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STEROLS OF SCALLOP

I. APPLICATION OF HYDROPHOBIC SEPHADEX DERIVATIVES TO THE RESOLUTION OF A COMPLEX MIXTURE OF MARINE STEROLS*

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SUMMARY

Column chromatography on a hydroxyalkoxypropyl derivative of Sephadex LH-20 and on Anasil B has been applied to the resolution of a complex marine sterol mixture in combination with argentation thin-layer chromatography and gas chromatography. This approach permits isolation in quantity of individual sterols from a complex mixture and separation of sterol mixtures that were not resolved without the modified Sephadex step. Seventeen sterols were detected in the scallop *Placopecten magellanicus*. 24-Methyl-cholesterol, 24-ethyl-cholesterol, 24-methyl-22-dehydrocholesterol and 24-ethyl-22-dehydrocholesterol, *i.e.* sterols whose configuration at C-24 had not been definitively established, were isolated in sufficient quantities for further study by nuclear magnetic resonance spectroscopy.

INTRODUCTION

Extensive studies of sterol composition were first carried out in marine invertebrates long before our technology was sufficient to separate or identify the compounds present. Many early studies on marine invertebrates were either not sufficient to identify any of the sterols or could only identify one or two of them due to the complexity of the mixture[1,2]. With the recent application of gas–liquid chromatography (GLC) and thin-layer chromatography (TLC) to marine sterol mixtures by Idler and co-workers[3,4] the number of sterols identified from marine sources has risen sharply, although the isolation of some of these sterols has sometimes required the use of preparative GLC[5,6]. Although many recent studies have not actually isolated sterols identified from marine sources, it has become increasingly apparent that this is de-

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sirable. This is especially true in organisms containing isomers, or sterols of a homologous series. Recently interest in the orientation about C-24 has increased[7], after a report by Thompson *et al.*[8] of the first effective method of distinguishing between 24*R* and 24*S* isomers. Although many sterols have been identified from marine organisms even to the point of C-24 alkyl orientation, in most cases, assignment at C-24 was made on the basis of insufficient data.

The recent synthesis of a hydrophobic derivative of Sephadex LH-20 by Ellingboe *et al.*[9], and demonstrations that it is effective in separating sterols of a homologous series[10,11], led us to investigate whether this method could be of use in the separation of the highly complex marine sterol mixtures. If Sephadex derivatives can be used effectively to isolate each of the components of a marine sterol mixture, along with previous methods of column and thin-layer chromatography, complete identifications could be made. Previous studies[3,4] have shown that the scallop contains a very complex sterol mixture containing most of the usual sterols of marine origin, and the separation of the sterols of this mixture was considered an indication of the results which might be achieved with other marine sterol mixtures.

MATERIALS AND METHODS

The hydroxyalkoxypropyl derivative of Sephadex LH-20 was prepared as described by Ellingboe *et al.*[9] using Needox 1114 (Ashland Chemical, Columbus, Ohio, U.S.A.). Samples (50–200 mg) were chromatographed on a 80-cm column using 100 × 2.5 cm I.D. solvent-resistant columns (Pharmacia, Uppsala, Sweden) eluted with 5% hexane in methanol. Samples were passed through a single time or were recycled using the technique of Hyde and Elliott[10]. A Buchler minipump was used to pump the solvent through the column at a rate of 40 ml/h. Resolution of the sterol mixture was diminished at greater flow-rates. Fractions (15 ml) were collected by an LKB Ultrovac 7000 fraction collector, solvent was evaporated under nitrogen in an N-EVAP evaporator (Organomation) and the fractions were analysed on a 1% SE-30 column at 235° on a Packard Model 878 gas chromatograph.

Separations using Anasil B (Analabs, North Haven, Conn., U.S.A.) were made on similar solvent-resistant columns used for Sephadex separations. A slurry of Anasil B in hexane was used to pack an 80-cm column, a solution of the sterol acetates in hexane was applied, and the column was developed with 4% diethyl ether in hexane. Fractions were handled and assayed as with the Sephadex column fractions.

Silver ion TLC of sterol acetates was accomplished using procedures described previously[4].

