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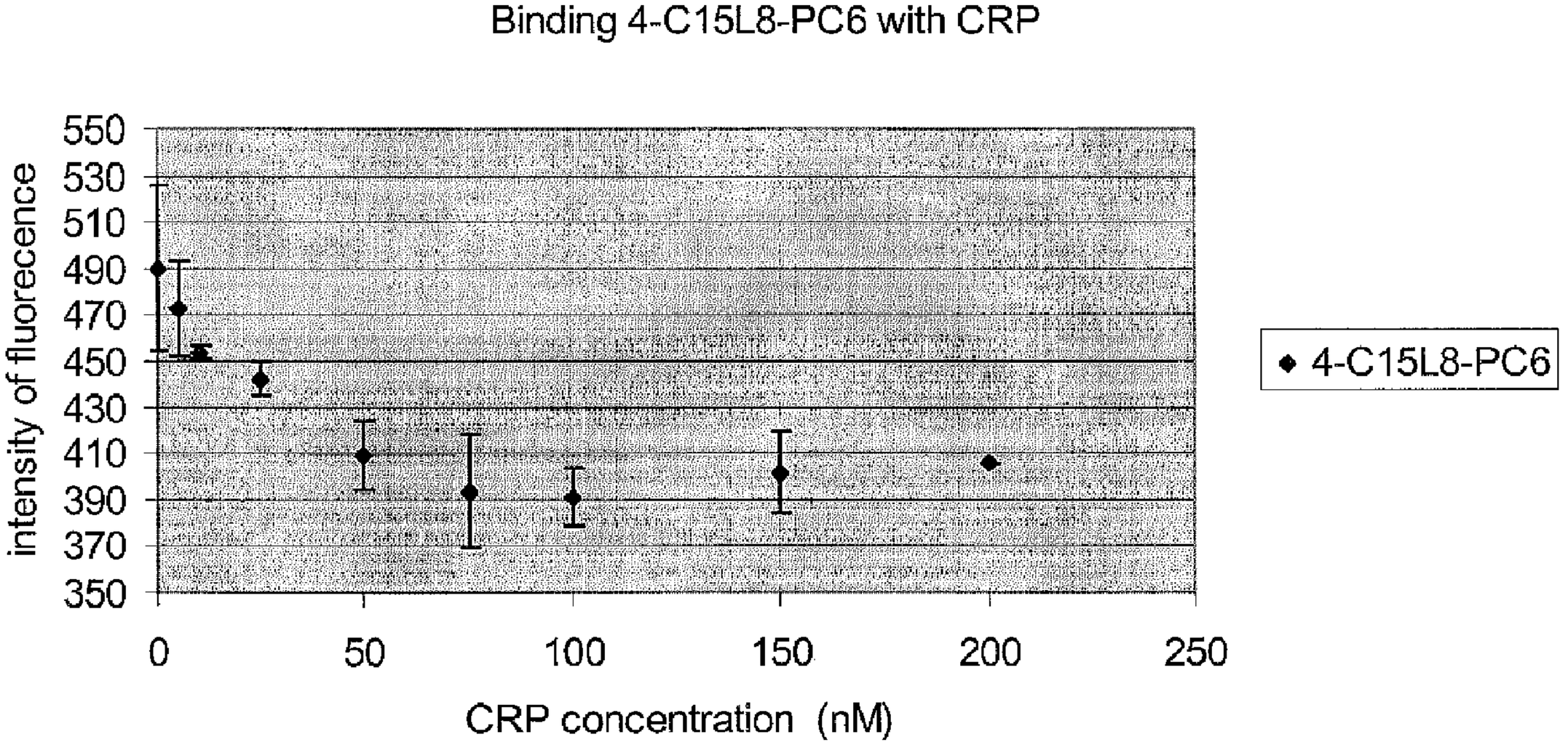
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(54) Title: BINDER FOR C-REACTIVE PROTEIN



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The invention relates to a polypeptide dimer, wherein both protomers have a sequence according to SEQ ID NO: 1, wherein at least one phosphocholine derivative is attached to said polypeptide. Said polypeptide shows a specific binding for C-reactive protein (CRP). The invention further relates to use of the polypeptide in assays for determineing the concentration of CRP, purification of CRP and compositions comprising CRP.

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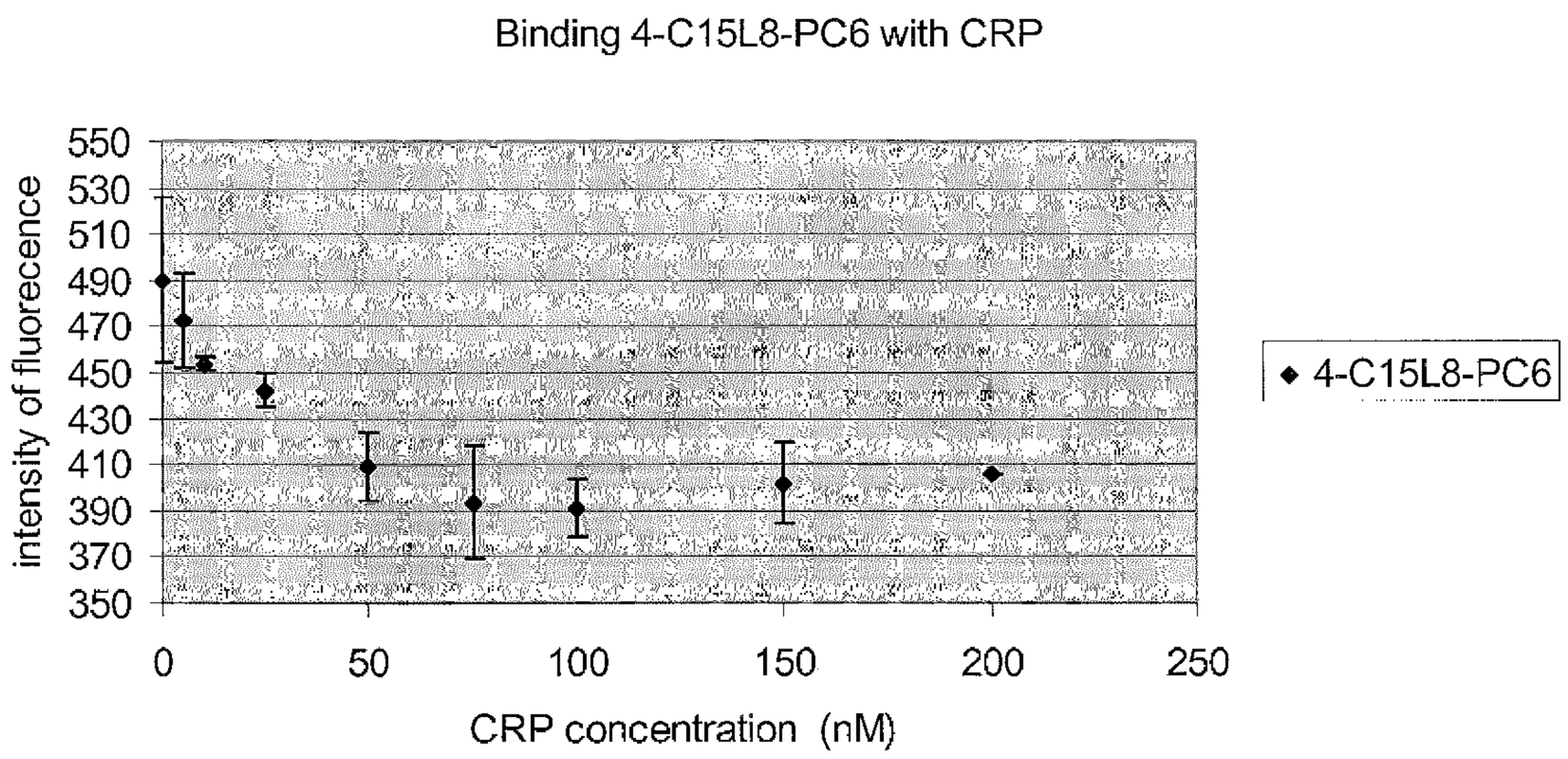
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(54) Title: BINDER FOR C-REACTIVE PROTEIN



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CECI EST LE TOME 1 DE 2
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THIS IS VOLUME 1 OF 2
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BINDER FOR C-REACTIVE PROTEIN

Field of the invention

The present invention relates to the field of molecules binding specifically to a certain protein, in this case C-reactive protein, and uses thereof.

Background of the invention

The *de novo* design of folded polypeptides aims at improving our understanding of protein structure, and also provides a platform for the engineering of new proteins with tailored functions. Designed, folded polypeptides that undergo pH-controlled, site-selective self-functionalization with ligands constitute an excellent toolbox for the construction of various complex molecular systems, e.g. model glycoproteins or complex receptors.

The object of international patent publication WO03/080653 was to provide folded, ligand modified helix-loop-helix polypeptide scaffolds that connect the key biosensing events of recognition and reporting. The well characterized interaction between the enzyme human carbonic anhydrase II, HCAII, and its inhibitor 4- carboxybenzenesulfonamide was selected for a proof of principle demonstration.

C-reactive protein is a plasma protein that circulates in the blood stream in increased amounts during inflammation and after tissue damage. CRP-levels are measured for assessment of i.a. inflammation and increased risk of cardiovascular disease. It is also of interest to measure CRP-levels during heart surgery.

Summary of the invention

The present invention relates to polypeptide scaffolds further modified to specifically bind to C-Reactive Protein (CRP). Such modified polypeptide scaffolds are hereinafter referred to as “CRP-specific binders”, “CRP-binders” or simply “binders”, which terms are used interchangeably unless otherwise indicated.

In a first aspect, the invention relates to CRP-specific binders comprising a polypeptide having a sequence according to SEQ ID NO: 1, wherein at least one phosphocholine derivative is attached to said polypeptide and to a polypeptide scaffold consisting of a four-helix bundle formed of dimers of polypeptides having the sequence according to SEQ ID NO: 1, which binders are modified by incorporation of a binding moiety comprising a phosphocholine derivative and optionally a reporter group that can give a detectable signal. The two protomers of the dimer may be covalently attached to each other through, for example, a disulfide bond, but may also be non-covalently bound to each other. The individual protomers may also be used as binders.

The invention also relates to a method for producing the polypeptide according to the first embodiment, said method comprising the steps

- synthesizing a polypeptide having the sequence according to SEQ ID NO: 1;
- bringing a phosphocholine derivative in contact with an unblocked lysine of said polypeptide under conditions suitable for the attachment of the phosphocholine derivative to the lysine;
- optionally bringing a reporter group in contact with an unblocked lysine of said polypeptide under conditions suitable for the attachment of the reporter group to the lysine.

In a further aspect the invention relates to the use of binders according to the first aspect in therapeutic and diagnostic applications, such as the incorporation of such binders in pharmaceutical or diagnostic compositions, assays for determining the concentration of CRP in a sample, e.g. from a patient undergoing heart surgery or suspected of having an inflammation, and in biotechnological applications, such as protein purification or binding assays.

Brief description of the figures

Figure 1 is an overview of a library of peptide scaffolds used in the present invention.

Figure 2 shows the affinity of a CRP-binder of the present invention to CRP.

Figure 3 is a schematic view of a CRP detection assay using the CRP-binder of the present invention.

Figure 4 shows the activity of native pyruvate kinase, pyruvate kinase bound to a linker, and pyruvate kinase bound to a CRP-binder, respectively.

Figure 5 is a comparison of signals generated by kinase-bound CRP-binder of the present invention and kinase-bound CRP-specific antibody, respectively.

Figure 6 is a comparison of signals generated by a kinase-bound CRP-binder of the present invention used for detection of CRP from human, horse and dog.

Detailed description of the invention

It has been surprisingly shown by the present inventors that the binding affinity between CRP and a polypeptide scaffold may be dramatically enhanced by attaching a phosphocholine derivative in certain positions. Phosphocholine alone has a binding affinity of ~1 μM and the scaffold polypeptides have a binding affinity of ~100-1000 μM, while the polypeptides modified with a phosphocholine derivative have a binding affinity for CRP in the nanomolar range.

The unmodified scaffold binds to the surface of CRP and the phosphocholine derivative should be introduced in a position within, or in close proximity of, the binding site on the polypeptide scaffold. In order to reach its binding site on CRP, the phosphocholine derivative may comprise a spacer of 1-12 carbon atoms, i.e. 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 or 12 carbon atoms.

A further surprising advantage of the binders of the present invention is that they bind with high affinity to CRP from different mammals, in contrast to CRP-

specific antibodies that usually bind with high affinity to CRP from one mammalian species but with lower affinity to CRP from other species.

The polypeptide scaffold may also be varied with regard to charge in order to optimize binding affinity to CRP. This is preferably done by exchanging a charged amino acid residue for an uncharged residue, or a residue with different charge, in the polypeptide, or vice versa. For example, a Val or Ala (uncharged) can be exchanged for a Glu (charge -1) or an Arg (charge +1).

According to one embodiment of the present invention, the polypeptide scaffold has at least two lysine residues to which a CRP-binding moiety and a reporter group, respectively, may be attached.

The basic polypeptide scaffold protomer has the sequence according to SEQ ID NO: 1. This protomer can be produced by any method known to the person skilled in the art, such as by recombinant technology or conventional automated solid phase peptide synthesis. One or more positions should carry a lysine. These positions are preferably selected from positions 8, 10, 15, 17, 22, 25, 34 and 37. The CRP-binding moiety and the optional reporter group are then attached to the lysines according to the method discussed below.

The reporter group may be any group that can be attached to a lysine residue and give a detectable signal. Preferred examples of reporter groups are fluorescent probes such as dansyl, coumarin, fluorescein, rhodamine and Oregon Green derivatives. The reporter group may also be an enzyme such as phosphoenolpyruvate kinase or a detectable particle such as a gold nanoparticle. The reporter group is attached to the lysine residue according to the supplier's instructions or by other conventional methods. Preferably, the reporter group is attached to the protomer while the newly synthesized protomer is still on the resin.

The CRP-binding moiety is a phosphocholine derivative comprising a phosphocholine moiety and a spacer. The length of the spacer depends on

where the ligand is attached to the scaffold and where the ligand binds to CRP. The spacer is usually an aliphatic chain of 1-12 carbon atoms optionally substituted with hydrophilic groups to enhance solubility.

The phosphocholine derivative is attached to the protomer by bringing the protomer into contact with an active ester of the phosphocholine derivative. The active ester has the general formula

$$OP(OH)_2O(CH_2)_2N^+(CH_3)_2(CH_2)_nCOOR^1 \qquad (I)$$

wherein $1 \leq n \leq 12$ and R^1 is a leaving group with a pK_a of about 6-8. The synthesis of active esters wherein n is 4, 6 or 11 and R^1 is p-nitro-phenyl are described in the experimental section below.

The phosphocholine derivative is incorporated into the polypeptide scaffold as disclosed in the experimental section.

The CRP-binder of the present invention may also be bound to a solid support, optionally through a linker. A number of solid supports, such as chips, plates, beads and membranes, and linkers are commercially available and may be combined by the skilled person depending on the circumstances the CRP-binder of the invention is to be used under.

