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Efficient synthesis of RGD-containing cyclic peptide–porphyrin conjugates by ring-closing metathesis on solid support

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Abstract—We report the efficient solid-phase synthesis of a new family of peptidic porphyrins containing a constrained RGD moiety obtained by ring-closing metathesis.

Photodynamic therapy (PDT) is a new method for the treatment of cancer that has received considerable attention in recent years.[1] This technique relies upon the selective accumulation of photosensitizing molecules such as porphyrins into tumors followed by irradiation of the affected area with visible light. Upon irradiation, the excited state of the photosensitizer generates singlet oxygen that induces cell damage and ultimately leads to cell death.[2] A major side effect of PDT is long-lasting skin photosensitivity due to a lack of porphyrin selectivity and a slow clearance rate of the photosensitizer. As a result, more selective photosensitizers are desired. To this end, we have devised a synthetic route to porphyrin derivatives designed for targeting tumors and more specifically neovascularization that nourish cancer cells.

Endothelial cells are crucial in angiogenesis, the process of new blood vessel formation associated with tumor growth and metastasis. $\alpha_v\beta_3$-Integrin, a heterodimeric transmembrane glycoprotein receptor, is overexpressed in actively proliferating endothelial cells in and around tumor tissues.[3] This integrin may therefore represent a promising target for the delivery of drugs such as photosensitizers.[4] Small peptide ligands for the $\alpha_v\beta_3$-integrin have been identified that share an RGD (Arg-Gly-Asp) motif as a common sequence. Cyclic peptides containing an RGD motif adopt conformations showing an increased affinity for integrins.[5a,b] In particular, the cyclic dithiopentapeptide CRGDC, containing a S–S link described by Ruoslahti and co-workers, presents an excellent selectivity for $\alpha_v\beta_3$-integrin.[6] In order to increase the plasmatic life time of the targeting molecule, the enzymatically-sensitive disulfide bond of the thiopeptide can be substituted with a C–C bond and, for this purpose, ring-closing metathesis appeared as a very attractive strategy due to the mild reaction conditions and the exceptional functional group tolerance of the Grubbs ruthenium-based catalysts.[7,8]

In connection with our research program on porphyrins designed for cancer phototherapy, we report in this paper the efficient solid-phase synthesis of glucosylated porphyrins **6a** and **6b** bearing a cyclic pseudopentapeptide incorporating a RGD sequence obtained by ring-closing metathesis. The general procedure for the synthesis of these porphyrins displayed in Schemes 1 and 2 consists of two steps: the cyclization of a diolefinic linear precursor, containing the RGD moiety flanked by two *allyl*glycine residues, on the solid phase and its connection to porphyrins bearing a spacer arm with a carboxylic end group, again on solid phase.

Ring-closing metathesis (RCM) reactions were carried out on a Rink Amide MBHA resin bearing the *allyl*Glycine-RGD-*allyl*Glycine linear pentapeptide (prepared according to conventional solid phase peptide synthesis using the Fmoc strategy), in which the β-carboxyl function of aspartate is protected with *tert*-butyl and the guanidino function of arginine protected

