Regular Article

Structure and Stereochemistry of Amphidinolide N Congeners from Marine Dinoflagellate Amphidinium Species

Masashi Tsuda,*a,b Mai Akakabe,c3 Mika Minamida,d Keiko Kumagai,c Masayuki Tsuda,c Yuko Konishi,c Akira Tominaga,e Eri Fukushima,f and Jun Kawabataf

a Center for Advanced Marine Core Research, Kochi University; Nankoku, Kochi 783–850, Japan; b Department of Agriculture and Marine Science, Kochi University; Nankoku, Kochi 783–850, Japan; c Science Research Center, Kochi University; Nankoku, Kochi 783–850, Japan; d Department of Applied Science, Kochi University; Nankoku, Kochi 783–850, Japan; * Graduate School of Kuroshio Science and Kochi Medical School, Kochi University; Nankoku, Kochi 783–850, Japan; and † Research Faculty of Agriculture, Hokkaido University; Sapporo 060–8589, Japan.

Received September 15, 2020; accepted October 24, 2020

Two highly potent cytotoxic 26-membered macrolides, isocaribenolide-I (1) and a chlorohydrin 2, together with known amphidinolide N (3), have been isolated from a free-swimming dinoflagellate Amphidinium species (KCA09053 and KCA09056 strains) collected off Iriomote Island, Japan. The structures of 1 and 2 were determined to be a congener of 3 with an isobutyl terminus and the chlorohydrin form of 3, respectively, by detailed analyses of spectroscopic data. The relative stereochemistries of 1 and 2 were elucidated by the conformational analyses based on NMR data.

Key words macrolide; dinoflagellate; Amphidinium; cytotoxic activity

Introduction

Marine dinoflagellates of the genus Amphidinium have been recognized as a valuable source of polyketides with novel chemical structures and interesting bioactivities.1) The macrolide group represented by amphidinolides are unique natural products with cytotoxic activity.2,3) In 1994, Kobayashi and colleagues had reported a 26-membered macrolide, amphidinolide N,4) isolated from marine symbiotic dinoflagellate Amphidinium species. Amphidinolide N exhibits the most potent cytotoxic activity among the amphidinolide macrolides. Soon after, Shimizu and colleagues isolated an antitumor 26-membered macrolide, named caribenolide-I,5) from the marine free-swimming dinoflagellate Amphidinium gibbosum.6,7) Thereafter, Kobayashi and colleagues reported a structural revision of the dihydroxy form at C-21 and C-24 into a tetrahydrofuran structure, and the relative stereochemistry of amphidinolide N was proposed.8) Although the planar structure of amphidinolide N is the same as that of caribenolide-I, it is unclear whether amphidinolide N is identical to caribenolide-I or not, because the spectral data of both compounds have not been compared.

As synthetic approaches to determine the structure of amphidinolide N and/or caribenolide-I, Nicolaou et al. accomplished the synthesis of several analogs of amphidinolide N and caribenolide-I in 2006.9,10) The total synthesis of the 7,10-epimer of amphidinolide N was reported by Hayashi and colleagues,11,12) and several synthetic studies for this amphidinolide N/caribenolide-I have been carried out.13–17) Recently, Trost et al. accomplished the synthesis of des-epoxy amphidinolide N,18) and proposed that amphidinolide N and caribenolide-I are identical on the basis of the chemical shift differences between natural specimens and their synthetic product. However, the stereostructures of amphidinolide N and caribenolide-I have not yet been elucidated.

During our continuing search for new cytotoxic metabolites from extracts of marine dinoflagellate Amphidinium species,19,20) we investigated the cytotoxic components contained in extracts of Amphidinium KCA09053 and KCA09056 strains, and found two new highly potent cytotoxic macrolides, isocaribenolide-I21,22) (1) and a chlorohydrin 2,22,23) together with a known amphidinolide N (3) (Fig. 1). The structures of 1 and 2 were confirmed by spectral data analyses, indicating that compounds 1 and 2 correspond to a congener with the isobutyl terminus of 3 and chlorohydrin form of 3, respectively. The relative stereochemistries of these compounds were elucidated by conformational analyses based on NMR data. Herein, we describe the isolation and provide a structural elucidation of 1 and 2. We also report that the physicochemical properties of compound 3 were identical to those of both amphidinolide N and caribenolide-I.

Results and Discussion

The dinoflagellate Amphidinium species (strain number KCA09053) was separated monocolonally from benthic sea sands collected off Iriomote Island, Japan.24) The dinoflagellates were cultivated in Muroto deep seawater containing 1% Provasoli’s enriched supplement for 14 d with mechanical stirring and illumination. The algal cells of the KCA09053 strain (dry weight 10.3 g from 150 L culture) were extracted using a methanol (MeOH)–toluene solvent system. The toluene-soluble materials of the algal extract were subjected to a silica gel column chromatography eluted by chloroform (CHCl3)–MeOH. The crude fraction, which exhibited >90% cell-growth inhibition of human cervix adenocarcinoma HeLa cells at 1 µg/mL, was chromatographed by octadeccysil...
ODS and amino silica gel column chromatography and then ODS HPLC to afford isocaribenolide-I (1, 0.006% from dry weight) together with iriomoteolides-10a, 12a, 25) and 13a 24) and amphirionin-5. 26,27) The Amphidinium KCA09056 strain was separated from the surface of the seagrass collected off Iriomote Island. 28) The toluene-soluble materials of the micro-algal extract were subjected to silica gel column chromatography, and then the crude fraction showing potent cytotoxic activity was chromatographed using an ODS column and then ODS HPLC to afford two cytotoxic components, compounds 2 (0.0054%) and 3 (0.0024%).

