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DESIGN AND SYNTHESIS OF 1,3-BIS(3-(TRIFLUOROMETHYL)-DIAZIRIN-3-YL)PHENYLALANINE FOR EFFICIENT PHOTO CROSS-LINKING

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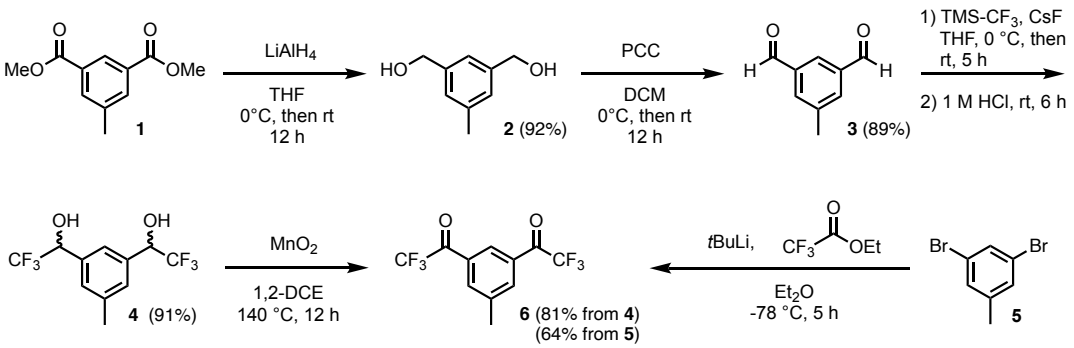
Abstract – Photoreactive α-amino acids are powerful chemical tools for elucidating interactions in protein networks. Typically, photophores are required for stability and generation of high-reactive species. Additionally, orientation of photophores is one of the significant issues for performing photoaffinity labeling. Therefore, we rationally designed a bis-diazirinyl-phenylalanine for which photo cross-linking efficiency could be increased. In this study, we synthesized a new 1,3-bis(3-(trifluoromethyl)diazirin-3-yl)phenylalanine and demonstrated further study in terms of photoactivation.

Elucidation of target biomolecules of a bioactive compound is invaluable in the field of life science for a deep understanding of complex biological mechanisms. Various strategies including a genetic approach and 3D structure determination involving solution-state NMR and X-ray crystallography have been used for that purpose. Although these approaches can provide detailed information on the structure of a target biomolecule, an abundant pure molecule is required. As an alternative method, affinity-based chemical probes have been used to identify and visualize target biomolecules in complex systems. Photoaffinity labeling is one of the powerful chemical approaches used for identifying the interaction, where it can conduct binding site mapping, protein-protein interaction and visualizes live-cell imaging by a photoinduced covalent bond between a ligand and its target molecule.[1] The selection of photophores is

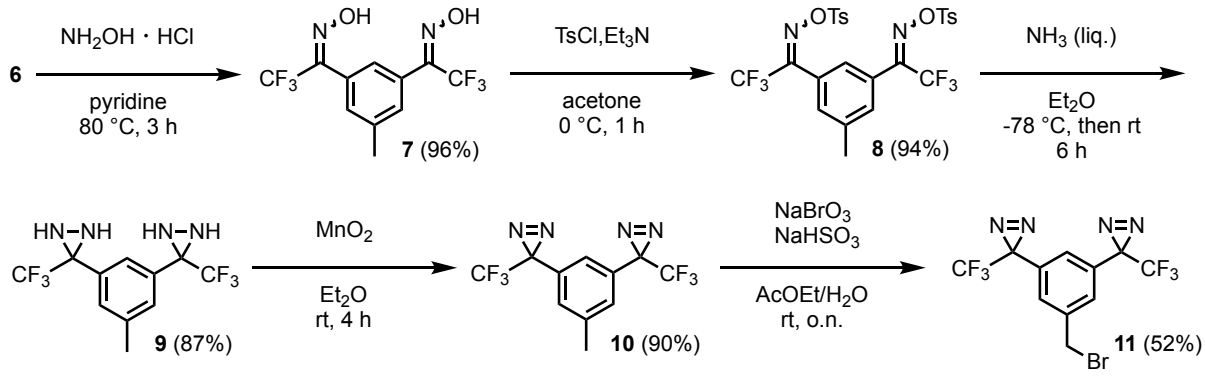
important for successful photoaffinity labeling experiments. (3-Trifluoromethyl)phenyldiazirine (TPD)[1d,2] is one of the most reliable photophores in photoaffinity labeling because it has a number of advantages over other commonly used photo-cross-linkers such as phenyl azides,[3] benzophenones[4] and the most recently discovered 2-aryl-5-carboxytetrazole.[5] Particularly, TPD has some advantage factors such as the stability against high temperature, nucleophiles, reducing agents, acidic and basic conditions. In addition to this, TPD can also generate an active species carbene to cross-link in a short time with a high wavelength (≈ 365 nm), which can prevent damage of target molecules. Furthermore, due to their high reactivity, carbenes are often rapidly quenched by water, which could be an advantage as it minimizes non-specific binding.[2a,6] Despite the promising characteristics of TPD, orientation of the diazirine on TPD also strongly affects the success of photoaffinity labeling and the labeling position towards target molecules.[7] Therefore, control of the change of photo-cross-linking efficiency depending on the orientation of photophores is a significant issue in photoaffinity labeling.[1d] Previously, in order to permit multiple cross-linking of alkyl chain polymers under mild conditions and without unwanted branching or fragmentation, a bis-diazirine was prepared by Lepage's group.[8] We established a novel bis-diazirinyl-phenylalanine (Phe) because photoreactive amino acids are powerful tools to validate the possibility of multiple photo-cross-linking.

