Chapter 5. Caged phosphoamino acids

5.1 Introduction

One of the lessons of the work presented in the previous chapter is that the great complexity inside a cell requires chemical tools to be as precise as possible. Caged tyrosine allows some control over the phosphorylation state of a particular side chain, but the downstream activity of tyrosine kinases remains out of the hands of the experimenter. By introducing caged phosphoamino acids, an additional degree of control may be exerted. Here, the phosphoamino acid is masked as a large, neutral residue. Irradiation itself reveals the wild-type phosphoamino acid, thus placing the phosphorylation state of the protein under direct experimental control. Needless to say, this phosphorylation may be reversed through the activity of phosphatases once the wild-type phosphoamino acid has been revealed. This reversibility may prove to be a useful null control, in that reversion to the non-phosphorylated phenotype can act as an intrinsic confirmation that an observed effect is due to phosphorylation. Indeed, the synthesis of non-hydrolyzable mimics may provide important insight into the kinetics of phosphatases *in vivo*. The three unnatural amino acids discussed here, caged phosphorylatable residues, caged phosphoamino acids, and caged non-hydrolyzable phosphoamino acid mimics, constitute a complete set for analysis of phosphorylation of a particular side chain in a protein.

5.1.1 Design of caged phosphoamino acids

The most straightforward design for caged phosphoamino acids involves protecting the phosphate oxygens with photo-removable protecting groups. (Figure 5.1) This chemistry has a relatively long history, as ATP has been a common target for caging.[1-3]

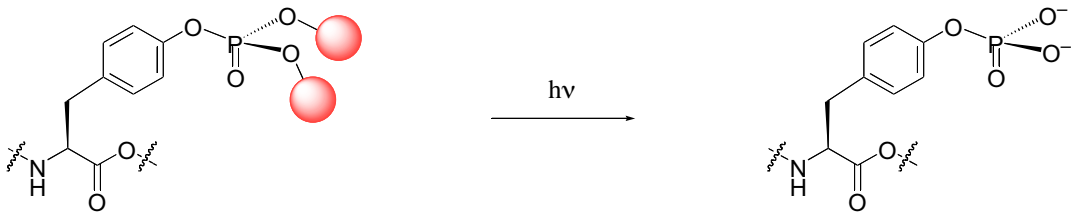


Figure 5.1 Schematic for the design of a phosphoamino acid where the side chain is caged. Photo-removable protecting groups are indicated as red spheres.

In addition to caged nucleotides, caged lipids have been widely reported, as has a caged phosphate-containing drug.[3-21] In addition to their synthetic relevance, a number of these studies provide important information on de-caging rates and conditions.[4,11,12,17-21] More recently, caged phosphoamino acid analogs have been developed and introduced into proteins and peptides.[22-25] Additionally, an interesting study has reported a caged mechanism-based inhibitor of tyrosine phosphatases, which is reversible only upon de-caging.[26] Solid-phase peptide synthesis using nitrophenethyl-caged phosphotyrosine, serine, and threonine has been accomplished very recently.[23] Although no application of these peptides has yet been reported, the de-caging efficiencies in water were determined. In the studies involving proteins, caging was performed by reaction of kinase-introduced thiophosphoryl groups with electrophilic reagents.[22,24,25] (Figure 5.2)

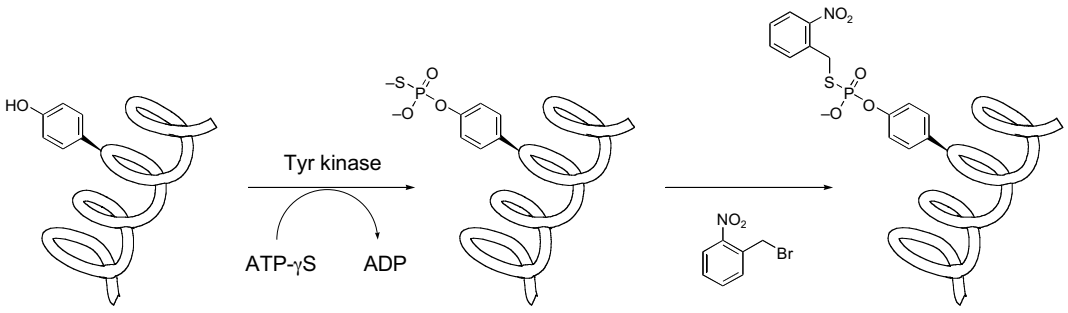


Figure 5.2 A strategy which has been reported for the incorporation of caged phosphoamino acids into proteins and synthetic peptides.[22,24,25]

Because of the requirement that the peptide or protein be modified with exogenously applied reagents, this technique is limited in both its specificity and *in vivo* application. Semi-synthetic peptides and proteins containing caged phosphoamino acids require transport into cells to be used to investigate signal transduction.

The introduction of caged phosphoamino acids by unnatural amino acid mutagenesis has the advantages of both site-specificity and compatibility with usage *in situ*. However, it has the potential drawback that steric bulk may be incompatible with ribosomal protein synthesis. It is encouraging that a number of very large residues have been incorporated via unnatural amino acid mutagenesis, notably the large hydrophobic residues introduced into streptavidin by the group of Sisido.[27] Nonetheless, caged phosphoamino acids are bulkier than any ribosomally incorporated amino acid. (Figure 5.3)

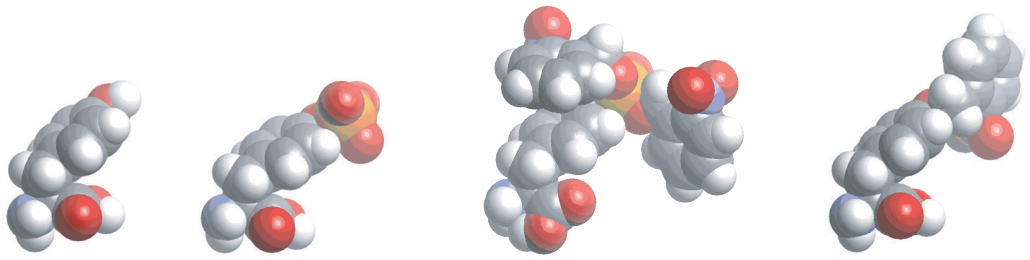


Figure 5.3 CPK models of tyrosine, phosphotyrosine, caged phosphotyrosine, and caged tyrosine, demonstrating the steric bulk of caged phosphotyrosine.

One potential solution to this problem of bulk would be to cage one, but not both of the phosphate oxygens. Indeed, in the studies cited above employed singly caged phosphoamino acids. However, the effects of phosphorylation are often successfully mimicked by replacing a potentially phosphorylated serine or threonine with a monoanionic residue, such as aspartate. Thus, a more certain means of ensuring that the caged residue is functionally equivalent to its non-phosphorylated form prior to photolysis is to utilize the neutral, doubly caged phosphodiester. By analogy to

isopropylidene or pivaloyl protection of vicinal diols, it might be possible to design a caged phosphate where both oxygens could be caged by a single photo-removable protecting group. Indeed, some compounds similar to this are known.[28] (Figure 5.4)

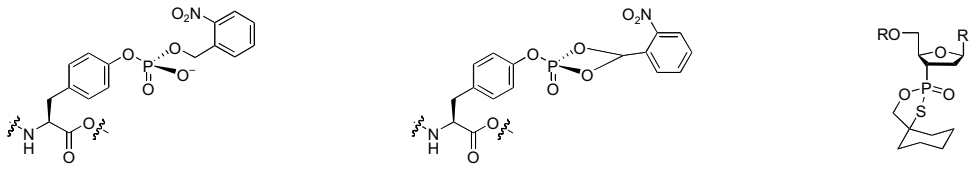


Figure 5.4 Possible solutions to the problem of caging group bulk. Two different mono-protected phosphoamino acid strategies are compared to the known oxathiaphosphalane ring system.[28]

However, for the initial experiments, the doubly caged phosphoamino acids represent a logical starting point. There is a large number of caging groups which may be utilized.[2,29-36] In fact, a flexible synthetic route which permits incorporation of a variety of these groups is desirable. The initial amino acid design relied on nitrobenzyl esters, which are well precedented, have optical properties which are appropriate for use in the oocyte system, and which are relatively sterically compact.

5.1.2 Non-hydrolyzable analogs

As indicated, another attribute which is desirable from a design perspective is stability to enzymatic removal. Phosphate groups are, of course, dynamically removed in a cell by phosphatases. Replacement of the O-P connectivity by a methylene unit is a common strategy for circumventing hydrolysis. The phosphonate which results from this replacement has rather different acidity than the parent phosphate. In order to more accurately mimic the natural phosphate group, difluoromethylene units are often utilized. (Figure 5.5)

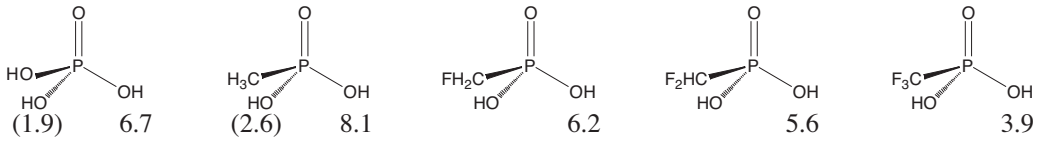


Figure 5.5 Values of phosphate pKa for phosphonate analogs containing fluorine.[37]

There is little question that the ionization state of difluorophosphonates more closely resemble that of phosphates, in that they are dianionic at physiological pH.[37-40] Whether or not phosphonates or difluorophosphonates are more effective analogs of phosphoamino acids, however, continues to be debated. A rather extensive literature on this subject suggests that context plays an important role. In some experiments, phosphonates have tighter binding, or are more effective than their difluorophosphonate congeners, while in others, the opposite trend is observed. (reviewed in Berkowitz)[41] No pattern has emerged such that an *a priori* evaluation may be made. Thus, the synthetic strategy for caged, non-hydrolyzable phosphoamino acid analogs should permit the synthesis of both phosphonate and difluorophosphonate analogs.

5.1.3 Mechanism-based phosphatase inhibitors

While difluorophosphonates act as non-hydrolyzable phosphate mimics, singly halogenated phosphonates have been shown to irreversibly inhibit phosphatases.[42,43] Covalent linkage of unnatural amino acid-containing proteins to phosphatases which act on them could provide a means for specifically identifying these phosphatases. While this methodology perhaps awaits the development of sensitive means for isolating and assaying the relevant phosphatases, the design of such molecules is straightforward and requires no great modification to the synthetic route to caged non-hydrolyzable phosphoamino acid analogs.

