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Preparation of isodesmosine-KLH conjugate for ELISA system

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Abstract

Chronic obstructive pulmonary disease (COPD) is a degenerative condition with limited diagnostic detection efficiency. Currently with no available cure, COPD is associated with irreversible elastic tissue degradation in lungs, which results in release of unusual amino acids, isodesmosine and desmosine. These biomarkers are potential key elements in enzyme-linked immunosorbent assay (ELISA), an analytical method, which can detect certain compounds including antigens and proteins in easy and affordable manner. In order to target a biomarker with ELISA, it is necessary to prepare its specific antibody, which can be achieved by immunization of host organism with appropriate antigen containing the biomarker. Although preparation of these types of conjugates has been published, desmosine and isodesmosine used by researchers are obtained from natural sources such as animal tissues. Here, we report the first synthetic preparation of isodesmosine and keyhole limpet hemocyanin (KLH) conjugate from commercially available chiral amino acids and carrier protein. Formation of the core pyridinium of isodesmosine was achieved through key reaction—Chichibabin pyridinium synthesis—to deliver a 1,2,3,5-tetrasubstituted pyridinium amino acid selectively. Further modifications involving KLH and maleimide linker provided the target conjugate, which could potentially invoke an immune response to produce anti-isodesmosine antibody for the ELISA system.

KEYWORDS

amino acids, Chichibabin pyridinium synthesis, conjugate, COPD

1 | INTRODUCTION

A term chronic obstructive pulmonary disease (abbreviated as COPD) embraces several types of respiratory system diseases, including chronic bronchitis and emphysema.[1] It is known to be a progressive, degenerative condition characterized by limited airflow to the lungs resulting from irreversible degeneration of their mature elastin structures. According to World Health Organization (WHO),[2] COPD became the third most common cause of death globally as of 2016, having killed more than 3 million of people over a wide range of differently developed countries. In addition to lack of treatment, COPD may be difficult to diagnose due to the nature of its symptoms, which initially do not indicate seriousness of condition. Even at present, it tends to be only recognized in late stages, which greatly decreases patient's chances for survival. For that reason, an alternative method for detection of early onset

Professor Koji Nakanishi contributed equally to this work.

of this disease is strongly needed to date. Solution to this problem might be found in COPD biomarkers, isodesmosine and desmosine (Figure 1), which are crosslinkers of elastin.[3]

With onset of the sickness, elastin structures in lungs become increasingly damaged, and both compounds are released into the patient's body, where their rising levels can be detected in most bodily fluids like blood, sputum, and urine by liquid chromatography-mass spectrometry (LC-MS or LC-MS/MS) analysis.[4,5] Easily accessible for analysis, these biomarkers have a potential usage as key elements in enzyme-linked immunosorbent assay (ELISA), a promising analytical method, which can detect certain compounds including antigens and proteins in an easy and affordable manner.

Successful performance of ELISA requires an antibody able to detect the antigen of interest with high specificity.[6] Although there have been previous reports regarding application of ELISA for detection of COPD biomarkers desmosines,[7,8] there has been no previous chemical synthesis of the conjugate from readily available chiral amino acid starting materials, without involving animal tissues in the process. Moreover, most of the published studies focus their research on desmosine as the immune response-inducing antigen component, without giving much attention to other crosslinkers (despite both biomarkers existing in vivo in approximately same abundance).

The aim of this study is to prepare an isodesmosine and keyhole limpet hemocyanin (KLH) conjugate-based solely on synthetic isodesmosine, which can be formed from chiral amino acids by lanthanide-promoted Chichibabin pyridinium synthesis.[9,10] The prepared conjugate can be useful as an immunogen (antigen) for preparation of antibody, which could pave the way towards ELISA-supported detection method of COPD. Meanwhile, the ability to produce an antigen conjugate purely out of commercially available organic compounds could open up a cleaner and more controlled formation process.

