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Synthesis, Conformational Study, and Spectroscopic Characterization of the Cyclic $C^{\alpha,\alpha}$-disubstituted Glycine 9-Amino-9-fluorenecarboxylic Acid

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Abstract: A series of terminally blocked peptides (to the pentamer level) from L-Ala and the cyclic $C^{\alpha,\alpha}$-disubstituted Gly residue Afc and one Gly/Afc dipeptide have been synthesized by solution method and fully characterized. The molecular structure of the amino acid derivative Boc-Afc-OMe and the dipeptide Boc-Afc-Gly-OMe were determined in the crystal state by X-ray diffraction. In addition, the preferred conformation of all of the model peptides was assessed in deuterochloroform solution by FT-IR absorption and ^{1}H-NMR. The experimental data favour the conclusion that the Afc residue tends to adopt either the fully-extended (C_5) or a folded/helical structure. In particular, the former conformation is highly populated in solution and is also that found in the crystal state in the two compounds investigated. A comparison with the structural propensities of the strictly related $C^{\alpha,\alpha}$-disubstituted Gly residues Ac_5c and DΦg is made and the implications for the use of the Afc residue in conformationally constrained analogues of bioactive peptides are briefly examined. A spectroscopic (UV absorption, fluorescence, CD) characterization of this novel aromatic $C^{\alpha,\alpha}$-disubstituted Gly residue is also reported. Copyright © 1999 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: 9-amino-9-fluorenecarboxylic acid; conformational analysis; peptide synthesis; X-ray diffraction; spectroscopy

INTRODUCTION

$C^{\alpha,\alpha}$-disubstituted Gly residues have been described as a useful new type of conformational constraint in peptides (for leading review articles see References [1–5]). Through this backbone modification information may be obtained on the active conformation of a peptide molecule at the receptor site and biological activity may be improved because of enhanced resistance to enzyme degradation. The replacement of both C^{α}-hydrogens in Gly peptides by alkyl moieties has profound structural consequences. The inherent interest in peptides rich in Aib (Figure 1), the prototypical $C^{\alpha,\alpha}$ symmetrically disubstituted glycine, results not only from the restricted conformational space that this residue is allowed to explore but also from its propensity to adopt a set of φ, ψ backbone torsion angles typical of regular type-III (III′) β-bend [6–8] and $3_{10}/\alpha$-helices [9]. In this connection, it was also shown that the conformational behaviour of the cycloaliphatic sub-family of

Abbreviations: Ac_nc, 1-aminocycloalkane-1-carboxylic acid; AcOEt, ethyl acetate; Afc, 9-amino-9-fluorenecarboxylic acid; DMSO, dimethylsulphoxide; DΦg, $C^{\alpha,\alpha}$-diphenylglycine; EEDQ, 1-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline; MeCN, acetonitrile; NMM, *N*-methylmorpholine; TEMPO, 2,2,6,6-tetramethylpiperidinyl-l-oxy.

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the $C^{\alpha,\alpha}$-symmetrically disubstituted Gly residues, generally referred to as Ac_nc (for the medium-sized ring Ac_5c residue, Figure 1, see References [10–14]), closely parallels that of Aib. By contrast, the fully-extended C_5 conformation [7,15–17] is markedly favoured over the folded/helical structures when two side-chain C^{β} atoms are symmetrically substituted but not interconnected in a cyclic system (for the DΦg residue, Figure 1, see References [18–22]).

As a part of a programme aimed at investigating the conformational preferences of $C^{\alpha,\alpha}$-symmetrically disubstituted Gly residues, we report here the synthetic aspects, the crystal-state and solution conformational properties (by using X-ray diffraction, FT-IR absorption and ^{1}H-NMR) and the spectroscopic (UV absorption, fluorescence, and CD) characterization of a series of peptides, to the pentamer level, based on Afc (Figure 1), a novel aromatic $C^{\alpha,\alpha}$-symmetrically substituted Gly residue, which may be considered a structural combination of the cyclic, folded/helical structure forming, Ac_5c residue and the aromatic, fully-extended structure supporting, DΦg residue.

To the best of our knowledge, the only papers dealing with the Afc residue describe the synthesis and chemical characterization of the linear derivatives H-Afc-OMe and Ac-Afc-OMe, but not of any Afc peptide [23,24].

MATERIALS AND METHODS

Synthesis and Characterization of Peptides

The analytical ^{1}H-NMR spectra were recorded on a Bruker (Karlsruhe, Germany) model AC-300 spectrometer in 0.04–0.08 M $CDCl_3$ solutions at 24°C. Because of the concomitant presence of *trans* (*anti*) and *cis* (*syn*) rotamers [25] in carbamates involving the N-terminal Boc-Afc sequence, chemical shift assignments in these compounds were only tentative. TLC runs were performed on precoated aluminium sheets of silica gel 60 F_{254} (Merck, Darmstadt, Germany) and purifications were obtained by chromatography on Kieselgel 60 (Merck) columns. The following elution systems were used: A, petroleum ether (40–65°C)/AcOEt (5:10); B, petroleum ether/AcOEt (3:10); C, petroleum ether/AcOEt (2:10); D, petroleum ether/AcOEt (1:1); E, petroleum ether/AcOEt (10:2); F, petroleum ether/AcOEt (10:4); G, petroleum ether/AcOEt (10:5); H, AcOEt/MeOH (10:0.2); I, AcOEt/MeOH (10:0.5).

Boc-Afc-OMe. H-Afc-OMe [23,24] (2.39 g,10 mmol) dissolved in MeCN (100 mL) was heated at 60°C for 48 h in the presence of 1 equivalent of $(Boc)_2O$. The mixture was evaporated to dryness and the solid residue was dissolved in fresh MeCN (100 mL) and heated for an additional 48 h with a second equivalent of $(Boc)_2O$. After evaporation of the solvent, the crude solid product was dissolved in AcOEt, washed with 1 M $KHSO_4$ and water, and dried over $MgSO_4$. The product crystallized from a concentrated AcOEt solution in 80% yield; m.p. 168–169°C. Found: C, 70.76; H, 6.39; N, 4.09%; $C_{20}H_{21}NO_4$ (339.39) requires C, 70.78; H, 6.24; N, 4.13%. ^{1}H-NMR: δ 7.75–7.29 (m, 8H, aromatic CH); 6.26 and 5.83 (s, 1H, *syn* and *anti* NH); 3.59 (s, 3H, OMe CH_3); 1.40 and 0.90 (s, 9H, *anti* and *syn* Boc CH_3).

