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A photoswitchable thioxopeptide bond facilitates the conformation-activity correlation study of insect kinin

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Abstract: Thioxopeptide bond ψ[CS–N], a nearly isosteric modification of the native peptide bond, was introduced into insect kinin active core pentapeptide to evaluate the impact of backbone *cis*/*trans* photoswitching on bioactivity. The thioxo analog Phe^1-Tyr^2-ψ[CS–N]-Pro^3-Trp^4-Gly^5-NH_2 (ψ[CS–N]^2-kinin), was synthesized by Fmoc solid-phase peptide strategy. The reversible photoswitching property was characterized via spectroscopic methods and HPLC, which showed that the *cis* conformer increased from 15.7 to 47.7% after 254 nm UV irradiation. A slow thermal reisomerization ($t_{1/2} = 40$ min) permitted us to determine the cockroach hindgut myotropic activity of the thioxopeptide in the photostationary state. The results indicated that the activity increased significantly after UV irradiation and recovered to the ground level after thermal re-equilibration. In the present study, by utilizing the phototriggered isomerization in a specific position of peptide backbone, we revealed that the *cis* ψ[CS–N]^2-kinin conformer is the active conformation when interacting with kinin receptor on cockroach hindgut. Copyright © 2008 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: insect kinin; thioxopeptide bond; photoisomerization; conformer

INTRODUCTION

Owing to the flexibility of polypeptide chain peptides and proteins have numerous conformers in solution. However, the recognition, reactivity, and stability of peptides and proteins are conformer specific; the modulation of the backbone conformation of these biopolymers has attracted great attention [1–4]. Photoswitching of the backbone conformation of peptides is an ideal method for studying the conformer specific interaction because the only external stimulus needed is light irradiation. Although, the natural peptide backbone itself provides a *cis*/*trans* ratio increase in the photostationary state, the wavelength range (<220 nm) of peptide absorbance, which must be used for photoswitching, is not compatible with the photochemical stability of proteins [5]. In addition, fast re-equilibration rates prevent the photoinduced *cis* population from applying isomer-specific effects on a coupled reaction. Some bulky organic moieties, such as photochromic azobenzene or spiropyran groups, were reported for photoswitching of peptide conformations [3,6–9]. However, the inherent disadvantages of these approaches are the limited biocompatibility of the hybrid peptide and the problem that the final effects imposed on the backbone by the isomerizing moiety are unpredictable. Recently, the thioxopeptide bond –CS–NR–(R = H, alkyl), which represents an isosteric replacement of the normal peptide bond with only a slight change in the electron distribution in the ground state [10,11], has attracted great interest. Most importantly, the thioxopeptide bond has been shown to be photoswitchable (Figure 1) [12–14]. The *cis* conformer increased dramatically by irradiation at 254 nm or 337 nm UV light, and has a moderate slow thermal re-equilibration rate [12–14]. In particular, the O/S exchange causes minor effects to the peptide backbone, and does not greatly affect the secondary structure formation [15–17]. Biologically, the activity of the thioxopeptide derivative of ligands was found to be similar to that of the native oxopeptide counterpart [18–20].

Considering such a special property of the thioxopeptide bond, we have used it for analysis of the conformation–activity relationship of insect kinin. The insect kinins share a highly conserved *C*-terminal pentapeptide sequence Phe-Xaa-Yaa-Trp-Gly-NH_2 where Xaa can be Tyr, His, Ser or Asn, and Yaa can be Ala but is generally Ser or Pro [21]. These kinins have been isolated from a number of insects, including species of *Dictyoptera, Lepidoptera*, and *Orthoptera*. The first members of this insect neuropeptide family were isolated on the basis of their ability to stimulate contractions of the isolated cockroach hindgut [22], but they are also potent diuretic peptides that stimulate the secretion of primary urine by Malpighian tubules [23]. In addition, insect kinins or related analogs have been reported to inhibit weight gain by larvae of the tobacco budworm (*Heliothis virescens*) [24] and corn earworm (*Helicoverpa zea*) [25]. More recently, the insect kinin peptides have been shown to have distinct and selective modulatory actions on the pyloric rhythm [26].

