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A New Strategy for Regioselective Interstrand Disulfide Bridging of Multiple Cysteine Peptides

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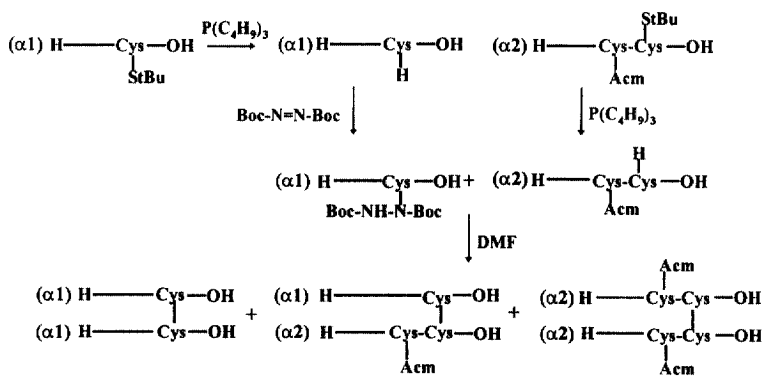
Abstract: Based on existing methods in cysteine chemistry a new strategy is described for repeating regioselective interstrand disulfide bridging of multiple cysteine peptides into heteromeric constructs crosslinked by a ladder of disulfides. The efficacy of this new approach is demonstrated by the successful assembly of three different collagenous peptides into heterotrimers in the defined α1/α2/α1′ order. © 1999 Elsevier Science Ltd. All rights reserved.

For the *de novo* design of folded proteins with multiple helix or helix/β-sheet domains, and built-in functionalities, scaffolds and templates of different chemical properties have been proposed and applied in the past years.[1,2] Thereby induction and stabilization of the secondary structure elements and their assembly into a defined three-dimensional array still remains the most challenging task. Nature often uses for such purpose more or less complex cystine networks. To mimic nature in this context we have elaborated a new synthetic strategy that allows for regioselective crossbridging of multiple polypeptide strands via disulfides into defined heteromers.

Since the pioneering work of Hiskey and Ward[3] and Kamber[4] on the regioselective cysteine pairing of two peptides to heterodimers as intermediates in the synthesis of human insulin, significant advances were achieved in the field by optimizing existing methods and developing new procedures based mainly on the activation of one cysteine residue as alkyloxycarbonylsulfenyl, arylsulfenyl or sulfenohydrazide derivative that is susceptible to nucleophilic attack by the second cysteine component to produce the unsymmetrical cystine peptides.[5,6] Efficient syntheses were reported with these regioselective disulfide bridging methods for heterodimeric cystine peptides, particularly of the insulin family of hormones.



Scheme 1. Regioselective disulfide bridging of two cysteine peptides by the sulfenohydrazide procedure.

Direct application of this existing methodology for the regioselective assembly of three different cysteine peptides (α1, α2 and α1′) into a heterotrimer proved to be exceedingly difficult. To build-up a C-terminal artificial cystine knot for crossbridging of three peptide chains, a selective chemistry had to be applied on the cysteine residues, particularly of the α2 chain, as shown in Scheme 1. Correspondingly, the thiol functions were protected orthogonally as acetamidomethyl (Acm) and *tert*-butylthio (StBu) derivatives, respectively. First