A key point in our synthesis of bis-diazirinyl-Phe is condensation of the 3,5-bis-TPD unit and an alanine derivative. Initially, 3,5-bis-trifluoroacetyltoluene **6** was prepared as reported by Hayes's group with slight modification.[9] Compound **1** was treated with $LiAlH_4$ to obtain 1,3-benzenedimethanol **2** in high yield. Compound **2** was oxidized with pyridinium chlorochromate (PCC) to form di-formyl moieties **3**. Subsequently, a trifluoromethyl group was subjected to installation into the formyl group **4**, followed by oxidation in the presence of activated MnO_2 at 140 °C in a sealed tube to produce trifluoroacetyl moiety **6** with sequential steps in good yield. Compound **6** was able to be prepared in another way; commercially available 3,5-dibromotoluene **5** was treated with four equivalents of *tert*-BuLi followed by treatment with ethyl trifluoroacetate to obtain **6** in a one-step reaction with a moderate yield of 64% (Scheme 1).[10]



Scheme 1. Synthesis of 3,5-bis-trifluoroacetyltoluene **6**

Next, compound **6** was converted to the oxime **7** with hydroxylamine hydrochloride, and then tosylation was performed with tosyl chloride to obtain the tosyl oxime **8** with a yield of over 90% in a two-step reaction. These steps were able to proceed by using two or more equivalents of each reagent that can be used in common diazirinyl synthetic methods. Compound **8** was subjected to conversion to diaziridine **9** with liquid ammonia, followed by oxidation of **9** to 3,5-bis-diazirinyl-toluene **10** with activated MnO_2 in high yield. Subsequently, monobromination at the benzyl position of **10** was conducted. Previously, Sheena's group used *N*-bromosuccinimide in the presence of 2,2'-azobisisobutyronitrile (AIBN) to selectively prepare monobromomethyl TPD at 70 °C in high yield.[11] However, dibromination was also observed for **10**. To avoid the production of a dibromomethyl derivative due to difficulty for separation, we tested some reagents and conditions. $NaBrO_3$ as a resource of bromine was shown to be selective monobromination **11** in moderate yield without dibromination (Table 1). Also, the remaining material **10** was able to be reused for bromination (Scheme 2).



