Convenient Syntheses of Fluorenylmethyl-Based Side Chain Derivatives of Glutamic and Aspartic acids, Lysine, and Cysteine

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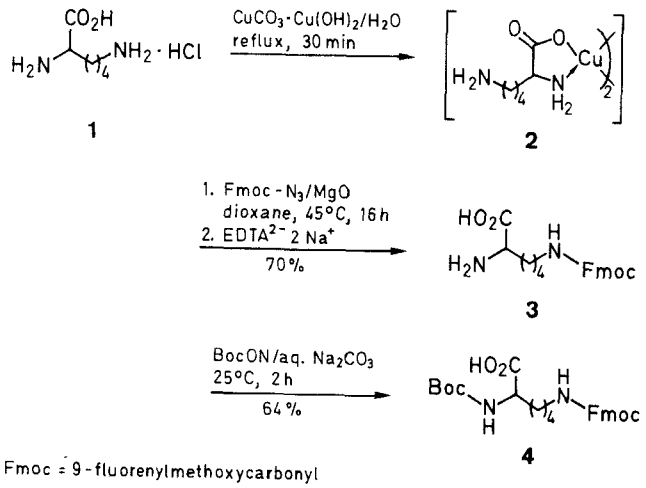
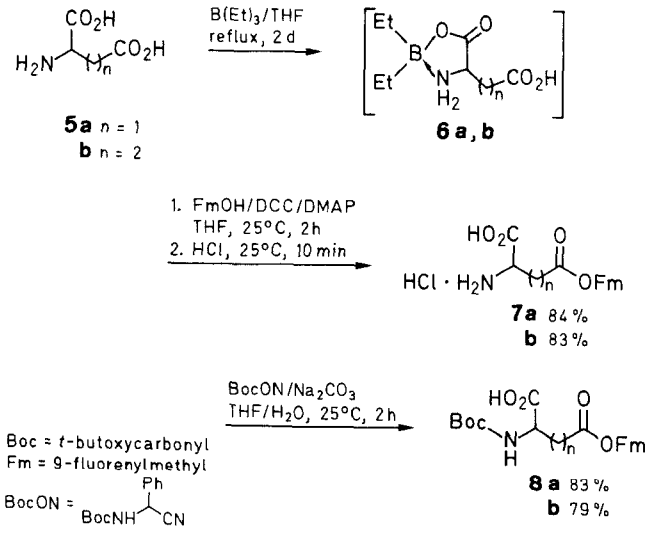
Efficient and practical one-pot syntheses of the fluorenylmethyl-based side chain derivatives of glutamic and aspartic acids, lysine, and cysteine are described. Likewise, stability/lability of these derivatives towards solvents and reagents used in solid phase peptide synthesis are discussed.

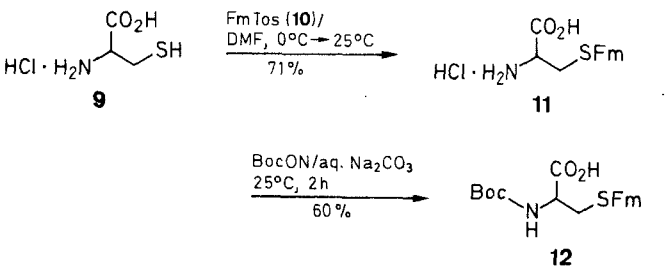
In this paper, we describe an efficient and practical one-pot syntheses of the fluorenylmethyl (Fm) based side chain derivatives of glutamic and aspartic acids, lysine, and cysteine, starting from the free amino acids. This type of protecting group, together with the *tert*-butoxycarbonyl (Boc)[2] for the α-amino function and the photocleavable *o*-nitrobenzamidobenzyl (Nbb)-resin,[3] exhibit three independent dimensions of orthogonality.[4] The stability of these derivatives, towards solvents and reagents used in solid-phase peptide synthesis, is also discussed.



The protection of amino acid side chains requires, preferably, stepwise protection of amino and carboxyl functions. In the case of lysine (**1**) this can be achieved by formation of a copper(II) complex **2**,[5] which is reacted with fluorenylmethoxycarbonyl azide (Fmoc-azide)[6] and decomplexed with ethylenediamine tetraacetic acid disodium salt ($EDTA^{2-}$, $2Na^{+}$) to give N^{ε}-Fmoc-lysine (**3**). The use of Fmoc-azide instead of Fmoc-chloride avoids some side reactions, such as the formation of Fmoc-dipeptides.[7,8] Reaction of **3** with 2-(*tert*-butoxycarbonyloxyamino)-2-phenylacetonitrile (BocON)[9] gave N^{α}-Boc-N^{ε}-Fmoc-lysine **4**.

