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Conformation-Dependent Side Reactions in Interstrand-Disulfide Bridging of Trimeric Collagenous Peptides by Regioselective Cysteine Chemistry

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Abstract: Conversion of single-chain or disulfide-bridged dimeric collagenous peptides into Cys(Npys) derivatives as activated species for subsequent regioselective thiol/disulfide exchange reactions leads to side products whose origin and nature was determined by HPLC and ESI-MS. In both cases the high tendency of the educts to self-associate into triple-helical homotrimers, as assessed by their dichroic properties in the reaction media, is responsible for the failure of this well established cysteine chemistry. Only by optimizing the synthetic strategy or by exploiting a kinetic control of the reaction, could these conformation-dependent limitations be more or less efficiently bypassed for the regioselective assembly of heterotrimeric collagen model peptides crosslinked with artificial cystine knots.

Keywords: collagen peptides; heterotrimers; synthesis; cystine knot; conformation

INTRODUCTION

Despite our previous positive experience gained in the regioselective interstrand disulfide bridging of multiple cysteine peptides via the Cys(Npys) /thiol exchange reaction [1], an application of this synthetic strategy to the synthesis of heterotrimeric collagen peptides containing the 457–469 adhesion epitope of collagen type IV led to unexpected difficulties. In contrast to the design of the collagenase cleavage site of collagen type I [2], in which five (Gly-Pro-Hyp) repeats were positioned at the *N*-terminus to stabilize the triple helix, in the case of collagen type IV the adhesion epitope of both α1 and α2 chains was extended *N*- and *C*-terminally with three and two (Gly-Pro-Hyp) repeats, respectively (see Figure 1). Moreover, the amino acid composition of collagen type IV in this region would suggest a higher tendency for a triple helical fold [3,4], thus conferring to the single chains a strongly enhanced tendency to self-association [5]. Consequently, first attempts to assemble the chains into the heterotrimers A and B (Figure 1) were accompanied by low yields, mainly because of several side products and their difficult separation by chromatographic procedures. In fact, major problems were encountered during activation of the cysteine residues as *S*-nitropyridylsulphenyl derivatives, both at the level of the single α chains and of the heterodimers. Therefore, the origin and nature of the side products were analysed in detail to possibly minimize such side reactions and to improve the overall synthetic strategy. Comparatively, an additional trimer (I) was synthesized in which peptide chains consisting of solely (Gly-Pro-Hyp) repeats were *C*-terminally crosslinked by the identical cystine knot (Figure 1). Since this type of collagenous chain should exhibit an even higher propensity for self-association into a

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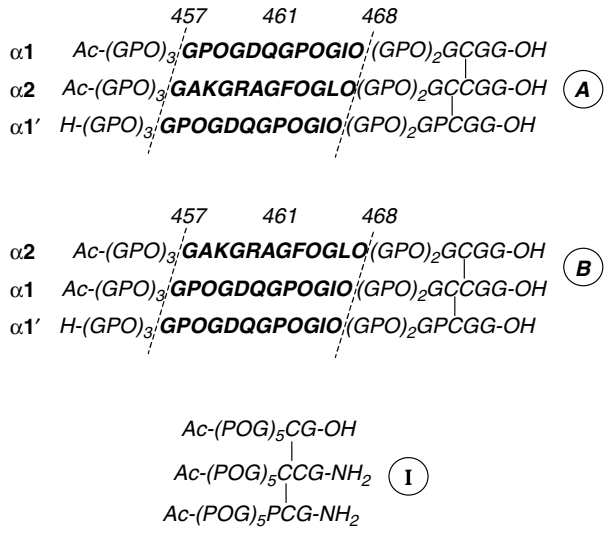

Figure 1 Synthetic heterotrimeric collagenous peptides A and B and collagenous model peptide I.

triple helix, the synthesis of this trimer was expected to confirm whether our assumption of a primarily conformation-dependent failure of the regioselective cysteine chemistry was correct. Indeed, the high propensity of these peptide chains for self-aggregation was found to markedly aggravate the synthetic difficulties. The results confirmed the conformation-dependent limitations of the regioselective thiol chemistry which were at least only partly bypassed with an exact kinetic control of the reactions involved.

