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Synthesis and Structural Characterization of Carboxyethylpyrrole-Modified Proteins: Mediators of Age-related Macular Degeneration

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Abstract

Protein modifications in which the ε-amino group of lysyl residues is incorporated into a 2-(ω-carboxyethyl)pyrrole (CEP) are mediators of age-related macular degeneration (AMD). They promote both angiogenesis into the retina (“wet AMD”) and geographic retinal atrophy (“dry AMD”). Blood levels of CEPs are biomarkers for clinical prognosis of the disease. To enable mechanistic studies of their role in promoting AMD, e.g., through the activation of B- and T-cells, interaction with receptors, or binding with complement proteins, we developed an efficient synthesis of CEP derivatives, that is especially effective for proteins. The structures of tryptic peptides derived from CEP-modified proteins were also determined. A key finding is that 4,7-dioxoheptanoic acid 9-fluorenylmethyl ester reacts with primary amines to provide 9-fluorenylmethyl esters of CEP-modified proteins that can be deprotected *in situ* with 1,8-diazabicyclo[5.4.0]undec-7-ene without causing protein denaturation. The introduction of multiple CEP-modifications with a wide variety of CEP:protein ratios is readily achieved using this strategy.

Introduction

Although it is relatively scarce in most tissues, docosahexaenoic acid (DHA) is essential for the growth, functional development and maintenance of the brain and is most abundant in photoreceptor cell membranes in the retina. Owing to the presence of the six homoconjugated C=C bonds in DHA, it is exquisitely sensitive to oxidative damage. Oxidative cleavage of

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phospholipids containing DHA produces reactive electrophilic phospholipid fragments, e.g., 4-hydroxy-7-oxohept-5-enoates, that convert the primary amino group of protein lysyl residues into 2-(ω -carboxyethyl)pyrrole (CEP) derivatives.¹ CEPs are especially abundant in ocular tissues from individuals with age-related macular degeneration (AMD), a slow, progressive disease² that is the major cause of untreatable loss of vision among the elderly in developed countries.³ Roughly 11% of people in the United States have AMD, and owing to increases in human life span, AMD is expected to nearly double in the next 25 years.⁴ Proteomic characterization of drusen, extracellular deposits that accumulate between the retina and the blood bearing choriocapillaris, revealed that CEP adducts are more abundant in AMD than in normal eyes.⁵ CEPs are also elevated in the blood of individuals with AMD.⁶ CEPs are not simply benign markers of oxidative damage, but also promote the growth of capillaries (neovascularization), and possibly contribute to choroidal neovascularization, also known as “wet” AMD.⁷ Such neovascularization is responsible for ~90% of the loss of vision associated with AMD. Remarkably, CEPs initiate an autoimmune response that may contribute to retinal degeneration. It had been observed that immunoglobins and complement components accumulate in subretinal neovascular membranes from AMD patients^{8, 9} and that autoantibodies against CEPs are elevated in the blood of individuals with AMD⁶ and in rodents exposed to intense light.¹⁰ Recently it was shown that mice immunized with CEP-modified serum albumin also develop autoantibodies to this hapten, fix complement component-3 in Bruch’s membrane, accumulate drusen below the retinal pigment epithelium during aging, and develop lesions in the retinal pigment epithelium mimicking geographic atrophy, the blinding end-stage condition characteristic of the “dry” form of AMD.¹¹ Apparently, these mice are sensitized to the generation of CEP adducts in the outer retina, where DHA is abundant and conditions for oxidative damage are permissive.

Generating proteins with various levels of CEP-modification has proven difficult, especially for higher CEP to protein molar ratios. We now report an efficient synthesis of CEP-modified proteins that was used to create the above mouse model of AMD. This synthesis and the characterization of tryptic peptides derived from the CEP-modified proteins will also enable mechanistic studies of their role in promoting AMD, for example through possible activation of B- and T-cells or interaction with complement proteins and as ligands for CEP receptors. A key contribution of the present report is that the 9-fluorenylmethyl ester of 4,7-dioxoheptanoic acid reacts with lysyl ϵ -amino groups to provide esters of CEPs that can be deprotected with 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) without causing protein denaturation. The introduction of multiple CEPs into proteins is readily achieved using this strategy. In addition, the preparation of CEPs tethered to proteins through an ω -amino hexanoate linker and their strong binding with anti-CEP antibodies is described. To characterize the CEP-modified proteins, the structures of tryptic peptides derived from CEP-modified proteins were also determined.

Results

Paal-Knoor synthesis using 4,7-dioxo-heptanoic acid is ineffective for the preparation of CEPs

The reaction of γ -keto aldehydes with primary amines, the Paal-Knoor synthesis¹², is generally an efficient method for the preparation of pyrroles. We previously successfully applied this reaction to the generation of carboxyheptylpyrrole and carboxypropylpyrrole derivatives through the reactions of 9,12-dioxododecanoic or 5,8-dioxooctanoic acid with proteins.¹³ However, attempts at preparing the corresponding carboxyethylpyrrole derivatives of proteins by treatment with 4,7-dioxoheptanoic acid (DOHA) generally caused precipitation, and in the few instances that precipitation did not occur, the ratio of pyrrole to protein, e.g. 1.6:1 for human serum albumin⁶, was much lower than we had obtained previously for the longer chain

carboxyalkylpyrroles. Another distinguishing feature of DOHA was the nearly complete absence of a signal for the aldehydic hydrogen in its ^1H NMR spectrum. We postulated that the unusual ^1H NMR spectrum and aberrant reactivity of DOHA are consequences of the proximity of the carboxyl group to the γ -ketoaldehyde array and that DOHA exists in equilibrium with the corresponding spiroacetal hemiacetal (Scheme 1). To obviate complications engendered by the carboxyl group, we sought a masked derivative that could be deprotected under conditions that would not lead to denaturation and consequent precipitation of proteins. This excluded acidic conditions. Therefore, we opted for a 9-fluorenylmethyl ester.

Synthesis of a 9-fluorenylmethyl (Fm) ester of DOHA

A Grignard reagent derived from 2-(2-bromoethyl)-1,3-dioxolane¹⁴ was previously acylated with 8-carbomethoxyoctanoyl chloride to selectively deliver a ketone.¹⁵ Following this precedent, we coupled this Grignard reagent with 3-carbomethoxypropionyl chloride and obtained the methyl ester **1a** (Scheme 2). The desired 9H-fluoren-9-ylmethyl ester 4,7-dioxoheptanoic acid (DOHA-Fm, **4**) was then obtained through saponification to afford the carboxylic acid **2a**, esterification with 9-fluorenylmethanol (FmOH), followed by hydrolysis of ethylene ketal in **3a**.

Subsequently we determined that the Grignard reagent from 2-(2-bromoethyl)-1,3-dioxane¹⁶ shows similar selectivity toward reaction with the acyl chloride but not the carbomethoxy group of 3-carbomethoxypropionylchloride to deliver **1b** in good yield. In addition, purification of the corresponding carboxylic acid **2b** is facilitated by the fact that it readily crystallizes. The keto aldehyde **3b** is also a crystalline solid. Initially, we used a large excess of FmOH (4 equivalents) and allowed the esterification to proceed for 3 days. However, the excess FmOH interfered with purification, requiring multiple column chromatographies. A more practical procedure, using 1.2 equivalents of FmOH and a 19 h reaction time, delivered **3b** in 85% yield after a single column chromatography on silica gel. This procedure was successfully and reproducibly applied on a multi-gram scale. Hydrolysis of the propylene acetal **3b** occurs more readily than the corresponding ethylene acetal **3a**, allowing shorter reaction times, and the isolation of pure DOHA-Fm (**4**) is more readily achieved from the acetal **3b** than from the corresponding ethylene acetal **3a**.

Synthesis of CEP-peptide and CEP-protein adducts by Paal-Knoor synthesis with DOHA-Fm

While the angiogenicity of CEPs was first detected in protein derivatives, the CEP dipeptide 2-(2-acetylamino-acetylamino)-6-[2-(2-carboxy-ethyl)-pyrrol-1-yl]-hexanoic acid methyl ester (**6**) was also found to be potently angiogenic.⁷ Therefore, we examined the applicability of the new synthetic method to prepare **6**. Paal-Knoor synthesis of the Fm ester **5** of a CEP dipeptide was readily achieved in 80 % yield by the condensation of methyl 6-amino-2-((2-acetylamino)acetyl)amino)hexanoate (Ac-Gly-Lys-OMe) with 1 equivalent of DOHA-Fm (Scheme 3). For peptide synthesis, the deprotection of 9-fluorenylmethyl esters is normally accomplished with piperidine in DMF. Piperidine serves both as a base to fragment the Fm group and as a scavenger to trap the dibenzofulvene released.¹⁷ The removal of an Fm group from an ester bound to a protein has not been reported. Piperidine was unsatisfactory, *vide infra*. This was readily determined by the persistence of a UV absorbtion at 265 nm that is characteristic of the fluorene group. Therefore, we examined the efficacy of a stronger base. Previously, a catalytic 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU)/octanethiol cocktail in THF was found effective for removal of flourenemethoxycarbonyl protecting groups from α -amino acids.¹⁷ We found that DBU successfully removed all Fm groups from protein adducts, *vide infra*, and this reagent was also applied to deprotection of the dipeptide Fm ester **5**. Removal of the Fm protecting group by treatment of **5** with DBU delivered the CEP dipeptide 2-(2-acetylamino-acetylamino)-6-[2-(2-carboxy-ethyl)-pyrrol-1-yl]-hexanoic acid methyl ester (**6**) in 86 % yield.

CEP-modified proteins are needed for immunoassays that measure levels of CEPs or anti-CEP autoantibodies *in vivo*.⁶ CEP-protein adducts are also essential for the recently described mouse model of AMD.¹¹ CEP-MSA is used as an antigen to immunize mice and elicit immune responses against CEP-protein adducts generated in the retina. We now find that the production of CEP-protein adducts can be readily accomplished by incubation of DOHA-Fm (**4**) with protein in 30 % DMF/phosphate-buffered saline (PBS) solution for 5 days at 37 °C followed by deprotection by addition of DBU to the reaction mixture and stirring for an additional 9 h. One equivalent of DOHA-Fm was used for each lysine group present in human serum albumin (HSA) or mouse serum albumin (MSA) (Scheme 4). Low molecular weight contaminants were removed by dialysis (*Mr* cutoff 14000) of the reaction mixture against 20% DMF in 10 mM PBS. An especially important feature of the use of Fm esters of DOHA is the ease with which residual Fm groups can be detected and their complete removal assured by UV spectroscopy, i.e., monitoring absorption at 265 nm. The final protein concentration was determined by Pierce bicinchoninic acid (BCA) protein assay¹⁸ or Bio-rad protein assay.¹⁹ The pyrrole concentration was determined by the generation of a characteristic chromophore through reaction with 4-(dimethylamino)benzaldehyde, the Ehrlich reagent²⁰, using the CEP dipeptide **6** as a quantitative standard. In contrast with the preparation of CEP-HSA by direct treatment with DOHA, that delivered a pyrrole to protein ratio of 1.6:1 for CEP-HSA⁶, the new synthetic method using DOHA-Fm provided CEP-HSA (**8a**) with a much higher pyrrole to protein ratio, 7.6 ± 1.1 to 1, and provided CEP-MSA (**8b**) with a pyrrole to protein ratio of 5.2 ± 1.0 to 1. To enable studies on the influence of pyrrole to protein ratio on biological activity, we incubated various amounts of the DOHA-Fm reagent with BSA to prepare CEP-BSA (**8c**) with pyrrole to protein ratios of 10.1 ± 1.0 , 7.0 ± 1.1 , 5.1 ± 1.0 and 3.7 ± 0.8 . The similar preparation of CEP-modified chicken egg albumin, rabbit myoglobin, and glycerol-3-phosphate dehydrogenase are detailed in the Experimental Procedures.

