Research Article

Journal of Labelled Compounds and Radiopharmaceuticals

Received 11 January 2011, Revised 28 March 2011, Accepted 20 April 2011 Published online 11 August 2011 in Wiley Online Library

(wileyonlinelibrary.com) DOI: 10.1002/jlcr.1900

Synthesis of a stable-isotope-labeled biotinylated pentasaccharide conjugate (EP217609), a dual-effect anticoagulant drug

Sébastien Richard,[a] Ahmed El Hadri,[a]* Marcel Schreuder Goedheijt,[b] Michel Heisen,[b] Jan Vader,[b] Peter Wiegerinck,[b] and Maurice Petitou[a]

EP217609 is a neutralizable dual-effect anticoagulant under clinical investigation in cardiopulmonary bypass during cardiac surgery. Stable-isotope-labeled EP217609 was synthesized as an internal standard for mass spectrometry in support of bioassays. EP217609 was obtained in six steps starting from three building blocks in an overall yield of 42%, with a chemical purity of >99%. Thus, coupling between the N-protected labeled biotin-lysine 4, prepared in three steps from [^{13}C,^{15}N]-L-lysine 2, and the pentasaccharide-spacer-amine 6 was first performed. Removal of the Cbz protective group to 8 followed by coupling of the activated peptidomimetic building-block 10 gave the immediate precursor of EP217609, which could be obtained after catalytic hydrogenolysis of 1,2,4-oxadiazol-5(2*H*)-one into amidine.

Keywords: antithrombotic; anticoagulant; isotope labeling; biotin; labeled lysine; pentasaccharide; heparin mimetic

Introduction

Compounds labeled with stable isotopes are commonly used as internal standards for mass spectroscopy (MS) studies of biological fluids during development of new drugs, wherein using isotopically labeled compound allows a more accurate determination of the concentration of the drug.[1–11] The stable isotope labels used in these studies are typically C-13, N-15, and H-2. The oxygen isotope O-18 is seldom used due to the propensity of oxygen atoms to exchange with water; however, in a few elegant studies, O-18-labeled compounds have been utilized for mechanistic elucidation.[12] The stable-isotope-labeled (SIL) compound is generally used as an internal standard to quantitate the parent compound in biological matrixes. A known amount of the SIL drug is added to the biological sample, and after processing to prepare the sample for liquid chromatography-MS (LC-MS), the ratio of SIL to unlabeled drug is determined. As by adding the SIL compound before processing, the loss of unlabeled compound due to these manipulations is accounted for, this ratio allows the calculation of the amount of unlabeled drug present in the biological sample.

A number of precautions have to be taken when designing an SIL version of a drug candidate. These particularly deal with the location of the label in the structure and the difference in atomic mass. Concerning the location of the label in the structure, as the molecule will be studied in LC-MS/MS, it is necessary to have the isotopic label in the daughter fragment. The minimum mass increase required for pentasaccharide analogs to achieve appropriate resolution is 6, while 8 or more is preferred. The isotopes incorporated are not limited to one or two elements. A key consideration and requirement of the labeling synthesis is that the isotopically labeled material must not contain (or only very low levels in the range of <0.1%) any unlabeled material.

Other considerations are less relevant, such as improving the signal-to-noise ratio as the same signal-to-noise ratio can be obtained by the addition of more SIL compounds to the biological sample. Among the stable isotopes used, C-13 and N-15 are preferred by bioanalysts because the labeled molecules seldom, if ever, fractionate from their unlabeled isotopomers under typical chromatographic conditions, in contrast to H-2-labeled compounds, which can have different retention times in LC if the label is not properly located.[13] This effect can be minimized by avoiding placing the deuterium label within two bonds of nitrogen.[13] The H-2 label must also be placed in a location that is nonexchangeable (e.g. not alpha to a carbonyl).

Although the starting materials for preparing SIL compounds are expensive, a wide range is commercially available. The synthesis of SIL compounds is very similar to unlabeled synthesis with a few notable exceptions. The synthesis often needs to be altered from the previously developed routes to allow for late-stage incorporation of isotope labeling into the target molecule. This allows for a higher chemical yield because of fewer reaction steps and generates less label material waste.

In this article, we report the synthesis and characterization of (^{13}C,^{15}N)-labeled EP217609, a dual-action anticoagulant, which contains a pentasaccharide analog, fondaparinux, covalently bound to a peptidomimetic thrombin inhibitor and to a biotin

[a]*Endotis Pharma, Biocitech, 102 Avenue Gaston Roussel, 93230 Romainville, France*

[b]*MSD, Department of Process Chemistry, Molenstraat 110, 5340 BH Oss, the Netherlands*

**Correspondence to: Dr. Ahmed El Hadri, Chemistry Department, Endotis Pharma, 102 Avenue Gaston Roussel, 93230 Romainville, France.*
E-mail: ahmed.elhadri@endotis.com

entity.[14,15] This new anticoagulant drug is currently under clinical trial in cardiopulmonary bypass during cardiac surgery, a clinical setting requiring a potent, rapidly neutralizable anticoagulant. The fast neutralization of the anticoagulant activity of EP217609 is carried out by injection of avidin, a protein displaying high affinity for biotin (Kd ~10^{15} M^{-1}).

Results and discussion

On the basis of the molecular weight of EP217609 and its MS spectrum, it was concluded that the minimum atomic molecular mass increase required was at least 6 in order to avoid any interference with the unlabeled material. Considering the structure, it is particularly attractive to use a commercially available (^{13}C,^{15}N) lysine that allows adding 8 amu (atomic mass units) at the very end of the building block assembly process.

EP217609 was originally synthesized in about 60 steps by De Kort and Van Boeckel.[15] In this route, the final coupling step was performed between the pentasaccharide building block **6** and a building block containing the peptidomimetics and the biotin entity (Scheme 1). As a small amount of undesired racemisation product was formed at the lysine alpha-carbon during the late step of this coupling reaction, a new approach to the synthesis of isotopically labeled EP217609 has been explored (Schemes 2–4), in which the final coupling involves the activation of a carboxylate function in alpha-position to a methylenic carbon.

