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FINAL 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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2

DESCRIPTION OF THE RESEARCH CARRIED OUT.

In the following pages we describe the work that has been done in our laboratory during the last 28 months in relation to the cicatrizant properties of the plant extract Sangre de Grado. The report is divided into three main parts:

The first part includes findings on the collection of the Sangre de Grado extract and on the extraction, purification, characterization and quantitation of the alkaloids from Sangre de Grado as well as new findings on the extraction, purification and characterization of the alkaloids present in the leaves of Croton lechleri.

The persons that collaborated on this part of the research were Dr. Ramon Ferreyra (Botanist), Gerald B. Hammond, Ph.D (Chemist) and the students Maria del Carmen Mustiga and Lily Carlin.

The second part includes experiments on the mechanism of action of Sangre de Grado and the active principle extracted from it. The students that collaborated on this part of the work were Maria del Carmen Planas, Marcos Milla, and Carlos Regalado.

The third part includes experiments on the possible mutagenic carcinogenic and/or tumor promoter activities of Sangre de Grado and its active principle.

The persons that collaborated on this part of the work were the student Jose Luis Cordova and Dr. Elba Rosas de Agusti (Pathologist).

While working on this project we were able to locate in Iquitos a plantation owned by Dr. Hamilton Mencher, that had trees of the species we were working with and who allowed us to do long term studies on the same plants without having to worry about the plants being torn down.

31

ABSTRACT.

Sangre de Grado extract as well as leaves and cortex were collected from trees of the species Croton lechleri growing in the peruvian jungle.

The Sangre de Grado extract and the cortex, were found to contain principally one alkaloid that was identified as Taspine. The leaves were found to contain three alkaloids from which we were able to obtain only one in crystalline form. This alkaloid was identified as Sinoacutina. Taspine was present in the Sangre de Grado extract at concentrations between 3.7 and 0.63 mg/ml, while in the cortex of the tree it was only present in trace amounts. Sinoacutina on the other hand, was also present in trace amounts in the leaves of the plant.

Taspine was shown to be the active cicatrizing principle in the Sangre de Grado extract using the cicatrization test with mice. For these "in vivo" tests we used the chlorhydrate form of taspine because its free base was insoluble in water. Taspine Chlorhydrate (Taspine-HCl) was found to accelerate the cicatrization process when applied locally.

"In Vitro" experiments with taspine-HCl in order to study its mechanism of action in tissue culture systems, showed that the alkaloid was nontoxic to human foreskin fibroblasts at concentration below 200 ng/ml and that it had no effect on cell proliferation. On the other hand taspine-HCl was found to inhibit the contraction of fibroblast-collagen lattices and to increase the migration of human foreskin fibroblasts to areas on the plates clear of cells.

Mutagenicity studies using the Ames test showed that Sangre de Grado was a weak mutagen while taspine-HCl had no mutagenic activity. Studies on the effect of Sangre de Grado and taspine-HCl on sister chromatid exchange (SCE) showed that taspine-HCl increased the number of SCEs in V79/AP4 cells, but no increase was evident when CHO cells were used. Using the two-stage mouse skin carcinogenesis system we have shown that neither Sangre de Grado nor taspine-HCl were carcinogenic or tumor promoters after one year of treatment.

RESULTS AND DISCUSSION.

I. 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 had 40 trees which were shown later to belong to the species we were working with. On this first trip we were able to obtain the botanical samples from seven of these trees and we also extracted Sangre de Grado at different times of the day from seven different trees, in order to corroborate reports from the natives that earlier times are better for the extraction of Sangre de Grado. In Table 1 we show that the amount of Sangre de Grado obtained varies from tree to tree but there was 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.

These determinations were done measuring the weight of the crude alkaloid extracted from the Sangre de Grado samples. A second method for determining the alkaloid content of Sangre de Grado is also shown in Table 1, and this time we made use of the extinction coefficient of taspine at 347 nm to calculate the concentration of taspine on the samples. This latter method was simpler than the first one and as we can see on Table 1, the results of both methods were very similar.

Two more trips to this same location took place in February, 1985 and in May, 1985. During these trips Sangre de Grado was collected from several trees and the content of alkaloid was determined. In Table 2. we present the results from these determinations, and what they show is that the yield of alkaloid was higher in February. By comparing these results to the ones on Table 1, we can conclude that the yield of alkaloid from the trees is much higher during the month of December.

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 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 Dragendorff reagent according to Munier and Machenboeuf (Thin Layer Chromatography by E. Stahl. 2nd Edition. Springer-Verlag, 1969).
- The white amorphous 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 were intense while the other three with rfs of 0.28, 0.19 and 0.05 respectively were in trace amounts. From all these spots only the one with an rf of 0.439 gave a positive reaction with the Dragendorff reagent according to Munier and Machenboeuf

1.1. 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 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 NH₄OH 28%.

170 ml of CH_2Cl_2 : MeOH (10:7) made basic with 2 ml NH₄OH 28%.

200 ml of CH_2Cl_2 : 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 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 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.

1.2. 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 chlorhydrate 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 68: 124-126, 1979) and Shamma and Moniot (Chem. Commun. pp.1065, 1971).

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. J. Pharm. Sci. 68: 124-126, 1979).

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. pp.1065, 1971).

The ultraviolet spectrum of the chlorhydrate 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.

1.3. PREPARATION OF TASPINE CHLORHYDRATE (TASPINE-HCl).-

HCl gas was passed through a chloroform solution of Taspine (yellow alkaloid crystal) until no further precipitation was observed. The alkaloid chlorhydrate 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 chlorhydrate of the white amorphous solid.

2. EXTRACTION PURIFICATION AND CHARACTERIZATION OF THE ALKALOIDS FROM THE LEAVES OF THE Croton lechleri.

2.1. 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 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 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.

2.2. CHARACTERIZATION OF THE ALKALOIDS FROM THE LEAVES OF THE Croton lechleri.

The crystalline 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 compound had a melting point of 200-202 C. 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 δ 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).

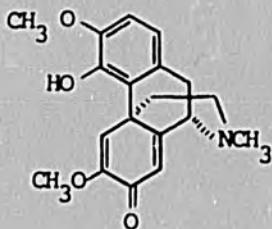
A sample of the alkaloid B was sent to Dr. Gerald B. Hammond (Department of Chemistry, University of Iowa, USA), were it was analyzed with a gas chromatographer attached to a mass spectrometer (GC-MS) Varian in order to verify the degree of purity of the crystals and to get an idea of the molecular weight and type of fragmentation of the sample.

From the mass spectrum (Figure 13.) the molecular ion was visible at m/e 327 (89.5%) and the peaks of high intensity were 312 (48%), 299 (66.3%), and 284 (100%).

This information discarded the presence of Glaucine or Taliporphine reported by Bettolo R.M., and Scarpatti M.L. (Phytochem. 18: 520, 1979) to be present in *Croton draconoides* but ¹H RMN spectrum indicated certain relation with this class of aporphinoids. After considering other parameters of the genera *Croton* from the Euphorbiaceae family and the spectroscopic data, the possibilities were reduced to two compounds: Salutaridine or its enantiomer Sinoacutina. The optical activity of the sample was measured and found to be: $[\alpha]_{D}^{25} = -132^{\circ}$ (c 0.33, methanol)

This value was similar to the one reported for Sinoacutina: $[\alpha]_{D}^{25} = -115^{\circ}$ (c 0.55, methanol) by Kametani et al. (J. Chem. Soc. (C), 2030, 1969). The most significative part was that the signs coincided.

Our conclusion was that alkaloid B was Sinoacutina (Chemical Abstract Index: 4090-18-0) :



3. EXTRACTION AND PURIFICATION OF THE ALKALOIDS FROM THE CORTEX OF THE *Croton lechleri*.

250 grams of cortex were grinded and then macerated in 1.5 l of 95% Ethanol. The ethanolic solution was concentrated in vacuo and then dissolved in ethyl acetate. This was followed by extraction with a 4% tartaric acid solution. The tartaric acid phase containing the alkaloids was taken then to pH 9.5 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 40°C.

Thin layer chromatography of the chloroform extract on silica gel HF 254 using as eluent Dichloromethane:Methanol (3:1) resolved one spot that comigrated with purified taspine.

II. STUDIES TOWARDS THE ELUCIDATION OF THE MECHANISM OF ACTION OF THE ACTIVE PRINCIPLE FROM SANGRE DE GRADO.

The empirical applications of Sangre de Grado together with the preliminary work of Chirinos J., Aguilar A., y Scarpatti M.L. (*Anales de V Congreso Latinoamericano de Farmacología y Terapéutica Lima, Peru. Page 227, Octubre de 1974.*) suggested that Sangre de Grado might be affecting cellular proliferation or tissue cell interactions. It is because of this that we have studied the effect of the active principle from Sangre de Grado on cell proliferation, cell migration, and on the contraction of cell-collagen lattices.

In the previous section we have purified and characterized from the Sangre de Grado extract the alkaloid Taspine which we show below, in our "in vivo" experiments, to be the active cicatrizing principle. It is also important to mention at this point that since Taspine was insoluble in water we have been working mainly with its chlorhydrate both for the "in vivo" and "in vitro" experiments.

1. "IN VIVO" EXPERIMENTS ON THE CICATRIZANT ACTIVITY OF THE ACTIVE PRINCIPLE FROM SANGRE DE GRADO.

We have tested Sangre de Grado as well as the alkaloid Taspine and the chlorhydrate we prepared from it for cicatrizing activity.

The protocol for a typical "in vivo" cicatrization test was as follows: Male mice (Strain Sencar) of 2-3 months of age whose weights oscillated between 25-30 grams were maintained in a room at 22-25°C and received food and water ad libitum. Before making the wounds, the back of the mice was shaved with surgical clippers at the level of the scapular waist (an area that could not be scratched by the mouse). This same area was then depilated using Opilca(Hoescht). 48 hours later the weight of the mice was measured and they were distributed in groups at random so as the average and the standard error of the average of the weights of the groups were homogeneous. Each mouse was placed in a separate cage with a card that had the following information: number of the animal, initial weight, starting hour and date, duration of the experiment, final weight, wound breaking strength (WBS), observations if any and treatment it received. The latter information was recorded on the card after measuring the WBS of the mouse and right before the statistical analysis was made. All this information was also recorded in a notebook that was utilized at the time the different treatments were administered. The purpose of this procedure was to prevent the operator from making subjective errors when measuring the WBS.

Next, mice were anesthetized with vapors of diethyl ether and a 1 cm incision was made perpendicular to the axis of symmetry of

the animal and the two borders of the wound were stitched together at its center. Treatment was started immediately, and every 12 hours the compound being tested was applied to the wound, for this purpose 0.05 ml of the solution being tested was applied slowly and directly to the wound by means of a micropipete. The controls received only the solvent in which the compound was diluted.

48 hours later, mice were sacrificed with an ether overdose and the WBS were quantitated by fixing one of the borders of the wound (after cutting the stich) while applying a measurable force to the other one.

Once all the WBS were measured and recorded on their respective cards, we recorded the treatment received by each mouse on the same cards. The data was then subjected to statistical analysis using the Student's-t-Test. Values were significant when $p < 0.05$. (Daniel W. Bioestadística. Editorial Limusa. Mexico, 1979).

The percentage of activity was calculated according to the following formula:

$$\% \text{ Activity} = \frac{\text{WBS}_t - \text{WBS}_c}{\text{WBS}_c} \times 100$$

WBS_t = Average of the force necessary to open the wound of a treated mouse.

WBS_c = Average of the force necessary to open the wound of an untreated mouse (Control).

In Table 3, we show the results from an experiment in which we tested the cicatrizant effect of the Sangre de Grado extract and the alkaloid extracted from it, which was shown in the previous section to be Taspine. We tested both, the free base of taspine which is insoluble in water and the chlorhydrate we prepared from it which is soluble in water. As we can observe on the Table, both Sangre de Grado and taspine chlorhydrate had significant cicatrizant effect. The free base of taspine had very low cicatrizant activity and this was probably due to difficulties in applying the right concentration of the compound because it was not possible to make a uniform suspension of the alkaloid in water.

The data shows that taspine is the active cicatrizant principle in the Sangre de Grado extract from Croton lechleri.

1.1 DOSE-RESPONSE CURVE FOR THE CICATRIZANT EFFECT OF TASPINE CHLORHYDRATE (TASPINE-HCl).

Once we demonstrated that taspine was the active principle from Sangre de Grado, we studied the effect of different

concentrations of the alkaloid chlorhydrate on the cicatrization process.

Table 4, shows the result of this experiment. As we can observe there is a significant cicatrizing activity of taspine-HCl at doses between 0.050 to 0.20 mg/ml., being 0.066 mg/ml the optimum concentration. Since these mice received four applications of 0.050 ml of a 0.066 mg/ml solution of taspine-HCl, the total dose of alkaloid they received was 0.0132 mg (0.4 ug/gr weight, since the average weight of these mice was 28 grams).

