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

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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 us to do long term studies on the same plants without having to worry about the plants being torn down.

RESULTS AND DISCUSSION.

1. ISOLATION, 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 analyzed in order to 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 optimum collection season.

The extraction procedure of the alkaloids from Sangre de Grado is practically 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, is the one being used now 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.

This extraction procedure yields an alkaloid mixture from which two different solids could be separated: A yellow crystalline 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 r_f s 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 r_f of 0.485, which fluoresced when exposed to short wave UV light (254 nm) and gave positive reaction with the Dragendorff reagent according to Munier and Machenboeuf.

- The white amorphous solid gave several spots which fluoresced when exposed to short wave UV light. From these spots the ones with an r_f of 0.439 and 0.12 were intense while the other three with r_f s of 0.28, 0.19 and 0.05 respectively were in trace amounts. From all these spots only the one with an r_f of 0.439 gave a positive reaction with the Dragendorff 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 and Medium Pressure Liquid Chromatography were used obtaining the results that we describe below.

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.

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Eluent:

- 1100 ml of CH₂ Cl₂ : MeOH (3:1) made acid with 10 ml acetic acid.
- 160 ml of CH₂ Cl₂ : MeOH (5:3) made acid with 2 ml acetic acid.
- 180 ml of CH₂ Cl₂ : MeOH (1:1) made acid with 2 ml acetic acid.
- 500 ml of CH₂ Cl₂ : MeOH (85:15) made basic with 5 ml NH₄OH 28%.
- 170 ml of CH₂ Cl₂ : MeOH (10:7) made basic with 2 ml NH₄OH 28%.
- 200 ml of CH₂ Cl₂ : MeOH (1:1) made basic with 2 ml NH₄OH 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 fractions were collected, the volume of each of these fractions oscillated between 10-60 ml. Each fraction 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 R_f 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 peak 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: H₂O (4:6)

Methanol: H₂O (8:2)

Methanol pure

Methanol: CH₃CN (7:3)

Methanol: CH₃CN: H₂O (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), 1985).

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 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 amorphous 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 amorphous 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 dried in vacuo at 70 C.

An identical procedure was followed for obtaining the

hydrochloride of the white amorphous solid.

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

30 kg of dried 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 ammonium 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 r_f of 0.63, spot B with an r_f of 0.51, and spot C with an r_f of 0.29 gave a positive reaction to the Dragendorff reagent according to Munier and 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 crystalline 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 recrystallized in 75% ethanol and 30 mg of white crystals were obtained.

The crystalline compound obtained showed a single spot positive to the Dragendorff reagent and with an r_f of 0.51 on silica gel HF 254 TLC using Chloroform:Methanol (9:1) as eluent. This compound 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 sent 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 Minimal 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% trypsin (T-EDTA, 10 ml per gram of tissue), then allowed to sediment and the supernatant was discarded. This trypsinization 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 typhimurium 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 reversion.

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 dissolved 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 chromatid 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 chinese 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 detachment 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% CO₂ 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 inocuous 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 cytotoxic 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 cytotoxic while concentration of 0.264 ug/ml and lower were not cytotoxic 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 (3×10^6 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 humidified atmosphere of 5% CO₂ 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 - deoxyuridine to a final concentration of 3 ug/ml of medium. Plates were then incubated for an additional 22 hours in a humidified atmosphere of 5% CO₂ 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 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 6. What we observe is that Taspine HCl at non cytotoxic 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

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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) anthracene (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-Tetradecanoyl 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% aqueous 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% aqueous 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.

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 ammonium hydroxide and extracted exhaustively with chloroform. Each chloroform extract was concentrated in a rotavapor then dried 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.

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TABLE 2

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

Taspine-HCl ng/ml	11 hours		42 hours	
	No. Cells	% Dead	No. Cells	% 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

10⁵ 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% CO₂ and 95% air. At 11 hours and at 42 hours sets of plates were collected by trypsinization 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 FIBROBLASTS.

Taspine-HCl. ng/ml	60 Hours		88 Hours	
	No. Cells	% Dead	No. Cells	% 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% CO₂ 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 trypsinization 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).

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 %					
0.021	363	27	99	295	7
0.058	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.

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 %					
0.025	86	37	59	23	23
0.050	88	41	85	30	22
0.110	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
0.0220	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.

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TABLE 6

EFFECT OF TASPINE HCl ON THE FREQUENCY OF SISTER CHROMATID EXCHANGE

Treatment	SCE/Methaphase
Control	7.22 \pm 1.86
Taspine HCl 0.088 ug/ml	8.14 \pm 1.99
Taspine HCl 0.264 ug/ml	11.40 \pm 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 supplemented with 5% fetal bovine serum and 0.5 mg/ml of gentamicin and incubated in a humidified atmosphere of 5% CO₂, 95% air at 37C for 24 hours. Then different concentrations of taspine HCl or medium were added to the corresponding plates simultaneously with 5-Bromodeoxyuridine 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.

