[JOINT CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY OF WAYNE STATE UNIVERSITY AND THE RESEARCH LABORATORIES OF SYNTEX, S.A.]

Naturally Occurring Oxygen Heterocyclics. IX.[1] Isolation and Characterization of Genipin[2]

CARL DJERASSI,[3] J. D. GRAY, AND FRED A. KINCL

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From the ripe fruit of *Genipa americana* L. there was isolated the "active" principle as a crystalline solid, which has been named genipin. Genipin corresponds to $C_{11}H_{14}O_5$, the oxygen atoms having been characterized in the form of an allylic primary alcohol, a secondary alcohol, and a carbomethoxy function which is conjugated with a cyclic enol ether moiety. Genipin is bicyclic and attention is called to the ready formation of bluish-violet dyes with amino acids.

Genipa americana L. (fam., *Rubiaceae*, subfamily *Cinchonoidea*) is a tree which is widely distributed throughout the Americas from Mexico[4] and the Caribbean region to South America.[5] Its indigenous use has been mentioned from time to time[4,5a] and particularly striking is the report[4] that the juice of the fruit imparts a dark-violet color upon every object with which it comes in contact. In fact, as early as the eighteenth century, Oviedo[6] writes "from the fruit is obtained a clear juice, in which the Indians bathe their limbs and sometimes the whole body, when tired. And also for their pleasure they paint themselves with the juice, which . . . turns everything it touches as black as fine and polished jet, or even blacker; and this dye cannot be removed for fifteen or twenty days or more. . . ." As will be mentioned below, the present three authors can testify from personal experience on their skin to the correctness of these statements.

The above reports as well as the reputed antitubercular activity[7] of extracts of the fruit of *Genipa americana* L. prompted us to undertake a detailed phytochemical study[8] of this fruit. As recorded in the experimental section, the inner part (seeds and connective tissue) of the ripe, fresh fruit afforded by a very simple procedure in nearly 1% yield a beautifully crystalline substance, which we have named genipin.[8] Genipin itself is colorless, but if brought in contact with the skin, it rapidly produces an indelible bluish-violet color. There is little question, therefore, that genipin is responsible for the indigenous use of the fruit. Furthermore, *in vitro* experiments with pure genipin confirmed[9] its antitubercular activity, but *in vivo* results in infected guinea pigs proved negative.

Genipin (m.p. 120–121°) possesses the empirical formula $C_{11}H_{14}O_5$ and is optically active ($[\alpha]_D$ +135°). Functional group analysis indicated the absence of C-alkyl groups, but the presence of one methoxyl substituent. Its infrared spectrum exhibited very strong bands at 5.90 and 6.13 μ as well as hydroxyl absorption, while the ultraviolet absorption spectrum was characterized by a maximum at 240 mμ (log ϵ 4.12).

As indicated above, genipin produces a bluish-violet color on the skin. In fact, this reaction also occurs very readily with amino acids (*e.g.* glycine, leucine, glutamic acid) and the great instability of genipin towards alkali, acid and ketonic reagents—all of them resulting in unidentified colored polymeric material—precluded standard characterization of the carbonyl chromophore responsible for the spectral behavior. However, the optical rotatory dispersion curve of genipin was plain,[10] which strongly suggested that it was an ester rather than a ketone.

Attention was next directed towards the hydroxyl function(s), whose presence was indicated

(1) Paper VIII, R. A. Finnegan, B. Gilbert, E. J. Eisenbraun, and C. Djerassi, *J. Org. Chem.*, in press.

(2) The work at Wayne State University was supported by the National Heart Institute (grant No. H-2574) of the National Institutes of Health, U. S. Public Health Service.

(3) Inquiries and reprint requests should be addressed to Department of Chemistry, Stanford University, Stanford, California.

(4) (a) P. C. Standley, *Trees and Shrubs of Mexico*, Contribution from the U. S. Herbarium, Government Printing Office, Washington, D. C., 1926, Vol. 23, p. 1371. (b) F. Miranda, *La Vegetacion de Chipas*, Departamento de Prensa y Turismo, Tuxtla Gutierrez, Chiapas, 1952, Part II.