1.2 KINETICS OF CICATRIZATION IN THE PRESENCE AND IN THE ABSENCE OF TASPINE CHLORHYDRATE.

Knowing the optimum concentration of taspine-HCl to be used in an "in vivo" cicatrization experiment, it was important to investigate the kinetics of the cicatrization process in the presence of the alkaloid chlorhydrate in order to get an approximation to how it was working.

For this purpose one hundred mice were divided into ten groups of ten mice each. In each group, five mice received treatment (0.050 ml of a 0.066 mg/ml solution of taspine-HCl every 12 hours) while the other five remained as controls.

At 12 hours, the wound breaking strength (WBS) of the first group of mice was measured, and from then on the same procedure was repeated with one group at a time at 12 to 24 hour intervals.

The result of this experiment is shown in Table 5, and in Figure 14. What we observe is that as early as 12 hr there is a significant increase in the wound breaking strength of wounds treated with taspine-HCl as compared to the untreated wounds. These significant increase in cicatrization was evident during the first 60 hours of the experiment. At 72 hours the difference in the wound breaking strength of the wounds of the treated and untreated mice were no longer statistically significant, and from then on until the end of the experiment these values remained very close.

What these data suggest is that the final product, that is the healed wound, is similar for both the treated and untreated mice. What is different between the taspine-HCl-treated and untreated mice, is the rate at which the wound reaches a point where it is more resistant to be opened. In other words, in the early stages of cicatrization, taspine-HCl accelerates the process, so the wound is more resistant to be opened..

2. CELL CULTURE STUDIES ON THE MECHANISM OF ACTION AND TOXICITY OF TASPINE CHLORHYDRATE (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 Eagle's 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 by trypsinization. These fibroblast were then frozen in liquid nitrogen and were our stock of cells for the experiments.

2.1. CELL PROLIFERATION AND TOXICOLOGICAL STUDIES OF TASPINE-HCl ON HUMAN FORESKIN FIBROBLASTS.

It was important for us to find out first the maximum dose at which we could use taspine-HCl in our fibroblast cultures. In doing so we studied also the effect of taspine-HCl on fibroblast proliferation. For this purpose two sets of 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 6, and what we observe is that we should use concentrations below 250 ng/ml of taspine-HCl, since at higher concentrations the percentage of dead cells increased dramatically.

The second set of experiments were carried out in order to measure the effect of taspine-HCl on subconfluent cells. The results of these experiments are shown in Table 7, and what we observe is that concentrations of 200 ng/ml and lower could be used safely since the percentage of dead cells due to the alkaloid 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 below 200 ng/ml. An experiment was carried out in order to verify this observation and the results are shown in Table 8, as we can observe taspine-HCl did not have an effect on cell proliferation.

Another method to study the effect of taspine-HCl on cell proliferation is by determining if it increases the number of cells that divide in a culture that has entered the stationary phase (Go). In Table 9, we show the result of this experiment, and as we can observe taspine-HCl at the concentrations tested did not increase the number of cells entering mitosis.

2.2. EFFECT OF TASPINE-HCl ON THE MIGRATION OF HUMAN FORESKIN FIBROBLASTS.

The migration of fibroblasts to the wound area could be accelerated by a cicatrizing compound. It is because of this that we studied the effect of taspine-HCl on this parameter, and the method we followed was the one described by Burk R.R. (PNAS 70:369-372, 1973). Cells were inoculated to 60 mm tissue culture dishes and when they reached confluence, a superficial line was made by placing a razor blade perpendicular to the surface of the plate and then by moving the razor blade towards the right the cell sheet was pushed in order to "clear" an area to the right of the line. Plates were then washed with PBS in order to eliminate all the cell debries and media without serum (Minimal essential medium Eagles with Hanks salts + 10mM Hepes, 50 ug/ml gentamycin) and with the different concentrations of taspine-HCl was added. Plates were then screened and the areas along the line clear of cells, were marked with the point of a fine bisturi. Plates were then placed at 37 C in a humidified atmosphere of 5% CO₂ and 95% air and at the time specified on the Table, the number of cells/cm that crossed the line along the marked areas were counted under a phase contrast microscope. What this method actually measures is the migration of cells to areas of the plate that have been cleared out of cells.

Our results are shown in Table 10, and as we can observe there was a significant effect of taspine-HCl on the migration of the fibroblasts to the cleared areas of the plate. This effect was observed at very low concentrations of taspine-HCl (2 ng/ml). Table 11. shows the results of an experiment similar to the one described in the previous Table, but here the medium was supplemented with 1% fetal bovine serum. Here it was also evident that taspine-HCl had a significant effect on the migration of the fibroblasts at concentrations between 2 and 8 ng/ml.

2.3. EFFECT OF TASPINE-HCl ON THE CONTRACTION OF LATTICES MADE OF HUMAN FORESKIN FIBROBLASTS AND COLLAGEN.

Wound contraction is another important parameter in the cicatrization process that we have studied using as a model system the contraction of cell-collagen lattices described by Bell and collaborators (Proc. Natl. Acad. Sci. USA 76:1274-1278, 1979).

Collagen-fibroblast lattices were prepared on 60 mm bacteriological glass dishes, so cells would not attach to the bottom. Each dish contained 1.4 ml of a collagen solution, 0.4 ml 5X Minimal essential medium Eagles with Hanks salts (MEM-H), 0.025 ml of 1M Hepes, 0.075 ml gentamycin (2 mg/ml), 1 ml of fetal bovine serum, 0.1 ml 1M NaOH, and 0.005 ml of the taspine-HCl solution. All these was kept in an ice bath until the foreskin fibroblasts were added in 0.5 ml of MEM-H. Once the cells were added, the plates were incubated at 37 C in a humidified atmosphere of 5% CO₂ and 95% air. Lattice diameters on the dishes were measured at different times.

The results of two different experiments utilizing different concentrations of cells and collagen and each one, two different concentrations of taspine-HCl are shown in Figure 15, and in Figure 16. As we can observe, the lowest concentration of taspine-HCl that is 52 ng/ml had no significant effect on the contraction of fibroblast-collagen lattices, while the concentration of 130 ng/ml decreased the rate at which the lattices contracted. This decrease, an even inhibition at one point of the rate of contraction of the lattices by the higher concentration of taspine-HCl was not the result of cell damage since in earlier experiments it was shown that those concentrations of alkaloid were non-toxic to the cells.

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

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

For this purpose we have used 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.

This Salmonella test has been validated for the detection of carcinogens as mutagens by studying a large number (about 300) of organic chemicals, of many chemical classes, which have been tested in the conventional animal carcinogenicity tests (McCann J., Choi E., Yamasaki E., Ames B.N. Proc. Natl. Acad. Sci. USA. 72:5135-5139. 1975).

The strains that we used were: TA 102, TA 100, TA 98, TA 97, TA 1538, TA 1537 and TA 1535, and were obtained from the Laboratory of Dr. Bruce N Ames.

It is also interesting to point out that since it is known how the original mutations originated (Base-pair substitutions for strains TA 1535 and TA 100, frameshift mutations for the other strains), the method provides the mechanism by which the mutagen is causing the reversion.

The method 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.

A chemical would be considered to have a negative response in the test if the number of induced revertants compared to the spontaneous was less than 2-fold when at least 0.5 mg or its maximum non-inhibitory level had been tested on the plate.

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 maximum concentrations we used were 0.22 % for Sangre de Grado and 0.045 mg/ml of Taspine-HCl since previous experiments

have shown that higher concentrations were inhibitory for bacteria.

In Table 12, 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. What we observe, is that Sangre de Grado was weakly mutagenic at concentrations of 0.11% and 0.22% for tester strain TA97 and at a concentration of 0.22% for tester strain 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 13, 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.

Taking together these results we can conclude that under the experimental conditions tested, taspine-HCl was not a mutagen while Sangre de Grado was a weak mutagen.

Taspine is not the weak mutagen present in Sangre de Grado, since from Table 1, we can assume that the content of Taspine-HCl in a 0.22% suspension of Sangre de Grado is between 0.0022 and 0.0066 mg/ml. These concentrations are below the concentration of Taspine-HCl tested on Table 12, and which showed no mutagenic activity. From these we can assume that something different from Taspine-HCl is the mutagenic component of Sangre de Grado.

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

Sister chromatide exchanges are easily visualized in metaphase chromosomes. These sister chromatic exchanges can be induced by subtoxic doses of carcinogens and mutagens, and it is because of this that they are considered sensitive indicators of chromosome damage and have been used as indicators of mutagenesis (Latt S.A. Proc. Natl. Acad. Sci. USA 70: 3395 - 3399, 1973. Carrano A.V., Thompson L.H., Lindl P.A., Minkler J.L. Nature 271:551-554, 1978).

The cell lines that we used for these experiments were V79/AP4 and CHO. Both cell lines were derived from embryos of the chinese hamster Cricetulus griseus. The V79/AP4 were lung fibroblasts and the CHO were ovary cells.

2.1 Evaluation of Citotoxicity of Taspine-HCl on V79/AP4 cells

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

a. Cellular 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 0.05 mg/ml of gentamicin and incubated in a humidified atmosphere of 5% CO₂ and 95% air at 37°C. 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 concentrations of 0.264 and 0.088 ug/ml of Taspine-HCl were innocuous for the cells.

b. Simultaneous determination of the integrity of the cell membrane and the activity of intracellular esterases.-

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. 104:1303-1306, 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 esterases 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 were treated with the two dyes and observed under UV light in a fluorescent microscope, healthy cells showed a bright green fluorescence, unhealthy cells showed a light green fluorescence and damaged and dead cells showed 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 concentrations of 0.264 ug/ml and lower were not citotoxic and were the ones utilized in the experiments described below.

2.2 Evaluation of Cytotoxicity of Taspine-HCl and Sangre de Grado on CHO cells.

Similar experiments to the ones described above showed that CHO cells were more resistant to higher concentrations of taspine-HCl and the concentrations to be utilized on these cells would be 0.88 ug/ml of taspine-HCl and 0.001% of Sangre de Grado.

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

For the purpose of these experiments we utilized two different cell lines: V79/AP4 and CHO cells.

For the test with V79/AP4, cells that were growing exponentially were seeded on 60 mm tissue culture petri dishes (3×10^5 cells per plate) using Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum and 0.05 mg/ml of gentamicin and incubated in a humified atmosphere of 5% CO₂ and 95% air at 37 C. 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'-Bromodeoxyuridine to a final concentration of 3 ug/ml of medium. Plates were then incubated for and 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 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 14. What we observe is that Taspine HCl at non cytotoxic concentrations increases the frequency of sister chromatid exchanges on V79/AP4 cells.

For the test with CHO, cells that were growing exponentially were seeded on 60 mm tissue culture petri dishes (3×10^5 cells per plate) using Hamm's F12 medium supplemented with 10% fetal bovine serum and 0.05 mg/ml of gentamicin and incubated in a humified atmosphere of 5% CO₂ and 95% air at 37 C. Twenty four hours later one set of plates received Taspine-HCl to a final concentration of 0.88 ug/ml, another set of plates received Sangre de Grado at a final concentration of 0.001%, and a third set of plates did not receive any Taspine-HCl or Sangre de Grado and remained as control. Simultaneously with the treatment the three sets of plates received 5'-Bromodeoxyuridine to a final concentration of 3 ug/ml of medium. Plates were then incubated for and additional 22

hours in a humidified atmosphere of 5% CO₂ 95% air at 37°C (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 15 and what we observe is that neither Taspine-HCl nor Sangre de Grado increased the frequency of sister chromatid exchanges. On the contrary, Taspine-HCl decreased significantly the frequency of sister chromatid exchanges.

From the experiments described above we can conclude that Taspine-HCl and Sangre de Grado did not produce chromosome damage.

3. STUDIES ON THE CARCINOGENIC AND/OR TUMOR PROMOTER POTENTIALITIES OF SANGRE DE GRADO AND TASPINE-HCl.

For this purpose we have used a two-stage mouse skin carcinogenesis system, and the method we followed was similar to the one described by Slaga and collaborators (Slaga T.J., Klein-Szanto A., Fischer S.M., Weeks C.E., Nelson K., and Major S. Proc. Natl. Acad. Sci. USA. 77:2251-2254, 1980):

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 were housed 10 per cage and food and water were provided ad libitum. There were 30 animals per each experimental group.

- The carcinogenic compound used was 7,12-Dimethylbenz(a)antracene (DMBA) and right before use it was dissolved in acetone under low light conditions. The carcinogenic single dose was 100 nmoles and the initiator single dose was 10 nmoles.

- The known tumor promoter compound used was 12-O-Tetradecanoyl phorbol 13-acetate (TPA) and the concentration used was 2 µg in 0.1 ml of acetone applied twice weekly. One week after the application of the initiator dose of DMBA, treatment with TPA (Group 5), Sangre de grado (Group 6), or Taspine-HCl (Group 8), was started, and continued during the whole length of the experiment.

The groups we have worked with were:

Group 1: No treatment.

Group 2: 100 nmol of DMBA in 0.1 ml acetone

Group 3: 10 nmol of DMBA in 0.1 ml acetone

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

Group 1. was our control for the formation of spontaneous tumors.

Group 2. was our control for DMBA as a carcinogen.

