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Regioselective synthesis of folic acid conjugates from diether-type archaeal lipid analogues

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ABSTRACT

A regioselective access to both α- and γ-folic acid conjugates derived from archaeal lipid analogues is described. The synthetic approach is based on conveniently protected glutamates that led first to α- and γ-glutamate derivatives. The final reconstruction of the folic acid moiety was achieved through the reaction of a protected/activated pteroate followed by a simple deprotection step. These α- and γ-folic acid conjugates would permit to establish the importance of a regiocontrolled introduction of folic acid on the folic acid/folate receptor interaction in the case of a targeted drug/gene delivery.

1. Introduction

Efficient drug/gene delivery should reach a series of requirements including targeting capability.[1–3] Thus recent developments are focused on the introduction of cell recognition ligands on different types of vectorization systems based on nanoparticle technologies (lipids or polymers). Due to its particular interest in cancer therapy, folic acid is one of the most attractive ligands. Indeed folate receptors (FR) are highly expressed on cancer cells leading to a good differentiation from other types of cells.[4–8] Folic acid is a small molecule bearing two carboxylic acid functions typically used for its incorporation into the vectorization system. However, the presence of these two acid functions usually led to a mixture of α- and γ-regioisomers (Fig. 1).

Recently, we developed the synthesis of archaeal lipid analogues bearing cell recognition ligands (sugar[9] or folate[10]) through a PEGylated spacer. Archaeal lipids and synthetic analogues exhibit atypical molecular structures (diether- and tetraether-type lipids) that provide highly stable liposomes (archaeosomes).[11–14] In fact, these membrane lipids play a key role in the remarkable capabilities of archaebacteria to survive under extreme environmental conditions (high or low temperature, high pressure, high or low pH). In particular, diether-type lipids were found to exhibit fusogenic membrane properties and the presence of branching methyl groups hinders the ordered packing of the hydrocarbon tails, which in turn improves membrane fluidity.[15] Our previous synthetic pathway for the preparation of folate derivatives was based on the direct coupling of folic acid with an amino group present at the terminal end of the diether-linked PEGylated chain. Even if this strategy led to a mixture of α- and γ-folates, these novel lipids have shown significant and ligand-mediated transfection of HeLa cells (Fig. 2).[10] As γ-conjugates have already shown to be better recognized by the FR on KB cells,[16] it should be interesting to have the two regioisomers of our folate conjugates at one's disposal with the aim to evaluate the impact of the α- or γ-folate introduction on the ligand/receptor affinity. Thus, we describe herein the access to both α- and γ-diether folate derivatives in a regioselective manner.

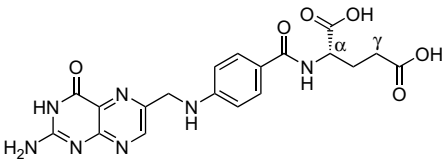

Figure 1. Structure of folic acid.

2. Results and discussion

Our regioselective strategy is based on a retrosynthetic cleavage at the pteroate/glutamate amide bonds leading to a α- or γ-glutamate conjugate from which an α/γ regioselective control would be easier (Scheme 1, only γ-conjugate shown). Indeed a suitable protected glutamate could ensure the isolation of well defined α- or γ-coupling products. This retrosynthetic scheme also applies for the α-regioisomer (not shown in Scheme 1). The pteroic acid **2** is readily accessible by enzymatic cleavage of folic acid using carboxypeptidase G[17] and PEG_{570}-diether derivative **4** was efficiently prepared as described in our recent paper.[10]

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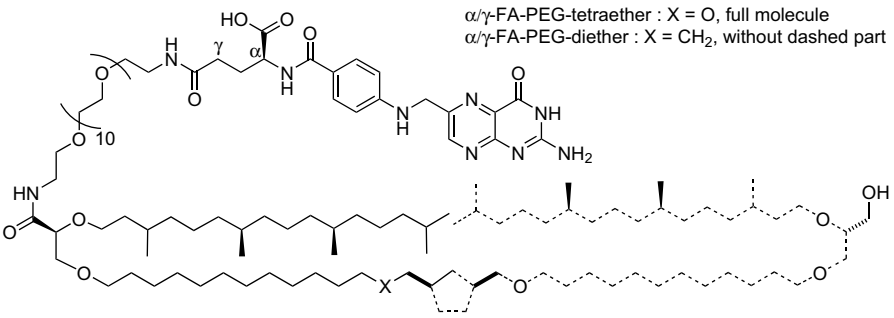


Figure 2. Folate equipped archaeal lipid analogues (diether or tetraether) (only γ-derivative is represented).

2.1. Synthesis of the α- and γ-glutamate diether conjugates

The reaction of readily or commercially available protected glutamates (**α-5**[18] and **γ-5**) with the PEGylated diether **4** was performed in the presence of TBTU/DIEA to furnish the corresponding amide compounds in moderate to good yields (50–76%) (Scheme 2). It is noteworthy that if the α-glutamate reacted at 40 °C (DCM reflux), the γ-glutamate required higher reaction temperature ($CHCl_3$ reflux). This difference can be explained by the in situ formation of the pyroglutamate **7**[19] after the action of the TBTU/DIEA on **γ-5**, which was isolated from the crude mixture.

