Supplementary information

A fluorescent probe for the quantification of heparin in clinical samples with minimal matrix interference

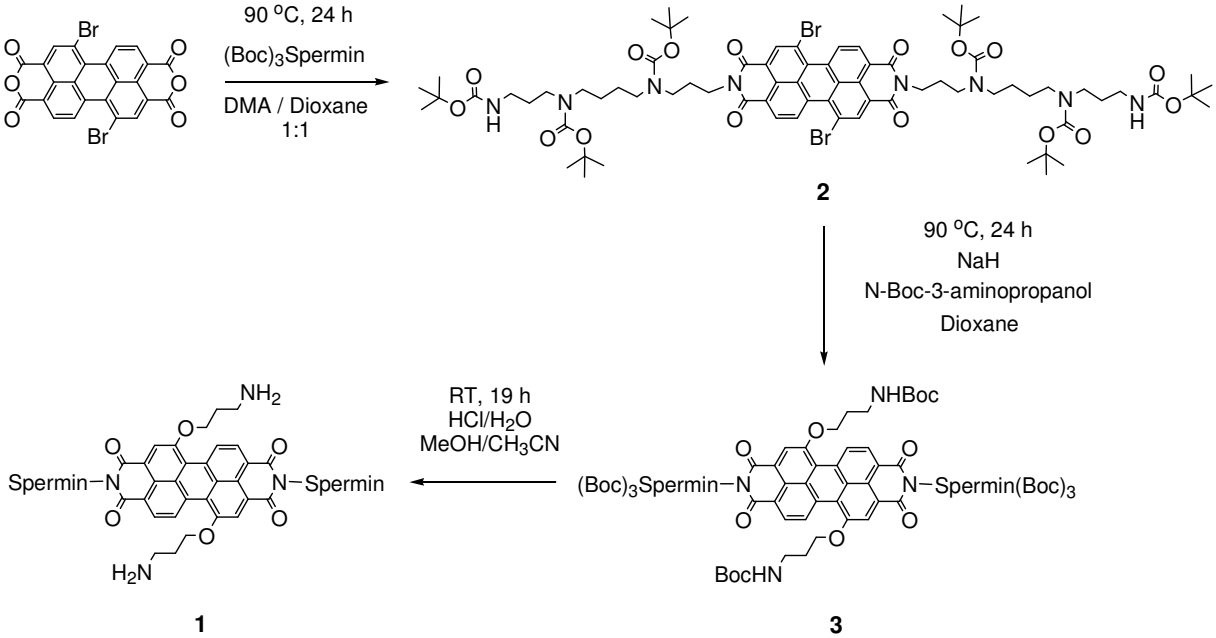
Helga Szelke,[1] Sarah Schübel,[1] Job Harenberg,[2] Roland Krämer[1]

[1]Universität Heidelberg, Anorganisch-Chemsiches Institut, Im Neuenheimer Feld 270, 69120 Heidelberg, Germany; [2]Universität Heidelberg, Medizinische Fakultät Mannheim, Institut für Experimentelle und Klinische Pharmakologie und Toxikologie, Maybachstr. 14, 68169 Mannheim

