Abstract:
Because of the enormous diversity of biological functions demonstrated by steroidal molecules, polyhydrox-ylated sterols from marine organisms are potential sources of compounds with significant biological activity. In addition to the interest shown in these molecules because of this activity, many researchers are interested in determining the biological pathways which marine organisms utilize to produce these compounds. In order to study polyhydrox-ylated sterols, however, better methodology is required to separate and then to determine the structure of these molecules.

In this investigation thirteen polyhydroxylated sterols from the marine sponge Dysidea etheria were isolated and their structures determined by NMR and MS techniques. A B-cyclodextrin HPLC column was utilized for the difficult separations of these closely related compounds. This is the first time that this unique column packing has been used for the isolation of natural product compounds. An additional problem with the analysis of polyhydroxylated sterols is the difficulty of obtaining a molecular ion with most mass spectroscopic techniques. Various MS techniques including electron impact, positive chemical ionization, negative ion chemical ionization, fast atom bombardment, and liquid chromatography-mass spectrometry were compared and contrasted for their ability to provide molecular weight information as well as structural information. The results of this study revealed that liquid chromatography-mass spectrometry was superior to the other methods tested for providing molecular weight information.

Additional studies were performed to determine the biological significance of these polyhydroxylated sterols to D. etheria. Biological activity screens were run that tested these compounds for: cytotoxicity, antimicrobial activity, insect growth activity, plant growth regulation, and ionophore capability. The results of this last study revealed a possible link between the ionophore activity of these sterols and the ability of D. etheria to incorporate calcium carbonate detrital material into the spongin skeleton of the sponge.

POLYHDROXYLATED STEROLS FROM THE MARINE SPONGE,

DYSIDEA ETHERIA

by

Robert Raymond West

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APPROVAL

of a thesis submitted by

Robert R. West

This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

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ABSTRACT

Because of the enormous diversity of biological functions demonstrated by steroidal molecules, polyhydroxylated sterols from marine organisms are potential sources of compounds with significant biological activity. In addition to the interest shown in these molecules because of this activity, many researchers are interested in determining the biological pathways which marine organisms utilize to produce these compounds. In order to study polyhydroxylated sterols, however, better methodology is required to separate and then to determine the structure of these molecules.

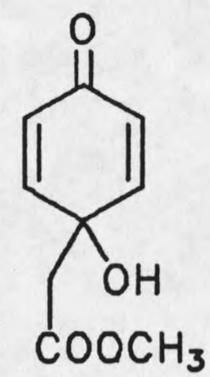
In this investigation thirteen polyhydroxylated sterols from the marine sponge *Dysidea etheria* were isolated and their structures determined by NMR and MS techniques. A ß-cyclodextrin HPLC column was utilized for the difficult separations of these closely related compounds. This is the first time that this unique column packing has been used for the isolation of natural product compounds. An additional problem with the analysis of polyhydroxylated sterols is the difficulty of obtaining a molecular ion with most mass spectroscopic techniques. Various MS techniques including electron impact, positive chemical ionization, negative ion chemical ionization, fast atom bombardment, and liquid chromatography-mass spectrometry were compared and contrasted for their ability to provide molecular weight information as well as structural information. The results of this study revealed that liquid chromatography-mass spectrometry was superior to the other methods tested for providing molecular weight information.

Additional studies were performed to determine the biological significance of these polyhydroxylated sterols to *D. etheria*. Biological activity screens were run that tested these compounds for: cytotoxicity, antimicrobial activity, insect growth activity, plant growth regulation, and ionophore capability. The results of this last study revealed a possible link between the ionophore activity of these sterols and the ability of *D. etheria* to incorporate calcium carbonate detrital material into the spongin skeleton of the sponge.

INTRODUCTION

The interest shown towards marine natural products has steadily increased since the early 1960's and the areas of interest seem to be expanding.[1] The pharmaceutical industry, for example, has become more involved in this research field, and new products will undoubtedly result from studies of the pharmacological activities of some of these compounds. The National Cancer Institute has also shown a vigorous new interest in marine natural products and has increased its commitment to developing new anticancer drugs from marine organisms.[2]

The roles of these natural products in the marine environment are just beginning to be understood. In the past, many studies of the secondary metabolites of marine organisms focused upon the defensive role of these compounds. More recent studies, however, have revealed the intricate ecological interplay of marine organisms to a much greater extent than ever before and some of the more subtle biological activities of some of these molecules are now being examined. An example of this emerging understanding of the ecological relationships between organisms was the observation that metamorphosis of the larvae of *Pecten maximus* occurred more readily in the presence of a species of red algae. Extracts from this alga revealed that a single compound, jacaranone, 1, was responsible for this settling behaviour.[3] Abalone larvae have shown a similar response upon exposure to crustose red algae, γ-aminobutyric acid (GABA) and its analogs being responsible in this case.[4] Other studies of some shell-less marine molluscs, known as nudibranchs, have demonstrated that many of these animals obtain metabolites from dietary sources and then incorporate them into defensive secretions.[5,6,7,8,9,10] These are just a few examples of the many ways that secondary metabolites of marine organisms aid in the defense, communication, and metabolic regulation of these creatures.



