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Cyanogen Iodide: A New Reagent for Disulfide Bond Formation in Peptides

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Abstract: Three cyclic disulfides were formed from linear analogs of calcitonin, epidermal growth factor (EGF), and a fragment of the DNA-binding Cro protein using a new reagent: cyanogen iodide (ICN). Each peptide contained two S-protected cysteine derivatives [Cys(Acm)] which were deprotected with concurrent disulfide bond formation. A comparison with I_2, a common reagent for this reaction, is included.

Disulfide bonds between cysteine residues are one of the most important means for stabilizing protein and peptide conformations.[1] Many peptide hormones contain disulfides; disulfide-linked, cyclic peptide analogs with a well-defined, comparatively rigid conformation have been used for studying structure-activity relationships and conformational properties of peptides.[2] Due to the complex functionality and reactivity of the individual amino acid sidechains it is important to have a range of reagents available for disulfide bond formation. Despite their importance, synthetic methods for disulfide bond formation in peptides are still very limited.[3]

In this paper we report a new reagent, ICN, for forming intramolecular disulfide bonds in three peptide analogs based on calcitonin (**1**), EGF (**2**), and a fragment of the DNA-binding Cro protein (**3**) in which each peptide contained two S-acetamidomethyl protected cysteine derivatives [Cys(Acm)]. These peptides were used to study the ease of formation of macrocyclic disulfides of various sizes. Additionally peptide **1** was used to test if ICN would react with the methionine sidechain and cause chain cleavage as is observed with cyanogen bromide.[4] ICN is highly soluble in a mixed aqueous-organic solvent system and as such is a convenient source of I^+ in solution.

NH_2-Ala-Cys(Acm)-Gly-Asn-Leu-Ser-Thr-Cys(Acm)-Met-Ala-OH (1)

AcNH-Asn-Cys(Acm)-Val-Val-Gly-Tyr-Ile-Gly-Glu-Arg-Cys(Acm)-Gln-Tyr-Arg-Asp-Leu-NH_2 (2)

AcNH-Gln-Thr-Lys-Thr-Cys(Acm)-Lys-Asp-Leu-Gly-Val-Tyr-Gln-Ser-Ala-Cys(Acm)-Asn-Lys-Ala-Ile-His-Ala-NH_2 (3)

The Cys-protected peptide analogs were prepared by solid phase methods on the *p*-alkoxybenzylalcohol resin (for the free carboxy terminus)[5] or the Rink resin (for a primary amide on the carboxy terminus)[6] using a conventional Fmoc-based strategy.[7] Removal of the Acm protecting groups and oxidation to the disulfide was accomplished in one step (Figure 1).[3c]

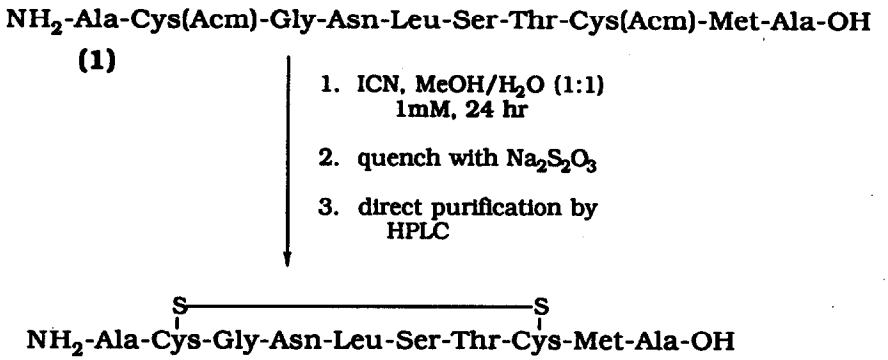

Figure 1. Deprotection and Disulfide Bond Formation with Calcitonin Analog 1

Peptides **1**, **2** and **3** were reacted with I_2 and ICN under a variety of conditions at high dilution, and intramolecular disulfide bond formation was monitored by HPLC (Table 1). All reported yields are isolated yields after preparative HPLC.[8]

Table 1. Conditions and Yields for Intramolecular Disulfide Bond Formation

Peptide	Conc.[a]	Reagent	Eq.	Solvent[b]	Time	Yield[c]
1	1mM	I_2	8	MeOH/H_2O	12 hr	15%
1	1mM	I_2	8	MeOH/H_2O	24 hr	39%
1	1mM	I_2	8	MeOH/H_2O	48 hr	26%
1	1mM	ICN	50	MeOH/H_2O	24 hr	41%
1	1mM	ICN	50	MeOH/H_2O	48 hr	48%
2	1mM	I_2	8	MeOH/H_2O	24 hr	16%
2	1mM	ICN	50	MeOH/H_2O	24 hr	69%
2	1mM	ICN	25	MeOH/H_2O	30 hr	N.R.
3	0.05mM	I_2	8	TFE/H_2O	9 hr	0%
3	0.05mM	ICN	80	TFE/H_2O	48 hr	14%

[a]Final concentration of peptides.
[b]1:1 mixture of organic solvent in H_2O.
[c]Isolated yields of peptides after preparative HPLC.

