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Synthesis and antifungal activities of cyclic octa-lipopeptide burkholdine analogues

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ABSTRACT

Synthesis and antifungal activity of cyclic octapeptide derivatives of burkholdines are described. To construct cyclic octapeptides, the combination of Fmoc-SPPS and cyclization with DIC/HOBt in the solution phase was employed. Synthesized peptides were evaluated for antifungal activity with MIC values against *Saccharomyces cerevisiae*, *Aspergillus oryzae*, and *Candida viswanathii*. As a result, the lipid side chain and the stereochemistry of each amino acid of Bk-1097 analogues significantly affected antifungal activity.

Burkholdines (Bks) were isolated from a culture of *Burkholderia ambifaria 2.2N* by Schmidt's group.[1] Bk-1097 (**1**), one of the burkholdines, is a cyclic octapeptide composed of eight amino acids, including β-hydroxytyrosine, β-hydroxyasparagine and new fatty acyl amino acid (FAA). Although the absolute stereochemistry of β-hydroxytyrosine residue and FAA moiety of Bk-1097 (**1**) has recently been reported to be 2*R*,3*R* and 3*R*,5*R*,6*S*,7*S*, respectively,[2] there is no complete assignment for identification using NMR experimentation with synthetic compounds and/or X-ray analysis. In contrast, potent biological activity of 1.6 μg/ml has been shown against the yeast *Saccharomyces cerevisiae* and the fungus *Aspergillus niger*. These activities are 16-fold more potent than amphotericin B,[3,4] which is used in antifungal therapy. Burkholdines, together with xylocandins[5] and occidiofungins,[6,7] are promising antifungal agents that produce fungal cell membrane defects, although the inhibitory mechanism and target enzyme are unresolved. Since there are many peptides with a variety of bioactivities, we are interested in the appearance of potent antifungal activities by the combination of amino acids.[8,9] Especially, cyclic peptides tend to show the potent activities because of employment of the rigid framework and preservation of sidechain trajectories. We herein planned to synthesize and evaluate various simplified Bk-1097 analogues to find cyclic peptides with more potent antifungal activity (Fig. 1).

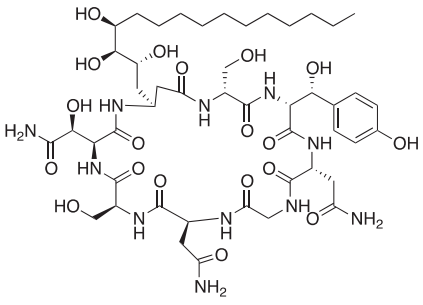

Figure 1. Bk-1097 (**1**).

Bk-1097 analogues were synthesized via Fmoc solid phase peptide synthesis (Fmoc-SPPS) using L- or D-usual amino acids in place of β-hydroxytyrosine, β-hydroxyasparagine and FAA. Synthesized peptides were evaluated by the minimum inhibitory concentration (MIC) against *Saccharomyces cerevisiae*, *Aspergillus oryzae* and *Candida viswanathii* (Fig. 2).

As depicted in Scheme 1, the designed cyclic peptides were synthesized. Linear precursors were prepared by Fmoc-SPPS using 2-chlorotrityl chloride resin[10,11] followed by cleavage from the resin with 20% HFIP/CH_2Cl_2. Intramolecular cyclization of the linear peptide and then final deprotection yielded designed cyclic lipopeptide after purification by preparative HPLC.

