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Structure-based hybridization of the bioactive natural products rhizonin A and ternatin leading to a selective fat-accumulation inhibitor against 3T3-L1 adipocytes

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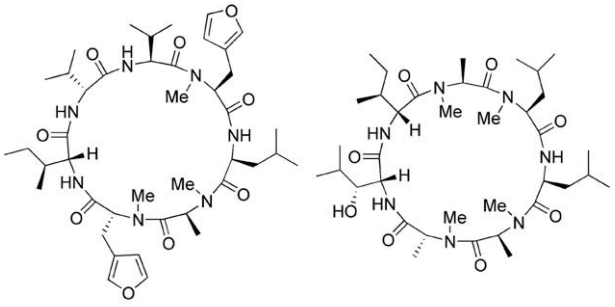
ABSTRACT

Based on the structural similarity between the naturally occurring cyclic heptapeptides rhizonin A and ternatin, two novel analogues were designed. The synthetic analogues were assessed with regard to their fat-accumulation inhibitory effect against 3T3-L1 adipocytes, and this led to the discovery of a potent and selective fat-accumulation inhibitor compared to the parent compound rhizonin A.

Rhizonin A (**1**) and ternatin (**2**) are novel bioactive cyclic heptapeptides that were isolated from the fungus *Rhizopus microsporus* van Tieghem[1] and the mushroom *Coliorus versicolor*,[2,3] respectively, (Fig. 1). They share common structural features in that they contain D-, N-methylated amino acids as well as unusual amino acids, for example, *N*Me-FurAla [*N*Me-3-(3-furyl)alanine] or β-OH-D-Leu [(2*R*,3*R*)-3-hydroxy-Leu]. Interestingly, they show extremely high structural homology: (i) identical sequence of DDDLLLL amino-acid configurations, (ii) positions of N-methylation, (iii) contiguous L-Leu[4]-*N*Me-L-Ala[5] moieties. An analysis of the X-ray crystal structures of **1** and **2** demonstrated that the solid-state conformation of **1** resembles that of **2**, in particular with regard to a β-turn structure in the region of positions 4 → 7, which might be mainly due to (i).[4] Therefore, these two compounds could be classified as closely related cyclic peptides. However, their reported biological activities are quite different. Recently, we discovered that **2** significantly inhibited fat-accumulation both in vitro[2,3] and in vivo.[5] Meanwhile, **1** has only been investigated in vivo and is known to exhibit a potent hepatotoxic effect in rat which leads to death in rat and ducklings.[6,7] To further examine the biological activity of **1**, we recently achieved the chemical synthesis of **1** and evaluated its fat-accumulation inhibitory activity against 3T3-L1 adipocytes.[8] While **1** exhibited a weak inhibitory effect at high concentration (IC_{50} 55 μM), it was also quite cytotoxic at the same concentration. To obtain a better understanding of structure–activity relationships (SAR) of **1** as well as of the relation between **1** and **2**, we developed a synthesis of novel rhizonin A analogues, which led to the discovery of a selective fat-accumulation inhibitor against 3T3-L1 adipocytes.

First, we focused on the two *N*Me-FurAla moieties in **1**, which do not exist in **2**. Uniquely, rhizonin A (**1**) (and its sister compound rhizonin B[1]) is the only natural product that contains an unusual FurAla moiety (including N-methylated variant) as a structural component. Thus, we designed the *di*-phenylalanine-substituted



Rhizonin A (**1**)
cyclo-[D-Val[1]-L-Val[2]-*N*Me-L-FurAla[3]-L-Leu[4]-*N*Me-L-Ala[5]-*N*Me-D-FurAla[6]-D-*allo*-Ile[7]]

Ternatin (**2**)
cyclo-[D-*allo*-Ile[1]-*N*Me-L-Ala[2]-*N*Me-L-Leu[3]-L-Leu[4]-*N*Me-L-Ala[5]-*N*Me-D-Ala[6]-β-OH-D-Leu[7]]

Figure 1. Structures of rhizonin A (**1**) and ternatin (**2**).

