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Synthetic Route to Human Relaxin-2 via Iodine-Free Sequential Disulfide Bond Formation

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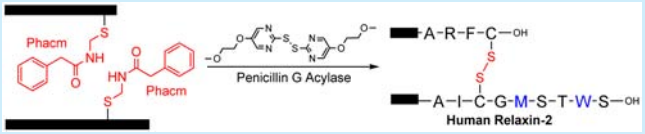
Supporting Information

ABSTRACT: A new synthetic route to human relaxin-2 has been established through a sequential disulfide bond formation process in the absence of iodine. It is enabled by a combination of cysteine protection with penicillin G acylase-labile Phacm and a newly identified thiol activator bis(5-(2-methoxyethoxy)-2-pyrimidinyl disulfide. The long-standing challenges in relaxin B-chain assembly and its poor solubility have been solved by the insertion of two isoacyl dipeptide segments. The overall yield was 25% from the B chain and 5.8% from the B-chain starting resin.



Human relaxin-2 (H2 relaxin) serves as a centrally important reproductive hormone regulating mammalian pregnancy and displays antifibrotic and vasodilatory activity.[1] As a member of the insulin superfamily, H2 relaxin shares the signature insulin structural motif that consists of two chains connected by three disulfide bonds. It possesses 24 residues in the A chain and 29 residues in the B chain (Figure 1). Two

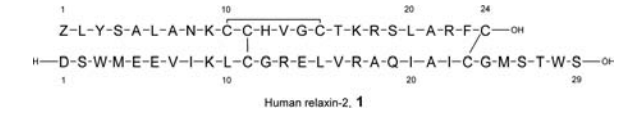
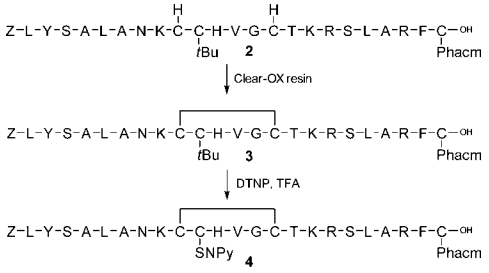


Figure 1. Human relaxin-2 sequence (Z: pyroglutamate).

general approaches have been established for the chemical synthesis in the insulin-like family of peptides, including H2 relaxin.[2] They include chain combination through sequential disulfide bond formation[3] or concerted, native protein conformational folding.[4] The former is preferred to facilitate structure−activity relationship studies among analogues that struggle to naturally fold. A limitation in the sequential approach is the customary use of iodine oxidation when applied to conversion of cysteine-Acm to cystine, as it is problematic for Met and Trp residues,[5] which both appear twice in H2 relaxin. An additional challenge in relaxin B-chain preparation is the poor physical properties that undermine its assembly and solubility.[2a−d] We report here a novel synthetic route to H2 relaxin that circumvents these limitations to provide a total yield superior to prior reports.

In a recent communication,[6] Liu et al. established a sequential route to disulfide bond formation for an insulin analogue synthesis in which iodine oxidation was replaced by a method utilizing a combination of a penicillin G-acylase (PGA) labile thiol-protecting group, Phacm,[7] and Ellman's reagent[8] (5,5′-dithiobis(2-nitrobenzoic acid), DTNB). The method appears to be independent of native structure as it was highly effective in preparation of insulin analogues with significantly reduced conformational stability. To apply this strategy to H2 relaxin, the synthesis of the A chain was started by coupling Fmoc-Cys(Phacm)-OH to NovoSyn TGA resin under Mitsunobu conditions[9] to minimize racemization. The side chains of other A-chain Cys residues were protected as trityl (Trt) at A10 and A15, and *tert*-butyl (*t*Bu) at A11. Peptide-chain assembly was performed by conventional methods, and the cleavage of the peptide from the resin was conducted with 95% trifluoroacetic acid (TFA), 2.5% H_2O, and 2.5% triisopropylsilane (TIS). The crude peptide was purified by C8 RP-HPLC to provide A-chain **2** in 23% yield (Scheme 1).

Scheme 1. Synthesis of Relaxin-2 A-Chain



The intra-A-chain disulfide bond was formed by treating A-chain **2** with Clear-OX resin[10] in aq 50% ACN at pH 6.8 for 2 h to yield A-chain **3**. The crude A-chain **3** was dried and treated with 10 equiv of 2,2′-dithiobis(5-nitropyridine) (DTNP) in TFA at rt for 4 h to remove the CysA11-*t*Bu protection and simultaneously activate the liberated thiol as *S*-nitropyridine (SNPy) to provide A-chain **4**. This one-pot S-*t*Bu to S-SNPy transformation originally established by Hondal and col-

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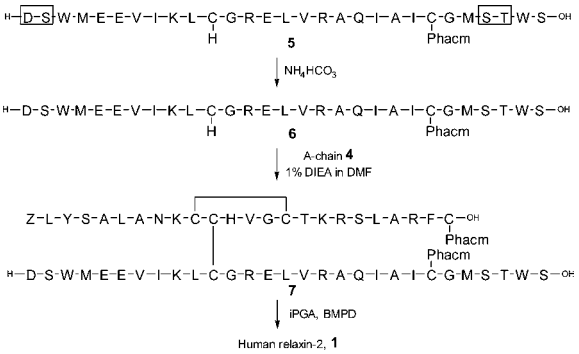
leagues[11] proved to be highly effective and applicable to the synthesis of other cystine-rich peptides.

