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Side Reactions during Photochemical Cleavage of an α-Methyl-6-nitroveratryl-based Photolabile Linker

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Abstract: The mechanisms of reactions causing irreversible inhibition of the activity of enzymes when irradiated in the presence of the recently developed α-methyl-6-nitroveratryl-based photolinker [Holmes CP. *J. Org. Chem.* 1997; **62**: 2370–2380] have been investigated. Several experiments based on the interaction of the photolinker with model peptides or *n*-butylamine have been accomplished. A complexity of products, resulting from the side reactions competing with the 'normal' photocleavage of the linker, have been found. The amino and thiol groups of the molecules present in the solvents upon irradiation were recognized as having a major influence on the course of photolysis. Some of these side products resulting from the interaction with amines were identified and the mechanisms by which they can be generated are discussed. The mechanism of the interaction of the thiol groups present in peptides or proteins with the photolinker is unclear and it remains to be further elucidated. It was found that the undesirable effects are favored by a basic pH and are largely reduced by a slightly acidic pH, together with the presence of dithiothreitol. Significant positive effects of dithiothreitol have been observed on the rate as well as the yield of the photocleavage. These results demonstrate that the use of photolabile linkers in biological media can be accompanied by undesired effects, which can be largely reduced by choosing appropriate conditions and additives.

Keywords: amine; enzyme; nitroveratryl; peptide; photocleavage; photolabile linker; side reactions; thiol

INTRODUCTION

The rapid development of solid phase combinatorial chemistry over the past few years has initiated the advancement of new methods for the screening of large combinatorial libraries for the identification of novel active compounds [1]. Libraries can be screened as immobilized compounds tethered to a solid support [2,3]. This approach is often complicated by the potential interference of target molecules with the surface of the support. The use of resin bound compounds is often problematic in the case of cell-based essays. On the other hand, the screening of soluble libraries [4–7], especially of a pseudo-peptide or non-peptide nature, can be negatively influenced by the limited solubility of such compounds. The ideal linker/resin should allow a complete synthesis, resist during the cleavage of the protecting groups and finally permit a non-destructive release of the final compounds.

Many laboratories have focused their efforts on the introduction of novel linkers based on photolabile groups releasing the desired molecules upon UV irradiation [8]. Photolytic cleavage offers a mild

Abbreviations: Ac, acetyl; Acm, acetamidomethyl; Ada, adamantyl; DIPEA, *NN*-diisopropylethylamine; DMF, *NN*-dimethylformamide; DMSO, dimethyl sulfoxide; Dnp, 2,4-dinitrophenyl; DTT, dithiothreitol; FAB MS, fast atom bombardment mass spectrometry; Fmoc, 9-fluorenylmethoxycarbonyl; HBTU, *O*-benzotriazol-1-yl-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high-performance liquid chromatography; Mcc, 7-methoxycoumarin-3-carboxylyl; TFA, trifluoroacetic acid; UV, ultra violet.

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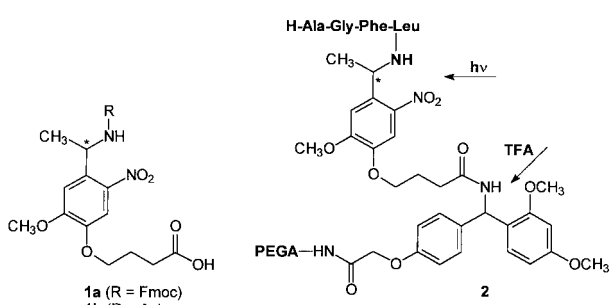
method for the release of synthesized compounds from the support, especially if the photolinkers (PLs) are stable under both acidic and basic conditions, and thus allow the removal of the protecting groups and thorough washing of the resin. This approach enables the release of bioactive ligands from only a few beads in a concentration sufficient for recognition by a target molecule, and thus overcomes the problem of the solubility of compounds that are poorly soluble in higher concentrations.

The recently developed and already commercially available PL **1a** [4-(4-(1-(9-fluorenylmethoxycarbonylamino)ethyl)-2-methoxy-5-nitrophenoxy)butanoic acid)] (Figure 1) represents a valuable tool for a solid phase synthesis of peptidic molecules as well as other organic structures [9,10]. The nitro group, together with two alkoxy groups on the benzene ring, facilitate the photolytic cleavage with >350 nm UV light, which is less harmful for proteins and peptides than UV light of shorter wavelengths. The introduction of the α-methyl group onto the benzylic carbon atom greatly enhances the PL cleavage kinetics rates even in aqueous media [9]. Thus, the PL **1a** provides improved properties compared with previously developed and used PLs [11,12].

These suitable properties of PL **1a** enable a rapid release of tethered compounds into biological media containing proteins; thus allowing *in situ* biological assays. This strategy was successfully applied for the screening of carboxyalkyl peptide inhibitor libraries of matrilysin [13].



