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# New analogue of arenastatin A, a potent cytotoxic spongean depsipeptide, with anti-tumor activity

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**Abstract**—Two analogues possessing steric hindered substituents on C-15 of arenastatin A (**1**), a potent cytotoxic spongean depsipeptide, were synthesized and shown to enhance stability in mouse serum. Notably, 15-*tert*-butylanalogue (**6**) with higher cytotoxicity exhibited in vivo anti-tumor activity through iv administration different from **1**. Additionally, conformation analysis among the two analogues and arenastatin A (**1**) indicated that the torsion angle from C-14 to C-20 is a conclusive factor for the potent cytotoxicity of **1**.


In the course of our search for new biologically active principles from marine metabolites, arenastatin A (**1**) was isolated from the marine sponge *Dysidea arenaria* as a minute amount of a substance with extremely potent cytotoxicity against a tumor cell line ($IC_{50}$: 5 pg/mL, KB cells).[1] Thereafter, we achieved the total synthesis of **1**[2] and elucidated the cytotoxicity of **1** to be ascribable to inhibition of microtubule assembly.[3] However, arenastatin A (**1**) displayed little in vivo anti-tumor activity through intravenous (iv) administration because of its lability in mouse serum. Furthermore, synthesis of the three amide analogues revealed the 15,20-ester linkage in **1** to be cleaved in mouse serum,[4] indicative of the significant reduction of biological potency in vivo. On the basis of this metabolic behavior, we next synthesized the carbaanalogue (**3**) and the deoxoanalogue (**4**) in anticipation of potent cytotoxicity as well as robustness in serum. In spite of acquisition of nearly complete stability in serum for **3** and **4**, their cytotoxic activities were fairly weaker than that of **1**.[5,6] This finding discloses that the 15,20-ester group in **1** must be essential for the potent cytotoxicity of **1**. In this context, we considered that steric hindered substituents on C-15 would prevent metabolism of the ester function. This report describes 15-*tert*-butylanalogue (**6**) of arenastatin A (**1**) not only shows enhanced stability in serum but also displays in vivo anti-tumor activity (Chart 1).

In order to protect the 15–20 ester function of arenastatin A (**1**) from metabolism in blood, one way would be to add steric hindrance in the neighborhood of this function. In our previous study[7] of the structure–activity relationship of **1**, each of the synthetic epimers of **1** at the position of 7,8-epoxide, 6-methyl, and OMe-tyrosine did not show any cytotoxicity at concentration below 0.1 μg/mL. On the other hand, 15-*epi*-arenastatin A,[8] which has been synthesized by the method[7] using D-leucine, was shown to exhibit moderate cytotoxicity ($IC_{50} = 20$ ng/mL). Therefore, exchange of the isobutyl group at the C-15 position for bulky substituents was assumed to keep the reduction of activity to a minimum. According to this working hypothesis, we designed the 15-*i*-propylanalogue (**5**) and the 15-*tert*-butylanalogue (**6**). The synthetic protocol for **5** and **6** was the same as for the synthesis of **1**, as shown in Scheme 1. Namely, introduction of the 7,8-epoxy portion was carried out in the final stage and a precursory cyclic depsipeptide was constructed from the four segments A–D. As the starting material for the synthesis of segment C, (*S*)-leucinic acid for **1** was replaced with 2-(*S*)-hydroxy-3-methylbutylic acid (**7**) for **5** and 3,3-dimethyl-2-(*S*)-hydroxybutylic acid (**8**) for **6**, respectively.

After protecting the carboxyl group in the segment C (**7** or **8**) as benzyl esters (**9** or **10**), condensation of **9** or **10**

