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Solid-Phase Synthesis of Peptidyl Thioacids Employing a 9-Fluorenylmethyl Thioester-Based Linker in Conjunction with Boc Chemistry

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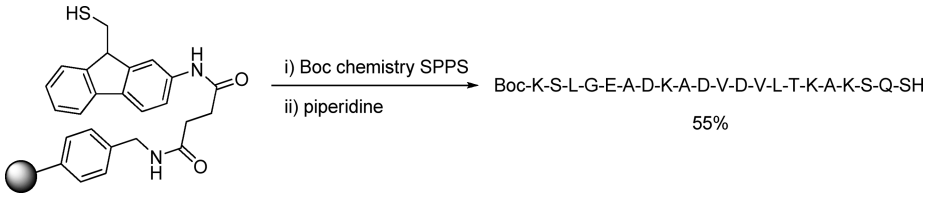

A method for the synthesis of peptidyl thioacids is described on the basis of the use of the *N*-[9-(thiomethyl)-9*H*-fluoren-2-yl]succinamic acid and cross-linked aminomethyl polystyrene resin. The method employs standard Boc chemistry SPPS techniques in conjunction with 9-fluorenylmethyloxycarbonyl protection of side-chain alcohols and amines and 9-fluorenylmethyl protection of side-chain acids and thiols. Cleavage from the resin is accomplished with piperidine, which also serves to remove the side-chain protection and avoids the HF conditions usually associated with the resin cleavage stage of Boc chemistry SPPS. The so-obtained thioacids are converted to simple thioesters in high yield by standard alkylation according to well-established methods.

Introduction

Thioacids [RC(=O)SH][1] are a fascinating but underappreciated class of compounds with a unique reactivity profile[2] that arises in part from their pK_a and the consequent ability of the conjugate base to act in a highly selective manner as nucleophile in aqueous media at pH 3−6. Not surprisingly, therefore, most applications of thioacids have been in the field of peptide chemistry where they have been employed in amide bond forming reactions either directly[3] or indirectly through their facile conversion to thioesters, key intermediates in a variety of chemical and enzymic amide ligation processes.[4]

The instability of thioesters toward the typical conditions of 9-fluorenylmethyloxycarbonyl (Fmoc) chemistry solid-phase peptide synthesis (SPPS), particularly the treatment

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with organic bases employed in the cleavage of Fmoc groups, led to an initial reliance on *tert*-butyloxycarbonyl-based (Boc) chemistry for the synthesis of peptidyl thioesters,[4o,q,5] but the HF conditions typically required for cleavage from the resin following Boc chemistry SPPS limit the use of this chemistry. Fmoc chemistry-based methods have subsequently been developed that replace piperidine in the Fmoc removal step by cocktails of 1-methylpyrrolidine, hexamethylenimine, and 1-hydroxybenzotriazole, but it has been found that these methods are prone to racemization at the thioester position.[6] The backbone amide linker (BAL) strategy enables Fmoc chemistry SPPS with subsequent incorporation of a C-terminal thioester prior to cleavage from the resin but requires careful control of reaction conditions to avoid epimerization on introduction of the thioester to the C-terminal end of the peptide chain.[7] To circumvent these problems, numerous methods have been developed according to which, after completion of the peptide synthesis by Boc or Fmoc methods, the linker to the resin is activated in such a way as to permit its displacement by a thiol or thiolate resulting in the liberation of the peptide in the form of the desired thioester or thioacid.[8] A variant on the BAL strategy, which avoids the epimerization problem, carries a C-terminal trithioorthoester through the Fmoc chemistry SPPS sequence before converting it to the required thioester by controlled hydrolysis.[9] More recently, a number of strategies have been developed in which thioesters are generated by O−S or N−S shifts of mercapto esters and mercapto amides following unmasking of a protected thiol group.[10] Despite the considerable ingenuity that has been deployed in the development of the above methods, none combine the directness that obviously results from the use of a simple C-terminal thioester-based linker with a method for release from the resin that avoids the use of HF. Previously, we introduced the 9-fluorenylmethyl thioesters from which thioacids are liberated by simple treatment with piperidine, that is, under the conditions usually employed for the cleavage of Fmoc groups in Fmoc chemistry SPPS.[11] We conceived that a linker based on the 9-fluorenylmethyl thioester would be compatible with the general conditions of Boc chemistry SPPS and that following peptide assembly treatment of the resin with piperidine would release the Boc-protected peptide into solution in the form of a C-terminal thioacid that could be readily transformed into a thioester by simple alkylation. Of essence, this method, whose reduction to practice we report here, employs conditions no more forcing than those encountered in standard Boc and Fmoc chemistry SPPS protocols and circumvents the terminal HF treatment that limits most Boc chemistry SPPS methods. We further conceived that the utility of this method would be enhanced by the application of side-chain protection strategies involving either a third orthogonal system enabling retention of side-chain protecting groups post cleavage[12] or a system according to which all protecting groups would be removed concomitantly with cleavage of the thioacid from the resin, depending on the ultimate application envisaged for the peptidyl thioacid.

