The synthesis and reactivity of optically pure amino acids bearing side-chain thioamides

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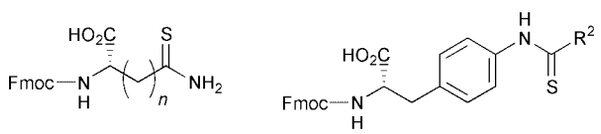
The synthesis and reactivity of fully protected thioamide analogues of asparagine and glutamine are described. A key feature of the synthetic strategies employed was the ability to perform selective thiations on multiple carbonyl-containing substrates. Also described are the preparations of thioamide derivatives of phenylalanine. The utility of these amino acid derivatives for solid-phase peptide synthesis is discussed.

Introduction

One of the most important techniques for studying the interaction of small biologically active molecules with their receptors is photoaffinity labelling.[1] This methodology is particularly important where high-resolution structures from X-ray or NMR data are not available for the receptor, as is often the case for large membrane proteins. During the course of our work directed towards the synthesis of photoactivatable analogues of peptides by solid-phase methods, it became apparent that all of the photoactivatable moieties currently used are based around aromatic (phenylalanine) systems. Whilst this is acceptable for peptides which naturally contain residues with aromatic side-chains, it is less than ideal for peptides which are highly polar in nature, or contain no aromatic residues. We found it desirable therefore, to have access to a non-aromatic, more polar amino acid that would be suitable as a photoaffinity tool and could be incorporated directly into peptides by solid-phase synthetic methods. We decided to explore the known photochemistry of the thiocarbonyl group[2] by synthesising thioamide derivatives of asparagine **1** and glutamine **2** in a protected form that would subsequently allow their incorporation into peptides by solid-phase synthetic strategies. Additionally, we desired thioamide-modified amino acids **3** and **4**, analogues of phenylalanine, in order to extend the range of photoactivatable groups available for the preparation of peptide analogues (Fig. 1).

As part of our design strategy, protection of the primary thioamide functionality present in **1** and **2** was considered essential during peptide synthesis to avoid unwanted side-reactions at the thioamide moiety, most notably acylation of the sulfur atom during amino acid coupling. Our initial protecting group of choice was the bulky, acid-labile trityl group, by analogy with the side-chain protection strategy employed for asparagine and glutamine. All of our synthetic strategies centred on selective thiations of enantiopure asparagine or glutamine analogues, thereby avoiding the requirement for an enantioselective step or resolution of a racemic mixture in the synthetic route to the final compounds. In the case of the aromatic thioamide analogues, *N*-thioacyl derivatives of (4-aminophenyl)alanine were prepared and used without protection.



1; $n = 1$
2; $n = 2$

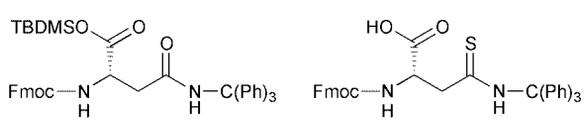
3; $R^2 = Ph$
4; $R^2 = CH_2Ph$

Fig. 1 Target amino acids incorporating a thiocarbonyl functional group.

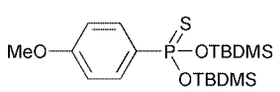
Results and discussion

Preparation of thioamide analogues of asparagine and glutamine

Initial experiments centred on a commercially available Fmoc-asparagine derivative for peptide synthesis, in which the carboxamide of the side chain was protected by a trityl group. This would have allowed direct access to a protected thioamide analogue of asparagine. Due to the acid lability of the trityl group and the base lability of the Fmoc group, an orthogonal means of protecting the carboxy functionality of this derivative prior to thiation was required. Thus reaction with TBDMS chloride furnished the pure silyl ester **5** in good yield following recrystallisation. Reaction of ester **5** with phosphorus penta-



5 **6**



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sulfide in THF under ultrasound irradiation, and *in situ* deprotection of the silyl ester with wet tetra-*n*-butylammonium fluoride produced a material with spectroscopic characteristics consistent with the desired target compound **6**. In spite of this, satisfactory elemental analyses and mass spectroscopic data could not be obtained for this compound.[3] Reaction of **5** with Lawesson's reagent furnished phosphonothioate **7** as the sole characterisable compound.

Attention was therefore directed towards an alternative synthetic strategy to **1** and **2** based on Fmoc-protected asparagine **8** and glutamine **9** respectively, with introduction of the protecting group at the final stage of the syntheses (Scheme 1). Clearly, **8** and **9** contain three different types of carbonyl group, of which the amide function was required to undergo selective

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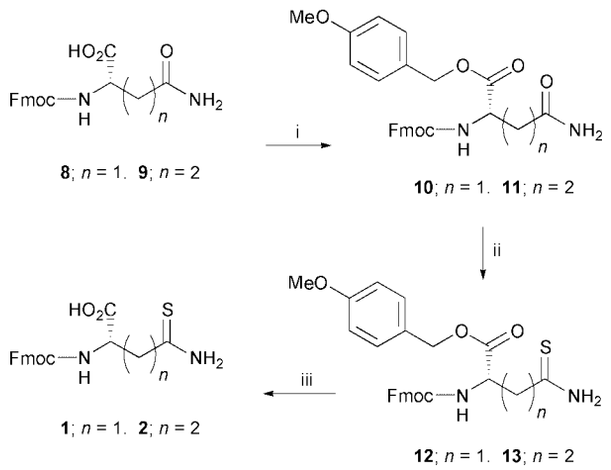
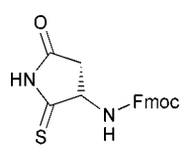


