CONSTITUENTS OF KOUSSO FLOWERS

by

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Thesis presented for the Degree of Ph.D.,

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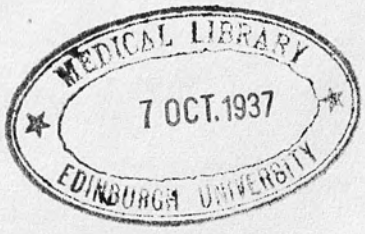


April 1937.

Contents

INTRODUCTION

An extract of the flowers of the Kousso tree has been used in Abyssinia for several centuries as a specific against tapeworm. This tree (Hagenia Abyssinica Willd. or Brayera Anthelminthica Kunth) grows to a height of about 60 feet and is found over the entire table-land of Abyssinia between 3,000 and 8,000 feet above sea level. It belongs to the family Rosaceae, and the flowers, from which the active anthelmintic extract is prepared, grow in broad panicles of 10-12 inches in length. The stalk of the panicle from which the flowers branch is clothed with hairs and dotted with small glands.

For the preparation of pharmacologically active extracts, the whole panicle is dried and extracted, although it is known that in the case of Rottlera Tinctoria, which is also used as an anthelmintic, the active principle is concentrated in the hairs and glands. No information is, however, available as to the distribution of the active principle in Kousso flowers. Although their medicinal properties/

properties had been known for two centuries in the East, Kousso flowers were first introduced into Europe by a Frenchman about 1850. In 1864 an official preparation of Kousso was included in the British Pharmacopoeia.

Chemical investigation of Kousso flowers was first undertaken by Wittstein (1) who, by extraction with various solvents, found the usual constituents, chlorophyll, fat, wax, and also a bitter substance which however he failed to isolate.

No further advance was made until the appearance of the work of Pavesi (2) and Vee (3). These authors, working independently of one another, but using approximately the same methods, announced the isolation of a substance which they called kussin. They ground the dried flowers with an alcoholic solution of lime, distilled off the alcohol and acidified with acetic acid. The resultant precipitate was filtered off, dissolved in alcohol and reprecipitated by water. This method of preparation is similar to that used by Calloud (4) in the isolation of santonin from Artemisia maritima.

In 1859 a communication by Bedall (5) was published/

published in which the preparation of kussin according to Pavesi and Vée was confirmed. Bedall gave the melting point of kussin as 193-5°, and its formula as $C_{26}H_{22}O_6$.

The firm of Merck, Darmstadt, in 1870, placed on the market a crystalline substance which they called kosin. The method of preparing this drug is not accurately known but some alkaline extraction medium was almost certainly used. The substance, which was examined by Fluckiger (6) forms short yellow needles of m.p. 142°. To it he assigned the formula $C_{31}H_{38}O_{10}$.

Until 1894 it was considered that kosin was the active principle of the flowers although no knowledge as to its chemical constitution had been gained. In that year, however, Leichsenring (7) published the results of an investigation which indicated that kosin was a breakdown product of the naturally occurring active principle, produced during the extraction of the plant by alkalies.

He exhausted the dried ground flowers with ether and evaporated the ethereal extract to small bulk. The thick green residue was then extracted with/

with light petrol ether and the solution decanted from the insoluble residue and evaporated to dryness. A hot alcoholic solution of the residue, on cooling, deposited a waxy substance which was filtered off, the filtrate being then evaporated to dryness. The residue was taken up in ether, extracted with dilute Na_2CO_3 solution, and the alkaline solution acidified and re-extracted with ether. After distillation of the solvent the residue was re-crystallised several times from alcohol. From the alcohol a substance, which Leichsenring called protokosin, crystallised in white glistening needles of m.p. 176°, to which he attributed a molecular formula $C_{29}H_{38}O_9$.

Leichsenring pointed out the similarity of protokosin to kosin, from which however it differed in colour and melting point. Both substances give a brown red colour on adding $FeCl_3$ to an alcoholic solution, and both, on warming with concentrated sulphuric acid give a cherry red colour and a smell of isobutyric acid.

From the alcoholic mother liquors by evaporation, solution in dilute sodium carbonate, and precipitation with acetic acid, an amorphous substance/

stance, m.p. 80° was obtained which Leichsenring called kosotoxin.

He showed that injections of sodium carbonate solutions of protokosin into the lymph sac of frogs were not poisonous, whereas similar experiments with kosotoxin rapidly produced paralysis and death. Only a small amount of protokosin was obtained and no chemical investigation of this substance is reported. A large amount of kosotoxin was isolated, however, and it was shown that by treatment with baryta this material yielded Merck kosin. By warming with concentrated sulphuric acid, kosotoxin was shown to yield isobutyric acid which was identified as the silver salt. Leichsenring also analysed Merck kosin and found it to have the formula $C_{23}H_{30}O_7$.

Five years later, Kondakov and Schatz (8) repeated some of the previous investigations of Kousso flowers without, however, contributing anything to our knowledge of the chemistry of the drug. They found for kosin, prepared by different methods, analytical figures corresponding to $C_{22}H_{30}$ or $_{32}O_7$.

The first real advance in the chemistry of these/

these compounds was made by Lobeck (9) in 1901. By repeated recrystallisation of Merck kosin from methyl alcohol he isolated two substances which he called α and β-kosin.

α-Kosin formed long citron yellow needles from alcoholic solution of m.p. 160°, and β-kosin short prisms of m.p. 120°. By analysis he showed that these two kosins were isomeric, and his analytical figures agreed with the formula proposed by Leichsenring, namely $C_{23}H_{30}O_7$. He also showed that both α and β-kosin contained two methoxyl groups.

By heating α-kosin with strong alkali and with concentrated sulphuric acid, Lobeck obtained methyl phloroglucinol-monomethyl ether and also a volatile fatty acid which, however, he did not identify.

From Merck's crude Kousso extract, by treatment with milk of magnesia, he obtained α-kosin, protokosin in very small amount, a new substance which he called kosidin and also kosotoxin. The only one of these substances which he obtained in sufficient quantity to permit of chemical investigation was kosotoxin. From this by heating with alkali and zinc he isolated/

isolated α-kosin and trimethylphloroglucinol. Later, Lobeck also attempted to repeat Leichsenring's work without success, since he was unable to isolate any crystalline substance from an ethereal extract of the dried flowers.

The above summary surveys the work done on kousso flowers up to the present date, no further publications having appeared on the subject since 1901.

THEORETICAL

(1) Protokosin

A survey of the methods previously used for the extraction of kousso flowers indicates that, if possible, the use of alkaline extraction media is to be avoided. In particular it has been shown by Leichsenring (loc. cit.) that the naturally occurring principle of the flower is destroyed in 20 minutes by hot dilute baryta. Prolonged contact with aqueous or alcoholic solutions of lime followed by evaporation, which most of the older methods of extraction involved, is therefore strongly contra-indicated.

