

- I. THE STRUCTURE OF THE ORANGE PIGMENT  
FROM PSEUDOMONAS AUREOFACIENS
- II. THE BIOGENESIS OF STRYCHNINE
- III. BECKMANN FISSION OF A  $\gamma$ -KETO OXIME

Thesis by  
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## ABSTRACT

### PART I

The structure of the orange pigment from Pseudomonas aureofaciens was investigated and found to be 1-carboxy-2-hydroxyphenazine. A degradation product, 1-methyl-2-methoxyphenazine, was shown to be identical to a synthetic sample.

### PART II

The biogenesis of the indole alkaloid, strychnine, was studied by administration of C<sup>14</sup> labelled precursors to Strychnos nux-vomica plants and degradations of the radioactive strychnine isolated from the plants. The results indicate an acetate origin for the non-tryptophan portion and a formate origin for the "berberine bridge" carbon of strychnine.

### PART III

A Beckmann fission of the tosylate of the  $\gamma$ -keto oxime, 3-acetoxy-lanostane-7,11-dione-7-oxime, was carried out with sodium t-butoxide, and the expected cleavage of ring B and formation of a nitrile and unsaturated ketone occurred.

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# THE STRUCTURE OF THE ORANGE PIGMENT FROM PSEUDOMONAS AUREOFACIENS

## Introduction

Naturally occurring coloring materials have been used by man for thousands of years, and just as the desire to procure these coloring matters led to an expansion of trade and commerce, the efforts of early chemists to determine the structures of these coloring matters stimulated the growth of organic chemistry in the middle of the nineteenth century. Later as synthetic dyes occupied the interests of commerce and chemistry, both trade and research in the natural pigments lagged. More recently, chemists and biochemists have become especially interested in the relationship of these natural pigments to biological systems.

It has been a little over one hundred years since the first phenazine pigment, pyocyanine, was isolated from a bacterial culture (1), although it was not established as a phenazine derivative until 1929 (2). Now many phenazine pigments from species of Pseudomonas and closely related bacteria and from the mold Streptomyces are known (Table I).

Several of the naturally occurring phenazine pigments exist in an oxidized or reduced form. For example, iodinin is a di-N-oxide and chlororaphine is a phenazhydrin. In fact one of the intriguing features of phenazines is that the phenazine-dihydrophenazine system is similar to the quinone-hydroquinone system (3, 4). The reduction of phenazines proceeds by one electron steps (5); thus semireduced phenazine free radicals may exist as well as the meriphenazines (phenazhydrins).



Table Ia

## Naturally Occurring Phenazine Pigments of Known Structure

| Pigment                 | Color             | Structure   | Microorganism   | References   |
|-------------------------|-------------------|---|---|--------------|
| Pyocyanine              | dark blue         | 5-methyl-1(5)-phenazine-<br>inone   | <u>Pseudomonas aeruginosa</u><br><u>Cyanococcus chromospirans</u> | 1, 2         |
| Chlororaphine           | green             | 3:1 molecular compound<br>of phenazine carbox-<br>amide and its dihydro<br>derivative | <u>Ps. chlororaphis</u>   | 11, 12       |
| Oxychlororaphine        | yellow            | phenazine carboxamide   | <u>Ps. chlororaphis</u>   | 12           |
| Iodinin                 | purple            | 1, 6-dihydroxyphenazine-<br>5, 10-dioxide   | <u>Chromobacterium iodinum</u>                                    | 13           |
| 1-Phenazinol            | orange            |   | <u>Ps. aeruginosa</u>   | 14           |
| 1-Carboxyphenazine      | yellow            |   | <u>Ps. aureofaciens</u><br><u>Streptomyces misa-<br/>kiensis</u>  | 15, 16<br>44 |
| 1, 6-Dihydroxyphenazine | yellow            |   | <u>S. thioluteus</u>  | 17           |
| Griseolutein A          | reddish<br>yellow | 1-methoxy-4-[(2-<br>hydroxyacetoxy) methyl]-<br>9-carboxyphenazine                    | <u>S. griseoluteus</u>  | 18           |
| Griseolutein B          | yellow            | 1-methoxy-4-[(1, 2-di-<br>hydroxyethoxy) methyl]-<br>9-carboxyphenazine               | <u>S. griseoluteus</u>  | 18           |

Table Ib

## Naturally Occurring Phenazine Pigments of Unknown Structure

| Pigment                                | Color | Empirical Formula or Partial Structure                       | Microorganism   | References |
|--|-------|--|---|------------|
| Pigment II from <i>P. aureofaciens</i> | red   | $C_{13}H_8O_3N_2$  | <i>Ps. aureofaciens</i>   | 15, 16     |
| Pigment A from <i>P. aeruginosa</i>    | red   | carboxy-2-amino-10-methylphenazinium hydroxide hydrochloride |   | 19         |
| Pigment B                              | red   | $C_{13}H_{15}O_6N_3S$  | <i>Ps. aeruginosa</i>   | 19         |
| Purple Pigment                         |       | $C_{16}H_{12}O_6N_2$   | <i>Pseudomonas</i> sp. from Trinidad  | 20         |
| Blue Pigment                           |       | $C_{14}H_{18}O_4N_2$ (?)                                     | <i>Ps. lemonieri</i>  | 21, 22     |
| Green Fluorescent Pigment              |       |  | <i>Ps. lemonieri</i><br><i>Ps. aeruginosa</i><br><i>Ps. eisenbergii</i><br><i>Ps. fluorescens</i> | 22, 23     |
| Tubermycin A                           |       | $C_{17}H_{16}O_2N_2$   | <i>S. misakiensis</i>   | 44         |

Several investigations have been carried out to determine whether the above phenomenon might be significant in biological systems as a means of lowering the energy barrier to atmospheric oxidation (6, 7, 8).

From the point of view of biogenesis, the occurrence of carbon substituents only at the 1 and 6 positions of the known phenazine pigments is noteworthy. However nothing definitive had been said concerning the biogenesis of phenazine pigments until the recent finding of Carter and Richards (8, 9), that anthranilic acid is a precursor of chlororaphine. The mechanism of anthranilic acid condensation to phenazine in bacteria and the biogenesis of the hydroxylated phenazines are still open to speculation. Conceivably the structures and oxidation states of recently discovered and incompletely characterized bacterial pigments might furnish a clue to these questions. For example, isolation of a phenazine mono-N-oxide might indicate a mechanism similar to that of the Wohl-Aue condensation, which is known to involve a nitroso intermediate (10).

A phenazine pigment of undetermined structure from Pseudomonas aureofaciens seemed to be promising with respect to such a mechanistic implication, and hence its structural elucidation was undertaken.

### Historical Background of the Orange Pigment

In 1956, a Dutch chemist, H. Bouman, first isolated from the mud of the river Maas, a strain of Pseudomonas, from whose liquid cultures he was able to isolate a yellow crystalline pigment, later shown to be l-carboxyphenazine (15). The same l-carboxyphenazine producing bacteria was isolated from soils in this country by Haynes and coworkers (16). This unusual bacterium was named Pseudomonas aureofaciens because of its characteristic golden color on agar media.

In addition to the yellow l-carboxyphenazine, both Kluyver (15) and Haynes (16) isolated a red acidic pigment to which they assigned the formula  $C_{13}H_8O_3N_2$ , although the carbon analysis was 1.7 percent low. The pigment was shown to have a phenazine nucleus by zinc dust distillation, hence this pigment seemingly is an oxidized form of l-carboxyphenazine. Since this pigment is produced in extremely small yield, neither Kluyver nor Haynes were able to isolate enough material for a structural determination.

### Pigment Production and Isolation

A strain of Ps. aureofaciens, NRRL B-1543P from the soils of Ohio, was obtained from Haynes and slants and culture media were prepared by his method. The cultures in 2.8 liter Fernback flasks were shaken for five days at room temperature. The mixture of crude pigments was isolated from the culture media by filtration of the acidified media through diatomaceous earth and extraction of the residue with chloroform. Lipids were separated from the pigments by washing the chloroform extract with sodium hydroxide solution, acidifying the bright red basic solution, and extracting again with chloroform.

Neither the chromatographic method of Kluyver (15) nor the precipitation with carbon dioxide technique of Haynes (16) was effective in separating the pigments. In fact no useful chromatographic method was found, and no difference in the solubilities of the pigments in sodium bicarbonate solution could be found. A clean separation was however effected by adding a very small amount of ten percent sodium hydroxide solution to the mixture of pigments so as to form a paste, then filtering by suction. The red filtrate contained none of the yellow 1-carboxy-phenazine, since it is apparently much less soluble in sodium hydroxide than the red pigment.

Acidification of the red filtrate and extraction with chloroform yielded the red pigment, which was crystallized from benzene. Tiny orange needles were obtained in a yield of about 10 mg. per liter of media. Crystals of impure pigment were red rather than orange, due to the presence of very tiny amounts of three other pigments (see page 19).

The pure orange compound darkens at about 220°, melts and decomposes at 225-226°, at which time yellow needles form above the decomposing pigment. These newly formed needles decompose at 236-237°. Kluyver reported a melting point near 225°, with gas evolution, and discoloration at 200°.

### Characterization

The most striking characteristic of the orange pigment is its brilliant red basic solution, which contrasts conspicuously with the yellow basic solution of 1-carboxyphenazine. The pKa of the pigment in 66.8 percent dioxane-water was found to be 7.61, a value reasonable for a carboxy group in this system (24). A neutralization equivalent of about 240 was obtained, based on titration in 66.8 percent dioxane-water. The red color did not appear at the end point, but rather when about twice the equivalent amount of base had been added; hence the phenazine carboxylate anion is probably not responsible for the red color. Since 1- and 2-phenazinol form red basic solutions, the presence of a phenol was inferred.

That the pigment is a phenazinol was confirmed by positive ceric nitrate and ferric chloride tests. A Tollens test was negative. However the impure red pigment gave a positive Tollens test. The formula  $C_{13}H_8O_3N_2$  assigned by Haynes (16) was found to be consistent with the microanalyses.

The ultraviolet spectrum in ninety-five percent ethanol exhibits bands at 252 m $\mu$  (log  $\epsilon$  4.60) and 363 m $\mu$  (log  $\epsilon$  4.04), which with added base change to 287 m $\mu$  (log  $\epsilon$  4.66), 363 m $\mu$  (log  $\epsilon$  3.97) and 490 m $\mu$  (log  $\epsilon$  3.90). These absorptions are consistent with those observed for 1- and 2-phenazinol and 1-carboxyphenazine shown in Table II.

The infrared spectrum shows no OH stretching absorptions above 3000  $cm^{-1}$ ; hence the phenol and carboxy hydrogens must both be intramolecularly hydrogen-bonded. A carbonyl band is present at

Table II  
Ultraviolet Absorptions of Phenazines

| <u>2-Phenazinol*</u>    |       |                         |       |                         |                |
|-------------------------|-------|-------------------------|-------|-------------------------|----------------|
| $\lambda_{\text{max.}}$ | log e | $\lambda_{\text{max.}}$ | log e | $\lambda_{\text{max.}}$ | log e          |
| absolute alcohol        |       | 50% alcohol             |       | water                   |                |
| 258                     | 4.85  | 255                     | 4.80  | 255                     | 4.72           |
| 355                     | 3.92  | 280                     | 4.20  | 280                     | 4.30           |
| 410                     | 3.85  | 365                     | 3.92  | 370                     | 3.92           |
|                         |       | 415                     | 3.75  | 415                     | 4.67           |
|                         |       |                         |       | 500                     | 3.4 (shoulder) |

1-Phenazinol\*

absolute alcohol

|     |      |
|-----|------|
| 265 | 4.72 |
| 360 | 3.82 |
| 370 | 3.90 |

1-Carboxyphenazine

95% ethanol

|     |      |
|-----|------|
| 251 | 4.85 |
| 365 | 4.12 |

---

\*Determined from published ultraviolet spectra (25).



1676  $\text{cm}^{-1}$ . The CH out-of-plane bending region has a very intense peak at 760  $\text{cm}^{-1}$ , presumably due to four adjacent hydrogens, and weaker peaks at 670  $\text{cm}^{-1}$  and 875  $\text{cm}^{-1}$  for which definite assignments have not been made.

The orange pigment (I) formed a bright yellow derivative  $\text{C}_{15}\text{H}_{12}\text{O}_3\text{N}_2$  (II) on treatment with diazomethane. The nuclear magnetic resonance spectrum of this yellow compound indicates the presence of two methoxy groups. The carbonyl band in the infrared was shifted up to 1737  $\text{cm}^{-1}$ . If the carbonyl of the orange pigment had been hydrogen-bonded to an ortho phenolic hydrogen, methylation of the phenol would account for this large hypsochromic shift.

Bands in the ultraviolet were observed in ninety-five percent ethanol at 257  $\text{m}\mu$  ( $\log e$  4.82) and 360  $\text{m}\mu$  ( $\log e$  3.95). 2-Methoxyphenazine in absolute alcohol exhibits bands at 255  $\text{m}\mu$  ( $\log e$  4.72) and 360  $\text{m}\mu$  ( $\log e$  4.00).

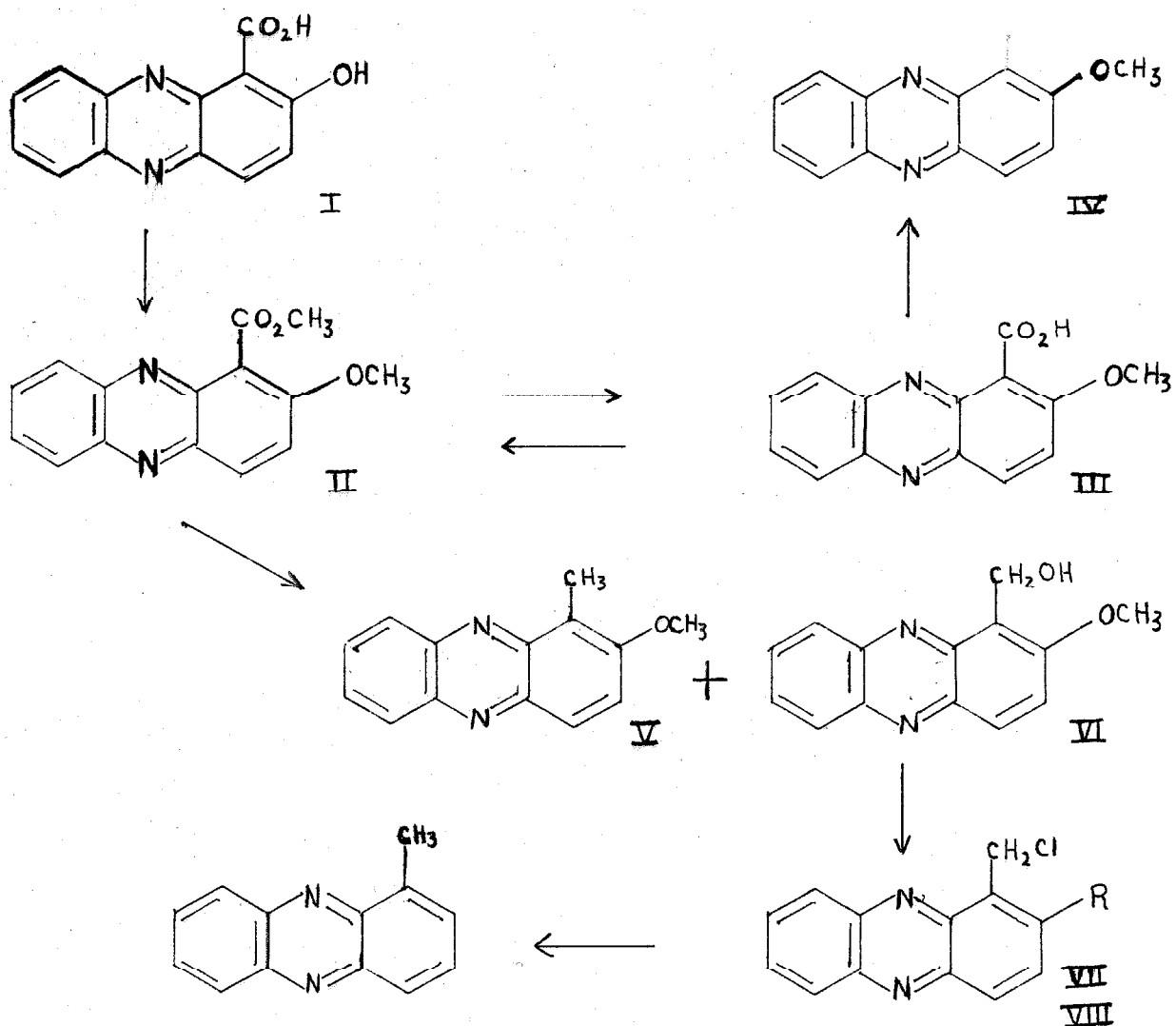
On refluxing in ten percent sodium hydroxide, the dimethyl derivative was converted to the carboxylic acid  $\text{C}_{14}\text{H}_{10}\text{O}_3\text{N}_2$  (III). Methylation with diazomethane reconverted this acid to the dimethyl derivative II. Decarboxylation of the acid III gave 2-methoxyphenazine (IV), whose identity was confirmed by mixed melting point and comparison of its infrared spectrum with an authentic sample, prepared by the method of Vivian (26). The decomposition of the orange pigment at the melting point is undoubtedly a thermal decarboxylation to 2-phenazinol, which when pure melts at 253-254° (27).

Reduction of the dimethyl derivative with lithium aluminum hydride in ether gave two compounds separated by chromatography on

alumina. A yellow fluorescent band was eluted with benzene-ether, and from it was obtained bright yellow crystals, which melted at 123-124°. The nuclear magnetic resonance spectrum indicates the presence of one methyl and one methoxyl group. The compound has no OH stretching or carbonyl bands in the infrared, hence was presumed to be a methyl-2-methoxyphenazine (V). Reduction of a carbomethoxyphenazine to a methylphenazine with lithium aluminum hydride in tetrahydrofuran was previously reported by Nakamura (18).

The second band, which was eluted with acetone, gave yellow crystals of a hydroxymethyl-2-methoxyphenazine (VI), which melted at 173-174°. This compound was treated with thionyl chloride, and an inseparable mixture believed to be composed of chloromethyl-2-methoxyphenazine (VII) and chloromethyl-2-chlorophenazine (VIII) was obtained. Reduction of the mixture with lithium aluminum hydride in ether and chromatography of the products gave pale yellow crystals of 1-methylphenazine. From this evidence, the orange pigment must be 1-carboxy-2-hydroxyphenazine.

1-Methyl-2-methoxyphenazine was synthesized and shown to be identical to the product (V) of the reduction of the dimethyl derivative (II) with lithium aluminum hydride. 3-Chloro-2-methyl-2'-nitrodiphenylamine, prepared by heating 3-chloro-2-methylaniline with o-chloronitrobenzene and sodium acetate at 250°, was reduced with ferrous oxalate at 270° to give 1-methyl-2-chlorophenazine. An N-oxide, presumably the 5-oxide, was obtained by oxidation with hydrogen peroxide in acetic acid at 50°. Refluxing this with potassium hydroxide in methanol yielded 1-methyl-2-methoxyphenazine-N-oxide and 1-methyl-2-hydroxyphenazine-



N-oxide. The 1-methyl-2-methoxyphenazine-N-oxide was reduced to 1-methyl-2-methoxyphenazine, m.p. 125°, with lithium aluminum hydride in ether. A mixed melting point of the synthetic 1-methyl-2-methoxyphenazine with the reduction product V was not depressed, and the infrared spectra were identical.

Methanolysis of 1-methyl-2-chlorophenazine to 1-methyl-2-methoxyphenazine directly was unsuccessful. Many attempts to oxidize the methyl group of the above compounds to a carboxy group were similarly unsuccessful.

## Reactions of 1-Carbomethoxy-2-methoxyphenazine

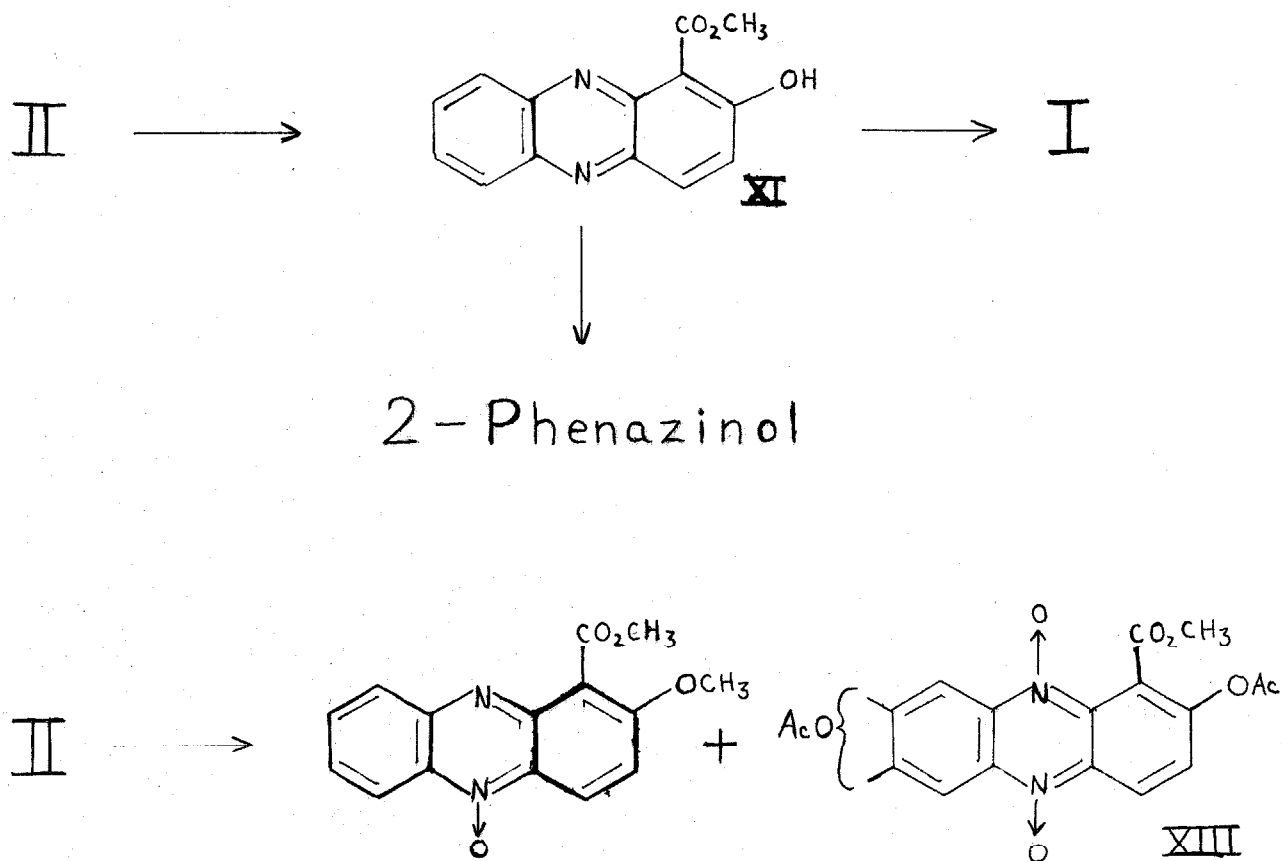
Two unusual reactions were carried out on the dimethyl derivative of the orange pigment. These were not essential to the structural proof, but did provide some of the more interesting chemistry of this problem.

When the dimethyl compound (II) was treated with stannous chloride in concentrated hydrochloric acid at 0°, the ether was cleaved in a few seconds giving the phenazinol (XI), m.p. 141°. This was partially soluble in sodium bicarbonate and gave a positive ferric chloride test. The carbonyl band in the infrared is shifted down to 1655 cm.<sup>-1</sup> by hydrogen-bonding. Methylation with diazomethane reconverted the phenazinol (XI) to the dimethyl compound (II). When the phenazinol (XI) was refluxed with sodium hydroxide, the red color characteristic of the orange pigment was observed. After a few hours, the color changed to orange, and a yellow compound (XII), which proved to be 2-phenazinol, was isolated. If the hydrolysis was stopped after one hour, crystals of the orange pigment (I) were isolated.

Previously phenazine ethers were cleaved by refluxing with aluminum chloride in benzene (26). Indeed, the dimethyl derivative (II) could be cleaved with aluminum chloride, and also by refluxing in concentrated hydrochloric acid or stannic chloride, however the more facile cleavage with cold stannous chloride is noteworthy. 2-Methoxyphenazine was similarly cleaved in good yield with stannous chloride.

Oxidation of the dimethyl derivative (II) with hydrogen peroxide in acetic acid at 50° resulted in two pigments, separable on an alumina column. One was the N-oxide of II and the other was a bright red compound having the approximate formula C<sub>18</sub>H<sub>14</sub>O<sub>8</sub>N<sub>2</sub>. The nuclear

magnetic resonance spectrum shows only one methoxyl group, but two methyl groups are present. The infrared peak at  $760\text{ cm}^{-1}$  is not present, but three bands appear between  $810$  and  $835\text{ cm}^{-1}$ , ruling out CH out-of-plane bending due to three or four adjacent hydrogens. The red compound did not form a darkly colored complex with stannous chloride as did all the 2-methoxyphenazines, however the stannous chloride readily converted the compound to a phenolic material. The most likely structure then for this product is 1-carbomethoxy-2, 7- or 8-diacetoxyphenazine-5,10-dioxide (XIII). N-Oxide rearrangements have not previously been reported in the phenazine series.



## Biogenetic Speculation

Since the possibility entertained at the outset that the orange pigment might be a phenazine-N-oxide was demonstrated not to be the case, there is no reason then to suspect a Wohl-Aue oxidative condensation of anthranilic acid units, proceeding through a nitroso intermediate. Phenazines have in fact been prepared by a great variety of condensations, oxidations, reductions, pyrolytic and photolytic reactions from amines; hence the mechanism occurring in the microorganisms might resemble any of these in vitro reactions.