Scheme 2. Preparation of 3,5-bis-diazirinyl-benzyl bromide **11**

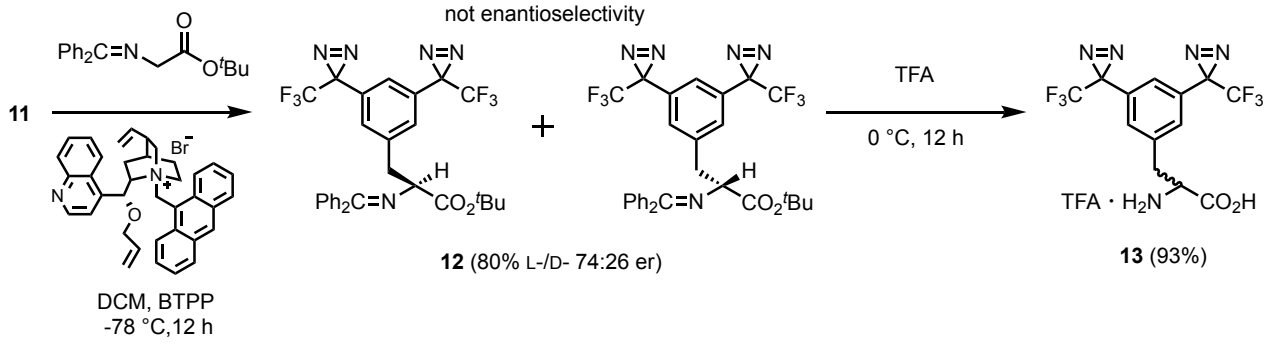
Table 1. Bromination conditions of **10**

entry	temp.	time (h)	Br donor (eq.)	AIBN (mol%)	conversion ratio (%)		
					mono-	di-	comp. **10**
1	70 °C	3	NBS (0.8)	2.5	50	20	30
2	70 °C	3	NBS (1.0)	2.5	65	30	5
3	70 °C	3	NBS (1.5)	0.6	55	25	10
4	50 °C	overnight	NBS (1.5)	2.5	30	15	55
5	rt	overnight	$NaBrO_3$ (12)	–	63	0	37

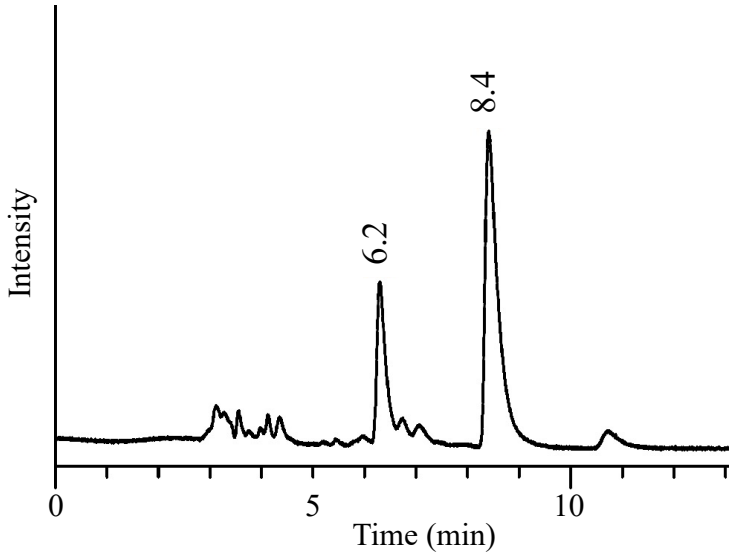
calculated by ^{1}H-NMR

In condensation of the alanine derivative (*tert*-butylglycinate benzophenone imine) with bromide **11**, we examined an asymmetric synthesis with a catalyst based on cinchonidine salt[12] in the presence of the phosphazene base P1-*tert*-buthyltris(tetramethylene) (BTPP) to obtain bis-diazirinyl-Phe derivative **12** (Scheme 3). However, unfortunately, the optical purity of **13** after deprotection corresponded to an er L-74% and D-26%, as analyzed by chiral HPLC (Figure 1). As the reason for this, due to the substitution

of bulky bis-diazirinyl moieties on aromatics, the enantiomeric allylation transition state with **11** mediated by the cinchonidinium catalyst might not be able to form.[13]



Scheme 3. Synthesis of 3,5-bis-diazirinyl-Phe **13**



	Time (min)	Peak Area (%)
Bis-diazirinyl-D-Phe	6.2	25.9
Bis-diazirinyl-L-Phe	8.4	74.1

Figure 1. HPLC profiles of bis-diazirinyl-Phe **13**. HPLC conditions: DAICEL CHIRALPAK ZWIX (–) (4.6×250 mm); 50 mM formic acid+25 mM DEA in MeOH/MeCN/H_2O, 49%/49%/2%; flow rate, 0.8 mL/min; detection at 350 nm.

