

A Defined α -Helix in the Bifunctional O-Glycosylated Natriuretic Peptide TcNP_a from the Venom of *Tropidechis carinatus***

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Abstract: Natriuretic peptides (NP) play important roles in human cardiac physiology through their guanylyl cyclase receptors NPR-A and NPR-B. Described herein is a bifunctional O-glycosylated natriuretic peptide, TcNP_a, from *Tropidechis carinatus* venom and it unusually targets both NPR-A and NPR-B. Characterization using specific glycosidases and ETD-MS identified the glycan as galactosyl- β (1-3)-N-acetyl-galactosamine (Gal-GalNAc) and was α -linked to the C-terminal threonine residue. TcNP_a contains the characteristic NP 17-membered disulfide ring with conserved phenylalanine and arginine residues. Both glycosylated and nonglycosylated forms were synthesized by Fmoc solid-phase peptide synthesis and NMR analysis identified an α -helix within the disulfide ring containing the putative pharmacophore for NPR-A. Surprisingly, both forms activated NPR-A and NPR-B and were relatively resistant towards proteolytic degradation in plasma. This work will underpin the future development of bifunctional NP peptide mimetics.

The natriuretic peptide (NP) family has traditionally been associated with regulation of cardioprotective roles but are also becoming appreciated as a family of endogenous hormones and para- and autocrine factors affecting physiological systems.^[1] The mammalian NP family (Table 1)

Table 1: Sequence alignment of selected natriuretic peptides. Bold residues are important for receptor binding.

Peptide	Sequence
hANP ^[11]	SLRRSSCFGGRMDRIGAQSGLGCSNFRY
hBNP ^[11]	SPKMVQSGSCFGRKMDRISSSSGLGCKVLRH
hCNP ^[11]	GLSKGCFGLKLDRIQSGMSGLGC
TNPc ^[12]	SDSKIGNGCFGFPDLRIGSVSGLGCSNRIMQNPPKFSGE
DNP ^[13]	EVKYDPCFQGHKIDRINHVSNLGCPSLRDRPNAPSTSA
CD-NP ^[14]	GLSKGCFGLKLDRIQSGMSGLGCPSLRDRPNAPSTSA
TcNP _a	GDGCFGLPIDRIGSASGMCGSVPKPTPGGS

comprises atrial natriuretic peptide (ANP), B-type natriuretic peptide (BNP), and C-type natriuretic peptide (CNP). Their biological effects are mediated through the natriuretic peptide receptors NPR-A and NPR-B which are membrane-bound guanylyl cyclases and elicit their responses through cGMP production.^[2] ANP and BNP bind to NPR-A, thus resulting in the renal and cardiovascular effects of vasorelaxation, natriuresis, diuresis, and endothelial permeability,^[3] but also induce metabolic responses such as lipolysis,^[4] brown fat thermogenesis^[5] and skeletal muscle mitochondrial biogenesis, respiration, and lipid oxidation.^[6] NPR-B is the principal receptor of CNP which mediates biological responses of vasorelaxation,^[7] a protective role preventing cardiac hypertrophy^[8] and fibroblast proliferation.^[9] These hormonal actions associated with cardiorenal disease have reinvigorated interest in natriuretic peptides which led to the mimetic CD-NP, a chimeric peptide which consists of CNP plus the C-terminal tail of *Dendroaspis* natriuretic peptide (DNP). This bifunctional peptide acts at NPR-A and NPR-B by stimulating natriuresis and diuresis effects from the DNP–NPR-A interaction and vasodilatory effects from CNP–NPR-B binding. In addition CNP is less hypotensive than ANP/BNP and remains active in the failing heart.^[10]

We describe the discovery and characterization of the bifunctional O-glycosylated natriuretic peptide TcNP_a, (Table 1) from the venom of the Australian snake *Tropidechis carinatus* (*T. carinatus*), which targets NPR-A and NPR-B. It is also the first member of the NP family that possesses significant secondary structure, an α -helix, and maintains strong resistance to proteolytic degradation in plasma.

