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Total Synthesis of Methotrexate-γ-TRIS-Fatty Acid Conjugates

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Several routes for the regiospecific synthesis of lipophilic γ-conjugates of methotrexate are described. Coupling of methotrexate-α-*tert*-butyl ester with glycyl-TRIS-(mono-/di-/tri-)palmitate followed by trifluoroacetic acid cleavage of the α-protection afforded the target methotrexate-γ-glycyl-TRIS-(mono-/di-/tri-)palmitate derivatives. Methotrexate-α-benzyl-γ-glycyl-TRIS-tripalmitate was prepared but no method was found to selectively cleave the α-ester. A method where the diaminopteridinylmethyl moiety was attached last was successful, but was low yielding in the final step. Coupling of 4-amino-4-deoxy-N^{10}-methylpteroic acid with glutamoyl-γ-glycyl-TRIS-palmitate derivatives efficiently afforded the desired conjugates.

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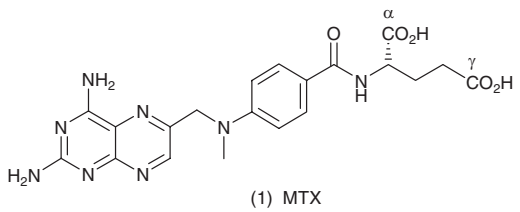
Introduction

Methotrexate (MTX) [(1), see Diagram 1], a widely used drug, has cytotoxic and anti-inflammatory activities and is used to treat a range of diseases including cancer,[1] rheumatoid arthritis,[1] and psoriasis.[2] Whilst its anti-inflammatory mechanism is not well understood, the drug inhibits cell replication through its action as a folate antagonist, preventing the conversion of folate into tetrahydrofolate via dihydrofolate by dihydrofolate reductase (DHFR). MTX also inhibits other folate-dependent enzymes.[3]

The carboxyl groups of the glutamyl moiety of MTX have important biological roles.[3] Derivatization of the carboxyl groups, particularly the α-carboxyl, impairs transport into the cells and binding to target enzymes such as DHFR. The addition of two to five glutamyl groups to the γ-carboxyl group (polyglutamylation) in vivo increases the binding of MTX to appropriate enzymes and prevents MTX efflux via transport systems. Polyglutamate formation and accumulation has unwanted effects in vivo where prolonged intracellular retention leads to long-term toxicity in organs such as the liver and kidney. Therefore, γ-substituted derivatives are less cytotoxic as they are unable to form polyglutamates.

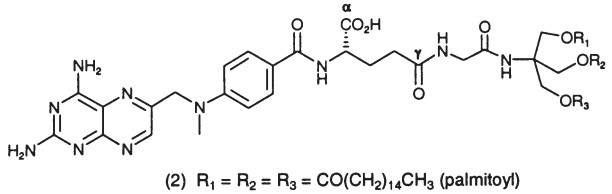
Studies in our laboratories have developed a method of conjugating fatty acids to compounds using 2-amino-2-hydroxymethylpropane-1,3-diol (TRIS).[3–10] We applied this method to methotrexate and prepared fatty acid conjugates (2)–(5) (Diagram 2) where the γ-carboxyl group of MTX is selectively connected by an amide bond to a conjugate group consisting of a glycyl-TRIS-palmitate ester(s) linkage.[3,6,8,9]

These conjugates were biologically evaluated in the areas of in vitro DHFR inhibition,[3,9] in vitro cytotoxicity,[3,9] and with rats or mice for anti-tumour activity,[8,9] delayed type hypersensitivity (immune) response,[9] and adjuvant-induced arthritis inhibition.[9] The tripalmitate (2) was also evaluated as a potential topical treatment for psoriasis in a proof-of-concept clinical trial in humans.[9]



(1) MTX

Diagram 1



(2) $R_1 = R_2 = R_3 = CO(CH_2)_{14}CH_3$ (palmitoyl)
(3) $R_1 = R_2$ = palmitoyl, $R_3 = H$
(4) R_1 = palmitoyl, $R_2 = R_3 = H$
(5) $R_1 = R_2 = R_3 = H$

Diagram 2

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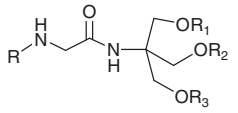
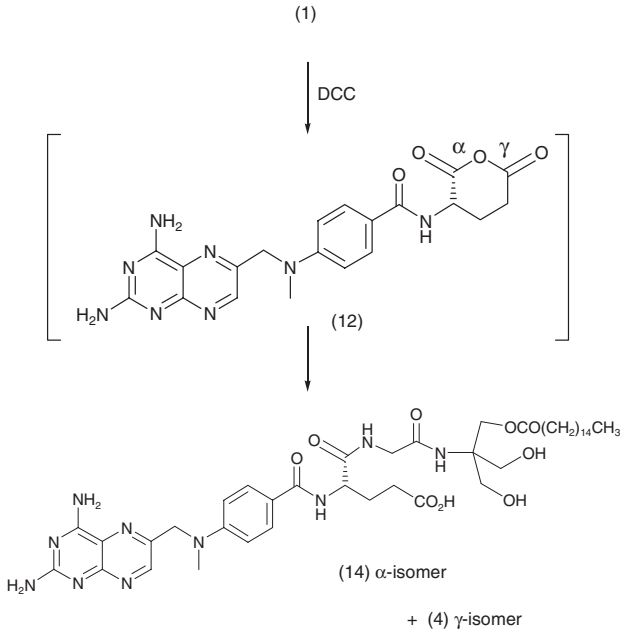
This paper details improved synthetic methodology (over that originally described[3,6,8]) for preparation of the methotrexate conjugates (2)–(5).

We have reported syntheses of *N*-protected precursors of the necessary amines [i.e. (6), (7), (8), and (9), see Diagram 3].[10]



(6) R = BnOCO, $R_1 = R_2 = R_3$ = palmitoyl
(7) R = BnOCO, $R_1 = R_2$ = palmitoyl, R_3 = H
(8) R = BnOCO, R_1 = palmitoyl, $R_2 = R_3$ = H
(9) R = BnOCO, $R_1 = R_2 = R_3$ = H
(10) R = H, $R_1 = R_2 = R_3$ = palmitoyl
(11) R = H, $R_1 = R_2$ = palmitoyl, R_3 = H
(12) R = H, R_1 = palmitoyl, $R_2 = R_3$ = H
(13) R = H, $R_1 = R_2 = R_3$ = H

Diagram 3



Scheme 1

Results and Discussion

Initially, direct coupling of glycyl-TRIS-palmitate moieties to (1) was investigated. This was expected to produce a mixture of the α- and γ-isomers, from which the desired γ-derivative could be isolated.

The required amines (10), (11), (12), and (13) (see Diagram 3) were liberated from their *N*-protected precursors[10] (6), (7), (8), and (9), respectively, by standard hydrogenolysis* over palladium-on-carbon catalyst as they were required.

Treatment of (1) with 1.2 equivalents of 1,3-dicyclohexylcarbodiimide (DCC) and *N*-hydroxy-succinimide (HOSu) to provide a mix of active esters, followed by reaction with amine (12) afforded an approximately 1 : 1 mixture of α- and γ-isomers which was practically chromatographically homogeneous.

We briefly studied reaction of (12) with the anhydride of the glutamic acid moiety of (1) to determine if an amine nucleophile would attack preferentially at the sterically less hindered γ-carbonyl group of the anhydride, leading to the γ-conjugate (see Scheme 1). The MTX anhydride intermediate, formed with DCC in *N*,*N*-dimethylformamide (DMF), was treated with monopalmitate (12) providing a mixture of α- and γ-isomers (14) and (4), respectively, in a ratio of 7 : 3.

An analogous experiment with tripalmitate (10) provided a near identical isomer ratio. We were unable to separate the isomers on a preparative scale.

During the preparation of this manuscript, a report[11] appeared describing regioselective opening of *N*-benzyloxycarbonyl-glutamic anhydride with the dibenzyl ester of 5-aminoisophthalic acid in the presence of one equivalent of 4-dimethylaminopyridine (DMAP) to give the γ-amide product. Other bases did not allow such high selectivity (if any at all) and in the absence of base, an 85 : 15 α : γ ratio was observed.[11]

We briefly revisited our MTX-anhydride opening with (10) employing one equivalent of DMAP in an ice-cooled reaction. Unfortunately, as before, a 7 : 3 α : γ ratio was observed. The greater γ-regioselectivity of the aniline compared with our primary amine may be due to the reduced nucleophilicity and greater steric bulk.[12]

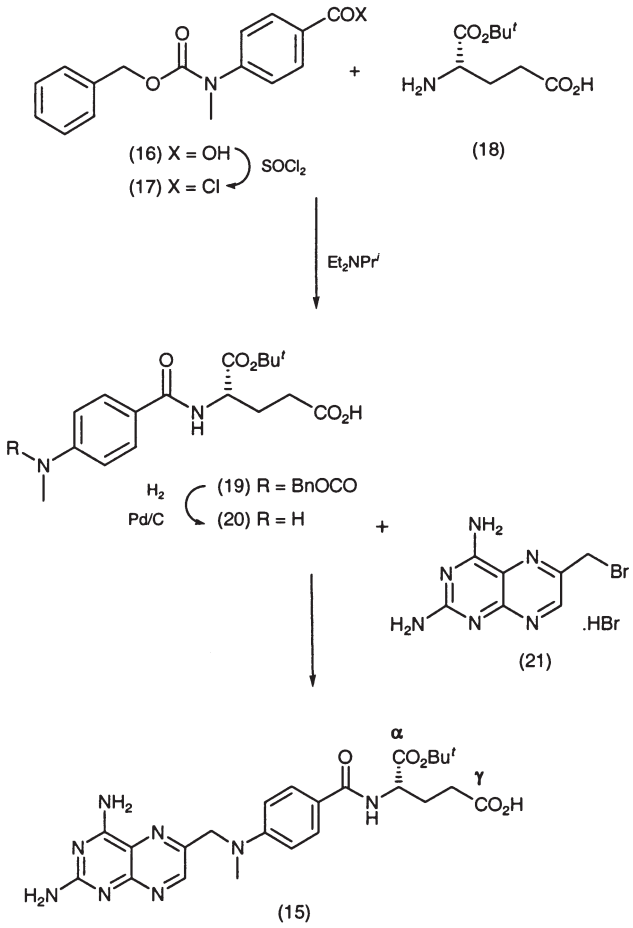
Since selective attachment of the conjugate groups to the γ-carboxyl moiety of the intact MTX molecule was not achieved, nor separation of the α- and γ-isomer mixtures on preparative scale, investigations into total syntheses to unambiguously produce the MTX derivatives (2)–(5) were undertaken.

The first method centred on the selectively protected MTX-α-*tert*-butyl ester (15), initially prepared using reported procedures.[13–15] A more efficient synthetic method for (15), which avoided chromatography, is outlined below (Scheme 2).

The *N*-protected amino acid (16)[16,17] was converted into its acid chloride (17) and reacted with α-*tert*-butyl-L-glutamate (18) to give (19). The benzyloxycarbonyl group was cleaved by standard hydrogenolysis to afford amine (20). Reaction of amine (20) with 6-bromomethyl-2,4-diaminopteridine hydrobromide (21)[18] provided (15).

Ester (15) was also synthesized from (22) (see Diagram 4),[14,15] and (18) using 2-(2-oxo-1(*2H*)-pyridyl)-

* 'Standard hydrogenolysis' refers to use of 10% palladium-on-carbon (Pd/C) catalyst with ethanol as solvent and a hydrogen atmosphere from a hydrogen-filled balloon. In the tripalmitate case (conversion of (6) into (10)), ethyl acetate was added as co-solvent.



