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[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY AND CHEMICAL ENGINEERING OF STANFORD UNIVERSITY]

Bitter Principles from *Echinocystis Fabacea*[1]

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From ether extracts of the juice of *Echinocystis fabacea*, two crystalline compounds of the apparent molecular formula $C_{30}H_{44}O_8$ have been isolated. One of these compounds is identical with curbitacin B and the other is a new compound which has been named *fabacein*. Both compounds have at least three hydroxyl groups, a readily saponified acetate group, and at least two carbonyl groups, one of which is conjugated with a double bond. Catalytic hydrogenation using a palladium-on-carbon catalyst saturates the carbon-carbon double bond in both compounds. Mild saponification of the hydrogenated compounds yields the same product having the formula $C_{28}H_{44}O_6$. Refluxing the hydrogenated compounds with methanolic alkali gives a compound, $C_{28}H_{42}O_6$, which appears to be a conjugated dienone. These compounds and their derivatives are characterized by the difficulty with which crystalline products can be obtained, and by the ease with which rearrangements apparently take place.

Recent publications on the bitter principles of members of the *Cucurbitaceae*[2] and a revival of interest in elaterin[3] and other compounds obtained from *Ecballium elaterium*,[4] because of their necrotizing action on tumors,[5] has prompted us to report the results of our work thus far on the bitter principles present in another member of the *Cucurbitaceae*, *Echinocystis fabacea*, the common man-root of central coastal California. It has been reported[6] that decoctions of the root of this plant were used by the California Indians to poison fish and to commit suicide, and by their medicine men to poison aged people when they became sick and decrepit. In the native practice of medicine both the seeds and the root were highly valued as a specific against rheumatism and venereal diseases. The root also was said to have a strong cathartic action. It is reported that extracts were used in California to make "Stoughton's Bitters."[7]

Previous work has shown that acid hydrolysis of the juice from the root yields a dark brown insoluble humus-like material from which a crystalline sapogenin, called echinocystic acid, was isolated.[8] Subsequently the structure of this compound was shown to be 16-hydroxyoleanolic acid.[9] Attempts to isolate a pure saponin from the juice have not been successful, but have indicated that the saponin is not responsible for the bitterness of the plant. The latter substances can be obtained directly from the fresh juice of the plant by extraction with chloroform or ether. From ether extracts, three crystalline products and an amorphous fraction have been isolated. The crystalline products are only slightly soluble in water, and the bitter taste is hardly discernible unless one tastes an alcoholic solution of the crystals. The amorphous fraction has the extremely bitter taste of the plant, the disagreeable effect at the back of the tongue and throat being very characteristic.

One of the crystalline compounds, m.p. 201–202°, $[\alpha]_D^{25}$ + 36° in ethanol was named fabacein I, and the other, m.p. 178–179°, $[\alpha]_D^{25}$ + 87° in ethanol, was called fabacein II. The third product, m.p. 230–231°, was called fabacein III.[10] Since then it has been found by paper chromatography that although fabaceins I and II are homogeneous, the material called fabacein III may be a mixture of products or convertible to a mixture of products on standing. Moreover, direct comparison of faba-

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(1) This work was supported by grant NSF-G-2367 from the National Science Foundation.

(2) (a) P. R. Enslin, *J. Sci. Food Agr.*, **5**, 410 (1954); (b) P. R. Enslin, S. Rehm, and D. E. A. Rivett, *J. Sci. Food Agr.*, **8**, 673 (1957).

(3) D. Lavie and S. Szinai, *J. Am. Chem. Soc.*, **80**, 707 (1958).

(4) D. Lavie and D. Willner, *J. Am. Chem. Soc.*, **80**, 710 (1958).

(5) M. Belkin, D. B. Fitzgerald, and G. W. Cogan, *J. Nat. Cancer Inst.*, **13**, 139 (1952).

(6) V. K. Chestnut, *Contr. U. S. Nat. Herbarium*, **7**, 390 (1902).

(7) J. G. Cooper, *Reports of Explorations and Surveys*, 35th Congress, 2nd Session, Senate Ex. Doc. No. 46, Part I, p. 57 (1859).

(8) I. Bergsteinsson and C. R. Noller, *J. Am. Chem. Soc.*, **56**, 1403 (1934); C. R. Noller, *J. Am. Chem. Soc.*, **56**, 1582 (1934).

(9) W. R. White and C. R. Noller, *J. Am. Chem. Soc.*, **61**, 983 (1939); D. Frazier and C. R. Noller, *J. Am. Chem. Soc.*, **66**, 1267 (1944); B. Bischoff, O. Jeger, and L. Ruzicka, *Helv. Chim. Acta*, **32**, 1911 (1949).

(10) W. O. Eisenhut and C. R. Noller, Abstracts of Papers Presented at the San Francisco Meeting of the American Chemical Society, April 1958.

cein II with a sample of cucurbitacin B kindly supplied by Dr. P. R. Enslin, showed that they are identical and fabacein II will be referred to henceforth by the latter name. Fabacein I, however, is different from any of the cucurbitacins reported thus far[2b] and the name *fabacein* is retained.