Preliminary experiments suggested that a much simpler and less drastic method of extraction would lead to the isolation of the active principle in a pure state. A substance possessing the recorded properties of protokosin may be obtained from an ethereal extract of the ground, dried flowers by purification with light petrol ether followed by recrystallisation from alcohol. This protokosin is/

is undoubtedly the substance isolated by Leichsenring using essentially the same method, and by Lobeck (though in very small yield) using a different method. The yield, however, is very much higher than that reported by any previous worker being 0.4% of the weight of the ground dried flowers.

A search was made for kosidin, which had been isolated by Lobeck, and which he asserted to be different from any of the other substances isolated by him, without success. The properties of kosidin as described by Lobeck, both physical and chemical, are remarkably similar to those of protokosin, and its preparation is almost identical with the method used by myself to isolate that substance. It would seem possible, then, that kosidin is simply somewhat impure protokosin. Kosotoxin has also been isolated in crystalline form, all previous preparations having been amorphous, but so far sufficient has not been obtained for detailed chemical investigation. Kosotoxin is very similar to protokosin in chemical properties; its alcoholic solution gives a reddish brown colour with $FeCl_3$, and on warming a fragment with concentrated sulphuric acid a cherry red colour is produced, and the smell of a volatile fatty acid may

be/

be detected. It differs from protokosin, however, in its melting point, which is 40° lower, and in its solubility in alcohol, in which it dissolves readily even in the cold.

As the result of several analyses and molecular weight determinations, the formula of protokosin is shown to be $C_{22}H_{28}O_7$, rather than that proposed by Leichsenring, namely $C_{29}H_{38}O_9$. It contains one methoxy group, and side chain methyl estimations, by the Kuhn-Roth method, indicate the presence of four $C\text{-}CH_3$ groups.

Previous workers had pointed out that the properties of kosin and protokosin were similar to those of the substances isolated from the anthelmintic Aspidium filix mas, which had been investigated by Boehm (10, 11, 12), and it is now found that certain analytical inconsistencies found in these compounds are paralleled in the Kousso group. The relationship between these two groups of compounds will be dealt with in more detail later. Here it is sufficient to point out that in spite of repeated determinations, satisfactory methoxyl values for protokosin could not be obtained and that a similar difficulty was experienced in the case if certain filix mas derivatives (cf. 11).

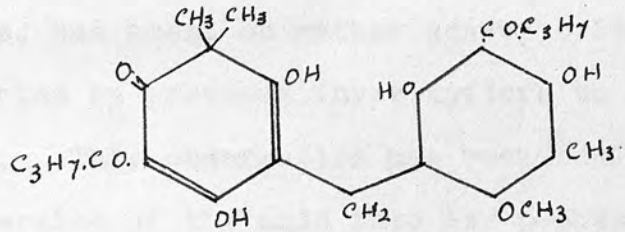
Although/

Although protokosin possesses phenolic properties, all attempts to prepare a crystalline derivative of the hydroxyl groups have been so far unsuccessful. Benzoyl chloride, p-nitro- and p-bromobenzoyl chloride, and acetic anhydride under various conditions have all failed to yield a derivative capable of being purified. By treatment with a large excess of acetic anhydride in cold pyridine, however, a crude acetyl derivative was obtained which on analysis indicated the presence of three acetyl groups. Estimations of active hydrogen by Zerewitinoff's method also show the presence of three hydroxyl groups, although as will be shown later, it is possible in this case that there is present a phenolic hydroxyl which does not react under the conditions of the estimation.

A considerable amount of evidence as to the chemical nature of protokosin is obtained by treatment with alkalies under various conditions. Following Leichsenring's (loc. cit.) method of degradation of kosotoxin by baryta, kosin was obtained from protokosin together with a volatile fatty acid. By fractionation of the crude kosin from methyl alcohol, two substances were obtained, one forming long slender yellow needles of melting point/

point 160°, and the other short yellow prisms of melting point 120°. These two substances possessed the chemical and physical properties described for α- and β-kosin respectively, by Lobeck. Messrs Merck of Darmstadt have kindly sent me a specimen of "Merck kosin" of melting point 142° which after re-crystallisation was shown to be identical with α-kosin by a mixed melting point, which showed no depression. Analyses and molecular weight determinations of the kosins showed that they were isomeric with one another and with protokosin, a point which had been missed by all previous investigators. The kosins differ from protokosin, however, in that each possesses an additional methoxy group, and that both of the kosins are optically inactive, whereas the mother substance is dextro-rotatory. The rather remarkable conversion of protokosin into α and β-kosin on treatment with alkali is paralleled by the behaviour of aspidin (I), a member of the filix mas group which under similar conditions yields an isomeric, yellow, alkali stable compound containing an/

an additional methoxyl group (12).

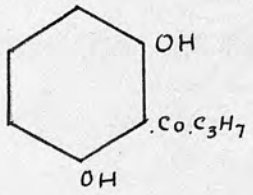


I

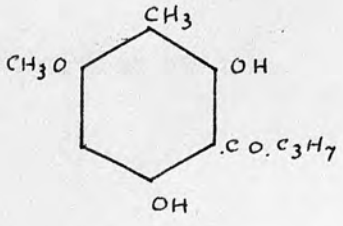
The mechanism of this reaction which apparently involves the migration of a methyl group from carbon to oxygen is, however, by no means clear. With the exception of the above mentioned instance, no similar intramolecular change is known. It was later found that the yield of kosin could be considerably improved by heating protokosin with 20% NaOH in the presence of zinc dust for a few minutes, rather than by the use of baryta. In this way about 35% of the protokosin used may be converted into the mixed kosins. Furthermore, it was found that in these experiments besides the fatty acid, phloroglucinols were produced. From the mixture of phloroglucinols, however, there could be separated in pure condition only one, which was identified as C-trimethyl phloroglucinol by comparison with a synthetic sample of that compound.

The fatty acid, which is invariably produced when/

when protokosin is warmed with strong alkalies or acids, had been, on rather scanty evidence, reported by previous investigators to be isobutyric acid. This observation has been confirmed by the conversion of the acid into its p-phenyl-phenacyl ester (13), and also into the p-toluidide. These derivatives were compared directly with specimens prepared from an authentic sample of isobutyric acid. In view of the deep seated changes attending the formation of this acid by hydrolytic reagents it seemed unlikely that the acid was originally present in ester combination in the molecule. Since, moreover, phloroglucinol derivatives form a large proportion of the protokosin molecule it is probable that the acid is formed by hydrolytic cleavage of a ketone such as II. Aspidinol (III) whose structure is known with certainty, (14, 15) undergoes this type of hydrolysis and it is assumed that a similar grouping is present in protokosin.



II



III

Determinations were made of the amount of isobutyric acid formed on hydrolysis both with 20% caustic, and with 15% sulphuric acid, and the figure obtained corresponds approximately to the presence of one isobutyryl group in the molecule.

