Synthesis of [1-8-NαC]-zanriorb A1, alanine-containing analogues, and their cytotoxic and anti-inflammatory activity

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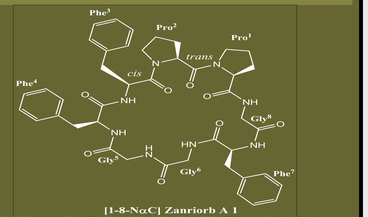
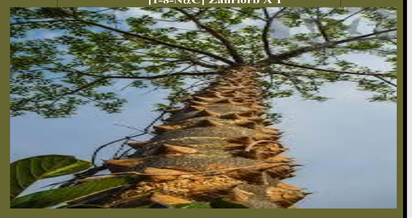
3rd Commonwealth Chemistry Posters
Building Networks to Address the Goals
28-29 September 2022

ABSTRACT

The synthesis of the orbitide[1-8-NαC]-zanriorb A1, isolated from the medicinal plant Zanthoxylum riedelianum, was investigated by solution-phase macrocyclization of a linear peptide and on-resin solid-phase macrocyclization with an acylsulfonamide safety-catch linker. The solution-phase route produced a mixture of proline rotamers, and the main component was assigned as the trans, cis rotamer, identical to the natural product. The on-resin cyclization was less successful, producing mainly a linear peptide, and minor cyclic products as rotameric mixtures. Although the natural product was reported to be significantly cytotoxic against Jurkat leukemia T cells, our synthetic peptides were inactive, suggesting the presence of other rotamers or impurities in the naturally isolated material. Additional analogues of zanriorb A1 were synthesized in which proline and glycine residues were replaced with alanine. While these analogues were not cytotoxic, several of them inhibited the production of nitric oxide in lipopolysaccharide (LPS)-stimulated macrophages. The most active compound, cyclic[Ala5,6,8]-zanriorb A1 had an IC50 of 22 μM and was more potent compared with the standard NG-monomethyl-L-arginine acetate (L-NMMA) with an IC50 of 98 μM, indicating their strong anti-inflammatory potential.



[1-8-NαC] Zanriorb A 1



Background

The plant Zanthoxylum *riedelianum* Engl. has been used as an analgesic for toothache and reported folk uses of this species has been confirmed by the in-vitro anti-inflammatory and anti-nociceptive activities of the leaves and stem bark [1].

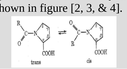
There is only one study that reported the presence of cyclic peptides in the genus Zanthoxylum[1]. Characterization of a new orbitide named [1-8-NαC]-zanriorb A1 **(1)** was reported from the leaves of Zanthoxylum riedelianum using NMR and mass spectrometry. When evaluated against three cancer cells lines, leukemia cell lines including K562 (chronic myeloid leukemia), Kasumi-1 (acute myeloid leukemia), and Jurkat (T-cell leukemia), and also normal mononuclear cells from human healthy donors, zanriorb A exhibited cytotoxic activity only against Jurkat cells with a low IC^{50} value of 218 nM [1, 2]. We also note that reported cyclic peptide is trans and cis rotameric forms at Pro1-Pro2 and Pro2-Phe3 that is Pro1, Δδ Cβ (28.4) − Cg (24.6) = δ 3.8 and Pro2 Δδ Cβ (31.4) − Cg (21.7) = δ 9.7 [2].

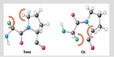
Most of cyclic peptides are rich in proline amino acid moieties, which may be in either cis or trans conformation. 13C chemical shifts and ROSEY or NOSEY interactions for Pro in *cis* and *trans* configurations vary significantly both in small peptides and in proteins [3, 4].

The signals of the β-C and γ-C atoms are more separated in *cis*-proline than in *trans*-proline peptide bond 13C chemical shifts for Pro in *cis* and *trans* configurations vary significantly both in small peptides and in proteins [3, 4].

The signals of the β-C and γ-C atoms are more separated in *cis*-proline than in *trans*-proline peptide bond. Proline peptide bonds are *trans* as shown by the small 13C NMR chemical shifts difference of Pro βC-γC and *cis* as indicated by the large 13C chemical shift difference of Pro βC-γC [3].

In NOSEY or ROSEY spectra *cis*-proline moiety give α-protons interaction with next amino acid moiety and *trans*-proline peptide bond identified by α-protons interaction of next amino acid with g-proton of proline moiety as shown in figure [2, 3, & 4].





