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Synthesis of Immunocomponents for the Measurement of Lead (Pb) by Fluorescence Polarization Immunoassay

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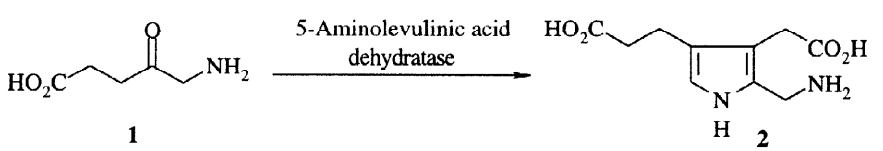
Summary: *Three novel haptens (**5b, 9b, 11b**) were synthesized from porphobilinogen (**2**) and conjugated to bovine serum albumin to afford immunogens (**6, 10, 12**). Five fluorescent tracers (**14, 17, 19, 29, 32**) were prepared from porphobilinogen (**2**), or haptens (**9b, 11b, 20b**) in >98% purity. The immunogens (**6, 10, 12**) were utilized for generation of polyclonal antibodies and the best tracer-antibody combination was selected to construct the calibration curve for the measurement of lead.*

The useful metal lead (Pb) exhibits distinct toxicity. Historical widespread use of lead in a variety of industries (e.g. paint, gasoline, batteries, printing, shipbuilding, automobile industries) has distributed lead throughout the environment.[1] Human intake of lead can occur by variety of pathways such as inhalation of airborne particles, water, food, alcoholic drinks and absorption through skin.[1a] The human body is able to process some amount of lead without adverse health effects, however, higher concentrations results in lead accumulation and toxicity. In the body, the absorbed lead is initially distributed in blood and soft tissues (e.g. kidney, liver, brain) in small amounts, and the remaining (about 95%) becomes lodged in bone with age.[2] Lead reduces the mitochondrial oxidative phosphorylation, alters calcium metabolism, and may affect the transmembrane transport of sodium, potassium and calcium ATPases.[4] Also, studies have demonstrated that lead inhibits the activity of several enzymes in heme synthesis, particularly 5-aminolevulinic acid dehydratase (ALAD).[3] ALAD enzyme catalyzes the transformation of 5-aminolevulinic acid (5-ALA, **1**) to porphobilinogen (PBG, **2**, Figure 1), the key building block in the biosynthesis of the "pigments of life" such as porphyrins, heme and vitamin B_{12}.[3]

Figure 1



1 **2**

The safety margin between unavoidable contact with lead and levels which constitute a significant risk to health is small.[1e] A wide variety of adverse health effects arise from lead poisoning which include headaches, sleeplessness, memory loss, irritability, weakness, anemia, weight loss, muscle paralysis and mental retardation.[1,5] These effects are especially pronounced in children with blood lead concentrations >800 ng/mL.[5a] In addition, recent studies have linked even moderately increased levels of lead in early childhood to reading disabilities and increased school drop-out rates.[6] In response to these studies, the US Public Health Service's Centers for Disease Control and Prevention (CDC) has gradually lowered the acceptable level of lead in blood

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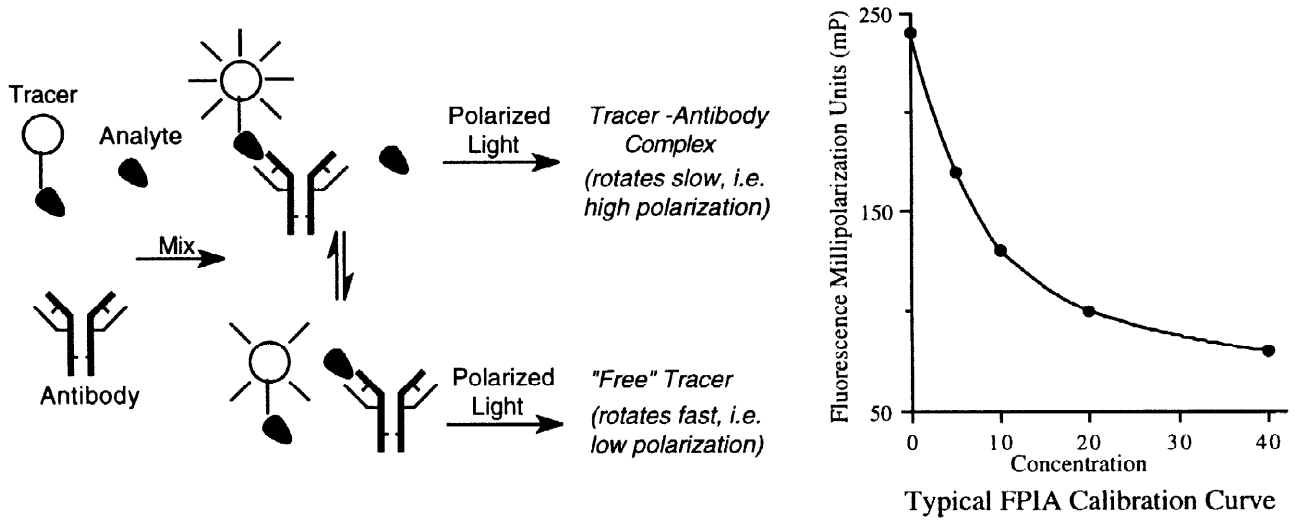
from 600 ng/mL in 1969 to 100 ng/mL in 1991. Additionally, it was recommended that prevention efforts focus on removing lead from the environment and improvement in methods for measuring lead.[7]

Measurement of lead (Pb): A variety of techniques (e.g. electrothermal atomic absorption spectro scopy,[8] anodic stripping voltammetry,[9] gas chromatography/mass spectrometry,[10] graphite furnace analysis[11]) have been reported in the literature for the measurement of lead. Recently, immunoassay methods have also been reported to estimate lead by measuring the activity of 5-aminolevulinic acid dehydratase (ALAD), the enzyme which converts 5-ALA (**1**) to PBG (**2**).[12] The amount of lead (Pb) is then indirectly estimated by measuring the amount of PBG (**2**) formed, which is inversely correlated to the lead concentration.[12] An essential part of these literature immunoassay methods involve the chemical derivatization of PBG (**2**) with Ehrlich's reagent (4-*N*,*N*-dimethylbenzaldehyde)[13] and its colorimetric analysis.

Our novel approach for the measurement of lead by immunoassay also utilizes the inhibition of ALAD by lead but involves quantification of the formed PBG (**2**) by fluorescence polarization (FPIA). FPIA is a widely used method for the measurement of low molecular weight (<2000 Da)[14] compounds and is based (Figure 2) on the principle[14] that a fluorescent molecule when excited with polarized light, will produce fluorescence inversely proportional to the rate of its rotation. In a competitive FPIA, the analyte competes with the fluorescent tracer for a limited number of antibody binding sites and therefore as the concentration of the analyte increases the polarization decreases. This inverse relationship between analyte concentration and net fluorescence polarization is illustrated by a typical FPIA calibration curve in Figure 2. Using such calibration curve, one can measure the amount of analyte present in an unknown sample.[14] The development of a fluorescence polarization immunoassay requires two key reagents: antibodies for binding and a signal generating material (fluorescent tracer) for detection. In this paper, we describe the synthesis of immunogens (**6**, **10**, **12**) required for the production of antibodies, fluorescent tracers (**14**, **17**, **19**, **29**, **32**) and preliminary data for the feasibility of lead measurement by a fluorescence polarization immunoassay.

Figure 2: Principle of Fluorescence Polarization Immunoassay



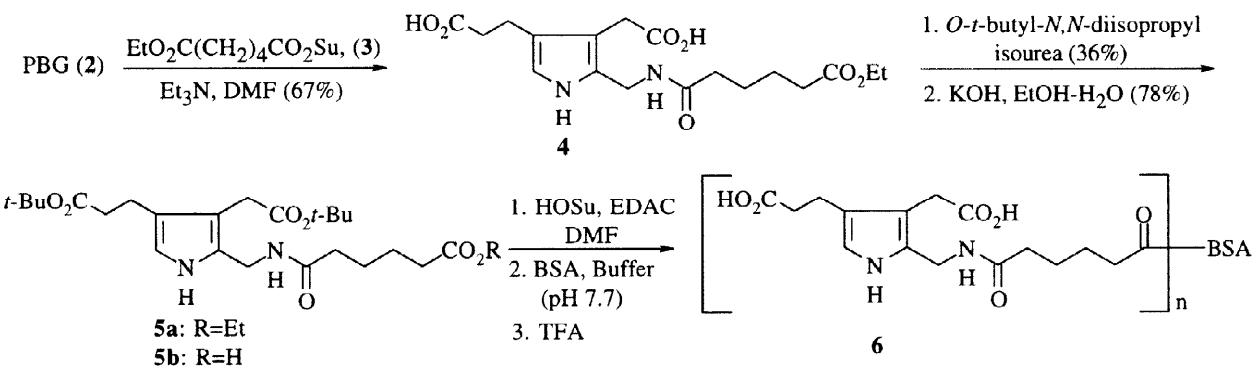
Typical FPIA Calibration Curve

Synthesis of Immunogens: Since low molecular weight compounds (e.g. PBG, **2**) do not as such induce the formation of antibodies, they need to be conjugated to a carrier protein, thus forming an

immunogen.[15,16] Immunogens are used for inoculation of an animal for the production of antibodies. Antibodies are proteins, approximate molecular weight 150 kDa, produced by B cell lymphocytes in response to the invasion of foreign molecule (antigen) in the body.[15] Antibodies are circulated in blood and lymph where they bind to specific antigens and this complex (antibody-antigen) is then removed from circulation primarily through phagocytosis.[15] Bovine serum albumin (BSA, molecular weight: 66,430 Da) is the most commonly used carrier protein for the preparation of immunogens because of its solubility in various aqueous buffers and high content of primary amines (59 lysines and the terminal amine) available for conjugation to hapten (modified analyte). The design of the chemical structure of the hapten plays a critical role in tailoring the specificity of produced antibodies. Traditionally, the hapten is conjugated to the carrier protein through a site that is remote from the critical determinants, e.g. carboxylic acids, amines. Also, the structure of the linking arm and the choice of method used to conjugate the hapten to carrier protein are also critical for obtaining analyte specific antibodies.[16] However, it is generally necessary to prepare a number of immunogens for a given analyte to probe the host animal's immune system to produce antibodies recognizing different native portions (epitopes) of the analyte. This is especially critical for an analyte with multiple determinants. PBG (**2**) has several critical determinants, therefore we prepared three haptens (**5b, 9b, 11b**) which were then conjugated to BSA to afford immunogens (**6**, **10**, **12**), respectively.

