Preprint Title  Sugar Derived Oxazolone Pseudotetrapeptide as γ-Turn Inducer and Anion-Selective Transporter

Authors  Sachin S. Burade, Sushil V. Pawar, Tanmoy Saha, Navnath M. Kumbhar, Amol S. Kotmale, Manzoor Ahmad, Pinaki Talukdar and Dilip D. Dhavale

Article Type  Full Research Paper

Supporting Information File 1  Supporting Information.pdf; 2.3 MB

ORCID® iDs  Dilip D. Dhavale - https://orcid.org/0000-0001-8221-6347
Sugar Derived Oxazolone Pseudotetrapeptide as γ-Turn Inducer and Anion-Selective Transporter

Sachin S. Burade¹, Sushil V. Pawar¹, Tanmoy Saha², Navanath Kumbhar¹, Amol S. Kotmale¹, Manzoor Ahmad², Pinaki Talukdar*² and Dilip D. Dhavale*¹

Address: ¹ Garware Research Center, Department of Chemistry, Savitribai Phule Pune University (formerly University of Pune), Pune 411007, India, and ² Indian Institute of Science Education and Research, Pune, Pune 411008, India

Email: Dilip D. Dhavale - ddd@chem.unipune.ac.in; Pinaki Talukdar- ptalukdar@iiserpune.ac.in

* Corresponding author

Keywords
sugar amino acid, oxazolone, pseudo-peptides, peptidomimetics, ion transport

Abstract
The C₃-tetrasubstituted furanoid sugar amino acid derived linear amino acid dipeptide 8 and tetrapeptide 9 on intra-molecular cyclization afforded oxazolone pseudo-peptides 1 and 2a, respectively, with formation of oxazole ring at the C-terminus. Conformational study of 2a showed seven membered intramolecular C=O···HN (II) hydrogen bonding leading to the γ-turn conformation which is absent in the precursor tetrapeptide 9 thus indicating the role of oxazolone ring in γ-turn formation in 2a. This fact was supported by the molecular modeling studies. The oxazolone ring containing pseudo-tetrapeptide 2a was found to be better ion transporter than pseudodi-
peptide 1. The ion selectivity of 2a indicated Cl\textsuperscript{-} anion-selective transport via the antiport mechanism.

**Introduction**

Tetrasubstituted \( \alpha \)-amino acid (TAA) derived peptidomimetics offer well defined turn structures due to the presence of stereochemically stable quaternary carbon centre [1]. For example, Toniolo and co-workers reported cyclopropane based TAA derived peptides that showed \( \alpha \)-turn conformation and forms 3\textsubscript{10}-helice conformation in higher oligomers [2-3]. Tanaka and co-workers reported the synthesis of TAA derived peptides from L/D-dimethyl tartarate which showed 3\textsubscript{10} helical conformation [4]. König and co-workers have reported tetrahydrofuran TAA and converted to three dipeptides that demonstrated \( \beta \)-turn type conformation [5]. Amongst these, the use of sugar derived TAA in peptidomimetics is less explored. Fleet and co-workers incorporated spiropeptides as well as tri-/tetra-peptides in to the TAA at the anomeric position of mannaofructose [6-8]. Stick and co-workers have reported the synthesis of tetrasubstitiuted sugar furanoid amino acid (TSFAA) derived homologated di-, tri-, tetra- and penta-peptides from D-glucofuranose derivative of which the linear TSFAA peptide showed a well defined helical array that are stabilized by internal hydrogen bonds [9-11]. Our group has reported trans-vicinal D-glucofuranoroic-3,4-di-acid having TAA framework and incorporated it in to the \( N \)-terminal tetrapeptide sequence (H-Phe-Trp-Lys-Thy-OH) to get glycopeptide which acts as a \( \alpha \)-turn inducer [12]. Recently, our group has synthesized acyclic- and cyclic- fluorinatated peptides from C-3 fluorinated D-glucofuranoid amino acid and demonstrated their selective anion transport activity [13-14]. In continuation of our interest in developing new sugar derived cyclic peptides [15], we synthesized TFSAA derived dipeptide 8
and tetra-peptide 9 from 3-oxo-D-glucofuranose derivative and attempted intramolecular cyclization to get corresponding cyclic peptides I and II (Figure 1) for ion transport activity study. In this process, we obtained oxazolone pseudo-peptides 1 and 2a (Figure 1) and not cyclic peptides I/II. The NMR studies of oxazolone ring containing pseudotetra-peptide 2a indicated γ-turn conformation that was stabilized by the seven membered (II)NH…O=C intramolecular hydrogen bonding which was noticed to be absent in linear tetrapeptide 9. The oxazolone ring containing pseudo-tetrapeptide 2a demonstrated selective Cl⁻ transport activity while; the pseudodi-peptide 1 showed less and linear tetra peptide 9 did not exhibit any ion transport activity. To the best of our knowledge, this is the first report on the formation of oxazolone peptides from TSFAA that induces γ-turn and demonstrate ion transport activity.