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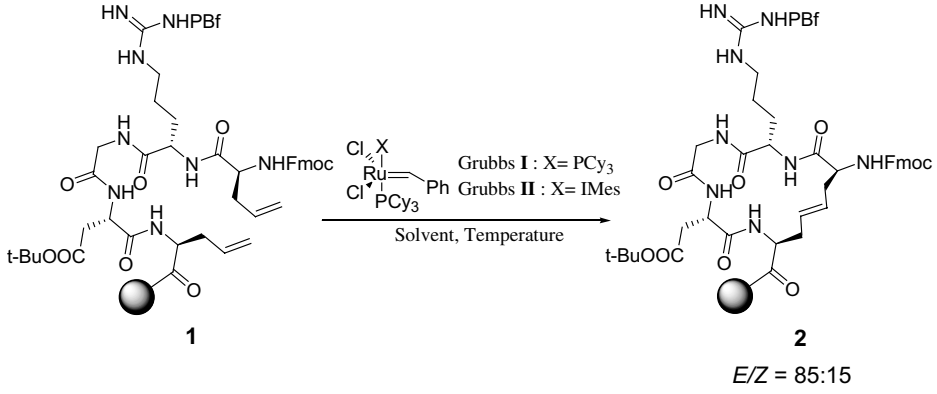
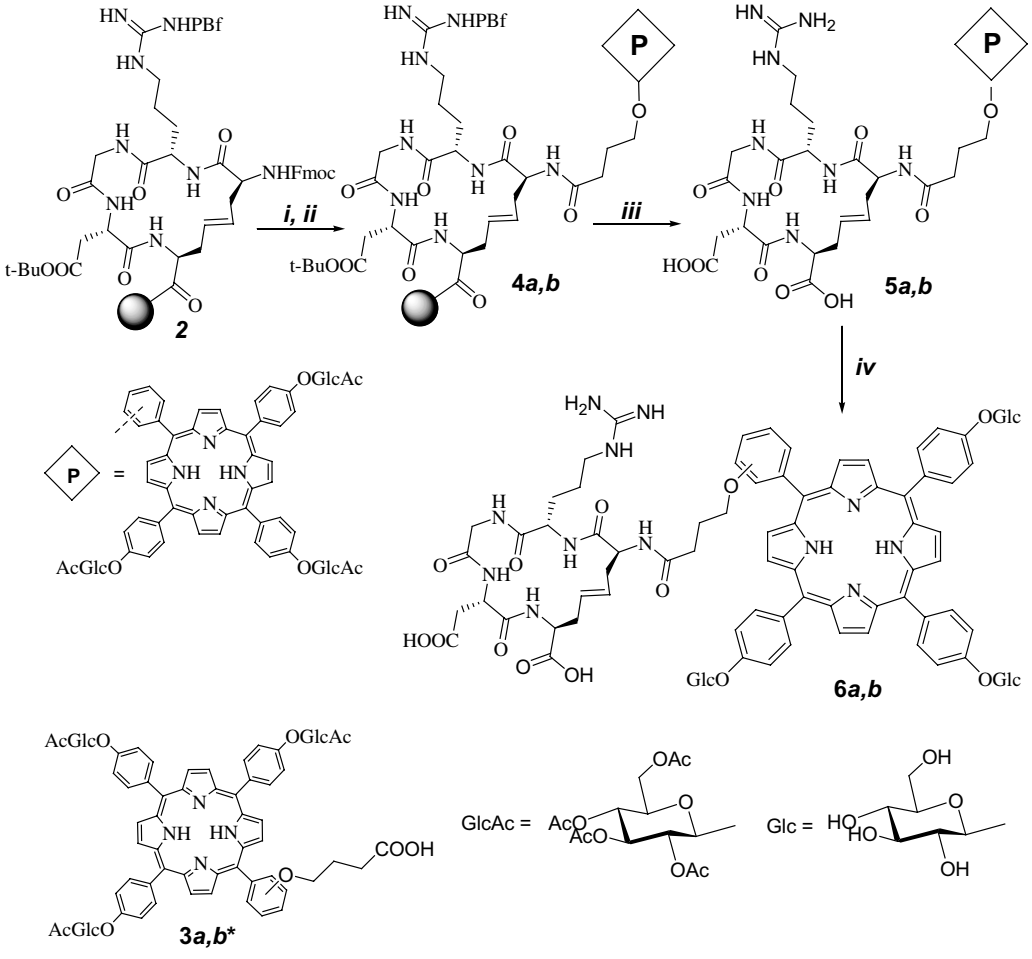


Scheme 1. Peptide cyclization by ring-closing metathesis on solid support.



Scheme 2. Reaction conditions: (i) DMF/piperidine 8:2, rt, 15 min; (ii) compounds **3a** or **3b** (2 equiv), DIC (2 equiv), HOBt (2 equiv), DMF/CH_2Cl_2 1:1, rt, 24 h; (iii) TFA/CH_2Cl_2/anisole 8.5:1:0.5, rt, 30 min; (iv) MeONa (0.5 M in MeOH), MeOH/CH_2Cl_2 8:2, rt, 1 h. *: synthesized from Ref. 8.

with a 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl (Pbf) group. In a typical experiment, the resin beads were allowed to swell in dry, degassed dichloromethane under an argon atmosphere and then Grubbs' catalyst (I or II) predissolved in dichloromethane was added with the help of a syringe. After reaction the beads were filtered, rinsed with CH_2Cl_2, DMF, and MeOH and then the product was cleaved and deprotected by TFA treatment of a fraction of resin for HPLC and MALDI analysis. Different conditions of temperature, reaction time, catalyst, or resin substitution were tested. Poor results were obtained using a resin with a substitution of 0.5 mmol g^{-1}. HPLC and mass spectrometry revealed the presence of several peaks corre-