Structurally, insect kinins require an intact *C*-terminal pentapeptide sequence for full cockroach

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myotropic and cricket diuretic activity, which was therefore designated as the active-core sequence [27]. Within the active-core pentapeptide, the aromatic residues Phe^1 and Trp^4 are of paramount importance for activity in both bioassay systems, whereas position 2 tolerates wide variations in the side-chain character ranging from acidic to basic or hydrophobic to hydrophilic [27]. NMR and molecular modeling studies of the cyclic insect kinin analog *cyclo*(Ala-Phe-Phe-Pro-Trp-Gly) and the linear analog H-Phe-Phe-Aib-Trp-Gly-NH_2 indicated that there are two distinct conformations with a β-turn encompassing the residues 1–4 and 2–5, respectively [27,28]. In addition, analogs containing either tetrazole or 4-aminopyroglutamte (APy) moieties that preferentially form the type VI β-turn were synthesized and found to retain significant activity in a cricket diuretic assay [24,29].

Although current structure–activity studies were most supportive of the 1–4 β-turn as the active receptor-bound conformation [27,29,30], the 2–5 β-turn could not be dismissed as a candidate. However, the fast interactive conversion rates prevented the isolation of the single isomers for detailed bioactivity assay. In order to obtain a more direct evidence for the active conformation, a kinin analog containing a thioxopeptide bond, which can be used as a photoswitch, was synthesized. As the Tyr^2–Pro^3 peptide bond within the core pentapeptide is important for its secondary structure formation [27], we selected this peptide bond as the thioxylating position, and prepared the thioxopeptide Phe-Tyr-ψ[CS–N]-Pro-Trp-Gly-NH_2. The photoswitching property of this thioxo analog was characterized, and the bioactivities in different states were evaluated by cockroach hindgut myotropic assay.

MATERIALS AND METHODS

Peptide Synthesis

The peptide, H-Phe-Tyr-Pro-Trp-Gly-NH_2 was synthesized manually by the solid-phase method, using the Fmoc strategy and starting from Rink Amide resin (Tianjin Nankai Hecheng Science and Technology Co., Ltd., China; 0.5 mM/g). A special protocol was used for the thioxopeptide as described elsewhere [31]. The Fmoc protecting group was removed using 20% piperidine in DMF. A twofold excess of the respective Fmoc amino acids was activated *in situ* using DIC (1 eq)/HOBt (1 eq) in DMF. Amino acid side chain protecting groups were tBu for Tyr and Boc for Trp. The coupling of Fmoc-Tyr(tBu)-ψ[CS–N]-6-nitrobenzotriazolide (Fmoc-Tyr(tBu)-TNB) took place over 12 h and at 277 K in CH_2Cl_2. Synthesis of the enantiomerically pure Fmoc-Tyr(tBu)-TNB was based on a modified method of Shalaby *et al* [32] starting with Fmoc-Tyr(tBu)-OH. The completeness of each coupling reaction during synthesis was monitored by the Kaiser test, except for the coupling of Fmoc-Tyr(tBu)-TNB which was monitored by chloranil test. Cleavage of the peptide from the resin with side-chain deprotection was performed by treatment with 95% TFA/2.5% H_2O/2.5% TIS (1.5 h) for the unmodified pentapeptide, and with 80% TFA/5% TIS/15% H_2O (2 h) for the thioxopeptide. The cleaved peptides were precipitated with 10–20 volumes of diethyl ether, filtered, washed successively with more ether and air-dried. The resulting crude peptides were extracted with water and lyophilized.

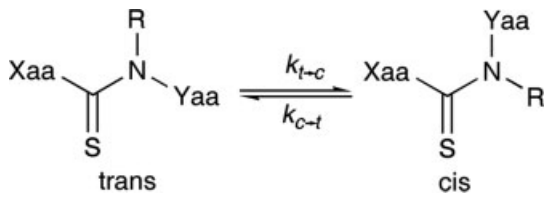

Figure 1 Schematic representation of *trans* and *cis* isomeric forms of a thioxopeptide bond (Xaa and Yaa: amino acid residues; R: H or alkyl).