Our attempts to extend this method to other amino acids were not successful due to the poor solubility of the copper(II) complexes. For example, we prepared several salts of aspartic acid copper(II) complexes: cesium, pyridinium, dimethylaminopyridinium, *N,N*-diisopropylneopentylammonium salts. However, these salts are insoluble in dimethylformamide and similar solvents and therefore not suitable for our purposes. Only the dicyclohexylammonium salt was soluble in dimethylformamide (1 g in 10 mL), but its reaction with fluorenylmethanol (FmOH) in the presence of *N,N*-diisopropylethylamine gave exclusively dibenzofulvene. Therefore we investigated the method described by Nefkens and Zwanenburg[10] for dual protection of amino and carboxyl groups. 2,2-Diethyl-5-oxotetrahydro-1,3,2-oxazaboroles derived from aspartic and glutamic acids **6a,b** were prepared from the corresponding amino acids **5a,b** and triethylborane in refluxing tetrahydrofuran. The resulting complexes **6a,b** were not isolated, and were allowed to react with fluorenylmethanol in presence of dicyclohexylcarbodiimide (DCC) and catalytic amounts of 4-dimethylaminopyridine (DMAP).[11] The ω-fluorenylmethyl ester of aspartic and glutamic acids were isolated as the hydrochloride salts **7a,b**, after bubbling hydrogen chloride gas through the crude reaction mixture. The introduction of the *tert*-butoxycarbonyl group was carried out in both cases as before using BocON to give **8a,b**.





The method described in the literature for the preparation of *S*-fluorenylmethylcysteine involves the reaction of cysteine with fluorenylmethyl chloride (FmCl) in the presence of *N,N*-diisopropylethylamine.[12] Although this reaction takes place in an acceptable yield, fluorenylmethyl chloride is obtained in a relatively poor yield (30%) from fluorenylmethanol and thionyl chloride.[6] However, fluorenylmethyl *p*-toluenesulfonate (**10**,

Table. Stability of Fluorenylmethyl Derivatives **4**, **8a, b** and **12**[a,b]

Product	TFA/CH_2Cl_2 3:7	Et_2NH/CH_2Cl_2 1:19	HF/*p*-Cresol[c] 9:1	piperidine/CH_2Cl_2 1:4	piperidine/CH_2Cl_2 1:1	piperidine/DMF 1:4	piperidine/DMF 1:1	0.1 M TBAF/DMF	Time (min)
4	+	+		+	±	±	−	±	1
	+	+		+	−	−		±	5
	+	+		±				±	20
	+	+	± (1 h)	−				−	240
8a	+	+		−	−	−	−	−	1
	+	+							5
	+	+							20
	+	+	± (1 h)						240
8b	+	+		±	−	−	−	−	1
	+	+		−					5
	+	+							20
	+	+	± (1 h)						240
12	+	+		+	+	+	±	+	1
	+	+		+	+	+	±	+	5
	+	+		+	+	±	±	+	20
	+	+	+ (1 h)	+	+	±	−	±	240

[a] (+), Stable (no unprotected product); (−), unstable (only unprotected product, no protected); (±), mixture of protected and unprotected products.
[b] Aliquots (50 µl) were removed at the indicated intervals and examined by TLC.
[c] Temperature 0 °C, 1 h, residue checked by amino acid analysis.

FmOTos) can be readily prepared with good purity and yield from fluorenylmethanol and tosyl chloride. The reaction of FmOTos with cysteine hydrochloride salt **(9)** in presence of *N,N*-diisopropylethylamine affords *S*-Fm-cysteine **11**, in similar yield as before. Further reaction of **11** with BocON gives the *N*-Boc-*S*-Fm-cystine **12**.

