Electronic Supplementary Material (ESI) for Chemical Communications.

Supplement information

Efficient Access to the Non-reducing End of Low Molecular Weight Heparin for Fluorescent Labeling

Zongqiang Wang, Chen Shi, Xuri Wu, Yijun Chen*

*To whom correspondence should be addressed.

E-mail: yjchen@cpu.edu.cn

Supplemental Methods

1. General procedures and instrumentation

Chemicals were purchased from sigma-Aldrich and J&K chemical company. Escherichia coli strains DH5α and BL21 (DE3) were obtained from Tiangen Biotech Co. Ltd. (Beijing, China). Analytic high performance chromatography (HPLC) was performed using LC2010A with UV detector monitoring at 232nm and fluorescent detector at 340 and 440 nm using ODS-2 hypersil column (4.6 x 250 mm, 5 μm). Mobile phase consisted of A (water) and B (acetonitrile). Samples of 10 μL were injected, and isocratic elution with 40% B was conducted for 12 min at 1 ml/min. Gas chromatography was performed using Agilent HP-6890 with Flame Ionization Detector. Compounds were analyzed using Agilent Hp-5Ms column (0.25 mm x 30 m). An initial oven temperature of 30℃ was maintained for 4 min and then programmed to raise from 30 to 35℃ at 0.5℃/min followed by a rise from 35 to 80℃ at 3℃/min, then raised to 289℃ and held for 15 min.[1] High-resolution electrospray ionization MS (HR-ESI-MS) was performed on Micromas Q-TOF Micro™, and HPLC-mass spectrometry (HPLC-MS) analysis was carried out on Agilent 1260-TOF. Nuclear magnetic resonance (NMR) data were recorded on a Bruker DRX-500 NMR spectrometer (Bruker, Germany). Fluorescent intensity was determined on TECAN Infinite M200 PRO. Infrared spectroscopy was recorded on Bruker TENSOR 27 (Bruker, Germany). Elementar Vario EL III and Melting Point Detector YRT-3 were used for elemental analysis and determination of melting points, respectively.

2. Synthesis of 2-MEAB labeled LMWH **8**

2.1 Synthesis of sulfhydryl acetaldehyde **2**

Chloroacetaldehyde (19.5 ml, 0.1 mol) was dissolved in 58.8 ml water and cooled to 0℃ by an external ice bath, 2 M Na_2CO_3 was used to adjust to pH 3. Sodium bisulfide (5.6 g, 0.16 mol) was dissolved in 33 ml water and cooled to 0℃ by an external ice bath. The sodium bisulfide solution was added to the chloroacetaldehyde solution and stirred until no further solid precipitation.[2] The precipitate was separated by filtration, washed with water and dried in vacuo. Sulfhydryl acetaldehyde **2** (14.7 g) was obtained at a yield of 75.3%. $[M+H]^+$ found 75.0, indicating a molecular formula of C_2H_4OS. The oxidized extent of compound **2** was determined to be 75% by Gas chromatography.

2.2 Synthesis of 2-(2-mercaptoethylamino)benzamide dimer **3**

Sulfhydryl acetaldehyde (2.81 g, 20 mmol) was dissolved in 120 ml anhydrous methanol, and 3Å molecular sieves was added to the solution. The suspension was stirred at room temperature for 0.5 h and then $ZnCl_2$ (0.25 g, 1 mmol) and $NaCNBH_3$ (0.50 g, 2 mmol) were added to the solution. The reaction mixture was stirred at room temperature for 16 h, and was then centrifuged at 7,500 rpm for 10 min to remove the 3 Å molecular sieves, and 140 ml of 0.1 N NaOH was added dropwise to the suspension with stirring. 2-(2-mercaptoethylamino)benzamide dimer **3** was precipitated. The precipitate was separated by filtration, and washed with 0.1 N NaOH and dried under reduced pressure to give a yield of 53.8%. HR-ESI-MS: $[M+H]^+$ 391.1348 and $[M-H]^-$ 389.1201, indicating a molecular formula of $C_{18}H_{22}N_4O_2S_2$. Melting Point: 199-201℃. Elemental analysis of **3** is shown in Table

S1. **^{1}H-NMR** (300 MHz, DMSO-d6): δ(ppm): 8.32 (s,1H), 7.81 (s,1H), 7.61 (d, J=8 Hz, 1H), 7.28 (t, J=7 Hz, 1H), 7.15 (s, 1H), 6.73 (d, J=8 Hz, 1H), 6.56 (t, J=7 Hz, 1H), 3.46 (t, J=6 Hz, 2H), 2.96 (d, J=6 Hz, 2H). (Fig S1). **^{13}C-NMR** (300 MHz, DMSO-d6): δ 171.5 ($CONH_2$), 149.1 (C-3), 132.6 (C-5), 129.1 (C-7), 114.2&114.1 (C6&C8), 110.9 (C-4), 40.9 (C-2), 36.9 (C-1) (Fig S2).

