Research Article

Journal of PeptideScience

Received: 11 December 2009 Revised: 25 January 2010 Accepted: 26 January 2010 Published online in Wiley Interscience: 26 February 2010

(www.interscience.com) DOI 10.1002/psc.1221

An optimized chemical synthesis of human relaxin-2

Kostas K. Barlos,[a] Dimitrios Gatos,[a] Zoe Vasileiou[a] and Kleomenis Barlos[a,b]*

Human gene 2 relaxin (RLX) is a member of the insulin superfamily and is a multi-functional factor playing a vital role in pregnancy, aging, fibrosis, cardioprotection, vasodilation, inflammation, and angiogenesis. RLX is currently applied in clinical trials to cure among others acute heart failure, fibrosis, and preeclampsia. The synthesis of RLX by chemical methods is difficult because of the insolubility of its B-chain and the required laborious and low yielding site-directed combination of its A (RLXA) and B (RLXB) chains. We report here that oxidation of the Met^{25} residue of RLXB improves its solubility, allowing its effective solid-phase synthesis and application in random interchain combination reactions with RLXA. Linear Met(O)^{25}-RLX B-chain (RLXBO) reacts with a mixture of isomers of bicyclic A-chain (bcRLXA) giving exclusively the native interchain combination. Applying this method Met(O)^{25}-RLX (RLXO) was obtained in 62% yield and was easily converted to RLX in 78% yield, by reduction with ammonium iodide. Copyright © 2010 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: chain combination; relaxin; solid-phase synthesis; 2-chlorotrityl resin

Introduction

Human relaxin-2 (RLX, **1a**) belongs to the insulin peptide family, which includes also insulin, relaxin-1 and relaxin-3/INSL7 [1–3], the insulin-like peptides INSL3-6 [4–8], IGF-1 [9], and IGF-2 [10]. Like insulin, RLX is the product of the enzymatic cleavage of its prohormone [11,12]. RLX is consisted of two peptide chains, the A-chain (RLXA) and the B-chain (RLXB) joined by two intermolecular cystine bridges (Figure 1). RLXA contains an additional intramolecular disulfide bond. RLX plays a vital homeostatic role in mammalian pregnancy optimizing the many physiological changes taking place during pregnancy [13–15]. RLX was found in a variety of tissues including several non-reproductive organs [16]. RLX acting as a pleiotropic hormone [17] protects heart [18–24], lungs [25], and kidney [26–28] due to its strong antifibrotic and vasodilator activity [29–35]. Furthermore, RLX may be useful in the treatment of pancreatitis [36], preeclampsia [37], inflammation [38], and in wound healing (angiogenesis) [39]. Surprisingly, relaxin is one of the main regulators of thirst acting also in the brain [40,41]. Because of its biological effects RLX has been applied in various clinical trials [42], among others to cure acute heart failure [43].

The previously reported chemical synthesis of RLX was performed by solid-phase synthesis of the A- and B-chains and subsequent chain combination by site-directed cystine bridge formation [44–46]. In that synthesis the A-chain was obtained by solid-phase synthesis using Fmoc-amino acids and cleaved from the resin in a partially protected form by TFA. For the protection of the thiol group of the Cys residues at position A10 and A15 the Trt group was used, while A11 and A24 were protected by Acm and MeBzl groups, respectively. The B-chain was solid-phase synthesized using Boc-amino acids and cleaved from resin by treatment with HF. The B11 Cys residue was side-chain protected by Acm, while B23 was electrophilically activated and simultaneously protected by Npys. Finally, regioselective building of the three intra- and interchain disulfide bonds was performed in three steps followed by the removal of the formyl protection of the Trp residues by treatment with base and reduction of Met-sulfoxide with ammonium iodide (Figure 2). RLX was thus obtained in a total yield of 1.4% calculated on the base of the applied B-chain.

Above method with slight changes, in particular by using different resins, has also been applied for the synthesis of several other insulin-like peptides [47,48]. This method, which requires two HF treatments and three reaction steps for chain combination, is rather laborious. In addition, the low yield achieved can be considered as economically not acceptable for larger scale syntheses. Random combination of RLXA (**5**) and RLXB could lead to a much simpler synthesis in higher yield. Unfortunately, the RLXB is reported to be very insoluble and its solid-phase synthesis using Fmoc-amino acids very complicated. So, an effective synthesis of

* *Correspondence to: Kleomenis Barlos, CBL-Patras, Patras Industrial Area, Block 1, Patras, Greece,. E-mail: barlos@cblpatras.gr*

a *Department of Chemistry, University of Patras, Rion-Patras, Greece*

b *CBL-Patras, Patras Industrial Area, Block 1, Patras, Greece*

Abbreviations used: *Acm, acetamidomethyl; AcN, acetonitrile; AcOH, acetic acid; Boc, tert.-butyloxycarbonyl; $(Boc)_2O$, di-tert-butyl dicarbonate; Clt, 2-chlorotrityl; CLTR, 2-chlorotrityl resin; DBU, 1,8-diazabicyclo[5.4.0]-undec-7-ene; DCM, dichloromethane; DEE, diethylether; DIC, N,N′-diisopropylcarbodiimide; DIEA, N,N-diisopropylethylamine; DMF, N,N-dimethylformamide; DMSO, dimethyl sulfoxide; DTT, 1,4-dithio-DL-threitol; ES-MS, electrospray mass spectrometry; Fmoc, 9-fluorenylmethoxycarbonyl; Gnd.HCl, guanidinium hydrochloride; HBTU, O-benzotriazolyl-N,N,N′,N′-tetramethyluronium hexafluorophosphate; HF, hydrogen fluoride; HOBt, 1-hydroxybenzotriazole; HPLC, high-performance liquid chromatography; IGF, insulin-like growth factor; INSL, insulin-like peptide; IPA, isopropanol; MeOH, methanol; Mmt, 4-methoxytrityl; MeBzl, 4-methylbenzyl; NMP, N-methyl-2-pyrrolidinone; Npys, 5-nitro-2-pyridinesulfenyl; Pbf, 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl; RP, reverse phase; RT, room temperature; SBS, step-by-step; SPPS, solid-phase peptide synthesis; SPS, solid-phase synthesis; tBu, tert-butyl; TES, triethylsilane; TFA, trifluoroacetic acid; TFE, trifluoroethanol; THF, tetrahydrofurane; TLC, thin layer chromatography; Trt, triphenylmethyl, trityl.*

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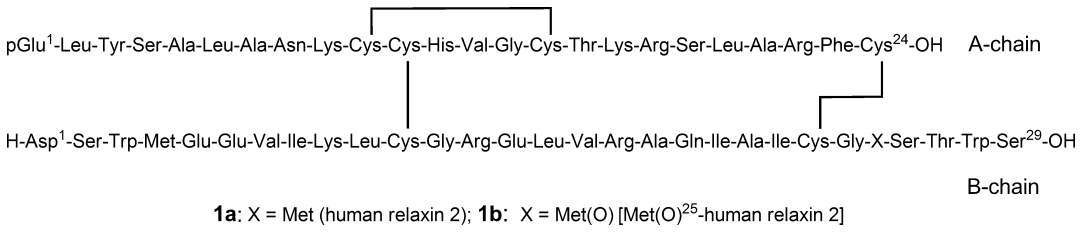

Figure 1. Structure of human relaxin-2 (RLX, **1a**) and Met(O)[25]-human relaxin-2 (RLXO, **1b**).