Binding assays

One aspect of this invention relates to binding assays using the binders of the present invention, and, more particularly, to a method for eliminating interference resulting from rheumatoid factors or human anti-mouse antibodies (HAMA) in biological samples.

Different types of sandwich assays are widely used. One of the most common is sandwich ELISA where an antibody is used to capture the antigen and another labelled antibody is used to detect the bound antigen. Both of these antibodies

are usually of mammalian origin. In such assays anti-mammalian IgG antibodies present in the samples, which often are derived from body fluids such as blood or serum, may simulate the behaviour of the antigen by linking the detection antibody to the capture antibody, thus causing false positive reactions. Such false positive reactions are observed in sandwich assays based on both polyclonal and monoclonal antibodies.

An increasing number of patients are given mouse monoclonal antibodies for diagnosis or therapy. Monoclonal antibody administration often induces an antibody response in the patients[12]; human anti-murine antibodies (HAMA) are produced. Sandwich-type assays based on mouse monoclonal antibodies are known to give false positive results when HAMA is present in the serum of the patient. This will be an increasing problem in the clinical chemistry laboratory as the number of patients treated with monoclonal antibodies increases.

Rheumatoid factor (RF) is also a major source of interference in many immunoassays. RF is an autoantibody that reacts with the Fc portion of mammalian IgG. RF can be of the IgA, IgG, or IgM classes. Between 60% and 80% of patients with Rheumatoid Arthritis (RA) have RF activity in their serum. RF has also been found in serum from patients with other connective tissue diseases and in many other diseases. Although RF reacts with the Fc-part of IgG, they display heterogeneity in their reactivity to IgG of different species. This reactivity to IgG of other species may produce false reactions in assays involving antibodies. The classical tests for RF are agglutination tests, in which the RF reacts with two (or more if it is an IgM-RF) different mammalian IgG molecules, thus causing agglutination. The same reaction will give a false-positive reaction in a sandwich ELISA, owing to the binding of detection antibody to capture antibody in the absence of the antigen that the assay was designed to detect.

One approach to the above problem is the use of Fab fragments in the assay, because the anti-IgG antibodies are usually directed against the Fc part of the IgG molecule. However, the digestion of IgG to produce pure Fab fragments is time consuming and usually results in loss of antibody titre and will not reduce

the interference caused by anti-Fab antibodies. Furthermore, addition of mouse IgG or mouse serum or chromatography to remove the interfering antibodies have been used to avoid the above mentioned problem. Addition of normal mouse IgG or normal mouse serum sometimes fails to eliminate the false positive reaction. Removal of interfering antibodies by chromatography is time consuming and less suitable for routine analysis.

One aspect of the present invention is therefore to change at least one of the antibodies in the assay to a binder according to the invention that is not bound to by HAMA or RF.

In one embodiment of this aspect, the invention relates to a method for assaying the concentration of C-reactive protein in a sample, comprising the steps

- bringing the sample in contact with a first polypeptide dimer according to any of claims 1-6, said first polypeptide being bound to a solid support;
- bringing the sample in contact with a second polypeptide dimer according to any of claims 3-6 having a reporter group attached to it; and
- detecting the presence or absence of the reporter group on said second polypeptide dimer.

Experimental section

Synthesis of Phosphocholine derivatives PC6, PC11 and PC4

The phosphocholine derivative to be attached to the polypeptide scaffold may have a spacer of 1-12 carbon atoms, as explained above. These are denoted PC, for phosphocholine, followed by a number giving the length of the spacer, i.e PC6 has a spacer of 6 carbon atoms.

For the synthesis of PC6 we originally chose a simple and short route as outlined in Scheme 1. In the first step 6-dimethylamino hexanoic acid was prepared by alkylation of the corresponding amino acid with formaldehyde under catalytic hydrogenation conditions in good yield. The resulting tertiary amine was simply purified with a strong acidic cation exchanger.[3] The carboxylic acid function was then esterified to form the methyl ester in essentially quantitative yield and no purification step was required. The subsequent alkylation with the bromide, which was easily prepared from diethyl chlorophosphate and 2-bromo ethanol,[4] proceeded usually with only moderate yields. After RP HPLC purification the methyl ester was cleaved with Lithium hydroxide and the carboxylic acid was obtained in almost quantitative yield and purification was not necessary. The generation of the active ester was done following a procedure of Kim et al. utilizing p-nitrophenyl chlorofomate, a base and DMAP.[5] The active ester was obtained after RP HPLC purification in 28% yield only. The final deprotection of the phosphate group was done under mild conditions with TMSBr without affecting the carboxylic ester.[6] On the other hand, the formation of side products for some substrates during this reaction is a known disadvantage of this procedure.[7] However, we obtained the desired phosphate in 25% yield besides the mono-deprotected phosphate and the quaternary amine without phosphate group.

For the preparation of PC11 we chose a synthetic route including some steps on solid phase in order to avoid cumbersome purification procedures and solubility problems (Scheme 2). Fmoc-protected undecanoic acid was therefore coupled to Wang resin utilizing the DIC method in 66% yield.[8] After deprotection with piperidine the dimethylation was accomplished with formic acid and sodium borohydride.[9] The alkylation step to the quaternary amine was a sluggish reaction and needed 10 equivalents of the bromide and heating for 4 days.[10] After cleavage from the resin the crude product was transformed to the active ester by the same method that we used for PC6. The final deprotection step of the phosphate group proceeded similarly to PC6 with formation of byproducts, but 27% of the desired product were obtained.

Scheme 1

PC6-pNitrophenylester Synthesis

Phosphoric acid 2-bromo-ethyl ester diethyl ester. A solution of 2-bromo ethanol (1.42 mL, 20 mmol) and dry pyridine (3.23 mL, 40 mmol) in dry dichloromethane (25 mL) was cooled to 0°C. Diethyl chlorophosphate (3.36 mL, 23 mmol) was added dropwise and the reaction mixture was stirred at room temperature for 24 h. Diethyl ether (40 mL) and 1 N HCl (40 mL) were added and the organic layer was separated and washed consecutively with 1 N HCl and saturated NaHCO3 and dried over MgSO4. After evaporation of the solvents the residue was purified by column chromatography (silica gel, ethyl acetate/pentane 1:1) to give the product (4.85g, 18.6 mmol, 93%) as light yellowish oil. ^{1}H NMR ($CDCl_3$, 400 MHz) δ = 1.34 (dt, 6H, 2 x CH_3, ^{3}J(H,H) = 7.2 Hz, ^{4}J(H,P) = 1.2 Hz); 3.52 (t, 2H, CH_2Br, ^{3}J = 6 Hz); 4.13 (dq, 4H, 2 x CH_2CH_3, ^{3}J(H,H) = 7.2 Hz, ^{3}J(H,P) = 7.2 Hz); 4.28 (dt, 4H, 2 x OCH_2CH_2Br, ^{3}J(H,H) = 6.4 Hz, ^{3}J(H,P) = 8.4 Hz). ^{13}C NMR ($CDCl_3$, 100.5 MHz) δ = 16.0 (d, 2 x CH_3, ^{3}J = 6.8 Hz); 29.4 (d, CH_2Br, ^{2}J = 7.6 Hz); 64.0 (d, OCH_2, ^{2}J = 5.3 Hz); 66.5 (d, OCH_2, ^{2}J = 5.3 Hz).

6-dimethylamino hexanoic acid. A solution of the amino acid (1.32 g, 10 mmol) in water was hydrogenated in a Parr apparatus in the presence of an excess of formaldehyde (4 mL) and 10% Pd/C (0.5g) at 90 psi of H2 at room temperature for 36h. The catalyst was removed by filtration and was washed twice with hot water. The combined aqueous layer was passed through a DOWEX 50WX2-100 ion exchanger column. After washing with water the column was eluted with 2% of aqueous NH4OH to give the pure product (1.39 g, 8.7 mmol, 87%) as colorless crystals.

6-dimethylamino hexanoic acid methyl ester. To an ice-cold solution of 6-dimethylamino hexanoic acid (1.37 g, 8.6 mmol) in dry methanol (50 mL) was added thionylchloride (9 mL) dropwise over 30 min. The reaction mixture was warmed to room temperature and was stirred over night. The solvent was evaporated under reduced pressure. Repeated addition of methanol and

subsequent evaporation gave the methyl ester in almost quantitative yield in pure form as colorless solid. ^{1}H NMR (DMSO d_6, 400 MHz) δ = 1.27 (m, 2H, C*H*$_2$); 1.53 (m, 2H, C*H*$_2$); 1.63 (m, 2H, C*H*$_2$); 2.31 (t, 2H, ^{3}J = 7.6 Hz, C*H*$_2$); 2.67 (s, 6H, N(C*H*$_3$)$_2$); 2.96 (t, 2H, ^{3}J = 8 Hz, C*H*$_2$); 3.57 (s, 3H, COOC*H*$_3$). ^{13}C NMR (DMSO d_6, 100.5 MHz) δ = 23.1; 23.8; 25.3; 32.9; 41.7; 51.2; 56.0; 173.1.

[2-Diethoxy-phosphoryloxy)-ethyl]-(5-methoxycarbonyl-pentyl)-dimethylammonium trifluoroacetate. The methyl ester (1.73 g, 10 mmol) was dissolved in dry acetonitrile (15 mL) and K_2CO_3 (1.38 g, 10 mmol) was added. This suspension was refluxed under nitrogen gas for 36 h. After cooling to room temperature water was added and the pH was adjusted to 2 by adding TFA. The solution was purified by rp HPLC (Supelco Discovery C18, 21.2 mm x 15 cm, 5µm, A: 5% IPA, 95% H_2O, 0.1% TFA, B: 90% IPA, 10% H_2O, 0.1% TFA, 0 to 15% B over 14 min, t_R = 11.8 min) and lyophilized to give the TFA salt of the quaternary amine as light yellow oil (1.06g, 3.0 mmol, 30%). LC-MS (Phenomenex Gemini, C18, 5µm, 150 x 3.0 mm, A: 0.1% formic acid in water, B: 0.1 % formic acid in acetonitrile, 10-50% B over 10 min) t_R = 6.4 min (LSD signal), m/z = 354.2 for $[M+H]^+$. ^{1}H NMR (CD_3OD, 400 MHz) δ = 1.41-1.50 (m, 8H, 2 x OCH_2CH_3 and C*H*$_2$); 1.77 (m, 2H, C*H*$_2$); 1.90 (m, 2H, C*H*$_2$); 2.44 (t, 2H, C*H*$_2$, ^3J = 7.2 Hz); 3.25 (s, 6H, N(C*H*$_3$)$_2$); 3.48 (m, 2H, C*H*$_2$); 3.72 (s, 3H, COOC*H*$_3$) 3.82 (m, 2H, C*H*$_2$); 4.26 (dq, 4H, 2 x C*H*$_2$CH$_3$, ^3J(H,H) = 6.8 Hz, ^3J(H,P) = 8.0 Hz); 4.58 (brs, 2H, C*H*$_2$). ^{13}C NMR (CD_3OD, 100.5 MHz) δ = 16.4 (d, 2 x OCH_2CH_3, ^3J = 6.1 Hz); 23.3 (CH_2); 25.3 (CH_2); 26.7 (CH_2); 34.4 (CH_2); 52.0 (CH_3); 52.2 (CH_3); 62.1 (d, CH_2, ^2J = 5.3 Hz); 64.8 (CH_2); 66.1 (d, CH_2, ^2J = 6.1 Hz); 66.7 (CH_2); 175.5 (CO).

(5-carboxy-pentyl)-[2-(diethoxy-phosphoryloxy)-ethyl]-dimethylammonium trifluoroacetate. The TFA salt of the quaternary amine (200 mg, 0.56 mmol) was dissolved in a mixture of *t*-butanol/water (2:1, 9 mL) and $LiOH{\cdot}H_2O$ (47 mg, 1.12 mmol) was added. The reaction mixture was stirred for 3 h at room temperature and the organic solvent was removed under reduced pressure. The pH of the aqueous solution was adjusted to 7 with 0.1 N HCl and the mixture was subsequently lyophilized and further used without purification. LC-MS

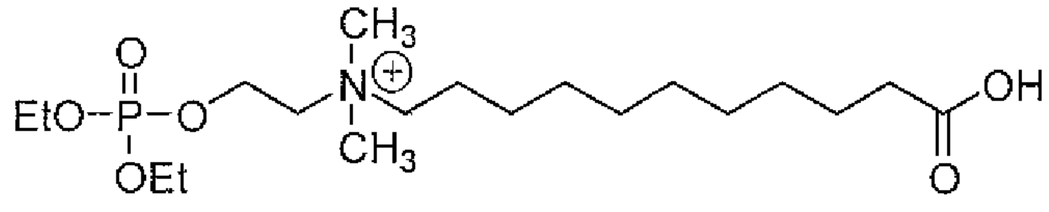
(Phenomenex Gemini, C18, 5µm, 150 x 3.0 mm, A: 0.1% formic acid in water, B: 0.1 % formic acid in acetonitrile, 10-40% B over 10 min) t_R = 6.8 min (LSD signal), m/z = 340.2 for $[M+H]^+$. ^1H NMR (CD_3OD, 400 MHz) δ = 1.34-1.42 (m, 8H, 2 x OCH_2CH_3 and CH_2); 1.67 (m, 2H, CH_2); 1.80 (m, 2H, CH_2); 2.20 (t, 2H, CH_2, ^3J = 7.6 Hz); 3.16 (s, 6H, $N(CH_3)_2$); 3.40 (m, 2H, CH_2); 3.72 (brs, 2H, CH_2); 4.18 (dq, 4H, 2 x CH_2CH_3, ^3J(H,H) = 7.0 Hz, ^3J(H,P) = 8.0 Hz); 4.50 (brs, 2H, CH_2). ^{13}C NMR (CD_3OD, 100.5 MHz) δ = 16.4 (d, 2 x OCH_2CH_3, ^3J = 6.8 Hz); 23.2 (CH_2); 26.5 (CH_2); 27.1 (CH_2); 37.9 (CH_2); 52.0 (CH_3); 62.1 (d, CH_2, ^2J = 4.6 Hz); 64.5 (CH_2); 66.1 (d, CH_2, ^2J = 6.1 Hz), 66.8 (CH_2); 181.3 (CO).

