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Synthesis and LC-MS/MS Analysis of Desmosine-CH_2, a Potential Internal Standard for the Degraded Elastin Biomarker Desmosine†

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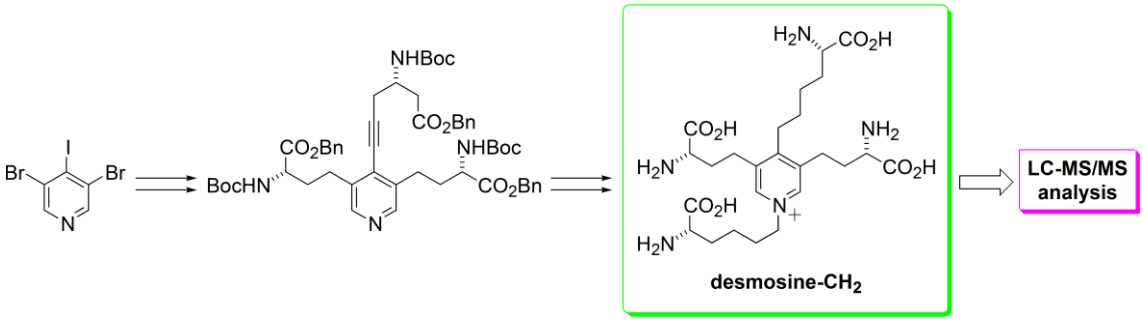
Graphical Abstract:



Abstract:

Desmosine-CH_2, an analog of the elastic tissue degradation biomarker desmosine, can be regarded as a potential internal standard for precise quantification of desmosines by LC-MS/MS. In this study, the chemical synthesis of desmosine-CH_2 was completed in 22% overall yield in five steps. The LC-MS/MS analysis of desmosine-CH_2 was also achieved.

† Electronic supplementary information (ESI) available: ^{1}H and ^{13}C NMR spectra for synthetic compounds (Supplementary information).

Introduction

Elastin is the main component of the elastic fibers that exist in vertebrate tissues and organs, such as the lungs, skin, and blood vessels. It exists as a crosslinking structures and forms sophisticated 3-D networks.[1,2] Desmosine (**1**, Fig. 1) and isodesmosine (**2**) are two major crosslinking pyridinium amino acids of elastin,[3] and exist only in the elastin matrix. The irreversible degradation of elastin-containing tissues that occurs in several widely prevalent diseases, such as atherosclerosis,[4] aortic aneurysms,[4] cystic fibrosis,[5,6] and chronic obstructive pulmonary disease (COPD),[7,8] have been associated with increased excretion of the peptides containing these two pyridinium compounds in the clinical samples.

In the lungs, elastin degradation occurs with the development of COPD. COPD is a respiratory disease mainly due to smoking and α_1-antitrypsin deficiency (AATD).[9] According to the World Health Organization (WHO), it is the fourth leading cause of death worldwide and is expected to become the third leading cause by 2030.[10] Despite the potential increase in COPD patients, the investigation of therapeutic agents that prevent the disease's progression is just beginning. The development of biomarkers that indicate the severity of COPD and the therapeutic response of patients would aid this effort.[11] In particular, excretion of **1** and **2** in body-fluids are expected to be useful biomarkers for the disease.[12,13]

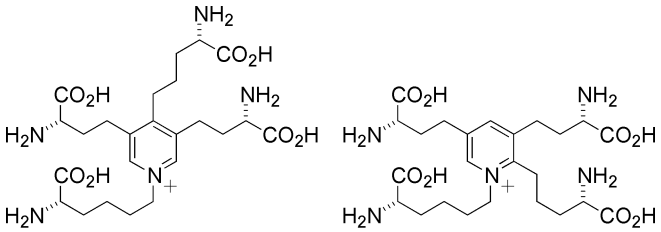


Fig. 1 Structures of desmosine **1** and isodesmosine **2**.

Liquid chromatography-mass spectrometry (LC-MS or LC-MS/MS) quantification methods for desmosines have been developed during the last decade, with deuterated desmosines, derived from the corresponding natural products via chemical exchange reactions used as an internal standard.[12] However, these standard compounds

were not stable enough in the acid hydrolysis reaction, which requires to release desmosine **1** and isodesmosine **2** from their peptide conjugate for the analysis. These standard compounds also have the potential to undergo D-H exchange during synthesis and hydrolysis before analysis. Recently, we have achieved the chemical synthesis of an acid-stable desmosine-d_4 (**3**, Fig. 2) and the precise quantification of desmosines via liquid chromatography tandem mass spectrometry utilizing **3** as the internal standard.[13d] Although **3** was demonstrated to be stable and retain its defined structure under acid hydrolysis conditions, some distribution of the deuteration ratio was observed in the electrospray ionization (ESI)-MS spectrum. The chemical synthesis of **3** involved complex multistep reactions and was obtained in only 60% deuterium isotope purity due to deuterium atom scrambling during the reaction.[13d] Thus, we proposed the use of a pseudo-desmosine, desmosine-CH_2 (**4**, Fig. 2), as a novel internal standard for quantification. The total synthesis of **1** utilizing palladium-catalyzed cross-coupling reactions was previously achieved in our laboratory,[14,15] and the synthesis of an alkyl-extended desmosine is possible following the same route. In order to investigate its applicability for the precise quantification of desmosines, we synthesized **4** via stepwise Sonogashira and Negishi cross-coupling reactions and its properties on LC-MS/MS were investigated.

