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The Fluorenylmethoxycarbonyl Group in Solid Phase Synthesis

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Abstract: The history of the fluorenylmethoxycarbonyl amino-protecting group since its introduction into solid phase peptide synthesis in 1978 is briefly traced.

Keywords: Fmoc; fluorenylmethoxycarbonyl protection

A hundred years ago, a new era in peptide and protein chemistry was opened when Fischer and Fourneau synthesized glycylglycine and recognized its relevance to the structure of proteins [1]. Modern peptide synthesis, however, probably did not begin until 30 years later when Bergmann and Zervas introduced the first easily removed urethane amino-protecting group [2]. It required another 30 years before the Merrifield solid phase method simplified the substantial experimental difficulties and made synthesis of larger peptides and proteins practical objectives [3].

Merrifield's first technique [3] (Figure 1a) used the Bergmann and Zervas [2] benzyloxycarbonyl (Z) group for amino protection, but it was cleaved by strong acid (hydrogen bromide in acetic acid) rather than the original mild catalytic hydrogenolysis. The supporting polystyrene resin was nitrated to provide a nitrobenzyl ester peptide-resin linkage which required even more vigorous acidic conditions for its ultimate cleavage. Less than a year later in his important paper describing the synthesis of bradykinin [4], this protecting group combination was replaced by the more easily cleaved t-butoxycarbonyl (Boc) and simple benzyl ester (Figure 1b), with a dramatic increase in overall efficiency. This point is worth making at the outset because it shows that from the start, Merrifield recognized the importance of mild reaction conditions if even moderately sized peptides, let alone proteins, were to be attainable by solid phase synthesis. This Boc/benzyl/polystyrene combination (Figure 1b) was widely and rapidly adopted and has become known as 'the Merrifield technique'.

Others were clearly still concerned about the vigour of reaction conditions in the revised Merrifield technique, and a number of alternative protecting groups was soon suggested [5], notably the very acid-labile trityl, *o*-nitrophenylsulphenyl, biphenylisopropxycarbonyl and α,α-dimethyl-3,5-dimethoxybenzyloxycarbonyl groups. Any one of these in combination with t-butyl or *p*-alkoxybenzyl derivatives for side chain protection and resin linkage could have provided distinctly milder overall reaction conditions, yet none displaced or even seriously competed with the established Boc derivatives. It may be that there were chemical disadvantages to each. Like the Boc/benzyl combination, none provided complete differentiation in the reaction conditions for cleavage of the temporary α-amino and more permanent side chain protecting groups (lack of orthogonality). The non-availability of commercial amino-acid derivatives, the wide measure of success and popularity of the established Merrifield

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technique, and general conservatism are also likely to have been powerful factors.

Our own work in this field began some 10 years later when a peptide chemistry group was established in the Medical Research Council Laboratory of Molecular Biology in Cambridge. We soon found that molecular biologists were seldom interested in short peptides. Proteins and nucleic acids were their working materials. Previous experience elsewhere with the 129-residue sequence of lysozyme had shown that stability of a natural protein to solid phase synthesis conditions, particularly to the final liquid hydrogen fluoride cleavage reaction of the Merrifield technique, might be misleading [6]. It is the stability of the unfolded, unstructured polypeptide chain which has to be considered. The observation that this may be distinctly less stable to strongly acidic reagents than the folded globular protein was later confirmed with the shorter pancreatic trypsin inhibitor. (E. Atherton, unpublished). Other small proteins, e.g. the 66-residue λ-cro repressor protein were unstable even to prolonged contact with trifluoroacetic acid [7]. Protein-like objectives would clearly require the mildest and most efficient reaction conditions if they were to become reliably attainable by solid phase (or any other) synthetic method.