Group 3. was our control for DMBA as an initiator.

Group 4. was our control for acetone.

Group 5. was our control for TPA as a tumor promoter. This control was very important since it would show as if our two-stage carcinogenesis protocol was working.

Group 6. was our test group for Sangre de Grado as a tumor promoter.

Group 7. was our test group for Sangre de Grado as a carcinogen

Group 8. was our test group for Taspine-HCl as a tumor promoter

Group 9. was our test group for Taspine-HCl as a carcinogen.

The experiment was started on December 2nd, 1984 and we report here data collected until December 31st, 1985. These data is summarized in Table 16, and what we can conclude is that our two-stage carcinogenesis protocol was working, since mice treated with DMBA as inducer and TPA as a promoter, developed papillomas three months after the initiation of the treatment (28 out of 29 mice). Also mice treated with carcinogenic doses of DMBA developed papillomas, but these ones started to appear at the end of November, 1985 and until now in only 10 out of 30 mice. With respect to our test groups 6 through 9, none of the mice on these groups have died or developed any papillomas until now. Autopsies were performed on five mice from each group and the results showed abnormalities only in groups 2 and 5:

Group 2: Gross examination: There were several papillomas from 3 to 5 mm in diameter (1 to 3 per mouse) and with a benign appearance.

Microscopic examination: Papillomatous lesions characterized by hyperplasia of the dermic papillae, which were elevated from the epidermic surface. The Malpighi stratus or spinous layer became thicker (acanthosis) with moderated hyperkeratosis. The epidermic basal layer had normal characteristics and we could not observe any malignant changes. The organs (kidney, liver, spleen, heart, lungs) did not present pathological alterations.

Group 5: Gross examination: There were several papillomas from 1 to 5 mm in diameter and with a benign appearance.

Microscopic examination: Papillomatous lesions characterized by hyperplasia of the dermic papillae, which were elevated from the epidermic surface. The Malpighi stratus or spinous layer became thicker (acanthosis) with accentuated hyperkeratosis and formation of corneal pseudocysts. The epidermic basal layer had normal characteristics and we could not observe any malignant changes. The organs did not present pathological alterations.

All these data suggests that neither Sangre de Grado nor Taspine-HCl are carcinogenic or tumor promoters.

We are planning to continue the experiment for at least another six months in order to be more conclusive, since 12 months might be too short a time, considering that the mice treated with the known carcinogen DMBA started to develop the tumors one month ago.

CONCLUSIONS

1. The alkaloid Taspine was the principal component present in Sangre de Grado extracted from Croton lechleri.
2. Taspine was present in trace amounts in extracts from the cortex of the Croton lechleri tree.
3. Three alkaloids were present in trace amounts in the leaves of the Croton lechleri, and the most abundant one was identified as Sinoacutina.
4. Taspine was the active cicatrizant principle present in Sangre de Grado extracted from Croton lechleri.
5. Taspine chlorhydrate acts at the beginning of cicatrization accelerating the wound healing process.
6. Taspine chlorhydrate was non-toxic to human foreskin fibroblasts at concentrations below 200 ng/ml, and it did not increase the rate of cell proliferation.
7. Taspine chlorhydrate was found to inhibit the contraction of cell-collagen lattices and to increase migration of human foreskin fibroblasts.
8. Mutagenicity studies using the Ames test showed that Sangre de Grado was a weak mutagen while taspine chlorhydrate had no mutagenic activity.
9. Studies on the effect of Sangre de Grado and taspine chlorhydrate on sister chromatid exchange (SCE) showed that Taspine chlorhydrate increased the number of SCEs in V79/AP4 cells, but no increase was observed in CHO cells.
10. Using the two-stage mouse skin carcinogenesis system we have shown that neither Sangre de Grado nor taspine-HCl were carcinogenic and/or tumor promoters after one year of treatment.

TABLE 1

EXTRACTION OF SANGRE DE GRADO FROM DIFFERENT TREES FROM IQUITOS
DURING THE FIRST TRIP

TREE N.	TIME OF EXTRACTION	VOLUME OF EXTRACTION	M-1 YIELD mg/ml	M-2 YIELD mg/ml
8	6-7 am	29 ml	3.4	3.7
1	7-8 am	30 ml	1.5	1.3
11	8-9 am	32 ml	1.6	1.8
2	9-10 am	13 ml	3.0	2.2
4	10-11 am	22 ml	2.0	2.5
6	11-12 am	33 ml	2.2	2.7
7	12-1 pm	17 ml	2.0	1.8

M-1= To 5 mls of each of the samples were added 5 ml of water, the mixture was then alkalized 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. $\bar{x}_1 = 2.24 \pm 0.65$

M-2= To 1 ml of each of the samples were added 1 ml of water, the mixture was then alkalized to pH 9 with ammonium hydroxide and extracted exhaustively with chloroform. Each chloroform extract was dried and then redissolved in 30 ml of ethyl alcohol and the absorbance at 347 nm was measured using a Zeiss Spectrofotometer. The extinction coefficient of taspine at 347 nm was 7380. $\bar{x}_2 = 2.28 \pm 0.72$

Statistical analysis using the Student's t-test showed that there was a significant difference between these groups and the groups on Table 2. ($P < 0.05$)

The collection date was December 19, 1984.

TABLE 2.

EXTRACTION OF SANGRE DE GRADO FROM DIFFERENT TREES FROM IQUITOS DURING THE SECOND AND THIRD TRIPS.

TREE No	Feb., 85	May, 85
	YIELD mg/ml	YIELD mg/ml
21	ND	0.80
22	1.56	1.14
30	0.81	0.75
31	1.16	0.99
32	1.56	1.28
36	1.46	0.94
39	0.89	0.78
42	1.25	0.63

Sangre de Grado was collected from the trees enumerated above on February 13 and 14, 1985; and on May 20, 1985. The alkaloid content was determined by the following method:

To 1 ml of each of the samples were added 1 ml of water, the mixture was then alkalized to pH 9 with ammonium hydroxide and extracted exhaustively with chloroform. Each chloroform extract was dried and then redissolved in 30 ml of ethyl alcohol and the absorbance at 347 nm was measured using a Zeiss Spectrofotometer. The extinction coefficient of taspine at 347 nm was 7380. $\bar{x}_{Feb.} = 1.24 \pm 0.28$; $\bar{x}_{May} = 0.91 \pm 0.20$.

Statistical analysis using the Student's-t-test showed that there was a significant difference between the two groups ($P < 0.05$).

TABLE 3.

CICATRIZANT ACTIVITY OF SANGRE DE GRADO, TASPINE AND TASPINE CHLORHYDRATE.

TREATMENT	WBS \pm Sd in grams	% ACTIVITY
Control (PBS)	45.15 \pm 8.25	-----
SG 10 %	59.15 \pm 11.28	31.0
Taspine 0.1 mg/ml	50.60 \pm 10.70	12.0
Taspine-HCl 0.1 mg/ml	71.43 \pm 8.41	58.2

Abbreviations: WBS = Wound breaking strength.
SG = Sangre de Grado
PBS = Phosphate buffered saline
Taspine-HCl = Taspine chlorhydrate.

Mice were anesthetized with vapors of diethyl ether and a 1 cm incision was made perpendicular to the axis of simmetry of the animal and the two borders of the wound were stiched together at its center. Treatment was started immediately, and every 12 hours the compound being tested was applied to the wound. For this purpose 0.05 ml of the solution being tested was applied slowly and directly to the wound by means of a micropipete. The controls received only the solvent in which the compound was diluted. 48 hours later, mice were sacrificed with an ether overdose and the WBS were quantitated by fixing one of the borders of the wound (after cutting the stich) while applying a measurable force to the other one. For more details see text.

Statistical analysis using the Student's-t-test showed that the difference between the control group and the SG group ($p<0.05$) and between the control group and the taspine-HCl group ($p<0.005$) were significant.

TABLE 4.

DOSE-RESPONSE CURVE FOR THE CICATRIZANT EFFECT OF TASPINE-HCl

TASPINE-HCl in mg/ml	WBS \pm Sd in grams	% ACTIVITY
Control (H ₂ O)	42.90 \pm 6.25	-----
0.002	47.56 \pm 12.90	10.80
0.004	48.66 \pm 8.18	13.40
0.050	59.80 \pm 6.98*	39.39
0.066	69.20 \pm 10.28**	61.30
0.100	66.28 \pm 7.60**	54.50
0.200	52.80 \pm 9.33*	23.10

Taspine-HCl = Taspine chlorhydrate.

WBS = Wound breaking strength.

Statistical analysis using the Student's-t-test showed:

* = The difference with the control was significative ($P < 0.05$)

** = The difference with the control was significative ($P < 0.01$)

For the methods see Table 3. and text. In this experiment we used 7 mice per each group.

TABLE 5.

KINETICS OF CICATRIZATION IN THE PRESENCE OF TASPINE CHLORHYDRATE

TREATMENT	TIME (Hours)	WBS \pm SD in grams	% ACTIVITY
Control	12	21.48 \pm 3.98	0
Taspine-HCl	12	35.67 \pm 13.36*	66.06
Control	24	35.50 \pm 4.18	0
Taspine-HCl	24	44.30 \pm 8.02*	24.87
Control	36	41.84 \pm 9.19	0
Taspine-HCl	36	62.60 \pm 16.85*	49.61
Control	48	51.80 \pm 10.99	0
Taspine-HCl	48	66.60 \pm 15.39**	32.43
Control	60	58.20 \pm 4.15	0
Taspine-HCl	60	75.28 \pm 16.34**	29.35
Control	72	70.40 \pm 7.13	0
Taspine-HCl	72	63.20 \pm 9.58	0
Control	96	104.6 \pm 14.89	0
Taspine-HCl	96	92.4 \pm 13.61	0
Control	120	102.2 \pm 11.05	0
Taspine-HCl	120	99.8 \pm 27.43	0
Control	144	119.7 \pm 22.95	0
Taspine-HCl	144	125.6 \pm 27.98	4.92
Control	168	146.7 \pm 26.95	0
Taspine-HCl	168	158.2 \pm 25.92	7.84

Taspine-HCl = Taspine chlorhydrate.

WBS = Wound breaking strength.

Statistical analysis using the Student's-t-test showed:

* = The difference with the control was significative ($P < 0.05$)

** = The difference with the control was significative ($P < 0.1$)

For the methods see Table 3. and text. In this experiment we used 5 mice per each group, and the concentration of taspine-HCl used was 0.066 mg/ml.

TABLE 6.

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

10⁶ 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 using 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.

Each point represents the average count of three plates.

TABLE 7.

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

TASPINE-HCl. ng/ml	42 Hours		60 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

10⁵ Foreskin fibroblasts were inoculated to 35 mm tissue culture dishes containing MEM-H + 10% fetal bovine serum, 10mM Hepes, 50 ug/ml streptomycin. 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. 42, and 60 hours later, 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/dish at 18 hours was 116,000 (7% of dead cells).

Each point represents the average count of three plates.

TABLE 2.

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

TASPINE-HCl. ng/ml	No. Cells	42 Hours		60 Hours	
		% Dead		% Dead	
50.0	167,000	9.5		159,000	9.1
10.0	132,000	9.1		151,000	8.6
2.0	116,500	8.1		262,000	4.8
0.5	74,000	14.9		334,000	3.3
0.1	122,000	9.2		124,000	9.3
0	161,000	5.6		278,000	4.3

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. 42, and 60 hours later, 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/dish at 18 hours was 104,000 (5.7% of dead cells).

Each point represents the average count of three plates.

TABLE 9.

EFFECT OF TASPINE-HCl ON THE MITOTIC INDEX OF CONFLUENT CULTURES OF HUMAN FORESKIN FIBROBLASTS.

TASPINE-HCl ng/ml	0 Hours	24 Hours	48 Hours	72 Hours	96 Hours
1000	911.1	1979.3	2073.5	785.4	DEAD
100	973.9	1853.6	1743.6	1508.1	1460.9
20	942.5	1727.9	1932.1	1602.3	1413.8
2	989.6	1932.1	2073.5	1837.9	1649.4
0.2	1225.2	1916.4	1853.6	1570.8	1696.5
0	973.9	2230.6	2073.5	1633.7	1602.2

10^5 Foreskin fibroblasts were inoculated to 60 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. Once the cells reached confluence (377,000 cells/plate) the number of mitosis per plate was counted in all the plates (0 hour), and the media was changed for media containing different concentrations of taspine-HCl. The number of mitosis/plate was also counted 24, 48, 72, and 96 hours later.

The following formula was applied in order to determine the number of mitosis per plate: Mt = (Mc x At) / Ac

Where: Mt = Mitosis/plate.

Mc = Mitosis counted (In 10 different areas of the plate).

At = Total area of the plate.

Ac = Area counted (Area of the 10 different areas counted).
Each point represents the average count of three plates.

TABLE 10.

EFFECT OF TASPINE CHLORHYDRATE ON THE MIGRATION OF HUMAN FORESKIN FIBROBLASTS.

