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# VASOPRESSIN TRISULPHIDE: SYNTHESIS, NMR STUDY AND AFFINITY STUDIES WITH $V_1$ AND $V_2$ SUBTYPES RECEPTORS.

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**Abstract** : A trisulfide-containing analogue of [arginine$^8$]-vasopressin has been synthesized from the corresponding dithiol derivative. Affinities for V1 and V2 receptors were determined. Characterization of the structural differences with the parent hormone was achieved by NMR analysis and a model was built from the disulphide derivative. © 1997 Elsevier Science Ltd. All rights reserved.

The oxidation state of cysteine residues plays a major role in the structure and function of many polypeptides and proteins. Oxidised cysteines form disulphide bonds which play an important part in many protein three-dimensional structure: they impose restrictions on the folding of the polypeptide chain and contribute to the stability of the native form. By contrast, there are also cases in which disulphide bridges must be reduced as a prerequisite for the biological function of a protein. As well as this, ratios of thiol to disulphide in small peptides such as glutathione are crucial for the maintenance of cellular redox states.

Discovery of trisulphide natural products endowed with interesting properties (calicheamicins[1], esperamicins[2], lissolinotoxins[3]) has generated significant interest during the past few years. Recently, we reported the formation of trisulphide derivatives as by-products during the chemical synthesis of cystine-containing peptides.[4] Also, a trisulphide derivative of the biosynthetic human growth hormone was detected during its purification from a recombinant *Escherichia coli* strain[5] at this time. These data show that trisulphide can be formed in a wide variety of conditions and support the hypothesis that trisulphide cystine-containing compounds exist *in vivo*. Whether such peptidic structures have the same biological properties as their disulphide counterparts is an interesting question. We first investigated whether trisulphides could be enzymatically reduced by disulphide reductases. Several glutathione-like trisulphides were synthesised. All of them were reduced to their corresponding thiols in two step reactions catalysed by either glutathione - or trypanothione reductase.[6a-b] The formation of a persulphide intermediate was demonstrated. Interestingly, one of these molecules was a significantly better substrate than its disulphide counterpart, which indicated that trisulphides may have different biological properties to their natural analogues. In this study, we considered the influence of the trisulphide link not in an enzyme-substrate system but in a ligand-receptor system. We required a model in which the disulphide bond plays a structural role essential for the existence of the biologically active conformation. Vasopressin (n = 0, Figure 1) and related hormones are products of major importance for therapeutic use.

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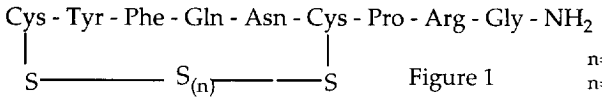


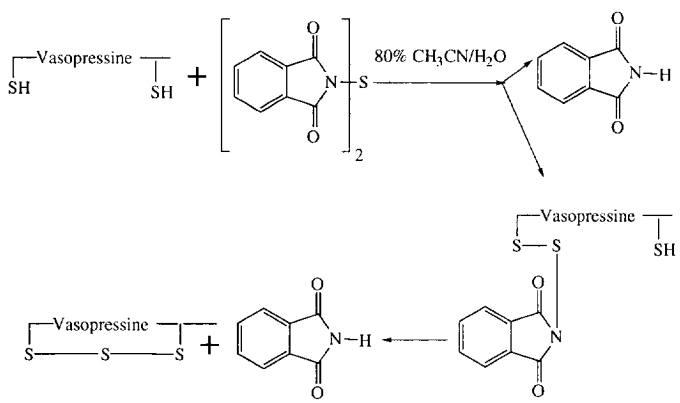

Figure 1

n=0 8-arginine vasopressin ($VS_2$)
n=1 8-arginine vasopressin trisulphide ($VS_3$)

They consist of nine residues and contain a disulphide bridge structurally involved in the physiological activity. Dicarba-deamino-vasopressin and oxytocin analogues have been previously described showing that the substitution of the disulphide bond by an ethylene linkage weakly preserves the biological activity.[7,8] We chose vasopressin for which a computer-generated 3D-model of its interaction with the Rat $V_{1a}$ receptor was recently published.[9] According to this model, in addition to steric constrains, the hormone disulphide bridge could play an "active" role in the binding to the receptor by interacting with a tryptophan residue. The presence of the more hydrophobic trisulphide bridge might modify such an interaction. Nevertheless, no structural data about trisulphides was available to predict the structural consequences of the presence of an extra sulphur. We report here the synthesis of the trisulphide analogue of vasopressin ($VS_3$), an estimation of its biological activity on both $V_1$ and $V_2$ vasopressin receptors and a NMR evaluation of its structural differences with the natural compound.

