Distribution of Unusual Cholesterol Precursors, 4-Methyl- and 4, 4-Dimethylsterols with $\Delta^8$ Unsaturation, in Gonads of Marine Archaeogastropods

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Abstract: In the present study, we show that four unusual cholesterol precursors, 5$\alpha$-cholesta-8,14-dien-3$\beta$-ol, 4-methyl-5$\alpha$-cholesta-8,24-dien-3$\beta$-ol (4-methylzymosterol), 4,4,14-trimethyl-5$\alpha$-cholesta-8,24-dien-3$\beta$-ol (lanosterol), and 4,4-dimethyl-5$\alpha$-cholesta-8,24-dien-3$\beta$-ol (4,4-dimethylzymosterol) are present in testes of the limpet Cellana grata, which is part of one of the most primitive gastropod families (Nacellidae Family). The distribution of these sterols in testes and ovaries of four dominant species of limpets, Cellana grata, Cellana toreuma, Nipponacmea concinna, and Nipponacmea fuscoviridis, was examined by capillary gas chromatography-mass spectrometry. Based on the data, we discuss about the biological roles and possible application as bio-resources of these sterols. This is the first identification of 5$\alpha$-cholesta-8,14-dien-3$\beta$-ol, 4-methylzymosterol, and 4,4-dimethylzymosterol in marine invertebrate gonads.

Key words: archaeogastropods, gonad, 4-methylsterol, 4,4-dimethylsterol

1 INTRODUCTION

Previous studies have shown significant differences in the proportion of both cholesterol and desmosterol between ovaries and testes of four different limpet species of archaeogastropods, Cellana grata, Cellana toreuma, Nipponacmea concinna, and Nipponacmea fuscoviridis$^{1,2}$, all of which are widely distributed on tidal rocky shores in northeastern Japan. Among the dominant species of limpets, the testes of C. grata and C. toreuma contained 5$\alpha$-cholesta-8-en-3$\beta$-ol (zymostenol)$^{1,2}$, 5$\alpha$-cholesta-8,24-dien-3$\beta$-ol (zymosterol)$^1$, cholesterol and desmosterol as major components. However, the biological and physiological roles of zymosterol and zymostenol in germ cells of marine invertebrates still remain unclear.

Interestingly, among the intermediates of cholesterol biosynthesis in germ cells of mammals, 4,4-dimethyl-5$\alpha$-cholesta-8,14,24-trien-3$\beta$-ol from human follicular fluid and 4,4-dimethyl-5$\alpha$-cholesta-8,24-dien-3$\beta$-ol (4,4-dimethylzymosterol) from bull testes, have been reported to activate meiosis in naked mouse oocytes$^3,4)$. Additionally, six other sterols with $\Delta^8$ unsaturation, 4,4-dimethyl-5$\alpha$-22-oxacholesta-8,14,24-trien-3$\beta$-ol, 4,4-dimethyl-5$\alpha$-23-oxacholesta-8,14,25-trien-3$\beta$-ol, 4,4-dimethylcholesta-8,14-dien-3$\beta$-ol, 4,4-dimethyl-5$\alpha$-cholest-8-en-3$\beta$-ol, 4-methylcholesta-8,24-dien-3$\beta$-ol, and zymosterol have been identified as activators of mouse oocyte meiosis$^5-8)$. The findings suggest the possibility of the presence of related bioactive cholesterol precursors in other animal gonads. The objective of this study is an attempt to clarify the presence of 4-methyl- and 4,4-dimethylsterols with $\Delta^8$ unsaturation in gonads of four dominant species of limpets by using capillary gas chromatography-mass spectrometry (GC-MS). These results could help us to understand the function of these cholesterol precursors in cellular development of marine mollusks and mammals.