RESULTS

The results of the chromatography of a 65-mg sample of crude scallop sterol (passed through the column one time) are seen in Fig. 1. Of the seventeen sterols now known to occur in the scallop, only two were obtained in pure form in this first step, but this step is essential to separate sterols of lower retention volumes from those with higher retention volumes in order to recycle samples in subsequent steps. Although better resolution was observed with samples smaller than 100 mg, samples as large as 200 mg were chromatographed as described above with only a moderate re-

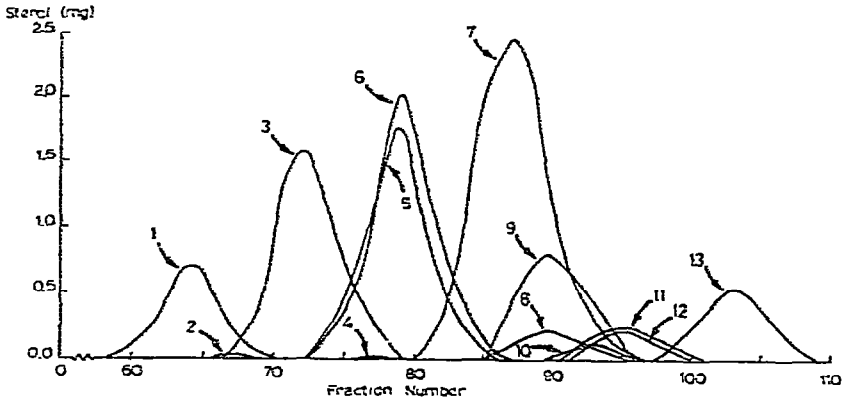

Fig. 1. Separation of crude scallop free sterols on hydroxyalkoxypropyl-Sephadex. 1 = 22-*trans*-24-norcholesta-5,22-dien-3β-ol; 2 = unidentified sterol (ST-I); 3 = *cis*- and *trans*-22-dehydrocholesterol, which includes unidentified sterol (ST-III); 4 = unidentified sterol (ST-II); 5 = 24-methylenecholesterol; 6 = 24-methyl-22-dehydrocholesterol; 7 = cholesterol and cholestanol; 8 = 24-ethyl-22-dehydrocholesterol; 9 = fucosterol and isofucosterol; 10 = unidentified sterol (ST-IV); 11 = 24-methyl-cholesterol; 12 = (*Z*)-24-propylidenecholesterol; 13 = 24-ethyl-cholesterol.

duction in resolution. The larger samples were necessary in order to isolate several of the minor scallop sterols

The following fractions were taken from this first separation: (1) Pure 22-*trans*-24-norcholesta-5,22-dien-3β-ol (tubes 58–66). These tubes were combined and called Compound A. (2) *cis*- and *trans*-22-dehydrocholesterol (tubes 71–72). These tubes were combined and called Compound B. (3) Tubes 67–70 and 73–87, containing mixtures of two or more of the following sterols: 22-*trans*-24-norcholesta-5,22-dien-3β-ol, *cis*- and *trans*-22-dehydrocholesterol, 24-methyl-22-dehydrocholesterol, 24-methylenecholesterol, cholesterol, cholestanol, 24-ethyl-22-dehydrocholesterol, fucosterol, isofucosterol, and three unidentified sterols (ST-I, ST-II, and ST-III). These tubes were combined and called Fraction M-1. (4) Tubes 88–101, containing mixtures of cholesterol, cholestanol, 24-ethyl-22-dehydrocholesterol, fucosterol, isofucosterol, 24-methyl-cholesterol, 24-propylidenecholesterol, an unidentified sterol (ST-IV), and 24-ethyl-cholesterol. These tubes were combined and called Fraction M-2. (5) Pure 24-ethyl-cholesterol (tubes 102–109). These tubes were combined and called Compound C.

