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Interplaying factors for the formation of photoswitchable β-hairpins: the advantage of a flexible switch

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A series of peptidomimetics intended to promote the β-hairpin motif have been studied. Structural variations include a turn region with and without a photoswitchable chromophore, and strands with amino acid side chains supporting various degrees of interstrand interactions for hairpin stabilisation. The propensity of the compounds to form β-hairpins was evaluated experimentally by NMR spectroscopy, translational self-diffusion studies and CD spectroscopy. In the presence of hairpin stabilising interstrand interactions, the structurally flexible stilbene chromophore appeared to be well compatible with the imposed secondary structure.

Supporting information may be found in the online version of this article

Keywords: peptidomimetics; conformational analysis; photoswitches; diffusion coefficients; CD

Introduction

The secondary structure of peptides is of pivotal importance for their biological function. Proteins are composed of a few regular structural elements, foremost α-helices, β-sheets and turns [1]. These building blocks are also involved in molecular recognition and are therefore potential candidates for drug design [2,3]. Compared with α-helices, β-hairpins have been studied less frequently [4–7]. β-Hairpins are the smallest structural motif in short β-strands, and are composed of two anti-parallel strands connected by a turn or a small loop. Since β-hairpins often are involved in protein–protein or protein–DNA interactions [8–11], model compounds for their conformational behaviour are of considerable interest. The formation of β-hairpins can be favoured by incorporation of appropriate turn-inducing segments such as ^{D}Pro-Gly into a linear peptide [12]. However, additional interactions between the amino acid side chains of the anti-parallel strands are also necessary [13].

We have recently shown that a stilbene moiety incorporated into a cyclic peptide with a hairpin-inducing motif facilitates photomodulation of β-hairpin formation [14].

A far more common and structurally related chromophore that has been utilised for the same purpose is azobenzene [15–18]. Photochemical properties, including detailed studies concerning both its photochemistry when incorporated into a peptide secondary structure, and its conformational properties, have been performed [19–24]. Interestingly, good β-turn-inducing properties in acyclic peptidomimetics were found [24].

The stilbene chromophore might be an interesting alternative to azobenzenes, because it is not thermoreversible or sensitive to reduction [25]. Recently, we have investigated conformational characteristics of the stilbene chromophore when incorporated into small model peptidomimetics [26]. Although turn-inducing properties were indicated by computations, no such motifs were detectable in solution. The primary objective of the present study is to identify structural factors that might support the formation of β-hairpin structural motifs in larger stilbene peptidomimetics.

Materials and Methods

Peptide Synthesis

Starting materials were purchased from commercial suppliers and were used without purification. SPPS for peptides **3** and **4** was carried out manually on Rink amide MBHA resin on a 500-mg scale (loading rate 0.73 mmol/g) using an Fmoc/*t*Bu protection scheme. Chain elongation was performed using the Fmoc-protected amino acids (110 μmol) with PyBOP (110 μmol) mediated coupling steps (2 h) in a mixture of diisopropylethylamine (220 μmol) and DMF (3.0 cm^3). Removal of the Fmoc groups was achieved by reaction with 20% piperidine in DMF for 5 + 10 min. After introduction of each amino acid, Kaiser's test [27] was performed and capping was carried out (30 min) by addition of acetic anhydride (1.5 cm^3) in dichloromethane (2.0 cm^3) and diisopropylethylamine (0.5 cm^3). Syntheses of peptidomimetics **5** and **6** were carried out on a PerSeptive Biosystems (Framingham, MA) Pioneer automated peptide synthesiser using standard Fmoc chemistry protocol and Fmoc-Ser-polyethylene glycol-polystyrene resin (Applied Biosystems, loading rate 0.16 mmol g^{-1}) or 2-chlorotrityl chloride resin (1.4 mmol g^{-1}), respectively. The chromophore-containing amino acid and the next amino acid in the sequence were coupled manually according to the procedure above. The synthesis of **5** was thereafter continued on the peptide synthesiser. Cleavage of the products was achieved by addition of 95% TFA in dichloromethane with 2.5% triethylsilane (TES) (1 h + 2 × 30 min), followed by filtration, concentration of the solutions under reduced pressure