5.2 Synthesis of caged phosphoamino acids

The conversion of alcohols to phosphates has become a routine transformation, due to its central role in nucleic acid synthesis.[44] Phosphoramidites provide a mild, high-yielding methodology to accomplish the conversion, which has been applied to both phosphopeptides and to phosphate-containing prodrugs with labile phosphate modifications.[44-52] Bis(nitrobenzyl) phosphoramidite **1** is precedented, for the synthesis of a nitrobenzyl-protected phosphotyrosine and a caged phospholipid.[15,53] In both cases, nitrobenzyl alcohol is added to commercially available diisopropyl phosphoramidous dichloride. (Figure 5.6)

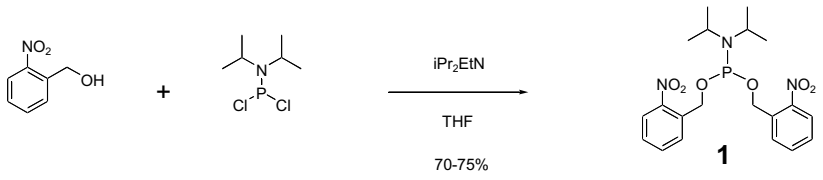


Figure 5.6 Synthesis of bis(nitrobenzyl) phosphoramidite **1**.[15,53]

The resulting phosphoramidite **1** may be conjugated to either aryl (tyrosine) or alkyl (serine or threonine) alcohols, catalyzed by 1*H*-tetrazole. The resulting trivalent intermediate is oxidized, to provide a mild, one-pot conversion of amino acid (e.g. **2**) to phosphoamino acid (e.g. **3**). (Figure 5.7)

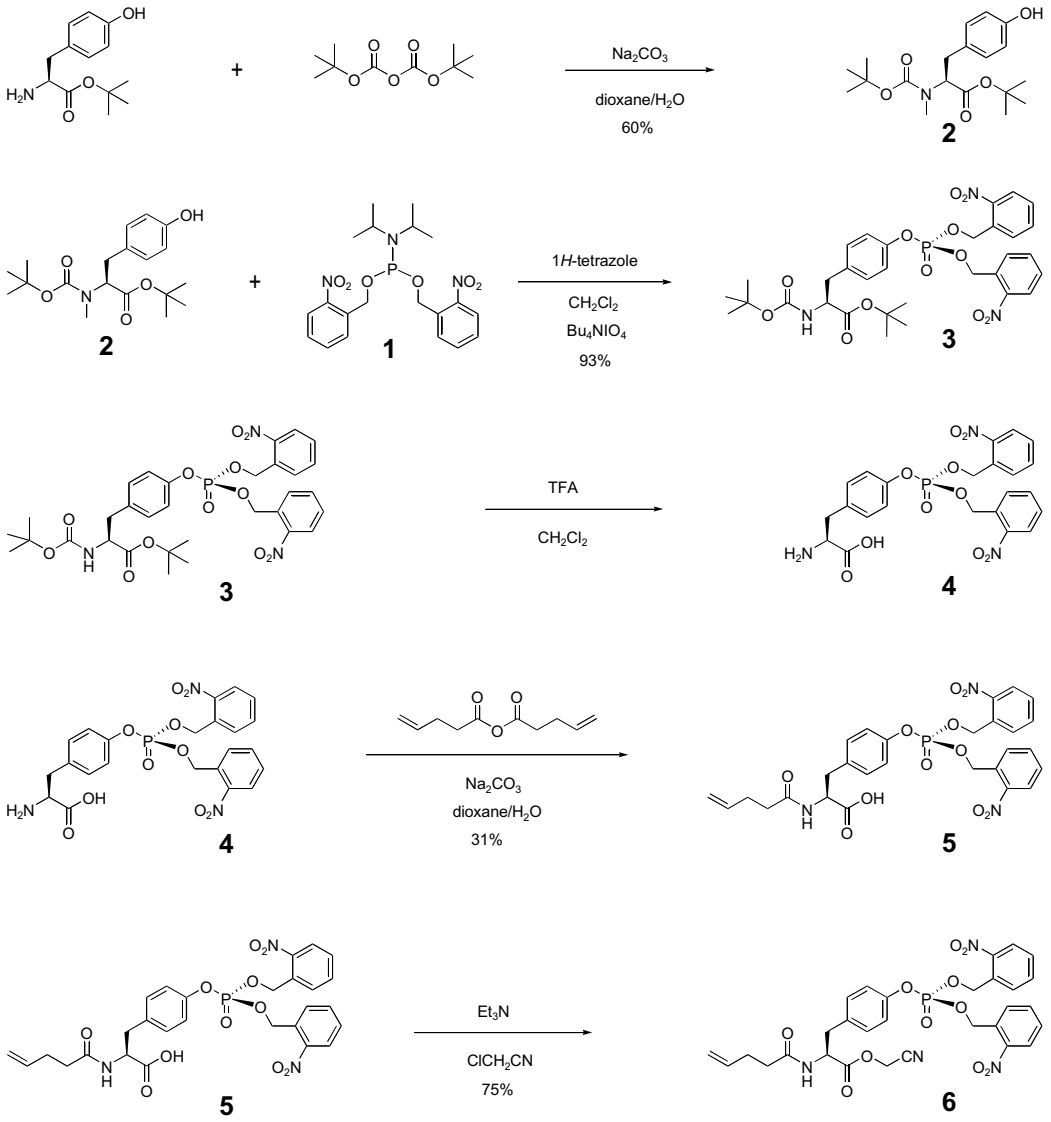


Figure 5.7 Synthesis of caged phosphotyrosine **6** from protected tyrosine **2** using bis(nitrobenzyl) phosphoramidite **1**.

Initially, it was feared that the 4-pentenoyl N-terminal protecting group would be unstable to the periodate oxidation involved in the final step of the phosphoramidite reaction. Thus, the amino acid was protected as the Boc t-butyl ester **3**, as shown in Figure 5.7. However, TLC experiments of 4PO-protected amino acids treated with tetrabutylammonium periodate at the appropriate conditions showed no apparent loss of

the protecting group. Thus, an even simpler route was subsequently used to generate caged phosphoserine **9** (Figure 5.8) and phosphothreonine **12** (Figure 5.9).

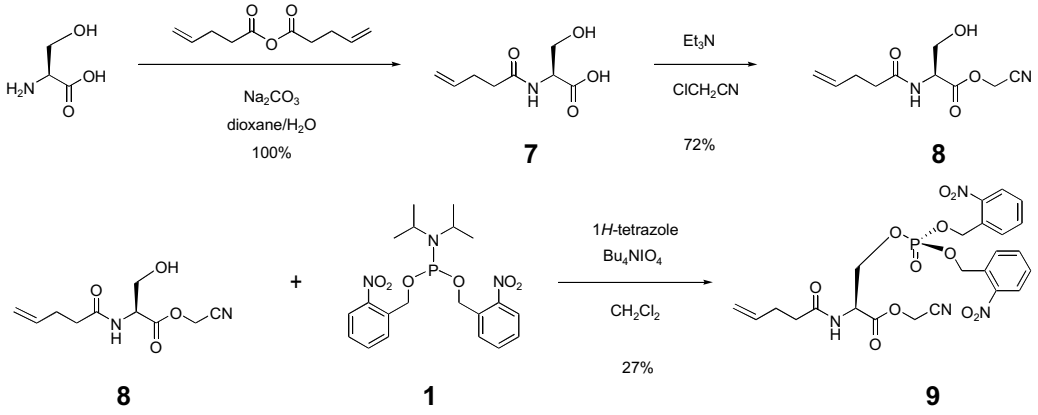


Figure 5.8 Synthesis of caged phosphoserine **9**.

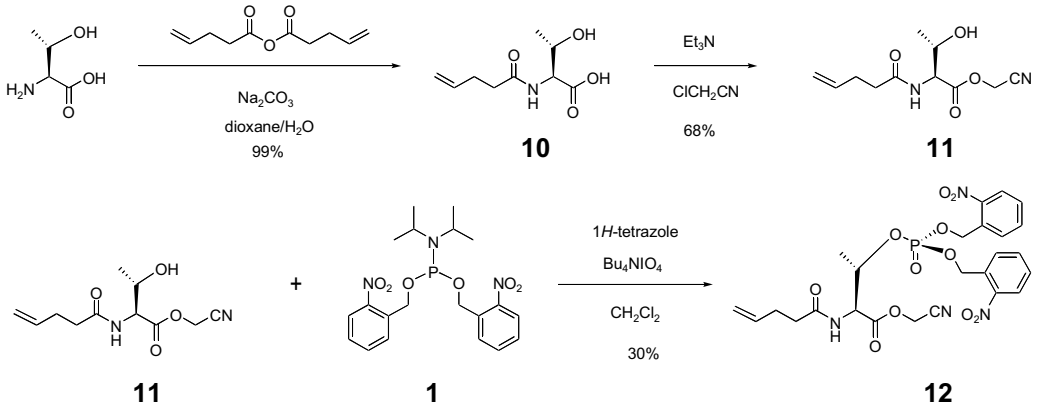


Figure 5.9 Synthesis of caged phosphothreonine **12**.