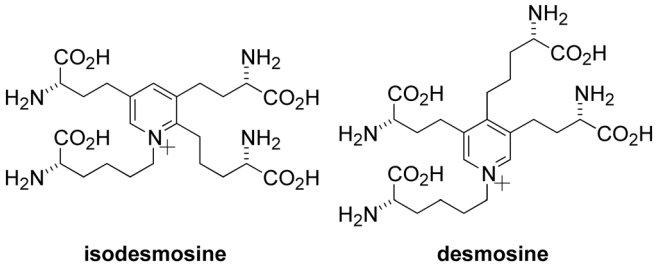


FIGURE 1 Structures of isodesmosine and desmosine

2 | MATERIALS AND METHODS

All nonaqueous reactions were conducted under an atmosphere of nitrogen with magnetic stirring unless otherwise indicated. All reagents were obtained from commercial suppliers and used without further purification unless otherwise stated. Analytical thin-layer chromatography (TLC) was performed on silica gel 60 F254 plates produced by Merck. Column chromatography was performed with acidic silica gel 60 (spherical, 40-50 μm) or neutral silica gel 60N (spherical, 40-50 μm) produced by Kanto Chemicals (Tokyo, Japan). High-performance liquid chromatography (HPLC) purification was performed with SHIMADZU LC-6AD, degasser unit DGU-20A3R, refractive index detector RID-10A, UV-Vis SPD-20A, valve unit FCV-12AH, and fraction collector FRC-10A. Removal of small amount of solvent was performed by Smart Evaporator CEV1-SQ-P2, CEV1-SK-P2, and CEV1A-GR-P2 (Kanagawa, Japan).

Optical rotations were measured on a JASCO P-2200 digital polarimeter at the sodium lamp ($\lambda = 589$ nm) D line and are reported as follows: $[\alpha]_D^T$ (c g cm^{-3}, solvent). ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded on a JEOL JNM-ECA 500 spectrometer (500 MHz). ^{1}H NMR data are reported as follows: chemical shift (δ, ppm), integration, multiplicity (s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet), coupling constants (J) in hertz, assignments. ^{13}C NMR data are reported in terms of chemical shift (δ, ppm). Electrospray ionization-mass spectrometer (ESI-MS) spectra were recorded on a JEOL JMS-T100LC instrument and are reported in mass-to-charge ratio (m/z).

2.1 | 1-*tert*-Butyl-2-*N*-benzyloxycarbonyl-L-lysine (5)

60% $HClO_4$ (0.064 mL, 0.59 mmol, 1.1 eq) has been added into flask containing Cbz-protected lysine 6 (150 mg, 0.53 mmol, 1.0 eq). After thorough distribution of acid, addition of *t*BuOAc (1.10 mL) has been done, and content was stirred magnetically for 48 hours, after which quench with saturated $NaHCO_3$ was done, followed by extraction using CH_2Cl_2. Crude was then checked with NMR, which revealed no major impurities; hence, column purification was abandoned. Product **5** (86.1 mg, 0.26 mmol, 48%) was obtained as yellow oil. ^{1}H NMR (500 MHz, $CDCl_3$, δ): 7.40-7.28 (5H, m, CH_2*Bn*), 5.37-5.26 (1H, m, NH), 5.16-5.06 (2H, m, *CH*$_2$Bn), 4.30-4.21 (1H, m, NH), 2.72-2.63 (2H, m, *CH*$_2$NH$_2$), 1.87-1.78 (1H, m, CH*CH*$_2$CH$_2$CH$_2$), 1.68-1.57 (5H, m, CH*CH*$_2$*CH*$_2$*CH*$_2$), 1.46 (9H, s, *t*Bu).

2.2 | 2-{16-(*tert*-Butoxycarbonyl)-16-(*S*)-[bis-(*tert*-butoxycarbonyl)-amino]-butyl}-3,5-bis-{20,24-(*tert*-butoxycarbonyl)-20,24-(*S*)-[bis-(*tert*-butoxycarbonyl)-amino] propyl}-1-{11-(*tert*-butoxycarbonyl)-11-(*S*)-[(carboxybenzyl)-amino]-pentyl}-pyridinium (3)