TASPINE-HCl ng/ml	No. CELLS/cm \pm SD		
	24 Hours	48 Hours	72 Hours
1000	1.5 \pm 1.63	1.7 \pm 2.56	0.0
200	24.7 \pm 1.66 ^b	28.2 \pm 2.56	41.9 \pm 4.38
20	21.4 \pm 0.63 ^a	23.7 \pm 0.65	38.7 \pm 7.96
2	27.0 \pm 1.83 ^b	35.2 \pm 1.42 ^c	70.7 \pm 5.67 ^b
0.2	22.7 \pm 2.03 ^b	26.3 \pm 3.90	43.2 \pm 3.46
0	15.6 \pm 2.2	22.9 \pm 3.08	38.5 \pm 5.88

10^5 Foreskin fibroblasts were inoculated to 60 mm tissue culture dishes containing Minimal essential medium Eagles with Hanks salts (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. When the cells reached confluence, a superficial line was made by placing a razor blade perpendicular to the surface of the plate and then by moving the razor blade towards the right the cell sheet was pushed in order to clear an area to the right of the line. Plates were then washed with PBS in order to eliminate all the cell debries and media without serum (Minimal essential medium Eagles with Hanks salts + 10mM Hepes, 50 ug/ml gentamycin) and with the different concentrations of taspine-HCl was added. Plates were then screened and the areas along the line clear of cells, were marked with the point of a fine bisturi. Plates were then placed at 37 C in a humidified atmosphere of 5% CO₂ and 95% air and at the time specified on the Table, the number of cells/cm that crossed the line along the marked areas were counted under a phase contrast microscope.

For each point 30 mm were read on each of three plates.

The data was subjected to statistical analysis using the Student's-t-test:

- ^a The difference with the control was significative ($P<0.05$)
- ^b The difference with the control was significative ($P<0.025$)
- ^c The difference with the control was significative ($P<0.01$)
- ^d The difference with the control was significative ($P<0.005$)

TABLE 11.

EFFECT OF TASPINE CHLORHYDRATE ON THE MIGRATION OF HUMAN FORESKIN FIBROBLASTS IN THE PRESENCE OF SERUM.

TASPINE-HCl ng/ml	No. CELLS/cm \pm SD	
	24 Hours	48 Hours
10	47.4 \pm 2.03	101.5 \pm 4.93
8	51.7 \pm 1.58 ^a	109.0 \pm 2.53 ^a
6	56.2 \pm 0.84 ^c	124.4 \pm 1.77 ^c
4	61.3 \pm 3.61 ^b	136.3 \pm 5.63 ^b
2	60.3 \pm 3.40 ^b	112.1 \pm 4.64 ^b
0	47.7 \pm 2.42	93.0 \pm 5.46

The methodology is the same as the one described in Table 10, except that this time the media with the different concentrations of taspine-HCl contained also 1% fetal bovine serum.

For each point 30 mm were read on each of three plates.

The data was subjected to statistical analysis using the Student's-t test:

- ^a The difference with the control was significative ($P<0.05$)
- ^b The difference with the control was significative ($P<0.02$)
- ^c The difference with the control was significative ($P<0.01$)
- ^d The difference with the control was significative ($P<0.005$)

TABLE 12.

v

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

	NUMBER OF REVERTANTS OF THE TESTER STRAIN						
	TA97	TA98	TA100	TA102	TA1535	TA1537	TA1539
S. P. E.	90-180	30-50	120-200	240-320	10-35	3-15	15-35
S. P. O.	212	28	107	127	29	15	30
S. G. %	,						
0.013	527	22	104	267	33	10	14
0.058	363	28	112	324	20	13	20
0.110	419	25	90	373	42	12	21
0.220	575	23	101	447	32	17	18
T. HCl mg/ml							
0.0047	175	28	84	165	26	13	20
0.0110	264	28	81	174	30	10	18
0.0230	123	30	96	185	24	9	19
0.0450	149	31	60	101	20	12	14

ABBREVIATIONS: 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 13.

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	TA1535	TA1537	TA1538
S P E	90-180	30-50	120-200	240-320	10-35	3-15	15-35
S P O	73	55	47	60	15	5	19
S G %							
0.023	81	42	72	46	21	10	21
0.058	84	38	69	52	17	8	18
0.110	90	50	90	41	26	9	15
0.220	81	34	71	30	ND	ND	15
T-HCl mg/ml							
0.0047	97	37	55	39	24	11	27
0.0110	84	42	63	43	27	13	26
0.0230	92	50	48	42	23	9	25
0.0450	72	47	52	39	25	15	24

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 14.

EFFECT OF TASPINE HCl ON THE FREQUENCY OF SISTER CHROMATID EXCHANGE

TREATMENT	SCE/METAPHASE
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 0.05 mg/ml of gentamicin and incubated in a humidified atmosphere of 5% CO₂, 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'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.

TABLE 15.

EFFECT OF TASPINE-HCl AND SANGRE DE GRADO ON THE FREQUENCY OF SISTER CHROMATID EXCHANGE

TREATMENT	SCE/METAPHASE
Control	14.72 ± 2.52
Taspine HCl 0.88 ug/ml	10.55 ± 3.27
Sangre de Grado 0.001 %	13.78 ± 2.63

SCE = Sister chromatid exchange.

3×10^5 CHO cells were inoculated into 60mm tissue culture dishes in Ham's F12 medium supplemented with 10% fetal bovine serum and 0.05 mg/ml of gentamicin and incubated in a humidified atmosphere of 5% CO₂, 95% air at 37°C for 24 hours. Then different concentrations of taspine HCl, Sangre de Grado 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.

For these experiment a total of 51 metaphases were read in each of the treatment groups.

Significance of the differences were calculated according to the Student-t test. Only the difference between the control and the taspine HCl group ($p < 0.05$) was significant.

TABLE 16.

TWO-STAGE CARCINOGENESIS EXPERIMENT TO TEST THE POTENTIAL CARCINOGENIC AND/OR TUMOR PROMOTER ACTIVITY OF SANGRE DE GRADO AND TASPINE-HCl.

GROUP	TREATMENT	No. OF MICE SURVIVING ^a	No. MICE WITH PAPILLOMAS
1	None	29 ^b	0
2	DMBAc	28 ^b	10 ^c
3	DMBA _i	30	0
4	ACETONE	30	0
5	DMBA _i + TPA	30 ^d	28 ^d
6	DMBA _i + SG	30	0
7	SG	30	0
8	DMBA _i + T-HCl	30	0
9	T-HCl	30	0

ABREVIATIONS: DMBA= 7-12 Dimethylbenz (α) antracene

DMBAc= Carcinogenic dosis of DMBA (1 of 100 nmol)

DMBA_i= Inducer dosis of DMBA (1 of 10 nmol)

TPA= 12-O-Tetradecanoyl phorbol 13-acetate (2 ug twice weekly)

SG= Sangre de Grado (0.1 ml of 50% solution twice weekly)

T-HCl= Taspine-HCl (0.2 mg twice weekly).

^a The experiment began on December 12, 1984 and the number of mice per group was 30, the ones reported on the table with the exception of group 5 correspond to the numbers on December 31, 1985. For group 5 it corresponds to May 13, 1985 (45 days after the papillomas appeared).

^b According to autopsy, Mice died of reasons not related to treatment.

^c Papillomas started to appear on November 25, 1985. An average of 3 papillomas/mouse.

^d Papillomas started to appear on April 1st, 1985. An average of 7 papillomas/mouse.

For the methodology see text.

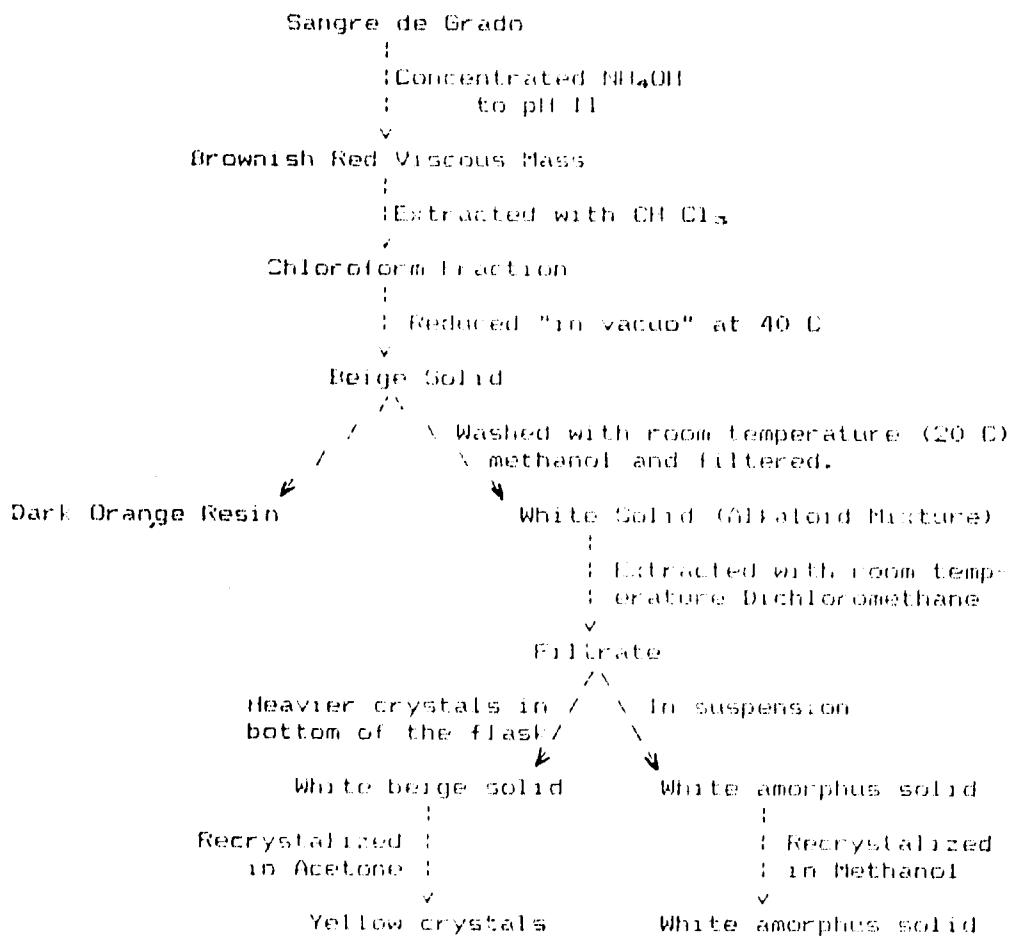


FIGURE 1. Extraction and Purification Procedure of the Alkaloid from Sangre de Grado.

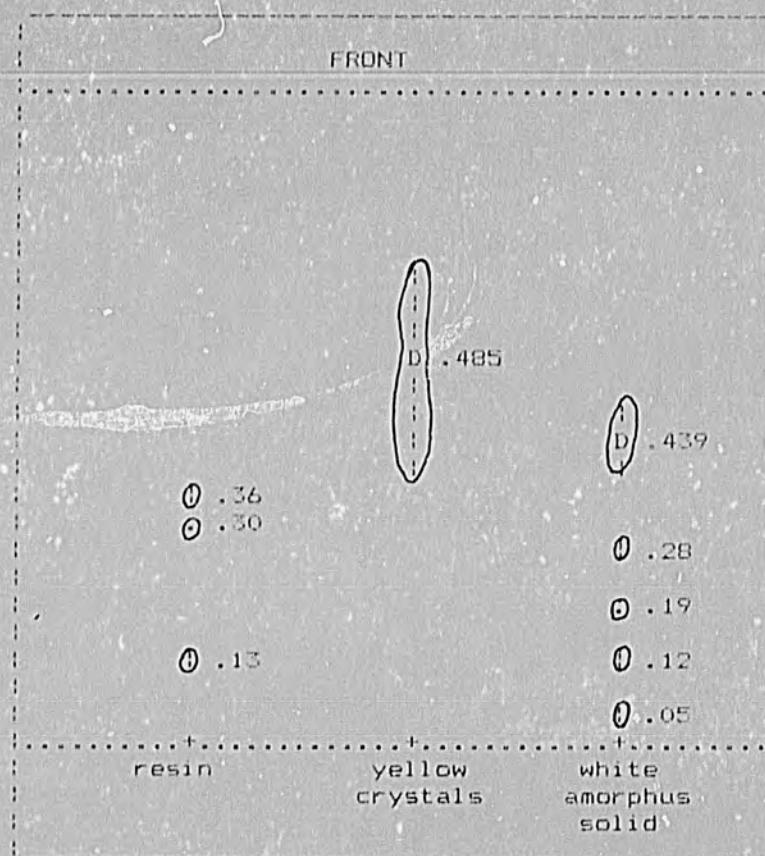
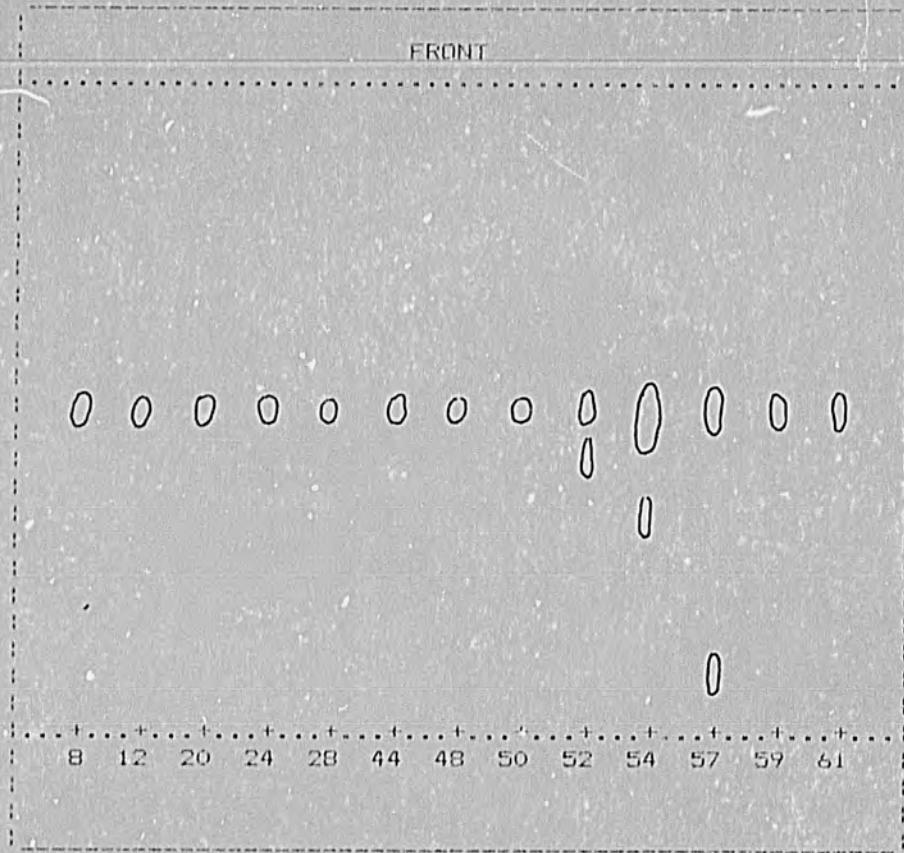


