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Photomodulation of Conformational States. Synthesis of Cyclic Peptides with Backbone-azobenzene Moieties

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Abstract: The search for photoresponsive conformational transitions accompanied by changes in physico-chemical and biological properties led us to the design of small cyclic peptides containing azobenzene moieties in the backbone. For this purpose, (4-aminomethyl)phenylazobenzoic acid (H-AMPB-OH) and (4-amino)phenylazobenzoic acid (H-APB-OH) were synthesized and used to cyclize a bis-cysteinyl-octapeptide giving monocyclic derivatives in which additional conformational restriction could be introduced by conversion to bicyclic structures with a disulphide bridge. While synthesis with H-AMPB-OH proceeded smoothly on a chlorotrityl-resin with Fmoc/*t*Bu chemistry, the poor nucleophilicity of the arylamino group of H-APB-OH required special chemistry for satisfactory incorporation into the peptide chain. Additional difficulties were encountered in the reductive cleavage of the *S*-*tert*-butylthio group from the cysteine residues since concomitant reduction of the azobenzene moiety took place at competing rates. This difficulty was eventually bypassed by using the *S*-trityl protection. Side-chain cyclization of the APB-peptide proved to be difficult, suggesting that restricted conformational freedom was already present in the monocyclic form, a fact that was fully confirmed by NMR structural analysis. Conversely, the methylene spacer in the AMPB moiety introduced sufficient flexibility for facile and quantitative side-chain cyclization to the bicyclic form. Both of the monocyclic peptides and both of the bicyclic peptides are photoresponsive molecules which undergo *cis*/*trans* isomerization reversibly. Copyright © 1999 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: azobenzene-peptides; cyclic peptides; bicyclic peptides; *cis*/*trans* isomerization; photoresponsive peptides

INTRODUCTION

The large changes in geometry and polarity of azobenzene which result from its light-induced *trans*→*cis* isomerization [1] have been exploited extensively for the design and synthesis of photoresponsive systems to do with the conformational, physico-chemical and biological properties of peptides, proteins and phospholipid bilayers [2,3]. Among these systems, poly-α-amino acids with a large number of built-in chromophores have allowed for photomodulating in a reversible manner the conformations and physical properties of the macromolecules. Because of the intrinsic chemical heterogeneity of these polymers, only crude pictures of the dynamics of the conformational transitions could be obtained, whilst defined local changes remained elusive. These, however, are more easily detectable if azobenzene groups are placed in topochemically defined positions of molecules that are more or less constrained in their conformational space [4–9]. Small cyclic peptides are relatively rigid structures that are extensively exploited for the design of libraries of conformers well suited for the restriction of bioactivities [10,11]. The incorporation

Abbreviations: H-AMPB-OH, (4-aminomethyl)phenylazobenzoic acid; H-APB-OH, (4-amino)phenylazobenzoic acid; DTT, dithiothreitol.

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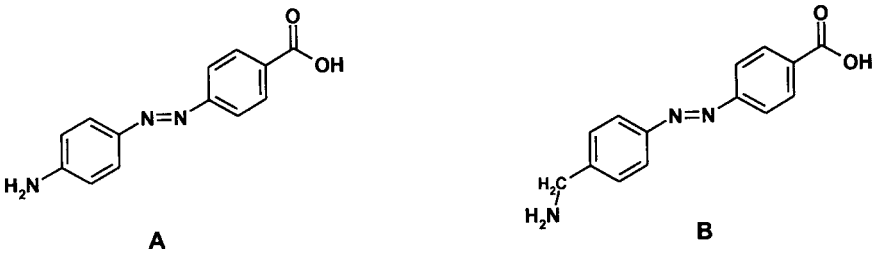


Figure 1 Structure of (4-amino)phenylazobenzoic acid (APB) (**A**) and (4-aminomethyl)phenylazobenzoic acid (AMPB) (**B**).

of an azobenzene moiety into the peptide backbones of such cyclic peptides is expected to give ideal systems for exploiting light-induced conformational transitions as on/off switches of biochemical and biophysical responses [8,12].

Previous studies of active-site fragments of thiol-protein oxidoreductases showed a full dependency of their redox potentials on the intervening dipeptide sequence of the Cys-Xaa-Yaa-Cys motif and on the preferred conformations of the disulphide-bridged loops [13,14]. Strong effects on the redox properties are, therefore, to be expected to result from the restriction of the conformational space of such bis-cysteinyl-peptides via backbone cyclization. A possible modulation of such restricted conformational space and, correspondingly, of the redox properties of the cyclic bis-cysteinyl-peptides, should be achieved by incorporation of photoresponsive moieties into the peptide backbone. To prove the efficiency of such a system, the active-site octapeptide fragment H-Ala-Cys-Ala-Thr-Cys-Asp-Gly-Phe-OH of thioredoxin reductase was chosen, as it is known to retain in the oxidized form the identical 3_{10}-helix turn structure [14,15] as in the intact enzyme [16], thus exhibiting a redox potential almost identical to that of the enzyme itself [13]. To analyse the optimal transfer of the changes in geometry of the azobenzene unit from the 9 Å distance between the two *p*-carbon atoms in the *trans*-configuration to 5.5 Å in the *cis*-configuration [17] onto the peptide backbone, the related amino acid (4-aminomethyl)phenylazobenzoic acid H-AMPB-OH, as suggested by Ulysse *et al.* [8], with a methylene group spacing the amino function from the azobenzene moiety, and (4-amino)phenylazobenzoic acid H-APB-OH having no spacer at either the amino or carboxyl group, were selected as backbone azobenzene units (Figure 1) [12,18].