5.3 Synthesis of caged non-hydrolyzable phosphoamino acid analogs

The synthesis of non-hydrolyzable analogs is substantially more complicated than that of phosphoamino acid analogs. Phosphono and difluorophosphono analogs of tyrosine, serine, threonine, and histidine are all well precedented.[54-66] The initial synthetic routes to the fluorinated tyrosine compounds relied on fluorination using DAST.[58,60,67] In more modern routes, this methodology has been superceded by the milder, safer, more

specific metal-catalyzed cross-coupling developed independently by Shibuya and Burton.[68-70] (Figure 5.10)

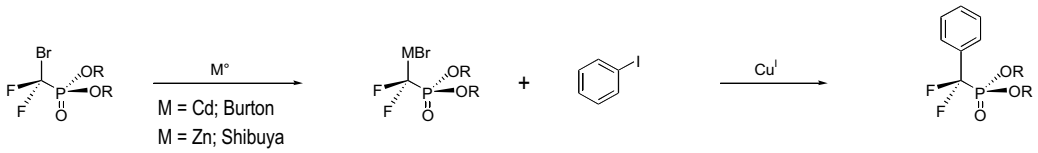


Figure 5.10 Methodology of Shibuya and Burton for the synthesis of difluorophosphonates from aryl iodides via copper(I)-catalyzed cross-coupling.[69,70]

For forming aliphatic carbon-phosphorus bonds, a number of methods exist.[67,71-81] Most of them are useful for generating phosphonates, although a number can be used to prepare difluorophosphonates.[67,71-81] The triflate displacement methodology employed by Berkowitz and others was selected for the synthesis of serine and threonine analogs.[41,61-65,82-90] (Figure 5.11)

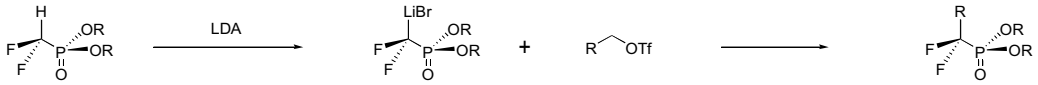


Figure 5.11 Berkowitz route to alkyl difluorophosphonates by direct nucleophilic displacement.[82]

In most cases, the phosphates are protected as the diethyl ester, although Berkowitz has reported benzyl- and alloc-protected difluorophosphonates.[41,83]

5.3.1 Difluorophosphonate intermediates

An important intermediate for the copper(I)-mediated crosslinking central to the routes of Shibuya and Burton is the dialkyl bromodifluorophosphonate. The diethyl version **15** is available from Aldrich, suggesting the possibility of coupling to suitably protected *p*-iodophenylalanine. The ethyl phosphodiester may be de-protected under the conditions of Rabinowitz, McKenna, and Jung (TMSI/TFA) to give the dianion.[55,56] Conditions analogous to those used in peptide coupling have been reported to generate

phosphodiesters.[91] Thus, coupling to nitrobenzyl alcohol may be possible, as indicated in Figure 5.12.

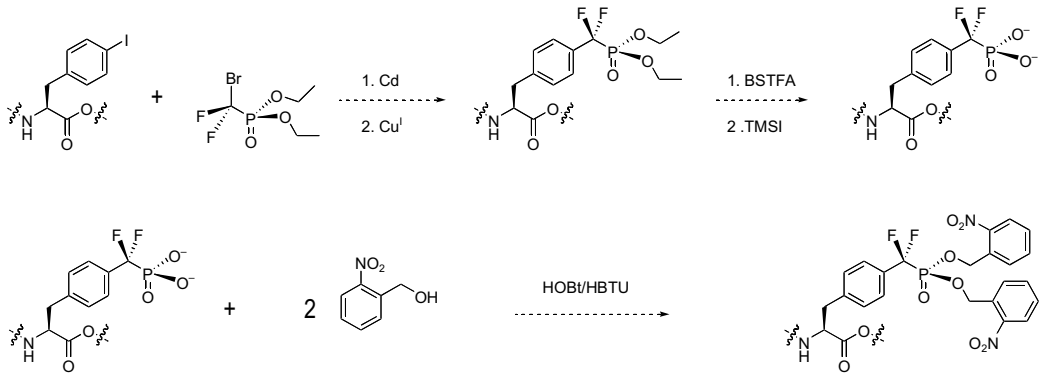


Figure 5.12 De-protection and re-protection scheme to replace ethyl esters of phosphate with nitrobenzyl esters.

Alternatively, the bis(nitrobenzyl) bromodifluorophosphonate **16** may be synthesized and utilized directly in the cross-coupling with protected *p*-iodophenylalanine. (Figure 5.13) The latter route, in fact, may be compatible with 4PO- (**13**) and cyanomethyl-protected iodophenylalanine **14**.

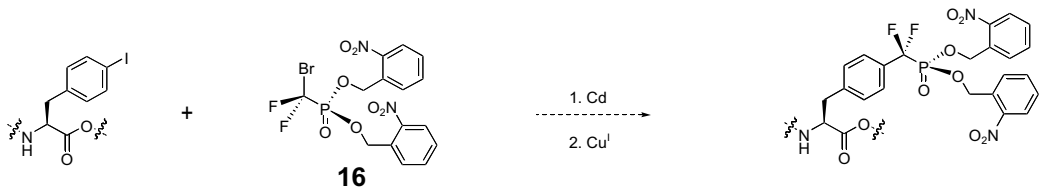
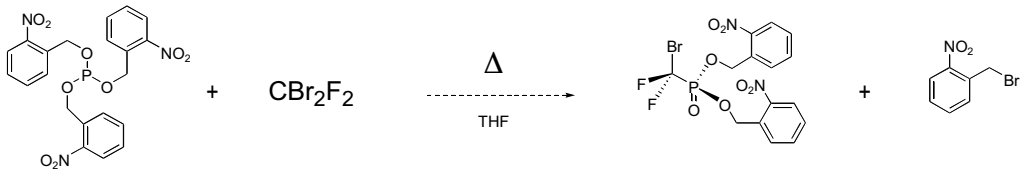


Figure 5.13 Installation of nitrobenzyl-protected phosphate.

A one-step synthesis of diethyl bromodifluorophosphonate from ethyl phosphite and CF_2Br_2 has been reported by the group of Savignac, following earlier syntheses of Burton.[81,92] Work by Mioskowski on the use of benzyl phosphites in the Arbuzov reaction suggests the necessity of *in vacuo* removal of the benzyl bromide product as it forms.[78] (Figure 5.14)



17 **16**

Figure 5.14 Proposed synthesis of bis(nitrobenzyl) bromodifluorophosphonate **16** following the route of Savignac to diethyl bromodifluorophosphonate.[81]

Nitrobenzyl phosphite **17** is easily available from the condensation of nitrobenzyl alcohol with PCl_3.[93] Pure material was re-crystallized from diethyl ether and fully characterized by NMR, although the compound proved unsuitable for electrospray mass spectrometry. However, nitrobenzyl phosphite appears to be significantly deactivated for Arbuzov chemistry relative to ethyl phosphite. The conditions of Savignac (60 °C, gently refluxing THF), gave no reaction. Heating the reaction to 100 °C overnight in a sealed tube also failed to give the desired product. The reactions were monitored by ^{31}P NMR. Because of fluorine-phosphorus coupling, the difluorophosphonate has a very distinctive triplet signal. (Figure 5.15)

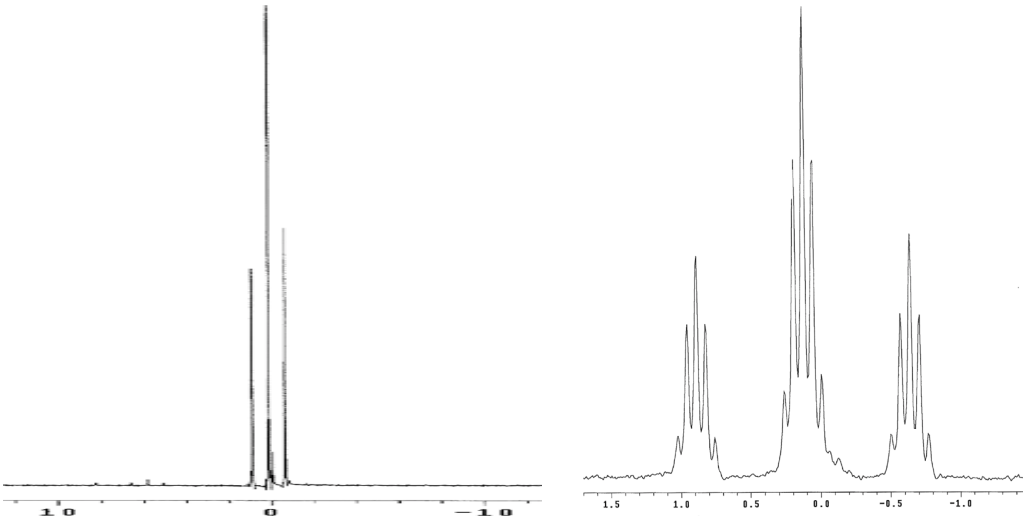


Figure 5.15 Characteristic triplet (J= 92 Hz) arising from $–CF_2$-P splitting (NMR of **15**).

The Aldrich diethyl bromodifluorophosphonate **15** provided an authentic sample in which this triplet was observed. Under the conditions of Savignac, the 134 ppm phosphite signal was still observed after overnight reaction, suggesting no formation of pentavalent phosphorus and, indeed, no reaction. After the sealed-tube reaction, pentavalent phosphorus signals were observed, although these could not be unequivocally assigned. The formation of nitrobenzyl phosphonate is a likely outcome of this reaction, although proton de-coupled phosphorus signals did not appear to correspond to the expected pattern from a methylene-phosphorus interaction.

An alternative route to dialkyl bromodifluorophosphonates was utilized by Berkowitz in a synthesis of bis(benzyl) bromodifluorophosphonoserine.[41] Here, a benzyl *H*-phosphonate is treated with sodium hydride or sodium HMDS and added to an electrophilic substrate. (Figure 5.16)

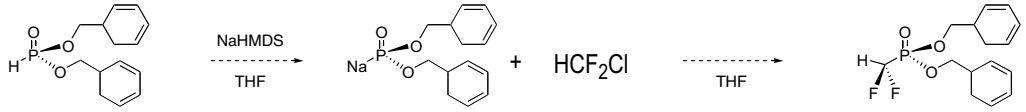


Figure 5.16 Alternative route to difluorophosphonates, involving benzyl *H*-phosphate.[41,74]

In the case of the Berkowitz reaction, $CHClF_2$ was used, and earlier reports show the addition of benzyl *H*-phosphonate to CH_2Cl_2.[41,74] To synthesize the bromodifluorophosphonate, addition to CBr_2F_2 would be required. As with nitrobenzyl phosphite **17**, nitrobenzyl *H*-phosphonate **18** was synthesized by simple modification of a preparation for the benzyl compound.[94] (Figure 5.17)

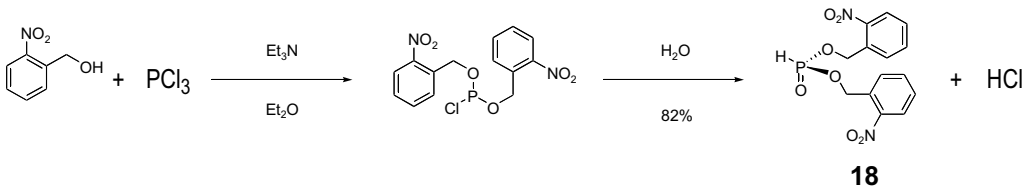


Figure 5.17 Route for preparation of nitrobenzyl *H*-phosphonate **18** from nitrobenzyl alcohol and phosphorus(III) chloride.[94]

The compound was re-crystallized from ethanol and characterized by carbon, proton, and phosphorus NMR. Again, it proved refractory to electrospray MS. The H-P interaction provides a very distinctive splitting pattern which was easily observed in proton-decoupled ^{31}P spectra. (Figure 5.18)

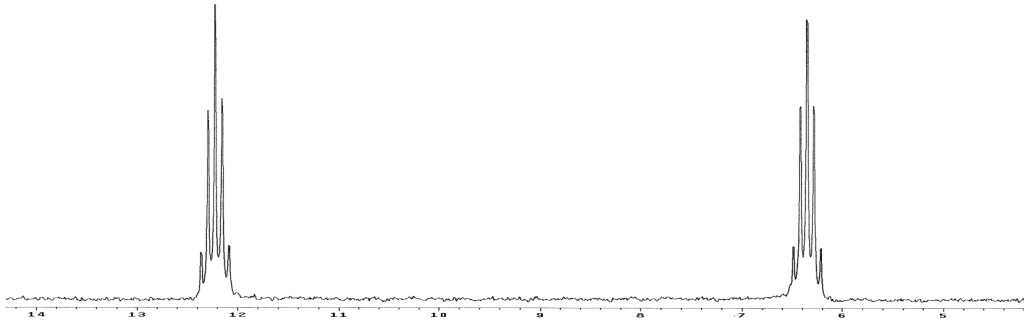


Figure 5.18 ^{31}P splitting pattern (J = 711 Hz) arising from H-P coupling in nitrobenzyl *H*-phosphonate **18**.

