Isolation of Scalarane-Type Sesterterpenoids from the Marine Sponge *Dysidea* sp. and Stereochemical Reassignment of 12-*epi*-Phyllactone D/E

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Abstract: The chemical investigation of the marine sponge *Dysidea* sp., which was collected from Bohol province in the Philippines, resulted in the identification of 15 new scalarane-type sesterterpenoids (1–14, 16), together with 15 known compounds. The chemical structures of the new compounds were elucidated based on NMR spectroscopy and HRMS. The structure of 12-*epi*-phyllactone D/E (15) isolated during this study was originally identified in 2007. However, careful inspection of our experimental 13C NMR spectrum revealed considerable discrepancies with the reported data at C-9, C-12, C-14, and C-23, leading to the correction of the reported compound to the C-12 epimer of 15, phyllactone D/E. The biological properties of compounds 1–16 were evaluated using the MDA-MB-231 cancer cell line. Compound 7, which bears a pentenone E-ring, exhibits significant cytotoxicity with a GI50 value of 4.21 μM.

Keywords: *Dysidea*; sesterterpenoid; scalarane; marine sponge; marine natural product; anticancer activity; stereochemistry reassignment

1. Introduction

Sesterterpenoids, such as the ophiobolins, sterigmatocystin, and hippolide A, have attracted a lot of attentions as potent pharmaceutical compounds because of their unique anti-inflammatory activity and cytotoxicity in various cancer cell lines [1]. These compounds are ubiquitous in a broad range of natural sources, from easily accessible terrestrial plants and insects to hard-to-access marine organisms. In marine nature, the scalarane-type scaffolds have emerged as one of the most prevalent structural features of the sesterterpenoids [2]. Since scalarin, a pentacyclic scalarane, was first isolated from the marine sponge *Cacospongia scalaris* in 1972 [3], a number of scalarane-type sesterterpenoids has been isolated from *Dysidea* sp. [4–5], *Phyllospongia* sp. [6–11], *Strepsichordia* sp. [12], *Cate-riospongia* sp. [13,14], *Smenospongia* sp. [15], and *Hyrtios* sp. [16], belonging to the order Dictyoceratida [17].

This family of scalarane derivatives is featured with a trans-fused 6/6/6/6 ring system and can be further categorized into three structural subgroups, namely scalarane, homoscalarane, and bishomoscalarane, based on the presence of single carbon substituents at C-20 and/or C-24 (Figure 1). Among them, bishomoscalarane exhibits an exceptionally broad range of diversity in the carbon framework, arising from two distinctive sites: C-20 and C-24/C-25 (Figure 2). Therefore, cyclopropane or alcohol/esters are frequently found at C-20 adjacent to the A ring [12]. The oxidation of C-24 and C-25 results in the formation of an extra E ring in the form of a lactone or cyclopentenone [18]; 24-oxo-25-norbishomoscalarane has also been identified as another feature of the D ring [8]. In addition, oxidation of the backbone usually occurs at C-3 [6], C-12 [19], and C-16 [12] to produce...
hydroxyl or ester substituents. A large group of bishomoscalarane derivatives found in nature is considered to be the outcome of these variations occurring in combinations.

Figure 1. Subtypes of the scalarane skeleton found in marine nature.

The marine sponge *Dysidea* sp. is known to be a rich source of scalaranes, which exhibits useful pharmacological properties, such as anticancer and antimicrobial activities [17,20–24]. In the course of our studies on bioactive natural products from marine organisms, we inspected the chemical components of *Dysidea* sp. collected from the Bohol province in the Philippines. As a result, we identified 15 new scalarane derivatives, including one scalarane, four 20,24-bishomo-25-norscalaranes, and 10 bishomoscalaranes (Figure 3), along with 14 known compounds (Figure S1, Supplementary Materials). In this report, we disclose the structural assignment of the new scalarane sesterterpenoids and their pharmacological properties as anti-cancer agents. In addition, the C-12 configuration of compound 15, which was assigned by Li in 2007 [11], was reinvestigated because of the significant differences observed between the reported and experimental $^{13}$C chemical shifts at C-9, C-12, C-14, and C-23.

Figure 2. Diversity of bishomoscalarane skeletons frequently found in nature.
Figure 3. The structures of compounds 1–16 isolated from Dysidea sp.

2. Results and Discussion

2.1. Structure Elucidation

Compound 1 was isolated as a colorless oil, and its molecular formula was determined to be C_{31}H_{39}O_{5} using HRESIMS (m/z [M + Na]^+ 523.3382, calcd 523.3394), corresponding to eight degrees of unsaturation (DOU). The 1H NMR spectrum of 1 exhibited three singlet methyl groups at δ_{H} 0.77, 0.82, and 1.04; three doublet methyl groups at δ_{H} 1.07, 1.23, and 1.37; and three oxymethines at δ_{H} 4.03, 4.19, and 5.41. Furthermore, unique upfield signals at δ_{H} 0.57 and −0.49 indicated the presence of a cyclopropane. Analysis of the 13C NMR and HSQC spectra revealed the presence of two ester carbons (δC 174.8 and 171.7), three oxymethine carbons (δC 80.4, 74.8, 64.5), 10 methylene carbons, six methine carbons, and six methyl groups. The HMBC data showed notable correlations between the singlet methyl groups and methines from δ_{H} 0.77 to δC 51.4/50.3, δ_{H} 0.82 to δC 54.3/51.4, and δ_{H} 1.04 to δC 54.3, which are known as characteristic correlations occurring from the ring junctions of scalarane-type 6/6/6/6 fused-cyclic systems (Figure 4). Additional HMBC correlations from the doublet methyl group at δ_{H} 1.37 to δC 80.4/44.9 and from the methine at δ_{H} 2.34 to δC 174.8/44.9 suggested the existence of a γ-valerolactone moiety. Therefore, our preliminary findings led to the hypothesis that compound 1 possessed a honulactone A-like scaffold (B+D type shown in Figure 2) [12].

