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The Effect of Linker Length on the Photomodification of Tyr Residue in *N*-(Tyrosyl)-*N*'-(5-azido-2-nitrobenzoyl)-diaminoalkanes

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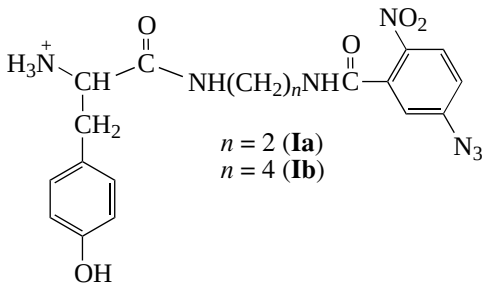
Abstract—*N*-(Tyrosyl)-*N*'-(5-azido-2-nitrobenzoyl)-1,4-diaminobutane containing a Tyr residue connected with the photoreactive aryl azide group through a diaminobutylene linker was synthesized as a model for studying the photomodification of Tyr residues in proteins. This compound and the compound with a shorter, 1,2-diaminoethylene linker, obtained previously, were subjected to a computer modeling to find their minimal energy conformations. The aromatic rings of Tyr and 5-azido-2-nitrobenzoic acid residues in the latter compound were localized in parallel planes at a distance of approximately 0.3 nm between them and were shown to be implicated in stacking interaction. On the contrary, the planes of aromatic rings of the former compound with a longer diaminobutylene linker were found to be situated perpendicularly to each other, with the distance between the centers of the rings being approximately 0.6 nm. The computer analysis was confirmed by experimental results: when studying the photomodification of the compound with the diaminobutylene linker, neither stable products of the Tyr photomodification nor unstable products capable of transformation into stable products in the dark were found. On the contrary, such products were previously identified in the case of the compound with diaminoethylene linker. The formation of amino, nitro, azoxy and azo derivatives was common for the photomodification of both compounds.

Key words: amino acid photoderivatives; tyrosine, 5-azido-2-nitrobenzoyl derivatives, photoderivatives

INTRODUCTION

The method of photoaffinity modification is widely used for studies of enzyme active sites as well as protein–nucleic acid and protein–protein interactions in supramolecular structures. The amino acid residues subjected to photomodification were determined upon the studies of protein binding sites by means of substrate analogs with aryl azide groups [1]. The substrate analogs containing a 5-azido-2-nitrobenzoic acid residue were shown to modify the Tyr residue in the majority of cases [1–3]. However, the modification products have not yet been identified, because it is hard to accumulate sufficient amounts of homogeneous photomodified peptides for the NMR study. Only electron absorption spectra were recorded for a few peptides containing Tyr residue modified by a derivative of 5-azido-2-nitrobenzoic acid. These spectra remain the only physicochemical characteristic of such products [2, 3].

Previously, we synthesized a model compound, *N*-(tyrosyl)-*N*'-(5-azido-2-nitrobenzoyl)-1,2-diaminoethane (**Ia**), in which the tyrosine and 5-amino-2-nitrobenzoic acid residues are connected by a diaminoethylene linker [4]. We demonstrated that the triplet 4-nitrobenzoylnitrene formed upon the irradiation of an (**Ia**) aqueous solution with the light with λ 313–365 nm can efficiently modify the Tyr residue in the same model molecule to give the stable product, cyclo-[1-(4'-nitro-3'-benzoyl)-2-(aminotyrosyl)-*N*,*N*'-ethylenediamine] (**IIa**). In addition, an unstable product **IIb** was



n = 2 (**Ia**)
n = 4 (**Ib**)

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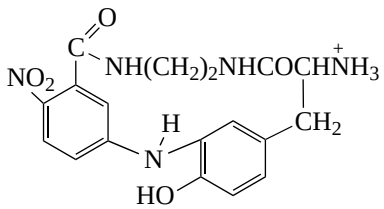
also found, which was almost completely converted into (**IIa**) in the dark. The electronic absorption spectra of (**IIa**) (λ_{max} 413 nm) and a peptide fragment of the Klenow fragment whose Tyr766 was modified by a 5-azido-2-nitrobenzoic acid residue introduced into the U residue of the primer of the primer–matrix duplex (λ_{max} 410 nm) [3] were similar in the area of 300–450 nm. Therefore, the structures of this stable product with a modified Tyr residue and (**IIa**) should be similar.



(**IIa**)

Since the Tyr and 5-azido-2-nitrobenzoic acid residues in (**Ia**) are connected by a short artificial linker, it was important to reveal the effect of an increase in the linker length on the intramolecular photomodification of the Tyr residue. In this study, we synthesized the model compound, *N*-(tyrosyl)-*N*'-(5-azido-2-nitrobenzoyl)-1,4-diaminobutane (**Ib**), whose linker is by two methylene groups longer than that of compound (**Ia**), and studied its photolysis.

