# Thioxylation as One-Atom-Substitution Generates a Photoswitchable Element within the Peptide Backbone**

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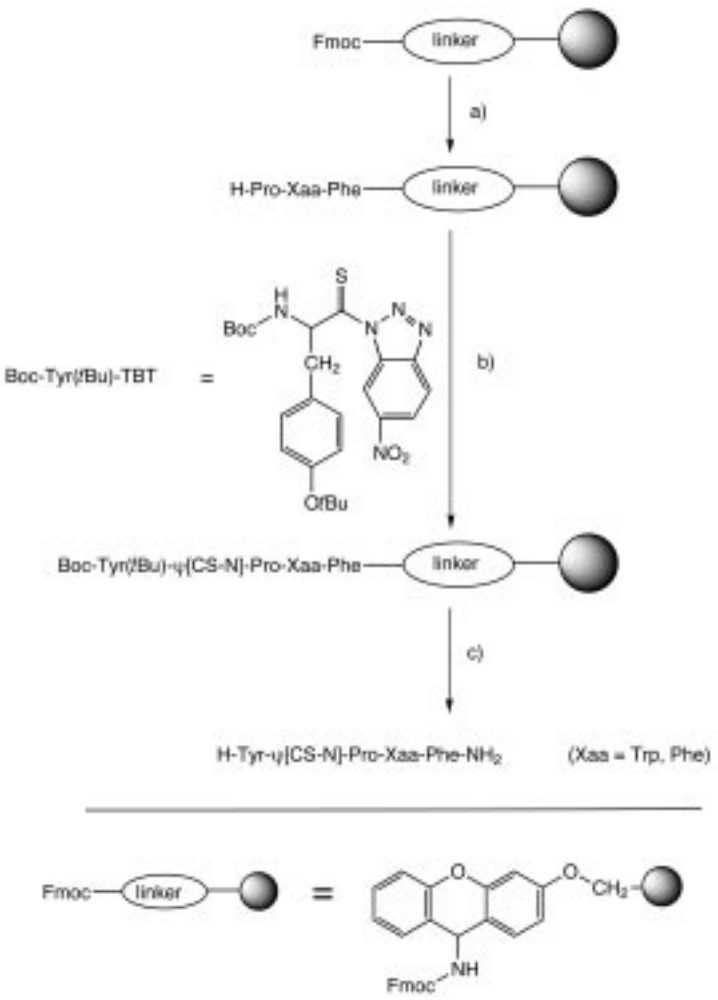
The thioxo peptide bond −CS−NR− (R = H, alkyl) represents an isosteric replacement of the peptide bond with only a slightly changed electron distribution in the ground state.[1] This O/S substitution in biologically active oligopeptides is of considerable interest because of the enhanced proteolytic stability[2] and the modulated activity and selectivity.[3]

Proline, the only gene-coded cyclic amino acid, plays a unique role for the backbone conformation. In oligopeptides the peptidyl–prolyl bond causes a conformationally heterogeneous ensemble of molecules consisting of *cis* and *trans* peptide bond isomers. The *cis*/*trans* isomerization follows first-order kinetics, characterized by half-lives between 10 and 100 s at room temperature.[4] Taken together these properties of the prolyl bond allow the detection of isomer-specific recognition in biological signaling.[5] Evaluating the isomeric state of the peptidyl–prolyl bond in signaling complexes provides a sensitive probe for the biologically active conformation. Moreover, "freezing" the dynamics of the backbone can be achieved by peptide bond thioxylation.[2a, 2c, 6] This further improves the analysis of isomer-specific interactions in biological recognition.