Characterization of CEP-modified proteins

To characterize the location of CEP modifications in proteins following reaction with DOHAFm and deprotection with DBU, the proteins were digested with trypsin (protein:trypsin = 50:1, w/w) and then analyzed by liquid chromatography tandem MS (LC MS/MS) with a CapLC system (Micromass, Beverly, MA) and a quadrupole time-of-flight mass spectrometer (QTOF2, Micromass).²¹ Peptides were separated on a 75 $\mu\text{m} \times 5$ cm Biobasic C18 column (New Objective, Cambridge, MA) by using aqueous formic acid/ acetonitrile solvents, a flow rate of 250 nL/min, and a gradient of 5-40% acetonitrile over 57 min followed by 80% acetonitrile for 2 min. Protein identifications from MS/MS data was accomplished with MASSLYNX 3.5 software (Waters), the Mascot search engine (Matrix Science) and the Swiss-protein and National Center for Biotechnology information protein sequence databases.^{22, 23} MS/MS analysis of modifications at specific *m/z* revealed peptide sequences with unambiguous CEP adducts on lysyl residues of the CEP-proteins. No CEPFm modifications were found after deprotection. We also analyzed CEPFm modified HSA before deprotection. We identified 3 CEPFm modifications at *m/z* 725.281, 660.289 and 776.820 on lysyl residues in the LC-MS/MS of HSA peptides ²⁵DAHKSEVAHR³⁴, ²³⁴AFKAWAVAR²⁴², and ²⁴⁷FPKAEFAEVSK²⁵⁷ in CEPFmHSA. We identified six CEP modifications at *m/z* 589.266, 571.260, 674.762, 881.401, 687.784, 636.252 on lysyl residues in the LC-MS/MS of HSA peptides ¹⁶¹KYLYEIR¹⁶⁸, ²³⁴AFKAWAVAR²⁴², ³⁵FKDLGEENFK⁴⁴, ⁴³⁸KVPQVSTPTLVEVSR⁴⁵², ²⁴⁷FPKAEFAEVSK²⁵⁷, and ²⁵DAHKSEVAHR³⁴ respectively. The similar LC-MS/MS analyses of CEP-modified chicken egg albumin, rabbit myoglobin, and glycerol-3-phosphate dehydrogenase are detailed in the Experimental Section.

CEP linked to proteins with an ω -aminohexanoyl tether

Direct coupling of DOHA-Fm to proteins results in a high yield of CEP modifications of lysyl residues. As an alternative approach for anchoring CEP haptens to proteins for use as coating agents to capture anti-CEP antibodies, we examined the utility of CEPs anchored to proteins through hexanoyl amides of protein lysyl residues. An Fm masked 2-carboxyethylpyrrole **9** was generated through the reaction of DOHA-Fm with 6-aminocaproic acid. After purification, **9** was activated by conversion into an N-hydroxysuccinimide ester **10**. Incubation of the active ester **10** with bovine serum albumin (BSA) followed by deprotection *in situ* by addition of DBU to the reaction mixture, delivered a 6-(2-carboxyethyl-1-pyrrolyl)hexanoyl amide derivative of BSA, CEPH-BSA (**11**, Scheme 5). Low molecular weight impurities were readily removed by dialysis (*M_r* cutoff 14000) with 20% DMF in 10 mM PBS 2 $\times$ 12 h and then with 10 mM PBS 2 $\times$ 12 h. The protein concentration was determined by a modified Lowry protein assay²⁴ using the Lowry protein assay reagent and Folin-Ciocalteu reagent. The pyrrole concentration was determined using Ehrlich assay. The pyrrole to BSA ratio in CEPH-BSA (**11**) was 5.4 ± 0.9 to 1.

The antibody binding affinity of CEPH-BSA (**11**) was determined by competitive enzyme-linked immunosorbent assay (ELISA)²⁵ using an anti-CEP-KLH polyclonal antibody (Figure 1). CEP-HSA was used as a coating agent and standard whose binding was inhibited by CEPH-BSA. The IC₅₀ of CEPH-BSA (1.93 pmol/mL) is lower than the IC₅₀ of CEP-HSA (3.02 pmol/mL) indicating that CEPH-BSA has a slightly higher affinity than CEP-HSA for binding anti-CEP-KLH antibody.

Synthesis of biotinylated CEP derivatives

A biotinylated CEP Fm ester (**15**) was prepared from DOHA-Fm and 4-amino butylbiotin (Scheme 6). Deprotection of the intermediate 3-(1-{4-[5-(2-Oxo-hexahydro-thieno[3,4-*d*]imidazol-4-yl)-pentanoylamino]-butyl}-1*H*-pyrrol-2-yl)-propionic acid 9*H*-fluoren-9-ylmethyl ester **15** by treatment with DBU in THF generated 3-(1-{4-[5-(2-Oxo-hexahydro-thieno[3,4-*d*]imidazol-4-yl)-pentanoylamino]-butyl}-1*H*-pyrrol-2-yl)-propionic acid (**16**).

All of the functionality in biotinylated CEP derivative **16** survived treatment with sodium hydroxide ethanol solution. This observation suggested the feasibility of simpler synthesis for preparing CEP derivatives of substrates that are stable to strong base. Thus, we prepared 3-(1-{4-[5-(2-oxo-hexahydro-thieno[3,4-*d*]imidazol-4-yl)-pentanoylamino]-hexyl}-1*H*-pyrrol-2-yl)-propionic acid (**20**) by reacting 4,7-dioxo-heptanoic acid methyl ester (DOHA-Me, **17**) with biotinyl-1,6-diaminohexane (**18**) followed by hydrolysis of the methyl ester **19** with ethanolic sodium hydroxide (Scheme 7).

Synthesis of ethanolamine phospholipid CEP derivatives

Phosphatidylethanolamines (PEs) are major components of certain membranes in the brain cells and in the photoreceptor cells of the retina. Because levels of PEs are strictly regulated, they are believed to have unique functional importance.²⁶ In view of the reactivity of the primary amino group of PEs and the abundance of DHA in brain and retina, we anticipate that DHA-derived oxidatively truncated phospholipids containing reactive electrophilic 4-hydroxy-7-oxohept-5-enoates convert the primary amino group of PEs into CEP-PE derivatives. We synthesized PE-CEPs to facilitate their detection and identification *in vivo*. Reaction of DOHA-Fm with 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE) or 1-palmitoyl-2-hydroxy-sn-glycero-3-phosphoethanolamine (lysoPE) followed by deprotection of the intermediate Fm esters **21** or **23** with DBU (Scheme 8) delivered the CEP-PEs **22** and **24**.

Synthesis of an active pentafluorophenyl ester of a lysyl CEP

Pentafluorophenyl esters of protected amino acids are widely used in peptide synthesis. We are interested in testing the ability of CEP modified peptides bound to major histocompatibility proteins to elicit an immune response to antigen specific T-cells. Furthermore, complexes of CEP modified peptides bound to constructs called “dimer X”, that have major histocompatibility proteins fused to immunoglobulin Fc constant regions, can be used to fluorescently label antigen specific T-cells, and consequently enable their quantitation by fluorescence activated cell sorting.²⁷ The pentafluorophenyl ester **26** of a CEP-Fm modified lysine was synthesized as a building block for construction of CEP modified peptides. Reaction of DOHA-Fm with 6-amino-2-(9H-fluoren-9-ylmethoxycarbonylamino)-hexanoic acid (Fmoc-Lys-OH) delivered **25**. The latter was then coupled with pentafluorophenol using the traditional DCC, DMAP method (Scheme 9).

Discussion

The CEP-modified tryptic peptides that we identified enabled determination of the specific lysyl groups that have been altered in various CEP-modified proteins. These tryptic peptides may also provide clues to the identity of the CEP-modified peptides involved in cellular immune responses. For example, major histocompatibility (MHC) class I proteins selectively bind peptides of 8-10 residues that have nonpolar “anchor residues” at or near their termini. Notably, the HSA tryptic nonapeptide ²³⁴AFKAWAVAR²⁴² amino acid sequence is very similar to the sequence ²³³ALKAWSVAR²⁴¹ in BSA and identical to the ²³⁴AFKAWAVAR²⁴² sequence in MSA (see Experimental Procedures). This sequence incorporates nonpolar amino acid residues on both ends and is appropriate for binding with MHC class I proteins. MS/MS analysis consistently detected modification on the ALKAWSVAR sequence of CEP-BSA in preparations generated with different CEP ratios, implying that this sequence is readily accessed and covalently altered by an aldehyde electrophile. Figure 2 shows a ball-and-stick model providing 3D visualization of HSA using PyMOL v0.99. X-ray crystallography of HSA reveals a dimer structure with two identical units. All lysines are shown as space-filling structures in the left unit while only the lysines that become incorporated into CEPs are shown as space-filling structures in the right unit. The lysine residue K236 in the ²³⁴AFKAWAVAR²⁴² sequence is marked. The 3D structure shows that this lysine is located at and protrudes from the surface of HSA. The physical environment and chemical characteristics of this specific lysyl residue probably facilitate access by DOHA-Fm and covalent adduction through Schiff base formation with its terminal aldehyde group leading to pyrrole formation. In vivo, pyrrole formation involves an analogous interaction between a protein and the aldehyde group of an oxidatively truncated phospholipid sn2-fatty acyl chain protruding like a whisker²⁸ from a membrane bilayer or the exterior of a lipoprotein particle. Based on the tryptic peptides identified above, further studies are underway to synthesize MHC-bound CEP modified peptides and to test their ability to elicit an immune response by antigen specific T-cells.

Immunoglobins and B-cells bind divalently through their variable (Fv) regions with haptens such as CEP. This can cause aggregation of proteins and/or cells displaying CEP modifications, and can activate B-cells. Furthermore, immunoglobins possess a constant (Fc) effector region that activates immune responses. In contrast, monoclonal scFv antibodies are monovalent constructs that contain only a single Fv binding region and no constant Fc region, and are therefore not expected to cause protein aggregation and deposition. Furthermore, monoclonal scFv antibodies are likely to favor penetration of the internal limiting membrane and access the subretinal space when injected intravitreally because of their relatively low molecular weight.²⁹ Therefore, we hypothesize that a monoclonal anti-CEP scFv antibody may be useful as a competitive inhibitor of CEP induced neovascularization. Specific human scFv antibodies

can be selected from engineered libraries of phage that display the antibody protein on their exterior. Selection is accomplished by using the corresponding hapten as “bait” to catch phage that produce (with the help of *E. coli*) and display antigen specific scFv on their exterior. One approach can exploit the CEP hapten described above, anchored through a biotin linker to streptavidin-coated magnetic beads.

Conclusions

The introduction of multiple CEP modifications of lysyl residues is readily achieved through reaction of proteins with DOHA-Fm, a 9-fluorenylmethyl ester of 4,7-dioxoheptanoic acid, followed by deprotection of intermediate Fm esters of CEPs, without causing protein denaturation, by treatment with DBU. CEP-modified mouse serum albumin available through this synthetic method was used to create a mouse model of AMD.¹¹ CEP-proteins and peptides prepared through this new methodology have also been used as biomarkers for clinical prognosis of AMD⁶, and for studies of their possible role in promoting pathological angiogenesis in “wet” AMD⁷, as well as mechanistic studies of their antigenicity, and apparent stimulation of complement accumulation and lesion formation in the “dry” form of AMD.¹¹ The medicinal and diagnostic utility of CEP derivatives described herein are under active investigation in our laboratories. We anticipate that the synthetic methodology reported above will facilitate studies on the activation of B- and T-cells by CEP-peptides. It will allow testing of the hypotheses that CEP derivatives represent a link between lipid oxidation and other manifestations of angiogenesis such as tumor growth or wound healing.

Experimental Section

6-[1,3]Dioxolan-2-yl-4-oxo-hexanoic acid methyl ester (1a)

A solution of 2-(2-bromoethyl)-1,3-dioxolane (1 g, 5.5 mmol) in anhydrous THF (2 mL) was added dropwise to a flame dried 100 mL flask with Mg turnings (150 mg, 6.25 mmol) and 4 mL THF and a small piece of I₂ under argon at room temperature to initiate the reaction. After adding a few drops, the reaction started as evidence by disappearance of the red-brown I₂ color. Then 5 mL THF was added to the flask. After completion of the addition, more THF (15 mL) was added. The reaction mixture was stirred for another 1 h and then cooled to -78 °C followed by slow addition of 3-carbomethoxypropionyl chloride (710 mg, 4.7 mmol) dissolved in 2.5 mL dry THF. The resulting mixture was stirred for another 40 min, then quenched with 30 mL of a saturated aqueous solution of NH₄Cl, and extracted with EtOAc (4 × 15 mL). The combined organic phase was washed with brine, dried with MgSO₄, and evaporated to obtain the crude product. The crude compound was purified by silica gel chromatography (30 % ethyl acetate in hexane, TLC: *R_f* = 0.3) to give 714 mg (60 %) of pure **1**. ¹H NMR (CDCl₃, 200 MHz) δ4.85 (t, *J* = 4.3 Hz, 1H), 3.7-3.90 (4H), 3.62 (s, 3H), 2.67 (m, 2H), 2.56 (t, *J* = 7.48 Hz, 4H), 1.93 (dt, *J* = 4.3, 7.48 Hz, 2H). ¹³C NMR (CDCl₃, 50 MHz, APT) δ207.81 (+) (CO), 173.09 (+) (COO), 103.06 (-) (CH), 64.83 (+) (CH₂), 51.61 (-) (CH₃), 36.87 (+) (CH₂), 36.30 (+) (CH₂), 27.62 (+) (CH₂), 27.41 (+) (CH₂). HRMS (FAB) (*m/z*) calcd for C₁₀H₁₇O₅ (MH⁺) 217.1076, found 217.1081.

