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Protease Inhibitors

Chemically Stable Diazo Peptides as Selective Probes of Cysteine Proteases in Living Cells

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Abstract: Diazo peptides have been described earlier, however, due to their high reactivity have not been broadly used until today. Here, we report the preparation, properties, and applications of chemically stable internal diazo peptides. Peptidyl phosphoranylidene-esters and amides were found to react with triflyl azide primarily to novel 3,4-disubstituted triazolyl-peptides. Nonaflyl azide instead furnished diazo peptides, which are chemically stable from pH 1–14 as amides and from pH 1–8 as esters. Thus, diazo peptides prepared by solid phase peptide synthesis were stable to final deprotection with 95 % trifluoroacetic acid. Diazo peptides with the recognition sequence of caspase-3 were identified as specific, covalent, and irreversible inhibitors of this enzyme at low nanomolar concentrations. A fluorescent diazo peptide entered living cells enabling microscopic imaging and quantification of apoptotic cells via flow cytometry. Thus, internal diazo peptides constitute a novel class of activity-based probes and enzyme inhibitors useful in chemical biology and medicinal chemistry.

The versatile reactivity of diazo peptides has inspired the imagination of chemists and biochemists ever since Curtius reported the N-terminal diazotization of a tetrapeptide in 1904.[1] C-terminal peptidyl diazo methyl ketones[2] (I) and N-terminal *N*-diazoacyl-peptides (II) have been prepared and investigated as irreversible enzyme inhibitors, photo-affinity protein probes, in cycloadditions, and as potential anti-cancer drugs (Figure 1).[3] N-terminal diazo peptides and C-terminal diazo methyl ketones, however, are unstable toward acid and react with various nucleophiles upon acidic activation, by irradiation, and by formation of transition metal carbenes.[4] Thus, the preparation of chemically stable diazo peptides, which tolerate standard conditions of solid phase peptide synthesis such as TFA cleavage, remained a challenge and bear high potential, as the obtained products might be suited to undergo selective reactions with protein surfaces and binding sites.[5] For that reason, we set the development of stable diazo peptides as our scientific aim and will report the results on this topic in the following.

We reasoned that an internal diazo peptide of general structure III, merged of the terminal structures I and II, (Figure 1) might be an attractive synthetic goal as double substitution of the diazoalkane motif with carbonyl groups should increase the stability of the product and enable versatile fine-tuning of the biological reactivity,[6] possibly resulting in highly specific activity-based protein probes (ABPP).[7] Bromo acetamides and acetate esters **1a**–**e** were employed to generate phosphonium salts **2a**–**g**, which provided peptidyl phosphoranes **3a**–**i** via C-acylation (Scheme 1) either with amino acid fluorides or amino acids activated with MSNT. Polymer-supported peptidyl phosphoranes have served as a source of diverse C-terminal peptide variations[8a] including peptidyl α-keto esters and amides,[8b] keto aldehydes,[8c,d] diketo esters,[8c] diketones,[8e]

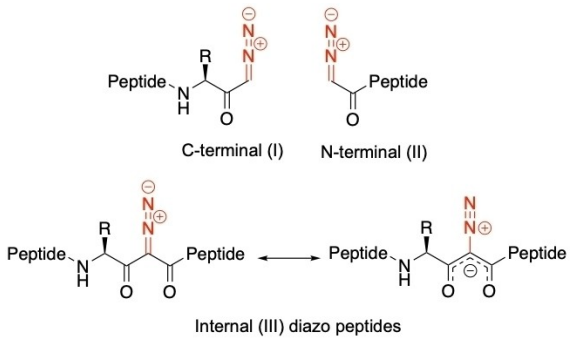

Figure 1. Diazo peptides of various structures and reactivities: C-terminal peptidyl diazo methyl ketones (I) and N-terminal diazo peptides (II) both suffer from limited stability under acidic conditions. Fused internal diazo peptides (III) were envisioned to possess increased stability due to mesomeric delocalization of the negative charge and thus might enable selective chemical probes.