Several attempts were made to demonstrate the keto group suspected to be present, by combination with various carbonyl reagents, but without success. This failure is perhaps not surprising in view of the heavily substituted nature of the molecule, derivatives of ketones of this type being always difficult to prepare.

When fused with potash, protokosin gave in addition to amorphous products, a small amount of a material identified as C-methyl-phloroglucinol by comparison with a synthetic specimen. It is doubtful, however, if any rigid conclusions may be drawn from the isolation of this substance since the experimental conditions under which it was formed do not exclude the possibility of its formation by demethylation of C-trimethyl-phloro-glucinol (16).

The position of the methoxyl group in protokosin/

protokosin has not been definitely ascertained but some evidence of its location is available. Boehm investigated the reaction between diazo-aminobenzene and several methylene-bisphloroglucinols (12) and found that all these substances reacted with this reagent to form azo-derivatives except those which had a methoxyl group in an ortho position to the linking methylene group.

Several attempts have been made to obtain an azo-derivative of protokosin but without success. In all cases the starting product was recovered unchanged. It seems possible then, that protokosin contains a methoxyl group ortho to such a methylene group. The substance is very resistant to demethylating agents, and the starting material may be recovered after boiling with concentrated hydriodic acid. At higher temperatures, however, an insoluble highly polymerised substance is obtained from these experiments and no identifiable product could be isolated.

Demethylation was also attempted using solutions of hydrobromic acid in glacial acetic acid. With this reagent, however, even in very low concentrations of hydrobromic acid, a red insoluble product/

product is formed. This substance acts like a quinone, being reduced by zinc dust and sulphur dioxide and reoxidised on exposure to air. It gives a pale yellow product on reductive acetylation, which is amorphous and could not be recrystallised. Protokosin is recovered quantitatively after heating on the water bath with 60% sulphuric acid and after prolonged refluxing with glacial acetic acid. A few mgm. of protokosin warmed with concentrated sulphuric acid produce so much of a red insoluble amorphous product that the reaction has not been further investigated.

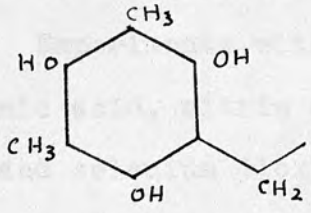
From the experimental evidence summarised above it seems reasonable to conclude that protokosin contains the groups II and IV. The large amounts of C-trimethyl-phloroglucinol obtained, and the relatively mild conditions under which it was formed, justify the assumption that no O-demethylation has occurred during the reaction.

Examination of their optical activity shows that protokosin is dextrorotatory whereas α and β-kosin are both optically inactive.

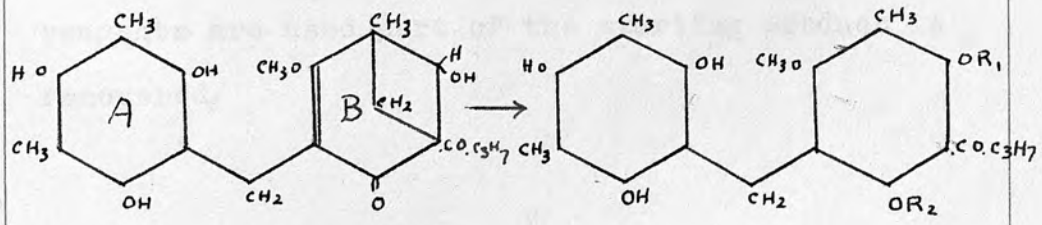
From the relationship between protokosin and the/

the Filix mas group, which also give phloroglucinols and butyric acid on hydrolysis, it is likely that the former is somewhat similarly constituted to the members of this group, namely as a methylene-bis-phloroglucinol.

For the structure of the second ring in the molecule no decisive evidence is available, but a suggestion as to its probable nature may be made. It must accommodate the isobutyryl side chain and the methoxyl group, and at the same time explain the optical activity of protokosin and its conversion into the two isomeric but optically inactive kosins. A possible formula which fulfils these requirements is given by V, the formation of the two kosins being represented by the reaction (V) ⟶ (VI) (R = H or CH_3; R_2 = CH_3 or H).



IV



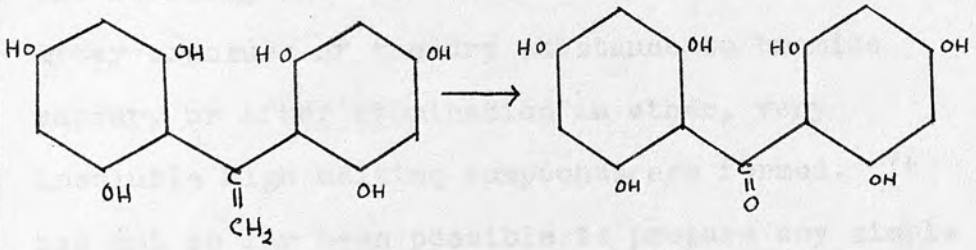
V VI

A fused ring system of type B has been predicated in filix acid and others of this group though no definite evidence of its presence has yet been obtained. A gem-dimethyl group such as is present in filicinic acid (gem-dimethyl phloroglucinol) is contra-indicated for several reasons. In the first instance the optical activity of protokosin would become more difficult to explain and in the second, several experiments known to permit of the isolation of compounds containing such a group, have failed to yield results. A search for filicinic butanone or filicinic acid by the methods described by Boehm (10, 11) failed to yield either of these substances, nor could dimethyl malonic acid be isolated by oxidation experiments in which it would have been formed.

Protokosin is very readily attacked by oxidising agents. Experiments with potassium permanganate, chromic acid, nitric acid, ozone, hydrogen peroxide and selenium dioxide, have all failed to yield a well defined intermediate oxidation product. When small molecular proportions of these reagents are used part of the starting product is recovered/

recovered and when larger proportions are used only low fatty acids can be isolated from the reaction mixture.

Direct methods of oxidation having failed to yield results, recourse was had to indirect methods. It was thought that the methylene bridge joining the two phloroglucinol rings might react with formaldehyde to give the grouping (VII), which could then be oxidised to (VIII).



VII VIII

It was found, however, that protokosin is unattacked by formaldehyde under any conditions, the starting product being recovered from condensations in neutral, acid and alkaline media and also after heating with trioxymethylene.

When protokosin in alcoholic solution is shaken in an atmosphere of hydrogen with platinum black there is no reaction; in the presence of platinum/

platinum oxide as a catalyst, however, hydrogen is rapidly absorbed, about 4 molecules being taken up After evaporation of the solvent a colourless, mobile oil is obtained which distils at 64° at 5 mm.

Experiments on the bromination of protokosin were unsatisfactory. When treated with one or two molecules of bromine in chloroform solution, the starting material is quantitatively recovered. After exposure of the dry substance to bromine vapour, or after bromination in ether, very insoluble high melting compounds are formed. It has not so far been possible to prepare any simple bromo-derivative of protokosin.

