Journal of Natural Products
Vol. 58, No. 2, pp. 172-176, February 1995

IN VIVO AND IN VITRO BIOSYNTHESIS OF SAPONINS IN SEA CUCUMBERS

RUSSELL G. KERR* and ZHENGJIAN CHEN

Department of Chemistry, Florida Atlantic University, Boca Raton, Florida 33431

ABSTRACT.—The triterpene precursor of saponins in sea cucumbers has been identified as parkeol [lanost-9(11)-en-3β-ol] [**1**]. Dissection of the sea cucumbers *Holothuria floridea* and *Actinopyga agassize* after incubations with radiolabeled parkeol demonstrated that saponin biosynthesis occurs exclusively in the Cuvier gland. This result was corroborated by incubating a cell-free extract of the Cuvier gland with labeled parkeol and observing transformation of the precursor to saponins.

Saponins are common metabolites of members of the phylum Echinodermata, particularly the sea cucumbers (Holothuroidea) and starfish (Asteroidea) (1). Holothurins, saponins from holothurians, are typically composed of carbohydrate and triterpenoid moieties, while asterosaponins are characteristically steroidal glycosides (1). In addition to the echinoderms, sponges, and coelenterates have also furnished a number of novel saponins (2).

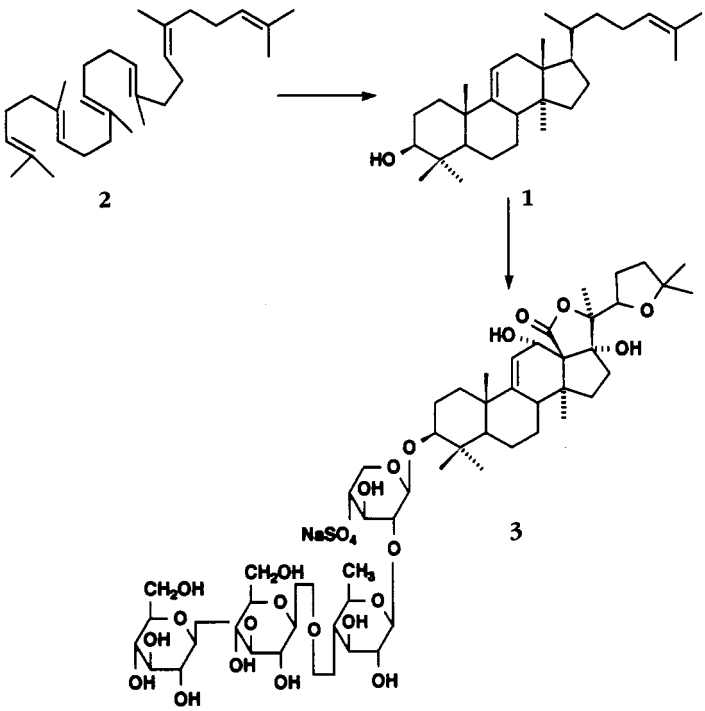
Most saponins exhibit potent hemolytic properties (3), while antitumor (4), antiinflammatory (5), and antibacterial (6) activities have also been reported. The saponins from several starfish have been shown to inhibit influenza virus multireplication (7). Recently, several triterpenoid glycoside sulfates have been isolated from the sea cucumber *Cucumaria echinata* (Cucumariidae) and the corresponding aglycones showed activity against L-1210 and KB cells (8). Due to the general toxicity of the saponins it is generally believed that these compounds act as chemical defense agents. The toxins are concentrated in specialized organs known as Cuvier glands which can be eviscerated to deter predators.

The triterpene portions of holothurins typically contain a $\Delta^{9(11)}$ double bond, which suggests that these compounds are derived from parkeol [**1**] [lanost-9(11)-en-3β-ol] (Scheme 1). A few reports have appeared in the literature concerning the metabolic origin of these echinoderm toxins, yet their biosynthetic precursors remain to be identified. Recently, Cordeiro and Djerassi reported that ^{3}H-labeled parkeol, lanosterol, and cycloartenol were not transformed into the saponins of the sea cucumbers *Bohadschia argus* and *Holothuria mexicana* (9,10). As these authors have indicated, it is puzzling that none of these triterpenes was transformed into saponins and this result may be due to some factor such as lack of transportation of precursor to the site of saponin biosynthesis, or the fact that the toxin is not continuously synthesized. Previous experiments with ^{14}C-labeled mevalonate (11) and acetate (12) resulted in low levels of incorporation into the crude saponins suggesting *de novo* biosynthesis does operate in sea cucumbers.