Accordingly, for the preparation of immunogen (**6**), the C-2 aminomethyl group in PBG (**2**) was used as a site of attachment to BSA through a six carbon linker (Scheme 1). First, PBG (**2**)[17] was treated with succinimidyl (5-ethoxycarbonyl) pentanoate (**3**) in DMF to obtain the ester (**4**) in 67% yield. Then, both free carboxylic groups in **4** were converted to *t*-butyl esters by treatment with *O*-*t*-butyl-*N,N'*-diisopropylisourea[18] to give **5a** in 36% yield after preparative HPLC purification. The ethyl ester in **5a** was selectively hydrolyzed using potassium hydroxide in ethanol-water medium to give the acid (**5b**) in 78% yield and >95% purity. The immunogen (**6**) was prepared by conjugating hapten (**5b**) to BSA and subsequently unmasking the carboxylic acid groups with trifluoroacetic acid.

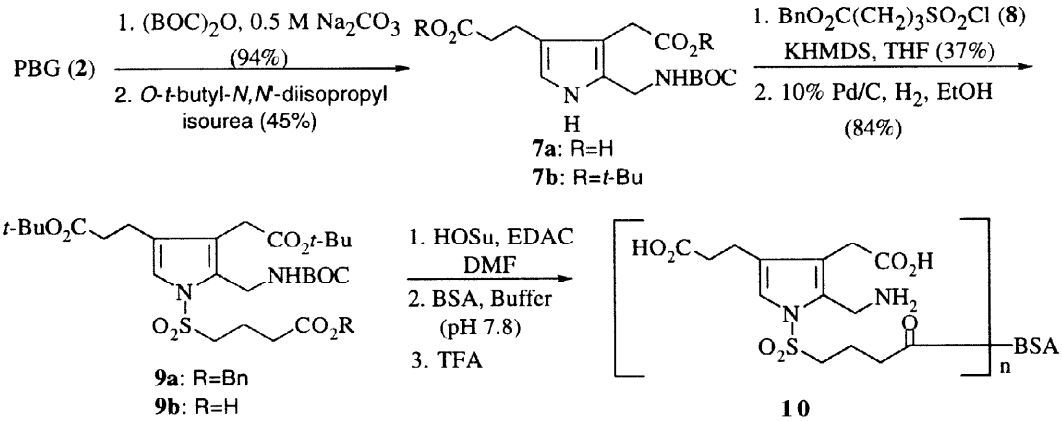
Scheme 1



The immunogen (**10**) was prepared (Scheme 2) by conjugating the pyrrole nitrogen of PBG (**2**) to BSA with use of a heterobifunctional linking group. In this design, the two carboxylic acids and primary amine are available for recognition by the immune system for antibody generation. Thus, the amino functionality in PBG (**2**) was protected as its *t*-butylcarbamate (BOC) by treatment with di-*t*-butyldicarbonate in 1.0 M aqueous sodium carbonate to give **7a** in excellent (93%) yield. The compound **7a** was then reacted with *O*-*t*-butyl-*N,N'*-

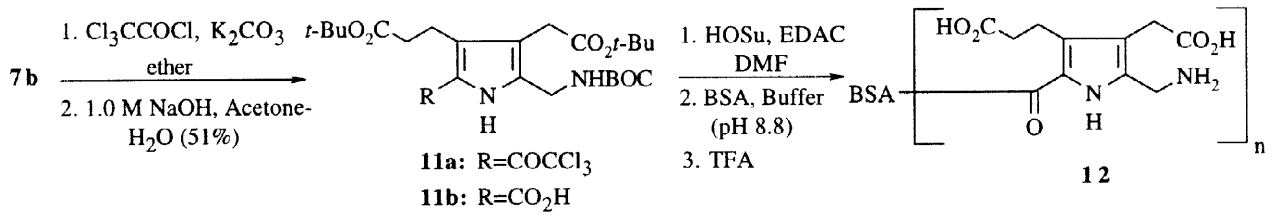
diisopropylisourea[18] in DMF to give the corresponding bis-*t*-butyl ester derivative (**7b**) in 36% yield. The heterobifunctional linking group was attached to the pyrrole nitrogen of **7b** via deprotonation[19] using potassium hexamethyldisilazide (KHMDS) in THF at 0 °C and treatment with benzyl-4-chlorosulfonyl butyrate (**8**)[16b] to afford the *N*-sulfonylated derivative (**9a**) in 45% yield after preparative HPLC purification. The benzyl group in **9a** was selectively removed by hydrogenolysis using 10% Pd/C in ethanol to give the acid (**9b**) in 84% yield. The hapten (**9b**) was then converted to the immunogen (**10**) by following the similar procedure developed for immunogen (**6**). Thus, **9b** was activated by treatment with HOSu to give the corresponding succinimidyl ester, conjugated to bovine serum albumin (BSA) and treated with trifluoroacetic acid to afford the immunogen (**10**).

Scheme 2



The immunogen (**12**) was prepared (Scheme 3) by conjugating the C5 position of pyrrole ring in PBG (**2**) via an amide functionality to BSA. Thus, the intermediate **7b** which was prepared from PBG (**2**) as depicted in Scheme 2, was functionalized (Scheme 3) by treatment with trichloroacetyl chloride[20] and anhydrous K_2CO_3 in ether to obtain the corresponding trichloromethylketone derivative (**11a**). The crude compound (**11a**) was hydrolyzed using 1.0 M NaOH in acetone-water and was purified by preparative HPLC to afford acid (**11b**) in 51% yield, for two steps. Finally, the acid functionality in hapten **11b** was transformed to the corresponding succinimidyl ester, conjugated to BSA and treated with trifluoroacetic acid to give the immunogen (**12**).

Scheme 3



A fundamental characteristic of an immunogen is the number of haptens covalently attached to the carrier protein (BSA) which can be determined from the new molecular weight of the modified protein. Several methods have been used to determine the extent of hapten incorporation.[21] The immunogens (**6**, **10**, **12**) were characterized by two of these methods: gel electrophoresis[22] and matrix assisted laser desorption (MALDI) mass spectrometry.[21] Gel electrophoresis separates proteins based on their molecular size and yields limited information about their molecular weight whereas MALDI directly measures the molecular weight of the protein. [21] Gel electrophoresis provided the information about the approximate molecular weight and showed the absence

of free BSA or BSA polymers. MALDI gave the molecular weight of immunogens (**6**, **10**, **12**; Table 1) and showed that an average of 15, 21 and 7 haptens were incorporated, respectively

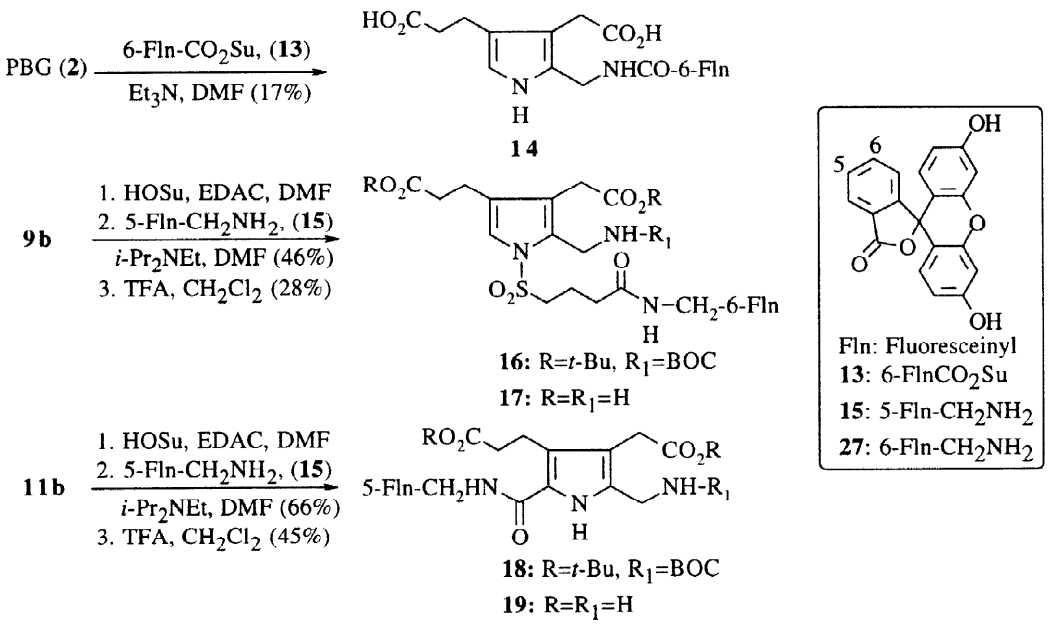
Table 1: Determination of molecular weight of immunogens (**6**, **10**, **12**) by MALDI mass spectrometry.

Entry	Immunogen	Molecular weight by MALDI[a,b]	Average number of haptens incorporated
1.	**6**	71,460	15
2.	**10**	73,400	21
3.	**12**	67,950	7

a. Determined on a Bruker reflex time of flight mass spectrometer with a sinapinic crystal matrix.
b. Molecular weight of bovine serum albumin (BSA) is 66,430

Synthesis of Fluorescent Tracers: The tracer, an important component used in the detection process in the immunoassay, is usually similar in structure to the analyte and contains a signal generating moiety.[14] For the preparation of tracers, it is customary to attach the fluorescein label to the same site in the hapten to which the carrier protein (BSA) was conjugated in preparing the immunogen.[23] Immunogen and tracer used in this approach are called homologous where the tracer mimics the overall topology of the hapten used to prepare immunogen. Homologous tracers usually have similar binding affinity with antibodies in comparison with analyte. Therefore, it can be difficult to displace the tracer from the antibody-tracer complex with analyte in a competitive assay format. Modification of the linking arm used for attachment of hapten to the fluorescent label can reduce the binding affinity of such tracers. An alternative approach would be the use of a tracer where the immunogen and tracer do not share the same site of attachment to the hapten (heterologous tracer). The heterologous tracer usually binds weaker with the antibodies than homologous tracers and can be of advantage in competitive assays.[24] Therefore, we prepared five fluorescent tracers (**14**, **17**, **19**, **29**, **32**) for use in different combinations with antibodies produced from immunogens (**6**, **10**, **12**) shown in Schemes 4, 5 and 6.

