Tetrahedron, 1964, Vol. 20, pp. 1781 to 1787. Pergamon Press Ltd. Printed in Northern Ireland

TWO NEW ANTIBIOTIC CYCLOPENTANOID MONOTERPENES OF PLANT ORIGIN

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(*Received* 16 *February* 1964; *in revised form* 14 *April* 1964)

Abstract—Two antibiotics have been isolated from *Genipa americana* L. The structure of the more abundant one, for which the name genipic acid is proposed, has been established as I by conversion to the known lactonic acid IV. The properties of the minor antibiotic, named genipinic acid, show that it is a carbomethoxyl derivative of genipic acid, and evidence is presented in support of its formulation as II.

THE presence of antibiotics in Puerto Rican jagua fruit [*Genipa americana* L., family Rubiaceae], long suspected because of its native uses and its resistance to rotting, has been confirmed experimentally by investigators at the University of Puerto Rico.[1] This report describes two broad spectrum antibiotics which have been isolated from the dried fruit in yields of 0·14% and 0·08% respectively and for which the names *genipic acid* (I) and *genipinic acid* (II) are proposed. Final separation and purification of the antibiotics were achieved by consecutive countercurrent distributions in a Craig–Post apparatus with two different solvent systems (Table 1). Homogeneity was established by the coincidence in the case of each antibiotic in each solvent system of the peaks located by titration, bioassay and residue weight determinations. Moreover, there was excellent agreement between the experimental curves plotted from residue weights and those calculated[2] for pure substances with the distribution coefficients given in Table 1 (e.g., see Fig 1). The products were obtained as hygroscopic amorphous white powders by removal *in vacuo* of the organic portion of the solvent from the combined contents of the tubes in the vicinity of the peaks and lyophilization of the remaining aqueous solutions.

The analytical data for genipic acid (I), its ammonium salt and its methyl ester all supported the molecular formula $C_9H_{12}O_4$ for I. The acid gave a positive Tollens test for an aldehyde group. The absence of the band expected for such a group in the 500–600 c/s region of its NMR spectrum and the occurrence instead of bands near 350 c/s for the parent acid and its derivatives (Table 2) suggested a hemiacetal structure for I. This was confirmed by the presence in the spectrum of the ammonium salt of a weak band at 575 c/s, the integration of which indicated the existence of about 20% of the free aldehyde form in the deuterium oxide solution, and by the mutarotation of an alkaline solution of I. The high frequency UV absorption band (λ_{max} 203, ε 2840) of genipic acid (I) established the presence of an isolated double bond. With the NMR band near 350 c/s accounted for by the hydrogen atom on the potential aldehydic carbon atom, there was no other NMR band attributable to a

[1] R. Cardova Marquez, J. H. Axtmayer and A. Brenes Pomales, *Bol. Asoc. Med. Puerto Rico* **46**, 375 (1954); *Chem. Abstr.* **49**, 2558 (1955).

[2] L. C. Craig and D. Craig, *Technique of Organic Chemistry* (Edited by A. Weissberger) Vol. III; Part I, p. 257. Interscience, New York, N.Y. (1956).

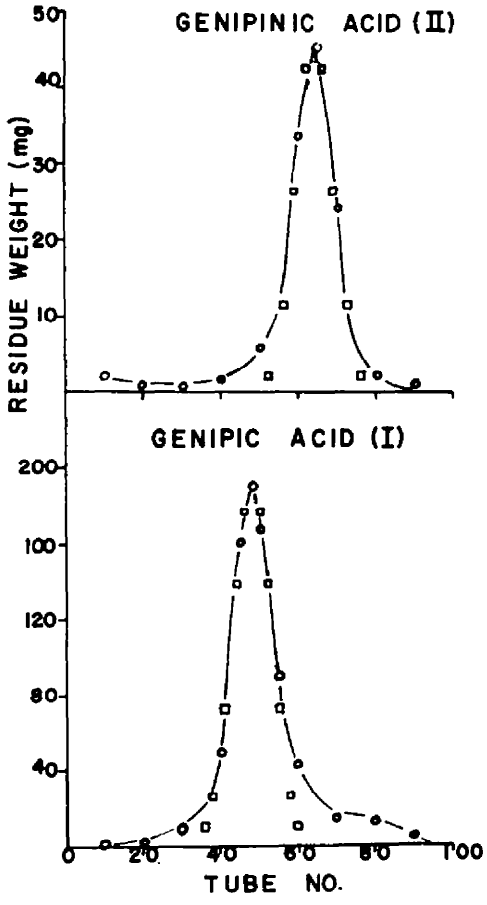


FIG. 1. Countercurrent distribution of jagua fruit antibiotics in solvent system B of Table 1. Experimental points are shown as circles and calculated[2] points as squares.