FIGURE 2. Thin Layer Chromatography of the Alkaloid and Resin Obtained during the Extraction of Sangre de Grado.-

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 rfs are shown next to each spot.



D = Positive to Dragendorff reagent.

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.

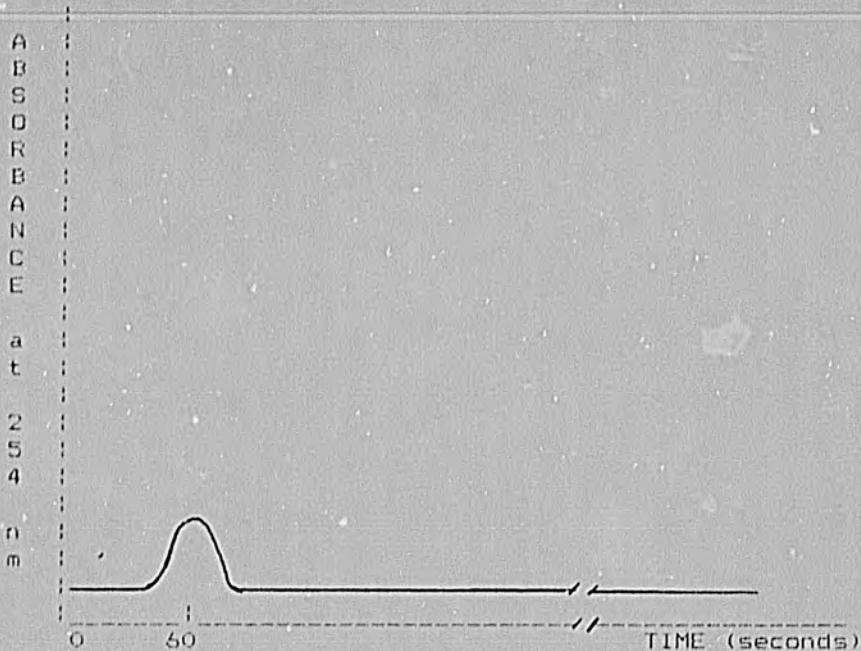


FIGURE 4. Elution Pattern of the Crude Alkaloid Mixture from Sangre de Grado from a Silica Gel 60 Medium Pressure Column of the Following Characteristics:
Stationary phase: silica gel 60
Dimension: 25 x 2 cm.
Detector: UV 254 nm lamp.
Eluent: Dichloromethane:methanol (3:1)
Column was run up to 4 hours.

FIGURE 5. Ultraviolet Spectra of the Chlorhydrate of the Yellow Crystals from Sangre de Grado (5A) and of the Reference Sample of Taspine Chlorhydrate Provided by Dr. Norman R. Farnsworth (5B).- Both spectra were run in Water.

Ident! Nr. U23119250

11/44

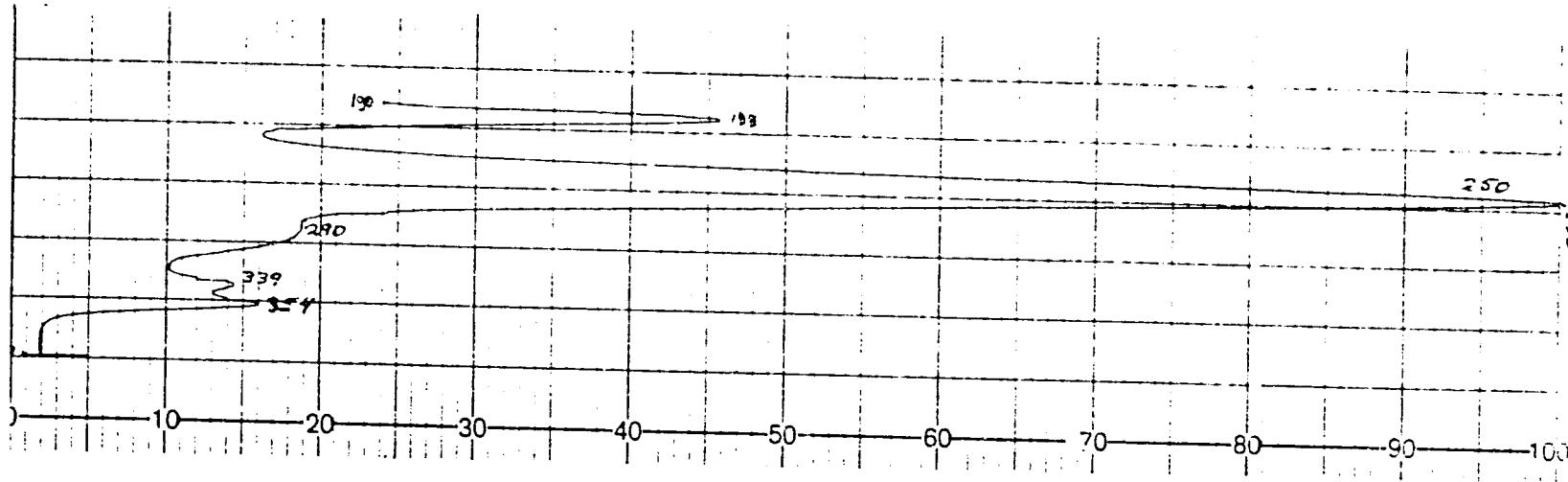


FIGURE 5▲

FIGURE 2B

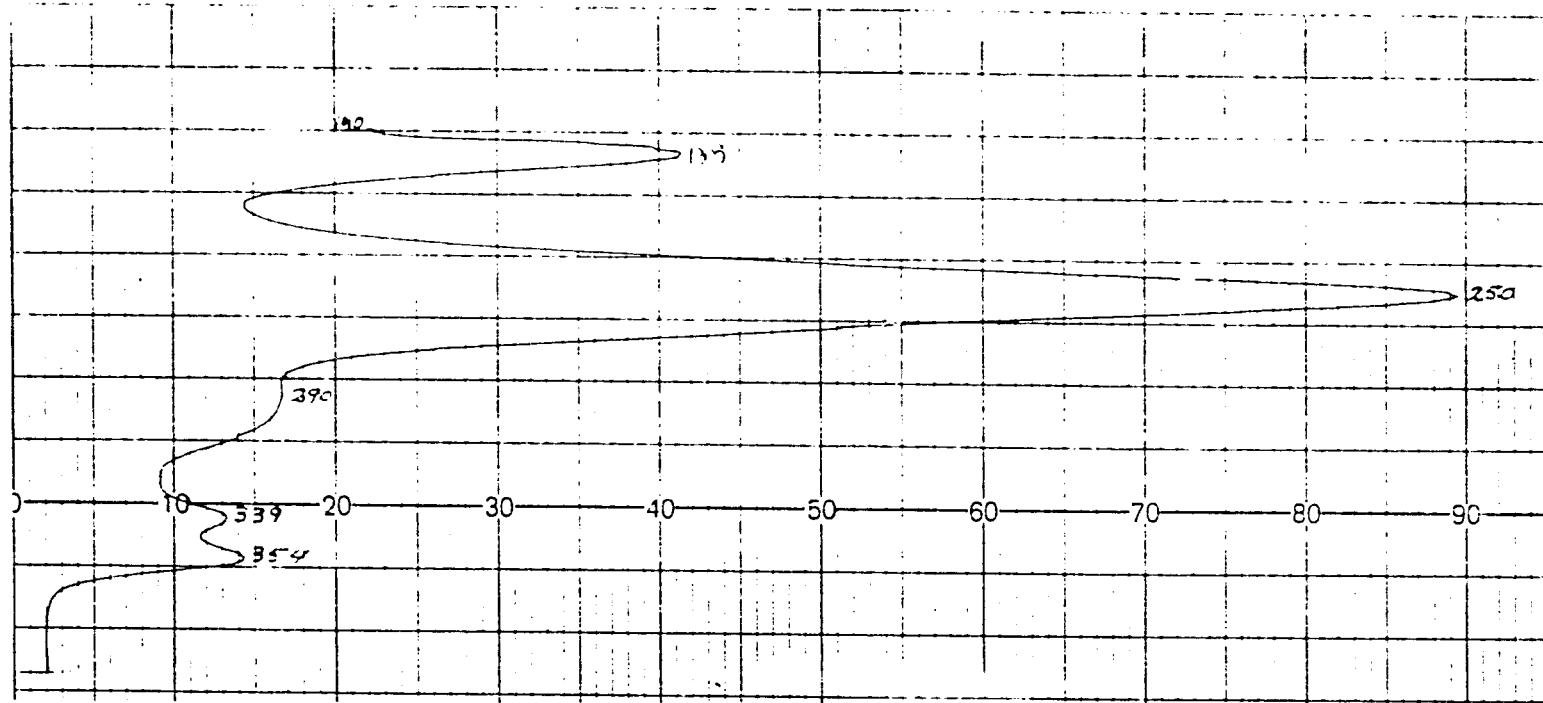
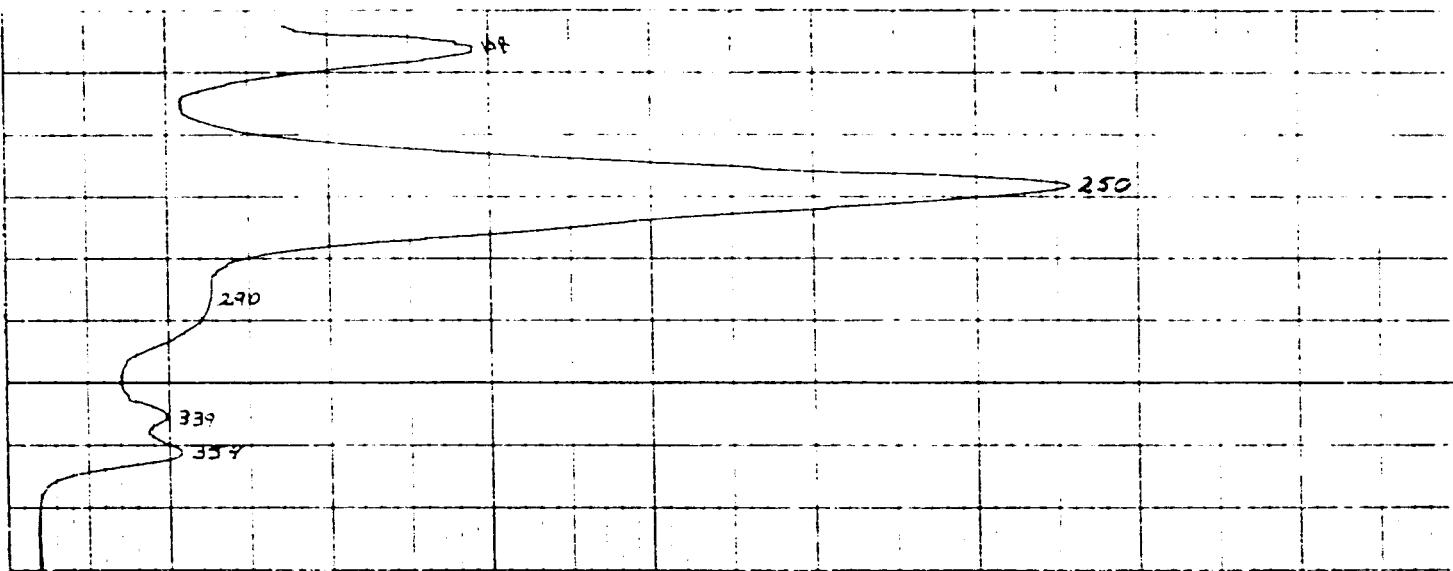