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and lyophilisation. Peptidomimetic **6** was cleaved from the resin with 0.5% TFA in dichloromethane. The products were purified on a Gilson 321 HPLC system connected to a Vydac Protein & Peptide C18 (218TP) column (10 μm, 22 × 250 mm) using a gradient of acetonitrile in 0.1% aq. TFA (10–85% MeCN, 75 min) at a flow rate of 5 cm^3 min^{-1} and UV detection at 230 nm (LKB 2151 absorbance detector), or on a Gilson system (Gilson 231 XL Injector, 118 UV/Vis detector, Gilson 402 Syringe pump, Gilson 333 and 334 pumps and Gilson FC204) connected to a Grace Vydac C18 column (22 × 250 mm, 5 μm) with a gradient of MeCN in 0.1% aq. TFA (20–60% MeCN, 60 min). The fractions were further analysed by LC-MS or by analytical HPLC using a Chromolith Performance RP-18e column (4.6 × 100 mm). Amino acid analyses were performed at the Department of Biochemistry and Organic Chemistry, Biomedical Centre, Uppsala, Sweden, on 24-h hydrolysates with a Biochrom 30 amino acid analyser, using ninhydrin detection. The stilbene derivative incorporated into **5** and the thioaurone derivative incorporated into **6** were prepared following literature procedures [26,28,29].

Photoisomerisation

Photoisomerisation of peptidomimetic **5** was performed for 90 min in DMSO-d_6 solution under N_2 gas flow using an Oriel 1000 W Xe ARC light source and a 300-nm Oriel UV filter. The thioaurone peptidomimetic **6** was isomerised in DMSO-d_6 solution for 15 min in an NMR-tube with a non-selective ACE Glass Incorporated 450 W UV-lamp connected to a 7830 Power supply.

NMR Measurements

NMR spectra were recorded on a Varian UNITY INOVA (^{1}H at 499.9 MHz), a Jeol EX- 400 (^{1}H at 399.8, and ^{13}C at 100.5 MHz) or a Jeol EX-270 (^{1}H at 270.2, and ^{13}C at 67.5 MHz) spectrometer. Chemical shifts are referenced indirectly to TMS via the ^{2}H lock signal. Assignment was made using COSY [30,31], NOESY [32,33], ROESY [34] and TOCSY [35] experiments. If necessary, solvent signals were suppressed using the WET presaturation scheme [35]. Mixing times between 0.3 and 1.0 s were used in NOESY and ROESY experiments. The pH values of the NMR samples were in the range of 2.1–2.2 for H_2O/D_2O, 3.2–3.5 for CH_3OH/CD_3OD and 4.6–4.8 for DMSO-d_6 solutions. Amide–proton temperature coefficients $\Delta\delta_{NH}/\Delta T$ (ppb/K) were measured for 3 mM samples in DMSO-d_6 (298–353/388 K), CH_3OH:CD_3OD (1 : 1, 188–328 K) and H_2O:D_2O (1 : 1, 283–358 K) solutions. The concentration dependency of chemical shifts and diffusion coefficients were monitored in the concentration interval of 60 μM to 3 mM. For LED-pulsed-field gradient spin echo (PGSE) diffusion experiments [36,37], *z*-gradients were employed, acquiring 16 transients for each value in an array of gradient pulse strengths (0–20 gauss/cm, 20 steps). A relaxation delay of 1 s, 9 ms gradient pulse duration, 20 ms diffusion delay and 5 ms storage delay were used. Diffusion coefficients were calculated based on the known coefficient of HDO (19.02×10^{-6} cm^2 s^{-1}) for compounds **3**, **4** and **6** and DMSO-d_5 (7.72×10^{-8} cm^2 s^{-1}) [38] for compound **5**.

CD Spectroscopy

CD spectra were measured on a JASCO J-810 spectropolarimeter from 190 to 400 nm using a 0.2-cm-path-length cell. Scans (3–5) were accumulated at ambient temperature with a scanning speed of 100 nm min^{-1}, using 0.56 mM methanol solutions for peptides **3** and **4** at 298 K. For peptidomimetic **5** a 0.25-mM methanol solution was analysed at 273–323 K, and for **6** a 0.5-mM methanol solution was analysed at 273–323 K.