TABLE 1. COUNTERCURRENT DISTRIBUTION OF JAGUA FRUIT ANTIBIOTICS

Antibiotic	Solvent system[a]	Distribution coefficient	Number of transfers	Peak tube
I	A	0·224	399	73
II	A	0·511	399	135
I	B	0·941	99	48
II	B	1·829	99	64

[a] A = ethyl acetate–water: 1–1; B = ethyl acetate–isobutanol–water: 3–1–4.

vinyl hydrogen atom. It was therefore concluded that the double bond was tetra-substituted, as predicted by the failure of I to absorb hydrogen in the presence of palladium on charcoal catalyst.

Demonstration of the cyclopentanoid 10-normonoterpene[3] carbon skeleton for genipic acid (I) came about as a consequence of an attempt to hydrogenate it with the aid of a platinum catalyst. Among the products isolated from the resulting complex

TABLE 2. NUCLEAR MAGNETIC RESONANCE DATA[a]

Sample	Solvent	H_a	H_b	OCH_3
Genipic acid (I)	$CDCl_3$	353	258	
Methyl ester of I	$CDCl_3$	353	250	222
NH_4^+ salt of I	D_2O	343	241	
Genipinic acid (II)	$CDCl_3$	350	260	227
Genipinic acid (II)	D_2O	353	245–253	222, 224

[a] Miss Diana Ede of this laboratory determined the NMR spectra using a Varian A-60 spectrometer. The data are given in c/s downfield from the reference band due to tetramethylsilane added as an internal standard in the case of the $CDCl_3$ solutions. Frequencies in the spectra obtained with D_2O solutions were calculated with the aid of the solvent band at 279 c/s. The integration curves were in agreement with the assignments given.

mixture was a lactonic acid (IV) which proved to be identical to the one obtained by Fujise, Ohara and Uda[4] by potassium permanganate oxidation of tetrahydroanhydrodeoxyaucubigenin (V) and recently[5] shown to have the structure indicated. The lactonic acid (IV) has the same molecular formula as genipic acid (I), and its formation from the latter is postulated to have proceeded through the enol form (III) *via* a rather unusual but not unprecedented[6,7] migration of the double bond under the influence of the hydrogenation catalyst. The transformation is undoubtedly favored by the driving force provided as a result of the considerable reduction in strain in the lactonic acid (IV) *vs.* genipic acid (I).

The occurrence of single relatively narrow two-hydrogen NMR bands near 250 c/s for genipic acid and the two derivatives listed in Table 2 is incompatible with the otherwise possible alternative structure Ia for the acid. The NMR spectra of Ia and corresponding derivatives would be expected to have bands in the 200–250 c/s region accounting for 4 hydrogen atoms, namely, those at C-8 (due to the additional deshielding influence of the double bond) as well as C-7. In addition, the two hydrogen atoms at C-7 interacting with the one at C-1 would produce a complex ABX pattern[8] similar to the one observed in the case of the lactonic acid (IV).

[3] The numbering of the cyclopentanoid monoterpene carbon skeleton followed in this paper is given completely in Formula II and is that used in the review by G. W. K. Cavill, *Rev. Pure Appl. Chem.* **10**, 169 (1960).

[4] S. Fujise, H. Ohara and H. Uda, *Chem. & Ind.* 289 (1960). The author is grateful for a sample of the lactonic acid from the Tohoku University laboratories generously supplied by Dr. Uda.

[5] K. Kurosawa and S. Fujise, *Chem. & Ind.* 1688 (1963).

[6] R. Delaby, *C.R. Acad. Sci., Paris* **182**, 140 (1926).

[7] R. Schroter, *Newer Methods of Preparative Organic Chemistry* p. 71. Interscience, New York, N.Y. (1948).

[8] L. M. Jackman, *Applications of Nuclear Magnetic Resonance Spectroscopy in Organic Chemistry* p. 90. Pergamon Press, New York, N.Y. (1959).

