Water-Soluble Pyrene Tags Enable the Detection of Carbohydrates by Label-Assisted Laser Desorption/ionisation Mass Spectrometry

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Matrix-assisted laser desorption/ionisation mass spectrometry (MALDI-MS) is widely used for the analysis of biomolecules. Label-assisted laser desorption/ionisation mass spectrometry (LALDI-MS) is a matrix-free variant of MALDI-MS, in which only analytes covalently attached to a laser desorption/ionisation (LDI) enhancer are detected. LALDI-MS has shown promise in overcoming limitations of MALDI-MS in terms of sample preparation and MS analysis. In this work, we have developed water-soluble pyrene-based LDI reagents (LALDI tags) that can be used for (in situ) labelling and LALDI-MS analysis of reducing carbohydrates from complex (biological) samples without the need for additional chemical derivatisation or purification. We have systematically explored the suitability of four pyrene-based LDI enhancers and three aldehyde-reactive handles, optimised sample preparation, and exemplified the use of a LALDI tag by the detection of lactose in cow’s milk. These results demonstrate that LALDI-MS is a promising technique for the analysis of reducing carbohydrates in biological samples, and pave the way for the development of LALDI-MS for glycomics and diagnostics.
Supplementary Information for:

**Water-soluble pyrene tags enable the detection of carbohydrates by label-assisted laser desorption/ionisation mass spectrometry**

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1.1 $N$-methylation reduces sensitivity of a 1-amidopyrene LDI enhancer.

Supplementary Figure 1: a) Chemical structure of $N$Me-1-APy S1. b) Recorded data for $N$Me-1-APy S1 i) UV-Vis, ii) LALDI-MS, iii) Determination of limits of detection of compound S1 by LALDI-MS. Bars represent the mean values for the sum of all positive mode ions related to S1, including the molecular ions (M$^+$), cationised molecules ([M+Na]$^+$, [M+K]$^+$), and major fragments ([M-CO]$^+$) detected by LALDI-MS of decreasing sample amounts. Error bars represent the standard deviation, where $n = 3$ (analysis of three individual spots from the same compound solution). c) i) Proposed conformational structure of $N$-methyl-1-amidopyrene S1. The pyrene ring is twisted out of the plane of the amide group to prevent steric clashing between the $N$-methyl group/carbonyl oxygen and the pyrenyl protons. ii) A schematic representation of the molecular orbitals in a planar and twisted conformation of an $N$-methyl-$N$-phenyl amide. The diagram shows that in the twisted conformation the molecular orbitals of the amide are orthogonal to the aromatic $\pi$-system so delocalisation cannot occur.
1.2 Poor reproducibility of LALDI-MS using ground-steel target plates may be due to ‘coffee ring’ effects during sample drying.

Supplementary Figure 2: a) LALDI-MS analysis of the lower detection limit for 1 (blue), 2 (green), and 3 (purple) using a ground-steel MALDI target plate. Bars represent the mean values for the sum of all positive mode ions related to each LALDI reagent, including the molecular ions (M**), cationised molecules ([M+Na]**, [M+K]**), and major fragments (2: [M-ketene]**, 3: [M-CO]**) detected by LALDI-MS of decreasing sample amounts. Error bars represent the standard deviation, where n = 3 (analysis of three individual spots from the same compound solution). b) SEM imaging of a ground-steel MALDI MTP 384 target plate ground steel spotted with 100 pmol (left) and 10 fmol (right) sample of 3. The
dark grey areas indicate where 3 has been deposited. c) EDAX elemental mapping analysis of the 10 fmol sample of 3. Dark grey vertical lines in the image show the grooves in the ground steel target plate. The trace overlaid on the magnified image of the plate’s surface shows the levels of carbon (purple) and oxygen (green) detected between the start and end points of the elemental analysis (blue crosshairs).
1.3 Evidence for the loss of CO from 1-APy derivatives in the MALDI source.

| Observed m/z | Proposed molecular formula | Expected monoisotopic mass (Da) | Mass accuracy (ppm) |
|--------------|----------------------------|---------------------------------|---------------------|
| 539.2523     | C_{30}H_{37}NO_8^{\ddagger\ddagger} | 539.2514                        | 1.67                |
| 451.1998     | C_{26}H_{23}NO_6^{\ddagger\ddagger} | 451.1989                        | 1.99                |
| 433.1891     | C_{26}H_{22}NO_5^{\ddagger\ddagger} | 433.1884                        | 1.62                |
| 407.1736     | C_{24}H_{21}NO_4^{\ddagger\ddagger} | 407.1727                        | 2.21                |
| 389.1630     | C_{24}H_{21}NO_4^{\ddagger\ddagger} | 389.1622                        | 2.06                |
| 363.1473     | C_{22}H_{19}NO_3^{\ddagger\ddagger} | 363.1465                        | 2.20                |
| 345.1368     | C_{22}H_{19}NO_3^{\ddagger\ddagger} | 345.1359                        | 2.61                |
| 319.1211     | C_{20}H_{15}NO_3^{\ddagger\ddagger} | 319.1203                        | 2.51                |
| 301.1105     | C_{20}H_{15}NO_2^{\ddagger\ddagger} | 301.1097                        | 2.66                |
| 275.0948     | C_{18}H_{13}NO_2^{\ddagger\ddagger} | 275.0941                        | 2.54                |

**Supplementary Figure 3**: CID product ion analysis of M^{\ddagger\ddagger}(3) (m/z 539.2523) (100 pmol). a) CID product ion spectrum of M^{\ddagger\ddagger}(3) with identified fragments annotated with their observed m/z. The precursor ion (m/z 539.2523) has been annotated with an asterisk (*). b) Proposed assignments for signals observed in (a). Bonds at which proposed fragmentations occur are indicated by blue dashed lines and are accompanied by the corresponding calculated monoisotopic mass for the fragment. Table shows the proposed molecular formulae, with the expected monoisotopic masses and mass accuracies, for each of the observed m/z peaks. Note that [M-28]\ddagger\ddagger was not detected in the CID product ion analysis of M^{\ddagger\ddagger}(3).
Supplementary Figure 4: LALDI-MS spectrum of 1-amidopyrene S2 (100 pmol) with the corresponding signals for the \( M^{+} \) (m/z 289.1099) and \([M-28]^{+}\) (m/z 261.1150) ions highlighted. These data suggest that \([M-28]^{+}\) results from a loss of CO from S2.
Observed m/z | Proposed molecular formula | Expected monoisotopic mass (Da) | Mass accuracy (ppm)
--- | --- | --- | ---
289.1089 | C_{19}H_{15}NO_{2}^{+} | 289.1097 | 0.77
258.0915 | C_{18}H_{12}NO^{-} | 258.0913 | 0.44
229.0887 | C_{17}H_{11}N^{+} | 229.0886 | 0.46
218.0965 | C_{16}H_{12}N^{+} | 218.0964 | 0.46

**Supplementary Figure 5**: a) CID product ion analysis of M***(S2) (m/z 289.1097) (100 pmol) with identified fragments annotated with their observed m/z. The parent ion (m/z 539.2523) has been annotated with an asterisk (*). b) Proposed assignments for signals observed in the CID product ion spectrum of M***(S2). Bonds at which proposed fragmentations occur are indicated by blue dashed lines and are accompanied by the corresponding calculated monoisotopic mass for the fragment. Table shows the proposed molecular formulae, with the expected monoisotopic masses and mass accuracy, for each of the observed m/z peaks. Note that [M-28]** was not detected in the CID product ion analysis of M***(S2).
| Observed m/z | Proposed molecular formula | Expected monoisotopic mass (Da) | Mass accuracy (ppm) |
|-------------|----------------------------|---------------------------------|--------------------|
| 261.1148    | C₁₈H₁₅NO⁺⁺                 | 261.1148                        | 0.00               |
| 230.0966    | C₁₇H₁₂N⁺                   | 230.0964                        | 0.87               |
| 229.0087    | C₁₇H₁₁N⁺⁺                  | 229.0086                        | 0.44               |
| 228.0810    | C₁₆H₁₂N⁺                   | 228.0808                        | 0.88               |

**Supplementary Figure 6**: CID product ion analysis of [M-28]⁺⁺(S₂) (m/z 289.1097) (100 pmol). a) CID product ion spectrum of [M-28]⁺⁺(S₂) with identified fragments annotated with their observed m/z. The precursor ion (m/z 261.1148) has been annotated with an asterisk (*). b) Proposed structure of [M-28]⁺⁺(S₂) with proposed assignments for signals observed in the CID product ion spectrum. Bonds at which proposed fragmentations occur are indicated by blue dashed lines and are accompanied by the corresponding calculated monoisotopic mass for the fragment. Table shows the proposed molecular formulae, with the expected monoisotopic masses and mass accuracy, for each of the observed m/z peaks. Note that the observed fragment with m/z 230.0966 is consistent with the loss of a methoxy radical, suggesting that the structure of [M-28]⁺⁺(S₂) is as shown in (b).
1.4 LALDI mass spectra of unreacted LALDI tags.

Supplementary Figure 7: LALDI-MS analysis of PyU-OEG-ONH₂ 6. a) LALDI mass spectrum of PyU-OEG-ONH₂ 6 (100 pmol) with identified fragments and cationised products annotated with their observed m/z. Inlaid spectra show expansion of the full spectrum at m/z 200-350 and m/z 520-545. b) Proposed assignments for fragments and cationised products observed in the LALDI mass spectrum. Bonds at which proposed fragmentations occur are indicated by blue dashed lines and are accompanied by the corresponding calculated monoisotopic mass for the fragment. Table shows the proposed molecular formulae, with the expected monoisotopic masses and mass accuracies, for each of the observed m/z peaks.
Supplementary Figure 8: LALDI-MS analysis of PyU-OEG-2AB 8. a) LALDI mass spectrum of PyU-OEG-2AB 8 (100 pmol) with identified fragments and cationised products annotated with their observed m/z. Inlaid graph shows expansion of the full spectrum at m/z 375-550. b) Proposed assignments for fragments and cationised products observed in the LALDI mass spectrum. Bonds at which proposed fragmentations occur are indicated by blue dashed lines and are accompanied by the corresponding calculated monoisotopic mass for the fragment. Table shows the proposed molecular formulae, with the expected monoisotopic masses and mass accuracy, for each of the observed m/z peaks.

| Observed m/z | Proposed molecular formula | Expected monoisotopic mass (Da) | Mass accuracy (ppm) |
|--------------|----------------------------|---------------------------------|--------------------|
| 681.2686     | C_{36}H_{42}KN_{4}O_{7}^+  | 681.2685                        | 0.15               |
| 665.2940     | C_{36}H_{42}N_{4}NaO_{7}^+ | 665.2946                        | -0.90              |
| 643.3132     | C_{36}H_{43}N_{4}O_{7}^+   | 643.3126                        | 0.93               |
| 524.2755     | C_{29}H_{38}N_{3}O_{6}^+   | 524.2755                        | 0.00               |
| 448.2055     | C_{20}H_{31}N_{3}NaO_{7}^+ | 448.2054                        | 0.22               |
| 426.2235     | C_{20}H_{32}N_{2}O_{7}^+   | 426.2235                        | 0.00               |
| 400.2442     | C_{19}H_{34}N_{2}O_{6}^+   | 400.2442                        | 0.00               |
| Observed m/z | Proposed molecular formula | Expected monoisotopic mass (Da) | Mass accuracy (ppm) |
|-------------|----------------------------|---------------------------------|--------------------|
| 634.2735    | C_{32}H_{41}N_3NaO_5^+     | 634.2735                        | 0.00               |
| 611.2837    | C_{32}H_{41}N_3O_9^{2+}    | 611.2837                        | 0.00               |
| 583.2889    | C_{31}H_{41}N_3O_8^{2+}    | 583.2888                        | 0.17               |
| 580.2541    | C_{32}H_{38}NO_8^+         | 580.2541                        | 0.00               |
| 539.2513    | C_{30}H_{37}NO_8^{2+}      | 539.2514                        | -0.19              |
| 525.2721    | C_{30}H_{37}NO_7^{2+}      | 525.2721                        | 0.00               |
| 511.2564    | C_{29}H_{37}NO_7^{2+}      | 511.2565                        | -0.2               |
| 301.1210    | C_{19}H_{15}N_2O^{2+}      | 301.1210                        | 0.00               |
| 289.1210    | C_{18}H_{15}N_2O^{2+}      | 289.1210                        | 0.00               |
| 274.1101    | C_{18}H_{14}N_2O^{2+}      | 274.1101                        | 0.00               |

Supplementary Figure 9: LALDI-MS analysis of 1-APy-OEG-Hz 9. a) LALDI mass spectrum of 1-APy-OEG-Hz 9 (100 pmol) with identified fragments and cationised products annotated with their observed m/z. Inlaid spectra show expansion of the spectrum at m/z 250-320 and m/z 510-640. b) Proposed assignments for fragments and cationised products observed in the LALDI mass spectrum. Bonds at which proposed fragmentations occur are indicated by blue dashed lines and are accompanied by the corresponding calculated monoisotopic mass for the fragment. Table shows the proposed molecular formulae, with the expected monoisotopic masses and mass accuracy, for each of the observed m/z peaks.
**Supplementary Table 1**: Summary of LALDI-MS analysis of LALDI tag reagents 3.5, 3.16 and 3.23. MS stability was assessed on the relative abundance of the molecular ion (M⁺) and cationised species ([M+Na]⁺, [M+K]⁺) compared to the fragments, degree of fragmentation, and ease of fragment assignment then scored using an arbitrary scale from + to −.

| LALDI tag reagent | Detected as molecular ion | MS stability | Limit of detection (pmol) |
|-------------------|---------------------------|--------------|--------------------------|
| PyU-OEG-ONH₂ 6    | Yes                       | +/-          | 10                       |
| PyU-OEG-2AB 8     | Yes                       | +            | 10                       |
| 1-APy-OEG-Hz 9     | Yes                       | –            | 100                      |
1.5 LALDI mass spectra of LALDI tag-labelled lactose derivatives.

| Observed m/z   | Proposed molecular formula | Expected monoisotopic mass (Da) | Mass accuracy (ppm) |
|----------------|-----------------------------|--------------------------------|---------------------|
| 917.3309       | C_{42}H_{38}KN_{2}O_{18}^+ | 917.3316                       | -0.76               |
| 901.3570       | C_{42}H_{38}N_{2}NaO_{18}^+ | 901.3577                       | -0.78               |
| 878.3673       | C_{42}H_{38}N_{2}O_{18}^{++} | 878.3679                       | -0.68               |
| 850.3723       | C_{41}H_{38}N_{2}O_{17}^{++} | 850.3730                       | -0.82               |
| 820.3618       | C_{40}H_{36}N_{2}O_{16}^{++} | 820.3624                       | -0.73               |
| 716.3146       | C_{38}H_{34}N_{2}O_{13}^{++} | 716.3151                       | -0.70               |
| 562.2408       | C_{30}H_{28}NNaO_{8}^+      | 562.2411                       | -0.53               |
| 539.2511       | C_{30}H_{32}NO_{8}^{++}     | 539.2514                       | -0.56               |
| 511.2562       | C_{29}H_{28}NO_{7}^{++}     | 511.2565                       | -0.59               |
| 495.2249       | C_{28}H_{33}NO_{7}^{++}     | 495.2252                       | -0.61               |
| 481.2457       | C_{28}H_{33}NO_{6}^{++}     | 481.2459                       | -0.42               |
| 467.2300       | C_{27}H_{33}NO_{6}^{++}     | 467.2302                       | -0.43               |
| 302.1175       | C_{20}H_{16}NO_{2}^{+}      | 302.1176                       | -0.33               |
| 217.0886       | C_{16}H_{11}N^{+}           | 217.0888                       | -0.92               |

Supplementary Figure 10: LALDI-MS analysis of LALDI tag-labelled lactose 10. a) LALDI mass spectrum of LALDI tag-labelled lactose 10 (100 pmol) analysed as a pure sample; identified fragments and cationised products are annotated with their observed m/z values. Inlaid spectra show expansion of the spectrum at m/z 460-500 and m/z 810-920. b) Chemical structure of LALDI tag-labelled lactose 10 with proposed assignments for fragments and cationised products observed in the LALDI mass spectrum. Bonds at which proposed fragmentations occur are indicated by blue dashed lines and are accompanied by the corresponding calculated monoisotopic mass for the fragment. Table shows the proposed molecular formulae, with the expected monoisotopic masses and mass accuracy, for each of the observed m/z peaks.
**Table**

| Observed m/z | Proposed molecular formula | Expected monoisotopic mass (Da) | Mass accuracy (ppm) |
|--------------|---------------------------|---------------------------------|---------------------|
| 902.3318     | C_{41}H_{57}KN_{3}O_{17}^+ | 902.3320                        | -0.22               |
| 886.3577     | C_{41}H_{57}N_{3}NaO_{17}^+ | 886.3580                        | -0.34               |
| 863.3674     | C_{41}H_{57}N_{3}O_{17}^{++} | 863.3682                        | -0.93               |
| 724.3052     | C_{35}H_{47}N_{3}NaO_{12}^+ | 724.3052                        | 0.00                |
| 701.3161     | C_{35}H_{47}N_{3}O_{12}^{++} | 701.3154                        | 1.00                |
| 669.2688     | C_{29}H_{36}N_{3}NaO_{17}^+ | 669.2689                        | -0.15               |
| 643.2895     | C_{29}H_{36}N_{3}O_{16}^{++} | 643.2896                        | -0.16               |
| 602.2836     | C_{25}H_{36}N_{3}NaO_{9}^+  | 602.2837                        | -0.17               |
| 588.2680     | C_{25}H_{36}N_{3}NaO_{9}^{++} | 588.2680                        | 0.00                |
| 579.2935     | C_{25}H_{36}N_{3}O_{9}^{++}  | 579.2939                        | -0.69               |
| 565.2778     | C_{25}H_{36}N_{3}O_{9}^{+}   | 565.2783                        | -0.88               |
| 547.2413     | C_{25}H_{36}N_{3}O_{9}^{+}   | 547.2415                        | -0.37               |
| 524.2513     | C_{25}H_{36}N_{3}O_{9}^{+}   | 524.2517                        | -0.76               |
| 503.2152     | C_{25}H_{36}N_{3}O_{9}^{+}   | 503.2147                        | 0.99                |
| 310.1077     | C_{25}H_{36}N_{3}O^{+}       | 310.1078                        | -0.32               |
| 287.1179     | C_{19}H_{15}N_{3}O^{+}       | 287.1179                        | 0.00                |

**Supplementary Figure 11**: LALDI-MS analysis of LALDI tag-labelled lactose 11. a) LALDI mass spectrum of LALDI tag-labelled lactose 11 (100 pmol) analysed as a pure sample; identified fragments and cationised products are annotated with their observed m/z values. Inlaid spectrum shows expansion of the spectrum at m/z 500-750. b) Chemical structure of LALDI tag-labelled lactose 11 with proposed assignments for fragments and cationised products observed in the LALDI mass spectrum. Bonds at which proposed fragmentations occur are indicated by blue dashed lines and are accompanied by the corresponding calculated monoisotopic mass for the fragment. Table shows the proposed molecular formulae, with the expected monoisotopic masses and mass accuracy, for each of the observed m/z peaks.
Supplementary Figure 12: LALDI-MS analysis of LALDI tag-labelled lactose 12. a) LALDI mass spectrum of LALDI tag-labelled lactose 12 (100 pmol) analysed as a pure sample; identified fragments and cationised products are annotated with their observed m/z values. Inlaid spectra show expansion of the spectrum at m/z 490-550 and m/z 665-850. b) Chemical structure of LALDI tag-labelled lactose 12 with proposed assignments for fragments and cationised products observed in the LALDI mass spectrum. Bonds at which proposed fragmentations occur are indicated by blue dashed lines and are
accompanied by the corresponding calculated monoisotopic mass for the fragment. Table shows the proposed molecular formulae, with the expected monoisotopic masses and mass accuracy, for each of the observed m/z peaks.
**Supplementary Figure 13:** LALDI-MS analysis of LALDI tag-labelled lactose 13. a) LALDI mass spectrum of LALDI tag-labelled lactose 13 (100 pmol) analysed as a pure sample; identified fragments and cationised products are annotated with their observed m/z values. Inlaid spectra show expansion of the spectrum at m/z 410-530 and m/z 740-840. b) Chemical structure of LALDI tag-labelled lactose 13 with proposed assignments for fragments and cationised products observed in the LALDI mass spectrum. Bonds at which proposed fragmentations occur are indicated by blue dashed lines and are accompanied by the corresponding calculated monoisotopic mass for the fragment. Table shows the proposed molecular formulae, with the expected monoisotopic masses and mass accuracy, for each of the observed m/z peaks.

| Observed m/z | Proposed molecular formula | Expected monoisotopic mass (Da) | Mass accuracy (ppm) |
|--------------|-----------------------------|---------------------------------|---------------------|
| 1007.3895    | C_{48}H_{64}KN_{4}O_{17}^+  | 1007.3898                       | -0.30               |
| 991.4151     | C_{48}H_{64}Na_{4}O_{17}^+  | 991.4159                        | -0.81               |
| 969.4336     | C_{48}H_{65}N_{4}O_{17}^+   | 969.4339                        | -0.31               |
| 829.3626     | C_{42}H_{54}Na_{4}O_{12}^+  | 829.3630                        | -0.48               |
| 807.3807     | C_{42}H_{55}N_{4}O_{12}^+   | 807.3811                        | -0.50               |
| 774.3263     | C_{32}H_{53}N_{3}NaO_{17}^+ | 774.3267                        | -0.52               |
| 748.3471     | C_{33}H_{53}N_{3}NaO_{16}^+ | 748.3475                        | -0.53               |
| 677.2943     | C_{29}H_{42}N_{3}NaO_{7}^+  | 677.2946                        | -0.44               |
| 524.2753     | C_{29}H_{38}N_{3}NaO_{6}^+  | 524.2755                        | -0.38               |
| 460.2054     | C_{21}H_{31}N_{3}NaO_{5}^+  | 460.2054                        | -0.22               |
| 438.2234     | C_{21}H_{32}N_{2}O_{7}^+    | 438.2235                        | -0.23               |
| 412.2441     | C_{20}H_{30}N_{3}O_{6}^+    | 412.2442                        | -0.24               |
| 287.1179     | C_{19}H_{26}N_{2}O^+        | 287.1179                        | 0.00                |
| Observed m/z | Proposed molecular formula | Expected monoisotopic mass (Da) | Mass accuracy (ppm) |
|--------------|-----------------------------|---------------------------------|---------------------|
| 580.2536     | C_{32}H_{38}NO_{9}^+         | 580.2541                        | -0.86               |
| 568.2776     | C_{31}H_{40}N_{2}O_{8}^{2+}  | 568.2779                        | -0.53               |
| 553.2665     | C_{31}H_{38}NO_{8}^{2+}      | 553.2670                        | -0.90               |
| 539.2510     | C_{30}H_{37}NO_{8}^{3+}      | 539.2514                        | -0.74               |
| 525.2721     | C_{29}H_{39}NO_{7}^{3+}      | 525.2721                        | 0.00                |
| 511.2561     | C_{29}H_{37}NO_{6}^{4+}      | 511.2565                        | -0.78               |
| 495.2612     | C_{29}H_{37}NO_{5}^{5+}      | 495.2615                        | -0.61               |
| 481.2456     | C_{28}H_{35}NO_{5}^{6+}      | 481.2459                        | -0.62               |
| 301.1209     | C_{19}H_{18}N_{3}O^{7+}      | 301.1210                        | -0.33               |
| 274.1100     | C_{18}H_{14}N_{2}O^{8+}      | 274.1101                        | -0.36               |