As shown in the Table, these fluorenylmethyl-based protecting groups **4, 8a, b and 12** are stable to the conditions used for the elongation of the peptide chain in a classical Boc/benzyl strategy of solid-phase peptide synthesis (trifluoroacetic acid/dichloromethane, 3:7; *N,N*-diisopropylethylamine/dichloromethane, 1:19). On the other hand, only the cysteine derivative **12** is completely stable to anhydrous hydrogen fluoride. Free amino acids (1–7%) were detected when derivatives of glutamic and aspartic acids **8a, b** and lysine **4** with fluorenylmethyl side chain protection were allowed to react with hydrogen fluoride for 1 h at 0° in presence of *p*-cresol. Likewise, all these groups can be removed by piperidine solutions, although the cysteine derivative **12** requires higher concentrations of piperidine in dimethylformamide and longer reaction times. Tetrabutylammonium fluoride can also remove, in a few minutes, the α-amino and carboxylic protecting groups,[13] but not from the ε-amino group of lysine nor from the thiol of cysteine.

The complete stability of *S*-Fm-cysteine **12** to anhydrous hydrogen fluoride allows the isolation, purification, characterization, and storage of peptides with the cysteine thiol function still protected, thus avoiding the side reactions usually derived from undesired partial cleavage of various cysteine protecting groups during reaction with hydrogen fluoride. The cleavage of the fluorenylmethyl group of cysteine in the presence of thiols (2-mercapto-1-ethanol or dithiothreitol) leads to free cysteine, in their absence direct oxidation to cystine takes place. Finally, *S*-Fm-cysteine **12** is stable to iodine/dimethylformamide (1:19) and 2-mercapto-1-ethanol/dimethylformamide (1:19). These results imply that fluorenylmethyl protection for cysteine is orthogonal with acetamidomethyl[14] and *tert*-butyl sulfide,[15] thus in principle allowing selective formation of disulfide bridges.

N^{ε}-Fmoc-L-lysine (3):

L-Lysine hydrochloride (**1**, 5 g, 27.4 mmol) is dissolved in H_2O (40 mL) and basic $CuCO_3$ (5 g, 45.2 mmol) is added. The mixture is refluxed for 30 min, and then the hot suspension is filtered and washed with H_2O. After cooling to 25 °C, the solution is basified with MgO (1.5 g) and Fmoc-azide (10.6 g, 40 mmol) dissolved in dioxane (75 mL) is added. After stirring at 45 °C for 16 h a bulky blue precipitate is formed. The whole mixture is stirred with 2N aq. AcOH (25 mL) for 1 h and then filtered. The residue is washed with H_2O (3 × 50 mL), dioxane (2 × 25 mL), and $CHCl_3$ (2 × 25 mL) in order to eliminate excess of azide, and dried to give the Cu(II) salt of N^{ε}-fluorenylmethyloxycarbonyl-L-lysine Cu(II) complex; yield: 6.98 g (64%); mp 211–213 °C (dec).

IR (KBr): ν = 3340, 3240, 2940, 2860, 1690, 1625, 1530, 1450, 1255, 1140, 760, 740 cm^{-1}.

The copper complex (5.19 g, 6.5 mmol) is finely powdered and added to a freshly supersaturated EDTA disodium salt solution [EDTA (2.53 g, 8.67 mmol) is added portionwise to a stirred solution of $NaHCO_3$ (1.42 g) in H_2O (20 mL)]. The suspension is vigorously shaken at 25 °C until the inital blue complex is decomposed, approximately 1 h, and a white solid separates. After filtering and washing with water a nearly quantitative amount of N^{ε}-Fmoc-L-lysine **(3)** is obtained; yield: 4.60 g (96%): mp 209–211 °C (dec).

^{1}H-NMR (CD_3OD/TMS): δ = 1.4–1.7 (m, 6H, H-3, H-4, H-5), 3.1–3.3 (m, 2H, H-6), 4.2–4.5 (m, 4H, H-2, CH-Fm, CH_2Fm), 7.3–7.9 (m, 8 H_{arom}).

^{13}C-NMR (DMSO-d_6): δ = 22.5 (C-4), 29.2 (C-3), 30.8 (C-5), 40.8 (C-6), 46.8 (CH-Fm), 54.2 (C-2), 65.2 (CH_2Fm), 120.0, 125.1, 127.0, 127.5 ($\underline{C}H_{arom}$, Fm), 140.7, 143.9 (C_{arom}, Fm), 156.1 (C-1), 170.2 (COFmoc).