$Pr(OTf)_3$ (16.5 mg, 28.0 μmol, 50 mol%) was added to the solution of **5** (18.9 mg, 56.1 μmol, 1.0 eq) and **4** (90.1 mg, 224.4 μmol, 4.0 eq) at room temperature and stirred for 24 hours in H_2O/DMF (2:1) solvent (0.56 mL). After 1 day, the content of flask was diluted with EtOAc and extracted. After silica gel column chromatography (hexane/EtOAc = 1/1, then EtOAc, then EtOAc/MeOH = 5/1), the product was obtained as yellow oil (27.5 mg, 16.8 μmol, 30%); R_f 0.50 (CH_2Cl_2/MeOH = 10/1); ^{1}H NMR (500 MHz, $CDCl_3$, δ): 8.84-8.81 (1H, s, H6), 7.91-7.94 (1H, s, H4), 7.39-7.26 (5H, m, CH_2*Bn*), 5.68-5.62 (1H, m, NH), 5.15-5.03 (2H, m, *CH_2*Bn), 4.72-4.36 (5H, m, H7/16/20/24), 4.22-4.14 (1H, m, H11), 3.19-3.05 (2H, m, H13), 2.97-2.74 (4H, m, H18/22), 2.46-2.14 (4H, m, H10/23), 2.13-1.92 (4H, m, H15/19), 1.83-1.67 (6H, m, H8/9/14), 1.49 (54H, s, Boc), 1.43 (36H, m, *t*Bu); ^{13}C NMR (125 MHz, $CDCl_3$, δ): 171.3, 169.2, 169.1, 169.1, 156.2, 153.2, 152.8, 152.6, 145.6, 144.6, 141.1, 140.5, 136.5, 128.4, 128.0, 127.9, 119.2, 83.4, 83.3, 83.3, 82.2, 82.0, 81.7, 81.42, 77.3, 77.0, 76.8, 66.8, 58.1, 57.9, 57.9, 53.7, 32.3, 31.6, 31.1, 29.9, 29.8, 29.7, 29.0, 28.5, 28.3, 28.1, 28.0, 28.0, 27.9, 22.1; high-resolution mass spectrometry (HRMS) (ESI, *m*/z): $[M]^+$ calcd for $C_{78}H_{126}N_5O_{22}^+$, 1,484.8895; found 1,484.8910.

2.3 | 2-{16-(*tert*-Butoxycarbonyl)-16-(S)-[bis-(*tert*-butoxycarbonyl)-amino]-butyl}-3,5-bis-{20,24-(*tert*-butoxycarbonyl)-20,24-(*S*)-[bis-(*tert*-butoxycarbonyl)-amino] propyl}-1-{11-(*tert*-butoxycarbonyl)-11-(*S*)-[(31-maleimido-26'-oxoheptyl)-amino]-pentyl}-pyridinium (2)

Pd/C (10.3 mg, 9.70 μmol, 1.1 eq) was added to solution of **3** (14.4 mg, 8.81 μmol, 1.0 eq) in MeOH (0.39 mL). Reaction system was subjected to hydrogen substitution, and content was stirred at room temperature overnight. Disappearance of Cbz group was confirmed by TLC as well as crude NMR, and solution was filtered through celite with MeOH. Silica gel column chromatography (EtOAc, then CH_2Cl_2/MeOH = 10/1) gave the product amine as yellow oil (9.5 mg, 6.3 μmol, 72%); R_f 0.45 (CH_2Cl_2/MeOH = 10/1). The amine (14.6 mg, 9.72 μmol, 1.0 eq) and maleimide linker **7** (3.3 mg, 10.21 μmol, 1.05 eq) were added into the reaction flask. Following nitrogen substitution, a solution of NMM in DMF (1:200, 140 μL) was injected, and reaction was stirred for 5 hours at room temperature. Funnel separation and drying gave crude, which was then purified with silica gel column chromatography (hexane/EtOAc 1:1, then EtOAc, then CH_2Cl_2/MeOH = 10/1). Product **2** was obtained as white oil in two separate fraction sets, depending on counter ion (total 10.1 mg, 60%).; R_f 0.55 (CH_2Cl_2/MeOH = 10/1); ^{1}H NMR (500 MHz, $CDCl_3$, δ): 9.85-9.76 (1H, s, H6), 7.95-7.91 (1H, s, H4), 6.69-6.66 (2H, m, H32,33), 4.96-4.65 (5H, m, H7/16/20/24), 4.31-4.25 (1H, m, H11), 3.52-3.47 (2H, m, H31), 3.20-3.06 (2H, m, H13), 3.00-2.70 (4H, m, H18/22), 2.51-1.80 (14H, m, H8/9/10/14/15/19/23/26/27/30), 1.68-1.54 (6H, m, H8/9/10/14/15/19/23/26/27/30), 1.53-1.48 (54H, m, Boc), 1.46-1.42 (36H, m, *t*Bu), 1.41-1.20 (4H, m, H28/29); ^{13}C NMR (125 MHz, $CDCl_3$, δ): 173.76, 171.59, 170.88, 169.19, 169.10, 169.07, 152.89, 152.77, 152.60, 152.56, 145.55, 145.34, 140.78, 140.60, 134.03, 83.48, 83.31, 82.00, 81.72, 81.53, 81.34, 58.01, 57.98, 57.86, 52.62, 37.92, 36.02, 31.98, 31.26, 30.84, 30.25, 29.84, 29.71, 29.38, 29.10, 28.76, 28.47, 28.10, 28.04, 27.93, 26.57, 26.20, 26.15, 25.63, 22.71, 22.51; HRMS (ESI, *m*/z): $[M]^+$ calcd for $C_{81}H_{133}N_6O_{23}^+$, 1,557.9422; found 1,557.9390.