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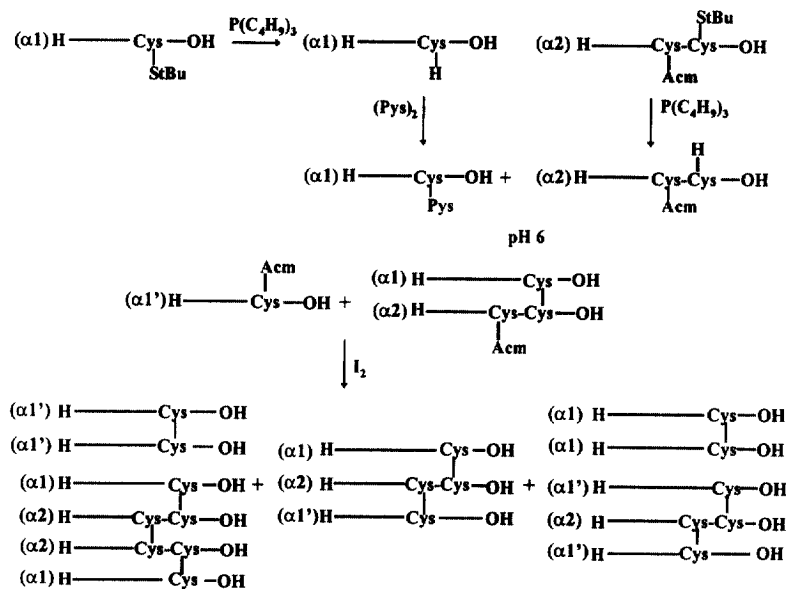
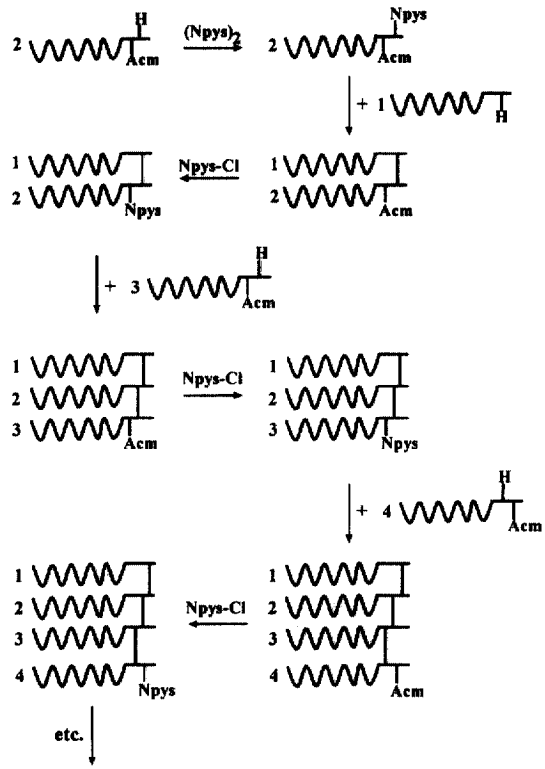
attempts to apply the sulfenohydrazide procedure[7,8] for the synthesis of the α1/α2 heterodimer (Scheme 1) failed both in terms of yields and reproducibility. Although the sulfenohydrazide derivative of the α1 chain could be isolated in homogeneous form, its reaction in DMF with the α2 chain containing a thiol-free cysteine residue and the second still protected as Acm derivative, led even under strict exclusion of air oxygen to a mixture of products that contains besides the desired heterodimer α1/α2(Acm) also the homodimers α1/α1 and α2(Acm)/α2(Acm). Since the cysteine peptides used in this study were collagenous peptides consisting mainly of Gly-Pro-Hyp repeats and thus were differing only minimally in their hydrophobic/hydrophilic properties, attempts to isolate the heterodimer α1/α2(Acm) resulted in heavy losses of material. Formation of the side products is attributed to the slightly basic conditions required for this method which, taking into account the relatively slow reaction rates of sulfenohydrazides with thiols, allow for thiol/disulfide exchange reactions to occur at significant extents. Homodimerization in the present case was additionally favoured by the known high tendency of collagenous peptides to self-association as well demonstrated by the improved yields obtained under more denaturing conditions such as chaotropic salts (e.g. 0.5 M LiCl) or higher temperature (40 °C).



Scheme 2. Regioselective disulfide bridging of two cysteine peptides into the heterodimer α1/α2, followed by random oxidation of the heterodimer and an excess of the third cysteine peptide α1′ with iodine.

As alternative procedure the α1(SH) chain was converted in 2-propanol/water (1:2) with 2-dipyridyldisulfide[9] to the pyridylsulfenyl derivative α1(Pys) which was then reacted with the α2(SH,Acm) chain in aqueous solution (pH 6.0) to produce in practically quantitative yield the heterodimer α1/α2(Acm) (Scheme 2). Subsequent cooxidation of the heterodimer and an excess of the third chain, i.e. α1′(Acm), with iodine in aqueous acetic acid[10] was expected to produce at least in statistical product distribution the heterotrimer α1/α2/α1′. Homodimerization of the excess α1′ chain and possibly at low extents even of the dimer α1/α2(Acm) was expected in this random oxidation reaction. However, besides traces of the target compound α1/α2/α1′, the homodimer $(\alpha1')_2$ as well as $(\alpha1\alpha2)_2$ are formed, but surprisingly even the homodimer $(\alpha1)_2$ and the heterotrimer α1′/α2/α1′ as well assessed by MALDI-TOF mass-spectrometric analysis. Moreover, side products deriving from reaction of the intermediately formed acetamidomethyl iodide with the hydroxyproline side chain were identified confirming previous similar observations at the level of serine and threonine residues upon I_2-mediated deprotection/oxidation of Cys(Acm) peptides.[11,12] A