6-[1,3]Dioxan-2-yl-4-oxo-hexanoic acid methyl ester (1b)

A solution of 2-(2-bromoethyl)-1,3-dioxane (16 g, 84 mmol) in anhydrous THF (32 mL) was added dropwise to a flame dried 1000 mL flask with Mg turnings (2.4 g, 100 mmol) and 64 mL THF and a small piece of I₂ under argon at room temperature to initiate the reaction. After adding a few drops, the reaction started as evidence by disappearance of the red-brown I₂ color. Then 50 mL THF was added to the flask. After completion of the addition, more THF (30 mL) was added. The reaction mixture was stirred for another 1 h and then cooled to -78 °C followed by slow addition of 3-carbomethoxypropionyl chloride (11.5 g, 74 mmol) dissolved in 40 mL

dry THF. The resulting mixture was stirred for another 40 min, then quenched with 200 mL of a saturated aqueous solution of NH_4Cl , and extracted with EtOAc (4×200 mL). The combined organic phase was washed with brine, dried with MgSO_4 , and evaporated to obtain the crude product. The crude compound was purified by distillation to give 13.4 g (78 %) of pure **1b**. ^1H NMR (CDCl_3 , 400 MHz) δ 4.57 (t, $J = 5.2$ Hz, 1H), 4.05-4.10 (m, 2H), 3.71-3.78 (m, 2H), 3.67 (s, 3H), 2.74 (t, $J = 6.6$ Hz, 2H), 2.59 (t, $J = 7.0$ Hz, 4H), 2.00-2.10 (m, 1H), 1.89-1.92 (m, 2H), 1.30-1.36 (m, 1H). ^{13}C NMR (CDCl_3 , 400 MHz) δ 208.51 (CO), 173.49 (COO), 101.03 (CH), 67.05 (CH_2), 52.01 (CH_3), 37.32 (CH_2), 36.82 (CH_2), 29.16 (CH_2), 27.96 (CH_2), 25.91 (CH_2). HRMS (FAB) (m/z) calcd for $\text{C}_{11}\text{H}_{19}\text{O}_5$ (MH^+) 231.1188, found 231.2631.

6-[1,3]Dioxolan-2-yl-4-oxo-hexanoic acid (**2a**)

Ester **1a** (390 mg, 1.8 mmol) in 10 mL of $\text{H}_2\text{O}/\text{MeOH}/\text{THF}$ (2:5:3, v/v/v) was stirred for 3 h with NaOH (367 mg, 9.2 mmol) at room temperature. The reaction mixture was then acidified with 3 N HCl to pH 3.0 and extracted with EtOAc (3×15 mL). The combined organic phase was washed with brine, dried with MgSO_4 , and evaporated to give acid acetal **2a** (360 mg, 90%), b.p. $129^\circ\text{C}/0.25$ mm Hg. ^1H NMR (CDCl_3 , 200 MHz) δ 4.85 (t, $J = 4.3$ Hz, 1H), 3.7-3.90 (4H), 2.5-2.64 (6H), 1.93 (dt, $J = 4.3$, 7.48 Hz, 2H). ^{13}C NMR (CDCl_3 , 100 MHz) δ 207.81 (CO), 178.03 (COOH), 103.17 (CH), 64.97 (CH_2), 36.76 (CH_2), 36.36 (CH_2), 27.71 (CH_2), 27.51 (CH_2). HRMS (FAB) (m/z) calcd for $\text{C}_9\text{H}_{15}\text{O}_5$ (MH^+) 203.0919, found 203.0917.

6-[1,3]Dioxan-2-yl-4-oxo-hexanoic acid (**2b**)

Ester **1b** (3.4 g, 14.8 mmol) in 90 mL of $\text{H}_2\text{O}/\text{MeOH}/\text{THF}$ (2:5:3, v/v/v) was stirred for 3 h with NaOH (3.1 g, 77.5 mmol) at room temperature. The reaction mixture was then acidified with 3 N HCl to pH 3.0 and extracted with EtOAc (3×80 mL). The combined organic phase was washed with brine, dried with MgSO_4 , and evaporated to give the acid acetal **2b** (2.9 g, 92%) that was crystallized from CH_2Cl_2 /hexane 1:4 to deliver white crystals, m.p. $98-9^\circ\text{C}$. ^1H NMR (CDCl_3 , 400 MHz) δ 4.57 (t, $J = 5.2$ Hz, 1H), 4.05-4.10 (m, 2H), 3.71-3.78 (m, 2H), 2.74 (t, $J = 6.6$ Hz, 2H), 2.55-2.67 (m, 4H), 2.00-2.10 (m, 1H), 1.89-1.92 (m, 2H), 1.30-1.36 (m, 1H). ^{13}C NMR (CDCl_3 , 400 MHz) δ 208.44 (CO), 178.79 (COO), 101.01 (CH), 67.05 (CH_2), 37.05 (CH_2), 36.75 (CH_2), 29.16 (CH_2), 28.01 (CH_2), 25.91 (CH_2). HRMS (FAB) (m/z) calcd for $\text{C}_{10}\text{H}_{15}\text{O}_5$ (M-H) 215.1006, found 215.6009.

6-[1,3]Dioxolan-2-yl-4-oxo-hexanoic acid 9H-fluoren-9-ylmethyl ester (**3a**)

(9H-Fluoren-9-yl)-methanol (373 mg, 1.9 mmol) in 3 mL dry CH_2Cl_2 was slowly added to the solution of dicyclohexylcarbodiimide (DCC, 295 mg, 1.425 mmol), dimethylamino pyridine (DMAP, 58 mg, 0.475 mmol) and the acid **2a** (96 mg, 0.475 mmol) in 5 mL dry CH_2Cl_2 . The resulting mixture was stirred for 72 h at room temperature. The solvent was removed. Flash chromatography of the residue (30 % ethyl acetate in hexane, TLC: $R_f = 0.3$) gave the ester **3a** (153 mg, 95 %) as a colorless oil. ^1H NMR (CDCl_3 , 200 MHz) δ 7.77 (d, $J = 6.74$ Hz, 2H), 7.60 (d, $J = 7.7$ Hz, 2H), 7.42 (dd, $J = 6.74$, 7.7 Hz, 2H), 7.29 (dd, $J = 6.74$, 7.7 Hz, 2H), 4.90 (t, $J = 4.3$ Hz, 1H), 4.40 (d, $J = 7.38$ Hz, 2H), 4.21 (t, $J = 7.38$ Hz, 1H), 3.80-3.96 (4H), 2.62-2.73 (4H), 2.56 (t, $J = 7.3$ Hz, 2H), 1.98 (td, $J = 7.3$, 4.3 Hz, 2H). ^{13}C NMR (CDCl_3 , 50 MHz, APT), 207.76 (+) (CO), 172.62 (+) (COO), 143.69 (+) (C), 141.19 (+) (C), 127.69 (-) (CH), 127.04 (-) (CH), 125.00 (-) (CH), 119.92 (-) (CH), 103.12 (-) (CH), 66.45 (+) (CH_2), 64.90 (+) (CH_2), 46.67 (-) (CH), 36.89 (+) (CH_2), 36.35 (+) (CH_2), 27.90 (+) (CH_2), 27.48 (+) (CH_2). HRMS (FAB) (m/z) calcd for $\text{C}_{23}\text{H}_{24}\text{O}_5$ (M^+) 380.1624, found 380.1614; calcd for $\text{C}_{23}\text{H}_{25}\text{O}_5$ (MH^+) 381.1702, found 381.1711.

6-[1,3]Dioxan-2-yl-4-oxo-hexanoic acid 9H-fluoren-9-ylmethyl ester (3b)

In a 25 mL flame-dried round-bottom flask equipped with a magnetic stirring bar, acid **2b** (331 mg, 1.53 mmol) was dissolved in 4 mL of dry dichloromethane and cooled to -20°C . A commercially available solution of dicyclohexylcarbodiimide (Aldrich, Milwaukee, 1.0 M in dichloromethane, 2 mL, 2 mmol) was added dropwise and the solution stirred at temperature for 20 min during which time a white precipitate formed. A solution of 9-fluorenemethanol (364 mg, 1.85 mmol) and N,N-dimethylaminopyridine (36 mg, 0.29 mmol) in dry dichloromethane (3 mL) in a flame-dried 15 mL round-bottom flask equipped with a magnetic stirring bar was added dropwise via cannula to the reaction mixture at -10°C and left stirring 18.5 h to slowly warm to room temperature. The solution was concentrated via rotary evaporation, dissolved in ethyl acetate, allowed to sit for 2 h at -20°C , and then filtered. The filtrate was concentrated and then purified by flash chromatography using 20% ethyl acetate/hexanes to give **3b** (512 mg, 85%) as a white crystalline solid, m.p. $67.5\text{--}69.5^{\circ}\text{C}$, ($R_f = 0.10$ in 25% ethyl acetate/hexanes). ^1H NMR (400 MHz, CDCl_3) δ 7.77 (dt, $J = 0.9, 7.6$ Hz, 5H), 7.60 (ddd, $J = 0.8, 1.9, 7.5$ Hz, 2H), 7.41 (tdd, $J = 0.6, 1.2, 7.5$ Hz, 2H), 7.32 (td, $J = 1.2, 7.5$ Hz, 2H), 4.56 (t, $J = 4.9$ Hz, 1H), 4.38 (d, $J = 7.2$ Hz, 2H), 4.22 (t, $J = 7.2$ Hz, 1H), 4.10–4.04 (m, 2H), 3.78–3.69 (m, 2H), 2.77–2.64 (4H), 2.57 (t, $J = 7.3$ Hz, 2H), 2.12–1.97 (m, 1H), 1.90 (td, $J = 4.9, 7.3$ Hz, 2H), 1.36–1.28 (ddt, $J = 1.2, 2.5, 13.4$, 1H). ^{13}C NMR (100 MHz, CDCl_3) δ 208.14, 172.70, 143.74, 141.24, 127.73, 127.08, 125.04, 119.97, 100.77, 77.32, 77.00, 76.68, 66.80, 66.51, 46.71, 37.02, 36.56, 28.93, 27.95, 25.70. HRMS (FAB) (m/z) calcd for $\text{C}_{24}\text{H}_{27}\text{O}_5$ (MH^+) 395.1814, found 395.1620.

4,7-Dioxo-heptanoic acid 9H-fluoren-9-ylmethyl ester (DOHA-Fm, 4)

Method A: ester **3a** (94 mg, 0.25 mmol) in 10 mL of $\text{AcOH}/\text{H}_2\text{O}$ (3:1, v/v) was stirred at 50°C for 5 h. TLC (100 % CHCl_3): $R_f = 0.5$ showed the completion of the reaction. The solvent was removed by rotary evaporation. Flash chromatography of the residue (25 % ethyl acetate in hexane) gave **4** (73 mg, 88 %). ^1H NMR (CDCl_3 , 400 MHz), δ 9.78 (s, 1H), 7.77 (d, $J = 6.74$ Hz, 2H), 7.60 (d, $J = 7.7$ Hz, 2H), 7.42 (dd, $J = 6.74, 7.7$ Hz, 2H), 7.30 (dd, $J = 6.74, 7.7$ Hz, 2H), 4.39 (d, $J = 7.38$ Hz, 2H), 4.20 (t, $J = 7.38$ Hz, 1H), 2.67–2.79 (8H). ^{13}C NMR (CDCl_3 , 100 MHz), δ 206.56 (CO), 200.27 (CHO), 172.53 (COO), 143.70 (C), 141.23 (C), 127.73 (CH), 127.07 (CH), 124.98 (CH), 119.96 (CH), 66.47 (CH_2), 46.70 (CH), 37.42 (CH_2), 36.87 (CH_2), 34.53 (CH_2), 27.93 (CH_2). HRMS (FAB) (m/z) calcd for $\text{C}_{21}\text{H}_{20}\text{O}_4$ (M^+) 336.1362, found 336.1361. **Method B:** Ester **3b** (94 mg, 0.25 mmol) in 10 mL of $\text{AcOH}/\text{H}_2\text{O}$ (3:1, v/v) was stirred at 50°C for 5 h. TLC (100 % CHCl_3): $R_f = 0.5$ showed the completion of the reaction. The solvent was removed by rotary evaporation. Flash chromatography of the residue (25 % ethyl acetate in hexane) gave **4** (73 mg, 88 %).

