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VIP Photoactive Bioisosters

Benzoylphosphonate-Based Photoactive Phosphopeptide Mimetics for Modulation of Protein Tyrosine Phosphatases and Highly Specific Labeling of SH2 Domains**

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The phosphorylation of tyrosine residues is an important posttranslational modification of proteins and regulates the activity of numerous signal transduction pathways, for example, in highly proliferating cells. Phosphotyrosine residues are located on membrane receptors,[1] adaptor proteins,[2] enzymes,[3] and transcription factors.[4] They are recognized by protein tyrosine phosphatases (PTPs) and phosphotyrosine binding domains exemplified by the src-homology domains type 2 (SH2 domains) first described in the human sarcoma protooncogene, src.[5] Temporally resolved and spatially resolved analysis of the presence and activity of phosphotyrosine binding sites is of fundamental relevance for describing cellular activation properly.

Photoactivated chemical probes have been used intensively to identify the interaction partners of small molecules in biological systems.[6] Usually, such probes are composed of a bioactive ligand component and a photoactive label, which contains an aryl azide, a diazirine, or a benzophenone residue. For targeting phosphotyrosine binding sites several bioisosteric groups have been described.[5] Benzyl phosphonates,[7] phenyl difluoromethyl phosphonates,[8] and isothiazolidinones[9] have found broader application. Recently, we have used several phosphotyrosine-mimicking fragments for the development of very specific PTP inhibitors[10] employing a variation of dynamic ligation screening.[11] In addition, covalent modifiers targeting the active sites of PTPs have been proposed as "activity-based probes", however, with limited selectivity and specificity.[12]

Here, we describe a conceptually different approach toward novel covalent protein probes targeting the active sites of phosphotyrosine binding proteins. Benzoylphosphonates of general structure **1** were envisaged as bioisosters of the phosphotyrosine residue capable of binding to phosphotyrosine recognition sites as phosphotyrosine mimetics. Owing to the carbonyl functionality directly adjacent to the phosphonic acid group, compounds of structure **1** were supposed to be activated by irradiation with light providing reactive radical species,[13] which might modify the target protein covalently and thereby modulate its activity (Scheme 1). To verify this hypothesis, benzoylphosphonates **1a**,**b** were prepared by Arbuzov acylation of triethyl phosphite with the respective acyl chlorides followed by deprotection with trimethylsilyl bromide (TMSBr).[14] The UV spectrum of compound **1a** in water displayed the n–π* transition at 368 nm in water with an extinction coefficient ε of 96 $cm^{-1}M^{-1}$.[15] Irradiation of **1a**,**b** dissolved in 2-propanol and water, a solvent mixture capable of hydrogen radical donation,[16] revealed a half-life of approximately 20 min. The major irradiation products of **1a**,**b** under these conditions were the isomeric photodimers *meso*-**2a**,**b** and *rac*-**2a**,**b**, corresponding to the *meso* and the racemic diols formed from recombination of monomeric radical intermediates. Minor by-products of the photoreaction of **1a**,**b** included the respective benzaldehydes **3a**,**b** and benzoic acid derivatives **4a**,**b**.

Without irradiation, compounds **1a**,**b** remained stable in 2-propanol/water solution. Irradiation in a non-hydrogen-radical-donating solvent like water led to only slow degradation of the benzoylphosphonate and provided no crosslinking product. Addition of the tetramethylpiperidinyl *N*-oxide radical (TEMPO) completely suppressed the formation of photodimerization products in 2-propanol/water. Mercaptans such as dithiothreitol and glutathione reduced the dimerization efficiency.[17] Products of the photodimerization reaction could be quantified when 4-methyl benzoylphosphonate (**1b**) served as the starting material since they display to better retention on the RP-18 column (Scheme 1, bottom). The half-life of **1b** was 15 min. After 40 min of irradiation, more than 90% of the recovered **2b** was found in the dimers *meso*- and *rac*-**2b**. The ratio of the two dimers was 2:1 in favor of the