RESULTS AND DISCUSSION

We presumed that the distance between the Tyr and 5-azido-2-nitrobenzoic acid residues in the new model (**Ib**) would be as short as in the previous model (**Ia**) [4], and this distance would be comparable with that between the reacting sites during the protein photoaffinity modification. At the same time, the elongation of the linker by two methylene groups in (**Ib**) could provide for a less dense spatial structure of the two interacting aromatic rings and allow the modification of not only *ortho*-position of Tyr residue [to give the product similar to (**IIa**)], but also other atoms in (**Ib**) (e.g., benzyl carbon atom in this residue). For example, it is known that the toluene photomodification by perfluoroaryl azides proceeds through the introduction of singlet arylnitrenes into *ortho*- and *para*-positions of the toluene aromatic ring and through the reaction of triplet arylnitrenes with benzyl carbon atom of toluene [5].

Aryl azide derivative of tyrosine (**Ib**) was prepared by acylation of *N*-(5-azido-2-nitrobenzoyl)-1,4-diaminobutane with *N*-succinimide ester of Boc-*DL*-Tyr(Boc) followed by the removal of Boc-protection. The structure of (**Ib**) was confirmed by ^{1}H NMR, UV, and IR spectroscopies and MALDI MS (the data are given in the Experimental section). ^{1}H NMR parameters of the Tyr and 5-azido-2-nitrobenzoic acid residues in (**Ib**) corresponded to the published data (cf. [6] and [4], respectively). Product (**Ib**) was stable at storage for six months at –20°C as a pellet and in methanol solution (50 mM) according to TLC and reversed-phase HPLC.

Before studying the photomodification of (**Ib**), the preferred conformations of models (**Ia**) and (**Ib**) were analyzed using computer modeling, in order to check the possibility of the intramolecular modification of Tyr residue by a highly reactive nitrene. The method of limited molecular mechanics and dynamics with regard to water environment [7] demonstrated that, in one of conformations of (**Ia**) corresponding to the minimum energy, the Tyr and 5-azido-2-nitrobenzoic acid residues are situated in parallel planes at a distance of approximately 0.3 nm and participate in stacking interaction. On the other hand, according to the same computer modeling, the aromatic rings of the reactive residues in (**Ib**) are disposed perpendicularly to each other at a distance of approximately 0.6 nm between the ring centers (Fig. 1). The distance between the nitrogen atom of aryl azide nearest to the aromatic ring and the hydrogen atom of Tyr in the *ortho*-position to its hydroxyl group is 0.48 nm in (**Ia**) and 0.70 nm in (**Ib**). These computation data suggest that a longer linker in the model (**Ib**) removes the reactive residue from the target to be modified and, therefore, decreases the probability of contact between them.

These results were experimentally verified by studying the photolysis of (**Ib**) in 0.15% aqueous methanol at concentrations of 0.031 and 0.062 mM by irradiation for 40 s. At 0.062 mM concentration, (**Ib**) was irradiated under aerobic (solutions both saturated and unsaturated with oxygen) and anaerobic conditions. Only aerobic conditions without saturation with oxygen were used at 0.031 mM concentration of (**Ib**). The resulting mixtures of products were analyzed by microcolumn reversed-phase HPLC immediately after irradiation and after storage in the dark at 20°C for 1–24 h.

Eight peaks were revealed in the mixture of photolysis products (0.062 mM, aerobic conditions without saturation with oxygen) immediately after the irradiation of (**Ib**) (Fig. 2a). Compounds corresponding to these peaks were identified as the photoconversion products (**IIIb**)–(**VIIb**) according to their spectral characteristics and MALDI MS (Scheme 1). Note that no stable products of the Tyr photomodification were found in the mixture of photolysis products.

The quantitative composition of the products changed after the reaction mixture storage in the dark at 20°C for 1–24 h (the dark stage of reaction) (Fig. 2b). It was found that the content of (**IIIb**), (**Vb**), and (**VIb**) (peaks *4*, *7*, and *8*, respectively) and of the compounds corresponding to peaks *2* and *3* increased due to the decomposition of the unstable compound (**VIIb**) (peak *6*), which practically disappeared 20 h after the beginning of the dark stage. The quantitative data are given in the table.

A comparison of the chromatographic profiles of the photolysis products of model compounds (**Ia**) (Fig. 2c)

