) and as expected, there was a significant increase (from 38 to 501) in the number of revertants with tester strain TA100.

2. EFFECT OF TASPINE-HCl ON THE FREQUENCY OF SISTER CHROMATID EXCHANGES.

Sister chromatide exchanges are easily visualized in metaphase chromosomes are considered sensitive indicators of chromosome damage and have been used as indicators of mutagenesis (Latt S.A. (1973) Proc. Natl. Acad. Sci. USA 70: 3395 - 3399).

The cell line that we used for this experiment was the V79/AP4 obtained by Ford and Yerganian in 1958 and were lung fibroblasts derived from a chinise hamster embryo Cricetulus griseus. This cell line has 22 chromosomes and was obtained from the Laboratorio de Mutagenesi e Defferenzamento. Pisa, Italy.

1.1 Evaluation of Citotoxicity of Taspine-HCl.

Our first task was to determine the non-citotoxic concentration of Taspine-HCl to be utilized and for this we used two criteria.

a. Celular destruction and/or dettachment of cells from the plates.

For this purpose 24 hours before the test 3×10^5 V79/AP4 cells were inoculated into 60 mm tissue culture petri dishes and grown in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum and 0.5 mg/ml of gentamicin and incubated in a humidified atmosphere of 5% CO_2 and 95% air at 37C. 24 hours later Taspine-HCl was added to the medium at different concentrations and plates were incubated for another 24 hours.

The results showed that concentrations of 1.76, 0.88 and 0.44 ug/ml of Taspine HCl were toxic for the cells, while concentratins of 0.264 and 0.088 ug/ml of Taspine HCl were inocuouss for the cells.

b. Simultaneous determination of the integrity of the cell membrane and the activity of intracelular estearases.- This method is a modification and combination of the methods of Rotman and Papermaster (Proc. Natl. Acad.Sci. USA 55: 134-141, 1966) and Edidin (J. Immunol 104: 1306-1970).

This technique makes use of two dyes:

- The first one a non polar non fluorescing compound fluorescein diacetate that could easily traverse the cell membrane. Once inside the cell, inespecific

estearases liberate the fluorescein which when exposed to UV light fluoresces with a green color.

- The second one is a polar fluorescent dye, etidium bromide which upon excitation with UV light fluoresces red. This dye could only enter the cells when the membrane is damaged.

The concentration of Fluorescein diacetate and etidium bromide used were 2 ugr/ml and 4 ugr/ml respectively. When cells are treated with the two dyes and observed under UV light in a fluorescent microscope, healthy cells will show a bright green fluorescence, unhealthy cells will show a light green fluorescence and damaged and dead cells will show a red fluorescence.

This method was also used in order to evaluate the citotoxic concentration of Taspine HCl and the results were in agreement with the ones described above,that is, concentrations of Taspine HCl between 1.76 and 0.44 ug/ml were citotoxic while concentration of 0.264 ug/ml and lower were not citotoxic and will be the ones utilized in the experiments that follows.

1.2 Evaluation of the effect of Taspine HCl on Sister Chromatid Exchange.-

For this test, V79/AP4 cells that were growing exponentially were seeded on 60 mm tissue culture petri dishes ($3x10^5$ cells per plate) using Dulbecco's modified Eagles's medium supplemented with 5% fetal bovine serum and 0.5 mg/ml of gentamicin and incubated in a humidied atmosphere of 5% CO_2 and 95% air at 37C.

Twenty four hours later two sets of plates received Taspine HCl to a final concentration of 0.264 ug/ml and 0.088 ug/ml respectively. A third set of plates did not receive any Taspine HCl and remained as control. Simultaneously with the treatment the three sets of plates received 5' Bromo - deoxiuridine to a final concentration of 3 ug/ml of medium. Plates where then incubated for and additional 22 hours in a humidified atmosphere of 5% CO_2 95% air at 37C (about two replicative rounds).

At the end of the 22 hours of incubation, Colcemid was added to the plates to a final concentration of 0.05 ug/ml and plates were incubated for and additional two hours. After this last incubation cells were fixed in ethanol: acetic acid (3:1) and stained with the Fluorochrome plus Giemsa technique of Perry and Wolff (Nature 251:156-158, 1974) and Latt (Proc. Natl. Acad. Sci. USA 70: 3395-3399, 1973).

The results are shown in Table 6. What we observe is that Taspine HCl at non citotoxic concentrations increases the frequency of sister chromatid exchanges.

2. STUDIES ON THE CARCINOGENIC AND/OR TUMOR PROMOTER POTENTIALITIES OF SANGRE DE GRADO AND TASPINE HCL.

We have just re-started our experiments on the carcinogenic

and/or tumor promoter potentialities of Sangre de Grado using the two stage mouse skin carcinogenesis system, since the experiment we started last June was ruined by two unpredictable accidents. For this experiment :
Female Sencar mice of 3 months of age were shaved with surgical clippers 4 days before treatment and those in the resting phase of the hair cycle were used. Mice are housed 10 per cage and food and water are available ad libitum. There are 30 animals per each experimental group.

- The carcinogenic compound used is 7-12 Dimethylbenz (α) antracene (DMBA). The carcinogenic single dose is 100 n moles and the initiator single dose is 10 n moles.
- The known tumor promoter compound used was 12-O-Tetra-decanoyl phorbol 13-acetate (TPA) and the concentration was 2 ug in 0.1 ml of acetone.

The groups we have already started are as follows:

Group 1:	100 n moles of DMBA in 0.1 ml acetone
Group 2:	10 n moles of DMBA in 0.1 ml acetone
Group 3:	2 ug of TPA in 0.1 ml of acetone twice weekly.
Group 4:	0.1 ml acetone twice weekly
Group 5:	0.1 ml of 50% aquous suspension of Sangre de Grado twice weekly.
Group 6:	0.2 mg of Taspine-HCl twice weekly.
Group 7:	10 n moles of DMBA in 0.1 ml acetone first week 2 ug TPA in 0.1 ml of acetone twice weekly thereafter.
Group 8:	10 n moles of DMBA in 0.1 ml acetone first week 0.1 ml of 50% aquous suspension of Sangre de Grado twice weekly thereafter.
Group 9:	10 n moles of DMBA in 0.1 ml acetone first week 0.2 mg of Taspine-HCl in 0.1 ml of water twice weekly thereafter.