Crude peptides were purified by preparative reversed-phase HPLC using a Waters Delta 600 system with a Waters Delta-pak semipreparative column C18 (7.8 × 300 mm, 300 Å, 15 μm) at 10 °C. Analytical HPLC was performed on a Waters Delta 600 system with a Delta-pak analytical column C18 (3.9 × 150 mm, 300 Å, 5 μm) at 10 °C. Analytical purity and retention time (t_R) of the peptides were determined at a flow rate of 1 ml/min using a linear gradient from 15 to 40% B for 40 min (solvent A = 0.1% TFA in H_2O; solvent B = 0.08% TFA in CH_3CN). All peptides showed >99% purity when monitored at 220 nm. The identities of the peptide analogs were characterized by ESI-TOF mass spectrometry: H-Phe-Tyr-Pro-Trp-Gly-NH_2 (t_R 20.3 min, calcd. mass: 667.3; exp. mass: 690.4 (M + Na); H-Phe-Tyr-ψ[CS–N]-Pro-Trp-Gly-NH_2 (t_{Rtrans} 22.8 min, t_{Rcis} 31.7 min, cald. mass: 683.3, exp. mass: 685.1 (M + H)).

UV-visible Absorption Spectra

Measurements were performed at 20 °C using a PerkinElmer Lambda 35 spectrometer. Slit width was set as 0.5 nm. Quartz UV cells of 1 cm path length were used. The UV–Vis spectra for 8.3×10^{-5} M thioxopeptide in 0.01 M sodium phosphate buffer (pH 7.2) were recorded before and after 5 min irradiation at 254 nm UV light with an intensity of 2 mW/cm^2; the spectrum of the peptide after four cycles of irradiation/re-equilibration was also measured.

Circular Dichroism (CD) Spectroscopy

The CD spectra of the thioxopeptide were recorded with a Jasco 815 spectrophotometer (Jasco, Tokyo, Japan). Measurements were performed at 20 °C with a peptide concentration of 1×10^{-4} M in 0.01 M sodium phosphate buffer (pH 7.2) with quartz CD cuvette of 5 mm path length. For the determination of the CD spectrum without irradiation, five accumulations were used. For the determination of the CD spectra of the irradiated peptide solution, however, only two accumulations were used and the irradiation/recording experiment was repeated three times. The final spectrum was the mean result of three raw spectra. All spectra were baseline corrected and smoothed. The temperature of the CD cuvette was controlled by circulating water from a thermostat.

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RP-HPLC Analysis of Photoisomerization

The photo-induced isomerization of the thioxopeptide was also analyzed by RP-HPLC. It was performed on a Waters Delta 600 system equipped with a Delta-pak analytical column C18 (3.9 mm × 150 mm, 300 Å, 5 μm) at 10 °C (solvent A = 0.1% TFA in H_2O; solvent B = 0.08% TFA in CH_3CN). The column was eluted at a flow rate of 1 ml/min with a linear gradient of 27% B to 90% B in 20 min. A standard laboratory UV lamp was used for irradiation at 254 nm and an intensity of 2 mW/cm^2. For determining the *cis*/*trans* ratio of the photostationary state, a sample containing 8.3×10^{-5} M thioxopeptide in 10 mM sodium phosphate buffer (pH 7.2) was irradiated with UV light at 254 nm for 5 min; then the measurement started. In order to calculate the relative amount of the *cis* and the *trans* conformers, the isosbestic point of the UV–vis absorptions of the *cis* and the *trans* conformers, 252 nm, was used. At this wavelength, the peak areas ratio of *cis* and *trans* conformer is directly related to the relative population of *cis* and *trans* conformers.

Determination of the *cis*/*trans* Isomerization Rate Constant

Measurement of the thermal relaxation was performed with a Mapada UV-1800 UV–vis spectrometer (Shanghai Mapada Instruments Co., Ltd., Shanghai, China) at 20 °C. The thioxopeptide solution (7.2×10^{-5} M in 0.01 M sodium phosphate buffer, pH 7.2) was irradiated with the UV lamp for 5 min. The measurement commenced, and the time course at 277 nm was monitored. The observed first-order rate constant k_{obsd} was calculated from the time course using a single-exponential equation fit.