A schematic diagram showing the approach used for the separation of scallop sterols is seen in Fig. 2. A second step in this separation involved the chromatography of Fraction M-1 using the recycling technique[10] to pass the sample through the modified Sephadex column three times. Sterols collected were: (1) 22-*trans*-24-norcholesta-5,22-dien-3β-ol (combined with Compound A); (2) a mixture of the above C_{26} sterol, an unknown sterol (called ST-I), *cis*- and *trans*-22-dehydrocholesterol, and traces of cholesterol, 24-methyl-22-dehydrocholesterol, and 24-methylenecholesterol (called Fraction M-1-1); (3) *cis*- and *trans*-22-dehydrocholesterol (combined with Compound B). (4) a mixture of two unknown sterols (called ST-II and ST-III), *cis*- and *trans*-22-dehydrocholesterol, 24-methyl-22-dehydrocholesterol and 24-methylenecholesterol (called Fraction M-1-2); (5) 24-methyl-22-dehydrocholesterol and 24-

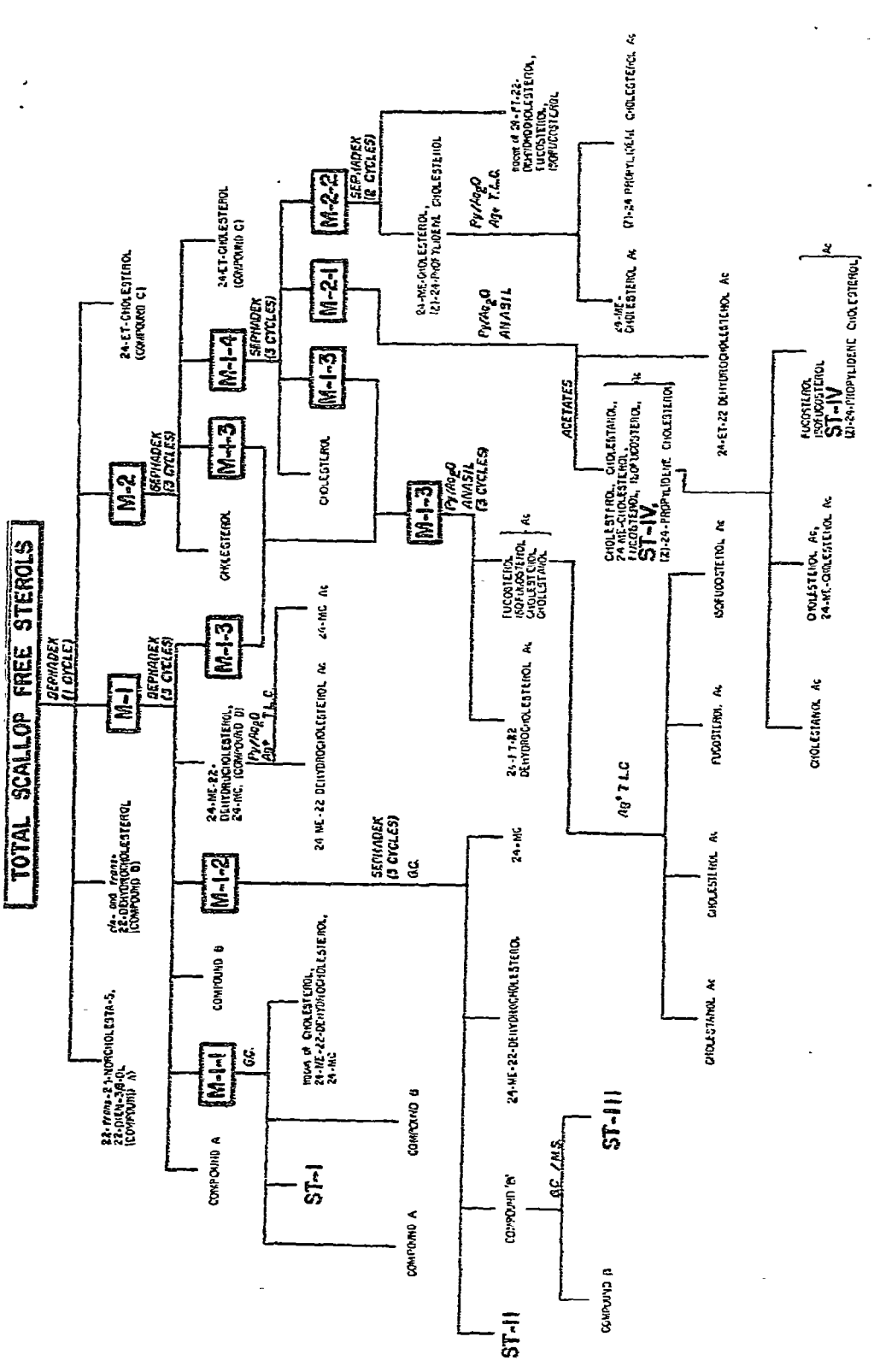

Fig. 2. Resolution of a complex mixture of marine sterols.

methylenecholesterol (called Compound D); (6) cholesterol, cholestanol, 24-ethyl-22-dehydrocholesterol, fucosterol and isofucosterol (called Fraction M-1-3).

Fractions M-1-1 and M-1-2 each contain less than 1 mg and the original samples must be 200 mg or more in order to continue the separation of these fractions. Continued separation was considered to be important since each of these fractions contained unidentified sterols.

The M-1-1 fractions from several separations were combined and purified on alumina. A structure has been proposed for sterol ST-I, on the basis of combined gas chromatographic and mass spectral (GC–MS) data (see Part II of this series[17]). Several M-1-2 fractions were likewise combined, purified on alumina and subjected to GC–MS analysis. Structures have been proposed for sterols ST-II and ST-III (see Part II of this series).

Compound D, containing 24-methylenecholesterol and 24-methyl-22-dehydrocholesterol, was acetylated and chromatographed on the phenylethylene oxide derivative of Sephadex LH-20[16]. A partial separation was achieved but complete separation was easily obtained by argentation TLC[4].