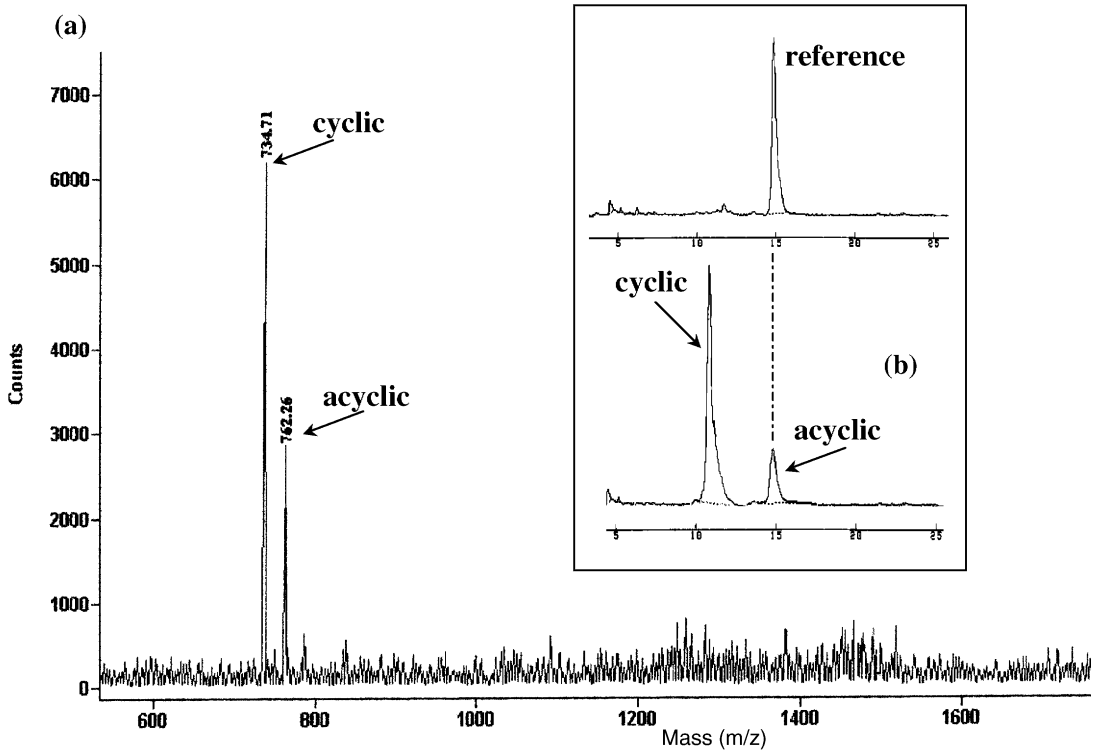


Figure 1. MALDI spectrum (a) (corresponding to the reaction conditions reported in Table 1, entry 7) and HPLC trace (b) of crude product of ring-closing metathesis from a resin with a substitution of 0.1 mmol g^{-1}. The HPLC analyses were carried out on a LDC/Milton Roy system with a spectrophotometer detector, wavelength 210 nm, using a reverse phase Supelcosil™ LC-18 column, (250×4.6 mm), with a flow rate of 0.6 mL min^{-1}, eluent: CH_3CN/H_2O (H_3PO_4/Et_3N 1:1 0.1%): 6:4.

sponding to the starting material, a minor amount of the desired ring-closed product and other compounds identified as products resulting from intermolecular cross metathesis (dimers and large macrocyclic peptides). In this case, the yield in expected cyclic pentapeptide **2** did not exceed 10%. The yield was significantly improved by reducing the resin substitution to 0.1 mmol g^{-1}. This low substitution loading avoided intermolecular reactions (Fig. 1) and, under the best conditions, when reaction was performed for 48 h in dichloromethane at reflux with 30 mol% of Grubbs' II catalyst added in three portions (Table 1, entry 8), we obtained the expected compound **2** in 71% yield. ^1H and ^{13}C NMR analysis confirmed the structure and showed that the reaction leads to a large proportion (>80%) of the *trans*-olefin stereoisomer.[11]