The sterols of sea cucumbers have been well characterized (1), and generally, the principal sterols have a $\Delta^{9(11)}$ 14α-methyl nucleus and thus appear to be metabolically related to the saponins. The biosynthetic precursor of this unusual sterol class has been demonstrated to be parkeol (9). Further, it was shown that squalene cyclizes directly to produce parkeol and not the isomeric lanosterol or cycloartenol. It thus seems reasonable that in sea cucumbers, parkeol [**1**] is the sole cyclization product of squalene [**2**] and that **1** is the precursor of both holothurins and sterols.

RESULTS AND DISCUSSION

The sea cucumbers used in this study were *Holothuria floridea* and *Actinopyga agassize*.



SCHEME 1. Biosynthesis of saponins in sea cucumbers.

These were collected off Long Key, Florida, and maintained in artificial tide pools with running sea water at the Keys Marine Laboratory. We have found that sea cucumbers survive for many weeks in this environment; however, if placed in relatively small aquaria, they generally eviscerate their Cuvier gland within a few hours. The organisms were left for about two weeks in the tide pools prior to incorporation experiments. It was our intent to provide as natural an environment as possible for the test sea cucumbers, to minimize stress and thus avoid evisceration which would result in loss of any labeled saponins.

The saponins from these sea cucumbers were isolated as previously described (13) and identified by comparison of their ^{13}C-nmr spectra with literature data (13). The main saponin from both *H. floridea* and *A. agassize* is holothurin A [**3**] (13).

In vivo biosynthetic experiments were conducted by injecting a solution of [24-^{3}H] parkeol in EtOH-H_2O (1:1) to the coelemic cavity of the sea cucumber. The labeled parkeol was synthesized as described previously (9). The injections were performed underwater to minimize stressing of the animal. The sea cucumbers were monitored for the first few hours to determine if the procedure would result in evisceration of the Cuvier gland. After an incubation period of seven days, the organisms were harvested and stored at $-20°$. To localize the site of saponin biosynthesis, the thawed sea cucumber was dissected into three components: (a) body wall, (b) Cuvier gland, and (c) coelemic fluid and internal organs. The three components were extracted separately by Soxhlet extraction with 95% EtOH. Purification of the saponins and parkeol was achieved by prep. tlc followed by repeated injection on reversed-phase hplc. The results of the parkeol feeding experiments with *H. floridea* and *A. agassize* are summarized in Table 1. With both sea cucumbers, radioactive parkeol and holothurin A [**3**] were recovered from the Cuvier gland, while with the body wall and coelemic fluid, the only radioactive fractions

TABLE 1. In Vivo Biosynthetic Experiments.

Sea cucumber part	Recovered radioactivity from the injection of 22×10^6 parkeol [1] (in dpm)			
	Holothuria floridea		*Actinopyga agassize*	
	1	3	1	3
Body wall.....	1.6×10^3	cold	6.7×10^3	cold
Cuvier gland	cold	1.9×10^3		
Coelemic fluid ...	1.7×10^2	cold	1.6×10^{3a}	1.3×10^{3a}

[a]The Cuvier gland could not be separated from the coelemic fluid and internal organs in *A. agassize* and were therefore worked up together.

were those associated with parkeol or sterols. These data suggest that parkeol is transported to the Cuvier gland and, in this organ, is then elaborated to the saponin.

To confirm that the location of saponin biosynthesis in sea cucumbers is the Cuvier gland, cell-free extracts of this organ from *H. floridea* and *A. agassize* were prepared and used in incubation experiments with labeled parkeol. The enzyme preparation was generated by grinding a fresh Cuvier gland in liquid N_2 to a fine powder in a large mortar and pestle and adding this to a buffer containing a mixture of protease inhibitors and protein stabilizers (phenyl methyl sulfonyl fluoride, Leupeptin, Pepstatin A, ethylene diamine tetraacetic acid, and dithiothreitol). Two sets of experiments with different buffers were used, as our experience with other systems has been that the choice of buffer greatly affects the activity of the cell-free extract. The buffers used were Tris and a phosphate buffer both at a pH of 7.7. The in vitro metabolic experiments were performed by incubating the cell-free extract with 1.1×10^6 dpm [24-^{3}H] parkeol at 27° for 6 h. Controls with both buffers were conducted in which the cell-free extracts were heated at 100° for 1 h prior to incubation with labeled parkeol.