Results and Discussion

The mercapto functionalized linker, *N*-[9-(tritylthiomethyl)-9*H*-fluoren-2-yl]succinamic acid (**5**), was prepared from commercially available 9*H*-fluoren-2-amine as shown in Scheme 1. The synthesis began with the conversion of 9*H*-fluoren-2-amine to the corresponding hydroxyl functional compound **1** following a literature procedure[13] involving formylation followed by reduction. Tosylation of **1** under standard conditions gave the sulfonate **2**, from which the amine **3** was liberated with trifluoroacetic acid. Reaction of **3** with succinic anhydride provided the hemisuccinate **4** that was subjected to treatment with tritylmercaptan and Hunig's base to give the protected linker **5** in excellent yield (Scheme 1). Treatment of **5** with diisopropyl carbodiimide and *N*-hydroxybenzotriazole in DMF gave an activated intermediate that was allowed to react with 1% divinylbenzene cross-linked aminomethylpoystyrene resin (0.41 mmol/g loading). Following washing with DMF, the functionalized resin was exposed to a 50% solution of TFA in dichloromethane to yield the desired resin-bound 9-fluorenylmethylthiol derivative **6** (Scheme 1). The attachment of linker **5** to the aminomethylpolystyrene resin was also accomplished in a satisfactory manner with the *O*-benzotriazolyltetramethyluronium hexafluorophosphate (HBTU) reagent[14] with the aid of diisopropylethylamine as base.

The preparation of a series of suitably protected amino acids was then undertaken. Thus, *N*-*tert*-butoxycarbonyl-L-serine and