However, base-mediated condensation of compound **18** with neither CF_2Br_2 nor CH_2Br_2 was observed. Subsequent discussion with former group members confirmed the experience that nitrobenzyl compounds are not typically compatible with strong base, as an apparent result of increased acidity at the benzylic position relative to benzyl compounds which lack the nitro ring substitution.[95]

5.3.2 Future prospects

The routes thus far adopted to generate the important bromodifluorophosphonate intermediate **16** for direct installation of the caged phosphonate have proven

unsuccessful. Two strategies remain viable for further work in this area. First, the de-protection and re-protection strategy proposed above may be implemented to convert the commercially available ethyl phosphodiester to the desired nitrobenzyl compound **16**. (Figure 5.)

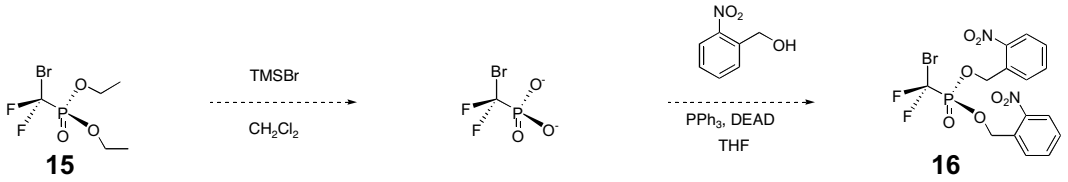


Figure 5.19 Generation of nitrobenzyl bromodifluorophosphonate **16** by de-protection of commercially available ethyl ester **15** followed by re-protection with nitrobenzyl alcohol.

As indicated previously, the conditions of Burton and Shibuya are expected to provide a mild and convenient synthesis of caged difluorophosphonotyrosine from this intermediate. (Figure 5.10) Given the difficulties encountered in using strong base in the presence of nitrobenzyl groups, the conditions of Berkowitz may prove incompatible with the direct installation of protected difluorophosphonate. One possible route is the use of metal-catalyzed addition to an α,β-unsaturated carboxylic acid, as in the report of Kawamoto *et al*.[96] (Figure 5.20)

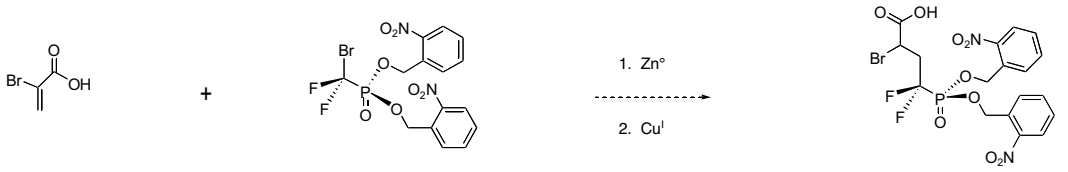


Figure 5.20 Proposal to employ Kawamoto's mild and selective metal-catalyzed bromodifluorophosphonate coupling on a vinyl halide substrate to provide an important intermediate for caged difluorphosphonoserine.[96]

In a second approach to synthesizing caged non-hydrolyzable phosphoamino acid analogs, the difluorophosphonate may be installed in its protected form. Subsequent de-protection of the difluorophosphonoamino acid may be accomplished and the desired

protecting group installed. Note that an unavoidable feature of this approach is the need for orthogonal protection of amine, carboxylate, and phosphate. Thus, a convenient means of simplifying this approach would be to synthesize a bromodifluorophosphonate with protecting groups orthogonal to those traditionally used for amino acid protection. An example is shown in Figure 5.21, where benzyl protection is utilized for formation of the caged non-hydrolyzable phosphoserine analog.

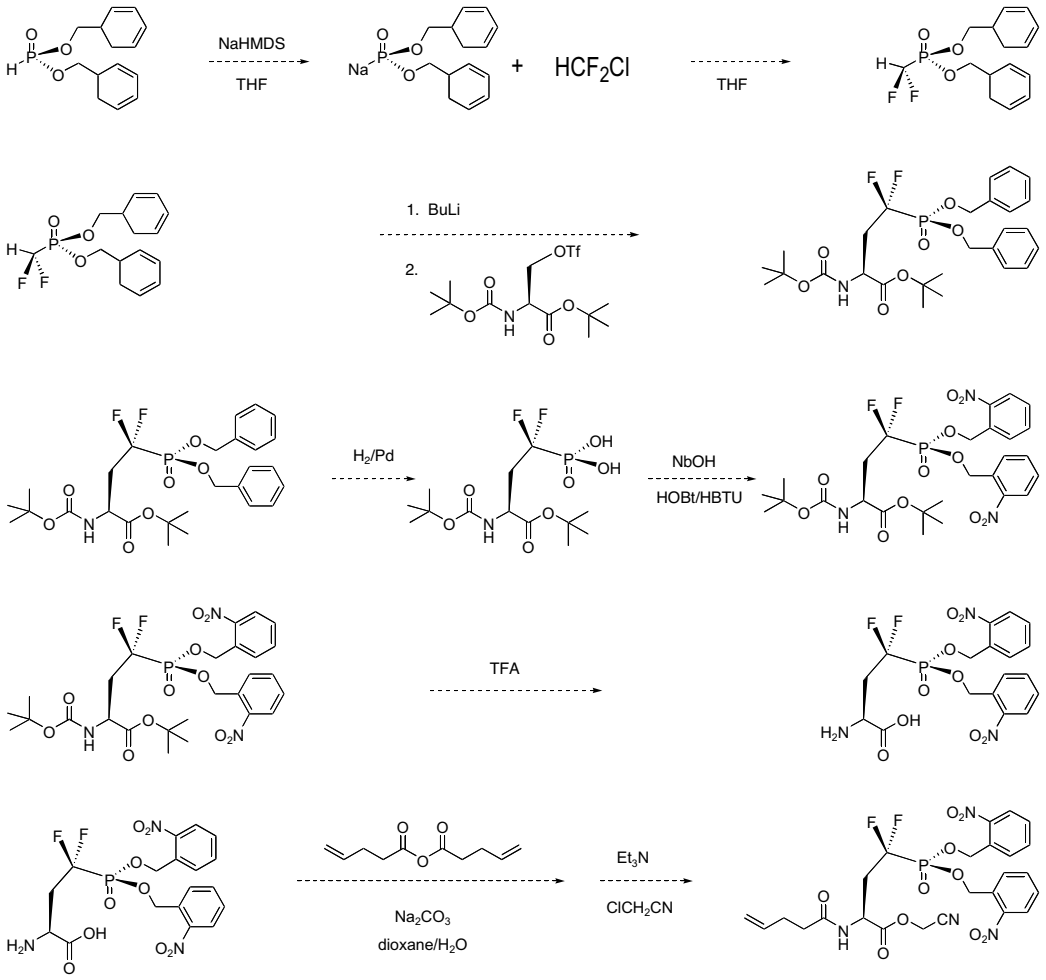


Figure 5.21 Proposed general scheme for synthesis of nitrobenzyl difluorophosphonoserine, involving installation of the protected difluorophosphonate followed by de-protection and re-protection with nitrobenzyl alcohol.

The 2-(trimethyl)silylethoxy (TSE) protecting group is also useful to consider, and is perhaps more desirable than benzyl because of its expected increased reactivity in Michaelis-Arbuzov chemistry.[97] This chemistry may be important in preparing phosphonate analogs. As indicated earlier, there is no good means of predicting whether difluorophosphonates or simple phosphonates will prove to be more effective phosphate mimics. Thus, the importance of developing synthetic schemes which permit phosphonate synthesis was cited. In general, there are two convenient routes for tyrosine analogs and one for serine or threonine analogs. In the case of tyrosine, the method of Shibuya and Burton could be used, employing a simple bromophosphonate. (Figure 5.22) Because the TSE phosphite is expected to be active in Michaelis-Arbuzov chemistry, the bromophosphonate may be generated via its condensation with dibromomethane, as in Figure 5.14. If desired, this compound could be de-protected and converted to a nitrobenzyl bromophosphonate by the Mitsonobu chemistry proposed above. (Figure 5.)

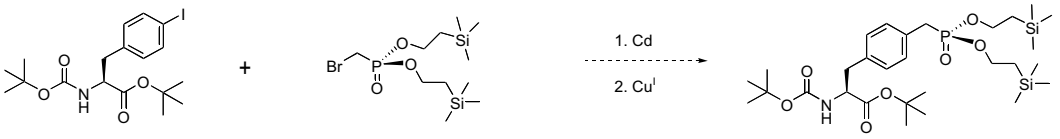


Figure 5.22 Proposed synthetic scheme for preparation of caged phosphonotyrosine, employing metal-mediated cross-coupling between an aryl iodide and bromophosphonate.