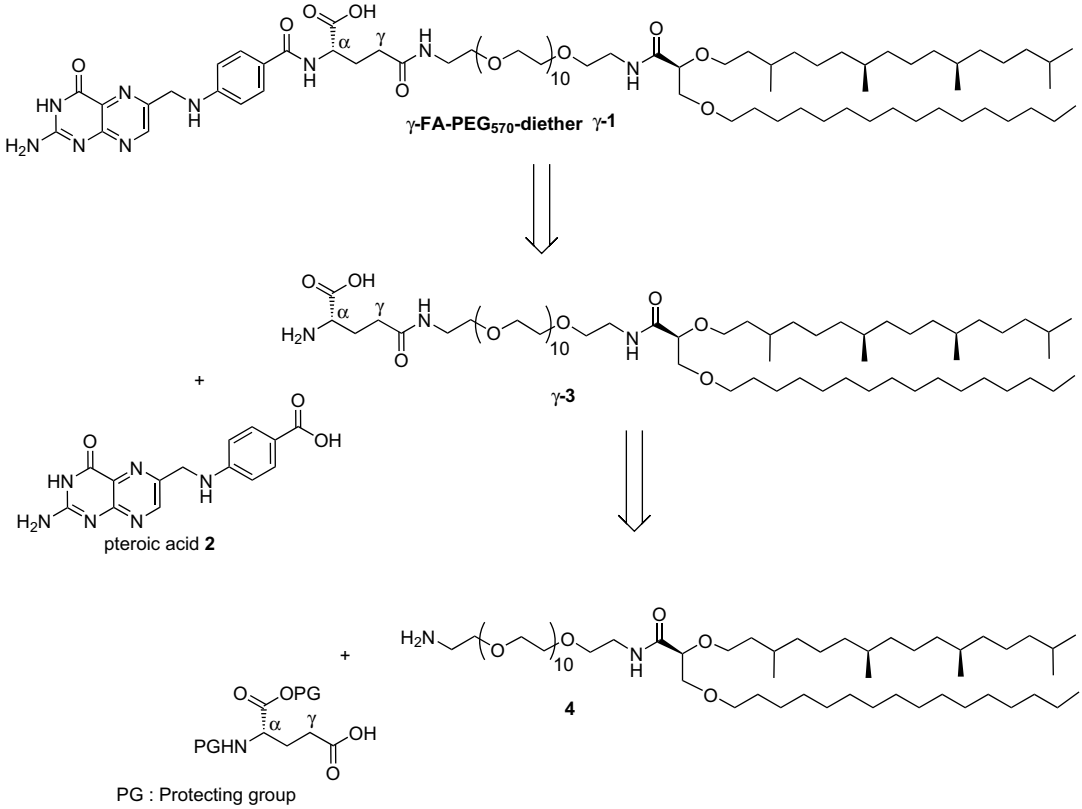
In fact this side reaction (less feasible in the case of **α-5**) did not prevent the formation of **γ-6** as we and others[20] have shown that pyroglutamates can be opened by primary amines such as benzylamine (Scheme 3) at room temperature in several days. These results suggest that **7** could play a role as a reaction intermediate to the formation of **γ-6**.

The simultaneous deprotection of both the primary amine and the carboxylic acid groups was achieved by hydrogenation in the presence of Pd/C in THF/MeOH (1:1). Deprotected glutamates **α-3** and **γ-3** were thus obtained in 67% and 59% yields, respectively (Scheme 4).

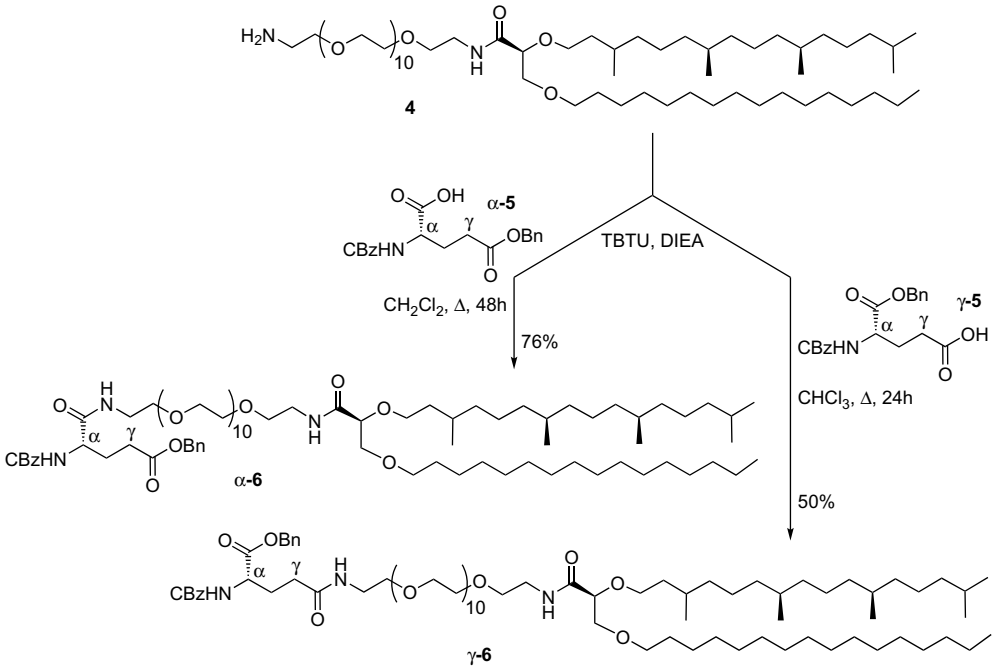
2.2. Reconstruction of the folate moiety

