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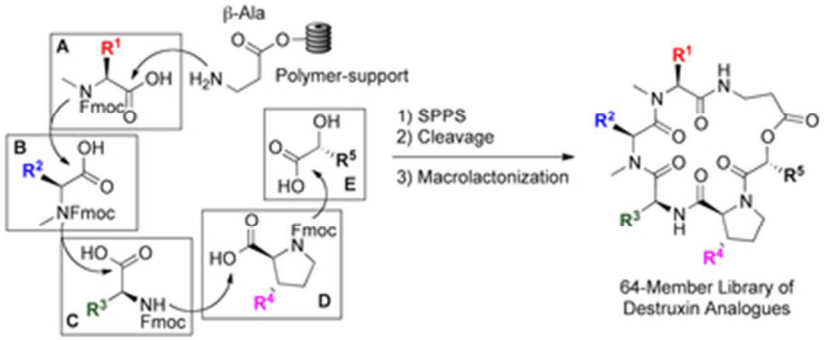

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Combinatorial Solid-Phase Synthesis and Biological Evaluation of Cyclodepsipeptide Destruxin B as a Negative Regulator for Osteoclast Morphology

Hiroshi Sato[1, 2], Masahito Yoshida[1], Hayato Murase[3], Hiroshi Nakagawa[3], and Takayuki Doi[, 1]*

[1]Graduate School of Pharmaceutical Sciences, Tohoku University, 6-3 Aza-Aoba, Aramaki, Aoba-ku, Sendai 980-8578, Japan; *[2]Mitsubishi Tanabe Pharma Corporation, 3-2-10, Dosho-machi, Chuo-ku, Osaka 541-8505, Japan*; *[3]Department of Applied Biological Chemistry, Chubu University, 1200 Matsumoto-cho, Kasugai, Aichi 487-8501, Japan*.

KEYWORDS: Cyclic Peptides, Cyclodepsipeptides, Solid-Phase Synthesis, Combinatorial Library, Osteoclasts

ABSTRACT: Combinatorial synthesis and biological evaluation of cyclodepsipeptide destruxin B have been achieved. The cyclization precursors were prepared by solid-phase peptide synthesis via a split and pool method utilizing SynPhase™ lanterns with colored tags and cogs, followed by cleavage from the polymer-support. Macrolactonization utilizing MNBA-DMAPO in solution-phase was successfully performed in parallel to afford the desired 64-member destruxin analogues in moderate to good yields. Biological evaluation of the synthesized analogues indicated that a MeAla residue for the building block **A** is required to induce the desired morphological changes in osteoclast-like multi-nuclear cells (OCLs), and introduction of the

substituent at the R^4 position of a proline moiety is tolerated by the morphology and may enable the preparation of a molecular probe for the target identification in the osteoclasts.

Introduction

A number of biologically active cyclic peptides have been isolated from marine sponges, fungi and bacteria in recent decades. These peptides exhibit a broad spectrum of attractive biological activities including antibiotic, antifungal, immunosuppressant, antitumor, and anti-inflammatory activities.[1] Because of their unique biological activities, naturally occurring cyclic peptides have recently been approved for clinical use and evaluated in clinical trials. For instance, the natural products cyclosporine A, daptomycin, and FK228 have already been used in clinical settings as an immunosuppressant, an antibiotic, and anticancer agent, indicating that this family of cyclic peptides may comprise a novel chemical space for discovering drug candidates because of their superior pharmacokinetics profiles relative to the corresponding linear peptides.[2]

Cyclodepsipeptide destruxins were first isolated from *Metarhizium anisopliae*, and consist of five amino acids (β-Ala, L-MeAla, L-MeVal, L-Ile and L-Pro) and one α-hydroxy acid derivative.[3] More than 30 destruxins have been isolated to date, most of which have been categorized into families A–E (**1a–e**) based on the structure of the α-hydroxy acid derivative (Figure 1).[4]

