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STRUCTURE OF SAPONINS A AND B FROM THE SEEDS OF *ACHYRANTHES ASPERA*

V. HARIHARAN and S. RANGASWAMI

Department of Chemistry, University of Delhi, Delhi-7, India

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Abstract—The structure of *Achyranthes* saponin A has been established as α-L-rhamnopyranosyl (1 → 4)-β-D-glucopyranosyl (1 → 4)-β-D-glucuronopyranosyl (1 → 3)-oleanolic acid (VI) and that of saponin B as the β-D-galactopyranosyl (1 → 28) ester of saponin A (VII).

THE PRESENCE of oleanolic acid glycosides has been reported from a number of plants; but the structure of the carbohydrate component has been elucidated only in a few cases.[1-4] We report here the complete structures of two glycosides of oleanolic acid which occur in the seeds of *Achyranthes aspera* L. (N.O. Amaranathaceae). The plant is an annual herb growing throughout India and the seeds are used in Indian medicine as a cure for renal dropsy. Khastgir *et al.*[5] hydrolysed the alcoholic extract and characterized the genin as oleanolic acid. Working with the purified saponin Gopalachari *et al.*[6] confirmed the presence of oleanolic acid as the aglycone and identified the sugars as galactose, glucose, rhamnose and xylose. The saponins have been re-examined by us now with a view to isolate the individual components and to elucidate the structure of the sugar part.

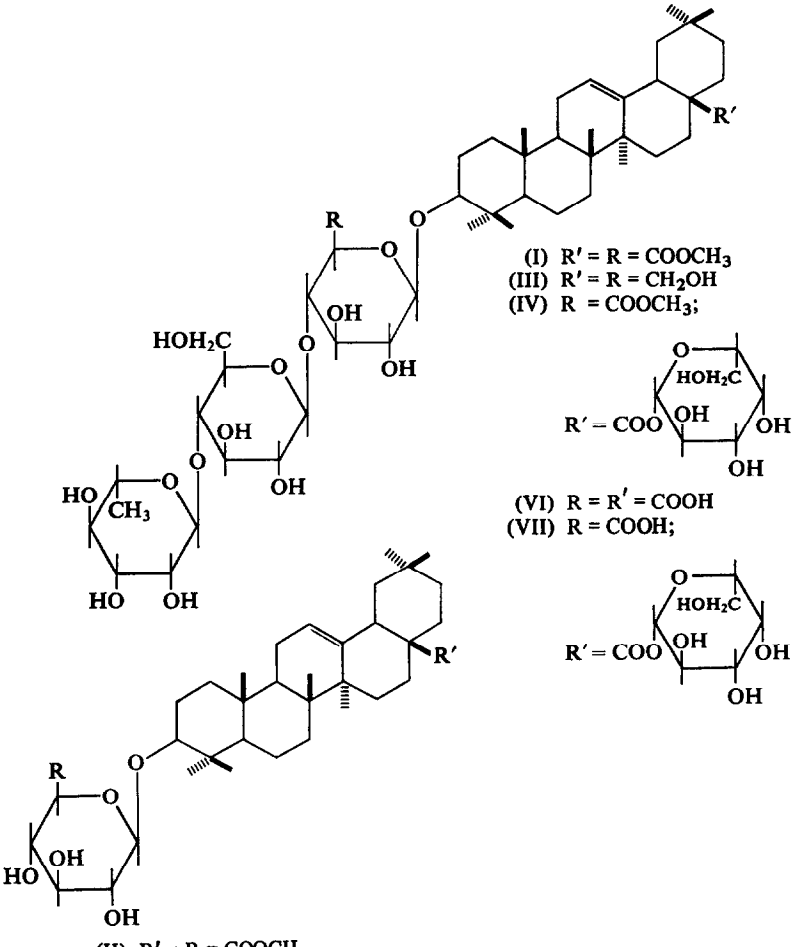
The dried seeds were extracted with petroleum ether and acetone to remove waxy and colouring material and then with alcohol. The saponin mixture obtained from the alcohol extract showed acidic reaction and did not contain any methoxyl group. It was treated with diazomethane and the product chromatographed. Two homogeneous compounds were obtained, the dimethyl ester and monomethyl ester respectively of two saponins which have been designated as saponin A and B. As a matter of convenience all the studies have been carried out with these two esters.