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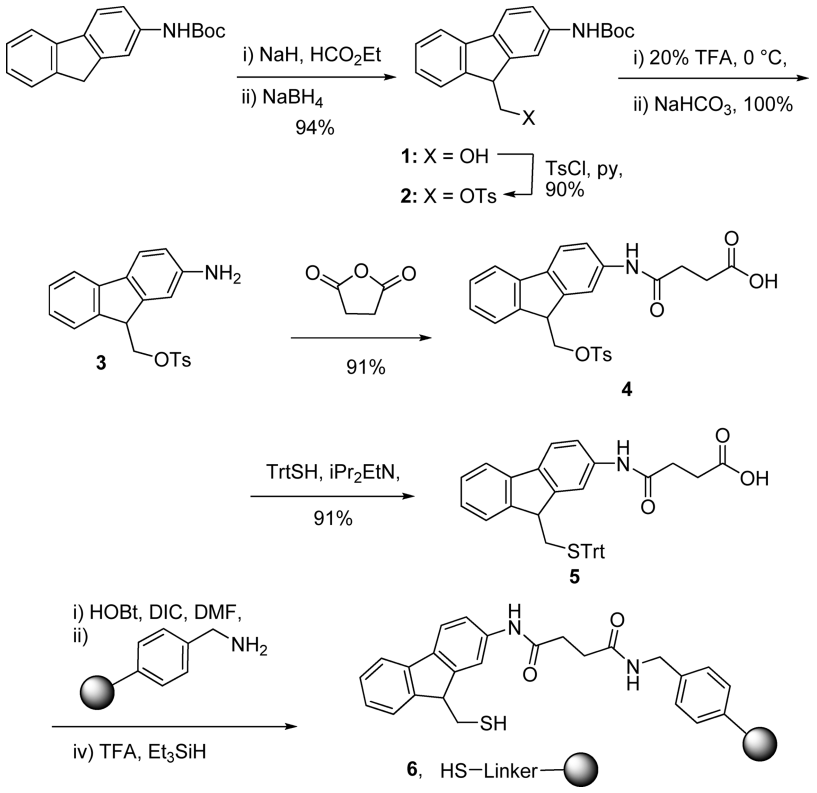
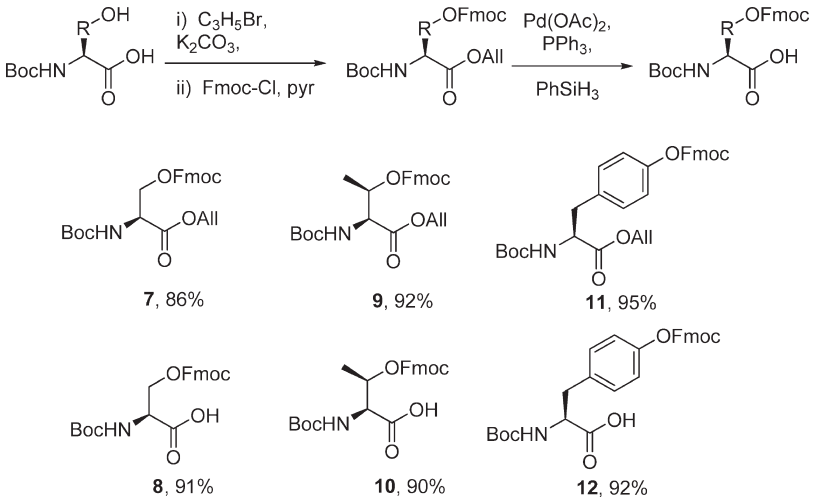
SCHEME 1. Preparation of a Functionalized Resin



SCHEME 2. Preparation of Protected Hydroxy Amino Acids



the analogous L-threonine and L-tyrosine derivatives were converted to their allyl esters with potassium carbonate and then to the 9-fluorenylmethyl carbonates with Fmoc chloride and pyridine. A combination of palladium(II) acetate, triphenylphosphine, and phenylsilane was the reagent of choice for the liberation the allyl esters required to complete this short sequence (Scheme 2).

Following a literature protocol,[15] treatment of powdered L-aspartic and L-glutamic acids with triethylborane in THF at reflux for 24 h, then with 9-fluorenylmethanol, dicyclohexylcarbodiimide, and 4-dimethylaminopyridine, and finally with gaseous hydrogen chloride gave the mono esters **13** and **14**. These HCl salts were then converted to the *N*-Boc derivatives in the standard manner (Scheme 3).

The corresponding mono allyl esters **17** and **18** were accessed according to the literature method,[16] as were the 9-fluorenylmethyl thioether of Boc-L-cysteine **19**,[15] the

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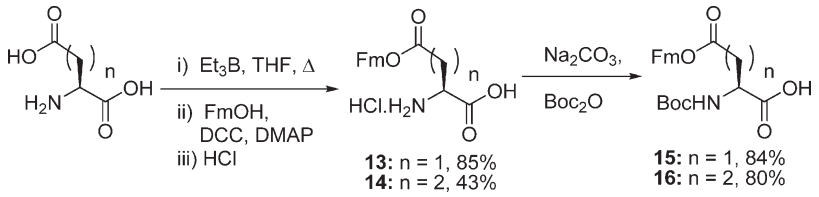
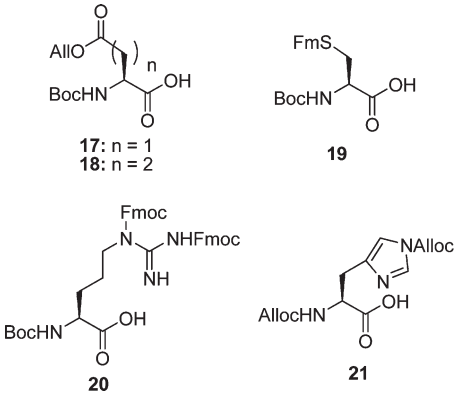
SCHEME 3. Preparation of Mono-9-fluorenylmethyl Esters of Aspartic and Glutamic Acid