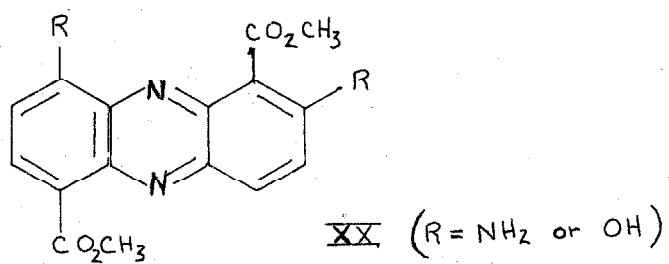
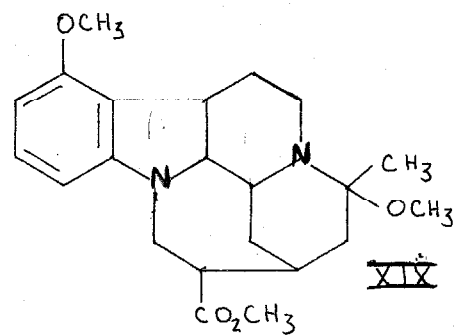
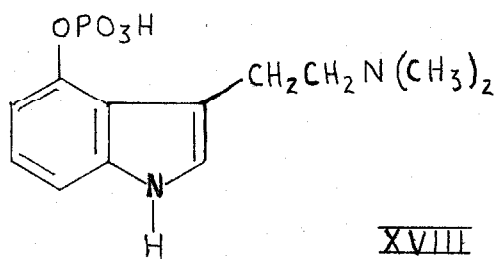
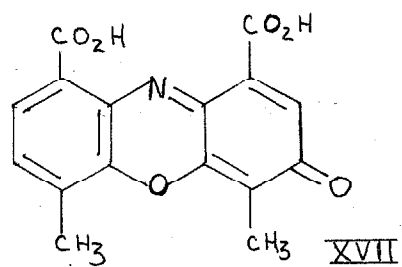
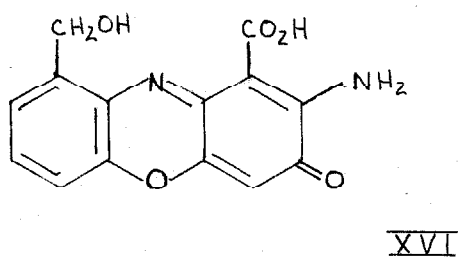
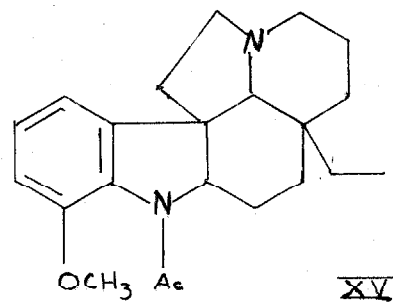
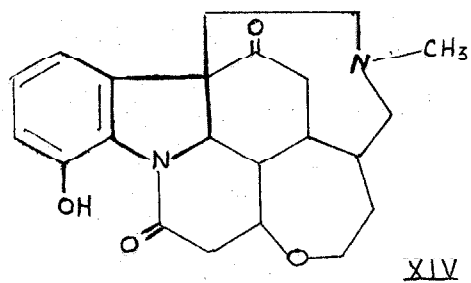
The phenazine hydroxy group might arise in four different ways, oxidative decarboxylation of a carboxyphenazine, electrophilic attack by a hydroxylating species, nucleophilic attack on the methyl phenazinium salt by hydroxide, or condensation of hydroxyanthranilic acids. Oxidative decarboxylations are known to occur in Pseudomonas bacteria; for example nicotinic acid is hydroxylated in this manner by Ps. fluorescens (28). In the case of the orange pigment and the griseolutein pigments, oxidative decarboxylation is unlikely, since 2 one-carbon substituents would have had to be present in one ring, ruling out any kind of anthranilic acid precursor. Phenazines are generally not attacked by electrophilic reagents, however hydroxylation of a dihydrophenazine or phenazine-N-oxide could have conceivably occurred in the biogenesis of these pigments.

Pyocyanine has been shown to result from treating 5-methylphenazinium methyl sulfate with base in the presence of visible light and air (29). 1-Phenazinol was also produced in small amounts in the reaction. When the same reaction was carried out in the dark, substitution occurred at the 2 position to give 10-methyl-2(10)-phenazinone.

This was easily converted to 2-phenazinol with hydrogen peroxide in base. Besides pyocyanine, at least three other partially characterized naturally occurring phenazine pigments lend support to a biogenesis involving a phenazinium intermediate. The purple pigment isolated from a Pseudomonas species of Trinidad was shown to have one N-methyl group (20). Holliman has shown that two pigments from Pseudomonas aeruginosa are 2-amino-10-methylphenazinium salts (19). Kehrman and Hovas (30) found that 2-amino-10-methylphenazinium methyl sulfate was formed by reacting 5-methylphenazinium methyl sulfate with ammonia in air.

The existence of many hydroxyindoles as well as hydroxyphenazines suggests that hydroxyanthranilic acids may be intermediates in the biogenesis of these two classes of compounds. Two hydroxyanthranilic acids are known to be involved in bacterial metabolism. 3-Hydroxyanthranilic acid, a metabolic product of tryptophan, was shown to be a precursor of nicotinic acid (31). Although it does not participate in the tryptophan-anthranilic acid cycle, it may give rise to 7-hydroxytryptophan in the same way that anthranilic acid is converted to tryptophan. The alkaloids, vomicine (XIV) (32) and aspidospermine (XV) (33), as well as the phenoxazone pigments, cinnabarin (XVI) (34) and actinocinin (XVII) (35), might be derived from this precursor. 5-Hydroxyanthranilic acid is known to promote the growth of Escherichia coli (36).

Neither 4- nor 6-hydroxyanthranilic acid are known precursors or metabolites, though these are the isomers which must be invoked in the case of the phenazine pigments. That 6-hydroxyanthranilic acid may be a progenitor is indicated by the structures of the hallucinagen psilocybin (XVIII) (37) and the alkaloid mitragenine (XIX) (38).





Incorporation of  $C^{14}$  labelled hydroxyanthranilic acids into their respective phenazine pigments by the phenazine-producing bacteria or mold would of course substantiate the latter of the biogenetic possibilities. Incorporation of  $O^{18}$  labelled water into the hydroxy group of phenazines would indicate that a nucleophilic attack on a phenazinium compound had occurred. An electrophilic oxidation might be tested by growing the bacteria in presence of  $O^{18}$  labelled oxygen to see if it is incorporated and by the effect of inhibitors on pigment production, such as  $\alpha, \alpha$ -dipyridyl, which complexes with iron and prevents the hydroxylating agents, ferryl and perferryl ions, from forming, and p-chloromercuribenzoate, which inhibits sulfhydryl hydroxylations (39).

## ISOLATION OF ADDITIONAL PIGMENTS

The methyl esters of three more pigments from Pseudomonas aureofaciens were isolated in very small quantities. Diazomethane in ether was added to a chloroform solution of a deep red batch of the crude orange pigment. Chromatography on alumina yielded a small amount of 1-carbomethoxyphenazine and a large amount of 1-carbomethoxy-2-methoxyphenazine. Elution with methanol-ether separated the remaining material into three bands of rose, brown, and violet hues.

The rose pigment has two carbonyl bands in the infrared at  $1740\text{ cm.}^{-1}$  and  $1660\text{ cm.}^{-1}$ . The higher band must be from a carbomethoxy group and the lower from a carbomethoxy group hydrogen-bonded to an ortho phenol or amino hydrogen. The CH out-of-plane bending absorption at  $760\text{ cm.}^{-1}$  for four adjacent hydrogens is not present; however several weaker peaks appear in this region. Two sharp peaks occur in the hydrogen stretching region at  $3495\text{ cm.}^{-1}$  and  $3470\text{ cm.}^{-1}$ , and weaker bands at  $3380\text{ cm.}^{-1}$  and  $3290\text{ cm.}^{-1}$ .

The violet pigment also has carbonyl peaks at  $1740\text{ cm.}^{-1}$  and  $1660\text{ cm.}^{-1}$ , but the hydrogen stretching absorptions are different, i. e. peaks at 3625, 3520, 3470, and  $3340\text{ cm.}^{-1}$ .

Not enough of the brown pigment was obtained for a good spectrum.

Possible structures for the pigments are represented by formulation (XX), although a dihydrophenazine or phenazhydrin structure cannot be ruled out. Further structural work on these pigments may turn up an interesting relationship with the partially characterized aminophenazines from Pseudomonas aeruginosa (11).

## EXPERIMENTAL

Pigment Production. Strain NRRL B-1543 P of Pseudomonas aureofaciens was obtained from William C. Haynes and was cultured by his method (15) by Dr. Ralph Pressman. The only modifications were that the inoculum flasks and 2.8 l. Fernbach flasks containing 800 ml. of culture medium were shaken on a horizontal shaker at 80-90 cycles per minute at 25-27° instead of on a rotary shaker at 28° C.

Isolation and Purification of the Orange Pigment. The culture medium (800 ml.) was acidified with 5.0 ml. of concentrated hydrochloric acid, and 10 g. of Celite was added. The suspension was filtered by suction after standing at least one hour. The Celite containing the pigments was allowed to dry, and the Celite pads from five cultures were boiled with 250 ml. of chloroform. After filtering, the pads were boiled again with 100 ml. of chloroform; this process was repeated six times. The combined chloroform filtrates were shaken with 50 ml. of 5% sodium hydroxide and the layers separated. This was repeated twice with 25 ml. of 5% sodium hydroxide. The combined red basic solutions were acidified with 1 N hydrochloric acid, and the pigments were extracted with 75 ml. of chloroform three times. The chloroform solutions were dried over sodium sulfate, filtered, and evaporated to give about 3.5 g. of crude pigments.

Sodium hydroxide solution (10%) was added to the crude pigments so as to form a thin paste, which was filtered by suction. Acidification of the red filtrate with 1 N hydrochloric acid, extraction with 50 ml. of chloroform three times, and evaporation of the chloroform after drying

over sodium sulfate yielded about 35 mg. of crude red pigment. The crude pigment was crystallized twice from benzene giving fine orange needles, darkening at about 220°, and melting at 225–226° with decomposition. Above the decomposing pigment, new yellow crystals formed which decomposed at 236–237°. Further treatment of the mixture of red and yellow pigments with sodium hydroxide as above gave another 25 mg. of red pigment, although less pure than the first material.

The orange pigment formed a gold-brown solution with ferric chloride in chloroform and pyridine (40), and a brown solution with ceric nitrate in dioxane (41). A negative Tollens test was given by the pure orange pigment; however the impure red pigment formed a silver mirror in the Tollens test.

The orange pigment (2.93 mg.) was dissolved in 10 ml. of 66.8% dioxane–water and titrated with 0.02343 N sodium hydroxide on a pH stat (42). The end point was observed at approximately 0.52 ml., giving a neutralization equivalent of about 240. The pKa in 66.8% dioxane–water was determined by titration with 0.02343 N sodium hydroxide on the pH stat, giving values of 7.60 and 7.61.

Anal. Calcd. for  $C_{13}H_8O_3N_2$ : C, 65.00; H, 3.36; N, 11.67.

Found: C, 64.88; H, 3.41; N, 11.75.

Ultraviolet spectrum in 95% ethanol:  $\lambda_{\text{max.}}$  252 m $\mu$  (log  $\epsilon$  4.60), 363 (4.04).

Ultraviolet spectrum in basic 95% ethanol:  $\lambda_{\text{max.}}$  287 m $\mu$  (log  $\epsilon$  4.66), 363 (3.97), 490 (3.90).

Infrared spectrum in chloroform:  $\nu_{\text{max.}}$  1675  $\text{cm.}^{-1}$ , 1608, 1567, 1483, 1445, 1365, 1300, 1275, 1140, 1125, 1009, 990, 961, 910, 865, 845.

Infrared spectrum in carbon disulfide:  $\nu_{\max}$ , 3005  $\text{cm}^{-1}$ , 2955, 2845, 1212, 1122, 925, 875, 760, 670.

Methylation of the Orange Pigment. A solution of 100 mg. of the orange pigment in 50 ml. of benzene was added to 25 ml. of an ether solution containing about 100 mg. of diazomethane prepared from 370 mg. of N-methyl-nitrosourea (43). The solution was stirred overnight with a magnetic stirrer, and acetic acid was added to destroy excess diazomethane. Evaporation under reduced pressure on a rotory evaporator left a yellow powder, which was chromatographed on an alumina (Merck) column. The bright yellow band was eluted with benzene-ether and crystallized from carbon tetrachloride. Yellow crystals (96 mg.), melting at 127-128°, were obtained. The yellow pigment was further purified by sublimation; the material collected between 110° and 120° at 1 mm. was recrystallized twice from carbon tetrachloride, giving yellow crystals, m.p. 129°.

Anal. Calcd. for  $\text{C}_{15}\text{H}_{12}\text{O}_3\text{N}_2$ : C, 67.15; H, 4.51; N, 10.44.  
Found: C, 67.09; H, 4.74; N, 10.30.

Ultraviolet spectrum in 95% ethanol:  $\lambda_{\max}$ , 257  $\text{m}\mu$  (log  $\epsilon$  4.82), 360 (3.95).

Infrared spectrum in chloroform:  $\nu_{\max}$ , 1737  $\text{cm}^{-1}$ , 1634, 1608, 1492, 1468, 1387, 1350, 1328, 1302, 1158, 1136, 1125, 1093, 969, 905.

Infrared spectrum in carbon disulfide:  $\nu_{\max}$ , 3060  $\text{cm}^{-1}$ , 3010, 2950, 2845, 828, 820, 800, 780, 760.

Nuclear magnetic resonance spectrum in carbon tetrachloride: doublet at 4.0 PPM below TMS.

Hydrolysis of the Dimethyl Derivative. A mixture of 44 mg. of the dimethyl compound (II) with 25 ml. of 10% sodium hydroxide solution was heated at 95° in an oil bath for seven hours. The orange basic solution was cooled, washed with 25 ml. of chloroform three times, and acidified with dilute hydrochloric acid. Extraction of the yellow suspension with 50 ml. of chloroform three times gave a yellow powder on drying and evaporation of the chloroform solution. Crystallization of the yellow material from benzene gave 25 mg. of the yellow acid (III), m.p. 204-205°.

Anal. Calcd. for  $C_{14}H_{10}O_3N_2$ : C, 66.13; H, 3.96; N, 11.02.

Found: C, 66.30; H, 4.01; N, 11.16.

Infrared spectrum in chloroform: 1717  $\text{cm}^{-1}$  (acid).

Decarboxylation of the Acid III. The yellow acid III (20 mg.) was heated with precipitated copper powder (0.5 g.) in 25 ml. of diphenyl ether at 250° in an oil bath. After four hours the mixture was cooled and placed on an alumina (Merck) column packed in petroleum ether (60-70). A yellow band was separated on the column with benzene and eluted from the column with chloroform. Evaporation of the chloroform left a yellow powder which was crystallized from water, giving 16 mg. of light yellow crystals, m.p. 121-122°. A mixed melting point with authentic 2-methoxyphenazine was not depressed. The infrared spectrum was identical to that of 2-methoxyphenazine.

2-Methoxyphenazine. 2-Nitro-4'-methoxydiphenylamine prepared from o-chloronitrobenzene and p-anisidine was reduced to 2-methoxyphenazine by the method of Vivian (26) without modification. The 2-

methoxyphenazine was chromatographed on alumina (Merck) in the same manner as above and crystallized from water, giving pale yellow crystals, m.p. 122-123°.

Methylation of the Acid III. To a solution of 20 mg. of the acid III in 10 ml. of chloroform was added a solution of about 100 mg. of diazomethane in 25 ml. of ether, prepared as before. The reaction was stirred with a magnetic stirrer overnight and acetic acid was added. Yellow crystals (15 mg.), m.p. 127°, were obtained by chromatographic separation on alumina and crystallization from carbon tetrachloride as in the previous methylation. A mixed melting point with the original dimethyl compound (II) was not depressed. The infrared spectra were identical.

Reduction of the Dimethyl Derivative. A solution of 100 mg. of the dimethyl compound (II) in 3 ml. of purified tetrahydrofuran was added dropwise to a stirred suspension of 100 mg. of lithium aluminum hydride (Metal Hydrides, Inc.) in 20 ml. of diethyl ether. The mixture was refluxed for three hours. The excess lithium aluminum hydride was destroyed by adding a saturated solution of sodium sulfate dropwise until no further reaction occurred. The ether was decanted and the white residue was washed with 20 ml. of ether twice. The combined ether solutions were evaporated and chromatographed on an alumina (Merck) column. A yellow band, brightly fluorescent in ultraviolet light, was eluted with benzene, and a second yellow band was eluted with ether-acetone.

Yellow material from the first band was purified by removing a small amount of yellow impurities by sublimation at 60° and 1 mm. pressure. Further sublimation at 80° and 1 mm. gave a yellow compound which was crystallized from ethanol, giving 22 mg. of yellow crystals, m.p. 123-124°. A mixed melting point with synthetic 1-methyl-2-methoxyphenazine was not depressed. When the reduction was carried out in tetrahydrofuran as a solvent, only 3 mg. of the methylphenazine were obtained.

Nuclear magnetic resonance spectrum in chloroform: singlets at 4.25 PPM and 2.93 PPM of equal area.

Sublimation of material from the second band at 100° and 1 mm. yielded yellow crystals of 1-hydroxymethyl-2-methoxyphenazine. Recrystallization from carbon tetrachloride furnished 61 mg. of the product, m.p. 173-174°.

Anal. Calcd. for  $C_{14}H_{12}O_2N_2$ : C, 69.99; H, 5.03; N, 11.66.  
Found: C, 70.09; H, 5.06; N, 11.60.

Chlorination of the Hydroxymethyl-2-methoxyphenazine (VI).

The hydroxymethyl-2-methoxyphenazine (50 mg.) was warmed with 3 ml. of thionyl chloride on a hot plate for 10 minutes. The solution was heated to boiling and the thionyl chloride evaporated. Petroleum ether (30-60) was added to the residue and then also evaporated. The residue was sublimed at 100° and 1 mm., giving some yellow material (7 mg.), which was recrystallized from carbon tetrachloride. As the bath temperature was raised to 140°, more material (36 mg.) was obtained. This was also recrystallized from carbon tetrachloride. Both purified



sublimates melted at 197–198°. The product seemed to decompose on alumina and silicic acid columns; no crystalline materials were eluted. Crystallization from ethanol also decomposed the product. Consistent analyses were not obtained; however the chlorine percentage indicated that a mixture of mono- and di-chlorinated products was present. Hence displacement of the 2-methoxy group by chloride must have occurred in some of the chloromethyl-2-methoxyphenazine.

Anal. Calcd. for 60%  $C_{14}H_{11}ON_2Cl$  plus 40%  $C_{13}H_8N_2Cl_2$ : C, 62.1; H, 3.8; N, 10.8; Cl, 18.8. Found: C, 62.26; H, 3.84; N, 11.82; Cl, 18.33.

Reduction of the Chloromethyl Compounds. A solution of 25 mg. of the product mixture of the chlorination reaction with thionyl chloride in 5 ml. of tetrahydrofuran was added dropwise to a stirred suspension of 50 mg. of lithium aluminum hydride in 20 ml. of diethyl ether. After refluxing for two hours, the suspension was cooled and a saturated sodium sulfate solution was added dropwise until the bubbling subsided. The ether solution was decanted and the white salts were washed with ether. The ether was evaporated and the residue chromatographed on alumina. Elution with benzene separated a pale yellow band, and sublimation of the material from this band gave light yellow crystals of 1-methylphenazine (7 mg.), m.p. 109°, lit. m.p. 108°. No other crystalline products could be obtained by further elution of the column.

Anal. Calcd. for  $C_{13}H_{10}N_2$ : C, 80.38; H, 5.19; N, 14.42. Found: C, 79.97; H, 5.43.

2-Nitro-3'-chloro-2'-methyldiphenylamine. o-Chloronitrobenzene (25 g.) (0.16 mole) was heated with 35 g. (0.25 mole) of 3-chloro-

2-methylaniline and 25 g. of sodium acetate (anhydrous) in a flask with a condenser at 270° for 20 hours. The black mixture was then steam distilled until no more organic material appeared in the distillate. The black oil remaining in the distillation flask was chromatographed on alumina (Merck). Elution with petroleum ether-benzene gave some orange material which was crystallized from ethanol. Yellow crystals (7.4 g.) (0.028 mole) of 2-nitro-3'-chloro-2'-methyldiphenylamine, m.p. 92-93°, were obtained; yield 18%.

Anal. Calcd. for  $C_{12}H_{11}O_2N_2Cl$ : C, 59.42; H, 4.22; N, 10.65; Cl, 13.50. Found: C, 59.75; H, 4.41; N, 10.90; Cl, 13.73.

Infrared spectrum in chloroform:  $\nu_{\max}$ , 3360  $\text{cm}^{-1}$ , 1620, 1578, 1505, 1414, 1383, 1348, 1326, 1283, 1151, 1123, 1083, 1017, 910, 812.

1-Methyl-2-chlorophenazine. A mixture of 5 g. (0.019 mole) of 2-nitro-3'-chloro-2'-methyldiphenylamine, 6.6 g. of ferrous oxalate prepared by stirring together equimolar solutions of ferrous sulfate and oxalic acid and drying the precipitate in air, and 50 g. of granulated lead was heated to 270° for 30 minutes. The mixture was cooled to 100° and sublimed at 1 mm. pressure. The yellow sublimate was chromatographed on alumina (Merck); petroleum ether-benzene eluted 2.4 g. of unreacted 2-nitro-3'-chloro-2'-methyldiphenylamine. The desired 1-methyl-2-chlorophenazine was eluted with ether and crystallized from ethanol. The yellow crystalline phenazine, m.p. 134°, was obtained in 70% yield (1.6 g.) (0.007 mole) based on unrecovered 2-nitro-3'-chloro-2'-methyldiphenylamine.

Anal. Calcd. for  $C_{13}H_9N_2Cl$ : C, 68.29; H, 3.96; N, 12.24; Cl, 15.51. Found: C, 68.36; H, 4.04; N, 12.22; Cl, 15.45.

1-Methyl-2-chlorophenazine-5-oxide. 1-Methyl-2-chlorophenazine (1.5 g.) (6.5 mmole) was dissolved in 75 ml. of warm acetic acid and 7.5 ml. of 30% hydrogen peroxide was added. The solution was heated at 50° for 24 hours. On addition of 75 ml. of water, the N-oxide crystallized out of solution and was collected by filtration under suction. Recrystallization from ethanol gave 1.4 g. (5.4 mmole) of bright yellow needles, m.p. 175-176°; yield 88%.

Anal. Calcd. for  $C_{13}H_9ON_2Cl$ : C, 63.76; H, 3.71; N, 11.52; Cl, 14.48. Found: C, 63.87; H, 3.62; N, 11.36; Cl, 14.60.

Infrared spectrum in chloroform:  $\nu_{\max}$ , 1342  $\text{cm}^{-1}$  (N-oxide).

1-Methyl-2-methoxyphenazine-5-oxide. To a hot solution of 1.7 g. (6.5 mmole) of 1-methyl-2-chlorophenazine-5-oxide in 125 ml. of methanol was added 25 ml. of an aqueous solution containing 9.0 g. of potassium hydroxide. This solution was stirred and refluxed for 24 hours. The resulting greenish solution was extracted with 100 ml. of diethyl ether three times. The ether extracts were evaporated and the residue was chromatographed on alumina. The fractions were collected with the aid of an ultraviolet lamp. Elution with benzene-ether separated the material into three bands. The first band yielded 120 mg. (0.53 mmole) of 1-methyl-2-chlorophenazine, presumably resulting from reduction of the N-oxide by methanol. The second band, which had a green fluorescence, was unreacted starting material (565 mg.) (2.2 mmole). The third band, which had a yellow fluorescence, gave 282 mg. (1.1 mmole) (25% yield based on unrecovered starting material) of yellow needles of 1-methyl-2-methoxyphenazine-5-oxide, recrystallized from ethanol.

Anal. Calcd. for  $C_{14}H_{12}O_2N_2$ : C, 69.99; H, 5.03; N, 11.66.

Found: C, 69.75; H, 5.31.

Infrared spectrum in chloroform:  $\nu_{\max}$ , 1342  $\text{cm}^{-1}$  (N-oxide).

The aqueous layer from the above procedure was acidified with dilute hydrochloric acid and extracted with ether. Evaporation of the ether left a small amount of 1-methyl-2-hydroxyphenazine-5-oxide, which could not be purified by crystallization or sublimation.

1-Methyl-2-methoxyphenazine. Reduction of 1-methyl-2-methoxyphenazine-5-oxide (100 mg.) (0.39 mmole) with lithium aluminum hydride was carried out in the same manner as for the dimethyl derivative of the orange pigment described before. After chromatographic separation, sublimation, and crystallization, only 35 mg. (0.16 mmole) (38% yield) of the 1-methyl-2-methoxyphenazine, m.p. 125°, were obtained.

Anal. Calcd. for  $C_{14}H_{12}ON_2$ : C, 74.99; H, 5.38; N, 12.49.

Found: C, 75.16; H, 5.57; N, 12.57.

Ether Cleavage of 1-Carbomethoxy-2-methoxyphenazine. To the red solution of 50 mg. (0.19 mmole) of the dimethyl compound in 20 ml. of concentrated hydrochloric acid was added two drops of a solution of 0.5 g. of stannous chloride in 2 ml. of concentrated hydrochloric acid (warmed with tin until clear) at 0°. A dark green complex formed immediately, and after two minutes, the solution turned yellow. The yellow solution was diluted with 30 ml. of water and extracted with 40 ml. of chloroform three times. The combined chloroform extracts were shaken with 25 ml. of 5% sodium hydroxide solution, giving an orange aqueous solution. This was acidified with hydrochloric acid and extracted

with 40 ml. of chloroform three times. The extract was washed with water, dried, and evaporated, giving an orange powder which was crystallized from a small volume of carbon tetrachloride and sublimed at 110° at 1 mm. The yellow sublimate was recrystallized from methanol (norite), giving 36 mg. (0.14 mmole) of yellow crystals, m.p. 140-141°; yield 77%. The compound was slightly soluble (about 0.5%) in sodium bicarbonate solution, but was easily extracted from the bicarbonate solution with chloroform. A ferric chloride test in water was negative, however in chloroform a red complex formed with ferric chloride (40), confirming the phenolic nature of the product XI.

Anal. Calcd. for  $C_{14}H_{10}O_3N_2$ : C, 66.13; H, 3.96. Found: C, 65.87; H, 3.89.

