Structure–Activity Relationship Study on Odoamide: Insights into the Bioactivities of Aurilide-Family Hybrid Peptide–Polyketides

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Supporting Information

ABSTRACT: Odoamide is a cytotoxic peptide–polyketide hybrid molecule isolated from the Okinawan marine cyanobacterium *Okeania* sp. For an efficient structure–activity relationship study of the peptide part of odoamide, a facile synthetic protocol was established using a solid-phase peptide synthesis. Among a series of peptides, the D-MeAla6 isomer exhibited a more potent cytotoxicity than natural odoamide. It was also demonstrated that the 26-membered macrocyclic natural odoamide and the 24-membered isomer with comparable cytotoxicities were slowly interconvertible, and both isomers contributed to the potent cytotoxicity of odoamide. Examination of the physicochemical properties revealed that the in vitro cytotoxicity was affected by the serum protein binding of odoamide derivatives, while the differences in the macrocyclic structures had no significant effect on the membrane permeability.

KEYWORDS: 1,3-Acyl transfer, cyclic depsipeptide, membrane permeability, odoamide, serum protein binding

Odoamide (**1a**) is a cyclic depsipeptide isolated from the Okinawan marine cyanobacterium *Okeania* sp. and exhibits highly potent cytotoxic activity against HeLa S₃ cell lines (Figure 1).¹ Recently, we reported the identification and characterization of odoamide through NMR analysis, chemical degradation, and total synthesis.^{1,2} Odoamide, with a 26-membered macrocycle scaffold, belongs to the aurilide-class of depsipeptides, which show potent cytotoxicities.^{3–7} Although aurilide-class depsipeptides share similar peptide and polyketide substructures, individual peptides have unique structural motifs. Aurilide (**2**) was the first depsipeptide discovered in this class and was isolated from the sea hare *Dolabella auricularia*.³ The configurations of the chiral centers in odoamide **1a** are identical to those of **2**, with three of the amino acids having different side-chains. Kulokekahlide-2 (**3**), which comprises similar amino acid components to odoamide **1a**, contains a D-Ala at the L-MeAla6 position of **1a**.⁴ Lagunamide C (**4**)⁶ and palau'amide (**5**)⁷ have 27-membered and 24-membered macrocycles, respectively. Twenty-four-membered cyclic peptides such as palau'amide **5** have shown highly potent cytotoxicities, despite the apparently alternative global conformations.^{7–9}

There have been several structure–activity relationship (SAR) studies of aurilide-class depsipeptides for the design of molecular probes for target identification, as well as to determine the absolute stereochemistry of the natural products.^{10–12} In these studies, the natural isomers were more cytotoxic compared with a number of depsipeptide epimers. A possible intramolecular ester exchange between the polyketide 5- and 7-hydroxy groups was also investigated in kulokekahlide-2 (**3**). Twenty-six-membered macrocycle (**3**) and the palau'amide-type 24-membered isomer were interconverted using an acyl transfer reaction in a low polarity

solvent such as CD₂Cl₂; no transformation was observed in polar solvents.¹³ The transformation of **3** and the derivatives was dependent on the presence of D-Ala6 and appropriate conformations.⁹ However, the stability of a series of peptides in aqueous media was not assessed because of their low solubility.⁹ We expected that an improved detection system would facilitate the evaluation of the postulated acyl transfers between two possible odoamide isomers (**1a** and **6**) under bioassay conditions (Figure 1). Based on the previous findings of aurilide-class depsipeptides, we investigated the structure–activity relationship of the cytotoxicities and physicochemical properties of odoamide derivatives, including serum protein binding and membrane permeability.

Recently, we achieved the total synthesis of odoamide using a convergent approach via conjugation of a tetrapeptide fragment with a polyketide portion.² The key macrocyclization between the D-allo-isoleucic acid and the L-Ala of the linear precursor proceeded smoothly; however, significant epimerization at the α-position of Ile was observed during the preparation of the linear precursor (see Supporting Information). Additionally, preparation of a variety of tetrapeptide fragments in advance was required for the diversification of the peptide substructure in odoamide. To overcome these disadvantages for the SAR study of odoamide, we initially set out to establish an efficient synthetic route for the late-stage derivatizations of odoamide analogues.