Figure 1: Pseudodi-peptide 1 and pseudotetra-peptide 2a with oxazolone ring.
Results and Discussion

D-Glucose was converted to C₃-tetrasubstituted furanoid sugar azido ester 3 as per our reported protocol [12]. Hydrolysis of ester functionality in 3 using LiOH in THF:H₂O:MeOH at room temperature afforded azido acid 4a (90%) while; hydrogenation of 3 using 10% Pd/C in MeOH at room temperature for 3 hours afforded amino ester 4b in 85% yield. The coupling of 4a and 4b using CMPI as coupling reagent in the presence of Et₃N in dichloromethane at 55 °C for 12 hours gave azido ester dipeptide 5 in 75% yield. Hydrolysis of 5 using LiOH in THF:H₂O:MeOH gave azido acid dipeptide 6a in 90% yield, while; hydrogenation of 5 using 10% Pd/C in methanol gave amino ester dipeptide 6b in 87% yield. The coupling of 6a and 6b using CMPI as coupling reagent in the presence of Et₃N in DCM afforded azido ester tetrapeptide 7 in 70% yield (Scheme 1) [16].

![Scheme 1: Synthesis of azido ester peptide 5 and 7.](image)

The azido ester dipeptide 5 and tetrapeptide 7 were individually converted to amino acid di- and tetra-peptides 8 and 9 respectively, using hydrolysis followed by hydrogenation reaction protocol (Scheme 2).
In order to get cyclic peptides I and II (Figure 1), an individual intramolecular coupling reaction of 8 as well as 9 was attempted. Thus, reactions of 8/9 with different coupling reagents such as HATU, TBTU, PyBOP, EDC.HCl under different solvent (DMF, acetonitrile, dichloromethane) and reaction conditions (25-80 °C for 24 h) were unsuccessful. This could be due to stable helical conformation of 8 and 9 in which reactive acid and amino functionalities are apart from each other. However, individual intramolecular coupling reaction of 8 and 9 using CMPI as a coupling reagent in the presence of Et3N in dichloromethane afforded oxazolone pseudodi- and pseudotetra-peptide 1 and 2a, respectively, with oxazolone ring formation at the C-terminal of the peptides [17-19]. The free amino group in 2a was acetylated with Ac2O, pyridine in DCM to get –NHAc derivative 2b. The single crystal formation of oxazolone pseudopeptides 1, 2a and 2b was unsuccessful under variety of solvent conditions.

The 1H and 13C-NMR spectra of 1, 2a and 2b showed sharp and well resolved signals in CDCl3 solution indicating the absence of rotational isomers (Figure S9, S11, S12 in SI). The oxazolone pseudo-dipeptide 1 is devoid of amide linkages and
is therefore not considered for conformational studies [20]. In case of 2a, the assignment of chemical shifts to different protons was made on the basis of $^1$H-$^1$H COSY, $^1$H-$^{13}$C-HMBC/HSQC, NOESY and $^1$H-$^{15}$N HSQC/HMBC studies (S14-S18 in SI) and values thus obtained are given in Table S1. The IR spectrum of 2a showed broad band at 3444 – 3421 cm$^{-1}$ indicating the presence of amino as well as amide NHs. The bands at 1740 and 1688 cm$^{-1}$ were assigned to the lactone carbonyl and amide (as well as imine) groups, respectively. In the $^1$H NMR spectrum, the downfield signals at δ 9.03 and 8.52 were assigned to the amide NH-I and NH-II, respectively. The signal at δ 1.80, integrating for two protons, was assigned to the presence of NH$_2$ functionality. In the $^{13}$C spectrum, appearance of signals at δ 170.8, 170.6 and 166.7 were assigned to the lactone/amide carbonyl functionalities. The signal at δ 163.0 was assigned to the -C=N functionality. The $^1$H-$^{15}$N HSQC and $^1$H-$^{15}$N HMBC spectra showed signal at δ 246.0 that was assigned to the imine (C=N-) nitrogen. The signal at δ 26.2 was assigned to the amine (NH$_2$) nitrogen. The signals at δ 112.8 and δ 114.1 were assigned to the nitrogen attached to the amide carbonyl (CONH) groups. This observed chemical shifts in the $^{15}$N NMR were suggestive for the formation of oxazolone ring [17-19] at the C-terminus supporting structure 2a.