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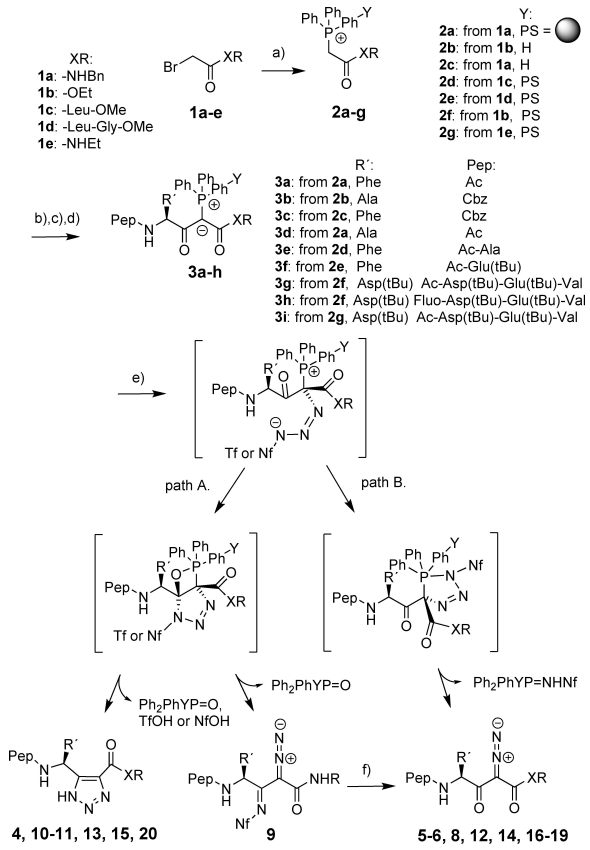


Scheme 1. Synthesis of 4,5-substituted triazolylpeptides and diazo peptides. Reaction conditions: a) PPh_3 or PPh_3 polystyrene resin (PS), Et_2O/THF 3:1 or DCM overnight, rt. b) Ylide formation: DIPEA. c) C-acylation: Fmoc-AA–F, DIPEA in CH_2Cl_2, or: Fmoc-AA–OH, MSNT, 2,6-lutidine, DCM overnight, rt, 48% - 91%. d) Fmoc-cleavage: 20% piperidine/DMF 2x10 min. Peptide elongation: DCM, Fmoc-AA–OH, TBTU, DIPEA, DMF, 2 h; Capping: Ac_2O, DIPEA, DCM, 1 h or: Fluo = 5,6-carboxy-fluorescein, DIC, HOBt. e) Path A. Cleavage: triflyl azide (Tf)/nonaflyl azide (Nf) in DCM 3 h-3 d. path B. Cleavage: nonaflylazide in DCM, 1–3 d; ev. followed by 95% TFA/water. f) Hydrolysis with 2% TFA in H_2O/ACN 50:50.

and vinylketones[8f] and were reported to react readily and regioselectively with azides furnishing linear[9a] and cyclic[9b] 1,5-disubstituted peptidyl-triazolyl-peptides. Treatment of polymer-supported N-acetyl-2-(L)-phenylalanyl-2-phosphoranylidene-N-benzylacetamide **3a** with trifluoromethylsulfonyl azide (triflyl azide, Tf–N_3) furnished the 4,5-disubstituted 1*H*-1,2,3-triazole **4** in an isolated yield of 34% (Scheme 1, Table 1). 5-Aminomethyl-1*H*-1,2,3-triazol-4-carboxamide (compound **4**), formed via dipolar cycloaddition of the phosphorus acyl-ylide to the azide followed by elimination of the phosphine-oxide and hydrolysis of the triflyl triazole (Scheme 1, path A), constitutes - to the best of our knowledge - the first representative of a new class of constrained γ-amino-acids.