**Supplementary Figure 14:** LALDI-MS analysis of LALDI tag-labelled lactose 14. a) LALDI mass spectrum of a LALDI tag-labelled lactose 14 (100 pmol) contaminated by an unknown impurity according to analytical HPLC; identified fragments and cationised products are annotated with their observed m/z values. Inlay shows expansion of the spectrum at m/z 470-590. b) Chemical structure of LALDI tag-labelled lactose 14 with proposed assignments for fragments and cationised products observed in the LALDI mass spectrum. Bonds at which proposed fragmentations occur are indicated by blue dashed lines and are accompanied by the corresponding calculated monoisotopic mass for the fragment. Table shows the proposed molecular formulae, with the expected monoisotopic masses and mass accuracy, for each of the observed m/z peaks. Note that no signals were detected for the parent ion of compound 14.
Supplementary Figure 15: Comparison of the LALDI-MS analysis of each LALDI tag labelled lactose 10-14 analysed as pure samples (100 pmol) illustrating the difference in sensitivity of each LALDI tag labelled glycan and the precision of the data collected using an AnchorChip target plate. Bars represent the mean values for the sum of all positive mode ions related to each LALDI reagent, including the molecular ions (M**), cationised molecules ([M+Na]**, [M+K]**), and major degradation products (10: [M-CO]**, 14: [M-C12H23NO10]**) detected by LALDI-MS at 100 pmol of each reagent. Error bars represent the standard deviation, where n = 3 (individual aliquots from the same compound solution analysed on 3 separate target spots).
1.6 LALDI-MS analysis of LALDI tag-labelled lactose derivatives from HBSS

Supplementary Figure 16: a) LALDI mass spectra of samples of LALDI tag-labelled lactose derivatives (100 pmol) in HBSS with molecular ions, cationised molecules, and identified fragments highlighted i) 10. ii) 11. iii) 12. iv) 13. b) An image showing a dried sample of LALDI tag-labelled lactose 10 (100 pmol) in HBSS captured by the LDI-MS laser target camera. The crystalline appearance of the sample shows the high abundance of buffer salts present in the analytical samples analysed during these experiments. c) Comparison of the LALDI-MS analysis of each of the LALDI tag labelled lactose analysed
from HBSS (100 pmol) illustrating the difference in LOD for each LALDI tag-labelled glycan and the precision of the data collected using an AnchorChip target plate. Bars represent the mean values for the sum of all positive mode ions related to each LALDI reagent, including the molecular ions (M$^{\text{+}}$), cationised molecules ([M+Na]$^{\text{+}}$, [M+K]$^{\text{+}}$), and major degradation products (10: [M-CO]$^{\text{+}}$) detected by LALDI-MS at 100 pmol of each reagent. Error bars represent the standard deviation, where n = 3 individual aliquots from the same compound solution analysed on 3 separate target spots).
Supplementary Table 2: Summary of the performance of LALDI tag-labelled lactose 10-13 in LALDI-MS experiments analysed from a background of HBSS.

| LALDI tag-labelled glycan | Molecular ion detected in LALDI-MS? | MS stability‡ | Limit of detection (pmol) |
|--------------------------|------------------------------------|----------------|--------------------------|
| 10                       | Yes                                | +/-            | 1                        |
| 11                       | Yes                                | +/-            | 10                       |
| 12                       | Yes                                | +              | 10                       |
| 13                       | Yes                                | +              | 10                       |

‡ Assessment of MS stability was based on i) the degree of fragmentation displayed by each analyte following LALDI-MS analysis, and ii) the relative abundance of the signals corresponding to the molecular ion (M•+) and cationised molecules ([M+Na]+, [M+K]+) compared to the relative abundance signals corresponding to fragments, then scored using an arbitrary scale from + to –.
### Supplementary Figure 17: LALDI-MS analysis of LALDI tag-labelled lactose 10 dissolved in Hanks’ buffered salt solution (HBBS).

a) LALDI mass spectrum of LALDI tag-labelled lactose 10 (100 pmol) analysed from a background of HBSS; identified fragments and cationised products are annotated with their observed m/z values. b) Chemical structure of LALDI tag-labelled lactose 10 with proposed assignments for fragments and cationised products observed in the LALDI mass spectrum. Bonds at which proposed fragmentations occur are indicated by blue dashed lines and are accompanied by the corresponding calculated monoisotopic mass for the fragment. Table shows the proposed molecular formulae, with the expected monoisotopic masses and mass accuracy, for each of the observed m/z peaks.

| Observed m/z | Proposed molecular formula | Expected monoisotopic mass (Da) | Mass accuracy (ppm) |
|--------------|----------------------------|---------------------------------|--------------------|
| 917.3305     | C₄₂H₅₈KN₂O₁₈⁺              | 917.3316                       | -1.20              |
| 901.3570     | C₄₂H₅₈N₂NaO₁₈⁺             | 901.3577                       | -0.78              |
| 878.3670     | C₄₂H₅₈N₂O₁₈⁻              | 878.3679                       | -1.03              |
| 850.3723     | C₄₁H₅₈N₂O₁₇⁻              | 850.3730                       | -0.82              |
| 716.3143     | C₄₂H₄₈N₂O₁₃⁻              | 716.3151                       | -1.12              |
| 562.2406     | C₃₀H₃₇NNaO₈⁺              | 562.2411                       | -0.89              |
| 539.2510     | C₃₀H₃₇NO₈⁺                | 539.2514                       | -0.74              |
| 511.2560     | C₂₉H₃₇NO₇⁺                | 511.2565                       | -0.98              |
| 302.1174     | C₂₀H₁₆NO₂⁺                 | 302.1176                       | -0.66              |
| Observed m/z | Proposed molecular formula | Expected monoisotopic mass (Da) | Mass accuracy (ppm) |
|--------------|---------------------------|---------------------------------|---------------------|
| 863.3674     | C_{41}H_{57}N_{3}O_{17}^{**} | 863.3682                        | -0.93               |
| 579.2935     | C_{32}H_{41}N_{3}O_{7}^{**}  | 579.2939                        | -0.69               |
| 565.2778     | C_{31}H_{39}N_{3}O_{7}^{**}  | 565.2783                        | -0.88               |
| 524.2513     | C_{29}H_{36}N_{2}O_{7}^{**}  | 524.2517                        | -0.76               |

**Supplementary Figure 18:** LALDI-MS analysis of LALDI tag-labelled lactose 11 dissolved in Hanks’ buffered salt solution (HBBS). a) LALDI mass spectrum of LALDI tag-labelled lactose 11 (100 pmol) analysed from a background of HBSS; identified fragments and cationised products are annotated with their observed m/z values. b) Chemical structure of LALDI tag-labelled lactose 11 with proposed assignments for fragments and cationised products observed in the LALDI mass spectrum. Bonds at which proposed fragmentations occur are indicated by blue dashed lines and are accompanied by the corresponding calculated monoisotopic mass for the fragment. Table shows the proposed molecular formulae, with the expected monoisotopic masses and mass accuracy, for each of the observed m/z peaks.
**Supplementary Figure 19:** LALDI-MS analysis of LALDI tag-labelled lactose 12 dissolved in Hanks’ buffered salt solution (HBBS). a) LALDI mass spectrum of LALDI tag-labelled lactose 12 (100 pmol) analysed from a background of HBSS identified fragments and cationised products are annotated with their observed m/z values. b) Chemical structure of LALDI tag-labelled lactose 12 with proposed assignments for fragments and cationised products observed in the LALDI mass spectrum. Bonds at which proposed fragmentations occur are indicated by blue dashed lines and are accompanied by the corresponding calculated monoisotopic mass for the fragment. Table shows the proposed molecular formulae, with the expected monoisotopic masses and mass accuracy, for each of the observed m/z peaks.

| Observed m/z | Proposed molecular formula | Expected monoisotopic mass (Da) | Mass accuracy (ppm) |
|--------------|----------------------------|---------------------------------|---------------------|
| 1022.3880    | C₄₉H₆₅KN₃O₁₈⁺             | 1022.3895                       | -1.47               |
| 1006.4146    | C₄₉H₆₅N₃NaO₁₈⁺            | 1006.4155                       | -0.89               |
| 670.3120     | C₃₈H₄₄N₃O₁₁⁺             | 670.3123                        | -0.45               |
| 539.2748     | C₃₀H₃₈N₂O₁⁺              | 539.2752                        | -0.74               |
| 413.2880     | C₂₀H₃₃N₂O₁⁺              | 413.2882                        | -0.48               |
| 217.0885     | C₁₆H₁₂N⁺                 | 217.0886                        | -0.46               |
**Supplementary Figure 20**: LALDI-MS analysis of LALDI tag-labelled lactose 13 dissolved in Hanks’ buffered salt solution (HBBS). a) LALDI mass spectrum of LALDI tag-labelled lactose 13 (100 pmol) analysed from a background of HBSS, identified fragments and cationised products are annotated with their observed m/z values. Inlays show expansion of the spectrum at m/z 275-325 and m/z 550-850. b) Chemical structure of LALDI tag-labelled lactose 13 with proposed assignments for fragments and cationised products observed in the LALDI mass spectrum. Bonds at which proposed fragmentations occur are indicated by blue dashed lines and are accompanied by the corresponding calculated monoisotopic mass for the fragment. Table shows the proposed molecular formulae, with the expected monoisotopic masses and mass accuracy, for each of the observed m/z peaks.
2. Supplementary methods

2.1 Synthetic routes

Supplementary Figure 21: Synthetic routes to the initial water soluble LALDI reagents 1-4.
Supplementary Figure 22: Synthetic route to NMe-1-APy S1

Supplementary Figure 23: Synthesis of 2-methoxy-N-methyl-N-(pyren-1-yl)acetamide S2.

Supplementary Figure 24: Synthetic route to hydroxylamine LALDI tag reagents 5 and 6.
Supplementary Figure 25: Synthetic route to 2-aminobenzamide LALDI tag reagents 7 and 8.

Supplementary Figure 26: Synthetic route to hydrazide LALDI tag reagents 9.
2.2 General comments

All reactions were carried out under an atmosphere of dry nitrogen unless otherwise stated, using anhydrous solvents from a solvent purification system (Innovative Technology Inc. PureSolv), with the exception of anhydrous DMF, which was purchased from Acros Organics. Acetic formic anhydride was prepared by combining acetic anhydride (1 equiv.) and formic acid (1.2 equiv.) with stirring at 55 °C for 2 h and used directly without purification. All other chemical starting reagents were purchased from commercial suppliers and used without further purification. Azeotropic distillation with toluene was used to remove persistent moisture from reagents with oligoethylene glycol chains before being used in a reaction. The identity and purity of known compounds was confirmed through the comparison of experimentally obtained data to values reported in the literature.

Thin layer chromatography (TLC) was carried out on Merck TLC Silica gel 60 F254 plates. Flash column chromatography was performed using Merck Geduran silica gel 60 (40–63 μm). All retention factors (Rf) are given to two decimal places along with the solvent system. Reversed-phase chromatography was performed using prepacked RediSep® Rf Reversed-phase C18 columns. Lyophilisation of compounds was performed using a Virtis Benchtop K freeze dryer.

The melting point of recrystallised solids was determined using a Griffin MFB-590 Melting Point Apparatus with a glass capillary melting point tube. Melting points (m.p.) are recorded as a range between the meniscus point and liquefaction with values given to the nearest degree in Celsius (°C).

\(^1\)H and \(^{13}\)C NMR spectra were recorded in deuterated solvents on a Bruker AVANCE 500 Ultrashield or Bruker AVANCE 400 Ultrasheild NMR Spectrometer. Chemical shifts are referenced to residual solvent peaks and are quoted in ppm. Coupling constants (J) are reported to the nearest 0.1 Hz. Assignment of spectra was based on expected chemical shifts and coupling constants, aided by DEPT, COSY, HMQC, and HMBC where appropriate.

High-resolution electrospray mass spectrometry (HR-ESI-MS) was performed using a Bruker MaXis Impact spectrometer; m/z values are reported to four decimal places. LC-MS was recorded on an Agilent Technologies 1200 series HPLC combined with a Bruker HCT Ultra ion trap using 50 × 20 mm C18 reversed-phase columns with a solvent system of increasing acetonitrile (5 to 95%) in water, each containing 0.1% formic acid. A flow rate of 1.5 cm³ min⁻¹ was used and m/z values are given to one decimal place. Retention times (Rt) are provided in minutes to the nearest two decimal places.

Infrared (IR) spectroscopy was carried out using a Bruker Alpha Platinum ATR. Samples were analysed neat and absorption maxima (vmax) are given in wave numbers (cm⁻¹) to the nearest whole
wavenumber. Signals are defined as either strong (s), medium (m), weak (w), or broad (br). UV-Vis absorption was measured using an Agilent Technologies Cary 100 UV-Vis Spectrophotometer with all samples were analysed as solutions in a 10 mm Hellma Analytics High Precision Quarts Suprasil cell. Absorption maxima ($\lambda_{\text{max}}$) are given in nanometers to the nearest whole nanometer with the corresponding molar extinction coefficient ($\varepsilon$) given in M$^{-1}$ cm$^{-1}$.

Scanning electron microscopy (SEM) was carried out using a FEI Nova NanoSEM450 Scanning Electron Microscope. SEM analysis of the LDI-MS target plate was carried out on a Bruker Daltonics ground-steel MALDI MTP 384 target plate. The LDI-MC target plate was mounted on an SEM stub using an adhesive copper tape and observed by field emission gun (FEG)-SEM, without coating, using Circular Backscatter (CBS) Detector operating at 5 kV with deceleration mode. Element analysis made by EDAX elemental mapping software (AMTEK) at 18kV.

2.3 Synthetic procedures
17-[[4-methylbenzenesulfonyl]oxy]-3,6,9,12,15-pentaoxaheptadecan-1-ol S4\textsuperscript{1,2}

Silver (I) oxide (1.23 g, 5.31 mmol), and potassium iodide (118 mg, 1.71 mmol) were added to a stirred solution of hexaethylene glycol S3 (1.00 g, 3.54 mmol) in DCM (20 mL) at 0 °C (ice bath). A solution of 4-toluenesulfonyl chloride (743 mg, 3.89 mmol) in DCM (20 mL) was then added drop-wise and the reaction mixture allowed to warm to room temperature and stir overnight (16 h). The reaction was filtered through a silica plug, eluted with EtOAc (250 mL), and concentrated in vacuo. The crude tosylate was subjected to flash chromatography (SiO$_2$; EtOAc–MeOH 95:5) to afford the product S4 as a colourless oil (1.37 g, 3.13 mmol, 88%). $R_t$ 0.18 (SiO$_2$; EtOAc–MeOH 95:5). \textsuperscript{1}H NMR (500 MHz, CDCl$_3$) $\delta$ 7.78 (d, 2H, J 8.3 Hz, CH-2', CH-7'), 7.33 (d, 2H, J 8.3 Hz, CH-3', CH-5'), 4.15 (t, 2H, J 4.81, CH$_2$-17), 3.71 – 3.57 (m, 22H, OCH$_2$CH$_2$O), 2.44 (s, 3H, CH$_3$-4). $^{13}$C NMR (125 MHz, CDCl$_3$) $\delta$ 144.9 (CCH$_3$-4'), 133.2 (CSO$_2$-1'), 129.9 (Aryl CH-3', CH-5'), 128.1 (Aryl CH-2', CH-7'), 72.7 (CH$_2$), 70.9 (CH$_2$), 70.7 (CH$_2$), 70.7 (CH$_2$), 70.6 (CH$_2$), 70.4 (CH$_2$), 69.4 (CH$_2$-17), 68.8 (CH$_2$), 61.8 (CH$_3$), 21.8 (CH$_3$-4). \textsuperscript{HR-ESI-MS} Calculated for C$_{19}$H$_{33}$O$_9$S: $m/z$ 437.1840 [M+H]$^{+}$; found 437.1841. \textsuperscript{LC-MS} Calculated for C$_{19}$H$_{33}$O$_9$S: $m/z$ 437.18 [M+H]$^{+}$; found 437.6. $R_t$ = 1.66. 1 × CH$_2$ from the OEG chain were unaccounted for in the $^{13}$C NMR spectra due to overlapping signals. Characterisation is consistent with data reported in the literature.\textsuperscript{1}
Sodium azide (726 mg, 11.2 mmol) was added to a stirred solution of 17-hydroxy-3,6,9,12,15-pentaoxaheptadecyl 4-methylbenzenesulfonate S4 (1.95 g, 4.47 mmol) in DMF (20 mL). The mixture was then heated to 60 °C with stirring for 20 h, then left to cool to room temperature. The reaction was diluted with EtOAc (30 ml), filtered through a pad of SiO$_2$, and eluted with EtOAc (300 mL). The solvent was removed under reduced pressure to afford the product S5 as a clear yellow oil (1.23 g, 4.01 mmol, 90%). $R_f$ 0.24 (EtOAc–MeOH 10:1). $^1$H NMR (500 MHz, CDCl$_3$) δ 3.70 – 3.69 (m, 2H, CH$_2$-1), 3.66 – 3.63 (m, 18H, OCH$_2$CH$_2$O), 3.59 – 3.57 (m, 2H, OCH$_2$CH$_2$O), 3.59 (t, 2H, J 5.2 Hz, CH$_2$-17). $^{13}$C NMR (125 MHz, CDCl$_3$) δ 72.7 (CH$_2$), 70.8 (CH$_2$), 70.8 (CH$_2$), 70.7 (CH$_2$), 70.7 (CH$_2$), 70.5 (CH$_2$), 70.2 (CH$_2$), 61.9 (CH$_2$OH-1), 50.8 (CH$_2$N$_3$-17). HR-ESI-MS Calculated for C$_{12}$H$_{26}$N$_3$O$_6$: m/z 308.1816 [M+H]$^+$; found 308.1823. LC-MS Calculated for C$_{12}$H$_{25}$N$_3$O$_6$: m/z 307.1738 [M]$^+$•; found 307.8, $R_t$ = 1.37. IR (Neat, $\nu_{\max}$/cm$^{-1}$) 3457 br s (OH hydroxy), 2099 s (N=N=N). 2 × CH$_2$ from the OEG chain were unaccounted for in the $^{13}$C NMR spectra due to overlapping signals. Characterisation is consistent with data reported in the literature.$^{56}$

Triphenylphosphine (939 mg, 3.58 mmol) was added to a stirred solution of 17-azido-3,6,9,12,15-pentaoxaheptadecan-1-ol S5 (1.00 g, 3.25 mmol) in THF (15 mL) at 0 °C (ice bath). The reaction mixture allowed to warm to room temperature and stir overnight (24 h). H$_2$O (1 mL) was added to the reaction mixture and left to stir (2 h) to hydrolyse the iminophosphorane intermediate. The reaction mixture was then concentrated under reduced pressure, diluted with H$_2$O (15 mL), and washed with toluene (3 × 15 mL). The aqueous layer was separated, and the water removed by lyophilisation to afford the product S6 as a yellow oil (915 mg, 3.25 mmol, Quant.). $^1$H NMR (500 MHz, CDCl$_3$) δ 3.70 – 3.69 (m, 2H, CH$_2$-1), 3.64 – 3.61 (m, 16H, OCH$_2$CH$_2$O), 3.59 – 3.57 (m, 2H, OCH$_2$CH$_2$O), 3.51 (t, 2H, J 5.1 Hz, CH$_2$-16), 2.85 (t, 2H, J 5.1 Hz, CH$_2$-17). $^{13}$C NMR (125 MHz, CDCl$_3$) δ 73.2 (CH$_2$), 73.0 (CH$_2$), 70.7 (CH$_2$), 70.7 (CH$_2$), 70.7 (CH$_2$), 70.6 (CH$_2$), 70.4 (CH$_2$), 70.3 (CH$_2$), 61.6 (CH$_2$OH-1), 41.8 (CH$_2$N$_3$-17). HR-ESI-MS Calculated for C$_{12}$H$_{24}$N$_3$O$_6$: m/z 282.1911 [M+H]$^+$•; found 282.1915. LC-MS found 282.0, $R_t$ 0.43.
IR (Neat, ν<sub>max</sub>/cm<sup>-1</sup>) 3420 br m (OH hydroxy), 3361 m (NH<sub>2</sub> asym), 3309 m (NH<sub>2</sub> sym). 1 × CH<sub>2</sub> from the OEG chain was unaccounted for in the <sup>13</sup>C NMR spectra due to overlapping signals. Characterisation is consistent with data reported in the literature.<sup>4</sup>

Methyl 4-(pyren-1-yl)butanoate S7<sup>i</sup> 5

Thionyl chloride (1.0 mL, 8.67 mmol) followed by dropwise addition of methanol (5 mL mmol) was added to a stirred suspension of pyrenebutyric acid S7 (500 mg, 1.73 mmol) in DCM (7.5 mL) at -15 °C (NaCl/ice bath). The reaction mixture was then allowed to stir at room temperature until determined complete by TLC (3 h). The reaction mixture was then concentrated in vacuo and the crude residue was purified by flash chromatography (SiO<sub>2</sub>; Hex–CHCl<sub>3</sub> 1:1 → 0:1) to afford S7i as a yellow oil that solidified to yellow needles on cooling (523 mg, 1.73 mmol, Quant.). R<sub>f</sub> 0.19 (SiO<sub>2</sub>; Hex–CHCl<sub>3</sub> 1:1).

m.p. = 46–47 °C (Hex : CHCl<sub>3</sub>). <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>) δ 8.31 (d, 1H, J 9.2 Hz, Aryl), 8.20 – 8.15 (m, 2H, Aryl), 8.12 (dd, 2H, J 8.5, 3.2 Hz, Aryl), 8.05 – 7.96 (m, 3H, Aryl), 7.86 (d, 1H, J 7.8 Hz, Aryl), 3.70 (s, 3H, OCH<sub>3</sub>), 3.40 (t, 2H, J 7.6 Hz, CH<sub>2</sub>-4), 2.47 (t, 2H, J 7.3 Hz, CH<sub>2</sub>-2), 2.21 (p, 2H, J 7.5 Hz, CH<sub>2</sub>-3). <sup>13</sup>C NMR (125 MHz, CDCl<sub>3</sub>) δ 174.1 (COOMe-1), 135.8 (Aryl C), 131.6 (Aryl C), 131.0 (Aryl C), 130.1 (Aryl C), 128.9 (Aryl C), 127.6 (Aryl CH), 127.5 (Aryl CH), 127.5 (Aryl CH), 126.9 (Aryl CH), 126.0 (Aryl CH), 125.2 (Aryl C), 125.1 (Aryl C), 125.1 (Aryl CH), 124.9 (Aryl CH), 124.9 (Aryl CH), 123.4 (Aryl CH), 51.7 (OCH<sub>3</sub>), 33.8 (CH<sub>2</sub>-2), 32.9 (CH<sub>2</sub>-4), 26.9 (CH<sub>2</sub>-3). HR-ESI-MS Calculated for C<sub>21</sub>H<sub>19</sub>O<sub>2</sub>: m/z 303.1379 [M+H]<sup>+</sup>; found 303.1383. LC-MS Calculated for C<sub>21</sub>H<sub>18</sub>NaO<sub>2</sub>: m/z 325.12 [M+Na]<sup>+</sup>; found 325.2, R<sub>t</sub> = 2.36. Characterisation is consistent with data reported in the literature.<sup>5</sup>
Methyl 4-(nitropyren-1-yl)butanoate S7ii

44% nitric acid (aq) (110 μL, 1.00 mmol) was added to a stirred solution of methyl 4-(pyren-1-yl)butanoate S7i (500 mg, 1.65 mmol) in acetic anhydride (11 mL) and stirred overnight. A second portion of 44% nitric acid (110 μL, 1.00 mmol) was added to the reaction and allowed to stir for another hour. The reaction mixture was then concentrated in vacuo and the crude residue was purified by flash chromatography (SiO\textsubscript{2}; CHCl\textsubscript{3}) to afford a 1:1:1 mixture of 3 regioisomers of S7ii as an orange oil (573 mg, 1.65 mmol, Quant). R\textsubscript{f} 0.38–0.50 (SiO\textsubscript{2}; CHCl\textsubscript{3}).

