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SYNTHESIS OF ISOTOPICALLY LABELED (+)-DEOXYPYRIDINOLINE

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Summary

An efficient synthesis of isotopically labeled (+)-deoxypyridinoline (**3**) was achieved via quaternization of (*S*,*S*)-(–)-**4** with (*S*)-(–)-iodide (**5**) and subsequent hydrolysis. The required (*S*)-(–)-iodide (**5**) was prepared from a commercially available (*S*)-5-(*tert*-butoxy)-4-[(*tert*-butoxycarbonyl)amino]-5-oxopentanoic acid (**6**) in seven steps and good overall yield.

Key words: bone collagen, cross-links, deoxypyridinoline, osteoporosis

Introduction

In recent years, the cross-links of bone collagen, (+)-pyridinoline (Pyd, **1**) (1) and (+)-deoxypyridinoline (Dpd, **2**) (2) (Figure 1) have attracted considerable attention due to their clinical utility (3, 4) in the diagnosis of osteoporosis (5, 6) and other metabolic bone diseases such as Paget's disease (7), hyperparathyroidism (8), and

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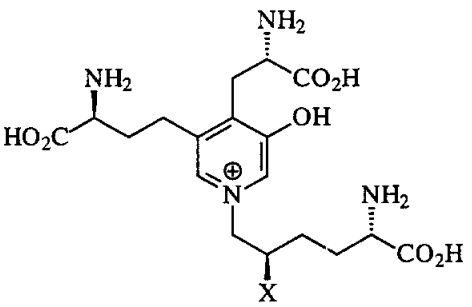
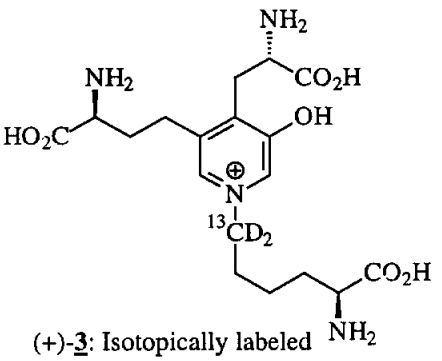
cancer (9). It was found that the cross-link (+)-Dpd (2) shows greater specificity for bone, and consequently it was accepted as a marker of choice for diagnosis of osteoporosis. A number of methods have been reported for quantification of cross-link (2), ex., amino acid analysis (10), HPLC (11, 12), and enzyme-linked immunosorbent assay (ELISA) (13, 14). Recently, we reported the synthesis of (+)-Dpd (2) and its analogs (15, 16, 17) which are needed for development of assays for osteoporosis. In this paper, we describe the synthesis of isotopically labeled (+)-deoxypyridinoline (3), which is necessary as an internal standard (IS) for quantification of cross-link (2) by mass spectrometry, and is critical for the correlation of various immunoassay methods.



(+)-1: X = OH, Pyridinoline
(+)-2: X = H, Deoxypyridinoline



(+)-3: Isotopically labeled deoxypyridinoline

Figure 1. Collagen cross-links (1, 2) and isotopically labeled (+)-Dpd (3)

Results and Discussion

Application of an internal standard (IS) is critical for achieving high precision and accuracy in the quantification of analytes by a mass spectrometry (18). There are number of important issues which need to be considered when choosing the IS, such as molecular weight, physicochemical properties, and the stability in assay conditions. Typically, isotopically labeled analyte is an ideal compound for use as an internal standard, because of its similar properties to the analyte except molecular weight. In preparing the isotopically labeled IS, it is important to incorporate the

isotope in the analyte (ex., **2**) at a position which is stable and not exchangeable. We envisioned (Figure 1) that an analog such as (+)-**3**, in which the 1-(2)-CH_2 is replaced by 1-(2)-$^{13}CD_2$ in the lysine chain, would meet this criterion. Thus, the strategy for the synthesis of labeled (+)-Dpd (**3**) (Figure 2) is based on the quaternization of (*S,S*)-(–)-**4** with iodide (*S*)-(–)-**5**, followed by hydrolysis (15). The labeled iodide (*S*)-(–)-**5** was prepared from a commercially available *L*-glutamic acid derivative, (*S*)-5-(*tert*-butoxy)-4-[(*tert*-butoxycarbonyl)amino]-5-oxo pentanoic acid (**6**) (16).

