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New Photoreactive Cleavable Reagents with Trifluoromethyldiazirine Group

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Abstract—Photoreactive crosslinking reagents that simultaneously contain a trifluoromethyldiazirine and an *o*-nitrobenzyl groups were synthesized for the first time. Photochemical properties of the reagents were studied, and the possibility of separate activation of the diazirine group and *o*-nitrobenzyl linker was shown.

Key words: *aryl(trifluoromethyl)diazirines, cleavable photoreactive reagents, photolysis*

INTRODUCTION

Photoaffinity modification is an effective approach for studying interactions between biological molecules [1].[2] Under irradiation, the photolabile group of one of the interacting molecules generates a highly reactive species that attacks neighboring groups with the formation of crosslinks. The study of the resulting covalent complexes furnishes one with the information on the structural organization of a system and on the nature of interacting molecules [2–4]. However, the difficulties in isolation and analysis of the cross-linked products hinder their successful identification. The use of photoreactive reagents that can be selectively cleaved after the covalent linking to a biological molecule may help approach to the solution of this problem (Fig. 1). Such reagents allow one to remove the useless part of the ligand molecule and, in this way, to facilitate the detection and isolation of the resulting products.

The photoreagents that, in addition to the trifluoromethyldiazirine group, contain some *chemically* cleavable groups (*cis*-diol, disulfide, or ester) are known [5–12]. Their cleavage proceeds under the action of chemical reagents, which imposes considerable limitations on their use for studying molecular interactions in biological systems. An alternative approach is based on the application of compounds that contain a *photocleavable* site, i.e., a group cleavable upon the UV irradiation. Fang *et al.* [13] were the first who synthesized a bifunctional reagent that contained two groups (*m*-nitrophenoxy and trifluoromethyldiazirine) capable of a selective cleavage at different pH on irradiation at 350 nm.

In this work, we suggest new photoreactive cleavable reagents (**I**)–(**IV**). They have two photolabile groups that are activated upon UV irradiation: *p*-trifluoromethyldiazirine group, which allows the covalent binding of the contacting molecules at the contact point; and *o*-nitrobenzyl group, which enables the splitting off of the useless part of the ligand. The photochemical properties of these compounds were studied, and a possibility of separate activation of diazirine and nitrobenzyl groups was shown.

RESULTS AND DISCUSSION

The choice of diazirine group as a photolabile function was not accidental. The photolysis of this group proceeds with the formation of the highly reactive carbene under mild conditions (irradiation at 350–360 nm) that are harmless for biological molecules. Moreover, this group is stable within a wide range of conditions, e.g., in thiol buffers with pH values varying from 0 to 14. The cleavable site, *o*-nitrobenzyl group, decomposes under the action of UV light of the same wavelengths; it is widely used for the synthesis of photolabile protecting groups, resins with photolabile linkers for the solid phase synthesis, and photocleavable conjugates of peptides with oligonucleotides [14–18].

Scheme 1 shows the synthesis of reagents (**I**) and (**II**). Starting 4-[3-(trifluoromethyl)-3*H*-diazirin-3-yl]toluene (**V**) was obtained by a modified Nassal procedure [19–21]. 2-Nitro-4-[3-(trifluoromethyl)-3*H*-diazirin-3-yl]toluene (**VI**) was synthesized by nitrating (**V**) with a HNO_3/H_2SO_4 mixture at 0°C. Carrying out the reaction at a low temperature allows one to avoid the formation of dinitroderivative and to obtain the target product (**VI**) with a good yield.

2-Nitro-4-[3-(trifluoromethyl)-3*H*-diazirin-3-yl]benzoic acid (**I**) was obtained by oxidation of (**VI**) by anal-

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[2] Abbreviations: NBS, *N*-bromosuccinimide.

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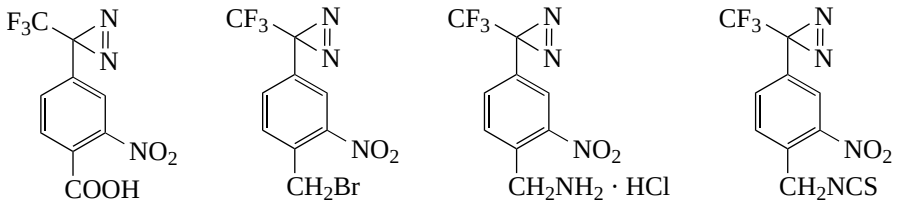


(I) **(II)** **(III)** **(IV)**

ogy with the method that was used for oxidation of the diazirine-containing reagents [19]. Potassium permanganate in aqueous pyridine was used as an oxidizing agent. A relatively low yield of the reaction product (31%) was apparently caused by a partial destruction of the diazirine cycle upon a long heating and by heavy losses upon purification.