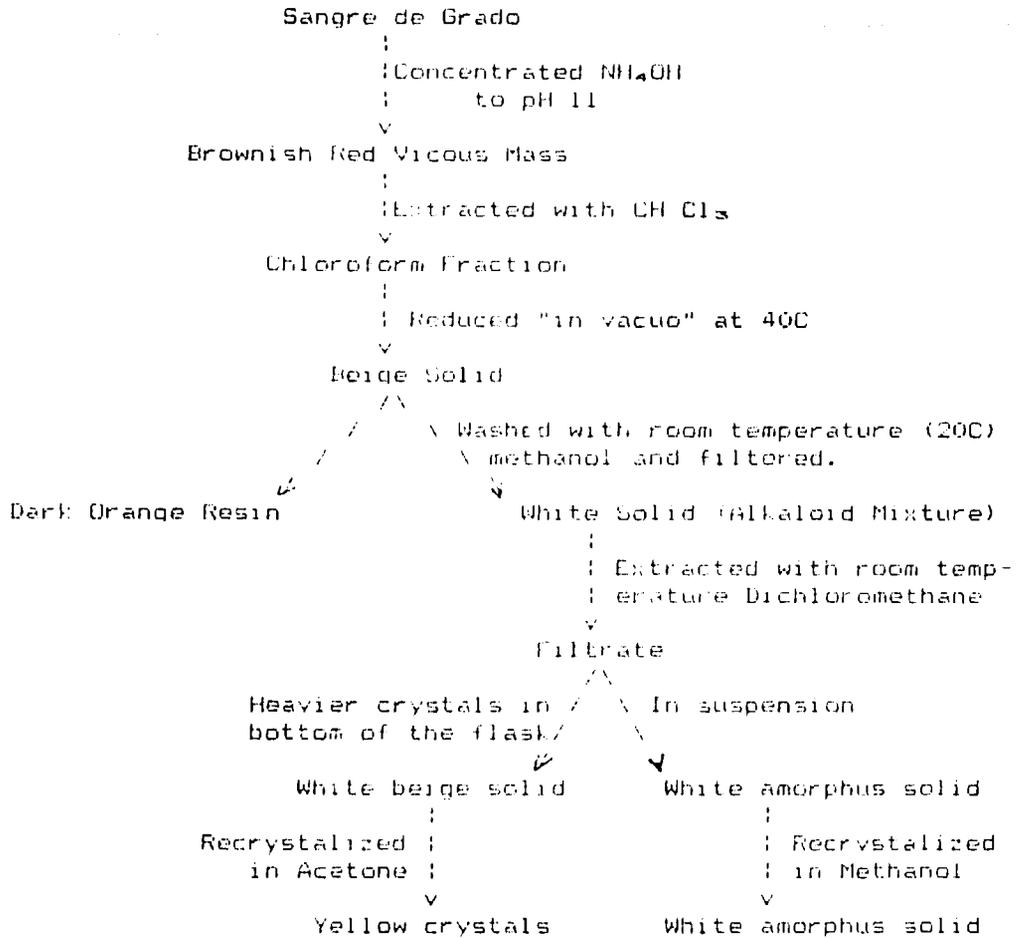
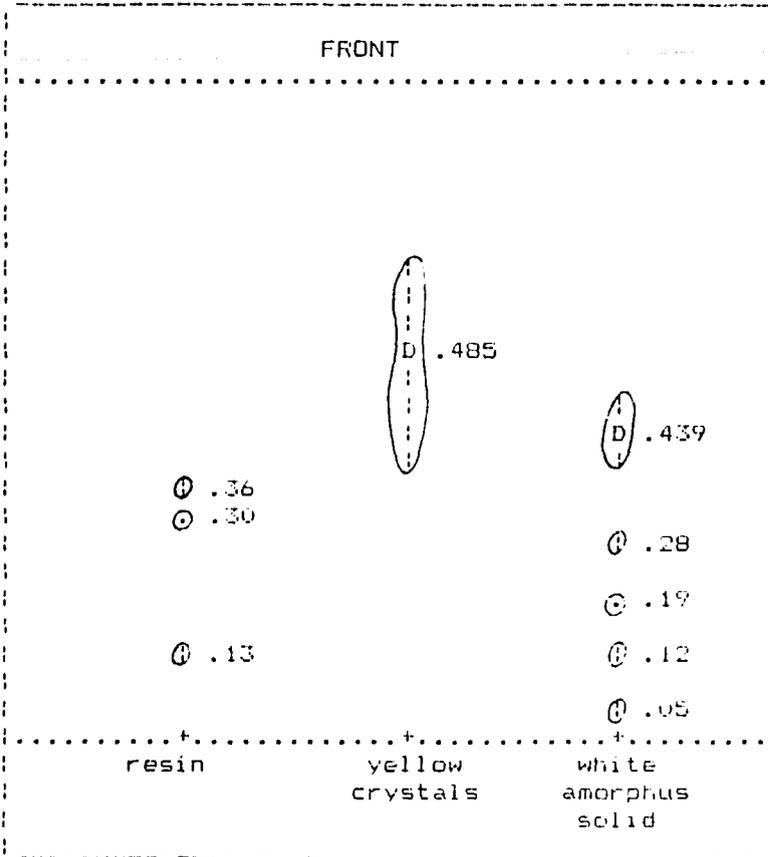


FIGURE 1.- Extraction and Purification Procedure of the Alkaloid from Sangre de Grado.



D = Positive to Dragendorff 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 dichloromethane: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 Rf's are shown next to each spot.