Fraction M-2 from the original separation was chromatographed on the modified Sephadex with recycling (three cycles), giving the following fractions: (1) cholesterol; (2) cholesterol, cholestanol, 24-ethyl-22-dehydrocholesterol, fucosterol and isofucosterol (combined with Fraction M-1-3); (3) cholesterol, cholestanol, fucosterol, isofucosterol, 24-ethyl-22-dehydrocholesterol, the unidentified sterol ST-IV, (*Z*)-24-propylidenecholesterol and 24-methyl-cholesterol (called Fraction M-1-4); (4) 24-ethyl-cholesterol (added to Compound C).

Fraction M-1-4 from above was subjected to another Sephadex separation (three cycles) and the following fractions were collected: (1) cholesterol; (2) cholesterol, cholestanol, fucosterol, isofucosterol and 24-ethyl-22-dehydrocholesterol (combined with previously obtained M-1-3); (3) cholesterol, cholestanol, 24-methyl-cholesterol, fucosterol, isofucosterol, unidentified sterol ST-IV, and (*Z*)-24-propylidenecholesterol (Fraction M-2-1); (4) 24-methyl-cholesterol and (*Z*)-24-propylidenecholesterol, traces of 24-ethyl-22-dehydrocholesterol, fucosterol, and isofucosterol (called fraction M-2-2).

The combined fractions M-1-3 were acetylated, purified on an alumina column, and chromatographed on an 80-cm Anasil column, using 4% diethyl ether in hexane as eluting solvent. This column effectively separates 24-ethyl-22-dehydrocholesterol acetate from other sterol acetates of the mixture. A similar separation was obtained using a mixture of hexane and benzene as eluting solvent. Cholesterol, cholestanol, fucosterol, and isofucosterol were separated as their acetates on argentation TLC. Fraction M-2-1 was handled the same way as Fraction M-1-3. It was acetylated, purified on alumina, and chromatographed on the Anasil column giving the acetates of cholesterol, cholestanol, 24-methyl-cholesterol (trace), fucosterol, isofucosterol, sterol ST-IV and (*Z*)-24-propylidenecholesterol in one fraction, and 24-ethyl-22-dehydrocholesterol acetate in a later fraction. The acetate mixture was further separated as before on argentation TLC, giving cholestanol, 24-methyl-cholesterol, cholesterol, fucosterol, isofucosterol, and the unidentified sterol ST-IV, and (*Z*)-24-propylidenecholesterol. Fraction M-2-2 was acetylated and pure samples of acetates of 24-methyl-cholesterol and (*Z*)-24-propylidenecholesterol were obtained from argentation TLC, after initial fractionation of free sterols on Sephadex.