[2-(diethoxy-phosphoryloxy)-ethyl]-dimethyl-[5-(4-nitro-phenoxycarbonyl)-pentyl]-ammonium trifluoroacetate. To a solution of the carboxylic acid (26 mg, 0.076 mmol) in dry acetonitrile (3 mL) were consecutively added triethylamine (0.01 mL, 0.084 mmol), p-nitrophenyl chloroformate (31 mg, 0.15 mmol) and DMAP (1 mg). The reaction was stopped after 4 h by addition of 0.1% TFA in water and subsequently purified by rp HPLC (Supelco Discovery C18, 21.2 mm x 15 cm, 5µm, A: 5% IPA, 95% H_2O, 0.1% TFA, B: 90% IPA, 10% H_2O, 0.1% TFA, 20 to 40% B over 12 min, t_R = 10.3 min) to give the p-nitrophenyl ester as colorless oil (10 mg, 0.022 mmol, 28%). LC-MS (Phenomenex Gemini, C18, 5µm, 150 x 3.0 mm, A: 0.1% formic acid in water, B: 0.1 % formic acid in acetonitrile, 10-90% B over 8 min) t_R = 4.3 min (UV signal), m/z = 461.5 for $[M+H]^+$. ^1H NMR ($CDCl_3$, 300 MHz) δ = 1.34 (t, 6H, 2 x OCH_2CH_3, ^3J = 7.2 Hz); 1.48 (m, 2H, CH_2); 1.81 (m, 4H, 2 x CH_2); 2.63 (t, 2H, CH_2, ^3J = 7.2 Hz); 3.27 (s, 6H, $N(CH_3)_2$); 3.51 (m, 2H, CH_2); 3.92 (m, 2H, CH_2); 4.14 (dq, 4H, 2 x CH_2CH_3, ^3J(H,H) = 7.5 Hz, ^3J(H,P) = 7.5 Hz); 4.47 (m, 2H, CH_2); 7.27 (d, 2H, ^3J = 9 Hz, Phe-H); 8.25 (d, 2H, ^3J = 9 Hz, Phe-H). ^{13}C NMR ($CDCl_3$, 75.4 MHz) δ = 16.0 (d, 2 x OCH_2CH_3, ^3J = 6.6 Hz); 22.4 (CH_2); 23.8 (CH_2); 25.4 (CH_2); 33.6 (CH_2); 51.6; 60.8 (d, CH_2, ^2J = 4.8 Hz); 63.3 (?); 64.7 (d, CH_2, ^2J = 5.9 Hz); 65.7; 122.5 (2 x Phe-CH); 125.2 (2 x Phe-CH); 145.3 (Phe-C); 155.3 (Phe-C); 170.8 (CO).

Dimethyl-[5-(4-nitro-phenoxycarbonyl)-pentyl]-(2-phosphonooxy-ethyl)-ammonium trifluoro acetate. The protected p-nitrophenyl ester (20 mg, 0.043 mmol) was dissolved in dry acetonitrile (3 mL) and trimethyl silylbromide (0.11

mL, 0.87 mmol) was added. The reaction mixture was stirred at room temperature for 24 h. After adding 0.1% TFA in water (1 mL) the solution was purified by rp HPLC (Supelco Discovery C18, 21.2 mm x 15 cm, 5µm, A: 0.1% TFA in water, B: 0.1% TFA in acetonitrile, 17 to 30% B over 14 min, t_R = 12.7 min) to give the phosphocholine derivative PC_6-pNitrophenylester (4.6 mg, 0.011 mmol, 25%) as TFA salt. LC-MS (Phenomenex Gemini, C18, 5µm, 150 x 3.0 mm, A: 0.1% formic acid in water, B: 0.1 % formic acid in acetonitrile, 10-90% B over 10 min) t_R = 3.8 min (UV signal), m/z = 405.5 for $[M+H]^+$.

Scheme 2



(10-Carboxy-decyl)-[2-(diethoxy-phosphoryloxy)-ethyl]-dimethyl-ammonium trifluoro acetate

Fmoc-undecanoic acid (635 mg, 1.5 mmol) was dissolved in dry DMF (2ml) and a solution of diisopropyl carbodiimid (0.12 ml, 0.75 mmol) in dry dichloromethane (10 ml) was added. The resulting yellow mixture was stirred at 0°C for 20 min. After removal of the volatile dichloromethane the solution of the symmetrical anhydride was added to the Wang resin (250 mg, 1.2 mmol/g, 0.3 mmol). DMAP (18 mg, 0.15 mmol) was added and the reaction mixture was stirred over night (W. C. Chan, P. D. White in *Fmoc Solid Phase Peptide Synthesis, A Practical Approach* (Eds.: W. C. Chan, P. D. White) Oxford University Press, Oxford **2000**, pp. 55-56.) The resin was washed with DMF (5 x 2 min), dichloromethane (5 x 2 min) and was shrunk with diethyl ether. The procedure was repeated one time and the final substitution level of 0.8 mmol/g (66%) was determined based on UV spectrophotometry of the Fmoc-piperidine adduct. (ibid., pp 62-63)

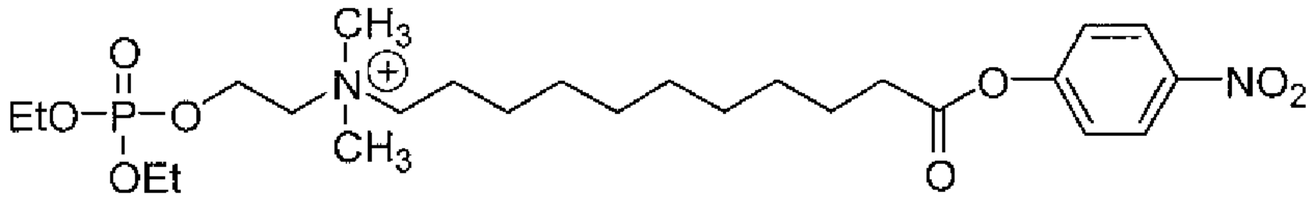
After Fmoc deprotection with 20% piperidine/DMF the resin was swelled in THF. A mixture of THF (2 ml), formaldehyde (40%, 0.7 ml, 1 mmol) and acetic acid (0.7 ml) were added and the resin was incubated for 5 min. Then sodium cyanoborohydrid (62 mg, 1 mmol) was added and the resin was stirred at room temperature over night. Afterwards the resin was washed successively with THF, H_2O and MeOH.

E. G. Brown, J. M. Nuss *Tetrahedron Lett.* **1997**, *38*, 8457-8460.

The resin was swelled in DMF and phosphoric acid diethyl ester-(2-bromo-ethyl ester) / phosphoric acid 2-bromo-ethyl ester diethyl ester (520 mg, 2 mmol) and DIEA (0.03 ml, 0.2 mmol) were added and the reaction mixture was heated to 60°C for 4 d. The resin was washed with DMF, DCM and was shrunk with MeOH.

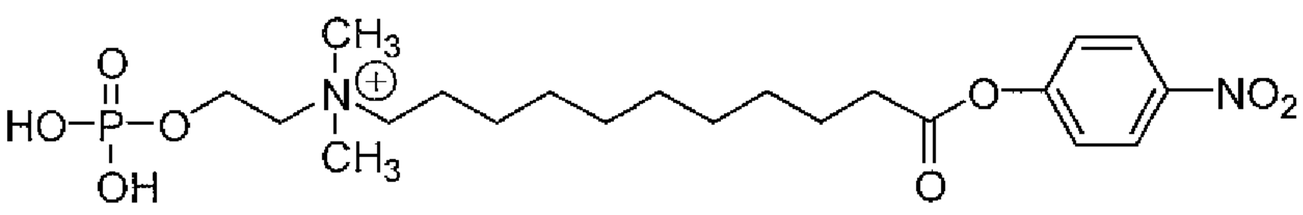
J. Cai, B. Wathey *Tetrahedron Lett.* **2001**, *42*, 1383-1385.

The product was finally cleaved from the resin by stirring it with a mixture of TFA/H_2O/TIS (95:2.5:2.5) for 2 h. The carboxylic acid was used after evaporation of the organic solvents, addition of water and lyophilization without further purification.



[2-(Diethoxy-phosphoryloxy)-ethyl]-dimethyl-[10-(4-nitro-phenoxycarbonyl)-decyl]-ammonium trifluoro acetate

To a solution of the carboxylic acid X in dry acetonitrile (5 ml) were consecutively added triethylamine (0.03 ml, 0.2 mmol), p-nitrophenyl chloroformate (80 mg, 0.39 mmol) and DMAP (7 mg). The reaction was stopped after 4 h by addition of 0.1% TFA in water and subsequently purified by RP HPLC (Supelco Discovery C18, 21.2 mm x 15 cm, 5µm, A: 0.1% TFA in IPA/H_2O 5:95, B: 0.1% TFA in IPA/H_2O 90:10, 15 to 45% B over 20 min, t_R = 16.5 min) to give the p-nitrophenyl ester X as colorless oil (4 mg, 0.007 mmol, %).
LC-MS (Phenomenex Gemini, C18, 5µm, 150 x 3.0 mm, A: 0.1% formic acid in water, B: 0.1 % formic acid in acetonitrile, 10-90% B over 10 min) t_R = 5.2 min (UV signal); M = 531.5 (M^+).



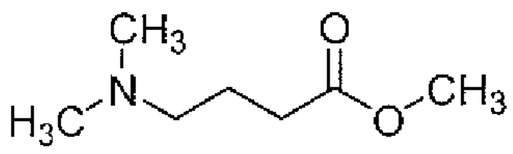
Dimethyl-[10-(4-nitro-phenoxycarbonyl)-decyl]-(2-phosphonooxy-ethyl)-ammonium trifluoro acetate

The protected p-nitrophenyl ester (4 mg, 0.007 mmol) was dissolved in dry acetonitrile (3 ml) and trimethyl silylbromide (0.11 ml, 0.07 mmol) was added after 0, 12 and 24 hours resp. The reaction mixture was stirred at room

temperature for totally 36 h. After adding 0.1% TFA in water (1 ml) the solution was purified by rp HPLC (Hichrom C8, 21.2 mm x 25 cm, 10µm, A: 0.1% TFA in ACN/H_2O 10:90, B: 0.1% TFA in ACN/H_2O 90:10, 40 to 60% B over 15 min, t_R = 14.2 min) to give the phosphocholine derivative (1.1 mg, 0.002 mmol, 27%) as TFA salt.

LC-MS (Phenomenex Gemini, C18, 5µm, 150 x 3.0 mm, A: 0.1% formic acid in water, B: 0.1 % formic acid in acetonitrile, 10-90% B over 10 min) t_R = 5.9 min (UV signal), M = 475.4 (M^+).

PC4



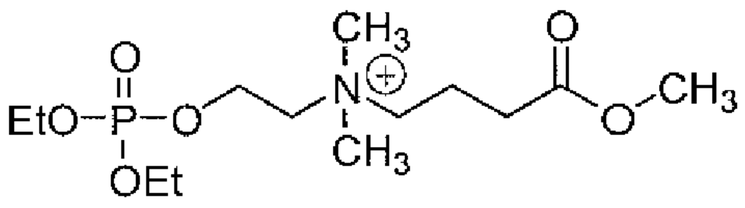
4-dimethylamino butyric acid methyl ester

same procedure as for PC6

^{1}H NMR (D_2O, 400 MHz) δ = 2.06 (m, 2H, CH_2); 2.56 (t, 2H, ^{3}J = 7.2 Hz, CH_2); 2.93 (s, 6H, N(CH_3)$_2$); 3.22 (m, 2H, CH_2); 3.75 (s, 3H, COOCH_3).

^{13}C NMR (CD_3OD, 100.5 MHz) δ = 20.9; 31.2; 43.5; 52.3; 58.1; 174.3

LC-MS (Phenomenex Gemini, C18, 5µm, 150 x 3.0 mm, A: 0.1% formic acid in water, B: 0.1 % formic acid in acetonitrile, 10-50% B over 10 min) t_R = 1.3 min (ELSD signal); M = 146.4 ((M+H)$^+$).

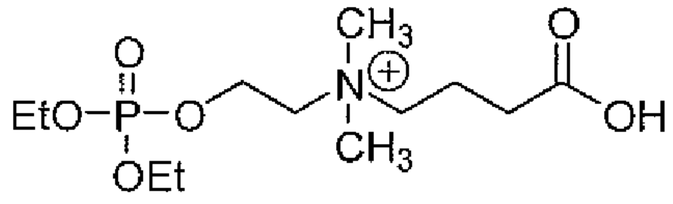


[2-Diethoxy-phosphoryloxy)-ethyl]-(3-methoxycarbonyl-propyl)-dimethylammonium trifluoroacetate

The methyl ester X (516 mg, 3.5 mmol) was dissolved in dry acetonitrile (8 ml) and K_2CO_3 (484 mg, 3.5 mmol) was added. This suspension was refluxed under nitrogen gas for 24 h. After cooling to room temperature water was added and the pH was adjusted to 2 by adding TFA. The solution was purified by rp HPLC (Supelco Discovery C18, 21.2 mm x 15 cm, 5µm, A: 0.1% TFA, 5% IPA in H_2O, B: 0.1% TFA, 10% H_2O in IPA, 0 to 10% B over 10 min, t_R = 8.8 min) and lyophilized to give the TFA salt of the quaternary amine as light yellow oil.

^{1}H NMR (CD_3OD, 400 MHz) δ = 1.36 (t, 6H, 2 x OCH_2CH_3, ^3J = 6.8Hz); 2.08 (m, 2H, CH_2); 2.47 (t, 2H, CH_2, ^3J = 7.2 Hz); 3.20 (s, 6H, N(CH_3)$_2$); 3.45 (m, 2H, CH_2); 3.70 (s, 3H, COOCH_3); 3.76 (t, 2H, CH_2, ^3J = 4.4 Hz); 4.18 (dq, 4H, OCH_2CH_3 ^3J(H,H) = 7.2 Hz, ^3J(H,H) = 7.2 Hz); 4.52 (bs, 2H, CH_2).

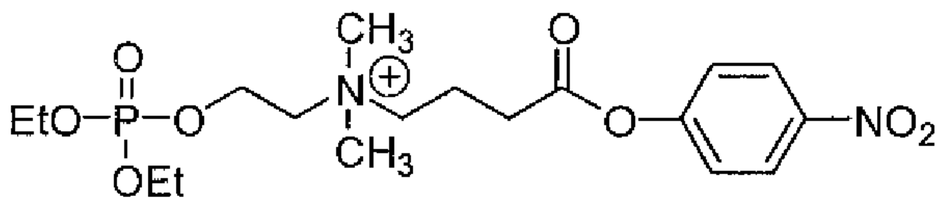
^{13}C NMR (CD_3OD, 100.5 MHz) δ = 16.4 (d, 2 x OCH_2CH_3, ^3J = 6.1 Hz); 19.0 (CH_2); 30.7 (CH_2); 52.3 (CH_3); 52.4(CH_3); 62.1 (d, CH_2, ^2J = 4.5 Hz); 64.4 (CH_2); 65.4 (CH_2); 66.1 (d, CH_2, ^2J = 6.0 Hz); 174.0 (CO).



(3-carboxy-propyl)-[2-(diethoxy-phosphoryloxy)-ethyl]-dimethylammonium trifluoroacetate

same procedure as for PC6

LC-MS (Phenomenex Gemini, C18, 5µm, 150 x 3.0 mm, A: 0.1% formic acid in water, B: 0.1 % formic acid in acetonitrile, 10-50% B over 10 min) t_R = 1.8 min (ELSD signal); M = 312.3 (M^+).