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TABLE 1

EXTRACTION OF SANGRE DE GRADO FROM DIFFERENT TREES FROM IQUITOS

TREE N.	TIME OF EXTRACTION	VOLUME OF EXTRACTION	YIELD mg/ml	TOTAL YIELD mg
8	6-7 am	29 ml	3.4	98.6
1	7-8 am	30 ml	1.5	40.5
11	8-9 am	32 ml	1.6	51.2
2	9-10 am	13 ml	3.0	39.0
4	10-11 am	22 ml	2.0	44.0
6	11-12 am	33 ml	2.2	72.6
7	12-1 pm	17 ml	2.0	34.0

To 5 mls of each of the samples were added 5 ml of water, the mixture was then alkalinized to pH 9 with amonium hydroxide and extracted exhaustively with chloroform. Each chloroform extract was concentrated in a rotavapor then dryed and the weight of the resulting alkaloid was measured in an analytical balance.

The collection dates were 12/19/84 and 12/20/84.
The diameter of the trees were 20 ± 5 cm.

TABLE 2

EFFECT OF TASPINE-HCl ON THE PROLIFERATION OF RECENTLY PLATED FORESKIN FIBROBLASTS.

Taspine-HCl ng/ml	11 hours No.Cells	11 hours % Dead	42 hours No.Cells	42 hours % Dead
2000	96,500	54	60,000	100
1500	91,500	46	79,500	96
1000	91,500	25	96,000	64
500	102,000	22	115,000	39
250	125,700	17	130,000	15
150	124,700	8	150,000	11
100	128,200	8	151,000	12
50	123,000	8	156,000	12
25	126,700	10	146,000	10
0	128,000	10	168,000	16

10^5 Foreskin fibroblasts were inoculated to 35 mm tissue culture dishes containing MEM-H + 10% fetal bovine serum, 10mM Hepes, 50 ug/ml gentamycin, and the concentration of taspine-HCl to be tested. The dishes were incubated at 37 C in a humidified atmosphere of 5% CO_2 and 95% air. At 11 hours and at 42 hours sets of plates were collected by tripsinization and counted.
We report here the total number of cells per plate as well as the percentage of dead cells calculated by the trypan blue exclusion method.

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TABLE 3.

EFFECT OF TASPINE-HCl ON THE PROLIFERATION OF SUBCONFLUENT CULTURES OF HUMAN FORESKIN FIBROBALSTS.

Taspine-HCl. ng/ml	60 Hours No.Cells	60 Hours % Dead	88 Hours No.Cells	88 Hours % Dead
2000	95,000	100	101,000	100
200	313,000	7	245,000	13
20	387,000	5	334,000	7
2	354,000	5	327,000	9
0.2	348,000	8	242,000	12
0	214,000	6	302,000	7

10^5 Foreskin fibroblasts were inoculated to 35 mm tissue culture dishes containing MEM-H + 10% fetal bovine serum, 10mM Hepes, 50 ug/ml gentamycin. The dishes were incubated at 37 C in a humidified atmosphere of 5% CO_2 and 95% air. At 18 hours the different concentrations of taspine-HCl were added to sets of them. At 60 hours and at 88 hours, sets of plates were collected by tripsinization and counted in an hemocytometer.

We report here the total number of cells per plate as well as the percentage of dead cells calculated by the trypan blue exclusion method.
The number of cells at 18 hours was 116,000 (7% of dead cells).

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TABLE 4

MUTAGENIC EFFECT OF SANGRE DE GRADO AND TASPINE-HCl ON THE TESTER STRAINS IN THE ABSENCE OF S-9 MIX.

	NUMBER OF REVERTANTS OF THE TESTER STRAIN				
	TA97	TA98	TA100	TA102	TA1538
S P E	90-180	30-50	120-200	240-320	15-35
S P O	237	20	113	196	5
S G %					
0.023	363	27	99	295	7
0.055	327	26	99	332	6
0.110	368	25	83	337	5
0.220	410	20	85	408	4
T-HCl mg/ml					
0.0047	240	24	92	140	9
0.0110	241	27	92	140	13
0.0230	19	26	85	106	13
0.0450	9	24	52	95	12

ABREVIATIONS: S P E : Spontaneous reversion expected
S P O : Spontaneous reversion observed
S G : Sangre de Grado
T-HCl : Taspine-HCl

For the methodology see text.

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TABLE 5

MUTAGENIC EFFECT OF SANGRE DE GRADO AND TASPINE-HCl ON THE TESTER STRAINS IN THE PRESENCE OF S-9 MIX.

	NUMBER OF REVERTANTS OF THE TESTER STRAIN				
	TA97	TA98	TA100	TA102	TA1538
S P E	90-180	30-50	120-200	240-320	15-35
S P O	68	42	38	31	23
S G %					
0.023	86	37	59	23	23
0.058	88	41	85	30	22
0.116	95	32	ND	29	ND
0.220	81	34	71	30	15
T HCl mg/ml					
0.0047	107	40	40	25	17
0.0110	91	38	44	31	22
0.0230	78	33	38	32	ND
0.0450	55	37	27	32	17

ABBREVIATIONS: S P E : Spontaneous reversion expected
S P O : Spontaneous reversion observed
S G : Sangre de Grado
T-HCl : Taspine-HCl
ND : Not done

For the methodology see text.

TABLE 6

EFFECT OF TASPINE HCl ON THE FREQUENCY OF SISTER CHROMATID EXCHANGE

Treatment	SCE/Methaphase
Control	7.22 ± 1.86
Taspine HCl 0.088 ug/ml	8.14 ± 1.99
Taspine HCl 0.264 ug/ml	11.40 ± 2.82

SCE = Sister chromatid exchange.

3×10^5 V79/AP4 cells were inoculated into 60mm tissue culture dishes in Dulbecco's modified Eagles medium suplemented with 5% fetal bovine serum and 0.5 mg/ml of gentamicin and incubated in a humidified atmosphere of 5% CO_2,95% air at 37C for 24 hours. Then different concentrations of taspine HCl or medium were added to the corresponding plates simultaneously with 5'Bromodeoxiuridine at a final concentration of 3 ug/ml of medium. 22 hours later colcemid to a final concentration of 0.05 ug/ml of medium was added to the plates and incubated for 2 hours. Then cells were fixed and stained. These data is the result of two separate experiments and a total of 57 metaphases were read in each of the treatment groups. Significance of the differences were calculated according to the student-t test. The difference between the control and the taspine HCl at 0.088 ug/ml group ($p<0.05$) and between the control and the taspine HCl at 0.264 ug/ml group ($p<0.01$) were significant.