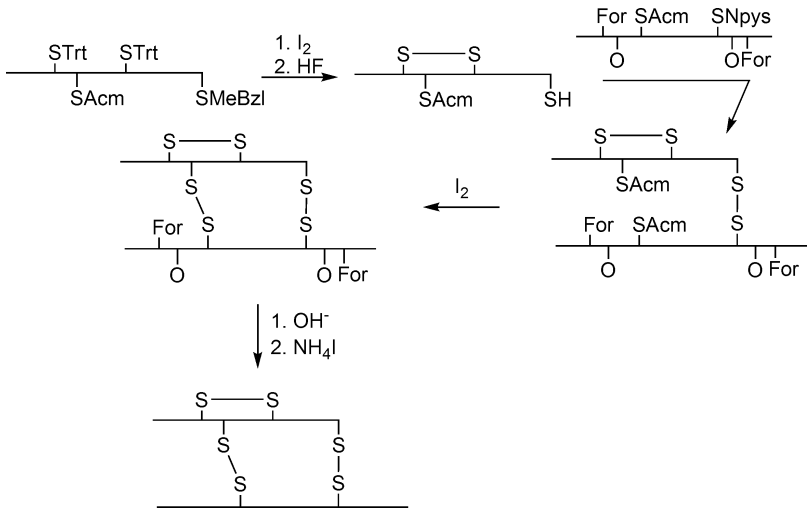

Figure 2. Site-directed disulfide bond formation during the synthesis of RLX.

RLX by random chain combination has not been described as yet. In contrary, if RLXB is extended by some residues or otherwise modified, it becomes possible to be synthesized and combined with RLXA [49]. An additional problem, not addressed in the previously published work, is the possible racemization of the *C*-terminal Cys residue of RLXA during its esterification on the resin and chain elongation. Due to above described problems only the recombinant human relaxin (rRLX) is used in the clinical trials performed for the evaluation of RLX as a therapeutic agent. However, the biological production of RLX is also complicated. So, prorelaxin is first produced by recombinant techniques as a single chain peptide. Then it is converted to RLX in four steps by oxidative folding, enzymatic cleavage at two positions using two different enzymes and finally conversion of the *N*-terminal Gln residue of RLXA to pyroglutamine by heating [50–56]. We decided, therefore, to explore the possibility to develop a more effective chemical synthesis of RLX using 2-chlorotrityl resin (CLTR) for the solid-phase synthesis of the chains and to improve their random chain combination.

Materials and Methods

2-Chlorotrityl chloride polystyrene resin (1% DVB, 100–200 mesh), Fmoc-amino acids, DIC, HOBt, and TFA were purchased from CBL-Patras (Patras, Greece). Solvents and other reagents used were purchased from Aldrich (Steinheim, Germany) and Merck (Darmstadt, Germany) and were of analytical grade. AcN used for HPLC was of gradient grade (Merck). TLC-analyses: TLC-aluminium sheets, silica gel 60, F_{254} (Merck). Analytical HPLC: Waters Alliance 2695 Separations Module combined with Waters 2996 photodiode array detector. Columns: (i) C-8 Purospher (particle size 5 µm, 125 × 4 mm) using linear gradients of 10–60% B over 30 min (conditions A), 20–100% B over 30 min (conditions B), 35–55% B over 30 min (conditions C); (ii) C-18 Nucleosil (particle size 5 µm, 125 × 4 mm) using linear gradients of 20–40% B over 30 min (conditions D); (iii) C-8 Purospher (particle size 5 µm, 250 × 4 mm) using linear gradients of 20–60% B over 30 min (conditions E) and 5–50% B over 30 min (conditions F). Eluent A was 0.08% TFA in water and eluent B was 0.08% TFA in AcN. Flow rate, 1 ml/min. UV detection at 214 nm. Purifications were carried out on a Waters 600E multisolvent delivery system, combined with Waters 996 photodiode array detector using a semipreparative C-8 Purospher column (particle size 5 µm, 250 × 10 mm) at a flow rate of 5 ml/min and linear gradients of 20–40% B over 45 min (conditions G), 30–30 over 20 min 30–55% B over 40 min (conditions H) and 20–60% B over 45 min (conditions I). UV detection at 214 and 290 nm. ES-MS spectra were recorded on a Waters Micromass ZQ 4000 mass detector (positive mode), controlled by the MassLynx 4.1 software, either by direct infusion using a syringe pump at a flow rate of 5 µl/min or in-line with a Waters Alliance 2695 LC system at a flow rate of 100 µl/min (using a T-flow splitter). Cone voltage was set at 30 V and scan time at 1 s, with interscan delay at 0.1 s.

Solid-Phase Peptide Assembly, General Protocol

Solid-phase peptide synthesis was carried out manually using plastic syringes equipped with porous polypropylene frits at RT. Amino acid side-chain protection was as follows: tBu for Asp, Glu, Tyr, Thr, Ser; Pbf for Arg; Boc for Lys and Trp; Trt for Asn and His. No side-chain protection was used for Gln. Cys[10,11,15,24] of the

A-chain and Cys[11,23] of the B-chain were incorporated as *S*-Mmt derivatives.

Preactivation of Fmoc-amino acids

The Fmoc-amino acid (4.5 mmol) and HOBt (5.8 mmol) were dissolved in NMP (4.5 ml) and cooled to 5 °C. Then, DIC (5.0 mmol) was added and the mixture was stirred for 15 min at 5 °C.

Coupling

The solution of the preactivated amino acid was added to the resin-bound peptide (5.0 g, starting loading 0.3 mmol/g) and shaken for 3 h at RT. A sample was taken to check the reaction completion by Kaiser test. In case of incomplete coupling (positive Kaiser test) recoupling was performed with a fresh solution of activated amino acid (6 mmol).

Fmoc-group removal and Fmoc-removal test

The resin-bound Fmoc-protected peptides were treated twice with 25% piperidine in NMP (25 ml each) for 30 min at RT. To check the completion of the Fmoc-removal a resin probe (approx. 2 mg) was taken, 25% piperidine in NMP (20 µl) was added and the mixture was heated for 5 min at 100 °C. From the resulting solution 10 µl were spotted on a TLC plate and checked under a UV-lamp for UV-absorbing material. The limit of the method was found to be 0.001 µmol. Alternatively, the solution was injected on HPLC and the Fmoc-material produced and released into solution was quantified at 265 nm. If the Fmoc-removal test remained positive (violet spot under UV) the piperidine treatment was prolonged or repeated until achieving negative test.

End-capping, of remaining N^{α}-amino functions, with $(Boc)_2O$

The resin-bound Fmoc-protected peptide was washed with NMP (×3) and reacted with a mixture of $(Boc)_2O$ (10 equiv.) and DIEA (5 equiv.) in NMP for 1 h at RT.