In the first step, [^{13}C,^{15}N]-L-lysine **2** was treated with *p*-anisaldehyde to form a Schiff base. Addition of benzyloxycarbonyl chloride in the presence of sodium hydroxide followed by direct hydrolysis afforded Cbz-Lys-OH **3** in a 78% yield (Scheme 2). Coupling between the activated D-(+)-biotin **1** and **3** was carried out in dimethylformamide (DMF) to afford the desired **4** in a practically quantitative yield.

The building block **4** was coupled to the oligosaccharide-spacer **6**[15] (Scheme 3) using a standard peptide coupling method (EDCI/HOBt) to give compound **7** in 88% yield. After hydrogenolysis, compound **8** was isolated quantitatively.

The peptidomimetic building-block **9**[16] was activated as pentafluorophenolyl ester and coupled with **8** to give compound **11** in 67% yield (Scheme 4). Deprotection of the amidine moiety of **11** by hydrogenolysis in the presence of Pd/C afforded EP217609 in quantitative yield.

In summary, ^{13}C- and ^{15}N-labeled EP217609 was synthesized in six steps starting from ^{13}C- and ^{15}N-labeled L-lysine. The overall yield of the labeling steps was 42% with a chemical purity higher than 99% (Figure 1) as determined by HPLC. This new approach for the synthesis of EP217609 allowed access to a pure labeled compound without racemisation of peptide reaction step and high isotopic enrichment.

The ESI-MS spectrum of labeled EP217609 (Figure 2) showed peaks corresponding to four and three multiple charged ions identical to that observed for the unlabeled compound with an increase of 2 and 2.6 amu, respectively, for molecular ions $[M-4H]^{4-}$ and $[M-3H]^{3-}$. Interestingly, no overlapping on peaks multiplicity of molecular ion between labeled and unlabeled compounds was observed.

Experimental

General

Labeled L-lysine was purchased from Sigma-Aldrich. The intermediates **5** and **9** were obtained from MSD, the manufacturer of EP217609. All reactions were carried out under an atmosphere of nitrogen unless stated otherwise. All compounds were homogeneous by thin layer chromatography (TLC) and had spectral properties consistent with their assigned structures. Purifications in organic solvents were performed by flash column chromatography on a Merck cartridge (GX0171511110LK [554–3646]; EVF D17, Si60 15–40 µm; 10 g) using the Isolera™ flash purification system (Biotage). Gel-permeation chromatography with aqueous eluents was performed using Sephadex G-25 (GE Healthcare). Compound purity was checked by TLC on silica gel 60 F_{254} (E. Merck) with detection by charring with sulfuric acid. The chemical purity of all labeled compounds was determined by HPLC and ESI-TOF or LC-MS. ^{1}H- and ^{19}F-NMR spectra were recorded on a Brucker 400-MHz instrument; chemical shifts were expressed relative to internal tetramethylsilane (TMS; spectra recorded in organic solvents) or trimethylsilyl propionate (TSP; spectra recorded in D_2O) unless stated otherwise. MS analyses were performed on a QSTAR® instrument 40 MCA Scans (MDS-Sciex and Applied Biosystems Inc.). Before analysis in D_2O, samples were passed through a Chelex (Bio-Rad) ion exchange column and lyophilized three times from D_2O. All prepared SIL compounds contained no unlabeled material by ESI-TOF MS analysis.

HPLC analysis

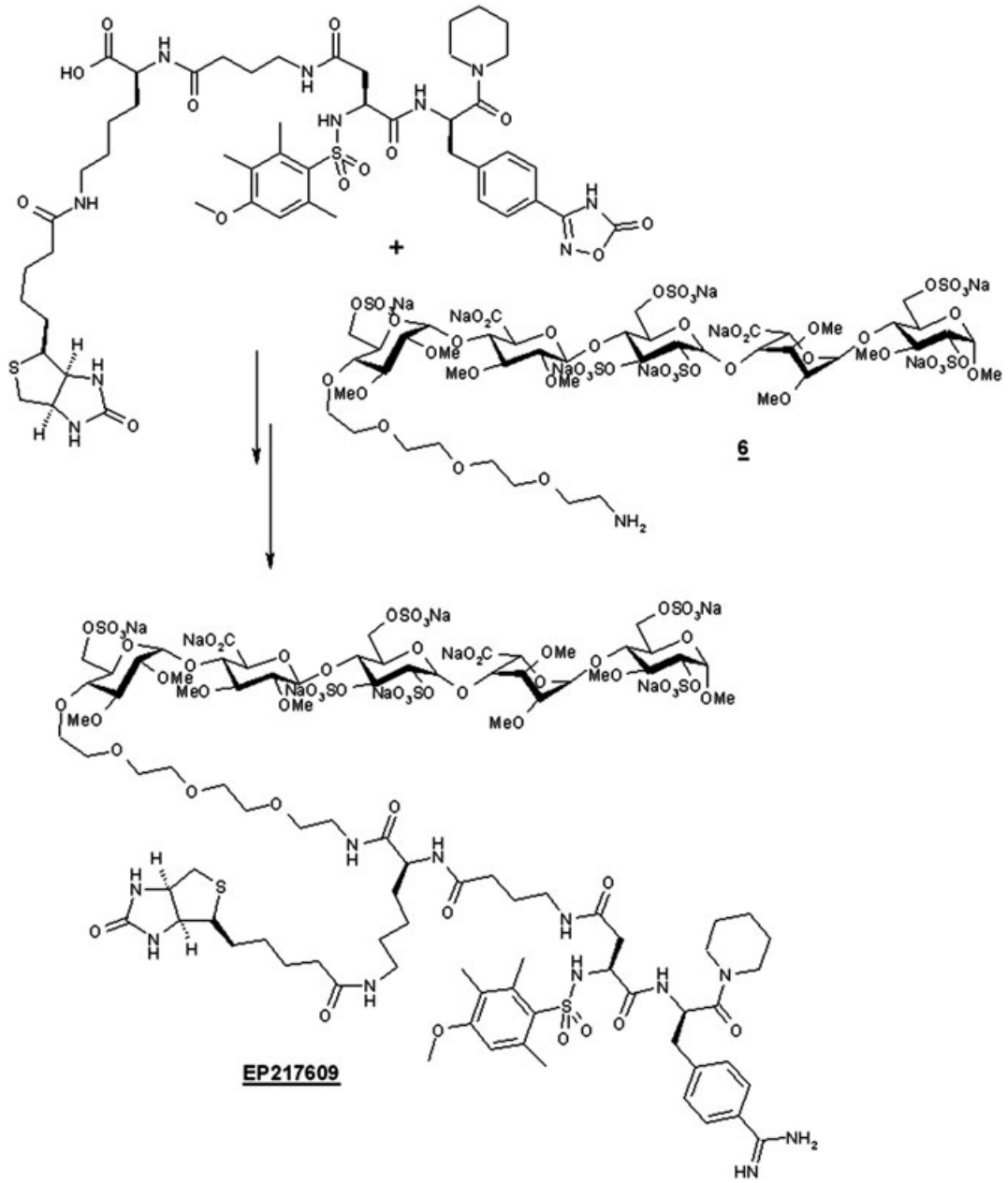
HPLC was performed on a Waters 600 Controller in combination with a Waters 2996 dual absorbance detector, set at 200 to 400 nm. An Altima 55–45 C18 (250 × 4.6 mm, 5 µm) column was used, and chromatograms were processed using MassLynx Soft. The following HPLC conditions were used: mobile phase A, 25 mM ammonium acetate; mobile phase B, 25 mM ammonium acetate in MeOH. Elution was carried out at 0.90 ml/min.

