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On the Synthesis of Orexin A: A Novel One-step Procedure to Obtain Peptides with Two Intramolecular Disulphide Bonds

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Abstract: An efficient strategy for the synthesis of orexin A, a recently discovered neuropeptide with two intramolecular disulphide bonds, was developed. Four different methods for the synthesis of peptides containing two disulphide bonds were compared and optimized with respect to reaction time, purity of the crude peptide and yield of the purified peptide. A new one-step cyclization method in solution is presented for fast, easy and high yield synthesis of orexin A, based on iodine oxidation in acetic acid/water and *S*-acetamidomethyl (*S*-Acm) and *S*-trityl (*S*-Trt) for side-chain protection of cysteine. Disulphide formation without selective side-chain protection leads to the formation of different mono- and bicyclic configurations of orexin A. These data stress the requirement of selective cysteine side-chain protection in the synthesis of orexin A. Copyright © 2000 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: orexin; hypocretin; disulphide; obesity; food intake; solid phase peptide synthesis; neuropeptides; cystine

INTRODUCTION

Two novel neuropeptides were discovered recently independently by two groups and identified as potent stimulators of food intake after intracerebroventricular administration [1,2]. Orexin A (also called hypocretin 1, because of its homology to secretin [3]) is a 33 amino acid residue peptide with two intramolecular disulphide bonds, connecting cysteine residues from positions 6 to 12 and 7 to 14, respectively. Orexin B consists of 28 residues and shares 46% identity with orexin A, which is mainly comprized at the *C*-terminus. The three-dimensional solution structure of orexin B, determined by 2D-NMR most recently, shows two α-helices, connected by a short linker sequence at position 20–23 [4]. The structure of orexin A is conserved among human, rat, mouse and cow, whereas rodent orexin B contains two amino acid substitutions compared to the human sequence: Pro instead Ser in position two and Asn instead of Ser in position 18. The orexins of *Xenopus laevis* show several amino acid exchanges, but the *C*-terminal decapeptide of orexin A and B and the positions next to the disulphide bonds in orexin A remain conserved (Figure 1), which suggests some importance in biological activity [5].

The two peptide amides derive from the same 130 amino acid precursor, prepro-orexin, whose mRNA is produced in defined regions of the lateral and perifornical hypothalamus (LH and PFH) and upregulated upon fasting. Orexin immunoreactive neurons are widely distributed in the brain, including the regions of the cerebral cortex, the medial groups of the thalamus, the circumventricular organs, the limbic system and the brain stem [6,7]. Further investigations suggest the involvement of these peptides into many physiological and behavioural activities that are involved in or associate with feeding

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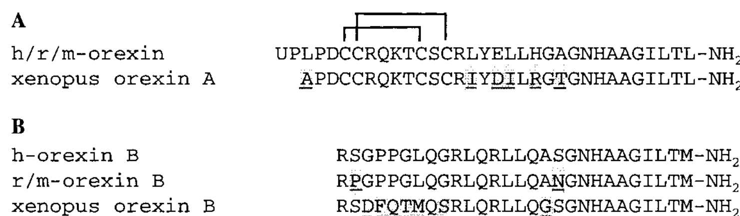

Figure 1 (A) Sequence of mature orexin A peptides. (B) Sequence of mature orexin B peptides. Deviations from the human sequences are underlined. U, pyroglutamic acid.

behaviour [8–11], and other functions like modulation of neuroendocrine function [12] or the sleep–wake cycle [13].

Orexin A and B are endogenous ligands of two closely related heptahelical G-protein-coupled receptors termed OX_1 and OX_2. Orexin A shows higher affinity to OX_1, whereas the binding affinity of the two peptides to OX_2 is in the same range.

Up to now, no structure–activity relationships of any of the two orexin peptides have been reported, nor any potent subtype selective antagonist or agonist is known to characterize the physiological and pharmacological role of the different receptors.

Solid phase peptide synthesis is a fast and convenient strategy to frequently obtain peptides of more than 40 amino acids. However, it is still a challenge to synthesize long peptides with intramolecular cycles, one or multiple disulphide bonds or sterically hindered sequences. The aim of this work was to establish and compare synthesis methods for fast and easy synthesis of orexin A by solid phase peptide synthesis based on the Fmoc-strategy. The obtained peptides were examined by HPLC and electrospray ionization mass spectrometry (ESI-MS).

EXPERIMENTAL PROCEDURES

Materials

The N^{α}-Fmoc-protected amino acids were purchased from Alexix (Läufelfingen, Switzerland). The side-chain protecting groups were: *tert*-butyl for Asp, Glu, Ser, Thr and Tyr, Boc for Lys, trityl for Asn, Gln and His, 2,2,5,7,8-pentamethylchroman-6-onyl (Pmc) for Arg. Cys was protected by Trt or Acm, according to the synthesis strategy. The 4-(2′,4′-dimethoxyphenyl-Fmoc-aminomethyl)-phenoxy (Rink amide) resin was obtained from Novabiochem (Läufelfingen, Switzerland). acetic acid (p.a.), *N*-hydroxybenzotriazole (HOBt), TFA, thioanisole, *p*-thiocresol, trimethylbromsilane, 1,2-ethanedithiol, piperidine, 1-methyl-2-pyrrolidinone, *tert*-butanol, DMF (p.a.), sodium hydrogenphosphate, potassium dihydrogenphosphate and iodine were obtained from Fluka (Buchs, Switzerland). *N*,*N*′-diisopropylcarbodiimide (DIC) was purchased from Aldrich (Buchs, Switzerland). DMF (pure), chloroform, dichlormethane, methanol and diethylether were purchased from Scharlau (La Jota, Barcelona, Spain). Acetonitrile was obtained from Romil (Cambridge, UK) and tetrachlormethane (p.a.) from Merck (Dietikon, Switzerland). *N*-(3-Maleinimidopropionyl)-biocytin (MPB) was synthesized according to [14].