Cucurbitacin B has been assigned the molecular formula $C_{32}H_{48}O_8$ from analyses and from the molecular weight calculated from the size of the unit cell determined by x-ray diffraction and from the density.[11] Numerous analyses of our product by two different laboratories gave values whose average is low in carbon by 1.16% and in hydrogen by 0.23% for this formula. Our analyses check better for $C_{30}H_{44}O_8$, although they are not as consistent as one would like them to be. Similarly the analyses of fabacein correspond to $C_{30}H_{44}O_6$ and do not agree with a C_{32} formula.

Cucurbitacin B reduces Tollens reagent, gives a positive Molish reaction, and gives a positive test for acetate.[12] It gives red colors when dissolved in acetic acid containing sulfuric acid, and in the Liebermann and Liebermann-Burchard tests, a Burgundy color with hydrogen bromide in acetic acid, and a purple color with antimony pentachloride in chloroform. The ferric chloride and Legal tests are negative. Thus far no crystalline products have been obtained by the action of diazomethane, methyl sulfate, and alkali either before or after refluxing with alkali, sulfuric acid in ether, *p*-toluenesulfonic acid in pyridine, thionyl chloride in pyridine, 2-methyl-2-ethyl-1,3-dioxolane in the presence of *p*-toluenesulfonic acid, sodium and 1-propanol, sodium borohydride, chromium trioxide in acetic acid, or Kiliani reagent. Dehydrogenation with palladium-on-charcoal or with selenium did not yield any aromatic compounds that could be detected by the infrared absorption spectra of the products.

Although Zerevitinov determinations indicate the presence of four to five reactive hydrogen atoms, presumably in hydroxyl groups, no crystalline acetates or other esters could be obtained with the exception of a monomethanesulfonate. This product was isolated in poor yield and was derived from a rearranged product as indicated by its infrared absorption spectrum. Similarly, although the infrared spectrum of cucurbitacin B indicates the presence of carbonyl groups, the carbonyl derivatives obtained were amorphous and could not be purified.

The ultraviolet absorption spectrum of cucurbitacin B has a maximum at 228 mμ, log ϵ 4.02, and an inflection at 270–290 mμ, log ϵ 2.32 at 280 mμ, indicating the presence of an α,β-unsaturated carbonyl system. Readily interpretable bands are present in the infrared absorption spectrum at 2.92 μ (OH), 5.82 and 5.92 μ (C=O), and at 6.17 μ (conjugated C=C). The strong absorption in the carbonyl region and at 6.17 μ supports the evidence from the ultraviolet absorption for the presence of a conjugated system, although carbon-carbon double bonds conjugated with ketone groups usually absorb between 6.18 and 6.30 μ.[13] Whether this double bond is conjugated with the carbonyl group responsible for the absorption at 5.82 μ or that at 5.92 μ is not clear. Ordinarily a ketone group conjugated with a double bond absorbs in the region 5.93–6.01 μ.[14] However, reactions of cucurbitacin B which destroy the α,β-conjugated system, destroy the absorption at 5.82 μ but do not affect the absorption at 5.92 μ. Hence we are inclined to associate the bands at 5.82 and 6.17 μ with the α,β-unsaturated carbonyl system. The low frequency of the 5.92 μ absorption, together with the persistence of this band after compounds have been subjected to strongly reducing conditions, lead us to suspect that the carbonyl group which gives rise to this absorption is highly hindered.

When cucurbitacin B was allowed to stand at room temperature in a standardized alcoholic solution of sodium hydroxide, 0.93 mole of alkali was consumed per mole of compound. The product could not be obtained crystalline. The infrared spectrum of the amorphous product did not show the bands at 5.82 and 6.17 μ, but strong absorption occurred at 6.02 μ. Thus the α,β-unsaturated carbonyl system has disappeared and a new chromophore has been introduced. The intense absorption at 5.94 μ was retained. Absorption at 6.02 μ has been associated with a diosphenol structure.[15] This assignment is supported by the fact that the saponified product now gives a positive ferric chloride test for enol.

When cucurbitacin B was heated to 250°, it lost acetic acid, which was recovered to the extent of 0.8 mole per mole of compound. The amorphous product isolated from the nonvolatile residue gave a color with ferric chloride. The infrared spectrum showed only one strong carbonyl band at 5.95 μ with very weak shoulders at 5.85 and 5.90 μ. No strong bands were present in the carbon-carbon double bond region.

When cucurbitacin B in ethyl acetate was hydrogenated using palladium on carbon as catalyst, from 1.0 to 1.36 moles of hydrogen was absorbed. Although crystalline products melting in the range 183–186° were obtained, analyses were not consistent. The ultraviolet absorption spectrum had only one maximum at 283 mμ, log ϵ 2.32, with high terminal absorption at 215 mμ, log ϵ 3.48, and a minimum at 268 mμ, log ϵ 1.83. The only distinct

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(12) M. Frerejacque, *Compt. rend.*, **240**, 1804 (1955).

(13) (a) L. J. Bellamy, *The Infrared Spectra of Complex Molecules*, John Wiley and Sons, Inc., New York, 1954, p. 37. (*b*) A. R. H. Cole, *Fortschr. Chem. Org. Naturstoffe*, **13**, 41 (1956).

(14) Ref. 13a, p. 114.

