Synthesis of Aureobasidin B Analogs and Their Antifungal Activity Against *Candida albicans*

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ABSTRACT: *Aureobasidins (Abs) are a class of cyclodepsipeptides with interesting antifungal properties but they are difficult to synthesize. This study aimed to synthesize analogs of aureobasidin B (AbB) by a combination of solid- and solution-phase synthesis and to investigate their antifungal properties. The linear peptides were synthesized on 2-chlorotrityl chloride resin with Fmoc strategy and a range of coupling reagents including HATU/HOAt, HBTU/HOBt, and BTC/sym-collidine. The cyclization step was undertaken in the solution phase. Four cyclic nonapeptides (NP1-NP4) and ten cyclic heptapeptides (HP1-HP2, HP4-HP11) were successfully synthesized and characterized. The analogs NP1, NP4, HP1, and HP2 demonstrated moderate antifungal activity against Candida albicans.*

KEYWORDS: *Aureobasidin B, Cycloheptapeptides, Cyclononapeptides, Solid-phase peptide synthesis.*

INTRODUCTION

Aureobasidins (Abs) are highly *N*-methylated cyclodepsipeptides first isolated from the black yeast *Aureobasidium pullulans* R106 [1]. Subsequently, approximately thirty congeners, including AbA and AbB (Fig. 1), have been isolated and characterized [2-7].

Aureobasidins possess antimicrobial activity against several pathogens including *Candida* species, *Histoplasma capsulatum*, *Blastomyces dermatitidis*, and *Cryptococcus neoformans* [8, 9]. Table 1 presents the antifungal properties of Abs against *Candida albicans* and *Cryptococcus neoformans* showing that AbA is the most active derivative against both pathogens with MIC values <0.05 μg/mL and 78 μg/mL, respectively [9]. Moreover, *Aureobasidium pullulans* is important for the potent biocontrol of postharvest diseases [10, 11], therefore further investigation of the aureobasidin class is warranted due to their biologically interesting properties.

The initial synthesis of aureobasidins was conducted in solution phase [12-14]. *Kurome et al*. (1996) reported the solution-phase synthesis of AbA using a *tert*-butyloxycarbonyl (Boc)- and phenacyl (Pac)-protection strategy and PyBrop coupling agent. *Jao et al*. (1996) used Boc and benzyl ester protection with mixed anhydride coupling agents, whereas *Schmidt et al*. (1998) used HATU to couple the segments. The linear nonapeptide of AbA was cyclized using PyBrop (*Kurome et al*. (1996); *Schmidt et al*. (1998)) or BOP *Jao et al*. (1996). Our research group reported the total synthesis of an AbL analog [*2S*,*3S*-Hmp]-AbL and *Leu* [6] -AbK using a combination of solid- and solution-phase methodology

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1021-9986/2022/12/4104-4116 13/$/6.03

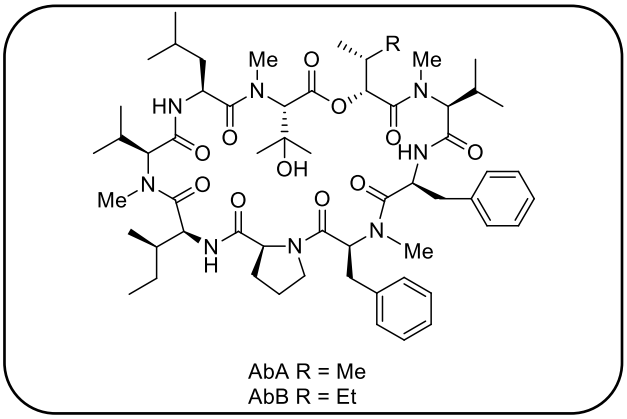


Fig. 1: Structure of AbA and AbB

that was more rapid but gave a low product yield [15, 16]. The synthesis was performed on 2-chlorotrytil resin with the Fmoc strategy and all amide bond formations were facilitated using HATU/HOAT, HBTU/HOBt, or BTC/sym-collidine. In *Maharani et al.* (2014), ester formation was undertaken in solution using DCC-based Steglich reaction before attachment to the resin, while in *Rahim et al.* (2020), the ester bond was constructed on resin using DIC. Both linear precursors were cyclized using HATU in a dilute solution.

Synthesis of these highly *N*-methylated depsinonapeptides is challenging, particularly the coupling of the hydroxy acid moiety and poor attachment of later residues onto the depsihexapeptidyl resin. Purification of the linear depsipeptides and their subsequent cyclization is problematic, with the process often culminating in the isolation of small amounts of very nonpolar cyclic products. Also, several residues are costly to obtain.

Due to some synthetic issues above, studies on aureobasidin analogues are considered as they may be easier and less expensive to generate but biologically active. Previous studies showed that analogues can give better biological properties [17, 18]. Another approach to preparing Abs analogs was reported by *Wuts et al.* (2015) using a commercially available AbA (Takara Bio Inc., Otsu, Japan) through the Suzuki coupling approach [19].

Design of Synthesis

Analysis of the primary structure of the known aureobasidins (shown in Table 1) revealed that six out of the nine positions of the cyclic depsipeptides were relatively inexpensive and usually unchanged between structures. The generalized aureobasidin structure in Fig. 2

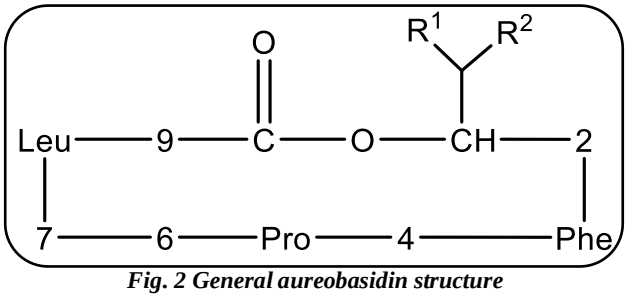


Fig. 2 General aureobasidin structure

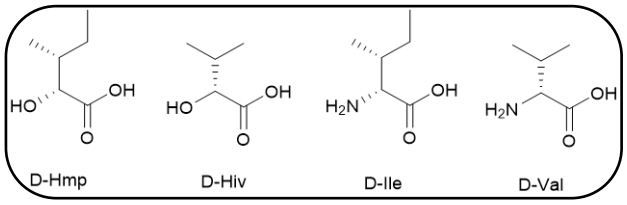


Fig. 3 Structures of D-Hmp, D-Hiv, D-Ile, and D-Val

shows that Phe3, Pro5, and Leu8 are conserved in all structures while a small amount of diversity was observed for MeVal2, MePhe4, and MeVal7 mostly due to the incorporation of the non-methylated variant.

Position 1 of AbA contains 2*R*-hydroxy-3*R*-methylpentanoic acid (D-Hmp) while AbB contains 2*R*-hydroxyisovaleric acid (D-Hiv) (Fig. 3), with AbA being more potent against *C. albicans* and *C. neoformans* than AbB [9]. No structure-activity relationship studies have been conducted to determine if the incorporation of a hydroxy acid is critical to the biological activity of the aureobasidins [3, 20].

In the synthesis of [2*S*,3*S*-Hmp]-AbL,[15] the hydroxy acid residue was attached to the resin as a depsidipeptide due to the difficulties of esterification during chain elongation. Unfortunately, hydrolysis of the ester bond during synthesis resulted in a low product yield. It was proposed that D-Hmp and D-Hiv could be replaced by their corresponding D-amino acids of D-isoleucine and D-valine, respectively (Fig. 3). Synthesis of cyclic peptides containing D-valine would be prioritized given that D-isoleucine is more expensive. It was expected that the synthesis of peptide analogs of the depsipeptides would be simpler avoiding hydrolysis of the ester bond.

