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RIVULARININ, A NEW SAPONIN FROM *ANEMONE RIVULARIS*

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Key Word Index—*Anemone rivularis*; Ranunculaceae; rivularinin; [α-L-arabinofuranosyl(1→2)-α-L-rhamnopyranosyl(1→4)-β-D-glucopyranosyl(1→4)-β-D-glucuronopyranosyl(1→3)]-3β-hydroxy-olean-12-en-28-oic acid; structural determination.

Abstract—A new saponin, rivularinin, has been isolated from the ethanolic extract of *Anemone rivularis* (Ranunculaceae). The saponin was shown to be [α-L-arabinofuranosyl(1→2)-α-L-rhamnopyranosyl(1→4)-β-D-glucopyranosyl(1→4)-β-D-glucuronopyranosyl(1→3)]-3β-hydroxy-olean-12-en-28-oic acid.

INTRODUCTION

Several saponins [1–4] have been isolated from species of the genus *Anemone*, but surprisingly, complete structures of these saponins have not been elucidated. We have therefore examined *Anemone rivularis* [5] with a view to a detailed study of the saponin and other constituents of the plant and have isolated a triterpenic acid [6] and a new saponin, rivularinin. The present paper presents the structure of rivularinin.

RESULTS AND DISCUSSION

Rivularinin, $C_{53}H_{84}O_{22}$ gave a copious lather when shaken with water, haemolysed red blood cells and responded to colour reactions characteristic of saponins [7]. It was hydrolysed with 7 % H_2SO_4 whereupon the sapogenin precipitated. After separating it from the sugar moiety by filtration, the sapogenin was purified by the usual potassium salt method [8].

Structure of the sapogenin

The sapogenin was characterized as 3β-hydroxy-olean-12-en-28-oic acid (oleanolic acid) by comparison of IR, PMR and MS data of the sapogenin and its acetyl and methyl ester derivatives with those reported in the literature [10–13] and by mmp and CO-TLC with an authentic sample.

Characterisation of the sugar moiety

The presence of D-glucuronic acid, D-glucose, L-arabinose and L-rhamnose in the hydrolysate was confirmed by co-PPC with authentic sugar samples (BuOH–HOAc–H_2O, 4:1:5); spray-aniline hydrogen phthalate, R_f 0.11, 0.17, 0.20 and 0.36, respectively).

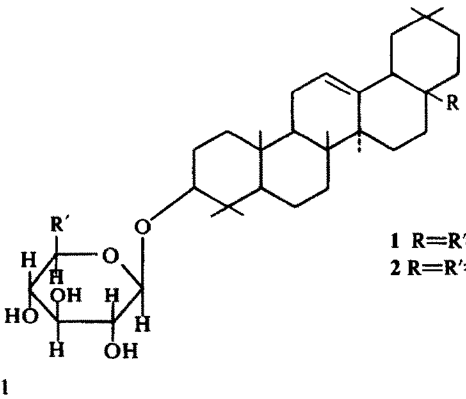
Quantitative hydrolysis of rivularinin indicated that the genin content was *ca* 42 % and the sugar moiety 58 %. A quantitative estimation [14] of the sugars present in the hydrolysate revealed that the four sugars were present in equimolar proportions. Therefore it was concluded that rivularinin contains one molecule each of sapogenin, D-glucuronic acid, D-glucose, L-rhamnose and L-arabinose.

Position of attachment of sugars to the sapogenin

In oleanolic acid, only the OH at C-3 and the COOH at C-17 are available for glycosidic linkage with sugar residues. From the following observations, it was concluded that all four sugars were linked as a tetroside unit to the C-3 hydroxyl group of the sapogenin. (i) The saponin was not hydrolysed with 5N NH_4OH which is a specific reagent [15] for hydrolysis of sugar esters without attacking other glycosidic linkages. Thus, sugars were not present in ester combination with the COOH group of the sapogenin. (ii) On methylation with CH_2N_2, the saponin furnished a dimethyl ester, $C_{55}H_{88}O_{22}$, which on hydrolysis with 7 % H_2SO_4 yielded oleanolic acid methyl ester and not oleanolic acid, indicating that there was a free COOH group in the genin.

