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α,α-Difluorophosphonomethyl Azobenzene Derivatives as Photo-Regulated Phosphoamino Acid Analogs. 1. Design and Synthesis

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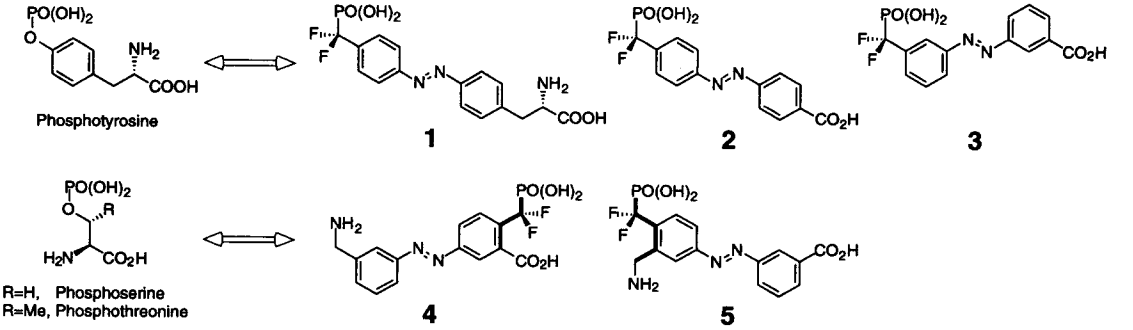
Abstract: A series of novel, photoregulated phosphoamino acid analogs based on an azobenzene core bearing an α,α-difluoromethylphosphonate as a hydrolytically stable phosphate isostere have been prepared with *N*-Fmoc protection for use in peptide synthesis. Classes of reagents analogous to both phosphotyrosine and phosphoserine/threonine were prepared by a common route employing a nitrosoarene/aniline condensation to form the azo linkage and the Cu(I)-promoted coupling of an iodoarene with (diethylphosphono)difluoromethyl cadmium bromide (Burton's method) to introduce the phosphonate moiety.

Keywords: amino acids and derivatives, azo compounds, peptide analogues/mimetics, phosphorylation

Reversible *O*-phosphorylation of proteins on serine, threonine, and tyrosine provides a central regulatory mechanism in biology. Our specific interest lies with the nuclear localization signal (NLS), a short peptide motif that directs proteins into the cell nucleus, and whose activity is often modulated by phosphorylation of flanking residues.[1] We seek to generate photoregulated derivatives of the NLS which could be used to effect extracellular control over nuclear transport and thereby over dependent processes such as gene transcription and cell division. For this task, we desired reagents that could mimic the effects of reversible phosphorylation through reversible photoisomerization. This letter describes the design and synthesis of a novel series of compounds (Scheme 1) intended for this purpose, and having wide potential applicability, that use the photo-isomerizable azobenzene chromophore to control the placement of a hydrolytically stable phosphate isostere. The use of these reagents in peptide synthesis, and their photochemical properties, will be reported in a forthcoming publication.

Azobenzene derivatives have been employed widely for the photoregulation of polypeptide function,[2,3] particularly through the modification of side chains, and artificial azobenzene-containing amino acids have also been prepared. For instance, 4-(phenylazo)phenylalanine (Pap)[4] has been applied to peptide antigens[5] and the ribonuclease S peptide[6] to bring intermolecular association under photo-control. Another amino acid, 4-aminomethyl-4'-carboxyazobenzene (p-Aza), was recently prepared[7] and installed into a cyclic peptide;[8] p-Aza

