Partial Characterization of a Biologically Active Steroid Glycoside Isolated from the Starfish *Marthasterias glacialis*

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1. A steroid glycoside (M_2), which induces avoidance and other reactions in the mollusc *Buccinum undatum*, has been isolated from extracts of the starfish *Marthasterias glacialis* by ion-exchange chromatography. 2. The steroid glycoside was homogeneous by t.l.c. and contained glucose, quinovose, fucose and sulphate in the molar proportions 1:2:1:1, in addition to a water-insoluble aglycone. 3. The aglycone was identified as a cholestane derivative containing an unusual $\Delta^{24,23}$ -ketone system, two secondary hydroxyl groups and an olefinic double bond, and had the molecular formula $C_{27}H_{42}O_3$. 4. The rates of release of sugars and sulphate suggested that fucose was at the non-reducing end of the oligosaccharide, with glucose glycosidically linked to the steroid. The sulphate group appeared to be linked to the other hydroxyl group of the steroid.

Certain molluscs, echinoderms and sea anemones exhibit marked avoidance or escape responses to the presence of predatory starfish in their environment, and this may serve to reduce predation of the responding animals (Feder, 1959, 1963, 1967; Feder & Christensen, 1966; Margolin, 1964a,b; Montgomery, 1967). The same response can also be elicited by extracts of starfish and the active principle appears to be secreted from the epidermis of the starfish (Feder & Lasker, 1964). The stability and some properties of the material were studied by Feder & Lasker (1964) for *Pisaster ochraceus* and by Feder & Arvidsson (1967) for *Asterias rubens* and *Marthasterias glacialis*. Using a sensitive bioassay procedure with *Buccinum undatum*, Mackie, Lasker & Grant (1968) were able to isolate and partially characterize surface-active steroid glycosides as the active principles in *A. rubens* and *M. glacialis*, and showed that some synthetic surface-active agents were equally active in the bioassay procedure. Solutions of these steroid glycosides of animal origin yield a stable foam when shaken and lyse human erythrocytes, and are therefore analogous to plant saponins. The most active steroid glycoside was obtained from extracts of *M. glacialis* and for this reason the present work is concerned with the isolation and partial characterization of the principal component.

MATERIALS AND METHODS

Animals. *M. glacialis* and *B. undatum* were obtained from the laboratory of the Scottish Marine Biological Association, Millport, Isle of Cumbrae, U.K. The starfish

were collected from the estuary of the River Clyde and stored at -20°C until required. *B. undatum* were dredged from areas around Millport and were maintained in a sea-water aquarium at 15°C .

Chemicals. L-Fucose (6-deoxy-L-galactose), D-glucose and mesityl oxide were obtained from British Drug Houses Ltd., Poole, Dorset, U.K.; D-quinovose (6-deoxy-D-glucose) was a gift from Dr J. S. D. Bacon, Macaulay Institute for Soil Research, Aberdeen, U.K. The Fermco Test S. F. G. (glucose oxidase) reagent was obtained from Hughes and Hughes, Brentwood, Essex, U.K.

Enzymes. Preparations of arylsulphatase (EC 3.1.6.1), maltase (α -D-glucosidase, EC 3.2.1.20) and β -D-glucosidase (EC 3.2.1.21) were obtained from Sigma (London) Chemical Co. Ltd., London S.W.6, U.K. β -D-Fucosidase (EC 3.2.1.38) from rat epididymis and *Patella vulgata* were a gift from Dr J. Conchie, Rowett Research Institute, Aberdeen, U.K.

Bioassay procedure with intact *Buccinum*. This procedure was carried out as described by Mackie *et al.* (1968).

Isolation of *Marthasterias* steroid glycoside M_2 . The steroid glycoside mixture was isolated from a butanol extract of the starfish as described by Mackie *et al.* (1968). In a typical preparation the mixture (400 mg) was dissolved in 5 mM-phosphate (Na_2HPO_4 - NaH_2PO_4) buffer, pH 7.85 (20 ml), and applied to a column (37 cm $\times$ 2.8 cm diam.) of DEAE-cellulose (Whatman DE-32). The column was eluted with starting buffer until the extinction at 245 nm rose to 0.2. Subsequently the active glycoside (M_2) was eluted with 0.1 M-phosphate buffer, pH 7.6. Fractions of volume 25 ml were collected.