Sequence of sugars in rivularinin

When rivularinin dimethyl ester (7) was subjected to partial hydrolysis (see Experimental) and the products methylated, oleanolic acid methyl ester and a dimethyl ester of oleanolic acid-β-D-glucuronopyranoside (1) was yielded. Thus, in rivularinin, D-glucuronic acid in its pyranose form was linked directly to the genin part. The structure of 1 has been confirmed by its reduction with $LiAlH_4$ to erythrodiol-3-β-D-glucoside(2) which on hydrolysis gave D-glucose and erythrodiol and on permethylation [16] followed by hydrolysis furnished 2,3,4,6-tetra-*O*-methyl-D-glucose indicating that C-1 of glucuronic acid was glycosidically linked to the sapogenin part of rivularinin.



1 R=R′=COOMe
2 R=R′=CH_2OH

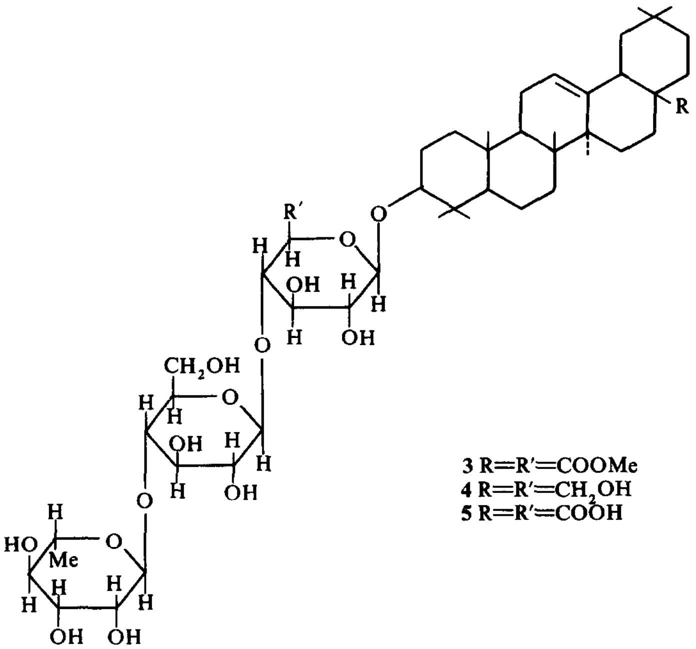
When rivularinin was subjected to partial hydrolysis [17] with 0.02 N H_2SO_4 and the products left for ten days, a prosapogenin (PS_1) was formed. Paper chromatographic examination of the hydrolysate indicated the presence of only L-arabinose. Complete hydrolysis of PS_1 with 7% H_2SO_4 produced oleanolic acid, D-glucuronic acid, D-glucose and L-rhamnose but not L-arabinose indicating that L-arabinose was present in rivularinin as an end sugar unit.

The prosapogenin (PS_1), on treatment with CH_2N_2, formed a dimethyl ester (3), which on reduction with $LiAlH_4$ gave a glycoside (4). Hydrolysis of 4 with Kiliani's mixture [18] produced erythrodiol, D-glucose and L-rhamnose. The erythrodiol content of 4 was 47.8% and the ratio of D-glucose to L-rhamnose, determined colorimetrically [14], was 2:1. This suggested that glycoside **4** was an erythrodiol trioside formed from one molecule of erythrodiol, one molecule of L-rhamnose and two molecules of D-glucose.