2-(2-Acetylamino-acetylamino)-6-{2-[2-(9H-fluoren-9-ylmethoxycarbonyl)-ethyl]-pyrrol-1-yl}-hexanoic acid methyl ester (5)

Methyl 6-amino-2-((2-acetylamino)acetyl)amino hexanoate (Ac-Gly-Lys-OMe, 25.6 mg, 0.08 mmol) in 1 mL methanol was added dropwise to DOHAFm (27 mg, 0.08 mmol) in 1.5 mL methanol. The solution was stirred for 9 h at room temperature under argon. The solvent was removed by rotary evaporation. TLC (4 % methanol in chloroform): $R_f = 0.34$. The crude compound was purified by silica gel chromatography (4 % methanol in chloroform) to give 35.8 mg (80%) of pure **5**. ^1H NMR (CDCl_3 , 400 MHz), δ 7.77 (d, $J = 7.6$ Hz, 2H), 7.60 (d, $J = 7.6$ Hz, 2H), 7.36–7.32 (dd, $J = 7.6, 7.2$ Hz, 2H), 7.27 (dd, $J = 7.6, 7.2$ Hz, 2H), 6.60 (dd, $J = 2.4, 1.6$ Hz, 1H), 6.54 (m, 1H), 6.28 (s, 1H), 6.09 (dd, $J = 3.6, 2.4$ Hz, 1H), 5.90 (m, 1H), 4.56–4.61 (m, 1H), 4.40 (d, $J = 7.2$ Hz, 2H), 4.21 (t, $J = 7.2$ Hz, 1H), 3.91 (dABq, $J = 5.2, 14$ Hz, 2H), 3.82 (t, $J = 7.2$ Hz, 2H), 3.76 (s, 3H), 2.65–2.86 (4H), 2.01 (s, 3H), 1.86 (m, 2H), 1.66 (m, 2H), 1.36 (m, 2H). ^{13}C NMR (CDCl_3 , 100 MHz), 172.98 (CO), 172.33 (CO), 170.58 (CO), 168.66 (CO), 143.74 (C), 141.31 (C), 130.73 (C), 127.80 (CH), 127.12 (CH), 125.01 (CH),

120.37 (CH), 120.04 (CH), 106.99 (CH), 105.26 (CH), 66.47 (CH₂), 52.53 (CH), 51.86 (CH₃O), 46.78 (CH), 46.09 (CH₂), 43.23 (CH₂), 33.22 (CH₂), 31.89 (CH₂), 30.67 (CH₂), 22.92 (CH₂), 22.40 (CH₂), 21.41 (CH₃). HRMS (FAB) (*m/z*) calcd for C₃₂H₃₈N₃O₆⁺ (MH⁺) 560.2755, found 560.2747.

2-(2-Acetylamino-acetylamino)-6-[2-(2-carboxy-ethyl)-pyrrol-1-yl]-hexanoic acid methyl ester (CEP-dipep, 6)

DBU (75 μ L) was added to **5** (27 mg, 0.047 mmol) in 2.5 mL THF. The system was stirred for 6 h under argon. After the removal of solvent, the crude compound was purified by silica gel chromatography (6 % methanol in chloroform) to give 21 mg (86 %) of CEP-dipep. TLC (10 % methanol in chloroform): *R_f* = 0.3; ¹H NMR (CDCl₃, 400 MHz), δ 7.18 (d, *J* = 8 Hz, 1H), 6.81 (m, 1H), 6.53 (m, 1H), 6.03 (dd, *J* = 3.2, 3.2 Hz, 1H), 5.87 (m, 1H), 4.53 (dt, *J* = 1H), 3.95 (dABq, *J* = 5.2, 13.6 Hz, 2H), 3.81 (t, *J* = 6.8 Hz, 2H), 3.70 (s, 3H), 2.65-2.86 (4H), 2.01 (s, 3H), 1.8-1.62 (4H), 1.36 (m, 2H). ¹³C NMR (CDCl₃, 100 MHz), δ 176.01 (COOH), 172.40 (COO), 171.35 (CO), 169.28 (CO), 131.40 (C), 119.92 (CH), 107.25 (CH), 105.00 (CH), 52.50 (CH), 52.00 (CH₃O), 45.66 (CH₂), 43.19 (CH₂), 32.98 (CH₂), 31.44 (CH₂), 30.90 (CH₂), 22.94 (CH₂), 22.07 (CH₂), 21.37 (CH₃). HRMS (FAB) (*m/z*) calcd for C₁₈H₂₈N₃O₆⁺ (MH⁺) 382.1978, found 382.1986.

Tryptic Digestion

CEP modified proteins were dissolved in NH₄HCO₃ buffer (8 M urea/2M NH₄HCO₃) to make a final concentration 2-3 pmol/ μ L. Trypsin solution (0.1 μ g/ μ L) was made by suspending sequencing grade modified porcine trypsin (20 μ g) (Promega, Cat. V511A) in trypsin resuspension buffer (Promega, Cat. V511A) (200 μ L). This trypsin solution was then added to protein solution in a ratio of 1:50 (w/w) enzyme/protein. After incubation at 37 °C for 24 h, the solution was centrifuged at 3000 rpm for 5 min. The upper layer (10 μ L) was subjected to LC-MS analysis on a CapLC system (Micromass, Beverly, MA) and a quadrupole time-of-flight mass spectrometer (QTOF2, Micromass) using MassLynx 3.5 software, the Mascot search engine (Matrix Science) and the Swiss-protein and National Center for Biotechnology information protein sequence databases..³⁰

Carboxyethylpyrrole-modified human serum albumin (CEP-HSA, 8a)

A solution of DOHA-Fm (2 mg, 0.006 mmol) in 750 μ L DMF was added to 1.5 mL 0.08 mM solution of HSA in PBS. The mixture was stirred under argon for 4 days. 200 μ L DBU was added to the system and stirred overnight under argon followed by two successive 12 h dialyses (*Mr* cutoff 14000) against 500 mL 20 % DMF in 10 mM PBS (pH 7.4) and two additional dialyses (12 h each) against 500 mL 10 mM PBS (pH 7.4) at 4 °C. The final protein concentration (1.80 mg/mL) was determined by the Pierce bicinchoninic acid (BCA) protein assay. The pyrrole concentration (187.14 μ M) was determined by Ehrlich assay. LC-MS/MS revealed six CEP modifications at *m/z* 589.266, 571.260, 674.762, 881.401, 687.784, 636.252 on lysyl residues of the HSA

peptides ¹⁶¹KYLYEIAR¹⁶⁸, ²³⁴AFKAWAVAR²⁴², ³⁵FKDLGEENFK⁴⁴, ⁴³⁸KVPQVSTPTLVEVS-R⁴⁵², ²⁴⁷FPKAEFAEVSK²⁵⁷, ²⁵DAHKSEVAHR³⁴ respectively. No CEPFm modifications were found after deprotection. Figure 3 shows the tandem MS spectrum of the doubly charged ion *m/z* 571.260 from a MS scan of tryptic digested CEP-HSA. The spectrum shows a series of fragment ions (b ions and y ions) sufficient to identify a CEP modification on Lys²³⁶ from HSA residues ²³⁴AFKAWAVAR²⁴². This particular sequence is very similar with the sequence ²³³ALKAWSVAR²⁴¹ in BSA and is exactly the same as in MSA. MS/MS analysis revealed unambiguous modification on the ALKAWSVAR sequence of CEP-BSA and the AFKAWAVAR sequence of MSA.

Carboxyethylpyrrole-modified mouse serum albumin (CEP-MSA, 8b)

A solution of DOHAFm (18.5 mg, 0.055 mmol) in 8 mL DMF was added slowly to the solution of 100 mg mouse serum albumin in 18 mL 10 mM PBS (pH 7.4). The mixture was stirred under argon for 4 days. 360 μ L DBU was added to the system and stirred overnight under argon followed by two successive 24 h dialyses (*Mr* cutoff 14000) against 1 L 20 % DMF in 10 mM PBS (pH 7.4) and two additional dialyses (24 h each) against 1 L 10 mM PBS (pH 7.4) at 4 °C. The final protein concentration (2.43 mg/mL) was determined by the Pierce bicinchoninic acid (BCA) protein assay. The pyrrole concentration (210 μ M) was determined by Ehrlich assay. LC-MS/MS revealed 15 CEP modifications at m/z 650.3296, 872.9611, 571.3492, 849.4302, 612.8407, 769.4069, 694.6888, 1047.0802, 857.4633 on lysyl residues of the MSA peptides ²⁵EAHKSEI AHR³⁴, ²⁰⁶LDGVKEKALVSSVR²¹⁹ (2 CEPs), ²³⁴AFKAWAVAR²⁴², ²⁴³LSQTFPNADF AEITKLATDLTK²⁶⁴, ²⁵⁸LATDLTKVNK²⁶⁷, ³⁷³LAKKYEATLEK³⁸³ (2 CEPs), ⁴³⁵YTQKAPQVSTPTLVEAAR⁴⁵², ⁵⁴⁴EKQIKKQTALAEVK⁵⁵⁸ (3 CEPs), ⁵⁵⁰QTA LAELVKHKPKATAEQLK⁵⁶⁹ (3 CEPs) respectively. The sequence coverage was 35%. No CEPFm modifications were found after deprotection. The tandem MS spectrum of AFKAWAVAR was shown in Figure 4.

Carboxyethylpyrrole-modified bovine serum albumin (CEP-BSA, 8c)

Eight different samples with CEP:BSA ratios from 0.6 to 10.0 were prepared. A solution containing various amounts (see Table 1) of DOHAFm in 8 mL DMF was added dropwise over 30 min to a solution of 100 mg BSA in 18 mL 10 mM PBS (pH 7.4). The mixture was stirred under argon for 4 days. Various amounts of DBU (see Table 1) were added and the resulting mixture was stirred overnight under argon followed by two successive 24 h dialyses (*Mr* cutoff 14000) against 2 L of 20 % DMF in 10 mM PBS (pH 7.4) and two additional dialyses (24 h each) against 2 L of 10 mM PBS (pH 7.4) at 4 °C. The final protein concentration was determined by the Pierce bicinchoninic acid (BCA) protein assay, while the pyrrole concentration was measured by Ehrlich assay. The final protein concentrations, pyrrole concentrations, and CEP:BSA ratios are shown in Table 1.

Carboxyethylpyrrole-modified chicken egg albumin (CEP-CEO)

A solution of DOHAFm (18 mg, 0.054 mmol) in DMF (3.2 mL) was added slowly to the solution of CEO (76 mg) in pH 7.4 PBS (10 mM, 7 mL). The mixture was stirred under argon for 4 days. DBU (140 μ L) was added and the resulting mixture was stirred overnight under argon followed by two successive 24 h dialyses (*Mr* cutoff 3500) against 1 L 20% DMF in 10 mM pH 7.4 PBS and two more dialyses (24 h each) against 1 L 10 mM pH 7.4 PBS at 4 °C. The final protein concentration (2.94 mg/mL) was determined by the Pierce bicinchoninic acid (BCA) protein assay. The pyrrole concentration (93 μ M) was determined by Ehrlich assay. LC-MS/MS revealed eight CEP modifications at m/z 540.2886, 801.7021, 839.3332, 808.0259, 610.2589, 630.8334, 459.1887 on lysyl residues of the CEO peptides ⁵¹TQINKVVR⁵⁸, ⁸⁵DILNQITKPNQVYSFSLASR¹⁰⁴, ¹⁸⁷AFK DEDTQAMPFR¹⁹⁹, ²⁰⁰VTEQESKPVQMMYQIGLFR²¹⁸, ²¹⁹VASMASEKMK²²⁸, ²⁷⁷KIK VYLPR²⁸⁴, ²⁸⁵MKMEEK²⁹⁰ respectively. The sequence coverage was 46%. No CEPFm modifications were found after deprotection.