Position 6 of aureobasidins typically contains L-*allo*isoleucine (L-*a*Ile) and aureobasidin I (AbI) with leucine (Leu) at position 6 has similar activity to aureobasidin A (AbA) against *C. albicans* (Table 1) [9]. In addition, aureobasidin C (AbC) and aureobasidin U2 (AbU2) have comparable antifungal activities to AbA 1 against *C.*

Table 1: Classification and antifungal activity of the aureobasidin family members

Classification	Residues						Antifungal activity (MIC, µg/mL) [10]	
	2	4	6	7	9	R^1, R^2	*Candida albicans*	*Cryptoccocus neoformans*
β-OHMeVal at position 8								
AbA	MeVal	MePhe	*a*Ile	MeVal	β-OHMeVal	CH_3, C_2H_5	<0.05	0.78
AbB	MeVal	MePhe	*a*Ile	MeVal	β-OHMeVal	CH_3, CH_3	0.1	1.56
AbC	MeVal	MePhe	Val	MeVal	β-OHMeVal	CH_3, C_2H_5	0.1	1.56
AbE	MeVal	β-OHMePhe	*a*Ile	MeVal	β-OHMeVal	CH_3, C_2H_5	<0.5	3.12
AbF	MeVal	MePhe	*a*Ile	Val	β-OHMeVal	CH_3, C_2H_5	0.2	25
AbI	MeVal	MePhe	Leu	MeVal	β-OHMeVal	CH_3, C_2H_5	<0.05	25
AbT1	MeVal	MePhe	*a*Ile	MeVal	β-OHMeVal	CH_3, C_2H_5	0.0125	1.56
AbT2	MeVal	MePhe	*a*Ile	MeLeu	β-OHMeVal	CH_3, C_2H_5	0.10	1.56
AbS3	MeVal	MePhe	*a*Ile	MeVal	β-OHMeVal	CH_2OH, C_2H_5	0.10	0.80
AbS4	MeVal	MePhe	*a*Ile	MeVal	β-OHMeVal	CH_3, $CH(CH_3)OH$	0.10	1.6
AbS2b	MeVal	MePhe	*a*Ile	MeVal	β-OHMeVal	CH_3, CH_2CH_2OH	0.2	1.6
AbS2a	MeVal	MeTyr	*a*Ile	MeVal	β-OHMeVal	CH_3, C_2H_5	0.4	3.1
AbS1	MeVal	MePhe	Met (O)	MeVal	β-OHMeVal	CH_3, C_2H_5 CH_3, C_2H_5	25	>25
N-MeVal at position 8								
AbG	MeVal	MePhe	*a*Ile	MeVal	MeVal	CH_3, C_2H_5	3.12	>25
AbK	MeVal	MePhe	*a*Ile	MeVal	MeVal	CH_3, CH_3	1.56	>25
AbL	MeVal	MePhe	Val	MeVal	MeVal	CH_3, C_2H_5	6.25	>25
AbM	MeVal	Phe	*a*Ile	MeVal	MeVal	CH_3, C_2H_5	6.25	>25
AbP	MeVal	MePhe	*a*Ile	Val	MeVal	CH_3, C_2H_5	3.12	>25
AbR	MeVal	β-OHMePhe	*a*Ile	MeVal	MeVal	CH_3, C_2H_5	25	>25
AbT3	MeVal	β-OHMePhe	*a*Ile	MeVal	MeVal	CH_3, CH_3	0.39	>25
AbT4	MeVal	β-OHMePhe	*a*Ile	MeVal	MeVal	CH_3, CH_3	0.10	1.56
AbU1	Val	β-OHMePhe	*a*Ile	MeVal	MeVal	CH_3, CH_3	0.78	>25
AbU2	Val	β-OHMePhe	Val	MeVal	MeVal	CH_3, CH_3	0.025	6.25
Other residues at position 8								
AbD	MeVal	MePhe	*a*Ile	MeVal	γ-OHMeVal	CH_3, C_2H_5	0.2	25
AbH	MeVal	MePhe	*a*Ile	MeVal	Val	CH_3, C_2H_5	1.56	>25
AbJ	MeVal	MePhe	*a*Ile	MeVal	N, β-MeAsp	CH_3, C_2H_5	0.78	25
AbN	MeVal	MePhe	*a*Ile	MeVal	DH3,4MeVal	CH_3, C_2H_5	1.56	>25
AbO	MeVal	MePhe	*a*Ile	MeVal	β-OHMePhe	CH_3, C_2H_5	12.5	>25
AbQ17	MeVal	MePhe	*a*Ile	MeVal	MePhe	CH_3, C_2H_5	12.5	>25

Key: MeVal = L-*N*-methylvaline, Val = L-valine, MePhe = L-*N*-methylphenylalanine, Phe = L-phenylalanine, β-OHMePhe = β-hydroxy- L-*N*-methylphenylalanine, MeTyr = L-*N*-methyltyrosine, *a*Ile = L-*allo*-isoleucine, Met(O) = L-methionine(O), β-OHMeVal = β-hydroxy- L-*N*-methylvaline, γ-OHMeVal = γ-hydroxy- L-*N*-methylvaline, *N*,β-MeAsp = L-*N*,β-dimethylaspartic acid, DH3,4-MeVal = L-*N*-methyl-3,4-didehydrovaline

albicans where valine (Val) is at position 6 (Table 1). Based on these examples, it was planned to replace the expensive L-*a*Ile with either the inexpensive Leu or Val residues.

The hydroxy group of the βOHMeVal residue at position 9 was suggested by Ishida *et al*. to be essential for receptor binding [21]. Studies by *Takesako et al*. showed that this residue was required for biological activity whereby substitution of this residue with MeVal caused the loss of antifungal properties against *C. albicans* and *C. neoformans* [20]. This loss can be seen by comparing the structures and activities of several aureobasidins such as AbA with AbG, AbB with AbK, AbC with AbL, and AbF with AbP (Table 1).

Replacement of βOHMeVal with an amino acid which is affordable but would retain the antifungal activity was desired. Threonine (Thr), containing a β-hydroxy group, was proposed as a suitable residue at position 9 for βOHMeVal. Using threonine which was not methylated

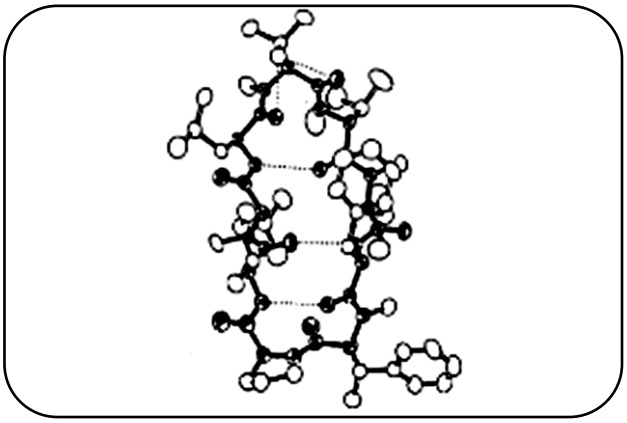


Fig. 4: Molecular conformation of AbE (structure taken from Ishida et al. [16]

would have the advantage of providing a more polar cyclic peptide for purification.

Some changes of biological function have been observed from the removal of a methylated residue as shown by comparing AbT4 with AbU1, AbA with AbF, and AbG with AbM (Table 1). However, this needs to be further explored to evaluate the balance between polarity and activity. The hydrophobicity of both the linear and cyclic depsipeptides had previously been a significant issue during their preparation, particularly during purification stages [20].

