Identification and Biosynthetic Origins of Sterols in the Marine Bryozoan *Bugula neritina*

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The sterols of *Bugula neritina* have been isolated and characterized spectroscopically. Cholesterol was found to be the predominant sterol with C_{28}, C_{29}, and C_{30} sterols recovered as minor components. In vivo biosynthetic experiments revealed that cholesterol is the only sterol produced by de novo biosynthesis, indicating that the other sterols are of dietary origin. Further, biosynthetic experiments using in vitro techniques indicated that 24-alkylated sterols (**4**, **5**, **7**, and **8**) are produced by alkylation of dietary sterols, while others are exclusively of dietary origin.

The sterols of marine invertebrates have become well characterized in recent years. It is now clear that unconventional structures are more common in the more primitive invertebrates, and that cholesterol is present in greater relative abundance in more advanced organisms.[1,2] Although there are numerous reports describing the structures and biosynthetic origins of sterols in many marine phyla,[1,2] there is a lack of information on members of the phylum Bryozoa. Specifically, there is a single report indicating the presence of dinosterol (4α,23,24-trimethylcholestanol) in the bryozoan *Bugula neritina*,[3] and no literature concerning the biosynthetic origin of sterols in these organisms.

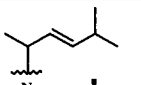
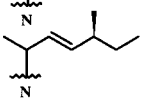
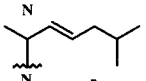
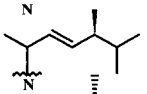
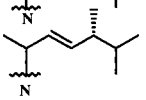
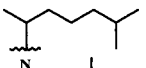
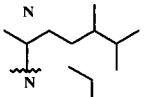
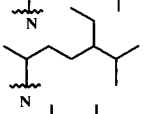
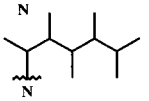
The bryozoan *B. neritina* is the source of the bryostatins, a family of polyketide macrolides with potent anticancer activity.[4] Bryostatin 1 is currently in clinical development and showing great promise as a treatment for leukemia and melanoma.[4] Due to difficulties encountered with obtaining sufficient quantities of *B. neritina* from nature, efforts directed toward the aquacultural production of the bryostatins are of key importance. Knowledge of limiting lipids in *B. neritina* is of great potential value in formulating enriched diets, as various groups have found that supplementing the diet of marine invertebrates with key lipids leads to enhanced growth rates.[5−8] This report describes the identification of sterols and presents a discussion of the sterol biosynthetic capabilities in *B. neritina*.

Results and Discussion

B. neritina is found in numerous temperate and subtropical regions worldwide; however, only a very few populations contain the clinical agent bryostatin 1.[9,10] In the study described herein, we examined the sterol metabolic capabilities of populations of *B. neritina* with and without bryostatin 1. Specimens of the bryozoan were collected from Palos Verdes, California (−10m), and from the Intracoastal Waterway at Boca Raton, Florida (−1m). The former group contains bryostatin 1, while the latter contains other congeners in this series.

Crude extracts of *B. neritina* were obtained from freeze-dried samples by repeatedly soaking in chloroform−methanol (1:1) until no further pigments were recovered. Flash chromatography over silica using a step gradient of hexane−ethyl acetate followed by normal-phase HPLC (hexane−ethyl acetate 8:2) afforded a mixture of sterols. Final purification was achieved by reversed-phase HPLC using methanol as eluent. The sterols were analyzed by comparison of NMR and GC−MS data with that from authentic standards. As described in Table 1, the two populations have similar sterol compositions. In both cases, the predominant sterol was cholesterol, with seven common marine sterols (**2**−**8**) present in smaller amounts. Both populations have small amounts of the unconventional sterols 22,23,24-trimethylcholesterol (**9**). Dinosterol, reported by Zielinski and co-workers,[3] was not present in the

