Synthesis of Two Analogues of Xylapeptide A and Their Potency as New Antimicrobial Agent

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Abstract: *Xylapeptide A, derived from the fungus* Xylaria *sp. x* Sophora tonkinensis, *exhibits potent and selective antimicrobial properties. Our research group has successfully synthesized xylapeptide A. In our recent work, two xylapeptide A analogues (An1 and An2) were synthesized using a combination of solid- and solution-phase synthesis methods. The linear precursors of An1 and An2 were synthesized on 2-CTC resin with the Fmoc strategy. The coupling reagents HBTU/HOBt and HATU/HOAt were employed. Subsequently, the linear precursor was cleaved from the resin using either 20% TFA or a TFE mixture, and then cyclized in solution phase with HBTU. The synthesized products were purified using semi-preparative RP-HPLC, giving the percent yields 16% for An1 and 12% for An2. Both compounds were then characterized by HR-ToF-MS,* ^{1}*H- and* ^{13}*C-NMR. The synthesized xylapeptide A and its analogues were evaluated against* Bacillus cereus, Bacillus subtilis, Staphylococcus aureus, Enterococcus faecalis, Escherichia coli, Klebsiella pneumoniae, *and* Candida albicans. *The result showed that An2, possessing arginine residue, exhibited higher activity compared to xylapeptide A and An1. This research suggests that xylapeptide A analogues hold great promise as novel antimicrobial agents.*

Keywords: *antimicrobial peptides; cyclopentapeptides; xylapeptide A; solid-phase peptide synthesis; peptide cyclization*

■ INTRODUCTION

Xu et al. [1] successfully isolated two novel cyclopeptides, xylapeptides A and B, from the fungus *Xylaria* sp. Both of these cyclopeptides, consisting of five amino acids, exhibit distinct structures: cyclo-L-Pip-L-Leu-D-Ala-L-Val-*N*-Me-L-Phe for xylapeptide A and cyclo-L-Pro-L-Leu-D-Ala-L-Val-*N*-Me-L-Phe for xylapeptide B. Notably, both xylapeptides contain *N*-methylphenylalanine. This amino acid belongs to the category of non-proteinogenic amino acids and is known to improve the oral bioavailability of cyclic peptides [2]. The key difference between the two cyclopeptides is pipecolic acid (Pip) in xylapeptide A, another non-proteinogenic amino acid, while xylapeptide B contains proline.

Xylapeptides A and B have been found to exhibit selective antibacterial activity against *Bacillus cereus* and *Bacillus subtilis*, with minimum inhibitory concentration (MIC) values of 12.5 µg/mL. Due to their biological properties, further exploration of these compounds is of interest, and chemical synthesis provides a viable approach. Chemical synthesis offers the advantage of faster access to compounds compared to direct isolation

from organisms, especially when the availability of the latter is limited. Kurnia et al. [3] and Muchlis et al. [4] successfully synthesized xylapeptides A and B using a combination of solid and solution phase methods, resulting in 21.0 and 8.9% yields, respectively.

In this study, the focus is on the preparation of analogues of xylapeptide A and the identification of candidates with potent antimicrobial activity. The structure of amino acids plays a critical role in determining their activity. Parameters such as hydrophobicity and cationic charge have been found to influence the activity of antimicrobial peptides [5-6]. Additionally, the presence of D-configured amino acids can enhance the stability and activity of antimicrobial peptides [7-8]. Structurally, the cyclic configuration of xylapeptide A enhances the peptide's stability by increasing its resistance to protease degradation. Substituting the valine residue with the cationic amino acid arginine is designed to elevate the overall cationic charge of the peptide, which may improve its biological activity. Additionally, the removal of the *N*-methyl group from *N*-methylphenylalanine is intended to elucidate its specific contribution to the compound's antimicrobial properties. The structures and residue sequence of xylapeptide A and its analogues can be seen in Fig. 1.

The synthesis of analogues of xylapeptide A follows that of the parent compound, but the active side chain present in arginine is protected with appropriate groups to prevent side reactions and requires different synthesis strategies. Analogues to xylapeptide A still have hydrophobic residues, which may trigger aggregation and potentially reduce yield during the synthesis of linear precursors in the solid phase due to the presence of alanine and valine residues [9]. Moreover, coupling the *N*-methylphenylalanine residue in the solid phase can be challenging, necessitating the use of suitable coupling reagents to ensure proper coupling reactions and high synthesis yield [10]. The side chain protective group must also be maintained during the release of the linear peptide from the resin, as the linear precursor will undergo cyclization, requiring the reactive group to remain protected, while providing C- and N-terminal ends.