X-ray crystallography studies by *Ishida et al.* showed an arrowhead structure where the βOHMeVal was considered important for interaction with the receptor while the proline was likely to be essential for the turn forming the other end of the cyclic depsipeptide [21, 22]. The structures of AbA and AbE were also shown to be stabilized by three hydrogen bonds, as shown in Fig. 4 for AbE.

Two of the intramolecular hydrogen bonds are between Phe3 and *a*Ile6 as Phe-NH--O=C-*a*Ile and Phe-C=O—HN-*a*Ile with the third bond observed between Leu8 and Hmp1 as Leu-NH --O=C-Hmp. Between these two pairs of hydrogen bonding residues are a third pair of residues, MeVal2 and MeVal7. Removing these three pairs of opposing residues in turn to produce three types of cyclic heptapeptides would allow the effects on hydrophobicity and activity to be evaluated. In addition, the synthesis of these compounds would be shorter and less expensive than the corresponding cyclic nonapeptides.

It was decided to prioritize less expensive but most altered, cyclic peptides. If improved activity was required, then the synthesis of the more time-consuming and expensive analogs using D-Ile, D-Hiv, D-Hmp, βOHMeVal and *a*Ile would be pursued.

The initial cyclic nonapeptides proposed were analogs of AbB requiring the hydroxy acid of D-Hiv to be replaced by the amino acid D-Val and βOHMeVal being substituted by Thr. The residue of *a*Ile6 present in AbB would be replaced by the less expensive residues leucine found in AbI and valine found in AbC and AbL to give the cyclononapeptide (NP) compounds NP1 and NP2. These amino acid changes would be compared to the original residue *a*Ile in NP3. The same strategy with D-Val and Leu but βOHvaline as a replacement for βOHMeVal would be followed for NP4. A summary of the planned analogs is shown in Table 2.

The strategy for the synthesis of the cyclic heptapeptide (HP) analogs was based on NP1, which was one of the most changed cyclic nonapeptides with three substituted residues. The first analog would eliminate a pair of residues, D-Val1 and Leu8 to give HP1. The second analog of HP2 would have MeVal2 and MeVal7 removed while Phe3 and Leu6 would be eliminated from HP3. Seven variations of HP2 (Table 2) were also synthesized, considering non-methylated Phe at residue 4 (HP5 and HP6), alternating Leu at position 6 to Ile and Val (HP7 and HP8) and replacement of Thr with Ser or Tyr (HP9-11).

It was decided to prioritize the synthesis of the less expensive, though most altered, cyclic nonapeptides starting with NP1 and HP1. If improved activity was required, then the synthesis of the more time-consuming and expensive analogs using D-Ile, D-Hiv, D-Hmp, βOHMeVal and aIle would be pursued.

The methods employed for the synthesis of [2*S*,3*S*-Hmp]-AbL would be applied to the synthesis of aureobasidin cyclic nonapeptide and cyclic heptapeptide analogs. Proline was still used as the first amino acid to be attached to the chlorotrityl resin due to its properties as a turn inducer. The Fmoc protection strategy and the use of HATU/HOAt, HBTU/HOBt, and BTC/collidine for coupling would also be employed.

EXPERIMENTAL SECTION

General procedures

Commercial-grade reagents and solvents were used without purification unless required. Solvents were purified or dried as described by *Perrin* and *Armarego* [25]. All Fmoc amino acids were purchased from Peptide Solutions Ltd. NMR spectra were performed on a Bruker

Table 2: Primary structure of aureobasidin B and proposed analogs

Compounds	Residues								
	5	4	3	2	1	9	8	7	6
Known aureobasidin									
AbB	Pro	MePhe	Phe	MeVal	D-Hiv	β-OHMeVal	Leu	MeVal	*a*lle
Cyclononapeptide analogs									
NP1	Pro	MePhe	Phe	MeVal	D-Val	Thr	Leu	MeVal	Leu
NP2	Pro	MePhe	Phe	MeVal	D-Val	Thr	Leu	MeVal	Val
NP3	Pro	MePhe	Phe	MeVal	D-Val	Thr	Leu	MeVal	*a*lle
NP4	Pro	MePhe	Phe	MeVal	D-Val	β-OHVal	Leu	MeVal	Leu
Cycloheptapeptide analogs									
HP1	Pro	MePhe	Phe	MeVal	-	Thr	-	MeVal	Leu
HP2	Pro	MePhe	Phe	-	D-Val	Thr	Leu	-	Leu
HP3	Pro	MePhe	-	MeVal	D-Val	Thr	Leu	MeVal	-
HP4	Pro	MePhe	Phe	Val	-	Thr	-	MeVal	Leu
HP5	Pro	Phe	Phe	-	D-Val	Thr	Leu	-	Leu
HP6	Pro	Phe	Phe	-	D-Val	Thr	Leu	-	Val
HP7	Pro	MePhe	Phe	-	D-Val	Thr	Leu	-	Ileu
HP8	Pro	MePhe	Phe	-	D-Val	Thr	Leu	-	Val
HP9	Pro	Phe	Phe	-	D-Val	Ser	Leu	-	Leu
HP10	Pro	MePhe	Phe	-	D-Val	Ser	Leu	-	Leu
HP11	Pro	MePhe	Phe	-	D-Val	Tyr	Leu	-	Leu

Avance 300 (300.13 MHz) instrument or a Bruker Avance 500 (500.19 MHz) instrument. ^{1}H-NMR was performed at 300.13 MHz or 500.19 MHz and ^{13}C-NMR was obtained at 75 MHz or 125 MHz. Deuterochloroform (Cambridge Isotope Laboratories Inc.) was used as a solvent and as an internal standard unless otherwise stated. Low Resolution-Electrospray Ionisation Mass Spectrometry (LR-ESI) was carried out using a Bruker Daltonics (Germany) Esquire 6000 ion trap mass spectrometer at 300ºC with a scan rate of 5500 m/z/sec. High Resolution-Electrospray Ionisation Mass Spectrometry (HR-ESI) was performed on an Agilent 6224 TOF LC/MS coupled to an Agilent 1290 Infinity (Agilent, Palo Alto, CA). All data were acquired and reference mass corrected via a dual-spray electrospray ionization (ESI) source. Analytical RP-HPLC was performed on a Shimadzu HPLC instrument I using LC-20AD Prominence Liquid Chromatography, SIL-20A HT Prominence Autosampler, and SPD-M20A Prominence Diode Array Detector and a Shimadzu HPLC instrument II using LC-20AD Prominence Liquid Chromatography, SIL-20A HT Prominence Autosampler, SPD-M20A Prominence Diode Array Detector, CTO-20A Prominence Column Oven and CBM-20A Prominence Communications Bus Module. Semi-preparative RP-HPLC was performed on a Beckman HPLC instrument using a System Gold 125 Solvent Module and System Gold 166 Detector and, on a Shimadzu HPLC, instrument I using LC-20AD Prominence Liquid Chromatography and SPD-M20A Prominence Diode Array Detector. The solvents were HPLC grade acetonitrile and ultrapure water with the addition of 0.1% TFA.

Solid-phase peptide synthesis of linear peptides

Solid-phase synthesis of linear peptides was performed on 2-chlorotrityl resin using a protocol for the synthesis of [2*S*,3*S*-Hmp]-AbL [15]. All Fmoc-deprotection steps were undertaken by employing 20% piperidine in DMF for 30 minutes. A range of coupling reagents such as HATU/HOAt, HBTU/HOBt, and BTC/sym-collidine were employed and the synthesis was manually monitored by LRMS. The resin cleavage step was completed using the same protocol as described, except for the peptidyl resin with a *t*-Bu protecting group.