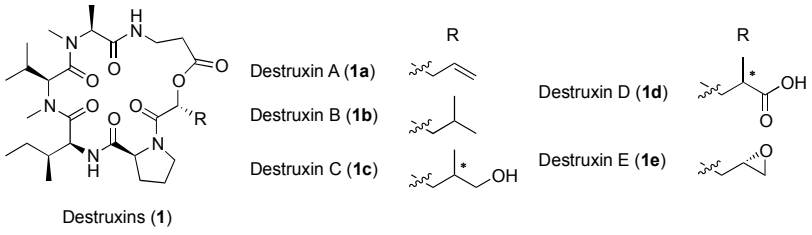


Figure 1. Naturally Occurring Destruxins

The destruxins exhibit valuable biological activities such as antibiotic, antitumor, and immunosuppressant.[5] In particular, Muroi *et al.* first reported that destruxin B (**1b**), which possess an *iso*-butyl moiety as a side-chain on an α-hydroxy acid derivative, reversibly inhibits vacuolar-type ATPase (V-ATPase) in a dose-dependent manner. In contrast, macrolide antibiotics, such as bafilomycin A1 and folimycin, irreversibly inhibit the V-ATPase activity.[6] In addition, Vázquez *et al.* also mentioned that the side chain of the α-hydroxy acid derivative is important for inducing the V-ATPase inhibitory activity and that destruxin E (**1e**) containing an epoxide in the side chain is the most potent destruxin.[7] Meanwhile, Nakagawa *et al.* have reported that destruxin B (**1b**) and destruxin E (**1e**) reversibly induce morphological changes in osteoclast-like multi-nuclear cells (OCLs) under concentrations lower than those affecting the V-ATPase activity to inhibit bone-resorbing activity without cell death.[8] In contrast, bisphosphonates, widely used in clinical contexts as anti-resorptive agents, are known to inhibit bone-resorbing activity by inducing apoptosis.[9] Therefore, it should be noted that destruxins are promising as a new type of anti-resorptive agents for use in osteoporosis therapies.

Destruxin analogues have been the subject of many synthetic studies because of their unique structural features and biological activities.[10] Recently, we reported the first total synthesis of destruxin E (**1e**) and structure-activity relationships (SAR) study based on the synthetic analogues of **1e**. In these works, we successfully showed that the stereochemistry of the epoxide in the side chain plays an important role in inducing the morphological changes[11] and that a steric effect around the MeAla residue and replacement of Ile with Phe residue negatively affect the morphological change of OCLs.[12] Based on these SAR results, we also attempted the synthesis of molecular probes for target identification. An analogue possessing an alkyl azide moiety instead of an Ile residue induced morphological changes in OCLs; however, its biological

activity was found to be 60-fold weaker than that of the natural product destruxin E (**1e**), indicating that further optimization is required for the preparation of an active molecular probe.

To better understand the SARs responsible for the induction of morphological changes in OCLs, we planned the synthesis of a combinatorial library based on destruxin B (**1b**), which is also a negative regulator for osteoclast morphology, as mentioned above. Unlike destruxin analogues, analogues based on destruxin B (**1b**) can be readily accessed because modification of the side chain on an α-hydroxy acid residue is not required after construction of the macrocycle. A variety of destruxin analogues containing various side chains on the constituent amino acids would help elucidate the effect of the side chain on the osteoclast morphology and facilitate the development of novel anti-resorptives agents in osteoporosis therapeutics. We herein report the combinatorial synthesis and biological evaluation of 64-member library of cyclodepsipeptides including the natural products destruxin A (**1a**), destruxin B (**1b**), roseotoxin B (**2**),[13] and roseocardin (**3**)[14] and elucidation of the SARs relevant to the morphological changes in OCLs (Figure 2).

