

# Sterols of *Testudodinium testudo* (formerly *Amphidinium testudo*): Production of the $\Delta^{8(14)}$ sterol gymnodinosterol and chemotaxonomic relationship to the Kareniaceae

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## Abstract

*Testudodinium testudo* is a peridinin-containing dinoflagellate recently renamed from *Amphidinium testudo*. While *T. testudo* has been shown via phylogenetic analysis of small subunit ribosomal RNA genes to reside in a clade separate from the genus *Amphidinium*, it does possess morphological features similar to *Amphidinium sensu stricto*. Previous studies of *Amphidinium carterae* and *Amphidinium corpulentum* have found the sterols to be enriched in  $\Delta^{8(14)}$  sterols, such as 4 $\alpha$ -methyl-5 $\alpha$ -ergosta-8(14),24(28)-dien-3 $\beta$ -ol (amphisterol), uncommon to most other dinoflagellate taxa and thus considered possible biomarkers for the genus *Amphidinium*. Here, we provide an examination of the sterols of *T. testudo* and show they are dominated not by amphisterol, but rather by a different  $\Delta^{8(14)}$  sterol, (24*R*)-4 $\alpha$ -methyl-5 $\alpha$ -ergosta-8(14),22-dien-3 $\beta$ -ol (gymnodinosterol), previously thought to be a major sterol only within the Kareniaceae genera *Karenia*, *Karodinium*, and *Takayama*. Also found to be present at low levels were 4 $\alpha$ -methyl-5 $\alpha$ -ergosta-8,14,22-trien-3 $\beta$ -ol, a sterol previously observed in *Karenia brevis* to be an intermediate in the production of gymnodinosterol, and cholesterol, a sterol common to many other dinoflagellates. The presence of gymnodinosterol in *T. testudo* is the first report of this sterol as the sole major sterol in a dinoflagellate outside of the Kareniaceae. The implication of this chemotaxonomic relationship to the Kareniaceae is discussed.

## KEYWORDS

*Amphidinium*, dinoflagellate, Dinophyceae, lipid, sterol, *Testudodinium*

THE photosynthetic, peridinin-containing dinoflagellate genus *Amphidinium* is a large and diverse one with well over 100 named species at the current time (Guiry and Guiry, 2021). Historically, species have been included in this athecate (naked) genus when they possess a characteristic epicone amongst other genus-specific morphological features, per the summaries given by Flø Jørgensen et al. (2004a, b). However, over the last couple decades, membership within the genus has become more restrictive wherein these morphological features are now

combined with phylogenetic analysis (Flø Jørgensen et al., 2004a; Karafas et al., 2017; Murray et al., 2012;). As such, the genus *Amphidinium sensu stricto* now includes species within two closely related clades, the Herdmanii Clade and the Operculatum Clade (Karafas et al., 2017).

It is not uncommon for dinoflagellates originally within the genus *Amphidinium* to be renamed as a new genus (and species combination) when phylogenetic characterization is used to augment morphology-based identification. For example, the genera *Bindiferia*

(Borchhardt et al., 2021), *Nusuttodinium* (Takano et al., 2014), *Prosoaulax* (Calado and Moestrup, 2005), and *Togula* (Flø Jørgensen et al., 2004b) are recently erected genera, which include species formerly within the genus *Amphidinium*. Included in this are also the species *Testudodinium testudo* (Herdman) Horiguchi, Tamura, Katsumata, et A. Yamaguchi (Horiguchi et al., 2012), which is renamed from *Amphidinium testudo* Herdman (Guiry and Guiry, 2021), and the new species *Testudodinium magnum* Pinto, Terada & Horiguchi (Pinto et al., 2017). Like these other renamed dinoflagellates, while *T. testudo* is a benthic, photosynthetic dinoflagellate possessing morphological features which resemble true members of the genus *Amphidinium*, sequence analysis of its gene for small subunit ribosomal RNA (SSU rRNA) places it elsewhere within the broader group of peridinin-containing dinoflagellates (Horiguchi et al., 2012; Pinto et al., 2017).