HPLC-MS of the reaction between resin **3a** and triflylazide revealed besides the main product **4** (MH^+ = 364 m/z) traces of a by-product **5** with slightly elongated retention time characterized by two mass signals at 365 and 337 m/z, respectively. These masses suggested formation of the internal diazo peptide **5**, which subsequently released elemental nitrogen in the gas phase. Mechanistically, compound **5** could be formed by a 2+3 cycloaddition of the phosphorus ylide to the azide followed by elimination of the triphenylphosphine imine (Scheme 1, path B). When nonafluorobutyl-sulfonyl (nonaflyl) azide was used instead of triflyl azide in this reaction, the isolated yield of the diazo peptide **5** was improved from 5% to 41% (SFigure 10). Apparently, the reduced electron density or the increased steric hindrance of the nonaflyl triazenium intermediate favored cyclization via attack of the phosphorus (path B) over cyclization via attack of the carbonyl carbon (path A).

For a systematic investigation of diazo peptide formation in solution, Cbz-protected amino acyl-phosphoranylidene ester **3b** and amide **3c** were employed. Reaction of **3b** with nonaflyl azide at concentrations >0.5 M proceeded to completion after 72 h in DCM at room temperature with 93% isolated yield of diazoaminoacyl ester **6** (Figure 2A). Other than diazo alkanes such as diazo methane, which are yellow, the diazo peptides reported here are colorless. A solution of **6** in MeOH displayed an absorption maximum at 252 nm ($\varepsilon = 8236\ lmol^{-1}dm^{-1}$). Irradiation at 254 nm induced rapid Wolff-rearrangement[10] to β-amino-malonic diester **7** in quantitative yield, presumably via an intermediary carbene and with a loss of the absorption maximum (Figure 2B). IR spectroscopy of **6** revealed a stretch vibration at 2150 cm^{-1} (SFigure 9) and the diazo carbon

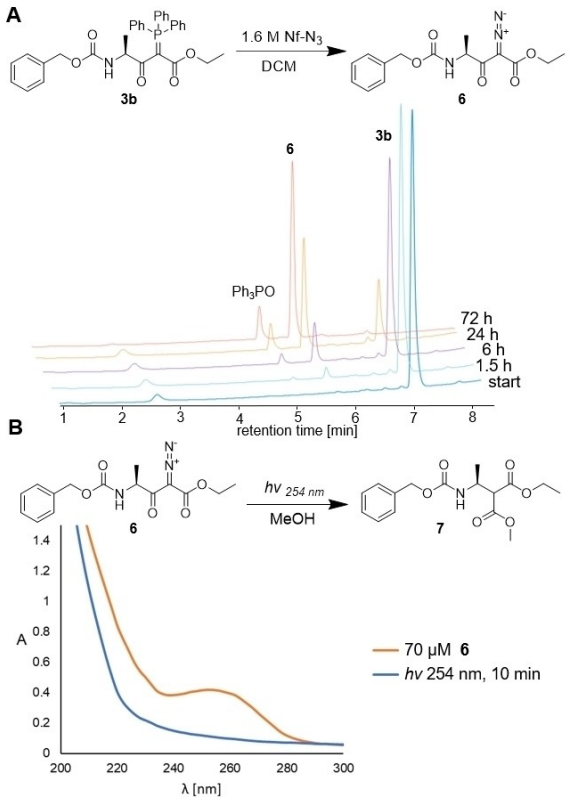


Figure 2. (A) HPLC traces of the reaction of **3b** with nonaflyl azide in solution monitored at 254 nm. (B) Irradiation of **6** at 254 nm leads to Wolff-rearrangement product **7** as followed by UV-spectroscopy.

Table 1: Synthesis of triazolyl-peptides and diazo peptides.