Since rivularinin and PS_1 both contained D-glucuronic acid which was not present in the glycoside (4), it may be concluded that one of the two D-glucose units present in **4** resulted from D-glucuronic acid during reduction of 3 and this glucose unit was, therefore, present in the pyranose form. Permethylation [16] of **4** followed by hydrolysis and examination of the hydrolysate by paper chromatography revealed the presence of 2,3,4-tri-*O*-methyl-L-rhamnose and 2,3,6-tri-*O*-methyl-D-glucose indicating that the L-rhamnose unit in **4** was present in its pyranose form at the terminal position and positions 1, 4 and 5 of both the D-glucose units were involved either in glycosidic linkage or in the formation of an inner oxide. Thus, the sequence of sugars in **4** was shown as L-rhamnose-D-glucose-D-glucose-erythrodiol. Accordingly, the sequence of sugars in PS_1 and rivularinin may be formulated respectively as L-rhamnose-D-glucose-D-glucuronic acid–oleanolic acid and L-arabinose-L-rhamnose-D-glucose-D-glucuronic–oleanolic acid.

Nature and position of glycosidic linkages in rivularinin

It has been shown that D-glucuronic acid was present as a pyranoside with the C-1 hydroxyl glycosidically linked to the C-3 hydroxyl of oleanolic acid. Therefore, the C-4 hydroxyl of the first D-glucopyranose unit (derived from a D-glucuronic acid unit) was the only available position for glycosidic linkage with the middle D-glucose unit in **4**. The ring size of the middle D-glucose unit and the positions of the glycosidic linkages in **4** were ascertained by periodate oxidation [19] which indicated that the middle D-glucose unit was the pyranose form and was linked to the first D-glucopyranose unit through 1 → 4 linkage and to terminal L-rhamnose unit through 4 → 1 linkage. Accordingly, **4**, **3** and PS_1(**5**) may be represented by the structures shown.



3 R=R′=COOMe
4 R=R′=CH_2OH
5 R=R′=COOH

Hydrolysis of PS_1 with the enzyme diastase yielded only L-rhamnose as free sugar indicating that only the terminal glycosidic linkage has the α-configuration. Thus, the glycosidic linkages in PS_1 were assigned the following configurations: L-rhamnose (1α → 4)-D:glucose (1β → 4)-D-glucuronic acid (1β → 3)-oleanolic acid.

The observed molecular rotation, $[M]_D$, for **3** was in good agreement with that calculated for the above configuration on the basis of Klyne's [20] rule (Table 1).

After elucidating the structure of PS_1, the problem of assigning a complete structure to rivularinin depended upon establishing the nature and position of attachment of the L-arabinose unit to PS_1. The easy elimination of L-arabinose during partial hydrolysis of rivularinin indicated that L-arabinose was present as a furanoside [21]. Furthermore, rivularinin on complete methylation followed by hydrolysis released L-arabinose as 2,3,5-tri-

Table 1. $[M]_D$ values for PS_1 dimethyl ester (3) and rivularin dimethyl ester (7)

Substance	$[\alpha]_D^{26}$ - (in MEOH)	$[M]_D$
Dimethyl ester of oleanolic acid -β-D-glucuronopyranoside	+12	+79
β-methyl-D-glucoside	−34	−66
α-methyl-L-rhamnoside	−62	−111
α-methyl-L-arabinoside	−125	−205
Calculated for 3 (+79 − 66 − 111)	—	−98
Observed for 3	−8.6	−83
Calculated for 7 (+79 − 66 − 111 − 205)	—	−303
Observed for 7	−26.4	−290