Synthesis Methods

General procedure. Orexin A was synthesized by four different methods of solid-phase technique in order to optimize reaction time, purity of the crude peptide and yield of the purified peptide (see Scheme 1). The peptides were synthesized by automated multiple solid phase peptide synthesis on a peptide synthesizer (Syro, MultiSynTech, Bochum, Germany) using Rink amide resin (30 mg, resin loading 0.6 mmol/g). Amino acids were attached by Fmoc-strategy in a double coupling procedure with tenfold excess of Fmoc-amino acid, HOBt, DIC in DMF (2×40 min). The Fmoc-deprotection step was accomplished with 40% piperidine in DMF for 3 min, 20% piperidine for 7 min and finally 40% piperidine for 5 min.

Synthesis of orexin A

Two-step cyclization of orexin A in solution. The linear resin-bound peptide was synthesized as described above with Cys(Acm) in positions 6 and 12 and Cys(Trt) in positions 7 and 14. The peptide was cleaved from the resin with a cleavage-cocktail of trifluoroacetic acid:thioanisole:ethandithiole (90:7:3), precipitated from ice-cold diethylether, collected by centrifugation, washed four times in

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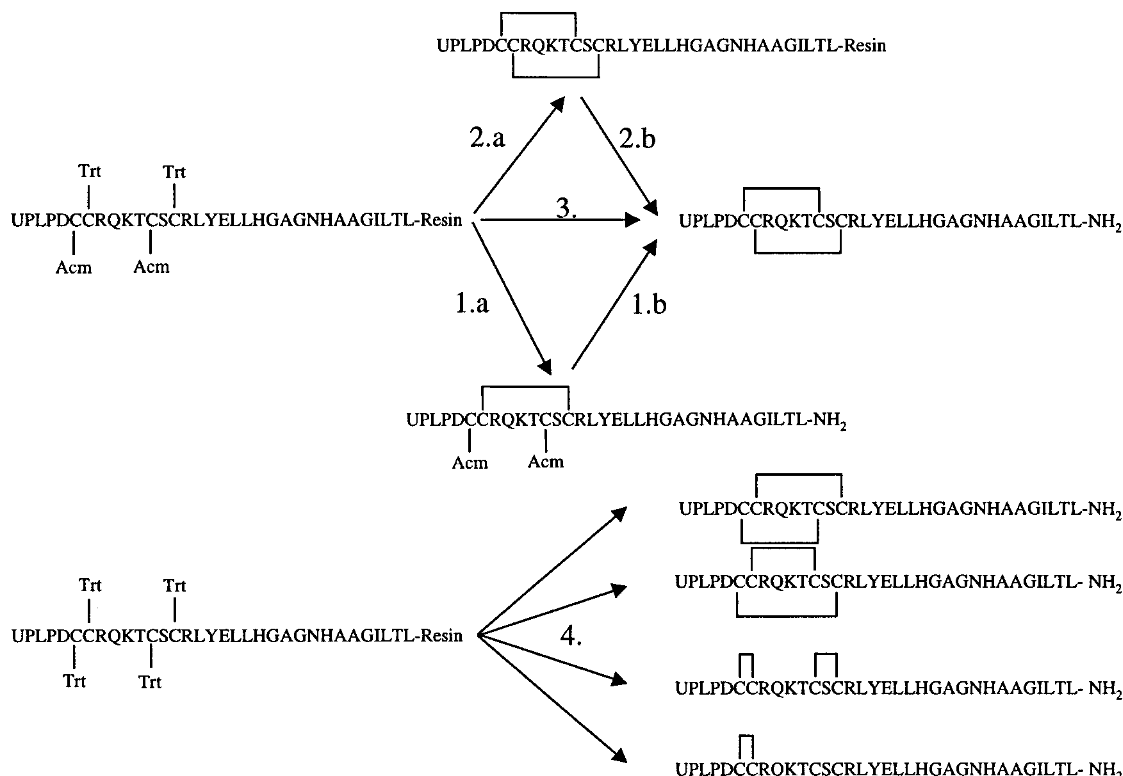

Scheme 1 Synthesis strategies of orexin A: (1) Two-step cyclization in solution; (a) Air oxidation in 0.1 M ammoniumhydrogencarbonat, (b) iodine oxidation in 50% acetic acid. (2) Selective disulphide formation by one-step cyclization on the resin; (a) cyclization by iodine treatement in methanol/chloroforme (1:1), (b) cleavage from the resin. (3) Selective disulphide formation by one-step cyclization in solution by iodine oxidation in aqueous acetic acid. (4) Unselective disulphide formation by iodine oxidation in 50% acetic acid might lead to different configurations of mono- or bicyclic structures. Four possible oxidation products are indicated.