All the attempts to obtain 2-nitro-4-[3-(trifluoromethyl)-3*H*-diazirin-3-yl]benzyl bromide (**II**) by the bromination of (**VI**) were unsuccessful. The conditions of rigorous radical bromination were tested, but the desired product was not detected; instead, only the starting compound was isolated from the reaction mixture. We suggest an alternative approach based on the synthesis of 4-[3-(trifluoromethyl)-3*H*-diazirin-3-yl]benzyl bromide (**VII**) by brominating the modified toluene (**V**) with subsequent nitration leading to the target (**II**). NBS was used to brominate (**V**) according to the procedure in [20].

Reagent (**II**) was prepared by nitrating substituted benzyl bromide (**VII**) with nitric acid. We have found that use of nitric acid containing nitrogen oxides favors the formation of large amounts of by-products. The same tendency was also observed when the reaction was carried out at the temperatures from 0 to +5°C. At the same time, the reaction was not completed at a substantially lower temperature (–20°C). On the basis of the model experiments, we determined the optimal conditions for the synthesis: the use of 90% nitric acid freed from nitrogen oxides and the reaction temperatures from –5°C to –10°C. Nevertheless, TLC showed the formation of three products with R_f 0.57, 0.4, and 0.3. The target product (**II**) (R_f 0.4) was isolated from the reaction mixture by a column chromatography on silica gel in yield of 33%. Its structure was proven by mass spectrometry and UV and IR spectroscopy.

2-Nitro-4-[3-(trifluoromethyl)-3*H*-diazirin-3-yl]benzylamine hydrochloride (**III**) (Scheme 2) was synthesized using the modified Delepine reaction [22]. The classical synthesis implies the interaction of a bromide with methenamine (hexamethylenetetramine) in ethanol followed by the decomposition of the resulting methenaminium salt by gaseous hydrogen chloride. This procedure gives the target amine with 40% yield. The use of chloroform as a solvent [23] instead of ethanol greatly increases the solubility of the reacting substances, which results in increase in yield of the reaction product: we obtained the methenaminium salt (**VIII**) a white crystalline substance in 90% yield. The salt was decomposed by two methods. The first of them consisted in the treatment with a 8 : 1 methanol–concentrate HCl mixture [24] and resulted in the target amine hydrochloride (**III**). The second method involved the decomposition of the methenaminium salt with sulfur dioxide to give 2-nitro-4-[3-(trifluoromethyl)-3*H*-diazirin-3-yl]benzylaminomethyl hydrogen sulfite (**IX**), which was stable on storage. It can be transformed into the amine by the treatment with 25% hydrochloric acid and water vapor [25].

2-Nitro-4-[3-(trifluoromethyl)-3*H*-diazirin-3-yl]benzyl isothiocyanate (**IV**) was obtained by the reaction of amine (**III**) with carbon disulfide in pyridine with DCC as a coupling reagent (Scheme 2). The reaction was carried out for 12 h at room temperature. The coupling reactions with the use of DCC are accompanied by the formation of dicyclohexylurea, which usually precipitates and can be easily removed from the reaction mixture. However, in our case, any precipitate was not