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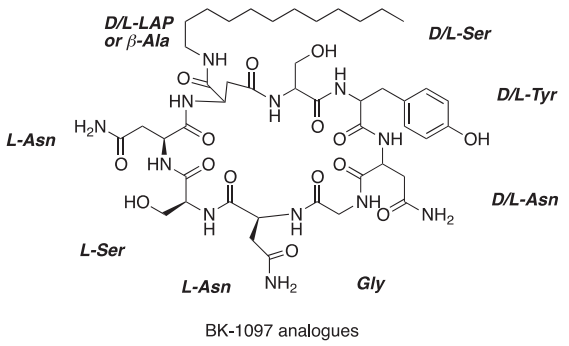


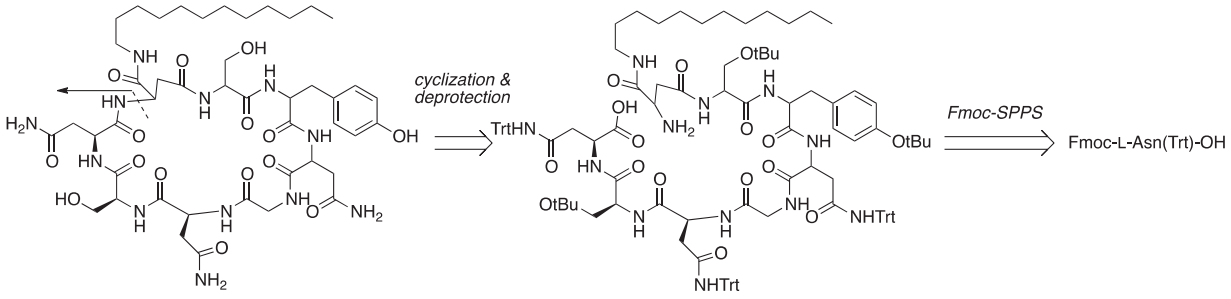
Figure 2. Bk-analogues with D/L-amino acids.

To replace the FAA residue with simplified lipo-β-amino acid, we designed *N*-lauryl-3-amino-4-carbamoylpropanoic acid (LAP) (**2**), which was coupled with linear peptides as the last component of the solid support. To prepare both enantiomers of LAP residues, Fmoc-L/D-Asp(*t*Bu)-OH was condensed with dodecylamine using BOP/Et_3N in DMF to give *N*-lauryl aspartic acid derivatives, and subsequent removal of *t*Bu by TFA afforded both enantiomers of Fmoc-LAP-OH (**3**) in moderate yield (Scheme 2).

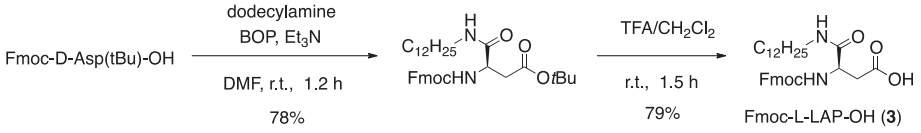
Synthesis of cyclic peptide (**7**) is shown in Scheme 3. Fmoc-Asp(Trt)-OH was linked onto 2-chlorotrityl chloride resin by esterification with DIPEA to obtain resin (**4**). After removing the Fmoc group by treatment with 20% piperidine/DMF, the remaining **7** amino acids were coupled by the general conditions. Resin was treated with 20% HFIP/CH_2Cl_2 to provide linear peptide (**5**) in 64% yield from loading on the resin. Linear peptide (**5**) was cyclized under the condition of DIC/DIPEA/HOBt·H_2O for 20 h in 55% yield. The macrocyclization between N-terminus and C-terminus was monitored by RP-HPLC and ESI TOF-MS. According to the HPLC profile, the hydrophilic peak as the linear peptide gradually dissolved for 20 h and a subsequent more hydrophobic peak appeared as a single compound. It showed no by-products as dimeric or trimeric peptides. Finally, the desired cyclic peptide (**7**) was obtained by deprotection using TFA/TIPS/H_2O (95:5:5) and subsequent purification of preparative RP-HPLC in 57% yield.[12,13] In addition to these anticipated issues, an unexpected problem arose when final deprotection with TFA was attempted, namely, the observation of three abundant by-products with masses 17, 34 and 51 daltons smaller than the desired product. We anticipated that intramolecular lactonizations between the amide groups of Asn side chains and the hydroxyl groups of Ser or Tyr residues proceeded to afford the hydrophobic products on a detectable scale. To attempt the several conditions for deprotection of the cyclic peptides, we found that TIPS as a scavenger was effective to avoid side reactions. Preparation of cyclic peptides (**8**–**25**) was similar to the method in Scheme 3 (Table 1).