diethylether and characterized by reversed phase HPLC and mass spectrometry. The peptide (32 mg/9 μmol) was dissolved in 0.1 M ammoniumhydrogencarbonat (50 ml) to give a 1.8×10^{-4} M solution and vigorously shaken at room temperature for 72 h to obtain the first disulphide bond by air oxidation. The reaction was terminated after control by analytical RP-HPLC and mass spectrometry. After lyophilization, the peptide was dissolved in 50% acetic acid (10^{-3} M) and iodine (50 mM) was added. The solution was shaken for 60 min and the iodine was extracted with tetrachlormethane six times. The aqueous phase was threefold diluted with water and lyophilized. Purification of the peptide was achieved by preparative HPLC on a C-18 column (Waters, 5 μm, 25 × 300 mm) with a linear gradient of 20–40% A in B over 50 min at a flow rate of 15 ml/min, the relevant fractions were collected. The crude (see Figure 2) and the pure (data not shown) peptide were characterized by ESI-MS (SSQ 710, Finnigan MAT, Bremen, Germany) and by analytical reversed-phase HPLC on a LiChrospher RP-18-column (5 μm, 3 × 125 mm, Merck, Darmstadt, Germany) using 0.08% TFA in acetonitrile (A) and 0.1% TFA in water (B) as the eluting system (20–40% A over 30 min at a flow rate of 0.6 ml/min).

One-step cyclization of orexin A on the resin. The linear resin bound peptide was synthesized as described above ('General procedure'). Side-chain protection groups were identical to method 1. Fifteen equivalents of iodine (0.135 mmol, 34.3 mg) were solved in 2 ml of methanol:chloroform (1:1). This solution was given to the pre-swollen resin (94 mg, resin loading 0.6 mmol/g) and shaken for 4 h. The resin was washed with methanol, dichlormethane, DMF and diethylether (4 × 1 ml each). The peptide was cleaved from the resin with a mixture of trifluoroacetic acid:thioanisole:water (90:5:5) for 3 h. The fully deprotected peptide was precipitated from ice-cold diethylether, centrifuged and washed in diethylether four times. The crude peptide was

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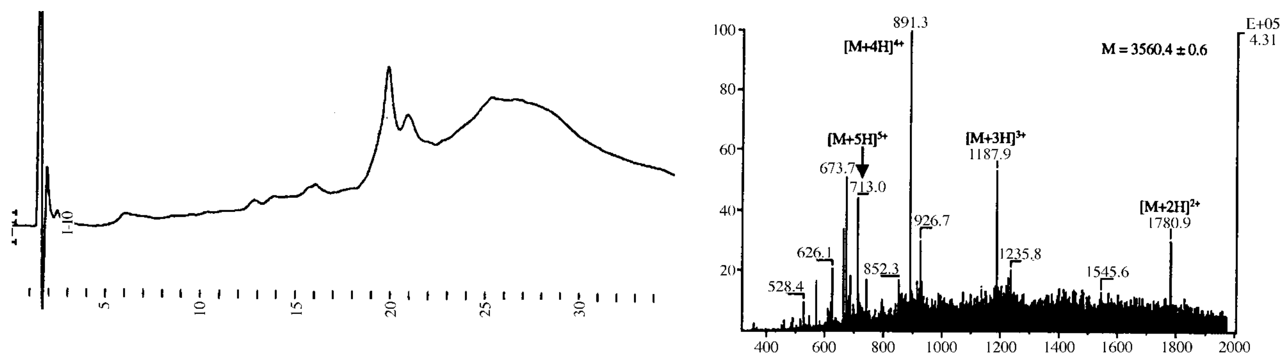

Figure 2 HPLC and mass spectrum of crude orexin A after two-step cyclization in solution. Expected mass: 3560 Da, found main product: 3561.4 ± 0.6 Da.

dissolved in *tert*-butanol:water (1:3 v/v), lyophilized and characterized by mass spectrometry and by analytical reversed-phase HPLC. The mass spectrum of the crude product showed the expected masses of orexin A (expected: 3560 Da, found: 3560.4 ± 1.0) (Figure 3). Purification of the peptide was performed by preparative HPLC and the pure peptide was characterized by HPLC and mass spectrometry. Analytical data were found as expected ($MW_{ecpected}$: 3560 Da, MW_{found}: 3560.5 ± 1.1 Da).

One-step cyclization of orexin A in solution. The linear peptide was synthesized as described above ('General procedure'), using Cys(Acm) in positions 6 and 12 and Cys(Trt) in positons 7 and 14. The peptide was cleaved from the resin with trifluoroacetic acid:thioanisole:1,2-ethanedithiole (90:7:3), precipitated from ice-cold diethylether, washed four times in diethylether and characterized by ESI-MS and by analytical reversed-phase HPLC. For cyclization 0.009 mmol (32 mg) of the peptide were dissolved in 9 ml of acetic acid (10^{-3} M) and a solution of 10 equivalents iodine (90 µmol, 23 mg) in 150 µl of methanol was added for fast building of the first disulphide bond. After 60 min of stirring, 2.25 ml of water was added to accelerate the half-time of *S*-Acm cleavage. The solution was shaken for another 90 min. To follow the cleavage of the *S*-Acm group and the building of the second disulphide bond, the solution was monitored by mass spectrometry 0, 20, 40 and 90 min after the addition of water (see Figure 4). After the reaction was complete, water (9 ml) was added and the iodine was extracted with tetrachlormethane (6 × 20 ml). The aqueous phase was diluted threefold with water and lyophilized. The crude peptide was characterized by analytical HPLC (Figure 5). The peptide was purified by preparative HPLC and characterized by ESI-MS and reversed phase HPLC on a LiChrospher RP-18 column. Analytical data were found as expected ($MW_{ecpected}$: 3560 Da, MW_{found}: 3560.2 ± 0.6 Da).