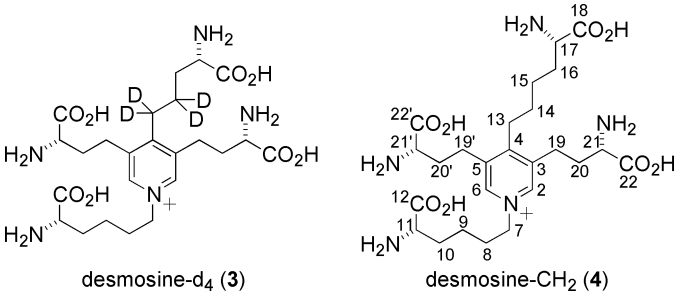


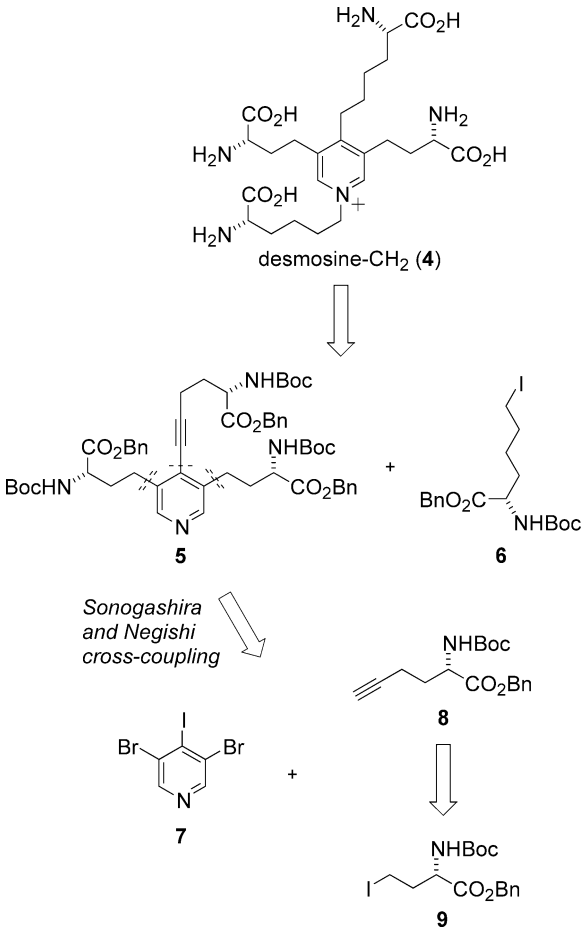
Fig. 2 Structures of desmosine-d_4 **3** and desmosine-CH_2 **4**.

Result and discussion

Chemical synthesis of desmosine-CH_2

The structure of target molecule **4** is similar to that of **1**. However, the carbon chain of the 4-alkyl amino acid moiety is one carbon longer than that of **1**. As illustrated in Scheme 1, it was thus envisioned that **4** would be retrosynthetically derived from

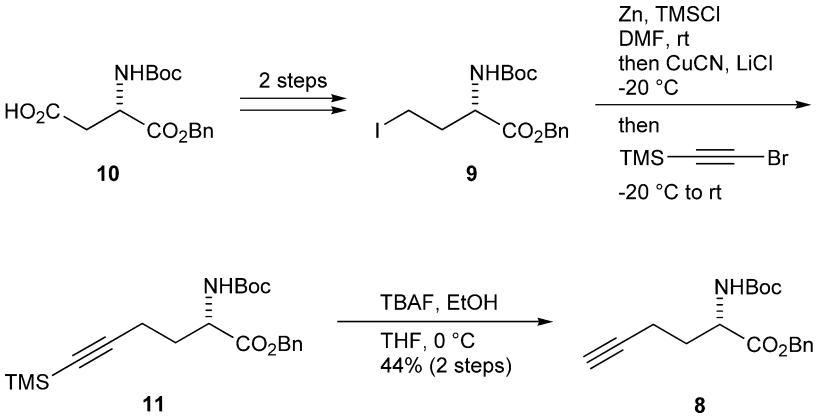
3,4,5-trisubstituted pyridine **5** and iodo amino acid (ω-iodobutyl L-glycine) derivative **6**[16] through late-stage formation of the pyridinium salt. Synthesis of **5** would then involve chemo- and regioselective palladium-catalyzed Sonogashira and Negishi cross-coupling reactions between trihalogenated pyridine **7**,[14b,17] the terminal alkyne **8**, and iodo amino acid **9**.[16] The protected alkyne **8** could be formed from **9**.



Scheme 1 Retrosynthesis of desmosine-CH_2 **4**.

Since preparation of 3,5-dibromo-4-iodopyridine **7**, iodo amino acids **6**, and **9** had previously been achieved from commercially available 4-aminopyridine,[17] benzoxyl-(*S*)-4-[(*tert*-butoxycarbonyl)amino]-5-oxopentanoic acid, and benzyl 2-(*S*)-[(*tert*-butoxycarbonyl)amino]-4-hydroxybutanoate (**10**),[16] respectively, our

synthesis commenced with the preparation of terminal alkyne **8** (Scheme 2). The coupling reaction of an organocopper compound and (2-bromoethynyl)trimethylsilane was achieved as follows. The organozinc reagent was prepared from **9** by treatment of Zn with trimethylsilyl chloride (TMSCl). The coupling reaction between the organocopper substrate (RCu(CN)ZnI),[18] prepared from this organizinc reagent, CuCN and LiCl, and freshly distilled (2-bromoethynyl)trimethylsilane afforded product **11**[19,20] and a byproduct derived from the organocopper reagent, that was inseparable from **11** due to their similar polarity. Removal of the TMS group in **11** as a crude mixture using tetrabutylammonium fluoride (TBAF) in the presence of EtOH provided the desired terminal alkyne **8** in 44% isolated yield from **9**.[21]