The results of all in vitro biosynthetic experiments are summarized in Table 2. Although the level of transformation varied between the two sea cucumbers, radioactive holothurin A [3] was obtained from both cell-free extracts. The recovered saponin from the controls was not radioactive in both cases.

With all in vivo and in vitro biosynthetic experiments, the intact saponin was purified by prep. tlc, followed by reversed-phase hplc using MeOH as mobile phase. The saponin fraction from the hplc injection was reinjected using the same conditions, and a small portion analyzed by a scintillation counter. To confirm that the observed radioactivity was due to holothurin A rather than an impurity that co-eluted on tlc and hplc, the purified saponin was hydrolyzed with aqueous HI and the resulting triterpene

TABLE 2. In Vitro Biosynthetic Experiments.

Type of buffer	Recovered radioactivity from the incubation of 1.1×10^6 parkeol [1] with cell-free extract (in dpm)			
	Holothuria floridea		*Actinopyga agassize*	
	1	3	1	3
Phosphate buffer .	4.4×10^5	4.6×10^3	6.6×10^5	2.8×10^3
Control	1.3×10^5	cold	1.2×10^6	cold
Tris buffer	4.4×10^5	5.4×10^3	9.8×10^5	1.2×10^3
Control	1.3×10^6	cold	1.2×10^6	cold

portion purified by prep. tlc. The recovered radioactivity of the resulting aglycone (*H. floridea*, 1900 dpm; *A. agassize*, 1300 dpm) was very similar to that of the portion of the saponin used in the hydrolysis (2000 dpm in each case), indicating that the observed radioactivity was due to the saponin.

The results obtained in this investigation have identified the triterpene precursor of saponins as parkeol [**1**] and, through dissection of sea cucumbers following in vivo experiments and by examining the metabolic capacity of a cell-free extract of Cuvier glands, the site of saponin biosynthesis has been established.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Hplc was performed using a refractive index detector, and either an Ultra-Si normal-phase column (10 mm i.d.×25 cm) using 8% EtOAc/hexanes, or a reversed-phase Ultrasphere column (10 mm i.d.×25 cm) with aqueous MeOH as mobile phase. Prep. tlc was performed with precoated Si plates (1000 μm thickness) using berberine chloride as the spray reagent. All solvents were distilled prior to use. Radioactivity was determined using a liquid scintillation counter and a toluene-based scintillation fluid.

ANIMAL MATERIAL.—*H. floridea* and *A. agassize* were collected off Long Key, Florida, at depths of 1–2 m. Voucher specimens have been kept in the author's laboratory at Florida Atlantic University. They were maintained at the Keys Marine Laboratory (Long Key) in running sea water prior to transporting to Florida Atlantic University.

WHOLE ORGANISM BIOSYNTHETIC EXPERIMENTS.—Whole organism biosynthetic experiments were performed by injecting an EtOH solution of radiolabeled parkeol into the coelemic cavity of the sea cucumber and the organism left for 7 days, flash frozen in liquid N_2, and stored at −20°.

EXTRACTION OF SEA CUCUMBERS.—The thawed sea cucumber (ca. 500 g) was dissected into its (a) body wall, (b) Cuvier gland, and (c) coelemic fluid and internal organs. Each of these three portions was extracted separately in a Soxhlet apparatus with 200 ml 95% EtOH for 48 h.

PURIFICATION OF TRITERPENES AND SAPONINS.—Parkeol (5 mg) was added as cold carrier to the crude extract as it is only present in trace quantities in the sea cucumbers. The EtOH extract was concentrated *in vacuo* and partitioned between Et_2O and H_2O. The organic fraction was shown to contain the triterpene parkeol [**1**] by tlc and was purified by prep. tlc using precoated silica plates (1000 μm thickness) and a mobile phase of hexanes-Et_2O (1:1). Subsequent purification by normal-phase hplc (8% EtOAc/hexanes) followed by reversed-phase hplc (MeOH) resulted in a parkeol fraction that was shown to be pure by capillary gc.