Quantitative sugar and sulphate analyses. Samples of the glycoside (5 mg) were heated with 1 ml volumes of $\text{m-H}_2\text{SO}_4$ in stoppered test tubes placed in a boiling-water bath. After the appropriate time solid BaCO_3 was added and the precipitated BaSO_4 removed by centrifugation

and washed with water. The supernatant solution and water washings were passed through a column (3.5 cm $\times$ 0.5 cm diam.) of Dowex 50 (H^+ form). The eluate was evaporated to dryness under reduced pressure and transferred quantitatively to a sheet of Whatman 3MM paper. Spots of D-glucose, L-fucose and D-quinovose were applied as markers and the chromatogram was developed with the solvent system *G*. After drying, guide strips were cut out and the positions of the sugars determined by spraying with aniline hydrogen phthalate solution.

The areas of the unsprayed chromatogram corresponding to the marker sugars were cut out and eluted with water. The amount of sugar was determined by using the cysteine- H_2SO_4 reagent (Dische & Shettles, 1948). Blank determinations were carried out on sheets of Whatman 3MM paper developed with the same solvent system, and the recovery of sugars was determined by applying known amounts of the three sugars to another chromatogram.

For the determination of sulphate (Dodgson, 1961) 5 mg samples were hydrolysed in 2M-HCl and acid was removed by using a rotary film evaporator.

Partial acid hydrolysis. Samples (2 mg) of the steroid glycoside M_2 were hydrolysed with 2 ml volumes of 0.05M-HCl in stoppered test tubes placed in a boiling-water bath. After the appropriate time the hydrolysates were neutralized to pH 7 by the addition of 0.2M- $NaHCO_3$ and made to a final volume of 5 ml. Total reducing sugars were determined with the alkaline ferricyanide reagent (Park & Johnson, 1949) and glucose was measured with a glucose oxidase (EC 1.1.3.4) reagent. The quantity of deoxy sugars could then be determined by difference. Preliminary experiments showed that glucose oxidase was without detectable effect on authentic samples of fucose and quinovose.

Isolation of degradation products of glycoside M_2 . Glycoside M_2 (30 mg) was heated in 0.05M-HCl (30 ml) for 4 h in a boiling-water bath under a reflux condenser. The hydrolysate was neutralized by the addition of 0.2M- $NaHCO_3$ and evaporated to dryness under reduced pressure. The residue was redissolved in water (20 ml) and extracted into water-saturated butan-1-ol (4 $\times$ 15 ml). The butanol extracts were combined, washed with water (2 $\times$ 5 ml) and evaporated to dryness under reduced pressure. The product was dissolved in 5 mM-phosphate buffer, pH 7.85 (2 ml), and applied to a column (13 cm $\times$ 1.8 cm diam.) of DEAE-cellulose. The column was eluted with 5 mM-phosphate buffer, pH 7.85 (fraction 1), and 0.1M-phosphate buffer pH 7.6 (fraction 2). The contents of tubes 5–22 (fraction 1) and 30–60 (fraction 2) were pooled and extracted into butanol after being concentrated under reduced pressure. Fraction 2 yielded approx. 5 mg of degraded steroid glycoside. A further sample of glycoside M_2 was hydrolysed for 7 h and fractions 1 and 2 were isolated as described above.

Preparation of the aglycone. Glycoside M_2 (250 mg) was dissolved in 2M-HCl (50 ml) and heated for 3 h under a reflux condenser in a boiling-water bath. The water-insoluble aglycone, after being washed with water, was dried at room temperature and dissolved in methanol. The solution was transferred to a tared container and evaporated to dryness under reduced pressure. The aglycone (77 mg) was stored *in vacuo* over KOH pellets until required.

Acetylation of the aglycone. The aglycone (30 mg) was

dissolved in anhydrous pyridine (1 ml) and treated dropwise with redistilled acetic anhydride (75 mg) and the solution stood at room temperature for 20 h. Excess of acetic anhydride was hydrolysed by the addition of water and the mixture evaporated to dryness under reduced pressure. Benzene (2 ml) was added and the solution again evaporated to dryness, giving the crude product as a pale-yellow oil (38 mg). Chromatography on silica gel yielded the acetylated product (22 mg).

