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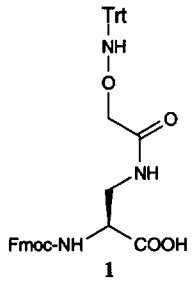
Analogues of Oxytocin with an Oxime Bridge using Chemoselectively Addressable Building Blocks

Franck Wahl and Manfred Mutter*

Institute of Organic Chemistry, University of Lausanne, BCH-Dorigny, CH-1015 Lausanne, Switzerland

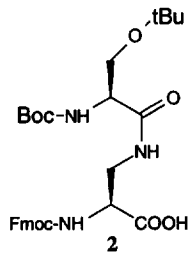
Abstract: The synthesis of two novel amino-acid building blocks for the chemoselective ligation of unprotected peptides via oxime bond formation and their application in the solid phase peptide synthesis of oxytocin analogues with the disulfide altered to an oxime bridge are described.

Disulphide bridges are often found in biologically active peptides and proteins where they play a crucial role in peptide folding and structural stabilisation. The artificial introduction of additional (non native) disulphide bridges into peptides or proteins results in conformational constraints improving the overall thermodynamic stability. However, beside being metabolically unstable, the chemical synthesis of disulphide containing peptides often relies on a judicious choice of cysteine protecting groups and the corresponding deblocking conditions. Over the past decades, many efforts have been devoted towards replacing disulphide bridges in peptides while still retaining their full bioactivity. For example, peptides containing thioether bridges (lanthionines[1]), lactam bridges[2] and isosteric linkages such as the "monocarba" or "dicarba" cystine analogues[3] have been successfully synthesised. Yet, these techniques usually suffer from a complicated strategy involving multiple orthogonal protecting groups.



1

Fmoc-Dap(Trt-Aoa)-OH



2

Fmoc-Dap(Boc-Ser(tBu))-OH

Here, we present a methodology for introducing a proteolytically stable intramolecular bridgehead in a single step with no extra orthogonal protecting groups. The recently developed chemoselective ligation techniques[4] allow for the condensation of unprotected peptide segments in aqueous media. We elaborate in the present work this convenient technique to substitute the disulphide bridge of neurohypophyseal hormone

oxytocin (OT) **3** by a more stable oxime bond (Figure 1) in a single cyclisation step. The oxime bond is formed selectively between an aldehyde and an aminooxy functionalised amino-acid side-chain in the presence of the remaining unprotected side-chains.

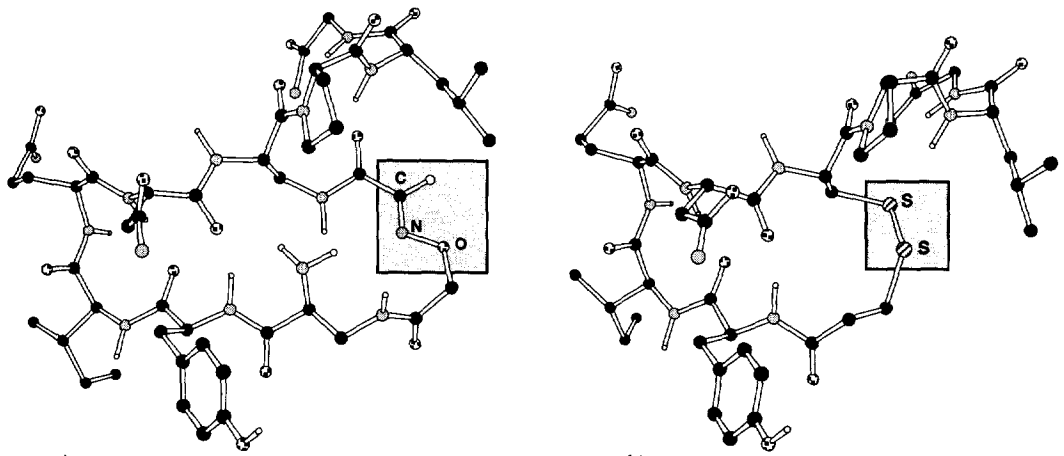


Fig. 1. Design by molecular modelling of: a) oxytocin analogue OT [oxime 1-6] with the disulphide bridge (S-S) altered to an oxime bridge (C=N-O); b) de-amino-oxytocin[5].