Experimental

Commercially available chemicals from Sigma-Aldrich (Germany) were used without purification. (N^1, N^4, N^9-tri-*tert*-butoxycarbonyl)-1,12-diamino-4,9-diazadodecane[S1] and 1,7-dibromoperylene-3,4,9,10-tetracarboxylic acid[S2, S3] were synthesized following literature methods. The latter was obtained as a 4:1 mixture of the 1,7- and 1,6-dibromo isomers [S3] and was used in the following steps without further purification. NMR spectra were obtained on Bruker Avance 200, 400 or 600 MHz spectrometers, chemical shifts are given in ppm relative to Me_4Si, and coupling constants are given in Hz. ESI mass spectra were measured on a Q-TOF Ultima ESI-MS instrument (Waters-Micromass). Semipreparative and analytical HPLC was performed on a Shimadzu liquid chromatograph equipped with a UV-visible detector and a column oven. Fluorescence spectra were acquired on a Varian Cary Eclipse fluorescence spectrophotometer, UV-Vis experiments were performed on a Varian Cary 100 Bio UV-Vis spectrophotometer using 1 cm optical path disposable PMMA cuvettes with a sample volume of 2 mL. Fluorescence titration experiments were performed using a portable fluorimeter (Nordantec Trilogy), equipped with a filter system for detection of fluorescence above 665 nm on excitation at 485 nm using disposable PMMA cuvettes with a sample volume of 1 mL.
Low molecular weight heparin (sodium salt from porcine intestinal mucosa, molecular weight given as 4000-6000) was obtained from Sigma-Aldrich, product number H8537. A mean molecular weight 5000 of this product was confirmed by polyacrylamide gel electrophoresis in a recent study.[S4] An anti-factor Xa activity of 140 IU/mg of this LMWH sample was determined using the S2222 chromogenic assay (Chromogenix, Milano, Italy) in combination with a Dynatech MR 7000 Spectrophotometer. Assays were calibrated using a reference standard of known anti-Xa activity.
Venous blood samples were obtained by clean vein puncture into serum separator tubes for serum collection or plastic vials containing 3.8% sodium citrate (1/9, v/v, citrate/blood) for plasma collection. Blood samples for serum preparation were allowed to clot for 30 minutes, then centrifuged at 1800 x g for 15 min. Samples for plasma preparation were centrifuged immediately at 1800 x g for 10 min. Both sample types were shock frozen and stored at -25°C.

Synthesis of 1.



Scheme S1. Synthesis of **1**

_N, N'_-Bis-(N^1, N^4, N^9-tri-tert-butoxycarbonyl-12-amino-4,9-diazadodecyl)-1,7-dibromoperylene-3,4:9,10-tetracarboxydiimide (2)

A mixture of 1,7-dibromo perylene-3,4,9,10-tetracarboxylic acid[S2] (0.66 g, 1.2 mmol) and (N^1, N^4, N^9-tri-*tert*-butoxycarbonyl)-1,12-diamino-4,9-diazadodecane (1.4 g, 2.8 mmol) in *N*, *N'*-dimethylacetamide (DMA, 10 mL) and 1,4-dioxane (10 mL) was stirred at 90°C for 24 hours. Upon addition of water (20 mL) a red precipitate was obtained that was washed with water, filtered off and dried in vacuum. This raw product was purified by column chromatography (silica gel, 1 % MeOH in CH_2Cl_2) to give the product as a mixture of the 1,7- and 1,6-dibromo isomers (0.42 g, 23 %). The two isomers could not be separated economically in this synthetic step, only a small amount (28 mg) of the major **2a** isomer could be isolated by repeated chromatography. As the ^{1}H NMR signals of the minor (1,6) isomer are superimposed with those of the major (1,7) isomer, their ratio could not be determined accurately at this stage. ^{1}H NMR (600 MHz, $CDCl_3$) δ 1.42 (br s, 54 H, CH_3), 1.45-1.51 (m s, 4 H, CH_2), 1.62-1.69 (m, 8 H, CH_2), 1.95-1.99 (m, 4 H, CH_2), 3.01-3.36 (m, 20 H, CH_2), 4.18-4.22 (m, 4 H, CH_2), 5.31 (br s, 2 H, CO-N*H*), 8.68 (d, 2 H, *J* = 7.0, Ar), 8.89 (s, 2 H, Ar), 9.47 (d, 2 H, *J* = 7.2, Ar); MS (ESI$^+$) m/z $[H\mathbf{2}]^+_{found}$ 1519.4, $[H\mathbf{2}]^+_{calc}$ 1519.6 (calculated for monoprotonated **2**, $C_{74}H_{103}Br_2N_8O_{16}$); UV/Vis ($CH_2Cl_2$) λ_{max} (ε / $M^{-1}\cdot cm^{-1}$) = 391 nm (7015), 460 nm (15640), 490 nm (33760) 528 nm (47910); λ_{em} = 548, 582 (sh) nm.

