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BIOSYNTHETIC STUDIES OF ECTEINASCIDINS IN THE MARINE TUNICATE *ECTEINASCIDIA TURBINATA*

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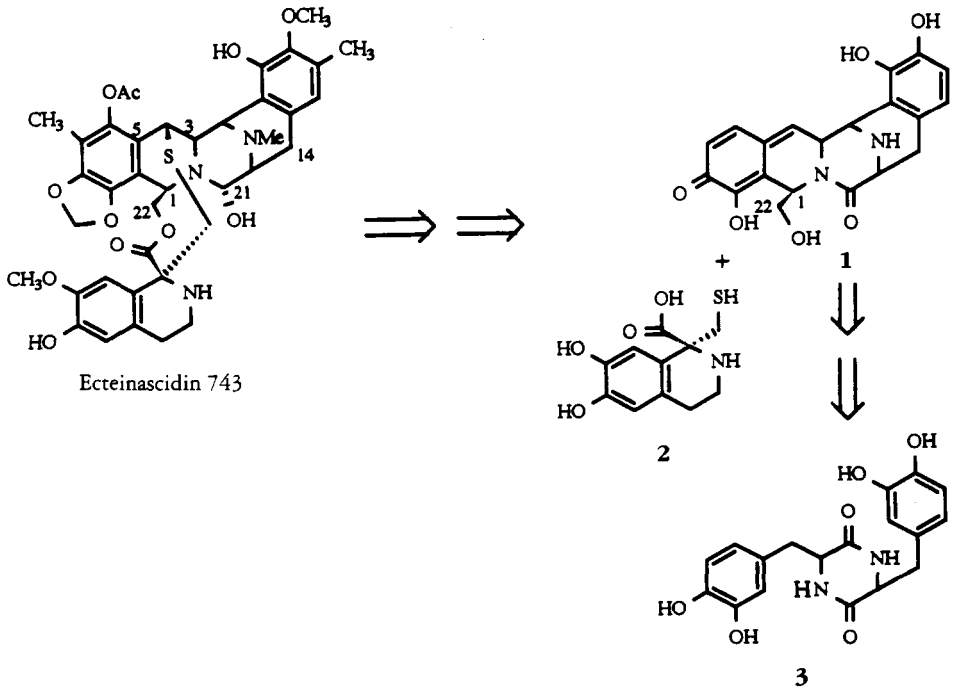
ABSTRACT.—A viable cell-free extract of the marine tunicate *Ecteinascidia turbinata* has been generated that is capable of transforming amino acids into the ecteinascidin antitumor alkaloids. Tyrosine and cysteine were identified as two building blocks used in ecteinascidin production. The enzyme preparations in the in vitro studies were fortified with ATP, Mg^{2+}, NADH, and NADPH.

The ecteinascidins are a family of alkaloids present in trace quantities in the marine tunicate *Ecteinascidia turbinata* (1–5) which exhibit potent activity against various tumor models in mice (5). Ecteinascidins have demonstrated protection against P-388 lymphoma, B16 melanoma, M5076 ovarian sarcoma, Lewis carcinoma, and the LX-1 human lung and MX-1 human mammary carcinoma xenografts. Ecteinascidin 743 (Et 743) is the most abundant of this family of alkaloids and is present in a 1×10^{-4}% w/w yield (see Scheme 1).

Ecteinascidins are tetrahydroisoquinoline derivatives which are structurally related to the safracins (6), saframycins (7,8), and naphthyridinomycins (9) from microbes, as well as the sponge metabolites, the renieramycins (10), and xestomysin (11). As suggested by Rinehart and co-workers (2), examination of the structures of these alkaloids seems to indicate that the ecteinascidin skeleton is composed of a functionalized diketopiperazine [**1**] and fragment **2**. Compound **1** is likely derived from diketopiperazine **3**, which is either a condensation product of two molecules of



SCHEME 1. Biogenesis of ecteinascidin 743.