\textbf{\textsuperscript{1}H NMR} (500 MHz, CDCl\textsubscript{3}) δ 8.87 (d, 1H, J 9.7 Hz, Aryl), 8.76 (d, 2H, J 9.4 Hz, Aryl), 8.57 (t, 2H, J 8.1 Hz, Aryl), 8.50 (d, J = 9.8 Hz, 1H, Aryl), 8.45 – 8.41 (m, 2H, Aryl), 8.29 – 8.15 (m, 8H, Aryl), 8.11 (d, J = 9.8 Hz, 1H, Aryl), 8.07 – 8.02 (m, 4H, Aryl), 7.96 (d, 1H, J 8.8 Hz, Aryl), 7.90 (d, 2H, J 7.8 Hz, Aryl), 3.65 (s, 3H, OCH\textsubscript{3}), 3.38 – 3.30 (m, 2H+2H+2H, 3 × CH\textsubscript{2}-4), 2.42 (m, 2H+2H+2H, 3 × CH\textsubscript{2}-2), 2.17 – 2.09 (m, 2H+2H+2H, 3 × CH\textsubscript{2}-3).

\textbf{\textsuperscript{13}C NMR} (125 MHz, CDCl\textsubscript{3}) δ 173.9 (COOMe\textsubscript{-1}), 173.8 (COOMe\textsubscript{-1}), 173.8 (COOMe\textsubscript{-1}), 143.0 (2 × Aryl C), 142.7 (Aryl C), 142.7 (Aryl C), 142.6 (Aryl C), 142.6 (Aryl C), 139.1 (Aryl C), 138.7 (Aryl C), 138.7 (Aryl C), 136.4 (Aryl C), 135.6 (Aryl C), 135.0 (Aryl C), 133.2 (Aryl C), 131.8 (Aryl CH), 131.1 (Aryl CH), 131.0 (2 × Aryl CH), 130.9 (Aryl C), 130.5 (Aryl C), 129.8 (Aryl C), 129.1 (Aryl C), 128.8 (Aryl C), 128.7 (Aryl CH), 128.7 (Aryl CH), 128.1 (Aryl C), 127.8 (Aryl CH), 127.7 (Aryl CH), 127.6 (Aryl CH), 127.3 (Aryl CH), 127.2 (Aryl CH), 127.0 (Aryl CH), 126.8 (Aryl CH), 126.4 (Aryl CH), 125.7 (Aryl C), 125.4 (Aryl C), 125.3 (Aryl C), 124.9 (Aryl C), 124.4 (Aryl C), 124.2 (Aryl C), 124.2 (Aryl C), 124.1 (Aryl CH), 123.6 (Aryl C), 123.6 (Aryl CH), 123.0 (Aryl CH), 122.9 (Aryl CH), 122.8 (Aryl CH), 121.9 (Aryl CH), 121.8 (Aryl CH), 121.1 (Aryl CH), 51.8 (OCH\textsubscript{3}), 51.8 (2 × OCH\textsubscript{3}), 33.7 (2 × CH\textsubscript{2}-2), 33.6 (CH\textsubscript{2}-2), 33.1 (CH\textsubscript{2}-4), 32.9 (CH\textsubscript{2}-4), 32.9 (CH\textsubscript{2}-4), 27.2 (2 × CH\textsubscript{2}-3), 26.4 (CH\textsubscript{2}-3). HRF-ESI-MS Calculated for C\textsubscript{21}H\textsubscript{18}N\textsubscript{2}O\textsubscript{4}: m/z 348.1230 [M+H]\textsuperscript{+}; found 348.1233. LC-MS Calculated for C\textsubscript{21}H\textsubscript{17}NNaO\textsubscript{2}: m/z 370.11 [M+Na]\textsuperscript{+}; found 370.1, R\textsubscript{t} = 2.35. IR (Neat, ν\textsubscript{max}/cm\textsuperscript{-1}) 1729 s (C=O ester), 1584–1435 w (C=C aromatic), 1506 s (NO\textsubscript{2} asym), 1307 s (NO\textsubscript{2} sym). Characterisation is consistent with data reported in the literature.
Throughout the reaction and work up the reaction and products were shielded from light. Under a N₂ atmosphere, acetic acid (1.00 mL, 18.0 mmol) and 10% Pd/C (180 mg) was added to a stirred solution of methyl 4-(nitropyren-1-yl)butanoate S7ii (500 mg, 1.44 mmol) in EtOAc (20 mL). The inert atmosphere was then replaced with H₂ and stirred overnight (16h). The reaction was then filtered, and the residue washed with EtOAc then the filtrate was concentrated by azeotropic distillation (rotavap) with toluene. The crude residue was purified by flash chromatography (SiO₂; Toluene–EtOAc 99:1 → 97:3→ 95:5→ 93:7→ 9:1) to afford the product S8 as a yellow oil (101 mg, 0.318 mmol, 22%), Rᶠ 0.32 (SiO₂; Toluene–EtOAc 9:1) and a mixture of two regioisomers as an orange oil (232 mg, 0.731 mmol, 51%), Rᶠ 0.21 (SiO₂; Toluene–EtOAc 9:1). Characterisation data only given for 6-isomer of S8. ¹H NMR (500 MHz, CDCl₃) δ 8.04 – 7.87 (m, 6H, Aryl), 7.78 (d, 1H, J 7.7 Hz, Aryl), 7.38 (t, 1H, J 9.0 Hz, Aryl), 3.69 (s, 3H, OCH₃), 3.33 (t, 2H, J 7.7 Hz, CH₂-4), 2.46 (t, 2H, J 7.3 Hz, CH₂-2), 2.22 – 2.15 (m, 2H, CH₂-3).

¹³C NMR (125 MHz, CDCl₃) δ 174.1 (COOMe-1), 140.9 (Aryl C), 134.7 (Aryl C), 134.6 (Aryl C), 129.9 (Aryl C), 128.4 (Aryl C), 127.8 (Aryl CH), 127.6 (Aryl CH), 126.5 (Aryl C), 126.3 (Aryl CH), 126.0 (Aryl CH), 124.3 (Aryl C), 123.5 (Aryl CH), 119.8 (Aryl CH), 119.5 (Aryl CH), 117.3 (Aryl C), 114.2 (Aryl CH), 51.7 (OCH₃), 33.9 (CH₂-2), 33.0 (CH₂-4), 26.6 (CH₂-3). HR-ESI-MS Calculated for C₂₁H₂₀NO₂: m/z 318.1489 [M+H]+; found 318.1482. LC-MS found 318.2, Rₜ = 2.39. IR (Neat, νmax/cm⁻¹) 3465 m (NH₂ asym), 3373 m (NH₂ sym), 1720 s (C=O Ester), 1601–1498 m (C=C Aromatic). Characterisation is consistent with data reported in the literature.⁵

Acetic anhydride (36 μL, 0.38 mmol) was added to a stirred solution of methyl 4-(6-aminopyren-1-yl)butanoate S8 (100 mg, 0.315 mmol) in DCM (3mL) and the mixture was allowed to stir overnight (16 h). The pH of the reaction mixture was then adjusted to 7 with sat. NaHCO₃ (aq). The layers were
separated and the organic layer was washed with sat. NaHCO₃ (aq) (3 × 2 mL) and brine (5 mL). The organic layer was dried (Na₂SO₄) and solvent removed in vacuo. The crude residue was purified by flash chromatography (SiO₂; CHCl₃–Acetone 1:0 →10:1 → 5:1) to afford the product S8i as an amorphous off-white solid (94 mg, 0.27 mmol, 83%) R₁ 0.32 (SiO₂; CHCl₃–Acetone 9:1). m.p. = 196–197 °C (Amorphous). ¹H NMR (500 MHz, DMSO) δ 10.27 (br s, 1H, CONH-1”), 8.32 (t, 1H, J 5.8 Hz, Aryl), 8.25 (d, 3H, J 10.2 Hz, Aryl), 8.22 – 8.13 (m, 3H, Aryl), 7.93 (d, 1H, J 7.8 Hz, Aryl), 3.61 (s, 3H, OCH₃), 3.33 (t, 2H, J 7.7 Hz, CH₂-4), 2.48 (t, 2H, J 7.2 Hz, CH₂-2), 2.27 (s, 3H, CH₃CONH-2”), 2.07 – 2.00 (m, 2H, CH₂-3). ¹³C NMR (125 MHz, DMSO) δ 173.2 (COOMe-1), 169.0 (CONH-1”), 136.2 (Aryl C), 131.8 (Aryl C), 129.2 (Aryl C), 128.3 (Aryl C), 127.9 (Aryl C), 128.3 (Aryl C), 127.7 (Aryl CH), 127.2 (Aryl CH), 127.1 (Aryl CH), 124.7 (Aryl CH), 124.7 (Aryl CH), 124.4 (Aryl C), 123.9 (Aryl C), 123.9 (Aryl C), 123.3 (Aryl CH), 122.5 (Aryl CH), 122.5 (Aryl CH), 121.5 (Aryl CH), 51.2 (OCH₃), 32.9 (CH₂-2), 32.0 (CH₂-4), 26.6 (CH₂-3), 23.5 (CH₃CONH-2”). HR-ESI-MS Calculated for C₂₃H₂₁NNaO₃: m/z 382.1414 [M+Na]+; found 382.1418. LC-MS found 382.3, Rₜ = 1.98. IR (Neat, ν max/cm⁻¹) 3265 m (NH), 1731 s (C=O Ester), 1682 (C=O Amide), 1602–1448 m (C=C Aromatic). Characterisation is consistent with data reported in the literature.

5-4-(6-acetamidopyren-1-yl)butanoic acid S9

1 M LiOH (aq) (2.96 mL, 2.96 mmol) was added to a stirred solution of methyl 4-(6-acetamidopyren-1-yl)butanoate S8i (93 mg, 0.27 mmol) in THF (2 mL) and the mixture was allowed to stir overnight (24 h). The reaction mixture was then acidified to pH 3 using 1M HCl (aq) and extracted with EtOAc (5 × 15 mL). The organic layers were combined, washed with brine (10 mL), dried (Na₂SO₄) and solvent removed in vacuo. The crude residue was then dry loaded onto celite (0.6 g) and purified by flash chromatography (SiO₂; CHCl₃–Acetone 2:1 → 1:1 → 1:2 → 0:1) to afford the product S9 as an amorphous off-white solid. (45.5 mg, 0.132 mmol, 49%). R₁ 0.49 (SiO₂; CHCl₃–Acetone 1:1). m.p. = 253–254 °C (CHCl₃ : Acetone). ¹H NMR (500 MHz, DMSO) δ 12.11 (br s, 1H, OH), 10.28 (br s, 1H, CONH-1”), 8.33 (d, 1H, J 9.2 Hz, Aryl), 8.28 – 8.22 (m, 3H, Aryl), 8.22 – 8.14 (m, 3H, Aryl), 7.92 (d, 1H, J 7.8 Hz, Aryl), 3.35 – 3.32 (m, 2H, CH₂-4), 2.39 (t, 2H, J 7.2 Hz, 2H, CH₂-2), 2.28 (s, 3H, CH₃CONH), 2.05 – 1.98 (m, 2H, CH₂-3). ¹³C NMR (125 MHz, DMSO) δ 174.4 (COOH-1), 169.1 (CH₃CONH-1”), 136.4 (Aryl C), 131.8 (Aryl C), 129.2 (Aryl C), 128.4 (Aryl C), 127.9 (Aryl C), 127.7 (Aryl CH), 127.2 (Aryl CH), 127.1 (Aryl CH), 124.7 (Aryl CH), 124.6 (Aryl CH), 124.4 (Aryl C), 124.0 (Aryl C), 123.3 (Aryl CH), 122.5 (Aryl CH), 121.5 (Aryl CH), 121.5 (Aryl CH), 121.4 (Aryl C), 120.5 (Aryl CH), 32.9 (CH₂-4), 32.0 (CH₂-4), 26.6 (CH₂-3), 23.5 (CH₃CONH-2”). Characterisation is consistent with data reported in the literature.
121.5 (Aryl CH), 33.3 (CH₂-2), 32.1 (CH₂-4), 26.8 (CH₂-3), 23.5 (CH₂CONH-2). HR-ESI-MS Calculated for C₂₃H₂₁NO₃: m/z 346.1438 [M+H]+; found 346.1432. LC-MS found 346.5, Rₑ = 1.74. IR (Neat, νₘₐₓ/cm⁻¹) 3443 br m (OH acid), 3272 m (NH), 1713 s (C=O Acid), 1682 s (C=O Amide), 1553–1498 m (C=C Aromatic). 1 × aromatic C was unaccounted for in the ¹³C NMR spectra due to overlapping signals. Characterisation is consistent with data reported in the literature.⁵

**N-(17-hydroxy-3,6,9,12,15-pentaoxaheptadecan-1-yl)-4-(pyren-1-yl)butanamide 1**

Hydroxybenzotriazole (112 mg, 0.832 mmol), N-(3-dimethylaminopropyl)-N’-ethylcarbodiimide hydrochloride (119 mg, 0.624 mmol) and a solution of 17-amino-3,6,9,12,15-pentaoxaheptadecan-1-ol S₆ (107 mg, 0.624 mmol) in DCM (8.0 mL) were added sequentially to a stirred solution of pyrenebutyric acid S₇ (100 mg, 0.347 mmol) in dry DCM (8.0 mL). The reaction was stirred until determined complete by LC-MS (21 h). The reaction mixture was washed with sat. NH₄Cl (3 × 10 mL) and brine (10 mL). The organic layer was then dried (Na₂SO₄) and the solvent removed in vacuo. The crude residue was purified by flash chromatography (SiO₂; CHCl₃–MeOH 99:1 → 90:10) to afford the product 1 as a brown oil (163 mg, 0.295 mmol, 85%). Rₑ 0.08 (SiO₂; CHCl₃–MeOH 95:5). ¹H NMR (500 MHz, CDCl₃) δ 8.32 (t, 1H, J 7.2 Hz, Aryl), 8.18–8.13 (m, 2H, Aryl), 8.12–8.08 (m, 2H, Aryl), 8.04–7.96 (m, 3H, Aryl), 7.88 (d, 1H, J 7.8 Hz, Aryl), 6.60 (br s, 1H, CONH-1), 3.64 (m, 2H, OCH₂CH₂O), 3.59–3.44 (m, 22H, OCH₂CH₂O), 3.40 (t, 2H, J 7.6 Hz, CH₂-4), 2.33 (t, 2H, J 7.2 Hz, CH₂-2), 2.26–2.19 (m, 2H, CH₂-3). ¹³C NMR (125 MHz, CDCl₃) δ 173.0 (CONH-1), 136.3 (Aryl C), 131.6 (Aryl C), 131.1 (Aryl C), 130.0 (Aryl C), 129.0 (Aryl C), 127.6 (Aryl CH), 127.5 (Aryl CH), 127.5 (Aryl CH), 126.8 (Aryl CH), 126.0 (Aryl CH), 125.2 (Aryl C), 125.2 (Aryl C), 125.0 (Aryl CH), 124.9 (Aryl CH), 124.9 (Aryl CH), 123.7 (Aryl CH), 72.8 (CH₂), 70.7 (CH₂), 70.6 (CH₂), 70.6 (CH₂), 70.5 (CH₂), 70.5 (CH₂), 70.3 (CH₂), 70.2 (CH₂), 70.1 (CH₂), 61.8 (CH₂), 39.4 (CH₂), 36.1 (CH₂-2), 33.0 (CH₂-4), 27.7 (CH₂-3). HR-ESI-MS Calculated for C₃₂H₄₂N₂O₇: m/z 552.2956 [M+H]+; found 552.2961. LC-MS Calculated for C₃₂H₄₁N₂NaO₂: m/z 574.2775 [M+Na]+; found 574.2. Rₑ = 1.89. UV-Vis (MeOH) λₘₐₓ 234 nm (ε 25190 M⁻¹cm⁻¹), 243 nm (ε 41860 M⁻¹cm⁻¹), 265 nm (ε 15450 M⁻¹cm⁻¹), 276 nm (ε 28360 M⁻¹cm⁻¹), 326 nm (ε 17260 M⁻¹cm⁻¹), 342 nm (ε 25680 M⁻¹cm⁻¹), 355 nm (ε 730 M⁻¹cm⁻¹).
Supplementary Figure 27: Analytical HPLC trace for N-(17-hydroxy-3,6,9,12,15-pentaoxaheptadecan-1-yl)-4-(pyren-1-yl)butanamide 1.

Supplementary Figure 28: $^1$H NMR spectra (500 MHz, CDCl$_3$) for N-(17-hydroxy-3,6,9,12,15-pentaoxaheptadecan-1-yl)-4-(pyren-1-yl)butanamide 1.
Supplementary Figure 29: $^{13}$C NMR spectra (125 MHz, CDCl$_3$) for $N$-(17-hydroxy-3,6,9,12,15-pentaoxaheptadecan-1-yl)-4-(pyren-1-yl)butanamide 1.

4-(6-acetamidopyren-1-yl)-$N$-(17-hydroxy-3,6,9,12,15-pentaoxaheptadecan-1-yl)butanamide 2

Hydroxybenzotriazole (25 mg, 0.19 mmol), $N$-(3-dimethylaminopropyl)-$N'$-ethylcarbodiimide hydrochloride (25 mg, 0.14 mmol) and a solution of 17-amino-3,6,9,12,15-pentaoxaheptadecan-1-ol S6 (22 mg, 0.077 mmol) in dry DCM (2.5 mL) were added sequentially to a stirred suspension of 4-(6-acetamidopyren-1-yl)butanoic acid S9 (24 mg, 0.069 mmol) in dry DCM (2.5 mL). The reaction was stirred until determined complete by LC-MS (21 h). The reaction mixture was diluted with DCM (10 mL) and filtered to remove precipitate. The filtrate was washed with sat. NH$_4$Cl (3 × 10 mL) and brine (10 mL). The organic layer was then dried (Na$_2$SO$_4$) and the solvent removed in vacuo. The crude residue was purified by flash chromatography (SiO$_2$; CHCl$_3$–MeOH 95:5) to afford the product 2 as a yellow waxy solid (27 mg, 0.044 mmol, 63%). $R_f$ 0.08 (SiO$_2$; CHCl$_3$–MeOH 95:5). $^1$H NMR (500 MHz,
CDCl₃ δ 8.25 (d, 1H, Aryl), 8.21 – 8.17 (m, 1H, Aryl), 8.06 (d, 1H, J 8.2 Hz, Aryl), 8.01 – 7.94 (m, 3H, Aryl), 7.80 (d, 1H, J 7.8 Hz, Aryl), 6.79 (br t, 1H, J 5.1 Hz, CONH-1), 3.63 (d, 2H, J 4.4 Hz, OCH₂CH₂O), 3.58 – 3.44 (m, 22H, OCH₂CH₂O), 3.33 (t, 2H, J 7.6 Hz, CH₂-4), 2.38 (s, 3H, CH₃CONH-, 2'''), 2.32 (t, 2H, J 7.2 Hz, CH₂-2), 2.22 – 2.14 (m, 2H, CH₂-3). ¹³C NMR (125 MHz, CDCl₃) δ 173.1 (CONH-1), 169.5 (CH₃CONH-1'''), 136.7 (Aryl C), 130.3 (Aryl C), 129.6 (Aryl C), 129.1 (Aryl C), 129.0 (Aryl C), 128.0 (Aryl CH), 127.7 (Aryl CH), 127.3 (Aryl CH), 125.5 (Aryl C), 125.2 (Aryl C), 124.9 (Aryl CH), 124.4 (Aryl C), 123.1 (Aryl CH), 123.0 (Aryl CH), 119.9 (Aryl CH), 72.8 (CH₂), 70.6 (CH₂), 70.5 (CH₂), 70.4 (CH₂), 70.4 (CH₂), 70.1, (CH₂), 70.0 (CH₂), 61.7 (CH₂), 39.4 (CH₂), 36.0 (CH₂-2), 33.0 (CH₂-4), 27.6 (CH₂-3), 24.4 (CH₃CONH-2'''). HR-ESI-MS Calculated for C₃₄H₄₄N₂O₈Na+: m/z 631.2990 [M+Na]⁺; found 552.2961. LC-MS Calculated for C₃₄H₄₅N₂O₈: m/z 609.3170 [M+Na]⁺; found 609.35, Rₜ 0.52. IR (Neat, νmax/cm⁻¹) 3313 br m (OH hydroxy), 3269 m (NH amide), 1639 s (C=O amide), 1550 – 1459 m (C=C aromatic), 1093 s (C-O aliphatic ether). UV-Vis (MeOH) λmax 245 nm (ε 41029 M⁻¹cm⁻¹), 279 nm (ε 40023 M⁻¹cm⁻¹), 348 nm (ε 39430 M⁻¹cm⁻¹), 355 nm (ε 25090 M⁻¹cm⁻¹). 1 × aromatic CH and 2 × CH₂ from the OEG chain were unaccounted for in the ¹³C NMR spectra due to overlapping signals.

Supplementary Figure 30: Analytical HPLC trace for 4-(6-acetamidopyren-1-yl)-N-(17-hydroxy-3,6,9,12,15-pentaoxaheptadecan-1-yl)butanamide 2.
Supplementary Figure 31: $^1$H NMR spectra (500 MHz, CDCl$_3$) for 4-(6-acetamidopyren-1-yl)-$N$-(17-hydroxy-3,6,9,12,15-pentaoxaheptadecan-1-yl)butanamide 2.
Supplementary Figure 32: $^{13}$C NMR spectra (125 MHz, CDCl$_3$) for 4-(6-acetamidopyren-1-yl)-N-(17-hydroxy-3,6,9,12,15-pentaoxaheptadecan-1-yl)butanamide 2.

2-chloro-N-(pyren-1-yl) acetamide S12

Under an N$_2$ atmosphere, triethylamine (1.27 mL, 9.12 mmol) and chloroacetyl chloride S11 (550 µL, 6.91 mmol) were added dropwise and sequentially to a stirred solution of 1-aminopyrene S10 (1.00 g, 4.56 mmol) in THF (20 mL) at 0 °C (ice bath). The reaction was then allowed to warm to room temperature and stir until determined complete by TLC (5 h). The solvent was removed under reduced pressure and the residue taken up in DCM (250 mL) and washed with sat. NaHCO$_3$ (2 × 50 mL), H$_2$O (50 mL), 1M HCl (2 × 50 mL), H$_2$O (2 × 50 mL), and brine (20 ml). The organic layer was dried (Na$_2$SO$_4$) and concentrated in vacuo to afford the product S12 as a brown solid (1.32 g, 4.49 mmol, 98%). $R_f$ 0.65 (SiO$_2$; DCM–EtOAc 90:10). $^1$H NMR (500 MHz, CDCl$_3$) δ 8.99 (br s, 1H, CONH-1), 8.41 (d, 1H, J 8.3 Hz, Aryl), 8.22 – 8.14 (m, 3H, Aryl), 8.11 (d, 1H, J 9.2 Hz, Aryl), 8.06 – 7.98 (m, 4H, Aryl), 8.06 – 7.98 (m, 4H, Aryl), 4.43 (s, 2H, \text{CH}$_2$-2). $^{13}$C NMR (125 MHz, CDCl$_3$) 164.8 (CONH-1), 131.4 (Aryl C), 130.9 (Aryl C), 129.7 (Aryl C), 129.01 (Aryl C), 128.6 (Aryl CH), 127.4 (Aryl CH), 127.4 (Aryl CH), 126.4 (Aryl CH), 125.9 (Aryl CH), 125.5 (Aryl CH), 125.3 (Aryl CH), 125.2 (Aryl C), 124.8 (Aryl C), 123.8 (Aryl C), 121.9 (Aryl CH), 119.8 (Aryl CH), 43.5 (CH$_2$-2). HR-ESI-MS Calculated for C$_{18}$H$_{12}$ClN: $m/z$ 316.0500 [M+Na]$^+$; found 316.0494. LC-MS Calculated for C$_{18}$H$_{13}$ClNO: $m/z$ 294.06802 [M+H]$^+$; found 294.33. IR (Neat, $\nu$ max/cm$^{-1}$) 3233 m (NH amide), 1557–1409 m (C=C aromatic), 840 s (C-Cl alkyl). Characterisation is consistent with data reported in the literature.$^6$

20-hydroxy-N-(pyren-1-yl)-3,6,9,12,15,18-hexaoxaicosanamide 3

A solution of N-pyren-1-yl chloroacetamide S12 (500 mg, 1.70 mmol) in DMF (2.5 mL) was added dropwise to an ice-cooled and stirred solution of hexaethylene glycol S3 (1.28 mL, 5.09 mmol) and
60% NaH in mineral oil (204 mg, 5.10 mmol) in DMF (3.5 mL) and stirred for 30 minutes. The reaction was then allowed to warm to room temperature and stir overnight until determined complete by LC-MS (16 h). The reaction was quenched with 1M HCl (10 mL) then extracted with CHCl₃ (3 × 10 mL). The org layers were collected and washed with 1M LiCl (5 × 10 mL), and brine (10 mL). The organic layer was then dried (Na₂SO₄) and the solvent removed in vacuo to give a crude brown oil. The crude product was then purified by flash chromatography (SiO₂; DCM–EtOAc–MeOH 9:9:2) to yield the product 3 as a brown oil (597 mg, 1.11 mmol, 65%). Rf 0.35 (SiO₂; DCM–EtOAc–MeOH 9:9:2)

³¹H NMR (500 MHz, CDCl₃) δ 9.61 (br s, 1H, CONH-1), 8.48 (d, 1H, J 8.2 Hz, Aryl), 8.21–8.15 (m, 4H, Aryl), 8.11 (d, 1H, J 9.2 Hz, Aryl), 8.05–7.98 (m, 3H, Aryl), 4.36 (s, 2H, CH₂-2), 3.98–3.92 (m, 2H, OCH₂CH₂O), 3.85–3.80 (m, 2H, OCH₂CH₂O), 3.73–3.68 (m, 2H, OCH₂CH₂O), 3.68–3.63 (m, 4H, OCH₂CH₂O), 3.56–3.47 (m, 8H, OCH₂CH₂O), 3.45–3.41 (m, 2H, OCH₂CH₂O), 3.40–3.35 (m, 2H, OCH₂CH₂O), 3.27 (s, 4H, OCH₂CH₂O).

