LAWRENCE
LIVERMORE
NATIONAL
LABORATORY

UCRL-JRNL-231463

# Studies in Solid Phase Peptide Synthesis: A Personal Perspective

A. R. Mitchell

June 4, 2007

Biopolymers:Peptide Science

# Studies in Solid Phase Peptide Synthesis: A Personal Perspective*

Alexander R. Mitchell

*Energetic Materials Center, Lawrence Livermore National Laboratory, University of California, 7000 East Avenue, L-282, Livermore, CA 94550, USA.*

*Phone: (925) 422 7994, E-mail: mitchell4@llnl.gov*

*This paper is dedicated to the memory of Bruce Merrifield (1921 – 2006) and former members of his laboratory that predeceased him: Balz Gisin (1940 – 1982), Mark Riemen(1953 –1993), Bruce Erickson (1942 – 1998) and Daniel Caldi (1945 – 2002).

**Abstract**

By the early 1970s it had became apparent that the solid phase synthesis of ribonuclease A could not be generalized. Consequently, virtually every aspect of solid phase peptide synthesis (SPPS) was reexamined and improved during the decade of the 1970s. The sensitive detection and elimination of possible side reactions (amino acid insertion, $N^{\alpha}$–trifluoroacetylation, $N^{\alpha\varepsilon}$–alkylation) was examined. The quantitation of coupling efficiency in SPPS as a function of chain length was studied. A new and improved support for SPPS, the "PAM-resin," was prepared and evaluated. These and many other studies from the Merrifield laboratory and elsewhere increased the general acceptance of SPPS leading to the 1984 Nobel Prize in Chemistry for Bruce Merrifield.

**Key words:**

Solid phase peptide synthesis; SPPS; side reactions; amino acid insertions; $N^{\alpha}$–trifluoroacetylation; $N^{\alpha\varepsilon}$–alkylation; coupling efficiency in SPPS; deletion sequences; PAM-resin

## Introduction

Bruce Merrifield's concept of an insoluble resin–bound peptide chain, a soluble activated amino acid and solvent to effect solid phase peptide synthesis (SPPS), so patently obvious today, constituted a new, revolutionary approach to organic synthesis that was eventually recognized with a Nobel Prize in Chemistry (1984). The early research, characteristically understated by Bruce in his autobiography,[1] provided challenges of heroic proportions, with respect to both scientific hurdles and severe resistance by the synthetic organic community. Garland Marshall, Bruce Merrifield's first graduate student (1963 – 1966), recalled the early "vehement and vitriolic" critics in his discussion of SPPS as a paradigm shift.[2] Bruce, a man modest in demeanor but strong in character, persevered. The rest, of course, is history.[1]

My goal as a graduate student in Roger Roeske's laboratory (Indiana University, 1964-1969) was to modify the recently developed SPPS, originally developed for the synthesis of linear peptides, to the synthesis of a series of cyclic peptides designed to model the active sites of serine proteases (chymotrypsin, trypsin). Suffice it to say that this goal was not totally achieved, despite some effort. The linear precursors were ultimately prepared by SPPS after difficulties in coupling non-protein amino acids were resolved. Subsequent purification and cyclization in solution provided the desired enzyme model.[3-5] My mixed experiences with SPPS prior to joining the Merrifield laboratory in 1969 fueled some skepticism regarding the method. Consequently, much of my subsequent research in SPPS focused on gaining a better understanding of the process and hopefully improving the method so that even severe critics would be mollified. What follows is a record of such efforts.

## Amino Acid Insertions in SPPS

In SPPS, peptide bonds usually are formed through the reaction of excess N-protected amino

acid and a coupling reagent such as dicyclohexylcarbodiimide (DCC) with amino acid or peptide derivatives of polystyrene. Brenner stated that acylation of peptide bonds, followed by aminoacyl insertion, may be possible under such conditions of "overactivation" [6,7] (Figure 1). This presents an interesting paradox in that the use of excess acylation reagents in SPPS to efficiently promote peptide bond formation (increase product homogeneity) would also favor amino acid insertions (decrease product homogeneity) if Brenner is correct. As even a low-level occurrence of amino acid insertions during SPPS would be unacceptable, it was critical that a sensitive detection of this side reaction be developed. As I was very much aware of product heterogeneity in SPPS from my thesis research, I designed a simple model system to test for amino acid insertions as a possible source of product heterogeneity in SPPS [8].

It is appropriate, however, to first step back in time and consider what instrumentation was available for the sensitive detection of low-level side reactions during the first 15-20 years of SPPS. HPLC, mass spectrometry and high resolution NMR, tools that now enable detection of side-products (insertions, deletions, rearrangements) in target peptides, were not readily available during this period. Fortunately, amino acid analyzers employing ion-exchange chromatography for the separation and detection of amino acids[9] and derivatives[10] were common in peptide and protein chemistry laboratories. These instruments could also be used to separate and detect mixtures of small peptides,[11,12] thereby allowing the determination of relatively low-level side reactions ($\geq$ 0.1%) in model peptide systems.

The model system used to detect possible amino acid insertions during SPPS[8] is illustrated in Figure 2. Glycine was employed since insertion reactions should be most favored in the absence of bulky side chains. Large excesses (11–22 equiv) of Boc-Gly-OH and DCC were used to promote acylation of the peptide bond. An amino acid analyzer calibrated with H-($Gly_n$)-OH

(where n = 1 through 4) was used to detect the products obtained from the acidolytic cleavages (HBr-TFA) of peptide resin products **1** and **2**. Cleavage of **1** did not reveal anything larger than the expected H-$(Gly_2)$-OH (<0.1% H-$(Gly_3)$-OH and H-$(Gly_4)$-OH). Cleavage of **2** gave a trace peak eluting at the position of H-$(Gly_4)$-OH (0.2%) in addition to the expected H-$(Gly_3)$-OH. The presence of H-$(Gly_4)$-OH could result from amino acid insertion as postulated by Brenner and/or a "double insertion" reaction observed with glycine derivatives under certain conditions of SPPS.[13] At any rate, the simple model system showed that amino acid insertions, though possible, are not significant side reactions under the usual conditions of SPPS.

These early results were later substantiated when a sensitive mass spectrometric technique that showed no insertion peptides could be detected (< 0.03%) in a 21-residue peptide prepared by SPPS.[14] Model studies enable us to examine side reactions, whether real or imagined, and improve our understanding and use of SPPS.

**The Rockefeller University**

***Preliminaries***

In 1967 I sent letters of inquiry to Robert Schwyzer (ETH, Zurich, Switzerland) and Bruce Merrifield regarding postdoctoral research in their laboratories. The Schwyzer reply was negative (sorry, no funding available). Bruce was positive and asked that what sort of research I would like to do in his laboratory. I sent Bruce a lengthy letter summarizing my work using SPPS and gave him my view of future problems and prospects of SPPS. Bruce liked what he read and offered me a position in his laboratory beginning in 1969.

I officially started working in the Merrifield laboratory as a postdoctoral research associate on June 2, 1969. Unofficially, I jumped the gun and started a week earlier (Memorial Day, May 26) without remuneration, as I was ecstatic at the prospect of working in the Merrifield laboratory at

The Rockefeller University and living in New York City. Bruce has described The Rockefeller University, his scientific base for over 55 years, in rich detail.[1] The high concentration of internationally acclaimed scientists on a campus encompassed by only five city blocks on the East Side of Manhattan was quite impressive. Equally impressive was the generous collegiality and civility displayed by the senior faculty to young and unknown scientific arrivals on campus – certainly not what an outsider might expect in New York City.

***Beginnings***

What to do and where to start? A colleague in the Midwest described a certain famous chemist (Nobel Laureate) who exerted total and minute control over his research group. Every day his secretary would post instructions on what reaction was to be run by each postdoctoral researcher. The same chemist was also accessible only through appointments with the secretary. The Merrifield laboratory was the antithesis of such an environment. Bruce was always accessible and the only requirement he had was that each graduate student and postdoctoral research associate work on a project of mutual interest (mainly to fulfill grant requirements) with as much freedom as they were willing to accept.

Recall that in early 1969 Bernd Gutte and Bruce Merrifield published the use of SPPS to achieve the total synthesis of an enzyme with ribonuclease A (Rnase A) activity.[15] This achievement, coupled with a similar effort by the Merck group using classical solution chemistry[16] attracted global attention in the scientific and popular press. Gutte's single-handed achievement, published only ten years after the concept of SPPS was recorded in Merrifield's laboratory notebook, was the scientific equivalent of a "grand slam" in baseball (a home run hit with all the bases occupied). How does one match that feat? It was unclear to me at the time that other proteins of comparable size could be prepared in acceptable purity using existing SPPS methodology.

Consequently, I focused on the synthesis of cyclic peptides by SPPS, a project I had started before coming to New York.

**Synthesis of cyclic peptides by SPPS**

My interest in cyclic peptides began during graduate studies with Roger Roeske when we prepared a new class of enzyme models.[3-5] Our model incorporates p-aminobenzoyl residues into a cyclic peptide to provide a relatively apolar cavity that might serve as a substrate binding site in aqueous solution. The peptide bridges between the p-aminobenzoyl residues allow for the variation in ring size and placement of functional side chains to form a catalytic site (Figure 3). Our synthesis began using classical, solution techniques until Roger visited the Merrifield laboratory in 1964 to assess SPPS. Roger, a forward looking organic chemist who had done post-doctoral research with Vladmir Prelog and Vincent du Vigneaud, returned from New York enthused about the future of SPPS. I began to look for ways to use SPPS to provide a convenient and rapid synthesis of cyclic peptides.

***Polynitrophenol supports***

The first demonstration that solid supports could be used to prepare cyclic peptides was provided by Fridkin and co-workers[17] and is illustrated in Figure 4. The key steps are (I) attachment of a benzyloxycarbonyl (Z) protected peptide to a cross-linked poly-4-hydroxy-3-nitrostyrene resin **3** using a 3-fold excess of Z-peptide and DCC-DMF coupling, (II) deprotection of the Z-peptide resin **4** by HBr-HOAc and (III) neutralization of the peptide resin hydrobromide **5** in $Et_3N$-DMF with cyclization in the same solvent to produce the cyclic peptide product **6**. Steps (I) and (III**)** are problematic. The coupling of Z-peptide to the nitrophenol support in step (I) using DCC-DMF will be accompanied by racemization via the well-known formation of oxazolones observed during the activation and coupling of peptide fragments. Step (III) features the

neutralization and cyclization of a polymeric peptide nitrophenyl ester. The release of a peptide oxazolone from the polymer support prior to cyclization cannot be ruled out. The formation of a peptide oxazolone would of course nullify any advantage of site isolation as a peptide oxazolone, if formed, will be in solution and free to racemize as well as cyclize, dimerize and polymerize. Initially, the use of peptide derivatives of cross-linked polymers to favor intramolecular reactions (cyclizations) over competing oligomerization was thought possible as the reactant molecules were considered attached to the polymer at relatively large intermolecular distances thereby providing a situation termed as "infinite dilution at finite concentration"[18]. It was shown by others that copolystyrene-2% divinylbenzene is not a rigid polymer in which specific sites maintain their separation during reaction.[19] For example, when only 0.5% or 1 out of 200 phenyl residues in copolystyrene-2% divinylbenzene are substituted with carboxymethyl groups, 50% of the carboxymethyl groups are available to form inter-site symmetrical anhydrides. This research, in addition to other work cited by the authors, led to the conclusion that reports which imply site separation must be explained on the basis of favorable kinetic relationships between the desired reactions and competitive reactions. However, discussions regarding kinetics, site isolation [20] or combinations of both factors detract from the practical advantage that polymer-supported syntheses of cyclic peptides have over syntheses carried out in solutions. This advantage has motivated subsequent workers to explore extensions of Fridkin's findings for the preparation of cyclic peptides by SPPS.[21]

***General approach for the synthesis of cyclic peptides by SPPS***

A general approach to preparing cyclic peptides by SPPS is presented as a generalized scheme in Figure 5. The scheme combines the synthesis of a linear peptide followed by cyclization. The synthesis of the linear peptide begins with the attachment of the carboxyl group of the C-terminal

amino acid to the polymer support. Schemes utilizing the attachment of various amino acid side-chains to a polymer support[22] represent a less general approach and are not discussed here. The linker attached to the C-terminal amino acid serves a dual function. It must be stable to the conditions of SPPS that involve repeated couplings, deprotections and base neutralizations during the step-wise synthesis of the linear peptide. Then, upon completion of the linear sequence, the activated peptidyl-linker must be susceptible **only** to an intramolecular attack by the amino group of the N-terminal amino acid of the peptide to yield the desired cyclic peptide under conditions that preclude oxazolone formation and possible racemization. How can this be accomplished?