Compound 3 was found to have the same molecular formula (C$_{33}$H$_{52}$O$_{11}$) as amphidinolide N 4,8) and carbenolide-I 5) by high-resolution (HR) electrospray ionization (ESI)-MS data \( m/z 647.3400 (M^+ + Na)^{+}, \Delta −0.23 \text{ mmu} \). The 13C and 1H chemical shifts for 3 in benzene-\( d_6 \) (C$_6$D$_6$) (Table S1) were well consistent with those of amphidinolide N. 4) The absolute values of the 13C chemical shift differences between this sample and the reported data fell in the range of 0.03 ppm, and the 1H-NMR data of 3 and amphidinolide N were also close to each other. The specific rotation \( [\alpha]_{D}^{19} +30 (c = 0.49, \text{ MeOH}) \) of this sample showed the same positive sign as the literature value \( [\alpha]_{D}^{19} +91 (c = 0.13, \text{ CHCl}_3) \) for caribenolide-I, thus indicating that compound 3 and caribenolide-I are identical. Therefore, we concluded that amphidinolide N and caribenolide-I were the same substance.

**Structures of Isocaribenolide-I (1) and Chlorohydrin 2**

Compound 1 was obtained as a colorless amorphous solid and was optically active. The molecular formula of 1 was established as C$_{33}$H$_{52}$O$_{11}$ based on HR-ESI-MS data \( m/z 647.3398 (M + Na)^+ , \Delta −0.40 \text{ mmu} \). The 13C-NMR (Table 1) spectrum in C$_6$D$_6$ showed the presence of 33 carbon signals comprising one ketone carbonyl, one ester carbonyl, two \( sp^2 \) quaternary carbons, one \( sp^2 \) methine, one \( sp^2 \) methylene, one hemiketal, 13 \( sp^3 \) methines (10 of which were oxygenated), eight \( sp^3 \)

| Position | $^{13}$C | $^1$H |
|----------|---------|--------|
| 1        | 173.6   | C      |
| 2        | 44.8    | 2.74 dd, 7.0 |
| 3        | 72.9    | 3.87 dd, 2.0 |
| 4        | 62.4    | 3.13 dd, 4.8 |
| 5        | 54.5    | 3.59  d, 2.0 |
| 6        | 147.0   | C      |
| 7        | 69.6    | 4.86 m  |
| 8        | 45.1    | 2.98 dd, 10.4, 16.7 |
| 9        | 210.5   | C      |
| 10       | 47.9    | 3.26 dq, 10.6, 6.7 |
| 11       | 127.2   | 5.16 brd, 10.6 |
| 12       | 136.3   | C      |
| 13       | 40.2    | 2.48 dd, 13.5, 3.6 |
| 14       | 70.8    | 4.32 dd, 9.6, 3.6 |
| 15       | 98.1    | C      |
| 16       | 65.8    | 3.61 t, 2.5 |
| 17       | 26.8    | 2.21 m  |
| 18       | 25.6    | 1.39 m  |
| 19       | 65.8    | 4.22 m  |
| 20       | 41.5    | 1.44 dq, 10.6 |
| 21       | 74.4    | 4.16 m  |
| 22       | 32.6    | 1.67 m  |
| 23       | 28.0    | 1.59 m  |
| 24       | 26.0    | 1.42 m  |
| 25       | 25.6    | 1.01 m  |
| 26       | 24.7    | 1.73 m  |
| 27       | 23.6    | 0.88(d, 7.0 |
| 28       | 21.7    | 0.93(d, 7.0 |
| 29       | 14.1    | 1.22(d, 7.0 |
| 30       | 111.5   | 5.40 brs |
| 31       | 15.0    | 5.31 brs |
| 32       | 15.7    | 1.13(d, 7.0 |
| 33       | 15.8    | 1.79(s   |

Table 1. $^{1}$H- and $^{13}$C-NMR Data of Isocaribenolide-I (1) in C$_6$D$_6$

The optical rotation value \( [\alpha]_{D}^{19} +27 (c = 0.49) \) in dichloromethane (CH$_2$Cl$_2$) of our sample showed the same sign as that reported for the literature value \( [\alpha]_{D}^{25} +101 (c = 0.13, \text{ CHCl}_3) \) for caribenolide-I, thus indicating that compound 3 and caribenolide-I are identical. Therefore, we concluded that amphidinolide N and caribenolide-I were the same substance.
methylenes, and five methyl carbons. Four out of eight degrees of unsaturation were accounted for by two olefins and two carbonyl groups, thus implying that I possessed four rings.