The aqueous fraction was concentrated by azeotropic distillation with C_6H_6 and purified by prep. tlc (silica, 1000 μm thickness) using $CHCl_3$-MeOH-H_2O (65:35:10), followed by hplc purification under reversed-phase conditions using MeOH-H_2O (73:27) to yield holothurin A [**3**].

Hydrolysis of saponin.—A portion of the hplc-purified saponin (holuthurin A, **3**) (2.0×10^3 dpm) was hydrolyzed with 57% HI at 120–130° for 6 h. Following an aqueous workup, the resulting genin was purified by prep. tlc ($CHCl_3$-MeOH-H_2O, 65:35:10).

PREPARATION OF CELL-FREE EXTRACT.—The Cuvier glands of specimens of *H. floridea* and *A. agassize* were carefully and quickly dissected at 0°, and ground to fine powders in liquid N_2. These were added to two separate buffer systems, buffer A, a phosphate buffer, and buffer B, TES/Tris, both at a pH of 7.7. The buffers (100 ml) contained 5 mM dithiothreitol, 5 mM ethylenediamine tetraacetic acid, and 10% bovine serum albumin. Leupeptin (100 μg), pepstatin A (100 μg), and phenyl methyl sulfonyl fluoride (0.1 mM) were added to the buffered crude enzyme preparations, followed by Tween 20 (100 μl). Aliquots (5-ml) of the cell-free extracts were then incubated with 0.5 μCi [24-^{14}C] parkeol for 6 h at 27°.

Controls of the cell-free extracts were prepared by heating the enzyme preparation at 100° for 1 h prior to the 6-h incubation. The incubations were terminated by addition of 5 ml of EtOAc. The parkeol and saponin were purified chromatographically as described above.

PREPARATION OF RADIOLABELED PARKEOL.—[24-^{3}H] Parkeol was prepared from cycloartenol according to a previously reported procedure (9).

ACKNOWLEDGMENTS

Financial assistance was provided by the Donors of The Petroleum Research Fund, administered by

the American Chemical Society, and by a Bristol-Myers Squibb Company Award of Research Corporation. We acknowledge the generous assistance of the staff of the Keys Marine Laboratory, Layton, Florida, in the collection and maintenance of the sea cucumbers.

LITERATURE CITED

1. D.J. Burnell and J.W. ApSimon, in: "Marine Natural Products." Ed. by P.J. Scheuer, Academic Press, New York, 1983, Vol. 5, Chap. 6.
2. D.J. Faulkner, *Nat. Prod. Rep.*, **9**, 323 (1992), and previous reviews in this series.
3. Y. Hashimoto, "Marine Toxins and Other Bioactive Marine Metabolites," Japan Scientific Societies Press, Tokyo, 1979, pp. 268–288.
4. G.R. Pettit, J.F. Day, J.L. Hartwell, and H.B. Wood, *Nature*, **227**, 962 (1970).
5. L.A. Goldsmith and G.P. Carlson, in: "Food and Drugs from the Sea. Proceedings 1974." Ed. by H.H. Webber and G.D. Ruggieri, Marine Technology Society, Washington, DC, 1976, pp. 354–365.
6. G.D. Ruggieri, R.F. Nigrelli, in: "Bioactive Compounds from the Sea." Ed. by H. Humm and C. Lane, Marcel Dekker, New York, 1974, pp. 183–195.
7. Y. Shimizu, *Experientia*, **27**, 1188 (1971).
8. T. Miyamoto, K. Togawa, R. Higushi, T. Komori, and T. Sasaki, *Liebigs Ann. Chem.*, 453 (1990).
9. M.L. Cordeiro and C. Djerassi, *J. Org. Chem.*, **55**, 2806 (1990).
10. M.L. Cordiero, R.G. Kerr, and C. Djerassi, *Tetrahedron Lett.*, **29**, 2159 (1988).
11. S.S.N. Murthy and A. Der Marderosian, in: "Food and Drugs from the Sea. Proceedings." Ed. by R. Worthen, Marine Technology Society, Washington, DC, 1973, p. 181.
12. A. Kelecom, D. Daloze, and B. Tursch, *Experientia*, **32**, 2353 (1976).
13. G.B. Elyakov, T.A. Kuznetsova, V.A. Stonik, and R. Albores, *Comp. Biochem. Physiol.*, **52B**, 413 (1975).

Received 5 July 1994