2.3 Synthesis of 2-(2-mercaptoethylamino)benzamide **4**

2-(2-mercaptoethylamino)benzamide dimer (200 mg, 0.51 mmol) was dissolved in DMSO (4 ml). DL-dithiothreitol (157 mg, 1.02 mmol) was added to the solution, and the reaction mixture was stirred at room temperature for 20 min. The mixture was added to reversed phase silica gel column and eluted with 30% acetonitrile. HPLC analysis was performed to determine each fraction, and fractions containing the product were collected and rotary evaporated to remove acetonitrile. Residual water was removed by lyophilization to give 2-(2-mercaptoethylamino)benzamide **4** at a yield of 76.6%. HR-ESI-MS: $[M+H]^+$ 197.0740 and $[M-H]^-$ 195.0653, indicating a molecular formula of $C_9H_{12}N_2OS$. Melting point: 189-191℃. Element analysis of **4** is shown in Table S1. **^{1}H-NMR** (500 MHz, DMSO-d6): δ(ppm): 8.30 (s,1H), 7.78 (s,1H), 7.58 (d, J=8 Hz, 2H), 7.24 (t, J=7.5 Hz, 1H), 7.10 (s, 1H), 6.68 (d, J=8 Hz, 1H), 6.52 (t, J=7 Hz, 1H), 3.30 (m, J=7 Hz, 2H), 2.65 (t, J=6.5 Hz, 2H), 2.33 (s, 1H) (Fig S4).**^{13}C-NMR** (500 MHz, DMSO-d6): δ 171.4 ($CONH_2$), 149.2 (C-3), 132.5 (C-5), 129.1 (C-7), 114.2&114.1 (C6&C8), 111.0 (C-4), 45.3 (C-2), 2 3.3(C-1) (Fig S5).

2.4 Synthesis of LMWH benzethonium salt

LMWH sodium salt (100 mg, 0.02 mmol) was dissolved in 1ml water in flask A and

mixed by stirring. Benzethonium chloride (250 mg, 0.56 mmol) was dissolved in 3 ml deionized water in flask B. Then, the solution of flask B was added dropwise to flask A. The mixture was stirred for 1 h at room temperature and kept at room temperature for another hour to allow precipitation. The supernatant was discarded and then replaced with the same volume of deionized water (2.5 ml). The mixture was stirred for additional 15 min. The supernatant was discarded and then replaced with the same volume of deionized water (2.5 ml). The resulting mixture was stirred for 15 min and then filtered. The precipitate was washed for three times with 10 ml of deionized water. Residual water was removed by lyophilization to give 300 mg LMWH benzethonium salt.

2.5 Synthesis of LMWH benzyl ester **6**

LMWH benzethonium salt (1.18 g) was dissolved in CH_2Cl_2 (46.3 ml). Benzyl chloride at 1.1 g/ml (9.83 ml, 0.08 mmol) was added, and the mixture was stirred at 30℃ for 24 h. Then, 160 ml of 10% sodium acetate in methanol was added. The precipitate was filtered, washed for three times with 50 ml methanol, and then lyophilized to give 400 mg LMWH benzyl ester **6**.

2.6 Labeling **4** to LMWH benzyl ester **6**

LMWH benzyl ester **6** (45 mg) was dissolved in 10ml formamide at 50℃. 2-MEAB **4** (58.8 mg, 0.3 mmol) and boric acid (18.6 mg, 0.3 mmol) were then added. The mixture was stirred at 50℃ for 24 h, and then centrifuged at 12,000 rpm for10 min. The supernatant was dialyzed extensively against deionized water (MWCO 500) to remove unreacted 2-(2-mercaptoethylamino)benzamide **4** and formamide, and then

lyophilized to yield 40 mg 2-MEAB labeled LMWH benzyl ester **7**.

2.7 Synthesis of 2-MEAB labeled LMWH **8**.

2-MEAB labeled LMWH benzyl ester **7** (100 mg) was dissolved in 3 ml deionized water. The solution was heated to 60℃, and 0.1 N NaOH was added. The mixture was stirred at 60℃ for 1 h. Then, the reaction mixture was allowed slowly to reach room temperature. 1 N HCl was used to adjust to approximately pH 6.0, and 300 mg NaCl was added. Subsequently, 9 ml methanol was then added dropwise with stirring. The precipitate was filtered and washed for three times with 5 ml methanol, and then dried under reduce pressure. Finally, 44 mg of 2-MEAB labeled LMWH **8** was obtained.

