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Synthesis of immunoreagents for measurement of galactosylhydroxylysine

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Abstract

(2S,5*R*)-(+)-Hydroxylysine (**6**) was transformed into (−)-succinimidyl ester (**13**) and conjugated to BSA or KLH to form immunogens (**2** and **3**) for generation of anti-galactosylhydroxylysine antibodies. Additionally, treatment of (−)-**13** with 6-Fln-CH_2NH_2 (**16**) or acridinium derivative (**17**) and subsequent hydrolysis gave the fluorescent (**4**) and chemiluminescent (**5**) tracers, respectively. These immunoreagents (**3**,**4** and **5**,**6**) are essential for development of assays for diagnosis of osteoporosis.

1. Introduction

Galactosylhydroxylysine (GHL **1**, Fig. 1) is a structural component of bone collagen, which is formed by post-translational glycosylation of hydroxylysine.[1] The structure of GHL (**1**) was shown to have a monosaccharide unit, D-galactopyranose, in which the anomeric center is β-linked at the (5*R*)-position of L-lysine.[2] During the process of bone resorption, GHL (**1**) is released into the serum and excreted in urine.[3] Thus, GHL (**1**) has attracted considerable attention for use as a biochemical marker for diagnosis of osteoporosis and other metabolic bone diseases.[4,5] A number of tedious methods were developed for its measurement, for example using amino acid analyzer,[2b,6] ion-exchange chromatography,[7] HPLC,[8] and enzyme-linked immunosorbent assay.[9] Development of sensitive and high throughput immunoassays, such as fluorescent polarization immunoassy (FPIA) and chemiluminescent immunoassay (CLIA), for GHL (**1**) is critically important for diagnosis and management of osteoporosis. In this paper, we describe the synthesis of immunoreagents (immunogens **2**,**3** and tracers **4**,**5**) starting from (2*S*,5*R*)-(+)-hydroxylysine (**6**), which are essential for development of assays (FPIA and CLIA) for GHL (**1**).

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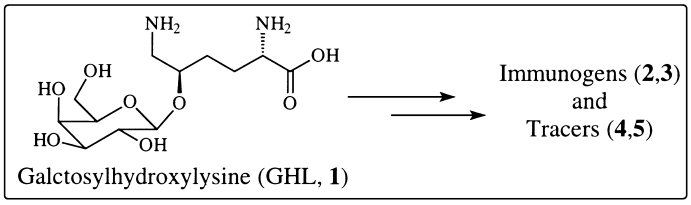


Figure 1.