Orexin A synthesis by unselective disulphide formation. Reduced orexin A was synthesized as described above ('General procedure'), using the *S*-Trt protecting group for the four cysteines. The peptide was

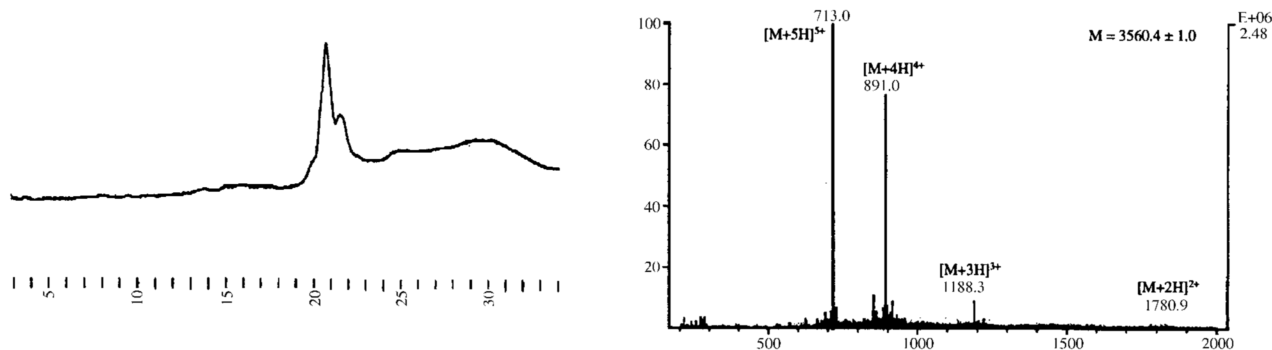

Figure 3 HPLC and mass spectrum of the crude product of the one-step orexin A synthesis on the resin. Expected mass: 3560 Da, found: 3560.4 ± 1.0 Da.

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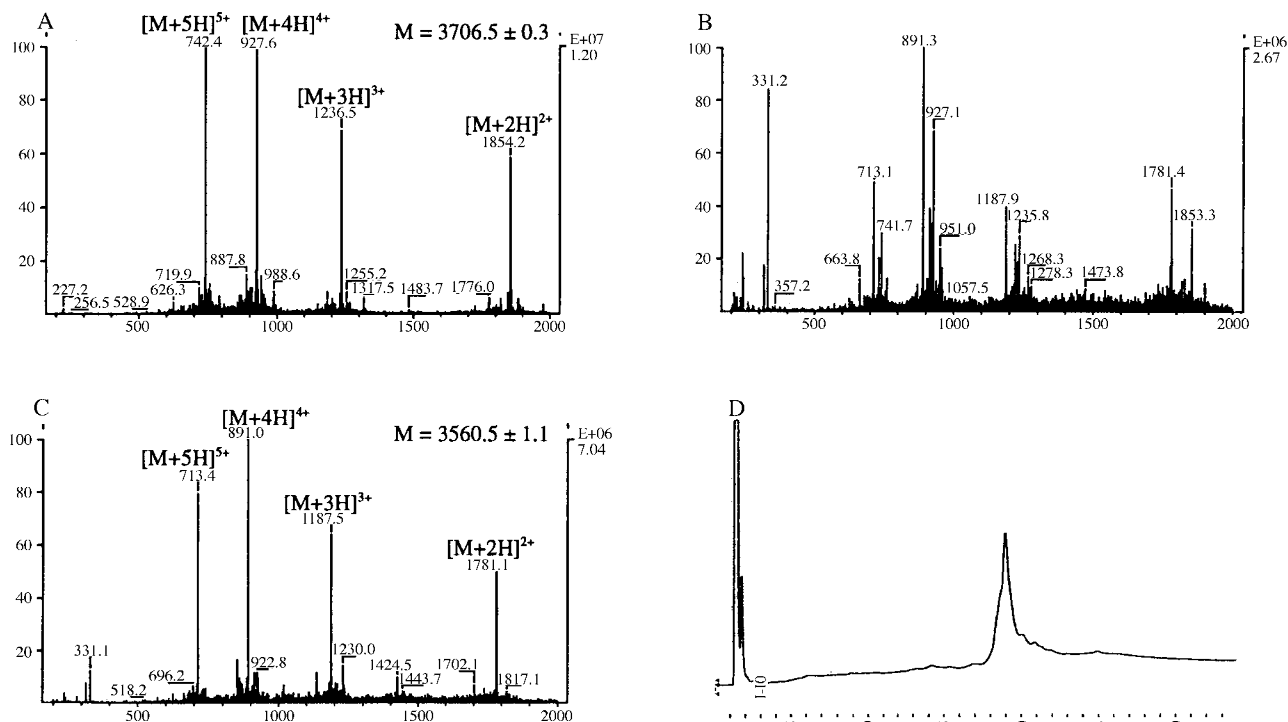