Scheme 1 — Relationship of Targets to Phosphoamino Acids



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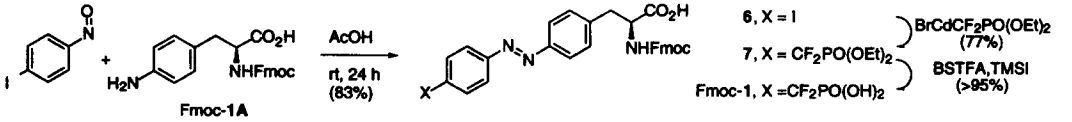
is the first amino acid that places an azobenzene unit within the peptide backbone. The azobenzene chromophore has also been combined effectively with transition-state analogs such as boronic acid and peptide aldehyde groups to generate photoregulated enzyme inhibitors.[9] Azobenzenes therefore provide a logical starting point for the development of photoregulated phosphoamino acid mimics. However, none of the available azobenzene-based amino acids or peptide modification reagents contain a phosphate or isosteric group which might allow it to function directly in this capacity. To fill this void, we designed and synthesized a series of novel azobenzene peptide reagents substituted with the α,α-difluoromethylphosphonate moiety,[10] a phosphate isostere that has met with great success in analogs of phosphotyrosine (pTyr),[11–13] phosphoserine (pSer),[14–16] and phosphothreonine (pThr).[16]

Two groups of reagents were designed (Scheme 1): amino acids (**1**, **4**, and **5**) for incorporation within the polypeptide backbone and capping reagents (**2** and **3**) for the N-termini of peptides. The conceptual relationship of **1** to pTyr is direct in that both are *para* substituted, and amino acid **1** bears the same relationship to phosphotyrosine that Pap does to phenylalanine. Amino acids **4** and **5** are intended to mimic pSer or pThr as best as possible within the confines of the azobenzene skeleton and shared synthetic chemistry with **1**. By placing the phosphonate moiety *ortho* to the backbone groups (carboxyl or benzylic amine), the close proximity of the phosphorus to the backbone is maintained. Reagent **4** exactly preserves the three-bond separation of C_α from P in pSer and pThr, whereas reagent **5** has a slightly longer four-bond separation (bold bonds in Scheme 1). These two reagents may be viewed as split phosphoamino acids where the disposition of the halves is controlled by azobenzene geometry. In this way, they are analogous to p-Aza, which splits the peptide backbone. The capping reagents are also most directly analogous to pTyr. These simple compounds may be useful for the modification of native peptides and proteins *via* coupling to the N-terminus or lysine side chains.

Scheme 2 — Synthesis of Fmoc-1



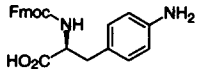
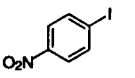
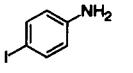
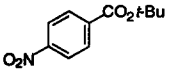
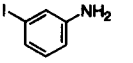
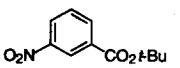
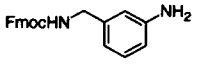
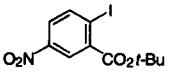
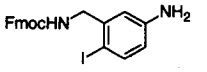
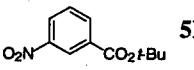
A synthetic strategy was developed that allows for the practical synthesis of all reagents, *N*-Fmoc[17] protected for peptide synthesis where appropriate, from inexpensive commercial materials using shared methodology; the general route is illustrated for Fmoc-**1** in Scheme 2. The syntheses involve two key steps: formation of the azo linkage and introduction of the phosphonate moiety. Unsymmetrical azobenzenes are readily prepared by the condensation of nitrosoarenes with anilines in glacial acetic acid;[4,7] this method proved entirely satisfactory, proceeding in 78–90% yield. The other key step, introduction of the phosphonate moiety, was conveniently effected using Burton's method of Cu(I)-promoted coupling of iodoarenes with BrCd–$CF_2PO(OEt_2)$.[18] We were gratified to discover that the reaction proceeds smoothly on iodoazobenzenes under the published conditions; moreover, it proved compatible with ester and urethane protecting groups as well as free carboxylic acids in the substrate. Therefore, the Burton coupling was performed as the penultimate step in all syntheses, followed only by final deprotection of the phosphonic- and carboxylic acid esters. Difficulty was encountered only in the synthesis of Fmoc-**5**, where sluggish and incomplete coupling was observed, perhaps due to steric factors.