Saponin A Dimethyl Ester. On complete hydrolysis it gave oleanolic acid methyl ester and not oleanolic acid, showing that in the parent saponin the —COOH at C_{17} of the genin was not esterified by any sugar. The sugars, when examined by paper chromatography, showed the presence of D-glucose, L-rhamnose and D-glucuronic acid. When saponin A dimethyl ester was subjected to partial hydrolysis with 1 per cent H_2SO_4, a mixture of products was obtained which were separated by chromatography after treatment with diazomethane. One of the chromatographic fractions was identified as oleanolic acid methyl ester and another

[1] N. K. KOCHETKOV, A. I. KHORLIN and V. E. VASKOVSKY, *Tetrahedron Letters* 713 (1962).
[2] N. K. KOCHETKOV, A. I. KHORLIN and V. E. VASKOVSKY, *Izv. Akad. Nauk. SSSR Ser. Khim.* **8**, 1398 (1963).
[3] N. K. KOCHETKOV, A. I. KHORLIN, V. E. VASKOVSKY and I. P. GUDKOVA, *Bull. Acad. Sci. USSR Div. Chem. Sci.* 1177 (1965).
[4] Z. KASPRZYK and Z. WOJCIECHOWSKI, *Phytochem.* **6**, 69 (1967).
[5] H. N. KHASTGIR, S. K. SEN GUPTA and P. SEN GUPTA, *J. Ind. Chem. Soc.* **35**, 693 (1958).
[6] R. GOPALACHARI and M. L. DHAR, *J. Sci. Ind. Res.* **17B**, 276 (1958).

as the dimethyl ester of oleanolic acid-β-D-glucoronopyranoside (II).[1, 2] The structure of (II) was confirmed by its reduction with $LiAlH_4$ to erythrodiol-3-β-D-glucoside (V). This, in turn, was characterized by hydrolysis to glucose and erythrodiol and by the formation of 2,3,4,6-tetra-*O*-methylglucose on permethylation[7] followed by hydrolysis.

Reduction of saponin A dimethyl ester with $LiAlH_4$ gave an erythrodiol glycoside whose aglycone content showed it to be a trioside. The sugars present were identified as D-glucose



and L-rhamnose by paper chromatography. A quantitative estimation showed they were present in the ratio 2:1. Permethylation of the above glycoside followed by hydrolysis and examination of the methylated sugars by paper chromatography gave only two compounds, viz. 2,3,4-tri-*O*-methylrhamnose and 2,3,6-tri-*O*-methylglucose. The former indicated the terminal position of the rhamnose unit and its pyranose configuration. The latter showed that in the two glucose units, positions 4 and 5 have been together involved in the formation of

[7] S. HAKOMORI, *J. Biochem.* **55**, 205 (1964).

inner oxide and glycoside. The glucuronic acid unit in (II) has been shown by Kochetkov *et al.*[1] to have pyranoside configuration, leaving position 4 for glycosidic linking. Direct proof for the pyranoside or furanoside configuration is lacking only for the middle sugar (glucose) unit. But, since furanoside configuration for a glucose moiety is very unusual among natural glycosides, it may be assumed that the middle glucose unit also is in pyranose form leaving its 4-OH group for glycoside linkage with the terminal rhamnose unit. Hence the $LiAlH_4$ reduction product may be represented by structure (III) and this was supported by the results of periodate titration in which 3·85 moles of the reagent were consumed (required 4 moles). Saponin A dimethyl ester itself can then be represented by (I), which is confirmed by the results of quantitative hydrolysis and determination of the percentage of the aglycone.

With regard to the configurations of the glycosidic linkages it is a general observation that D-sugars occur with β-glycosidic linkages and L-sugars with α-glycosidic linkages. One could therefore expect that the sugar chain in (III) would be L-Rha $1\overset{\alpha}{\rightarrow}4$ D-Glc $1\overset{\beta}{\rightarrow}4$ D-Glc $1\overset{\beta}{\rightarrow}$ and in saponin A dimethyl ester it would be L-Rha $1\overset{\alpha}{\rightarrow}4$ D-Glc $1\overset{\beta}{\rightarrow}4$ D-Glur $1\overset{\beta}{\rightarrow}$. The calculation of the molecular rotation of (I) on the basis of Klyne's rule[8] is shown below. The calculated and observed values for $[M]_D$ are of the same order of magnitude. Hence the glycosidic linkages are as shown in (I) for saponin A dimethyl ester and saponin A itself can be represented as (VI).