Figure 4 Mass spectrum of orexin A, (A) 0, (B) 60, (C) 150 min after dissolving the peptide in acetic acid and adding 10 equivalents of iodine. After 60 min of reaction time ('time B'), 20% water was added to the solution to accelerate *S*-Acm deprotection. (A) linear orexin A with two *S*-Acm groups on Cys 6 and 12: correct mass 3706 Da, found: 3706.5 ± 0,3 Da. (B) Partially oxidized orexin A. (C) Fully oxidized orexin A: correct mass 3560 Da, found: 3560.5 ± 1.1. (D) HPLC of the crude orexin A.

cleaved from the resin with trifluoroacetic acid:thioanisole:1,2-ethandithiol (90:7:3), precipitated from ice-cold diethylether, washed four times with diethylether, lyophilized and characterized by mass spectrometry and reversed phase HPLC. The peptide (32 mg, 0.009 mmol) were dissolved in 4.5 ml of water. Acetic acid (4.5 ml) and 11.4 mg of iodine (5 equivalents) in 200 μl of methanol were added and the solution shaken for 2 h. The solution was diluted to twice the volume with water and the iodine was extracted with tetrachlormethane (six times, equal volume). The aqueous phase was lyophilized and the peptide characterized by mass spectrometry and reversed phase HPLC (LiChrospher RP-18-column, 5 μm, 3 × 125 mm, Merck, Darmstadt, Germany) using 0.08% TFA in acetonitrile (A) and 0.1% TFA in water

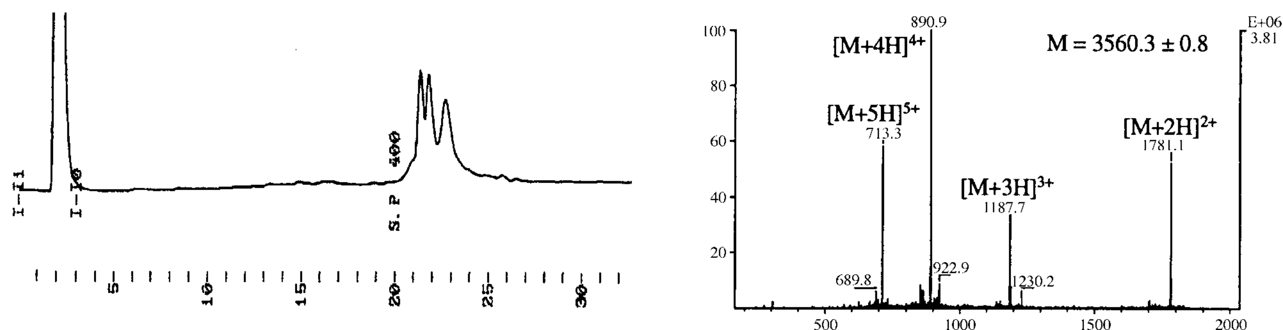

Figure 5 Mass spectrum and HPLC of unselectively oxidized orexin A. The HPLC shows multiple peaks, indicating different configurations. The mass spectrum gives the expected masses (expected: 3560 Da, found: 3560.3 ± 0.8 Da).

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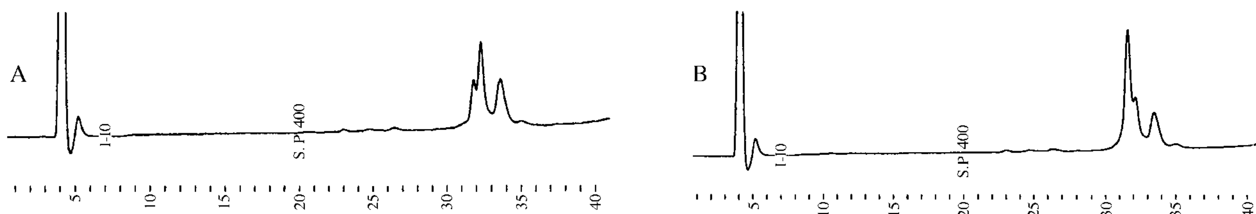

Figure 6 (A) Co-injection of orexin A synthesized by one-step cyclization in solution and unselectively oxidized orexin A leads to discrete enlargement of the peak at RT = 32.1 min. (B) Co-injection of the wrongly connected orexin A analogue (Cys6–14/7–12) and unselectively oxidized orexin A leads to discrete enlargement of the peak at RT = 31.5 min. This result indicates the building of different orexin A conformations during unselective orexin A oxidation.

(B) as the eluting system (20–40% A over 30 min at a flow rate of 0.6 ml/min). The mass spectrum showed the correct masses (expected: 3560 Da; found: 3560.3 ± 0.8 Da), the HPLC chromatogram revealed a mixture of at least three major products, a double peak at RT (retention time) = 21.28, 21.72 min and a third peak at 22.61 min (Figure 5).

To control the shift in retention time of the oxidized orexin A, a co-injection with reduced orexin A was performed, using an HPLC system as follows: RP-18 column (LiChrospher 100, 5 μm, 250 × 4 mm, Merck, Darmstadt, Germany), linear gradient of 20–40% A in B over 40 min, flow rate of 0.6 ml/min (A: 0.08% TFA in acetonitrile; B: 0.1% TFA in water).