The synthesis of **1** (Scheme 2) is representative. Its two halves were readily derived from commercial materials. *p*-Nitro-L-phenylalanine was Fmoc protected in 87% yield using Fmoc–OSu (Su = *N*-succinimido),[19] purified by recrystallization from EtOAc, and reduced in quantitative yield to aniline **1A** using H_2 (1 atm) with 10% Pd/C catalyst in methanol. Reaction of **1A** (400 mg, 1 mmol) with 4-iodonitrosobenzene (280 mg, 1.2 mmol, prepared as detailed below) in AcOH for 24 h at room temperature, followed by removal of the solvent *in vacuo* and recrystallization from EtOAc, afforded **6** in 83% yield. Introduction of the phosphonate moiety using Burton's reagent in DMF afforded diethyl phosphonate **7** as a yellow oil in 77% yield after chromatography on silica gel (1:2 hexanes/EtOAc, 1% v/v AcOH). Final deprotection (>95% yield in all cases) was performed with bis(trimethylsilyl) trifluoroacetamide (BSTFA) and TMSI essentially as described.[20,21] Thus, to 50 mg of **7** in 2 mL of dry CH_2Cl_2 was added 500 μL of BSTFA. After 1 h, the mixture was cooled to –20 °C, and TMSI (200 μL) was added. The mixture was stirred at –20° C for 30 min, 0 °C for 30 min, and room temperature for 1 h, then concentrated by rotary evaporation (30 mm Hg) to a brown oil. After removal of trace volatiles (0.03 mm Hg, 1 h), the residue was taken up in 2 mL of $CH_3CN/CF_3CO_2H/H_2O$ (8:1:2), stirred for 2.5

h, concentrated by rotary evaporation, and partitioned between EtOAc/0.1 M aq. HCl (10 mL each). The organic layer was washed with H_2O followed by brine, then dried over $MgSO_4$ and evaporated to dryness to afford Fmoc-**1** as an orange gum in >95% yield.[22]

The other reagents were prepared in direct analogy; a complete list of the intermediates and yields is found in the Table. In cases where the carboxyl half entered as the nitroso component, the acids were protected as their *t*-butyl esters to improve solubility and ease chromatographic purification of the nitroso intermediate. Acids were readily esterified in >95% yield using isobutylene/CH_2Cl_2 (1:2 v/v) with 0.5 mol % conc. H_2SO_4 as a catalyst by stirring them for 2–3 d at room temperature in a pressure tube (CAUTION: Use a safety shield). Completion of the reaction is indicated by dissolution of the acid, and the products were isolated by diluting with EtOAc and washing the organic layer with sat. aq. Na_2CO_3 followed by H_2O and brine; beyond removal of solvent *in vacuo*, no purification was necessary.

Table: Intermediates and Reaction Yields

Product	Aniline Component		Nitro Component		$NO_2 \rightarrow NO$ Reduction	Azo Coupling	Burton Coupling[a]
Fmoc-1		**1A**		**1N**	82%	83%	77%
2		**2A**		**2N**	79%	84%	76%
3		**3A**		**3N**	82%	90%	79%
Fmoc-4		**4A**		**4N**	65%	81%	63%
Fmoc-5		**5A**		**5N**	79%	78%	34% (64%)[b]

a) All couplings employed 2 equiv of Br–Cd–$CF_2PO(OEt)_2$ at room temperature for 3 hours except entry **3**, where 8 equiv at 40 °C were employed.
b) Yield in parentheses based on recovered starting material.

The components were derived easily from commercial materials. All iodoanilines **1A–5A** were prepared by reduction of the corresponding nitroarenes, either with H_2 as described above (**1A–4A**) or with Zn (4 equiv)–NH_4Cl (6 equiv) in 2-methoxyethanol (**5A**). Both nitro compound **4N** and aniline **5A** were prepared from 2-iodo-4-nitrobenzoic acid.[23] Iodide **4N** was prepared by esterification as described above, whereas aniline **5A** was prepared by a multistep sequence: conversion to the amide ($SOCl_2$, then NH_4OH; 64% after recrystallization from EtOH/H_2O), reduction to the benzylic amine (BH_3•THF;[24] 73%), protection with Fmoc-Cl/*i*-PrNEt_2 in THF (82%), and finally, reduction to the aniline (>95%). As **5A** is prone to decomposition, it was carried forward immediately. All compounds gave satisfactory ^{1}H-NMR, ^{13}C-NMR, and MS analyses.