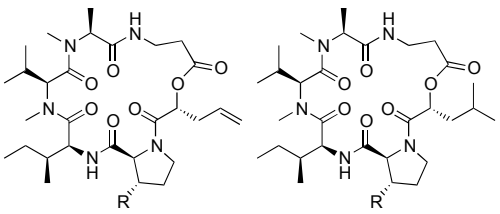


Figure 2. Structures of destruxin A (1a), B (1b), roseotoxin B (2), and roseocardin (3)

RESULTS AND DISCUSSION

To elucidate the effect of the side chain on a cyclodepsipeptide scaffold, we designed a 64-member analogues **5a–d** based on the structure of destruxin A (**1a**), destruxin B (**1b**), roseotoxin B (**2**), and roseocardin (**3**). We planned for the preparation of the cyclodepsipeptide library by solid-phase peptide synthesis (SPPS) similar to our recent total synthesis of destruxin E (**1e**),[11, 12] and the details of the synthesis are dipicted in Figure 3. The cyclization precursors **4a–d**, comprising building blocks *A* (two), *B* (two), *C* (four), *D* (two), and *E* (two), could be prepared by SPPS using a split and pool method with colored tags and cogs.[15] After cleavage from the polymer-support, 2-methyl-6-nitrobenzoic anhydride (MNBA)-mediated macrolactonization[16] in solution would furnish 64 analogues in parallel.

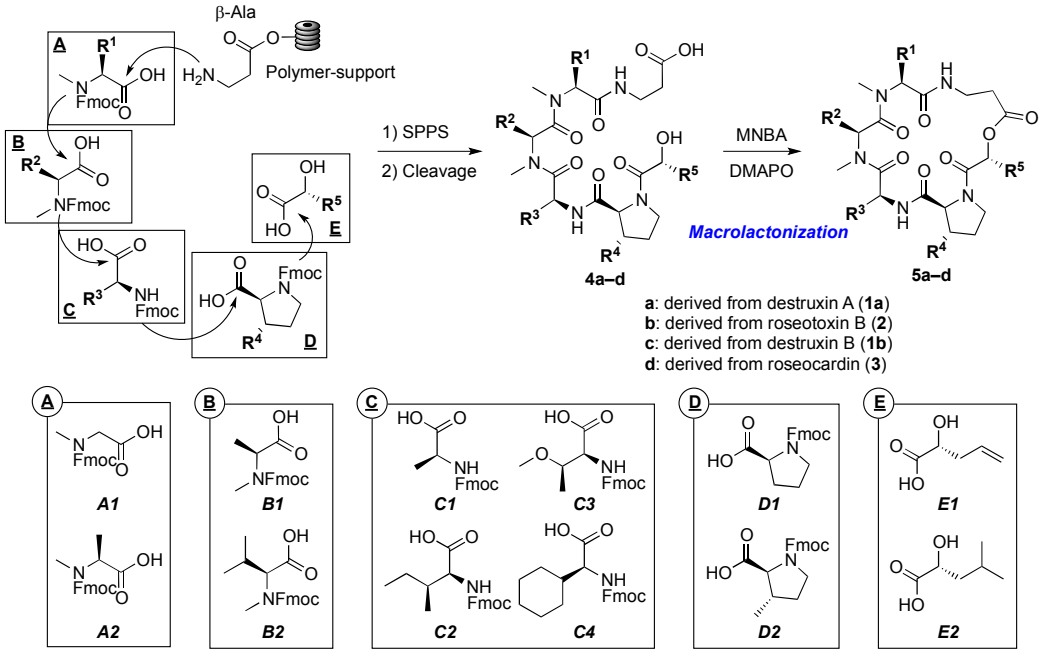
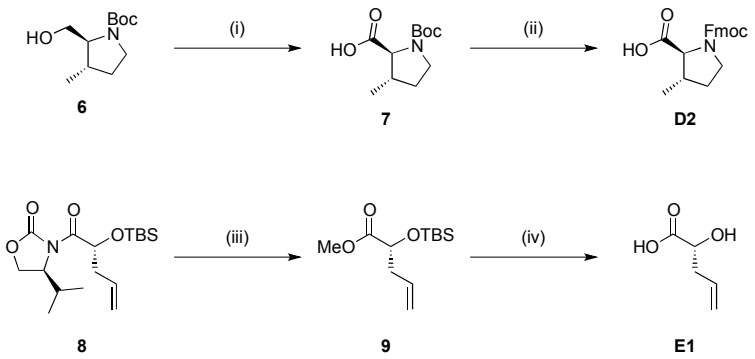