Fucosterol and isofucosterol were not satisfactorily separated on the Sephadex

columns used in this work. The same problem exists with the separation of *cis*- and *trans*-22-dehydrocholesterol. Fucosterol and *cis*-22-dehydrocholesterol were eluted slightly before their respective isomers but even recycling did not produce significant amounts of either isomer in pure form. A better, although still difficult separation is the argentation TLC of the acetates described previously. From the retention times described it is apparent that preparative GLC also can be used successfully in both cases[5]. Although laborious, both methods can produce small amounts of pure compounds from the isomers mentioned above.

DISCUSSION

The work of Bergmann and his colleagues[1,12] demonstrated that marine invertebrates usually contain complex sterol mixtures. Resolution of these complex mixtures into pure components was slow because of the unavailability of more refined techniques and this usually led to confusion in the characterisation of certain sterols of which 24-methylenecholesterol is a well known example[13]. 24-Methylenecholesterol was isolated as a pure component by Idler and Fagerlund[14] by column chromatography of the azoyl esters, a technique which was first introduced by Ladenburg *et al.*[15] in 1938 and fashioned into a viable experimental tool by Idler and Baumann[16]. With the development of GLC both analytical and preparative, silver ion TLC and mass spectrometry, many other components were isolated and characterised; for example, the scallop sterols had been resolved into at least eleven components by Idler and Wiseman[5] in 1971, including a C_{26} and C_{30} sterol as new sterols.

In this study the use of the modified Sephadex LH-20 has made it possible to concentrate very minor components present in the scallop sterol mixture and this has resulted in the detection of four new sterols by GC–MS. For example, it has long been suspected from GLC studies of the methyl ether of the scallop sterols that there were peaks corresponding to very minor sterol components, the structures of which were unknown. Preparative GLC would have been too laborious and maybe even impractical since collection and recovery by this method was significantly lower than the 100% recovery obtained by Sephadex. In their recent characterisation of the first C_{30} sterol isolated from the scallop, Idler *et al.*[6] separated this C_{30} from the C_{26} $\Delta^{5,22}$-sterol by preparative GLC (1.5 mg from 15 mg 60% mixture) having originally concentrated these two components by preparative silver ion TLC. Had the modified Sephadex been available, however, the separation would have been simplified, there would have been no loss due to thermal decomposition on the gas chromatographic column and the percentage recovery significantly increased.

Of the C-24 epimeric sterols present in the scallop, the absolute configuration of 24-methyl-cholesterol, 24-methyl-22-dehydrocholesterol, 24-ethyl-cholesterol and 24-ethyl-22-dehydrocholesterol was not definitively established since it was difficult to obtain some of these sterols in pure form; for example, silver ion TLC cannot separate 24-methyl-cholesterol from cholesterol when these sterols are present in the same mixture. The use of the modified Sephadex LH-20 as a complementary analytical tool has, however, made it possible to isolate these C-24 epimeric sterols in pure form and in quantities sufficient for resolution by nuclear magnetic resonance (NMR) spectroscopy (see Part III of this series[18]).

It must, however, be borne in mind that the modified Sephadex along with the

other available analytical methods is not the ultimate in the resolution of complex marine sterol mixtures. The modified Sephadex was unable to resolve the *cis* and *trans* isomers of 22-dehydrocholesterol, while the C-24 diastereoisomers fucosterol and isofucosterol were not clearly separable. The most satisfactory method for the resolution of both pairs of isomers is still preparative GLC[3] although silver ion TLC can provide an enriched fraction of each component. The recycling technique sometimes required to obtain the best resolution of a sterol mixture can be time consuming especially if a long column is in operation, although the ease of operation and the quantitative recovery of the materials under study usually compensate for this delay.

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