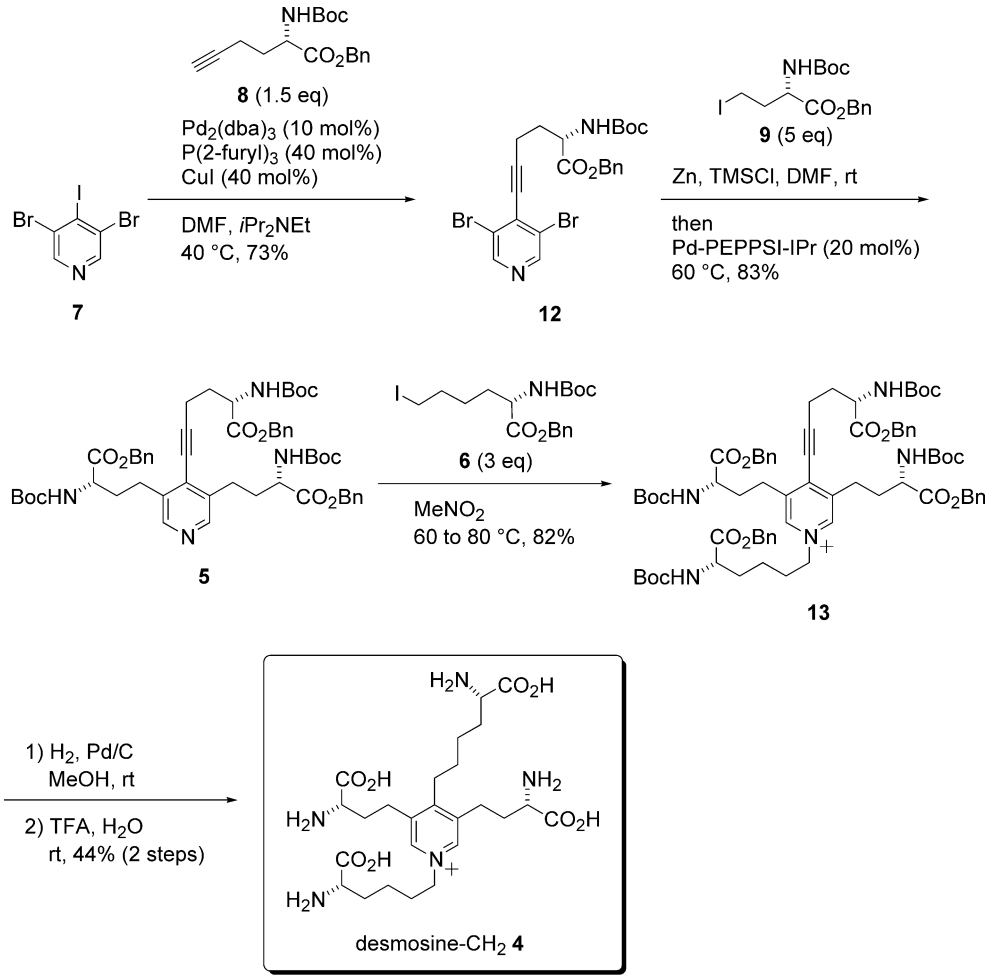


Scheme 2 Synthesis of terminal alkyne **8**.

With the coupling substrates **8** and **9** in hand, we explored C-C bond formation between 3,5-dibromo-4-iodopyridine **7** and the corresponding alkyne and iodo amino acids utilizing palladium-catalyzed cross-coupling reactions in order to achieve the synthesis of **4**. Based on the established synthetic strategy,[14] the Sonogashira cross-coupling reaction between the 4-position in **7** and alkyne **8** was performed using 10 mol% $Pd_2(dba)_3$, 40 mol% P(2-furyl)$_3$,[22] and 40 mol% CuI in DMF and *i*Pr_2NEt at 40 °C to give monocoupled pyridine **12** in 73% yield. The introduction of iodoalkylated L-glycine **9** at the 3,5-positions of the obtained **12** was then attempted via a Negishi cross-coupling reaction using the catalyst Pd-PEPPSI-IPr, which was developed by

Organ and co-workers.[23] The reaction was performed using an improved procedure,[24] that involved removal of the excess Zn from the organozinc reagent via centrifugation, followed by the coupling using 20 mol% Pd-PEPPSI-IPr catalyst at 60 °C to afford the tricoupled pyridine **5** in 83% yield without any dicoupled compound. Interestingly, the yields of the coupling reaction in the present study were greater than those obtained during the synthesis of desmosine **1** (Sonogashira: 58%, Negishi: 60%).[14b] Therefore, this result suggested a difference in reactivity, which may be due to the steric bulkiness of the *t*-butoxycarbonyl or benzyl group in the desmosine intermediates.

Formation of the pyridinium salt of **5** with ω-iodoalkylated L-glycine **6** in $MeNO_2$ at 60 °C to 80 °C afforded **13** in 82% yield. After reduction of the benzyl and alkyne groups with H_2 and Pd/C, the *t*-butoxycarbonyl protecting groups were successfully removed using trifluoroacetic acid (TFA) to give crude **4**. Purification via C18 column chromatography afforded desired desmosine-CH_2 **4** in 44% yield over two steps. The exact mass of the obtained **4** is 540 *m*/*z*, which means that the mass shifts 14 *m*/*z* comparing with natural desmosine **1** (526 *m*/*z*).