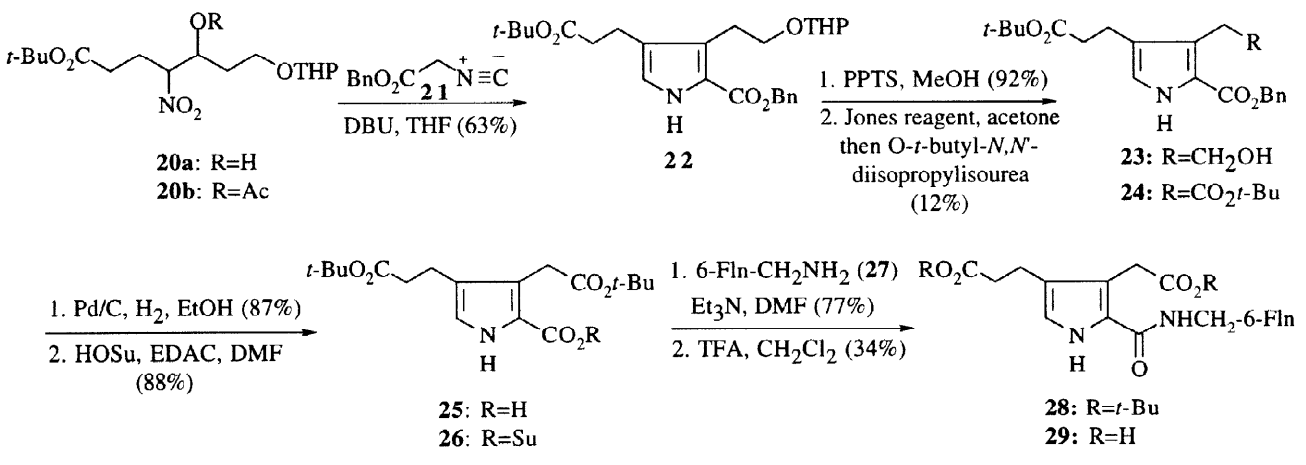
Scheme 4



The tracer (**14**) was prepared (Scheme 4) directly from PBG (**2**) by treatment with 6-carboxyfluorescein succinimidyl ester (**13**) in the presence of diisopropylethylamine in DMF and purified by preparative HPLC, to yield an orange powder. Tracer (**17**) was prepared from the intermediate (**9b**) which was used to prepare the immunogen (**10**) as shown in Scheme 4. Thus, the acid (**9b**) was converted to the corresponding succinimidyl active ester with HOSu in DMF and reacted with 5-aminomethylfluorescein hydrobromide[25] (**15**) in the presence of diisopropylethylamine in DMF to afford **16** in 46% yield after preparative HPLC purification. The *t*-butyl groups in **16** were removed using trifluoroacetic acid in CH_2Cl_2 and subsequently was purified to afford the fluorescent tracer (**17**) in 28% yield. The tracer (**19**) was prepared (Scheme 4) from the intermediate (**11b**) from which the immunogen (**12**) was prepared. Thus, the acid (**11b**) was transformed to the corresponding succinimidyl ester with HOSu in DMF and reacted with 5-aminomethylfluorescein hydrobromide (**15**) in the presence of diisopropylethylamine in DMF and purified by preparative HPLC to afford **18** in 66% yield. Finally, **18** was treated with trifluoroacetic acid in CH_2Cl_2 and purified by preparative HPLC to afford the fluorescent tracer (**19**) in 45% yield in >99% purity.

The two tracers (**29, 32**) were designed to take advantage of the increased stability of 2-substituted pyrroles containing an electron withdrawing group, such as an amide functionality. These tracers (**29, 32**) were prepared by following the strategy developed[17b] previously for preparation of 2-carboxy-3,4-substituted pyrrole derivatives. Thus, condensation (Scheme 5) of α-acetoxynitro compound (**20**)[17c] with benzyl isocyanoacetate (**21**)[26] in the presence of DBU afforded the pyrrole derivative (**22**) in 63% yield. The THP ether in **22** was then hydrolyzed using PPTS in methanol[17c] to give hydroxy compound (**23**) in excellent yield (92%). The alcohol

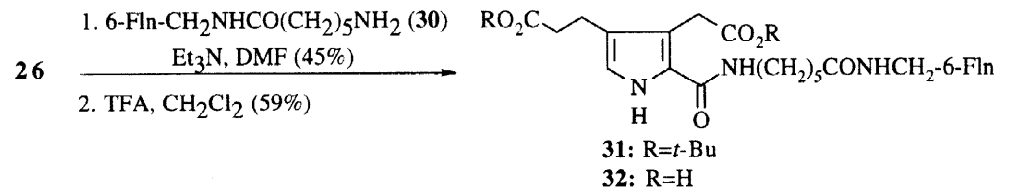
Scheme 5



(**23**) was transformed to the corresponding *t*-butyl ester by oxidation with Jones reagent followed by treatment of the resulting acid with *O*-*t*-butyl-*N,N'*-diisopropylisourea[18] to afford **24** in 12% yield, for two steps. Hydrogenolysis of compound (**24**) using 10% Pd/C in ethanol gave the acid (**25**) in 89% yield which upon activation with HOSu in DMF afforded the corresponding succinimidyl ester (**26**) in 88% yield. Treatment of the succinimidyl ester (**26**) with 6-aminomethylfluorescein hydrobromide (**27**)[25] in the presence of triethylamine in DMF followed by hydrolysis of the resulting intermediate (**28**) with trifluoroacetic acid afforded the fluorescent tracer (**29**) as an orange powder in >99% purity in 26% overall yield. Similarly, the tracer (**32**) was prepared

(Scheme 6) by reacting the active ester (**26**) with fluorescein derivative (**30**) in the presence of triethylamine and hydrolysis with trifluoroacetic acid in 59% yield and >99% purity.

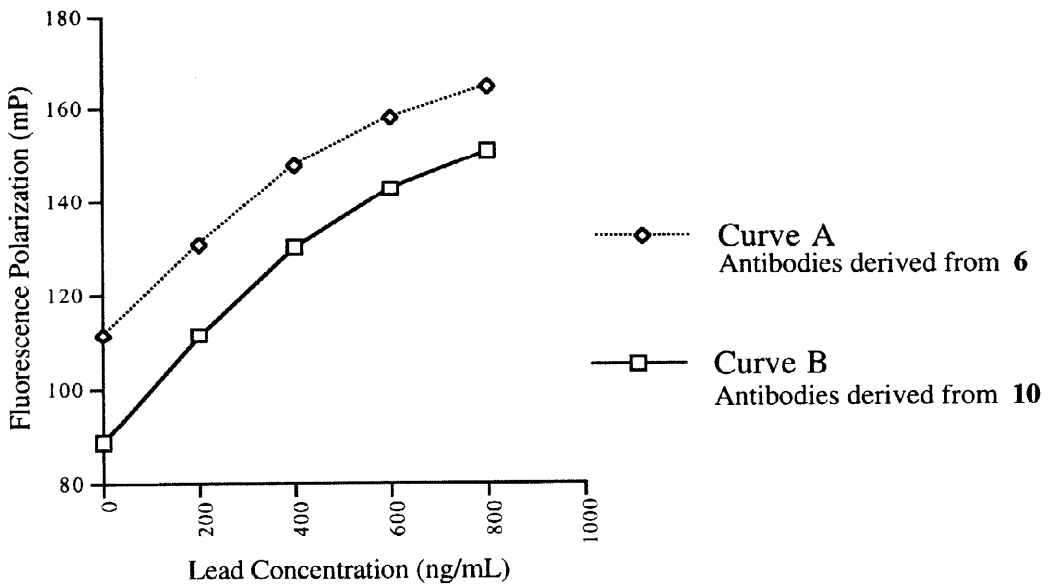
Scheme 6



31: R=*t*-Bu
32: R=H

The immunogens (**6**, **10**, **12**) were used for inoculation of rabbits and mice according to the standard protocol.[15b] The antisera obtained from the animals were evaluated for the presence of antibodies using all five tracers (**14**, **17**, **19**, **29**, **32**). Preliminary data indicated that the best response in producing anti-PBG antibodies was observed in serum of both groups of animals which had been inoculated with the immunogens **6** and **10**. Thus, calibration curves (Figure 3) were developed for the two best combinations of antisera and tracers (antisera derived from immunogen **6** with homologous tracer **14** and antisera derived from immunogen **10** with homologous tracer **17**, where both antisera were diluted 1:100, see experimental for details). An acidic solution of a known quantity of lead was added to a buffer (pH. 7.8) solution and to this mixture, 5-aminolevulinic acid dehydratase (ALAD) enzyme, a pre-diluted antisera solution, and a solution of 5-aminolevulinic acid (**1**) were added sequentially. The resulting mixture was incubated at 37 °C and a solution of fluorescent tracer (**14**) or (**17**) was added, followed by another incubation at 37 °C and finally the fluorescence polarization was measured in millipolarization units (mP). Five point calibration curves (A,B) were generated for each of the following tracer-antibody combinations [tracer **14**/A_b (immunogen **6**) and tracer **17**/A_b (immunogen **10**] using calibrators (I-V) containing 0 (I), 200 (II), 400 (III), 600 (IV), 800 (V) ng/mL of lead (Figure 3, curves A,B). Since the lead concentration is inversely proportional to the amount of porphobilinogen (**2**) formed, the calibration curves (Figure 3) are the inverse of the shape of "typical" FPIA calibration curve (Figure 2). As seen from the two

Figure 3. Lead Calibration Curves for Tracer **14**/A_b (Immunogen **6**) and Tracer **17**/A_b (Immunogen **10**)



curves A and B, an increase in the cocentration of lead resulted in a net increase in the fluorescence polarization and had dynamic ranges of 56 mP and 62 mP, respectively. We therefore, selected calibration curve B, which had the larger dynamic range, in order to establish the priliminary immunoassay parameters such as: dynamic concentration range, precision, sensitivity and correlation. The dynamic concentration range of the assay is 0-800 ng/mL which covers the lead concentration range recommended by the Centers for Disease Control and Prevention (CDC).[7] The precision of the method was found to be ±7.5% for low concentration (200 ng/mL), ±10% for medium concentration (400 ng/mL) and ±15% for high concentration (800 ng/mL) of lead. The sensitivity of the assay was determined by diluting the calibrator (II) and found to be 50 ng/mL, which is below the toxic level of lead in blood as outlined by the CDC. The apparent affinity constant for the polyclonal antibody used for the generation of curve A was 1.2×10^7 M^{-1} while for antibody used for generation of curve B was 1.3×10^7 M^{-1}, these values are not equilibrium affinity constants since the analyte, fluorescent tracer and antibody were combined in the Abbott IMx analyzer for a total time of 6.25 min. The FPIA preliminary results were compared to electrothermal atomic absorption spectroscopy and found to be ±10%. Thus, these initial results indicate that the immunocomponents described here are adequate for the further development of a clinically relevant assay for the measurement of lead.

In summary, three haptens (**5b**, **9b**, **11b**) were synthesized from porphobilinogen (**2**) and conjugated to bovine serum albumin to afford immunogens (**6**, **10**, **12**). Five fluorescent tracers (**14**, **17**, **19**, **29**, **32**) were prepared from porphobilinogen (**2**) or haptens (**9b**, **11b**, **20b**) in >98% purity. Our preliminary data demonstrates the feasibility of quantification of the heavy metal lead by FPIA utilizing these immunocomponents. We hope that our approach will open opportunities to measure other heavy, toxic elements by immunoassay which has inherent advantages of providing rapid, accurate and reliable results at a low cost.