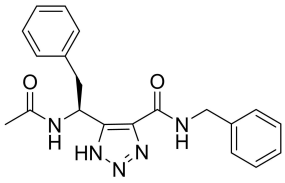
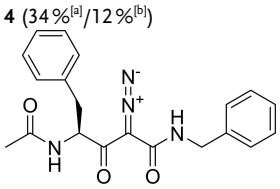
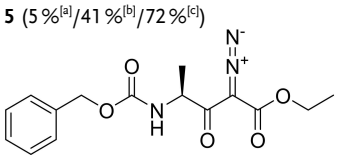
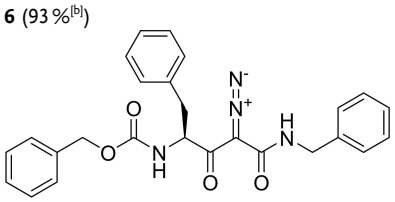
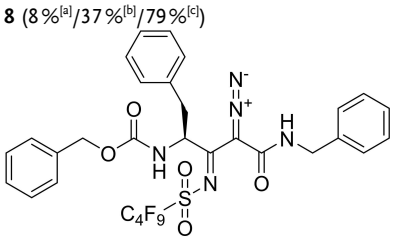
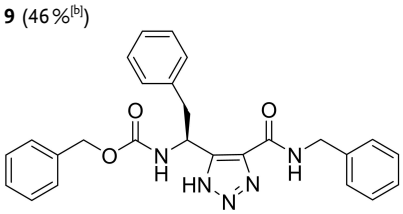
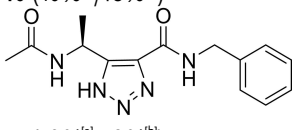
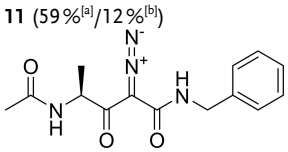
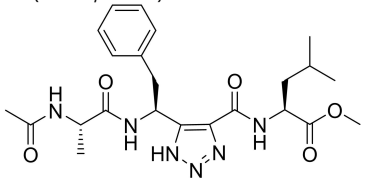
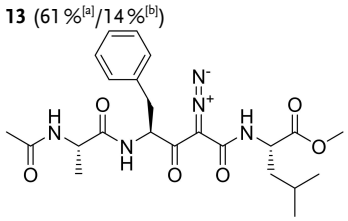
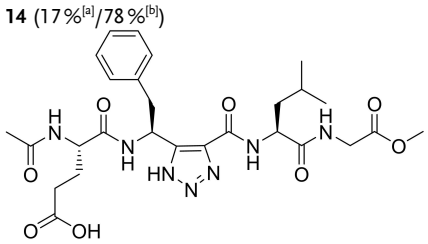
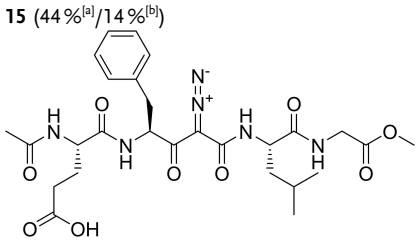
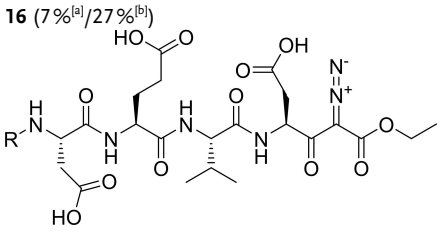
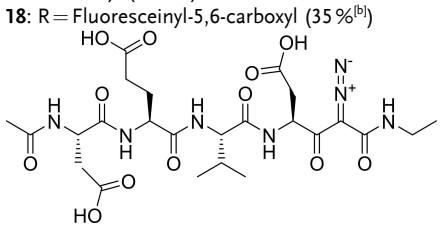
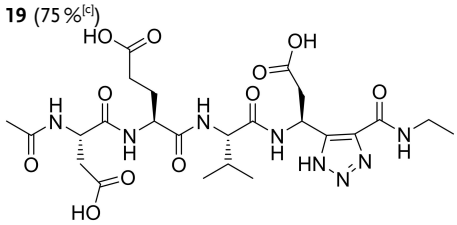
Starting material	Product (Yield)
3a	 **4** (34 %[a]/12 %[b])
3a	 **5** (5 %[a]/41 %[b]/72 %[c])
3b	 **6** (93 %[b])
3c	 **8** (8 %[a]/37 %[b]/79 %[c])
3c	 **9** (46 %[b])
3c	 **10** (40 %[a]/18 %[b])
3d	 **11** (59 %[a]/12 %[b])
3d	 **12** (23 %[a]/37 %[b])
3e	

Table 1: (Continued)