2a H-Ala-Gly-Phe-Leu-NH_2
3 H-Gly-Phe-Phe-Tyr-Thr-Pro-Lys-OH
4 H-Leu-Tyr-Gln-NH_2
5 Ac-Leu-Tyr-Lys-NH_2
6 H-*D*-Gln(Ada)-Cys-Tyr-*D*-Trp-Lys-Val-Cys(Acm)-Thr-NH_2
7 Ac-Gly-Cys-Ala-Phe-NH_2

Figure 1 PLs and model peptides used in the irradiation experiments. Bonds cleaved by UV irradiation ($h\nu$) or trifluroacetic acid (TFA) are indicated by arrows. The asterisks indicate the chiral atoms.

We have chosen PL **1a** and a similar approach to that of Schullek *et al.* [13] for the study of the interactions of the Zn-metalloenzyme thimet oligopeptidase, with the peptides (inhibitors) released from the PL containing structures. Because of the inexplicable loss of enzymatic activity upon UV irradiation observed only in the presence of structures bearing the PL without inhibitors, we focused on the possibility of side reactions of PL **1a** with compounds of a peptide/protein nature.

Experiments were performed using the *N*-acetylated form (**1b**) of PL **1a** in solution or using a model peptide synthesized on the PL attached to an insoluble support via the TFA labile rink linker (**2**, Figure 1). These structures were irradiated at 365 nm in the presence of the primary amine or model peptides containing groups such as primary amines or thiols; thus mimicking proteins in biological media. Our attention was focused on (i) the identification of major side reactions (side products), and (ii) their elimination by appropriate conditions or additives. We have identified the reactive groups of the PL and several side products resulting from the interactions of these reactive groups with the compounds present in the solution upon UV irradiation were disclosed and identified. The possible reaction mechanisms resulting in these side products are discussed and the conditions for the effective suppression of these undesired reactions are defined.

MATERIALS AND METHODS

Materials

Fmoc protected amino acids, resins, HBTU, photolinker **1a** and fluorogenic substrate Mcc-Pro-Leu-Gly-Pro-D-Lys(Dnp) were purchased from Calbiochem-Novabiochem (Switzerland). All other chemicals and solvents were supplied by Sigma-Aldrich (St. Louis, MO, USA).

Preparation of Rat Testes Homogenate (RTH)

Rat testes (25 g) were cut into small pieces and homogenized in 50 ml of ice-cold 20 mM Hepes/NaOH buffer, pH 7.4, containing 0.1 mM DTT, using a Teflon glass homogenizer. The homogenate was than centrifuged at $3500 \times g$ for 20 min. The pellet was discarded and the supernatant was centrifuged at $100\,000 \times g$ for 60 min. The final supernatant was checked for the thimet oligopeptidase activity with Mcc-Pro-Leu-GlyPro-D-Lys(Dnp) substrate as previously described [5]. The protein

concentration was determined, according to Bradford [14], to be about 15 mg/ml.

Irradiation Experiments

All experiments were done in a double-coated water-cooled glass container (volume about 1 ml) with an opened top. The container was irradiated from the top with a monochromatic UV lamp (UV Products, Upland, CA, model B 100 AP, 10 mW/cm^2 at 365 nm). All samples were purged with argon prior to UV irradiation.

Irradiation Experiments with RTH

About 1 μmol of the substituted PEGA resin was suspended in 300 μl of RTH (20 mM Hepes/NaOH buffer, pH 7.4) and gently stirred with a magnetic stirrer. The mixture was irradiated for 300 min as described above. The 5 μl samples of RTH were periodically checked for the enzymatic activity.

Photolysis of PL 1b in Solution in the Presence of Peptides 3, 4 and 5

Peptides **3**, **4** or **5** (1 μmol) and PL **1b** (1 μmol) were dissolved in 500 μl of a buffer containing 5% DMSO. The buffers used were 50 mM Hepes/NaOH buffer, pH 6.9 or 8.0 (peptides **3**–**5**) or 50 mM potassium phosphate buffer, pH 6.9 and 8.0 (peptide **3**). The irradiation was realized as described above. The reaction mixtures were periodically checked by HPLC (218 nm).

Photolysis of Resin 2 in the Presence of Peptides 3 and 6

Peptides **3** or **6** (2 μmol) and resin **2** (2 μmol) were suspended in 500 μl of 50 mM Hepes/NaOH buffer, pH 8.0, containing 25% methanol in the case of peptide **6** to achieve its solubility. In a parallel experiment, DTT was added (50 mM) to the mixture containing peptide **6**. The irradiation was arranged as described above. The reaction mixture was periodically checked by HPLC. After 90 min of irradiation, the resin was removed by filtration and thoroughly washed with the buffer, methanol, DMSO and dichloromethane (5 × 2 ml of each for 2 min). This resin was than cleaved for 1 h with 92.5% TFA containing 2.5% ethandithiol and 5% water (v/v). The cleavage mixture was evaporated to dryness *in vacuo* and analyzed by HPLC. Observed peaks were isolated and analyzed by mass spectrometry and amino acid analysis.