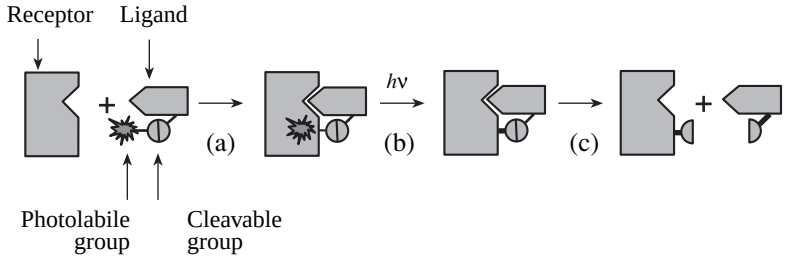
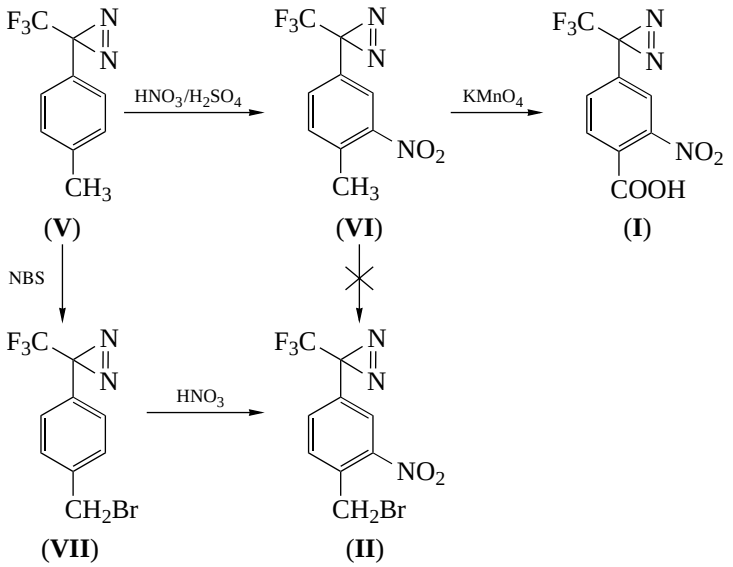
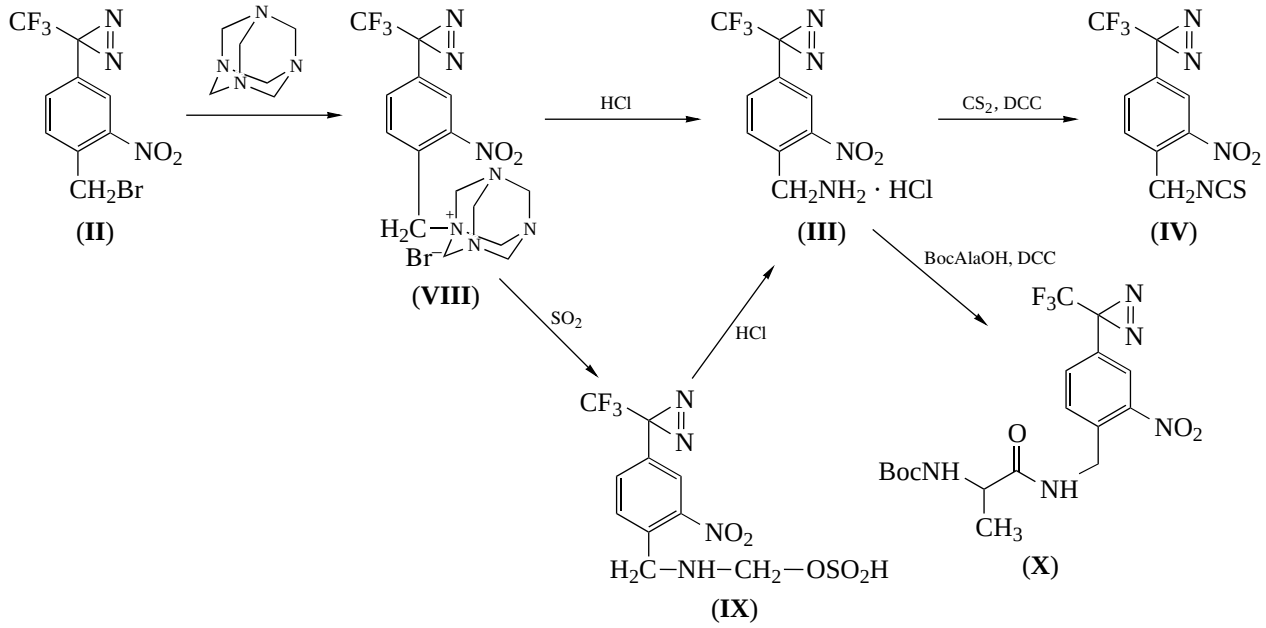


Fig. 1. The principle of the photoaffinity modification by the use of a cleavable reagent: (a) the formation of a ligand–receptor noncovalent complex, (b) the formation of a covalent complex upon the irradiation, (c) the cleavage (upon the action of light or chemical reagents) and the removal of the ballast part of the ligand from the ligand–receptor complex.