Infrared spectrum in chloroform:  $\nu_{\max}$ , 1665  $\text{cm}^{-1}$  (ester).

Methylation of the Phenazinol XI. The phenolic product XI was treated with diazomethane in exactly the same manner as the acid III, and the product was purified in the same way. A quantitative yield of 1-carbomethoxy-2-methoxyphenazine was obtained. A mixed melting point and comparison of infrared spectra proved the product's identity.

Hydrolysis of the Phenazinol XI. A solution of 15 mg. (0.06 mmole) of the phenazinol XI in 25 ml. of 10% sodium hydroxide solution was refluxed for 12 hours. After a few minutes the solution turned from orange to red. At the end of 12 hours, the solution was cooled and the red color changed to yellow-orange. The solution was acidified and extracted with chloroform (30 ml.) three times. The chloroform extract was dried and evaporated, leaving a yellow material. Crystallization

from ethanol (norite) gave a yellow powder (5 mg.) (0.017 mmole), m.p. 245-250°, lit. m.p. (2-phenazinol) 253-254°. A mixed melting point with synthetic 2-phenazinol, m.p. 250-251°, was not depressed. The infrared spectra of the naturally derived and synthetic 2-phenazinols were identical.

When a solution of 15 mg. of the phenazinol XI in 25 ml. of 10% sodium hydroxide was refluxed for one hour and then cooled, the red color remained. Acidification and extraction as above yielded an orange powder, which was crystallized twice from a small amount of benzene. About 3 mg. of orange needles, m.p. 218-221°, were obtained. The mixed melting point with the orange pigment was not depressed.

2-Phenazinol. A solution of 25 mg. (0.12 mmole) of 2-methoxyphenazine was treated with a concentrated hydrochloric acid solution of stannous chloride in the same manner as that described above for the dimethyl derivative of the orange pigment, giving 18 mg. (0.091 mmole) (78% yield) of 2-phenazinol, m.p. 250-251°, from ethanol.

Ether Cleavage of 1-Carbomethoxy-2-methoxyphenazine with Other Reagents. The dimethyl derivative of the orange pigment (15 mg. (0.06 mmole)) was dissolved in 25 ml. of benzene and 35 mg. of aluminum chloride was added. After refluxing for one hour, the brown suspension was cooled and added to ice. The benzene solution was shaken with 25 ml. of 10% sodium hydroxide solution. The orange aqueous layer was acidified with hydrochloric acid and extracted with chloroform (25 ml.) three times. The chloroform extracts yielded about 3 mg. of yellow phenolic material, m.p. 135-137°. No depression of this melting

point occurred when mixed with the phenazinol XI, although it is somewhat lower than the melting point of the pure phenazinol XI.

On reflux in 20 ml. of concentrated hydrochloric acid for five days, the dimethyl derivative (15 mg.) afforded a small yield of the same phenolic material, m.p. 134-137°, when isolated in the same manner as above. When 0.5 g. of stannic chloride was added to the refluxing solution of the dimethyl derivative in 20 ml. of hydrochloric acid, only one day of refluxing was necessary to obtain a yield of 8 mg. of the impure phenazinol XI, m.p. 134-137°.

Oxidation of 1-Carbomethoxy-2-methoxyphenazine. A solution composed of 50 mg. (0.20 mmole) of 1-carbomethoxy-2-methoxyphenazine and 3 ml. of superoxol in 10 ml. of acetic acid was heated at 50° for 20 hours. The solution was diluted with 50 ml. of water and extracted with 75 ml. of chloroform three times. The chloroform solutions were washed with 100 ml. of 5% sodium carbonate solution and 100 ml. of water, then dried over sodium sulfate and evaporated. The orange-red powder thus obtained was chromatographed on alumina (Merck). Elution with ether-acetone gave two compounds.

A yellow pigment was crystallized from carbon tetrachloride, giving 7 mg. (0.025 mmole) of yellow crystals, m.p. 155-156°. The mother liquor yielded 10 mg. of impure material. The yellow compound formed a dark complex with stannous chloride in concentrated hydrochloric acid which turned yellow in a few minutes and yielded a phenolic material; hence the methoxy group must still be present in the yellow pigment. The carbon analysis is .72% low for the mono-N-oxide of the

dimethyl derivative II, although this might result from contamination by a small amount of the di-N-oxide, which one would expect to be difficult to separate from the mono-N-oxide.

Anal. Calcd. for  $C_{15}H_{12}O_4N_2$ : C, 63.38; H, 4.26. Found: C, 62.66; H, 4.57.

Infrared spectrum in chloroform:  $\nu_{\max}$ , 1735  $\text{cm}^{-1}$  (ester), 1342 (N-oxide).

A bright red pigment was crystallized from carbon tetrachloride, yielding 15 mg. (0.039 mmole) of crystals, m.p. 184°. The red compound did not form a dark complex with stannous chloride in concentrated hydrochloric acid, but nevertheless gave a phenolic substance as a result of the treatment.

Anal. Calcd. for  $C_{18}H_{14}O_8N_2$ : C, 55.96; H, 3.65; N, 7.25. Found: C, 55.50; H, 3.81; N, 7.06.

Infrared spectrum in chloroform:  $\nu_{\max}$ , 1735  $\text{cm}^{-1}$  (ester), 1342 (N-oxide).

Infrared spectrum in carbon disulfide:  $\nu_{\max}$ , 832  $\text{cm}^{-1}$ , 817, 812, 708.

Nuclear magnetic resonance spectrum in deuterochloroform (microcell): singlets at 1.28 PPM and 4.13 PPM; ratio of areas is 2 : 1.

Isolation of Additional Pigments. A particularly dark red batch of the impure orange pigment (50 mg.) was treated with diazomethane in the manner described and chromatographed on alumina (Merck). First a small amount of 1-carbomethoxyphenazine (5 mg.) was eluted, then 35 mg. of 1-carbomethoxy-2-methoxyphenazine. Elution with



methanol-ether separated the remaining material into three bands. Not enough of these pigments were obtained for crystallization or analysis, hence only spectral data were gathered.

Infrared spectrum of the rose pigment (4 mg.) in carbon disulfide:  
 $\nu_{\text{max.}}$  3495  $\text{cm.}^{-1}$ , 3470, 3380, 3290, 2950, 2880, 1740, 1660, 1380, 1365, 1322, 1295, 1196, 1110, 1086, 1075, 1025, 955, 948, 875, 828, 818, 796.

Ultraviolet spectrum in 95% ethanol:  $\lambda_{\text{max.}}$  257  $\text{m}\mu$  ( $\log e$  4.9), 300 (shoulder), 363 (3.9), 485 (2.8).

Infrared spectrum of the violet pigment (3 mg.) in chloroform:  
 $\nu_{\text{max.}}$  3625  $\text{cm.}^{-1}$ , 3520, 3470, 3340, 2975, 2945, 2890, 1725, 1653, 1605, 1567, 1384, 1297, 1135, 1075, 1042, 875, 836, 826.

Ultraviolet spectrum in 95% ethanol:  $\lambda_{\text{max.}}$  240  $\text{m}\mu$  ( $\log e$  4.6), 255 (4.6), 282 (4.3), 293 (4.3), 364 (4.2), 535 (2.8).

Not enough of the brown pigment was obtained for a good spectrum.

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## THE BIOGENESIS OF STRYCHNINE

## Introduction

The origin of alkaloids and the reason for their existence have been the subjects of conjecture and investigations by chemists for a great number of years. The ontology of alkaloids is little understood; the simple suggestion that their sole function is that of providing a protective toxicity is not regarded seriously, since toxicity is a relative thing, and numerous examples of animals and plants attacking the alkaloid producing plants with immunity are known (1). That alkaloids might have some regulating function in the plant has been suggested (1). A more generally accepted proposal is that the formation of alkaloids is a mechanism for removing excess metabolites (2) or substances which are harmful to the alkaloid producing plant (1).

Exploring the biogenesis of alkaloids, on the other hand, has been a fruitful recreation. Two types of experiments have been successful in relating hypothetical progenitors to the natural product. Incorporation of radioactive substrates, such as amino acids, acetate, formate, and mevalonate, into the alkaloids has been studied, and in vitro syntheses of the alkaloids from amino acids and other proposed biogenetic intermediates under "physiological conditions" have been carried out.

Although a vast amount of speculation has been offered for the biogenesis of the indole alkaloids, and even structural predictions based on these rationalizations have been made (3), only a few biosynthetic experiments have been performed in this field. The intent of the present work has been to investigate the biogenesis of the classic indole alkaloid, strychnine.

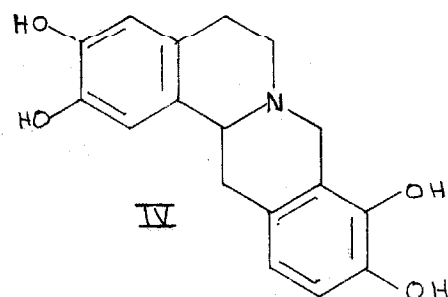
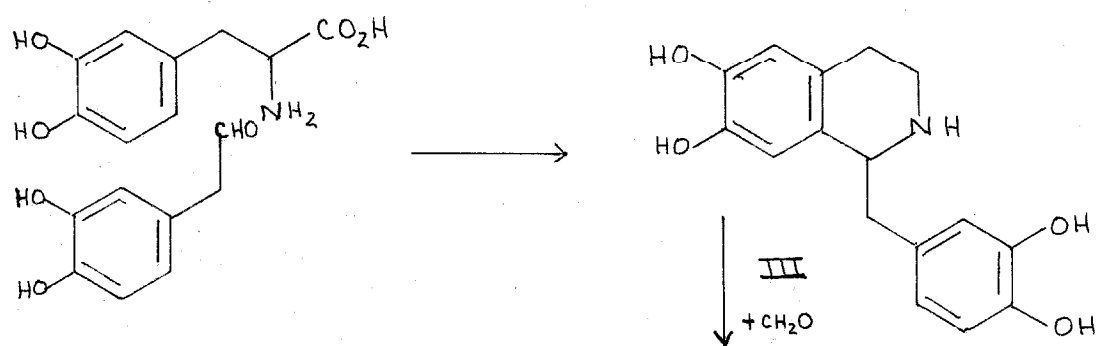
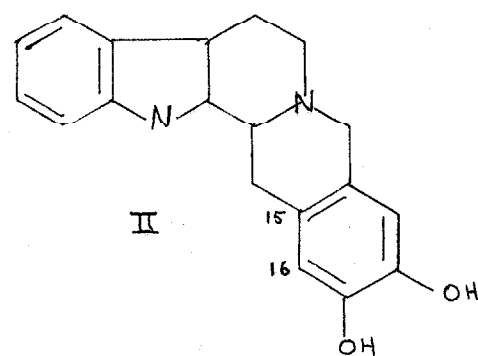
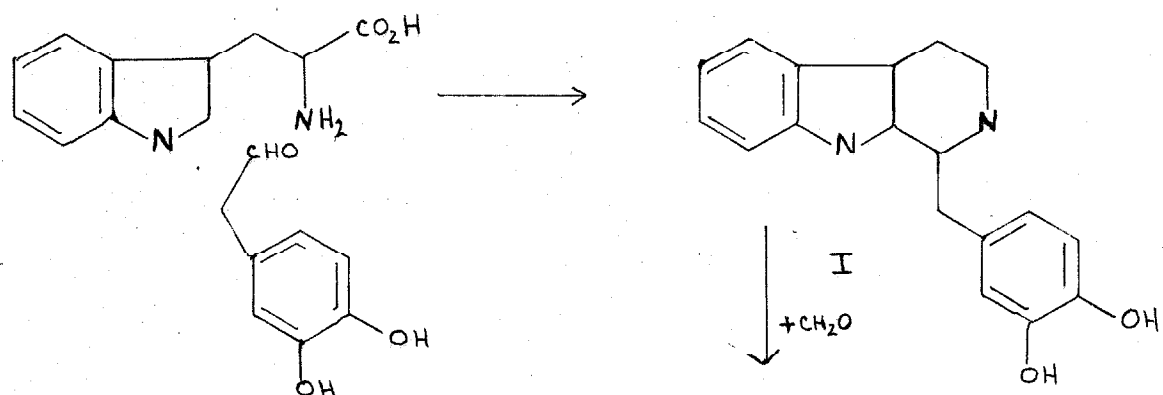
### History of the Theories of Indole Alkaloid Biogenesis

The first proposal for the biogenesis of an indole alkaloid was made by Barger (4) and Hahn (5), who suggested that the yohimbine alkaloids result from the condensation of 3,4-dihydroxyphenylacetaldehyde with tryptophan at its  $\alpha$ -position, forming the  $\beta$ -carboline (I), followed by a Mannich reaction with a formaldehyde unit, giving the yohimbine type structure (II). The alkaloid semperivine (6) has exactly this carbon skeleton; whereas yohimbine has a carbomethoxy group at C-16. Robinson (7) rationalized that a carbon atom might have been introduced ortho to the phenolic hydroxyl or that a tropolone intermediate may be involved. Hahn found that m-hydroxyphenylpyruvic acid did condense with tryptamine under "physiological-type" conditions and that decarboxylation and condensation with formaldehyde readily gave the carbon skeleton II.

The role of the Mannich reaction in biogenesis had been previously accepted in theory for the formation of norlaudanosoline (III) from 3,4-dihydroxyphenylalanine and 3,4-dihydroxyphenylacetaldehyde and the reaction of norlaudanosoline with formaldehyde in a second Mannich reaction to give protoberberine (IV). A considerable variety of alkaloids could be derived from these two intermediates (8). Tracer experiments have since shown that indeed labelled tyrosine\* is incorporated into morphine (9,10) and papaverine (11) in the expected manner.

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\*Tyrosine is a precursor of 3,4-dihydroxyphenylalanine and hence also 3,4-dihydroxyphenylacetaldehyde.



Moreover Rapoport showed that morphine is not formed by combination of two identical units derived from tyrosine and that a symmetrical structure never existed, since the two tyrosine derived fragments of morphine did not have the same activity (41). In fact one of the tyrosine derived fragments had twice the activity of the other in morphine obtained from plants fed with radioactive carbon dioxide. The difference in activity between the two "tyrosine" fragments was attributed to the different sizes of the "pools" of the two intermediates derived from tyrosine.

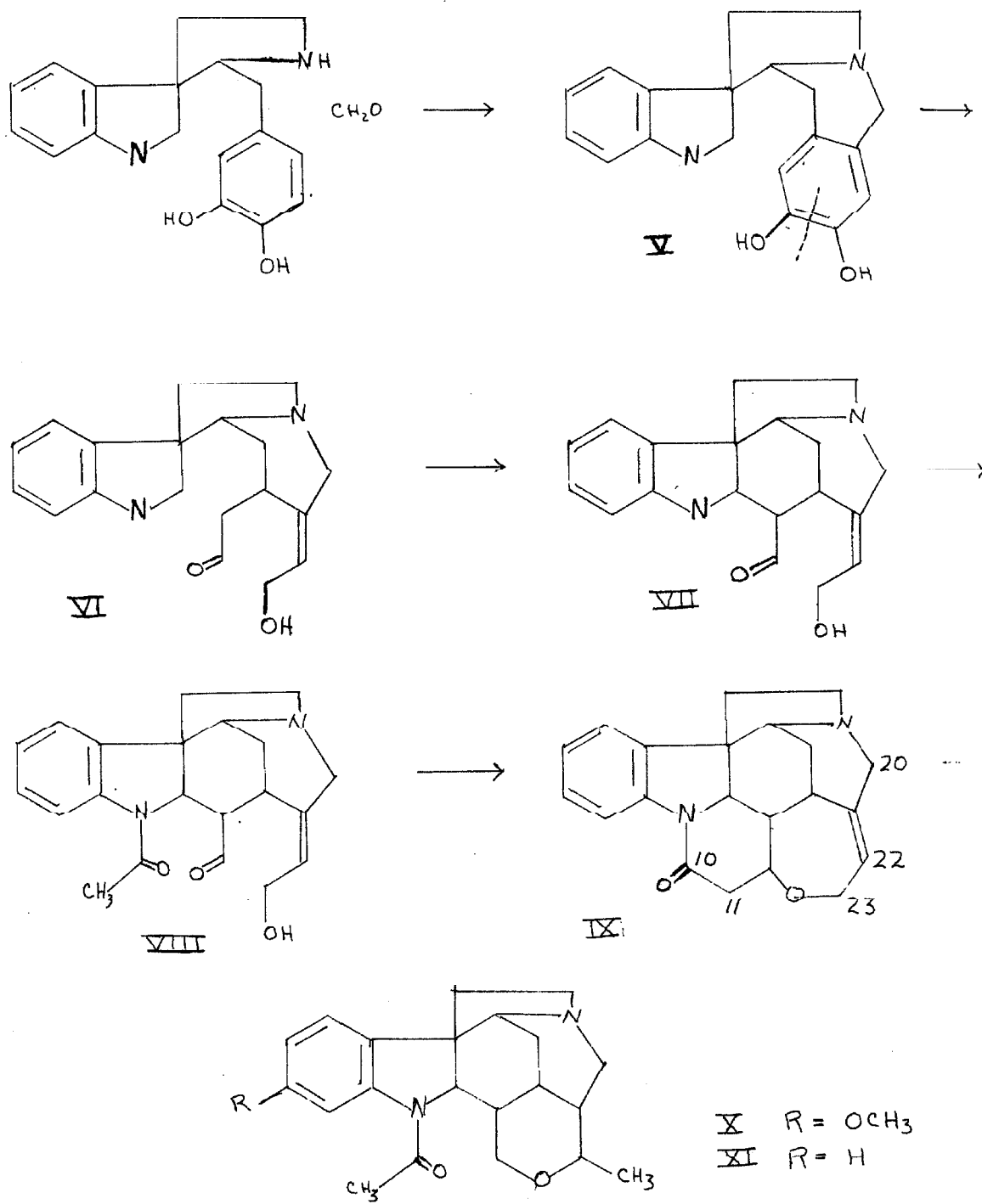
For the biogenesis of strychnine Woodward proposed a fission of the catchol ring of the precursor V, derived by condensation of 3,4-dihydroxyphenylacetaldehyde with the  $\beta$ -position of tryptophan followed by a Mannich reaction with formaldehyde. A third condensation in the fission product (VI) results in the Wieland-Gumlich aldehyde (VII), which would yield strychnine (IX) on acetylation (12).

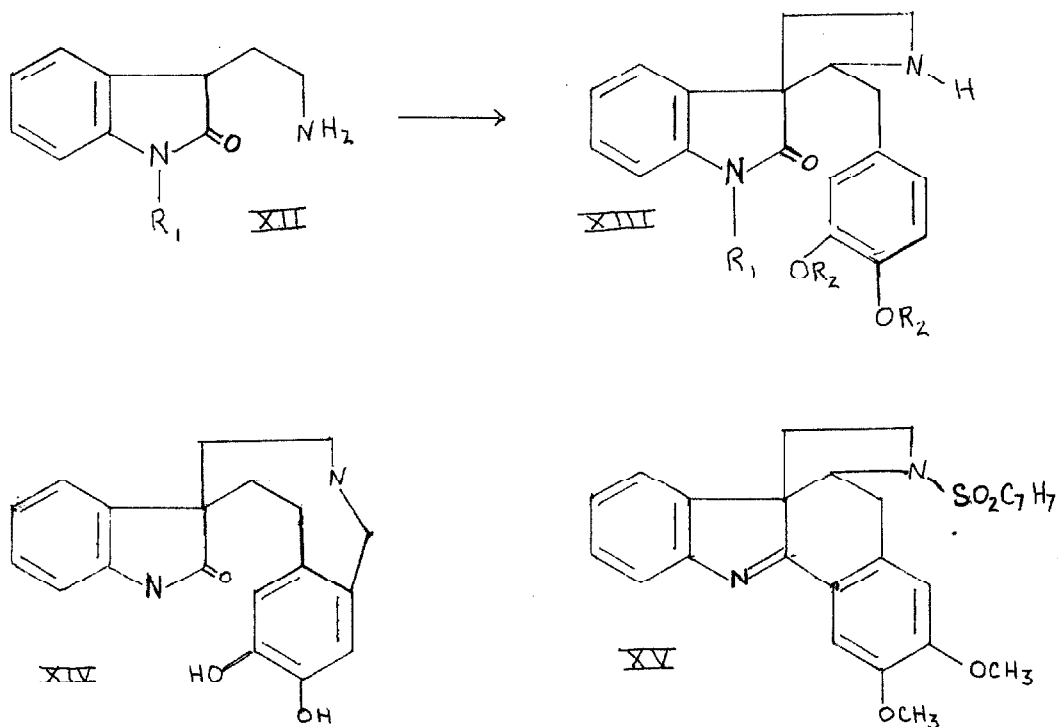
Several Strychnos alkaloids have been isolated which support the latter step of Woodward's scheme. Diaboline (13) is identical to the intermediate VIII, and strychnospermine (X) and spermostrychnine (XI) (14) closely resemble this intermediate. The Wieland-Gumlich aldehyde occurs in nature as one of the curare alkaloids, caracurine-VII (15), and as a dimer in caracurine-V (16).

When the Wieland-Gumlich aldehyde was heated with acetic anhydride, acetylation with ring closure did occur to give strychnine (17). Condensation of a substituted phenylacetaldehyde with an  $\alpha$ -oxindole (XII) resulted in an intermediate (XIII) containing ring E of strychnine (18, 19). Formylation led to the tetrahydroisoquinoline XIV (18), whereas



Bischler-Napieralski conditions converted the tosylate of XIII to the indolenine XV (19).





Fission of the  $\alpha$ -indole product (II) would similarly yield many alkaloids (20). The great success of the "Woodward fission" proposal in helping to elucidate the structures of emetine (31) and cinchonamine (21) proved it to be a very useful if not entirely correct concept.

Several limitations of the Barger-Hahn and Woodward proposals were later recognized (22). In addition to the proposal's failure to satisfactorily explain the origin of the C-16 carboxy group mentioned above, the proposal was questioned because in all the indole alkaloids with ring E intact, except alstoniline, ring E is aliphatic rather than aromatic; and a fission mechanism, which could give alkaloids with such a variety of oxidation states in the two chains resulting from the fission, did not seem feasible. Wenkert and Bringi (22) discovered that the absolute configuration at C-15 in the indole alkaloids is

unique\* and believed that the dihydroxyphenylacetaldehyde hypothesis did not readily offer an explanation for this stereospecificity. They formulated a biogenesis for the indole alkaloids involving a hydrated form of prephenic acid, which had been shown by Davis to be a precursor of phenylalanine (23).

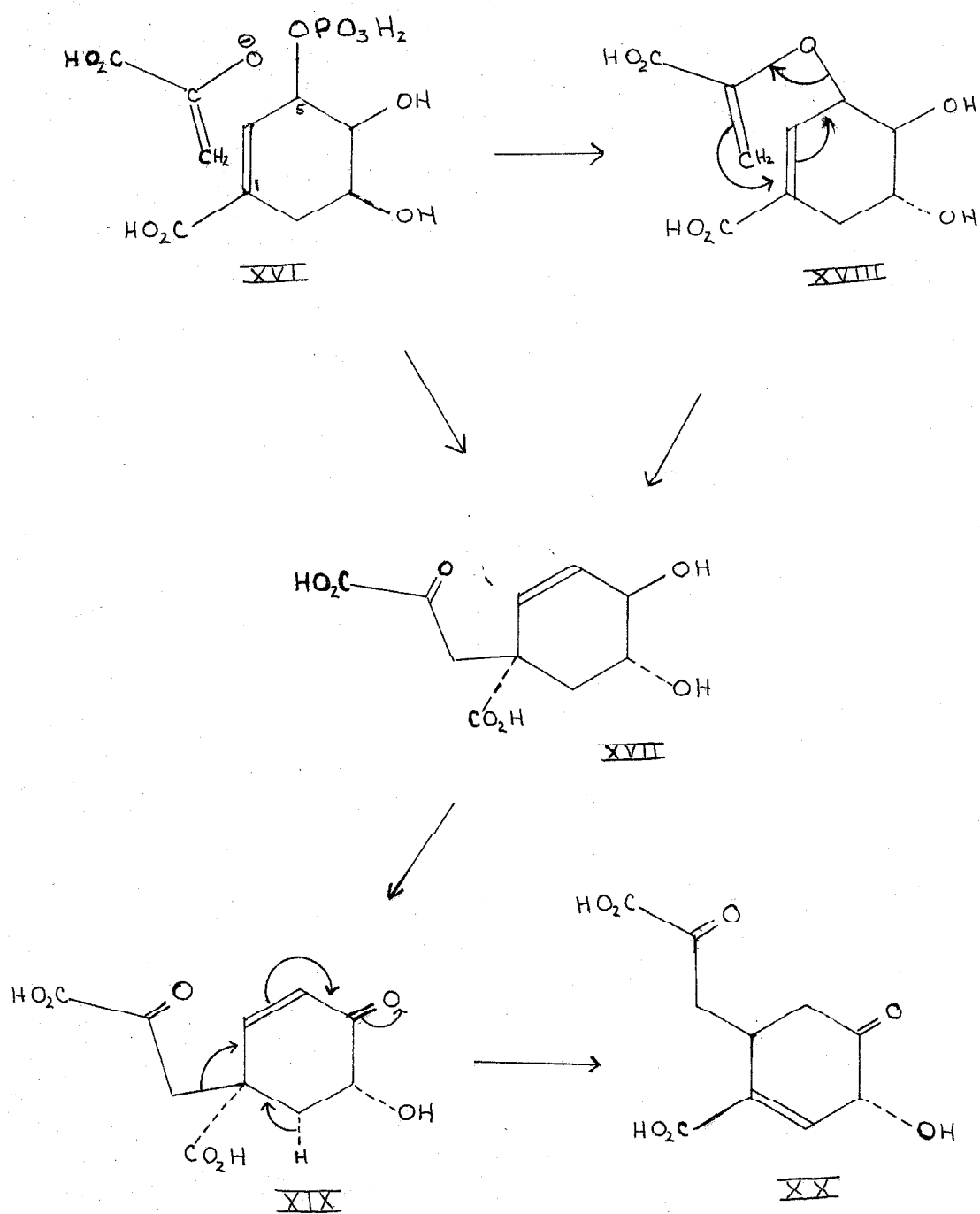
In this scheme, 5-phosphoshikimic acid (XVI) is attacked by enol pyruvate at either C-1, giving the hydrated form of prephenic acid (XVII), or at C-5, giving the pyruvate enol ether XVIII, which can undergo a Claisen type rearrangement to the acid XVII. The oxidized acid XIX rearranges by a 1,2 shift of the pyruvate group to the compound XX, which condenses with tryptophan and formaldehyde to give the yohimbine alkaloids. This theory then provides for the carboxy group at C-16 of yohimbine, as well as the  $\alpha$ -configuration of C-15 in all the indole alkaloids.