***Mercaptophenol supports***

Consider three polymer supports used for the synthesis of cyclic peptides (Figures 6-8). Support **7**, prepared by the reaction of 4-mercaptophenol with chloromethylpolystyrene-2%-divinylbenzene [23,24] is acylated with a Boc-amino acid using DCC or mixed anhydride coupling to provide derivative **8** which is elongated by conventional SPPS to the desired protected peptide derivative **9** (Figure 6). Oxidation of **9** with 3-chloroperbenzoic acid yields the corresponding sulfone derivative **10** which is deprotected (HCl-HOAc) to give the peptide hydrochloride derivative **11**. Suspension of **11** in excess $Et_3N$-DMF for 18 hours provides cyclic peptide **12**. The synthesis in Figure 6 extends the approach in Figure 5 in using a polymer support to effect **both** synthesis of the linear peptide sequence and subsequent cyclization to cyclic peptide. However, both approaches expose polymeric peptide active esters to $Et_3N$-DMF during cyclization reactions (Figure 4: **5** -> **6** and Figure 6: **11 -> 12** ). The potential for oxazolone formation and subsequent racemization prior to cyclization must be recognized and evaluated.

***Arylhydrazine supports***

Wieland and coworkers,[25] in a very thorough and detailed paper, extended the work of earlier researchers[26,27] in examining the use of peptide derivatives of phenylhydrazine in solution and solid phase chemistry. The aryl hydrazine support **13** was prepared by the esterification of Boc-4-aminobenzoic acid with chloromethylpolystyrene-2%-divinylbenzene (Figure 7). Deprotection of **13** and elongation using Boc/benzyl chemistry employed earlier on mercaptophenol support[23] (Figure 6) gave protected peptide derivative **14**. Transesterification of **14** ($Et_3N$-$CH_3OH$) afforded Boc-hexapeptide methyl ester **15**. Deprotection of **14** and **15** provided the corresponding peptide trifluoroacetates **16** and **17**, which were treated with NBS and pyridine in THF to give peptidyl-diazenes **18** and **19**. Treatment of a THF solution containing **19** (high dilution at ≈ 1 mM) with triethylamine (~ 60 equiv.) for 72 h gave cyclic peptide **20** in 10% yield after detection and isolation from a thin layer chromatogram calibrated with an authentic sample of **20** prepared by alternative synthetic routes. Treatment of resin- supported peptidyl-diazene **18** in an analogous fashion gave a crude reaction mixture with **20** detectable on a thin layer chromatogram but attempts to isolate **20** failed. Similarly, attempts to cyclize a linear peptide having the sequence of antaminide (cyclic decapeptide from the poisonous mushroom *Amanita phalloides*) failed to produce an isolable yield of antaminide using the same aryl hydrazine support. In contrast, antaminide is obtained in 25% yield when the linear precursor is cyclized in solution.[28] While the SPPS of linear peptide sequences on the aryl hydrazine support proceeded without incident, cyclizations went poorly, if at all, when compared with analogous cyclizations in solution. My attempts to cyclize peptides from peptidyl-diazene resins were also not encouraging as low yields of partially racemized cyclic products were invariably observed. It should be noted that earlier workers observed some racemization (≤ 4.5%) when peptidyl-hydrazides were oxidized and used in peptide coupling reactions.[26,27] Clearly, Wieland

demonstrated that the use of aryl hydrazine supports for the SPPS of cyclic peptides was contraindicated[25] and more promising alternatives should be investigated.

It should be observed, however, that 31 years later Rosenbaum and Waldmann reinvestigated Wieland's system and were able to prepare cyclic peptides by SPPS using aryl hydrazine supports.[29] One deciding factor appears to be an efficient purification tool (HPLC) not available to Wieland in 1970. Accordingly, a cyclic hexapeptide was prepared and isolated in 19% yield while a cyclic heptapeptide (isolated from the marine organism *Stylotella aurantium)* was obtained in 7% overall yield. The claim that intramolecular cyclization from the peptidyl-diazene support proceeds without racemization bears scrutiny, however. The possibility of partial racemization of the C-terminal amino acid after oxidative conversion to the peptidyl-diazene was modelled using Fmoc-L-Ala attached to an arylhydrazine support. Oxidation of the Fmoc-L-Ala-hydrazide support to the acyldiazene derivative followed by reaction with H-L-Phe-OMe gave only Fmoc-L-Ala-L-Phe-OMe (analysis by chiral HPLC) which "unambiguosly proved that epimerization had not occurred [29]". Agreed, but **only** in the model dipeptide system investigated. It is well-known in peptide chemistry that urethane-protected amino acids couple **without epimerization** and segment coupling of peptides not containing C-terminal glycine or proline couple with **with epimerization** to some degree.[30] The preparation, separation and detection of a cyclic peptide target and its corresponding diastereoisomer will help determine to what extent peptidyldiazenes epimerize during intramolecular cyclizations.

***Catechol supports***

The supports described above (Figures 4, 6 and 7) have inherent shortcomings, especially with respect to racemization potential during peptide cyclizations. Consequently, the preparation and use of peptide monoesters of catechol to prepare cyclic peptides via SPPS seemed very

promising. The remarkable resistance of peptide monoesters of catechol to racemization,[31,32] coupled with the high reactivity of 2-hydroxyphenyl esters when compared to the corresponding phenyl esters, led to their use for the preparation of peptide polymers.[33] Use of the monobenzyl ether of catechol allows the synthesis of relatively inert peptide phenyl ester monomers. Removal of the benzyl ether ("safety catch") provides the more reactive peptide monoesters of catechol used in polymerization reactions. This work suggested a route to the SPPS of cyclic peptides that might overcome the drawbacks of the approaches described in Figures 4, 6 and 7.

The new support would feature a catechol monobenzyl ether (*o*-benzyloxyphenol) attached to the usual copolystyrene-divinylbenzene support employed in SPPS (Figure 8). Kun[34] had prepared hydroquinone-quinone redox polymers through the Friedel-Crafts alkylation of hydroquinone and derivatives (1,4-diacetoxybenzene and 1,4-dimethoxybenzene) using chloromethylated polystyrene resins. The reaction is carried out in refluxing ethylene dichloride in the presence of zinc chloride and can be followed by the evolution of hydrogen chloride.

Analogously, catechol reacts with chloromethylated polystyrene to give catechol resin **21** while use of *o*-benzyloxyphenol[32] should give the monobenzyl-catechol support **22** (Figure 8**)**. Acylation of **22** with a Boc-amino acid will give **23** and peptide elongation using Boc/benzyl chemistry provides peptide derivative **24**. Treatment of **24** with HBr-HOAc removes the N-Boc and O-benzyl protecting groups to give the peptide-catechol monoester **25** which is reacted in $Et_3N$-DMF to provide cyclic peptide **26** and catechol resin **21**. Eventually it was found that the scenario in Figure 8 could not be realized due to the conditions of the Friedel-Crafts reaction used to prepare **22**. While reaction of *o*-benzyloxyphenol with chloromethylated polystyrene may initially provide support **22,** the release of hydrogen chloride in refluxing ethylene dichloride will convert support **22** to the debenzylated catechol resin **21** (Figure 9). Subsequent

efforts to selectively protect one of the two hydroxyl functions in resin **21** were unsuccessful. Monoacylated catechols can be obtained from the corresponding cyclic sulfite derivatives.[35] This allows the preparation of peptidyl-catechol derivatives **25** for use in cyclization studies (Figure 10). The use of Boc-$Pro_3$-OH to form the 9-atom cyclo-[$Pro_3$] provides the most meaningful model as oxazolone intermediates cannot be formed during the cyclization reaction. Rothe first prepared cyclo-[$Pro_3$] in 88% yield through the cyclization of H-$Pro_3$-OH in solution using the phosphite and *p*-nitrophenyl ester methods [36]. Preparations of cyclo-[$Pro_3$] using the $Pro_3$-catechol resin **25**, the H-$Pro_3$-*p*-nitrophenyl resin **26** and H-$Pro_3$- *p*-nitrophenyl ester **27** are compared in Figure 10. H-$Pro_3$-catechol resin **25** afforded cyclo-[$Pro_3$] in $\leq$ 5 % yield, while **26** furnished cyclo-[$Pro_3$] in 20-30% yield and **27** gave cyclo-[$Pro_3$] in 36% yield in an non-optimized solution synthesis. The low potential for peptide cyclizations to occur on catechol resin supports was confirmed in other model systems.[37] Jones, in later reports, also recognized the sluggish reactivity of *o*-hydroxyphenyl esters when compared with ordinary active esters ( *p*-nitrophenyl, pentachlorophenyl or succinimido), especially in dipolar aprotic solvents (DMF, DMSO) where aminolysis is even retarded somewhat.[38,39] Suffice it to say that the cyclic peptide project that I brought to New York became inordinately time consuming (when to stop?), inconclusive (just one more model study?) and less than satisfying. At any rate, there were other, more pressing issues to address in SPPS.

**Amino Acid Insertions Revisited**

Bruce has contrasted his objectives and tactics in the initial development of SPPS with later refined studies (see p. 177 in reference 1). The first stage was a qualitative effort to put together chemistry that would provide a viable scheme for SPPS. The next stage involved obtaining semi-quantitative data to demonstrate that the technique was basically satisfactory. The last stage

involved highly quantitative experiments designed to detect and eliminate low-level side reactions. An example of the last stage is illustrated in a study utilizing urethane-protected amino acids and mixed anhydride couplings by Merrifield, Mitchell and Clarke.[13] In 1973 Bruce was still able to spend some time in the laboratory and, together with Joan Clarke, one of Bruce's many outstanding technician assistants (see p. 97 in reference 1), we explored the possibility of amino acid insertions occurring in SPPS during mixed anhydride couplings. The $N^{\alpha}$-2-(4-biphenylyl)-propyloxycarbonyl (Bpoc) derivatives of Gly, Ala and -Leu were activated (ethyl chlorocarbonate and triethylamine in methylene chloride, 0° C) and subsequently used in coupling reactions (25° C) with H-Val-resin to produce the expected H-Leu-Ala-Gly-Val-OH as well as a by-product identified as H-Leu-Ala-Gly-Gly-Val-OH (4%). Initially, we thought this unexpected product arose from acylation of the urethane nitrogen of Bpoc-Gly-Val-resin and/or acylation of the amide nitrogen of Bpoc-Gly-Val-resin and subsequent insertion as postulated by Brenner.[6,7] Mitchell and Roeske[8] had earlier demonstrated that acylations of urethane and amide nitrogens do not significantly occur in a system employing Boc-Gly-OH and DCC couplings (Figure 1). The same finding was made in this study when it was shown that Bpoc-Gly-Val-resin was not acylated at the urethane nitrogen by symmetrical or mixed anhydrides of Bpoc-Gly, by Bpoc-Gly activated with dicyclohexylcarbodiimide, or by leucine-N-carboxyanhydride.[13] It was determined that urethane acylation occurred before the activated intermediate (Bpoc-Gly-$OCO_2Et$) was coupled to the H-Val-resin in the original synthesis. The mechanism for this side reaction involves disproportionation of the mixed anhydride of Bpoc-Gly-OH to the symmetrical anhydride, and intramolecular rearrangement of the latter to form N-Bpoc-$N^{\alpha}$-(Bpoc-Gly)-Gly-OH, which is subsequently activated by anhydride interchange and reacts with Val-resin to yield N-Bpoc-$N^{\alpha}$-(Bpoc-Gly)-Gly-Val-resin.[13] Rearrangement is

dependent on the temperature and time of mixed anhydride formation and is undetectable after activation at -15° C (10 min) and coupling at -15° C (2 h). No urethane acylation (< 0.1 mol %) was observed during coupling of Bpoc-Gly-OH with Val-resin when DCC was used under standard SPPS conditions.

**N-Alkylation during the Acidolytic Cleavage of Urethane Protecting Groups**

The motivation for this project came from a report of 20% N-alkylation during the acidolytic cleavage of a Bpoc group from a derivative of hydroxylamine.[40] An analogous N-alkylation during the acidolytic cleavage of Boc groups during SPPS could offer an explanation of the rise in background observed with picrate monitoring during SPPS.[41]

The deprotection of $N^{\alpha}$-Boc-peptide-resin **28** to yield small quantities (~ 0.1-1%) $N^{\alpha}$-*tert*-butyl peptide **29** during each deprotection step is undesirable because it would give rise to terminated chains or N-alkyl peptides (Figure 11). Also, the production of a variable amount of hindered secondary amines would give corresponding increases in background when picrate[41] or chloride[42] monitoring methods were used to follow the course of a solid phase peptide synthesis. I devised a model system (Figure 12) and found no evidence of $N^{\alpha}$-*tert*-butylation (< 0.05%, **32**).[43] The presence of 0.17% H-Gly-Lys(Bzl)-OH **34**, however, indicated that N-benzylation had occurred during partial deprotection of the $N^{\varepsilon}$-benzyloxycarbonyl (Z) group when **31** was treated in TFA-$CH_2Cl_2$. When Boc-Lys(Z)-resin **30** was treated with a variety of acidic cleavage reagents used to deprotect Boc-peptides, H-Lys(Bzl)-OH (**35**, ≤ 0.88%) was found. The extent of H-Lys(Bzl)-H production depended on the nature of the reagent and whether a carbonium ion scavenger was used. This side reaction did not occur when more acid-stable Z protecting groups such as the 2,4-Cl2-Z group[44] were used. N-Benzylation (0.47- 3.26%) also occurred under conditions of complete deprotection with Z-Gly-OH, Bzl-Lys-OH and H-Lys(Bzl)-OH in

refluxing TFA or ambient TFMSA-TFA. No N-benzylation (< 0.1%) could be detected when amino acid resins or free amino acids containing Z protecting groups were cleaved with anhydrous HF. In summary, $N^{\alpha}$-*tert*-butylation was not observed when model experiments using the Boc protecting group under conditions of SPPS were employed. N-Benzylation, a novel side reaction, was observed when Z groups were removed from Z derivatives of Gly and Lys by TFA or TFMSA-TFA. This side reaction was avoided when a more acid-stable Z protecting group was used for lysine.