During the following 5 years, we endeavoured to apply sound chemical principles to the development of efficient but mild solid phase procedures which might make small proteins more reliably accessible by synthesis [8]. The results[†] are summarized in Figure 2. The new features introduced were:

1. Use of Carpino's [9] base-labile fluorenylmethoxycarbonyl (Fmoc) amino-protecting group (1) with t-butyl derivatives for side chain protection. This provided for the first time a truly orthogonal combination of temporary and permanent protecting groups removable under exceptionally mild conditions.[‡]
2. A polar polyamide resin support well solvated (swollen) by a range of solvents (particularly dimethylformamide) which were also good solvating agents for protected peptides and oligonucleotides. Dimethylformamide is also a kinetically good solvent for many acylation and non-acidic deprotection reactions. The resin was prepared both as a soft beaded gel and in a more rigid, physically supported form enabling synthesis to be carried out under continuous flow conditions. Spectroscopic measurement of fluorene absorption in the flowing stream then enabled a useful degree of reaction and synthesizer monitoring to be achieved simply. This simple monitoring, especially of the deprotection reaction, later proved critical in understanding the phenomenon of 'difficult sequences' (see below).
3. A range of individual peptide-resin linkage agents enabling detachment of the completed peptide from the resin support with or without retention of side chain protecting groups. The

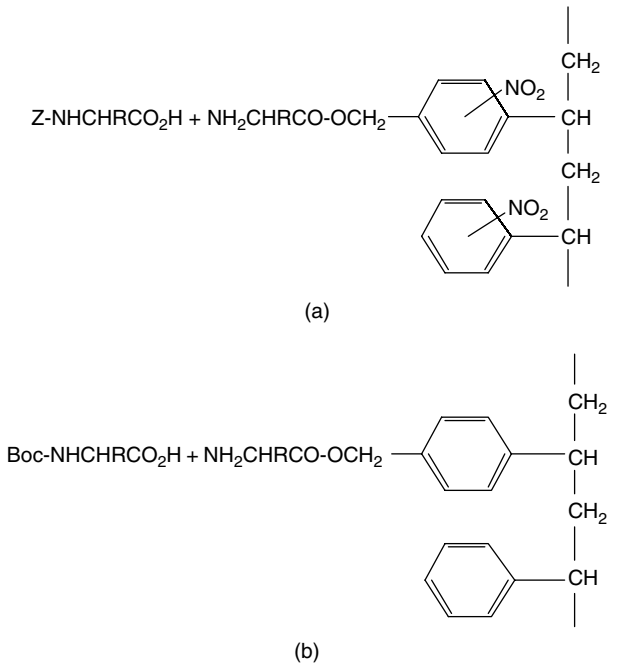


Figure 1 The Merrifield solid phase synthesis technique (b), and its forerunner (a).

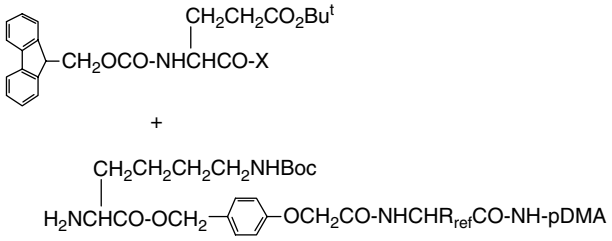


Figure 2 The Fmoc-polyamide technique. pDMA, Polar, poly(dimethylacrylamide) resin, physically supported for continuous flow synthesis; X, -OPfp or other activating groups.

[†] My long standing colleague Eric Atherton played a major role in the successful development of Fmoc-based solid phase synthesis. I am grateful to him and to all our other colleagues for their excellent work.

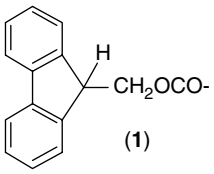
[‡] We found later that Johannes Meienhofer and his colleagues were simultaneously exploring use of the Fmoc-group in polystyrene-based solid phase synthesis [10].

p-alkoxybenzyl linker shown is cleaved by acids under the same conditions as side chain Boc and t-butyl groups.

4. Incorporation of a permanently bound, internal reference amino-acid facilitating analytical control of the synthesis through amino-acid analysis.
5. In addition, as the problem of 'difficult sequences' became more and more apparent, we later turned our attention to its detailed understanding and solution within the above framework (see below).

Most of these developments were completed 25 years ago and it would have been an indictment of organic chemistry if some had not been superseded by more recent innovations. Much, however, has withstood this test of time. The fluorenylmethoxycarbonyl/t-butyl protecting group combination continues to gain in popularity; this short review is concerned mostly with this aspect of present day solid phase synthesis [11]. The polydimethylacrylamide resin has been widely used but is now being replaced in many laboratories by more easily made alternatives based upon polyethylene glycol. The concept of a polar resin matrix solvated by a polar, kinetically good organic solvent remains sound. Novel peptide resin linkage agents continue to be enthusiastically devised and extensively used. The widespread availability of mass spectrometry has reduced dependence on amino-acid analysis of peptides and peptide resins and the value of internal reference amino acids.