METHODS AND MATERIALS

General procedure

Bruker Avance 600 and 800 MHz NMR spectrometer was used for NMR spectra, respectively. NMR data were collected at 25_C. FAB mass spectra were recorded on QSTAR XL MS/MS systems (Agilent, USA). Preparative RP-HPLC separation was performed by using a Jaigel ODS-MAT 80 (C18) column using elution with acetonitrile/water (60:40) in 0.08% TFA. The purity of peptides was established by ultra-performance liquid chromatography (UPLC) (Agilent 1260 Infinity Diode Array, C-4 reversed-phase analytical column, 5 μm, 150 _ 4.6 mm), and analytical reverse-phase high-performance liquid chromatography (Waters, 2696, mobile phase: acetonitrile/water (60:40) in 0.08% TFA). Optical rotation was measured with a JASCO DIP 360 polarimeter (Japan) at the sodium D line (path length 50 mm).

Synthesis of zanriorb A1 by Synthetic Route-1

Wang resin (1 g, 1.2 mmol/g) was soaked in DCM for 20 min, Fmoc-Gly-OH was loaded on Wang resin in presence of coupling reagents, i.e., PyBOP (5 equiv) and DIEA (8 equiv). After successful completion, the Fmoc group was removed by addition of 20% 4-methylpiperidine in DMF for 30 min and a second Fmoc-protected residue was coupled with the first amino acid attached to the Wang resin. The coupling was achieved by using 3 equivalents each of Fmoc-protected amino acids and coupling reagent at each successive step. Following, the above deprotection and coupling strategies, linear peptide was synthesized and cleaved from the resin by using TFA cocktail with a composition of 95% TFA in H2O. The crude product was purified by RP-HPLC. The linear peptide was cyclized by solution-phase macrocyclization. A mixture of linear peptide 2 (96 mmol), HATU (100 mmol) in DMF (20 ml) was added to a stirring solution of DIPEA (600 mmol) + HATU (10 mmol) in DMF (10 ml) at a rate of 0.1 ml/min. After addition of reagents, the reaction mixture was stirred for 24 h and reaction mixture was concentrated in vacuo and purified by RP-HPLC to obtain the synthetic cyclic peptide 1 (Scheme-1).

Synthesis of zanriorb A1 by Synthetic Route-2

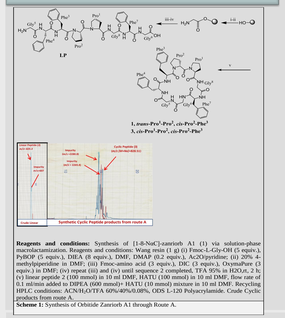
4-Sulfamyl butyryl-aminomethyl resin (0.7 mmol/g, Novabiochem, 1 g) was used in the synthesis of linear peptide precursor 4. Fmocamino acid (5 equiv.), PyBOP (5 equiv.), and DIEA (8 equiv.) in 5 ml of DMF were added to resin which was preswollen in DMF. The reaction mixture was kept on manual shaking for overnight. The peptidyl resin was deprotected by 20% 4-methyl piperidine in DMF for 20 min and then washed with (3 DMF) and deprotection step was monitored by the Kaiser test. The same coupling and deprotection steps were followed in the construction of peptide sequence 4. The terminal amino acid was protected with Boc, by treating the resin with (Boc)2O (6 equiv.) and DIEA (6 equiv.) for a duration of 2 h. The safety-catch sulfonamide linker was alkylated by reaction with ICH2CN (10 equiv.) in DIEA (12 equiv.) in NMP solvent for 24 h. The peptidyl resin was thoroughly washed with THF, DCM and DMF. The Boc-group of peptidyl resin was cleaved by using 95% TFA for 1 h, and then the resin was washed with 2% DIEA in DMF. The peptidyl resin pre-soaked in THF was treated with 2% DIEA/THF for 12 h in an inert atmosphere. The resin was then filtered and thouroughly washed with THF and DCM. The filtrate was concentrated in vacuo and the crude peptide product was precipitated with cold ether and products were purified by RP-HPLC (Scheme-2).

Synthesis of zanriorb A1 Analogs

Similarly the cyclic analogs 7-10 of zanriorb A1 were synthesis by adopting route-1 and cyclic analog 11 were synthesis by adopting route-2.