**Quantitation of synthetic efficiency in solid phase peptide synthesis as a function of chain length**

When Bruce began working in the Woolley laboratory in 1949 each postdoctoral fellow worked on separate projects and never as a group on a single problem (see p. 42 in reference 1). Woolley assigned research topics and discussed results on a frequent basis. When I arrived in 1969 one could propose their own research plans within the framework of SPPS, and although Bruce was never demanding with respect to the pace of research, he was always accessible for advice and consultation. Also, in the 1970s more of the individual researchers in the laboratory began teaming together on problems of mutual interest. An example of this is found in the development of the aminoacyl-4-(oxymethyl)phenylacetamidomethyl-resin (-O-$CH_2$-PAM-resin) or "PAM-resin" for short.[45,46] The PAM-resin was originally developed as part of a program to examine synthetic efficiency in solid phase peptide synthesis as a function of chain length, which will now be discussed.

Since the early days of the solid-phase there had been a general feeling that there must be resin-imposed steric limitations to stepwise solid-phase peptide synthesis.[47] Some workers thought that reactions will be less efficient close to the polymer backbone[48] while others thought that there

would be significant declines in yields as the peptide is elongated, due to temporary steric occlusion of peptide chains within the polymer network.[49] In 1971 I proposed an approach that would allow testing of coupling or synthetic efficiency in SPPS. A generalized version of this approach is given in Figure 13 where a well-characterized model peptide is synthesized at increasing distances from the solid support by using a polyamino acid spacer of varying lengths. The model peptide Leu-Ala-Gly-Val is used as a chromatographic system that separates all possible deletion and termination peptides from the parent peptide at a 0.1% detection level.[13]

***Use of an aryl hydrazine resin***

An early chemical formulation of the approach outlined above (Figure 13) is presented in Figure 14. Chloromethylated polystyrene is reacted with the cesium salt of Boc-Phe-OH to provide the starting Boc-Phe resin which is deprotected, neutralized and reacted with Boc-Lys(Tos)-OH and DCC to provide Boc-Lys(Tos)-Phe resin. Repetitions of deprotection, neutralization and coupling (n= 0, 10, 20 ...) provide peptide spacers of varying chain lengths designated as Boc-$[Lys(Tos)]_{n+1}$-Phe-resin **36**. Valine, the C-terminal amino acid of the model peptide is then added as part of a linker or handle comprising Boc-Val and *p*-hydrazinobenzoic acid (PHBA). Acylation of **36** with Boc-Val-PHBA provides **37** which is subsequently elongated by SPPS to give support **38.** Cleavage of **38** by oxidation in the presence of acetic acid-water followed by deprotection should afford H-Leu-Ala-Gly-Val-OH and related deletion peptides. Preliminary studies on the oxidative cleavage of a Boc-Leu-Val-PHBA-Phe-resin provided H-Leu-Val-OH in low yield that contained ~10 % of the H-Leu-D-Val-OH diastereoisomer. The production of target peptide, deletion peptides, target peptide diastereoisomer and deletion peptide diastereoisomers would clearly overwhelm the analytical system used for the detection of H-Leu-Ala-Gly-Val-OH and related deletion peptides.[13] The combination of an inefficient

oxidative cleavage coupled with racemization clearly indicated the need for better chemistry. Other iterations of the scheme in Figure 12 were tested after the aryl hydrazine experiments and found lacking. Rather than trying to optimize a less than promising system, it was decided not to pursue this project, at least until some better ideas surfaced. Work on some of the projects described above, especially cyclic peptides, was continued.

***Phenylacetamidomethyl(PAM) resin - early phase***

The Merrifield laboratory had numerous visitors seeking practical laboratory training in SPPS. Virtually every member of the laboratory took turns at instructing visitors on a one to one basis for one to two week periods. Late in 1973 Bruce described an exchange program sponsored by the National Academy of Sciences and asked if I would like to take on a Russian scientist for 4 months beginning in 1974. I initially hesitated, thinking I didn't have time to entertain a possible science bureaucrat who hadn't worked in a laboratory for years. I finally agreed to do it as my small contribution to easing American - Soviet relations during the Brezhnev era of the Cold War. My fears regarding Mikail Nikolaich Ryabtsev were unfounded as he was a hands-on chemist, eager to work and learn new techniques. It was also time to launch another attack on the coupling efficiency problem via a PAM resin. As he lived on campus and I lived across the street it was easy to mesh our schedules, which usually meant working in the laboratory Mondays through Saturdays with most evenings included. Sundays were reserved for touring New York City, visiting museums, etc. As I felt responsible for Mikail's personal safety I made certain that he knew to avoid the South Bronx and similar areas having high adventure potential. He was soon on his own with no untoward incidents to report. Back to the lab and how to execute the general approach outlined in Figure 13?

The scheme in Figure 15 evolved after related variations tested in the laboratory showed little

promise. The insertion of a phenylacetamidomethyl (PAM) bridge between the polystyrene matrix and peptide sites was predicted to make the resulting peptide esters significantly more stable (between 25 and 400 times) than peptide chains bound to the then commonly used oxymethyl-poly(styrene-co-divinylbenzene) resin (see p.155 in reference 1). The key to the scheme is Boc-valyl-4-(oxymethyl)phenylacetic acid **39** which is initially used to acylate aminomethyl-poly(styrene-co-divinylbenzene) resin **40**. Aminomethyl-resin **40** was prepared by the hydrazinolysis of phthalimidomethyl-resin that was obtained from the reaction of potassium phthalimide with chloromethyl-resin.[45] The acylation of **40** with **39** provides **41** which is elongated by SPPS to give tetrapeptide resin **42**. Acidolytic cleavage (HF) of **42** will yield H-Leu-Ala-Gly-Val-OH and related deletion peptides. A second cycle of H-Leu-Ala-Gly-Val-OH synthesis begins with Boc-deprotection of **42** followed by neutralization and coupling with **39** to provide the new Boc-Valyl-4-(oxymethyl)phenylacetamidomethyl resin **43** while terminating the tetrapeptide resin **42** obtained from the first synthetic cycle. Resin **43** is elongated in the usual fashion to provide resin **44** which upon cleavage should provide H-Leu-Ala-Gly-Val-OH and related deletion peptides from **only** the second cycle. Additional cycles (n) provide resin **45** containing [Leu-Ala-Gly-Val-4-(oxymethyl)phenylacetamide]$_{n+2}$ resin. The yields of H-Leu-Ala-Gly-Val-OH and deletion peptides are determined after every synthetic cycle to provide information on coupling efficiency as a function of peptide chain length from the polystyrene matrix.

The synthesis of Boc-valyl-4-(oxymethyl)phenylacetic acid **39**, a key component in our scheme, was problematic. Briefly stated, the reaction of Boc-Val-OH cesium salt with 4-(chloromethyl)phenylacetic acid[50] yields **39** in addition to unreacted 4-(chloromethyl)-phenylacetic acid and polymers deriving of 4-hydroxymethylphenylacetic acid containing

variable amounts of esterified Boc-Val . Attempts to purify **39** by crystallization, by column chromatography on Sephadex LH-20 or silica gel, and by countercurrent distribution were not entirely satisfactory. Ultimately, after considerable trial and error, **39** was obtained analytically pure after repeated preparative layer chromatography in 9: 1 hexane-AcOH and isolation as a cyclohexylammonium salt. A general, and more convenient preparation of Boc-aminoacyl-4-(oxymethyl)phenylacetic acids was developed later and reported in a subsequent PAM-resin paper.[46]

An alternative scheme for the general preparation of Boc-aminoacyloxymethyl-PAM-resins is given in Figure 16 using Boc-Val-4-(oxymethyl)phenylacetamidomethyl-resin **41** as an example. 4-(Acetoxymethyl)phenylacetic acid **46**, obtained from the reaction of 4-(chloromethyl)-phenylacetic acid with sodium acetate, is reacted with aminomethyl-resin **40** to provide 4-(acetoxymethyl)phenylacetamidomethyl-resin **47**. Hydrazinolysis of **47** provides 4-(hydroxymethyl)phenylacetamidomethyl-resin **48** which is acylated with a Boc-amino acid, in this case Boc-Val, to provide Boc-Val-4-(oxymethyl)phenylacetamidomethyl resin **41** which can be elongated by SPPS to give tetrapeptide resin **42** (Figure 15).

The third route for preparation of Boc-aminoacyloxymethyl-PAM-resins shown in Figure 17 employs 4-(halomethyl)phenylacetic acid (X = Cl, Br) **49**. This route furnished a model tetrapeptide in less satisfactory purity than did the previous routes. Alhough the predominant reaction was N-acylation of the aminomethyl-resin by the DCC-activated 4-(halomethyl)phenylacetic acid to give 4-(halomethyl)phenylacetamidomethyl resin **50** , N-benzylation of some aminomethyl sites by the halomethylphenylacetic acid may also have occurred. In addition, halomethyl-PAM sites that did not react with the first Boc-amino acid may have participated in undesirable benzylation reactions later in the synthesis. A report by

Sparrow [51] described the preparation of a Boc-aminoacyl-4-(oxymethyl)phenylacetamido-11-undecanoylaminomethyl-resin in a variation of Figure 17. The use of the aminoundecanoyl spacer was thought necessary to overcome possible steric hindrance by the polystyrene backbone to peptide synthesis[52] but the acid stability of this resin was not investigated.

In addition to the H-Leu-Ala-Gly-Val-OH test peptide, a deca-lysyl-valine test peptide was prepared using a Boc-Val-oxymethyl-PAM-resin. The crude product obtained after HF cleavage was analyzed by ion-exchange chromatography on carboxymethylcellulose and the chromatogram was nearly identical to a chromatogram obtained earlier using Boc-Val-$OCH_2$-resin.[53] We compared the acid stabilities of tetrapeptide-$OCH_2$-PAM-resin **42** with a conventional tetrapeptide-$OCH_2$-resin[13] in 50% TFA-$CH_2Cl_2$ and found that the loss of peptide chains followed apparent first order kinetics for both resins.[45] The H-Leu-Ala-Gly-Val-$OCH_2$-PAM resin was found to be about 100 times more stable than the conventional Leu-Ala-Gly-Val-$OCH_2$-resin. The loss of peptide chains per 20-min $N^{\alpha}$-deprotection step was calculated to be 0.7% for the usual resin but only 0.007% for the PAM-resin. When assembly of the desired peptide is complete, however, the benzyl ester bond of the PAM-resin is readily cleaved in high yield (87%) by treatment with anhydrous HF. Thus the advantages of increased acid stability of the anchoring bond can be achieved without sacrificing the high yield of peptide obtained by HF cleavage. The greater acid stability of the PAM-resin will result in substantially higher yields of large polypeptides such as ribonuclease (124 residues) where the loss of peptide chains from the support should be reduced from 80% to about 4%. Also, the late initiation and growth of shorter peptide by-products on the resulting hydroxymethyl sites will be decreased.[54]

***Phenylacetamidomethyl(PAM) resin - intermediate phase***

Colleagues and I sometimes wondered what criteria were used to select members of the

Merrifield laboratory. Strangely enough, no one ever bothered to ask Bruce the question, "why am I here?" Bruce has commented on the role of chance in science and luck being a large factor in the selection of problems (see p. 248 in reference 1) I suspect similar considerations were in play in determining how Bruce staffed the laboratory. Equally important, Bruce's impeccable integrity and decency provided the foundation on which the laboratory operated. I felt extremely privileged to work with such congenial and competent persons whether they were the glassware washer, secretary, technicians, students or postdoctoral fellows. I can list only a few, some in cited publications, others in work described in this paper. At any rate, I think both Bruce and SPPS were very fortunate to have Stephen (Steve) B. H. Kent and James (Jimmy) P. Tam join the laboratory as postdoctoral research associates. Although we overlapped at Rockefeller (Mitchell, 1969-77; Kent, 1974-81; Tam, 1976-92), Steve and I didn't begin working together on problems of mutual interest until 1976 and interactions with Jimmy were limited to stimulating discussions regarding future directions and improvements in SPPS. Kent and Tam have developed remarkably prolific, independent careers in peptide and protein chemistry since their beginnings in the Merrifield laboratory in the 1970s.