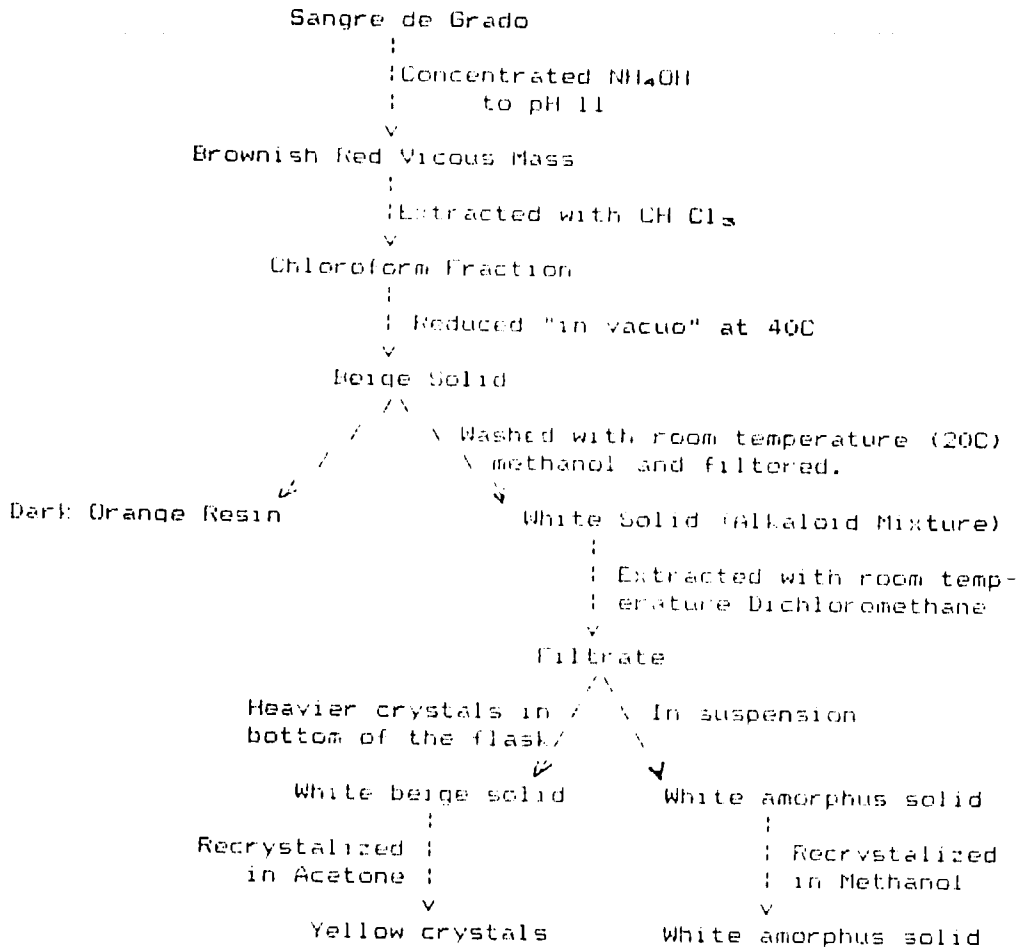
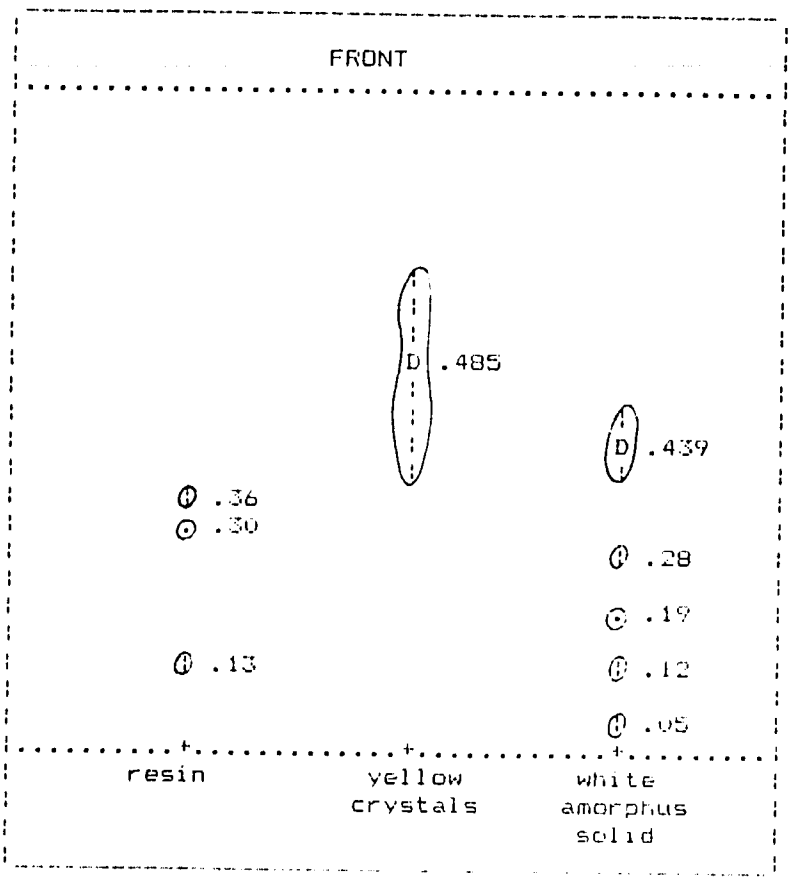


FIGURE 1.- Extraction and Purification Procedure of the Alkaloid from Sangre de Grado.



D = Positive to Dragendorf reagent.

FIGURE 2.- Thin layer chromatography of the alkaloid and resin obtained during the extraction of Sangre de Grado. TLC was performed on Silica Gel 60 F254 using as eluent a mixture of dichlorometane:methanol (3:1). The resin spots fluoresced when excited with long wave UV light of 366 nm and all other spots with short wave UV light of 254 nm. The rfs are shown next to each spot.