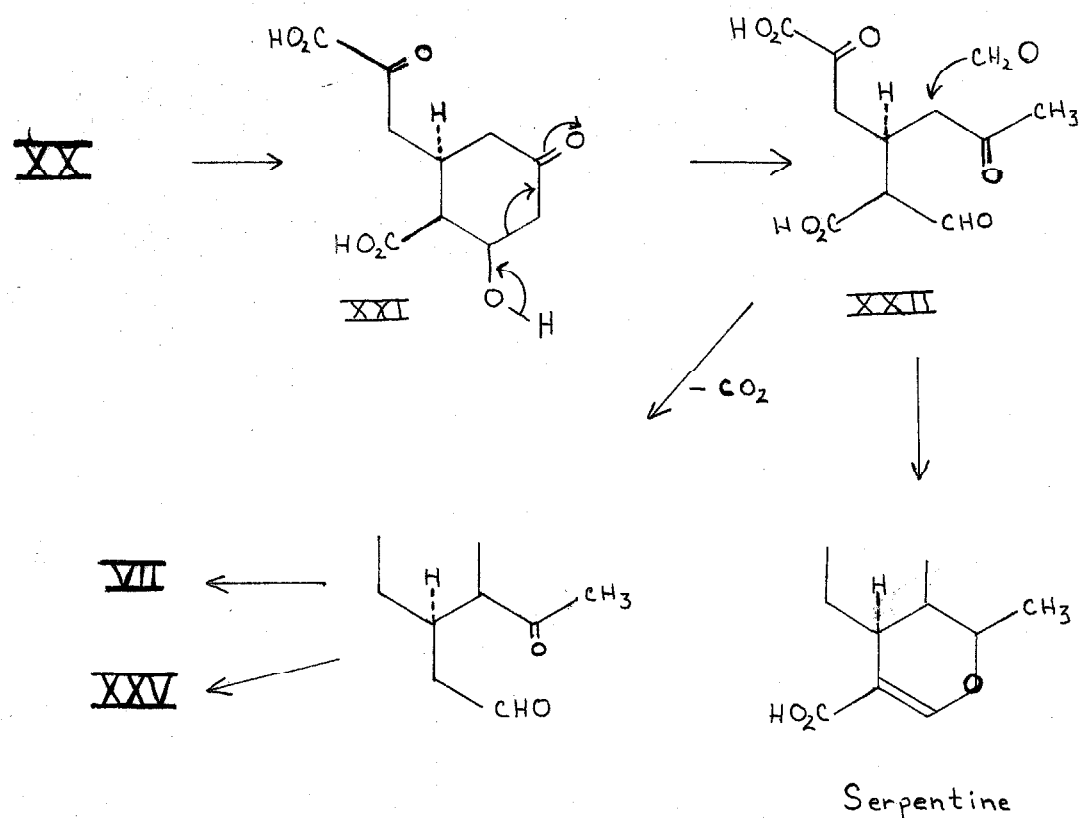
Other types of skeletons were postulated to result by conversion of the hydroaromatic compound XX to a reduced compound XXI. Ring opening of the reduced compound XXI by a retro-aldol cleavage leads to the important precursor XXII, from which many of the classes of indole alkaloids may be derived as shown in the diagram. Wenkert cleverly extended his prephenate hypothesis even to the Iboga and Aspidosperma alkaloids by a series of rearrangements of the precursor XXII (24).

A third biogenetic proposal for the indole alkaloids was advanced by Thomas (25). He suggested that the non-tryptophan part is derived

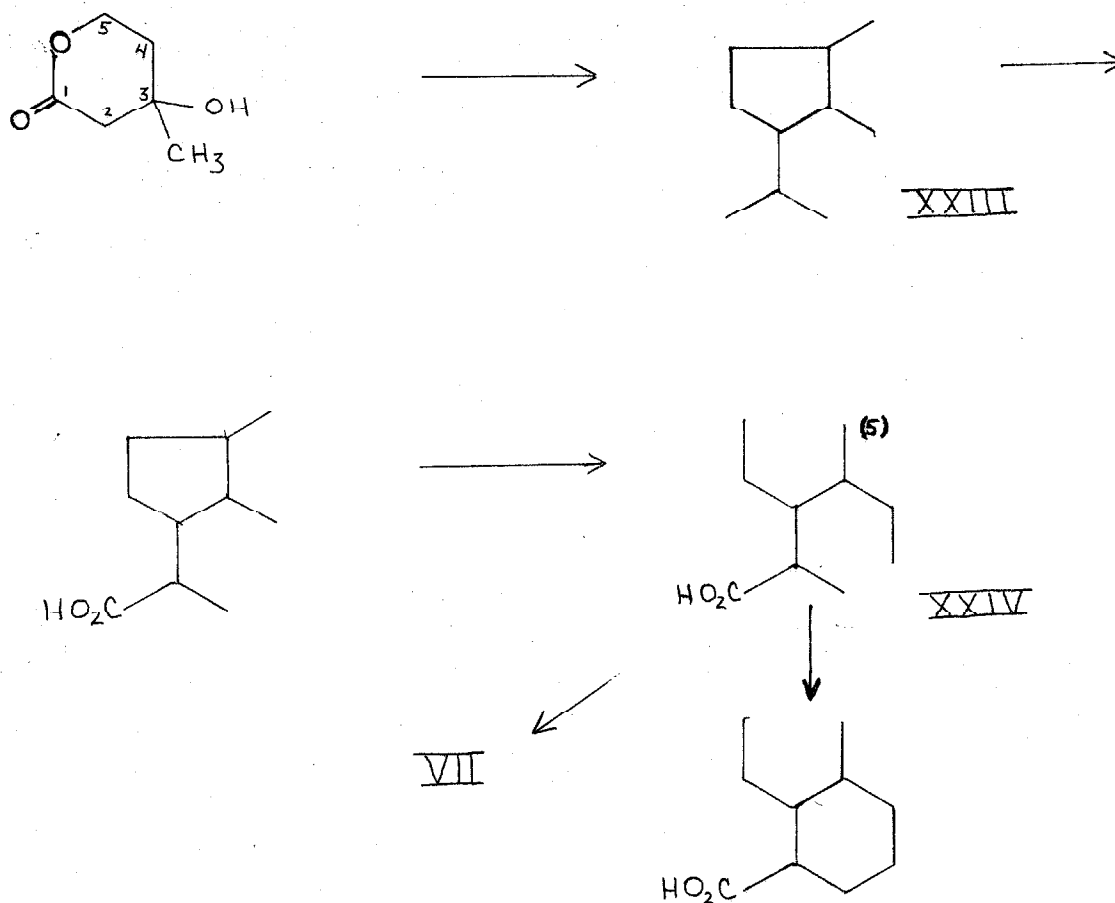
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\*The only exception is  $\psi$ -akuammicine, which is a racemate of akuammicine.





from a cyclopentanoid monoterpene (XXIII), composed of two units of mevalonate. This carbon skeleton is present in the pyridine alkaloids, actinidine (26) and skytanthine (27), as well as in the monoterpenes, verbenalin (28), genepin (29), loganine (30), and asperuloside (31). Formation of the non-tryptophan moiety proceeds as shown in the diagram. The acyclic intermediate (XXIV) would cyclize to give the homocyclic ring E alkaloids, as in the conversion of swertiamarin to erythrocentaurin (32). Other indole alkaloids arise in the same manner as from Wenkert's intermediate. The "berberine bridge" carbon would be derived from a C-5 of mevalonate, rather than from formate.



Leete has shown that tryptophan-2- $C^{14}$  was incorporated by Rauwolfia serpentina into ajmaline (XXV), with the activity appearing at C-5 as expected (33). Leete also found that sodium formate- $C^{14}$  was incorporated into ajmaline with 12% of the activity at C-21, the "berberine bridge" carbon, and 48% in the N- $CH_3$  group (34). The large value for the N- $CH_3$  group was expected because a 1-carbon unit intermediate (formate) is involved in the neogenesis of methionine, which trans-methylates the indole nitrogen. Formate is known to be incorporated also into serine, a precursor of the side chain of tryptophan (35), and

is readily oxidized to carbon dioxide, which would distribute labelling throughout the molecule; hence the 12% activity at C-21 definitely supports the 1-carbon unit origin of the "berberine bridge" carbon. The fact that different percentages of activity appear at the presumed 1-carbon unit carbons is obviously a result of different "pool" sizes of the formate which is involved in the Mannich reaction, the formate which becomes the methyl group of methionine, and the formate which is incorporated into serine.

Some experimental evidence was accumulated at this point which seemed to rule out the biogenetic possibilities previously suggested. Tyrosine-2-C<sup>14</sup> was administered to Rauwolfia serpentina, and the alkaloids isolated were found to be inactive. Hence the 3,4-dihydroxyphenyl-alanine intermediate was apparently not involved in the biosynthesis (36). The terpene precursor, mevalonic acid-2-C<sup>14</sup>, similarly yielded no radioactive ajmaline from Rauwolfia serpentina (36) or radioactive strychnine from Strychnos nux-vomica (37). Since alanine is readily converted to pyruvate, which becomes the side chain of Wenkert's pre-phenic acid intermediate, D, L-alanine was fed Rauwolfia serpentina plants; however only 2% of the activity of ajmaline occurred at C-3, which would have been derived from C-2 of alanine (36).

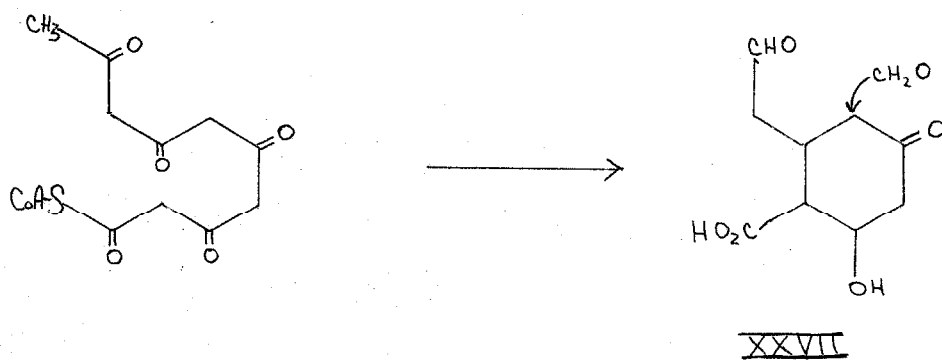
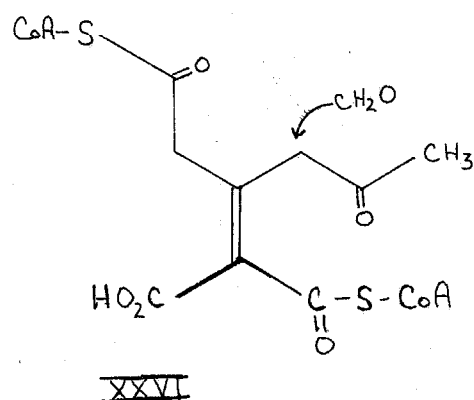
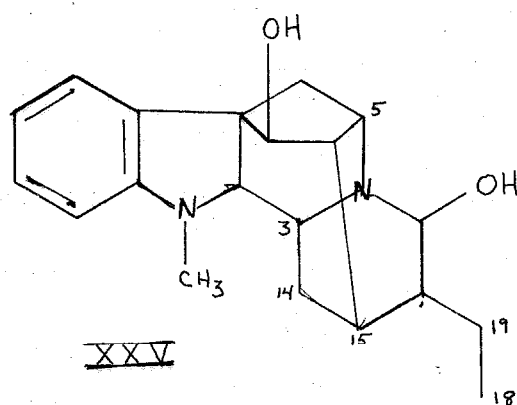
Caution should be exercised in interpreting these negative results. Nonincorporation might mean only that the intended precursor was not able to reach the site of biosynthesis or that the alkaloid was not even being produced when the tracer was added.

That acetate units are important in the biogenesis of indole alkaloids is a fourth possibility. The Lycopodium alkaloids appear to

be constructed of polyacetyl chains (38) and parts of many pyridine and tropane alkaloids have been shown to be indirectly derived from acetate units (39). Leete has proposed that the non-tryptophan portion of indole alkaloids is formed by a linear condensation of three units of acetyl-coenzyme A, followed by attachment of the "berberine bridge" carbon and condensation with a molecule of malonyl-coenzyme A (36). Leete's experimental results indicate that acetate is incorporated into ajmaline (XXV) by Rauwolfia serpentina, with one-fourth of the activity appearing at C-3 and one-fourth at C-19, while C-14 and C-18 were inactive. This confirmation of an acetate origin represents an important breakthrough in the biogenesis of indole alkaloids.

Richards and Hendrickson (40) have since objected to Leete's intermediate (XXVI) and pointed out that condensation of malonyl-coenzyme A with the acetate chain should occur with decarboxylation, that chain branching of this sort is uncommon, and that the intact ring E of some indole alkaloids implies that their precursor is cyclic. The intermediate which they proposed is derived from five acetate units. Cyclization followed by oxidation of the end-methyl group gives a precursor (XXVII), which happens to be identical to the prephenate type of intermediate utilized by Wenkert (22).





### Approach to the Problem

The biosynthesis of strychnine, while not expected to differ essentially from that of ajmaline as far as precursors are concerned, was undertaken to provide evidence supplemental to the findings of Leete, indicating an acetate origin for the non-tryptophan portion of indole alkaloids, and a formate origin for the "berberine bridge" carbon. The relationship of the additional acetate unit of strychnine to the rest of the non-tryptophan portion was of special interest. Obviously the intermediates proposed by Leete (36) and by Richards and Hendrickson (40) would result in the same labelling in acetate-1-C<sup>14</sup> labelled strychnine.

Strychnine had been customarily isolated from the seeds of the Strychnos nux-vomica plants. That year old seedlings could incorporate labelled substrates into strychnine and that the strychnine could then be isolated by maceration of the whole plant was demonstrated by Hall and Willner (37), who grew Strychnos plants in an atmosphere of carbon dioxide-C<sup>14</sup> and obtained radioactive strychnine.

Further investigations by Willner indicated that 67% of the activity of strychnine labelled with sodium acetate-1-C<sup>14</sup> occurs at C-10 and that C-11 is inactive. He decomposed the hydrochloride (XXX) in aqueous sodium acetate and isolated the hydrogen cyanide and carbon dioxide evolved as silver cyanide and barium carbonate respectively. Attempts to purify the organic product, the Wieland-Gumlich aldehyde (VII), were unsuccessful.

### I. C-10

Verification of Willner's results was important because of the remarkably large percentage of activity which he found in the barium carbonate. Various derivatives of the Wieland-Gumlich aldehyde were prepared, but only the picrate was susceptible to purification by recrystallization; hence Willner's organic residue was treated with picric acid in ethanol and the picrate recrystallized twice from ethanol. The activity was found to be  $31 \pm 2\%$ \* of the activity of the strychnine.

The degradation performed by Willner was repeated on a smaller amount of the same batch of acetate labelled strychnine which Willner used, only no attempt was made to isolate the carbon dioxide and hydrogen cyanide evolved in the last step. The radioactive strychnine was refluxed with isoamyl nitrite and sodium in ethanol, giving the oxime (XXIX) (14). The hydrochloride of the oxime was prepared and treated with thionyl chloride. The Beckmann rearrangement product (XXX) was then refluxed in sodium acetate solution, and the Wieland-Gumlich aldehyde (42) thus obtained was recrystallized from acetone twice, giving a reasonably pure product. An activity of  $36 \pm 2\%$  of strychnine was obtained.

### II. C-22 + C-23

In order to demonstrate an acetate biogenesis for the rest of the non-tryptophan portion of strychnine, the activity of C-22 and C-23 was determined. Sodium acetate-1-C<sup>14</sup> was administered to the year old Strychnos nux-vomica plants by absorption of an aqueous solution of the

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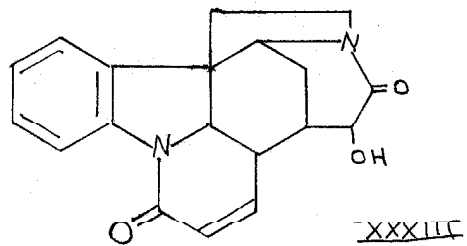
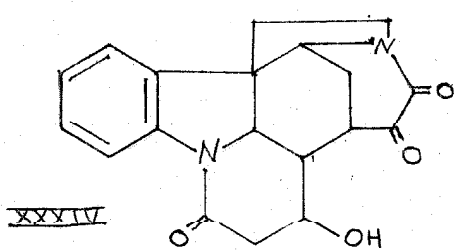
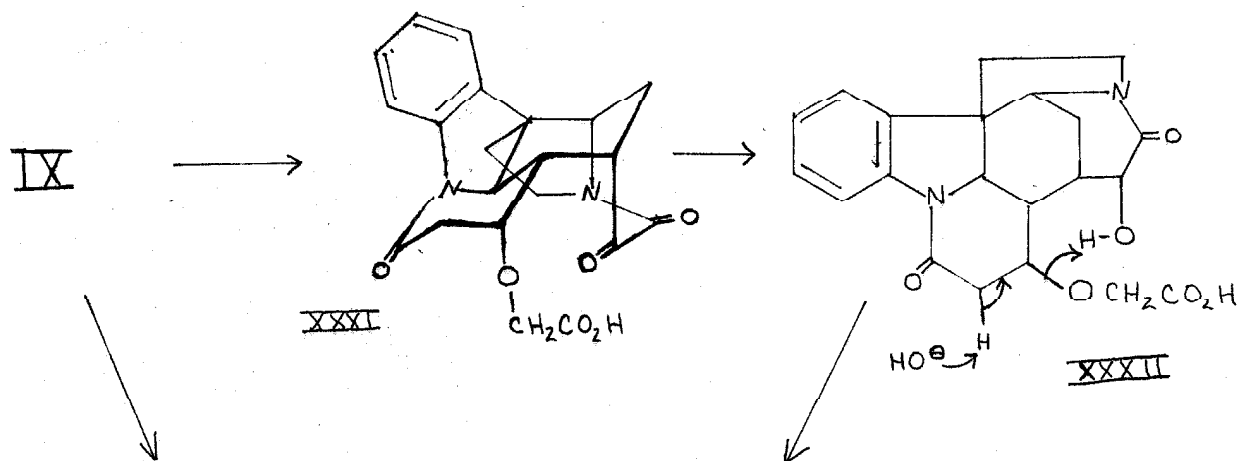
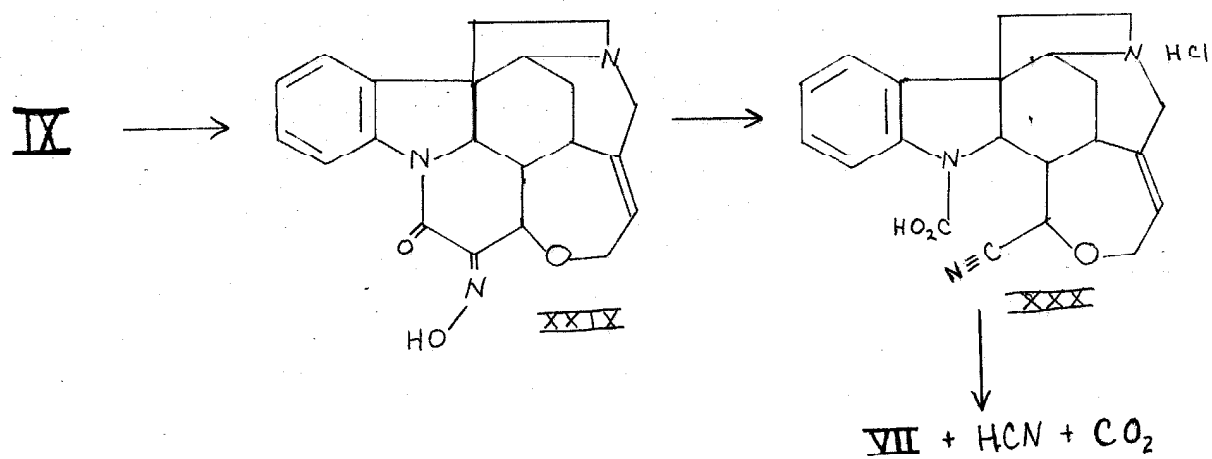
\*All errors reported are the probable errors.

substrate through a slit cut in the stem. After four weeks the plants were washed and ground in a Waring blender. The plant material was mixed with calcium hydroxide, dried in an oven, and extracted with petroleum ether. Crude alkaloids were extracted from the ground plant material with chloroform and were then chromatographed. The middle fractions were evaporated and triturated with ethanol-water to remove brucine. The radioactive strychnine was diluted with crystallized unlabelled strychnine and recrystallized several times from ethanol.

The acetate labelled strychnine was oxidized to strychninonic acid (XXXI) with potassium permanganate in acetone (43). Since a proton-donating solvent molecule is prevented by the strychnine cage structure from approaching the ether oxygen, strychninonic acid does not eliminate glycolic acid, containing the desired C-22 and C-23, when treated with hydroxide. Therefore the strychninonic acid was reduced with sodium amalgam to strychninolic acid (XXXII), in which the newly formed hydroxyl hydrogen can assist in the elimination of glycolic acid by stabilizing the leaving glycolate ion (44). Treatment of strychninolic acid with 1 N sodium hydroxide thus afforded strychninolone-a (XXXIII), containing all the carbons except C-22 and C-23. Its activity was measured in solution and found to be  $78 \pm 3\%$  of strychnine. This value was checked by isolation of a neutral substance (XXXIV) (45), a by-product of the permanganate oxidation of strychnine. The activity of this compound was  $77 \pm 4\%$  of strychnine.

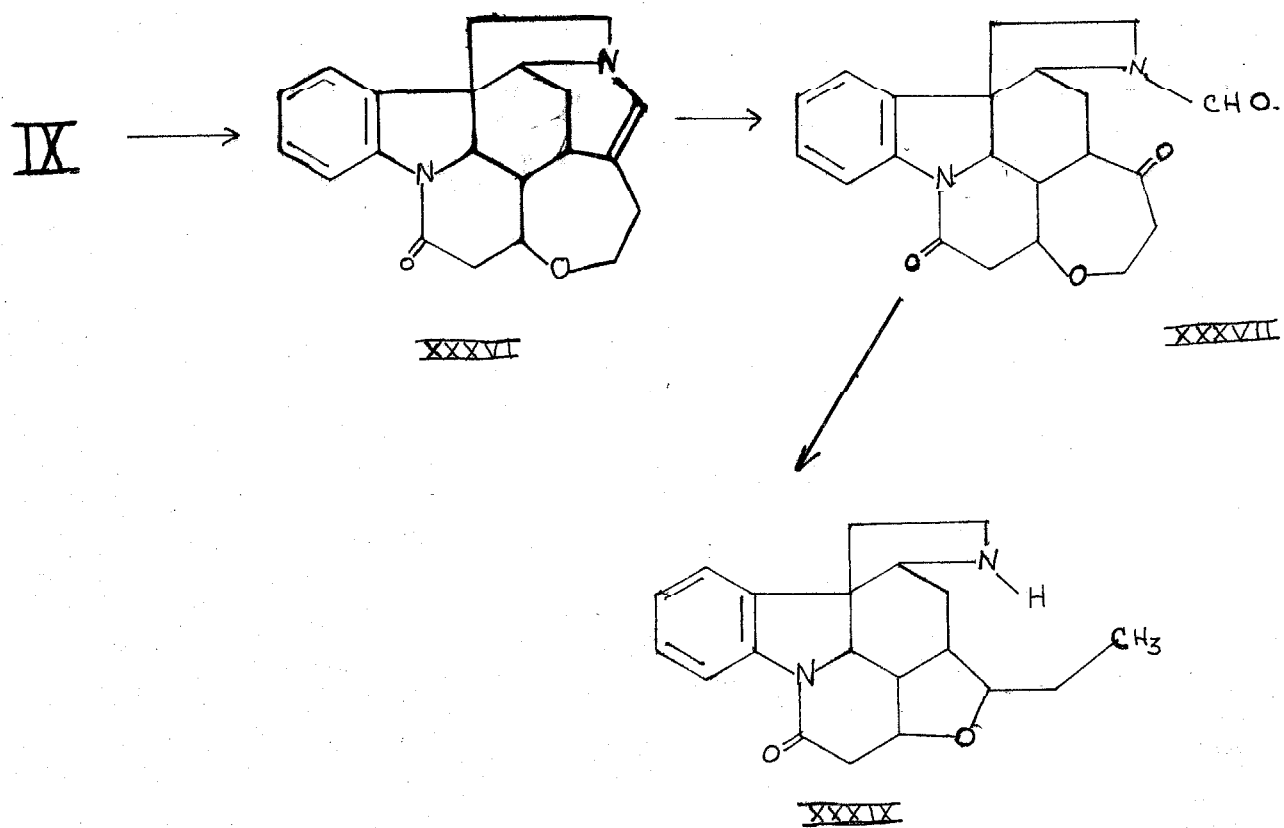
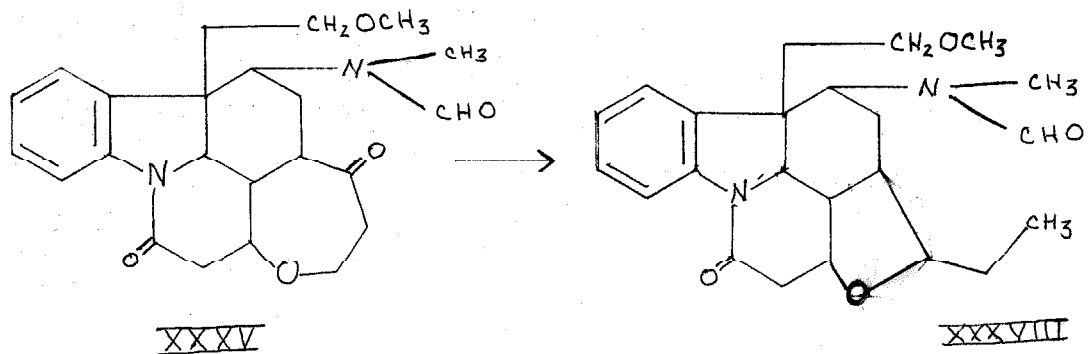
### III. C-20

Several possible related degradations were available for isolation of C-20, the "berberine bridge" carbon. In these schemes of degradation,



C-20 may be removed from the molecule in the last step as formic acid. The major problem in each of these was carrying out the hydrolysis of the sterically hindered formyl compounds. Woodward was not able to hydrolyze the N-formyl compound, methoxymethylchanodihydrostrychnone (XXXV) (46); however reduction of the keto group by a Clemmensen reaction, hydrogenation, or treatment of the ethyl mercaptal with Raney nickel gave formyl compounds which were easily hydrolyzed. Woodward's degradation was attempted but it did not give sufficient yields of formic acid or the des-formyl compound to justify its use in degrading radioactive strychnine.