Starting material	Product (Yield)
3e	**13** (61 %[a]/14 %[b]) 
3f	**14** (17 %[a]/78 %[b]) 
3f	**15** (44 %[a]/14 %[b]) 
3g	**16** (7 %[a]/27 %[b])  **17**: R = acetyl (67 %[b]) **18**: R = Fluoresceinyl-5,6-carboxyl (35 %[b])
3h	 **19** (75 %[c])
3h	 **20** (23 %[b])

[a] Cleavage with triflyl-azide. [b] Cleavage with nonaflyl-azide without hydrolysis. [c] Cleavage with nonaflyl-azide followed by hydrolysis with 2 % TFA in H_2O/ACN 50:50.

showed a chemical shift of 76 ppm in the ^{13}C NMR spectrum. Phosphoranylidene amide **3c** reacted more rapidly with nonaflyl azide and was completely consumed after

3 h (SFigure 11). Main isolated product, however, was not the expected diazo peptide **8**, but 2-diazo-3-nonaflylimine peptide **9**, which was identified by mass ions at 738 and 710 m/z, indicating the loss of nitrogen during ionization, and its ^{1}H, ^{13}C, and ^{19}F NMR spectra. Generation of diazo peptide amide **9** can be rationalized from the cycloaddition product formed via path A, followed by elimination of phosphine oxide and cleavage of the N1–N2 bond yielding **9** with an isolated yield of 46 %. The nonaflyl-imine in **9** was hydrolyzed with 2 % TFA in H_2O/ACN 50:50 furnishing diazo peptide **8** in a drastically improved yield of 79 %. With triflyl azide, major product of soluble **3c** was the 4,5-disubstituted 1,2,3-triazol **10** isolated in 40 % yield.

Reaction conditions used for soluble phosphoranes **3b**,**c** were also applied to the polymer-supported phosphoranylidene amides **3d**,**e**,**f** and furnished the alanine-derived 1,2,3-triazole **11** and diazo-amide **12**, the internal tripeptide triazol **13** and the internal diazo tripeptide **14**, the internal triazolyl-tetrapeptide **15** and the internal diazo-tetrapeptide **16**.

Chemical stability of diazo peptides was investigated for the model compounds **6** and **8** (STable 1). Diazo peptide ester **6** remained unaffected between pH 1–8, even with 0.25 EtSH, while its C-terminal ethyl ester was cleaved at pH 9 and higher. Even more forcing acidic conditions like 95 % trifluoroacetic acid or HF-pyridine complex, however, did not modify the compound. Diazo peptide amides like compound **8** were also stable under strongly basic conditions. Most interestingly, the diazoalkane moiety was not even cleaved at 0.25 M sodium ethyl thiolate, a strong nucleophile with comparable reactivity as the thiolate found in the active site of cysteine proteases. Diazo ester **6** was converted slowly to the hydrazone with reducing agents such as 100 mM dithiothreitol (10 % after 72 h) and triphenylphosphine (100 % after 72 h). Both diazo peptides were stable in 100 mM glutathione for 48 h.