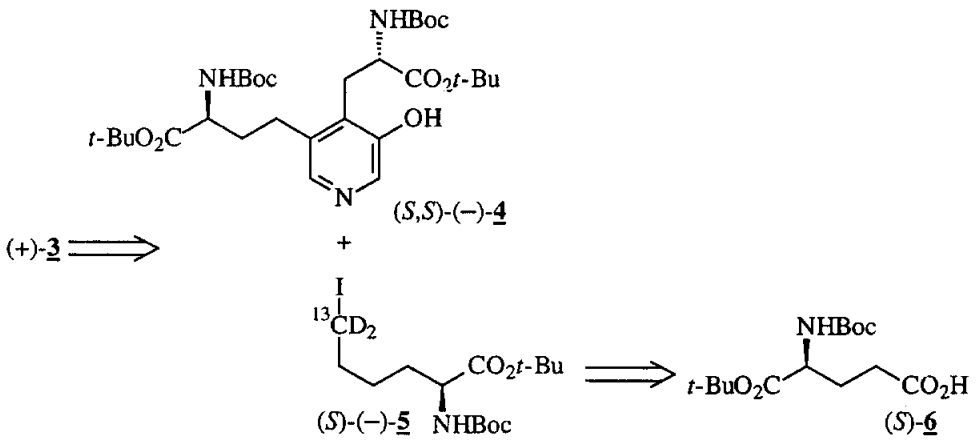
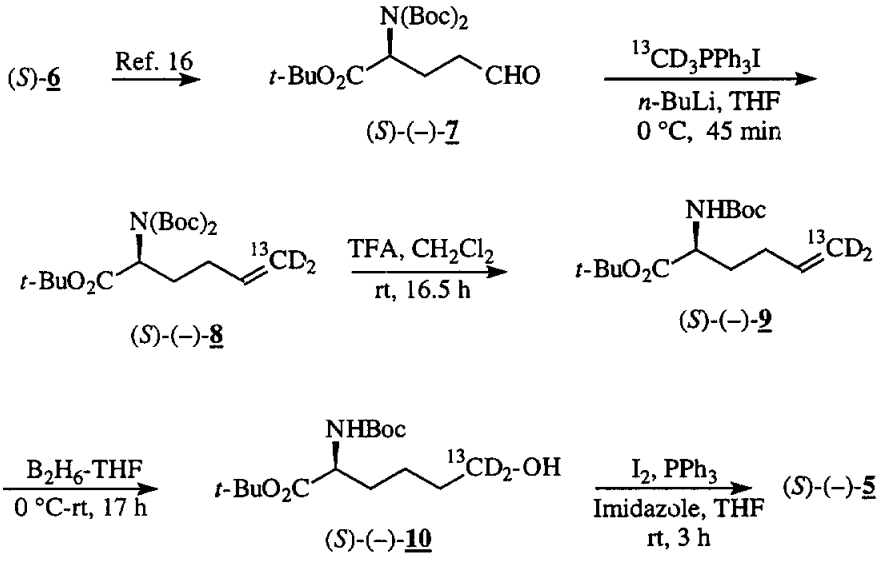


Figure 2: Retrosynthesis of labeled (+)-deoxypyridinoline (**3**)