An alternative strategy for synthesis of tyrosine phosphonate would employ Arbuzov chemistry directly on a benzyl bromide analog of tyrosine.[53] (Figure 5.23)

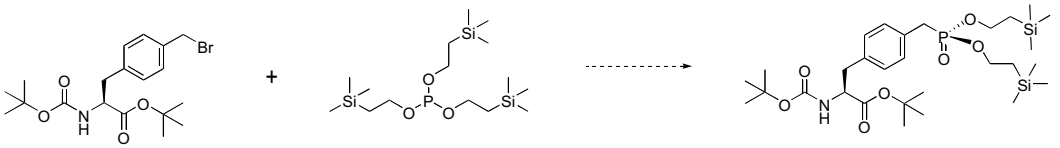


Figure 5.23 Proposed generalized Michaelis-Arbuzov scheme for synthesis of phosphonotyrosine.

A similar route is available for serine phosphonate analogs. (Figure 5.)

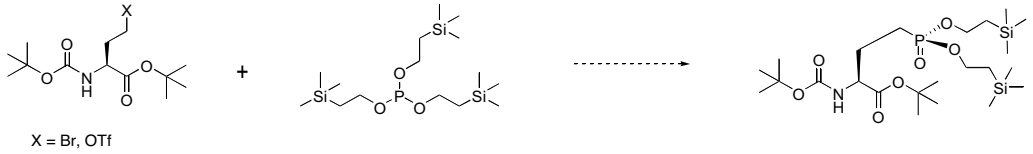


Figure 5.24 Proposed route to the synthesis of caged phosphonoserine.

A final strategy which would ameliorate problems associated with the nitrobenzyl group is to utilize different caging chemistry. Phenacyl groups, for instance, should behave more like benzyl substituents in both Arbuzov and dialkyl phosphate chemistry than do nitrobenzyl groups. (Figure 5.25)

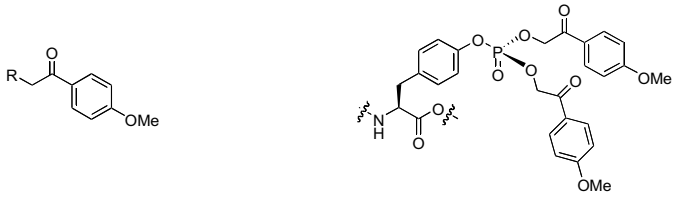


Figure 5.25 The *p*-methoxyphenacyl group, a synthetically tractable phototrigger for alcohols and carboxylic acids, and a phosphotyrosine analog showing the use of phenacyl as a phosphate caging group.[34,36]

To date, both phosphoserine and phosphothreonine have been synthesized as the active, protected amino acids **9** and **12**. However, coupling to dCA has yet to be performed. One possible difficulty which may be anticipated is β-elimination of the phosphate from the side chain.[98-100] Such elimination was observed by Fahmi *et al*. with a glycosylated serine derivative upon coupling to dCA, but the conditions of the coupling were able to be altered in such a way as to permit formation of the desired product.[98] Also, the solid-phase conditions for preparing peptides in the presence of singly caged phosphoamino acids involve piperidine in DMF, to which the linkage was apparently stable.[23] If elimination is a complicating factor in dCA coupling, the switch to acetonitrile from DMF reported by Fahmi will be employed in an effort to overcome the

problem.[98] If elimination persists, alternative coupling methodologies may need to be investigated.

Attempts to circumvent the synthetic problems outline above and prepare caged non-hydrolyzable phosphoamino acid analogs are ongoing.

5.4 Identification of appropriate biological systems for analysis by caged pAA

Both tyrosine and serine phosphorylation are highly important to the functional regulation of ion channels. Direct observation of phosphorylated side chains has been difficult, as noted in Chapter 4. The unequivocal introduction of a phosphorylated side chain by the method outlined here presents a unique opportunity to study the regulation of channels by serine, threonine, or tyrosine phosphorylation. The initial experiments to establish the proof of principle should target channels for which a very distinctive change occurs upon phosphorylation.

5.4.1 Tyrosine phosphorylation

The work on Kir2.1 described in Chapter 4 makes this ion channel a very attractive target for preliminary studies of tyrosine phosphorylation. Revealing the wild-type tyrosine residue at 242 results in current decrement with a time course of approximately 11 minutes. It would be very interesting to observe the effect of uncaging phosphotyrosine at this position. An immediate decrease in current would imply that the events taking place over minutes that are observed by uncaging tyrosine correspond to phosphorylation itself. If the kinetics of tyrosine kinases in a living cell could be observed in this fashion, it would represent a unique kind of information that may be obtained by this technique. Alternatively, if the de-caging of phosphotyrosine results in a

current decrease over a similarly long time course it may suggest that the kinetics of a protein-protein interaction are being observed, although it is possible that a very slow conformational change could be occurring. In either case, suppression of Kir2.1 with phosphotyrosine analogs provides a valuable test case for observing the effects of tyrosine phosphorylation on ion channel activity.

5.4.2 Serine phosphorylation

Recently, a striking change accompanying serine phosphorylation has been observed in the potassium channel KCNK2.[101] Phosphorylation of a single C-terminal serine residue causes the channel to acquire voltage dependence. (Figure 5.26) The presence of a such a dramatic phenotype makes this channel an excellent system for studying phosphoserine analogs.[102]

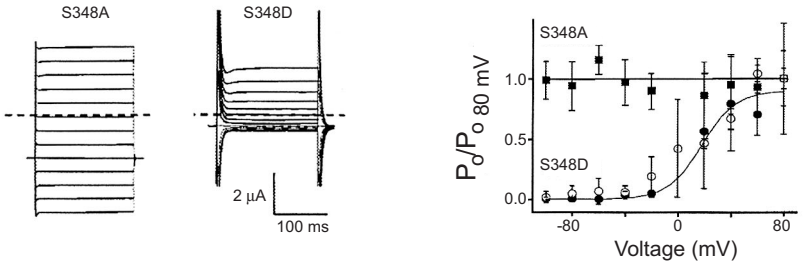


Figure 5.26 Voltage dependence of KCNK2 upon introduction of negative charge at Ser348 [adapted from Bockenhauer, *Nat. Neurosci.* **4**, 486 (2001)]. The effects of PKA were successfully mimicked by mutagenesis of Ser to Asp at position 348. The left panel illustrates the voltage dependence of the S348D but not S348A channels expressed in *Xenopus* oocytes, in response to voltage steps in 100 mM external K^+. The right panel shows the dependence of channel open probability on voltage for S348D (circles) relative to wild-type (squares) channels.[101]

5.5 Progress toward controlling phosphorylation with unnatural amino acids

5.5.1 Tyrosine phosphorylation

One important prerequisite for the use of phosphotyrosine analogs to control tyrosine phosphorylation is that they can be employed in suppression experiments. Encouraging results have been obtained in this regard in the nAChR. Mouse muscle receptors containing A122, I123, or F124TAG mutations in the alpha subunit were suppressed with pTyr(ONb)$_2$ and Tyr. As shown in Figure 5.27, significant currents were obtained, suggesting that the residue is competent for ribosomal translation. However, suppression efficiency of the caged phosphotyrosine was significantly diminished relative to the wild-type residue, which produced five to ten times the current of pTyr(ONb)$_2$ suppression at these sites.

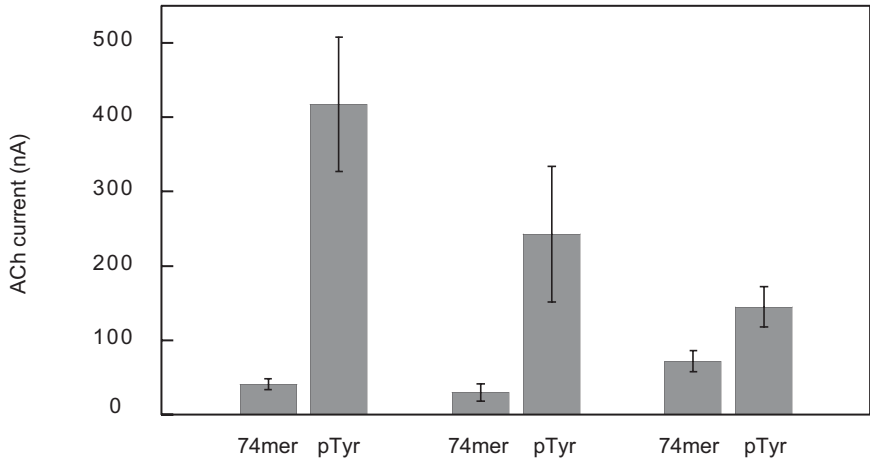


Figure 5.27 Suppression at nAChR αA123, αI123, and αF124 with 74mer and pTyr(ONb)$_2$. Currents elicited by 200 μM ACh were measured 36 hr after the injection of 2 ng total mRNA in the ratio of 10:1:1:1 α:β:γ:δ.

Early experiments in Kir2.1 provided little evidence for incorporation at Y242, even though Tyr(ONb) was successfully suppressed at this position. (Figure 5.28) However, it

may well be the case that this situation may be remediated by optimization of suppression conditions.

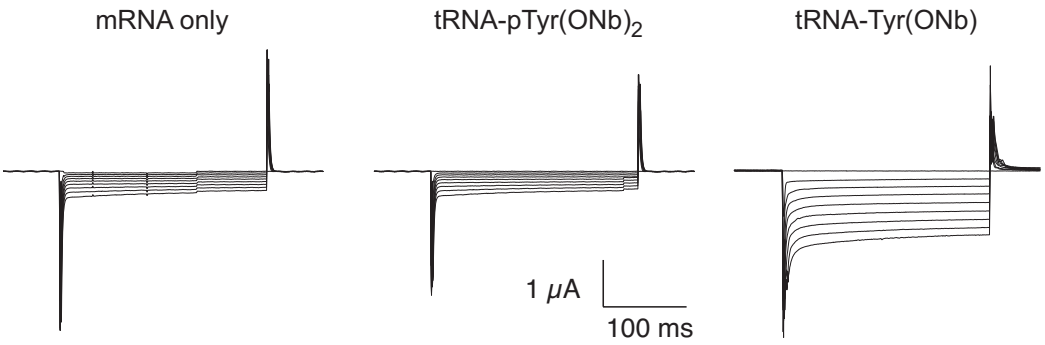


Figure 5.28 Attempted suppression at Kir2.1 Y242 with pTyr(ONb)$_2$. Currents were measured in High K^+ solution 48 hr after the injection of 12.5 ng total mRNA.

