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ANANASIC ACID

A NEW TRIHYDROXYTRITERPENECARBOXYLIC ACID FROM PINEAPPLE STEMS

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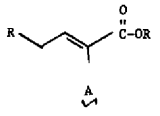
Abstract—From the stems of commercially grown pineapple (*Ananas comosus* var Cayenne) we have isolated 3β,11α,15α-trihydroxycycloart-24-en-26-oic (ananasic) acid (**6**) and determined its structure by spectral determinations and by comparison with the known 3β-hydroxycycloart-24-en-26-oic (mangiferolic) acid (**3a**).

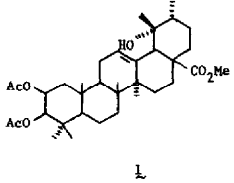
The stems of the commercially grown pineapple plant, *Ananas comosus* var. Cayenne (Bromeliaceae), have been the source of the proteolytic enzyme bromelain.[1] During routine bromelain production it had been noted[2] that contamination of the enzyme by phenolic acids adversely affected proteolytic activity. Furthermore, there have been reports[3,4] of the use of bromelain in human therapy that cannot be rationalized on the basis of enzymatic properties, but might well be explained by the presence in commercial bromelain of small bioactive substances. It seemed therefore worthwhile to examine pineapple stems in the hope of clarifying the phenomena which have been ascribed to the presence of contaminating small molecules. In the course of this work we have isolated a new triterpene carboxylic acid, ananasic acid, which is the subject of this report.

We have isolated this acid from the dried and milled pineapple stumps in a scheme designed for separation of acidic compounds. After defatting the plant material with ligroin we extracted it with acetone. The resulting residue was taken up in ether from which the acids were removed with dilute aqueous bicarbonate. Acidification and extraction into ether rendered the mixture of acids suitable for fractionation by Sephadex LH-20. Scanning of the PMR spectra of the eluates revealed that we had achieved only partial separation of fatty acid and phenolic acid fractions because of troublesome tailing. In order to allow chromatographic separation without interference from carboxyl groups[5] we treated the fractions with diazomethane and rechromatographed the methyl esters on Bio-Sil A without, however, achieving significant separation. The bulk of the sample was eluted again in a single fraction with methanol–chloroform. We therefore acetylated the mixture of methyl esters, which had OH bands in the IR, and again chromatographed on BioSil A. In this fashion, we finally isolated the acetylated triterpene ester after additional purification by preparative TLC on silica gel in 0.0007% yield from pineapple stumps. Mass spectral data showed that we had in hand a methyl ester diacetate of composition $C_{35}H_{54}O_7$, which corresponds to a molecular formula of $C_{30}H_{48}O_5$ for the parent compound. This composition, coupled with PMR resonances reminiscent of steroidal Me groups clearly implied that we were dealing with a triterpene. Interestingly, the diacetate still had free OH absorption in the IR. During a subsequent isolation run we succeeded in obtaining the free acid, m.p. 194–197.5°, $[\alpha]_D$ +4.23°, which we esterified with diazomethane. The ester could be crystallized from hexane, but apparently as a solvate since it softened over a wide range prior to melting at 131–133°. The methyl ester had a small molecular ion at *m/e* 502 ($C_{31}H_{50}O_5$)and a more substantial one at M^+-18, ν_{max} 1715 cm^{-1}, a UV maximum at 219 nm. The PMR spectrum revealed a broad three proton singlet at δ 1.83 indicative of an olefinic Me and only one signal in the olefinic region, a broad one proton triplet at δ 6.77. Decoupling experiments at 300 MHz showed that the olefinic proton was coupled to the Me group and to a two proton multiplet near δ 2.15. These data are consistent with part structure **A**. Because of the small amount of com-



A

pound available to us the intensities of the CMR signals were not fully explicit. Clearly, though, the compound possessed only three low field C atoms, at δ 168.468, 142.489 and 127.254 ppm, certainly none in addition to those defined by part structure **A**. Since the molecular formula requires seven elements of unsaturation, the triterpene had to be pentacyclic. There are at least nine known pentacyclic trihydroxytriterpenecarboxylic acids recorded in the literature,[6] all of which belong either to the oleanane or the ursane series. While the PMR spectrum of our ester and the spectrum of the ursane derivative, methyl diacetyltormetate (**1**),[7] exhibited a good deal of correspondence, the mass spectrum of our ester was strikingly different from that of a representative of the oleanane group, methyl entagenate (**2**).[8] In compound **2** the olefinic



