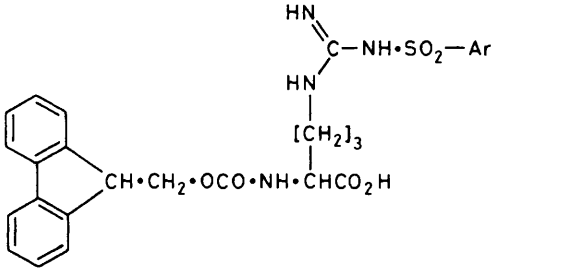
Side Chain Protected N^{α}-Fluorenylmethoxycarbonylamino-acids for Solid Phase Peptide Synthesis. N^{α}-Fluorenylmethoxycarbonyl-N^{G}-4-methoxy-2,3,6-trimethylbenzenesulphonyl-L-arginine

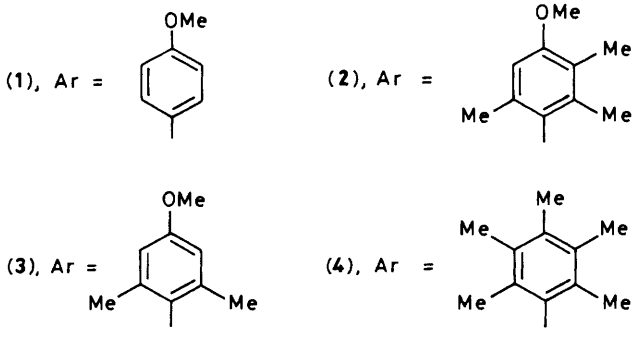
Eric Atherton, Robert C. Sheppard, and John D. Wade

Laboratory of Molecular Biology, The Medical Research Council Centre, Hills Road, Cambridge CB2 2QH, U.K.

The preparation and use in solid phase synthesis of N^{α}-fluorenylmethoxycarbonyl-N^{G}-4-methoxy-2,3,6-trimethylbenzenesulphonyl-L-arginine is described.

Protection of the guanidine side chain of arginine derivatives presents a continuing problem in both classical (solution) and solid phase peptide synthesis. In the latter, the introduction[1,2] of N^{α}-fluorenylmethoxycarbonyl (Fmoc) amino-acids[3] as alternatives to the customary t-butoxycarbonyl (Boc) derivatives has presented new requirements for stability and lability. Earlier studies[4] using the N^{α}-Fmoc derivatives of N^{G}-*p*-methoxybenzenesulphonylarginine (**1**) and N^{G},N^{G}-bisadamantyloxycarbonylarginine were not completely satisfactory. The former required excessively vigorous reaction conditions ($MeSO_3H$) for its removal; the latter is exceptionally difficult to prepare, tends in peptide synthesis to give significant ornithine-containing byproducts, and in our polar polyamide system is undesirably lipophilic. N^{α}-Fmoc-N^{G}-Boc-Arginine gave in our hands low yields in coupling reactions and appeared to be inadequately stable.† Protonation of the guanidine function as in Boc-Arg-HCl[1] does not provide protection appropriate to the Fmoc series. Substantial deprotonation of guanidinium salts occurs during simulated Fmoc deprotection [20% piperidine–dimethylformamide(DMF)], and subsequent reaction with acylamino-acid anhydride occurs at the guanidine nitrogens. These results prompted a further search for Fmoc-arginine derivatives suitable for solid phase peptide synthesis, and especially for solid phase fragment condensation strategies.





The N^{G}-arylsulphonyl group is placed arbitrarily on the guanidine function.

† See R. Colombo, *Int. J. Pept. Protein Res.*, 1982, **19**, 71, for a successful application.