3 Labeling of LMWH with double fluorophores

2-MEAB labeled LMWH **8** (12 mg) and sodium cyanoborohydride (25 mg, 0.40mmol) were dissolved in 560 μl deionized water, and mixed with a solution of 2-aminoacridone (12mg, 0.06 mmol) dissolved in 158 μl of acetic acid-DMSO (3:17). The mixture was incubate at 37℃ for 16 h and then dialyzed against deionized water (MWCO 500) for 24 h to remove unreacted 2-aminoacridone, and then lyophilized. Finally, 10 mg of double fluorophore labeled LMWH was obtained.

4 Expression and purification of Heparinase II.

Recombinant hepainase II was overexpressed in *Escherichia coli* BL21(DE3) cells (Novagen) and purified by column chromatography on Hi-trap-FF (GE-Healthcare) and SP-FF (GE-Healthcare) as previously described[3]. Briefly, the gene encoding heprinase II was synthesized and cloned in pET28a vector, and then transformed to *E.*

coli BL21(DE3). One liter cultures were grown at 15℃ in LB medium supplemented with 40 μg/ml kanamycin, and protein expression was induced by addition of 0.5 mM isopropyl 1-thio- β -D-galactopyranoside at OD_{600} of 0.9. The cultures were allowed to grow for 8~12 h at 37℃ prior to harvest. The cells were harvested by centrifugation and disrupted using high pressure cell disruption (Constant Systems, UK), and the supernatant was obtained by centrifugation. Heparinase II was purified using two chromatographic steps, including His-trap-FF (GE-Healthcare) and SP-FF (GE-Healthcare). The purified protein was concentrated to 3 mg/ml by ultrafiltration using a centricon YM-100 concentrator (Millipore Corp., USA) in 50 mM sodium phosphate buffer (pH 7.4). The purified protein was examined by 12% SDS-PAGE with commassie blue staining.

5 Heparinase II assay by FRET

A 100 μl portion of 2-MEAB-LMWH-2-AMAC at 1 mg/ml in 50 mM sodium phosphate buffer (pH 7.4) was added to each well in a 96-well assay plate (black color with flat bottom, Corning Inc, USA), and 100 μl of purified heparinase II (3 mg/ml) was then added to each well. The plate was incubated at 37℃ for 24 h. The HTRF value in each well was measured using a Victor multilabel counter.

Supplemental Table and Figures

Table S1. Element Analysis of 2-(2-mercaptoethylamino)benzamide dimer **3** and 2-(2-mercaptoethylamino)benzamide **4**

	2-(2-mercaptoethylamino) benzamide **3**	2-(2-mercaptoethylamino) benzamide dimer **4**
N (%)	11.36	13.26
C (%)	48.34	50.59
H (%)	5.67	5.49