¹³C NMR (125 MHz, CDCl₃) δ 169.3 (CONH-1), 131.5 (Aryl C), 131.0 (Aryl C), 129.3 (Aryl C), 128.0 (Aryl CH), 127.5 (Aryl CH), 127.0 (Aryl CH), 126.3 (Aryl CH), 125.5 (Aryl CH), 125.4 (Aryl CH), 125.3 (Aryl C), 125.1 (Aryl CH), 124.9 (Aryl C), 123.9 (Aryl C), 122.4 (Aryl CH), 121.0 (Aryl CH), 72.7 (CH₂), 71.7 (CH₂), 71.2 (CH₂), 70.8 (CH₂), 70.6 (CH₂), 70.5 (CH₂), 70.4 (CH₂), 70.3 (CH₂), 70.3 (CH₂), 61.8 (CH₂). HR-ESI-MS Calculated for C₃₀H₃₈N₂O₁₀: m/z 540.2591 [M+H]+; found 540.2600.

LC-MS found 540.30, Rₜ 0.65. IR (Neat, νmax/cm⁻¹) 3417 br m (OH hydroxy), 3269 m (NH amide), 1639 s (C=O amide), 1550–1458 m (C=C aromatic), 1093 s (C-O aliphatic ether). UV-Vis (MeOH) λmax 242 nm (ε 35512 M⁻¹cm⁻¹), 276 nm (ε 26195 M⁻¹cm⁻¹), 340 nm (ε 24603 M⁻¹cm⁻¹), 355 nm (ε 10870 M⁻¹cm⁻¹). 2 × CH₂ from the OEG chain were unaccounted for in the ¹³C NMR spectra due to overlapping signals.

Supplementary Figure 33: Analytical HPLC trace for 20-hydroxy-N-(pyren-1-yl)-3,6,9,12,15,18-hexaoxaicosanamide 3.
Supplementary Figure 34: $^1$H NMR spectra (500 MHz, CDCl$_3$) 20-hydroxy-N-(pyren-1-yl)-3,6,9,12,15,18-hexaoaicosanamide 3.

Supplementary Figure 35: $^{13}$C NMR spectra (125 MHz, CDCl$_3$) 20-hydroxy-N-(pyren-1-yl)-3,6,9,12,15,18-hexaoaicosanamide 3.
Triphosgene (298 mg, 1.00 mmol) was added portion-wise slowly to a stirred solution of 1-aminopyrene S10 (200 mg, 0.912 mmol) in DCM (10 mL) at 0 °C (ice bath) and allowed to stir for 1 hr. The reaction was then allowed to warm to room temperature with continued stirring (24 h). The reaction mixture was then dry loaded onto celite (200 mg) and purified by filtration through a short silica plug, eluting with DCM. Solvent was removed in vacuo to afford the product S13 as a yellow solid (214 mg, 0.880 mmol, 96%).

\[^{1}H\text{NMR}\](500 MHz, CDCl\textsubscript{3}) \(\delta 8.29\) (d, 1H, J 9.1 Hz, Aryl), 8.20 (d, 2H, J 7.6 Hz, Aryl), 8.15 (d, 1H, J 9.1 Hz, Aryl), 8.10 (d, 1H, J 8.1 Hz, Aryl), 8.07 – 8.01 (m, 3H, Aryl).

\[^{13}C\text{NMR}\](125 MHz, CDCl\textsubscript{3}) \(\delta 131.5\) (Aryl C), 131.2 (Aryl C), 129.4 (Aryl C), 128.6 (Aryl CH), 127.5 (Aryl CH), 127.17 (Aryl CH), 127.0 (NCO-1'), 126.7 (Aryl CH), 125.6 (Aryl CH), 125.5 (Aryl CH), 125.4 (Aryl C), 125.5 (Aryl CH), 123.4 (Aryl C), 123.2 (Aryl CH), 122.0 (Aryl CH). \[^{1}R\text{IR}\](Neat, \(\nu_{\text{max}}/\text{cm}^{-1}\)) 2255 s (N=C=O). 2 \times\) aromatic Cs were unaccounted for in the \(^{13}C\) NMR spectra due to overlapping signals. Characterisation is consistent with data reported in the literature.\(^{7}\)

A solution of 17-amino-3,6,9,12,15-pentaoxaheptadecan-1-ol S6 (180 mg, 0.740 mmol) in THF (1.0 mL) was added dropwise to a stirred solution of 1-isocyanopyrene S13 (228 mg, 0.812 mmol) in THF (10 mL) at 0 °C (ice bath) and allowed to stir for 30 min. The reaction was then allowed to warm to room temperature and stir overnight (21 h). Solvent was then removed under reduced pressure and the crude residue purified by flash chromatography (SiO\textsubscript{2}; DCM–MeOH 95:5) to afford the product 4 as a brown waxy solid (217 mg, 0.414 mmol, 56%).

\[^{1}H\text{NMR}\](400 MHz, CDCl\textsubscript{3}) \(\delta 8.35\) (d, 1H, J 8.3 Hz, Aryl), 8.18 (d, 1H J 9.2 Hz, Aryl), 8.10 – 8.00 (m, 4H, Aryl), 7.94 – 7.88 (m, 4H, Aryl + NH-1), 6.25 (br s, 1H, NH-3), 3.66 – 3.42 (m, 24H, OCH\textsubscript{2}CH\textsubscript{2}O), 3.15 (br s, 1H, OH). \[^{13}C\text{NMR}\](100 MHz, CDCl\textsubscript{3}) \(\delta 157.2\) (NHCONH-2), 132.7 (Aryl C), 131.5 (Aryl C), 131.1 (Aryl C), 128.0 (Aryl C), 125.5 (Aryl CH), 125.4 (Aryl C), 125.3 (Aryl CH), 123.4 (Aryl C), 123.2 (Aryl CH), 122.0 (Aryl CH).
127.4 (Aryl CH), 127.1 (Aryl CH), 126.1 (Aryl CH), 126.0 (Aryl CH), 125.4 (Aryl CH), 125.3 (Aryl C), 124.9 (Aryl CH), 124.9 (Aryl C), 124.5 (Aryl CH), 123.5 (Aryl C), 122.0 (Aryl CH), 121.4 (Aryl CH), 72.6 (CH₂), 70.6 (CH₂), 70.6 (CH₂), 70.5 (CH₂), 70.4 (CH₂), 70.2 (CH₂), 70.2 (CH₂), 61.6 (CH₂), 40.4 (CH₂). **HR-ESI-MS**

Calculated for C₂₉H₃₇N₂O₇: m/z 525.2595 [M+H]+; found 525.2591. **LC-MS** found 525.54, Rₜ 0.55. **IR** (Neat, νmax/cm⁻¹) 3359 br m (OH hydroxy), 3285 m (NH urea), 1626 s (C=O urea), 1525–1416 m (C=C aromatic). **UV-Vis** (MeOH) λmax 223 nm (ε 133386 M⁻¹cm⁻¹), 278 nm (ε 23040 M⁻¹cm⁻¹), 341 nm (ε 21640 M⁻¹cm⁻¹), 355 nm (ε 17180 M⁻¹cm⁻¹). 3 × CH₂ from the OEG chain were unaccounted for in the ¹³C NMR spectra due to overlapping signals.

**Supplementary Figure 36:** Analytical HPLC trace for 3-(17-hydroxy-3,6,9,12,15-pentaoxaheptadecan-1-yl)-1-(pyren-1-yl)urea 4.
Supplementary Figure 37: $^1$H NMR spectra (400 MHz, CDCl$_3$) for 3-(17-hydroxy-3,6,9,12,15-pentaoxaheptadecan-1-yl)-1-(pyren-1-yl)urea 4.

Supplementary Figure 38: $^{13}$C NMR spectra (100 MHz, CDCl$_3$) for 3-(17-hydroxy-3,6,9,12,15-pentaoxaheptadecan-1-yl)-1-(pyren-1-yl)urea 4.
**N-(pyren-1-yl)formamide S14**

Acetic formic anhydride (55 µL, 0.69 mmol) was added to a stirred solution of 1-aminopyrene S10 (100 mg, 0.460 mmol) in THF (1 mL) and allowed to stir until determined complete by LC-MS (3 h). Solvent removed under reduced pressure to yield the product S14 as an off-white solid (113 mg, 0.460 mmol, Quant.). Rf 0.19 (SiO2; DCM–EtOAc 9:1). \(^1^H\) NMR (400 MHz, DMSO) δ 10.85 (d, 2H, J 10.4 Hz, NH), 10.69 (br s, 5H, NH), 8.81 (d, 2H, J 10.5 Hz, CHO-1), 8.61 (s, 4H, CHO-1), 8.57 (d, 5H, J 8.3 Hz, Aryl), 8.38 (d, 7H, J 9.3 Hz, Aryl), 8.30 – 8.21 (m, 28H, Aryl), 8.17 – 8.02 (m, 24H, Aryl). \(^1^C\) NMR (100 MHz, DMSO) δ 164.5 (CHO-1), 160.4 (CHO-1), 131.0 (Aryl C), 130.9 (Aryl C), 130.4 (Aryl C), 127.9 (Aryl C), 127.5 (Aryl CH), 127.3 (Aryl CH), 127.2 (Aryl CH), 126.6 (Aryl CH), 126.5 (Aryl CH), 125.6 (Aryl CH), 125.3 (Aryl CH), 125.1 (Aryl CH), 124.9 (Aryl CH), 124.4 (Aryl CH), 123.9 (Aryl C), 122.1 (Aryl C), 121.8 (Aryl CH), 121.4 (Aryl CH), 121.3 (Aryl CH), 120.0 (Aryl CH). HR-ESI-MS Calculated for C\(_{17}\)H\(_{12}\)NO: 246.0913 m/z [M+H]\(^+\); found 246.0908. \(^2\) LC-MS found 246.30, R\(_t\) 0.70. IR (Neat, \(\nu_{\text{max}}/\text{cm}^{-1}\)) 3269 m (NH amide), 1696 s (C=O amide), 1550–1458 m (C=C aromatic). From the experimentally obtained data it was noted that S14 appears as a 2:5 mixture of rotamers in the \(^1^C\) and \(^1^H\) NMR spectra.

**N-methylpyren-1-amine S15**

Under an N\(_2\) atmosphere, 1M LiALH\(_4\) (377 µL, 0.377 mmol) was added dropwise to a rapidly stirred suspension of N-(pyren-1-yl)formamide S14 (75 mg, 0.31 mmol) in THF (2.0 mL) at 0 °C (ice bath). The reaction was then allowed to warm to room temperature and stir until determined complete by LC-MS (2 h). The reaction was then cooled to 0 °C and quenched with EtOAc (10 mL). Na\(_2\)SO\(_4\)•10H\(_2\)O and Celite (1:1 w/w) (1.00 g) was added to the reaction mixture and allowed to stir (10 min) then filtered and washed thoroughly with EtOAc (100 mL). The solvent was removed under reduced pressure to yield the product S15 as a brown waxy solid (70 mg, 0.30 mmol, 99%). Rf 0.18 (SiO2; DCM–Hex 40:60). \(^1^H\) NMR (400 MHz, CDCl\(_3\)) δ 8.12 – 7.87 (m, 7H, Aryl), 7.77 (d, 1H, J 8.8 Hz, Aryl), 7.32 (d, 1H, J 8.4 Hz,
Aryl), 4.85 (br s, 1H, NH), 3.16 (s, 3H, CH$_3$-1'). $^{13}$C NMR (100 MHz, CDCl$_3$) δ 143.6 (Aryl C), 132.6 (Aryl C), 131.8 (Aryl C), 127.9 (Aryl CH), 126.6 (Aryl CH), 126.0 (Aryl CH), 125.9 (Aryl CH), 125.9 (Aryl C), 123.9 (Aryl CH), 123.3 (Aryl CH), 123.1 (Aryl C), 119.5 (Aryl CH), 116.6 (Aryl C), 108.4 (Aryl CH), 31.3 (CH$_3$-1').

HR-ESI-MS Calculated for C$_{17}$H$_{14}$N: 232.1121 m/z [M+H]$^+$; found 232.1115. LC-MS found 232.33, R$_t$ 0.85. IR (Neat, $\nu_{\text{max}}$/cm$^{-1}$) 3267 m (NH amine), 1555 – 1455 m (C=C aromatic). 1 × aromatic C and 1 × aromatic CH were unaccounted for in the $^{13}$C NMR spectra due to overlapping signals.

2-chloro-N-methyl-N-(pyren-1-yl) acetamide S16

[Chemical structure image]

Under an N$_2$ atmosphere, triethylamine (255 µL, 1.83 mmol) and chloroacetyl chloride S11 (100 µL, 1.26 mmol) were added sequentially to a stirred solution of N-methylpyren-1-amine S15 (200 mg, 0.912 mmol) in THF (5.0 mL) at 0 °C (ice bath). The reaction was then allowed to warm to room temperature and stir until determined complete by TLC (1 h). The solvent was removed under reduced pressure and the residue taken up in DCM (30 mL) and washed with sat. NaHCO$_3$ until the aqueous layer remained basic (pH 8). The organic phase was washed with H$_2$O (30 mL × 2), and brine (20 ml), then dried (Na$_2$SO$_4$) and concentrated in vacuo to afford the product S16 as an amorphous yellow solid (280 mg, 0.911 mmol, Quant.). R$_t$ 0.65 (SiO$_2$; DCM–EtOAc 90:10). $^1$H NMR (400 MHz, CDCl$_3$) $^1$H NMR (400 MHz, CDCl$_3$) δ 8.33 – 8.20 (m, 4H, Aryl), 8.17 (d, 1H, J 8.8 Hz, Aryl), 8.14 – 8.06 (m, 2H, Aryl), 8.01 (d, 1H, J 9.2 Hz, Aryl), 7.91 (d, 1H, J 8.1 Hz, Aryl), 7.38 (d, 1H, J 13.4 Hz, CH-2), 3.73 (d, 1H, J 13.4 Hz, CH-2), 3.56 (s, 3H, CH$_3$-1'). $^{13}$C NMR (100 MHz, CDCl$_3$) δ 167.4 (CONR$_2$-1), 135.6 (Aryl C), 136.5 (Aryl C), 131.8 (Aryl C), 131.2 (Aryl C), 130.9 (Aryl C), 130.0 (Aryl CH), 128.8 (Aryl CH), 127.8 (Aryl C), 127.1 (Aryl CH), 126.9 (Aryl CH), 126.5 (Aryl CH), 126.3 (Aryl CH), 125.8 (Aryl CH), 125.6 (Aryl CH), 124.6 (Aryl C), 120.9 (Aryl CH), 42.1 (CH$_2$-2), 38.4 (CH$_3$-1”). HR-ESI-MS Calculated for C$_{19}$H$_{15}$ClNO: 308.0837 m/z [M+H]$^+$; found 308.0830. LC-MS found 308.33, R$_t$ 0.8. IR (Neat, $\nu_{\text{max}}$/cm$^{-1}$) 1660 s (C=O amide), 1557–1409 m (C=C aromatic), 843 s (C-Cl alkyl).
A solution of 2-chloro-N-methyl-N-(pyren-1-yl) acetamide S16 (133 mg, 0.432 mmol) in DMF (1 mL) was added dropwise to an ice-cooled and stirred solution of hexaethylene glycol S3 (325 µL, 1.30 mmol) and 60% NaH in mineral oil (57 mg, 1.4 mmol) in DMF (1 mL) and stirred for 30 minutes. The reaction was then allowed to warm to room temperature and stir overnight until determined complete by LC-MS (16 h). The reaction was quenched with 1M HCl (1 mL) then extracted with CHCl₃ (3 × 2 mL). The org layers were collected and washed with 1M LiCl (5 × 2 mL), and brine (2 mL). The organic layer was then dried (Na₂SO₄) and the solvent removed in vacuo to give a crude brown oil. The crude product was then purified by flash chromatography (SiO₂; DCM–MeOH 95:5) to yield the product S1 as a brown oil (3 mg, 0.005 mmol, >1%). Rf 0.17 (SiO₂; DCM–MeOH 95:5). HR-ESI-MS Calculated for C₃₁H₄₀NO₈: 554.2748 m/z [M+H]⁺; found 554.2758. LC-MS found 554.65, Rf 0.75. UV-Vis (MeOH) λmax 242 nm (ε 20980 M⁻¹cm⁻¹), 265 nm (ε 12280 M⁻¹cm⁻¹), 276 nm (ε 17470 M⁻¹cm⁻¹), 312 nm (ε 6210 M⁻¹cm⁻¹), 326 nm (ε 12200 M⁻¹cm⁻¹), 341 nm (ε 16360 M⁻¹cm⁻¹), 355 nm (ε 3350 M⁻¹cm⁻¹).

Supplementary Figure 39: Analytical HPLC trace for 20-hydroxy-N-methyl-N-(pyren-1-yl)-3,6,9,12,15,18-hexaoxaicosanamide S1
2-methoxy-N-(pyren-1-yl) acetamide S2

![Structure of 2-methoxy-N-(pyren-1-yl) acetamide S2]

2-chloro-N-(pyren-1-yl) acetamide S12 (128 mg, 0.436 mmol) was dissolved in a methanolic solution of NaOMe (25% w/w, 10 mL, 131 mmol) and allowed to stir at room temperature until determined complete by TLC (16 h). The pH of the reaction mixture was quenched with 2M HCl (40 ml) and extracted with DCM (5 × 30 mL). The organic layers were then combined, washed with brine (30 ml) and dried (Na₂SO₄). The solvent was then removed under reduced pressure to give the product S2 as a brown oil (126 mg, 0.436 mmol, Quant.) Rf 0.09 (SiO₂; DCM).

1H NMR (400 MHz, CDCl₃) δ 9.03 (s, 1H, CONH), 8.54 (d, 1H, J 8.3, Aryl), 8.17 – 8.11 (m, 3H, Aryl), 8.06 (d, 1 H, J 9.2, Aryl), 7.99 – 7.95 (m, 4H, Aryl), 4.23 (s, 2H, CH₂-2), 3.64 (3H, OCH₃).

13C NMR (100 MHz, CDCl₃) δ 168.1 (CONH), 131.3 (Aryl C), 130.8 (Aryl C), 129.6 (Aryl C), 129.0 (Aryl C), 128.0 (Aryl CH), 127.3 (Aryl CH), 126.8 (Aryl CH), 126.1 (Aryl CH), 125.5 (Aryl CH), 125.3 (Aryl CH), 125.1 (Aryl C), 125.0 (Aryl CH), 124.7 (Aryl C), 122.8 (Aryl C), 121.2 (Aryl CH), 119.7 (Aryl CH), 72.5 (CH₂-2), 59.5 (OCH₃). HR-ESI-MS Calculated for C₁₉H₁₆NO₂: m/z 290.1176 [M+H]+; found 290.1171. LC-MS found, 290.31 Rₜ 0.65. IR (Neat, νmax/cm⁻¹) 3266 m (NH amide), 1668 s (C=O amide), 1515–1429 m (C=C aromatic).

20-[(1,3-dioxo-2,3-dihydro-1H-isoyindol-2-yl)oxy]-N-(pyren-1-yl)-3,6,9,12,15,18-hexaoxaicosanamide S18

![Structure of 20-[(1,3-dioxo-2,3-dihydro-1H-isoyindol-2-yl)oxy]-N-(pyren-1-yl)-3,6,9,12,15,18-hexaoxaicosanamide S18]

A solution of 20-hydroxy-N-(pyren-1-yl)-3,6,9,12,15,18-hexaoxaicosanamide 3 (210 mg, 0.389 mmol) in THF (3 mL), and N-hydroxyphthalimide S17 (190 mg, 1.17 mmol) were added sequentially to an ice-cooled solution of DIAD (230 µL, 1.17 mmol) and triphenylphosphine (306 mg, 1.17 mmol) in THF (5 mL) and stirred for 10 min. The reaction was then allowed to warm to room temperature and stir overnight (20 h). The reaction mixture was then concentrated in vacuo. The crude residue was purified by flash chromatography (SiO₂; EtOAc–MeOH 100:0 → 90:10) to afford the product S18 as a clear orange oil (221 mg, 0.341 mmol, 88%). Rf 0.23 (SiO₂; EtOAc–MeOH 90:10). 1H NMR (400 MHz, CDCl₃)
δ 9.57 (br s, 1H, CONH-1), 8.49 (d, 1H, J 8.3 Hz, Aryl), 8.21 – 8.15 (m, 5H, Aryl), 8.12 (d, 1H, J 9.2 Hz, Aryl), 8.06 – 7.98 (m, 4H, Aryl), 7.80 – 7.76 (m, 2H, CH-4', CH-7'), 7.72 – 7.65 (m, 2H, CH-5', CH-6'), 4.36 (s, 2H, CH-2), 3.98 – 3.95 (m, 2H, OCH-2), 3.85 – 3.81 (m, 2H, OCH-2), 3.61 – 3.58 (m, 2H, OCH-2), 3.46 – 3.42 (m, 2H, OCH-2), 3.31 – 3.24 (m, 2H, OCH-2).

13C NMR (100 MHz, CDCl3) δ 169.2 (CONH-1), 163.54 (CONHCO-1', CONHCO-3'), 134.5 (CH-5', CH-6'), 131.5 (Aryl C), 131.0 (Aryl C), 130.2 (Aryl C), 129.3 (Aryl C), 128.0 (Aryl CH), 127.5 (Aryl CH), 127.0 (Aryl CH), 126.3 (Aryl CH), 125.4 (Aryl CH), 125.0 (Aryl CH), 124.9 (Aryl C), 123.8 (Aryl C), 123.6 (CH-4', CH-7'), 122.3 (Aryl CH), 70.9 (CH2), 70.8 (CH2), 70.6 (CH2), 70.5 (CH2), 70.4 (CH2), 69.3 (CH2).

HR-ESI-MS Calculated for C38H41N2O10+: m/z 685.2756 [M+H]+; found 685.2765. LC-MS found 685.58, Rf 0.75.

