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An efficient synthesis of 5-aminolaevulinic acid (ALA)-containing peptides for use in photodynamic therapy

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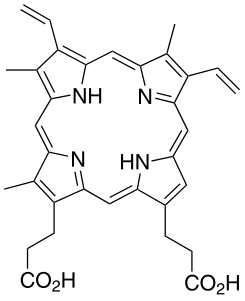
Abstract—An efficient and cost-effective procedure has been devised for the preparation of urethane-protected 5-aminolaevulinic acid (5-ALA) dipeptide ester derivatives which avoids problems associated with the instability of 5-ALA under basic conditions. The procedure is also applicable to the direct synthesis of *N*-(α)-acetyl amino acid-ALA dipeptides in high enantiomeric purity as potential novel prodrugs for photodynamic therapy (PDT).

1. Introduction

Photodynamic therapy (PDT) is a non-thermal technique for inducing tumour destruction with light following administration of a light-activated photosensitising drug.[1,2] Provided that the drug may be selectively introduced and retained in cancerous cells relative to normal adjacent tissue, necrosis is selective. A promising approach in PDT involves the exogenous administration of 5-aminolaevulinic acid (ALA), a naturally occurring compound present in mammalian cells which can be metabolised to a porphyrin photosensitiser, protoporphyrin IX (PpIX) via the haem biosynthetic pathway (Fig. 1).[3] Following accumulation of PpIX within the affected tissue, PDT treatment is then carried out using red laser light, activating PpIX and leading to the production of cytotoxic reactive oxygen species. The main clinical application of ALA-PDT at present is for the treatment of skin cancers via topical application of ALA at the appropriate place on the skin,[4] but the technique is also particularly suited to the visualization and treatment of early tumours in hollow organs where damage to underlying muscle must be minimized.



PpIX

Figure 1.

A significant drawback to ALA-PDT is the fact that ALA is a zwitterion at physiological pH resulting in low lipid solubility and limiting passage through biological barriers such as cellular membranes. To overcome this problem, various lipophilic ALA ester derivatives or other novel prodrugs and formulations have been investigated. In this regard, we[5] and others[6] have conjectured that incorporation of 5-ALA into a short peptide derivative, would provide a suitable means of both facilitating transdermal delivery and also improved targeting into cancerous cells. Release of ALA once incorporated would then be mediated by intracellular peptidase and esterase activities.

We report herein an efficient route for the synthesis of a

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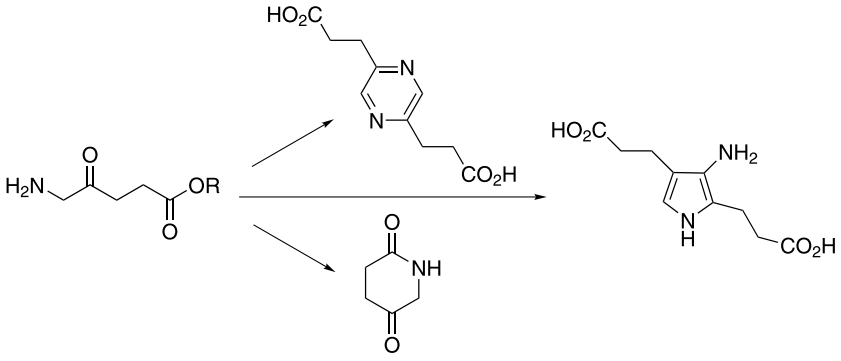


Figure 2. Possible degradation products of 5-ALA derivatives at neutral or basic pH.

range of 5-ALA peptide prodrug derivatives, suitable for evaluation in skin explant and cellular assays.

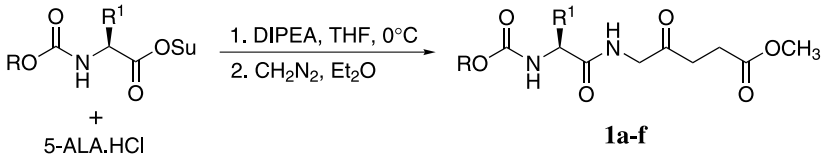
2. Results and discussion

Although Bertozzi et al.[7] have reported the solid phase synthesis of peptides containing 2-ALA, the preparation of 5-ALA-containing peptides is non-trivial. Acylation of 5-ALA derivatives is fraught with potential difficulties, chiefly associated with the instability of ALA in solution above pH 4.[8] It has been shown that around pH 7, 5-ALA dimerises to give pyrazine derivatives,[9] while at higher pH, pseudo-porphobilinogen may be formed.[10] Conversion to ester derivatives appears to exacerbate these problems of instability, introducing the potential for formation of lactam-type derivatives (Fig. 2).

All these processes are typically associated with a significant darkening of the ALA solution, and indeed we have found that using conventional peptide synthesis methodology, coupling of equimolar quantities of *N*-(α)-protected amino acids to esters of ALA via carbodiimide/1-hydroxybenzotriazole (HOBt) activation generally leads to complex mixtures and uniformly low yields of the desired peptides.[11]

Berger et al.[6] have reported two separate approaches for the preparation of 5-ALA-containing peptides, however both employ either an excess of the activated amino acid components or a 5-ALA derivative. In the first case,[6a] 1 equiv of a Boc-protected amino acid was preactivated with 5 equiv each of *N*-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide hydrochloride (EDC·HCl) and HOBt and coupled with 5 equiv of an ester derivative of 5-ALA. Although apparently effective, the expense of 5-ALA makes this approach less attractive for large scale preparations of such 5-ALA-containing dipeptides. In a more recent report,[6b] 5-ALA containing peptides were obtained via reaction of in situ formed symmetrical anhydrides of di- or tripeptide derivatives, necessitating the use of 2 equiv of the latter relative to the 5-ALA component.

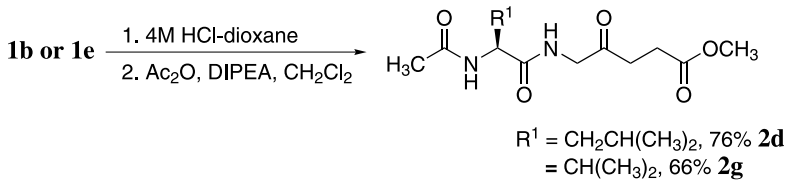
For our studies in this area, we have developed an alternative approach that provides access to various orthogonally protected ALA dipeptides, derivatisable at either N- or C-terminus, once the key ALA pseudopeptide bond is formed, but crucially employing only 1 equiv of both 5-ALA and the activated amino acid derivative. To achieve this, and avoid competing decomposition of 5-ALA, we chose to react the latter, in the form of its hydrochloride salt, with a urethane-protected amino acid active ester in THF solution (in which 5-ALA hydrochloride is insoluble). Initiation of the coupling reaction is then effected by slow addition of base (DIPEA) such that any 5-ALA released into solution is immediately intercepted by the acylating agent (in excess) before competing side reactions can intervene (Scheme 1). This general strategy of simultaneous deprotection and coupling has been utilized previously in peptide chemistry to overcome the problem of



Scheme 1.