(15) R. J. W. Le Fevre, F. Maramba, and R. L. Werner, *J. Chem. Soc.*, 2496 (1953); *cf.* Ref. 2b and 3.

band in the double bond region of the infrared spectrum is at 5.92 μ, although a shoulder is present at 5.84 μ. Thus the α,β-unsaturated carbonyl system has been removed. The hydrogenated product still reduces Tollens reagent slowly. The ferric chloride test for enols, the Molish test, the Legal test, and the Frerejaque test for acetate are negative. However, on saponification with standard alkali at room temperature, from 0.3 to 0.6 mole of alkali is consumed and pyrolysis of one product gave 0.7 mole of acetic acid. Despite the small amount of alkali consumed during saponification, the yields of crystalline saponified product were 75–80%. Evidently partial hydrogenolysis of the acetate group takes place along with hydrogenation of a double bond.

The product of saponification at room temperature of the hydrogenated compound crystallized well and melted at 233–234°. Analyses indicate the empirical formula $C_{28}H_{44}O_7$, which would be expected if a dihydro derivative of $C_{30}H_{44}O_8$ lost one acetyl group on saponification. Five terminal methyl groups and four active hydrogen atoms also are indicated. A maximum in the ultraviolet absorption spectrum occurs at 280 mμ, log ϵ 2.21, a minimum at 255 mμ, log ϵ 1.99, and high terminal absorption at 215 mμ, log ϵ 3.40. The infrared spectrum shows two strong carbonyl bands at 5.83 and 5.92 μ. Reaction with hydroxylamine gave only an amorphous product. Its infrared spectrum showed strong absorption at 5.92 μ but the absorption of the original product at 5.83 μ had disappeared. It is likely that the absorption of the oxime is superposed on the original absorption at 5.92 μ.[16]

When the product of reduction of cucurbitacin B with hydrogen in the presence of palladium on charcoal was refluxed with standard methanolic alkali, again less than one mole of alkali (0.6 mole) was consumed. The saponification product was adsorbed on alumina containing 6% water and eluted first with chloroform and then with chloroform-methanol mixture. The first fractions could be crystallized from 2-propanol and gave a product identical with one obtained by the saponification of hydrogenated fabacein. The more strongly adsorbed fractions gave a strong ferric chloride test. They were oily and could not be obtained in a pure form.

Pyrolysis of one sample of hydrogenated cucurbitacin B gave 0.7 mole of acetic acid. The residue was surprisingly similar to the pyrolysis product of cucurbitacin B. The single strong band in the double bond region of the infreared spectrum is at 5.93 μ.

When cucurbitacin B in methanol was hydrogenated in the presence of Adams catalyst approximately one mole of hydrogen was absorbed. It was difficult to obtain reproducible results, possibly because of poisoning of the catalyst. The crystalline product, m.p. 198°, gave a positive ferric chloride test. The α,β-unsaturation was retained as shown by the ultraviolet spectrum (λ_{max} 229, log ϵ 3.85, with a shoulder at 275 mμ, log ϵ 2.75). Strong bands were present in the infrared spectrum at 5.93 and 6.18 μ together with a shoulder at 5.87 μ. At least two moles of hydrogen were absorbed when the hydrogenation was carried out in glacial acetic acid. The yield of crystalline product was only 33%. Here, as in the hydrogenations using a palladium catalyst, the α,β-unsaturation was lost, the infrared spectrum showing only one strong band at 5.93 μ.

Reduction of cucurbitacin B with sodium in 1-propanol or with sodium borohydride gave products which could not be crystallized. However the infrared spectra showed that the α,β-unsaturated carbonyl system was lost, although the chromophore responsible for the absorption at 5.92 μ was unchanged. Oxidation of either cucurbitacin B or of the palladium-catalyzed hydrogenation product with chromium trioxide in pyridine or with Kiliani mixture in acetone gave mixtures of neutral and acidic products from which crystalline compounds have not yet been isolated.

During the course of this work it was observed that some roots yield a compound which is different from cucurbitacin B. This compound has been named *fabacein*. It is less soluble in methanol than cucurbitacin B and melts at 201–202°. Analyses agree with the formula $C_{30}H_{44}O_8$ and chemical evidence indicates that it is an isomer of cucurbitacin B. In the color tests and with most reagents it behaves in the same way as cucurbitacin B. However, Zerevitinov determinations indicate three reactive hydrogens instead of four or five. Moreover, three absorption bands occur in the carbonyl region of the infrared spectrum at 5.79, 5.85, and 5.93 μ. The hydroxyl band is sharper than that for cucurbitacin B and is at 2.88 μ, and the conjugated double bond absorption is at 6.14 μ. Other minor differences occur in other regions of the spectrum. In the ultraviolet absorption spectrum λ_{max} 230 mμ is somewhat stronger (log ϵ 4.08) than the maximum at 228 mμ for cucurbitacin B (log ϵ 4.02) and a distinct maximum occurs at 293 mμ (log ϵ 2.26) instead of the inflection at 280 mμ. A Kuhn-Roth determination indicates the presence of six methyl groups capable of yielding acetic acid on oxidation.

When fabacein is hydrogenated in ethyl acetate in the presence of palladium on carbon, from 1 to 1.1 mole of hydrogen was absorbed, and the products melted from 174 to 189°. The ultraviolet spectrum had a single maximum at 287 mμ, log ϵ 2.21, with a minimum at 250 mμ, log ϵ 1.75, and high terminal absorption at 215 mμ, log ϵ 3.40, and the infrared spectrum had bands only at 5.81 and 5.88 μ, indicating loss of the α,β-unsaturated carbonyl system. When the various samples were saponified at room temperature, from 0.4 to 1.0 mole of alkali was consumed, indicating that partial hydrogenolysis of the acetate group had occurred. The saponification

(16) Ref. 13a, p. 226.

product, however, was obtained crystalline in good yields and appears from melting point, mixture melting point, and infrared spectra to be identical with the product of saponification of hydrogenated cucurbitacin B.