2.4 | 2-[16-(*S*)-Amino-16-carboxy-butyl]-1-[11-(*S*)-(31-maleimido-26′-oxoheptyl)-amino-11-carboxy-pentyl]-3,5-bis-[20,24-(*S*)-amino-20,24-carboxy-propyl]-pyridinium (8)

A solution of TFA/CH_2Cl_2 (95:5, 1.81 mL) was injected into nitrogen-purged flask containing starting material **2** (13.5 mg, 7.9 mmol, 1.0 eq). Content was stirred for 2 hours at room temperature, and change of color of the solution was observed from clear to very light pink-orange. Evaporation of acid and solvent was then done using rotary evaporator, and reverse phase silica column purification (H_2O, then H_2O/MeOH 1:1) gave crude product. Desired **8** (7.6 mg, 8.97 mmol, quant) was then obtained after HPLC purification (Conditions: Cosmosil 5C_{18}-AR-II column (10 mm × 250 mm); mobile phase: HPLC-grade distilled H_2O/MeOH (gradient, solvent A: MeOH with 0.1% TFA; solvent B: H_2O with 0.1% TFA, 0-2 min: A/B = 2/98; 2-4 min: A/B = 2-10/98-90; 4-9 min: A/B = 10:90; 9-30 min: A/B = 10-50/90-50; 30-35 min: A/B = 50/50; 45-47 min: A/B = 50-98/50-2), flow rate: 1.0 mL/min; detection: 278 nm; injection amount: 20 μL; temperature: 40°C; R_f 0.2 (H_2O/MeOH = 9/1); $[\alpha]_D^{25}$ = 4.1 (c = 0.003 g cm^{-3} in MeOH); ^{1}H NMR (500 MHz,

MeOH-d_4, δ): 8.80-8.71 (1H, s, H6), 8.38-8.31 (1H, s, H4), 6.82-6.74 (2H, m, H32/33), 4.65-4.51 (2H, m, H7), 4.46-4.39 (1H, dd, J = 9.4, 5.0, H11), 4.14-4.09 (1H, t, J = 6.1 Hz, H16/20/24), 4.09-4.05 (1H, t, J = 6.6 Hz, H16/20/24), 4.05-4.01 (1H, t, J = 6.4 Hz, H16/20/24), 3.50-3.45 (2H, t, J = 7.1 Hz, H31), 3.23-2.92 (6H, m, H13/18/22), 2.4-1.46 (10H, m, H8/9/10/14/15/19/23/26/27/30), 1.40-1.25 (4H, m, H28/29); ^{13}C NMR (125 MHz, MeOH-d_4, δ): 176.4, 175.3, 172.7, 172.7, 155.5, 147.4, 145.1, 142.3, 140.3, 135.4, 135.3, 59.7, 52.86, 38.5, 36.7, 32.1, 31.9, 31.8, 31.7, 31.4, 29.7, 29.7, 29.4, 28.7, 28.6, 27.4, 26.8, 26.8, 25.3, 23.6; HRMS (ESI, m/z): $[M]^+$ calcd for $C_{35}H_{53}N_6O_{11}^+$, 733.3772; found 733.3750.