[2-(Diethoxy-phosphoryloxy)-ethyl]-dimethyl-[3-(4-nitro-phenoxycarbonyl)-propyl]-ammonium trifluoroacetate

To a solution of the carboxylic acid in dry acetonitrile (5 ml) were consecutively added triethylamine, p-nitrophenyl chloroformate and DMAP. The reaction was stopped after 4 h by addition of 0.1% TFA in water and subsequently purified by RP HPLC (Supelco Discovery C18, 21.2 mm x 15 cm, 5µm, A: 0.1% TFA, 5% IPA in H_2O, B: 0.1% TFA 10% H_2O in IPA, 10 to 30% B over 13 min, t_R = 11.8 min) to give the p-nitrophenyl ester as colorless oil.

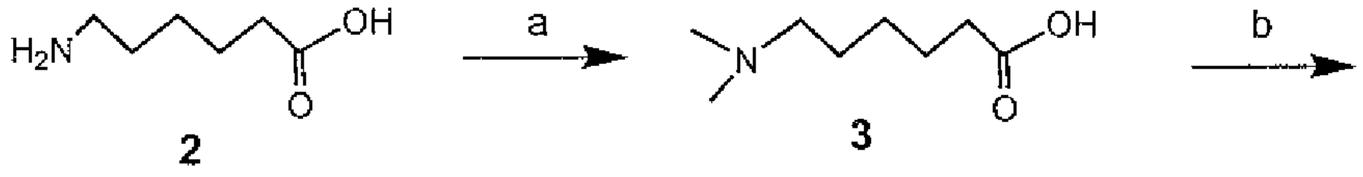
LC-MS (Phenomenex Gemini, C18, 5µm, 150 x 3.0 mm, A: 0.1% formic acid in water, B: 0.1 % formic acid in acetonitrile, 10-90% B over 10 min) t_R = 3.1 min (UV signal); M = 433.2 (M^+).

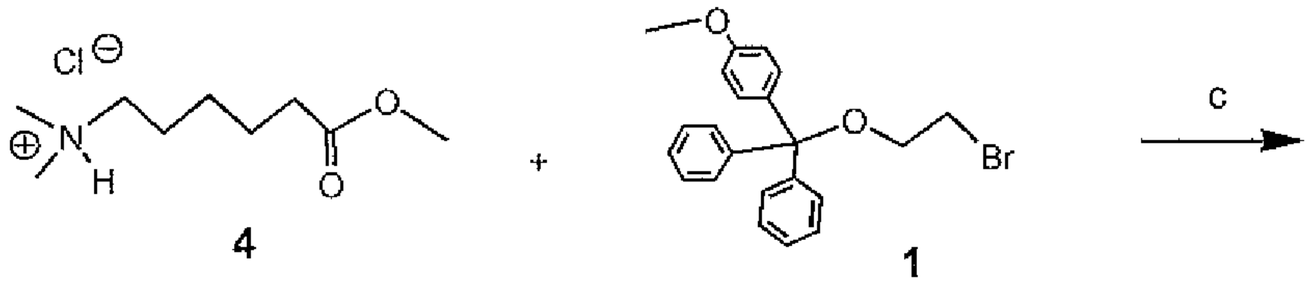
Alternative scheme for preparation of PC6

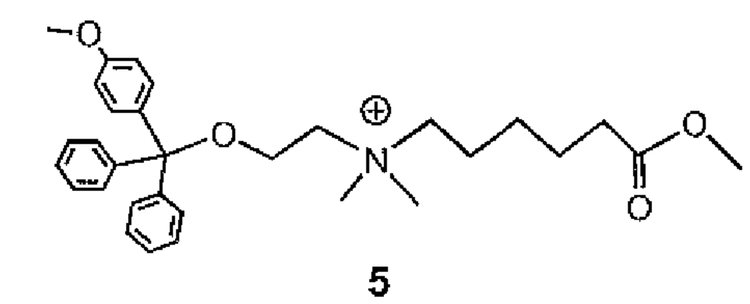
During the priority year, an alternative strategy for preparing the phosphocholine ligand PC6 was developed. This is outlined below.

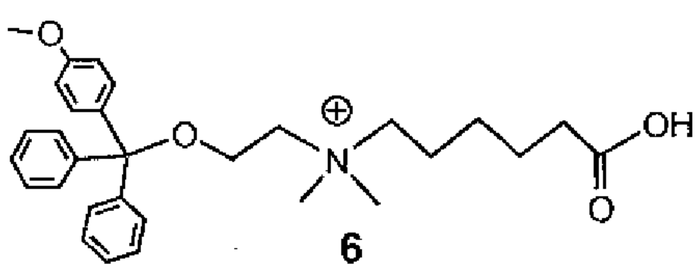
This strategy for preparation of PC6 (compound **9**) is outlined in scheme 3. Phosphorylation was chosen as the last step, since this function was considered troublesome, even in a protected form, to carry through other necessary transformation steps.

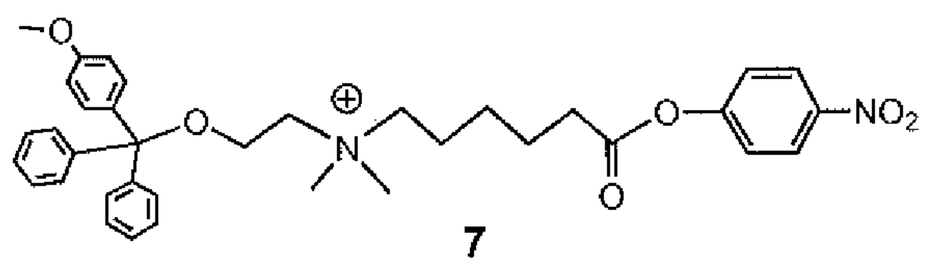
Commercially available aminohexanoic acid **2** was N,N-dimethylated[11 12] by an Eschweiler-Clarke-type procedure (treatment with formaldehyde and hydrogen/palladium/carbon), and the resulting product (**3**, 86%) was esterified by treatment with methanolic hydrogen chloride to give the methyl ester **4** (96 %). Quartenarization was effected by reacting **4** with monomethoxytrityl-protected bromoethanol **1**, forming derivative **5** (73%). Unprotected bromoethanol could also be reacted with **4**, but the reaction yield was lower and the product was more difficult to detect and purify. Monomethoxytrityl protection conferred lipophilicity and TLC detectability (UV visible and acid stainable) to the crucial intermediate **5**. Saponification of **5** with lithium hydroxide/water/t-butanol gave the corresponding acid **6** (95%), which was treated without isolation with p-nitrophenol and diisopropyl carbodiimide in pyridine to give the p-nitrophenyl ester **7** (86%). Brief treatment of **7** with formic acid gave the corresponding alcohol **8**, which was used directly in the next step, because of its sensitivity to self-condensation. Reaction of crude **8** at 0°C in acetonitril with first an excess of phosphorous oxychloride and triethylamine, then water gave the desired monophosphate **9** in 22% yield from **7** , after HPLC purification. Compound **9** gave the expected NMR and LC-MS spectra, and was stable in aqueous solution at acidic pH (0.1 % TFA). For convenient storage, however, the material was kept as a DMSO stock solution in the freezer.

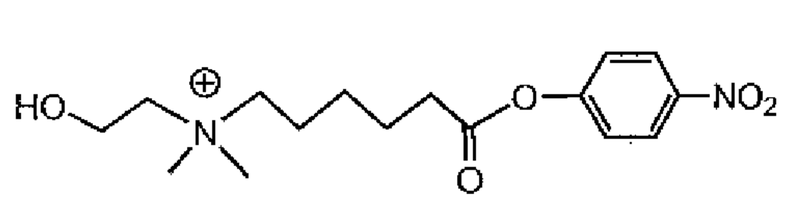


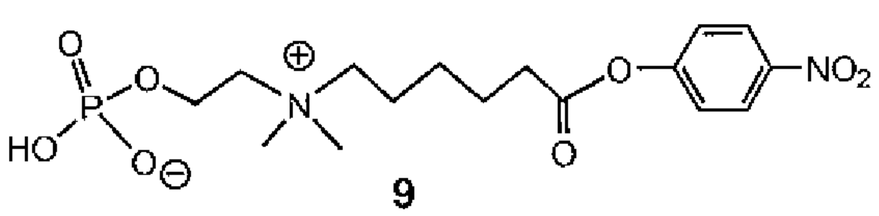












Scheme 3, reagents, conditions, and yields: a: CH_2O, H_2, Pd/C (86%); b: CH_3OH, HCl (96%); c: Na_2CO_3, CH_3CN (73%); d: LiOH, t-BuOH/H_2O (95%); e: DIPCDI, pyridine (86%); f: HCOOH/H_2O; g: $POCl_3$, TEA; h: H_2O (22% from **7**).

General methods – Concentrations were performed at reduced pressure (bath temperature <40°C). NMR spectra were recorded at 298° K with a Varian Unity 500 MHz spectrometer. Only selected NMR data are reported. Assignments were corroborated by appropriate 2-D experiments. Electrospray mass spectroscopy (ES-MS) for dilute methanol or acetonitril solutions was performed in the direct-inlet mode using a Perkin-Elmer SCIEX API 150-EX mass spectrometer. The positive-ion mode spectra were recorded and processed using the manufacturer software on an Apple MacIntosh Quadra computer.

TLC was performed on Silica Gel F254 (Merck, Darmstadt, Germany) plates with detection by UV-light and/or by staining with either 5 % ninhydrin in butanol or 5% sulfuric acid in ethanol. Column chromatography was performed on Matrex silica gel 60 Å (35-70 μm, Amicon) unless otherwise stated. Preparative HPLC was carried out using a Varian HPLC system consisting of a 9012 gradient pump, a 9065 diode array detector, and a PC computer system running the Varian Star HPLC software. Isolute C-18 EC reversed phase silica (40-70 micron particle size) from International Sorbent Technology Ltd (Mid Glamorgan, UK) was packed and eluted with the specified solvents in open glass columns. Other reagents and solvents were purchased with high commercial quality and were used without further purification unless otherwise stated.

2-Monomethoxytrityloxy-1-bromoethanol (1) - This compound was prepared analogously to the corresponding trityl derivative[13]. Briefly, monomethoxytrityl chloride (3.09 g, 10 mmol) was added to a solution of bromoethanol (1.0 g, 8.0 mmol) in dry pyridine (5 mL) at room temperature. After 12 hrs, TLC (hexane-ethyl acetate, 9:1) indicated presence of two UV-absorbing spots, one (major) with rf 0.9, one (minor) with rf 0.7. Water (0.1 mL) was added, and after 15 min stirring at r.t. the mixture was partitioned between ethyl acetate and aq 2M sulfuric acid, the organic layer was washed with aq. sodium bicarbonate and concentrated. The residue was purified by column chromatography on silica gel (160 g) packed in pentane-ethyl acetate 95:5 and eluted with a pentane-ethyl acetate 95:5 – 90:10 stepwise gradient. Collection of the main band gave **1** as a syrup (2.75 g, 87 %), which crystallized on standing. Alternatively, chromatography could be omitted and the crude syrup crystallized directly from

methanol to give pure crystals in a yield of 40-50%. Direct-inlet ES-MS showed a large peak at m/z 273 (monomethoxytrityl cation) but only two very small peaks at 419/421 (M+Na). H-NMR data ($CDCl_3$): 3.44/3.48 (2 multipets, 2+2 H, OCH2CH2Br), 3.83 (s, 3H, ArOCH3), 6.88 (d, 2H, ArH), 7.24-7.52 (m, 12H, Ar-H).

6-(N,N-Dimethylamino)-N-[2-(monomethoxytrityloxy)-ethyl]-hexanoic acid methyl ester (5) -

A solution of 6-aminohexanoic acid (**2**, 13.2 g) in aquoeus formaldehyde (37%, 40 mL) was mixed with Pd/C (1.0 g) and the mixture was hydrogenated in a Parr stainless steel apparatus with magnetic stirring overnight at room temperature and 50 bar. A check by TLC (ethyl acetate-methanol-acetic acid-water, 6:3:3:2, ninhydrin detection) revealed almost complete conversion from starting material (rf 0.7) to a slower-migrating product (rf 0.5, browner color). The reaction mixture was filtered through a bed of Celite, the filter bed was washed with water (20 mL), diluted (60 mL), and the solution was slowly passed through a column of Dowex-50 Wx2-100 mesh (H+ form, 0.7 meq/mL, 150 mL, carefully pre-washed with milli-Q water). The column was then washed with milli-Q water (200 + 100 mL), and then the product was eluted with 2% aq ammonia (400 mL). The eluate was monitored by TLC (ethyl acetate-methanol-acetic acid-water, 6:3:3:2, ninhydrin detection). The appropriate fractions were partially evaporated, then lyophilized to leave a semi-solid residue (13.7 g, 86 %). Direct inlet ES-MS revealed a strong peak at m/z 160.2 (M+H), corresponding to the N,N-dimethylated product (**3**).

A fraction of this crude material (3.97 g, 25 mmol) in methanol (75 mL) was cooled in ice and stirred while thionyl chloride (10 mL, 134 mmol) was added dropwise during 30 min, after which the cooling was removed and the mixture was left at room temperature overnight. A check by TLC (ethyl acetate-methanol-acetic acid-water, 10:3:3:2, ninhydrin detection) revealed conversion to a slightly faster-moving compound with weaker staining and a different tint. There were also minor faster-moving impurities carrying over from the previous step. After 16 hrs, the reaction mixture was concentrated and co-concentrated several times with methanol to give a residue (5.05 g) which crystallized partially

on standing. Direct-inlet ES-MS of this material revealed a strong peak at m/z 174.0 (M+H), corresponding to 6-(N,N-dimethylamino)-hexanoic acid methyl ester (**4**).

A fraction of this crude material (1.12 g, purity approximately 75 %, 4.0 mmol), dry acetonitril (25 mL), 2-(monomethoxytrityloxy)-2-bromoethanol (**1**, 1.60 g, 4.0 mmol), and solid anhydrous sodium carbonate (800 mg) was refluxed (bath temp 70°C) overnight, after which TLC showed only partial conversion, so more **1** (800 mg, 2.0 mmol) and sodium carbonate (500 mg) was added, and reflux was continued for another 24 hrs. TLC then showed that the majority of starting material had disappeared, and that there was a new, faster-migrating, ninhydrin-staining and UV-absorbing spot. The mixture was partitioned between dichloromethane and water (the desired material was in the organic phase), the aq. phase was washed with a little dichloromethane, and the combined organic layers were washed with a little water, then concentrated to a small volume (10 mL), which was partitioned between methyl-t-butyl ether and water (the desired material was now in the aqueous phase). The organic phase was washed with a little water, and the combined aqueous layer was washed with a little methyl-t-butyl ether. The combined aqueous solution was rotavapored shortly to remove non-aqueous solvents (approx. 1/3 of volume was removed) and the residual solution was passed through a Bond-Elut C-18-EC column (35 g, packed in methanol and then equilbrated with water), washed after with water, eluted with first 20 %, 50 and then finally 60 % methanol in water. The desired material eluted as a pure band with the 60% methanol eluent. Approprate fractions were collected, a few drops of pyridine were added, and the solution was lyophilized, leaving behind compound **5** as a white solid (1.53 g, 73 %). ES-MS showed a strong m/z 490 peak (M+). H-NMR data (CDCl3): 1.31 (m, 2H, H-4), 1.65 (m, 2H, H-3), 1.75 (m, 2H, H-5), 2.31 (t, 2H, H-2), 3.30 (s, 6H, (CH3)2N), 3.47 (m, 2H, H-6), 3.62 (m, 2H, OCH2C*H2*N), 3.68 (s, 3H, COOCH3), 3.82 (s, 3H, ArOCH3), 3.93 (m, 2H, OC*H2*CH2N), 6.89 (d, 2H, ArH), 7.24-7.41 (m, 12H, ArH).