Carboxyethylpyrrole-modified myoglobin (CEP-myoglobin)

A solution of DOHAFm (12 mg, 0.036 mmol) in DMF (3.6 mL) was added slowly to the solution of myoglobin (32.38 mg) in pH 7.4 PBS (10 mM, 5 mL). The mixture was stirred under argon for 4 days. DBU (140 μ L) was added and the resulting mixture was stirred overnight under argon followed by two successive 24 h dialyses (*Mr* cutoff 3500) against 500 mL 20% DMF in 10 mM pH 7.4 PBS and two more dialyses (24 h each) against 500 mL 10

mM pH 7.4 PBS at 4 °C. The final protein concentration (0.65 mg/mL) was determined by Bio-Rad protein assay. The pyrrole concentration (91 µM) was determined by Ehrlich assay. LC-MS/MS revealed CEP modifications at *m/z* 671.2667, 604.9635, 815.2087, 557.4288, 785.6061, 899.6666, on lysyl residue of the myoglobin peptides ³²LFTGHPETLEKFDKF KHLK⁵⁰ (3 CEP modifications), ⁴⁸HLKTEAEMK⁵⁶, ⁶³KHGT VVLTAALGGILK⁷⁷, ⁷⁹KGHHEAELKPLAQSHATK⁹⁶, ¹¹⁹HPGDFGADAQGAMTKALE LFR¹³⁹, ¹⁴⁰NDIAAKYKELGFQG¹⁵³ (2 CEP modifications). The peptide sequence coverage achieved 91%. No CEPFm modifications were found after deprotection.

Carboxyethylpyrrole-modified GPDH (CEP-GPDH)

GPDH (30 mg) was added in 10 mL of 10 mM PBS (pH 7.4). After vortexing for 5 min and centrifugation (4 °C) at 3000 rpm for 10 min (GPDH does not dissolve well in PBS), 4 mL of clear solution was taken and DOHAFm (5.8 mg, 0.017 mmol) in DMF (4 mL) was added slowly to it. The mixture was stirred under argon for 4 days. DBU (120 µL) was then added and the resulting mixture was stirred overnight under argon followed by two successive 24 h dialyses (*M_r* cutoff 3500) against 500 mL 20% DMF in 10 mM pH 7.4 PBS and two more dialyses (24 h each) against 500 mL 10 mM pH 7.4 PBS at 4 °C. The final protein concentration (0.29 mg/mL) was determined by the Bio-Rad protein assay. The pyrrole concentration (65 µM) was determined by Ehrlich assay. LC-MS/MS revealed six CEP modifications at *m/z* 655.3343, 669.3289, 923.9847, 639.3413, 740.8362 on lysyl residues of the GPDH peptides ¹⁰⁵AGAHLKGGAKR¹¹⁵, ¹⁸⁴TVDGPSGKLWR¹⁹⁴, ¹⁹⁸GAAQNIIPASTGAAK AVGK²¹⁶, ²⁵⁸VVKQASEGPLK²⁶⁸, ³²¹VVDLMVHMASKE³³² respectively. The peptide sequence coverage achieved 45%. No CEPFm modifications were found after deprotection.

6-{2-[2-(9*H*-Fluoren-9-ylmethoxycarbonyl)-ethyl]-pyrrol-1-yl}-hexanoic acid (9)

6-aminocaproic acid (10.8 mg, 0.082 mmol) in 400 µL water was slowly added to DOHA-Fm (21 mg, 0.0625 mmol) in 600 µL methanol. The solution became cloudy with the addition. The heterogeneous system was stirred for 48 h under argon at room temperature and became homogenous. The solution was extracted with CH₂Cl₂, washed with brine, dried with Na₂SO₄ and evaporated, the yellowish residue was loaded to the silica gel in a filter with chloroform and washed with 15 mL chloroform, 15 mL 10 % ethyl acetate in hexane, 15 mL 20 % ethyl acetate in hexane and 60 mL 50 % ethyl acetate in hexane successively. The 50 % ethyl acetate/hexane washed fractions were collected and dried to give 21.8 mg (81 %) acid **9**. TLC (ethyl acetate/hexane, 2:3 v/v): *R_f* = 0.22. ¹H NMR (CDCl₃, 400 MHz), δ 7.77 (d, *J* = 6.74 Hz, 2H), 7.58 (d, *J* = 7.7 Hz, 2H), 7.42 (dd, *J* = 7.7, 7.2 Hz, 2H), 7.30 (dd, *J* = 6.74, 7.2 Hz, 2H), 6.58 (dd, *J* = 3.2, 3.6 Hz, 1H), 6.06 (dd, *J* = 3.2, 3.2 Hz, 1H), 5.87 (m, 1H), 4.42 (d, *J* = 7.2 Hz, 2H), 4.22 (t, *J* = 7.2 Hz, 1H), 3.79 (t, *J* = 7.2 Hz, 2H), 2.72-2.84 (m, 4H), 2.34 (t, *J* = 7.2 Hz, 2H), 1.75-1.34 (6H). ¹³C NMR (CDCl₃, 100 MHz), δ 177.40 (COOH), 172.90 (COO), 143.73 (C), 141.20 (C), 130.66 (C), 127.78 (CH), 127.10 (CH), 125.00 (CH), 120.34 (CH), 120.04 (CH), 106.88 (CH), 105.20 (CH), 66.40 (CH₂), 46.79 (CH₂), 46.28 (CH), 33.45 (CH₂), 33.20 (CH₂), 31.01 (CH₂), 26.23 (CH₂), 24.26 (CH₂), 21.44 (CH₂). HRMS (FAB) (*m/z*) calcd for C₂₇H₃₀NO₄ (MH⁺) 432.2175, found 432.2190.

6-{2-[2-(9*H*-Fluoren-9-ylmethoxycarbonyl)-ethyl]-pyrrol-1-yl}-hexanoic acid 2,5-dioxo-pyrrolidin-1-yl ester (CEPFmSu, 10)

Acid **9** (15 mg, 0.035 mmol) and N-hydroxysuccinimide (4.5 mg, 0.039 mmol), DCC (7.5 mg, 0.036 mmol) were dissolved in dry CH₂Cl₂ (7.5 mL) under Argon. The clear solution became cloudy after 15 minutes. The reaction mixture was stirred for 3.5 h. Solvent was removed by evaporation. The crude product was purified by silica gel chromatography with ethyl acetate/hexane (2:3, v/v) to deliver 16.6 mg (90 %) active ester **10**. TLC (ethyl acetate/hexane, 2:3): *R_f* = 0.25. ¹H NMR (CDCl₃, 400 MHz), δ 7.77 (d, *J* = 6.74 Hz, 2H), 7.58 (d, *J* = 7.7 Hz, 2H),

7.42 (dd, $J = 7.7, 7.2$ Hz, 2H), 7.30 (dd, $J = 6.74, 7.2$ Hz, 2H), 6.58 (dd, $J = 3.2, 3.6$ Hz, 1H), 6.06 (dd, $J = 3.2, 3.2$ Hz, 1H), 5.87 (m, 1H), 4.42 (d, $J = 7.2$ Hz, 2H), 4.22 (t, $J = 7.2$ Hz, 1H), 3.79 (t, $J = 7.2$ Hz, 2H), 2.72-2.86 (m, 8H), 2.60 (t, $J = 7.2$ Hz, 2H), 1.81-1.70 (m, 4H), 1.48-1.40 (m, 2H). ^{13}C NMR (CDCl_3 100 MHz), δ 172.79 (COO), 169.10 (COO), 168.39 (CO), 143.75 (C), 141.29 (C), 130.69 (C), 127.77 (CH), 127.10 (CH), 125.01 (CH), 120.34 (CH), 120.01 (CH), 106.92 (CH), 105.24 (CH), 66.36 (CH_2), 46.79 (CH_2), 46.21 (CH), 33.31 (CH_2), 30.86 (CH_2), 30.72 (CH_2), 25.87 (CH_2), 25.55 (CH_2), 24.25 (CH_2), 21.44 (CH_2). HRMS (FAB) (m/z) calcd for $\text{C}_{31}\text{H}_{33}\text{N}_2\text{O}_6$ (MH^+) 529.2338, found 529.2340.

6-(2-Carboxyethyl-1-pyrrolyl)-hexanoyl BSA amide (CEPH-BSA, 11)

A solution of 1.4 mg CEPFmSu in 150 μL DMF was added to 1 mL of 10 mM pH 7.4 PBS containing 3 mg/mL of BSA. The cloudy solution became homogenous with overnight stirring. After 2 days, 25 μL DBU was added and the resulting mixture stirred overnight under argon followed by two successive 12 h dialysis (M_r cutoff 14000) against 500 mL 20 % DMF in 10 mM PBS (pH 7.4) and two additional dialysis (12 h each) against 500 mL 10 mM PBS (pH 7.4) at 4 $^\circ\text{C}$. The final protein concentration (1.34 mg/mL) was determined by modified Lowry protein assay. The pyrrole concentration (134 μM) was determined by Ehrlich assay. LC-MS/MS revealed nine CEPH modifications at m/z 594.9934, 618.8188, 541.8060, 937.9577, 526.7835, 612.3604, 689.4319, 765.3984, 964.5088 corresponding to the BSA peptides $^{400}\text{LKHLVDEPQNLIK}^{412}$, $^{233}\text{ALKAWSVAR}^{241}$, $^{242}\text{LSQKFPK}^{248}$, $^{437}\text{KVPQVSTPTLVEVSR}^{451}$, $^{452}\text{SLGKVGTR}^{459}$, $^{490}\text{TPVSEK}^{498}$, $^{548}\text{KQTALVELLK}^{557}$, $^{246}\text{FPKAEFVEVTK}^{256}$, $^{249}\text{AEFVEVTKLV TDLTK}^{263}$ respectively and unambiguously confirmed CEPH adducts on lysyl residues. No CEPHFm modifications were found after deprotection.

6-(2-Carboxyethyl-1-pyrrolyl)-hexanoyl MSA amide (CEPH-MSA)

A solution of CEPFmSu (630 μg) in DMF (150 μL) was added in MSA (2 mg/mL, 1 mL) in PBS (10 mM, pH 7.4). The cloudy solution became clear with overnight stirring. After 2 days, DBU (25 μL) was added and the resulting mixture was stirred overnight under argon followed by two successive 12 h dialyses (M_r cutoff 3500) against 500 mL 20% DMF in PBS (10 mM, pH 7.4) and two more dialyses (12 h each) against 500 mL PBS (10 mM, pH 7.4) at 4 $^\circ\text{C}$. The final protein concentration (2.06 mg/mL) was determined by modified Lowry protein assay. The pyrrole concentration (69.5 μM) was determined by Ehrlich assay. LC-MS/MS revealed four CEPH modifications at m/z 512.2829, 627.8604, 608.8177, 732.3707 on lysyl residues of the MSA peptides $^{206}\text{LDGVKEK}^{212}$, $^{234}\text{AFKAWAVAR}^{242}$, $^{376}\text{KYEATLEK}^{383}$, $^{435}\text{YTQKAPQVSTPTLVEAAR}^{452}$, respectively. No CEPHFm modifications were found after deprotection.

6-(2-Carboxyethyl-1-pyrrolyl)-hexanoyl CEO amide (CEPH-CEO)

A solution of CEPFmSu (1.0 mg) in DMF (150 μL) was added in CEO (4 mg/mL, 1 mL) in PBS (10 mM, pH 7.4). After the coupling underwent for 2 days, the solution was still not clear until the addition of DBU (25 μL). The system was stirred overnight under argon followed by two successive 12 h dialyses (M_r cutoff 14000) against 500 mL 20% DMF in PBS (10 mM, pH 7.4) and two more dialyses (12 h each) against 500 mL PBS (10 mM, pH 7.4) at 4 $^\circ\text{C}$. The final protein concentration (3.46 mg/mL) was determined by modified Lowry protein assay. The pyrrole concentration (85.4 μM) was determined by Ehrlich assay. LC-MS/MS revealed a CEPH modification at m/z 596.8715 on a lysyl residue of the CEO peptide $^{51}\text{TQINKVVR}^{58}$. No CEPHFm modifications were found after deprotection.