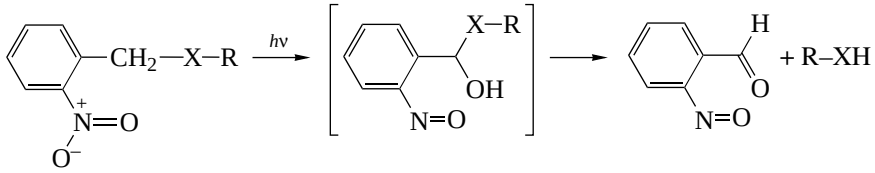
Scheme 1. The synthesis of 2-nitro-4-[3-(trifluoromethyl)-3*H*-diazirin-3-yl]toluene (**VI**), 2-nitro-4-[3-(trifluoromethyl)-3*H*-diazirin-3-yl]benzoic acid (**I**), and 2-nitro-4-[3-(trifluoromethyl)-3*H*-diazirin-3-yl]benzyl bromide (**II**).



Scheme 2. The synthesis of 2-nitro-4-[3-(trifluoromethyl)-3*H*-diazirin-3-yl]benzylamine hydrochloride (**III**), 2-nitro-4-[3-(trifluoromethyl)-3*H*-diazirin-3-yl]benzyl isothiocyanate (**IV**), and 2-nitro-4-[3-(trifluoromethyl)-3*H*-diazirin-3-yl]benzylamide of N^{α}-Boc-alanine (**X**).

formed, because dicyclohexylthiourea is easily soluble in organic solvents. We solve this problem by the dicyclohexylthiourea removal by a column chromatography on silica gel (see the Experimental section).

2-Nitro-4-[3-(trifluoromethyl)-3*H*-diazirin-3-yl]benzylamine (**III**) was coupled with N^{α}-Boc-alanine in the presence of DCC to give (**X**). After the elimination of Boc group, ninhydrin test of the reaction product showed the presence of amino group in the resulting compound, and an absorption band corresponding to the diazirine ring (363 nm, ε 170 M^{-1} cm^{-1}) was observed in its UV spectrum.



Scheme 3. The cleavage of *o*-nitrobenzyl derivative under the UV light action.

A characteristic band in the region of 342–353 nm (ε 160–640 M^{-1} cm^{-1}) is observed in the electronic absorption spectra of ethanol solutions of diazirines (**I**)–(**IV**) (Table 1), which is in a good agreement with literature data [19, 26, 27]. The UV irradiation with a wavelength ~360 nm results in the photolytic decomposition of the diazirine group to give a carbene. In this case, the intensity of the light absorption at the maximum corresponding to the diazirine ring decreases along with an increase in the irradiation time. The half-decay time is determined as the time necessary for half of the molecules to undergo a photolytic decomposition. If the intensity of the light irradiation is known, the quantum yield (φ) for the decomposition of a photolabile group may be determined as:

$$\varphi = \Delta m/\Delta n,$$

where Δn is the number of light quanta absorbed in a definite time, and Δm is the number of the mols of diazirine reacted for the time. These values can be specified as follows:

$\Delta n = I_{absorbed} t = I_0 \varepsilon l c t$, where $I_{absorbed}$ is the intensity of light absorbed by a substance (quanta/s), I_0 is the intensity of the light irradiating the reaction vessel (quanta/s), ε is the molar absorption coefficient (M^{-1} cm^{-1}), c is concentration of a substance (mol/l), l is a thickness of the absorbing layer (cm), and t is the irradiation time (s);

$\Delta m = \Delta c N_A V = \Delta A/\varepsilon N_A V$, where ($\Delta c$ is a change in a substance concentration (mol/l), ΔA is the change in the optical absorption, N_A is Avogadro's number (mol^{-1}), and V is the volume of solution (ml).

Table 1. The UV spectra of the synthesized photoreagents (**I**)–(**IV**)