Finally, the NMR spectra of deuterochloroform solutions of genipic acid (I) contained a band which shifted from 308 c/s to 385 c/s when the concentration was increased from 5% to 20%. This concentration dependent band integrating for two deuterium oxide exchangeable[9] hydrogen atoms reflected interaction between the carboxyl and hydroxyl groups *via* hydrogen bonding, which requires a *cis* relationship between the C-3 and C-4 substituents.

The analytical data for genipinic acid (II) were in agreement with the molecular formula $C_{11}H_{14}O_6$, and the titration data indicated the presence of an ester as well as a carboxyl group. The NMR spectrum of II in deuterochloroform after deuterium oxide exchange[9] was virtually identical to that of genipic acid (I) obtained under the same conditions except for the appearance in the former of a sharp methoxyl band at 227 c/s. After correction for the different molecular weights, the integrated area of the carbonyl band in an IR spectrum of genipinic acid (II) with the 5·50–6·10 μ region expanded five-fold was 2·04 times that in a spectrum of genipic acid (I) obtained with the same instrument (Beckman IR4) and exactly the same procedure. These facts support the view that genipinic acid (II) is a carbomethoxyl derivative of genipic acid (I), and to conform with the typical cyclopentanoid monoterpene carbon skeleton, the carbomethoxyl group is considered to be attached at C-8.[3] Formula IIa shows the close relationship of the free aldehyde form of genipinic acid (II) to that (VIa) of genipin (VI), which was previously isolated from Mexican *Genipa americana* by other investigators.[10,11]

Two outstanding properties observed for genipinic acid (II) are consistent with the structure formulated. First, as indicated in Table 2 the very sharp methoxyl band in the NMR spectrum of a deuterochloroform solution of II was split into two sharp bands when deuterium oxide was used as the solvent. The IR ($CHCl_3$) and NMR ($CDCl_3$) spectra of the sample recovered from the deuterium oxide solution showed that it was unchanged, and additional countercurrent distribution of the material from which the sample had been taken gave results identical to those obtained initially (Table 1). The explanation for the splitting of the methoxyl NMR band in deuterium oxide is that genipinic acid (II) is actually a mixture of C-8 epimers, and the interconversion of these epimers is more rapid than their equilibration between the two solvent phases during countercurrent distribution.

The second outstanding property of genipinic acid (II), first observed after an attempt to prepare an ammonium salt, was its conversion in the presence of alcoholic alkali to a strongly UV absorbing chromogen (λ_{max} 274, ε 15,800 in 0·01N ethanolic KOH). The reaction was instantaneous and reversible, the absorption band at 274 mμ being immediately and completely removed by acidification and restored by rebasification. It did not occur when the treatment of II with alkali in alcohol was preceded by methylation of the carboxyl group with diazomethane or saponification of the carbomethoxyl group. Similarity with the UV absorption spectra of certain substituted malonic esters (e.g., IX for which λ_{max} 272, ε 18.500[12]) favors formulation

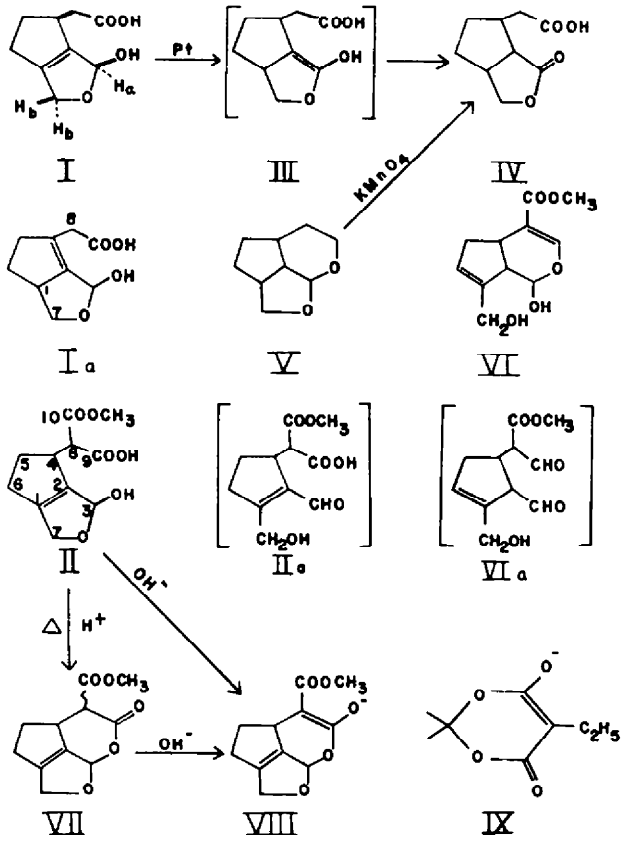
[9] H. M. Fales and A. V. Robertson, *Tetrahedron Letters* No. 3, 111 (1962).