A final test that was performed with the phosphotyrosine analog involved recognition of the residue by anti-phosphotyrosine antibodies. A dot blot of 6 nmol of dCA-pTyr(ONb)$_2$ was performed. Development of the blot with BAbCo PY72 anti-phosphotyrosine antibody reveals that only irradiated dCA-amino acid is recognized by the antibody. (Figure 5.29)

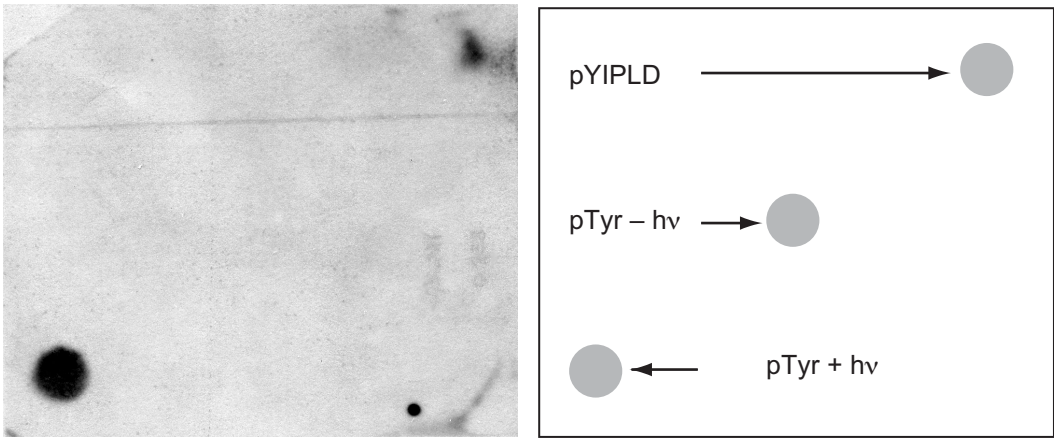


Figure 5.29 Dot blot of irradiated and non-irradiated dCA-pTyr(ONb)$_2$ **21** with the anti-phosphotyrosine antibody PY72. The right panel shows the pattern of spotting, where pYIPLD is a positive control peptide.

This experiment confirms that the residue, when caged, is inequivalent to phosphotyrosine, but that the irradiated residue reflects the properties of native pTyr.

5.6 Synthetic methods

5.6.1 General experimental procedures

All reactions were run under positive argon pressure, except where indicated. Anhydrous solvents were obtained from BakerDRY™ drums and were used without further purification. Amino acids and most commercially available reagents were obtained from Sigma-Aldrich. Thin-layer chromatography was performed using Whatman glass plates pre-coated with 60 Å silica gel. Nuclear magnetic resonance spectroscopy was performed on divisional Bruker spectrometers at the following frequencies: ^{1}H (300 MHz), ^{13}C (75 MHz) ^{31}P (121 MHz), ^{19}F (282 MHz). Mass spectrometry data were obtained from the divisional facility. Analytical and preparative HPLC was carried out using a Millipore system with fixed-wavelength and diode-array UV detection fitted with a C_{18} reverse-phase column.

5.6.2 Bis(nitrobenzyl) diisopropyl phosphoramidite 1

Diisopropyl phosphoramidous chloride (1 eq, 5 mmol) was thawed and dissolved in 4 mL anhydrous THF. In a flame-dried reaction vessel, nitrobenzyl alcohol (2 eq, 10 mmol) and dry diisopropylethylamine (3 eq, 15 mmol) were combined in 10 mL dry THF. The flask was fitted with an addition funnel and cooled to 0 °C in a water/ice bath. The addition funnel was charged with the phosphoramidous chloride solution, and it was added dropwise to the reaction flask. After 30 min reaction at 0 °C, the ice bath was removed and the reaction permitted to stir an additional 30 min at room temperature.

TLC (1:1 ethyl acetate:hexane, R_f = 0.8) confirmed reaction, although some starting nitrobenzyl alcohol remained. Filtration of the precipitate in a coarse fritted funnel, followed by washing with 25 mL ethyl acetate produced a clear, golden filtrate. The filtrate was washed with 25 mL saturated $NaHCO_3$ and 25 mL saturated brine. The organic layer was dried over $MgSO_4$ and concentrated to an amber solid by rotary evaporation. The residue was brought up in ether and purified by flash column chromatography (1:20 triethylamine:ether, R_f = 0.9) to give a pale yellow solid in 70-75% yield. ^{1}H NMR ($CDCl_3$) δ = 1.25 (d, *J* = 6.9 Hz, 12H), 3.75 (m, 2H), 5.17 (m, 2H), 7.44 (t, *J* = 9 Hz, 2H), 7.67 (t, *J* = 9 Hz, 2H), 8.10 (d, *J* = 8.1 Hz). ^{13}C NMR ($CDCl_3$) δ = 25.0, 25.1, 43.6, 43.7, 62.6, 62.9, 124.9, 128.0, 128.7, 134.0, 136.2, 136.3, 146.9. ^{31}P NMR ($CDCl_3$) δ = 151.7. MS (ESI) Calcd for $C_{20}H_{27}N_3O_6P$ $(M+H)^+$: 436.16. Found 458.2 $(M+Na^+)^+$, 474.2 $(M+K^+)^+$.

5.6.3 Boc-Tyrosine-OtBu 2

Commercially available Tyr-OtBu (1 eq, 3.46 mmol) was weighed into a flask and dissolved in 25 mL dioxane:water (5:1). Boc anhydride (1.2 eq, 4.16 mmol) was added, which dissolved readily. Triethlyamine (1.2 eq, 4.16 mmol) was added, without overt effervescence. After overnight stirring at room temperature, the reaction was monitored by TLC (1:1 ethyl acetate:hexane, R_f = 0.6) and shown to be complete. The reaction was quenched by the addition of 50 mL of 1 *M* $NaHSO_4$ and 50 mL CH_2Cl_2. The reaction mixture was then extracted thrice with CH_2Cl_2 (15 mL portions) and dried over Na_2SO_4. After rotary evaporation, the crude product was dissolved in a minimum volume of ethyl acetate:hexane (1:1). Flash column chromatography (1:1 ethyl acetate:hexane) gave the pure compound in approximately 60% yield. ^{1}H NMR (CD_3CN) δ = 1.43 (s, 18H), 2.93

(ABM, J = 25.2, 15, 7.8 Hz, 2H), 5.58 (d, J = 8.1 Hz, 1H), 6.78 (d, J = 6 Hz, 2H), 7.07 (d, J = 8.4 Hz, 2H). ^{13}C NMR (CD_3CN) δ = 27.5, 27.9, 28.3, 37.1, 56.2, 79.4, 81.6, 115.3, 115.5, 128.2, 130.5, 130.8, 155.7, 155.9, 171.4.

5.6.4 Boc-pTyr(ONb)$_2$-OtBu 3

N- and C-terminally protected Boc-tyrosine-OtBu **2** (1 eq, 0.78 mmol) was combined with freshly sublimed tetrazole (1.8 eq, 1.51 mmol) in a flame-dried flask fitted with a stirbar. Dry acetonitrile (10 mL) was added to solubilize the tetrazole, followed by 38 mL dry CH_2Cl_2. The reaction vessel was cooled to 0 °C in a water/ice bath. A solution of bis(nitrobenzyl) phosphoramidite **1** (1.5 eq, 1.15 mmol, in 2 mL dry CH_2Cl_2) was added dropwise via syringe. After 5 min mixing at 0 °C, the ice bath was removed and the reaction allowed to proceed 2.5 hr at room temperature. TLC (1:1 ethyl acetate:hexane, R_f = 0.7) revealed possible product formation. Oxidation was performed with tetrabutylammonium periodate (1.5 eq, 1.15 mmol) added in 5 mL dry THF. After 10 min reaction at room temperature, TLC (1:1 ethyl acetate:hexane, R_f = 0.4) indicated that the starting nitrobenzyl alcohol had been consumed. The reaction was quenched with 200 mL CH_2Cl_2 and washed twice (50 mL saturated $NaHCO_3$, 50 mL saturated NaCl). The organic layer was dried over Na_2SO_4 and concentrated to a thin yellow oil under reduced pressure. Flash column chromatography (1:1 ethyl acetate:hexane) gave the purified title compound in 93% yield. ^{1}H NMR ($CDCl_3$) δ = 1.36 (s, 18H), 3.05 (m, 2H), 4.42 (m, 1H), 5.1 (d, J = 12.9 Hz, 1H), 5.61 (d, J = 7.5 Hz, 4H), 7.17 (s, 4H), 7.52 (m, 2H), 7.7 (m, 4H), 8.13 (d, J= 8.1 Hz, 2H). ^{31}P NMR ($CDCl_3$) δ = -3.96.

5.6.5 pTyr(ONb)$_2$ 4

Deprotection of N- and C-protected caged pTyr **3** was performed with TFA. Boc-pTyr(ONb)$_2$-OtBu (1 eq, 0.5 mmol) and *p*-methoxybenzene (6 eq, 3 mmol) were added to a dry reaction vessel. Dry CH_2Cl_2 (5 mL) was added to produce a clear yellow solution. A 5 mL portion of trifluoroacetic acid was added. The reaction was stirred 60 min at room temperature, resulting in a solution of variable color, ranging from salmon to green. TLC (1:20 triethlyamine:ether, R_f = 0.1, and 1:20 acetic acid:ethyl acetate, R_f = 0.2, developed with ninhydrin) revealed the removal of both protecting groups. Volatiles were removed under high vacuum, after which 10 mL toluene were added and the flask returned to vacuum. The resulting compound was taken on to the subsequent steps without further purification or characterization.

5.6.6 4PO-pTyr(ONb)$_2$ 5

De-protected pTyr(ONb)$_2$ **4** was dissolved in 6 mL *p*-dioxane and 6 mL water. Na_2CO_3 (1.4 eq, 0.67 mmol) was added. Complete solubilization of reactants was achieved through *ad libitum* addition of dioxane (approximately 10 mL). 4PO-anhydride (1.4 eq, 0.67 mmol) was added in one portion and reaction allowed to proceed 4 hr at room temperature. Starting material appeared completely converted by TLC (1:20 acetic acid:ethyl acetate, R_f = 0.4). The reaction was quenched with 25 mL of 1 *M* $NaHSO_4$ and 25 mL CH_2Cl_2. Extraction with CH_2Cl_2 (3x, 10 mL each) was followed by drying over Na_2SO_4. After rotary evaporation, the thin yellow oil was brought up in 1:20 acetic acid:ethyl acetate and purified by flash column chromatography (1:20 acetic acid:ethyl acetate). Under the conditions used, a stiff gel formed in certain column fractions, resulting in some loss of product. Thus, the purified title compound was obtained in 31%

yield. ^{1}H NMR (CD_3CN) δ = 2.22 (m, 4H), 3.06 (ABM, *J* = 50.6, 14.1, 4.8 Hz, 2H), 4.64 (m, 1H), 4.94 (m, 2 H), 5.58 (d, *J* = 7.8 Hz, 4H), 5.75 (m, 1H), 6.87 (m, 2H), 7.25 (m, 4H), 7.58 (m, 2H), 8.09 (d, *J*= 7.5 Hz, 2H). ^{13}C NMR (CD_3CN) δ = 29.6, 35.1, 36.5, 67.0, 68.0, 114.9, 117.6, 119.8, 120.3, 124.8, 125.6, 126.4, 128.1, 128.7, 129.0, 130.0, 131.3, 133.4, 134.5, 135.0, 135.5, 147.2, 149.2, 149.3, 172.8, 172.9. ^{31}P NMR (CD_3CN) δ = -8.5. MS Calcd for $C_{28}H_{28}N_3O_{11}P$: 613.15 Found: (ESI$^+$) 614.4 $(M+H^+)^+$, 636.4 $(M+Na^+)^+$, 652.0 $(M+K^+)^+$. Found: (ESI$^-$) 612.0 $(M-H^+)^-$.