Figure 3. Synthetic strategy, and the building blocks *A*, *B*, *C*, *D* and *E* for the combinatorial synthesis of the 64-member library of cyclodepsipeptides 5

Synthesis of the Building Blocks

The building blocks ***C3*** and ***E2*** were readily prepared by previously reported procedures.[17] The building blocks (2*S*, 3*S*)-Fmoc-3-methylproline ***D2*** and (*S*)-2-hydroxy-4-pentenoic acid ***E1*** were synthesized as illustrated in Scheme 1. Oxidation of alcohol **6**, prepared from the commercially available L-pyroglutamic acid,[18] using $TEMPO^{+}BF_4^{-}$[19] provided acid **7** in 90% yield. After the removal of the Boc group in **7** under acidic conditions, protection of the resulting amine with an Fmoc group furnished ***D2*** in 90% yield. ***E1*** was prepared as follows: solvolysis of **8** using MeOMgBr/MeOH afforded the corresponding methyl ester **9** in 94% yield.[20] Hydrolysis of the methyl ester, followed by the removal of the TBS group furnished the desired ***E1*** in 67% yield.[21]

Scheme 1. Preparation of the building blocks *D2* and *E1*[a]



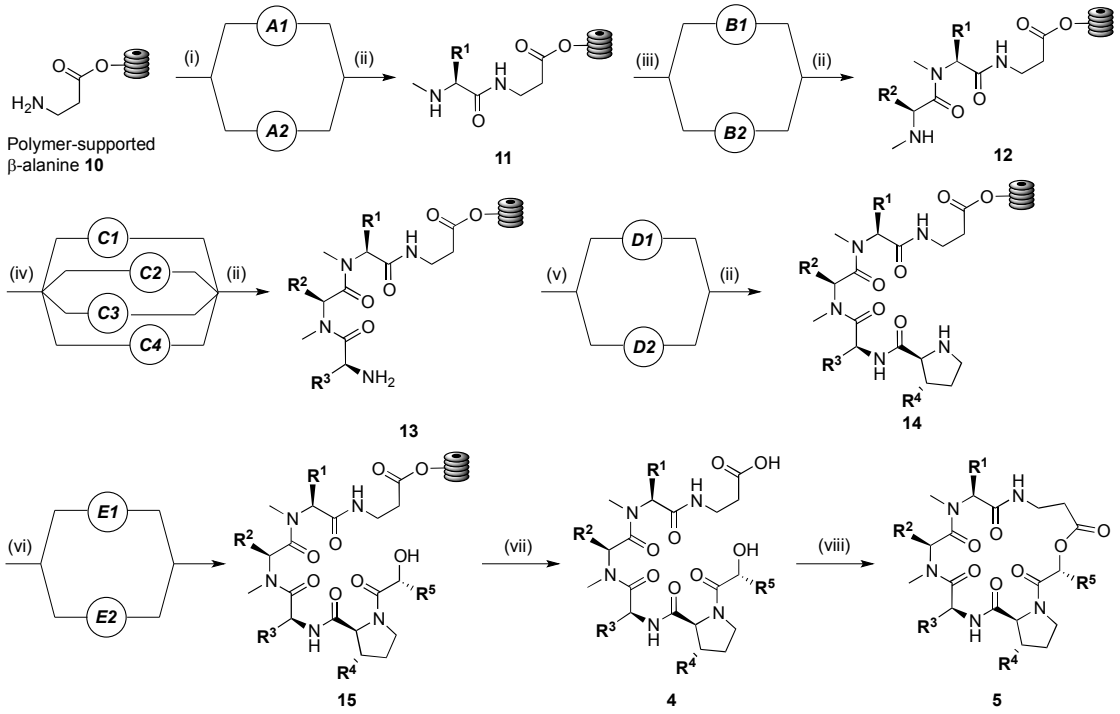
[a]*Reagent*: (i) $TEMPO^{+}BF_4^{-}$, $NaClO_2$, MeCN–Phosphate Buffer, rt, 12 h, 90%; (ii) a) TFA–CH_2Cl_2, 0 °C, 1.5 h; b) FmocCl, aqueous Na_2CO_3, 1,4-dioxane, rt, 12 h, 90% (2 steps); (iii) MeOMgBr, MeOH, rt, 18 h, 94%; iv) a) $LiOH{\bullet}H_2O$, THF–H_2O, rt, 4 h; b) TBAF, THF, rt, 6 h, 67% (2 steps)