The synthesis of the H2 relaxin B chain began with attachment of Fmoc-Ser(*t*Bu)-OH to ChemMatrix HMPB resin[12] by the symmetric anhydride method (Scheme 2). This

Scheme 2. Synthesis of Human Relaxin-2[a]



[a]The isoacyl segment is highlighted with a box.

was followed by conventional peptide chain assembly, and an analysis of the first 10 residues (B20−B29) provided successful results. The subsequent couplings of B10−B19 revealed a peptide of poor quality, and continued assembly of the entire B-chain failed to provide any meaningful product (Figure 2). The

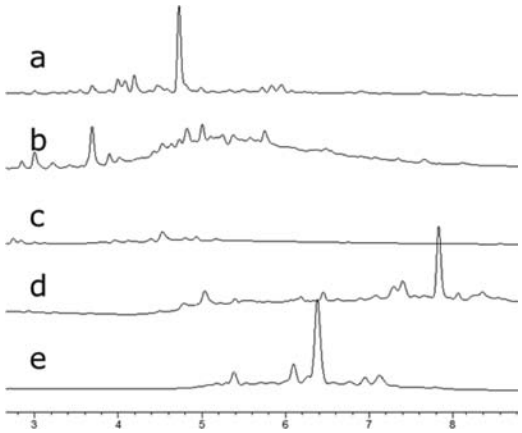


Figure 2. HPLC chromatograms of the crude products of relaxin B-chain synthesis: a, B20−29; b, B10−29; c, B1−B29 (B-chain **6**) assembled as all-amide backbone; d, B1−B29 assembled with isoacyl Ser-Thr; e, B1−B29 assembled with isoacyl Asp-Ser and Ser-Thr (B-chain **5**).

poor results were presumed to be caused by inefficient couplings and Fmoc removal in the mid-region of the sequence, consistent with prior reports.[2,4d] The crude final B-chain product had extremely poor solubility in 0.1% TFA aq ACN. The introduction of an isoacyl element has proven to be a highly effective approach to addressing such problems in hydrophobic peptides such as insulin A-chain[13] and β amlyoid 1−42.[14] To apply this approach to B-chain synthesis, an initial isoacyl dipeptide was inserted as Ser-Thr at B26−B27. It was coupled as a commercially available dipeptide building block using the standard protocol. This single substitution at the C-terminus greatly improved the quality of peptide assembly using the same coupling conditions as previously employed (Figure 2). The solubility of the isoacyl B26−B27 B chain was greatly is superior to that of the all-amide B chain but still challenging in 0.1% TFA aq ACN, and consequently, the recovery from C8 RP-HPLC chromatography was low. The final yield was disappointingly only 4%, despite a good quality peptide assembly. To further enhance the solubility, a second isoacyl dipeptide of Asp-Ser was introduced at B1−B2, and it served to significantly increase the yield of isoacyl B chain **5** after purification to 23%. An AspB1 deletion was observed in the double-isoacyl B chain when the crude peptide was exposed to acidic conditions for an extended duration. The side reaction is an acid-mediated succinic anhydride formation at the N-terminus. To mitigate this issue, once purified and lyophilized the double isoacyl B chain was immediately resuspended in 50 mM ammonium bicarbonate buffer to facilitate the quantitative O−N acyl shift and freeze-dried. This provided the all-amide B-chain **6** as a stable powder.

The formation of first interchain disulfide bond was conducted through SNPy-directed thiolysis by mixing A chain **4** and B chain **6** in 1% *N*,*N*-diisopropylethylamine (DIEA)-containing DMF for 30 min. It provided the crude heterodimer **7** after ether precipitation in a yield of nearly 90%. The heterodimer had limited solubility in phosphate buffer at pH 5−8, and solvent optimization revealed that the inclusion of 2 M guanidine promoted complete dissolution. Cosolvents such as DMSO and acetonitrile were also attempted; however, poor reactivity of iPGA subsequently resulted. The addition of Ellman's reagent (DTNB, 5 equiv) and iPGA (5.5 unit/μmol Phacm) to heterodimer **7** at pH 6.9 (2 M guanidine) smoothly produced H2 relaxin **1** as the main product; however, free A chain and B chain were also detected in significant amounts (Figure 3). The complete consumption of heterodimer **7** within

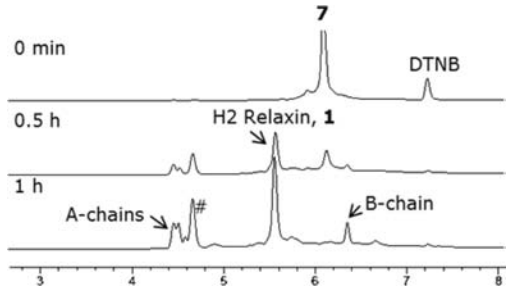


Figure 3. HPLC chromatograms of relaxin-2 synthesis by use of DTNB as the activator (#: nonpeptide species).