The determination of the number of hydroxyl groups in the compounds of the kousso group has offered some difficulty. Analysis of the acetyl derivatives of protokosin, and α and β-kosin shows the presence of three acetyl groups. Estimation of hydroxyl groups by Zerewitinoff analysis, however, gives rather anomalous results. While protokosin/

protokosin and α-kosin give figures approximating to three hydroxyl groups when the estimation is carried out at room temperature or at 90°, β-kosin shows three hydroxyl groups at room temperature and four at the higher temperature. As a result of these determinations it seems likely that in all cases four hydroxyl groups are present but that one of them is masked under ordinary conditions.

When distilled in vacuo with zinc dust, protokosin yields a very viscous oil which is insoluble in alkali and readily soluble in all organic solvents. No crystalline fraction could be isolated from the distillate. When however the crude oil is warmed for a few minutes with nitrating mixture a substance is obtained which is insoluble in boiling alcohol but which can be recrystallised from acetone. The yield of this substance, which on analysis is found to be a saturated hydrocarbon, $C_{17}H_{36}$, is extremely low and it is doubtful if any significance can be attributed to the observation.

(2) Kosotoxin

From the alcoholic mother liquors of protokosin and kosin by evaporation and replacement of the alcohol by petrol ether, a crystalline substance separates on prolonged standing. After repeated recrystallisation from petrol ether this substance melts at 140°. In chemical properties it is very similar to protokosin. Its alcoholic solution gives a brownish red colour with ferric chloride, and on warming a particle with concentrated sulphuric acid a cherry red colour is produced. It differs from protokosin however in melting point and in its solubility in alcohol, in which it dissolves readily even in the cold. The analytical figures agree best with the formula $C_{24}H_{32}O_7$ although this cannot be regarded as definitely settled. I propose to refer to this substance as kosotoxin, although it will be seen that it is very different from the substance referred to under that name by Leichsenring. The substance isolated by him was amorphous of melting point about 80°. The melting point for a similar product obtained by Lobeck was 65°. Both these investigators obtained kosin by heating this material/

material with alkali, but I have failed to confirm this observation using conditions under which α-kosin should have been isolated if it had been formed. There was no trace whatsoever of any alkali stable yellow substance.

It has been reported by Leichsenring that on injection, in carbonate solution, into the lymph sac of a frog, kosotoxin is much more poisonous than protokosin. I am indebted to Dr Raventos of the Pharmacology Department of this University for some experiments on the toxicity of both protokosin and kosotoxin towards frogs, in which he finds that protokosin is much more toxic than kosotoxin.

A critical survey of the yields and methods of isolation of these products by previous investigators, suggests that their "kosotoxin" is largely impure protokosin. I have found that a period of several months is required for the complete deposition of protokosin from crude alcoholic solutions. This time was not allowed by previous workers, and also the yield of this substance obtained in the present investigation is much higher than that previously reported. The discrepancy observed however in the toxicity of the various samples of protokosin is, as yet, inexplicable though further pharmacological experiments/

experiments may afford an explanation.

Several other methods of isolating kosotoxin in increased yield have been tried but so far too small an amount of the substance has been obtained to permit of extensive chemical investigation.

Experimental /

EXPERIMENTAL.

Isolation of Protokosin

25 kilos. of ground dry Kousso flowers are thoroughly extracted by percolation with ether and the ethereal extract evaporated to small bulk (about 4 litres).

The following method of extraction refers to 500 c.c. of the concentrated ethereal extract.

500 c.c. of the extract are poured into 2 litres of petrol ether of boiling point range 40-60° and the liquid refluxed for 4 hours. The petrol extract is decanted from the thick green residue, which is extracted by a further litre of the solvent. The combined petrol ether extracts are evaporated and the residue dissolved by boiling with 1 litre alcohol. On cooling, a waxy deposit separates, and after 48 hours is filtered and washed with cold alcohol. The alcoholic extract is evaporated to 200-300 c.c. and allowed to stand. Protokosin begins to separate after 24-48 hours and is filtered off at intervals of 3-4 days, till the deposition of crystals/

crystals becomes very slow. The residue of protokosin on the filter is thoroughly washed with cold alcohol, which removes most of the green coloured impurities. It is then recrystallised by dissolving in a small amount of hot $CHCl_3$ and pouring in boiling alcohol. After a few hours, the protokosin begins to separate in rosettes of short thick needles.

Pure protokosin forms colourless short needles, m.p. 182°, very soluble in $CHCl_3$, moderately soluble in hot benzene, acetone, petrol ether and alcohol, and sparingly soluble in ether. Prolonged boiling is necessary to effect solution in alcohol. 10-12 Gm. protokosin are obtained from 500 c.c. of ethereal extract. The alcoholic solution however continues to deposit protokosin slowly for several months and the total yield of purified material amounts to 90-100 gm. Protokosin in alcoholic solution gives a brownish colour on the addition of a drop of $FeCl_3$ solution, and on warming with conc. H_2SO_4 a deep cherry red colour is produced and the smell of isobutyric or some similar fatty acid may be detected. Protokosin is not wetted by water and is insoluble in Na_2CO_3 solution/

solution. Protokosin is soluble in NaOH solution but only slowly, unless wetting is promoted by touching it with alcohol. A solution in strong NaOH solution turns deep brownish red on boiling.

Analysis of protokosin.

C, 65.4; 65.4%. $C_{21}H_{25}O_6(OCH_3)$, C, 65.4%.

Found: H, 7.0; 7.0; 7.0%. Requires H, 6.9%.

CH_3O, 8.1; 8.0; 8.1%. CH_3O, 7.6%.

Active H, 11.6; 11.7 OH $(OH)_3$ 12.6%.

M.W. 412, 396, 399. M.W. 404

Mols. H.A. (Kuhn-Roth)
3.0; 3.3.

Estimation of % Volatile Fatty Acid.

(a) 210 mg. protokosin are boiled for 20 minutes with 20 c.c. 10% NaOH. The liquid is cooled, carefully acidified with H_3PO_4 and steam distilled. The steam distillate is titrated with 0.1 N NaOH, 4.7 c.c. required.

Calculated as isobutyric acid the % C_3H_7CO = 15.1.

(b) /

(b) 202 mg. protokosin in 6 c.c. 15% H_2SO_4 are heated for 6 hours at 160° in a sealed tube, After cooling the tube is opened, carefully washed into a distilling flask and steam distilled. The distillate required 4.6 c.c. 0.1 N NaOH for neutralisation. Calculated % C_3H_7CO = 15.1.

The theoretical % C_3H_7CO required to produce one molecule of isobutyric acid on hydrolysis is 17.6.

Attempts to prepare a derivative of protokosin.

Methylation.

1. A few mg. of protokosin are heated with K_2CO_3 in xylene. The solution becomes yellow due to the formation of kosins (cf. later), so that methylation could not be carried out in this medium.