Having the two regiocontrolled glutamates in hand, we envisaged the final reconstruction of the folate moiety by a coupling reaction with an activated pteroate. The reaction conditions described by Nomura et al.[21] efficiently provided imidazolide **9** (Scheme 5). It is noteworthy that carbonyl diimidazole (CDI) reacted in two reaction sites (carboxylic acid and guanidine sites) leading to the protected derivative **9** after reaction with trimethylsilyl ethanol.

The crucial step in the preparation of the two folate regioisomers involved the coupling reaction between **9** and glutamates **3**, which was carried out in the presence of tetramethylguanidine in refluxing chloroform. Unprecedentedly to our knowledge, the corresponding products were deprotected by using



Scheme 1. Retrosynthetic scheme for the preparation of the γ-regioisomer.



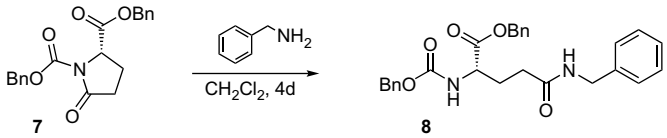
Scheme 2. Regioselective synthesis of protected α- and γ-glutamate derivatives.

cesium fluoride. These conditions applied to **α-3** and **γ-3** led to an uncompleted coupling reaction and afforded after deprotection (CsF) a mixture of folate derivative **1**, accompanied by its corresponding glutamate **3** (**α-1**/**α-3** 15:85; **γ-1**/**γ-3** 36:64) (Scheme 6).

Even if this coupling reaction was not complete, a dialysis purification allowed to isolate pteroic acid free samples, which is required for their further biological evaluation. Only small amounts of ligand are needed and recommended to exhibit effective ligand/receptor interactions,[22] the α/γ differences would therefore be evaluated in binding tests for instance. It is noteworthy that our experience in the synthesis of such folate derivatives has shown that the use of a large excess of pteroate or folate derivatives, which could increase the completion of the coupling step, would lead to additional difficulties in its dialysis removal (loss of product during long dialysis).[10] However, the application of the methodology described here to other lipid systems that are easily retained during the dialysis process, would allow the use of a large excess of **9** and would furnish the corresponding α- and γ-folate derivatives in higher yields.

3. Conclusion

We described herein the synthesis of regiocontrolled folate equipped archaeal lipid analogues (diether) based on a retrosynthetic cleavage of the folate group into pteroate and glutamate derivatives. The functionalization of appropriately protected glutamates permitted to achieve this goal. The reconstruction of the folate moiety involved an imidazolide pteroate derivative and a final deprotection step. Therefore regioncontrolled α- and γ-FA-PEG$_{570}$-diether compounds were prepared and would be involved in comparative biological assays.



Scheme 3. Ring opening of the pyroglutamate by benzylamine.