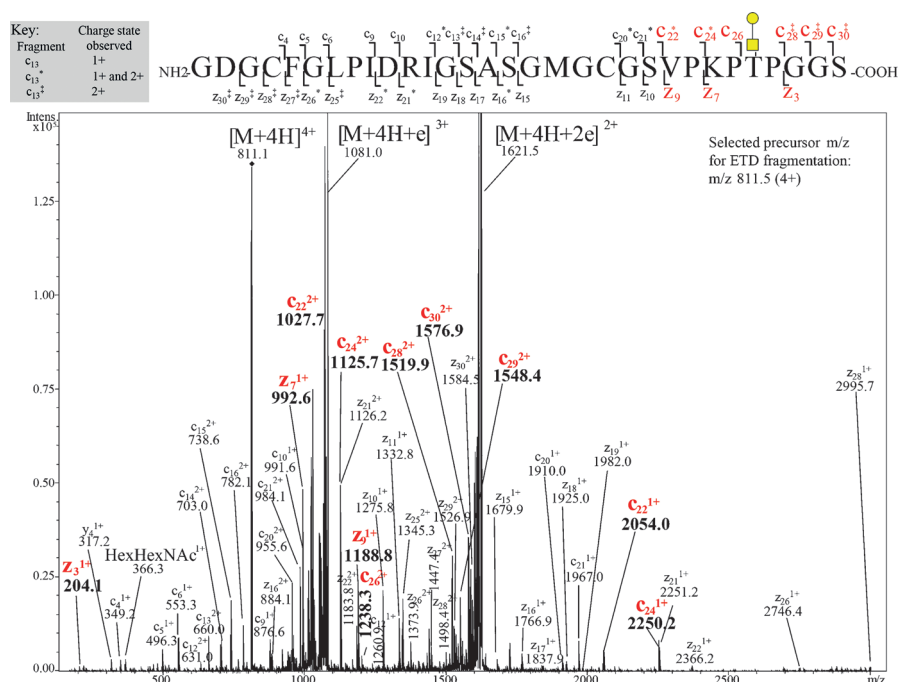
Glycopeptide TcNP_a was isolated from *T. carinatus* venom using ion-exchange chromatography and comprised 2.2 % of the crude venom. Subsequent electrospray ionization

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Scheme 1. Localization of the TcNPα O-linked glycan. De novo sequencing of the glycosylated NP and localization of the glycan using ion trap ETD MS/MS. Fragment ions supporting the glycosylated post-translational modification on the threonine residue are highlighted in red.

(ESI) LC-MS/MS (SI 4.2) showed marker ions of m/z 204.07 (*N*-acetylhexosamine) and m/z 366.10 (hexose-*N*-acetylhexosamine) in the MS/MS spectra, thus indicating the presence of *O*-linked glycosylation, which is consistent with a hexose-*N*-acetylhexosamine (Hex-HexNAc) structure (see Figure S2A in the Supporting Information). Using optimized ionization conditions to avoid premature in-source fragmentation, the primary structure of the glycosylated NP was sequenced de novo and comprised 31 amino acid residues with a native nonreduced mass of 3239.23 Da (see Figure S2B in the Supporting Information). This sequence derives from an earlier reported cDNA sequence (TcNPα, calculated mass 3647.71 Da) which consisted of 39 amino acid residues without glycosylation.^[15] The translated primary structure was determined using electron-transfer dissociation mass spectrometry (ETD-MS) and the threonine residue (Thr27) at the C-terminus was identified as the *O*-linked glycosidic residue (Scheme 1).

An advantage of using ETD-MS is that the *c*- and *z*-type ions generated give a more complete series of peptide fragment ions while preserving labile side-chain modifications, including *O*-linked glycosylation.^[16] The m/z value corresponding to the highest charge state (m/z 811.5 $[M+4H]^{4+}$) of TcNPα was manually selected for ETD MS/MS fragmentation. The ETD MS/MS spectra confidently matched theoretical fragment ions, produced computationally, of the Thr27-modified TcNPα using a previously reported methodology.^[17] *O*-linked glycosylation appears to be free of peptide motifs but it does have a preference for proximate proline residues near glycosylated serine or threonine residues.^[18] Of the four putative modification sites, the C-terminal threonine residue (Thr27, surrounded by three proline

residues) was identified as the glycosylated residue using ETD-MS/MS (Scheme 1).

To characterize the linkage and topology of the *O*-linked glycan moiety, TcNPα was treated with the specific enzymes *O*-glycosidase (endo- α -*N*-acetylglucosaminidase) and β (1-3) galactosidase (Figure 1). *O*-Glycosidase catalyzes the specific removal of the α -linked Core 1 (Gal β (1-3)GalNAc) and Core 3 (GlcNAc β (1-3)GalNAc) glycans from either serine or threonine residues.^[19] This enzyme-based degradation approach led to the conclusion that the TcNPα *O*-linked glycan was the Core 1 mucin-type *O*-glycosylation, galactosyl- β (1-3)-*N*-acetylglucosamine (Gal-GalNAc). This natriuretic peptide from *T. carinatus* is the first mature NP known to be glycosylated (Figure 1).