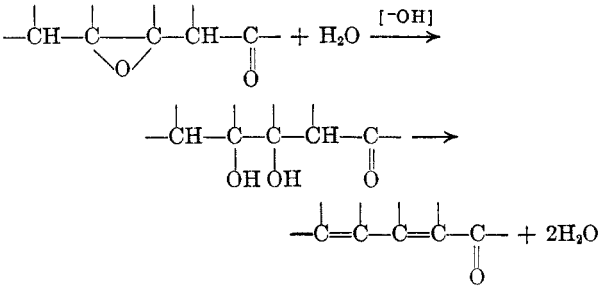
When the hydrogenated fabacein was refluxed with methanolic sodium hydroxide, from 0.6 to 1.0 mole of alkali was consumed. When the saponification product was chromatographed on alumina, only a single compound was obtained which was identical with the crystalline portion of the saponification product of hydrogenated cucurbitacin B. Analyses of this compound indicate that its formula is $C_{28}H_{42}O_6$. It has four reactive hydrogens or one more than the original fabacein, and the same number of terminal methyl groups (five) if one allows for the methyl group present as the acetate group in fabacein. The absorption spectrum of this product, however, is entirely different from that of the hydrogenated fabacein. It has regained an α,β-unsaturated carbonyl system, although one that is different from that present in fabacein or cucurbitacin B. In the ultraviolet region a maximum occurs at 270 mμ, log ϵ 3.92. For the end absorption at 210 mμ, log ϵ is 3.48 and a minimum occurs at 232 mμ, log ϵ 3.04. In the double bond region of the infrared spectrum strong absorption occurs at 5.85 and 6.17 μ, and a very strong band is present at 5.93 μ, whereas hydrogenated fabacein has only two intense bands at 5.81 and 5.88 μ. When cucurbitacin B or fabacein is refluxed with methanolic alkali under the same conditions, the infrared spectrum of the product shows only one strong band at 5.95 or 5.93 μ, respectively, with shoulders at 5.90 and 5.89 μ and slight peaks at 6.03 and 6.02 μ.

Like cucurbitacin B, fabacein in glacial acetic acid absorbed two moles of hydrogen in the presence of Adams catalyst. However no crystalline product could be isolated.

DISCUSSION

Work on the determination of the structures of cucurbitacin B and of fabacein has been extremely difficult because of the ease with which the compounds rearrange and the poor success of attempts to obtain pure crystalline derivatives or degradation products. From the results thus far, the following tentative conclusions may be drawn. Starting with fabacein, $C_{30}H_{44}O_8$, three oxygen atoms probably are present as hydroxyl groups (Zerevitinov), two as carbonyl groups (IR spectrum), and two in the acetate group, leaving one additional oxygen atom. The hydrogen deficiency is 18 indicating nine rings or double bonds. Two double bonds are present as carbonyl groups, one as a carbon-carbon double bond (IR spectrum and hydrogenation), and one in the acetate carbonyl, indicating five rings. The extra oxygen atom may be either in an open-chain ether, in a third carbonyl group or in an oxide ring. Thus four or five carbocyclic rings are indicated.

Catalytic hydrogenation followed by saponification at room temperature gives a product whose analysis corresponds to $C_{28}H_{44}O_7$. Saturation of the carbon-carbon double bond and hydrolysis of the acetate group leads to an additional hydroxyl group (Zerevitinov). Refluxing the hydrogenated fabacein with alkali gives $C_{28}H_{42}O_6$, which differs in composition from the product of saponification at room temperature by a molecule of water and the reintroduction of a conjugated system. The long wave length at which the compound absorbs in the ultraviolet (270 mμ) and the high intensity of the absorption (log ϵ 3.92) suggest the presence of a conjugated dienone system. Conceivably this system could arise by a reaction such as



Four-, five-, or six-membered oxide rings properly joined to a carbonyl group also could lead to a conjugated dienone. Thus the evidence so far suggests the presence of an oxide ring and four carbocyclic rings. Although too much confidence should not be placed in terminal methyl analyses, the values obtained for these compounds are consistent, namely that, exclusive of the acetate group, five moles of acetic acid are formed on oxidation. This result could be accommodated by the carbon skeleton present in ergosterol but not by that of the usual skeletons of tetracyclic triterpenes.

The Zerevitinov determinations indicate that cucurbitacin B has at least one and probably two more hydroxyl groups than fabacein. If this is true, it is difficult to fit cucurbitacin B into the above picture. However, the results for cucurbitacin B were considerably more erratic than for the other compounds, and it is possible that the high values for reactive hydrogen may have been due to enolization of one or both of the carbonyl groups. Hence for the time being it is assumed that cucurbitacin B and fabacein may be isomeric compounds having the same functional groups.

EXPERIMENTAL

Isolation of cucurbitacin B and fabacein. Roots of *Echinocystis fabacea* (man-root, man-in-the-ground, or wild cucumber vine) weighing up to 50 kg. were cut in pieces and put through a Hobart juice press. The plant juice was obtained in a yield of 60–80% of the root. The yield varied with the moisture content of the root. No attempt was made to remove all of the juice and the moist pulp was discarded.

