[CONTRIBUTION FROM THE NATIONAL INSTITUTE OF ARTHRITIS AND METABOLIC DISEASES, NATIONAL INSTITUTES OF HEALTH, PUBLIC HEALTH SERVICE, U. S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE]

STEVIOSIDE. II. THE STRUCTURE OF THE AGLUCON

ERICH MOSETTIG AND WILLIAM R. NES

Received March 7, 1955

Bridel and Lavieille (1) have shown in a series of excellent investigations that stevioside (I),[1,2] the principal constituent of *Stevia Rebaudiana* Bertoni (the Paraguayan kaà-hê-é), is hydrolyzed by enzymatic material prepared from *Helix pomatia* to "steviol" (IV) and with dilute sulfuric acid to an isomeric aglucon, "isosteviol" (II). "Isosteviol" was also obtained by boiling "steviol" in dilute ethanolic sulfuric acid. By careful analysis of the glucoside and the aglucon the French investigators were able to formulate the hydrolysis as:

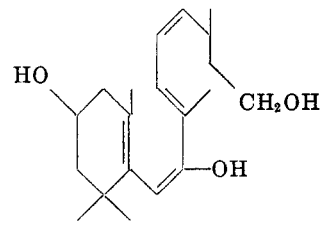
$$C_{38}H_{60}O_{18} + 3H_2O \rightarrow C_{20}H_{30}O_3 + 3C_6H_{12}O_6$$

The sugar moiety was recognized as consisting of three D-glucose units, while "steviol" and "isosteviol" were found to be weakly acidic compounds forming well-defined salts. From the aqueous, alkaline solutions they could be precipitated with carbon dioxide which led the French authors to assume that they are either phenols or compounds containing an acidic hydroxyl group. Since, in contrast to the aglucon and its isomer, the glucoside contained no titratable group, it was concluded that it is the acidic group of "steviol" to which the sugar moiety is attached. The authors did not venture to comment on the nature of the acid-catalyzed isomerization, but they did show that the ultraviolet spectrum of "isosteviol" has a maximum at about 305 mμ[3], while the curve of "steviol" is lacking in a maximum.

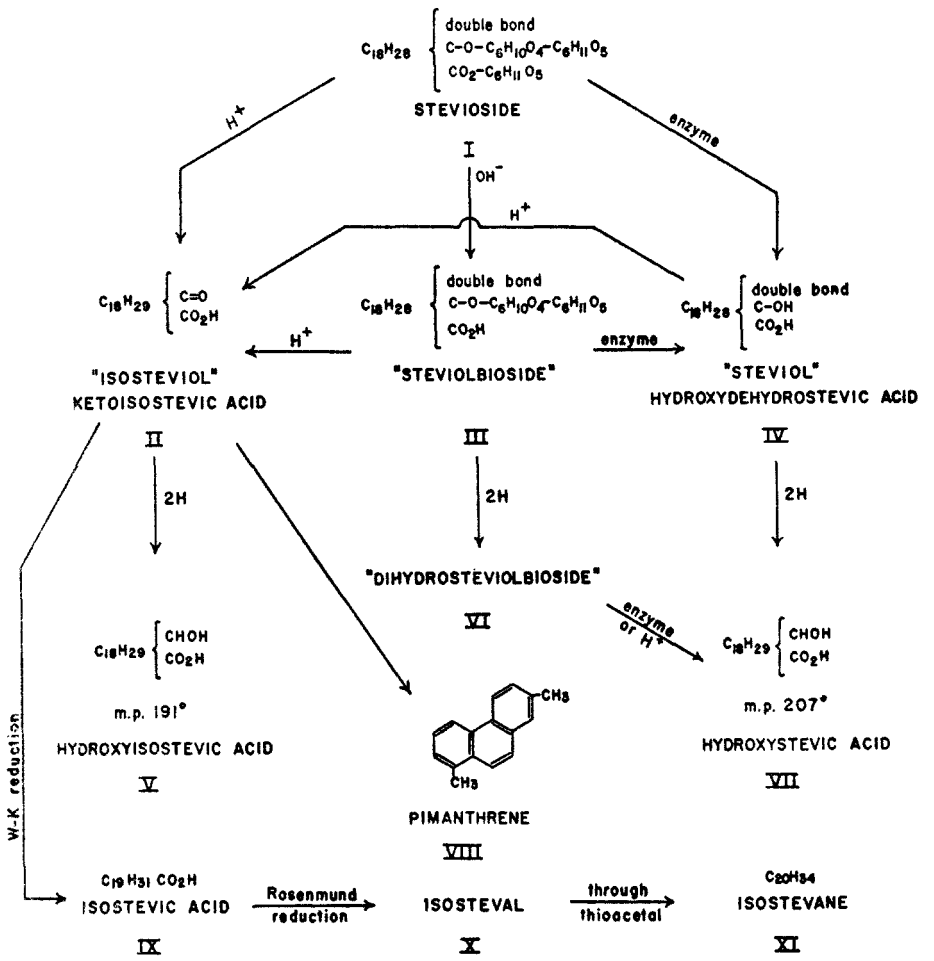
The major part of our investigations concern "isosteviol" (II). This proved to be exceedingly stable; it sublimed readily *in vacuo* and could be distilled under atmospheric pressure without decomposition. It readily formed a semicarbazone indicating the presence of a carbonyl group. That a carboxyl group is responsible for the acidity of the molecule has been shown with diazomethane by the formation of an ester which could be reduced with lithium aluminum hydride

[1] For a short historical review of this glucoside, see the foregoing paper by Wood, Allerton, Diehl, and Fletcher (2).

[2] Very recently Bell (3) has drawn attention to "Stevioside, a unique sweetening agent" and suggested further investigation. He proposes (entirely speculatively) for the aglucon a formula of the type:



[3] ϵ is not given; see ensuing discussion for our $\lambda_{max.}$ and ϵ values.



to an alcohol. By boiling "isosteviol" with acetic anhydride only a relatively unstable mixed anhydride ($\lambda_{max}^{isooctane}$ 225 mμ) with acetic acid was obtained, indicating the absence of an alcoholic hydroxyl group. The presence of the carbonyl group and the carboxyl group and the absence of a hydroxyl group have been confirmed spectroscopically ($\nu_{max}^{CS_2}$ 1742 and 1695 cm.$^{-1}$, $\lambda_{max}^{alc.}$ 293 mμ (ϵ 21). Failure of the ester to hydrolyze under rather drastic conditions indicates a sterically hindered position for the carboxyl group. The maxima at 293 mμ and 1742 cm.$^{-1}$ indicate very strongly that the carbonyl is in a 5-membered ring (4, 5). Neither by chemical nor spectroscopic means could an ethylenic bond be detected in "isosteviol". These findings together with the well-established empirical formula, $C_{20}H_{30}O_3$, allow the conclusion that "isosteviol" is a keto acid (II) derived from a saturated four-ring hydrocarbon.[4]

[4] The realization that "isosteviol" is a keto acid makes it advisable, particularly in view of the difunctional derivatives, to modify the original names introduced by Bridel and Lavieille (1) and, for the time being, to call "isosteviol" ketoisostevic acid as derived from the parent hydrocarbon isostevane ($C_{20}H_{34}$).

