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Simultaneous Cyclization and Derivatization of Peptides Using Cyclopentenediones

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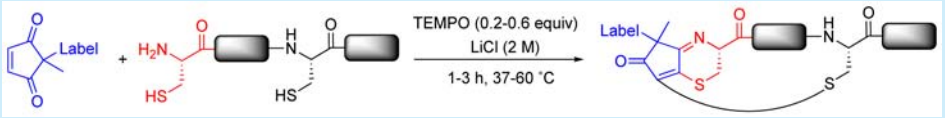
Supporting Information



ABSTRACT: Unprotected linear peptides containing *N*-terminal cysteines and another cysteine residue can be simultaneously cyclized and derivatized using 2,2-disubstituted cyclopentenediones. High yields of cyclic peptide conjugates may be obtained in short reaction times using only a slight excess of the cyclopentenedione moiety under TEMPO catalysis and in the presence of LiCl.

Cyclic peptides have found wide application in the biological and medicinal fields. These compounds have been investigated to inhibit amyloid fibrillation,[1] as antibacterial[2] and antifungal[3] agents, in the treatment of tumors[4] and to regulate protein−protein interactions through epitope mimicking,[5] among others.

Further functionalization of cyclic peptides can ameliorate their properties or those of the molecule to which they are attached. For example, conjugation to proteins[6] may enhance protein transduction, and conjugation to fluorophores[7] is important to obtain traceable systems. As for cyclic RGD peptides, which are widely utilized to selectively target some cancer cells, they need to be conjugated to drugs, other biomolecules, or radiolabels to be used in cancer therapy and diagnosis.[8] For these reasons, methodologies allowing for the simultaneous cyclization and derivatization of peptides are of great interest, and in the past decade different groups have conducted research in this area.[9]

We have recently described that 2,2-disubstituted cyclopent-4-ene-1,3-diones (CPDs) selectively react with *N*-terminal cysteines in the presence of other cysteines.[10] After formation of the Michael-type adduct (with a mass of **M** Da), intramolecular imine formation furnishes an intermediate (**M**-18 Da adduct), which is subsequently oxidized (likely by oxygen) to generate a conjugated structure with a mass of **M**-20 Da that exhibits a UV maximum at 330 nm (Scheme 1).

Since the **M**-20 conjugated adducts may undergo a second nucleophilic addition, we decided to explore the possibility of cyclizing peptides through reaction with another cysteine in the same peptide chain (Scheme 2a).

This manuscript describes different steps of the optimization of the cyclization reaction, which may provide labeled cyclic peptides in cases where suitably derivatized CPDs are used. Parameters affecting the reaction outcome and kinetics have been optimized, and product stability has been examined. In all cases formation of a cyclic molecule was confirmed by mass spectrometry and UV spectroscopy, and NMR proof was obtained for one peptide. The **M**-20 cyclic precursor (Scheme 2a) was occasionally detected (HPLC-MS).

Scheme 1. Outcome of the Reaction between CPDs and *N*-Terminal Cysteines

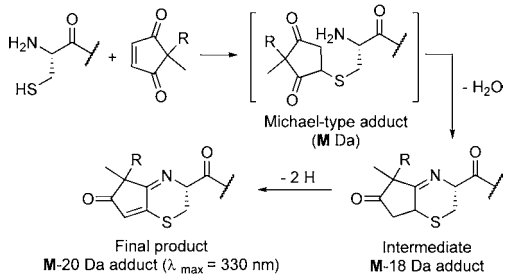


Figure 1 assembles the structures of the CPDs and peptides utilized in the different cyclization reactions. In addition to model compounds such as peptides **1**−**4** and CPD **a**, CPDs carrying biotin and dansyl labeling moieties (**b** and **c**, respectively), the linear precursors of oxytocin[11] (**5**) and a bombesin agonist[12] (**6**), and epimeric peptides **7**−**9** (see below for an explanation) and **10** were used.