MATERIALS AND METHODS

Melting points were determined on capillary melting point apparatus (Büchi, Switzerland) and are uncorrected. Optical rotations were measured in a thermostated 1-dm cell on a Perkin-Elmer polarimeter (Model 141). Precoated silica gel 60 TLC plates were from Merck AG (Darmstadt, Germany) and compounds were visualized with the chlorine/tolidine or permanganate reagent. Analytical HPLC was carried out with Waters equipment on Nucleosil 150/C18 columns (Macherey and Nagel, Düren, Germany) using a linear gradient of CH_3CN/2% H_3PO_4 from 5:95 to 80:20 in 12 min at a flow rate of 1.5 ml/min. FAB-MS spectra were recorded on a Finnigan MAT 900 and ESI-MS on a Perkin-Elmer API 165. Amino acid analyses of the acid hydrolysates (6 M HCl, 110°C; 24 h) were performed on a Biotronic analyzer (LC 6001). The racemization of amino acids was determined by gas chromatographic enantiomeric resolution according to Frank *et al.* [19] (cysteine side chains were derivatized with 4-vinylpyridine as described by Siedler *et al.* [20]), using a Carlo Erba gas chromatograph (HRGC 4160).

Chlorotrityl-resin was purchased from PepChem GmbH (Tübingen, Germany). All reagents and solvents used in the synthesis were of the highest quality commercially available. Amino acid derivatives were purchased from Alexis (Grünberg, Germany). Fmoc-Phe-F was obtained from Fmoc-Phe-OH with cyanuric fluoride in 78% overall yield; m.p. 117–118°C (Lit [21]: m.p. 118–120°C); characterized as methyl ester: homogeneous in TLC (petroleum ether/EtOAc, 4:1, R_f 0.7; $CHCl_3$/MeOH/AcOH, 9:1:0.1, R_f 0.9) and in HPLC (t_R 12.07 min); ESI-MS: $m/z = 402.2$ $[M + H^+]$; $M_r = 401.4$, calcd. for $C_{25}H_{23}NO_4$.

(4-Amino)phenylazobenzoic Acid (H-APB-OH) (1)

The title compound was prepared following essentially the procedure described by Schündehütte [22]. A mixture of 4-nitrobenzoic acid (20 g; 100 mmol) and 4,4′-phenylenediamine (16.7 g; 180 mmol) in 3% $NaOH/H_2O$ (500 ml) was heated under reflux at 95°C. After 2 h the solution was cooled to room temperature; the precipitate was collected and washed with water and acetone; yield: 9.5 g (36%); m.p. 232°C; homogenous on TLC (CH_3CN/H_2O, 4:1, R_f 0.4; $CHCl_3$/MeOH/AcOH, 4:1:0.1, R_f 0.7) and HPLC (t_R 6.32 min); ESI-MS: $m/z = 242.0$ $[M + H^+]$; $M_r = 241.2$, calcd. for $C_{13}H_{11}N_3O_2$.

H-APB-Ala-O*t*Bu (2)

To a suspension of H-APB-OH (1.2 g; 5 mmol) in 100 ml dioxane DIEA (1.9 ml; 11.5 mmol), HOBt (780 mg; 5.75 mmol) and EDCI (1.1 g; 5.75 mmol) were successively added. After 12 h the solvent was removed and the residue was extracted from water with EtOAc; the combined organic layers were washed with 5% $NaHCO_3$, 5% $KHSO_4$ and water and dried (Na_2SO_4). The solution was evaporated and the residue was chromatographed on silica gel (100 g silica gel 60; eluent: $CHCl_3$/cyclohexane/CH_3CN, 25:10:10); yield: 2.1 g (90%); homogenous on TLC ($CHCl_3$/cyclohexane/CH_3CN, 25:10:10, R_f 0.6; petroleum ether/EtOAc, 1:1, R_f 0.4); FAB-MS: $m/z = 369.4$ $[M + H^+]$; $M_r = 368.4$, calcd. for $C_{20}H_{24}N_4O_3$.

Fmoc-Phe-APB-Ala-O*t*Bu (3)

To compound **2** (2.0 g; 5.43 mmol) in THF, *N*,*O*-bis(trimethylsilyl)acetamide (2.6 ml; 10.8 mmol) and DIEA (2.8 ml; 16.3 mmol) were added followed by Fmoc-Phe-F (6.3 g; 16.3 mmol). The reaction mixture was kept overnight at 40°C. The solvent was evaporated and the crude product chromatographed on silica gel (150 g silica gel 60; eluent: $CHCl_3/CH_3CN$, 10:1); yield: 2.4 g (62%); homogenous on TLC ($CHCl_3/CH_3CN$, 10:1, R_f 0.75; $CHCl_3$/MeOH, 30:1, R_f 0.8) and HPLC (t_R 12.50 min); FAB-MS: $m/z = 738.2$ $[M + H^+]$; $M_r = 737.3$, calcd. for $C_{44}H_{43}N_5O_6$.