3 × CH2 from the OEG chain were unaccounted for in the 13C NMR spectra due to overlapping signals.

20-hydroxylamino-N-(pyren-1-yl)-3,6,9,12,15,18-hexaoxaicosanamide 5

80% hydrazine hydrate (46 µL, 0.76 mmol) was added dropwise to a rapidly stirred solution of 20-[(1,3-dioxo-2,3-dihydro-1H-isindol-2-yl)oxy]-N-(pyren-1-yl)-3,6,9,12,15,18-hexaoxaicosanamide S18 (164 mg, 0.253 mmol) in THF (6 mL) until determined complete by LC-MS (3h). The reaction mixture was then filtered through celite and washed through with THF (20 mL). The solvent was then removed under reduced pressure to give the product 5 as a brown oil. (134 mg, 0.242 mmol, 96%) Rf 0.16 (SiO2; EtOAc–MeOH 80:20). 1H NMR (500 MHz, CDCl3) δ 9.60 (br s, 1H, CONH-1), 8.48 (d, 1H, J 8.2 Hz, Aryl), 8.21 – 8.15 (m, 4H, Aryl), 8.11 (d, 1H, J 9.2 Hz, Aryl), 8.05 – 7.98 (m, 3H, Aryl), 4.35 (s, 2H, CH2-2), 3.97 – 3.92 (m, 2H, OCH2CH2O), 3.85 – 3.81 (m, 2H, OCH2CH2O), 3.80 – 3.76 (m, 2H, OCH2CH2O), 3.72 – 3.68 (m, 2H, OCH2CH2O), 3.61 – 3.58 (m, 2H, OCH2CH2O), 3.56 – 3.53 (m, 2H, OCH2CH2O), 3.52 – 3.48 (m, 4H, OCH2CH2O), 3.46 – 3.42 (m, 2H, OCH2CH2O), 3.40 – 3.36 (m, 2H, OCH2CH2O), 3.31 – 3.24 (m, 4H, OCH2CH2O). 13C NMR (125 MHz, CDCl3) δ 169.3 (CONH-1), 131.4 (Aryl C), 131.0 (Aryl C), 130.2 (Aryl C), 129.3 (Aryl C), 127.9 (Aryl CH), 127.4 (Aryl CH), 127.0 (Aryl CH), 126.3 (Aryl CH), 125.5 (Aryl CH), 125.3 (Aryl CH), 125.1 (Aryl CH), 124.8 (Aryl C), 123.8 (Aryl C), 122.3 (Aryl CH), 120.9 (Aryl CH), 71.8 (CH2), 71.2 (CH2), 70.9 (CH2), 70.6 (CH2), 70.5 (CH2), 70.4 (CH2), 69.3 (CH2).
74.8 (CH₃), 71.7 (CH₂), 71.1 (CH₂), 70.8 (CH₂), 70.5 (CH₂), 70.4 (CH₂), 70.4 (CH₂), 70.4 (CH₂), 70.3 (CH₂).

**HR-ESI-MS** Calculated for C₃₀H₃₉N₂O₈: m/z 555.2701 [M+H]⁺; found 555.2698. **LC-MS** found 555.61, Rₗ 0.55. 4 × CH₂ from the OEG chain were unaccounted for in the ¹³C NMR spectra due to overlapping signals.

3-{17-[{1,3-dioxo-2,3-dihydro-1H-isoindol-2-yl}oxy]-3,6,9,12,15-pentaoxaheptadecan-1-yl}-1-(pyren-1-yl)urea S₁₉

![Chemical Structure](image)

A solution of 3-(17-hydroxy-3,6,9,12,15-pentaoxaheptadecan-1-yl)-1-(pyren-1-yl)urea 4 (500 mg, 0.953 mmol) in THF (3 mL), and N-hydroxypythalimide S₁₇ (466 mg, 2.86 mmol) were added sequentially to an ice-cooled solution of DIAD (563 µL, 2.86 mmol) and triphenylphosphine (750 mg, 2.86 mmol) in THF (5 mL) and stirred for 10 min. The reaction was then allowed to warm to room temperature and stir overnight (20 h). The reaction mixture was then concentrated in vacuo. The crude residue was purified by flash chromatography (SiO₂; EtOAc–MeOH 100:0 → 90:10) to afford the product S₁₉ as a brown waxy solid (390 mg, 0.582 mmol, 61%). Rₗ 0.38 (SiO₂; EtOAc–MeOH 90:10).

**¹H NMR** (400 MHz, CDCl₃) δ 8.28 (d, 1H, J 8.3 Hz, Aryl), 8.13 (d, 1H, J 9.2 Hz, Aryl), 8.02 (d, 2H, J 7.5 Hz, Aryl), 7.96 (d, J 8.5 Hz, Aryl), 7.89 – 7.80 (m, 4H, Aryl + NH-1), 7.71 – 7.65 (m, 2H, CH-4”, CH-7”), 7.65 – 7.55 (m, 2H, CH-5”, CH-6”), 6.20 (br s, 1H, NH-3), 4.22 – 4.14 (m, 2H, OCH₂CH₂O), 3.64 – 3.43 (m, 22H, OCH₂CH₂O). **¹³C NMR** (100 MHz, CDCl₃) δ 163.4 (CONHCO-1”, CONHCO-3”), 157.2 (NHCONH-2), 134.4 (CH-5”, CH-6”), 132.5 (Aryl C), 131.4 (Aryl C), 130.9 (Aryl C), 128.8 (C-3b”, C-7b”), 128.0 (Aryl C), 127.3 (Aryl CH), 127.1 (Aryl CH), 126.1 (Aryl CH), 125.9 (Aryl CH), 125.3 (Aryl CH), 125.2 (Aryl C), 124.9 (Aryl CH), 124.7 (Aryl C), 124.5 (CH-4”, CH-7”), 123.6 (Aryl C), 123.4 (Aryl CH), 122.0 (Aryl CH), 121.3 (Aryl CH), 70.6 (CH₂), 70.5 (CH₂), 70.4 (CH₂), 70.3 (CH₂), 70.2 (CH₂), 69.1 (CH₂), 40.3 (CH₂).

**HR-ESI-MS** Calculated for C₃₇H₄₀N₃O₉: 670.2759 m/z [M+H]⁺; found 670.2765. **LC-MS** found 670.52, Rₗ 0.65. 4 × CH₂ from the OEG chain were unaccounted for in the ¹³C NMR spectra due to overlapping signals.
3-\{17-\text{aminooxy}\}-3,6,9,12,15-pentaoxaheptadecan-1-yl\}-1-\{pyren-1-yl\}urea 6

80% hydrazine hydrate (45 µL, 0.75 mmol) was added dropwise to a rapidly stirred solution of 3-\{17-\[1,3-\text{dioxo}-2,3-\text{dihydro}-1H-\text{isoindol-2-yl}oxy\]-3,6,9,12,15-pentaoxaheptadecan-1-yl\}-1-\{pyren-1-yl\}urea S19 (100 mg, 0.149 mmol) in THF (1 mL) until determined complete by LC-MS (5 h). The solvent was then removed under reduced pressure and the crude residue purified by flash chromatography (SiO$_2$; DCM–MeOH 95:5) to give the product 6 as a brown waxy solid. (64 mg, 0.12 mmol, 80%) $R_f$ 0.24 (SiO$_2$; DCM–MeOH 95:5).

$^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 8.38 (d, 1H, J 8.3 Hz, Aryl), 8.23 (d, 1H, J 9.3 Hz, Aryl), 8.13 – 8.03 (m, 3H, Aryl), 8.01 – 7.87 (m, 5H, Aryl + NH-1), 6.29 (br s, 1H, NH-3), 3.72 – 3.67 (m, 2H, OCH$_2$CH$_2$O), 3.66 – 3.43 (m, 22H, OC$_2$H$_2$O).

$^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ 157.1 (NHCONH-2), 132.7 (Aryl C), 131.6 (Aryl C), 131.1 (Aryl C), 128.1 (Aryl C), 127.5 (Aryl CH), 127.2 (Aryl CH), 126.2 (Aryl CH), 126.1 (Aryl CH), 125.5 (Aryl CH), 125.4 (Aryl C), 125.0 (Aryl CH), 125.0 (Aryl C), 124.6 (Aryl CH), 123.6 (Aryl C), 122.0 (Aryl CH), 121.5 (Aryl CH), 74.7 (CH$_2$), 70.6 (CH$_2$), 70.5 (CH$_2$), 70.4 (CH$_2$), 70.3 (CH$_2$), 70.2 (CH$_2$), 69.6 (CH$_2$), 40.4 (CH$_2$).

HR-ESI-MS Calculated for C$_{29}$H$_{37}$N$_3$O$_7$: 540.2524 m/z [M+Na]$^+$; found 562.2526. LC-MS Calculated for C$_{29}$H$_{38}$N$_3$O$_7$: 540.27 m/z [M+H]$^+$; found 540.46, $R_t$ 0.60.

3 $\times$ CH$_2$ from the OEG chain were unaccounted for in the $^{13}$C NMR spectra due to overlapping signals.

Supplementary Figure 40: Analytical HPLC trace for 3-\{17-\text{aminooxy}\}-3,6,9,12,15-pentaoxaheptadecan-1-yl\}-1-\{pyren-1-yl\}urea 6.
Supplementary Figure 41: $^1$H NMR spectra (400 MHz, CDCl$_3$) for 3-[(17-aminooxy)-3,6,9,12,15-pentaoxaheptadecan-1-yl]-1-(pyren-1-yl)urea 6.

Supplementary Figure 42: $^{13}$C NMR spectra (100 MHz, CDCl$_3$) for 3-[(17-aminooxy)-3,6,9,12,15-pentaoxaheptadecan-1-yl]-1-(pyren-1-yl)urea 6.
20-azido-\textit{N}-({pyren-1-yl})-3,6,9,12,15,18-hexaoxaicosanamide S20

Under an N\textsubscript{2} atmosphere, 60\% NaH in mineral oil (200 mg, 5.00 mmol) was added to an ice-cooled solution of 17-azido-3,6,9,12,15-pentaoxaheptadecan-1-ol S5 (450 mg, 1.46 mmol) in DMF (5 mL), followed by the dropwise addition of a solution of 2-chloro-\textit{N}-pyren-1-yl acetamide S12 (368 mg, 1.25 mmol) in DMF (5 mL). The reaction was then allowed to warm to room temperature and stirred until determined complete by LC-MS (21 h). The reaction was diluted by EtOAc (50 mL), and washed with 1M HCl (10 mL), 1M LiCl (5 × 25 mL). The aqueous layers were then combined, extracted with EtOAc (5 × 10 mL). Then the organic layers were combined, washed with brine (10 mL), and dried (\text{Na}_2\text{SO}_4). The solvent removed in vacuo to give a crude brown oil which was purified by flash chromatography (SiO\textsubscript{2}; DCM–MeOH 1:0 → 95:5) to yield the product S20 as a brown oil (594 mg, 1.05 mmol, 84\%). R\text{f} 0.42 (SiO\textsubscript{2}; SiO\textsubscript{2}; DCM–MeOH 95:5). \textsuperscript{1}H NMR (500 MHz, CDCl\textsubscript{3}) \(\delta\) 9.56 (br s, 1H, CONH-1), 8.50 (d, 1H, J 8.3 Hz, Aryl), 8.21 – 8.16 (m, 4H, Aryl), 8.12 (d, 1H, J 9.2 Hz, Aryl), 8.07 – 7.97 (m, 3H, Aryl), 4.36 (s, 2H, CH\textsubscript{2}-2), 3.97 – 3.94 (m, 2H, OCH\textsubscript{2}CH\textsubscript{2}O), 3.85 – 3.82 (m, 2H, OCH\textsubscript{2}CH\textsubscript{2}O), 3.72 – 3.69 (m, 2H, OCH\textsubscript{2}CH\textsubscript{2}O), 3.61 – 3.58 (m, 2H, OCH\textsubscript{2}CH\textsubscript{2}O), 3.57 – 3.54 (m, 2H, OCH\textsubscript{2}CH\textsubscript{2}O), 3.54 – 3.50 (m, 4H, OCH\textsubscript{2}CH\textsubscript{2}O), 3.48 – 3.45 (m, 2H, OCH\textsubscript{2}CH\textsubscript{2}O), 3.41 – 3.38 (m, 2H, OCH\textsubscript{2}CH\textsubscript{2}O), 3.33 (t, 2H, J 5.1 Hz, CH\textsubscript{2}-20), 3.29 (s, 4H, OCH\textsubscript{2}CH\textsubscript{2}O). \textsuperscript{13}C NMR (125 MHz, CDCl\textsubscript{3}) \(\delta\) 169.2 (CONH-1), 131.5 (Aryl C), 131.0 (Aryl C), 129.3 (Aryl C), 128.0 (Aryl C), 127.5 (Aryl CH), 127.0 (Aryl CH), 126.3 (Aryl CH), 125.5 (Aryl CH), 125.3 (Aryl C), 125.3 (Aryl C), 125.1 (Aryl CH), 124.9 (Aryl C), 123.8 (Aryl C), 122.2 (Aryl CH), 120.9 (Aryl CH), 120.9 (Aryl C), 71.8 (CH\textsubscript{2}), 71.2 (CH\textsubscript{2}-2), 70.9 (CH\textsubscript{2}), 70.7 (CH\textsubscript{2}), 70.6 (CH\textsubscript{2}), 70.6 (CH\textsubscript{2}), 70.5 (CH\textsubscript{2}), 70.4 (CH\textsubscript{2}), 70.4 (CH\textsubscript{2}), 50.8 (CH\textsubscript{2}-20). HR-ESI-MS Calculated for C\textsubscript{30}H\textsubscript{37}N\textsubscript{4}O\textsubscript{7}: m/z 565.2657 [M+H]\textsuperscript{+}; found 565.2661. LC-MS found 565.62, R\text{f} 0.67. IR (Neat, \(\nu_{\text{max}}/\text{cm}^{-1}\)) 3304 m (NH amide), 2098 m (N=N=N), 1689 s (C=O amide), 1518–1416 m (C=C aromatic). 1 × CH\textsubscript{2} from the OEG chain was unaccounted for in the \textsuperscript{13}C NMR spectra due to overlapping signals.
A solution of triethylamine (1090 µL, 7.820 mmol) in dry THF (1 mL) was added dropwise to a stirred solution of hexaethylene glycol S3 (1.00 g, 3.54 mmol) and methanesulfonyl chloride (610 µL, 7.89 mmol) in dry THF (4 mL) at 0 °C (ice bath) and then the mixture was allowed to stir for 1 h. The ice bath was then removed, and the reaction allowed to stir at room temperature for 4 h. The reaction mixture was then diluted with H₂O (5 mL) and the pH adjusted to 8 using NaHCO₃. NaN₃ (510 mg, 7.737 mmol) was then added to the reaction mixture and the resulting mixture was heated at reflux for 18 h. The reaction was allowed to cool and Et₂O (10 mL) was added and the layers separated. The aqueous layer was saturated with NaCl and extracted with Et₂O (5 × 10 mL). The organic layers were combined, washed with brine (10 mL) and dried (MgSO₄) and solvent removed in vacuo to yield the product S21 as a colourless oil (986 mg, 2.80 mmol, 79%). ¹H NMR (500 MHz, CDCl₃) δ 3.67 – 3.64 (m, 20H, OCH₂CH₂O), 3.37 (t, 4H, J 5.0 Hz, CH₂-1,17). ¹³C NMR (125 MHz, CDCl₃) δ 70.8 (CH₂), 70.8 (CH₂), 70.8 (CH₂), 70.8 (CH₂), 70.7 (CH₂), 70.1 (CH₂-2, 16), 50.8 (CH₂N₃-1, 17). HR-ESI-MS Calculated for C₁₂H₂₄N₆NaO₅+: m/z 355.1700 [M+Na]⁺; found 355.1717. LC-MS found 355.1, Rₜ = 1.68. IR (Neat, νmax/cm⁻¹) 2120 s (N=N=N). Characterisation is consistent with data reported in the literature.⁸

17-azido-3,6,9,12,15-pentaoxaheptadecan-1-amine S22 ⁸

A solution of triphenylphosphine (719 mg, 2.74 mmol) in Et₂O (5 mL) was added dropwise to a vigorously stirred suspension of 1,17-diazido-3,6,9,12,15-pentaoxaheptadecane S21 (985 mg, 2.80 mmol) in 5% HCl (aq) (10 mL) then the mixture was allowed to stirred for 24 h. The reaction mixture was then washed with dichloromethane (3 × 40 mL) and the aqueous phase was cooled in an ice bath and the pH adjusted to 12 using KOH pellets. The water was removed by lyophilisation and product was taken up in dichloromethane (25 mL). The organic phase was dried (Na₂SO₄) and the solvent removed in vacuo to yield the product S22 as a yellow oil that was used without further purification (779 mg, 2.54 mmol, 91%). ¹H NMR (500 MHz, CDCl₃) δ 3.67 – 3.59 (m, 20H, OCH₂CH₂O), 3.49 (t, 2H, J 5.2 Hz, OCH₂CH₂O), 3.37 (t, 2H, J 5.2 Hz, CH₂-17), 2.84 (t, 2H, J 5.2 Hz, CH₂-1). ¹³C NMR (125 MHz, CDCl₃) δ 73.6 (CH₂), 70.8 (CH₂), 70.8 (CH₂), 70.7 (CH₂), 70.7 (CH₂), 70.7 (CH₂), 70.4 (CH₂), 70.1 (CH₂), 50.8
(CH$_2$N$_2$-17), 41.9 (CH$_3$NH$_2$-1). **HR-ESI-MS** Calculated for C$_{12}$H$_{26}$NaNO$_5$$: m/z$ 329.1795 [M+Na]$; found 329.1795. **LC-MS** Calculated for C$_{13}$H$_{27}$NaO$_5$: $m/z$ 307.20 [M+H]$; found 307.20, $R_t$ = 1.96. **IR** (Neat, $\nu_{\text{max}}$/cm$^{-1}$) 3375 w (NH$_2$ asym), 3312 w (NH$_2$ sym), 2101 m (N=N=N).

Characterisation is consistent with data reported in the literature.

3-(17-azido-3,6,9,12,15-pentaoxaheptadecan-1-yl)-1-(pyren-1-yl)urea **S23**

A solution of 17-azido-3,6,9,12,15-pentaoxaheptadecan-1-amine **S22** (399 mg, 1.30 mmol) in THF (3.0 mL) was added dropwise to a stirred solution of 1-isocyanopyrene **S13** (288 mg, 1.18 mmol) in THF (2.0 mL) at 0 °C (ice bath) and allowed to stir for 30 min. The reaction was then allowed to warm to room temperature and stir overnight (21 h). Solvent was then removed under reduced pressure and the crude residue purified by flash chromatography (SiO$_2$; DCM–MeOH 95:5) to afford the product **S23** as a yellow waxy solid (560 mg, 1.057 mmol, 89%). $R_f$ 0.34 (SiO$_2$; DCM–MeOH 95:5). **$^1$H NMR** (400 MHz, CDCl$_3$) $\delta$ 8.37 (d, 1H, J 8.3 Hz, Aryl), 8.21 (d, 1H, J 9.2 Hz, Aryl), 8.13 – 8.04 (m, 3H, Aryl), 8.00 – 7.92 (m, 4H, Aryl), 7.90 (br s, 1H, NH-1), 6.15 (br t, 1H, J 4.8 Hz, NH-3), 3.63 – 3.46 (m, 18H, OCH$_2$CH$_2$O), 3.41 – 3.34 (m, 4H, OCH$_2$CH$_2$O), 3.17 – 3.11 (m, 2H, OCH$_2$CH$_2$O). **$^{13}$C NMR** (100 MHz, CDCl$_3$) $\delta$ 157.1 (NHCONH-2), 132.6 (Aryl C), 131.6 (Aryl C), 131.1 (Aryl C), 128.1 (Aryl C), 127.8 (Aryl CH), 127.3 (Aryl CH), 126.3 (Aryl CH), 126.1 (Aryl CH), 125.5 (Aryl CH), 125.4 (Aryl C), 125.1 (Aryl CH), 124.9 (Aryl C), 124.6 (Aryl CH), 123.6 (Aryl C), 122.0 (Aryl CH), 121.3 (Aryl CH), 70.6 (CH$_2$), 70.5 (CH$_2$), 70.4 (CH$_2$), 70.2 (CH$_2$), 69.9 (CH$_2$), 50.6 (CH$_2$), 40.4 (CH$_2$). **HR-ESI-MS** Calculated for C$_{29}$H$_{38}$N$_5$O$_6$: $m/z$ 550.2660 [M+H]$^+$; found 550.2660. **LC-MS** found 550.40, $R_t$ 0.65. **IR** (Neat, $\nu_{\text{max}}$/cm$^{-1}$) 3287 m (NH urea), 2098 m (N=N=N), 1625 s (C=O urea), 1523–1415 m (C=C aromatic). 5 × CH$_2$ from the OEG chain were unaccounted for in the $^{13}$C NMR spectra due to overlapping signals.
Under an N\textsubscript{2} atmosphere, a solution of triphenylphosphine (294 mg, 1.12 mmol) in THF (3.0 ml) was added to a solution of 20-azido-N-(pyren-1-yl)-3,6,9,12,15,18-hexaoxaicosanamide S20 (575 mg, 1.019 mmol) in THF (7.0 mL) at 0 °C (ice bath). The reaction was then allowed to warm to room temperature and stirred until determined complete by TLC (24 h). H\textsubscript{2}O was added (5.0 mL) and the reaction mixture allowed to stir for 1 h to hydrolyse the iminophosphorane intermediate. The solvent was then removed in vacuo and the crude product purified by flash chromatography (SiO\textsubscript{2}; DCM–sat. NH\textsubscript{3} in MeOH 1:0 → 95:5 → 90:10) to yield the product S24 as a yellow oil (435 mg, 0.808 mmol, 79%).