_N, N'_-Bis-(N^1, N^4, N^9-tri-tert-butoxycarbonyl-12-amino-4,9-diazadodecyl)-1,7-bis-(N^1-tert-butoxycarbonyl-3-hydroxypropyl)-perylene-3,4:9,10-tetracarboxydiimide (3)

A mixture of *tert*-butyl N-(3-hydroxypropyl)carbamate (0.60 g, 3.42 mmol) and sodium hydride (2.6 mg, 0.07 mmol) stirred at RT for 20 minutes. Then **2** (40 mg, 0.03 mmol) in 1,4-dioxane (2.5 mL) was added to the reaction mixture and stirred at 90°C for 19 hours. After removal of solvent the remaining oil was dissolved in a minimum amount of ether and the product was precipitated with pentane. This raw product was purified by column chromatography (silica gel, 2 % MeOH in CH_2Cl_2) to afford the product as a purple solid (8.8 mg, 20 %). A ~2:1 ratio of the 1,7- and 1,6-dialkoxy isomers was determined by integration of selected ^{1}H NMR signals of the perylene core (see Figure S1). **3a** (5.3 mg, 17 %) was prepared as described above using **2a** as a starting material. (1,7)-isomer (major): ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.37-1.49 (m, 72 H, CH_3), 1.38-1.52 (m, 4 H, CH_2), 1.61-1.70 (m, 4 H, CH_2), 1.94-2.05 (m, 4 H, CH_2), 2.23-2.34 (m, 4 H, CH_2), 2.99-3.35 (m, 20 H, CH_2), 3.37-3.49 (m, 4 H, CH_2), 4.15-4.22 (m, 4 H, CH_2), 4.43-4.51 (m, 4 H, CH_2), 4.96 (br s, 2 H, CO-N*H*), 5.33 (m, 4 H, CO-N*H*), 8.28 (s, 2 H, Ar), 8.54 (d, 2 H, *J* = 8.2, Ar), 9.48 (d, 2 H, *J* = 8.4, Ar); (1,6)-isomer (minor): ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.37-1.49 (m, 72 H, CH_3), 1.38-1.52 (m, 4 H, CH_2), 1.61-1.70 (m, 4 H, CH_2), 1.94-2.05 (m, 4 H, CH_2), 2.23-2.34 (m, 4 H, CH_2), 2.99-3.35 (m, 20 H, CH_2), 3.37-3.49 (m, 4 H, CH_2), 4.15-4.22 (m, 4 H, CH_2), 4.43-4.51 (m, 4 H, CH_2), 4.96 (br s, 2 H, CO-N*H*), 5.33 (m, 4 H, CO-N*H*), 8.30 (s, 2 H, Ar), 8.62 (d, 2 H, *J* = 8.4, Ar), 9.42 (d, 2 H, *J* = 8.8, Ar); UV/Vis (CH_2Cl_2) λ_{max} (ε / $M^{-1}\cdot cm^{-1}$) = 400 nm (3940), 493 nm (sh), 530 nm (17090) 571 nm (24490); λ_{em} = 595, 632 (sh) nm.

All three ^{1}H NMR signals of the perylene core H atoms are downfield shifted by ~0.3 ppm compared with a previously reported 1,7-di(dodecyloxy) substituted perylene diimide (9.14 d, J=8.4; 8.28 d, J=8.4; 7.92 s)[S5] in $CDCl_3$, claimed to be isolated as a pure isomer.[S6] Note that comparable shifts of perylene ^{1}H signals have been reported to result solely from variations of the concentration of perylene diimides.[S7]

_N_, _N_'-Bis-(12-amino-4,9-diazadodecyl)-1,7-bis-(3-hydroxypropyl)-perylene-3,4:9,10-tetracarboxydiimide (1)

A solution of **3** (8.8 mg, 5 µmol) in 10 mL of methanol-acetonitrile (1:1) was treated with 3 mL of 16 % hydrochloric acid for 3 hours at room temperature. After removal of solvent under reduced pressure the remaining solid was taken up in methanol and precipitated with acetone to afford the product as a purple solid in the form of the salt (H_8**1**)Cl_8 (5.9 mg, 98 %). HR-MS (ESI$^+$) m/z $[H\mathbf{1}]^+_{found}$ 907.5561, $[H\mathbf{1}]^+_{calc}$ 907.5558 (calculated for monoprotonated **1**, $C_{50}H_{71}N_{10}O_6$). Elemental analysis calcd (%) for $C_{51}H_{88}Cl_8N_{10}O_{10}$ (**1**·8HCl·3H_2O·MeOH): C 47.67, H 6.90, N 10.90; found: C 47.96, H 6.58, N 10.67; UV/Vis (H_2O) λ_{max} (ε / $M^{-1}\cdot cm^{-1}$) = 405 nm (7500), 542 nm (36500), 576 nm (37700); λ_{em} = 615 nm.