The synthesis began with key alcohol intermediate **7**² in which the D-allo-isoleucic acid (D-allo-Hila) and polyketide 5-

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Figure 1. Structures of odoamide (**1a**) and aurilide-class depsipeptides.

hydroxy group were protected with phenacyl (Pac) and methylthiomethyl (MTM) groups, respectively (Scheme 1).¹⁴ Acylation of the polyketide 7-hydroxy group in **7** with Fmoc-MeAla-Cl gave a fully protected amine **8**. Deprotection of the phenacyl ester in **8** followed by loading the resulting carboxylic acid onto Cl-(2-Cl)Trt resin provided resin **9**, which was amenable to solid-phase peptide synthesis. After deprotection of the 9-fluorenylmethoxycarbonyl (Fmoc) group in **9**, Fmoc-Ile-OH was used in the coupling reaction on resin **10**. However, significant elimination of a diketopiperazine consisting of MeAla and Ile dipeptide was observed during the subsequent piperidine treatment for removal of the Fmoc group.^{15,16} To avoid the simultaneous formation of the diketopiperazine under the basic conditions, allyloxycarbonyl (Alloc) protection was chosen for the Ile, as it can be removed under neutral conditions.¹⁷ After the coupling of Alloc-Ile-OH with amine **10**, the Alloc group was removed by treatment with Pd(PPh₃)₄ and PhSiH₃. The sequential coupling of Fmoc amino acids followed by cleavage from the resin provided the linear precursor **11**. Macrocyclization of **11** with HATU–HOAt–collidine using a reported protocol² gave odoamide **1a**. A series of odoamide analogues (**1b**, **1c**, **1e**, **6**, and **12–17**) were also synthesized using this protocol (Figures 2–4, see Supporting Information).

The cytotoxicities of the resulting odoamide analogues against A549 cells were evaluated using an MTS assay. The cultured cells were exposed to each peptide for 72 h. Initially, we investigated odoamide epimers **1b–e**, which exhibited different global conformations of the macrocycles compared with that of natural **1a** (Figure 2). D-Ala2 epimer **1b** and D-allo-Ile5 epimer **1d** showed significantly less potent cytotoxicities (IC₅₀ **1b** = 1.55 μM; IC₅₀ **1d** = 645 nM). Substitution of D-

Scheme 1. Synthesis of Odoamide (**1a**) via Solid-Phase Peptide Synthesis^a

^aReagents and conditions: (a) Fmoc-L-MeAla-Cl, (*i*-Pr)₂NEt, 1,2-dichloroethane, 40 °C; (b) Zn, AcOH, rt; (c) Cl-(2-Cl)Trt resin, (*i*-Pr)₂NEt, CH₂Cl₂, rt; (d) 20% piperidine/DMF, rt; (e) Alloc-L-Ile-OH, *N,N'*-diisopropylcarbodiimide (DIC), HOAt, DMF, rt; (f) Pd(PPh₃)₄, PhSiH₃, CH₂Cl₂, rt; (g) Fmoc-Sar-OH, HOBt·H₂O, DIC, DMF, rt; (h) Fmoc-D-MePhe-OH, HATU, (*i*-Pr)₂NEt, DMF, rt; (i) Fmoc-L-Ala-OH, HATU, (*i*-Pr)₂NEt, DMF, rt; (j) 30% 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP)/CH₂Cl₂, rt; (k) HATU, HOAt, collidine, DMF, rt; (l) AgNO₃, 2,6-lutidine, THF, H₂O, rt to 70 °C. Abbreviations: MTM, methylthiomethyl; Pac, phenacyl; Sar, sarcosine.

MePhe3 with L-MePhe3 also led to a 10-fold decrease in the bioactivity (IC₅₀ **1c** = 48 nM). Significant decrease or loss of the cytotoxicity by the stereoinversion at these positions in kulokekahilide-2 and aurilide have been previously reported,^{8,9,11} indicating that L-amino acids at positions 2 and 5 and a D-amino acid at position 3 are needed for potent bioactivity of the aurilide family peptides. In contrast, D-MeAla6 epimer **1e** exhibited slightly more potent bioactivity (IC₅₀ = 1.9 nM) compared with odoamide **1a**. Interestingly, the arrangement of the stereochemical configurations in **1e** are identical to that of kulokekahilide-2 (**3**), which has an unmethylated D-Ala at the L-MeAla6 position of **1a**. In a previous SAR study of kulokelahilide-2 derivatives, natural kulokekahilide-2 (**3**) had better cytotoxic profiles against a number of cell lines than the L-Ala6 epimer.⁹ This implies that the D-configuration at the MeAla6 position, regardless of the presence or absence of the N-methyl group, gives the appropriate global conformation for the potent cytotoxicities of aurilide family peptides.