RESULTS AND DISCUSSION

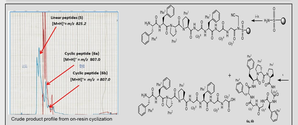
SYNTHETIC ROUTE 1



Reagents and conditions: Synthesis of [1-8-NαC]-zanriorb A1 (1) via solution-phase macrocliatcanization. Reagents and conditions: Wang resin (1 g) (i) Fmoc-L-Gly-OH (5 equiv.), PyBOP (5 equiv.), DIEA (8 equiv.), DMF, DMAP (0.2 equiv.), Ac2O/pyridine; (ii) 20% 4-methylpiperidine in DMF; (iii) Fmoc-amino acid (4 equiv.), DIC (3 equiv.), OxymaPure (3 equiv.) in DMF; (iv) repeat (iii) and (iv) until sequence 2 completed, TFA 95% in H2O,t, 2 h; (v) linear peptide 2 (100 mmol) in 10 ml DMF, HATU (100 mmol) in 10 ml DMF, flow rate of 0.1 ml/min added to DIPEA (600 mmol)+ HATU (10 mmol) mixture in 10 ml DMF. Recycling HPLC conditions: ACN/H2/TFA 60%/40%/0.08%, ODS L-120 Polyacrylamide. Crude Cyclic products from route A.

Scheme 1: Synthesis of Orbitide Zanriorb A1 through Route A.

SYNTHETIC ROUTE 2



Reagent and conditions: Attempted synthesis of [1-8-NαC]-zanriorb A1 by on-resin macrocyclization. Reagent and conditions: Sulfamylbutyryl resin (1 g), (i) Fmoc-L-Gly-OH (5 equiv.), PyBOP (5 equiv.), DIEA (8 equiv. in DMF); (ii) 20% 4-methylpiperidine in DMF; (iii) Fmoc-L-amino acid (5 equiv.), PyBOP (5 equiv.), DIEA (8 equiv.) in DMF; (vi) 20% 4-methylpiperidine in DMF; (v) repeat (iii) and (iv) until sequence completed, 20% 4-methylpiperidine in DMF; (vii) (Boc)2O (6 equiv.), DIEA (6 equiv.) in DCM, rt., 2 h; (viii) ICH2CN (10 equiv.), DIEA (12 equiv.) in 5 ml NMP, 2_ 12 h; (ix) 95% TFA in H2O; (x) 2% DIEA/THF for 12 h

Scheme 2: Route B for the Synthesis of Orbitide Zanriorb A1 (1).

Synthesis From Route A:

- **Linear Peptide (LP):** Yellowish liquid yield 55% (320 mg); tR 3.199 min; [α]25 D = 157.1 (c 0.0024, MeOH); FABMS m/z 825.4 [M+H]+ (C43H53N8O9).
- **Synthetic Zanriorb A1:** Yellowish liquid yield 22% (217 mg); tR 3.41 minFABMS m/z 807.3, HR-FABMS m/z = 807.3842 [M+H]+ (calcd. for $C_{43}H_{50}O_8N_8$, 807.3830).

Synthesis From Route B:

- **Cyclic peptide 6a:** Yellowish liquid yield 12% (130 mg); tR 3.41 min, HR-FABMS m/z = 807.3812 [M+H]+ (calcd. for 807.3830, $C_{43}H_{50}O_8N_8$).
- **Cyclic peptide 6b:** Yellowish liquid yield 2-3% ; HR-FABMS m/z = 807.3842 [M+H]+ (calcd. for 807.3830, $C_{43}H_{50}O_8N_8$).

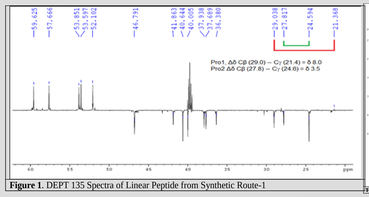


Figure 1. DEPT 135 Spectra of Linear Peptide from Synthetic Route-1

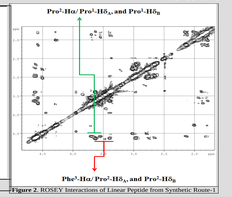


Figure 2. ROSEY Interactions of Linear Peptide from Synthetic Route-1

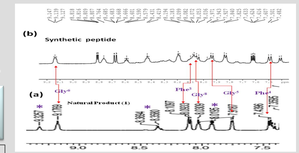


Figure 3. **(a)** ^{1}H NMR spectra of natural product **1** (amide region)* * shows unassigned peak in natural product spectrum. **(b)** ^{1}H NMR spectra of **synthetic peptide** (amide region) (from route A).

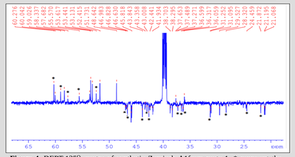


Figure 4. DEPT-135[2] spectra of synthetic Zanriorb A1from route-1. * represent the 13C-NMR shifts of natural product in DEPT-135[2] spectrum of synthetic Zanriorb A1from route-1.