Scheme 2

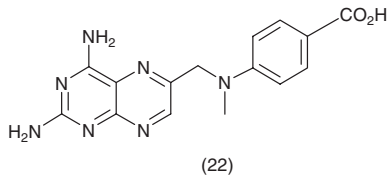


Diagram 4

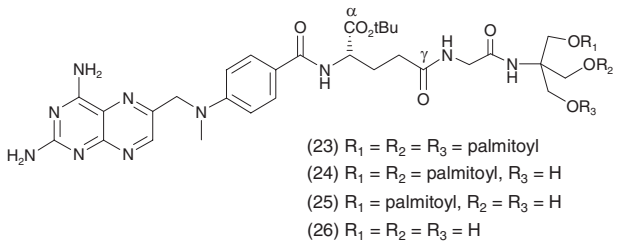


Diagram 5

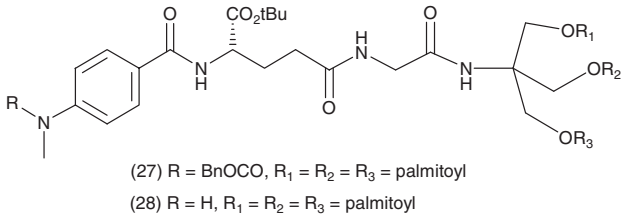


Diagram 6

1,1,3,3-tetramethyluronium tetrafluoroborate (TPTU) as the coupling agent.

Coupling of (15) with amine (10) was accomplished with DCC and catalytic DMAP in dichloromethane (DCM)/DMF to give the α-protected conjugate (23). Cleavage of the α-*tert*-butyl ester moiety of (23), see Diagram 5, was achieved with trifluoroacetic acid (TFA) in DCM to afford the tripalmitate conjugate (2).

Analogous syntheses from (15) with amines (11), (12), and (13) provided conjugates (3), (4), and (5), respectively, via the corresponding α-*tert*-butyl esters (24), (25), and (26), see Diagram 5.

The tripalmitate (23) was also prepared in moderate yield from the bromomethylpteridine (21)[18] and amine (28), see Diagram 6. Amine (28) was prepared by DCC-mediated coupling of (10) and (19) to give (27), followed by hydrogenolysis.

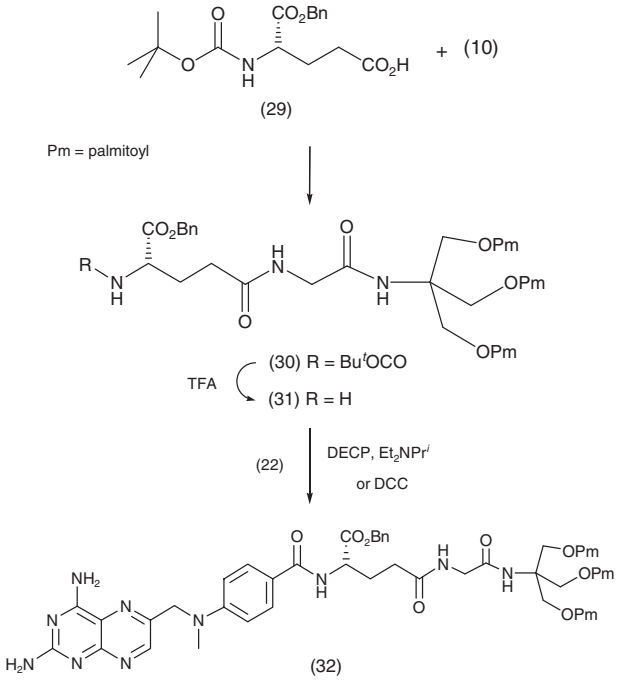
Synthesis of the desired conjugates using the corresponding α-*tert*-butyl esters required two consecutive and inefficient (particularly in solvent volume) chromatographic purification procedures that were not amenable to scale-up. Multi-gram quantities of the desired conjugates were required to enable comprehensive investigations of their therapeutic potential and scale-up of two inefficient procedures was not desired. Therefore, we investigated another route utilizing benzyl ester protection for the α-carboxyl group instead of *tert*-butyl ester; a modification we hoped would result in a milder and cleaner α-deprotection step.

The glutamate (29) was coupled with amine (10) using DCC and catalytic DMAP to give (30). The *tert*-butyloxycarbonyl group was removed using TFA in DCM providing (31) (Scheme 3). Coupling of (31) to (22), using either DCC or diethyl cyanophosphonate (DECP), produced (32) in moderate yield (max. 50% with DECP).

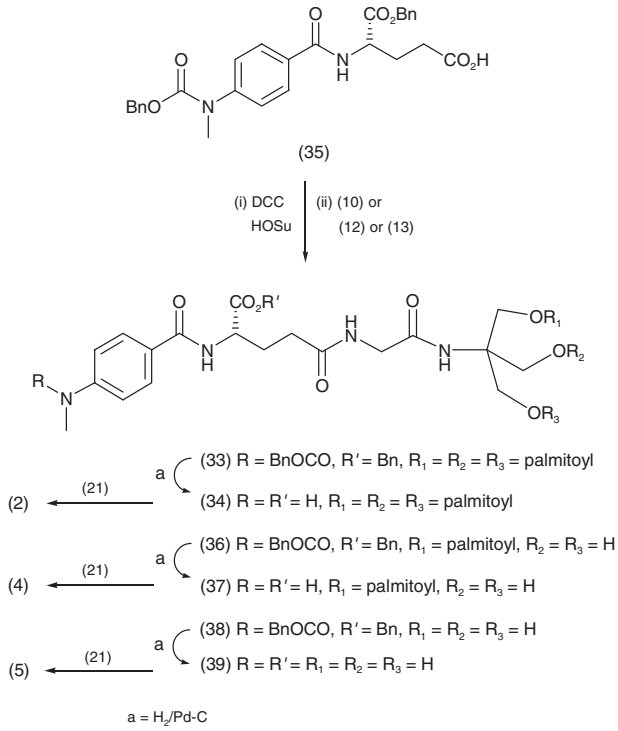
Cleavage of the α-benzyl ester of (32) to give the tripalmitate conjugate (2) proved to be problematic. Standard hydrogenolysis of (32) in ethanol, or ethyl acetate, containing a small amount of acetic acid, resulted in minimal reaction. Treatment with either palladium-on-carbon or platinum oxide catalyst in acetic acid resulted in a complex mixture of products.

A literature search suggested the hydrogenolysis would have problems. Hydrogenations (of double bonds) in folate-related molecules have been achieved[19–21] but with difficulty. Reduction of the heterocyclic ring occurred as a side reaction.[19,20] Interestingly, none of these substrates had a nitrogen atom at the 10-position (unlike our case).

We briefly investigated hydrolytic cleavage of (32) to (2). The α-benzyl ester group in glutamoyl moieties in similar molecules has been hydrolysed with barium hydroxide in



Scheme 3



Scheme 4

aqueous ethanol[13] or sodium hydroxide in aqueous methanol.[22] However, we were unable to find conditions to hydrolyse the benzyl ester whilst leaving the palmitate esters intact.

Another route, utilizing chemistry developed by Piper and co-workers,[17] also involves benzyl protection, but avoids the need to carry out hydrogenolysis in the presence of the (pteridinylmethyl)methylamino moiety. Treatment of amine (31) with acid chloride (17) provided (33). Standard hydrogenolysis of this compound cleaved both the benzyloxycarbonyl protecting group and the benzyl ester, providing (34). Reaction of this compound with the pteridine bromide (21)[18] afforded the tripalmitate conjugate (2) in moderate yield (Scheme 4). Compound (33) was prepared more efficiently by coupling of amine (10) to the γ-carboxyl group of (35).[17] The chemistry described above was repeated with monopalmitate (12) to provide (4) via intermediates (36) and (37). Use of aminotriol (13) afforded (5) via (38) and (39).

The yield of (36) from coupling of (12) to (35)[17] was lower than desired (ca. 40%) and we suspected side reactions were occurring at the two free hydroxyl groups of (12) The process was improved by the coupling of (35)[17] with the monopalmitate/acetonide (41) (liberated from (40),[10] see Diagram 7), which has the non-palmitoylated hydroxyl groups blocked.

This modification resulted in a 73% yield of (42). The acetonide group was cleaved by acid-catalysed hydrolysis affording (36) and the sequence continued as before.

The last step of coupling amine (37) with pteridine bromide (21)[18] was low yielding (20–40%). When aminotriol (13) was used as the conjugate group, the yield from the corresponding step was very poor (less than 10%). Hence another synthetic method was required.

A more efficient synthetic route involved the pteroic acid (22),[14,15] which we had in plentiful supply from earlier work. Treatment of α-benzyl-*N*-benzyloxycarbonyl-L-glutamate (43) with DCC/HOSu and then aminotriol (13) provided (44). Analogous reactions with monopalmitate (12), dipalmitate (11), and tripalmitate (10) afforded (45), (46), and (47), respectively (Scheme 5). Hydrogenolysis of (44) provided free amino acid (48). TPTU-mediated coupling of (48) to (22)[14,15] produced (5). Analogous chemistry with (45) afforded monopalmitate (4) via (49), and with tripalmitate (45) gave (2) via (50) (Schemes 5 and 6).

We were unable to isolate dipalmitate (51) from hydrogenolysis of (46). Thin-layer chromatography (TLC)

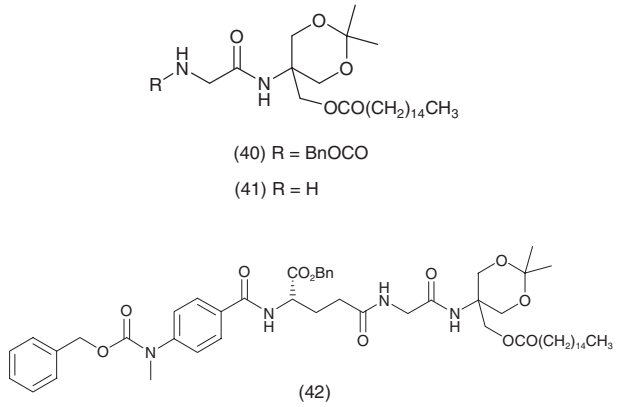
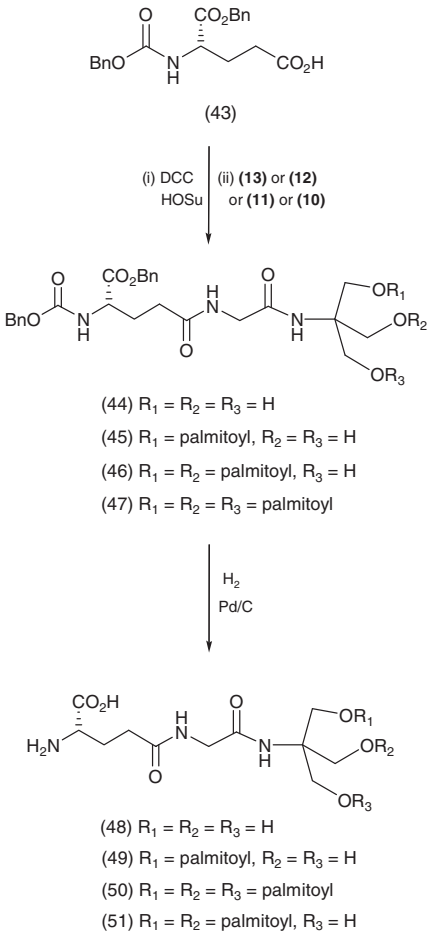


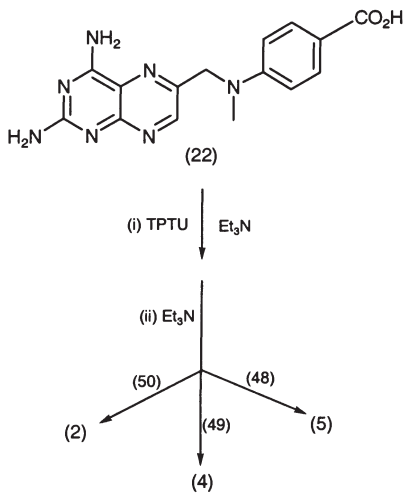
Diagram 7



Scheme 5

analysis suggested that the starting material had been consumed, but we could not extract the product into a solvent to enable the catalyst to be filtered off. This was despite using catalytic palladium on carbon, alumina, or barium sulfate supports, and a wide range of hot extraction solvents.