Cockroach Hindgut Myotropic Assay

Periplaneta americana cockroaches (Department of Vector Biology and Control, Center for Disease Control and Prevention, Gansu Province, China) were taken from stock colonies maintained at 27 °C and fed with dry dog food *ad libitum*. Hindguts isolated from the CNS tissue were dissected and prepared for recording of myotropic activity as previously described [33,34]. The hindgut was fixed in a vertical glass chamber containing 10 ml insect saline (NaCl 8.19 g l^{-1}, KCl, 0.37 g l^{-1}, $CaCl_2$, 0.56 g l^{-1}, $MgCl_2 \cdot 6H_2O$, 0.2 g l^{-1}, glucose, 0.9 g l^{-1}, HEPES, 2.4 g l^{-1}, pH 7.25). Isometric responses were recorded using a strain gauge transducer (Institute of Space Medical-Engineering, Beijing, China) linked to a 6240B recorder system (Machine Equipment Corporation of Chengdu, Chengdu, China). The hindgut was allowed to equilibrate until a stable baseline of spontaneous activity was observed. At this time, the spontaneous contraction was recorded over a 5 min period. Then, a known desired quantity of peptide solution was added to the bioassay chamber containing the hindgut and recorded for 5 min. The effect of the peptide was obtained by subtracting the 5 min spontaneous contraction area from the 5 min peptide stimulated area of the contract curve. This procedure was repeated for each concentration of peptide.

For kinin thioxopentapeptide, 300 μl stock solutions in insect saline were prepared. From this, 100 μl of each stock solution was taken for myotropic assay. The rest 200 μl of each stock solution was irradiated with 254 nm UV lamp (2 mW/cm^2 light intensity) for 10 min. Aliquots (100 μl) of the irradiated stock solutions were diluted with cold insect saline to the desired concentration for myotropic assay. The rest of the 100 μl of each irradiated sample was kept in the dark overnight, and the myotropic activity was measured. The unmodified parent kinin pentapeptide was used as positive control, and the maximal response obtained in 1.2×10^{-7} M concentration was designated 100%.

RESULTS

Biophysical Characterization of ψ(CS–N)^2-Kinin

The UV-visible absorption, CD and Raman spectra of the kinin thioxopeptide are different for the *cis* and *trans* conformers in agreement with previous results obtained with other thioxopeptides [12,14,35]. The UV-visible absorption spectrum of ψ[CS–N]^2-kinin shows an absorption increase at 277 nm under irradiation at 254 nm, with an isobestic point at 252 nm (Figure 2). The spectrum of the re-equilibrated peptide solution can be fully restored and retained as unchanged after several cycles of irradiation/re-equilibration. This observation indicates that the photoisomerization is reversible and there is no noticeable photochemical degradation during several photoisomerization cycles. By monitoring the time-dependent decrease of absorbance at 277 nm (inset of Figure 2) the rate constant of the reversible thioxopeptide bond *cis*/*trans* isomerization was extracted. At 20 °C, the first-order rate constant for thioxopeptide bond isomerization of ψ[CS–N]^2-kinin was $K_{obs} = (2.9 \pm 0.1) \times 10^{-4}$ s^{-1}.

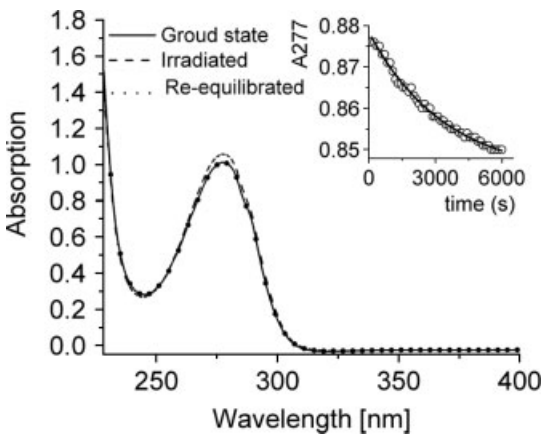

Figure 2 UV-visible absorption spectra of the thioxopeptide (c = 8.3×10^{-5} M in 0.01 M sodium phosphate buffer, pH 7.2, 20 °C): ground state (———), after 5 min of irradiation at 254 nm (- - - -), and upon thermal re-equilibration after four cycles of irradiation (·····). Inset: the time dependence of the *cis*/*trans* isomerizaton of the thioxokinin peptide (c = 8.3×10^{-5} M in 0.01 M sodium phosphate buffer, pH 7.2, 20 °C). The time dependence of the absorbance at 277 nm follows a first-order reaction characterized by a rate constant $k_{obs} = (2.9 \pm 0.1) \times 10^{-4}$ s^{-1}.