Figure 4. COSY and HMBC correlations observed for compounds 1, 5, and 7.
While the $\Delta^{17,18}$-olefin in homulanolactones is considered one of the structural features that forms the unsaturated lactone E-ring, the initially identified $\gamma$-valerolactone and DOU suggest the possibility of a saturated terminal lactone in compound 1. This speculation was confirmed by the $^1$H-$^1$H COSY cross peak observed for H$_2$-15–H$_2$-16–H-17–H-18, as well as HMBC correlations from CH$_3$-23 (δ$_H$ 1.04) to C-18 (δ$_C$ 52.5) and from H-18 (δ$_H$ 2.34) to C-13 (δ$_C$ 38.7). In addition, the cyclopropane moiety inferred from the $^1$H NMR data was positioned at C-4 based on the HMBC correlations from H$_2$-19 (δ$_H$ 0.57, and -0.49) to C-3 (δ$_C$ 33.2) and from CH$_3$-27 (δ$_H$ 1.07) to C-4 (δ$_C$ 22.7), and the spin system for CH$_3$-27–H-20 (δ$_H$ 0.72)–H$_2$-19 (δ$_H$ 0.57, -0.49) in the $^1$H-$^1$H COSY spectrum. Interpretation of the remaining HMBC correlations from CH$_3$-4' (δ$_H$ 1.23) to C-2' (δ$_C$ 43.3)/C-3' (δ$_C$ 64.5), H$_2$-2' (δ$_H$ 2.49/2.42) to C-1' (δ$_C$ 171.7)/C-3', and H-12 (δ$_H$ 5.41) to C-1' elucidated the 3-hydroxyl butanoate group at C-12.

The trans-fused cyclic scaffold in 1 was determined from the NOESY cross peaks observed between H-11β (δ$_H$ 1.71) and CH$_3$-21 (δ$_H$ 0.82)/CH$_3$-22 (δ$_H$ 0.77), and CH$_3$-23 and CH$_3$-21/H-17 (δ$_H$ 1.86) (Figure 5). The NOESY correlations between H-12 and CH$_3$-23, and H-18 and H-14 (δ$_H$ 1.23)/H-24 (δ$_H$ 4.03) suggested the $\beta$-orientations of H-12 and CH$_3$-26, respectively. Moreover, the 205° configuration of CH$_3$-27 was determined based on the NOESY signals observed between H-19$_{cis}$ (δ$_H$ -0.49) and H-3$\beta$ (δ$_H$ 1.24)/CH$_3$-27, and H-19$_{trans}$ (δ$_H$ 0.57) and H$_2$-6.

Figure 5. NOESY correlations observed for compound 1.

Compound 2 was isolated as a colorless oil, and its molecular formula was determined to be C$_{33}$H$_{46}$O$_6$ by HRESIMS (m/z [M + Na]$^+$ 537.3167, calcd 537.3187), corresponding to nine degrees of unsaturation. Analysis of the 1D and 2D NMR spectra obtained for 2 indicated a similar carbon framework to 1, but the higher oxidation state of the lactone in E ring appeared as a major difference. HMBC correlations from CH$_3$-23 (δ$_H$ 1.22) to C-18 (δ$_C$ 133.7) and from CH$_3$-26 (δ$_H$ 1.56) to C-17 (δ$_C$ 162.9) revealed an a,$\beta$-unsaturated lactone in the E ring, which was responsible for the one degree higher DOU than that of 1. In addition, the $^{13}$C chemical shift of C-24 (δ$_C$ 104.4) was characteristic of a ketal carbon atom, of which the position was confirmed by HMBC correlations from CH$_3$-26 to C-24. The $\beta$-configuration of OH-24 was determined by the NOESY correlation observed between H-16α (δ$_H$ 2.28) and CH$_3$-26 (Figure S3, Supplementary Materials).

Compound 3 was isolated as a mixture of two inseparable epimers. The molecular formula of 3 was deduced to be C$_{31}$H$_{46}$O$_6$ by HRESIMS (m/z [M + Na]$^+$ 535.3011, calcd 535.3030), corresponding to 10 degrees of unsaturation. An initial inspection of the $^{13}$C NMR spectrum revealed that most of the peaks were split into a doublet-like shape, indicating a 1:1 mixture of diastereomers. The 1D and 2D NMR spectra obtained for compound 3 exhibited most of the structural features of 2, except for one more disubstituted olefin observed at δ$_H$ (6.38/6.37)/δ$_C$ (138.84/138.80) and δ$_H$ (6.29/6.25)/δ$_C$ (118.6/118.4). The location of the double bond was determined to be $\Delta$15,16 using the consecutive $^1$H-$^1$H COSY correlations observed for H-14 (δ$_H$ 2.69/2.62)–H-15 (δ$_H$ 6.38/6.37)–H-16 (δ$_H$ 6.29/6.25). The splittings observed in the $^{13}$C NMR spectrum were most prominent at CH$_3$-26 (Δδ$_C$ 1.13 ppm), informing a mixture of C-24 epimers. This phenomenon has often been observed in the case of 24-homoscalaranes, which possess both an $\Delta$15,16.
olefin and 24-hydroxy pentenolide E-ring [25,26]. Since the $\Delta^{15,16}$-olefin increases the
planarity of the D-ring and renders the C-24 stereocenter more isolated, the 24R$^*$ and 24S$^*$
diastereomers exhibit almost identical spectroscopic and chromatographic behaviors to
give an inseparable mixture.

Compound 4 was isolated as an inseparable mixture and its molecular formula was
determined to be C$_{32}$H$_{46}$O$_6$ by HRESIMS (m/z [M + Na]$^+$ 549.3163, calcd 549.3187), indicating
10 degrees of unsaturation. The NMR spectra of 4 were only discriminated from those of
3 by the extra methylene group observed at $\delta_H$ 1.50 and $\delta_C$ 29.5/29.4, which was also supported
by the mass difference of +14. The extra methylene group was observed in the ester side chain located at C-12, which formed a 3-hydroxypentanoate moiety, as supported
by the spin system for H$_2$-2$'$ ($\delta_H$ 2.35)–H-3$'$ ($\delta_H$ 3.90/3.86)–H-2$'$–H-4$'$ ($\delta_H$ 1.50)–CH$_2$-5$'$ ($\delta_H$ 0.95) in the $^1$H–$^1$H COSY spectrum.