Instead the sodium formate-C<sup>14</sup> labelled strychnine was converted to neostrychnine by refluxing with Raney nickel. The neostrychnine was then oxidized with osmium tetroxide and potassium periodate to the N-formyl compound, neostrychnone (XXXVII), which had previously been prepared by ozonization of neostrychnine (47). When neostrychnone was refluxed with concentrated hydrochloric acid and amalgamated zinc, hydrolysis of the formyl group of the reduced compound took place, and an amine was isolated. The white material which precipitated from ethanol on addition of acetone was treated with picric acid in ethanol, and the picrate obtained was crystallized from acetone-ethanol. Since a Kuhn-Roth oxidation of the amine gave part of a mole (59%) of acetic acid, a structure analogous to that of desformylmethoxymethylchanodihydrostrychnane (XXXVIII) was assumed for the desformylneostrychnane (XXXIX). The picrate of the desformyl amine (XXXIX) was counted on a planchet and its activity found to be  $82 \pm 2\%$  of strychnine.



Neostrychnine, like methoxymethylchanodihydrostrychnone, was not hydrolyzed under any conditions. The oxime of neostrychnone, prepared from neostrychnone and also by addition of nitrous acid to the double bond of neostrychnine, was similarly not hydrolyzed.

Table of Results

| Compound                         | Type of Sample Counting | Activity c/min.-mmol.       | % Activity in Fragment |
|----------------------------------|-------------------------|-----------------------------|------------------------|
| Strychnine - I                   | planchet                | $9.53 \pm 0.27 \times 10^2$ |                        |
| Wieland-Gumlich Aldehyde picrate | "                       | $2.99 \pm 0.05 \times 10^2$ | $31 \pm 2\%$           |
| Strychnine - I                   | liquid                  | $2.51 \pm 0.03 \times 10^3$ |                        |
| Wieland-Gumlich Aldehyde         | "                       | $8.98 \pm 0.05 \times 10^2$ | $36 \pm 2\%$           |
| Strychnine - II                  | liquid                  | $2.23 \pm 0.05 \times 10^3$ |                        |
| Strychninolon-a                  | "                       | $1.74 \pm 0.04 \times 10^3$ | $78 \pm 3\%$           |
| Neutral Product                  | "                       | $1.72 \pm 0.05 \times 10^3$ | $77 \pm 4\%$           |
| Strychnine - III                 | planchet                | $1.73 \pm 0.03 \times 10^3$ |                        |
| Desformylstrychnone picrate      | "                       | $1.42 \pm 0.03 \times 10^3$ | $82 \pm 2\%$           |

% Activity  
in Carbons

|             |              |
|-------------|--------------|
| C-10        | $69 \pm 2\%$ |
|             | $64 \pm 2\%$ |
| C-22 + C-23 | $22 \pm 3\%$ |
|             | $23 \pm 4\%$ |
| C-20        | $18 \pm 2\%$ |



## Discussion

I. C-10

The results for the activity of the Wieland-Gumlich aldehyde differ slightly, but the average of the values corresponds quite well with the data obtained by Willner, that 67% of the activity resides in C-10 of acetate-1-C<sup>14</sup> labelled strychnine. This relatively large value might be interpreted in two ways.

1. Carbon atoms C-10 and C-11 are the only acetate-derived carbons of strychnine, and the residual of the activity, i.e. 33%, is randomly distributed in the rest of the molecule and results from incorporation of radioactive carbon dioxide formed by decarboxylation of the acetate-1-C<sup>14</sup>.

2. The C-10 acetate unit is derived from a different "pool" of acetate than the proposed acetate-derived C<sub>9</sub> precursor. If the radioactive acetate, which was incorporated into the plant and utilized by the plant both in making the C<sub>9</sub> precursor and in acetylating the indole nitrogen, were diluted equally with the plant's own supply of acetate in both cases, then C-10 and each of the four acetate carboxy-derived carbons of the C<sub>9</sub> precursor should have equal activity. But if the radioactive acetate which forms the C<sub>9</sub> precursor is diluted with more of the plant's acetate than the labelled acetate in the C-10 pool, then C-10 obviously has a greater activity.

Several reasons may be conceived to explain the greater activity of the C-10 acetate "pool" relative to the acetate used in the C<sub>9</sub> precursor. That the formation of the various fragments which are later combined to make the alkaloid occurs in different parts of the plant has been

suggested by grafting experiments (1). Hence it is possible that the administrated acetate may not have been able to reach the site of acetate-malonate condensation as readily as the site of N-acetylation. A related explanation might be that the biosynthesis of the C<sub>9</sub> polyacetate moiety from acetate units derived from pyruvate is proximate to the site of decarboxylation of pyruvate, and the radioactive acetate introduced does not penetrate this system effectively. A third possibility is that a great amount of malonate existed in the plant at the time the experiment was conducted. This would have in effect diluted the labelled acetate going through the polyacetate condensation. Finally, the plant might not have been producing much of the polyacetate fragment at the time of feeding the labelled acetate. The effect of factors such as these would be expected to vary somewhat and hence a wide range of values might be obtained for the activity of C-10. (See section II below.)

## II. C-22 and C-23

The activities of strychninolone-a and the neutral product correspond nicely and indicate that about 22% of the activity of the other batch of acetate-1-C<sup>14</sup> labelled strychnine occurs in C-22 and C-23. This value is indeed large enough to rule out possibility 1. above, and definitely indicates an acetate origin for the non-tryptophan precursor. Although this value represents roughly 1/5 of the activity of the strychnine, which is of course exactly what one would calculate if all the acetate in the plant were equally diluted with labelled acetate, this value is regarded as fortuitous in view of the preceding experiment. In this case, if the four acetate derived carbons of the C<sub>9</sub> precursor are equally labelled,

then C-10 must have less than 20% of the activity of the strychnine. It is unfortunate that not enough of the second batch of acetate labelled strychnine was available for a determination of the activity of C-10.

At least two factors were different in the two feeding experiments, and these might be manifested in the presumed difference in the activities of C-10 in the two cases. The plants in the first experiment were 2 years old and those in the second 1 year old, and in the first experiment each plant was fed 6.7 mg. of sodium acetate-C<sup>14</sup> and in the second about 1 mg. of sodium acetate-C<sup>14</sup>. Although no experiments concerned with the ontogeny of alkaloids in Strychnos nux-vomica have been carried out, studies on other plants have indicated that the amount and kind of alkaloids produced varies with the age of the plant (1). Conceivably the older Strychnos plants may have stored a greater amount of the C<sub>9</sub> precursor or malonate. Since the plants in the first experiment were fed more acetate, their metabolism might have been affected much more than the plants in the second experiment. Too much acetate might have "triggered" the plant to eliminate it through alkaloid biosynthesis and hence forced the plant to call upon its resources and use up its supply of polyacetate precursors.

Another factor might have been the phase of the various cycles operating in the plants. No attention was paid to this factor in the above experiments although it may have been important, since the amount of alkaloids produced by a plant varies directly with the growth of new tissue, flowering, and budding (1).

III. C-20

The 18% incorporation of sodium formate at C-20 certainly substantiates the one carbon unit origin proposed. As mentioned previously, formate incorporated into the side chain of tryptophan accounts for a large amount of the activity.

### Experimental

Liquid samples were counted on a 3 channel Nuclear Chicago 720 Series liquid scintillation system. Ten to fifteen milligram samples of the pure compounds were weighed on a Cahn electrobalance and dissolved in 10 ml. of absolute ethanol and 10 ml. of scintillating solution was added. The scintillating solution was prepared by dissolving 25.8 g. of PPO\* and 0.738 g. of POPOP† in 3.6 l. of toluene. The samples were then counted at -44° for 200 to 300 minutes. Quenching factors were determined using 1,1'-dicyanobicyclohexyl-C<sup>14</sup>.

Solid samples were counted on a Nuclear Chicago system composed of a Model 115 Automatic Sample Changer and Detector, Model 181 A Scaler, and Model 11B Printing Timer. This system had a very low background count (2 counts/minute). The flow detector was used. Two to three milligram samples were ground with acetone and spread over the surface of 2 cm. diameter ground glass plates in a thin layer. The glass plates were slipped inside metal holders and stacked in the counter. The time was registered for 200 counts for each cycle.

Administration of Labelled Substrates. Slits 1 1/4 inches long were cut in the stems of one year old Strychnos nux-vomica plants, and one inch vials were attached to the stems so that the slits reached the bottom of the vials. An aqueous solution of the substrate (5 ml.) was poured into each vial. Within two or three days the plant had absorbed

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\*2,5-Diphenyloxazole.

†1,4-bis-2-(5-phenyloxazolyl)-benzene.

the solution. The plants were grown at 30° and 70% humidity for one month. Soil was then washed from the roots, and the entire plants were cut up and ground in a Waring blender.

Sodium acetate-1-C<sup>14</sup> (2 mc.) was fed to 23 plants, and sodium formate-C<sup>14</sup> (1 mc.) was fed to 24 plants.

Isolation of Labelled Strychnine. The moist macerated plant material (1.2 kg.) was mixed with 116 g. of calcium hydroxide and dried in an oven at 80°. The dried material was extracted with one gallon of petroleum ether (60-70) in a Soxhlet extractor for 14 hours. This was followed by extraction with one gallon of chloroform for 48 hours. The chloroform extract was concentrated to 50 ml. and shaken with 10% sulfuric acid (25 ml.) four times. The aqueous solution was basified with ammonium hydroxide, cooled, extracted with chloroform (50 ml.) six times. The chloroform extracts were dried over sodium sulfate and evaporated. The formate labelled plants gave 710 mg. of crude alkaloids, and the acetate labelled plants gave 468 mg.

The formate labelled alkaloids were mixed with 700 mg. of recrystallized unlabelled strychnine and dissolved in 20 ml. of a 1:1 mixture of benzene-chloroform. The alkaloids were chromatographed on 45 g. of alumina (Merck). Elution with benzene-chloroform (7:3) gave insignificant amounts of oils and films. Elution with chloroform gave several fractions of a slightly yellow powder. These were combined and triturated with 10 ml. of 25% ethanol. The ethanolic solution was removed by filtration and the residue was crystallized from ethanol, giving 737 mg. of white crystals, m.p. 280-283; lit. m.p. 286-288°.

The activity of this material was determined by counting in toluene-ethanol solution and was found to be 14,600 counts/min.-mmol. The incorporation of radioactive formate by the plant was thus 0.002%. The labelled strychnine was further diluted with 1.3 g. of cold strychnine and recrystallized twice from ethanol. Pure strychnine (1.873 g.), m. p. 286-288°, was obtained.

The acetate labelled strychnine was treated in the same manner; radioactive alkaloids (468 mg.) were mixed with 473 mg. of cold strychnine, chromatographed, triturated with 25% ethanol, and crystallized, giving 507 mg. of strychnine, activity 11,700 counts/min.-mmol. Incorporation was 0.0005%. The radioactive strychnine was mixed with 1.000 g. of cold strychnine and recrystallized twice from ethanol.

### The Wieland-Gumlich Aldehyde

Radioactive strychnine ( $2.51 \pm 0.03 \times 10^3$  counts/min., -mmol.) was obtained from Dr. Willner and was converted to the Wieland-Gumlich aldehyde by essentially the method of Anet and Robinson (14).

Hydroxyiminostrychnine. The radioactive strychnine (300 mg.) (0.90 mmole) was suspended in 7 ml. of absolute ethanol in a 100 ml. three-necked round bottom flask, which was fitted with a cold finger condenser filled with dry ice-acetone. The mixture was stirred with a magnetic stirrer, and 1 ml. of isoamyl nitrite was added. The suspension was then heated to 70° in an oil bath, and slowly a sodium ethoxide solution prepared from 75 mg. of sodium in 8 ml. of absolute ethanol was added. Continued heating at 70° for 4 hours caused all the strychnine to react and go into solution. The solution was evaporated to dryness under vacuum in a water bath. The residue was dissolved in 5 ml. of water and acidified with acetic acid. Isoamyl alcohol was removed by extraction with 5 ml. of ether. The ether extract was washed four times with 2 ml. of dilute acetic acid, and the combined aqueous layers were heated with 10 mg. of norite and filtered. The filtrate was basified with ammonium hydroxide, and the yellow precipitate was collected by filtration under reduced pressure. On drying, 302 mg. (0.83 mmole) of a yellow powder were obtained. The yellow product, hydroxyiminostrychnine, was heated in 1.5 ml. of water containing 0.08 ml. of concentrated hydrochloric acid. The hydrochloride crystallized out on cooling. It was collected and dried at 100° to a yellow powder (241 mg.) (0.61 mmole).



Beckmann Rearrangement. The hydroxyiminostrychnine hydrochloride (241 mg.) (0.61 mmole) was added slowly to 2 ml. of thionyl chloride at 26°. After 20 minutes the clear brown solution was added to a small amount of ice. On standing a few minutes, yellow crystals formed. These were filtered under reduced pressure and washed with 1 ml. of methanol and 1 ml. of ether, giving 160 mg. (0.40 mmole) of the nitrile hydrochloride.

Wieland-Gumlich Aldehyde. The above hydrochloride (160 mg.) (0.40 mmole) was refluxed with 100 mg. of sodium acetate in 10 ml. of water under nitrogen for 1 1/2 hours. The solution was basified with ammonium hydroxide and extracted with chloroform (20 ml.) three times. The chloroform extracts were dried over sodium sulfate and evaporated under reduced pressure at 50°. The crude Wieland-Gumlich aldehyde (38 mg.) (0.12 mmole) was crystallized twice from acetone, giving white crystals, m.p. 214°; lit. m.p. 216°. Samples were counted in toluene-ethanol solution; activity  $8.98 \pm 0.05 \times 10^2$  counts/min.-mmol.

Picrate of Wieland-Gumlich Aldehyde. The impure Wieland-Gumlich aldehyde obtained from Willner was dissolved in 4 ml. of ethanol and a saturated solution of picric acid in ethanol was added until no further precipitation took place. The picrate was filtered, and recrystallized from ethanol. After two more recrystallizations, the yellow crystals melted at 214-215°. Samples were plated on planchets and counted; activity  $2.99 \pm 0.05 \times 10^2$  counts/min.-mmol.

### Strychninolone-a

Acetate-1- $C^{14}$  labelled ( $2.23 \pm 0.05 \times 10^3$  counts/min.-mmol.) was converted to strychninolone-a through the classic intermediates of Leuchs (43, 44), and the neutral product  $C_{19}H_{18}O_4N_2$  (45) was also isolated.

Strychninonic Acid. Radioactive strychnine (830 mg.) (2.50 mmole) was dissolved in 7 ml. of chloroform and added dropwise to 40 ml. of acetone conjunctively with 1.6 g. (10 mmole) of potassium permanganate in small portions over a period of 1 hour. After 15 minutes, the brown precipitate was filtered and washed with 10 ml. of acetone. The brown residue was mixed with cold water and filtered. This was repeated until the filtrate was colorless. About 150 ml. of water was used. The filtrate was extracted with chloroform (50 ml.) three times and acidified with dilute hydrochloric acid (1 N). The acidified solution was extracted with chloroform (50 ml.) three times. The chloroform solutions were dried and evaporated, leaving a white foam. Recrystallization from water gave 262 mg. (0.66 mmole, 27% yield) of white crystals, m.p. 263-265°.

Neutral product. The chloroform extract of the basic filtrate from the above oxidation was dried and evaporated. The slightly yellow foam was crystallized from ethanol (norite), giving almost white crystals (13 mg.) (0.038 mmole), m.p. 330-332°; lit. m.p. 332°. The black manganese dioxide residue was stirred with hot water, filtered, and the filtrate extracted with chloroform. Evaporation of the chloroform gave more of the neutral product, but it was less pure and harder to crystallize.

A sample of the pure neutral product, m.p. 330-332°, was counted in toluene-ethanol solution; activity  $1.74 \pm 0.04 \times 10^3$  counts/min.-mmol.

Strychninolic Acid. The strychninonic acid (262 mg.) (0.66 mmole) was dissolved in 5 ml. of sodium hydroxide (1 N) and stirred in a 25 ml. Erlenmeyer flask by a magnetic stirrer. Hydrochloric acid (1 N) was added until the strychninonic acid began to precipitate. A small amount of sodium amalgam (2.5%) was added and the suspension stirred. Whenever the suspension cleared, more hydrochloric acid was added to reprecipitate the organic acids. Additional sodium amalgam was added whenever the suspension failed to clear. After 3 hours the acidic solution was extracted with chloroform (25 ml.) three times. The chloroform solution was dried over sodium sulfate and evaporated, giving a white frothy mixture of strychninolic and strychninonic acids. The ketone carbonyl band in the infrared was small, so most of the strychninonic acid presumably was reduced.

Strychninolone-a. To the impure strychninolic acid (199 mg.) (0.50 mmole) was added 3 ml. of 1 N sodium hydroxide. After standing at room temperature for 24 hours under nitrogen, the basic solution was extracted three times with 15 ml. of chloroform. On drying and evaporation, 68 mg. (0.21 mmole) of a white froth of strychninolone-a were obtained. The strychninolone-a was crystallized twice from ethanol-water, giving 15 mg. (0.05 mmole) of white crystals, m.p. 234-235°; lit. m.p. 236°. This material was similarly counted in toluene-ethanol solution; activity  $1.74 \pm 0.04 \times 10^3$  counts/min.-mmol.

### Desformylneostrychnone

Formate- $C^{14}$  labelled strychnine ( $1.73 \pm 0.03 \times 10^3$  counts/min.-mmol.) was degraded to neostrychnone, which, under Clemmenson conditions, lost its formyl group.

Neostrychnine. Strychnine (1.300 g.) (3.90 mmole) was refluxed in 13 ml. of xylene with 1 ml. of Raney nickel, which was prepared by the method of Vogel (48) and was washed free of ethanol with xylene. The refluxing was continued for 24 hours with rapid stirring. The suspension was then filtered while hot, and nearly all the xylene was removed under reduced pressure. The oily residue was crystallized from ethanol (norite), giving 0.975 g. (2.92 mmole) of neostrychnine, m.p. 213-215°; lit. m.p. 228-229°.

Neostrychnone. Neostrychnine (0.975 g.) (2.92 mmole) was dissolved in 45 ml. of purified dioxane. An aqueous solution (15 ml.) of osmium tetroxide (45 mg.) was added, and the solution turned black. Ground sodium periodate (6.6 g.) was introduced in small portions over 45 minutes, while the solution was stirred. The solution was diluted with 50 ml. of water after another 45 minutes and extracted with 50 ml. of chloroform three times. The chloroform solutions were dried and quickly evaporated under reduced pressure. The dark residue was crystallized from ethanol (norite) twice. Grey crystals of neostrychnone, m.p. 233-235°, were obtained; lit. m.p. 235-237°, from ozonization of neostrychnine (47).

Desformylneostychnane. Amalgamated zinc (1.5 g.) was added to 358 mg. (0.98 mmole) of neostychnone dissolved in 7 ml. of cold concentrated hydrochloric acid, and after the initial foaming had ceased the mixture was stirred and heated to reflux. An additional 2.5 g. of amalgamated zinc and 10 ml. of concentrated hydrochloric acid were added in three portions over 3 hours. The mixture was then refluxed for two more hours. The solution was decanted from the unreacted zinc and diluted with 25 ml. of water. The zinc was washed with 5 ml. of 1 N hydrochloric acid. The acidic solution was shaken with 30 ml. of chloroform and filtered under reduced pressure to break the emulsion. The aqueous layer was then extracted twice more with 30 ml. of chloroform and basified with 20% sodium hydroxide solution. Extraction with 75 ml. of chloroform three times and evaporation of the chloroform gave a small amount of oily material. This was taken up in 3 ml. of ethanol and filtered. Addition of acetone precipitated the white amorphous desformyl compound. The white material was again dissolved in 3 ml. of ethanol, and a saturated solution of picric acid in ethanol was added dropwise until no further picrate came out of solution. The picrate was crystallized twice from acetone-ethanol. Both the desformyl compound and its picrate melted above  $310^{\circ}$ . A N-H stretching band was observed in the infrared spectrum, but no ketone carbonyl or aldehyde C-H stretching peak appeared. A Kuhn-Roth determination gave .59 mole of acetic acid.

Anal. Calcd. for  $C_{26}H_{27}O_9N_5$ : C, 56.41; H, 4.92. Found: C, 56.50; H, 4.80.

The picrate was plated on a planchet and counted; activity  $1.42 \pm 0.03 \times 10^3$  counts/min. -mmol.

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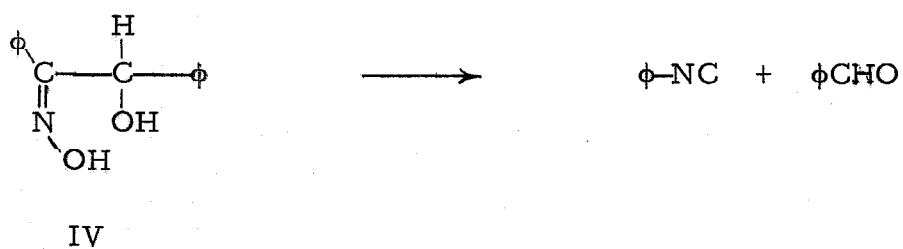
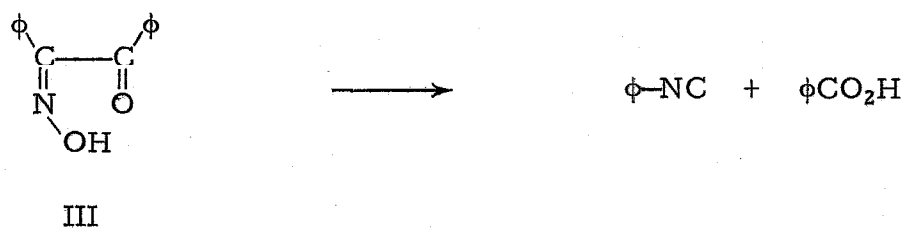
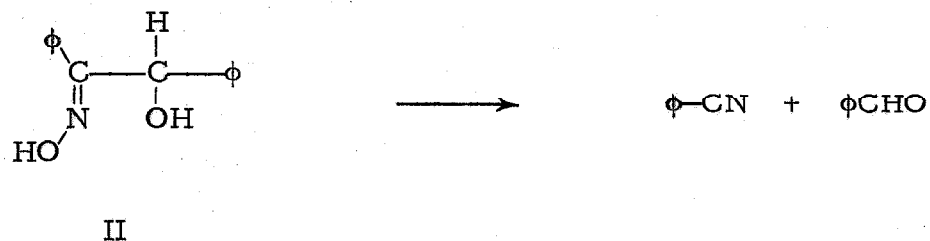
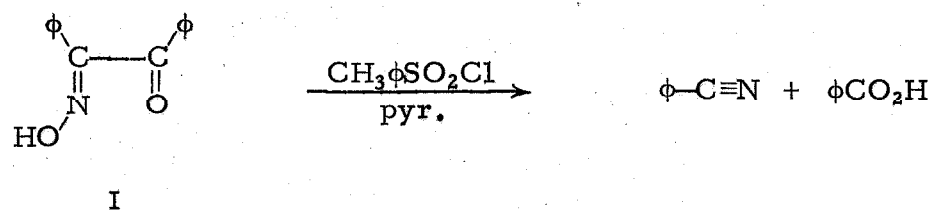
BECKMANN FISSION OF A  $\gamma$ -KETO OXIME

## Introduction

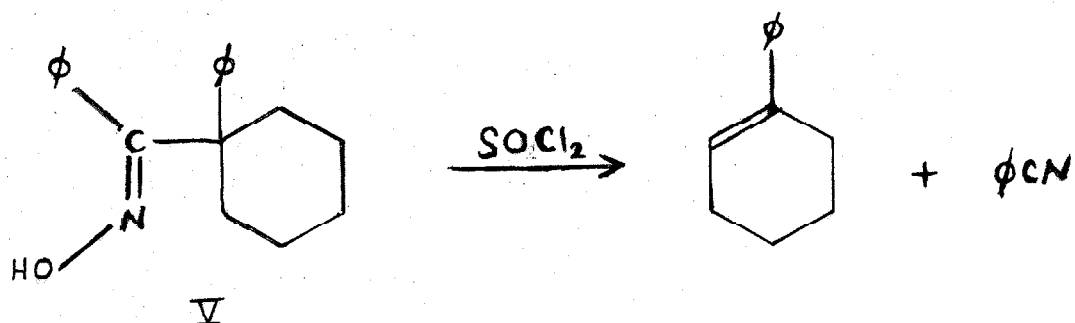
One of the favorite reactions used by natural products chemists in some of their elegant structural elucidations is the second order Beckmann rearrangement or Beckmann fission. This reaction is characterized by cleavage of a carbon-carbon single bond and formation of a nitrile, and hence differs from the Beckmann rearrangement in that only a pair of electrons migrates rather than a carbon atom. Whenever the group  $\alpha$  to the oxime carbon can stabilize a positive charge, this type of reaction occurs; hence  $\alpha$ -keto oximes,  $\alpha$ -hydroxy oximes,  $\alpha$ -amino oximes, and  $\alpha, \alpha$ -disubstituted oximes undergo the Beckmann fission.