Eluent	Time (min)					
	0	45	50	55	60	75
% Ammonium acetate (A)	100	45	40	5	55	55
% Ammonium acetate in MeOH (B)	0	55	60	95	45	45

Chemistry

*N^{α}-Benzyloxycarbonyl-L-($^{13}C_6$,$^{15}N_2$)lysine (Cbz-Lys-OH, **3**)*

To an aqueous LiOH solution (2N, 0.58 mL) at room temperature, isotope-labeled L-lysine monohydrochloride (**2**; 0.2 g, 1.05 mmoL) was added, and the mixture was cooled below 4 °C. *p*-Anisaldehyde (0.16 g, 1.15 mmoL) was added in three portions of about 50 mg each. The reaction mixture was stirred for 3 h, maintaining the temperature below 4 °C; then the thick paste was kept overnight below 5 °C. After filtration and washing with cold acetonitrile (0.3 mL), the wet cake of N^{ε}-anisilidine-L-lysine Lys[N=CH-(p-C_6H_4OMe)]-OH was used in the next step without further purification. It was suspended at 0 °C in a 1N aqueous NaOH/ethanol mixture [1:1 (v/v), 1.9 mL], cooled to −20 °C, and benzyloxycarbonyl chloride (0.64 g, 3.75 mmoL) and a cold 1N aqueous NaOH/ethanol solution [2:2.5 (v/v), 2 mL] were then added in portions while the temperature of the reaction mixture was maintained at −20 °C. The reaction mixture was stirred for about 2 h, allowing the temperature to rise to

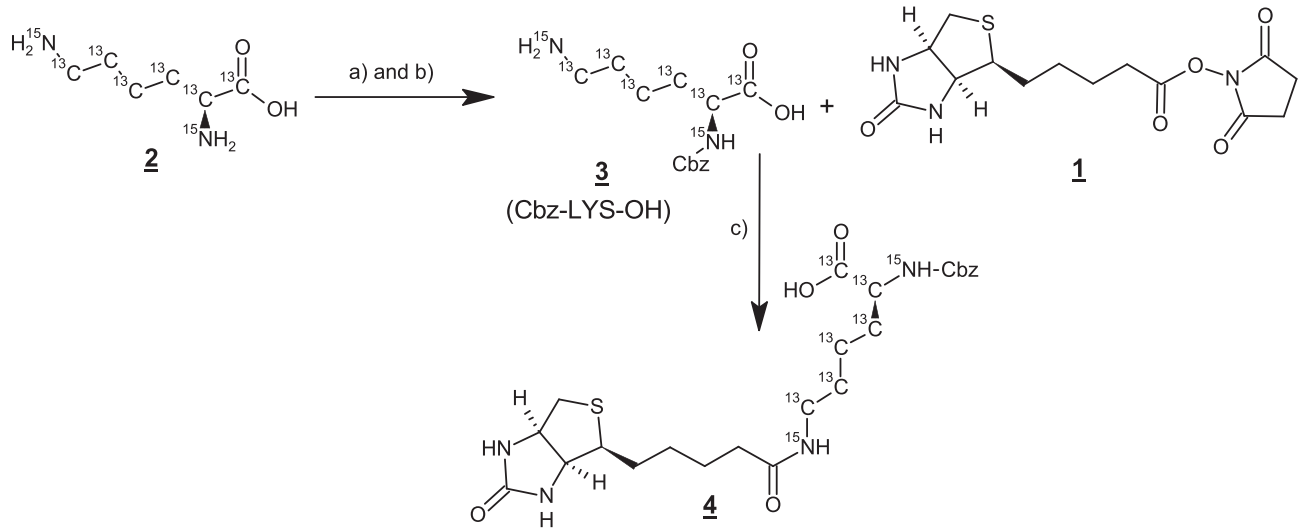


Scheme 1. Original synthesis of unlabeled EP217609.[15]

about −5 °C. The pH of the reaction mixture was then adjusted to 2 by adding concentrated HCl (0.23 mL) to hydrolyze the intermediate Schiff base. Ethanol was then evaporated at around 50 °C, and the aqueous phase was then washed with dichloromethane (4 × 5 ml) and concentrated. The pH was then adjusted to 6.5–7 using aqueous 1N NaOH, and the solution was concentrated under vacuum. Purification on silica gel column using dichloromethane/methanol (9:1) as eluent afforded **3** (0.21 g, 78%). ^{1}H NMR (400 MHz, D_2O) δ 1.13–1.88 (m, 6 H), 2.82 (m, 2 H), 3.97 (m, 1 H,), 4.98 (s, 2 H), 4.99 (d, 1 H, J = 24 Hz, NH-CO-O), 7.29 (m, 5 H).