In summary, regiospecific routes have been developed for the synthesis of MTX-γ-conjugates (2), (3), (4), and (5).



Scheme 6

Experimental

General

General experimental conditions have been described previously.[10]

For high-pressure liquid chromatography (HPLC) analysis of di- and tri-palmitate derivatives, a gradient system was used commencing with 50% acetonitrile/37.5% tetrahydrofuran/12.5% water (containing 0.05% TFA) for 3 min followed by a 22 min gradient to 50% acetonitrile/50% tetrahydrofuran (containing 0.05% TFA). For the mono-palmitate derivatives, an isocratic mobile phase of 80% methanol/water (containing 0.05% TFA) was used. For triol (5), a gradient system was used commencing with 20% acetonitrile/80% aqueous ammonium formate solution (0.05 M, adjusted to pH 3.5with formic acid) for 2 min followed by a 13 min gradient to 80% acetonitrile/20% aqueous ammonium formate solution (0.05 M, adjusted to pH 3.5with formic acid). For MTX-α/γ-tripalmitate mixtures, a mobile phase of 50% acetonitrile/35.5% tetrahydrofuran/14.5% water (containing 0.05% TFA) was used on a 250 × 4.6 mm Alltima C18 column. For MTX-α/γ-monopalmitate mixtures, a mobile phase of 50% acetonitrile/50% water (containing 0.05% TFA) on a 250 × 10 mm Alltima C18 column with a flow rate of 4 mL/min was used. Preparative, reversed-phase HPLC of (5) was performed with a Waters μBondapak C18, 40 × 100 mm column, using a flow rate of 40 mL/min. A gradient system was used starting with water for 10 min, followed by a 25 min gradient to 8% methanol/water.

In the ^{1}H NMR spectra of compounds prepared in this work, NH_2 signals usually appeared as very broad signals, which were difficult to discern. They are quoted only when clearly observed.

Synthesis

N-*[2-[(1-Oxohexadecyl)oxy]-1,1-bis[[(1-oxohexadecyl)oxy]methyl]ethyl]glycinamide (10)*

The *N*-protected tripalmitate (6)[10] (13.38 g, 13.02 mmol) was dissolved with warming in a mixture of ethyl acetate (80 mL) and ethanol (200 mL). Palladium-on-carbon (10%, 1.00 g) was added. The reaction mixture was vigorously stirred under hydrogen gas (balloon) at 40°C for 20 h, cooled, and then filtered. The filtrate was evaporated to yield the *title compound* (10) (11.16 g, 96%) as a white, waxy solid, which was used without further purification. ^{1}H NMR δ ($(CD_3)_2SO$+ and some $CDCl_3$) 7.56, s, NH; 4.43, br, s, 3 × CH_2O; 3.15, br s, CH_2N; 2.32, t, *J* 7.2 Hz, 3 × CH_2CO; 1.69–1.50, m, 3 × C**H**$_2$$CH_2CO$; 1.28, br, 36 × CH_2; 0.88, t, *J* 6.5 Hz, CH_3. Mass spectrum (ESI$^+$) *m/z* 894 (M + H, 100%), 916 (M + Na, 17).

The following compounds were prepared by employing the appropriate *N*-protected substrate in the above procedure, but using ethanol as sole solvent (this procedure in 100% ethanol is hereinafter referred to as 'the standard hydrogenolysis procedure'). The three crude products described in this section were of sufficient purity to be used without further purification.

N-*[1-Hydroxymethyl-2-[(1-oxohexadecyl)oxy]-1-[(1-oxohexadecyl)oxymethyl]ethyl] glycinamide (11)*

Obtained 12.13 g, 97%, from (7)[10] at 45°C for 19 h. ^{1}H NMR δ ($CDCl_3$) 7.95, s, TRIS-NH; 4.35 and 4.16, AB, *J* 11.3 Hz, 2 × CH_2O; 3.84, s, HOC**H**$_2$; 3.57, s, CH_2N; 2.36, t, *J* 7.5 Hz, 2 × CH_2CO_2; 1.71–1.51, m, 2 × C**H**$_2$$CH_2CO_2$; 1.28, br s, 24 × CH_2; 0.88, t, *J* 6.3 Hz, 2 × CH_3. Mass spectrum (ESI$^+$) *m/z* 656 (M + H, 100%).

N-*[1,1-Bis(hydroxymethyl)-2-[(1-oxohexadecyl)oxy]ethyl]glycinamide (12)*

Obtained 7.71 g, 98%, from (8)[10] at room temperature for 4.5 h. ^{1}H NMR δ ($CDCl_3$) 8.19, br, NH; 4.28, s, CH_2O; 3.71, d, and 3.54, AB, *J* 12.5 Hz, 2 × HOC**H**$_2$; 3.37, s, CH_2N; 2.36, t, *J* 6.8 Hz, CH_2CO_2; 1.74–1.53, m, C**H**$_2$$CH_2CO_2$; 1.24, br s, 12 × CH_2; 0.88, t, *J* 6.9 Hz, CH_3. Mass spectrum (CI$^+$) *m/z* 417 (M + H, 62%), 399 (M – H_2O, 100).

N-*[2-Hydroxy-1,1-bis(hydroxymethyl)ethyl]glycinamide (13)*

Obtained 4.84 g, 100%, from (9)[10] at room temperature for 19 h. ^{1}H NMR δ ($(CD_3)_2SO$) 7.95, br s, NH; 4.96, br s, 3 × OH; 4.37, br, NH_2; 3.55, s, 3 × CH_2O; 3.24, s, NCH_2CO.

Direct Conjugation of MTX (1).* N$^{\alpha}$-*[[4-[[(2,4-Diamino-6-pteridinyl)-methyl]methylamino]benzoyl]-L-(α- and γ-)glutamyl]-*N*-[1,1-bis-(hydroxymethyl)-2-[(1-oxohexadecyl)oxy]ethyl]glycinamide (14) and (4)

Method A

MTX (1) (200 mg, 0.44 mmol), HOSu (56 mg, 0.48 mmol) and DCC (110 mg, 0.53 mmol) were dissolved in acetonitrile (6 mL) and DMF (6 mL). The resulting mixture was stirred at room temperature overnight. A solution of (12) (200 mg, 0.48 mmol) in DMF (3 mL) was added and the mixture stirred at room temperature for 6 h. Water (5 mL) was added, the mixture stirred, and the yellow precipitate was filtered off. The solid was washed with water and acetonitrile and purified on a silica flash column eluting with chloroform/methanol/water (70 : 30 : 0 to 65 : 30 : 5) affording a mixture of α- and γ-isomers in a ratio of 56 : 44 (by HPLC).

Method B

A similar reaction to above, except omitting HOSu (allowing formation of the MTX-internal anhydride intermediate), gave a mixture of α- and γ-isomers in a ratio of 7 : 3 (by HPLC).

Use of the tripalmitoylated amine (10) (dissolved in DCM) in method B also gave an α-to-γ ratio of 7 : 3.

The above reaction was repeated, with the additional step[11] of adding DMAP (54 mg, 0.44 mmol) in DCM (0.5 mL) before adding (10). The mixture was ice-cooled during and after these additions and worked up by evaporation and by washing a DCM solution of the residue with 10% aqueous citric acid followed by solvent removal. HPLC analysis of the crude product revealed a mixture of α- and γ-isomers in a 7 : 3 ratio.

***α*-t-Butyl-N-[4-[(benzyloxycarbonyl)methylamino]benzoyl]-L-glutamate (19)**

A mixture of (16)[17] (2.82 g, 9.9 mmol), thionyl chloride (5 mL) and toluene (56 mL) was heated under reflux for 4 h. The mixture was cooled and evaporated to afford the acid chloride (17) as a cream solid, which was evacuated at room temperature overnight. The crude acid chloride (17) was dissolved in dioxan (18 mL) and the resulting solution added dropwise to an ice-cooled solution of (18) (1.928 g, 9.5 mmol) and diisopropylethylamine (4.4 mL) in a mixed solvent of dioxan (48 mL) and water (36 mL). The reaction was stirred in an ice bath for 40 min and then at room temperature for 24 h. Dioxan was removed under vacuum and the remaining solution poured into water (80 mL). The aqueous solution was acidified with 10% acetic acid to pH 4.5 and then extracted three times with ethyl acetate. The organic phase was washed three times with saturated, aqueous, sodium chloride solution and dried. Removal of the solvent gave the *title compound* (19) (4.34 g, 99%) as a white solid. A portion was flash chromatographed on silica gel, eluting with 2–3% methanol in DCM. M.p. 156.5-157.5°C (Found: C, 63.7; H, 6.5; N, 6.0%. $C_{25}H_{30}N_2O_7$ requires C, 63.8; H, 6.4; N, 6.0%). ^{1}H NMR δ ($CDCl_3$) 7.79, d, *J* 8.5 Hz, 2 × ArH; 7.35, d, *J* 8.4 Hz, 2 × ArH; 7.31, m, C_6H_5; 6.99, d, *J* 7.6 Hz, NH; 5.17, s, OCH_2; 4.71, ddd, *J* 4.5, 7.9, 8.5 Hz, α-H; 3.34, s, NCH_3; 2.61–2.41, m, γ-CH_2; 2.41–2.20, m, and 2.15–1.91, m, β-CH_2; 1.48, s, $C(CH_3)_3$. Mass spectrum (APCI$^-$) *m/z* 469 (M – H, 100%).

***α*-t-Butyl-N-[4-(methylamino)benzoyl]-L-glutamate (20)**

Prepared from (19) by the standard hydrogenolysis procedure in 99% yield as a colourless solid, which was used without further purification. ^{1}H NMR δ ($CDCl_3$): 7.70, d, *J* 8.8 Hz, 2 × ArH; 6.91, d, *J* 7.5 Hz, NH; 6.62, d, *J* 9.0 Hz, 2 × ArH; 4.71, dt, *J* 4.5, 8.0 Hz, α-H; 2.88, s, NCH_3; 2.55–2.42, m, γ-CH_2; 2.42–2.20, m, and 2.13–1.92, m, β-CH_2, 1.48, s, $C(CH_3)_3$.

***α*-t-Butyl-N-[4-[[(2,4-diamino-6-pteridinyl)methyl]methyl-amino]benzoyl]-L-glutamate (15)**

Method A

Pteridine bromide.HBr (21)[18] (100 mg, 0.25 mmol) and (20) (87 mg, 0.25 mmol) were mixed in DMF (3 mL) and the resulting solution stirred at room temperature for 31 h. The solution was concentrated under vacuum and the residue dissolved in water (20 mL). The solution was adjusted to pH 4 with 0.33 N NaOH and the resulting precipitate was collected, rinsed with water and dried under vacuum at 40°C. This provided the crude title compound (15) (110 mg. 86%) as a yellow powder. ^{1}H NMR δ ($(CD_3)_2SO$) 12.05, br, CO_2H; 8.63, s, H-7; 8.21, d, *J* 7.5 Hz, glu-NH; 8.04, br, NH_2; 7.73, d, *J* 8.9 Hz, 2 × ArH; 7.11, br, NH_2; 6.82, d, *J* 9.0 Hz, 2 × ArH; 4.82, s, CH_2N; 4.35–4.18, m, glu-α-H; 3.22, s, NCH_3; 2.32, t, *J* 7.4 Hz, glu-γ-CH_2; 2.11–1.75, m, glu-β-CH_2; 1.39, s, $C(CH_3)_3$. Mass spectrum (ESI$^+$) *m/z* 511 (M + 1, 44%), 533 (M + Na, 100).