Mass Spectrometry

ESI-mass spectra were obtained with a Finnigan ThermoQuest AQA mass spectrometer (ESI 30 eV, probe temperature 100 °C) equipped with a Gilson 322-H2 Gradient Pump system and using a Chromolith Performance RP-18e column (4.6 × 100 mm). A H_2O:MeCN:formic acid (0.05%) mobile phase was used with a gradient of MeCN (20–100% MeCN, 3–5 min). MALDI-TOF-MS spectra were obtained with an Applied Biosystems Voyager-DE PRO.

Results and Discussion

Design of Peptides and Peptidomimetics

Compounds **1**–**5** (Scheme 1) were designed for examination of intrinsic factors governing β-hairpin folding. The Ile-Val-Leu-Leu and Leu-Ala-Thr-Thr anti-parallel strands in peptides **1**–**3** are extracted from the S4 sheet sequence of the TATA-box-binding protein, known to fold in its native environment [39]. Compounds **1**, **2** and **7** have been previously reported [14] and are included here for comparison. β-Branched amino acids are frequently found in strand segments of native β-hairpins [40,41] and have been described to be optimal for stabilisation of β-hairpin conformations since they promote formation of hydrophobic clusters [42,43]. To increase solubility in water, a diethylaminoacetyl tail was attached to the N-terminus of peptides **1**–**4**. Peptidomimetic **1** has the ^{D}Pro-Gly turn inducer [12] and an optimal sequence for hairpin formation [14]. The stilbene chromophore has been reported to have potential turn-inducing properties in small peptidomimetics, according to computational studies [26]. For synthetic reasons, methylene linkers were placed between the stilbene moiety and the carboxyl and amino groups, respectively. An analogue without these linkers exhibits markedly reduced reactivity during the coupling step of peptide synthesis [26], and therefore also was unsuitable for SPPS procedures. Peptide **3** contains the same amino acids as **1**, but the ^{D}Pro-Gly sequence is inverted (i.e. to Gly-^{D}Pro) to provide a peptide lacking a turn-inducing loop. Therefore, **3** was expected to adopt random coil conformation in solution. Peptide **4** was designed to incorporate a good turn inducer (^{D}Pro-Gly as in **1**) but with one of the strands lacking attractive interchain hydrophobic interactions by replacing all amino acids with Gly. Peptidomimetic **5** combines an amino acid sequence resembling the previously reported tryptophan zipper peptides [13,44], known to support β-hairpin conformations, with a stilbene chromophore.

Thioaurones are a further type of photoswitchable compounds, which have recently been used in peptidomimetics [28,29]. Since our conformational studies indicated that thioaurone derivatives are considerably more rigid than the stilbene amino acid used here [26], we prepared peptidomimetic **6**, a congener of the previously reported cyclic stilbene peptidomimetic **7** [14], to test the compatibility of this chromophore with the β-hairpin motif (Scheme 2).