Table 1. Sterol Compositions of *Bugula neritina*

	structure		Relative Abundance (%) California	Relative Abundance (%) Florida
1		24-nor-22-dehydrocholesterol	2.6	0.8
2		occelasterol	13.7	5.5
3		22-dehydrocholesterol	4.3	11.0
4		brassicasterol	7.5	8.8
5		epibrassicasterol	2.1	3.0
6		cholesterol	62.5	55.1
7		24-methylcholesterol	2.2	11.2
8		24-ethylcholesterol	3.5	2.6
9		22,23,24-trimethylcholesterol	1.5	1.8

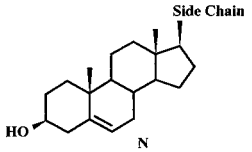


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Table 2. Recovered Radioactivity (dpm) in Sterols **4**, **5**, **7**, and **8** from Incubation with ^{3}H-SAM and Desmosterol

source of *B. neritina*	**4**	**5**	**7**	**8**
California	12 400	18 950	24 500	8000
Florida	87 200	60 800	147 900	9400

populations of *B. neritina* examined in this study. However, the 22,23,24-trimethylcholesterol is biosynthetically related to dinosterol, perhaps suggesting a relatedness between these collections.

There are three potential sources of sterols in *B. neritina*, de novo biosynthesis, assimilation from the diet, and modification of dietary sterols. Experiments have been conducted to evaluate the relative contributions of these factors to the sterol composition of this bryozoan. Biosynthetic experiments were carried out using both in vivo and in vitro techniques. In vivo biosynthetic experiments with *B. neritina* were performed by adding a solution of the radiolabeled precursor to ca. 25 g of the bryozoan (2−3 colonies) in 500 mL aerated seawater in a 1-L beaker. (The use of liposome-encapsulated precursor did not increase the level of incorporation.) The seawater was changed every 12 h, and the bryozoans were fed a commercially available diet. After a total of 48 h, the bryozoans were extracted and the sterols purified as described above.

In vitro biosynthetic experiments were carried out using a modification of our previously reported method.[11] Freshly collected *B. neritina* was cleaned of extraneous material, flash frozen, and stored at −80 °C. The frozen tissue was ground to a fine powder in a large, chilled mortar and pestle, and the powder added to a phosphate buffer at pH 7.7 containing leupeptin (0.1 mM), pepstatin A (0.1 mM), phenylmethylsulfonylfluoride (0.1 mM), EDTA (5 mM), and DTT (5 mM). The enzyme preparation was then incubated with radiolabeled precursor for 4 h at 27 °C and then quenched by the addition of ethyl acetate.

To determine which sterols, if any, of *B. neritina* are produced de novo, the metabolism of [C_{24}-^{3}H] lanosterol was investigated under our in vivo conditions. The labeled lanosterol (10 μCi) was incubated with a 25-g colony of the bryozoan for 48 h. The sterols of the bryozoan were isolated by HPLC as described above, and the radioactivity of each sterol was determined by scintillation counting. Only cholesterol was radioactive in both the California and Florida samples (41 400 dpm and 32 000 dpm respectively), indicating that this is the sole sterol produced de novo by *B. neritina*. The remaining sterols are of an exogenous origin.

The question of possible modification of dietary sterols in *B. neritina* was addressed by examining the capacity for sterol side-chain alkylation and the less common dealkylation. Desmosterol (24-dehydrocholesterol) and ^{3}H-labeled (*S*)-adenosylmethionine (SAM, 2.2 × 10^6 dpm) were incubated with cell-free extracts of *B. neritina* samples from California and Florida. With both samples of the bryozoan, radioactive 24-methylcholesterol (**7**), 24-ethylcholesterol (**8**), brassicasterol (**4**), and epibrassicasterol (**5**) were recovered (Table 2). Thus, these four 24-alkylated sterols are produced, at least in part, by the alkylation of exogenous desmosterol.