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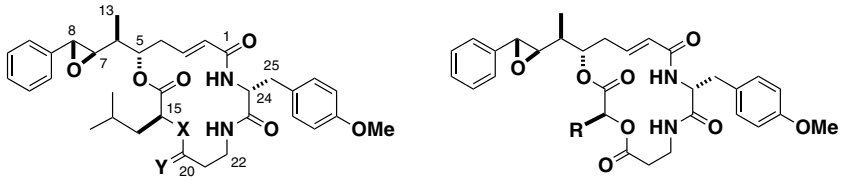
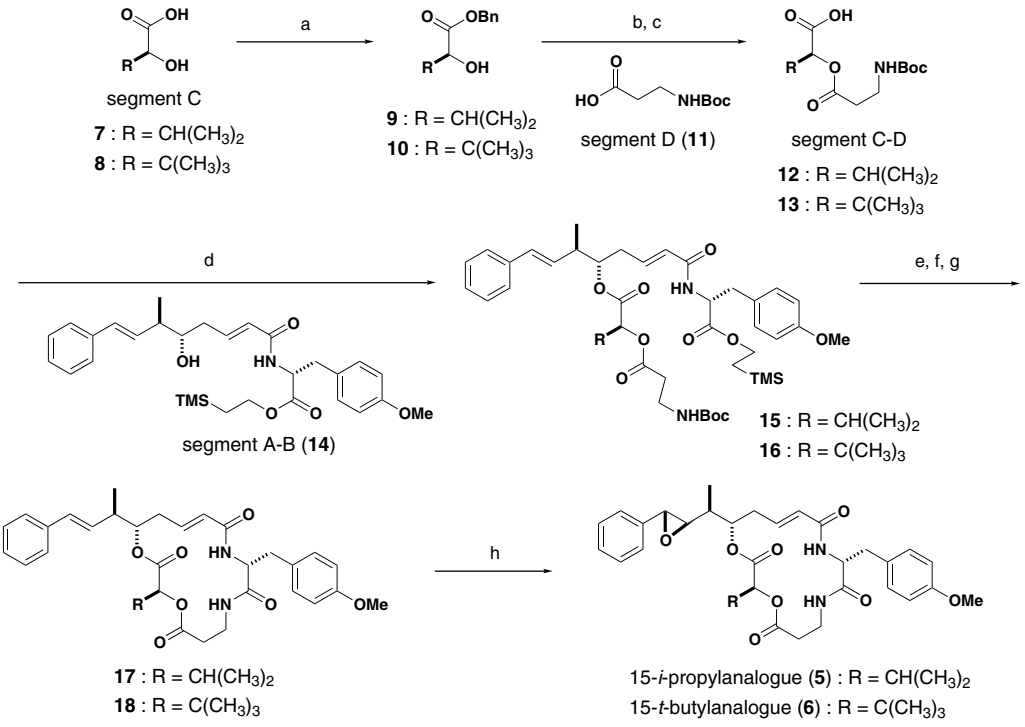


arenastatin A (**1**) : X = Y = O
triamideanalogue (**2**) : X = NH, Y = O
carbaanalogue (**3**) : X = $CH_2$, Y = O
deoxoanalogue (**4**) : X = O, Y = $H_2$

15-*i*-propylanalogue (**5**) : R = $CH(CH_3)_2$
15-*t*-butylanalogue (**6**) : R = $C(CH_3)_3$

**Chart 1.**



**Scheme 1.** Synthesis of 15-*i*-propylanalogue (**5**) and 15-*tert*-butylanalogue (**6**). Reagents and conditions: (a) BnBr, $NaHCO_3$, TBAI, $CH_2Cl_2$–$H_2O$; (b) **11**, EDCI·HCl, DMAP, $CH_2Cl_2$; (c) $H_2$, Pd–C, MeOH, 66% (**12**, three steps), 89% (**13**, three steps); (d) **14**, EDCI·HCl, DMAP, $CH_2Cl_2$, quant. (**15**), 69% (**16**); (e) TFA, $CH_2Cl_2$, 0 °C; (f) HCl–$Et_2O$; (g) DPPA, $NaHCO_3$, DMF, 0 °C, 64% (**17**), 48% (**18**); (h) dimethyldioxirane, $CH_2Cl_2$, 0 °C, 65% (**5**), 52% (**6**).

and the segment D (**11**) in the presence of 4-dimethylaminopyridine (DMAP) and 3-(3-dimethylaminopropyl)-1-ethylcarbodiimide hydrochloride (EDCI·HCl) followed by deprotection of the benzyl ester by use of Pd–C under hydrogen atmosphere gave a segment C–D (**12** or **13**) in 66% or 89% yield for three steps. Subsequently, the segments A–B (**14**) and C–D (**12** or **13**) were connected by using the same method to afford a coupled product (**15** or **16**) quantitatively or in 69% yield, respectively. Cleavage of the two protective groups of **15** or **16** followed by HCl treatment gave a *seco* amino acid as an HCl salt, which was further subjected to intramolecular macrolactamization using diphenylphosphorus azide (DPPA) and $NaHCO_3$ to furnish a cyclic depsipeptide (**17** or **18**) in 64% or 48% yield, respectively. Finally, epoxidation of **17** was successfully accomplished by using dimethyldioxirane to afford a 15-*i*-propylanalogue (**5**)[9] in 65% yield as a major product. Similarly, the 15-*tert*-butylanalogue (**6**)[10] was also prepared from **18** in 52% yield.