Finally, bis-diazirinyl-Phe **13** was irradiated under black light to evaluate its photoreactive properties. Irradiation of 1 mM of a solution of **13** under black light (100 W) in water confirmed a decrease in the characteristic diazirinyl absorbance around 350–365 nm[7,14] as the irradiation time proceeded (Figure 2a). As shown Figure 2b, both diazirinyl moieties on compound **13** reacted with water exhibiting the strongest intensity peak of water adducts (*m*/*z* 362) and its decarboxylic peak (*m*/*z* 316) after 10 minutes of photoirradiation (Figure 2b). Based on these results, the half-life of both diazirinyl moieties on compound

13 were calculated to be 3.5 min under black light (100 W). Therefore, bis-diazirinyl-Phe **13** has sufficient reactivity for photoaffinity labeling.

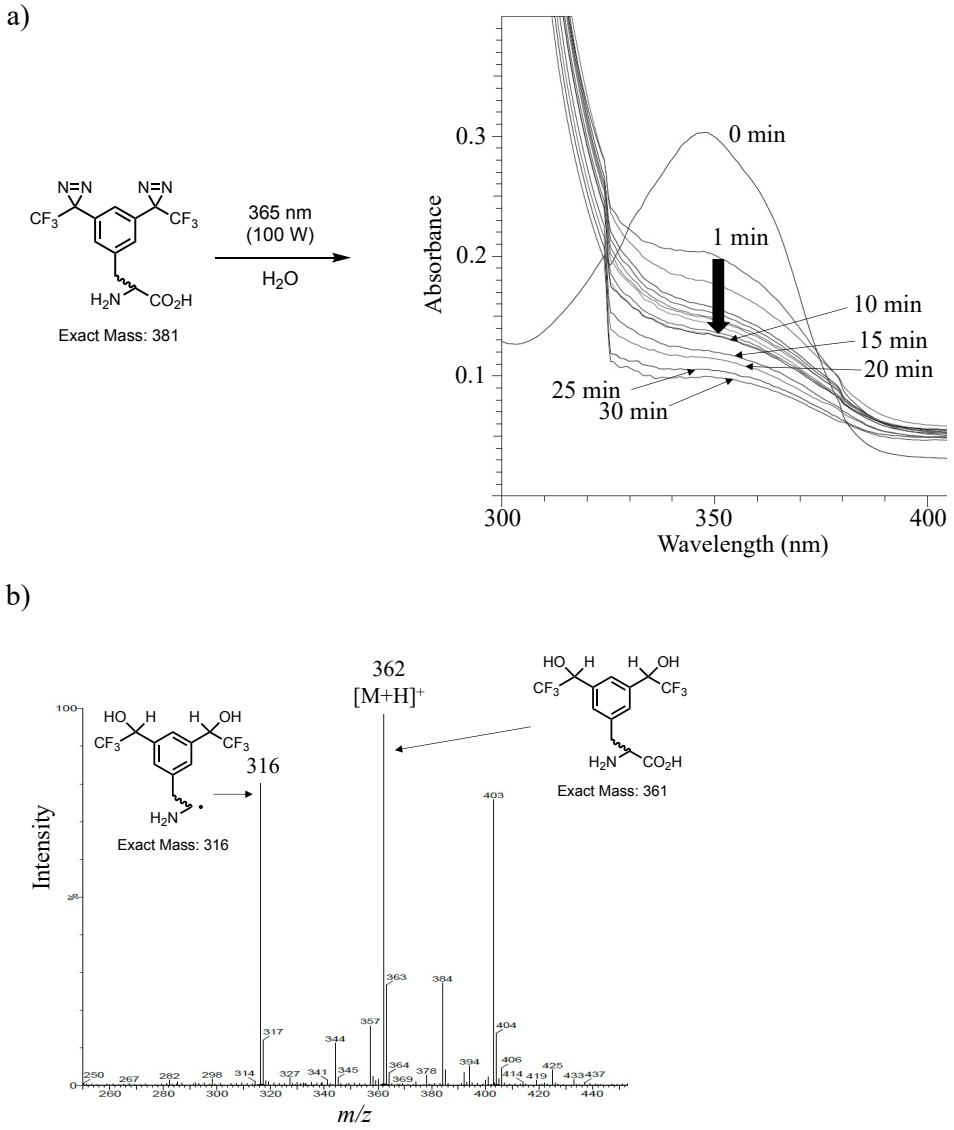


Figure 2. a) Photolysis of bis-diazirinyl-Phe **13** in H_2O (1 mM) under black light (100 W). UV spectra of the photolysis were recorded every minute for 10 min. b) ESI-MS analysis of a mixture of products after photoirradiation of compound **13** for 10 minutes.