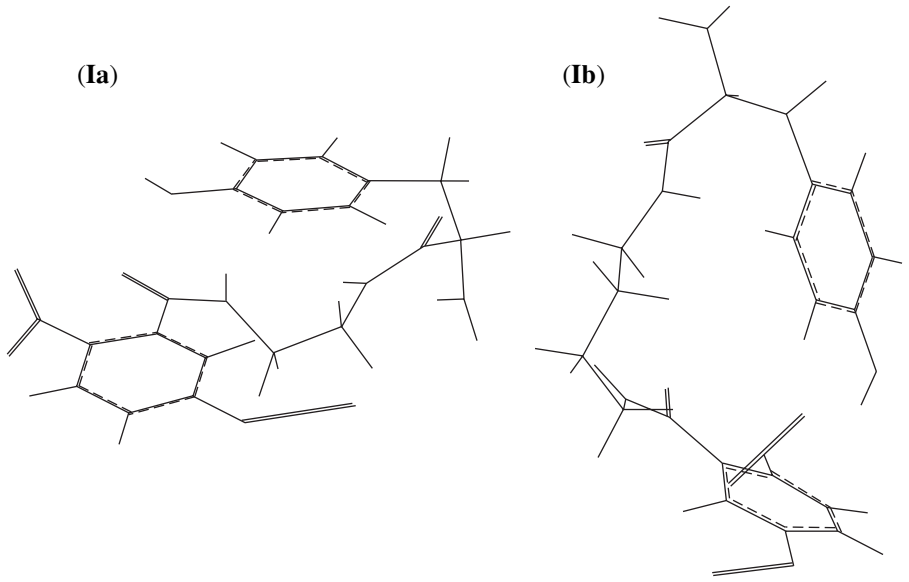
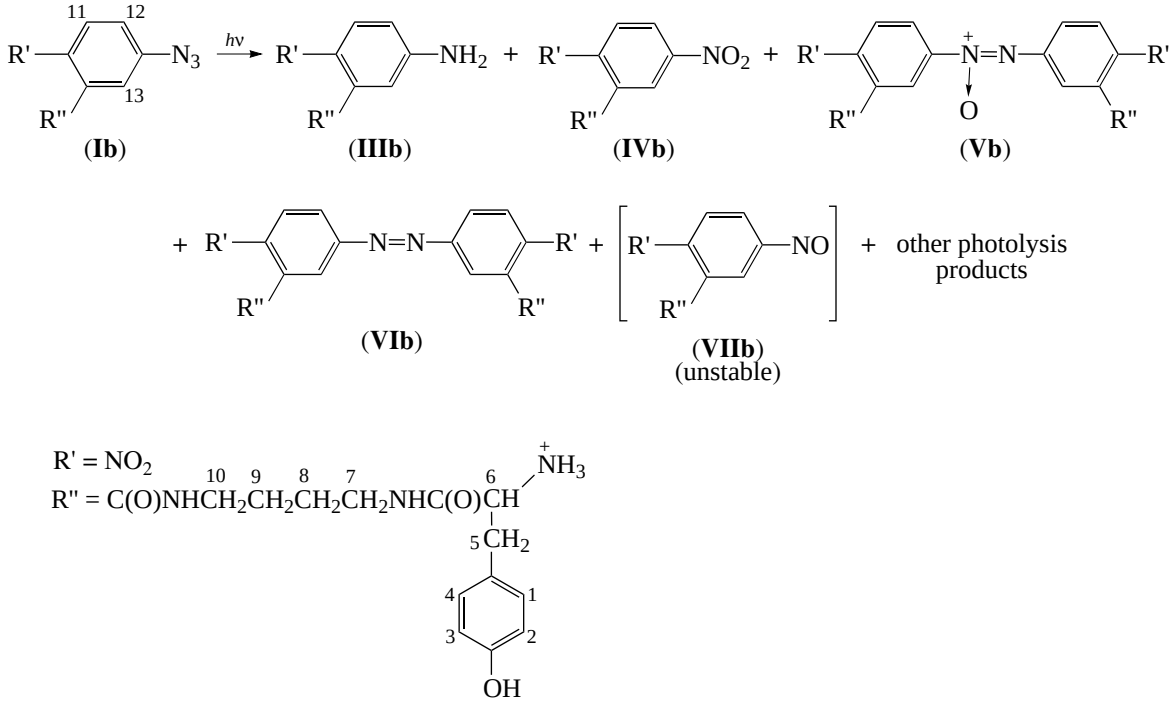


Fig. 1. Spatial structures of the model compounds (**Ia**) and (**Ib**) corresponding to the minimal-energy conformations under aqueous conditions (water molecules are not shown).

and (**Ib**) (Fig. 2a) demonstrated that the photolysis processes of these compounds differ. The main difference was found in the group of peaks *1–6* (Fig. 2*c*), which contained the product of intramolecular photomodification of Tyr (**IIa**) (peak *6*). No stable products of the Tyr photomodification were found among the compounds corresponding to peaks *1–4* (Fig. 2a). It should be noted that the compounds corresponding to peaks *2* and *3*



Scheme 1. The photolysis products (**Ib**). Here and further in the Experimental section, atoms are numerated for convenience of the interpretation of ^{1}H NMR spectra.