It was apparent from earlier work on PAM resin (*vide supra*)[45] that this support would provide larger peptides in higher yields and purity than the conventional peptidyl-$OCH_2$-resin. A more convenient preparation of PAM resin was needed and the first task involved an improved preparation of the aminomethyl-resin **40** mentioned earlier (Figures 15-17). The use of either hydroxymethyl- or chloromethylphthalimide with a Friedel-Crafts catalyst to effect direct amidomethylation of the unsubstituted polystyrene matrix via a Tscherniac-Einhorn reaction[55] provides a phthalimidomethyl-resin which upon hydrazinolysis provides the desired aminomethyl-resin **40**.[56] This preparation of **40** involves one less step than the earlier preparation

[45] and eliminates the need for chloromethylated polystyrene and the carcinogenic chloromethyl methyl ether used in its preparation.

The original synthesis of Boc-Val-4-(oxymethyl)phenylacetic acid **39** from Boc-Val and 4-(chloromethyl)phenylacetic acid was tedious and could not be generalized for the preparation of other Boc-aminoacyl-4-(oxymethyl)phenylacetic acids.[45] Martin Engelhard, a knowledgeable organic chemist from Germany with Old World *savoir faire*, joined Steve and I in developing a convenient and general preparation of Boc-aminoacyl-4-(oxymethyl)phenylacetic acids for use in PAM resins.

Our preferred general preparation of Boc-aminoacyl-4-(oxymethyl)phenylacetic acids (Figure 18) begins with the reaction of 4-(bromomethyl)phenylacetic acid **49**[51,57] with bromoacetophenone to provide 4-(bromomethyl)phenylacetic acid phenacyl ester **51**. Condensation of a Boc-amino acid salt with **51** yields the Boc-aminoacyl-4-(oxymethyl)phenylacetic acid phenacyl ester **52**. The phenacyl group is removed by Zn-AcOH acid reduction at room temperature, without cleaving the Boc or benzyl ester, to give the desired Boc-aminoacyl-4-(oxymethy1)phenylacetic acid **53**. The route shown in Figure 18 can be used for a variety of protected amino acids as most of the commonly used protecting groups are stable to the reductive cleavage conditions. We also explored the use of 4-(bromomethyl)phenylacetic acid N-hydroxysuccinimide ester hoping that the N- hydroxysuccinimide ester would serve as a carboxyl protecting group during the formation of the benzyl ester bond, and then serve as an active ester to allow the acylation of aminomethyl-resin to give Boc-aminoacyl-$OCH_2$-PAM-resin. Unfortunately, the reaction of Boc-Val-OH cesium salt with 4-(bromomethyl)phenylacetic acid N-hydroxysuccinimide ester proceeded poorly and yielded a multiplicity of products in addition to the desired Boc-valyl-4-(oxymethyl)phenylacetic acid N-hydroxysuccinimide ester

which could not be isolated as a pure compound.

An improved preparation of Boc-aminoacyl-4-(oxymethy1)phenylacetic acids was developed by Tam and Kent by using potassium fluoride as the base in the two esterification reactions in Figure 18.[58] The stage was set to now utilize PAM resins for the synthesis of larger peptides.

***Phenylacetamidomethyl (PAM) resin - mature phase***

The work described for the early and intermediate phases of PAM resin development[45,46,56,58] set the stage for utilizing PAM resins in the synthesis of larger peptides. This work was entrusted to other capable hands as it was time to heed Horace Greeley's admonition, "Go West, young man," leave Rockefeller University (1969-77) and return to California after a 16-year absence (military service, graduate and postgraduate work). The quantitation of synthetic efficiency in solid phase peptide synthesis as a function of chain length, the initial *raison d'etre* for PAM resin, was extended and completed by Sarin and Kent using the scheme in Figure 15 as a starting point.[59] Briefly stated, the model test peptide H-Leu-Ala-Gly-Val-OH was synthesized by SPPS at increasing distances from a 1% cross-linked polystyrene support. The efficiency of the synthesis was evaluated by quantitatively measuring the deletion peptides H-Leu-Ala-Val-OH and H-Leu-Gly-Val-OH that were produced during the synthesis of the tetrapeptide.[13] The insertion of an oxymethylphenylacetyl group between the test peptide and the peptide chains used to provide spacers from the support made it possible to selectively evaluate the quality of the tetrapeptide without interference by the spacer. Low and constant levels of deletion peptides were found and no significant effect of distance from the support or of peptide loading on the synthetic efficiency could be detected up to a chain length of 60 residues and a peptide-to-resin weight ratio of 4 to 1. The observation of high synthetic efficiency even up to 60 residues and 80% peptide content clearly demonstrated the lack of intrinsic limitations to stepwise solid-phase synthesis over an

extreme range of peptide loading. This study demonstrated that the poor synthetic results obtained in certain applications of SPPS by other laboratories have chemical rather than resin-related physical explanations.

The synthesis of mammalian glucagon, a 29-amino acid peptide hormone secreted by the pancreas, was considered a landmark achievement when Erich Wünsch and coworkers described the preparation of fully active, crystalline material in 1968.[60] The synthetic glucagon was prepared in solution using classical fragment condensation methods by a large, skilled team over a period of several years.[61] Wünsch in a review on the synthesis of naturally occurring polypeptides, reflected on the problems of synthetic peptide research circa 1971.[48]

Using the glucagon synthesis as a model, he contended that conventional (solution) synthesis with maximum use of protective groups allows the synthesis and subsequent coupling of peptide fragments with almost total avoidance of by-products. This approach would be considered very good for the synthesis of peptide sequences containing up to 30 amino acid residues and, barring solubility problems, the maximum sequence possible would be 30-50 amino acid residues. A large portion of the review is devoted to solid-phase synthesis, which despite the surprising simplicity of the idea and the possibility of automation, exhibits "inborn defects" with respect to peptide synthesis and inadequacy of analytical methods to monitor synthetic progress. Wünsch concluded that SPPS in 1971 was "unsuitable for the satisfactory synthesis of higher natural peptides (with more than 15 amino acid residues)." Benefiting from hindsight,[1] we can toss much of Wünsch's litany of concerns and faults with SPPS into the dustbin of peptide history. Again, using the glucagon synthesis as a model, the resources and manpower required for the solution synthesis of glucagon analogs to support an extensive study of structure-activity relationships would be enormous and could not be undertaken.

A cursory reading of Bruce's scientific autobiography[1] and this paper will inform the reader that the Merrifield laboratory was not sitting on its collective hands after the 1969 publication of Gutte's synthesis of an enzyme with Rnase A activity.[15] In addition to considerable methodological work on SPPS, parallel synthetic efforts on biologically active peptides such as glucagon were being pursued. A renewed interest in glucagon physiology and its role in diabetes mellitus developed in response to the bihormonal hypothesis of Unger.[62] Questions about the mechanism of action of glucagon could best be answered by total synthesis of the hormone and of appropriate analogues. Recall that glucagon, with its 29 amino acid sequence containing many trifunctional amino acids, was considered by Wünsch to be beyond the capability of SPPS with its many "inborn defects".[48] Svetlana Mojsov, one of the many talented graduate students in the Merrifield laboratory, took up the challenge.

The first stepwise solid phase synthesis of mammalian glucagon was briefly described in 1977 as part of a report on recent developments in SPPS.[63] The detailed synthesis that was reported later described the preparation of fully active, crystalline glucagon using an alkoxybenzyl alcohol resin (Wang resin) with the biphenylylisopropyloxycarbonyl group (Bpoc) used for temporary $\alpha$-amino protection.[64] The crude synthetic material was purified by gel filtration and ion-exchange chromatography followed by crystallization of the 29-residue hormone from water. The synthetic glucagon was homogeneous and indistinguishable from natural bovine glucagon by gel electrophoresis, ion-exchange chromatography, reverse-phase high-pressure liquid chromatography, fluorescence spectroscopy, amino acid analysis and it was fully active in the rabbit hyperglycemia assay.

An improved synthesis of crystalline mammalian glucagon was subsequently developed by Svetlana using a PAM resin with $N^{\alpha}$-*t*-butoxycarbonyl and benzyl-based side-chain protection

for most of the trifunctional amino acids.[65] The cyclohexyl-protecting group was used for the β-carboxyl of aspartic acid to minimize aspartimide formation.[66] Cleavage of the 29-residue peptide from the resin using an improved HF procedure[67] provided crude synthetic glucagon in 75% yield. A **one-step purification** using preparative $C_{18}$ reverse-phase chromatography gave pure material (48% overall yield), which was crystallized from aqueous solution at pH 9.2. The overall 48% isolated yield of homogeneous glucagon based on the starting C-terminal residue is much higher than the yield obtained in the earlier stepwise solid-phase synthesis of glucagon in which more acid-labile protecting groups were used.[64] It is also higher than the yield reported for synthesis by solution methods.[60,61] The high yield obtained in the synthesis and the subsequent ease of purification of synthetic glucagon made it feasible for the first time to approach structure-function studies of the glucagon molecule through the total synthesis of selected analogues in a rapid and cost-effective manner.

Over 200 analogues (agonists, antagonists) of glucagon had been synthesized in the Merrifield laboratory by 2006. An overview of research probing the glucagon receptor has been provided by Cecilia Unson, Bruce's long term collaborator and colleague for 28 years.[68]

Presently, a 1 to 2 person-week of effort is required for the preparation and purification of a glucagon analogue using the chemistry outlined above.[69]

Again, to belabor the very obvious, a comparable study of structure-function relationships based on the availability of glucagon analogues from solution synthesis [60,61] is unthinkable with respect to manpower, cost and time required. **This is precisely why Bruce Merrifield invented SPPS and his colleagues labored to improve upon the method as originally conceived.**

What about the SPPS of peptides larger than glucagon, say 90 to 100 residues? Steve Kent and co-workers carefully re-examined the synthetic protocols used with PAM resins and introduced

*in situ* neutralization into SPPS using Boc/Benzyl chemistry for the rapid, efficient synthesis of difficult sequences.[70] In addition, several side reactions were examined and eliminated. The resulting improved chemistry and protocols were utilized in the synthesis of the L and D enantiomers of the 99 residue HIV-1 protease (1-99).[71] Whether PAM resins with more improved chemistry can be routinely employed for the synthesis of peptides ≥ 90 to 100 residues remains to be established. However, the development of native chemical ligation methods where purified fragments without side-chain protection can be coupled together has provided strategies for the synthesis of larger peptides (> 100 residues).[72,73] Recently, Torbeev and Kent reported the covergent chemical synthesis of a 203 residue "Covalent Dimer" of HIV-1 protease enzyme using native chemical ligation methods. The resulting enzyme molecule showed full catalytic activity and a high resolution crystal structure was reported.[74] In that PAM resins were used to produce the free $^{\alpha}$carboxyl fragments employed in this synthesis, it is pleasing to know that there is still a role for a resin support developed over 30 years ago in the Merrifield laboratory.

**Other projects**

The Merrifield group meetings usually occurred every Monday at 1 PM when students, postdocs and guest investigators presented their experimental findings, problems, and ideas. It was also a good opportunity to candidly run inexplicable results past the group for comments and insight. Collaborations resulted when 2 or 3 members of the group were attracted to problems of mutual interest. These efforts were usually informal, short term and provided pleasant respites from some of the longer-term work described earlier (*vide supra*).

***Mechanisms and Prevention of Trifluoroacetylation in SPPS***

In a study on the occurrence of trifluoroacetylation in SPPS I had observed that when a sample of Boc-Lys(Z)-$OCH_2$-resin was deprotected (TFA), neutralized (tertiary amine) and cleaved

(HBr-TFA or HF) a low level of $N^{\alpha}$-trifluoroacetyl-lysine (~ 1%) was observed. When Z-Lys(Boc)-$OCH_2$-resin was treated in identical fashion, $N^{\varepsilon}$-trifluoroacetyl-lysine (~10%) was observed. Repetition of the deprotection, neutralization and cleavage steps again gave trifluoroacetyl-lysine products. When neutralization was omitted, no trifluoroacetyl-lysine products were observed. Also, when a primary amine was used for neutralization the production of trifluoroacetyl-lysine products was suppressed. Both of the resins were prepared from a chloromethyl-resin by the cesium salt procedure.[75] ***Trifluoroacetylation in the absence of a coupling step ran contrary to conventional wisdom*** which held that residual TFA, whatever the source, must be activated (DCC or other activating reagents) to allow for subsequent trifluoroacetylation of α-amino groups in SPPS.[76] Steve Kent, joined by Martin Engelhard, tackled the project in a very detailed, thorough manner and conclusively showed that trifluoroacetylation occurred when hydroxymethyl groups were present in the resin.[77] The esterification of hydroxymethyl-resins occurs upon standing in TFA and is observed by the appearance of a band at 1785 $cm^{-1}$ in the IR. The formation of trifluoroacetoxymethyl-resin and transfer of the TFA group to free $N^{\alpha}$-amino group during neutralization is shown in Figure 19. Hydroxymethyl groups can be preexisting (as in some commercial resins) or they can be formed during SPPS by the slow loss of peptide chains during deprotection steps. About 1 to 2 % of TFA peptide is generated per synthetic cycle when conventional peptidyl-$OCH_2$-resins are used. Use of the more acid stable peptidyl-PAM-resin, which is free of hydroxyl groups, reduces $N^{\alpha}$-trifuoroacetylation to < 0.02 % per synthetic cycle. Since peptidyl-PAM-resins are 100 times more acid-stable than conventional peptidyl-$OCH_2$-resins, the generation of new hydroxymethyl sites is greatly suppressed.