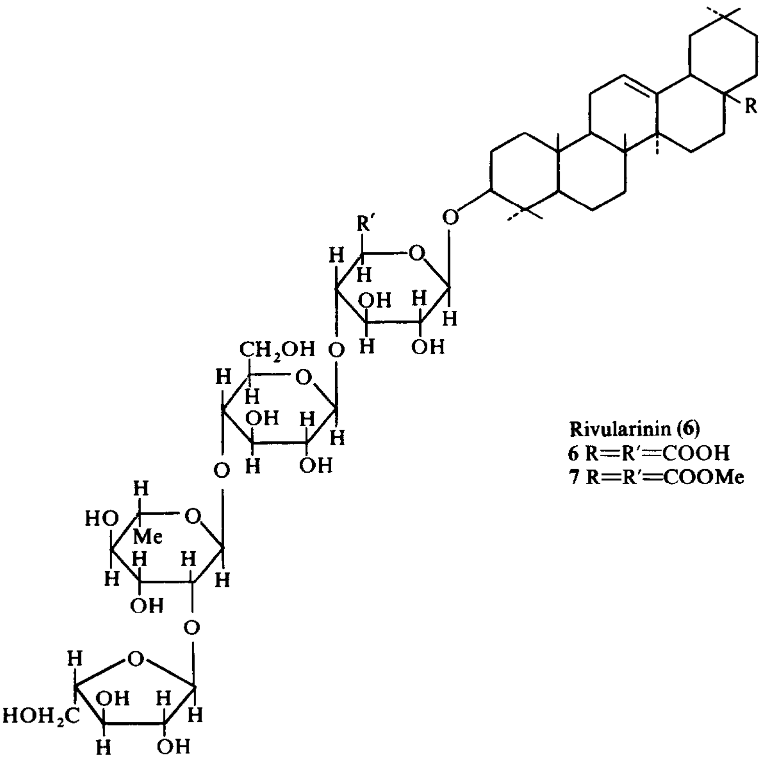
O-methyl-L-arabinose and L-rhamnose as 3,4-di-O-methyl-L-rhamnose (identified by PC [22] with authentic samples), whereas completely methylated erythrodiol trioside released L-rhamnose as 2,3,4-tri-O-methyl-L-rhamnose. This suggested that in rivularinin, the OH at C-2 of the L-rhamnopyranose unit was involved in a glycosidic linkage. Release of 2,3,5-tri-O-methyl-L-arabinose clearly suggested that the L-arabinose unit in rivularinin was present as a furanoside leaving only its anomeric OH for glycosidic linkage with the L-rhamnose unit. Therefore, the L-arabinose unit was linked to the L-rhamnose unit through a 1 → 2 linkage. Since enzymatic hydrolysis of rivularinin with diastase yielded only L-rhamnose and L-arabinose, these two sugars were involved in α-glycosidic linkages. Hence, rivularinin (6) is [α-L-arabinofuranosyl (1 → 2)-α-L-rhamnopyranosyl (1 → 4)-β-D-glucopyranosyl (1 → 4)-β-D-glucuronopyranosyl(1 → 3)]-3β-hydroxy-Δ^{12}-oleanene-28-oic acid; rivularinin dimethyl ester may be represented by 7.

The above nature of the glycosidic linkages in rivularinin was also supported by the calculated $[M]_D$ value [20] for structure 7 which was in good agreement with the experimental value (Table 1).

EXPERIMENTAL

Extraction. The defatted powdered plant (5 kg) was exhaustively extracted with EtOH. The EtOH extract (4.5 l.) was concd (1.5 l.) under red. pres. and kept in a refrigerator for a week when a light brown ppt. was observed. The ppt. was separated by filtration and identified as betulinic acid [6]. The filtrate was further concd and the residual amount of EtOH was removed *in vacuo*. The residue thus obtained was successively washed with Et_2O, $CHCl_3$ and Me_2CO and was finally dissolved in MeOH, filtered and the filtrate poured into excess Et_2O whereby a light brown mass was pptd. This process was repeated several times and the product was decolourized by passing its MeOH soln through a bed of activated charcoal. The MeOH soln was then taken to dryness, redissolved in a small quantity of MeOH and poured into a large quantity of dry Me_2CO to give a colourless ppt. The ppt. on repeated crystallization from MeOH yielded colourless crystals (11.6 g) of rivularinin, mp 179–81°. The purity of rivularinin was checked by PPC ($BuOH–HOAc–H_2O$, 4:1:5, spray—25% CCl_3 . COOH in Et_2O; yellow spot, R_f 0.51). Found: C, 59.52; H, 8.06, $C_{53}H_{84}O_{22}$ requires: C, 59.32, H, 7.83%.