Another challenge in the synthesis of xylapeptide A analogues lies in the cyclization of linear precursors to form cyclic products. Cyclization can be difficult, especially when β-turn residues or residues forming *cis*-amide conformers are absent. Dimerization and epimerization are also potential hurdles during cyclization. Fortunately, the presence of D-alanine and/or *N*-methylphenylalanine in xylapeptide A analogues is advantageous, as these residues have been reported to form *cis*-amide conformers, facilitating cyclization [11-13]. However, the potential for dimerization and epimerization needs to be addressed in this synthesis, given that xylapeptide analogue A comprises five residues, which generally possess the potential for dimerization or oligomerization [14].

■ EXPERIMENTAL SECTION

Materials

The research involved the use of various materials, including amino acid residues (Fmoc-D-Ala-OH, Fmoc-L-Arg(Pbf)-OH, Fmoc-L-*N*-Me-Phe-OH, Fmoc-L-Phe-OH, Fmoc-L-Pip-OH, Fmoc-L-Leu-OH), coupling

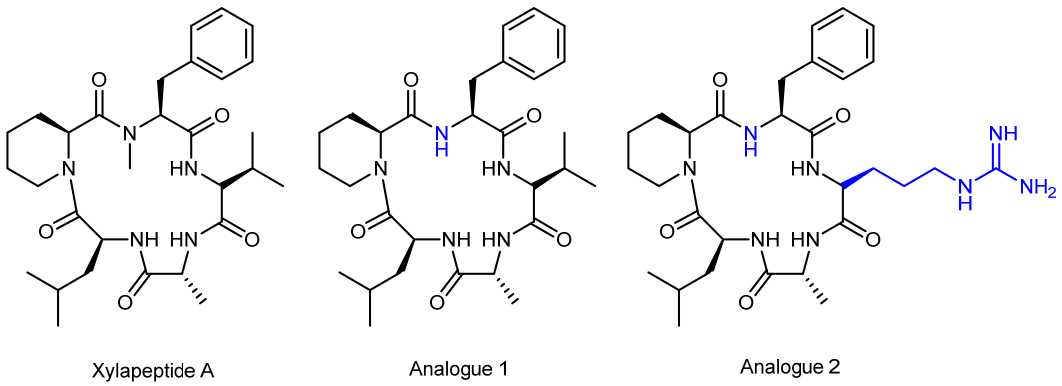


Fig 1. Structures of xylapeptide A, analogue 1 (An1), and analogue 2 (An2)

reagents (*O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyl uronium hexafluorophosphate (HATU), *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyl uronium hexafluorophosphate (HBTU), 1-hydroxy-7-azabenzotriazole (HOAt), and 1-hydroxybenzotriazole (HOBt)), and 2-chlorotrithyl chloride (2-CTC) resin with a concentration of 0.15 mmol/100 g, obtained from GL-Biochem Ltd. (China). Solvents and reagents used included trifluoroacetic acid (TFA), trifluoroethanolic acid (TFE), acetic acid, distilled water, acetonitrile, dichloromethane (DCM), dimethyl sulfoxide (DMSO), *N*,*N*-dimethylformamide (DMF), *N*,*N*-diisopropylethylamine (DIPEA), piperidine, acetaldehyde, and *p*-chloranyl, obtained from Sigma-Aldrich. All materials used were of analytical grade quality.

For the antimicrobial activity test, nutrient agar (NA) and Mueler-Hinton agar (MHA) growth media, as well as nutrient broth (NB) and Mueller Hinton broth (MHB) were employed. The test involved bacterial strains of *Bacillus cereus* (ATCC 11778), *Bacillus subtilis*, *Enterococcus faecalis* (ATCC 29212), *Staphylococcus aureus* (ATCC 6538), *Escherichia coli* (ATCC 11229), *Klebsiella pneumoniae* (ATCC 2357), and *Candida albicans* (ATCC 10231) fungal strains. Ciprofloxacin, vancomycin, and nystatin were used as positive controls during the antimicrobial activity assessment.

Instrumentation

The research was conducted using some laboratory equipments, including a solid-phase peptide synthesis (SPPS) reactor, rotary suspension mixer, Buchi rotary evaporator, Waters 1525-Series RP-HPLC with photodiode array detector for both analytical and semipreparative purposes, Jupiter C18 and VDSpher PUR 100 C18-SE columns for HPLC, Tecan Metro 200 Spectrophotometer, Waters Q-ToF-ESI Mass Spectrometry (MS), Agilent 500 MHz Nuclear Magnetic Resonance (NMR) with DD2 Console System, air pump, and standard laboratory glassware. These instruments were used for peptide synthesis, purification, and characterization throughout the research.