Fmoc-protected L-arginine derivative **20**,[17] and Alloc-protected L-histidine **21**.[18]



With all building blocks in hand, attention was turned to SPPS using standard Boc techniques with DIC/HOBt as the coupling agent and TFA to liberate the N-terminus of the growing chains from their Boc derivatives. A number of peptides were assembled in this manner as shown in Table 1 (entries 1−5). As with the preparation of the resin-bound thiol **6** (Scheme 1), this methodology is not limited to carbodiimide chemistry but is perfectly adaptable to the other methods as evidenced by the application of the HBTU protocol (Table 1, entry 6). After completion of the on-resin procedure, treatment with a solution of piperidine in DMF or, to enable direct loading of the reaction mixture to the HPLC column, acetonitrile released the desired thioacids protected at the N-terminal ends in the form of the Boc derivatives,[19] which were typically obtained with a high degree of purity as determined by ESI-TOF and HPLC methods. Similar treatment of individual beads was used to systematically monitor the individual reaction steps during the course of the peptide assembly sequence. Although mass spectrometry was the method of choice for monitoring these SPPS reactions, the Kaiser ninhydrin test also performed in a perfectly satisfactory manner for both the coupling and Boc removal steps. While the thioacid **22** was a simple model tetrapeptidyl thioacid (Table 1, entry 1), the peptidyl thioacids **23**, **24**, **25**, and **27** are all natural sequences. The sequences of thioacids **23** and **24** (Table 1, entries 2 and 3) were selected from the Glucagon-like peptide-1 (GLP-1)[20] and represent segments 7−16 and 17−26, respectively, of that peptidyl hormone. Peptide **25** (Table 1, entry 4) represents the 94−101 segment of Human Secretory Phospholipase A_2 (hsPLAA$_2$),[21] and peptide **27** (Table 1, entry 5) is the 65−84 unit of Human Parathyroid Hormone (hPTH).[22]

The ^{1}H and ^{13}C NMR spectra of the crude tetrapeptidyl thioacid **22** as obtained on simple release from the resin, acidification with HCl, and drying are presented in the Supporting Information (Figures 1 and 2) to illustrate the high degree of purity typically obtained by this method. In a similar vein, the ESI-TOF mass spectrum of the peptidyl thioacid **27**, immediately after release from the resin and prior to purification by HPLC is presented in the Supporting Information as Figure 3.

Particular attention is drawn to entries 4 and 5 of Table 1 in which the C-terminal amino acid is asparagine and glutamine, respectively. The amino acid building blocks for these residues were employed without protection of the side-chain amide functionality, and it is noteworthy that cyclization of these amides onto the resin-bound thioester with premature peptide release in the form of an imide did not occur to any significant extent.[23] In general, the strategy of employing Fmoc protection for side-chain amines and hydroxyl groups, coupled with the protection of side-chain carboxylates and thiols, ensures clean chemistry while eliminating the need for extra deprotection steps pre- or post-cleavage of the peptidyl thioacid from the resin. Nevertheless, should the retention of side-chain protection be required on cleavage from the resin, this may be conveniently accomplished through the use of building blocks whose side chains are covered with either the allyl or allyloxycarbonyl system[12] depending on the case (Table 1, entries 2 and 3).