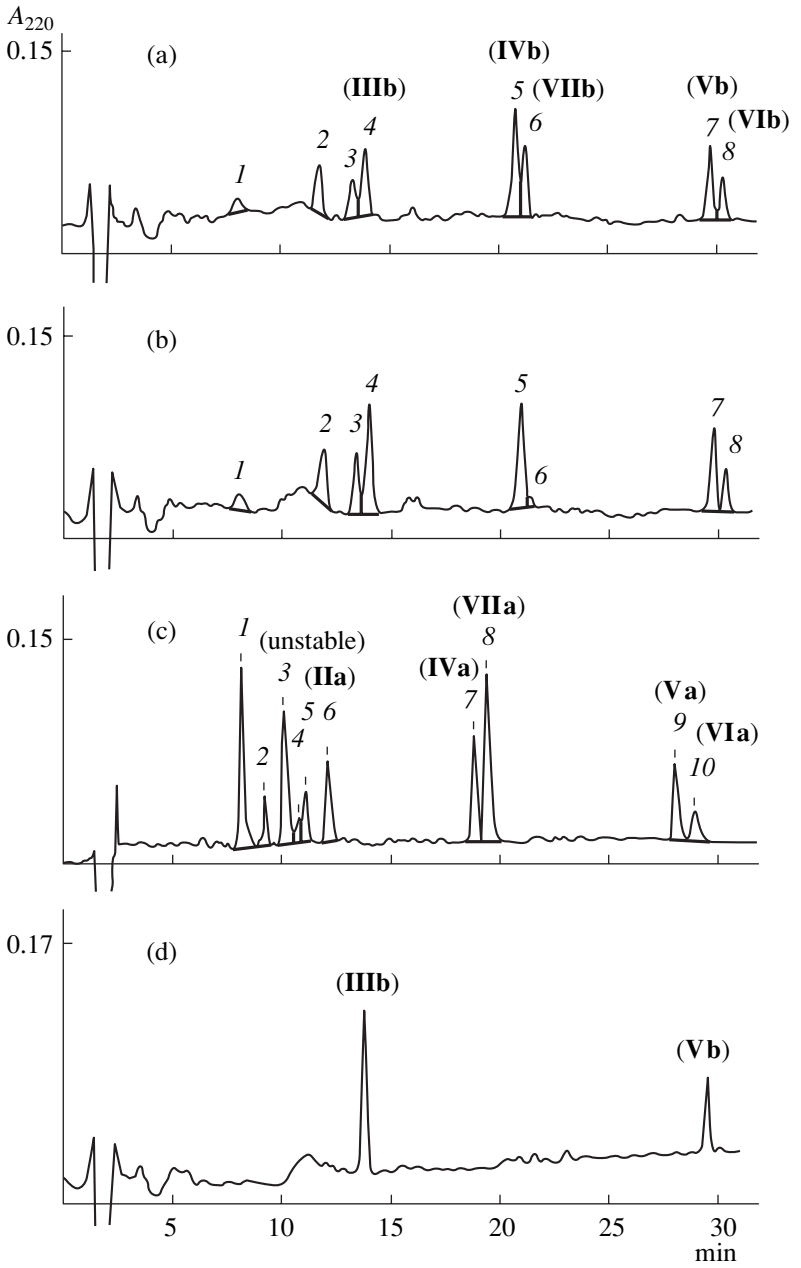


Fig. 2. Analysis of the products of 40-s photolysis of (c) (**Ia**) and (a, b, d) (**Ib**) (in concentration of 0.062 mM in 0.15% aqueous methanol) (a) just after the irradiation, (b) after the storage in the dark for 20 h at 20°C, and (d) after the concentration of the combined fractions *2* and *3* (Fig. 2a) by the microcolumn reversed-phase HPLC in a linear gradient of acetonitrile (5 to 20%) in 0.1% TFA for 32 min.

were unstable upon the concentration and their structures were not determined. The retention times of peaks *7–10* in Fig. 2c and peaks *5–8* in Fig. 2a were close to each other, which suggests that the corresponding compounds probably have similar chemical structures. Previously, we identified substances corresponding to peaks *7*, *9*, and *10* in Fig. 2c as nitro (**IVa**), azoxy (**Va**), and azo (**VIa**) derivatives of (**Ia**), respectively [4]. On the basis of electronic spectroscopy and quantitative analysis of stable products formed after irradiation of (**Ia**) at various concentrations, structure (**VIIa**) of nitroso derivative of (**Ia**) was presumably ascribed to the unstable product from peak *8* in Fig. 2c, which almost completely disappears at storage in the dark for 20 h. Accordingly, compounds eluted as peaks *5–8* (Fig. 2a) probably are nitro, nitroso, azoxy, and azo derivatives of (**Ib**). Therefore, the same numbers [e.g., (**IVa**) to the substance from peak *7* in Fig. 2c and (**IVb**) to that from peak *5* in Fig. 2a] were attributed to the photolysis products of (**Ia**) and (**Ib**), which can be con-

Relative content of the products (A) immediately after photolysis of (**Ib**) and (B) after 20-h storage in the dark (according to reversed-phase HPLC*)

Concentration of (**Ib**), mM	Products and their relative content, %													
	(**IIIb**) peak *4*		(**IVb**) peak *5*		(**Vb**) peak *7*		(**VIb**) peak *8*		(**VIIb**) (unstable) peak *6*		unidentified compounds peak *2*		peak *3*	
	A	B	A	B	A	B	A	B	A	B	A	B	A	B
0.062	14.8	23.0	20.1	20.2	14.0	15.8	7.6	9.0	13.1	–	12.2	14.8	8.5	12.0
0.062 saturation with O_2	9.65	17.3	45.7	45.5	–	–	1.5	4.6	28.5	7.0	3.9	12.6	2.2	8.3
0.062 saturation with Ar	19.2	19.2	–	–	–	–	62.4	62.4	–	–	–	–	–	–
0.031	14.5	18.8	20.6	20.6	7.1	7.0	4.0	3.9	35.8	–	–	28.7	–	7.46