Table 1. Urethane-protected 5-ALA dipeptide derivatives

	R	R^1	Yield/%	Name
1a	Bu^t	CH_3	65	Boc-Ala-ALA-OCH_3
1b	Bu^t	$CH_2CH(CH_3)_2$	87	Boc-Leu-ALA-OCH_3
1c	Bu^t	$(CH_2)_4NHZ$	85	Boc-Lys(Z)-ALA-OCH_3
1d	Bu^t	CH_2Ph	79	Boc-Phe-ALA-OCH_3
1e	Bu^t	$CH(CH_3)_2$	32	Boc-Val-ALA-OCH_3
1f	CH_2Ph	H	80	Z-Gly-ALA-OCH_3



Scheme 2.

diketopiperazine formation on coupling to an Xaa-Pro-OMe dipeptide[12] and has also been exploited more recently as part of a highly efficient tandem deprotection-coupling methodology using *N*-(α)-allyloxycarbonyl protected amino acid derivatives.[13]

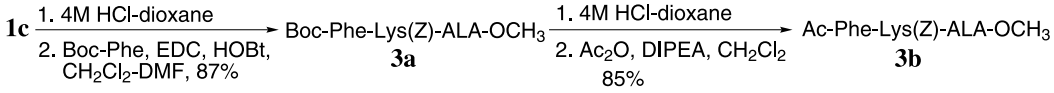
As shown in Table 1, when a variety of amino acid succinimidyl ester derivatives, were coupled with 5-ALA as described above, the corresponding dipeptides were isolated in generally very good yields, via conversion to the methyl esters by treatment with ethereal diazomethane to facilitate isolation. The sole exception was the Boc-Val derivative which coupled rather slowly and ultimately gave a modest yield of 32%. These results compare very favourably with those reported by Berger et al.[6] for the preparation of similar dipeptides on the same scale of synthesis (e.g., **1a**, **1d**[6a]) and it should be noted again that they provide a significant economy in terms of the use of 5-ALA. All the dipeptides were obtained in analytically pure form following isolation by chromatography or crystallization.

We have found that the most appropriate 5-ALA dipeptide substrates for PDT studies are those in which 5-ALA is coupled to an *N*-(α)-acetyl amino acid derivative, since they provide a useful balance of lipophilicity and water solubility.[14] As expected, Boc-protected derivatives such as **1b** and **1e**, were readily transformable into novel prodrug entities as illustrated in Scheme 2. Treatment of **1b** or **1e** with 4 M HCl–dioxane provided the corresponding hydrochloride salts which were then treated with acetic anhydride in the presence of DIPEA to give the corresponding acetylated peptides in good yield. No degradation of the 5-ALA peptides was observed under these conditions with the key acylation step having already been performed.

To confirm that suitably protected 5-ALA dipeptides may be further elaborated by standard peptide chemistry, the lysine-containing derivative **1c** was transformed into the corresponding pseudotripeptides **3a** and **3b** as shown in Scheme 3. Once again, no degradation of the 5-ALA unit was observed either during acidolytic cleavage of *N*-(α)-Boc protection or subsequent acylation reactions in the presence of tertiary amine. The dipeptide fragment Ac-Phe-Lys has previously been employed to create lysosomally-cleavable prodrugs of the anticancer agent doxorubicin[15] showing the potential value of expedient access to building blocks such as **1a–f** for the preparation of peptide prodrugs that are susceptible to cleavage by specific proteases or targeted to particular cellular transporters.[16]

In view of the effectiveness of our protocol for the coupling of Boc or Z-protected amino acids to 5-ALA we were interested to explore the possibility of the direct synthesis of *N*-(α)-acetylated derivatives by this method in order to facilitate rapid biochemical screening of such prodrugs. When the *N*-(α)-acetyl succinimidyl ester derivatives of Gly, D- or -L-Ala, D- or L-Phe, and L-Val were reacted with 5-ALA hydrochloride as before, followed by esterification (Scheme 4), the desired dipeptides were obtained in moderate yields (26–43%, Table 2, entries 1, 2, 4, 8–10) and with surprisingly good optical purity.

Coupling with activated *N*-(α)-acetyl amino acids is rarely used in peptide synthesis since the former are highly prone



Scheme 3.

Table 2. Direct preparation of Ac-Xaa-ALA-OCH_3 derivatives

	Entry	L or D	R^1	Dilute base	ee[a]/%	Yield/%
2a	1	—	H	N	—	32
2b	2	L	CH_3	N	46	26
	3	L	CH_3	Y	>98	51
2c	4	D	CH_3	N	41	47
	5	D	CH_3	Y	>98	73
2d	6[b]	L	$CH_2CH(CH_3)_2$	N	0	37
	7	L		Y	90	47
2e	8	L	CH_2Ph	N	>98	43
2f	9	D	CH_2Ph	N	>98	41
2g	10	L	$CH(CH_3)_2$	N	64	33
	11	L		Y	>98	38

[a] ee determined by ^1H NMR with the chiral shift reagent $Eu(hfc)_3$ (hfc, 3-(heptafluoropropylhydroxy-methylene)-(+)-camphorate).
[b] Coupling via the pentafluorophenyl ester.

to racemisation via oxazolone formation in the presence of base,[17] but for the Phe derivatives **2e** and **2f** (entries 8 and 9) the desired peptide was obtained essentially as a single enantiomer. Enantiomeric purities were assessed using chiral shift ^{1}H NMR with Eu(hfc)$_3$; addition of the reagent led to a splitting of the resonance corresponding to the ester methyl group, presumably via diastereoisomeric chelate formation involving the ester and keto carbonyls of the ALA residue. Integration of these signals then gave a direct estimation of the enantiomeric purity of the peptide.

The direct acylation of 5-ALA with *N*-(α)-acetyl derivatives offers some advantages in terms of speed and economy, particularly for cases such as Val, where coupling of the Boc-protected derivative proceeds in a somewhat lower yield (see Table 1). As anticipated, when the rate of addition of base was carefully controlled (in dilute THF solution), not only the yield but also the optical purity of the peptides (Table 2, entries 3, 5, 7, 11) was significantly improved, since controlled base addition limits the concentration of free 5-ALA, the species required for coupling which may also participate in polymerization or dimerisation reactions etc (see above),[8–10] but moreover minimises the amount of base available to promote oxazolone formation and racemisation. Preliminary studies with other active esters such as pentafluorophenyl (entry 6) and 4-nitrophenyl (not shown), did not suggest any significant advantage in terms of yield relative to the readily available succinimidyl esters which formed the basis of our study, however racemisation appeared to be much more significant with these species.