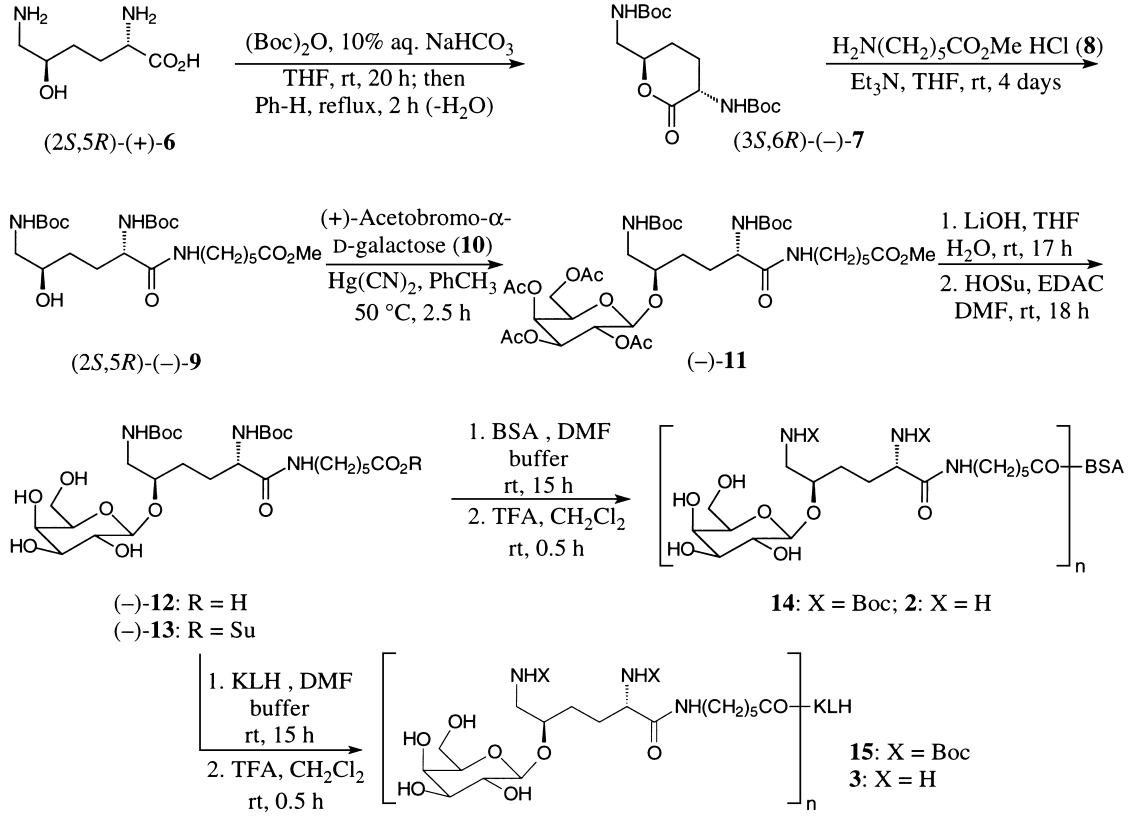
2. Results and discussion

In recent years there has been a tremendous surge in the development of new immunoassay technologies and innovative automated analyzers to meet the numerous challenges faced by the clinical laboratories.[10] The automated immunoassay analyzers developed by Abbott Laboratories, for example TDx and AxSYM, utilize FPIA,[11] whereas the recently launched ARCHITECT instrument is based on CLIA[12] technology. Both FPIA and CLIA methods require two key reagents, antibodies for binding and a signal generating material (tracer) for detection. Since the low molecular weight analytes (e.g. GHL, **1**) do not, as such, induce the formation of antibodies, they need to be conjugated to a carrier protein, thus forming an immunogen.[13] These immunogens are then used for inoculation of an animal (for example mouse, hamster, rabbit, sheep) for production of antibodies. Thus, the first phase in the development of immunoassay is design and synthesis of immunogens and tracers.

There are a number of important issues which need to be considered when preparing the immunogens, such as: site of the analyte modification, selection of carrier protein, selection of linking arm, method of conjugation and the hapten density.[14] More importantly, the chirality of optically active analytes (e.g. GHL, **1**), need to be preserved in preparing the immunoreagents. In order to allow the host animal immune system to recognize both chiral centers in the backbone of GHL (**1**), we envisioned introducing aminohexanoic acid as a spacer between GHL (**1**) and carrier protein. This is important, since in the case of thyroxine (T_4),[15] we observed that the anti-thyroxine antibodies, which were raised from an immunogen prepared by direct conjugation of the carboxylic acid of T_4 to carrier protein, were blind to the chiral center. Bovine serum albumin (BSA, molecular weight: 66 430 Da) and keyhole limphet hemocyanin (KLH, molecular weight: 450–13 000 kDa) are the two commonly used carrier proteins for the preparation of immunogens. Among these, BSA is well suited as carrier protein because of its solubility in various aqueous buffers and high content of primary amines (59 lysines and a terminal amine) available for conjugation with hapten. Although the immunogens prepared using KLH as carrier protein are difficult to characterize due to the poor solubility and variable mass, they are also frequently used for production of antibodies. Since the antibodies produced for carrier protein are also present in the host animal bleeds, an alternative hapten-carrier protein conjugate such as KLH-immunogen, is required to select the anti-analyte antibodies. Therefore, the key hapten, (−)-acid (**12**), a modified GHL (**1**) with linking arm, was envisioned from (2*S*,5*R*)-(+)-hydroxylysine (**6**).

Thus, (2*S*,5*R*)-(+)-**6** was treated (Scheme 1) with $(Boc)_2O$ in 10% aq. $NaHCO_3$ and THF followed by refluxing the resulting crude bis-Boc-acid in benzene for 2 h, to produce the (3*S*,6*R*)-(−)-lactone (**7**) in 94% yield. Opening of the (−)-lactone (**7**) with linking arm, methyl-6-aminohexanoate (**8**) in THF afforded the (2*S*,5*R*)-(−)-ester (**9**) in 78% yield. The next step in the synthesis of hapten (**12**) was to install the galactosyl unit from the hydroxyl group of (−)-**9**. Therefore, (−)-**9** was treated with 2.0 equiv. of (+)-acetobromo-α-D-galactose (**10**) and 2.2 equiv. of mercury(II) cyanide[16] in toluene at 50°C for 2.5 h. The product was purified by preparative reverse phase HPLC and lyophilized to give the glycosylated product (−)-**11** in >99% purity, but in low yield (13%). Our attempts to improve the yield for a glycosylation step