With one equivalent of lithium aluminum hydride or catalytically with platinum, ketoisostevic acid (II, "isosteviol") was reduced to a hydroxyisostevic acid (V), and with an excess of lithium aluminum hydride to a dihydroxyisostevane. By the Huang-Minlon modification of the Wolff-Kishner reduction the keto acid II was transformed to isostevic acid (IX). This acid formed a very stable chloride which in the Rosenmund reduction gave isosteval (X) in good yields. This aldehyde showed a remarkable behavior in that after 24 hours the original crystals had converted to a colorless oil; the presence of a carboxyl and a hydroxyl group were apparent spectroscopically. The nature of this spontaneous reaction is being investigated. The aldehyde (X) readily formed a semicarbazone and a diethylthioacetal. The latter strongly resisted desulfurization with Raney nickel. Only with a large excess of the catalyst and by prolonged heating (22 hours in dioxane) could we obtain a sulfur-free product (XI) in a rather poor yield. Although this compound, forming well-developed, sharply melting crystals, is very likely the desired hydrocarbon ($C_{20}H_{34}$, isostevane, XI), we wish to record this experiment with reservation, since lack of material did not allow the desirable repetition. Our hydrocarbon does not appear to be identical with any known hydrocarbon of this composition.

By hydrolysis of stevioside with various snail-enzyme preparations we were able to secure small amounts of the primary aglucon agreeing in every respect with the "steviol" (IV) described by Bridel and Lavieille (1). In agreement with these authors we have found that this compound is isomerized to "isosteviol" (II) with acid. The Zerewitinoff determination showed the presence of two active hydrogens in "steviol" (IV). The infrared spectrum clearly showed the carboxyl group ($\nu_{max}^{CCl_4}$ 1695 cm.$^{-1}$) and, moreover, revealed a hydroxyl group ($\nu_{max}^{CCl_4}$ 3610 and broad absorption 3521–3333 cm.$^{-1}$) and the absence of a keto group. The methyl ester exhibited only the free hydroxyl absorption at 3610 cm.$^{-1}$ (2.77 μ) together with a band at 1730 cm.$^{-1}$ (5.78 μ) for the ester grouping (Fig. 1).

The ultraviolet absorption of "steviol" (IV) is shown in Figure 2 together with that of phyllocladene.[5] A double bond is clearly indicated in "steviol" which cannot be more highly substituted than that in phyllocladene. The extinction coefficients of these two absorptions suggest (6) a di- or trisubstituted ethylenic linkage. Since by ozonization experiments phyllocladene has been shown (7) to have a disubstituted ethylenic linkage $\left(\diagdown\!\!\diagup C{=}CH_2\right)$, it follows that the double bond in "steviol" is also disubstituted. This deduction was substantiated by infrared absorption studies which, furthermore, indicated that the substitution is asymmetric. Phyllocladene showed bands at 3058, 1773, 1656, 1427, and 874 cm.$^{-1}$ in good agreement with the values assigned[6] for the asymmetrically disubstituted ethylenic linkage. All of these bands could also be ascertained in the

[5] Dr. W. I. Taylor of the University of New Brunswick kindly supplied us with a pure sample of phyllocladene.

[6] See reference 4, p. 31.

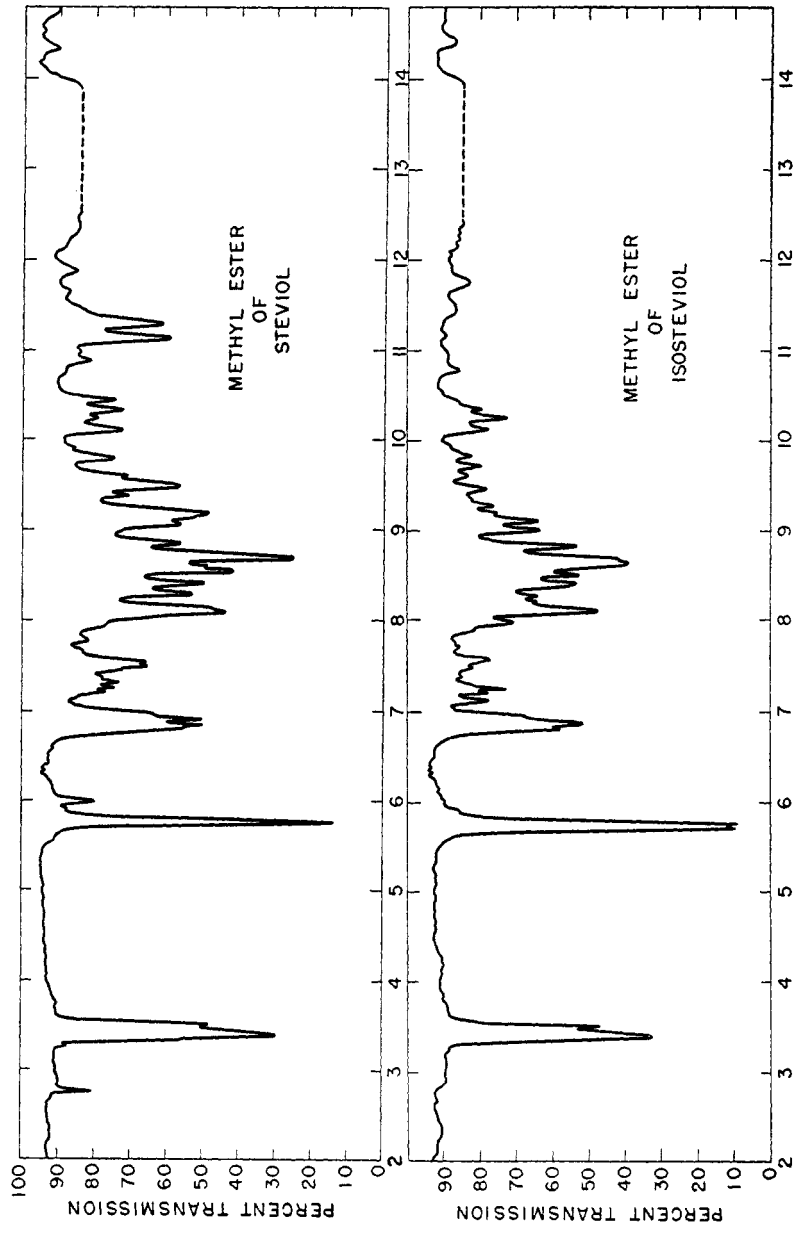


FIG. 1. INFRARED SPECTRA IN CARBON TETRACHLORIDE SOLUTION.

spectra of "steviol" and/or its methyl ester, namely, at 3058 (3.27 μ), 1799 (5.56 μ), 1664 (6.01 μ), 1425 (7.02 μ), and 886 cm.$^{-1}$ (11.29 μ), while the spectra of hydroxystevic acid (VII) and "isosteviol" (II) and its derivatives did not possess this series of bands. We also found that in the unsaturated compounds (phyllocladene, "steviol" and the methyl ester of "steviol") there is a band at 1460 cm.$^{-1}$ (6.85 μ) and one at 1445 cm.$^{-1}$ (6.92 μ), while in the compounds such as "isosteviol" and hydroxystevic acid there is a band only at 1452 cm.$^{-1}$ (6.88 μ). This may be related to the findings of Wiesner, *et al.* (8a) who report that a band at 1440 cm.$^{-1}$ in garryine (possessing the $\diagdown\!\!\diagup C{=}CH_2$ grouping) disappears on hydrogenation. In Figure 1 the spectra of the methyl esters of "steviol" and "isosteviol" are shown and it will be seen that the bands for the various functional groups are quite evident. [The band at 1425 cm.$^{-1}$ (7.02 μ) which is present in the spectrum of "steviol" is obscured in the spectrum of the methyl ester by carbomethoxyl absorption (9).] Since steviol does not appear to be an enol (no