Fmoc-Phe-APB-Ala-OH (4)

Compound **3** (2.3; 3.1 mmol) was dissolved in 100 ml of TFA/CH_2Cl_2 (1:1). After 60 min, the solution was taken to dryness and the residue was triturated with methyl *tert*-butyl ether; yield: 1.9 g (90%); homogenous on TLC ($CHCl_3$/MeOH, 4:1, R_f 0.6; CH_3CN/H_2O, 4:1, R_f 0.3) and HPLC (t_R 11.07 min); FAB-MS: $m/z = 682.2$ $[M + H^+]$; $M_r = 681.2$, calcd. for $C_{40}H_{35}N_5O_6$.

N-Fmoc-(4-aminomethyl)phenylazobenzoic Acid (Fmoc-AMPB-OH) (5)

The title compound was synthesized following essentially the procedure reported by Ulysse and Chmielewski [23].

***N*-Fmoc-(4-amino)benzylamine**. To a solution of 4-aminobenzylamine (5 ml; 44 mmol) and TEA (6.1 ml; 44 mmol) in 50 ml of CH_3CN and 5 ml of DMF a solution of Fmoc-OSu (14.9 g; 44 mmol) in 100 ml of CH_3CN was added dropwise. The product was precipitated with water, filtered off and dried. The product was washed with methyl *tert*-butyl ether/trifluoroethanol (1:1); yield: 76%; homogenous on TLC (CH_3CN/H_2O, 10:1, R_f 0.85; petroleum ether/EtOAc, 2:1, R_f 0.4) and HPLC (t_R 7.68 min); ESI-MS: $m/z = 345.4$ $[M + H^+]$; $M_r = 344.4$, calcd. for $C_{22}H_{20}N_2O_2$.

For the residual steps the known procedure was applied; overall yield: 11%; homogenous on TLC ($CHCl_3$/MeOH/AcOH, 4:1:0.1, R_f 0.6; CH_3CN/H_2O, 4:1, R_f 0.3) and HPLC (t_R 12.01 min); ESI-MS: $m/z = 478.2$ $[M + H^+]$; $M_r = 477.4$, calcd. for $C_{29}H_{23}N_3O_4$.

Solid-Phase Synthesis of Linear APB- and AMPB-Peptides

The syntheses were performed manually or on the automatic peptide synthesizer (Advanced ChemTech) on a trichlorotrityl-resin loaded with Fmoc-Gly (loading: 0.56 mmol/g) according to the protocol of Table 1. The syntheses were carried out in a 0.12 mmol scale on the automatic synthesizer or 1.0 mmol scale on the manual synthesizer, using the following amino acid derivatives: Fmoc-Asp(O*t*Bu)-OH, Fmoc-Cys(S*t*Bu)-OH or Fmoc-Cys-(Trt)-OH, Fmoc-Thr(*t*Bu)-OH, Fmoc-Ala-OH, H-APB-OH or Fmoc-AMPB-OH, and Fmoc-Phe-OH. For all couplings, Fmoc-cleavage and washing steps, freshly distilled NMP was used as solvent. Coupling efficiency was monitored by the Kaiser test [24] or by the chloranil test [25]. For coupling of Fmoc-Cys(S*t*Bu)-OH or Fmoc-Cys(Trt)-OH the amount of the auxiliary base DIEA was reduced from 8 to 5 equivalents. The amino acid H-APB-OH was used in unprotected form. For acylation of the resin-linked H-APB-peptide the arylamine group was activated by silylation with 2 equivalents of

Table 1 Protocol of Peptide Synthesis on Fmoc-Gly-TCP Resin (Loading: 0.50 mmol/g)

Synthetic step	Reagents and solvents	Reaction time
Fmoc cleavage	20% (v/v) piperidine/NMP	3 + 15 min
Coupling	Stepwise amino-acylation in sequence mode with 4 equivalents of the amino acid derivatives: Fmoc-Asp(O*t*Bu)-OH, Fmoc-Thr(*t*Bu)-OH, Fmoc-Ala-OH, Fmoc-Ala-OH, H-APB-OH or Fmoc-AMPB-OH/4 equivalents HBTU/4 equivalents HOBt/8 equivalents DIEA in NMP; for coupling of the cysteine derivatives 4 equivalents Fmoc-Cys(S*t*Bu)-OH or Fmoc-Cys(Trt)-OH/4 equivalents HBTU/4 equivalents HOBt/5 equivalents DIEA in NMP	2 × 30 min
Coupling on H-APB-peptide-resin	Silylation with 2 equivalents *N*,*O*-bis(trimethylsilyl)acetamide; 3 equivalents Fmoc-Phe-F	2 × 45 min
Acidolytic cleavage	CH_2Cl_2/AcOH/trifluoroethanol/triethylsilane 8/1/1/0.15 (v/v)	2 × 30 min

N,*O*-bis(trimethylsilyl)acetamide and acylation was performed with Fmoc-Phe-F (3 equivalents). For incorporation of the AMPB moiety standard procedures were applied. Peptide cleavage from the resin was carried out with CH_2Cl_2/trifluoroethanol/AcOH/triethylsilane (8:1:1:0.15), in two steps of 30 min each at room temperature. The resin was filtered off, washed with CH_2Cl_2 and the filtrate was evaporated. The residual solids were dissolved in small amounts of trifluoroethanol and the crude products were precipitated with ether. The precipitates were collected by filtration or centrifugation and dried. The following peptides were obtained by this procedure.