(5) (a) F. C. Hoehne, *Plantas e Substancias Vegetais Toxicas e Medicinaes*, Graphicars, Sao Paulo, Brazil, 1939, p. 273. (b) A. Weberbauer, *El Mundo Vegetal de los Andes Peruanos*, Ministerio de Agricultura, Lima, Peru, 1945, p. 599.

(6) B. V. Oviedo, *Cualidades y Riquezas del Nuevo Reino de Granada*, eighteenth century manuscript published by L. A. Cuervo, Biblioteca de Historia Nacional, Bogota, 1930.

(7) Private communication from Lic. A. L. Monserrate, San Juan, Puerto Rico (1955).

(8) T. Peckolt, *Z. Oesterr. Apoth.-Ver.*, **34**, 227 (1896) has examined this plant in Brazil and reported the occurrence of mannitol, and a variety of unidentified crude materials. Peckolt also mentions the isolation of some crystals to which the name genipin was given. As the latter was not characterized and no melting point was recorded, we feel justified in employing this name for the pure constituent isolated by us.

(9) Private communication from Dr. C. Casas Campillo (Syntex, S.A.).

(10) See C. Djerassi, *Optical Rotatory Dispersion. Applications to Organic Chemistry*, McGraw-Hill Book Co., New York, 1960.

by the infrared spectrum. Acetylation produced a noncrystalline diacetate, which now lacked further hydroxyl absorption in the infrared. Attempts to saponify this diacetate were unsuccessful, because of the extreme lability of genipin towards base or indeed even boiling with water. Eventually a crystalline bis-3,5-dinitrobenzoate was secured whose analysis confirmed definitely the presence of two hydroxyl groups in the molecule. Further insight was gained by treatment of genipin with triphenylmethyl bromide in pyridine solution, leading in excellent yield to a crystalline monotrityl ether, which in turn could be acetylated to a crystalline monotrityl ether monoacetate. It follows that genipin possesses one primary hydroxyl group, while the other one is presumably secondary in view of its ease of acetylation.

Genipin trityl ether was considerably more stable than genipin. Thus it was recovered unchanged upon oxidation with manganese dioxide[11] in chloroform and no reaction was observed upon treatment with hydroxylamine hydrochloride in pyridine solution. It can be concluded that the secondary hydroxyl group of genipin is not allylic and that no ketonic function is present in the molecule, a conclusion in accordance with the rotatory dispersion behavior. Combined with the methoxyl analysis, the carbonyl group can now be attributed tentatively to a carbomethoxy function and, on the assumption that there is present no other carbonyl group in the molecule (*viz.*, negative reaction of genipin trityl ether with hydroxylamine), the infrared and ultraviolet absorption spectra then require that this carbomethoxy group be conjugated. Consequently, attempts were made to hydrogenate genipin in order to obtain further information about the unsaturated carbonyl chromophore and to determine at the same time the number of unsaturated linkages.

Hydrogenation with palladium charcoal in methanol solution resulted in the uptake of approximately two equivalents of hydrogen. The resulting product represented a mixture which at this stage was not separated, but its ultraviolet absorption spectrum was hardly affected ($\lambda_{max}^{C_2H_5OH}$ 258 mμ) and the infrared spectrum still exhibited two very strong bands at 5.88 and 6.10 μ, indicating that the unsaturated ester chromophore was not attacked. On the other hand, the crude product by Kuhn-Roth oxidation now showed the presence of one C-methyl group (the starting material possesses none), leading to the conclusion that genipin contains an allylic primary alcohol grouping which had undergone hydrogenolysis as well as reduction of the double bond. When genipin was exposed to platinum oxide in glacial acetic acid, then, depending upon the conditions, between two to four equivalents of hydrogen were consumed and the resulting material exhibited no high selective ultraviolet absorption, indicating the final reduction of the α,β-unsaturated carbonyl chromophore.