Synthesis of Linear A-chain (RLXA, 5)

H-Cys(Mmt)-O-CLTR (5 g, 1.5 mmol) was chain-elongated according to the procedures described above. All couplings were completed within 1.5 h or less, as indicated by the negative Kaiser test. The finally obtained peptidyl-resin was washed with DCM (3 × 20 ml) and then treated with a solution of 1.5% TFA in DCM/TES/DTT (94 : 3 : 3) (2 × 20 ml × 5 min) and washed with DCM (5 × 20 ml). The combined filtrates were concentrated on a rotary evaporator and the obtained oily partially protected linear A-chain **4** was precipitated by the addition of cold DEE (200 ml), washed with DEE (5 × 50 ml) and dried at 35 °C under vacuum. Yield: 6 g (88%). Peptide **4** (2 g, 0.44 mmol) was subsequently treated with prechilled TFA/DCM/TES/DTT (91 : 3 : 3 : 3) (200 ml) for 4 h at 0 °C. The solution was concentrated in vacuum and the product was precipitated with the addition of cold DEE (150 ml), washed with cold DEE (10 × 30 ml) and dried in vacuum to a constant weight. Yield: 1.08 g (92%) of crude RLXA (**5**). ES-MS: $M_{calcd.}$ = 2654.7 Da, M_{found} = 2655.4 Da.

Preparation of Bicyclic A-chain (bcRLXA, 6–8)

Crude RLXA (1.08 g, 0.41 mmol) was oxidized with 20% DMSO in ammonium acetate buffer at pH = 6.0 (300 ml) for 48 h, acidified with TFA to pH 3.0 and purified by semipreparative HPLC (conditions G) to obtain, after lyophilization, 497 mg (46%) of a mixture of bicyclic A-chain isomers of 99% purity. ES-MS: $M_{calcd.}$ = 2650.9 Da, M_{found} = 2651.8 Da.

Synthesis of the Linear Met(O)[25] - Relaxin 2 B-Chain (RLXBO, 12b)

H-Ser(tBu)-O-CLTR (5 g, 1.5 mmol) was chain-elongated according to the procedures described above. The obtained Fmoc–Met–Ser(tBu)-Thr(tBu)-Trp(Boc)-Ser(tBu)-O-CLTR **(19)** was then washed with THF (3 × 20 ml) and a solution of 10% hydrogen peroxide in THF (20 ml) was added to the resin. The mixture was shaken at RT for 6 h, until oxidation completion was verified by HPLC analysis and the resin was washed with THF (5 × 10 ml) and NMP (3 × 10 ml) to give Fmoc-Met(O)-Ser(tBu)-Thr(tBu)-Trp(Boc)-Ser(tBu)-O-CLTR **(20)**. This was then elongated stepwise to give resin-bound protected Met(O)[25] B-chain. After the incorporation of Ala[18] and until Val[7] the Fmoc-removal was slower and required 2 h at RT for completion. From Glu[14] to Leu[10] double couplings were required to achieve negative Kaiser test. At Glu[14] and Arg[13] end-capping with $(Boc)_2O$ was necessary for negative Kaiser test. The finally obtained peptidyl-resin was washed with DCM (6 × 20 ml) and treated with a solution of 2% TFA in DCM/DTT (95 : 5) (8 × 20 ml × 2 min) and DCM (5 × 20 ml). The filtrates were combined and concentrated on a rotary evaporator. The cleaved product was precipitated by the addition of cold DEE (200 ml), filtered, washed with DEE (10 × 50 ml) and dried under vacuum to afford 5.8 g (85%) of the partially protected B-chain **11b**. A portion of this material (500 mg, 109 µmol) was globally deprotected by treatment with prechilled TFA/H_2O/DTT (90 : 5 : 5) (50 ml) for 1 h at 0 °C and 3 h at RT. The deprotection solution was concentrated and the product was precipitated by the addition of cold DEE (75 ml), washed with DEE (10 × 15 ml) and dried to a constant weight. Yield: 332 mg (92%) of crude **12b**. This material (332 mg, 100 µmol) was purified by semipreparative HPLC (conditions H) and freeze dried. Yield 113 mg (34%) of **12b** of 93% purity. A sample of 5 mg of this material was further purified by a second HPLC run to afford after lyophilization 2.8 mg of **12b** with 99% purity. ES-MS: $M_{calcd.}$ = 3326.8 Da, M_{found} = 3327.3 Da.

Random Folding of the Relaxin A-Chain with Met(O)[25] B-Chain. Synthesis of RLXO (1b)

bcRLXA mixture of isomers (31.2 mg, 12 µmol) were dissolved in 6 M Gnd.HCl (1 ml) and 0.1 M sodium glycinate buffer at pH 10.8 (5 ml). To the resulting solution, RLXBO of 93% purity (35.5 mg, 10 µmol) dissolved in DMSO (900 µl), was added dropwise. The mixture was vigorously stirred for 16 h at 20 °C. A solid material, formed during stirring, was collected by centrifugation and washed with IPA/water (2 : 1) (5 × 10 ml). This material was dissolved in 6 M Gnd.HCl and combined with the main solution. The resulting solution was acidified with 5% aqueous TFA (2 ml) to pH 3 and subjected to semipreparative HPLC purification (conditions I) to yield, after lyophilization, RLXO (**1b**) (37 mg, 62%) of 99% purity. $M_{calcd.}$ = 5975.9 Da, M_{found} = 5976.3 Da.

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Reduction of RLXO (1b) with Ammonium Iodide. Synthesis of RLX (1a)

RLXO (18 mg, 3 μmol) was treated with a 0.5 M solution of NH_4I in TFA/water (9 : 1) (1.8 ml) for 20 min at 0 °C. The reaction was quenched by the addition of 1 M ascorbic acid in water (900 μl) and directly injected on semipreparative HPLC (conditions I) to afford, after lyophilization, RLX (**1a**) (14 mg, 78%) of 99% purity. The overall yield relative to the purified RLXBO was 48%. ES-MS: $M_{calcd.} = 5959.9$ Da, $M_{found} = 5960.2$ Da.

Tryptic Digestion of RLX

RLX (100 μg) was dissolved in 50 μM Tris-HCl buffer/5 μM $CaCl_2$ pH = 8 (100 μl). The mixture was added to a tryspin solution in the same buffer (10 μl/10 μg). After incubation for 45 min at 37 °C, 100 μl of the mixture were injected in analytical HPLC (conditions F). Fragment **24**: $M_{calcd.} = 1295.2$ Da, $M_{found} = 1295.3$ Da; Fragment **26**: $M_{calcd.} = 1532.8$ Da, $M_{found} = 1533.5$ Da.