Compound 5 was isolated as a colorless oil. Its molecular formula was determined to be C$_{32}$H$_{48}$O$_6$ by HRESIMS (m/z [M + Na]$^+$ 551.3310, calcd 551.3343), corresponding
to nine degrees of unsaturation. Our initial analysis of the $^1$H NMR spectrum obtained for
compound 5 indicated the presence of the scalarane-type scaffold: five singlet methyl
groups at $\delta_H$ 0.80, 0.84, 1.06, 2.02, and 2.22; two doublet methyl groups at $\delta_H$ 1.08 and 1.25;
three oxymethines at $\delta_H$ 4.19, 5.11, and 5.76; unique cyclopropane signals at $\delta_H$ 0.59 and
-0.49; and one singlet olefin at $\delta_H$ 6.72. The $^{13}$C NMR and HSQC spectra showed one ketone
($\delta_C$ 197.7), two ester carbons ($\delta_C$ 172.0, 170.2), one trisubstituted olefin carbon ($\delta_C$ 153.2, 135.1), three oxymethine carbons ($\delta_C$ 76.8, 65.3, 64.6), eight methylenes, four methines, and
seven methyl groups. In addition, the HMBC correlation from the singlet methyl at $\delta_H$ 2.22
to $\delta_C$ 197.7 suggested the presence of a methyl ketone moiety, instead of the lactone E-ring
observed in compounds 1–4, leading to the conclusion that 5 had a B+F type skeleton, as
shown in Figure 2.

Detailed interpretation of the combined spectral data of 5 revealed that the features
related to the A-B-C ring system were identical to those of 1–4. As anticipated, the methyl
ketone was positioned at C-17 to form an unsaturated ketone in the D ring on the basis
of HMBC correlations between CH$_3$-26 ($\delta_H$ 2.22) and C-17 ($\delta_C$ 135.1), and H-18 ($\delta_H$ 6.72)
and C-17/C-24 ($\delta_C$ 197.7) (Figure 4). Moreover, the $^1$H–$^1$H COSY cross peak for H-14 ($\delta_H$
1.76)–H$_2$-15 ($\delta_H$ 1.89, 1.61)–H-16 ($\delta_H$ 5.76) and HMBC correlations from $\delta_H$ 2.02 (CH$_3$CO$_2$–)
to $\delta_C$ 170.2 (CH$_3$CO$_2$–) and from H-16 to $\delta_C$ 170.2 positioned an acetate substituent at C-16,
of which the relative configuration was assigned to be $\alpha$-orientation based on the small
coupling constants between H$_2$-15 and H-16 (dd, $J_{H-15-H-16}$ = 4.3, 1.6 Hz).

Compound 6 was isolated as an amorphous solid. Its molecular formula was determined
to be C$_{30}$H$_{44}$O$_5$ by HRESIMS (m/z [M + Na]$^+$ 509.3215, calcd 509.3237), correspon-
ding to eight degrees of unsaturation. The $^1$H and $^{13}$C NMR spectra obtained for
compound 6 were almost identical to those of 5. However, the absence of one ester carbon
and the singlet methyl group at $\delta_H$ 2.02 suggested deacetylation from 5, which was further
supported by an upfield shift of H-16 ($\delta_H$ 4.62). The relative configuration of OH-16 was
assigned as $\beta$-orientation based on the large coupling constant observed between H-16 and
H-15$^\beta$ (dd, $J_{H-15-H-16}$ = 9.6, 5.1 Hz).

Compound 7 was isolated as a yellow oil. Its molecular formula was determined to be C$_{31}$H$_{44}$O$_4$ by HRESIMS (m/z [M + Na]$^+$ 503.3113, calcd 503.3132), correspon-
ding to 10 degrees of unsaturation. Preliminary analysis of the $^1$H and $^{13}$C NMR data revealed that
the scalarane-type scaffold had a cyclopropane substituent on the A ring. Interpretation of
the $^{13}$C NMR and HSQC spectra exhibited the sp$^2$ carbons in the enone systems: three sp$^2$
methines at $\delta_H$ 7.38/$\delta_C$ 157.5, $\delta_H$ 6.34/$\delta_C$ 137.4, and $\delta_H$ 6.64/$\delta_C$ 130.4; and one trisubstituted
sp$^2$ carbon atom at $\delta_C$ 136.4. Therefore, HMBC correlations observed from H-25 ($\delta_H$
7.38) to C-17 ($\delta_C$ 136.4)/C-18 ($\delta_C$ 49.3)/C-24 ($\delta_C$ 195.9), H-26 ($\delta_H$ 6.34) to C-17/C-24/C-25 ($\delta_C$
157.5), and H-18 ($\delta_H$ 3.35) to C-17/C-23 ($\delta_C$ 14.5), as well as the $^1$H–$^1$H COSY cross peak for
H-18–H-25–H-26, confirmed the presence of a $\Delta^{25,26}$-cyclopenten-24-one subunit for the
E-ring and the trisubstituted double bond at $\Delta^{16,17}$ (Figure 4).
Compound 8 was isolated as a yellow oil, and its molecular formula was determined to be C_{34}H_{52}O_{8} by HRESIMS (m/z [M + Na]^+ 611.3541, calcd 611.3554), corresponding to nine degrees of unsaturation. The $^1$H NMR spectrum obtained for compound 8 showed similar patterns to that of 5. However, the upfield peaks observed for the cyclopropane moiety in 5 were substituted by an oxymethine at $\delta_H$ 5.35, a methyl singlet at $\delta_H$ 1.09, and an acetate at $\delta_H$ 2.03, suggesting the C+F type scaffold shown in Figure 2. Therefore, the connectivity of C-27–C-20–C-4–C-19 was determined using the HMBC correlations observed from CH$_3$-19 ($\delta_H$ 0.99) to C-20 ($\delta_C$ 73.2) and from CH$_3$-27 ($\delta_H$ 1.09) to C-4 ($\delta_C$ 39.4)/C-20 (Figure 6). In addition, the acetate at $\delta_H$ 2.03 exhibited a HMBC correlation with C-20 to be located at C-20. The relative configuration at C-20 was assigned as 20$^R$ from the NOESY correlations observed between H-20 ($\delta_H$ 5.35) and H-2$\beta$ ($\delta_H$ 1.47)/CH$_3$-22 ($\delta_H$ 0.87), and H-3$\beta$ ($\delta_H$ 1.67) and CH$_3$-27 (Figure 7). Similarly, the configuration of the acetate group at C-16 was assigned as a-orientation based on the small coupling constant observed for H-16 (dd, $J_{H-15-H-16}$ = 4.3, 1.6 Hz).