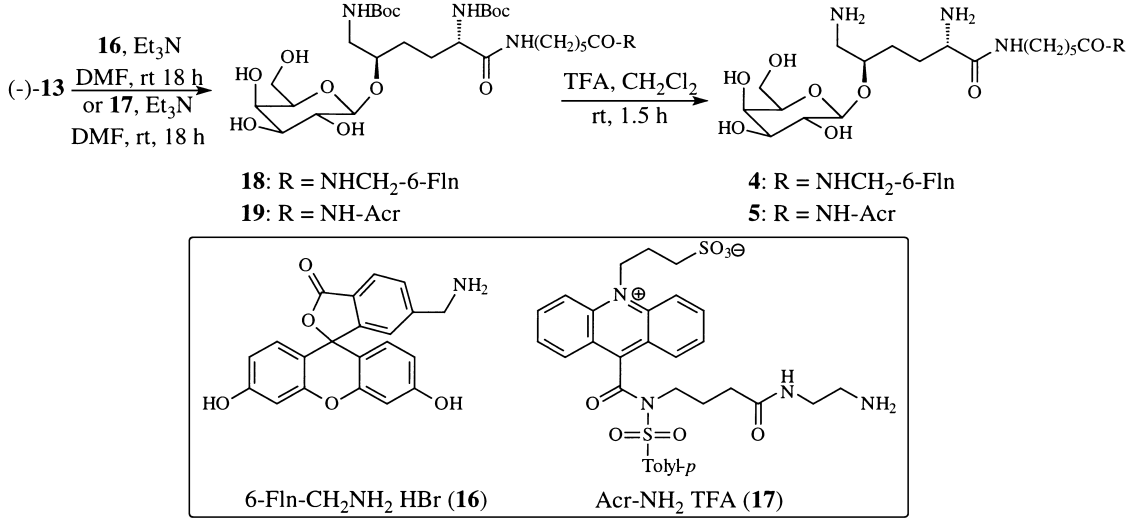
by changing the ratios of reagents and reaction conditions were unsuccessful. Finally, alkaline hydrolysis of (−)-**11** using LiOH in THF–H_2O followed by HPLC purification afforded the key hapten, (−)-acid (**12**), in 63% yield.



Scheme 1.

Since the in situ activation and coupling of acid hapten [for example (−)-**12**] to the carrier protein using reagents such as EDAC, can lead to the undesired modification of carrier protein, we found that it is critical to use pure activated ester [for example (−)-succinimidyl ester (**13**)] for preparation of the immunogen. Therefore, the (−)-acid (**12**) was converted to the corresponding (−)-succinimidyl ester (**13**) using *N*-hydroxysuccinimide and EDAC in DMF, in 65% yield and >98% purity. Conjugation of the activated ester (−)-**13** with carrier protein, BSA, was carried out in DMF and buffer (pH: 8.0) at room temperature. After 15 h, the crude reaction mixture was purified by dialysis to remove the hydrolyzed hapten (**12**) and other low molecular weight impurities. Lyophilization of the product gave an immunogen precursor (**14**), in which the Boc protected amino groups of the hapten were unmasked using trifluoroacetic acid in CH_2Cl_2. Immunogen (**2**) was purified by dialysis and isolated as a white powder after lyophilization. Analysis of the immunogen (**2**) by gel electrophoresis[17] showed a single narrow band indicating its homogeneity and the absence of free BSA. The resolution of gel electrophoresis is inadequate to determine the hapten density. Therefore, the molecular weight of the immunogen (**2**) was determined by MALDI[18] and found to be 77 465, which represents an average of 16 haptens incorporated per BSA. This substitution rate (26%) was consistent with 16 haptens per BSA, as observed for its precursor **14**. The immunogen (**3**) was prepared from (−)-**13** and KLH by following the procedure developed for immunogen (**2**).

In order to allow the tracer to mimic overall topology of the hapten, which was used to prepare the immunogen, the fluorescent and chemiluminescent labels were directly attached to hapten (−)-**12**. Thus, the active ester (−)-**13** was treated (Scheme 2) with 6-aminomethylfluorescein (6-Fln-CH_2NH_2, **16**) in DMF in the presence of triethylamine at room temperature to afford **18** in 69% yield. Hydrolysis of the Boc groups in **18** using trifluoroacetic acid in CH_2Cl_2 at room temperature followed by HPLC purification afforded the fluorescent tracer (**4**) in 84% yield and >99% purity as a yellow powder. Similarly, the active ester (−)-**13** was treated with acridinium derivative (Acr-NH_2, **17**)[19] and triethylamine in DMF at room temperature to afford **19** in 72% yield after purification by preparative reverse phase HPLC. Finally, hydrolysis of **19** using trifluoroacetic acid in CH_2Cl_2 followed by HPLC purification afforded the chemiluminescent tracer (**5**) in 77% yield and >99% purity as a yellow powder.



Scheme 2.

In summary, two immunogens (**2**,**3**) were prepared via conjugation of (−)-succinimidyl ester (**13**) to the carrier protein (BSA or KLH) followed by hydrolysis, for generation of anti-galactosylhydroxylysine antibodies. The key (−)-succinimidyl ester (**13**) was synthesized from a commercially available (2*S*,5*R*)-(+)-hydroxylysine (**6**) in five steps. Additionally, treatment of (−)-**13** with 6-aminofluorescein (**16**) or acridinium derivative (**17**) followed by hydrolysis gave the fluorescent (**4**) and chemiluminescent (**5**) tracers, respectively, which are essential for development of assays for diagnosis of osteoporosis.