Previous research on *T. carinatus* venom has identified the group D

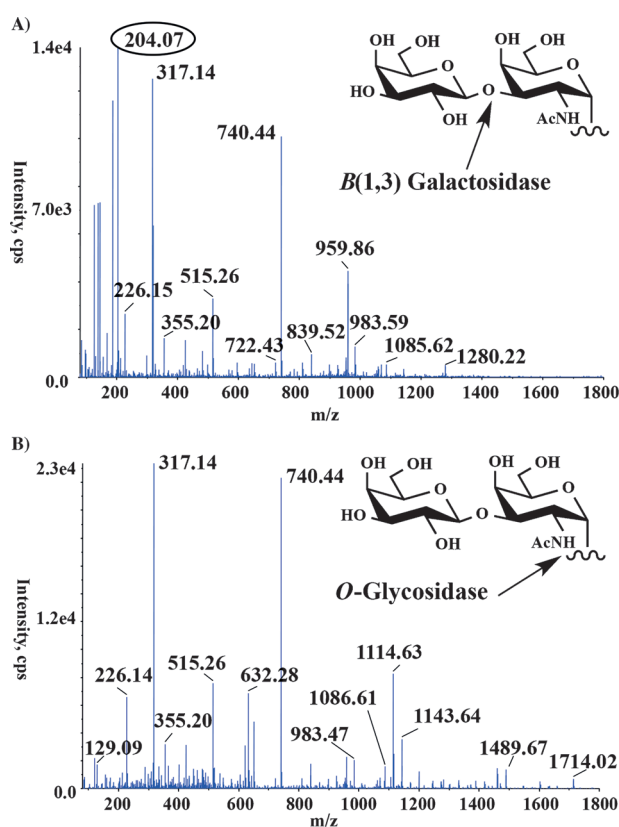


Figure 1. Characterization of the TcNPα O-linked glycan. A) ESI MS/MS spectra of β (1-3) galactosidase treatment highlighting the *N*-acetylhexosamine marker ion (m/z 204.07). B) ESI MS/MS spectra from the *O*-glycosidase assay highlighting the absence of glycan marker ions.

prothrombin activator trocarn as being *O*-glycosylated at Ser45 on the light chain.^[20] Nevertheless, snake toxins are predominantly reported to possess N-linked glycosylation, especially serine proteases, where the glycan may modulate the protease catalytic activity.^[21] An *O*-glycosylated conotoxin (contulakin-G) from *Conus geographus* is a potent analgesic which targets G-protein coupled receptors and reached phase II clinical trials for post-operative pain. The glycan, Gal-GalNAc, is linked to inhibiting proteolytic degradation and gave two orders of magnitude greater potency as an analgesic than the nonglycosylated form.^[22]

The nonglycosylated (TcNPa-ng) and glycosylated (TcNPa) peptides were synthesized using standard Fmoc solid-phase peptide synthesis on Fmoc-serine-Wang resin (see Figure S3 in the Supporting Information). Evaluation of their secondary structures was undertaken by circular dichroism (CD) analysis in 50 % TFE/H₂O and 50 % HFIP/H₂O. Both NP forms showed a maximum signal at $\lambda = 190$ nm, and negative bands at $\lambda = 208$ nm and 222 nm, which are indicative of an α -helical structure (see Figure S4 in the Supporting Information).

Previously, CD and Fourier transform infrared (FTIR) spectroscopic studies of ANP, BNP, and their various analogues have not shown the presence of α -helical structures though a mixture of β -turn and β -sheet conformations have been suggested.^[23] NMR spectra of both NP forms (1 mM) were recorded in 50 % deuterated TFE/water (pH 5.0). A complete backbone assignment was possible despite the limited dispersion in the amide-bond region. A family of 20 structures, which satisfies the distance and angle restraints, was calculated for TcNPa-ng and TcNPa. The structural statistics are given in Table S1 in the Supporting Information. The major element of secondary structure is an α -helix extending from residues Pro8-Gly13. The proline-rich C-terminal region, containing the glycosylation site, is disordered and consistent with the secondary shifts which are generally within ± 0.1 ppm of random coil values. The polypeptide structures for TcNPa-ng and TcNPa are very similar as shown in Figure 2.