Procedure

The procedure for synthesizing xylapeptide A analogues (An1 and An2) is based on the synthesis procedure for xylapeptides A and B, c-PLAI and c-PLAI analogues, nocardiotide A, and petriellin A [3-4,6,15-17] using a combination of solid phase and solution phase synthesis methods.

Binding of the first amino acid to the resin

The procedure started by adding 250 mg of 2-chlorotrithyl chloride resin to the reactor along with 5 mL of DCM. The mixture was then shaken for 30 min using a rotary suspension mixer, and the filtrate was removed using an air pump until the resin became dry. Subsequently, a mixture of Fmoc-AA1-OH (0.375 mmol), DIPEA (0.375 mmol), and 4 mL DCM solution were added to the developed resin. The reactor was shaken for 4 h at room temperature using the rotary suspension mixer. Finally, the resin was filtered, washed with DCM and DMF, and dried using an air pump.

Calculation of resin loading value

To determine the resin loading value, 0.6 mg of resin was weighed and placed into a vial. Then, 3 mL of a 20% piperidine solution in DMF was added to the vial, and the solution was thoroughly mixed until it became homogeneous. The mixture was allowed to stand for 30 min. Afterward, the absorbance of the solution was measured using a UV spectrophotometer at a wavelength of 290 nm. Based on the absorbance obtained, the resin loading value was calculated as a reference for the number of moles of amino acids attached to the resin per gram of resin used (Eq. (1)).

$$\text{Loading resin} = \frac{(\text{sample abs.} - \text{blank abs.}) \times \text{V(mL)}}{5800 \times \text{mass of resin(g)}} \qquad (1)$$

Capping resin

To perform the capping of the resin, a mixture of 5 mL of MeOH:DCM:DIPEA (15:80:5) was added to the reactor. The mixture was shaken for 15 min, and this step was repeated twice. Afterward, the resin was filtered and washed with DCM and DMF. Finally, the resin was dried using an air pump to obtain dry Fmoc-AA1-resin.

Fmoc deprotection

To remove the protective group Fmoc from the first amino acid, a 4 mL solution of 20% piperidine in DMF was added to the resin. The mixture was shaken for 5 min, and this step was repeated twice. After deprotection, the resin was filtered and washed with DCM and DMF. Additionally, to ensure the successful removal of the Fmoc group, a control test known as the chloranyl test was performed. The appearance of a blue/green color indicated successful deprotection, whereas a yellow color indicated that the deprotection reaction was not successful.

Amino acid coupling/pentapeptide linear extension

In a round-bottom flask, a mixture containing Fmoc-AA_2-OH (3 equivalents), HBTU (3 equivalents), and HOBt (3 equivalents) was dissolved in 4 mL of DMF, along with DIPEA (6 equivalents). The solution was sonicated for 5 min to ensure thorough mixing. Next, the solution was added to the dry peptide resin-AA1-NH_2 and shaken for 4 h to facilitate the coupling of the second amino acid. After the coupling reaction, the resin was filtered and washed with DCM and DMF. Subsequently, the resin was dried. A chloranyl test was performed to confirm the success of the coupling of the second amino acid.

Chloranyl test

The chloranyl test involves 2% acetaldehyde in DMF (solution 1) and 2% *p*-chloranyl in DMF (solution 2). A few drops of the peptide resin are placed into a small test tube, and then 2–5 drops of solutions 1 and 2 are added to the tube. The mixture is shaken briefly and then left at room temperature for 5 min. During this time, the color of the resin grains is observed. If the deprotection reaction of the Fmoc protective group has been successful, the chloranyl resin will exhibit a blue/green color. This color change indicates that the Fmoc group has been removed successfully, and the amino group on the resin is now available for further coupling reactions. However, if the deprotection reaction is not successful, the chloranyl resin test will turn yellow. This color change suggests that the Fmoc group has not been completely removed, and further deprotection steps might be required to ensure the proper coupling of subsequent amino acids.

Release of linear pentapeptide from resin

The peptide resin (NH_2-AA_5-AA_4-AA_3-AA_2-AA_1-resin) was subjected to two different deprotection conditions based on the presence or absence of side chain protecting groups. For the peptide chain without a side chain protecting group (analogue 1), 5 mL of a 20% TFA solution in DCM was added. Meanwhile, for the peptide chain with a side chain protecting group (analogue 2), 5 mL of AcOH:TFE:DCM (2:2:2) was used. The mixture was shaken for 60 to 90 min at room temperature to facilitate the deprotection reaction, resulting in the formation of NH_2-AA_5-AA_4-AA_3-AA_2-AA_1-OH. The successful release of the peptide from the resin was indicated by a change in the color of the resin to red. This deprotection step was performed twice to ensure complete removal of the protecting groups and obtain the desired product. Afterward, the resin was filtered and washed with DCM, and the filtrate was concentrated using a rotary evaporator to further purify the product.