3. Experimental

3.1. General methods and materials

^1H and ^{13}C NMR spectra were recorded on a Varian Gemini spectrometer (300 MHz), the chemical shifts (δ) were reported in ppm relative to TMS and coupling constants (*J*) were reported in hertz. Electrospray ionization mass spectrometry (ESI-MS) was carried out on a Perkin–Elmer (Norwalk, CT) Sciex API 100 Benchtop system employing Turbo Ionspray ion source and HRMS was performed on Nermang 3010 MS-50, JEOL SX102-A mass spectrometers. Thin layer chromatography was performed on pre-coated Whatman MK6F silica gel 60 Å plates (layer thickness: 250 μm) and visualized with UV light and/or using a $KMnO_4$ solution [$KMnO_4$ (1.0 g), NaOH (8.0 g) in water (200 mL)]. Column

chromatography was performed on silica gel, Merck grade 60 (230–400 mesh). THF and CH_2Cl_2 were freshly distilled from sodium benzophenone and CaH_2, respectively, under nitrogen. All reagents were purchased from Aldrich Chemical Co. (Milwaukee, WI) or Sigma Chemical Co. (St. Louis, MO) and used without purification, except where noted. All the solvents employed were of HPLC grade purchased from EM Science (Gibbstown, NJ) and used as received. Analytical reverse phase (RP) HPLC was performed using a Waters, μBondapak, RCM, C18, 10 μ (8×100 mm) column (solvents ratio v/v reported) unless otherwise stated. Preparative reverse phase (RP) HPLC was performed using a Waters, μBondapak, RCM, C18, 10 μ (40×100 mm) column (solvents ratio v/v reported) unless otherwise stated. Optical rotations were measured on an Autopol III polarimeter, Rudolph Research, Flanders, NJ. Melting points were recorded in open capillary tubes on an Electrothermal apparatus and were uncorrected.

*3.2. (−)-*tert*-Butyl-(3*S*,6*R*)-6-{[(*tert*-butoxycarbonyl)amino]methyl}-2-oxotetrahydro-2*H*-pyran-3-ylcarbamate (**7**)*

(2*S*,5*R*)-(+)-Hydroxylysine dihydrochloride monohydrate (**6**, 0.253 g, 1.0 mmol) was dissolved in 10% aq. $NaHCO_3$ (10 mL) and THF (5 mL). To this mixture, $(Boc)_2O$ (0.872 g, 4.0 mmol, 4.0 equiv.) was added at room temperature with vigorous stirring in two portions, at 4 h intervals. After stirring the mixture for 16 h, the pH was adjusted to 3.5 using 6.0N HCl and extracted with $CHCl_3$ (3×40 mL). The combined organic layers were washed with brine (15 mL), dried ($MgSO_4$) and concentrated on a rotary evaporator. The residue was dissolved in benzene (20 mL) and refluxed for 2 h while removing the water azeotropically using a Dean–Stark apparatus. The solvent was removed on a rotary evaporator and the crude product purified by silica gel column chromatography (50–80% EtOAc in hexanes) to afford 0.324 g of (3*S*,6*R*)-(−)-lactone (**7**) in 94% yield as a colorless glassy material. R_f: 0.58 (80% EtOAc in hexanes); mp 126–127°C; analytical RP HPLC: MeCN:0.1% aq. trifluoroacetic acid=55:45, 2.0 mL/min at 225 nm, t_R: 3.88 min, >99%; $[\alpha]_D^{20}$ −18.1 (*c* 0.56, $CHCl_3$); ^{1}H NMR ($CDCl_3$): δ 5.28–5.18 (m, 1H), 5.02–4.94 (m, 1H), 4.52–4.42 (m, 1H), 4.12–4.02 (m, 1H), 3.54–3.44 (m, 1H), 3.26–3.17 (m, 1H), 2.38–2.46 (m, 1H), 2.03–1.74 (m, 3H), 1.45 (s, 9H), 1.44 (s, 9H); ^{13}C NMR ($CDCl_3$): δ 170.6, 155.9, 155.5, 81.9, 80.4, 79.8, 77.2, 51.5, 44.7, 28.3, 28.2, 27.2, 25.7; ESI-MS (*m/z*): 345 $(M+H)^+$, 362 $(M+NH_4)^+$, 367 $(M+Na)^+$, 706 $(2\times M+NH_4)^+$, 711 $(2\times M+Na)^+$.