As the sequence of octapeptides, intramolecular cyclization of linear peptides was effective with D-Ser-D-Tyr (entries 8 and 10) or D-Tyr-D-Asn (entry 15) motifs. A couple of D-amino acids on the peptide sequences induce the formation of the turn structure and subsequent intra-molecular macro-cyclization proceeded smoothly. However, cyclization of linear peptides with D-Ser-D-Tyr-D-Asn sequences (entries 3, 17 and 19) gave moderate yield. Stereochemistry of LAP units, which are the sole β-amino acid residues, did not affect the cyclization processes (Table 1).

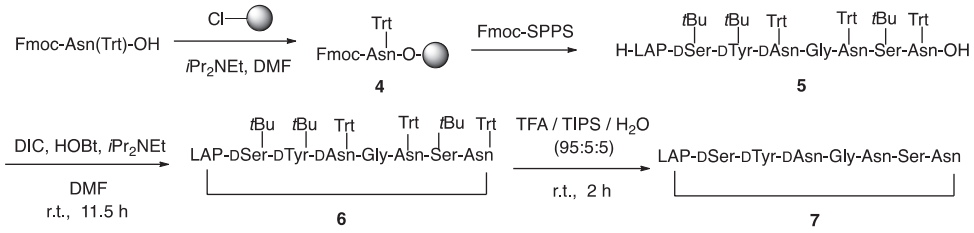
The antifungal activities of burkholdine analogues (**7**–**25**) against *S. cerevisiae*, *A. oryzae* and *C. viswanathii* are shown in Table 2[14]



Scheme 1. Synthetic plan for cyclic lipopeptides.

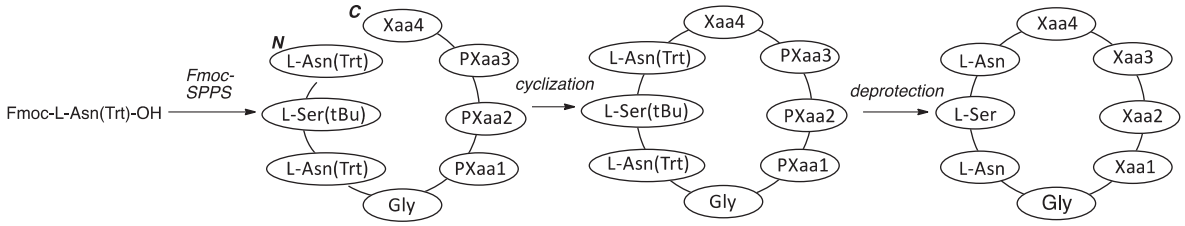


Scheme 2. Synthesis of lipo-amino acids, Fmoc-LAP-OH (**3**).



Scheme 3. Synthesis of cyclic octalipopeptide (**7**).