\textit{Rf} 0.13 (SiO\textsubscript{2}; DCM–sat. NH\textsubscript{3} in MeOH 95:5). \textit{\textsuperscript{1}H NMR} (400 MHz, CDCl\textsubscript{3}) \(\delta\) 9.57 (br s, 1H, CONH-1), 8.49 (d, 1H, J 8.3 Hz, Aryl), 8.21 – 8.14 (m, 4H, Aryl), 8.11 (d, 1H, J 9.2 Hz, Aryl), 8.06 – 7.97 (m, 3H, Aryl), 4.35 (s, 2H, CH\textsubscript{2}-2), 3.94 (dd, 2H, J 3.2 Hz, OCH\textsubscript{2}CH\textsubscript{2}O), 3.82 (dd, 2H, J 3.2 Hz, OCH\textsubscript{2}CH\textsubscript{2}O), 3.70 (dd, 2H, J 3.8 Hz, OCH\textsubscript{2}CH\textsubscript{2}O), 3.54 – 3.48 (m, 6H, OCH\textsubscript{2}CH\textsubscript{2}O), 3.47 – 3.41 (m, 4H, OCH\textsubscript{2}CH\textsubscript{2}O), 3.39 (dd, 2H, J 3.5 Hz, OCH\textsubscript{2}CH\textsubscript{2}O), 3.28 (s, 4H, OCH\textsubscript{2}CH\textsubscript{2}O), 2.80 (t, 2H, J 5.1 Hz, CH\textsubscript{2}-20). \textit{\textsuperscript{13}C NMR} (100 MHz, CDCl\textsubscript{3}) \(\delta\) 169.2 (CONH-1), 131.4 (Aryl C), 131.0 (Aryl C), 130.2 (Aryl C), 129.3 (Aryl C), 127.9 (Aryl CH), 127.5 (Aryl CH), 127.0 (Aryl CH), 126.3 (Aryl CH), 125.5 (Aryl CH), 125.3 (Aryl CH), 125.1 (Aryl CH), 124.9 (Aryl C), 123.8 (Aryl C), 122.3 (Aryl CH), 120.9 (Aryl CH), 73.3 (CH\textsubscript{2}), 71.7 (CH\textsubscript{2}), 71.1 (CH\textsubscript{2}), 70.8 (CH\textsubscript{2}), 70.5 (CH\textsubscript{2}), 70.4 (CH\textsubscript{2}), 70.3 (CH\textsubscript{2}), 41.8 (CH\textsubscript{2}-20). \textit{HR-ESI-MS} Calculated for C\textsubscript{30}H\textsubscript{39}N\textsubscript{2}O\textsubscript{7}: \(m/z\) 539.2752 [M+H]+; found 539.2749. \textit{LC-MS} found 539.43, \textit{Rt} 0.55. \textit{IR} (Neat, v\textsubscript{max}/cm\textsuperscript{-1}) 3377 m (NH\textsubscript{2} asym), 3303 m (NH\textsubscript{2} sym), 1688 s (C=O amide), 1518–1416 m (C=C aromatic). 3 × CH\textsubscript{2} from the OEG chain were unaccounted for in the \textsuperscript{13}C NMR spectra due to overlapping signals.
A solution of triphenylphosphine (490 mg, 0.675 mmol) in THF (3.0 ml) was added to a solution of 20-azido-\(N\)-(pyren-1-yl)-3,6,9,12,15,18-hexaoxaicosanamide S23 (513 mg, 0.533 mmol) in THF (5.0 mL) at 0 °C (ice bath). The reaction was then allowed to warm to room temperature and stirred until determined complete by TLC (24 h). \(\text{H}_2\text{O}\) was added (2.0 mL) and the reaction mixture allowed to stir for 2 h to hydrolyse the iminophosphorane intermediate. The solvent was then removed in vacuo and the crude product purified by flash chromatography (SiO\(_2\); DCM–sat. \(\text{NH}_3\) in MeOH 1:0 → 95:5 → 90:10) to yield the product S25 as a yellow waxy solid (342 mg, 0.653 mmol, 70%). \(\text{Rf}\ 0.38\) (SiO\(_2\); DCM–sat. \(\text{NH}_3\) in MeOH 9:1). \(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta\ 8.40\ (d, 1\text{H}, J \ 8.3\ \text{Hz}, \text{Aryl}), 8.24\ (d, 1\text{H}, J \ 9.2\ \text{Hz}, \text{Aryl}), 8.16 - 8.02\ (m, 4\text{H}, \text{Aryl}), 8.00 - 7.90\ (m, 4\text{H}, \text{Aryl + NH}_3), 6.33\ (br\ t, 1\text{H}, J \ 4.5\ \text{Hz}, \text{NH}-1), 3.64 - 3.48\ (m, 18\text{H}, \text{OCH}_2\text{CH}_2\text{O}), 3.41\ (dd, 2\text{H}, J \ 5.8, 3.4\ \text{Hz}, \text{OCH}_2\text{CH}_2\text{O}), 3.29\ (t, 2\text{H}, J \ 5.2\ \text{Hz}, \text{OCH}_2\text{CH}_2\text{O}), 2.69\ (t, 2\text{H}, J \ 5.2\ \text{Hz}, \text{CH}_2-17).\n
\(^{13}\)C NMR (100 MHz, CDCl\(_3\)) \(\delta\ 157.1\ (\text{NHCONH-2}), 132.8\ (\text{Aryl C}), 131.6\ (\text{Aryl C}), 131.1\ (\text{Aryl C}), 128.0\ (\text{Aryl C}), 127.5\ (\text{Aryl CH}), 127.2\ (\text{Aryl CH}), 126.2\ (\text{Aryl CH}), 126.1\ (\text{Aryl CH}), 125.5\ (\text{Aryl CH}), 125.4\ (\text{Aryl C}), 125.0\ (\text{Aryl CH}), 124.5\ (\text{Aryl CH}), 123.4\ (\text{Aryl C}), 121.9\ (\text{Aryl CH}), 121.4\ (\text{Aryl CH}), 73.1\ (\text{CH}_2), 70.6\ (\text{CH}_2), 70.6\ (\text{CH}_2), 70.5\ (\text{CH}_2), 70.5\ (\text{CH}_2), 70.2\ (\text{CH}_2), 70.1\ (\text{CH}_2), 41.7\ (\text{CH}_2-17), 40.4\ (\text{CH}_2).\n
HR-ESI-MS Calculated for \(\text{C}_{29}\text{H}_{38}\text{N}_3\text{O}_6\)\(^+\): \(m/z\ 524.2755\) [M+H]\(^+\); found 524.2757. LC-MS found 524.49, \(\text{Rf}\ 0.55.\) IR (Neat, \(\nu_{\text{max}}/\text{cm}^{-1}\)) 3390 m (NH\(_2\)), 1625 s (C=O urea), 1524 – 1416 m (C=C aromatic). 1 × aromatic C and 2 × CH\(_2\) from the OEG chain were unaccounted for in the \(^{13}\)C NMR spectra due to overlapping signals.

20-[(2-nitrophenyl)formamido]-\(N\)-(pyren-1-yl)-3,6,9,12,15,18-hexaoxaicosanamide S27

Under an \(\text{N}_2\) atmosphere, hydroxybenzotriazole (176 mg, 1.30 mmol), \(N\)-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (199 mg, 1.04 mmol) and a solution of 20-amino-\(N\)-(pyren-1-yl)-3,6,9,12,15,18-hexaoxaicosanamide S24 (280 mg, 0.520 mmol) in DCM (5.0 mL) were added
sequentially to a stirred solution of 2-nitrobenzoic acid S26 (96 mg, 0.57 mmol) in DCM (5.0 mL). The reaction was stirred until determined complete by LC-MS (15 h). The solvent removed in vacuo and the crude residue was purified by flash chromatography (SiO$_2$; DCM–MeOH 1.0 → 95:5) to afford the product S27 as a brown oil (350 mg, 0.509 mmol, 98%). R$_f$ 0.32 (SiO$_2$; DCM–MeOH 95:5). $^1$H NMR (400 MHz, CDCl$_3$) δ 9.58 (br s, 1H, CONH-1), 8.48 (d, 1H, J 8.3 Hz, Aryl), 8.20 – 8.17 (m, 4H, Aryl), 8.12 (d, 1H, J 9.2 Hz, Aryl), 8.07 – 7.99 (m, 3H, Aryl), 7.93 (dd, 1H, J 8.3, 1.0 Hz, CH-3”), 7.50 (td, 1H, J 7.6, 1.0 Hz, CH-5”), 7.45 (dd, 1H, J 7.6, 1.6 Hz, CH-6”), 7.39 (ddd, 1H, J 8.3, 7.6, 1.6 Hz, CH-4”), 6.90 (br s, 1H, CONH-1’), 4.41 – 4.32 (m, 2H, OCH$_2$CH$_2$O), 3.98 – 3.93 (m, 2H, OCH$_2$CH$_2$O), 3.85 – 3.79 (m, 2H, OCH$_2$CH$_2$O), 3.67 – 3.60 (m, 2H, OCH$_2$CH$_2$O), 3.53 – 3.48 (m, 2H, OCH$_2$CH$_2$O), 3.46 (dd, 2H, J 3.9 Hz, OCH$_2$CH$_2$O), 3.43 – 3.38 (m, 2H, OCH$_2$CH$_2$O), 3.30 (dd, 2H, J 3.7 Hz, OCH$_2$CH$_2$O), 3.24 – 3.16 (m, 2H, OCH$_2$CH$_2$O). $^{13}$C NMR (100 MHz, CDCl$_3$) δ 169.3 (CONH-1), 166.6 (CONH-1’), 146.6 (C-1”), 133.5 (CH-5”), 133.2 (C-2”), 131.4 (aryl C), 130.9 (aryl C), 130.2 (CH-4”), 129.2 (aryl C), 128.9 (CH-6”), 127.9 (aryl CH), 127.4 (aryl CH), 127.0 (aryl CH), 126.3 (aryl CH), 125.5 (aryl CH), 125.3 (aryl CH), 125.3 (aryl C), 125.1 (aryl CH), 124.8 (aryl C), 124.4 (CH-3”), 123.8 (aryl C), 122.3 (aryl CH), 120.9 (aryl CH), 71.7 (CH$_2$), 71.1 (CH$_2$), 70.8 (CH$_2$), 70.4 (CH$_2$), 70.4 (CH$_2$), 70.4 (CH$_2$), 70.3 (CH$_2$), 70.2 (CH$_2$), 69.6 (CH$_2$), 40.1 (CH$_2$). 

HR-ESI-MS Calculated for C$_{37}$H$_{45}$N$_4$O$_{10}$+ m/z 705.3130 [M+NH$_4$]$^+$; found 705.3135. LC-MS found 705.47, R$_f$ 0.65. IR (Neat, $v_{max}$/cm$^{-1}$) 3291 m (NH amide), 1659 s (C=O amide), 1523 s (NO$_2$ asym), 1347 m (NO$_2$ sym), 1554–1438 m (C=C aromatic). 1 × aromatic C and 1 × CH$_2$ from the OEG chain were unaccounted for in the $^{13}$C NMR spectra due to overlapping signals.

2-nitro-N-(17-[[pyren-1-yl]carbamoyl]amino)-3,6,9,12,15-pentaoxaheptadecan-1-yl)benzamide S28

Hydroxybenzotriazole (73 mg, 0.54 mmol), N-(3-dimethylaminopropyl)-N’-ethylcarbodiimide hydrochloride (83 mg, 0.43 mmol) and a solution of 3-(17-amino-3,6,9,12,15-pentaoxaheptadecan-1-yl)-1-(pyren-1-yl)urea S25 (114 mg, 0.216 mmol) in DCM (2.0 mL) were added sequentially to a stirred solution of 2-nitrobenzoic acid S26 (40 mg, 0.24 mmol) in DCM (3.0 mL). The reaction was stirred until determined complete by LC-MS (15 h). The solvent removed in vacuo and the crude residue was purified by flash chromatography (SiO$_2$; DCM–MeOH 95:5) to afford the product S28 as a yellow waxy
solid (124 mg, 0.184 mmol, 85%). Rf 0.24 (SiO2; DCM–MeOH 95:5). 1H NMR (400 MHz, CDCl3) δ 8.35 (d, 1H, J 8.3 Hz, Aryl), 8.18 (d, 1H, J 9.3 Hz, Aryl), 8.09 (dd, 1H, J 7.5, 0.8 Hz, Aryl), 8.06 – 8.01 (m, 3H, Aryl), 7.96 – 7.88 (m, 4H, Aryl + NH-1’”), 7.80 (dd, 1H, J 8.1, 0.8 Hz, CH-3’), 7.37 (td, 1H J 7.3, 0.8 Hz, CH-5’), 7.32 (dd, 1H, J 7.3, 1.8 Hz, CH-6’), 7.27 (dd, 1H, J 8.1, 7.3, 1.8 Hz, CH-4’), 7.00 (br t, 1H, J 5.2 Hz, CONH-1), 6.28 (br t, 1H, J 5.2 Hz, NH-3’”), 3.60 – 3.45 (m, 24H, OCH2CH2O).

13C NMR (100 MHz, CDCl3) δ 166.9 (CONH-1), 157.1 (NHCONH-2’”), 146.4 (C-1’), 133.4 (CH-5’), 133.0 (C-2’), 132.8 (Aryl C), 131.6 (Aryl C), 131.1 (Aryl C), 130.0 (CH-4’), 127.8 (Aryl C), 127.4 (Aryl CH), 127.1 (Aryl CH), 126.1 (Aryl CH), 125.5 (Aryl CH), 125.3 (Aryl C), 125.0 (Aryl CH), 124.9 (Aryl C), 124.5 (Aryl CH), 124.2 (CH-3’), 123.1 (Aryl C), 121.5 (Aryl CH), 121.3 (Aryl CH), 70.6 (CH2), 70.5 (CH2), 70.4 (CH2), 70.3 (CH2), 70.2 (CH2), 69.6 (CH2), 40.4 (CH2), 40.1 (CH2). HR-ESI-MS Calculated for C36H41N4O9+: m/z 673.2868 [M+H]+; found 673.2866. LC-MS found 673.49, Rf 0.7.

IR (Neat, νmax/cm⁻¹) 3279 m (NH urea), 1626 s (C=O urea/amide), 1562 – 1450 m (C=C aromatic).

1 × aromatic C and 2 × CH2 from the OEG chain were unaccounted for in the 13C NMR spectra due to overlapping signals.

20-[(2-aminophenyl)formamido]-N-(pyren-1-yl)-3,6,9,12,15,18-hexaoxaicosanamide 7

Fe powder (406 mg, 7.27 mmol) was added to a stirred solution of 20-[(2-nitrophenyl)formamido]-N-(pyren-1-yl)-3,6,9,12,15,18-hexaoxaicosanamide S27 (100 mg, 0.145 mmol) in glacial acetic acid (5.0 mL) then heated to 60 °C and stirred (2 h). The reaction was then allowed to cool, and the reaction basified (pH 8) using sat. NaHCO3. The reaction mixture was extracted with EtOAc (4 × 30 mL). The organic layers were combined, washed with sat. NaHCO3 (20 mL), brine (10 mL), and dried (Na2SO4). The solvent removed under reduced pressure to afford the product 7 as a brown oil (86 mg, 0.13 mmol, 90%). Rf 0.17 (SiO2; DCM–MeOH 96:4). 1H NMR (500 MHz, CDCl3) δ 9.61 (br s, 1H, CONH-1’), 8.48 (d, 1H, J 8.2 Hz, Aryl), 8.21 – 8.16 (m, 4H, Aryl), 8.12 (d, 1H, J 9.2 Hz, Aryl), 8.04 – 7.99 (m, 3H, Aryl), 7.35 (dd, 1H, J 7.9, 1.4 Hz, CH-6’), 7.15 (ddd, 1H, J 8.2, 7.9, 1.4 Hz, CH-4’), 6.83 (br s, 1H, CONH-1’), 6.63 (dd, 1H, J 8.2, 1.0 Hz, CH-3’), 6.59 (td, 1H, J 7.9, 1.0 Hz, CH-5’), 5.51 (br s, 2H, NH2), 4.35 (s, 2H, CH2-2), 3.95 – 3.92 (m, 2H, OCH2CH2O), 3.82 – 3.78 (m, 2H, OCH2CH2O), 3.70 – 3.61 (m, 4H, OCH2CH2O), 3.59 – 3.54 (m, 4H, OCH2CH2O), 3.49 – 3.44 (m, 4H, OCH2CH2O), 3.42 – 3.40 (m, 2H, OCH2CH2O), 3.37 – 3.32 (m, 2H, OCH2CH2O), 3.26 – 3.21 (m, 4H, OCH2CH2O). 13C NMR (125 MHz, CDCl3) δ 169.5 (CONH-1’), 169.3 (CONH-1), 148.8 (C-1’), 132.2 (CH-4’), 131.5 (Aryl C), 131.0 (Aryl C), 130.2
(Aryl C), 129.3 (Aryl C), 128.0 (Aryl CH), 127.8 (CH-6"), 127.5 (Aryl CH), 127.0 (Aryl CH), 126.3 (Aryl CH), 125.5 (Aryl CH), 125.4 (Aryl CH), 125.3 (Aryl C), 125.2 (Aryl CH), 124.9 (Aryl C), 123.9 (Aryl C), 122.4 (Aryl CH), 121.0 (Aryl CH), 117.3 (CH-3"), 116.6 (CH-5"), 116.4 (C-2"), 71.7 (CH₂), 71.2 (CH₂), 70.8 (CH₂), 70.5 (CH₂), 70.4 (CH₂), 70.3 (CH₂), 70.2 (CH₂), 70.0 (CH₂), 39.5 (CH₂). 

HR-ESI-MS Calculated for C₃₇H₄₄N₃O₈: 658.3123 m/z [M+H]+; found 658.3118. LC-MS found 658.47, Rₜ 0.65. IR (Neat, νmax/cm⁻¹) 3434 m (NH₂), 3322 m (NH amide), 1631 m (C=O amide), 1554–1415 m (C=C aromatic).

Fe powder (125 mg, 2.23 mmol) was added to a stirred solution of 2-nitro-N-[17-[[pyren-1-yl]carbamoyl]amino]-3,6,9,12,15-pentaoxaheptadecan-1-yl]benzamide S28 (93 mg, 0.14 mmol) in glacial acetic acid (5.0 mL) then heated to 60 °C and stirred (2 h). The reaction was then allowed to cool, and the reaction basified (pH 8) using sat. NaHCO₃. The reaction mixture was extracted with EtOAc (4 × 30 mL). The organic layers were combined, washed with sat. NaHCO₃ (20 mL), brine (10 mL), and dried (Na₂SO₄). The solvent removed under reduced pressure to afford the product 8 (88 mg, 0.14 mmol, 99%); Rₜ 0.28 (SiO₂; DCM–MeOH 94:6). ¹H NMR (500 MHz, CDCl₃) δ 8.42 (d, 1H, J 8.3 Hz, Aryl), 8.26 (d, 1H, J 9.2 Hz, Aryl), 8.16–8.06 (m, 3H, Aryl), 8.02–7.93 (m, 4H, Aryl), 7.93 (br s, 1H, NH-1''), 7.30 (d, 1H, J 7.8 Hz, CH-6''), 7.14 (td, 1H, J 7.8, 1.0 Hz, CH-4''), 6.84 (br s, 1H, CONH-1), 6.62 (d, 1H, J 8.1 Hz, CH-3''), 6.56 (t, 1H, J 7.5 Hz, CH-5''), 6.31 (br s, 1H, NH-3''), 5.47 (br s, 2H, NH₂), 3.64–3.56 (m, 15H, OCH₂CH₂O), 3.53–3.48 (m, 4H, OCH₂CH₂O), 3.46–3.39 (m, 6H, OCH₂CH₂O). ¹³C NMR (125 MHz, CDCl₃) δ 169.6 (CONH-1), 157.1 (NHCONH-2''), 148.7 (C-1''), 132.8 (Aryl C), 132.2 (CH-4''), 131.6 (Aryl C), 131.1 (Aryl C), 128.1 (Aryl C), 127.8 (CH-6''), 127.6 (Aryl CH), 127.3 (Aryl CH), 126.3 (Aryl CH), 126.2 (Aryl CH), 125.6 (Aryl CH), 125.5 (Aryl C), 125.1 (Aryl CH), 124.9 (Aryl C), 124.6 (Aryl CH), 123.5 (Aryl C), 121.9 (Aryl CH), 121.4 (Aryl CH), 117.3 (CH-3''), 116.7 (CH-5''), 116.4 (C-2''), 70.6 (CH₂), 70.5 (CH₂), 70.4 (CH₂), 70.2 (CH₂), 69.9 (CH₂), 40.4 (CH₂), 39.4 (CH₂). HR-ESI-MS Calculated for C₅₆H₄₂N₄NaO₇: 665.2946 m/z [M+Na]+; found 665.2944. LC-MS found 665.47, Rₜ 0.7. IR (Neat, νmax/cm⁻¹) 3457 w (NH₂), 3326 m (NH urea), 1626 s (C=O urea/amide), 1545–1459 m (C=C aromatic). 3 × CH₂ from the OEG chain were unaccounted for in the ¹³C NMR spectra due to overlapping signals.
**Supplementary Figure 43**: Analytical HPLC trace for 2-amino-N-(17-[[pyren-1-yl]carbamoyl]amino)-3,6,9,12,15-pentaoxaheptadecan-1-yl)benzamide 8.

**Supplementary Figure 44**: $^1$H NMR spectra (500 MHz, CDCl$_3$) for 2-amino-N-(17-[[pyren-1-yl]carbamoyl]amino)-3,6,9,12,15-pentaoxaheptadecan-1-yl)benzamide 8.
Supplementary Figure 45: $^{13}$C NMR spectra (125 MHz, CDCl$_3$) for 2-amino-N-{{[pyren-1-yl]carbamoyl}amino}-3,6,9,12,15-pentaoxaheptadecan-1-yl)benzamide 8.

Methyl 1-[[pyren-1-yl]carbamoyl]-2,5,8,11,14,17,20-heptaoxadocosan-22-oate S30

Methyl bromoacetate S29 (125 µL, 1.32 mmol) was added dropwise to a stirred solution of 20-hydroxy-N-{pyren-1-yl}-3,6,9,12,15,18-hexaoxaicosanamide 3 (473 mg, 0.877 mmol) and 60% NaH in mineral oil (53 mg, 1.325 mmol) in DMF (2 mL) and stirred for 30 minutes. The reaction was then allowed to warm to room temperature and stir until determined complete (3 h). The reaction was diluted with EtOAc (20 mL) then washed with 1M LiCl (5 × 15 mL) and brine (10 mL). The organic layer was then dried (Na$_2$SO$_4$) and the solvent removed in vacuo to give a crude brown oil. The crude product was then purified by flash chromatography (SiO$_2$; DCM–EtOAc–MeOH 6:3:1) to yield the product S30 as a brown oil (262 mg, 0.428 mmol, 49%). $R_f$ 0.07 (SiO$_2$; EtOAc–MeOH 8:2). $^1$H NMR (500
1 M LiOH (2.0 mL, 0.0 mmol) was added to a stirred solution of methyl 1-[(pyren-1-yl)carbamoyl]-2,5,8,11,14,17,20-heptaoxadocosan-22-oate S30 (109 mg, 0.371 mmol) in THF (2 mL) and allowed to stir until determined complete by TLC (2 h). The reaction was then acidified with 1 M HCl (1 mL), organic solvent removed under reduced pressure, and the product extracted with EtOAc (5 × 3 mL). The organic layers were combined, washed with brine, and dried (Na₂SO₄). The solvent was removed to yield the product S31 as a brown oil (105 mg, 0.176 mmol, 99%). Rf 0.19 (SiO₂; DCM–MeOH 95:5).

**1H NMR** (500 MHz, CDCl₃) δ 8.26 (d, 1H, J 3.2 Hz, Aryl), 8.25 (d, 1H, J 3.4 Hz, Aryl), 8.23 – 8.18 (m, 3H, Aryl), 8.15 (d, 1H, J 9.0 Hz, Aryl), 8.13 (d, 1H, J 9.2 Hz, Aryl), 8.10 – 8.05 (m, 2H, Aryl), 5.12 (d, 1H, J 17.1 Hz, CH₂-2), 4.04 (d, 1H, J 17.1 Hz, CH₂-2), 3.99 (d, 1H, J 15.4 Hz, CH₂-21), 3.77 (d, 1H, J 15.4 Hz, CH₂-21), 3.74 (dd, 2H, J 5.0, 3.9 Hz, OCH₂CH₂O), 3.67 – 3.63 (m, 8H, OCH₂CH₂O), 3.63 – 3.57 (m, 8H, OCH₂CH₂O), 3.57 – 3.50 (m, 6H, OCH₂CH₂O). **13C NMR** (125 MHz, CDCl₃) δ 171.2 (COOH-22), 171.0 (CONH-1), 134.4 (Aryl C), 131.8 (Aryl C), 131.2 (Aryl C), 130.9 (Aryl C), 129.9 (Aryl C), 128.7 (Aryl CH), 127.9 (Aryl C), 127.2 (Aryl CH), 126.8 (Aryl CH), 126.6 (Aryl CH), 126.3 (Aryl CH), 126.2 (Aryl CH), 125.7 (Aryl CH), 125.6 (Aryl CH), 124.6 (Aryl CH), 124.5 (Aryl CH), 121.1 (Aryl CH), 72.7 (CH₂), 70.8 (CH₂), 70.6 (CH₂), 70.5 (CH₂), 70.5 (CH₂), 70.3 (CH₂), 69.4 (CH₂-21), 61.8 (CH₃), 52.4 (OCH₃), 51.6 (CH₂-2). **HR-ESI-MS** Calculated for: 612.2803 m/z [M+H]+; found 612.2810. **LC-MS** found, 612.67 Rₚ 0.65. **IR** (Neat, νmax/cm⁻¹) 3264 m (NH), 1748 m (C=O ester), 1672 m (C=O amide), 1506–1437 m (C=C aromatic). 2 × CH₂ from the OEG chain were unaccounted for in the **13C NMR** spectra due to overlapping signals.