*3.3. (−)-Methyl 6-({(2*S*,5*R*)-2,6-bis[(*tert*-butoxycarbonyl)amino]-5-hydroxyhexanoyl}amino)hexanoate (**9**)*

Triethylamine (0.106 mL, 6.0 mmol, 4.0 equiv.) was added to a suspension of 6-aminohexanoic acid hydrochloride (**8**, 0.545 g, 3.0 mmol, 2.0 equiv.) and THF (8 mL) at room temperature under nitrogen. To this mixture, a solution of (3*S*,6*R*)-(−)-lactone (**7**, 0.514 g, 1.5 mmol) in THF (8 mL) was added via a double ended needle and stirred for 4 days. The reaction mixture was concentrated on a rotary evaporator and the residue dissolved in EtOAc (125 mL) and water (25 mL). The organic layer was separated and the aqueous layer extracted with EtOAc (50 mL). The combined organic layers were washed with water (25 mL) and brine (2×25 mL), dried ($MgSO_4$) and the solvent was removed on a rotary evaporator. The crude compound was purified by silica gel column chromatography (70% EtOAc in hexane to 3% MeOH in EtOAc) to afford 0.573 g of (−)-ester (**9**) in 78% yield as a colorless gummy material. R_f: 0.15 (80% EtOAc in hexanes); analytical RP HPLC: MeCN:water=15:85, 2.0 mL/min at 225 nm, t_R: 2.34 min, 98.2%; $[\alpha]_D^{20}$ −9.6 (*c* 0.82, $CHCl_3$); ^{1}H NMR ($CDCl_3$): δ 6.43 (dist. t, 1H), 5.42 (d, 1H, *J*=7.5 Hz), 5.06 (dist. t, 1H), 4.20–4.10 (m, 1H), 3.76–3.66 (m, 1H), 3.66 (s, 3H), 3.55–3.48 (m, 1H), 3.32–3.21 (m, 3H), 3.07–2.98 (m, 1H), 2.31 (t, 2H, *J*=7.5 Hz), 1.91–1.31 (m, 10H), 1.44 (s, 9H), 1.43 (s, 9H); ^{13}C

NMR ($CDCl_3$): δ 174.1, 171.9, 81.9, 79.8, 77.2, 71.4, 51.6, 46.6, 39.2, 33.8, 30.1, 29.4, 29.1, 28.4, 28.3, 26.2, 24.4; ESI-MS (*m/z*): 490 $(M+H)^+$; HRMS (FAB, *m/z*): calcd for $C_{23}H_{43}N_3O_8$: 490.3128 $(M+H)^+$; observed: 490.3110.

*3.4. (−)-Tetraacetate (**11**)*

(+)-Acetobromo-α-D-galactose (**10**, 3.02 g, 7.362 mmol, 2.0 equiv.) and mercury(II) cyanide (2.08 g, 8.28 mmol, 2.2 equiv.) were added sequentially to a solution of (−)-ester (**9**, 1.80 g, 3.681 mmol) dissolved in toluene (55 mL) under nitrogen. The mixture was heated at 50°C for 2.5 h, and concentrated on a rotary evaporator. The residue was purified by silica gel column chromatography (50% EtOAc in hexanes to 3% MeOH in EtOAc) to afford 1.077 g of material, which was further purified by preparative RP HPLC (MeCN:water=50:50, 45.0 mL/min at 225 nm). The product was lyophilized to afford 0.395 g of (−)-tetraacetate (**11**) in 13% yield as a white powder. R_f: 0.36 (80% EtOAc in hexanes); analytical RP HPLC: MeCN:water=55:45, 2.0 mL/min at 225 nm, t_R: 7.56 min, >99%; $[\alpha]_D^{20}$ −9.1 (*c* 0.53, $CHCl_3$); ^1H NMR ($CDCl_3$): δ 6.43 (br. s, 1H), 5.10–5.34 (m, 2H), 5.22–5.15 (m, 2H), 5.00 (dd, 1H, *J*=10.2, 3.3 Hz), 4.55 (d, 1H, *J*=7.5 Hz), 4.21–4.30 (m, 2H), 4.02–3.92 (m, 2H), 3.74–3.64 (m, 1H), 3.66 (s, 3H), 3.38–3.08 (m, 4H), 2.31 (t, 2H, *J*=7.2 Hz), 2.16 (s, 3H), 2.06 (s, 3H), 2.05 (s, 3H), 1.98 (s, 3H), 1.66–1.31 (m, 10H), 1.45 (s, 9H), 1.44 (s, 9H); ^{13}C NMR ($CDCl_3$): δ 174.1, 171.7, 170.3, 170.2, 170.1, 169.6, 156.5, 155.8, 101.3, 81.5, 80.1, 79.5, 77.2, 70.8, 70.7, 68.9, 67.0, 61.4, 51.5, 44.0, 39.2, 33.8, 29.1, 29.0, 28.6, 28.5, 28.3, 26.2, 24.2, 20.8, 20.7, 20.6, 20.5; ESI-MS (*m/z*): 820 $(M+H)^+$, 837 $(M+NH_4)^+$, 1639 $(2\times M+H)^+$; HRMS (FAB, *m/z*): calcd for $C_{37}H_{61}N_3O_{17}$: 842.3893 $(M+Na)^+$; observed: 842.3892.