[10] C. Djerassi, J. D. Gray and F. A. Kincl, *J. Org. Chem.* **25**, 2174 (1960). Repetition of the procedure described for the isolation of genipin with the fruit used in the present investigation produced no trace of the compound. This may reflect either a geographic or seasonal variation of the plant.

[11] C. Djerassi, T. Nakano. A. N. James, L. H. Zalkow, E. J. Eisenbraun and J. N. Shoolery, *J. Org. Chem.* **26**, 1192 (1961).

[12] M. I. Kabachnik, S. T. Ioffe, E. M. Popov and K. V. Vatsuro, *Tetrahedron* **12**, 76 (1961).

of the chromogen as VIII, and additional support for this was obtained in the following manner: When genipinic acid (II) was either inadvertently or intentionally warmed or subjected to mild acid treatment, some of it was converted to a mixture of lactones, which could be separated from unchanged II by countercurrent distribution in solvent system B of Table 1, the broad peak of the mixture occurring near tube 180 after 199 transfers. Immediately after this mixture of lactones (VII) was dissolved in alcoholic alkali, an UV absorption spectrum identical to that described above for II under the same conditions was obtained.



EXPERIMENTAL

All m.ps were taken on a Kofler Micro Hot Stage. IR spectra were obtained with a Beckman IR-4 Spectrophotometer and UV spectra with a Beckman DK-2 Spectrophotometer. For detecting the high frequency UV band due to isolated double bonds, 0·005% solutions in J. T. Baker No. 9400 reagent grade ethyl alcohol were used. In accordance with the suggestion of T. H. Applewhite and R. A. Micheli, *Tetrahedron Letters* No. 16, 560 (1961), the instrument and technique were calibrated with cyclohexene (λ_{max} 202, ε 1280) and cholesteryl acetate (λ_{max} 202, ε 4950). The mol wt of the methyl ester of genipic acid was determined with a benzene solution at 37° in a Vapor Pressure Osmometer, Model 301A (Mechrolab Inc., Mountain View, California).

Isolation and properties of the antibiotics. The fresh fruit was collected in Puerto Rico in September, 1960, and stored in deep freeze until used. The extreme thermal lability of the antibiotics and the apparent release from the ground fruit tissue of enzymes which destroyed them made certain steps in the isolation procedure critical. In a typical experiment 26 kg of the frozen fruit was allowed to

thaw only slightly and then was rapidly thinly sliced with a commercial vegetable slicing apparatus (Hobart Manufacturing Company, Troy, Ohio). The slices were spread on screens, placed in a forced warm air draft oven while still in a semi-frozen condition and dried for 16 hr at 60°. The dried slices (6 kg) were ground with a standard Model No. 2 Wiley Mill with a 1 mm mesh screen and extracted 6 hr in a Soxhlet apparatus with chloroform. The residue weight of an aliquot of the extract showed that a total of 134 g of material had been extracted. The chloroform solution was thoroughly extracted with water and the combined aqueous layers concentrated *in vacuo* to 80 ml. This final aqueous concentrate, which contained 23 g of total solute, was subjected to 399 transfers in a 40 ml per phase Craig–Post countercurrent distribution apparatus with pre-equilibrated solvent system A of Table 1. The peaks, located by residue weights, titration and assay of antibiotic activity, were at tubes 73 and 135. The contents of tubes 60–85 and a 40 ml methanol rinse of each tube were combined, and the organic portion of the solvent was removed *in vacuo*. Lyophilization of the remaining aqueous solution gave 8·10 g of genipic acid (I). Similarly, the combined contents of tubes 115–150 afforded 4·54 g of genipinic acid (II). Portions of both products were subjected to countercurrent distribution in pre-equilibrated solvent system B of Table 1, giving the results shown in Fig. 1.