Resin cleavage of peptidyl resin with a t-Bu protecting group

A cleavage cocktail of 95% of trifluoroacetic acid in water (10 mL) was added to the peptidyl-trichlorotrityl resin (0.45 mmol) and the yellow resin turned bright red. It was shaken for one hour, then washed with the cleavage cocktail and dry dichloromethane. The combined fractions were evaporated and the resulting residue was dissolved in acetonitrile:water (1:1, 5 mL). The solution was freeze-dried to obtain a brown solid. A sample of the crude solution (20 µl,1 mg/1mL in 50% acetonitrile in water) was subjected to analytical RP-HPLC on a Phenomenex Jupiter 10 µ, C18 column (250 mm × 4.6 mm), 20–90% acetonitrile in water over 30 min, with a flow rate of 1 mL/min at 40°C and monitored at 240 nm.

Cyclization

The linear precursor (0.0014 mmol) was dissolved in dry dichloromethane (0.27 mM), then 2-(7-aza-1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (6.3 µmol) and *N,N*-diisopropylethylamine (0.013 mmol) were added. The solution was stirred under nitrogen for 24 hours, concentrated *in vacuo* at room temperature, and purified by semi-preparative RP-HPLC (acetonitrile:water in the presence of 0.1% TFA, 50% ACN over 40 minutes, followed by 50-90% ACN over 40 minutes, 2 mL/min) to obtain the cyclic product as a white solid. ^{1}H-NMR data was collected when a sufficient sample was available.

NP1: ESI-MS *m/z* $[M+Na]^+$ 1080.6; HR-MS (ESI) *m/z* Calcd. for $C_{58}H_{89}N_9O_{10}$ requires $[M+Na]^+$ 1080.6474 Found 1080.6473.

NP2: ESI-MS *m/z* $[M+H]^+$ 1044.7; HR-MS (ESI) *m/z* Calcd. for $C_{58}H_{89}N_9O_{10}$ requires $[M+H]^+$ 1044.6419 Found 1044.6497

NP3: ESI-MS *m/z* $[M+H]^+$ 1058.7; HR-MS (ESI) *m/z* Calcd. for $C_{58}H_{89}N_9O_{10}$ requires $[M+Na]^+$ 1080.6474 Found 1080.6477

NP4: ESI-MS *m/z* $[M+H]^+$ 1072.7; HR-MS (ESI) *m/z* Calcd. for $C_{58}H_{89}N_9O_{10}$ requires $[M+Na]^+$ 1094.6630 Found 1094.6636

HP1: ESI-MS *m/z* $[M+H]^+$ 846.5; HR-MS (ESI) *m/z* Calcd. for $C_{58}H_{89}N_9O_{10}$ requires $[M+Na]^+$ 846.5129 Found 846.5135

HP2: ESI-MS *m/z* $[M+H]^+$ 832.06; HR-MS (ESI) *m/z* Calcd. for $C_{45}H_{65}N_7O_8$ requires $[M+H]^+$ 832.4973 Found 832.4979; ^{1}H-NMR (400 MHz, $CDCl_3$): δH 0.60 (3H, d, J= 8.0), 0.85 (3H, d, *J*=8.0), 0.91-0.99 (12H, m), 1.29 (3H, d, *J*=4.00), 1.61-1.70 (2H, m), 1.79-1.89 (4H, m), 2.37 (2H, m), 2.72-2.77 (1H, m), 3.07-3.18 (4H, m), 3.07 (3H, s, N-Me), 3.45-3.50 (2H, m), 3.65 (1H, m), 3.97 (1H, m), 4.14 (1H, m), 4.32-4.40 (5H, m), 4.52 (1H, m), 4.87-4.90 (1H, m), 5.13 (1H, q, *J*=8), 6.16 (1H, bs), 7.15-7.34 (10H, m), 7.40 (1H, bs), 8.28 (1H, bs).

HP4: ESI-MS *m/z* $[M+H]^+$ 832.5; HR-MS (ESI) *m/z* Calcd. for $C_{45}H_{65}N_7O_8$ requires $[M+H]^+$ 832.4973 Found 832.4972; ^{1}H-NMR (400 MHz, $CDCl_3$): δH 0.71 (3H, d, *J*= 4.0), 0.94 (9H, d, *J*=8.0), 0.89 (3H, t, *J*=4.0), 1.11 (3H, d, *J*= 8.0), 1.66-1.72 (2H, m), 1.33 (3H, d, 8.00), 1.89-1.94 (1H, m), 2.46-2.58 (2H, m), 2.89-2.96 (2H, m), 2.97 (3H, s, N-Me), 3.01-3.05 (2H, m), 3.11 (3H, s, N-CH3), 3.11-3.16 (2H, m), 3.22-3.25 (2H, m), 3.52 (1H, m), 3.73 (1H, m), 4.07 (1H, d, *J*=8), 4.06-4.09 (1H, m), 4.52-4.55 (1H, m), 4.55-4.57 (1H, m), 5.05-5.06 (3H, m), 5.08-5.09 (1H, d, *J*=4), 6.61 (1H, bs), 7.00 (1H, bs), 7.17-7.37 (10H, m), 8.02 (1H, bs), 8.49 (1H, bs).

HP5: ESI-MS *m/z* $[M+H]^+$ 818.03; HR-MS (ESI) *m/z* Calcd. for $C_{45}H_{65}N_7O_8$ requires $[M+H]^+$ 818.4816 Found 818.4817; ^{1}H-NMR (400 MHz, $CDCl_3$): δH 0.55 (3H, d, *J*=8), 0.71-0.73 (3H, d, *J*=8), 0.84-1.00 (12H, m), 1.19 (3H, d, *J*=4.00), 1.62-1.75 (2H, m), 1.91-2.07 (2H, m), 2.07-2.19 (4H, m), 2.20-2.29 (1H, m), 3.03-3.07 (4H, m), 3.11-3.17 (2H, m), 3.49-3.51 (2H, m), 4.16 (1H, m), 4.22-4.25 (1H, m), 4.31 (1H, m), 4.62-4.65 (2H, m), 4.82 (1H, m), 5.13-5.12 (2H, m), 6.85-7.35 (10H, m), 7.49 (1H, bs), 7.67 (1H, bs), 7.71 (1H, d, *J*=8), 7.85 (1H, s), 7.86 (1H, d, *J*=4), 8.60 (1H, s).

HP6: ESI-MS *m/z* $[M+H]^+$ 804.00; HR-MS (ESI) *m/z* Calcd. for $C_{45}H_{65}N_7O_8$ requires $[M+H]^+$ 804.4660 Found 804.4666; ^{1}H-NMR (400 MHz, $CDCl_3$): δH 0.62 (3H, d, *J*=8), 0.81 (3H, d, *J*=8), 0.85-1.05 (12H, m), 1.22 (3H, d, 8.00), 1.45 (1H, d, *J*=4), 1.71-1.77 (2H, m), 1.89-2.07 (2H, m), 2.09-2.33 (1H, m), 2.52-2.54 (1H, m), 2.83 (2H, bs), 2.94-2.99 (2H, m), 3.15-3.16 (2H, m), 3.44-3.63 (2H, m), 4.15 (1H, bs), 4.30 (1H, m), 4.30 (1H, m), 4.43 (2H, m), 4.55 (1H, m), 4.71 (1H, m), 5.10-5.15 (1H, m), 6.26 (1H, d, *J*=4), 7.09-7.36 (10H, m), 8.00 (1H, s).