We performed comparative stability studies of both NP forms in rat plasma (Figure 3). Greater than 60 % of both natriuretic peptides remained intact after 48 hours of incubation. Hence the high resistance cannot be attributed to the C-terminal glycan alone. Recent research has shown DNP is also resistant to proteolysis and that addition of the DNP tail to CNP confers proteolytic resistance to the chimeric peptide in the presence of proteases associated with human kidney membranes.^[24]

To determine the functional properties of the TcNPa, NPR-A and NPR-B plasmids were transfected into CHO cells and the EC₅₀ value, based on cGMP production for each peptide, was determined using a competitive ELISA assay. Similar EC₅₀ values for TcNPa and TcNPa-ng (Table 2) suggest that the O-linked glycan is not directly involved in receptor binding. Surprisingly, both peptide forms were shown to activate both NPR-A and NPR-B unlike other native natriuretic peptides such as ANP, BNP, and DNP which target NPR-A and produce natriuretic, diuretic, and vasodilatory effects as a result of myocardial stretch and over-

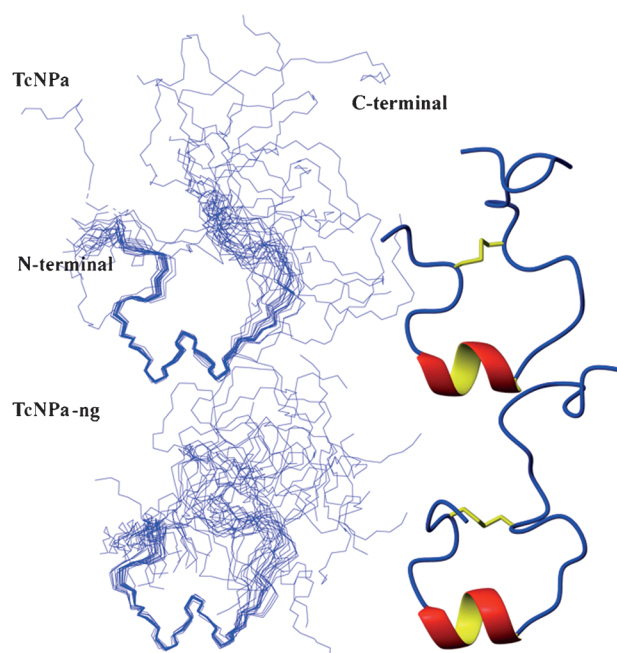


Figure 2. NMR structure of synthetic TcNPa-ng and TcNPa. Superposition of the 20 lowest-energy structures over the backbone atoms of residues 7–14 for both structures are shown on the left. The lowest-energy structure is shown in ribbon form on the right. The helical regions are shown with thickened ribbons and the disulfide bond is in stick format. The figure was made using MOLMOL.^[25]

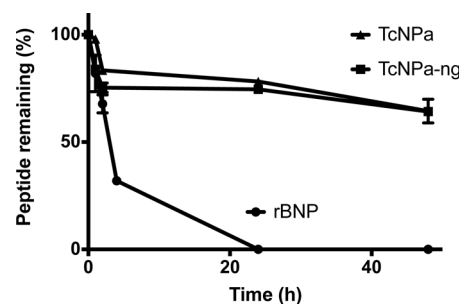


Figure 3. Plasma stability of synthetic TcNPa-ng, TcNPa and rBNP.

load.^[13] Synthetic forms of ANP (Carperitide) and BNP (Nesiritide) are approved for the treatment of acute decompensated heart failure in Japan and the US, respectively, though both have been linked with unwanted side effects such as excessive hypotension and impaired renal function.^[26–28] The bifunctionality of both the nonglycosylated and glycosy-

Table 2: EC₅₀ values of synthetic TcNPa and TcNPa-ng at NPR-A and NPR-B.

Peptide	Receptor EC ₅₀ [nM]	
	NPR-A	NPR-B
ANP	42.71	–
CNP	–	74.60
TcNPa	1080.0	328.60
TcNPa-ng	672.90	261.0

lated variants of TcNP_a may provide leads to molecules that will potentially limit the aforementioned side effects.^[14]

In conclusion, we have isolated and characterized a unique natriuretic peptide from *T. carinatus*. The mature 31 residue peptide contains a mucin-type Gal-GalNAc *O*-linked glycan on the C-terminal threonine residue. NMR and CD analysis identified an α -helical secondary structure in both synthetic glycosylated and nonglycosylated NPs and may prove invaluable in determining the full natriuretic peptide pharmacophore to aid the design of smaller helical mimetics.^[29,30] While NMR structural analysis and stability testing did not infer a structural role for the glycan, the cGMP assay showed both variants are bifunctional in stimulating both NPR-A and NPR-B in a fashion similar to that of the designed chimera CD-NP. Thus, TcNP_a represents the first report of a nonhuman *O*-glycosylated NP which is bifunctional and serves as a lead molecule to a novel therapeutic class.

Keywords: glycopeptide synthesis · helical structures · natriuretic peptide TcNP_a · snake toxin · *Tropidechis carinatus*

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