Photolysis of Resin 2 in the Presence of *n*-Butylamine

n-Butylamine (100 μmol) was added to resin **2** (1 μmol) in 500 μl of 50% methanol in water (pH > 10). The mixture was irradiated for 90 min as described above. The photolysis was checked by HPLC. The photo-released peaks were isolated and analyzed by mass spectrometry, amino acid analysis and ^{1}H-NMR. After the irradiation, the resin was washed and cleaved with TFA as described before. The TFA cleavage mixture was evaporated and analyzed by HPLC.

Photolysis of Resin 2 in the Presence of Peptide 7

Peptide **7** (10 μmol) was added to resin **2** (5 μmol) in 1500 μl of 50 mM Hepes/NaOH buffer, pH 8.0, containing 8% methanol and 8% DMSO (to achieve solubility). The mixture was irradiated for 350 min as described above. In parallel, resin **2** was irradiated under the same conditions alone or with peptide **7** in the presence of 50 mM DTT. The reactions were checked by HPLC. The resin was washed and cleaved with TFA as described above. Some of the peaks of the TFA cleavage were isolated by HPLC and characterized by mass spectrometry and amino acid analysis.

Syntheses

PL 1b. PL **1a** was anchored to the 2-chlorotrityl resin according to Barlos [15]. The Fmoc protecting group was removed with 20% piperidine in DMF. The *N*-terminal acetylation was performed with 5% acetic anhydride and 1% DIPEA in DMF. The resin was treated (2 h) with the mixture of acetic acid, trifluoroethanol and dichloromethane (2:2:6 v/v). The cleavage mixture was evaporated to dryness *in vacuo*. The purity of PL **1b** was checked by HPLC.

Ac-(βAla)$_3$-PEGA and Ac-(βAla)$_2$-PL-PEGA. The structures were synthesized on the amino-PEGA resin with Fmoc-βAla and PL **1a** using HBTU/DIPEA *in situ* strategy [16]. Fmoc protecting group was removed with 20% piperidine in DMF. The *N*-terminal acetylation was accomplished with acetic anhydride. The completeness of the coupling reactions was followed by the ninhidrine test [17] and by quantitative determination of the Fmoc group [18].

Resin 2. The structure was synthesized on the amino-PEGA resin with Fmoc protected amino acids, PL **1a** and Fmoc-rink linker using HBTU/DIPEA *in situ* strategy as described above.

Peptide 3. Peptide **3**, as a part of the *C*-terminus of the porcine insulin B-chain, was prepared by trypsinolysis of porcine insulin [19] and purified by HPLC and characterized by amino acid analysis and mass spectrometry.

Peptides 4–7. The peptides were prepared by a solid-phase synthesis using the rink amide AM resin and Fmoc/HBTU/DIPEA *in situ* strategy as described above. The peptides were cleaved from the resin with 92.5% TFA containing 2.5% triisopropylsilane, 2.5% ethanedithiol and 2.5% water (v/v), then extracted with diethylether, lyophilized, purified by HPLC and characterized by amino acid analysis and mass spectrometry.

Analyses

HPLC. HPLC was performed on the SP 8800 HPLC apparatus (Spectra Physics) using a linear gradient of 8–80% acetonitrile in water (0.1% TFA, 25 min). Analyses were performed using the Nucleosil 120 C18 5 μm column (Watrex, Prague) and peptides were purified on the Vydac 218TP510 semi-preparative column.

Mass spectrometry. The mass spectra were measured on the ZAB-EQ device (VG Analytical, Manchester, UK) by the FAB technique (Xe 8 kV).

Amino acid analysis. The samples for amino acid analysis were hydrolyzed with 6 M HCl at 110°C for 20 h (tryptophane containing samples were hydrolyzed with the addition of 4% thioglycolic acid). Cysteine was determined as cysteic acid after the oxidation with peroxoformic acid (1 h). The analysis was performed on the Biochrom 20 apparatus (Pharmacia, Uppsala, Sweden).

NMR. The ^{1}H-NMR spectrum was obtained from the FT-NMR spectrometer Varian UNITY-500 MHz. The sample was dissolved in d_6-DMSO.

RESULTS AND DISCUSSION

Oligopeptidase Activity upon UV Irradiation

In order to check the enzymatic stability upon UV irradiation we performed several experiments in which a crude RTH (pH 7.4) containing thimet oligopeptidase (EC 3.4.24.15) [20] was irradiated alone or in the presence of structures containing the PL tethered to a solid support (Table 1). As a solid support we chose the PEGA resin [3,21–23]. For the blank experiments we have used the substituted support without inhibitor (the structure formed by *N*-acetylated β-alanines attached to the PEGA resin via PL **1a**). The data in Table 1 clearly show that the thimet oligopeptidase is almost stable when irradiated alone as well as in the presence of resin without the PL. We detected a linear decrease of the enzymatic activity in the presence of the structure *N*-Ac-(βAla)$_2$-PL-PEGA, which resulted in a loss of the activity of about 60% after 300 min of irradiation. The addition of 50 mM semi-carbazide or 5% DMSO/5 mM DTT significantly improved the enzyme stability.

