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Facile Reduction in the Synthesis of Phosphorylcholine Affinity Columns

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Abstract: Reduction of p-nitrophenylphosphorylcholine to the amine utilizing ammonium formate and 10% Pd/C in methanol, followed by *in situ* generation of the diazonium salt resulted in the synthesis of *p* -diazophenylphosphorylcholine. This reaction was simpler than the usual approach utilizing hydrogen gas for the reduction to *p*-aminophenylphosphorylcholine. The diazonium salt then successfully reacted with the amino acid tyrosine, which had been previously attached to an agarose bead. Alternatively the diazonium salt was coupled to BSA and the resulting conjugate used to coat ELISA plates. Selective PC-binding antibodies recognized the antigen, and bound specifically to both the column and plates.
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With the goal of facilitating the preparation of affinity columns **1** and enzyme-linked immunosorbent assay (ELISA)[1] plates, this paper reports the fast and convenient reduction of *p* -nitrophenylphosphorylcholine **4** to *p* -aminophenylphosphorylcholine **5**, catalyzed by palladium using ammonium formate in methanol. The resulting amino compound **5** was treated *in situ* with sodium nitrite in hydrochloric acid to give the desired diazonium salt **6** used in the preparation of affinity columns and protein conjugates. Phosphorylcholine (PC, **10**), an important component of cell membranes, has been used as a test antigen to screen antibodies secreted by murine myelomas[2,3]. Affinity columns provided a convenient way to select active antigen-binding recombinants during studies on the solid-phase synthesis of antibodies.[4] The PC antigen has been successfully linked to a solid support in an orientation that does not interfere with antibody binding (**1**, Figure **1**) by controlling the chemistry of the attachment reaction to a spacer molecule linked to an agarose bead.

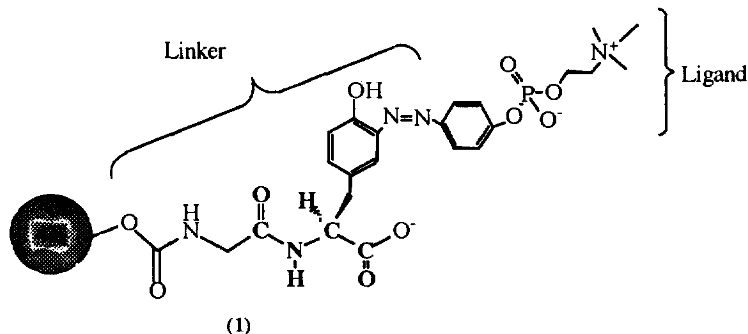
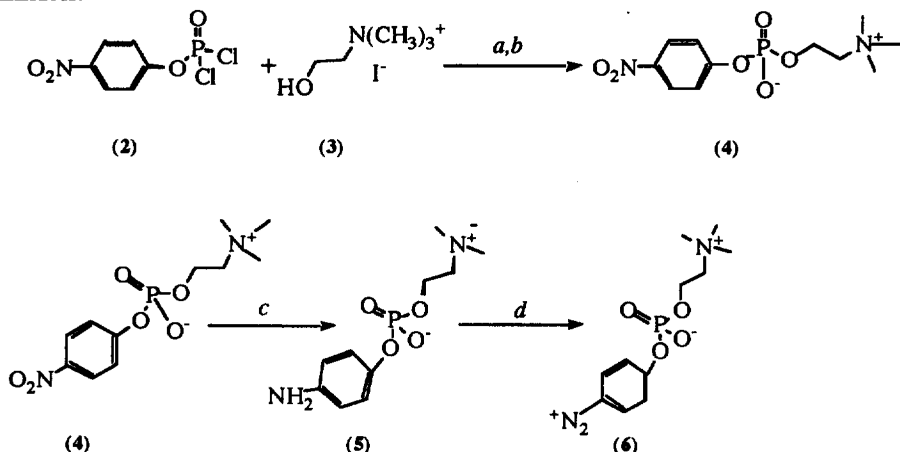


Fig. 1. Synthetic target **1** showing solid support (Sepharose® 4B beads), linker and ligand.

Chesebro and Metzger's original synthesis of p-diazophenylphosphorylcholine **6** (DPPC) utilized atmospheric hydrogenation with hydrogen gas for one h at room temperature in the presence of 10% Pd/C.[5] Herein, we report that the preparation of these columns is considerably faster and easier using ammonium

formate and the product columns give the same separation and exhibit identical antibody selectivity as those generated by the previous method.