In a typical ICN reaction the peptide (2.5 mmol) was dissolved in a 1:1 mixture of MeOH/H_2O (2.5 ml for the 1 mM reaction conditions). The reaction vessel was covered with aluminum foil and ICN (0.124 mmol) was added to this solution as a solid.[9] The reaction was monitored by analytical HPLC until complete and was quenched with a 0.1 M solution of sodium thiosulfate. The reaction mixture was purified directly by preparative

HPLC.[8,10] With peptides 1 and 2 the reactions with I_2 and ICN were both complete after 24 hrs. If the reactions were allowed to progress to 48 hrs there was a slight increase in the cyclic disulfide yields with ICN, whereas yields with I_2 dropped dramatically (Figure 2).

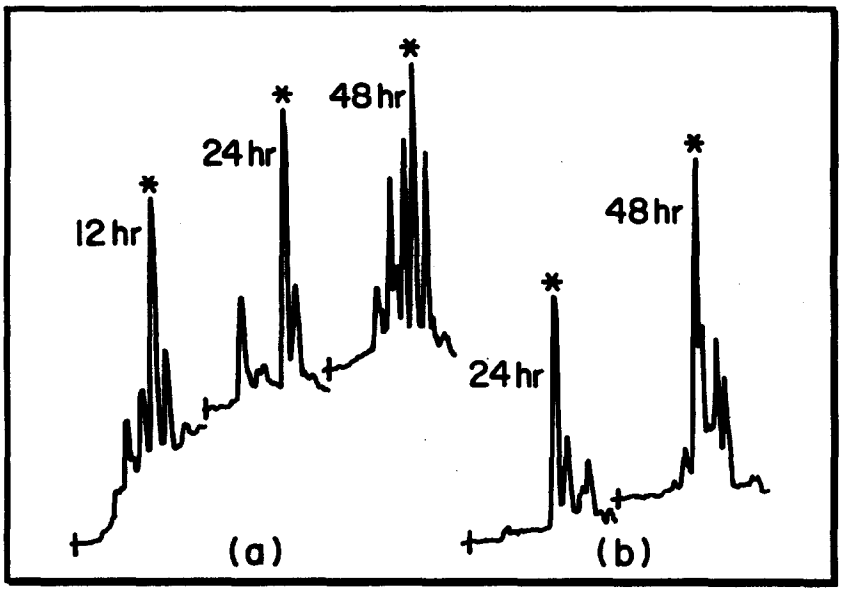

Figure 2. Time course of the reaction of calcitonin analog 1 with a) I_2 and b) ICN as monitored by HPLC.[8] The product disulfide is marked with an asterisk.

Although the isolated yields of the cyclic products was very sequence dependent, all of the peptides studied under equivalent conditions gave higher yields of cyclic disulfides with ICN than I_2. The reactions with ICN were also less prone to product decomposition with extended reaction times. No cleavage of peptide 1 at methionine was detected under any reaction conditions with ICN. With peptide 3 a very dilute solution was used due to the tendency of this peptide to aggregate at concentrations greater than 50 μM. In this case only a complex mixture of unidentifiable products was obtained with I_2 which contained none of the desired cyclic disulfide, whereas ICN gave a very modest yield of the desired material. ICN is also more convenient to use in a variety of solvent systems as it is very soluble in MeOH, TFE and mixed aqueous-organic systems, whereas I_2 is only sparingly soluble in TFE and the mixed solvent systems.

In all ICN is a better reagent for intramolecular disulfide bond formation in peptides than I_2 due to its higher yields, much slower product decomposition, and high solubility in a variety of solvents, and as such it is an important addition to the range of reagents available for disulfide bond formation.[11]

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Abbreviations: Acm = acetamidomethyl, Ac = acetyl, EGF = epidermal growth factor, TFA = trifluoroacetic acid, TFE = trifluoroethanol

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8. HPLC conditions: All reactions were monitored and purified by HPLC using Vydac-C_8 reverse phase columns (analytical - 1 x 25 cm, preparative - 2.2 x 25 cm) with mobile phase A (100% CH_3CN/0.1% TFA) and mobile phase B (100% H_2O/0.1% TFA) with linear gradients (5-60% A for peptide **1**, 5-70% A for peptide **2** and 15-40% A for peptide **3**) of 30 min (analytical) or 60 min (preparative).

9. Methods for using iodine: a) Reactions in TFE - peptides were dissolved in 1:1 TFE/H_2O and I_2 was added via a 1M stock solution in MeOH. b) Reactions in MeOH - equal volumes of a 16 mM I_2 solution in MeOH and a 2 mM peptide solution in H_2O were added together.

10. All HPLC purified products were identified by mass spectroscopy (FAB, glycerol matrix).

11. ICN mirrored the reactivity of I_2 with respect to the removal of sulfhydryl protecting groups and disulfide formation with a series of cysteine derivatives. Acm and trityl were the most reactive, and benzyl, *t*-butyl and methoxybenzyl showed low reactivity.

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