By way of example, two of the peptidyl thioacids obtained in this manner were converted to the corresponding *S*-benzyl thioesters by simple alkylation with benzyl bromide and *sym*-collidine in DMF (Scheme 4).[5c]

Finally, as a demonstration of the broad scope of the chemistry of thioacids, a single decapeptidyl thioacid was subjected to reaction with a sulfonyl azide, under conditions described by the Williams and Liskamp groups[24] for much

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(23) This is evident simply from the yields of the isolated thioacids **25** and **27**, which require average minimum coupling yields of >93% and >97%, respectively, for each coupling deprotection cycle. For comparable reasons premature peptide cleavage by diketopiperazine formation at the level of deprotection of the dipeptide and the migration of side-chain protecting groups to N-terminal amines on neutralization do not appear to be major concerns, at least for the examples provided.

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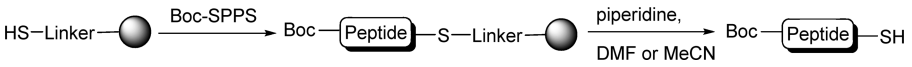
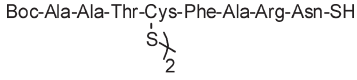
TABLE 1. Boc Chemistry SPPS of Peptidyl Thioacids[a]



Entry	Resin bound peptide	Peptide α-thioacid	% Yield[b]
1	Boc-Met-Ala-Val-Ala-S-linker-	Boc-Met-Ala-Val-Ala-SH (**22**)	95[c]
2	Alloc-His(Alloc)-Ala-Glu(OAll)-Gly-Thr(OFmoc)-Phe-Thr(OFmoc)-Ser(OFmoc)-Asp(OAll)-Val-S-linker-	Alloc-His-Ala-Glu(OAll)-Gly-Thr-Phe-Thr-Ser-Asp(OAll)-Val-SH (**23**)	80[c,d]
3	Boc-Ser(OFmoc)-Ser(OFmoc)-Tyr(OFmoc)-Leu-Glu(OAll)-Gly-Gln-Ala-Ala-Lys(Alloc)-S-linker-	Boc-Ser-Ser-Tyr-Leu-Glu(OAll)-Gly-Gln-Ala-Ala-Lys(Alloc)-SH (**24**)	78[c]
4	Boc-Ala-Ala-Thr(OFmoc)-Cys(Fm)-Phe-Ala-Arg(Fmoc)$_2$-Asn-S-Linker-	Boc-Ala-Ala-Thr-Cys-Phe-Ala-Arg-Asn-SH (**25**)	57[e,f,g]
5	Boc-Lys(Fmoc)-Ser(OFmoc)-Leu-Gly-Glu(OFm)-Ala-Asp(OFm)-Lys(Fmoc)-Ala-Asp(OFm)-Val-Asp(OFm)-Val[8]-Leu-Thr(OFmoc)-Lys(Fmoc)-Ala-Lys(Fmoc)-Ser(OFmoc)-Gln-S-Linker-	Boc-Lys-Ser-Leu-Gly-Glu-Ala-Asp-Lys-Ala-Asp-Val-Asp-Val[8]-Leu-Thr-Lys-Ala-Lys-Ser-Gln-SH (**27**)	55[f,h]
6	Boc-Met-Ala-Val-Ala-S-linker-	Boc-Met-Ala-Val-Ala-SH (**22**)	88[c,i]