The juice was strained through cheesecloth to remove fines and extracted immediately three times with peroxide-free ether, using volumes equal to the volume of juice. Emulsified portions of the extract were clarified by centrifugation, and the aqueous centrifugate was extracted twice with ether. The combined ether extracts were washed three times with water, dried over sodium sulfate and concentrated by distillation on the steam bath until the solution became cloudy. The remaining solvent was removed under reduced pressure using a water bath whose temperature did not exceed 40°. The residue was dissolved in a minimum volume of petroleum ether (30–60°) and methanol (1v.:1v.). Sufficient water was added to cause separation into two layers and to give a petroleum ether layer approximately equal to the volume used originally. The layers were separated, and the aqueous methanol layer was extracted five times with equal volumes of petroleum ether. Concentration of the petroleum ether extract gave a fatty oil, amounting to about 0.15% of the weight of the juice, which has not as yet been examined.

Concentration of the aqueous methanol extract to dryness gave solids amounting to about 0.6% of the weight of the juice. For the isolation of pure products, the aqueous methanol solution was concentrated on the steam bath at 20-mm. pressure to a semiviscous sirup and placed in the cold room at 2° to crystallize. After 24 to 48 hr. the crystals were removed by filtration. Occasionally crystallization occurred when the original ether extract was concentrated. When this happened the crystals were removed and, after no more crystals could be obtained, the distribution between aqueous methanol and petroleum ether was applied to the residue. Extracts from some roots could not be made to crystallize even after long standing.

Repeated recrystallization from methanol gave either a product melting at 178–179° or one melting at 201–202°. The yield of purified material was 0.05 to 0.15% of the weight of the juice. Roots collected in August when the plants were dormant seemed more likely to yield the higher-melting compound, whereas those collected during the growing season (February to June) usually gave the lower melting compound. This compound has been isolated also from the green vine and leaves of the plant. One root of the species *Echinocystis oregana* dug in the vicinity of Corvallis, Ore., in May[17] gave only the lower-melting compound.

Originally the higher-melting compound was called fabacein I and the lower-melting fabacien II.[10] Direct comparison of fabacein II with cucurbitacin B,[18] however, showed the two to be identical.[19] The higher-melting compound appears to be different from any previously recorded compound, and the name *fabacein* is assigned to it.

Concentration of the mother liquors from the initial crystallizations yields a considerable amount of an amorphous product which is extremely bitter and which gives an infrared absorption spectrum similar to that of cucurbitacin B and fabacein. However, thus far all attempts to obtain a crystalline product from it have failed. Occasionally when the concentrated aqueous methanol extract failed to crystallize, chromatographing on deactivated alumina gave crystalline material melting at 230° which at first was thought to be pure. Its paper chromatogram, however, shows that it is a mixture of several compounds.[19]

Cucurbitacin B. Our sample of cucurbitacin B melted at 178–179°; $[\alpha]_D^{25}$ +87° (c. 0.89 in abs. ethanol). The previously reported values[2a] are m.p. 180–182°; $[\alpha]_D^{25}$ +87.5°. Mixture melting point, infrared spectra, and comparative paper chromatograms of our product with an authentic sample of cucurbitacin B[18] indicate that the two samples are identical.[19]

Numerous analyses of various samples of our material prepared by different persons and crystallized from different solvents have been made in two laboratories.[20]

Anal. Calcd. for $C_{30}H_{44}O_8$: C, 67.64; H, 8.33; for $C_{32}H_{48}O_8$: C, 68.54; H, 8.63. Found: C, 67.31, 66.96, 68.08, 67.90, 67.64, 67.81, 67.80, 68.64, 67.30, 67.22, 65.07, and 66.85; H, 8.37, 8.36, 8.50, 8.56, 8.44, 8.31, 8.34, 8.54, 8.29, 8.27, 8.24, and 8.58. Average of all values: C, 67.38; H, 8.40.

It does not appear to be possible to account for the difference between our results and the values for the $C_{32}H_{48}O_8$ formula[11] on the assumption that our product is contaminated by moisture or solvent (methanol, ethyl acetate, or hexane).

Ultraviolet absorption spectrum[21]: γ_{max} 228 mμ, log ε 4.02; marked inflection between 270 and 290 μ, log ε 2.32 at 280 mμ. Infrared spectrum[21]: strong absorption maxima at 2.92, 5.82, 5.92, and 6.17 μ.[22] Zerevitinov determinations: 0.824 and 0.824% H; for molecular weight 533, equivalent to 4.4 reactive hydrogens per molecule.

A monomethanesulfonate was obtained by mixing 0.533 g. (0.001 mole) with 0.638 g. (0.006 mole) of methanesulfonyl chloride at 0° and adding 0.8 cc. of pyridine. The tube was sealed and then removed from the cold bath and shaken. The solid dissolved, the mixture warmed to about 12°, and the solution became orange in color. The mixture was cooled again to prevent a further rise in temperature and was alternately warmed and cooled until heat no longer was liberated. It then was allowed to stand at room temperature for 1 hr. and the product worked up by pouring the mixture onto ice, washing, and crystallizing from aqueous methanol. The yield of product, m.p. 155°, was 70 mg.