51-

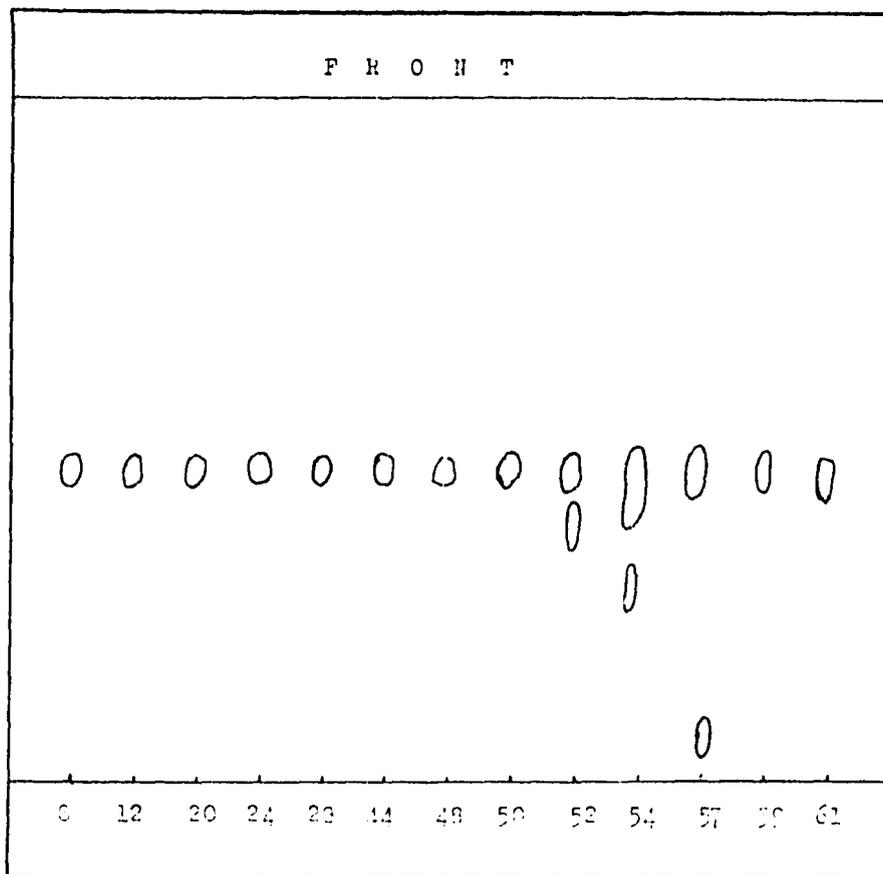


FIGURE 3.- Thin layer chromatography of representative fractions eluted from the dry column. The system was Silica gel 60 F254 Merck and the eluent was dichloromethane: Methanol (3:1) made acid with acetic acid.

52

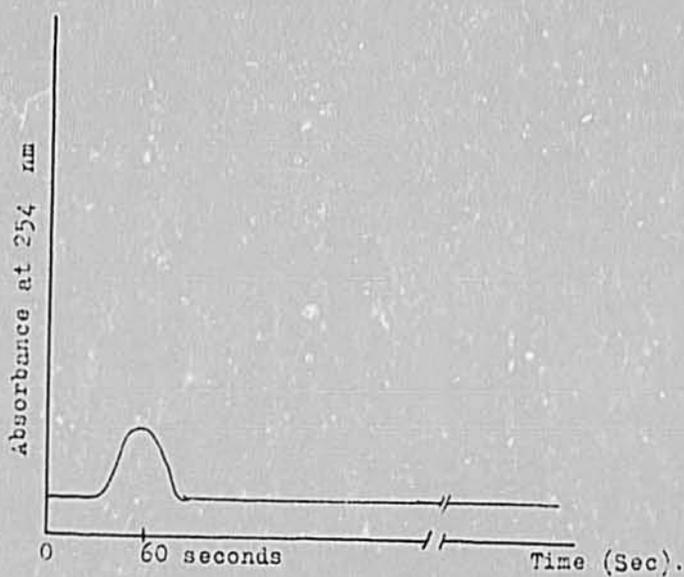


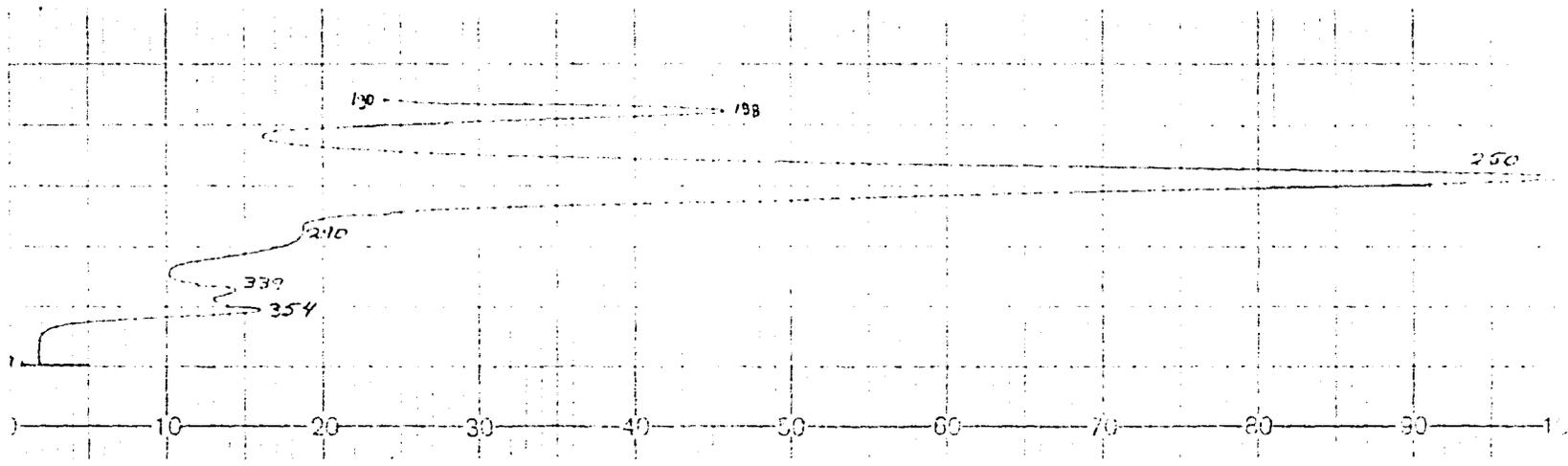
FIGURE 4.- Elution pattern of the crude alkaloid mixture from Sangre de Grado from a Silica gel 60 Medium Pressure Column of the following characteristics:
Stationary phase Silica gel 60
Dimension: 25 x 2 cm.
Detector: UV 254 nm lamp
Eluent: Dichloromethane: Methanol (3:1).
Column was run up to 4 hours.