## Chemistry

We first attempt to synthesize $VS_3$ using the method described for the preparation of glutathione trisulphide from glutathione disulphide.[4] Vasopressin disulphide ($VS_2$) was reacted with elemental sulphur (10 eq.) in EtOH / $CHCl_3$ / $CS_2$ / $NH_4OH$ 28% (45:5:2:2). This protocol produced mixtures of trisulphide, tetrasulphide and pentasulphide derivatives associated with dimeric and trimeric peptides containing variable numbers of additional sulphur atoms. After several purification steps, purity and yield of $VS_3$ were insufficient to perform NMR and biological assays. Good yield and excellent purity were finally obtained using the method described by Lundin *et al.* .[10] Briefly, as shown in scheme 1, one equivalent of dihydrovasopressin[11] were reacted with one equivalent of commercial N, N'-thiobisphtalimide in acetonitrile / water (8:2).




Scheme 1

High dilution conditions (0.07 mM) were used to avoid the formation of dimers. The formation of the trisulphide derivative could be easily monitored by Plasma Desorption Mass Spectrometry performed directly on the reaction medium (Figure 2).

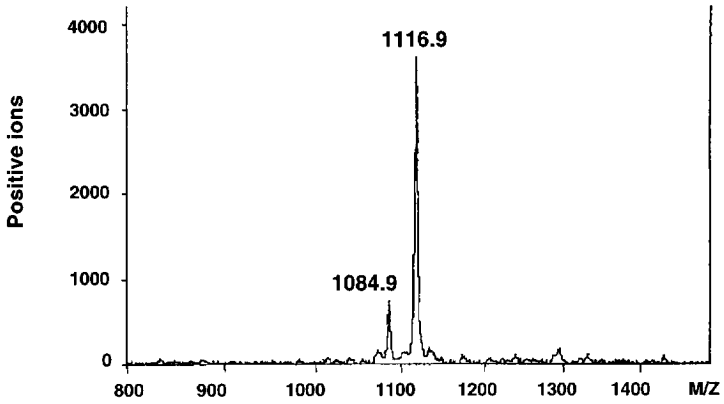



Figure 2 : Plasma desorption mass spectrum of the reaction mixture : (M+H+) = 1116.9 for vasopressin trisulphide

After evaporation, crude extract was submitted to gel filtration (G15 from Pharmacia) followed by preparative reverse-phase HPLC.[12] $VS_3$ was obtained with a yield of 48%; its purity was established by analytical HPLC[12] and capillary electrophoresis. The trisulphide bond was stable for more than two weeks at pH 2. At pH 7, $VS_3$ degradation began to be detectable after three days (appearance of $VS_2$ identified by Plasma Desorption Mass Spectrometry). At pH 9, $VS_3$ disappeared in three days producing $VS_2$.