Table 1 Oligopeptidase Activity in RTH upon UV Irradiation (365 nm, 300 min) under Different Conditions

Conditions	UV	Relative enzymatic activity (% ± 5)
RTH	−	100
RTH	+	96
RTH + Ac-(βAla)$_3$-PEGA	+	96
RTH + Ac-(βAla)$_2$-PL-PEGA	−	97
RTH + Ac-(βAla)$_2$-PL-PEGA	+	38
RTH + Ac-(βAla)$_2$-PL-PEGA + 50 mM semicarbazide	+	86
RTH + Ac-(βAla)$_2$-PL-PEGA + 5% DMSO/5 mM DTT	+	92

These results led us to consider the role of the PL moiety upon UV irradiation in the deactivation of the oligopeptidase. Several authors have described covalent modifications of *m*- and *p*-alkoxy substituted nitrobenzenes by strong nucleophilic agents, such as primary amines [24,25] or thiols [26]. Kaplan *et al.* [27] have observed a time-dependent inhibition of Na, K-ATPase activity when irradiated in the presence of 1-(2-nitro)phenylethyl phosphate. This inhibition was effectively suppressed by the addition of the thiol agents glutathione and sodium bisulfite. We designed several model experiments to investigate the possibility of these reactions.

Photolysis of PL 1b in Solution in the Presence of Peptides 3, 4 and 5

Peptides **3**–**5** (Figure 1) possessing primary amino groups (either α- or ε-, or both) were irradiated in solution in the presence of an acetylated form of PL **1b**. We examined the influence of pH and the type of buffer on the photolytical reactions. We observed a significant time-dependent decrease in the concentration of the peptides during UV irradiation (Table

2). The most important loss (47%) was detected in the Hepes buffer pH 8.0 in the case of peptide **3** possessing both types of amino groups. The difference in the loss of peptides **4** and **5** signalizes that α- and ε-amino groups differ in their reactivity. A higher pH clearly favors the loss in the peptide concentration. This may indicate that the amino group deprotonation increases the possible side reactions. No significant differences in the photolysis products were observed in the irradiated samples containing PL **1b** alone or in the presence of the peptides (three major peaks, data not shown). No precipitation was observed in the samples. We cannot exclude that the peptides form very hydrophobic compounds with the PL **1b** moiety upon UV irradiation. These could be irreversibly retained on the reverse-phase C18 column. The major peaks in the reaction mixtures containing peptide **3** were isolated and analyzed by FAB MS. We did not find any signal corresponding to the hypothetical adduct of peptide **3** and PL **1b**. We also did not find a signal corresponding to the final product of the PL cleavage (compound **1c**, Figure 2) [9,28] among the m/z signals obtained from the isolated peaks. Nevertheless, we detected the m/z signal of 547.0 for which we propose a structure consisting of two photolyzed molecules forming a dimer via –NO=N– bond (theoretical $MH^+ = 546.5$) (compound **1d**, Figure 2). The formation of this type of compound under similar conditions was observed by other authors [29,30].

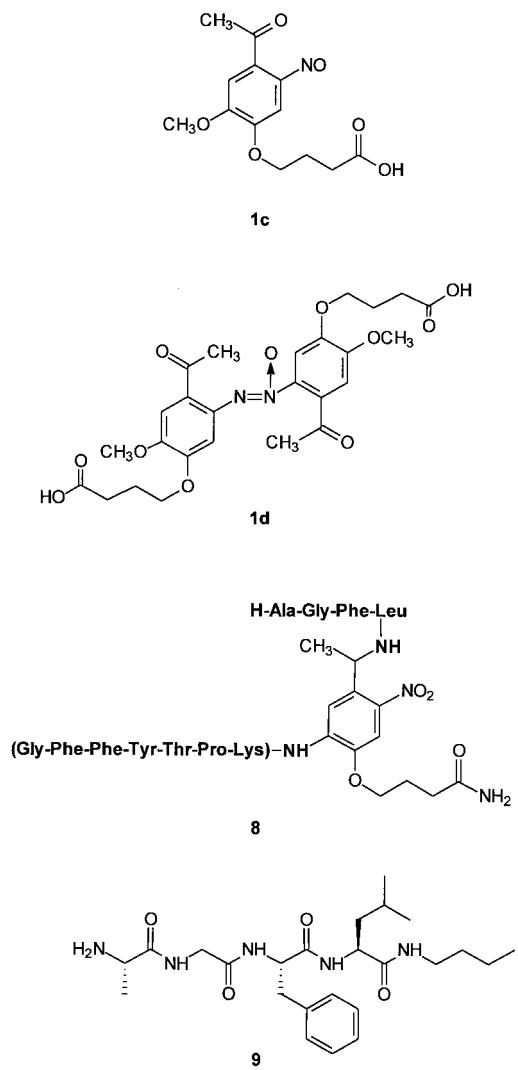


Figure 2 The proposed structures for some of the m/z signals identified by the MS analysis.