Here we report on the use of the thioxo prolyl bond as a probe of the isomeric state and on the ability to photoswitch between *cis* and *trans* isomers when this bond is present in the backbone of endomorphins.[7] The thioxylated endomorphins Tyr-$\psi$[CS-N]-Pro-Trp-Phe-$NH_2$ (**1**) and Tyr-$\psi$[CS-N]-Pro-Phe-Phe-$NH_2$ (**2**) are effective $\mu$-receptor agonists whose affinity for the $\mu$-receptor is comparable to that of the natural endomorphins (unpublished results). The thioxo peptides **1** and **2** were synthesized by thioacylation of the resin-bound tripeptides Pro-Xaa-Phe using thioxylated tyrosyl-6-nitrobenzotriazolide (Boc-Tyr(*t*Bu)-TNB; Boc = *t*BuOCO).[8] To circumvent the problems usually occurring during acidolytical deprotection of thioxo peptides,[9] we used a newly developed method[10] for the mild detachment of the thioxylated products from the resin. This procedure simultaneously deprotects the tyrosyl side chain in the presence of thioxo peptides bonds (Scheme 1).

UV/Vis and CD spectroscopy of peptides **1** and **2** in aqueous solution showed the characteristic absorption band




Scheme 1. Synthesis of peptides **1** and **2** on Sieber amide resin (linker): a) Fmoc amino acid (5 equiv), coupling conditions: TBTU/HOBt/DIEA (1/1/2) in NMP; Fmoc deprotection: 20% piperidine in NMP; b) Boc-Tyr(*t*Bu)-TBT (2 equiv), DIEA (6 equiv), 277 K, $CH_2Cl_2$; c) 2 M $SnCl_4$ in $CH_3CN$, $CH_2Cl_2$, 1 h at 298 K, or 2.2 M $ZnCl_2 \cdot Et_2O$ complex, $CH_2Cl_2$, 10 h at 298 K. Xaa = Trp (peptide **1**), Phe (peptide **2**); TBTU = *O*-(1-benzotriazolyl)tetra-*N*-methyluronium; HOBt = 1-hydroxy-1*H*-benzotriazole; DIEA = diisopropylethylamine; NMP = *N*-methylpyrrolidone.

for the $^1\pi - {}^1\pi^*$ transition of thioxo peptide bonds (Figure 1) and a negative Cotton effect (Table 1, Figure 2). Moreover, we found a positive Cotton effect for the $^1n - {}^3\pi^*$ transition (Table 1, Figure 2), which is indicative of an asymmetric center in close proximity to the chromophore.[11]

The time dependence of the light-induced *cis*/*trans* photoisomerization of the Tyr-$\psi$[CS-N]-Pro-bond (by excitation of the $^1n - {}^3\pi^*$ transition with a $N_2$ laser at 337 nm; Figure 3) demonstrates the existence of an isosbestic point at 251 nm, as could be expected for a uniform transition between two conformers (shown in Figure 1 for **2**). In the case of thioxylated oligopeptides without aromatic side chains we were also able to demonstrate *cis*/*trans* photoisomerization by excitation of the $^1\pi - {}^1\pi^*$ transition. As depicted in Figure 1 this photoisomerization is reversible and occurs without photodecomposition. Even after four cycles of excitation/reequilibration the UV/Vis spectrum of the thioxo peptide **1** remains unchanged. This is in contrast to the results obtained

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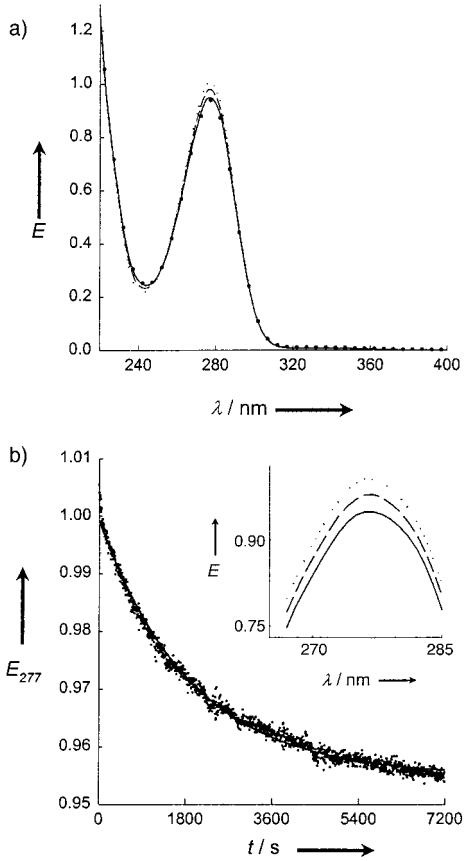