In summary, we have achieved for the first time a synthesis of bis-diazirinyl-phenylalanine for efficient photoaffinity labeling. However, the stereoselective construction of **13** is still underway. This simple preparation will be acceptable for all biochemists and contribute to a deeper understanding of peptide-receptor interactions and **13** can be directly incorporated at specific positions in the polypeptide chain.[15]

EXPERIMENTAL

*(5-Methyl-1,3-phenylene)dimethanol **2***

Dimethyl 5-methylisophthalate **1** (1.50 g, 7.17 mmol) was dissolved in THF (20 mL), and LAH (1.00 g, 26.4 mmol) was added to the THF solution at 0 °C. The reaction was warmed to room temperature and stirred for 12 h. The reaction was cooled to 0 °C, and 1 M HCl was added to the reaction until an acidic condition was reached. Insoluble materials were removed with celite filtration, and the filtrate was evaporated. The residue was purified by column chromatography (AcOEt/hexane 1/2) to yield (5-methyl-1,3-phenylene)dimethanol **2** (1.00 g, 92%) as a colorless oil. Analytical data were identical to those reported in the literature.[16]

*5-Methylisophthalaldehyde **3***

(5-Methyl-1,3-phenylene)dimethanol **2** (1.00 g, 6.62 mmol) and PCC (3.20 g, 15.2 mmol) were dissolved in CH_2Cl_2 at 0 °C. The reaction mixture was stirred for 12 h at room temperature, and insoluble materials were removed with celite filtration. The filtrate was evaporated, and the residue was purified by column chromatography (CH_2Cl_2 to AcOEt/hexane 1/2) to yield 5-methylisophthalaldehyde **3** (873 mg, 89%) as a white-colored amorphous mass. Analytical data were identical to those reported in the literature.[17]

*1,1'-(5-Methyl-1,3-phenylene)bis(2,2,2-trifluoroethanol) **4***

5-Methylisophthalaldehyde **3** (714 mg, 5.32 mmol) was dissolved in THF (17 mL). Trifluoromethyltrimethylsilane (1.66 g, 11.7 mmol) and CsF (20 mol%) were added to the THF solution at 0 °C. The reaction mixture was stirred for 5 h at room temperature, and 1 M HCl was added and then stirred for 3 h at the same temperature. The organic compound was extracted with AcOEt, washed with brine, and dried over $MgSO_4$. The crude product was purified by column chromatography (AcOEt/hexane 1/3) to yield 1,1'-(5-methyl-1,3-phenylene)bis(2,2,2-trifluoroethanol) **4** (1.32 g, 91%) as a pale yellow oil. ^{1}H-NMR (270 MHz, CD_3OD) δ : 7.33 (1H, s), 7.23 (2H, s), 4.90 (2H, q, J = 7.0 Hz), 2.27 (3H, s). ^{13}C-NMR (68 MHz, CD_3OD) δ: 139.3, 137.1, 129.9, 126.2 (q, J = 281.0 Hz), 125.4, 72.9 (q, J = 31.3 Hz), 21.3. ^{19}F-NMR (470 MHz, CD_3OD) δ : -79.5. MS was not detected.

*1,1'-(5-Methyl-1,3-phenylene)bis(2,2,2-trifluoroethanone) **6***

1,1'-(5-Methyl-1,3-phenylene)bis(2,2,2-trifluoroethanol) **5** (989 mg, 3.43 mmol) and activated MnO_2 (excess) were dissolved in 1,2-dichloroethane (15 mL) in a sealed tube. The reaction was warmed to 140 °C and stirred for 12 h. The solvent was evaporated, and the residue was purified by column chromatography (AcOEt/hexane 1/6) to yield 1,1'-(5-methyl-1,3-phenylene)bis(2,2,2-trifluoroethanone) **6** (789 mg, 81%) as a pale yellow oil.

^{1}H-NMR (270 MHz, $CDCl_3$) δ: 8.54 (1H, s), 8.20 (2H, s), 2.59 (3H, s). ^{13}C-NMR (68 MHz, $CDCl_3$) δ: 179.5 (q, *J* = 35.5 Hz), 140.8, 136.5, 130.7, 128.7, 116.3 (q, *J* = 290.5 Hz), 21.3. ^{19}F-NMR (470 MHz, $CDCl_3$) δ: -71.7. MS was not detected.