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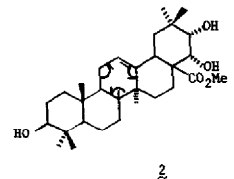

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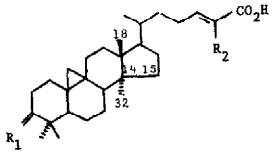
linkage gives rise to a *retro* Diels–Alder fragmentation (see arrows) with a prominent peak at *m/e* 294 (corresponding to $C_{17}H_{26}O_4$), which further fragments to *m/e* 235 by loss of the angular carbomethoxy function, and to *m/e* 217 by ensuing loss of water. The fragment at *m/e* 294 ($C_{17}H_{26}O_4$)

†From the Ph.D Dissertation, University of Hawaii (1975).

is observed in all Δ^{12} ursenes and oleanenes[8] and arises directly from the molecular ion. The mass spectrum of the pineapple stump ester exhibits a prominent fragment ion at *m/e* 293, which was shown to have a composition of $C_{22}H_{29}$ by high resolution data. It arises from the molecular ion $C_{31}H_{50}O_5$ (*m/e* 502) by consecutive loss of three molecules of water (*m/e* 448), followed by loss of $C_9H_{15}O_2$ (155) as a single neutral fragment. While loss of such a fragment is typical of fragmentation under electron impact that is observed with steroidal side chains, it cannot be rationalized in case of an ursane or oleanane skeleton.

A number of tetracyclic triterpenoids do possess steroidal side chains, notably those belonging to the euphane, dammarane, protostane, lanostane, or cucurbitane groups. The pineapple constituent could belong to one of these classes only if a second double bond were present. Such a structural feature, as has been pointed out, appeared unlikely. Our attention was thus drawn to the only *penta*cyclic triterpenoid class that also possesses a steroidal side chain: the cycloartane group. Moreover, several members of this group,[9] mangiferolic (**3a**),[10] mangiferonic (**3c**),[11]† isomangiferolic (**3b**)[12] and hydroxymangiferonic (**3d**),[13] had been isolated from *Mangifera indica*.[10,14]

All share with our acid a side chain terminating in an



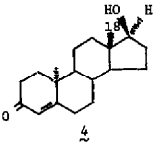
3a: R_1 = HO, H ; R_2 = Me
3b: R_1 = H, HO ; R_2 = Me
3c: R_1 = =O, R_2 = Me
3d: R_1 = =O, R_2 = CH_2OH

α-substituted acrylic acid. The 100 MHz PMR spectrum of our acid failed to show high field signals attributable to cyclopropane protons. A 300 MHz spectrum, however, revealed two doublets at δ 0.475 and 0.645 (J = 4.5 Hz), which had been obscured in the 100 MHz spectrum by side bands. By comparison, the cyclopropane protons of mangiferolic acid (**3a**) resonate at δ 0.37 and 0.58 with a coupling constant of 4 Hz,[10] and those of other cycloartenols at δ 0.31 and 0.56.[15] Since the pineapple constituent bears three OH groups, proximity of one of these to the cyclopropane would readily explain the downfield shift of these protons.