H-Phe-APB-Ala-Cys(StBu)-Ala-Thr(tBu)-Cys(StBu)-Asp(OtBu)-Gly-OH (8). Yield: 54%; HPLC: t_R 12.17 min; ESI-MS: $m/z = 1298.4$ $[M + H^+]$; $M_r = 1297.5$, calcd. for $C_{60}H_{87}N_{11}O_{13}S_4$; amino acid analysis: Asp 1.00 (1), Thr 0.76 (1), Gly 1.00 (1), Ala 1.94 (2), Cys 1.64 (2), Phe 0.74 (1); peptide content: 95%.

H-Phe-AMPB-Ala-Cys(StBu)-Ala-Thr(tBu)-Cys(StBu)-Asp(OtBu)-Gly-OH (14). Yield: 50%; HPLC: t_R 12.02 min; ESI-MS: $m/z = 1312.8$ $[M + H^+]$; $M_r = 1311.4$, calcd. for $C_{61}H_{88}N_{11}O_{13}S_4$; amino acid analysis: Asp 1.00 (1), Thr 0.83 (1), Gly 1.02 (1), Ala 1.97 (2), Cys 1.66 (2), Phe 0.93 (1); peptide content: 96%.

H-Phe-APB-Ala-Cys(Trt)-Ala-Thr(tBu)-Cys(Trt)-Asp-(OtBu)-Gly-OH (16). Yield: 54%; HPLC: t_R 12.30 min; ESI-MS: $m/z = 1606.8$ $[M + H^+]$; $M_r = 1605.4$, calcd. for $C_{90}H_{99}N_{11}O_{13}S_2$.

H-Phe-AMPB-Ala-Cys(Trt)-Ala-Thr(tBu)-Cys(Trt)-Asp-(OtBu)-Gly-OH (17). Yield: 50%; HPLC: t_R 12.94 min; ESI-MS: $m/z = 1621.8$ $[M + H^+]$; $M_r = 1619.8$, calcd. for $C_{91}H_{101}N_{11}O_{13}S_2$.

c(Phe-APB-Ala-Cys(StBu)-Ala-Thr-Cys(StBu)-Asp-Gly) (10). To an ice-cold solution of PyBOP (122 mg; 0.23 mmol), HOBt (32 mg; 0.23 mmol) and DIEA (40 µl; 0.23 mmol) in freshly distilled DMF (100 ml) was added dropwise a solution of **8** (150 mg; 0.12 mmol) in DMF (200 ml) to reach a final peptide concentration of 4×10^{-4} M. After 12 h, the solvent was evaporated, the residue was dissolved in DMF (2 ml) and the product was precipitated with cold water. The crude product was collected by centrifugation, washed with water and dried. The residue was taken up in EtOAc, unsoluble material was filtered off and the filtrate was evaporated to dryness to yield the side-chain protected monocyclic peptide **9**. Deprotection of **9** was performed by dissolving the solid in TFA/CH_2Cl_2/triethylsilane (95:3.5:1.5; 20 ml). After 45 min, the reaction mixture was evaporated and the residue was intensively washed with MeOH; overall yield: 61 mg (45%); HPLC: t_R 10.44 min; ESI-MS: $m/z = 1168.8$ $[M + H^+]$; $M_r = 1167.4$, calcd. for $C_{52}H_{69}N_{11}O_{12}S_4$; amino acid analysis: Asp 1.01 (1), Thr 0.85 (1), Gly 1.00 (1), Ala 1.96 (2), Cys 1.65 (2), Phe 0.99 (1); peptide content: 93%.

c(Phe-AMPB-Ala-Cys(StBu)-Ala-Thr-Cys(StBu)-Asp-Gly) (15). The title compound was prepared from the linear precursor **14** as described for **10**, by cyclization with PyBOP and deprotection of the monocyclic

intermediate with TFA/CH_2Cl_2 (95:5); yield: 70%; HPLC: t_R 11.24 min; ESI-MS: $m/z = 1182.6$ [M + H$^+$]; $M_r = 1182.4$, calcd. for $C_{53}H_{71}N_{11}O_{12}S_4$; amino acid analysis: Asp 1.09 (1), Thr 0.95 (1), Gly 1.00 (1), Ala 2.10 (2), Cys 1.88 (2), Phe 0.99 (1); peptide content: 89%.