Perhaps the most surprising very recent trend has been the decline in Fmoc-based continuous flow synthesis brought about by the reduced availability of commercial flow synthesizers. The method brought the advantages of simplicity and economy, was efficient and popular amongst its users, and had the facility for monitoring both coupling and deprotection reactions and the correctness of synthesizer function. This degree of control is not easily achieved in batchwise synthesizers. True automation (feedback analytical control of both coupling and deprotection reactions) has only been implemented in continuous flow instruments. The decline of the flow method seems to have been a consequence of commercial rather than scientific factors. Both the pioneering companies in the field (LKB and Cambridge Research Biochemicals) were acquired by larger organizations, and there were further mergers and take-overs before the last continuous flow instrument (the Perseptive Pioneer) was very recently squeezed from the market place. Perhaps it should be emphasized that it has proved very straightforward to construct flow synthesizers in laboratory workshops, and that considerable detail of early instruments has been published [8,29].



(**1**)

Carpino and Han introduced the fluorenylmethoxycarbonyl group (**1**) into solution peptide synthesis in 1972 [9]. Its exceptional lability to bases, especially to secondary amines, is a consequence of the activation of the ring proton β to the urethane oxygen by participation in a potential cyclopentadienide system. Cleavage probably then follows an E1cb elimination mechanism. Carpino's suggestion initially fell upon stony ground and had little immediate effect on the practice of peptide synthesis. The reason for this is easy to see. The primary products of the cleavage reaction (Figure 3) are the carbamate salt (**3**) and dibenzofulvene (**2**). The latter is a reactive, involatile solid (c.f. the volatile and easily removed products of Boc and Z cleavage) which may undergo polymerization and addition reactions, especially with the cleaving amine. These side products may not be easy to separate when the synthesis is carried out in solution as Carpino suggested. In solid phase synthesis, however, they may be simply washed away. This is especially true in continuous flow synthesis where the side products are removed from contact with the resin as they are formed, also effectively preventing significant back addition of the amino-component or its carbamate salt (**3**). The immediate fate of the latter has not been investigated. It probably exchanges with the large excess of secondary amine reagent present. In contrast to acidic cleavage of the Boc group, no free carbon dioxide is evolved and in continuous flow synthesis there is correspondingly no disruption of the resin bed.

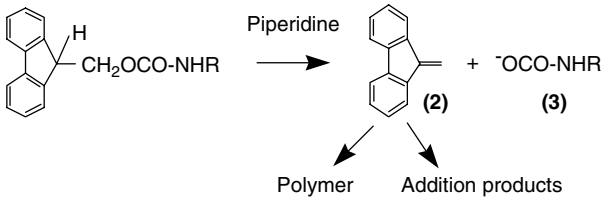


Figure 3 Cleavage of Fmoc-derivatives by secondary amines.

Not all the properties of the Fmoc group are advantageous. Fmoc-amino-acid derivatives are not always easy to obtain pure using Carpino's fluorenylmethyl chloroformate reagent. We received a number of early reports from other laboratories of difficulties experienced in using the Fmoc-technique which were most probably due to the use of impure Fmoc-amino-acid derivatives. This problem was effectively solved by improved laboratory practice and the introduction of the O-Fmoc-hydroxysuccinimide (Fmoc-OSu) reagent [12]. The Fmoc group is particularly hydrophobic and amino-acid derivatives have lower solubility than their Boc analogues. Activated derivatives (particularly symmetrical anhydrides) may crystallize from solution during their preparation. The hydrophobicity of the Fmoc group may conceivably contribute to the formation of 'difficult sequences', though these appear to be primarily determined by amino acid sequence and side chain protecting groups [13]. Some side reactions induced by the cleaving base, notably sequence dependent formation of succinimide derivatives from asparagine and β-t-butyl aspartate residues, have been noted [11]. Complete solutions to the 'difficult sequence' and aspartimide problems are described later.