Scheme 1 *Reagents and conditions*: i, $ClCH_2PhOMe$, NaI, DMA, RT, 17 h, 93% (**10**), 93%) (**11**); ii, P_4S_{10}, THF, ultrasound, 88% (**12**), 88% (**13**); iii, TFA, $HS(CH_2)_2SH$, PhSMe, 91% (**1**), 67% (**2**).

thiation. By first protecting the carboxy groups as their 4-methoxybenzyl esters **10** and **11** however, using conditions similar to those previously described[4] for the attachment of amino acids to resins as their 4-alkoxybenzyl esters, a highly selective thiation could be achieved using phosphorus pentasulfide[5] in THF under ultrasound irradiation in excellent yield to give thioamides **12** and **13** respectively. Thiation of **10** with Lawesson's reagent[6] in toluene at 50 °C was less selective, giving rise to unacceptable quantities of thiomaleimide **14**



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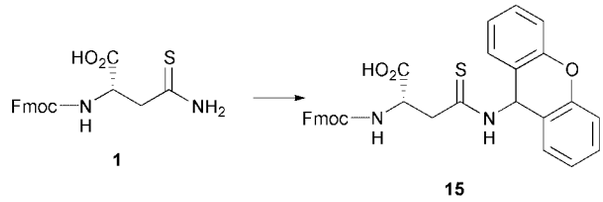
(10%), which was absent from the phosphorus pentasulfide thiation. Proof of the chemoselectivity of the thiations was obtained *via* analysis using IR and ^{13}C NMR spectroscopies. In the IR spectra of **12** and **13**, the carbonyl stretching vibrations of the amide groups of **10** and **11** were clearly absent. The ^{13}C NMR spectra of **13** showed a clear resonance for the thiocarbonyl carbon at δ 209 ppm, with corresponding absence of the amide carbonyl resonance (present at δ 159.9 ppm in compound **11**). Additional model thiation experiments with *N*-Fmocbenzylamine illustrated that no reaction occurred with the urethane carbonyl under these conditions. Deprotection of the carboxy moiety was then readily achieved without alkylation of the thioamide by using TFA in the presence of thioanisole and ethane-1,2-dithiol as cation scavengers to yield compounds **1** and **2**. Previous experiments directed towards the synthesis of thioasparagine analogues[7] had illustrated the susceptibility of the thioamide sulfur to alkylation by alkyl cations generated during the strong acid cleavage of amine protecting groups. These problems were not encountered by us.

As the final step of the syntheses, it was intended to incorporate trityl protecting groups onto the thioamide nitrogens using trityl alcohol in the presence of sulfuric acid as dehydrating agent, conditions previously described for the *N*-alkylation of thioacetamide.[8] Despite our best attempts however, the *N*-alkylated product could not be isolated. We therefore considered the xanthen-9-yl moiety[9] as an alternative protecting group, and in the case of asparagine, we were able to incorporate this group successfully by heating **1** with two equivalents of 9-hydroxyxanthene in acetic acid to produce **15** (Scheme 2). With thioglutamine **2**, material giving suitable elemental analysis could not be obtained. *N*-Xanthenyl thioacetamide[9] was also prepared as a model compound, and was

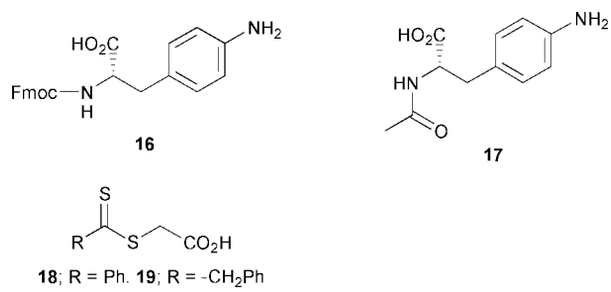


Scheme 2 *Reagents and conditions*: 9-hydroxyxanthene, acetic acid, △, 60%.