In preliminary experiments, aqueous solutions of CPD **a** and peptides **1** or **2** were heated (60 °C), and formation of the two expected diastereomeric cyclic adducts (differing in the configuration of C2 in the CPD ring) was confirmed by the appearance of two peaks with a mass of **M**-22 Da (HPLC-MS analysis) and a UV maximum around 375 nm (see Scheme 2a). However, both crudes, and especially that of **1a**, were rather

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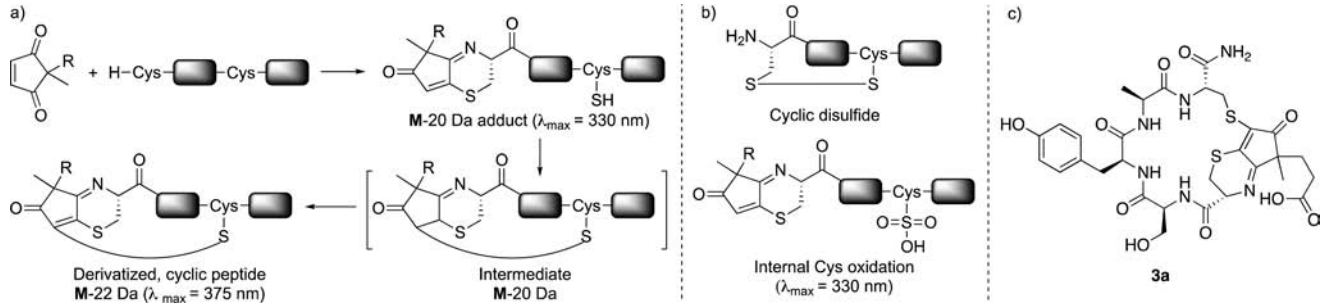
Scheme 2. (a) Different Steps in the Intramolecular Cyclization Reaction;[a] (b) Major Side Products Formed;[b] (c) Cyclic Peptide 3a



[a]**M** is the mass of the Michael-type adduct that would be generated by a simple CPD-thiol addition; see Scheme 1. R may incorporate a labeling moiety; see Figure 1. [b]Oligomers were not observed, probably because of the high dilution reaction conditions.

complex, which evidenced that cyclization conditions had to be fine-tuned.

In an attempt to accelerate the reaction, **1** and **a** were reacted under conditions enhancing the nucleophilicity of thiol groups, at pH = 8 (phosphate buffer) and in the presence of 1−2 equiv of different bases (NaOH, Et_3N). Yet, these experiments rendered complex crudes, with the undesired disulfide as the main product (Scheme 2b).

Prompted by the fact that some thiol-involving conjugate additions are radical reactions,[13] the addition of 2,2,6,6-tetramethyl-1-piperidinyloxy (TEMPO) and O_2 (balloon) was assayed. TEMPO provided better results than O_2, although formation of the cyclic disulfide and the product with the internal cysteine oxidized (see Scheme 2b) were observed as competing side reactions. Therefore, the protocol for TEMPO addition (amount and timing) together with peptide concentration, CPD excess, and reaction temperature were adjusted to reduce the amount of byproducts.

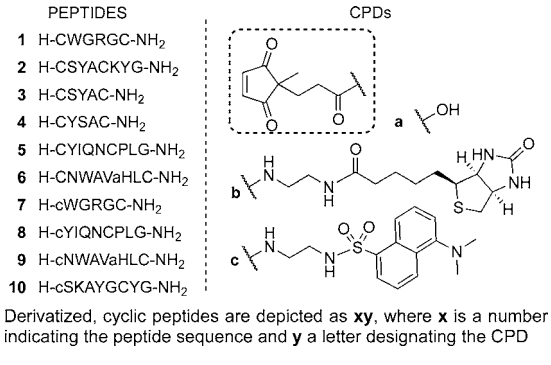


Figure 1. Structures of the different peptides and CPDs used in this work. Lower case letters describe D-amino acids.