Method B

The pteroic acid (22)[15] (2.01 g, 6.18 mmol) was suspended in DMF (40 mL). Triethylamine (1.75 mL, 12.4 mmol) was added with stirring and the solid dissolved. TPTU (1.84 g, 6.17 mmol) was added and the reaction mixture was stirred at room temperature under nitrogen for 2.5 h. In a separate vessel, triethylamine (1.05 mL) and (18) (1.40 g, 6.89 mmol) were suspended in DMF (30 mL). The active ester mixture was added slowly to the triethylamine/(18) suspension (rinsed in with DMF (3 mL)) and the resulting mixture was stirred at room temperature under nitrogen for 20 h. The mixture was concentrated and the syrupy residue triturated with ethyl acetate. The resulting solid was collected by filtration and stirred in chloroform (ca. 70 mL). The solid was collected by filtration, washed with fresh chloroform, and dried at room temperature under vacuum providing the title compound (15) (2.80 g, 89%) as a dark, orange solid, which was of lower purity (by NMR and HPLC) than samples of (15) prepared by the earlier method.

N$^{\alpha}$-*[[4-[[(2,4-Diamino-6-pteridinyl)methyl]methylamino]benzoyl]-L-(α-*t*-butoxy)glutamoyl]-*N*-[2-[(1-oxohexadecyl)oxy]-1,1-bis[[(1-oxohexadecyl)oxy]methyl]ethyl]glycinamide (23)

Method A

The MTX α-ester (15)[13] (11.00 g, 21.5 mmol) was dissolved in DMF (86 mL). DCM (200 mL) was added. DCC (5.50 g, 26.7 mmol) was then added and the solution was stirred for 20 min. Amine (10) (21.32 g, 23.9 mmol) in DCM (70 mL) was then added dropwise over 10 min. DMAP (258 mg, 2.1 mmol) was then added and the resulting mixture stirred at room temperature under nitrogen for 24 h. The mixture was filtered and concentrated (rotary/oil pump, 50°C) to remove DMF. The residue was dissolved in DCM, refiltered and evaporated. The residue was dissolved in ethyl acetate/DCM (150/20 mL), washed with saturated, aqueous, sodium chloride solution, dried and evaporated to dryness. The residue was flash chromatographed on silica gel, eluting with 4–5% methanol in DCM, affording the *title compound* (23) (22.01 g, 74%) as a bright yellow solid. M.p. 91-93°C (Found: C, 67.3; H, 9.7; N, 10.0%. $C_{78}H_{132}N_{10}O_{11}$ requires C, 67.6; H, 9.6; N, 10.1%). ^{1}H NMR δ ($(CD_3)_2SO/CDCl_3$, 5 : 2) 8.55, s, H-7; 8.25, d, *J* 6.8 Hz, glu-NH; 8.03, t, *J* 6.2 Hz, gly-NH; 7.87, s, TRIS-NH; 7.72, d, *J* 9.4 Hz, 2 × ArH; 7.44, br, NH_2; 6.80, d, *J* 9.4 Hz, 2 × ArH; 6.63, br, NH_2; 4.80, s, CH_2N; 4.29, m, 7H, glu-α-H and 3 × CH_2O; 3.69, d, *J* 6.1 Hz, NCH_2CO; 3.21, s, NCH_3; 2.26, m, 8H, 3 × CH_2CO and glu-γ-CH_2; 2.08–1.89, m, glu-β-CH_2; 1.58–1.40, m, C**H**$_2CH_2CO$; 1.36, s, $C(CH_3)_3$; 1.22, br, 36 × CH_2; 0.85, t, *J* 6.6 Hz, CH_3; Mass spectrum (APCI$^+$) *m/z* 1386 (M + H, 100%), 1408 (M + Na, 89).

Method B

Pteridine bromide (21)[18] (0.220 g, 0.55 mmol) was dissolved in DMF (8 mL). DCM (12 mL) was added. Amine (28) (0.654 g, 0.54 mmol) was dissolved in DCM (12 mL) and added dropwise to the pteridine solution. The resulting solution was stirred at room temperature overnight. The solution was concentrated under vacuum. The residue was then dissolved in DCM and the solution was washed twice with aqueous sodium bicarbonate solution (0.5%), twice with water, dried and evaporated. The residue was flash chromatographed on silica gel, eluting with 2–5% methanol in DCM, which provided the title compound (23) (0.46 g, 61%) as a yellow powder.

N^{α}-*[[4-[[(2,4-Diamino-6-pteridinyl)methyl]methylamino]benzoyl]*-L-(α-t-butoxy)glutamoyl]-N-*[1-hydroxymethyl-2-[(1-oxohexadecyl)-oxy]-1-[(1-oxohexadecyl)oxymethyl]ethyl]glycinamide (24)

The MTX α-ester (15)[13] (3.04 g, 5.95 mmol) was dissolved, with stirring, in DMF (40 mL). HOSu (0.91 g, 7.80 mmol) was added and stirred until dissolution occurred. DCC (1.82 g, 8.82 mmol) was then added and the resulting mixture stirred at room temperature under nitrogen for 16 h. In a separate vessel, amine (11) (4.29 g, 6.55 mmol) and triethylamine (1.1 mL, 0.81 g, 8.0 mmol) were dissolved in DCM (35 mL). This solution was added to that of the MTX active ester and the resulting mixture stirred for 5 h. The mixture was evaporated and the residue extracted with DCM. The resultant suspension was filtered through glass wool (to remove the urea by-product) and the filtrate was evaporated. The residue was flash chromatographed on silica gel. Elution with 3–8% methanol in DCM afforded the *title compound* (24) (4.17 g, 61%) as a yellow solid. M.p. 97–100°C (Found (sample dried under vacuum to constant weight): C, 62.9; H, 8.9; N, 12.1%. $C_{62}H_{102}N_{10}O_{10}.2H_2O$ requires C, 62.9; H, 9.0; N, 11.8%). ^{1}H NMR δ ($(CD_3)_2SO$) 8.55, s, H-7; 8.24, d, *J* 6.2 Hz, glu-NH; 8.01, *J* 5.1 Hz, gly-NH; 7.73, d, *J* 9.5 Hz, 2 × ArH; 7.52, s, TRIS-NH; 6.80, d, *J* 9.5 Hz, 2 × ArH; 6.67, br, NH_2; 4.92, t, *J* 5.8 Hz, OH; 4.79, s, $ArCH_2N$; 4.31–4.14, m, 5H, 2 × CH_2O and glu-α-H; 3.73–3.58, m, 4H, NCH_2CO and C**H**$_2$OH; 3.21, s, NCH_3; 2.32–2.18, m, 6H, 2 × CH_2CO and glu-γ-CH_2; 2.07–1.90, m, glu-β-CH_2; 1.59–1.41, m, 2 × C**H**$_2CH_2CO$; 1.38, s, $C(CH_3)_3$; 1.22, br, 24 × CH_2; 0.84, t, *J* 6.6 Hz, 2 × CH_3. Mass spectrum (ESI^+) *m/z* 1169 (M + Na, 100%); (ESI^-) *m/z* 1181 (M + Cl^-, 100%).

The following compounds were prepared by employing the appropriate amine in the above procedure. The two products described in this section were obtained as yellow solids.

N^{α}-*[[4-[[(2,4-Diamino-6-pteridinyl)methyl]methylamino]benzoyl]*-L-(α-t-butoxy)glutamoyl]-N-*[1,1-bis(hydroxymethyl)-2-[(1-oxohexadecyl)oxy]ethyl]glycinamide (25)*

Obtained 0.934 g, 61%. M.p. 107–110°C (Found (sample dried under vacuum to constant weight): C, 59.5; H, 8.0; N, 14.9%. $C_{46}H_{73}N_{10}O_9.H_2O$ requires C, 59.6; H, 8.0; N, 15.1%) (Found: *m/z* 909.554. $C_{46}H_{73}N_{10}O_9$ requires *m/z* 909.556). ^{1}H NMR δ ($(CD_3)_2SO$) 8.58, s, H-7; 8.25, d, *J* 6.3 Hz, glu-NH; 8.05, t, *J* 5.3 Hz, gly-NH; 7.75, d, *J* 9.5 Hz, 2 × ArH; 7.27, s, TRIS-NH; 6.80, d, *J* 9.5 Hz, 2 × ArH; 6.78, br, NH_2; 4.86–4.71, m, 4H, $ArCH_2N$ and 2 × OH; 4.35–4.18, m, glu-α-H; 4.20, s, CH_2O; 3.65, d, *J* 6.3 Hz, NCH_2CO; 3.57, d, *J* 5.5 Hz, 2 × C**H**$_2$OH; 3.21, s, NCH_3; 2.36–2.18, m, 4H, CH_2CO and glu-γ-CH_2; 2.10–1.84, m, glu-β-CH_2; 1.60–1.39, m, C**H**$_2CH_2CO$; 1.35, s, $C(CH_3)_3$; 1.25, br s, 12 × CH_2; 0.85, t, *J* 6.3 Hz, CH_3. Mass spectrum ($APCI^+$) *m/z* 931 (M + Na, 22%), 909 (M + H, 100); ($APCI^-$) *m/z* 943 (M + Cl^-, 100%).

N^{α}-*[[4-[[(2,4-Diamino-6-pteridinyl)methyl]methylamino]benzoyl]*-L-(α-t-butoxy)glutamoyl]-N-*[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycinamide (26)*

Obtained 0.33 g, 57% (DMF sole reaction solvent), m.p. > 140°C (gradual dec.) (Found (sample dried under vacuum to constant weight): C, 50.3; H, 6.2; N, 19.0%. $C_{30}H_{42}N_{10}O_8.3H_2O$ requires C, 49.7; H, 6.2; N, 19.3%). ^{1}H NMR δ ($(CD_3)_2SO$) 8.56, s, H-7; 8.27, d, *J* 6.8 Hz, glu-NH; 8.10, t, *J* 6.2 Hz, gly-NH; 7.46, br s, NH_2; 7.14, s, TRIS-NH; 7.72and 6.82, AA′BB′, *J* 9.4 Hz, 4 × ArH; 6.62, br s, NH_2; 4.79, s, $ArCH_2N$; 4.69, t, *J* 6.2 Hz, 3 × OH; 4.34–4.15, m, glu-α-H; 3.70, d, *J* 6.3 Hz, gly-NCH_2CO; 3.52, d, *J* 6.2 Hz, 3 × C**H**$_2$OH; 3.21, s, NCH_3; 2.37–2.15, m, glu-γ-CH_2; 2.09–1.80, m, glu-β-CH_2; 1.39, s, $C(CH_3)_3$. Mass spectrum (ESI^+) *m/z* 671 (M + H).