Assessment of stability of the two synthesized analogues (**5**, **6**) in mouse serum was carried out in the same manner as that in our previous report[4] and the result is shown in Figure 1. Although both **5** and **6** were gradually metabolized, they were shown to be more stable than **1**. Notably, **5** and **6** displayed moderate cytotoxicities against KB-3-1 cells ($IC_{50}$; **5**: 30 ng/mL, **6**: 10 ng/mL), respectively.[11]

Next, the 15-*tert*-butylanalogue (**6**) with both higher stability and stronger cytotoxicity was assessed for antitumor activity in vivo against Lewis lung carcinoma subcutaneously (sc) implanted in mice.[12] As shown in

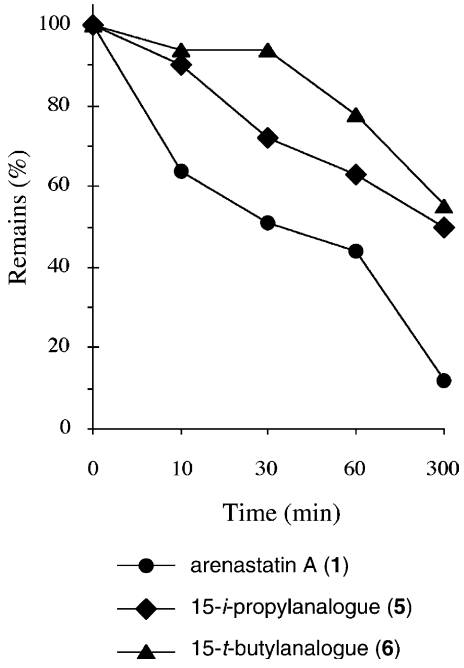



**Figure 1.** Stability of 15-*i*-propylanalogue (**5**) and 15-*tert*-butylanalogue (**6**). Each sample (10 μL of 0.1 mg/mL solution) was treated with fresh mouse serum (100 μL) and incubated at 37 °C for 0, 10, 30, 60, and 300 min, respectively. After extraction of the reaction mixture with EtOAc, each extract was analyzed by reversed-phase HPLC to determine the remaining amounts of **1**, **5**, and **6**, respectively.

Table 1, 15-*tert*-butylanalogue (**6**) reduced tumor volume in a dose-dependent manner through iv administration. It is noteworthy that **6** significantly inhibited the growth of tumor volume at the dose of 10 mg/kg without any signs of acute toxicity such as body weight loss, diarrhea, and mortality.

Previously, conformation analysis of the four compounds (**1**–**4**) by molecular dynamic calculation with distance restraints revealed both the spatial orientation of the C-20 carbonyl group and the conformation of the 16-membered ring of **1** to be significantly associated to potent cytotoxicity.[6] The foregoing large difference in cytotoxic outcome among arenastatin A (**1**) and the two analogues (**5**, **6**) was presumed to be attributable to the difference in conformations of the 16-membered ring portion. Thus, conformation analysis of **5** and **6** was examined by using SYBYL 6.5 (Tripos Ass., St. Louis, MO) in the same manner as our previous report.[6] NOE cross peaks in the $^1$H NMR spectra of **5** or **6** were classified into three classes depending on their intensities as strong, medium, or weak and then translated into distance restraints (upper bound of distance: 2.8, 3.5, and 5.0 Å), respectively. Consequently, an initial structure was built with the aid of the NOE data with a complete random array of atoms and refined by energy minimization using the BFGS method.[13] After this refined structure was subjected to 50 cycles of molecular dynamic calculation, the 50 structures obtained were refined by energy minimization once again. Among the resulting conformers, acceptable conformers were selected on the basis of criteria that torsion angles of the amide linkages and enone moieties were within ±15°.

The average value of pairwise root-mean-square distance deviation (rmsd) for the backbone (16-membered ring) heavy atoms and the restraint violations of arenastatin A (**1**) and the two analogues (**5**, **6**) are summarized in Table 2. Each compound exhibited not only good convergence on the 16-membered ring portions but also adequate compatibility with NOE information. Because of the crucial participation of the two ester linkages in **1** for the potent cytotoxicity, we compared the conformation of the 16-membered ring portion of each compound focusing on the two torsion angles (C4–C5–O–C14 and C14–C15–O–C20).