Chromatography. T.l.c. of steroid glycosides and aglycones was carried out on a silica gel support (Camag DO; Merck GF254) with one or more of the following solvent systems: solvent *A*, upper phase of the system butan-1-ol-ethyl acetate-water (4:1:2, by vol.); solvent *B*, butan-1-ol-acetic acid-water (12:3:5, by vol.); solvent *C*, dichloromethane-acetone (4:1, v/v); solvent *D*, ethyl acetate-ethanol (4:1, v/v); solvent *E*, ethyl acetate-acetone (4:1, v/v); solvent *F*, ethyl acetate-benzene (1:19, v/v).

The steroid glycosides and aglycones were detected by spraying with a 10% (w/v) solution of phosphotungstic acid in ethanol (Yasumoto, Tanaka & Hashimoto, 1966; Smith, 1969), by exposure to iodine vapour or by u.v. absorption.

Chromatography of sugars was carried out on Whatman no. 1 or 3MM paper with one or more of the following solvent systems: solvent *G*, ethyl acetate-pyridine-water (10:4:3, by vol.); solvent *H*, phenol-water (5:2, w/v); solvent *J*, upper phase of the system butan-1-ol-ethanol-water (5:1:4, by vol.). Authentic samples of D-glucose, L-fucose and D-quinovose were used as markers and the sugars were detected by spraying with the standard aniline hydrogen phthalate reagent.

Infrared spectra. These were measured for Nujol mulls on a Perkin-Elmer model 237 grating spectrophotometer.

Ultraviolet spectra. These were measured in ethanol on Perkin-Elmer model 137 UV and Unicam SP.800 spectrophotometers.

Nuclear-magnetic-resonance spectra. These were determined for solutions in deuteriochloroform, with chloroform and tetramethylsilane as internal references, on Varian A60 and HA100 spectrometers.

Mass spectra. These were recorded on an AEI MS902 spectrometer operating at 70 eV and with a source temperature of 170°C.

RESULTS

Steroid glycosides were isolated from *M. glacialis* by butanol extraction as described by Mackie *et al.* (1968) and freed from traces of amino acids and other amines by treatment with Dowex 50 (H^+ form). The material had an extinction maximum at 245 nm and contained two major components as judged by t.l.c., both migrating towards the anode on paper electrophoresis at pH 7.8. Both components were strongly adsorbed on Dowex 1 (acetate form, at pH 4.5) but a clear separation could be effected by ion-exchange chromatography on DEAE-cellulose by using a step-wise elution procedure (Fig. 1).

The material eluted with 5 mM-phosphate buffer (fraction 1, Fig. 1; about 10% of the material

Fig. 1. Elution diagram of steroid glycoside mixture on a column of DEAE-cellulose (37 cm $\times$ 2.8 cm). The column was eluted with 0.1M-phosphate buffer, pH 7.6, at the point indicated by the arrow. Fractions of volume 25ml were collected and fractions within the horizontal bars were pooled. Fraction 1 was eluted by 5mM-phosphate buffer, and fraction 2 by 0.1M-phosphate buffer.

applied to the column) had no significant absorption at 245nm and was found by t.l.c. to contain one major component giving an orange-brown colour with phosphotungstic acid spray reagent, together with at least two other minor components. This mixture was designated glycoside M_1 but has not been further characterized.

The material in the pooled fractions absorbing at 245nm and eluted with 0.1M-phosphate buffer (fraction 2, Fig. 1; about 40% of the material applied to the column) migrated as a single component on t.l.c. in solvent systems *A* and *B*, gave a green colour with phosphotungstic acid spray reagent and for convenience was designated glycoside M_2 . This material was eluted from the column as an extremely broad peak, which initially suggested heterogeneity. However, t.l.c. failed to show any evidence of heterogeneity, and it is assumed that the broad peak is due to aggregation of the molecules of M_2 on the DEAE-cellulose. These aggregates may have been broken down when the column was eluted with 0.1M-phosphate buffer.

On bioassay with *B. undatum*, glycoside M_2 proved to be about ten times as active (on a dry-weight basis) as glycoside M_1 , and glycoside M_2 was also active in eliciting avoidance reactions in the scallops *Pecten maximus* and *Chlamys opercularis* and in the brittle-star *Ophiothrix fragilis*.