Boc-Afc-OH. A 2 mL solution of 2 N NaOH was added during 1 h to Boc-Afc-OMe (2 mmol) dissolved in MeOH (20 mL) and the mixture was left stirring at room temperature. The progress of the saponification reaction was followed by TLC in eluant E. After 24 h the starting methyl ester disappeared and the reaction mixture consisted of the free acid (Boc-Afc-OH, R_F 0.00 in E) and about 10–15% of the decarboxylated product (R_F 0.55 in E). The reaction mixture was evaporated to dryness, the residue was taken up in AcOEt and washed with 1 M $KHSO_4$. The free acid was extracted with saturated aqueous $NaHCO_3$ and the resulting alkaline solution was acidified to pH 2 with 1 M $KHSO_4$. The product separated out and crystallized in 80% yield; m.p. 153–155°C. Found: C, 69.88; H, 6.14; N, 4.26%; $C_{19}H_{19}NO_4$ (325.37) requires C, 70.14; H, 5.89; N, 4.30%. ^{1}H-NMR: δ 8.72 (s, 1H, COOH);

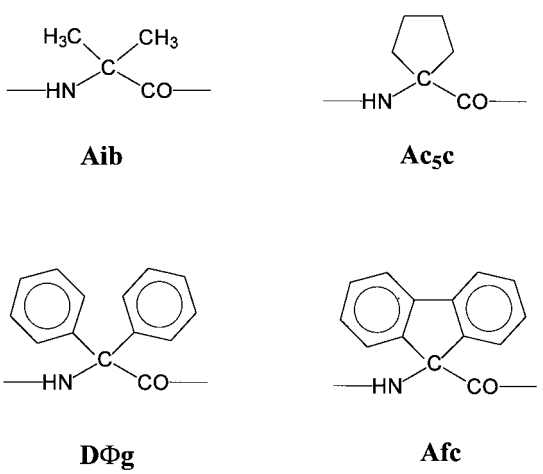

Figure 1 The four $C^{\alpha,\alpha}$-disubstituted glycines discussed in this work.

7.66–7.36 (m, 9H, 8 aromatic CH and 1 NH); 0.60 (s, 9H, *syn* Boc CH_3).

Ac-Afc-OMe. H-Afc-OMe (2 mmol) dissolved in anhydrous MeCN (20 mL) was heated at 50–60°C overnight under argon in the presence of $(Ac)_2O$ (2.2 mmol). Upon cooling the acetylated product crystallized out in 44% yield. Additional product (37% yield) was obtained by evaporation of the filtrate and crystallization from AcOEt. Total yield 81%; m.p. 238–239°C; ninhydrin negative (the starting material H-Afc-OMe has a very close R_F value but it is ninhydrin positive). Found: C, 71.76; H, 5.61; N, 4.92%; $C_{17}H_{15}NO_3 \cdot 0.2H_2O$ (284.92) requires C, 71.66; H, 5.45; N, 4.91%. ^{1}H-NMR: δ 7.69–7.28 (m, 8H, aromatic CH); 6.60 (s, 1H, NH); 3.61 (s, 3H, OMe CH_3); 1.97 (s, 3H, Ac CH_3).

Boc-Afc-Gly-OMe. Boc-Afc-OH (3 mmol) dissolved in CH_2Cl_2 (10 mL) was pre-activated with EEDQ [26] (3.3 mmol) at room temperature for 1 h. HCl·H-Gly-OMe (4.5 mmol) was added, followed by NMM (4.5 mmol) slowly over 1 h. The reaction was left stirring overnight, the solvent was evaporated, and the residue dissolved in AcOEt. The resulting solution was washed as usual with 1 M $KHSO_4$, saturated aqueous $NaHCO_3$ and water, and dried over $MgSO_4$. TLC in eluant G showed the presence of some decarboxylation product of Boc-Afc-OH with R_F 0.75 and of the dipeptide with R_F 0.30. Column chromatography in eluant G gave Boc-Afc-Gly-OMe in 71% yield after crystallization from AcOEt/*n*-hexane; m.p. 104–106°C. Found: C, 65.91; H, 6.18; N, 6.71%; $C_{22}H_{24}N_2O_5 \cdot 0.3H_2O$ (401.85) requires C, 65.75; H, 6.17; N, 6.97%. ^{1}H-NMR: δ 7.75–7.32 (m, 8H, aromatic CH); 6.51 (s, 1H, Afc NH); 5.50 (s, 1H, Gly NH); 3.79 (d, 2H, Gly CH_2, $J = 5.5$ Hz); 3.60 (s, 3H, OMe CH_3); 1.30 and 0.81 (s, 9H, *anti* and *syn* Boc CH_3).

Boc-Afc-L-Ala-OMe. Boc-Afc-OH (2 mmol) in anhydrous CH_2Cl_2 (10 mL) was cooled to -30°C under argon, then NMM (2 mmol) was added, followed by ethyl chloroformate (2 mmol). After 2 min an excess of HCl·H-L-Ala-OMe (3 mmol) in cold CH_2Cl_2 (10 mL) was added followed by NMM (3 mmol). The reaction was left stirring at -10°C under argon for 1 h and at room temperature overnight. After the usual work up, a column chromatography in eluant F gave a fraction with R_F 0.75 (37% yield) of the decarboxylation product of Boc-Afc-OH, and a fraction with R_F 0.30 containing the dipeptide in 61% yield. Upon evaporation of an ethereal solution of this compound, a hard foam was obtained having an indefinite m.p. in the range 38–50°C; $[\alpha]_D^{25} = -20.8°$ ($c = 0.35$; MeOH). Found: C, 66.84; H, 6.66; N, 6.71%; $C_{23}H_{26}N_2O_5 \cdot 0.2H_2O$ (414.07) requires C, 66.71; H, 6.42; N, 6.76%. ^{1}H-NMR: δ 7.75–7.30 (m, 8H, aromatic CH); 6.50 (s, 1H, Afc NH); 5.55 (s, 1H, Ala NH); 4.34 (m, 1H, Ala αCH); 3.55 (s, 3H, OMe CH_3); 1.26 and 0.84 (s, 9H, *anti* and *syn* Boc CH_3); 1.10 (d, 3H, Ala, βCH_3, $J = 7.2$ Hz).

Boc-L-Ala-Afc-L-Ala-OMe. Boc-Afc-L-Ala-OMe (2 mmol) was treated with neat TFA at 20°C for 2 h. The reaction mixture was evaporated to dryness, the residue was taken up in CH_2Cl_2 and repeatedly evaporated to constant weight. The yield of H-Afc-L-Ala-OMe trifluoroacetate was close to 100%. This product was dissolved in cold THF (10 mL) in the presence of NMM (2 mmol) and the solution was added to an excess of mixed anhydride obtained at -20°C from Boc-L-Ala-OH (3 mmol), NMM (3 mmol) and ethyl chloroformate (3 mmol) in anhydrous THF (10 mL). The reaction mixture was left stirring at -10°C for 1 h and at 20°C overnight. After evaporation of the solvent the residue was taken up in AcOEt, washed with 1 M $KHSO_4$, saturated aqueous $NaHCO_3$ and water, and dried over $MgSO_4$. Column chromatography in eluant D gave a fraction containing the tripeptide (R_F 0.35 in D) in 75% yield obtained as a powder, through repeated evaporations of a CH_2Cl_2 solution, with an indefinite m.p. in the range 71–82°C; $[\alpha]_D^{25} = -52.0°$ ($c = 0.22$; MeOH). Found: C, 64.35; H, 6.91; N, 8.53%; $C_{26}H_{31}N_3O_6 \cdot 0.2H_2O$ (485.16) requires C, 64.37; H, 6.52; N, 8.66%. ^{1}H-NMR: δ 7.84 (s, 1H, Afc NH); 7.75–7.31 (m, 8H, aromatic CH); 5.66 (d, 1H, Ala NH, $J = 7.3$ Hz); 5.04 (d, 1H, Ala NH); 4.39 (m, 1H, Ala αCH); 4.20 (m, 1H, Ala αCH); 3.60 (s, 3H, OMe CH_3); 1.43 (s, 9H, Boc CH_3); 1.31 (d, 3H, Ala, βCH_3, $J = 7.0$ Hz); 1.15 (d, 3H, Ala βCH_3, $J = 7.2$ Hz).