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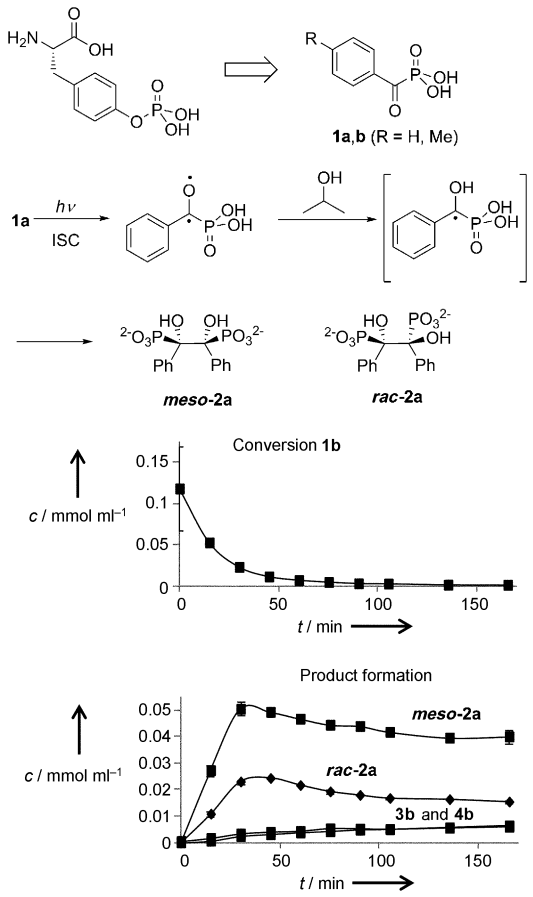


Scheme 1. Top: Photoactivation and photocrosslinking of benzoyl phosphonate **1a**,**b** as phosphotyrosine bioisosters. Bottom: Products of the reaction with the starting material **1b** were quantified by LC–MS. **3b**: 4-methylbenzaldehyde, **4b**: 4-methylbenzoic acid. ISC = intersystem crossing.

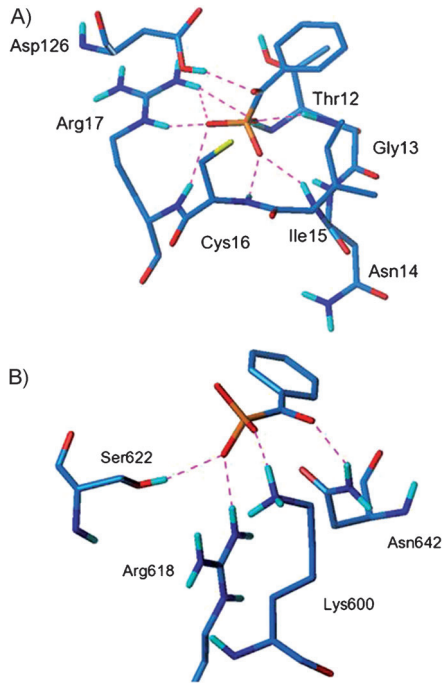


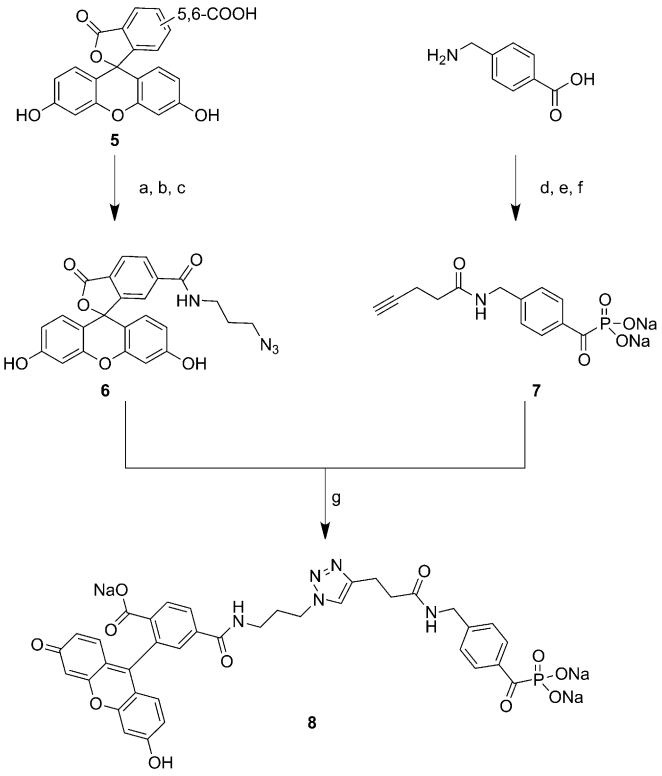
Figure 1. Docking experiments with benzoylphosphonate **1a** in the phosphotyrosine binding site of the protein tyrosine phosphatase from *Mycobacterium tuberculosis* (MptpA) (A) and the SH2 domain of STAT5b (modeled from STAT5a; B).