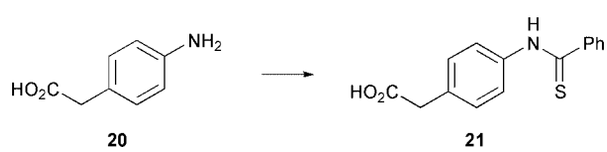
found to be stable to all of the reagents encountered during peptide synthesis and was smoothly deprotected to thioacetamide in 5 h using TFA.[3]

Preparation of thioamide analogues of phenylalanine

Phenylalanine derivatives **3** and **4** were prepared by manipulation of the (*S*)-enantiomer of phenylalanine. All our attempts to acylate the amino group of the Fmoc-protected 4-aminophenyl substituted alanine **16**[10] in DMF at 50–60 °C were unsuccessful due to *in situ* deprotection of the α-amino group by the primary aromatic amine. This was supported in one case by the isolation of (*S*)-2-acetylamino-3-(4-aminophenyl)propionic acid **17** following attempted acetylation using



acetic anhydride. Reactions at lower temperatures yielded only unreacted starting materials. As an alternative approach, we considered thioacylation of the aromatic amino group using dithioesters **18**[11b] and **19**[11a] as active thioacylating agents. Model experiments clearly illustrated that **18** was capable of thioacylating the amino group of 2-(4-aminophenyl)acetic acid **20** under mild conditions to produce the thioamide **21** in good yield (Scheme 3). Repetition of this reaction with phenylalanine



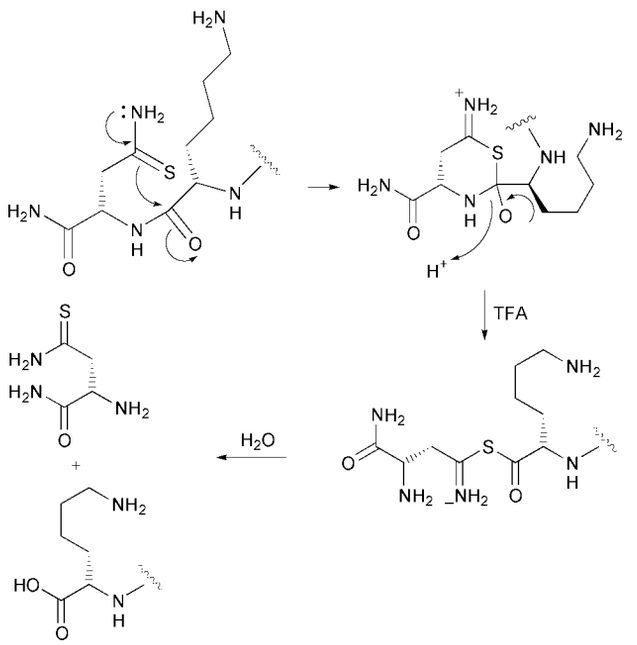
Scheme 3 *Reagents and conditions*: **18**, NaOH, H_2O, 76%.

derivative **16** proceeded smoothly to the target compounds **3** and **4**. The retention of the optical purity of these compounds was illustrated by chiral TLC on the Fmoc-deprotected derivatives, where no traces of the (*R*)-isomers were detectable.

Preparation of peptides

In order to demonstrate the applicability of **15** for peptide synthesis, we synthesised the peptide analogue Arg-Lys-Ile-Cys-Gly-Lys-Asn* (where Asn* indicates γ-thioasparagine **15**), corresponding to the seven *C*-terminal residues of mast cell degranulating peptide (MCDP),[12] using conventional Fmoc solid-phase coupling strategies. Analysis of the synthesis data indicated that derivative **15** was efficiently coupled onto the peptide chain using *N*,*N*-diisopropylcarbodiimide as the carboxy activating agent. All other couplings using both pentafluorophenyl esters and diimides, and all Fmoc deprotections, occurred smoothly. Deprotection of the resin following synthesis was performed using TFA in the presence of scavengers,

and the crude peptide was analysed by MALDI-MS. This produced no ions corresponding to the desired peptide (*m*/*z* 833.1), but peaks of *m*/*z* 886.0 ± 1.0, 818.8 ± 0.9 and 704.1 ± 0.9. A similar deprotection was performed with a modified version of the cleavage reagent used above containing an excess of thioacetamide as an additional scavenger. In this case, a similar ion distribution was observed, with the exception that the 2,2,5,7,8-pentamethylchroman-6-ylsulfonyl (Pmc) protecting group of arginine remained intact. Consistent with these observations, we were able to propose a mechanism whereby the thioamide moiety of thioasparagine reacts in an intramolecular fashion with *tert*-butyl cations derived from the neighbouring lysine during the cleavage reaction. The resulting *S*-*tert*-butyliminothiolate is then either partially hydrolysed under these conditions to the corresponding aspartate (*m*/*z* 818.8), or rearranges to the more stable *N*-*tert*-butylthioamide (*m*/*z* 889.5). Interestingly, the peak of *m*/*z* 704.1, lacking the *C*-terminal thioasparagine residue, is best accounted for by intramolecular reaction of the thioamide with the peptide backbone, in a similar fashion to the process occurring during Edman degradation [13] (Scheme 4). Production of this peptide by



Scheme 4 Proposed mechanism for cleavage of the peptide backbone by the thioamide.

a failure of the coupling reaction during peptide synthesis was ruled out by the feedback data from the synthesiser, and the presence of a capping step before coupling of the second amino acid.