L-Glutamic Acid δ-Fluorenylmethyl Ester (7b); Typical Procedure:
L-Glutamic acid (**5b**, 1.60 g, 11 mmol) is suspended in THF (20 mL), and a 1 M solution of BEt_3 in THF (13 mL, 13 mmol) is added. The mixture stirred at reflux until the amino acid has dissolved (2 d). The solution is filtered to remove small particles and FmOH (2.35 g, 12 mmol), DCC (2.47 g, 11 mmol), and DMAP (0.15 g, 1.2 mmol) dissolved in a small amount of THF (total volume 32 mL) are added. After 2 h at 25 °C, TLC ($CHCl_3$/AcOH, 19:1) indicates that all complex **6b** has reacted. The mixture is filtered in order to remove dicyclohexylurea, concentrated by rotary evaporation, and the residue is diluted with EtOAc (25 mL). HCl gas is passed through the solution for about 10 min at 25 °C and **7b** is collected by filtration and washed with EtOAc (2 × 10 mL). No further purification is required; yield: 3.28 g (83 %); mp 155–156 °C.

^{1}H-NMR (CD_3OD/TMS): δ = 2.3–2.5 (m, 2 H, H-3), 2.81 (t, 2 H, H-4), 4.19 (t, 1 H, CH-Fm), 4.3–4.4 (m, 1 H, H-1), 4.5–4.7 (m, 2 H, CH_2Fm), 7.4–7.8 (m, 8 H_{arom}).

^{13}C-NMR (DMSO-d_6): δ = 26.6 (C-3), 30.6 (C-4), 48.1 (CHFm), 53.0 (C-2), 67.5 (CH_2Fm); 121.0, 126.0, 128.2, 128.9 ($\underline{C}H_{arom}$, Fm), 142.5, 145.0 ($\underline{C}_{arom}$, Fm), 172.0 (C-1), 173.5 (C-5).

MS (*m/z*): 343 ([M + 18 − 36]), 308, 282, 231, 214, 164 (100 %), 147.

L-Aspartic Acid γ-Fluorenylmethyl Ester (7a)
Starting from L-aspartic acid and hydrochloride (**5a**) following the typical procedure for **7b**, gives **7a** in 84 % yield; mp 220–222 °C (dec).

^{1}H-NMR (CD_3OD/TMS): δ = 3.30 (d, 2 H, H-3), 4.47 (t, 1 H, H-2), 4.52 (t, 1 H, CH-Fm), 4.5–4.7 (m, 2 H, CH_2Fm), 7.5–8.0 (m, 8 H_{arom}).

^{13}C-NMR (CD_3OD): δ = 34.2 (C-3), 46.1 (CH−Fm), 48.5 (C-2), 66.5 (CH_2Fm), 120.2, 125.3, 127.3, 127.9 ($\underline{C}H_{arom}$, Fm), 140.7, 143.5 ($\underline{C}_{arom}$, Fm); 169.5 (C-1); 169.6 (C-4).

MS (*m/z*): 329 ([M + 18 − 36]), 312, 268 (100 %), 213, 196, 151, 107.

Fluorenylmethyl *p*-Toluenesulfonate (10)
TosCl (19.6 g, 0.1 mol) in anhydrous pyridine (16.1 mL, 0.2 mol) is added portionwise to a solution of FmOH (19.6 g, 0.1 mol) in $CHCl_3$ (100 mL), cooled in an ice-bath. After 2 h stirring, TLC (CH_2Cl_2) indicates that all FmOH has reacted. The solution is washed with 10 % aq. $NaHCO_3$ (2 × 25 mL), sat. brine (2 × 25 mL), and dried ($MgSO_4$). After filtration the solvent is evaporated *in vacuo*, and the product is recrystallized by dissolving in $CHCl_3$, adding hexane to incipient turbidity and allowing to stand at r.t. overnight to give **10** as a colorless solid; yield: 28.9 g (83 % yield); mp 115 °C.

$C_{21}H_{18}O_3S$	calc.	C 71.98	H 5.17	S 9.14
(350.43)	found	71.97	5.49	8.89

^{1}H-NMR ($CDCl_3$/TMS): δ = 2.40 (s, 3 H, CH_3), 4.1–4.5 (m, 3 H, CH-Fm, CH_2Fm), 7.2–7.8 (m, 8 H_{arom}).

^{13}C-NMR ($CDCl_3$): δ = 21.6 (CH_3), 46.6 (CHFm), 71.8 (CH_2Fm), 120.0, 125.1, 127.2, 127.8 ($\underline{C}H_{arom}$-Fm), 128.0, 129.8 ($\underline{C}H_{arom}$-Tos), 132.5, 142.4 (C_{arom}-Tos), 141.2, 144.5 (C_{arom}-Fm).

MS: (*m/z*) = 350 [(M)], 178 (100 %), 165.