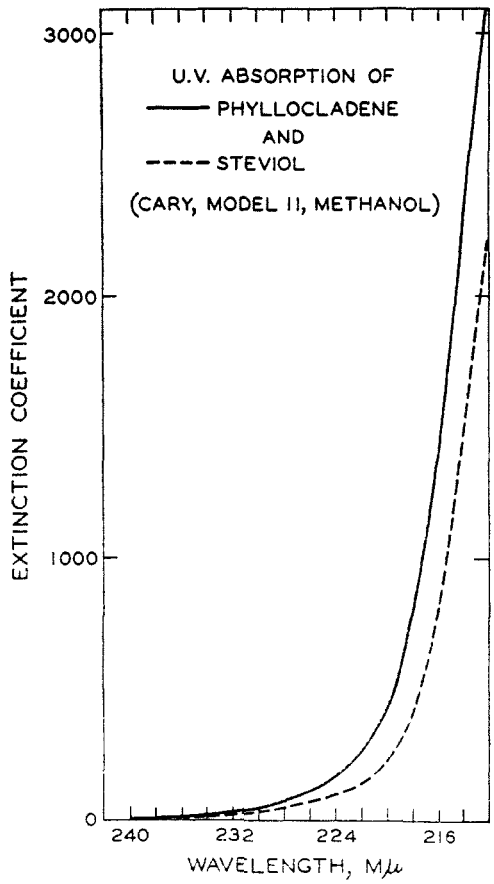


FIG. 2. ULTRAVIOLET SPECTRA IN METHANOL SOLUTION.

color reaction with ferric chloride), these data lead to the partial formula IV, namely a hydroxydehydrostevic acid. Catalytic hydrogenation gave a hydroxystevic acid (VII) isomeric with hydroxyisostevic acid (V) which is obtained in the analogous manner from ketoisostevic acid (II). By either acid or enzymatic hydrolysis of "dihydrosteviolbioside" (VI) we obtained a hydroxystevic acid which could not be differentiated in its infrared absorption from the material (VII) obtained from steviol by reduction. The various samples of hydroxystevic acid, however, differed somewhat in melting point and rotation and their steric homogeneity is therefore in question. The infrared spectrum of hydroxyisostevic acid (V) is quite different from that of VII.

Dehydrogenation of ketoisostevic acid (II, "isosteviol") at 370° with 10% palladium-charcoal yielded pimanthrene[7] (VIII). The melting point of pimanthrene and the melting points of a series of its complex compounds are extremely close to those of 1-ethyl-2-methylphenanthrene. The unambiguous differentiation between these two substituted phenanthrenes is important in that the isolation of 1-ethyl-2-methylphenanthrene would indicate a 17-keto steroid[8] while the isolation of pimanthrene would indicate a terpenoid. We found three distinct differences between authentic samples of the two hydrocarbons: (a) The *s*-trinitrobenzene complex of pimanthrene undergoes a complete polymorphic change from needles to flakes before it melts, while the analogous complex of 1-ethyl-2-methylphenanthrene undergoes only a slight polymorphic change. (b) The U.V. absorption of both hydrocarbons, while similar, can be distinguished. In isoöctane pimanthrene shows a maximum at 326 mμ and an inflexion at 328 mμ. The reverse is true of 1-ethyl-2-methylphenanthrene. Both hydrocarbons show maxima at 340 and 344 mμ, but in pimanthrene the 340 mμ is of higher intensity, while in 1-ethyl-2-methylphenanthrene these maxima are of the same intensity. The most significant difference in the ultraviolet absorption lies in the ratio of intensities of the 350 mμ and 328 mμ bands $\left(\frac{\epsilon 350}{\epsilon 328}\right)$ which is 0.67 for pimanthrene and 1.1 for 1-ethyl-2-methylphenanthrene.[9] (c) The infrared spectra are entirely different (see Figure 3). In all three respects the comparison of our dehydrogenation product (VIII) with authentic samples of these two hydrocarbons leaves no doubt about its identity with pimanthrene.[10]

[7] The total of spectroscopically identifiable phenanthrenes amounted to only *ca.* 5% suggesting quaternary carbon atoms in "isosteviol."

[8] Bachmann and Dreiding (10) have shown that 17-keto steroids are dehydrogenated to give 1-ethyl-2-methylphenanthrene readily and in good yields, the carbon atom of the carbonyl group being lost.

[9] Examination of the literature shows that to a certain extent this observation may be generalized, *i.e.*, the 1,7-dialkylphenanthrenes have a smaller ratio $\left(\frac{\epsilon 350}{\epsilon 328}\right)$ than the 1,2-dialkylphenanthrenes (see experimental section, Table II). This generalization suffers the limitation that the size of the substituent influences this ratio. With increasing size of the alkyl group the ratio decreases.

[10] An extraneous absorption maximum in the spectrum of our sample of pimanthrene at 13.06 μ may be related to an extraneous band at 377 mμ. Askew (11) has called attention to