The 9-fluorenylmethoxycarbonyl (Fmoc) protective group was easily removed in quantitative yield under very mild basic conditions using 20% piperidine in DMF. Reaction was monitored by UV–vis spectroscopy by measurement of the quantity of dibenzofulvene–piperidine adduct liberated. The synthesis and characterization of carboxy-glucosylporphyrins **3a**,**b**, which were condensed on peptide, have been thoroughly described in a previous paper.[9] Formation of these compounds proceeds in three steps: the synthesis of monohydroxyphenyltriglucosylporphyrins using the Lindsey protocol, followed by alkylation with *tert*-butyl 4-bromobutyrate and then hydrolysis.[10]

A general procedure to covalently bind carboxy-functionalized porphyrins **3a**,**b** to the NH_2 of the cyclic pentapeptide consists in activation of the carboxyl group with diisopropylcarbodiimide (DIC)/1-hydroxybenzotriazole (HOBt) system. In a typical experiment, the resin bearing the cyclic peptide (nominal loading 0.1 mmol g^{-1}) was swelled in DMF/CH_2Cl_2 and then

Table 1. Reaction conditions for ring-closing metathesis on solid support (resin substitution: 0.1 mmol g^{-1})

Entry	Solvent	Temperature (°C)	Catalyst (mol%)	Time (h)	Yield (%)
1	CH_2Cl_2	20	I (30)	24	31
2	CH_2Cl_2	20	II (30)	24	31
3	CH_2Cl_2	40	I (30)	24	40
4	CH_2Cl_2	40	II (30)	24	43
5	CH_2Cl_2	40	I (30)	48	51
6	CH_2Cl_2	40	II (30)	48	52
7	CH_2Cl_2	40	I (30) added in three portions	48	65
8	CH_2Cl_2	40	II (30) added in three portions	48	71

porphyrin **3a**,**b** (2 equiv) was added with DIC (2 equiv) and HOBt (2 equiv). After 24 h, the resin was filtered, washed with DMF and CH_2Cl_2, and then dried under vacuum overnight. Peptidic porphyrins **4a** and **4b**, grafted on resin, were obtained in 82% and 79% yield, respectively.

Porphyrin derivatives **5a**,**b** were isolated by simultaneous detachment and deprotection of the peptidic-porphyrin conjugates from supports **4a**,**b** by reaction with TFA/CH_2Cl_2/anisole (85:10:5, v/v) for 30 min. After evaporation of solvents, neutralization by $NaHCO_3$, and purification on silica gel PLC (eluent $CHCl_3$/EtOH 80:20 with 1% TFA) the expected compounds **5a** and **5b** were obtained in 70% yield. Removal of the acetate protective groups on the glucosylporphyrins **5a** and **5b** with 0.5 M sodium methoxide in MeOH/CH_2Cl_2 (8:2) gave water-soluble porphyrins **6a** and **6b** in 80% yield, after purification by preparative reverse-phase chromatography (RP-18 phase [10 μm], H_2O/THF 75:25).

Mass spectrometry of all porphyrins derivatives was performed using the MALDI technique and for all compounds, spectra gave the expected quasi molecular peaks $[M+H]^+$ with a minor contribution of the radical cation $M^{+\cdot}$. In addition, these porphyrins show standard absorption spectra with a Soret band near 420 nm and four less intense visible Q bands with an etio outline. Finally, acetylated RGD-triglucosylporphyrins gave satisfactory ^1H and ^{13}C NMR data and structures of compounds **6a**,**b** were confirmed by HRMS analysis.[11]

In order to determine the photosensitizing properties of porphyrins **6a** and **6b**, trapping reactions of $^1O_2$ with ergosterol acetate were carried out. Reference experiments with eosin or hematoporphyrin (HP) as sensitizers gave ergosterol acetate endoperoxide in nearly quantitative yields. Under the same experimental conditions, porphyrins **6a** and **6b** showed the same efficiency for $^1O_2$ production than HP, which is known as a photosensitizer that produces singlet oxygen. Biological evaluation of compounds **6a**,**b** for their anticancer activity by PDT is currently in progress in our laboratory and will be reported elsewhere.