Abbreviations: EI, electron impact; GC, gas chromatography; GC-MS, gas chromatography-mass spectrometry; MS, mass spectrum; TMS, trimethylsilyl
2 EXPERIMENTAL PROCEDURES

2.1 Extraction of lipids and sterols from limpet gonads

Adults from four dominant species of limpets, C. grata, C. toreuma, N. concinna, and N. fusceoviridis, were collected in the coast of Funakosi and Otsuchi Bays, Iwate, northeastern Japan, from July to November, 2008 and 2009. The preparation of limpet gonads (1.0 – 2.0 g) and lipid extraction were performed as described elsewhere. Total lipids (4 – 5 mg) were saponified as described previously. After extraction of sterols, they were basically converted into their trimethylsilyl (TMS) derivatives by treatment with 0.3 mL of Sylon (Supelco Inc., Bellefonte, PA, USA) at 80°C for 1 hr. The reaction mixture was evaporated under a gentle stream of nitrogen at 40°C, and the derivatives were redissolved in 0.1 mL of n-hexane. The TMS derivatives of sterols were analyzed as described below.

2.2 Identification of sterols

Identification of sterols was based on their retention times relative to cholesterol (RRTs) calculated by GC, their mass spectra, the analysis of the Wiley Registry of Mass Spectral Data (7th edition) and literature citations.

2.3 GC and GC-MS Analyses

Sterols and their TMS derivatives were analyzed by using GC with a Shimadzu GC-1700 chromatograph equipped with an FID and an SAC-5 (30 m×0.25 mm i.d., 0.25 μm film thickness; Supelco, Bellefonte, PA, USA), as described previously. GC-MS analyses were performed by using a Shimadzu GCMS-QP2010 coupled with a Shimadzu 2010 GC-MS with a 30 m×0.25 mm i.d., 0.25 μm film thickness; Supelco, Bellefonte, PA, USA. The rest of operating conditions of GC-MS were as described elsewhere. In this study, it was not possible to discriminate between 4α- and 4β-methylzymosterol. The sterol samples were analyzed in duplicate.

3 RESULTS AND DISCUSSION

Table 1 lists data regarding the electron impact-mass spectra (EI-MS) of 4-demethyl-, 4-methyl-, and 4,4-dimethylsterols originated from testes of the limpet Cellana grata. Fig. 1 shows total ion current (TIC) chromatogram of the TMS derivatives of these sterols. As far as we know, the presence of 5α-cholesta-8,14-dien-3β-ol, 4-methylzymosterol, and 4,4-dimethylzymosterol in marine invertebrate gonads have not been reported in the literature.

The identification of 5α-cholesta-8, 14-dien-3β-ol was done by capillary GC-MS analysis of its underivatized (free) and trimethylsilylated sterols. Analysis of the EI-MS data indicates that the 5α-cholesta-8, 14-dien-3β-ol underivatized sterol has a molecular ion peak at m/z 384, which fits with the molecular weight of a diunsaturated C27 sterol depicting characteristic ions at m/z 369 [M – CH3]++, 351 [M – CH3 – H2O]++. The results are in agreement with the 5α-cholesta-8, 14-dien-3β-ol data obtained from the Wiley Mass Spectral Database. The MS of the TMS derivative of 5α-cholesta-8, 14-dien-3β-ol showed diagnostic ion peaks at m/z 456 [M]+, 441 [M – CH3]+, 351 [M – TMSOH – CH3]+ (base peak), and 182 [M]+. The MS data for this sterol were identical to those obtained from published literature. The identity of 5α-cholesta-8, 14-dien-3β-ol was also verified by comparing the RRT value of 5α-cholesta-8, 14-dien-3β-ol with that of zymostenol from published studies. Therefore, according to the above results, we conclude that the sterol with the RRT value of 1.023 shown in Table 1 is 5α-cholesta-8, 14-dien-3β-ol.

As for the only 4-methylsterol detected in the present study, the MS of underivatized 4-methylzymosterol showed a molecular ion peak at m/z 398, which fits with the molecular weight of a diunsaturated C28 sterol with key diagnostic ions at m/z 383 [M – CH3]++, 365 [M – CH3 – H2O]++, 285 [M – side chain – 2H]++, and 227 [M – side chain – C15 to C17 – H2O]++. The MS data for 4-methylzymosterol shown in Table 1 are in agreement with those reported by Kuchta et al. and Darnet and Rahier. The molecular structure of 4-methylzymosterol was confirmed by characteristic ion peaks at m/z 470 [M]+, 455 [M – CH3]+, 380 [M – TMSOH]++, and 365 [M – TMSOH – CH3]++ present in the MS of the TMS derivative of 4-methylzymosterol. These MS data are very similar to those found in the Wiley Mass Spectral Database. Additionally, the identity of 4-methylzymosterol was verified by comparing the RRT value of 4-methylzymosterol with that of ergosterol obtained from published studies. Consequently, the sterol with the RRT value of 1.277 shown in Table 1 was defined as 4-methylzymosterol.