Figure 6. COSY and HMBC correlations observed for compounds 8, 10, and 14.

Compound 9 was isolated as a colorless oil, and its molecular formula was determined to be C_{30}H_{48}O_{6} by HRESIMS (m/z [M + NH$_4$]$^+$ 522.3810, calcd 522.3789) corresponding to seven degrees of unsaturation. Analysis of the 1D and 2D NMR data provided almost identical features to those of 8 to determine the carbon skeleton of compound 9. In this case, only one ester carbon atom ($\delta_C$ 172.2) was observed in the $^{13}$C NMR spectrum, and the acetate groups shown in the $^1$H NMR spectrum of 8 disappeared. This information indicated that compound 9 was the deacetylation product of 8. Accordingly, the upfield shifts of H-20 ($\delta_H$ 4.32) and H-16 ($\delta_H$ 4.55) were the major differences, compared to compound 8.

Compound 10 was isolated as a yellow oil, and its molecular formula was determined to be C_{33}H_{50}O_{8} by HRESIMS (m/z [M + Na]$^+$ 597.3404, calcd 597.3398), corresponding to nine degrees of unsaturation. Preliminary inspection of the $^{13}$C NMR and HSQC data of 10 identified four singlet methyl groups ($\delta_C$ 16.6, $\delta_H$ 0.87/$\delta_C$ 16.8, $\delta_H$ 0.96/$\delta_C$ 23.3, $\delta_H$ 1.13/$\delta_C$ 19.8), three doublet methyl groups ($\delta_H$ 1.07/$\delta_C$ 16.0, $\delta_H$ 1.18/$\delta_C$ 22.5, $\delta_H$ 1.39/$\delta_C$ 18.2), and one acetate group ($\delta_H$ 2.03/$\delta_C$ 22.0), indicating a honulactone C-like scaffold (C+D type shown in Figure 2) [12]. A detailed analysis of the $^1$H NMR spectrum identified
an oxymethine group at δ_H 4.44 as a major difference from honulactone C. The location of the oxymethylene was determined to be C-16, as indicated by the HMBC correlations from H-16 (δ_H 4.44) to C-17 (δ_C 162.1)/C-18 (δ_C 135.6) and 1H-1H COSY cross peak for H2-15 (δ_H 1.91, 1.84)-H-16 (Figure 6). The configuration of the OH-16 group was assigned as α-orientation based on the small coupling constant observed for H-16 (dd, J_H-15-H-16 = 4.7, 1.4 Hz), and compound 10 was named as 16α-hydroxyhonulactone C [12].

Compound 11 was isolated as a yellow oil. Its molecular formula was determined as C33H40O13 by HRESIMS (m/z [M + Na]^+ 597.3396, calcld 597.3398), corresponding to nine degrees of unsaturation. The 1H and 13C NMR data of 11 were almost identical to those of 10, but a ketal moiety (δ_C 104.4) was observed instead of one doublet methyl group and two oxymethines in compound 10. As shown in compounds 2-4, the hemiketal functionality in the scalarane-type scaffold usually occurs at C-24 in the E-ring, which was also applicable in this case, as indicated by the HMBC correlations from CH2-26 (δ_H 1.56) to C-17 (δ_C 162.9)/C-24 (δ_C 104.4). The α-orientation of the hydroxyl group at C-24 was determined by the NOESY correlation between H-16β (δ_H 2.33) and CH3-26. Thus, compound 11 was named 24α-hydroxyhonulactone C [12].

Compound 12 was isolated as an inseparable mixture. Its molecular formula was determined as C33H44O13 by HRESIMS (m/z [M + Na]^+ 595.3241, calcld 595.3241), corresponding to 10 degrees of unsaturation. Compared to 11, two more sp2 methines at δ_C 138.9/138.7 and δ_H 6.38, and δ_C 118.44/118.35 and δ_H 6.28/6.26 were observed in the 13C NMR and HSQC spectra, indicating the presence of a disubstituted double bond. These sp2 protons were involved in a spin system for H-14 (δ_H 2.66/2.62)-H-15 (δ_H 6.38)-H-16 (δ_H 6.28/6.26) in the 1H-1H COSY spectrum and used to confirm the presence of the Δ15,16-olefin, which was further supported by HMBC correlations from H-15 to C-13 (δ_C 40.1/40.0)/C-14 (δ_C 53.96/53.90)/C-17 (δ_C 157.3) and from H-16 to C-14/C-18 (δ_C 130.9). As discussed in the cases of 3 and 4, the presence of the olefin at Δ15,16 and the hemiketal at C-24 rendered compound 12 an inseparable mixture of C-24 epimers.

Compound 13 was isolated as an amorphous solid. Its molecular formula was determined as C31H48O6 by HRESIMS (m/z [M + Na]^+ 539.3325, calcld 539.3343), corresponding to eight degrees of unsaturation. Inspection of the 1H NMR spectrum of 13 revealed most of the structural features of the bishomoscalarane-type skeletons. Precise analysis of the 13C NMR and HSQC data revealed the presence of a triplet methyl group (δ_H 0.67/δ_C 8.80) and ketal carbon (δ_C 104.4), suggesting the A+D type skeleton shown in Figure 2. While most of the spectral data of 13 were identical to phyllofolactone H, the ketal carbon indicated the oxidation of C-24 to give a 24-hydroxy pentenolide E ring. This insight can be confirmed by the HMBC correlation from CH3-26 (δ_H 1.48) to C-17 (δ_C 163.0)/C-24 (δ_C 104.4). The configuration of OH-24 was determined to be α-orientation by the NOESY correlation between H-16β (δ_H 2.33) and CH3-26. Thus, compound 13 was named 24α-hydroxyphyllofolactone H [19].