Acknowledgements

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11. All new compounds reported here gave analytical and spectral data consistent with the assigned structures. NMR spectra were recorded on a Brüker AM-400 spectrometer. Chemical shifts are reported in ppm downfield from tetramethylsilane (TMS). Selected analytical data: compound **2** (after cleavage from resin): *(3S,9S,12S,14E,17S)-17-carbamoyl-12-((9-fluorenylmethoxycarbonyl)amino)-9-(3-guanidinopropyl)-2,5,8,11-tetraoxo-1,4,7,10-tetraazacycloheptadec-14-enyl-3-acetic acid*: ^1H NMR (400 MHz, DMSO-d_6): 1.48–1.52 (m, 2H), 2.31–2.33 (m, 2H), 2.37–2.42 (m, 4H), 2.53–2.52 (m, 2H), 3.70–3.73 (m, 2H), 4.11–4.13 (m, 1H), 4.20–4.24 (m, 4H), 4.31–4.32 (m, 2H), 4.46–4.49 (m, 1H), 5.35–5.40 (m, 2H), 7.32 (t, 2H, $J = 7.3$ Hz), 7.33–7.35 (m, 1H), 7.35–7.37 (m, 1H), 7.41 (br t, 2H, $J = 7.3$ Hz), 7.63–7.64 (m, 1H), 7.69 (br d, 2H, $J = 7.0$ Hz), 7.87 (br d, 2H, $J = 7.8$ Hz), 8.01–8.03 (m, 1H), 8.28–8.29 (m, 1H). ^{13}C NMR (100 MHz, DMSO-d_6): 24.6, 33.9, 34.8, 35.7, 39.3, 42.5, 45.5, 46.5, 49.9, 51.8, 51.8, 52.1, 65.5, 120, 125.1, 126.9, 127.5, 140.9, 144, 156.8, 157.1, 170.3, 171.1, 172.5, 172.5, 174.3; HPLC: 11.87 min (CH_3CN/H_2O (H_3PO_4/Et_3N 1:1 0.1%): 6:4). MS MALDI: calculated for $C_{35}H_{43}N_9O_9$: $m/z = 733.31$; found: $m/z = 734.71$ $[M+H]^+$.
Compound **5a**: TLC R_f: 0.32 ($CHCl_3$/EtOH/TFA 8:2:0.1). UV–vis: [$CHCl_3$/MeOH 8:2; λ (nm), ($\varepsilon \times 10^{-3}$ ($L\,mol^{-1}\,cm^{-1}$))]: 420 (312.0), 516 (12.5), 552 (6.9), 592 (4.2), 648 (4.0). MS MALDI: $m/z = 2248.74$ $[M+H]^+$. ^1H NMR (400 MHz, DMSO-d_6): 1.61–1.65 (m, 2H), 1.69–1.72 (m, 2H), 2.12 (s, 9H), 2.13 (s, 9H), 2.23 (s, 9H), 2.26 (s, 9H), 2.30 (quint., 2H, $J = 6.7$ Hz), 2.48–2.53 (m, 4H), 2.61 (t, 2H, $J = 7.3$ Hz), 2.63–2.66 (m, 2H), 3.18–3.21 (m, 2H), 4.14–4.18 (m, 5H), 4.19–4.22 (m, 2H), 4.30 (dd, 3H, $J = 2.0$ Hz, $J = 12.3$ Hz), 4.30–4.32 (m, 2H), 4.42 (dd, 3H, $J = 2.0$ Hz, $J = 12.3$ Hz), 4.49–4.51 (m, 1H), 5.30 (t, 3H, $J = 9.6$ Hz), 5.46–5.50 (m, 6H), 5.58 (d, 3H, $J = 7.2$ Hz), 5.60–5.68 (m, 2H), 7.31 (d, 2H, $J = 8.2$ Hz), 7.42 (d, 6H, $J = 8.2$ Hz), 8.11 (d, 2H, $J = 8.2$ Hz), 8.15 (d, 6H, $J = 8.2$ Hz), 8.85–8.90 (m, 8H). ^{13}C NMR (100 MHz, DMSO-d_6): 20.7, 20.9, 24.7, 26.0, 33.0, 34.1, 34.5, 35.9, 37.9, 41.0, 44.5,51.4, 52.8, 53.7, 62.6, 67.7, 68.9, 72.0, 72.5, 73.5, 99.3, 113.3,115.5, 119.7, 119.8, 120.7, 128.8, 128.9, 130.9, 134.9, 136.0, 137.2, 148.0, 157.2, 157.9, 170.5, 170.6, 170.7, 172.6, 172.9, 174.1, 174.7, 175.3, 178.1.
Compound **5b**: TLC R_f: 0.37 ($CHCl_3$/EtOH/TFA 8:2:0.1). UV–vis: [$CHCl_3$/MeOH 8:2; λ (nm), ($\varepsilon \times 10^{-3}$ ($L\,mol^{-1}\,cm^{-1}$))]: 420 (333.2), 516 (13.4), 552 (7.2), 592 (4.4), 648 (4.1). MS MALDI: $m/z = 2248.64$ $[M+H]^+$. ^1H

