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ISOLATION AND REACTIONS OF 22-DIHYDROSTIGMASTEROL

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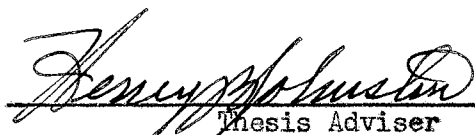
ISOLATION AND REACTIONS OF 22-DIHYDROSTIGMASTEROL

ELDRED T. SMITH

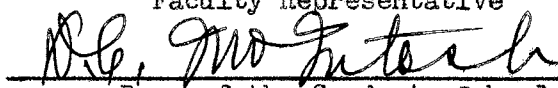
MASTER OF SCIENCE

1950

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ISOLATION AND REACTIONS OF 22-DIHYDROSTIGMASTEROL

By

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## I N T R O D U C T I O N

When plant sterols were proved structurally similar to sex hormones, Vitamin D<sub>3</sub>, and bile salts, their importance as a source of raw material was recognized. Tall oil pitch, commercially available as a by-product of the Kraft Process in the paper industry, was selected as a probable source of such a sterol. Preliminary observations indicated that the sterol content was sufficient to warrant further investigations; therefore, the present work was undertaken to establish methods of isolation and purification along with some possible uses. The sterol isolated was identified as 22-dihydrostigmasterol along with negligible amounts of other sterols. The prepared derivatives were then used in oxidative reactions which might eventually lead to sex hormones, Vitamin D<sub>3</sub>, or other physiologically active compounds.

## H I S T O R I C A L

Sterols (8) may be defined as the saturated or unsaturated alcohols derived from cyclopentanoperhydrophenanthrene. By this definition, some of the sex hormones and certain adrenal substances are sterols. In nature the sterols are widely distributed, both free and combined as the esters or glycosides. Esters are common to both plant and animal life, but the glycosides are found only in plants. On basis of occurrence, the sterols are classified as zoosterols (animal), phytosterols (plant), and mycosterols (yeast).

The sterols are usually isolated in the form of well crystallized, waxy-feeling compounds from the unsaponifiable portion of fat extracts. Since there are sometimes several sterols present in any natural product, separation is often difficult. The occurrence of mixed crystals and of molecular compounds makes the purification of the free alcohol by crystallization impracticable. In this case, advantage is taken of the differential solubilities of derivatives such as the dibromides of unsaturated sterols and the digitonides.

Because of the difficulty of obtaining pure compounds, many of the analyses of the older literature are erroneous. Esters, rather than the free sterol, are often used in the various analyses in order to better interpret the results.

The investigation of sterols, including many oxidative degradation products, has been well discussed by Windaus and Wieland (24). In 1932, Rosenheim and King (15) brought attention to material that established the cycloperhydrophenanthrene nucleus. This structure for the sterols, along

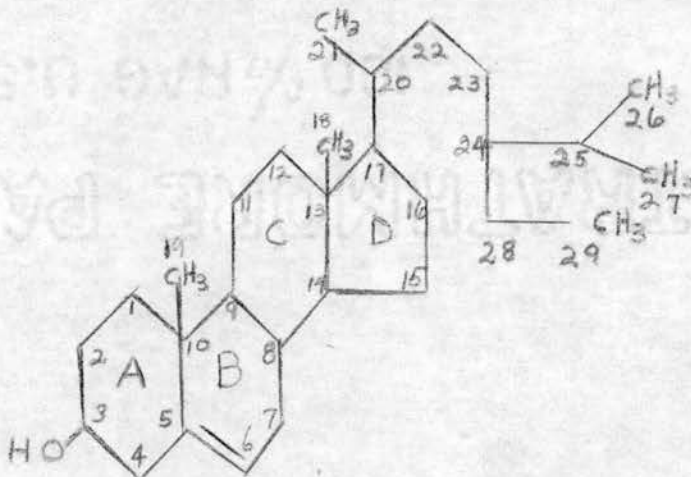
with bile acids, hormones and related compounds, was immediately compatible with the vast amount of experimental material which had been accumulated. Finally, in 1939-1940, Bachman (9) and co-workers carried out a total synthesis of one of the sex hormones. Thus, both by degradation and synthesis, the structure of the nucleus has been established.