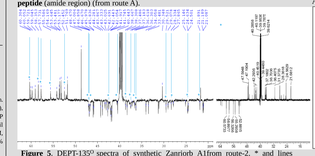


Figure 5. DEPT-135[2] spectra of synthetic Zanriorb A1from route-2. * and lines represent the 13C-NMR shifts of natural product in DEPT-135[2] spectrum of synthetic Zanriorb A1from route-2. The right side is DEPT-135[2] spectra of reported natural product

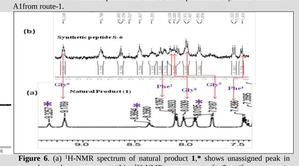


Figure 6. (a) ^{1}H-NMR spectrum of natural product **1**.* shows unassigned peak in natural product spectrum (b) ^{1}H-NMR spectrum of **6a** (from on-resin macrocyclization) Arrows indicate the chemical shifts of **6a** similar to natural products. The unassigned chemical shifts represent the unknown conformer..

TABLE-1: ^{13}C (125 MHz) NMR data for natural product and synthetic product 1, and rotamer 3 in DMSO-d_6

		Reported ^{13}C data	^{13}C data for synthetic peptides	
Residue	Position	trans Pro^1, cis Pro^2 (1)	trans Pro^1, cis Pro^2 (1)	cis Pro^1 cis Pro^2 (3)
Pro^1	α	60.6	60.2	60.0
	β	28.4 } trans	28.3 } trans	27.5 } cis
	γ	24.6 } trans	24.5 } trans	21.1 } cis
	δ	47.2	46.9	45.8
	CO	172.0	172.0	173.4
Pro^2	α	58.9	59.0	58.9
	β	31.4 } cis	31.2 } cis	28.0 } cis
	γ	21.7 } cis	21.5 } cis	21.0 } cis
	δ	47.6	46.8	45.8
	CO	172.1	171.1	
Phe^3	NH			
	α	55.3	55.5	53.1
	β	37.2	37.4	38.2
	1	138.8	137.8	
	2-6	126.8- 130.0	126.2- 129.3	
	CO	170.7	170.8	
Phe^4	NH			Amide
	α	52.8	52.4	51.7
	β	36. 7	36.3	38.2
	1	138.1		137.6
	2-6	126.8- 130.0		126.3- 129.3
	CO	170.3	170.1	169.6
Gly^5	NH			
	α	42.3	42.4	41.9
	CO	169.0	169.5	
Gly^6	NH			
	α	43.8	43.0	42.6
	CO	169.2	169.1	168.9
Phe^7	NH			
	α	56.3	57.6	53.6
	β	36.4	36.0	38.7
	1	137.6		137.3
	2-6	126.8- 130.0		126.3- 129.3
	CO	173.9	173.4	
Gly^8	NH			
	α	43.2	43.8	43.8
	CO	171.8	171.8	

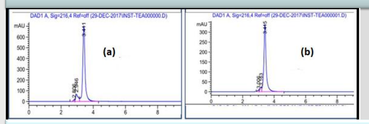
Table-3: ^{1}H (800 MHz) and ^{13}C (125 MHz) data for 6b and natural peptide 1 in DMSO-d_6.

Residue	Position	Synthetic Peptide 1-8		Natural Product[1]	
		^{1}H	^{13}C	^{1}H	^{13}C
Pro^1	α	3.61	60.2	4.28, 4.60 (1, 10.5)	60.7
	β	2.01 trans	28.3	2.01	28.2 trans
	γ	1.81	24.5	1.82 m	24.7
	δ	3.68, 3.42	47.2	3.42 m	47.2
	CO		172.0		172.0
Pro^2	α	4.47	59.0	4.42-4.46 (1, 10.9)	58.9
	β	1.85, 1.59 cis	31.2	1.83 m	31.2 cis
	γ	0.87	21.5	1.32 m	21.5
	δ	3.80 3.55m	46.8	3.42	47.6
	CO		170.1		172.1
Phe^3	NH	8.91		8.16 m	
	α	4.48	55.5	4.59 m	55.3
	β	3.08	37.4	3.08 m	37.2
	1	3.28			
	2,6	7.17-7.25	126.2-129.3	7.30 m	126.8-130.0
	CO	170.6			170.7
Phe^4	NH	7.72		7.46 (8.2)	
	α	4.73	57.6	4.67 m	52.4
	β	3.05, 2.73	36.0	2.96 m	36.7
	1	3.73			
	2,6	7.17-7.25	126.2-129.3	7.30 m	126.1-130.0
	CO		170.2		170.3
Gly^5	NH	8.3		7.91 (8.8)	
	α	3.67	42.0	3.71 m	42.3
	CO		169.4		169.8
Gly^6	NH	8.78		8.37 (8.8)	
	α	3.75	43.0	3.75 m	43.8
	CO		168.3		169.2
Phe^7	NH	7.98		7.72 m	
	α	4.35	53.6	4.38 m	56.1
	β	3.01-2.73 m	38.7	3.06 m	36.4
	1		137.3		137.6
	2,6	7.16 - 7.25	126.3-129.3	7.30 m	126.8-130.0
	CO		173.1		
Gly^8	NH	8.01		8.01 (8.2)	
	α	3.72	43.2	3.75 m	43.2
	CO		171.8		171.8