The only step requiring special care is the nitro to nitroso reduction, which was effected by the two-step/one-pot sequence of reduction to the hydroxylamine with zinc followed by oxidation with $FeCl_3$.[7] The hydroxylamine is inevitably contaminated by some unreacted nitro compound and some of the corresponding aniline, formed by over-reduction. This complication cannot be avoided completely, and because reaction times vary considerably among substrates (0.5–3 h), the reaction must be monitored closely by TLC, as described below. The aniline must be removed prior to the azo coupling; this is best accomplished by chromatographic purification of the much less polar nitroso compound. As the nitroso compounds are unstable, they must be chromatographed quickly and used immediately. Finally, experience in this laboratory showed that the nitro to nitroso transformation is problematic for Fmoc-protected compounds. Therefore, the fragment bearing the amino terminus (if present) was always selected as the aniline.

A representative procedure for the nitro to nitroso reduction is as follows. To a solution of 4-iodonitrobenzene (**5N**, 2.0 g, 8 mmol) in 2-methoxyethanol (60 mL) was added Zn dust (1.2 g, 18.4 mmol, 2.3 equiv) and NH_4Cl (685 mg, 12.8 mmol, 1.6 equiv). The reaction mixture was stirred at room temperature and

monitored closely by TLC (silica gel F_{254}, 5:1 hexanes/EtOAc; the aniline is readily distinguished from the hydroxylamine by its slightly higher mobility and rapid, intense staining with ninhydrin). When the large majority (75–90%) of the nitroarene had been consumed, hydroxylamine formation slowed, and aniline formation began to accelerate. At this point (ca. 30 min for **5N**), the solution was promptly cooled to 0 °C, and $FeCl_3$ (3.89 g, 24 mmol, 3 equiv) in 72 mL of H_2O/ethanol (5:1) was added. After 3 h at 0 °C, the reaction mixture was extracted with EtOAc (3 × 80 mL). The combined extracts were washed with brine, dried over $MgSO_4$, concentrated under reduced pressure, and chromatogaphed rapidly on silica gel, eluting with hexanes/EtOAc (50:1). Removal of the solvent *in vacuo* afforded 4-iodonitrosobenzene (1.53 g, 6.56 mmol, 82%) as a green solid.

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22. MS (ESI, 0.1% NH_4OH): *m/z* calc'd for $C_{31}H_{25}F_2N_3O_7P$ $(M-H)^-$ = –620.140, found –619 (2.6%), –620 (4.2%), –621 (2.9%). ^{31}P NMR (121.4 MHz, CD_3OD, vs. external H_3PO_4): δ 4.74 (t, J = 102 Hz); ^{19}F NMR (282.2 MHz, CD_3OD, vs. external $CFCl_3$ in acetone-d_6): δ 68.3 (d, J = 104 Hz); ^1H NMR (299.9 MHz, CD_3OD; vs. $\underline{H}CD_2OD$ = 3.30): 2.87–3.07 (m, 2H), 4.11 (br t, 1H, J = 7 Hz), 4.16–4.26 (m, 1H), 4.31 (dd, 1H, J = 7 Hz, 10 Hz), 4.48 (dd, 1H, J = 5 Hz, J = 10 Hz), 7.19–7.28 (m, 2H), 7.29–7.38 (m, 2H), 7.41 (d, 1H, J = 8 Hz), 7.55 (d, 1H, J = 8 Hz), 7.75 (t, 2H, J = 8 Hz), 7.83 (d, 1H, J = 8 Hz), 7.93 (d, 1H, J = 8 Hz); ^{13}C NMR (125.7 MHz, CD_3OD, vs. $\underline{C}D_3OD$ = 49.0): δ 38.43, 56.48, 67.93, 120.85, 123.39, 124.07, 126.13, 126.23, 128.12, 128.48, 128.71, 131.30, 142.51, 143.04, 145.13, 145.18, 152.73, 154.91, 158.36, 174.81.
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