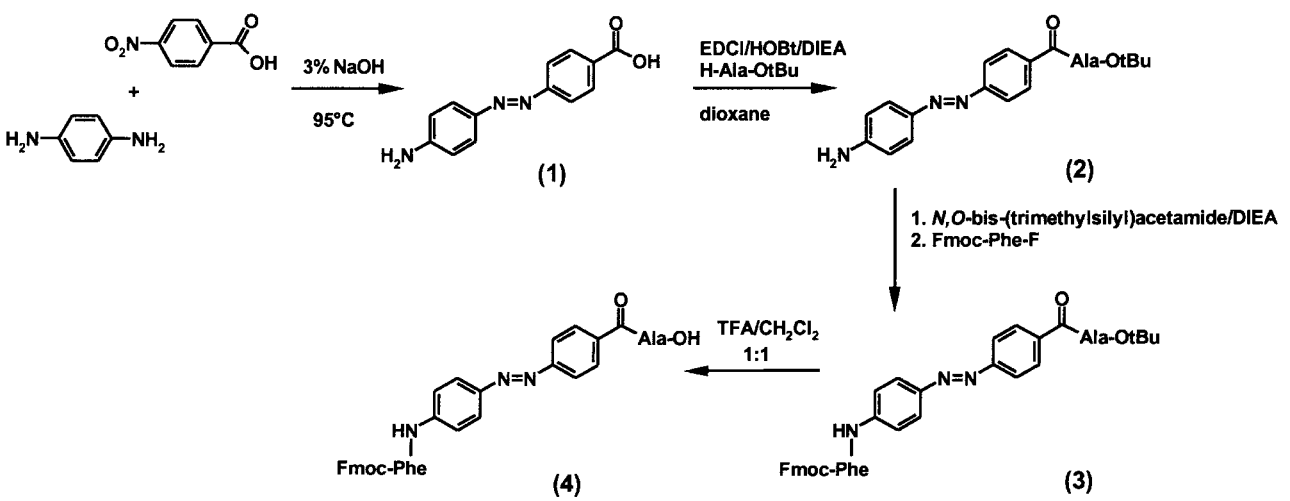
c(Phe-APB-Ala-Cys-Ala-Thr-Cys-Asp-Gly-) (18). The *S*-Trt-protected linear peptide **16** was cyclized as described for the synthesis of compound **10**. After deprotection with TFA/CH_2Cl_2/triethylsilane (95:3.5:1.5) the reaction mixture was evaporated and the residue washed with MeOH and dissolved in DMF at 10^{-4} M concentration. The apparent pH of the solution was adjusted to 8 with TEA, and 0.5 equivalents of di-*tert*-butyl azodicarboxylate were then added. During oxidation the solution was irradiated at 360 nm with a xenon lamp 450 XBO (Osram, München) at a light intensity of 0.5 mW/cm^2. After12 h, the solvent was evaporated, the product was dissolved in *tert*-butanol/water (1:1) and lyophylized. The crude product containing 10–15% bicyclic compound was purified by preparative HPLC on a 250/21 Nucleosil 300-5 C8 (Macherey and Nagel) by linear gradient elution from CH_3CN/0.1% TFA (1:9) to CH_3CN/0.08% TFA (8:2); yield: 7%; HPLC: t_R (*trans*) = 8.97 min, t_R (*cis*) = 9.10 min; ESI-MS: $m/z = 990.6$ [M + H$^+$]; $M_r = 989.4$, calcd. for $C_{44}H_{51}N_{11}O_{12}S_2$; amino acid analysis: Asp 0.89 (1), Thr 0.98 (1), Gly 1.09 (1), Ala 2.00 (2), Cys 1.39 (2), Phe 0.86 (1); peptide content: 85%.

c(Phe-AMPB-Ala-Cys-Ala-Thr-Cys-Asp-Gly-) (19). The *S*-Trt protected linear AMPB-peptide **17** was cyclized as described for the preparation of compound **10**. The resulting fully protected cyclic product was deprotected with TFA/CH_2Cl_2 (95:5) containing 6 equivalents of triethylsilane. The reaction mixture was evaporated and the residue was washed extensively with MeOH. The bis-cysteinyl-intermediate was then oxidized in DMF at 10^{-4} M concentration with 0.5 equivalents of di-*tert*-butyl azodicarboxylate, without the need of irradiation as described for **18**. The crude product containing 75–80% bicyclic compound was purified by HPLC as described for **18**; yield: 44%; HPLC: t_R (*cis*) = 8.55 min, t_R (*trans*) = 9.00 min; ESI-MS: $m/z = 1004.2$ [M + H$^+$]; $M_r = 1003.4$, calcd. for $C_{45}H_{53}N_{11}O_{12}S_2$; amino acid analysis: Asp 1.16 (1), Thr 0.96 (1), Gly 1.12 (1), Ala 2.00 (2), Cys 1.43 (2), Phe 1.00 (1); peptide content: 83%.