HP7: ESI-MS *m/z* $[M+H]^+$ 831.5; HR-MS (ESI) *m/z* Calcd. for $C_{45}H_{65}N_7O_8$ requires $[M+H]^+$ 832.4973 Found 832.4972; ^{1}H-NMR (400 MHz, $CDCl_3$): δH 0.54 (3H, bs), 0.73-0.90 (9H, m), 0.88-0.90 (3H, m), 1.21 (3H, m), 1.32 (3H, d, *J*=4.00), 1.66-1.71 (3H, m), 1.80-1.81 (1H, m), 1.97-2.02 (1H, m), 2.32 (2H, m), 2.32 (1H, m), 2.55-2.59 (1H, m), 2.96 (3H, s, N-Me), 3.05 (4H,bs), 3.42 (2H, m), 3.53-3.58 (2H, m), 3.87 (1H, m), 4.12-4.19 (1H, m), 4.34-4.38 (2H, m), 4.46 (1H, bs), 4.72 (1H, m), 4.86 (1H, m), 5.01 (1H, m), 5.90 (1H, bs, NH), 7.03-7.23 (10H, m), 7.45 (1H, bs), 8.18 (1H, bs).

HP8: ESI-MS *m/z* $[M+H]^+$ 818.47; HR-MS (ESI) *m/z* Calcd. for $C_{45}H_{65}N_7O_8$ requires $[M+H]^+$ 818.4816 Found 818.4816; ^{1}H-NMR (400 MHz, $CDCl_3$): δH 0.54 (3H, d, *J*= 8.0), 0.84 (3H, d, *J*=8.0), 0.96-1.03 (12H, m), 1.32 (3H, d, *J*=4.00), 1.62 (1H, m), 1.80-1.91 (2H, m), 2.43 (2H, m), 2.67-2.72 (2H, m), 3.08 (3H, s, N-Me), 3.11-3.16 (4H, m), 3.48 (2H, m), 3.65 (2H, m), 3.95 (1H, m), 4.12-4.19 (1H, m), 4.30 (1H, m), 4.34 (1H, bs), 4.59 (2H, m), 4.86 (1H, m), 5.10-5.12 (1H, m), 6.03 (1H, bs), 7.12-7.33 (10H, m), 7.57 (1H, bs), 8.21 (1H, bs).

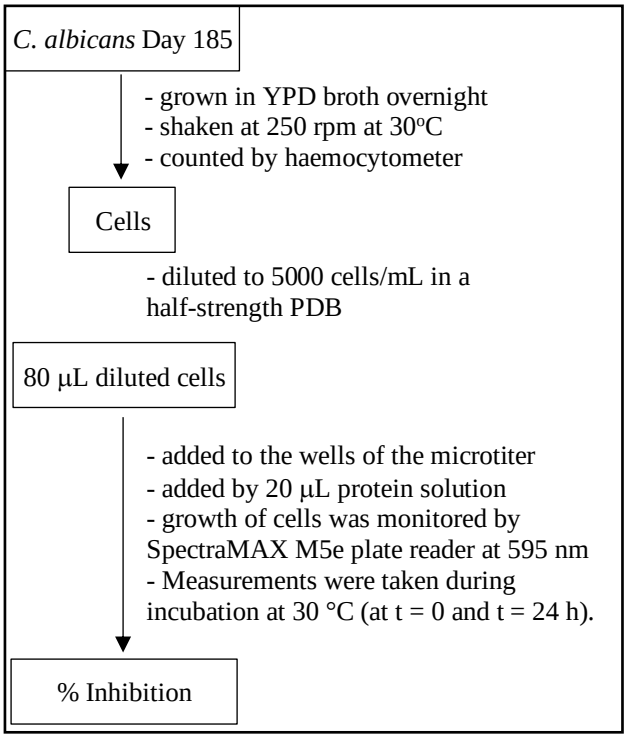
HP9: ESI-MS *m/z* $[M+H]^+$ 804.00; HR-MS (ESI) *m/z* Calcd. for $C_{45}H_{65}N_7O_8$ requires $[M+H]^+$ 804.4660 Found 804.4662; ^{1}H-NMR (400 MHz, $CDCl_3$): δH 0.31 (3H, d, *J*= 4.0), 0.65-0.90 (15H, m), 1.60-1.67 (3H, m), 1.86-1.91 (2H, m), 1.92-1.99 (2H, m), 2.10-2.20 (1H, m), 2.89-2.95 (2H, m), 2.92-2.95 (1H, m), 3.06-3.11 (3H, m), 3.39-3.49 (2H, m), 4.07-4.17 (2H), 4.17-4.23 (1H, m), 4.23-4.32 (1H, m), 4.47-4.50 (1H, m), 4.47-4.50 (1H, m), 4.79 (2H, m), 5.04-5.09 (1H, m), 6.13 (1H, d, J=8), 6.36 (1H, bs), 6.75 (1H, bs), 6.94-7.32 (10H, m), 7.85 (1H, bs), 8.17 (1H, bs), 8.55 (1H, bs, NH).

HP10: ESI-MS *m/z* $[M+H]^+$ 818.50; HR-MS (ESI) *m/z* Calcd. for $C_{45}H_{65}N_7O_8$ requires $[M+H]^+$ 818.4816 Found 818.4818; ^{1}H-NMR (400 MHz, $CDCl_3$): δH 0.65 (3H, d, *J*= 8.0), 0.82 1 (3H, d, *J*=8.0), 0.90-1.02 (12H, m), 1.63-1.67 (2H, m), 1.73-1.75 (4H, m), 2.35-2.38 (2H, m), 2.88 (1H, m), 3.04 (3H, s, N-Me), 3.11-3,17 (4H, m), 3.46 (2H, m), 3.50 (2H, m), 3.79 (1H, m), 4.10-4.12 (2H, m), 4.30 (2H, m), 4.38 (1H, CH), 4.47 (1H, m), 4.80 (1H, m), 5.12 (1H, m), 6.55 (1H, bs, NH), 7.12-7.33 (10H, m), 7.55 (1H, bs); 8.59 (1H, bs).

HP11: ESI-MS *m/z* $[M+H]^+$ 894.45; HR-MS (ESI) *m/z* Calcd. for $C_{45}H_{65}N_7O_8$ requires $[M+H]^+$ 894.5129 Found 894.5126; ^{1}H-NMR (400 MHz, $CDCl_3$): δH 0.42 (3H, d, *J*= 8.0), 0.78-0.98 (15H, m), 1.59-1.72 (1H, m), 1.64-1.72 (2H, m), 1.85-1.90 (4H, m), 2.10-2.20 (1H, m), 2.65-2.70 (1H, m), 3.03 (3H, s, N-Me), 3.03-3.14 (8H, m), 3.45-3.49 (1H, m), 3.59-3.64 (1H, m), 4.14-4.16 (1H, m), 4.20 (2H, m), 4.38-4.42 (1H, m), 4.58 (1H, m), 4.87-4.91 (1H, m), 5.05-5.11 (1H, m), 6.82-7.28 (14H, m), 7.60 (1H, bs), 8.35 (1H, bs).

Antifungal activity

The antifungal activity was assessed as previously reported [26]. Briefly, *C. albicans* DAY185 was grown in YPD broth overnight and shaken at 250 rpm at 30°C, then diluted to 5000 cells/mL in half-strength potato dextrose broth (PDB, Becton Dickinson) and 80 μL of the diluted cells were added a microtitre plate together with a protein solution (20 μL) to obtain a final concentration of 0–100 μg/mL. Growth was monitored by a SpectraMAX M5e plate reader (Molecular Devices) with a 9-well scan and absorbance at 595 nm at 30°C (at t = 0 and t = 24 h). The peptide samples were dissolved in methanol which resulted in a final methanol concentration of 5% for the top concentration of peptide. Methanol (5%) was also added to the no-treatment control.