Compound 14 was isolated as an inseparable mixture. Its molecular formula was determined as C31H46O6 by HRESIMS (m/z [M + Na]^+ 537.3175, calcld 537.3187), corresponding to nine degrees of unsaturation. Similar to compound 3, the 13C NMR spectrum of 14 showed a 1:1 splitting pattern corresponding to a mixture of two diastereomers. The distinctive spectral features of 14, differentiated from 13, were observed as the two sp2 methines at δ_H 6.40/6.38 and 6.28/6.27, suggesting an unsaturated derivative of 13. The methines belonged in the 1H-1H COSY correlation for H-14 (δ_H 2.68/2.62)-H-15 (δ_H 6.40/6.38)-H-16 (δ_H 6.28/6.27) to identify the olefin at C-15 (Figure 6). In addition, 14 was determined to be a mixture of C-24 epimers, considering the largest splitting observed at CH3-26 (Δδ_C 1.13 ppm).

Compound 15 was isolated as an inseparable mixture. Its molecular formula was determined to be C32H48O6 by HRESIMS (m/z [M + Na]^+ 551.3366, calcld 551.3343), corresponding to nine degrees of unsaturation. The MS data indicated an additional methylene relative to 14, which was further supported by the change observed in the coupling pattern of the terminal methyl group of the side chain at C-12 from a doublet to triplet. The 13C
NMR and HSQC data identified the methylene group at $\delta_H$ 1.51/1.25 and $\delta_C$ 29.5/29.4, which were involved in the spin system for $H_2$-2’-H-3’-H$_2$-4’-CH$_3$-5’ in the $^1$H-$^1$H COSY spectrum to confirm the presence of the 3-hydroxypentanoate side chain. The orientation of the ester at C-12 was assigned as $\alpha$ by the NOESY signal between H-12 ($\delta_H$ 5.55/5.49) and CH$_3$-23 ($\delta_H$ 1.06/1.05), as well as the small coupling constant observed for H-12 (dd, $J = 2.3, 1.8$ Hz), to identify 12-$\alpha$-phyllactone D/E.

Interestingly, the identified structure was previously isolated as a mixture of C-24 epimers by Li et al. in 2007 [11], but our experimental $^{13}$C NMR data showed some discrepancies with the previously reported data at C-9 ($\Delta 4.28$ ppm), C-11 ($\Delta 2.7$ ppm), C-12 ($\Delta 2.08$ ppm), C-14 ($\Delta 4.46$ ppm), and C-23 ($\Delta 4.35$ ppm) (Figure 8a). In addition, another identification of 12-epi-phyllactone D/E was reported by Andersen et al. in 2009 [13]. Although they acquired almost identical experimental NMR data with ours rather than those reported by Li, the isolated compound was estimated to be same as Li’s without consideration of the differences in NMR data (Tables S17 and S18, Supplementary Materials). Therefore, we investigated the variations in $^{13}$C chemical shifts depending on the orientation of the substituents at C-12.

![Figure 8](image_url)

**Figure 8.** (a) The differences observed between the experimental and reported $^{13}$C chemical shifts (Li et al.) of 15 in CDCl$_3$. (b) The deviations in the $^{13}$C NMR chemical shifts observed for reported (Li et al.) and isolated 15 relative to phyllactone D and phyllactone E. (c) The structures of phyllactone D and E.

Phyllactone D (17) and E (18), the reported 12$\beta$-epimers of 15, were selected for comparison [25]. While C-12 in phyllactones D and E was observed at $\delta_C$ 75.1 and 75.8,
respectively, the corresponding chemical shifts of the reported and isolated 15 were observed at δ_C 75.3 and 73.2/73.1, respectively. The deviations observed for isolated 15 from phyllactone D/E became more obvious at C-9, C-14, and C-23 (Figure 8b). However, the reported chemical shifts for 15 were better aligned with those of phyllactone D/E. Furthermore, the differences in the ^13_C NMR chemical shifts observed between isolated 15 and compounds 3, 4, 12, and 14, which share an identical substructure for the B-E ring system, showed negligible values (< 0.5 ppm) around the C-ring (Table S19, Supplementary Materials). Accordingly, isolated 15 is more likely to be the 12α-epimer. Even though Li determined the 12α-configuration observing the NOESY signal between H-12 and CH3-23 and J_H-12–H-13 calculation (3.0, 2.5 Hz), the NMR database suggests that the compound previously reported by Li is presumed to be a mixture of phyllactone D (17) and E (18).

Compound 16 was isolated as a yellowish oil. Its molecular formula was determined as C27H40O5 by HRESIMS (m/z [M + Na]+ 467.2762, calc 467.2768), corresponding to eight degrees of unsaturation. The ^1H NMR spectrum of 16 revealed five singlet methyl groups at δ_H 0.73, 0.74, 0.79, 0.85, and 0.86; one acetate group at δ_H 1.95; one oxymethine at δ_H 4.80; one olefin at δ_H 7.30; and one aldehyde at δ_H 9.41. The ^13_C and HSQC NMR spectra showed characteristic peaks for the aldehyde carbon atom at δ_C 196.4, two carbonyl carbons at δ_C 169.6 and 169.6, one trisubstituted olefin at δ_C 145.8 and 124.2, and one oxymethine at δ_C 76.9. The HMBC correlation between the two methyl groups at δ_C 33.3 and 21.4 was identified as a characteristic feature of the 4-dimethyl-sesterterpenoid scaffold (Figure 9). The aldehyde at δ_H 9.41 exhibited a HMBC correlation with C-18 (δ_C 58.7) to be located at C-25. Additional HMBC correlations from H-18 (δ_H 3.07) to C-16 (δ_C 145.8)/C-17 (δ_C 124.2)/C-24 (δ_C 169.6), along with the ^1H-^1H COSY cross peak for H-14-H2-15-H-16, indicated the presence of the acid at C-24 and trisubstituted olefin at C-16. The acetate group (δ_C 21.3/δ_H 1.95) was positioned at C-12, as indicated by the HMBC correlation from H-12 (δ_H 4.80) to C-1′ (δ_C 169.6) and ^1H-^1H COSY cross peak for H2-11-H-12. Thus, the planar structure of 16 was found to be the deacetalization product of scalarin (19) [3]. The NOESY correlations between CH3-23 (δ_H 0.86) and H-12/H-18 determined the configuration of the C-12 acetate and C-18 formyl groups as α.