Synthesis of Orexin A analogue with 6–14 and 7–12 disulphide bonds. The peptide was synthesized using the one-step cyclization method as described above ('One-step cyclization of orexin A in solution'). Cysteine protecting groups were chosen as follows: Cys(Acm) in positions 6 and 14 and Cys(Trt) in positions 7 and 12, respectively. The peptide was purified by preparative reversed phase HPLC and characterized by mass spectrometry and analytical HPLC. Analytical data were found as expected ($MW_{ecpected}$: 3560 Da, MW_{found}: 3559.8 ± 1.07 Da).

Control of Complete Oxidation and Correct Folding of Orexin A

Completeness of oxidation was investigated by mass spectrometry. Five micrograms of the purified orexin A (one-step cyclization in solution) were dissolved in 70 μl of water:*tert*-butanol (1:3) and subsequently 5 μg of MPB was added. After 2 h of shaking, the product was characterized by mass spectrometry. For the control the same experiment was performed with reduced orexin A.

The mass spectra that were measured after the reaction of MPB with orexin A, still showed the mass of oxidized orexin A (expected: 3560 Da, found: 3559.9 ± 0.7 Da), whereas for the reduced orexin A the addition of four MPB was found as expected (expected: 5658 Da, found: 5657.2 ± 0.3 Da).

Control of the correct bridging of orexin A was achieved by HPLC co-injection of orexin A (one-step cyclization in solution) with the wrongly bridged orexin A analogue and each of them with the unselectively oxidized orexin A (see Figure 6). The following HPLC system was used: LiChrospher 100, 5 μm, 250 × 4 mm, Merck, Darmstadt, Germany and a linear gradient of 20–40% A in B within 40 min (A: 0.08% TFA in acetonitrile; B: 0.1% TFA in water).

RESULTS

Synthesis of Orexin A

Orexin A is a 33 amino acid peptide with two intramolecular disulphide bonds, connecting the four cysteine residues from positions 6 to 12 and 7 to 14, respectively. The peptide was synthesized by four different methods of solid-phase technique to optimize reaction time, purity of the crude peptide and yield of the purified peptide (see Scheme 1). Selective and unselective formation of the two disulphide bonds was performed and compared (Table 1). Subsequently, the peptides were purified by preparative HPLC in each case in a single step. ESI-MS and analytical HPLC was used to confirm peptide purity and identity.

Two-step synthesis of orexin A in solution. During the cleavage of the peptide from the resin under reductive conditions (1,2-ethanedithiole), all side chains were removed, except for S-Acm in positions 6 and 12. The linear orexin A showed the expected mass of 3706 Da as demonstrated by mass spectrometry and a purity of about 85% (data not shown). Complete building of the first disulphide

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Table 1 Comparison of the Cyclization Strategies for Two Intramolecular Disulphide Bonds in Orexin A Synthesis

Method	Yield of purified orexin A	Time exposure	Instrumental and material effort
Two-step cyclization in solution	~10%; many side products, polymerization	Several days	High, peptide has to be lyophilized twice, toxicity of CCl_4, used for I_2 extraction
One-step cyclization on the resin	~10%; high polymerization	1 day	Very low, easily to handle, easy removal of chemicals
One-step cyclization in solution	~30–35%	1 day	Low, toxicity of CCl_4, used for I_2 extraction
Unselective oxidation in solution	n.d.; building of different configurations	1 day	Low, toxicity of CCl_4, used for I_2 extraction

bridge after 72 h of air oxidation in 0.1 M ammoniumhydrogencarbonate was verified by HPLC and mass spectrometry (data not shown). After lyophilization of the peptide, cleavage of the *S*-Acm group and connection of the second disulphide bond was performed by iodine oxidation in 50% acetic acid. Analytical HPLC and mass spectrum of the oxidized peptide are shown in Figure 2. The main peak of the HPLC chromatogram (retention time (RT) = 19.8 min) derived from orexin A and the smaller peak at RT = 20.8 min corresponded to orexin A with two *S*-Acm groups, as proven by mass spectrometry of the peak fractions. A big heap of polymers and side products led to the peaks of longer retention time (> 23 min). The peptide was purified by preparative HPLC and the pure peptide was characterized by analytical HPLC and mass spectrometry (data not shown). The overall yield from peptide–resin was only about 10%, because of the high amount of polymerization and incomplete cleavage of the Acm group.

One-step cyclization of orexin A on the resin. The selective formation of the two disulphide bonds on the resin in one step was achieved by the use of the iodine-labile cysteine protecting groups *S*-Acm and *S*-Trt with Cys(Acm) in positions 6 and 12 and Cys(Trt) in positions 7 and 14. The advantage of these protecting groups in peptide synthesis is, that their half-time in the presence of iodine differs dramatically, depending on the solvent. This allows selective formation of two disulphide bonds by selection of suitable reaction conditions [15]. Remarkable for the selective formation of the two disulphide bonds in solution is, that the *S*-Acm group is stable to trifluoroacetic acid cleavage, whereas *S*-Trt is cleaved by trifluoroacetic acid during cleavage of the peptide from the resin [16,17].

The HPLC of the crude peptide, that was cleaved from the resin after cyclization, showed a main product at a RT = 20.6 min, corresponding to the desired orexin A (see Figure 3). A smaller peak at RT = 21.45 min originated from the peptide, still contained the two *S*-Acm protecting groups, as verified by mass spectrometry (data not shown). The large peaks at higher RT derive from a large amount of side products and polymers. Purification of the crude peptide by preparative HPLC led to highly pure product. The overall yield of this synthesis method from peptide–resin was in the range of 10%.