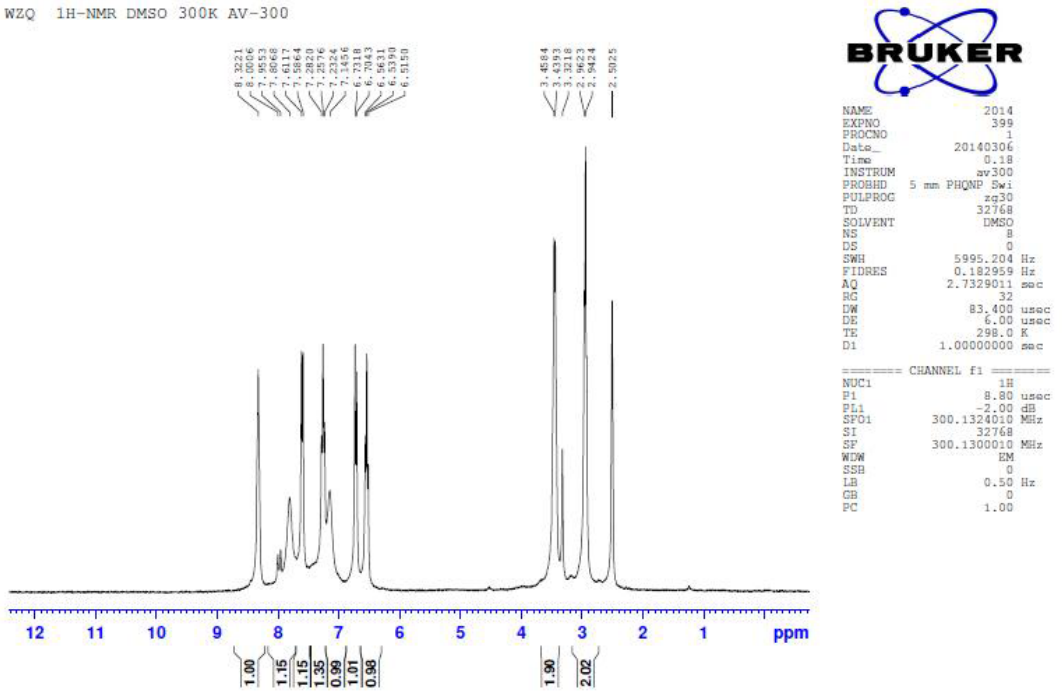

Figure S1. [1]H-NMR of 2-(2-mercaptoethylamino)benzamide dimer **3** in DMSO-d6 at room temperature.

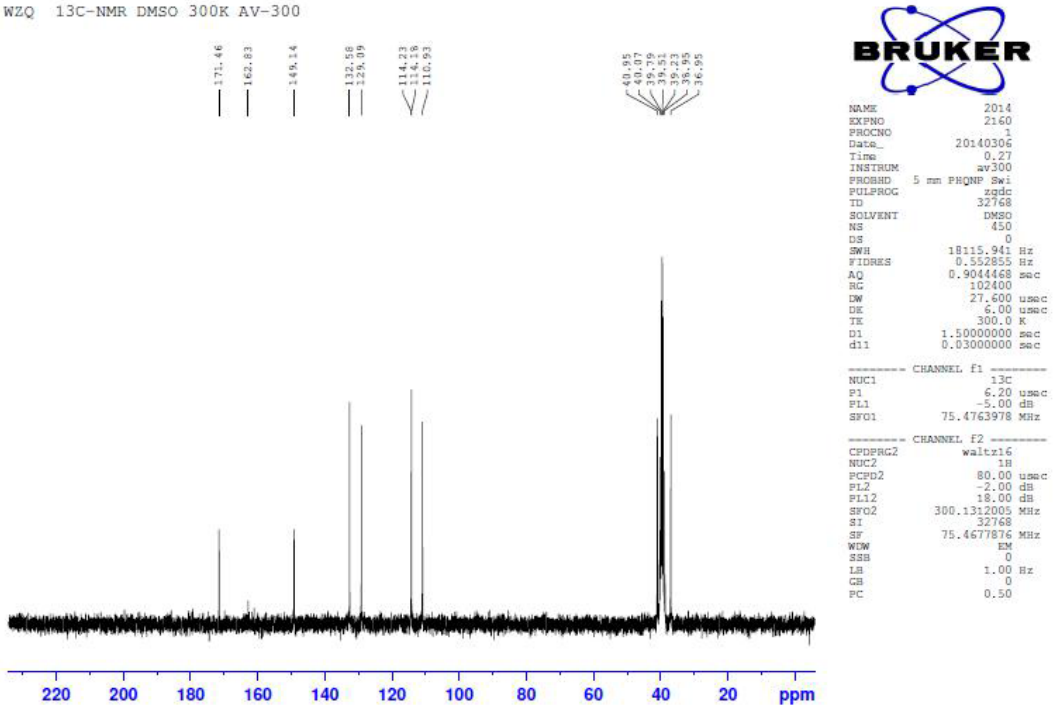

Figure S2. [13]C-NMR spectrum of 2-(2-mercaptoethylamino)benzamide dimer **3** in DMSO-d6 at room temperature.