Table 1
Sequences of synthesized cyclic octapeptides and their chemical yields



Entry	Peptide	Xaa4	Xaa3	Xaa2	Xaa1	Fmoc-SPPS (%)	Cyclization		Deprotection (%)	Total yield (%)
							(h)	(%)		
1	**8**	β-ALa	Ser	Tyr	Asn	86	62	55	25	12
2	**9**	β-ALa	D-Ser	Tyr	D-Asn	55	30	45	44	11
3	**10**	β-ALa	D-Ser	D-Tyr	D-Asn	68	25	46	6	2
4	**11**	D-LAP	D-Ser	Tyr	Asn	38	23	63	54	13
5	**12**	D-LAP	Ser	Tyr	Asn	57	21	46	68	8
6	**13**	L-LAP	D-Ser	Tyr	Asn	51	13	35	64	4
7	**14**	L-LAP	Ser	Tyr	Asn	45	19	32	97	8
8	**15**	D-LAP	D-Ser	D-Tyr	Asn	32	18	73	54	18
9	**16**	D-LAP	Ser	D-Tyr	Asn	60	22	54	41	21
10	**17**	L-LAP	D-Ser	D-Tyr	Asn	61	15	66	24	14
11	**18**	L-LAP	Ser	D-Tyr	Asn	23	17	59	76	8
12	**19**	D-LAP	Ser	Tyr	D-Asn	15	23	64	76	21
13	**20**	D-LAP	Ser	D-Tyr	D-Asn	50	22	69	27	30
14	**21**	L-LAP	Ser	Tyr	D-Asn	62	14	69	42	18
15	**22**	L-LAP	Ser	D-Tyr	D-Asn	52	18	79	27	11
16	**23**	D-LAP	D-Ser	Tyr	D-Asn	50	26	34	50	5
17	**24**	D-LAP	D-Ser	D-Tyr	D-Asn	45	21	46	47	8
18	**25**	L-LAP	D-Ser	Tyr	D-Asn	54	16	64	55	19
19	**7**	L-LAP	D-Ser	D-Tyr	D-Asn	64	20	55	57	20

Analogues (**8**), (**9**) and (**10**) with the involvement of β-Ala shown no antifungal activities against *S. cerevisiae*. Analogues (**20**), (**25**) and (**7**) exhibited great potency with the MIC values of 50, 50, and 25 μg/mL, respectively. It is suggested that D-form stereochemisty of Asn residue in Xaa1 position was essential and, additionally, stereochemistry of Ser residue in Xaa3 and LAP was likely to show opposite stereochemistry. Antifungal activities of other sequences were weak and therefore we predicted that there was a correlation between the stereochemistry of naturally produced burkholdines and those of analogues. Although the MIC values of *A. oryzae* have the same tendency as those of *S. cerevisiae*, antifungal activities against *C. viswanathii* were not shown. Burkholdines also tended to result in lower antifungal activities against *Candida* than against *Saccharomyces* or *Aspergillus* (Table 2).

For more potent antifungal activity, replacement with LLAP instead of β-Ala or D-LAP gave antifungal compounds. In contrast, comparison of L-Tyr and D-Tyr produced no significant difference in antifungal activities (Fig. 3).

In conclusion, we synthesized 29 Bk-1097 analogues through the use of Fmoc-SPPS. From the results of the MIC assay, the lipid chain at the Xaa4 position and stereochemistry at Xaa1, Xaa3, and Xaa4 positions were found to be important for antifungal activity. We will plan a structure–activity relationship study for more potent antifungal activity.

Table 2
MIC values for cyclic peptides (**7**–**25**)

Peptide	MIC (μg/mL)[a]			Peptide	MIC (μg/mL)[a]		
	S. cerevisiae	*A. oryzae*	*C. viswanathii*		*S. cerevisiae*	*A. oryzae*	*C. viswanathii*
8	>800	>800	800	**17**	>800	800	>800
9	800	>800	>800	**18**	>800	400	800
10	800	800	>800				
				19	800	*800*	>800
11	400	>800	>800	**20**	50	100	>800
12	200	>800	>800	**21**	>800	400	800
13	400	>800	>800	**22**	800	400	>800
14	800	200	>800				
				23	400	400	>800
15	800	800	>800	**24**	400	800	>800
16	800	>800	>800	**25**	50	100	400
				7	25	50	800

[a] MIC value of Amphotericin B control against *S. cerevisiae* was 0.8 μg/ml.