Photolysis of Resin 2 in the Presence of Peptides 3 and 6

The previous results led us to arrange further model experiments. PL **1a** bearing model peptide **2a** (Ala-Gly-Phe-Leu-NH_2) was attached to the PEGA resin via the TFA labile rink linker (**2**). The irradiation of this structure in the presence of peptides **3** and **6** thus mimics the conditions under which the PL moiety anchored to the resin is photolyzed in the presence of proteins. The goal of this approach was to isolate (after TFA cleavage from the resin) and characterize hypothetical products of the interaction of PL with peptides **3** and **6**. All the irradiation experiments were done at pH 8.0, which favors the observed side reactions as demonstrated in Table 2.

Table 2 The Loss of Model Peptides upon UV Irradiation (365 nm, 300 min) in Solution in the Presence of PL **1b** Dependent on the Used pH and Buffer

	Loss of peptide (% ± 5)			
Conditions	Hepes pH 6.9	Hepes pH 8.0	Phosphate pH 6.9	Phosphate pH 8.0
Peptide **3** (GFFYTPK)	17	47	9	24
Peptide **4** (LYQ-NH_2)	2	22		
Peptide **5** (Ac-LYK-NH_2)	0	10		

Figure 3 shows the HPLC analyses of materials obtained by TFA cleavage of resin **2** irradiated in the presence of peptides **3** or **6** (photo-released peptide **2a** and added peptides **3** or **6** were removed from the irradiated resins by washing steps prior to TFA cleavage as described in the Materials and Methods section). Significantly more peaks appeared in the cleavage mixtures of resin **2** irradiated in the presence of peptide **3** (d) or peptide **6** without DTT (c) than in cleavage mixtures obtained from resin **2** irradiated alone (a) or with peptide **6** in the presence of 50 mM DTT (b).

Peaks 1, 2, 3 and 4 (Figure 3) were isolated and analyzed by MS and amino acid analysis. Because of the evident complexity of photoreactions occurring during our experiments we were able to explain only some of the m/z signals of the mass spectra obtained (data not shown). We were able to identify compound **8** (Figure 2) ($MH^+ = 1514.3$) and its fragments in peak 1 (Figure 3, d). This compound is the result of *ipso* nucleophilic substitution of the methoxy group of the PL moiety present in resin **2** by α-(Gly) or ε-(Lys) amino group of peptide **3** [24–26]. The mass spectra of peaks 2 and 3 revealed the presence of low molecular weight m/z signals. The appearance of the m/z signal of 406.4 in peak 2 indicates the presence of peptide **2a** ($MH^+ = 406.5$), which is the normal product of the photocleavage of resin **2** and it should not be found in the TFA cleavage mixture. Peaks 2 and 3 were not present when resin **2** was irradiated alone.

Peak 4 isolated from TFA cleavage mixture after irradiation of resin **2** in the presence of peptide **6** (Figure 3, c) rendered a complex spectrum of m/z signals from 651.3 to 2698.6. We observed the signal of 686.4, which fits in with the theoretical MH^+ of the unphotolyzed peptide–linker structure cleaved by TFA from resin **2** (686.8). Amino acid analysis of peak 6 provided the composition of amino acids offering the relative ratio between peptides **6** and **2a** approximately 2:1.

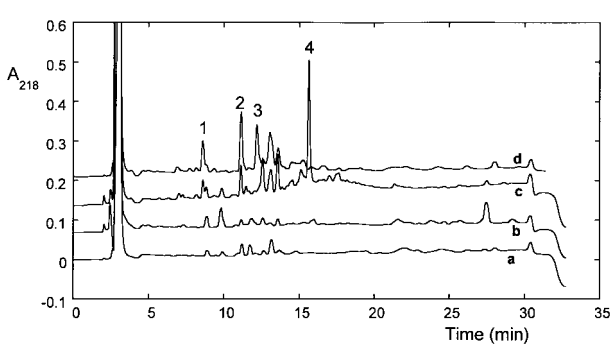


Figure 3 HPLC analyses of products of the TFA cleavage of the irradiated resin **2**. Resin **2** irradiated alone (a), in the presence of peptide **6** with 50 mM DTT (b), in the presence of peptide **6** without DTT (c), and in the presence of peptide **3** (d).