The planar structure of I was elucidated on the basis of detailed NMR studies, including $^1$H–$^1$H correlated spectroscopy (COSY), total correlation spectroscopy (TOCSY), heteronuclear multiple-quantum coherence (HMQC), methine- and methylene-selected editing heteronuclear single quantum coherence (CH- and CH$_2$-sel E-HSQC), heteronuclear multiple-quantum correlation (HMBC), and nuclear Overhauser effect spectroscopy (NOESY) spectra recorded in CD$_6$. Analyses of $^1$H–$^1$H COSY and TOCSY spectra revealed five proton–proton networks from H$_2$-2 to H-5 and H$_2$-30, from H-7 to H$_2$-8, from H-10 to H-11 and H$_2$-32, from H$_2$-13 to H-14, and from H-16 to H$_2$-28 and H$_2$-29 (Fig. 2). The presence of the trans epoxide at C-4–C-5 was deduced from the $^{13}$C chemical shifts of C-4 and C-5 ($\delta_C$ 62.4 and 54.5, respectively) and the $^1$H–$^1$H coupling constant values (H-4/H-5: 2.0 Hz). The E-geometry of the C-11–C-12 olefin was assigned from NOESY correlations for H-10/H$_2$-33 and H-11/H$_2$-13b as well as the relatively high-field chemical shift for C-33 ($\delta_C$ 15.8). Connections of these five networks and C-31 and C-33 via four quaternary carbons (C-6, C-9, C-12, and C-15) were suggested by HMBC correlations as follows: C-6/H$_2$-31, C-7/H$_2$-31, C-9/H$_2$-8, C-9/H-10, C-11/H$_2$-33, C-12/H$_2$-33, C-13/H$_2$-33, C-15/H-14, and C-16/H-14. HMBC correlations for C-1/H-2 and C-1/H-25 revealed that an ester linkage existed between C-1 and C-25, suggesting the presence of a 26-membered macrolactone ring. One of two residual rings was determined to be a tetrahydrofuran ring at C-21–C-24 from the HMBC correlations for C-21/H-24. The relatively low-field carbon resonance ($\delta_C$ 30.4) in the deuterium secondary isobutyl portion for C-26–C-29. Therefore, the gross structure of I possessed the same macrocyclic portion at C-1–C-25 with four C$_1$ branches (C-30, C-31, C-32, and C-33) as that of amphidinolide N (3) and an isobutyl terminus for C-26–C-29. Therefore, the gross structure of I was concluded to be an analog of amphidinolide N (3) with an isobutyl side-chain as shown in Fig. 1.

The $^1$H- and $^{13}$C-NMR data (Table 2) in CDCl$_3$ and CD$_6$ were similar to those of I and 3. The $^{13}$C-NMR spectra of 2 disclosed the presence of a total of 33 carbons (one ketone carbonyl, one ester carbonyl, three quaternary carbons, one $sp^3$ methine, one $sp^2$ methylene, 12 $sp^2$ methines, 10 $sp^3$ methylenes, and four methylenes). ESI-MS of 2 in MeOH showed pseudo-molecular ion peaks [(M + Na$^+$)] at m/z 683 and 685 (approx. 3:1), indicating that I had a chlorine atom in the molecule, and the molecular formula of I was established as C$_{62}$H$_{53}$ClO$_{11}$ with seven degrees of unsaturation based on HR-ESI-MS data [m/z 683.3179 (M + Na$^+$), $\Delta$ +1.06 mmu]. Because four double bonds in the molecule accounted for four out of seven degrees of unsaturation, 2 was inferred to possess three rings, indicating that 2 has one ring less than 1 or 3. The deuterio-substituted (M + Na$^+$) peaks at m/z 689 and 691 observed for ESI-MS data in MeOH-d$_4$ suggested the presence of six hydroxyl groups in the molecule, one more hydroxyl group than 3. Detailed analysis of NMR data in CDCl$_3$ revealed that the C-1–C-3 and C-8–C-29 portions in 2 were the same as those in amphidinolide N (3) (Fig. 3). The presence of the resonances of C-4 ($\delta_C$ 3.77, $\delta_H$ 74.0, CDCl$_3$) and C-5 ($\delta_C$ 4.68, $\delta_H$ 56.7, CDCl$_3$) of 2 indicated that 2 lacked the epoxy ring at C-4–C-5 in 3. In the deuteration secondary isotope shifts of the $^{13}$C-NMR data measured in CD$_6$ (Table S2), the relatively large shift for C-4 ($\Delta\delta_C$ +0.07) was observed compared to that for C-5 ($\Delta\delta_C$ +0.02), suggesting that C-4 and C-5 were adjacent to a hydroxyl group and chlorine atom, respectively. Therefore, the gross structure of 2 was concluded to be the chlorohydrin form of the epoxide at C-4–C-5 of 3, as shown in Fig. 1.

Relative Stereochemistry of Chlorohydrin 2 To elucidate the relative stereochemistry of 13 chiral centers in these amphidinolide N type macrolides, we first demonstrated the conformation analysis of 2, without an allyl epoxide portion at C-4–C-6, based on NOESY data and scalar coupling constants in CDCl$_3$. The $^1$H–$^1$H coupling constants were estimated by analysis of the resolution-enhanced $^1$H-NMR spectrum, while the $^{2,3}J(C/H)$ values were obtained by employing the hetero half-filtered TOCSY (HETLOC) spectrum. The intensities of NOESY correlations were normalized with the integration value of the H-2/H$_2$-30 cross-peak as 100 (Table S3), and the correlations were categorized into dominant nuclear Overhauser effects (NOEs) or not. Bond rotation analyses assisted by J-based configuration analysis$^{20}$ indicated that the relative configurations for C-2–C-3, C-3–C-4, C-4–C-5, and C-24–C-25 bonds were erythro, threo, erythro, and threo relatives, respectively (Figs. 4a–4d). A couple of methine–methylenic portions in the C-7–C-8 and C-13–C-14 bonds were implied to possess bond rotations with anti-relations for
H-7–H-8a, 7-OH–H-8b, H-13a–14-OH, and H-13b–H-14, as shown in Figs. 4e and 4f.