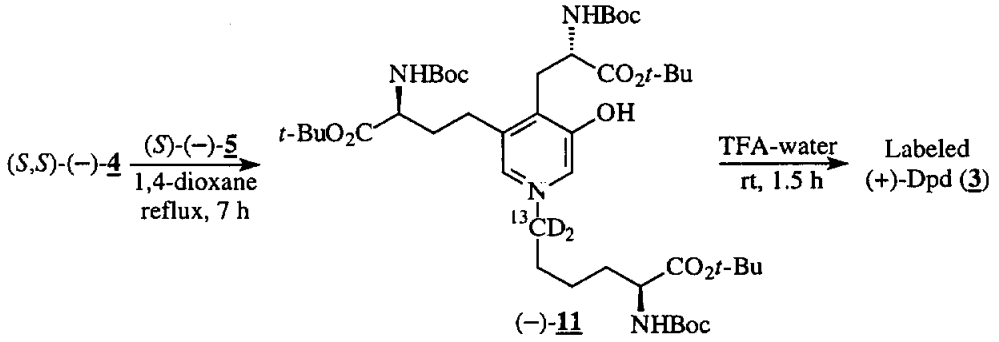
Thus (*S*)-**6** was converted (Scheme 1) to the aldehyde (*S*)-(–)-**7** (16), which upon Wittig reaction with the ylide generated from a labeled ($^{13}CD_3$)-methyl triphenylphosphonium iodide (19) and *n*-BuLi in THF, gave the olefin (*S*)-(–)-**8** in 46% yield after silica gel column chromatography. Analysis of olefin (*S*)-(–)-**8** by ^1H NMR, ^{13}NMR, ESI-MS and HRMS indicated that deuterium in $^{13}CD_2$-label was not exchanged during the protic work-up. Selective removal of one of the Boc groups in (*S*)-(–)-**8** was accomplished by treatment with 1.5 equiv. of trifluoroacetic acid in methylene chloride to give the mono-Boc compound (*S*)-(–)-**9** in excellent yield (88%). The next step in the synthesis of iodide (*S*)-(–)-**5** was the hydroboration of olefin (*S*)-(–)-**9** using borane-THF complex to afford the alcohol (*S*)-(–)-**10** in 48%

yield after purification by silica gel column chromatography. Finally, the hydroxyl group in (*S*)-(–)-**10** was converted to the desired labeled iodide (*S*)-(–)-**5** using triphenylphospine, iodine and imidazole in 78% yield.



Scheme 1: Synthesis of labeled (*S*)-(–)-iodide (**5**)

Quaternization (Scheme 2) of (*S,S*)-(–)-**4** with the labeled iodide (*S*)-(–)-**5** was carried out (15) in refluxing anhydrous 1,4-dioxane for 7 h, which afforded the pyridinium compound (–)-**11** in 40% yield after purification by silica gel column



Scheme 2: Completion of the synthesis of labeled (+)-deoxypyridinoline (**3**)

chromatography. Finally, hydrolysis of Boc and *tert*-butyl protective groups in (–)-**11**, using TFA-water, followed by purification of the crude product by preparative reverse phase HPLC, afforded the labeled (+)-deoxypyridinoline (**3**) in 84% yield.

In summary, an efficient chiral synthesis of isotopically labeled (+)-deoxypyridinoline (**3**) was achieved via quaternization of (*S*,*S*)-(–)-**4** with (*S*)-(–)-iodide (**5**), and subsequent hydrolysis.

Experimental

General methods and materials: ^{1}H and ^{13}C NMR spectra were recorded on a Varian Gemini spectrometer (300 MHz) and the chemical shifts (δ) were reported in ppm relative to TMS and coupling constants (*J*) were reported in Hz. Electrospray ionization mass spectrometry (ESI-MS) were carried on a Perkin-Elmer (Norwalk, CT) Sciex API 100 Benchtop system employing Turbo IonSpray ion source and the HRMS were obtained on a Nermang 3010 MS-50, JEOL SX102-A. 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)] or phosphomolybdic acid reagent (20 wt% solution in ethanol) or 0.2% ninhydrin in ethanol. Column chromatography was performed on silica gel, Merck grade 60 (230–400 mesh). Anhydrous solvents were freshly distilled [(THF from a purple solution of sodium and benzophenone) and (CH_2Cl_2 from CaH_2)] 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 reversed phase (RP) HPLC was performed using a Waters (RCM, C18, Symmetry 7.0 μm, 8 x 100 mm) column (solvents ratio v/v reported) unless otherwise stated. Optical rotations were measured on Autopol III polarimeter, Rudolph Research, Flanders, NJ.

(*S*)-(–)-1-*tert*-Butyl-2-[bis-(*tert*-butoxycarbonyl)amino]-5-oxopentanoate (7) was prepared from commercially available (*S*)-5-*tert*-butoxy-4-[(*tert*-butoxycarbonyl) amino]-5-oxopentanoic acid (6) in three steps (16). The pyridinium derivative, (–)-*tert*-butyl-(2*S*)-4-(4-{(2*S*)-3-(*tert*-butoxy)-2-[(*tert*-butoxycarbonyl)amino]-3-oxopro pyl}-5-hydroxy-3-pyridinyl)-2-[(*tert*-butoxycarbonyl)amino]butanoate (4) was prepared according to our reported procedure (15).