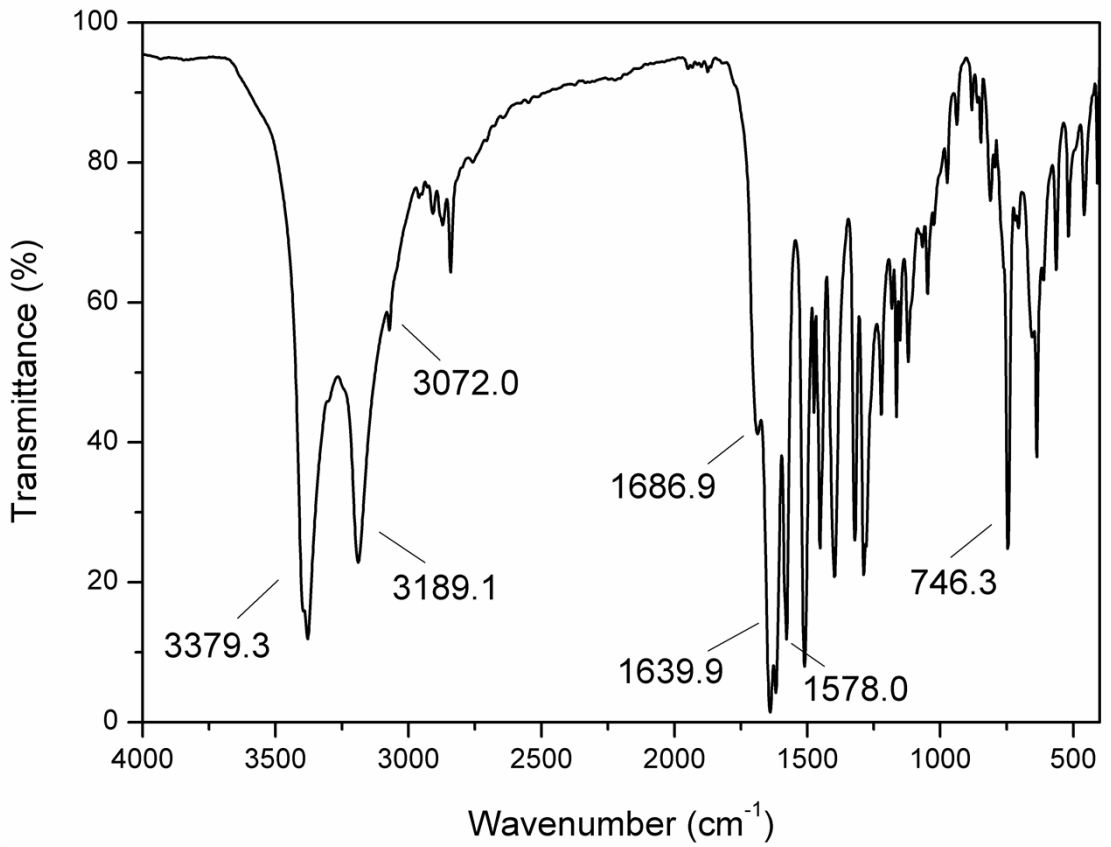

Figure S3. IR Spectrum of 2-(2-mercaptoethylamino)benzamide **3**. $CONH_2$: $\nu_{(N-H)}$= 3379.3 cm^{-1} and 3189.1 cm^{-1}; $\nu_{(C=O)}$=1686.9 cm^{-1}; $\delta_{(N-H)}$ =1639.9 cm^{-1}; C_6H_4: $\nu_{(C-H)}$= 3072.0 cm^{-1}; $\nu_{(C=C)}$=1578.0 cm^{-1}; δ=$_{(C-H)(o-)}$=746.3 cm^{-1}.