Figure 5a illustrates the conformation of the C-1–C-13 portion, and the sequential four chiral centers from C-2 to C-5 of 2 were revealed to have \(2^S\), \(3^R\), \(4^S\), and \(5^R\) configurations from the bond rotation analyses described above. Considering the bond rotation for C-7–C-8 and NOESY correlations for H-5/H-2, the relative configuration at C-7 was deduced to be \(R^*\) through the exomethylene unit at C-6. The NOESY correlation was observed for H-8b/H-10 through the ketone carbonyl at C-9, implying a \(\beta\)-orientation and \(10^S\) configuration for the 32-methyl group. The anitperiplanar relationship for H-10–C-33 and H-11–C-13b was deduced from NOESY correlations for H-10/H-33 and H-11/H-13b, suggesting axial orientations for H-13b and the 33-methyl group.

For the C-12–C-26 portion of 2 (Fig. 5b), NOESY correlations for H-13a/H-16 and H-14/H-33 indicated that the hydroxyl group at C-14 was oriented toward the inside of the macrocyclic ring. The rather small \(J(H-16/H-17a)\) and \(J(H-16/H-17b)\) and relatively large \(J(H-17a/H-18a)\) and \(J(H-18a/H-19)\) values and the NOESY correlation for H-17a/H-19 disclosed the chair conformation of the tetrahydropyran ring at C-15–C-19 with axial orientations for 16-OH and H-19. An \(\alpha\)-axial orientation for 15-OH on the tetrahydropyran ring at C-15–C-19 was presumed, because it also seems to be favorable from the point of view of the anomeric effect. Molecular calculation studies of simple C-13–C-19 models with \(\alpha\)- and \(\beta\)-orientations for 15-OH revealed that the minimized energy for the model with an \(\alpha\)-hydroxyl group was lower than for that with a \(\beta\)-one.30) Thus, the stereochemistries of C-14, C-15, C-16, and C-19 was elucidated to be \(S^*\), \(S^*\), \(R^*\), and \(R^*\), respectively.

### Table 2. \(^1\)H- and \(^13\)C-NMR Data of Chlorohydrin 2 in CDCl\(_3\) and C\(_6\)D\(_6\)

| Position | CDCl\(_3\) | \(^1\)H | CDCl\(_3\) | \(^1\)H |
|----------|------------|--------|------------|--------|
| 1        | 174.2      | C      | 174.2      |        |
| 2        | 45.1       | CH     | 2.84       | dq, 8.5, 7.0 |
| 3        | 73.5       | CH     | 4.28       | brd, 8.5 |
| 4        | 74.0       | CH     | 3.77       | brd, 9.7 |
| 5        | 57.6       | CH     | 4.68       | d, 9.7  |
| 6        | 149.5      | C      |            |        |
| 7        | 72.2       | CH     | 4.71       | dd, 3.2, 9.5 |
| 8        | 45.4       | CH\(_2\) | 3.09     | dd, 17.3, 9.5 |
| 9        | 212.3      | C      |            |        |
| 10       | 47.3       | CH     | 3.36       | dq, 9.4, 7.0 |
| 11       | 126.7      | CH     | 5.03       | brd, 9.4 |
| 12       | 136.5      | C      |            |        |
| 13       | 39.6       | CH\(_2\) | 2.34     | dd, 13.6, 3.1 |
| 14       | 71.3       | CH     | 4.03       | dd, 9.5, 3.1 |
| 15       | 97.8       | C      |            |        |
| 16       | 65.6       | CH     | 3.64       | t, 2.5  |
| 17       | 27.1       | CH\(_2\) | 2.17     | m       |
| 18       | 25.1       | CH\(_2\) | 1.56     | m       |
| 19       | 66.1       | CH     | 4.14       | m       |
| 20       | 41.5       | CH\(_2\) | 1.61\(^a\) | m       |
| 21       | 74.8       | CH     | 4.23       | m       |
| 22       | 32.1       | CH\(_2\) | 2.05     | m       |
| 23       | 27.6       | CH\(_2\) | 2.01     | m       |
| 24       | 80.2       | CH     | 4.02       | m       |
| 25       | 75.3       | CH     | 4.81       | m       |
| 26       | 30.6       | CH\(_2\) | 1.55\(^a\) | m       |
| 27       | 27.3       | CH\(_2\) | 1.30\(^a\) | m       |
| 28       | 22.5       | CH\(_2\) | 1.31\(^a\) | m       |
| 29       | 13.9       | CH\(_2\) | 0.88\(^b\) | t, 7.0  |
| 30       | 14.0       | CH\(_2\) | 1.24\(^b\) | d, 7.0  |
| 31       | 117.7      | CH\(_2\) | 5.49     | s       |
| 32       | 15.5       | CH\(_2\) | 1.13\(^b\) | d, 7.0  |
| 33       | 16.7       | CH\(_2\) | 1.78\(^b\) | brs     |

\(^a\) 2H. \(^b\) 3H.
The relative stereochemistry between C-19 and C-21 was considered from an equivalent signal pattern of H-20 $\delta_{\text{H}}$ 1.61 (2H) in CDCl$_3$ and 1.44 (2H) in C$_6$D$_6$, which might be due to the chemical equivalence of these protons; H-20$\beta$ was gauche-oriented against H-19 and 21-O and anti-oriented against H-21 and 19-O, while H-20$\alpha$ was anti-oriented against H-19 and 21-O and gauche-oriented against H-21 and 19-O. The rotating frame nuclear Overhauser effect spectroscopy (ROESY) correlations for H-18$\beta$/H (2)-20 and H (2)-20/H-22$\alpha$ observed in CDCl$_3$ also supported this stereostructure. The NOESY correlation for H-21/H-25 implied an anti-relationship for H-21–H-24 of the tetrahydropyran ring at C-21–C-24. Considering the bond rotation for C-24–C-25, the stereochemistries of C-21, C-24, and C-25 were all concluded to be R$\ast$. Therefore, the total relative configuration of 2 was concluded to be 2S$\ast$, 3R$\ast$, 4S$\ast$, 5R$\ast$, 7R$\ast$, 10S$\ast$, 14S$\ast$, 15S$\ast$, 16R$\ast$, 19R$\ast$, 21R$\ast$, 24R$\ast$, and 25R$\ast$, as shown in Fig. 1.