**Affinity of $VS_3$ towards $V_1$ and $V_2$ subtypes vasopressin receptors**

The physiological effects of vasopressin are known to be mediated by two types of cell surface receptor which are distinguished by their differences in affinity for various vasopressin analogues and by the second messenger systems they activate. Activation of $V_1$ subtype vasopressin receptors, found in smooth muscle cells, hepatocytes and platelets, results in the formation of inositol 1,4,5-triphosphate and diacylglycerol which increase intracellular $Ca^{2+}$ and protein kinase C activity. Activation of $V_2$ subtype vasopressin receptors, present in kidney epithelial cells, stimulates adenylate cyclase increasing intracellular cAMP content. Competition binding assays were performed on $V_1$ subtype receptors obtained from membrane preparations of A7r[5] cells (smooth muscle of rat embryonic thoracic aorta) and on $V_2$ subtype receptors obtained from LLCPK1 cells (kidney pig). For $V_1$ receptors, [$^3$H][d($CH_2$)$^5$, tyr(Me)$^2$ arginine-vasopressin was used as radioligand at 0.3 nM and d($CH_2$)$^5$, tyr(Me)$^2$ as non specific ligand at 1 µM.[13] For $V_2$ receptors, [$^3$H] ($VS_2$) was used as radioligand at 3 nM and arginine-vasopressin at 2 µM.[14] Specific binding was obtained by subtracting non-specific binding determined in the presence of an excess of non radioactive ligand from the total binding. $IC_{50}$ values ( concentrations of trisulphide derivative inhibiting 50 % of the specific binding of the radioligand ) were determined from the percentages of specific binding for ten concentrations of $VS_3$ (Figures 3 and 4) and are reported in Table 1.

| Compounds | $V_1$ subtype receptor $IC_{50}$ (nm) | $V_2$ subtype receptor $IC_{50}$ (nm) |
|---|---|---|
| vasopressin ($VS_2$) | 10 | 3.3 |
| vasopressin trisulphide ($VS_3$) | 9.5 | 9.5 |

Table 1 : $IC_{50}$ values for vasopressin trisulphide and reference vasopressin for $V_1$ and $V_2$ subtype receptors

$[^3H][d(CH_2)^5, Tyr(Me^2)]$ 8-arginine vasopressin

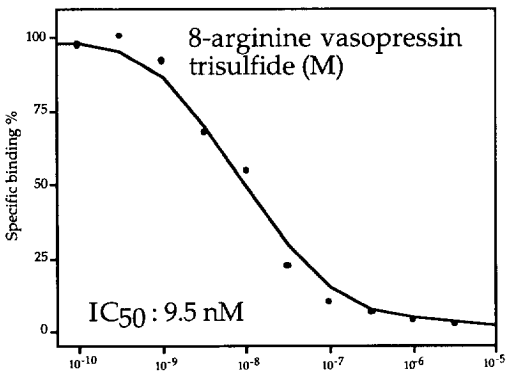



Figure 3 : Trisulfide analogue : competition binding data towards V1 receptors

$[^3H]AVP$

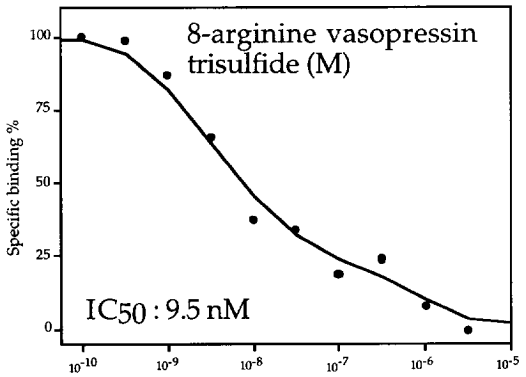



Figure 4 : Trisulfide analogue : competition binding data towards V2 receptors

Affinities of $VS_3$ and the natural disulphide analogue for the $V_1$ receptor are identical. On the other hand, the presence of the third sulphur atom leads to a threefold diminishment for the vasopressin affinity towards the $V_2$ subtype receptor. However this difference might not be significant due to the experimental error made on the $IC_{50}$ determination (estimated to 1/2 Log in this case). These results clearly illustrate that the introduction of an additional sulphur in the disulphide bridge of vasopressin has no relevant physiological consequences for the affinity of the hormone for its receptors.

**NMR analysis of vasopressin trisulphide**

NMR spectra were recorded at 300°K on a Bruker 600 MHz DMX spectrometer in the conditions previously described for vasopressin disulphide $VS_2$[15] : 2 mM $VS_3$ in 500 µl DMSO. The 1D spectrum appeared to contain different minority forms. The combined TOCSY and NOESY spectra allowed a complete assignment of the different resonances of the majority form (Table 2).