57

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 .

55

FIGURE 5 A



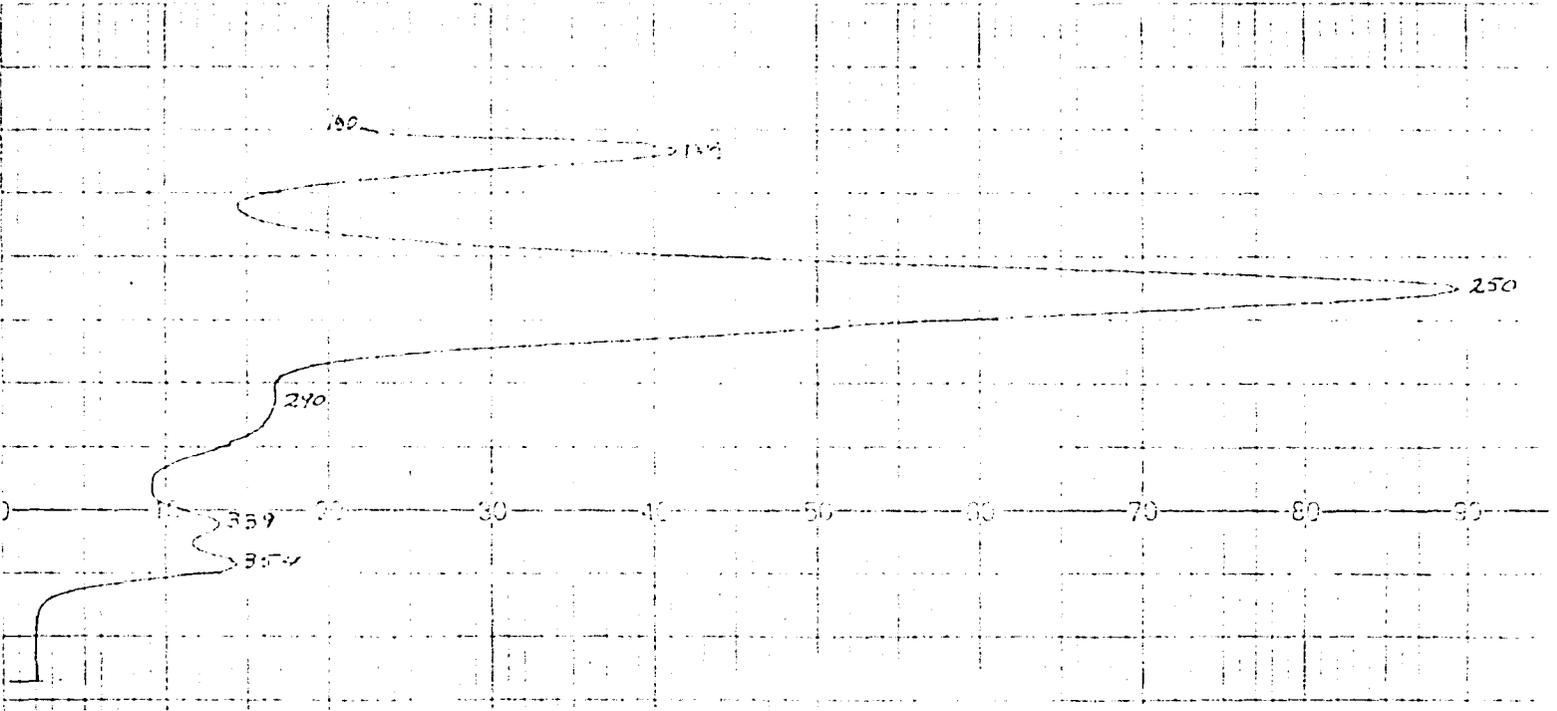


FIGURE 5 B

0

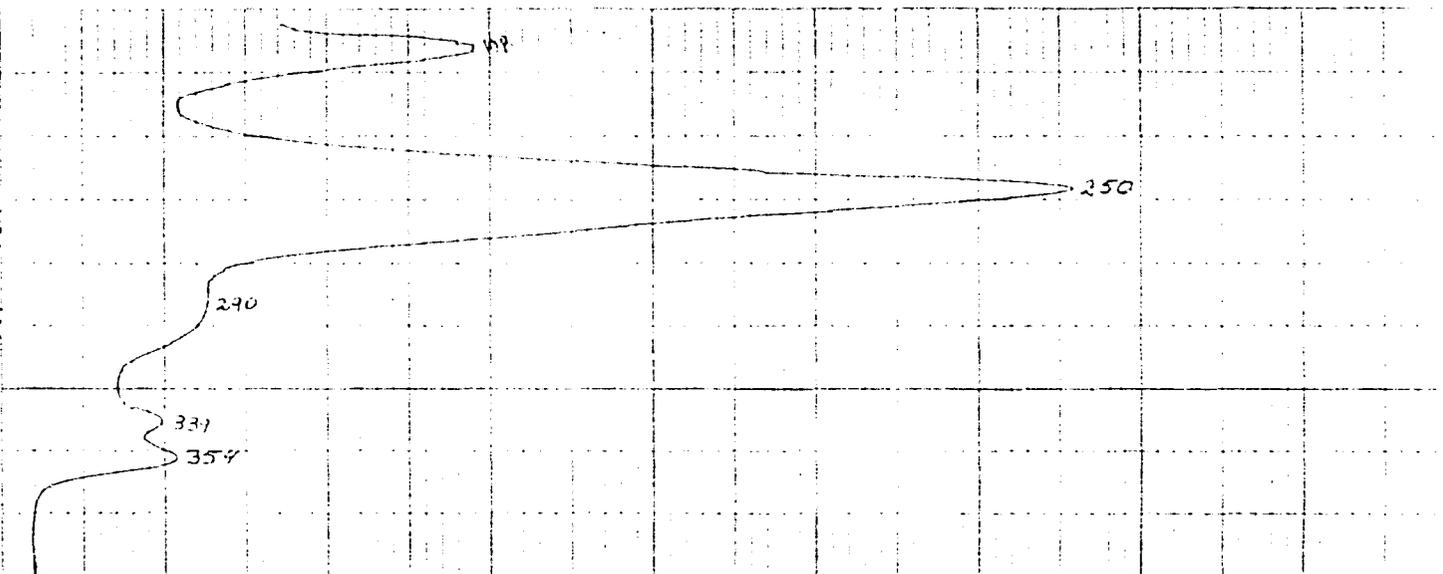