Combinatorial Synthesis of a 64-Member library of Cyclodepsipeptides 5

After preparing all the building blocks were ready for the synthesis of a cyclodepsipeptide library **5**, we performed the SPPS of cyclization precursors **4a–d** by a split and pool method as depicted in Scheme 2. The 64 β-Ala-OH-attached SynPhase™ lanterns 10,[22] tagged by colored stems and cogs, were initially divided into two groups and first acylations with amino acids ***A***{*1–2*} were performed utilizing DIC/HOBt, respectively (Scheme 2, step 1). After the completion of the acylation on the polymer-supports, as detected by the Kaiser test,[23] all of the polymer-supported dipeptides were gathered and washed with organic solvents to remove excess reagents. The removal of the Fmoc group with 20% piperidine in DMF provided the polymer-supported *N*-methylamines **11**, which were subsequently split into two groups. The second coupling with the amino acids ***B***{*1–2*} was performed by treatment with PyBroP[24]/DIEA, repeatedly due to steric hindrance of the *N*-methylamines. The resulting polymer-supports were again pooled for washing, and the removal of the Fmoc group afforded the polymer-supported amines **12**, which were divided into four groups for the acylation with the acids ***C***{*1–4*}. Similar to the synthesis of tripeptide **12**, double coupling with the acids ***C*** was required to provide the desired tetrapeptides **13**. After washing the polymer-supports and removing the Fmoc group, acylations with ***D***{*1–2*} in separate vessels followed by the removal of the Fmoc group provided pentapeptides **14**. The polymer-supports were again divided into two groups, and coupling with acid derivatives ***E***{*1–2*} was performed to afford hexapeptides **15**. The polymer-supports were split into independent vessels, and cleavage from the polymer-support by treatment with 30% 1,1,1,3,3,3-hexafluoroisopropanol (HFIP)/CH_2Cl_2[25] furnished 64-member cyclization precursors **4** with good to excellent purities (81–98% determined at UV 214 nm). Finally, all of the resulting cyclization precursors were subjected to macrolactonization using MNBA/4-

(dimethylamino)pyridine *N*-oxide (DMAPO) in parallel to afford the desired cyclodepsipeptides **5**, including four natural products in 46–77% isolated yields.

Scheme 2. Combinatorial synthesis of a 64-member library of cyclodepsipeptides 5 using a split and pool method[a]



[a]*Reagent*: (i) Amino acids ***A{1–2}***, DIC, HOBt, DMF, rt, 12 h; (ii) 20% piperidine/DMF, rt, 1 h; (iii) Amino acids ***B{1–2}***, PyBroP, DIEA, DMF, rt, 12 h; (iv) Amino acids ***C{1–4}***, PyBroP, DIEA, DMF, rt, 12 h (twice); (v) Amino acids ***D{1–2}***, PyBroP, DIEA, DMF, rt, 12 h; (vi) β-Hydroxy acids ***E{1–2}***, PyBroP, DIEA, DMF, rt, 12 h; (vii) 30% HFIP/CH_2Cl_2, rt, 12 h; (viii) MNBA, DMAPO, CH_2Cl_2, 30 °C, 48 h