N^{α}-*[[4-[[(2,4-Diamino-6-pteridinyl)methyl]methylamino]benzoyl]*-L-γ-glutamyl]-N-*[2-[(1-oxohexadecyl)oxy]-1,1-bis[[(1-oxohexadecyl)-oxy]methyl]ethyl]glycinamide (2)

Method A

TFA (1.7 mL, 22 mmol) was added dropwise to an ice-cooled, stirred solution of (23) (0.86 g, 0.62 mmol) in DCM (5 mL) and the resulting mixture allowed to warm to room temperature at which it was stirred, under nitrogen, for 5.5 h. The mixture was placed in a refrigerator (4°C) overnight then diluted with DCM (25 mL) and washed with aqueous, sodium bicarbonate solution (5% w/v, 35 mL) (pH > 7). Acetic acid (glacial, 1.8 mL) was added and the mixture shaken (pH ca. 4). The organic layer was washed with water (2 × 25 mL), dried and evaporated. The residue was flash chromatographed on silica gel, eluting with 8-30% methanol in DCM to afford the *title compound* (2) (742 mg, 90%) as a yellow solid. When this reaction was performed on a 15 g scale, a 78% yield was obtained. M.p. > 220°C (dec.) (Found (sample dried under vacuum to constant weight): C, 65.1; H, 9.6; N, 10.3%. $C_{74}H_{124}N_{10}O_{11}.2H_2O$ requires C, 65.1; H, 9.4; N, 10.3%) (Found: *m/z* 1329.959. $C_{74}H_{125}N_{10}O_{11}$ requires *m/z* 1329.953). ^{1}H NMR δ ($(CD_3)_2SO/CDCl_3$, 5 : 2) 8.51, s, H-7; 7.99–7.88, m, 2H, gly-NH and glu-NH; 7.91, s, TRIS-NH; 7.67, d, *J* 8.7 Hz, 2 × ArH; 7.17, br, NH_2; 6.75, d, *J* 8.7 Hz, 2 × ArH; 6.42, br, NH_2; 4.75, s, CH_2N; 4.26, m, 7H, glu-α-H and 3 × CH_2O; 3.66, d, *J* 4.7 Hz, NCH_2CO; 3.19, s, NCH_3; 2.25, m, 8H, 3 × CH_2CO and glu-γ-CH_2; 2.14–1.84, m, glu-β-CH_2; 1.63–1.36, m, C**H**$_2CH_2CO$; 1.20, br, 36 × CH_2; 0.85, t, *J* 6.5 Hz, CH_3. Mass spectrum (ESI^+) *m/z* 1330 (M + H, 100%), 1352 (M + Na, 22); (ESI^-) *m/z* 1328 (M − 1, 100%).

Method B

The amine (34) (0.287 g, 0.25 mmol) was dissolved with stirring in DCM (9 mL) and, in a separate vessel, bromomethyl pteridine (21)[18] (0.10 g, 0.25 mmol) was dissolved with stirring in DMF (3 mL). The pteridine bromide solution was added to the amine solution and the resulting mixture stirred at room temperature under nitrogen for 67 h. The mixture was evaporated under vacuum and the residue dissolved in DCM (12 mL). This solution was washed with aqueous sodium bicarbonate solution (1%, 10 mL) (pH 7–8). Without separating the layers, aqueous acetic acid (10%) was added dropwise with shaking to adjust the pH back to 4–5. The organic layer was washed with water (20 mL), dried and evaporated. The residue was purified by radial chromatography on a 1 mm silica gel plate. Elution with 8% methanol/0.8% water in DCM to 15% methanol/1% water in DCM gave the title compound (2) (0.161 g, 48%) as a yellow solid, identical to that prepared by the earlier method.

Method C

The pteroic acid (22)[15] (58 mg, 0.18 mmol) was dissolved in DMF (3 mL). Triethylamine (50 μL, 0.37 mmol) was added with stirring and the solid dissolved. TPTU (54 mg, 0.18 mmol) was added and the reaction mixture was stirred at room temperature under nitrogen for 4.5 h. In a separate vessel, triethylamine (30 μL) and (50) (0.20 g, 0.20 mmol) were dissolved in DCM (3 mL). The active ester mixture was added slowly to the (50)/triethylamine solution and the resulting mixture (pH 8) stirred at room temperature under nitrogen for 18 h, and at 40°C for 7.5 h. The mixture was evaporated and the residue radially chromatographed, eluting with 92 : 8 : 0.4 dichloromethane/methanol/water to give the title compound (2) (0.111 g, 47%) as a yellow solid.

N^{α}-*[[4-[[(2,4-Diamino-6-pteridinyl)methyl]methylamino]benzoyl]*-L-γ-glutamyl]-N-*[1-hydroxymethyl-2-[(1-oxohexadecyl)oxy]-1-[(1-oxohexadecyl)oxymethyl]ethyl]glycinamide (3)

Treatment of *tert*-butyl ester (24) using 'Method A' above (chromatography mobile phase 0–1% water in 1 : 4 methanol/DCM) afforded the *title compound* (0.31 g, 56%) as a yellow solid. M.p. > 200°C (dec.) (Found (sample dried under vacuum to constant weight): C, 60.5; H, 8.4; N, 12.4%. $C_{58}H_{94}N_{10}O_{10}.3H_2O$ requires C, 60.8; H, 8.8; N, 12.2%). ^{1}H NMR δ ($(CD_3)_2SO$) 8.52, s, H-7; 8.05, t, *J* 5.8 Hz, gly-NH; 7.82–7.59, m, 5H, 2 × ArH and NH_2 and TRIS-NH (D_2O exchange revealed 7.67, d, *J* 8.9 Hz, 2 × ArH); 6.78, d, *J* 8.9 Hz, 2 × ArH; 4.75, s, $ArCH_2N$; 4.30–4.41, m, 5H, glu-α-H and 2 × CH_2OPm; 3.57–3.73, m, 4H, NCH_2CO and C**H**$_2$OH; 3.17, s, NCH_3; 2.10–2.30, m, 6H, 2 × CH_2CO and glu-γ-CH_2; 1.80–2.09, m, glu-β-CH_2; 1.36–1.57, m, 2 × C**H**$_2CH_2CO$; 1.07–1.33, br, 24 × CH_2; 0.82, t, *J* 6.6 Hz, 2 × CH_3. Mass spectrum (ESI^-) *m/z* 1089 (M − H, 100%).

N^{α}-*[[4-[[(2,4-Diamino-6-pteridinyl)methyl]methylamino]benzoyl]-L-γ-glutamyl]*-N-*[1,1-bis(hydroxymethyl)-2-[(1-oxohexadecyl)oxy]-ethyl]glycinamide (4)

Method A

Subjecting *tert*-butyl ester (25) to the above procedure (chloroform as extraction solvent instead of DCM, chromatography mobile phase 78 : 20 : 2 to 60 : 35 : 5 chloroform/methanol/water) gave the *title compound* (0.66 g, 79%) as a yellow solid. M.p. > 170°C (dec.) (Found (sample dried under vacuum to constant weight): C, 55.8; H, 7.8; N, 15.2%. $C_{42}H_{65}N_{10}O_9.3H_2O$ requires C, 55.6; H, 7.8; N, 15.4%) (Found: *m/z* 853.492. $C_{42}H_{65}N_{10}O_9$ requires *m/z* 853.494). ^{1}H NMR δ ($(CD_3)_2SO$) 8.57, s, H-7; 8.16, d, *J* 6.2 Hz, glu-NH; 8.07, t, *J* 6.3 Hz, gly-NH; 7.75, d, *J* 8.8 Hz, 2 × ArH; 7.37, s, TRIS-NH; 6.84, d, *J* 8.9 Hz, 2 × ArH; 6.62, br, NH_2; 4.79, s, $ArCH_2N$; 4.32–4.12, m, glu-α-H; 4.20, s, CH_2O; 3.67, d, *J* 6.3 Hz, NCH_2CO; 3.57, s, 2 × C**H**$_2$OH; 3.20, s, NCH_3; 2.35-2.15, m, 4H, CH_2CO and glu-γ-CH_2; 2.13–1.85, m, glu-β-CH_2; 1.60–1.37, m, C**H**$_2CH_2CO$; 1.25, br, 12 × CH_2; 0.85, t, *J* 6.3 Hz, CH_3. Mass spectrum (ESI^+) *m/z* 853 (M + H, 100%).

Method B

Prepared from amine (37) and bromomethyl pteridine (21)[18] following 'Method B' for compound (2) (but with DMF as sole solvent), which afforded the title compound (4) (0.264 g, 36%) as a yellow solid.

Method C

TPTU-mediated coupling of pteroic acid (22)[15] with amine (49) as described in 'Method C' for compound (2) (but with DMF as sole solvent) afforded the title compound (4) (2.67 g, 54%) as a yellow solid.

N^{α}-*[[4-[[(2,4-Diamino-6-pteridinyl)methyl]methylamino]benzoyl]-L-γ-glutamyl]*-N-*[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycinamide (5)

Method A

The *tert*-butyl ester (26) (0.459 g, 0.68 mmol) was dissolved, with stirring, in TFA (3 mL). The solution was stirred at room temperature for 1.5 h and the solvent was evaporated under vacuum. The residue was dissolved in water and the solution pH (ca. 2) was adjusted to 6 with aqueous sodium bicarbonate solution (1 g in 75 mL). Aqueous acetic acid (10%) was added until pH 4 was reached. The solution was freeze-dried and the residue purified by flash chromatography and then radial chromatography on silica gel. Elution with 1–3% water in 3 : 5 methanol/chloroform afforded the *title compound* (5) (0.086 g, 21%) as a yellow solid. M.p. > 180°C (dec.) (Found (sample dried under vacuum to constant weight): C, 46.5; H, 6.0; N, 20.8%. $C_{26}H_{35}N_{10}O_8.3H_2O$ requires C, 46.7; H, 6.0; N, 21.0%) (Found: *m/z* 615.263. $C_{26}H_{35}N_{10}O_8$ requires *m/z* 615.264). ^{1}H NMR δ ($(CD_3)_2SO$) 8.58, s, H-7; 8.28, d, *J* 6.3 Hz, NH; 8.11, t, *J* 6.2 Hz, NH; 7.73, d, *J* 8.5 Hz, 2 × ArH; 7.78–7.62, br, and 7.55–7.40, br, NH_2; 7.15, s, NH; 6.83, d, *J* 8.5 Hz, 2 × ArH; 6.63, s, br, NH; 4.80, s, ArC**H**$_2$N; 4.94–4.57, br, 3 × OH; 4.35–4.20, m, glu-α-H; 3.70, d, *J* 5.8 Hz, NC**H**$_2$; 3.53, s, 3 × C**H**$_2$OH; 3.22, s, NCH_3; 2.20-2.32, m, glu-γ-CH_2; 1.80-2.15, m, glu-β-CH_2. Mass spectrum (ESI^+) *m/z* 615 (M + H, 100%).

Method B

Prepared from amine (39) and pteridine bromide (21)[18] using 'Method B' for compound (4), which afforded the *title compound* (5) (8%) as a yellow solid.

Method C

The pteroic acid (22)[15] (2.88 g, 8.86 mmol) was dissolved in DMF (100 mL). TPTU (2.58 g, 8.67 mmol) and triethylamine (2.49 mL, 17.6 mmol) were added portionwise alternately. The mixture was stirred at room temperature for 2 h, then added dropwise to a stirred suspension of (48) (3.00 g, 9.79 mmol) and triethylamine (1.00 mL) in DMF (100 mL). The pH was adjusted to 8 with triethylamine and the mixture stirred overnight. Most of the DMF (ca. 135 mL) was removed under vacuum and the concentrated solution adjusted to pH 8 with triethylamine. Further stirring at room temperature for 32 h and concentration under vacuum gave a dark red, oily residue, which was triturated with acetonitrile (180 mL) to give a yellow solid. The solid was dissolved in DMF (20 mL), re-precipitated by dropwise addition of acetonitrile (100 mL), filtered off, rinsed with acetonitrile and ether, and then air-dried over night. Purification by reversed-phase preparative HPLC in 500 mg portions (eluting with 0-8% MeOH/H_2O, see general experimental section), followed by freeze-drying, afforded the title compound (5) (2.472 g, 52%) as a bright yellow solid.