Ammonium formate has gained attention as a selective, mild and effective reducing agent for the catalytic hydrogenation of azides, nitro groups and nitriles (reviewed by Ram).[6] Ammonium formate has also been successfully used in the dehydrohalogenation of aromatic chlorocarbons and for the selective removal of the carbobenzyloxy group from protected peptides.[7,8] In addition to the nitro group, by increasing the amount of ammonium formate added and the reaction time, heterocyclic rings were also reduced in 5-nitro-6-methoxy-4-methylquinoline.[9] This simple method for preparation of an activated phosphorylcholine ligand was useful both for preparation of affinity columns and also for preparation of the PC-bovine serum albumin (BSA) conjugates used in ELISAs.

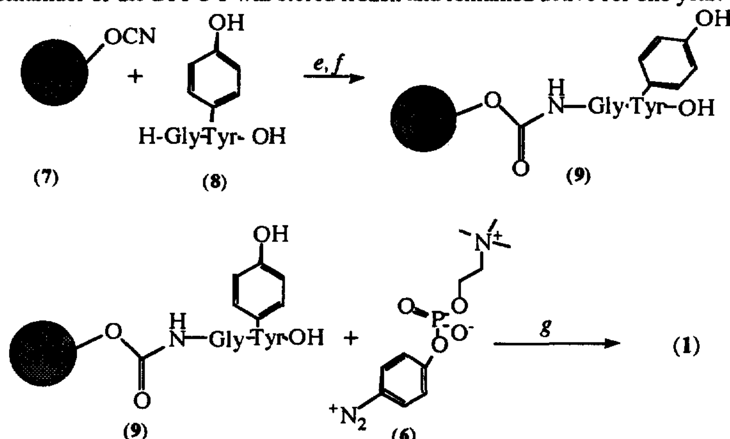


Scheme 1. *a*. Quinoline, CH_3CN 0°. *b*. Pyridine, H_2O, Amberlite® MB-3 resin (IWT®TMD-8). *c*. HCO_2NH_4, 10% Pd/C, CH_3OH. *d*. $NaNO_2$, 0.1 N HCl, 0°.

The availability of the key intermediate, DPPC **6** (Scheme 1), allowed convergent synthesis of the desired affinity column following the reaction sequence in Scheme **2**. DPPC **6** was prepared from choline and 4-nitrophenylphosphorodichloridate. Choline iodide **3** reacted with 4-nitrophenylphosphorodichloridate **2** in the presence of quinoline (0.1 equivalents) in acetonitrile at 0° for 5 h, followed by quenching with pyridine-water in the presence of IWT®-TMD-8 ion exchange resin (added until no further color change was observed). The product mixture was filtered and the filtrate dried to isolate the desired nitrophenyl phosphate ester **4**.[10] Nitro compound **4** was then reduced to amine **5** using the title reaction, with ammonium formate as a hydrogen donor.

In a typical experiment: to a suspension of p-nitrophenylphosphorylcholine **4** (1.76 mmol) in dry methanol, 10%Pd/C (0.15 g) was added under nitrogen, followed by addition of ammonium formate (8.74 mmol) with stirring. Gas evolved and quickly subsided in one min. A silica gel TLC plate eluted with isopropanol : ammonium hydroxide : water (7:2:1 v/v/v) showed the reduction to be essentially complete (R_f = 0.45). After filtration through celite to remove the catalyst, the solvent was removed by evaporation, resulting in a yellow oil. The amine **5** was analyzed by NMR in CD_3OD (which showed an upfield shift of the aromatic region protons from δ= 8.28, 7.36 to 7.0, 6.8). The crude oil **5** was dissolved in ice cold hydrochloric acid (0.1 N, 10 mL, 0°) for diazotization. With stirring at room temperature, small amounts of sodium nitrite were added behind an explosion shield until starch-KI paper showed >50 mg/L excess nitrite (the nitrite was allowed to react for a few min before testing). Aliquots of this solution were immediately coupled

(17.5 mL gel **7** : 0.06 mmol Gly-Tyr **8** : 0.18 mmol DPPC **6**) to the glycyl-tyrosine linkers(Sigma) attached to Sepharose® 4B beads (Pharmacia) that had been prepared according to scheme 2. After gently shaking overnight at room temperature the resin was extensively washed with 10% sodium hydroxide and high-salt borate buffer before use. The remainder of the DPPC **6** was stored frozen and remained active for one year.



Scheme **2**. *e*. Coupling buffer 0.1 M $NaHCO_3$ / 0.5 M NaCl pH 8-9. *f*. 1 M Ethanolamine in coupling buffer for capping. *g*. BBS.

PC-binding IgA myeloma antibody McPC603[11] bound specifically to these columns in borate buffer (0.2 M boric acid, 0.16 M NaCl, pH 8.18 with NaOH (1.6 g/L, leading to a total $Na^{+}{}_{(aq)}$ concentration of 0.2 M, Figure 2). Serum proteins did not bind to the column. McPC603 was specifically eluted with borate containing PC (**1 0**, 10^{-3} M Calcium PC chloride, pH 8.18, Figure 2) after extensive washing with the high salt buffer to demonstrate the specificity of binding. The columns were then used to study synthetic and native recombinant antibodies as well as murine antibodies.