Such resistance of an α,β-unsaturated ester toward hydrogenation is unexpected; furthermore, the ultraviolet absorption maximum at 240 mμ is much too high for an ordinary unsaturated ester[12] and the infrared double bond peak near 6.10 μ much too intense. On the other hand, esters of type —O—C=C—CO_2CH_3 are characterized by precisely this behavior[13]: their ultraviolet absorption spectra are contain by an absorption maximum in the 240 mμ region, they exhibit a very strong infrared band around 6.1 μ associated with the enol ether grouping and finally such a double bond shows considerable resistance toward standard hydrogenating conditions.

If genipin contained the grouping —O—C(=)—C—CO_2CH_3 then together with the two hydroxyl groups discussed above, this would account for all five oxygen atoms of the molecule. Consequently, the above hydrogenations were repeated in the following fashion without attempting a major purification of the initially formed complex reaction mixture:

Genipin was reduced with a palladium catalyst, which should result in hydrogenolytic removal of the primary hydroxyl group. Chromatography on alumina effected a rough separation between nonpolar and polar material and the former was now submitted to hydrogenation with platinum oxide and acetic acid at room temperature. Saponification of the total reduction product afforded an acid—the first direct chemical evidence for the presence of an ester function in genipin—which was re-esterified with diazomethane and fractionally distilled. The lower boiling material represented an oil, whose analysis corresponded to $C_{11}H_{18}O_3$ with one methoxyl and one C-methyl group. The infrared spectrum now contained its principal carbonyl band at 5.76 μ without any hydroxyl absorption, indicating that the second hydroxyl group was also lost by hydrogenolysis. A shoulder at 5.85 μ and a relatively small band at 6.08 μ suggested that the unsaturated ester had not been reduced completely and when the hydrogenation with platinum oxide was repeated at 30°, these two bands disappeared. Repeated saponification of this ester (no ultraviolet absorption maximum) now led to an acid, which formed a beautifully crystalline *S*-benzylthiuronium salt and whose analysis was completely compatible with a salt of an acid of empirical formula $C_{10}H_{16}O_3$. The ester of this acid exhibited no hydroxyl absorption in the infrared and could be recovered unchanged upon oxidation with chro-

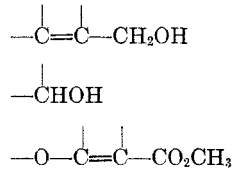
(11) R. M. Evans, *Quart. Rev.*, **13**, 61 (1959).

(12) A. J. Nielsen, *J. Org. Chem.*, **22**, 1539 (1957).

(13) See F. E. Bader, *Helv. Chim. Acta*, **36**, 215 (1953); O. Halpern and H. Schmid, *ibid.*, **41**, 1109 (1958).

mium trioxide. We can conclude, therefore, that the third oxygen atom is involved in an ether linkage. As the acid $C_{10}H_{16}O_3$ is now completely saturated, *genipin must be bicyclic.*

The above reported experiments can now be summarized in terms of the following partial structure for genipin:



which accounts for all of the oxygen atoms and for eight out of the eleven carbon atoms of the substance. These structural features must be incorporated into a bicyclic structure (the ether oxygen being involved in one of the rings) and the correct juxtaposition of the various functional groups will have to account for the remarkable chemical properties of genipin: its lability to acid or base, the formation of colored complexes with amino acids, and finally its apparently rather complex behavior upon hydrogenation. In connection with the last point, it may be pertinent to mention that the reaction of genipin with other reducing agents such as lithium aluminum hydride is exceedingly complicated and leads to a mixture of hitherto unidentified C_{10} products ranging in oxygen composition from three oxygen atoms to one. While the last mentioned product is formed in only small amounts, the loss of four oxygen atoms in such a hydride reduction is remarkable.

Further studies on genipin are in progress.