2. 0.35 gm. Ag_2CO_3, (excess), 0.2 gm. protokosin and 0.2 c.c. (4 mols.) methyl sulphate are refluxed until there is no further apparent reaction. Protokosin is recovered.

Protokosin is also recovered when methyl iodide is used in the above experiment in place of methyl sulphate.

3./

3. 0.5 gm. protokosin in ether is added to an ethereal solution of diazomethane. After standing 24 hours, the ether is removed by evaporation. The remaining gum solidifies in the ice chest but could not be recrystallised. The methylated product is insoluble in NaOH and an alcoholic solution gives a slate gray colour on the addition of $FeCl_2$.

Acetylation, etc.

1. 100 mg. of protokosin are heated for 1 hour on the water bath with excess acetic anhydride and sodium acetate. The mixture is poured into water, extracted with ether, and the ethereal extract washed with $NaHCO_3$ solution and dried. After evaporation of the ether, a gum was obtained which could not be crystallised.

2. 0.5 gm. protokosin is dissolved in a mixture of 5 c.c. pyridine and 5 c.c. acetic anhydride and allowed to stand at room temperature for 36 hours. At the end of this period the reaction mixture is poured into 80 c.c. of ice water and stirred. On standing a few hours a solid forms which is filtered and washed thoroughly with water. The substance, however/

however, cannot be recrystallised. It rapidly forms a gum on warming with solvents, which does not resolidify. The crude substance is insoluble in caustic soda solutions and melts between 90-100°. Addition of $FeCl_3$ to an alcoholic solution gives no colour reaction. The crude acetyl derivative is analysed for CH_3CO groups as follows:

0.1 gm. is boiled for 2 hours with 50 c.c. alcoholic 2% KOH. Most of the alcohol is distilled off on the water bath and the residue cooled, acidified with H_3PO_4 and steam distilled. The steam distillate is titrated with 0.025 N NaOH. 21.0 c.c. were required.

% CH_3CO found = 22.6. % CH_3CO required for triacetyl derivative = 24.0.

Attempts were also made to prepare a p-nitrobenzoyl or p-brombenzoyl derivative by Einhorn's method without success. In each case an amorphous substance insoluble in alkali was obtained.

Chlorination.

Protokosin reacts with thionyl chloride on gentle warming but the product, after destruction of the excess thionyl chloride by alcohol, is amorphous.

Phenyl/

Phenyl urethane.

Protokosin is dissolved in benzene (dry) and refluxed for two hours with slightly more than the theoretical amount of phenyl isocyanate required to react with one hydroxyl group. At the end of the two hours the benzene is evaporated to small bulk. From the solution on cooling unchanged protokosin crystallises. Unchanged protokosin is also recovered after 6 hours boiling in benzene solution. Nothing crystalline can be recovered after heating 50 mg. of protokosin with 0.12 gm. of the pyridine complex with chlorcarbonyl di-phenylamine in pyridine on the water bath.

Ketonic Reagents.

Numerous reactions were carried out to obtain a derivative of protokosin with reagents acting on a keto group. Hydroxylamine, phenyl hydrazine, p-nitro and di-nitro-phenyl hydrazine and aniline under the usual conditions all failed to react with the substance. In most cases the starting material was recovered unchanged.

It is believed that the failure to demonstrate a C = O group is due to powerful steric hindrance. It/

It is well known that derivatives of di-ortho substituted ketones are very difficult to prepare.

Azo derivative.

Protokosin does not react in alcoholic solution with diazoaminobenzene either in the cold or after a short period of heating. From these experiments the starting material may be recovered, If the period of heating is prolonged (1 hour), the reaction mixture becomes very dark and from it nothing can be recovered.

Treatment with alkalies.

1. 1 Gm. protokosin is boiled for 20 minutes with 2.5% baryta. The solution turns reddish brown in colour. After cooling, the liquid is filtered and from the insoluble residue 0.45 gm. of the starting material may be recovered by recrystallisation from alcohol. The filtrate is acidified with acetic acid and extracted with ether. The ethereal extract (which is yellow) is dried with Na_2SO_4, filtered, evaporated, and the residue recrystallised from MeOH. After several recrystallisations the m.p. is 147°. This substance, which is α-kosin forms yellow needles very sparingly soluble in cold alcohol/

alcohol but soluble on boiling for a few minutes.

I am indebted to Messrs Merck of Darmstadt for 1 gm. of Merck kosin (m.p. 142°). After two recrystallisations from MeOH, this substance melts at 148° and a mixed m.p. with the above substance shows no depression. This material is impure α-kosin. The correct m.p. of α-kosin is 160°, but it is very difficult to attain this degree of purity by recrystallisation, due to contamination with small amounts of β-kosin which has very similar solubilities (see below).

2. 300 Mg. of protokosin are boiled one minute with 15 c.c. 20% NaOH and 150 mg. zinc dust. The solution becomes red and a reddish precipitate is formed. Water is added to redissolve the precipitate and the solution is cooled, filtered from zinc and acidified. The acid liquid, which smells strongly of a butyric acid, is extracted with ether and the ethereal extract washed with $NaHCO_3$ solution, dried with Na_2SO_4, filtered and evaporated. The residue after evaporation of the ether is recrystallised several times from MeOH. After purification, the above α-kosin is obtained in a yield of 11 mg.

From/

From the methyl alcoholic mother liquors on concentration, a second product separates, which, after repeated recrystallisation from MeOH and finally from petrol ether, melts at 120° and forms short yellow prisms. This substance is almost certainly the fraction obtained by Lobeck from Merck kosin by fractionation, which he called β-kosin.

Analysis of the kosins.

α-kosin

% C,	65.11	% H,	6.98	% MeO,	13.19	M.W.	393
	65.32		6.81		13.45		

β-kosin

65.25	7.01	13.4	350	(Rast)

$C_{22}H_{28}O_7$ requires:

65.34	6.93	15.3 ($2CH_3O$)	404

Mols. of HA found by Kuhn-Roth $C.CH_3$ determination:

2.7: 2.5.

Acetyl products of α- and β-kosin.

100 mg. of α- or β-kosin are heated for two hours on a boiling water bath with 2 c.c. of acetic anhydride and a little anhydrous sodium acetate. The reaction mixture is poured into water and extracted/

extracted with ether. After removal of the solvent, the residue is recrystallised from petrol ether (B.P. 80-100°).

M.P. acetyl α-kosin 123°; acetyl β-kosin 155°

Analysis:

Acetyl α-kosin.

Found: % C, 63.7; H, 6.75; CH_3CO, 24.48.

Acetyl β-kosin.

Found: % C, 63.81; H, 6.79; CH_3CO, 24.46.

$C_{22}H_{25}O_7(CH_3CO)_3$ requires:

C, 63.58; H, 6.41; CH_3CO, 23.8.

Optical Activity.