*N^{α}-Benzyloxycarbonyl-[D-(+)-biotinyl]-L-($^{13}C_6$,$^{15}N_2$)lysine (**4**)*

In a 50-mL round-bottomed flask, compound **1** (0.23 g, 0.66 mmoL) was suspended in 6 mL of anhydrous DMF to give a white suspension. N^{α}-Benzyloxycarbonyl-L-lysine **3** (0.2 g, 0.69 mmoL) and diisopropyl-ethylamine (0.73 mL, 4.16 mmoL, 6 equivalents) were added, and the reaction mixture was stirred overnight at room temperature. After 20 h, TLC analysis (dichloromethane/methanol 9:1) of the yellow solution indicated a complete reaction. Water (3 mL) was added, and the reaction mixture was stirred for 4 h at room temperature (slight rise in temperature after addition water). The white precipitate was filtered, washed twice with water (1 mL) and twice with ether (2 mL), and dried under vacuum at 40 °C to afford **4** (0.32 g, 95%). LC-MS (ESI): *m/z* 515.3, purity 99%; ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.13–1.61 (m, 12 H), 2.04 (t, 2 H, J = 8.0 Hz), 2.57 (d, 1 H, J = 12.0 Hz), 2.82 (m, 2 H), 3.08 (m, 1 H), 4.10 (m, 3 H), 4.30 (m, 1 H), 5.03 (s, 2 H), 6.35 (bs, 1 H), 6.42 (bs, 1 H), 7.34 (m, 5 H), 7.63 (bs, 1 H), 7.86 (bs, 1 H).



Scheme 2. (a) *p*-Anisaldehyde, 2N LiOH, 4 °C, overnight, not isolated; (b) 1N NaOH/EtOH, Cbz-Cl, −20 °C to −5 °C, conc. HCl, 78% over a and b; (c) DIEA, DMF, 95%.

*Methyl O-2,3-di-O-methyl-4-O-(12-aza-3,6,9-trioxa-dodecyl)-6-O-sulfo-alpha-D-glucopyranosyl-(L->4)-O-2,3-di-O-methyl-beta-D-glucopyranuronosyl-(L->4)-O-2,3,6-tri-O-sulfo-alpha-D-glucopyranosyl-(L->4)-O-2,3-di-O-methyl-alpha-L-idopyranuronosyl-(L->4)-3-O-methyl-2,6-di-O-sulfo-alpha-D-glucopyranoside octakis sodium salt (**6**)*

To a solution of compound **5** (0.15 g, 0.077 mmoL) in water (10 mL) was added 10% Pd/C (0.07 g), and the mixture was stirred at room temperature under a continuous stream of hydrogen. After 4 h, the catalyst was removed by filtration and the filtrate was concentrated. Lyophilisation gave pure **6** (0.138 g, 100%), which was used for the next step without further purification.

*Methyl O-2,3-di-O-methyl-4-O-(12-[N^{α}-benzyloxycarbonyl-{N^{ε}-(D-(+)-biotinyl)}-L-($^{13}C_6$,$^{15}N_2$)lysyl]-aza-3,6,9-trioxa-dodecyl)-6-O-sulfo-alpha-D-glucopyranosyl-(L->4)-O-2,3-di-O-methyl-beta-D-glucopyranuronosyl-(L->4)-O-2,3,6-tri-O-sulfo-alpha-D-glucopyranosyl-(L->4)-O-2,3-di-O-methyl-alpha-L-idopyranuronosyl-(L->4)-3-O-methyl-2,6-di-O-sulfo-alpha-D-glucopyranoside octakis sodium salt (**7**)*

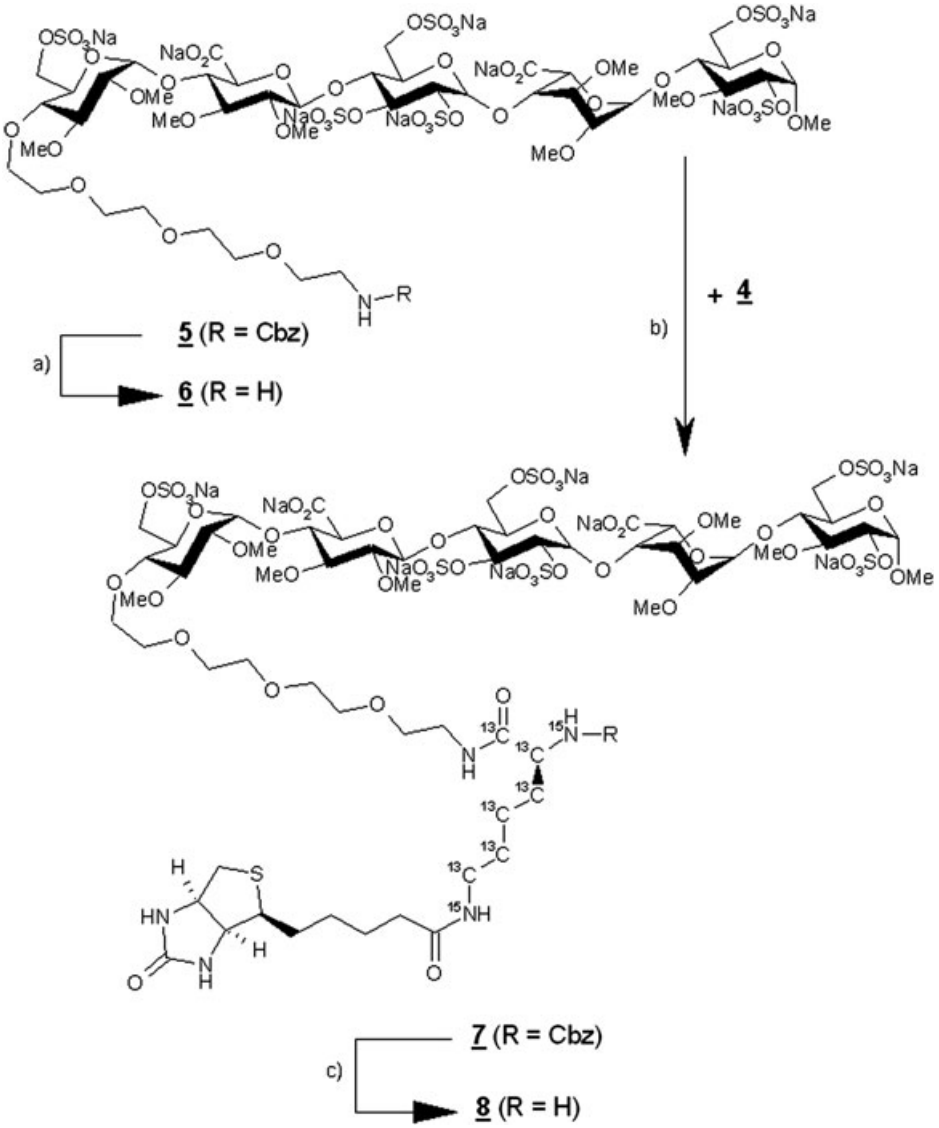
To a suspension of compound **4** (0.039 g, 0.075 mmoL) in anhydrous DMF (1 mL) in a 5-mL round-bottomed flask under nitrogen, 1-hydroxybenzotriazole (0.011 g, 0.079 mmoL) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (0.015 g, 0.079 mmoL) were added, and the mixture was stirred for 1 h at room temperature. A solution of compound **6** (0.09 g, 0.05 mmoL) in anhydrous DMF (3 mL) was added dropwise, and the mixture was stirred for 2 h at room temperature. TLC (spraying with 10% H_2SO_4 in ethanol) and HPLC analyses showed complete reaction. The mixture was poured into ethyl acetate (2 mL), stirred for 15 min at room temperature, and filtered. The cake was washed twice with 2 mL of ethyl acetate dissolved in water and desalted by passing through Sephadex G-25 to afford **7** (0.101 g, 88%) in 93% purity; ESI-MS: *m/z* calcd for $C_{70}H_{109}N_5O_{54}S_7$, 528.8558; found, 528.8385 $[M-4H]^{4-}$; calcd for $C_{70}H_{110}N_5O_{54}S_7$, 705.4745; found, 705.4567 $[M-3H]^{3-}$; ^1H NMR (400 MHz, D_2O) δ 1.01–1.58 (m, 12 H), 2.08 (t, 2 H, J = 7.0 Hz), 2.58 (d, 1 H, J = 12.0 Hz), 2.80 (m, 2 H), 3.07–3.64 (m, 58 H), 3.67–3.86 (m, 4 H), 3.92–4.01 (m, 2 H), 4.07–4.28 (m, 6 H), 4.38–4.52 (m, 2 H), 4.90 (s, 2 H), 4.91–5.04 (m, 2 H), 5.22 (d, 1 H, J = 4.0 Hz), 5.30 (d, 1 H, J = 4.0 Hz), 7.28 (m, 5 H).