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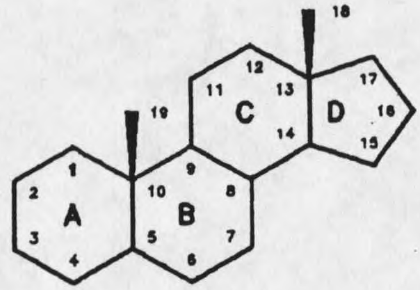
The competition for survival is so intense along the relatively narrow confines of the coral reefs that reef dwelling organisms have developed a complex system of antibiosis, which involves the limitation or exclusion of certain forms of life by diffusable substances produced by other living species.[11] Some organisms, such as sponges, are primarily filter feeders whose dietary intake requires the straining of myriads of bacteria and microplankton from seawater. Sponges are apparently resistant to the action of this multitude of microorganisms that enter their complex system of canals and cavities, and were therefore postulated to produce antibiotic compounds to prevent infection. As early as 1952, the antibiotic effects of some subtropical sponges were demonstrated by placing fresh sponge fragments on inoculated Petri dishes.[11] In addition to this demonstrated antibacterial activity, sponges, because of their sessile habit and porous structure, must also deal with a large number of organisms that frequently dwell in close proximity. One can easily imagine, therefore, that a sessile marine organism such as a sponge might have to produce compounds to protect itself from overgrowth by plants and other invertebrates, as well as from predation by crustaceans and other marine organisms.

It is precisely this fabulous array of biological activities that makes marine natural products so inviting to someone interested in developing compounds with new or different modes of action. The unique chemical environment of sea water has enabled marine organisms to elaborate so many new and unusual types of compounds in comparison to their terrestrial counterparts, and these new natural products quite often demonstrate biological activities that are different from those elicited by compounds from terrestrial sources. These are some reasons, then, why one might decide to study marine natural products: 1) to determine the delicate chemical ecological relationships that exist between marine organisms; and 2) to elucidate the structures of new and unusual compounds that demonstrate activity in one or more of a variety of biological screens designed to test for compounds with pharmacological or other unique biochemical significance.

Many of the secondary metabolites produced by marine organisms fall into the same categories as those produced by their terrestrial counterparts, even though the modifications might be very unusual. One class of compounds whose presence in marine organisms has been studied quite extensively is the sterols. The study of sterols in general dates back to the latter part of the eighteenth century, when the organic nature of gallstones was first discovered. In 1816, Chevreul named the principal component of these stones "cholesterine" to indicate that it was a fatty (-ine) solid (stereos) of bile (chole).[12] When Berthelot[13] demonstrated in 1859 that this compound was an alcohol, its name was changed to cholesterol to indicate that it was in this class of compounds. The term sterol came to be used for all compounds closely related to cholesterol, and the term steroids describes the entire array of naturally occurring and synthetic compounds that are derived from sterols. Therefore, an entire class of compounds of great physiological and biochemical importance has been grouped under the rather unimaginative name of "solids", if the closest English equivalent were to be used.[14]