The position of the hydroxyl group at C<sub>3</sub> was soon established based on the experimental evidence furnished by several workers. The point of attachment, C<sub>17</sub>, of the principal side chain to the nucleus was suggested from x-ray diffraction and surface film measurements (2), and confirmed by formation of methylcholanthrene from 12-ketocholanic acid. After satisfying the carbon and hydrogen requirements of the nucleus and the side chain, two carbon and six hydrogen atoms remained. After much work on degradation by-products (21), the two methyl groups were proved to be attached at C<sub>10</sub> and C<sub>13</sub>.

22-dihydrostigmasterol, also known as  $\beta$ -sitosterol, is the principal sterol found in cottonseed oil (19) and Calycanthus oil (4). It has been isolated from tall oil (17), wheat germ oil (1), and crepe rubber (10). Many other plants contain this same sterol. 22-dihydrostigmasterol is a monounsaturated alcohol whose empirical formula is C<sub>29</sub>H<sub>50</sub>O (23). It has been obtained from stigmasterol by selective hydrogenation of the double bond in the side chain (3). Dirscherl and Nahn (6) succeeded in isolating (+)-6-methyl-5-ethyl-2-heptanone from the chromic acid oxidation of  $\beta$ -sitosterol. They claimed that this compound could have come only from the side chain attached at C<sub>17</sub>, thus proving the side chain to be a ten carbon radical,  $-\text{CH}(\text{CH}_3)\text{CH}_2\text{CH}_2\text{CH}(\text{C}_2\text{H}_5)\text{CH}(\text{CH}_3)_2$ , and establishing  $\beta$ -sitosterol and 22-dihydrostigmasterol as identical compounds. Unsaturation



occurs in the 5,6 position. Structurally, the following representation is possible:

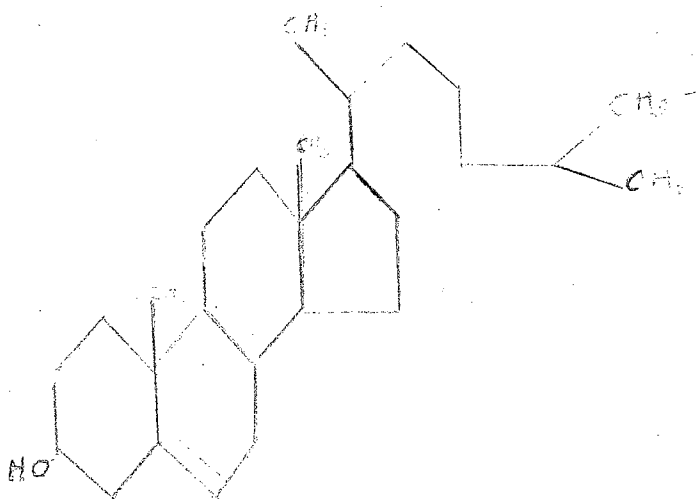


22-dihydrostigmaterol

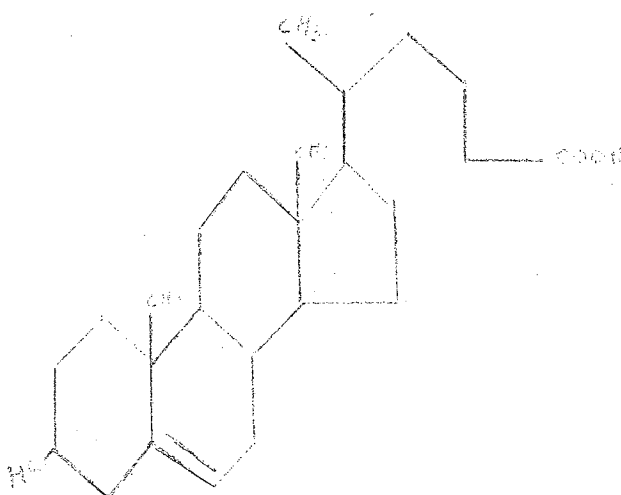
This planar representation fails to show the possibilities of isomerization exhibited by sterols and related compounds. The stereochemical arrangement of the hydroxyl group at  $C_3$  with respect to the  $C_5$  hydrogen atom was inferred by Ruzicka (16) while working with cholestanol. Windaus (22) recognized the possible differences in the juncture between rings A and B which is analogous to cis and trans decalin. Similar possibilities exist between rings B,C and C,D. To carry the idea of isomerism a step further, the methyl groups at  $C_{10}$  and  $C_{13}$  can assume varying configurations in many compounds. The  $C_{17}$  side chain also has been the subject of speculation as to its possible configuration when positions of the  $C_{20}$  methyl group and the  $C_{24}$  substituent are in question. In the field of sex hormones, stereochemical relationships are important and have been investigated extensively in connection with possible effect on biological activity.