4. Experimental

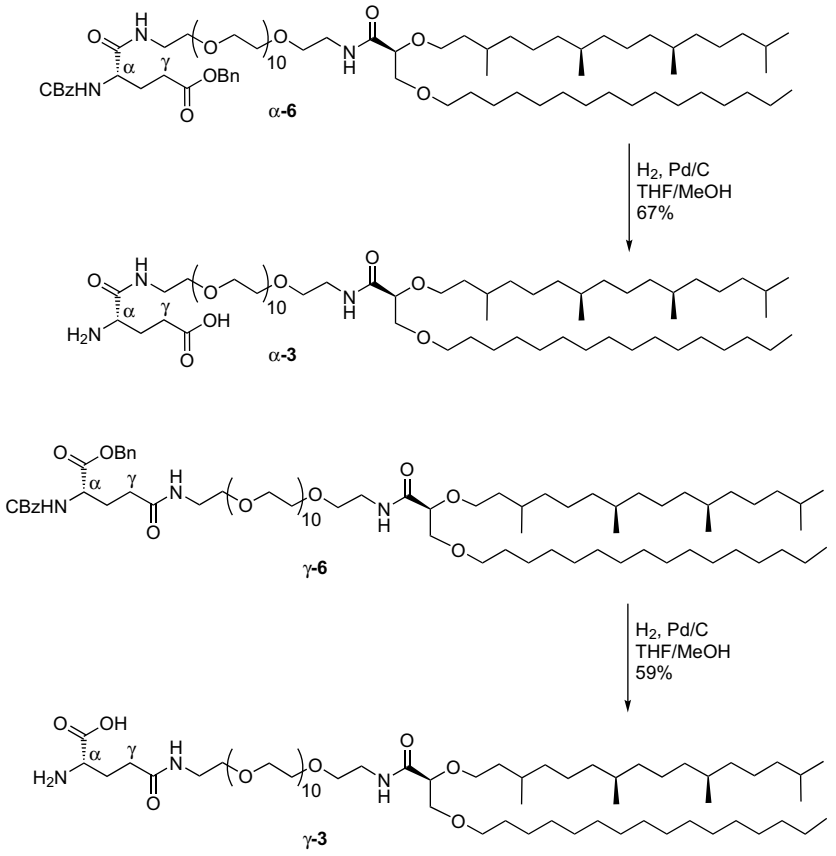
4.1. General

Commercially available chemicals were used without further purification, and solvents were carefully dried and distilled prior to use. Unless otherwise noted, nonaqueous reactions were carried out under a nitrogen atmosphere. Analytical TLC was performed on Merck 60 F_{254} silica gel nonactivated plates. A solution of 5% H_2SO_4 in EtOH was used to develop the plates. Merck 60 H (5–40 μm) silica gel was used for column chromatography. ^{1}H and ^{13}C NMR spectra were recorded at 400 and 100 MHz, respectively, on a Bruker Avance III spectrometer.

4.2. Synthetic procedures

4.2.1. CBzGlu-α-PEG$_{570}$-diether ***α-6***

To a mixture of the protected glutamate **α-5** (140 mg, 0.380 mmol, 1.2 equiv) and TBTU (132 mg, 0.410 mmol, 1.3 equiv) in 20 mL of dry CH_2Cl_2 was added DIEA (68 μL, 0.410 mmol, 1.3 equiv) under nitrogen atmosphere. After stirring for 20 min at room temperature under nitrogen atmosphere, the mixture was added to the PEG$_{570}$-diether derivative **4** (360 mg, 0.316 mmol, 1 equiv). The resulting mixture was refluxed for 48 h under nitrogen atmosphere. Aqueous HCl (1 N) was added and the organic phase was washed with water. The combined organic layers were dried with $MgSO_4$ and concentrated under reduced pressure. The product was purified by flash chromatography on silica gel (CH_2Cl_2/MeOH 96:4). Yield: 76%; $[\alpha]_D^{20}$ −3.3 (*c* 1, CH_2Cl_2); R_f (CH_2Cl_2/MeOH 96:4) 0.17. IR (cm^{-1}): 3424, 3322, 3033, 2925–2855, 1731, 1673, 1529, 1456, 1377, 1348, 1250, 1110, 951, 737–667. ^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm): 0.83–0.88 (m, 18H), 1.00–1.54 (m, 52H), 1.92–1.99 (m, 1H), 2.12–2.21 (m, 1H), 2.39–2.54 (m, 2H), 3.42–3.47 (m, 6H), 3.52–3.55 (m, 6H), 3.57–3.63 (m, 40H), 3.74–3.75 (m, 1H), 3.77–3.78 (m, 1H), 3.88–3.90



Scheme 4. Deprotection of α- and γ-glutamate derivatives.

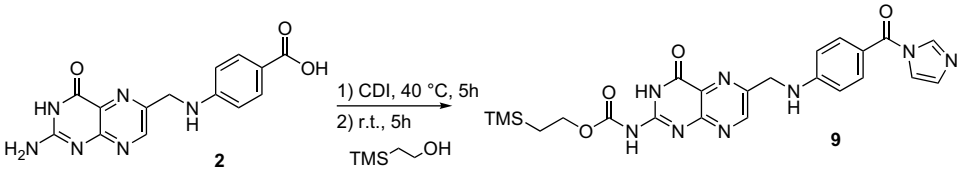
(dd, *J*=2.5, 5.9 Hz, 1H), 4.22–4.28 (m, 1H), 5.08 (s, 2H), 5.09 (s, 2H), 5.73–5.75 (m, 1H), 6.89 (m, 1H), 7.03–7.04 (m, 1H), 7.29–7.35 (m, 10H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ (ppm): 14.11, 19.58, 19.66, 19.73, 22.61, 22.65, 22.67, 22.71, 24.36, 24.46, 24.78, 25.52, 26.03, 27.95, 28.41, 29.34, 29.46, 29.52, 29.63, 29.68, 29.85, 30.25, 32.77, 37.26, 37.36, 37.39, 37.44, 38.57, 38.68, 39.35, 54.04, 66.45, 66.88, 69.52, 69.73, 69.83, 70.45–70.56, 70.22, 70.29, 71.50, 71.69, 80.49, 128.01, 128.11, 128.20, 128.24, 128.48, 128.53, 135.71, 136.22, 156.07, 170.57, 170.98, 172.93. ESI-MS (*m*/*z*) calcd for $C_{83}H_{147}O_{19}N_3$ $[M+Na]^+$: 1513.0527, found: 1513.0505; $[M+K]^+$: 1529.0266, found: 1529.0268. Anal. Calcd for $C_{83}H_{147}O_{19}N_3 \cdot 2H_2O$: C, 65.28; H, 9.97; N, 2.75. Found: C, 65.11; H, 9.80; N, 2.80.