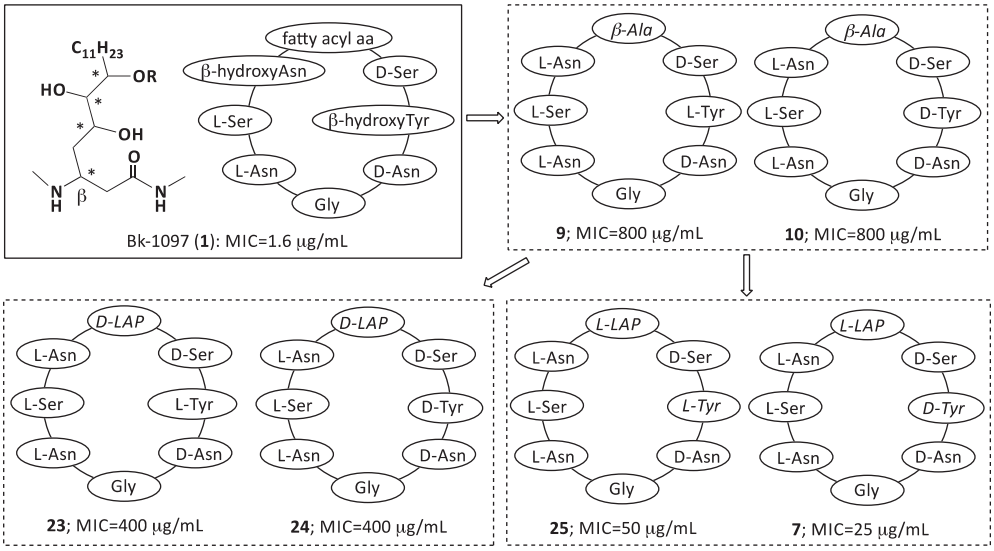


Figure 3. Structure–activity relationship study of Bk analogues.