5.6.7 4PO-pTyr(ONb)$_2$ cyanomethyl ester 6

A dry flask was charged with 4PO-pTyr(ONb)$_2$ **5** (1 eq, 0.15 mmol), 1.5 mL dry DMF, and 1.5 mL chloroacetonitrile (160 eq, 24 mmol). Triethlyamine (3 eq, 0.45 mmol) was added and the reaction stirred 8.5 hr at room temperature. Progress was monitored by TLC (1:1 ethyl acetate:hexane, R_f = 0.1 and ethyl acetate, R_f = 0.7). The reaction was quenched with 50 mL ethyl acetate and washed twice (25 mL saturated $NaHCO_3$ and 25 mL saturated NaCl). The organic portion was dried over Na_2SO_4 and the solvent removed *in vacuo*. Flash column chromatography (1:1 ethyl acetate:hexane) gave the purified compound, typically in approximately 75% yield. ^{1}H NMR ($CDCl_3$) δ = 2.32 (m, 4H), 3.13 (d, *J* = 3.9 Hz, 2H), 4.75 (q, *J* = 35.7, 15.6 Hz, 2H), 5.01 (m, 2H), 5.63 (d, *J* = 6.0 Hz, 4H), 6.06 (d, *J* = 7.8 Hz, 1H), 7.18 (m, 2H), 7.53 (m, 2H), 7.66 (m, 4H), 8.14 (d, *J*= 7.8 Hz, 2H). ^{13}C NMR ($CDCl_3$) δ = 29.6, 35.6, 37.1, 49.3, 53.1, 67.1, 67.2, 116.1, 120.5, 120.6, 125.3, 128.7, 128.8, 129.4, 130.9, 132.7, 134.4, 136.8, 170.3, 172.3. ^{31}P NMR ($CDCl_3$) δ = -4.2. MS Calcd for $C_{30}H_{29}N_4O_{11}P$: 652.16. Found: (ESI$^+$) 653.2 $(M+H^+)^+$, 675.2 $(M+Na^+)^+$, 691.0 $(M+K^+)^+$. Found: (ESI$^-$) 516.0 $(M-Nb)^-$.

5.6.8 4PO-Serine 7

Serine (1 eq, 4.76 mmol) and Na_2CO_3 (1.4 eq, 6.66 mmol) were dissolved in 60 mL water, 30 mL hexane. A solution of 4-pentenoyl anhydride (1.4 eq, 6.66 mmol) in 15 mL dioxane was added in one portion. After 2 h at room temperature, TLC (10:10:1 ethyl acetate: hexanes: acetic acid, R_f = 0.7, developed with iodine and ninhydrin) revealed complete conversion of starting amino acid. Reaction was stopped by addition of 250 mL of 1 *M* $NaHSO_4$ and 250 mL CH_2Cl_2. After extraction with CH_2Cl_2 (3x, 100 mL each), the aqueous layer was treated with 350 mL saturated NaCl solution and extracted twice with 100 mL portions of THF. The organic layers were combined and dried over Na_2SO_4. Solvent was removed by rotary evaporation and the residue brought up in CH_2Cl_2. Flash column chromatography (CH_2Cl_2 followed by 1:1 CH_2Cl_2:ethyl acetate) was carried out, and the product isolated in nearly quantitative yield. ^{1}H NMR (CD_3CN) δ = 2.38 (m, 4H), 3.89 (ABM, *J* = 26.7, 11.4, 3.9 Hz, 2H), 4.56 (m, 1H), 5.03 (m, 2H), 5.83 (m, 1H), 7.51 (d, *J* = 7.8 Hz, 1H). ^{13}C NMR (CD_3CN) δ = 29.8, 35.2, 54.9, 62.1, 115.3, 137.2, 172.4, 174.8.

5.6.9 4PO-Serine cyanomethyl ester 8

4PO-Serine **7** (1 eq, 4.5 mmol) was dissolved in 25 mL dry DMF. To this solution were added 25 mL chloroacetonitrile (88 eq, 395 mmol) and triethylamine (3 eq, 13.5 mmol). Overnight stirring at room temperature gave complete reaction (TLC in ethyl acetate, R_f = 0.5). Volatiles were removed under vacuum and the residue was dissolved in ethyl acetate with a small amount of methanol. Flash column chromatography (ethyl acetate) gave the compound in 72% yield. ^{1}H NMR (CD_3CN) δ = 2.34 (m, 4H), 3.86 (dd, *J* = 23.4, 6 Hz, 2H), 4.56 (m, 1H), 4.84 (s, 2H), 5.05 (m, 2H), 5.86 (m, 1H), 7.25 (d, *J* = 7.8

Hz, 1H). ^{13}C NMR (CD_3CN) δ = 29.7, 35.2, 54.2, 61.9, 115.2, 117.9, 137.6, 169.9, 173.3.

5.6.10 4PO-pSer(ONb)$_2$ cyanomethyl ester 9

In a dry flask, 4PO-Ser cyanomethyl ester **8** (1 eq, 0.6 mmol) and tetrazole (1.8 eq, 1.08 mmol, used without purification) were combined. Dry acetonitrile (7 mL) and dry CH_2Cl_2 (30 mL) were added. After complete dissolution, the flask was cooled to 0 °C in a water/ice bath. A solution of bis(nitrobenzyl) phosphoramidite **1** (1.5 eq, 0.92 mmol, in 2 mL dry CH_2Cl_2) was added dropwise via syringe. The reaction was mixed for 5 min at 0 °C and then returned to room temperature. After 2.5 hrs at room temperature, TLC (1:1 ethyl acetate:hexane, R_f = 0.3) confirmed that the starting material had been completely converted. After an additional 2.5 hr, the oxidant mCPBA (1.1 eq, 0.66 mmol, in 5 ml dry CH_2Cl_2) was added and the reaction allowed to stir 15 min at room temperature. After TLC (1:1 ethyl acetate:hexane, R_f = 0.2), the reaction stirred another 15 min and was then quenched with 50 mL CH_2Cl_2 and washed twice (20 mL saturated $NaHCO_3$ and 20 mL brine). The aqueous phase was extracted with CH_2Cl_2 and the combined organic phases dried over Na_2SO_4. Rotary evaporation gave a yellow oil, which was purified by flash column chromatography (1:1 ethyl acetate:hexane, to ethyl acetate after fraction 12) in 27% yield. ^{1}H NMR ($CDCl_3$) δ = 2.35 (m, 4H), 3.99 (m, 2H), 4.47 (m, 1H), 4.81 (s, 2H), 5.51 (m, 4H), 5.79 (m, 1H), 7.06 (d, *J* = 7.5 Hz, 1H), 7.51 (m, 2H), 7.66 (m, 4H), 8.11 (d, *J* = 8,1 Hz, 2H). ^{13}C NMR ($CDCl_3$) δ = 29.5, 35.5, 67.1, 67.6, 67.7, 114.4, 115.9, 125.4, 129.0, 129.6, 129.9, 131.5, 134.4, 136.8, 147.0, 167.9, 172.9. ^{31}P NMR ($CDCl_3$) δ = -0.52.

5.6.11 4PO-Threonine 10

A reaction vessel was charged with threonine (1 eq, 4.20 mmol) and Na_2CO_3 (1.4 eq, 5.89 mmol) along with 60 mL water and 30 mL *p*-dioxane. A solution of 4-pentenoyl anhydride (1.4 eq, 5.89 mmol) was prepared in 15 mL dioxane and added to the reaction vessel in a single portion. TLC (10:10:1 ethyl acetate: hexanes: acetic acid, R_f = 0.7, developed with iodine and ninhydrin) subsequent to 2 h stirring at room temperature showed complete reaction. The reaction was quenched (250 mL of 1 *M* $NaHSO_4$ and 250 mL CH_2Cl_2) and extracted thrice with CH_2Cl_2 (100 mL portions). Considerable amounts of product remained in the aqueous layer, so it was treated with an equal volume of brine and extracted twice with THF (100 mL each time). The combined organic layers were dried over Na_2SO_4 and concentrated. A flash column was prepared in CH_2Cl_2 and the residue re-dissolved in CH_2Cl_2. Chromatography (CH_2Cl_2, followed by 1:1 CH_2Cl_2:ethyl acetate) produced purified 4PO-threonine in 99% yield. ^{1}H NMR (CD_3CN) δ = 1.17 (d, *J* = 6.3 Hz, 3H), 2.40 (m, 4H), 4.34 (d, *J* = 4.2 Hz, 1H), 4.51 (d, *J* = 6.3 Hz, 1H), 5.02 (m, 2H), 5.85 (m, 1H), 7.40 (d, *J* = 8.4 Hz, 1H). ^{13}C NMR (CD_3CN) δ = 19.7, 29.9, 35.2, 57.9, 67.7, 115.3, 137.3, 172.7, 174.6, 175.0.

5.6.12 4PO-Threonine cyanomethyl ester 11

To a flame-dried flask were added 4PO-threonine **10** (1 eq, 4.5 mmol), 25 mL dry DMF, and 25 mL chloroacetonitrile (88 eq, 395 mmol). After addition of triethylamine (3 eq, 13.5 mmol), the reaction was stirred overnight. TLC (ethyl acetate, R_f = 0.6) indicated complete reaction, and the solvent was removed under vacuum. The residual solids were brought up in a minimal volume of ethyl acetate with a few drops of methanol. Flash column chromatography (ethyl acetate) was performed and the compound isolated in

68% yield. ^{1}H NMR (CD_3CN) δ = 1.16 (d, *J* = 6.3 Hz, 3H), 2.36 (m, 4H), 4.29 (m, 2H), 4.52 (dd, *J* = 8.4, 3 Hz, 1H), 4.83 (s, 2H), 5.05 (m, 2H), 5.87 (m, 1H), 7.06 (d, *J* = 8.4 Hz, 1H). ^{13}C NMR (CD_3CN) δ = 20.4, 29.8, 35.4, 50.3, 57.4, 67.3, 115.4, 117.8, 170.1, 173.6.