MATERIALS AND METHODS

The synthesis of the α chains and their assembly into the heterotrimers A and B was described in the preceeding article [5], whereas the synthesis of the trimer I will be reported elsewhere. Analytical RP-HPLC was carried out on 125/4 Nucleosil 100-5 C_8 (Macherey & Nagel, Düren, Germany) or on 150/3.9 X-Terra MS 300-5 C_8 (Waters) columns using a linear gradient of ACN/2% H_3PO_4 from 5 : 95 to 90 : 10 in 15 min at a flow rate of 1.5 ml/min and monitoring at 210 nm. Electro spray ionization mass spectrometry (ESI-MS) was performed on a PE Sciex API 165 from Perkin-Elmer.

The CD spectra were recorded on a Jasco J-715 spectropolarimeter equipped with a thermostatted cell holder and connected to a data station for signal averaging and processing. All spectra were recorded in the range 190–250 nm, employing quartz glass cuvettes of 0.1 cm optical path length. An average of 10 scans is reported and expressed in terms of ellipticity units per mole of peptide residues ($[\theta]_R$). The measurements were performed on peptide solutions pre-equilibrated at 4 °C for at least 12 h, at a concentration of 3×10^{-5} M in trifluoroethanol. The concentrations were determined by weight and peptide content as obtained from the quantitative amino acid analysis of the peptides.

RESULTS AND DISCUSSION

Synthesis of the Cys(Npys) Activated α Chains

Attempts to convert the S-deprotected cysteine residue of the $\alpha1$ chain, i.e. $\alpha1$(SH) (A) and $\alpha1$(Acm,SH) (B) for the assembly of the trimers A and B, respectively, into the related *S*-Npys derivatives by reaction with di-(3-nitro-2-pyridine)disulfide (DTNP) were found to be accompanied by the formation of the related homodimers to a considerable extent even when taking great care to avoid air oxygen (Figure 2a, data shown only for the assembly of trimer B). Conversely, the identical reaction was found to proceed smoothly in the case of the $\alpha2$(Acm,SH) (A) and $\alpha2$(SH) (B) chains (Figure 2b, data shown only for the trimer B), and the related $\alpha2$(Acm,Npys) (A) and $\alpha2$(Npys) (B) derivatives were sufficiently homogeneous to be used directly in the thiol/disulfide exchange reaction with the $\alpha1$(SH) (A) and $\alpha1$(Acm,SH) (B) chains, respectively, for production of the corresponding heterodimers [5].

Both α chains are built up with the same number of (Gly-Pro-Hyp) repeats at the *C*- and *N*-terminal extensions; however, the $\alpha1$ chain contains in the adhesion epitope two additional, although non-consecutive, (Gly-Pro-Hyp) repeats which lead to a significantly enhanced tendency to self-association into homotrimers. In fact, the CD spectrum and the thermal denaturation curve of the $\alpha1$(StBu) (A) chain [5] and similarly of the $\alpha1$(Acm,StBu) (B) (data not shown) are fully consistent with the onset of a triple-helical structure. This process of self-association facilitates the reaction of the *S*-Npys activated $\alpha1$ chain with a second $\alpha1$(SH) chain in the aggregate, thus competing kinetically with the reaction of the thiol groups with DTNP. Consequently, significant amounts of homodimers are formed.

While for the synthesis of the trimers A and B this drawback was readily bypassed using the $\alpha2$ chains for cysteine activation, in the case of