With what were optimal conditions at this stage (protocol 1, Supporting Information (SI), section 4) cyclic peptides **1a** and **2a** could be satisfactorily obtained employing only 0.4 equiv of TEMPO (80−85% HPLC-based yield; see entries 1 and 2 of Table 1). In parallel with these experiments, the **3** + **a** reaction was carried out at a higher scale (SI, section 5). Both diastereomers of cyclic peptide **3a** (Scheme 2c, **M**-22 Da products) were isolated by HPLC and fully characterized by NMR. HMBC experiments allowed the correlation between the olefin carbons of the CPD ring and the β-hydrogens of both the *C*- and *N*-terminal cysteines to be observed, confirming that cyclization had taken place.

The conjugated system generated upon cyclization was found to contribute to the peptide absorbance at 280 nm (UV-based yields exceeded 100%), which ruled out quantification of the cyclic conjugates from the absorbance of Tyr and/or Trp.[14] To allow for amino acid independent cyclic peptide quantification, the molar absorption coefficient of the 4,5-disubstituted CPD ring around 375 nm was determined (12 215 M^{-1} cm^{-1} at 373 nm in water, and 12 645 M^{-1} cm^{-1} at 365 nm in methanol) from the UV spectra of solutions of **3a** (quantified by NMR using 1,4-dioxane as the internal standard[15]).

Table 1. Reaction Times and Yields for the Cyclizations Carried out Using the First Set of Fine-Tuned Conditions[a]

entry	reaction[a]	product	time (h)[b]	yield (%)[c]
1	**1** + **a**	**1a**	2.75	81
2	**2** + **a**	**2a**	2.25	84
3	**1** + **b**	**1b**	5.5	64
4	**1** + **c**	**1c**	6.5	49
5	**5** + **a**	**5a**	4[d]	<30

[a]Cyclization reactions were carried out using protocol 1 (SI, section 4). [b]Time needed for the complete disappearance of the corresponding **M**-20 Da adduct (the **M**-22 Da cyclic peptide is then completely formed). [c]HPLC-based cyclization yield at that time (280 nm). Products were not isolated. [d]A large amount of side products was observed (SI, section 7).

At that point, more challenging reactions such as those involving CPDs **b** and **c** and the linear precursor of oxytocin (**5**) were carried out (Table 1, entries 3−5).

Use of CPD **a** to cyclize different peptides (**1**, **2**, and **5**, entries 1, 2, and 5 in Table 1) showed that the smallest cycle (19-membered ring, **2** + **a** reaction) was the one formed more rapidly. Yet, reaction **1** + **a** was faster than **5** + **a**, which indicates that peptide sequence, in addition to ring size (both reactions afford 22-membered rings in this case), influences the kinetics of the cyclization. Peptides **3** and **4**, which only differ in the amino acid sequence, were also reacted with CPD **a** (see SI, section 8). **3** cyclized more rapidly than **4**, which may suggest that aromatic amino acids adjacent to the *N*-terminal Cys have a negative impact on the cyclization rate.

Reaction rates were also affected by the substituent at the 2 position of the CPD, as shown by the experiments in which peptide **1** was reacted with the three CPDs (**a**, **b**, and **c**; cf. entries 1, 3, and 4 in Table 1). These results suggest that large substituents have a negative impact on cyclization, but this

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Table 2. Reaction Yields for the Cyclization and Derivatization Reactions Carried out Using the Finally Optimized Procedure, and Stability of the Isolated Products to Bases