Once again, derivatives such as **2a** were readily transformable into other potential prodrugs by standard chemistry with the key Xaa-ALA peptide bond now in place. For example (Scheme 5), saponification of **2a** with aq LiOH proceeded in quantitative yield whereupon the dipeptide acid was converted to the corresponding hexyl ester in 65% yield by DMAP-catalysed esterification[18] with DCC. This further emphasizes the potential of derivatives such as **1a–f** and **2a–g** as synthons for the preparation of more elaborate 5-ALA-containing peptides and also highlights the possibility of employing other chemistries in the esterification stage of our general procedure once the critical acylation has been achieved.

3. Conclusion

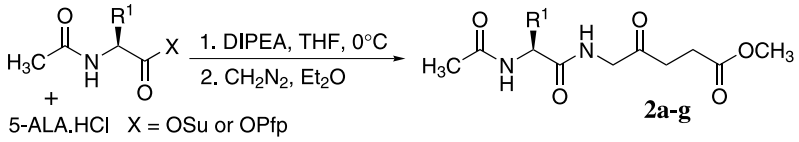
We have developed highly efficient and economic preparations of 5-ALA peptides that overcome the known instability of the amino acid under basic conditions. The derivatives obtained may be elaborated into a wide range of 5-ALA prodrug derivatives using conventional peptide chemistry for use as novel PDT agents. Details of these studies will be reported shortly.[14]

4. Experimental

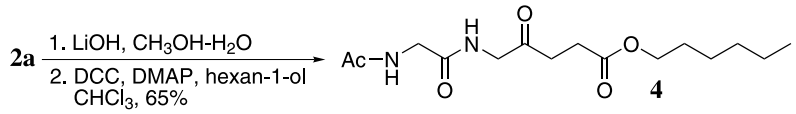
4.1. General

Melting points were recorded on an Electrothermal IA9000 series digital melting point apparatus and are quoted uncorrected. NMR spectra were recorded on a Varian Gemini 300 (^{1}H, 300 MHz; ^{13}C, 75.4 MHz;) or Bruker Avance DPX 300 FT-spectrometers. Chemical shifts (δ) are expressed in ppm and coupling constants (J) are given in Hz. Mass spectra were recorded on a VG AUTOSPEC mass spectrometer in electron impact (EI) mode, a VG Quattro triple quadrupole instrument in positive electrospray (ES) mode, or a TofSpec 2E instrument (MALDI-TOF). Elemental analyses were performed in the Department of Chemistry, University of St. Andrews, or by MEDAC Ltd, Brunel Science Centre, Surrey UK. Optical rotations were measured at 20 °C in a 10 cm path length cell using a Perkin Elmer 343 polarimeter and are quoted in 10^{-1} deg. cm^2 g^{-1}. Flash chromotography was performed according to the method of Still et al.,[19] on columns of silica gel (Fluka Silica Gel 60; 35–70 μm mesh, or Fluorochem; 40–60 μm mesh). Ethereal diazomethane was prepared from Diazald®. Petroleum ether bp 40–60 °C was distilled through a vigreux column prior to use. All other solvents were of Analar quality or were dried using standard procedures.

Amino acid active esters. Urethane and acetyl-protected amino acid *N*-hydroxysuccinimidyl ester derivatives were either commercially available (Calbiochem or SigmaAldrich), or were prepared by standard DCC-mediated esterifications.[20,21] Ac-L-Leu-OPfp was prepared by DCC-mediated esterification of Ac-L-Leu with pentafluorophenol.



Scheme 4.



Scheme 5.

4.2. Preparation of ALA 'dipeptides'

Urethane derivatives (typical procedure). A suspension of the amino acid active ester (1.5 mmol) and 5-ALA·HCl (0.25 g, 1.5 mmol) in dry THF (20 mL) was cooled to −5 °C under argon. A solution of DIPEA (0.26 mL, 1.5 mmol) in dry THF (10 mL) was slowly added over 120 min, then the reaction mixture was stirred overnight under cooling. The solvent was evaporated and an excess of freshly prepared ethereal diazomethane (40 mL) was added with cooling in an ice bath. The reaction mixture was stirred at room temperature for 2 h, then the solvent was carefully evaporated. The crude product was redissolved in EtOAc (40 mL) and was washed with 5% aq citric acid, 5% aq $NaHCO_3$, H_2O, and saturated aq NaCl (40 mL each). The organics were dried ($MgSO_4$) and the solvent was evaporated to give the crude product which was purified by column chromatography (EtOAc/40–60 petrol or MeOH/CH_2Cl_2 gradient) to give white solid or colourless oils. For **1f**, the product was purified by recrystallisation.

Acetyl derivatives (Method A). The coupling reaction and esterification were performed as for the urethane-protected derivatives, except that the base was added directly rather than in THF solution. The crude products were pre-absorbed onto silica using MeOH as solvent and purified by column chromatography using MeOH/CH_2Cl_2 (CH_2Cl_2 to 10% MeOH/CH_2Cl_2) as eluent, followed by recrystallisation from EtOAc/40–60 petrol to give the dipeptides as white solids.

Method B. The coupling reaction and esterification were performed exactly as for the urethane-protected derivatives. The crude products were pre-absorbed onto silica using MeOH as solvent and purified by column chromatography using MeOH/CH_2Cl_2 (CH_2Cl_2 to 10% MeOH/CH_2Cl_2) as eluent. If required, the dipeptides were further purified by recrystallisation from EtOAc/40–60 petrol as previously.

4.3. ^{1}H NMR chiral shift experiments with *N*-(α)-acetylated dipeptides

Optimum resolution was obtained when a molar ratio of Eu(hfc)$_3$ versus 5-ALA 'dipeptide' of approximately 1:4 was employed. In a typical experiment, a solution of the 5-ALA 'dipeptide' (10 mg, ca. 30 μmol) in dry $CDCl_3$ (0.7 mL) was treated with Eu(hfc)$_3$ (10 mg, ca. 8 μmol). The method was verified by examining mixtures of Ac-D-Phe-ALA-OMe and Ac-L-Phe-ALA-OMe. Integral ratios for each enantiomer were found to be consistent with the ratio of each in solution.