A ~2:1 ratio of the 1,7- and 1,6-dialkoxy isomers was determined by integration of selected ^{1}H NMR signals of the perylene core (Figure S1) and was confirmed by analytical HPLC (Figure S2). A highly enriched sample of isomer **1a** was obtained by removal of the Boc protecting groups of **3a** (5.3 mg), as described for **3** to afford the product (H_8**1a**)Cl_8 (3.3 mg, 91 %). A ~8:1 ratio of the 1,7- and 1,6-dialkoxy isomers was determined by integration of selected ^{1}H NMR signals (Figure S1) and was confirmed by analytical HPLC.

(H_8**1a**)Cl_8 (1,7-isomer, major): ^{1}H NMR (600 MHz, D_2O) δ 1.64-1.70 (m, 8 H, CH_2), 1.91-1.95 (m, 4 H, CH_2), 2.06-2.10 (m, 4 H, CH_2), 2.35-2.40 (m, 4 H, CH_2), 2.93-3.03 (m, 16 H, CH_2), 3.10-3.13 (m, 4 H, CH_2), 3.22-3.25 (m, 4 H, CH_2), 4.16-4.21 (m, 4 H, CH_2), 4.60-4.62 (m, 4 H, CH_2), 8.50 (s, 2 H, Ar), 8.55 (d, 2 H, J = 8.4, Ar), 9.52 (d, 2 H, J = 8.4, Ar);

(H_8**1b**)Cl_8 (1,6-isomer, minor): ^{1}H NMR (600 MHz, D_2O) δ 1.64-1.70 (m, 8 H, CH_2), 1.91-1.95 (m, 4 H, CH_2), 2.06-2.10 (m, 4 H, CH_2), 2.35-2.40 (m, 4 H, CH_2), 2.93-3.03 (m, 20 H, CH_2), 3.10-3.13 (m, 4 H, CH_2), 4.16-4.21 (m, 4 H, CH_2), 4.60-4.62 (m, 4 H, CH_2), 8.40 (s, 2 H, Ar), 8.65 (d, 2 H, J = 8.4, Ar), 9.46 (d, 2 H, J = 8.4, Ar).