5.6.13 4PO-pThr(ONb)$_2$ cyanomethyl ester 12

In a flame-dried flask, 4PO-Thr cyanomethyl ester **11** (1 eq, 0.77 mmol) was combined with sublimed tetrazole (1.8 eq, 1.38 mmol). By syringe, dry acetonitrile (10 mL) and dry CH_2Cl_2 (34 mL) were added. The flask was cooled to 0 °C in a water/ice bath after the solids had completely dissolved. A solution of bis(nitrobenzyl) phosphoramidite **1** (1.5 eq, 1.15 mmol, in 2 mL dry CH_2Cl_2) was slowly added. After 5 min at 0 °C, the reaction was allowed to warm to room temperature. After 2.5 hrs at room temperature, TLC (1:1 ethyl acetate:hexane, R_f = 0.3) suggested that reaction was complete. Tetrabutylammonium periodate (1.5 eq, 1.15 mmol, in 5 ml dry THF) was added and the reaction stirred 15 min at room temperature. TLC (1:1 ethyl acetate:hexane, R_f = 0.2) indicated complete conversion, and quenching with 60 mL CH_2Cl_2 was followed by washing the organic layer (40 mL saturated $NaHCO_3$ and 40 mL brine). The combined organic phases were dried over Na_2SO_4. White needles were observed upon standing. Rotary evaporation gave a yellow oil, which was purified by flash column chromatography (7:3 ethyl acetate:hexane) in 30% yield. ^{1}H NMR ($CDCl_3$) δ = 1.23 d, *J* = 6.3 Hz, 3H), 2.42 (m, 4H), 3.31 (m, 2H), 4.47 (m, 1H), 4.84 (s, 2H), 5.5 (m, 4H), 5.9 (m, 1H), 6.6 (d, *J* = 7.5 Hz, 1H), 7.6 (m, 2H), 7.8 (m, 4H), 8.2 (d, *J* = 8,1 Hz, 2H). ^{31}P NMR ($CDCl_3$) δ = -0.62. Found: (ESI$^+$) 590.8 $(M+H^+)^+$, 613.0 $(M+Na^+)^+$.

5.6.14 Nitrobenzyl phosphite 17

In a 500 mL RBF, 1 mL PCl_3 (1 eq, 11.4 mmol, Acros Organics) was added to 300 mL dry ether. The reaction was cooled to –78 °C and 4.95 mL triethylamine (3.1 eq, 35.5 mmol) were slowly added via syringe. A small amount of white vapor was produced during the addition. After approximately 5 min stirring, a solution of 5.26 g nitrobenzyl alcohol (3 eq, 34.3 mmol) in 60 mL dry ether were added over 10 min through an addition funnel. Reaction continued for 2 hr at –78 °C, after which the reaction was allowed to come to room temperature and stir overnight. The copious cream-colored precipitate was removed by filtration through a fine frit. The solid was washed with ether and the filtrate evaporated *in vacuo* to give 4.6 g yellowish solid with a single ^{31}P resonance (crude yield 82%). Crystallization from ether gave 2.1 g of pure nitrobenzyl phosphite as white crystals. Only one crop was collected, then the filtrate was rotovapped to recover the remaining crude product. ^{1}H NMR ($CDCl_3$) δ = 5.36 (d, J = 6.6 Hz, 6H),7.44 (t, J = 7.8 Hz, 3H), 7.63 (m, 3H), 7.77 (m, 3H), 8.06 (d, J = 8.4 Hz, 3H). ^{13}C NMR ($CDCl_3$) δ = 61.8, 124.5, 129.6, 133.2, 135.0, 146.8. ^{31}P NMR ($CDCl_3$) δ = 143.13. MS Calcd for $C_{21}H_{18}N_3O_9P$: 487.08. Found: (ESI$^+$) 488.2 $(M+H^+)^+$, 510.2 $(M+Na^+)^+$, 256.0 $(M+K^+)^+$.

5.6.15 Nitrobenzyl H-phosphonate 18

Similarly to the synthesis of nitrobenzyl phosphite **17**, 1 mL PCl_3 (1 eq, 11.4 mmol, Acros Organics) and 3.34 mL triethylamine (2.1 eq, 23.9 mmol) were combined in 300 mL dry ether at -78 °C. Nitrobenzyl alcohol was added (2 eq, 22.8 mmol, a solution of 3.50 g in 60 mL anhydrous ether) over 10 min via addition funnel. The reaction was stirred 2 hr at -78 °C and allowed to warm to room temperature overnight. After

approximately 12 hr total reaction time, 100 mL water were added and the reaction stirred 30 min at room temperature. The crystals were filtered out and washed with water. The filtrate was washed with 100 mL 5% K_2CO_3 and 100 mL water. After drying over Na_2SO_4, the ether was removed *in vacuo* to give 3.3 g orange solid (82% crude yield). Crystallization from ethanol gave 1.3 g pure nitrobenzyl *H*-phosphonate as off-white crystals. The remaining product was recovered from the mother liquor. ^{1}H NMR ($CDCl_3$) δ = 5.53 (sept, $J \approx 6.2$ Hz, 4H),7.14 (d, $J_{P\text{-}H} = 716.7$ Hz, 1H), 7.75 (m, 2H), 7.68 (m, 4H), 8.09 (d, $J = 7.2$ Hz 2H). ^{13}C NMR ($CDCl_3$) δ = 64.5, 126.0, 128.0, 128.6, 129.6, 130.1, 132.2, 135.3, 146.8. ^{31}P NMR ($CDCl_3$) δ = 9.8 (m). [H-coupled]: 9.3 (dquint, $J_{P\text{-}H}$ = 711 Hz, $J_{\text{-}CH\text{-}O\text{-}P} = 8.3$ Hz).

5.6.16 4PO-Tyrosine 19

Tyrosine (1 eq, 4.14 mmol) and sodium carbonate (1.4 eq, 5.7 mmol) were weighed into a 250 mL round-bottom flask equipped with a stirbar. A total of 60 mL water and 60 mL dioxane were added, until both solids were nearly completely dissolved. 4-Pentenoic anhydride (1.4 eq, 5.7 mmol) was added in a single portion and the reaction stirred at room temperature. Progress was monitored by TLC (1:20 acetic acid:ethyl acetate, R_f = 0.5, developed with iodine and ninhydrin). After 4 h, 10 mL of 1 *N* HCl were added (resulting in a pH of approximately 4.5) and the reaction stirred 15 min to hydrolyze any pentenoyl ester formed from side chain reactivity. The reaction was quenched with 100 mL of 1 *M* $NaHSO_4$ and 200 mL CH_2Cl_2. The organic layer was removed, and the aqueous layer was treated with 200 mL saturated NaCl and extracted thrice with 50 mL portions of THF. The combined organic layers were dried over Na_2SO_4 and solvent evaporated *in vacuo*. The crude mixture was re-dissolved in a minimal volume of ethyl

acetate with a small amount of methanol and loaded on a silica gel column. Flash column chromatography (1:20 acetic acid:ethyl acetate) gave purified product in 75% yield. ^{1}H NMR (CD_3CN) δ = 2.25 (m, 4H), 2.99 (ABM, *J* = 30.9, 14.1, 5.7 Hz, 2H), 4.62 (m, 1H), 4.96 (m, 2H), 5.7 (m, 1H), 6.75 (d, *J* = 6.6 Hz, 2H), 6.93 (d, *J* = 8.1 Hz, 1H), 7.06 (d, *J* = 4.5 Hz, 2H).

5.6.17 4PO-Tyrosine cyanomethyl ester 20

A solution of 4PO-tyrosine **19** (1 eq, 0.44 mmol) was prepared in 2 mL dry DMF and 2 mL chloroacetonitrile (107 eq, 31.6 mmol). Triethylamine (3 eq, 1.32 mmol) was added and the reaction stirred 6 h at room temperature. TLC (ethyl acetate, R_f = 0.5) showed the reaction to be complete. Solvent was removed under high vacuum and the residue dissolved in 10:1 CH_2Cl_2:ethyl acetate with a few drops of methanol. Flash column chromatography (ethyl acetate) produced the title compound in 56% yield. ^{1}H NMR (CD_3CN) δ = 2.24 (m, 4H), 2.99 (ABM, *J* = 30.9, 14.1, 5.7 Hz, 2H), 4.64 (m, 1H), 4.77 (s, 2H), 5.00 (m, 2H), 5.8 (m, 1H), 6.74 (d, *J* = 4.8 Hz, 2H), 6.85 (d, *J* = 7.5 Hz, 1H), 7.06 (d, *J* = 8.7 Hz, 2H). MS Calcd for $C_{16}H_{18}N_2O_4$: 302.13. Found (ESI$^-$): 301.2 $(M\text{-}H^+)^-$, 337.0 $(M+Cl)^-$.

5.6.18 dCA-4PO-pTyr(ONb)$_2$ 21

4PO-pTyr(ONb)$_2$ cyanomethyl ester **6** (1 eq, 0.05 mmol) was dissolved in 0.8 mL dry DMF in a flame-dried 5 mL conical flask fitted with a stirbar. The dinucleotide dCA (10 mg) was added in one portion, and the reaction was allowed to continue 3 h at room temperature. The reaction was monitored by analytical HPLC (gradient: 25 mM ammonium acetate, pH 4.5 to CH_3CN). The addition of tetrabutylammonium acetate was

necessary to promote reaction. Upon completion of the reaction, the pure compound was obtained by preparative HPLC (gradient: 25 mM ammonium acetate, pH 4.5 to CH_3CN). MS (ESI) Calcd for $C_{47}H_{52}N_{11}O_{23}P_{33}$: 1231.25. Found ($ESI^-$): 546.0 $(M\text{-}Nb)^{2-}$, 806.2 (M-Nb-pdC)$^-$. Found ($MALDI^-$): 1265.1 $(M+Cl^-)^-$. Found ($MALDI^+$): 1097.2 $(M\text{-}Nb)^+$, 962.2 $(M\text{-}2Nb)^+$.

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