6-(N,N-Dimethylamino)-N-[2-(monomethoxytrityloxy)-ethyl]-hexanoic acid p-nitrophenyl ester (7) – A solution of compound **5** (420 mg, 0.86 mmol) in 2:1

butanol-water (6.0 mL) was mixed with lithium hydroxide (72 mg) and the solution was stirred at room temperature for 3 h, after which TLC (ethyl acetate-methanol-acetic acid-water, 12:3:3:2) showed complete conversion into a compound with a slightly lower rf., An ES-MS spectrum at this stage showed m/z 476.0 and 482.3 peaks (M+H and M+Li, respectively). The mixture was diluted with water (10 mL), the pH was adjusted to 7.5 with aq 0.1 M hydrochloric acid, and then the solution was lyophilized to give crude **6** (510 mg, contaminated with lithium chloride). This material (approximately 0.86 mmol) was dissolved in in dry pyridine (6.0 mL), and then p-nitrophenol (400 mg, 2.86 mmol), and diisopropylcarbodiimide (270 microL, 0.176 mmol) was added. After stirring for 3 hrs at RT another portion ofdiisopropyl carbodiimide (200 microL) was added, and the mixture was stirred for another 24 hrs at r.t., after which TLC (ethyl acetate-methanol-acetic acid-water, 15:3:3:2) indicated complete reaction. The mixture was evaporated and coevaporated x3 with dichloromethane-toluene to remove pyridine. The residue was partitioned between aqueous 0.2 M triethylammonium acetate (pH 5.0) and methyl t-butyl ether (about 100 mL of each solvent and heavy shaking was necessary to dissolve everything), TLC (ethyl acetate-methanol-acetic acid-water, 10:3:3:2, UV and ninhydrin detection) showed that the product was exclusively in the aq. phase, whereas most of the p-nitrophenol and also other UV-absorbing impurities were in the organic phase. The organic phase was washed with a little aq. buffer, and the combined aqueous phases were washed with methyl t-butyl ether, then extracted with dichloromethane (TLC now indicated complete transfer of product into the organic phase). The aqueous layer was washed with a little dichloromethane, and the combined organic layers were washed with a small amount of buffer, and carefully separated. At this, TLC revealed presence of a very pure material, and H-NMR also confirmed this. The material was kept in the freezer in solution, but could, if necessary, be concentrated to give syrupy, crude **7** (440 mg, 86 %). In this form, the material also remain unchanged for weeks at freezer temperature. An ES-MS of the material showed a strong signal at m/z 596.8 (M+).

H-NMR data (CDCl3): 1.41 (m, 2H, H-4), 1.75-1.85 (m, 4H, H-3, H-5), 2.63 (t, 2H, H-2), 3.24 (s, 6H, (CH3)2N), 3.49 (m, 2H, H-6), 3.62 (m, 2H, OCH2C*H*2N),

3.81 (s, 3H, ArOCH3), 3.84 (m, 2H, OC*H2*CH2N), 6.89 (d, 2H, ArH), 7.24-7.41 (m, 12H, ArH), 8.28 (d, 2H, ArH).

6-(N,N-Dimethylamino)-N-[(2-phosphoryloxy)-ethyl]-hexanoic acid p-nitrophenyl ester (9) – A solution of compound **7** (55 mg, 0.10 mmol) was dissolved in formic acid (2 mL) and kept at RT for 60 min, then it was concentrated and co-concentrated x 3 with dry acetonitril. The residual compound **8**, showing a reasonable purity by TLC (ethyl acetate-methanol-acetic acid-water, 6:3:3:2, ninhydrin and UV detection) and ES-MS (m/z 325.2, M+), was dissolved in dry acetonitril (0.5 mL) and added to a cooled (0°C) solution of phosphorous oxychloride (0.019 mL, 0.2 mmol, freshly distilled) and triethylamine (0.069 mL, 0.5 mmol) in dry acetonitril (0.5 mL). After 1 h at 0°C, more phosphorous oxychloride (0.05 mL) and triethylamine (0.05 mL) was added, and after another hour water (0.05 mL) was added and the mixture was further stirred at 0°C for 1 hour, after which TLC showed disappearance of most starting material, and appearance of a UV-absorbing spot with very low (rf 0.05) mobility. The reaction mixture was diluted with 0.2% aq TFA (3.8 mL), washed with methyl t-butylether (3.5 mL), the aq phase was carefully separated, rotavapored shortly at 35°C (down to approx. 2/3 volume) and the resulting solution was injected in 400 microL portions on a Supelco Discovery C-18 column (15 x 21.2 cm, 5 microM), using a flow of 8.0 mL/min and a gradient over 40 min from 82% to 58% water in acetonitril (a 0.1 % TFA concentration was maintained throughout). One major and one minor UV-absorbing (300 nm) peak were seen at 18-20 min and 24-26 min respectively, corresponding to desired product **9** and starting material **8,** respectively. Fractions containing **9** were pooled, concentrated to a small volume, and lyophilized to give pure **9** as an amorphous material (9 mg, 22 % from **7**). An LC-MS spectrum showed a dominating m/z 405.2 signal (M+). H-NMR data (DMSO-d6): 1.38 (m, 2H, H-4), 1.67-1.78 (m, 4H, H-3, H-5), 2.69 (t, 2H, H-2), 3.01 (s, 6H, (CH3)2N), 3.35 (m, 2H, H-6), 3.60 (m, 2H, OCH2C*H2*N), 4.24 (m, 2H, OC*H2*CH2N), 7.44 (d, 2H, ArH), 8.31 (d, 2H, ArH). H-NMR data (D2O, HOD = 4.68): 1.35 (m, 2H, H-4), 1.66 (m, 2H, H-3 or H-5), 1.72 (m, 2H, H-3 or H-5), 2.59 (t, 2H, H-2), 3.02 (s,

6H, (CH3)2N), 3.28 (m, 2H, H-6), 3.52 (m, 2H, OCH2C*H2*N), 4.19 (m, 2H, OC*H2*CH2N), 7.22 (d, 2H, ArH), 8.19 (d, 2H, ArH).

Peptide Synthesis

A library of sixteen 42-residue peptides (Figure 1) was prepared by solid-phase peptide synthesis. The components of the library varied with regards to the number of charged residues and the position of incorporation of the ligand. The synthesis procedure was the same in each case and is exemplified here by that of 3-C15L8Cys24 (TA4Cys(Acm)).

The polypeptide was synthesized on a Pioneer automated peptide synthesizer (Applied Biosystems) using standard Fmoc chemistry. The synthesis was performed on 0.1 mmol scale on Fmoc-PAL-PEG-PS resin with a substitution level of 0.2 mmol/g. The following amino acid derivatives (Novabiochem AG) were used: Fmoc-Ala-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Asp(tBu)-OH, Fmoc-Cys(Acm)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Glu(tBu)-OH, Fmoc-Gly-OH, Fmoc-His(Trt)-OH, Fmoc-Ile-OH, Fmoc-Leu-OH, Fmoc-Lys(Aloc)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Nle-OH, Fmoc-Phe-OH, Fmoc-Pro-OH, Fmoc-Val-OH. The side chain of Lys 15 was Aloc protected, whereas Boc protection was used for Lys 8. A fourfold excess of amino acid derivative was used for each coupling step. TBTU (*O*-(7-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate, 0.5M in DMF) was used as coupling reagent in combination with DIEA (diisopropyl ethyl amine, 1M in DMF). A standard coupling time of 60 min was used, except for Gln, Cys, Pro and His (90 min) and the first residue, Arg, and Asn (2h). After the conventional automated solid phase peptide synthesis was completed, the free N-terminus was acetylated with acetic anhydride treating the resin for 2 h with a mixture of acetic anhydride/dichloromethane (3:1) and the resin was washed with dichloromethane (6 x) and shrunk with diethyl ether. The Aloc protecting group was removed with 3 eq. of tetrakis(triphenyl)palladium(0) in chloroform/acetic acid/NMM (*N*-methyl morpholine (18:2:1) for 2,5h. The resin was consecutively washed with DIEA (0.5% in DMF), sodium diethyl dithiocarbamic acid (0.5% in

DMF), DMF, dichloromethane and shrunk with diethyl ether. 7-methoxycoumarin-3-carboxylic acid (3 eq.) was coupled to the side chain of Lys 15 with HBTU (3eq.), HOBt (3 eq.) and DIEA (6 eq.) in DMF for 4h. The resin was washed with DMF, dichloromethane and shrunk with diethyl ether. The peptide was cleaved from the resin with TFA, TIS (triisopropyl silane) and water (95:2.5:2.5, 1 ml per 100 mg of resin) over 2h at room temperature. The TFA was evaporated under reduced pressure and the peptide was precipitated by addition of cold diethyl ether, centrifuged, washed and lyophilized. The crude product was purified by RP HPLC (Hichrom C8, 21.2 mm x 25 cm, 10µm, A: 0.1% TFA, 10% ACN in H_2O, B: 0.1% TFA 10% H_2O in ACN, 38 to 46% B over 18 min, t_R = 16.0 min).

Preparation of peptide scaffold dimers.

Disulfide bond formation (H. Tamamura, A. Otaka, J. Nakamura, K. Okubo, T. Koide, K. Ikeda, T. Ibuka, N. Fuji *Int. J. Peptide Protein Res.* **1995**, *45*, 312-319.):

The peptide (2.4 mg (0.51µmol) of 3-C15L8Cys(Acm)24) was dissolved in TFA (200 µl) and silver triflate (5 mg, 20 µmol) and anisole (1 drop) were added. The reaction mixture was incubated in the refrigerator at 4°C for 24h. After addition of diethyl ether the peptide precipitated and was washed 3 times with cold diethyl ether. To the precipitate were added DMSO (0.25 ml) and 1 N HCl (1 ml). The reaction mixture was stirred at room temperature for 22h. The suspension was centrifuged and the precipitate was discarded. The liquid was neutralized with diluted NaOH and analyzed by RP-HPLC (analytical run: Varian C18, 150 x 4.6 mm, 5µm, A: 0.1% TFA, 10% ACN in H_2O, B: 0.1% TFA 10% H_2O in ACN, 30 to 60% B over 30 min, t_R = 23.4 min.

Reaction of PC_6-pNitrophenylester with TA_4+Cys(Acm).

This is a specific example of a protocol for incorporating a phosphocholine derivative into a peptide, in this case TA_4+ with a protective group Acm on the Cys-residues.

To a solution of TA_4+Cys(Acm) peptide (500 µg, 0.1 µmole) in 100 µL of 0.1 M phosphate buffer pH8 were added 2.8 µL of 0.1 M PC6-pNitrophenylester in DMSO (0.3 µmole, 3 eq.). The reaction mixture was incubated at 4°C. LC-MS (Phenomenex Gemini, C18, 5 µm, 150 x 3.0 mm, A: 0.1% formic acid in water, B: 0.1 % formic acid in acetonitrile, 10-90 % B over 10 min) t_R = 4.1 min (UV signal), m/z = 670.5 for $[M+8H]^{8+}$, 766.1 for $[M+7H]^{7+}$, 893.3 for $[M+6H]^{6+}$, 1071.9 for $[M+5H]^{5+}$.

Immobilization of TA4+PC6Cys(Acm) Peptide on Porous Polystyrene Coated with F108-2-Pyridyl Disulfide

To a solution of TA_4+PC6Cys(Acm) peptide (90 µg, 18 nmol) in 18 µL of 0,1 M phosphate buffer pH 8 were added 1.8 µL of 5 mg/mL iodine in 50% MeOH in water (8.6 µg, 34 nmol, 2-fold excess relative to thiol groups). The reaction mixture was incubated at 25°C for 20 min, cleaned with PepClean C18 Columns (Pierce) and added directly to a 50 µL suspension containing 10 mg of F108-PDS-coated porous particles in phosphate buffer.

Kinase-peptide conjugation

To a solution of TA_4+PC6Cys(Acm) peptide (90 µg, 18 nmol) in 18 µL of 0,1 M phosphate buffer pH 8 were added 1.8 µL of 5 mg/mL iodine in 50% MeOH in water (8.6 µg, 34 nmol, 2-fold excess relative to thiol groups). The reaction mixture was incubated at 25°C for 20 min, cleaned with PepClean C18 Columns (Pierce) and added directly to a 1.5 mL of 1.3 mg/ml Kinase-PDS in phosphate buffer.

Formation of TA_4PC6CysH

Reaction of PC_6-pNitrophenylester with TA_4Cys(Acm). To a solution of TA_4Cys(Acm) peptide (1 mg, 0.2 µmol) in 1 mL of 0.1 M phosphate buffer pH8 were added 10 µL of 0.1 M PC6-pNitrophenylester in DMSO (0.8 µmol, 5 eq.). The reaction mixture was incubated at 4°C for 2 days, purified by gel filtration using NAP-10 columns (GE Healthcare) to have 1.5mL of TA_4PC6Cys(Acm) in water and finaly lyophilized. LC-MS (Phenomenex Gemini, C18, 5 µm, 150 x 3.0 mm, A: 0.1% formic acid in water, B: 0.1 % formic acid in acetonitrile, 10-90 %

B over 10 min) t_R = 4.1 min (UV signal), m/z = 856.0 for $[M+6H]^{6+}$, 1026.8 for $[M+5H]^{5+}$, 1282.6 for $[M+4H]^{4+}$.

Cysteine deprotection. $TA_4PC6Cys(Acm)$ was dissolved in 1 mL of TFA/anisole (99:1) and 6 mg of AgOTf were added (20 µmol, 100 eq.). After 2 hours at 4°C, the peptide silver salt was precipitated with cold ether, isolated by centrifugation and washed 2 times with ether. 116 µL of 10 mg/ml DTT in 1 M acetic acid were added and the solution volume was extended to 1 mL with 1 M acetic acid. After one night at 25°C the peptide was purified by gel filtration using NAP-15 columns to have 1.5 mL of TA4PC6CysH in water and lyophilized. LC-MS (Phenomenex Gemini, C18, 5 µm, 150 x 3.0 mm, A: 0.1% formic acid in water, B: 0.1 % formic acid in acetonitrile, 10-90 % B over 10 min) t_R = 4.6 min (UV signal), m/z = 843.5 for $[M+6H]^{6+}$, 1012.3 for $[M+5H]^{5+}$, 1264.6 for $[M+4H]^{4+}$.