conclusions from these studies have been extended to related fields, and similar methods employed to establish the importance of spatial isomerism.

Chemical investigation initiated over a century ago on each of two series of physiologically important substances, the sterols and bile acids, eventually met on common ground. Correlation has now been established between the sterols and bile acids after years of arduous research during which information necessary to establish the structure of key compounds in both series was presented. By 1934 the following structures were accepted:

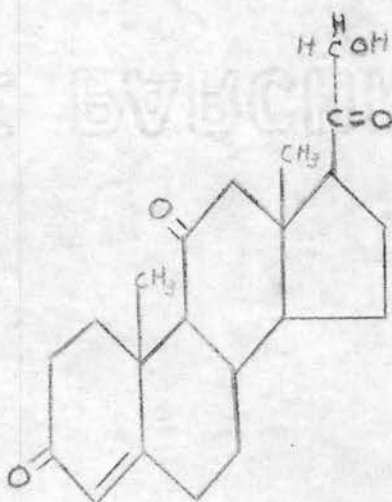


Cholesterol

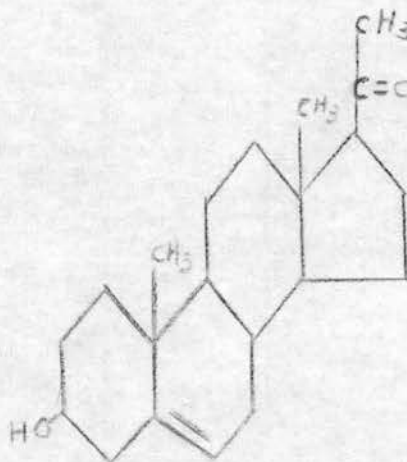


Cholic Acid

Sex hormones are related to the sterols and bile acids. They possess physiological activity useful for remedial treatment in many types of diseases. Cortisone and pregnenolone (13) are two of the synthetic hormones that have attained importance recently.