Classic examples of this reaction are the cleavage of anti-benzil monoxime (I) to benzonitrile and benzoic acid (1) and the cleavage of anti-benzoin oxime (II) to benzonitrile and benzaldehyde (2), on treatment with p-toluenesulfonyl chloride in pyridine. The syn isomers of benzil and benzoin oximes (III and IV) gave benzoisonitriles in addition to benzoic acid and benzaldehyde, presumably via phenyl migration followed by electron migration.

Early in the study of the structure of strychnine a Beckmann fission was carried out by H. Wieland (3). The illustrious Wieland-Gumlich aldehyde was prepared from hydroxyiminostrychnine by treatment with thionyl chloride, followed by hydrolysis. (See Part II of this thesis.)



Oximes with a tertiary  $\alpha$ -carbon have been cleaved to the olefin and nitrile on treatment with thionyl chloride. The oxime V has been converted to 1-phenylcyclohexene and benzonitrile in this manner (4).

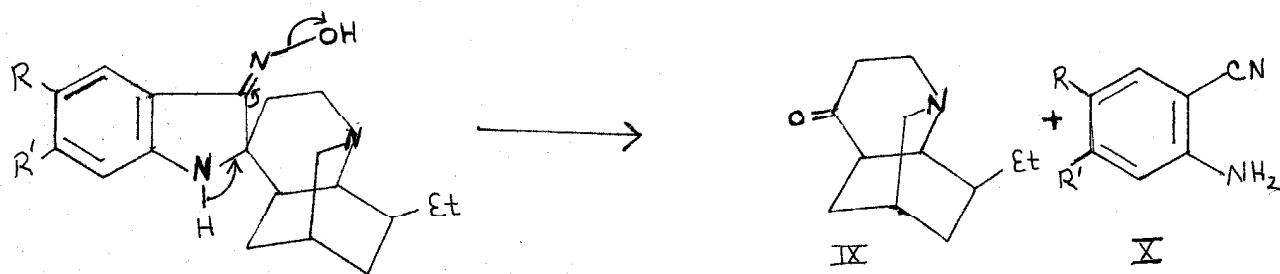
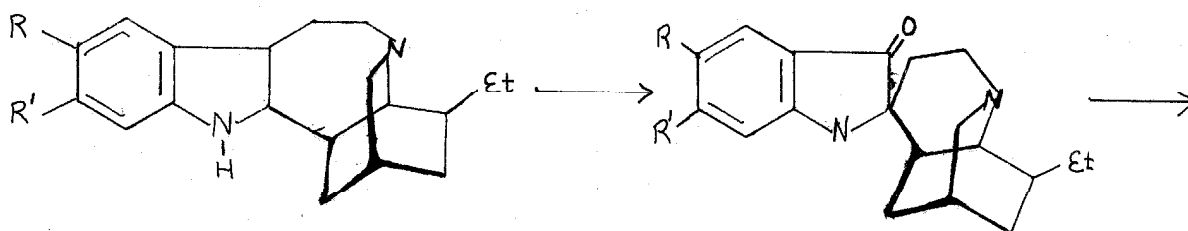
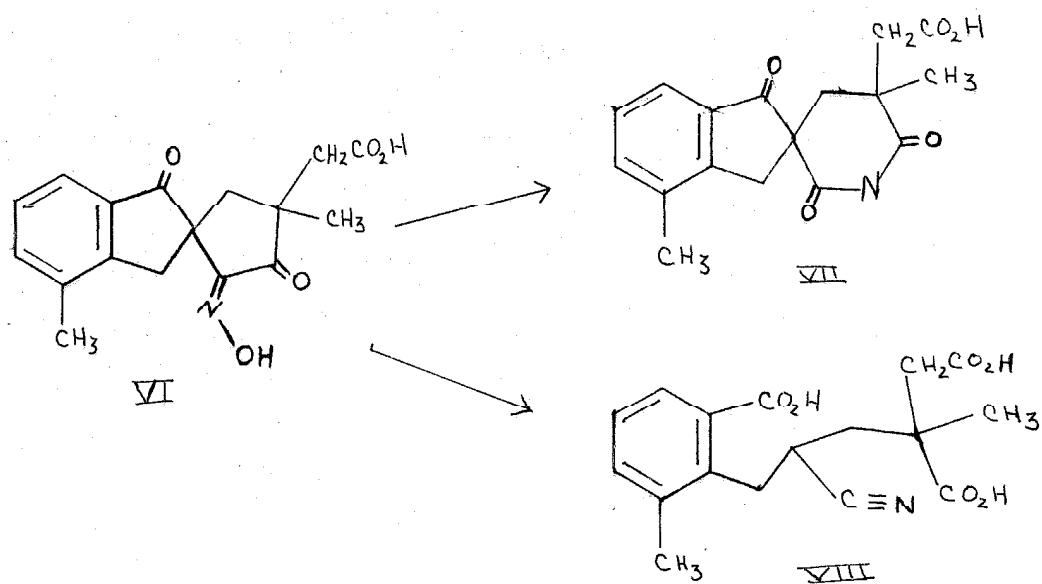


Two clever applications of the Beckmann fission reaction in the degradation of natural products have been reported recently. Polyphosphoric acid converted the gibberic acid derivative VI to the imide VII in the normal Beckmann rearrangement; however on treatment of the oxime VI with p-toluenesulfonyl chloride in sodium hydroxide solution, the tri-carboxylic acid VIII was obtained via the Beckmann fission course. This evidence established the presence of a methylene carbonyl bridge in gibberic acid (5).

A Beckmann fission reaction was used to transform the alkaloids from Tabernanthe iboga to one common derivative IX plus the three anthranilonitriles (X) whose relationship was shown (6).

Three possible mechanisms have been considered for the Beckmann fission of  $\alpha$ -keto oximes.

1. Separation of the conjugate base of a strong acid from the nitrogen, combined with migration of an electron pair forming a stable cation,  $\text{RCO}^+$ .
2. Attack of base on the carbonyl, followed by electron pair migration and the leaving of the conjugate base from the nitrogen.
3. A concerted attack on the carbonyl and leaving of the conjugate base.

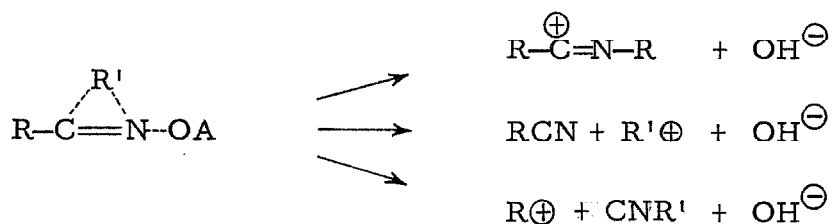


| alkaloid      | R                | R'               |
|---------------|------------------|------------------|
| ibogaine      | OCH <sub>3</sub> | H                |
| ibogamine     | H                | H                |
| tabernanthine | H                | OCH <sub>3</sub> |

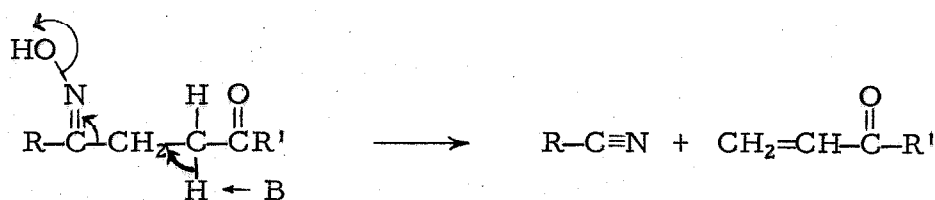
Ferris (7) found that the Beckmann cleavage of  $\alpha$ -keto oxime acetates in ethanol is catalyzed by amines and sodium acetate and that in reactions carried out with a large amount of an amine present, the product is mostly the ethyl ester with only a small amount of amide; hence he claims that the reaction goes through the oxocarbonium ion  $\text{RCO}^+$ , which attacks the alcohol and a small amount of the amine (mechanism 1), rather than proceeding by attack of a base on the carbonyl (mechanism 2) or a concerted mechanism (mechanism 3).

Studies by Grob (8) indicate that the ease of fission of  $\alpha$ -amino oximes varies with the electron releasing character of the N-substituent indicating an electron deficient transition state in the rate determining step as in mechanism 1.

The type of intermediate envisioned for the Beckmann rearrangement may be involved in the Beckmann fission as well. Then the various products obtained depend upon the ability of the  $\alpha$ -carbon to stabilize a positive charge and the geometry of the oxime.

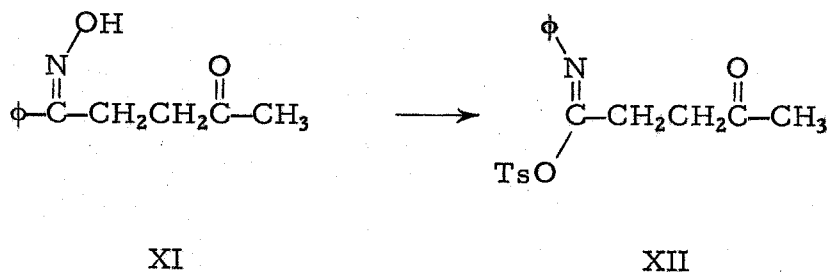


A  $\gamma$ -keto oxime might be expected to form an  $\alpha, \beta$ -unsaturated ketone in addition to the nitrile in a base catalyzed elimination fission as shown.

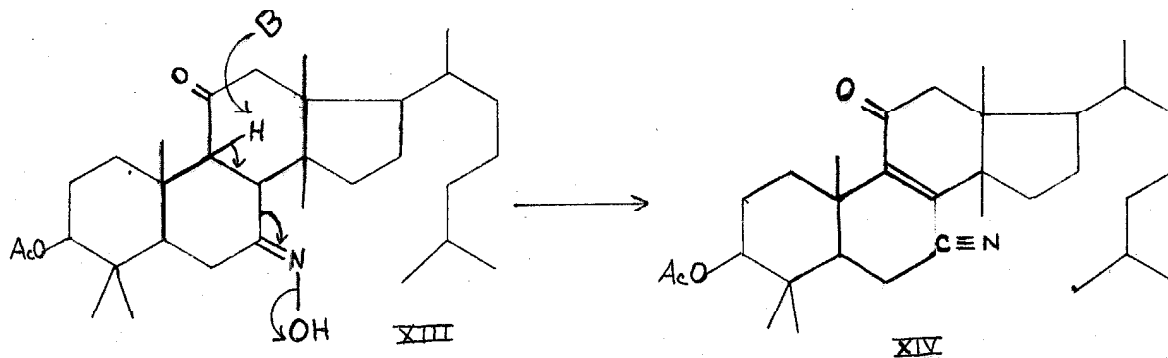


A  $\gamma$ -keto oxime unsubstituted at the  $\alpha$  position would not be expected to undergo a Beckmann cleavage via mechanism 1, although the normal Beckmann rearrangement would be possible. However in this type of compound, the reaction might be initiated by abstraction of an  $\alpha$ -hydrogen by strong base, followed by electron rearrangement and leaving of the conjugate base from the nitrogen.

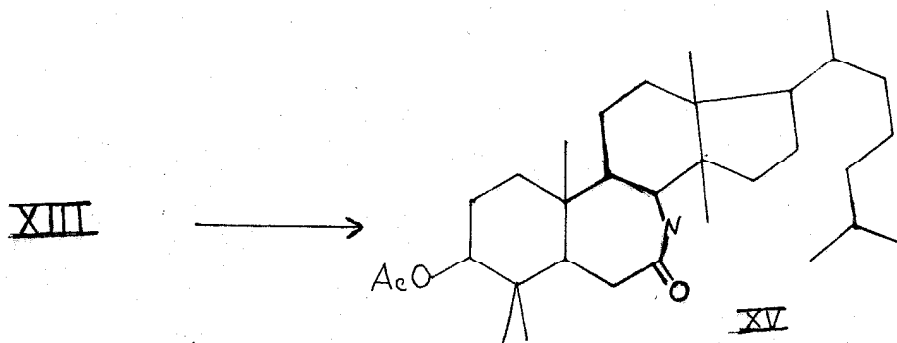
At the time of investigation, the only known example of the Beckmann fission of a  $\gamma$ -keto oxime was when 1-oximino-1-phenylpentan-4-one (XI) was treated with *p*-toluenesulfonyl chloride in sodium hydroxide solution and the imidolyl sulfonate (XII) was isolated (9). This product was further converted to phenyl isocyanide. Since an imidolylsulfonate was isolated, conditions were not suitable for an elimination type of reaction; hence the formation of an  $\alpha, \beta$ -unsaturated ketone by a Beckmann fission could not compete with phenyl migration under these conditions.



An interesting substrate for demonstrating a base-catalyzed cleavage is the lanosterol derivative, 3-acetoxylanostane-7, 11-dione-7-oxime (XIII), prepared from 3-acetoxylanostane-7, 11-dione by refluxing with excess hydroxylamine hydrochloride and pyridine in alcohol for 6 hours (10). Suitable conditions could then result in the opening of ring B of this triterpene derivative, giving the nitrile XIV.



Barton (11) and Voser (12) rearranged the monoxime XIII to the lactam XV, with phosphorus pentachloride. Since the carbon  $\alpha$  to the oximino function is not tertiary, the conventional lactam was the expected product.



The oxime XIII was reacted with thionyl chloride in dry benzene solution. A white crystalline product, identical to the normal Beckmann product obtained by Barton (11) and Voser (12), was isolated by chromatography.

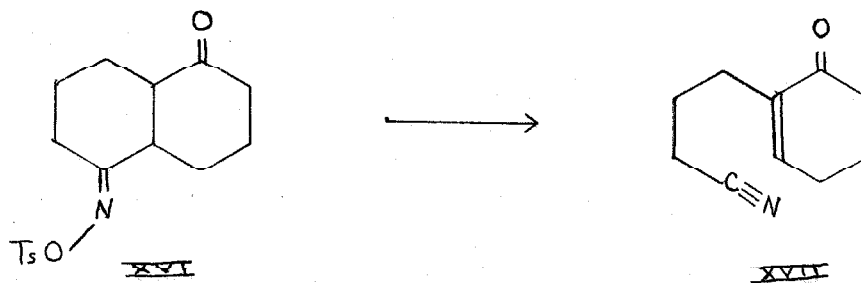
The p-toluenesulfonate of the oxime was prepared by refluxing the oxime with sodium hydride in ether, then refluxing the sodium salt of the oxime with p-toluenesulfonyl chloride. The crystalline tosylate was obtained in reasonably good yield, since the sodium hydride removes only the oximino hydrogen and does not initiate the cleavage reaction. The tosylate was then refluxed with sodium t-butoxide, water was added, and the white suspension extracted with ether. Chromatographic separation of the oily material obtained and crystallization from ligroin gave a small amount of white crystals, m.p. 240–242°, in 29% yield. The infrared and ultraviolet spectra and the analysis indicated that the desired unsaturated ketone nitrile (XIV) was formed.

The low yield may result from formation of the conventional lactam and the Neber rearrangement, a common reaction of oxime arylsulfonates, in which a hydrogen  $\alpha$  to the oxime carbon is abstracted by base and an azirine ring forms. Hydrolysis of the azirine ring results in an  $\alpha$ -amino ketone. Although no crystalline amines were isolated, this product might account for some of the oily material obtained. Due to the steric hindrance of the methyl groups in the lanosterol derivative, abstraction of the hydrogen  $\alpha$  to the ketone might not have occurred as readily as abstraction of the hydrogen  $\alpha$  to the oxime function; hence the normal Beckmann rearrangement and the Neber rearrangement presumably competed extensively with the Beckmann fission.

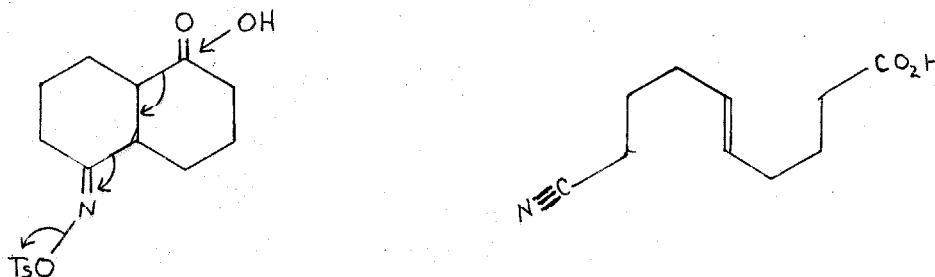


The monoxime XIII was refluxed with toluenesulfonyl chloride in pyridine; however only starting material was recovered. Also the monoxime tosylate was refluxed with sodium hydride in diglyme and none of the nitrile was formed. Apparently only a strong base can initiate this cleavage reaction by attacking the hydrogen  $\alpha$  to the ketone.

The cleavage of a  $\gamma$ -keto oxime to a nitrile and unsaturated ketone was recently confirmed by Grob (13). The monoxime tosylate of 1,5-diketotransdecalin (XVI) gave the cyclohexenone nitrile (XVII) with sodium methoxide or potassium *t*-butoxide. Heating in 80% ethanol gave the normal Beckmann lactam.



When the oxime was reacted with *p*-toluenesulfonyl chloride and 1 N sodium hydroxide in aqueous dioxane, a nitrile acid was isolated, which they presumed to be XVIII. This must have resulted from a cleavage analogous to the Beckmann fission of  $\alpha$ -keto oximes.



## Experimental

3-Acetoxy- $\Delta^8$ -lanostene. Lanosteryl acetate (5 g.) (10.6 mmole) was dissolved in 300 ml. of acetic acid and 1 g. of platinum oxide was added. The mixture was hydrogenated at 50 lbs. pressure and 70° for two days. The warm acetic acid solution was filtered to remove the catalyst and the filtrate cooled. Water (300 ml.) was added to precipitate the dihydro compound. The white precipitate was filtered with suction and recrystallized from ethyl acetate twice. White flakes (4.3 g.) (9.2 mmole) of 3-acetoxy- $\Delta^8$ -lanostene, m.p. 118-119°, were obtained in 86% yield; lit. m.p. 118-120°.

3-Acetoxy- $\Delta^8$ -lanostene-7,11-dione. The procedure of Ruzicka (14) was used without modification. 3-Acetoxy- $\Delta^8$ -lanostene (4.0 g.) (9.1 mmole) was oxidized to 2.5 g. (5.3 mmole) of 3-acetoxy- $\Delta^8$ -lanostene-7,11-dione; 58% yield.

3-Acetoxylanostane-7,11-dione. The procedure of Ruzicka (14) was again used. 3-Acetoxy- $\Delta^8$ -lanostene-7,11-dione (2.0 g.) (4.2 mmole) was reduced to 1.6 g. (3.4 mmole) of 3-acetoxylanostane-7,11-dione; 81% yield.

3-Acetoxylanostane-7,11-dione-7-oxime. The procedure is essentially that of Voser (12). To 488 mg. (1.00 mmole) 3-acetoxylanostane-7,11-dione in 30 cc. absolute ethanol were added 1.0 g. (15 mmole) of hydroxylamine hydrochloride and 12 cc. of pyridine, and the solution was refluxed for 6 hours. The solution was then neutralized with dilute sulfuric acid and extracted with benzene. Chromatographic separation

on alumina and crystallization from ethanol gave 383 mg. (76.0%) of white needles, m.p. 211-213°; lit., m.p. 213-214°.

Anal. Calcd. for  $C_{32}H_{53}O_4N$ : C, 74.52; H, 10.36; N, 2.72.

Found: C, 74.36; H, 10.40; N, 2.62.

Lactam Product. Thionyl chloride (5.0 g.) in 10 ml. of dry benzene was added dropwise to a solution of 100 mg. (0.2 mmole) of the above oxime in 10 ml. of benzene. The solution was stirred at room temperature for 3 hours, then water was added and the mixture extracted with benzene. Chromatography on alumina and crystallization from ethanol gave white crystals of the lactam, m.p. 247-249°; lit., m.p. 249-250°. Yield, 42 mg. (0.082 mmole) 42%.

Infrared spectrum in chloroform:  $\nu_{\max}$  3420  $\text{cm}^{-1}$  (N-H), 1727 (ester, ketone), 1667 (amide I), 1280 (amide II).

3-Acetoxylanostane-7,11-dione-7-oximetosylate. A solution of 100 mg. (0.20 mmole) of 3-acetoxylanostane-7,11-dione-7-oxime in 10 ml. of dry ether was stirred and cooled in ice while 15 mg. (0.30 mmole) of a 50% suspension of sodium hydride in mineral oil were added. The solution was refluxed under nitrogen with vigorous stirring for 24 hours. A solution of 36 mg. (0.19 mmole) of p-toluenesulfonyl chloride in 10 ml. of dry ether was added dropwise. The ether suspension was stirred for 3 hours at room temperature. The ether solution was drawn up through a piece of cotton into a capillary pipette. Great care was taken to exclude air and moisture. The resulting clear solution was evaporated under reduced pressure in a rotary evaporator. The white needles thus obtained were dissolved in a tiny amount of carbon tetrachloride and 10 ml. of

ligroin (30-60) were added. The solution was seeded with a few of the needles obtained, and upon cooling, 45 mg. of white needles of the tosylate were obtained. Concentration of the ligroin mother liquor yielded an additional 10 mg. of product, m.p. 243-246°; 41% yield. A second reaction using the same procedure gave a somewhat higher yield of 70%.

Anal. Calcd. for  $C_{39}H_{59}O_6SN$ : C, 70.00; H, 8.83; S, 4.79; N, 2.09. Found: C, 69.77; H, 8.91; S, 4.84; N, 1.83.

Nitrile Product. To a solution of 35 mg. (0.052 mmole) of the oxime tosylate in 10 ml. of t-butyl alcohol was added dropwise 5 ml. of a sodium t-butoxide suspension prepared from 50 mg. of sodium. The suspension was refluxed for 1 hour. Water was added dropwise with stirring and the suspension neutralized with dilute hydrochloric acid. Extraction with ether gave an oil which was chromatographed on alumina. The pale yellow oil obtained in intermediate fractions was crystallized from ligroin (30-60) to give a white substance, m.p. 240-242°. Further recrystallization from ligroin yielded the pure nitrile, 7 mg. (0.015 mmole), m.p. 246-246.5°; 29% yield.

Infrared spectrum in chloroform:  $\nu_{\max}$ , 2245  $\text{cm}^{-1}$  (nitrile), 1725 (ester), 1658 (unsaturated ketone), 1602 ( $C=C$ ).

Ultraviolet spectrum in ethanol:  $\lambda_{\max}$ , 246 m $\mu$ ,  $\log e = 3.83$ .

Anal. Calcd. for  $C_{32}H_{51}O_3N$ : C, 77.21; H, 10.33; N, 2.82. Found: C, 77.46; H, 10.51; N, 3.04.

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## PROPOSITION I

## The Biogenesis of Indole Alkaloids

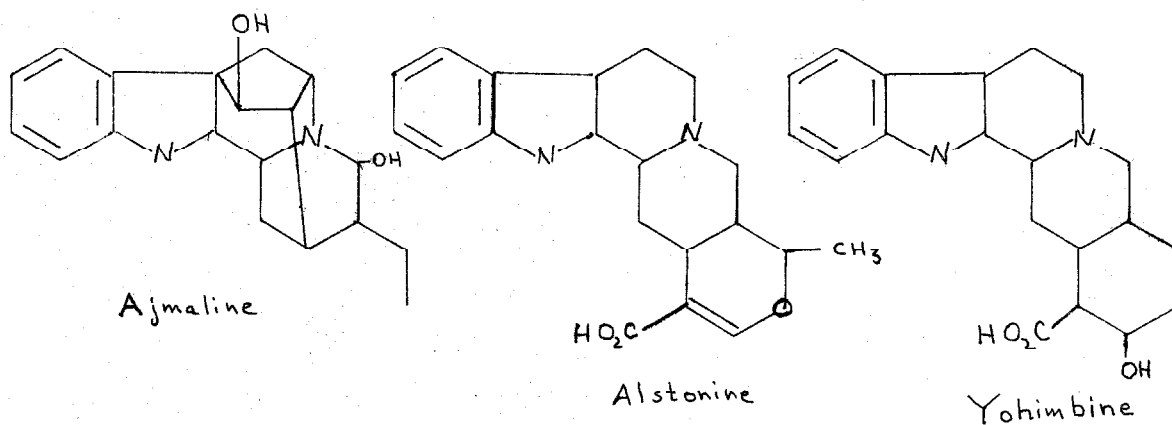
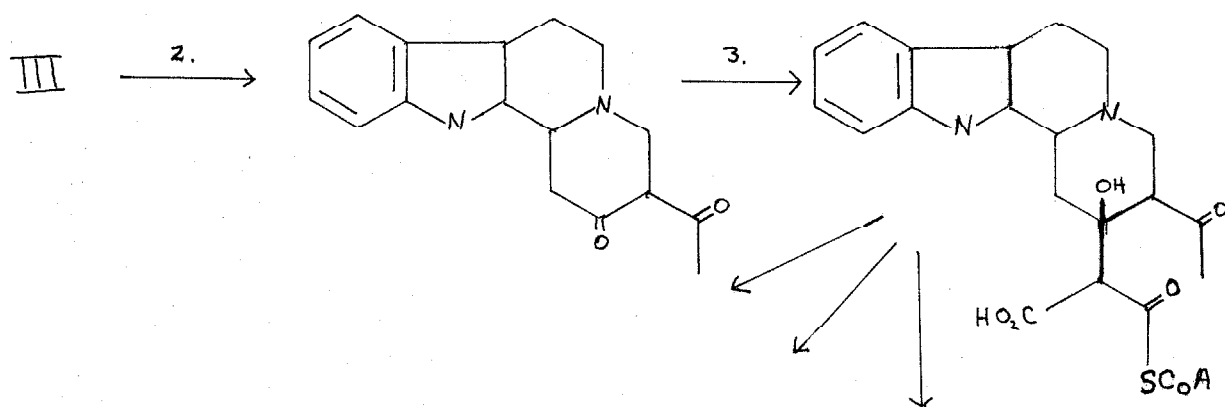
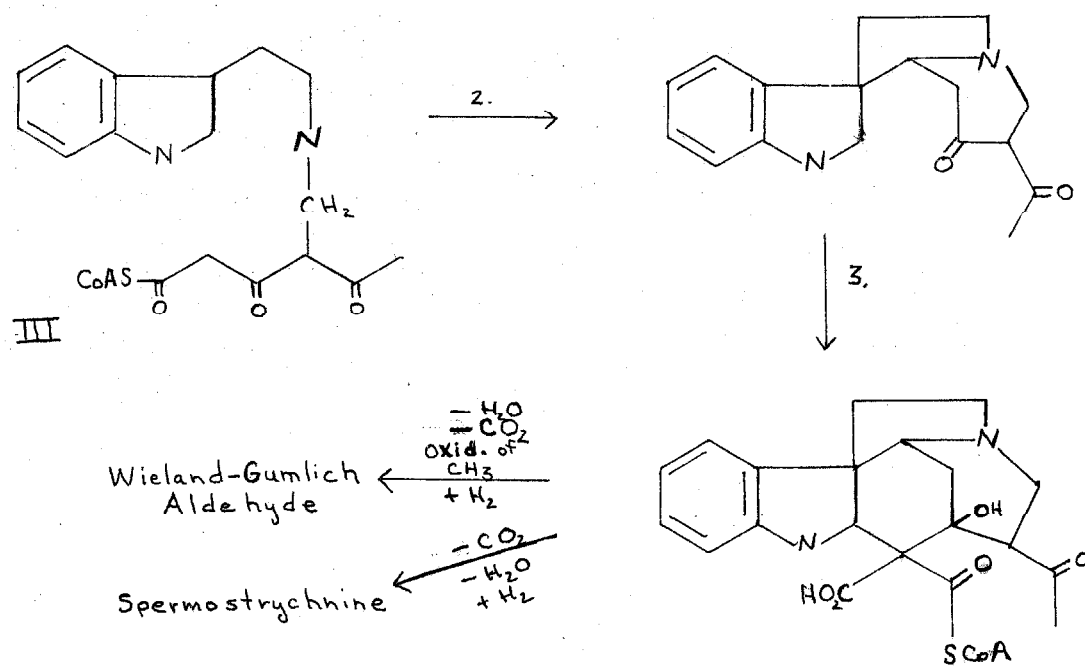
The acetate derived C<sub>9</sub> intermediate involved in the biogenesis of indole alkaloids needs clarification. As mentioned in Part II of this thesis, two recent proposals for the origin of the C<sub>9</sub> precursor have been made. Leete (1) suggested a linear C<sub>6</sub> acetate-derived chain condensing with a molecule of malonyl Coenzyme A (XXVI, pg. 50). Richards and Hendrickson (2) pointed out that this was not likely to happen since condensation of malonyl Coenzyme A occurs with concomitant decarboxylation, and hence chain branching of this sort is virtually unknown. Their third point that the intact ring E in some alkaloids suggests a cyclic precursor rather than Leete's acyclic precursor may be irrelevant. In view of the large number of alkaloids without ring E, it is seemingly more economical to use an acyclic precursor for the C<sub>9</sub> fragment, rather than the cyclic one (XXVII, pg. 50) which Richards and Hendrickson proposed, which at the least must lose one end methyl group and undergo a reverse aldol reaction to give the acyclic precursors.