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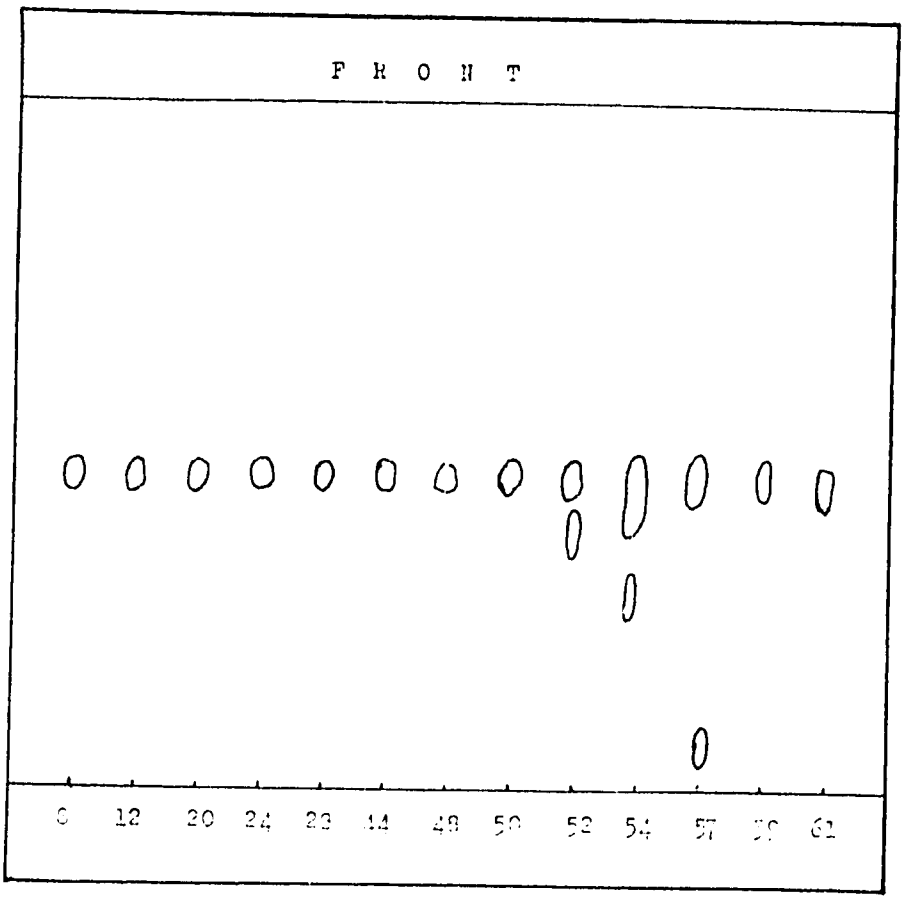

FIGURE 3.- Thin layer chromatography of representative fractions eluted from the dry column. The system was Silica gel 60 F254 Merck and the eluent was dichlcromethane: Methanol (3:1) made acid with acetic acid.

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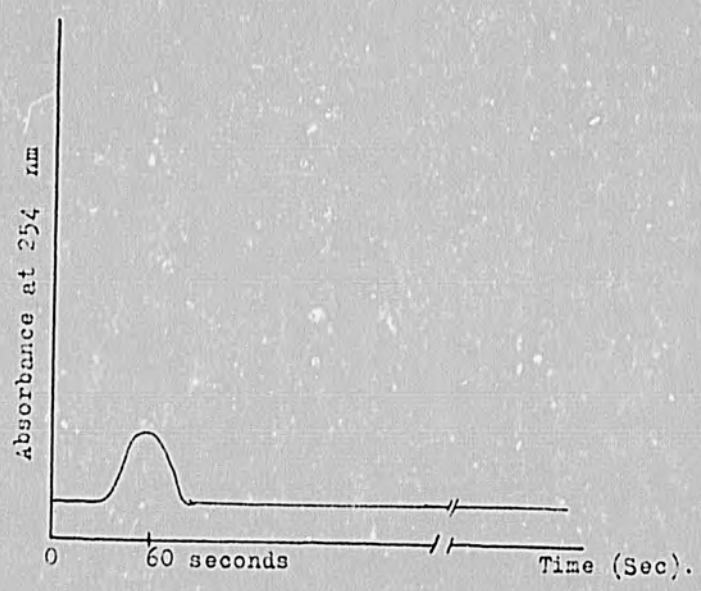

FIGURE 4.- Elution pattern of the crude alkaloid mixture from Sangre de Grado from a Silica gel 60 Medium Pressure Column of the following characteristics:
Stationary phase Silica gel 60
Dimension: 25 x 2 cm.
Detector: UV 254 nm lamp
Eluent: Dichloromethane: Methanol (3:1).
Column was run up to 4 hours.

FIGURE 5.- Ultraviolet Spectra of the hydrochloride of the yellow crystals from Sangre de Grado (5A) and of the reference sample of Taspine hydrochloride provided by Dr. -- Norman R. Farnsworth (5 B) Both spectra were run in H_2O.