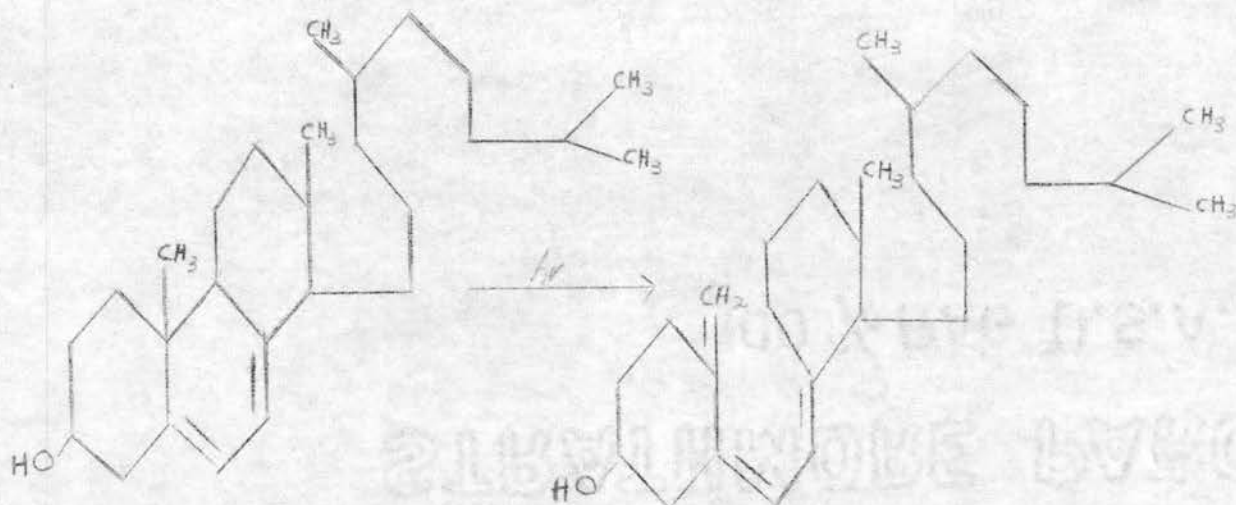
Cortisone



Pregnenolone

Extensive use of synthetic cortisone is limited by the exorbitant production costs. The first production price was estimated at \$70,000 per pound. As a result of improved production methods, cortisone is currently available to hospitals approved by the American Medical Association at \$95 per gram (5). Pregnenolone is much cheaper to produce and seems equally effective for many purposes.

Vitamin D<sub>3</sub> is also related to the sterol family. Upon exposure to ultraviolet light, 7-dehydrocholesterol is converted to Vitamin D<sub>3</sub>.



7-dehydrocholesterol

Vitamin D<sub>3</sub>

These serve as examples illustrating the importance of sterols as possible raw materials in synthetic production of related compounds commercially in demand.

Tall oil pitch was chosen as a possible source of 22-dihydrostigmasterol. It has been known for some time that tall oil, a by-product of the Kraft Process in the paper industry, contained from 5 to 7 per cent of unsaponifiable materials. In 1931 Sandquist and Bengtsson (17) isolated  $\beta$ -sitosterol from the unsaponified portion.  $\beta$ -sitosterol was later proved structurally identical with 22-dihydrostigmasterol (6). Tallene (11), West Virginia Pulp and Paper Company's trade name for the residue remaining in the stillpot after the initial purification of tall oil by distillation, contains a high percentage of unsaponifiable material. Crude analysis of Tallene indicates an unsaponifiable content of 22 to 32 per cent. Purification of tall oil, and separation (20) into its main components, has been the subject of much investigation. The most generally accepted method for partial

separation and recovery of the sterol portion is that of alkali saponification combined with solvent extraction.

## E X P E R I M E N T A L

## ISOLATION OF 22-DIHYDROSTIGMASTEROL

Tallene, residue from initial purification of tall oil by distillation, was used as a possible source of 22-dihydrostigmasterol. Crude analysis showed 25 to 32 per cent unsaponifiable content. A modification (12) of the usual saponification-extraction method was used for isolation of the sterol fraction.

Procedure: To a solution of 15.4 grams of potassium hydroxide in 113 ml. of methanol, 100 grams of Tallene were added. An additional 50 ml. of methanol was used to wash down any Tallene adhering to the reaction flask which was a one liter round bottom flask fitted with a reflux condenser. The mixture was boiled under gentle reflux for one hour, cooled and diluted with 257 ml. of distilled water. Ethyl ether was used as the extracting solvent. The resulting ethereal solution was evaporated to approximately one-third of the original volume and cooled in an ice bath whereupon plate-like crystals precipitated. The crystals were removed by suction filtration. A solvent recovery apparatus was used in the ether evaporation. Gentle suction was necessary to prevent matting of the funnel by the plate-like crystals. The resulting platelets, contaminated with an oily, yellow material, were purified by recrystallization from hot ether. Four to five recrystallizations were necessary to produce white needle-like crystals melting at  $135-136.5^{\circ}$  which is in excellent agreement with the literature value,  $135.5-136^{\circ}$  (7). Five grams of pure sterol were recovered. Another five grams were estimated to be in the filter paper and mother liquor. Extensive solvent extractions gave a slightly higher recovery of sterol.