EXPERIMENTAL[14]

Isolation of genipin. The ripe[15] fresh fruit of *Genipa americana* L. was collected in May 1955 by Sr. S. Fuentes (Botanical Department, Syntex, S.A.) near Tuxtepec, State of Oaxaca, and separated manually into the outer part (*ca.* 40–50% by weight) and the inner portion. The latter, consisting of seeds and connective tissue, was not dried but rather extracted thoroughly with ether. Evaporation of the ether to dryness and digestion with hexane to remove adhering oily material afforded in 0.9–1.0% yield crude genipin, m.p. 108–112°. Genipin can be chromatographed on 1:1 silica-Celite and eluted with benzene-ether (8:2), but purification is best accomplished by recrystallization from ether-methanol and pure methanol, whereupon it is obtained in the form of colorless prisms with the following properties: m.p. 120–121°, $[\alpha]_D$ +135° (methanol), plain rotatory dispersion curve[16] in dioxane (*c*, 0.10): $[\alpha]_{650}$ +60°, $[\alpha]_{589}$ +105°, $[\alpha]_{500}$ +172°, $[\alpha]_{400}$ +395°, $[\alpha]_{350}$ +700°, $[\alpha]_{300}$ +1720°, $[\alpha]_{275}$ +3400°; $\lambda_{max}^{C_2H_5OH}$ 240 mμ, log ε 4.12, $\lambda_{max}^{CHCl_3}$ 2.78, 2.99, 5.90 (s), 6.13 (s) μ. Genipin gives no color with alcoholic ferric chloride, but when placed on the skin it produces an indelible blue stain. Alternatively, genipin can be dissolved in methanol, a 1% aqueous solution of an amino acid (glycine, leucine, glutamic acid) added and the mixture warmed on the steam bath, whereupon a color develops from yellow through deep red to blue and violet; ultimately a fine blue precipitate separates.

Anal. Calcd. for $C_{11}H_{14}O_5$: C, 58.40; H, 6.24; O, 35.36; methoxyl, 13.72. Found: C, 58.55; H, 6.57; O, 35.08; methoxyl, 13.69; no C-methyl group.

Genipin diacetate was prepared by leaving a sample of genipin in acetic anhydride–pyridine (2:1) solution overnight at room temperature. After processing in the usual manner, the product was chromatographed on Merck acid-washed alumina and eluted with benzene. As the substance failed to crystallize it was distilled twice at 150°/0.2 mm. before being submitted for analysis; $[\alpha]_D$ +60° (methanol), $\lambda_{max}^{CHCl_3}$ 5.65 (inflect.), 5.70, 5.81, and 6.02 μ; $\lambda_{max}^{C_2H_5OH}$ 234.5 mμ, log ε 3.94. The hypsochromic shift in the ultraviolet absorption spectrum suggests some interaction between the chromophore and one of the acetate groups.

Anal. Calcd. for $C_{15}H_{18}O_7$: C, 58.06; H, 5.85; O, 36.09; methoxyl, 10.0. Found: C, 57.97; H, 5.82; O, 35.77; methoxyl, 10.36.

The *bis-3,5-dinitrobenzoate* was obtained by treating 200 mg. of genipin in benzene solution with an excess of 3,5-dinitrobenzoyl chloride in the same solvent, adding 1 cc. of pyridine and heating the mixture at 80° for 30 min. Ether was added, the solution was washed with dilute acid, dilute sodium hydroxide, water, dried, and evaporated. Recrystallization of the residue from methanol afforded the desired derivative, m.p. 149–150°.

Anal. Calcd. for $C_{25}H_{18}N_2O_{15}$: C, 48.90; H, 2.95; N, 9.13; O, 39.10; methoxyl, 5.05. Found: C, 49.00; H, 3.11; N, 9.36; O, 38.90; methoxyl, 5.29.

Genipin trityl ether. A solution of 200 mg. of genipin was added to a 10% excess of triphenylmethyl bromide dissolved in pyridine and the mixture was heated on the steam bath for 30 min. It was then poured into water, extracted with ether, and washed thoroughly with water. The solvent was removed and the residue was maintained at 30°/2.5 mm. before chromatographing on Merck acid-washed alumina. Elution with hexane-benzene (1:1) removed triphenylcarbinol while benzene-ether (1:1) provided the trityl ether in over 90% yield. Recrystallization from hexane-benzene or from methanol afforded colorless crystals, m.p. 175.6–176.5°, plain optical rotatory dispersion curve (*c*, 0.073 in dioxane): $[\alpha]_{589}$ +55°, $[\alpha]_{500}$ +90°, $[\alpha]_{400}$ +165°, $[\alpha]_{350}$ +284°, $[\alpha]_{300}$ +500°, $[\alpha]_{287.5}$ +685°.