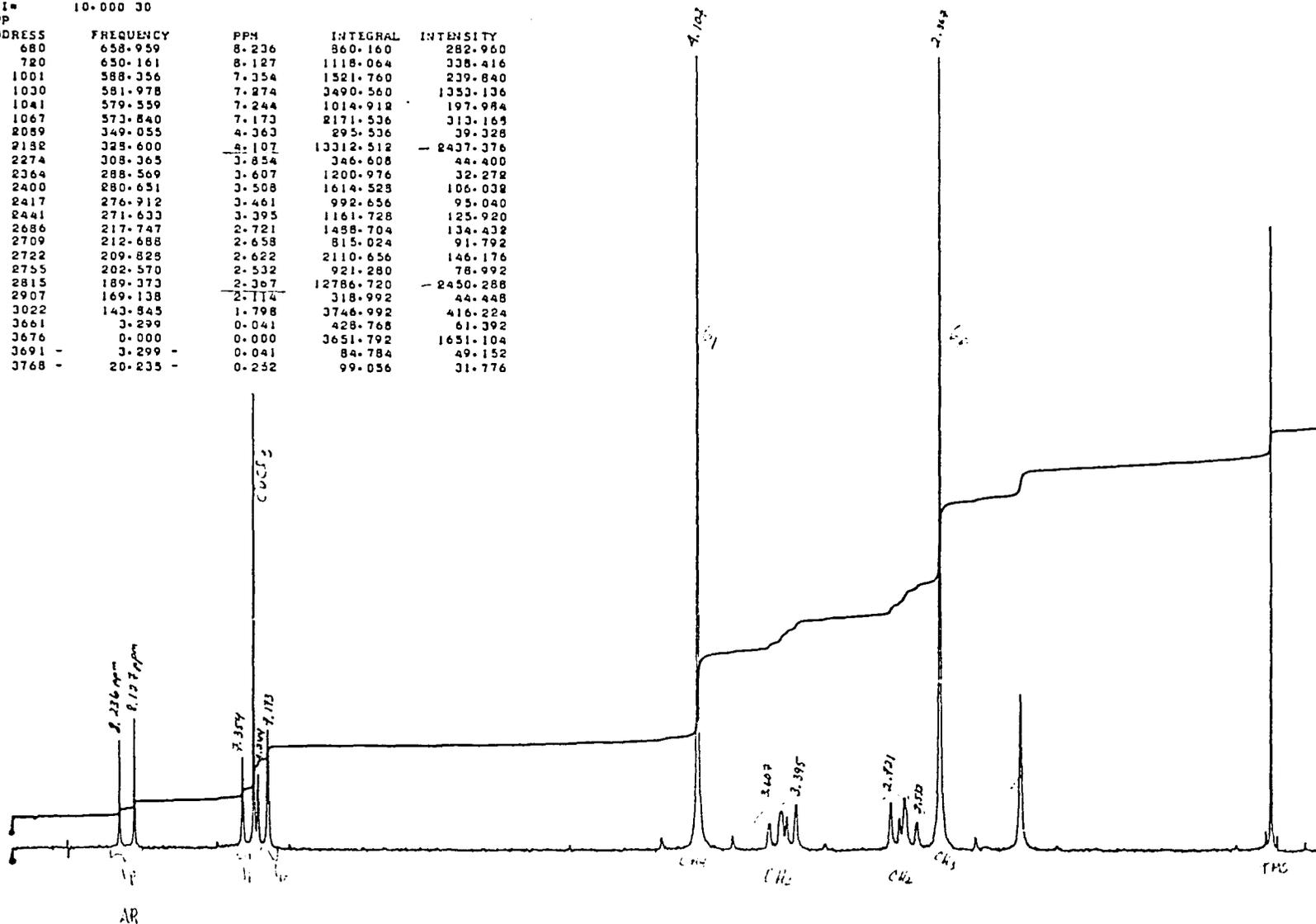


FIGURE 6 UV Spectrum of the Hydrochloride of the White Amorphous Solid from Sangre de Grado in H₂O.