^{1}H NMR spectra of 3, 3a, 1 and 1a

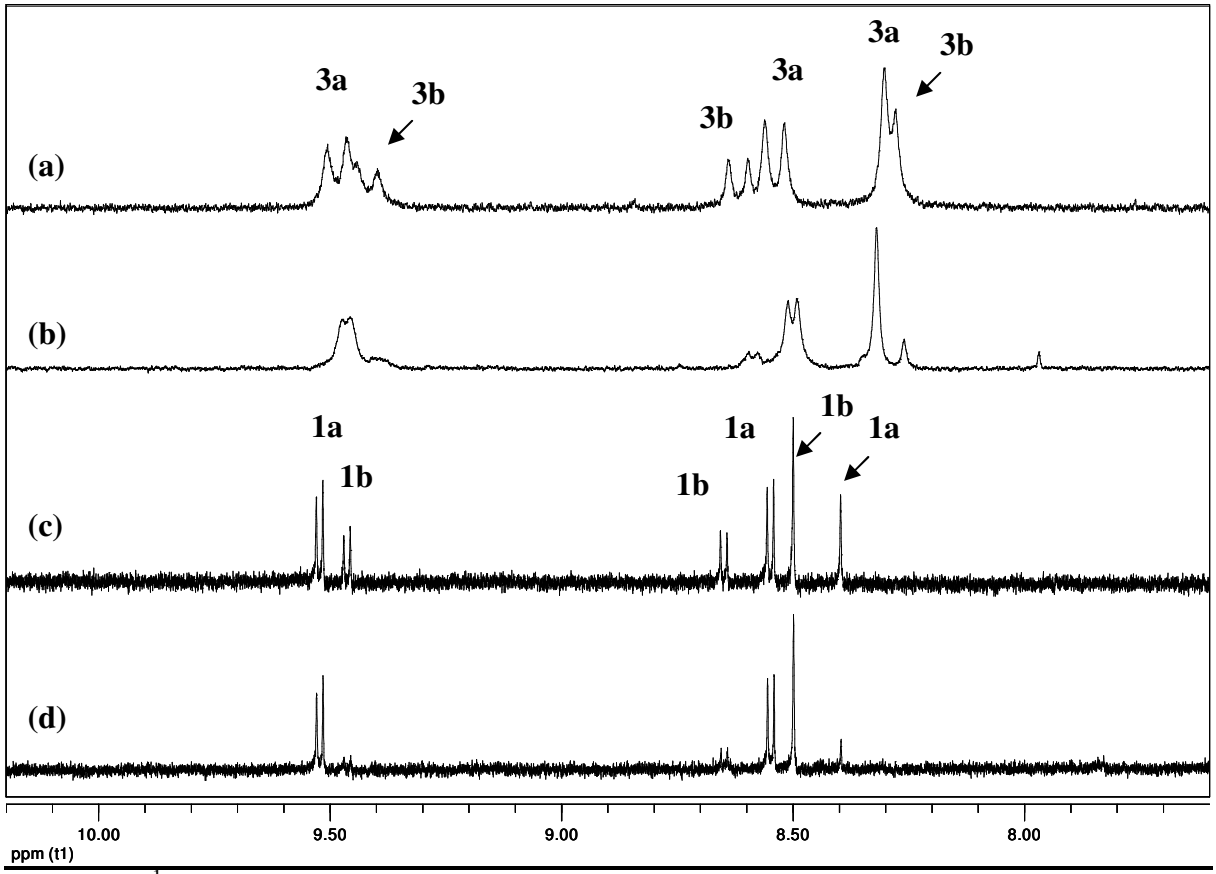
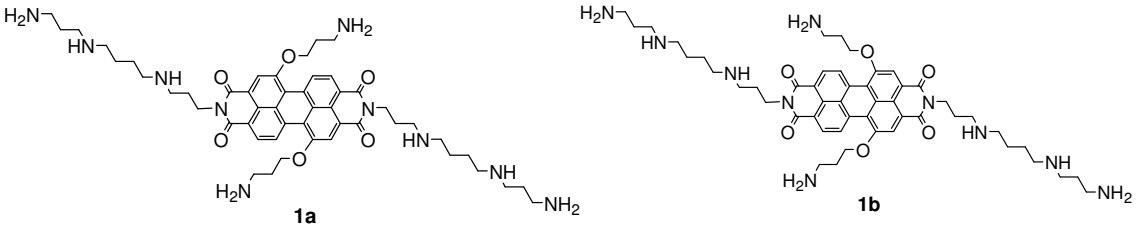


Figure S1. ^{1}H NMR signals of the perylene core H atoms of (a) Boc-protected precursor **3**, signals attributed to a 2:1 mixture of **3a** and **3b** (200 MHz, 10 mM in $CDCl_3$); (b) enriched sample of **3a** (400 MHz, 5 mM in $CDCl_3$); (c) **1**, signals attributed to a 2:1 mixture of **1a** and **1b** (600 MHz, 0.1 mM in D_2O) and (d) isolated regioisomer **1a** (600 MHz, 0.1 mM in D_2O).

HPLC separation, visible and fluorescence spectra of isomers 1a and 1b



Scheme S2. Structure of the two isomers of **1**

Small quantities of the isomers could be isolated by semipreparative HPLC using a Macherey-Nagel Nucleosil C4 250 x 4.6 mm column with gradients of CH_3CN (solvent B, containing 0.1% trifluoroacetic acid) in water (solvent A, containing 0.1% trifluoroacetic acid): 25 ^{o}C, 0 % B for 5 min, in 30 min to 35 % B, in 10 min to 90 % B, 90 % B for 10 min. R_t (**1a**)= 24.4 min, R_t (**1b**)= 25.6 min. HR-MS (ESI^+) m/z $[H_2\mathbf{1a}]^{2+}_{found}$ 454.2813, $[H_2\mathbf{1b}]^{2+}_{found}$ 454.2814, $[H_2\mathbf{1}]^{2+}_{calc}$ 454.2813 (calculated for diprotonated **1**, $C_{25}H_{36}N_5O_3$).