Results and Discussion

Solid-Phase Synthesis of RLXA

The successful solid-phase synthesis of a peptide containing a Cys residue at its *C*-terminal position requires the epimerization-free esterification of the Cys residue onto the resin. Electrophilic activation of the Cys carboxyl function which is known to lead to extensive racemization should be avoided [57–59]. Therefore, we used the CLTR [60]. Indeed, the analysis of the resin-bound Cys(Mmt) gave <0.2% of D-enantiomer indicating its racemization-free esterification. Starting from CLTR-bound Cys(Mmt), the SPS of RLXA was performed using Fmoc-amino acids, side-chain protected with TFA-labile protecting groups (Figure 3). Their activation was mediated by DIC/HOBt. Besides Cys-24, the three other Cys residues contained in RLXA were also protected at their thiol function with the very acid-sensitive Mmt group [61]. The chain extension was performed without facing any problem by applying a threefold molar excess of activated amino acids and using 25% piperidine in NMP for the Fmoc-removal. The resin-bound peptide was then treated with a mixture of 1.5% TFA in DCM/TES/DTT (94 : 3 : 3) for 5 min (twice) at RT to cleave it from the resin. Under these conditions the *S*-Mmt groups were removed simultaneously from the side-chain of the Cys residues. The partially protected crude RLXA (**4**) was obtained after precipitation with diethyl ether in 88% yield. The addition of scavengers, such as TES during this step is absolutely necessary. Otherwise the liberated thiol groups of the peptide react with the CLTR cations formed during the cleavage and remain bound on the resin. The deprotection of multiple Cys-containing peptides, such as RLXA, is challenging because the free Cys thiols act as internal scavengers for the electrophilic species, which are generated during the acidic treatment. Therefore, sophisticated mixtures have been proposed as deprotection cocktails [62,63]. Nevertheless, the deprotection of every individual peptide requires optimization. This is often the crucial factor for the obtained peptide yield and purity. Thus, we treated the partially protected RLXA with various mixtures in order to choose the most suited deprotection cocktail. Our results are summarized in Table 1. We found that cleavage mixtures containing TFA/DCM/TES/DTT (Table 1, runs 5–10) are better suited for the deprotection of RLXA than those containing water instead of DCM (Table 1, runs 1–4). Higher dilution of the peptide and low temperature during the deprotection increase also the content of RLXA in the crude mixture. Best results were obtained by treating the protected peptide with TFA/DCM/TES/DTT (91 : 3 : 3 : 3) for 4 h at 0 °C under high dilution. In this case the content of RLXA in the product mixture was 82% (Figure 4(b)), in comparison to 17% obtained by treatment with TFA/H_2O/DTT (94 : 3 : 3) and high concentration (Figure 4(a)). HPLC purification of the obtained mixture gave RLXA in 40% total yield calculated on the base of the starting H-Cys(Mmt)-CLTR resin (**2**) and 97% purity (Figure 4(c)). The identity of the obtained RLXA was confirmed with ES-MS analysis (Figure 4(d)).

Table 1. Deprotection of 1 mg of crude protected RLXA under various conditions

Run	Cocktail	Volume (μl)	Temperature (°C)	Time (h)	HPLC purity (%)
1	TFA/H_2O/DTT (94 : 3 : 3)	30	22	3	17
2	TFA/H_2O/TES/DTT (91 : 3 : 3 : 3)	30	0	4	24
3	TFA/H_2O/TES/DTT (91 : 3 : 3 : 3)	250	0	4	35
4	TFA/H_2O/TES/DTT (91 : 3 : 3 : 3)	1000	0	4	45
5	TFA/DCM/TES (94 : 3 : 3)	30	0	4	27
6	TFA/DCM/TES/DTT (91 : 3 : 3 : 3)	30	22	3	56
7	TFA/DCM/TES/DTT (91 : 3 : 3 : 3)	30	0	4	63
8	TFA/DCM/TES/DTT (91 : 3 : 3 : 3)	250	0	4	68
9	TFA/DCM/TES/DTT (91 : 3 : 3 : 3)	250	0	4	72
10	TFA/DCM/TES/DTT (91 : 3 : 3 : 3)	1000	0	4	82

Synthesis of a Mixture of Isomers of Bicyclic RLXA (bcRLXA, 6–8) by DMSO Oxidation of RLXA

The combination of the RLX A- and B-chains is believed to follow a pathway similar to that of the analogous insulin chains. Due to the complexity of the biological events, the mechanism of the folding still remains controversial. It is assumed that the folding of RLX begins with the oxidation of the A-chain **5** to form the bicyclic intermediates **6–8** [49]. To follow this method for the production of RLX we prepared the bcRLXA (Figure 3). Thus, we treated RLXA with DMSO [64] in an aqueous buffer at pH = 6. After 12 h at RT we obtained three bcRLXA isomers in 1 : 4 : 10 molar ratio. The HPLC analysis showed that bcRLXA isomers are more polar than most of the impurities in the product mixture. We assumed therefore that it could be easier to purify by RP-HPLC the bcRLXA isomers than the RLXA. So, we converted crude RLXA to a mixture of bcRLXA isomers by DMSO oxidation for 2 day at RT. After HPLC-purification we obtained a mixture of the two mainly produced bcRLXA isomers in 46% yield.

Synthesis of Met(O)^{25} - Relaxin B-Chain (12b)

To improve the synthesis of RLX a better method of preparing the RLXB (**12a**) was necessary. So, in order to develop an effective and simpler synthetic method than that utilizing the site-directed chain

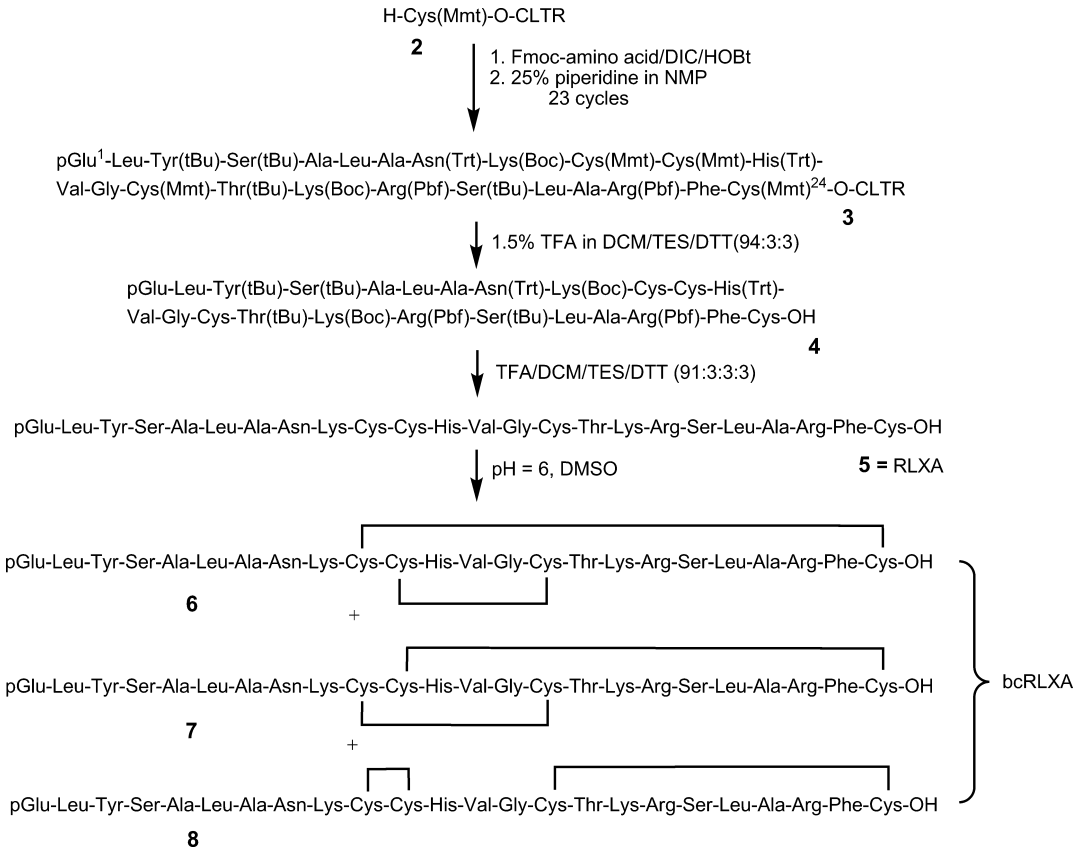

Figure 3. Solid-phase synthesis of RLXA (**5**) and a mixture of bicyclic isomers (bcRLXA, **6**–**8**).