Formation of $TA_4^+PC6CysH$

Reaction of PC_6-pNitrophenylester with $TA_4^+Cys(Acm)$. To a solution of $TA_4^+Cys(Acm)$ peptide (1 mg, 0.2 µmol) in 1 mL of 0.1 M phosphate buffer pH8 were added 10 µL of 0.1 M PC6-pNitrophenylester in DMSO (0.8 µmol, 5 eq.). The reaction mixture was incubated at 4°C for overnight, purified by gel filtration using NAP-10 columns (GE Healthcare) to have 1.5mL of $TA_4^+PC6Cys(Acm)$ in water and finally lyophilized. LC-MS (Phenomenex Gemini, C18, 5 µm, 150 x 3.0 mm, A: 0.1% formic acid in water, B: 0.1 % formic acid in acetonitrile, 10-90 % B over 10 min) t_R = 4.0 min (UV signal), m/z = 670.3 for $[M+8H]^{8+}$, 766.1 for $[M+7H]^{7+}$, 893.4 for $[M+6H]^{6+}$.

Cysteine deprotection. $TA_4^+PC6Cys(Acm)$ was dissolved in 1 mL of TFA/anisole (99:1) and 6 mg of AgOTf were added (20 µmol, 100 eq.). After 2 hours at 4°C, the peptide silver salt was precipitated with cold ether, isolated by centrifugation and washed 2 times with ether. 116 µL of 10 mg/ml DTT in 1 M acetic acid were added and the solution volume was extended to 1 mL with 1 M acetic acid. After one night at 25°C the peptide was purified by gel filtration using NAP-15 columns to have 1.5 mL of $TA_4^+PC_6CysH$ in water and lyophilized. LC-MS (Phenomenex Gemini, C18, 5 µm, 150 x 3.0 mm, A: 0.1% formic acid in

water, B: 0.1 % formic acid in acetonitrile, 10-90 % B over 10 min) t_R = 4.6 min (UV signal), m/z = 661.5 for $[M+8H]^{8+}$, 755.9 for $[M+7H]^{7+}$, 881.6 for $[M+6H]^{6+}$.

Incorporation of ligand into each polypeptide scaffold of the peptide library.

A more general protocol for incorporation of a phosphocholine derivative is given below. A person skilled in the art will be able to adapt this protocol in order to optimize it for specific conditions that may apply.

Each peptide from the library is dissolved in 1 mL 50 mM phosphate buffer, pH 8, to a concentration of 1 mM to form a stock solution. The amount of peptide needed to form the stock solution is calculated from its molecular weight assuming a water content of 25%. The concentration of each stock solution is determined using an exctinction coefficient of 20300 M^{-1} cm^{-1} at 355 nm. 100 µL of each stock solution is transferred to a well of a microtitre plate and two eq. of the active ester of the ligand to be tested dissolved in DMSO at an initial concentration of 100 mM is added and left to react for 1 hr.

Affinity measurements

In a separate set of wells of the microtitre plate the C-reactive protein (CRP) was dissolved to a concentration of 500 nM in 10 mM HEPES, 150 mM NaCl and 5 mM $CaCl_2$ at pH 7.4. Each one of the polypeptides functionalized with ligand and fluorophore was diluted and added to each well containing the target protein, to give a final concentration of peptide of 500 nM.

As a negative control, in a parallell experiment the same procedure was applied using polypeptides with fluorophore but without ligand. The microtitre plate was introduced into a fluorescence based plate reader set for excitation at 355 nm and emission at 420 nm. Peptides for which the fluorescence emission was different between polypeptide funtionalized with ligand and polypeptide without ligand were considered as “hits” and selected for more careful analysis.

An experimental protocol for careful analysis of affinity using a fluorimeter is given in Table I and Figure 2. The polypeptide used has the sequence named 3-C15L8. Although the analysis of the fluorescent data is complicated by the fact that C-reactive protein has five binding sites for phosphocholine it is clear that the affinity for the functionalized polypeptide in the presence of 5 mM $CaCl_2$ is compatible with a dissociation constant of low nM or better.

The corresponding experiment with the polypeptide carrying fluorophore but no ligand showed no change in fluorescence as a function of the concentration of CRP, showing that the polypeptide scaffold itself has very low affinity for CRP. Consequently, the combination of polypeptide and phosphocholine derivative forms a binder for CRP where cooperative interactions beween the components of the functionalized polypeptide and CRP gives rise to a very high overall affinity.

Table 1

Peptide	Buffer	Conc peptide (nM)	Conc CRP (nM)	Vol CRP	Vol peptide	Vol buffer	Intensities			Average	Error
4-C15L8-PC6	Bis Tris 20 mM, 5 mM CaCl2 pH7,2	100	0	0	10	490	456,78	486,4	527,63	490,27	35,58319
		100	5	0,5	10	489,5	449,62	477,97	489,83	472,4733	20,66086
		100	10	1	10	489	456,17	455,1	450,21	453,8267	3,177489
		100	25	2,5	10	487,5	446,88	433,51	446,39	442,26	7,581682
		100	50	5	10	485	423,14	393,56	409,77	408,8233	14,81271
		100	75	7,5	10	482,5	406,08	365,75	408,88	393,57	24,13347
		100	100	10	10	480	399,31	381,79		390,55	12,38851
		100	150	15	10	475	413,76	389,49		401,625	17,16148
		100	200	20	10	470	405,77			405,77	
4-C15L8	Bis Tris 20 mM, 5 mM CaCl2 pH7,2	100	0	0	10	490					
		100	5	0,5	10	489,5					
		100	10	1	10	489					
		100	25	2,5	10	487,5					
		100	50	5	10	485					
		100	75	7,5	10	482,5					
		100	100	10	10	480					
		100	150	15	10	475					
		100	200	20	10	470					
-	Bis Tris 20 mM, 5 mM CaCl2 pH7,2	0	0	0	0	500					
-	Bis Tris 20 mM, 5 mM CaCl2 pH7,2	0	1500	15	0	485					

WO 2007/117215 PCT/SE2007/050227
CA 02647271 2008-09-24
31

Solid phase CRP detection assay

One embodiment of a CRP detection assay having the binder of the present invention bound to a solid phase is schematically shown in Figure 3.

In this assay the capture of CRP is accomplished by a CRP-binder attached to latex particles and the recognition signal is generated by the binder conjugated to the enzyme pyruvate kinase. The kinase phosphorylates ADP, with the help of added PEP (phosphoenolpyruvate), to form ATP. The ATP, in turn, forms a complex with the enzyme luciferase which enables it to catalyze the oxidation of luciferin to oxyluciferin. This reaction generates light. The light intensity becomes a direct measure of the amount of kinase in the sample, which in turn is a measure of the amount of CRP bound.

Preparation of CRP-binders bound to latex particles

Monodisperse polystyrene latex particles with a diameter of 239 nm were suspended in ultra-pure (MilliQ) water containing the polymeric surfactant Pluronic F108, containing pyridyl disulfide (PDS) conjugated end-groups to allow linking of thiol containing ligands. The surfactant molecules adsorb to the particles via their hydrophobic center blocks[14]. The number of Pluronic molecules taken up, through over-night adsorption, by each particle was determined by sedimentation field-flow fractionation, SdFFF to be 16500[15].The PDS groups were then replaced by either the Cys-containing CRP-binder. A protocol for immobilising a binder (Ta4+) is given above.The analysis by SdFFF indicated that each particle had taken up 2560 binders.

The enzyme pyruvate kinase (type III from Rabbit muscle; Sigma-Aldrich product nr. P9136-5KU) was assayed, either as received, or after treatment with the bifunctional linker molecule, SPDP (Pierce Biotechnology, product nr. 21857), or after attachment to the binder via a disulfide bridge (exemplary protocol given above). The three forms of the enzyme were assayed for their respective ability to phosphorylate ADP in the following manner: aliquots containing 0.36 mg kinase in 50 mM glycine-TRIS buffer of pH 7.6 were each added to the "luminescence reagent", i.e. a mixture of 40 µL luciferase ("Thermostable", from BioThema AB, Stockholm) at 0.5 mg/mL, 10µL PEP

(Sigma-Aldrich) at 8.9 mg/mL, 10□L 15 mM luciferin (Sigma-Aldrich). At time t = zero aliquots of 10 μL ADP (Sigma-Aldrich, 2.6 mg/mL) were added to each sample mixture and the light emission was followed with a CCD camera for 300 sec. The results are shown in Figure 4. The three forms were largely of comparable activity. The commercial product was unexpectedly somewhat less active than its modified forms, probably caused by an (unintended) removal of some minor inhibitor during the processing.

The photon flux was measured by a CCD camera from a reaction mixture of the type illustrated in Fig. 3 and, for comparison, from a reaction mixture having an CRP-specific IgY antibody as CRP-binding agent instead of the CRP-binder of the present invention. The reagent concentrations and total amounts were the same as described for the "luminescence reagent" in Fig. 4. The samples were either CRP-free serum or serum containing 12 mg CRP/L. The highest photon flux was that provided by a mixture wherein CRP-binders on the particle surfaces had captured CRP from the concentrated serum sample. After 3 cycles of washing and centrifugation the composite sample was allowed to take up binder-kinase conjugate; it was then mixed with the above luminescence reagent and allowed to generate its photon flux. Similarly, particles containing antibodies (IgY anti-CRP, a gift from Prof. A. Larsson, Uppsala Academic Hospital) were allowed to bind CRP from the serum of known (12 mg/L) concentration. An antibody-kinase conjugate was added to generate the photon flux, as described. The CRP-free serum was used as blank. In addition to the four samples just described, there was one that consisted only of surfactant coated nanoparticles of the same size as those carrying binders or antibodies in the four actual samples. The results are shown in Figure 5. The figure clearly shows that the binder–based detection of CRP gives a strong signal, while the binder-based signal from CRP-free serum gives a straight baseline of zero light intensity. The antibody-based detection gives a somewhat weaker signal in the CRP-containing serum, but demonstrates a higher non-specific adsorption than the binder-analogue to judge from the signal resulting from the CRP-free serum. The surfactant-coated particles are clear blanks in this study.

An experiment analogous to that of Fig. 5 was performed on binder-conjugated latex particles (10 μL, 10 % w/v polystyrene particles with 239 nm diameter). The particles were added to 100 μL of serum from dog, horse, or human subjects where the former two had unknown contents of CRP and the latter contained 12 mg/L. After 10 minutes of incubation the particles were washed three times with centrifugation and then incubated with binder-kinase conjugate for 15 minutes. After a three-fold wash procedure, and addition of the "luminescence reagent" described under Fig. 4, the samples were scanned by a luminometer from BioThema which measures cumulative photon generation and computes rates of ATP generation as a measure of kinase concentration, in turn proportional to the amount of CRP in the sample. The results are shown in Figure 6 and Table 1. From this analysis it is clear that the binders have the ability to capture CRP not only from humans, but from dog and horse as well.

Table 2

Sample	Reaction rate (pmol/min)
Human CRP	0.045±0.007
Dog CRP	0.084±0.009
Horse CRP	0.026±0.007

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DEMANDE OU BREVET VOLUMINEUX

LA PRÉSENTE PARTIE DE CETTE DEMANDE OU CE BREVET COMPREND PLUS D'UN TOME.

CECI EST LE TOME 1 DE 2
CONTENANT LES PAGES 1 À 35

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JUMBO APPLICATIONS/PATENTS

THIS SECTION OF THE APPLICATION/PATENT CONTAINS MORE THAN ONE VOLUME

THIS IS VOLUME 1 OF 2
CONTAINING PAGES 1 TO 35

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NOM DU FICHIER / FILE NAME :

NOTE POUR LE TOME / VOLUME NOTE:

CLAIMS

1. Polypeptide having a sequence according to SEQ ID NO: 1, wherein at least one phosphocholine derivative is attached to said polypeptide.

2. Polypeptide consisting of two dimerized polypeptides according to claim 1, wherein the two protomers have the same or different amino acid sequences.

3. Polypeptide according to claim 1 or 2 wherein the phosphocholine derivative is attached to a lysine in position 8, 17, 22 or 34 of one of the protomers.

4. Polypeptide according to any of claims 1-3, further having a reporter group attached to said polypeptide, preferably to a lysine in position 10, 15, 25 or 37 of one of the protomers.

5. Polypeptide according to any of claims 1-4, wherein the phosphocholine derivative is attached to the polypeptide through a spacer comprising a carbon chain with a length of 1-12 carbon atoms.

6. Polypeptide according to any of claims 1-5, having a binding affinity for C-reactive protein of less than 100 nanomolar.

7. Polypeptide according to any of claims 1-6, wherein said protomers have the same sequence or different sequences, and said sequences are selected from SEQ ID NOS: 3-18.

8. Method for producing the polypeptide dimer according to any of claims 2-7, said method comprising the steps

- synthesizing at least two polypeptides having the sequence according to SEQ ID NO: 1;
- bringing a phosphocholine derivative in contact with an unblocked lysine of one of said polypeptides under conditions suitable for the attachment of the phosphocholine derivative to the lysine;
- optionally bringing a reporter group in contact with an unblocked lysine of one of said polypeptides under conditions suitable for the attachment of the reporter group to the lysine, and
- allowing the polypeptides to form a polypeptide dimer.

9. Method for assaying the concentration of C-reactive protein in a sample, said method comprising the step of bringing the sample in contact with a polypeptide according to any of claims 1-7.

10. Method according to claim 9, wherein the sample is derived from a mammalian patient.

11. Method according to any of claims 9-10 , wherein said polypeptide dimer is bound to a solid support.

12. Method according to claim 11, comprising the steps
- bringing the sample in contact with a first polypeptide according to any of claims 1-7, said first polypeptide being bound to a solid support;
- bringing the sample in contact with a second polypeptide according to any of claims 4-7 having a reporter group attached to it; and
- detecting the presence or absence of the reporter group on said second polypeptide.

13. Method for purification of C-reactive protein comprising bringing a composition comprising C-reactive protein in contact with a polypeptide according to any of claims 1-7.