*Methyl O-2,3-di-O-methyl-4-O-(12-[{N^{ε}-(D-(+)-biotinyl)}-L-($^{13}C_6$,$^{15}N_2$)lysyl]-aza-3,6,9-trioxa-dodecyl)-6-O-sulfo-alpha-D-glucopyranosyl-(L->4)-O-2,3-di-O-methyl-beta-D-glucopyranuronosyl-(L->4)-O-2,3,6-tri-O-sulfo-alpha-D-glucopyranosyl-(L->4)-O-2,3-di-O-methyl-alpha-L-idopyranuronosyl-(L->4)-3-O-methyl-2,6-di-O-sulfo-alpha-D-glucopyranoside octakis sodium salt (**8**)*

To a solution of **7** (0.088 g, 0.038 mmoL) in water (8 mL) was added 10% Pd/C (0.04 g), and the mixture was stirred at room temperature under a continuous stream of hydrogen. After 4 h, the catalyst was removed by filtration, and the filtrate was concentrated and lyophilized to give **8** (0.082 g, 96%). ESI-MS: *m/z* calcd for $C_{62}H_{104}N_5O_{52}S_7$, 660.7955; found, 660.8155 $[M-3H]^{3-}$; ^1H NMR (400 MHz, D_2O) δ 1.02–1.68 (m, 12 H), 2.10 (t, 2 H, J = 7.2 Hz), 2.62 (d, 1 H, J = 15.6 Hz), 2.85 (m, 2 H), 3.07–3.66 (m, 58 H), 3.72–3.87 (m, 4 H), 3.94–4.02 (m, 2 H), 4.07–4.19 (m, 4 H), 4.26 (m, 2 H), 4.45–4.52 (m, 2 H), 4.90 (d, 1 H, J = 4.0 Hz), 5.13 (s, 1 H), 5.23 (d, 1 H, J = 4.0 Hz), 5.31 (d, 1 H, J = 4.0 Hz).

*4-[4-[[(1R)-1-[[4-(1,2,4-Oxadiazol-5(2H)-one)-phenyl]methyl]-2-oxo-2-(1-piperidinyl) ethyl]amino]-3-[[(4-methoxy-2,3,6-trimethylphenyl)sulfonyl]amino]-1,4(S)-dioxobutyl] amino]-butanoic acid pentafluorophenyl ester (**10**)*

To a solution of **9** (0.15 g, 0.206 mmoL) in anhydrous DMF (1.5 mL) in a 5-mL round-bottomed flask under nitrogen, 2,3,4,5,6-pentafluorophenol (0.042 g, 0.226 mmoL) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (0.051 g, 0.268 mmoL) were added, and the mixture was stirred for 16 h at room temperature. TLC and HPLC analyses showed complete reaction. Water (2 mL) was added to the mixture, and **10** was extracted with CH_2Cl_2 (3 × 15 mL). The organic layer was concentrated and purified on a silica gel column to afford **10** (0.152 g, 87%). ^1H NMR (400 MHz, $CDCl_3$) δ 1.60–1.69 (m, 6 H), 1.86 (m, 2 H), 1.99 (t, 2 H, J = 8.0 Hz), 2.15 (s, 3 H, Me), 2.57 (s, 3 H, Me), 2.67 (s, 3 H, Me), 2.76 (t, 2 H, J = 8.0 Hz), 2.82 (m, 1 H), 3.06 (dd, 1 H, J = 20.0 and 4.0 Hz), 3.42 (dd, 2 H, J = 16.0 and 8.0 Hz), 3.61 (m, 4 H), 3.88 (s, 3 H, OMe),