The change in the concentration of peptide **6** upon photolysis in the presence of resin **2** was monitored by HPLC (data not shown). Our findings suggest that DTT is able to protect peptide 6 upon irradiation or partially recover its concentration. We suppose that the reversible effect is due to the formation of the dimer of peptide 6. Nevertheless, these data demonstrate that also some irreversible processes independent of DTT take part during photolysis. The protective role of DTT on the stability of peptides will be discussed later.

Photolysis of Resin 2 in the Presence of *n*-Butylamine

The photoreaction of *n*-butylamine with 3,4-dimethoxy-1-nitrobenzene leading to the substitution of the alkoxy groups by amine was already documented [24,25]. Thus, we chose the strong nucleophile *n*-butylamine to study its possible interaction with resin **2** upon UV irradiation.

Resin **2** was irradiated in the presence of *n*-butylamine and the photo-released peptides were analyzed by HPLC. Surprisingly, besides peptide **2a**, the normal photocleavage product of resin **2**, we observed the appearance of a more hydrophobic peak. This peak was isolated and identified by FAB MS, amino acid analysis and ^{1}H-NMR as Ala-Gly-Phe-Leu-butylamide (compound **9**, Figure 2). The ratio between the photo-released peptide **2a** and the respective peptide-butylamide was approximately 1:2. This compound is the result of the UV light catalyzed transamidation reaction between the C-terminally tethered peptide **2a** and the strong nucleophile butylamine. This type of interaction was observed during the photolysis of a similar PL in the presence of methylamine [31]. We did not find any significant HPLC peak after the TFA cleavage of resin **2** irradiated in the presence of *n*-butylamine.

Photolysis of Resin 2 in the Presence of Peptide 7

Several authors have noticed the possibility of interactions of the cysteine thiol group with aromatic photoactivable compounds [26,27,32,33]. We synthesized peptide **7** (Figure 2) to elucidate the role of the -SH groups in the interaction with PL **1a**. Peptide **7** was irradiated in the presence of resin **2**. The change in the concentration of the peptide **7**

monomer and dimer (disulfide) upon photolysis was monitored by HPLC (data not shown). The equilibria of both forms of peptide **7**, monomeric and dimeric, were present in the reaction mixture before irradiation due to the presence of DMSO [34]. Monomeric peptide **7** almost completely and rapidly disappeared after the beginning of irradiation and the concentration of the dimer remained almost unchanged during the irradiation time (200 min). After the irradiation was finished, the addition of DTT (50 mM) to the reaction mixture increased the concentration of the monomer but it was compensated by the decrease in the concentration of the dimer. These results are consistent with those observed by Jelenc *et al.* [26].

The HPLC analysis of the peaks obtained by the TFA cleavage of resin **2** irradiated in the presence of peptide **7** is displayed in Figure 4 (photo-released peptide **2a** and added peptide **7** were removed from the irradiated resin by washing steps prior to TFA cleavage as described in Materials and Methods section). Peaks 5–11 were isolated and characterized by MS and amino acid analysis.

We identified peptide **2a** and its fragments by FAB MS and amino acid analysis of the peak 6. The amino acid composition of peaks 7–9 showed the presence of Cys, Gly, Ala and Phe amino acids but not Leu. This result indicates the presence of some forms of peptide **7**. FAB MS analyses did not help to reveal the origin of the respective peaks. Nevertheless, the analysis of the peak 8 revealed the m/z signal of 873.3, which fits in well with the theoretical m/z value of the peptide **7** dimer (disulfide) ($MH^+ = 873.0$). We suppose that the presence of peptide **2a** in peak 6 and the dimer of peptide **7** in peak 8 resulted from the TFA mediated destruction of some more complicated molecules containing peptide **7** and the intermediates of the cleavage of resin **2**.

Both peaks 10 and 11 contain Ala, Gly, Phe and Leu amino acids and no Cys. The mass spectra revealed the m/z signals of 406.2 and 686.3 in both peaks. The former signal should be that of peptide **2a** resulting from the fragmentation of the unphotolyzed peptide–linker structure cleaved from resin **2** by TFA (see Figure 1), which corresponds to the latter m/z signal (calculated $MH^+ = 686.8$).

We analyzed only a subset of the peaks obtained by the TFA cleavage from resin **2** photolyzed in the presence of peptide **7** (Figure 4). We observed the presence of peptide **7** containing structures on the resin after UV irradiation and thorough washing. As the only reactive moiety in peptide **7** is the cysteine thiol group, its mode of interaction with the PL moiety still remains to be investigated.

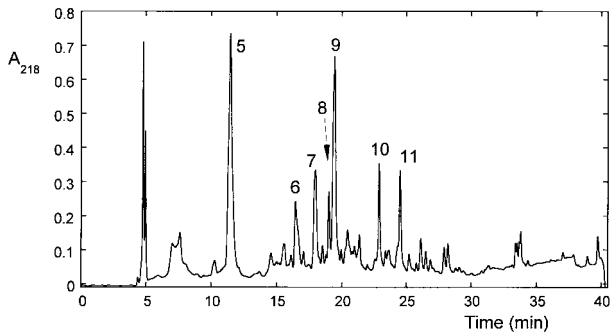


Figure 4 HPLC analysis of products of the TFA cleavage of resin **2** irradiated in the presence of peptide **7**.