*3.5. (−)-Acid (**12**)*

Lithium hydroxide monohydrate (0.199 g, 4.76 mmol, 10.0 equiv.) and water (6.0 mL) were added sequentially to a solution of (−)-tetraacetate (**11**, 0.390 g, 0.1 mmol) dissolved in THF (16 mL) at room temperature. After stirring the mixture for 17 h, the solvent was removed on a rotary evaporator to dryness. The residue was dissolved in MeCN:0.1% aq. trifluoroacetic acid (10 mL, ratio 35:65) and the pH of the mixture was adjusted to 5.6 using 1N HCl. The product was purified by preparative RP HPLC (MeCN:0.1% aq. trifluoroacetic acid=30:70, 45.0 mL/min at 225 nm). The product was lyophilized to afford 0.191 g of (−)-acid (**12**) in 63% yield as a white powder. Analytical RP HPLC: MeCN:0.1% aq. trifluoroacetic acid=35:65, 2.0 mL/min at 225 nm, t_R: 3.35 min, >99%; $[\alpha]_D^{20}$ −7.9 (*c* 0.38, MeOH; ^1H NMR (CD_3OD): δ 4.28 (d, 1H, *J*=7.2 Hz), 4.00–3.93 (m, 1H), 3.84–3.62 (m, 3H), 3.55–3.41 (m, 3H), 3.26–3.10 (m, 5H), 2.29 (t, 2H, *J*=7.5 Hz), 1.98–1.86 (m, 1H), 1.70–1.31 (m, 9H), 1.44 (s, 18H); ESI-MS (*m/z*): 638 $(M+H)^+$, 655 $(M+NH_4)^+$, 660 $(M+Na)^+$; HRMS (FAB, *m/z*): calcd for $C_{28}H_{51}N_3O_{13}$: 660.3314 $(M+Na)^+$; observed: 660.3316.

*3.6. (−)-Succinimidyl ester (**13**)*

N-Hydroxysuccinimide (0.069 g, 0.6 mmol, 2.0 equiv.) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDAC, 0.086 g, 0.45 mmol, 1.5 equiv.) were added sequentially to a solution of (−)-acid (**12**, 0.191 g, 0.3 mmol) dissolved in DMF (3.0 mL) at room temperature under nitrogen. After stirring the mixture for 16 h, the solvent was removed on a rotary evaporator under vacuum. The residue was dissolved in MeCN:water (8 mL, ratio 75:25) and purified by RP HPLC (MeCN:0.1% aq. trifluoroacetic acid=35:65, 45.0 mL/min at 225 nm). The product was lyophilized to afford 0.142 g of (−)-succinimidyl ester (**13**) in 65% yield as a white powder. Analytical RP HPLC: MeCN:0.1% aq.

trifluoroacetic acid=35:65, 2.0 mL/min at 225 nm, t_R: 5.53 min, >98%; $[\alpha]_D^{20}$ −5.0 (*c* 0.18, MeOH); ^{1}H NMR (CD_3OD): δ 4.28 (d, 1H, *J*=6.9 Hz), 4.00–3.94 (m, 1H), 3.86–3.62 (m, 3H), 3.56–3.42 (m, 3H), 3.28–3.10 (m, 5H), 2.83 (s, 4H), 2.64 (t, 2H, *J*=7.2 Hz), 1.98–1.86 (m, 1H), 1.80–1.30 (m, 9H), 1.44 (s, 18H); ESI-MS (*m/z*): 735 $(M+H)^+$, 752 $(M+NH_4)^+$, 757 $(M+Na)^+$; HRMS (FTMS, *m/z*): calcd for $C_{32}H_{54}N_4O_{15}$: 757.3477 $(M+Na)^+$; observed: 757.3478.

*3.7. BSA-immunogen (**2**)*

A solution of BSA (0.200 g, 0.003006 mmol) dissolved in buffer (pH: 8.00, 10 mL) was added to a solution of (−)-succinimidyl ester (**13**, 0.0507 g, 0.06914 mmol, 23.0 equiv.) dissolved in DMF (2 mL) at room temperature. The mixture was stirred for 15 h and dialyzed in a solution of 0.05N phosphate buffer (1.8 L) and MeOH (200 mL) twice by changing to fresh buffer–MeOH mixture for a total of 8 h. The mixture was further dialyzed in deionized (DI) water (4.0 L) four times by changing to fresh DI water every 5 h. Lyophilization of the mixture afforded 0.197 g of immunogen precursor (**14**) with Boc protective groups, as a white powder. Analysis of this immunogen precursor (**14**) by gel electrophoresis showed a single narrow band and an absence of free BSA. MALDI mass spectrum: 76587 [BSA with 16 haptens (**12**)]. A mixture of TFA (9.5 mL) and water (0.5 mL) was added to this intermediate immunogen (0.192 g) at room temperature and stirred for 0.5 h. The solvent was removed on a rotary evaporator to dryness and the residue was diluted with water (15 mL). The pH of the mixture was adjusted to 8.5 using 1N aq. NaOH. The mixture was dialyzed in 0.05N phosphate buffer (4.0 L) twice by changing to a fresh buffer solution, for a total of 15 h. The mixture was further dialyzed in DI water (4.0 L) twice by changing to fresh DI water every 3 h. Lyophilization of the mixture afforded 0.179 g of BSA-immunogen (**2**) as a white powder. Analysis of immunogen (**2**) by gel electrophoresis showed a single narrow band and an absence of free BSA. MALDI mass spectrum: 73645 [BSA with 16 haptens].