Pentapeptide linear cyclization

The cyclization of the linear pentapeptide was performed in solution phase to produce a cyclopentapeptide. The cyclization was carried out using the HBTU reagent (3 equivalents) in DCM with a dilute concentration of 1.25 mM. Additionally, 1% (v/v) DIPEA was added to facilitate the reaction. The mixture was left to react for 72 h at room temperature. To monitor the progress of the reaction, a control was performed using a GF254 nm silica TLC plate. Once the cyclization was complete, the next step involved releasing the Pbf protective group, using 95% TFA in water at 0 °C. After cyclization and deprotection, the resulting product was purified using semi-preparative RP-HPLC to isolate the cyclopentapeptide. The purity of the product was then assessed using HR-TOF-MS, ^{1}H-NMR, and ^{13}C-NMR analytical RP-HPLC.

Antimicrobial activity test with microdilution method

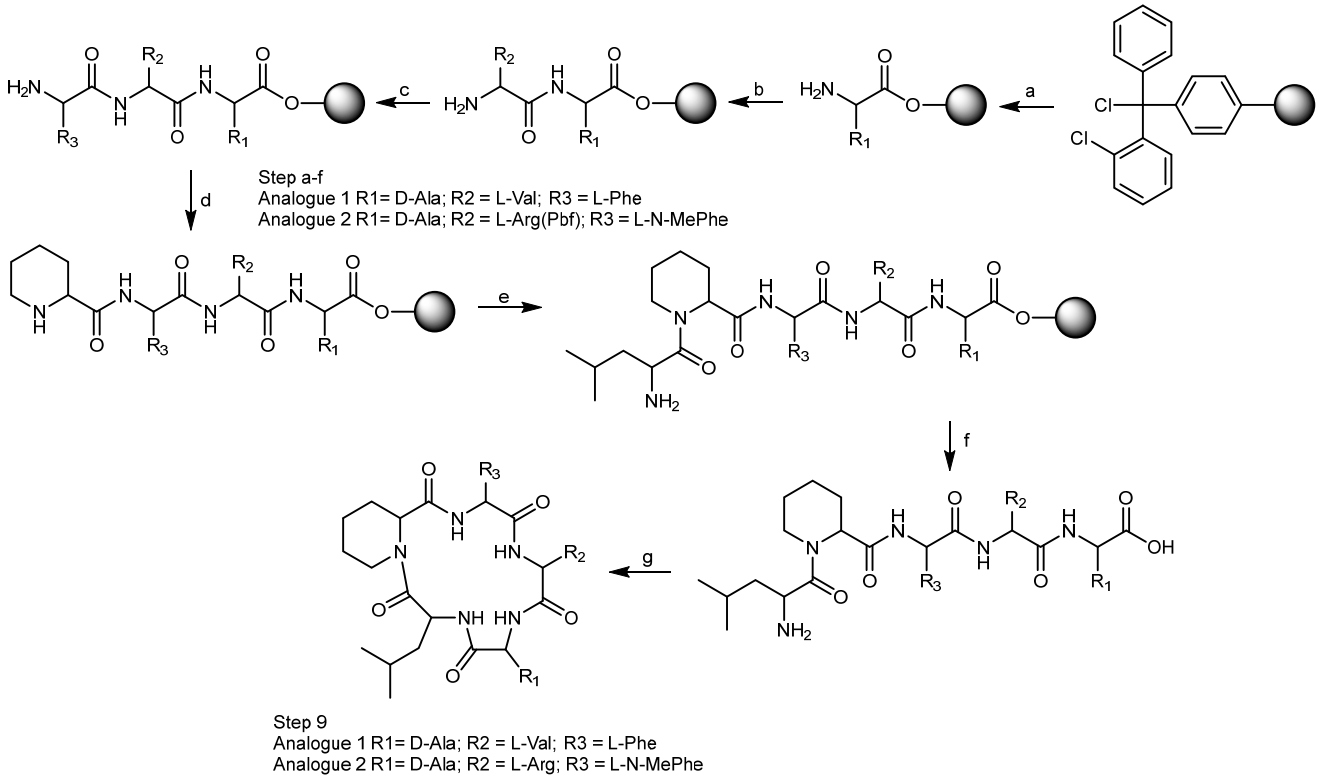
The antimicrobial activity test was conducted using the microdilution method to determine the minimum concentration of the antimicrobial agent required to inhibit bacterial growth. Bacterial strains, including *B. cereus*, *B subtilis*, *E. faecalis*, *S. aureus*, *E.*