4.3.1. Boc-L-Ala-ALA-OMe (1a). 1.10 mmol scale. Yield: 65% (colourless oil). $[\alpha]_D$ −17.3 (c=0.54 $CHCl_3$); δ_H ($CDCl_3$) 1.39 (3H, d, J=7.1 Hz, CH_3-Ala), 1.47 (9H, s, Bu^t), 2.66–2.69 (2H, m, $COCH_2CH_2$), 2.75–2.79 (2H, m, $COCH_2CH_2$), 3.70 (3H, s, CO_2CH_3), 4.21–4.29 (3H, m, $NHCH_2CO$, $CHCH_3$), 5.01 (1H, br, urethane NH), 6.83 (1H, br, amide NH). δ_c 18.8 ($CHCH_3$), 28.0 ($COCH_2CH_2$), 28.7 ($C(CH_3)_3$), 34.9 ($COCH_2CH_2$), 49.5 ($NHCH_2CO$), 50.5 ($CHCH_3$), 52.3 (CO_2CH_3), 80.7 ($C(CH_3)_3$), 155.8 (urethane C=O), 173.2 (2 signals, amide and ester C=O), 203.9 (ketone C=O); *m/z* (ES) 317 (100%, $[M+H]^+$), 339.2 (23, $[M+Na]^+$); (Found: C, 53.07; H, 7.29; N, 8.75. $C_{14}H_{24}N_2O_6$ requires: C, 53.15; H, 7.65; N, 8.85%).

4.3.2. Boc-L-Leu-ALA-OMe (1b). 2.98 mmol scale. Yield 87% (colourless oil). $[\alpha]_D$ −17.0 (c=1.62 $CHCl_3$); δ_H ($CDCl_3$) 0.95–0.98 (6H, m, CH_3-Leu), 1.47 (9H, s, Bu^t), 1.55–1.73 (3H, m, $CHCH_2(CH_3)_2$, $CHCH_2(CH_3)_2$) 2.66–2.70 (2H, m, $COCH_2CH_2$), 2.75–2.79 (2H, m, $COCH_2CH_2$), 3.70 (3H, s, CO_2CH_3), 4.21 (2H, d, J=4.7 Hz, $NHCH_2CO$), 4.13–4.29 (1H, m, $CHCH_2CH(CH_3)_2$), 4.90 (1H, br, urethane NH), 6.80 (1H, br, amide NH); δ_c 22.1, 23.4 (CH_3-Leu), 25.1 ($CHCH_2(CH_3)_2$), 27.9 ($COCH_2CH_2$), 28.7 ($C(CH_3)_3$), 34.9 ($COCH_2CH_2$), 41.8 ($CHCH_2CH(CH_3)_2$), 49.4 ($NHCH_2CO$), 52.3 (CO_2CH_3), 53.4 ($CHCH_2$-$CH(CH_3)_2$), 80.5 ($C(CH_3)_3$), 156.1 (urethane C=O), 173.3 (amide C=O), 173.4 (ester C=O), 204.1 (ketone C=O); *m/z* (ES) 359 (100%, $[M+H]^+$), 381 (16, $[M+Na]^+$); (Found: C, 56.63; H, 8.13; N, 8.10. $C_{17}H_{30}N_2O_6$ requires C, 56.97; H, 8.44; N, 7.82%).

4.3.3. Boc-L-Lys(Z)-ALA-OMe (1c). 1.50 mmol scale. Yield: 85% (white solid). Mp: 85–87 °C (from CH_2Cl_2/40–60 petrol); $[\alpha]_D$ −9.6 (c=0.87 $CHCl_3$); δ_H ($CDCl_3$) 1.32–1.70 (4H, m, $CHCH_2CH_2CH_2CH_2NH$), 1.72–1.88 (2H, m, $CHCH_2(CH_2)_3NH$), 1.45 (9H, s, Bu^t), 2.64–2.68 (2H, m, $COCH_2CH_2$), 2.72–2.76 (2H, m, $COCH_2CH_2$), 3.18–3.24 (2H, m, CH_2NH), 3.69 (3H, s, CO_2CH_3), 4.14–4.25 (1H, m, $CH(CH_2)_4NH$), 4.19 (2H, d, J=4.8 Hz, $NHCH_2CO$), 5.00 (1H, br, CH_2NH), 5.11 (2H, s, OCH_2Ph), 5.11–5.19 (1H, m, Bu^{t}OCONH), 6.85 (1H, br, amide NH), 7.30–7.38 (5H, m, Ph); δ_c 22.8 ($CHCH_2CH_2$-$(CH_2)_2NH$), 27.9 ($COCH_2CH_2$), 28.7 ($C(CH_3)_3$), 29.8 ($CH(CH_2)_2CH_2CH_2NH$), 32.4 ($CHCH_2(CH_2)_3NH$), 34.9 ($COCH_2CH_2$), 40.8 ($CH(CH_2)_3CH_2NH$), 49.4 ($NHCH_2CO$), 52.3 (CO_2CH_3), 54.7 ($CH(CH_2)_4NH$), 67.0 (CH_2Ph), 128.5, 128.9, 137.0 (Ph), 156.1, 157.0 (urethane C=O), 172.7 (amide C=O), 173.3 (ester C=O), 204.1 (ketone C=O); *m/z* (ES) 508 (100%, $[M+H]^+$), 530 (36, $[M+Na]^+$), 546 (21, $[M+K]^+$); (Found: C, 59.13; H, 7.49; N, 8.23. $C_{25}H_{37}N_3O_8$ requires C, 59.16; H, 7.35; N, 8.27%).

4.3.4. Boc-L-Phe-ALA-OMe (1d). 1.10 mmol scale. Yield: 79% (colourless glass, Lit.,[6a] Mp: 83–85 °C); $[\alpha]_D$ −1.4 (c=0.89 $CHCl_3$); δ_H ($CDCl_3$) 1.33 (9H, s, Bu^t), 2.57–2.65 (4H, m, $COCH_2CH_2$), 3.01–3.12 (2H, m, CH_2Ph), 3.60 (3H, s, CO_2CH_3), 3.98–4.17 (2H, m, $NHCH_2CO$), 4.35 (1H, br, $CHCH_2Ph$), 4.85 (1H, br, urethane NH), 6.50 (1H, br, amide NH), 7.11–7.25 (5H, m, Ph); δ_c 28.0 ($COCH_2CH_2$), 28.6 ($C(CH_3)_3$), 34.9 ($COCH_2CH_2$), 38.8 ($CHCH_2Ph$), 49.5 ($NHCH_2CO$), 52.3 (CO_2CH_3), 56.1 ($CHCH_2Ph$), 80.7 ($C(CH_3)_3$), 127.4, 129.1, 129.7, 136.9 (Ph), 155.8 (urethane C=O), 171.8 (amide C=O), 173.2 (ester C=O), 203.6 (ketone C=O); *m/z* (ES) 393 (100%, $[M+H]^+$), 415 (26, $[M+Na]^+$), 431 (13, $[M+K]^+$); (Found: C, 61.07; H, 7.26; N, 7.17. $C_{20}H_{28}N_2O_6$ requires C, 61.21; H. 7.19; N. 7.14%).