2.5 | Isodemosine-KLH conjugate (1)

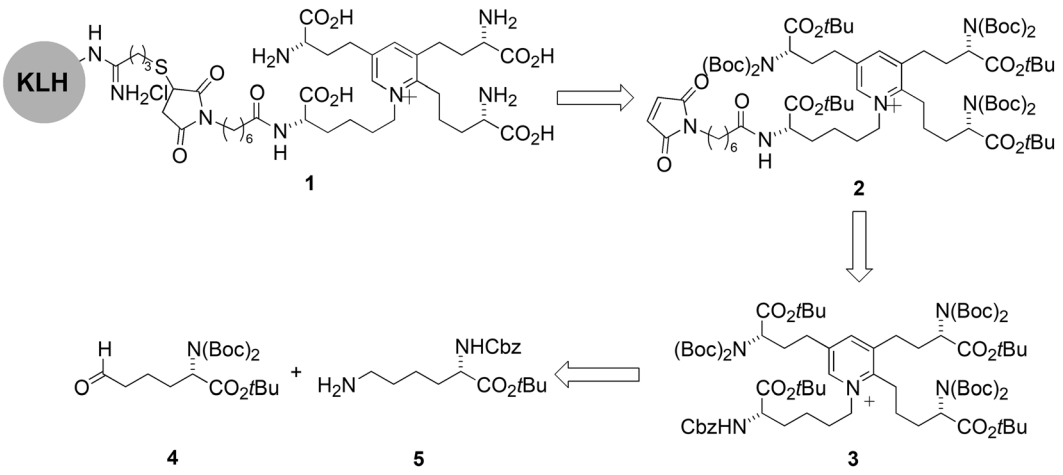
8 (2.0 mg, 2.36 μmol) was dissolved in thiolated KLH (**9**) solution (348 μL, in phosphate-buffered saline [PBS] containing 1 mM EDTA) and was stirred for 2 hours at room temperature. When the sample has completely entered the bed, it was eluted with PBS (pH, 7.4) and the solution was collected. The procedure of Ellman assay is as follows: blank solution for calibration was prepared in 1.5 mL of Eppendorf tube. 200 μL of distilled water and 200 μL of PBS buffer were injected into the tube using automatic micropipette, along with 4 μL of DTNB solution. Calibration using the blank was made on JASCO V-730BIO spectrophotometer. After that, sample solution was prepared in another Eppendorf tube. 200 μL of distilled water, 160 μL of PBS buffer, 4 μL of DTNB, and 40 μL of sample were injected into the tube. Such prepared solution was then analyzed with the spectrophotometer, indicating lack of compounds containing free thiol groups.

3 | RESULTS AND DISCUSSION

Overall design of suitable conjugate involved selection of segments aimed at strengthening the immunizing potential. Besides use of KLH (which itself is highly immunogenic), a selective sulfhydryl-based linkage at one amine position of isodesmosine was envisioned in hopes it would enable full exposure of hapten. According to retrosynthetic route (Scheme 1), target **1** could be obtained from **2** through trifluoroacetic acid (TFA)-mediated global deprotection of acid-liable groups, followed by Michael addition with thiolated KLH protein. **2** on the other hand would be accessible from protected isodesmosine **3** after selective deprotection of benzyloxycarbonyl (Cbz) group and attachment of maleimide segment. Pyridinium core of **3** would be formed through key reaction, Chichibabin pyridinium synthesis,[9,10] using 4 equivalents of aldehyde **4** and 1 equivalent of lysine **5**. It has been previously reported that in appropriately adjusted conditions, this reaction can yield an isodesmosine-type compound selectively.[10b]