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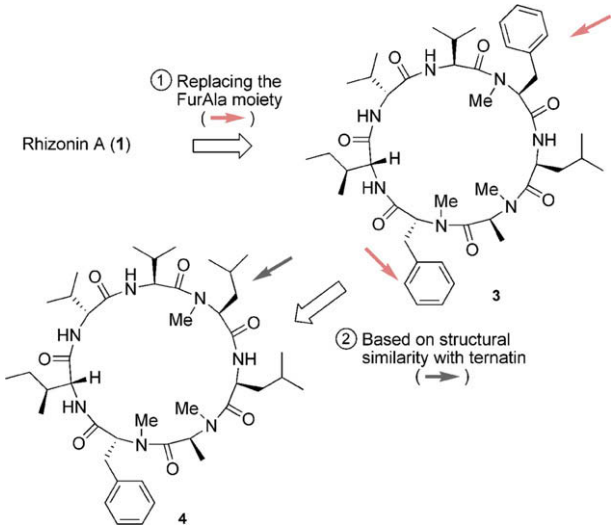


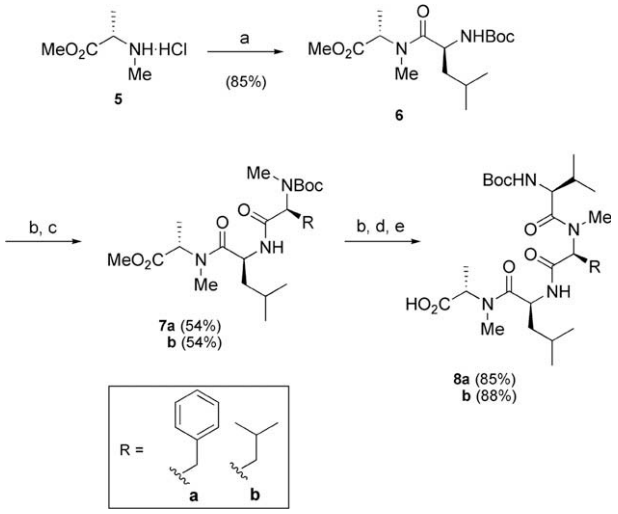
Figure 2. Design of rhizonin A analogues **3** and **4**.

analogue [*N*Me-L-Phe[3], *N*Me-D-Phe[6]]rhizonin A (**3**) to examine the influence of unusual *N*Me-FurAla moieties on bioactivity (Fig. 2). Moreover, with the concept of hybridization in mind, we sought to synthesize other analogue [*N*Me-L-Leu[3], *N*Me-D-Phe[6]]rhizonin A (**4**), which has three amino-acid sequences as in **2** on the right side of the chemical structure.

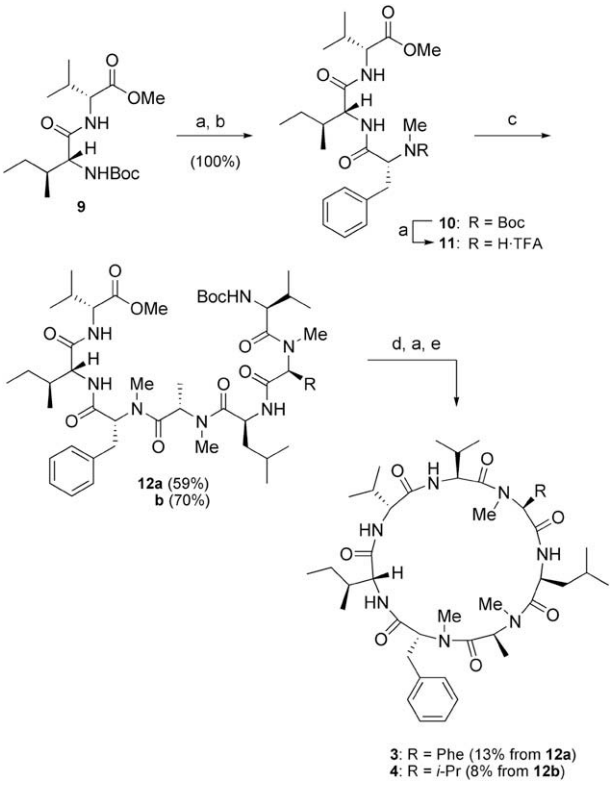
The syntheses of analogues **3** and **4** were slightly modified from the synthetic route toward **1**. First, we prepared the right fragments **8a** and **8b**, which are shown in Scheme 1. The starting material *N*Me-L-Ala-OMe hydrochloride (**5**) was coupled with Boc-L-Leu-OH in the presence of PyBroP to give dipeptide **6**.

Removal of the Boc group in 50% TFA/CH_2Cl_2 followed by amide-bond formation with the corresponding Boc-amino acids provided tripeptides **7a** and **7b**, respectively. Finally, sequential Boc deprotection, coupling, and methyl ester hydrolysis afforded the desired **8a** and **8b**.