***S*-Fm-L-cysteine (11):**
L-Cysteine hydrochloride (**9**, 5.3 g, 34 mmol) and FmOTos (**10**, 15 g, 43 mmol) are dissolved in DMF (150 mL). The mixture is cooled in an ice-bath and diisopropylethylamine (17 mL, 102 mmol) is added portionwise, with formation of a white precipitate. The suspension is stirred for 16 h at 25 °C and then EtOAc (150 mL) is added. The solid is filtered, washed with EtOAc (2 × 50 mL) and the product is recrystallized from 1 N HCl to give **11**; yield: 8.1 g (71 %); mp 210 °C.

^{1}H-NMR (DMSO-d_6): δ = 2.7–3.1 (m, 4 H, $C\underline{H}_2$SFm, CH_2Fm), 4.1–4.4 (m, 2 H, H-2, CH-Fm), 7.2–7.9 (m, 8 H_{arom}).

^{13}C-NMR (DMSO-d_6): δ = 32.4 (C-3), 36.0 (CH_2Fm), 46.4 (CH-Fm), 52.1 (C-2), 120.0, 125.1, 127.0, 127.5 ($\underline{C}H_{arom}$, Fm), 140.6, 145.7 (C_{arom}, Fm), 169.6 (C-1).

MS: (*m/z*) = 300 [(M + 1)], 124 (100 %).

***N*-Boc-L-Glutamic Acid δ-Fluorenylmethyl Ester (8b); Typical Procedure:**
Ester **7b** (1.81 g, 5 mmol) is dissolved in THF/H_2O (1:1) (15 mL), and the resulting solution is brought to pH 9.5 by adding 10 % aq. Na_2CO_3. The solution is cooled in an ice bath and BocON (1.36 g, 5.5 mmol) in dioxane (5 mL) is added. After 15 min of stirring in an ice bath, the reaction is continued at 25 °C and kept at pH 9.5 by adding further Na_2CO_3 solution. After 2 h, the TLC ($CHCl_3$/AcOH 19:1) shows that all **7b** has reacted. The mixture is washed with Et_2O (2 × 50 mL), acidified with 1 N aq. HCl (to pH 2), and extracted with EtOAc (3 × 50 mL). The organic layer is washed with H_2O (2 × 25 mL), dried ($MgSO_4$) and after filtration the solvent is evaporated *in vacuo*. The product is recrystallized by dissolving in a few drops of EtOAc, adding hexane to incipient turbidity and allowing to stand overnight at − 20 °C affording **8b** a colorless solid; yield 1.67 g (79 %); mp 129–131 °C; $[\alpha]_D$ + 10.7° (*c* = 1, $CHCl_3$).

$C_{24}H_{27}NO_6$	calc.	C 67.14	H 6.12	N 3.40
(425.48)	found	67.54	6.44	3.05

^{1}H-NMR ($CDCl_3$/TMS): δ = 1.43 (s, 9 H, $C(CH_3)_3$), 1.9–2.2 (m, 2 H, H-3), 2.5–2.7 (m, 2 H, H-4), 4.0–4.4 (m, 4 H, CH-Fm, H-2, CH_2Fm), 7.3–7.9 (m, 8 H_{arom}).

^{13}C-NMR (DMSO-d_6): δ = 27.3 (C-3), 28.3 ($C(\underline{C}H_3)_3$), 30.4 (C-4), 46.7 (CHFm), 52.9 (C-2), 66.7 (CH_2Fm), 80.0 ($\underline{C}(CH_3)_3$), 120.0, 125.0, 127.1, 127.8 ($\underline{C}H_{arom}$, Fm), 141.3, 143.7 (C_{arom}, Fm), 156.0 (CO), 173.0 (C-1), 176.0 (C-5).

MS: (*m/z*) = 443 ([M + 18), 399, 343, 231, 214, 164 (100 %), 147.

***N*-Boc-L Aspartic Acid γ-Fluorenylmethyl Ester (8a):**
From **7a** (1.00 g, 2.9 mmol) following typical procedure to give **8a**; yield: 0.99 g (83 %); mp 137–138 °C; $[\alpha]_D$ + 26.9° (*c* = 1, $CHCl_3$).