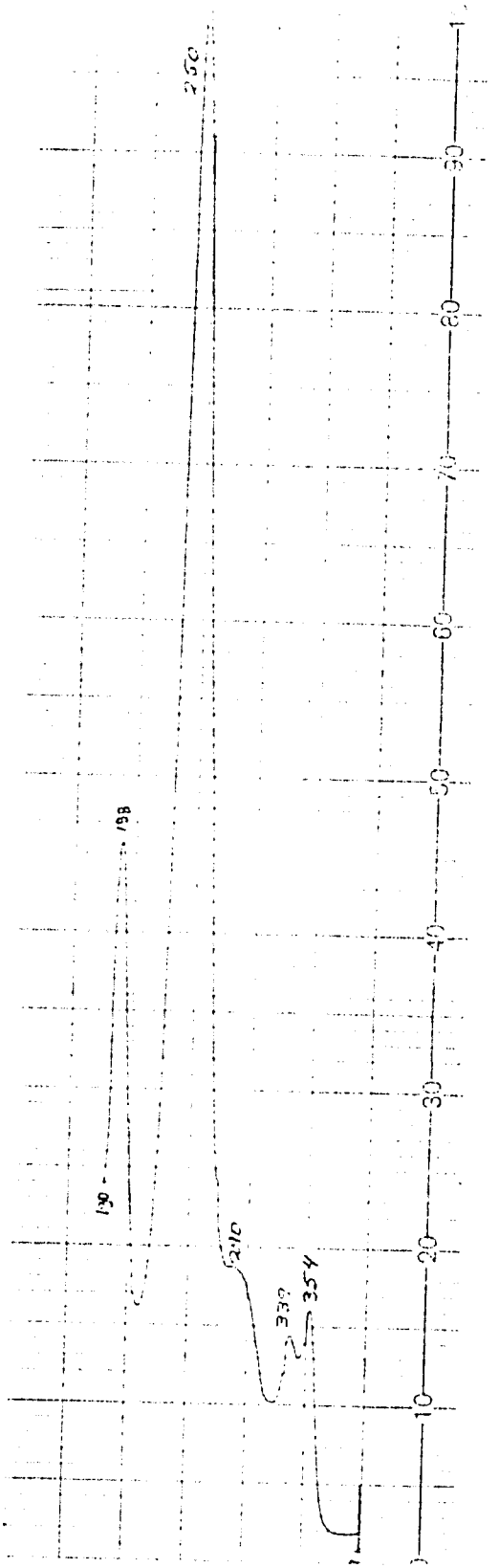

FIGURE 5 A

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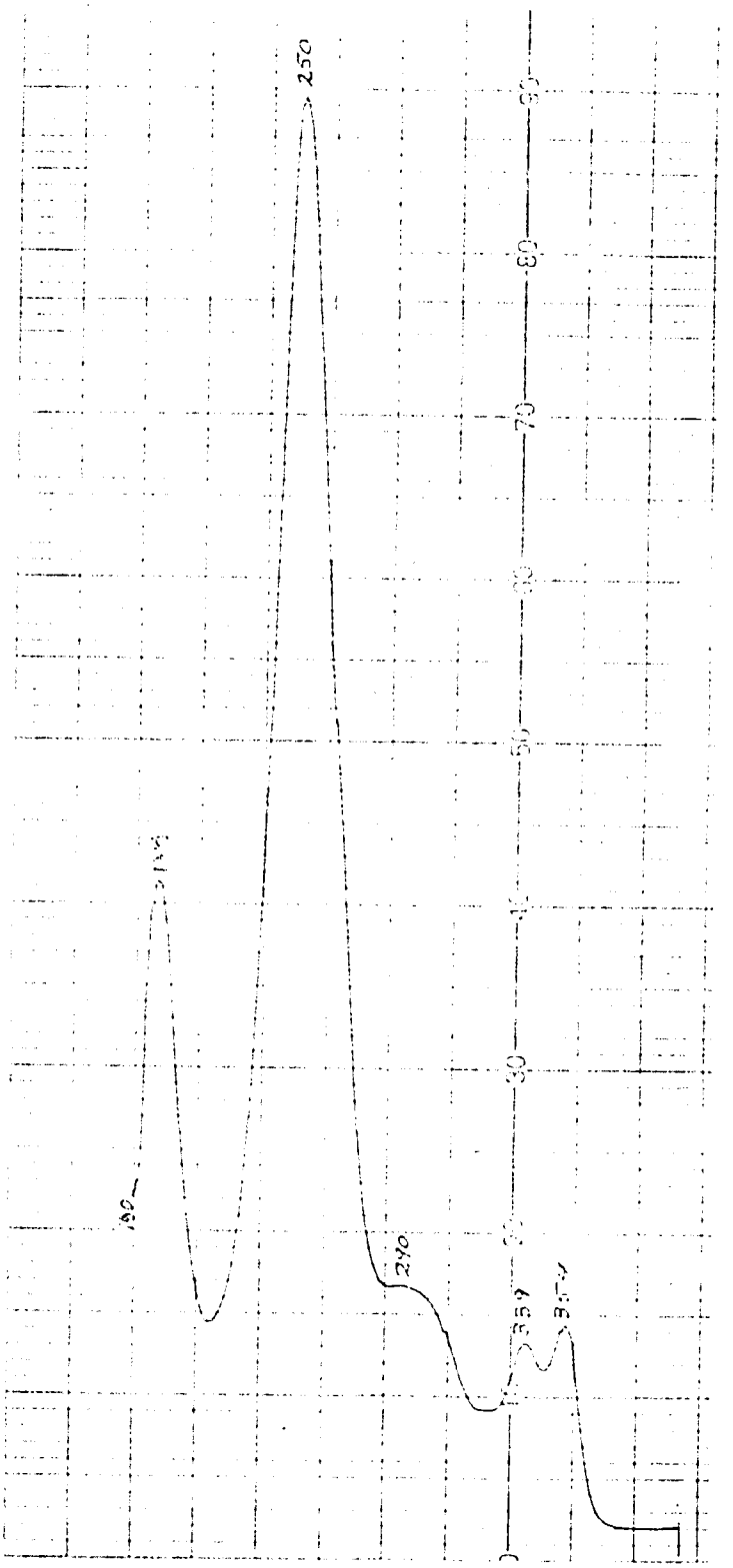