Figure 9. COSY, HMBC, and NOESY correlations observed for compound 16.

Whereas scalarin (19) exists only in its hemiacetal form, the formation of 18-epi-19 or 19 via the acetalization of 16 was not observed. To rationalize the observed difference in reactivity, 18-epi-16 was proposed as a plausible precursor of scalarin, and geometrical optimization of 16 and 18-epi-16 was performed at the B3LYP/6-31G** level of theory. The atomic distance between O-24 to C-25 was calculated to be 3.37 Å for 16 and 2.68 Å for 18-epi-16 (Figure 10). This result suggests that 18-epi-16 can undergo acetalization to form scalarin because the β-orientation of C-25 increases its proximity to the acid at C-24. However, the acetalization of the 25α-formyl group in 16 will be restricted due to its remoteness to OH-24 to exist as its aldehyde form.
2.2. Biological Activity

The cytotoxicity of compounds 1–16 against MDA-MB-231 (a human breast cancer cell line) was evaluated to elucidate their potential as anticancer agents. Compounds 1–6, 8, 11, and 13–15 exhibited moderate cytotoxicity with GI50 values ranging from 40 to 72 µM. Compounds 9, 10, 12, and 16 were inactive toward the cancer cell line (Table 1). Among the bishomoscalaranes, the highest anticancer activity was exhibited by compound 7, which has a relatively rare cyclopentenone E-ring (B+E type scaffold in Figure 2), with a GI50 value of 4.2 µM.

Table 1. The results of the cytotoxicity tests against MDA-MB-231 (human breast cancer cell line) obtained for compounds 1–16.

| Compound | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|----------|---|---|---|---|---|---|---|---|
| GI50 (µM) | 69.94 | 43.38 | 72.49 | 54.02 | 53.58 | 50.8 | 4.21 | 53.55 |

| Compound | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 |
|----------|---|---|---|---|---|---|---|---|
| GI50 (µM) | >100 | >100 | 71.14 | >100 | 50.71 | 63.54 | 40.82 | >100 |

1 Cisplatin (Sigma-Aldrich, St. Louis, MO) was used as a positive control (GI50 = 1.31 µM).

The highly diversified structures of the isolated scalaranes provided some information on their structure–activity relationship (SAR). The presence of the △15,16-olefin generally had a detrimental effect that reduced the cytotoxicity in the range of 12–30 µM, as shown by the sets of 2 and 3 (B+D type), 13 and 14 (C+D type), and 13 and 14 (A+D type). Comparing 3 with 4 and 14 with 15, the homologation of one methylene group at C-4′ was beneficial toward increasing the activity to ~20 µM. A series of compounds 2, 11, and 13, which only differ at the C-4 substituent, indicated the disadvantageous effect of oxidation at C-20 on the anticancer activity. The negative effect of oxidation at C-20 was also observed in the inactive series of compounds 9, 10, and 12.

3. Materials and Methods

3.1. General Experimental Procedures

Specific optical rotations were collected on a Rudolph Research Analytical (Autopol III) polarimeter (Rudolph Research Analytical, Hackettstown, NJ, USA). IR spectra were
measured on a JASCO FT/IR-4100 spectrophotometer (JASCO Corporation, Tokyo, Japan). The 1D and 2D NMR spectra were taken in CDCl$_3$ using a Bruker 600 MHz spectrometer (Bruker BioSpin GmbH, Rheinstetten, Germany) at 297.1 K. $^1$H NMR spectra were collected after 64–128 scans, and $^{13}$C NMR spectra were collected at a range of 10,000–15,000 scans depending on the sample concentrations. The mixing time for NOESY experiments was set as 0.3 s. Chemical shifts were reported in parts per million relative to CHCl$_3$ residue ($\delta_H$ 7.26, $\delta_C$ 77.1) in CDCl$_3$. High resolution mass-spectra were obtained on a Sciex X500R Q-TOF spectrometer (Framingham, MA, USA) equipped with an ESI source. MPLC was performed using the TELEDYNE ISCO CombiFlash Companion with the TELEDYNE ISCO RediSep Normal-phase Silica Flash Column (Teledyne ISCO, Lincoln, NE, USA). HPLC was performed on a PrimeLine Binary pump (Analytical Scientific Instruments, Inc., El Sobrante, CA, USA) utilizing silica columns (YMC-Pack Silica, 250 × 10 mm I.D., or 250 × 4.6 mm I.D., 5 μm; YMC Co., Ltd., Kyoto, Japan), the Shodex RI-101 (Shoko Scientific Co. Ltd., Yokohama, Japan), or the UV-M201.

3.2. Biological Material

The marine sponge used in this study was collected in March 2016 from the Bohol province in the Philippines (N 9°43′31.39″ E 124°32′19.86″) at a depth of 15 m using scuba diving. The sponge was directly kept frozen at −20 °C until identified as Dysidea sp. and chemically analyzed. A voucher sample (163PIL-267) has been stored at the Marine Biotechnology Research Center, Korea Institute of Ocean Science & Technology (KIOST).

3.3. Extraction and Isolation

The lyophilized specimen (wt. 1.5 kg) was extracted with MeOH (2.0 L × 3) and CH$_2$Cl$_2$ (4.0 L × 2) at room temperature. The combined extracts were concentrated under reduced pressure. The dried residue (89.5 g) was partitioned with n-butanol (5.0 L) and water (5.0 L). The n-butanol layer was concentrated and further partitioned between n-hexane (3.0 L) and 15% aqueous methanol (3.0 L). A portion (12.2 g) of the concentrated 15% aqueous methanol fraction (31.7 g) was subjected to flash column chromatography over C18 (YMC Gel ODS-A, 60 Å, 230 mesh (YMC Co, Ltd., Kyoto, Japan)) with a stepwise gradient solvent system (50%, 60%, 70%, 80%, 90%, and 100% MeOH, acetone, and EtOAc). The 80% MeOH fraction (612.7 mg) was further separated using MPLC on C18 with a gradient solvent system from 70% MeOH to 100% MeOH over 40 minutes to yield 4 fractions. The third subfraction (250.1 mg) was separated using HPLC (elucent 65% MeOH) to yield 8 (3.9 mg, $t_R$ = 38 min), 9 (2.5 mg, $t_R$ = 42 min), 10 (2.3 mg, $t_R$ = 58 min), honulactone C (9.8 mg), and honulactone D (9.0 mg). The fourth subfractions (175.3 mg) was separated using HPLC (elucent 70% MeOH) to yield 11 (1.8 mg, $t_R$ = 28 min), 12 (1.4 mg, $t_R$ = 28 min), and honulactone I+J mixture (1.6 mg).