The final products were hygroscopic amorphous white powders. They were extremely labile and tended to darken rapidly when warmed or treated with acid or base. Both gave positive Tollens tests for aldehyde groups. For genipic acid (I), $\lambda^{CH}_{CHCl_3}$ 2·92, $\lambda^{C=O}_{CHCl_3}$ 5·80, λ_{max} 203 (ε 2840), $[\alpha]^{27}_D$ −105° (*c* 1·0 in ethanol). (Found: C, 58·5; H, 6·7; Eq. W. 190. $C_9H_{12}O_4$ requires: C, 58·7; H, 6·6%; M.W. 184). The specific rotation at 27° of a solution of 37 mg of genipic acid in 4 ml of 50% ethanol–water containing 0·1 ml piperidine gradually changed from −79° initially to −126° after 1 hr and then remained constant at the latter value. In addition to the NMR data given in Table 2, the spectra of deuterochloroform solutions of genipic acid contained a band which integrated for two hydrogen atoms, shifted from 308 c/s to 385 c/s when the concentration was increased from 5% to 20% and was removed to deuterium oxide exchange.[9] For genipinic acid (II), $\lambda^{CH}_{CHCl_3}$ 2·92, $\lambda^{C=O}_{CHCl_3}$ 5·71–5·78 (broad), λ_{max} 203 (ε 3200), $[\alpha]^{26}_D$ −126° (*c* 1·0 in ethanol). (Found: C, 54·7; H, 6·1; Eq. W. 221 by direct titration with base, 133 by addition of excess base and back-titration with acid after 4 hr. $C_{11}H_{14}O_6$ requires: C, 54·5; H, 5·8%; M.W. 242). After correction for the different mol wts, the integrated area of the carbonyl band in an IR spectrum of a chloroform solution of genipinic acid with the 5·50–6·10 μ region expanded five-fold was 2·04 times that in a spectrum of genipic acid in chloroform obtained the same day with the same instrument operated at the same speed, gain, period, slit schedule and scale. Instead of appearing as a distinct band as in the case of genipic acid, the absorption due to the two exchangeable hydrogen atoms in the NMR spectrum of genipinic acid in deuterochloroform was spread from 250 to 400 c/s. The data given in Table 2 for the latter compound were taken from the much cleaner spectrum obtained after deuterium oxide exchange.[9] A deuterium oxide solution of genipinic acid also gave a clean NMR spectrum from which the additional data given in Table 2 and discussed in the text were obtained.

Both antibiotics inhibited the growth *in vitro* of a wide variety of Gram-negative and Gram-positive bacteria, a representative fungus (*Trichophyton mentagrophytes*), an alga (*Chlorella vulgaris*) and a protozoan (*Tetrahymena gelleii*). Quantitatively, genipic acid was about one hundredth and genipinic acid about one sixtieth as active as penicillin *vs. Bacillus subtilis in vitro*.

Ammonium salt of genipic acid. Anhydrous ammonia was bubbled through a solution of 1 g genipic acid in 5 ml absolute ethanol in an ice bath. The resulting precipitate was removed by centrifugation, thoroughly washed with chilled absolute ethanol, and dried (P_2O_5) at room temp to provide 0·8 g white crystalline ammonium genipate, m.p. 125–130° dec. (Found: C, 53·6; H, 7·4; N, 7·2. $C_9H_{15}O_4N$ requires: C, 53·7; H, 7·5; N, 7·0%). In addition to the bands listed in Table 2, there was present in the NMR spectrum of the salt a band at 575 c/s integrating for approximately 0·2 hydrogen atom.

Methyl ester of genipic acid. An ether solution of diazomethane[13] was added dropwise to 137 mg genipic acid in 1 ml methanol until the evolution of gas ceased and the yellow color of the reagent persisted. The solvent was removed and the residue dried to provide 137 mg of an oil, methyl genipate, $\lambda^{OH}_{CHCl_3}$ 2·85, $\lambda^{C=O}_{CHCl_3}$ 5·77, λ_{max} 203 (ε 2340), NMR data given in Table 2. (Found: C, 60·5; H, 7·5; M.W. 222. $C_{10}H_{14}O_4$ requires: C, 60·6; H, 7·1; M.W. 198.)