51

.MI= 10.000 30

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
2132	328.600	4.107	13312.512	2437.376
2274	308.365	3.854	346.608	44.400
2364	288.560	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	1488.704	134.432
2709	212.688	2.658	815.024	91.792
2722	209.825	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	0.299	0.041	428.768	61.392
3676	0.000	0.000	3651.792	1651.104
3691	0.299	0.041	84.784	49.152
3768	20.235	0.232	99.056	31.776



80 MHz
 CUCS
 CH2
 CH3
 TMS

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

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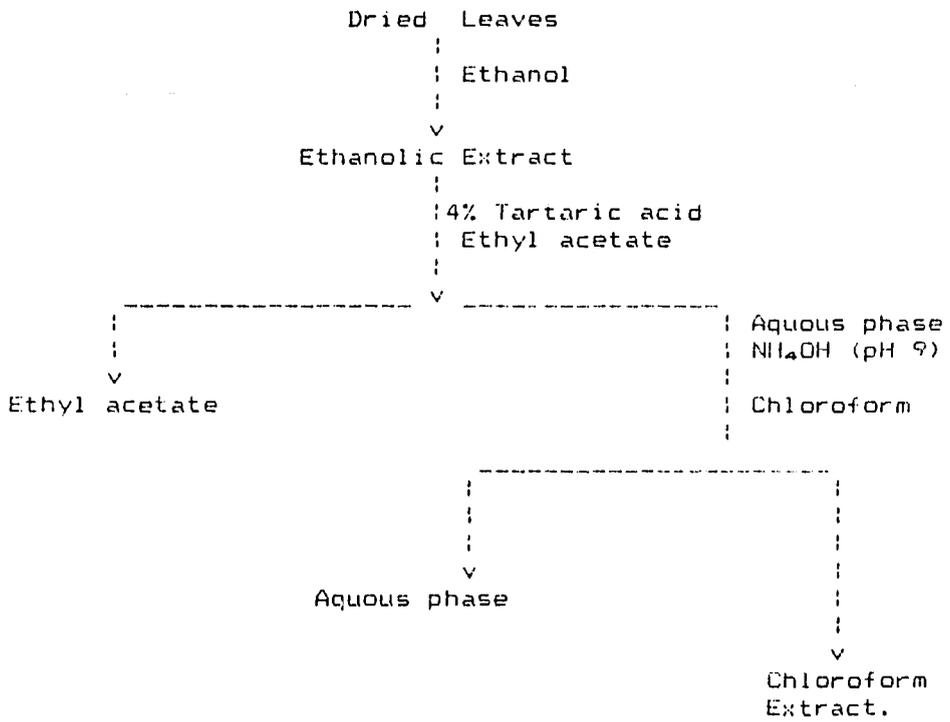


FIGURE 3.- Extraction and purification procedure for the leaves of the Croton lechleri.

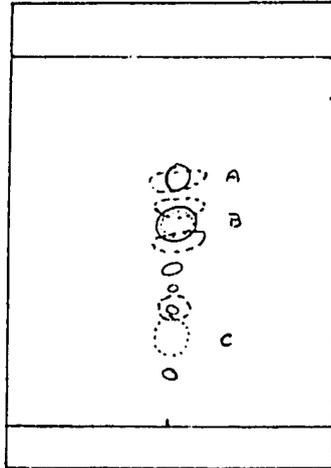


FIGURE 9.- Thin layer chromatography of the chloroform extract from the leaves of the Croton lechleri.

TLC was performed 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 (.....)