FIGURE 5 B

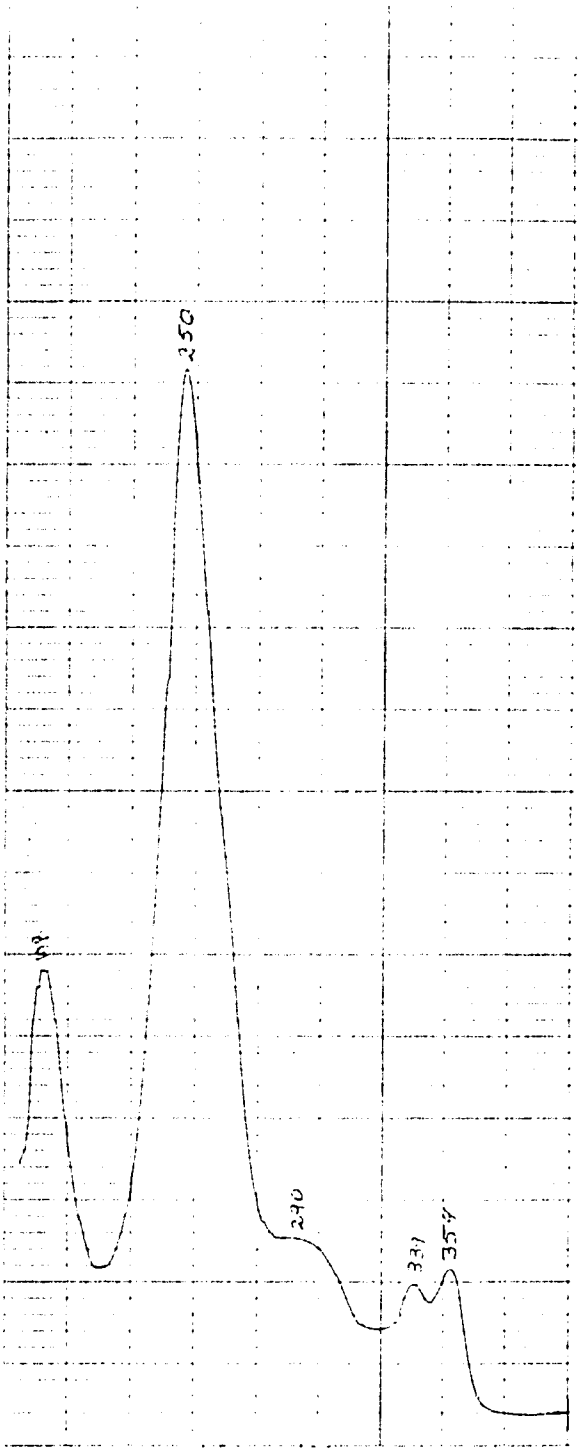

FIGURE 6 UV Spectrum of the Hydrochloride of the White Amorphus Solid from Sangre de Grado in H_2O.

•MI= 10.000 30
•PP

ADDRESS	FREQUENCY	PPM	INTEGRAL	INTENSITY
680	658.959	8.236	860.160	282.960
720	650.161	8.127	1118.064	338.416
1001	588.356	7.354	1521.760	239.840
1030	581.978	7.274	3490.560	1353.136
1041	579.559	7.244	1014.912	197.984
1067	573.840	7.173	2171.536	313.168
2089	349.055	4.363	295.536	39.328
2182	328.600	4.107	13312.512	- 2437.376
2274	308.365	3.854	346.608	44.400
2364	288.569	3.607	1200.976	32.272
2400	280.651	3.508	1614.528	106.032
2417	276.912	3.461	992.656	95.040
2441	271.633	3.395	1161.728	125.920
2686	217.747	2.721	1458.704	134.432
2709	212.688	2.658	815.024	91.792
2722	209.828	2.622	2110.656	146.176
2755	202.570	2.532	921.280	78.992
2815	189.373	2.367	12786.720	- 2450.288
2907	169.138	2.114	318.992	44.448
3022	143.845	1.798	3746.992	416.224
3661	3.299	0.041	428.768	61.392
3676	0.000	0.000	3651.792	1651.104
3691 -	3.299 -	0.041	84.784	49.152
3768 -	20.235 -	0.252	99.056	31.776

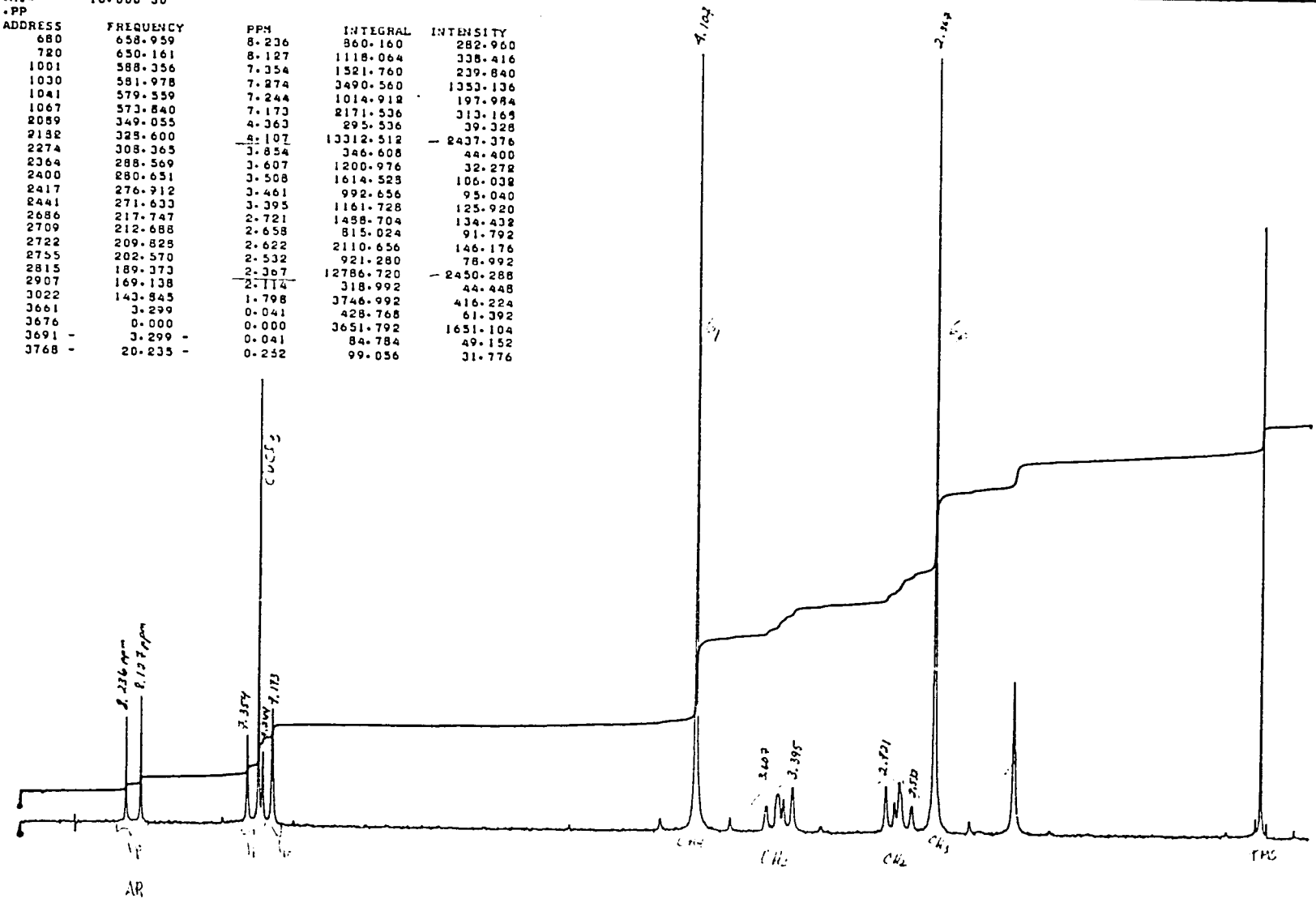

FIGURE 7. NMR Spectrum of the yellow crystals obtained during the purification procedure of Sangre de Grado.