Since instead of esterifying a hydroxyl group, the methanesulfonyl chloride may have replaced an acetyl group, calculated values are given for both possible products and for both the C_{30} and the C_{32} formulas for cucurbitacin B.

Anal. Calcd. for $C_{31}H_{46}O_{10}S$: C, 60.96; H, 7.59; S, 5.25; for $C_{33}H_{50}O_{10}S$: C, 62.04; H, 7.89; S, 5.02; for $C_{29}H_{44}O_9S$: C, 61.25; H, 7.80; S, 5.62; for $C_{31}H_{48}O_9S$: C, 62.39; H, 8.11; S, 5.36. Found: C, 61.37; H, 7.98; S, 5.11.

Thus the analysis agrees best with the composition of a monosulfonate of a C_{30} compound. However, considerable rearrangement has taken place. The maxima of the absorption bands in the infrared spectrum now lie at 5.78 and 5.92 μ, and the intense band at 6.17 μ has been replaced by weak absorption at 6.12 μ. The strong maxima at 7.40 and 8.52 μ may be due to the sulfonate group. If the fabacein is dissolved first in pyridine and then methanesulfonyl chloride is added, a different product is formed which has not yet been purified.

Fabacien. Fabacien is less soluble in methanol than cucurbitacin B and melts at 201–202°; $[\alpha]_D^{25}$ +36° (c. 0.515 in abs. ethanol). Although the melting point is in the neighborhood of those of cucurbitacins A and C, the rotation is different, and it behaves very differently on formamide impregnated paper.[19] Thus with benzene as an irrigant it has an R_F value of 0.8–0.85. On the same strip cucurbitacins A, B, and C have R_F values of about 0.45. With benzene–ethyl acetate (2v.:3v.) as irrigant, fabacein has an R_F

(17) We are indebted to Prof. A. N. Steward of Oregon State College for supplying us with this specimen.

(18) We wish to thank Dr. P. R. Enslin of the National Chemical Research Laboratory of South Africa for sending us authentic samples of cucurbitacins A, B, C, and E (elaterin).

(19) This work was done by Dr. A. Melera at Stanford University. The paper chromatograms were prepared according to the procedure of P. R. Enslin, T. G. Joubert, and S. Rehm, *J. S. African Chem. Inst.*, **7**, 131 (1954).

(20) Microanalyses by Berkeley Analytical Laboratories, Berkeley, Calif., or Huffman Microanalytical Laboratories, Wheatridge, Colo.

(21) All ultraviolet absorption spectra were taken in 95% alcohol using a Beckman Model DU spectrophotometer. All infrared absorption spectra were made using a Perkin-Elmer Model 21 spectrophotometer. The samples were dispersed in potassium bromide disks.

(22) *Cf.* ref. 2b.

value of 0.90, whereas cucurbitacins A, B, and C have values of 0.38, 0.80, and 0.56, respectively.

Anal. Calcd. for $C_{30}H_{44}O_8$: C, 67.64; H, 8.33; for $C_{32}H_{48}O_8$: C, 68.54; H, 8.63. Found: C, 67.86, 66.90, 67.33, 67.62, 67.69, 67.77, 67.88, 67.57, 67.54, and 67.47; H, 8.22, 8.07; 8.19, 8.17, 8.27, 8.08, 8.75, 7.85, 8.34, and 8.31. Average of all analyses: C, 67.56; H, 8.23.

Ultraviolet absorption spectrum: λ_{max} 230 mμ, log ϵ 4.08; 293 mμ, log ϵ 2.26. Infrared spectrum: strong absorption at 2.88, 5.79, 5.85, 5.93, and 6.14 μ. Zerevitinov determinations: 0.549 and 0.508% H; for mol. wt. 533, equivalent to 2.9 and 2.7 reactive hydrogens per molecule. Terminal methyl: 14.4, 14.6%. Assuming a molecular weight of 533 and 85% recovery,[23] these values are equivalent to 6.0 and 6.1 methyl groups per molecule that are capable of yielding acetic acid on oxidation.

Pyrolyses. During attempts to dehydrogenate cucurbitacin B or fabacein with palladium on charcoal, it was observed that a volatile acid was eliminated at the beginning of the reaction. The same acid was lost when these compounds were heated to 250° in the absence of a catalyst and was identified as acetic acid by its Duclaux numbers. Titration of the evolved acid from a weighed sample showed the evolution of 0.78 mole of acetic acid per mole of compound. The residues from the pyrolyses were amorphous and could not be crystallized. They gave a color with ferric chloride. The product from cucurbitacin B gave only a single strong absorption maximum in the infrared at 5.95 μ with weak shoulders at 5.85 and 5.90 μ; that from fabacein gave a broad absorption band in the carbonyl region with slight dips at 5.85 and 5.95 μ, and the double bond absorption at 6.17 μ likewise was lost.