Boc-L-Ala-L-Ala-Afc-L-Ala-OMe. Boc-L-Ala-Afc-L-Ala-OMe (2 mmol) was treated with neat TFA at 20°C for 2 h. The reaction mixture was evaporated to dryness, the residue was taken up in CH_2Cl_2 and repeatedly evaporated to constant weight. The yield of H-L-Ala-Afc-L-Ala-OMe trifluoroacetate was close to 100%. This product was dissolved in cold CH_2Cl_2 (10 mL) in the presence of NMM (2 mmol) and the solution was added to an excess of mixed anhydride obtained at -20°C under argon from Boc-L-Ala-OH (2.5 mmol), NMM (2.5 mmol) and ethyl chloroformate (2.5 mmol) in anhydrous THF (8 mL). The reaction mixture was left stirring at -10°C for 1 h and at room temperature overnight. The product was worked up as usual and chromatographed on a

Kieselgel column in eluant B to give a fraction containing the tetrapeptide in 87% yield with R_F 0.51 in eluant C. The product was obtained as a powder, through repeated evaporations of a CH_2Cl_2 solution, with an indefinite m.p. in the range 92–108°C; $[\alpha]_D^{25} = -72.9°$ ($c = 0.32$; MeOH). Found: C, 61.07; H, 6.56; N, 9.79%; $C_{29}H_{36}N_4O_7 \cdot 1H_2O$ (570.65) requires C, 61.04; H, 6.71; N, 9.82%. ^{1}H-NMR: δ 7.90 (s, 1H, Afc NH); 7.74–7.31 (m, 8H, aromatic CH); 6.67 (d, 1H, Ala NH, $J = 7.2$ Hz); 5.64 (d, 1H, Ala NH, $J = 7.3$ Hz); 5.08 (d, 1H, Ala NH); 4.52 (m, 1H, Ala αCH); 4.39 (m, 1H, Ala αCH); 4.09 (m, 1H, Ala αCH); 3.58 (s, 3H, OMe CH_3); 1.38 (s, 9H, Boc CH_3); 1.34 (d, 3H, Ala βCH_3, $J = 7.0$ Hz); 1.27 (d, 3H, Ala βCH_3, $J = 7.0$ Hz); 1.14 (d, 3H, Ala βCH_3, $J = 7.2$ Hz).

Boc-Afc-L-Ala-L-Ala-Afc-L-Ala-OMe. Boc-L-Ala-L-Ala-Afc-L-Ala-OMe (0.5 mmol) was treated with neat TFA at 20°C for 2 h. H-L-Ala-L-Ala-Afc-L-Ala-OMe trifluoroacetate (yield close to 100%) was obtained as usual and then dissolved in CH_2Cl_2 (4 mL) in the presence of NMM (0.5 mmol). Then, the solution was added to an excess of mixed anhydride obtained at −20°C under argon from Boc-Afc-OH (0.85 mmol), NMM (0.85 mmol) and ethyl chloroformate (0.85 mmol) in anhydrous CH_2Cl_2 (4 mL). After l h at −10°C the reaction mixture was left stirring overnight at room temperature. The product was worked up as usual and then chromatographed on a Kieselgel 60 column in eluant B. After a fast moving band corresponding to a relevant amount of the decarboxylation product of Boc-Afc-OH (R_F 0.90 in eluant B), the product eluted at R_F 0.40 in B in a 52% yield. Repeated evaporations of a CH_2Cl_2 solution gave the product as a powder with an indefinite m.p. in the range 120–130°C; $[\alpha]_D^{25} = -68.1°$ ($c = 0.26$; MeOH). Found: C, 66.34; H, 6.01; N, 8.91%; $C_{43}H_{45}N_5O_8 \cdot 1H_2O$ (777.88) requires C, 66.39; H, 6.09; N, 9.00%.

Boc-Afc-L-Ala-L-Ala-OMe. A mixed anhydride was formed from Boc-Afc-OH (1 mmol), NMM (1 mmol) and ethyl chloroformate (1 mmol) in anhydrous CH_2Cl_2 (4 mL) at −20°C under argon. To this reagent a cold slurry of HCl·H-L-Ala-L-Ala-OMe (0.7 mmol) and NMM (0.7 mmol) in CH_2Cl_2 (4 mL) was added. After 1 h at −10°C, the reaction mixture was left stirring at room temperature overnight and the product was worked up as usual. A column chromatography in eluant A gave, after a fast running band corresponding to the decarboxylation product of Boc-Afc-OH, the tripeptide with R_F 0.45 in A. Repeated evaporations of a CH_2Cl_2 solution gave the product as a powder in 60% yield with an indefinite m.p. in the range 75 − 88°C; $[\alpha]_D^{25} = -26.7°$ ($c = 0.22$; MeOH). Found: C, 64.51; H, 6.64; N, 8.49%; $C_{26}H_{31}N_3O_6$ (481.56) requires C, 64.85; H, 6.49; N, 8.73%. ^{1}H-NMR: δ 7.80–7.30 (m, 8H, aromatic CH); 7.00–5.40 (broad peaks, 3H, 3 NH); 4.30 (m, 2H, Ala αCH); 3.63 (s, 3H, OMe CH_3); 1.24 and 0.80 (s, 9H, *anti* and *syn* Boc CH_3); 1.24 (d, 3H, Ala βCH_3, $J = 7.0$ Hz); 1.02 (d, 3H, Ala βCH_3, $J = 7.0$ Hz).

Ac-Afc-L-Ala-L-Ala-OMe. Boc-Afc-L-Ala-L-Ala-OMe (0.5 mmol) was treated with neat TFA at 20°C for 2 h. The reaction mixture was evaporated to dryness, the residue was taken up in CH_2Cl_2 and repeatedly evaporated to constant weight. After dissolution of H-Afc-L-Ala-L-Ala-OMe trifluoroacetate in anhydrous MeCN (5 mL), NMM (1 mmol) and $(Ac)_2O$ (1 mmol) were added and the reaction mixture was heated to 50–60°C under stirring for 4 h. After evaporation to dryness, the residue was taken up in AcOEt and washed with 1 M $KHSO_4$ and water. A column chromatography in eluant H gave the product (R_F 0.30) in 74% yield. Repeated evaporations of the CH_2Cl_2 solution gave the product as a powder with an indefinite m.p. in the range 88–106°C; $[\alpha]_D^{25} = +3.5°$ ($c = 0.22$; MeOH). Found: C, 63.69; H, 6.32; N, 9.29%; $C_{23}H_{25}N_3O_5 \cdot 0.5H_2O$ (432.48) requires C, 63.87; H, 6.06; N, 9.71%. ^{1}H-NMR: δ 7.80–7.30 (m, 8H, aromatic CH); 6.99 (s, 1H, Afc NH); 6.89 (d, 1H, Ala NH, $J = 7.3$ Hz); 5.75 (d, 1H, Ala NH, $J = 7.7$ Hz); 4.39 (m, 2H, Ala αCH); 3.71 (s, 3H, OMe CH_3); 1.98 (s, 3H, Ac CH_3); 1.32 (d, 3H, Ala βCH_3, $J = 7.2$ Hz); 1.11 (d, 3H, Ala βCH_3, $J = 7.2$ Hz).