The identification of 4, 4, 14a-trimethyl-5α-cholesta-8, 24-dien-3β-ol (lanosterol) as one of the 4,4-dimethyl sterols was based on a molecular ion and characteristic ion peaks present in its underivatized and trimethylsilylated sterol forms. The identity of lanosterol was also confirmed by its RRT, which coincided to that of an authentic sample (CO427, Tokyo Chemical Industry Co., Ltd.). The MS of 4,4-dimethylzymosterol displayed a molecular ion peak at m/z 412, which corresponds to the molecular weight of a diunsaturated C29 sterol, and key diagnostic ion peaks at m/z 397 [M – CH3]++, 379 [M – CH3 – H2O]++, 299 [M – side chain – 2H]++, and 241 [M – side chain – C15 to C17 – H2O]++. These MS data are in agreement with those reported by Kuchta et al. and Darnet and Rahier. In a similar way, the structure of the TMS derivative of 4, 4-dimethylzymosterol was confirmed by a molecular ion at m/z 484 and characteristic ions at m/z 469 [M – CH3]++, 394 [M – TMSOH]++, 379 [M – TMSOH – CH3]++, and 69 (base peak). The MS data are in agreement with those de-
Table 1  EI-MS diagnostic data for 4-demethyl-, 4-methyl-, and 4,4-dimethylsterols from gonads of the limpet Cellana grata.

<table>
<thead>
<tr>
<th>Sterol class</th>
<th>RRT</th>
<th>m/z (relative ion intensity)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Underivatized</td>
</tr>
<tr>
<td>Sterols with Δ8 unsaturation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4-Demethylsterol</td>
<td>1.023</td>
<td>384 ([M]+, 100), 369 (91), 351 (50), 325 (6), 271 (18), 238 (28), 211 (11), 196 (14), 169 (15)</td>
</tr>
<tr>
<td>5α-cholesta-8,14-dien-3β-ol</td>
<td>1.040</td>
<td>386 ([M]+, 100), 371 (43), 353 (11), 273 (21), 255 (14), 229 (28), 213 (23), 161 (15), 147 (28), 119 (22), 107 (32), 95 (29), 81 (22), 69 (16)</td>
</tr>
<tr>
<td>Zymosterol</td>
<td>1.124</td>
<td>384 ([M]+, 93), 369 (100), 351 (21), 341 (6), 299 (13), 258 (11), 246 (25), 231 (22), 229 (32), 215 (14), 213 (44), 211 (12), 147 (49), 131 (38), 119 (46), 107 (61), 95 (54), 81 (49), 69 (96)</td>
</tr>
<tr>
<td>Zymostenol</td>
<td>1.277</td>
<td>398 ([M]+, 100), 383 (84), 365 (17), 385 (20), 258 (21), 245 (28), 227 (25), 215 (15), 161 (24), 145 (29), 131 (18), 121 (35), 95 (35), 81 (29), 69 (39)</td>
</tr>
<tr>
<td>4-Methylsterol</td>
<td>1.541</td>
<td>426 ([M]+, 39), 411 (80), 393 (50), 259 (13), 187 (33), 147 (31), 135 (18), 119 (48), 109 (68), 95 (41), 81 (27), 69 (100)</td>
</tr>
<tr>
<td>4-Methylzymosterol</td>
<td>1.575</td>
<td>412 ([M]+,100), 397 (72), 379 (43), 345 (16), 299 (15), 281 (32), 259 (28), 241 (24), 215 (41), 135 (64), 119 (37), 107 (34), 95 (51), 81 (31), 69 (76)</td>
</tr>
</tbody>
</table>

*RRT: Retention time relative to free cholesterol was calculated by running the samples for 19.541 min on a Supelco SAC-5 column (30 m × 0.25 mm i.d., 0.25 µm film thickness) at 270℃.