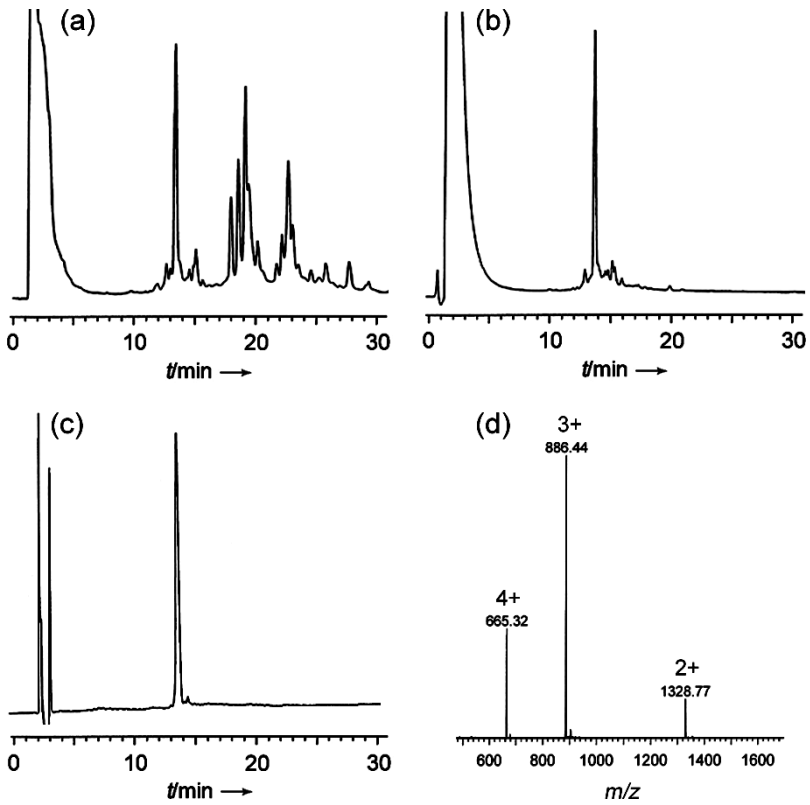

Figure 4. (a) Analytical HPLC profile of the crude product mixture obtained after deprotection of the A-chain **4** by treatment with (a) TFA/H_2O/DTT (94 : 3 : 3) for 3 h at 22 °C, (b) TFA/DCM/TES/DTT (91 : 3 : 3 : 3) for 4 h at 0 °C, (c) analytical HPLC profile of purified **5** (Conditions A); and (d) ES-MS of RLXA.

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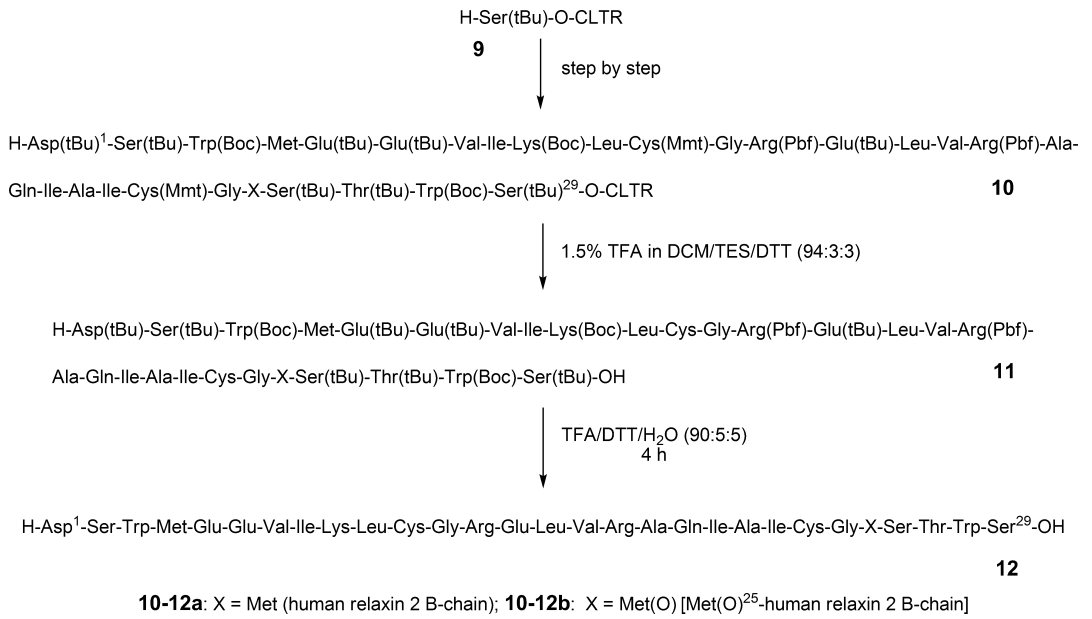

Figure 5. Solid-phase synthesis of relaxin-2 B-chain.

combination procedure, we have undertaken several attempts to prepare the B-chain. Thus, efforts were made to synthesize it by employing the step-by-step (SBS) [65], the fragment condensation [66] and the native chemical ligation approaches [67,68].

We started with SBS by using the CLTR resin **9** and applying Fmoc-amino acids side-chain protected with TFA-labile protecting groups for the chain assembly (Figure 5). The activation of the amino acids was performed with DIC/HOBt in NMP. We used 25% piperidine in NMP for the Fmoc-removal. At the beginning of the synthesis no problem was encountered. Problems started by the incorporation of Ala-21. After this point we steadily faced difficult coupling and deprotection steps. Couplings were incomplete even after repeated reactions with a fourfold molar excess of activated amino acids. In addition, it became almost impossible to completely remove the Fmoc-group by piperidine treatment. Furthermore, the HPLC-analysis of the intermediates proved to be very difficult. In fact very broad HPLC-peaks were observed independently if we analyzed the protected, the totally deprotected or the side-chain deprotected Fmoc-intermediates. LC-MS-analysis of the finally obtained product mixture gave us the possibility to identify RLXB inside a broad peak. Due to the extensive overlapping with closely eluted impurities no attempt was made to separate it from that mixture.

After above disappointing attempts to synthesize RLXB by the SBS method we tried to synthesize it by the fragment condensation approach. In this method the target peptide is divided into smaller protected fragments. These are then condensed sequentially in solution or on solid support. It is favorable to use fragments, which contain Gly as the *C*-terminal amino acid to avoid possible racemization during the condensation [69].