These data clearly showed that we were dealing with the carbon frame and side chain of mangiferolic acid (**3a**), with location and configuration of three OH groups to be determined. Mass spectral evidence indicated that no OH group could be on the side chain.‡ As was mentioned earlier, a key $C_{22}H_{29}$ (*m/e* 293) fragment arises from consecutive loss of three molecules of water followed by loss of the entire side chain. A second fragmentation sequence, molecular ion (*m/e* 502) minus water (*m/e* 484) minus side chain (*m/e* 329) minus water (*m/e* 311) minus water (*m/e* 293) supports this interpretation. Typical cycloartenol fragmentation, on the other hand, is triggered by initial cleavage of the 3-membered ring with subsequent loss of rings A and B, which constitute a $C_9H_{16}O$ fragment of 140 mass units.[16,17] In full analogy with these data, the mass spectrum of our methyl ester displays a significant peak (25%) at *m/e* 344, corresponding to a loss of 140 mass units from M^+-18 (*m/e* 484). High resolution data assign a composition of $C_{22}H_{32}O_3$ to the *m/e* 344 ion. If the analogy with cycloartenol holds, one OH group is lost with the A/B fragment and consequently the remaining two OH groups must be situated at rings C and/or D. The *m/e* 484 ion apparently represents the loss of one of these two OH's. These two OH's cannot be present as hydroxymethylenes since all quaternary Me signals are clearly observed at higher field than δ 1.2 in the PMR spectrum. The two OH's must therefore be situated at C-11, C-12, C-15, C-16 or C-17. The third OH must be at C-1, C-2 or C-3 of ring A in agreement with cycloartenol fragmentation data.

Expansion of the δ 3–4 ppm region of the 300 MHz PMR spectrum provides substantial evidence for further refinement of these data. Three one-proton multiplets at δ 3.32, 3.64 and 3.81 represent the three carbinol methine protons. We assign the signal at highest field to a 3β-OH by comparison with a chemical shift of δ 3.18 in the spectrum of mangiferolic acid (**3**).[10] It is a broad signal because of virtual coupling to the C-1 protons.[18] In full agreement with a 3β-configuration of the ring A OH is our assignment of two high field 3-proton singlets at δ 0.82 and 0.95 to C-29 and C-28 Me's. By comparison, the corresponding resonances of methyl mangiferolate (**3a**, methyl ester) occur at δ 0.84 and 0.96. Furthermore, PMR data for a large number of cycloartane derivatives have been correlated. In cycloartenol the relevant Me signals are observed at δ 0.80 and 0.97, and in 3β,11α-dihydroxycycloartane at δ 0.82 and 0.97.[19]

While the high field (δ 3.32) hydroxymethine signal is a broad multiplet and has been firmly assigned to a 3α-proton, the remaining two hydroxymethine signals exhibit four lines each, presumably doublets of doublets. Each of these protons is thus coupled to two non-equivalent protons and only C-11 or C-12, and C-15 can be sites for these OH's. The shape and location of the signal at δ 3.64 are very similar to the corresponding resonance of the C-17



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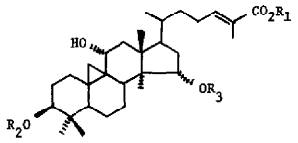
methine of testosterone (**4**), which occurs at δ 3.67.[20] In this model (**4**) the C-17 OH and the 18-Me are β-oriented, while a vicinal Me (C-32) at C-14 of mangiferolic acid (**3a**) has α-configuration. By analogy with **4**, the C-15 OH in ananasic acid should therefore also be α-oriented. This assignment is further supported on biogenetic grounds as 15α-OH substituents are of common occurrence in the cycloartenol series.[19]

The remaining hydroxymethine proton (δ 3.81, dd) must be 11β- or 12α-oriented, which would correspond to an 11α- or 12β-configuration for the third OH. The outer lines of the signal at δ 3.81 are 15 Hz apart; it is the X portion of an ABX pattern and thus implies a coupling constant of $\geq$8 Hz for the AX or BX protons, which is appropriate for *trans*-diaxial vicinal hydrogens.[21] We favor the 11α-OH assignment for these reasons. The downfield shift of the cyclopropane protons relative to those of mangiferolic (**3a**) are more compatible with an 11α- than a 12β-OH group, as was mentioned above. In the related 5α-14α-androstane (**5**) series, where extensive compilations have been made[22] of the influence of 11- or 12-OH groups on the chemical

†Mangiferonic acid (**3c**) has also been reported from *Shorea acuminata* (Dipterocarpaceae).[12]

‡Since hydroxymangiferonic acid (**3d**) designates a side chain hydroxy, the trivial name ananasic acid seems appropriate for the new acid.