When comparing these assignments with those previously published for the native vasopressin, an excellent agreement between the chemical shift values of the two molecules was observed, indicating a high degree of structural similarity. At the level of $H^{\alpha}$ and NH protons chemical shifts, no difference superior to 0.16 ppm was

observed. Even the ß protons of the two cysteine residues flanking the trisulphide bridge were not shifted by more than 0.2 ppm from their value in the native $VS_2$ molecule, confirming the minor structural modifications induced by the trisulphide bridge.

| Residue | $H^N$ | $H^\alpha$ | $H^\beta$ | Others |
|---|---|---|---|---|
| Cys1 | 8.32 | 4.10 | 3.56/3.05 | - |
| Tyr2 | 8.94 | 4.39 | 2.78/2.68 | δ 6.95 ε 6.65 |
| Phe3 | 8.29 | 4.35 | 3.23/2.93 | δ 7.29 ε/ζ 7.33 |
| Gln4 | 8.54 | 3.94 | 1.96/1.92 | γ 2.18 |
| Asn5 | 8.10 | 4.52 | 2.66 | N δ 2 7.48/7.01 |
| Cys6 | 8.24 | 4.88 | 3.26/2.98 | - |
| Pro7 | - | 4.29 | 2.04/1.95 | γ 1.86 δ 3.72/3.61 |
| Arg8 | 8.25 | 4.22 | 1.83/1.72 | γ 1.58 δ 3.13 ε 7.52 |
| Gly9 | 7.90 | 3.62/3.68 | - | - |

Table 2 : $^1H$ chemical shift values of vasopressin trisulphide $VS_3$ in $(CD_3)_2SO$ at 300°K. No stereospecific assignment was made for the ß methylene protons.

A 3D model of $VS_2$ was generated from the crystallographic structure of deamino-oxytocin[16] by sequence replacement using the SYBYL 6.2 Molecular Modelling Package. The compound was energy minimised using the TRIPOS Force Field included in the SYBYL MAXIMIN 2 module. The partial atomic charges were computed using the Pullman method. A distance independent dielectric constant $\varepsilon = 40$ was used in calculations to mimic experimental conditions.[17] NMR experimental interproton distances obtained for vasopressin by Schmidt *et al.*[15] were used as geometrical constraints for energy minimisation. $VS_3$ was built from $VS_2$ and minimised in the same conditions. This model (figure 5) cannot be seen as an « ab initio » structure generation. NMR data suggest similar structures for vasopressin disulphide and vasopressin trisulphide. The model just shows that the presence of a third sulphur atom is compatible with the vasopressin structure without introducing geometrically impossible constraints.

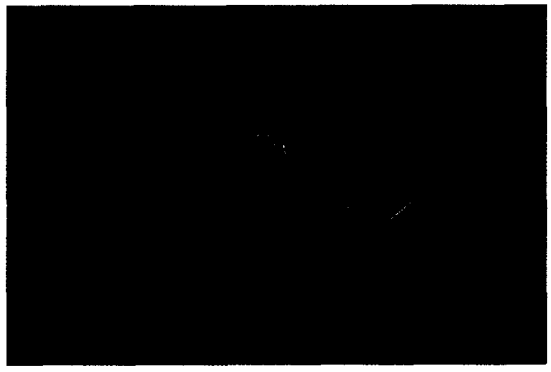


Figure 5 Superimposition of $VS_2$ (purple) and $VS_3$ (orange) models; Sulphur atoms are coloured in yellow.

**Conclusion**: The affinity studies towards the two receptors and the NMR analysis agree very well on the limited influence of the trisulphide bridge. This suggests that the trisulphide bond is sufficiently flexible for the peptide to adopt a conformation which mimics the natural one. In this case, the di/trisulphide bond belongs to a loop which is a poorly constrained structure in which the third sulphur atom can occupy a large range of conformations. It can be assumed that the introduction of a trisulphide bridge in more constrained structures such as α-helix or ß-sheets would have more drastic physiological and structural effects. This could be demonstrated by for example, studying trisulphides analogues of toxins which possess fairly rigid structures.

**Acknowlegments**

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12. HPLC conditions: Nucleosil C18 reverse-phase columns; solvent A ($H_2O$, TFA 0.05%), solvent B ($CH_3CN/H_2O$ 60%, TFA 0.05%). Preparative HPLC: 0 to 15% B (10 min), 15% to 60% B (3h), 60 % to 100% B (20 min). Analytical HPLC: linear gradient, 0 to 100% B (30 min)
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