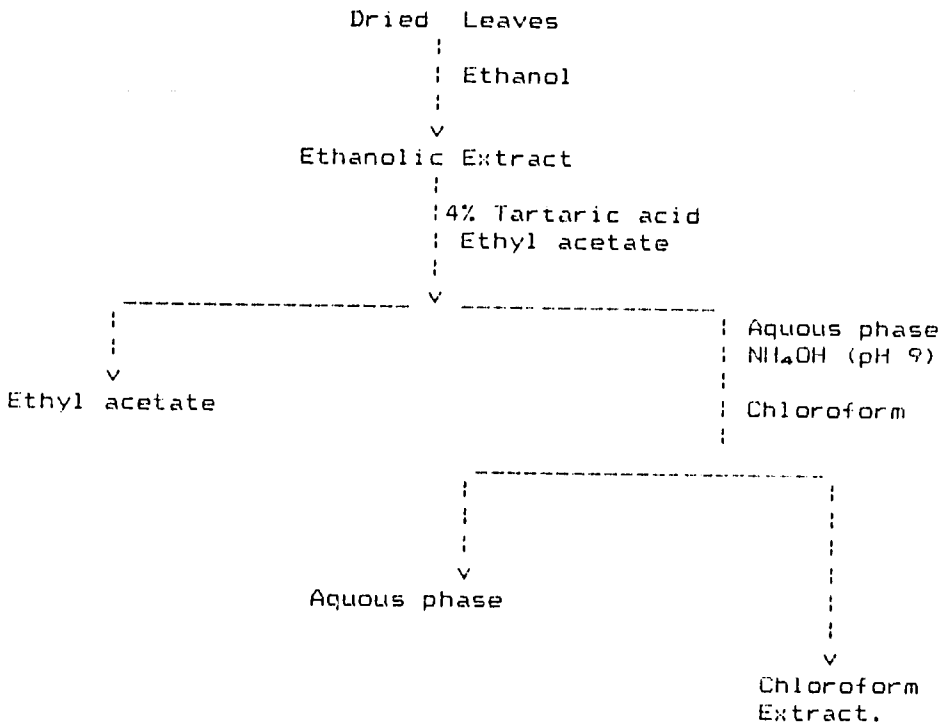

FIGURE 8.- Extraction and purification procedure for the leaves of the *Croton lechleri*.

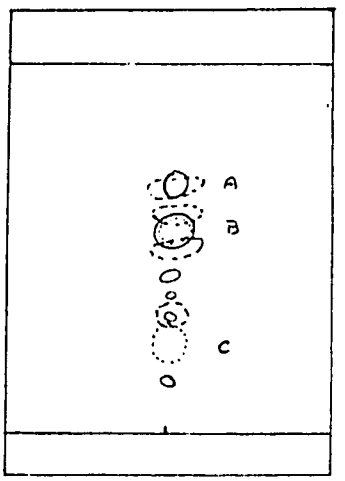

FIGURE 9.- Thin layer chromatography of the chloroform extract from the leaves of the Croton lechleri.

TLC was perfomed on silica gel 60 HF 254 using as eluent a mixture of Chloroform: Methanol (9:1). Spots A, B and C with rfs of 0.63, 0.51 and 0.29 respectively were positive to the Dragendorff reagent.

Fluorescence at 254 nm (————————)

Fluorescence at 366 nm (----------)

Dragendorff positive (.....)