N^{α}-*[[4-[(Benzyloxycarbonyl)methylamino]benzoyl]-L-(α-*t*-butoxy)-glutamoyl]*-N-*[2-[(1-oxohexadecyl)oxy]-1,1-bis[[(1-oxohexadecyl)-oxy]methyl]ethyl]glycinamide (27)

DCC (0.69 g, 3.36 mmol) and DMAP (22 mg) were added to an ice-cooled solution of (19) (1.05 g, 2.24 mmol) and (10) (2.00 g, 2.24 mmol) in DCM (30 mL). The mixture was stirred at room temperature for 24 h, filtered and the filtrate concentrated under vacuum. The residue was purified on a silica flash column (MeOH/DCM, 1 : 100 to 2 : 100) to afford the *title compound* (27) (1.748 g, 58%) as a white solid. M.p. 40-41°C (Found: C, 70.2; H, 9.9; N, 4.4%. $C_{79}H_{132}N_4O_{13}$ requires C, 70.5; H, 9.9; N, 4.2%). ^{1}H NMR δ ($CDCl_3$) 7.82, d, *J* 8.8 Hz, 2 × ArH; 7.37, d, *J* 7.5 Hz, 2 × ArH; 7.35, m, C_6H_5; 7.21, d, *J* 7.4 Hz, glu-NH; 6.79, t, *J* 5.3 Hz, gly-NH; 6.56, s, TRIS-NH; 5.19, s, $PhCH_2$; 4.72–4.56, m, α-CH; 4.41, s, 3 × OCH_2; 3.90, dd, *J* 5.2, 16.7 Hz, and 3.76, dd, *J* 5.1, 17.3 Hz, NCH_2; 3.36, s, NCH_3; 2.47–2.31, m, γ-CH_2; 2.31, t, *J* 7.0 Hz, 3 × CH_2CO; 2.14–1.95, m, β-CH_2; 1.70–1.52, m, 3 × C**H**$_2CH_2CO$; 1.49, s, $C(CH_3)_3$; 1.36–1.18, m, 36 × CH_2; 0.88, t, *J* 6.5 Hz, 3 × CH_3. Mass spectrum (ESI^+) *m/z* 1346 (M + H, 2%), 387 (22), 309 (24), 225 (100).

N^{α}-*[[(4-Methylamino)benzoyl]-L-(α-*t*-butoxy)glutamoyl]*-N-*[2-[(1-oxohexadecyl)oxy]-1,1-bis[[(1-oxohexadecyl)oxy]methyl]ethyl]glycinamide (28)

Prepared from (27) by the standard hydrogenolysis procedure in 98% yield as a white solid, the title compound was used without further purification. ^{1}H NMR δ ($CDCl_3$) 7.69, d, *J* 8.7 Hz, 2 × ArH; 7.19, t, *J* 5.7 Hz, gly-NH; 6.95, d, *J* 6.4 Hz, glu-NH; 6.72, s, TRIS-NH; 6.64, d, *J* 8.8 Hz, 2 × ArH; 4.69–4.55, m, α-CH; 4.42, s, 3 × OCH_2; 3.91, dd, *J* 5.6, 16.7 Hz, and 3.80, dd, *J* 5.4, 16.6 Hz, NCH_2; 2.90, s, NCH_3; 2.47–2.21, m, 8H, 3 × CH_2CO and γ-CH_2; 2.06–1.86, m, β-CH_2; 1.68–1.51, m, 3 × C**H**$_2CH_2CO$; 1.49, s, $C(CH_3)_3$; 1.31–1.25, m, 36 × CH_2; 0.88, t, *J* 6.7 Hz, 3 × CH_3. Mass spectrum (ESI^+) *m/z* 1233 (M + Na, 30%), 565 (15), 225 (100).

N^{α}-*[*N-(*t*-Butyloxycarbonyl)-L-(α-benzyloxy)glutamoyl]*-N-*[2-[(1-oxohexadecyl)oxy]-1,1-bis[[(1-oxohexadecyl)oxy]methyl]ethyl]-glycinamide (30)

α-Benzyl-*N*-(*t*-butyloxycarbonyl)-L-glutamate (29) (2.86 g, 8.4 mmol) and (10) (7.56 g, 8.4 mmol) were dissolved with stirring in DCM (60 mL). DCC (2.08 g, 10.08 mmol) was added followed by DMAP (50 mg) and the resulting mixture stirred at room temperature under nitrogen for 18.5 h. The mixture was filtered and the filtrate evaporated. The residue was extracted with hot petroleum spirit (60–80) and filtered again. The filtrate was evaporated and the residue recrystallized from petroleum spirit (60–80) to give the *title compound* (30) (9.31 g, 91%) as a white solid. M.p. 56–56.5°C (Found: C, 70.6; H, 10.1; N, 3.8%. $C_{71}H_{125}N_3O_{12}$ requires C, 70.3; H, 10.4; N, 3.5%). ^{1}H NMR δ ($CDCl_3$) 7.35, br s, C_6H_5; 6.55–6.45, m, 2H, gly-NH and TRIS-NH; 5.32, d, *J* 6.9 Hz, glu-NH; 5.21, AB *J* 12.0 Hz and 5.13, AB, *J* 12.2 Hz, CH_2Ph; 4.49–4.22, m, 7H, glu-α-H and 3 × CH_2O; 3.84, d, *J* 5.3 Hz, gly-CH_2; 2.31, t, *J* 7.5 Hz, 8H, 3 × CH_2CO and glu-γ-CH_2; 2.40–2.10, m and 2.06–1.72, m, glu-β-CH_2; 1.72–1.48, m, 3 × C**H**$_2CH_2CO$; 1.43, s, $C(CH_3)_3$; 1.26, br s, 36 × CH_2; 0.88, t, *J* 6.7 Hz, 3 × CH_3. Mass spectrum (ESI^+) *m/z* 1235 (M + Na, 34%), 1113 (21), 122 (100).

N^{α}-*[L-(α-Benzyloxy)glutamoyl]*-N-*[2-[(1-oxohexadecyl)oxy]-1,1-bis[[(1-oxohexadecyl)oxy]methyl]ethyl]glycinamide (31)

TFA (0.85 mL, 11 mmol) was added slowly to an ice-cooled, stirred solution of (30) (0.85 g, 0.70 mmol) in DCM (2 mL) under nitrogen.

The resulting mixture was stirred at room temperature under nitrogen for 2.5 h. The mixture was evaporated under vacuum without heating. The residue was dissolved in DCM (8 mL) and washed with saturated, aqueous, sodium bicarbonate solution (5 mL) (pH 8–9) (CAUTION: gas evolution). The organic layer was dried and evaporated without heating to afford the title compound (31) (0.77 g, 100%) as an off-white coloured solid which was used without further purification. ^{1}H NMR δ ($CDCl_3$) 7.35, br s, C_6H_5; 6.80–6.72, m, gly-NH; 6.43, s, TRIS-NH; 5.24, s, CH_2Ph; 5.15, d, *J* 5.5 Hz, glu-NH; 4.49–4.31, m, 7H, 3 × CH_2O and glu-α-H; 3.85, d, *J* 5.2 Hz, gly-CH_2; 2.41–2.18, m, 8H, 3 × CH_2CO and glu-γ-CH_2; 2.14–1.70, br, glu-β-CH_2; 1.70–1.44, m, 3 × C**H**$_2$$CH_2CO$; 1.25, br s, 36 × CH_2; 0.88, t, *J* 6.7 Hz, 3 × CH_3. Mass spectrum (ESI$^+$) *m/z* 1135 (M + Na, 13%), 1113 (M + H, 31), 1037 (100).

N$^\alpha$-*[[4-[[(2,4-Diamino-6-pteridinyl)methyl]methylamino]benzoyl]-L-(α-benzyloxy)glutamoyl]*-N-*[2-[(1-oxohexadecyl)oxy]-1,1-bis[[(1-oxohexadecyl)oxy]methyl]ethyl]glycinamide (32)*

Method A

The pteroic acid (22)[15] (88 mg, 0.27 mmol) was dissolved with warming and stirring in DMA (3.5 mL). The solution was cooled to room temperature and amine (31) (0.30 g, 0.27 mmol) was added. Upon dissolution, DCC (67 mg, 0.32 mmol) was added followed by DMAP (4 mg, 0.03 mmol). The resulting mixture was stirred at room temperature under nitrogen for 47 h. More DCC (30 mg) was added and the mixture stirred for 5 days. DCM (15 mL) was added and the mixture was washed with water (3 × 25 mL). The organic layer was dried and evaporated. The residue was purified by radial chromatography on a silica gel 1 mm plate, eluting with 1–4% methanol in DCM to give the *title compound* (32) (0.154 g, 40%) as a yellow solid. M.p. 128–128.5°C (Found: C, 68.2; H, 9.3; N, 9.8%. $C_{81}H_{130}N_{10}O_{11}$ requires C, 68.5; H, 9.2; N, 9.9%). ^{1}H NMR δ ($(CD_3)_2SO/CDCl_3$, 5 : 2) 8.55, s, H-7; 8.41, d, *J* 7.6 Hz, glu-NH; 7.98, t, *J* 5.6 Hz, gly-NH; 7.84, s, TRIS-NH; 7.74, d, *J* 8.9 Hz, 2 × ArH; 7.35, br s, C_6H_5; 7.32, br, NH_2; 6.79, d, *J* 8.9 Hz, 2 × ArH; 6.75, br, NH_2; 5.10, s, CH_2Ph; 4.79, s, CH_2N; 4.51–4.33, m, glu-α-H; 4.23, m, 3 × CH_2O; 3.67, d, *J* 5.7 Hz, NCH_2CO; 3.21, s, N-CH_3; 2.37–2.15, m, 8H, 3 × CH_2CO and glu-γ-CH_2; 2.15–1.86, m, glu-β-CH_2; 1.61–1.37, m, 3 × C**H**$_2$$CH_2CO$; 1.21, br, 36 × CH_2; 0.84, t, *J* 6.7 Hz, 3 × CH_3. Mass spectrum (ESI$^+$) *m/z* 1442 (M + Na, 100%), 1420 (M + H, 79).

Method B

Diisopropylethylamine (0.2 mL, 1.1 mmol) was added to a stirred solution of (22)[15] (0.20 g, 0.55 mmol) in DMF (8 mL) under nitrogen. DECP (0.15 mL, 1.1 mmol) was added and the resulting mixture stirred for 55 min. Diisopropylethylamine (0.2 mL, 1.1 mmol) was added followed by a solution of (31) (1.22 g, 1.1 mmol) in DMF (3 mL) and the mixture stirred for 42.5 h. Sodium bicarbonate (100 mg) was added and the mixture evaporated. The residue was dissolved in DCM (25 mL) and washed with half-saturated aqueous sodium bicarbonate solution (25 mL) then water (2 × 25 mL). The organic layer was dried and evaporated. The residue was purified by radial chromatography on a silica gel 4 mm plate, eluting with 3–5% methanol in DCM to give the *title compound* (32) (0.394 g, 50%) as a yellow solid.

N$^\alpha$-*[[4-[(Benzyloxycarbonyl)methylamino]benzoyl]-L-(α-benzyloxy)glutamoyl]*-N-*[2-[(1-oxohexadecyl)oxy]-1,1-bis[[(1-oxohexadecyl)oxy]methyl]ethyl]glycinamide (33)*

Method A

The amine (31) (0.74 g, 0.67 mmol) was dissolved in DCM (8 mL). Diisopropylethylamine (0.2 mL) was added and the stirred solution was cooled in an ice-bath. A solution of chloride (17) in dry DCM (3 mL) was added slowly. The resulting mixture was allowed to warm to room temperature and stirred for 5 h. The mixture was transferred to a separating funnel and washed with water (2 × 20 mL). The organic layer was dried and evaporated. The residue was purified by radial chromatography on a silica gel 2 mm plate, eluting with 0–2% methanol in DCM to give the *title compound* (33) (0.62 g, 68%) as a white solid. M.p. 37.5–38°C (Found: C, 71.2; H, 9.5; N, 4.3%. $C_{82}H_{130}N_4O_{13}$ requires C, 71.4; H, 9.5; N, 4.1%). ^{1}H NMR δ ($CDCl_3$) 7.83, d, *J* 8.4 Hz, 2 × ArH; 7.49, d, *J* 7.1 Hz, glu-H; 7.43–7.28, m, 2 × C_6H_5; 7.70, d, *J* 8.4 Hz, 2 × ArH; 6.58, t, *J* 5.1 Hz, gly-NH; 6.53, s, TRIS-NH; 5.25 and 5.18, AB, *J* 12.1 Hz, CH_2Ph (ester); 5.19, s, CH_2Ph (Z); 4.86–4.71, m, glu-α-H; 4.41, s, 3 × CH_2O; 3.73, dd, *J* 4.5, 16.5 Hz and 3.89, dd, *J* 5.1, 16.5 Hz, gly-CH_2; 3.36, s, NCH_3; 2.41–2.22, m, 8H, 3 × CH_2CO and glu-γ-CH_2; 2.19–1.85, m, glu-β-CH_2; 1.70–1.44, m, 3 × C**H**$_2$$CH_2CO$; 1.25, br s, 36 × CH_2; 0.88, t, *J* 6.2 Hz, 3 × CH_3. Mass spectrum (APCI$^+$) *m/z* 1380 (M + H, 100%), 1402 (M + Na, 37); (APCI$^-$) *m/z* 1414 (M + Cl^-, 100%).