Substance	$[\alpha]_D$ in degrees	$[M]_D$ in degrees
Dimethyl ester of oleanolic acid β-D-glucuronopyranoside	+15	+99
β-Methyl-D-glucoside	−34	−64
α-Methyl-L-rhamnoside	−62	−111
Calculated for (I)		−76
Observed for (I)	−3·7	−36

Saponin B methyl ester. On complete hydrolysis the aglycone was found to be oleanolic acid and not its methyl ester, indicating that the —COOH group of the oleanolic acid moiety in the parent saponin was not free to accept diazomethane and should therefore exist in ester combination with some group which is removed on subsequent hydrolysis. The sugars were identified as D-glucose, D-galactose, D-glucuronic acid and L-rhamnose by paper chromatography. When saponin B methyl ester was hydrolysed with 5 N NH_4OH for 1 hr and the sugar portion examined by paper chromatography, only galactose was found to be present. This indicates that the galactose is present in ester combination with the —COOH of the aglycone. Since the saponin methyl ester was non-reducing the anomeric hydroxyl of the galactose should be involved in this ester link. Saponin B methyl ester was hydrolysed with 15 per cent aq. KOH to break the ester links and the resulting progenin was treated with diazomethane. The product was found to be identical with saponin A dimethyl ester. Further, the $LiAlH_4$ reduction product of saponin B methyl ester was identical with erythrodiol trioside (III) obtained from saponin A dimethyl ester, the COO-galactose link having been reductively cleaved by the reducing agent and the $—COOCH_3$ in glucuronic acid unit having been reduced to $—CH_2OH$. Hence saponin B methyl ester can be represented as the 28-galactosyl ester (IV).

[8] W. KLYNE, *Biochem. J.* **47**, xli 4 (1950).

This structure received confirmation from the following two observations: (1) When saponin B methyl ester was subjected to partial hydrolysis with 1 per cent H_2SO_4, and processed further the dimethyl ester of oleanolic acid-β-D-glucuronopyranoside (II) was obtained. (2) When saponin B methyl ester was permethylated and hydrolysed, 2,3,4-tri-*O*-methylrhamnose, 2,3,6-tri-*O*-methylglucose and 2,3,4,6-tetra-*O*-methylgalactose were identified by paper chromatography. The detection of the last compound confirms the earlier conclusion that the anomeric hydroxyl of the galactose unit is involved in linkage with—COOH of the aglycone.

The configuration at the anomeric carbon of the galactose moiety in saponin B methyl ester as β has again been deduced by the application of Klyne's rule. This rule, which was originally enunciated for true glycosidic linkages and has been applied to innumerable cases off cardiac glycosides by Reichstein and his group, has recently been applied also to sugar esters involving the anomeric hydroxyl of a sugar and the —COOH of another molecule.[3,9] The calculations regarding saponin B methyl ester are shown below:

Substance	$[\alpha]_D$ in degrees	$[M]_D$ in degrees
Saponin B methyl ester	−8	−92
Saponin A dimethyl ester	−3·7	−36
$[M]_D$ contribution of galactose		−56
α-Methyl-D-galactoside		+380
β-Methyl-D-galactoside		+1

In their work on the chemistry of aralosides A, B and C, Kochetkov *et al.*[1−3] have made use of the difference in the molecular rotations between the glycosides of the general formula

$$\begin{array}{l}\text{Sugar}_1\text{-Glur-O-(3)-oleanolic acid-COO-sugar}_3\\ \qquad\quad |\\ \qquad\text{Sugar}_2\end{array}$$

and their $LiAlH_4$ reduction products, namely

$$\begin{array}{l}\text{Sugar}_1\text{-Glc-O-3-erythrodiol}\\ \qquad\quad |\\ \qquad\text{Sugar}_2\end{array}$$

to calculate the rotational contribution of sugar$_3$ and therefrom the configuration at its anomeric carbon atom. On the same analogy, the difference between the molecular rotations of saponin B methyl ester (−92°) and III (−67°), namely −25°, should give the molecular rotatory contribution of the D-galactose moiety in saponin B methyl ester. The value of this difference (−25°) also shows that a β-galactosidic (value +1°) and not an α-galactosidic (value + 380°) linkage is involved.

EXPERIMENTAL

For paper chromatography, Whatman No. 1 filter paper and the following solvents were employed: *n*-BuOH–HOAc–H_2O, 4:1:5 upper layer (system A); *n*-BuOH–pyridine–H_2O, 6:4:3 (system B); *n*-BuOH–pyridine–H_2O–benzene, 5:3:3:1 upper layer (system C); *n*-BuOH–EtOH–H_2O, 5:1:4, upper layer (system D).

[9] J. POLONSKI, E. SACH and E. LEDERER, *Bull. Soc. Chim. France* 880 (1959).