Properties of the steroid glycoside M_2 . Glycoside M_2 did not yield an insoluble adduct with cholesterol (Chanley, Ledeen, Wax, Nigrelli & Sobotka, 1959). It showed an absorption maximum at 245nm ($E_{1\%}^{1\text{cm}}$ 60 in water) and was essentially non-reducing (less than 10 $\mu\text{mol/g}$ dry wt.). The glycoside yielded colours with the following spray reagents: phosphotungstic acid (green), phosphomolybdic acid (blue), antimony trichloride (lavender),

Table 1. Mobility of glycoside M_2 , aglycone and aglycone diacetate in various solvent systems

Experimental details are given in the text

Solvent ...	R_F				
	<i>A</i>	<i>B</i>	<i>C</i>	<i>D</i>	<i>E</i>
Glycoside M_2	0.1	0.4	—	—	—
Aglycone	0.7	0.9	0.05	0.5	0.3
Aglycone diacetate	—	—	0.7	0.75	—

trichloroacetic acid (bluish-white fluorescence under u.v. light) and 2,4-dinitrophenylhydrazine (orange-yellow). On acid hydrolysis (2M-hydrochloric acid at 100°C for 3h) the steroid glycoside yielded a water-insoluble aglycone, reducing sugars and inorganic sulphate. Quantitative determination of the water-soluble components after hydrolysis for various times established that the molar proportions glucose:quinovose:fucose:sulphate were 1:2:1:1.

Nature of aglycone. The water-insoluble aglycone obtained by acid hydrolysis (2M-hydrochloric acid at 100°C for 3h) was homogeneous as judged by t.l.c. with several solvent systems (Table 1) and showed a strong absorption band at 237nm ($E_{1\%}^{1\text{cm}}$ 112 in ethanol). The absorption spectrum in concentrated sulphuric acid showed, after 2h, maxima at 298nm ($E_{1\%}^{1\text{cm}}$ 310) and 392nm ($E_{1\%}^{1\text{cm}}$ 210) with inflexions at 375nm and 415nm. This is consistent with the presence of hydroxyl and keto groups in the steroid nucleus (Smith & Bernstein, 1963).

The i.r. spectrum contained bands at 1695 cm^{-1} and 1610 cm^{-1} characteristic of an $\alpha\beta$ -unsaturated ketone.

The n.m.r. spectrum of the aglycone, although not well resolved, showed two high-field singlets (τ 9.36 and 9.04) characteristic of angular methyl groups of the steroid nucleus. Two broader singlets at lower field (τ 8.10 and 7.85) suggested the presence of methyl groups attached to a double bond, and broad multiplets at τ 6.40, 4.65 and 3.87 indicated that the molecule contained primary or secondary hydroxyl groups and two distinct kinds of olefinic double bond. The purity of the material available at this stage did not allow integrated proton intensities to be determined with sufficient accuracy. In addition, the exchangeable hydroxyl protons were submerged in the aliphatic proton envelope. The number of hydroxyl groups in the aglycone was therefore determined by acetylation with an excess of acetic anhydride in pyridine.

Although the aglycone and its diacetate migrated as a single component in solvents *C* and *D*, another solvent system (solvent *F*) resolved the diacetate into a major component and a trace (less than 10%) of a second component. The major component

Fig. 2. N.m.r. spectrum of aglycone diacetate. The chemical shifts shown are τ values relative to the absorption line of tetramethylsilane.

(R_F 0.44 in solvent *F*; three developments) was isolated by preparative t.l.c. on silica gel, by using multiple development. It showed an absorption maximum at 237 nm (ϵ 11170) consistent with an $\alpha\beta$ -unsaturated ketone chromophore. Its i.r. spectrum confirmed that the hydroxyl groups had been completely acetylated, and absorption in the carbonyl region at 1715 cm^{-1} was superimposed on the original unsaturated ketone absorption.

The n.m.r. spectrum (Fig. 2) of this material showed a new six-proton singlet at τ 7.96, indicating that two acetoxyl groups had been introduced. Other features of the spectrum were unchanged, apart from a downfield shift of one angular methyl group signal. Integrated intensities for this compound suggested that both hydroxyl groups were secondary (two-proton multiplet now at τ 5.3) and the low-field multiplet could now be assigned to the α -proton of an $\alpha\beta$ -unsaturated system identical with that of mesityl oxide (Jackman & Wiley, 1960) (I).