kinetically and thermodynamically favored *meso* product. To demonstrate whether crosslinking of benzoylphosphonate **1a** with peptides was feasible, the model peptide FKIAG-NH_2 was irradiated in the presence of **1a** for 4 h. LC/ToF-MS measurements revealed the formation of a benzoyl adduct only upon irradiation, which can be formed by crosslinking of **1a** and subsequent elimination of phosphonic acid. It is not clear whether elimination of phosphonic acid occurred during irradiation in buffer or during ionization in the mass spectrometer.

Next, benzoylphosphonate **1a** was investigated in the presence of the phosphotyrosine binding sites of *Mycobacterium tuberculosis* protein tyrosine phosphatase A (MptpA) and the SH2-containing transcription factor STAT5b as model proteins. Docking experiments suggested favorable binding interactions with the P-loop of MptpA, similar to those predicted for phosphotyrosine (Figure 1 A). Docking experiments with the SH2 domain of STAT5b (modeled from the crystal structure of STAT5a) also indicated a potential for binding (Figure 1 B). In both cases the carbonyl group acted as hydrogen-bond acceptor of an amino acid side chain of aspartic acid and asparagine, respectively.

Binding predictions for the phosphatase MptpA were verified by measuring the inhibitory activity of **1a**. Employing *p*-nitrophenol phosphate (*p*NPP) as the substrate, the enzyme reaction of MptpA was monitored colorimetrically at 405 nm by means of detection of the nitrophenoxide anion. Compound **1a** inhibited MptpA with an IC_{50} value of approximately 1 mM. Taking into account a K_M value of 2.3 mM for *p*NPP, this IC_{50} value corresponds to a K_I value of 186 μM, assuming a competitive mode of inhibition.[18] Irradiation of **1a** with the protein MptpA in tris(hydroxymethyl)aminomethane (Tris) buffer for 45 min and subsequent measurement of PTP activity resulted in a decreased IC_{50} value of 84 μM, indicating the deactivation of the phosphatase by irradiated benzoylphosphonate. For comparison, irradiation of phosphatase and substrate alone did not change the activity.

Additionally, the binding of benzoylphosphonates **1** to one of its target proteins was investigated by using a fluorescently labeled derivative of benzoylphosphonate to visualize the photocrosslinking product (Scheme 2). Starting from 4-aminomethylbenzoic acid, the pentynoic acid amide **7** of 4-aminomethylbenzoyl phosphonate was prepared in three steps (see the Supporting Information). Dipolar cycloaddition with the 6-fluoresceinylcarboxamide derivative **6** of 3-azidoprop-1-yl amine yielded triazole **8** under copper(I) catalysis.[19] Triazole **8** was first investigated as a photoactive inhibitor of PTP1B (Figure 2 A). Inhibition was amplified from an IC_{50} value of 1 mM to a value of 28 μM by irradiation for 45 min. Next photocrosslinking products of **8** with protein tyrosine phosphatase PTP1B were generated at different concentrations in Tris buffer and were subsequently separated



Scheme 2. Preparation of the fluorescein-labeled benzoyl phosphonate **8**. Reaction conditions: a) isobutyryl chloride, pyridine, CH_2Cl_2, 0 °C, 85 %, after isomer separation the 6-isomer was obtained in 41 % yield; b) 3-azidoprop-1-yl amine, DCC, HOBt, THF, 88 %; c) TFA, 60 °C, 93 %; d) pent-4-ynoic acid-OSu, Na_2CO_3, 87 %; e) 1. $(COCl)_2$, [DMF], CH_2Cl_2, 2. $P(OBn)_3$,toluene, 75 %; f) TFA, 0 °C, 92 %; g) 1. **6**, **7**, NaAsc, $CuSO_4$, THF/H_2O, basic buffered HPLC, 2. Amberlite IR-120 cation-exchange resin, Na^+ form, H_2O, 40 %. Asc = ascorbate, DCC = *N*,*N*′-dicyclohexylcarbodiimide, HOBt = 1-hydroxybenzotriazole, OSu = 1-oxysuccinimide, TFA = trifluoroacetic acid.