Scheme 3 Synthesis of **4**.

LC-MS/MS analysis using desmosine-CH$_2$ as the internal standard

With compound **4** in hand, analytical studies of desmosine-CH$_2$ **4** were then performed. The ESI mass spectrum of desmosine-CH$_2$ (**4**, DES-CH2) is shown in Fig. 3. The spectrum shows a molecular ion at 540 *m*/*z*. The most aboundant transisition ion at 495 *m*/*z* is selected for reaction ion monitoring (RIM) in the LC-MS/MS analysis. It was found that the compound **4** is stable toward acid hydrolysis and can be successfully used

as an internal standard for the analysis for LC-MS/MS analysis of desmosine (**1**, DES) and isodesmosine (**2**, IDES) in biological fluids. We compared the analysis of **1** and **2** using DES-CH2 with that of previously developed internal standard demosine-d_4 (**3**, DES-d4).[13d] The result is shown in the LC-MS/MS chromatogram in Fig 4. Meanwhile, the uses of DES-CH2 and DES-d4 as the internal standards for DES and IDES analysis are shown in Fig 5. In Figs. 5A and 5B, the calibration curves were obtained with the ratios to the internal standard DES-CH2, which gave the excellent linearity. On the other hand, Figs. 5C and 5D are with that of using DES-d4 as the internal standards. As a result, both internal standards demonstrated good linearity for the analysis of **1** and **2**.

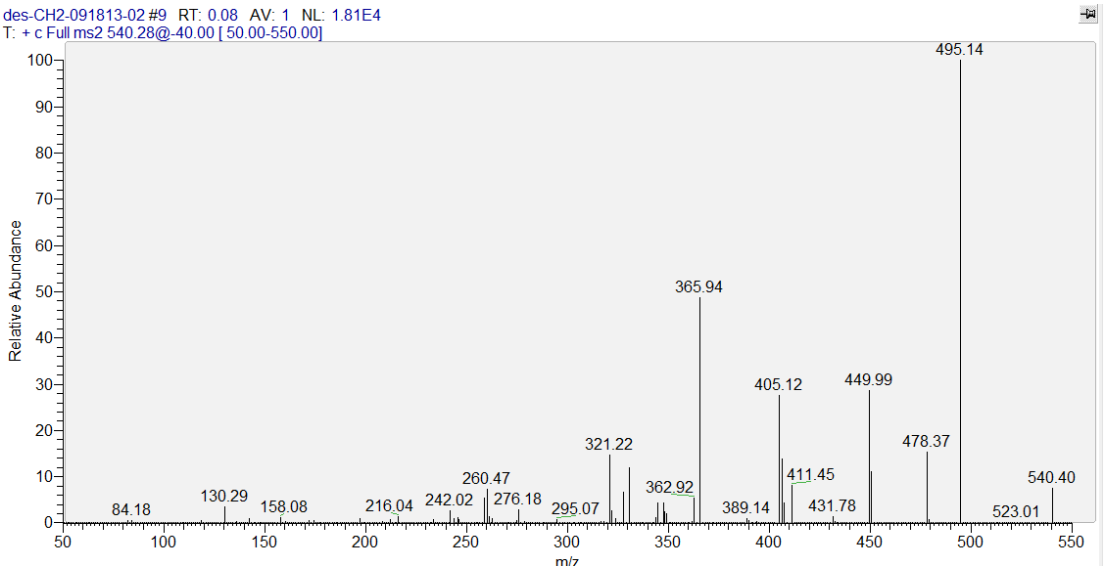


Fig. 3 ESI mass spectrum of desmosine-CH_2 **4** (DES-CH2).

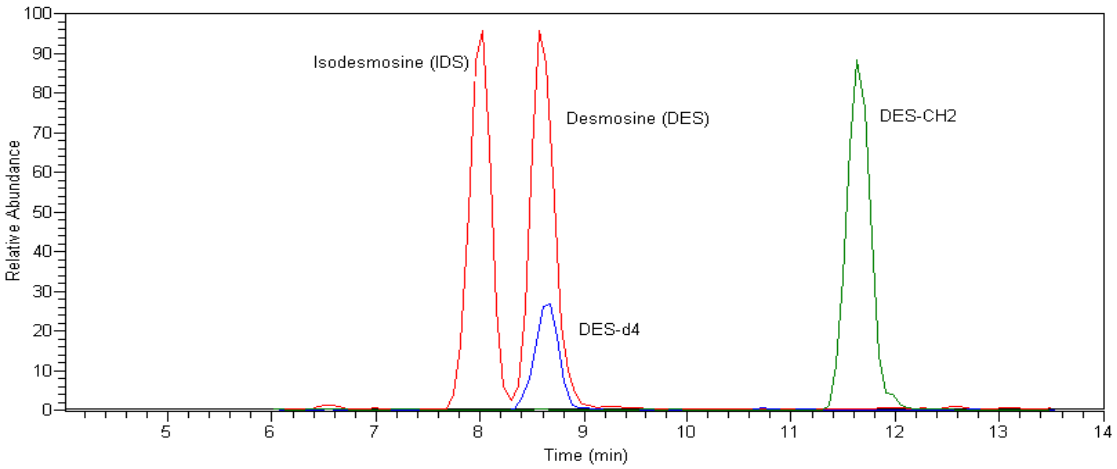


Fig. 4 RIM mass chromatogram of DES, IDS, DES-d4, and DES-CH2.