## REACTIONS

Reaction of 22-dihydrostigmasterol with sodium hypochlorite: The reaction vessel was a 250 ml. three-necked round bottom flask fitted with mechanical stirrer, gas inlet and gas outlet tubes. One gram 22-dihydrostigmasterol was suspended in 20 ml. 6N aqueous sodium hydroxide. Chlorine gas from a cylinder was passed into the solution at regular intervals. Extreme caution was exercised to ascertain that the solution remained basic to litmus at all times. This procedure was continued for 2½ hours. The mixture was then boiled on a steam bath for two hours with pH of 9 being maintained. By this time a major portion of the solid sterol had disappeared. The reaction mixture was cooled, the solids removed by filtration and the basic filtrate extracted with ether. No solid or liquid materials were obtained after evaporation of the ether. The basic solution was acidified with sulfuric acid and liberated a yellowish gas which was assumed to be a mixture to chlorine and hydrogen chloride. After standing overnight, the solution was extracted with ether. A pungent, volatile liquid remained after evaporation of the ether. It gave a positive Beilstein halogen test. The acid solution was then evaporated to dryness and extracted with acetone (C.P.). A brownish discoloration developed in the solution as the evaporation of the acetone progressed. Finally, a black precipitate was left in the container. No ketonic compounds were identifiable from this residue.

Preparation of 22-dihydrostigmasteryl acetate: 25 grams of 22-dihydrostigmasterol were placed in a one liter round bottom flask fitted with a reflux condenser. Just enough hot glacial acetic acid was used to dissolve the sterol. Nine grams of acetic anhydride were added and the mixture gently refluxed for one hour. White crystals formed on cooling. These were removed by filtration and washed with cold methanol. The observed melting point was 118-119° as compared with the literature value of 118.5-119.5 (7) Recrystallization from methanol did not change the melting point.

Nitric acid oxidation of 22-dihydrostigmasteryl acetate: One gram of 22-dihydrostigmasteryl acetate and five ml. of clear concentrated nitric acid were placed in an eight inch test tube. After standing for 36 hours in the absence of light, two layers appeared. The upper layer was a gummy, yellow solid which became very brittle when washed with distilled water. This solid was difficultly soluble in sodium hydroxide. A yellow precipitate formed when the basic solution was acidified. This precipitate, collected by filtration, gave a melting point of 60-71°. The lower layer, a clear yellow liquid, was made basic with sodium hydroxide and extracted with ether. The ether layer remained clear, extracting none of the yellow color from the basic solution. No solid or liquid material remained after the ether was evaporated. The clear yellow basic layer was then made acid with sulfuric acid and extracted with ether. The yellow color moved into the ether layer. Evaporation of the ether left a yellow amorphous powder.

Preparation of 5,6-dibromo-22-dihydrostigmasteryl acetate: 10.5 grams of 22-dihydrostigmasteryl acetate were placed in a one liter round bottom



flask and glacial acetic acid added until solution was effected at room temperature. Five grams of liquid bromine were added dropwise with vigorous shaking. The mixture was then cooled in an ice bath. A yellow crystalline precipitate formed and was removed by filtration. On drying, a tan colored solid resulted which was decolorized with charcoal. The resulting white crystals melted at 112-114°. There was a pronounced shrinkage at 105°.

Chromic acid oxidation of 5,6-dibromo-22-dihydrostigmasteryl acetate:

Run I (14). One gram of 5,6-dibromo-22-dihydrostigmasteryl acetate was dissolved in 10 ml. of boiling glacial acetic acid and treated dropwise over a period of six hours with a solution of three grams of chromium trioxide in 10 ml. 50% acetic acid. As the reaction progressed an oily liquid formed on top of the reaction mixture. Upon cooling, this liquid solidified into white granular crystals with a yellow tint. Decolorizing charcoal was used to remove the contaminating material. The white crystalline solid obtained on purification melted from 74-76°. The yield was so low that further reactions were carried out in order that enough compound might be obtained for proper identification. Several recovery methods were tried assuming that a high molecular weight ketone was present in the reaction mixture. Only small amounts of very impure compound were recovered, due either to the low yield of the ketonic compound or shortcomings of the methods used.