For the aromatic-based thioamide systems **3** and **4**, the applicability of these in solid-phase peptide synthesis was demonstrated by their efficient incorporation into the neurokinin A (NKA) peptide sequence to yield photolabile analogues of NKA having the sequence His-Phe*-Lys-Thr-Asp-Ser-Phe-Val-Gly-Leu-Met-NH_2 (where Phe* corresponds to the position of incorporation of **3** and **4** respectively).[14] The NKA system is a naturally occurring hormone and has been implicated in a large number of important biological processes.[15] The coupling efficiency of both of the amino acids **3** and **4** was in excess of 97%. Following purification using reverse-phase HPLC, the purified peptides were characterised using mass spectrometry and were both found to have the correct molecular weights of 1415.1 and 1455.7 respectively.[14]

In conclusion, we have developed efficient methods for the preparation of thiated amino acids based on both aromatic and non-aromatic systems. Although the aromatic-based systems are efficiently incorporated into peptides using standard solid-phase techniques, it was not possible to incoporate a thioasparagine-based system into peptides due to degradation during the solid-phase synthesis. This work does, however, give access to a new class of peptide analogue with potential as photochemical probes.

Experimental

General

Ultrasound experiments were carried out in a Kerry PUL55 ultrasonic bath with a power input of 55 W. Chiral TLC was performed using chiral TLC plates purchased from Aldrich, with methanol–water–acetonitrile (1 : 1 : 4) as the mobile phase and DL-phenylalanine as reference. Spot detection was performed using ninhydrin. Melting points were recorded on a Reichert hot stage and are uncorrected. All infra-red spectra were recorded as Nujol mulls unless otherwise stated. ^{1}H NMR spectra were recorded with TMS as internal standard unless otherwise stated. Coupling constants are in Hz. The solvent signal was used as internal standard for ^{13}C NMR experiments.

Peptide synthesis and analysis

Peptide synthesis was performed on a Milligen 9050 peptide synthesiser using the 'Express' software application. Standard Fmoc-compatible coupling and deprotection cycles were performed throughout. The side-chain functional groups of arginine, lysine and cysteine were protected as their 2,2,5,7,8-pentamethylchroman-6-ylsulfonyl (Pmc), Boc and trityl derivatives, respectively. Capping of the resin following coupling of compound **15** was carried out using *N*-acetylimidazole. Peptide chain assembly was performed on Novasyn KR 100 amide resin (0.1 meq g^{-1}), with final cleavage performed on 50 mg of resin using a mixture of TFA (7 ml), ethane-1,2-dithiol (0.5 ml), thioanisole (0.75 ml), triisopropylsilane (0.5 ml), phenol (0.5 g) and water (0.5 ml) for 4 to 5 h. A modified mixture was also used containing thioacetamide (0.15 g, 2 mmol). The crude peptides were precipitated with diethyl ether (15 ml) and analysed directly by MALDI-MS. Mass spectra of peptides were determined on a VG Organic TofSpec MALDI mass spectrometer using Opus 3.1 software. The matrix used was α-cyano-4-hydroxycinnamic acid, with adrenocorticotropic hormone (ACTH, fragment 1–13, *m*/*z* 1623.8) as external standard. Desorption was performed using a pulsed nitrogen laser at 337 nm.

(*S*)-N^{α}-(Fluoren-9-ylmethoxycarbonyl)-N^{γ}-tritylasparagine *tert*-butyldimethylsilyl ester (5)

To a solution of N^{α}-(fluoren-9-ylmethoxycarbonyl)-N^{γ}-tritylasparagine (1.00 g, 1.68 mmol), *N*,*N*-diisopropylethylamine (320 µl, 1.84 mmol) and 4-dimethylaminopyridine (0.02 g, 0.2 mmol) under argon in dichloromethane (7.5 ml) at 0 °C, was added *tert*-butyldimethylsilyl chloride (0.278 g, 1.84 mmol) portion-wise with stirring. Following the addition, the mixture was stirred at 0 °C for 15 min and then at room temperature for 70 min before being diluted with dichloromethane (10 ml) and washed with water (2 × 10 ml). Following drying of the organic layer ($MgSO_4$) and removal of the drying agent by filtration, the solvent was removed *in vacuo* to give the crude product as a colourless solid. Recrystallisation from chloroform–pentane gave the title compound **5** (1.13 g, 95%) as a colourless solid, mp 108–112 °C; $[\alpha]_D^{30}$ +12 (*c* 0.6 in chloroform) (Found: C, 74.4; H, 6.55; N, 4.2. $C_{44}H_{46}N_2O_5Si$ requires: C, 74.3; H, 6.52; N, 3.9%); ν_{max}/cm^{-1} 3640, 3430, 2930, 1715s, 1490s, 1210s, 835; δ_H(300 MHz, $CDCl_3$) 0.21 and 0.24 (6 H, s, Si-CH_3), 0.89 (9 H, s, Si-C(CH_3)$_3$), 2.84 (1 H, dd, *J* 11.0 and 2.8, CHC*H*HC(O)NH(Trt)), 3.09 (1 H, dd, *J* 11.0 and 2.8, CHCH*H*C(O)NH(Trt)), 4.15–4.21 (1 H, m, -CH_2C*H* Fmoc), 4.26–4.58 (2 H, m, -CH_2CH Fmoc), 4.54 (1 H, m, C*H*CH_2C(O)NH(Trt)), 6.15

(1 H, d, *J* 8.8, N*H* Fmoc), 6.66 (1 H, s, C(O)N*H*(Trt)), 7.14–7.32 (17 H, m, Ar), 7.33–7.43 (2 H, m, Ar), 7.59 (2 H, d, *J* 7.8, Ar), 7.76 (2 H, d, *J* 8.1, Ar); *m*/*z* (EI) 710 (M^+, 1.5%), 532 (5), 243 (100).