Relative Stereochemistry of Isocaribenolide-I (1) and Amphidinolide N (3)

Elucidating the stereostructure of isocaribenolide-I (1) and amphidinolide N (3) with an allyl epoxide may have a higher degree of difficulty than elucidating that of 2, owing to the conformation change described below. We performed the conformation analysis of 1 by applying the similar data analysis method described above. Magnitudes or values for $J$(H/H) needed for stereochemical assignments were obtained from resolution-enhanced 1H-NMR spectra and selective population transfer (SPT)$^{31}$ experiments. The intensities of NOESY correlations were normalized with the integration value of H-2/H 3-30 cross-peak as $\pm$100. Black arrows show correlations with intensities of 40 or more, while gray ones show correlations with intensities less than 40.

The relative stereochemistry between C-19 and C-21 was considered from an equivalent signal pattern of H-20 $\delta_{\text{H}}$ 1.61 (2H) in CDCl$_3$ and 1.44 (2H) in C$_6$D$_6$, which might be due to the chemical equivalence of these protons; H-20$\beta$ was gauche-oriented against H-19 and 21-O and anti-oriented against H-21 and 19-O, while H-20$\alpha$ was anti-oriented against H-19 and 21-O and gauche-oriented against H-21 and 19-O. The rotating frame nuclear Overhauser effect spectroscopy (ROESY) correlations for H-18$\beta$/H (2)-20 and H (2)-20/H-22$\alpha$ observed in CDCl$_3$ also supported this stereostructure. The NOESY correlation for H-21/H-25 implied an anti-relationship for H-21–H-24 of the tetrahydropyran ring at C-21–C-24. Considering the bond rotation for C-24–C-25, the stereochemistries of C-21, C-24, and C-25 were all concluded to be R$\ast$. Therefore, the total relative configuration of 2 was concluded to be 2S$\ast$, 3R$\ast$, 4S$\ast$, 5R$\ast$, 7R$\ast$, 10S$\ast$, 14S$\ast$, 15S$\ast$, 16R$\ast$, 19R$\ast$, 21R$\ast$, 24R$\ast$, and 25R$\ast$, as shown in Fig. 1.

Amphidinolide N (3) Elucidating the stereostructure of isocaribenolide-I (1) and amphidinolide N (3) with an allyl epoxide may have a higher degree of difficulty than elucidating that of 2, owing to the conformation change described below. We performed the conformation analysis of 1 by applying the similar data analysis method described above. Magnitudes or values for $J$(H/H) needed for stereochemical assignments were obtained from resolution-enhanced 1H-NMR spectra and selective population transfer (SPT)$^{31}$ experiments. The intensities of NOESY correlations were normalized with the integration value of H-2/H 3-30 cross-peak as $\pm$100. Black arrows show correlations with intensities of 40 or more, while gray ones show correlations with intensities less than 40.

All of the J-values and NOESY correlations were recorded in CDCl$_3$. The intensities of NOESY correlations were normalized with the integration value of H-2/H 3-30 cross-peak as $\pm$100. Black arrows show correlations with intensities of 40 or more, while gray ones show correlations with intensities less than 40. Dashed arrows show NOESY correlations. $^1$H–H coupling constants (Hz) (H/H): 8.5 (2/3), <1 (3/4), 9.7 (4/5), 9.5 (7/8a), 3.2 (7/8b), 9.4 (10/11), 3.1 (13a/14), 9.5 (13b/14), 2.5 (16/17a), 2.5 (16/17b), >8 (17a/18a), <3 (17b/18a), >8 (18a/19), <3 (18b/19), and 8.4 (2/25).

Amphidinolide N (3) Elucidating the stereostructure of isocaribenolide-I (1) and amphidinolide N (3) with an allyl epoxide may have a higher degree of difficulty than elucidating that of 2, owing to the conformation change described below. We performed the conformation analysis of 1 by applying the similar data analysis method described above. Magnitudes or values for $J$(H/H) needed for stereochemical assignments were obtained from resolution-enhanced 1H-NMR spectra and selective population transfer (SPT)$^{31}$ experiments. The intensities of NOESY correlations were normalized with the integration value of H-2/H 3-30 cross-peak as $\pm$100. Black arrows show correlations with intensities of 40 or more, while gray ones show correlations with intensities less than 40.