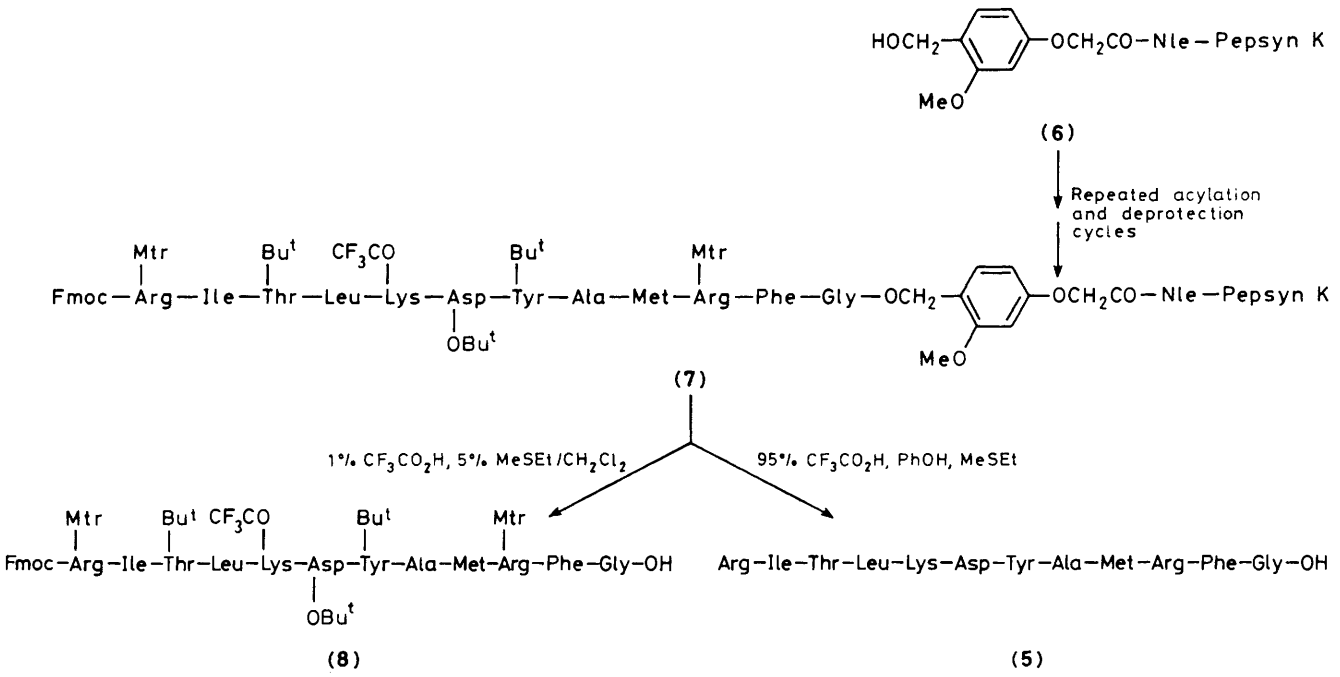
Encouraged by the report of Fujino *et al.*[5] that N^{α}-unprotected-N^{G}-4-methoxy-2,6-dimethylbenzenesulphonyl(Mds)-arginine showed appreciably greater lability to acid than did the simpler 4-methoxy derivatives (cleaved by CF_3CO_2H–thioanisole mixtures at 50 °C) we began a study of N^{α}-Fmoc derivatives of other side-chain substituted arylsulphonyl-arginines which might have even greater lability. The symmetrical 2,4,6-trimethoxy and 2,3,4,5,6-pentamethyl derivatives were selected in order to avoid problems of isomer formation.[6] While this was in progress, a further more comprehensive study by Fujino and his colleagues appeared.[7] 4-Methoxy-2,3,6-trimethyl(Mtr)arginine was the most labile (cleaved by CF_3CO_2H–thioanisole in *ca.* 1 h); the 2,4,6-trimethoxybenzenesulphonyl derivative was marginally more stable and the pentamethyl compound distinctly more so. Our studies now show that the fluorenylmethoxycarbonyl derivative of N^{G}-Mtr-arginine (**2**) is a promising reagent for use in solid phase synthesis.

Amorphous N^{α}-Fmoc-N^{G}-Mtr-Arg (**2**), m.p. 68—72 °C, is prepared by the reaction of N^{G}-Mtr-Arg[7] with fluorenylmethyl chloroformate in chloroform solution containing diisopropylethylamine, and purified by silica chromatography and precipitation from ethyl–light petroleum.‡ The yield is 50—65%. N^{α}-Fmoc-N^{G}-Mds-Arg (**3**), m.p. 127—129 °C, and the pentamethyl derivative (**4**), m.p. 123—125 °C, may be obtained similarly. The Mtr group appears to be completely stable to the condtions of Fmoc-group removal (20% piperidine–DMF). No cleavage was detected (h.p.l.c.) after 3.5 h, equivalent to 21 deprotection cycles. Complete removal of the Mtr group by CF_3CO_2H–thioanisole (5%) at room temperature was obtained after 2—2.5 h; reaction was slower in the presence of other scavengers (ethanedithiol, ethyl methyl sulphide, phenol, or a mixture of the last two), requiring *ca.* 5 h for completion, but see below.