(*S*)-(–)-[(6)-$^{13}CD_2$]-1-*tert*-Butyl-2-[bis-(*tert*-butoxycarbonyl)amino]-5-hexe noate (8): *n*-BuLi (2.5 M soln in hexane, 5.0 mL, 12.5 mmol, 1.1 equiv.) was added dropwise to a suspension of ($^{13}CD_3$)-methyltriphenylphosphonium bromide (5.12 g, 12.5 mmol, 1.1 equiv.) in THF (100 mL) at room temperature under nitrogen. After stirring the mixture for 30 min, the resulting orange ylide solution was cooled to 0 °C and a solution of (*S*)-(–)-7 (4.42 g, 11.4 mmol, 1.0 equiv.) dissolved in THF (40 mL) was added via double ended needle. The reaction mixture was stirred for 45 min at 0 °C and then quenched with saturated aq NH_4Cl solution (100 mL). The mixture was diluted with water (100 mL) and extracted with EtOAc (3 x 150 mL). The combined organic layers were dried (Na_2SO_4) and the solvent removed on a rotary evaporator. The crude compound was purified by silica gel column chromatography (10% EtOAc in hexanes) to afford 1.564 g of (*S*)-(–)-8 in 46% yield as a thick oil. R_f: 0.62 (15% EtOAc in hexanes); $[\alpha]^{23}_D$ –15.9 (c 1.48, MeOH); Analytical RP HPLC: MeCN:0.1% aq trifluoroacetic acid/70:30; 2.0 mL/min at 225 nm, R_t: 2.43 min, 99%; ^1H NMR ($CDCl_3$): δ 5.88–5.80 (m, 1H), 4.81–4.70 (m, 1H), 2.22–2.06 (m, 3H), 2.00–1.89 (m, 1H), 1.54 (s, 18H), 1.44 (s, 9H); ^{13}C NMR ($CDCl_3$): δ 170.0, 152.6, 137.6, 114.6 (quin), 82.7, 81.1, 58.3, 30.4, 28.6, 27.98, 27.9; ESI-MS (m/z): 389 (M + H)$^+$, 411 (M + Na)$^+$; HRMS (FAB, m/z): calcd for $C_{20}H_{33}D_2NO_6$, 389.2589 (M + H)$^+$; observed, 389.2582.

(*S*)-(–)-[(6)-$^{13}CD_2$]-1-*tert*-Butyl-2-[(*tert*-butoxycarbonyl)amino]-5-hexenoate (9): Trifluoro acetic acid (0.43 mL, 5.65 mmol, 1.5 equiv.) was added to a solution of

(*S*)-(–)-**8** (1.464 g, 3.77 mmol) dissolved in CH_2Cl_2 (40 mL) at room temperature and stirred for 16.5 h. The mixture was diluted with ether (120 mL) and washed with 10% aq NaOH (60 mL) and brine (60 mL). The organic layer was dried (Na_2SO_4) and the solvent was removed on a rotary evaporator. The crude compound was purified by silica gel column chromatography (10% EtOAc in hexanes) to afford 0.956 g of (*S*)-(–)-**9** in 88% yield as a thick oil. R_f: 0.39 (15% EtOAc in hexanes); $[\alpha]^{23}_D$ –20.2 (c 1.41, MeOH); Analytical RP HPLC: MeCN:0.1% aq trifluoroacetic acid/70:30, 2.0 mL/min at 215 nm, R_t: 2.60 min. 98%; ^{1}H NMR ($CDCl_3$): δ 5.87–5.74 (m, 1H), 5.08–4.97 (m, 1H), 4.19 (q, 1H, *J*=12.9, 7.5 Hz), 2.18–2.03 (m, 2H), 1.93–1.81 (m, 1H), 1.74–1.62 (m, 1H), 1.48 (s, 9H), 1.45 (s, 9H); ^{13}C NMR ($CDCl_3$): δ 172.1, 155.5, 137.4, 114.8 (quin), 81.8, 79.6, 53.5, 32.2, 29.3, 28.2, 27.9; ESI-MS (m/z): 289 $(M + H)^+$; 306 $(M + NH_4)^+$; HRMS (FAB, m/z): calcd for $C_{15}H_{25}D_2NO_4$, 289.2065 $(M + H)^+$; observed, 289.2058.