***Tests for racemization***

Reports suggesting that low levels of racemization occur during the stepwise synthesis of peptides[78] prompted the search for a sensitive model to test such claims. Kent and Mitchell teamed with George Barany, the youngest ever graduate student at Rockefeller University, to develop a sensitive test for the occurrence of racemization during SPPS.[79] Diastereomers of the model peptide H-Leu-Ala-Gly-Val-OH were prepared and chromatographed on a standard amino acid analyzer using a single column of sulfonated polystyrene resin with ninhydrin detection. The single D-amino acid diastereomers, H-L-Leu-D-Ala-Gly-L-Val-OH and H-D-Leu-L-Ala-Gly-L-Val-OH, were separated from one another and from the all L-amino acid tetrapeptide. The determination of the D-amino acid- containing diastereomers was accurate $\geq$ 0.1 % for a loading of 4 mmol of tetrapeptide and the limit of detection was less than 0.01% for a 12-mmol loading. The analysis was applied to the crude cleavage products obtained from the stepwise synthesis of Leu-Ala-Gly-Val and no D-amino acid-containing diastereomers were detected (<0.02 %). The model system developed here can be used to study racemization in both solution and solid phase methods of peptide synthesis.

In a related study we modified the Manning-Moore procedure[11] used to establish the stereochemical purity of D- and L-amino acids and derivatives prepared from these amino acids.[79] The chemical synthesis of optically pure peptides requires starting materials that have high stereochemical purity, hence the need for a procedure that allows the detection and quantitation of less than one part D-amino acid in the presence of 1000 parts L-amino acid. The Manning-Moore procedure, designed for the precise determination of the D and L isomers in a given sample of a amino acid, is based upon chromatographic separation of the diastereoisomeric dipeptides obtained by derivatization with an L-amino acid N-carboxyanhydride (NCA). The derivatization step is rapid, proceeding in 2 min at pH 10.4 in about 90% yield. Conditions of

elution are known for each of 21 individual amino acids and the resulting pairs of LD and LL dipeptides on a amino acid analyzer using ion exchange chromatography. L-leucine N-carboxyanhydride (L-Leu NCA) is used to prepare, without racemization, [L-Leu$^1$]dipeptides of the acidic and neutral amino acids. The [L-Leu$^1$]dipeptides of the aromatic and basic amino acids are strongly retarded on the amino acid column and glutamic acid NCA is used to prepare resolvable dipeptides of these amino acids. Under the proper conditions as little as 0.01% of the opposite enantiomer can be detected in an amino acid. The success of the Manning-Moore procedure is contingent upon the availability of L-Leu NCA and L-Glu NCA. Although we had used the Manning-Moore procedure with considerable success,[81] the syntheses of L-Leu NCA and L-Glu NCA require the use of phosgene (poison gas in WWI) and give variable yields. In addition, the commercial availability of these labile compounds had been erratic. Also, L-Glu NCA and L-Leu NCA deteriorate at room temperature and must be stored dry at -20$^o$ C to retard decomposition and polymerization. We replaced both L-Glu NCA and L-Leu NCA with the readily prepared and commercially available Boc-L-leucine N-hydroxysuccinimide ester (Boc-L-Leu-OSu). Boc-L-Leu-OSu reacts with amino acids to give diastereomeric [L-Leu$^1$]dipeptides which are suitable, after deprotection in TFA, for chromatographic separation. The standard protocol involves the reaction of amino acid (1 equiv) with Boc-L-Leu-OSu (2 equiv) and sodium bicarbonate (2 equiv) in tetrahydrofuran-water (1:1) at room temperature for 1 h, followed by trifluoroacetic acid deprotection. The stock solution of Boc-L-Leu-OSu in tetrahydrofuran is stable at room temperature for at least 1 week, and at least 1 month if stored at 4$^o$ C. Coupling yields, as measured by the disappearance of amino acid, varied from 94.5 to 99.8% without detectable racemization (< 0.1%). A larger excess Boc-L-Leu-OSu (10 equiv) was employed in the conversion of DL-lysine to $N^{\alpha}$,$N^{\varepsilon}$-(di-L-Leu)-DL-Lys, which gave a

coupling yield of 97 %. As the separation of the [L-Leu$^1$]dipeptides containing acidic or neutral D- and L-aminoacids had already been reported,[11,81] it was necessary only to derive conditions for the separation of the [L-Leu$^1$]dipeptides containing the aromatic or basic D- and L-amino acids. We achieved this by the use of shorter ion exchange columns and/or more basic buffers that allowed the detection of less than one part (0.1%) of D-amino acid in the presence of 1000 parts of L-amino acid.[80]

***Quantitative Evaluation of Methods for Coupling Asparagine in SPPS***

The dehydration of amides to nitriles is known to occur with $N^{\alpha}$-protected asparagine and glutamine, both in solution and in solid-phase peptide synthesis, during coupling with DCC, mixed anhydride, pyrophosphite, and other activation methods. Dehydration occurs while these amino acids are activated and not during subsequent coupling steps after asparagine or glutamine has been incorporated. The proposed mechanism for dehydration involves nitrile compound formation through a cyclic isoimide intermediate.[82] Dehydration had been shown to be prevented when 1-hydroxybenzotriazole was added together with DCC for activation and coupling of $N^{\omega}$-protected asparagine and glutamine in solution[83] and solid phase synthesis.[84] As the sensitivity of detection for dehydration in these studies was > 5%, there was a definite need to know the extent of this reaction at the 0.1% level and also how to best avoid this reaction during SPPS of molecules such as glucagon that contain asparagine and glutamine.[64,65]

Svetlana Mojsov and I developed a quantitative procedure employing ion exchange chromatography to detect the formation of β-cyanoalanylglycine and other side products (β-aspartamidinoacetic acid, α-aspartylglycine, β-aspartylglycine) resulting from the coupling of Boc-Asn to Gly-resins.[85] Coupling of Boc-Asn with Gly-$OCH_2$-(1,4-phenylene)-$OCH_2$-resin (Wang resin) using DCC gave Asn-Gly (54.8%), Ala(CN)-Gly (39.2%) and β-aspartamidino

acid (5.5%) after TFA cleavage. Activation of Boc-Asn by DCC plus hydroxybenzotriazole or by the nitrophenyl ester gave 98 to 99% of Asn-Gly with low levels of byproducts (0.5% nitrile and 0.2% amidine). The use of Boc-Asn with the 4,4'-dimethoxybenzhydryl amide protecting group completely prevented nitrile formation during DCC coupling. Pure Ala(CN)-Gly quantitatively reconverts to Asn-Gly by HF treatment and rehydration of the nitrile group also occurs in 50% TFA in $CH_2Cl_2$, but much more slowly.

**Postscript**

I returned to California to work at Lawrence Livermore National Laboratory (LLNL) after 8 very interesting years in the Merrifield laboratory (1969-1977). I left with mixed emotions as Steve Kent and I were preparing to undertake the first synthesis of an all-D enzyme, specifically the 124 residue D-RNase A (see pp. 139-141 in reference 1). D-RNase A was never synthesized as, and this is my conjecture, the technology to accomplish such a feat was immature at the time. Later, however, Steve and colleagues elsewhere accomplished a similar goal by synthesizing the L and D enantiomers of the 99 residue HIV-1 protease using stepwise SPPS.[71]

My initial years at LLNL involved the synthesis of fluorescent peptide substrates (peptidyl-aminocoumarins) for the sensitive detection of various proteases.[86,87] These materials could only be prepared using solution chemistry. A collaboration with Julio Camarero (LLNL) 25 years later made the peptidyl-aminocoumarins and related materials accessible by SPPS.[88,89] Although the bulk of my time at LLNL has been devoted to energetic materials research, I have maintained an interest in peptide chemistry and kept in frequent contact with Bruce over the years. I was greatly honored when Bruce asked me to critically review the manuscript for his scientific biography, *Life During a Golden Age of Peptide Chemistry. The Concept and Development of Solid-Phase Peptide Chemistry* (see p. 253 in reference 1).

**Bruce Merrifield**

How to best describe the man and his science? Garland Marshall assessed the scientific impact of Bruce's work in his incisive review "Solid-Phase Synthesis: A Paradigm Shift".[2] Solid-phase synthesis as used for the synthesis of biopolymers (peptides, proteins, nucleic acids), synthesis of natural products, chemical ligation and materials development has indeed provided a paradigm shift in the molecular biology, biotechnology and chemistry communities.

The man who emerges from the pages of *Life During a Golden Age of Peptide Chemistry,* and the man his colleagues knew and respected, was tough and dedicated but also caring and modest. He deeply cared about his two families, the family at home and the family in the laboratory (see pp. 208-227 in reference 1). Libby Merrifield, his wife, friend and colleague for over 55 years provided the bedrock for his career. He did not voice anger when the early critics maligned him and his work, just as he did not complain about a long-term progressively invasive skin cancer and the increasingly draconian medical treatments. It would have been out of character and a waste of energy that could be better used in the laboratory. Early in 2003, prior to the final combinations of treatments (surgery, chemotherapy, and radiation), I asked Bruce if he had considered retirement as an option. He smiled and said, "sure, I think I'll retire just about 2 minutes before I drop dead in the lab." Bruce, thank you for your life, your work and your inspiration to all who knew you.

**Acknowledgments**

This review was prepared under the auspices of the U.S. Department of Energy by the University of California, Lawrence Livermore National Laboratory, under Contract No. W-7405-Eng-48. I am especially indebted to Dr. Julio Camarero for a critical reading of the manuscript and to Dr. Yolanda Palom for preparing the figure illustrations.

**Figures**

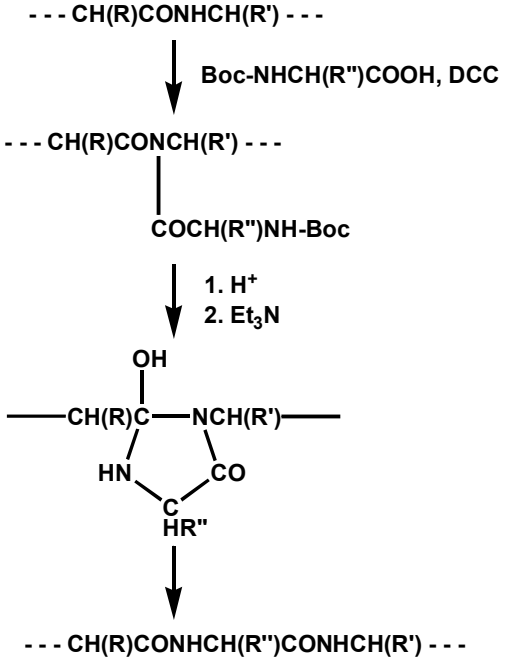


**Figure 1.** Acylation of peptide bonds followed by aminoacyl insertion as postulated by Brenner.[6,7]

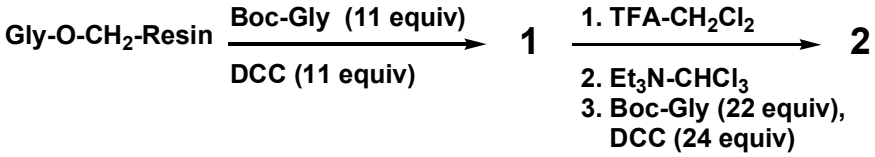


**Figure 2.** Model system to test amino acid insertions during SPPS.[8]

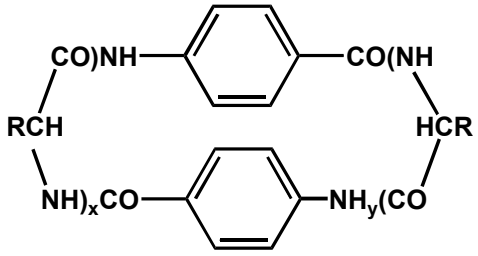


**Figure 3.** Proposed enzyme model with peptide bridges between p-aminobenzoyl residues to allow placement of functional side chains.[3-5]