(a) Protokosin in 10% solution in $CHCl_3$ (1 Dm.tube)
Zero 0.15.
Reading 0.95

Specific rotation of protokosin + 8.0.

(b) α-kosin 10% $CHCl_3$ solution - optically inactive.

(c) β-kosin 10% $CHCl_3$ solution - do. do.

(3) /

(3) 5 gm. protokosin are dissolved in 30 c.c. of 20% NaOH solution and 5 gm. of zinc dust added. The mixture is heated at 100° for 4 hours. The reaction mixture slowly turns red, and as the reaction progresses, again becomes lighter in colour. At the end of 4 hours the zinc is filtered off, the filtrate acidified with H_2SO_4 and steam distilled. The residue in the flask after steam distillation is extracted thoroughly with ether, and the extract dried and distilled as usual. The gum left after evaporation of the ether, on grinding with cold MeOH, forms a yellow crystalline solid, which is filtered off and from which α- and β-kosin are obtained by fractionation from MeOH. Water is added to the methyl alcoholic filtrate which is again extracted with ether. After removal of the solvent by evaporation, the residue is warmed with benzene, the insoluble portion being filtered off and recrystallised from water.

The substance recrystallises from water in needles of m.p. 184°. An aqueous solution gives a blue colour with $FeCl_3$ which is rapidly bleached, and a white precipitate forms. It reduces $AgNO_3$ solution but gives no colour with a pine chip moistened with HCl.

Analysis/

Analysis:

Found: C, 64.8; H, 7.0.

$C_9H_{12}O_3$ requires C, 64.3; H, 7.1.

A mixed m.p. of this substance with a synthetic specimen if trimethyl-phloroglucinol showed no depression.

The steam distillate from the above experiment is made alkaline, evaporated to small bulk, acidified with H_2SO_4 and extracted with ether. Part of the ethereal extract is evaporated and the residual fatty acid converted into the p-phenylphenacyl ester in the usual fashion. After recrystallisation from aqueous alcohol this ester showed no depression in melting point when mixed with p-phenyl-phenacyl isobutyrate, m.p. 72°.

From a second part of the ethereal extract, the p-toluidide of the acid is prepared in the following manner: Part of the ether is evaporated and a few drops of thionyl chloride added. After warming for a few minutes to convert the acid to acid chloride, an excess of p-toluidine is added to the reaction mixture, which is heated for some time longer. Excess of p-toluidine is now removed by extraction with/

with dilute HCl, and the ethereal solution of the p-toluidide dried and evaporated.

After repeated recrystallisation of this derivative from petrol ether, a fraction was obtained of melting point 102°, which was undepressed on mixing with a specimen of isobutryl p-toluidide (m.p. 105°). No other fraction of definite m.p. could be isolated from the crude p-toluidides.

(4) To 10 gm. KOH melted with a little water, are added 5 gm. of protokosin in several portions. The mixture froths and turns red at 150°. At 250° the temperature begins to rise rapidly and reaches 320° with extensive charring. After cooling, the melt is dissolved in water, acidified, steam distilled and extracted with ether. Nothing crystallises from a methyl alcoholic solution of the residue left after removal of the ether. The alcoholic solution is then diluted with water and re-extracted with ether. After evaporation of the ether, the residue is dissolved in water and the solution decolorised with charcoal. On standing for some time, the concentrated aqueous solution deposits crystals of m.p. 208°. This substance sublimes/

sublimes in a high vacuum at 290-300°, is soluble in acetic acid, from which it is precipitated by adding xylene. An aqueous solution on addition of $FeCl_3$ gives a blue colour which turns to brown after a few minutes.

A mixed melting point with a synthetic specimen of methyl-phloroglucinol (m.p. 210°) shows no depression.

(5) 10 gm. NaOH are melted with a little water in a nickel basin. To the melt is added in portions a mixture of 5 gm. protokosin and 10 gm. zinc dust. The mixture turned brown at 150° but not much charring was observed. After cooling, the melt was dissolved in water, acidified, steam distilled and extracted with ether in a continuous extraction apparatus for 60 hours.

After evaporation of the ether, a gelatinous substance is formed which separates in an amorphous condition from a hot methyl alcoholic solution. The product has a melting point 69°, but could not be crystallised. Methylation of this material with diazomethane gave a white amorphous product from MeOH of m.p. 57°.

Acetylation/

Acetylation with acetic anhydride also failed to yield a crystalline derivative.

(6) 2 Gm. protokosin and 4 gm. zinc dust are boiled for 5 minutes with 10 c.c. 20% NaOH. After the excess of zinc has been removed by filtration, the filtrate is acidified, steam distilled and extracted with ether (Extract I). The aqueous solution is then continuously extracted with ether for 24 hours to remove any substance only sparingly soluble in ether (Extract II).
Extract I: α and β-kosin are obtained as usual by fractionating the residue after evaporation of the ether from methyl alcohol.
Extract II: This portion of the ethereal extract yields no residue after removal of the solvent.

(7) 20 gm. protokosin are boiled for 15 minutes with 600 c.c. 20% NaOH and 40 gm. of zinc dust. The excess of zinc is removed as usual and the filtrate acidified and extracted with ether. After shaking with $NaHCO_3$ solution to remove volatile fatty acids, the ether is distilled, and the residue dissolved in the minimum amount of boiling MeOH, and then cooled in a freezing mixture. The mixture/

mixture of kosins is filtered off and fractionated as usual from MeOH. After purification, 4.3 gm. α-kosin and 0.8 gm. β-kosin were isolated. Water is added to the methyl alcoholic filtrate from the crude mixture of kosins and the liquid extracted with ether several times. After decolorising the extract with charcoal, it is dried with Na_2SO_4 and evaporated to dryness. The almost colourless residue is boiled with benzene till no more is dissolved.

Recrystallisation of the insoluble part from water and then from ethyl acetate petrol ether mixture, shows this to be trimethyl-phloroglucinol, m.p. and mixed m.p. 184-5°. Yield 2 gm.

From the hot benzene extract on cooling, a little more trimethyl-phloroglucinol separates. Addition of petrol ether to the concentrated benzene solution, from which nothing separates on cooling, precipitates an oil, from which the supernatant liquid is decanted. Evaporation of the supernatant liquid also yields an oil. Both of these residues were submitted to distillation in a high vacuum but no identifiable compound could be isolated.

Bromination/

Bromination

(1) 100 mg. protokosin are dissolved in 2 c.c. $CHCl_3$ and allowed to stand overnight with 1 mol. Br_2. The colour of the bromine solution disappears. After evaporation of the chloroform, the residue is recrystallised from MeOH and found to consist of unchanged protokosin. 50 Mg. of starting material were recovered but no crystalline derivative could be isolated from the alcoholic mother liquors.

(2) Finely powdered protokosin is exposed to excess bromine vapour. HBr is evolved and a red solid forms which separates from a hot alcoholic solution as an amorphous substance having a m.p. about 300°.