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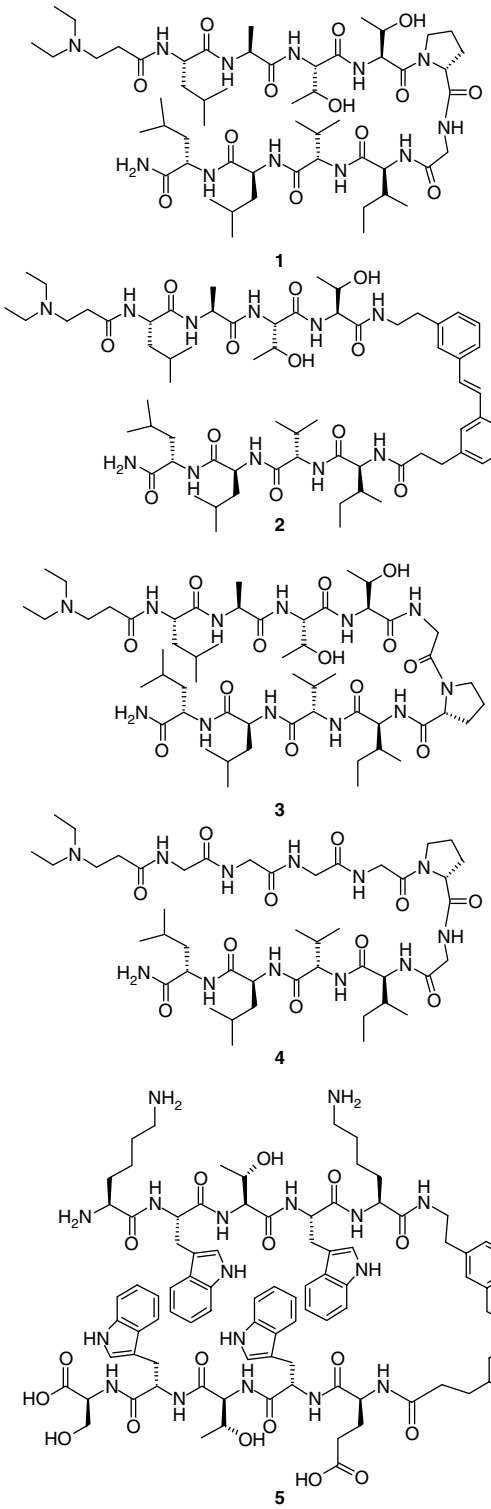
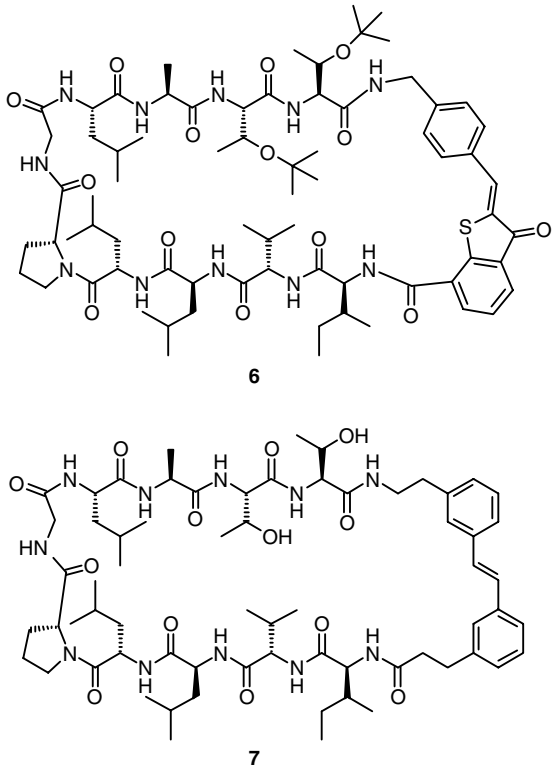


Scheme 1. Acyclic peptides and peptidomimetics used in this study. Only one isomer is shown for the photoswitchable compounds.



Scheme 2. Cyclic peptidomimetics discussed in this study.

Synthesis

Preparation of compounds **1**–**5** followed routine SPPS protocols [14,19]. For the cyclic peptidomimetic **6**, the initial strategy was to cyclise its linear precursor linking the thioaurone amino acid and Thr, but after several attempts, varying coupling reagents, concentrations and reaction times, this approach appeared unfeasible. Instead, cyclisation was accomplished by shifting the coupling position by two amino acids, thus linking Thr and Ala using HATU. Initially, Thr-sidechain-protecting groups were removed by a mixture of TFA : water : TES (95 : 2.5 : 2.5), but after this deprotection step, the peptide could no longer be detected by LC-MS. Because of these problems with the chemical stability of the thioaurone switch [45], the *tert*-butyl protecting groups were left in place and the protected peptidomimetic **6** was used in the further studies.

Photochemistry

Photoisomerisation of *E*-**5** was carried out for DMSO-d_6 solution using selective irradiation at the absorption maximum (λ_{max} = 300 nm) of the *E*-stilbene double bond. The percentage of *Z*-isomer at the photostationary state after 90 min of irradiation was 76%. Attempts to separate the isomers on preparative reversed-phase HPLC were unsuccessful. Photoisomerisation of *Z*-**6** was performed for DMSO-d_6 solution by non-selective irradiation for 15 min. The amount of *E*-isomer obtained at the photostationary state was 78%. Since the isomers were not baseline separated on preparative HPLC, no further attempts were carried out to separate the *E*- and *Z*-isomers. Relative amounts of *E*- and *Z*-isomers were determined by ^{1}H NMR spectroscopy. Conformational studies on *Z*-**5** and *E*-**6** were performed on the photostationary mixtures, as has been reported by others [20,44].