We decided to divide RLXB into three fragments, namely the resin-bound 25–29 (**13**) fragment and to condense it with the 13–24 (**14**) and 1–12 (**15**) protected fragments, both containing Gly as the *C*-terminal amino acid (Figure 6). By using this method we hoped to overcome the problems in the region 18–23, which contains two Ala and two Ile residues and, therefore, it was expected to be a difficult sequence. After the condensation of the 13–24 fragment more facile coupling and deprotection steps

Fmoc-Met[25]-Ser(tBu)-Thr(tBu)-Trp(Boc)-Ser(tBu)[29]-O-CLTR **13**

Fmoc-Arg(Pbf)[13]-Glu(tBu)-Leu-Val-Arg(Pbf)-Ala-Gln-Ile-Ala-Ile-Cys(Mmt)-Gly[24]-OH **14**

Boc-Asp(tBu)[1]-Ser(tBu)-Trp(Boc)-Met-Glu(tBu)-Glu(tBu)-Val-Ile-Lys(Boc)-Leu-Cys(Mmt)-Gly[12]-OH **15**

H-Cys[23]-Gly-Met-Ser-Thr-Trp-Ser[29]-OH **16**

Thz[11]-Gly-Arg-Glu-Leu-Val-Arg-Ala-Gln-Ile-Ala-Ile[22]-SR **17**

H-Asp[1]-Ser-Trp-Met-Glu-Glu-Val-Ile-Lys-Leu[10]-SR **18**

$R = CH_2\text{-}CH_2\text{-}CO_2CH_3$

Figure 6. Peptide fragments required for the fragment condensation (peptides **13**–**15**) and 'ligation' (peptides **16**–**18**) synthesis of RLXB (**12a**).

were expected. In fact, we made similar observations during the synthesis of the atrial natriuretic peptide [70]. The protected fragments required should be prepared using the CLTR. The peptides are cleaved from this resin with intact remaining side-chain protection of the tBu type by treatment with mild acids, such as acetic acid or 1% TFA [71,72].

Unfortunately, similarly to our efforts to synthesize the whole chain by the SBS procedure, the synthesis of the 13–24 protected fragment **14** was not successful. So, after the incorporation of Val-16 the removal of the Fmoc-group could not be completed even after a 4 h treatment with 25% piperidine. The subsequent coupling with Fmoc-Leu-OH to incorporate the Leu-15 residue was also not possible to be driven to completion. Similar difficulties were observed in the incorporation of the next amino acids. Finally, the fragment cleaved from the resin was impure and insoluble to be analyzed or to be applied in fragment condensations.

Next, we considered the possibility to obtain RLXB by the native chemical ligation approach. In this method a *C*-terminal fragment which contains a Cys as the *N*-terminal amino acid is condensed with the thioester of the next fragment. The fragments

are prepared separately, deprotected and purified prior to ligation. The required peptide thioesters could be prepared by solid-phase methods using mercaptoacid derivatives bound on PAM or MBHA-resins and cleaved from the resin by treatment with HF [73]. An alternative method, which avoids the use of the hazardous and toxic HF, is to produce the required protected fragment on the CLTR and to condense it in solution with a suitable mercapto acid derivative to the required peptide thioester [74,75].

We therefore decided to study the ligation of the thiazolidine derivative of segment 11–22 (**17**) with the segment 23–29 (**16)** and after treatment with methoxylamine to liberate Cys-11, to ligate the obtained 11–29 fragment with the (1–10)-thioester (**18)** (Figure 6). The fragments should be synthesized on CLTR. The protected fragments 1–10 and 11–22 should be subsequently converted to their corresponding thioesters by condensing with mercaptopropionic acid methylester in solution. However, we were again not able to obtain RLXB by this method because the synthesis of the 11–22 protected fragment was impossible. So, after the incorporation of Val-16 the synthesis could not be continued.

Although our results were completely disappointing, it was interesting to observe that a sharp peak of an early eluting side-product was present in the most of the HPLC-analyses performed during the attempted synthesis of RLXB. MS indicated that it was a product with +16 Da in mass, in respect to the expected mass of RLXB and its intermediates. We concluded therefore that it was the Met-25 sulfoxide. Sulfoxides are often formed during the acidic deprotection of Met-containing peptides, which is not performed under complete exclusion of air. The detected side-product was not the Met-4 sulfoxide since its identification occurred before the incorporation of that residue. The sharpness of the peaks of the Met(O)-containing intermediate peptides and of the final Met(O)-25 B-chain (Figure 7(a) and (b)) was very promising, indicating that oxidation of the Met-25 residue of RLXB to the corresponding RLXBO could significantly improve its solubility and its HPLC elution behavior. We expected similar improvements in the intermediate fragments during the peptide elongation. This change in solubility and elution properties could allow a better analysis of the intermediates obtained and so the possibility to optimize the synthesis. In addition, we expected an easier purification by HPLC and handling of the crude and purified RLXBO, in comparison to the native chain. Therefore, we preferred to explore this possibility before using expensive pseudoprolines with questionable success. Incorporation of pseudoprolines in the peptide chain improves the performance of the coupling and deprotection steps during solid-phase synthesis. This is possible because β-turns of the peptide chain are disrupted [76]. However, even if the assembly of RLXB could be successfully performed by using pseudoprolines, the final product after global deprotection would be the native chain which is reported to be rather insoluble. In addition, we were very interested to get RLXBO, since it is reported that RLXO is more active than the native hormone in the up regulation of cAMP production in human uterine endometrial cells [77]. So, RLXBO **12b** was finally prepared by the Fmoc/tBu-based methodology.

Starting from resin **9** two methods for the incorporation of Met(O)-25 were tested. In the first and simpler method we used Fmoc-Met(O)-OH, while in the second we performed an on-resin oxidation of the Met-25 residue using hydrogen peroxide in THF as oxidant (Figure 8). The reaction proceeding was followed by treating small resin samples with a mixture of DCM/TFE/AcOH (7 : 2 : 1) for 5 min at RT and analyzing the product mixture released by HPLC and ES-MS. We observed that the conversion of the resin-bound peptide **19** to its corresponding sulfoxide **20** was completed within 5 h at RT. The purity of this peptide (98%) was identical to that obtained by the direct Met(O) incorporation.

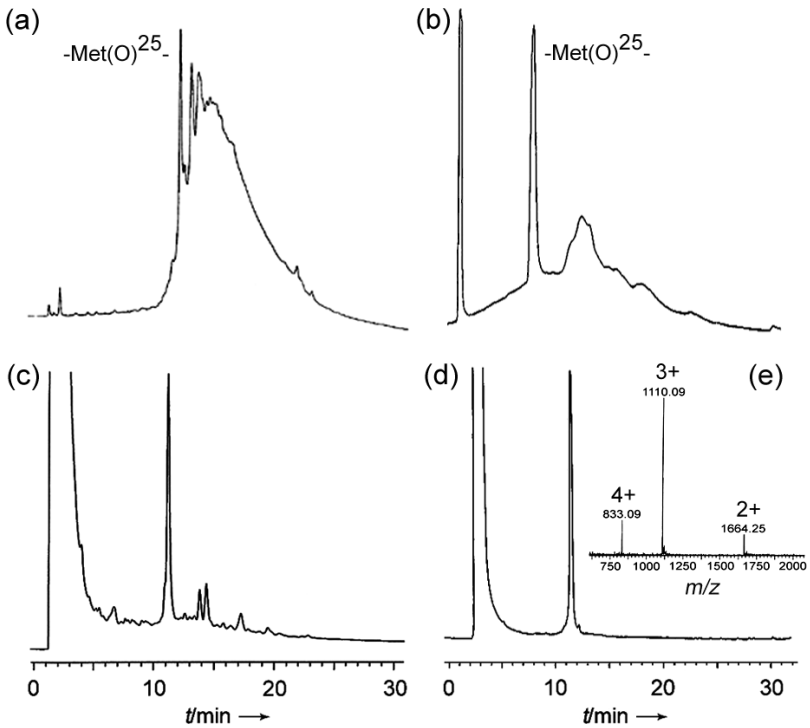

Figure 7. Analytical HPLC of (a) side-chain deprotected Fmoc-(17–29)-OH of RLXB, (b) crude deprotected RLXB* (conditions B), (c) crude deprotected RLXBO, (d) purified RLXBO (**18b**) (conditions C), and (e) ES-MS of RLXBO. * The sample was dissolved in 2 M LiBr and 20% IPA was added.