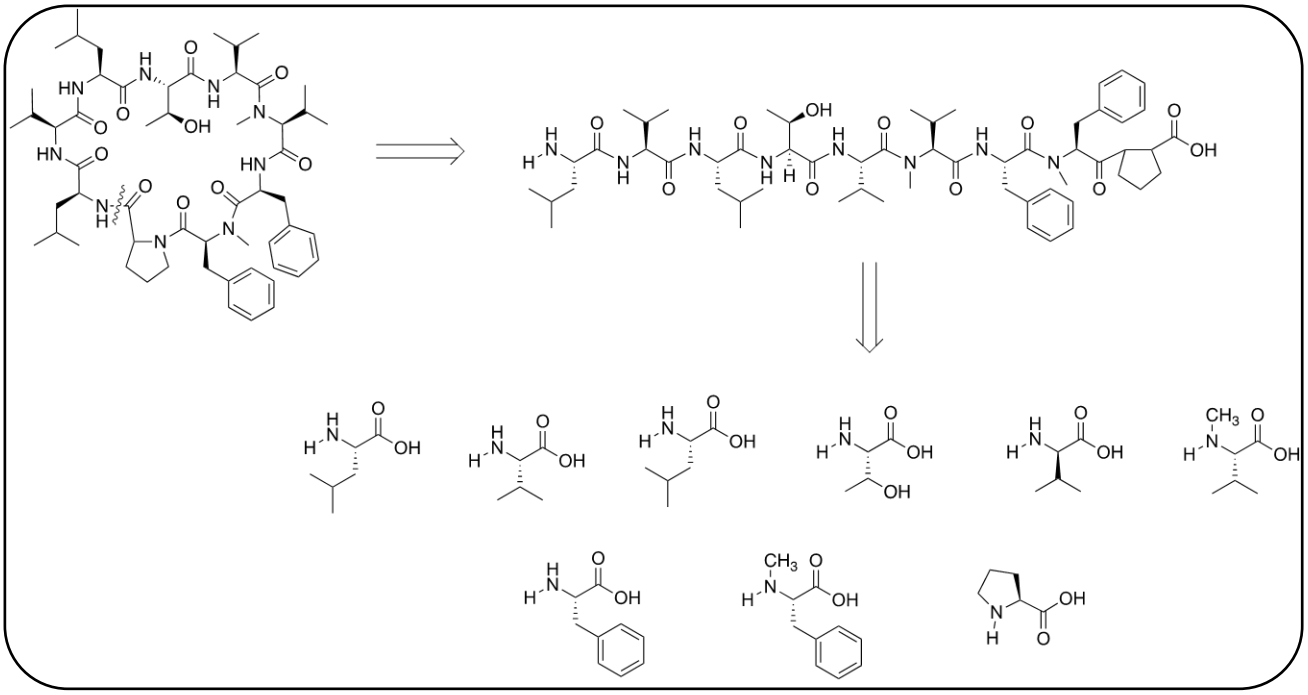


Scheme 1. Antifungal assay

RESULTS AND DISCUSSION

The linear precursors were prepared on 2-chlorotrityl resin using a Fmoc protection strategy. Proline was the first amino acid attached to the resin due to the ability of the proline to resist racemization and act as a turn inducer during cyclization [12, 23]. Retrosynthetic analysis of one of the analogs is presented in Scheme 2.

Fmoc deprotection was undertaken by employing a cocktail of 20% piperidine in DMF for 30 minutes. Coupling reactions were mostly facilitated by HBTU/HOBt or HATU/HOAt. BTC/*sym*-collidine was successfully employed for any coupling reaction involving *N*-methylated residues. HATU/HOAt was particularly effective for coupling reactions involving either the addition of or elongation from D-Val. A protocol of double coupling was required due to the β-branching of the D-Val.



Scheme 2: Retrosynthetic analysis of NP1

Coupling of Fmoc-L-Thr onto the tetrapeptidyl resin using HATU/HOAt was also difficult but improved by double coupling. Threonine and β-OHvaline were sometimes attached without the protection of the hydroxyl group, as reported by Fischer *et al*. for threonine [24]. However, some analogs were prepared using *t*-butyl protected threonine (HP7 and HP8) and *t*-butyl protected tyrosine (HP11). Fmoc-L-Val and Fmoc-L-*a*lle were attached to the octapeptidyl resin with a double-coupling protocol using HATU/HOAt but only a single coupling was needed when Fmoc-L-Leu was attached.

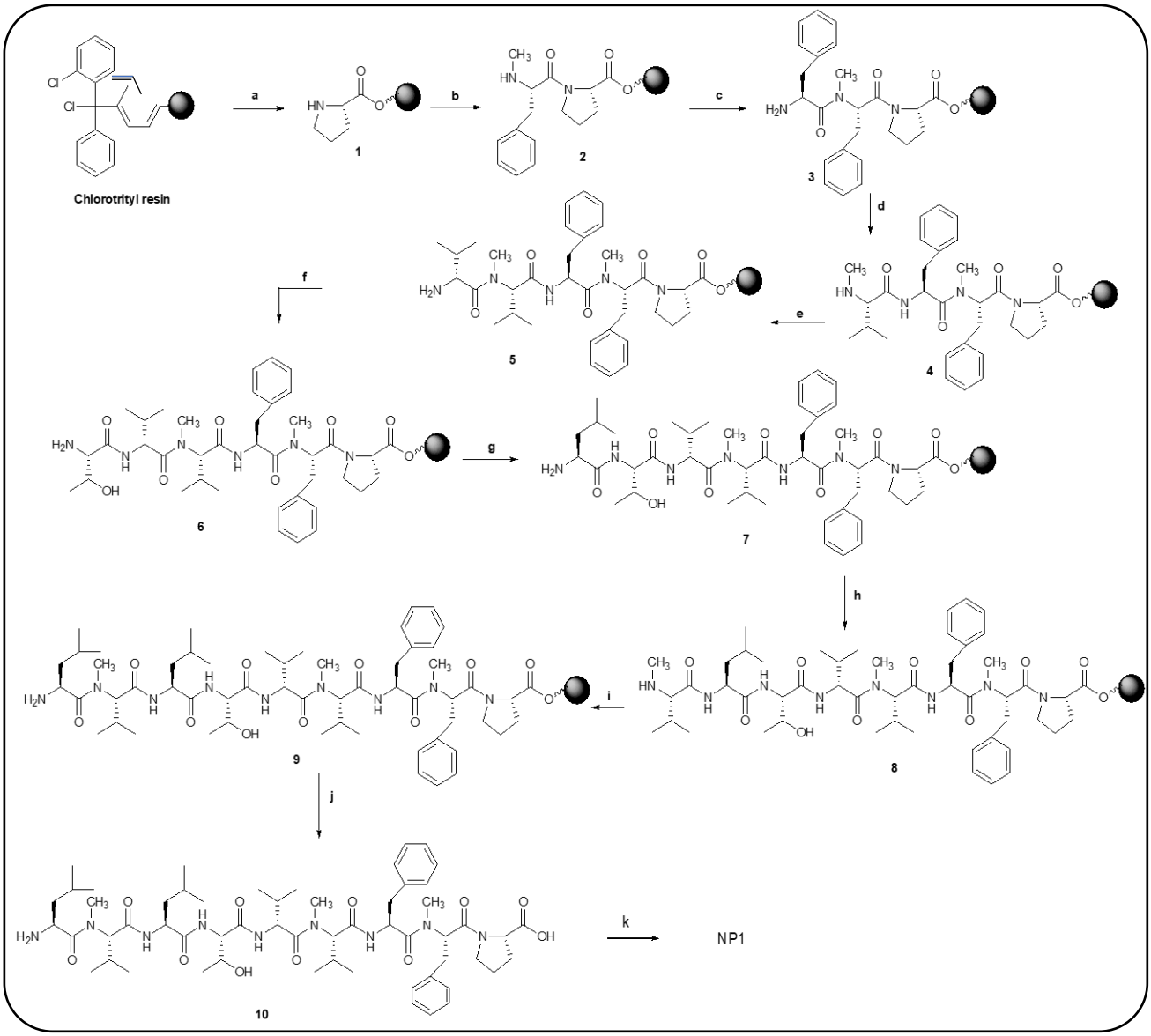
Unfortunately, the synthesis of HP3 was unsuccessful due to problems with the fourth coupling which could not be resolved. Double coupling using HATU/HOAt and a further double coupling using BTC/collidine reagent was undertaken but this only resulted in a minor component of the desired tetrapeptide and a major component of the unreacted tripeptide as determined by analytical RP-HPLC and ESI-MS. It seemed likely that this coupling failure was caused by the presence of two adjacent *N*-methylated residues but further investigations as to whether the methylation of this residue is necessary could be undertaken.