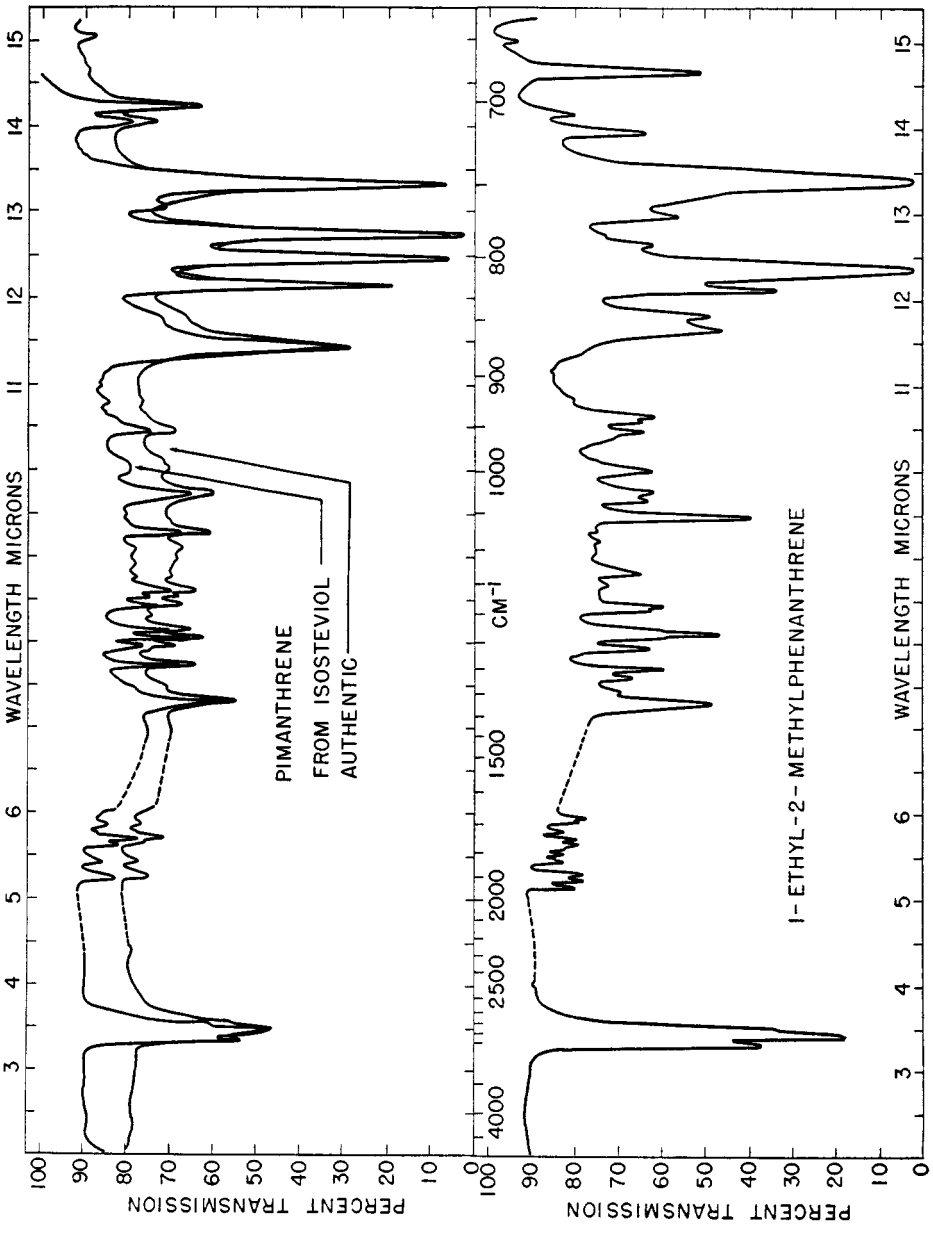
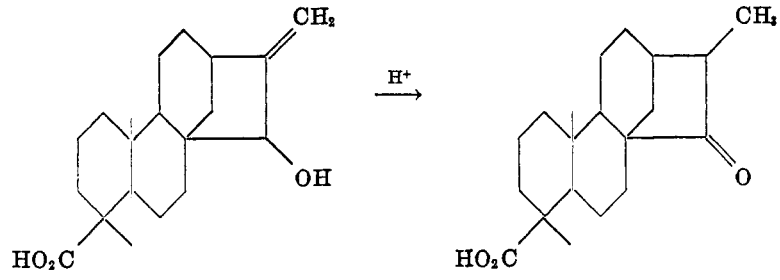


FIG. 3. INFRARED SPECTRA IN CARBON DISULFIDE SOLUTION.

The evidence now at hand indicates that isosteviol possesses the perhydrophenanthrene skeleton to which is attached a five-membered ring which is not, however, at the 1,2-positions as in the steroids; on the assumption that the transformation from "steviol" to "isosteviol" does not involve a carbon migration, we tentatively propose the same basic skeleton for "steviol", *viz.*, A, as for "isosteviol", B.

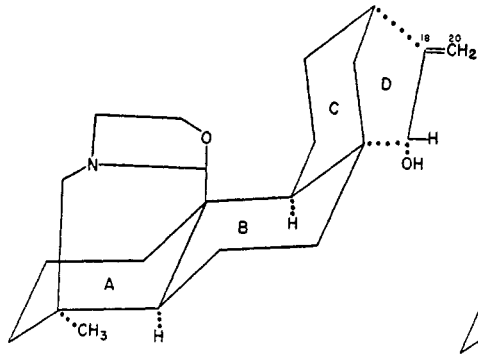


A B

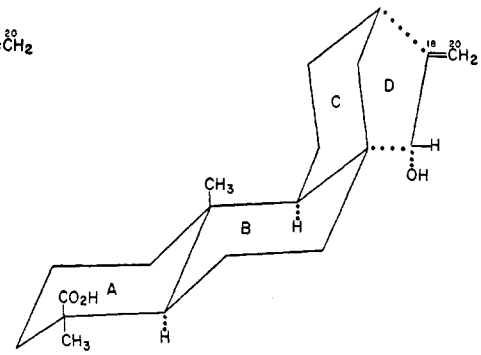
This tentative formulation of the five-membered ring finds an analogy in the assumed structures for the diterpenoid hydrocarbons phyllocladene and isophyllocladene (7) as well as with the diterpenoid alkaloids (8).[11] The position of

such additional bands in the ultraviolet spectra of pimanthrene and other aromatic hydrocarbons which were otherwise quite pure.

[11] Formulas A and B correspond to the skeleton of phyllocladene. Isostevane (XI) does not appear to be a dihydrophyllocladene as our tentative formulation would indicate. This anomaly may be a result of differing skeletal configurations, but further work remains to settle this question.



XII



XIII

On the basis of the ease with which both steviol and laurifoline are isomerized to a ketone, Dr. Djerassi (private communication) has suggested that steviol and laurifoline have the same stereochemistry for rings C and D. Since laurifoline may be represented by XII (Djerassi, Smith, Lippman, Figdor, and Herran, forthcoming paper), steviol may be represented by XIII, if the fusion of rings A and B is the same as in the diterpenoid acids. This interpretation allows an explanation for the nonidentity of hydroxystevic acid (VII) and hydroxyisostevic acid (V). Inspection of molecular models reveals that the β-orientation of the methyl group on C-18 is less hindered than the α-orientation, and, therefore,

the carboxyl is placed as shown only by analogy with the known diterpenoid acids.[12] Despite uncertainties as to the nature of the 5-membered ring and the positions of the functional groups, it seems very likely that steviol is a diterpenoid acid, and as such is remarkable in that it exists in nature as a glucoside.

These studies are being continued in order to throw further light on the structures of "steviol" and "isosteviol".

EXPERIMENTAL[13]

Ketoisostevic acid ["isosteviol", (II)], employed in the following reactions, was obtained essentially according to the direction of Bridel and Lavieille (1) by hydrolysis of stevioside[14] with 10% aq. H_2SO_4. By recrystallization from ether-pentane (3:2), transparent prisms, m.p. 231–233° were obtained and from ethanol, or ethanol-water flakes, melting somewhat lower (230–231°) with a change in crystal form prior to melting; $[\alpha]_D$ −79.3° (95% ethanol); λ_{max}^{EtOH} 293 mμ (ε 21); $\nu_{max}^{CS_2}$ 1742, 1695 cm.$^{-1}$; lit. (1) m.p. 234° (Maquenne block), $[\alpha]_D$ −78° (95% ethanol).

Anal. Calc'd for $C_{20}H_{30}O_3$: C, 75.43; H, 9.50; Active H, 1.0.
Found: C, 75.53; H, 9.53; Active H, 0.96.

The *semicarbazone* melted with decomposition at 288–290°, sintering at 275–280°, after recrystallization from ethanol (sparingly soluble).

Anal. Calc'd for $C_{21}H_{33}N_3O_3$: C, 67.17; H, 8.86; N, 11.19.
Found: C, 67.02; H, 8.83; N, 11.34.