Bis(*tert*-butyldimethylsilyl) 4-methoxyphenylthiophosphonate (7)

A solution of ester **5** (0.62 g, 0.88 mmol) and Lawesson's reagent (0.21 g, 0.52 mmol) in toluene (4 ml) was stirred under argon at 45 °C for 17 h before the solvent was removed *in vacuo*. Purification by flash column chromatography (CH_2Cl_2–hexane; 1:1) gave the title compound **7** (0.33 g, 87%) as a yellow oil (Found: C, 52.75; H, 8.7; S, 7.8. $C_{19}H_{37}O_3PSSi_2$ requires: C, 52.74; H, 8.6; S, 7.4%); ν_{max}(film)/cm^{-1} 2920s, 1590, 1470, 1250s, 1110s, 825s, 715; δ_H(300 MHz, $CDCl_3$) 0.10 and 0.31 (12 H, s, Si-(CH_3)$_2$), 0.91 (18 H, s, SiC(CH_3)$_3$), 3.86 (3 H, s, CH_3OPh), 6.93 (2 H, dd, *J* 8.3 and 1.8, Ar), 7.85 (2 H, dd, *J* 8.3 and 13.9, Ar); *m*/*z* (EI) 432 (M^+, 2%), 417 (5), 375 (100), 319 (18), 73 (25).

(*S*)-N^{α}-(Fluoren-9-ylmethoxycarbonyl)asparagine 4-methoxybenzyl ester (10)

A solution of N^{α}-(fluoren-9-ylmethoxycarbonyl)asparagine **8** (4.90 g, 13.8 mmol), 4-methoxybenzyl chloride (2.80 ml, 16.4 mmol), sodium iodide (3.11 g, 20.1 mmol) and *N*,*N*-diisopropylethylamine (5.0 ml, 28 mmol) in *N*,*N*-dimethylacetamide (40 ml) was stirred under argon at room temperature for 17 h before being diluted with chloroform (500 ml) and washed with water (2 × 500 ml). Following drying of the organic solution ($MgSO_4$), the drying agent was removed by filtration and the solvent removed *in vacuo* to give the crude product as a yellow oil. This oil was triturated with diethyl ether (100 ml) and left for 1 h, producing a colourless precipitate. The solid was collected by filtration, washed with diethyl ether (2 × 100 ml) and recrystallised from chloroform–hexane to give the title compound (6.10 g, 93%) as a colourless solid, mp 172–174 °C; $[\alpha]_D^{30}$ −11 (*c* 0.2 in *N*,*N*-dimethylformamide) (Found: C, 68.05; H, 5.25; N, 6.0. $C_{27}H_{26}N_2O_6$ requires: C, 68.34; H, 5.52; N, 5.9%); ν_{max}/cm^{-1} 3400, 3300s, 3200, 1740s (C=O ester), 1685s (C=O Fmoc), 1645s (C=O amide), 1505, 730; δ_H(300 MHz, $CDCl_3$) 2.77 (1 H, dd, *J* 4.1 and 15.4, CHCH_2C(O)NH_2) and 2.97 (1 H, dd, *J* 4.1 and 15.5, CHCH_2C(O)NH_2), 3.77 (3 H, s, CH_3Ph), 4.19–4.22 (1 H, m), 4.28–4.34 (1 H, m), 4.38–4.42 (1 H, m), 4.44–4.63 (1 H, m, CHCH_2C(O)NH_2), 5.14 (2 H, s, CH_3OPhCH_2), 5.37 and 5.51 (2 H, br s, CONH_2), 6.07 (1 H, d, *J* 7.9, N*H* Fmoc), 6.84 (2 H, d, *J* 7.6, Ar), 7.26–7.32 (4 H, m, Ar), 7.40 (2 H, t, *J* 7.5, Ar), 7.58 (2 H, d, *J* 7.1 Ar), 7.76 (2 H, d, *J* 7.4 Ar); *m*/*z* (EI) 473 (M^+ − H, 0.02%), 217 (2), 196 (2), 178 (100), 165 (19), 121 (37).