Eukaryotes possess membrane-reinforcing, ringed lipids known as sterols (Dufourc, 2008). Dinoflagellates as a class have been demonstrated to produce a wide variety of sterols *de novo*, ranging from the C<sub>27</sub> desmethyl cholesterol (cholest-5-en-3 $\beta$ -ol) to the C<sub>30</sub> 4 $\alpha$ -methyl-substituted dinosterol (4 $\alpha$ ,23,24-trimethyl-5 $\alpha$ -cholest-22E-en-3 $\beta$ -ol; Leblond et al., 2010; Volkman, 1986, 2003; Volkman et al., 1998). Given the large number of species in the genus *Amphidinium*, comparatively few have had their sterol compositions determined, but *Amphidinium*, along with members of the Kareniaceae, is one of the few dinoflagellate genera where some species exhibit sterols characterized by a  $\Delta^{8(14)}$  nuclear unsaturation (Giner et al., 2003; Kokke et al., 1981; Leblond and Chapman, 2002; Mooney et al., 2007). More specifically, *Amphidinium carterae* Hulburt and *Amphidinium corpulentum* Kofoid & Swezy have been observed to produce 4 $\alpha$ -methyl-5 $\alpha$ -cholest-8(14)-en-3 $\beta$ -ol and 4 $\alpha$ -methyl-5 $\alpha$ -ergosta-8(14),24(28)-dien-3 $\beta$ -ol (amphisterol; Kokke et al., 1981; Leblond and Chapman, 2002), which can be considered possible biomarkers of the genus when compared to many other non- $\Delta^{8(14)}$  sterol-producing dinoflagellate genera (Leblond et al., 2010). Similarly, the Kareniaceae genera *Karenia*, *Karlodinium*, and *Takayama* have been observed to produce (24*R*)-4 $\alpha$ -methyl-5 $\alpha$ -ergosta-8(14),22-dien-3 $\beta$ -ol (gymnodinosterol) and its 27-*nor* isomer (brevesterol); (Giner et al., 2003; Leblond and Chapman, 2002; Mooney et al., 2007).  $\Delta^{8(14)}$  Sterols are rarely found outside of *Amphidinium* and the Kareniaceae (see review by Leblond et al. 2010). For example, brevesterol has been found as one of the more abundant sterols in *Scrippsiella trochoidea* (F.Stein) A.R.Loeblich III, but it is present amongst a number of common 4 $\alpha$ -methyl-substituted dinoflagellate sterols (Leblond and Chapman, 2002). Thus, *S. trochoidea* does not fit into the same chemotaxonomic group as the Kareniaceae, which do not exhibit these same sterols as the major sterols (Leblond et al. 2010).

The objective of our study was to characterize the sterols of *T. testudo* to provide additional chemotaxonomic evidence for or against its exclusion from the genus *Amphidinium* as *T. testudo*. More specifically, we sought to examine whether *T. testudo* produces the  $\Delta^{8(14)}$  sterols indicative of the genus *Amphidinium* or sterols, such as cholesterol and dinosterol, more commonly found in other dinoflagellates distributed broadly throughout the class Dinophyceae. To this end, we present a characterization of the sterols of *T. testudo*, which is one of the few commercially available dinoflagellates renamed from the genus *Amphidinium*.