*3.8. KLH-immunogen (**3**)*

A solution of KLH (0.200 g), dissolved in buffer (pH: 8.00, 10 mL) was added to a solution of (−)-succinimidyl ester (**13**, 0.0507 g, 0.06914 mmol) dissolved in DMF (2 mL) at room temperature. The mixture was stirred for 15 h and dialyzed in a solution of 0.05 N phosphate buffer (1.8 L) and MeOH (200 mL) twice by changing to fresh buffer–MeOH mixture every 4 h. The mixture was further dialyzed in deionized (DI) water (4.0 L) four times by changing to fresh DI water every 5 h. Lyophilization of the mixture afforded 0.213 g of an immunogen precursor (**15**) with Boc protective groups. A mixture of TFA (9.5 mL) and water (0.5 mL) was added to this precursor **15** (0.213 g) at room temperature and stirred for 0.5 h. The solvent was removed on a rotary evaporator to dryness, the resulting residue was diluted with water (15 mL) and the pH was adjusted to 8.5 using 1N aq. NaOH. The mixture was dialyzed in 0.05 N phosphate buffer (4.0 L) twice by changing to a fresh buffer solution, for a total of 15 h. The mixture was further dialyzed in DI water (4.0 L) twice by changing to fresh DI water every 3 h. Lyophilization of the mixture afforded 0.194 g of KLH-immunogen (**3**) as a pale gray powder.

*3.9. 6-Fluorescenyl-conjugate (**18**)*

6-Fln-CH_2NH_2 HBr (**16**, 0.00992 g, 0.0225 mmol, 1.5 equiv.) and triethylamine (0.012 mL, 0.088 mmol, 8.0 equiv.) were added sequentially to a solution of (−)-succinimidyl ester (**13**, 0.011 g, 0.015 mmol) dissolved in anhydrous DMF (0.3 mL) at room temperature under nitrogen. After stirring the reaction mixture for 20 h, the solvent was removed on a rotary evaporator under vacuum to dryness. The residue was dissolved in MeCN:0.1% aq. trifluoroacetic acid (3.0 mL, ratio 1:1) and purified

by preparative RP HPLC (MeCN:0.1% aq. trifluoroacetic acid=35:65, 45.0 mL/min at 225 nm). The product was lyophilized to afford 0.0102 g of **18** in 69% yield as a yellow powder. Analytical RP HPLC: MeCN:0.1% aq. trifluoroacetic acid=35:65, 2.0 mL/min at 225 nm, t_R: 8.39 min, >99%; ^{1}H NMR (CD_3OD): δ 8.54–8.48 (m, 45/100H), 7.99 (d, 1H, *J*=7.8 Hz), 7.92–7.86 (m, 55/100H), 7.64–7.60 (m, 1H), 7.12 (s, 1H), 6.78–6.58 (m, 6H), 4.46–4.42 (m, 2H), 4.27 (d, 1H, *J*=7.2 Hz), 3.99–3.92 (m, 1H), 3.84–3.40 (m, 6H), 3.28–3.02 (m, 5H), 2.18 (t, 2H, *J*=7.2 Hz), 1.98–1.18 (m, 10H), 1.43 (s, 9H), 1.42 (s, 9H); ESI-MS (*m/z*): 982 $(M+H)^+$; HRMS (FAB, *m/z*): calcd for $C_{49}H_{64}N_4O_{17}$: 1003.4158 $(M+Na)^+$; observed: 1003.4159.