Acknowledgments

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References and notes

1. Tawfik, K.; Jeffs, P.; Bray, B.; Dubay, G.; Falkinham, J. O., III; Mesbah, M.; Youssef, D.; Khalifa, S.; Schmidt, E. W. *Org. Lett.* **2010**, *12*, 664.
2. Lin, Z.; Falkinham, J. O., III; Tawfik, K. A.; Jeffs, P.; Bray, B.; Dubay, G.; Cox, J. E.; Schmidt, E. W. *J. Nat. Prod.* **2012**, *75*, 1518.
3. (a) Croatt, M. P.; Carreia, E. M. *Org. Lett.* **2011**, *13*, 1390; (b) Volmer, A. A.; Szpilman, A. M.; Carreira, E. M. *Nat. Prod. Rep.* **2010**, *27*, 1329.
4. Ernst, B.; Magnani, J. L. *Nat. Rev. Drug Disc.* **2009**, *8*, 661.
5. Bisacchi, G. S.; Hockstein, R. D.; Koster, W. H.; Parker, W. L.; Rathnum, M. L.; Unger, S. E. *J. Antibiot.* **1987**, *40*, 1520.
6. Gu, G.; Smith, L.; Liu, A.; Lu, S. *Appl. Environ. Microbiol.* **2011**, *77*, 6189.
7. Lu, S.; Novak, J.; Austin, F. W.; Gu, G.; Ellis, D.; Kirk, M.; Wilson-Stanford, S.; Tonelli, M.; Smith, L. *Biochemistry* **2009**, *48*, 8312.
8. Seto, Y.; Takahashi, K.; Matsuura, H.; Kogami, Y.; Yada, H.; Yoshihara, T.; Nabeta, K. *Biosci., Biotechnol., Biochem.* **2007**, *71*, 1470.
9. Luo, J.; Wang, X.; Ma, L.; Kong, L. *Bioorg. Med. Chem. Lett.* **2007**, *17*, 4460.
10. Kikuchi, M.; Watanabe, Y.; Tanaka, M.; Akaji, K.; Konno, H. *Bioorg. Med. Chem. Lett.* **2011**, *21*, 4865.
11. Kikuchi, M.; Nosaka, K.; Akaji, K.; Konno, H. *Tetrahedron Lett.* **2011**, *52*, 3872.
12. *Synthesis of cyclic peptide (**7**):* To a suspension of 2-Cl-trityl chloride resin (40 mg, 1.6 mmol/g) in DMF (1 ml) were added Fmoc-Asn(Trt)-OH (115 mg, 0.192 mmol) and DIPEA (22 μl, 0.128 mmol). The mixture was stirred for 2 h at 25 °C. To a resultant resin was added 20% piperidine in DMF and the mixture was stirred for 30 min at 25 °C. Fmoc-Ser(*t*Bu)-OH (74 mg, 0.192 mmol), Fmoc-Asn(Trt)-OH (115 mg, 0.192 mmol), Fmoc-Gly-OH (57 mg, 0.192 mmol), Fmoc-D-Asn(Trt)-OH (115 mg, 0.192 mmol), Fmoc-D-Tyr(*t*Bu)-OH (88 mg, 0.192 mmol), Fmoc-D-Ser(*t*Bu)-OH (74 mg, 0.192 mmol), Fmoc-LAP-OH (111 mg, 0.192 mmol) were successively condensed to this resin using DIC (30 μl, 0.192 mmol)/HOBt (29 mg, 0.192 mmol)/DIPEA (22 μl, 0.192 mmol) for 2 h. The resulting resin was treated with HFIP/CH_2Cl_2 (1:4, 5 ml) for 2 h and the mixture was filtrated. After evaporation of the HFIP, ether was added, and the resulting precipitate was purified by preparative HPLC (CH_3CN/H_2O = 88:12) to yield octapeptide (**5**) (79 mg, 0.0409 mmol, 64%) as a white powder. To a solution of octapeptide (**5**) (79 mg, 0.0409 mmol) in DMF (4.5 ml) were added DIC (32 μl, 0.204 mmol), HOBt (31 mg, 0.204 mmol) and DIPEA (21 μl, 0.123 mmol) and the mixture was stirred for 20 h at room temperature. After adding AcOEt and H_2O, the mixture was separated. The organic layer was washed with brine, dried over $MgSO_4$, filtered, and concentrated in vacuo. The residue was purified by column chromatography on silica gel (95:5 $CHCl_3$/MeOH) to give the cyclic peptide (**6**) as a white powder. Cyclicpeptide (**6**) (43 mg, 0.0225 mmol) was added to TFA/TIPS/H_2O (95:5:5; 2 ml) and the mixture was stirred for 2 h at room temperature. After evaporation of TFA, ether was added, and the resulting precipitate was purified by preparative HPLC (CH_3CN/H_2O = 55.8:44.2) to yield a cyclicoctapeptide (**7**) (13 mg, 0.0129 mmol, 57%) as a white powder.
13. *Analytical HPLC using a Cosmosil 5C18 (10 mm × 250 mm) column: linear peptide (**5**):* rt 11.96 min (CH_3CN gradient; 80–100% in 30 min). ESI TOF-MS. Calcd 1933.36 for $C_{114}H_{138}N_{12}O_{16}Na$; found: 1933.36 for $[M+H]^+$. Cyclic peptide (**6**): 32.59 min (CH_3CN gradient; 80–100% in 30 min and then 100% CH_3CN for 10 min). ESI TOF-MS. Calcd 1936.01 for $C_{114}H_{136}N_{12}O_{15}Na$; found: 1936.34 for $[M+Na]^+$. Cyclic peptide (**7**): 7.06 min (CH_3CN gradient; 40–70% in 30 min). ESI TOF-MS. Calcd 1041.50 for $C_{45}H_{70}N_{12}O_{15}Na$; found: 1041.59 for $[M+H]^+$.
14. *Assay protocol:* The minimal inhibitory concentration (MIC) of burkholdine analogues was determined with broth by the usual twofold serial dilution method. Growth media for *S. cerevisiae* strain X2180-1A, *A. oryzae* strain 1-Murasaki-1 (Bio's) and *C. viswanathii* strain NBRC10321 were YPD medium. Before starting the MIC assay, the microbial cells were grown in broth for 2 days. The concentration of the burkholdine analogues started with 1 mg/L in the medium containing 0.1% DMSO, and underwent twofold serial dilution. Then, the serial twofold dilution solution was poured into a 96-well microtiter plate and inoculated with microbial suspensions of about 5.0×10^3 cells. The MIC was defined as the lowest concentration of antimicrobial agent that inhibited the development of visible growth after 24 h of incubation at 30 °C in each case.