Ac-Afc-L-Ala-L-Ala-Afc-L-Ala-OMe. Boc-Afc-L-Ala-L-Ala-Afc-L-Ala-OMe (0.36 mmol) was treated with neat TFA at 20°C for 2 h. The reaction mixture was evaporated to dryness, the residue was taken up in CH_2Cl_2 and repeatedly evaporated to constant weight. After dissolution of H-Afc-LAla-L-Ala-Afc-L-Ala-OMe trifluoroacetate in anhydrous MeCN (4 mL) in the presence of NMM (0.4 mmol), $(Ac)_2O$ (0.4 mmol) was added and the reaction mixture was heated to 50–60°C overnight. After evaporation of the solution to dryness, the residue was taken up in AcOEt and washed with 1 M $KHSO_4$ and water. A column chromatography in eluant I gave the product, R_F 0.40 (I), in 88% yield. Repeated evaporations of the CH_2Cl_2 solution gave the product as a powder with an indefinite m.p. in the range 124–145°C; $[\alpha]_D^{25} = -72.7°$ ($c = 0.27$; MeOH). Found: C, 62.05; H, 5.58; N, 8.98%; $C_{40}H_{39}N_5O_7 \cdot 4H_2O$

Table 1 Crystallographic Data and Structure Refinement for the Afc Derivative and Peptide

Parameter	Boc-Afc-OMe	Boc-Afc-Gly-OMe
Empirical formula	$C_{20}H_{21}NO_4$	$C_{22}H_{24}N_2O_5 \times 1/8C_6H_{14}$
Formula weight (a.m.u)	339.4	407.2
Crystal system	Monoclinic	Triclinic
Space group	$P2_1/c$	$P\bar{1}$
a (Å)	17.422(3)	14.563(2)
b (Å)	10.871(2)	16.085(2)
c (Å)	20.618(3)	10.046(2)
α (°)	90.0	101.4(1)
β (°)	109.7(1)	94.9(1)
γ (°)	90.0	99.9(1)
Volume (Å^3)	3676.4(11)	2255.1(6)
Z (molecules/unit cell)	8	4
Density (calc.) (g cm^{-3})	1.226	1.199
Absorption coefficient (mm^{-1})	0.697	0.697
F(000)	1440	865
Reflections collected	5611	7036
Independant reflections	5449 [R(int) = 0.06]	6438
Solved by	SHELXS 86 [27]	SHELXS 86
Refinement method	Full-matrix least-squares on F^2	Full-matrix least-squares on F^2
Refined by	SHELXL 93 [28]	SHELXL 93
Goodness-of-fit on F^2	0.976	0.992
Data/restraints/parameters	5445/18/452	6433/1/529
Final R indices [$I > 2\sigma(I)$]	$R_1 = 0.057$, $wR_2 = 0.149$	$R_1 = 0.071$, $wR_2 = 0.219$
R indices (all data)	$R_1 = 0.089$, $wR_2 = 0.165$	$R_1 = 0.114$, $wR_2 = 0.247$
Temperature (K)	293	293
Radiation (λ, Å)	CuK_α (1.54178)	CuK_α (1.54178)
Scan method	$\theta/2\theta$	$\theta/2\theta$
θ range (°)	2.7–60.0	2.9–60.3
Index ranges	$-19 < h < 18$, $0 < k < 12$, $0 < l < 23$	$-16 < h < 16$, $-18 < k < 17$, $0 < l < 11$
Crystallization solvent	Acetonitrile	Ethyl acetate-n-hexane (vapour diffusion)
Crystal size (mm)	$0.4 \times 0.3 \times 0.2$	$0.3 \times 0.3 \times 0.2$
$\Delta\rho_{max}$ and $\Delta\rho_{min}$ (e·Å^{-3})	0.257/−0.280	0.923/−0.246

(773.85) requires C, 62.08; H, 6.12; N, 9.05%. ^{1}H-NMR: δ 7.90 (s,1H, Afc[4] NH); 7.75–7.28 (m, 16H, aromatic CH); 7.17 (d, 1H, Ala[3] NH, $J = 7.7$ Hz); 6.67 (s, 1H, Afc[1] NH); 6.01 (d, 1H, Ala[5] NH, $J = 7.2$ Hz); 5.75 (d, 1H, Ala[2] NH, $J = 6.4$ Hz); 4.43 (m, 2H, Ala[3] and Ala[5] αCH); 4.12 (m, 1H, Ala[2] αCH); 3.59 (s, 3H, OMe CH_3); 1.72 (s, 3H, Ac CH_3); 1.35 (d, 3H, Ala[3] βCH_3, $J = 7.2$ Hz); 1.21 (d, 3H, Ala[5] βCH_3, $J = 7.2$ Hz); 1.12 (d, 3H, Ala[2] βCH_3, $J = 7.2$ Hz).

Infrared Absorption

The infrared solution spectra were obtained using a Perkin-Elmer (Norwalk, CT) model 1720X FT-IR spectrophotometer, nitrogen flushed, equipped with a sample-shuttle device, at 2 cm^{-1} nominal resolution, averaging 100 scans. Cells with path lengths of 0.1, 1.0 and 10 mm (with CaF_2 windows) were used. Spectrograde deuterochloroform (99.8% d) was purchased from Fluka (Buchs, Switzerland). Solvent (baseline) spectra were recorded under the same conditions.

^{1}H-Nuclear Magnetic Resonance

The ^{1}H-NMR spectra were recorded with a Bruker model AM 400 spectrometer. Measurements were carried out in deuterochloroform (99.96% d; Acros, Geel, Belgium) and deuterated dimethylsulphoxide (99.96% d_6; Acros) with tetramethylsilane as the internal standard. The free radical TEMPO was purchased from Fluka.

X-Ray Diffraction

Colourless single crystals of the amino acid derivative Boc-Afc-OMe and the dipeptide Boc-Afc-Gly-

OMe were grown from the solvents listed in Table 1. Data collections were performed on a Philips (Eindhoven, The Netherlands) model PW 1100 four-circle diffractometer. Unit cell determination was carried out by the least-squares refinement of the setting of 25 high angle reflections accurately centred. No significant variations were observed in the intensities of the standard reflections monitored at regular intervals during data collection, thus implying electronic and crystal stabilities. Lorentz and polarisation corrections were applied to the intensities, but no absorption corrections were made. Crystal data are listed in Table 1.

The two structures were solved by direct methods and refined by the full-matrix least-squares procedure on F^2, using all data. All non-hydrogen atoms of the two structures were refined anisotropically. Planarity restraints were applied to the phenyl rings.

The hydrogen atoms of the two independent molecules in the asymmetric unit of Boc-Afc-OMe were calculated at idealized positions and during the refinement they were allowed to ride on their carrying atom, with U_{iso} set equal to 1.2 (or 1.5 for methyl groups) times the U_{eq} of the carrying atom.

Also, in the case of Boc-Afc-Gly-OMe there are two independent molecules in the asymmetric unit. However, in this structure also, some co-crystallized disordered solvent molecules are found near the cell origin. The disordered solvent was eventually modelled as one half of a *n*-hexane molecule (the second half is generated by symmetry through the inversion centre), which was isotropically refined with occupancy factor 0.5. It is worth noting that the overall geometrical features of the solvent molecule, and in particular the bond distances, are far from the ideal values. In addition, some significant residual density is observed in the same region, including a peak of 0.92 e $Å^{-3}$ at the origin, but the data did not allow a deeper understanding of the disorder. In any case, it is our contention that the geometrical and conformational parameters of the peptide molecules are firmly established.

UV Absorption

Ultraviolet absorption spectra were obtained on a Perkin-Elmer model Lambda 5 UV/VIS spectrophotometer. A quartz cell (Hellma, Müllheim, Germany) of 10 mm path length was used. Spectrograde MeOH (Acros) was used as solvent.