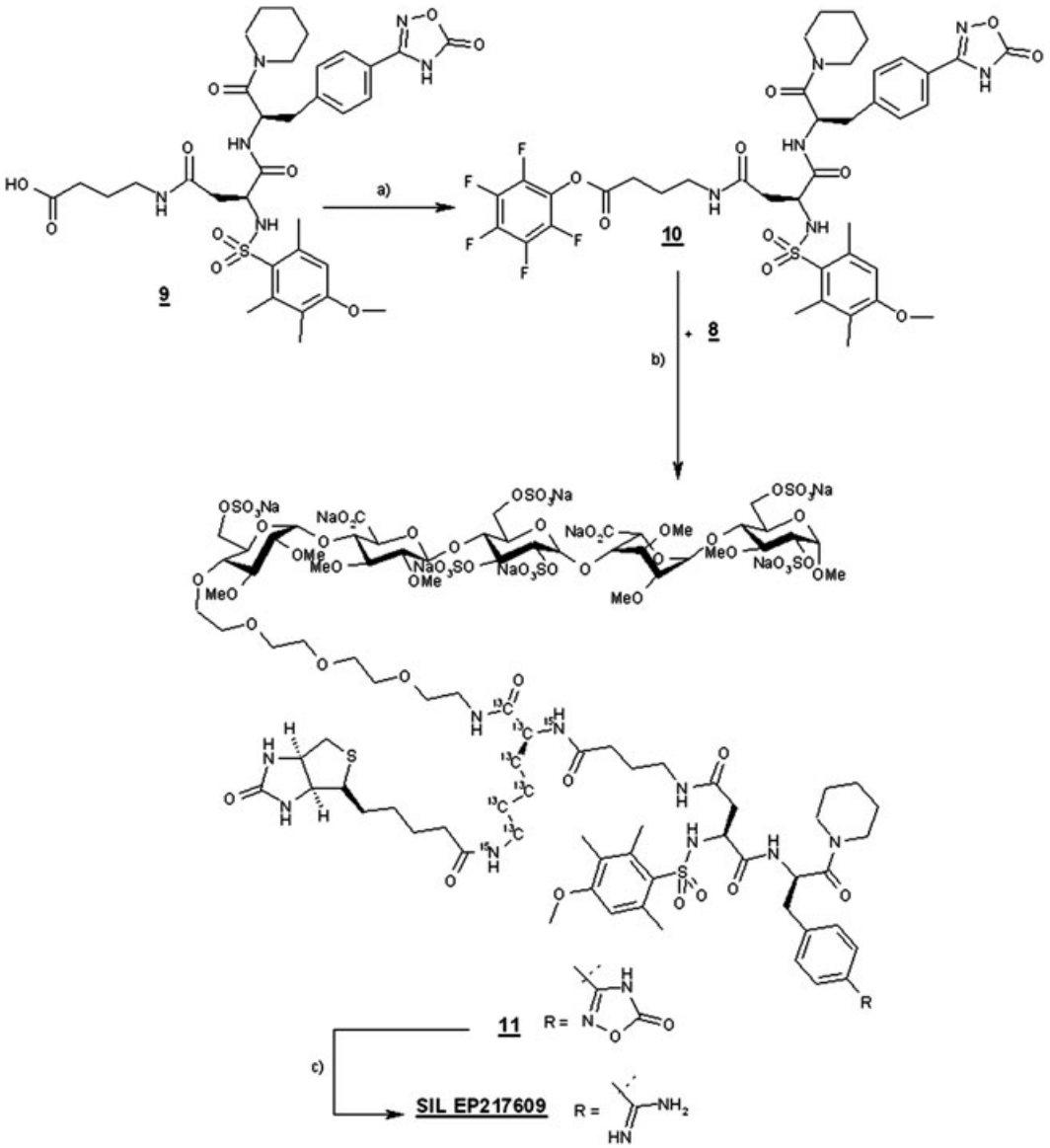
Scheme 3. (a) H_2, 10% Pd/C, H_2O, room temperature, 4 h, 100%; (b) EDCI/HOBt, DMF, room temperature, 2 h, 88%; (c) H_2, 10% Pd/C, H_2O, room temperature, 4 h (96%).

4.12 (m, 1 H), 5.20 (m, 1 H), 5.34 (s, 2 H), 6.50 (bs, 1 H), 6.61 (s, 1 H), 7.12 (bs, 1 H), 7.30 (d, 2 H, $J = 12.0$ Hz), 7.55 (d, 2 H, $J = 12.0$ Hz). ^{19}F NMR ($CDCl_3$, 400 MHz) δ −162.4 (dt, 2 F, $J = 28.57$ and 3.2 Hz), −158.1 (t, 1 F, $J = 28.57$ Hz), −152.6 (dd, 2 F, $J = 28.57$ and 3.2 Hz).

*Methyl O-2,3-di-O-methyl-4-O-«<12-N-«N$^{\varepsilon}$-(D-(+)-biotinyl)-N-<)]-{4-[[4-[[(1R)-L-[(4-[1,2,4-oxadiazol-5(2H)-one]-phenyl)methyl]-2-oxo-2-(L-piperidinyl)ethyl]amino]-3-[[(4-methoxy-2,3,6-trimethylphenyl)sulfonyl]amino]-l,4-(S)-dioxo-butyl]amino]-butanoyl}-L-($^{13}C_6$,$^{15}N_2$)lysyl»-12-aza-3,6,9-trioxa-dodecyl>»-6-O-sulfo-alpha-D-glucopyranosyl-(L->4)-O-2,3-di-O-methyl-beta-D-glucopyranuronosyl-(L->4)-O-2,3,6-tri-O-sulfo-alpha-D-glucopyranosyl-(L->4)-O-2,3-di-O-methyl-alpha-L-idopyranuronosyl-(L->4)-3-O-methyl-2,6-di-O-sulfo-alpha-D-glucopyranoside octakis sodium salt (**11**)*

To a solution of **10** (0.051 g, 0.057 mmoL) in 4 mL of anhydrous DMF, in a 5-mL round-bottomed flask, compound **8** (0.076 g, 0.035 mmoL) and diisopropyl-ethylamine (0.01 mL, 0.056 mmoL, 1.6 equivalent) were added, and the reaction mixture was stirred at 40 °C for 16 h. HPLC and TLC analyses showed complete reaction. The mixture was poured into ethyl acetate (10 mL) and stirred for 10 min at room temperature. After filtration and washing with ethyl acetate (2 mL), the solid was dissolved in water and desalted by passage through Sephadex G-25 to afford **11** (0.067 g, 67%) as a white solid. HPLC purity 92%; ESI-MS: *m/z* calcd for $C_{96}H_{145}N_{11}O_{61}S_8$, 672.9150; found, 673.1746 $[M-4H]^{4-}$; ^{1}H NMR (400 MHz, D_2O) δ 1.01–1.73 (m, 20 H), 2.04 (s, 3 H), 2.08 (t, 2 H, $J = 8.0$ Hz), 2.19 (m, 2 H), 2.25 (m, 2 H), 2.41 (s, 3 H), 2.51 (s, 3 H), 2.64 (m, 2 H), 2.70–2.99 (m, 5 H), 3.10–3.68 (m, 57 H), 3.75–3.83 (m, 8 H), 3.91 (m, 2 H), 4.02–4.10 (m, 3 H), 4.17–4.34 (m, 7 H), 4.46 (m, 2 H), 4.58 (m, 1 H), 4.70 (m, 1 H), 4,81 (m, 1 H), 4.98 (m, 1 H), 5.30 (m, 1 H), 5.37 (d, 1 H, $J = 4.0$ Hz), 6.75 (s, 1 H), 7.19 (d, 2 H, $J = 8.0$ Hz), 7.65 (d, 2 H, $J = 8.0$ Hz).