All of the J-values and NOESY correlations were recorded in CDCl$_3$. The intensities of NOESY correlations were normalized with the integration value of H-2/H 3-30 cross-peak as $\pm$100. Black arrows show correlations with intensities of 40 or more, while gray ones show correlations with intensities less than 40. Dashed arrows show NOESY correlations. $^1$H–H coupling constants (Hz) (H/H): 8.5 (2/3), <1 (3/4), 9.7 (4/5), 9.5 (7/8a), 3.2 (7/8b), 9.4 (10/11), 3.1 (13a/14), 9.5 (13b/14), 2.5 (16/17a), 2.5 (16/17b), >8 (17a/18a), <3 (17b/18a), >8 (18a/19), <3 (18b/19), and 8.4 (2/25).
Isocaribenolide-I (1) was deduced to be different from that for 2. Both intermediate values for \( \Delta J(H-2/H-3) \) (7.0 Hz) and \( \Delta J(H-3/H-4) \) (4.8 Hz) suggest that the C-2–C-3 and C-3–C-4 bonds are rotating. The former corresponds to an average value of the coupling constant when \( \text{anti} \) (approx. 9 Hz) and \( \text{gauche} \) (approx. 3 Hz) relations for H-2–H-3 are 2:1, while the latter value is suitable for the average at a 1:2 ratio of \( \text{anti} \) and \( \text{gauche} \)-relations for H-3–H-4. The normalized integration values of dominant NOESY correlations (Table S4) showed incredible pairs of NOESY correlations, such as H-4/H-2 (inversion value: 18.40) and H-5/H-2 (21.64) and H-5/H-7 (18.07), and H-4/H-13 (14.12) and H-5/H-30 (10.43), around the \( \text{trans} \) epoxide conjugated with an exomethylene. Considering the bond rotations associated with NOESY correlations, three plausible conformations could be proposed for the C-1–C-9 portion of 1 (Fig. 7). In conformation A with H-2–H-3 \( \text{anti} \) and H-3–H-4 \( \text{gauche} \)-relations, H-4 and H-13,30, via five bonds, came close enough to give rise to the corresponding NOESY correlation, while H-5 and H-13,30, via six bonds, drew together in conformation B with double \( \text{anti} \)-interactions for H-2–H-3 and H-3–H-4. The NOESY correlation for H-2/H-5 may occur in conformation B, and the NOESY correlation for H-2/H-5 may be seen for conformation C with double \( \text{gauche} \) interactions between H-2/H-3 and H-3/H-4. In conformations A/C and B, H-7 closes with H-4 and H-5, respectively. This coexistence of NOESY correlations for H-4/H-7 and H-5/H-7 suggests the presence of a major conformation change, plausibly derived from a flip of the \( \text{trans} \) epoxide. Therefore, the relative stereochemistry of 1 is suggested as \( 2^\ast, 3^R, 4^5^R, 5^5^R, 7^R^* \), \( 10^S^*, 14^5^R^*, 15^5^S^*, 16^R^*, 19^R^*, 21^R^*, 24^R^*, \) and \( 25^R^* \), which is not inconsistent with the relative configurations predicted by the structural correlation between isocaribenolide-I (1) and 2.

Because amphinadinolide N (3) was isolated at the same time as chlorohydrin 2, we assumed that 3 may be structurally and biosynthetically related to 2. The structural relationship between 3 and the corresponding chlorohydrin 2 indicates that 2 may be generated from 3 through the nucleophilic addition of a chloride anion from behind the C-5 epoxy carbon and cleavage of the O–C-5 bond. Considering the stereochemical inversion at C-5, the relative stereochemistry of 3 is presumed to be the same as that of 1. To clarify the stereochemistry of 3, the \( ^{13} \)C chemical shifts of isocaribenolide-I (1) in \( \text{C}_6\text{D}_6 \) were compared with those of 3, because the difference in planar structures between 3 and 1 is only a side-chain. Figure 8a shows the values of \( ^{13} \)C chemical shift difference between 1 and 3 (\( \Delta \delta \) in ppm) = \( \delta (\text{in } \text{C}_6\text{D}_6) - \delta (\text{in } \text{C}_6\text{D}_6) \) for the macrocyclic portion of C-1–C-25 with four C-1 branches (C-30, C-31, C-32, and C-33). Relatively large \( \Delta \delta \) values were observed for C-2 (–0.5 ppm), C-24 (+0.4 ppm), and C-25 (–1.8 ppm), probably due to the neighboring side-chain structural differences. The absolute \( \Delta \delta \) values for the residual carbons were extremely small (\( \leq 0.3 \) ppm), strongly indicating that the relative stereochemistry of 11 chiral carbons for C-2, C-3, C-4, C-5, C-7, C-10, C-14, C-15, C-16, C-19, and C-21 in 3 were common to those in 1 at least. In contrast, Fig. 8b describes the \( ^{13} \)C chemical shift differences between 2 and 3 (\( \Delta \delta \) in ppm) = \( \delta (\text{in } \text{C}_6\text{D}_6) - \delta (\text{in } \text{C}_6\text{D}_6) \) for the C-1–C-29 portion with four C-1 branches (C-30, C-31, C-32, and C-33). The absolute \( \Delta \delta \) values for C-21–C-29 were so small (\( \leq 0.2 \) ppm), suggesting that the relative stereochemistries of C-21, C-24, and C-25 were the same as those of 2. Interestingly, the relatively large \( \Delta \delta \) values were observed for C-6 (+2.7 ppm), C-7 (+2.3 ppm), C-9 (+1.0 ppm), C-31 (+6.4 ppm), and C-33 (+1.2 ppm), which might be derived from a deshielding effect, probably due to the neighboring electron-rich chloride atom at C-5. Therefore, the relative stereochemistry of 3 was concluded to be

---

**Fig. 6.** Stereostructures for C-6–C-26 Portion in Isocaribenolide-I (1)

All of the \( J \)-values and NOESY correlations were recorded in \( \text{C}_6\text{D}_6 \). The intensities of NOESY correlations were normalized with the integration value of H-2/H-13,30 cross-peak as \( +100 \). Black arrows show correlations with intensities of 20 or more, while gray ones show those with intensities of less than 20. \( ^1\text{H}–^1\text{H} \) coupling constants (Hz) (H/H): 10.4 (7/8a), 2.2 (7/8b), 10.6 (10/11), 3.6 (13a/14), 9.6 (13b/14), 2.5 (16/17a), 2.5 (16/17b), <3 (17a/18a), <3 (17b/18a), >8 (18a/19), >8 (18a/19), and 8.2 (24/25).