## MATERIALS AND METHODS

### Culturing

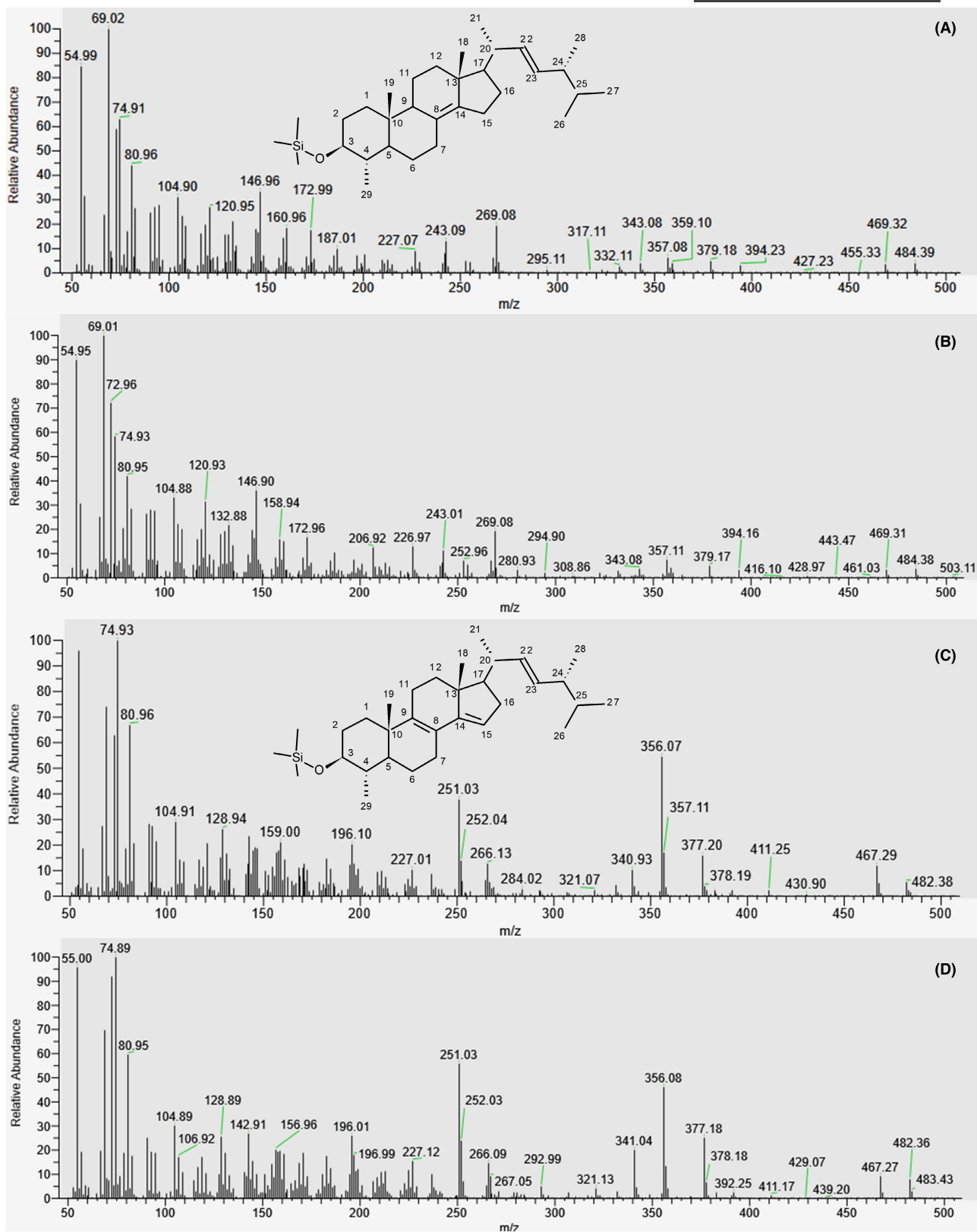
*Testudodinium testudo* RCC 1981 was acquired from the Roscoff Culture Collection (Roscoff, France) and grown in triplicate in *f/2* medium (Guillard, 1975; Guillard and Ryther, 1962;) at 20°C, a light/dark cycle of 14/10 h, and a light intensity of approximately 50  $\mu\text{M photons m}^{-2} \text{ s}^{-1}$  using a combination of cool white fluorescent and LED bulbs. Cells were harvested via filtration onto Whatman 934-AH glass microfiber filters (GE Healthcare) during the exponential phase of growth when cells were at a concentration of approximately 10<sup>4</sup> cells/ml. Filters were preserved at -80°C until lipid extraction.