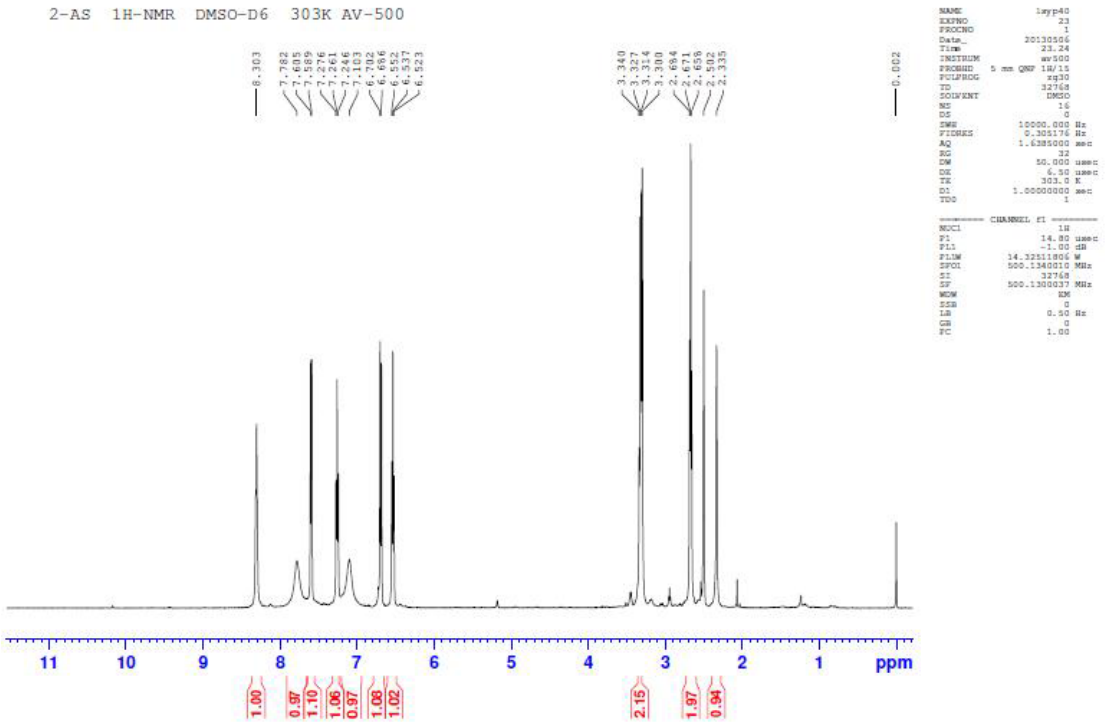

Figure S4. [1]H-NMR of 2-(2-mercaptoethylamino)benzamide **4** in DMSO-d6 at room temperature.

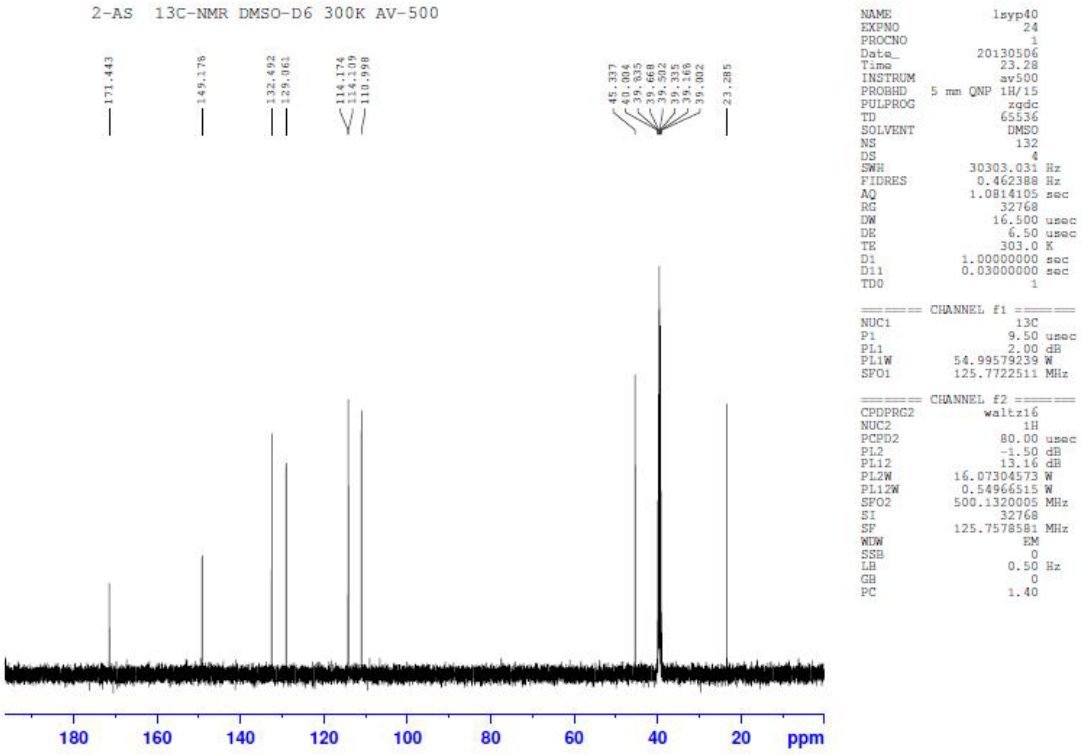

Figure S5. [13]C-NMR spectrum of 2-(2-mercaptoethylamino)benzamide **4** in DMSO-d6 at room temperature.