To this end, two suitably protected amino-acid building blocks **1** and **2** were designed to allow for the post-synthetic chemoselective cyclisation of a fully deprotected peptide via oxime bond formation after Fmoc/tBu[6] stepwise solid phase assembly. Compound **1** was obtained starting from commercially available *N*α-Fmoc-2,3-diaminopropionic acid (Fmoc-Dap-OH) and *N*-trityl-aminooxyacetic acid hydroxysuccinimide ester (Trt-Aoa-OSu[7]). The aldehyde function required for oxime formation is generated upon periodate oxidation of 1,2-aminoalcohol present in building block **2** as a side chain functionality. **2** was prepared in a similar fashion starting from Fmoc-Dap-OH and Boc-Ser(tBu)-OSu. The chemoselectively addressable building blocks **1** and **2** were readily obtained in 80% and 85% yield, respectively and proved to be very soluble in solvents such as DMF and DCM.

3 Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH_2 Oxytocin

S——S

4 Dap-Tyr-Ile-Gln-Asn-Dap-Pro-Leu-Gly-NH_2 Oxytocin [Dap(Aoa)-1, Dap(Ser)-6]

NH
O=C-CH_2ONH_2

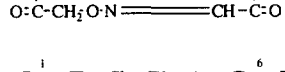
NH
HO—CH(C=O)
NH_2

5 Dap-Tyr-Ile-Gln-Asn-Dap-Pro-Leu-Gly-NH_2 Oxytocin [Dap(Aoa)-6, Dap(Ser)-1]

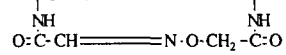
NH
O=C—CH(OH)
NH_2

NH
H_2N-O-CH_2-C=O

6 Dap-Tyr-Ile-Gln-Asn-Dap-Pro-Leu-Gly-NH_2 Oxytocin [Oxime 1-6]



7 Dap-Tyr-Ile-Gln-Asn-Dap-Pro-Leu-Gly-NH_2 Oxytocin [Oxime 6-1]



The suitability of both building blocks during SPPS was demonstrated by the chemical synthesis of two linear oxytocin analogues **4** and **5**. Assembly of the oxytocin sequences was accomplished by Fmoc/tBu based chemistry on Rink amide MBHA™ support (resin loading = 0.49 mmol/g) using 2 eq. of PyBOP for activation and 20% piperidine/DMF for Fmoc deprotection. Removal of the N-terminal Fmoc group was followed by cleavage and deprotection under standard conditions (85% TFA, 5% TIS, 5% water, 5% phenol, 90 min) to give the crude linear peptides **4** and **5**. The absence of branched sequences due to acylation of the aminoxy nitrogen was confirmed by systematic ESI-MS analysis of the by-products in the crude. The crude peptides were purified by RP-HPLC to single peak products and characterised by ESI-MS (Figure 2). Cyclisation of the linear peptides was performed by oxidation of the 1,2-aminoalcohol side-chain with sodium periodate and subsequent *in situ* oxime bond formation at pH=3.5[8]. These two reaction steps proceeded rapidly and with high chemoselectivity pointing to an elevated reactivity of aminooxy groups at acidic pH and to the propensity of the sequence for cyclisation. Peptides **6** and **7** were characterised by RP-HPLC (Figure 2), ESI-MS (m/z=1086.5) and NMR spectroscopy (not shown). Of the two possible isomers only the trans oxime isomer was observed by NMR.