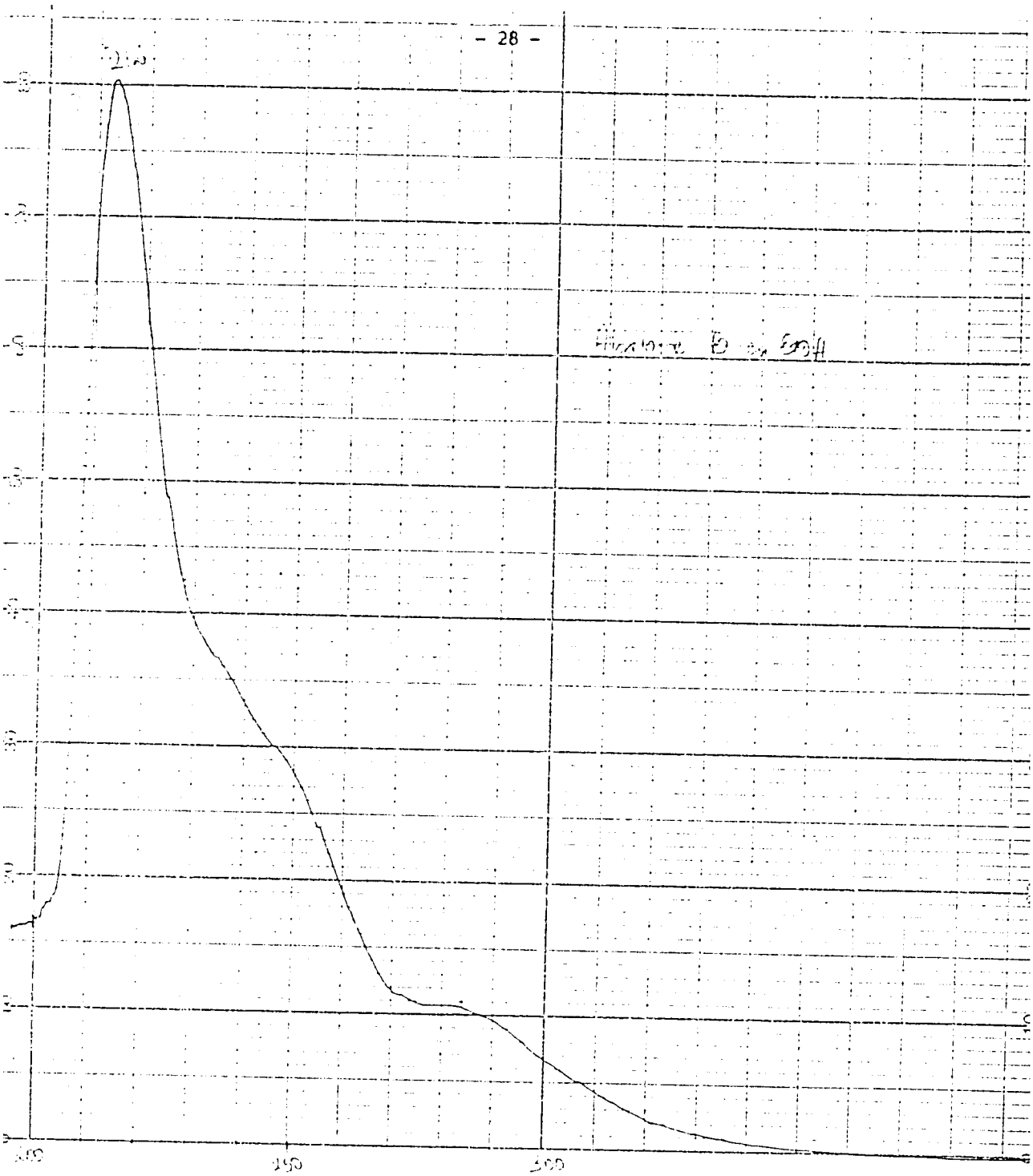

FIGURE 10.- Ultraviolet Spectrum of alkaloid B from the leaves of Croton lechleri in Ethanol.

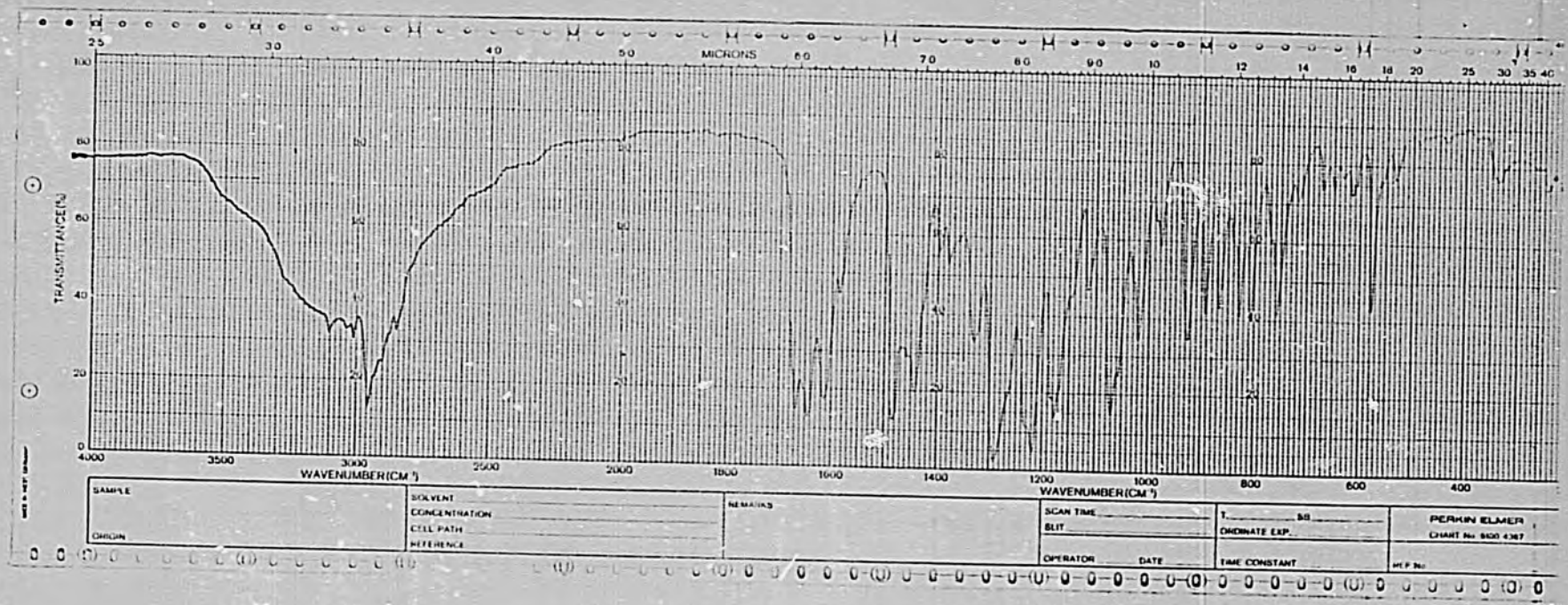

FIGURE 11.- Infrared Spectrum of alkaloid B from, Croton lechleri in KBr.

ADDRESS	FREQUENCY	PPM	INTEGRAL	INTENSITY
920	606.172	7.577	594.720	114.432
932	603.533	7.544	4557.504	629.760
1031	581.758	7.271	16314.560	6942.176
1191	546.567	6.832	485.856	132.384
1229	538.709	6.727	4411.776	950.240
1240	535.789	6.697	4351.424	538.112
1375	506.096	6.326	5577.760	891.392
1409	493.618	6.232	4299.136	754.176
2260	311.444	3.893	14262.144	3223.008
2309	300.666	3.758	16518.176	2191.424
2342	293.408	3.667	3729.152	507.496
2490	260.856	3.260	3562.880	930.752
2547	248.319	3.103	4127.520	181.728
2730	208.069	2.600	2678.240	193.688
2749	203.890	2.548	2777.248	154.400
2774	198.391	2.479	1621.216	228.160
2785	195.972	2.449	16511.232	2532.448
2831	185.854	2.323	1469.728	138.512
2843	183.215	2.290	3170.240	234.080
3078	131.528	1.644	8584.800	1076.384
3565	24.414	0.305	430.976	117.632
3660	3.319	0.043	1060.160	166.528
3676	0.000	0.000	13163.136	4597.936
3691 -	3.299 -	0.041	301.568	116.832

FIGURE 12.- NMR Spectrum of alkaloid B from Croton lechleri in $CDCl_3$.

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