coli, *K. pneumoniae*, and *C. albicans*, were inoculated into both NB and MHB media, using a McFarland standard of 0.5 as a reference for bacterial density. Test samples were dissolved in 2% DMSO at a 1000 µg/mL concentration and then diluted in several stages. Subsequently, twelve sample solutions, amoxicillin as the positive control, and 2% pure DMSO as the negative control were placed in 96-well microplates and incubated at 37 °C for 18 h. After incubation, data readings were taken using a spectrophotometer at a wavelength of 600 nm. The MIC was determined by calculating the percentage of bacterial inhibition and the percentage of cell death for each sample. This method allows for the assessment of the antimicrobial effectiveness of the test samples against the different bacterial strains and *C. albicans*.

■ RESULTS AND DISCUSSION

The linear precursors of An1 and An2 were synthesized using the same method as xylapeptide A. In this process, D-alanine was chosen as the C-terminal amino acid, and L-leucine was selected as the N-terminal amino acid for all the analogues (Scheme 1). The selection was applied due to the fact that D-amino acids are commonly used as turn-inducing elements in peptide design to promote head-to-tail cyclization and improve yields [18].

The loading resin value of each analogue, which represents the amount of the first amino acid successfully bound to the resin, was found to be 0.5–0.6 mmol/g. A resin loading value between 0.2 and 0.8 mmol/g is



Scheme 1. Scheme of synthesis of An1 and An2. (a) (1) Fmoc-D-Ala-OH, DIPEA, DCM, 4 h, (2) methanol:DCM:DIPEA (15:80:5), (3) 20% piperidine in DMF, (b) (1) Fmoc-L-Val-OH (analogue 1) or Fmoc-L-Arg(Pbf)-OH (analogue 2), HBTU, HOBt, DIPEA, DMF, 4 h, (2) 20% piperidine in DMF, (c) Fmoc-L-Phe-OH (analogue 1) or Fmoc-L-*N*-Me-Phe-OH (analogue 2), HBTU, HOBt, DIPEA, DMF, 4 h, (2) 20% piperidine in DMF, (d) Fmoc-L-Pip-OH, HATU, HOAt, DIPEA, DMF, 4 h; (2) 20% piperidine in DMF; (e) Fmoc-L-Leu-OH, HATU, HOAt, DIPEA, DMF, 4 h; (2) 20% piperidine in DMF; f) 20% TFA in DCM (An1); AcOH:TFE:DCM (2:2:6) (An2), (g) (1) HBTU, DIPEA in 1.25 mM DCM (An1); (1) HBTU, DIPEA in 1.25 mM DCM (2) 95% TFA in water, 0 °C (An2)

considered ideal for linear synthesis using the solid-phase method [19]. After the successful binding of the first amino acid to the resin, the capping resin stage was carried out to prevent any unreacted sites on the resin. Subsequently, the Fmoc group was deprotected, and the subsequent amino acids were coupled to the resin, with the reaction progress monitored using the chloranyl test. This led to the formation of a pentapeptide bound to the resin. Once the pentapeptide bound to the resin was obtained, the next step involved releasing the peptide chain from the resin. For the linear precursor without side chain protective groups, this was achieved using a solution of 20% TFA in dichloromethane. On the other hand, for the linear precursor of analogue 2, the TFE mixture was used to release the peptide chain from the resin while retaining the side chain protecting group that is still required for the subsequent cyclization step.

After obtaining the crude precursors of the synthesized An1 and An2, HR-ToF-MS analysis was conducted to confirm the formation of the analogues. The mass spectroscopy results revealed the presence of a molecular peak ion for each xylapeptide. A linear precursor compound is indicated in Table 1. This analysis provides evidence that the analogues have been successfully synthesized, and the mass spectrometry data allow for the verification of their molecular compositions.

The cyclization of An1 and An2 linear precursors was performed directly from the crude product. The cyclization of linear peptide precursors follows the head-to-tail method, wherein the reaction occurs between the C-terminal end and the N-terminal end of the linear peptide. Xylapeptide A analogues possess five amino acid residues. Short chain cyclization involving less than seven amino acid residues is susceptible to cyclodimerization and epimerization at the C-terminus [18]. The cyclization of linear precursors of An1 and An2 was carried out at a dilute concentration to avoid cyclodimerization, which can occur with short-chain cyclization. The reaction was performed using HBTU coupling reagent in a 1.25 mM solution of dichloromethane with the addition of basic DIPEA at room temperature. The reaction was monitored by thin-layer chromatography on a TLC silica GF plate at 254 nm with ninhydrin spray and mass spectrometry analysis to confirm the formation of the cyclic product. These controlled conditions ensured successful cyclization of the xylapeptide A analogue linear precursor while minimizing the formation of undesired byproducts.