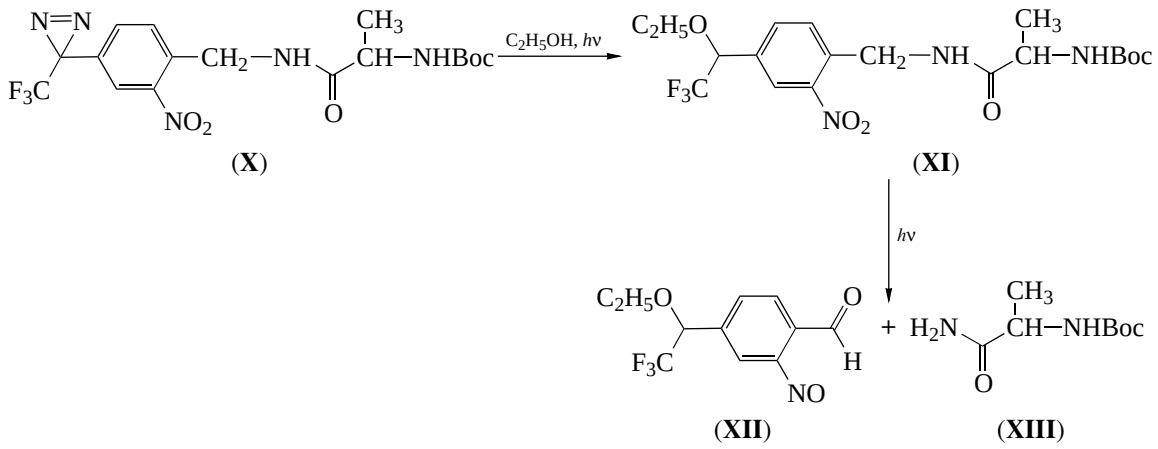
Compound	λ_{max}, nm	ε, M^{-1} cm^{-1}
(**I**)	256	19900
	291	1300
	343	200
(**II**)	251	12600
	295	1300
	342	160
(**III**)	220	5500
	260	1800
	353	330
(**IV**)	220	8400
	246	11600
	353	640

The photolysis of compounds containing *o*-nitrobenzyl group proceeds according to the mechanism of intramolecular oxidation–reduction as shown in Scheme 3. In this case, *o*-nitrosobenzaldehyde is formed, which follows from the appearance of a peak in the UV-range of 300–400 nm corresponding to the absorption of nitroso group [28].

We studied the kinetics of the selective photodecomposition of diazirine group and nitrobenzyl linker by the example of 2-nitro-4-[3-(trifluoromethyl)-3*H*-diazirin-3-yl]benzylamide of N^{α}-Boc-alanine (**X**), which is a simplified model of the photoreactive protein molecule that can be used for studying the nucleic acid–protein and protein–protein interactions.

The kinetic study was carried out by recording the UV spectra of an ethanolic solution of (**X**) after its irradiation for a definite time. A mercury lamp emitting monochromatic light at 365 nm was used as a light source for irradiation. An intensity of the absorption at 363 nm corresponding to diazirine group was observed to decrease during the first 25 min of photolysis (Fig. 2a). Clear isobestic points observed in the spectra indicate a photochemical reaction of the first order. As known [29], the products of carbene insertion into O–H bond are formed upon the photolysis of diazirines in alcohols. Therefore, 2-nitro-4-(2,2,2-trifluoromethyl-1-ethoxyethyl)benzylamide of N^{α}-Boc-alanine (**XI**) seems to be the major product in the initial stage of the photolysis of (**X**) (Scheme 4). The absorption maximum at 358 nm corresponding to nitroso group was observed to appear and to grow at longer irradiation times (Fig. 2b). This indicated the decomposition of nitrobenzyl group, which presumably resulted in the formation of 2-nitroso-4-(2,2,2-trifluoro-1-ethoxyethyl)benzaldehyde (**XII**) and another product, amide of N^{α}-Boc-alanine (**XIII**), which was detected by TLC in the irradiated solution.

The data that characterize the photochemical properties of the diazirine and nitrobenzyl groups in (**X**) are presented in Table 2. One can see that these groups sig-



Scheme 4. Scheme 4. The scheme of the UV photolysis of 2-nitro-4-[3-(trifluoromethyl)-3*H*-diazirin-3-yl]benzylamide of N^{α}-Boc-alanine (**X**).

nificantly differ in their half-decay times ($\tau_{1/2}$; 7.4 and 67 min, respectively) and quantum yields (φ) of the photochemical decomposition. This means that the photolysis of (**X**) is selective: diazirine group first forms a short-lived carbene and then the nitrobenzyl linker is degraded.

Thus, new photoreactive cleavable reagents that include a trifluoromethyldiazirine group are here described. The presence of chemically active groups [carboxyl in (**I**), bromomethyl in (**II**), amino in (**III**), and isothiocyanate in (**IV**)] helps use these reagents for the modification of amino, carboxyl, and sulfhydryl groups as the ligands within biological molecules that may be useful to study their intermolecular contacts by the method of photoaffinity modification.

EXPERIMENTAL

TLC was carried out on the precoated Silica gel 60 F_{254} plates (Merck, Germany) using the following solvent systems: (A) 65 : 10 petroleum ether–chloroform, (B) 4 : 1 hexane–ethyl acetate, (C) 2 : 1 chloroform–hexane, (D) 50 : 25 : 2 benzene–acetone–acetic acid; and (E) hexane. Substances were detected by their UV absorption. Column chromatography was performed on Silica gel 60 (70–230 mesh; Merck, Germany).