Run II. Using a one liter three-necked round bottom flask fitted with reflux condenser and dropping funnel as the reaction vessel, 25 grams of 5,6-dibromo-22-dihydrostigmasteryl acetate were dissolved in 200 ml. of boiling glacial acetic acid and treated dropwise with a solution of 75 grams of chromium trioxide in 230 ml. of 50% acetic acid. After cooling, the mixture was

diluted with distilled water and a gummy precipitate settled to the bottom of the reaction flask. Thirty ml. of 12N sulfuric acid were added and the entire mixture extracted with benzene. A yellowish color was imparted to the benzene layer, but disappeared when washed with 6N sodium hydroxide. No solid or liquid materials were left after evaporating the clear benzene layer.

Run III. Using a 250 ml. round bottom flask, fitted as in Run II, one gram of 5,6-dibromo-22-dihydrostigmasteryl acetate was dissolved in 10 ml. of boiling glacial acetic acid and treated with three grams of chromium trioxide in 10 ml. of 50% acetic acid. The resulting mixture was made slightly basic with sodium hydroxide and extracted with ether. A yellow color moved into the ether solution and upon evaporation a yellowish impure solid was recovered in small quantity. The use of decolorizing charcoal did not change the color of this compound.

Run IV. One gram of 5,6-dibromo-22-dihydrostigmasteryl acetate, using reaction vessel as described in previous runs, was dissolved in 10 ml. of boiling glacial acetic acid and treated dropwise with a solution of three grams of chromium trioxide in 10 ml. of 50% acetic acid. The solution was then made basic with sodium hydroxide and evaporated to a thick, viscous liquid. This liquid was dissolved in ethyl alcohol and two grams of semicarbazide hydrochloride were added. By means of a water bath, the temperature was maintained at 70° for two hours. The precipitate that formed was removed by filtration, washed with ether and then boiled in water. The resulting mixture was filtered, and the precipitate warmed with hydrochloric and acetic acids. The mixture was cooled and extracted with ether. The ether layer was washed with sodium hydroxide and evaporated. A minute quantity of off-white crystals was recovered.

Run V. One gram of 5,6-dibromo-22-dihydrostigmasteryl acetate, using apparatus as in previous run, was dissolved in sufficient glacial acetic acid to effect solution at 70°. This temperature was maintained throughout the reaction. Three grams of chromium trioxide in 10 ml. of 50% acetic acid were added dropwise. No oily layer appeared, and the recovery methods used in Runs II and III netted only a few impure yellow crystals.

Reaction of 5,6-dibromo-22-dihydrostigmasteryl acetate in liquid

ammonia: 1.8 grams 5,6-dibromo-22-dihydrostigmasteryl acetate were placed in liquid ammonia and stirred at intervals. The ammonia soon boiled away at room temperature leaving a dry mass of crystals. Apparently, no reaction had taken place since the melting point was the same as the starting material, nitrogen was not present in a sodium fusion test, and the compound gave a negative result in the diazo test for primary amines.

Reaction of 5,6-dibromo-22-dihydrostigmasteryl acetate with a mixture

of ammonium carbonate and ammonium chloride: Four grams ammonium carbonate, one gram ammonium chloride and one gram of the ester were fused in a porcelain evaporating dish. The lowest possible fusion temperature was used. The resulting black, gummy mass was washed with water. This wash water showed positive results for water soluble bromides. The classical test was used. The black mass was then extracted with 10 ml. of hot concentrated hydrochloric acid. After cooling, the solution was made basic with sodium hydroxide. Needle-like crystals formed, but in such small amounts that identification could not be attempted.

Reaction of 5,6-dibromo-22-dihydrostigmasteryl acetate with sodium in liquid ammonia: Ammonolysis reaction mixture was used, and one gram metallic sodium was dissolved in the liquid ammonia. A blue color appeared and after the ammonia had evaporated, the reaction mixture showed positive test for water soluble bromide. If sodium amide were used in liquid ammonia, it might be possible to replace the bromine atoms with an amino group.