disproportionation of disulfide bonds with iodine has previously been observed only in the synthesis of the human IgG1 hinge fragment in antiparallel alignment, and it was attributed to the high propensity of this portion of the IgG molecule for a parallel alignment.[6] This side reaction has to result from an attack of the intermediately formed sulfenyl iodides on disulfides or by the direct reaction of disulfides with iodine.



Scheme 3. Regioselective disulfide bridging of three or more cysteine peptides under acidic conditions to prevent scrambling of formed disulfides via thiol/disulfide exchange reactions.

These experimental results clearly revealed that for the successful crossbridging of three cysteine peptides with a cystine knot methods and experimental conditions had to be elaborated that strictly prevent undesired thiol/disulfide exchange reactions. For such purpose the synthetic route outlined in Scheme 3 was developed which allowed to considerably improve our first approach to the synthesis of heterotrimeric collagen peptides that was based on a different cysteine protection type.[13] The α2(SH,Acm) chain was converted in DMF/AcOH (95:5) with 2,2′-dithio-di-(5-nitro)pyridine (DTNP)[14] into the corresponding 5-nitropyridyl-2-sulfenyl (Npys) derivative which was then reacted in a 1.05:1 ratio with the α1(SH) chain in degassed and argon-saturated ammonium acetate buffer. Due to the better leaving-group character of the nitropyridine-2-thione if compared to pyridine-2-thione,[15,16] the reaction can be performed at pH 4.5-5.5 where a thiol/disulfide exchange reaction at the newly formed α1/α2(Acm) disulfide bridge is fully suppressed. Spectroscopic monitoring of the reaction at 420 nm confirmed quantitative formation of the heterodimer α1/α2(Acm). This was then reacted in acetic acid/trifluoroacetic acid (2:1) with freshly prepared 3-nitropyridyl-2-sulfenyl chloride[17] which in analogy to the 2-nitrophenyl-sulfenyl chloride[18] or alkyloxycarbonyl-sulfenyl chlorides[4] displaces the Acm group with concomitant formation of the activated unsymmetrical disulfide α1/α2(Npys). Thereby reaction of the sulfenyl chloride with the preformed disulfide was not observed to occur, a fact that is more supportive for a direct reaction of iodine with disulfides to induce the disproportionation observed above. Since the amino termini of

the heterodimer α1/α2(Acm) were acetylated, N^{α}-sulfenylation did not occur; but this would be prevented even in the case of unprotected peptide chains under the acidic conditions used. Finally, reaction of the S-activated heterodimer α1/α2(Npys) in ammonium acetate (pH 4.5-5.5) with α1′(SH) in a 1:1 ratio was found to proceed quantitatively with formation of the heterotrimer α1/α2/α1′. The yields of isolated materials both at the level of the intermediates and the heterotrimers were affected only by the gel-chromatographic steps required for separation of the reagents and were in the range of 70-80 % of analytically well characterized compounds. With this synthetic route protein-sized (about 10 kDa) heterotrimeric collagen-like molecules were synthesised in which the single peptide chains are aligned in a defined order to form with an one-residue shift the right-handed triple-helical coiled coil as the characteristic 3D structure of collagens.[19]

Following Scheme 3, in principle an unlimited number of cysteine-containing peptide chains that differ in sequence and conformational preferences, can be disulfide bridged in the desired order and in parallel or antiparallel alignments into suprastructures. Such artificial cystine ladders could represent an interesting alternative to templates and scaffolds for the design and production of polypeptide structures that mimick natural proteins or part of them. Even the assembly of multiple-helix bundles of transmembrane proteins should be feasible by this new approach. The sole limitation of this synthetic strategy is the high reactivity of the tryptophan side chain toward sulfenyl halides under acidic conditions,[20] although even this drawback may partly be bypassed by the use of an enzymatically cleavable thiol protection like the S-phenylacetamidomethyl derivative.[21]

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