Fluorescence

Steady-state fluorescence spectra were recorded on a Perkin-Elmer model MPF-44 spectrofluorimeter. Excitation in the near-UV region was achieved by irradiating at 281 nm, with excitation and emission slit values set at 4 and 2.5 nm, respectively. All experiments were carried out in a quartz cell (Hellma) using spectrograde MeOH (Acros) as solvent.

Circular Dichroism

The circular dichroism spectra were recorded on a Jasco (Tokyo, Japan) model J-715 spectropolarimeter equipped with a Haake (Karlsruhe, Germany) thermostat. A rectangular quartz cell (Hellma) of 1 mm path length and a cylindrical fused quartz cell (Hellma) of 0.2 mm path length were employed. Spectrograde MeOH (Acros) was used as solvent. The values are expressed in terms of $[\theta]_T$, the total molar ellipticity (deg·cm^2 dmol^{-1}).

RESULTS AND DISCUSSION

Peptide Synthesis

The classical Bücherer–Berg method could not be used for the synthesis of the free amino acid H-Afc-OH, as the alkaline hydrolysis of the spirohydantoin from 9-fluorenone leads to the decarboxylated product 9-fluorenylurea [29]. DuPriest *et al.* synthesized the ester H-Afc-OMe by treatment of a 9-fluorenone Schiff base anion with methylchlorocarbonate [24]. This reaction is easier to perform than the amination of the anions of 9-fluorenecarboxylates [23,30]. Therefore, we exploited the DuPriest method for the synthesis of the starting ester H-Afc-OMe.

Acylation of the Afc α-amino group and peptide bond formation did not present any particular difficulty. In general, Boc- and Ac- incorporation, as well as coupling with amino acid residues through the mixed anhydride or the EEDQ methods, led to good yields of the desired products. While saponification of Boc-Afc-OMe in aqueous MeOH using NaOH proceeds in a satisfactory yield, giving approximately 80% of the free acid (Boc-Afc-OH) and about 15% of the decarboxylated product Boc-9-aminofluorene, we found that Boc-Afc-OH decarboxylates in a short period of time in the presence of tertiary amines in organic solvents. Therefore, carboxyl activation with EEDQ, which avoids the use of

one equivalent of tertiary amine [26], proved to be the best coupling method. Probably, quinoline, that is formed in this latter reaction, is too weak as a base to induce decarboxylation.

Crystal-state Conformational Analysis

The molecular and crystal structures of one Afc derivative, Boc-Afc-OMe, and one dipeptide, Boc-Afc-Gly-OMe, were determined by X-ray diffraction. Each compound has two independent molecules (**A** and **B**) in the asymmetric unit. The molecular structures with the atomic numbering schemes are illustrated in Figures 2 and 3, respectively.

Bond lengths and bond angles are in general agreement with previously reported values for the geometry of the *tert*-butyloxycarbonylamino [31] and methyl ester [32] groups, the peptide unit [33,34], and the fluorenyl moiety [35,36]. In particular, the $(sp^2)C-C(sp^2)$ bond, directly connecting the two phenyl rings (in the range 1.47–1.48 Å) is typical of a biphenyl system, and the two $(sp^3)C^{\alpha}-C^{\beta}(sp^2)$ bonds (in the range 1.51–1.53 Å) are reminiscent of the corresponding bonds in the benzyl moiety [37]. Preliminary evidence for the presence of the strain introduced by the fully-extended (C_5) structure in the Afc residues is provided by the values of the conformationally critical $N-C^{\alpha}-C'$ (τ) bond angle [7,15–17,34,38], experimentally found in the range 104–106°, very much compressed if compared with the tetrahedral value of 109.5°. Interestingly, the complementary $C^{\beta 1}-C^{\alpha}-C^{\beta 2}$ (τ') bond angle, usually expanded to 111–117° to compensate for the low value of τ [18,21,22], is also significantly compressed (100–102°) as it is internal to the cyclopentadienyl ring. In these Afc-containing compounds angular compensation arises from the wider than usual $N-C^{\alpha}-C^{\beta 1}$ and $N-C^{\alpha}-C^{\beta 2}$ bond angles (114–116°). The other bond angles of the Afc residues internal to the cyclopentadienyl system are in the range 108–112°, remarkably smaller than the classical bond angles at a sp^2 carbon (120°), but

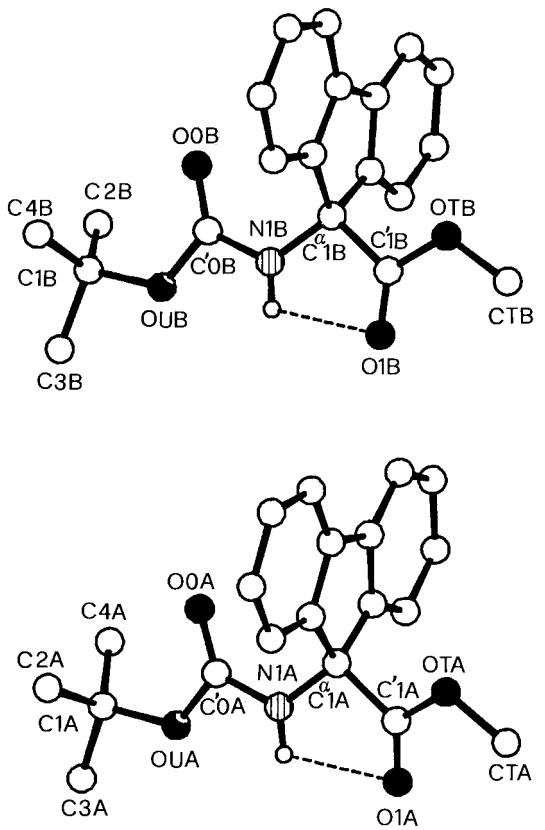

Figure 2 X-ray diffraction structures of the two independent molecules **A** and **B** in the asymmetric unit of Boc-Afc-OMe with numbering of the atoms (for clarity only the backbone atoms are labelled). The intramolecular H-bond is represented by a dashed line.

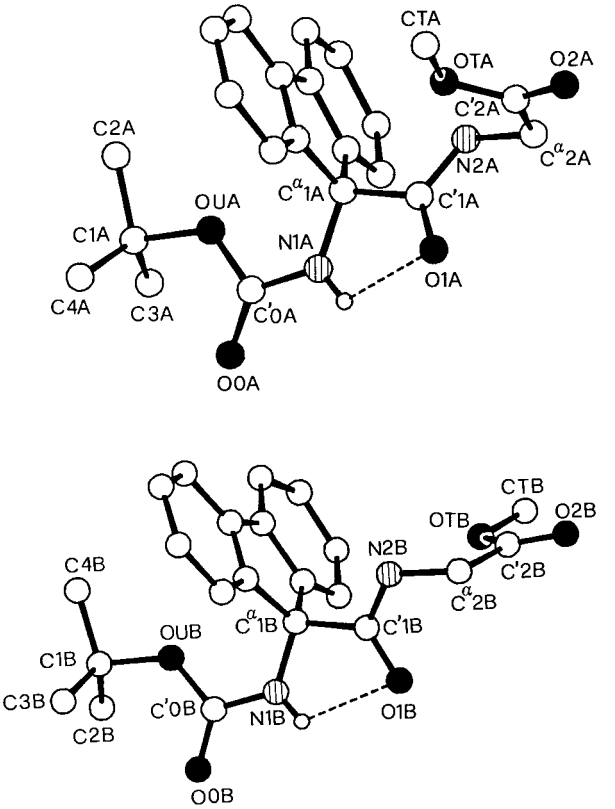

Figure 3 X-ray diffraction structures of the two independent molecules **A** and **B** in the asymmetric unit of Boc-Afc-Gly-OMe with numbering of the atoms (for clarity only the backbone atoms are labelled). The intramolecular H-bond is represented by a dashed line.

larger than those typical of a saturated cyclopentanyl system (104–107°) [13]. In the two Afc compounds some very large bond angles at sp^2 carbons (128–132°) are also found: C^{α}–$C^{\beta 1}$–$C^{\gamma 11}$, C^{α}–$C^{\beta 2}$–$C^{\gamma 21}$, $C^{\delta 12}$–$C^{\gamma 12}$–$C^{\gamma 22}$ and $C^{\gamma 12}$–$C^{\gamma 22}$–$C^{\delta 22}$ (with $C^{\gamma 11}$, $C^{\gamma 21}$, $C^{\delta 12}$, $C^{\delta 22}$ external to the cyclopentadienyl ring).