Methyl O-2,3-di-O-methyl-4-O-«<12-N-«N$^{\varepsilon}$-(D-(+)-biotinyl)-N-<)]-{4-[[4-[[(1R)-L-[[4- (aminoiminomethyl)phenyl]methyl]-2-oxo-2-(L-piperidinyl)ethyl]amino]-3-[[(4-methoxy- 2,3,6-trimethylphenyl) sulfonyl]amino]-l,4-(S)-dioxo-butyl]amino]-butanoyl}-L-($^{13}C_6$,$^{15}N_2$) lysyl»-12-aza-3,6,9-trioxa-dodecyl>»-6-O-sulfo-alpha-D-



Scheme 4. (a) Pentafluorophenol, EDCI, DMF, room temperature, 16 h, 87%; (b) DMF, 40 °C, 16 h, 67%; (c) H_2, 1% Pd/C, H_2O, room temperature, 4 d (quantitative).

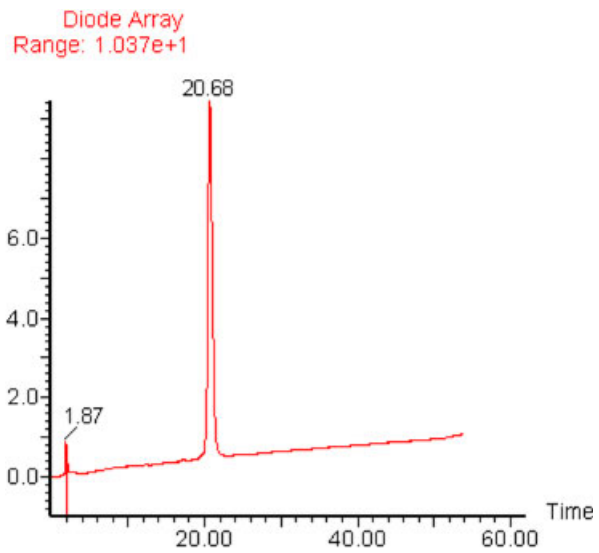


Figure 1. HPLC profile of synthetic stable-isotope-labeled EP217609.

glucopyranosyl-(L->4)-O-2,3-di-O-methyl-beta-D-glucopyranuronosyl-(L->4)-O-2,3,6-tri-O-sulfo-alpha-D-glucopyranosyl-(L->4)-O-2,3-di-O-methyl-alpha-L-idopyranuronosyl-(L->4)-3-O-methyl-2,6-di-O-sulfo-alpha-D-glucopyranoside octakis sodium salt (EP217609).

To a solution of **11** (0.017 g, 0.006 mmoL) in water (2 mL) was added 1% Pd/C (0.01 g), and the mixture was stirred at room temperature under a continuous stream of hydrogen for 4 days. HPLC analysis showed complete reaction. The catalyst was removed by filtration, and the filtrate was desalted by passage through a G-25 Sephadex column. The combined fractions of interest were pooled and concentrated. Lyophilisation afforded pure EP217609 as a white solid in quantitative yield. HPLC purity >99%; ESI-MS: *m/z* calcd for $C_{95}H_{147}N_{11}O_{59}S_8$, 662.4214; found, 662.6471 $[M-4H]^{4-}$; calcd for $C_{95}H_{148}N_{11}O_{59}S_8$, 883.5619; found, 883.8674 $[M-3H]^{3-}$; ^1H NMR (400 MHz, D_2O) δ 1.03–1.83 (m, 19 H), 1.90 (m, 2), 2.01 (s, 3 H), 2.02–2.08 (m, 4 H), 2.20–2.32 (m, 2 H), 2.40 (s, 3 H), 2.51 (s, 3 H), 2.65 (d, 1 H, $J = 17.1$ Hz), 2.76 (m, 1 H), 2.80–2.90 (m, 2 H), 2.92–3.07 (m, 2 H), 3.07–3.70 (m, 52 H), 3.70–3.88 (m, 8 H), 3.92 (m, 2 H),