(*S*)-N^{α}-(Fluoren-9-ylmethoxycarbonyl)glutamine 4-methoxybenzyl ester (11)

This was prepared from N^{α}-(fluoren-9-ylmethoxycarbonyl)-glutamine **9** (5.0 g, 13.6 mmol) using the procedure outlined for the preparation of ester **10**, to give the title compound (6.10 g, 93%) as a colourless solid, mp 172–174 °C (Found: C, 68.6; H, 5.7; N, 5.8. $C_{28}H_{28}N_2O_6$ requires: C, 68.8; H, 5.8; N, 5.7%); ν_{max}/cm^{-1} 3490, 3420, 3200, 1790s (C=O ester), 1685s (C=O Fmoc), 1645s (C=O amide), 1505, 1170, 750, 730; δ_H(400 MHz, $CDCl_3$) 2.01 (1 H, m, CHC(*H*)CH$_2$C(O)NH_2), 2.20 (3 H, m, CHC(H)-*H*CH_2C(O)NH_2), 3.78 (3 H, s, CH_3OPh), 4.20 (1 H, m, C*H* Fmoc), 4.40 (3 H, m, C*H*CH$_2$CH$_2$C(O)NH_2 and CH_2 Fmoc), 5.11 (2 H, s, CH_3OPhCH_2), 5.66 (1 H, d, *J* 7.5, N*H*-Fmoc), 5.70 (1 H, m, NH_2), 6.86 (2 H, d, *J* 8.5, Ar), 7.32–7.26 (4 H, m, Ar), 7.48 (2 H, t, *J* 7.5, Ar), 7.57 (2 H, d, *J* 7.5, Ar), 7.76 (2 H, d, *J* 7.5, Ar); δ_C(100 MHz, $CDCl_3$) 28.4, 31.6, 47.2, 53.4, 55.3, 67.0, 67.2, 114.0, 119.9, 125.0, 127.0, 127.3, 127.7, 130.2, 141.3, 143.6, 143.8, 156.7 (*C*=O ester), 159.9 (*C*=O amide), 171.7 (*C*=O urethane); *m*/*z* (FAB) 488 (M^+ − H, 0.02%), 350 (1), 275 (2), 196 (100), 178 (96), 165 (40), 121 (65).

(*S*)-N^{α}-(Fluoren-9-ylmethoxycarbonyl)-γ-thioasparagine 4-methoxybenzyl ester (12)

A mixture of amide **10** (0.20 g, 0.4 mmol) and phosphorus pentasulfide (0.08 g, 0.2 mmol) in tetrahydrofuran (10 ml) was irradiated with ultrasound under an argon atmosphere for 1 h, care being taken to maintain the temperature at ≤40 °C. Following concentration to a volume of approximately 2 ml *in vacuo*, the mixture was filtered to remove any precipitate. The filtrant was washed with small portions of hot chloroform, which were combined with the tetrahydrofuran filtrate and purified directly by flash column chromatography (CH_2Cl_2–pentane–EtOAc; 3:3:2) to give the title compound (0.18 g, 88%) as a colourless solid, mp 136–137 °C; $[\alpha]_D^{30}$ −7 (*c* 0.3 in chloroform) (Found: C, 66.05; H, 5.4; N, 5.7; S, 6.6. $C_{27}H_{26}N_2O_5S$ requires: C, 66.11; H, 5.3; N, 5.7; S, 6.5%); ν_{max}/cm^{-1} 3330, 3200, 1730s (C=O ester), 1690s (C=O Fmoc), 1610, 1510, 750, 730; δ_H(300 MHz, $CDCl_3$) 3.20–3.23 (2 H, m, CHCH_2C(S)NH_2), 3.76 (3 H, s, CH_3OPh), 4.17 (1 H, t, *J* 6.7, C*H* Fmoc), 4.37 (2 H, d, *J* 6.5, CH_2 Fmoc), 4.65–4.73 (1 H, m, C*H*CH$_2$C(S)NH_2), 5.12 (2 H, s, CH_3OPhCH_2), 6.13 (1 H, d, *J* 7.0, N*H* Fmoc), 6.83 (2 H, d, *J* 8.5, Ar), 7.26–7.32 (4 H, m, Ar), 7.40 (2 H, t, *J* 7.3, Ar), 7.57 (2 H, d, *J* 7.3, Ar), 7.76 (2 H, d, *J* 7.4, Ar); *m*/*z* (EI) 490 (M^+, 0.03%), 352 (0.3), 178 (100), 165 (28), 121 (30).

(*S*)-N^{α}-(Fluoren-9-ylmethoxycarbonyl)-δ-thioglutamine 4-methoxybenzyl ester (13)

This was prepared from ester **11** (1.0 g, 2.05 mmol) using the method described above for the preparation of thioamide **12**, yielding the title compound (0.86 g, 88%) as a colourless solid, mp 134–136 °C (Found: C, 66.6; H, 5.5; N, 5.5; S, 6.4. $C_{28}H_{28}N_2O_5S$ requires: C, 66.7; H, 5.6; N, 5.6; S, 6.4%); ν_{max}/cm^{-1} 3330, 3200, 1730s (C=O ester), 1690s (C=O Fmoc), 1610, 1510, 750, 730; δ_H(300 MHz, $CDCl_3$) 2.05–2.01 (1 H, m, CHC(*H*)HCH$_2$C(O)NH_2), 2.39–2.35 (1 H, m, CHC(H)*H*CH$_2$-C(O)NH_2), 2.60–2.57 (1 H, m, CHCH$_2$C(*H*)HC(O)NH_2), 2.67–2.63 (1 H, m, CHCH$_2$C(H)*H*C(O)NH_2), 3.79 (3 H, s, CH_3OPh), 4.19 (1 H, m, C*H* Fmoc), 4.47 (3 H, m, C*H*CH$_2$CH$_2$C(O)NH_2 and CH_2 Fmoc), 5.12 (2 H, s, CH_3OPhCH_2), 5.59 (1 H, d, *J* 7.5, N*H* Fmoc), 6.87 (2 H, d, *J* 8.5, Ar), 7.50–7.25 (6 H, m, Ar), 7.57 (3 H, m, NH_2 and Ar), 7.76 (2 H, d, *J* 7.5, Ar), 7.94 (1 H, m, NH_2); δ_C(100 MHz, $CDCl_3$) 32.9, 40.7, 47.1, 52.9, 55.3, 67.1, 67.5, 114.0, 120.0, 124.9, 127.05, 127.1, 127.8, 130.3, 141.2, 143.7, 146.6, 159.8 (*C*=O ester), (*C*=O Fmoc), 171.5, 209.0 (*C*=S); *m*/*z* (FAB): 504 (M^+, 0.03%), 383 (1), 326 (2), 265 (21), 178 (66), 121 (100).