Use of the new arginine derivative in solid phase synthesis is illustrated by assembly of the dodecapeptide (**5**) comprising residues 4—15 of the cro repressor protein sequence. The continuous flow variant[8] of the Fmoc-polyamide method[1] was used, with a new more highly substituted kieselguhr-supported polydimethylacrylamide gel functionalised with sarcosine methyl ester (0.23 mequiv./g including the inert mineral support).§ A permanently bound internal reference amino-acid (norleucine) was first introduced in the usual manner,[1] followed by the very acid labile dialkoxybenzyl alcohol linkage agent[9] giving the starting resin (**6**). Thereafter the Fmoc-amino-acid anhydrides in 3-fold excess were added sequentially. Reaction of the first residue (glycine) with the resin-bound linkage agent was catalysed by 4-dimethylaminopyridine.

‡ Details of this preparation may be obtained from the authors prior to full publication.

§ Cross-linked polydimethylacrylamide gel support in macroporous kieselguhr (Pepsyn K) is available from Cambridge Research Biochemicals Ltd., Button End Industrial Estate, Harston, Cambridge CB2 5NX, and from Omnifit Ltd., 51 Norfolk Street, Cambridge CB1 2LD.



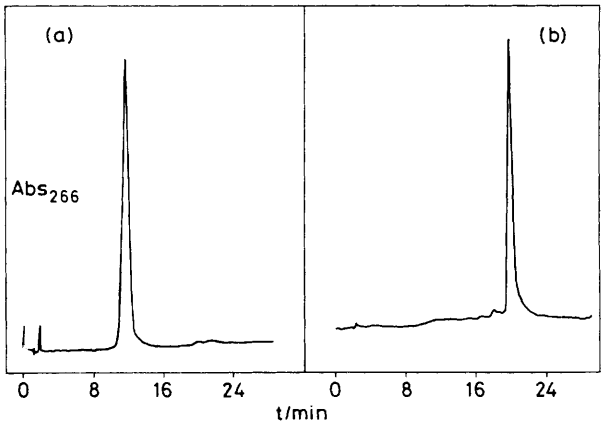


Figure 1. H.p.l.c. of total crude protected dodecapeptide (**8**). Conditions: (a) RadialPAK SiO_2 column; solvent A, $CHCl_3$; solvent B, $CHCl_3$–MeOH–AcOH (75:15:15); linear gradient of 20 to 80% B over 30 min at 3 ml min^{-1}. (b) μ-Bondapak C_{18} column; solvent A, 0.01 M NH_4OAc pH 4.5; solvent B, MeCN; linear gradient of 40 to 100% B over 30 min at 1.5 ml min^{-1}.

Intermediate Fmoc-peptide resins were deprotected with 20% piperidine–DMF. Tyrosine, threonine, and aspartic acid were side-chain protected by t-butyl ether, or ester, lysine as the N^{ε}-trifluoroacetyl derivative, and both arginine residues as Mtr derivatives. Methionine was unprotected. Ninhydrin and trinitrobenzenesulphonic acid colour tests for residual amine were negative after 25 min in all cases except for the two arginine residues. The first coupling (to phenylalanine-14) was complete after longer (overnight) reaction; the second (to isoleucine-5) still gave weakly positive colour tests after this period and the acylation was repeated. It was then complete within 1 h. Thus the formation or coupling rate of Fmoc-Arg-(Mtr) anhydride is slower than for most Fmoc-amino-acids, although in earlier experiments using the original less substituted (0.11 meguiv./g) kieselguhr-supported resin, reaction was apparently complete after the usual 25 min period.¶