(*S*)-(–)[(6)-$^{13}CD_2$]-*tert*-Butyl-2-[(*tert*-butoxycarbonyl)amino-6-hydroxyhexanoate (10): (*S*)-(–)-**9** (0.914 g, 3.17 mmol) in THF (16 mL) was cooled to 0 °C and a solution of borane-THF complex (1.0 M soln in THF, 4.12 mL, 4.12 mmol, 1.3 equiv.) was added under nitrogen. The cooling bath was removed, the mixture allowed to warm to room temperature and stirred for 17 h. The reaction was then cooled to 0 °C, 1N aq. NaOH (4.77 mL, 4.75 mmol, 1.5 equiv.) added followed by 30% H_2O_2 (4.0 mL), and the mixture stirred for 30 min. The reaction mixture was diluted with water (30 mL) and extracted with EtOAc (3 x 50 mL). The combined organic layers were dried (Na_2SO_4) and concentrated on a rotary evaporator. The crude product was purified by silica gel column chromatography (40% EtOAc in hexanes) to afford 0.450 g of (*S*)-(–)-**10** in 48% yield as a colorless oil. R_f: 0.23 (40% EtOAc in hexanes); Analytical RP HPLC: MeCN:0.1% aq trifluoroacetic acid/50:50, 2.0 mL/min at 215 nm, R_t: 4.0 min, 98%; $[\alpha]^{23}_D$ –27.1 (c 1.32, MeOH); ^{1}H NMR ($CDCl_3$): δ 5.05 (d, 1H, *J*=8.1 Hz), 4.20–4.09 (m, 1H), 1.80–1.60 (m, 6H),

1.46 (s, 9H), 1.44 (s, 9H); ^{13}C NMR ($CDCl_3$): δ 172.1, 155.6, 81.7, 79.6, 61.5 (quin), 53.7, 32.6, 32.0, 28.2, 27.9, 21.2; ESI-MS (m/z): 306 $(M + H)^+$, 324 $(M + NH_4)^+$; HRMS (FAB, m/z): calcd for $^{12}C_{14}{}^{13}CH_{27}D_2NO_5$, 307.2283 $(M + H)^+$; observed, 307.2290.

(*S*)-(–)-[(6)-$^{13}CD_2$]-*tert*-Butyl-2-[(*tert*-butoxycarbonyl)amino]-6-iodohexa noate (5): Triphenylphosphine (0.495 g, 1.89 mmol, 1.5 equiv.), imidazole (0.136 g, 2.0 mmol, 1.6 equiv.) and iodine (0.480 g, 1.89 mmol, 1.5 equiv.) were added sequentially to a solution of (*S*)-(–)-**10** (0.387 g, 1.26 mmol) dissolved in THF (15 mL) at room temperature under nitrogen. After stirring the mixture for 3 h, the solvent was removed on a rotary evaporator to dryness and the crude product was purified by silica gel column chromatography (10% EtOAc in hexanes) to afford 0.415 g of labeled iodide (*S*)-(–)-**5** in 78% yield as colorless thick oil. R_f: 0.40 (20% EtOAc in hexanes); Analytical RP HPLC (Waters, RCM, C18, Novapak, 4.0μm, 8 x 100 mm): MeCN:water:TFA/20:80:0.1, 2.0 mL/min at 215 nm, R_t: 3.44 min, >99%; $[\alpha]^{23}_D$ –13.1 (c 1.22, MeOH); ^1H NMR ($CDCl_3$): δ 5.04 (d, 1H, *J*=8.1 Hz), 4.16 (q, 1H, *J*=13.5, 7.2 Hz), 1.96–1.60 (m, 6H), 1.46 (s, 9H), 1.43 (s, 9H); ^{13}C NMR ($CDCl_3$): δ 171.6, 155.1, 81.7, 79.4, 53.6,32.8, 31.8, 28.2, 27.9, 25.9, 6.2 (quin); ESI-MS (m/z): 417$(M + H)^+$, 434 $(M + NH_4)^+$; HRMS (FAB, m/z): calcd for $^{12}C_{14}{}^{13}CH_{26}D_2NO_4I$, 417.1300 $(M + H)^+$; observed, 417.1298.