### Lipid processing and sterol analysis

Total lipids were extracted and fractionated according to polarity using the procedure described by Leblond and Chapman (2000). The sterol ester and free sterol fractions were saponified and derivatized to form trimethylsilyl (TMS)-ether derivatives of sterols according to published procedures (Leblond and Chapman 2002). The derivatives were analyzed via gas chromatography/mass spectrometry (GC/MS) with a Thermo TSQ Quantum GC/MS and a Restek Rxi-5Sil MS column (30 m  $\times$  0.25  $\mu\text{m}$  film thickness, Restek Corp.) in positive-ion electron impact (EI) mode using the instrument conditions described by Houle et al. (2019). Relative retention times (RRT) to cholesterol were calculated using retention times (RT) according to the methodology of Jones et al. (1994).

## RESULTS AND DISCUSSION

*Testudodinium testudo* was observed to produce three sterols: (1) cholesterol (C<sub>27:1</sub>, *m/z* 458 as its TMS-ether derivative, RT of 37.30 min, RRT of 1.000, relative percentages of 1.2  $\pm$  0.3% of free sterols and 1.7  $\pm$  1.7% of sterol esters, respectively), (2) gymnodinosterol (C<sub>29:2</sub>, *m/z*



**FIGURE 1** Mass spectra of (A) gymnodinosterol and (C) 4 $\alpha$ -methyl-5 $\alpha$ -ergosta-8,14,22-trien-3 $\beta$ -ol from *Testudodinium testudo* RCC 1981. Panels B and D are mass spectra of these same two sterols, respectively, as produced by *Karenia brevis* strain EPA-JR1

484 as its TMS-ether derivative, RT of 38.97 min, RRT of 1.492, relative percentages of 95.3 $\pm$ 1.6% of free sterols and 98.3 $\pm$ 1.3% of sterol esters, respectively), and (3)

4 $\alpha$ -methyl-5 $\alpha$ -ergosta-8,14,22-trien-3 $\beta$ -ol (C<sub>29:3</sub>, *m/z* 482 as its TMS-ether derivative, RT of 39.08 min, RRT of 1.525, relative percentages of 3.6 $\pm$ 1.3% of free sterols and

0% of sterol esters, respectively). Free sterols constituted approximately 83% and sterols as sterol esters approximately 17%, respectively, of the total sterols.

Mass spectra of gymnodinosterol and 4 $\alpha$ -methyl-5 $\alpha$ -ergosta-8,14,22-trien-3 $\beta$ -ol are shown in [Figure 1](#), along with mass spectra of these same sterols from *Karenia brevis* (C.C.Davis) Gert Hansen & Moestrup. While spectra for these sterols from *K. brevis* were originally published in Leblond and Chapman (2002) and Leblond et al. (2011), respectively, they are included again here as run on the same instrument as the *T. testudo* sterols. Their retention times matched those of the *T. testudo* sterols, and, as evidenced in [Figure 1](#), their mass spectra were also identical. Thus, we conclude that *T. testudo* produces a  $\Delta^{8(14)}$  sterol common to the Kareniaceae, and its presumed biosynthetic intermediate (see Leblond et al., 2011 for discussion on the biosynthesis of gymnodinosterol and brevesterol in *K. brevis*), rather than a  $\Delta^{8(14)}$  sterol specific to *Amphidinium*. It should be noted, however, that while the genus *Amphidinium* is one of the few dinoflagellate taxa where the sterol composition can be dominated by  $\Delta^{8(14)}$  sterols, exceptions include the photosynthetic *Amphidinium massartii* Biecheler, which has instead been observed to reside in a cluster of dinoflagellates which produce cholesterol as a dominant sterol in the absence of  $\Delta^{8(14)}$  sterols (Leblond et al., 2010), and heterotrophic *Amphidinium longum* Lohmann, which has also been observed to produce cholesterol (Chu et al., 2009). These two species indicate that the genus *Amphidinium* deserves a fuller survey of its members' sterol composition diversity.