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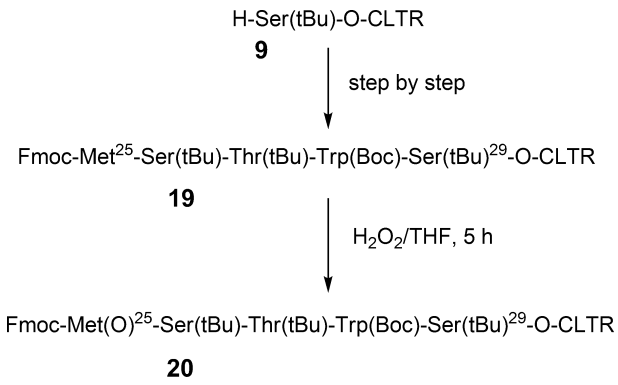

Figure 8. On-resin oxidation of the 25–29 protected fragment (**20**) of B-chain.

Although the on-resin oxidation seems to be more complicated than the direct incorporation of Met(O) in the peptide chain, it can be useful in several cases. So, if during the chain elongation unexpected difficulties are met in couplings and in the removal of the Fmoc-groups, the oxidation of an already incorporated Met-residue could be helpful in overcoming those problems. The obtained resin-bound Met(O)-containing peptide **20** was then chain-elongated as usual by the SBS procedure. No problem in its synthesis was encountered until Ala-18. Although the incorporation of this residue was complete, as indicated by the negative Kaiser test [78] and HPLC analysis, only 95% of its Fmoc-group could be removed by a 4 × 30 min treatment with 25% piperidine in NMP. After the incorporation of Glu-14 the synthesis became more difficult than before. Double couplings were required with a fourfold molar excess of activated amino acids and prolonged piperidine treatments for the completion of the reactions. Fortunately, after the incorporation of the Val-7 residue the peaks of the intermediates became again sharp and couplings and deprotections were fast and complete. After the chain assembly the peptide was cleaved from the resin by treatment with 2% TFA in DCM/DTT (95 : 5) and deprotected with TFA/H_2O/DTT for 1 h at 0 °C and 3 h at RT. In this case anhydrous TFA deprotection cocktails which gave excellent results during the deprotection of RLXA could not be used because the concurrent reduction of the Met-sulfoxide to Met was observed during the acidic treatment. Although not optimized, this first synthesis gave a crude product mixture which consisted, besides other impurities, of 39% RLXBO (Figure 7(c)). As expected, the obtained product exhibited good solubility in various solvent mixtures. So, it was soluble at a concentration of 20 mg/ml in DMSO and 4 mg/ml in DMF/water (80 : 20). This fact allowed its simple purification by semipreparative HPLC, which gave RLXBO in 26% yield and 99% purity (Figure 7(d)). The correct molecular mass of the obtained product was determined by ES-MS. To improve the total yield on RLXO without losing any RLXBO quantity we used a semipurified product which contained 93% RLXBO for the subsequent chain folding experiments. This material was contaminated with ca. 5% of the closely eluting desCys-11 failure sequence, which was formed due to the incomplete removal of the Fmoc-group of the Gly-12 residue. We increased in that way the yield of RLXBO to 34%.

Chain Folding

Like insulin, relaxin can be obtained by oxidative folding of the two individual chains. However, reported yields were very low

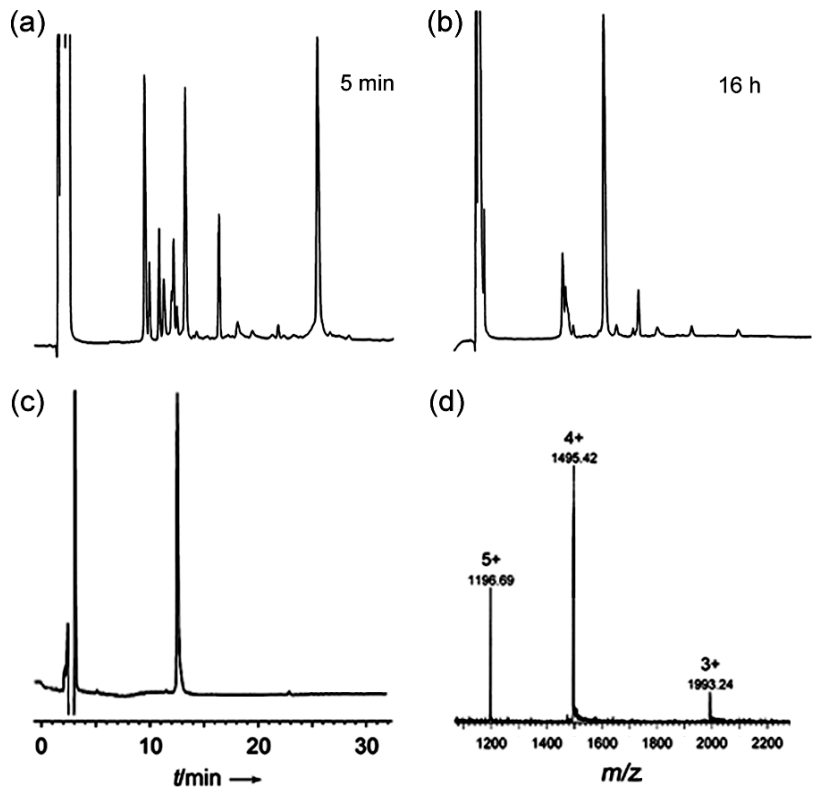

Figure 9. (a), (b) Analytical HPLC profiles of the product mixture obtained during combination of bcRLXA with RLXBO at 5 min and 16 h, respectively in a 1.2 : 1 molar ratio, (c) analytical HPLC, and (d) ES-MS of the purified RLXO (conditions E).

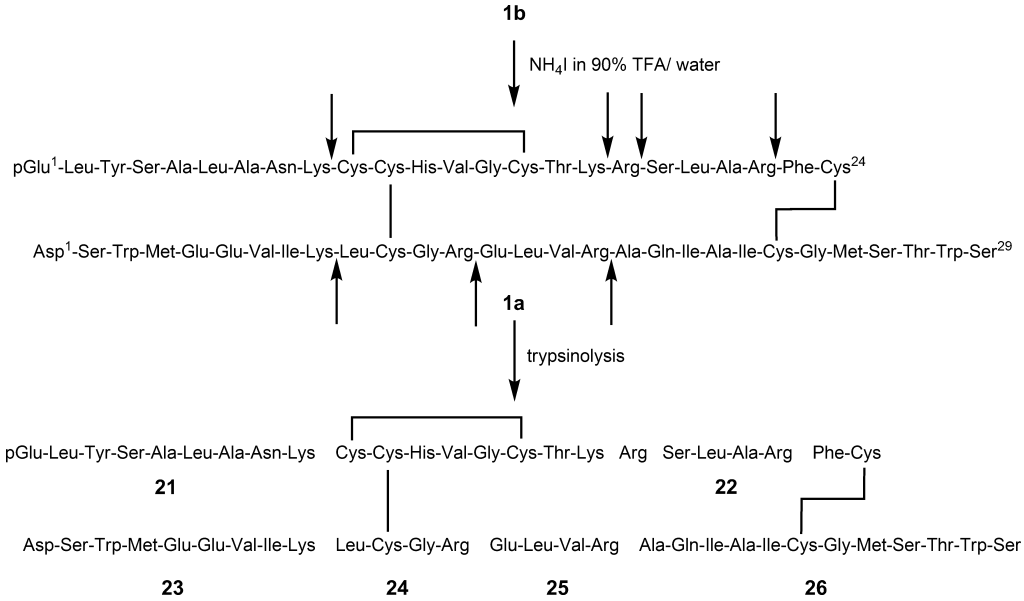

Figure 10. Reduction of RLXO (**1b)** to human relaxin-2 (**1a)** with ammonium iodide and the products of its trypsinolysis.