The 100% MeOH fraction (612.7 mg) was further separated using MPLC on C18 with a gradient solvent system from 70% MeOH to 100% MeOH over 40 minutes to yield 4 fractions. The second subfraction (250.1 mg) was directly separated using MPLC on SiO$_2$ with a gradient solvent system from 70% HX to 100% EtOAc over 80 minutes to yield 8 subfractions (based on TLC analysis). Scalarin (19, 213.0 mg) was recrystallized from the second subfraction (572.8 mg) under the HX-EtOAc solvent conditions. The residue (250.0 mg) of the second subfraction was separated using HPLC (HX/acetone = 7/1) to yield 4 (5.2 mg, $t_R$ = 54 min), 15 (5.5 mg, $t_R$ = 48 min), phyllofolactone H (5.7 mg), and phyllofolactone I (11.5 mg). The third subfraction (295.5 mg) was separated using HPLC (HX/acetone = 7/1) to yield 1 (3.4 mg, $t_R$ = 34 min), 3 (7.0 mg, $t_R$ = 76 min), 14 (6.0 mg, $t_R$ = 66 min), 13 (2.3 mg, $t_R$ = 60 min), 16 (4.5 mg, $t_R$ = 45 min), honulactone A (21.6 mg), honulactone B (26.2 mg), honulactone E+F mixture (21.4 mg), and phyllofolactone J+K (2.7 mg). The fourth subfraction (380.0 mg) was separated using HPLC (HX/acetone = 5/1) to yield 2 (3.1 mg, $t_R$ = 36 min), 5 (1.6 mg, $t_R$ = 31 min), 6 (5.6 mg, $t_R$ = 32 min), 7 (5.0 mg, $t_R$ = 30 min), and phyllofenone C (2.3 mg).
3.4. Assay

Human breast cancer MDA-MB-231 cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and were cultured in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Gibco, Carlsbad, CA, USA), 1 × antibiotic-antimycotic solution (Thermo Fisher Scientific, Waltham, MA, USA), and 25 mM HEPES (Gibco). Cultures were maintained in a humidified atmosphere of 95% air/5% CO₂ at 37 °C.

Cell viability was determined using a CCK-8 (Cell Counting Kit-8, Dojindo Laboratory, Kumamoto, Japan) assay according to the manufacturer’s instructions. MDA-MB-231 cells were seeded at 5 × 10⁵ cells/well into a 96-well plate and then were treated with various concentrations of compounds 1–16. Following treatment for 48 h, the cells were incubated with the CCK-9 solution, and the absorbance was measured at 450 nm using a SpectraMax i3 microplate reader ( Molecular Devices, Sunnyvale, CA, USA). GS₅₀ values were calculated from a non-linear regression fit using GraphPad Prism version 9.2.0 (GraphPad Software, La Jolla, CA, USA).

1: colorless oil; [α]D²⁰ + 20.0 (c 0.2, CHCl₃); IR (ATR) νmax 3131, 2954, 2929, 2581, 1770, 1734, 1452, 1381, 1261, 1176, 1027 cm⁻¹; ¹H NMR and ¹³C NMR, see Tables S1 and S2, Supplementary Materials; HRESIMS m/z 523.3382 [M + Na]⁺ (calcd for C₃₁H₅₉O₇Na, 523.3394).

2: colorless oil; [α]D²⁰ + 40.0 (c 0.2, CHCl₃); IR (ATR) νmax 3735, 2954, 2925, 2851, 1731, 1689, 1452, 1374, 1278, 1176, 1014 cm⁻¹; ¹H NMR and ¹³C NMR, see Tables S1 and S2, Supplementary Materials; HRESIMS m/z 537.3167 [M + Na]⁺ (calcd for C₃₁H₅₉O₇Na, 537.3187).

3: colorless oil; [α]D²⁰ + 45.0 (c 0.2, CHCl₃); IR (ATR) νmax 3727, 2957, 2922, 2865, 2848, 1738, 1657, 1458, 1371, 1286, 1621, 1173, 1031 cm⁻¹; ¹H NMR and ¹³C NMR, see Tables S1 and S2, Supplementary Materials; HRESIMS m/z 535.3011 [M + Na]⁺ (calcd for C₃₁H₄₇O₆Na, 535.3030).

4: colorless oil; [α]D²⁰ + 48.3 (c 0.2, CHCl₃); IR (ATR) νmax 3735, 2954, 2922, 2869, 2855, 1731, 1685, 1452, 1374, 1286, 1173, 1021 cm⁻¹; ¹H NMR and ¹³C NMR, see Tables S1 and S2, Supplementary Materials; HRESIMS m/z 549.3163 [M + Na]⁺ (calcd for C₃₂H₄₆O₇Na, 549.3187).

5: colorless oil; [α]D²⁰ − 20.0 (c 0.1, CHCl₃); IR (ATR) νmax 3727, 2961, 2929, 2851, 1734, 1678, 1452, 1367, 1254, 1173, 1027 cm⁻¹; ¹H NMR and ¹³C NMR, see Tables S1 and S2, Supplementary Materials; HRESIMS m/z 551.3310 [M + Na]⁺ (calcd for C₃₂H₄₅O₇Na, 551.3343).

6: amorphous powder; [α]D²⁰ + 45.0 (c 0.2, CHCl₃); IR (ATR) νmax 3735, 2971, 2929, 2865, 1724, 1678, 1657, 1452, 1371, 1296, 1173, 1080, 1027 cm⁻¹; ¹H NMR and ¹³C NMR, see Tables S1 and S2, Supplementary Materials; HRESIMS m/z 509.3215 [M + Na]⁺ (calcd for C₃₀H₄₆O₅Na, 509.3237).