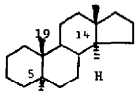


6 : $R_1 = R_2 = R_3 = H$

7 : $R_1 = Me$; $R_2 = R_3 = H$

8 : $R_1 = Me$; $R_2 = R_3 = Ac$

shift of the 19-Me, 11β-substituents cause a greater shift (0.26 ppm) than we observe when going from mangiferolic acid (**3a**) to our acid (0.06–0.10 ppm). Influence of 12-substituents in either orientation is negligibly small.[22] And again, on biogenetic grounds, 11α-substituents are often observed in cycloartane derivatives.[9] All these considerations lead to 3β,11α,15α-trihydroxycycloart-24-en-26-oic acid (11α,15α-dihydroxymangiferolic acid, (**6**) for ananasic acid.



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Our diacetate, on this basis, is the 3β,15α-derivative. This is in accord with the known unreactive character of 11-substituents and with the PMR spectrum of the acetate, where a broad resonance at δ 4.5 replaces the hydroxymethine resonances at δ 3.32 and 3.64.

Jones oxidation of **7** furnished a triketone, the physical properties of which were in full accord with the proposed structure. Similarly, a monoketone was obtained upon Jones oxidation of **8**.

EXPERIMENTAL

M.ps determined on a Fisher–Johns apparatus and uncorrected. Rotations on a Bendix–Ericksson ETL-NPL polarimeter, type 114 A. NMR data on Varian A-60, HA-100, XL-100 instruments except where noted. Mass spectra on a Hitachi Perkin–Elmer RMU 6D instrument except where noted.

Isolation of **8**. Cleaned and vacuum-dried pineapple stumps (Dole Co.) were milled to provide a brown granular meal. This material (1.4 kg) was defatted with ligroin and then extracted with acetone in a Ciereszko extractor.[23] The residue of the acetone extract (0.39 g) was taken up in ether, washed with dil HCl and then extracted with 5% $NaHCO_3$. The aqueous base, after an ether wash, was neutralized with dil HCl and extracted with ether, yielding 0.184 g residue.

This residue was chromatographed on Sephadex LH-20 (85 g) and eluted with MeOH. The fractions were scanned by PMR. All those bearing carboxyl groups were treated with diazomethane. The resulting mixed methyl esters were chromatographed on Bio-Sil A (2.3 × 27 cm) with ether–hexane, 5:95. The solvent was changed to 10:90 and 25:75 solvent ratios. The bulk of the charge was finally eluted with MeOH–$CHCl_3$, 25:75, as a yellow band (0.713 g). This fraction was treated with Ac_2O/pyridine overnight and the oily residue after solvent removal was partitioned between benzene and water. The benzene soln was dried (Na_2SO_4), filtered, and stripped, yielding 87 mg of a yellow oil. This oil was not homogeneous by TLC and was further purified on BiO-Sil A (1.5 × 55 cm, $CHCl_3$), followed by TLC on silica gel HF-254 + 366 with pet ether-ether-acetic acid, 70:30:2. The largest of the six TLC bands (10.1 mg, 7.2×10^{-4}%) was characterized.

Compound **8** was homogeneous on silica gel HF-254 + 360 (E. Merck) with the previous solvent system ($R_f = 0.31$) or with MeOH-chloroform, 2:98 ($R_f = 0.53$); visualization with EtOH–H_2SO_4, 1:1, followed by heating. Highest mass peak, 526.3646 for $C_{33}H_{50}O_5$, ($M^+ - C_2H_2O$).

Isolation of **6**. The free acid was isolated from 10 kg dry stump with the following changes. The residue of the acetone extract was dissolved in MeOH and filtered through Sephadex LH-20 (5.5 × 4.5 cm) to remove polymers. The combined early fractions were chromatographed on Bio-Sil A (332 g) and eluted with chloroform, then chloroform-MeOH (95:5, then 90:10), finally MeOH. Combined fractions 8 and 9 (125 ml each, 24.3 g combined residues) were further purified by TLC (silica gel HF-254, chloroform-MeOH, 95:5). Resulting bands were screened by PMR. The triterpenoid fraction was once more put through an identical TLC step, $R_f = 0.17$. This fraction, after recrystallization from MeOH, yielded colorless prisms (4.7 mg), m.p. 194–197.5°, $[\alpha]_D^{25°} + 4.23°$ (*c* 0.47, MeOH). PMR (100 MHz): δ0.87 (3 H, s); 0.99 (3 H, s); 1.03 (3 H, s); 1.19 (3 H, s); 1.88 (3 H, broad s); 6.77 (1 H, broad t, J ~ 6 Hz); other unresolved signals between δ 1 and 3.6. MS *m/e* (rel. int) 488 (2, M^+), 470 (21, M–H_2O), 434 (8, M–3H_2O).