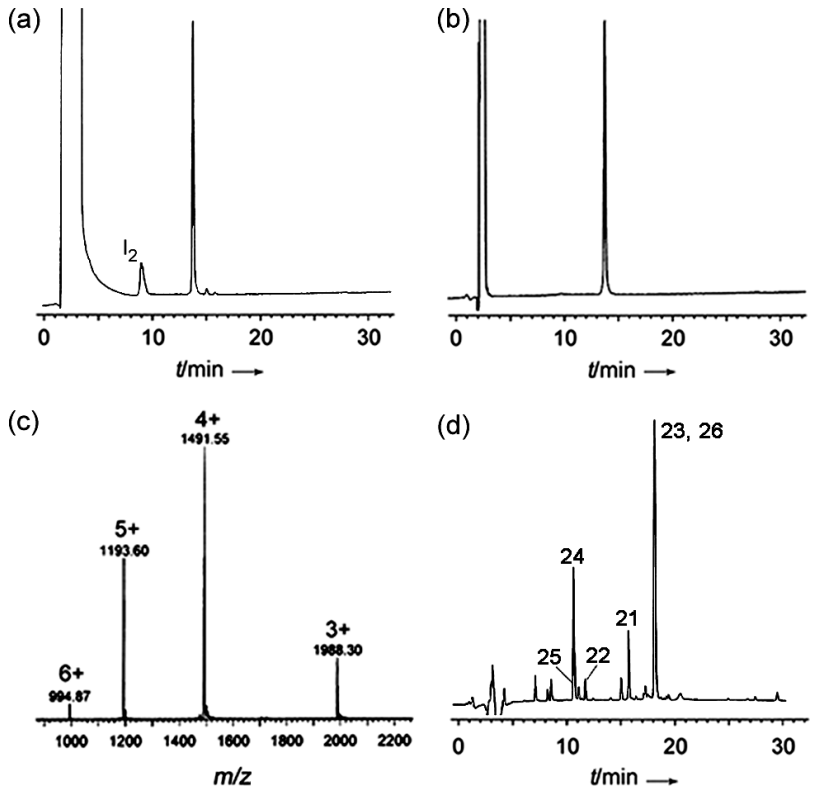

Figure 11. Analytical HPLC profiles of (a) crude and (b) purified RLX (conditions E), (c) ES-MS of purified RLX, (d) analytical HPLC profile of the product mixture obtained after the trypsinolysis of synthetic human relaxin-2 (conditions F).

due to the poor solubility of the RLXB [79] and its intramolecular oxidation during the folding [57]. To aid random chain combination studies, a more soluble RLXB extended at its *C*-terminus by four residues has been prepared and reacted with excess RLXA [49]. The analysis of these experiments led to the conclusion that the first molecular event during the oxidative folding was the fast independent formation of a stable bcRLXA reaction intermediate. Therefore, to obtain RLXO we performed the chain combination by using a mixture of bcRLXA isomers and RLXBO of 93% purity in a molar ratio of 1.2 : 1 in 15% DMSO at pH = 10.8. The reaction was complete after 16 h at RT (Figure 9(b)). The main products of the reaction were RLXO and cyclic B-chain (cRLXBO), which were formed in 9 : 1 molar ratio and were identified by ES-MS. RLXO was isolated from that mixture by semipreparative HPLC in 62% yield. The analytical HPLC profile of the purified RLXO is shown in Figure 9(c) and its ES-MS in Figure 9(d).

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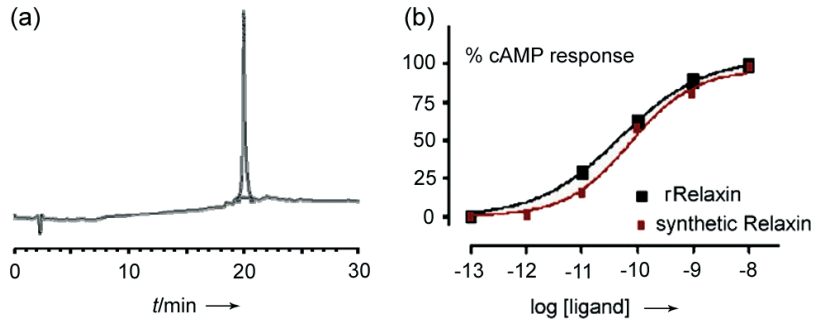

Figure 12. (a) Analytical HPLC profile of the coelution of a standard of recombinant human relaxin-2 (rRLX) and synthetic RLX and (b) comparison of the biological potency of recombinant and synthetic RLX.

Preparation of Human Relaxin-2 (RLX, 1a) by the Reduction of RLXO (1b)

Methionine sulfoxides can be selectively reduced to methionine in the presence of disulfide bonds by treatment with ammonium iodide in acidic media. This method has been applied for the production of RLX during its synthesis by site-directed chain combination (Figure 2) where the Met(O)^{4,25} relaxin disulfoxide was reduced effectively to native relaxin. Therefore, we treated **1b** with a tenfold molar excess of ammonium iodide in 90% TFA for 20 min at 0 °C (Figure 10). We obtained **1a** by this method in 99% purity (Figure 11(a)). Finally, we isolated the formed RLX in 78% yield after semipreparative HPLC-purification and lyophilization (Figure 11(b)). Its expected molecular mass was determined by ES-MS (Figure 11(c)). The correct orientation of the A and B chains was determined by trypsinolysis (Figure 10) and peptide mapping. After incubation with trypsin the obtained tryptic fragments were recorded and their mass was determined by LC-MS. Besides the identified fragments **21**–**23** and **25**, which have less diagnostic value we identified the fragments **24** and **26** which prove the correct orientation of the folded chains (Figure 11(d)). The correct identity of the obtained human relaxin-2 was further supported by its co-elution on HPLC with an authentic sample of recombinant human relaxin-2 (Figure 12(a)). The synthetic RLX was determined to be equipotent to the recombinantly derived relaxin-2 standard in the LGR7 relaxin receptor cAMP activation assay (Figure 12(b)).

Conclusions

An improved synthesis of human relaxin-2 in 48% total yield, calculated according to the applied B-chain, was performed based on the facile random folding of the bicyclic A-chain with the Met(O)^{25} B-chain. This or similar approaches could provide further insulin-like peptides or other heterodimeric peptides.

Acknowledgement

We thank Dr Ross Bathgate (Howard Florey Institute, University of Melbourne), for the chemical and biological comparison of synthetic with recombinant relaxin-2.

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