**Fig. 7.** Three Plausible Conformations for C-1–C-9 Portion in Isocaribenolide-I (1); Conformations A: \textit{Anti}– and \textit{Gauche}–, B: Both \textit{Anti}–, and C: Both \textit{Gauche}–Relations for H-2–H-3 and H-3–H-4

All of the \( J \)-values and NOESY correlations were recorded in \( \text{C}_6\text{D}_6 \). The means of black and gray arrows are the same as described in Fig. 7. \( ^1\text{H}–^1\text{H} \) coupling constants (Hz) (H/H): 7.0 (2/3), 4.8 (3/4), and 2.0 (4/5).
2S*, 3R*, 4S*, 5S*, 7R*, 10S*, 14S*, 15S*, 16R*, 19R*, 21R*, 24R*, and 25R*. Nevertheless, the absolute stereochemistry of 1–3 remains undetermined because the α-methoxy-α-(trifluoromethyl)-phenylacetyl (MTPA) esters and degradation product were not prepared.

In this study, we isolated three 26-membered macrolides from marine dinoflagellate Amphidinium species and elucidated the structures of two new compounds, isocaribenolide-I (1) and compound 2 together with known amphidinolide N (3). Isocaribenolide-I (1) is an analog of 3 with an isobutyl side-chain. Compound 2 is a chlorohydrin form of 3, and it may be an artifact generated in its extraction and/or separation. Moreover, we concluded that amphidinolide N (3) and calibenolide-I are the same substance, and proposed the relative stereochemistry of 3 on the basis of the NMR data for the chlorohydrin form (2) of 3. Compounds 1, 2, and 3 exhibited extremely potent cytotoxic activity against human cervix adenocarcinoma HeLa cells (IC50: 0.02, 0.06, and 0.01 nM, respectively). The cytotoxicity of compound 2 was less potent than that of 1 and 3, suggesting that the presence of the epoxide ring in 2 may be essential for the powerful cytotoxicity of this macrolide.

**Experimental**

**General Experimental Procedures** Optical rotation and IR data were measured on a JASCO DIP-370 polarimeter and a JASCO FT/IR-5300 spectrophotometer, respectively. NMR data were recorded using 2.5 mm microcells (Shigemi Co., Ltd., Japan). NMR spectra were measured on a Bruker AMX-500 spectrometer or Varian-NMR500 spectrometer equipped with a triple resonance PFG cold probe or CH “Xsens” PFG cold probe. Chemical shifts were reported in ppm with reference to the residual proton and carbon signals of C6D6 (δH 7.20 and δC 128.0, respectively) and CDCl3 (δH 7.26 and δC 77.0, respectively). NMR data reconstruction and analysis were performed with Mnova NMR 7.0.3 (Mestrelab Research, Spain). The ESI-MS spectra of 1 and 3 were measured on a ThermoFisher Exacta spectrometer, and that of 2 was recorded on a JEOL JMS-T100LC spectrometer.

**Extraction and Isolation** One dinoflagellate Amphidinium species (strain number KCA09053) was separated monoclonaually from benthic sea sands collected off Iriomote Island, Japan. Another dinoflagellate Amphidinium species (strain KCA09056) was monoclonaually separated from the surface of a seagrass collected off Iriomote Island, Japan. Information on the biomaterials and cultivation are described in previous papers. Dried algal cells (at a total weight of 10.3 g) of the KCA09053 strain obtained from 150 L of the medium were extracted with MeOH/toluene (3:1) and partitioned between toluene and water. The toluene-soluble materials (1.15 g) of the
extract were subjected to SiO\textsubscript{2} gel CC using a stepwise elution with CHCl\textsubscript{3} (200 mL) and CHCl\textsubscript{3}/MeOH (98:2). The fraction eluted with CHCl\textsubscript{3}/MeOH, 98:2 was chromatographed successively by using an ODS [Cosmosil 140C\textsubscript{8}-PREP, Nacalai Tesque Inc., Japan; eluent: acetonitrile (CH\textsubscript{3}CN)/H\textsubscript{2}O, 7:3] to afford compounds 1, 0.006%, retention time (\(t_R\)) 8.6 min).

The dried algell cells (40.95g) of the KCA09056 strain obtained from 250L of the culture were extracted with MeOH/toluene (3:1). The toluene-soluble materials (15.28g) of the extract were subjected to silica gel CC using a gradient elution of 0–2% MeOH in CHCl\textsubscript{3}.

Cytotoxicity was performed at 37 °C in 5% carbon dioxide, at a density of 5000 cells per well in 96-well plates, using Dulbecco’s modified eagle medium containing 10% fetal calf serum. The viability of the treated groups was estimated as a percentage of that of the control groups. The cytotoxicity is expressed as IC\textsubscript{50}.

Acknowledgments We thank Ai Tokumitsu and Seiko Oka of the Equipment Management Center, Hokkaido University, for the measurement of MS spectra, and Satoru Ibuki, Keita Ikede, and Takahiro Tsushima of the Kochi Prefectural Deep Seawater Laboratory for the supply of deep seawater and assistance with dinoflagellate cultivation. This study was partially supported by a Grant-in-Aid for Scientific Research (No 26670045 to M.T.) from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan.

Conflict of Interest The authors declare no conflicts of interest.