The remarkable chemical stability of the diazo peptides **6** and **8** encouraged us to investigate applications of these compounds under physiological conditions and in living cells. For this purpose, diazo peptides **17**, **18**, and **19** were designed containing the substrate recognition motif of the cysteine protease human caspase-3 (hC-3).[11] The characteristic tetrapeptide sequence was prepared as diazo peptide ester or amide, respectively, using Fmoc-based solid phase peptide synthesis on *O*-ethyl 2-triphenylphosphoranylidene acetate resin **2f** and *N*-ethyl 2-triphenylphosphoranylidene acetamide resin **2g**. The N-terminus was capped with *N*-acetyl or *N*-fluoresceinyl-carboxyl, respectively, and the diazo peptides were isolated after cleavage with nonaflyl azide and 95 %TFA/water in preparatively useful yields (Table 1). The stability of the tetrapeptides was not altered by the presence of side chain functionalities like carboxylic acids. Next, binding and inhibition of the protein target was investigated in an enzyme inhibition assay. Active human caspase-3 was obtained from recombinant pro-caspase-3 produced in a bacterial expression system. Processing of the proenzyme occurred spontaneously during the protein purification process.[12] The enzyme activity was assayed using the fluorogenic substrate Ac-DEVD-AMC.[13] Enzyme activities plotted against logarithmic inhibitor concentrations indicated IC_{50} values of 23 nM for the ester **17**, 33 nM for **18**, and 0.56 μM for the amide **19**. The flattening course of the fluorescence readout over time indicated time-dependent inhibition.[14] So when the assay was conducted with fixed incubation times (t) of protease and diazo peptides **17** and **19** (0 min, 10 min, 1 h, 2 h, 4 h) adding the fluorogenic substrate thereafter the IC_{50} values reduced from 23 nM (t = 0 min) to 4 nM (t = 4 h) for the ester **17** (Figure 3A) and from 0.56 μM (t = 0 min) to 0.05 μM (t = 4 h) for the amide **19** (SFigure 2). The pseudo first order inactivation rates k_{obs} were determined for a range of inhibitor concentrations and delivered k_{inact}/K_I values of 255,000 ± 8000 $M^{-1}s^{-1}$ for **17** and 2,803 ± 59.9 $M^{-1}s^{-1}$ for **19** (SFigure 3).[15] Thus, **17** was more than 100 times more reactive than the cellularly active fluoromethylketone inhibitor Z-DEVD-fmk, for which a k_{inact}/K_i value of 2,000 $M^{-1}s^{-1}$ was reported.[16]

An enzyme sample treated with 10x IC_{50} concentration of inhibitors **17** and **19** was diluted 1: 100 with buffer containing no inhibitor in a jump dilution assay.[17] The enzyme was not re-activated after the dilution, an observation suggesting irreversible inhibition (Figure 3B). Irreversible covalent modification of the protein was confirmed by protein mass spectrometry. Deconvolution of a mass spec-

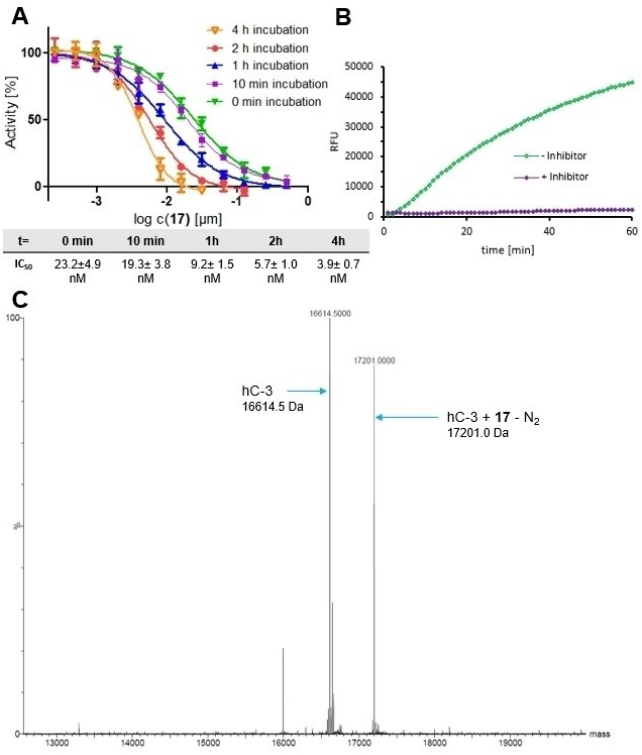


Figure 3. (A) Time-dependent inhibition of human caspase-3 (hC-3) by diazo peptide ester **17**. Error bars indicate the standard error of each data point measured in triplicates. IC_{50}-values and 95 % confidence intervals were determined via nonlinear regression und computed from the standard errors. (B) Jump dilution assay indicating irreversible inhibition of hC-3 by **17**. (C) Deconvoluted protein-MS of hC3 incubated with **17** showing the covalent protein-inhibitor-complex (hC3 + **17**-N_2) but also the native protein despite no caspase activity was left.

trum of caspase-3 incubated with diazo peptide **17** revealed two protein species, the unmodified protein with a mass of 16,615 Da and a second protein with a mass of 17,201 Da (Figure 3C). The mass difference between both protein peaks of 586 Da corresponded to the mass of diazo peptide **17** (614 Da) minus 28 Da for the release of one molecule of nitrogen and about 47 % of the original enzyme preparation was modified. Since no enzymatic activity remained in the sample, this finding suggested that diazo peptide **17** is strongly selective for the active protease, while the inactive enzyme was not modified covalently.