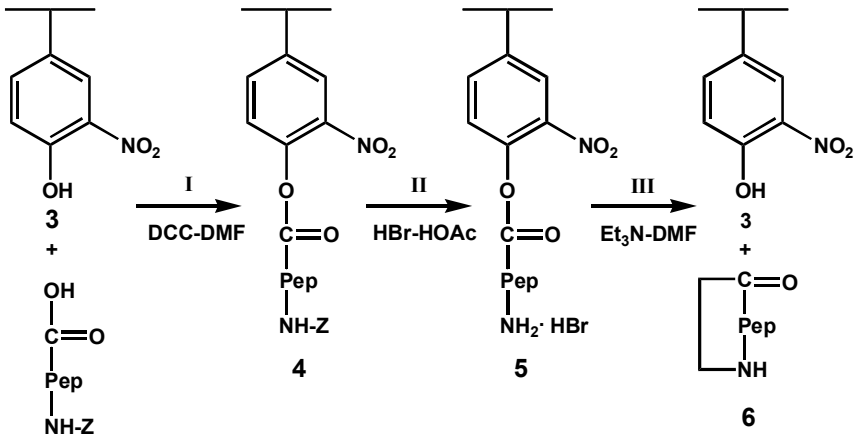


**Figure 4.** Synthesis of cyclic peptides using polynitrophenol supports.[17]

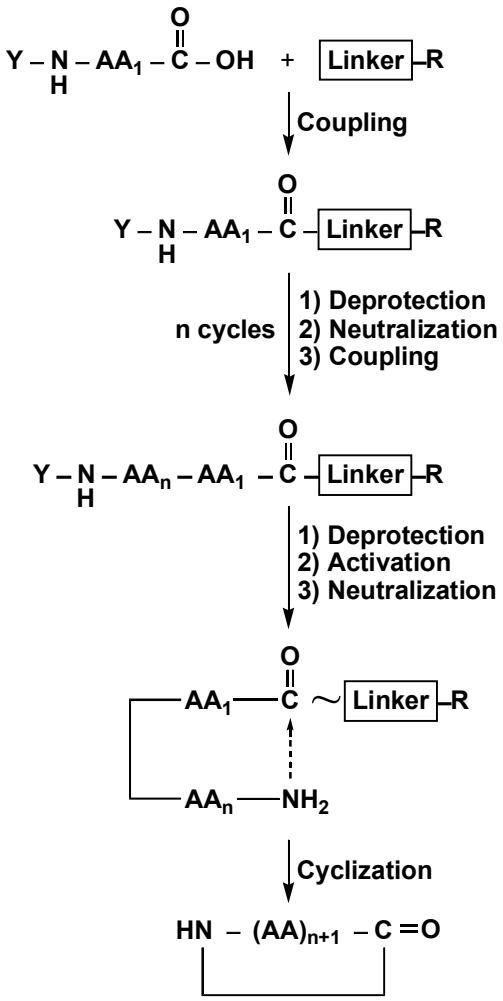


**Figure 5.** General scheme for the SPPS of homodetic cyclic peptides.

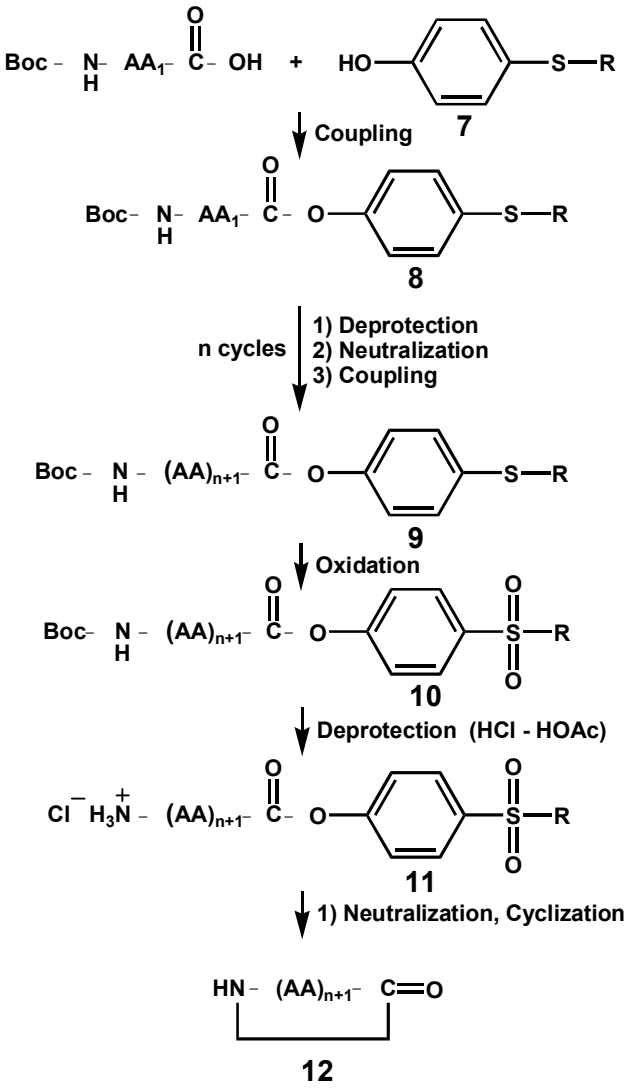


**Figure 6.** Use of mercaptophenol resin for the SPPS of cyclic peptides.[23]

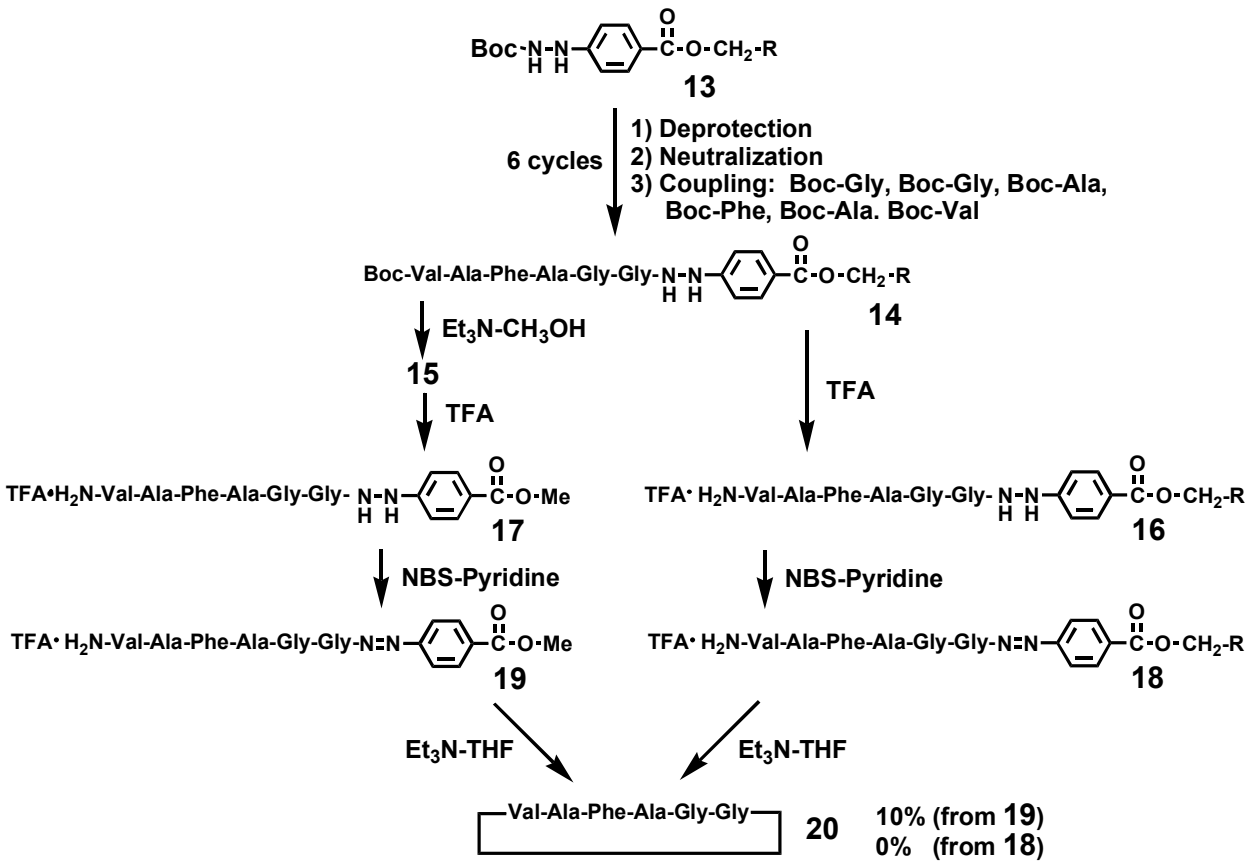


**Figure 7.** Comparative cyclizations of peptidyl-diazenes in solution and from a resin support according to Wieland.[25]

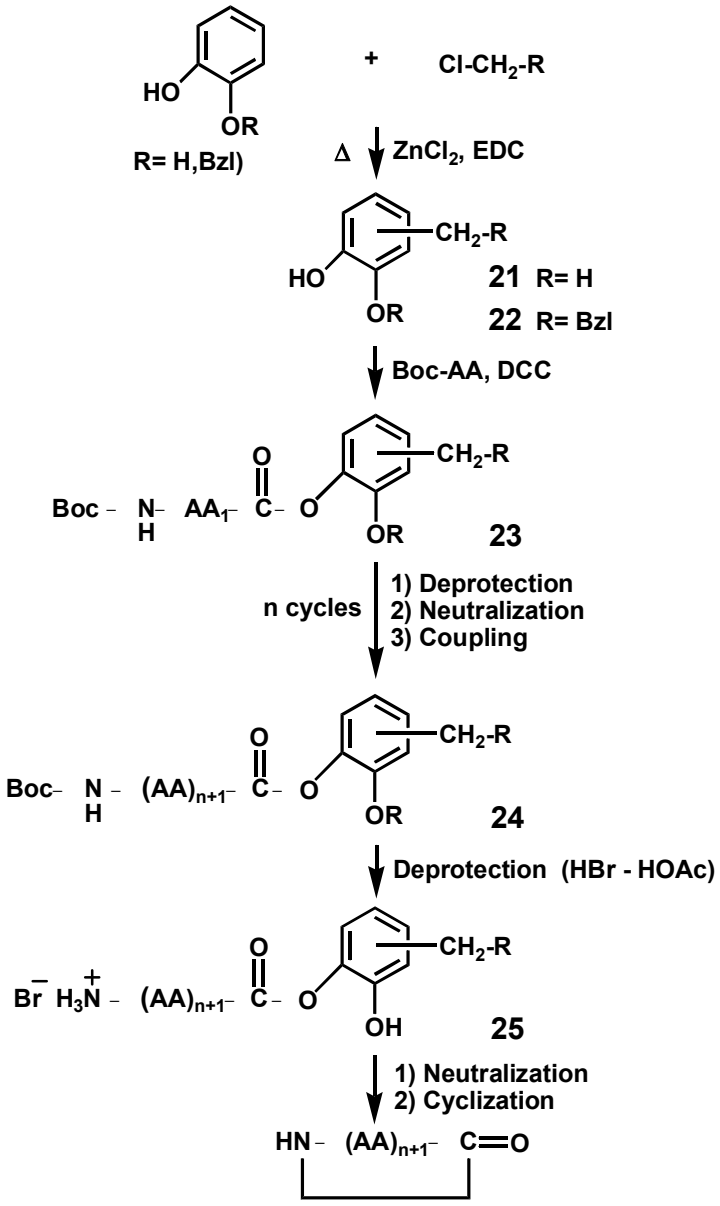


**Figure 8.** Proposed synthesis of cyclic peptides using a catechol resin.

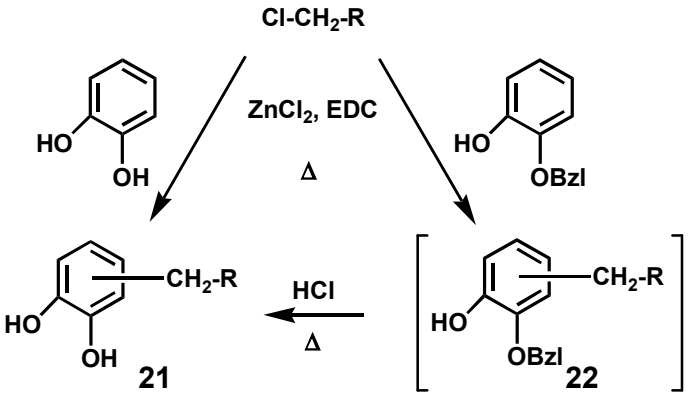


**Figure 9.** Friedel-Crafts reaction of catechol or *o*-benzyloxyphenol with chloromethyl resin to provide catechol resin.