* For HPLC conditions, see the Experimental section. The amounts of photoproducts were estimated by integration of the corresponding chromatographic peaks obtained at 220 nm. A, the chromatographic profiles were obtained just after the irradiation of (**Ib**) (see Fig. 2a); B, the chromatographic profiles were obtained after the storage of the photolysis mixture for 20 h in the dark at 20°C (see Fig. 2b).

sidered to be the corresponding derivatives of the same chemical nature.

The stable products of the photolysis of (**Ib**) were obtained in the amounts sufficient for their identification by ^{1}H NMR, UV-spectroscopy, and MALDI MS and by the comparison with the analogous products of the photolysis of (**Ia**) (see [4] and the Experimental section).

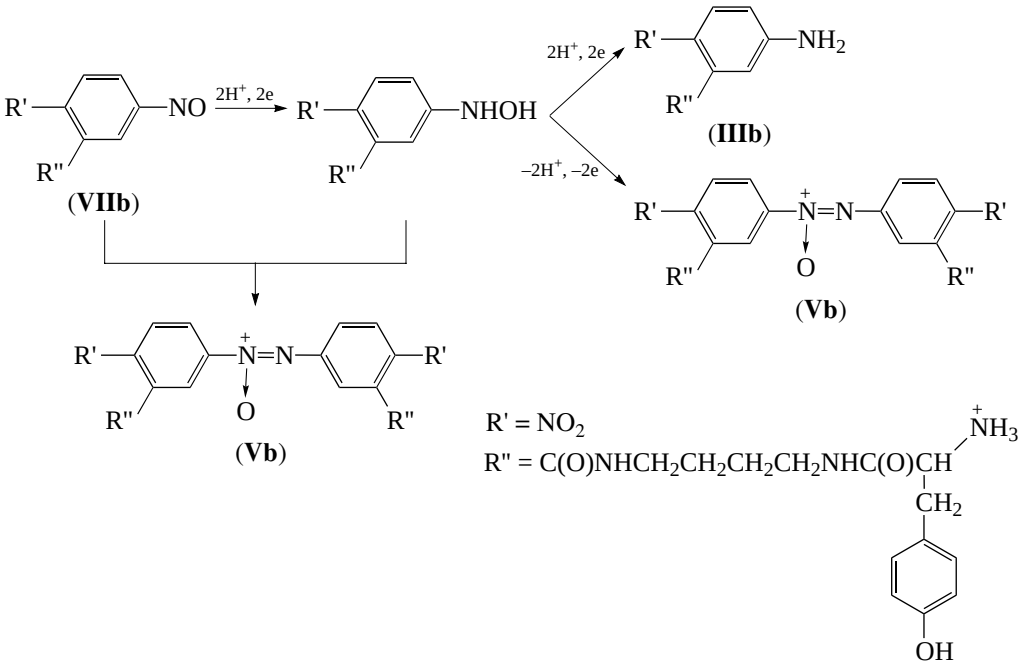
The structure of aromatic amine (**IIIb**) was ascribed to the product in peak *4* of Fig. 2a, because it contained a Tyr residue similar to that in (**Ib**) and changes in the chemical shifts of H11, H12, and H13 in (**IIIb**) were characteristic of an amino substituted derivative [8]. The electronic absorption spectra of (**IIIa**) (λ_{max} 384 nm [4]) and (**IIIb**) (λ_{max} 385 nm) are similar.

Products eluted in fractions *2* and *3* (Fig. 2a) and compound (**VIIb**) from peak *6* could not be identified because of their instability. It was established that all the unstable compounds appeared only in the course of photolysis under aerobic conditions (see table). It is known that the irradiation of 4-nitrophenyl azide results in the triplet nitrene, which can intensively interact with oxygen to give (4-nitrophenyl)nitroso oxide in the form of a biradical or a zwitterion. Rapid dimerization of this nitroso oxide proceeds through an intermediate unstable cyclic diperoxide and results in 1,4-dinitrobenzene and 1-nitroso-4-nitrobenzene [9, 10]. We ascribed to (**VIIb**) a probable nitroso structure, since the electron absorption spectra of (**VIIa**) (λ_{max} 278 nm, [4]) and (**VIIb**) (λ_{max} 278 nm) practically coincided and were similar to that of 1-nitroso-4-nitrobenzene (λ_{max} 282 nm, [11]). It was found by the quantitative analysis of the photolysis products that the saturation of the solutions (0.062 mM) of (**Ib**) with oxygen and subsequent irradiation under aerobic conditions results in a more than twofold increase in the content of nitro derivatives (**IVb**) and (**VIIb**). The two compounds were absent among the products of the anaerobic irradiation of (**Ib**) (see table).