To evaluate the capability of *B. neritina* to degrade phytosterols to cholesterol, [C_3−^{3}H] 24-methylenecholesterol (2.2 × 10^6 dpm) was incubated under our in vitro conditions. The degradation of $\Delta^{24(28)}$ sterols was first observed by Ikekawa[12] in insects, but has since been observed in marine invertebrates.[13] After the incubation of the cell-free extract of *B. neritina*, no radioactivity was recovered in any C_{27} sterol, indicating a lack of degradation of 24-methylenecholesterol. Thus, dealkylation of phytosterols appears to be absent in this bryozoan.

The above-described biosynthetic experiments indicate that cholesterol is the single sterol produced de novo; sterols **4**, **5**, **7**, and **8** are the result of alkylation of dietary sterols and **1**, **2**, **3**, and **9** are assimilated directly from the diet. Because cholesterol is synthesized from lanosterol via desmosterol, the lack of production of alkylated sterols in the lanosterol experiment indicates that de novo sterol biosynthesis must be compartmentalized and thus separated from the site of dietary modification of exogenous sterols. It is interesting to note that the sterol composition and biosynthetic origins of the two populations of *B. neritina* are very similar, even though the samples were collected from such distinct geographic environments.

Experimental Section

General Experimental Procedures. HPLC was performed using a refractive index detector and a normal-phase column (Altex, Ultrasil-Si, 10 mm i.d. × 25 cm) with 8% EtOAc in hexane as mobile phase followed by use of a reversed-phase Ultrasphere column (10 mm × 25 cm i.d.), with MeOH as mobile phase. The purity of HPLC fractions was determined by GC using a capillary column (DB-5, 25 m) and an FID with a temperature program of 280 °C (1 min), 5 °C/min to 290 °C (30 min). All solvents were Optima grade or distilled prior to use. [^{3}H−C_{24}]-Lanosterol was synthesized as previously reported,[14] and ^{3}H-labeled SAM was purchsed from DuPont. Radioactivity was determined using a liquid-scintillation counter and a toluene-based scintillation fluid.

Collection of *Bugula neritina* and In Vivo Biosynthetic Experiments. *B. neritina* was collected from Palos Verdes, California, at a depth of 10 m and shipped in seawater at 15 °C to Florida Atlantic University. The Californian bryozoan was maintained at 18 °C in 1-L beakers of seawater and the seawater changed every 12 h. *B. neritina* from Florida was collected at a depth of 1 m in the Intracoastal Waterway in Boca Raton. It was maintained at 22 °C and the seawater changed every 12 h. [C_{24}−^{3}H]-Lanosterol was added as an EtOH solution to the seawater, and seawater changes were made every 12 h for a total incubation time of 48 h. At this time, the bryozoan was lyophilized and extracted repeatedly with $CHCl_3$−MeOH (1:1). The extract was chromatographed on Si gel with the sterols eluting with 20% EtOAc in hexane. Further purification with normal-phase HPLC (20% EtOAc in hexane) followed by reversed-phase HPLC (MeOH) afforded sterols that were homogeneous by capillary GC.

Cell-Free Extract Preparation and Incubations. The *B. neritina* used for in vitro work was flash frozen in liquid N_2 and stored at −80 °C. The frozen material (250 g) was ground to a fine powder in a large, chilled mortar and pestle, and the powder was added to a phosphate buffer (500 mL) at pH 7.7, containing leupeptin (0.1 mM), pepstatin A (0.1 mM), EDTA (5 mM), DTT (5 mM), and 10% BSA. The homogenate was centrifuged at 5000 × *g* for 15 min and the supernatant stored at −80 °C. Aliquots (40 mL) were incubated with the precursor (^{3}H-SAM and desmosterol or ^{3}H-24-methylenecholesterol) in a shaker water bath at 30 °C for 8 h. The sterols were extracted from the aqueous mixture with EtOAc, and purified as described above.

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