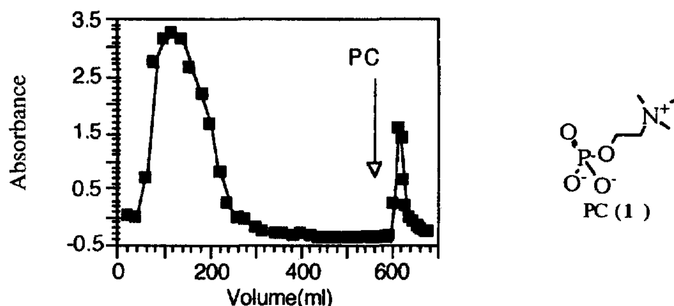


Fig. 2. Elution profile of a representative affinity column run monitored at 220 nm.

The diazonium salt was also coupled to BSA and the PC-BSA conjugate was used to coat ELISA plates for the titration of anti-PC antibodies. In a typical experiment BSA (high purity fraction V, 600 mg) was dissolved in BBS (20 mL) overnight at 4°C. Insoluble components were removed by centrifugation at 1000 RPM for 10 min. The supernatant was stirred on ice and DPPC **6** (0.18 mmol, 1 mL aliquot in 0.1 N HCl prepared as above) was added dropwise over 30 min. The solution was mixed at 4°C overnight and then dialyzed extensively against 25 mM tris, 0.15 M NaCl, pH 8. Filter sterilization (0.45 µm) was performed on the conjugates before use and samples were frozen at -70° for later use. The ELISA plates were coated with conjugate solution

overnight at 4°C (0.1 mL/well of the appropriate dil. of the PC-BSA in BBS) in a humidified atmosphere.[12] The plates were washed with BBS (3X), unreacted sites coated with 1% BSA in BBS for 1 h at RT, then washed again with BBS (3X) before treating with a PC-specific antibody (McPC603, 0.3 mg/mL, off affinity column, dil. x 1:1000 in BBS) for 4 h at RT. After washing with BBS (3X) once more, final visualization utilized an alkaline phosphatase-rat monoclonal to mouse kappa conjugate (1:500 in BBS, Zymed, So. San Francisco), with p-nitrophenyl phosphate as the substrate. All dilutions of PC-BSA (from 1 to 1:10,000) used in coating the plates gave average absorbances of 0.3 at 405 nm. The specificity of the interaction was confirmed by a measured absorbance of 0.006 for unrelated rat antibodies and 0.095 for purified kappa light chains from McPC603 (which should not bind to PC).

This paper described the extremely fast and effective reduction of p-nitrophenylphosphorylcholine with ammonium formate and 10% palladium on carbon in methanol applied to the total synthesis of an affinity column. Diazotization of the amine to generate DPPC **6** followed by reaction with Sepharose® 4B-glycyl-tyrosine produced effective affinity columns for our studies. Typically 1 mg of antibody could be bound per mL of swollen Sepharose® after this reaction.[13] DPPC **6** was also coupled with BSA to generate conjugates for ELISA plate binding studies. This PC-type of ligand attached to a solid support via the phosphate end of the molecule effectively acts as an antigen mimic because the trimethylammonium portion of the native antigen is buried in the antibody binding pocket. This convenient synthesis of the versatile reactive intermediate, DPPC **6**, makes PC **10** more accessible for immunological studies and provides an example of the use of a diazonium salt to add functionality to a solid support.

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REFERENCES AND NOTES

1. Abbreviations used in this manuscript: ELISA, enzyme-linked immunosorbent assay; PC, phosphorylcholine; DPPC, 4-diazophenylphosphorylcholine; BSA, bovine serum albumin; IgA, immunoglobulin A (alpha heavy chain); BBS, borate-buffered saline; TLC, thin-layer chromatography.
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10. Purity >90%, 6.81 min by RP-HPLC(220 nm) on Rainin C-18, 3 μm particles, 4.6 mm ID x 5 cm L column, isocratic 60% acetonitrile/water, 1 mL/min. UV absorbance λ_{max} at 285 nm in 0.01 M phosphate buffer pH=7. R_f = 0.78 (Starting material R_f = 0.28) in isopropanol/NH_4OH/H_2O (7:2:1) on silica gel, UV visualization. MP 239.8-240.4°. ^{1}H NMR (360 MHz, D_2O/DSS) δ 8.28 (d, 2H), 7.36 (d, 2H), 4.76 (HOD), 4.45 (m, 2H), 3.70 (m, 2H), 3.20 (s, 9H).
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12. The ELISA procedure used was recommended by the Scharff laboratory.
13. The maximum binding capacity was never reached in our samples as evidenced by the lack of PC-binding activity in the protein which flowed through the column.

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