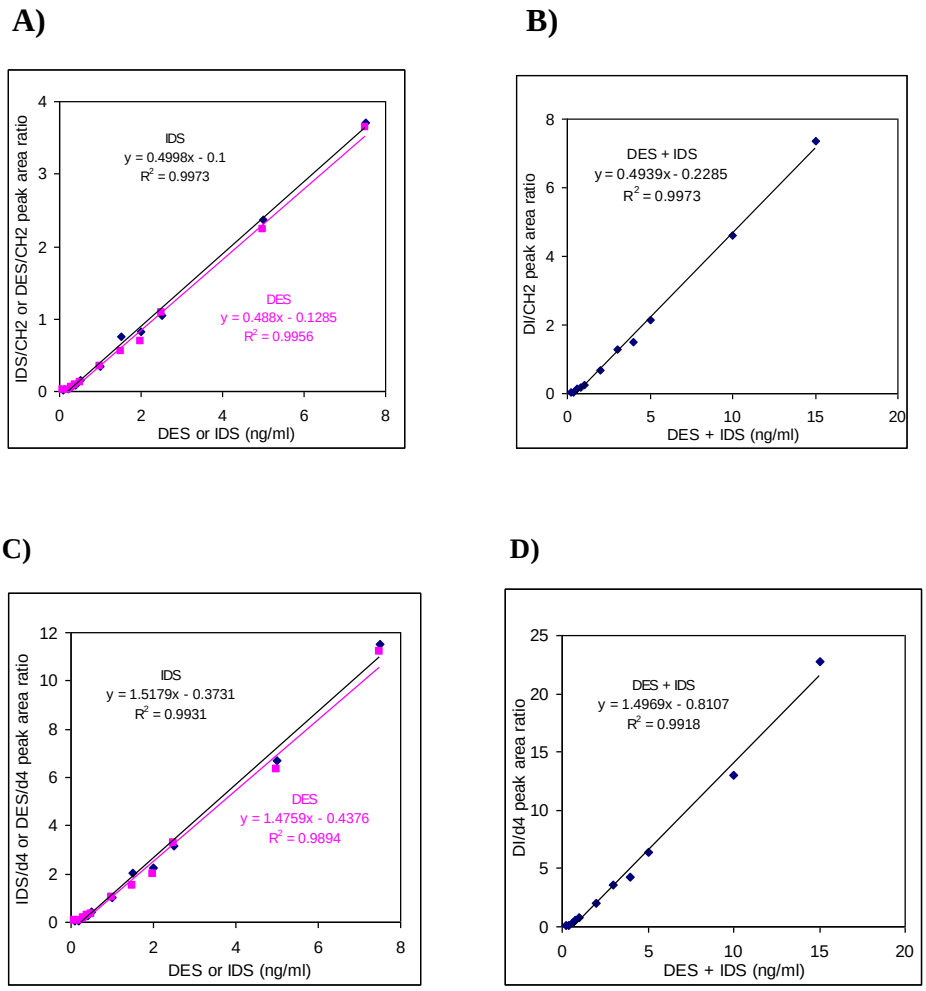


Fig. 5 Comparison of DES and IDES analysis using DES-CH2 and DES-d4 as the internal standards.

Conclusion

Synthesis of desmosine-CH_2 (**4**) was achieved via chemo- and regioselective Sonogashira and Negishi cross-coupling reactions as key steps in 22% yield over five steps from **7**. We also examined the LC-MS/MS analysis of **4** and found that desmosine-CH_2 has applicability as a novel internal standard for desmosines, which are biomarkers of elastin degradation. This achievement should enable further studies on the precise quantification of desmosines via LC-MS/MS and development of a rapid diagnostic method for disease that induce elastic tissue degradation.

Experimental

General procedures

All non-aqueous reactions were conducted under an atmosphere of nitrogen with magnetic stirring unless otherwise indicated. *N*,*N*-Dimethylformaide (DMF) was dried by distillation by $MgSO_4$ and stored over activated molecular sieves. Triethylamine (Et_3N) was distilled over CaH_2. Other solvents, such as methanol (MeOH), ethanol (EtOH), nitromethane ($MeNO_2$), and tetrahydrofuran (THF) were purchased from commercial suppliers and stored over activated molecular sieves. All reagents were obtained from commercial suppliers and used without further purification unless otherwise stated. The enantiomerically pure (>99.5%) protected amino acids, starting materials of iodo amino acid **6**, and **9**, were purchased from Wananabe Chemical Industries, Ltd (Hiroshima, Japan). Analytical thin layer chromatography (TLC) was performed on Silica gel 60 F_{254} 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).

Melting points were measured by an AS one ATM-01 apparatus. Optical rotations were measured on a JASCO P-2200 digital polarimeter at the sodium lamp (λ = 589 nm) D line and are reported as follows: $[\alpha]_D^T$ (*c* g/100 mL, solvent). UV spectra were recorded on a JASCO V-560 UV/VIS spectrophotometer and are reported in wavelengths (nm). Infrared (IR) spectra were recorded on a JASCO FT-IR 4100 spectrometer and are reported in wavenumbers (cm^{-1}). ^1H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded on a JEOL JNM-EXC 300 spectrometer (300 MHz) or on a JEOL JNM-ECA 500 spectrometer (500 MHz). ^1H 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 Hz, assignments. ^{13}C NMR data are reported in terms of chemical shift (δ, ppm). FAB-MS spectra were observed on the JEOL JMS-700. 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*). JASCO HPLC systems PU-2085, MD-2010, and CO-2060 were used for the purification of **4**.