(–)-[1-(2)-$^{13}CD_2$]-Pyridinium compound (11): A solution of labeled iodide (*S*)-(–)-**5** (0.210 g, 0.505 mmol, 2.5 equiv.)] dissolved in anhydrous 1,4-dioxane (2.0 mL) was added to a solution 3-hydroxypyridine derivative (*S,S*)-(–)-**4** (15) (0.150 g, 0.252 mmol) which was dissolved in anhydrous 1,4-dioxane (5.0 mL) under nitrogen. The mixture was gently refluxed for 7 h. The solvent was removed on a rotary evaporator to dryness and the crude compound was purified by silica gel column chromatography (3-5% MeOH in CH_2Cl_2) to afford 0.103 g of the pyridinium

compound (–)-**11** in 40% yield as a pale yellow solid. Analytical RP HPLC: MeCN:0.1% aq trifluoroacetic acid/80:20, 2.0 mL/min at 215 nm, R_t: 3.08 min, 98.2%; $[\alpha]^{23}_D$ –9.7 (c 1.28, MeOH); ^{1}H NMR ($CDCl_3$): δ 7.52 (s, 1H), 7.03 (s, 1H), 5.20 (d, 1H, *J*=7.1 Hz), 5.11 (d, 1H, *J*=7.2 Hz), 4.20–4.06 (m, 3 H), 3.41–3.31 (m, 2H), 3.00–2.64 (m, 3H), 2.08–1.22 (m, 8H), 1.47 (s, 9H), 1.46 (s, 18H), 1.45 (s, 18H), 1.39 (s, 9H); ESI-MS (m/z): 884 $(M)^+$; HRMS (FAB, m/z): calcd for $^{12}C_{44}{}^{13}CH_{75}D_2N_4O_{13}$, 884.5641 $(M)^+$; observed, 884.5630.

Labeled (+)-deoxypyridinoline (3): A mixture of trifluoroacetic acid (9.5 mL) and water (0.5 mL) was added to the pyridinium compound (–)-**11** (0.095 g, 0.063 mmol)] at room temperature, and stirred the mixture for 1.5 h. The solvent was removed on a rotary evaporator (<40 °C bath temperature) to dryness, and the residue was dissolved in MeCN-0.1% aq TFA (10 mL, ratio, 1:99). The product was purified by preparative RP HPLC [Waters, C18, RCM, Novapak, 4μ (40 x 100 mm) using MeCN:0.1% aq trifluoroacetic acid/1:99, 20 mL/min at 215 nm]. The solvent was removed on a rotary evaporator to about 200 mL volume, and lyophilized to afford 0.080 g of labeled (+)-Dpd (**3**) in 92% purity as determined by analytical HPLC. The 0.072 g of this material was dissolved in MeCN-0.1% aq TFA (8 mL, 1:99 ratio) and further purified by preparative RP HPLC [Waters, C18, RCM, μBondpak, 10 μm (40 x 100 mm) using MeCN:0.1% aq trifluoroacetic acid/1:99, 20 mL/min at 215 nm]. The solvent was removed on a rotary evaporator to about 120 mL volume, and lyophilized to afford 0.061 g of isotopically labeled (+)-deoxypyridinoline-TFA salt (**3**) as a colorless gummy material in 74% yield. Analytical RP HPLC: MeCN:0.1% aq trifluoroacetic acid/1:99; 1.0 mL/min at 215 nm, R_t: 3.16 min, 97%; $[\alpha]^{23}_D$ +36.6 (c 1.31, MeOH); ^{1}H NMR (CD_3OD): δ 8.41 (br s, 1H), 8.33 (br s, 1H), 4.29 (t, 1H, *J*=6.9 Hz), 4.04–3.92 (m, 2H), 3.54–3.38 (m, 2H), 3.18–2.98 (m, 2H), 2.40–2.14 (m, 2H), 2.11–1.90 (m, 4H), 1.66–1.44 (m, 2H); ^{13}C NMR (CD_3OD): δ 171.9, 171.7, 171.6, 158.0, 142.7, 142.5, 136.7, 130.1, 61.8 (quin), 53.8, 53.4, 52.8, 31.4, 30.9, 30.8, 29.0, 27.0, 22.6; ESI-MS (m/z): 416 $(M)^+$; HRMS (FAB, m/z): calcd for $^{12}C_{17}{}^{13}CH_{27}D_2N_4O_7$, 416.2195 $(M)^+$; observed, 416.2195.

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