The observed covalent modification of active caspase-3 by inhibitor **17** was surprising considering the stability of **6** toward thiolate anions. Since there was no reason to assume that the protein-bound cysteine thiolate was more nucleophilic than an excess of sodium ethyl thiolate, the observed reactivity of inhibitor **17** must have been caused by interaction with the protease. Molecular modelling of the complex of **17** with caspase-3 starting from a crystal structure of the enzyme with the peptide aldehyde inhibitor Ac-DEVD–H (PDB: 2H5I) revealed binding of the 3-ketocarbonyl group of **17** to the oxo-anion hole of caspase-3 (Figure 4Bi). Binding of the keto-oxygen to this pocket lined with hydrogen bond donors (Gly122, Cys163) obviously polarized the stable diazo ketone and thus enabled covalent attack of the cysteine thiolate residue at the keto-carbon atom (Figure 4Bii) and then the 1,2-migration to the diazo-carbon (Figure 4A, Biii) leading to the extrusion of a nitrogen molecule, possibly supported by protonation via the imidazole-NH of His121 (SFigure 9).

Finally, fluorescent diazo peptide **18** was investigated in HeLa cells, a human cervix carcinoma cell line. Cells were incubated for 13 h with 5 μM staurosporine, a pan-kinase inhibitor,[18] or with DMSO for control. Subsequently, the fluorescent diazo peptide **18** (5 μM) was added for 3 h, and fixed cells were inspected with confocal fluorescence microscopy (Figure 5). While control cells showed virtually no fluorescence and regular shape (Figure 5A, left), staurosporine-treated cells were stained intensively with the fluorophore and displayed irregular cell morphologies, suggesting apoptosis (Figure 5A, right). In addition, cells were stained with an antibody raised against caspase-3 products showing massive formation of caspase-3 cleaving products (SFigure 7). For

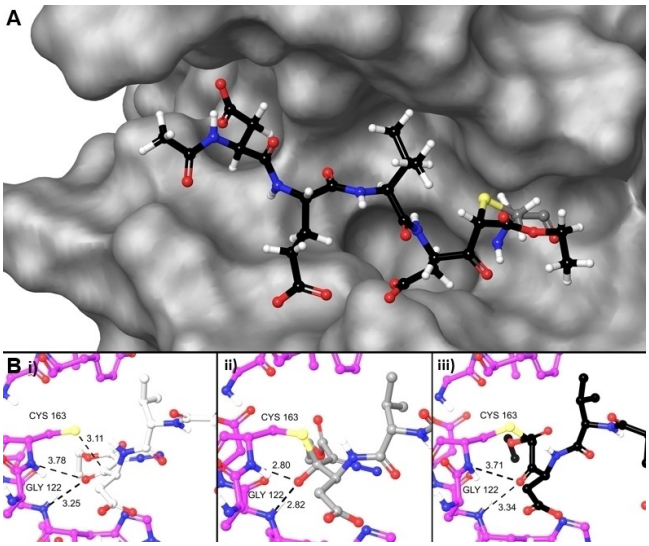


Figure 4. (A) Molecular modeling of the covalent complex formed via the irreversible reaction of diazo peptide **17** with the active site surface of caspase 3 (inhibitor backbone in black, Cys163 indicated with grey backbone). (B) Close-ups of the different stages of binding: i) docked inhibitor **17**, ii) reversible binding of **17** as hemithioketal, iii) irreversible binding of Cys163 to C2 after the expulsion of nitrogen. Distances between atoms of Gly122 and Cys163 and atoms of **17** are shown in dashed lines.