(*S*)-N^{α}-(Fluoren-9-ylmethoxycarbonyl)-γ-thioasparagine (1)

Thioamide **12** (0.158 g, 0.32 mmol) was treated with a mixture of thioanisole (0.1 ml) and ethane-1,2-dithiol (0.1 ml) in trifluoroacetic acid (1 ml) for 10 min at room temperature. Following the addition of a diethyl ether–pentane mixture (1:1; 15 ml), the mixture was left to stand for 3.5 h at 0 °C and then centrifuged at 3000 rpm for 5 min. After removal of the supernatant by decantation, the remaining colourless precipitate was washed with ice-cold diethyl ether (2 × 2 ml) and recrystallised from ethyl acetate–petrol (bp 80–100 °C) to give the title compound (0.108 g, 91%) as a colourless solid, mp 156–159 °C; $[\alpha]_D^{30}$ −25 (*c* 0.1 in ethanol) (Found: C, 61.35; H, 4.7; N, 7.3. $C_{19}H_{18}N_2O_4S$ requires: C, 61.61; H, 4.9; N, 7.6%); λ_{max} (EtOH)/nm 318 ($\log_{10}\varepsilon$ 1.86), 299 (3.77), 286 (3.87), 264 (4.42); ν_{max}/cm^{-1} 3300, 3140, 1690s, 1525s, 1260s, 1055, 755, 740s; δ_H(300 MHz, $(CD_3)_2SO$) 2.79 (1 H, dd, *J* 5.0 and 14.0, CHC(*H*)HC(S)NH_2), 2.90 (1 H, dd, *J* 5.0 and 14.2, CHC(H)*H*C(S)NH_2), 4.21–4.27 (3 H, m, C*H* and CH_2 Fmoc), 4.63–4.65 (1 H, m, C*H*CH$_2$C-(S)NH_2), 7.32 (2 H, t, *J* 6.8, Ar), 7.41 (2 H, t, *J* 7.3, Ar), 7.62 (1 H, d, *J* 8.1, N*H* Fmoc), 7.69 (2 H, d, *J* 7.0, Ar), 7.88 (2 H, d, *J* 7.5, Ar), 9.16 and 9.52 (2 H, br s, NH_2); *m*/*z* (EI) 371 (M^+ + H, 50%), 332 (4), 191 (9), 179 (100), 121 (30).

(*S*)-N^{α}-(Fluoren-9-ylmethoxycarbonyl)-δ-thioglutamine (2)

This was prepared from ester **13** (1.20 g, 2.4 mmol) using the method described above for the preparation of thioamide **1** to give the title compound (0.89 g, 67%) as a colourless, amorphous solid, mp 152–154 °C (Found: C, 62.5; H, 5.4; N, 7.3. $C_{20}H_{20}N_2O_4S$ requires: C, 62.5; H, 5.2; N, 7.3%); ν_{max}/cm^{-1} 3300, 3140, 1690s (C=O Fmoc), 1525s, 1325s, 1260s, 1055, 755, 740s; δ_H(300 MHz, ($CDCl_3$) 2.10–2.02 (1 H, m, CHC(*H*)-$HCH_2C(O)NH_2$), 2.41–2.36 (1 H, m, CHC(H)*H*$CH_2C(O)$-NH_2), 2.61–2.57 (1 H, m, $CHCH_2C(H)HC(O)NH_2$), 2.69–2.65 (1 H, m, $CHCH_2C(H)HC(O)NH_2$), 4.19 (1 H, m, C*H*-Fmoc), 4.71–4.3 (3 H, m, C*H*$CH_2CH_2(O)NH_2$ and CH_2 Fmoc), 7.76–7.25 (8H, m, Ar-Fmoc); *m*/*z* (FAB) 384 (M^+, 14%), 352 (14), 298 (21), 178 (100), 166 (90), 121 (82).