Figure 1. a) UV/Vis absorption spectra of peptide **2** in 0.01M sodium phosphate buffer (pH 7.4), $7 \times 10^{-5}$M, 313 K. Equilibrated peptide before irradiation at 337 nm by $N_2$ laserlight (——), peptide after 30 min of irradiation (····), peptide after 40 min of irradiation (– – –), peptide after four cycles of irradiation/reequilibration (••••). By quantitative analysis of the $^1$H NMR spectra under the same conditions a *cis* content of 21 % for the peptide **2** was obtained. After irradiation a *cis* content of 58 % was found. b) Time dependence of the *cis*/*trans* isomerization of peptide **2**: 0.01M sodium phosphate buffer (pH 7.4), $7 \times 10^{-5}$M, 313 K. The time dependence of the absorbance at 277 nm follows a first-order reaction characterized by a rate constant $k_{obs} = (5.23 \pm 0.03) \times 10^{-4}\,s^{-1}$. Inset: Enlargement of the UV spectra between 265 and 285 nm.

Table 1. UV/Vis and CD spectroscopic parameters of the thioxo prolyl bond of *cis*-**2** and *trans*-**2** dissolved in 0.01M sodium phosphate buffer (pH 7.4) at 277 K.

| Conformer | UV/Vis λ [nm] (ε [cm²mmol⁻¹]) | | CD λ [nm] (θ [cm²dmol⁻¹]) | |
|---|---|---|---|---|
| | $^1\pi-^1\pi^*$ | $^1n-^3\pi^*$ | $^1\pi-^1\pi^*$ | $^1n-^3\pi^*$ |
| *trans*-**2** | 277 (13780) | 335 (104) | 277 (−7366) | 341 (1962) |
| *cis*-**2** | 276 (15153) | 334 (122) | 277 (8988) | 340 (1219) |

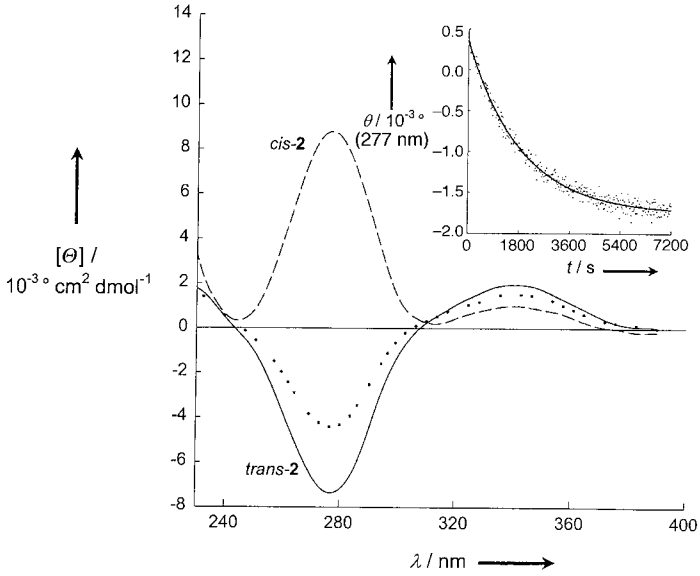


Figure 2. CD spectra of the pure conformers of peptide **2** and the equilibrated solution in 0.01M sodium phosphate buffer (pH 7.4), $2 \times 10^{-4}$M, 313 K; equilibrated peptide (••••), *trans*-**2** (——), and *cis*-**2** (– – –). Inset: The time dependence of the *cis*/*trans* isomerization of peptide **2**: 0.01M sodium phosphate buffer (pH 7.4), $2 \times 10^{-4}$M, 313 K. The ellipticity at 277 nm follows a first-order reaction with a rate constant of $k_{obs} = (5.38 \pm 0.02) \times 10^{-4}\,s^{-1}$.