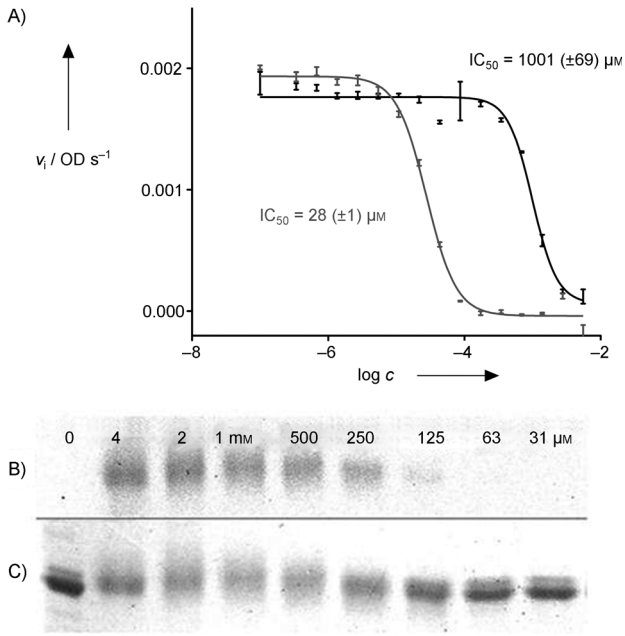
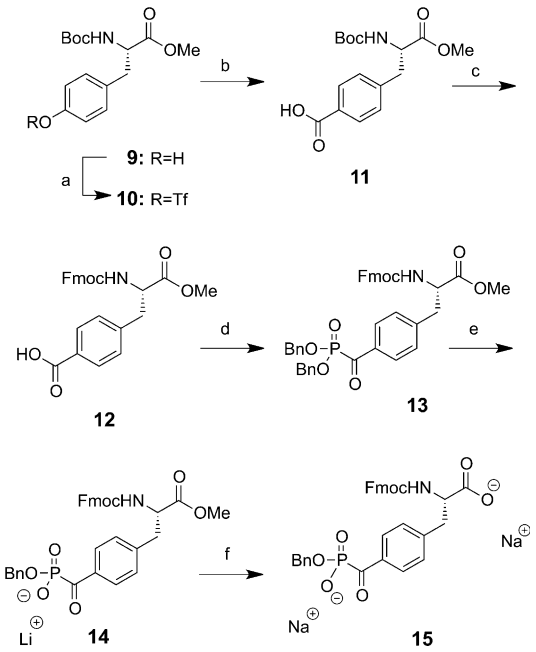


Figure 2. A) Inhibition (IC_{50} values) of protein tyrosine phosphatase PTP1B with benzoylphosphonate **8** in the enzymatic pNPP assay with (gray) and without (black) irradiation. Bottom: B) Fluorescence of PTP1B with **8** as detected in gel electrophoresis (PAGE) after irradiation for 45 min. C) Same gel stained with Coomassie Blue. (For time-dependent experiments see Figure S5 in the Supporting Information.)