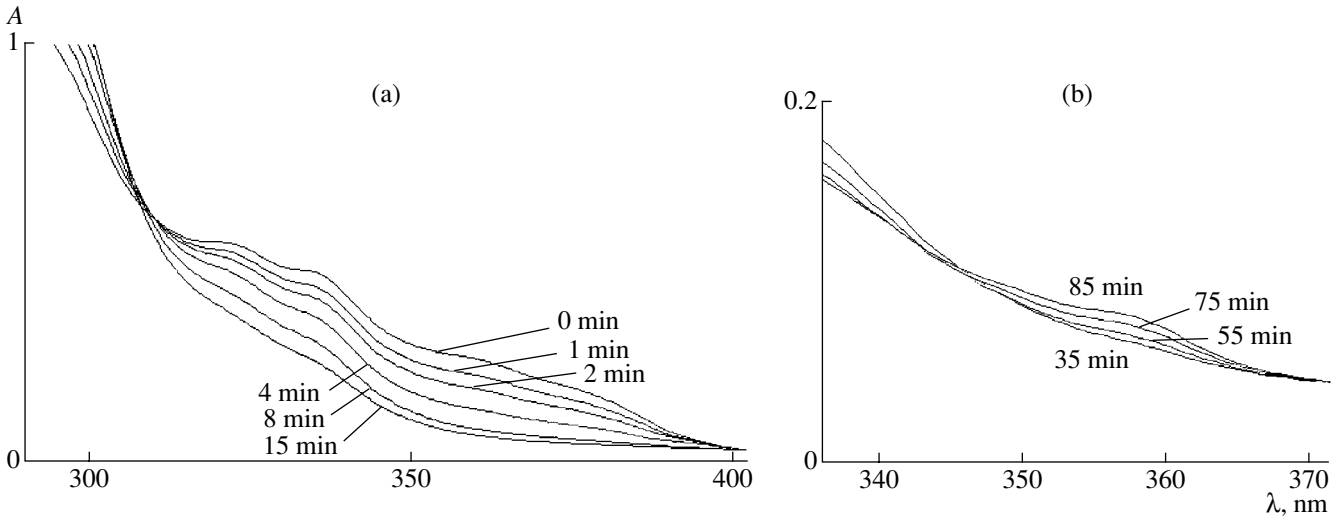
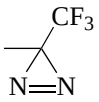
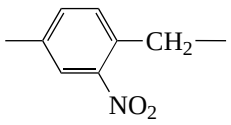


Fig. 2. The kinetics of the 2-nitro-4-[3-(trifluoromethyl)-3*H*-diazirin-3-yl]benzylamide of N^{α}-Boc-alanine (**X**) photolysis. The absorption spectra of the solution of (**X**) in ethanol (*c* 1.35 mM) are shown at various irradiation times: (a) a decrease in the intensity of the peak at 363 nm, corresponding to diazirine group for 0–15 min; (b) an increase in the absorption at 358 nm (this peak corresponds to nitroso group) for 35–85 min.

Table 2. The kinetic characteristics of the photolysis of 2-nitro-4-[3-(trifluoromethyl)-3*H*-diazirin-3-yl]benzylamide of N^{α}-Boc-alanine (**X**)

Photolabile group	$\tau_{1/2}$, min	k, min^{-1}	φ
	7.4 ± 1.4	$(9.3 \pm 1.7) \times 10^{-2}$	0.98 ± 0.33
	66.9 ± 15.8	$(1.03 \pm 0.23) \times 10^{-2}$	0.109 ± 0.032

IR spectra were recorded on a Specord 40 instrument (Germany). Mass spectra were determined using a Finnigan MAT INCOS 50 instrument using the electron impact ionization. UV spectra were measured in a single beam mode on an Aminco DW 2000 spectrophotometer at the wavelength range of 200–500 nm and scanning rate of 3 nm/s in quartz cells with an optical path length of 1 cm.

Syntheses of substances containing trifluoromethyldiazirine group were carried out under the conditions of moderate illumination.

4-[3-(Trifluoromethyl)-3*H*-diazirin-3-yl]toluene (**V**) was synthesized as described in [19]. 4-[3-(Trifluoromethyl)-3*H*-diazirin-3-yl]benzyl bromide (**VII**) was obtained according to the method [20] in 74% yield.