Method B

The carboxylic acid (35)[17] (0.51 g, 0.97 mmol), HOSu (0.13 g, 1.16 mmol), and DCC (0.30 g, 1.45 mmol) were dissolved with stirring in DCM (13 mL) and the resulting mixture stirred for 3 h. Amine (10) (0.87 g, 0.97 mmol) was added followed by DMAP (ca. 6 mg). The resulting mixture was adjusted to 8 with diisopropylethylamine and stirred at room temperature under nitrogen for 21 h then filtered through glass wool and the filtrate was evaporated. The residue was redissolved in petroleum spirit (60–80) and filtered again. The filtrate was evaporated and the residue purified by radial chromatography on a silica gel 4 mm plate, eluting with 0–2% methanol in DCM to give the title compound (1.03 g, 77%) as a white solid.

The following compounds were prepared by employing the appropriate amine in analogous procedures, except omitting the DMAP.

N$^\alpha$-*[[4-[(Benzyloxycarbonyl)methylamino]benzoyl]-L-(α-benzyloxy)-glutamoyl]*-N-*[2-Hydroxy-1,1-bis(hydroxymethyl)ethyl]glycinamide (38)*

Obtained 151 mg, 52%, as a colourless gum (using a DCM/DMF mixed solvent system and ethyl acetate in place of petroleum spirit) (Found: *m/z* 687.263. $C_{34}H_{40}N_4O_{10}Na$ (M + Na^+) requires *m/z* 687.264). ^{1}H NMR δ ($CDCl_3$) 7.78, d, *J* 8.1 Hz, 2H, 2 × ArH; 7.55, d, *J* 6.8 Hz, NH; 7.4–77.20, m 12H, 2 × C_6H_5 and 2 × ArH; 7.13, s, NH; 5.22–5.05, m, 2 × OCH_2Ph; 4.90–4.65, m, glu-α-H; 4.04–3.68, m, NCH_2; 3.67, br s, 3 × OCH_2; 3.37, s, NCH_3; 2.46–2.20, m, glu-γ-CH_2; 2.12–1.86, m, glu-β-CH_2. Mass Spectrum (APCI$^+$) *m/z* 665 (M + H, 55%), 647 (81), 557 (57), 539 (71), 134 (100).

N$^\alpha$-*[[4-[(Benzyloxycarbonyl)methylamino]benzoyl]-L-(α-benzyloxy)-glutamoyl]*-N-*[2,2-dimethyl-5-[(1-oxohexadecyl)oxymethyl]-[1,3]di-oxan-5-yl]glycinamide (42)*

Obtained 10.23 g, 73%, as a white, waxy solid. M.p. 52–53°C (Found: C, 67.2; H, 8.0; N, 6.0%. $C_{53}H_{74}N_4O_{11}$ requires C, 67.5; H, 7.9; N, 5.9%). ^{1}H NMR δ ($CDCl_3$) 7.82, d, *J* 8.5 Hz, 2 × ArH; 7.52, d, *J* 7.3 Hz, glu-NH; 7.45–7.27, m, 12H, 2 × C_6H_5 and 2 × ArH; 6.59–6.42, m, 2H, gly-NH and TRIS-NH; 5.24 and 5.17, AB, *J* 12.1 Hz, CH_2Ph; 5.18, s, CH_2Ph; 4.88–4.78, m, glu-α-H; 4.43, s, CH_2O-palmitoyl; 4.24 and 4.22, AB, *J* 12.1 Hz, CH_2O; and 3.92, dd, *J* 5.5, 12.1 Hz, and 3.82–3.49, m, 4H, CH_2O and gly-CH_2; 3.35, s, NCH_3; 2.44–2.22, m, 4H, CH_2CO and glu-γ-CH_2; 2.21–2.02, m, glu-β-CH_2; 1.71–1.51, m, C**H**$_2$$CH_2CO$; 1.47, s, CH_3; 1.40, s, CH_3; 1.24, br, 12 × CH_2; 0.87, t, *J* 6.7 Hz, CH_3. Mass spectrum (APCI$^+$) *m/z* 943 (M + H, 28%), 885 (99), 777 (100); (APCI$^-$) *m/z* 977 (M + Cl^-, 100).

N$^\alpha$-*[[4-[(Benzyloxycarbonyl)methylamino]benzoyl]-L-(α-benzyloxy)glutamoyl]*-N-*[1,1-bis(hydroxymethyl)-2-[(1-oxohexadecyl)oxy]ethyl]glycinamide (36)*

Obtained 0.19 g, 42%, as a clear, colourless, viscous oil (Found: C, 66.3; H, 7.7; N, 6.2%. $C_{50}H_{70}N_4O_{11}$ requires C, 66.5; H, 7.8; N, 6.2%). ^{1}H NMR δ ($(CD_3)_2SO$) 8.84, d, *J* 7.4 Hz, glu-NH; 8.07, t, *J* 5.5 Hz, gly-NH; 7.87, d, *J* 8.7 Hz, 2 × ArH; 7.44, d, *J* 8.7 Hz, 2 × ArH; 7.40–7.30, m, 10H, 2 × C_6H_5; 7.28, s, TRIS-NH; 5.13, s, 2 × CH_2Ph; 4.79, t, *J* 5.5 Hz, 2 × OH; 4.53–4.37, m, glu-α-H; 4.18, s, CH_2O; 3.68, d, *J* 5.5 Hz, gly-CH_2; 3.56, d, *J* 5.8 Hz, 2 × C**H**$_2$OH; 3.29, s, NCH_3; 2.39–2.20, m, 4H, CH_2CO and glu-γ-CH_2; 2.20–1.85, m, glu-β-CH_2; 1.62–1.36, m, C**H**$_2$$CH_2CO$; 1.22, br, 12 × CH_2; 0.84, t, *J* 6.6 Hz, CH_3. Mass spectrum (APCI$^+$) *m/z* 903 (M + H, 69%), 885 (M – H_2O, 100); (APCI$^-$) *m/z* 937 (M + Cl^-, 100).

Compound (36) was also prepared by the hydrolysis of acetonide (42) by the following method. The substrate (42) (1.24 g, 1.31 mmol) was dissolved in dioxan (30 mL) with stirring. Water (6 mL) was added to the stirred solution, followed by *p*-toluenesulfonic acid monohydrate (60 mg). The mixture was heated at 45°C for 5 h, stored in a freezer overnight then stirred at 45–50°C for 2 h. The mixture was concentrated under vacuum and the residue partitioned between DCM and half-concentrated, saturated, sodium chloride solution. The aqueous layer was extracted with DCM (× 2), and the combined organic layers washed with saturated, aqueous, sodium chloride solution, dried, and then evaporated. The residue was radially chromatographed on a 4 mm silica gel plate, eluting with 2% methanol in DCM to return the starting material (42) (50 mg, 4%) and give the title compound (36) (0.91 g, 77%) as a clear, colourless, viscous oil.

N-*(2,2-Dimethyl-5-[(1-oxohexadecyl)oxymethyl]-[1,3]dioxan-5-yl)-glycinamide (41)*

Prepared from (40)[10] by the standard hydrogenolysis procedure in quantitative yield as a white, waxy solid, which was used without further purification. ^{1}H NMR δ ($CDCl_3$) 7.60, s, TRIS-NH; 4.48, s, CH_2O; 4.32 and 3.79, AB, *J* 12.4 Hz, 2 × CH_2O; 3.31, s, CH_2N; 2.54, br, NH_2; 2.34, t, *J* 7.3 Hz, $OCOCH_2$; 1.72–1.54, m, C**H**$_2$$CH_2CO$; 1.50, s, CH_3; 1.41, s, CH_3; 1.25, br, 12 × CH_2; 0.87, t, *J* 6.5 Hz, CH_3. Mass spectrum (APCI$^+$) *m/z* 457 (M + H, 32%), 399 (100); (APCI$^-$) *m/z* 491 (M + Cl$^-$, 100).

N$^\alpha$-*[(4-methylamino)benzoyl-L-γ-glutamyl]*-N-*[2-[(1-oxohexadecyl)-oxy]-1,1-bis[[(1-oxohexadecyl)oxy]methyl]ethyl]glycinamide (34)*

Prepared from (33) by the standard hydrogenolysis procedure in 99% yield as a white solid, which was used without further purification. ^{1}H NMR δ ($(CD_3)_2SO$/$CDCl_3$, 5 : 2) 8.11, d, *J* 7.5 Hz, glu-NH; 7.99, t, *J* 5.5 Hz, gly-NH; 7.82, s, TRIS-NH; 7.66, d, *J* 8.5 Hz, 2 × ArH; 6.48, d, *J* 8.5 Hz, 2 × ArH; 6.05, m, NH; 4.26, m, 7H, glu-α-H and 3 × CH_2O; 3.67, d, *J* 5.5 Hz, gly-CH_2; 2.71, d, *J* 4.0 Hz, NCH_3; 2.24, t, *J* 7.1 Hz, 8H, glu-γ-CH_2 and 3 × CH_2CO; 2.30–1.83, m, glu-β-CH_2; 1.62–1.37, m, 3 × C**H**$_2$$CH_2CO$; 1.25, br s, 36 × CH_2; 0.83, t, *J* 6.8 Hz, 3 × CH_3. Mass spectrum (APCI$^+$) *m/z* 1156 (M + H, 100%), 1178 (M + Na, 79); (APCI$^-$) *m/z* 1154 (M + Cl$^-$, 100%).

The following compounds were prepared by employing the appropriate *N*-benzyloxycarbonyl and benzyl ester protected substrate in the above procedure. The two crude products described in this section were of sufficient purity to be used without further purification.

N$^\alpha$-*[(4-Methylamino)benzoyl-L-γ-glutamyl]*-N-*[1,1-bis(hydroxy-methyl)-2-[(1-oxohexadecyl)oxy]ethyl]glycinamide (37)*

Obtained 4.26 g, 97%, as a white solid. ^{1}H NMR ($(CD_3)_2SO$) δ 8.16, d, *J* 7.5 Hz, glu-NH; 8.07, t, *J* 5.8 Hz, gly-NH; 7.67, d, *J* 8.8 Hz, 2 × ArH; 7.29, s, TRIS-NH; 6.52, d, *J* 8.8 Hz, 2 × ArH; 6.28–6.14, m, N**H**CH_3; 4.37–4.20, m, glu-α-H; 4.18, s, CH_2O; 3.67, d, *J* 5.5 Hz, gly-CH_2; 3.56, s, 4H, 2 × C**H**$_2$OH; 2.70, d, *J* 4.9 Hz, NCH_3; 2.26, t, *J* 7.5 Hz, 4H, glu-γ-CH_2 and CH_2CO; 2.15–1.80, m, glu-β-CH_2; 1.60–1.38, m, C**H**$_2$$CH_2CO$; 1.22, br s, 12 × CH_2; 0.84, t, *J* 6.5 Hz, CH_3. Mass spectrum (APCI$^+$) *m/z* 679 (M + H, 79%), 661 (100); (APCI$^-$) *m/z* 677 (M + Cl$^-$, 100).