Experimental

General Procedure: ^{1}H NMR spectra were recorded at 300 MHz on a Varian Gemini spectrometer. Mass spectra were obtained on a Nermag 3010 MS-50 or JEOL SX102-A mass spectrometers or Perkin-Elmer Sciex API III electrospray mass spectrometer. MALDI mass spectra were obtained on a Bruker reflex time of flight mass spectrometer with a sinapinic crystal matrix. Column chromatography was performed on silica gel, Merck grade 60 (230-400 mesh). Thin layer chromatography was performed on pre-coated Whatman MK6F silica gel 60 Å plates (layer thickness: 250 μm) and were visualized with UV light and/or using $KMnO_4$ solution ($KMnO_4$ (1.0 g), NaOH (8.0 g) in water (200 mL) unless otherwise noted. THF was freshly distilled under nitrogen from a purple solution of sodium and benzophenone. CH_2Cl_2 was freshly distilled from CaH_2 under nitrogen. All reagents were purchased from Aldrich Chemical Co., Milwaukee, WI or Sigma Chemical Co., St. Louis, MO and were used without further purification, except where noted. All solvents employed were of HPLC grade, purchased from EM Science, Gibbstown, NJ and were used as received. Analytical HPLC was performed using a Waters μBondapak C_{18} 10μ (8 mm x 100 mm) reversed phase column eluting at 2 mL/min with acetonitrile (MeCN)/0.1% aqueous formic acid with UV detection at 225 nm unless otherwise stated. Preparative HPLC was performed using a Waters μBondapak C_{18} 10 μ (40 mm × 100 mm) reversed phase column eluting at 45 mL/min with MeCN/0.1% aqueous formic acid with UV detection at 225 nm. Benzyl-4-chlorosulfonylbutyrate (**8**),[16b] *t*-butyl-4-nitrobutyrate[28] 3-[(tetrahydro-2H-pyran-2-yl)oxy]-propanal,[17c] and

benzyl isocyanoacetate (**21**)[26] were prepared by following literature procedures. A standard solution of lead was purchased from GFS Chemicals, Columbus, OH.

Succinimidyl 5-(ethoxycarbonyl)pentanoate (**3**): Dicyclohexylcarbodiimide (DCC, 28.3 g, 137 mmol, 1.05 equiv.) was added to a mixture of adipic acid monoethyl ester (22.8 g, 131.0 mmol) and *N*-hydroxysuccinimide (HOSu, 15.7 g, 137 mmol, 1.05 equiv.) in anhydrous dimethylformamide (DMF, 100 mL) at room temperature under nitrogen atmosphere. The reaction was stirred for 20 h, filtered and the solvent was removed on a rotary evaporator. The resulting crude compound was dissolved in ethyl acetate (100 mL) and the undissolved material was separated by filtration. The filtrate was concentrated on a rotary evaporator and the crude material was purified by silica gel column chromatography (50% EtOAc in hexane) to afford 30.6 g of **3** in 85% yield as an oil. ^{1}H NMR (DMSO-d_6): δ 4.05 (q, J = 7.1 Hz, 2H), 2.80 (s, 4H), 2.75–2.65 (m, 2H), 2.40–2.27 (m, 2H), 1.70–1.55 (m, 4H), 1.16 (t, J=7.1 Hz, 3H); ES–MS: 272 $(M+H)^+$.

2-[*N*-(5-ethoxycarbonylpentanamido)methyl]-3-carboxymethyl-1H-pyrrole-4-propanoic acid (4): PBG (**2**, 0.800 g, 3.28 mmol) was dissolved in a mixture of 0.10 M NaH_2PO_4 buffer (30 mL) and 1M Na_2CO_3 (2.4 mL) solution. To this mixture, a solution of succinimidyl 5-(ethoxycarbonyl)pentanoate (**3**, 2.67 g, 9.83 mmol, 3.0 equiv.) in anhydrous DMF (30 mL) was added at room temperature under nitrogen. The reaction flask was covered with aluminum foil and after stirring for 6 h the solvents were removed on a rotary evaporator. The crude product was purified by preparative HPLC (MeCN:0.1% aqueous formic acid/25:75) to give 0.840 g of **4** in 67% yield. Analytical HPLC: (MeCN:0.1% aqueous formic acid/25:75), R_t: 5.66 min, 97%; ^{1}H NMR (DMSO-d_6): δ 10.24 (s, 1H), 7.93 (t, J = 6.4 Hz, 1H), 6.39 (s, 1H), 4.12 (d, J = 4.9 Hz, 2H), 4.05 (q, J = 7.1 Hz, 2H), 3.27 (s, 2H), 2.60–2.00 (m, 8H), 1.58–1.38 (m, 4H), 1.17 (t, J = 7.1 Hz, 3H); ES–MS: 383 $(M+H)^+$.

***t*-Butyl-2-[*N*-(5-ethoxycarbonylpentanamido)methyl]-3-(*t*-butoxycarbonyl)methyl-1H-pyrrole-4-propanoate (5a)**: *O*-*t*-Butyl-*N,N'*-diisopropylisourea[18] (4.7 g, 23.5 mmol, 14.0 equiv.) was added to a 0-5 °C solution of compound **4** (0.64 g, 1.67 mmol) in DMF (6.0 mL) at room temperature under nitrogen. After stirring the reaction mixture for 44 h, THF (15 mL) was added and filtered. The filtrate was concentrated on a rotary evaporator and purified the crude product by preparative HPLC (MeCN:0.1% aqueous formic acid/60:40) to afford 0.30 g of **5a** in 36% yield. Analytical HPLC: (MeCN:0.1% aqueous formic acid/60:40), R_t: 8.09 min, 93%; ^{1}H NMR ($CDCl_3$): δ 8.67 (s, 1H), 6.62 (t, J = 5.3 Hz, 1H), 6.41 (s, 1H), 4.29 (d, J = 5.6 Hz, 2H), 4.11 (q, J = 7.1 Hz, 2H), 3.32 (s, 2H), 2.72–2.10 (m, 8H), 1.70–1.55 (m, 4H), 1.44 (s, 9H), 1.43 (s, 9H), 1.24 (t, J = 7.1 Hz, 3H); ES–MS: 495 $(M+H)^+$.

***t*-Butyl 2-[*N*-(5-carboxypentanamido)methyl]-3-(*t*-butoxycarbonyl)methyl-1H-pyrrole-4-propanoate (5b)**: Aqueous potassium hydroxide (0.26 M, 7.0 mL, 1.82 mmol, 3.0 equiv.) was added to an ice cooled solution of triester (**5a**, 0.30 g, 0.607 mmol) in EtOH (7.0 mL) under argon atmosphere. The mixture was allowed to warm to room temperature, covered with aluminum foil and stirred for 3 h. The solvent was removed, on a rotary evaporator, the residue was dissolved in H_2O (150 mL) and acidified to pH = 2.8 using 1.5 M H_3PO_4. The mixture was extracted with ethyl acetate (4 × 100 mL), combined extracts were washed with brine (50 mL) and dried (Na_2SO_4). The solvent was removed on a rotary evaporator to afford 0.22 g of hapten **5b** in 78% yield. ^{1}H NMR ($CDCl_3$): δ 8.90 (s, 1H), 6.89 (t, J = 5.6 Hz, 1H), 6.44 (s, 1H), 4.30 (d, J = 5.7 Hz, 2H),

3.33 (s, 2H), 2.68 (t, J = 7.9 Hz, 2H), 2.45 (t, J = 7.9 Hz, 2H), 2.33 (t, J = 6.7 Hz, 2H), 2.20 (t, J = 6.7 Hz, 2H), 1.70–1.55 (m, 4H), 1.44 (s, 9H), 1.43 (s, 9H); ES–MS: 467 $(M+H)^+$; HRMS (FAB + KI): calcd for $(C_{24}H_{38}N_2O_7 + K)^+$ 505.2316, found 505.2308.

Immunogen (6): A solution of 3-(dimethylamino)propyl carbodiimide (EDAC, 0.183 g, 0.955 mmol, 4.0 equiv.) was added to a mixture of acid **5b** (0.11 g, 0.236 mmol) and *N*-hydroxysuccinimide (HOSu, 0.11 g, 0.956 mmol, 4.0 equiv.) in DMF (6.0 mL) at room temperature under argon atmosphere. Reaction flask was covered with aluminum foil and the mixture was stirred for 20 h. After removing solvents on a rotary evaporator, the residue was dissolved in a Et_2O (50 mL) and 0.05 M NaH_2PO_4 buffer (pH=5.5) solution (50 mL) and extracted with ether (4 × 50 mL). The combined extracts were washed with brine (50 mL) and dried (Na_2SO_4). Solvents were removed on a rotary evaporator to yield 0.105 g of the corresponding succinimidyl ester in 80% yield. Analytical HPLC: (MeCN:0.1% aqueous formic acid/60:40), R_t: 5.76 min, 85%.

The above prepared crude succinimidyl ester of **5b** (0.037 g, 0.066 mmol) was dissolved in DMF (16.0 mL) and added to an ice cooled solution of BSA (0.210 g) in 0.1 M NaH_2PO_4 buffer (pH=7.7, 20.0 mL). The reaction mixture was warmed to room temperature, covered with aluminum foil and stirred for 40 h. Purification of the mixture by gel filtration [60 g G-25 Sephadex, 20% MeOH in H_2O containing 100 mM ammonium acetate] followed by lyophilization afforded 0.28 g of intermediate. To this intermediate conjugate, CH_2Cl_2 (10.0 mL). and trifluoroacetic acid (TFA, 10.0 mL) were added sequentially at room temperature. The reaction mixture was stirred for 20 minutes and then solvents removed on a rotary evaporator. The residue was dissolved in H_2O (80 mL), neutralized with 1M NH_4OH and the mixture was lyophilized to afford a solid which was dissolved in H_2O (100 mL), neutralized with 1M NH_4OH and lyophilized. The resulting solid was dialyzed in H_2O (4 L) for 6 h and lyophilized to give 0.24 g of the immunogen (**6**). MALDI mass spectrum: 71,456 [BSA + 15 haptens (**5b**)].

2-[*N*-(*t*-Butoxycarbonyl)amidomethyl]-3-carboxymethyl-1H-pyrrole-4-propanoic acid (7a): A solution of di-*t* butyl dicarbonate (BOC_2O, 2.15 g, 9.4 mmol, 1.00 equiv.) in THF (15.0 mL) was added to a solution of PBG (**2**, 2.3 g, 9.4 mmol) in a mixture of (30 mL) 0.50 M aqueous sodium carbonate (15.0 mL, 15.0 mmol) and THF (15.0 mL) at room temperature under a nitrogen. The reaction mixture was stirred for 4 h and added an additional 0.50 M sodium carbonate solution (10 mL) and a solution of BOC_2O (2.1 g, 9.4 mmol) in THF (15.0 mL). After stirring the mixture for an 4 h, the reaction mixture was concentrated on a rotary evaporator and the residue was dissolved in a mixture of water (200 mL) and 1M NaOH (5 mL). The resulting mixture was washed with ether (50 mL) and separated the aqueous layer. The aqueous layer was acidified with 1 M H_3PO_4 to pH=3 and extracted with ethyl acetate (3 × 100 mL). The combined ethyl acetate extracts were dried (Na_2SO_4) and solvent was removed on a rotary evaporator to afford 2.86 g of bis-acid (**7a**) in 94% yield as a thick gum. Analytical HPLC: (MeCN:0.1% aqueous formic acid/50:50), R_t: 2.41 min: 97%; ^1H NMR (DMSO-d_6): δ 12.02 (br s, 1H), 11.98 (br s, 1H), 10.14 (br s, 1H), 6.81 (s, 1H), 6.36 (s, 1H), 4.01 (d, J = 5.6 Hz, 2H), 3.45 (s, 2 H), 2.77 (t, J = 7.1 Hz, 2H), 2.56 (t, J = 7.1 Hz, 2H), 1.43 (s, 9H); ES–MS: 324 $(M)^+$.