DOPA or is the result of hydroxylation of the diketopiperazine from tyrosine. The two-carbon unit (C-1,C-22), is presumably derived from glyoxylate (or a glycoaldehyde), as has been documented with anhalamine (12). The glyoxylate could, in turn, be produced from either serine or glycine. Fragment **2** appears to be derived from the condensation of β-mercaptopyruvic acid with dopamine. It is also conceivable that **1** could condense with β-mercaptopyruvic acid and this subsequently combines with dopamine. Thus, while it seems reasonable to suggest which building blocks are involved in ecteinascidin production, the order in which they are assembled is not yet clear.

Specimens of *Ecteinascidia turbinata* were collected from mangrove roots in a shallow channel by Long Key, Florida. Colonies ranged in size from 250 ml to 1000 ml and were all collected at a depth of 1 m. The tunicate was transported back to the lab in aerated sea water.

Biosynthetic experiments were performed with both live organisms and enzyme preparations. To prepare a crude cell-free extract, freshly collected *E. turbinata* was cleaned of extraneous matter, flash frozen in liquid nitrogen and ground to a fine powder in a large, chilled mortar and pestle. The powder was added to a buffer containing several protein inhibitors and stabilizers (phenyl methyl sulfonyl floride 0.1 mM, leupeptin 0.1 mM, pepstatin A 0.1 mM, EDTA 5 mM, DTT 5 mM, and BSA 10%). The resulting crude enzyme extract was centrifuged at 10,000×*g* to remove cell debris. Two separate buffer systems were evaluated as our past experience indicated significant differences in effectiveness of buffers. We used a phosphate buffer and Tris/TES in separate experiments.

Because the natural products are present in trace quantities, we reasoned that the enzymes responsible for their production would also be present in low concentrations. To help facilitate an in vitro conversion of amino acids to the alkaloids, the supernatants from both buffers were fortified with the coenzymes ATP, Mg^{2+}, NADH, and NADPH. The fortified cell-free extracts were incubated with radiolabeled amino acid for 12 h at 27° and then quenched by the addition of EtOAc. The ecteinascidins were purified by a reversed-phase flash column followed by hplc as reported by Wright and co-workers (1). Ecteinascidin 743 was identified in the hplc by comparison of retention times with that of an authentic sample, and by co-injection with the standard. Radiochemical purity was determined by reinjection of the Et 743 peak from the first hplc injection using the initial solvent system (15% MeCN-85% H_2O-0.1% TFA) and subsequently with a different composition of mobile phase (10% MeCN-90% H_2O-0.1% TFA). In addition to collecting the ecteinascidin peak, fractions prior to and following this peak were also collected. In all experiments in Table 1 reporting radioactive ecteinascidins, the ecteinascidin peak was radioactive while fractions before and after this were at background level (<50 dpm).

The results of incubating [^{14}C(U)] tyrosine, [^{35}S] cysteine and [^{14}C(U)] serine

TABLE 1. In vitro Incorporations into Ecteinascidin 743.

Buffer (conditions)	Recovered radioactivity (dpm) in Et 743 from incubation of labeled amino acid with cell-free extract		
	[^{14}C(U)]tyrosine 5.5×10^6 dpm	[^{35}S] cysteine 11×10^6 dpm	[^{14}C(U)] serine 5.5×10^6 dpm
Phosphate	3.0×10^3 dpm	2.1×10^3 dpm	bkgnd[a]
Tris/TES	8.5×10^2 dpm	5.5×10^2 dpm	bkgnd[a]
Phosphate (control)	[a]	[a]	bkgnd[a]

[a]Background radioactivity is taken as <50 dpm.

with Tris/TES and a phosphate buffer are summarized in Table 1. Controls were performed to establish that any observed transformation of the labeled amino acids was due to enzymatic activity. The controls were identical to the experiments described above except that the crude cell-free extracts were heated at 100° for 1 h prior to addition of the labeled precursor.