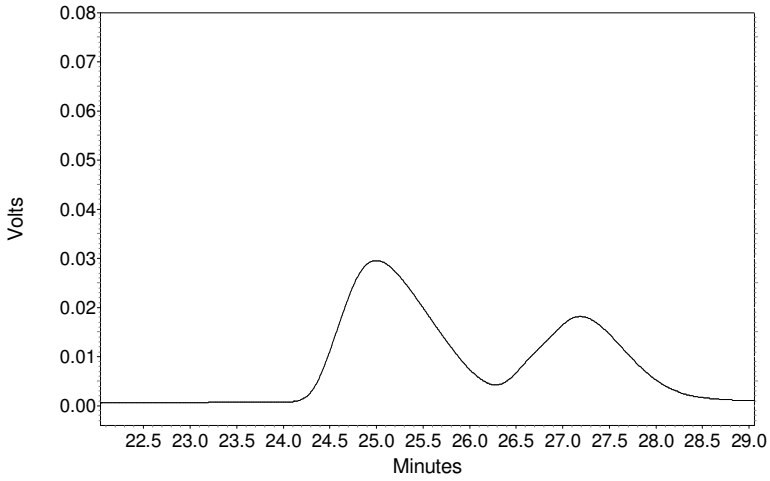


Figure S2. HPLC elution profile of **1** (2:1 mixture of **1a** and **1b**)

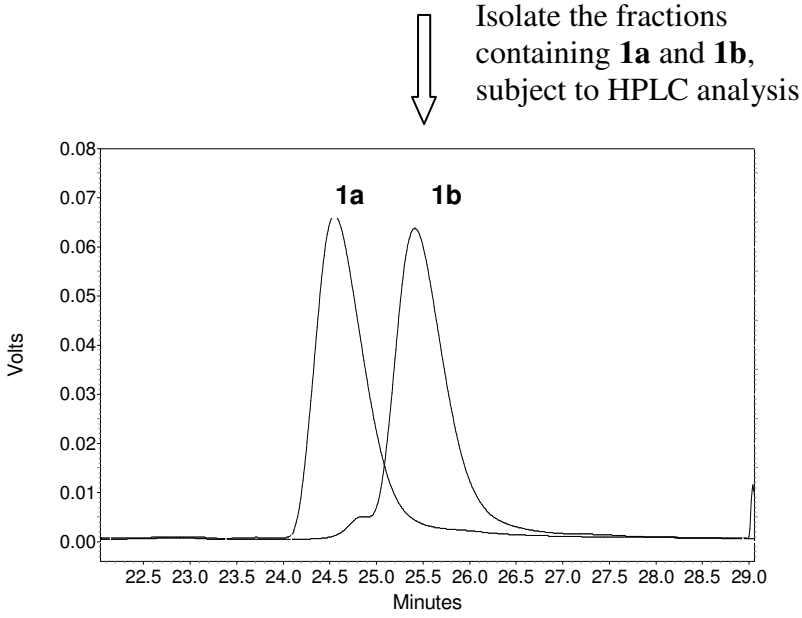


Figure S3. The two HPLC elution profiles of isolated **1a** (solid) and **1b** (dashed) fractions. Re-mixing the isolated fractions and HPLC analysis of the mixture results in an elution profile comparable to that in figure S2.