[a]All reactions used ~0.1 mmol of aminomethyl polystyrene resin (244 mg, resin loading 0.41 mmol/g). All cleavage steps used 25% TFA in CH_2Cl_2 (2 × 1.5 mL, 2 × 30 min) after which the resin was washed with CH_2Cl_2 (3 × 2 mL) and neutralized with 5% DIPEA in CH_2Cl_2 (5 mL). All coupling reactions with the exception of entry 6[i] used Boc-L-amino acids (0.4 mmol) preactivated with HOBt (54 mg, 0.4 mmol) and DIC (62 μL, 0.4 mmol) in DMF (1 mL) for 30 min. The preactivated amino acid was added to the resin with an additional DMF (1 mL) and shaken for 3 h. After coupling, the resin was washed with DMF (3 × 2 mL) and CH_2Cl_2 (3 × 2 mL). [b]With the exception of **22**, which required no purification, yields are for compounds isolated and purified by RP-HPLC and are based on the substitution level of the aminomethyl polystyrene resin, taking into account the aliquots removed for monitoring of reaction progress. [c]The peptidyl thioacid was released from the resin by treatment with 20% piperidine in DMF for 20 min. [d]The Alloc group was cleaved from the side chain of the histidine residue in the course of the treatment with piperidine. [e]After incorporation of cysteine into the peptide sequence, triethylsilane (0.5%) was included in the TFA/CH_2Cl_2 solution used for Boc-removal in all subsequent coupling steps. [f]The peptidyl thioacid was released from the resin by the treatment with 50% piperidine in acetonitrile for 30 min. Directly analogous results could be obtained with piperidine in DMF, but the use of acetonitrile enables the reaction mixture to be loaded directly onto the HPLC for purification. [g]In addition to the desired peptide α-thioacid **25**, 15% of a disulfide **26** corresponding to oxidative dimerization of thioacid was isolated by RP-HPLC. This structure is written as a symmetric cystine derivative rather than as the alternative diacyl sulfide as no such dimers were seen in any of the other examples. [h]After introduction of the eighth amino acid in the sequence and the removal of the Boc group it was found necessary to conduct the neutralization with a 5% solution of DIPEA in *N*-methylpyrrolidine. All subsequent coupling and deprotection steps in this sequence also employed *N*-methylpyrrolidine as solvent.[25] [i]This reaction sequence employed HBTU and *i*Pr_2NEt for the coupling reactions in place of DIC/HOBt.



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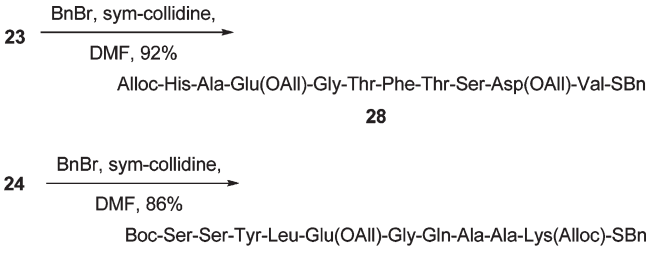
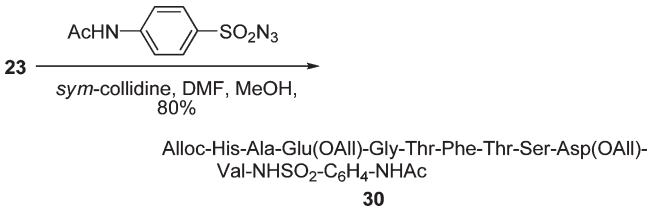
SCHEME 4. Peptidyl Thioester Synthesis



simpler substrates, resulting in the isolation of a C-terminal sulfonylamide (Scheme 5).

(25) Fields, G. B.; Fields, C. G. *J. Am. Chem. Soc.* **1991**, *113*, 4202–4207.

SCHEME 5. Formation of a Sulfonylamide



Overall, we describe the successful implementation of a straightforward method for the SPPS of peptidyl thioacids using standard Boc chemistry with release from the resin using conditions typically used for Fmoc removal during the course of Fmoc chemistry SPPS. In conjunction with the protection of side chain amino and hydroxyl groups as Fmoc carbonates, and of side-chain acids and thiols as 9-fluorenyl-

methyl esters and thioethers this chemistry provides a very convenient and mild means of access to peptidyl thioacids and, by simple alkylation, of their *S*-esters.