1-[(pyren-1-yl)carbamoyl]-2,5,8,11,14,17,20-heptaoxadocosan-22-oic acid S31
(Aryl C), 124.6 (Aryl C), 121.1 (Aryl CH), 72.7 (CH₂), 70.6 (CH₂), 70.6 (CH₂), 70.5 (CH₂), 70.5 (CH₂), 70.3 (CH₂), 69.4 (CH₂-21), 61.6 (CH₂), 52.0 (CH₂-2). HR-ESI-MS Calculated for C₆₀H₄₀NO₁₀: 598.2647 m/z [M+H⁺]; found 598.2640. LC-MS found 598.35., Rₖ 0.55. IR (Neat, ν_max/cm⁻¹) 3439 br m (OH acid), 1734 m (C=O acid), 1670 m (C=O amide), 1507 – 1438 m (C=C aromatic).

3 × CH₂ from the OEG chain were unaccounted for in the ¹³C NMR spectra due to overlapping signals.

1-{N’-[[tert-butoxy]carbonyl]hydrazinecarbonyl}-N-{pyren-1-yl}-2,5,8,11,14,17,20-heptaoxadocosan-22-amide S33

Hydroxybenzotriazole (11 mg, 0.081 mmol), N-(3-dimethylaminopropyl)-N’-ethylcarbodiimide hydrochloride (13 mg, 0.068 mmol) and tert-butylcarbazate S32 (5 mg, 0.04 mmol) were added sequentially to a stirred solution of 1-{[pyren-1-yl]carbamoyl}-2,5,8,11,14,17,20-heptaoxadocosan-22-oic acid S31 (19 mg, 0.032 mmol) in DCM (1 mL). The reaction was stirred until determined complete by TLC (16 h). The reaction mixture was then concentrated in vacuo and the crude residue was purified by flash chromatography (SiO₂; DCM – MeOH 9:1) to afford the product S33 as a brown oil (19 mg, 0.027mmol, 85%). Rf 0.43 (SiO₂; DCM – MeOH 9:1).

¹H NMR (500 MHz, CDCl₃) δ 8.71 (br s, 1H, CONH-22), 8.28 – 8.25 (m, 2H, Aryl), 8.22 (d, 1H, J 2.1 Hz, Aryl), 8.20 (d, 1H, J 3.4 Hz, Aryl), 8.17 – 8.15 (m, 2H, Aryl), 8.11 – 8.05 (m, 3H, Aryl), 6.73 (br s, 1H, CONH-1”), 5.05 (d, 1H, J 14.9 Hz, CH₂-21), 4.05 (d, 1H, J 14.9 Hz, CH₂-21), 3.99 (d, 1H, J 15.8 Hz, CH₂-1), 3.77 (d, 1H, J 15.8 Hz, CH₂-1), 3.70 – 3.67 (m, 2H, OCH₂CH₂O), 3.65 – 3.40 (m, 22H, OCH₂CH₂O), 1.54 – 1.42 (m, 9H, OC(CH₃)₃-7”). ¹³C NMR (125 MHz, CDCl₃) δ 172.1 (CONH-1), 168.7(CONHNH-1”), 155.4 (NHCOOᵗBu-4”), 153.8 (Aryl C), 133.8 (Aryl C), 132.0 (Aryl C), 131.2 (Aryl C), 130.9 (Aryl C), 130.1 (Aryl CH), 128.8 (Aryl CH), 127.6 (Aryl C), 127.2 (Aryl CH), 126.8 (Aryl CH), 126.6 (Aryl CH), 126.4 (Aryl CH), 126.2 (Aryl CH), 125.9 (Aryl CH), 125.7 (Aryl C), 124.6 (Aryl C), 120.8 (Aryl CH), 81.7 (OC(CH₃)₃-6”), 72.8 (CH₂), 70.8 (CH₂), 70.7 (CH₂), 70.6 (CH₂), 70.6 (CH₂), 70.6 (CH₂), 70.5 (CH₂), 70.5 (CH₂), 70.4 (CH₂), 69.4 (CH₂-1), 61.8 (CH₂), 53.1 (CH₂-21), 28.4 (OC(CH₃)₃-7”). HR-ESI-MS Calculated for C₃₇H₅₀N₃O₁₁: 712.3440 m/z [M+H⁺]; found 712.3441. LC-MS Calculated for C₃₇H₅₃N₄O₁₁: 729.37 m/z [M+NH₄⁺]; found 729.46, Rₖ 0.55. IR (Neat, ν_max/cm⁻¹) 3439 br m (OH acid), 1734 m (C=O acid), 1670 m (C=O amide), 1507 – 1438 m (C=C aromatic). 2 × CH₂ from the OEG chain were unaccounted for in the ¹³C NMR spectra due to overlapping signals.
Trifluoroacetic acid (300 mL, 60.0 mmol) was added to a stirred solution of 1-[N’-[(tert-butoxy)carbonyl]hydrazinecarbonyl]-N-(pyren-1-yl)-2,5,8,11,14,17,20-heptaoxadocosan-22-amide S33 (90 mg, 0.010 mmol) in DCM (1 mL) and allowed to stir until determined complete by TLC (2 h). The reaction was diluted with DCM (4 mL), washed with sat. NaHCO₃ (3 × 2 mL), brine (2 mL), and dried (Na₂SO₄). The solvent was removed under reduced pressure and the residue purified by reversed-phase chromatography (C₁₈; H₂O (+0.1% CH₃COOH)–MeCN (+0.1% CH₃COOH) 95:5→5:95) to give the product 9 as a brown oil (46 mg, 0.076 mmol, 60%).

**¹H NMR** (500 MHz, CDCl₃) δ 8.20 (t, 2H, J 6.7 Hz, Aryl), 8.15 (d, 1H, J 4.0 Hz, Aryl), 8.13 (d, 1H, J 2.8 Hz, Aryl), 8.09 (d, 1H, J 8.9 Hz, Aryl), 8.05–7.98 (m, 3H, Aryl), 7.95 (d, 1H, J 9.1 Hz, Aryl), 4.92 (d, 1H, J 15.1 Hz, CH₂-21), 3.99 (d, 1H, J 15.1 Hz, CH₂-21), 3.91 (d, 1H, J 15.6 Hz, CH₂-1’), 3.68 (d, 1H, J 15.6 Hz, CH₂-1’), 3.64–3.59 (m, 2H, OCH₂CH₂O), 3.58–3.35 (m, 22H, OCH₂CH₂O).

**¹³C NMR** (125 MHz, CDCl₃) δ 171.7 (CONH-1), 169.1 (CONH-1’), 134.0 (Aryl C), 132.0 (Aryl C), 131.2 (Aryl C), 130.8 (Aryl C), 130.1 (Aryl CH), 128.8 (Aryl CH), 127.7 (Aryl C), 127.2 (Aryl CH), 126.9 (Aryl CH), 126.5 (Aryl CH), 126.4 (Aryl CH), 126.3 (Aryl CH), 125.8 (Aryl CH), 125.7 (Aryl C), 124.6 (Aryl C), 120.8 (Aryl CH), 72.8 (CH₃), 70.8 (CH₂), 70.6 (CH₂), 70.6 (CH₂), 70.5 (CH₂), 70.5 (CH₂), 70.3 (CH₂), 69.5 (CH₂-1’), 61.7 (CH₂), 53.0 (CH₂-21).

**HR-ESI-MS** Calculated for C₃₂H₄₂N₃O₉: 612.2916 m/z [M+H]+; found 612.2908. **LC-MS** found 612.35, R₉ 0.50. 2 × CH₂ from the OEG chain were unaccounted for in the ¹³C NMR spectra due to overlapping signals.

**Supplementary Figure 46**: Analytical HPLC trace for 1-(hydrazinecarbonyl)-N-(pyren-1-yl)-2,5,8,11,14,17,20-heptaoxadocosan-22-amide 9.
Supplementary Figure 47: $^1$H NMR spectra (500 MHz, CDCl$_3$) for 1-(hydrazinecarbonyl)-N-(pyren-1-yl)-2,5,8,11,14,17,20-heptaoxadocosan-22-amide 9.

Supplementary Figure 48: $^{13}$C NMR spectra (125 MHz, CDCl$_3$) for 1-(hydrazinecarbonyl)-N-(pyren-1-yl)-2,5,8,11,14,17,20-heptaoxadocosan-22-amide 9.
24,25,27,28-tetrahydroxy-N-(pyren-1-yl)-26-[[3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxy]-3,6,9,12,15,18,21-heptaoxa-22-azaoctacos-22-enamide 10

3, 5-diaminobenzoic acid (31 mg, 0.20 mmol) was added to a stirred solution of lactose (349 mg, 1.02 mmol) and 20-hydroxylamino-N-(pyren-1-yl)-3,6,9,12,15,18-hexaoaicosanamide 5 (113 mg, 0.204 mmol) in a 1:1 mixture of MeCN : 0.1 M citrate buffer pH 3 (5 mL) and stirred until confirmed complete by LC-MS (72 h). The solvent was then removed under reduced pressure and purified by reversed-phase high-pressure liquid chromatography (C18; H2O (+0.1% CH3COOH)–MeCN (+0.1% CH3COOH) 95:5→50:50) to afford the product 10 as a brown oil (33 mg, 0.038 mmol, 19%) \( R_f \) 0.05 (SiO2; EtOAc–MeOH 80:20). **HR-ESI-MS** Calculated for C42H58N2NaO18: \( m/z \) 901.3577 [M+Na]+; found 901.3575. **LC-MS** Calculated for C42H59N2O18+: \( m/z \) 879.38 [M+H]+; found 879.43.

**Supplementary Figure 49:** Analytical HPLC trace for 24,25,27,28-tetrahydroxy-N-(pyren-1-yl)-26-[[3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxy]-3,6,9,12,15,18,21-heptaoxa-22-azaoctacos-22-enamide 10.
1-(pyren-1-yl)-3-[(21,22,24,25-tetrahydroxy-23-[(3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxy]-3,6,9,12,15,18-hexaoxa-19-azapentacos-19-en-1-yl]urea 11

3, 5-diaminobenzoic acid (19 mg, 0.13 mmol) was added to a stirred solution of lactose (190 mg, 0.555 mmol) and 3-[17-(aminooxy)-3,6,9,12,15-pentaoxaheptadecan-1-yl]-1-(pyren-1-yl)urea 6 (60 mg, 0.11 mmol) in a 1:1 mixture of MeCN : 0.1 M citrate buffer pH 3 (0.5 mL) and stirred until confirmed complete by LC-MS (24 h). The solvent was then removed under reduced pressure and purified by reversed-phase high-pressure liquid chromatography (C18; H2O (+0.1% CH3COOH)–MeCN (+0.1% CH3COOH) 95:5→50:50) to afford the product 11 as brown waxy solid (4.6 mg, 0.05 mmol, 5%). HR-ESI-MS Calculated for C41H57N3NaO17+: m/z 886.3580 [M+Na]+; found 886.3580. LC-MS Calculated for C41H58N3O17+: m/z 864.38 [M+H]+; found 864.25.

Supplementary Figure 50: Analytical HPLC trace for 1-(pyren-1-yl)-3-[(21,22,24,25-tetrahydroxy-23-[(3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxy]-3,6,9,12,15,18-hexaoxa-19-azapentacos-19-en-1-yl]urea 11.
Lactose (21 mg, 0.061 mmol) was dissolved in a stirred solution of 20-[(2-aminophenyl)formamido]-\(N\)-(pyren-1-yl)-3,6,9,12,15,18-hexaoxaicosanamide 7 (20 mg, 0.030 mmol) and NaBH\(_3\)CN (5.5 mg, 0.09 mmol) in 30% AcOH in DMSO (86.8 µL) then heated to 60 °C and stirred (2h). The reaction mixture was then allowed to cool and purified by reversed-phase high-pressure liquid chromatography (C\(_{18}\); H\(_2\)O (+0.1% CH\(_3\)COOH)–MeCN (+0.1% CH\(_3\)COOH) 95:5→5:95) to afford the product 12 as a white solid (10.2 mg, 0.010 mmol, 34%). HR-ESI-MS Calculated for C\(_{49}\)H\(_{66}\)N\(_3\)O\(_{18}\): \(m/z\) 984.4336 [M+H]\(^{+}\); found 984.4335. LC-MS found 984.39.

**Supplementary Figure 51:** Analytical HPLC trace for \(N\)-(pyren-1-yl)-20-[(2-[(2,3,5,6-tetrahydroxy-4-[[3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxy]hexyl]amino)phenyl]formamido]-3,6,9,12,15,18-hexaoxaicosanamide 12.
Lactose (7.2 mg, 0.02 mmol) was dissolved in a stirred solution of 2-amino-N-{17-[(pyren-1-yl)carbamoyl]amino}-3,6,9,12,15-pentaoxaheptadecan-1-yl)benzamide 8 (6.7 mg, 0.01 mmol) and NaBH₃CN (1.9 mg, 0.31 mmol) in 30% AcOH in DMSO (30 µL) then heated to 60 °C and stirred (2h). The reaction mixture was then allowed to cool and purified by reversed-phase high-pressure liquid chromatography (C₁₈; H₂O (+0.1% CH₃COOH)–MeCN (+0.1% CH₃COOH) 95:5→5:95) to afford the product 13 as a white solid (5.0 mg, 0.01 mmol, 68%). **HR-ESI-MS** Calculated for C₄₈H₆₅N₄O₁₇⁺: m/z 969.4339 [M+H]⁺; found 984.4335. **LC-MS** found 969.51.

**Supplementary Figure 52:** Analytical HPLC trace for N-{17-[(pyren-1-yl)carbamoyl]amino}-3,6,9,12,15-pentaoxaheptadecan-1-yl)2-{[2,3,5,6-tetrahydroxy-4-[[3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxy]hexyl]amino}benzamide 13.
N-(pyren-1-yl)-1-\{N’-\{2,3,5,6-tetrahydroxy-4-\{\{3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl\}oxy\}hexyl\}hydrazinecarbonyl\}2,5,8,11,14,17,20-heptaoxadocosan-22-amide 14

Lactose (25 mg, 0.07 mmol) was dissolved in a stirred solution of 1-\{hydrazinecarbonyl\}-N-(pyren-1-yl)-2,5,8,11,14,17,20-heptaoxadocosan-22-amide 9 (24 mg, 0.04 mmol) and NaBH₃CN (5.9 mg, 0.09 mmol) in 30% AcOH in DMSO (47 µL) then heated to 60 °C and stirred (2 h). The reaction mixture was then allowed to cool and subject to reversed-phase high-pressure liquid chromatography (C₁₈; H₂O (+0.1% CH₃COOH)–MeCN (+0.1% CH₃COOH) 95:5→5:95). Purification was incomplete and 14 was isolated as an impure compound (18.9 mg) and analysed without further purification. **HR-ESI-MS**

Calculated for C₄₄H₆₄N₃O₁₉+: m/z 938.4129 [M+H]+; found 938.4134. **LC-MS** found 938.49.

**Supplementary Figure 53:** Analytical HPLC trace for N-(pyren-1-yl)-1-\{N’-\{2,3,5,6-tetrahydroxy-4-\{\{3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl\}oxy\}hexyl\}hydrazinecarbonyl\}2,5,8,11,14,17,20-heptaoxadocosan-22-amide 14.
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Water-soluble pyrene tags enable the detection of carbohydrates by label-assisted laser desorption/ionisation mass spectrometry

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Abstract

Matrix-assisted laser desorption/ionisation mass spectrometry (MALDI-MS) is widely used for the analysis of biomolecules. Label-assisted laser desorption/ionisation mass spectrometry (LALDI-MS) is a matrix-free variant of MALDI-MS, in which only analytes covalently attached to a laser desorption/ionisation (LDI) enhancer are detected. LALDI-MS has shown promise in overcoming limitations of MALDI-MS in terms of sample preparation and MS analysis. In this work, we have developed water-soluble pyrene-based LDI reagents (LALDI tags) that can be used for (in situ) labelling and LALDI-MS analysis of reducing carbohydrates from complex (biological) samples without the need for additional chemical derivatisation or purification. We have systematically explored the suitability of four pyrene-based LDI enhancers and three aldehyde-reactive handles, optimised sample preparation, and exemplified the use of a LALDI tag by the detection of lactose in cow’s milk. These results demonstrate that LALDI-MS is a promising technique for the analysis of reducing carbohydrates in biological samples, and pave the way for the development of LALDI-MS for glycomics and diagnostics.

Introduction

Matrix-assisted laser desorption/ionisation (MALDI) is a soft ionisation technique for mass spectrometry (MS), in which analytes are co-crystallised with a suitable matrix that facilitates absorption of laser light as well as transfer of energy and charge to analytes.1,2 MALDI-MS is a powerful analytical tool for studying high molecular weight biomolecules, such as oligonucleotides, lipids, and glycoconjugates.3 Because of their low limits of detection and high-throughput capabilities, MALDI-MS instruments are increasingly being exploited as platforms for glycomics, proteomics, and point-of-care diagnostics.4-7 Despite the advantages and wide-spread use of MALDI-MS, the presence of a matrix also brings with it several disadvantages: analytes need to be able to mix and co-crystallise with the matrix, co-crystallised samples can contain significant inhomogeneities (“hot spots”) leading to challenges with reproducibility and quantification, and matrix peak charge-sharing clusters can obscure low molecular weight (below ~800-1000 Da) analytes.8 Moreover, because of the indiscriminate desorption/ionisation of species present in the matrix, contaminants such as salts, detergents, or more-readily ionised species can result in complicated spectra and analyte signal suppression, meaning purification of analytes may be needed before MALDI-MS analysis.9 Analysis of biological species can be especially difficult because reagents commonly used in biological studies,
such as buffers, salts and detergents, can affect both the co-crystallisation and ionisation.\textsuperscript{10} High salt concentrations can completely obscure or suppress analyte ionisation, necessitating the use of volatile buffers or sample clean up prior to MALDI-MS analysis. These limitations have prompted the development of various matrix-free LDI-MS approaches.\textsuperscript{11} A particularly promising approach is label-assisted laser desorption/ionisation mass spectrometry (LALDI-MS), in which chemical tags (LDI enhancers) cause the selective desorption/ionisation from complex mixtures of the analytes to which they are attached, without the need for purification or an external matrix.\textsuperscript{11}

Pyrenes have proven particularly suitable as LDI-enhancing tags. Amano et al. analysed oligosaccharides and glycopeptides derivatised with pyrenebutanoic hydrazide or 1-pyrenyl diazomethane by MALDI-MS.\textsuperscript{12–15} In these experiments, analytes were first purified and then labelled in organic solvent on the MALDI target plate, before co-crystallisation with a matrix. In contrast, Kozmin et al. demonstrated the direct detection of pyrene-labelled small molecules, as their stable (radical) cations, by matrix-free LDI in a MALDI-MS instrument equipped with a standard 355 nm laser.\textsuperscript{16} This enabled high-throughput reaction discovery by LALDI-MS, in which nanomoles of the pyrene-labelled substrates and products could be detected selectively against a complex background of reagents, catalysts and additives upon evaporation of the reaction solvents. Subsequently, the group of Basak demonstrated the use of pyrene-based reagents in LALDI-MS to detect zinc ions,\textsuperscript{17} cis-1,2-diols and catechols,\textsuperscript{18} and biogenic amines,\textsuperscript{19} Kita & Kigoshi and co-workers used LALDI-MS to analyse tryptic peptides,\textsuperscript{20–22} and Guo and co-workers described pyrene-based peptide probes for the measurement of protease activity.\textsuperscript{23}

Carbohydrates are the most ubiquitous and structurally diverse group of biomolecules, and their detection and analysis by mass spectrometry is notoriously difficult.\textsuperscript{24–27} Unlike proteins and nucleic acids, the biosynthesis of carbohydrates is not template-driven or under transcriptional control. Therefore, artificial amplification of a specific carbohydrate cannot be achieved and ultimately restricts the available material to the often minute heterogeneous quantities obtained from natural sources.\textsuperscript{20} Compared to peptides, carbohydrates are not easily ionised. To circumvent this issue, chemical derivatisation, such as permethylation\textsuperscript{20} or, less commonly, the addition of reducing-terminal functional groups\textsuperscript{29} is often employed to promote ionisation, enable purification and/or separation, and improve the stability and MS analysis of glycans.\textsuperscript{24} Such approaches have led MALDI-MS to be widely used for analysis and quantification of carbohydrates such as N- and O-glycans.\textsuperscript{25,29–32}

We hypothesised that LALDI-MS could be a suitable method for the direct detection of carbohydrates from complex biological samples. Such an approach would require the in situ derivatisation of carbohydrates with suitable LDI enhancers, which we call LALDI tags (Figure 1). Here, we report the development of water-soluble, pyrene-based LALDI tags for (in situ) labelling of reducing carbohydrates, and their subsequent detection by LALDI-MS. We have synthesised and characterised four different pyrene-based LDI enhancers, and optimised sample preparation, in order to achieve reproducible LALDI-MS measurements with good limits of detection. In addition, we have explored the use of different biorthogonal handles for the labelling of a reducing carbohydrate, lactose. Using LALDI-MS, the resulting pyrene-tagged lactose derivatives could be detected in complex aqueous samples without the need for purification, desalting or derivatisation. In addition, we have exemplified the use of LALDI tags for the direct detection (after in situ derivatisation) of lactose in cow’s milk.
Figure 1: Concept: LALDI tags as a tool for MS-based glycomics. The figure describes the general workflow for the analysis of glycans (in this study: lactose) from a biological sample using LALDI-MS. The inset shows the general LALDI tag design, incorporating an LDI-enhancing label (dark blue) to facilitate matrix-free laser desorption/ionisation, a linker (orange) to improve aqueous solubility of the LALDI tag, and a reactive handle (pink) that provides a quick and reliable method to selectively label glycans.

Results

Development of water-soluble pyrene-based ionisation enhancers

In order to develop water-soluble LALDI tags with good sensitivity and stability in LALDI-MS, we first considered the nature of the pyrene group and its linkage to a solubilising oligoethylene glycol (OEG) linker. Most previous LALDI-MS studies used LDI enhancers based on pyrene butyramide (PyBA). In addition, Yoneda et al. reported that compounds based on 6-amidopyrene (6-APy) have increased absorption at 355 nm (the wavelength of lasers commonly used in MALDI instruments), and that some 6-APy compounds could be observed by LALDI-MS with limits of detection down to 10 pmol. However, the 6-APy tag readily fragmented to lose ketene, and the work suggested that careful design of linker chemistry is essential to avoid complex fragmentation patterns in LALDI-MS or subsequent tandem MS analysis. Therefore, we designed and synthesised four different pyrene-OEG conjugates for analysis in LALDI-MS. Two were based on the previously reported PyBA (1) and 6-APy (2) moieties, and two on the novel 1-amidopyrene (1-APy; 3) and pyrene urea (PyU; 4) moieties (Figure 2a). The synthesis, purification and chemical analysis of these compounds are described in the Supplementary Methods. Compounds 1 and 2 exhibited UV/vis absorption profiles identical to those reported by Yoneda et al., with 6-APy derivative 2 exhibiting a bathochromic shift of the pyrene absorption band compared to PyBA derivative 1 (Figure 2b). 1-APy derivative 3 and PyU derivative 4 exhibited similar bathochromic shifts to 2, consistent with the extended conjugation of compounds 2-4 compared to 1. In addition, this shift was absent in an N-methylated variant of 3, compound S1 (see Supplementary Figure 1), suggesting that N-methylation results in steric hindrance preventing full conjugation between amide and pyrene.
Compounds 1-4 were then analysed by LALDI-MS, comparing their stabilities (i.e. level of in-source fragmentation), lower limits of detection and ease of spectral interpretation. A 9.4 T (Bruker solariX XR) high-performance MALDI-FT-ICR mass spectrometer was used, allowing unambiguous assignment of elemental formulae of molecular ions, cationised molecules, and decomposition products with low- or sub-ppm mass accuracy (Figure 2c,d). Compounds were dissolved in methanol, a known amount of each sample was spotted onto a target plate, and solvent was allowed to evaporate, resulting in films containing 1 fmol – 100 pmol of each compound on the target plate. LALDI-MS analysis was performed on these films without addition of any other reagents, matrices or surface materials, with three independent spots measured for each sample (see Methods for details). Measurements were initially performed using samples spotted onto standard ground steel MALDI target plates, but low shot-to-shot reproducibility was obtained (Supplementary Figure 2a). This was most likely the result of heterogeneities in the sample films. Indeed, scanning electron microscopy analysis of the sample films revealed the formation of ‘coffee ring’ patterns and accumulation of analytes in grooves on the target plate upon sample drying (Supplementary Figure 2b,c). Instead, use of a micro-focusing target plate designed to localise hydrophilic samples onto a smaller area resulted in LALDI-MS measurements with improved limits of detection of 10 fmol – 10 pmol, excellent shot-to-shot reproducibility, and good correlation between amount of analyte and signal intensity (Figure 2c,d). Therefore, this micro-focusing target plate was used for all subsequent LALDI-MS experiments.