Isolation of **7**. The methyl ester of **6** was obtained by treating the early eluates of the Sephadex LH-20 filtration (see isolation of **6**) with ethereal diazomethane. TLC analysis of the Bio-Sil A chromatography showed the triterpenoid in fraction 32. This and the next fraction were combined, rechromatographed on Bio-Sil A with chloroform-MeOH, 95:5. PMR scans showed that fractions 6–9 contained the characteristic high field Me peaks; they were chromatographed on silica gel HF-254, $R_f = 0.30$. Rechromatography on a small Sephadex LH-20 column and again TLC gave **7**, $R_f = 0.29$, identical with **7** obtained by diazomethane treatment of **6**. PMR (100 MHz): δ 0·48 (1 H, d, J = 4.5), 0.65 (1 H, d, J = 4.5), 0.88 (3 H, s); 1.00 (3 H, s); 1.04 (3 H, s); 1.07 (3 H, d, J 6 Hz); 1.20 (3 H, s); 1.89 (3 H, br s); 2.6 (1 H, m); 3.32 (1 H, m); 3.64 (1 H, m); 3.78 (3 H, s); 3.84 (1 H, m); 6.80 (1 H, br t, J = 6 Hz). CMR low field peaks observed at δ 168.5, 142.5, 127.3 ppm downfield from TMS. IR 3620, 3500, 2940, 2860, 1715, 1650, 1610, 1380, 1270, 1145, 1100, 1005, 950, 840 cm^{-1}. MS (70 eV): *m/e* (rel. int.) 502 (5, M^+), 484 (59, M–H_2O), 466 (100, M–2H_2O), 451 (22), 448 (25, M–3H_2O), 379 (35), 344 (25), 327 (33), 311 (54), 293 (27). UV λ_{max}^{MeOH} 200 (ϵ 12,800), 277 nm (ϵ 384).

Transformation of **7** *to* **8**. A 12 mg sample of **7** was treated with Ac_2O and pyridine. The product was chromatographed on Sephadex LH-20 (1.7 × 100 cm) with $CHCl_3$–MeOH, 50:50. Preparative TLC of fractions 5–8 on silica gel HF-254, developed with $CHCl_3$ yielded **8**, $R_f = 0.54$, identical with **8** obtained earlier.

Jones oxidation of **7**. The methyl ester (13.8 mg) was treated as previously described.[24] After work-up the product was purified on silica gel HF-254 with $CHCl_3$–MeOH, 95:5, $R_f = 0.8$. IR 2950, 2870, 1735 (sh), 1705, 1650, 1460, 1440, 1385, 1270, 1110, 1050 cm^{-1}. PMR (100 MHz): δ 0.74 (1 H, d, J = 4.5), 0.92 (3 H, s), 1.08 (3 H, s), 1.11 (3 H, s), 1.38 (3 H, s) 1.84 (3 H, br s), 3.76 (3 H, s), 6.73 (1 H, br t, J = 7). MS *m/e* (rel int.) 496 (5), 464 (9), 436 (3), 421 (1), 409 (2), 287 (9).

Jones oxidation of **8**. A sample of **8** (8.3 mg) was oxidized as above and purified by Sephadex LH 20 and preparative TLC on silica gel HF-254 with $CHCl_3$–MeOH, 98:2, $R_f = 0.55$. PMR δ 0.50 (1 H, d, J = 4.5), 0.90 (3 H, s), 0.91 (3 H, s), 0·92 (3 H, s), 1.34 (3 H, br s), 1.84 (3 H, br s), 2.08 (6 H, s), 3.76 (3 H, s), 6.75 (1 H, br t, J = 6). MS *m/e* 524 (M-60) was observed.

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