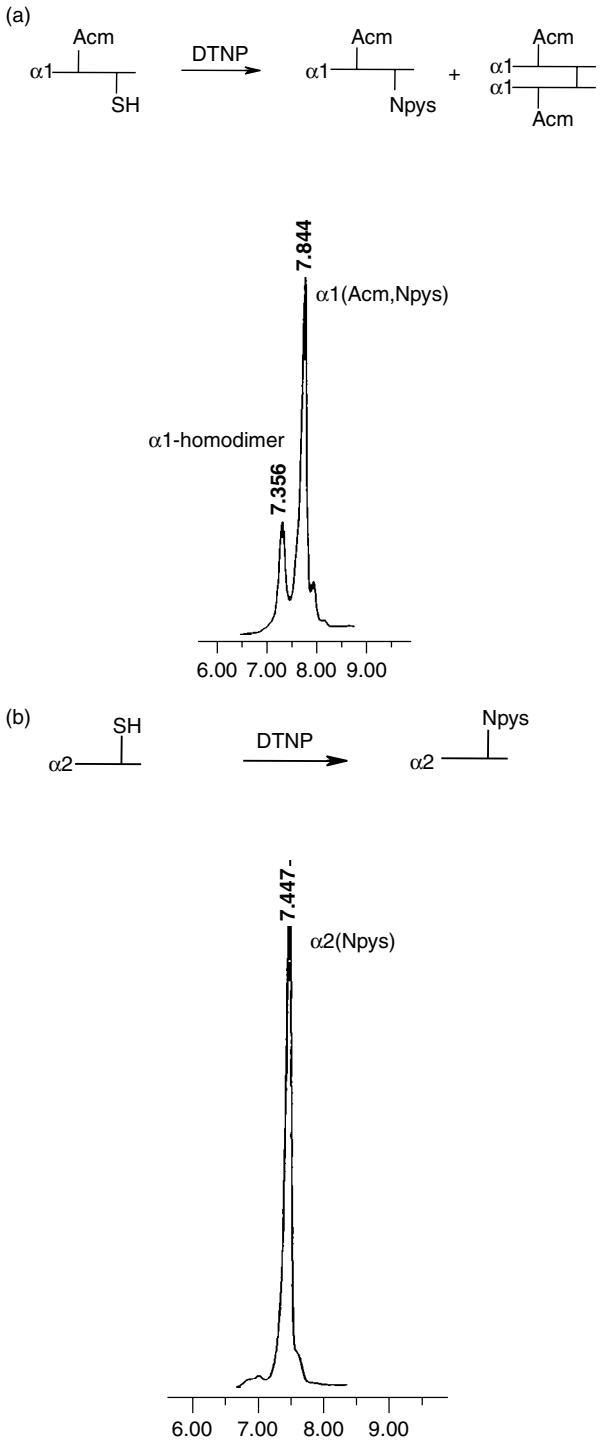

Figure 2 (a) Activation of the α1(Acm,SH) (B) chain with di-(3-nitro-2-pyridine)disulfide (DTNP). The product α1(Acm,Npys) (B) is contaminated by considerable amounts of the homodimer, as shown by the HPLC profile. (b) Activation of the α2(SH) (B) chain with di-(3-nitro-2-pyridine)disulfide (DTNP). The reaction proceed straightforwardly without formation of side products, as confirmed by the HPLC elution profile.

the trimer I high contents of homodimers were formed independently of the chains used in the Cys-activation step since these peptides are built-up exclusively by (Gly-Pro-Hyp) repeats. Again the CD spectra of the single chains were supportive of triple-helical structures (data not shown), e.g. with a T_m of 15°–17 °C for (Gly-Pro-Hyp)$_5$-containing chains. Because of the extreme difficulties encountered in the purification both by HPLC and gel filtration, the assembly of the dimer was finally successfully performed by activation of the *S*-Acm protected cysteine residue on a solid support with Npys-Cl, followed by acidic cleavage of the α(Npys) chain derivative from the resin prior to its reaction with the second α(SH) chain (details of this synthesis will be reported elsewhere).

Synthesis of the Cys(Npys) Derivatives of the Heterodimers

Once the first disulfide bridge is formed, crossbridging with the third α1'(SH) chain requires preactivation of the *S*-Acm protected cysteine residue of the heterodimer as outlined in Figure 3. For this purpose in our previous syntheses of collagen heterotrimers [1] we made use of the 3-nitropyridyl-2-sulphenyl chloride (Npys-Cl). Unexpectedly, difficulties were encountered because of non-reproducible results and the formation of side products. Thus, alternative reagents were examined such as the more reactive 5-nitropyridyl-2-sulphenyl chloride [6]. The pattern of side products obtained in this case was even more complex, suggesting as possible bypass a kinetic control. For this purpose less reactive sulphenyl halides such as the 2-nitrophenylsulphenyl chloride [7] and the 2,4-dinitrophenylsulphenyl chloride were used to displace the *S*-Acm group with concomitant formation of unsymmetric disulfides; however, both reagents failed. To gain insight into the problematic reaction with Npys-Cl, the effect of solvent, excess of reagent and reaction time was analysed with the heterodimer $\alpha 2\alpha 1$(Acm) (B). Because of the presence of reactive amino groups acidic conditions were required and thus, the use of AcOH, 5% AcOH in DMF or TFE was attempted, but without success. Only with AcOH/TFA (3 : 2) as the solvent the reaction was observed to occur, and this was attributed to the excellent solvation properties of trifluoroacetic acid which could also interfere with hydrogen-bonding patterns as present in triple helices. Indeed, the CD spectra of the heterodimers in aqueous solution were supportive of such ordered