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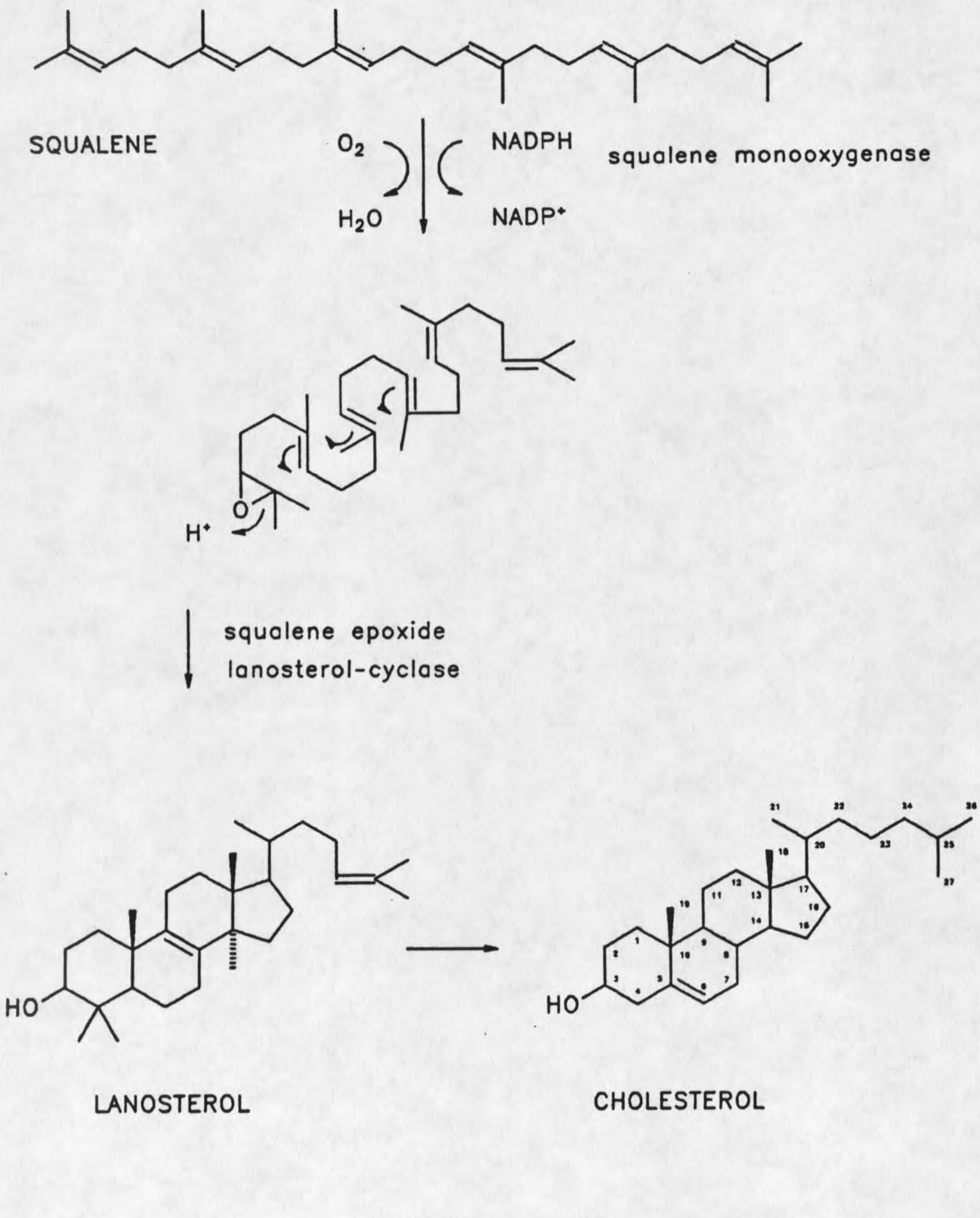
The structural feature common to all steroids is the saturated tetracyclic hydrocarbon perhydrocyclopentanophenanthrene, 2, shown with the commonly employed numbering system. Steroids differ in the number and position of double bonds; in the type, location, and number of substituent groups; in the configuration of these groups in relationship to the steroid nucleus; and in the configuration of the rings relative to one another. All steroids originate from the linear triterpene squalene, which cyclizes readily to form lanosterol through an extraordinary series of concerted 1,2-methyl group and hydride shifts, as well as π-electron migrations, along the squalene chain (Figure 1). Lanosterol can then undergo conversion to cholesterol by the removal of two methyl groups at C-4 and one from C-14, saturation of the double bond in the side chain, and shift of the double bond from the $\Delta^{8(9)}$ to the

$\Delta^{5(6)}$ position in ring B. The sterols of fungi and plants, such as ergosterol and stigmasterol, are synthesized by mechanisms similar to the above.[15]

Figure 1. Biosynthesis of cholesterol from squalene.



By the end of the 1800's, cholesterol had been isolated from virtually all animal sources tested and was therefore thought to be the universal sterol of the animal world. During the first decade of the present century, however, it was discovered that in certain animals cholesterol was replaced totally or in part by closely related compounds. It was from a sponge that Henze first isolated a sterol other than cholesterol from an animal.[16] Dorée (1909) was the first to describe the possible significance of this variation among animal sterols, and his work can be thought of as a classic in the field of comparative biochemistry.[17] Dorée isolated sterols from one or two representatives of the larger phyla of the animal kingdom, and since he found sterols in all the specimens, he inferred their presence in all animals. He obtained cholesterol not only from warm-blooded animals, but from vertebrates and invertebrates, such as sea anemones, as well. Although he did not find a sterol typical of each phylum, Dorée showed that in at least two invertebrates, a sponge and the common starfish, cholesterol was replaced by another substance. By 1909, a sufficient body of evidence had accumulated to demonstrate that there existed a diversity of sterols, at least among invertebrates.

With the advent of more sophisticated instrumentation and better separation techniques, enough information had been gathered about sterols and their structures to permit Bergmann, in two reviews (1949, 1962), to survey the growing body of data from the standpoint of comparative biochemistry.[14,18] In these studies, he examined the connections between an animal's sterol content and its classification, and speculated about the evolutionary aspects of sterols. Using sterols as taxonomic markers, Bergmann was able to make a good case for the reclassification of some organisms whose sterol content did not quite match with those from the same genus.

From the very early reports of Henze and Dorée, through Bergmann and other subsequent sterol researchers, sponges were recognized as being a rich source of new sterols. When Bergmann first began work on invertebrate sterols, the first four sponges that he investigated each yielded new sterols. These astonishing preliminary results prompted a comprehensive survey of sponge sterols of more than fifty species of sponges. Individual, identifiable sterols were obtained from about one-half of the species studied. Although the results were not as dramatic as the initial studies had indicated, there was still ample evidence from this body of research to suggest that sponges contained a great diversity of sterols. Comparative studies such as these indicated to many researchers that, in the animal kingdom, the more primitive species contain a greater variety of sterols,