If a condensation with malonate occurs after the triacetate chain has condensed with the tryptamine and formate (III) rather than at the site of polyacetate condensation, then the synthetase enzyme, which customarily decarboxylates malonyl coenzyme A either just before or concerted with condensation with acyl coenzyme A, may not function here. Claisen condensation of an anionic malonate would then give a carbinol which is dehydrated in some manner.

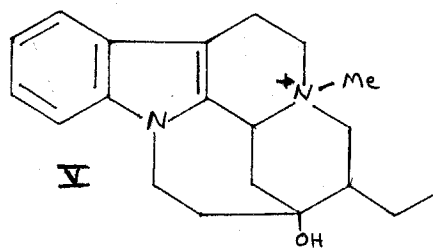
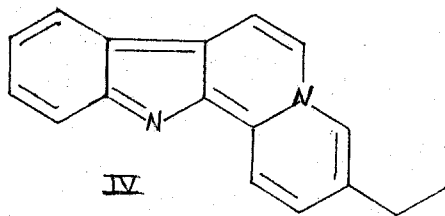
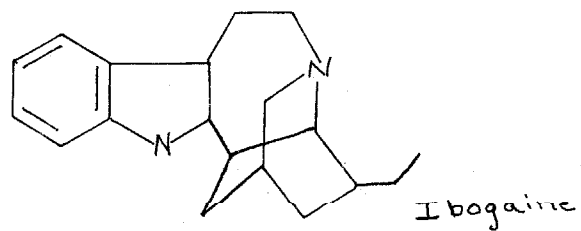
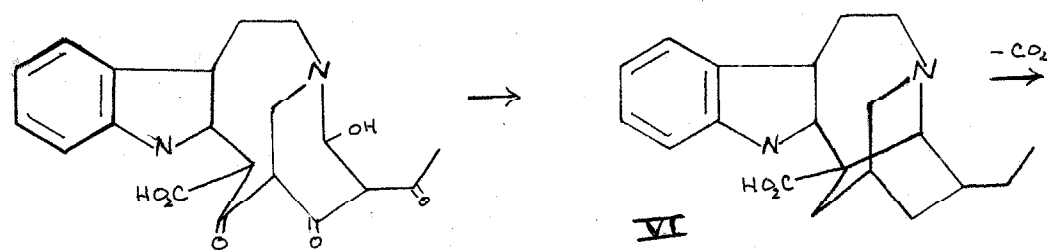
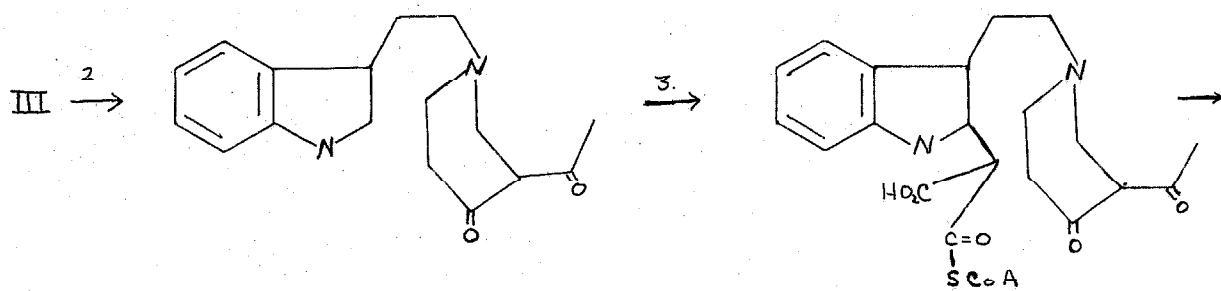
The following steps then might be written for indole alkaloid biosynthesis.

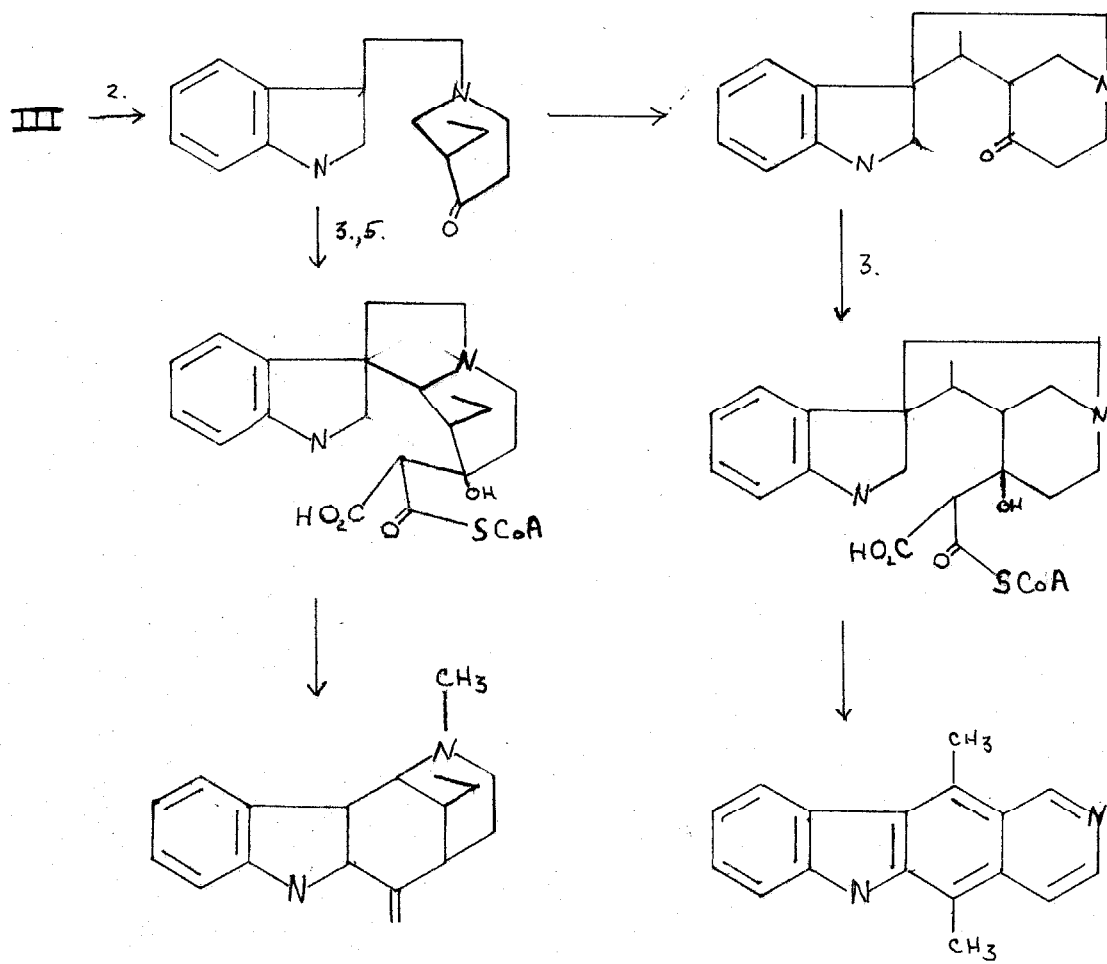
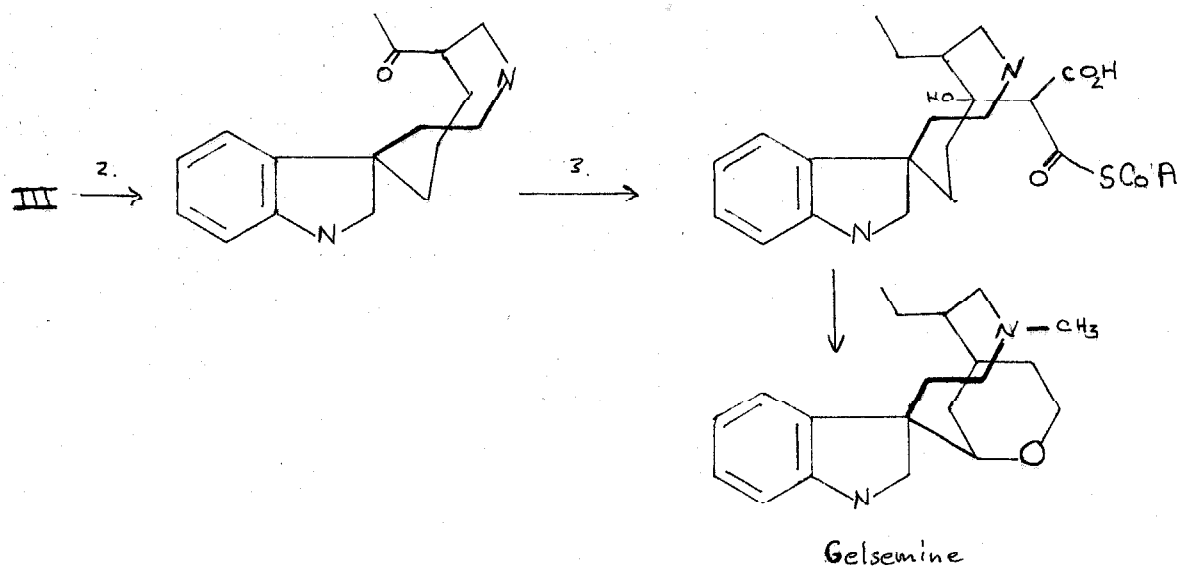
1. A Mannich reaction of tryptamine and a formate unit with a triacetate unit, always at the C-4 methylene of the C<sub>6</sub> triacetate unit.
2. Condensation of the larger end of the triacetate chain (carboxy end) with the nitrogen and in many cases also with the 2 (ajmaline, alstonine, yohimbine) or 3 (strychnine) positions of the indole or oxindole ring. In gelsemine the carboxy end of the triacetate does not condense with the nitrogen but only with the 3 position of the indole ring.
3. Condensation of C-2 of malonate with either the middle keto group of the triacetate unit or the 1, 2, or 3 position of the indole ring.
4. Dehydration and reduction of the double bond.
5. In some cases oxidation of the formate-derived carbon and possibly further attachment of this carbon to the indole ring (aspido-spermine, eburnamenine, uleine) or malonate unit (ibogaine).
6. In some cases condensation of coenzyme A malonate ester carbonyl with a methylene of the triacetate unit (eburnamenine, aspido-spermine, ibogaine).

The structures of two alkaloids are significant with respect to the proposed scheme. Flavopereirine (IV) seems to result from the triacetate unit without the malonate unit attached. Mavacurine (V) retains the hydroxyl group presumably resulting from condensation at a keto group.





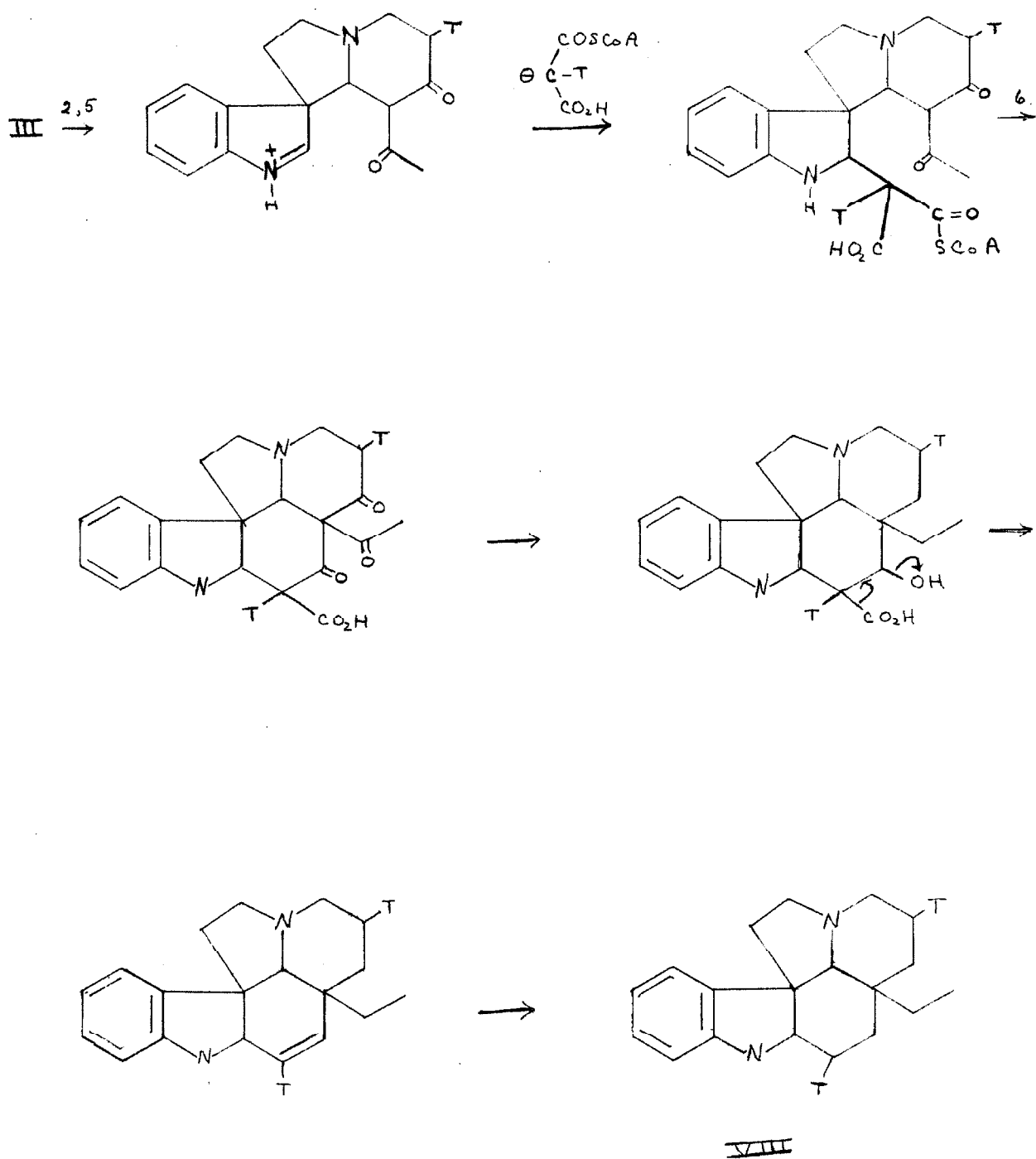




Obviously either the pathway suggested by Richards and Hendrickson or the malonate pathway would give the same labelling pattern in acetate-1-C<sup>14</sup> labelled ajmaline and this pattern corresponds to the experimental results (1). A simple experiment would rule out the biogenesis proposed by Richards and Hendrickson. Their pathway predicts that the carboxy group of acetate-1-C<sup>14</sup> labelled voacangine (VI) would contain 1/5 of the activity of the molecule, whereas the pathway involving a malonate precursor would not predict any activity for the carboxy group, since it must arise from carbon dioxide. Voacangine is easily decarboxylated to ibogaine (3).

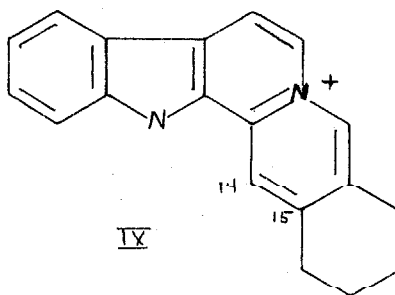
A second experiment might demonstrate at what stage the malonate condenses. If malonate condenses with the triacetate chain before it gets involved with the tryptamine, then the fragment must rearrange in order to form the aspidospermine structure (VII) by a mechanism such as Wenkert suggested (4). Condensation at a later time need not involve a rearrangement.

The administration of malonate-1-C<sup>14</sup>-2-T to Aspidosperma plants and the measurement of the C<sup>14</sup> to T ratio in aspidospermine (VIII) is proposed. If the malonate condenses with the triacetate chain initially, then none of the tritium would remain on C-2 of the presumed malonate-derived unit. However if malonate condenses with the indole ring first, then loss of tritium from C-2 of the malonate unit may not necessarily occur, since a double bond need not arise between C-2 of malonate and C-2 of the indole ring. Further condensation with the triacetate methylene also need not cause loss of tritium from C-2 of malonate, since decarboxylation can occur at this point.



Significantly no carboxylated Aspidosperma bases are known. The argument that decarboxylation of a C<sub>9</sub> precursor, formed by condensation of malonate and triacetate, occurs in this case weakens the idea of universal precursors for indole alkaloid biosynthesis.

A similar experiment might be tried with sempervirine (IX), which may have retained one of its malonyl hydrogens, since dehydration of the carbinol might give the double bond between C-14 and C-15.



#### References

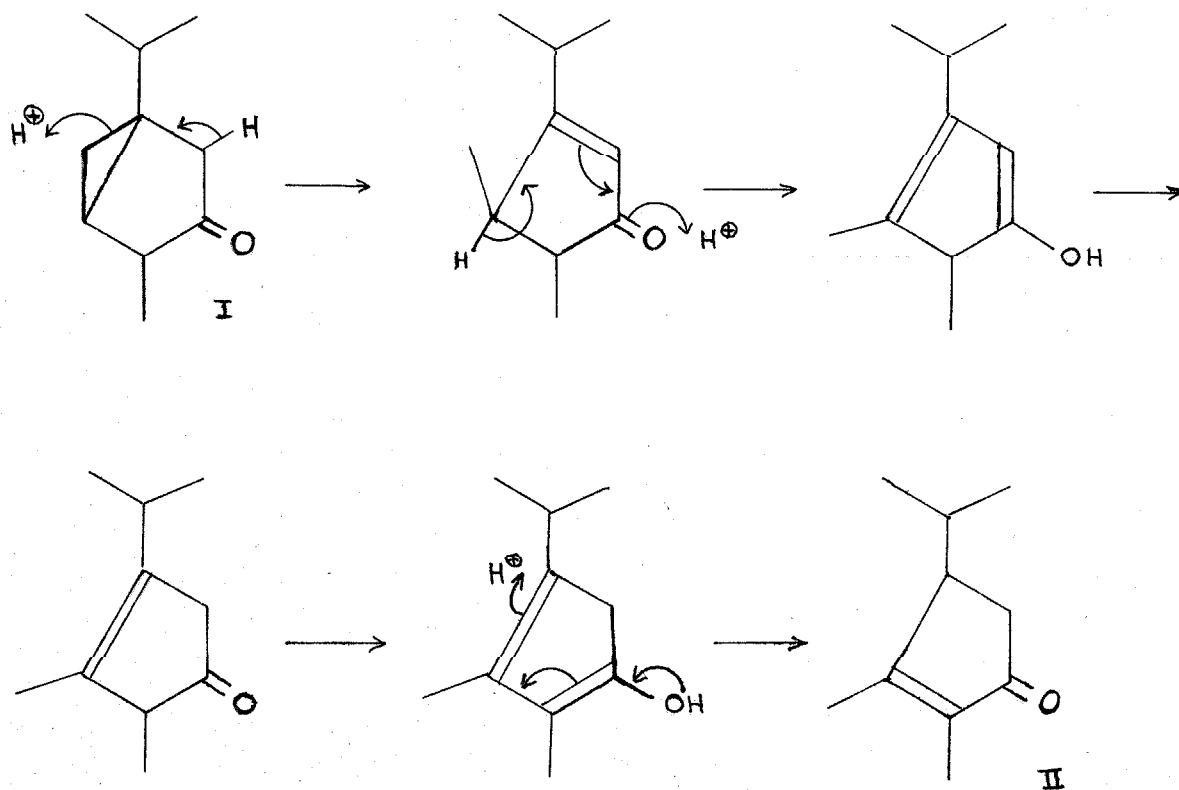
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## PROPOSITION II

## The Rearrangement of Thujone

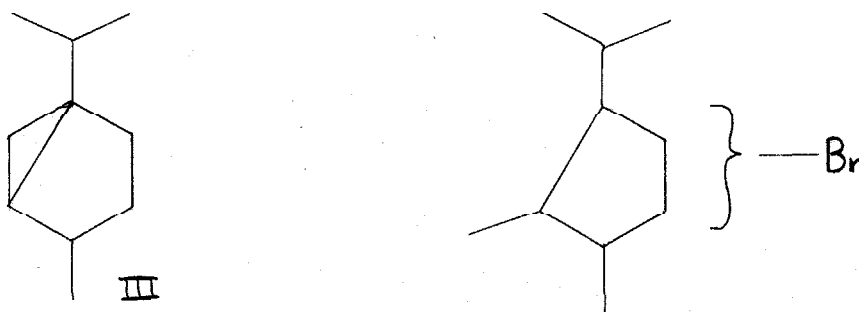
A study of the rearrangement of thujone to "isothujone" is proposed. The nature of the transformation and its stereochemical implications are discussed.

Thujone (I) isomerizes to the cyclopentenone "isothujone"\* (II) in cold concentrated sulfuric acid (1). The following mechanism has been written (2) for this transformation to a conjugated ketone,

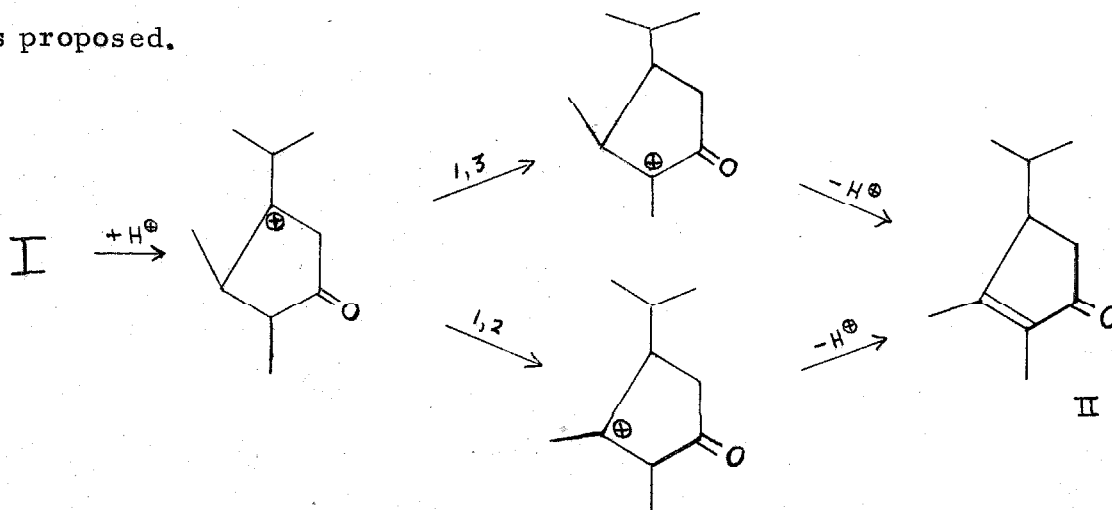


\*The quotation marks are used to distinguish the rearrangement product from d-isothujone, a naturally occurring geometrical isomer of thujone.

That the rearrangement is initiated by protonation of the cyclopropane ring rather than by protonation of the carbonyl, which would appear to be more basic, is suggested by the fact that thujane (III) is converted to a cyclopentane halide on treatment with hydrobromic acid or hydroiodic acid. Presumably the ring opening proceeds in accordance with the Markownikoff rule; the least substituted (most basic) carbon is protonated, opening the ring to form the most stable carbonium ion, which is attacked by the halide ion. The oxime of thujone is similarly rearranged to "isothujone" oxime.