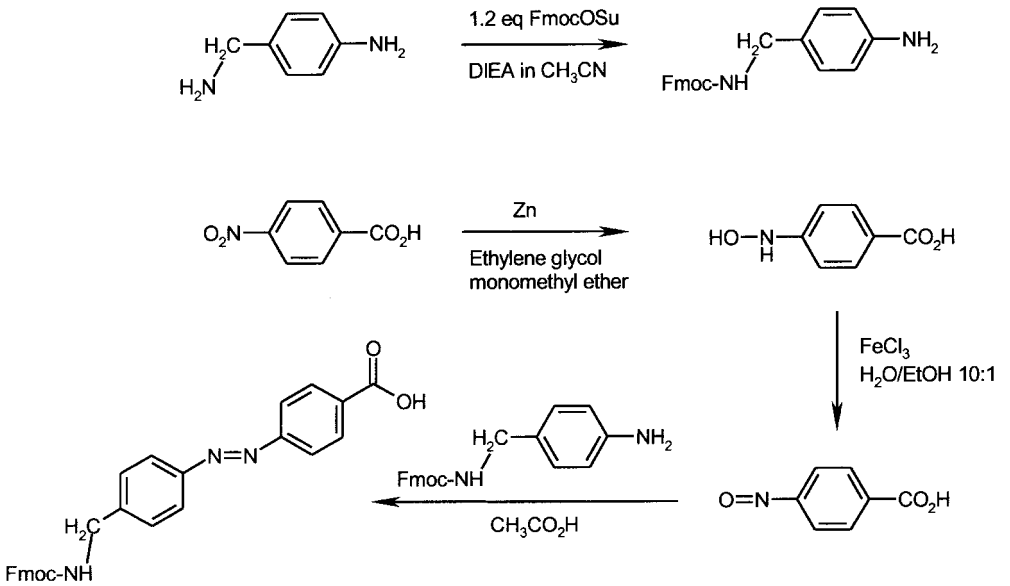
RESULTS AND DISCUSSION

Synthesis of the Monocyclic Azobenzene-Peptides in Solution

(4-Amino)phenylazobenzoic acid (H-APB-OH) (**1**) is readily accessible from 4-nitrobenzoic acid and 4,4-phenylendiamine, as shown in Scheme 1, following essentially the method of Schündehütte [22]. For the synthesis of (4-aminomethyl)phenylazobenzoic acid (H-AMPB-OH) the procedure described by Ulysse and Chmielewski [23] was partially modified since hydrogenation of *N*-Fmoc-(4-nitro)benzylamine in MeOH to produce *N*-Fmoc-(4-amino)benzylamine is accompanied by significant losses of the Fmoc group. This observation fully agrees with the reported instability of *N*-Fmoc protection toward hydrogenation over Pd catalyst [26]. Although by performing the hydrogenation in EtOAc the extent of Fmoc-cleavage could be minimized, the low nucleophilicity of arylamines [27,28] allows for

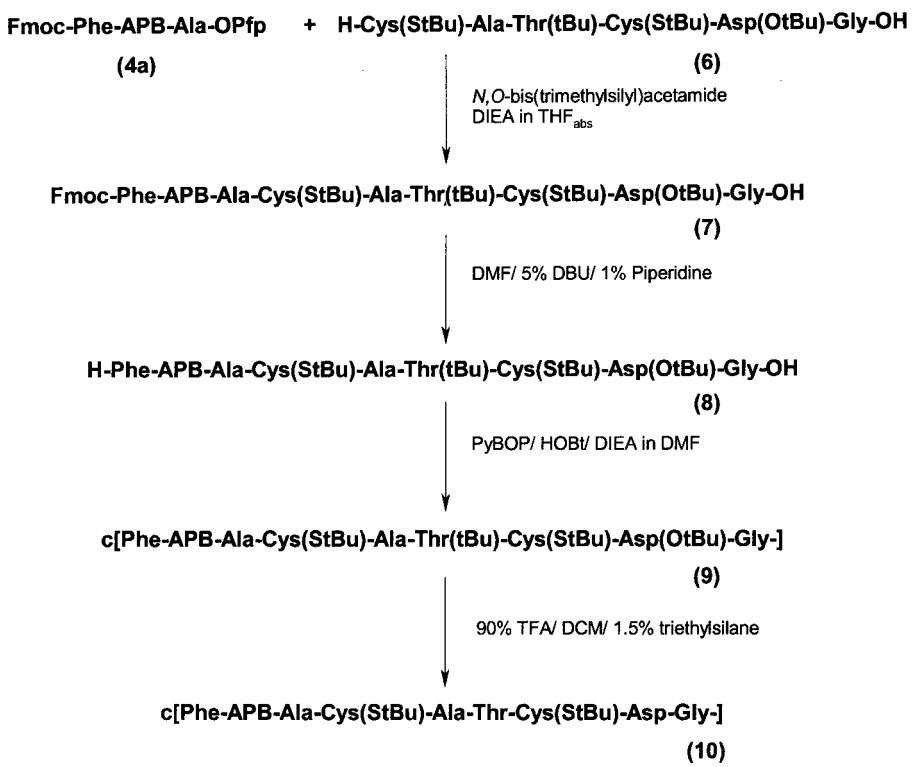


Scheme 1 Synthesis of the tripeptide **4** in solution.

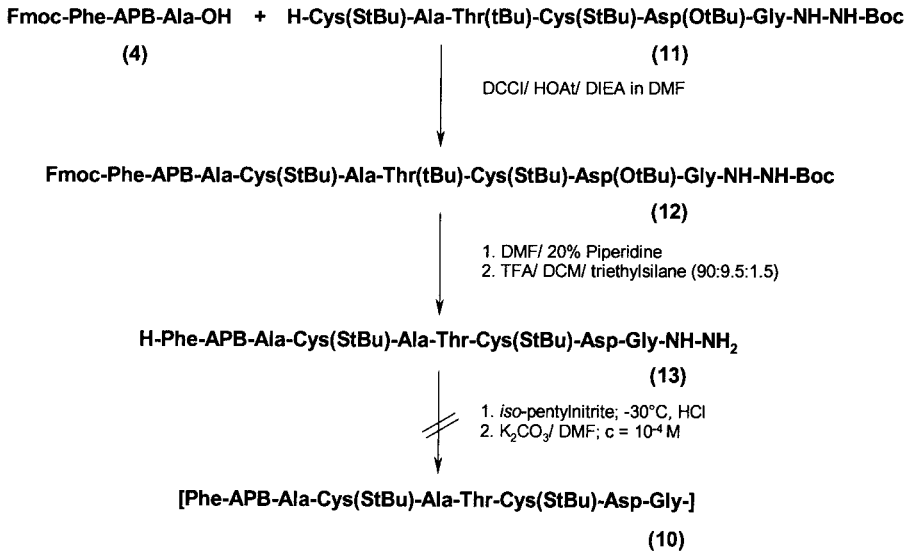


Scheme 2 Synthesis of *N*-Fmoc-4-(aminomethyl)phenylazobenzoic acid (**5**).

selective acylation of (4-amino)benzylamine with Fmoc-OSu to produce *N*-Fmoc-(4-amino)benzylamine (Scheme 2). In the subsequent steps the procedure of Ulysse and Chmielewski [23] was applied and Fmoc-AMPB-OH (**5**) was obtained in satisfactory overall yields.