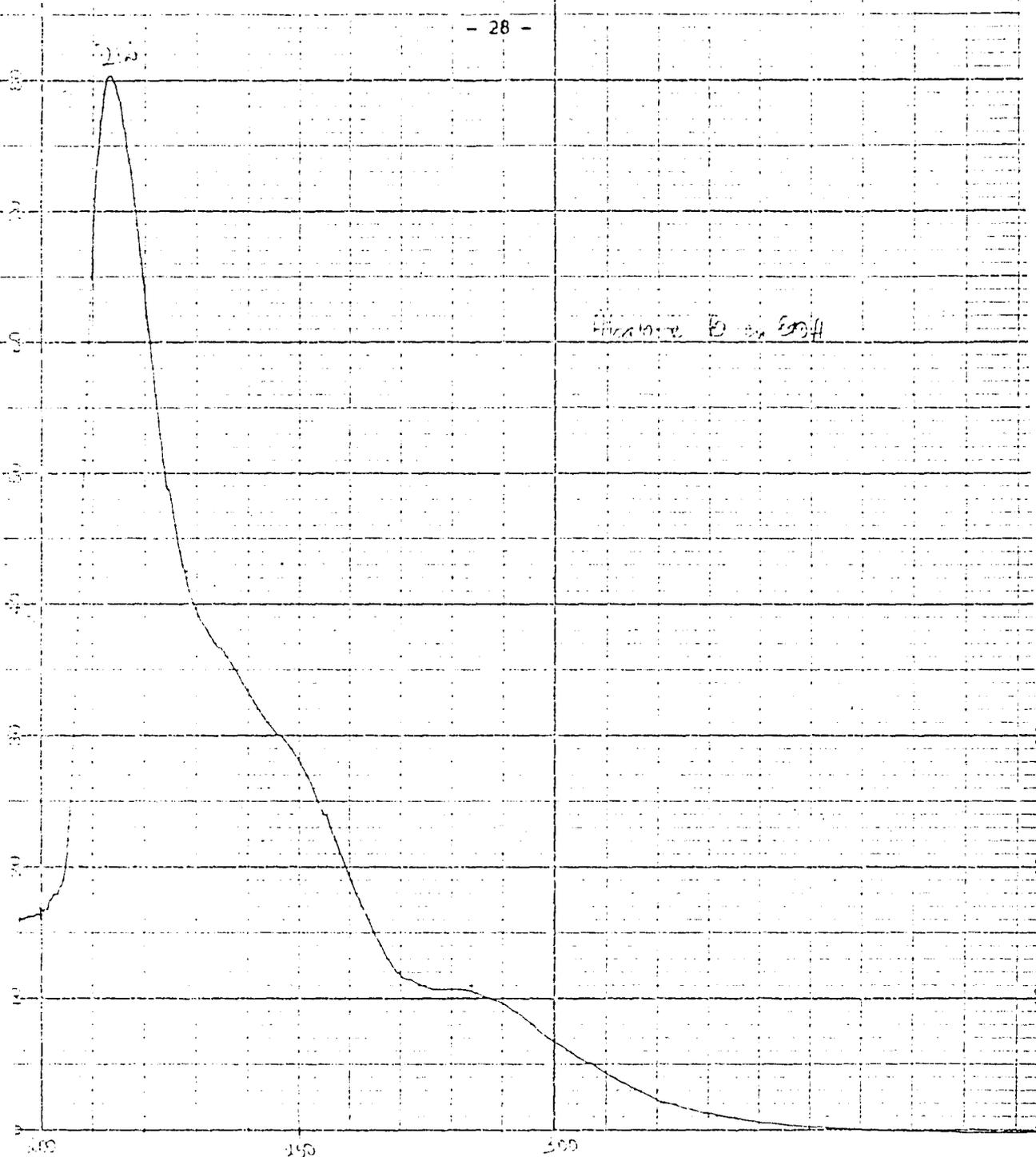


FIGURE 10.- Ultraviolet Spectrum of alkaloid B from the leaves of Croton lechleri in Ethanol.

611

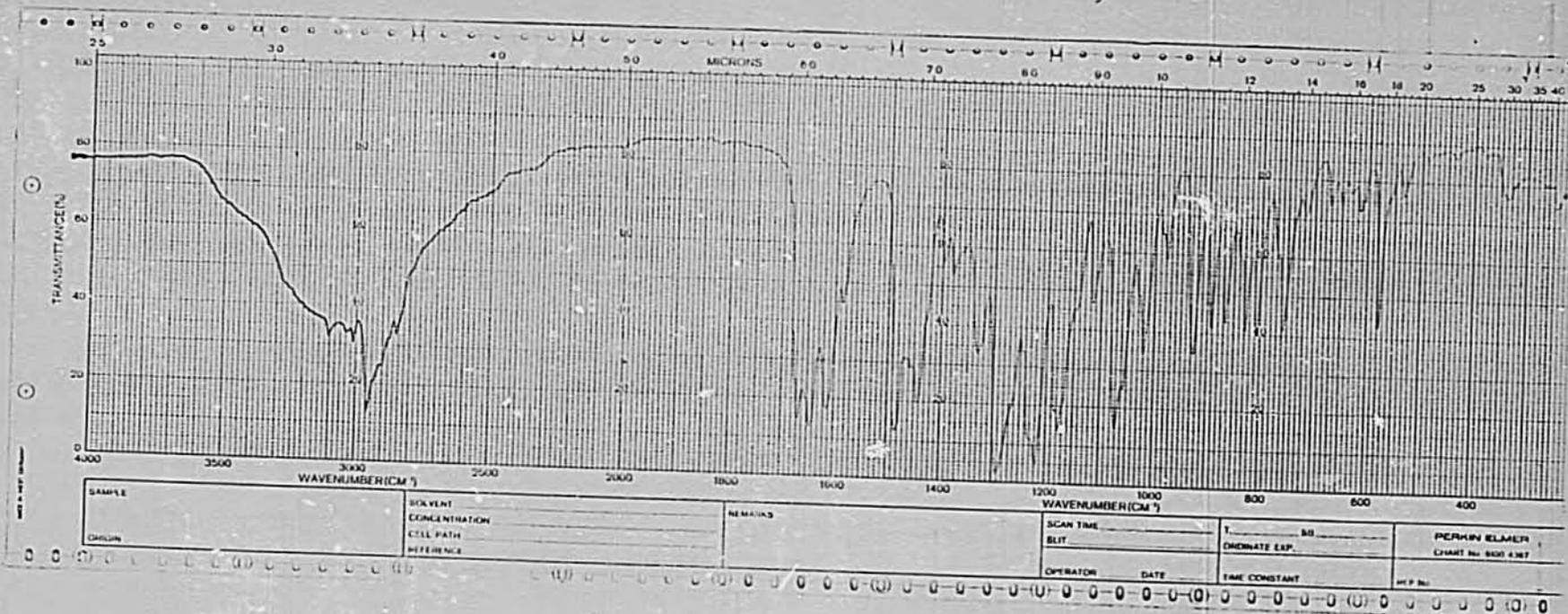


FIGURE 11.- Infrared Spectrum of alkaloid B from, Croton lechleri in KBr.