The $^1$H NMR spectra of N-acylated compound 2b showed three downfield signals at δ 8.24, 8.19 and 8.09 that were assigned to three amide NHs. An additional singlet at δ 2.0, integrating for three protons, was assigned to the NHCOCH$_3$. In the $^{13}$C NMR spectrum, appearance of five signals in the downfield region at δ 171.6, 170.9,167.5,165.0, and 164.0 indicated the presence of three amide, lactone carbonyl and imine carbon (-C=N) suggesting the presence of oxazolone ring in 2b.
Conformational Study of 2a

The downfield shift of amide NH protons >δ 7.5 in 2a suggested the possible involvement of intra-molecular hydrogen bonding [21]. The observed NOESY cross peaks of NH(I)←NH₂ indicated closer proximity and are oriented on the same side (Figure 2). This is likely to involve (I)NH⋯NH₂ weak intramolecular hydrogen bonding. The amide NH(II) showed strong cross peaks with H-2, H-5 of ring C and H-4 of ring B and weak cross peaks with H-1, H-6 proton of ring C, indicating closer proximity and orientation on the same side of these protons. Appearance of strong NOE between NH(II)←H-4 and weak NOE between NH(II)←H-2 protons of ring B indicated that the NH(II) is bending towards the ring A carbonyl with the formation of seven membered ring intramolecular hydrogen bonding leading to the γ-turn conformation (Figure 2).

![Diagram of molecule with NOE peaks](image)

**Figure 2:** NOESY spectrum of 2a and characteristic NOE.

The involvement of amide NHs in intramolecular H-bonding was supported by the DMSO-d₆ titration studies. Thus, 5 μL of DMSO- d₆ was sequentially added (up to 50 μL) to the CDCl₃ solution of 2a and change in δ value of NH protons was monitored by the ¹H NMR [22]. The NH(I) proton showed higher change in chemical shift Δδ =
0.2 ppm indicating weak (I)NH···NH$_2$ intramolecular H-bonding, and NH(II) showed smaller $\Delta \delta = 0.13$ ppm suggesting strong (II)NH···O=C H-bonding (Figure 3).

![DMSO titration study of 2a.](image)

Figure 3: DMSO titration study of 2a.

This fact was further supported by temperature dependent $^1$H-NMR study [23-27]. The temperature dependent $^1$H NMR of 2a in CDCl$_3$ solvent at 283 – 323 °K was recorded that showed higher $\Delta \delta/\Delta T$ value of $6.2 \times 10^{-3}$ ppm/K for NH(I) indicating its involvement in weak intramolecular H-bonding. For NH(II) the lower $\Delta \delta/\Delta T$ value of $3.7 \times 10^{-3}$ ppm/K supported its association in strong intra-molecular hydrogen bonding with C=O leading to the $\gamma$-turn conformation (Figure 4).

![$^1$H NMR temperature study of 2a.](image)

Figure 4: $^1$H NMR temperature study of 2a.
The $^1$H NMR dilution study of 2a in CDCl$_3$ solution showed negligible change ($\Delta \delta = 0.01$) in the chemical shift of NH(I) and (II) protons (Figure S17), further supporting their intramolecular hydrogen bonding with the free NH$_2$ and C=O, respectively. These studies thus supported the presence of $\gamma$-turn helical type conformation of 2a.