***t*-Butyl 2-[*N*-(*t*-butoxycarbonyl)amidomethyl]-3-(*t*-butoxycarbonyl)methyl-1H-pyrrole-4-propanoate (7b):** *O*-*t*-Butyl-*N,N'*-diisopropylisourea[18] (19.6 g, 98.0 mmol, 11.4 equiv.) was added to an ice cooled solution of diacid (**7a**) (2.8 g, 8.6 mmol) in DMF (15 mL) under nitrogen atmosphere. The mixture was warmed to room temperature and stirred for 16 h. The reaction was poured into a mixture of water and phosphate buffer (500 mM, pH=6.0) (1:1 ratio, 100 mL) and extracted with ether (3 × 125 mL). The combined ether

extracts were washed with brine (70 mL), dried (Na_2SO_4) and the solvent was removed on a rotary evaporator. The crude compound was purified by preparative HPLC (MeCN:0.1% aqueous formic acid/ 58:42) to afford 1.7 g of **7b** as an oil in 45% yield. Analytical HPLC: (MeCN:0.1% aqueous formic acid/65:35), R_t: 6.99 min, 92%; ^1H NMR ($CDCl_3$): δ 8.58 (br s, 1H), 6.45 (s, 1H), 5.26 (br s, 1H), 4.15 (d, J = 6.1 Hz, 2H), 3.30 (s, 2 H), 2.69 (t, J = 5.5 Hz, 2H), 2.45 (t, J = 5.5 Hz, 2H), 1.43 (s, 27 H); ES–MS: 438 $(M)^+$; HRMS (FAB) calcd for $(C_{23}H_{38}N_2O_6)^+$: 438.2730, found: 438.2711.

***t*-Butyl 2-[*N*-(*t*-butoxycarbonyl)amidomethyl]-3-(*t*-butoxycarbonyl)methyl-N_1-[(3-benzyl oxycarbonylpropyl)sulfonyl]-pyrrole-4-propanoate (9a):** Potassium hexamethyldisilylazide (KHMDS, 0.50 M, 1.2 mL, 0.60 mmol, 1.2 equiv.) was added to an ice cooled solution of ester (**7b**, 219 mg, 0.50 mmol) in THF (5 mL) under nitrogen atmosphere and stirred for 30 min. A solution of benzyl-4-chlorosulfonylbutyrate[13b] (**8**, 0.166 g, 0.6 mmol, 1.2 equiv.) in THF (1 mL) was added at 0-5 °C to the reaction mixture, allowed to warm to room temperature and stirred for 14 h. The reaction was quenched by pouring it into a mixture of water (50 mL) and saturated sodium bicarbonate (5.0 mL). This mixture was extracted with ethyl acetate (3 × 75 mL), combined extracts were dried (Na_2SO_4) and solvent was removed on a rotary evaporator. Crude product was purified by preparative HPLC (MeCN:0.1% aqueous formic acid/ 70:30) to afford 0.128 g of the N_1-sulfonyl compound (**9a**) in 37% yield. Analytical HPLC: (MeCN:0.1% aqueous formic acid/65:35), R_t: 16.46 min, 99%; ^1H NMR ($CDCl_3$): δ 7.39 (br s, 5H), 6.84 (s, 1H), 5.33 (br s, 1H), 5.11 (s, 2H), 4.36 (d, J = 6.2 Hz, 2H), 3.52 (s, 2H), 3.31 (t, J = 7.5 Hz, 2H), 2.63 (t, J = 7.5 Hz, 2H), 2.51–2.39 (m, 4H), 2.03 (q, J = 7.5 Hz, 2H), 1.43 (s, 27 H); ES–MS: 679 $(M+H)^+$; HRMS (FAB + KI) calcd for $(C_{34}H_{50}N_2O_{10}S + K)^+$: 717.2823, found: 717.2839.

***t*-Butyl 2-[*N*-(*t*-butoxycarbonyl)aminomethyl]-3-(*t*-butoxycarbonyl)methyl-N_1-[(3-carboxy propyl)sulfonyl]-pyrrole-4-propanoate (9b):** To the compound **9a** (0.110 g, 0.16 mmol) dissolved in absolute ethanol (25 mL), was added palladium on carbon (10% Pd/C, 0.060 g) and stirred under hydrogen (1 atm) atmosphere for 16 h. The mixture was filtered and the solvent was removed on a rotary evaporator to afford 0.092 g of hapten (**9b**) in 84% yield. Analytical HPLC: (MeCN:0.1% aqueous formic acid/65:35), R_t: 13.84 min, 98%; ^1H NMR ($CDCl_3$): δ 7.90 (br s, 1H), 6.87 (s, 1H), 5.35 (br s, 1H), 4.37 (d, J = 5.8 Hz, 2H), 3.56 (br s, 2H), 3.36 (t, J = 7.3 Hz, 2H), 2.63 (t, J = 7.6 Hz, 2H), 2.46 (t, J = 7.6 Hz, 2H), 2.39 (t, J = 7.2 Hz, 2H), 2.02 (q, J=7.2 Hz, 2H), 1.44 (s, 27H); ES–MS: 589 $(M+H)^+$; HRMS (FAB) calcd for $(C_{27}H_{44}N_2O_{10}S + H)^+$: 589.2795, found: 589.2796.

Immunogen (10): The hapten (**9b**, 0.070 g, 0.12 mmol) was dissolved in DMF (3.0 mL) and added sequentially HOSu (0.136 g, 1.20 mmol, 10.0 equiv.) followed by EDAC (0.115 g, 0.60 mmol, 5.0 equiv.) at room temperature under nitrogen. After stirring the mixture for 14 h, it was poured into Et_2O (70 mL) and washed with phosphate buffer (500 mM, pH=6.0, 30 mL). The organic layer was separated, dried (Na_2SO_4) and the solvent was removed on a rotary evaporator to afford 0.067 g of the succinimidyl ester of **9b** in 82% yield. ^1H NMR ($CDCl_3$): δ 6.88 (s, 1H), 5.32 (br s, 1H), 4.38 (d, J = 5.9 Hz, 2H), 3.53 (s, 2H), 3.40 (t, J = 7.2 Hz, 2H), 2.83 (s, 4H), 2.74 (t, J = 7.2 Hz, 2H), 2.63 (t, J = 7.2 Hz, 2H), 2.20–2.08 (m, 4H), 1.43 (s, 27H); ES–MS: 686 $(M+H)^+$.

The above prepared crude succinimidyl ester of **9b** (0.044 g, 0.064 mmol, 19 equiv.) was dissolved in DMF (2 mL) and added to a solution of bovine serum albumin (BSA, 0.220 g) in a mixture of phosphate buffer (50 mM, pH=7.8, 22 mL) and DMF (6 mL) at room temperature. The reaction mixture was then stirred for 15 h, the crude product was purified by Sephadex G-25 column chromatography (20% MeOH in H_2O containing 100 mM ammonium acetate) and lyophilized to afford 0.278 g of an intermediate conjugate. To this intermediate conjugate, methylene chloride (12 mL) and trifluoroacetic acid (12 mL) were added sequentially at room temperature. The mixture was stirred for 30 min and concentrated on a rotary evaporator. The residue was dissolved in aqueous ammonium acetate (100 mM, 40 mL), stirred for 15 h and the resulting homogeneous solution was lyophilized. The material was dissolved in water (50 mL) and lyophilized and resulting material was subjected to this water treatment/lyophilization process two more times to give 0.210 g immunogen (**10**). MALDI mass spectrum: 73,400 [BSA + 21 haptens (**9b**)].

***t*-Butyl 2-[*N*-(*t*-butoxycarbonyl)amidomethyl]-3-(*t*-butoxycarbonyl)methyl-1H-pyrrole-5-carboxy-4-propanoate (11b)**: To the 0 °C cooled solution of compound **7b** (0.675 g, 0.50 mmol) in anhydrous ether (15 mL), were added anhydrous potassium carbonate (2.24 g, 16 mmol) and trichloroacetyl chloride (0.207 mL, 1.85 mmol, 3.6 equiv.) under nitrogen atmosphere. The reaction mixture was stirred for 30 min and poured into a mixture of ether (200 mL), water (100 mL) and saturated aqueous sodium bicarbonate (100 mL). Organic layer was separated and the aqueous layer was extracted with ether (50 mL). The combined organic layers were dried (Na_2SO_4) and the solvent was removed on a rotary evaporator to afford 0.805 g of crude trichloromethyl ketone (**11a**). ES–MS: 602 $(M+ NH_4)^+$.

The crude ketone (**11a**, 0.805 g, 1.5 mmol) was dissolved in a mixture of acetone (30 mL) and water (6 mL) and added aqueous sodium hydroxide solution (1.0 M, 3.5 mL, 3.5 mmol, 2.3 equiv.) at room temperature under nitrogen. Reaction mixture was stirred for 25 min and solvent was removed using rotary evaporator. The residue was diluted with water (100 mL) and washed with ether (50 mL). Aqueous layer was adjusted to pH = 2.5 using 1.0 M H_3PO_4 and extracted with ethyl acetate (3 × 100 mL). The combined ethyl acetate extracts were washed with brine (30 mL), dried (Na_2SO_4), and solvent was removed using a rotary evaporator to give a dark oil which was purified by preparative HPLC (MeCN:0.1% aqueous formic acid/60:40) to afford 0.122 g of **11b** in 51% yield for two steps. Analytical HPLC: (MeCN:0.1% aqueous formic acid/65:35), R_t: 5.33 min, 96%; ^{1}H NMR ($CDCl_3$ + 1 drop CD_3OD): δ 9.98 (br s, 1H), 5.52 (br s, 1H), 4.28 (d, J = 5.4 Hz, 2H), 3.40 (s, 2H), 3.02 (t, J = 7.8 Hz, 2H), 2.49 (t, J = 7.8 Hz, 2H), 1.44 (s, 27H); ES–MS: 483 $(M+H)^+$; HRMS calcd for $(C_{24}H_{38}N_2O_8 + K)^+$ 521.2265, found: 521.2256.

Immunogen (12): The hapten (**11b**, 0.122 g, 0.25 mmol) was dissolved in DMF (3.3 mL) and added HOSu (0.288 g, 2.5 mmol, 10.0 equiv.) followed by EDAC (289 mg, 1.25 mmol, 5.0 equiv.) at room temperature under nitrogen atmosphere. Reaction mixture was stirred for 14 h, poured into ether (100 mL) and washed with phosphate buffer (500 mM, pH=6.0, 50 mL) and water (50 mL). The ether layer was dried (Na_2SO_4) and concentrated on a rotary evaporator to afford 0.143 g of the succinimidyl ester of **11b** in 98% yield. ^{1}H NMR ($CDCl_3$): δ 9.82 (br s, 1H), 5.33 (br s, 1H), 4.23 (d, J = 6.1 Hz, 2H), 3.40 (s, 2H), 3.05 (t, J = 7.6 Hz, 2H), 2.87 (s, 4H), 2.51 (t, J = 7.6 Hz, 2H), 1.45 (s, 27H); ES–MS: 597 $(M+H)^+$.