## DISCUSSION

This work has established the fact that the still-pot residues from the distillation of tall oil contain a significant amount of 22-dihydrostigmasterol. Tallene, a commercial still-pot residue, contains 22-32 per cent of an unsaponifiable material. The major portion of the unsaponified material in Tallene is 22-dihydrostigmasterol, and moderate extraction gives a 25-50 per cent recovery. No appreciable amount of interfering sterols was found, nor were isomeric configurations of the 22-dihydrostigmasterol isolated, probably because the vigorous distillation process would have altered the configuration of the isomeric compounds. The only evidence of a contaminant was a persistent yellow color from small amounts of an unknown compound. This compound existed in such small quantities that it was not isolable.

Tallene, as a source of 22-dihydrostigmasterol has a market value of about \$40 per ton and is considered by-product waste. On the basis of extractive methods developed here, the cost of the pure sterol from tall oil pitch is \$0.25 per pound. This compares favorably, in a competitive way, with other sources from which some sterols are produced at a cost of \$2.50 per pound.

The first oxidation run, using chromic acid, gave a ketonic material in about 15% yield. It has a melting point in the range predicted by comparison with dibromo derivatives of other compounds having a phenanthrene nucleus and a 17-keto group. The amount of material isolated did not permit rigid identification. However, the results of this first run could not be duplicated. Some previous oxidations of sterols by chromic acid have shown yields of 3 per cent. The problem in Run I is to determine

the vital, but obscure, step necessary to produce the high yields.

Replacement of the bromine atom by an amino group in the typical ammonolysis reactions failed to produce appreciable amounts of the amine.

Oxidation by nitric acid and by sodium hypochlorite produced acidic materials which were not desired. It was hoped that such oxidizing agents might give reactions on the side chain. However, this did not prove to be true.

In summary, it is believed that the following investigations would be profitable: (a) search for the key conditions which control the high yield of the ketone secured in Run I of the chromic acid oxidation reaction; (b) methods of substituting an amino group on the nucleus; to date, such derivatives have not been reported; (c) better methods of isolating and characterizing the ketonic compound; and (d) possible methods of entrance into the side chain in order to avoid chromic acid oxidation as means of total degradation of the side chain.

## S U M M A R Y

This work is concerned chiefly with the isolation and reactions of 22-dihydrostigmasterol. Tall oil pitch was selected as the source of this sterol, and isolation was accomplished by using a modification of the usual alkali saponification-solvent extraction method. From the chromic acid oxidation of 5,6-dibromo-22-dihydrostigmasteryl acetate small amounts of a ketonic material were isolated. A compound soluble in concentrated hydrochloric acid was isolated from the ammonolysis reaction. In both instances, the small amounts of material isolated did not permit rigorous identification.

Reagents used in the oxidation reactions were: (a) cold, clear, concentrated nitric acid in absence of light; (b) sodium hypochlorite; (c) chromic acid in boiling glacial acetic acid, and (d) chromic acid in glacial acetic acid at 70°.

Methods of isolating the ketonic material from the oxidation reactions were: (a) separation by filtration of the material crystallizing in the reaction flask; (b) acidifying the reaction mixture with 12N aqueous sufluric acid, extracting with benzene, then washing the benzene layer with 6N sodium hydroxide and evaporating; (c) making the mixture basic and extracting all organic materials with ether, and (d) isolating as the semicarbazone and regenerating the ketone.

The methods of ammonolysis tried on the dibromo derivative of 22-dihydrostigmasteryl acetate were: (a) reaction in liquid ammonia, and (b) fusion with ammonium carbonate and ammonium chloride.

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## B I O G R A P H Y

Eldred Troy Smith was born in Tioga, Louisiana on May 20, 1923. He attended Louisiana public grade schools and was graduated from Tioga High School in June, 1941. In the fall of 1941, he entered Louisiana College at Pineville, Louisiana, and attended school there until February, 1943, at which time he enlisted in the United States Army Air Corps. He remained on active duty until March, 1946, when he received his honorable discharge. In June, 1946, he re-entered Louisiana College, and received his Bachelor of Science degree in May, 1948. On July 31, 1948, he married Shirley Leona Botzong of Tioga, Louisiana. In September, 1948, he entered the graduate school at Oklahoma Agricultural and Mechanical College.

During his advanced study at Oklahoma Agricultural and Mechanical College, he served as a graduate fellow in the Chemistry Department.

Typed by Mrs. J. O. Richardson