Molecular modeling studies

In order to corroborate our results obtained from the NMR studies, the molecular modeling study was performed using Spartan'14 software [28-29]. The initial geometry of 2a, generated from the NOESY study, was subjected for geometry optimization using semi-empirical PM6 method. The resulted optimized structure of 2a indicated considerable crowding due to the presence of oxazolone ring and two acetonide rings of the sugar ring D (Figure 5A). To accumulate the oxazolone ring, the sugar ring C is pushed towards sugar rings B and A forming $\gamma$-turn conformation that is stabilized by seven membered intramolecular (II) NH···O=C hydrogen bonding (bond distance ($d$) = 2.61 Å and bond angle (NH···O) = 114.06°). To understand the role of oxazolone ring in stabilizing the $\gamma$-turn, we performed geometry optimization on TFSAA linear tetrapeptide amino acid 9 (Figure 5C). The optimized geometry of 9 showed change in helical conformation to release the crowding due to acetonide groups wherein; the N- and C-terminals are further away- thus precluding the $\gamma$-turn conformation (bond distance ($d$) = 3.11 Å), and bond angle (NH···O) = 98.90°. The comparison of geometrically optimized models of 2a and 9 showed small structural changes with respect to helical pitch length. The distance between C=O···N(II) is 3.18 Å in 2a and 3.43 Å in 9. The distance between C$_{\alpha 1}$···C$_{\alpha 4}$ is 9.67 Å in 2a and 9.84 Å in 9 (Figure S27 in SI). Similarly, distance between N$_1$···C$_4$ is 9.44 Å in 2a and 10.47 Å in 9. This suggested elongated helical structure of linear tetrapeptide 9 than 2a thus supporting the compact helical architecture for 2a due to the presence of oxazolone
ring leading to γ-turn conformation. The molecular modelling study of N-acetylated compound 2b also indicated the presence of seven membered hydrogen bonding between NH(II) and –C=O ((bond distance \(d\) = 2.74 Å). and bond angle (NH···O) = 112.98°) suggesting the presence of γ-turn conformation (Figure 5B) due to the presence of oxozolone ring.

![Figure 5: Optimized helical conformations of (A) 2a, (B) 2b and (C) 9.](image)

**Ion transport activity**

The cation and anion transport across lipid bilayer membrane plays a crucial role in various biological processes [30-32]. Amongst these, the transport of anions is useful in regulating intracellular pH, membrane potential, cell volume, and fluid transport [33]. Any dysfunction in these processes led to various diseases such as cystic fibrosis, Dent disease, Bartter syndrome, and epilepsy [34-41]. In order to mimic the regulatory functions in living systems, a wide range of anion transporters have been investigated that include peptides [42-51], oligoureas [52-53], anion-π slides [54-57], steroids [58-61], calixpyrroles [62-64], calixarenes [65-67], and other scaffolds [68-70]. In particular, peptide based transmembrane anion transporters have attracted
great interest. For example, Ghadiri [42], Ranganathan [43], and Granja [44] have independently reported different types of cyclic peptides as anion transporters. Gale, Luis and coworkers [45-46] have separately reported the linear pseudopeptides as a receptors and transporters of chloride and nitrate anions.

Inspired with our recent ion transport studies with acyclic and cyclic fluorinated sugar derived peptides [13-14], we investigated the ion transport activity of 1 and 2a across lipid bilayer membrane. In this study, the collapse of the pH gradient (pH_{out} = 7.8 and pH_{in} = 7.0), created across egg yolk L-α-phosphatidylcholine (EYPC) vesicles with entrapped 8-hydroxypyrene-1,3,6-trisulfonic acid trisodium salt (HPTS) dye (i.e., EYPC-LUVs⇒HPTS) [71-78] was monitored by measuring the fluorescence intensity of the dye at λ_{em} = 510 nm (λ_{ex} = 450 nm) with time (Figure S11). Thus, addition of 2a (10 µM) resulted in the significant increase in HPTS fluorescence within 200 s (Figure 6B), while oxazolone pseudodi-peptide 1 was found to be lesser active (Figure 6A).

Figure 6: Ion transport activity (A) for 1, (B) for 2a, across EYPC-LUVs HPTS.

From the dose response data of 2a, the calculated effective concentration EC_{50} = 0.72 µM indicated good ion transport activity of 2a (Figure S12 in SI). The Hill coefficient n value of 1.26 indicated that one molecule of 2a is involved in the formation of the active transporter. The promising ion transport activity of 2a encouraged us to explore its cation and anion selectivity study by varying either cations (for MCl, M^+ = Li^+, Na^+, K^+, Rb^+, and Cs^+) or anions (for NaA, A^−, F^−, Cl^−, Br^−, I^−, NO_3^−, SCN^−, AcO^− and ClO_4^−) of the extra-vesicular salt, respectively. Thus,
variation of external cations, in the presence of 2a (0-10 µM), showed minor changes in the transport activity with the sequence: Na\(^+\) > Rb\(^+\) > Li\(^+\) > K\(^+\) ~ Cs\(^+\) (Figure 7A), which suggest lesser influence of alkali metal cations in the transport process. However, variation of extra-vesicular anions demonstrated the changes in the transport behaviour with the following selectivity sequence: Cl\(^-\) >> AcO \(^-\) ~ SCN\(^-\) ~ F\(^-\) > NO\(_3\)\(^-\) >> Br\(^-\) ~ I\(^-\), showing highest selectivity for the Cl\(^-\) ion (Figure 7B). This data indicated the presence of Cl\(^-\)···HNH···Cl\(^-\) interactions with anion. The absence of change in chemical shift of amide NH’s is probably due to their involvement in strong intramolecular hydrogen bonding.