Isolation of Saponins

The alcoholic extract mentioned in the introductory part was concentrated, diluted with water and extracted successively with ether and *n*-BuOH. The syrupy residue of the butanol extract was taken in methanol and added to a large volume of ether. The brown precipitate consisting of crude saponin was filtered and purified twice by dissolving in methanol and precipitating with ether (yield of colourless saponin mixture ~ 1%). Zeisel's estimation showed the absence of methoxyl. The saponin mixture was dissolved in methanol and treated with excess of ethereal diazomethane. The product was chromatographed over silica gel. Elution with $CHCl_3$–MeOH (94:6) gave saponin A dimethyl ester and elution with $CHCl_3$–MeOH (85:15) gave saponin B monomethyl ester.

Saponin A Dimethyl Ester (I)

Melting point 187–191°, $[\alpha]_D^{27}$ −3·7° (c = 0·9, methanol). (Found: C, 62·1; H, 8·8; OCH_3, 7·1. $C_{50}H_{80}O_{18}$ required: C, 62·0; H, 8·3; OCH_3 (2), 6·4%.)

Hydrolysis

Saponin A dimethyl ester was refluxed with 7% H_2SO_4 in 80% aq. methanol for 4 hr. After addition of water and removal of solvent, the genin was quantitatively extracted with ether (yield 46·4; calc. 48·5%) and identified as methyl oleanolate (m.p., m.m.p., TLC and i.r.). The aqueous mother liquor, when examined on paper in solvents A–C, showed the presence of D-glucose, L-rhamnose and D-glucuronic acid. The hydrolysis was repeated using Kiliani's mixture (HOAc:HCl:H_2O, 35:15:50) and heating in a sealed tube at 100° for 3 hr. The product was diluted with water and the genin extracted with $CHCl_3$. The aqueous residue showed the same sugars as above.

Partial Hydrolysis: Isolation of (II)

Saponin A dimethyl ester (300 mg) was refluxed with 1% H_2SO_4 in 80% aq. methanol for 3 hr and the methanol was removed with the addition of water. The precipitate was filtered, dissolved in methanol and treated with excess of ethereal CH_2N_2. The product was chromatographed over silica gel. $CHCl_3$ eluted oleanolic acid methyl ester (50 mg) (m.p., m.m.p. and TLC). $CHCl_3$–MeOH (98:2) eluted dimethyl ester of oleanolic acid glucuronoside (II) (30 mg) which crystallized as needles from methanol–ether, m.p. 201–204°, $[\alpha]_D^{27}$ + 15° (c = 0·5, methanol). (Found: C, 69·7; H, 9·4. $C_{38}H_{60}O_9$ required: C, 69·1; H, 9·2%.)

Hydrolysis of (II)

Structure (II) was hydrolysed with Kiliani's mixture in a sealed tube at 100° for 3 hr and the product worked up as before. The genin was identified as oleanolic acid methyl ester (TLC) and the sugar as glucuronic acid.

Reduction of (II) to Erythrodiol Glucoside (V)

Structure (II) was reduced with $LiAlH_4$ in boiling tetrahydrofuran for 16 hr. After decomposition of excess of $LiAlH_4$ with moist ethyl acetate, the product was acidified and extracted with *n*-BuOH. The solvent was removed and the residue crystallized from methanol–ether (yield 80%), m.p. 107–108°, $[\alpha]_D^{27}$ + 27° (c = 0·7, methanol).

Permethylation of (V) and Hydrolysis

A mixture of sodium hydride dispersion in oil (50%, 50 mg) and dimethylsulphoxide (DMSO) (5 ml) was kept at 80° for 1 hr, (V) (20 mg) dissolved in DMSO (5 ml) was added to the above mixture and it was kept at 80° for 1 hr. After cooling, 0·5 ml of MeI was added and the mixture left overnight. The product was poured into ice-cold water and extracted with $CHCl_3$. The syrup obtained on evaporation of the solvent was hydrolysed with Kiliani's mixture and the product worked up as usual. The sugar portion yielded only 2,3,4,6-tetra-*O*-methylglucose [identified by direct comparison on paper in solvent D and on TLC in diisopropyl ether:methanol, (5:1)].

Reduction of Saponin A Dimethyl Ester (I) to (III)

Structure (I) was reduced with $LiAlH_4$ in boiling tetrahydrofuran. The product was purified by preparative TLC on silica gel with $CHCl_3$–MeOH (9:1); m.p. 210–215°, $[\alpha]_D^{27}$ − 7·1° (c = 0·8, methanol). (Found: C, 61·9; H, 9·3. $C_{48}H_{82}O_{17}$ required: C, 61·9; H, 8·8%.)