6-(2-Carboxyethyl-1-pyrrolyl)-hexanoyl GPDH amide (CEPH-GPDH)

GPDH (10 mg) was added in PBS (10 mM, 10 mL). After vortexing for 5 min and centrifugation (4 °C) at 3000 rpm for 10 min, the upper clear solution (2 ml) was taken and CEPFmSu (770 µg) in DMF (300 µL) was added. The cloudy solution became clear after 2 days. Then DBU (25 µL) was added and the resulting mixture was stirred overnight under argon followed by two successive 12 h dialyses (*Mr* cutoff 3500) against 500 mL 20% DMF in PBS (10 mM, pH 7.4) and two more dialyses (12 h each) against 500 mL PBS (10 mM, pH 7.4) at 4 °C. The final protein concentration (0.32 mg/mL) was determined by modified Lowry protein assay. The pyrrole concentration (32.5 µM) was determined by Ehrlich assay. LC-MS/MS revealed CEPH modifications at *m/z* 635.0367, 712.5500, 919.7650, 572.9763, 899.6923, 726.0422, 980.7797, 731.1005, 941.7422, 579.9560, 508.9000, 696.1018, 797.5823 on lysyl residues of the GPDH

peptides ¹VKVG VNGFGR¹⁰, ⁵³FHGTVK AENGK⁶³, ⁶⁴LVI NGKAITIFQER⁷⁷, ¹⁰⁵AGAHL KGGAK¹¹⁴, ¹⁶⁰VIHDHFGIVEGLMTTVHAITA TQKTVDGPGSGK¹⁹¹, ¹⁸⁴TVDGPGSKLWR¹⁹⁴, ¹⁹⁸GAAQNIIPASTGAAKAVGK²¹⁶, ²¹³AVGKVIPELNGK²²⁴, ²¹⁷VIPELNGKLTGMAFR²³¹, ²⁴⁹AAKYDDIK²⁵⁶, ²⁵²YDDIK K²⁵⁷, ²⁵⁸VVKQASEGPLK²⁶⁸, and ³²¹VVDLMVHMASKE³³². No CEPHm modifications were found after deprotection. The peptide sequence coverage achieved 68%.

{4-[5-(2-oxo-hexahydro-thieno[3,4-d]imidazol-4-yl)-pentanoylamino]-butyl}-carbamic acid tert-butyl ester (13)

N-Boc-1,4-butanediamine (20 mg, 0.106 mmol) in 2 mL CH₂Cl₂ was added in *d*-biotin *p*-nitrophenyl ester (38 mg, 0.10 mmol) in 2 mL CH₂Cl₂. The system was stirred for 10 h. TLC (30 % ethyl acetate in hexane): *R_f* = 0.25. After removal of the solvent by rotary evaporation, the crude compound was purified by flash chromatography (30 % ethyl acetate in hexane) to give 28 mg (80 %) **13**. ¹H NMR (CD₃OD, 400 MHz), δ4.47-4.50 (m, 1H), 4.28-4.31 (m, 1H), 3.2 (m, 1H), 3.16 (t, *J* = 7.2 Hz, 2H), 3.03 (t, *J* = 6.8 Hz, 2H), 2.92 (dd, *J* = 12.4, 4.8 Hz, 1H), 2.68-2.71 (d, *J* = 12.8 Hz, 1H), 2.19 (t, *J* = 7.6 Hz, 2H), 1.44-1.7 (10H), 1.42 (s, 9H).

5-(2-Oxo-hexahydro-thieno[3,4-d]imidazol-4-yl)-pentanoic acid (4-amino-butyl)-amide (14)

100 µL trifluoroacetic acid was added in 6 mg **13** in 400 µL CH₂Cl₂. After stirring for 3 h, TLC (20% methanol in chloroform, *R_f* = 0.1) showed the reaction was completed. The solvent was removed by rotary evaporation. The yellowish residue was neutralized by 1N NaOH to pH 8.5. After removal of the solvent, the residue was treated with methanol and the methanol extraction was concentrated and dried to give 4 mg (90%) **14**. ¹H NMR (CD₃OD, 400 MHz), δ4.46-4.51 (m, 1H), 4.28-4.31 (m, 1H), 3.2 (m, 1H), 3.16 (t, *J* = 7.2 Hz, 2H), 2.9 (dd, *J* = 12.4, 4.8 Hz, 1H), 2.68-2.71 (d, *J* = 12.4 Hz, 1H), 2.65 (t, *J* = 7.2 Hz, 2H), 2.19 (t, *J* = 7.6 Hz, 2H), 1.34-1.7 (10H).

3-(1-{4-[5-(2-Oxo-hexahydro-thieno[3,4-d]imidazol-4-yl)-pentanoylamino]-butyl}-1H-pyrrol-2-yl)-propionic acid 9H-fluoren-9-yl-methyl ester (15)

Amine **14** (4 mg, 0.012 mmol) in 150 µL methanol was added in DOHA-Fm (4 mg, 0.0119 mmol) in 150 µL methanol. The solution was stirred for 9 h at room temperature under argon. The solvent was removed by rotary evaporation. TLC (5 % methanol in CHCl₃): *R_f* = 0.2. The crude compound was purified by silica gel chromatography (5 % methanol in chloroform) to give 6 mg (84 %) of pure **15**. ¹H NMR (CDCl₃, 400 MHz), δ7.70 (d, *J* = 7.6 Hz, 2H), 7.50 (dd, *J* = 7.6, 1.2 Hz, 2H), 7.34 (dd, *J* = 7.6, 7.2 Hz, 2H), 7.27-7.23 (ddd, *J* = 7.6, 7.2, 1.2 Hz, 2H), 6.51 (dd, *J* = 1.6, 2.8 Hz, 1H), 5.98 (dd, *J* = 2.8, 3.6 Hz, 1H), 5.79 (m, 1H), 5.74 (m, 1H), 5.58 (s, 1H), 4.82 (s, 1H), 4.40 (m, 1H), 4.36 (d, *J* = 7.2 Hz, 2H), 4.20 (m, 1H), 4.15 (t, *J* = 7.2 Hz, 1H), 3.76 (t, *J* = 7.2 Hz, 2H), 3.15 (m, 2H), 3.05 (m, 1H), 2.84-2.79 (dd, *J* = 4.8, 12.8 Hz, 1H), 2.78-2.76 (m, 2H), 2.71-2.66 (m, 2H), 2.64-2.61 (d, *J* = 12.8 Hz, 1H), 2.06-2.10 (dt, *J* = 3.6,

7.2 Hz, 2H), 1.72-1.36 (10H). HRMS (FAB) (m/z) calcd for $C_{35}H_{43}N_4O_4S^+$ (MH^+) 615.3005, found 615.2995.

3-(1-{4-[5-(2-Oxo-hexahydro-thieno[3,4-*d*]imidazol-4-yl)-pentanoylamino]-butyl}-1*H*-pyrrol-2-yl)-propionic acid (16)

5 μ L DBU was added to **15** (6 mg, 0.01 mmol) in 500 μ L THF and stirred for 2 h. TLC (10 % methanol in chloroform, R_f = 0.3) showed the completion of the reaction by disappearance of the UV active starting spot. The corresponding acid was purified by flash chromatography (7 % methanol in chloroform) to provide 3.7 mg (85 %) acid **16**. 1H NMR (CD_3OD , 400 MHz), δ 6.57 (dd, J = 2.8, 1.6 Hz, 1H), 5.92 (dd, J = 3.2, 2.8 Hz, 1H), 5.79 (m, 1H), 4.60 (3H), 4.48 (m, 1H), 4.27 (m, 1H), 3.86 (t, J = 7.2 Hz, 2H), 3.19-3.15 (3H), 2.94-2.89 (dd, J = 4.8, 12.8 Hz, 1H), 2.86-2.82 (m, 2H), 2.71-2.67 (d, J = 12.8 Hz, 1H), 2.60-2.56 (m, 2H), 2.20-2.17 (t, J = 7.2 Hz, 2H), 1.72-1.36 (10H). HRMS (FAB) (m/z) calcd for $C_{21}H_{33}N_4O_4S^+$ (MH^+) 437.2222, found 437.2193; calcd for $C_{21}H_{31}N_4O_3S^+$ (M^+-OH) 419.2117, found 419.2102.

4,7-Dioxo-heptanoic acid methyl ester (DOHA-Me, 17)

Ester **1** (22.5 mg, 0.104 mmol) in 5 mL of $AcOH/H_2O$ (3:1, v/v) was stirred at 50 °C for 5 h. TLC ($CHCl_3$): R_f = 0.45 showed the completion of the reaction. The solvent was removed by rotary evaporation. Flash chromatography of the residue (25 % ethyl acetate in hexane) gave **17** (15.6 mg, 87 %). 1H NMR ($CDCl_3$, 200 MHz), δ 9.78 (s, 1H), 3.67 (s, 3H), 2.56-2.81 (8H); HRMS (FAB) (m/z) calcd for $C_8H_{11}O_4^+$ (M^+-H) 171.0658, found 171.0656; calcd for $C_8H_{13}O_5^+$ (M^++OH) 189.0763, found 189.0783.

5-(2-Oxo-hexahydro-thieno[3,4-*d*]imidazol-4-yl)-pentanoic acid (6-amino-hexyl)-amide (18)

1,6-diaminohexane (270 mg, 2.324 mmol) in 10 mL pyridine- H_2O (9:1, v/v) was added slowly to *d*-biotin *p*-nitrophenyl ester (Sigma) (100 mg, 0.274 mmol) in 20 mL pyridine- H_2O (9:1, v/v). The clear yellow solution was stirred for 24 h at room temperature. The solvent was removed by rotary evaporation. TLC (30% NH_3 -saturated methanol in $CHCl_3$, v/v), R_f = 0.24. The crude compound was purified by silica gel chromatography (30 % methanol saturated with NH_3 in chloroform) to give 84 mg (90 %) of pure **18**. 1H NMR (CD_3OD , 400 MHz), δ 4.46-4.51 (m, 1H), 4.28-4.31 (m, 1H), 3.20 (m, 1H), 3.16 (t, J = 7.2 Hz, 2H), 2.9 (dd, J = 12.4, 4.8 Hz, 1H), 2.68-2.71 (d, J = 12.4 Hz, 1H), 2.65 (t, J = 7.2 Hz, 2H), 2.19 (t, J = 7.6 Hz, 2H), 1.34-1.7 (14H). ^{13}C NMR (CD_3OD , 100 MHz), δ 175.97 (CO), 166.10 (CO), 63.36 (CH), 61.60 (CH), 57.01 (CH), 42.3 (CH_2), 42.0 (CH_2), 40.2 (CH_2), 36.8 (CH_2), 33.2 (CH_2), 30.50 (CH_2), 29.8 (CH_2), 29.5 (CH_2), 28.8 (CH_2), 28.6 (CH_2), 28.0 (CH_2). HRMS (FAB) (m/z) calcd for $C_{16}H_{31}N_4O_2S^+$ (MH^+) 343.2168, found 343.2165.

3-(1-{6-[5-(2-Oxo-hexahydro-thieno[3,4-*d*]imidazol-4-yl)-pentanoylamino]-hexyl}-1*H*-pyrrol-2-yl)-propionic acid methyl ester (19)

Amine **18** (45 mg, 0.13 mmol) in 1 mL methanol was added to DOHA-Me (23 mg, 0.13 mmol) in 1.5 mL methanol. The solution was stirred for 9 h at room temperature under argon. The solvent was removed by rotary evaporation. The crude compound was purified by silica gel chromatography (5 % methanol in chloroform, TLC: R_f = 0.18) to give 47 mg (75 %) of pure **19**. 1H NMR (CD_3OD , 400 MHz), δ 6.52 (dd, J = 2.0, 2.8 Hz, 1H), 5.9 (dd, J = 3.2, 2.8 Hz, 1H), 5.75 (m, 1H), 4.46-4.49 (dd, J = 8.0, 4.0 Hz, 1H), 4.29 (dd, J = 8.0, 4.8 Hz, 1H), 4.28-4.3 (m, 1H), 3.82 (t, J = 7.6 Hz, 2H), 3.65 (s, 3H), 3.1-3.2 (3H), 2.9 (dd, J = 12.4, 4.8 Hz, 1H), 2.85 (t, J = 7.2 Hz, 2H), 2.68-2.71 (d, J = 12.4 Hz, 1H), 2.65 (t, J = 7.2 Hz, 2H), 2.19 (t, J = 7.6 Hz, 2H), 1.34-1.7 (14H).