14. Pharmaceutical composition comprising a polypeptide according to any of claims 1-7.

15. Diagnostic composition comprising a polypeptide according to any of claims 1-7.

Figure 1

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42		
1-C15L8	KE2-	N	E	A	D	L	E	A	K	I	R	H	L	A	E	K	L	E	A	R	G	P	E	D	C	E	Q	L	A	E	Q	L	A	R	A	F	E	A	F	A	R	A	G	COOH	
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2-C15L8	KE2	N	E	A	D	L	E	A	K	I	R	H	L	A	E	K	L	A	A	R	G	P	V	D	C	A	Q	L	A	E	Q	L	A	R	A	F	E	A	F	A	R	A	G	COOH	
		0	-1	0	-1	0	-1	0	1	0	1	0	0	0	-1	1	0	0	0	1	0	0	0	-1	0	0	0	0	0	-1	0	0	0	1	0	0	-1	0	0	0	1	0	0	-1	-2
3-C15L8	TA4	N	A	A	D	J	E	A	K	I	R	H	L	A	E	K	J	A	A	R	G	P	V	D	C	A	Q	J	A	E	Q	L	A	R	R	F	E	A	F	A	R	A	G	CONH2	
		0	0	0	-1	0	-1	0	1	0	1	0	0	0	-1	1	0	0	0	1	0	0	0	-1	0	0	0	0	0	-1	0	0	0	1	1	0	-1	0	0	0	1	0	0	0	1
4-C15L8	TA4+	N	A	A	D	J	E	A	K	I	R	H	L	R	E	K	J	A	A	R	G	P	R	D	C	A	Q	J	A	E	Q	L	A	R	R	F	E	R	F	A	R	A	G	CONH2	
		0	0	0	-1	0	-1	0	1	0	1	0	0	1	-1	1	0	0	0	1	0	0	1	-1	0	0	0	0	0	-1	0	0	0	1	1	0	-1	1	0	0	1	0	0	0	4

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1-C10L17	KE2-	N	A	A	D	L	E	A	A	I	K	H	L	A	E	A	L	K	E	R	G	P	E	D	C	E	Q	L	A	E	Q	L	A	R	A	F	E	A	F	A	R	A	G	COOH	
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2-C10L17	KE2	N	A	A	D	L	E	A	A	I	K	H	L	A	E	A	L	K	A	R	G	P	V	D	C	A	Q	L	A	E	Q	L	A	R	A	F	E	A	F	A	R	A	G	COOH	
		0	0	0	-1	0	-1	0	0	0	1	0	0	0	-1	0	0	1	0	1	0	0	0	-1	0	0	0	0	0	-1	0	0	0	1	0	0	-1	0	0	0	1	0	0	-1	-2
3-C10L17	TA4	N	A	A	D	J	E	A	R	I	K	H	L	A	E	R	J	K	A	R	G	P	V	D	C	A	Q	J	A	E	Q	L	A	R	A	F	E	A	F	A	R	A	G	CONH2	
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4-C10L17	TA4+	N	A	A	D	J	E	A	R	I	K	H	L	R	E	R	J	K	A	R	G	P	R	D	C	A	Q	J	A	E	Q	L	A	R	A	F	E	R	F	A	R	A	G	CONH2	
		0	0	0	-1	0	-1	0	1	0	1	0	0	1	-1	1	0	1	0	1	0	0	1	-1	0	0	0	0	0	-1	0	0	0	1	0	0	-1	1	0	0	1	0	0	0	4

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1-C25L22	KE2-	N	E	A	D	L	E	A	A	I	R	H	L	A	E	A	L	E	A	R	G	P	K	D	C	K	Q	L	A	E	Q	L	A	R	A	F	E	A	F	E	R	A	G	COOH	
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2-C25L22	KE2	N	E	A	D	L	E	A	A	I	R	H	L	A	E	A	L	A	A	R	G	P	K	D	C	K	Q	L	A	E	Q	L	A	R	A	F	E	A	F	A	R	A	G	COOH	
		0	-1	0	-1	0	-1	0	0	0	1	0	0	0	-1	0	0	0	0	1	0	0	1	-1	0	1	0	0	0	-1	0	0	0	1	0	0	-1	0	0	0	1	0	0	-1	-2
3-C25L22	TA4	N	A	A	D	J	E	A	A	I	R	H	L	A	E	R	J	A	A	R	G	P	K	D	C	K	Q	J	A	E	Q	L	A	R	A	F	E	A	F	A	R	A	G	CONH2	
		0	0	0	-1	0	-1	0	0	0	1	0	0	0	-1	1	0	0	0	1	0	0	1	-1	0	1	0	0	0	-1	0	0	0	1	0	0	-1	0	0	0	1	0	0	0	1
4-C25L22	TA4+	N	A	A	D	J	E	A	R	I	R	H	L	R	E	R	J	A	A	R	G	P	K	D	C	K	Q	J	A	E	Q	L	A	R	A	F	E	R	F	A	R	A	G	CONH2	
		0	0	0	-1	0	-1	0	1	0	1	0	0	1	-1	1	0	0	0	1	0	0	1	-1	0	1	0	0	0	-1	0	0	0	1	0	0	-1	1	0	0	1	0	0	0	4

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42		
1-C37L34	KE2-	N	E	A	D	L	E	A	A	I	R	H	L	A	E	R	L	E	A	R	G	P	A	D	C	A	Q	L	A	E	Q	L	A	A	K	F	E	K	F	A	R	A	G	COOH	
		0	-1	0	-1	0	-1	0	0	0	1	0	0	0	-1	1	0	-1	0	1	0	0	0	-1	0	0	0	0	0	-1	0	0	0	0	1	0	-1	1	0	0	1	0	0	-1	-3
2-C37L34	KE2	N	A	A	D	L	E	A	A	I	R	H	L	A	E	R	L	A	A	R	G	P	V	D	C	A	Q	L	A	E	Q	L	A	A	K	F	E	K	F	A	R	A	G	COOH	
		0	0	0	-1	0	-1	0	0	0	1	0	0	0	-1	1	0	0	0	1	0	0	0	-1	0	0	0	0	0	-1	0	0	0	0	1	0	-1	1	0	0	1	0	0	-1	-1
3-C37L34	TA4	N	A	A	D	J	E	A	A	I	R	H	L	A	E	R	J	A	A	R	G	P	V	D	C	A	Q	J	A	E	Q	L	A	R	K	F	E	K	F	A	R	A	G	CONH2	
		0	0	0	-1	0	-1	0	0	0	1	0	0	0	-1	1	0	0	0	1	0	0	0	-1	0	0	0	0	0	-1	0	0	0	1	1	0	-1	1	0	0	1	0	0	0	1
4-C37L34	TA4+	N	A	A	D	J	E	A	K	I	R	H	L	R	E	R	J	A	A	R	G	P	R	D	C	A	Q	J	A	E	Q	L	A	R	K	F	E	K	F	A	R	A	G	CONH2	
		0	0	0	-1	0	-1	0	1	0	1	0	0	1	-1	1	0	0	0	1	0	0	1	-1	0	0	0	0	0	-1	0	0	0	1	1	0	-1	1	0	0	1	0	0	0	4

Figure 2

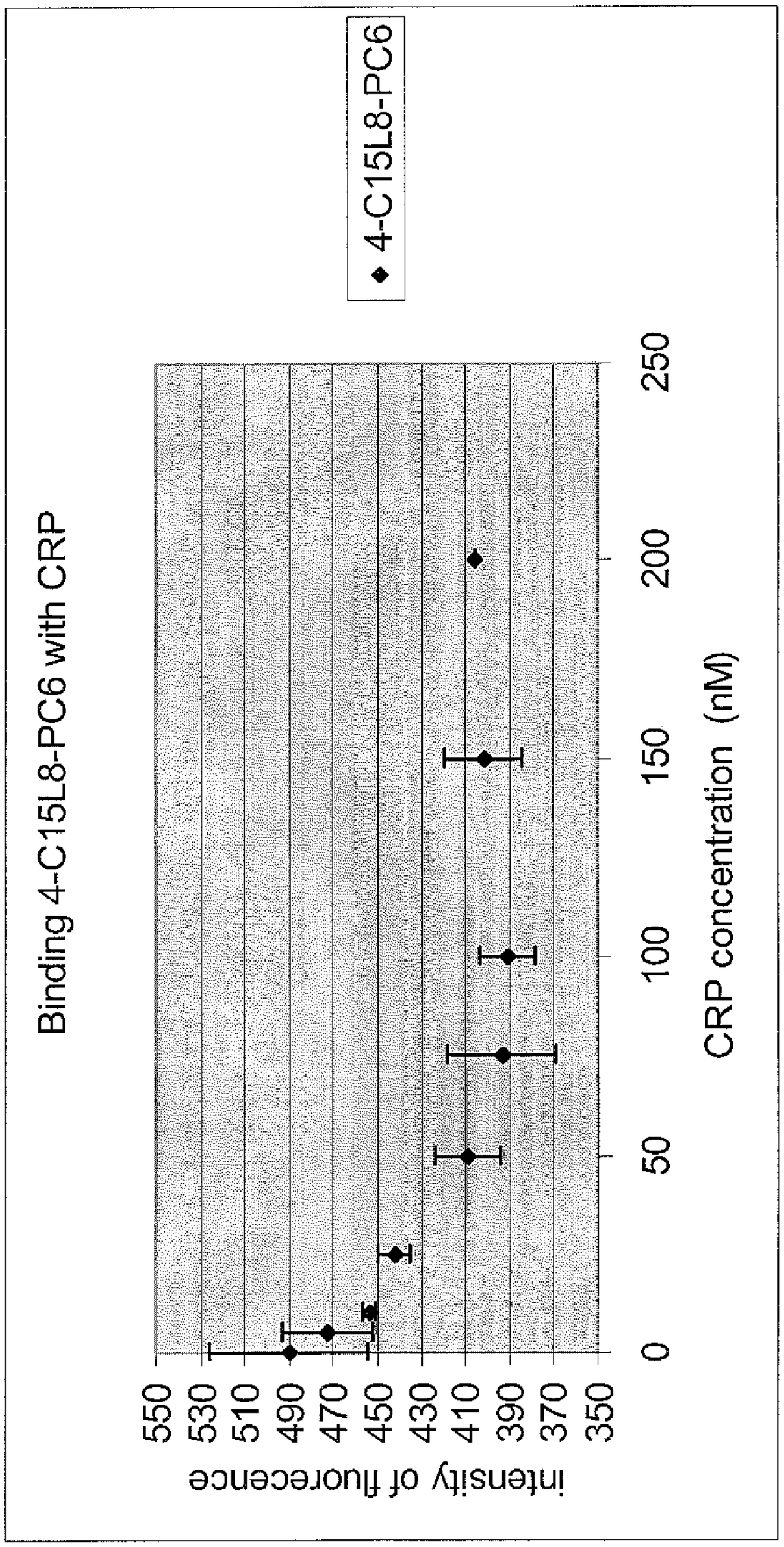

4-C15L8-PC6
Binding 4-C15L8-PC6 with CRP
intensity of fluorecence
550
530
510
490
470
450
430
410
390
370
350
CRP concentration (nM)
0
50
100
150
200
250