4.2.2. Glu-α-PEG$_{570}$-diether ***α-3***

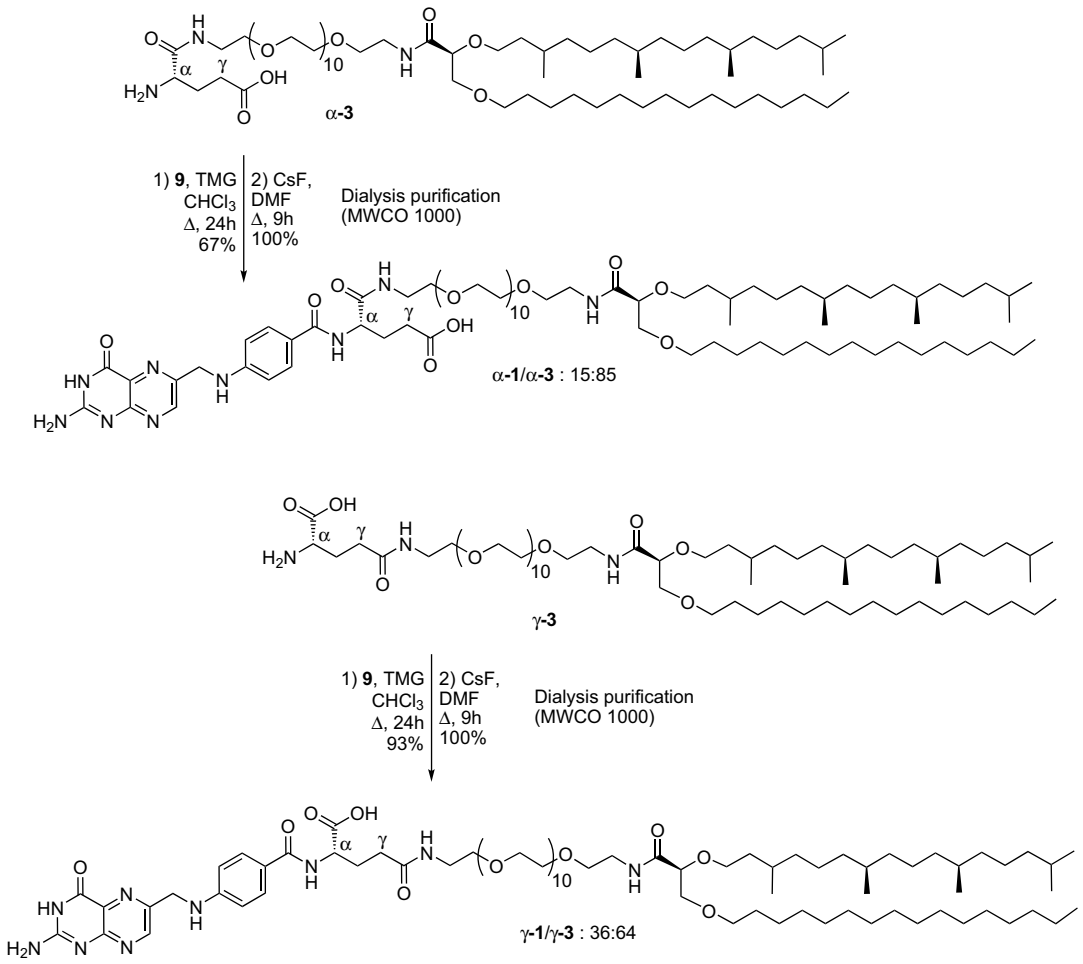
To a solution of **α-6** (90 mg, 0.060 mmol, 1 equiv) in 4 mL of THF/CH_3OH (1:1) was added palladium on activated carbon (15 mg, 17%). After stirring for 5 h 30 min under hydrogen atmosphere, the suspension was filtered over Celite®. The solvent was evaporated under reduced pressure and the product was purified by flash chromatography on silica gel (CH_2Cl_2/MeOH/Et_3N 9:1:0.1). Yield: 67%; $[\alpha]_D^{20}$ +4.6 (*c* 1, CH_2Cl_2); R_f (CH_2Cl_2/MeOH 9:1) 0.14. IR (cm^{-1}): 3582, 3419, 2924–2854, 1674, 1526, 1462, 1377, 1349, 1258, 1114, 922, 804–666; ^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm): 0.83–0.89 (m, 18H), 1.00–1.54 (m, 52H), 1.79–1.83 (m, 1H), 1.93–2.02 (m, 1H), 2.32–2.43 (m, 2H), 3.40–3.47 (m, 6H), 3.50–3.56 (m, 6H), 3.60–3.64 (m, 40H), 3.74–3.75 (m, 1H), 3.77–3.78 (m, 1H), 3.88–3.90 (dd, *J*=2.5, 5.9 Hz, 1H), 4.27–4.32 (m, 1H), 7.02–7.07 (m, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ (ppm): 14.11, 19.58, 19.68, 19.74, 22.63, 22.69, 22.73, 24.38, 24.49, 24.79, 25.28, 26.05, 27.96, 29.35, 29.54, 29.59, 29.65, 29.70, 29.86, 31.91, 32.79, 37.28, 37.38, 37.41, 37.47, 37.53, 38.57, 38.70, 39.34, 39.35, 49.21, 69.74, 69.86–70.46, 71.53, 71.71, 80.48, 170.58, 170.65, 179.80. ESI-MS (*m*/*z*) calcd for $C_{68}H_{135}O_{17}N_3$ $[M+Na]^+$: 1288.9689, found: 1288.9698.