After 72 h of cyclization, a cyclic product was obtained, and an analogue with a side chain protecting group was then subjected to a reaction with 95% TFA in water. The low-temperature release condition was applied for the deprotection step of the side chain protecting group from the protected An2 crude, which contained a Pbf protective group on the arginine residue. Based on the mass spectral data, it can be confirmed that the target product (the unprotected cyclopentapeptide of An2) has been obtained. The crude products were then subjected to a purification step using semipreparative RP-HPLC. After obtaining the purified fractions, HR-ToF-MS analysis (Table 1) was performed to confirm the presence of the target compounds in the collected fractions. The HR-ToF-MS spectra showed molecular ion peaks at *m/z* $[M+H]^+$ 542.3347 (calculated *m/z* 542.3342) for An1 and *m/z* $[M+H]^+$ 613.3848 (calculated *m/z* 613.3826) for An2, corresponding to the mass of the desired peptides. The purity analysis was conducted using analytical RP-HPLC. The MS spectra and the analytical RP-HPLC chromatogram of An1 and An2 are shown in the Supplementary Material.

Based on the results of the analytical RP-HPLC chromatogram, it was observed that the retention time of xylapeptide A and its analogues (An1 and An2) differed. This variation in retention time is attributed to the distinct amino acid compositions of the peptides, which directly influence their retention on the column.

Table 1. Mass spectrometry data of xylapeptide A analogues, An1 and An2

	HR-ToF-MS		
Compound	Observed (*m/z*)	Calculated (*m/z*)	Molecular Formula
An1	542.3347	542.3342	$C_{29}H_{44}N_5O_5$
An2	613.3848	613.3826	$C_{31}H_{49}N_8O_5$

Notably, xylapeptide A exhibited a sharp peak at 22.337 min, while analogue 1 had a retention time of 19.766 min. This discrepancy in retention time can be attributed to the presence of a methyl group on the Nα-amino in xylapeptide A. The removal of this methyl group on the phenylalanine residue decreased its hydrophobicity, resulting in a faster retention time compared to the main compound xylapeptide A. Similarly, an observation was made for An2 containing a cationic arginine residue. This residue possesses free amine groups in its side chain, rendering it 2 more polar in nature. Therefore, the presence of free amine groups influenced the retention time in the analytical RP-HPLC results. An2 exhibited a retention time of 13.759 min, differing from the retention time of xylapeptide A.

The NMR characterization of purified An1 and An2 was performed to verify the synthesized structures and compare them with the structure isolated by Xu et al. [1]. An1 and An2 were characterized using ^{1}H-NMR (500 MHz) and ^{13}C-NMR (125 MHz) in $CDCl_3$ as the solvent. The NMR characterization results for An1 and An2 are shown in Table 2. The ^{1}H-NMR spectra of An1 and An2 showed 44 and 49 proton signals, respectively, including signals of secondary amide protons, aromatic protons, α-protons, aliphatic methines, aliphatic methylenes, and aliphatic methyls. The ^{13}C-NMR spectra showed 29 signals for An1 and 31 signals for An2, including carbon signals of carbonyls, quarternary carbons, aromatic carbons, α-carbons, aliphatic methine carbons, aliphatic methylene carbons, and aliphatic methyl carbons. NMR analysis revealed that the data are consistent with the structures of An1 and An2. Upon comparing the ^{13}C-NMR characterization results of xylapeptide A with its analogues, several differences were observed, confirming the successful formation of the analogues. For An1, the removal of the methyl group from the Nα-amino on the phenylalanine residue resulted in the loss of the sp^3 methyl carbon signal at δ_C 31.6 ppm in the ^{13}C-NMR spectrum. Meanwhile, An2, containing arginine, was characterized by a distinct chemical shift at δ_C 157.43 ppm, arising from the side chain carbon of the arginine residue (R-NH-C-$NHNH_2$) in the ^{13}C-NMR spectrum. These differences in chemical shifts and signals further validate the successful synthesis of An1 and An2.