In each compound the Afc residue of both molecules **A** and **B** is characterized by an intramolecularly H-bonded, fully-extended C_5-ring structure [7,15–17] with the following sets of φ, ψ backbone torsion angles [39]: 179.8(2)°, −167.9(2)° and 178.8(2)°, −176.2(2)° for molecules **A** and **B** of Boc-Afc-OMe; 179.0(3)°, 157.0(3)° and 177.6(4)°, −158.4(3)° for molecules **A** and **B** of Boc-Afc-Gly-OMe. The N1···O1 intramolecular separation is 2.619(4) Å and 2.618(4) Å for molecules **A** and **B** of the amino acid derivative, while 2.624(5) Å and 2.588(5) Å for molecules **A** and **B** of the dipeptide.

In the four molecules of the two Afc compounds, characterized by the *tri*cyclic fluorenyl moiety, the dihedral angle between normals to the average planes of the two phenyl rings is $< 3.4(1)°$, indicating a good planarity for this system. By contrast, this angle is in the range 55–128° for the *bi*cyclic diphenyl system of the DΦg residue [18,21,22]. The side-chain χ^1 (N–C^{α}–C^{β}–C^{γ}) torsion angles of the Afc residues have values that can be grouped in two ranges, ±52.8(5)–56.0(5)° and ±124.8(4)–129.1(3)°. The dihedral angles between the normals to the planes of the fluorenyl and the C_5-ring systems are 92.5(1)° and 88.9(1)° for molecules **A** and **B** of the amino acid derivative, and 96.5(1)° and 94.6(2)° for molecules **A** and **B** of the dipeptide, i.e. the two planes are essentially orthogonal to each other.

The C-terminal Gly residue of the dipeptide is found in the *bridge* region of the conformational space [40] with φ, ψvalues of −79.2(4)°, 1.2(5)° for molecule **A** and 83.1(5)°, −3.2(6)° for molecule **B**. In the four molecules of the two compounds: (i) the ester –C(=O)–O– bonds [32] are *trans*, with a deviation from planarity not exceeding 3°; (ii) the peptide bond [33,34] (in Boc-Afc-Gly-OMe) is distorted *trans* planar, the ω_1 torsion angle being 164.5(3)° for molecule **A** and −169.9(3)° for molecule **B**; (iii) the urethane C(=O)–NH– bond [31] is *trans* in Boc-Afc-OMe, with ω_0 173.3(2)° for molecule **A** and 176.2(2)° for molecule **B**. However, this bond is *cis* in Boc-Afc-Gly-OMe, with ω_0 0.1(5)° for molecule A and −1.2(6)° for molecule **B**; (iv) the θ^1 torsion angle of the *tert*-butyloxycarbonylamino group [31] is *trans* planar, with a deviation of less than 10° from 180°; (v) the ester conformation with respect to the preceding C^{α}–N bond [41] is *synperiplanar* in the amino acid derivative, but it is *antiperiplanar* in the dipeptide.

A further, although indirect support to the occurrence of the intramolecularly H–bonded C_5 conformation in the Afc residues of the two compounds is based on the observation that the pertinent urethane N–H and peptide C′=O groups are *not* involved in the intermolecular H-bonding schemes. The crystal packing of the dipeptide is characterized by (peptide) N2A–H2A···O0B=C′0B (urethane) (x, y, z) and (peptide) N2B–H2B···O0A=C′0A (urethane) ($x-1$, y, z) intermolecular H-bonds, linking together molecules **A** to molecules **B**. The N2A···O0B and N2B···O0A separations are 2.898(4) Å and 2.886(5) Å, respectively, i.e. within the average range observed for a large number of N–H···O=C intermolecular H-bonds in peptide structures [42–44].

Solution Conformational Analysis

The preferred conformations of the N- and C-blocked Afc derivatives and peptides were assessed in a solvent of low polarity ($CDCl_3$) over the concentration range of 10–0.1 mM by using FT-IR absorption and ^{1}H-NMR.

The conformationally informative 3500–3300 cm^{-1} region of the FT-IR absorption spectra is unusually complex for an oligopeptide series based on a $C^{\alpha,\alpha}$-disubstituted Gly residue (Figure 4 and Table 2). We assign: (i) the high-frequency band (> 3430 cm^{-1}) to free, solvated, N–H stretching vibrators; (ii) the medium-frequency bands (in the range 3425–3395 cm^{-1}) to weakly intramolecularly H-bonded N–H stretching vibrators of fully-extended (C_5) conformers; (iii) the low-frequency band (< 3380 cm^{-1}) to strongly intramolecularly H-bonded N–H stretching vibrators of folded conformations [18,45]. No significant variation is observed in the spectra by changing derivative or peptide concentration. The assignment of the C_5 band is corroborated by its presence even in the simple amino acid derivatives, too short to form intramolecularly H–bonded folded forms. In the 1770–1620 cm^{-1} region bands are seen at 1753–1739 cm^{-1} (methyl ester C=O stretching vibrator), 1724–1688 cm^{-1} (Boc urethane C=O stretching vibrator), and 1684–1641 cm^{-1} (amide and peptide C=O stretching vibrators) [46].

A preliminary conformational conclusion, which may be immediately extracted from this IR absorp-