Two different cleavage cocktails were used to remove the peptides from the resin. A solution of 2% TFA in dichloromethane was used to cleave the linear peptides without protecting groups, whereas the linear peptides with *t*-butyl protecting groups were cleaved from the resin using 95% TFA in water. Purification of crude linear peptides (NP2-NP4) was problematic due to the significant amount of impurities present, thus the linear precursors were not always completely pure when cyclized.

Cyclizations were undertaken using either HATU or HBTU in dichloromethane and the presence of DIPEA. Once the cyclic product was observed by ESI-MS, the reaction mixture was concentrated under reduced pressure, then the resulting crude material was purified using semi-preparative RP-HPLC. The synthesis of one of the analogs is shown in Scheme 3.

The purification was relatively straightforward for both the cyclononapeptides (Fig. 5) and cycloheptapeptides (Fig. 6). All analogs were characterized using mass spectrometry techniques and analytical RP-HPLC, with retention times shown in Table 3. HP1 was found to be the most hydrophobic cycloheptapeptide due to the presence of three N-methylated residues.

Several cycloheptapeptide analogs, when sufficient material was available, were also studied by ^{1}H-NMR spectroscopy. The ^{1}H-NMR spectra of analogs HP2 and HP4-HP11 showed the expected proton signals differentiating between analogs with and without the *N*-methyl functionality. It was noted that the proton signals are present as single peaks in all the spectra, particularly



Scheme 3: Synthesis of NP1, (a) i. Fmoc-L-Pro, CH_2Cl_2, DIPEA, ii. Methanol, iii. 20% piperidine in DMF (b) i. Fmoc-L-N-methylphenylalanine, HBTU/HOBt, HATU/HOAt, or BTC/collidine, DIPEA, CH_2Cl_2: DMF (1:1), ii 20% piperidine in DMF (c) i. Fmoc-L-phenylalanine, HATU/HOAt, DIPEA, CH_2Cl_2: DMF (1:1), ii. 20% piperidine in DMF (d) i. Fmoc-L-methylvaline, HBTU/HOBt or HATU/HOAt, DIPEA, CH_2Cl_2: DMF (1:1), ii. 20% piperidine in DMF. (e) i. Fmoc-D-valine, HATU/HOAt, DIPEA, CH_2Cl_2: DMF (1:1), ii. 20% piperidine in DMF (f) i. Fmoc-L-threonine, HATU/HOAt, DIPEA, CH_2Cl_2: DMF (1:1), ii. Piperidine:DBU:DMF (2:2:96) (g) i. Fmoc-L-leucine, HATU/HOAt, DIPEA, CH_2Cl_2:DMF (1:1), ii. Piperidine:DBU:DMF (2:2:96) (h) i. Fmoc-L-N-methylvaline, HATU/HOAt, DIPEA, CH_2Cl_2:DMF (1:1), ii. Piperidine:DBU:DMF (2:2:96) (i) i. Fmoc-L-leucine, HATU/HOAt, DIPEA, CH_2Cl_2:DMF (1:1), ii. Piperidine:DBU:DMF (2:2:96), (j) 2.5% TFA in dichloromethane, 10 minutes, repeated twice (k) HATU in DCM.

for the *N*-methyl signals, indicating that only a single conformer is present. This observation was somewhat surprising as rotational isomerism was observed in the spectra of AbA and [2*S*,3*S*-Hmp]-AbL which revealed two conformers in a ratio of 57:43 and 61:39, respectively [11,14]. However, not all NH signals were revealed in the spectra due to the small amounts of the sample available for NMR analysis. The low yield of the final cyclic products is probably due to the poor attachment of Fmoc-D-Val, particularly when it was attached to the *N*-methylated peptidyl resin. Optimization of this step could include further investigation of the performance of other coupling reagents and the replacement of Fmoc-D-Val with Fmoc-D-Ile (D-Hmp analog) may be more successful.

Table 3: Retention times of all AbB analogues

Analogue	Retention time (min)	Method[a]
NP1	18.7 min	A
NP2	17.6 min	A
NP3	19.8 min	A
NP4	19.9 min	A
HP1	23.3 min	B
HP2	16.2 min	B
HP4	17.9 min	B
HP5	15.7 min	B
HP6	15.3 min	B
HP7	15.8 min	B
HP8	14.6 min	B
HP9	15.3 min	B
HP10	15.3 min	B
HP11	19.1 min	B

a General conditions: acetonitrile:water in the presence of 0.1% TFA, 1mL/min; Method A: 50% ACN for 10 mins then 50-90% ACN over 10 mins; Method B: 30-50% ACN over 10 mins, then by 50-90% ACN over 10 mins.