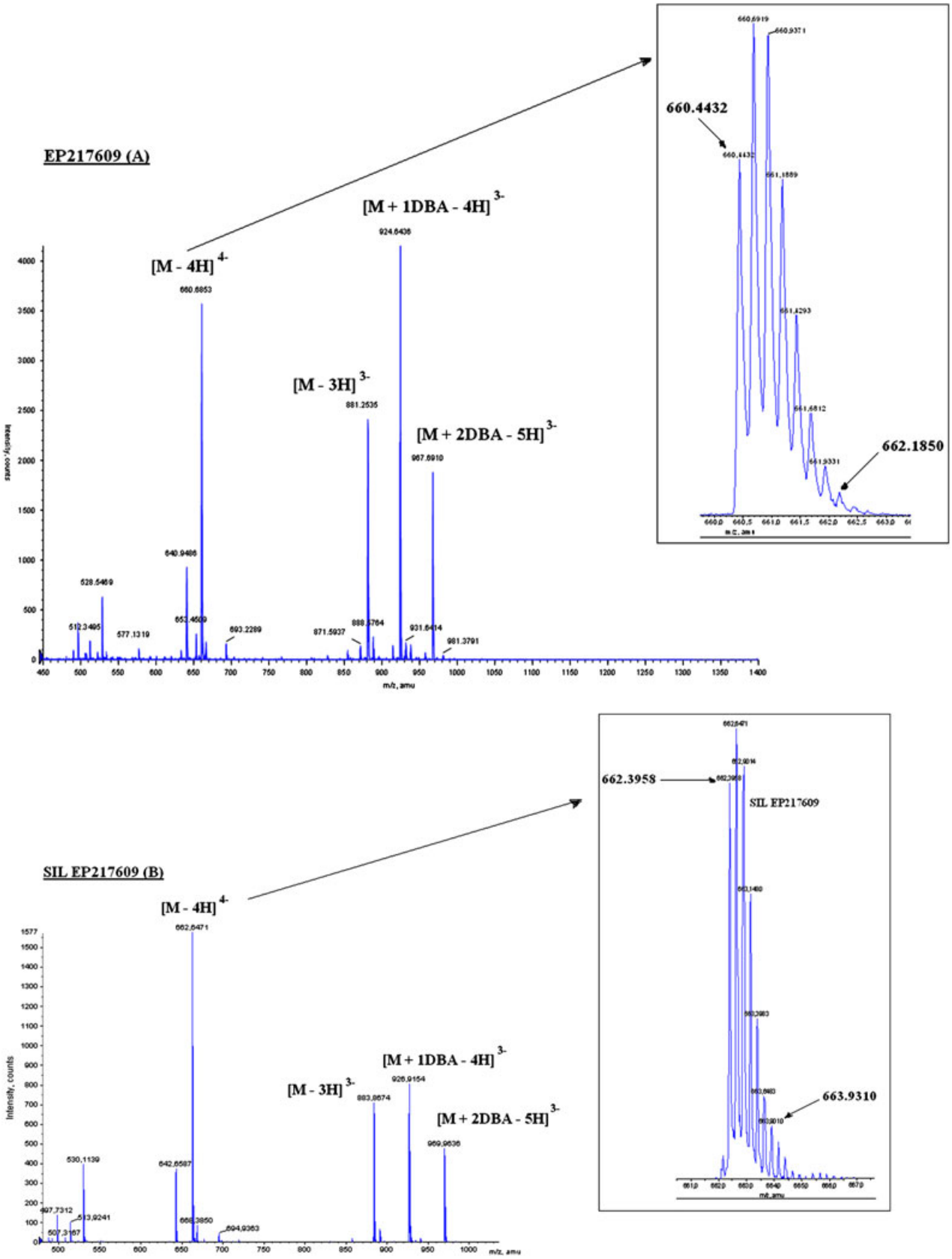


Figure 2. ESI-MS spectra of unlabeled **(A)** and labeled EP217609 **(B)** with an example of expanded region of molecular ion *m/z* $[M-4H]^{4-}$ showing peaks from 660.4432 to 662.1850 for unlabeled compound **(A)** and peaks from 662.3958 to 664.1537 for labeled compound **(B)**. DBA, dibutylamine.

3.99–4.13 (m, 5 H), 4.13–4.44 (m, 8 H), 4.48 (m, 2 H), 4.60 (m, 1 H), 4.70 (m, 1 H), 4,84 (m, 1 H), 4.98 (m, 1 H),5.25 (m, 1 H), 5.36 (m, 1 H), 6.74 (s, 1 H), 7.30 (d, 2 H, $J = 8.51$ Hz), 7.66 (d, 2 H, $J = 8.51$ Hz).

Acknowledgements

The authors thank Djamel Abderrahmani for mass spectrometry and Francisco Alvarez for NMR spectroscopy.

References

[1] M. Preininger, *Dev. Food Sci.* **1998**, *40*, 87–97.
[2] C. Rambal, C. Pachiaudi, S. Normand, J. P. Riou, P. Louisot, A. Martin, *Carbohydr. Res.* **1992**, *236*, 29–37.
[3] C. M. Chavez-Eng, M. L. Constanzer, B. K. Matuszewski, *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.* **2002**, *767*(1), 117–129.
[4] E. Buncel, J. R. Jones, *Eur. J. Med. Chem.* **1988**, *23*(1), 107.
[5] L. Wolska, M. Gdaniec-Pietryka, P. Konieczka, J. Namieśnik, *Talanta* **2009**, *78*(3), 730–735.
[6] L-C. Lin, H-Y. Wu, V. Shin-Mu Tseng, L-C. Chen, Y-C. Chang, P-C. Liao, *J. Am. Soc. Mass Spectrom.* **2010**, *21*(2), 232–241.
[7] S. Niwayama, S. Kurono, H. Cho, H. Matsumoto, *Bioorg. Med. Chem. Lett.* **2006**, *16*(23), 6054–6057.
[8] L. B. Nilsson, G. Eklund, *J. Pharm. Biomed. Anal.* **2007**, *43*(3), 1094–1099.
[9] M. G. Horning, W. G. Stillwell, J. Nowlin, K. Lertratanangkoon, D. Carroll, I. Dzidic, R. N. Stillwell, E. C. Horning, R. M. Hill, *J. Chromatogr. A* **1974**, *91*, 413–423.
[10] T. A. Baillie, J. R. Jones, Third International Symposium on the Synthesis and Applications of Isotopically Labeled Compounds, Innsbruck, Austria. *International Journal of Radiation Applications and Instrumentation. Part A. Appl. Radiat. Isot.* **1988**, *39*(6), 520.
[11] M. Palermo, C. Gomez-Sanchez, E. Roitman, C. H. L. Shackleton, *Steroids* **1996**, *61*(10), 583–589.
[12] T. A. Baillie, R. A. Halpin, B. K. Matuszewski, L. A. Geer, C. M. Chavez-Eng, D. Dean, M. Braun, G. Doss, A. Jones, T. Marks, D. Melillo, and K. P. Vyas, *Drug Metab. Dispos.* **2001**, *29*, 1614–1628.
[13] A. Valleix, S. Carrat, C. Caussignac, E. Léonce, A. Tchapla, *J. Chromatogr. A* **2006**, *1116*(1–2), 109–126.
[14] M. Petitou, V. Nancy-Portebois, G. Dubreucq, V. Motte, D. Meuleman, M. de Kort, C. A. van Boeckel, G. M. Vogel, J. A. Wisse, *Thromb. Haemost.* **2009**, *102*(5), 804–810.
[15] M. De Kort, C. A. Van Boeckel, *Patent* **2006**, WO/2006/067173
[16] C. A. Van Boeckel, C. M. Tromp, T. T. Geertsen, *Patent* **2001**, WO/2001/042262