Experimental Section

General Methods. Unless otherwise stated, ^{1}H and ^{13}C NMR were recorded in $CDCl_3$ solution and optical rotations in $CHCl_3$ solutions. All organic extracts were dried over sodium sulfate and concentrated under aspirator vacuum. Chromatographic purifications were carried out over silica gel. All peptide thioacid syntheses were carried out on a 0.1 mmol scale employing 1% DVB cross-linked aminomethyl polystyrene resin (244 mg, resin loading 0.41 mmol/g) in a 10 mL manual synthesizer glass reaction vessel with a Teflon-lined screw cap. The peptide resin was shaken during both N^{α}-*tert*-butoxycarbonyl deprotection and coupling steps. After each coupling step, formation of the desired peptide thioacid was confirmed by cleavage of a small amount (~5 mg) of resin using a 20% solution of piperidine in DMF for 20 min, followed by examination by ESI-TOF mass spectrometry. Isolated yields were determined on the basis of the theoretical yield calculated for the use of 0.1 mmol of resin with a loading of 0.41 mmol/g. These yields take no account of the aliquots removed for monitoring and are therefore minimum yields.

[2-(*tert*-Butoxycarbonylamino)-9*H*-fluoren-9-yl]methyl 4-Methylbenzenesulfonate (2). To a stirred solution of [2-(*tert*-butoxycarbonylamino)-9*H*-fluoren-9-yl]methanol[13] (1.8 g, 5.8 mmol) and 4-methylbenzenesulfonyl chloride (1.65 g, 8.7 mmol) in $CHCl_3$ (20 mL) was added pyridine (0.9 mL, 11.6 mmol) at 0 °C. The reaction mixture was stirred at room temperature for 6 h, and then the organic layer was washed with 1 M HCl, water, and brine, dried, and concentrated. Chromatographic purification using 30% ethyl acetate in hexane afforded **2** (2.42 g, 90%): yellowish syrup; ^{1}H NMR (500 MHz) δ 7.78−7.76 (d, J = 8.0 Hz, 2H), 7.66−7.61 (dd, J = 8.5, 12.8 Hz, 2H), 7.57 (s, 1H), 7.51−7.50 (d, J = 7.5 Hz, 1H), 7.38−7.35 (t, J = 7.0 Hz, 2H), 7.31−7.29 (d, J = 8 Hz, 2H), 7.25−7.22 (t, J = 7.5 Hz, 1H), 6.61 (s, 1H), 4.31−4.24 (m, 2H), 4.20−4.17 (t, J = 7.5 Hz, 1H), 2.43 (s, 3H), 1.56 (s, 9H); ^{13}C NMR (125 MHz) δ 153.0, 145.1, 143.7, 142.5, 141.3, 138.0, 136.5, 133.0, 130.1, 128.3, 128.2, 126.7, 125.3, 120.7, 119.8, 118.8, 115.6, 80.9, 72.0, 46.9, 28.6, 21.8; ESI-HRMS calcd for $C_{26}H_{27}NO_5S$ [M + Na]$^+$ 488.1508, found 488.1486.

(2-Amino-9*H*-fluoren-9-yl)methyl 4-Methylbenzenesulfonate (3). To a stirred solution of **2** (2.4 g, 5.2 mmol) in CH_2Cl_2 (16 mL) was added TFA (4 mL) dropwise at 0 °C. The reaction mixture was stirred at the same temperature for 20 min before it was neutralized at 0 °C by saturated aqueous $NaHCO_3$. The organic layer was washed with water and brine, dried, and concentrated. Chromatographic purification using 40% ethyl acetate in hexane afforded **3** (1.9 g, 100%): light yellow syrup; ^{1}H NMR (400 MHz) δ 7.78−7.76 (d, J = 8.4 Hz, 2H), 7.57−7.55 (d, J = 7.2 Hz, 1H), 7.50−7.48 (d, J = 8.0 Hz, 1H), 7.44−7.42 (d, J = 7.2 Hz, 1H), 7.34−7.29 (m, 3H), 7.17−7.13 (t, J = 6.4 Hz, 1H), 6.86 (s, 1H), 6.71−6.68 (dd, J = 1.6, 8.4 Hz, 1H), 4.26−4.18 (m, 2H), 4.13−4.09 (t, J = 7.2 Hz, 1H), 3.67 (br s, 2H), 2.41 (s, 3H); ^{13}C NMR (100 MHz) δ 146.4, 145.1, 144.7, 142.0, 141.7, 133.1, 132.3, 130.1, 128.2, 128.1, 125.7, 125.1, 121.1, 119.0, 115.2, 112.2, 72.5, 46.8, 21.9; ESI-HRMS calcd for $C_{21}H_{19}NO_3S$ [M + H]$^+$ 366.1164, found 366.1152.