4.3.5. Boc-L-Val-ALA-OMe (1e). 1.50 mmol scale. Yield: 32% (white solid). Mp: 73.5–77 °C; $[\alpha]_D$ −21.2 (c=1.32 $CHCl_3$); δ_H ($CDCl_3$) 0.84 (3H, d, J=6.8 Hz, CH_3-Val), 0.90 (3H, d, J=6.8 Hz, CH_3-Val), 1.38 (9H, s, Bu^t), 2.05–2.16 (1H, m, $CH(CH_3)_2$), 2.56–2.61 (2H, m, $COCH_2CH_2$), 2.66–2.70 (2H, m, $COCH_2CH_2$), 3.61 (3H, s, CO_2CH_3),

3.89–3.99 (1H, m, C*H*CH(CH_3)$_3$), 4.14 (2H, d, J=4.8 Hz, NHCH_2CO), 4.95 (1H, br, urethane NH), 6.62 (1H, br, amide NH); δ_c 17.95, 19.6 (CH_3-Val), 27.9 (COCH_2CH_2), 28.7 (C(CH_3)$_3$), 31.2 (CH(CH_3)$_2$), 34.9 (COCH_2CH_2), 49.4 (NHCH_2CO), 52.3 ($CO_2C H_3$), 60.2 (CHCH(CH_3)$_2$), 80.3 (C(CH_3)$_3$), 156.2 (urethane C=O), 172.2 (amide C=O), 173.2 (ester C=O), 204.0 (ketone C=O); *m/z* (ES) 345 (100%, $[M+H]^+$), 367 (100, $[M+Na]^+$), 348 (48, $[M+K]^+$); (Found: C, 55.45; H, 8.13; N, 8.10. $C_{16}H_{26}N_2O_6$ requires: C, 55.80; H, 8.20; N, 8.13%).

4.3.6. Z-Gly-ALA-OMe (1f). 1.10 mmol scale. Yield 80% (white solid). Mp: 103–105 °C; δ_H ($CDCl_3$) 2.66–2.70 (2H, m, COCH_2CH_2), 2.74–2.79 (2H, m, COCH_2CH_2), 3.70 (3H, s, CO_2CH_3), 3.94 (2H, d, J=5.6 Hz, NHCH_2CONH), 4.23 (2H, d, J=4.6 Hz, NHCH_2CO) 5.16 (2H, s, OCH_2Ph), 5.51 (1H, br, urethane NH), 6.78 (1H, br, amide NH), 7.32–7.44 (5H, m, Ph); δ_c 27.9 (COCH_2CH_2), 34.9 (COCH_2CH_2), 44.7 (NHCH_2CONH), 49.4 (NHCH_2CO), 52.4 ($CO_2C H_3$), 67.6 (OCH_2Ph), 128.5, 128.6, 128.9, 136.6 (Ph), 157.1 (urethane C=O), 169.8 (amide C=O), 173.3 (ester C=O), 204.2 (ketone C=O); *m/z* (ES) 337 (100%, $[M+H]^+$), 359 (35, $[M+Na]^+$), 375 (12, $[M+K]^+$); (Found: C, 57.11; H, 6.00; N, 8.35. $C_{16}H_{20}N_2O_6$ requires C, 57.14; H, 5.99; N, 8.32%).

4.3.7. Ac-Gly-ALA-OMe (2a). *Method A*. 2.30 mmol scale. Yield: 32%. Mp: 192–194 °C. δ_H ($CDCl_3$) 2.06 (3H, s, $COCH_3$), 2.64–2.69 (2H, m, COCH_2CH_2), 2.73–2.78 (2H, m, COCH_2CH_2), 3.67 (3H, s, CO_2CH_3), 3.99 (2H, d, J=5.0 Hz, NHCH_2CONH), 4.22 (2H, d, J=5.0 Hz, NHCH_2-CO), 6.33 (1H, br, CH_3CONHCH_2), 6.76 (1H, br, NHCH_2-COCH_2CH_2); δ_c 23.4 (COCH_3), 28.1 (COCH_2CH_2), 35.0 (COCH_2CH_2), 43.5 (NHCH_2CONH), 49.6 (NHCH_2CO), 52.5 ($CO_2C H_3$), 169.7, 171.5 (amide C=O), 173.4 (ester C=O), 204.1 (ketone C=O); *m/z* (EI) 244 (11%, M^+), 213 (7, $[M-OMe]^+$), 171 (8) 130 (45), 115 (78), 100 (100, $[M-ALA\text{-}OCH_3]^+$); (Found: C, 49.14; H, 6.60; N, 11.44. $C_{10}H_{16}N_2O_5$ requires C, 49.18; H, 6.60; N, 11.47%).

4.3.8. Ac-L-Ala-ALA-OMe (2b). *Method A*. 1.50 mmol scale. Yield: 26%. Mp: 102–106 °C. δ_H ($CDCl_3$) 1.38 (3H, d, J=6.9 Hz, CH_3-Ala), 2.01 (3H, s, $COCH_3$), 2.63–2.67 (2H, m, COCH_2CH_2), 2.72–2.77 (2H, m, COCH_2CH_2), 3.70 (3H, s, CO_2CH_3), 4.18 (2H, d, J=4.8 Hz, NHCH_2CO), 4.50–4.60 (1H, m, CHCH_3), 6.31 (1H, d, J=6.6 Hz, CH_3CONH), 6.94 (1H, br, NHCHCH_3CO); δ_H ($CDCl_3$/Eu(hfc)$_3$) 3.709 (2.2H, s, CO_2CH_3-L-Ala), 3.742 (0.8H, s, CO_2CH_3-D-Ala); δ_c 17.9 (CH*C*H$_3$), 22.4 (CH_3CO), 27.0 (COCH_2CH_2), 33.9 (COCH_2CH_2), 48.5 (NHCH_2CO), 48.9 (*C*HCH$_3$), 51.4 ($CO_2C H_3$), 170.2, 172.6 (amide C=O), 173.0 (ester C=O), 204.0 (ketone C=O); *m/z* (MALDI-TOF) 281 (100%, $[M+Na]^+$), 297 (23, $[M+K]^+$); (Found: C, 51.30; H, 6.87; N, 11.08. $C_{11}H_{18}N_2O_5$ requires C, 51.19; H, 7.03; N, 10.85%).