4.2.3. CBzGlu-γ-PEG$_{570}$-diether ***γ-6***

To a mixture of the protected glutamate **γ-5** (9.7 mg, 0.026 mmol, 1.2 equiv) and TBTU (9.1 mg, 0.028 mmol, 1.3 equiv) in 1 mL of dry $CHCl_3$ was added DIEA (4.7 μL, 0.028 mmol, 1.3 equiv) under nitrogen atmosphere. After stirring for 20 min at room temperature under nitrogen atmosphere, the mixture was added to the PEG$_{570}$-diether derivative **4** (24.7 mg, 0.022 mmol, 1 equiv). The resulting mixture was refluxed for 24 h under nitrogen



Scheme 5. Preparation of the pteroate imidazolide.



Scheme 6. Preparation of **α-1** and **γ-1**.

atmosphere. Aqueous HCl (1 N) was added and the organic phase was washed with water. The combined organic layers were dried with $MgSO_4$ and concentrated under reduced pressure. The product was purified by flash chromatography on silica gel (CH_2Cl_2/MeOH 96:4). Yield: 50%; $[\alpha]_D^{20}$ −4.1 (*c* 1, CH_2Cl_2); R_f (CH_2Cl_2/MeOH 96:4) 0.2. IR (cm^{-1}): 3414, 3335, 2924–2854, 1722, 1667, 1531, 1463, 1377, 1357, 1257, 1112, 700–666. ^1H NMR ($CDCl_3$, 400 MHz) δ (ppm): 0.83–0.88 (m, 18H), 1.00–1.54 (m, 52H), 1.98–2.06 (m, 1H), 2.16–2.27 (m, 3H), 3.37–3.47 (m, 6H), 3.49–3.56 (m, 6H), 3.57–3.68 (m, 40H), 3.74–3.75 (m, 1H), 3.77–3.78 (m, 1H), 3.88–3.90 (dd, *J*=2.5, 5.9 Hz, 1H), 4.35–4.40 (m, 1H), 5.09 (s, 2H), 5.16 (s, 2H), 5.93–5.95 (m, 1H), 6.46–6.47 (m, 1H), 7.03–7.04 (m, 1H), 7.30–7.38 (m, 10H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ (ppm): 14.12, 19.58, 19.66, 19.73, 22.61, 22.65, 22.71, 22.67, 24.37, 24.47, 24.79, 25.61, 26.03, 27.95, 29.35, 29.47, 29.52, 29.64, 29.69, 29.85, 31.90, 32.78, 37.26, 37.37, 37.40, 37.46, 37.51, 38.57, 38.68, 39.34, 53.79, 66.90, 67.16, 69.51, 69.68, 69.74, 69.83, 70.14, 70.23, 70.29, 70.50–70.57, 71.49, 71.70, 80.48, 128.07, 128.10, 128.27, 128.41, 128.46, 128.58, 135.26, 136.20, 170.59, 170.61, 171.87, 171.91. ESI-MS (*m*/*z*) calcd for $C_{83}H_{147}O_{19}N_3$ $[M+Na]^+$: 1513.0527, found: 1513.0505; $[M+K]^+$: 1529.0266, found: 1529.0268. Anal. Calcd for $C_{83}H_{147}O_{19}N_3 \cdot H_2O$: C, 66.06; H, 9.95; N, 2.78. Found: C, 65.92; H, 10.15; N, 2.76.

4.2.4. *Glu-γ-PEG$_{570}$-diether* **γ-3**

To a solution of **γ-6** (53 mg, 0.036 mmol, 1 equiv) in 4 mL of THF/CH_3OH (1:1) was added palladium on activated carbon (20 mg, 37%). After stirring for 18 h under hydrogen atmosphere, the suspension was filtered over Celite®. The solvent was evaporated under reduced pressure and the product was purified by flash chromatography on silica gel (CH_2Cl_2/MeOH/Et_3N 9:1:0.1). Yield: 59%; $[\alpha]_D^{20}$ +10.1 (*c* 1, CH_2Cl_2); R_f (CH_2Cl_2/MeOH 9:1) 0.3. IR (cm^{-1}): 3582, 3424, 2924–2854, 1668, 1527, 1463, 1377, 1349, 1251, 1111, 951, 754–666. ^1H NMR ($CDCl_3$, 400 MHz) δ (ppm): 0.82–0.88 (m, 18H), 1.03–1.70 (m, 52H), 1.84–1.99 (m, 2H), 2.27–2.55 (m, 2H), 2.93–2.98 (q, *J*=7.2 Hz, 2H), 3.39–3.49 (m, 6H), 3.51–3.56 (m, 4H), 3.57–3.63 (m, 40H), 3.77–3.74 (m, 1H), 3.76–3.77 (m, 1H), 3.87–3.89 (dd, *J*=2.5, 5.9 Hz, 1H), 4.01–4.08 (m, 1H), 7.02–7.05 (m, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ (ppm): 14.10, 19.56, 19.65, 19.72, 22.60, 22.65, 22.66, 22.70, 24.34, 24.45, 24.76, 25.42, 26.02, 27.93, 29.45, 29.51, 29.61, 29.66, 29.78, 29.83, 31.88, 32.75, 37.24, 37.34, 37.37, 37.42, 37.48, 38.67, 39.32, 44.87, 55.77, 69.70, 69.81, 69.95–70.53, 71.49, 71.67, 80.45, 170.59, 170.60, 177.43.