The olefinic methyl groups of this compound resonate at τ 8.12 and 7.88 respectively, each being

broadened by allylic coupling to the α -proton (τ 3.95). These chemical shifts agree closely with those recorded for the aglycone diacetate (τ 8.10 and 7.85 for the methyl groups and τ 3.94 for the olefinic proton). In addition, the u.v. spectra of the two compounds are closely similar, showing that the major chromophore of the aglycone and its diacetate is the same as that of mesityl oxide.

The mass spectrum (Fig. 3) of the diacetate confirmed that the aglycone was a derivative of cholestane. The molecular ion appeared at m/e 498 and accurate mass measurement established the molecular formula as $\text{C}_{31}\text{H}_{46}\text{O}_5$. With this information the aglycone may be deduced to be $\text{C}_{27}\text{H}_{42}\text{O}_3$.

Sequence of sugar residues and sulphate. Fig. 4 shows the rates of release of sugars and sulphate on hydrolysis with 0.05M-hydrochloric acid. After 2 h over 70% of the deoxy sugars were liberated, whereas less than 15% of the glucose was released (glucose oxidase method). Similarly, after 4 h 30% of the glucose and essentially all the deoxy sugars were released. Paper chromatography showed that fucose was the first detectable reducing sugar to appear, followed by quinovose (Table 2). Free inorganic sulphate was obtained only after the liberation of approx. 40% of the glucose.

The products obtained by hydrolysing glycoside M_2 for 4 h with 0.05M-hydrochloric acid were fractionated on a column of DEAE-cellulose, yielding fractions 1 and 2 (Fig. 5). Hydrolysis for 7 h gave an increased amount of fraction 1, which consisted of the aglycone (R_F 0.7 in solvent *A*), plus

Fig. 3. Mass spectrum of aglycone diacetate. The intensity of ions above m/e 390 has been multiplied by 5.

Fig. 4. Rates of release of sugars and sulphate from steroid glycoside M_2 with 0.05M-HCl at 100°C. ○, Glucose (mmol/g of glycoside M_2); ■, sulphate (mmol/g of glycoside M_2); ●, deoxy sugar (mmol/g of glycoside M_2).

another component (R_F 0.55), which contained glucose but no sulphate. The absence of sulphate is consistent with the fact that this component was unadsorbed on DEAE-cellulose. Fraction 2 accounted for approx. 20% of the original glucose, and t.l.c. showed the presence of a major component (R_F 0.25 with solvent A) and a small quantity of a second component (R_F 0.4). The material with R_F 0.25 contained glucose and sulphate in the molar ratio 1:1.1, and the material with R_F 0.4 contained sulphate but no detectable glucose. The small amount of material precluded the determination of the steroid/sulphate molar ratio.

Biological activity of degradation products obtained by partial acid hydrolysis. *Buccinum*-foot-withdrawal assays performed on samples of glycoside

M_2 hydrolysed with 0.05M-hydrochloric acid showed that the biological activity was relatively labile to acid (Table 2). Thus the range of minimal effective dose increased from its original value of 0.3–0.6 μ g to 2.5–5 μ g after 3 h. A comparison of the effect of acid on biological activity with the rates of release of sugars and sulphate indicates that the overall surface-activity of the steroid glycoside is more important than its negative charge.

DISCUSSION

The present paper confirms that the principal substance present in extracts of *M. glacialis* that induces an avoidance reaction in *B. undatum* is a steroid glycoside or a saponin-like substance. The first identification of a saponin from a source other than plants was made independently by Nigrelli (1952) and Yamanouchi (1955) during studies on the toxic substances of sea cucumbers (Holothuroidea). Subsequent work on echinoderms established that saponins were present in a variety of sea cucumbers (Chanley, Perlstein, Nigrelli & Sobotka, 1960; Yasumoto, Nakamura & Hashimoto, 1967; Habermehl & Volkwein, 1968) and starfish (Rio, Stempien, Nigrelli & Ruggieri, 1965; Yasumoto, Tanaka & Hashimoto, 1966). A variety of biological properties have been described for these starfish saponins, but they have not been previously identified as the substances responsible for the avoidance reactions of various marine organisms to predatory starfish.