***N*-[9-(Tosyloxymethyl)-9*H*-fluoren-2-yl]succinamic Acid (4).** To a stirred solution of **3** (1.8 g, 4.9 mmol) in THF (10 mL) was added succinic anhydride (590 mg, 5.9 mmol) portionwise over a period of 10 min at room temperature. The reaction mixture was stirred at room temperature for 1 h, then the reaction mixture was concentrated and subjected to chromatographic purification using 5% methanol in dichloromethane when it afforded **4** (2.1 g, 91%): white solid, crystallized from chloroform/hexane; mp 165.8−166.2 °C; ^{1}H NMR (400 MHz) δ 7.65−7.63 (m, 3H), 7.55−7.51 (m, 3H), 7.37−7.7.36 (d, J = 7.2 Hz, 1H), 7.26−7.2 (m, 4H), 7.14−7.11 (t, J = 7.2 Hz, 1H), 4.20−4.13 (m, 2H), 4.07−4.04 (t, J = 7.2 Hz, 1H), 2.67−2.62 (m, 4H), 2.32 (s, 3H); ^{13}C NMR (100 MHz) δ 175.6, 171.1, 145.3, 143.3, 142.4, 141.1, 137.7, 137.3, 132.5, 130.1, 128.2, 128.0, 126.8, 125.1, 120.5, 120.1, 120.0, 119.8, 116.8, 72.0, 46.8, 31.7, 29.4, 21.7; ESI-HRMS calcd for $C_{25}H_{23}NO_6S$ [M + Na]$^+$ 488.1144, found 488.1120.

***N*-[9-(Tritylthiomethyl)-9*H*-fluoren-2-yl]succinamic Acid (5).** To a stirred solution of **4** (2.0 g, 4.3 mmol) and triphenylmethanethiol (1.5 g, 5.4 mmol) in DMF (15 mL) was added diisopropylethylamine (1.8 mL, 10.8 mmol) at 0 °C. The reaction mixture was stirred at room temperature for 15 h, after which time the DMF was removed under high vacuum and the crude mixture was dissolved in EtOAc, washed with water and brine, dried, and concentrated. Chromatographic purification of the residue using 4% methanol in dichloromethane afforded **5** (2.23 g, 91%): light brown solid, crystallized from chloroform/hexane; mp 84−85 °C; ^{1}H NMR (500 MHz) δ 7.80 (br s, 1H), 7.57−7.53 (m, 4H), 7.43−7.41 (m, 6H), 7.31−7.24 (m, 8H), 7.21−7.7.17 (m, 4H), 3.57−3.54 (t, J = 7.0 Hz, 1H), 2.74−2.70 (m, 3H), 2.67−2.62 (m, 3H); ^{13}C NMR (125 MHz) δ 177.5, 170.5, 147.2, 146.1, 144.9, 140.5, 137.5, 136.8, 130.0, 128.2, 127.7, 127.0, 126.7, 124.9, 120.4, 119.9, 119.7, 116.9, 67.6, 47.2, 36.1, 32.0, 29.7; ESI-HRMS calcd for $C_{37}H_{31}NO_3S$ [M + Na]$^+$ 592.1922, found 592.1892.

Acknowledgment. We thank the NIH (GM62160) for support of this work.

Supporting Information Available: Complete experimental details, copies of the ^{1}H and ^{13}C NMR spectra of compounds **2**−**5**, **7**−**12**, and **22**−**30**, and HPLC traces and mass spectra of peptidyl thioacids **22**−**27**. This material is available free of charge via the Internet at http://pubs.acs.org.