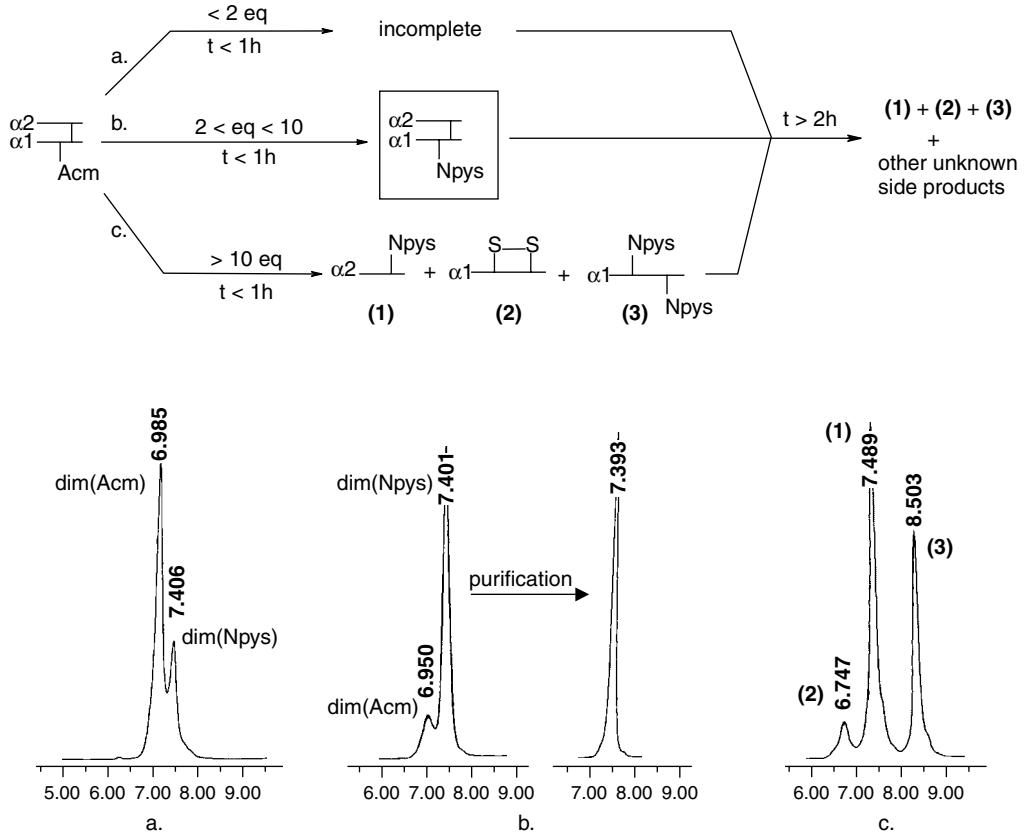

Figure 3 Reaction of the heterodimer $\alpha 2\alpha 1$(Acm) (B) in AcOH/TFA (3 : 2) with increasing excesses of Npys-Cl: (a) <2 eq, (b) 2–10 eq and (c) >10 eq, and a reaction time <1 h as monitored by HPLC. Upon longer reaction times (>2 h), additional unidentified side products are formed.

structures (*vide infra*). To uncoil ordered structures if present, even denaturing media, e.g. 8 M urea or Gdn·HCl, were considered, but the instantaneous hydrolysis of Npys-Cl in aqueous solution prevented their use.

By examining the effect of excess reagent in AcOH/TFA it was observed that more than 2 equiv Npys-Cl were required to initiate the reaction, although with a 10-fold excess at least three side products were obtained (Figure 3). These were identified by ESI-MS as the $\alpha 2$(Npys) (B), the intramolecularly disulfide bridged $\alpha 1$ (B) and the $\alpha 1$(Npys,Npys) (B). This pattern of side products can only arise from an electrophilic attack of the Npys-Cl on the disulfide with the concomitant disproportionation of the heterodimer into its components. In fact, using (Z-Cys-OH)$_2$ as the model compound and performing the reaction with Npys-Cl under identical conditions, the Z-Cys(Npys)-OH derivative was found to be produced at detectable extents, as assessed by LC-MS. Monitoring by HPLC the reaction of the heterodimer $\alpha 2\alpha 1$(Acm) (B) with excess Npys-Cl as a function of time clearly revealed that in addition to the desired product $\alpha 2\alpha 1$(Npys) (B), the side products $\alpha 2$(Npys) (B) and the disulfide bridged $\alpha 1$ monomer were the first to be formed. The latter was then converted to the $\alpha 1$(Npys,Npys) (B) which again must result from an attack of the Npys-Cl on the intramolecular disulfide. This reaction may derive from the constraints of the disulfide bridge between adjacent cysteine residues. Indeed, it is known that such bridging is difficult to achieve as it requires *trans*→*cis* isomerization of the Cys-Cys peptide bond [8] and thus even an opening of the ring may be facilitated.