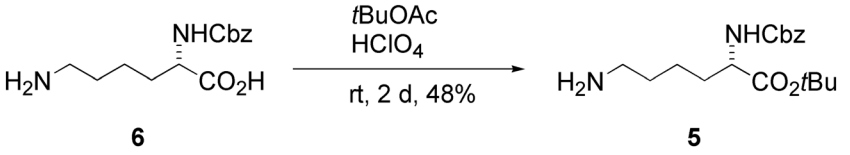
Synthesis of desired aldehyde **4** was accomplished using foundations of the synthetic route established previously, starting from commercially available chiral glutamic acid with protecting groups.10a, 11 To assemble second key substrate **5**, commercially available chiral lysine **6** was *tert*-butylated in the presence of *t*BuOAc and $HClO_4$ (Scheme 2) in 48% yield.[12]

With both **4** and **5** in hand, Chichibabin pyridinium synthesis was conducted in the presence of 50 mol% Pr$(OTf)_3$ catalyst and in DMF/H_2O = 1/2 solvent system for 24 hours at room temperature (Scheme 3).10b Desired tetrasubstituted pyridinium **3** was selectively obtained in 30% yield. In further step, selective deprotection of Cbz



SCHEME 1 Retrosynthetic route of target compound **1**

SCHEME 2 Synthesis of lysine segment **5**



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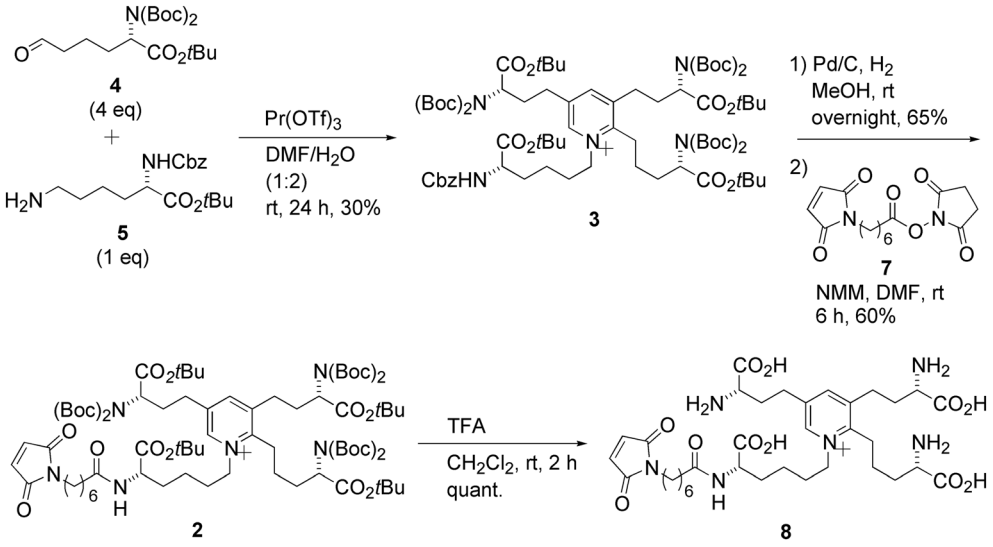
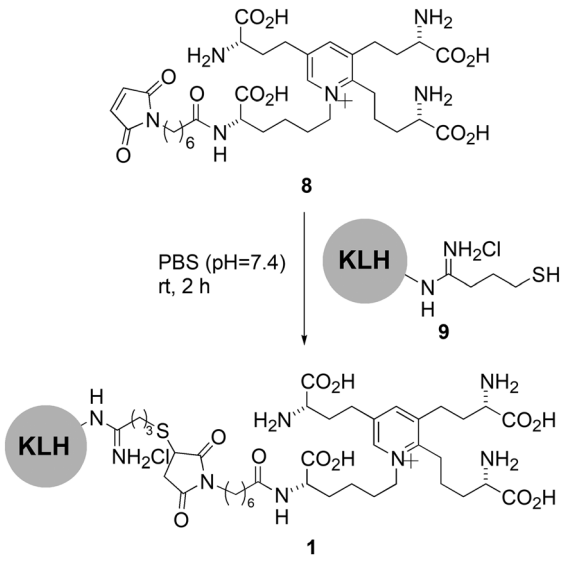
SCHEME 3 Synthetic scheme towards **8**