Saponifications. The easy loss of acetic acid on pyrolysis led to attempts to saponify the compounds under mild conditions. Weighed amounts of cucurbitacin B or fabacein were dissolved in methanol cooled to 10°, and an excess of 0.159*N* potassium hydroxide solution was added. The container was swept with nitrogen, stoppered, and allowed to rise to room temperature over 5 to 14 hr. During this time the solution turned yellow. Titration with standard acid indicated that 0.93 to 0.99 mole of alkali was used per mole of compound. The solvent was removed at 40° under reduced pressure and the residue taken up in ether. The ether solution was washed with water, dried, and the ether removed. The products were amorphous and could not be crystallized. The infrared spectrum of the product from cucurbitacin B showed absorption at 5.94 and 6.02 μ whereas that from fabacein absorbed strongly at 5.93 μ with a dip at 5.85 μ and no absorption at 6.02 μ. Neither product absorbed at 6.17 μ, indicating that the α,β-unsaturated carbonyl system was lost.

When either cucurbitacin B or fabacein were refluxed with alcoholic alkali, no further utilization of alkali took place, and the infrared spectra of the products were not appreciably different from those of the products of saponification at room temperature.

Acetylations. Attempts to acetylate both cucurbitacin B and fabacein were carried out with acetic anhydride in pyridine at room temperature for 24 hr., with refluxing for 3 hr., and with the addition of *p*-toluenesulfonic acid as catalyst; with acetic anhydride in acetic acid at room temperature with *p*-toluenesulfonic acid, sulfuric acid, or perchloric acid as catalyst; and with acetic anhydride in acetic acid and sodium acetate as catalyst. When the reaction mixtures were poured onto ice, an oil was obtained which solidified when stirred. The infrared spectra no longer showed the presence of hydroxyl groups, but the α,β-unsaturated carbonyl system was retained. Attempts to crystallize the products from various solvents and solvent mixtures were unsuccessful. Similarly chromatographing on alumina deactivated with 3 to 6% water did not yield any crystallizable fractions.

The product of acetylation of fabacein with acetic anhydride in acetic acid in the presence of *p*-toluenesulfonic acid deserves special mention. In contrast with the behavior of fabacein, the amorphous acetylated product, when allowed to stand with alkali at room temperature, did not consume any base. Nevertheless, the infrared spectrum of the amorphous product had only one strong band at 5.80 μ, with dips at 5.90, 6.02, and 6.14 μ. Thus deacetylation, rearrangement, and reacetylation must have taken place with elimination of the easily saponified acetyl group and rearrangement of the unsaturated system.

Palladium-catalyzed hydrogenations. In a typical run, 100 mg. of 5% palladium on activated charcoal powder (Baker and Co.) suspended in 25 cc. of reagent grade anhydrous ethyl acetate was saturated with hydrogen in a hydrogenation apparatus. To this mixture a solution of 2 g. of cucurbitacin B in 50 cc. of ethyl acetate was added and stirring was continued for 40 min., after which time the absorption of hydrogen had stopped completely. The volume of hydrogen absorbed corresponded to 1.02 mole per mole of compound. In other runs up to 1.36 mole of hydrogen was absorbed. Removal of the catalyst and solvent and crystallization from ethyl acetate–hexane gave products melting from 183° to 186°. Analyses indicated 66.66–70.30% carbon and 8.00–9.09% hydrogen. Frequently the odor of acetic acid could be detected when the solvent was being removed from the hydrogenated product. It appeared therefore that hydrogenolysis of the acetate group was taking place simultaneously with hydrogenation of a double bond. The infrared spectrum showed strong absorption at 5.92 μ with a shoulder at 5.84 μ and loss of the absorption at 6.17 μ.

When different samples were saponified with standard alkali at room temperature, consumption of alkali varied from 0.3 to 0.6 mole per mole of compound, assuming that the hydrogenated product still retained the acetyl group. Evidently partial hydrogenolysis does occur.

The product from the saponification of the hydrogenated cucurbitacin B crystallized well from methanol or 2-propanol. The yield after the first crystallization from methanol was 740 mg. from 1 g. of hydrogenated material. Four more crystallizations from 2-propanol gave 455 mg., m.p. 233–234°; $[\alpha]_D^{25}$ +65.5 (c. 0.67 in abs. ethanol).

Anal. Calcd. for $C_{28}H_{44}O_7$: C, 68.26; H, 9.00; for $C_{30}H_{48}O_7$: C, 69.20; H, 9.29. Found: C, 67.83, 68.04; H, 8.78, 9.10.

Terminal methyl: 13.6%; equivalent to 5.2 methyl groups per molecule capable of yielding acetic acid, assuming 85% recovery.[23] Zerevitinov determination: 0.825 and 0.767% H; equivalent to 4.0 and 3.8 reactive hydrogens per molecule. Ultraviolet absorption: λ_{max} 280, log ϵ 2.21. Infrared absorption: 5.83 and 5.92 μ.

When fabacein was hydrogenated under the above conditions, from 1 to 1.1 mole of hydrogen was absorbed. The products melted from 174 to 189° although the individual samples melted over a 1° range. Carbon analyses varied from 67.28 to 68.53% and hydrogen from 8.05 to 8.83%. When samples were saponified at room temperature, from 0.41 to 0.96 mole of alkali were consumed, assuming the product to be the hydrogenated acetate. The saponification product, however, always was the same and melted after crystallization from 2-propanol at 233–234°.

Anal. Calcd. for $C_{28}H_{44}O_7$: C, 68.26; H, 9.00; for $C_{30}H_{48}O_7$: C, 69.20; H, 9.29. Found: C, 68.25, 68.20, 67.88, 68.37, 68.11; H, 8.74, 9.07, 8.51, 8.44, 8.57; av., C, 68.16; H, 8.67.