4.2.5. *α-Benzyl-L-glutamate-γ-amidobenzyl* ***8***

A mixture of the pyroglutamate **7** (10.4 mg, 0.029 mmol, 1 equiv) and benzylamine (4 μL, 0.040 mmol, 1.3 equiv) in 250 μL of dry CH_2Cl_2 was stirred for 4 days at room temperature under nitrogen atmosphere. The solvent was evaporated under reduced pressure to afford crude **8**. ^1H NMR ($CDCl_3$, 400 MHz) δ (ppm): 1.17–1.24 (m, 1H), 1.90–1.97 (m, 1H), 2.12–2.18 (m, 2H), 4.38–4.40 (d, *J*=5.5 Hz, 2H), 5.08 (s, 2H), 5.14–5.17 (m, 1H), 5.30 (s, 2H), 5.72–5.74 (d, *J*=7.5 Hz, 1H), 6.05–6.07 (m, 1H), 7.23–7.37 (m, 15H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ (ppm): 28.7, 32.4, 43.8, 53.6, 67.2, 67.4, 127.6–138.2, 156.4, 171.7, 171.8.

4.2.6. (6-([4-(Imidazole-1-carbonyl)-phenylamino]-methyl)-4-oxo-3,4-dihydro-pteridin-2-trimethylsilylethyl)-carbamic acid ethyl ester ***9***[21]

A mixture of pteroic acid (26 mg, 0.088 mmol, 1 equiv), CDI (54 mg, 0.333 mmol, 4 equiv), and dry triethylamine (75 μL, 0.333 mmol, 4 equiv) in 430 μL of dry DMSO was stirred for 5 h at 40 °C under nitrogen atmosphere. At room temperature, trimethylsilyl ethanol (95 μL, 0.666 mmol, 8 equiv) was added under nitrogen atmosphere. After stirring for 5 h at room temperature under nitrogen atmosphere, the mixture was precipitated in 3 mL of AcOH/Et_2O/H_2O (1:20:35). The precipitate was filtered and purified by flash chromatography on silica gel ($CHCl_3$/MeOH 9:1). Yield: 55%; ^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm): 0.05–0.06 (m, 9H), 1.02–1.06 (m, 2H), 4.27–4.31 (m, 2H), 4.65–4.67 (m, 2H), 6.77–6.80 (m, 2H), 7.09 (m, 1H), 7.61–7.63 (m, 3H), 8.15 (s, 1H), 8.88 (s, 1H). ESI-MS (*m*/*z*) calcd for $C_{23}H_{26}O_4N_8Si$ $[M+Na]^+$: 529.1744, found: 529.1747; $[M+K]^+$: 545.1483, found: 545.1493.

4.2.7. FA-α-PEG$_{570}$-diether ***α-1***

4.2.7.1. TeocFA-α-PEG$_{570}$-diether. A mixture of **9** (28 mg, 0.055 mmol, 1.4 equiv), **α-3** (50 mg, 0.040 mmol, 1 equiv), and dry TMG (15 μL, 0.012 mmol, 3 equiv) in 400 μL of dry $CHCl_3$ was refluxed for 24 h under nitrogen atmosphere. After evaporation under reduced pressure, the solid was purified by dialysis (MWCO 1000) against DMSO and lyophilized to afford compounds TeocFA-α-PEG$_{570}$-diether and **α-3** (15:85). Yield: 67 mol%; ^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm): 0.06–0.07 (m, 9H), 0.83–0.88 (m, 18H), 0.99–1.73 (m, 54H), 1.92–2.09 (m, 2H), 2.24–2.43 (m, 2H), 3.38–3.50 (m, 6H), 3.52–3.57 (m, 6H), 3.60–3.69 (m, 40H), 3.74–3.75 (m, 1H), 3.77–3.78 (m, 1H), 3.88–3.90 (dd, *J*=2.5, 5.9 Hz, 1H), 4.03–4.14 (m, 1H), 4.32–4.37 (m, 2H), 4.69–4.70 (d, *J*=6.4 Hz, 2H), 6.66–6.68 (d, *J*=8.8 Hz, 2H), 7.05 (m, 2H), 7.34–7.35 (t, *J*=4.4 Hz, 1H), 7.85–7.87 (d, *J*=8.4 Hz, 2H), 8.82 (s, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ (ppm): 1.00, 14.11, 16.79, 19.58, 19.67, 19.74, 22.62, 22.68, 22.66, 22.71, 24.34, 24.38, 24.48, 24.79, 26.05, 27.96, 29.18, 29.35, 29.48, 29.51, 29.54, 29.60, 29.64, 29.69, 29.84, 29.88, 31.91, 32.79, 37.28, 37.38, 37.42, 37.46, 37.48, 38.72, 39.25, 39.36, 63.45, 69.67, 69.75, 69.85–70.58, 71.52, 71.72, 80.52.