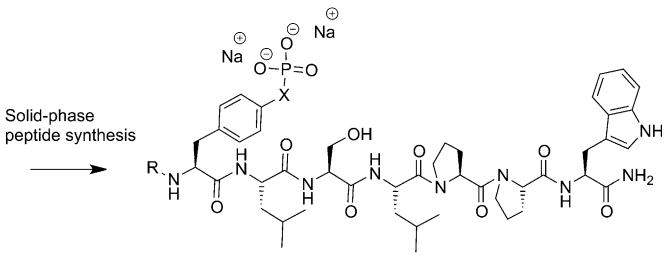
by electrophoresis using a sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) (Figure 2B,C). For comparison, the gel was stained with Coomassie Brilliant Blue in order to visualize the entire protein. Fluorescently labeled PTP1B was found at concentrations of triazole **8** of 125 μM and higher. The fluorescence intensity correlated with the concentrations of the fluorescent label and protein and confirmed the covalent modification of the PTP1B protein. Fluorescent protein bands were detected only after irradiation in the presence of **15**. The degree of covalent protein modification was investigated at three irradiation times (20, 40, 60 min) each at three concentrations (0.25, 0.5, and 1 mM), and protein fluorescence was found to increase over time (see Figure S5 in the Supporting Information). Prolonged irradiation times and increased probe concentrations, however, led to a defocusing of the protein band and therefore shorter irradiation at moderate probe concentrations was preferable.

Phosphotyrosine (pTyr)-containing peptides are the native and in many cases potent ligands of phosphotyrosine receptors. Therefore, the incorporation of benzoylphosphonate into peptides using the unnatural amino acid 4-(phosphonocarbonyl)phenylalanine (pcPhe) as a building block (Scheme 1) was hypothesized as a strategy to enhance the affinity, crosslinking efficiency, and specificity of the photoactive phosphotyrosine isoster toward its target proteins. In order to test this hypothesis *N*-Fmoc-4-(*O*-benzyl-phosphonocarbonyl)phenylalanine building block **15** was designed for the direct integration of the benzoylphosphonate into peptides (Scheme 3).

Enantiomerically pure *N*-Boc-L-tyrosine methyl ester **9** was sulfonylated with *N*-phenylbis(trifluoromethylsulfonylimide) yielding *N*-Boc-*O*-triflyltyrosine methyl ester **10**.[20] Compound **10** was carboxylated employing gaseous carbon monoxide and palladium acetate with 1,3-bis(diphenylphosphino)propane (dppp) as a catalyst in 89 % yield.[21] The resulting *N*-Boc-4-carboxyphenylalanine methyl ester **11** was N-deprotected with TFA and reprotected employing Fmoc-succinimide (FmocOSu) to furnish **12**, which was activated as carboxylic acid chloride and used in a Michaelis–Arbuzov acylation of tribenzyl phosphite to provide the 4-(*O*,*O*′-dibenzyl-phosphonocarbonyl) derivative **13** of *N*-Fmoc-phenylalanine methyl ester.[22] As the dibenzylphosphonate **13** was cleaved rapidly by piperidine[23] under the conditions used for Fmoc removal, nucleophilic monodebenzylation of **13** was accomplished with lithium bromide, yielding the monobenzyl phosphonate **14**.[24] The monobenzyl-protected benzoylphosphonate was sufficiently stable toward the basic conditions required during Fmoc cleavage. Next, the methyl ester **14** was saponified enzymatically with subtilisin Carlsberg ("alcalase") at pH 8.[25] The remaining protons in the two acidic positions of the molecule were replaced by sodium ions using sodium-loaded Amberlite IR-120 ion-exchange resin; the resulting *N*-Fmoc-protected 4-(*O*-benzylphosphonocar-



Scheme 3. Reaction conditions: a) *N*-phenylbis(trifluoromethylsulfonylimide), Et_3N, CH_2Cl_2, 0°C, 96%; b) dppp, $Pd(OAc)_2$, CO(g), DIPEA, DMF/H_2O 3:1, 70°C, 89%; c) TFA, CH_2Cl_2; FmocOSu, Na_2CO_3, acetone/H_2O, 0°C, 87%; d) $(COCl)_2$, DMF, CH_2Cl_2; $P(OBn)_3$, toluene, 75%; e) LiBr, MeCN; f) subtilisin Carlsberg, aq. NH_4HCO_3, pH 8; Amberlite IR-120, Na^+ form, H_2O, 89%. Bn = benzyl, Boc = *tert*-butoxycarbonyl, DIPEA = diisopropylethyl amine, Fmoc = 9-fluorenylmethyloxycarbonyl, FmocOSu = Fmoc-succinimide.