Another route

1,3-Dibromo-5-methylbenzene **5** (814 mg, 3.25 mmol) was dissolved in abs. Et_2O (20 mL) and cooled to -78 °C under N_2. A solution of *tert*-BuLi (7.87 mL of 1.65 M pentane solution, 13.0 mmol) was added dropwise over a period of 15 min and stirred for 20 min. Ethyl trifluoroacetate (1.12 mL, 9.43 mmol) was added at −78 °C and the mixture was stirred for 5 h and then quenched saturated ammonium chloride (7 mL) and extracted with Et_2O. The organic layer was washed with brine and dried over $MgSO_4$. The residue was purified by column chromatography (AcOEt/hexane 1/7 to CH_2Cl_2) to yield **6** (593 mg, 64%)

2,2,2-Trifluoro-1-(3-methyl-5-(2,2,2-trifluoro-1-(hydroxyimino)ethyl)phenyl)ethanone oxime 7

1,1'-(5-Methyl-1,3-phenylene)bis(2,2,2-trifluoroethanone) **6** (693 mg, 2.40 mmol) and hydroxylamine hydrochloride (1.00 g, 14.4 mmol) were dissolved in pyridine (6 mL). The reaction mixture was stirred at 80 °C for 3 h and concentrated. The residue was dissolved in Et_2O (20 mL). The organic layer was washed with 1 M HCl and brine, dried over $MgSO_4$, filtrated, and concentrated. The residue was purified by column chromatography (CH_2Cl_2 to AcOEt/hexane 1/2) to yield 2,2,2-trifluoro-1-(3-methyl-5-(2,2,2-trifluoro-1-(hydroxyimino)ethyl)phenyl)ethanone oxime **7** (723 mg, 96%, mixture of *syn*- and *anti*- isomers) as a white amorphous mass.

^{1}H-NMR (270 MHz, CD_3OD) δ: 7.38 (2H, s), 7.32 (1H, s), 2.40 (3H, d, *J* = 2.3 Hz). ^{13}C-NMR (68 MHz, CD_3OD) δ: 146.6 (q, *J* = 34.1 Hz), 140.2, 131.8 (m), 129.1, 127.1 (m), 120.3 (q, *J* = 270.3 Hz), 21.3. ^{19}F-NMR (470 MHz, CD_3OD) δ: -63.7, -67.5, -67.6. HRMS-ESI (*m*/*z*) $[M + H]^+$ calcd for $C_{11}H_9F_6N_2O_2$ 315.0568, found 315.0581.

*2,2,2-Trifluoro-1-(3-methyl-5-(2,2,2-trifluoro-1-(tosyloxyimino)ethyl)phenyl)ethanone O-tosyl oxime **8***

2,2,2-Trifluoro-1-(3-methyl-5-(2,2,2-trifluoro-1-(hydroxyimino)ethyl)phenyl)ethanone oxime **7** (644 mg, 2.05 mmol) was dissolved in acetone (16 mL) and cooled to 0 °C. Triethylamine (830 mL) and *p*-toluenesulfonyl chloride (820 mg, 4.30 mmol) were successively added to the reaction mixture. The reaction mixture was stirred for 1 h at the same temperature and concentrated in vacuo, and the residue was purified by column chromatography (CH_2Cl_2/hexane 1/4 to CH_2Cl_2) to yield 2,2,2-trifluoro-1-(3-methyl-5-(2,2,2-trifluoro-1-(tosyloxyimino)ethyl)phenyl)ethanone *O*-tosyl oxime **8** (1.19 g, 94%) as a colorless oil.

^{1}H-NMR (270 MHz, $CDCl_3$) δ: 7.90 (4H, m,), 7.41-7.13 (7H, m), 2.52-2.41 (9H, m). ^{13}C-NMR (68 MHz, $CDCl_3$) δ: 179.5 (q, *J* = 36.3 Hz), 140.8, 136.5, 136.5, 130.8, 128.7, 128.7, 128.7, 116.3 (q, *J* = 291.3 Hz), 21.3 (3C). ^{19}F-NMR (470 MHz, $CDCl_3$) δ: -61.4, -61.5, -66.8. HRMS-ESI (*m*/*z*) $[M + H]^+$ calcd for $C_{25}H_{21}F_6N_2O_6S_2$ 623.0745, found 623.0768.