TABLE-2: List of biological activity and %yield all cyclic peptides.

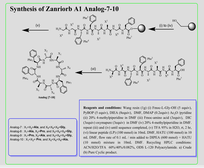
S. No	Structures	m/z	HRMS	Activity IC50 (μM±SD)	R_f (Mix)	Yield (%)
1	Synthetic Zanriorb A1 from **route-1** ($C_{43}H_{50}O_8N_8$) Cyclo[-Phe^3-Pro^1-Pro^2-Gly^5-Phe^4-Gly^6-Phe^7-Gly^8-]	807.4	807.3842	Inactive	3.411	22
2	Synthetic Zanriorb A1 from **route-2** ($C_{43}H_{50}O_8N_8$) Cyclo[-Phe^3-Pro^1-Pro^2-Gly^5-Phe^4-Gly^6-Phe^7-Gly^8-]	807.4	807.3812	Inactive	3.415	12
3	($C_{39}H_{47}O_8N_7$) Cyclo[-Phe^3-Pro^1-Ala^5-Ala^4-Gly^6-Phe^7-Gly^8-]	755.4	755.3525	Inactive	3.36	61
4	8 ($C_{37}H_{45}O_8N_7$) Cyclo[-Phe^3-Pro^1-Phe^7-Ala^5-Pro^2-Gly^6-Phe^4-Gly^8-]	781.3	781.3673	65.0±3.5	3.23	39
5	9 ($C_{37}H_{45}O_8N_7$) Cyclo[-Phe^3-Pro^1-Phe^7-Pro^2-Ala^5-Gly^6-Phe^4-Gly^8-]	781.3	781.3656	Inactive	3.32	32
6	10 ($C_{40}H_{49}N_8O_8$) Cyclo[-Phe^3-Pro^1-Phe^7-Pro^2-Ala^5-Phe^4-Ala^6-Ala^8-]	849.4	849.4331	22.2±0.2	4.80	52
7	11 ($C_{40}H_{49}N_8O_{10}$) Cyclo[-Phe^3-Pro^1-Phe^7-Glu^5-Pro^2-Ala^4-Phe^6-Ala^8-]	881.4	881.4233	39.6±1.1	3.06	19

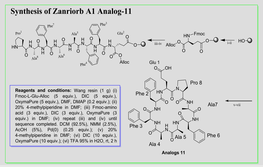
UPLC Profile

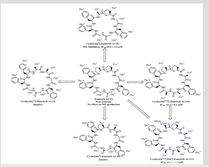


UPLC condition for pure products from routes A and B: C4 reversed-phase analytical column, ACN/H2O/TFA 60 % /40 % / 0.08 %. a) Cyclic product from route A with retention time 3.411min. b) Cyclic product (6a) from route B with retention time 3.415 min.

Synthesis of Analogs







CONCLUSIONS

The cyclic orbitide [1-8-NαC]-zanriorb A-1 was obtained as mixture of conformers by using two different routes towards the synthesis of natural product **1**. None of the cyclic rotamers or analogs of [1-8-NαC]-zanriorb A-1 exhibited any cytotoxicity as reported for natural product. Alanine substitution at position 5, 6, and 8 effects on conformation, substitution on position 2 affects the biological activity of analogs whereas alanine substitution at position 1 does not affects the nitric oxide inhibitory activity. The cyclic[Ala5,6,8]-zanriorb A1 (10) was identified as a potent inhibitor of nitric oxide production in LPS activated J774.2 macrophages and non-toxic against normal fibroblast NIH-3T3 cells which can act as a safe lead anti-inflammatory molecule. The Analog cyclic[Ala5,6,8]-zanriorb A1 was identified as an inhibitor of nitric oxide production which is more active than the standard L-NMMA[2].

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Acknolegment

This work was supported by a grant 5738/Sindh/NRPU/R&D/ HEC/2016 from Higher Education Commission Pakistan, and a grant PSF/NSLP/S-HEJ (290) from the Pakistan Science Foundation.