The carbon numbering on ^1H NMR of all compounds is corresponding with desmosine-CH_2 **4** in Fig. 2.

Benzyl 17-(*S*)-[(*tert*-butoxycarbonyl)amino]hex-14-ynoate (8).

CuCN (322.5 mg, 3.58 mmol, 1.0 eq) and LiCl (309.3 mg, 7.16 mmol, 2.0 eq) were dried together by heating under vacuum at 150 °C for 2 h in one flask. While the CuCN and LiCl were drying, zinc powder (1.45 g, 22.2 mmol, 6.2 eq) dried under vacuum with heating using a heat gun over 5 min. To the flask containing the zinc powder were added DMF (1.5 mL) and trimethylsilyl chloride (0.14 mL, 1.1 mmol, 5 mol% to Zn) at room temperature. After stirring for 30 min at room temperature, to this suspension was slowly added a solution of benzyl 2-(*S*)-[(*tert*-butoxycarbonyl)amino]-4-iodobutanoate (**9**) (1.5 g, 3.58 mmol, 1.0 eq) in DMF (1.0 mL, washed with 0.5 mL × 2). After confirming generation of **9-Zn** judged on TLC, to another flask containing the CuCN and LiCl in DMF (7.5 mL) cooling to -5 °C was added a solution of **9-Zn** over 30 min. After stirring for 15 min at -5 °C, the solution was then cooled to -20 °C. Freshly prepared bromo(trimethylsilyl)acetylene (1.0 mL, 7.16 mmol, 2.0 eq) was added dropwise to the reaction mixture over 5 min. The mixture was then allowed to slowly warm to room temperature and continue stirring for 17 h. The mixture was diluted with EtOAc, and quenched with saturated NH_4Cl solution. The aqueous layer was then extracted with EtOAc. The combined organic layers were washed with brine, dried over Na_2SO_4, and concentrated *in vacuo*. Purification on silica gel column chromatography (hexane/EtOAc = 20/1→10/1) afforded **11** with inseparable byproduct (870.4 mg);

To a solution of the crude product in THF (30.0 mL) and EtOH (654 μL) cooled to 0 °C was added TBAF (607 μL, 1.0 M solution in THF). After stirring at 0 °C for 1.5 h, the reaction mixture was diluted with EtOAc, and quenched with saturated NH_4Cl solution. The aqueous layer was then extracted with EtOAc. The combined organic layers were washed with brine, dried over Na_2SO_4, and concentrated *in vacuo*. Purification on silica gel column chromatography (hexane/EtOAc = 15/1) afforded **8** as a white solid (496.4 mg, 1.18 mmol, 44% (2 steps)); R_f 0.29 (hexane/EtOAc = 8/1); $[\alpha]_D^{20}$ +5.7 (*c* 0.1, $CHCl_3$); mp 61-62 °C; IR (ATR, cm^{-1}) 3400, 3326, 1755, 1683, 1514, 1451, 1368, 1295, 1254, 1213, 1160, 1052, 959, 755, 695, 638; ^1H NMR (300 MHz, $CDCl_3$) δ 7.37-7.33 (5H, m, Bn), 5.22-5.17 (2H, m, Bn), 5.13 (1H, s, NH), 4.43 (1H, m, H17), 2.29-2.23 (2H, m, H15), 2.13-2.04 (1H, m, H16), 1.96 (1H, t, J = 2.8 Hz, H13), 1.92-1.79 (1H, m, H16), 1.43 (9H, s, *t*Bu); ^{13}C NMR (75 MHz, $CDCl_3$) δ 171.8, 155.0, 135.0, 128.3, 128.2, 128.0, 82.5, 79.7, 69.0, 66.9, 52.6, 31.1, 28.0, 14.5; FAB-MS (*m/z*) calcd for $C_{18}H_{24}NO_4$ $[M+H]^+$ 318.17, found 318.25.

(*S*)-Benzyl-17-(*tert*-butoxycarbonylamino)-13-(3,5-dibromo-pyridin-4-yl)-hex-14-ynoate (12).

A solution of 3,5-dibromo-4-iodopyridine (**7**) (249.1 mg, 0.69 mmol, 1.0 eq), benzyl 17-(*S*)-[(*tert*-butoxycarbonyl)amino]hex-14-ynoate (**8**) (327.1 mg, 1.03 mmol, 1.5 eq), $Pd_2(dba)_3$ (64.2 mg, 70.0 µmol, 10 mol%), P(2-furyl)$_3$ (64.9 mg, 0.28 mmol, 40 mol%), and CuI (55.7 mg, 0.28 mmol, 40 mol%) in DMF (34.5 mL) was degassed by freeze/pump/thaw techniques. *i*Pr$_2$NEt (6.9 mL) was added to the resulting solution. After stirring at room temperature for 17 h, the reaction mixture was diluted with EtOAc and quenched with saturated NH_4Cl solution. The aqueous layer was then extracted with EtOAc. The combined organic layers were washed with brine, dried over Na_2SO_4, and concentrated *in vacuo*. Purification by flash column chromatography (hexane/EtOAc = 5/1) afforded the pure **12** (276.3 mg, 0.50 mmol, 73%) as a white solid; R_f 0.27 (hexane/EtOAc = 5/1); $[\alpha]_D^{21}$ +9.9 (*c* 0.1, $CHCl_3$); mp 114-115 °C; IR (ATR, cm^{-1}) 3348, 1753, 1682, 1518, 1448, 1360, 1303, 1215, 1166, 1055, 959, 752, 694, 606; ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.61 (2H, s, H2/6), 7.37-7.36 (5H, m, Bn), 5.24-5.18 (2H, m, Bn), 5.14 (1H, m, NH), 4.52 (1H, m, H17), 2.67-2.61 (2H, m, H15), 2.32-2.21 (1H, m, H16), 2.08-1.96 (1H, m, H16), 1.42 (9H, s, *t*Bu); ^{13}C NMR (75 MHz, $CDCl_3$) δ 172.0, 155.5, 149.7, 135.3, 134.7, 128.8, 128.6, 128.4, 123.5, 104.3, 80.2, 78.1, 67.4, 53.1, 31.3, 28.4, 16.7; ESI-HRMS (*m/z*) calcd for $C_{23}H_{24}Br_2N_2NaO_4$ $[M+Na]^+$ 574.9980, found 574.9980.