One-step cyclization of orexin A in solution. In order to improve the yield of orexin A synthesis by avoiding polymer formation, selective disulphide formation was performed in highly diluted solution. The peptide was cleaved from the resin under reductive conditions (1,2-ethandithiole). The *S*-Acm groups remained bound to the peptide whereas all other protecting groups were cleaved. The linear orexin A intermediate showed a purity of > 95% (HPLC) and the expected mass of 3706 Da was verified by mass spectrometry. The deprotection of the *S*-Acm group by iodine was monitored by mass spectrometry at 0, 60, 80, 100, and 150 min after iodine addition. One hour after iodine addition, 20% of water was added to the solution in order to accelerate the speed of *S*-Acm deprotection dramatically. The mass spectra, that follow *S*-Acm deprotection and the HPLC of the crude orexin A after lyophilization, demonstrate the completeness of the oxidation procedure (Figure 4). The lyophilized peptide was purified by preparative RP-HPLC and the relevant fractions investigated by analytical HPLC and mass spectrometry. The overall yield from peptide resin was 35%.

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Orexin A synthesis by unselective disulphide formation. Many peptides that are found in nature and show multiple disulphide bonds, have a preferred conformational structure that might lead to a selective disulphide bond formation even under unselective oxidation conditions [18].

The peptide was synthesized by iodine oxidation of linear orexin A in 50% acetic acid using a concentration of 10^{-3} M. After 2 h of reaction time, the iodine was extracted and the peptide was lyophilized. HPLC interestingly showed three major peaks: a double peak at RT = 21.28, 21.72 min and a third peak at 22.61 min, whereas the mass spectrum showed the expected masses (Figure 5). In order to identify the peaks, co-injection of the obtained mixture was performed with reduced orexin A, first. All three major peaks revealed a shorter retention time (32.1, 32.7, 33.9 min) than reduced orexin A (36.2 min), suggesting that a partial oxidation had taken place. The formation of two different configurations was proven by co-injection of the mixture with orexin A (disulphide bonds Cys6–12/7–14) and an analogue with opposite disulphide bonds (Cys6–14/7–12). Both peaks of the double peak of unselectively oxidized orexin A could be raised exclusively, by co-injection with orexin A and its analogue with reverse disulphide bonds, respectively (Figure 6).

Control of Complete Oxidation and Correct Folding of Orexin A

Control of the complete oxidation was performed by reaction of oxidized (one-step cyclization in solution) and reduced orexin A with MPB. MPB reacts selectively with free thiol groups and therefore can be used as specific biotinylating reagent for detection of SH groups in protein and peptide chemistry [14]. As expected, the mass spectra, that were measured after the reaction of MPB with the peptides, showed no addition of MPB to the oxidized orexin A, and an addition of four MPB to the reduced orexin A, according to the number of free thiol groups of the peptides. This confirms the complete oxidation of orexin A synthesized by one-step cyclization in solution and the applicability of MPB to detect free thiol groups simply by solving it in an aqueous solution together with the peptide and subsequent analysis by mass spectrometry or dot blot.

For verification of the correct building of the disulphide bonds during orexin A synthesis, an orexin A analogue with wrongly connected cysteines (Cys6–14/7–12) was synthesized. This peptide was co-injected with previously synthesized orexin A (one-step cyclization in solution). The retention time differed significantly, and co-injection led to a double peak in HPLC similar to the double peak of the unselectively oxidized orexin A (Figure 5). Interestingly, the wrongly connected orexin A analogue displayed the shorter retention time.

This result, together with the proof of complete oxidation, suggests on the one hand the correct folding of orexin A during one-step cyclization in solution was obtained. On the other hand, it shows, that unselective oxidation of orexin A leads to a mixture of different configurations, which indicates the necessity of regioselective cysteine pairing strategies in the synthesis of correctly folded orexin A.

DISCUSSION

The disulphide bond is one of the weakest types of covalent bonding in peptides and proteins. Intrachain disulphide bonds serve to confer a discrete three dimensional structure and conformational stability. Disulphide bond formation reaction is a key step in the synthesis of cystine-containing peptides. Air oxidation and iodine oxidation are amongst the general methods that are employed, as demonstrated in recent reviews [16,19]. Air oxidation is one of the mildest methods to build a disulphide bond, but usually requires a very long time, up to several days [20]. Hydrophobic or basic peptides tend to aggregate and precipitate since the reaction has to be carried out in aqueous solution of slightly basic pH and high dilution to prevent polymerization [21,22]. In contrast, disulphide bonds can be formed within a short time (minutes to hours), using iodine oxidation. Iodine oxidation needs particularly controlled conditions, since several nucleophilic amino acids, e.g. Met, Tyr, His and Trp, are susceptible to iodine and frequently overoxidized. Application of the *S*-Acm and *S*-Trt protecting groups allow the regioselective synthesis of two intramolecular disulphide bonds because of their significant differences in half-time depending on the solvent (see [15]). This fact was used to optimize the synthesis strategy of peptides with two intra-chain disulphide bonds in the example of the recently discovered neuropeptide orexin A. The common way to synthesize two intramolecular disulphide bonds is to liberate two cysteine side chains, followed by mild oxidation to the disulphide. Formation of the second cystine is most frequently done by iodine

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oxidation of an S-Acm protected pair of cysteines, because S-Acm is acid stable, hydrohpilic and can be converted directly to cystine without interfering with existing disulphide bonds [23]. The combination of S-Trt and S-Acm as Cys-protecting groups, as performed in the present work, has the advantage, that S-Trt can be cleaved by trifluoroacetic acid and iodine, whereas S-Acm is stable to trifluoroacetic acid cleavage. On the one hand S-Trt could be selectively removed during cleavage of the peptide from the resin, whereas S-Acm remained bound, on the other hand the different half-times of the cleavage reaction under iodine oxidation allowed selective disulphide formation of the peptide still bound on the resin.