In each compound, a characteristic cluster consisting of predominant conformers appeared as depicted in Figure 2. Furthermore, the percentage abundance of conformers belonging to each cluster was estimated by Boltzmann distribution. Apparently, the two analogues (**5**, **6**) adopted the torsion-1 angle around −70° different from arenastatin A (**1**). This conformational feature can account for the difference in the cytotoxic scores of **1**, **5**, and **6**. On the other hand, the lower ratio of the conformer similar to **1** and the presence of a second dominant cluster with respect to **5** may give rise to weaker cytotoxicity than that of **6**.

In conclusion, substitution of the steric hindered *tert*-butyl group for the isobutyl group on C-15 of arenastatin A (**1**) provided anti-tumor efficacy in vivo through iv administration as well as enhancement of robustness in serum. Conformational analysis by molecular

**Table 1.** In vivo anti-tumor activity of 15-*tert*-butylanalogue (**6**)

| Treatment | Dose[a] (mg/kg) | Tumor volume[b] ($cm^3$, day 14) | T/C[c] |
|---|---|---|---|
| **6** | 2.5 | 6.6 ± 1.1 | 0.96 |
| | 5 | 7.1 ± 1.0 | 1.03 |
| | 10 | 4.9 ± 1.5* | 0.71 |
| Mitomycin C | 1.65 | 4.2 ± 0.9* | 0.62 |
| | 3.3 | 3.3 ± 0.7* | 0.48 |
| Vehicle | | 6.9 ± 1.3 | 1.00 |

*$p < 0.05$ against vehicle control (Dunnett method).
[a] Iv administration × 3 (days 3, 7, 11).
[b] Mean ± SD.
[c] Treated/control.

**Table 2.** Structural statistics and distance restraint violations

| | Arenastatin A (**1**) | 15-Isopropyl (**5**) | 15-*tert*-Butyl (**6**) |
|---|---|---|---|
| Number of accepted conformers | 43 | 45 | 46 |
| Rmsd (Å) for backbone heavy atom[a] | 0.62 ± 0.22 | 0.58 ± 0.19 | 0.63 ± 0.22 |
| Restraint violation (kcal/mol)[a,b] | 0.18 ± 0.27 | 1.63 ± 0.38 | 1.99 ± 0.46 |

[a] The given numbers are the mean ± SD. The backbone means 16-membered ring.
[b] When the calculated distance is greater than the distance restraint, the energy incurred is $200(d_{measure} - d_{restrain})$.[2]

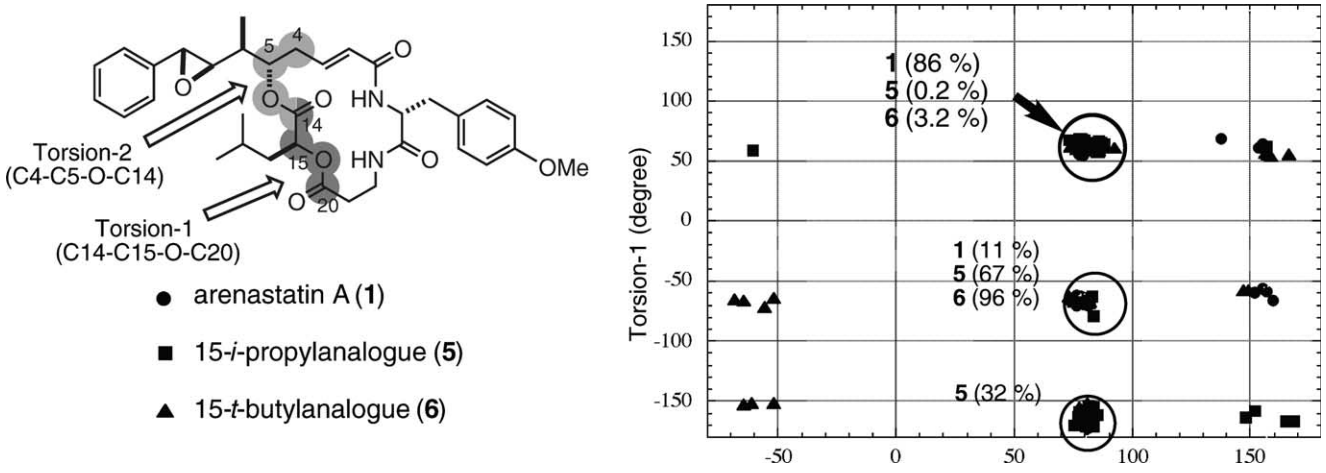



**Figure 2.** Distribution of conformers of **1**, **5**, and **6** obtained by molecular dynamic calculation.

dynamic calculation of arenastatin A (**1**) and the two analogues **5**, **6** established the torsion angle from C-14 to C-20 as a conclusive factor for the potent cytotoxicity of **1**.