Influence of DTT on Photocleavage Rates and Yields

We compared the rates of the photolytic release of peptide **2a** from resin **2** (i) alone, (ii) in the presence of peptide **7**, and (iii) in the presence of 50 mM DTT. The time-dependent release of peptide **2a** was monitored by HPLC and is shown in Figure 5. We observed apparent differences in the release of peptide **2a** in these three parallel experiments. The highest yield of the photoproduct was observed in the presence of DTT with the estimated $t_{1/2}$ of about 13 min (Figure 5, a). The photocleavage of resin **2** without DTT was much slower ($t_{1/2} > 80$ min) and rendered only about 65% of peptide **2a** after 350 min compared with the experiment accomplished in the presence of DTT (Figure 5, b). Finally, the photocleavage in the presence of peptide **7** (Figure 5, c) proceeded with the estimated $t_{1/2}$ of about 13 min, which is the same as in the presence of DTT.

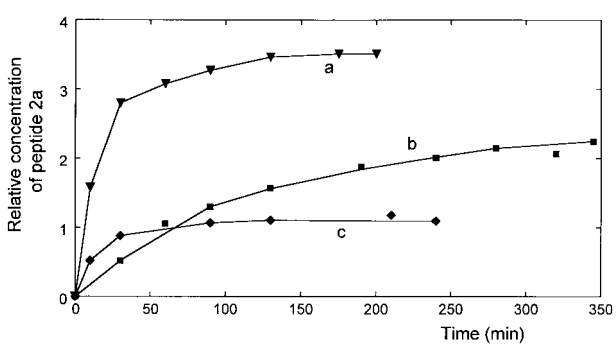


Figure 5 Influence of the presence of DTT and peptide **7** on rates and yields of photocleavage of resin **2**. Photolysis of resin **2** in the presence of DTT (a), alone (b), and in the presence of peptide **7** (c).

However, the final yield of peptide **2a** was only about 30% of the yield observed in the presence of 50 mM DTT (Figure 5, a).

These data indicate that DTT has a clearly positive effect on the photocleavage of resin **2**. The presence of DTT accelerates the cleavage and also leads to higher yields of the released peptide **2a**. On the other hand, the presence of peptide **7**, containing one thiol group, accelerates the cleavage in similar way as DTT but with a significantly lower final yield of peptide **2a**. This significant influence of DTT on the rate of photoclevage of PL **1a** is in contradiction to the data published by Holmes [9]. He observed only a weak acceleration of the photocleavage of the *N*-acetylated form of PL **1a** in the presence of DTT.

The mechanism of the action of DTT on the acceleration of photolysis of 1-(2-nitro)phenylethyl phosphates was proposed by Walker *et al.* [32]. They observed a DTT mediated disappearance of the photocleavage nitrosoketone product. The authors proposed that the reaction of the nitrosoketone product with the thiol groups could be the cause of the inhibition of enzymes present upon photolysis [27]. Nevertheless, this theory does not explain why peptide **7** decreased the yield of photolysis (Figure 5, c).

We suppose that both DTT and peptide **7** can act as scavengers of free radicals quenching the photocleavage (e.g. singlet oxygen) or react with the nitrosoketone product (compound **1c**, Figure 2) [32]. Nevertheless, we are convinced the thiol group of peptide **7** is also able to interact covalently with some of the intermediates of the PL photolysis and not exclusively with the final nitrosoketone product. Such a structural change of the PL moiety thus stops effectively its photocleavage. On the other hand, the thiol groups of DTT do not cause this effect, probably because of its tendency to preferential intramolecular cyclization leading to the disulfide bond (resulting six-membered ring is an optimal arrangement).