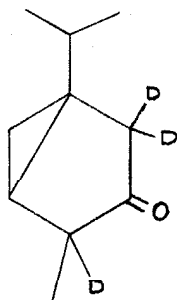


A more economical mechanism than deMayo's for the isomerization of thujone might be considered. That the carbonium ion resulting from protonation of the cyclopropane ring rearranges by a 1,2 or 1,3 hydride shift to a second carbonium ion, which collapses to the conjugated ketone, is proposed.



This mechanism might be distinguished from deMayo's mechanism by carrying out the reaction in  $D_2SO_4$  and comparing the nuclear magnetic spectrum of the product with "isothujone" prepared in  $H_2SO_4$ . Since intramolecular hydride migration occurs without undergoing exchange with solvent molecules, hydrogen rather than deuterium at C-3 would indicate a hydride migration. If deuterium were observed at C-3, it would be necessary to determine whether exchange with  $D_2SO_4$  occurs in thujone or the product "isothujone." This could be done by isolating unrearranged thujone from the reaction, and by treating "isothujone" with  $D_2SO_4$ .

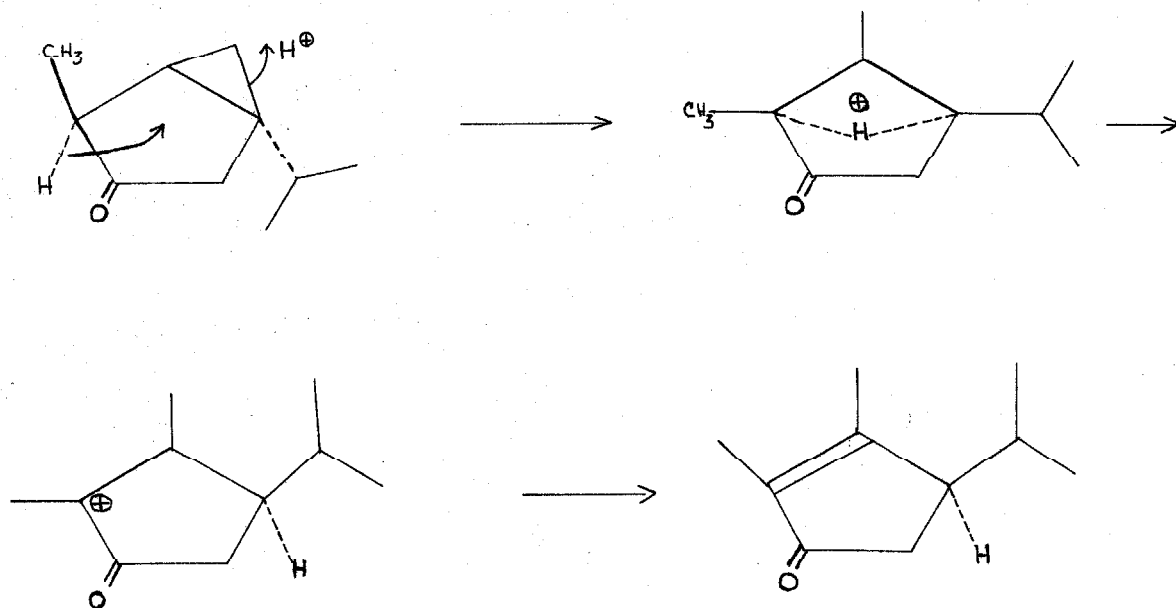
A 1, 2 hydride shift might be distinguished from a 1, 3 hydride shift by using a deuterium substituted thujone (IV) and analyzing the nuclear magnetic resonance spectrum of the "isothujone" obtained.



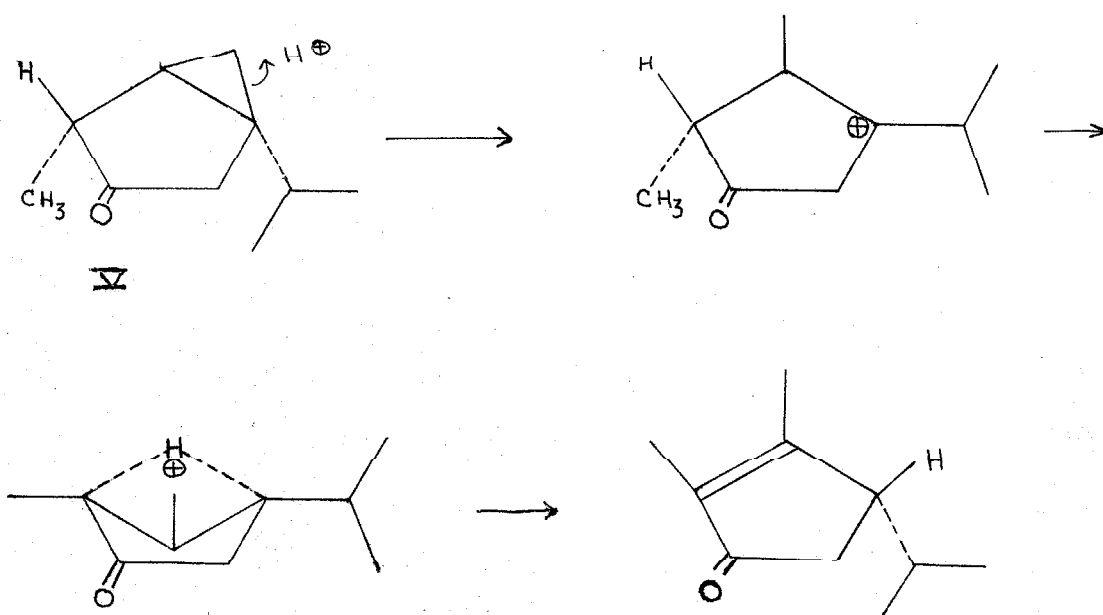
IV

The stereochemistry of thujone is not known with certainty; however it is believed to be one of the possible enantiomers with a trans configuration of the methyl and isopropyl groups, since it has a lower density and refractive index than d-isothujone (Auwers-Skita rule) (3). A hydride shift would result in inversion of configuration at C-3, whether or not the shift were concerted with protonation of the cyclopropane ring or a tertiary carbonium ion at C-3 had fully developed.





The possibility of anchimeric assistance of cyclopropane fission by incipient 1,3-hydride shift might be demonstrated by carrying out the rearrangement of *d*-isothujone, the *cis* isomer (V). If this isomer is shown to rearrange by a 1,3 hydride shift, then the full development of a tertiary carbonium ion at C-3 is required prior to hydride migration, and a concerted mechanism is ruled out.



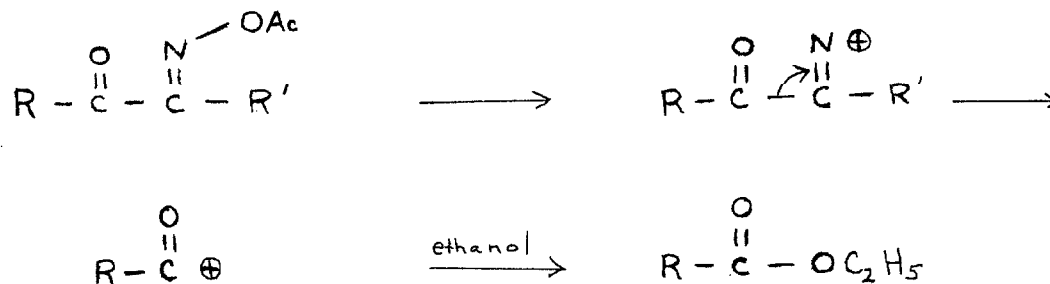
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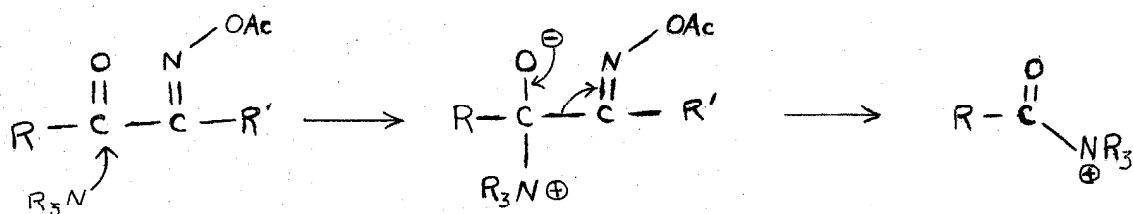
## PROPOSITION III

The Mechanism of the Beckmann Fission of  $\alpha$ -Keto Oximes

In trying to determine the mechanism of the Beckmann fission reaction, Ferris (1) catalyzed the cleavage of  $\alpha$ -keto oxime acetates in ethanol with amines and sodium acetate, carbonate, and cyanide, and found that when the amine was present in large amounts, only a very small amount of amide was formed in relation to the ethyl ester. He concluded that the reaction is initiated by the leaving of the acetate from the nitrogen, followed by electron pair migration and fission of the carbon-carbon bond, leaving the oxocarbonium ion  $\text{RCO}^+$ , which he stated attacks the solvent (ethanol) to give the ester.

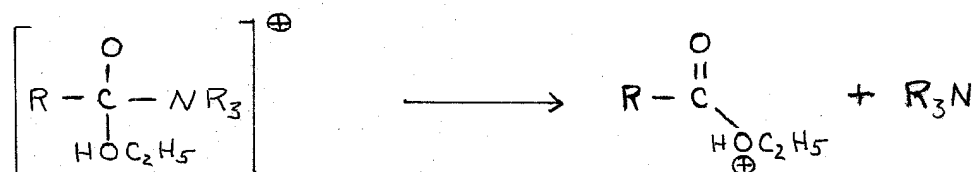
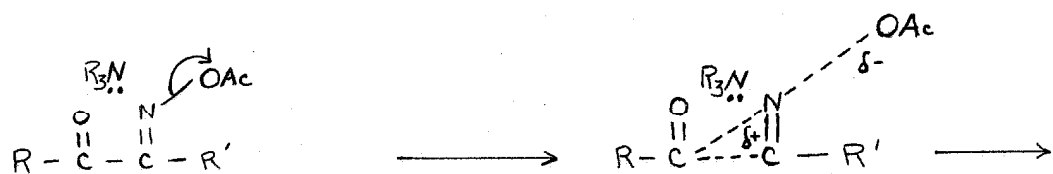


He rejected the other possible mechanisms, attack of nucleophile on the carbonyl, followed by bond cleavage and leaving of the acetate, and a concerted attack on the carbonyl and leaving of the acetate, because he was not able to isolate much of the amide.



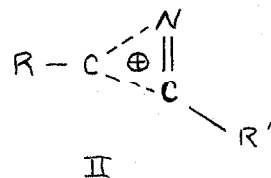
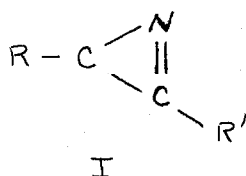
Two features of this work require clarification. What is the nature of the catalysis? Is an amide stable in these conditions?

Seemingly the function of the nucleophile (amine or weak basic salt) in catalyzing the reaction in the first mechanism considered is to assist in the ionization, that is to help stabilize the electron deficient transition state formed as the acetate leaves the nitrogen. If an acylium ion is then formed, it would react with a nearby amine molecule rather than a solvent molecule perhaps somewhat farther away. Now the conjugate acid of the amide just formed would be attacked by the solvent to give the ethyl ester. Presumably the excess of alcohol drives the equilibrium to the right.

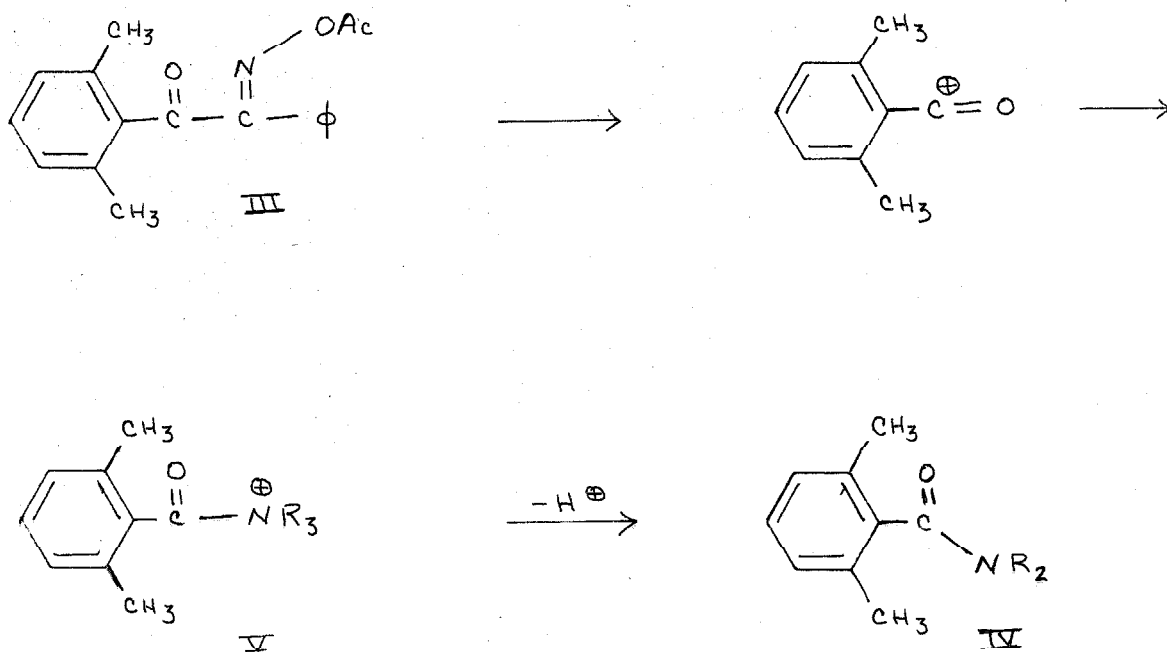


Since the other mechanistic possibility, that the amine or acetate attacks the carbonyl to initiate the reaction, would give the same N-acylammonium ion immediate, this would also be expected to undergo ethanolysis in the same manner. Hence the conclusion advanced by Ferris is unfounded.

The related Neber rearrangement of oxime tosylates has been shown to involve an azirine ring intermediate (I) (2). The formation of this three membered ring bears a significant relationship to the Beckmann rearrangement, in which the existence of the bridged intermediate (II) is postulated (3).



If a syn-phenyl 2,6-dimethylbenzoyl ketoxime acetate (III) were treated with ethanol and a primary or secondary amine, the amide (IV) should be isolated in good yield if the mechanism does involve the acylium ion, since N-(2,6-dimethylbenzoyl)-ammonium ion (V) is prevented sterically from being attacked by the ethanol. If the cleavage must be initiated by the attack of a nucleophile on the keto group, then the Beckmann cleavage of the acetate III will not occur, since the nucleophile is prevented from attacking the carbonyl.



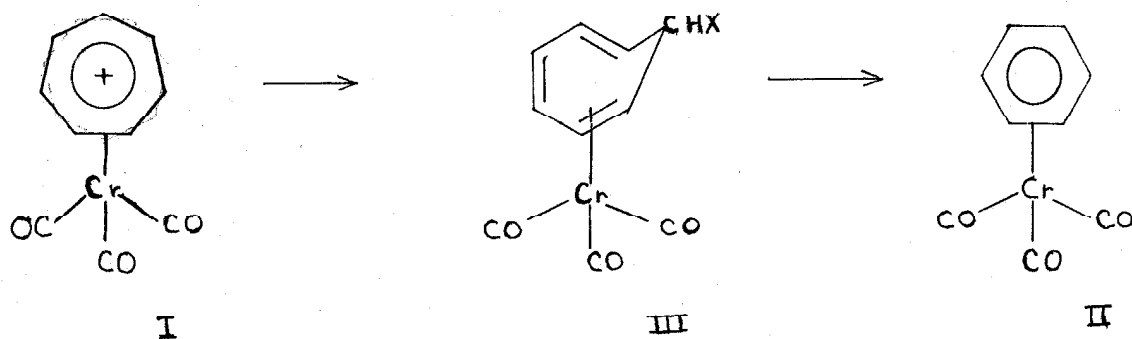
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## PROPOSITION IV

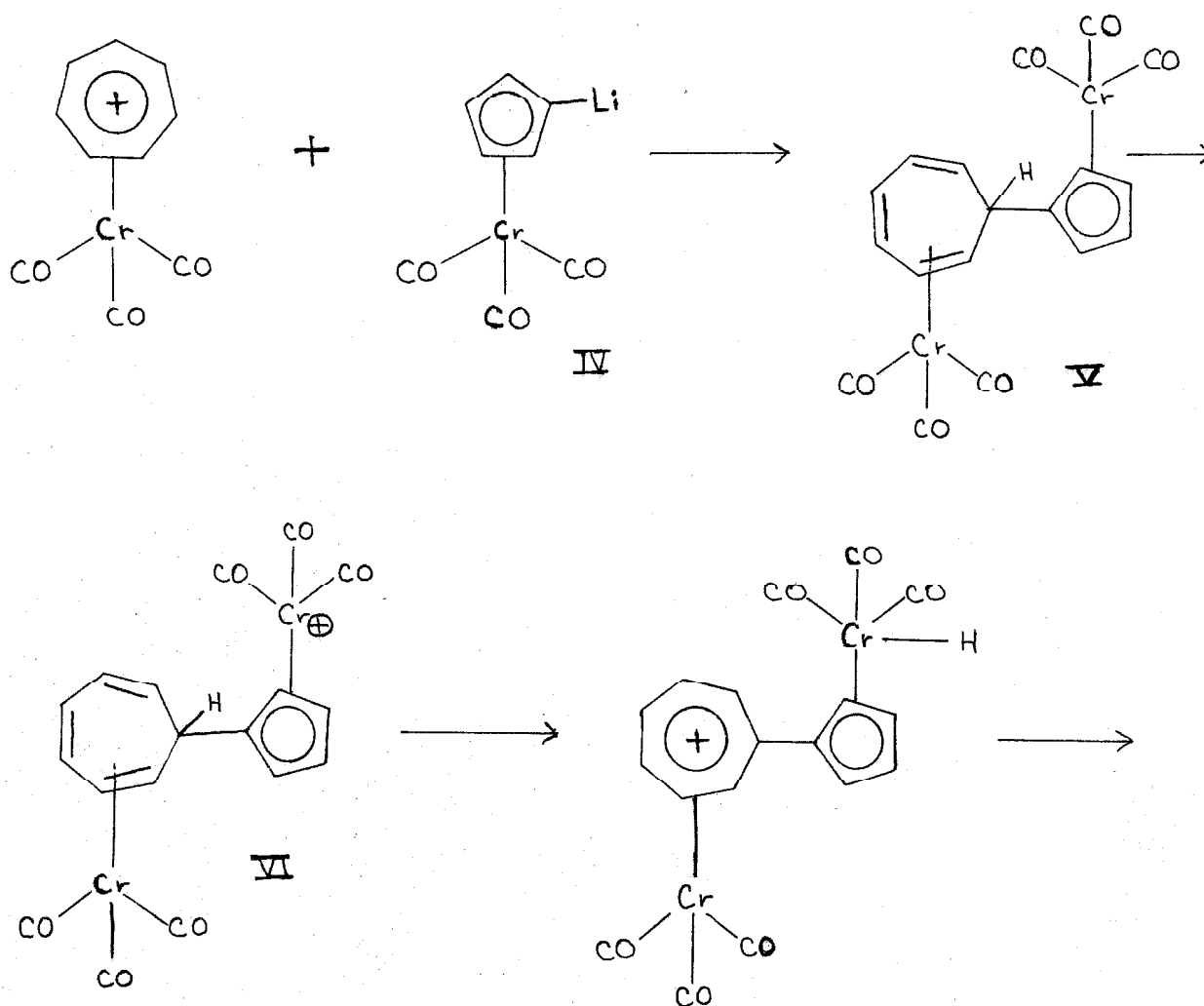
## A Synthesis of Sesquifulvalene

When cyclopentadienide was reacted with tricarbonyltropylium-chromium (I), an unexpected product, tricarbonylbenzenechromium (II), was isolated in 50% yield (1). The mechanism for this reaction is unknown, although the intermediate III is believed to have formed, since *t*-butylcyclopentadienyl lithium gives such a product ( $X=C_5H_5Bu^t$ ), which they presumed was stabilized by steric hindrance of some sort. That the benzene chromium complex product arises from the tropylium complex by ring contraction was established.

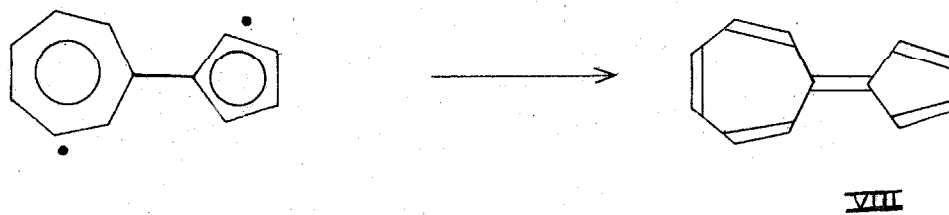


Since other bases (2) lead to the cycloheptatriene complex (III) ( $X=CN$ , OMe, SH, H), the ring contraction may be attributed to the extreme reactivity of the cyclopentadienyl system. In fact, the tropylium iron complex gives an ethylazulene complex when treated with cyclopentadienyl anion.

It is proposed that for the synthesis of sesquifulvalene, this reaction be modified by attacking the tropylium complex with the lithium derivative of a  $\pi$ -cyclopentadienylchromium complex (IV) rather than cyclopentadienyl lithium. The resulting compound (V) may be stable to ring contraction, since the  $\pi$ -cyclopentadienyl complex protects the cyclopentadienyl ring. Oxidation of the product with air might give the complex (VI), which would yield the tropylium complex (VII) by hydride migration. This tropylium complex would be expected to react with triphenylphosphine (1) giving sesquifulvalene (VIII)!







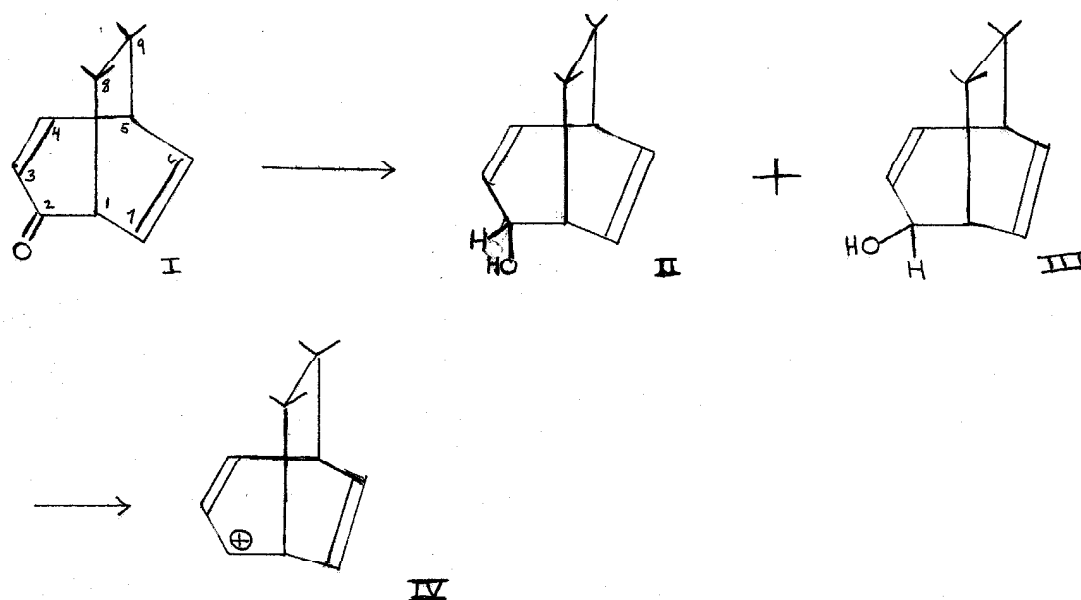
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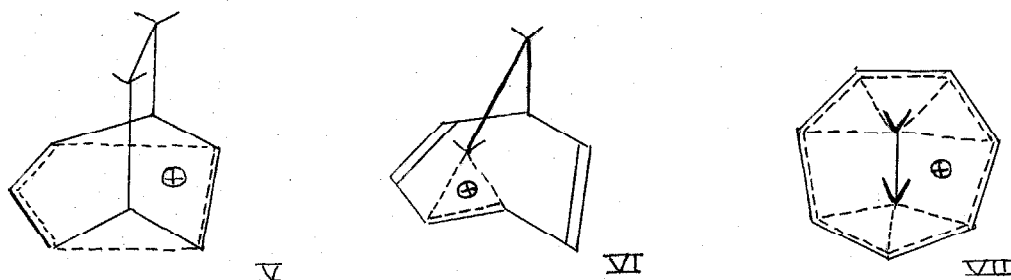
## PROPOSITION V

## Bicyclo[3.2.2]nonadiene Carbonium Ions

Diels-Alder adducts of tropone (I) have been prepared (1). It is proposed that one of these be reduced to the carbinol and anchimeric stabilization of the carbonium ion (IV) be studied.



In addition to the allylic stabilization, one might expect stabilization by the 6, 7 double bond (V) as observed in the norbornene carbonium ions (2). Also stabilization involving C-8 and C-9 as in norbornane carbonium ions (3) seems feasible. This would involve rehybridization at C-1 and C-5, and the non-classical intermediates such as VI might be written. A combination of these two effects leads to a "bishomotropylium" ion (VII).



One of the ways of studying this ion would be by analysis of a nuclear magnetic resonance spectrum of a salt of this ion or a sulfuric acid solution of the ketone (I) or carbinol. This should distinguish VII from V and VI, since all the tropylium ring carbons in VII share the positive charge, and hence the tropylium ring hydrogens are nearly identical.

The bishomotropylium ion would not necessarily anchimerically assist solvolysis of an ester of II or III, but might only form after the allylic carbonium ion formed. Conceivably anchimeric assistance to solvolysis of an endo isomer II might result from structure VI and the exo isomer III from structure V.

If either the bishomotropylium ion or structure VI is involved, labelling should be distributed equally among the seven tropylium ring positions. Since  $C^{14}$  labelling would require extensive degradation, deuterium labelling and nuclear magnetic resonance analysis of the product isolated from the solvolysis would be more feasible.

The bishomotropylium ion might dissociate to tropylium ion plus dienophile. If this happened rapidly at a low temperature, then of course the bishomotropylium ion could not be observed directly by nuclear magnetic resonance spectroscopy.

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