Supplementary Materials The online version of this article contains supplementary materials. The spectral data of compounds 1–3 and Tables S1–S4 are available as supplementary materials.

References and Notes
1) Carroll A. R., Copp B. R., Davis R. A., Keyzers R. A., Prinsep M. R., Nat. Prod. Rep., 37, 175–223 (2020).
2) Kobayashi J., Tsuda M., Nat. Prod. Rep., 21, 77–93 (2004).
3) Kobayashi J., Kubota T., J. Nat. Prod., 70, 451–460 (2007).
4) Ishibashi M., Yamaguchi N., Sasaki T., Kobayashi J., J. Chem. Soc. Chem. Commun., 1455–1456 (1994).
5) Bauer I., Maranda L., Young K. A., Shimizu Y., Fairchild C., Correll L., MacBeth J., Huang S., J. Org. Chem., 60, 1084–1086 (1995).
6) Maranda L., Shimizu Y., J. Physiol., 32, 873–879 (1996).
7) Murray S., Jørgensen M. F., Daugaeb J., Rhodes L., J. Physiol., 40, 366–382 (2004).
8) Takahashi Y., Kubota T., Imachi M., Wälchli M. R., Kobayashi J., J. Antibiot., 66, 277–279 (2013).
9) Nicolaou K. C., Brenzovich W. E., Bulger P. G., Francis T. M., Org. Biomol. Chem., 4, 2199–2157 (2006).
10) Nicolaou K. C., Bulger P. G., Brenzovich W. E., Org. Biomol. Chem., 4, 2158–2183 (2006).
11) Ochiai K., Kuppusamy S., Yasui Y., Okano T., Matsumoto Y., Gupta N. R., Takahashi Y., Kubota T., Kobayashi J., Hayashi Y., Chem. Eur. J., 22, 5282–5286 (2016).
12) Ochiai K., Kuppusamy S., Yasui Y., Harada K., Gupta N. R., Takahashi Y., Kubota T., Kobayashi J., Hayashi Y., Chem. Eur. J., 22, 5287–5291 (2016).
13) Seck M., Franck X., Seon-Meniel B., Hoqueeniller R., Figadère B., Tetrahedron Lett., 47, 4175–4180 (2006).
14) Jolicoeur Y., Franck X., Seon-Meniel B., Hoqueeniller R., Figadère B., Tetrahedron Lett., 47, 5905–5908 (2006).
15) Trost B. M., J. Org. Chem., 74, 5632–5635 (2012).
16) Kawashima Y., Toyoshima A., Fuwa H., Sasaki M., Org. Lett., 14, 2232–2235 (2016).
17) Toyoshima A., Sasaki M., Tetrahedron Lett., 57, 3532–3535 (2016).
18) Trost B. M., Bai W.-J., Stivala C. E., Hohn C., Pooceck C., Heinrich M., Xu S., J. Am. Chem. Soc., 140, 17316–17326 (2018).
19) Kumagai K., Tsuda M., Fukushima E., Kawabata J., Masuda A., Tsuda M., J. Nat. Med., 71, 506–512 (2017).
20) Tsuda M., Makihara R., Tsuda M., Suzuki T., Chem. Pharm. Bull., 68, 864–867 (2020).
21) Tsuda M., Kumagai K., Akakabe M., Tominga A., Konishi Y., Tsuda M., Japan Patent, 1, 2011–184437 (2011).
22) Compound 2 is identical to the compound previously named neocarboxinone-I: Kumagai K., Akakabe M., Miinama M., Nishikawa T., Tsuda M., Konishi Y., Tsuda M., Tominga A., Symposium papers for 53rd Symposium on the Chemistry of Natural Products, Osaka, 53, 359–354 (2011).
23) Compounds 2 and 3 were assigned as stereoisomers of C-7, C-10, C-14, C-15, C-16, C-19, C-21, C-24, and C-25 in Doctor’s thesis of...
M. Minamida Kochi University (2015).

24) Akakabe M., Kumagai K., Tsuda M., Konishi Y., Tominaga A.,
Tsuda M., Fukushi E., Kawabata J., *Tetrahedron*, 70, 2962–2965 (2014).

25) Akakabe M., Kumagai K., Tsuda M., Konishi Y., Tominaga A.,
Kaneno D., Fukushi E., Kawabata J., Masuda A., Tsuda M., *Chem.
Pharm. Bull.*, 64, 1019–1023 (2016).

26) Akakabe M., Kumagai K., Tsuda M., Konishi Y., Tominaga A.,
Tsuda M., Fukushi E., Kawabata J., *Tetrahedron Lett.*, 55, 3491–
3494 (2014).

27) Kanto M., Sato S., Tsuda M., Sasaki M., *J. Org. Chem.*, 81, 9105–
9121 (2016).

28) Tsuda M., Makihara R., Minamida M., Tsuda M., Akakabe M.,
Kumagai K., Fukushi E., Kawabata J., Suzuki T., *Heterocycles*,
1678–1685 (2020).

29) Matsumori N., Kaneno D., Murata M., Nakamura H., Tachibana K.,
*J. Org. Chem.*, 64, 866–876 (1999).

30) Minimum energies of the C-13–C-19 models with $\alpha$- and
$\beta$-orientations for 15-OH were calculated as 59.02 and 70.05 kJ/mol,
respectively, by applying the MM2 minimization in the program
Chem 3D (Chembridge Soft).

31) Hoffman R. A., Forsén S., *Prog. Nucl. Magn. Reson. Spectrosc.*, 1,
15–204 (1966).