Scheme 3 Synthesis of the monocyclic APB-peptide (**10**) in solution.



Scheme 4 Synthesis of the monocyclic APB-peptide (**10**) by the hydrazide method.

The amino acid H-APB-OH is a fully conjugated system which was characterized in its photochromic properties [29]. The very poor nucleophilicity of H-APB-OH allows for its use as carboxyl component without the need of *N*-protection in acylation steps. As shown in Scheme 1, even upon *C*-terminal derivatization of the amino acid H-APB-OH, the amino group still remains a poor nucleophile and therefore strong acylation procedures were required. In fact, coupling of Fmoc-Phe-OH with the dipeptide H-APB-Ala-O*t*Bu (**2**) was achieved only by silylation of the amino group and using the fluoride method [21]. Upon *C*-terminal deprotection of the intermediate **3** the tripeptide derivative Fmoc-Phe-APB-Ala-OH (**4**) was obtained as a possibly useful synthon.

For assembly of the linear nonapeptide **7** according to Scheme 3, the hexapeptide derivative **6** was synthesized in solution essentially following previous syntheses in this field [30]. Fragment condensation was performed, upon silylation of the hexapeptide **6** with *N*,*O*-bis(trimethylsilyl)acetamide, via the pentafluorophenyl ester of the synthon **4** without auxiliary base. With this condensation procedure, racemization of the *C*-terminal alanine residue did not occur as determined by the gas chromatographic racemization test [19]. Because of unfavourable solubility properties of both the *N*-protected APB-nonapeptide **7** and its N^{α}-deprotected form **8**, unsatisfactory yields were obtained. To possibly bypass this problem, the nonapeptide hydrazide **13** was prepared by condensation of the synthon **4** with the hexapeptide derivative **11** (Scheme 4). Although the fully protected intermediate **12** was obtained in good yields and conversion to the nonapeptide hydrazide **13** proceeded almost quantitatively, cyclization via the azide procedure failed. Besides Curtius rearrangement products, side reactions involving the APB moiety were observed at extents that prevented a successful purification of the desired cyclic peptide **10**. Conversely, cyclization of the side-chain protected APB-nonapeptide **8** via the PyBOP/HOBt/DIEA procedure [31] was found to produce in good yields the monocyclic APB-peptide **9** (Scheme 3). Thus the problem to be solved was a reasonably good accessibility of the linear precursor.

Synthesis of the Linear Azobenzene-Peptides on Solid Support

In previous syntheses of bis-cysteinyl-octapeptides related to the active sites of thiol protein oxidoreductases, racemization of the cysteine residues, whatever the thiol protecting group, was found to occur at extents of up to 30% using the standard HBTU/HOBt/DIEA (1:1:2) coupling procedure [30,32]. This high degree of racemization of cysteine residues was fully confirmed recently by a detailed study by Han *et al.* [33]. It can, however, be largely suppressed by the use of pentafluorophenyl esters [30,33]. For the synthesis of the

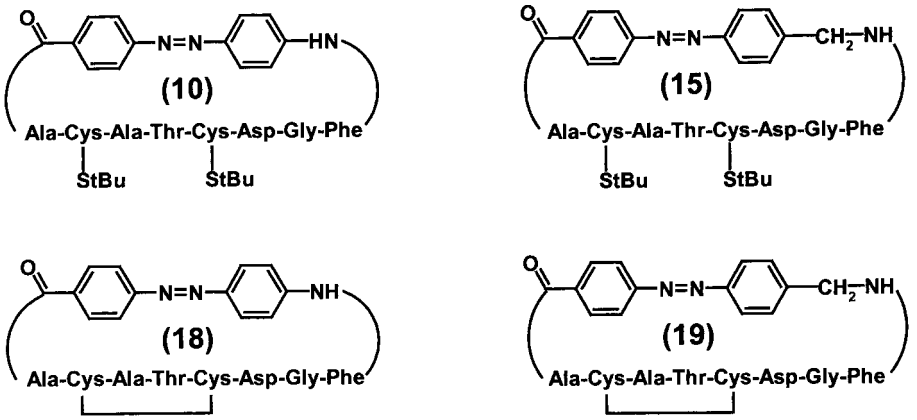


Figure 2 Structures of the mono- (**10**) and bicyclic (**18**) APB-peptide and of the mono- (**15**) and bicyclic (**19**) AMPB-peptide containing the active-site sequence of thioredoxin reductase; the *cis* or *trans* azo-configuration is deliberately not shown.

side-chain protected nonapeptide **8** on a solid support the chlorotrityl-resin [34] was chosen and the Fmoc/*t*Bu strategy [35]. Since pentafluorophenol is known to be relatively acidic ($pK_a = 5.53$ in water), its release in acylation steps with pentafluorophenyl esters was expected to cleave the growing peptide chain from the chlorotrityl-resin to some extent. Although acylation of the resin-bound hexapeptide **6** with Fmoc-Phe-APB-Ala-OH (**4**) was found to proceed in satisfactory manner, high losses of peptide material were observed in the acylation steps with Fmoc-Cys(S*t*Bu)-OPfp. Consequently, unsatisfactory yields of the desired linear nonapeptide **8** were obtained.