Table 2. ^{1}H and ^{13}C-NMR spectral data of An1 and An2

Amino acid	An1 [$CDCl_3$, 500 MHz]		Amino acid	An2 [$CDCl_3$, 500 MHz]	
	δH [ppm], mult, *J* [Hz]	δC [ppm]		δH [ppm], mult, *J* [Hz]	δC [ppm]
Phenylalanine			Phenylalanine		
CO		169.60	CO		169.90
α	4.34, (*d*), 15.00	64.10	α	4.36, (*d*), 15.00	63.70
β	3.21	36.30	β	2.79	33.70
β'	3.28, (*m*)		β'	3.43, (*d*), 13.70	
γ	-	136.80	γ	-	138.10
H Phe	7.19, (*d*), 7.50	129.30	H Phe	7.19, (*m*)	129.50
	7.27, (*t*), 5.00	129.20		7.28, (*t*), 7.42	129.10
	7.23, (*m*)	126.90		7.23, (*d*), 7.30	127.20
Me	-	-	Me	2.84, (*s*)	30.70
NH	7.31, (*m*)		NH	-	-
Valine			Arginine		
CO		171.80	CO		172.00
α	4.13, (*t*), 7.42	59.60	α	4.46, (*d*), 7.53	56.50
β	2.25, (*m*)	29.30	β	2.56, (*m*)	28.80
γ	0.93, (*d*), 5.50	18.40	γ	2.03, (*m*)	23.00
γ'		19.90	δ	3.26, (*m*)	41.10
NH	7.33, (*t*), 7.43		C=NH	7.94, (*s*)	157.40
Alanine			HN-C	2.53, (*s*)	

Amino acid	An1 [$CDCl_3$, 500 MHz]		Amino acid	An2 [$CDCl_3$, 500 MHz]	
	δH [ppm], mult, *J* [Hz]	δC [ppm]		δH [ppm], mult, *J* [Hz]	δC [ppm]
CO		172.20	H_2NC	7.94, (*s*)	
α	4.60, (*m*)	46.10	NH	7.31, (*d*), 7.36	
β	1.35, (*d*), 6.94	14.70	Alanine		
NH	6.38, (*d*), 10.00		CO		172.70
Leucine			α	4.57, (*m*)	48.00
CO		173.10	β	1.27, (*d*), 6.86	14.20
α	4.95, (*m*)	48.40	NH		
β	1.20, (*m*)	40.70	Leucine		
β'	1.47, (*m*)		CO		173.30
γ	1.77, (*m*)	25.10	α	4.74, (*t*), 7.34	48.50
δ	0.87, (*d*), 5.00	21.70	β	1.18, (*m*)	41.10
δ'		23.30	β'	1.48, (*m*)	
NH	6.84, (*d*), 9.15		γ	1.65, (*m*)	24.80
Pipecolic			δ	0.88, (*m*)	21.40
CO		171.40	δ'		21.40
α	2.30, (*m*)	57.90	NH	7.11, (*d*)	
β	0.77, (*m*)	28.10	Pipecolic		
β'	1.42, (*m*)		CO		171.30
γ	0.80, (*m*)	22.90	α		53.00
γ	1.60, (*m*)		β	0.76, (*m*)	28.10
δ	1.27, (*d*), 7.02	25.20	β'	1.40, (*m*)	
δ'	1.47, (*m*)		γ	0.83, (*m*)	23.70
ε	2.46, (*m*)	47.70	γ	1.55, (*m*)	
ε'	3.81, (*m*)		δ	1.32, (*m*)	25.00
NH	-		δ'	1.48, (*m*)	
			ε	2.76, (*m*)	47.20
			ε'	3.80, (*m*)	
			NH	-	

Based on the characterization results of ^{1}H-NMR and ^{13}C-NMR, it can be concluded that the structures of An1 and An2 were successfully confirmed, indicating the successful synthesis of these two analogues. The percentage yield of the two analogues obtained from the synthesis process is 16% and 12%, respectively. These low yields are commonly obtained in the synthesis of cyclic peptides as is also found in the synthesis of petriellin A and nocardiotide A [16-17]. Overall, the combination of NMR characterization, mass spectrometry, and purity testing through analytical RP-HPLC allows for comprehensive confirmation of the successful synthesis of An1 and An2.

The synthesized An1 and An2 together with xylapeptide A were subjected to *in vitro* antimicrobial activity testing using the microdilution method to assess their effectiveness against various pathogens (Table 3). The tested pathogens included four Gram-positive bacteria (*B. cereus*, *B. subtilis*, *S. aureus*, and *E. faecalis*), two Gram-negative bacteria (*E. coli* and *K. pneumoniae*), and one fungal strain (*C. albicans*).