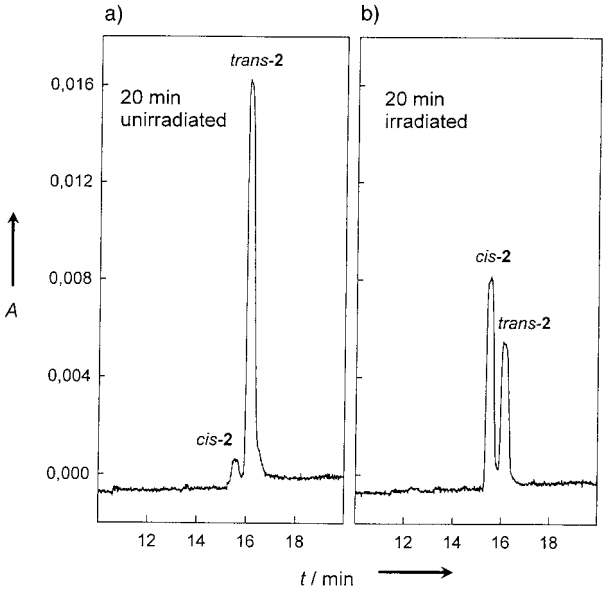


Figure 3. a) Electropherogram of *trans*-**2** 20 min after dissolution in 0.05M sodium phosphate buffer (pH 7.4) at 298 K. b) Electropherogram of *trans*-**2** after 20 min of irradiation prior with a $N_2$ laser (wavelength 337 nm, pulse length 500 ps, pulse frequency 30 Hz, 400 μJ per pulse, 298 K). CE conditions: 0.05M sodium phosphate buffer (pH 2.5), 285 K, quartz capillary: 60 cm × 50 μm, 30 kV, UV detection at 200 nm.

by irradiation of *N*-methylthioacetamide with UV radiation or laser excitation at different wavelengths.[12]

Using Raman and UV spectroscopy, these authors were able to demonstrate that the quantum yield for the *cis*/*trans* photoisomerization is comparable to that of the photodecomposition.[12c] Thus, modification of the thioxo peptide bond does not provide a suitable probe for the detection of isomer-specific reactions of secondary amide bonds. However, the thioxylated tertiary amide bond of the thioxo prolyl moiety proved to be useful as a photoswitchable element within the oligopeptide backbone (Figure 3). The reequilibration after irradiation follows first-order kinetics with $k_{obs} = k_{cis \rightarrow trans} + k_{trans \rightarrow cis}$. Temperature- and time-dependent investigations of this reaction at 277 nm allow the determination of the rate constants of the *cis*/*trans* isomerization (Figure 1). The *cis*/*trans* isomerization of the Tyr-$\psi$[CS-N]–Pro bond in **2** is 245

times slower than isomerization of the Tyr–Pro bond in the oxo form of **1**. Thus, separation of *cis*/*trans* conformers of thioxo prolyl bonds using HPLC might become possible.

Up to now *cis*/*trans* conformers of thioxo prolyl bonds could be separated only in analytical amounts using capillary electrophoresis (CE).[13] We now succeeded in separating *cis*-**1**, *trans*-**1**, *cis*-**2**, and *trans*-**2** on a preparative scale by reverse-phase HPLC. The lyophylized fractions are stable as pure conformers at 253 K for more than four weeks.