We were fortunate that at least two companies, Cambridge Research Biochemicals in Cambridge, UK, and Chemical Dynamics, New Jersey, were quick to recognize the potential of Fmoc-amino-acid derivatives and soon made them available commercially. There is no doubt that this encouraged their rapid and widespread adoption, though even now they are still distinctly more costly than their Boc analogues. On a molar basis, Fmoc-glycine is twice as expensive as Boc-glycine, and Fmoc–t-butyl glutamate six times more than Boc-benzyl glutamate.[§] In fact, the cost of amino-acid derivatives may not be the dominant factor in peptide synthesis. A detailed analysis of a recent large scale synthesis showed that the cost of solvents and other reagents may be equally or more significant [14], even in continuous flow synthesis which is especially economical in solvent usage.

It is difficult to gauge accurately the extent to which Fmoc-based solid phase synthesis has now replaced the standard Merrifield technique. Much synthesis is now carried out in commercial laboratories and is often unpublished. Regrettably it is more and more exceptional to find in the literature detailed descriptions of synthetic methods used. Nevertheless, a survey of five recent volumes of the two prime journals, The *Journal of Peptide Chemistry* and the *Journal of Peptide Science* (official publications of the American and European Peptide Societies, respectively) yielded nearly 300 papers using peptides prepared by solid phase synthesis in which their origin from Boc or Fmoc techniques was indicated. The results are summarized in Table 1.

In the European Society's journal, nearly two and half times as many papers used Fmoc-chemistry as Boc; in the American only just over one and a half times. Both journals are, of course international in their scope, and too much weight should not be given to these results as indicating national trends, though it is certain that Fmoc chemistry was more rapidly and enthusiastically adopted in Europe than in the United States. It may be that these figures actually underestimate the present popularity of the Fmoc group. In the UK, a major supplier of both Fmoc and Boc-amino-acid derivatives reported a 9 : 1 sales ratio in favour of the former. Probably the uptake of the Fmoc-method was substantially increased by a very favourable comparative report [15] of the Association of Biomolecular Research Facilities. The conclusion of the present survey is that both techniques are still operated alongside each other and in forms largely unchanged since their introduction. The Merrifield technique has been in use for 40 years and Fmoc-based synthesis for more than 25. Just as in Boc-based solid phase synthesis where many alternative but similarly conceived acid-labile groups were suggested but failed to compete, a similarly large number of base-labile protecting groups, e.g. (**4**)–(**12**), have been advocated as alternatives to Fmoc. The simple methylsulphonyl-ethoxycarbonyl group (4, R = Me) [17] was included in our initial survey [16], but was considered to be insufficiently base-labile. The

Table 1 Solid Phase Synthesis Techniques used in Recent Publications of the *Journal of Peptide Research* and *Journal of Peptide Science*. Five Volumes of Each Journal were Scanned

	J. Pept. Res.	*J. Pept. Sci.*
Total papers using SPPS	170	128
Using Boc-benzyl chemistry	64 (38%)	37 (29%)
Using Fmoc-t-butyl chemistry	106 (62%)	91 (71%)

[§] Source: Novabiochem catalogue, 2002/3.

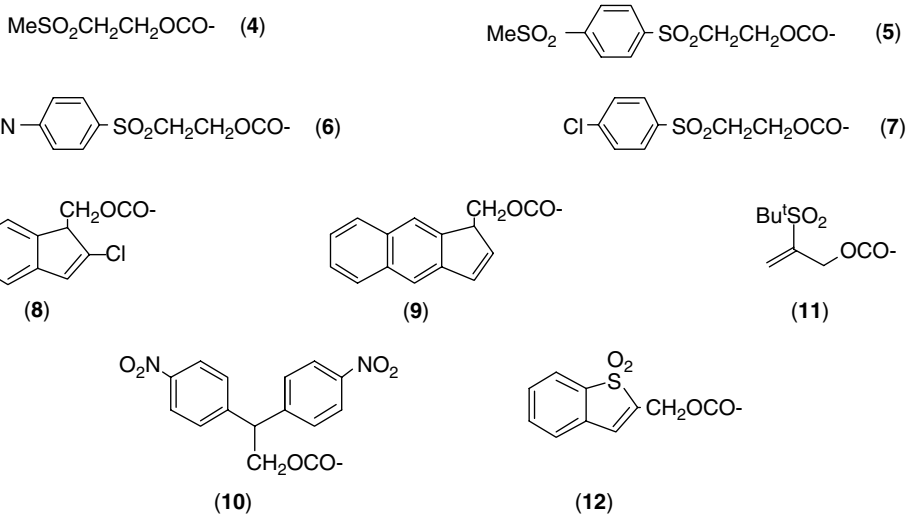
three more recent related compounds (**5**) [18], (**6**) [19,20], and (**7**) [21] represent attempts to increase this reactivity by replacement of the terminal methyl group by various negatively substituted aromatics. The chloroindene (**8**) and benzoindene (**9**) derivatives are variants by Carpino [22] on the original Fmoc cyclopentadiene structure. Compound (**10**) is a simple negatively substituted diphenylmethane [23]. All of the foregoing rely for their base lability on the presence of an activated hydrogen β to the urethane oxygen. Two more original structures (**11**, **12**), also from Carpino's laboratory, generate intermediate anions through Michael addition to activated double bonds [24,25].