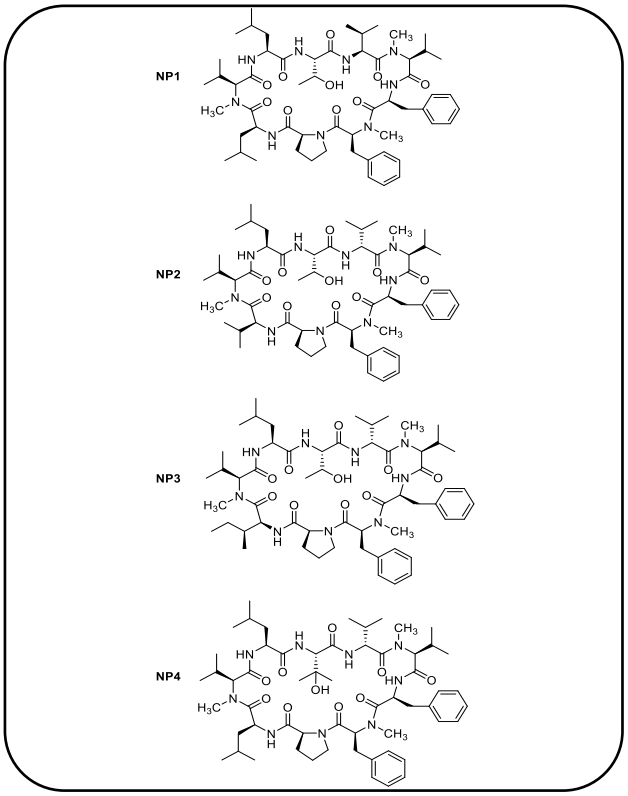


Fig. 5: Structure of cyclononapeptide analogs NP1-NP4

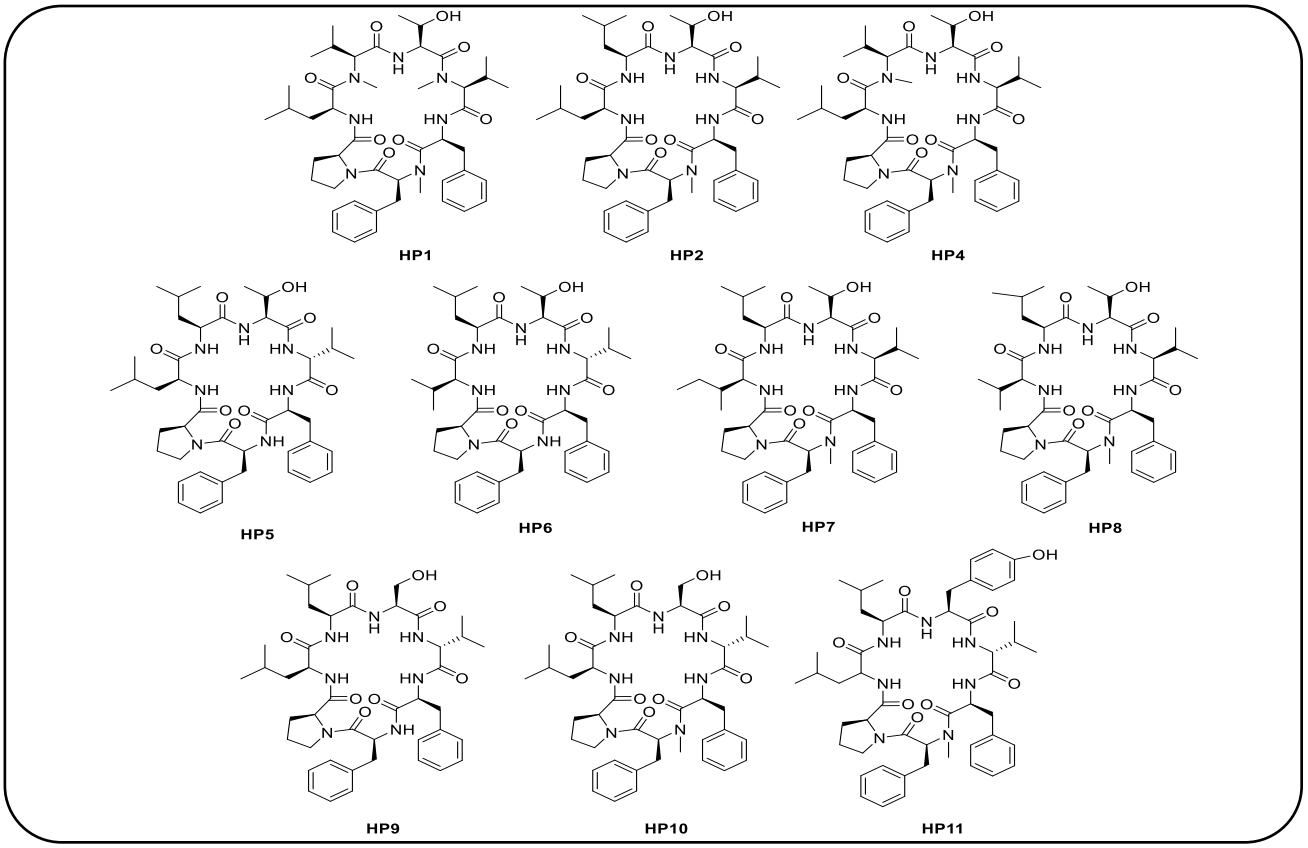


Fig. 6: Structures of the cycloheptapeptide analogs HP1, HP2, and HP4-11

Table 4: Antifungal activity of the cyclic peptide aureobasidin analogs against C. albicans at 50 and 100 µg/mL

Compounds	Growth (% relative to untreated control)	
	50 µg/mL	100 µg/mL
NP1	66.5	35.7
NP2	101.9	61.9
NP3	137.5	65.2
NP4	116.9	29.5
HP1	149.1	33.5
HP2	128.211	15.1
AbA	0	0

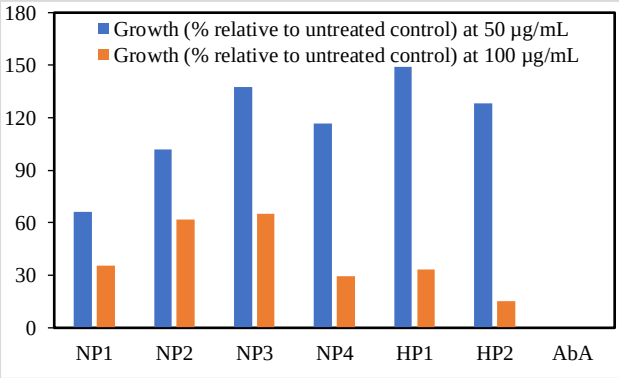


Fig. 7: Antifungal activity of aureobasidin analogues against Candida albicans at 50 and 100 µg/mL

Biological assays

The antifungal activity of the synthesized aureobasidin analogs was assessed against the human pathogen *C. albicans* DAY185 with the percentage growth (% growth) for each of the analogs at 50 and 100 µg/mL shown in Table 4. Unfortunately, cycloheptapeptides HP4-HP11 did not show activity when tested.

Some of the synthesized aureobasidin analogs had better antifungal activity than the first synthesized aureobasidin derivative, [2*S*,3*S*-Hmp]-AbL, which had an IC_{50} of >100 µg/mL. These analogs were NP1, NP4, HP1, and HP2, which were found to inhibit the growth of *C. albicans* by 74.3, 70.5, 66.5%, and 84.9% relative to the untreated control when applied at 100 µg/mL. In particular, cyclononapeptide NP1 showed some promising antifungal activity against *C. albicans* with an IC_{50} of 80 µg/mL. Analogs containing valine in NP2 and *allo*isoleucine in NP3 instead of leucine at the 6-position were not as active, with an IC_{50} >100 µg/mL. Threonine was shown to be an agreeable substitution for the more expensive residue β-OHvaline with NP4 having an IC_{50} of 88 µg/mL but the need for *N*-methylation of this residue still needs to be determined. The smaller cycloheptapeptide analog HP2 showed comparable activity to NP1 (IC_{50} 85 µg/mL) which is particularly valuable since this analog was the most straightforward to synthesize and purify. Based on these findings, several aureobasidin analogs including cyclononadepsipeptides and cycloheptadepsipeptides were identified for future synthesis to further explore and optimize the biological activity of this class of antifungal compounds.

CONCLUSIONS

Fourteen analogs of AbB were successfully synthesized using a combination of solid- and solution-phase methods. All linear peptides were prepared on 2-chlorotrytil chloride resin via a Fmoc strategy. HATU/HOAt was the most effective coupling agent to facilitate amide bond formation involving D-Val residue compared to HBTU/HOBt and BTC/sym-collidine. All cyclizations in solution phase were carried out using either HBTU or HATU without any issue including the purification. Modest antifungal activity was observed for four of the peptides tested, two cyclononapeptides (NP1 and NP4) and two cycloheptapeptides (HP1 and HP2) necessitating further investigation of the antifungal activity of these aureobasidin analogs.

Acknowledgments

We are greatly appreciative of the support given to this work by the La Trobe Institute for Molecular Science, La Trobe University. Special thanks are given to Dr. Belinda Abbott, Catherine S. Meister, Chamodi K. Gardhi, and Dr. Oscar Liu for their help during synthesis and characterization. I would also like to thank Dr. Brigitte M. Hayes, Dr. Nicole L. van der Weerdend, and Prof. Marilyn A. Anderson for evaluation of the biological activity of our peptides. We would also like to acknowledge the *La Trobe University-Comprehensive Proteomics Platform* for providing infrastructure and expertise. ATH and RM are grateful to the Indonesian government for the research grant of PDUPT-Kemenristekdikti 2021 (1207/UN6.3.1/PT.00/2021) and ALG (Prof. Ace Tatang Hidayat)-2021-Universitas Padjadjaran.

Supplementary Information

The datasets supporting this article have been uploaded as part of the electronic supplementary material.

Received: Nov. 16, 2021 ; Accepted: Jan. 17, 2022

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