By optimizing the kinetic control of these side reactions the desired $\alpha 2\alpha 1$(Npys) (B) derivative could finally be obtained as the major product for the successive assembly of the heterotrimer B. Likewise with this procedure the $\alpha 1\alpha 2$(Npys) (A) derivative was produced for the subsequent assembly of the heterotrimer A [5].

Conversely, such optimization trials failed in the case of trimer I, for which the protection scheme had to be changed by replacing the Acm group with Trt. This allowed the production of the $\alpha 1\alpha 2$(SH) by acid treatment and its subsequent reaction with the $\alpha 1'$(Npys) chain for the assembly of the trimer (details of this synthesis are reported elsewhere).

Conformational Properties of the Heterodimers

As reported in the preceeding article [5], the single $\alpha 1$ chains of the trimers A and B exhibit dichroic properties supportive of a triple-helical conformation in aqueous solution; most surprisingly even the heterodimers, e.g. $\alpha 1\alpha 2$(Acm) (A) are folded into triple helices in aqueous solution as well evidenced by the Rpn value of 0.093 and a cooperative thermal transition with a T_m of 33 °C (Table 1). Such self-association is consistent only with an assembly of the stretched dimers into homotrimers, a fact that would foresee an interruption of the triple helix at the disulfide juncture and consequently, a bulging out of this part of the peptide chain. The low reactivity of the dimers toward Npys-Cl suggests that this type of aggregation would have to sequester in particular the Cys(Acm) residue into buried positions to account for the sterically hindered access. A very low reactivity of this residue was observed in AcOH or TFE. CD spectra in AcOH used to analyse whether such homotrimers are formed in this solvent, could not be recorded because of its strong absorbance in the far UV. However, AcOH is routinely used to solubilize native collagen for biochemical studies and thus should not be of particular harm to the native triple-helical structure of collagen. Alcohols such as MeOH, EtOH, ethylene glycol, glycerol and several others are commonly used for studies of the conformational properties of collagen and collagen model peptides [9,10]. Thereby it was observed that, in contrast to monohydric alcohols, the addition of polyols enhances the melting temperature of collagen [11,12], with the hydroxyalkyl chain length of polyols playing a decisive role in stabilizing the triple-helical structure. Consequently, TFE as a monohydric alcohol should exert marginal effects on the triple helix, although direct evidence to our knowledge has not been reported in the literature. On the other hand, it is well established that TFE disrupts coiled coils [13,14]. In order to investigate the effect of TFE on the triple-helical structure and to account for the non-reactivity of the heterodimers with Npys-Cl in this solvent, the dichroic properties of the collagenous peptides were determined and the related parameters are reported in Table 1. The dichroic properties of the $\alpha 1\alpha 2$(Acm) (A) heterodimer are fully consistent with a triple-helical structure and very similar to the values obtained in aqueous solution (see Table 1). The lack of effects of TFE on the triple-helical conformation is further supported by the CD parameters of the heterotrimer A which are almost identical to those determined for the same compound in aqueous buffer (Table 1). Conversely, a destabilizing effect is noted for the $\alpha 1$(StBu) (A) chain whose dichroic properties are more consistent with a poly(Pro)-II like conformation with a Rpn value of 0.069 (Table 1). On the contrary, the same peptide chain in physiological buffer and at the same concentration was found to be folded as a homotrimer into a triple-helical structure, with a Rpn value of 0.116 [5].

These spectroscopic data confirm that conformational aspects account for the lack of reactivity of the heterodimers and that only with the strongly protonating and thus solvating properties of TFA, is

Table 1 CD Parameters of Collagenous Peptides Containing the Adhesion Epitope 457–469 of Collagen Type IV at 3×10^{-5} M Concentration in TFE and in the Case of the Dimer $\alpha 1\alpha 2$(Acm) (A), for Comparison, also in 50 mM Tris·HCl, 50 mM NaCl, 10 mM $CaCl_2\cdot 2H_2O$ (pH 7.4); the CD spectra were recorded at 4 °C, after 12 h Pre-equilibration at the Same Temperature

Peptide	max (nm; θ_R)	min (nm; θ_R)	Rpn
$\alpha 1\alpha 2$(Acm) (A) in aqueous buffer	224; 1640	199; −17659	0.093
$\alpha 1$(StBu) (A) in TFE	223; 1686	198; −24514	0.069
$\alpha 1\alpha 2$(Acm) (A) in TFE	223; 3133	199; −32360	0.097
Heterotrimer A in aqueous buffer [5]	224; 3831	198; −34317	0.112
Heterotrimer A in TFE	224; 3464	198; −30299	0.114

the triple helix disrupted to an extent that allows for access of reagents to the Cys(Acm) residue.