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As the *cis*/*trans* isomerization of the thioxopeptide bond occurs in the proximity of chiral centers, the CD spectrum has special advantages for exploration of such conformational environment change produced by thioamide bond rotation. The CD spectra of the kinin thioxopentapeptide showed two thioxo group derived bands in the near-UV CD spectra, representing the $\pi - \pi^*$ and $n - \pi^*$ transition at 276 and 346 nm, respectively (Figure 3). After irradiation, the molar ellipticity at 276 nm increased from −40 018 to −31 028° cm^2 dmol^{-1}. This finding suggests that the molecular geometry around the chromophore (C=S) changed significantly upon irradiation. After sufficient re-equilibration, the original CD spectrum was fully recovered.

The slow *cis*/*trans* isomerization rate ($t_{1/2} = 40$ min) permitted us to directly determine the *cis*/*trans* ratio in the photostationary state by RP-HPLC (Figure 4). Before irradiation, two peaks were observed in the chromatogram, with the first peak ($t_{R1} = 3.8$ min) corresponding to 84.3% and the second ($t_{R2} = 8.1$ min) to 15.7% of the peak areas. After 5 min irradiation, the contents of the second peak increased from 15.7 to 47.7%. The ratio of these two peaks reached the starting value after sufficient re-equilibration time. Accordingly, the second peak corresponded to the *cis* conformer, whose content had increased by UV irradiation in the reversible manner [12–14].

Cockroach Hindgut Myotropic Bioassay

The biological activity of H-Phe-Tyr-ψ[CS–N]-Pro-Trp-Gly-NH_2 in the ground, irradiated, and thermally relaxed state was evaluated with *P. americana* cockroach hindgut myotropic assay. In all the three states agonist activity was observed, with a maximal response

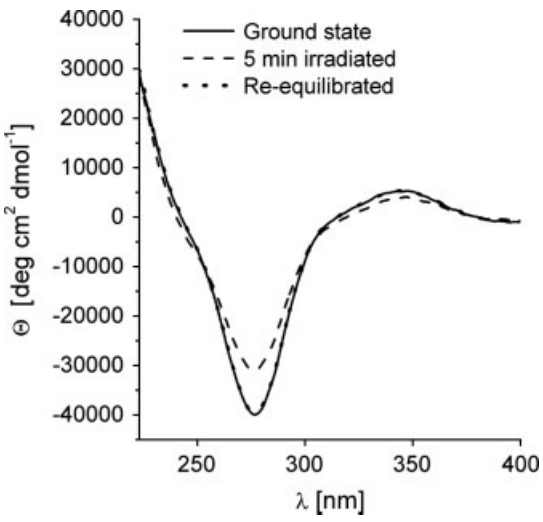

Figure 3 CD spectra of the thioxopeptide (c = 2.0×10^{-4} M in 0.01 mM sodium phosphate buffer, pH 7.2, 20 °C) in ground state (———), after 5 min irradiation (- - - -), and thermal relaxation (·····).