Peptides containing L-Cys at the *N*-terminal position						Peptides containing D-Cys at the *N*-terminal position					
				nonepimerized product (%)[c]						nonepimerized product (%)[c]	
entry	reaction[a]	product	yield (%)[b]	isomer 1	isomer 2	entry	reaction[a]	product	yield (%)[b]	isomer 1	isomer 2
1	**1** + **b**	**1b**	76/34	64	83	8	**7** + **b**	**7b**	76/40	95	93
2	**1** + **c**	**1c**	75/37	n.d.	n.d.	9	**7** + **c**	**7c**	66/40	n.d.	n.d.
3	**5** + **a**	**5a**	80	87	6	10	**8** + **a**	**8a**	72	94	99
4	**5** + **b**	**5b**	65	n.d.	n.d.	11	**8** + **b**	**8b**	77/33	n.d.	n.d.
5	**5** + **c**	**5c**	84/33	n.d.	n.d.	12	**8** + **c**	**8c**	82/45	n.d.	n.d.
6	**6** + **a**	**6a**	36[d]	45	59	13	**9** + **a**	**9a**	63	54	40
7	**6** + **b**	**6b**	25[d]	n.d.[e]	n.d.[e]	14	**9** + **b**	**9b**[f]	74/22	100	36
						15	**10** + **a**	**10a**	68/47[g]	n.d.	n.d.

[a]Cyclization reactions were carried out using protocol 2 (SI, section 4; 2 h reaction time). Water was used as solvent except in experiments corresponding to entries 2, 4, 5, 9, and 12, where a 1:1 MeOH/H_2O mixture was employed. [b]HPLC-based cyclization yield after 2 h (280 nm)/Isolated yield (after HPLC purification, SI section 11). [c]Stability determined at pH 7.4 after 12 h of incubation (n.d. = not determined). [d]Several side reactions took place upon cyclization of peptide **6**. [e]These products could not be isolated due to the complexity of the crude. [f]Isomer 2 of product **9b** was isolated as a mixture of epimers at the *N*-terminal Cys. [g]Reaction time was 3.5 h in this case (total amount of TEMPO: 1.4 equiv).

trend is not reproduced by results assembled in Table 2 (see below, entries 3−5 and 10−12).

In all cases, longer reaction times correlated with lower cyclization yields, as slower rates cause longer exposure of the internal thiol to the free radical TEMPO, which favors oxidation to sulfonic acid.

To overcome this problem, the effect of a chaotropic agent such as LiCl in the reaction mixture was evaluated. Here, the troublesome **5** + **a** reaction was selected as model system. Different amounts of LiCl were added to the reaction mixture, and the conversion of the **M**-20 Da adduct to the cyclic product was studied (see SI, section 9). The positive effect of LiCl was demonstrated, and a clear correlation between the amount of LiCl and the cyclization rate could be observed. Although the best results were obtained with the highest salt content, a 2 M LiCl concentration was deemed the best option to accelerate the reaction while reducing possible elution problems during HPLC purification. The cyclization protocol was therefore reoptimized in the presence of LiCl. This resulted in an improved procedure (protocol 2, section 4 of the SI) in which peptide, CPD, and TEMPO (1:1.5:0.2 molar ratio) are incubated at 60 °C[16] in 2 M aq. LiCl for 1 h. 0.2 equiv of TEMPO is then added, and every 30 min until completion of the reaction (as assessed by HPLC). Cyclizations normally take place in less than 2 h. Solvent composition (water or water/methanol mixtures) has to be adjusted for each CPD on the basis of its solubility.

Protocol 2 was then used to synthesize different cyclic peptides, generally with satisfactory results (Table 2; in the **10** + **a** reaction a longer reaction time was required to attain a satisfactory cyclization yield). Yet, a large amount of contaminants with the same mass and UV profile but different retention times was found to accompany purified compounds **1b**, **1c**, **5b**, and **5c** (and other byproducts, in the case of **5b**). Revision of the isolation procedure revealed that these peptides had been lyophilized at the same time as NH_3- or Et_3N-containing solutions. We surmised that these impurities resulted from epimerization of the *N*-terminal cysteine, which is likely favored by the high resonance stabilization of the intermediate anion (see SI, page S2). Studies carried out with peptide **1b** showed that the cyclic peptides remained stable while in the reaction crude at room temperature, at acidic pH, and in water or aqueous organic solvents, but not under basic conditions or after prolonged heating.