NMR (400 MHz, DMSO-d_6): 1.10–1.15 (m, 2H), 1.25–1.27 (m, 2H), 1.30–1.33 (m, 4H), 1.89–1.93 (m, 4H), 2.11 (s, 9H), 2.12 (s, 9H), 2.13 (s, 9H), 2.23 (s, 6H), 2.26 (s, 3H), 2.62–2.65 (m, 2H), 2.79–2.81 (m, 2H), 3.77–3.79 (m, 1H), 3.82–3.86 (m, 1H), 3.94–3.96 (m, 1H), 4.00–4.03 (m, 2H), 4.14–4.18 (m, 4H), 4.31 (dd, 3H, $J = 12.4$ Hz, $J = 2.1$ Hz), 4.42 (dd, 3H, $J = 12.2$ Hz, $J = 5.6$ Hz), 5.00–5.06 (m, 2H), 5.30 (t, 3H, $J = 9.4$ Hz), 5.46–5.50 (m, 6H), 5.57 (d, 3H, $J = 7.1$ Hz), 7.34 (d, 1H, $J = 8.2$ Hz), 7.34–7.35 (m, 1H), 7.42 (d, 6H, $J = 8.2$ Hz), 7.76 (dt, 1H, $J = 7.4$ Hz, $J = 1.2$ Hz), 8.10 (d, 1H, $J = 8.2$ Hz), 8.15 (d, 6H, $J = 8.2$ Hz), 8.85–8.88 (m, 8H). ^{13}C NMR (100 MHz, DMSO-d_6): 20.6, 20.7, 20.8, 20.9, 24.3, 25.7, 32.5, 33.4, 33.9, 35.7, 37.8, 40.5, 44.3, 51.1, 52.1, 53.0, 53.1, 62.1, 67.7, 68.4, 71.3, 72.3, 72.8, 99.1, 115.1, 115.5, 119.6, 119.7, 120.5, 128.6, 128.7, 130.7, 131.3, 135.1, 135.6, 136.9, 137.0, 146.5, 155.8, 156.6, 157.1, 170.3, 170.6, 171.2, 171.4, 172.6, 172.9, 174.1, 174.7, 175.1, 178.1.

Compound **6a**: *(3S,9S,12S,14E,17S)-17-carbamoyl-12-{4-(10,15,20-tris(4-[β-D-glucopyranosyloxy]-phenyl)porphyrin-5-yl)phenyloxybutanalamido}-9-(3-guanidinopropyl)-2,5,8,11-tetraoxo-1,4,7,10-tetraazacycloheptadec-14-enyl-3-acetic acid*: TLC R_f: 0.37 (MeOH/H_2O 7:3), UV–vis: [H_2O; λ (nm), ($\varepsilon \times 10^{-3}$ (L mol^{-1} cm^{-1}))]: 419 (282.0), 516 (9.3), 552 (7.2), 592 (3.7), 648 (2.2). HRMS (ESI): calculated for $C_{86}H_{97}N_{13}O_{27}$: $m/z = 1743.6615$, found: $m/z = 1744.6710$ [M+H]$^+$.

Compound **6b**: *(3S,9S,12S,14E,17S)-17-carbamoyl-12-{2-(10,15,20-tris(4-[β-D-glucopyranosyloxy]-phenyl)porphyrin-5-yl)phenyloxybutanalamido}-9-(3-guanidinopropyl)-2,5,8,11-tetraoxo-1,4,7,10-tetraazacycloheptadec-14-enyl-3-acetic acid*: TLC R_f: 0.41 (MeOH/H_2O 7:3), UV–vis: [H_2O; λ (nm), ($\varepsilon \times 10^{-3}$ (L mol^{-1} cm^{-1}))]: 417 (256.0), 516 (11.3), 552 (8.1), 592 (3.6), 648 (2.1). HRMS (ESI): calculated for $C_{86}H_{97}N_{13}O_{27}$: $m/z = 1743.6615$, found: $m/z = 1767.6496$ [M+H+Na]$^+$.