The dramatic isomer specificity of the Cotton effect for the $^1\pi-{}^1\pi^*$ transition (Figure 2) observed can be used for kinetic investigations of the *cis/trans* isomerization of thioxo peptides. We determined rate constants of $5.4 \times 10^{-4}\ s^{-1}$ at 313 K for the sum of the isomerization in the forward and reverse directions (Figure 2), which is in good agreement with the values of $5.2 \times 10^{-4}$ and $5.3 \times 10^{-4}\ s^{-1}$ calculated from UV spectroscopic measurements (Figure 1) and $^1$H NMR jump experiments, respectively. With CE measurements we calculated quantum yields of 0.05 for *cis*-**2** and 0.19 for *trans*-**2**. These quantum yields are smaller than those of the often used but sterically more demanding azobenzoles.[14]

In summary, thioxo prolyl bonds represent sensitive probes for detecting isomer specificity in biological signaling, allowing the induction of well-defined changes of the backbone conformation by *cis*/*trans* photoisomerization. Kinetic and thermodynamic analysis of the *cis*/*trans* isomerization becomes possible because of the marked spectroscopic differences of the thioxo peptide bond isomers. In contrast to the oxo peptide bonds present in native proteins the bathochromic shift of the UV/Vis absorption of the thioxo peptide bond allows sensitive UV/Vis measurements of prolyl bond isomerization in a proteinaceous environment. Results concerning isomer specificity of the interaction of peptides **1** and **2** with the opioid $\mu$-receptor from neuroglioma SH-SY5Y cells will be reported elsewhere.

## *Experimental Section*

Peptides **1** and **2** were synthesized by 9-fluorenylmethoxycarbonyl (Fmoc) chemistry on Sieber amide resin (Calbiochem-Novabiochem, Bad Soden, Germany). Coupling of Boc-Tyr(tBu)-$\psi$[CS-N]-6-nitrobenzotriazolide took place over 12 h and at 277 K in $CH_2Cl_2$. The structures of the thioxylated compounds have been confirmed by $^1$H NMR spectra.

Peptide **1**: Simultaneous removal of the protecting groups and the peptide from resin: 2 M $SnCl_4$ in $CH_3CN$, 1 h, 298 K. Purification was performed on a HPLC gradient system (Sykam (LiChrospher, Nucleosil RP-18, 250 × 25 mm). We used 0.05 % trifluoroacetic acid (TFA) as mobile phase A and acetonitrile with 0.05 % TFA as mobile phase B. MS: $m/z = 627$ [$M^+$], calcd for $C_{34}H_{38}N_6O_4S_1 = 626$ (monoisotopic).

Peptide **2**: Simultaneous removal of the protecting groups and the peptide from resin: 2.2 M $ZnCl_2 \cdot Et_2O$ complex in $CH_2Cl_2$, 10 h, 298 K. Purification: HPLC analogous to peptide **1**. MS: $m/z = 588$ [$M^+$], calcd for $C_{32}H_{37}N_5O_4S_1 = 587$ (monoisotopic).

Separation of the conformers: To increase the amount of *cis* conformer in the thioxo peptide solution the sample was irradiated with a $N_2$ laser immediately before separation (wavelength 337 nm, pulse length 500 ps, pulse frequency 30 Hz, 400 μJ per pulse, 1 h, 277 K). Conditions of HPLC separation: LiChrospher, Nucleosil RP 18, 250 × 25 mm, 29 % acetonitrile, 0.05 % TFA; 277 K. The pure conformers ($t_{R,\ trans\text{-}\mathbf{1}} = 12.3$ min, $t_{R,\ cis\text{-}\mathbf{1}} = 19.8$ min, $t_{R,\ trans\text{-}\mathbf{2}} = 10.4$ min, $t_{R,\ cis\text{-}\mathbf{2}} = 17.2$ min) were collected in liquid nitrogen and lyophylized.

Determination of quantum yields by CE: the laser beam was fixed in a right angle 15 cm in front of the capillary. Using a light conductor cable we were able to excite the separated conformers inside the capillary. The *cis*/*trans* photoisomerization was measured at 251 nm (isosbestic point of the interconverting isomers). Correction of peak areas by migration time lead to reduced peak areas and allowed calculation of individual quantum yields.

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