CONCLUSIONS

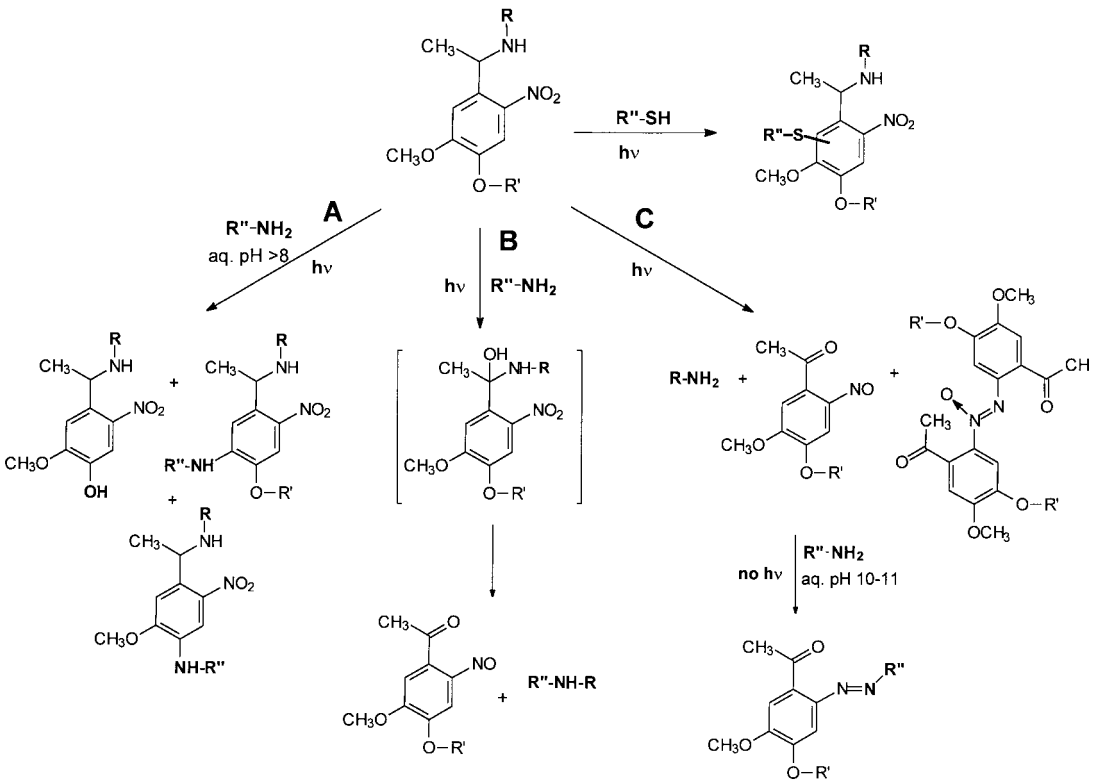
We have analyzed UV light mediated interactions of model peptides and *n*-butylamine with the recently developed α-methyl-6-nitroveratryl-based PL. We have found a complexity of products resulting from the side reactions competing with the normal photocleavage of the linker. The possible concurrent reactions of the PL groups are summarized in Scheme 1. The amino and thiol groups of the molecules occurring in the solvent upon photolysis have a major effect on the course of the photoreaction. The mechanism of the interaction of thiols with the photoreactive moiety is unclear and remains to be further investigated.

Photolysis under basic pH cannot be recommended because of the high risk of the nucleophilic substitution on the aromatic ring (Scheme 1, A) by molecules carrying a free amino group (either photo-released peptide or some compounds in the solution). This type of reaction is well documented [24–26]. Our experiments provided only indirect evidence of such side products (Figure 2, compound **8**) as the analysis of the complex reaction mixtures (complicated by a low concentration of single side products) was beyond the limit of our analytical capabilities. Walker *et al.* have demonstrated that the photolysis of a related photolabile compound is an acid catalyzed process [32] and thus slightly acidic conditions upon photolysis of the PL **1a** are recommended, which is in agreement with our findings.

Another side reaction can cause a modification of the *C*-terminus of a released peptide (Scheme 1, B). Its *C*-terminal amide bond (tethered form) can be attacked upon irradiation by an amine from the solution and thus the products of photolysis are both the 'normally' photo-released peptide and its transamidated derivative (Figure 2, compound **9**). This side reaction was already mentioned by Hammer *et al.* [31]. The authors described a peptide–methylamide side product upon photolysis in the presence of methylamine. We propose that by the same principle Hammer *et al.* successfully accelerated the photolysis by the addition of aqueous ammonia. In fact, in such a way, the concurrent reaction provided a peptide–amide, the same product as that of the 'normal' photocleavage.

The third type of possible side reactions can be caused by the reactivity of the products of the 'normal' photolysis (Scheme 1, C). These reactions are mediated by basic pH and the literature offers a number of examples [29,30,32,35]. We did not obtain clear evidence of these side effects, but we found supporting indices provided by the analyses of the photolyzed materials (Figure 2, compound **1d**).

Our general observation is that molecules, both the photo-released peptides and/or similar species in solution, are able to attack the PL moiety upon UV irradiation (both the photolyzed and



Scheme 1 Concurrent reactions occurring upon UV irradiation of α-methyl-6-nitroveratryl-based PL. R is an acyl group, R′ is a linking moiety (e.g. butyryl) and R″ is an alkyl group.

unphotolyzed) and thus produce a variety of side products leading to a less effective photolysis. We have found that the undesirable side reactions are favored in general by basic pH and largely reduced by slightly acidic pH, together with the presence of DTT, which can act as a radical scavenger. We believe this study could be useful for future applications of PL **1a**, which can be a powerful tool for the studies of interactions between proteins and ligands.

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