The commonly used two-step cyclization, with initial air oxidation of the S-Trt deprotected peptide followed by iodine oxidation of the Cys(Acm), interestingly resulted in a quite low yield of about 10% because of polymerization and incomplete Acm deprotection. Details in the experimental procedure seemed to play a very important role for the correct building of the two disulphide bonds in high quantities. The choice of other protecting groups, like the combination of S-*tert*-butylsulphenyl (StBu) groups with S-Acm or S-2,4,6-trimethoxybenzyl (Tmob) with S-Acm, as it was performed in the synthesis of conotoxin [24,25] might open other possibilities in two-step cyclization of two intramolecular disulphide bonds. The TFA stable StBu protecting group gives on the one hand the possibility to obtain a fully Cys side-chain protected peptide in solution, on the other hand an additional cleavage step (thiols [26] or trialkylphosphines [27,28]) is necessary to remove the StBu groups from the peptide. Accordingly, the use of S-Trt or Tmob in combination with S-Acm is favoured. Nevertheless, StBu is a well known Cys-protecting group and successfully used in synthesis of single or multiple disulphide bonds [29].

Because air oxidation requires a long reaction time of up to several days, our optimization steps led to the combination of Cys(Acm) and Cys(Trt), by applying their different behaviour under iodine oxidation.

The most elegant method, with respect to the handling, is cyclization of the peptide on the resin, because chemicals and reagents can be removed easily. Iodine cleavage of S-Trt from the peptide is about 500-fold faster than S-Acm cleavage, using a solvent mixture of methanol:chloroform (1:1) [15]. This allows selective formation of two disulphide bonds on the resin in a so called one-step cyclization. After only 4 h of iodine oxidation, the disulphide formation was complete. Unfortunately, the yield of this synthesis method was only in the range of 10%. The use of other peptide resins, e.g. NovaSyn TG Sieber Resin with low resin loading, could not improve the result, neither the exchange of S-Acm and S-Trt in order to close the disulphide bridges in the appropriate order. Despite a high polymerization rate and formation of side products, the low yield might also be caused by the solvent, since chloroform is known to suppress disulphide formation [30].

To keep the short reaction time and simultaneously raise the yield by avoiding polymerization, the one-step cyclization was transformed from resin into solution with surprisingly good results. The TFA labile S-Trt group was removed during cleavage of the peptide from the resin. Selective building of the first disulphide bridge of orexin A was achieved by performing the iodine oxidation reaction in acetic acid, where the half time of S-Acm is about 45 min [15]. Addition of 20% water after 1 h raised the S-Acm half-time up to 50–60 s which shortened the reaction time dramatically for the building of the second disulphide bond. The progress of the S-Acm cleavage and the oxidation reaction could be easily followed by mass spectrometry. This synthesis method provided high yield ($\sim$35%) of the correctly folded orexin A as proven by the synthesis of the wrongly connected orexin A analogue associated with a short reaction time, easy handling and the possibility of on-line control of the reaction.

It is important to note, that best synthesis results for peptides containing multiple disulphide bonds might be obtained by random oxidation, because the desired biologically-active isomer is generally the most thermodynamically stable. However, unselective iodine oxidation of orexin A in 50% acetic acid led to the building of at least three different products (see Figure 5). Two of them corresponded to orexin A and its wrongly connected analogue. The third one, with a HPLC retention time between the reduced open chained orexin A and the two double bridged analogues, might derive from partial oxidation (only one disulphide bridge closed) or from a bicyclic analogue, connecting Cys6–7/12–14. Studies on air oxidation of bis-cysteinyl-peptides H-Cys-(Gly)$_n$-Cys-OH with $n = 0$–15 have shown that under conditions of high dilution (10^{-3}–10^{-4} M) and $n \geq 4$, oxidation products are largely dictated by the probability of collision of the thiol groups and intra-chain-bridged monomers were predominant, whereas for $n < 4$ formation of dimers and oligomers

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besides intra-chain monomers was observed due to insufficient flexibility of the peptide chain [19]. Nevertheless, it is possible to form even small disulphide loops, whereby the equilibrium constant K_c for loop closing increases in the rank order $n = 1$, 3, 0, 5, 4 and 2 [31].

This work compares four different synthesis methods for the synthesis of peptides with multiple disulphide bonds and presents a one-step cyclization method for fast and easy synthesis of orexin A with high yield. Furthermore, this work indicates the building of different mono- or bicyclic configurations of orexin A after unselective disulphide formation, indicating the necessity of selective formation of the two disulphide bonds. The exact structure of the different products that are built after unselective oxidation besides orexin A and its wrongly connected analogue and whether milder oxidation conditions might lead to a higher yield of the correctly folded orexin A remain topics for further investigation.

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