[(21*S*,21'*S*)-Benzyl 19,19'-19-{(*S*)-18-(benzyloxy)-17-(*tert*-butoxycarbonylamino)-18-oxopent-13-ynyl}pyridine-3,5-diyl]bis-21-(*tert*-butoxycarbonylamino)butanoate (5).

Zinc dust (198 mg, 3.0 mmol) was placed in a nitrogen-purged 1.5 mL microtube. Dry DMF (150 µL) and trimethylsilyl chloride (60.0 µL, 0.47 mmol) were added, and the resulting mixture was stirred vigorously for 15 min at room temperature. Stirring was stopped, and the solution was removed by micro syringe. The remaining solid was dried using a hot air gun at reduced pressure. The activated zinc was cool to room temperature, and a solution of benzyl 2-(*S*)-[(*tert*-butoxycarbonyl)amino]-4-iodobutanoate (**9**) (217.5 mg, 0.5 mmol, 5.0 eq) in dry DMF (150 µL and rinsed with 100 µL DMF) was added to the activated zinc. The reaction mixture was stirred at room temperature for 1 h, after which time TLC analysis (hexane/EtOAc = 5/1) revealed that no starting material remained. Stirring was stopped and the zinc duct was allowed to settle using a centrifuge separator. The solution was removed from the activated zinc via micro syringe with 200 µL DMF and add to a 10

mL flask, containing Pd-PEPPSI-IPr (13.2 mg, 20 mol%) and **12** (55.2 mg, 0.1 mmol, 1.0 eq). Stirring was continued for 1.5 h at 60 °C, the reaction mixture was diluted with EtOAc and quenched with a saturated NH_4Cl solution. The aqueous layer was then extracted with EtOAc. The combined organic layers were washed with brine, dried over Na_2SO_4, and concentrated *in vacuo* to give the crude product as yellow oil. Purification by flash column chromatography (hexane/EtOAc = 2/1→1/1) afforded the pure **6** (77.8 mg, 0.83 mmol, 83%) as a yellow oil; R_f 0.42 (hexane/EtOAc = 1/1); $[\alpha]_D^{20}$ +23.5 (*c* 0.1, $CHCl_3$); IR (ATR, cm^{-1}) 3783, 3465, 3084, 2976, 2361, 1713, 1514, 1452, 1367, 1254, 1168, 1053, 859, 748, 697; ^1H NMR (300 MHz, $CDCl_3$) δ 8.18 (2H, s, H2/6), 7.33-7.30 (15H, m, Bn), 5.38 (1H, s, 17NH), 5.32-5.30 (2H, m, NH), 5.19-5.09 (6H, m, Bn), 4.43 (1H, m, H17), 4.38-4.36 (3H, m, H11/21/21'), 2.77-2.68 (4H, m, H19/19'), 2.58-2.54 (2H, m, H16), 2.21-1.84 (6H, m, H16/20/20'), 1.43-1.40 (27H, m, *t*Bu); ^{13}C NMR (75 MHz, $CDCl_3$) δ 172.3, 172.1, 155.5, 149.4, 147.7, 135.4, 135.3, 128.7, 128.6, 128.4, 128.3, 80.0, 67.3, 67.1, 53.5, 53.0, 33.0, 28.4, 28.1, 16.5; ESI-MS (*m/z*) calcd for $C_{55}H_{68}N_4NaO_{12}$ $[M+Na]^+$ 999.47, found 999.44.; ESI-HRMS (*m/z*) calcd for $C_{55}H_{68}N_4NaO_{12}$ $[M+Na]^+$ 999.4731, found 999.4744.

(3,5-bis((*S*)-22-(Benzyloxy)-21-(*tert*-butoxycarbonylamino)-22-oxobutyl)-4-((*S*)-18-(benzyloxy)-17-(*tert*-butoxycarbonylamino)-18-oxopent-13-ynyl)-1-((*S*)-12-(benzyloxy)-11-(*tert*-butoxycarbonylamino)-12-oxohexyl)pyridinium iodide) (13).