None of the foregoing yet show signs of rivalling the Fmoc group, though in most cases their originators point out possible advantages.

Mention has been made earlier of the problem of 'difficult sequences' in solid phase synthesis. These are sequence dependent regions in a synthetic objective characterized by a sudden reduction in the rates of both deprotection and coupling reactions [26]. The latter are frequently prevented from proceeding to completion causing the synthesis to fail. They are believed to be a consequence of internal association of the peptide chains within the resin matrix. Possibly hydrogen bonding in β-sheet type structures are involved. The polar solvent, polar resin combination was introduced to minimize association effects, and there is little doubt that this is effective in comparison with earlier apolar polystyrene/dichloromethane-based techniques, but this minimization is not complete. Any widely applicable method of solid phase synthesis must be able to handle such 'difficult' sequences.

Structures relying on hydrogen bonding for their stability might be disrupted by replacement of the peptide bond hydrogen by a suitable substituent. Replacement of all the N–Hs, i.e. essentially use of secondary rather than primary amino acids throughout, is barely a feasible approach in view of the serious steric hindrance of acylation associated with many secondary residues. In our examination of the difficult sequence phenomenon using the spectrophotometric monitoring capability afforded by the Fmoc-group in continuous flow synthesis [13], we were relieved to find that a single N–H replacement was capable of dissociating a substantial length of peptide chain. Only about every sixth residue needed to be substituted to prevent association in a susceptible sequence. Since difficult sequences usually span only a restricted length, commonly less than 15 residues beginning 5 or 6 residues from the carboxy terminus, the design of a suitable reversible *N*-substituent immediately offered a feasible approach.

N-benzyl groups were obvious candidate substituents but there were conflicting requirements. To maintain the mild reaction conditions which was such an important feature of the Fmoc-solid phase method, a ring substitution pattern was required which allowed easy removal at the end of the synthesis. Two ring methoxy groups permitted acidic cleavage under the same conditions as the side chain t-butyl groups and an alkoxybenzyl peptide-resin linkage. However, terminal *N*-2,4-dimethoxybenzyl peptides presented unacceptable steric hindrance



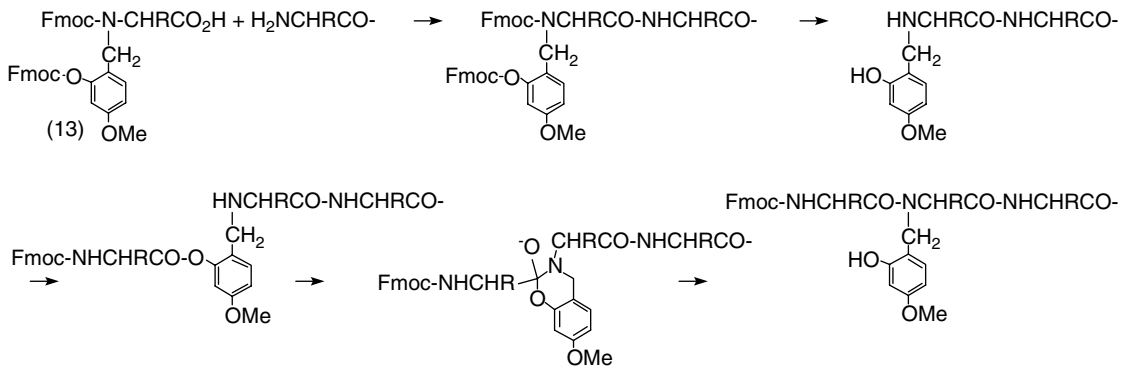
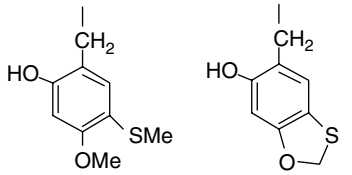