In addition to sterols, photosynthetic dinoflagellates are also characterized by the fatty acid-containing galactolipids mono- and digalactosyldiacylglycerol (MGDG and DGDG, respectively) that provide structural support for plastid membranes (Gray et al., 2009; Hölzl and Dörmann, 2019; Leblond and Lasiter, 2009). Within peridinin-containing dinoflagellates, two clusters have been observed based on MGDG and DGDG composition in which Cluster 1 species are rich in C<sub>18</sub>/C<sub>18</sub> (*sn*-1/*sn*-2 regiochemistry) MGDG and DGDG, and those in Cluster 2 are rich in C<sub>20</sub>/C<sub>18</sub> MGDG and DGDG (Gray et al., 2009). We previously identified 20:5/18:4 MGDG and DGDG as the main galactolipids produced by multiple *Amphidinium* species, and 18:5/18:4 MGDG and DGDG as the main galactolipids of *T. testudo* (Leblond et al., [in press](#)). Based on these characterizations, *Amphidinium* species were placed within Cluster 2, whereas *T. testudo* was placed within Cluster 1. Although placement of species within the clusters does not necessarily reflect phylogenetic relatedness, the lack of C<sub>20</sub>/C<sub>18</sub> galactolipids and *Amphidinium*-specific sterols produced by *T. testudo* provides additional lipid biochemistry-based evidence supporting the removal of *T. testudo* from the genus *Amphidinium*.

Because *T. testudo* has been removed from the genus *Amphidinium* per Horiguchi et al. (2012) due to

its disparate SSU rRNA gene sequence, we were initially surprised to observe production of a  $\Delta^{8(14)}$  sterol, especially one that is considered a biomarker for the Kareniaceae. We instead expected to observe cholesterol as a major sterol and the presence of non- $\Delta^{8(14)}$ , 4 $\alpha$ -methyl-substituted sterols common to other photosynthetic dinoflagellates (see Leblond et al. 2010 for a review of the distribution of sterols across many dinoflagellate genera). However, the SSU rRNA phylogeny presented by Pinto et al. (2017) places members of the genus *Testudodinium* close to two species of *Karenia*. Given this, it is thus perhaps not surprising that *T. testudo* produces gymnodinosterol and its presumed biosynthetic intermediate 4 $\alpha$ -methyl-5 $\alpha$ -ergosta-8,14,22-trien-3 $\beta$ -ol rather than either more common dinoflagellate sterols or the  $\Delta^{8(14)}$  sterols found in *Amphidinium*. *Karenia*, which has a haptophyte-derived plastid rather than a peridinin-containing one, has MGDG and DGDG enriched in C<sub>16</sub> and C<sub>14</sub> fatty acids at the *sn*-2 position and does not fit within either cluster of peridinin-containing dinoflagellates (Leblond and Lasiter, 2009; Leblond et al., 2019). There is no evidence to suggest that the sterol composition of dinoflagellates is influenced by plastid type as evidenced by aberrant plastid-containing dinoflagellates (Leblond and Lasiter, 2012; Leblond and Vandergrift, 2022). Thus, this chemotaxonomic relationship to *Karenia* with regard to sterol composition is in line with the observation of Leblond et al. (2010) that dinoflagellates with phylogenetically close SSU rRNA sequences often produce similar sets of sterols amongst all the possible sterols (over 50) produced by the class Dinophyceae as a whole. This observation of gymnodinosterol production by *T. testudo* broadens our knowledge base to include a new genus of peridinin-containing dinoflagellate that produces a Kareniaceae biomarker as a major sterol.

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