Hydrolysis of III

This was done using Kiliani's mixture. The genin was identified as erythrodiol (m.p., m.m.p. and TLC) and the sugars as glucose and rhamnose.

Quantitative Sugar Estimation

A known quantity of (III) was hydrolysed with 7% H_2SO_4 in a sealed tube and both the aglycone and the sugar were estimated quantitatively. Aglycone found 45·8% (required 47·5%). The syrup containing the sugars was chromatographed on paper, sprayed with aniline hydrogen phthalate, the sugar spots eluted with 60% aq. acetic acid and estimated colorimetrically. Average of four experiments gave the Glc:Rha ratio as 2:1.

Permethylation of (III) and Hydrolysis

This was carried out using CH_3I and NaH exactly as described under the permethylation of (V). The product was hydrolysed with 7% H_2SO_4 and the genin filtered out. The aqueous part was purified as usual and examined for methylated sugars on paper with solvent D. Expressed in terms of R_G values in which the R_f of 2,3,4,6 tetra-*O*-methylglucose was taken as 1, two spots with R_G values 1·01 and 0·84 were obtained. According to literature[10] these are the R_G values of 2,3,4-tri-*O*-methylrhamnose and 2,3,6-tri-*O*-methylglucose, respectively. The identities were further confirmed by direct comparison with authentic 2,3,4-tri-*O*-methylrhamnose (prepared from rhamnose) and 2,3,6-tri-*O*-methylglucose (prepared from lactose by permethylation and hydrolysis) and 2,3,4-tri-*O*-methylglucose (prepared from rutin by permethylation and hydrolysis).

Saponin B Methyl Ester

Melting point 200–205°, $[\alpha]_D^{27} - 8°$ ($c = 1·2$, methanol). (Found: C, 57·7; H, 8·0; OCH_3, 3·3. $C_{55}H_{90}O_{24}$ required: C, 58·2; H, 7·8; OCH_3 (1), 2·7%.)

Hydrolysis

Saponin B methyl ester was hydrolysed by boiling with 7% H_2SO_4 in 80% aq. methanol. The genin was identified as oleanolic acid (m.p., m.m.p. and TLC) and its methyl ester as methyl oleanolate (m.p., m.m.p. and TLC). The mother liquor contained glucuronic acid, galactose, glucose and rhamnose.

Hydrolysis with 1% H_2SO_4

Saponin B methyl ester was refluxed with 1% H_2SO_4 for 3 hr, the separated solid was filtered, washed free of acid, dissolved in methanol and treated with excess of ethereal CH_2N_2. The product, when tested by TLC, showed four spots. The major spot had the same R_f as that of the dimethyl ester of oleanolic acid-β-D-glucuronoside (II) described earlier under saponin A dimethyl ester.

Hydrolysis with 5 N Ammonium Hydroxide

Saponin B methyl ester (20 mg) was heated with 5 N NH_4OH (1 ml) in a sealed tube for 1 hr at 100°, acidified with dil. HOAc and extracted with *n*-BuOH. The aqueous part was neutralized with ammonia and evaporated to dryness. The residual syrup was passed through charcoal; galactose was the only sugar present.

Permethylation of Saponin B Methyl Ester (IV)

This was carried out using CH_3I and NaH exactly as described under the permethylation of (V) and the product was hydrolysed with 7% H_2SO_4. The genin was filtered out. The aqueous part was examined for methylated sugars as usual. 2,3,4,6-Tetra-*O*-methylgalactose (R_G 0·88), 2,3,6-tri-*O*-methylglucose (R_G 0·84) and 2,3,4-tri-*O*-methylrhamnose (R_G 1·01) were identified employing authentic samples (solvent D).

Conversion of saponin B monomethyl ester to saponin A dimethyl ester. Saponin B monomethyl ester was heated with 15% aq. KOH for 75 min at 100°. The mixture was acidified and extracted with *n*-BuOH. The butanol extract was washed neutral, evaporated to dryness, residue dissolved in methanol and treated with excess of ethereal CH_2N_2. The product, after chromatography over silica gel, crystallized from methanol–ether as needles, m.p. 190–191°. It was identical with saponin A dimethyl ester (m.m.p., TLC and i.r.).

Reduction of Saponin B methyl Ester (IV) to (III)

Structure IV was reduced with Li AlH_4 in boiling tetrahydrofuran for 18 hr. The product, isolated in the usual manner, was crystallized from methanol–ether as needles, m.p. 208–210°. It was identical with (III) (m.m.p. and TLC).

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[10] W. LEDERER and M. LEDERER, *Chromatography*, p. 249, Elsevier Publishing Company (1957).