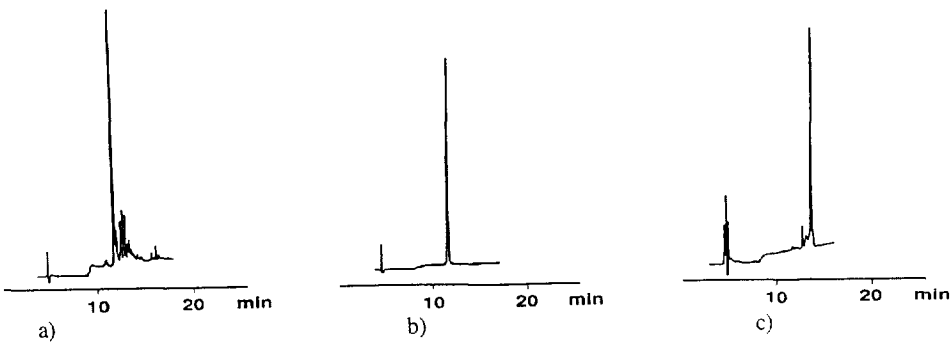


Fig. 2. Analytical RP-HPLC of: a) crude peptide **5** R_t=12.41 min. b) purified peptide **5**, m/z=1135.4. c) crude oxidised and cyclised peptide **7** after 20 min, R_t=15.04 min, m/z=1086.5. HPLC column Nucleosil 300-5C_{18} (4x250mm), buffer A 0.1% aq. TFA, 90% aq. acetonitrile; buffer B 0.1% aq. TFA; linear gradient 10-50% A over 30 min, 1 ml/min, 214 nm.

In conclusion, this presented strategy offers an elegant way for introducing oxime bonds as mimetics of disulphide bridges in the total synthesis of bioactive peptides. Most notably, the cyclisation step proceeds smoothly in high yields, resulting in a kinetically and metabolically stable oxime bond. The readily accessible chemoselectively addressable building blocks **1** and **2** proved to be compatible with standard Fmoc-based SPPS and should become versatile tools for introducing conformational constraints into peptide backbones via side-chain cross-linking. The further elaboration of the synthetic and biological implications of the novel strategy is presently under investigation.

Acknowledgements

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6. Abbreviations: Aoa, aminooxy acetic acid; DAP, 2,3-diaminopropionic acid; DCM, dichloromethane; DMF, N,N,dimethylformamide; Fmoc, 9-fluorenylmethyloxycarbonyl; OSu, O-succinimide ester; PyBOP, benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate; SPPS, Solid Phase Peptide Synthesis.
7. Triethylamine (4 equiv.) was added slowly to a cooled suspension of commercially available O-(carboxymethyl)hydroxylamine and trityl bromide (2 equiv.) in DCM/DMF (2:1). Then it was heated to reflux for 2h under argon. After cooling the solvents were evaporated and the residue extracted with ethyl acetate, washed with water, brine and dried over magnesium sulfate. N-Trityl aminooxy acetic acid was recrystallised in ethyl acetate to give 77% of pure white crystals. Of which the OSu ester was prepared by addition of N-hydroxysuccinimide (1 equiv.) and dicyclohexylcarbodiimide (1 equiv.) in dioxane/ethyl acetate (2:1) and stirred overnight at 4°C. After filtration and evaporation, the solid residue was recrystallised in isopropanol to give the *N*-Trityl-aminooxyacetic acid hydroxysuccinimide ester (Trt-Aoa-OSu) in 72% yield. Fmoc-Dap-OH was refluxed in dichloromethane in the presence of trimethylsilyl chloride (2 equiv.) for 2 h 30 min, then after cooling, diisopropylethylamine (2 equiv.) and *N*-Trityl-aminooxyacetic acid hydroxysuccinimide ester (Trt-Aoa-OSu) were added slowly at room temperature and stirred for 6h. Methanol was added to quench the reaction, the solvents evaporated and the residue extracted with DCM, washed with 10% aq. citric acid, brine and dried over magnesium sulphate. The product was purified by silica gel flash chromatography with DCM/methanol to give **1** as a white solid (80% yield).
8. Linear peptide **4** or **5** were dissolved in water to a concentration of 1×10^{-4}M, pH 3.5 (TFA salt), and treated with a 2-fold molar excess of sodium periodate for 20 min at room temperature. The reaction was quenched by addition of ethylene glycol (2 eq.) and the reaction mixture lyophilised before RP-HPLC purification.

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