A sample of the final peptide-resin (**7**) (Found: Asp, 0.92; Thr, 0.92; Gly, 1.00; Ala, 0.98; Met, 0.94; Ile, 0.81; Leu, 0.92; Tyr, 0.91; Phe, 0.96; Lys, 0.95; Arg, 1.77; initial Gly/Nle, 0.90; final Gly/Nle, 0.87) was cleaved with 1% CF_3CO_2H in dichloromethane containing methyl ethyl sulphide (5%) to yield the amino and side chain protected dodecapeptide (**8**). The crude product was passed down Sephadex LH20 to remove residual scavengers and CF_3CO_2H–DMF complexes. Both amino-acid analysis (Found: Asp, 0.98; Thr, 1.05; Gly, 1.00; Ala, 1.04; Met, 1.00; Ile, 0.94; Leu, 1.02; Tyr, 1.05; Phe, 1.05; Lys, 0.95; Arg, 1.86) and h.p.l.c. on reversed phase [Figure 1(b)] and Radialpak silica columns [Figure 1(a)] indicated a very satisfactory assembly. A second sample of resin was deprotected and cleaved by treatment with 20% piperidine–DMF and then CF_3CO_2H–phenol–methyl ethyl sulphide (95:2.5:2.5, 6 h). Scavengers were removed by extraction with ethyl acetate, and the remaining N^{ε}-trifluoroacetyl protecting group removed with 1 M aqueous piperidine. After desalting on Sephadex G25, ion-exchange chromatography on carboxymethylcellulose gave the elution profile of Figure 2. Dodecapeptide (**5**) from the main peak (Found: Asp, 0.98; Thr, 1.00; Gly, 1.00; Ala, 0.95; Met, 0.95; Ile, 0.85; Leu, 0.95; Tyr, 0.94; Phe, not determined; Lys, 0.95; Arg, 1.88) gave the h.p.l.c. profiles shown in Figures 3(a) and (b). The overall yield was 50%. The forerunning impurity has the same elution time as the dodecapeptide sulphoxide formed by hydrogen peroxide oxidation of the mixture [Figure 3(c)].

Our results indicate that Fmoc-Arg(Mtr) is a useful reagent for the introduction of arginine residues in solid phase peptide synthesis. Cleavage of the side-chain protecting group requires

¶ Recent quantitative studies on rates of acylation using kieselguhr-supported polydimethylacrylamide resins have shown that, for other amino-acids, reaction is more than 80% complete after a single passage through the column (contact time *ca.* 1 min) (Dryland and Sheppard, to be published).

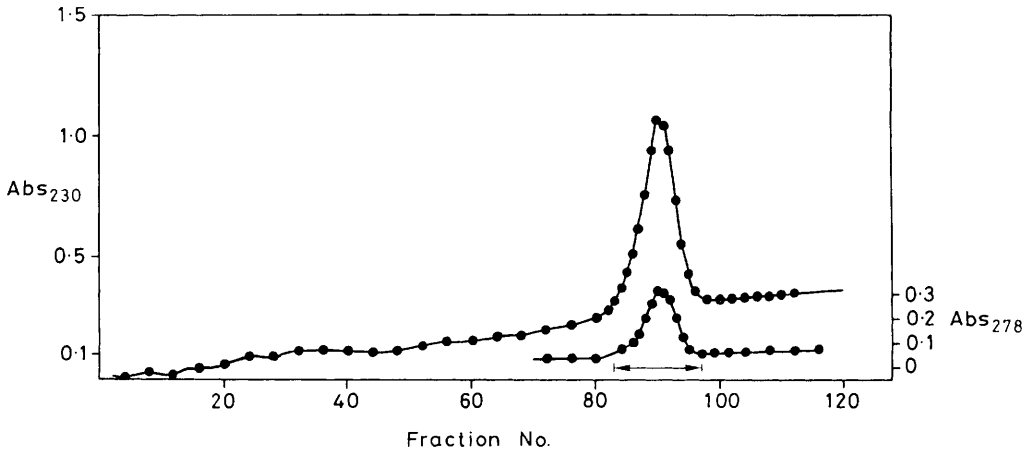


Figure 2. Ion-exchange chromatography of dodecapeptide (**5**). Conditions: CM52 column 11.6 × 1 cm diameter; linear gradient of 0.01 to 0.20 M NH_4OAc pH 4.5 (250 ml of each). Fraction size 2.9 ml.