CONCLUSION

In recent years great advances have been made in the synthesis of multiple cystine peptides, and in this context the activation of single suitably protected cysteine residues by reaction with Npys-Cl represents a preferred procedure [15]. The present study, however, clearly revealed the limitations of the regioselective cysteine chemistry that may derive from sterically hindered accesses resulting from preferred conformational states even in non-aqueous media.

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REFERENCES

1. Ottl J, Moroder L. A new strategy for regioselective interstrand disulfide bridging of multiple cysteine peptides. *Tetrahedron Lett.* 1999; **40**: 1487–1490.
2. Ottl J, Moroder L. Disulfide-bridged heterotrimeric collagen peptides containing the collagenase cleavage site of collagen type I. Synthesis and conformational properties. *J. Am. Chem. Soc.* 1999; **121**: 653–661.
3. Kramer RZ, Bella J, Mayville P, Brodsky B, Berman HM. Sequence dependent conformational variations of collagen triple-helical structure. *Nature Struc. Biol.* 1999; **6**: 454–457.
4. Persikov AV, Ramshaw JAM, Kirkpatrick A, Brodsky B. Amino acid propensities for the collagen triple-helix. *Biochemistry* 2000; **39**: 14960–14967.
5. Saccà B, Moroder L. Synthesis of heterotrimeric collagen peptides containing the $\alpha 1\beta 1$ integrin recognition site of collagen type IV. *J. Peptide Sci.* preceeding article.
6. Rabanal F, DeGrado WF, Dutton PL. Use of 2,2′-dithiobis(5-nitropyridine) for the heterodimerization of cysteine containing peptides. Introduction of the 5-nitro-2 pyridinesulphenyl group. *Tetrahedron Lett.* 1996; **37**: 1347–1350.
7. Moroder L, Marchiori F, Borin G, Scoffone E. Studies on cytochrome-c. 2. Synthesis of protected heptapeptide (sequence 17-23) of bakers-yeast iso-1-cytochrome-c. *Biopolymers* 1973; **12**: 493–505.
8. Sukumaran DK, Prorok M, Lawrence DS. A molecular constraint that generates a cis peptide bond. *J. Am. Chem. Soc.* 1991; **113**: 706–707.
9. Feng Y, Melacini G, Taulane JP, Goodman M. Acetyl-terminated and template-assembled collagen-based polypeptides composed of Gly-Pro-Hyp sequences. I. Synthesis and conformational analysis by circular dichroism, ultraviolet, absorbance, and optical rotation. *J. Am. Chem. Soc.* 1996; **118**: 10351–10358.
10. Engel J, Chen HT, Prockop DJ, Klump H. The triple helix in equilibrium with coil conversion of collagen-like polytripeptides in aqueous and nonaqueous solvents. Comparison of the thermodynamic parameters and the binding of water to $(L\text{-Pro-L-Pro-Gly})_n$ and $(L\text{-Pro-L-Hyp-Gly})_n$. *Biopolymers* 1977; **16**: 601–622.
11. Gekko K, Koga S. Increased thermal stability of collagen in the presence of sugars and polyols. *J. Biochem.* 1983; **94**: 199–205.
12. Harrap BS. The effect of aliphatic alcohols on the thermal stability of tropocollagen under acidic conditions. *Int. J. Protein Res.* 1969; **1**: 245–252.
13. Fermandjian S, Maroun RS, Amekraz B, Jankowski CK. Self-association of an amphipathic helix peptide inhibitor of HIV-1 integrase assessed by electro spray ionization mass spectrometry in trifluoroethanol/water mixtures. *Rapid Comm. Mass Spectrometry* 2001; **15**: 320–324.
14. Monera OD, Zhou NE, Kay CM, Hodges RS. Comparison of antiparallel and parallel two-stranded α-helical coiled-coils. Design, synthesis, and characterization. *J. Biol. Chem.* 1993; **268**: 19218–19227.
15. Andreu D, Albericio F, Solé NA, Munson MC, Ferrer M, Barany G. Formation of disulfide bonds in synthetic peptides and proteins. *Meth. Mol. Biol.* 1994; **35**: 91–169.