3,3'-(5-Methyl-1,3-phenylene)bis(3-(trifluoromethyl)diaziridine) ***9***

2,2,2-Trifluoro-1-(3-methyl-5-(2,2,2-trifluoro-1-(tosyloxyimino)ethyl)phenyl)ethanone *O*-tosyl oxime **8** (928 mg, 1.49 mmol) was dissolved in Et_2O. In a sealed tube, liquid ammonia (excess) was added at −78 °C and the etherial solution of tosyl oxime was added. The reaction mixture was warmed to room temperature and then stirred for 6 h at the same temperature. After excess ammonium gas had been removed in a draft chamber, the residual solution was concentrated. The crude residue was purified by column chromatography (AcOEt/hexane 1/3) to yield 3,3'-(5-methyl-1,3-phenylene)bis-(3-(trifluoromethyl)diaziridine) **9** (405 mg, 87%) as a colorless amorphous mass.

^{1}H-NMR (270 MHz, $CDCl_3$) δ: 7.65 (1H, d, *J* = 6.3 Hz), 7.51 (2H, s), 2.86 (2H, d, *J* = 9.2 Hz), 2.41 (3H, s), 2.33 (2H, d, *J* = 9.2 Hz). ^{13}C-NMR (68 MHz, $CDCl_3$) δ: 139.5, 132.4 & 132.4, 130.5 & 130.4, 125.1 & 124.9, 123.3 (q, *J* = 277.7 Hz), 57.7 (q, *J* = 36.0 Hz), 21.1. ^{19}F-NMR (470 MHz, $CDCl_3$) δ: -75.3, -75.4. HRMS-ESI (*m*/*z*) $[M + H]^+$ calcd for $C_{11}H_{11}F_6N_4$ 313.0888, found 313.0862.

3,3'-(5-Methyl-1,3-phenylene)bis(3-(trifluoromethyl)-3H-diazirine) ***10***

3,3'-(5-Methyl-1,3-phenylene)bis(3-(trifluoromethyl)diaziridine) **9** (237 mg, 0.760 mmol) and activated MnO_2 (500 mg) were suspended in Et_2O (15 mL). The reaction mixture was stirred at room temperature for 4 h and then insoluble material was filtrated. The filtrate was concentrated, and the residue was purified by column chromatography (CH_2Cl_2) to yield 3,3'-(5-methyl-1,3-phenylene)bis(3-(trifluoromethyl)-3*H*-diazirine) **10** (210 mg, 90%) as a colorless oil. ^{1}H-NMR (270 MHz, $CDCl_3$) δ: 7.07 (2H, s), 6.76 (1H, s), 2.38 (3H, s). ^{13}C-NMR (68 MHz, $CDCl_3$) δ: 139.9, 130.1, 128.5, 121.8 (q, *J* = 274.9 Hz), 121.7, 28.1 (q, *J* = 40.8 Hz), 21.4. ^{19}F-NMR (470 MHz, $CDCl_3$) δ: -65.2. MS was not detected.

3,3'-(5-(Bromomethyl)-1,3-phenylene)bis(3-(trifluoromethyl)-3H-diazirine) ***11***

To a solution of $NaBrO_3$ (1.24 g, 8.25 mmol) in water (3 mL) was added 3,3'-(5-methyl-1,3-phenylene)bis(3-(trifluoromethyl)-3*H*-diazirine) **10** (213 mg, 0.688 mmol) in AcOEt (4 mL), followed by a solution of $NaHSO_3$ (0.858 g, 8.25 mmol) in water (6 mL) over a period of about 10 min. The reaction mixture was stirred for 24 h at room temperature. The reaction was poured into Et_2O. The ethereal solution was washed with brine, dried over $MgSO_4$, and evaporated. The residue was purified by

column chromatography (hexane to CH_2Cl_2/hexane 1/9) to yield 3,3'-(5-(bromomethyl)-1,3-phenylene)bis(3-(trifluoromethyl)-3*H*-diazirine) **11** (139 mg, 52%) as a colorless oil.