Next, the left fragment **11** was prepared from the known dipeptide **9**[9] in the same manner (Scheme 2). Removal of the Boc group in **9** followed by coupling with Boc-*N*Me-D-Phe-OH afforded tripeptide **10**, which was then subjected to Boc deprotection to give



Scheme 1. Synthesis of the right fragments **8a** and **8b**. Reagents and conditions: (a) Boc-L-Leu-OH, PyBroP, DIPEA, CH_2Cl_2; (b) 50% TFA/CH_2Cl_2; (c) Boc-*N*Me-L-Phe-OH for **7a**, Boc-*N*Me-L-Leu-OH for **7b**, HATU, DIPEA, CH_2Cl_2; (d) Boc-L-Val-OH, HATU, DIPEA, CH_2Cl_2; (e) LiOH, *t*-BuOH, THF, H_2O.



Scheme 2. Synthesis of rhizonin A analogues **3** and **4**. Reagents and conditions: (a) 50% TFA/CH_2Cl_2; (b) Boc-*N*Me-D-Phe-OH, HATU, DIPEA, CH_2Cl_2; (c) **8a** (1.0 equiv) for **3**, **8b** (1.0 equiv) for **4**, HATU, DIPEA, CH_2Cl_2 (d) LiOH, *t*-BuOH, THF, H_2O; (e) HATU, HOAt, DIPEA, DMF, CH_2Cl_2 (1.5 mM).

11. Fragment couplings of **11** with **8a** and **8b** were conducted between the carboxyl group in the L-Leu[4] moiety and the amino group in the *N*Me-L-Ala[5] moiety to give heptapeptides **12a** and **12b** in moderate yield. Sequential methyl ester hydrolysis and Boc deprotection of **12a** and **12b** provided the corresponding cyclic precursors. Finally, the key macrolactamizations between the carboxyl group in the D-Val[1] moiety and the amino group in the L-Val[2] moiety were performed in the presence of HATU (2.0 equiv) and HOAt (2.0 equiv) at low concentration (1.5 mM). After HPLC purification of the crude materials, rhizonin A analogues **3**[10] and **4**[11] were obtained in 13% yield from **12a** and 8% yield from **12b** (in three steps), respectively.

The results of the in vitro fat-accumulation-inhibition assay for synthetic rhizonin A analogues **3** and **4**, as well as rhizonin A (**1**) and ternatin (**2**), are shown in Table 1. Cell viability was calculated independently to exclude undesired fat-accumulation inhibition arising from the toxicity of the tested compounds.

Based on the results, both **3** and **4** more strongly inhibited fat-accumulation than the parent compound **1**, though **2** was the most bioactive among the compounds tested. Hybrid compound **4** was

Table 1
Inhibitory effects of synthetic analogues on fat-accumulation against 3T3-L1 adipocytes and their cell viability[a]

Compound	Fat-accumulation inhibitory effect: IC_{50} (μM)	Cell viability: IC_{50} (μM)
Rhizonin A (**1**)	55 ± 3.7	70% at 62[b] (μM)
3	42 ± 1.1	>240
4	5.6 ± 2.1	>240
Ternatin (**2**)	0.027 ± 0.003	0.28 ± 0.03

[a] Values are means of quadruplicate determinations.
[b] Not tested at higher concentrations.

10-fold more potent than **1**. The overall potency of inhibitory effect is in the order (**2**>) **4** > **3** > **1**. Thus, modifications on the right half of **1** that imitates the sequence of ternatin (**2**) should greatly strengthen the bioactivity.

On the other hand, the toxic profiles of the synthetic analogues showed an opposite tendency. Interestingly, **3** and **4** were found to be less toxic (IC_{50} > 240 μM), while **1** showed considerable cytotoxicity (the expected IC_{50} value for cell viability is about 62–176 μM) at IC_{50} value for fat-accumulation inhibition. The selectivity indices [SI; IC_{50} value for cytotoxicity/that for fat-accumulation] of **3** and **4** were >12.3 and >43, and therefore higher than that of **1** (the expected SI is about 1–3). This result strongly suggests that the two FurAla moieties are responsible for the potent cytotoxicity of **1** against 3T3-L1 adipocytes. Moreover, hybrid analogue **4** had a greater SI than **2** (SI = 10). Therefore, **4** may be a plausible candidate that possesses a selective and/or effective fat-accumulation inhibitory effect against 3T3-L1 murine adipocytes.