Method B. 1.50 mmol scale. Yield: 51%. δ_H ($CDCl_3$/Eu(hfc)$_3$) single enantiomer.

4.3.9. Ac-D-Ala-ALA-OMe (2c). *Method A*. 1.50 mmol scale. Yield: 47%. Mp: 102–104 °C. δ_H ($CDCl_3$/Eu(hfc)$_3$) 3.709 (0.9H, s, CO_2CH_3-D-Ala), 3.742 (2.1H, s, CO_2CH_3-L-Ala); (Found: C, 51.05; H, 7.06; N, 10.76. $C_{11}H_{18}N_2O_5$ requires C, 51.19; H, 7.03; N, 10.85%).

Method B. 1.30 mmol scale. Yield 73%. δ_H ($CDCl_3$/Eu(hfc)$_3$) single enantiomer.

4.3.10. Ac-L-Leu-ALA-OMe (2d). *Method A* (*using the pentafluorophenyl ester*). 1.50 mmol scale. Yield: 37%. Mp: 74–76 °C. δ_H ($CDCl_3$) 0.92–0.95 (6H, m, CH_3-Leu), 1.50–1.70 (3H, m, CHCH_2(CH_3)$_2$, CHCH_2(CH_3)$_2$), 2.01 (3H, s, $COCH_3$), 2.63–2.67 (2H, m, COCH_2CH_2), 2.72–2.76 (2H, m, COCH_2CH_2), 3.68 (3H, s, CO_2CH_3), 4.08–4.24 (2H, m, NHCH_2CO), 4.51–4.56 (1H, m, CHCH_2CH(CH_3)$_2$), 6.25 (1H, d, J=5.2 Hz, CH_3CONH), 7.00 (1H, br, NHCHCH_2-CH(CH_3)$_2$CO);); δ_H ($CDCl_3$/Eu(hfc)$_3$) 3.767 (1.5H, s, CO_2CH_3-L-Leu), 3.813 (1.5H, s, CO_2CH_3-D-Leu); δ_c 22.0, 22.8 (CH_3-Leu), 23.0 (COCH_3) 24.7 (CH*C*H$_2$(CH_3)$_2$), 27.4 (COCH_2CH_2), 34.4 (COCH_2CH_2), 41.2 (CHCH_2-CH(CH_3)$_2$), 49.0 (NHCH_2CO), 51.5 ($CO_2C H_3$), 51.8 (*C*HCH$_2$CH(CH_3)$_2$), 170.4, 172.6 (amide C=O), 173.0 (ester C=O), 203.7 (ketone C=O); *m/z* (EI) 300 (3%, M^+), 269 (7, $[M-OCH_3]^+$), 156 (62, $[M-ALA\text{-}OCH_3]^+$), 86 (100); (Found: C, 56.20; H, 7.97; N, 9.23. $C_{14}H_{24}N_2O_5$ requires C, 55.99; H, 8.05; N, 9.33%).

Method B (*using the succinimidyl ester*). 1.50 mmol scale. Yield 47%. δ_H ($CDCl_3$/Eu(hfc)$_3$) 3.611 (2.85H, s, CO_2CH_3-L-Leu), 3.6689 (0.15H, s, CO_2CH_3-D-Leu).

From Boc-L-Leu-OCH$_3$. **1b** (0.796 g, 2.22 mmol) was treated with 4 M HCl in dioxane (14 mL) and the resulting solution was stirred at room temperature for 40 min. The solvent was evaporated and the crude hydrochloride salt was dried thoroughly in vacuo. A suspension of the hydrochloride salt in CH_2Cl_2 (30 mL) was cooled in an ice bath and was treated with DIPEA (0.46 mL, 2.64 mmol), followed by acetic anhydride (0.42 mL, 4.45 mmol). The reaction mixture was allowed to attain room temperature overnight, then it was diluted with CH_2Cl_2 (30 mL) and was washed with 5% aq $NaHCO_3$, 5% aq citric acid and saturated aq NaCl (30 mL each). The aqueous layers were back-extracted with CH_2Cl_2 (4×30 mL) and the combined organics were dried ($MgSO_4$) and the solvent evaporated to give a colourless oil which was purified by column chromatography using MeOH/CH_2Cl_2 (CH_2Cl_2 to 7% MeOH/CH_2Cl_2) as eluant. This gave a white solid (0.508 g, 76%), indistinguishable (^{1}H, ^{13}C NMR) from the samples prepared by Methods A and B.

4.3.11. Ac-L-Phe-ALA-OMe (2e). *Method A*. 1.38 mmol scale. Yield: 43%. Mp: 129–130 °C. δ_H ($CDCl_3$) 1.95 (3H, s, $COCH_3$), 2.60–2.70 (4H, m, COCH_2CH_2), 3.00–3.15 (2H, m, CH_2Ph), 3.68 (3H, s, CO_2CH_3), 4.00–4.20 (2H, m, NHCH_2CO), 4.70–4.80 (1H, br, CHCH_2Ph), 6.20 (1H, br, CH_3CONH), 6.70 (1H, br, NHCHCH_2Ph); δ_H ($CDCl_3$/Eu(hfc)$_3$) single enantiomer; δ_c 23.1 (COCH_3) 27.5 (COCH_2CH_2), 35.0 (COCH_2CH_2), 38.3 (CH_2Ph), 49.0 (NHCH_2CO), 52.0 ($CO_2C H_3$), 56.1 (CHCH_2Ph), 127.0, 128.6, 129.2, 136.3 (Ph), 170.1, 170.9 (amide C=O), 172.8 (ester C=O), 203.2 (ketone C=O); *m/z* (EI) 334 (11%, M^+), 303 (3, $[M-OCH_3]^+$), 275 (15, $[M-CO_2CH_3]^+$), 219 (5), 190 (20), 171 (8), 120 (100); (Found: C, 60.75; H,

6.68; N, 8.15 $C_{17}H_{22}N_2O_5$ requires C, 61.07; H, 6.61; N, 8.38%).

4.3.12. Ac-D-Phe-ALA-OMe (2f). *Method A*. 1.38 mmol scale. Yield: 41%. Mp: 126–128 °C. δ_H ($CDCl_3$/Eu(hfc)$_3$) single enantiomer. (Found: C, 60.78; H, 6.63; N, 8.36. $C_{17}H_{22}N_2O_5$ requires C, 61.07; H, 6.61; N, 8.38%).