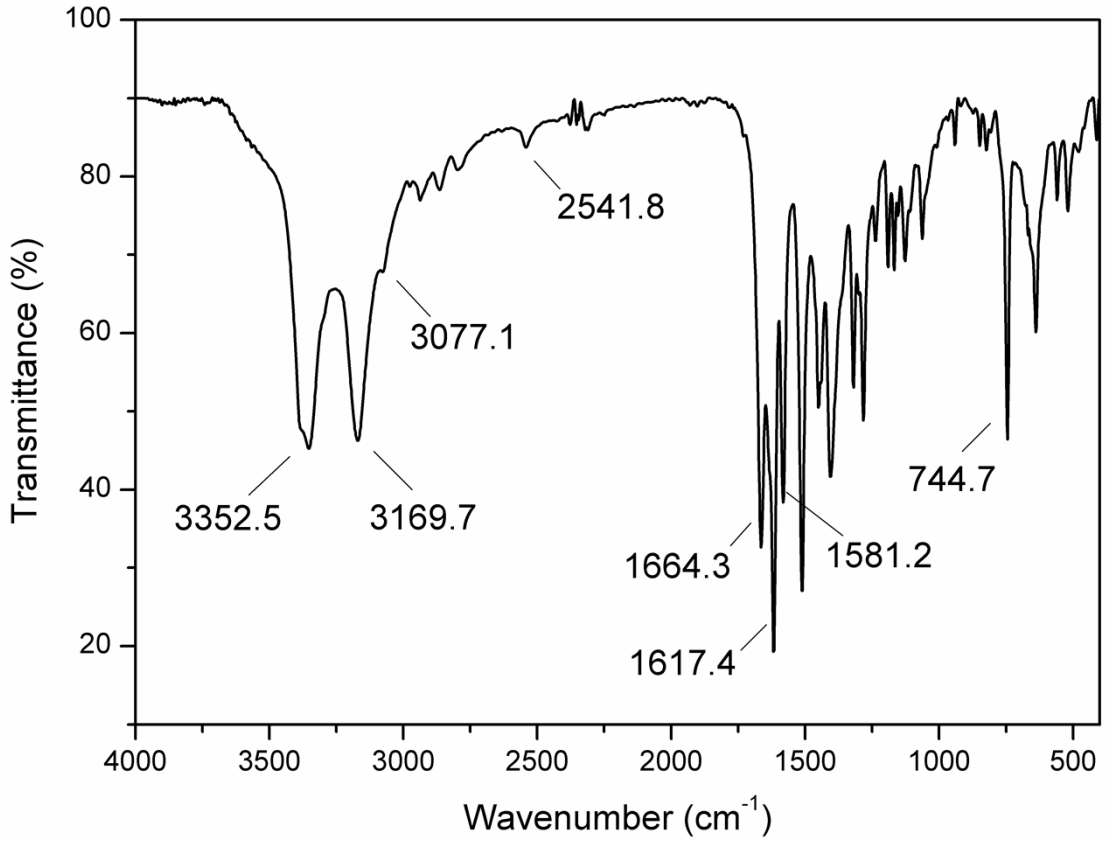

Figure S6. IR Spectrum of 2-(2-mercaptoethylamino)benzamide **4**. $CONH_2$: $\nu_{(N-H)}$= 3352.5 cm^{-1} and 3169.7 cm^{-1}; $\nu_{(C=O)}$=1664.3 cm^{-1}; $\delta_{(N-H)}$ =1617.4 cm^{-1}; SH: $\nu_{(S-H)}$=2541.8 cm^{-1}; C_6H_4: $\nu_{(C-H)}$=3077.1 cm^{-1}; $\nu_{(C=C)}$=1581.2 cm^{-1}; δ=$_{(C-H)(o-)}$=744.7 cm^{-1}.

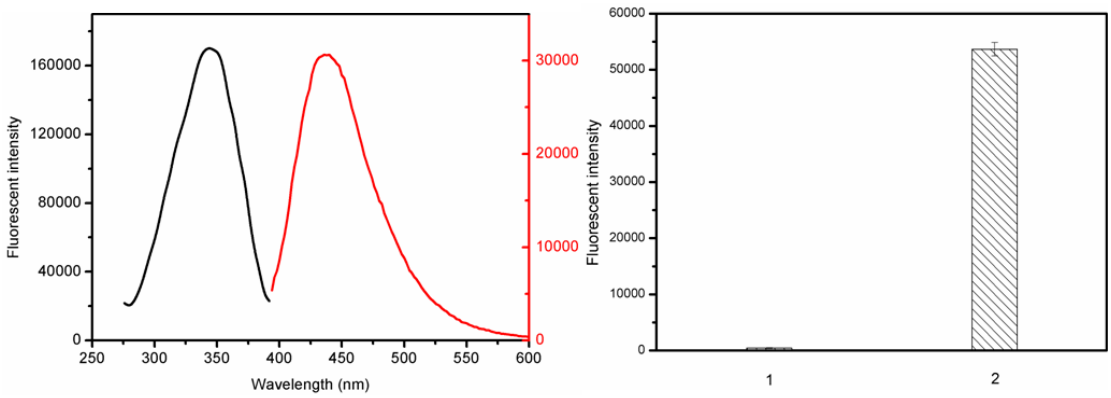

Figure S7. The excitation and emission wavelengths of 2-(2-mercaptoethylamino)benzamide **4 (a)** and the fluorescent intensity of LMWH labeled 2-MEAB (column 2) compared to LMWH (column 1) at same concentration of 1 mg/ml **(b)**.