16 : X = O, R = 5,6-fluoresceinylcarboxy(CF)-glycinyl: 34%
17 : X = O, R = acetyl: 32%
18 : X = C=O, R = 5,6-carboxyfluorescein(CF)-glycinyl: 29%
19 : X = C=O, R = acetyl-glycinyl: 36%

Scheme 4. Synthesis of phosphotyrosine peptides **16** and **17** and the corresponding 4-(phosphonocarbonyl)phenylalanine derivatives **18** and **19** for use in binding assays and for covalent photomodification of proteins.

Table 1: Binding affinities of tested phosphotyrosine- and benzoylphosphonate-based peptides toward STAT5b.[a]

	Peptide	K_D, K_I[a]
16	CF-GpYLSLPPW-NH_2	0.042 (±0.003) μM
17	Ac-pYLSLPPW-NH_2	0.604 (±0.11) μM
18	CF-GpcFLSLPPW-NH_2	0.896 (±0.44) μM
19	Ac-GpcFLSLPPW-NH_2	11 (±1.9) μM

[a] Without irradiation. K_I values were calculated from the IC_{50} values with a formula by Nikolovska-Coleska et al.[31] pcF = 4-phosphoncarbonylphenylalanine.

bonyl)phenylalanine building block **15** was suitable for use in solid-phase peptide synthesis. Ion exchange was essential for obtaining a stable product **15** ($[\alpha]_{D,25°C} = +25.0°$, $c = 1.0$, H_2O), which could be stored for months without racemization or hydrolysis of the carbonyl–phosphonate bond.

As the first target protein for photoactive phosphotyrosine peptide mimetics, we selected the STAT5b protein, which contains a phosphotyrosine-recognizing SH2 domain. In the cellular context STAT5 is phosphorylated at a tyrosine residue predominantly by JAK2 kinase,[26] which is associated with several receptors including the erythropoietin[26,27] and interleukin 2 receptors;[26,28,29] this reaction results in the formation and subsequent dimerization of phospho-STAT5.[26,29,30] Phospho-STAT5 dimers are transported into the nucleus where they act as transcription factors. Constitutive activation or overexpression of STAT5 has been firmly established for several types of leukemia. For our experiments, STAT5b was recombinantly expressed conjugated with maltose-binding protein (MBP) as a purification tag, resulting in a protein construct of 120 kDa.[14]

Several phosphotyrosine peptides derived from STAT5-binding proteins were synthesized and investigated as ligands of STAT5. For binding studies the peptides were N-terminally labeled with 5,6-carboxyfluorescein (CF). The octapeptide *N*-CF-Gly-pTyr-Leu-Ser-Leu-Pro-Pro-Trp-NH_2 (**16**) from the β-subunit of the GM-CSF receptor was identified as the most potent ligand of STAT5; it displayed a dissociation constant (K_D) of 42 nM (Scheme 4, Table 1). The N-acylated derivative of **16**, peptide **17**, was capable of competing with **16** for binding of STAT5 and displayed a K_I value of 604 nM. Benzoylphosphonate **1a** had an IC_{50} value of 840 μM which corresponds to a K_I value of 326 μM.[31] Irradiation of the probe led to a significant increase of measured affinity over time up to an IC_{50} of roughly 457 μM after 4 h (Table 2).

Table 2: IC_{50} values of benzoylphosphonate **1** against MptpA, PTP1B, and STAT5b without and with irradiation.

	Without irradiation	With irradiation
MptpA (**1a**)	993 (±46) μM	84 (±9) μM[a]
STAT5b (**1a**)	840 (±126) μM	457 (±47) μM[b]
PTP1B (**1a**)	ca. 4 mM	8.9 (±0.3) μM
PTP1B (**15**)	1001 (±69) μM	28 (±1) μM

[a] Irradiation for 45 min. [b] Irradiation for 240 min.