4.2.7.2. FA-α-PEG$_{570}$-diether ***α-1***. A mixture of TeocFA-α-PEG$_{570}$-diether (30 mg, 0.018 mmol, 1 equiv) and cesium fluoride (13.5 mg, 0.088 mmol, 5 equiv) in 1 mL of dry DMF was refluxed for 9 h under nitrogen atmosphere. After evaporation under reduced pressure, the solid was purified by dialysis (MWCO 1000) against DMSO and lyophilized to afford compounds **α-1** and **α-3** (15:85). Yield: 100 mol%; ^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm): 0.83–0.84 (m, 18H), 1.06–1.54 (m, 52H), 2.14–2.17 (m, 2H), 2.41–2.46 (m, 2H), 3.20–3.26 (m, 6H), 3.30–3.45 (m, 6H), 3.49–3.53 (m, 40H), 3.59–3.61 (m, 1H), 3.66–3.67 (m, 1H), 3.80–3.82 (m, 1H), 3.97–4.01 (m, 1H), 4.08–4.12 (m, 2H), 6.44–6.46 (m, 2H), 7.54–7.55 (m, 2H), 7.68–7.79 (m, 2H), 8.03–8.04 (m, 1H), 8.26–8.31 (m, 1H), 8.53 (s, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ (ppm): 14.35, 15.08, 22.58, 22.90, 24.19, 25.97, 29.52, 31.61, 32.42, 37.10, 37.42, 38.55, 39.03, 69.39, 70.00, 70.37, 80.24.

4.2.8. FA-γ-PEG$_{570}$-diether ***γ-1***

4.2.8.1. TeocFA-γ-PEG$_{570}$-diether. A mixture of **9** (28 mg, 0.055 mmol, 1.7 equiv), **γ-3** (42 mg, 0.033 mmol, 1 equiv), and dry TMG (15 μL, 0.012 mmol, 3 equiv) in 400 μL of dry $CHCl_3$ was refluxed for 24 h under nitrogen atmosphere. After evaporation under reduced pressure, the solid was purified by dialysis (MWCO 1000) against DMSO and lyophilized to afford compounds TeocFA-γ-PEG$_{570}$-diether and **γ-3** (36:64). Yield: 93 mol%; ^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm): 0.06–0.07 (m, 9H), 0.83–0.89 (m, 18H), 1.03–1.71 (m, 54H), 2.05–2.21 (m, 2H), 2.26–2.39 (m, 2H), 3.38–3.49 (m, 6H), 3.52–3.56 (m, 6H), 3.58–3.66 (m, 40H), 3.74–3.75 (m, 1H), 3.77–3.78 (m, 1H), 3.88–3.90 (dd, *J*=2.4, 6.0 Hz, 1H), 4.02–4.19 (m, 1H), 4.37–4.41 (m, 2H), 4.70–4.72 (d, *J*=6.4 Hz, 2H), 6.65–6.67 (d, *J*=8.8 Hz, 2H), 7.06 (m, 2H), 7.33–7.34 (m, 1H), 7.87–7.89 (d, *J*=8.8 Hz, 2H), 8.86 (s, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ (ppm): 14.11, 19.60, 19.67, 19.74, 22.62, 22.66, 22.68, 22.72, 24.38, 24.49, 24.79, 26.05, 27.97, 29.35, 29.49, 29.55, 29.65, 29.70, 29.84, 29.88, 32.79, 37.29, 37.39, 37.42, 37.46, 37.48, 37.50, 38.72, 39.25, 39.36, 69.75, 69.85, 70.23–70.54, 71.52, 71.72, 80.52, 170.66, 170.72.

4.2.8.2. FA-γ-PEG$_{570}$-diether ***γ-1***. A mixture of previous TeocFA-γ-PEG$_{570}$-diether (10 mg, 0.003 mmol, 1 equiv) and cesium fluoride (2 mg, 0.013 mmol, 5 equiv) in 1 mL of dry DMF was refluxed for 9 h under nitrogen atmosphere. After evaporation under reduced pressure, the solid was purified by dialysis (MWCO 1000) against DMSO and lyophilized to afford compounds **γ-1** and **γ-3** (36:64). Yield: 100 mol%; ^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm): 0.80–0.82 (m, 18H), 1.04–1.54 (m, 52H), 1.65–1.80 (m, 2H), 2.00–2.02 (m, 2H), 3.16–3.25 (m, 6H), 3.29–3.44 (m, 6H), 3.45–3.56 (m, 41H), 3.65–3.67 (m, 1H), 3.79–3.80 (m, 1H), 4.12–4.17 (m, 1H), 4.52–4.54 (m, 2H), 6.66–6.72 (m, 2H), 7.33–7.34 (m, 2H), 7.56–7.57 (m, 2H), 7.66–7.70 (m, 2H), 8.05–8.11 (m, 2H), 8.31 (s, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ (ppm): 14.17, 19.67, 22.50, 22.67, 24.33, 26.17, 27.83, 28.50, 29.50, 29.67, 31.67, 32.50, 37.17, 38.33, 39.17, 40.17, 69.32, 70.11, 70.80, 71.82, 72.39, 80.45.

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