Methyl ketoisostevate was obtained by treating II with excess ethereal diazomethane solution. Recrystallized from acetone-pet. ether or methanol it formed prisms melting at 202–203°, several characteristic transformations of the crystals taking place below the m.p.; λ_{max}^{EtOH} 293 mμ (ε 36).

Anal. Calc'd for $C_{21}H_{32}O_3$: C, 75.85; H, 9.70.
Found: C, 76.05; H, 9.71.

The ester was recovered unchanged after refluxing in ten parts of 10% methanolic KOH solution for 12 hours.

The *semicarbazone* crystallized from ethanol in well-developed prisms melting gradually over the range of 220–255° with decomposition.

Anal. Calc'd for $C_{22}H_{35}N_3O_3$: C, 67.83; H, 9.06; N, 10.79.
Found: C, 67.57; H, 8.97; N, 10.78.

Ketoisostevic-acetic acid anhydride was obtained by boiling II with acetic anhydride for

---

the β-orientation should be assigned to the isomerization product isosteviol (II) and its dihydro derivative (V). On the other hand, the opposite configuration (α) for the methyl group of VII would be expected on hydrogenation of the $\Delta^{18,20}$-double bond of IV, since a hydrogen atom would enter on the less hindered (β) side.

[12] See reference 7(a), p. 374.

[13] All melting points were determined on a Kofler block, and are recorded as read. Rotations were determined at 20° in *ca.* 1% solutions and are accurate to within about 2°. Ultraviolet spectra were determined on a Cary recording spectrophotometer, Model 11, with the assistance of Mrs. Anne Wright. Infrared spectra were determined on a Perkin-Elmer double beam spectrophotometer, Model 21, by Mrs. Phyllis Smeltzer, Mrs. Alma Hayden, and Mr. H. K. Miller. Analyses are by the Analytical Service Laboratory of this Institute under the direction of Dr. William C. Alford.

[14] Highly purified stevioside (I), "steviolbioside" (III), and a large sample of ketoisostevic acid (II) (recryst. from ether-pet. ether) were supplied by Dr. H. G. Fletcher of this laboratory.

3 hours. The excess reagent was removed *in vacuo*, and water was added to the residue. By extraction with ether, and washing the ethereal solution with water and aqueous sodium carbonate solution, the anhydride was obtained and finally recrystallized from benzene-pet. ether giving flat needles, m.p. 123–126°; $\lambda_{max}^{isooctane}$, 225, 279, 297, 307, 318 mμ (ϵ 167, 19, 22, 20, 12). The compound reconverted slowly to II.

Anal. Calc'd for $C_{22}H_{32}O_4$: C, 73.30; H, 8.95.

Found: C, 73.74; H, 9.42.

Hydroxyisostevic acid (V). (*a*) *By catalytic reduction of ketoisostevic acid* (II). A solution of 455 mg. of II in 20 ml. of glacial acetic acid (distilled from CrO_3) was hydrogenated in the presence of 124 mg. of platinum oxide at room temperature and atmospheric pressure. One molecular equivalent of hydrogen was absorbed in 12 hours. The hydroxy acid crystallized from methanol-water as colorless needles, melting at 191–193°, with previous loss of solvent, and a change to another crystalline form beginning at about 145°; yield 310 mg.

(*b*) *By reduction of* II *with lithium aluminum hydride.* To a solution of 220 mg. of II in 25 ml. of ether was added 0.40 ml. of 1.2 *M* ethereal lithium aluminum hydride in portions. A precipitate formed immediately. The solution was stirred 10 minutes at room temperature and 6 *N* aqueous HCl was added gradually until two clear layers resulted. After addition of water the ethereal layer was separated, washed, and dried. The hydroxy acid was crystallized from methanol-water and gave 115 mg. of colorless needles melting at 189–190°. Recrystallization raised the m.p. to 190–191° (previous loss of solvent, and resolidification above 150°). The compound retained its solvent when dried at 80° *in vacuo*, but it was obtained solvent-free by drying for 2 hrs. at 140° at 0.1 mm. This material, when heated did not change below the melting point and showed $[\alpha]_D$ −64° (EtOH). The solvated form showed: ν_{max}^{Nujol} 1704 cm.$^{-1}$ (carboxyl), *ca.* 3400 cm.$^{-1}$ (hydroxyl) and 1653 cm.$^{-1}$. The solvent-free form did not show the latter maximum indicating that this band is attributable to hydrogen bonding on the carboxyl group.

Anal. Calc'd for $C_{20}H_{32}O_3$: C, 74.95; H, 10.06.

Found: C, 75.13; H, 10.22.

The samples of hydroxyisostevic acid from procedures (a) and (b) gave no melting point depression on admixture and had the same infrared spectra.

The *methyl ester* was prepared with excess ethereal diazomethane. It crystallized from methanol-water in fine colorless needles melting at 163–166°.

Anal. Calc'd for $C_{21}H_{34}O_3$: C, 75.40; H, 10.25.

Found: C, 75.26; H, 10.32.

Dihydroxyisostevane. To a solution of 300 mg. of II in 30 ml. of ether was added slowly at room temperature 5 ml. of a 1.2 *M* ethereal lithium aluminum hydride solution, and the reaction mixture was kept refluxing for 24 hrs. The heavy precipitate, which formed immediately, gradually increased. The reaction mixture was decomposed with water and dilute HCl. The ethereal layer was treated twice with dilute KOH, in water to remove acidic material. The dialcohol obtained by evaporation of the ethereal solution was purified by sublimation *in vacuo* and by recrystallization from methanol-water. In neither operation was the melting point of the crude material changed essentially. The m.p. of the analytical sample (by sublimation) was unsharp at about 155°, at least one other crystalline modification being formed before melting.

Anal. Calc'd for $C_{20}H_{34}O_2$: C, 78.38; H, 11.18; active H, 2.0.

Found: C, 78.28; H, 11.15; active H, 2.2.

Isostevic acid (IX). A mixture of 4.0 g. of II, 50 ml. of triethylene glycol, 10 ml. of 95% hydrazine, and 5.0 g. of KOH was distilled until about 5 ml. were removed. The reaction mixture then was kept refluxing at 150° ± 5° for 22 hrs. The condenser was removed, and the temperature was raised rapidly to 200° and maintained for 2 hrs. By pouring the cooled reaction mixture into 650 ml. of water a colored clear solution was obtained. The somewhat gelatinous precipitate which formed on acidification was filtered, dissolved in ether, and the ethereal solution was washed twice with water and dried. The residue obtained by evapora-

tion was recrystallized from methanol-water to give 1.8 g. of clusters of crystals melting at 183–187°. By further recrystallization needles were obtained melting at 191–192° (at *ca.* 150° transition to another needle form), $[\alpha]_D$ −46° (EtOH). The last traces of solvent could be removed only by sublimation (140°/0.08 mm.), but the m.p. was not raised thereby.

Anal. Calc'd for $C_{20}H_{32}O_2$: C, 78.88; H, 10.60.
Found: C, 79.09; H, 10.90.

The *methyl ester* was prepared with diazomethane, and was recrystallized from methanol-water. It formed needles, m.p. 143–144° (transition to another crystal form at *ca.* 135°), $[\alpha]_D$ −50° (EtOH).

Anal. Calc'd for $C_{21}H_{34}O_2$: C, 79.19; H, 10.76.
Found: C, 79.12; H, 10.71.