The unstable substances eluted in fractions *2* and *3* (Fig. 2a) were formed from (**VIIb**) at the dark stage processes. These products were absent just after the irradiation of the low-concentration (0.031 mM) solution of (**Ib**), but they appeared after the dark stage of the decomposition reaction of (**VIIb**) (table). These products also formed immediately after the irradiation of the concentrated (0.062 mM) solution of (**Ib**), and their content also increased after the dark stage due to the decomposition of (**VIIb**) (Figs. 2a, 2b, table).

The mixture formed after the irradiation of 0.062 mM solution of (**Ib**) under aerobic conditions without saturation with oxygen was kept in the dark at 20°C for 4 days and analyzed by the reversed-phase HPLC. In this case, products corresponding to peaks *2* and *3* were absent from the mixture, while the amounts of amino derivative (**IIIb**) (peak *4*) and azoxy derivative (**Vb**) (peak *7*) increased by 15.3 and 8.3%, respectively, in comparison with their amounts just after the irradiation of (**Ib**). At the same time, a new peak with the retention time of 22.49 min was observed in the chromatogram (data not shown). Practically complete conversion of these compounds into the amine (**IIIb**) (λ_{max} 385 nm) and azoxy derivative (**Vb**) (λ_{max} 342 nm (Fig. 2d) also proceeded upon the concentration of the combined fractions *2* and *3* according to reversed-phase HPLC, UV-spectroscopy, and MALDI MS.

Nitrosobenzene is known to be easily reduced to form phenylhydroxylamine. The interaction of nitrosobenzene with phenylhydroxylamine leads to azoxybenzene, and the disproportionation of phenylhydroxylamine gives aniline and azoxybenzene [12]. We can presume that (**VIIb**) would also undergo similar conversions in the presence of methanol as a reducing agent (Scheme 2). It is probable that the compound eluted in fraction *3* corresponds to *N*-(tyrosyl)-



Scheme 2. Conversions of the hypothetic nitroso compound (**VIIb**) in the presence of reducing agent (methanol).

N'-[*N*-(2-nitro-5-hydroxylaminobenzoyl)]-1,4-diaminobutane, because its electronic absorption spectrum is similar to that of *N*-(4-nitophenyl)hydroxylamine (λ_{max} 356 nm) [13]. Data about the products of interaction of phenols and their analogs with aromatic nitroso compounds lack in the literature. At the same time, the nitroso group is known to be analogous to the carbonyl group and enters similar reactions. Since the reaction with alcohols with the formation of unstable hemiacetals is characteristic of carbonyl-containing compounds, we can presume that the compound eluted in fraction *2* results from a similar interaction of the nitroso group of (**VIIb**) with the Tyr hydroxyl group.

It is well known that the irradiation of aromatic azides results in the splitting off of a nitrogen molecule and the formation of a singlet arylnitrene [10, 14]. However, the irradiation of 4-nitrophenyl azide easily gives a triplet 4-nirtophenylnitrene due to the extremely rapid transition of the singlet 4-nitrophenylnitrene into its basic triplet state [9]. As follows from the studies of photolysis of the model compounds (**Ia**) [4] and (**Ib**), their irradiation results in the reactions characteristic of the triplet 4-nitrophenylnitrene [9]: dimerization with formation of substituted azobenzenes (**VIa**) and (**VIb**), reduction to the substituted 4-nitroanilines (**IIIa**) and (**IIIb**), and interaction with oxygen giving rise to nitro products (**IVa**) and (**IVb**) and substituted azoxybenzenes (**Va**) and (**Vb**) (see Scheme 1). However, the analysis of the photolysis products of (**Ib**) did not reveal products of the photomodification of the Tyr residue: neither stable (analogs of **IIa**) nor unstable analogs [4], capable of converting into stable analogs during the dark stage.

The importance of an optimal linker length for the effective intramolecular modification of the tyrosine phenyl group in various compounds was demonstrated in a number of publications [15–18]. For example, the intramolecular alkylation was found to proceed in the compounds of the general formula $Ph(CH_2)_nCH_2Cl$, where $n = 2$ or 3; in the case of a longer linker it gave a high yield of the cyclization product, whereas only its trace amounts formed in the case of a shorter linker [15]. For compounds of the general formula $PhCO(CH_2)_nCH_2Cl$ ($n = 4$–8), the short linker ($n = 4$) ensured a high yield of the cyclization product, whereas the successive elongation of the linker to $n = 5$–8 resulted only in the intermolecular alkylation products [16].

In our case, the linker elongation by two methylene groups inhibited the modification of the *ortho*-C–H bond in the Tyr residue; the corresponding product (**IIa**) arose only under the irradiation of the model compound (**Ia**) with the shorter linker. No other modification variants were found either. On the basis of calculations and experimental data, we can conclude that the linker elongation by two methylene groups prevents the realization of the molecule conformation in which the Tyr and 5-azido-2-nitrobenzoic acid residues are in an optimal position for the formation of a stable modification product.