To confirm the cysteine epimerization hypothesis, the two diastereomers of cyclic peptides **1b** and **7b** (**1** and **7** differ in the configuration of the *N*-terminal Cys) were isolated. Next, each isomer of **1b** was incubated at pH 9.0 for 20 min, and the resulting crude was analyzed by HPLC. A new compound with the same mass and UV properties had appeared in both cases. Coinjection experiments revealed that each of the newly formed products coeluted with one of the isomers of **7b**, thus proving that epimerization had occurred (Figure 2).

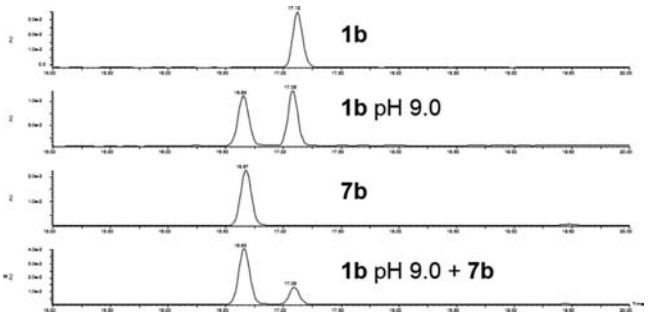

Figure 2. HPLC-MS traces (280 nm) showing that one of the isomers of **1b** is partially transformed into one of the isomers of **7b** after treatment with a base (AU = absorbance units).

On the basis of these data, D-Cys was employed for the synthesis of different labeled cyclic peptides, which were formed in good yields (see Table 2, entries 8−15), and could be isolated as stereoisomerically pure compounds except in one case.

Additional stability experiments were carried out with each of the two isomers of cyclic peptides **5a**, **6a**, **8a**, **9a**, and **9b** (incubation at pH 7.4 PBS buffer for 12 and 24 h, and at pH 9.0 for 20 min). HPLC monitoring revealed that the extent of epimerization was largely compound-dependent, although in general L-Cys-containing products tended to epimerize faster than those containing D-Cys. Table 2 shows that the amount of nonepimerized product is usually higher for the peptides with D-Cys than for those with L-Cys (cf. entries 1 and 8, 3 and 10, and 6 and 13; see SI, section 10).

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Even though it yields stereoisomeric compounds, we believe that this methodology may find application, since synthetic methods providing mixtures of isomers have found use in bioconjugation (inverse electron demand Diels–Alder cycloadditions, reactions between azides and strained cyclooctynes) and in therapy (three phosphorothioate oligonucleotides have been approved as drugs).

In conclusion, this work has demonstrated that CPDs can be used to simultaneously cyclize and derivatize peptides with two cysteine residues, with one at the *N*-terminus. This should not be a limitation, since several methods (including SPPS) allow this residue to be introduced into peptides and proteins.[17]

Cyclizations have furnished 19-, 22-, 25-, and 31-membered rings and take place in good yields and relatively short reaction times using TEMPO as the catalyst and in the presence of LiCl. Different data (MS, UV, and also NMR) have confirmed that cyclization does take place.

Structural parameters affecting the reaction rate and outcome as well as the stability of the conjugated cyclic peptides have been examined. Our results indicate the peptide sequence as an influential factor in the cyclization kinetics and show that peptides with D-Cys at the *N*-terminus furnish cycles generally more stable. For this reason, introduction of D-Cys at the *N*-terminal position is recommended. However, structural studies out of the scope of this work should be performed to establish the ultimate reasons behind the observation that both the CPD and peptide nature have a profound effect on the reaction rate, as well as the different chemical behaviors of the L- and D-epimers.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.6b03825.

Experimental procedures, compound characterization data, and HPLC profiles (PDF)

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Notes

The authors declare no competing financial interest.

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