Figure 3

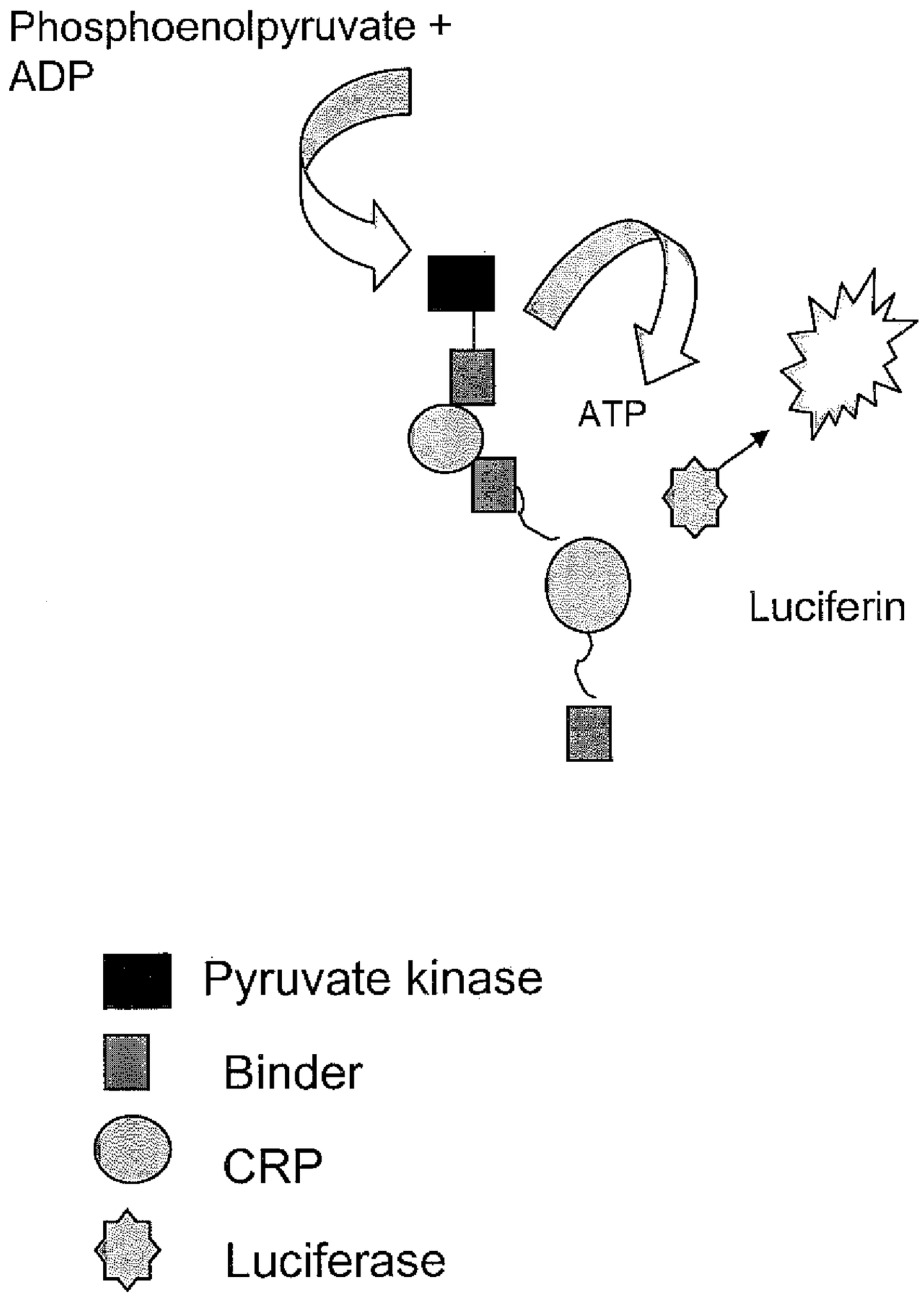

Phosphoenolpyruvate +
ADP
ATP
Luciferin
Pyruvate kinase
Binder
CRP
Luciferase

Figure 4

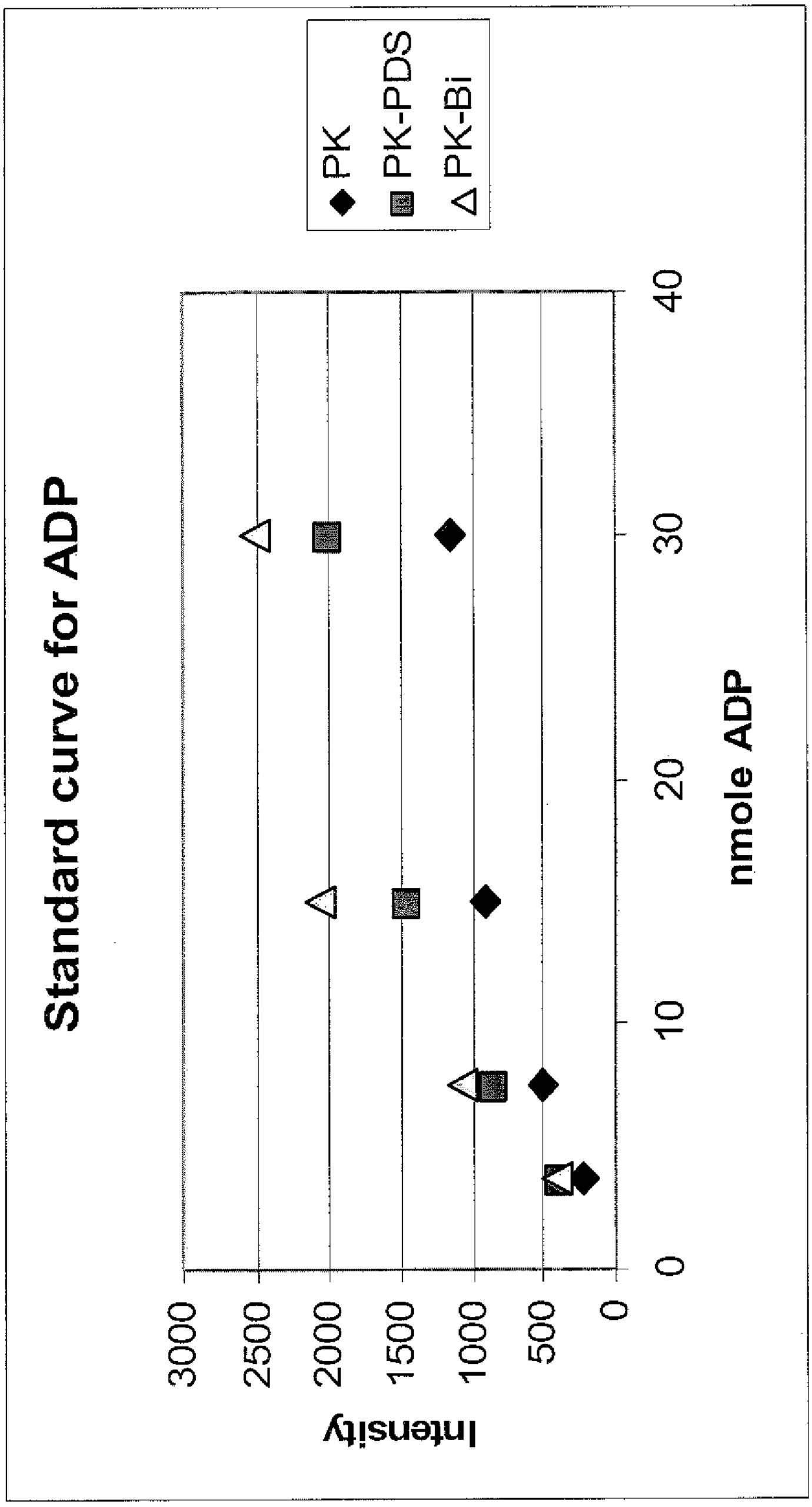
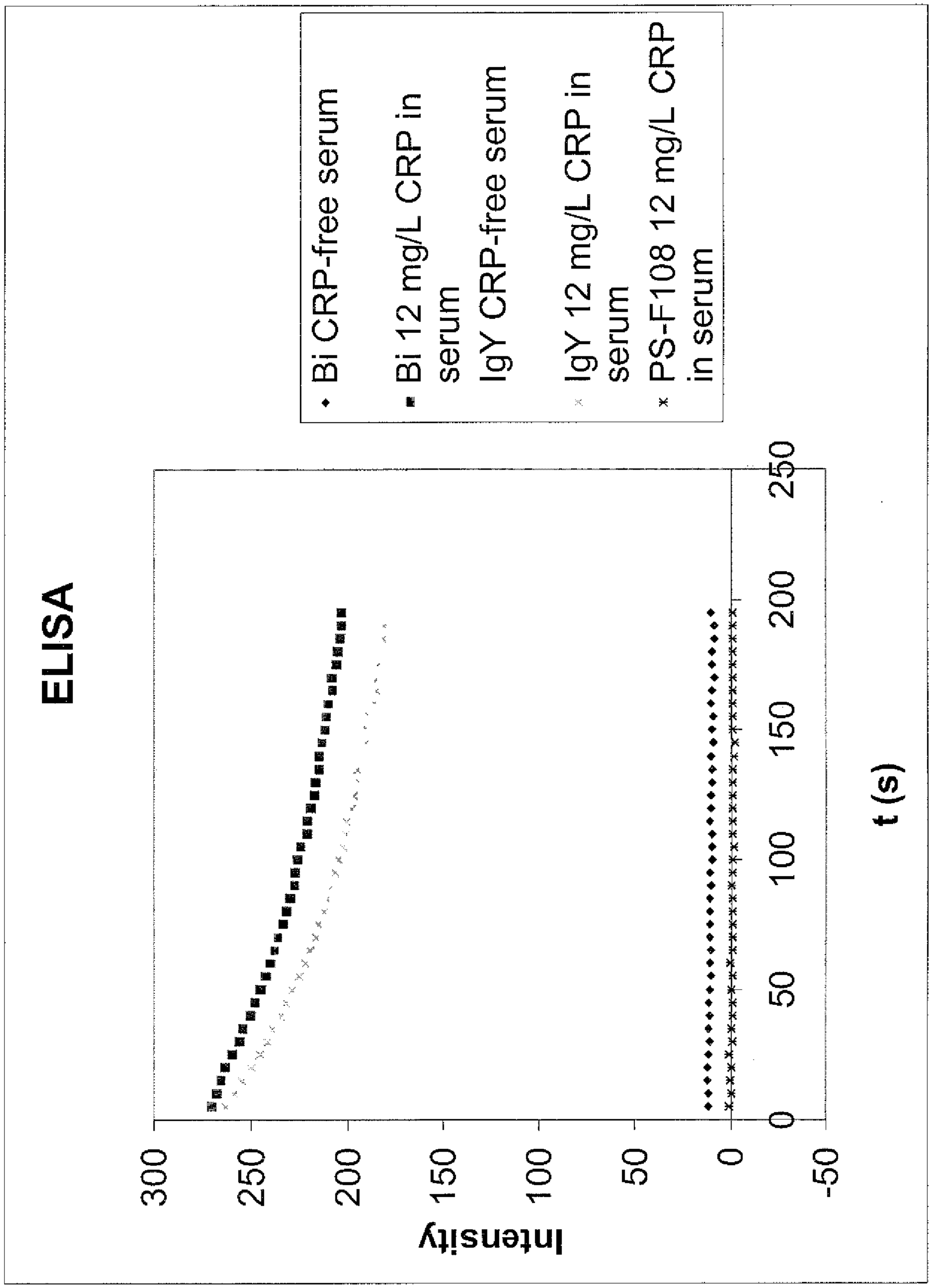

PK
PK-PDS
PK-Bi
Standard curve for ADP
Intensity
3000
2500
2000
1500
1000
500
0
0
10
20
30
40
nmole ADP

Figure 5


ELISA
Bi CRP-free serum
Bi 12 mg/L CRP in serum
IgY CRP-free serum
IgY 12 mg/L CRP in serum
PS-F108 12 mg/L CRP in serum
Intensity
t (s)
300
250
200
150
100
50
0
-50
0
50
100
150
200
250

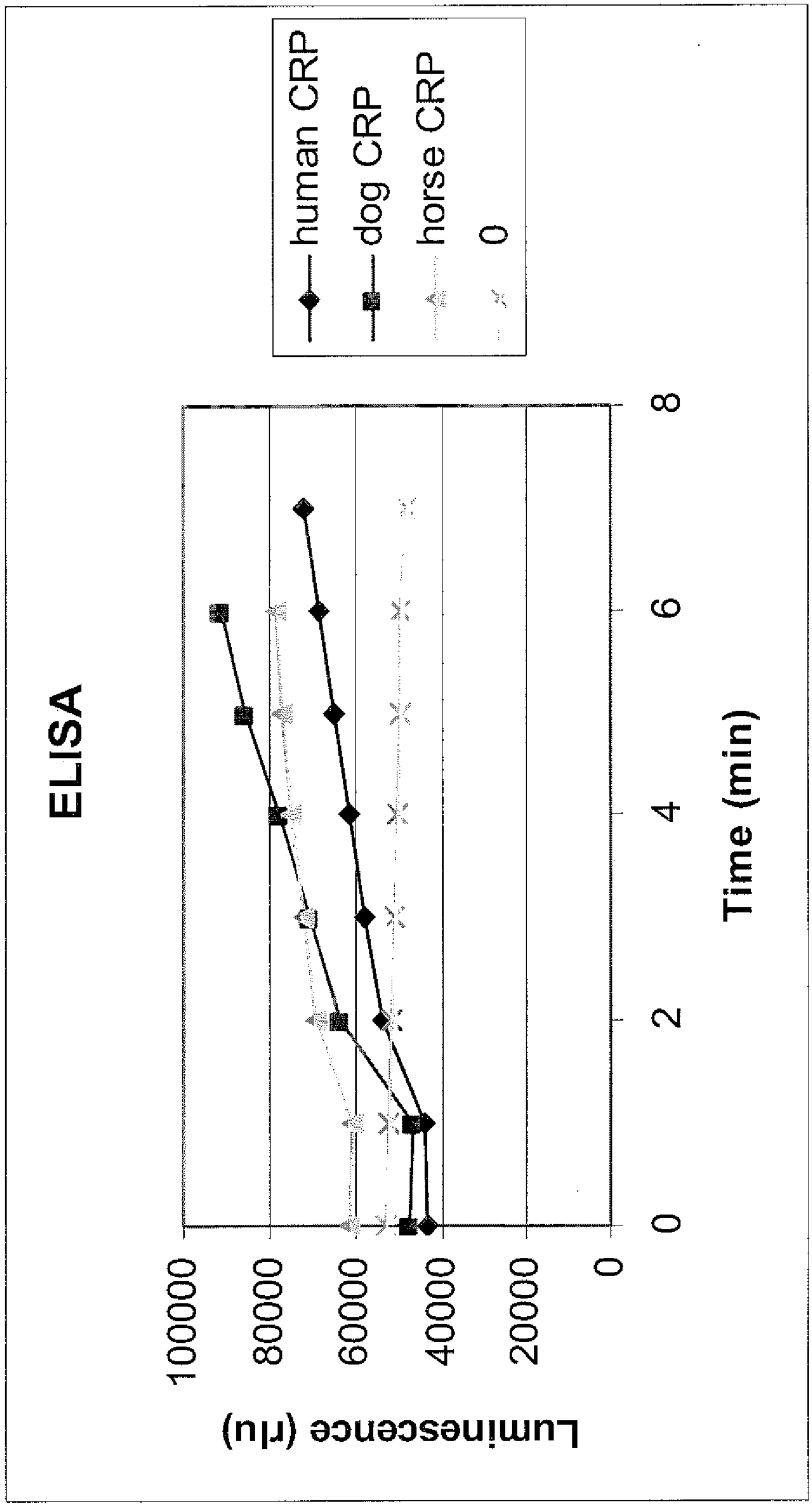
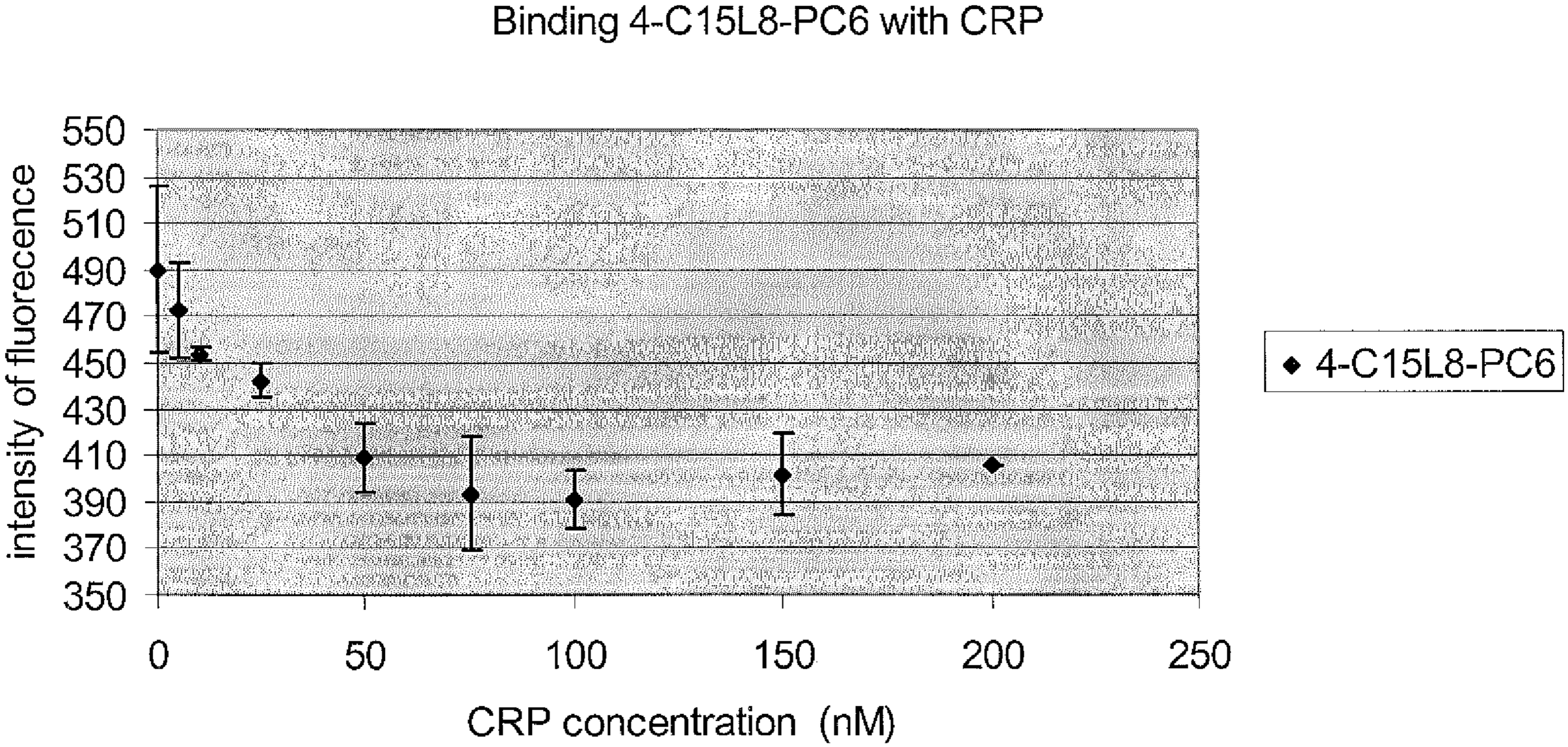

ELISA
Luminescence (rlu)
100000
80000
60000
40000
20000
0
0
2
4
6
8
Time (min)
human CRP
dog CRP
horse CRP
0

Figure 6


Binding 4-C15L8-PC6 with CRP
intensity of fluorecence
550
530
510
490
470
450
430
410
390
370
350
0
50
100
150
200
250
CRP concentration (nM)
4-C15L8-PC6