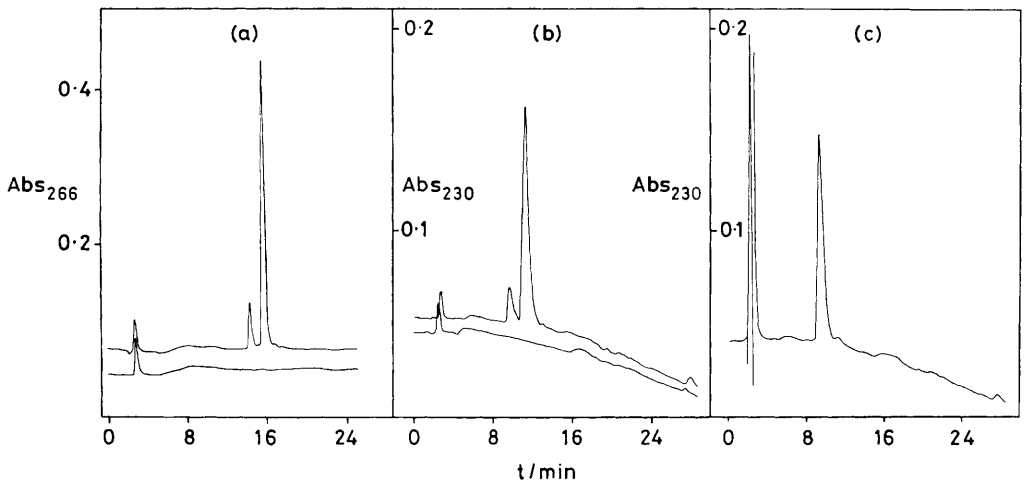


Figure 3. H.p.l.c. of dodecapeptide (**5**) after ion-exchange chromatography. Conditions: μ-Bondapak C_{18} column; (a) solvent A, 0·083 M triethylamine phosphate pH 3.2; solvent B, 40% A–60% MeCN; linear gradient 20 to 80% B over 30 min at 1.5 ml min^{-1}. (b) Solvent A, 0.01 M NH_4OAc pH 4.5; solvent B, 10% A–90% MeCN; linear gradient 20 to 80% B over 30 min at 1.5 ml min^{-1}. (c) As in (b) 10 min after treating the dodecapeptide (16 nmol) in H_2O (25 μl) with 30% H_2O_2 (2 μl). In (a) and (b), the lower traces are solvent blanks.

protracted treatment with trifluoroacetic acid, but in the example cited, this did not apparently result in serious (irreversible) side reactions. The conditions adopted differ from those found by ourselves and by Fujino *et al.*[7] (CF_3CO_2H containing 10% thioanisole) to give the most rapid cleavage of Mtr groups, but in our experience give purer products even though contact time is extended. This is particularly noteworthy for peptides containing side-chain-protected cysteine residues, which may be substantially destroyed by CF_3CO_2H–thioanisole mixtures.[10]

Received, 9th June 1983; Com. 761

References

1 E. Atherton, C. J. Logan, and R. C. Sheppard, *J. Chem. Soc., Perkin Trans.* 1, 1981, 538.
2 J. Meienhofer, M. Waki, E. P. Heimer, T. J. Lambros, R. C. Makofski, and C.-D. Chang, *Int. J. Pept. Protein Res.*, 1979, **13**, 35.
3 L. A. Carpino and G. Y. Han, *J. Am. Chem. Soc.*, 1970, **92**, 5748; *J. Org. Chem.*, 1972, 3404.
4 E. Atherton, V. Woolley, and R. C. Sheppard, *J. Chem. Soc., Chem. Commun.*, 1980, 970.
5 M. Fujino, O. Nishimura, M. Wakimasu, and C. Kitada, *J. Chem. Soc., Chem. Commun.*, 1980, 668.
6 C. W. Smith, G. Skala, and J. R. Boal, *J. Chem. Soc., Chem. Commun.*, 1981, 1243.
7 M. Fujino, M. Wakimasu, and E. Kitada, *Chem. Pharm. Bull. Jpn.*, 1981, **29**, 2825.
8 E. Atherton, E. Brown, and R. C. Sheppard, *J. Chem. Soc., Chem. Commun.*, 1981, 1151; R. C. Sheppard, *Chem. Br.*, 1983, 402.
9 R. C. Sheppard and B. J. Williams, *Int. J. Pept. Protein Res.*, 1982, **20**, 451.
10 E. Atherton and P. Ward, to be published.