7: colorless oil; [α]D²⁰ + 33.3 (c 0.1, CHCl₃); IR (ATR) νmax 3735, 2957, 2918, 2848, 1727, 1702, 1657, 1458, 1371, 1254, 1176, 1038 cm⁻¹; ¹H NMR and ¹³C NMR, see Tables S1 and S2, Supplementary Materials; HRESIMS m/z 503.3113 [M + Na]⁺ (calcd for C₃₁H₄₄O₄Na, 503.3132).

8: colorless oil; [α]D²⁰ + 8.3 (c 0.2, CHCl₃); IR (ATR) νmax 3727, 2961, 2929, 2851, 1738, 1721, 1671, 1505, 1452, 1374, 1246, 1031 cm⁻¹; ¹H NMR and ¹³C NMR, see Tables S1 and S2, Supplementary Materials; HRESIMS m/z 611.3541 [M + Na]⁺ (calcd for C₃₄H₅₂O₈Na, 611.3554).

9: colorless oil; [α]D²⁰ − 6.7 (c 0.1, CHCl₃); IR (ATR) νmax 2961, 2925, 2851, 1745, 1727, 1505, 1265, 1031 cm⁻¹; ¹H NMR and ¹³C NMR, see Tables S1 and S2, Supplementary Materials; HRESIMS m/z 522.3810 [M + NH₄]⁺ (calcd for C₃₀H₃₅NO₄Na, 522.3789).

10: colorless oil; [α]D²⁰ + 73.3 (c 0.1, CHCl₃); IR (ATR) νmax 3477, 3388, 2966, 2923, 2866, 1729, 1457, 1368, 1250 cm⁻¹; ¹H NMR and ¹³C NMR, see Tables S1 and S2, Supplementary Materials; HRESIMS m/z 597.3404 [M + Na]⁺ (calcd for C₃₅H₅₃O₈Na, 597.3398).
11: colorless oil; [α]D20^ + 40.0 (c 0.1, CHCl3); IR (ATR) vmax 2965, 2918, 2855, 1731, 1649, 1458, 1374, 1250, 1169, 1035 cm⁻¹; 1H NMR and 13C NMR, see Tables S1 and S2, Supplementary Materials; HRESIMS m/z 597.3396 [M + Na]^+ (calcd for C33H50O8Na, 597.3398).

12: colorless oil; [α]D20^ + 71.7 (c 0.2, CHCl3); IR (ATR) vmax 3392, 2946, 2925, 2858, 1734, 1455, 1367, 1243, 1180 cm⁻¹; 1H NMR and 13C NMR, see Tables S1 and S2, Supplementary Materials; HRESIMS m/z 595.3241 [M + Na]^+ (calcd for C33H49O8Na, 595.3241).

13: amorphous powder; [α]D20^ + 6.7 (c 0.2, CHCl3); IR (ATR) vmax 3727, 2957, 2929, 2848, 1727, 1657, 1455, 1374, 1278, 1176 cm⁻¹; 1H NMR and 13C NMR, see Tables S1 and S2, Supplementary Materials; HRESIMS m/z 539.3325 [M + Na]^+ (calcd for C31H46O6Na, 539.3343).

14: colorless oil; [α]D20^ + 31.7 (c 0.2, CHCl3); IR (ATR) vmax 3717, 2961, 2925, 2872, 1727, 1649, 1458, 1374, 1275, 1257, 1176 cm⁻¹; 1H NMR and 13C NMR, see Tables S1 and S2, Supplementary Materials; HRESIMS m/z 537.3175 [M + Na]^+ (calcd for C31H46O6Na, 537.3187).

15: colorless oil; [α]D20^ + 30.0 (c 0.2, CHCl3); IR (ATR) vmax 3735, 2957, 2925, 2869, 1731, 1448, 1363, 1278, 1176, 1014 cm⁻¹; 1H NMR and 13C NMR, see Tables S1 and S2, Supplementary Materials; HRESIMS m/z 551.3366 [M + Na]^+ (calcd for C32H46O6Na, 551.3343).

16: colorless oil; [α]D20^ − 70.0 (c 0.2, CHCl3); IR (ATR) vmax 3277, 2961, 2922, 2851, 1738, 1646, 1452, 1381, 1225, 1021 cm⁻¹; 1H NMR and 13C NMR, see Tables S1 and S2, Supplementary Materials; HRESIMS m/z 467.2762 [M + Na]^+ (calcd for C27H30O3Na, 467.2768).

4. Conclusions

A total of 15 novel scalaranes 1–14 and 16, including 14 bishomoscalaranes and one scalarin derivative, has been isolated from the marine sponge, Dysidea sp., and characterized using a combination of 1D and 2D NMR spectroscopy. The isolation and structural identification of compound 15 resulted in the reassignment of the previously characterized 12-epi-phyllactone D/E. The actual structure of the reported 12-epi-phyllactone D/E was determined to be a mixture of known phyllactones D and E through the precise analysis of the experimental and reported 13C chemical shifts. In addition, the effect of the C-18 configuration in 16 on the formation of the hemiacetal E-ring was rationalized by measuring the atomic distances between C-25 and O-24 in the experimental and reported 13C chemical shifts. In 16, the evaluation of the anticancer activities of compounds 1–16 and 18-epi-16 revealed that compound 7 exhibited significant cytotoxicity with a GI50 value of 4.2 µM. Detailed studies to elucidate the biological mechanism of 7 are currently underway in our laboratory.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/md19110627/s1, I. Experimental procedure; Figure S1: Structures of known compounds isolated from Dysidea sp., Tables S1–S16: 13C/1H chemical shifts for 1–16, Figure S2: Key 1H-1H COSY, HMBC correlations of 1–16, Figure S3: Key NOESY correlations of 1–16, Tables S17–S19, Figures S4 and S5: Comparison of 13C/1H chemical shifts for 12β epimer (I7, 18) and 12α epimer (15) reported by Li et al., Andersen et al., and our experiment, II. Computational methods; Supporting Information II; Figures S1–S–112: 1H NMR, 13C NMR, COSY, HSQC, HMBC, NOESY, and HRMS spectra of 1–16.

Author Contributions: A.-Y.S. worked on isolation and structure elucidation. A.S. and C.C. performed the biological evaluation. J.L. collected the marine sponge and supervised the whole research work. All authors have read and agreed to the published version of the manuscript.

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