Conformational Analysis by NMR Spectroscopy

One of the most significant tools for determining the conformation of peptides in solution is the detection of non-sequential NOEs [46]. Because of solubility problems at the concentrations needed for NMR analysis, compounds **3**–**6** were investigated in DMSO

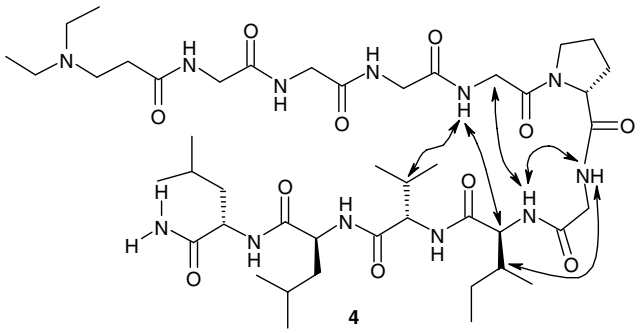


Figure 1. Summary of non-sequential NOEs observed for **4** in methanol solution at 25 °C. ROESY, mixing time 400 ms.

solution, which is in line with many previous studies [47,48]. Interstrand NOEs were observed for peptide **4**, but unlike for compound **1** [14], only in the region closest to the turn (Figure 1). This can be attributed to the absence of interstrand hydrophobic interactions for **4**. Non-sequential NOEs were absent for peptide **3** which is lacking the turn-inducing sequence of **1**. Also **5** was devoid of non-sequential NOEs. The cyclic peptidomimetics displayed an interesting disparity, i.e. whereas interstand NOEs were present for **7** [14], no such effects could be detected for **6**.

Analysis of amide–proton temperature coefficients showed similar values and patterns for all acyclic compounds, i.e. they do not provide useful information regarding secondary structures. This would also be expected for conformationally flexible peptides, especially when they are involved in equilibria with random coil conformers [49]. The *Z*-isomers of the cyclic compounds **6** and **7** showed lower values than their *E*-isomers. Because of the indicated rather dynamic conformational properties of these peptidomimetics, a detailed computational analysis was not performed.

The absence of aggregation of the investigated compounds was confirmed by the concentration independence of the amide proton chemical shifts, as well as the observation of well-resolved ^{1}H NMR signals. Translational self-diffusion measurements using PGSE NMR methods have previously been used to follow conformational changes in polypeptides [14,50–54]. Diffusion coefficients are known to be affected by molecular weight and shape, as well as solvation phenomena such as hydration [52,53], and have been used to probe aggregation of β-hairpin mimetics [14,37,44]. Possible differences in diffusion coefficients of photoisomers (mimetics **2, 5–7**, Table 1) were believed to be indicative of conformational effects [37,55]. Initially, the absence of aggregation was supported by observation of concentration independent diffusion coefficients for **5**. The effect of conformational change on the diffusion coefficient is nicely illustrated by **1** (hairpin) and **3** (random coil), which have the same molecular weight but significantly different diffusion coefficients. A lower diffusion coefficient was obtained for *Z*-**5** than for *E*-**5**, possibly indicating a random coil conformation for *E*-**5** ($D = 9.7 \times 10^{-7}$ m^2 s^{-1}) and the presence of hairpin conformers for *Z*-**5** ($D = 7.7 \times 10^{-7}$ m^2 s^{-1}). The diffusion coefficients for the *E*/*Z*-isomers of cyclic peptidomimetic **6** were very similar, whereas a larger difference was observed for its stilbene congener, i.e. the cyclic peptidomimetic **7** [14]. The cyclic peptidomimetics have larger diffusion coefficients than the acyclic compounds, which is likely the result of a more compact conformation and possibly also a lower extent of solvatisation of the latter. Likewise, the photoisomers of the stilbene peptidomimetics **5** as well as **7** show such a significant difference. Finally, no difference is shown for the photoisomers of **2**. We conclude that in the peptidomimetics **5** and **7**, the stilbene switch results in a photoinduced conformational change. In contrast, the more rigid thioaurone switch (peptidomimetic **6**) does not produce such differences. Interestingly, also photoisomers of previously reported peptidomimetics with azobenzene chromophore (AMPP-Trp-zip, Table 1) had very similar diffusion coefficients.