The *acid chloride* was prepared by refluxing the acid in excess thionyl chloride for 45 min. After crystallization from light petroleum ether it melted at 125–126°.

Anal. Calc'd for $C_{20}H_{31}ClO$: Cl, 10.98. Found: Cl, 10.88.

The acid chloride did not appear to hydrolyze readily, for in subsequent experiments chromatographic fractions which were eluted from an alumina column with methanol showed a band at 1805 cm.$^{-1}$ which is characteristic of —COCl and the material gave a positive Beilstein test for halogen.

Hydroxyisostevane. A solution of methyl isostevate and excess lithium aluminum hydride in ether was refluxed for one hour; no precipitate formed. The reduction product was isolated as described for hydroxyisostevic acid (V). Recrystallization from methanol-water gave colorless needles, melting at 130–131° (transition to another form of fine needles at about 110°), $[\alpha]_D$ +2° (EtOH). The crystals became opaque on drying at 78° *in vacuo*, but the m.p. remained unaltered.

Anal. Calc'd for $C_{20}H_{34}O$: C, 82.69; H, 11.80.
Found: C, 82.70; H, 12.05.

The acid itself, although being in solution, was reduced only to a small extent under these conditions.

The *tosylate* was prepared by allowing a solution of the alcohol and *p*-toluenesulfonyl chloride in pyridine to remain at room temperature for 2 days. The product was obtained by recrystallization from pet. ether in two polymorphic forms, the main portion as fine needles, m.p. 106–107° (resolidifying and remelting at 118–119°), the lesser portion as rosettes, m.p. 118–119°.

Anal. Calc'd for $C_{27}H_{40}O_3S$: C, 72.93; H, 9.07.
Found: C, 72.61; H, 9.08.

Isosteval (X). (*Rosenmund reduction*). Hydrogen was passed through a refluxing mixture of 1.21 g. of the chloride of isostevic acid (IX), 1.03 g. of 5% Pd-$BaSO_4$, and 25 ml. of dry xylene. The rate of evolution of HCl was followed by titration. After 41 minutes 95% of the calculated amount of HCl had been evolved and the reaction was stopped. The semi-crystalline residue, obtained by evaporating the filtered cooled solution, was dissolved in 25 ml. of light pet. ether and adsorbed on a column of 30 g. of alumina (Woelm, "almost neutral", pH *ca.* 6). After elution of a little oily material with 25 ml. of benzene, the aldehyde (608 mg.) was eluted with 75 ml. of the same solvent. Another fraction of 100 ml. eluted an additional 63 mg. of aldehyde. Crystallization from methanol-water yielded 520 mg. of colorless needles melting at 68–70°; $\nu_{max}^{CHCl_3}$ 1715 cm.$^{-1}$, 2732 cm.$^{-1}$. The m.p. did not change appreciably on *rapid* recrystallization. The sample was analyzed within one hour of its preparation.

Anal. Calc'd for $C_{20}H_{32}O$: C, 83.27; H, 11.18.
Found: C, 83.30; H, 11.28.

On standing, the crystalline aldehyde formed oil droplets within a few hours, and had become completely oily after 24 hrs. In chloroform solution this oil showed a sharp band at 3509 cm.$^{-1}$ for free, alcoholic hydroxyl and broad absorption at 3390–2381 cm.$^{-1}$ for carboxylic hydroxyl; the carboxyl also was evident at 1695 cm.$^{-1}$, and a shoulder at 1709 cm.$^{-1}$ may represent some unchanged aldehyde.

The *semicarbazone* was formed readily and melted at 224–226°, when the sample was placed on the heating stage at 200°. Otherwise the m.p. was *ca.* 3° lower.

Anal. Calc'd for $C_{21}H_{35}N_3O$: N, 12.16. Found: N, 12.54.

The *diethyl thioacetal* was prepared by passing dry HCl gas through a solution of 446 mg. of the aldehyde (a few hours after its preparation) in 2.5 ml. of ethyl mercaptan for 30 minutes at 0°. The solution was allowed to stand at room temperature overnight, diluted with chloroform and ether, and successively washed with 0.1 *N* aq. HCl, aq. Na_2CO_3, and water. The dried solution on evaporation left an oil which crystallized from acetone to give 410 mg. of lustrous elongated plates melting at 63–65°. One recrystallization from acetone-water raised the m.p. to 65–66°.

Anal. Calc'd for $C_{24}H_{42}S_2$: C, 73.03; H, 10.73; S, 16.24.

Found: C, 73.14; H, 10.84; S, 16.19.

Isostevane (XI) *(by desulfurization of the diethyl thioacetal)*. A solution of 360 mg. of the above compound in 10 ml. of dioxane was heated under reflux and with stirring with *ca.* 6 g. of wet (EtOH) Raney nickel for 17 hrs. After filtration of the nickel, the dioxane was evaporated *in vacuo* leaving 242 mg. of oil which on treatment with acetone-ethanol-water gave 234 mg. of crystalline material melting unsharply from 49–53°. Two more recrystallizations from acetone-ethanol, and from ethanol raised the m.p. to 60–61°. Further recrystallization did not change this m.p. The compound showed $[\alpha]_D$ +12° ($CHCl_3$) and still contained sulfur. No reasonable empirical formula could be computed from the analytical figures (Found: C, 80.79, 80.75; H, 11.09, 10.93); this substance is possibly a mixture.

The mother liquors from the various recrystallizations were combined, and again treated with a large excess of Raney nickel yielding 65 mg. of an oil which on crystallization from acetone-ethanol-water gave lustrous plates melting sharply at 39–40°, $[\alpha]_D$ +4° ($CHCl_3$). Recrystallization from ethanol-water did not change the melting point.

Anal. Calc'd for $C_{20}H_{34}$: C, 87.51; H, 12.49.

Found: C, 87.21; H, 12.73.

"Dihydrosteviolbioside" (VI). The bioside III (5 g.) was introduced into a flask containing 300 ml. of ethanol and 2.0 g. of 10% palladium-on-charcoal which had previously been saturated with hydrogen. The mixture was shaken in a hydrogen atmosphere at atmospheric pressure and room temperature, and within 20 min. 162 ml. of hydrogen was absorbed (Calc'd 190 ml.). The product which was crystallized from methanol melted at 183–186°. Recrystallization from methanol raised the melting point to 186–188°, $[\alpha]_D$ −37° (EtOH).

Anal. Calc'd for $C_{32}H_{52}O_{13}$: C, 59.61; H, 8.13.

Found: C, 59.49; H, 8.15.

Conversion of hydroxydehydrostevic acid (IV, *"steviol"*) *to ketoisostevic acid* (II, *"isosteviol"*). A solution of 74 mg. of IV in 5.0 ml. of ethanol, 10.0 ml. of water and 0.75 ml. of conc'd sulfuric acid was refluxed for one hour. A few drops of water were added to cloud the solution. The long plates which precipitated melted at 230–231° and weighed 57 mg. The infrared spectrum taken in carbon tetrachloride solution was identical with that of II obtained by hydrolysis of stevioside (I).