Careful optimization of the HBTU/HOBt/DIEA [36] procedure in the Fmoc-Cys(S*t*Bu)-OH coupling steps with determination of epimerization by the gas chromatographic racemization test [20] led to the conditions reported in Table 1. Reduction of the auxiliary base DIEA to 1.25 equivalents was found to suffice for largely preventing racemization at the two crucial cysteine coupling steps. Moreover, adaptation of the experiences collected in the solution synthesis of the synthon **4** to the solid phase synthesis led to incorporation of the APB moiety by the standard HBTU/HOBt/DIEA procedure without *N*-protection and, upon silylation of the amino group with *N*,*O*-bis(trimethylsilyl)-acetamide, acylation was performed with Fmoc-Phe-F [21]. Subsequent mild acidolytic cleavage of the APB-nonapeptide from the resin with CH_2Cl_2/AcOH/trifluoroethanol/triethylsilane (8:1:1:0.15) [37] produced the side-chain protected linear precursor **8** in sufficiently homogeneous form for its direct use in the cyclization step. This was performed by the PyBOP method and upon acidolytic deprotection, the bis-*S*-*tert*-butylthio derivative of the monocyclic APB-peptide **10** was isolated in satisfactory yields as an analytically well-characterized compound (Figure 2).

The linear nonapeptide containing as azobenzene unit the AMPB derivative **14** was synthesized without particular difficulties on the chlorotrityl-resin using Fmoc-AMPB-OH (**5**) and following the procedures reported in Table 1. Cyclization of **14** by the PyBOP method followed by acidolytic deprotection afforded the monocyclic AMPB-peptide **15** (Figure 2) as a homogenous and well-characterized compound.

Synthesis of the Bicyclic Azobenzene-Peptides

In the conversion of the thiol-protected monocyclic azobenzene-peptides to the thiol-free forms additional difficulties were encountered due to the facile reduction of the azo moiety by strongly reducing thiols, e.g. DTT, by excesses of tributylphosphine, and even by equivalent amounts of (tris-carboxyethyl)phosphine [38], all in aqueous DMF. As reduction of the azo moiety proceeds at rates comparable to those of the reductive cleavage of the *S*-*tert*-butylthio group, the *S*-trityl group was selected as alternative thiol-protection. The linear APB- and AMPB-peptides (**16**, **17**) were synthesized according to the protocol of Table 1, cleaved from the resin in the side-chain protected form and finally cyclized by the PyBOP method. Acidolytic deprotection of the monocyclic APB-peptide was performed in the presence of

triethylsilane to capture the trityl cations. The monocyclic bis-cysteinyl APB-peptide was obtained without detectable reduction of the azo group by the trialkylsilane scavenger. In order to avoid side reactions at the azobenzene moiety di-*tert*-butyl azodicarboxylate [39] was used for oxidation to the bicyclic form **18** (Figure 2). Even though the reaction was performed at high dilution, and under irradiation at 360 nm to induce *trans*→*cis* isomerization of the azobenzene unit and thus to facilitate the side-chain cyclization as predicted by molecular dynamics calculations, the bicyclic compound **18** was formed only in 10–15% yield as monitored by HPLC, the other products being mainly oligomers. Applying the identical strategy, the cyclic side-chain protected bis-*S*-Trt-monocyclic AMPB-peptide was obtained and then deprotected using only 10 equivalents of triethylsilane. In fact, the AMPB moiety was found to be more susceptible to reduction than the APB group. However, the amount of scavenger used proved to be sufficient to capture the trityl cations quantitatively. Finally, the resulting monocyclic bis-cysteinyl-AMPB-peptide was oxidized in DMF, again with di-*tert*-butyl azodicarboxylate, to produce in almost quantitative manner the bicyclic AMPB-peptide **19** (Figure 2), even in the absence of irradiation.

CONCLUSION

The mono- and bicyclic APB- and AMPB-peptides are photoresponsive systems with the *cis*/*trans* isomerization occurring in a reversible manner. Although the peptides are not water-soluble and thus measurement of the redox potentials was impossible, ^{1}H-NMR conformational analysis of the monocyclic peptides clearly revealed that the monocyclic APB-peptide **10** relaxes from the highly rigid structure of the *trans*-azo-isomer into an ensemble of less constrained conformers of the *cis*-azo-isomer via a well defined light-switchable two-state conformational transition [40]. On the other hand, the monocyclic AMPB-peptide **15** which contains the methylene group as spacer at the amino function, has greater conformational freedom, the *trans*-azo-isomer exhibiting two conformers (populated 3:1). These results suggest that the APB-peptide represents a more suitable template than the AMPB-peptide for the design of water-soluble photoresponsive redox-active molecules.

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