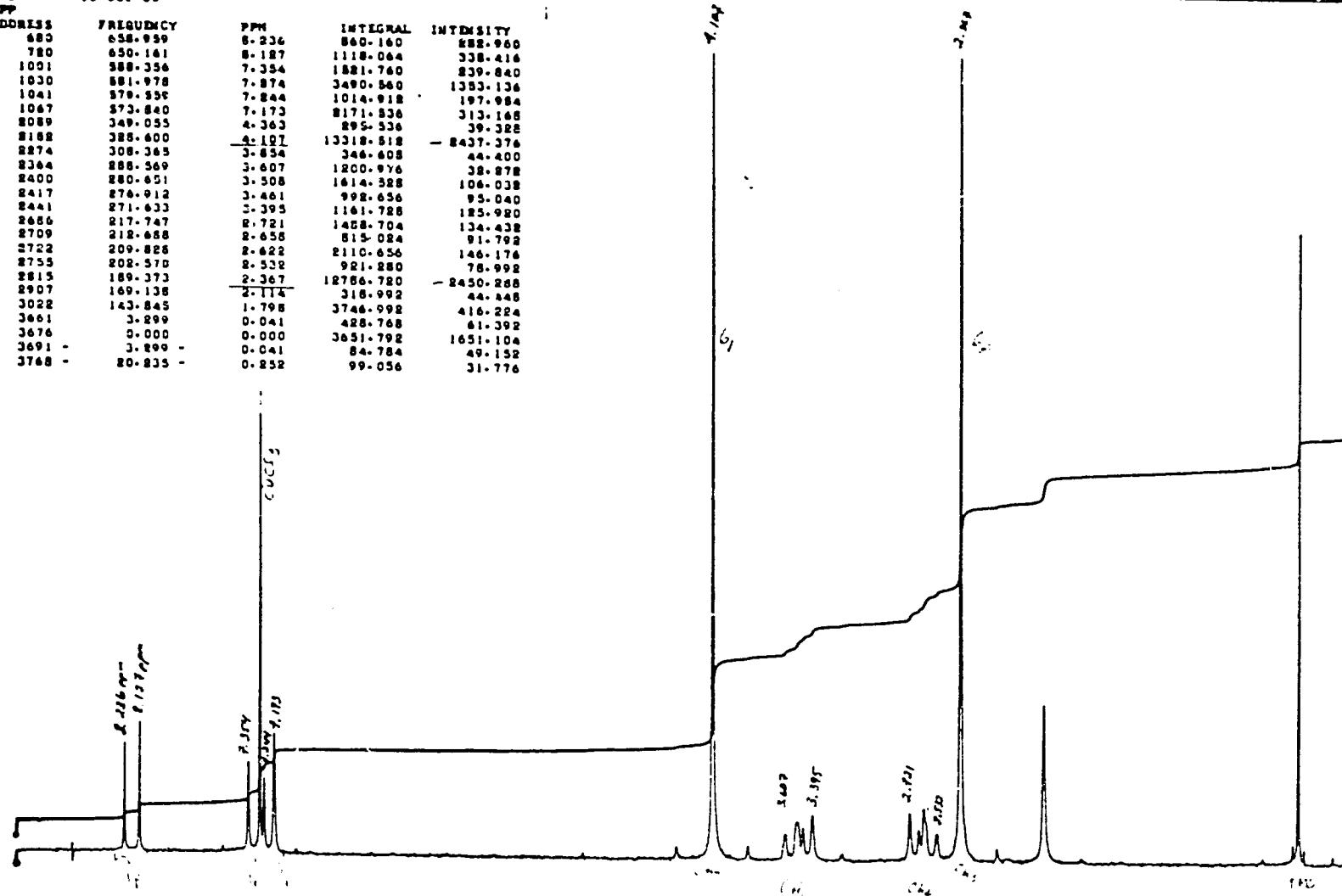
FIGURE 6 UV Spectrum of the Hydrochloride of
the White Amorphous Solid from Sangre
de Grado in H₂O.



-M1- 10:00P 38

•

ADDRESS	FREQUENCY	PPM	INTEGRAL	INTENSITY
680	658.939	5.236	860.160	862.960
720	650.161	5.127	1118.064	338.416
1001	588.356	7.354	1821.760	239.840
1030	581.978	7.874	3490.560	1353.136
1041	579.555	7.844	1014.918	197.984
1067	573.840	7.173	8171.836	313.168
2089	349.055	4.363	295.536	39.328
2182	388.600	4.191	13318.818	- 8437.376
2274	308.345	3.654	346.408	44.400
2364	288.569	3.607	1200.976	38.876
2400	280.651	3.508	1614.388	106.038
2417	276.912	3.481	998.656	95.040
2441	271.633	3.395	1161.728	185.920
2686	217.747	2.721	1458.704	134.438
2709	212.658	2.658	515.024	91.792
2722	209.826	2.622	2110.656	146.176
2755	202.370	2.532	921.280	78.952
2815	189.373	2.367	12786.720	- 2450.288
2907	169.138	2.114	318.992	44.448
3022	143.845	1.798	3746.998	416.224
3661	3.299	0.041	428.768	61.392
3676	3.000	0.000	3651.792	1651.104
3691	3.299	0.041	84.784	49.152
3768	20.815	0.252	99.056	31.774



80 MHz ^1H

CDG13 11/1

PM 1545

sw 900

— 188 —

142

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• 118 •

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

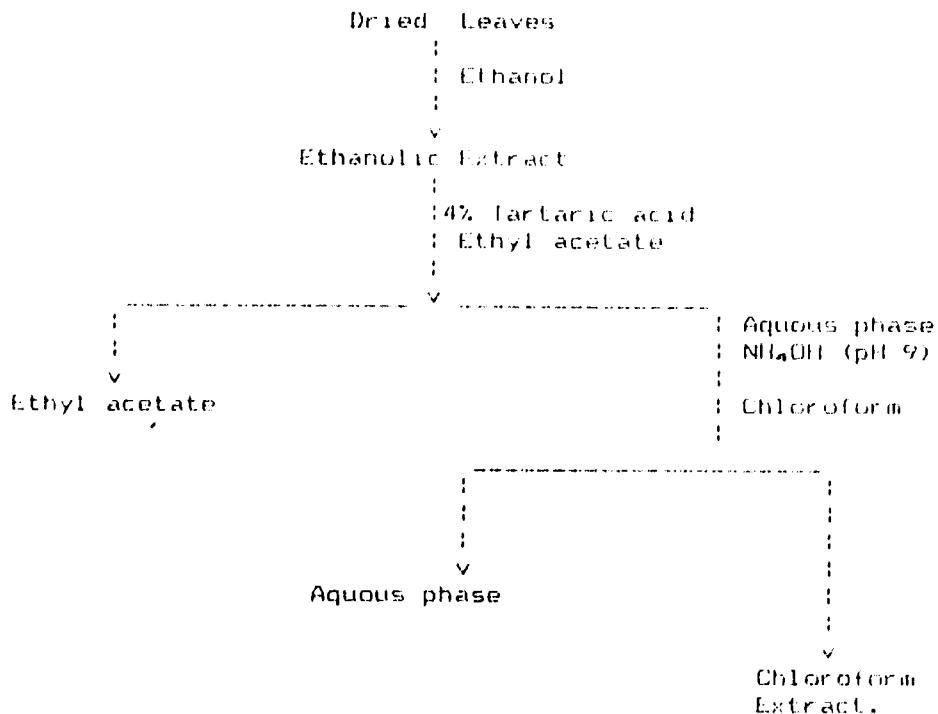


FIGURE 8. Extraction and Purification Procedure for the Leaves of the Croton lechleri.

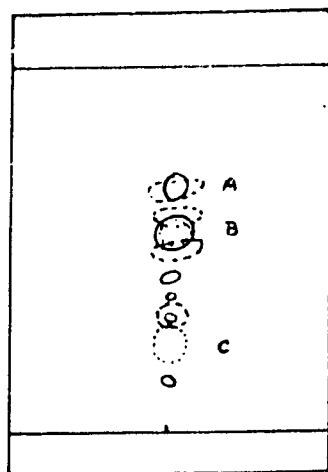


FIGURE 9.- Thin layer chromatography of the chloroform extract from the leaves of the Croton lechleri.

TLC was 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 (.....)

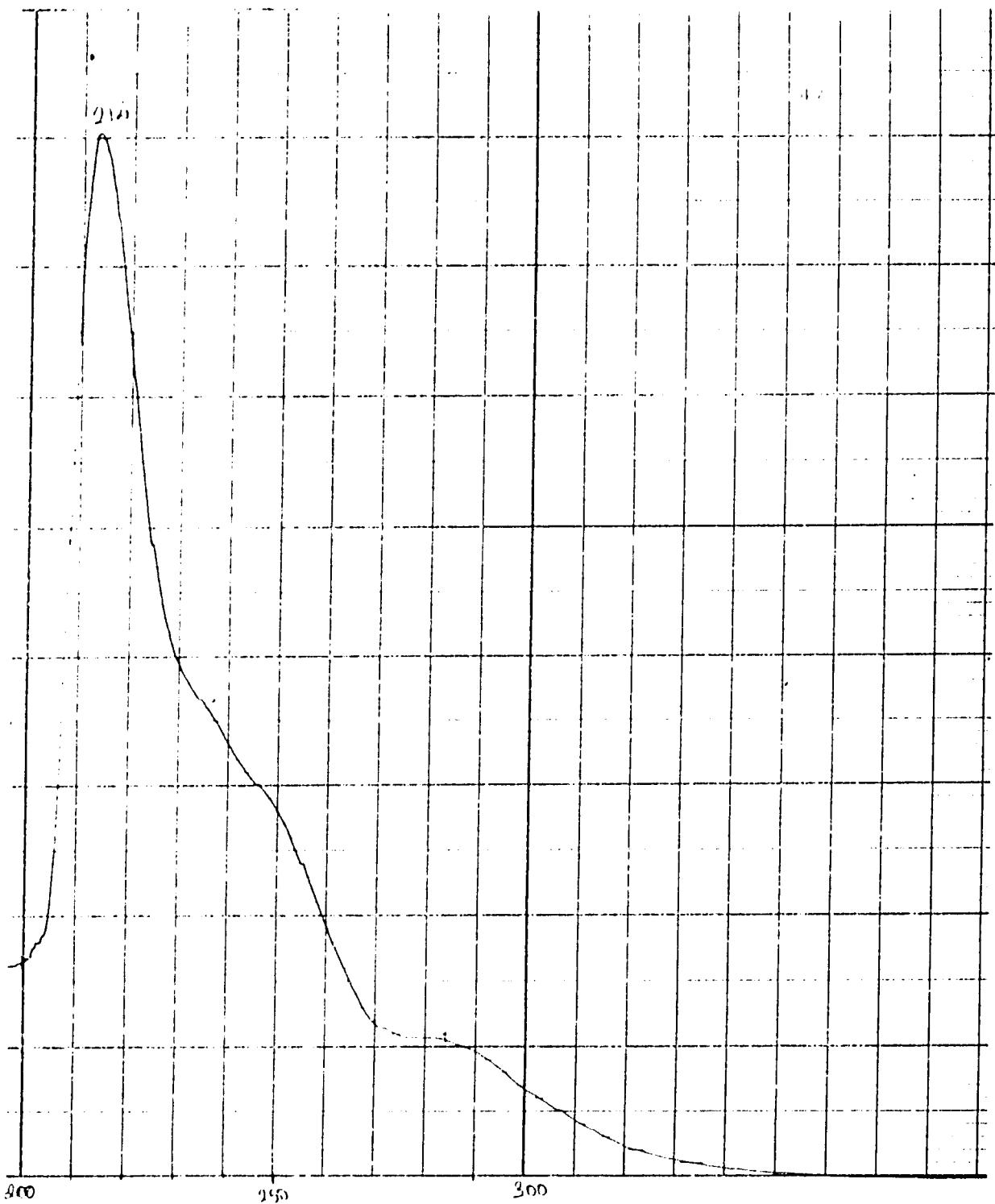


FIGURE 10.- Ultraviolet Spectrum of alkaloid B from the leaves of Croton lechleri in Ethanol.