Table 1. Overview of diffusion coefficients (DMSO-d_6 solution) for the compounds discussed in this work

Compound	M (g mol^{-1})	D (cm^2 s^{-1})	References
1	1122	9×10^{-7}	Ref. 14
2	1246	$E: 7 \times 10^{-7}$	Ref. 14
		$Z: 7 \times 10^{-7}$	
3	1122	1.9×10^{-6}	
4	964	2.4×10^{-6}	
5	1713	$E: 9.7 \times 10^{-7}$	
		$Z: 7.7 \times 10^{-7}$	
6	1383	$E: 1.4 \times 10^{-6}$	
		$Z: 1.3 \times 10^{-6}$	
7	1255	$E: 1.4 \times 10^{-6}$	Ref. 14
		$Z: 1.8 \times 10^{-6}$	
AMPP-Trp-zip	1688	$E: 2.0 \times 10^{-6}$	Ref. 44, CD_3OH
		$Z: 1.9 \times 10^{-6}$	solutions

Conformational Analysis by CD

CD spectroscopy provides sensitive overall secondary structure information for peptides in solution [46,55]. CD data for **1** indicated β-hairpin conformation, whereas data for *Z*- and *E*-**2** were inconclusive. In the present study, peptide **3**, which has no β-hairpin inducer in its turn, gives rise to a spectrum indicating random coil conformation (minimum at 204 nm). Peptide **4**, containing the ^{D}Pro-Gly β-turn inducer and a polyglycine strand, gave rise to a spectrum similar to that of **3**, however, with an additional shoulder at higher frequency (210–230 nm) (Supporting information).

The CD spectrum of peptidomimetic **5** (Figure 2, top) reveals the formation of a β-hairpin, exhibiting the typical appearance reported for Trp-zippers [13,44]. The characteristic maximum at 228 nm and the minimum at 215 nm are indicative of the interactions taking place between the aromatic chromophores of the tryptophans. Furthermore, bands around 280–300 nm (Supporting information) indicate the presence of a defined secondary structural environment, as opposed to a collapsed hydrophobic environment [13].

Estimation of hairpin content of tryptophan zipper peptidomimetics has been based upon the work of Cochran [13]. Thus, CD spectroscopy gives an estimated 40% β-hairpin content for the *Z*-isomer of **5**, according to previous reasoning [44]. For the *E*-isomer of **5**, a lower hairpin content of 24% was indicated. The temperature dependence of the CD signal (Figure 2, bottom) of both isomers reveals thermal unfolding of the hairpin at approximately 28 °C. CD investigations indicated the presence of hairpin conformations for *Z*-**7**, and the absence of this structural motif for **2** and *E*-**7** [14]. The CD spectra of **6**, when compared with those reported for **7**, support the absence of a hairpin motif.

The structural parameters addressed in this study, and their effect on overall peptide or peptidomimetic secondary structure,

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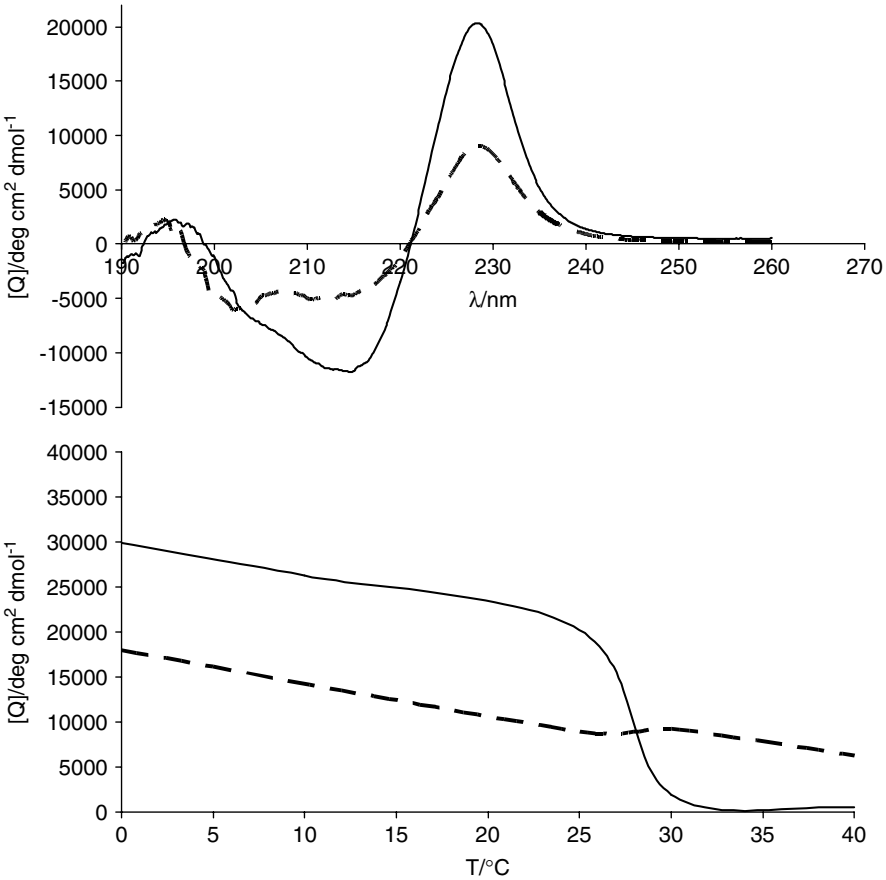