Anal. Calcd. for $C_{30}H_{28}O_5$: C, 76.90; H, 6.02; O, 17.08; methoxyl, 6.63. Found: C, 77.41; H, 6.02; O, 16.77; methoxyl, 6.83.

The trityl ether was recovered unchanged when it was stirred at room temperature for 60 hr. in chloroform solution with ten times its weight of manganese dioxide or when 100 mg. of it was heated under reflux for 2 hr. with 400 mg. of hydroxylamine hydrochloride, 2 cc. of pyridine, and 2 cc. of ethanol.

The *trityl ether acetate* was obtained in nearly quantitative yield after letting the trityl ether stand overnight with pyridine–acetic anhydride; m.p. 192.5–193.5° after recrystallization from ethanol.

Anal. Calcd. for $C_{32}H_{30}O_6$: C, 75.27; H, 5.92; O, 18.80. Found: C, 74.94; H, 6.00; O, 18.48.

Catalytic hydrogenation of genipin. A methanolic solution of 5.0 g. of genipin was hydrogenated at room temperature and atmospheric pressure in the presence of 1.25 g. of 10% palladized charcoal, 2 molar equivalents of hydrogen having been consumed within 3 hr. While chromatography on silica gel effected no separation, chromatography on Fisher alumina was more effective and benzene eluted 1.525 g. of oil which still possessed the typical intense infrared bands at 5.85 and 6.08 μ. An additional 1.5 g. was eluted with

(14) Melting points were determined on the Kofler block. The infrared spectra are due to Mrs. Dolores Phillips, while the microanalyses were performed by A. Bernhardt, Mülheim, Germany, and J. F. Alicino, Metuchen, New Jersey.

(15) Unripe fruit did not contain any appreciable quantity of genipin, but rather a blue dye, which may be derived from genipin.

more polar solvents, but this material was not used for the following transformation.

The benzene eluted 1.5-g. sample was dissolved in acetic acid and shaken overnight at room temperature with 0.4 g. of platinum oxide catalyst. The product was saponified with 5% aqueous ethanolic potassium hydroxide for 24 hr., concentrated, and water added. Extraction with ether left no residue and acidification of the ether solution followed by extraction with ether, extraction with bicarbonate, acidification, and re-extraction with ether furnished, after drying and evaporation, 0.86 g. of acid.

A 0.55-g. sample of this acid was esterified with ethereal diazomethane solution and the crude ester was fractionated by distillation affording 0.31 g. of material with b.p. 60°/0.01 mm. and 0.20 g. with b.p. 93°/0.01 mm. The lower boiling fraction exhibited no infrared hydroxyl absorption, a strong band at 5.76 μ, a shoulder at 5.85 μ and a relatively weak band at 6.08 μ.

Anal. Calcd. for $C_{11}H_{18}O_3$: C, 66.64; H, 9.15; O, 24.21; C-methyl, 7.57; methoxyl, 15.66. Found: C, 66.81; H, 8.80; O, 24.70; C-methyl, 6.48; methoxyl, 16.30.

As the infrared spectrum indicated the presence of some remaining unsaturated ester, 250 mg. of the above analytical sample was again hydrogenated with acetic acid and platinum oxide at 30° and the ester isolated in the usual way; $\lambda_{max}^{capill.}$ 5.75 μ, no selective ultraviolet absorption. A 200-mg. aliquot of the ester was saponified by heating under reflux for 5 hr. with 5% ethanolic potassium hydroxide affording 120 mg. of acid, which was dissolved in 2.5 cc. of water; 2 drops of phenolphthalein indicator was added followed by 1*N* aqueous sodium hydroxide until a faint pink color persisted after heating to about 80°. Two drops of 0.1*N* hydrochloric acid was added to make the solution slightly acidic followed by the dropwise introduction of 10.5 g. of *S*-benzylthiourea in 2.5 cc. of water. The *S*-benzylthiuronium salt precipitated immediately and, after cooling in ice, it was filtered and recrystallized from water; m.p. 135.5–136.5°.