Hydrogenation of genipic acid. A solution of 1 g genipic acid in 20 ml ethanol failed to absorb

[13] F. Arndt, *Organic Synthesis* (Edited by A. H. Blatt) Vol 11; p. 165. J. Wiley, New York, N.Y.

any hydrogen in the presence of 100 mg 5% Pd-C catalyst at room temp and atm. press. However, when 100 mg pre-reduced PtO_2 was added, a mole H_2 was absorbed in 2 hr under the same conditions. The catalyst was removed by filtration, the solution concentrated *in vacuo*, and the residue chromatographed on silicic acid (Mallinckrodt No. 2847) with chloroform as the eluting solvent. The principal product (300 mg, $\lambda^{OH}_{CHCl_3}$ 2·82, $\lambda^{C=O}_{CHCl_3}$ 5·78, 5·83, no significant UV absorption down to 190 mμ) failed to crystallize. It gave a negative Tollens test for an aldehyde group, and its NMR spectrum (Table 2, note *a*) in deuterochloroform showed a typical secondary methyl group doublet at 61 and 67 c/s as well as a carboxyl band at 520 c/s removable by deuterium oxide exchange.[9] The only other product eluted from the column in significant quantity was recrystallized from ethyl acetate–cyclohexane to provide 160 mg of *lactonic acid* IV, m.p. 86–87° (reported[4] 86·5–87·5°), mixed m.p. with an authentic sample 86–87°, $[\alpha]_D^{26}$ +42° (*c* 0·5 in $CHCl_3$), no significant UV absorption down to 190 mμ. (Found: C, 58·8; H, 6·4; Eq. W. 189 by direct titration with base, 98 by addition of excess base and back-titration with acid 1 hr later. Calc. for $C_9H_{12}O_4$: C, 58·7; H, 6·6; M.W. 184.) The IR spectrum was identical to that of the authentic sample and showed $\lambda^{C=O}_{CHCl_3}$ 5·65, 5·81. The NMR spectrum (see Table 2, note *a*) of IV in deuterochloroform was quite complex and showed an ABX pattern[8] for the C-7 methylene hydrogen atoms with components at 231, 234, 241, 245, 255, 263, 270 and 279 c/s.

Further studies on genipinic acid (II). In an effort to discover the explanation for the splitting of the methoxyl NMR band of genipinic acid in deuterium oxide (Table 2), a drop of water was added to the NMR sample solution, which was then lyophilized. The IR spectrum of the residue in chloroform and the NMR spectrum of a similarly prepared residue in deuterochloroform were identical to those obtained initially. Repeat countercurrent distribution of the material from which the samples of genipinic acid for the NMR spectra in deuterium oxide had been taken gave results identical to those given in Table 1.

When genipinic acid was obtained after the first countercurrent distribution (solvent system A of Table 1) by removing the water from the final aqueous concentrate *in vacuo* at 60° rather than by lyophilization, the second countercurrent distribution (solvent system B of Table 1) provided, in addition to unchanged genipinic acid, a small quantity of material which peaked at tube 180 after 199 transfers. The same material was obtained from a chloroform solution of genipinic acid which had been allowed to stand until a slight acidity developed. The width of the band for this material in the countercurrent distribution weight curve was greater than expected for a pure compound, and the material appeared to be a mixture of lactones (VII), $\lambda^{OH}_{CHCl_3}$ none, $\lambda^{C=O}_{CHCl_3}$ 5·73 (Found: C, 58·1; H, 5·9; insignificant quantity of base consumed by direct titration, Eq. W. 208 by addition of excess base and back titration 1 hr later.[14] $C_{11}H_{12}O_5$ requires: C, 58·9; H, 5·4; M.W. 224).

Both genipinic acid and the above described mixture of lactones were immediately converted into a strongly UV absorbing chromogen (VIII, λ_{max} 274, ε 15,800) when dissolved in 0·01 N ethanolic KOH. The absorption band was immediately and completely removed by acidification with HCl aq and restored by rebasification with KOH. The chromogen was not formed from genipic acid, the crude product of esterification of genipinic acid with diazomethane,[13] or the product of saponification of genipinic acid with $Ba(OH)_2$ and removal of the reagent as the sulfate.

Acknowledgements—The author wishes to thank Mr. A. J. Damascus and his assistants of this laboratory for all of the infrared and ultraviolet spectra and optical rotation determinations and Dr. R. D. Muir of G. D. Searle & Co., Division of Biological Research for the antibiotic activity determinations.

[14] Under identical back-titration conditions, genipinic acid itself gave an Eq. W. of 196.