Fig. 1  Total ion current (TIC) chromatogram of the TMS derivatives of sterols in testes of the limpet Cellana grata. The chromatogram was obtained on a Supelco SAC-5 column (30 m × 0.25 mm × 0.25 µm film thickness).
Fig. 2  EI-MS of the TMS derivatives of 5α-cholesta-8,14-dien-3β-ol (A), 4-methylzymosterol (B), and 4,4-dimethylzymosterol (C) in testes of the limpet Cellana grata.

Table 2  Distribution of 4-demethyl-, 4-methyl-, and 4,4-dimethylsterols in gonads of four different dominant limpet species from northeastern Japan.

<table>
<thead>
<tr>
<th>Sterol class</th>
<th>Testis</th>
<th>Ovary</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sterols with Δ8 unsaturation</td>
<td>C. grata</td>
<td>C. toreuma</td>
</tr>
<tr>
<td>4-Demethylsterol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5α-Cholesta-8,14-dien-3β-ol</td>
<td>&lt;0.1*</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Zymostenol</td>
<td>14.6</td>
<td>1.5</td>
</tr>
<tr>
<td>Zymosterol</td>
<td>4.6</td>
<td>10.0</td>
</tr>
<tr>
<td>4-Methylsterol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4-Methylzymosterol</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>4,4-Dimethylsterol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lanosterol</td>
<td>&lt;0.1</td>
<td>–</td>
</tr>
<tr>
<td>4,4-Dimethylzymosterol</td>
<td>&lt;0.1</td>
<td>–</td>
</tr>
</tbody>
</table>

Mean values (% of total sterols) of duplicate analyses performed on different samples.

* The sterol identity was confirmed by capillary GC-MS analysis of underivatized and TMS derivative sterol forms on a Supelco SAC-5 column (30 m × 0.25 mm × 0.25 μm film thickness).

b Not detected.
scribed by Bard et al.\(^\text{13}\) and Singh and Porter\(^\text{14}\). In addition, the identity of 4,4-dimethylzymosterol was also verified by comparing the RRT value of 4,4-dimethylzymosterol with that of lanosterol obtained from published studies\(^\text{10-13}\).

On the basis of the above information it can be said that the sterol with the RRT value of 1.575 listed in Table 1 is 4,4-dimethylzymosterol.

In previous studies, the two dominant species of Cellana (Cellana grata and Cellana toreuma) exhibited significant differences in sterol components with Δ8 unsaturation, namely, zymostenol and zymosterol, but the reason is still unclear. In this study, the presence of unusual lanosterol and 4,4-dimethylzymosterol was confirmed for the first time. Interestingly, the testes of C. grata are extremely rich in zymostenol, zymosterol, 4-methylzymosterol, and 4,4-dimethylzymosterol. Although zymosterol occurred in limpet gonads, it is structurally homologous (sterols containing Δ8,24 unsaturated moieties) to 4-methylzymosterol, lanosterol and 4,4-dimethylzymosterol.

Furthermore, we determined the composition and proportion of sterols with Δ8 unsaturation in gonads of dominant species of Cellana in order to assess whether differences in their sterol composition could be attributable to sexual differences. Although only small amounts of the sterol components were detected in this study, the presence of at least 4-methyl- and 4,4-dimethylsterols was mainly verified in testes rather than in ovaries (Table 2). Elucidation of the function of these minor unusual sterols could provide us with new possibilities for bio-resources of limpet widely distributed on tidal rock shores. Our results indicate that a large variety of structural forms of sterols with Δ8 unsaturation can be found as minor components of ovaries and testes of four different archaeogastropods. The exception was zymostenol and zymosterol, which were found only in testes of C. grata and C. toreuma\(^\text{1}\). Our intense interest in the physiological function of cholesterol precursors came from articles of Byskov et al.\(^\text{4,6}\) describing the bioactive properties of sterols with Δ8 unsaturation, especially zymosterol, 4-methylzymosterol and 4,4-dimethylzymosterol, in mammalian germ cells. The present findings suggest that some sterols with Δ8 unsaturation might play an important role in the reproductive cycle of marine invertebrates. However, the physiological function of the type of sterols in marine invertebrate gonads remains unclear. Much work is still needed to determine whether zymosterol, 4-methylzymosterol and 4,4-dimethylzymosterol are directly involved in the reproductive cycle and cellular development of marine archaeogastropods.

4 ACKNOWLEDGMENTS

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