Anal. Calcd. for $C_{18}H_{26}N_2O_3S$: C, 61.75; H, 7.43; N, 8.00; O, 13.72; S, 9.15. Found: C, 61.55; H, 7.40; N, 8.27; O, 13.64; S, 8.97.

DETROIT, MICH.
MEXICO, D. F.

[CONTRIBUTION FROM THE PROCESS DEVELOPMENT AND RESEARCH DEPARTMENT OF THE SCHERING CORP.]

Microbiological Transformation of Steroids. VIII. 16β-Hydroxylation and Other Transformations of Testosterone by *Wojnowicia graminis*

HERSHEL L. HERZOG, MARGARET JEVNIK GENTLES, ALVIN BASCH, W. COSCARELLI, MARY ELLEN ATNARISH ZEITZ, AND WILLIAM CHARNEY

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Incubation of testosterone with *Wojnowicia graminis* (CBS) affords a variety of transformation products, including 16β-hydroxytestosterone, 16α-hydroxytestosterone, 16-ketotestosterone, 6β-hydroxytestosterone, 6β-hydroxy-4-androstene-3,17-dione, 14α-hydroxy-4-androstene-3,17-dione, 4-androstene-3,17-dione, and two additional, incompletely characterized crystalline products, one of which appears to be hydroxylated at 12α-. From a limited study of the action of the same organism on 4-androstene-3,17-dione, testosterone, and 16α-hydroxytestosterone were isolated. This is the first example of microbiological 16β-hydroxylation.

From the work of McAleer and Dulaney[1] it is known that *Wojnowicia graminis* (CBS) hydroxylates progesterone at C_{21}, affording thereby desoxycorticosterone. We speculated that hydroxylation of a steroidal substrate of only 19 carbon atoms might also occur preferentially at a methyl site rather than a methylene or methylidyne site, and we might thereby effect 18- or 19-hydroxylation. With this in mind we have incubated testosterone (I) with *W. graminis* in a 1% yeast extract-1% cerelose medium for seventy-two hours and isolated the steroidal products by extraction with chloroform.

After very careful chromatography over Florisil and in a toluene–propylene glycol partition system, we were able to isolate nine crystalline transformation products (in addition to recovered starting material), and to identify completely seven of them. These, in order of increasing polarity with respect to partition in the toluene–propylene glycol paper chromatographic system, were: 4-androstene-3,17-dione (II), uncharged testosterone (I), 16-ketotestosterone (III), 6β-hydroxy-4-androstene-3,17-dione (IV), 14α-hydroxy-4-androstene-3,17-dione (V), 16β-hydroxytestosterone (VI), 6β-hydroxytestosterone (VII), and 16α-hydroxytestosterone (VIII).

Identifications of I and II were easily accomplished by comparison of melting point, paper chromatographic mobilities and infrared spectra with those of authentic samples.

Our sample of III had m.p. 153–155°, $[\alpha]_D^{24}$ −54° (chloroform), $\lambda_{max}^{CH_3OH}$ 240 mμ (ϵ = 16,600). A five-membered ring carbonyl band was noted in the infrared spectrum at 5.70 μ. Both blue and red tetrazolium reagents gave positive tests with III, indicating the presence of an α-ketol system. Since the ultraviolet spectrum of III did not shift in alkaline solution,[2] the ketol must have been in the D-ring. Meyer and Lindberg[3] describe 16-ketosterosterone, m.p. 152–158°, $[\alpha]_D^{25}$ −52° (chloroform). Upon

(1) W. J. McAleer and E. L. Dulaney, *Arch. Biochem. Biophys.*, 62, 109 (1956).

(2) A. S. Meyer, *J. Org. Chem.*, 20, 1240 (1955).

(3) A. S. Meyer and M. C. Lindberg, *J. Am. Chem. Soc.*, 76, 3033 (1954).