A solution of the succinimidyl ester of **11b** (0.0655 g, 0.113 mmol, 48 equiv.) in DMF (2 mL) was added to a solution of bovine serum albumin (BSA, 0.150 g) which was dissolved in a mixture of phosphate buffer (50

mM, pH = 8.8, 50 mL) and DMF (10 mL). The mixture was then stirred for 3 days at room temperature. Separation of the crude mixture by Sephadex G-25 column chromatography (20% MeOH in H_2O containing 100 mM ammonium acetate) and lyophilization afforded 0.207 g of an intermediate to which methylene chloride (10 mL) and trifluoroacetic acid (10 mL) were added at room temperature. After stirring the mixture for 30 min, the solvent was removed on a rotary evaporator. The crude immunogen was dissolved in aq. ammonium acetate (100 mM, 40 mL) and stirred for 15 h at room temperature. The resulting homogeneous solution was lyophilized, the resulting material was dissolved in water (50 mL) and lyophilized. The aqueous treatment/lyophilization process was repeated twice to give 0.153 g of immunogen (**12**). MALDI mass spectrum: 67,950 [BSA + 7 haptens (**11b**)].

2-[(*N*-amidomethyl-6-fluoresceinyl)aminomethyl]-3-carboxymethyl-1H-pyrrole-4-propanoic acid (14): To a solution of 6-carboxyfluorescein (0.034 g, 0.090 mmol, 1.1 equiv.) in DMF (1.7 mL) was added DCC (0.018 g, 0.086 mmol, 1.05 equiv.) and HOSu (0.011 g, 0.087 mmol, 1.05 equiv.) at room temperature under nitrogen atmosphere. The reaction flask was covered with aluminum foil and stirred for 20 h. Reaction mixture was filtered to afford a solution of 6-carboxyfluorescein succinimidyl ester (**13**) which was added to a solution of porphobilinogen (**2**, 0.020 g, 0.082 mmol) in 0.10 M phosphate (pH=7.7, 4.0 mL) at room temperature under nitrogen atmosphere. The reaction was covered with aluminum foil and stirred for 20 h. Solvents were removed on a rotary evaporator and the crude product was purified twice by preparative HPLC (MeCN:0.1% aqueous formic acid/ 30:70) to give 0.008 g of fluorescent tracer (**14**) in 17% yield as an orange powder. Analytical HPLC: (MeCN:0.1% aqueous formic acid/30:70) R_t: 8.14 min, 98%; ^1H NMR (CD_3OD): δ 8.15–8.00 (m, 2H), 7.65 (s, 1H), 6.70–6.40 (m, 7H), 4.45 (s, 2H), 3.45 (s, 2H), 2.75–2.40 (m, 6H); ES–MS: 585 $(M+H)^+$; HRMS calcd for $(C_{31}H_{24}N_2O_{10} + H)^+$ 585.1509, found: 585.1509.

2-Aminomethyl-3-carboxymethyl-N_1-{[(*N*-amidomethyl-6-fluoresceinyl)carboxypropyl] sulfonyl}-pyrrole-4-propanoic acid (17): EDAC (0.192 g, 0.10 mmol, 3.3 equiv.) was added to a mixture of acid (**9b**, 0.020 g, 0.034 mmol) and HOSu (0.0196 g, 0.17 mmol, 5.0 equiv.) dissolved in DMF (1.0 mL) at room temperature under nitrogen atmosphere and stirred for 14 h. The reaction mixture was diluted with ether (50 mL), washed with phosphate buffer (500 mM, pH = 6.0, 30 mL). The ether layer was dried (Na_2SO_4) and concentrated using rotary evaporator to afford 0.023 g of the corresponding succinimidyl active ester in 99% yield. ^1H NMR ($CDCl_3$): δ 6.88 (s, 1H), 5.32 (br s, 1H), 4.38 (d, J = 5.9 Hz, 2H), 3.53 (s, 2H), 3.40 (t, J = 7.2 Hz, 2H), 2.83 (s, 4H), 2.74 (t, J = 7.2 Hz, 2H), 2.63 (t, J = 7.2 Hz, 2H), 2.20–2.08 (m, 4H), 1.43 (s, 27H); ES–MS: 686 $(M+H)^+$.

The above prepared succinimidyl active ester (0.023 g, 0.033 mmol) was dissolved in DMF (1 mL) and added 5-aminomethylfluorescein hydrobromide[25] (**15**, 0.025 g, 0.048 mmol, 1.45 equiv.) followed by diisopropylethylamine (0.044 mL, 0.25 mmol, 7.6 equiv.) at room temperature under nitrogen. After stirring the reaction mixture for 18 h, the solvent was removed on a rotary evaporator. The crude product was purified by preparative HPLC (MeCN:0.1% aqueous formic acid/40:60) to afford 0.014 g of compound **16** in 46% yield. Analytical HPLC: (MeCN:0.1% aqueous formic acid/65:35), R_t: 8.05 min, 96%; ^1H NMR ($CDCl_3$ + 2 drops CD_3OD): δ 7.88–7.80 (m, 1H), 7.52–7.40 (m, 1H), 7.05–6.92 (m, 1H), 6.87 (s, 1H), 6.70–6.30 (m, 6H), 5.40 (br s, 1H), 4.52–4.28 (m, 4H), 4.11–4.02 (m, 2H), 3.65–3.52 (m, 2H), 3.35–3.20 (m, 2H), 2.65–2.52 (m, 2H), 2.51–2.42 (m, 4H), 2.10–1.95 (m, 2H), 1.43 (s, 27H); ES–MS: 932 $(M)^+$.

Trifluoroacetic acid (1 mL) was added to a suspension of the above prepared fluorescein derivative (**16**, 0.014 g, 0.015 mmol) in methylene chloride (1 mL) at room temperature and stirred 30 min. The solvent was then removed on a rotary evaporator and the residue was purified by preparative HPLC (MeCN:0.1% aqueous formic acid/25:75) and lyophilization gave 0.0031 g of the fluorescent tracer (**17**) in 28% yield as an orange powder. Analytical HPLC: (MeCN:0.1% aqueous formic acid/30:70) R_t: 6.78 min, 98%; ^{1}H NMR (CD_3OD + 5 drops $CDCl_3$): δ 8.03 (s, 1H), 7.76 (d, J = 8.0 Hz, 1H), 7.26 (d, J = 8.0 Hz, 1H), 7.14 (s, 1H), 6.97 (s, 2H), 6.95 (d, J = 8.9 Hz, 2H), 6.80 (d, J = 8.9 Hz, 2H), 4.55 (s, 2H), 4.36 (s, 2H), 3.63 (s, 2H), 3.60–3.46 (m, 2H), 2.71 (t, J = 7.1 Hz, 2H), 2.55 (t, J = 7.1 Hz, 2H), 2.01 (t, J = 7.2 Hz, 2H); ES–MS: 720 $(M+H)^+$; HRMS calcd for $(C_{35}H_{33}N_3S_1O_{12} + H)^+$ 720.1863, found: 720.1851.

2-Aminomethyl-3-carboxymethyl-5-[(*N*-amidomethyl-6-fluoresceinyl)carbonyl]-1H-pyrrole-4-propanoic acid (19): EDAC (0.0421 g, 0.22 mmol, 5.4 equiv.) was added to a mixture of acid (**11b**) (0.020 g, 0.041 mmol) and HOSu (0.0517 g, 0.45 mmol, 11 equiv.) dissolved in DMF (3.3 mL) at room temperature under nitrogen and stirred for 14 h. The reaction mixture was diluted with ether (50 mL), washed with phosphate buffer (500 mM, pH = 6.0, 50 mL), water (50 mL) and dried (Na_2SO_4). The solvent was removed on a rotary evaporator to afford 0.0245 g of the corresponding succinimidyl ester of **11b** in 98% yield. Analytical HPLC using MeCN:0.1% aqueous formic acid/55:45) R_t: 22.78 min: 99%; ^{1}H NMR ($CDCl_3$): δ 10.02 (br s, 1H), 5.50 (br s, 1H), 4.24 (d, J = 5.5 Hz, 2H), 3.42 (s, 2H), 3.05 (t, J = 7.6 Hz, 2H), 2.82 (s, 4H), 2.51 (t, J = 7.6 Hz, 2H), 1.45 (s, 27H); ES–MS: 597 $(M+H)^+$.

The above prepared succinimidyl active ester of **11b** (0.024 g, 0.042 mmol) was dissolved in DMF (0.80 mL) and added 5-aminomethylfluorescein hydrobromide (**15**, 0.050 g, 0.085 mmol, 2 equiv.) followed by additon of diisopropylethylamine (0.073 mL, 0.42 mmol) at room temperature under nitrogen. The reaction mixture was stirred at 50 °C for 3.5 days and solvents were removed on a rotary evaporator. Preparative HPLC purification (MeCN:0.1% aqueous formic acid/54:46) of the crude mixture gave 0.023 g of fluorescein derivative (**18**) in 66% yield. Analytical HPLC using MeCN:0.1% aqueous formic acid/55:45) R_t: 22.78 min: 99%; ^{1}H NMR ($CDCl_3$ + 2 drops CD_3OD): δ 7.99 (s, 1H), 7.71 (d, J = 6.1 Hz, 1H), 7.12 (d, J = 6.1 Hz, 1H), 6.68 (s, 2H), 6.60–6.43 (m, 4H), 4.74 (s, 2H), 4.23 (s, 2H), 3.35 (s, 2H), 3.00–2.92 (m, 2H), 2.62 (t, J = 7.1 Hz, 2H), 1.47 (s, 18 H), 1.38 (s, 9H); ES–MS: 826 $(M)^+$.

Trifluoroacetic acid (3.5 mL) was added to a suspension of above prepared fluorescein derivative (**18**, 0.023 g, 0.028 mmol) in methylene chloride (3.5 mL) at room temperature and stirred 30 min. Toluene (5 mL) was added to the reaction mixture and the solvents azetropically removed on a rotary evaporator. The crude product was purified by preparative HPLC (MeCN:0.1% aqueous formic acid/ 25:75) and lyophilized to afford 0.0078 g of fluorescent tracer (**19**) in 45% yield as an orange powder. Analytical HPLC: (MeCN:0.1% aqueous formic acid/ 30:70) R_t: 5.20 min, 99%; ^{1}H NMR (CD_3OD): δ 7.99 (s, 1H), 7.78 (d, J = 6.9 Hz, 2H), 7.17 (d, J = 6.9 Hz, 2H), 6.66 (d, J = 2.4 Hz, 2H), 6.64 (d, J = 8.7 Hz, 2H), 6.52 (dd, J = 8.7, 2.4 Hz, 2H), 4.73 (s, 2H), 4.09 (s, 2H), 3.42 (s, 2H), 3.04 (t, J = 6.3 Hz, 2H), 2.65 (t, J = 6.3 Hz, 2H); ES–MS: 613 $(M)^+$; HRMS calcd for $(C_{32}H_{27}N_3O_{10} + H)^+$ 614.1775, found: 614.1766.