N$^\alpha$-*[(4-Methylamino)benzoyl-L-γ-glutamyl]*-N-*[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycinamide (39)*

Obtained 286 mg, 97%, as a white foamy solid (^{1}H n.m.r. displayed the desired product mixed with ethanol in ca. 1 : 1 mole ratio). ^{1}H NMR δ ($(CD_3)_2SO$) 8.18, d, *J* 6.9 Hz, glu-NH; 8.12, t, *J* 6.3 Hz, NH; 7.68, d, *J* 8.8 Hz, 2 × ArH; 7.17, s, NH; 6.53, d, *J* 8.7 Hz, 2 × ArH; 6.30–6.20, m, NH; 4.40–4.23, m, α-CH: 3.72, d, *J* 6.3 Hz, glu-CH_2; 3.53, s, 3 × OCH_2; 2.72, d, *J* 6.2 Hz, NCH_3; 2.28, t, *J* 6.3 Hz, γ-CH_2; 2.17–1.85, m, β-CH_2.

N$^\alpha$-*[N-(Benzyloxycarbonyl)-L-(α-benzyloxy)glutamoyl]*-N-*[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycinamide (44)*

The acid (43) (4.04 g, 11 mmol) and HOSu (1.87 g, 16 mmol) were dissolved in acetonitrile (60 mL) at room temperature. A solution of DCC (3.35 g, 16 mmol) in acetonitrile (10 mL) was added. The reaction was stirred at room temperature for 3.5 h. The suspension was filtered and the collected solid was rinsed with acetonitrile. The filtrate was mixed with triethylamine (1.00 mL) and added dropwise to a solution of freshly prepared (13) (17.06 mmol) in DMF (50 mL). The reaction pH was adjusted to 8 with triethylamine. The mixture was stirred at room temperature overnight and filtered through a glass filter paper, rinsing the collected solid with acetonitrile. The filtrate was concentrated under vacuum to a volume of about 15 mL. More acetonitrile (100 mL) was added and a white precipitate formed. The suspension was stirred at room temperature until all the gum-like material broke up. The mixture was filtered and the filtrate was concentrated to give a colourless gum, which was purified on a silica flash column (5 cm × 20 cm, preconditioned with DCM, eluted with MeOH/DCM 5 : 100) to afford the *title compound* (44) (3.77 g, 65%) as a white wax (Found: *m/z* 532.232. $C_{26}H_{34}N_3O_9$ requires *m/z* 532.229). ^{1}H NMR δ ($(CD_3)_2SO$) 8.07, t, *J* 6.3 Hz, NH; 7.82, d, *J* 6.3 Hz, NH; 7.45–7.22, m, 10H, 2 × C_6H_5; 7.16, s, NH; 5.13, s, $PhCH_2$; 5.04, m, $PhCH_2$; 4.68, t, *J* 6.3 Hz, 3 × OH; 4.18–4.04, m, glu-α-H; 3.70, d, *J* 6.3 Hz, NCH_2; 3.52, d, *J* 6.3 Hz, 3 × C**H**$_2$OH; 2.25, t, *J* 6.2 Hz, glu-γ-CH_2; 2.12–1.68, m, glu-β-CH_2. Mass Spectrum (APCI$^+$): *m/z* 532 (M + H, 15%), 514 (100), 406 (50), 91 (27).

N$^\alpha$-*[L-γ-Glutamyl]*-N-*[2-Hydroxy-1,1-bis(hydroxymethyl)ethyl]-glycinamide (48)*

Prepared from (44) by the standard hydrogenolysis procedure in quantitative yield as a white solid, which was used without further purification. ^{1}H NMR δ ($(CD_3)_2SO$) 8.30, t, *J* 5.6 Hz, gly-NH; 7.47, s, TRIS-NH; 5.60–4.90, br, NH_2; 3.71, and 3.62 AB, *J* 5.9, 16.9 Hz, NHC**H**$_2$; 3.60–3.12, m, 7H, 3 × OCH_2 and glu-α-CH; 2.31, t, *J* 6.5 Hz, glu-γ-CH_2; 1.98–1.80, m, glu-β-CH_2.

N$^\alpha$-*[N-(Benzyloxycarbonyl)-L-(α-benzyloxy)glutamoyl]*-N-*[1,1-bis-(hydroxymethyl)-2-[(1-oxohexadecyl)oxy]ethyl]glycinamide (45)*

The carboxylic acid (43) (2.51 g, 6.76 mmol) and HOSu (1.18 g, 10.25 mmol) were dissolved in DCM (63 mL). DCC (2.01 g, 9.74 mmol) was added and the resulting mixture was stirred for 2 h. The reaction mixture was filtered and the filtrate was mixed with triethylamine (0.5 mL) and added dropwise into a stirred solution of (12) (3.14 g, 7.54 mmol) in DCM (63 mL). Triethylamine (0.5 mL) was added and the reaction mixture was stirred at room temperature for 3 h. More triethylamine (2 drops) was added and the reaction mixture was stirred at 4°C overnight. The reaction mixture was evaporated under vacuum to give a semisolid which was purified by radial chromatography on silica gel, eluting with 0–2.5% methanol in DCM to give the *title compound* (45) (4.07 g, 66%) as a white wax (Found: C, 65.2; H, 8.2; N, 5.4%. $C_{42}H_{63}N_3O_{10}$ requires C, 65.5; H, 8.3; N, 5.5%) (Found: *m/z* 770.462. $C_{42}H_{64}N_3O_{10}$ requires *m/z* 770.459). ^{1}H NMR δ ($CDCl_3$) 7.37, br s, 2 × C_6H_5; 6.83, s, TRIS-NH; 6.44–6.31, m, gly-NH; 5.72, d, *J* 8.5 Hz, glu-NH; 5.18, s, CH_2Ph; 5.10, s, CH_2Ph; 4.52–4.36, m, glu-α-H; 4.29, s, CH_2O; 3.99–3.49, m, 6H, CH_2N and 2 × C**H**$_2$OH; 2.39–2.18, m, 4H, CH_2CO and glu-γ-CH_2; 2.02–1.82, m, glu-β-CH_2; 1.75–1.42, m, C**H**$_2$$CH_2CO_2$; 1.28, br s, 12 × CH_2; 0.88, t, *J* 6.8 Hz, CH_3. Mass spectrum (APCI$^+$) *m/z* 770 (M + H, 53%), 752 (M – H_2O, 100); (APCI$^-$) *m/z* 804 (M + Cl$^-$, 100%). An 82% yield was obtained on a smaller (800 mg) scale.

The following compounds were prepared by employing the appropriate amine in the above procedure.

N$^\alpha$-*[N-(Benzyloxycarbonyl)-L-(α-benzyloxy)glutamoyl]*-N-*[1-hydroxymethyl-2-[(1-oxohexadecyl)oxy]-1-[(1-oxohexadecyl)-oxymethyl]ethyl]glycinamide (46)*

Obtained 1.00 g, 83%, as a white solid. M.p. 43-44°C (Found: C, 69.0; H, 9.6; N, 4.3%. $C_{58}H_{93}N_3O_{11}$ requires C, 69.1; H, 9.3; N, 4.2%). ^{1}H NMR δ ($CDCl_3$) 7.35, br s, 10H, 2 × C_6H_5; 6.69, s, TRIS-NH; 6.40, m, gly-NH; 5.70, d, *J* 8.0 Hz, glu-NH; 5.17, s, CH_2Ph; 5.10, s, CH_2Ph; 4.50–4.34, m, glu-α-H; 4.38 and 4.23, AB, *J* 11.5 Hz; 2 × CH_2O; 3.98–3.69, m, gly-CH_2; 3.77, s, C**H**$_2$OH; 2.39–2.17, 4H, m, CH_2CO and glu-γ-CH_2; 2.07–1.82, m, glu-β-CH_2; 1.69–1.49, m, C**H**$_2$$CH_2CO$; 1.25, br s, 24 × CH_2; 0.88, t, *J* 6.4 Hz, 2 × CH_3. Mass spectrum (APCI$^+$) *m/z* 1008 (M + H, 55%), 990 (100); (APCI$^-$) *m/z* 1042 (M + Cl$^-$, 100%).

N^{α}-[N-(Benzyloxycarbonyl)-L-(α-benzyloxy)glutamoyl]-N-[2-[(1-oxohexadecyl)oxy]-1,1-bis[[(1-oxohexadecyl)oxy]methyl]ethyl]-glycinamide (47)

Obtained 1.30 g, 87%, as a white wax (Found: C, 71.4; H, 10.2; N, 3.4%. $C_{74}H_{123}N_3O_{12}$ requires C, 71.3; H, 9.9; N, 3.4%). ^{1}H NMR δ ($CDCl_3$) 7.35, br s, 2 × C_6H_5; 6.50, s, TRIS-NH; 6.29, br, gly-NH; 5.68, d, *J* 7.9 Hz, glu-NH; 5.17, s, CH_2Ph; 5.11, s, CH_2Ph; 4.41, m, 7H, glu-α-H and 3 × CH_2O; 3.87–3.76, m, gly-CH_2; 2.38–2.12, m, 8H, 3 × CH_2CO and glu-γ-CH_2; 2.12–1.84, br, glu-β-CH_2; 1.69–1.46, m, C**H**$_2CH_2CO$; 1.25, br s, 36 × CH_2; 0.9, t, *J* 6.5 Hz, 3 × CH_3. Mass spectrum ($APCI^+$) *m/z* 1246 (M + H, 78%), 1171 (27), 1138 (100); ($APCI^-$) *m/z* 1282 (M + Cl^-, 35%), 1172 (68), 1154 (100).

N^{α}-[L-γ-Glutamyl]-N-[1,1-bis(hydroxymethyl)-2-[(1-oxohexadecyl)-oxy]ethyl]glycinamide (49)

Prepared from (45) by the standard hydrogenolysis procedure in 93% yield as a light grey solid, which was used without further purification. ^{1}H NMR δ ($(CD_3)_2SO$) 8.26, t, *J* 5.8 Hz, gly-NH; 7.56, s, TRIS-NH; 5.35, b, NH_2; 4.31 and 4.18, AB, *J* 10.7 Hz, CH_2O; 3.75–3.25, m, 7H, glu-α-H and CH_2N and 2 × C**H**$_2$OH; 2.40–2.13, m, 4H, CH_2CO_2 and glu-γ-CH_2; 2.04–1.74, m, glu-β-CH_2; 1.62–1.37, m, C**H**$_2CH_2CO_2$; 1.27, br s, 12 × CH_2; 0.88, t, *J* 6.7 Hz, CH_3. Mass spectrum (ESI^+) *m/z* 546 (M + H, 100%).

N^{α}-[L-γ-Glutamyl]-N-[2-[(1-oxohexadecyl)oxy]-1,1-bis[[(1-oxohexa-decyl)oxy]methyl]ethyl]glycinamide (50)

Prepared from (47) by the standard hydrogenolysis procedure as a white solid (0.32 g, 97%). ^{1}H NMR δ ($(CD_3)_2SO/CDCl_3$, 2 : 1) 8.12, m, gly-NH; 7.93, s, TRIS-NH; 4.27, s, 3 × CH_2O; 3.90–3.51, m, glu-α-H and CH_2N; 2.40–2.18, m, 8H, 3 × CH_2CO_2 and glu-γ-CH_2; 2.10–1.91, m, glu-β-CH_2; 1.60–1.35, m, 3 × C**H**$_2CH_2CO_2$; 1.22, br s, 36 × CH_2; 0.84, t, *J* 6.7 Hz, 3 × CH_3. Mass spectrum (ESI^+) *m/z* 1023 (M + H, 100%).

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