EXPERIMENTAL

Trifluoroacetic acid, 5-azido-2-nitrobenzoic acid (Sigma, United States), and 2,5-dihydroxybenzoic acid (Aldrich, United States) were used in this study. *N*-(5-Azido-2-benzoyl)-1,4-diaminobutane and *N*-succinimide ester of Boc-*DL*-Tyr(Boc) were synthesized in the technological laboratory of NIBKh, Siberian Division, Russian Academy of Sciences by the methods described in [4] and [19], respectively. Acetonitrile, 1,4-dioxane, *N*,*N*-dimethylformamide, benzene, pyridine, acetone, methanol, ethanol, methylene chloride, diethyl ether, ethyl acetate, and *n*-hexane were purified according to the standard procedures. All the reagents used were of the reagent grade.

The electronic absorption spectra were recorded on a Shimadzu UV-2100 spectrophotometer (Japan) in quartz cells with a 0.1-cm optical pathway. IR spectra were recorded on a Bruker IFS 66 spectrometer (Germany) in KBr pellets. ^{1}H NMR spectra (δ, ppm, *J*, Hz) were recorded on a Bruker AM 400 spectrometer (Germany) at the working frequency of 400.13 MHz in D_2O at 25°C. The chemical shifts were calculated relative to the residual solvent signal (water, δ 4.80 ppm). The NMR spectra were interpreted with the use of a Sadtler catalog of NMR spectra (Sadtler Research Laboratories) and other widely used sources of the NMR spectral information.

Mass spectra were registered on a VISION 2000 MALDI time-of-flight mass spectrometer (Thermo Bio Analysis, UK) using a VSL-337ND nitrogen laser (337 nm, impulse of 3 ns, energy of 150–200 μJ/imp) for ionization. 2,5-Dihydroxybenzoic acid was used as a matrix. The samples for the MALDI TOF MS were prepared according to the following procedures: aqueous solutions of the tested compounds (0.1–0.5 mM, 0.3 μl) were mixed with a solution (0.3 μl) of the matrix (20 mg/ml) in 20% aqueous acetonitrile, dried, and analyzed. The spectra were obtained by registering positive ions in the reflection regime.

TLC was performed on DC-Alufolen Kieselgel 60 F_{254} precoated plates (Merck, Germany) in the following chromatographic systems: (A) 4 : 1 methanol–ammonia and (B) 9 : 1 methylene chloride–methanol.

Analytical microcolumn reversed-phase HPLC was performed on a MiliChrom A-02 microcolumn liquid chromatograph (EcoNova, Russia) [20]. The photolysis products were isolated on a MiliChrom-4 microcolumn liquid chromatograph (Orel, Russia).

The spatial structures of (**Ia**) and (**Ib**) were calculated by the method of limited molecular mechanics and dynamics using the Amber 4.0 program [7]. Visualization of the structures and rotation around some chemical bonds were performed with the use of the HyperChem program (HyperCube). All the calculations were performed on personal Pentium computers.

***N*-(*DL*-Tyrosyl)-*N*'-(5-azido-2-nitrobenzoyl)-1,4-diaminobutane (Ib).** A solution of *N*-(5-azido-2-nitrobenzoyl)-1,4-diaminobutane (36 mg, 0.11 mmol) in anhydrous dioxane (2 ml) was added to a solution of Boc-*DL*-Tyr(Boc) *N*-succinimide ester (57.5 mg, 0.12 mmol) in anhydrous dioxane (2 ml). The reaction mixture was stirred for 10 min in the dark, evaporated to dryness, and treated with water (5 ml). The product was extracted with ethyl acetate (2 × 2 ml). The extract was dried over anhydrous Na_2SO_4 for 24 h, filtered, and evaporated. The oily residue was dissolved in dioxane (1 ml), and the solution was dropwise added to water (5 ml). The water layer was decanted, and the oily residue was dried in a desiccator over P_2O_5 for several days, dissolved in methanol (1 ml), and treated with methanol (0.4 ml) saturated with HCl. The solution was kept for 48 h at room temperature and poured into cool ether (14 ml). The oily residue was several times washed with ether, dissolved in methanol (1 ml) and precipitated with ether (10 ml). The precipitate was filtered, washed with ether (2 ml), and dried in a vacuum to give (**Ib**); yield 30 mg (47%); R_f 0.82 (A), 0.1 (B); RT 21.91 min (for HPLC conditions, see below); UV (10% aqueous methanol), λ_{max}, nm (ε, M^{-1} cm^{-1}): 318 (8800); IR (KBr, ν_{max}, cm^{-1}): 1342, 1517 (NO_2), 1653 ($C{=}O_{amide}$), 2123 (N_3); ^{1}H NMR: 8.21 (1 H, d, *J* 9.0, H11), 7.38 (1 H, dd, *J* 9.0 and 2.5, H12), 7.22 (1 H, d, *J* 2.5, H13), 7.14 (1 H, d, *J* 8.5, H1 and H4), 6.82 (1 H, d, *J* 8.5, H2 and H3), 3.3–3.2 (4 H, m, H7 and H10), 3.18 (1 H, d, *J* 7.0, H6), 3.1–3.0 (2 H, m, H5), 1.6–1.5 (4 H, m, H8 and H9); MALDI,[2] *m*/*z*: 386.6 $[M + H - NO]^+$, and 400.5 $[M + H - O]^+$, 416.5 $[M + H]^+$, and 438 $[M + Na]^+$ for (**IIIb**).