Figure 4 Use of the *N*-(2-hydroxy-4-methoxybenzyl) (Hmb) group in peptide synthesis.

to acylation (chain elongation). Replacement of the benzene ring by thiophene or furan (effectively eliminating one of the ortho substituents) did not provide a useful less hindered substituent. However, replacement of the *o*-methoxy by a hydroxy group provided an N-benzyl amino acid derivative which retained the acid lability of the dimethoxy analogue but which also provided a markedly less hindered coupling mechanism (Figure 4). Acylation of the secondary amino group could now occur through initial, internally base-catalysed reaction with the unhindered phenolic oxygen followed by O-to-N migration. The *N*,*O*-bisFmoc amino acid derivatives (**13**) have provided a very practical route to the synthesis of *N*-(2-hydroxy-4-methoxybenzyl) peptides and further chain extension, provided that the activation method and location of the substituent in the peptide chain were chosen carefully [26].

The rate limiting step in *N*-acylation of *N*-(2-hydroxy-4-methoxybenzyl) derivatives appeared to be the *O*-to-*N* rearrangement. Molecular modelling indicated significant steric crowding in the 6-membered cyclic intermediate. The acylation rate might be usefully enhanced by increasing the reactivity of the initially formed phenyl ester (Figure 4), but again there is a substitution pattern conflict. Increased reactivity of the phenyl ester required electron withdrawing substituents in the ring which would reduce the acid lability of the group at the end of the synthesis. In our hands, *N*-nitrobenzyl substituents could not be cleaved under usefully mild acidic reaction conditions.[¶] A possible solution to this dilemma is application of the 'safety catch' principle in which an electron withdrawing, rate enhancing ring substituent is modified at the end of the synthesis to become electron donating and favouring easy cleavage. An attractive candidate is the reduction of electron withdrawing sulphoxide to electron donating sulphide,[#] Two *N*-substituents (14,15) have been developed incorporating this feature but have yet to be extensively tested in peptide synthesis [30,31].



N-2-hydroxy-4-methoxybenzyl (Hmb) amino-acids are readily prepared by reductive alkylation using the appropriate benzaldehyde. In a number of applications [26,27] using the bisFmoc derivative (**13**), introduction of Hmb substituents has substantially eliminated or reduced the problems caused by internal association and improved the quality of the solid phase synthesized products. Incorporation in the residue on the carboxy side of aspartic acid also eliminates the possibility of succinimide formation. The presence of a free hydroxyl group after incorporation provides added versatility. Simple acylation, for example, effectively abolishes the acid-lability so that peptides retaining *O*-acyl-Hmb substituents may be cleaved from the resin. These peptides are commonly much more soluble than the free peptides facilitating chromatographic purification. The acyl substituent (a phenyl ester) may then be simply

[¶] Miranda *et al.* have pointed out that a 2-hydroxy-4-nitrobenzyl substituent might be cleaved photochemically [32].

[#] For a very early application of this safety catch in solid phase synthesis, see ref [29].

cleaved along with the terminal Fmoc group by treatment with piperidine or another nucleophile, and the Hmb substituent(s) removed with acid in the usual manner. Similarly, they may be used for the preparation of soluble fully protected fragments suitable for fragment condensation synthesis. Thus not only do Hmb derivatives facilitate solid phase synthesis, they also offer a solution to the very long-standing problems associated with the common insolubility of protected peptide fragments in solution synthesis.

Fmoc-based synthesis is now firmly established alongside the Merrifield technique. It has proved both efficient and versatile, is useful for both stepwise (single amino acid addition) and fragment condensation procedures, and has provided analytical techniques which were seriously lacking in other procedures. It encouraged the development of continuous flow synthesizers, ultimately with feed back analytical control and full automation [28]. It has been applied to the synthesis of modified peptides including lipopeptides and phosphorylated and glycosylated derivatives, to the multiple synthesis of peptides and in combinatorial techniques. The associated Hmb derivatives can suppress side reactions, facilitate synthesis of 'difficult sequences' and the purification of solid phase-synthesized peptides, and contribute to solution synthesis by opening a route to more soluble fragments. Until the ingenuity of organic chemists provides something better, Fmoc-chemistry is clearly the procedure of choice for peptide synthesis in the twenty-first century.

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