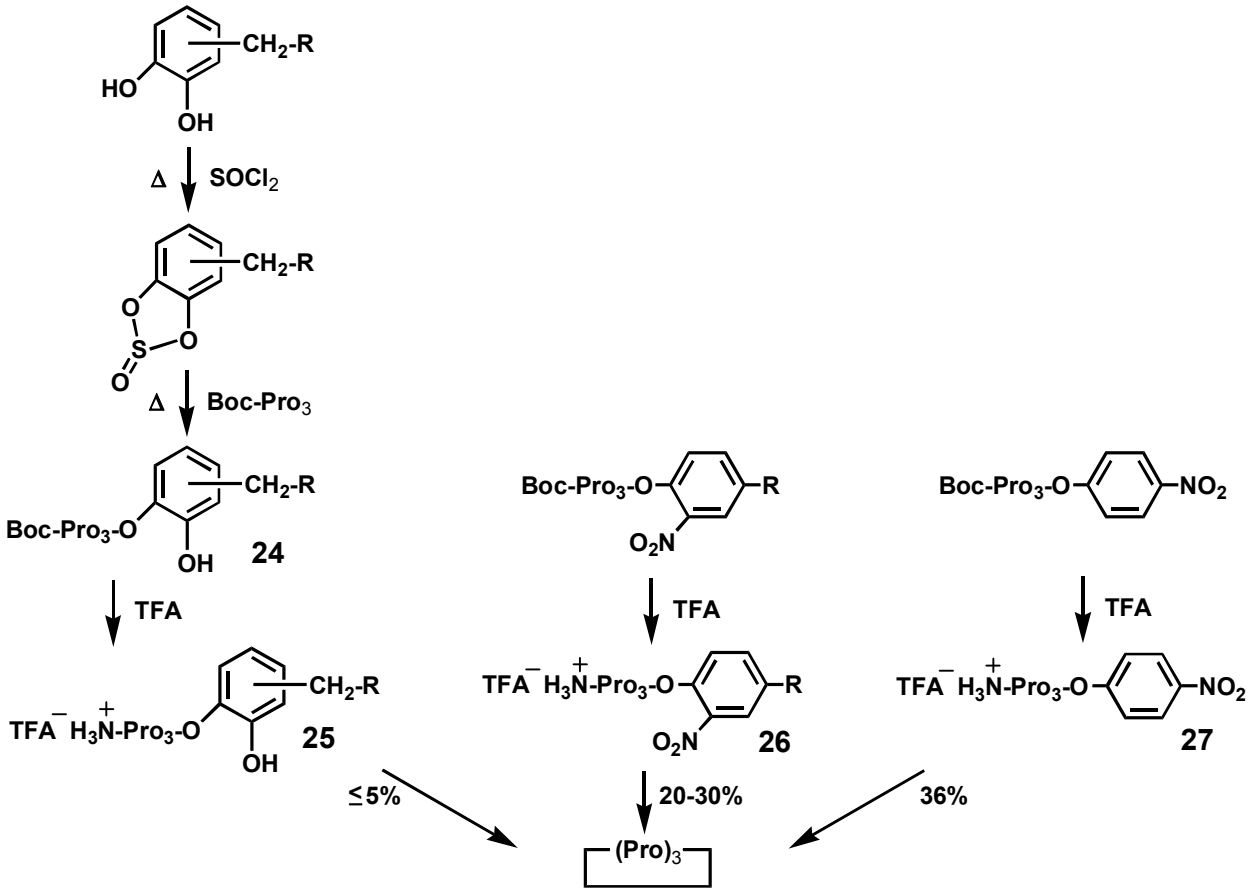


**Figure 10.** Comparison of cyclizations of $Pro_3$ using catechol and nitrophenol supports and solution synthesis.

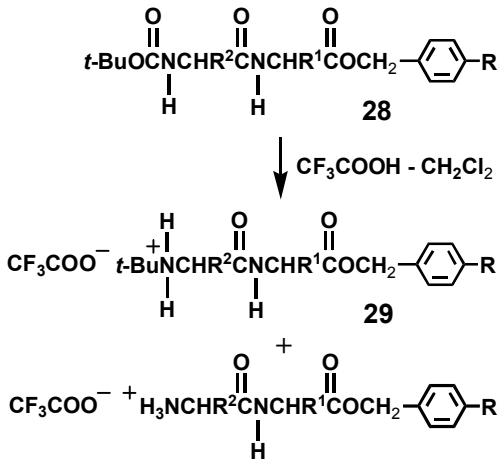


**Figure 11.** Ocurrence of N-alkylation during the acidolytic cleavage of Boc-protecting group.

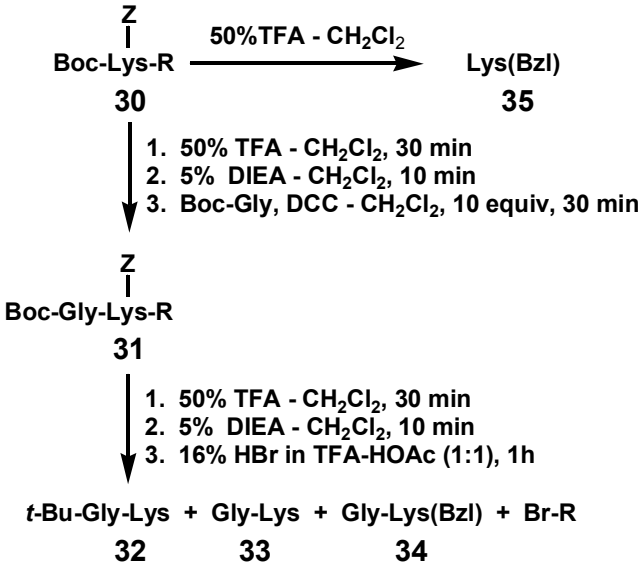


**Figure 12.** Model system for the detection of $N^{\alpha}$-*tert*-butylation and $N^{\varepsilon}$-benzylation during SPPS.

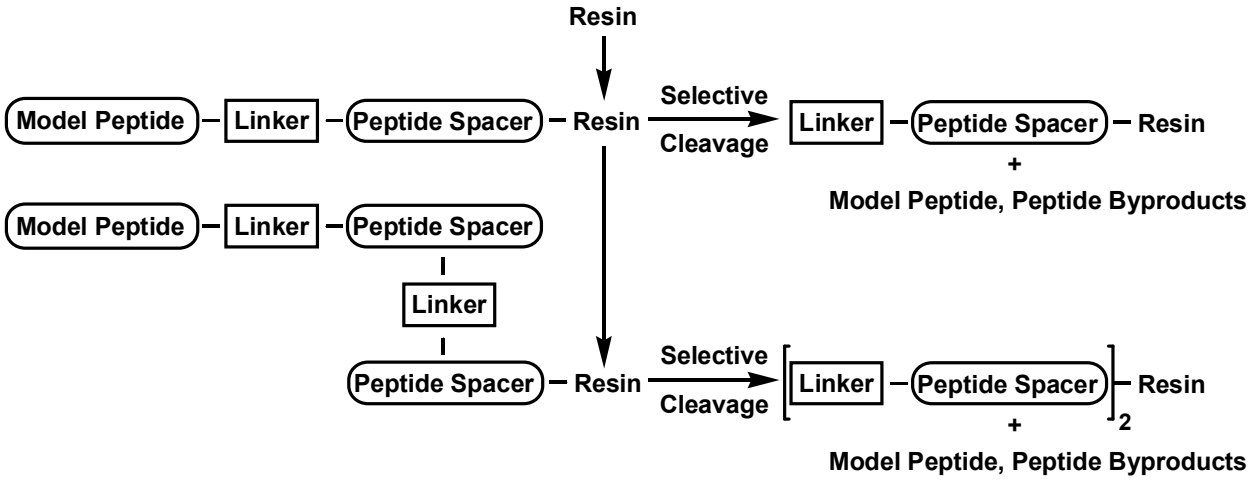


**Figure 13.** General scheme for measuring synthetic efficiency of SPPS as a function of chain length.

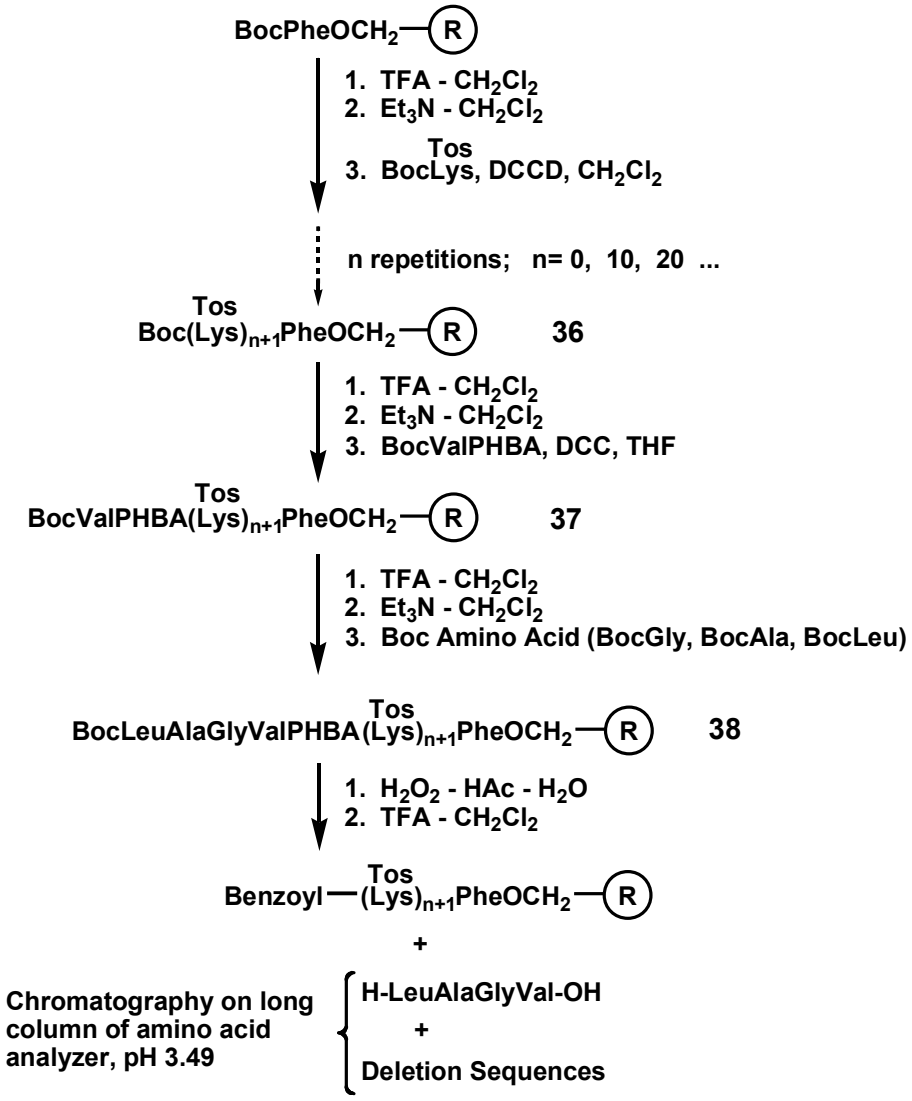


**Figure 14.** Determination of synthetic efficiency of SPPS using phenylhydrazine resin. PHBA is *p*-hydrazinobenzoic acid.