The steroid glycosides of *M. glacialis* consist mainly of two components, designated glycosides

Table 2. *Lability of sulphate, sugars and biological activity to 0.05M-hydrochloric acid at 100°C*

Experimental details are given in the text. Abbreviations: dGal, fucose; dGlc, quinovose; Glc, glucose.

Time (h)	% of sulphate released	% of glucose released	Reducing sugars released	Range of minimal effective dose in bioassay (μ g)
0	0	0	—	0.3–0.6
0.5	0	0	dGal	0.3–0.6
1.5	0	10	dGal, dGlc, Glc	2.5–5
3.0	0	25	dGal, dGlc, Glc	2.5–5
6.5	20	60	dGal, dGlc, Glc	10–20

Fig. 5. Elution diagram of steroid glycoside degradation products on a column of DEAE-cellulose (13 cm $\times$ 1.8 cm). The column was eluted with 0.1M-phosphate buffer, pH 7.6, at the point indicated by the arrow. Fractions of volume 6 ml were collected and fractions within the horizontal bars were pooled.

(II) (plus two hydroxyl groups and one double bond unassigned)

(III) ($\lambda_{\max}$, 238.6 nm; ϵ 13170)

M_1 and M_2 , glycoside M_2 being the most active in eliciting the avoidance reaction of *B. undatum*. Glycoside M_2 was homogeneous by t.l.c. and was distinct from the steroid glycosides isolated by Yasumoto & Hashimoto (1965, 1967) and Chanley *et al.* (1960). Hydrolysis yielded reducing sugars, inorganic sulphate and a water-insoluble aglycone.

The aglycone was essentially homogeneous and a study of its structure revealed some novel features. Molecular features recognized in the n.m.r. spectrum could be assigned as in the structure (II). This leaves two hydroxyl groups and one double bond to be located in the steroid nucleus. The observed downfield shift of one of the angular methyl group signals on acetylation suggests that one at least of the hydroxyl groups lies close to the C-19 angular methyl group, and that both are remote from the C-18 angular methyl group. The almost universal occurrence of a hydroxyl group in the C-3 position in naturally occurring steroids suggests that one of the hydroxyl groups is in this position.

Clearly the only position available for the $\alpha\beta$ -unsaturated ketone system is in the side chain, i.e., the Δ^{24} -23-ketone shown (structure II). The olefinic methyl groups are at C-26 and C-27. These

deductions are confirmed by the mass-spectral fragmentation pattern. The major fragmentation (see Scheme 1) involves cleavage of the side chain at C-20–C-22 with the loss of six carbon atoms. The side-chain fragment with m/e 98 is the base peak in the mass spectrum and the fragmentation apparently occurs both in the molecular ion and from the $[M-60]$ and $[M-120]$ ions formed by elimination of one or two molecules of acetic acid. The Δ^{24} -23-ketone is not an artifact of acid hydrolysis, since the glycoside M_2 showed an absorption maximum at 245 nm characteristic of such a system.

The presence of such a Δ^{24} -23-ketone system has not, as far as we are aware, been previously reported in any naturally occurring steroid or steroid glycoside. Entwistle & Pratt (1968) have described the isolation of 23- ξ -hydroxylanosterol from a common fungus. Oxidation of this material with chromium trioxide afforded the diketone (III), which displayed n.m.r. features analogous to the aglycone diacetate studied here.

The aglycones of saponins from sea cucumbers

Scheme 1.

and starfish differ in structure. Those from sea cucumbers contain a five-membered ring lactone absent from those of starfish (Yasumoto & Hashimoto, 1965; Yasumoto *et al.* 1967). The i.r. spectrum of the aglycone of *M. glacialis* glycoside M_2 showed that such a lactone system was absent. The aglycone of one of the saponins of the Pacific starfish *Asterias amurensis* also appeared to contain an $\alpha\beta$ -unsaturated ketone (Yasumoto & Hashimoto, 1967), but the two apparently homogeneous saponins were probably mixtures. This can be seen from the fact that both saponins gave, on hydrolysis, two identical aglycones.