Isolation and study of sapogenin from rivularinin. Rivularinin (3 g) was hydrolysed by refluxing with 7% H_2SO_4 (400 ml) for 5 hr on a steam bath. The aglycone part was separated from the aq. hydrolysate and was purified by the K salt method [8]. It was crystallized from $CH_3OH–CHCl_3$ (1:1) into colourless crystals (1.07 g), mp 308–10°, $[\alpha]_D^{26}$ + 78° ($CHCl_3$). The purity of sapogenin was established by TLC ($CHCl_3–C_6H_6$–AcOEt, 1:2:3; spray 30% $SbCl_3$ in $CHCl_3$ R_f 0.35). Found: C, 79.09; H, 10.53, MW 456 (MS). $C_{30}H_{48}O_3$ requires C, 78.94; H, 10.52%. IR ν_{max}^{KBr} cm^{-1}: 3420, 2900, 2840, 1701, 1464, 1390, 1366, 1347, 1325, 1305, 1264, 828, 818, 804; MS *m/e*:



Rivularinin (6)
6 R=R′=COOH
7 R=R′=COOMe

456 (M^+), 441, 411, 410, 395, 300, 248, 207, 203 (base peak), 189, 175, 133. Acetate: $C_{30}H_{47}O_2 . OCOCH_3$, mp 266–7°, $[\alpha]_D^{26}$ + 73° ($CHCl_3$); MS *m/e*: 498 (M^+), 483, 452, 438, 423, 301, 249, 248, 203 (base peak), 189, 133. Methyl ester; $C_{31}H_{50}O_3$, mp 198–9°, MS *m/e*: 470 (M^+), 455, 411, 410, 262, 249, 207, 203 (base peak), 189, 133; PMR (in $CDCl_3$): δ 0.75 (3H), 0.80 (3H), 0.97 (6H), 1.00 (6H), 1.16 (3H), 3.60 (3H), 5.28 (1H).

Quantitative estimation of sugars in the saponin hydrolysate. The ratio of sugars in the saponin was determined colorimetrically [15] in a Klett-Summerson photoelectric colorimeter using a blue filter (420 nm) with the help of standard curves of authentic sugars. Ten solns (10, 20, 30 ... 100 µg in 0.02 ml H_2O) of each of four sugars, D-glucuronic acid, D-glucose, L-arabinose and L-rhamnose were applied on Whatman No. 1 filter papers (50 × 55 cm, spot distances 3.75 cm). The chromatograms were developed by descending technique with BuOH–HOAc–H_2O (4:1:1) for 24 hr, dried in air, sprayed with aniline hydrogen phthalate on both the sides, and dried at 105° for 15 min. The coloured spots were cut out in equal rectangles eluted by immersion in 50% HOAc (5 ml each) and the colour intensity of each eluate measured.

Estimation of sugars in saponin hydrolysate. The saponin (60 mg) was hydrolysed by refluxing with 7% H_2SO_4 (20 ml) for 5 hr on a steam bath. The reaction mixture was extracted with $CHCl_3$ to yield the sapogenin (25.2 mg). The hydrolysate was neutralized with $BaCO_3$, filtered and concd to a syrup (1 ml). A very small portion of the syrup was dissolved in 1 ml H_2O and aliquots applied on Whatman No. 1 filter paper. The chromatograms were developed, sprayed, dried and the coloured spots were cut out in equal rectangles, eluted separately and assayed as described above.

Dimethyl ester (**7**) *of rivularinin.* Rivularinin (2.4 g) in MeOH was treated with an Et_2O soln of CH_2N_2 till a permanent yellow colour was obtained. The reaction mixture was kept overnight at 0°, the product isolated and crystallized from $CHCl_3$–MeOH (1:2) into colourless plates (2.25 g), mp 108–10°, $[\alpha]_D^{26}$ −26.4° (MeOH). Found: C, 66.28; H, 8.23; $C_{55}H_{88}O_{22}$ requires: C, 60.00; H, 8.00%.