(3) Tp an ethereal solution of protokosin is added an excess of bromine. After removal of the solvent, the residue is dissolved in benzene and precipitated with petrol ether. This process is repeated, and the substance then separates from a hot CCl_4 solution as an amorphous, coherent mass, which decomposes about 165°.

(4)/

(4) A solution of 100 mg. of protokosin in chloroform is allowed to stand at room temperature for 3 hours with 0.023 c.c. of bromine (2 mols.) After evaporation of the solvent in vacuo, the residue is recrystallised from MeOH and is found to consist of unchanged starting material.

(5) 100 Mg. of protokosin are heated for 15 mins. at the boiling point with 2 mols. of bromine in chloroform solution. After removal of the solvent by evaporation, the residue is recrystallised from MeOH, and again consists of unchanged starting material.

Reduction.

(1) To a warm solution of 100 mg. protokosin in 20% alcoholic HCl, is added excess of zinc dust in small portions. From the hot, filtered, alcoholic solution, an amorphous yellow substance separates, which could not be crystallised.

(2) 100 Mg. of protokosin are dissolved in dilute NaOH solution (2%), 0.1 gm. platinum black is added and the liquid shaken in an atmosphere of H_2. No hydrogen/

hydrogen is absorbed and from the acidified reaction mixture the starting product is recovered almost quantitatively.

(3) 100 Mgm. of protokosin are dissolved in cold glacial acetic acid and shaken in a hydrogen atmosphere with platinum black. 19 c.c. H_2 were absorbed. Water is added to the acetic acid solution after filtration, and the precipitated solid is filtered off. This is recrystallised from alcohol and consists of unchanged starting material (60 mgm.). Nothing could be recovered from the dilute acetic acid mother liquors.

(4) 0.5 Gm. of protokosin are dissolved in 60 c.c. alcohol and shaken in an atmosphere of hydrogen with 0.1 gm. PtO_2 as catalyst. 110 c.c. of H_2 are rapidly absorbed corresponding to about 3 mols. of H_2. After filtering and distilling off most of the solvent, 50 mg. of protokosin were recovered. The mother liquors were evaporated and distilled in vacuum, a fraction boiling, after redistillation, at 64° at 5 mm. being collected. Yield 0.25 gm.

The/

The residue in the distilling flask consisted of a substance which was deposited in an amorphous form from a hot alcoholic solution.

Analysis:

Found: % C, 61.2 % H, 10.8.

$C_{12}H_{26}O_4$
requires C, 61.0 H, 11.11.

Oxidation.

(1) 300 Mg. protokosin are dissolved in 15 c.c. 2% NaOH and 75 mg. $KMnO_4$ (equivalent to 3 atoms of oxygen) added. After standing overnight at room temperature, the liquid is filtered and the filtrate acidified. A white precipitate is formed, which cakes together and is filtered off. On recrystallisation from MeOH, this proves to be the starting material which is largely recovered. Nothing else could be isolated.

(2) 2 Gm. of protokosin are dissolved in acetone and an aqueous acetone solution of 2 gm. (12 atoms of oxygen) $KMnO_4$ added in several portions. The reaction mixture is allowed to stand overnight and filtered. After acidification both filtrate and residue/

residue are extracted with ether, and the extract dried, filtered and evaporated. A yellow amorphous substance very soluble in organic solvents and insoluble in water, but dissolved by Na_2CO_3 solution, remains. It is dissolved in benzene and precipitated by petrol ether several times. The product could not be crystallised from any solvent.

(3) 2 Gm. protokosin are dissolved in a mixture of 20 c.c. acetone and 20 c.c. cold 10% NaOH. 4% aqueous $KMnO_4$ solution is added in 8 c.c. portions (2 oxygen atoms) with shaking. Oxidation is very rapid. In all, 80 c.c. of $KMnO_4$ solution are added in 20 minutes. The reaction mixture is acidified with H_2SO_4 and extracted with ether. From the extract a substance very similar to that from experiment 2 is obtained, along with a quantity of low fatty acids. On further oxidation of this amorphous material with $KMnO_4$ in Na_2CO_3 solution, a mixture of low fatty acids is again obtained.

(4) 500 Mg. protokosin are dissolved in 15 c.c. alcohol and refluxed with 250 mg. SeO_2 for 2 hours. On cooling the filtered liquid, protokosin recrystallises out. This is redissolved, a further gram/

gram of SeO_2 added and the mixture again boiled for two hours. At the end of this period, the liquid is filtered from SeO_2 and Se, and the solvent removed in vacuo. After evaporation of the alcohol, the residue is dissolved in hot petrol ether, and filtered from a little insoluble selenium. On cooling, an amorphous substance separates out, which cannot be crystallised.

(5) 100 Mg. protokosin are dissolved in a few c.c. of warm glacial acetic acid. An acetic acid solution of CrO_3 (50 mg.) is added, and the mixture warmed until all the red colour disappears. Nothing separates on cooling. The reaction mixture is diluted with water and extracted with $CHCl_3$. Nothing, however, could be isolated from the extract.

(6) 100 Mg. of protokosin are boiled with a 20% solution of nitric acid in acetic acid for 2 hours. A gelatinous substance separates on cooling. It is soluble in Na_2CO_3 solutions, but could not be crystallised.

Experiments/

Experiments with Hydriodic Acid.

(1) Protokosin is heated on the water bath with HI (s.g. 1.5) and red phosphorus until the solution is decolorised. The cooled liquid is extracted with ether, and the residue left after evaporation of the dried extract a recrystallised from acetone. The starting material is almost quantitatively recovered.

(2) 500 Mg. of protokosin are boiled with 10 c.c. of HI (s.g. 1.5) and red phosphorus, the liquid being treated as in experiment 1. The starting material is again almost quantitatively recovered.

(3) 1 Gm. of protokosin with 25 c.c. of HI (s.g. 1.9) are heated for 2 hours in a sealed tube at 200°. After cooling, the reaction mixture is thoroughly extracted with ether, and the extract washed with water and with $Na_2S_2O_3$ solution. From the concentrated extract nothing could be recovered, the material being almost completely destroyed.

Condensation/

Condensation with Formaldehyde

(1) 100 Mg. of protokosin are dissolved in MeOH containing excess of formaldehyde. After the liquid has been refluxed for 3 hours, it is allowed to cool, when the starting material crystallises out almost quantitatively.

(2) After heating a 1% alcoholic HCl solution of protokosin on the water bath for 10 minutes with excess of formaldehyde, the protokosin is quantitatively recovered.

(3) 100 Mg. protokosin are dissolved in 20% alcoholic HCl, excess formaldehyde added, and the mixture warmed for 6 hours on the water bath. Nothing separates on cooling, nor can any product be recovered from the reaction mixture by diluting and extracting with ether.