In the antimicrobial activity test, positive controls (ciprofloxacin, vancomycin, and nystatin) were used. Additionally, a negative control using 2% DMSO solvent was employed. The MIC value of xylapeptide A and its analogues, An1 and An2, against the test pathogens was

Table 3. MIC values of xylapeptide A and analogues (An1 and An2)

Compound	Minimum inhibitory concentration (MIC) (μg/mL)						
	Gram-positive bacteria				Gram-negative bacteria		Mold
	B. cereus	*B. subtilis*	*S. aureus*	*E. faecalis*	*E. coli*	*K. pneumoniae*	*C. albicans*
Xylapeptide A	125.00	125.00	500.00	>500.00	>500.00	>500.00	500.00
An1	62.50	62.50	250.00	250.00	>500.00	>500.00	250.00
An2	15.60	31.30	15.60	31.30	31.30	125.00	62.50
Positive control							
Ciproflaxacin	0.20	0.20	0.20	0.78	0.10	0.10	-
Vancomycin	0.78	3.13	6.25	12.50	50.00	3.125	-
Nystatin	-	-	-	-	-	-	0.20

determined by measuring the lowest concentration of the samples that inhibited the growth of the pathogens. According to da Silva et al. [20], MIC values are categorized as active (<100 μg/mL), moderate (100–500 μg/mL), weak (500–1000 μg/mL), or inactive (>1000 μg/mL). The results of the antimicrobial test showed that the synthesized xylapeptide A exhibited weak and inactive activity against *C. albicans, S. aureus*, *E. faecalis*, *E. coli*, and *K. pneumoniae*, with MIC values ranging from 500 to >500 μg/mL. However, it displayed moderate antimicrobial activity against *B. cereus* and *B. subtilis*, with an MIC value of 125 μg/mL. It's worth noting that the results of this antimicrobial test differed from those of the isolated xylapeptide A studied by Xu et al. [1]. The isolated xylapeptide A showed strong activity against *B. subtilis* and *B. cereus* strains, with an MIC value of 12.5 μg/mL. These variations in test results could be attributed to differences in test conditions, such as the use of different strain cultures and growth conditions, as reported by Napolitano et al. [21].

The antimicrobial activity testing of the synthesized An1 and An2 revealed interesting variations compared to the parent compound xylapeptide A. An1, which lacked the *N*-methyl group on the phenylalanine residue, demonstrated enhanced antimicrobial activity compared to xylapeptide A against all five pathogens. The reduction in hydrophobicity of xylapeptide A by eliminating the *N*-methyl group seemed to contribute to its increased antimicrobial activity. While the hydrophobicity of a compound influences its antimicrobial properties, An1 remains hydrophobic even after removing a single methyl group. However, this modification may alter the peptide's conformation, which could ultimately impact its antimicrobial activity. The adoption of specific conformations enables peptides to interact effectively with bacterial membranes, thereby enhancing their antimicrobial properties [22-23]. The substitution of valine residues with a cationic arginine residue in An2 led to further improvements in antimicrobial activity compared to xylapeptide A and An1. The replacement with arginine residues showed a significant effect on antimicrobial activity against *S. aureus*, *E. faecalis*, *E. coli*, and *K. pneumoniae* strains, with MIC values ranging from 15.6 to 125 μg/mL. The MIC values are similar to several coumarin conjugates showing broad-spectrum antimicrobial properties [24-25]. The presence of arginine residues in analogue 2 exerted a profound effect on increasing antimicrobial activity. This can be attributed to the cationic nature of the arginine residue, which increases the overall charge on xylapeptide A. This enhanced charge can facilitate interactions with pathogenic cell membranes and potentially cause damage to these membranes, leading to increased antimicrobial efficacy [26-29]. Overall, the modifications made in An1 and An2 demonstrated various impacts on their antimicrobial activity. These findings highlight the importance of understanding the structure-activity relationship of peptides and how specific changes in amino acid residues can influence their biological activity. The results provide valuable insights for the design and development of novel antimicrobial peptides with improved efficacy against different pathogens.

■ CONCLUSION

Two analogues of xylapeptide A, An1 and An2, were successfully synthesized using a combination of solid and solution phase peptide synthesis methods, with the percentage yields obtained for each analogue being 16% (An1) and 12% (An2). The two synthesized analogues exhibited higher antimicrobial activity than xylapeptide A against various pathogens, including *B. cereus*, *B. subtilis*, *S. aureus*, *E. faecalis*, *E. coli*, *K. pneumoniae*, and *C. albicans*. The presence of cationic residue (argininine) in An2 enhances the antimicrobial efficacy of the analogues, making them more effective in inhibiting the growth of the tested pathogens.

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■ CONFLICT OF INTEREST

All authors declare no financial/commercial conflicts of interest.

■ AUTHOR CONTRIBUTIONS

Rani Maharani and Handi Nugraha Muchlis designed the study and wrote the manuscript. Handi Nugraha Muchlis synthesized and evaluated the biological activity of all compounds. Rani Maharani, Handi Nugraha Muchlis, Ace Tatang Hidayat, and Jamaludin Al-Anshori interpreted the data. Desi Harneti, Nurlelasari, Tri Mayanti, Kindi Farabi, and Unang Supratman contributed to purification. All authors gave final approval for publication.

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