*3.10. Fluorescent tracer (6-Fln-tracer, **4**)*

Dichloromethane (3.0 mL) and trifluoroacetic acid (3.0 mL) were added sequentially to 6-fluorescenyl-conjugate (**18**) (0.010 g, 0.0102 mmol) at room temperature and stirred for 1.5 h. The solvent was removed on a rotary evaporator to dryness and dissolved in MeCN:0.1% aq. trifluoroacetic acid (5 mL, ratio 30:70). The residue was purified by preparative RP HPLC (MeCN:0.1% aq. trifluoroacetic acid=25:75, 45.0 mL/min at 225 nm). The product was lyophilized to afford 0.0086 g of fluorescent tracer-TFA salt (6-Fln-tracer, **4**) in 84% yield as a yellow powder. Analytical RP HPLC: MeCN:0.1% aq. trifluoroacetic acid=25:75, 2.0 mL/min at 225 nm, t_R: 4.61 min, >99%; ^{1}H NMR (CD_3OD): δ 7.95 (d, 1H, *J*=7.5 Hz), 7.62–7.58 (m, 1H), 7.09 (s, 1H), 6.67 (d, 2H, *J*=2.1 Hz), 6.59–6.50 (m, 4H), 4.42 (s, 2H), 4.36 (d, 1H, *J*=7.5 Hz), 4.02–3.94 (m, 1H), 3.87 (t, 1H, *J*=6.6 Hz), 3.81–3.41 (m, 5H), 3.22–3.07 (m, 3H), 2.98–2.90 (m, 2H), 2.19 (t, 2H, *J*=7.5 Hz), 2.14–1.22 (m, 10H), 1.43 (s, 9H), 1.42 (s, 9H); ESI-MS (*m/z*): 781 $(M+H)^+$, 803 $(M+Na)^+$; HRMS (FAB, *m/z*): calcd for $C_{39}H_{48}N_4O_{13}$: 803.3111 $(M+Na)^+$; observed: 803.3110.

*3.11. Acridinium-conjugate (**17**)*

Acr-NH_2 TFA (**17**, 0.0204 g, 0.03 mmol, 1.5 equiv.) and triethylamine (0.022 mL, 0.16 mmol, 8.0 equiv.) were added sequentially to a solution of (−)-succinimidyl ester (**13**, 0.0146 g, 0.02 mmol) dissolved in anhydrous DMF (0.4 mL) at room temperature under nitrogen. After stirring the mixture for 15 h, the solvent was removed on a rotary evaporator under vacuum. The residue was dissolved in MeCN:0.1% aq. trifluoroacetic acid (5.0 mL, ratio 1:2) and purified by preparative RP HPLC (MeCN:0.1% aq. trifluoroacetic acid=33:67, 45.0 mL/min at 225 nm). The product was lyophilized to afford 0.018 g of **19** in 72% yield as a yellow powder. Analytical RP HPLC: MeCN:0.1% aq. trifluoroacetic acid=35:65, 2.0 mL/min at 225 nm, t_R: 4.83 min, >99%; ^{1}H NMR (CD_3OD): δ 8.99–8.92 (m, 2H), 8.48–8.43 (m, 2H), 8.19–7.61 (m, 6H), 7.21–7.17 (m, 2H), 5.78–5.74 (m, 2H), 4.32–4.24 (m, 2H), 4.00–3.92 (m, 1H), 3.86–3.04 (m, 15H, merged with CD_3OD), 2.86–2.12 (m, 8H), 2.58 and 2.38 (two s, 3H), 2.00–1.28 (m, 12H), 1.43 (s, 9H), 1.42 (s, 9H); ESI-MS (*m/z*): 1247 $(M+H)^+$, 1269 $(M+Na)^+$; HRMS (FAB, *m/z*): calcd for $C_{58}H_{83}N_7O_{19}S_2$: 1268.5094 $(M+Na)^+$; observed: 1268.5077.

*3.12. Chemiluminescent tracer (Acr-tracer, **5**)*

CH_2Cl_2 (4.0 mL) and trifluoroacetic acid (4.0 mL) were added sequentially to **19** (0.018 g, 0.015 mmol) at room temperature and stirred for 1.5 h. The solvent was removed on a rotary evaporator and the residue was dissolved in MeCN:0.1% trifluoroacetic acid (8.0 mL, ratio 1:1). Purification of the crude product by preparative RP HPLC (MeCN:0.1% aq. trifluoroacetic acid=25:75, 45.0 mL/min at 225 nm) followed by lyophilization afforded 0.00121 g of Acr-tracer-TFA salt (**5**) in 77% yield as a yellow

powder. Analytical RP HPLC: MeCN:0.1% aq. trifluoroacetic acid=25:75, 2.0 mL/min at 225 nm, t_R: 5.02 min, >99%; ^{1}H NMR (CD_3OD): δ 8.98–8.92 (m, 2H), 8.50–8.42 (m, 2H), 8.22–7.61 (m, 6H), 7.22–7.18 (m, 2H), 5.80–5.70 (m, 2H), 4.44–4.26 (m, 3H), 4.10–3.06 (m, 15H, merged with CD_3OD), 3.00–2.10 (m, 8H), 2.58 and 2.38 (two s, 3H), 1.80–1.20 (m, 12H); ESI-MS (*m/z*): 1047 $(M+H)^+$, 1069 $(M+Na)^+$; HRMS (FAB, *m/z*): calcd for $C_{48}H_{67}N_7O_{15}S_2$: 1046.4215 $(M+H)^+$; observed: 1046.4209.

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