(4) A few milligrams of protokosin are ground with a large excess of trioxymethylene, and heated in a metal bath at 170° till all the trioxymethylene has colatilised. Unchanged starting material is recovered/

recovered from the residue by recrystallisation from alcohol.

(5) After heating protokosin in a sealed tube with trioxymethylene for 1 hour at 150°, a very dark, charred, melt is formed, from which nothing can be recovered by extraction with boiling alcohol.

Experiments with Acetic Acid.

(1) 1 Gm. protokosin is dissolved in 10 c.c. of hot acetic acid containing 1 c.c. HI (s.g. 1.5), and heated for 1 hour on a boiling water bath. The solution becomes deep red in colour, and on cooling a scarlet amorphous precipitate forms. A further quantity of the same substance is obtained by diluting the mother liquor, and extracting with a large volume of ether, in which it is only sparingly soluble. This material forms a highly insoluble reddish powder which dissolves in caustic alkalies giving a crimson solution. An alcoholic ammonia solution of the material shows a very faint green fluorescence in ultra-violet light. No solvent could be found from which the material could be crystallised.

That the substance is a quinone is shown by the following reactions:-

(a) /

(a) An alcoholic solution is decolorised on boiling with zinc dust but the colour returns on exposure to air.

(b) On passing SO_2 into an alcoholic solution, a colourless amorphous precipitate is formed which redissolves if the current of gas is continued.

When the compound is boiled for half an hour with acetic anhydride and zinc dust, and the reaction mixture poured into water, a pale yellow substance is produced, which separates amorphous from a hot alcoholic solution, but which does not re-oxidise on exposure to air.

This quinone-like substance is always produced when protokosin is treated with acetic acid containing a mineral acid. The following experiments were done with a few milligrams of protokosin to find conditions in which this substance is not formed.

(a) Protokosin dissolved in glacial acetic acid containing 33% HBr. The colour develops immediately on warming.

(b) In acetic containing 1.5% HBr, the red colour develops after warming on the water bath for 5 mins.

(c) /

(c) In acetic acid containing 0.3% HBr, the colour develops in 20 minutes.

(2) 200 Mg. protokosin are dissolved in glacial acetic acid and refluxed for 7½ hours. From the reaction mixture, part of the protokosin recrystallises on cooling, and the rest may be recovered by diluting with water, extracting with ether, and recrystallising the residue from alcohol.

Zinc Dust Distillation.

200 mg. of protokosin are intimately mixed with 4 gm. of zinc dust and distilled in vacuo. A thick oil distils, which is dissolved in ether and the solution decolorised with charcoal. After evaporation of the solvent, an oil remains which does not solidify on freezing. This oil is soluble in organic solvents but insoluble in water or alkalies. It does not form a picrate.

The zinc dust distillate from 1 gm. of protokosin is warmed for two minutes on the water bath with 2 c.c. of nitration mixture. After dilution with water and cooling, the mixture is filtered, the residue washed with water and recrystallised from acetone. The substance formed is insoluble in water/

water or boiling alcohol but recrystallises from acetone in short white glistening needles melting sharply at 53°.

Analysis.

Found: % C, 85.11; % H, 14.89; M.W. 237.

$C_{17}H_{36}$
requires C, 85.0. H, 15.0; M.W. 240.

Grignard Reaction.

To the Grignard reagent in ether, formed from 2.6 gm. Mg and ethyl iodide, is added an ethereal solution of 2 gm. of protokosin. The mixture is allowed to stand for 36 hours and is then decomposed as usual by ice cold H_2SO_4.

From the dried ethereal layer a gum was obtained which could not be crystallised. It contained no protokosin. The material decomposed completely when an attempt was made to distil a little in a high vacuum. No crystalline product could be obtained after acetylation of the gum with acetic anhydride and sodium acetate.

Alkali/

Alkali Treatment of α-Kosin.

(1) 4.5 Gm. α-kosin are boiled for 4 hours with 100 c.c. of 40% NaOH and 5 gm. zinc dust in an atmosphere of N_2. A white solid separates and is filtered off with the excess of zinc dust. The filtrate is acidified and thoroughly extracted with ether. Only a very small residue was left by this extract on evaporation. The residue of zinc dust is thoroughly washed with boiling water, and the filtrate cooled and acidified. From an ethereal extract of this acidified filtrate, 2.5 gm. α-kosin were recovered after recrystallisation.

(2) 2.5 Gm. α-kosin are dissolved in hot 5% NaOH (80 c.c.). The liquid is maintained at the boiling point by adding solid NaOH to a concentration of 80%, and is then boiled for a further ten minutes. When the concentration of NaOH reaches about 30% the sodium salt of α-kosin begins to separate out. From this experiment 2.0 gm. α-kosin were recovered. The melting point of the kosin has now been raised to 158-9°.

Kosotoxin

100 c.c. of the alcoholic mother liquors from the isolation of protokosin are evaporated to small bulk in vacuo and diluted to 200 c.c. with petrol ether, b.p. 80-100°. After standing for several weeks the crystalline deposit is filtered off and recrystallised several times from petrol ether, b.p. 80-100°. This substance forms white needles of m.p. 140-1°. It is very soluble in alcohol, acetone and chloroform, sparingly soluble in cold high boiling petrol ether and insoluble in light petrol ether. An alcoholic solution gives a brownish red colour on the addition of $FeCl_3$ and the solid, on warming with conc. H_2SO_4 gives a cherry red colour.

Analysis.

Found: % C, 66.80, 66.56; % H, 7.59, 7.45.

$C_{24}H_{32}O_7$ requires 66.6. 7.46.

Treatment with Alkalies.

50 mg. of kosotoxin are dissolved in 2 c.c. of 20% NaOH, 200 mg. zinc dust added and the mixture refluxed for 5 minutes. The liquid turns brown and then rapidly decolorises. After filtration from the zinc, cooling and acidifying the liquid, which smells of isobutyric acid, is extracted with ether. The colourless extract is washed with $NaHCO_3$ solution, dried with Na_2SO_4 and evaporated. Only a small amount of a gum, which could not be crystallised, remained, there being no trace of an alkali stable, yellow degradation product.

Toxicity of kosotoxin and protokosin.

Experiment	Wt. of frog in gm.	Total dose in mg.	Dose/gm. in γ	Result
1% solution of kosotoxin in alcoholic saline carbonate solution. pH approx. 10.				
A	37	0.19	5	Survived
B	42	0.42	10	do.
C	27	0.675	25	do.
D	30	1.5	50	Died in 6-8 hours
1% solution of protokosin in alcoholic saline carbonate solution. pH approx. 10.				
A	33	0.15	5	Alive after 24 hours
B	25	0.25	10	Died within 24 hours
C	25	0.625	25	Died in 6 hours
D	25	1.0	40	Died in 3 hours

Post mortem: in most cases haemorrhage at the site of injection; in a few contracted heart; in all hyperexcitable sciatic nerve. Probably death due to central paralysis?

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