1 h demonstrated that the Phacm group can be efficiently removed by using 5.5 units of iPGA per μmol Phacm. The degradation of the intermediate heterodimer suggested that the capping of the iPGA-liberated free thiol by 5 equiv of DTNB was not sufficiently fast to completely suppress the intramolecular disulfide scrambling; 20 equiv of DTNB was also examined, but it led to no improvement. This was unanticipated from its application to insulin synthesis and indicates that the homologous heterodimers, despite being structurally similar, are uniquely different. Consequently, we explored a small set of thiol reagents that serve to cap and activate the enzymatically liberated thiol, which included Aldrich thiol, 6,6′-dithiodinicotinic acid, bis(5-(2-methoxyethoxy)-2-pyrimidinyl disulfide (BMPD), and dithiobis(pyridine *N*-oxide) (Figure 4). It was found that BMPD and dithiobis(pyridine *N*-oxide) as replacements for DTNB provided the best crude product profile. In a semipreparative synthesis using

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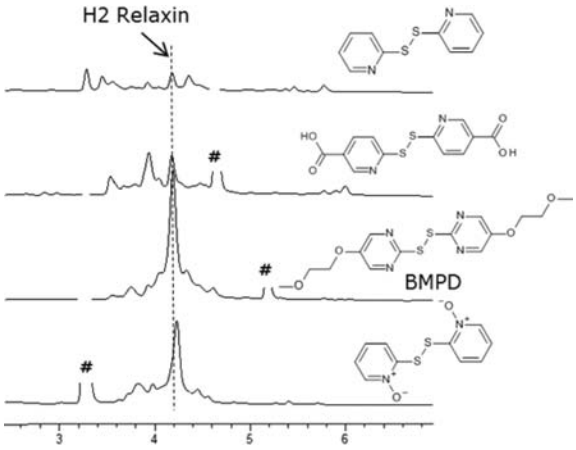

Figure 4. HPLC chromatograms in screen of thiol-activators (peaks marked with # are activators).

BMPD, H2 relaxin **1** was obtained in 25% yield (calculated from the B chain) with a single chromatographic purification after the sequential connection of two interchain disulfide bonds (Figure 5). The overall yield in H2 relaxin synthesis,

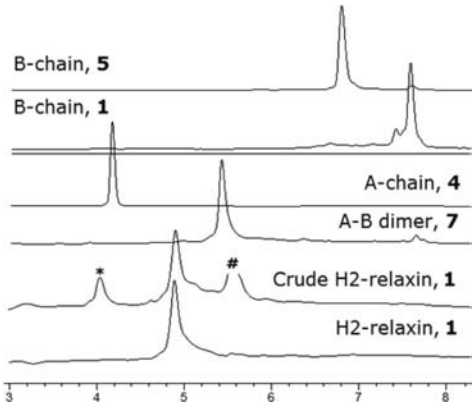

Figure 5. HPLC chromatographs obtained in preparing relaxin-2 by using BMPD. (# is BMPD, and the main portion of peak * is a nonpeptide species).

based upon the resin substitution for the synthetically challenging B chain, was 5.8%. This is the most efficient route to disulfide bond directed synthesis reported to date.

The assessment of in vitro biological activity was performed in an engineered cell overexpressing relaxin 1 receptor. Firefly luciferase reporter gene assay was designed to indirectly measure cAMP production. The potency of the synthetic peptide and native hormone control was assessed to be nearly identical (Figure S7). This indirectly confirms the integrity in disulfide bond formation as incorrectly paired isomers in the insulin-family are of much lower potency.

In conclusion, we have established a novel and efficient synthetic route to H2 relaxin highlighted by the double isoacyl-optimized B-chain synthesis and sequential iodine-free disulfide bond-formation process. This synthetic strategy dramatically enhances B-chain assembly and its physical handling as a synthetic intermediate. The absence of iodine oxidation completely avoids the potential issues well-recognized when used in the presence of Trp and Met residues. Furthermore, this work advances our previously communicated use of iPGA/Phacm while identifying BMPD as a novel and more effective synthetic reagent. The collective, successful application to relaxin broadens the conviction that this approach can be applied to other Met- and Trp-containing cysteine-rich peptides.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.6b02751.

Solid-phase synthesis of the individual chain; stepwise disulfide bond formation in solution; LC−MS spectra of the key peptides (PDF)

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Notes

The authors declare no competing financial interest.

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