3-(1-{4-[5-(2-Oxo-hexahydro-thieno[3,4-d]imidazol-4-yl)-pentanoylamino]-hexyl}-1H-pyrrol-2-yl)-propionic acid (20)

Sodium hydroxide (16 mg, 0.4 mmol) was added to **19** (47 mg, 0.1 mmol) in 1 mL absolute ethanol and stirred for 4 h at room temperature. TLC (10 % methanol in chloroform, R_f = 0.3) showed the completion of the reaction by disappearance of the starting spot. After removal of the solvent, the residue was neutralized with 3 N HCl to pH = 3 and extracted with ethyl acetate. The ethyl acetate extract was washed with brine, dried with $MgSO_4$, and concentrated to afford 38 mg (84 %) **20**. 1H NMR (CD_3OD , 400 MHz), δ 6.57 (dd, J = 2.0, 2.4 Hz, 1H), 5.93 (dd, J = 2.4, 3.6 Hz, 1H), 5.80 (m, 1H), 4.46 (dd, J = 7.6, 5.2 Hz, 1H), 4.29 (dd, J = 4.8, 7.6 Hz, 1H), 3.84 (t, J = 7.6 Hz, 2H), 3.1-3.2 (3H), 2.9 (dd, J = 12.4, 4.8 Hz, 1H), 2.85 (t, J = 7.2 Hz, 2H), 2.68-2.71 (d, J = 12.4 Hz, 1H), 2.61 (t, J = 7.2 Hz, 2H), 2.19 (t, J = 7.2 Hz, 2H), 1.34-1.7 (14H). ^{13}C NMR (CD_3OD , 100 MHz), δ 176.82 (CO), 175.97 (CO), 166.10 (CO), 132.00 (C), 121.22 (CH), 107.52 (CH), 106.08 (CH), 63.36 (CH), 61.60 (CH), 57.01 (CH), 47.19 (CH₂), 41.03 (CH₂), 40.22 (CH₂), 36.82 (CH₂), 34.48 (CH₂), 32.54 (CH₂), 30.30 (CH₂), 29.78 (CH₂), 29.51 (CH₂), 27.62 (CH₂), 27.44 (CH₂), 26.95 (CH₂), 22.55 (CH₂). HRMS (FAB) (m/z) calcd for $C_{23}H_{37}N_4O_4S^+$ (MH^+) 465.2530, found 465.2524.

Octadec-9-enoic acid 2-[(2-{2-[2-(9H-fluoren-9-ylmethoxycarbonyl)-ethyl]-pyrrol-1-yl}-ethoxy)-hydroxy-phosphoryloxy]-1-hexadecanoyloxymethyl-ethyl ester (21)

Triethylamine (TEA) (25 μ L, 0.247 mmol) was added to POPE (50 mg, 0.065 mmol) in 500 μ L $CHCl_3$, then DOHAFm (22 mg, 0.065 mmol) in 500 μ L $CHCl_3$ was added to the mixture. The system was stirred 20 h under Argon. After evaporation of solvent, the crude product was purified by silica gel chromatography (10 % methanol in chloroform, TLC: R_f = 0.2) to give 50 mg (76 %) of pure **21**. 1H NMR ($CD_3OD:CDCl_3$ = 1:1, 400 MHz), δ 7.74 (d, J = 7.6 Hz, 2H), 7.53 (d, J = 7.6 Hz, 2H), 7.36 (dd, J = 7.6, 7.6 Hz, 2H), 7.27 (dd, J = 7.6, 7.6 Hz, 2H), 6.63 (dd, J = 2.8, 1.6 Hz, 1H), 5.94 (dd, J = 3.2, 2.8 Hz, 1H), 5.77 (m, 1H), 5.28 (m, 2H), 4.36 (d, J = 6.8 Hz, 2H), 4.28 (m, 1H), 4.17 (t, J = 6.8 Hz, 1H), 4.05-3.98 (6H), 3.68 (t, J = 6.4 Hz, 2H), 2.86-2.82 (t, J = 7.2 Hz, 2H), 2.73-2.69 (t, J = 7.2 Hz, 2H), 2.25-2.21 (4H), 2.0-1.94 (4H), 1.57 (4H), 1.21 (44H), 0.84 (t, J = 6.4 Hz, 6H). ^{13}C NMR ($CD_3OD:CDCl_3$ = 1:1, 50 MHz, APT), δ 175.21 (+) (CO), 174.84 (+) (CO), 174.81 (+) (CO), 145.08 (+) (C), 142.68 (+) (C), 132.30 (+) (C), 131.24 (-) (CH), 130.95 (-) (CH), 129.13 (-) (CH), 128.53 (-) (CH), 126.31 (-) (CH), 121.30 (-) (CH), 121.22 (-) (CH), 108.49 (-) (CH), 106.77 (-) (CH), 71.86 (-) (CH), 67.96 (+) (CH₂), 66.62 (+) (CH₂), 64.60 (+) (CH₂), 64.05 (+) (CH₂), 48.10 (+) (CH₂), 47.82 (-) (CH), 35.47 (+) (CH₂), 35.34 (+) (CH₂), 34.66 (+) (CH₂), 33.27 (+) (CH₂), 31.03 (+) (CH₂), 30.85 (+) (CH₂), 30.70 (+) (CH₂), 30.66 (+) (CH₂), 30.61 (+) (CH₂), 30.49 (+) (CH₂), 28.49 (+) (CH₂), 26.24 (+) (CH₂), 26.20 (+) (CH₂), 23.98 (+) (CH₂), 22.64 (+) (CH₂), 15.14 (-) (CH₃). HRMS (FAB) (m/z) calcd for $C_{60}H_{91}NNa_2O_{10}P^+$ [(M-H)Na₂⁺] 1062.6177, found 1062.6.

Octadec-9-enoic acid 2-[(2-{2-(2-carboxy-ethyl)-pyrrol-1-yl}-ethoxy)-hydroxy-phosphoryloxy]-1-hexadecanoyloxymethyl-ethyl ester (PE-CEP, 22)

DBU (20 μ L, 0.13 mmol) was added to **21** (24 mg, 0.025 mmol) in 500 μ L $CHCl_3$. After 3 h, the reaction was completed and the system was diluted by 1.5 mL $CHCl_3$. The solution was washed with 2 mL phosphate buffer of pH 5.5. The organic phase was washed with brine, dried over magnesium sulfate, then vacuum-filtered, concentrated, purified by flash chromatography (15 % methanol in chloroform, TLC: R_f = 0.18) to yield 19 mg (90 %) of pure **22**. 1H NMR ($CD_3OD:CDCl_3$ = 1:1, 400 MHz), δ 6.56 (m, 1H), 5.96 (m, 1H), 5.83 (m, 1H), 5.30 (m, 2H), 5.15 (m, 1H), 4.33 (m, 1H), 4.05-3.98 (6H), 3.81 (m, 2H), 2.83 (m, 2H), 2.58 (m, 2H), 2.27 (t, J = 7.2 Hz, 4H), 2.0-1.94 (4H), 1.57 (4H), 1.21 (44H), 0.84 (t, J = 6.4 Hz, 6H). ^{13}C NMR ($CD_3OD:CDCl_3$ = 1:1, 100 MHz), δ 180.36 (COOH), 174.49 (CO), 174.08 (CO), 133.54 (C), 130.46 (CH), 130.14 (CH), 120.98 (CH), 107.26 (CH), 105.05 (CH), 71.00 (CH), 65.84

(CH₂), 64.03 (CH₂), 63.11 (CH₂), 47.29 (CH₂), 34.70 (CH₂), 34.56 (CH₂), 32.40 (CH₂), 32.42 (CH₂), 30.23 (CH₂), 30.17 (CH₂), 30.00 (CH₂), 29.80 (CH₂), 29.63 (CH₂), 27.66 (CH₂), 25.40 (CH₂), 23.13 (CH₂), 14.31 (CH₃). HRMS (FAB) (*m/z*) calcd for C₄₆H₈₂NNaO₁₀P⁺ (MNa⁺) 862.5574, found 862.5550; calcd for C₄₆H₈₁NNa₂O₁₀P⁺ [(M-H)Na₂⁺] 884.5394, found 884.5291.

Hexadecanoic acid 3-[(2-{2-[2-(9H-fluoren-9-ylmethoxycarbonyl)-ethyl]-pyrrol-1-yl}-ethoxy)-hydroxy-phosphoryloxy]-2-hydroxy-propyl ester (23)

Triethylamine (TEA) (12 μ L, 0.116 mmol) was added to lyso-PE (42 mg, 0.093 mmol) in 500 μ L CHCl₃, then DOHAFm (26 mg, 0.077 mmol) in 500 μ L CHCl₃ was added to the mixture. The cloudy system was stirred 20 h under Ar. After evaporation of solvent, the crude product was purified by silica gel chromatography (10 % methanol in chloroform, TLC: *R_f* = 0.2) to give 40 mg (69 %) of pure **23**. ¹H NMR (CDCl₃, 400 MHz), δ 7.70 (d, *J* = 7.6 Hz, 2H), 7.52 (d, *J* = 7.6 Hz, 2H), 7.35 (dd, *J* = 7.6, 7.6 Hz, 2H), 7.25 (dd, *J* = 7.6, 7.6 Hz, 2H), 6.58 (m, 1H), 5.94 (m, 1H), 5.74 (m, 1H), 5.28 (m, 2H), 4.31 (d, *J* = 6.8 Hz, 2H), 4.14 (t, *J* = 6.8 Hz, 1H), 4.03-3.60 (9H), 2.82-2.78 (t, *J* = 7.2 Hz, 2H), 2.69-2.65 (t, *J* = 7.2 Hz, 2H), 2.21-2.17 (t, *J* = 7.2 Hz, 4H), 1.54-1.45 (m, 2H), 1.23-1.19 (24H), 0.86 (t, *J* = 6.8 Hz, 3H). ¹³C NMR (CDCl₃, 100 MHz), δ 173.69 (CO), 173.42 (CO), 143.69 (C), 141.22 (C), 131.07 (CH), 127.77 (CH), 127.09 (CH), 125.02 (CH), 120.97 (CH), 119.98 (CH), 107.20 (CH), 105.34 (CH), 77.20 (CH), 69.07 (CH₂), 66.66 (CH₂), 65.37 (CH₂), 64.30 (CH₂), 46.65 (CH₂), 46.35 (CH), 33.97 (CH₂), 33.11 (CH₂), 31.92 (CH₂), 29.73 (CH₂), 29.67 (CH₂), 29.58 (CH₂), 29.37 (CH₂), 29.23 (CH₂), 24.83 (CH₂), 22.69 (CH₂), 21.23 (CH₂), 14.11 (CH₃). HRMS (FAB) (*m/z*) calcd for C₄₁H₅₈NNaO₉P⁺ (MNa⁺) 776.3904, found 776.3939; calc. for C₄₁H₅₇NNa₂O₉P⁺ (MNa₂⁺) 798.3724, found 798.3717.

Hexadecanoic acid 3-(pp-hydroxy-phosphoryloxy)-2-hydroxy-propyl ester (lysoPE-CEP, 24)

DBU (20 μ L, 0.13 mmol) was added to **23** (24 mg, 0.03 mmol) in 500 μ L CHCl₃. After 3 h, the reaction was completed and the system was diluted by 1.5 mL CHCl₃. The solution was washed with 2 mL phosphate buffer of pH 5.5. The organic part was washed with brine, dried over magnesium sulfate and vacuum-filtered, concentrated, purified by flash chromatography (CHCl₃:MeOH:H₂O = 65:25:4, v/v, TLC: *R_f* = 0.18) to yield 16 mg (95 %) of pure lysoPE-CEP. ¹H NMR (CD₃OD:CDCl₃ = 1:1, 400 MHz), δ 6.60 (m, 1H), 5.96 (m, 1H), 5.83 (m, 1H), 4.10-4.0 (5H), 3.74-3.6 (m, 2H), 3.6-3.5 (m, 2H), 2.8-2.9 (m, 2H), 2.68-2.65 (m, 2H), 2.32 (t, *J* = 6.8 Hz, 2H), 1.57 (m, 2H), 1.23 (24H), 0.85 (t, *J* = 7.2 Hz, 3H). ¹³C NMR (CD₃OD:CDCl₃:D₂O = 50:50:1, 100 MHz), δ 175.20 (CO), 133.00 (C), 121.23 (CH), 107.36 (CH), 105.56 (CH), 70.80 (CH), 66.05 (CH₂), 65.63 (CH₂), 47.17 (CH₂), 34.61 (CH₂), 33.29 (CH₂), 32.48 (CH₂), 30.22 (CH₂), 30.05 (CH₂), 29.90 (CH₂), 29.88 (CH₂), 29.72 (CH₂), 27.04 (CH₂), 25.42 (CH₂), 23.21 (CH₂), 14.38 (CH₃). HRMS (FAB) (*m/z*) calcd for C₂₇H₄₈NNaO₉P⁺ (MNa⁺) 598.3121, found 598.3053.