SCHEME 4 Formation of conjugate **1**

group was performed using Pd/C condition in hydrogen atmosphere in 65% yield. Free amine was then reacted with maleimide segment **7** in presence of mild base to obtain **2** in 60% yield.[13] In the next step, isodesmosine-maleimide intermediate **2** was subjected to deprotection of acid-liable Boc and *t*Bu groups, and finally, **8** was purified by HPLC in quantitative yield. The optical rotation value was observed to $[\alpha]_D^{25} = 4.1(c = 0.003$ g cm^{-3}, MeOH), suggesting that the obtained compound **8** was not racemic.

As the last step towards target compound, Michael addition of **8** and thiolated KLH protein **9** was performed in PBS (pH, 7.4).[14] After stirring for 2 hours at room temperature, white precipitation arose gradually, indicating formation of conjugate. Successful conjugation was confirmed with Ellman assay.

4 | CONCLUSION

In summary, synthesis of target conjugate **1** has been finalized starting from commercially available protected glutamic acid and protected lysine **6** through use of key reaction, Chichibabin pyridinium synthesis, which furnished the main core of isodesmosine. Further selective deprotection revealed free amine group that reacted with maleimide substrate to give **2**. TFA-mediated global deprotection of acid-liable groups delivered **8**, which reacted with thiolated KLH protein **9**, forming desired immunoreagent **1**. As of current state, immunization using the antigen on animal host organism is being investigated.