^{1}H-NMR (270 MHz, $CDCl_3$) δ: 7.28 (2H, s), 6.91 (1H, s), 4.42 (2H, s). ^{13}C-NMR (68 MHz, $CDCl_3$) δ: 140.0, 131.0, 128.3, 124.3, 121.7 (q, J = 280.5 Hz), 30.8, 28.1 (q, J = 39.1 Hz). ^{19}F-NMR (470 MHz, $CDCl_3$) δ: -65.1. MS was not detected. The starting material was able to be recovered (66.8 mg, 31%).

*tert-Butyl 3-(3,5-bis(3-(trifluoromethyl)-3H-diazirin-3-yl)phenyl)-2-(diphenylmethyleneamino)propanoate **12***

tert-Butylglycinate benzophenone imine (78.5 mg, 0.266 mmol) and *O*-allyl-*N*-9-anthracenylmethylcinchonidium bromide (21.9 mg, 36.2 mmol) were dissolved in CH_2Cl_2 (3 mL). 3,3'-(5-(bromomethyl)-1,3-phenylene)bis(3-(trifluoromethyl)-3*H*-diazirine) **11** (93.5 mg, 0.241 mmol) was added to the mixture and was cooled at –78 °C. To the solution was slowly added BTPP (81.8 mL, 0.362 mmol) followed by stirring for an additional 12 h. After removal of the solvent, the residue was purified by column chromatography (AcOEt/hexane 1:7) to yield *tert*-butyl 3-(3,5-bis(3-(trifluoromethyl)-3*H*-diazirin-3-yl)phenyl)-2-(diphenylmethyleneamino)propanoate **12** (116 mg, 80%) as a colorless oil.

^{1}H-NMR (270 MHz, $CDCl_3$) δ: 7.62-7.29 (8H, m), 6.99 (2H, s), 6.85 (1H, s), 6.61 (2H, d, J = 6.9 Hz), 4.07 (1H, t, J = 6.6 Hz), 3.19 (2H, d, J = 6.3 Hz), 1.45 (9H, s). ^{13}C-NMR (68 MHz, $CDCl_3$) δ: 171.1, 169.9, 140.8, 138.8, 136.0, 128.6, 128.2, 127.9, 127.2, 122.5, 121.7 (q, J = 274.9 Hz), 81.6, 66.8, 39.2, 28.0 (q, J = 40.8 Hz), 27.9. ^{19}F-NMR (470 MHz, $CDCl_3$) δ: -65.1. HRMS-ESI (m/z) $[M + H]^+$ calcd for $C_{30}H_{26}F_6N_5O_2$ 602.1991, found 602.1984.

*2-Amino-3-(3,5-bis(3-(trifluoromethyl)-3H-diazirin-3-yl)phenyl)propanoic acid **13***

tert-Butyl 3-(3,5-bis(3-(trifluoromethyl)-3*H*-diazirin-3-yl)phenyl-2-(diphenylmethyleneamino)propanoate **12** (116 mg, 0.192 mmol) was dissolved in TFA (3 mL) at 0 °C. The mixture was stirred for 12 h at room temperature. TFA was evaporated and the residue was purified by column chromatography (CH_2Cl_2 to AcOEt/MeOH/H_2O 4/1/1) to yield 2-amino-3-(3,5-bis(3-(trifluoromethyl)-3*H*-diazirin-3-yl)phenyl)propanoic acid **13** (88.4 mg, 93%) as a colorless amorphous mass.

^{1}H-NMR (270 MHz, CD_3OD) δ: 7.28 (2H, s), 7.16 (1H, s), 3.87 (1H, s), 3.34 (1H, dd, J = 7.3, 3.6 Hz), 3.11 (1H, dd, J = 13.8, 7.6 Hz). ^{13}C-NMR (68 MHz, CD_3OD) δ: 176.4, 140.5, 131.7, 130.3, 124.8, 123.3 (q, J = 274.3 Hz), 56.7, 37.6, 29.3 (q, J = 40.8 Hz). ^{19}F-NMR (470 MHz, CD_3OD) δ: -66.9. HRMS-ESI (m/z) $[M + H]^+$ calcd for $C_{13}H_{10}F_6N_5O_2$ 382.0739, found 382.0714.

*Photolysis of compound **13** in H_2O*

1 mM of a solution of bis-diazirinyl-Phe **13** in H_2O was placed in a quartz cuvette. Photolysis was carried out with a 100 W black light at a distance of 1 cm from the surface of the light source.

Spectra were measured after each minute, and then the half-life was calculated from the decrements of the absorbance around 360 nm.

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