2-(9H-Fluoren-9-ylmethoxycarbonylamino)-6-{2-[2-(9H-fluoren-9-ylmethoxycarbonyl)-ethyl]-pyrrol-1-yl}-hexanoic acid (25)

6-amino-2-(9H-fluoren-9-ylmethoxycarbonyl amino)-hexanoic acid (40 mg, 0.1 mmol) was suspended in 10 mL methanol with DOHAFm (30 mg, 0.089 mmol). Then 20 μ L of acetic acid was added. The suspension dissolved gradually and a light yellow oil was generated at the bottom of the flask as the reaction proceeded. The system was stirred 24 h under Ar. After the removal of solvent, the crude compound was purified by silica gel chromatography (4 % methanol in chloroform) to give 45 mg (75 %) of light yellow oil **25**. TLC (4 % methanol in chloroform): *R_f* = 0.15; ¹H NMR (CDCl₃, 400 MHz), δ 7.75 (d, *J* = 7.2 Hz, 4H), 7.55 (d, *J* = 7.6 Hz, 4H), 7.39 (dd, *J* = 7.2, 7.6 Hz, 4H), 7.30 (dd, *J* = 7.2, 7.6 Hz, 4H), 6.57 (m, 1H), 6.06 (dd, *J* = 3.2, 3.2 Hz, 1H), 5.87 (m, 1H), 5.4 (m, 1H), 4.41 (d, *J* = 6.8 Hz, 2H), 4.39 (m, 1H),

4.20 (t, $J = 6.8$ Hz, 2H), 3.78 (t, $J = 6.8$ Hz, 2H), 2.85-2.73 (4H), 1.91-1.39 (6H). ^{13}C NMR (CDCl_3 , 100 MHz), δ 176.58 (COOH), 173.08 (COO), 156.04 (CO), 143.75 (C), 143.66 (C), 141.25 (C), 130.58 (C), 127.75 (CH), 127.69 (CH), 127.07 (CH), 127.03 (CH), 125.01 (CH), 124.95 (CH), 119.99 (CH), 106.99 (CH), 105.23 (CH), 67.04 (CH_2), 66.46 (CH_2), 53.49 (CH), 47.07 (CH), 46.71 (CH), 46.04 (CH_2), 33.18 (CH_2), 31.84 (CH_2), 30.73 (CH_2), 22.40 (CH_2), 21.37 (CH_2). HRMS (FAB) (m/z) calcd for $\text{C}_{42}\text{H}_{41}\text{N}_2\text{O}_6^+$ (MH^+) 669.2959, found 669.2949.

2-(9H-Fluoren-9-ylmethoxycarbonylamino)-6-{2-[2-(9H-fluoren-9-ylmethoxycarbonyl)-ethyl]-pyrrol-1-yl}-hexanoic acid pentafluorophenyl ester (26)

10 mL freshly distilled CH_2Cl_2 was added to the mixture of pentafluorophenol (50 mg, 0.27 mmol), dicyclohexylcarbodiimide (DCC, 41.5 mg, 0.201 mmol), dimethylamino pyridine (DMAP, 8 mg, 0.067 mmol) and acid **25** (45 mg, 0.067 mmol). The resulting mixture was stirred for 72 h at room temperature. The solvent was removed. Flash chromatography of the residue (15 % ethyl acetate in hexane, TLC: $R_f = 0.2$) gave low melting white solid **26** (50 mg, 90 %). ^1H NMR (CDCl_3 , 200 MHz) δ 7.75 (dd, $J = 6.8, 6.8$ Hz, 4H), 7.56 (dd, $J = 7.2, 7.2$ Hz, 4H), 7.38 (dd, $J = 7.2, 7.6$ Hz, 4H), 7.29 (4H), 6.58 (dd, $J = 3.2, 1.6$ Hz, 1H), 6.06 (dd, $J = 3.2, 3.2$ Hz, 1H), 5.87 (m, 1H), 5.38 (d, $J = 8.2$ Hz, 1H), 4.39-4.45 (5H), 4.17-4.22 (m, 2H), 3.84 (t, $J = 7.2$ Hz, 2H), 2.86 (t, $J = 6.8$ Hz, 2H), 2.77 (t, $J = 6.8$ Hz, 2H), 2.0-1.46 (6H). ^{13}C NMR (CDCl_3 , 100 MHz), δ 173.14 (COO), 168.96 (COO), 156.09 (COO), 143.93 (C), 143.82 (C), 141.57 (C), 141.51 (C), 130.94 (C), 128.03 (CH), 127.32 (CH), 125.22 (CH), 120.57 (CH), 120.25 (CH), 107.41 (CH), 105.62 (CH), 67.46 (CH_2), 66.73 (CH_2), 53.83 (CH), 47.32 (CH), 46.99 (CH), 46.31 (CH_2), 33.40 (CH_2), 32.18 (CH_2), 30.99 (CH_2), 22.72 (CH_2), 21.66 (CH_2). HRMS (FAB) (m/z) calcd for $\text{C}_{48}\text{H}_{40}\text{F}_5\text{N}_2\text{O}_6^+$ (MH^+) 835.2801, found 835.2813.

Competitive ELISA for inhibition of anti-CEP antibody binding to CEP-HSA by CEPH-BSA

CEP-HSA was used as a coating agent and a standard, CEPH-BSA was used as an inhibitor. A blank, a positive control containing no inhibitor, and up to 8 serial dilutions of the inhibitor and 8 serial dilutions of the CEP-HSA standard were run. Each well of the ELISA plate was coated with CEP-HSA solution (100 μL), prepared by diluting a solution containing 187.14 nmol/mL HSA-bound CEP in PBS to 187.14 pmol/mL with pH 7.4 PBS (10 mM). The plate was incubated at 37 °C for 1 h, then washed with 10 mM PBS ($3 \times 300 \mu\text{L}$), and then blocked by incubating 1 h at 37 °C with 300 μL of 1 % chicken ovalbumin (COA) in 10 mM PBS. The plate was then rinsed with 0.1 % COA in 10 mM PBS (300 μL). Eight serial dilutions of CEPH-BSA inhibitor or CEP-HSA standard (120 μL each with a dilution factor of 0.2) were preincubated at 37 °C for 1 h with anti-CEP-KLH antibody solution (120 μL) that was prepared by adding 5 μL of protein G column-purified antibody (1.8 mg/mL) in PBS to 10 mL of 0.2% COA in 10 mM PBS. The initial inhibitor and standard concentrations were 1162 pmol/mL and 935 pmol/mL, respectively. These were prepared by diluting a CEPH-BSA solution (116.2 nmol/mL) or CEP-HSA solution (187.14 nmol/mL) with 10 mM PBS, respectively. Blank wells were filled with 0.1 % COA (100 μL). Positive control wells were filled with the diluted antibody solution (50 μL) and PBS (50 μL). The antibody-antigen complex solutions (100 μL) were then added in duplicate to their respective halves of the plate, which was then incubated at room temperature with gentle agitation on a shaker for 1 h. After the supernatant was discarded, the wells were washed with 0.1 % COA ($3 \times 300 \mu\text{L}$), and then 100 μL of goat anti-rabbit IgG-alkaline phosphatase solution (Boehringer-Mannheim, Indianapolis, Indiana) which was prepared by adding 10 μL of the commercial enzyme-linked secondary antibody in 10 mL of 1 % COA was added. The plate was then incubated at room temperature with gentle agitation for 1 h and washed with 0.1 % COA ($3 \times 300 \mu\text{L}$). 100 μL of a solution of 1.0 mg/mL of *p*-nitrophenyl phosphate in 0.2 M Tris buffer (Sigma-Aldrich, Milwaukee, WI, Cat. Sigma N1891) was added. The plate was then incubated at room temperature for 20 min until the maximum absorbance reached 0.6-0.8. The development was terminated by adding 3 N NaOH (50 μL) to each well before measuring the final absorbance values. The absorbance in each

well was measured with a dual-wavelength Bio-Rad 450 microplate reader with detection at 405 nm relative to 655 nm. Absorbance values for duplicate assays were averaged and scaled to make the maximum curve fit value close to 100 percent. The averaged and scaled percent absorbance values were plotted against the log of concentration. Theoretical curves for each plot were fit to the absorbance data with a four parameter logistic function, $f(x) = (a-d)/[1+(x/c)^b] + d$ using SigmaPlot 9.0 (Jandel Scientific Software, San Rafael, CA). Parameter a = the asymptotic maximum absorbance, b = slope at the inflection point, c = the inhibitor concentration at the 50 % absorbance value (IC₅₀), and d = the asymptotic minimum absorbance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

AMD, age-related macular degeneration
BSA, bovine serum albumin
CEP, carboxyethylpyrrole
CEPH, 6-(2-carboxyethyl-1-pyrrolyl)-hexanoyl
COA, chicken ovalbumin
DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene
DHA, docosahexaenoic acid
DMF, N,N-dimethylformamide
DOHA, 4,7-dioxoheptanoic acid
ELISA, enzyme-linked immunosorbent assay
FmOH, 9-florenylmethanol
GPDH, glyceraldehyde phosphate dehydrogenase
HSA, human serum albumin
MSA, mouse serum albumin
PBS, phosphate buffered saline
THF, tetrahydrofuran

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Scheme 1.

Scheme 2.

Scheme 3.

Scheme 4.

Scheme 5.

Scheme 6.

Scheme 7.

Scheme 8.

Scheme 9.

Figure 1.
Inhibition curves showing cross-reactivity of the anti-CEP-KLH antibody for CEP-HSA (•) and CEPH-BSA (◆) against CEP-HSA as coating agent.

Figure 2.

A ball-and-stick model 3D structure for HSA. All the lysines are shown as space-filling structures in the left unit. Only lysines that become CEP modified are shown in the right unit. The lysine (K236) in the sequence, which is common to HSA, BSA and MSA, is marked.

Figure 3.

Tandem MS characterization of the doubly charged ion m/z 571.260 from a MS scan of tryptic digested CEP modified HSA shows series of fragment ions sufficient to unambiguously identify a CEP modification on the lysyl residue (K236). Asterisks denote fragment ions with a modified lysyl residue.

Figure 4.

Tandem MS characterization of the doubly charged ion m/z 571.3492 from a MS scan of tryptic digested CEP modified MSA shows series of fragment ions sufficient to unambiguously identify a CEP modification on the lysyl residue (K236). Asterisks denote fragment ions with a modified lysyl residue.

Table 1

Generation of various CEP:BSA ratios.

Sample	DOHA-Fm (mg)	DBU (μ L)	[BSA] (mg/mL)	[pyrrole] (μ M)	CEP:BSA (mean $\pm$ SD) [†]
1	36	720	3.37	496	10.1 $\pm$.0
2	18	360	3.14	348	7.0 $\pm$ 1.1
3	9	180	3.13	248	5.1 $\pm$ 1.0
4	5	90	2.97	167	3.7 $\pm$ 0.8
5	2.5	45	2.80	91.1	2.2 $\pm$ 0.1
6	1.25	22.5	2.96	66.9	1.5 $\pm$ 0.3
7	0.6	10.8	2.78	43.6	1.0 $\pm$ 0.6
8	0.3	5.4	2.99	26.1	0.6 $\pm$ 0.03

[†]The pyrrole concentration for each sample was measured 3 times over 1 h and the CEP:BSA ratio mean $\pm$ SD was calculated.