Partial hydrolysis of dimethyl ester(**7**): *isolation of dimethyl ester of oleanolic acid-β-D-glucuronopyranoside*(**1**). **7** (1.2 g) was refluxed with 1% H_2SO_4 in 80% MeOH (100 ml) for 4 hr. MeOH was removed and the reaction mixture diluted with H_2O. The ppt. was separated by filtration, dissolved in MeOH and treated with excess of ethereal CH_2N_2. The product was chromatographed on a column of Si gel. Elution with $CHCl_3$ yielded oleanolic acid methyl ester (190 mg) while elution with $CHCl_3$–MeOH (95:5) furnished the dimethyl ester of oleanolic acid glucuronoside (160 mg), crystallized from Et_2O–EtOH (1:2), mp 202–5°, $[\alpha]_D^{26}$ + 12° (MeOH). Found: C, 69.23; H, 9.31; $C_{38}H_{60}O_9$ requires: C, 69.09; H, 9.09%.

Reduction of the dimethyl ester of oleanolic acid glucuronoside (**1**) *to erythrodiol glucoside*(**2**): **1** (20 mg) was reduced under reflux with $LiAlH_4$ in Et_2O–THF for 10 hr. The excess $LiAlH_4$ was decomposed with moist EtOAc. The product was acidified (HCl) and extracted with BuOH. The solvent was removed and the residue recrystallized from Et_2O–EtOH (1:2) into colourless flakes (95 mg), mp 207–9°, $[\alpha]_D^{26}$ +29° (MeOH).

Hydrolysis of erythrodiol glucoside(**2**): *isolation of erythrodiol.* Erythrodiol glucoside (20 mg) was hydrolysed with Killiani's mixture (HOAc–HCl–H_2O, 35:15:50) in a sealed tube at 100° for 3 hr. The genin was isolated in the usual way and crystallized from $CHCl_3$–MeOH (1:1) mp 330–2°.

Permethylation of erythrodiol glucoside(**2**) *and hydrolysis of the permethyl derivative.* 60 mg of glucoside (**2**) were treated with MeI (1 ml) and Ag_2O (1 g) in DMF (4 ml) for 48 hr at room temp. The contents were filtered and the residue washed with a little dimethyl formamide. The filtrate was evapd to dryness and the residue taken in $CHCl_3$ (30 ml). The syrup obtained after removal of $CHCl_3$ was hydrolysed with Killiani's mixture and the product worked up in the usual way. The hydrolysate contained 2,3,4,6-tetra-*O*-methyl-D-glucose (PPC with authentic sample, BuOH–EtOH–H_2O, 5:1:4).

Isolation of PS_1. Rivularinin (5.3 g) was treated with 0.02 N H_2SO_4 (500 ml) and the reaction mixture kept at room temp. (26–38°) for 10 days. It was then extracted with BuOH and concd to a syrup. The syrup was crystallized from MeOH into an amorphous powder (3.76 g).

Dimethyl ester of PS_1. Dimethyl ester (**3**) was prepared from PS_1 (2.12 g) by the method described for the prepn of dimethyl ester of rivularinin. The product obtained was crystallized from $CHCl_3$–MeOH (1:2) into colourless needles (2.5 g), mp 188–90°, $[\alpha]_D^{26}$ −8.6° (MeOH). Found: C, 62.2, H, 8.5; $C_{50}H_{80}O_{18}$ requires: C, 61.9; H, 8.2%.

Reduction of **3** *to erythrodiol trioside*(**4**). 2.0 g of **3** was reduced with $LiAlH_4$ in Et_2O–THF. The product was purified by PLC with $CHCl_3$–MeOH (7:) and was crystallized from MeOH as a colourless solid (1.07 g), mp 212–216°. Found: C, 63.32; H, 9.01; $C_{48}H_{80}O_{16}$ requires: C, 63.1; H, 8.7%.

Periodate oxidation of erythrodiol trioside(**4**). Periodate oxidation of **4** was carried out by the method of Jones *et al.* [21]. 50 mg of **4** were dissolved in 25 ml EtOH and 25 ml 0.15 M sodium metaperiodate soln were added to it. The oxidation was allowed to take place at room temp. for 60 hr. Aliquots (5 ml) were withdrawn in duplicate from the reaction mixture at different intervals of time and analysed for periodate and formic acid.

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