4.3.13. Ac-L-Val-ALA-OMe (2g). *Method A*. 1.50 mmol scale. Yield: 33%. Mp: 147–148 °C. δ_H ($CDCl_3$) 0.94 (3H, d, J=4.6 Hz, CH_3-Val), 0.96 (3H, d, J=4.6 Hz, CH_3-Val), 2.00–2.12 (4H, m, $COCH_3$, C*H*$(CH_3)_2$), 2.62–2.67 (2H, m, $COCH_2CH_2$), 2.72–2.77 (2H, m, $COCH_2CH_2$), 3.67 (3H, s, CO_2CH_3), 4.10–4.28 (2H, m, $NHCH_2CO$) 4.34–4.39 (1H, m, C*H*$CH(CH_3)_3$), 6.35 (1H, br, CH_3CONH), 6.70 (1H, br, N*H*$CH(CH_3)_2CO$); δ_H ($CDCl_3$/Eu(hfc)$_3$) 3.723 (2.45H, s, CO_2CH_3-L-Val), 3.775 (0.55H, s, CO_2CH_3-D-Val); δ_c 18.0, 19.0 (CH_3-Val), 23.1 ($COCH_3$), 27.4 ($COCH_2CH_2$), 31.0 ($CH(CH_3)_2$), 34.4 ($COCH_2CH_2$), 49.0 ($NHCH_2CO$), 52.0 (CO_2CH_3), 58.3 ($CHCH(CH_3)_2$), 170.3, 172.2 (amide C=O), 172.9 (ester C=O), 203.6 (ketone C=O); *m/z* (EI) 286 (3%, M^+), (5, M-OCH_3), 142 (40, [M−ALA-OCH_3]$^+$), 114 (100), 72 (95); (Found: C, 54.86; H, 7.92; N, 9.71. $C_{13}H_{22}N_2O_5$ requires C, 54.53; H, 7.74; N, 9.78%).

Method B. 1.50 mmol scale. Yield: 38%. δ_H ($CDCl_3$/Eu(hfc)$_3$) single enantiomer.

From Boc-L-Val-OCH$_3$. **1e** (0.164 g, 0.476 mmol) was treated with 4 M HCl in dioxane (2 mL) and the resulting solution was stirred at room temperature for 1 h. The solvent was evaporated and the crude hydrochloride salt was dried thoroughly in vacuo. A suspension of the hydrochloride salt in CH_2Cl_2 (4 mL) was cooled in an ice bath and was treated with DIPEA (0.11 mL, 0.63 mmol), followed by acetic anhydride (96 μL, 1.02 mmol). The reaction mixture was allowed to attain room temperature overnight, then it was diluted with CH_2Cl_2 (25 mL) and was washed with 5% aq $NaHCO_3$, 5% aq citric acid and saturated aq NaCl (25 mL each). The aqueous layers were back-extracted with CH_2Cl_2 (4×25 mL) and the combined organics were dried ($MgSO_4$) and the solvent evaporated to give an off-white solid which was recrystallised from CH_2Cl_2-hexane to give a white solid (90 mg, 66%), indistinguishable (^{1}H, ^{13}C NMR) from the samples prepared by Methods A and B.

4.3.14. *N*-(*tert*-Butoxycarbonyl)-L-phenylalanyl-N^{ε}-(benzyloxycarbonyl)-L-lysinyl-5-aminolaevulinic acid methyl ester (Boc-L-Phe-L-Lys(Z)-ALA-OMe) (3a). 1c (0.141 g, 0.278 mmol) was treated with 4 M HCl in dioxane (2.5 mL) and the resulting solution was stirred at room temperature for 30 min. The solvent was evaporated and the crude hydrochloride salt was dried thoroughly in vacuo. A stirred solution of Boc-L-Phe (74 mg, 0.279 mmol) and 1-hydroxybenzotriazole monohydrate (58 mg, 0.429 mmol) in CH_2Cl_2 (1.5 mL) and DMF (1.5 mL) was cooled in an ice bath and EDC·HCl (53 mg, 0.276 mmol) was added. After 40 min, a solution of the preceding hydrochloride salt in DMF (2 mL) was added, followed by DIPEA (0.11 mL, 0.63 mmol) and the reaction mixture was allowed to attain room temperature overnight. After evaporation of the solvents, the residue was dissolved in EtOAc (20 mL) and was washed with 5% aq citric acid, 5% aq $NaHCO_3$, H_2O, and saturated aq NaCl (20 mL each). The organic layer was dried ($MgSO_4$) and the solvent was evaporated to give an off-white solid which was purified by column chromatography using MeOH/CH_2Cl_2 (5–9% MeOH/CH_2Cl_2) as eluant. This gave a white solid (0.163 g, 89%). Mp: 103.5–107.5 °C; $[\alpha]_D$ −17.0 (c=1.0 $CHCl_3$); δ_H ($CDCl_3$ 0.83–1.54 (4H, m, $CHCH_2CH_2CH_2CH_2NH$), 1.31 (9H, s, Bu^t), 1.70–1.90 (2H, m, $CHCH_2(CH_2)_3$), 2.55–2.57 (2H, m, $COCH_2CH_2$), 2.61–2.63 (2H, m, $COCH_2CH_2$), 2.93–3.10 (4H, m, $CH(CH_2)_3CH_2NH$, $CHCH_2Ph$), 3.59 (3H, s, CO_2CH_3), 4.01–4.10 (2H, m, $NHCH_2CO$), 4.32–4.37 (2H, m, C*H*$(CH_2)_4NH$, C*H*CH_2Ph), 4.95–5.10 (4H, m, OCH_2Ph, 2×urethane NH), 6.60–6.67 (2H, m, 2×amide NH), 7.10–7.34 (10H, m, 2× Ph); δ_c 22.6 ($CHCH_2CH_2CH_2CH_2NH$), 27.9 ($CHCH_2CH_2CH_2CH_2NH$), 28.6 ($C(CH_3)_3$), 29.7 ($COCH_2CH_2$), 31.8 ($CHCH_2(CH_2)_3$-NH), 34.6 ($COCH_2CH_2$), 38.4 ($CHCH_2Ph$), 40.8 ($CH(CH_2)_3CH_2NH$), 49.4 ($NHCH_2CO$), 52.3 (CO_2CH_3), 53.3 ($CH(CH_2)_4NH$), 56.4 ($CHCH_2Ph$), 67.0 (OCH_2Ph), 80.9 ($C(CH_3)_3$), 127.4, 128.5, 128.9, 129.1, 136.7, 137.0 (Ph), 156.1, 157.0 (urethane C=O), 171.7, 172.0 (amide C=O), 173.3 (ester C=O), 203.0 (ketone C=O); *m/z* (ES) 655 (57%, $[M+H]^+$), 677.4 (16, $[M+Na]^+$); (Found: C, 62.32; H. 7.26; N, 8.47. $C_{34}H_{46}N_4O_8$ requires C, 62.37; H, 7.08; N, 8.55%).