In summary, two novel analogues of rhizonin A (**1**) were designed on the basis of structural hybridization with the potent fat-accumulation inhibitor ternatin (**2**). The modification of the two *N*Me-FurAla moieties in the structure of **1** led to analogues with more potent and selective fat-accumulation inhibitory activities. Further chemical and biological studies on **1**, including its SAR, are now underway.

Acknowledgments

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10. *Spectroscopic data for* ***3***: $[\alpha]^{23}_{D}$ −15.4° (*c* = 0.15, $CHCl_3$); IR ($CHCl_3$) 3326, 2933, 2337, 1650, 1506 cm^{-1}; ^1H NMR (400 MHz, C_6D_6) δ 7.85 (d, *J* = 9.6 Hz, 1H), 7.77 (d, *J* = 9.2 Hz, 1H), 7.41 (d, *J* = 7.2 Hz, 1H), 7.35 (s, 1H), 7.22–6.93 (m, 10H), 5.93 (dd, *J* = 12.4, 5.2 Hz, 1H), 5.89 (d, *J* = 10.0 Hz, 1H), 5.70–5.62 (m, 1H), 4.90 (t, *J* = 9.2 Hz, 1H), 4.71 (t, *J* = 9.2 Hz, 1H), 4.38 (q, *J* = 6.8 Hz, 1H), 4.00–3.96 (m, 1H), 3.89 (dd, *J* = 15.2, 5.2 Hz, 1H), 3.66–3.58 (m, 1H), 3.40 (dd, *J* = 8.4, 4.8 Hz, 1H), 2.80 (dd, *J* = 15.2, 12.4 Hz, 1H), 2.71 (s, 3H), 2.69 (s, 3H), 2.63 (s, 3H), 2.33–2.25 (m, 1H), 2.24–2.12 (m, 1H), 1.90–1.75 (m, 2H), 1.55–1.47 (m, 1H), 1.37–1.27 (m, 2H), 1.25 (d, *J* = 6.8 Hz, 6H), 1.18–1.13 (m, 1H), 1.05 (d, *J* = 6.8 Hz, 3H), 1.02 (d, *J* = 6.8 Hz, 3H), 0.96 (t, *J* = 7.2 Hz, 3H), 0.94 (d, *J* = 6.8 Hz, 3H), 0.91 (d, *J* = 6.4 Hz, 3H), 0.79 (d, *J* = 6.8 Hz, 3H), 0.47 (d, *J* = 7.2 Hz, 3H); HRMS (FAB) calcd for $C_{46}H_{69}N_7O_7Na$ $(M+Na)^+$ 854.5196, found 854.5175.
11. *Spectroscopic data for* ***4***: $[\alpha]^{23}_{D}$ −2.5° (*c* = 0.15, $CHCl_3$); IR ($CHCl_3$) 3300, 2966, 2881, 2363, 1644, 1499 cm^{-1}; ^1H NMR (400 MHz, C_6D_6) δ 7.87 (d, *J* = 9.2 Hz, 1H), 7.62 (d, *J* = 9.2 Hz, 1H), 7.36 (s, 1H), 7.18–6.90 (m, 5H), 6.85 (d, *J* = 6.4 Hz, 1H), 6.09–5.90 (m, 2H), 5.71–5.62 (m, 1H), 5.43–5.26 (m, 1H), 4.98 (t, *J* = 9.2 Hz, 1H), 4.79 (t, *J* = 9.2 Hz, 1H), 4.39 (q, *J* = 7.2 Hz, 1H), 3.48–3.35 (m, 2H), 3.06 (s, 3H), 2.65 (s, 3H), 2.58 (s, 3H), 2.54–2.45 (m, 2H), 2.33–2.25 (m, 1H), 2.05–1.77 (m, 4H), 1.32 (d, *J* = 6.8 Hz, 3H), 1.23 (d, *J* = 6.8 Hz, 3H), 1.06–0.80 (m, 4H), 1.05 (d, *J* = 6.8 Hz, 3H), 1.00 (d, *J* = 6.8 Hz, 3H), 0.99 (d, *J* = 6.8 Hz, 3H), 0.96 (t, *J* = 4.8 Hz, 3H), 0.92 (d, *J* = 6.4 Hz, 3H), 0.87 (d, *J* = 6.4 Hz, 3H), 0.84 (d, *J* = 6.4 Hz, 3H), 0.82 (d, *J* = 6.4 Hz, 3H), 0.75 (d, *J* = 6.4 Hz, 3H); HRMS (FAB) calcd for $C_{43}H_{71}N_7O_7Na$ $(M+Na)^+$ 820.5313, found 820.5291.