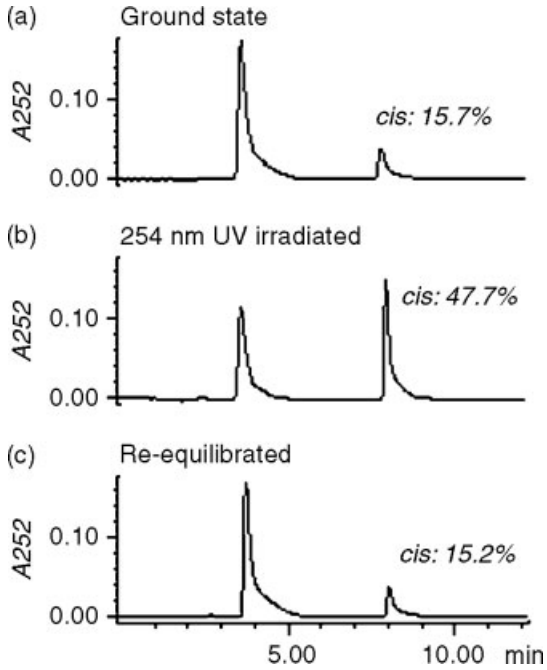

Figure 4 RP-HPLC analysis of thioxopeptide; (a) before irradiation; (b) after 5 min irradiation, and (c) after re-equilibration. Eluting condition: 25–90 B%, 20 min, 10 °C, monitoring wavelength 252 nm. A sample containing 8.3×10^{-5} M thioxopeptide in 10 mM sodium phosphate buffer (pH 7.2) was irradiated with UV light at 254 nm until the photostationary state was reached and then chromatographed. The *cis* content was calculated by integration of the respective peak area ($t_{Rtrans} = 3.8$ min, $t_{Rcis} = 8.1$ min).

of about 80% of positive control H-Phe-Tyr-Pro-Trp-Gly-NH_2 (Table 1). In ground state, the synthetic ψ[CS–N]^2-kinin stimulated the cockroach hindguts contraction with an EC_{50} of $(4.1 \pm 0.2) \times 10^{-7}$ M. After irradiation at 254 nm, the myotropic activity increased to an EC_{50} value of $(1.1 \pm 0.2) \times 10^{-7}$ M (Figure 5). After thermal re-equilibration, the activity returned to the basal level of EC_{50} of $(3.9 \pm 0.3) \times 10^{-7}$ M. This value is not statistically different from that of the ground state ($P > 0.05$), while in the photostationary state, the activity was statistically different from the ground and re-equilibrated state ($P < 0.05$).

DISCUSSION

Owing to the partial double-bond character of peptide bonds, both *cis* and *trans* conformers can be found particularly for Xaa-Pro in peptides and proteins, and this spatial arrangement difference can play an important role in the bioactive conformation of peptides [1,36–41]. In natural linear peptides such proline bonds are in a dynamic equilibrium between the two isomers in aqueous solution, and the fast interconversion rates prevent the isolation of single isomers for analyzing isomer-specific effects on coupled processes. However,

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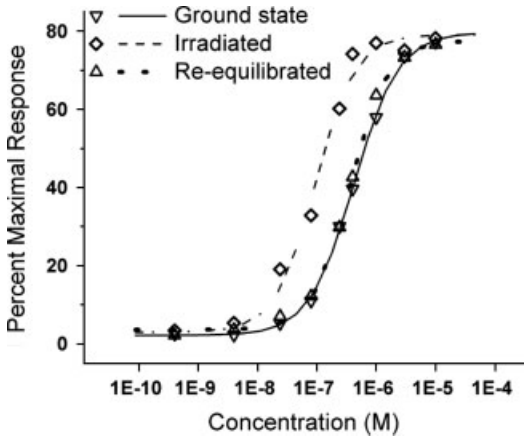

Figure 5 A comparison of the dose–response curves for the three different states of the thioxokinin analog H-Phe-Tyr-ψ[CS–N]-Pro-Trp-Gly-NH_2 in cockroach hindgut myotropic assay. Data points are the means of four to eight determinations. (———): activity in ground state; (- - - -) : activity at the photostationary state of irradiation; (· · · · · ·): activity after re-equilibration. The y-axis represents contract activity expressed as a percentage of the maximal response observed for the thioxopeptide against the parent kinin pentapeptide H-Phe-Tyr-Pro-Trp-Gly-NH_2 as positive control. The values at the photostationary state and ground state are statistically different ($P < 0.05$), while those at the ground state and after re-equilibration are not statistically different ($P > 0.05$).

combining a photoswitch into the peptide backbone is an ideal approach to solve this problem. For these hybrid peptides, the specific conformer can be controlled with a defined wavelength light and thus, the conformer-specific interaction can be evaluated. This facilitates identification of geometries and conformations adopted in ligand–receptor interaction. In this context thioxopeptide analogs have the special advantages of only one atom replacement compared to the native peptide bond, without introduction of bulky photochromic moieties.