## Acknowledgements

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9. **5**: white powder, $[\alpha]_D^{22}$ +35.2 (*c* 0.66, $CHCl_3$). IR (KBr): 3297, 2924, 1744, 1674, 1514 $cm^{-1}$. $^1H$ NMR (500 MHz, $CDCl_3$) $\delta$: 7.24–7.38 (5H, m, Ph), 7.11 (2H, d, $J = 8.5$ Hz, 27-H), 6.93 (1H, br, 22-NH), 6.81 (2H, d, $J = 8.5$ Hz, 28-H), 6.68 (1H, ddd, $J = 4.3$, 9.8, 15.3 Hz, 3-H), 5.69 (1H, d, $J = 15.3$ Hz, 2-H), 5.66 (1H, d, $J = 8.5$ Hz, 24-NH), 5.19 (1H, ddd, $J = 1.8$, 4.9, 11.0 Hz, 5-H), 4.75 (1H, ddd, $J = 5.5$, 7.3, 8.5 Hz, 24-H), 4.73 (1H, d, $J = 4.3$ Hz, 15-H), 3.77 (3H, s, 30-H), 3.68 (1H, d, $J = 1.8$ Hz, 8-H), 3.54 (1H, m, 22a-H), 3.40 (1H, m, 22b-H), 3.15 (1H, dd, $J = 5.5$, 14.0 Hz, 25a-H), 3.00 (1H, dd, $J = 7.3$, 14.0 Hz, 25b-H), 2.88 (1H, dd, $J = 1.8$, 7.3 Hz, 7-H), 2.55 (3H, m, 4a, 21-H), 2.40 (1H, ddd, $J = 9.8$, 11.0, 14.0 Hz, 4b-H), 1.90 (1H, m, 6-H), 1.79 (1H, m, $J = 6.7$ Hz, $CH(CH_3)_2$), 1.15 (3H, d, $J = 6.7$ Hz, 13-H), 0.88, 0.74 (both 3H, d, $J = 6.7$ Hz, $CH(CH_3)_2$). FABMS *m/z*: 593 $(M+H)^+$. FAB-HRMS *m/z*: calcd for $C_{33}H_{41}O_8N_2$: 593.2863. Found: 593.2861.
10. **6**: white powder, $[\alpha]_D^{22}$ +55.1 (*c* 0.48, $CHCl_3$). IR (KBr): 3279, 2926, 1740, 1678, 1514 $cm^{-1}$. $^1H$ NMR (500 MHz, $CDCl_3$) $\delta$: 7.24–7.38 (5H, m, Ph), 7.11 (2H, d, $J = 8.5$ Hz, 27-H), 7.03 (1H, m, 22-NH), 6.82 (2H, d, $J = 8.5$ Hz, 28-H), 6.68 (1H, ddd, $J = 6.9$, 7.9, 15.3 Hz, 3-H), 5.71 (1H, d, $J = 15.3$ Hz, 2-H), 5.65 (1H, d, $J = 7.9$ Hz, 24-NH), 5.39 (1H, dt, $J = 9.2$, 4.3 Hz, 5-H), 4.70 (1H, ddd, $J = 5.5$, 7.9, 7.9 Hz, 24-H), 4.64 (1H, s, 15-H), 3.78 (3H, s, 30-H), 3.71 (1H, d, $J = 2.4$ Hz, 8-H), 3.56 (1H, m, 22a-H), 3.39 (1H, m, 22b-H), 3.14 (1H, dd, $J = 5.5$, 14.6 Hz, 25a-H), 3.02 (1H, dd, $J = 7.9$, 14.6 Hz, 25b-H), 2.91 (1H, dd, $J = 2.4$, 6.7 Hz, 7-H), 2.57 (3H, m, 4a, 21-H), 2.49 (1H, m, 4b-H), 1.88 (1H, m, 6-H), 1.15 (3H, d, $J = 6.7$ Hz, 13-H), 0.98 (9H, s, $C(CH_3)_3$). FABMS *m/z*: 607 $(M+H)^+$. FAB-HRMS *m/z*: calcd for $C_{34}H_{43}O_8N_2$: 607.3019. Found: 607.3041.
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the back of BDF1 mice. The analogue **6** or mitomycin C was administered through iv to the tumor-bearing mice at various doses on days 3, 7, and 11. Each group consisted of six mice. On day 14, the tumor volumes were determined as $0.5a^2b$, where $a$ and $b$ are the minor and major tumor axes. In this study, the statistical significance in comparison with the nontreated group and the treated groups was evaluated using Dunnett's test.

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