Having successfully obtained the desired cyclodepsipeptides **5**, we then evaluated their effects on the morphological changes in polarized OCL (see Figure S1). The results are summarized in Table 1 and Figure 4. The analogues **5c** and **5d** which contain an *iso*-butyl moiety at the α-hydroxy acid, display greater biological activity than the analogues **5a** and **5b**, which

contain an allyl group. Thus, it is conceivable that the side chain of the α-hydroxy acid moiety has an important effect on the osteoclast morphology. The desired biological activity was lost when Sar ***A1*** was used as building block ***A*** and MeVal ***B2*** was replaced by MeAla ***B1*** as building block **B**, such as in **5a{a–d}** (entries 1–4), **5b{a–d}** (entries 17–20), **5c{a–d}** (entries 33–36), and **5d{a–d}** (entries 49–52). On the other hand, the analogues **5{a-d}m** consisting of {***A2***, ***B2***} (entries 13, 29, 45, and 61) induced the morphological changes at 50 μM, whereas no effect was observed after treatment by the analogues **5{a–d}e** with {***A2***, ***B1***} (entries 5, 21, 37, and 53) and **5{a–d}i** with {***A1***, ***B2***} (entries 5, 21, 37, and 53). The above results indicate that the R^1 and R^2 moieties must be hydrophobic to induce the morphological changes.

In addition, the introduction of Ile ***C2*** or L-cyclohexylglycine (Chg) ***C4*** residue retained the biological activity in the range of 12.5 to 50 μM when the analogues possessed MeAla ***A2*** for **5{a–d}f** (entries 6, 22, 38, and 54) and **5{a–d}h** (entries 8, 24, 40 and 56), or MeVal ***B2*** for **5{a–d}j** (entries 10, 26, 42, and 58) and **5{a–d}l** (entries 12, 28, 44, and 60). In particular, the natural products destruxin A (**1**) (as **5an**), roseotoxin B (**2**) (as **5bn**), destruxin B (**1b**) (as **5cn**), and roseocardin (**3**) (as **5dn**) were the most potent inducers of the morphological changes, and the analogues **5{a–d}p** possessing Chg ***C4*** instead of Ile ***C2*** also induced changes in the osteoclast morphology at a similar concentration to the natural products.

On the other hand, replacement of Ile ***C2*** by a polar Thr(Me) ***C3*** significantly diminished the biological activity; therefore, it is conceivable that a hydrophobic substituent for the R^3 moiety may be crucial for inducing the desired morphological change in OCLs. Notably, the analogues **5b** and **5d**, which possess a methyl group at the R^4 position, were found to retain the biological activity similar to the corresponding analogues **5a** and **5c**. Therefore, the introduction of the substituent at the R^4 position should be tolerated by the osteoclast morphology, and this position

is a potential candidate for the introduction of a chemical tag to prepare a molecular probe for target identification in the osteoclasts.

Table 1. Biological evaluation of the cyclodepsipeptide library on the osteoclast morphology

Entry	**5**	{*A*, *B*, *C*, *D*, *E*}	Yield [%][a]	Minimum Concentration [μM][b, c]
1	**5aa**	{*1*, *1*, *1*, *1*, *1*}	62	–
2	**5ab**	{*1*, *1*, *2*, *1*, *1*}	61	25
3	**5ac**	{*1*, *1*, *3*, *1*, *1*}	51	–
4	**5ad**	{*1*, *1*, *4*, *1*, *1*}	48	–
5	**5ae**	{*2*, *1*, *1*, *1*, *1*}	65	–
6	**5af**	{*2*, *1*, *2*, *1*, *1*}	69	50
7	**5ag**	{*2*, *1*, *3*, *1*, *1*}	71	–
8	**5ah**	{*2*, *1*, *4*, *1*, *1*}	72	25
9	**5ai**	{*1*, *2*, *1*, *1*, *1*}	63	–
10	**5aj**	{*1*, *2*, *2*, *1*, *1*}	64	50
11	**5ak**	{*1*, *2*, *3*, *1*, *1*}	57	–
12	**5al**	{*1*, *2*, *4*, *1*, *1*}	68	50
13	**5am**	{*2*, *2*, *1*, *1*, *1*}	67	50
14[d]	**5an**	{*2*, *2*, *2*, *1*, *1*}	66	1.56
15	**5ao**	{*2*, *2*, *3*, *1*, *1*}	58	25
16	**5ap**	{*2*, *2*, *4*, *1*, *1*}	58	3.13
17	**5ba**	{*1*, *1*, *1*, *2*, *1*}	56	–
18	**5bb**	{*1*, *1*, *2*, *2*, *1*}	63	–