2-Nitro-4-[3-(trifluoromethyl)-3*H*-diazirin-3-yl]-toluene (VI). A 95% H_2SO_4 (1.4 ml) was carefully added dropwise to a stirred cooled (0°C) 4-[3-(trifluoromethyl)-3*H*-diazirin-3-yl]toluene (**V**) (400 mg, 2 mmol). Then a nitrating mixture [97% HNO_3–fuming HNO_3–concentrate H_2SO_4 (0.29 + 0.1 + 0.55 ml, respectively)] was dropwise added. The reaction mixture was stirred for 1 h, poured on ice and extracted with diethyl ether (2 × 10 ml). The extract was washed with 10% sodium bicarbonate (2 × 10 ml) and water (2 × 10 ml), dried over anhydrous sodium sulfate, and evaporated to give (**VI**) as an yellowish oil; yield of 350 mg (70%); R_f 0.75 (A) and 0.22 (E); UV (diethyl ether), λ_{max}, nm ($\log\varepsilon$): 250 (4.0), 288 (3.0), and 358 (2.3); MS, *m*/*z*: 245 $[M]^+$.

2-Nitro-4-[3-(trifluoromethyl)-3*H*-diazirin-3-yl]benzoic acid (I). Potassium permanganate (1 g, 5.6 mmol) was added to a stirred solution of (**VI**) (350 mg, 1.4 mmol) in a 10 : 7 pyridine–water mixture (17 ml). The reaction mixture was stirred at 50°C for 15 h, cooled to room temperature, diluted with water (50 ml), acidified to pH 2 with 1 N sulfuric acid, discolored by the addition of saturated $Na_2S_2O_5$ solution, and then extracted with diethyl ether (2 × 100 ml). The organic phase was alkalized with 1 N NaOH to pH 10 and extracted with water. The water phase was acidified with 1 N sulfuric acid to pH 2 and extracted with diethyl ether. The extract was dried over anhydrous sodium sulfate and evaporated. The residue was dissolved in ethanol (8 ml) and precipitated with water (25 ml) to give (**I**) as a white crystalline substance; yield 120 mg (31%); R_f 0.27 (B); UV (hexane, λ_{max}, nm ($\log\varepsilon$): 256 (4.3), 291 (3.1), and 343 (2.3); MS, *m*/*z* 247 $[M - N_2]^+$, 167 $[M + H - N_2 - CCF_3]^+$.

2-Nitro-4-[3-(trifluoromethyl)-3*H*-diazirin-3-yl]benzyl bromide (II). 4-[3-(Trifluoromethyl)-3*H*-diazirin-3-yl]benzyl bromide (**VII**) (1.36 g, 4.87 mmol) was added dropwise to a cooled (–10°C) 90% nitric acid (9 ml, 0.17 mmol) depleted of nitrogen oxides. The reaction mixture was extracted with diethyl ether (2 × 20 ml); the extract was washed with a saturated sodium bicarbonate solution and water, dried over sodium sulfate, filtered, and evaporated on a rotary evaporator. The resulting yellow oil was dissolved in a solvent system A (1 ml) and chromatographed on silica gel using system A as an eluent. Three fractions were collected with R_f 0.57, 0.4, and 0.3. Target product (**II**) was in fraction with R_f 0.4; yield 511 mg (33%); R_f 0.4 (A) and 0.13 (E); UV (ethanol, λ_{max}, nm ($\log\varepsilon$): 251 (4.1), 295 (3.1), and 342 (2.2); IR (ν, cm^{-1}): 1704 (NO_2), 1608 (N=N); MS, *m*/*z*: 297 $[M + H - N_2]^+$, 295 $[M - H - N_2]^+$, 251 $[M + H - N_2 - NO_2]^+$, 249 $[M - H - N_2 - NO_2]^+$.

2-Nitro-4-[3-(trifluoromethyl)-3*H*-diazirin-3-yl]benzylamine hydrochloride (III). *Method 1.* Compound (**II**) (372 mg, 1.15 mmol) was added to a solution of methenamine (170 mg, 1.2 mmol) in chloroform (2 ml). The reaction mixture was stirred for two days, and the precipitated methenaminium salt (483 mg, 90%) was filtered and decomposed by the treatment with a 1 : 8 conc. HCl–methanol mixture (10 ml) for three days at room temperature. The precipitated ammonium chloride was filtered off, and the filtrate was evaporated to dryness to yield 306 mg (90%) of (**III**).