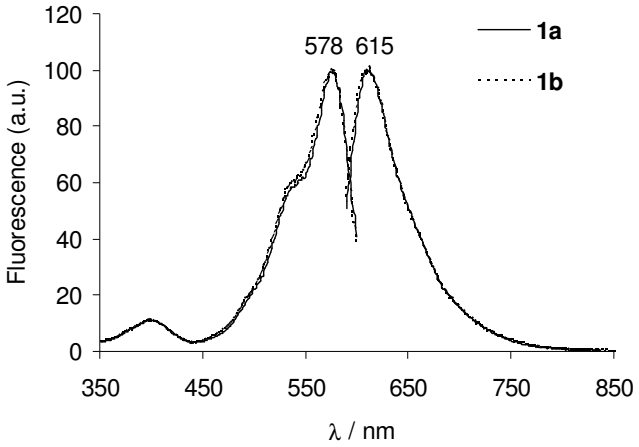


Figure S4. HPLC separated fractions of **1a** and **1b** (trifluoroacetate salts) were taken up in water and solutions adjusted to the same 578 nm absorbance level. The figure compares excitation spectra (for λ_{em} = 615 nm, curves on the left) and, respectively, emission spectra (for λ_{ex} = 578 nm, curves on the right) of **1a** and **1b**. The shape of fluorescence emission band is independent of emission wavelength in the range 350-580 nm. To our knowledge, we describe here for the first time the photophysical properties of a dialkoxy-1,6-isomer of a perylene diimide which are found to be very similar to those of the corresponding 1,7-isomer. The previously described 1,7- isomer of a di(dodecyloxy) substituted perylene diimide [S5] has a fluorescence emission maximum at 597 nm in $CHCl_3$.

Response of 1, 1a and 1b to heparin

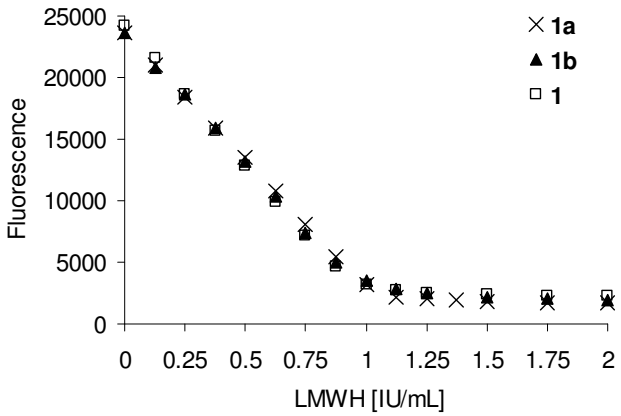


Figure S5. Comparison of the fluorescence response of **1** (as obtained from synthesis), **1a** and **1b** (HPLC fractions) to LMWH. Concentration of **1** is 1 µM. Solutions of **1a** and **1b** were adjusted to have the same 485 nm absorbance as the 1 µM solution of **1**. Water with 20 vol% human blood serum, pH 7.0 (buffer 60 mM MOPS), I ≈ 0.2 M (200 mM sodium tosylate), T = 20°C. Excitation at 485 nm, emission recorded >665 nm.

In view of the very similar response of **1**, **1a** and **1b** to heparin, we consider a separation of the isomers unnecessary in the context of heparin quantification.

References

[S1] A. J. Geall, I. S. Blagbrough, *Tetrahedron,* 2000, **56**, 2449.

[S2] (a) U. Rohr, P. Schlichting, A. Bohm, M. Gross, K. Meerholz, C. Brauchle, *Angew. Chem. Int. Ed.,* 1998, **37**, 1434. (b) A. Bohm, H. Arms, G. Henning, P. Blaschka, US Patent 6,143,905, 2000.

[S3] F. Würthner, V. Stepanenko, Z. Chen, C. R. Saha-Möller, N. Kocher, D. Stalke, *J. Org. Chem.*, 2004, **69**, 7933.

[S4] H. S. Garg, H. Mrobat, L. Yu, C. Freeman, B. Li, F. Zhang, R. J. Linhardt, C. A. Hales, *Carbohydrate Res*., 2008, **343**, 2406.

[S5] C. Zhao, Y. Zhang, R. Li, X. Li, J. Jiang, *J. Org. Chem*., 2007, **72**, 2402

[S6] W. Su, Y. Zhang, C. Zhao, X. Li, J. Jiang, *ChemPhysChem*, 2007, **8**, 1857.

[S7] A. D. Shaller, W. Wang, H. Gan, A. D. Q. Li, *Angew. Chem. Int Ed*., 2008, **47**, 7705.