4.3.15. *N*-Acetyl-L-phenylalanyl-N^{ε}-(benzyloxycarbonyl)-L-lysinyl-5-aminolaevulinic acid methyl ester (Ac-L-Phe-L-Lys(Z)-ALA-OMe) (3b). 3a (0.122 g, 0.186 mmol) was treated with 4 M HCl in dioxane (2 mL) and the resulting solution was stirred at room temperature for 1 h. The solvent was evaporated and the crude hydrochloride salt was dried thoroughly in vacuo. A suspension of the hydrochloride salt in CH_2Cl_2 (4 mL) was cooled in an ice bath and was treated with DIPEA (40 μL, 0.23 mmol), followed by acetic anhydride (35 μL, 0.37 mmol). The reaction mixture was allowed to attain room temperature overnight at which point a gelatinous white solid had separated out. The reaction mixture was diluted with CH_2Cl_2 (25 mL) to dissolve the solid and was washed with 5% aq $NaHCO_3$, 5% aq citric acid and saturated aq NaCl (25 mL each). Drying ($MgSO_4$) and evaporation of the solvent gave an off-white solid which was precipitated from CH_2Cl_2/hexane to give a white powder (95 mg, 85%). Mp: 144–146 °C; $[\alpha]_D$ −6.9 (c=1.0 CH_3OH); δ_H ($CDCl_3$) 1.20–1.91 (6H, m, $CHCH_2CH_2CH_2CH_2NH$), 1.87 (3H, s, $COCH_3$), 2.55–2.58 (2H, m, $COCH_2CH_2$), 2.62–2.66 (2H, m, $COCH_2CH_2$), 2.92–3.10 (4H, m, $CH(CH_2)_3CH_2NH$, $CHCH_2Ph$), 3.60 (3H, s, CO_2CH_3), 4.01–4.08 (2H, m, $NHCH_2CO$), 4.30–4.40 (1H, m, C*H*$(CH_2)_4NH$), 4.65 (1H, ABq, C*H*CH_2Ph) 5.01–5.18 (4H, m, OCH_2Ph, 2× urethane NH), 6.25 (1H, d, J= 7.2 Hz, CH_3CONH) 6.55–6.60 (1H, m, amide NH), 6.68 ((1H, d, J=7.7 Hz, amide NH), 7.12–7.32 (10H, m, 2× Ph); δ_c 22.6 ($CHCH_2CH_2CH_2CH_2NH$), 23.3 ($COCH_3$) 27.9 ($CHCH_2CH_2CH_2CH_2NH$), 29.7 ($COCH_2CH_2$), 32.4 ($CHCH_2(CH_2)_3NH$), 34.9 ($COCH_2CH_2$), 38.8 ($CHCH_2Ph$), 40.9 ($CH(CH_2)_3CH_2NH$), 49.5 ($NHCH_2CO$), 52.3 (CO_2CH_3), 53.3 ($CH(CH_2)_4NH$), 54.8 ($CHCH_2Ph$), 66.9 (OCH_2Ph), 127.3, 128.4, 128.9, 129.7, 136.9, 137.1 (Ph), 157.1 (urethane C=O), 170.9, 171.9 (2 signals) (amide C=O), 173.3 (ester C=O), 204.3 (ketone C=O); *m/z* (ES) 597 (100%, $[M+H]^+$), 619 (27, $[M+Na]^+$); (Found: C,

62.02; H. 6.73; N, 9.31. $C_{34}H_{46}N_4O_8$ requires C, 62.40; H, 6.76; N, 9.39%).

4.3.16. *N*-Acetylglycyl-5-aminolaevulinic acid hexyl ester (Ac-Gly-ALA-OHex) (4). A stirred solution of **2a** (0.152 g, 0.53 mmol) in CH_3OH (1.2 mL) and H_2O (1.8 mL) was treated with LiOH (16 mg, 0.66 mmol). The reaction mixture was stirred at room temperature for 40 min, then it was applied to a column of Amberlyst IR 120 resin (plus) cation exchange resin which was eluted with 60% aq CH_3OH. Acidic fractions were collected and the solvent was evaporated to give the crude acid as a white solid (0.182 g). A solution of the acid (70 mg, 0.25 mmol) in $CHCl_3$ (10 mL) was treated with DCC (58 mg, 0.28 mmol), DMAP (5 mg, 40 μmol) and hexan-1-ol (40 μL, 0.32 mmol). The reaction mixture was stirred at room temperature for 21 h, then it was filtered to remove DCU and the solvent was evaporated. The crude product was purified by column chromatography using 5% MeOH/CH_2Cl_2 as eluant, then recrystallised from CH_3OH/diethyl ether to give a white solid (52 mg, 65%); Mp: 124–125 °C; δ_H ($CDCl_3$) 0.82 (3H, t, $J=6.7$ Hz, $(CH_2)_5CH_3$), 1.10–1.35 (6H, m, $(CH_2)_2CH_2CH_2CH_2CH_3$), 1.40–1.70 (2H, m, $CH_2CH_2(CH_2)_3CH_3$), 1.99 (3H, s, $COCH_3$), 2.57–2.68 (4H, m, $COCH_2CH_2$), 3.91 (2H, d, $J=5.2$ Hz, $NHCH_2CONH$), 4.16 (2H, d, $J=4.7$ Hz, $NHCH_2CO$), 6.10 (1H, br, CH_3CONH), 6.50 (1H, br, $NHCH_2COCH_2CH_2$); δ_c 14.0 ($(CH_2)_5CH_3$), 22.5, 25.5, 27.8, 28.5 $(CH_2)CH_2CH_2CH_2CH_3$, $COCH_2CH_2$), 31.3 ($CH_2CH_2(CH_2)_3CH_3$), 34.5 ($COCH_2CH_2$), 43.0 ($NHCH_2CONH$), 49.1 ($NHCH_2CO$), 65.1 ($CH_2(CH_2)_4CH_3$)), 169.1, 170.1 (amide C=O), 172.5 (ester C=O), 203.6 (ketone C=O); [Found: (EI) 314.18417, $C_{15}H_{26}N_2O_5$ requires 314.18351]; *m/z* (ES) 315 (70%, $[M+H]^+$), 337 (100, $[M+Na]^+$), 353 (10, $[M+Na]^+$).

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References and notes

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