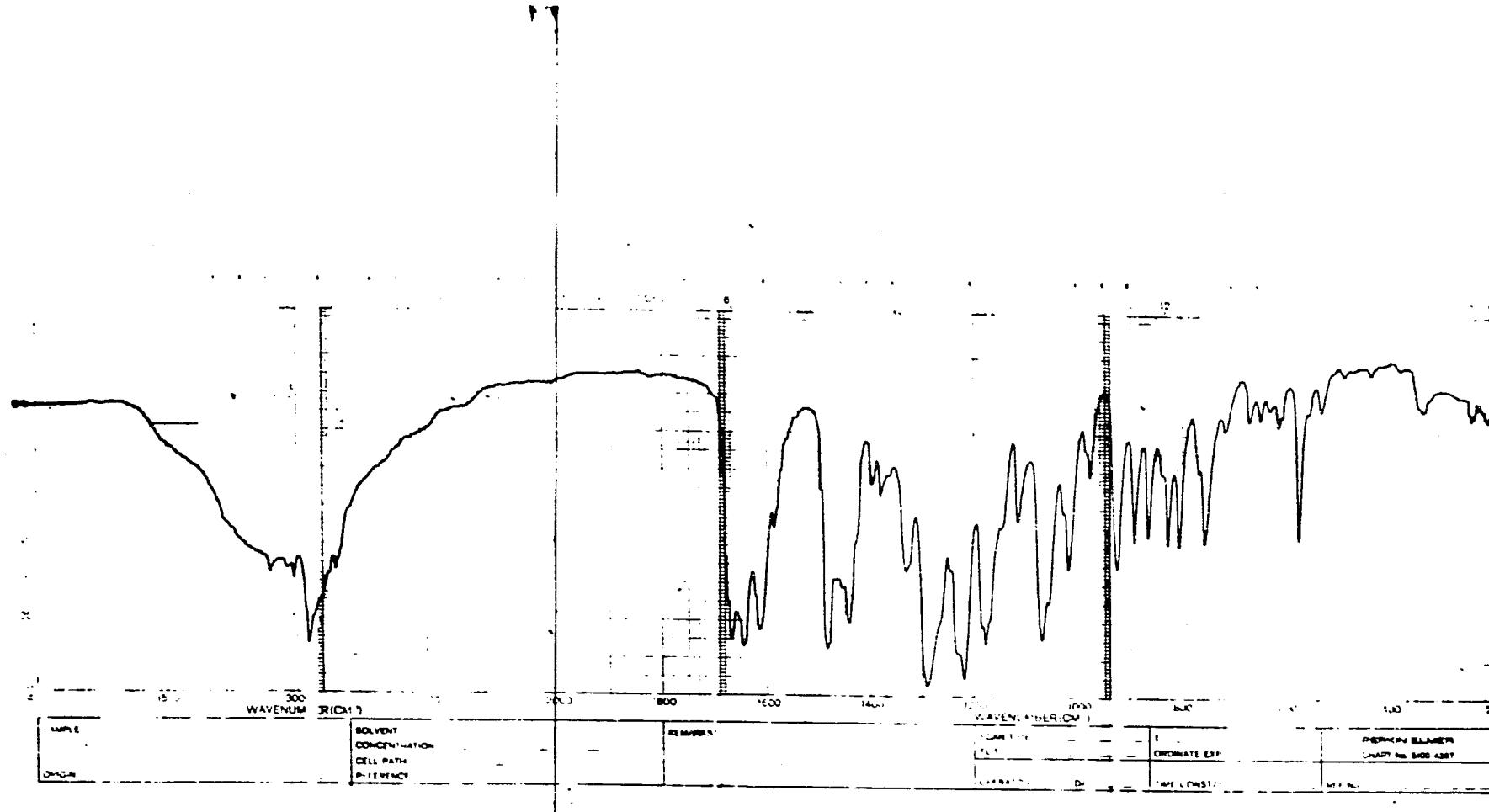


FIGURE 11.- Infrared Spectrum of alkaloid B from the leaves of Croton lechleri in KBr.

ADDRESS	FREQUENCY	PPM	INTEGRAL	INTENSITY
920	606.178	7.577	594.720	114.432
932	603.533	7.544	4557.504	629.760
1031	581.756	7.271	16314.560	6942.176
1191	546.567	6.832	485.856	138.384
1229	538.209	6.787	4411.776	950.040
1240	535.759	6.697	4351.424	538.112
1375	506.096	6.326	5577.760	891.398
1409	498.618	6.232	4299.136	754.176
2260	311.444	3.893	14262.144	3283.008
2309	300.666	3.758	16518.176	2191.424
2342	293.405	3.667	3729.152	202.496
2490	260.856	3.260	3562.850	230.752
2547	245.319	3.03	4127.520	151.728
2730	208.069	2.600	2678.240	198.688
2749	203.590	2.545	2777.248	154.400
2774	198.391	2.479	1621.216	228.160
2785	195.972	2.449	16511.232	2532.448
2831	155.854	2.323	1469.725	155.512
2843	157.845	2.290	3170.240	234.080
3078	151.528	1.644	8584.800	1076.384
3565	24.413	0.305	430.976	117.632
3660	3.319	0.043	1060.160	166.528
3676	0.000	0.000	13163.136	4057.936
3691	3.299	0.041	301.568	116.532

3C NH₂ H
 CDCl₃ -TMS
 Pd 45.417
 Si 500
 O₁ 2200
 N 100
 H2aw 20
 10.1121 14
 425

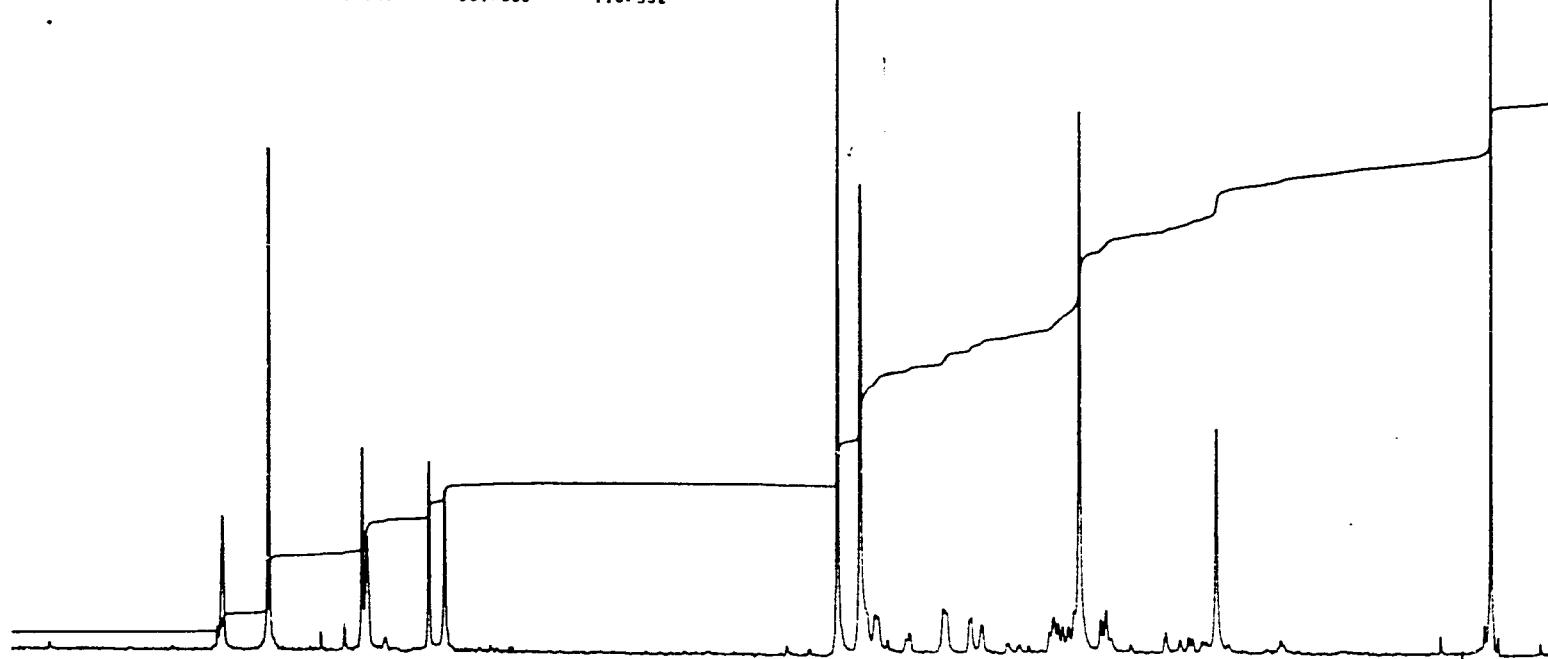


FIGURE 12.- NMR Spectrum of alkaloid B from the leaves of Croton lechleri in CDCl₃.

FIGURE 15. Mass Spectrum of Alkaloid B from the leaves of Croton Techleri.

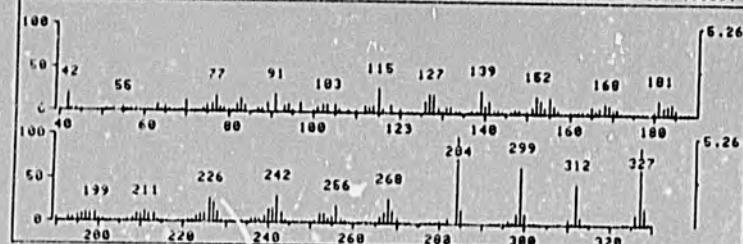
520

ALCALOID E, 50-260 010/M SCH RHC=40-600, EV=1
 J. HAMMOND 4-3-86 (HARTGRAVES)
 P^X 1.0 MASS RANGE 39.0, 447.0 TOTAL ABUND= 4269654.

TP: 200 202

SCAH # 20 40 60 80 100 120 140 160

* 96 RET. TIMEI 3.83 TOT ABUND= 328299. BASE PK/ABUND= 284.2/ 16833.



FHN 1350C, SPECTRUM # 96 RET. TIMEI 3.83, 251 PEAKS

M/Z	REL ABUND						
40	.5	110	10.5	185	7.6	240	.8
42	21.1	120	5.1	186	3.0	249	2.4
43	3.3	122	3.0	187	2.2	250	3.1
44	4.5	123	2.3	188	2.3	251	4.1
45	.9	124	3.0	189	1.5	252	12.4
50	1.6	126	15.5	190	5.1	253	11.0
51	4.7	127	23.0	191	1.9	254	6.8
52	1.3	128	22.7	192	1.7	255	> 0
53	3.5	129	6.9	193	6.3	256	20.6
54	2.2	130	3.8	194	5.1	257	5.4
55	4.7	131	8.8	195	9.2	258	3.5
56	3.9	132	8.1	196	10.3	259	> 0
57	4.3	133	4.7	197	11.0	260	< 1
58	4.1	134	4.0	198	10.3	261	< 1
59	1.1	136	.7	199	11.7	262	< 1
60	.2	137	4.4	200	5.2	263	1.1
61	.3	138	4.1	201	4.5	264	1.3
62	1.9	139	27.0	202	1.7	265	1.5
63	8.0	140	10.9	203	1.2	266	8.3
64	3.7	141	14.6	204	< 0	267	12.9
65	7.2	142	5.8	205	1.3	268	49.3
66	4.4	143	5.3	206	2.0	269	16.2
67	2.8	144	4.4	207	4.9	270	7.3
68	2.6	145	2.6	208	5.1	271	2.3
69	3.6	146	4.3	209	9.6	272	< 1
70	13.7	147	5.9	210	18.7	273	< 1
71	2.7	148	4.0	211	12.0	274	< 1
72	1.6	149	2.3	212	10.0	275	< 1
73	.8	150	3.0	213	9.7	276	.9
74	5.4	151	7.9	214	4.1	279	1.1
75	7.9	152	20.8	215	2.9	280	3.1
76	18.6	153	16.0	216	1.6	281	2.3
77	19.6	154	8.4	217	< 0	282	6.3
78	7.5	155	10.0	218	1.3	284	100.0
79	5.5	156	10.2	219	< 0	205	18.4
80	3.5	157	5.5	220	1.5	206	2.4
81	3.7	158	2.1	221	3.2	207	< 1
82	18.2	159	2.6	222	3.5	292	< 1
83	16.7	160	4.2	223	7.9	293	< 1
84	8.0	161	3.5	224	10.4	294	2.3
86	2.8	162	4.2	225	12.5	295	1.9
87	5.3	163	3.3	226	29.6	296	5.6
88	5.0	164	3.3	227	24.9	297	3.6
89	11.6	165	10.0	228	13.9	298	13.6
91	20.3	166	5.3	229	3.0	299	66.3
93	7.9	167	7.9	230	1.1	300	12.9
94	18.0	168	13.5	231	1.1	301	1.7
95	4.0	169	11.6	232	< 0	302	< 1
97	10.7	170	6.3	233	< 0	300	< 1
97	11.4	171	6.3	234	2.2	310	7.3
100	4.4	172	2.7	235	2.3	311	3.9
101	6.6	173	1.5	236	4.9	312	40.0
102	18.1	174	1.7	237	7.7	313	9.5
103	18.5	175	2.7	238	6.1	314	1.4
105	9.5	176	1.9	239	8.3	315	< 1
106	5.6	177	2.4	240	15.9	323	< 1
108	4.2	178	2.2	241	10.1	324	< 1
110	4.1	179	2.9	242	31.3	326	13.7
112	9.2	180	5.6	243	13.7	327	89.5
113	8.4	181	18.5	244	3.1	328	20.4
114	7.9	182	9.9	245	< 0	329	3.6
115	26.4	183	11.4	246	< 0	330	< 1
116	6.2	184	13.5	247	< 0		