PP 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	6.932	435.856	132.384
1229	538.709	6.727	4411.776	920.740
1240	532.734	6.697	4351.424	538.112
1375	506.094	6.326	5577.760	891.392
1409	498.618	6.232	4299.136	754.176
2260	311.444	3.893	14262.144	3223.008
2309	306.676	3.758	16513.176	2191.424
2342	293.408	3.667	4729.152	2074.496
2490	266.856	3.160	3562.880	530.720
2547	248.319	3.103	4127.120	181.728
2730	208.042	2.600	2672.240	194.888
2749	203.290	2.548	2777.248	154.400
2774	198.391	2.479	1621.216	228.160
2785	195.472	2.444	16511.232	2332.448
2831	185.854	2.325	1469.728	435.512
2843	183.215	2.290	3170.240	524.080
3078	131.555	1.644	8584.800	1076.384
3565	24.414	0.305	430.976	117.652
3660	3.319	0.062	1060.160	166.568
3676	0.000	0.000	13163.136	4587.936
3691	3.299	0.041	301.568	116.832

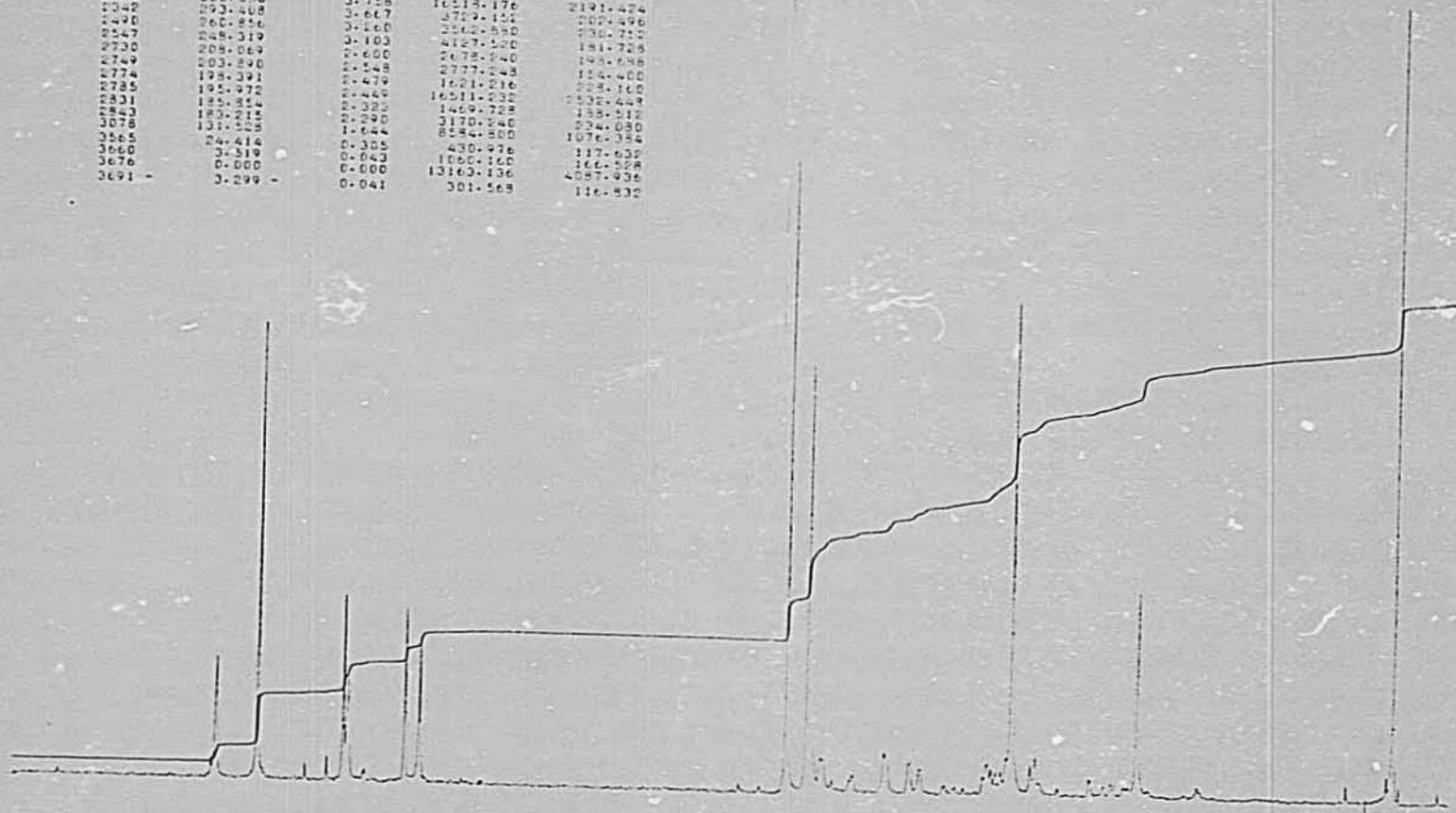


FIGURE 12.- NMR Spectrum of alkaloid B from Croton lechleri in $CDCl_3$.