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REFERENCES

1. Global Initiative for Chronic Obstructive Lung Disease (GOLD) "2019 Global Strategy for the Prevention, Diagnosis, and Management of COPD", 2019. Accessed at https://goldcopd.org/wp-content/uploads/2018/11/GOLD-2019-v1.7-FINAL-14Nov2018-WMS.pdf
2. WHO "The top 10 causes of death", 2018. at https://www.who.int/news-room/fact-sheets/detail/the-top-10-causes-of-death
3. (a) Patridge SM, Elsden DF, Thomas J. Constitution of the cross-linkages in elastin. *Nature*. 1963;197:1297-1298. (b) Thomas J, Elsden DF, Partridge SM. Degradation Products from Elastin: Partial Structure from Two Major Degradation Products from the Cross-linkages in Elastin. *Nature*. 1963;200:651-652.
4. (a) Kaga N, Soma S, Fujimura T, Seyama K, Fukuchi Y, Murayama K. Quantification of elastin cross-linking amino acids, desmosine and isodesmosine, in hydrolysates of rat lung by ion-pair liquid chromatography-mass spectrometry. *Anal. Biochem.* 2003;318(1):25-29. (b) Boutin M, Berthelette C, Gervais FG, et al. High-sensitivity nanoLC-MS/MS analysis of urinary desmosine and isodesmosine. *Anal. Chem.* 2009;81:1881-1887. (c) Shiraishi K, Matsuzaki K, Matsumoto A, Hashimoto Y, Iba K. Development of a robust LC-MS/MS method for determination of desmosine and isodesmosine in human urine. *J. Oleo Sci.* 2010;59(8):431-439. (d) Miliotis T, Lindberg C, Semb KF, van Geest M, Kjellstrom S. Quantitative high-performance liquid chromatography–tandem mass spectrometry method for the analysis of free desmosines in plasma and urine. *J. Chromatogr. A*. 2013;1308:73-78. (e) Albarbarawi O, Barton A, Miller D, et al. Characterization and validation of an isotope-dilution LC-MS/MS method for quantification of total desmosine and isodesmosine in plasma and serum. *Bioanal.* 2013;5:1991-2001. (f) Lamerz J, Friedlein A, Soder N, Cutler P, Dobeli H. Determination of free desmosine in human plasma and its application in two experimental medicine studies. *Anal. Biochem.* 2013;436(2):127-136. (g) Ongay S, Hendriks G, Hermans J, et al. Quantification of free and total desmosine and isodesmosine in human urine by liquid chromatography tandem mass spectrometry: a comparison of the surrogate-analyte and the surrogate-matrix approach for quantitation. *J. Chromatogr. A*. 2014;1326:13-19.
5. (a) Ma S, Lin YY, Turino GM. Measurements of desmosine and isodesmosine by mass spectrometry in COPD. *Chest*. 2007;131(5):1363-1371. (b) Ma S, Turino GM, Lin YY. Quantitation of desmosine and isodesmosine in urine, plasma, and sputum by LC–MS/MS as biomarkers for elastin degradation. *J. Chromatogr. B*. 2011;879:1893-1898. (c) Ma S, Turino GM, Hayashi T, Yanuma H, Usuki T, Lin YY. Stable deuterium internal standard for the isotope-dilution LC–MS/MS analysis of elastin degradation. *Anal. Biochem.* 2013;440(2):158-165. (d) Murakami Y, Suzuki R, Yanuma H, et al. Synthesis and LC-MS/MS analysis of desmosine-CH_2, a potential internal standard for the degraded elastin biomarker desmosine. *Org. Biomol. Chem.* 2014;12(48):9887-9894.
6. An example of small molecule and career protein conjugate is A2E and BSA, see, Abeywickrama C, Matsuda H, et al. Immunochemical recognition of A2E, a pigment in the lipofuscin of retinal pigment epithelial cells. *Proc. Natl. Acad. Sci. USA*. 2007;104:14610-14615.
7. Gunja-Smith Z. An enzyme-linked immunosorbent assay to quantitate the elastin crosslink desmosine in tissue and urine samples. *Anal. Biochem.* 1985;147:258-264.
8. Matsumoto T, Mizusaki S, Nishimura K, Oshima S. An enzyme-linked immunosorbent assay for desmosine. *Bull. Chest Dis. Res. Inst., Kyoto Univ*. 1986;19:9-22.
9. (a) Chichibabin AE. Synthesis of pyridine bases from saturated aldehydes and ammonia. *Zh. Russ. Fiz.-Khim. O-vA*. 1905;37:1229-1253. (b) Yu L, Chen D, Li J, Ramirez J, Wang PG, Bott SG. Lanthanide-promoted reactions of aldehydes and amine hydrochlorides in aqueous solution. Synthesis of 2,3-Dihydropyridinium and Pyridinium Derivatives. *J. Org. Chem.* 1997;62(1):208-211.
10. (a) Usuki T, Sugimura T, Komatsu A, Koseki Y. Biomimetic Chichibabin pyridine synthesis of the COPD biomarkers and elastin cross-linkers isodesmosine and desmosine. *Org. Lett.* 2014;16(6):1672-1675. (b) Tanaka N, Kurita M, Murakami Y, Usuki T. Chichibabin and isoChichibabin pyridinium syntheses of isodesmosine, desmosine, and their derivatives. *Eur. J. Org. Chem.* 2018;43:6002-6009.
11. Tanaka N, Usuki T. Preparation of protected $^{13}C_n$-labeled isodesmosines: mechanistic insight of isodesmosine formation. *Nat. Prod. Commun*. 2019;14,1934578X19849967.
12. Milewska MJ, Chimiak A. Oxidation of amino acid. Part V. A novel synthesis of N^6-acetyl-N^6-hydroxylysine from lysine. *Tetrahedron Lett.* 1987;28:1817-1819.
13. Ikeda M, Ochi R, Kurita YS, Pochan DJ, Hamachi I. Cooperative catalytic reactions using distinct transition-metal catalysts: ruthenium- and copper-catalyzed enantioselective propargylic alkylation. *Chem. Eur. J.* 2012;18(41):13091-13096.
14. Rudolf B, Palusiak M, Zakrzewski J, Salmain M, Jaouen G. Sulfhydryl-selective, covalent labeling of biomolecules with transition metallocarbonyl complexes. Synthesis of (η^5-C_5H_5)M$(CO)_3$(η^1-*N*-Maleimidato) (M = Mo, W), X-ray Structure, and Reactivity Studies. *Bioconjugate Chem.* 2005;16(5):1218-1224.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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