***t*-Butyl 4-nitro-5-acetoxy-7-[(tetrahydro-2H-pyran-2-yl)oxy]-heptanoate (20b):** 4-Dimethyl aminopyridine (DMAP) (1.18 g, 9.7 mmol, 1.0 equiv.) was added to a room temperature solution of *t*-butyl 4-

nitrobutyrate[28] (2.74 g, 14.5 mmol, 1.5 equiv.) and 3-[(tetrahydro-2H-pyran-2-yl)oxy] propanal[17c] (1.53 g, 9.7 mmol) in dry CH_2Cl_2 and stirred for 96 h. The solvent was removed on a rotary evaporator and the crude mixture was purified by silica gel column chromatography (20-35% EtOAc in hexane) to afford 2.45 g of α-hydroxynitro compound (**20a**) in 73% yield as a colorless thick oil. ^{1}H NMR ($CDCl_3$): δ 4.62–4.54 (m, 2H), 4.30–3.45 (m, 5H), 2.41–2.10 (m, 4H), 1.90–1.70 (m, 2H), 1.57–1.51 (m, 7H), 1.44 (s, 9H); ES-MS: 365 $(M+NH_4)^+$.

The above prepared α-hydroxynitro compound (**20a**, 4.45 g, 12.8 mmol) was dissolved in CH_2Cl_2 (50 mL), cooled to 0 °C and added anhydrous pyridine (1.15 mL, 19.2 mmol) followed by acetic anhydride (4.83 mL, 51.3 mmol) dropwise under nitrogen. After 2.0 h, the mixture was allowed to warm to room temperature and stirred for 14 h. Workup consisted of pouring the reaction mixture into 10% aq. $NaHCO_3$ (50 mL), separated layers and extracted the aqueous layer with CH_2Cl_2 (2 × 50 mL). The combined organic layers were washed with 5% HCl (20 mL), water (30 mL), brine (15 mL), dried ($MgSO_4$) and the solvents were removed on a rotary evaporator. Purification of crude compound by silica gel column chromatography (20% EtOAc in hexane) afforded 2.63 g of **20b** in 51% yield as a colorless thick oil. ^{1}H NMR ($CDCl_3$): δ 5.50-5.40 (m, 1H), 4.90-4.80 (m, 1H), 4.65–4.50 (m, 1H), 4.15-3.35 (m, 4H), 2.45-1.45 (m, 12 H), 2.08 and 2.04 (two s, 3H), 1.44 (s, 9H); ES-MS: 407 $(M+NH_4)^+$.

***t*-Butyl 2-(benzyloxycarbonyl)-3-{[2-(tetrahydro-2H-pyran-2-yl)oxy]ethyl}-1H-pyrrole-4-propanoate (22):** 1,8-Diazabicyclo[5.4.0]undec-7-ene (DBU, 0.76 mL, 5.08 mmol, 1.2 equiv.) was added to an ice cooled mixture of α-acetoxynitro compound (**20b**, 1.65 g, 4.24 mmol) and benzyl isocyanoacetate[26] (**21**, 0.890 g, 5.08 mmol, 1.2 equiv.) and THF (14 mL) under nitrogen. Reaction mixture was stirred for 30 min at 0-5 °C, allowed to warm to room temperature and stirred for 24 h. The resulting orange-red color solution was quenched with water (20 mL) and extracted with ethyl acetate (3 × 50 mL). The combined organic layers were washed with water (20 mL), brine (15 mL), dried ($MgSO_4$) and the solvents were removed on a rotary evaporator. Crude compound was purified by silica gel column chromatography (20% EtOAc in *n*-hexane) to afford 1.21 g of **22** in 63% yield as a colorless thick oil. ^{1}H NMR ($CDCl_3$): δ 8.86 (br s, 1H), 7.45–7.26 (m, 5H), 6.70 (s, 1H), 5.25–5.35 (m, 2H), 4.51 (br s, 1H), 3.88–3.70 (m, 2H), 3.56–3.38 (m, 2H), 3.04 (t, J = 7.2 Hz, 2H), 2.75 (t, J = 7.8 Hz, 2H), 2.47 (t, J = 7.5 Hz, 2H), 1.80–1.43 (m, 6H), 1.42 (s, 9H); ES-MS: 475 $(M+NH_4)^+$.

***t*-Butyl 2-(benzyloxycarbonyl)-3-(2-hydroxyethyl)-1H-pyrrole-4-propanoate (23):** In a dry single necked round bottom flask equipped with magnetic stir bar, nitrogen inlet was placed the THP-ether (**22**, 1.20 g, 2.18 mmol) in methanol (20 mL, added pyridinium *p*-toluenesulfonate (PPTS, 0.58 g, 2.29 mmol, 1.05 equiv.) at room temperature and stirred for 48 h. Solvent was removed *in vacuo* and the mixture was diluted with water (30 mL) and ethyl acetate (75 mL). The aqueous layer was separated and extracted with ethyl acetate (2 × 50 mL). The combined organic extracts were washed with brine (20 mL), dried ($MgSO_4$) and concentrated on a rotary evaporator. Purification of thecrude compound by silica gel column chromatography (30-40% ethyl acetate in hexane) gave 1.66 g of alcohol (**23**) in 92% yield. ^{1}H NMR ($CDCl_3$): δ 8.90 (br s, 1H), 7.42–7.27 (m, 5H), 6.71 (d, J = 2.7 Hz, 1H), 5.29 (s, 2H), 3.79–3.74 (m, 2H), 3.03 (t, J = 6.3 Hz, 2H), 2.73 (t, J = 7.8 Hz, 2H), 2.47 (t, J = 7.8 Hz, 2H), 2.15–2.05 (m, 1H), 1.41 (s, 9H); ES-MS: 374 $(M+H)^+$.

***t*-Butyl 2-(benzyloxycarbonyl)-3-(*t*-butoxycarbonylmethyl)-1H-pyrrole-4-propanoate (24):** Jones reagent (2.67 M, 0.64 mL, 1.69 mmol, 1.48 equiv.) was added in one portion to a 0-5 °C cooled solution of alcohol (**23**, 0.400 g, 1.13 mmol) in acetone (20 mL) and stirred for 1.5 h. The reaction mixture was quenched with isopropanol (3.0 mL) and stirred for 30 min at room temperature. The mixture was diluted with acetone (22 mL), filtered and the solid was washed with acetone (30 mL). The filtrate was concentrated on a rotary evaporator and the resulting crude acid was dissolved in DMF (0.5 mL), cooled to 0-5 °C and added a solution of *O*-*t*-butyl-*N,N'*-diisopropylisourea[18] (0.313 g, 5.0 eq.) in DMF (1.0 mL). The cooling bath was removed and the mixture was stirred for 43 h at room temperature. The solvents was removed on a rotary evaporator and the crude compound was purified by silica gel column chromatography (20% ethyl acetate in hexane) to afford 0.190 g of **24** in 12% for two steps. Analytical HPLC: (MeCN:0.1% aqueous formic acid/25:75), R_t: 4.15 min, 97%; ^{1}H NMR ($CDCl_3$): δ 8.88 (br s, 1H), 7.26– 7.41 (m, 5H), 6.73 (d, J = 3.0 Hz, 1H), 5.29 (s, 2H), 3.77 (s, 2H), 2.70 (t, J = 6.9 Hz, 2H), 2.48 (t, J = 6.9 Hz, 2H), 1.42 (s, 9H), 1.40 (s, 9H); ES-MS: 444 $(M + H)^+$; HRMS calcd for $(C_{25}H_{33}NO_6 + H)^+$: 444.2386; found: 444.2383.

***t*-Butyl 2-(carboxy)-3-(*t*-butoxycarbonylmethyl)-1H-pyrrole-4-propanoate (25):** Palladium on carbon (10% Pd/C, 0.029 g) was added to a solution of ester (**24**, 0.094 g, 0.212 mmol) in absolute ethanol (4 mL) and stirred under hydrogen (1 atm) for 2.0 h at room temperature. The mixture was diluted with ethanol (10 mL), filtered and the solvent was removed on a rotary evaporator to afford 0.065 g of acid (**25**) in 89% yield. Analytical HPLC: (MeCN:0.1% aqueous formic acid/75:25) R_t: 2.57 min, 96%; ^{1}H NMR (acetone-d_6): δ 10.52 (br s, 1H), 6.88 (d, J = 3.3 Hz, 1H), 3.84 (s, 2H), 2.71 (t, J = 8.4 Hz, 2H), 2.49 (t, J = 8.4 Hz, 2H), 1.47 (s, 9H), 1.45 (s, 9H); mass spectrum (DCI/NH_3):354 $(M + H)^+$; HRMS calcd for $(C_{18}H_{27}NO_6 + H)^+$: 354.1917; found: 354.1914; .

***t*-Butyl 2-(succinimidyloxycarbonyl)-3-(*t*-butoxycarbonylmethyl)-1H-pyrrole-4-propanoate (26):** EDAC (0.033 g, 0.175 mmol, 3.5 equiv.) was added to a room temperature mixture of compound **25** (0.017 g, 0.05 mmol) and HOSu (0.015 g, 0.125 mmol, 2.5 equiv.) in dry DMF (0.5 mL) at room temperature under nitrogen atmosphere and stirred for 36 h. The mixture was diluted with potassium phosphate (pH 6.0) buffer solution (3 mL) and extracted with ether (3 × 20 mL). The combined ethereal extracts were washed with water (5 mL), dried ($MgSO_4$) and concentrated on a rotary evaporator to afford 0.021 g of succinimidyl ester (**26**) in 88% yield. Analytical HPLC: (MeCN:0.1% aqueous formic acid/75:25) R_t: 2.68 min, 89%; ^{1}H NMR (CD_3CN): δ 10.32 (br s, 1H), 7.12 (s, 1H), 3.88 (s, 2H), 2.97 (s, 4H), 2.83 (t, J = 8.1 Hz, 2H), 2.59 (t, J = 7.8 Hz, 2H), 1.57 (s, 9H), 1.55 (s, 9H); ES-MS: 468 $(M+NH_4)^+$.