$C_{23}H_{25}NO_6$	calc.	C 67.14	H 6.12	N 3.40
(411.45)	found	66.61	6.20	3.08

^{1}H-NMR ($CDCl_3$/TMS): δ = 1.41 (s, 9 H, $C(CH_3)_3$), 2.8–3.1 (m, 2 H, H-3), 4.0–4.4 (m, 4 H, CHFm, H-2, CH_2−Fm), 7.2–7.8 (m, 8 H_{arom}).

^{13}C-NMR ($CDCl_3$): δ = 28.3 ($C(\underline{C}H_3)$, 36.5 (C-3), 46.6 (CH=Fm), 52.9 (C-2), 66.7 (CH_2Fm), 81.0 ($\underline{C}(CH_3)_3$), 120.1, 125.0, 127.2, 127.8 ($\underline{C}H_{arom}$, Fm), 141.3, 143.7 (C_{arom}, Fm), 155.6 (CO), 172.0 (C-1), 175.5 (C-5).

MS: (*m/z*) = 429 ([M + 18), 385, 368, 251, 231, 214 (100 %), 207, 179.

***N*$^{\alpha}$-Boc-*N*$^{\varepsilon}$-Fmoc-L-lysine (4):**
From **3** (4.23 g, 11.5 mmol) following typical procedure to give **4**; yield: 3.77 g (70 %); mp 88–91 °C; $[\alpha]_D$ − 1.4° (*c* = 1, MeOH).

$C_{26}H_{32}N_2O_6$	calc.	C 66.65	H 6.88	N 5.98
(468.55)	found	66.65	6.95	6.09

^{1}H-NMR ($CDCl_3$/TMS): δ = 1.45 (s, 9 H, $C(CH_3)_3$), 1.2–1.9 (m, 6 H, H-3, H-4, H-5), 3.15 (m, 2 H, H-6), 4.1–4.5 (m, 4 H, CH-Fm, H-2, CH_2Fm), 7.3–7.8 (m, 8 H_{arom}).

^{13}C-NMR ($CDCl_3$): δ = 22.4 (C-4), 28.3 ($C(\underline{C}H_3)_3$), 29.3 (C-3), 32.0 (C-5), 40.7 (C-6), 47.3 (CH-Fm), 53.2 (C-2), 66.9 (CH_2Fm), 80.1 ($\underline{C}(CH_3)_3$), 120.0, 125.0, 127.1, 127.7 ($\underline{C}H_{arom}$, Fm), 141.3, 143.9 (C_{arom}, Fm), 155.8 (CO-Boc), 156.7 (C-1), 176.3 (CO-Fmoc).

MS: (*m/z*) = 486 ([M + 18]), 425, 364, 323, 303, 264, 247, 231, 213 (100 %), 203, 196.

***N*-Boc-*S*-Fm-L-Cysteine (12)**
From **11** (2.99 g, 10 mmol) following typical procedure to give **12**; yield: 2.39 g (60 %); mp 74–75 °C; $[\alpha]_D$ − 10.1° (*c* = 1, DMF).

$C_{22}H_{25}O_4S$	calc.	C 66.14	H 6.30	N 3.50	S 8.02
(399.51)	found	65.98	6.26	3.37	7.52

^{1}H-NMR (DMSO-d_6/TMS): δ = 1.35 (s, 9 H, $C(CH_3)_3$), 2.7–2.9 (m, 2 H, H-3), 3.12 (d, 2 H, CH_2Fm), 4.1–4.5 (m, 2 H, CH-Fm, H-2), 7.2–7.8 (m, 8 H_{arom}).

^{13}C-NMR (DMSO-d_6): δ = 28.1 ($C(\underline{C}H_3)_3$), 33.8 (C-3), 35.7 (CH_2Fm), 46.4 (CHFm), 53.9 (C-2), 78.2 ($\underline{C}(CH_3)_3$), 119.9, 124.9, 126.9, 127.4 ($\underline{C}H_{arom}$, Fm), 140.4, 145.8 (C_{arom}, Fm), 155.3 (CO-Boc), 172.4 (C-1).

MS (*m/z*): 417 [(M + 1)], 177, 124 (100%).

Stability Experiments

Solutions (1 mmol) of protected amino acids in different reagents were prepared. Aliquots of the solution (50 µL) were removed at different times and checked by TLC ($CHCl_3$/AcOH, 19:1, for all reagents except for TFA/CH_2Cl_2 where BuOH/Py/AcOH/H_2O, 15:10:3:12, is used). HF reaction is carried out in the presence of *p*-cresol (9:1) at 0 °C, for 1 h, and after evaporation the residue is checked by amino acid analysis.

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