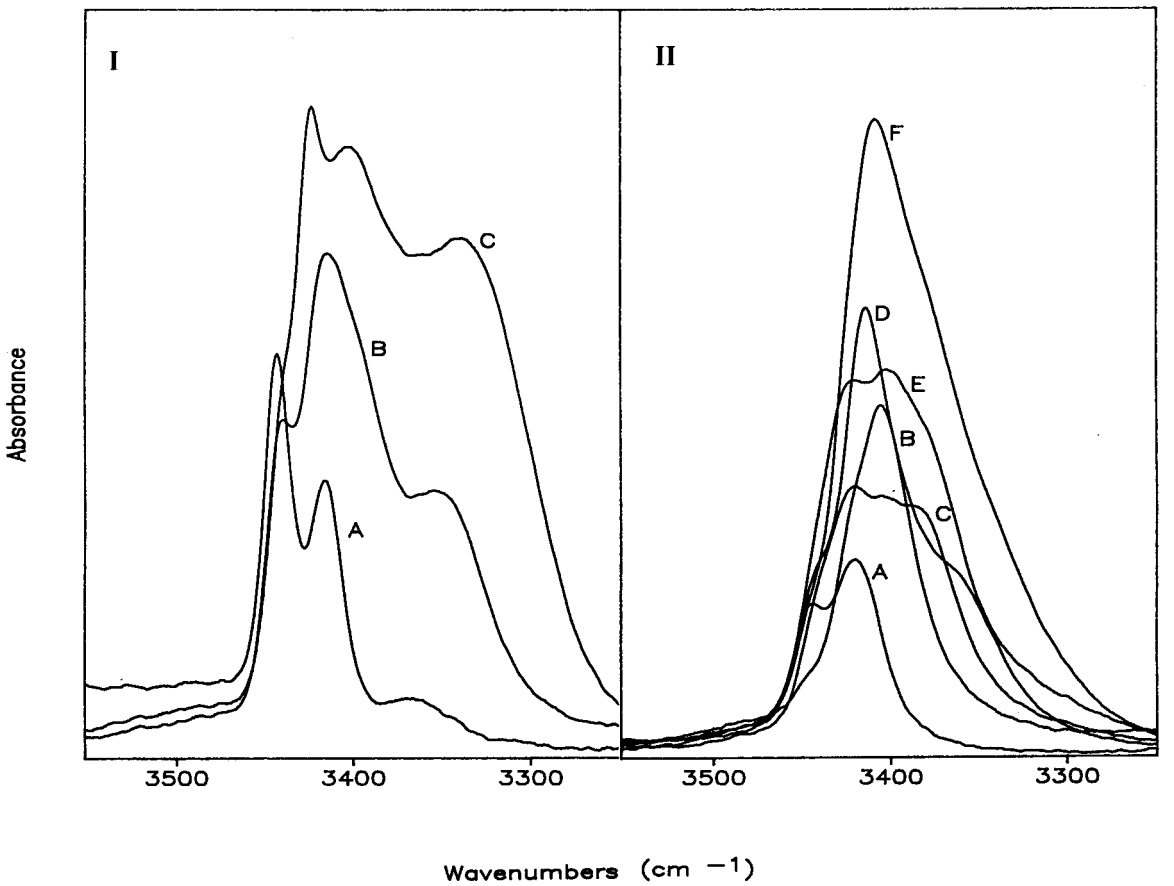

Figure 4 FT-IR absorption spectra (N–H stretching region) of: (I) the N^{α}-acetylated series, AcAfc-OMe (A), Ac-Afc-(L-Ala)$_2$-OMe (B), and Ac-Afc-(L-Ala)$_2$-Afc-L-Ala-OMe (C); and (II) the N^{α}-*tert*-butyloxycarbonylated series, Boc-Afc-OMe (A), Boc-Afc-L-Ala-OMe (B), Boc-L-Ala-Afc-L-Ala-OMe (C), Boc-Afc-(L-Ala)$_2$-OMe (D), Boc-(L-Ala)$_2$-Afc-L-Ala-OMe (E), and Boc-Afc-(L-Ala)$_2$-Afc-L-Ala-OMe (F). Peptide concentration 1 mM in $CDCl_3$ solution.

tion investigation is that, in general, the tendency of Afc peptides to give folded/helical structures in solution is rather low, and more specifically it is remarkably decreased with respect to that of the Ac_5c peptides of corresponding main-chain length. Peptides, where bands associated with bend/helical structures are clearly seen, are those characterized by an *internal* Afc residue. If the Afc is *N-terminal*, however, the fully-extended structure seems to largely predominate.

The delineation of inaccessible (or intramolecularly H-bonded) NH groups of the Afc peptides by ^{1}H-NMR was carried out using: (i) solvent dependence of NH chemical shifts, by adding increasing amounts of the strong H-bonding acceptor solvent DMSO [47,48] to the $CDCl_3$ solution and (ii) free-radical (TEMPO) induced line broadening of NH resonances [49]. As an example, Figure 5 illustrates the behaviour of the NH resonances of the pentapeptide Ac-Afc-(L-Ala)$_2$-Afc-L-Ala-OMe upon addition of DMSO and TEMPO. The NH proton assignment was performed in $CDCl_3$ solution with standard 2D homonuclear ^{1}H-NMR techniques. Two classes of NH protons were observed. Class (i) includes Afc^1 and Ala^3 protons, whose chemical shifts are sensitive, but only moderately if compared with other series of peptides based on cyclized $C^{\alpha,\alpha}$-disubstituted Gly residues, to the addition of DMSO and whose resonances broaden upon addition of TEMPO. Class (ii) includes Ala^2, Afc^4, and Ala^5 protons displaying a behaviour typical of shielded protons (relative insensitivity of chemical shifts to solvent composition and line widths to the presence of TEMPO). The same two classes of protons were also identified from an analysis of the spectra as a function of peptide concentration over the range 10–0.1 mM (not shown). The unusually high-field shift observed for the Ala^2 and Ala^5 NH proton

Table 2 FT-IR Absorption Data for the Afc Derivatives and Peptides

Compound	3500–3300 cm^{-1}	1770–1620 cm^{-1}
Boc-Afc-OMe	3447, *3420*[b]	1742, 1724, *1710*, 1690
Boc-Afc-L-Ala-OMe	3423[c], *3406*	1741, 1723, 1710, *1679*, 1641
Boc-Afc-(L-Ala)$_2$-OMe	3444, *3415*, 3361	1742, 1723, *1688*, *1670*
Boc-L-Ala-Afc-L-Ala-OMe	3442, *3423*, 3405, 3378	1742, 1707, 1696, *1677*
Boc-(L-Ala)$_2$-Afc-L-Ala-OMe	*3427*, *3401*, 3377	1741, 1698, *1673*
Boc-Afc-(L-Ala)$_2$-Afc-L-Ala-OMe	3448, 3424, *3413*	1741, 1724, 1698, *1680*
Ac-Afc-OMe	*3443*, 3415	*1739*, *1683*
Ac-Afc-(L-Ala)$_2$-OMe	3443, *3419*, 3409, 3395, 3344	1740, 1692, 1676, *1667*
Ac-Afc-(L-Ala)$_2$-Afc-L-Ala-OMe	3442, *3425*, 3401, 3338	1740, 1697, *1673*
Boc-Afc-Gly-OMe	3431, *3409*	1753, 1724, 1710, *1684*

[a] In $CDCl_3$ solution (peptide concentration 1 mM).
[b] Strong band.
[c] Very weak band.

resonances, as compared with other N^{α}-blocked peptides rich in different types of $C^{\alpha,\alpha}$-disubstituted Gly residues [50], may possibly be determined by the ring-current effect of the fluorenyl moiety of the two preceding Afc^1 and Afc^4 residues, respectively.

In summary, these ^{1}H-NMR results allow us to conclude that in $CDCl_3$ solution the conformational distribution in the Afc-rich peptides is distinctly different from that of peptides based on 3_{10}-helical forming $C^{\alpha,\alpha}$-disubstituted Gly residues [50], where protons assigned to residues 1 and 2 are *remarkably* sensitive to addition of DMSO and TEMPO, but rather provide evidence for populations of β-bend/3_{10}-helical conformations and fully-extended structures concomitantly occurring in the equilibrium mixtures of the Afc-rich peptides. These conclusions are in agreement with those extracted from the IR absorption study discussed above.