Next, we investigated the intramolecular ester exchange at the polyketide 1,3-diol unit in odoamide **1a** under aqueous conditions. In previous research,¹³ organic solvents such as CH₂Cl₂ and DMSO were used to dissolve the peptides at high concentrations, which was necessary for detection by UV absorbance but are not physiological conditions. We chose liquid chromatography–mass spectrometry (LC–MS) for quantitative analysis to analyze the interconversion between 26- and 24-membered isomers in a range of concentrations of biological evaluations without any pretreatment. When natural **1a** (300 nM) was incubated in Dulbecco's modified Eagle's

Figure 2. Structures and cytotoxicities of odoamide derivatives with an amino acid epimer. IC_{50} values are the concentrations for 50% growth inhibition of A549 cells ($n = 3$).

medium (DMEM) at 37 °C for 72 h, 31% of **1a** was converted into 24-membered isomer **6** (see Supporting Information).¹⁸ Conversely, 26% of **6** (300 nM) was subjected to ring-expansion to provide 26-membered **1a** under the same condition. These results suggest that odoamide is in a slow equilibrium between 26-membered form **1a** and 24-membered form **6**. The 24-membered macrocycle **6** exhibited highly potent cytotoxicity ($IC_{50} = 4.5$ nM) by 72 h exposure to A549 cells (Figure 3), which correlated with the results for palau'amide (**5**) and the 24-membered analog of kulokekahilide-2.^{7,8} It is unlikely that the bioactivity of the 24-membered peptide **6** was solely derived from natural **1a** generated by acyl transfer of **6** because the potency difference between peptides **1a** and **6** was minimal. Indeed, calculated from their potencies and conversion yields after 72 h incubation, 24-membered **6** itself (without acyl transfer) displays a cytotoxic effect with an IC_{50} values of <4.7 nM. Therefore, we concluded that the natural 26-membered form **1a** and 24-membered isomer **6** both contribute to the potent cytotoxicity of odoamide.¹⁹

Two methoxy derivatives **12** and **13** were synthesized, which would prevent the polyketide component in **1a** and **6** from undergoing acyl transfer reactions. The 26-membered macrocycle **12** retained the cytotoxic activity of odoamide **1a** ($IC_{50} = 4.3$ nM), while the 24-membered isomer **13** showed significantly less potent activity ($IC_{50} = 3.38 \mu M$). These results suggest that the 5-hydroxy group of the polyketide component in odoamide **1a** is not essential for its bioactivity and that the 7-hydroxy group in **6** was essential. Given the acyl transfer toward the 7-hydroxy group is not needed for the

Figure 3. Structures and cytotoxicities of 26- and 24-membered macrocyclic odoamide derivatives. IC_{50} values are the concentrations for 50% growth inhibition of A549 cells ($n = 3$).

cytotoxicity of 24-membered macrocycle **6** as discussed above, this hydroxy group may have an important role for increasing membrane permeability and/or interaction with the target molecule(s). The polyketide 7-methoxy group of **13** would be unfavorable for the bioactivity due to the loss of the essential hydrogen bond and/or the increased steric hindrance.

Several other odoamide derivatives with alternative macrocyclic scaffolds were designed and synthesized (Figure 4). Peptide **14** without the polyketide 5-hydroxy group was prepared using a conjugated diene byproduct, which was obtained during the synthesis of the common polyketide intermediate (see Supporting Information). The slightly lower potency of **14** ($IC_{50} = 28$ nM) suggested that the 26-membered form **1a** was a bioactive structure even without acyl transfer

Figure 4. Structures and cytotoxicities of odoamide derivatives with modified macrocyclic scaffolds. IC_{50} values are the concentrations for 50% growth inhibition of A549 cells ($n = 3$).

toward the 24-membered isomer **6**, although the planarity at the polyketide 4,5-position was less favorable compared with the parent structure in **1a**. Substitution of sarcosine (Sar) with *N*-methyl- β -alanine (**15**) and glycine (**16**) also led to a slight decrease in the cytotoxicity (IC_{50} **15** = 74 nM; IC_{50} **16** = 31 nM). Subtle conformational changes by ring expansion to a 27-membered macrocycle (**15**) and loss of *N*-methyl group (**16**) did not improve the bioactivity of **1a**. Peptide **17**, with a *D*-Phe3 substituent, also gave a high cytotoxicity (IC_{50} = 5.4 nM). Even though the opposite chirality at this position in **1c** resulted in a decrease of the bioactivity, the loss of the *N*-methyl group at the same position in **17** was tolerable, suggesting that this *N*-methyl group in **1a** would have no effect on the global conformations and thereby the bioactivity.