(*S*)-N^{α}-(Fluoren-9-ylmethoxycarbonyl)-N^{γ}-xanthen-9-yl-γ-thioasparagine (15)

A solution of thioamide **1** (0.72 g, 1.9 mmol) and 9-hydroxyxanthene (0.77 g, 3.9 mmol) in acetic acid (25 ml) was heated under argon at 85 °C for 110 min. Following removal of the solvent *in vacuo*, the residue was triturated with diethyl ether (45 ml) and left for 30 min at room temperature, producing the crude product as a colourless precipitate. Following removal of the diethyl ether by decantation, the residue was washed with diethyl ether (3 × 20 ml) and re-crystallised from chloroform to give the title compound (0.63 g, 60%) as a colourless solid, mp 157–159 °C; $[\alpha]_D^{30}$ −5 (*c* 0.3 in *N*,*N*-dimethylformamide) (Found: C, 70.04; H, 5.01; N, 4.8. $C_{32}H_{26}N_2O_5S$ requires: C, 69.80; H, 4.76; N, 5.1%); ν_{max}/cm^{-1} 3360, 3310, 1730s, 1690s, 1450s, 1260s, 1140, 755, 745; δ_H(300 MHz, $(CD_3)_2SO$) 2.90–2.99 (1 H, m, CHC(*H*)HC-(O)NH), 3.12–3.21 (1 H, m, CHC(H)*H*C(O)NH), 4.02–4.08 (1 H, m, C*H* Fmoc), 4.16–4.25 (2 H, m, CH_2 Fmoc), 4.73–4.76 (1 H, m, C*H*CH_2C(O)NH), 7.00–7.41 (13 H, m, Ar, N*H* Fmoc and N*H* amide), 7.56 (2 H, d, *J* 8.1, Ar), 7.70 (2 H, d, *J* 7.3, Ar), 7.88 (2 H, d, *J* 7.3, Ar), 11.23 (1 H, br, CO_2H); *m*/*z* (EI) 550 (M^+, 0.04%), 334 (4), 309 (4), 181 (100), 165 (26).

2-(4-Thiobenzoylaminophenyl)acetic acid (21)

S-Carboxymethyl dithiobenzoate **18**[11b] (2.12 g, 1.0 mmol) was added to a solution of 2-(4-aminophenyl)acetic acid (1.5 g, 1.0 mmol) in aqueous sodium hydroxide (1 M, 5 ml) and the mixture stirred at room temperature for 6 h. The mixture was then acidified to pH 3 by the careful addition of concentrated hydrochloric acid, and extracted with ethyl acetate (2 × 20 ml). The combined organic extracts were dried ($MgSO_4$), filtered, and concentrated *in vacuo*. Purification of the crude product by flash column chromatography ($CHCl_3$–EtOAc–EtOH; 6:2:2) gave the title compound (1.85 g, 70%) as a yellow solid, mp 171–172 °C (Found: C, 66.3; H, 4.7; N, 5.2; S, 11.9. $C_{15}H_{13}NO_2S$ requires: C, 66.4; H, 4.8; N, 5.2; S, 11.8%); λ_{max} (MeOH)/nm 320 (ε/dm^3 mol^{-1} cm^{-1} 121), 290 (95); ν_{max}/cm^{-1} 3200–2800, 1700s (C=O), 1520, 860s; δ_H(400 MHz, $(CD_3)_2SO$) 3.72 (2 H, s, CH_2), 7.44 (2 H, d, *J* 8.5, Ar), 7.59 (2 H, t, *J* 7.0, Ar), 7.64 (1 H, m, Ar), 7.88 (2 H, d, *J* 8.5, Ar), 7.95 (2 H, d, *J* 7.0, Ar), 11.82 (1 H, br s, N*H*); δ_C(100 MHz, $(CD_3)_2SO$) 44.26, 127.9, 131.3, 131.9, 133.4, 135.8, 136.9, 142.5, 146.6, 174.5 (*C*=O), 201.4 (*C*=S); *m*/*z* 271 (M^+, 48%), 238 (30), 224 (27), 168 (8), 121 (100).

(*S*)-2-(Fluoren-9-ylmethoxycarbonylamino)-3-(4-thiobenzoyl-aminophenyl)propionic acid (3)

This was prepared from *S*-carboxymethyl dithiobenzoate **18**[11b] (0.052 g, 0.25 mmol) and 3-(4-aminophenyl)-2-(fluoren-9-ylmethoxycarbonylamino)propionic acid **16**[10] (0.1 g, 0.25 mmol) using the procedure described above for the preparation of thioamide **21** to give the title compound 0.86 g (74%) as a bright yellow solid, mp 145–146 °C. Full physical data have been previously described.[14]

(*S*)-2-(Fluoren-9-ylmethoxycarbonylamino)-3-[4-(2-phenylthio-acetylamino)phenyl]propionic acid (4)

This was prepared from *S*-carboxymethyl phenyldithioacetate **19**[11a] (0.056 g, 0.25 mmol) and 3-(4-aminophenyl)-2-(fluoren-9-ylmethoxycarbonylamino)propionic acid **16**[10] (0.1 g, 0.25 mmol) using the procedure described above for the preparation of thioamide **21** to give the title compound 0.86 g (74%) as a light yellow solid, mp 193 °C. Full physical data have been previously described.[14]

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