A mixture of **5** (13.3 mg, 13.6 µmol, 1.0 eq) and benzyl 2-(*S*)-((*tert*-butoxycarbonyl)amino)-6-iodohexanoate **6** (18.3 mg, 40.8 µmol, 3.0 eq) in $MeNO_2$ (1.0 mL) was heated at 60 °C for 23 h, then warmed up to 80 °C for 25 h. The reaction mixture was concentrated *in vacuo*. Purification on neutral silica gel column chromatography (hexane/EtOAc = 1/1 → CH_2Cl_2/MeOH = 10/1) yielded **13** (15.9 mg, 11.2 µmol, 82%) as a yellow oil; R_f 0.32 (CH_2Cl_2/MeOH = 10/1); $[\alpha]_D^{25}$ +10.9 (*c* 0.1, $CHCl_3$); IR (ATR, cm^{-1}) 3862, 3740, 3360, 2976, 2363, 2224, 1711, 1628, 1513, 1370, 1252, 1167, 1053, 861, 749, 698; ^1H NMR (300 MHz, $CDCl_3$) δ 8.95 (2H, s, H2/6), 7.34-7.31 (20H, m, Bn), 5.62-5.59 (2H, m, NH), 5.33 (1H, s, NH), 5.24 (1H, s, NH), 5.21-5.11 (8H, m, Bn), 4.73-4.66 (1H, m, H7), 4.57-4.53 (1H, m, H7), 4.40-4.39 (1H, m, H17), 2.97 (4H, t, *J* = 7.4 Hz, H19/19'), 2.64 (2H, t, *J* = 6.2 Hz, H15), 2.27-1.94 (8H, m, H8/16/20/20'), 1.91-1.65 (4H, m, H9/10), 1.42-1.39 (36H, m, *t*Bu); ^{13}C NMR (75 MHz, $CDCl_3$) δ 172.2, 155.7, 142.9, 135.4, 135.3, 135.2, 128.7, 128.5, 80.2, 67.5, 67.4, 67.2, 53.1, 29.7, 28.4, 17.1; ESI-HRMS (*m/z*) calcd for $C_{73}H_{94}N_5O_{16}$ $[M]^+$ 1296.6673, found 1296.6696.

4-(17'-(*S*)-Amino-17'-carboxy-pentyl)-1-(11"-(*S*)-amino-11"-carboxy-pentyl)-3,5-bis-(21"'-(*S*)-amino-21"'-carboxy-propyl)-pyridinium, desmosine-CH_2 (4).

A solution of **13** (33.3 mg, 23.4 µmol, 1.0 eq) in MeOH (1.0 mL) was treated with 10% Pd/C (125.6 mg, 116.9 µmol, 5.0 eq) and hydrogenated at balloon pressure. After stirring for 4 days at 40 °C, the insoluble was separated by filtration through a Celite pad on neutral silica gel eluting with MeOH. The filtrate (19.0 mg) in MeOH (0.6 mL) was treated with 10% Pd/C (71.6 mg) and hydrogenated at balloon pressure at 40 °C. After stirring for 2 days, the insoluble was separated by filtration through a Celite pad on neutral silica gel eluting with MeOH and the filtrate was then concentrated *in vacuo*. The filtrate (13.6 mg) was used to the next reaction without further purification;

A mixture of TFA and distilled water (2.9 mL, TFA/water = 95/5) was added to the filtrate at room temperature and stirred for 3 h. The solvent was removed *in vacuo*. Purification on C18 silica gel column chromatography (0.1% TFA in distilled water) yielded **4** as a yellow oil (6.7 mg, 10.3 µmol, 44% (2 steps)); R_f 0.22 [MeOH (0.1% TFA)/H_2O (0.1% TFA)]; $[\alpha]_D^{20}$ +16.4 (*c* 0.1, H_2O); ^1H NMR (500 MHz, D_2O) δ8.55 (2H, s, H2/6),4.1 (2H, t, *J* = 7.6 Hz, H7), 4.10 (2H, t, *J* = 6.1 Hz, H21/21'), 5.14 (1H, m, NH), 4.04-3.97 (2H, m, H11/17), 3.10-3.04 and 2.97-2.89 (4H, m, H19/19'), 2.97-2.89 (2H, m, H13), 2.26-2.21 (4H, m, H21/21'), 217-1.94 (8H, m, H8/10/15/16), 1.68-1.55 (4H, m, H9/14); ^{13}C NMR (75 MHz, D_2O) δ173.2, 173.1, 172.8 (C12/18/22/22'), 160.1 (C4), 142.3 (C2/6), 140.3 (C3/5), 61.2 (C7), 53.7, 53.6 (C11/17/20/20'), 31.0, 30.5, 30.1, 29.8 (C8/10/15/21/21'), 29.1 (C13), 26.1, 25.2 (C9/14), 21.6 (C16); ESI-HRMS (*m*/*z*) calcd for $C_{25}H_{42}N_4O_8$ $[M]^+$ 540.3033, found 540.3031.

LC-MS/MS analysis of desmosine-CH_2.

A TSQ Discovery electrospray tandem mass spectrometer (Thermo Fisher Scientific) was used for LC–MS/MS analysis. The high-performance liquid chromatography (HPLC) conditions used were as follows: 2 mm×150 mm dC18 (3 µm) column (Waters, MA); the mobile phase A (7 mM heptafluorobutyric acid (HFBA)/5 mM NH_4Ac in water) and B (7 mM HFBA/5 mM NH_4Ac in 80% acetonitrile) programmed linearly from 100% A to 75% A in 12 min. Quantitation was performed by reaction ion monitoring (RIM) of the transitions of desmosine-CH_2 (*m*/*z* 540 to *m*/*z* 495), with collision energy set at 45 V for both transition, collision gas pressure at 1.5 m Torr, tube lens at 132 V, sheath gas pressure set at 45 and auxiliary gas pressure at 6 units, and ion spray voltage at 3.8 kV. The scan time set at 1.00 ms and both quadrupoles (Q1

and Q3) were at 0.7 Da full-width half-maximum (FWHM). All the LC-MS/MS analyses are performed in triplicate as the standard practice.

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