Next, 4-phosphonocarbonyl peptides **18** (R = *N*-fluoresceinyl-carboxy-glycinyl) and **19** (R = *N*-acetyl-glycinyl), the hypothetic photoactive mimetics of phosphotyrosine peptides **16** and **17**, were synthesized (Scheme 4). Fmoc-protected amino acid building blocks were coupled on Rink amide linker by diisopropyl carbodiimide/HOBt activation. The protected 4-phosphonocarbonyl building block **15** and the subsequent amino acid building block were coupled following activation with *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate (TBTU) and DIPEA; the coupling proceeded with high efficiency as indicated by a negative

Kaiser test. After incorporation of **15**, subsequent removal of the Fmoc protecting groups was carried out with 2% 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) in DMF[32] (1 min, 10 min) in order to avoid partial cleavage of the phosphonate.[33] Peptides **18** and **19** were cleaved from the resin by treatment with neat TFA. Thiol nucleophiles were avoided at this point as they led to the formation of dithioketals. Following the cleavage, TFA was removed by evaporation and the crude peptides were dissolved in aqueous NH_4HCO_3. For purification HPLC was conducted with a basic ammonium bicarbonate buffer at pH 8 furnishing peptides **18** and **19** in yields of 29% and 36%, respectively. The products were stable under mildly basic and neutral conditions.

In order to exclude intramolecular photoreactions of the synthesized peptide probes **18** and **19**, we compared their photostability without the target protein. Samples were irradiated in 2-(4-(2-hydroxyethyl)-1-piperazinyl)-ethansulfonic acid (HEPES) buffer at 4°C and analyzed by HPLC at different time points. No significant differences could be detected. Next, binding of **18** to STAT5 protein was investigated and was found to proceed with a K_D value of 0.9 μM, as determined by a fluorescence polarization binding assay (Table 1). The binding of peptide **19** was evaluated in a competition experiment with ligand **16** and a K_I value of 11 μM ($IC_{50} = 28$ μM) was measured for the former. Upon irradiation a significant time-dependent decrease of the measured IC_{50} could be observed, resulting in an IC_{50} value of 13 μM after 4 h, while the inhibitory effect of phosphotyrosine peptide **17** remained unaffected.

Having established the recognition of peptides **18** and **19** by the MBP–STAT5b protein construct, we investigated the photocrosslinking potential of the ligand. For this purpose, solutions of protein with 4-(phosphonocarbonyl)phenylalanine peptide at different concentrations in HEPES buffer were irradiated in microtiter plates with 384 wells and a transparent bottom with light at 365 nm from below using a transilluminator. The resulting protein solutions were analyzed by SDS polyacrylamide gel electrophoresis. Excitation of the gel indicated labeling of the protein with fluorescein in the case of irradiated samples, whereas non-irradiated samples showed no fluorescence. When the gels were stained with Coomassie Blue as a control, the amount of protein and the appearance of the protein band were not altered by irradiation. Labeling was quantified using a Lumi-Imager and was found to be concentration-dependent with respect to the protein and the photocrosslinking peptide **18** (see Figure 3A and Figures S6 and S7 in the Supporting Information).

The photolabeling efficiency, given as the probe concentration at half-maximum labeling, was determined to be 18 μM. Time-dependent labeling was investigated over a time period of 120 min at different concentrations. At a low concentration (2.5 μM) of the photoactive modifier **18** an almost linear increase of the protein fluorescence was observed over the entire period, whereas at high concentration (40 μM) protein fluorescence went into saturation after 20–30 min (see Figures S14–S16 in the Supporting Information).

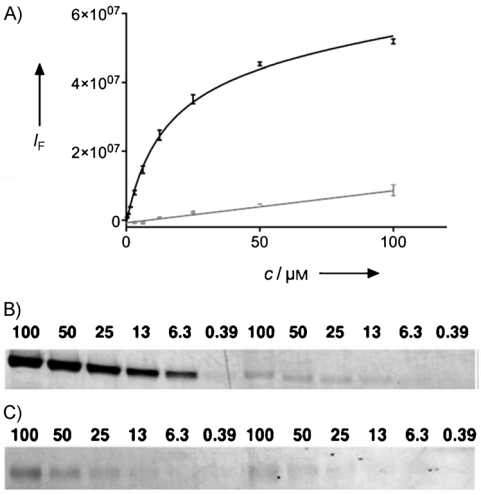
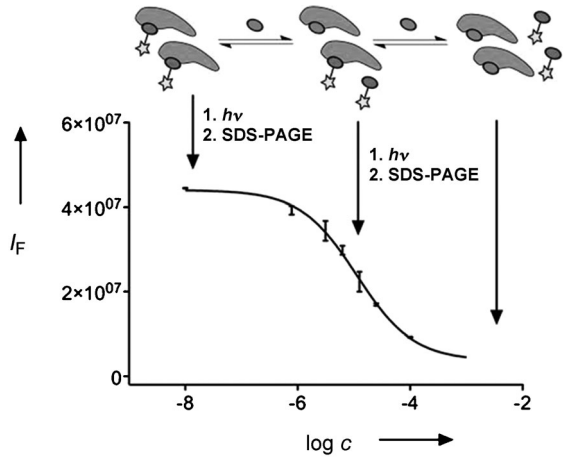