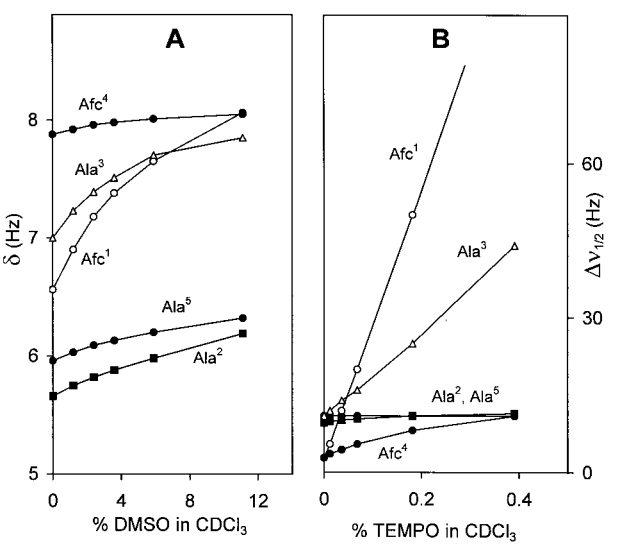

Figure 5 (A) Plot of NH chemical shifts in the ^{1}H-NMR spectrum of Ac-Afc-(L-Ala)$_2$-Afc-L-Ala-OMe as a function of increasing percentages of DMSO added to the $CDCl_3$ solution (v/v). (B) Plot of bandwidth of the NH signals of the same peptide as a function of increasing percentages of TEMPO (w/v) in $CDCl_3$. Peptide concentration 1 mM.

An additional striking feature of derivatives and peptides characterized by the N-terminal *Boc*-Afc sequence is noteworthy, namely the *trans* (*anti*)-*cis* (*syn*) CO–NH bond isomerization of the urethane (carbamate) moiety. Usually, the urethane *cis* rotamer exists in small amounts at room temperature and it is diffficult to identify ('hidden partner') [25]. This phenomenon, which, however, is surprisingly evident in the Boc-Afc peptides and results in rather complex ^{1}H-NMR spectra, is currently under investigation in our laboratories. It is interesting to note that neither a fully extended (C_5) conformation nor β-bend/3_{10}-helical structures involving the N-terminal sequence are compatible with a urethane *cis* rotamer.

Spectroscopic Characterization

We have spectroscopically characterized derivatives and peptides based on this novel aromatic $C^{\alpha,\alpha}$-disubstituted Gly residue using electronic absorption in the near UV region, fluorescence, and CD. Exciton coupling between the L_b benzenoid states of the fluorene chromophore [51,52] results in multiple absorption bands, which in Ac-Afc-OMe are seen at 305 nm ($\varepsilon = 1300$), 293 nm ($\varepsilon = 3200$), 281 nm ($\varepsilon = 11500$), and 270 nm ($\varepsilon = 14600$) (Figure 6). Afc is also suitable for internal fluorescence labelling of peptide molecules with its large quantum yield and

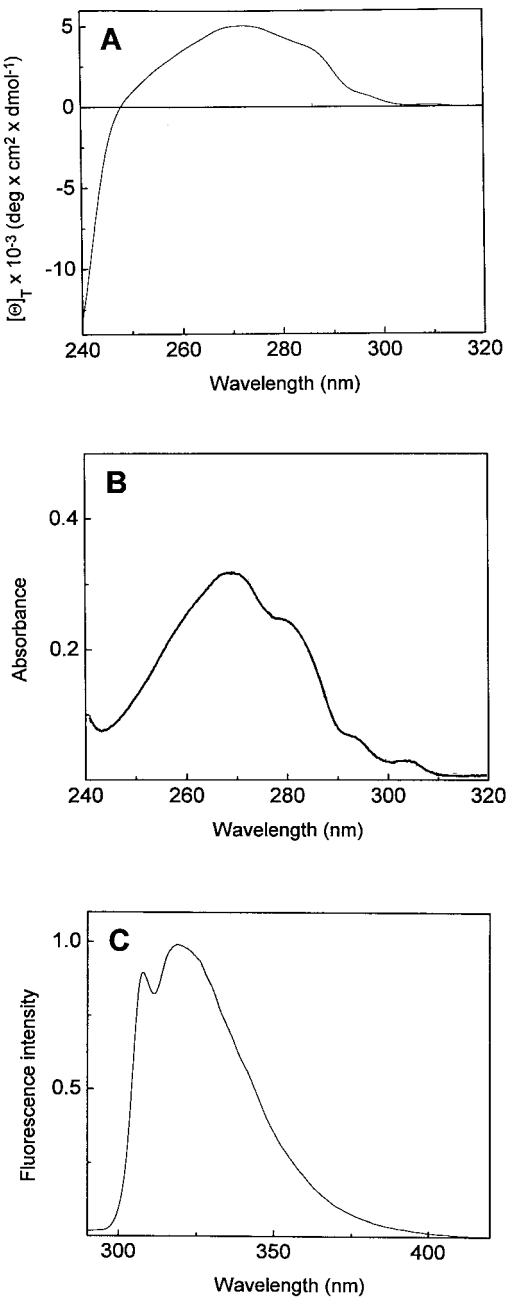

Figure 6 Near-UV spectroscopic properties of selected Afc derivatives and peptides in MeOH solution: (A) CD spectrum of Ac-Afc-(L-Ala)$_2$-OMe; (B) UV absorption spectrum of Ac-Afc-OMe; (C) fluorescence spectrum (excitation at 281 nm) of Ac-Afc-OMe.

a minor influence by the solvent composition, as already reported for other fluorene derivatives [53]. Figure 6 shows the emission spectrum of Ac-Afc-OMe with maxima at 328 and 308 nm following excitation at 281 nm. Finally, optical activity can be induced in the otherwise achiral Afc residue by incorporating this aromatic amino acid into an appropriate chiral peptide. In Figure 6 the near-UV chirospectroscopic properties of a representative Afc-containing peptide are shown. A broad Cotton effect of moderate intensity, characterized by an unresolved fine structure, centred at about 270 nm and assigned to the exciton split electronic transitions of the fluorene chromophore, is seen in the CD spectrum. It should be also noted that the UV absorption and fluorescence spectra of the Afc-based compounds discussed above parallel those published for peptides protected at the amino function(s) with the popular Fmoc group [53–56], that is characterized by a closely related chromophoric group.

CONCLUSIONS

The results of the present crystal-state and solution experimental investigation strongly support the view that the Afc residue, an additional member of the family of cyclic $C^{\alpha,\alpha}$-disubstituted Gly residues, favours conformations in both the fully-extended (C_5) and the folded/helical regions of the conformational space. Peptides, where a partial tendency to give folded/helical structures is found, are those with an internal Afc. However, when Afc is N-terminal, the fully-extended conformation appears to predominate. This overall conformational conclusion is not surprising in view of the observation that Afc represents a structural combination of: (i) Ac_5c, a $C_i^{\alpha} \leftrightarrow C_i^{\alpha}$ cyclized, $C^{\alpha,\alpha}$-disubstituted Gly residue with high bend/helix propensity [10–14], and (ii) DΦg, with $C^{\alpha,\alpha}$-symmetrically disubstituted, bulky side chains favouring the fully-extended conformation [18–22].

Considerable recent interest has been devoted to the development of conformationally restricted analogues of bioactive peptides [4,57–63]. The availability of highly active, structurally constrained agonist is of value in delineating the nature of the receptor-bound conformation. In this connection it may be expected that the introduction of Afc residues at carefully selected positions of bioactive peptides can be rewarding. We also believe that the fluorescence properties of Afc will represent a useful expansion of the arsenal of spectroscopic tools available to peptide chemists.

Acknowledgements

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