The data indicate that tyrosine and cysteine are employed in the biosynthesis of the ecteinascidins. The relatively low levels of incorporation are presumably due to the efficient use of these amino acids in polypeptide synthesis. The lack of significant conversion of serine to the ecteinascidins could have two possible explanations. First, serine may simply not be used, and the glyoxylate (or an equivalent) may be derived from some other source such as glycine. Second, if the glyoxylate is derived from serine, there are many more biosynthetic steps involved in the conversion of serine to the ecteinascidins than with the conversion of tyrosine or cysteine to the same compounds. Thus, one would expect lower recovered radioactivity in the ecteinascidins from the serine experiments than with the other amino acids. It is therefore conceivable that serine is involved in ecteinascidin production but at a level below our present detection limits. It is also clear from Table 1 that the phosphate buffer is the superior one for in vitro ecteinascidin biosynthesis. Importantly, however, a viable cell-free extract of *E. turbinata* has been developed, which is capable of transforming amino acids to the target alkaloid.

Biosynthetic experiments were also performed using live colonies of the tunicate. In these experiments, a solution of the labeled precursor was added to a colony (300–500 ml) of *E. turbinata* in 1000 ml of sea water. The tunicate was allowed to filter this sea water for 8 h, rinsed with sea water, and then placed in an aquarium of flowing sea water. The tunicates were harvested after 10 days, the ecteinascidins purified as previously described, and the radioactivity measured. In these experiments, only 0.8–1.5% of the labeled precursor was incorporated into the tunicate. However, the purified ecteinascidin 743 was found to be radioactive (2,050 dpm), corroborating the in vitro findings with tyrosine. We experienced difficulties in maintaining healthy colonies of *E. turbinata* in the controlled environments required for the in vivo work, and thus in the future will pursue our cell-free methodology. Experiments are currently underway using the in vitro techniques to elucidate more extensively the metabolic origin of the ecteinascidins.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Hplc was performed using a diode-array detector monitoring at 230 nm, with a C_{18} reversed-phase column using MeCN-H_2O-TFA (18:82:0.1) as mobile phase. All solvents were distilled prior to use. Radioactivity was determined using a liquid scintillation counter and a toluene-based scintillation fluid.

ANIMAL MATERIAL.—Colonies of the tunicate *Ecteinascidia turbinata* were collected at Long Key, Florida, and maintained at the Keys Marine Laboratory in flowing sea water prior to transporting to Florida Atlantic University. The specimens of *E. turbinata* were found at a depth of ca. 1 m attached to mangrove roots.

WHOLE ORGANISM BIOSYNTHETIC EXPERIMENTS.—In vivo biosynthetic experiments were conducted as follows. Colonies (300–500 ml) were placed in a 1000-ml beaker of sea water that was floated in a holding tank of flowing sea water by means of a polystyrene ring. A solution of labeled precursor was added to the beaker and after 12 h the tunicate was rinsed in sea water and placed in flowing sea water for 12 days. The organism was then frozen and stored at −20° for later analysis.

EXTRACTION AND ISOLATION.—The frozen tunicate (300–500 g) was lyophilized and extracted repeatedly in MeOH. The crude extract was chromatographed under reversed-phase conditions, eluting with a step gradient of MeCN/H_2O (all fractions contained 0.1% TFA). The 40% MeCN/60% H_2O/0.1% TFA fraction contained the majority of the ecteinascidins as determined by hplc. This fraction was further purified by hplc using an analytical C_{18} column eluted at 1 ml/min

with 15% MeCN/85% H_2O/0.1% TFA, followed by re-injection with 10% MeCN/90% H_2O/0.1% TFA. Elution was detected by uv at 230 nm.

PREPARATION OF CELL-FREE EXTRACTS.—Freshly collected colonies of *E. turbinata* (ca. 500 ml) were flash frozen in liquid N_2 and ground to a fine powder in a large, chilled mortar and pestle. The powder was added to two buffer systems, a phosphate buffer and Tris/TES, both at a pH of 7.7. The buffers (200 ml) contained 5 mM dithiothreitol, 5 mM ethylenediamine tetraacetic acid, and 10% bovine serum albumin. Leupeptin (100 μg), pepstatin A (100 μg), phenyl methyl sulfonyl fluoride (0.1 mM), ATP (1 mg), NADH (1 mg), and NADPH (1 mg) were added to the buffered crude enzyme preparations. The enzyme extracts were divided into 50-ml aliquots and incubated with labeled precursor for 12 h. Controls of the cell-free extracts were prepared by heating the enzyme preparation at 100° for 1 h prior to incubation. The incubations were terminated by the addition of 50 ml EtOAc.

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