Hydroxydehydrostevic acid ("steviol", IV). *(a) From stevioside.* In the enzymatic hydrolysis of stevioside the directions of Bridel and Lavieille (1) were modified as follows. A solution of 2.0 g. of stevioside in 250 ml. of water (boiled previously) to which had been added 5 ml. of freshly prepared "enzyme-juice"[15] and 10 ml. of toluene, was incubated at 34° for 6 days. The *p*H in different experiments varied from 4.8 to 5.3. The slightly colored, and clear solution became opaque and turbid after about 10 hrs., and a fine white apparently amorphous precipitate containing crystalline material appeared. The reaction mixture was exhaustively extracted with ether, and the residue of the washed and dried ethereal solution was recrystallized from ethanol-water or methanol and gave 0.43 g. (54%) of needles melting at 206–208°, $[\alpha]_D$ −74.0° (ethanol). Another crystallization from methanol gave 0.35 g.

[15] This preparation, as well as the "dry preparation" was obtained from the gut of *Helix pomatia* following the directions developed in Prof. Reichstein's laboratory (16).

(44%) of needles melting at 212–213° with a change above 180° to rhomboid plates, $[\alpha]_D$ −93.6° (ethanol), lit. (1) m.p. 215°, $[\alpha]_D$ −94.7° (ethanol). The analytical sample was dried at 100° in a high vacuum.

Anal. Calc'd for $C_{20}H_{30}O_3$: C, 75.43; H, 9.50; Active H, 2.00.

Found: C, 75.48; H, 9.60; Active H, 2.13.

For spectroscopic data see introduction and Figure 2.

(*b*) *From "steviolbioside"* (III). A solution of 0.5 g. of "steviolbioside" (III) in 500 ml. of water was incubated with 1.5 ml. of "enzyme-juice", and the steviol was isolated and purified as above.

The yields of practically pure "steviol" (IV) in the enzymatic hydrolysis of either stevioside (I) or "steviolbioside" (III) and of *hydroxystevic acid* (VII) in the hydrolysis of dihydrosteviolbioside (VI) varied from 50–70%. There was no appreciable difference in yields whether the freshly prepared "enzyme-juice" or a "dry preparation"[15] of the enzyme was used. In our early experiments we made enzyme preparations from the gut of a small Moroccan snail, *Helix lactea*. While we achieved hydrolysis with this preparation, this species is less desirable because of its smallness.

The *methyl ester* of IV, prepared using excess ethereal diazomethane, crystallized from methanol-water as needles which, after being air-dried, melted at 58–60° with recrystallization at 60–70° and a final melt at 111–112°. The compound held solvent tenaciously and only by drying at 100° in a vacuum for 8 hrs. could all solvent be removed. After this treatment the lower melting point was no longer observed.

Anal. Calc'd for $C_{21}H_{32}O_3$: C, 75.85; H, 9.70.

Found: C, 75.62; H, 9.75.

Hydroxystevic acid (VII). Hydroxydehydrostevic acid (IV, "steviol") (153 mg.) was allowed to drop into 20 ml. of ethanol containing 92 mg. of 10% palladium-on-charcoal which was saturated with hydrogen at 20° and 756 mm. pressure. In 40 minutes 13 ml. of hydrogen was absorbed after which no further uptake occurred. Calc'd for one molecular equivalent is 12 ml. The catalyst was filtered out, and the solvent was removed in a vacuum. The residue was crystallized from methanol-water yielding the product as fine needles, m.p. 202–203°, which after being dried at 140° in a vacuum for 13 hrs. melted at 206–208°; $[\alpha]_D$ −70° (EtOH). This material appeared from the analysis to be solvated.

Anal. Calc'd for $C_{20}H_{32}O_3 \cdot \frac{1}{4}H_2O$: C, 74.03; H, 10.02.

Found: C, 74.08; H, 10.19.

Sublimation failed to remove the solvate.

Found: C, 74.01; H, 10.04.

By enzymatic or acid hydrolysis of "dihydrosteviolbioside" (VI) samples having identical infrared spectra (mulls) as that of the above material were obtained. However, the melting points and rotational values were not reproducible to the desired degree of accuracy. Thus, hydrolysis of dihydrosteviolbioside (VI) in 5% aq. sulfuric acid for 2½ hrs. yielded a product melting at 197–200° (dried 18 hrs. at 140° in a vacuum), $[\alpha]_D$ −51° (EtOH) and in another run the product melted at 208–209° when dried similarly, $[\alpha]_D$ −69° ($CHCl_3$).

Anal. Calc'd for $C_{20}H_{32}O_3$: C, 74.95; H, 10.07.

Found: C, 74.85; H, 10.11.

By enzymatic hydrolysis [using the procedure described under the preparation of "steviol" (IV)] the sample which was obtained melted at 212–213° when dried as above, $[\alpha]_D$ −62° (EtOH).

The sample melting at 208–209° depressed the melting point of hydroxyisostevic acid (V), and the infrared spectrum of hydroxyisostevic acid (V) was quite different from that of hydroxystevic acid (VII).

Dehydrogenation of isostevic acid. [*Isolation of pimanthrene* (VIII)]. A mixture of 4.0 g. of II and 2.0 g. of 10% palladium-on-charcoal was heated at 370° in a slow stream of carbon dioxide until gas evolution had become very slow. Thus, in 4 hrs. 1200 ml. (room temp.

TABLE I

COMPARISON OF ULTRAVIOLET ABSORPTION SPECTRA

Isoöctane

λ_{max} (log ϵ)

Pimanthrene (from isosteviol)	Pimanthrene (authentic)	1-Ethyl-2-methylphenanthrene
377 (1.72)[10]	—	—
350 (2.21)	350 (2.12)	350 (2.37)
344 (2.29)	344 (2.20)	344 (2.25)
340 (2.31)	340 (2.23)	340 (2.25)
334 (2.48)	334 (2.46)	335 (2.54)
328‡(2.34)	328‡(2.30)	328 (2.33)
326 (2.34)	326 (2.30)	326‡(2.33)
320 (2.45)	320 (2.43)	320 (2.45)
300 (4.18)	301 (4.20)	300 (4.15)
288 (4.07)	288 (4.06)	288 (4.06)
280 (4.16)	280 (4.15)	280 (4.16)
258 (4.83)	258 (4.81)	258 (4.80)

‡ Inflection.

TABLE II

COMPARISON[a] OF RATIOS[b] OF INTENSITIES OF ULTRAVIOLET ABSORPTION BONDS FOR DISUBSTITUTED PHENANTHRENES

Substituted Phenanthrene	Ratio (ϵ 350/ϵ 328)
1,7-Dimethyl	0.67
1-Methyl-7-*i*-propyl	.53
1-Methyl-7-*sec*-butyl	.45
1-Ethyl-7-methyl	.73
1-Ethyl-7-*i*-propyl	.49
	Average for 1,7-disubstitution = 0.6
1,2-Dimethyl	1.2
1-Ethyl-2-methyl	1.1
1-Methyl-2-*i*-propyl	0.72
	Average for 1,2-disubstitution = 1.0

[a] See footnote 9. [b]Ratios are calculated from the data of (a) Table I, (b) ref. 11, or (c) ref. 15. The value given for pimanthrene is an average from the three sources.

atm. pressure) of gas was collected over 50% aq. KOH. The reaction mixture was extracted with ether, and an aliquot of the extracted material in isoöctane showed a typical phenanthrene spectrum in the ultraviolet region. The intensity of absorption at 258 mμ indicated the presence of *ca.* 0.25 g. of substituted phenanthrenes. The whole ether extract then was evaporated to dryness, dissolved in light pet. ether, and adsorbed on 100 g. of alumina. The material was eluted with two 1-l. portions of light pet. ether containing 5% benzene, and two 1-l. portions of light pet. ether containing 10% benzene. These four fractions contained (determined spectroscopically) 30, 114, 39, and 5 mg. respectively of substituted phenanthrenes. The richest fraction (containing 114 mg.) yielded, when crystallized from methanol-water, 68 mg. of colorless flakes melting at 67–71°. Recrystallization from the same

solvent gave 47 mg. of flakes of m.p. 75–76°. Two more recrystallizations raised the m.p. to a constant 78–79°. A mixture of this material with an authentic sample of 1,7-dimethylphenanthrene (pimanthrene) of m.p. 83–84°[16] melted at 81–82°.