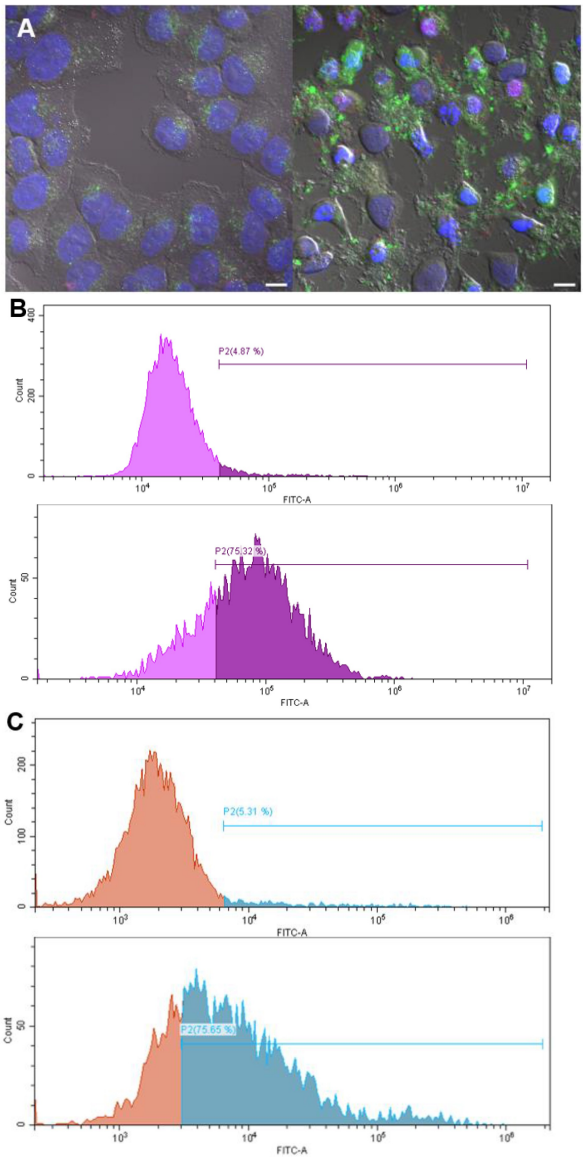


Figure 5. (A) Imaging of apoptosis in HeLa-cells using fluorescent diazo peptide **18**. Left: DMSO control with no staurosporine, right: cells treated with 5 μM staurosporine. Scale bar 10 μm. (B) Quantification of fluorescent cells with **18** and FACS analysis; upper image: no staurosporine (< 5 % fluorescent cells), lower image: 5 μM staurosporine (75.3 % fluorescent, apoptotic cells). (C) Quantification of apoptotic cells using FITC-labelled annexin-V; upper image: DMSO control, lower image: 5 μM staurosporine.

quantification, both cell populations were trypsinated and analyzed by flow cytometry[19] indicating an increase from <5% to about 75% of highly fluorescent, apoptotic cells (Figure 5B). For control, FITC-labeled annexin-V was added to HeLa cells treated with staurosporine or DMSO yielding strongly increased fluorescence only after treatment with staurosporine. Thus, the activity-based probe **18** enabled the imaging of cellular apoptosis via labeling of active caspase-3 in apoptotic cells, which could be quantified by flow cytometry.

In summary, in this work we have established synthetic access to chemically stable diazo peptides and to 4,5-disubstituted triazolyl-peptides. Both compound classes were accessible through dipolar cycloaddition reactions of soluble or polymer-supported acyl-phoshoranylidene esters and amides with electron-poor azides, followed by opening of the intermediary ring structures in case of diazo peptides. The remarkable chemical stability of diazo peptides enabled their application under physiological conditions and in living cells. Diazo peptides carrying the recognition sequence of human caspase-3 were identified as covalent, irreversible inhibitors with IC_{50} values in the low nanomolar range and with high selectivity for the active enzyme. A fluorescent diazo peptide of the same sequence was useful for the imaging and quantification of apoptotic cells. Our experiments establish diazo peptides as activity-based protein probes. This application might be extendable to further proteins with reactive nucleophiles in the active site including other cysteine and serine proteases. Further studies will investigate potential diagnostic and therapeutic applications of this class of molecules.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

Keywords: diazo compounds · diazo peptides · activity-based probes · irreversible protease inhibitors · apoptosis

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