In addition to eliciting an avoidance reaction in *B. undatum* and other molluscs, glycoside M_2 was haemolytic (Mackie *et al.* 1968) and caused contraction of isolated smooth muscle. This may be compared with the findings of Yasumoto & Hashimoto (1965) for asterosaponin A, which was found to cause irreversible blockage of contractural responses from a rat phrenic nerve-diaphragm preparation (Friess, Durant & Chanley, 1968). The exact role of the sulphate group of glycoside M_2 is still to be determined. The sulphate group of holothurin A is essential for blockage of contractural response (Friess, Durant, Chanley & Fash, 1967), but attempts to remove sulphate from glycoside M_2 with a sulphatase preparation have been unsuccessful. Similarly α - and β -D-glucosidase and β -D-fucosidase appeared to have no effect on the glycoside. However, the effect of acid hydrolysis on biological activity (foot withdrawal) suggests that the surface activity of the glycoside is more important than the negative charge. This view is supported by the fact that non-ionic surface-active agents also elicit avoidance reactions (Mackie *et al.* 1968).

Glycoside M_2 contained glucose, quinovose, fucose and sulphate in the molar proportions 1:2:1:1. Since the steroid glycoside and its degradation products are non-reducing, all the monosaccharides must be involved in glycosidic

(IV) (suggested relationship of constituent monosaccharides and sulphate to aglycone in steroid glycoside M_2 ; abbreviations: dGal, fucose; dGlc, quinovose; Glc, glucose)

linkage, both to each other and to the steroid. Fucose is the first monosaccharide released on hydrolysis, followed by quinovose and assuming a linear oligosaccharide, fucose must therefore be at the non-reducing end. The isolation of a non-reducing fragment containing approximately equimolar amounts of glucose and sulphate suggests that glucose is glycosidically linked to the steroid. Glucose is released more rapidly than sulphate (Fig. 4), which indicates that the sulphate group is linked to the steroid via an ester linkage to the other hydroxyl group. If the sulphate was attached to glucose, free glucose would not be obtained until after the release of sulphate. The release of glucose was determined by using the enzyme glucose oxidase. This enzyme is specific for glucose, and neither glucose 3-O-sulphate nor glucose 6-O-sulphate act as substrate (Lloyd, Large, Davies, Olavesen & Dodgson, 1968). The isolation of a fragment containing sulphate but no detectable glucose is further evidence for a linkage between steroid and sulphate.

An alternative structure might be a sulphate diester, with the sulphate acting as a bridge between glucose and steroid. However, such a fully covalent diester would be uncharged and is therefore excluded by the negative charge on the steroid glycoside, demonstrated by its behaviour during electrophoresis and ion-exchange chromatography. Assuming a linear oligosaccharide, the sequence must therefore be as shown (see structure IV). This structure is compatible with the presence of two hydroxyl groups in the steroid nucleus, and may be compared with the findings of Chanley, Feageson, Mezzetti & Rossi (cited by Chanley & Rossi, 1969), who concluded that the sulphate group of holothurin A, the glycoside from a sea cucumber, is attached to one of the sugars (xylose). No information is, however, available on the position of the sulphate group in other starfish steroid glycosides. The preponderance of deoxy sugars in the glycoside is in agreement with the results of work carried out on the saponins of Pacific starfish. Thus asterosaponin A contains 2 molar proportions of fucose and 2 molar proportions of quinovose, and asterosaponin B contains fucose, quinovose, xylose and galactose in the molar proportions 1:2:1:1, both saponins containing 1 molar proportion of sulphate (Yasumoto & Hashimoto, 1967).

The function of the starfish steroid glycosides in Nature is not known. It has been suggested that the starfish may use toxic substances to induce shell opening in certain molluscs, but there appears to be little evidence for this (Feder & Christensen, 1966). The steroid glycosides may serve as repellents against potential predators, as is the case with sea-cucumber glycosides (Bakus, 1968). The method of preparing the extracts involves freezing and thawing the starfish and this may result in the introduction of stomach contents and other contaminants. In the case of *A. amurensis* steroid glycosides were found in all the parts of the starfish examined, and the extract may therefore contain steroid glycosides not present in the surface epithelium. This is unlikely, since extraction of excised oral and aboral epithelia of *M. glacialis* yielded the same glycosides (M_1 and M_2) normally found in the exudate. The method of preparation could release hydrolytic and other enzymes that may result in changes in the structure of the steroid glycosides during isolation.

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