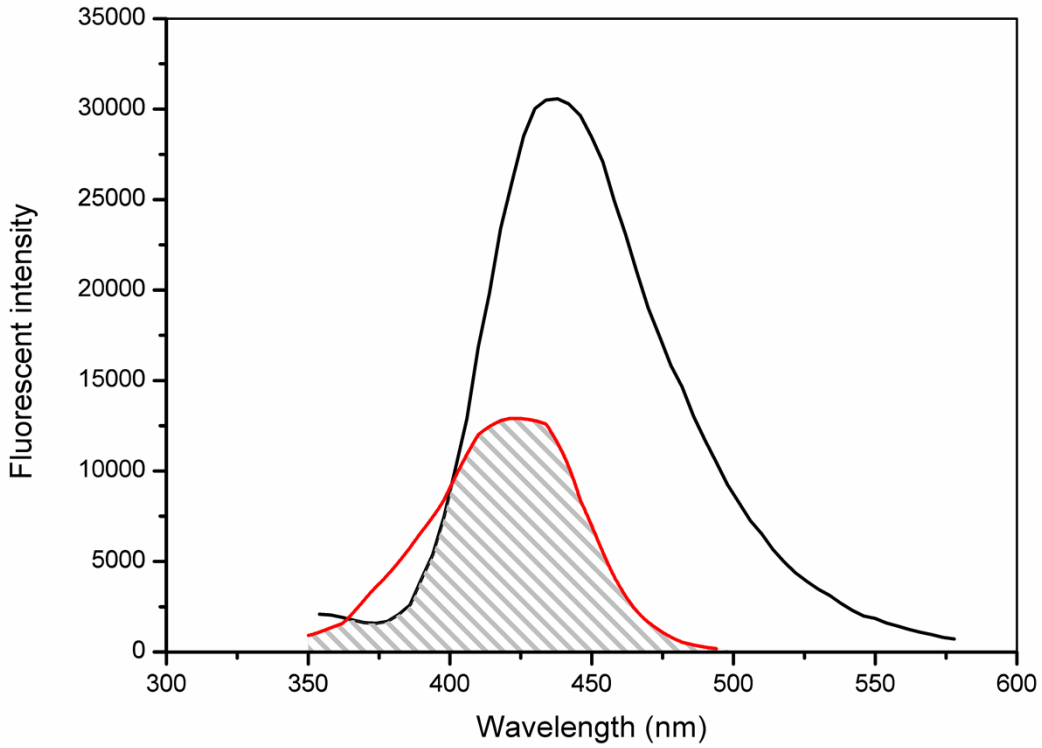

Figure S8. Overlay between emission wavelength of 2-MEAB and excitation wavelength of 2-aminoacridone. Black line is emission wavelength of 2-MEAB, and red line is the excitation wavelength of 2-AMAC. The gray shadow is the overlap of the two wavelengths.

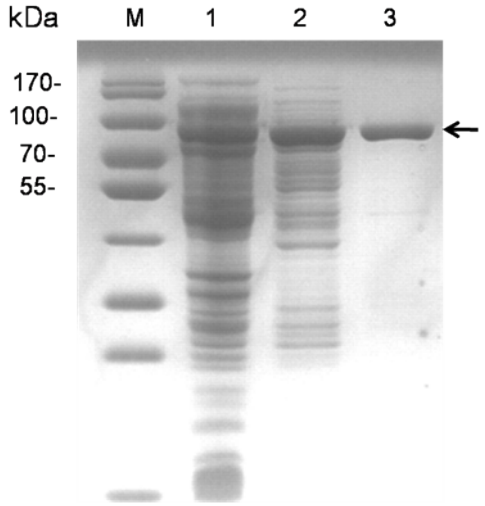

Figure S9. SDS-PAGE of purified recombinant heparinase II. Lane 1: crude extracts; Lane 2: after Histrap-FF column; Lane 3: after SP-FF column.

Supplemental References:

1. C. Vermeulen, S. Collin. *J. Arg. Food. Chem*., 2002, 50, 5654-5659.
2. A. P. Gaywood, H. McNab. *J. Org. Chem*., 2009, 74, 4278-4282.
3. D.Shaya, A. Tocilj, Y.Li, J. Myette, G. Venkataraman, R. Sasisekharan, and M. Cygler. *J. Biol. Chem*, 2006, 281, 15525-15535.