No depression was observed when this product was mixed with the similar product from cucurbitacin B, and the infrared spectra of the two products were identical.

When the hydrogenation product from fabacein was refluxed with standard alkali, from 0.63 to 1.0 mole of alkali was consumed. Unlike fabacein, however, the product was different from that obtained by saponification at room tem-

(23) *Cf.* W. G. Dauben and J. H. Richards, *J. Am. Chem. Soc.*, **78**, 5330 (1956); H. Roth in *Methoden der Organischen Chemie*, Georg Thieme Verlag, Stuttgart, Vol. 2, 1953, p. 276.

perature. It crystallized readily from 2-propanol and melted at 268–269°.

Anal. Calcd. for $C_{28}H_{42}O_6$: C, 70.85; H, 8.92; for $C_{30}H_{46}O_6$: C, 71.68; H, 9.22. Found: C, 70.55, 70.80, 70.80; H, 8.48, 8.47, 8.70.

Terminal methyl: 13.5%, equivalent to 5.0 methyl groups capable of yielding acetic acid, assuming 85% recovery. Zerevitinov determination: 0.774, 0.814, 0.807% H, equivalent to 3.67, 3.86, and 3.83 reactive hydrogen atoms per molecule.

Ultraviolet absorption spectrum: $\lambda_{max.}$ 270 mμ, log ε 3.92; end absorption at 210 mμ, log ε 3.48; minimum at 232 mμ, log ε 3.04. Infrared spectrum: maxima of strong intensity at 5.85 and 6.17 μ, and one of very high intensity at 5.93 μ.

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[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY OF THE OHIO STATE UNIVERSITY]

Chitosan Nitrate[1]

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The nitrate ester-salt, the nitrate and perchlorate salts, and the nitrate ester derivatives of chitosan (essentially *N*-deacetylated chitin) have been prepared in stable form. The perchlorate salt of chitosan nitrate was prepared but was quite unstable.

Chitin, a condensation polymer of 2-acetamido-2-deoxy-D-glucopyranose believed to be linked β-(1→4), when subjected to the action of strong alkali[2] affords the corresponding *N*-deacetylated polymer, chitosan. Most chitosan preparations still contain considerable amounts of the *N*-acetyl function. The configurationally *trans* orientation of the acetamido and hydroxyl groups on carbons two and three of the polymeric units makes the acetyl function extremely difficult to remove with alkali. However, considerable success has recently been achieved[3] in reducing the acetyl content of chitosan to a negligible value. Graded hydrolysis of the completely deacetylated chitosan has been employed for the isolation of the D-glucosamine oligosaccharides[3,4] which confirm the β-(1→4) character of the original chitin.

In our investigation of the polymeric nitrate derivatives of chitosan, we chose to employ a reprecipitated preparation of about 85% *N*-deacetylation rather than to subject the polymer to the degradative action required to raise this value significantly. Part of this work was, however, carried out with a chitosan of 97% *N*-deacetylation.

Suspensions of these chitosans in glacial acetic acid containing either perchloric or nitric acid afforded excellent yields of the corresponding water-soluble amine salts. In some cases, sufficient acetic anhydride was added to combine with the water introduced by the mineral acids.

Chitosan (85% free amine) was found to dissolve in absolute nitric acid but the resulting chitosan nitrate was nearly identical to that obtained employing absolute nitric acid admixed with acetic acid and acetic anhydride which reacts heterogeneously with chitosan. Both reaction media afforded the nitric acid salt of chitosan nitrate ester in which approximately 85% of the two available hydroxyl functions were esterified, corresponding to a degree of substitution of 1.7. The heterogeneous method resulted in somewhat higher yields and avoided difficulties in the isolation of the product from the reaction mixture. The conversion of the nitric acid salt of chitosan nitrate ester to the free amino nitrate (unchanged ester content) was accomplished in 80% yield by careful treatment of the salt, dissolved in 50% aqueous acetone, with dilute alkali.

The free amino chitosan nitrate ester was treated with an anhydrous solution of perchloric acid in acetic acid to form the perchlorate salt of chitosan nitrate ester, all samples of which were unstable at room temperature, decomposing slowly in most cases and in one instance with detonation.

Similar results were obtained when chitosan containing 97% free amine groups was employed. However, the drastic conditions utilized to obtain this chitosan were reflected by the deviations in the analyses obtained for the derivatives and by the decreased stability of the perchlorate salt of this chitosan nitrate ester.

At least two factors appear to contribute to the observed instability of the derivatives of chitosan nitrate ester. The polymeric derivatives are structurally related to the nitrate ester-salt derivatives of the mono-, di- and tri-ethanolamines, which monomeric crystalline substances decrease in stability in the order given.[5] The alkaline saponi-

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(2) G. W. Rigby, U. S. Patent **2,040,879** (1936).

(3) Sylvia T. Horowitz, S. Roseman, and H. J. Blumenthal, *J. Am. Chem. Soc.*, **79**, 5046 (1957).

(4) S. A. Barker, A. B. Foster, M. Stacey, and J. M. Webber, *J. Chem. Soc.*, 2218 (1958).

(5) J. Barbière, *Bull. soc. chim. France*, [5], **11**, 470 (1944); Aubry, *Mem. poudres*, **25**, 189 (1932); *Chem. Abstr.*, **27**, 4083 (1933).