19	**5bc**	{*1*, *1*, *3*, *2*, *1*}	46	–
20	**5bd**	{*1*, *1*, *4*, *2*, *1*}	53	–
21	**5be**	{*2*, *1*, *1*, *2*, *1*}	57	–
22	**5bf**	{*2*, *1*, *2*, *2*, *1*}	63	50
23	**5bg**	{*2*, *1*, *3*, *2*, *1*}	67	–
24	**5bh**	{*2*, *1*, *4*, *2*, *1*}	68	25
25	**5bi**	{*1*, *2*, *1*, *2*, *1*}	59	–
26	**5bj**	{*1*, *2*, *2*, *2*, *1*}	65	50
27	**5bk**	{*1*, *2*, *3*, *2*, *1*}	54	–
28	**5bl**	{*1*, *2*, *4*, *2*, *1*}	69	50
29	**5bm**	{*2*, *2*, *1*, *2*, *1*}	50	50
30[e]	**5bn**	{*2*, *2*, *2*, *2*, *1*}	67	1.56
31	**5bo**	{*2*, *2*, *3*, *2*, *1*}	53	25
32	**5bp**	{*2*, *2*, *4*, *2*, *1*}	64	3.13
33	**5ca**	{*1*, *1*, *1*, *1*, *2*}	65	–
34	**5cb**	{*1*, *1*, *2*, *1*, *2*}	69	–
35	**5cc**	{*1*, *1*, *3*, *1*, *2*}	51	–
36	**5cd**	{*1*, *1*, *4*, *1*, *2*}	77	–
37	**5ce**	{*2*, *1*, *1*, *1*, *2*}	64	–
38	**5cf**	{*2*, *1*, *2*, *1*, *2*}	70	25
39	**5cg**	{*2*, *1*, *3*, *1*, *2*}	63	–
40	**5ch**	{*2*, *1*, *4*, *1*, *2*}	77	25
41	**5ci**	{*1*, *2*, *1*, *1*, *2*}	77	–
42	**5cj**	{*1*, *2*, *2*, *1*, *2*}	66	50

43	**5ck**	{*1*, *2*, *3*, *1*, *2*}	55	–
44	**5cl**	{*1*, *2*, *4*, *1*, *2*}	73	50
45	**5cm**	{*2*, *2*, *1*, *1*, *2*}	70	50
46[f]	**5cn**	{*2*, *2*, *2*, *1*, *2*}	65	0.78
47	**5co**	{*2*, *2*, *3*, *1*, *2*}	67	12.5
48	**5cp**	{*2*, *2*, *4*, *1*, *2*}	63	3.13
49	**5da**	{*1*, *1*, *1*, *2*, *2*}	63	–
50	**5db**	{*1*, *1*, *2*, *2*, *2*}	63	–
51	**5dc**	{*1*, *1*, *3*, *2*, *2*}	51	–
52	**5dd**	{*1*, *1*, *4*, *2*, *2*}	70	–
53	**5de**	{*2*, *1*, *1*, *2*, *2*}	58	–
54	**5df**	{*2*, *1*, *2*, *2*, *2*}	63	50
55	**5dg**	{*2*, *1*, *3*, *2*, *2*}	62	–
56	**5dh**	{*2*, *1*, *4*, *2*, *2*}	71	12.5
57	**5di**	{*1*, *2*, *1*, *2*, *2*}	71	–
58	**5dj**	{*1*, *2*, *2*, *2*, *2*}	68	25
59	**5dk**	{*1*, *2*, *3*, *2*, *2*}	65	–
60	**5dl**	{*1*, *2*, *4*, *2*, *2*}	75	50
61	**5dm**	{*2*, *2*, *1*, *2*, *2*}	65	50
62[g]	**5dn**	{*2*, *2*, *2*, *2*, *2*}	64	0.78
63	**5do**	{*2*, *2*, *3*, *2*, *2*}	67	12.5
64	**5dp**	{*2*, *2*, *4*, *2*, *2*}	55	1.56