LALDI-mass spectra of compounds 1-4 all showed the radical cations (M⁺) as the main peaks, in addition to sodiated and potassiated molecules ([M+Na]⁺, [M+K]⁺) (Figure 2c); the latter are most likely the result of salts in the solvents and on the target plate. Because we developed LALDI tags for analyses from biological samples containing high salt concentrations, no attempts were made to suppress the detection of these cationised molecules. 6-APy 2 and 1-APy 3 achieved favourable lower limits of detection of 10 fmol (Figure 2c,d). However, both compounds also exhibited some in-source fragmentation. For 6-APy 2, a peak was observed at [M–42]⁺ correlating with fragmentation at the terminal amide and loss of ketene, consistent with reports by Yoneda et al. 20 For 1-APy 3, a peak was observed at [M–28]⁺, which according to LC-MS analysis was not present in the synthetic product 3. Accurate mass LALDI MS (Figure 2) and product ion analysis (Supplementary Figure 3), in combination with LALDI-MS analysis of a minimal 1-APy analogue S2 (Supplementary Figures 4-6) (which also displayed [M–28]⁺ peaks), revealed that these signals were the result of the loss of CO. Collision-induced dissociation (CID) product ion analysis of the molecular ion of 1-APy 3 resulted in multiple expected product ions arising from fragmentations along the OEG and amide linkers (Supplementary Figure 3a). However, the [M–28]⁺ peak observed on LALDI-MS of 1-APy 3 was absent in the CID product ion spectrum of the molecular ion of 3, suggesting that the loss of CO occurs in the MALDI source. N-methylated 1-APy derivative S1 displayed reduced LDI source stability compared to 1-APy 3, with an increased relative abundance of [M–28]⁺ and additional fragmentation observed in the LALDI mass spectrum (Supplementary Figure 1). PyBA 1 and PyU 4 displayed little in source fragmentation and so excellent stability, and could be detected down to 100 fmol and 1 pmol, respectively (Figure 2c,d). Qualitative comparison of compounds 1-4 in Table 1 suggests that 6-APy 2 and 1-APy 3 have the most favourable limits of detection, while PyBA 1 and PyU 4 have the highest stability. Because 1-APy derivatives are more stable (no loss of ketene) and easier to synthesise than 6-APy derivatives, and because PyBA derivatives can give complex fragmentation patterns in tandem
MS analysis, LDI enhancers 3 and 4 were selected for the development of LALDI tags for carbohydrate analysis.

Figure 2: Performance of four different pyrene derivatives in LALDI-MS experiments. a) Structures of water-soluble pyrene-based LALDI reagents. b) UV/vis absorption spectra for compounds 1-4. The red hashed line indicates the wavelength of excitation applied in the LDI-MS instrument (355 nm). Note that the UV/vis spectra of pyrenes in solid state have been reported to display broad absorption bands <391 nm. c) LALDI-mass spectra of compounds 1-4 (10 pmol samples). d) Determination of limits of detection of compounds 1-4 by LALDI-MS. Bars represent the mean values for the sum of all positive mode ions related to each LALDI reagent, including the molecular ions (M**), cationised molecules ([M+Na]**, [M+K]**), and major fragments (2: [M-ketene]**, 3: [M-CO]**)) detected by LALDI-MS of decreasing sample amounts. Error bars represent the standard deviation, where n = 3 (analysis of three individual spots from the same compound solution).

Table 1: Summary of the performance of LDI enhancers 2-4 to PyBA 1 in LALDI-MS experiments.

| LDI enhancer | MS stability | Limit of detection (pmol) |
|--------------|--------------|--------------------------|
| PyBA 1       | No major fragmentation | 0.1                      |
| 6-APy 2      | Additional peak at [M–42]** | 0.01                    |
| 1-APy 3      | Additional peak at [M–28]** | 0.01                    |
| PyU 4        | No major fragmentation | 1                       |

PyBA, pyrenebutyramid; 6-APy, 6-amidopyrene; 1-APy, 1-amidopyrene; PyU, pyrene urea.
Development of LALDI tags for reducing carbohydrates

To label reducing carbohydrates, such as N- or O-glycans released through enzymatic or chemical cleavage techniques, suitable reactive handles were required that would be efficient, selective, and form a stable conjugate product in the presence of a complex biological matrix. Inspired by common glycan labelling strategies, reviewed in, three reactive handles were chosen: hydroxylamine, hydrazide, and 2-aminobenzamide (2-AB). Five unique LALDI tags were synthesised, combining the two selected LDI enhancers and the three reactive handles through their solubilising linker (compounds 5-9, Table 2). The synthesis, purification and chemical analysis of these LALDI tags are described in the Supplementary Methods. Compounds 6, 8 and 9 were analysed by LALDI-MS to determine whether their detection and stability would be affected by the three different reactive handles (Supplementary Figures 7-9; Supplementary Table 1). Analysis of these unreacted LALDI tags would also provide insight relevant for the analysis of in situ labelling reactions, allowing distinctions to be made between signals relating to the products, unreacted LALDI tags, and other signals in the spectra. Each of the reagents was detected by LALDI-MS, but the compounds (especially 9) underwent more in-source fragmentation than the simple LDI enhancers 1-4. This is most likely because of the reactivity of the handles, and the instability of N-O and N-N bonds that we observe in our LALDI-MS experiments (see for example Supplementary Figure 10) consistent with previous reports of N-O bond fragmentation in LALDI-MS.

LALDI tags 5-9 were subsequently used for the labelling of lactose, which was considered an ideal model for the labelling reaction and LALDI-MS analysis because it is readily available, and because small glycans such as lactose can be difficult to detect by MALDI-MS without further chemical modifications (reviewed in), use of additives, or use of specialised nanoparticle supports. Lactose was labelled with each of the five LALDI tags 5-9, with high conversion assessed by LC-MS analysis (Table 2). The pyrene-labelled lactose derivatives 10-14 were purified by preparative HPLC to enable analysis of known quantities of well-defined analytes, in order to study performance of 10-14 in LALDI-MS analysis. According to analytical HPLC, compounds 10-13 were analytically pure (Supplementary Figures 49-52), while compound 14 contained contaminants resulting from the labelling reaction that could not be removed (Supplementary Figure 53). LALDI-MS analysis was carried out on these samples (Figure 3; Table 2).
Table 2: The use of LALDI tags 5-9 to detect lactose by LALDI-MS.

| LALDI tag | Ratio LALDI tag:lactose | Reaction Conditions* | Product | % Label converted† | Molecular ion detected in LALDI-MS? | MS stability‡ | Limit of detection (pmol) |
|-----------|-------------------------|----------------------|---------|-------------------|------------------------------------|---------------|--------------------------|
| 5\textsuperscript{54} | 1:5 | oxime ligation\textsuperscript{54} | A 10 | 100% | Yes | +/- | 1 |
| 6\textsuperscript{54} | 1:5 | oxime ligation\textsuperscript{54} | A 11 | 100% | Yes | + | 10 |
| 7\textsuperscript{55} | 1:2 | reductive amination\textsuperscript{55} | B 12 | 92% | Yes | ++ | 10 |
| 8\textsuperscript{55} | 1:2 | reductive amination\textsuperscript{55} | B 13 | 89% | Yes | ++ | 10 |
| 9\textsuperscript{56} | 4:7 | reductive hydrazination\textsuperscript{56} | B 14 | 64% | No | -- | n/a |

* Reaction conditions: A) 3,5-diaminobenzoic acid, MeCN : sodium citrate buffer (0.1 M, pH 3) (1:1), rt, 24 h; B) NaBH\textsubscript{3}CN, DMSO:AcOH (7:3), 60 °C, 2 h. Additional details of the reaction conditions, purification and chemical analysis of the labelled lactose are described in the Supplementary Methods. † Conversion was calculated by comparing integrals of UV absorbance peaks for starting materials 5-9 and products 10-14 in the LC-MS analysis of the reaction mixture. ‡ MS stability was assessed from i) the number of ions observed, in addition to the molecular species, on LALDI-MS analysis of each analyte, and ii) the relative abundance of the signals corresponding to the molecular ion (M\textsuperscript{+}) and cationised molecules ([M+Na]\textsuperscript{+}, [M+K]\textsuperscript{+}) compared to the additional ions (presumably fragment ions), then scored using an arbitrary scale from ++ to --.
Figure 3: LALDI mass spectra for samples of LALDI-tagged lactose derivatives (100 pmol) with molecular ions, cationised molecules, and identified fragments. a) 10 (from MeOH). b) 10 (from HBSS) c) 11 (from MeOH). d) 12 (from MeOH). e) 13 (from MeOH). f) 14 (from MeOH). HBBS = Hanks’ balanced salt solution.

LALDI-MS analysis of 10-14 resulted in reproducible detection (Figure 3; Supplementary Figures 10-15). Lactose oxime derivatives 10 and 11, and the 2-AB-linked lactose derivatives 12 and 13 gave signals with good signal-to-noise ratios (Figure 3a,c,e; Supplementary Figures 10-13). However, analysis of the hydrazide-linked lactose derivative 14 did not yield signals for the expected products; instead the spectrum was dominated by signals for low molecular mass species, presumably produced on in-source fragmentation occurring predominantly around the hydrazide linkage (Figure 3f; Supplementary Figure 14). Consistent with analysis of simple 1-APy LDI enhancer 3 (see above), loss of CO was clearly detected from 1-APy-labelled lactose derivative 10 (Figure 3a; Supplementary Figure 10). Loss of CO was also observed during analysis of 1-APy-labelled lactose 12, although only low intensity signals for [12–CO]⁺⁺ were observed (Supplementary Figure 12). Both oxime-linked lactose derivatives 10 and 11 displayed in-source fragmentation at the oxime linkage (Figure 3a,c; Supplementary Figures 10-11). 2-AB linked lactose derivatives 12 and 13 were clearly detected as [M+Na]⁺ (Figure 3d,e); only low intensity fragment ions – presumably arising on fragmentation around the benzamide and secondary amine groups – were observed (Figure 3d,e; Supplementary Figures 12-13). 1-APy 10 was found to have the lowest limit of detection, observed down to a sample size of
1 pmol, while labelled lactose derivatives 11-13 were found to have a lower limit of detection of 10 pmol. No lower limit of detection was determined for lactose hydrazide derivative 14 as neither the molecular ion nor cationised molecules were detected at any sample quantity. From these results, it was concluded that pyrene-labelled lactose compounds 12 and 13 were the most stable in LALDI-MS analysis (Table 2), while compound 10 resulted in the lowest limit of detection (while displaying N-O bond cleavage).

To test the detection of LALDI-tagged lactose derivatives in a complex sample matrix, LALDI-MS analysis of 10-13 was repeated with the samples dissolved in the cell culture buffer Hanks’ balanced salt solution (HBSS), which contains various buffer salts ([Na+] 142 mM, [K+] 5.80 mM, [Mg2+] 0.898 mM, [Ca2+] 1.26 mM), glucose (5.55 mM), and a phenol red indicator (26.6 µM). An image of the evaporated sample of LALDI-tagged lactose 10 in HBSS, captured by the mass spectrometer’s target camera is shown in Supplementary Figure 16b. The crystalline appearance of the sample is consistent with the high abundance of buffer salts present in the samples analysed during these experiments. While the limits of detection were affected by the presence of the buffer components (Supplementary Table 2), each of the pyrene-labelled lactose derivatives 10-13 was clearly detected in the presence of HBSS, with the spectra obtained (Figure 3b; Supplementary Figure 16a) being nearly identical to those obtained from pure samples (Figure 3a,c-f). These results highlight the advantage of the LDI-enhancing label in selectively promoting ionisation and MS detection of only the labelled species, even in the presence of buffer, contaminants and high concentrations of salts.

Use of a LALDI tag for selective labelling and detection of lactose in cow’s milk

To test if LALDI tags could be used to label reducing carbohydrates in situ in a complex sample matrix, LALDI tag 6 was used to label lactose directly in cow’s milk by adapting the previously optimised oxime ligation conditions (Table 2). Cow’s milk was chosen for this proof-of-concept because of its high lactose concentration (4–5% w/w) and complex matrix. Following labelling, the sample was diluted and centrifuged to remove curd to enable the sample solution to be transferred by pipette to the MALDI plate. The supernatant was analysed directly by LALDI-MS (Figure 4), giving notably clean LALDI mass spectra displaying peaks corresponding to lactose derivative 11 (m/z 896.3591 [M+Na]+ and 902.3328 [M+K]+). The most intense peak (m/z 524.2518) was assigned as a fragment ion (11') resulting from N-O bond cleavage. This fragment could also have been generated through fragmentation of the hydroxyl amine-functionalized LALDI tag 6 (N-O bond fragmentation was observed during LALDI-MS analysis of PyU-OEG-ONH2; 6; see Supplementary Figure 7), but it should be noted that the amount of 6 added to the milk sample was less than the estimated amount of lactose present, and that ions corresponding to intact 6 were not detected in this LALDI-MS experiment. Although different LALDI tags and in situ labelling conditions need to be explored in future studies, these results highlight the potential of LALDI tags to be used for the labelling and analysis of reducing carbohydrates directly in complex biological samples. Furthermore, it is important to note that detection of only pyrene-containing molecules indicates that, at the concentrations tested, the LDI enhancers did not act as a matrix for the many other non-carbohydrate species present in the samples.
Figure 4 LALDI-MS analysis of milk following glycan labelling with LALDI tag 6. a) Schematic representation of the general workflow for in situ labelling of lactose in milk and subsequent LALDI-MS analysis. b) LALDI mass spectrum obtained following direct LALDI-MS analysis of the labelling reaction mixture; identified fragments and molecular species are annotated with their observed m/z values. c) Chemical structures for LALDI tag-labelled lactose 11 and proposed major fragment ion 11'. Proposed assignments for fragments and molecular species observed in the LALDI mass spectrum in (b) have been annotated in the structure of 11, accompanied by their corresponding calculated monoisotopic masses.

Discussion

We have developed novel, water-soluble LDI-enhancers, exhibiting properties including low limits of detection, good stability, and predictable MS fragmentation. We introduced two new LDI enhancers, 1-APy and PyU, derivatives of which can be readily synthesised from commercially available 1-aminopyrene. By studying the behaviour of samples on different target plates, we optimised both limits of detection and reproducibility of LALDI-MS measurements. 1-APy 3 displayed lower limits of detection than PyBA derivative 1, and higher stability than 6-APy derivative 2 in LALDI-MS experiments. In contrast, PyU 4 showed no in-source fragmentation but had higher limits of detection. By combining pyrene-based LDI enhancers with handles for tagging reducing carbohydrates, we were able to develop a range of water-soluble LALDI reagents capable of labelling and detecting reducing carbohydrates (in this work: lactose) by LALDI-MS. We have demonstrated that
LALDI tagging can be performed in cow’s milk, and that pyrene-tagged lactose derivatives can be detected by LALDI-MS in complex (biological) matrices without the need for further purification, chemical modification, or additional MALDI matrices.

The correlation between increased UV absorption at 355 nm and (slightly) improved limit of detection in LALDI-MS experiments displayed by 6-APy 2 and 1-APy 3 compared to PyBA 1 is consistent with results reported by Yoneda et al. However, the improvement in limits of detection in our experiments was modest. Moreover, PyU 4 was found to have a higher limit of detection than PyBA 1, despite displaying a similar bathochromic shift to 2 and 3. These results emphasise that factors additional to optical properties of the pyrene tag (e.g., ease of ionisation, stability of molecular ion and cationised molecules, stability to in-source fragmentation) are also likely to influence limits of detection in LALDI-MS. It should also be noted that, in LALDI-MS, samples are analysed from a dried spot, not as solutions. Sharma et al. demonstrated that, in solution, the UV absorption profile of pyrene is identical to that of PyBA derivative 1, while solid pyrene exhibits a broader and red-shifted UV absorption profile with much higher absorbance at 355 nm. They proposed that the observed change in absorbance was due to increased r-stacking interactions in solid pyrene. Therefore, while information gained on the solution phase UV absorbance of 1-4 was useful for comparison of our compounds to previously reported pyrene-based LDI enhancers, it may not accurately represent the absorbance of an LDI enhancer during a LALDI-MS experiment. Indeed, Kigoshi & Kita et al. recently demonstrated that LDI enhancers based on differently substituted pyrenes (including PyBA, 6-APy and a new, highly sensitive N,N-dimethylaminopyrene) all displayed a broad absorbance band <391 nm when UV/vis was performed on solid samples. The authors observed that the sensitivity of three pyrene derivatives in LALDI-MS was inversely correlated with their fluorescence quantum yield, suggesting that performance of LDI enhancers is related to their heat emission. This observation should be taken into account in the design of future generations of LALDI tags with increased LALDI-MS sensitivity.

LALDI-MS was preceded by the development of fluorophore-assisted laser desorption/ionisation mass spectrometry (FALDI-MS), in which fluorescent dyes acted as the LDI enhancers. In addition, West et al. demonstrated that 2-aminobenzamide, a common fluorescent label for reducing carbohydrates, could be used for the FALDI-MS detection of the oligosaccharide maltoheptaose. However, the resulting spectrum featured unidentified higher mass signals and analysis required a relatively large amount of sample (~157 nmol). In addition, the fluorophores appeared to act as weak matrices, ionising impurities and species other than the carbohydrate analytes, thereby generating more complicated spectra than was the goal. In contrast, our results suggest that pyrene derivatives do not act as a general MALDI matrix under the conditions tested in this work, and result in ionisation of only covalently labelled species even when analysed against complex (biological) backgrounds.

Our first generation water-soluble LALDI tags for the labelling and detection of reducing carbohydrates incorporate reactive handles that are commonly used for carbohydrate derivatisation. LALDI tags with a hydroxylamine or 2-AB reactive handle were successfully used to label and detect lactose. The selection of LALDI tags for specific (biological) sample application will depend both on the compatibility of the samples with labelling conditions (e.g., solvent and pH) and the required stability: the 2-AB linked products displayed higher in-source stability than oxime-linked products, with the latter undergoing N-O fragmentation. The successful in situ labelling and detection of lactose in cow’s
milk suggests that LALDI tags can indeed be used for detection of carbohydrates in biological matrices, but it should be noted that carbohydrate ligation conditions need to be optimised further for the specific biological matrix. In addition, enrichment may be needed for the LALDI-MS analysis of less abundant carbohydrates. Interestingly, recent reports suggest that the affinity of pyrene derivatives for materials such as polystyrene\textsuperscript{23} and TSK-G3000S gel (styrene-divinylbenzene copolymer)\textsuperscript{21,22} may allow specific affinity enrichment of LALDI-tagged carbohydrates from complex mixtures before LALDI-MS analysis, which could be used to significantly improve the limit of detection.

Through this investigation we have gained a better understanding of how the structure and chemical functionality of LDI enhancers, linkers, and reactive handles affect the limits of detection and in-source stability of reagents analysed by LALDI-MS. Our findings highlight LALDI tags as promising reagents for the detection/analysis of carbohydrates as well as other biomolecules from complex biological environments. Important for glycomics and activity-based profiling approaches, the reproducibility and concentration-dependence of the LALDI-MS signal intensity may additionally allow (semi-)quantitative measurements, for example through careful external calibration or internal calibration using isotopically labelled internal standards.

**Methods**

**Synthesis and characterization.** Synthetic procedures, compound characterisation and relevant spectra of final compounds are available in the Supplementary Methods.

**UV/vis analysis.** UV/vis absorption was measured using an Agilent Technologies Cary 100 UV-Vis Spectrophotometer. All samples were analysed as solutions in a 10 mm Hellma Analytics High Precision Quarts Suprasil cell. Samples for UV/vis analysis were prepared from a 1 mM methanolic solution of the purified analytes, then adjusted to the desired concentration by serial dilution. Absorption maxima ($\lambda_{\text{max}}$) are given in nanometers to the nearest whole nanometer with the corresponding molar extinction coefficient ($\varepsilon$) given in M$^{-1}$ cm$^{-1}$.

**LALDI-MS analysis.** LALDI-MS was carried out on a Bruker Daltonics solariX XR FTMS 9.4T mass spectrometer with an Apollo II dual ESI/MALDI ion source. All optimised analysis was performed using a Bruker Daltonics MTP AnchorChip Target 384. Ionisation/desorption of the samples was achieved using a fixed wavelength (355 nm) Bruker Smartbeam II (Nd:YAG) laser. $m/z$ values are reported to four decimal places to reflect the mass accuracies obtained using external mass calibration with phosphorus red. LALDI-MS fragmentation assignment was carried out with the assistance of a web application developed by ChemCalc that provides a list of possible molecular formulae from a given monoisotopic mass.$^{60,61}$

Samples for LALDI-MS analysis were prepared from a 1 mM solution of the purified analytes in methanol, which was then adjusted to the desired concentration with either methanol or Hanks’ balanced salt solution. A known volume (1 µL) of each analytical sample was spotted onto the target plate and the solvent allowed to evaporate under ambient conditions. Evaporation of aqueous solutions was assisted using reduced pressure. LALDI-MS analysis was then performed directly on each sample (without addition of further reagents such as MALDI matrix or SALDI surface material) with 1600 laser shots per acquisition (8 scans, 200 shots, 200 Hz, medium laser focal size). Each sample was analysed in triplicate (three separate aliquots on separate targets) and the average values of the peak
intensities are reported. Mass spectra were acquired using ftmsControl software version 2.1 (Bruker Daltonics) and processed using flexAnalysis software version 3.0 (Bruker Daltonics).

In situ labelling of lactose from milk with hydroxylamine LALDI tag 5. 3,5-diaminobenzoic acid (3 mg, 0.02 mmol) was added to a stirred solution of 3-[17-(aminooxy)-3,6,9,12,15-pentaoxaheptadecan-1-yl]-1-(pyren-1-yl)urea 5 (10 mg, 0.02 mmol) in a 1:1 mixture of cow’s milk: 0.1 M citrate buffer pH 3 (0.5 mL) and stirred (12 h). The mixture was then diluted 10-fold with methanol, subjected to centrifugation (10000 g, 30 s), and 1 µL of the supernatant directly spotted onto an AnchorChip target plate, dried, and analysed by LALDI-MS.

Data availability. The authors declare that the data supporting the findings of this study are available within the article and Supplementary Information file, or from the corresponding author upon reasonable request.

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Author contributions

J.R.H. carried out synthesis and characterisation of reagents, and UV/vis and LALDI mass spectrometry experiments. E.T.B. contributed to LALDI mass spectrometry experiments. A.N.K. performed electron microscopy experiments. J.R.H, S.L.W., J.T.-O. and R.S.B. designed experiments. J.R.H. and A.N.K. analysed data. J.R.H. made figures. S.L.W., J.T.-O. and R.S.B. provided project supervision. R.S.B. conceived the project. J.R.H., J.T.-O. and R.S.B. wrote the manuscript. All authors commented on the manuscript.

Conflict of interest

The authors declare no conflict of interest.

Additional Information

Supplementary Information (Supplementary Figures, Methods and References, including details of the synthesis and characterisation of compounds)