Figure 3. Experiments to verify the specificity of the photolabeling of MBP-STAT5b with the photoactive mimetic CF-GpcFLSLPPW-NH_2 **18**. A) Concentration-dependent photolabeling of equal concentrations of STAT5b (black) and BSA (gray), respectively, with **18** as quantified from the SDS polyacrylamide gel (for inverted fluorescence and Coomassie pictures of the gel see Figures S8 and S9 in the Supporting Information). B) Photolabeling of MBP-STAT5b at different concentrations of **25** (in μM) without (left six lanes) and with protein denaturation using 6M urea (see Figures S10 and S11 in the Supporting Information). C) Photolabeling of BSA without (left six lanes) and with protein denaturation using 6M urea (see Figures S10 and S11 in the Supporting Information).

In contrast, irradiation of **18** in the presence of bovine serum albumine (BSA) as a control protein without phosphotyrosine recognition sites resulted in unspecific labeling which was not saturated and progressed linearly even at very high concentrations of the photoreactive probe (Figure 3A and Figures S8 and S9 in the Supporting Information). In addition, the specificity of the photolabeling was verified by denaturation of the target protein using 6M urea (Figure 3B,C and Figures S10 and S11 in the Supporting Information). Only correctly folded STAT5b was modified covalently, whereas denatured STAT5, BSA, and denatured BSA were not labeled. Finally, the specificity of the photolabeling was further confirmed by using the nonfluorescent, phosphotyrosine-containing control peptide **17** as a competing molecule; **17** suppressed the photolabeling with peptide **18** in a concentration-dependent manner while it had no effect on the unspecific photolabeling (Figure 4 and Figures S12 and S13 in the Supporting Information).

In summary, we have demonstrated that benzoylphosphonates are photoactive bioisosters of phosphotyrosine residues. Likewise, 4-(phosphonocarbonyl)phenylalanine-containing peptides were proven to be photoactive mimetics of native phosphotyrosine peptides. Both the bioisosteric fragments and the peptide mimetics bind reversibly to phosphotyrosine recognizing protein domains. Upon irradiation with light, photoactivation of the benzoylphosphonates resulted in the functional deactivation and the covalent modification of the target proteins STAT5b, PTP1B, and MptpA. The degree of covalent labeling correlated with concentration and irradiation time. Most significantly, phosphotyrosine peptide



PAM probe conc. 18	40 μM								
Ac-GpYLSLPPW-NH_2 17	0 μM	0.8 μM	1.6 μM	3.1 μM	6.3 μM	12.5 μM	25 μM	50 μM	100 μM
Fluorescence									

Figure 4. Competitive displacement of PAM probe **18** (PAM = photoactive mimetic) by acetylated phosphotyrosine peptide **17** results in the suppression of labeling. Photolabeling was quantified from the inverted fluorescence image of the SDS-polyacrylamide gel. (For the entire fluorescence picture see Figure S12, for Coomassie control staining see Figure S13).

mimetics labeled their target proteins with high specificity for the phosphotyrosine binding site. The photochemical ligation reaction was saturated in a concentration-dependent manner, and the labeling could be suppressed by protein denaturation and by using the native phosphotyrosine peptide as a competing ligand.

To our knowledge this work constitutes the first example of a photoactive bioisoster, a chemical moiety that combines photocrosslinking activity and biomimicry in one bioisosteric group. Photoactive bioisosters might find applications in functional cell biology, bioanalytics, and proteomic investigations.

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