To rationalize the structure–activity relationships of the odoamide derivatives, we evaluated the physicochemical properties under in vitro bioassay conditions (Figure 5 and

Figure 5. Relationships between physicochemical properties and cytotoxicity. (A) Scatter plot between ratio of unbound peptide and cytotoxicity IC_{50} . (B) Scatter plot between normalized IC_{50} and membrane permeability. ^a IC_{50} values are the concentrations for 50% growth inhibition of A549 cells. ^bRatio of unbound peptide was evaluated in medium supplemented with 10% (v/v) fetal bovine serum. ^cNormalized IC_{50} = $IC_{50} \times$ unbound peptide ratio. ^dMembrane permeability was evaluated by PAMPA (pH 7.4).

Supporting Information).^{20,21} We evaluated serum protein-binding of the representative peptides (odoamide **1a**; the epimers **1b**, **e**; and the macrocyclic variants **12**, **13**, **15**, and **16**) in cell culture medium supplemented with 10% (v/v) fetal bovine serum (see Supporting Information). No direct correlations were observed between the serum protein binding and the cytotoxic potency of the peptides (Figure 5A). For example, peptides **1a** and **1e** with relatively low serum protein binding (75.9 and 85.5%, respectively) had highly potent

cytotoxicity, while peptide **12** also exhibited bioactivity in spite of the high protein binding (97.2%). When the normalized IC_{50} values were calculated based on the ratio of unbound peptide (normalized IC_{50} = $IC_{50} \times$ unbound peptide ratio), the cytotoxic potencies under hypothetical serum-free conditions of peptides **1a**, **1e**, **12**, **15**, and **16** were similar (in the range of 0.12–1.5 nM), suggesting that these modifications would not have significant effect on altering the interaction with the potential target protein(s) of intact peptide **1a**. Additionally, we assessed the membrane permeability of these representative peptides using a parallel artificial membrane permeability assay (PAMPA). Interestingly, all peptides exhibited good passive diffusion [$>1.0 \times 10^{-6}$ cm/s,²² except for a slightly moderate value for peptide **13** (0.82×10^{-6} cm/s)] across the phospholipid layers, leading to similar concentrations of peptides across the extracellular and intracellular compartments. This indicated that the membrane permeability into the intracellular compartment had no significant effect on their cytotoxicities (Figure 5B). Accordingly, the significantly lower cytotoxicity of **1b** and **13** may be attributed to lower affinity to the target protein and high serum protein binding.

In summary, we have established an efficient synthetic procedure for odoamide derivatives using solid-phase techniques. Taking advantage of this protocol, SARs of the peptide part of odoamide were investigated. The amino acid configurations of *L*-Ala2, *D*-MePhe3, and *L*-Ile5 in odoamide **1a** were essential for potent cytotoxicity, while the *D*-MeAla6 isomer **1e** exhibited more potent bioactivity. This suggests that the kulokekahilide-2-type arrangement of chiral components is more favorable for a high potency of the odoamide derivatives. Additionally, it was demonstrated that the 26-membered odoamide **1a** and the 24-membered isomer **6**, which were slowly interconverted via an intramolecular ester exchange at the polyketide 1,3-diol moiety, both contributed to the highly potent cytotoxicity of odoamide. The assessment of physicochemical properties revealed that odoamide derivatives exhibited good membrane permeability but had different serum protein binding behaviors to affect their cytotoxicities. This information will be useful for further optimization of aurilide-class depsipeptides, including odoamide derivatives, in which the interaction with the molecular target(s) as well as lower serum protein binding should be considered.

■ ASSOCIATED CONTENT

Supporting Information

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Experimental details and NMR spectra of all new compounds (PDF)

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

Alloc, allyloxycarbonyl; DIC, *N,N'*-diisopropylcarbodiimide; DMEM, Dulbecco's modified Eagle's medium; Fmoc, 9-fluorenylmethyloxycarbonyl; HATU, *O*-(7-aza-1*H*-benzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate; HFIP, 1,1,1,3,3,3-hexafluoro-2-propanol; Hila, isoleucic acid; HOAt, 1-hydroxy-7-azabenzotriazole; LC-MS, liquid chromatography-mass spectrometry; MeAla, *N*-methylalanine; MePhe, *N*-methylphenylalanine; MTM, methylthiomethyl; Pac, phenacyl; PAMPA, parallel artificial membrane permeability assay; Sar, sarcosine

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