PAUSE

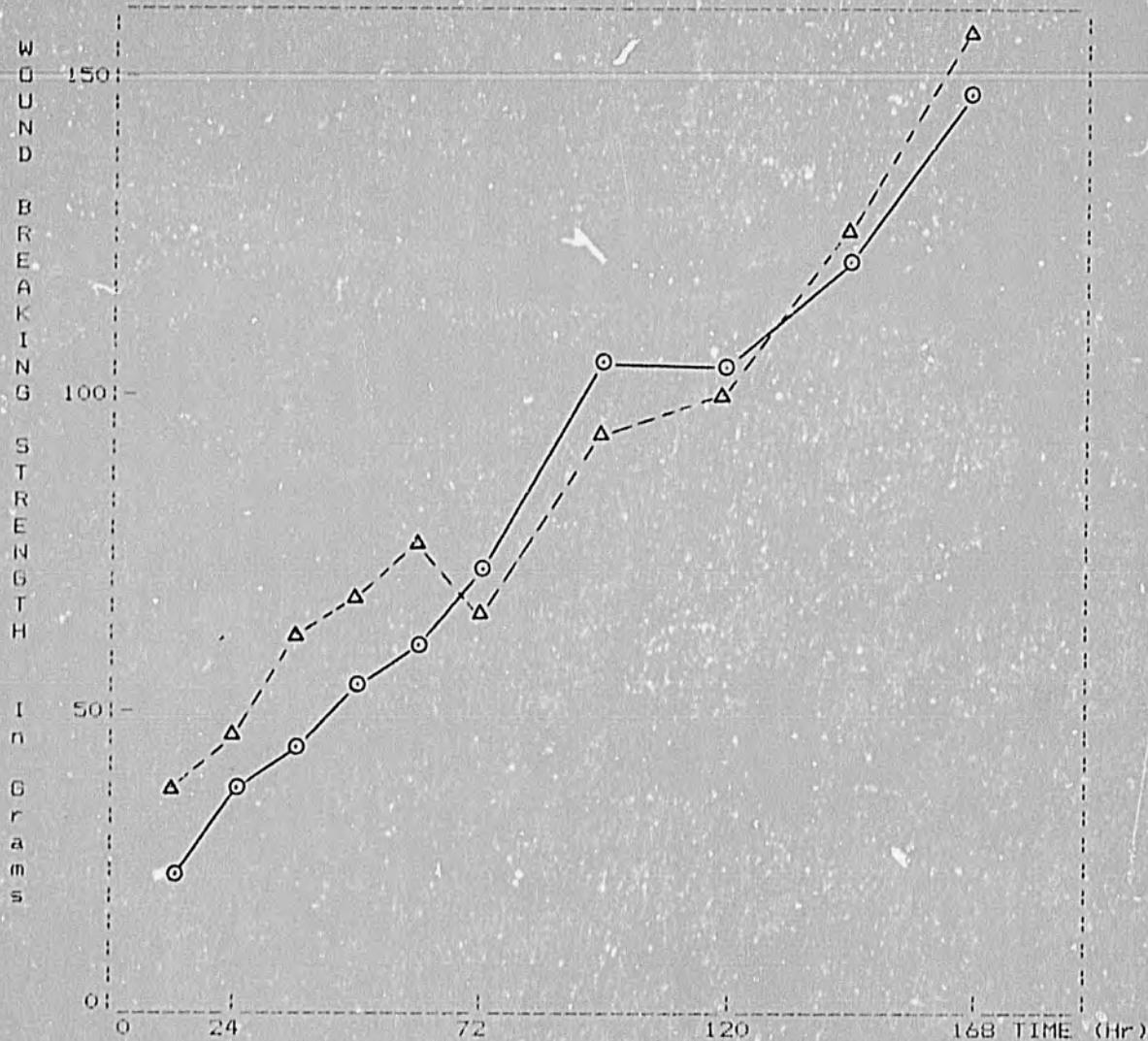


FIGURE 14. Kinetics of Cicatrization in the Presence and in the Absence of Taspine-HCl.-
For the methods see text and Table 3. In this experiment we used 5 mice per each group, and the concentration of taspine-HCl used was 0.066 mg/ml.
Taspine-HCl-treated (Δ --- Δ).
Control without taspine-HCl (\circ — \circ).

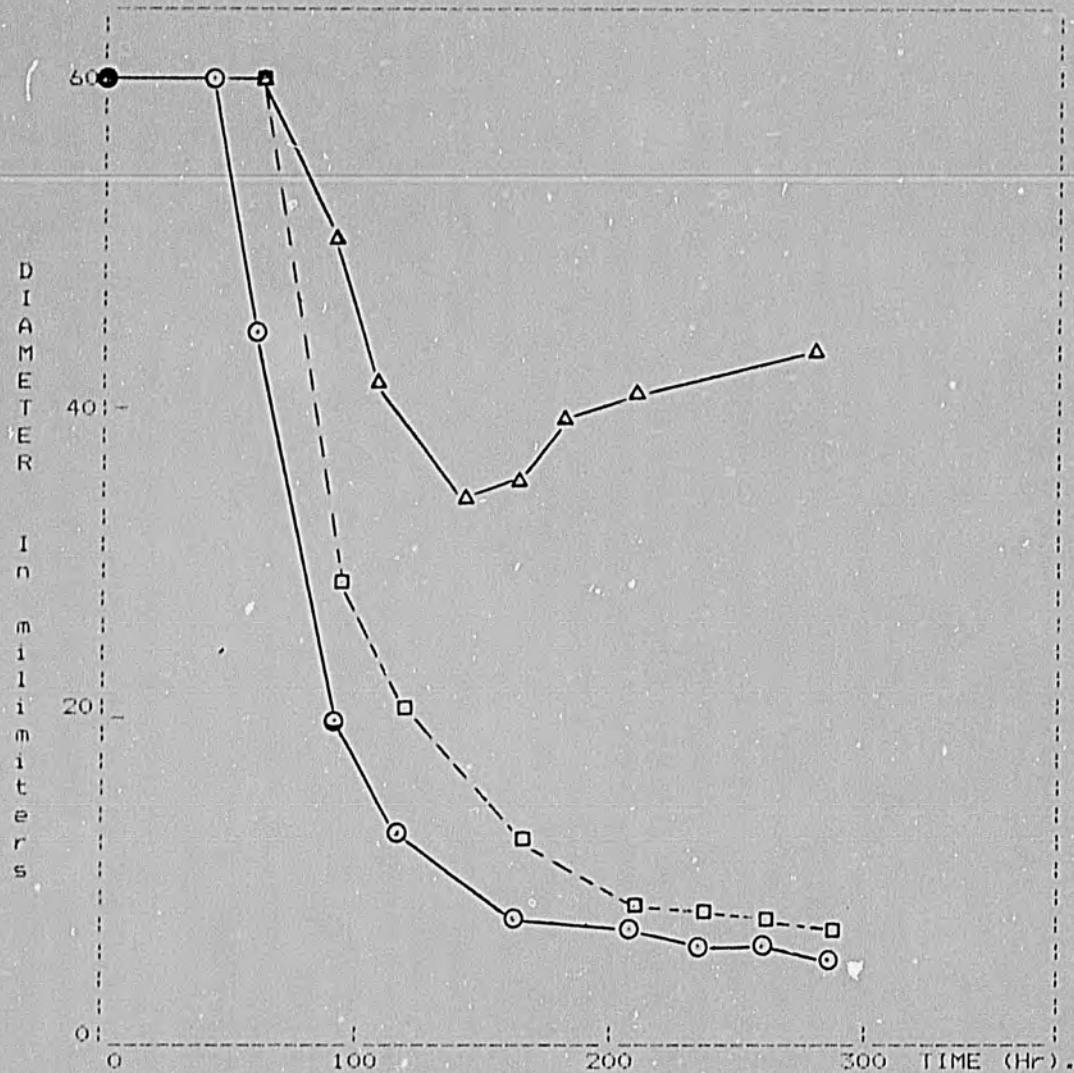


FIGURE 15. Effect of Taspine Chlorhydrate on the Contraction of Fibroblasts-Collagen Lattices.-
For the general methodology see text.
Laticces for this experiment were prepared with
 3×10^6 cells and contained 0.280 mg/ml collagen.
The concentrations of taspine-HCl tested were:
130 ng/ml (Δ); 52 ng/ml (\square); Control 0 ng/ml (\circ);
Control laticces without cells did not contract at all.
Each point corresponded to the average of three plates.
Deviations are not shown because they were very small.
(They were less than \pm 5% of each value).

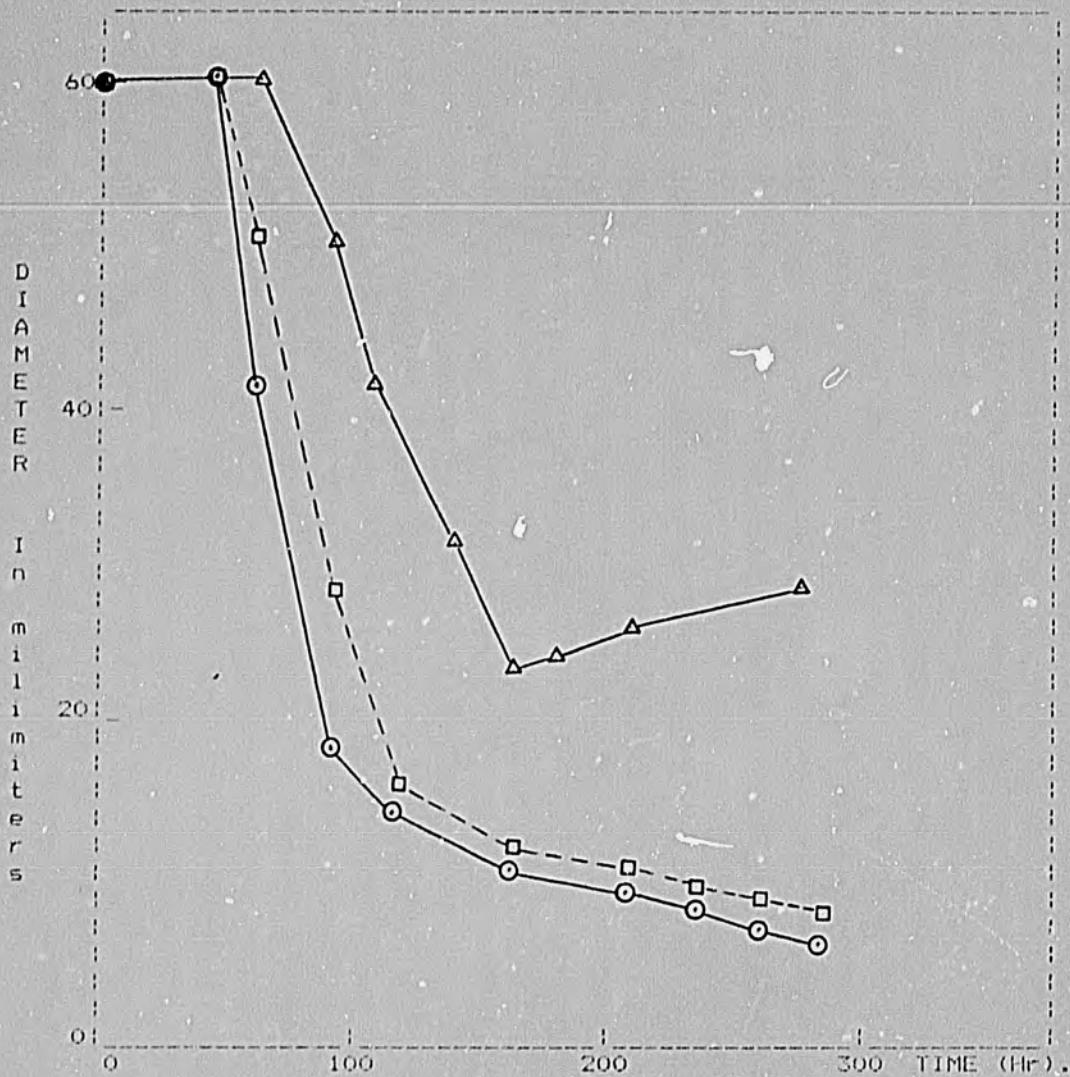


FIGURE 16. Effect of Taspine Chlorhydrate on the Contraction of Fibroblasts-Collagen Lattices.-
For the general methodology see text.
Laticces for this experiment were prepared with
 6×10^5 cells and contained 0.560 mg/ml collagen.
The concentrations of taspine-HCl tested were:
130 ng/ml (Δ); 52 ng/ml (\square); Control 0 ng/ml (\circ);
Control laticces without cells did not contract at all.
Each point corresponded to the average of three plates.
Deviations are not shown because they were very small.
(They were less than \pm 5% of each value).