Anal. Calc'd for $C_{16}H_{14}$: C, 93.15; H, 6.84.
Found: C, 92.50; H, 7.20.

The infrared and ultraviolet spectra of this sample and of an authentic sample of pimanthrene are given in Figure 3 and Table I.[10]

The *s-trinitrobenzene complex* melted at 161–162° with a prior change of the needles to flakes above 150°. The mixture with an authentic sample of the complex (m.p. 164–165°, change of crystal form above 150°) melted at 163–164° with a previous change of crystal form above 150°. Lit. (12) m.p. 159–160°.

Anal. Calc'd for $C_{22}H_{17}N_3O_6$: C, 63.00; H, 4.09.
Found: C, 62.67; H, 4.02.

The *picrate* melted at 129.5–130°, lit. (12) m.p. 130–131°.

Anal. Calc'd for $C_{22}H_{17}N_3O_7$: N, 9.65. Found: N, 9.46.

1-Ethyl-2-methylphenanthrene. *d,l*-1,2,3,4-Tetrahydrodesoxy-β-dihydroequilenin was prepared according to the directions of Bachmann, *et al.* (13) and dehydrogenated essentially according to the procedure used by Bachmann and Dreiding (10) for the *d*-enantiomorph. We did not isolate the intermediate *d,l*-desoxyisoequilenin. The dehydrogenation was carried out by heating first with 10% palladium-on-charcoal for about 20 minutes in a stream of carbon dioxide, and then raising the temperature to 360° until the evolution of gas had practically ceased (total heating period of 70 minutes). The 1-ethyl-2-methylphenanthrene crystallized from methanol-water in colorless flakes melting at 78–80°, lit. (10) m.p. 79–80°. A mixture with pimanthrene showed a melting point depression of only 2°. The infrared and ultraviolet spectra, however, were distinctly different from that of pimanthrene (see Figure 3 and Table I).

The *s-trinitrobenzene complex* was recrystallized from ethanol and melted at 154–155°, only a few of the original lemon-yellow needles transforming to flakes above 150°. Lit. (10) m.p. 152–153°. A mixture with the corresponding pimanthrene complex (from II) partially melted at 153–155° with the formation of flakes melting at 158–159°.

The *picrate* melted at 134–135° in agreement with the literature (10, 14).

Acknowledgment. We wish to express our thanks to Dr. R. Ph. Dollfus of the National Museum of Natural History, Paris and to Dr. A. Wettstein of CIBA, Basel for generous supplies of living vineyard snails.

We greatly appreciate the assistance of Robert B. Kostic, of this laboratory, particularly in carrying out extensive dehydrogenation experiments, in maintaining and dissecting the snails, in the various preparations of the enzyme and in the enzymatic hydrolysis experiments.

We wish to thank Dr. T. von Brand and Mrs. M. O. Nolan of the National Microbiological Institute for their help and advice in dissecting the snails.

SUMMARY

The aglucon of stevioside (I), "steviol" (IV), which is obtainable by enzymatic hydrolysis using the juice from the gut of *Helix pomatia* has been shown to be a hydroxy acid containing the $\diagdown C{=}CH_2$ group. "Isosteviol" (II) obtained by

[16] A very generous sample of pimanthrene was obtained through the courtesy of Professor O. Jeger. We purified this sample by recrystallization and chromatography.

acid hydrolysis of the glucoside I is a saturated keto acid with the carbonyl group in a five-membered ring.

From the dehydrogenation mixture of II pimanthrene has been obtained.

II has been subjected to the Wolff-Kishner reduction, and the resulting acid IX converted to the corresponding aldehyde X. Raney nickel desulfurization of the diethylthioacetal of X gives the saturated hydrocarbon $C_{20}H_{34}$.

It is proposed that "steviol" and "isosteviol" are diterpenoid acids with a 2,11-cyclopentanoperhydrophenanthrene skeleton.

BETHESDA 14, MARYLAND

REFERENCES

(1) BRIDEL AND LAVIEILLE, *J. pharm. chim.* [8] **14**, 99, 154, 321, 369 (1931).
(2) WOOD, ALLERTON, DIEHL, AND FLETCHER, *J. Org. Chem.*, **20**, preceding paper (1955).
(3) BELL, *Chemistry & Industry*, 897 (1954).
(4) BELLAMY, *The Infrared-Spectra of Complex Molecules*, John Wiley & Sons, Inc., New York, N. Y., 1954, page 128.
(5) DORFMAN, *Chem. Revs.*, **53**, 50 (1953).
(6) BLADON, HENBEST, AND WOOD, *J. Chem. Soc.*, 2737 (1952).
(7) *Cf.* (a) SIMONSEN, BARTON, AND OWEN, *The Terpenes*, Vol. III, Cambridge University Press, 1952, page 340; (b) BRANDT, *New Zealand J. Sci. Technol.*, **B34**, 46 (1952).
(8) See, for instance, (a) WIESNER, FIGDOR, BARTLETT, AND HENDERSON, *Can. J. Chem.*, **30**, 608 (1952); (b) WIESNER, ARMSTRONG, BARTLETT, AND EDWARDS, *J. Am. Chem. Soc.*, **76**, 6068 (1954); (c) PELLETIER AND JACOBS, *J. Am. Chem. Soc.*, **76**, 4496 (1954); (d) DJERASSI, SMITH, FIGDOR, HERRAN, AND ROMO, *J. Am. Chem. Soc.*, **76**, 5889 (1954); and (e) WIESNER, TAYLOR, FIGDOR, BARTLETT, ARMSTRONG, AND EDWARDS, *Chem. Ber.*, **86**, 800 (1953).
(9) JONES, *J. Org. Chem.*, **19**, 1252 (1954).
(10) BACHMANN AND DREIDING, *J. Am. Chem. Soc.*, **72**, 1323 (1950).
(11) ASKEW, *J. Chem. Soc.*, 509 (1935).
(12) RUZICKA, REY, AND SPILLMANN, *Helv. Chim. Acta*, **25**, 1375 (1942).
(13) BACHMANN, DREIDING, AND STEPHENSON, *J. Am. Chem. Soc.*, **73**, 2765 (1951).
(14) HAWORTH, *J. Chem. Soc.*, 454 (1934).
(15) HEILBRONNER, DANIKER, AND PLATTNER, *Helv. Chim. Acta*, **32**, 1723 (1949).
(16) HUBER, BLINDENBACHER, MOHR, SPEISER, AND REICHSTEIN, *Helv. Chim. Acta*, **34**, 46 (1951).