Figure 2. Top: The CD spectra of the photoswitchable peptidomimetic **5** (methanol solution, 25 °C). Bottom: Thermal unfolding of the hairpin conformation monitored by CD. *E*-**5** (dashed), *Z*-**5** (solid, photostationary mixture containing 24% *E*-isomer).

Table 2. Summary of factors pertaining to hairpin formation of the compounds in this work

Compound	Turn segment	Turn-inducing properties	Interacting side chains	Strand interaction
1	^{D}Pro-Gly	+	(+)[a]	Yes
2	Stilbene	(+)[b]	(+)[a]	No
3	Gly-^{D}Pro	−	(+)[a]	No
4	^{D}Pro-Gly	+	(+)[c]	No
5	Stilbene	(+)	+[d]	Yes[e]
6	Thioaurone/^{D}Pro-Gly[f]	(+)/+	(+)[g]	No
7	Stilbene/^{D}Pro-Gly[f]	(+)/+	(+)	Yes[e]

[a] Amino acids from the S4 region in TATA-box-binding protein, known to facilitate hairpin formation in its native form.
[b] According to conformational studies.
[c] In turn region.
[d] Hairpin-inducing tryptophan zipper sequence.
[e] Only for the *Z*-isomer.
[f] Cyclic peptidomimetic.
[g] Thr side chains protected.
+, strong; (+), modest; −, none.

are summarised in Table 2. The presence of interactions between opposed strands has been inferred primarily from the CD data. The reverse Gly-^{D}Pro in the peptide sequence generates a random coil, whereas the ^{D}Pro-Gly order promotes a β-hairpin. Peptide **4** indicates also that a turn-inducing segment is not sufficient for the formation of a β-hairpin if the amino acids in the strands cannot cooperate with their side chains.

Conclusions

We have demonstrated that a stilbene-type switch in a peptide sequence with hairpin-inducing amino acids facilitates a considerable content of β-hairpin conformers for its *Z*-isomer, in contrast to the *E*-isomer. The *Z*-isomer of the switch can, without inducing a β-turn itself, uphold the hairpin conformation generated by

a Trp-zipper. Conversely, the *E*-form disrupts the hairpin motif. The flexibility of the stilbene amino acid mimetics, which initially might appear to be a drawback for the formation of β-hairpins, has now proved to be its main strength because it can adopt to the overall conformation of a peptide. Interestingly, this seems to be in contrast with the reported β-turn-inducing ability of the azobenzene chromophore [24]. Although such capabilities were suggested by computational studies on model peptidomimetics with stilbene chromophores [26], these could not be verified experimentally. The *E*-isomer of stilbene, as well as both thioaurone isomers, appear to be disruptive, the latter most likely due to larger rigidity. Since the *Z*-stilbene switch does not induce hairpins itself, it relies upon other components in the peptide to induce a defined secondary structure. Examples are tryptophan side chains in the linear peptidomimetics, or ^{D}Pro-Gly in the cyclic peptidomimetics. Photoisomerisation to the *E*-isomer can then be used to disrupt these motifs. This opens up for an attractive option; instead of developing a photoswitch with motif-inducing properties, which might require laborious optimisations, it seems to be possible to employ a more flexible switch. The latter does not need to induce a specific motif, but is able to adopt itself to a peptide conformation which is generated by other structural factors. We have here and previously [14] proved that the stilbene switch is able to provide this function.

Supporting information

Supporting information may be found in the online version of this article.

Acknowledgements

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