The synthetic ψ[CS–N]^2-kinin analog confirmed the fully reversible photoisomerization with a slow re-equilibration rate (half life $t_{1/2} = 40$ min). In the myotropic assay, the analog retained in the ground state 80% of the maximal response obtained with the parent kinin pentapeptide (Table 1). This slight activity loss may result from the larger size of the sulfur atom [42,43], the longer carbon–sulfur bond length [44,45], and the different electron distribution of thioxopeptide bond ψ[CS–N] [46]. In the photostationary state, a fourfold enhanced myotropic activity was observed. After thermal relaxation the ground-state activity was recovered (Table 1). This is consistent with the HPLC analysis, which showed an increase of the *cis* ψ[CS–N]^2-kinin isomer from 15.7 to 47.7% upon UV irradiation at 254 nm and full thermal relaxation to the ground state. Thus, the enhanced activity has to be attributed to the increased content of *cis* conformer on irradiation.

Previous NMR and molecular modeling studies of the cyclic insect kinin analog *cyclo*(Ala-Phe-Phe-Pro-Trp-Gly) revealed the presence of two major turn conformations in aqueous solution: a 1–4 *cis*-Pro type VI β-turn (60%) and 2–5 *trans*-Pro type I β-turn (40%) [27]. The conformation-restricted tetrazole insect kinin analog H-Phe-Phe-ψ[CN_4]-Ala-Trp-Gly-NH_2 (L, L) [30] and the APy acid analog Ac-Arg-Phe-APy-Trp-Gly-NH_2 [29] showed significantly enhanced activity in a cricket diuretic assay. Both the tetrazole and APy moieties are *cis*-peptide bond type VI β-turn mimics. In the *cis* ψ[CS–N]^2-kinin isomer a Phe^1-Trp^4 type β-turn is likely to be formed. From previous studies, it is known that backbone conformations compatible with the three major types of regular secondary structures (α-helix, β-sheet, and β-turn) are accessible to thioxylated amino acids, although the bulkier thioxopepitde bond restricts the available ϕ and ψ angles of the amino acids flanking the thioamide group [15–17,47]. Furthermore, the sequence of *cis* ψ[CS–N]^2-kinin is consistent with the observation that aromatic side-chains flanking the Pro residue favor the *cis*-Pro conformation, thus enhancing the percentage of type VI β-turn population (*cis* Pro in the third position of the turn) [48,49]. Conversely, based on data analysis for turn occurrence in proteins

Table 1 Activity of kinin thioxopentapeptide H-Phe-Tyr-ψ[CS–N]-Pro-Trp-Gly-NH_2 in different states evaluated by cockroach hindgut myotropic assay

States of ψ[CS–N]^2-kinin	*Cis* (%)	Cockroach hindgut myotropic activity, EC_{50} (10^{-7} M)[a]	% Maximal response[a,b]
Before irradiation	15.7	4.1 ± 0.2	79 ± 7
254 UV irradiation	47.7	1.1 ± 0.2	81 ± 4
Re-equilibrated overnight	15.2	3.9 ± 0.3	79 ± 4

[a] Expressed in mean ± SEM.
[b] Maximal response is the maximal contract response of thioxopeptide expressed as a percentage of the maximal response of the parent pentapeptide H-Phe-Tyr-Pro-Trp-Gly-NH_2 at 1.2×10^{-7} M concentration.

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[50,51], the *trans* ψ[CS–N]^2-kinin, is much more likely to form β-turn within Tyr^2–Gly^5, which is probably not compatible with the insect kinin receptor interaction. Consequently, we suggest that a Phe^1–Trp^4 β-turn as favored by the the *cis*-Pro conformation represents the bioactive conformation of the insect kinin.

In conclusion, introduction of a photoswitchable thioxopepitde bond into the insect kinin core-pentapeptide allowed modulation of the myotropic activity and thus identification of structural elements of the bioactive conformation of this receptor ligand. In similar mode in the course of this work, Wildemann [52] reported the photoswitching property of a thioxopeptide in ribonuclease S, further confirming its unique advantage for structure-function studies.

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