Method 2. Compound (**II**) (100 mg, 0.31 mmol) was added to a solution of methenamine (43 mg, 0.31 mmol) in chloroform (1 ml). The reaction mixture was stirred for two days, and the precipitated methenaminium salt (143 mg, 90%) was filtered and then dis-

solved in water (2 ml). Gaseous SO_2 was passed through the solution for 1.5 h, which was accompanied with warming-up of the reaction mixture and the formation of a white precipitate 2-nitro-4-[3-(trifluoromethyl)-3*H*-diazirin-3-yl]benzylaminomethyl hydrogen sulfite (**IX**). The filtered precipitate was suspended in 25% HCl (5 ml) and aqueous vapor was passed though the suspension for 15 min. The resultant clear solution was cooled, and a white precipitate was formed. It was filtered and washed with an ice water (2×2 ml) to yield 68 mg (75%) of (**III**); R_f 0.4 (B); UV (ethanol, λ_{max}, nm ($\log\varepsilon$): 220 (3.74), 260 (3.24), and 353 (2.51).

2-Nitro-4-[3-(trifluoromethyl)-3*H*-diazirin-3-yl]benzyl isothiocyanate (IV). Compound (**III**) (50 mg, 0.17 mmol) was dissolved in pyridine (2.5 ml), and carbon disulfide (100 μl, 1.7 mmol) was added to the stirred solution. The reaction mixture was cooled to 0°C, treated with DCC (55 mg, 0.27 mmol), stirred for 4 h, and then evaporated. The residue was chromatographed on a silica gel column eluted with the system C to yield 30 mg (58%) of (**IV**); R_f 0.7 (B) and 0.9 (C); UV (ethanol, λ_{max}, nm ($\log\varepsilon$): 220 (3.92), 246 (4.06), and 353 (2.8); IR (cm^{-1}): 2080 (NCS), 1704 (NO_2), 1612 (N=N); MS, *m*/*z*: 269 $[M - H - S]^+$, 244 $[M - NCS]^+$, 229 $[M - NCS - N]^+$.

2-Nitro-4-[3-(trifluoromethyl)-3*H*-diazirin-3-yl]benzylamide of N^{α}-Boc-alanine (X). N^{α}-Boc-alanine (8.7 mg, 46 μmol) was dissolved in dichloromethane (1.5 ml) and treated with 1-hydroxybenzotriazole (9 mg, 67 μmol). The reaction mixture was cooled on ice, treated with (11 mg, 54 μmol) DCC, and stirred for 1 h. A solution of (**III**) (16 mg, 50 μmol) and diisopropylethylamine (20 μl, 54 μmol) in dichloromethane (1 ml) was added to the reaction mixture, and stirring was continued for one day. The precipitate of dicyclohexylurea was filtered off, the filtrate was evaporated, the residue was dissolved in ethyl acetate (10 ml), and the solution was washed with 10% sodium bicarbonate (2×5 ml), 0.1 N sulfuric acid (2×5 ml), water (2×5 ml), and a saturated solution of sodium chloride (2×5 ml). The ethyl acetate extract was dried over anhydrous sodium sulfate and evaporated to yield 10 mg (50%) of (**X**); R_f 0.73 (D); UV (ethanol, λ_{max}, nm ($\log\varepsilon$): 319 (3.9), 333 (2.52), and 363 (2.2). The presence of a free amino group was proven by the ninhydrin test after the removal of Boc group.

The photolysis of 2-nitro-4-[3-(trifluoromethyl)-3*H*-diazirin-3-yl]benzylamide of N^{α}-Boc-alanine (X). A 1.35 mM solution of (**X**) in ethanol was irradiated in a quartz cell with an optical path length of 1 cm. A Mineralight UVGL-58 mercury lamp (with an integrated filter), which emits a monochromatic light with a wavelength of 365 nm with intensity of 700 μW/cm^2, was used as a light source for the irradiation. The intensity of the incident light (in quanta/s) required to determine the quantum yield was measured by actinometry using the ferrioxalate–phenanthroline technique [30]. The measured intensity (I_0) was 1.5×10^{16} quanta/s. After irradiation for 75 min, N^{α}-Boc-alanine amide was detected in the solution by TLC, R_f 0.38 in 9 : 1 chloroform–methanol; its spot coincided with that for the reference compound. On spraying the plate with 0.2% ninhydrin in acetone containing 10% acetic acid with subsequent heating to 100°C, a purple staining characteristic of amino acids was observed.

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