2-[(*N*-Amidomethyl-6-fluoresceinyl)carbonyl]-3-carboxymethyl-1H-pyrrole-4-propanoic acid (29): In a dry single-necked round bottom flask equipped with magnetic stir bar was placed the succinimidyl ester (**26**, 8.0 mg, 0.017 mmol) in dry DMF (0.5 mL) and added 6-aminomethylfluorescein hydrochloride[25] (**27**, 6-AMF, 10.0 mg, 0.025 mmol, 1.5 equiv.) followed by triethylamine (0.018 mL, 0.13 mmol, 8.0 equiv) at room temperature under nitrogen. The mixture was stirred for 18 h and purified by preparative HPLC (MeCN:0.1% aqueous formic acid/45:55) to afford 0.0096 g of fluorescein derivative **28** in 77% yield. Analytical HPLC: (MeCN:0.1% aqueous formic acid/ 55:45), R_t: 6.43 min, >99%; ^{1}H NMR (CD_3OD): δ 7.96 (d, J = 7.8 Hz, 1H), 7.68 (d, J = 7.8 Hz, 1H), 7.21 (s, 1H), 6.69–6.48 (m, 7H), 4.59 (s,

2H), 3.52 (s, 2H), 2.67 (t, J = 7.5 Hz, 2H), 2.43 (t, J = 7.2 Hz, 2H), 1.38 (s, 9H), 1.34 (s, 9H); ES-MS (M + H)$^+$ 697.

Trifluoroacetic acid (1.0 mL) was added to a suspension of **28** (0.0085 g, 0.012 mmol) in dry CH_2Cl_2 (1.0 mL), and stirred at room temperature for 1.5 h. The mixture was concentrated on a rotary evaporator and purified by preparative HPLC (MeCN:0.1% aqueous formic acid/70:30) and lyophilized to afford 0.0024 g of fluorescent tracer (**29**) in 34% yield. Analytical HPLC: (MeCN:0.1% aqueous formic acid/30:70), R_t: 7.08 min, >99%; ^{1}H NMR (CD_3OD): δ 7.95 (d, J = 7.8 Hz, 1H), 7.72–7.68 (m, 1H), 7.21 (s, 1H), 6.67–6.51 (m, 7H), 4.61 (s, 2H), 3.37 (s, 2H), 2.77 (t, J = 7.8 Hz, 2H), 2.50 (t, J = 7.2 Hz, 2H); ES–MS: 584 (M)$^+$; HRMS calcd for ($C_{31}H_{24}N_2O_{10}$ + H)$^+$ 585.1509, found 585.1506.

1-(*N*-amidomethyl-6-fluoresceinyl)-5-aminopentane (30): In a dry single-necked round bottom flask equipped with magnetic stir bar was placed 6-(*t*-butoxycarbonylamino)-hexanoic acid (1.15 g, 5.0 mmol), HOSu (0.69 g, 6.0 mmol, 1.2 equiv.) and dry DMF (15 mL) and added EDAC (1.24 g, 6.5 mmol, 1.3 equiv.) at room temperature and stirred for 24 h under nitrogen. The solvent was removed on a rotary evaporator and diluted with water (25 mL). The mixture was extracted with ether (50 mL, 2 × 30 mL) and the combined etherial layers were washed with water (2 × 20 mL), brine (15 mL) and dried ($MgSO_4$). Removal of the solvent on a rotary evaporator afforded 1.52 g of of 6-*t*-butoxycarbonyl-amino-1-hexanoic acid succinimidyl ester in 93% yield. ^{1}H NMR ($CDCl_3$): δ 4.60 (br s, 1H), 3.14–3.05 (m, 2H), 2.97 (s, 4H), 2.83 (s, 4H), 2.61 (t, J = 7.2 Hz, 2H), 1.71–1.82 (m, 2H), 1.54–1.40 (m, 4H), 1.43 (s, 9H).

To the above prepared crude succinimidyl ester (0.032 g, 0.1 mmol, 2.0 equiv.) in dry DMF (0.4 mL) were added 6-(aminomethyl)fluorescein hydrochloride (**27**, 6-AMF) (0.020 g, 0.05 mmol) followed by triethylamine (0.049 mL, 0.35 mmol, 7.0 equiv.) at room temperature under nitrogen. The mixture was stirred for 24 h at room temperature and purified by preparative HPLC (MeCN:0.1% aqueous formic acid/32:68) and lyophilized to afford 0.026 g of 6-*t*-butoxycarbonylamidopentane-1-(6-carboxamidomethyl)fluorescein in 96% yield. Analytical HPLC: (MeCN:0.1% aqueous formic acid/40:60), R_t: 7.98 min, 96%; ^{1}H NMR (CD_3OD): δ 8.05 (d, J = 8.1 Hz, 1H), 7.69 (d, J = 6.9 Hz, 1H), 7.19 (s, 1H), 6.87–6.70 (m, 6H), 4.51 (d, J = 6.0 Hz, 2H), 3.12–3.05 (m, 2H), 2.30–2.20 (t, J = 7.8 Hz, 2H), 1.65–1.46 (m, 4H), 1.56 (s, 9H), 1.35–1.25 (m, 2H); ES-MS: 575 (M+H)$^+$.

The above prepared 6-*t*-butoxycarbonylamidopentane-1-(6-carboxamidomethyl)fluorescein (0.024 g, 0.042 mmol) in dry CH_2Cl_2 (1.0 mL) added trifluoroacetic acid (0.5 mL) and stirred at room temperature for 10 min. The mixture was concentrated on a rotary evaporator and purified by preparative HPLC (MeCN:0.1% aqueous formic acid/60:40) and lyophilized to give 0.020 g of 6-aminopentane-1-(6-carboxamidomethyl)fluorescein (**30**) in 95% yield. Analytical HPLC: (MeCN:0.1% aqueous formic acid/40:60), R_t: 2.54 min, >99%; ^{1}H NMR (CD_3OD): δ 7.96 (d, J = 8.1 Hz, 1H), 7.55 (d, J = 8.7 Hz, 1H), 7.10 (s, 1H), 6.79–6.53 (m, 6H), 4.86 (s, 2H), 2.83 (t, J = 8.1 Hz, 2H), 2.21 (t, J = 7.5 Hz, 2H), 1.62–1.50 (m, 4H), 1.38–1.29 (m, 2H); ES-MS: 475 (M+H)$^+$.

2-{[(*N'*-Amidomethyl-6-fluoresceinyl)-*N*-amidopentylcarbonyl]carbonyl}-3-carboxy methyl-1H-pyrrole-4-propanoic acid (32): Triethylamine (0.020 mL, 0.15 mmol, 11.5 equiv.) was added to a mixture of succinimidyl ester (**26**, 0.006 g, 0.013 mmol) and 6-aminopentane-1-(6-carboxamidomethyl)fluorescein (**30**, 0.007 g, 0.014 mmol, 1.08 mmol) and dry DMF (0.5 mL) at room temperature under nitrogen. The mixture was stirred for 20 h and purified by preparative HPLC (MeCN:0.1%

aqueous formic acid/45:55) and lyophilized to afford 0.0045 g of fluorescein derivative (**31**) in 45% yield. Analytical HPLC: (MeCN:0.1% aqueous formic acid/45:55), R_t: 6.27 min, 98%; ^1H NMR (CD_3OD): δ 7.97–7.94 (m, 1H), 7.80–7.78 (m, 1H), 7.08 (s, 1H), 6.68–6.53 (m, 7H), 4.40 (s, 2H), 3.62 (s, 2H), 2.70 (t, J = 7.5 Hz, 2H), 2.47 (t, J = 8.1 Hz, 2H), 2.20–2.16 (m, 2H), 1.60–1.20 (m, 8H), 1.42 (s, 9H), 1.40 (s, 9H); ES-MS: 810 $(M+H)^+$.

Trifluoroacetic acid (1.0 mL) was added to a 0-5 °C to a solution of **31** (0.004 g, 0.005 mmol) in dry CH_2Cl_2 (1.0 mL) and stirred at room temperature for 1.5 h. The mixture was concentrated and purified by preparative HPLC (MeCN:0.1% aqueous formic acid/60:40) and lyophilized to afford 0.002 g of fluorescent tracer (**32**) in 59% yield as an orange powder. Analytical HPLC: (MeCN:0.1% aqueous formic acid/60:40), R_t: 3.46 min, 99%; ^1H NMR (CD_3OD): δ 7.90 (d, J = 7.5 Hz, 1H), 7.60–7.55 (m, 1H), 7.07 (s, 1H), 6.69–6.50 (m, 7H), 4.42 and 4.39 (s, 2H), 3.53 (s, 2H), 2.78 (t, J = 7.5 Hz, 2H), 2.53 (t, J = 7.8 Hz, 2H), 2.17 (t, J = 6.9 Hz, 2H), 1.56–1.24 (m, 8H); ES-MS: 698 $(M+H)^+$; HRMS calcd for $(C_{37}H_{35}N_3O_{11} + H)^+$ 697.2350, found 697.2339.

Construction of calibration curve (A,B): A standard solution of lead (GFS Chemicals, Columbus, OH) was diluted to 200, 400, 600 and 800 ng/mL in water and adjusted to pH=0.5 with concentrated HNO_3. The resulting mixture was adjusted to pH=7.1 with a neutralizing buffer [pH=7.85 which contains 1.25 M 3–(*N*–morpholino)propanesulfonic acid (MOPS), 1.25M *N*–(2–hydroxyethyl)piperazine–*N*'–(2-ethanesulfonic acid) (HEPES), 30 mM hydroxyquinoline-5-sulfonic acid and 0.1% Neomycin Sulfate]. The enzyme, 5-aminolevulinic acid dehydratase (ALAD) and the prediluted (1:100) rabbit antisera solutions were diluted into a buffer [pH=7.0 which contains 0.25 M MOPS, 0.9 M ammonium sulfate, 0.5% polyethylene glycol 8K and 0.1% neomycin Sulfate). The substrate consisted of 50 mM 5-aminolevulinic acid (**1**), 25 mM aq. tris(2-carboxyethyl)phosphine hydrochloride and 250 mM aq. zinc chloride. The fluorescent tracer (**15** or **19**) was diluted in Abbott IMx FPIA buffer[28] to approximately 13 nM.

These experiments were run on the Abbott IMx[28] analyzer using the following steps to generate the calibration curves.

1. A blank read was taken on an empty cuvette.
2. The lead (Pb) calibrator (150 μL) was mixed with neutralizing buffer (90 μL) and Abbott IMx FPIA buffer (50 μL).
3. A portion (140 μL) of this mixture was combined with the enzyme/antisera solution (90 μL) and Abbott IMx FPIA (95 μL) buffer then incubated for 6.25 minutes in the cuvette.
4. The substrate (90 μL) and Abbott IMx FPIA buffer (150 μL) were added to the cuvette, incubated for 20 minutes followed by the addition of fluorescent tracer (220 μL) and Abbott IMx FPIA buffer (375 μL) with a final incubation of 6.25 minutes.
5. The polarization was measured in millipolarization units on the cuvette solution.

Lead calibrators (I-V) with concentration of 0, 200, 400, 600 and 800 ng/mL were run in replicates of 2 or 4 and generated a calibration curve see Figure 2.

A pretreatment solution (200 μL, a mixture of 9% aqueous trichloroacetic acid, 0.6 N HNO3 and 5 mM periodic acid) was added to a sample (200 μL) in a 1.5 mL microfuge tube which was capped and vortexed for 30 sec. Centrifugation of the mixture at 10,000 rpm for 2 min afforded a translucent supernatant. The concentration

of the lead (Pb) was measured by following the procedure described except addding the translucent supernatant solution insted of the lead (Pb) calibrator in step 2.

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