![Figure 7](https://example.com/figure7.png)

**Figure 7:** (A) Cation and (B) Anion transport activity of 2a.

**Chloride leakage study**

In order to know the role of free NH\(_2\) group in 2a for Cl\(^-\) recognition during the transport of the ion, we monitored the Cl\(^-\) transport activities of the amino compound 2a and its N-acylated derivative 2b. The influx of Cl\(^-\) ion by these transporters were monitored using EYPC-LUVs\(\rightleftharpoons\)Lucigenin. Additionally, compound 9, that has a free amino and a free carboxylic acid groups, was also subjected to the Cl\(^-\) transport study. The Lucigenin, a Cl\(^-\) sensitive dye, was entrapped within the lipid vesicles and the rate of quenching in fluorescence at \(\lambda_{em} = 535 \text{ nm} \) (\(\lambda_{ex} = 455 \text{ nm}\)) was monitored using transporter 2a by creating a Cl\(^-\) gradient across the lipid membrane by applying NaCl in the extravesicular buffer (Figure S14 in SI). The compound 2a showed a
significant decrease in the fluorescence rate of lucigenin and the change in fluorescence upon the addition of 2a (Figure 8A and 8B). We observed that the N-acetylated compound 2b to be inactive (Figure 8A) indicating that the free amine group is necessary for the transport activity. The compound 9 did not exhibit any transport activity even at very high concentration (Figure S16 in SI).

Further, the variation of cations in the extravesicular buffer using different salts of MCl (M⁺ = Li⁺, Na⁺, K⁺, Rb⁺, Cs⁺) does not make any change in the transport rate of 2a (20 µM) which excludes any role of cation in an overall transport process (Figure 8C). Finally, to evaluate the mechanism of ion transport, the transport of Cl⁻ using the compound 2a (20 µM) was monitored in presence and absence of valinomycin (a selective K⁺ transporter, 1 µM). There was a significant increase in the transport rate of 2a in presence of valinomycin confirming the transport process occurring through antiport mechanism via Cl⁻/NO₃⁻ exchange (Figure 8D). This study suggest that the fact that the presence of oxazolone ring and free amino group are responsible for the ion transport and selective Cl⁻ activity of 2a.

![Figure 8](image_url)

**Figure 8:** (A) Comparison of Ion Transport activity of 2a and 2b at 20 µM across EYPC-LUVs→Lucigenin (B) Concentration dependent activity of 2a across EYPC-
Lucigenin (C) Transport activity of 2a (20 µM) by changing extravesicular cations (D) Transport activity of 2a (20 µM) in the presence and absence of valinomycin (1 µM) across EYPC-LUVs → Lucigenin binding.

**Conclusion**

In conclusion, we have synthesized C3-TFSAA dipeptide 8 and tetrapeptide 9. The intramolecular cyclization of 8 and 9 led to the formation of oxazolone ring at the C-terminal giving pseudo-peptides 1 and 2a. The pseudo-tetrapeptide 2a showed γ-turn conformation that is stabilized by the seven membered intramolecular hydrogen bonding. The pseudo-tetrapeptide 2a was found to be selective ion transporter towards anions than alkali cations. The anion transport by 2a was facilitated by an anion-anion antiport mechanism. The absence of γ-turn conformation as well as ion transport activity in linear tetrapptide 9 – the precursor of 2a, suggest that the oxazolon ring in 2a is γ-turn inducer as well as responsive for selective anion transport activity.

**Supporting Information**

Supporting information (Experimental procedures, mp, ¹H and ¹³C NMR data, HRMS and 2D NMR) for this article is provided in supporting information

**Acknowledgements**

We are thankful to the Science and Engineering Research Board (SERB), New Delhi (File no. EMR/2014/000873) for financial support and Central Instrumentation Facility (CIF), SPPU, Pune for analytical services. S.S.B., S.P, T.S., A.K and N.K. are thankful to CSIR and UGC, DSK-PDF New Delhi for providing fellowships.
References


16. Synthesis of azido dipeptide 5 and tetrapeptide 7 is reported [11] using TsCl in pyridine as activating agent for carboxylic group. The same reaction at our hand gave dark brown colour product that on purification afforded ~30% yield while; the use of CMPI as coupling reagent gave pale yellow solid product that on purification gave ~75% yield of 5 and 7.


20. Further, reactions of 1 and 2 under different acidic/basic conditions gave complex mixture of products thus precluding extension of work.