[a]Isolated yield calculated from polymer-supported β-Ala-OH **10**; [b]Minimum concentration for morphological changes; [c]The bar indicates the morphological change was not observed at 50

μM. [d]**5an**: destruxin A (**1a**); [e]**5bn**: roseotoxin B (**2**); [f]**5cn**: destruxin B (**1b**); [g]**5dn**: roseocardin (**3**);

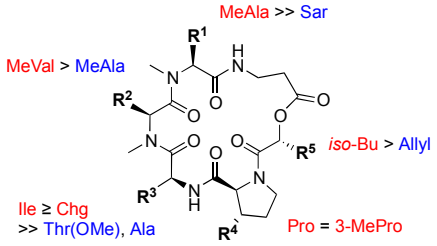


Figure 4. Summary of SARs study for destruxin analogues

In conclusion, we have demonstrated the combinatorial synthesis of 64-member library of destruxin analogues and evaluated their biological activities for morphological changes in OCLs. The 64-member cyclization precursors **4** were efficiently synthesized with good purities by SPPS on SynPhase™ lanterns with color cogs using a split and pool method, and the macrolactonization utilizing MNBA/DMAPO in parallel afforded the desired cyclodepsipeptides **5** in moderate yields. The biological activities of all analogues were found to be weaker than that of destruxin E (**1e**) possessing an epoxide-containing side chain; however, we identified interesting features in the SARs for morphological changes in OCLs. For example, based on the biological evaluation of the synthetic analogues, a MeAla residue for the building block ***A*** is required to induce morphological changes in OCLs, which is consistent with the results for the destruxin E analogues we had previously reported.[12] In addition, replacement of a MeVal residue with a MeAla residue as building block ***B*** significantly diminished the biological activity. This finding indicates that the R^1 and R^2 moieties must be hydrophobic for the induction of the morphological changes. The structural difference in the side chain on the α-hydroxy acid derivatives significantly affected the biological activity, indicating that the α-hydroxy acid

moiety has an important effect on the osteoclast morphology. In addition, a hydrophobic amino acid as building block ***D*** is required to retain the desired biological activity. Notably, the analogues **5b** and **5d**, which possess a methyl group at the R^4 position, induce morphological changes at similar concentrations to the analogues **5a** and **5c**, indicating that the introduction of a substituent at the R^4 position is tolerated by the morphology and may be used to prepare a molecular probe for the target identification in osteoclasts. This is the first example of the elucidation of the SARs of cyclodepsipeptides for osteoclast morphology, and the results are expected to be valuable in the development and discovery of new lead structures for anti-resorptives agents based on a cyclodepsipeptide scaffold.

ASSOCIATED CONTENT

Supporting Information.

The supporting information is available free of charge on the ACS publication website at DOI:. Further details on the experimental procedure, compound data, and copies of ^{1}H and ^{13}C NMR spectra for the synthesized compounds (PDF)

AUTHOR INFORMATION

Corresponding Author

Takayuki Doi, Tel +81-22-795-6865; e-mail: doi_taka@mail.pharm.tohoku.ac.jp

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Notes

The authors declare no competing financial interest.

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Graphical Abstract