Irradiation of the samples was performed using a DRSh-1000 Hg high pressure lamp (1000 W). Two spectral lines (313 and 365 nm) were selected by a combination of glass filters. The intensity of the incident light ($J = 1.35 \pm 0.01) \times 10^{15}$ photons s^{-1} cm^{-2}) was measured by a ferrioxalate Hatchard–Parker actinometer [21].

A 0.031 or 0.062 mM solution of (**Ib**) (accuracy was ±5%) in 0.15% aqueous methanol was irradiated. The molar extinction coefficient of (**Ib**) was determined as a sum of absorption coefficients of Tyr (ε_{275} 1230 M^{-1} cm^{-1} [22]) and aryl azide (ε_{318} 8800 M^{-1} cm^{-1} [2]).

Aerobic conditions of irradiation were provided for by simply using 0.062 mM solution of (**Ib**) in 0.15% aqueous methanol or blowing oxygen through the solution for 20 min at 0°C.

Anaerobic conditions were provided for by bubbling argon through 0.062 mM solution of (**Ib**) for 20 min at 0°C.

The photolysis products were analyzed by reversed-phase HPLC on a Nucleosil 100-5C18 microcolumn (2 × 75 mm, 5 μm (Macherey-Nagel, Ger-

[2] A photolysis of (**Ib**) with the formation of amine (**IIIb**) proceeds simultaneously with the laser ionization (λ 337 nm) of (**Ib**) in the MALDI method.

many)) in a linear gradient of acetonitrile (5 to 20%) in 0.1% TFA for 32 min at a flow rate of 100 μl/min just after the irradiation of (**Ib**) or after the storage of reaction solution in the dark at room temperature for 1–24 h. The photolysis products were characterized by their retention times and by their spectral characteristics: the ratio of absorption at 210, 274, 330, and 346 nm to that at 220 nm. The UV-spectra of the products were recorded during the chromatographic process at the "stop flow" conditions of the chromatograph. The relative quantities of products were estimated by integrating the chromatographic profile obtained at 220 nm.

The isolation of the photolysis products in necessary quantities was performed by reversed-phase HPLC on a column (2 × 64 mm) as described above.

Photolysis products were obtained by evaporation of the water–acetonitrile solutions on a rotor evaporator in a vacuum at 32°C.

Identification of the photolysis products of (Ib). The following products were isolated and identified:

3-(Tyrosylaminobutylaminocarbonyl)-4-nitroaniline (IIIb); RT 14.06 min; ^{1}H NMR: 8.09 (1 H, d, *J* 9.0, H11), 7.14 (1 H, d, *J* 8.0, H1 and H4), 6.86 (1 H, d, *J* 8.0, H2 and H3), 6.81 (1 H, dd, *J* 9.0 and 2.5, H12), 6.66 (1 H, d, *J* 2.5, H13), 4.11 (1 H, d, *J* 7.0, H6), 3.3–3.2 (4 H, m, α- and δ-CH_2 of Bu), 3.1–3.0 (2 H, m, H5), 1.5–1.4 (4 H, m, β- and γ-CH_2 of Bu); MALDI MS, *m*/*z*: 386.6 $[M + H - NO]^+$; UV (7% aqueous acetonitrile, 0.1% TFA): λ_{max} 385 nm.

1,4-Dinitro-3-(tyrosylaminobutylaminocarbonyl)benzene (IVb); RT 21.35 min; UV (9% aqueous acetonitrile, 0.1% TFA): λ_{max} 254 nm; MALDI MS, *m*/*z*: 439 $[M + Na - NO]^+$ and 452 $[M + Li]^+$.

[4,4'-Dinitro-3,3'-bis(tyrosylaminobutylaminocarbonyl)]-1,1'-azoxybenzene (Vb); RT 29.67 min; UV (18% aqueous acetonitrile, 0.1% TFA): λ_{max} 342 nm; MALDI MS, *m*/*z*: 783 $[M + H - 2\ NO]^+$ and 814 $[M + H - NO]^+$.

[4,4 Dinitro-3,3'-bis(tyrosylaminobutylaminocarbonyl)]-1,1'-azobenzene (VIb); RT 30.21 min; UV (18% aqueous acetonitrile, 0.1% TFA): λ_{max} 328 nm.

Product (VIIb) is unstable; it is probably 1-nitroso-3-(tyrosylaminobutylaminocarbonyl)-4-nitrobenzene; RT 21.72 min; UV (9% aqueous acetonitrile, 0.1% TFA): λ_{max} 278 nm.

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