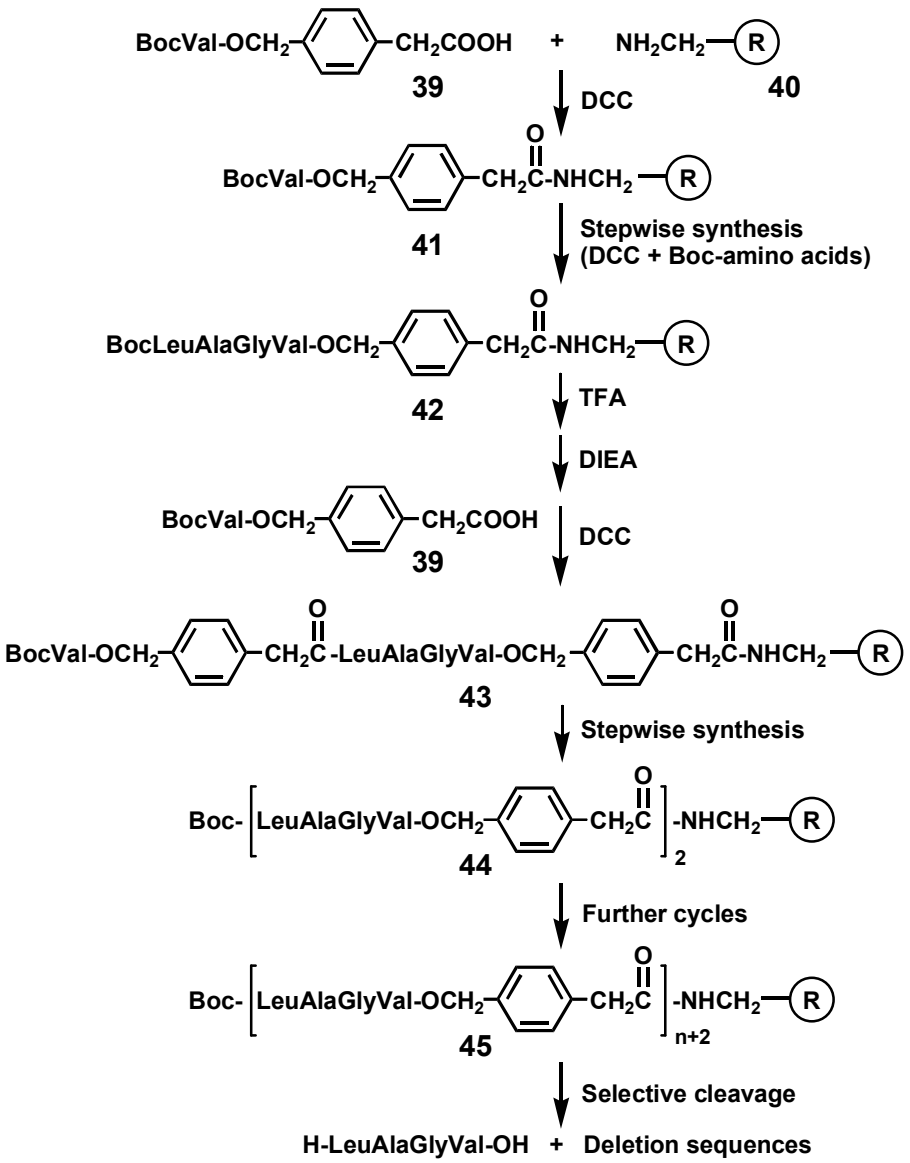


**Figure 15.** Use of PAM-resin to investigate synthetic efficiency in SPPS.

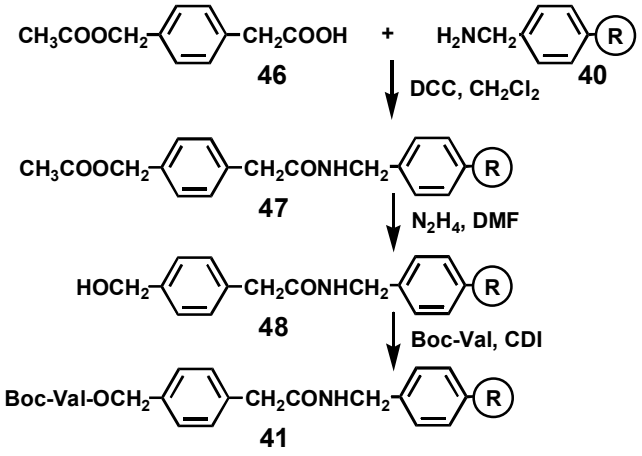


**Figure 16.** General preparation of Boc-aminoacyloxymethyl-PAM-resins using 4-(acetoxymethyl)phenyl acetic acid.

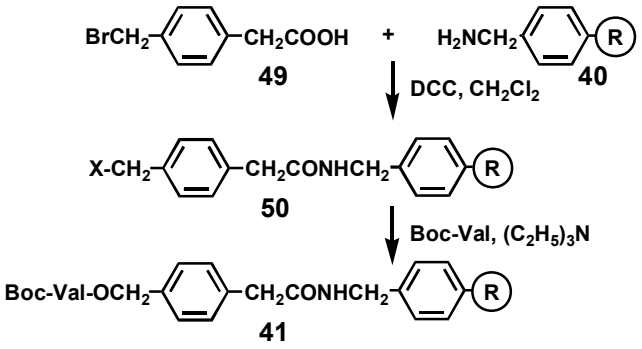


**Figure 17.** General preparation of Boc-aminoacyloxymethyl-PAM-resins using 4-(halomethyl)phenyl acetic acid (X= Cl, Br).

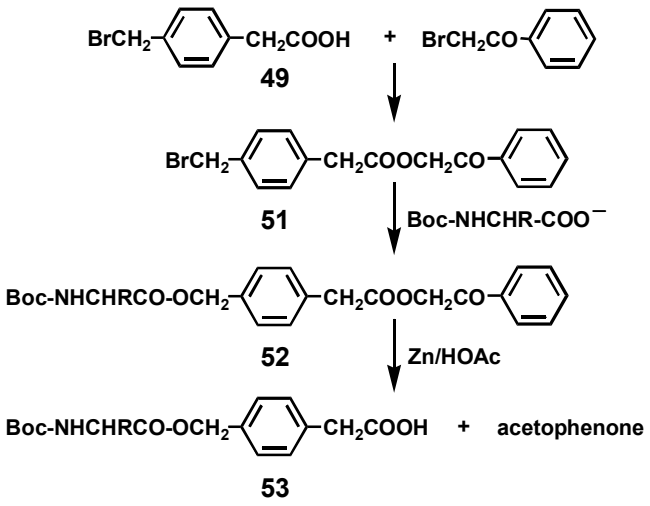


**Figure 18.** General preparation of Boc-aminoacyl-4-(oxymethyl)phenyl acetic acids.

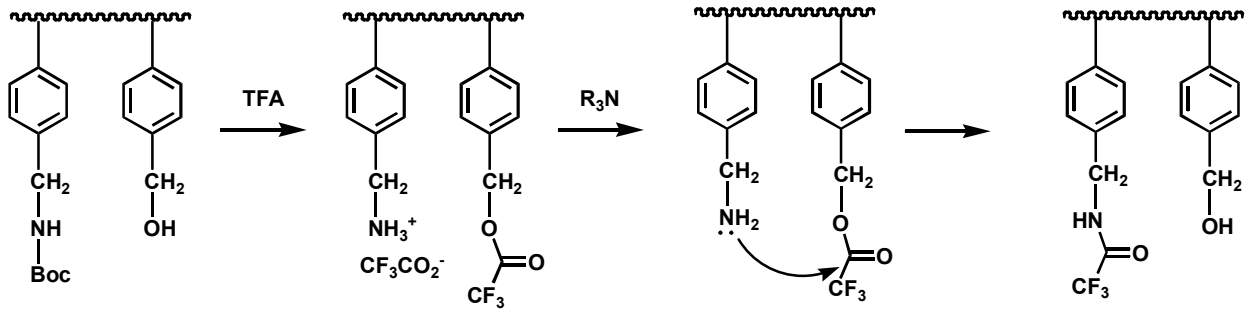


**Figure 19.** Formation of trifluoroacetoxymethyl-resin and transfer of trifluoroacetyl group to $N^{\alpha}$-amino groups during neutralization.

**References**


1. Merrifield, B. "Life During a Golden Age of Peptide chemistry : The Concept and Development of Solid-Phase Peptide Synthesis," American Chemical Society: Washington, DC, 1993.
2. Marshall, G. R. J Pept Sci 2003, 9, 534-544.
3. Mitchell, A. R.; PhD Thesis, Indiana University, 1969.
4. Roeske, R. W.; Mitchell, A. R.; Cladel, N. M.; Gupta, S. K in "Peptides: Chemistry and Biochemistry," Weinstein, B.; Lande, S., Eds.; Dekker: New York, 1970, p 55-64.
5. Mitchell, A. R.; Gupta, S. K.; Roeske, R. W. J Org Chem 1970, 35, 2877-2881.
6. Brenner, M. J Cell Comp Physiol 1959, 54, 221-230.
7. Brenner, M. in "Peptides," Beyerman, H. C.; Van De Linde, A.; Van Den Brink, W. M., Eds.; John Wiley & Sons, Inc.: New York, 1967, p 1-7.
8. Mitchell, A. R.; Roeske, R. W. J Org Chem 1970, 35, 1171-1172..
9. Spackman, D. H.; Stein, W. H.; Moore, S. Anal Chem 1958, 30, 1190-1206.
10. Mitchell, A. R.; Roeske, R. W. J Chromatog 1969, 43, 266-267.
11. Manning, J. M.; Moore, S. J Biol Chem 1968, 243, 5591-5597.
12. Dorman, L. C.; Markley, L. D.; Mapes, D. A. Anal Biochem 1971, 39, 492-497.
13. Merrifield, R. B.; Mitchell, A. R.; Clarke, J. E. J Org Chem 1974, 39, 660-668.
14. Merrifield, R. B.; Singer, J.; Chait, B. Anal Biochem 1988, 174, 399-414.
15. Gutte, B.; Merrifield, R. B. J AmChem Soc 1969, 91, 501-502.
16. Hirschmann, R.; Nutt, R. F.; Veber, D. F.; Vitali, R. A.; Varga, S. L.; Jacob, T. A.; Holly, F. W.; Denkewalter, R. G. J Am Chem Soc 1969, 91, 507-508.
17. Fridkin, M.; Patchornik, A.; Katchalski, E. J Am Chem Soc 1965, 87, 4646-4648.
18. Kraus, M. A.; Patchornik, A. Isr J Chem 1978, 17, 298-303.
19. Crowley, J. I.; Harvey, T. B.; Rapoport, H. J Macromol Sci Chem 1973, 7, 1117-1126.
20. Mitchell, A. R.; R.B. Merrifield, R. B. in "Peptides:Sructure and Function," Deber, C. M.; Hruby, V. J.; Kopple, K. D., Eds.; Pierce: Rockford, IL, 1985, p 289-292.
21. Blackburn, C.; Kates, S. A. Methods Enzymol 1997, 289, 175-198.
22. Valero, M.-L.; Giralt, E.; Andreu, D. Tetrahedron Lett 1996, 37, 4229-4232..
23. Flanigan, E.; Marshall, G. R. Tetrahedron Lett 1970, 1970, 2403-2406.
24. Marshall, D. L.; Liener, I. E. J Org Chem 1970, 35, 867-868.
25. Wieland, T.; Lewalter, J.; Birr, C. Liebigs Ann Chem 1970, 740, 31-47.
26. Wolman, Y.; Berger, A.; Patchornik, A.; Gallop, P. M. J Am Chem Soc 1962, 84, 1889-1892.
27. Milne, H. B.; Most, C. F. J Org Chem 1968, 33, 169-175.
28. Wieland, T.; J. Faesel, J.; Konz, W. Liebigs Ann Chem 1969, 722, 197-209.
29. Rosenbaum, C.; Waldmann, H. Tetrahedron Lett 2001, 42, 5677-5680.
30. Benoiton, N. L. "Chemistry of Peptide Synthesis," CRC Press: Boca Raton, Fl, 2006, p 93-124.
31. Kemp, D. S.; Chien, S. W. J Am Chem Soc 1967, 89, 2743-2745.
32. Jones, J. H.; Young, G. T. J Chem Soc 1968, 436-441.
33. Cowell, R. D.; Jones, J. H. J Chem Soc 1971, 1082-1090.

34. Kun, K. A. J Polymer Sci 1965, A3, 1833-1843.
35. Green, A. J. Chem Soc 1927, 500-504.
36. Rothe, M.; Steffen, K. D.; Rothe, I. Angew Chem 1965, 77, 347-348.
37. Mitchell, A. R.; Rahman, A.; Merrifield, R. B. in "Peptides 1990," Giralt, E.; Andreu, D., Eds,; Escom:Leiden, The Netherlands, 1991, p 39-40.
38. Jones, J. H. Chem & Ind 1974, 723-727.
39. Jones, J. H. In "Chemistry and Biochemistry of Amino Acids, Peptides and Proteins," Weinstein, B., Ed.; Dekker: New York, 1977, p 29-63.
40. Sheradsky, T.; Salemnick, G.; Frankel, M. Isr J Chem 1971, 9, 263-266.
41. Hodges, R. S.; Merrifield, R. B. Anal Biochem 1975, 65, 241-272.
42. Dorman, L. C. Tetrahedron Lett 1969, 2319-2321.
43. Mitchell, A. R.; Merrifield, R. B. J Org Chem 1976, 41, 2015-2019.
44. Erickson, B. W.; Merrifield, R. B. J Am Chem Soc 1973, 95, 3757-3763.
45. Mitchell, A. R.; Erickson, B. W.; Ryabtsev, M. N.; Hodges, R. S.; Merrifield, R.B. J Am Chem Soc 1976, 98, 7357-7362.
46. Mitchell, A. R.; Kent, S. B. H.; Engelhard, M.; Merrifield, R. B. J Org Chem 1978, 43, 2845-2852.
47. Bayer, E.; Eckstein, H.; Hagele, K.; Konig, W. A.; Bruning, W.; Hagenmaier, H. J Am Chem Soc 1970, 92, 1735-1738.
48. Wünsch, E. Angew Chem Int Ed Engl 1971, 10, 786-795.
49. Fankhauser, P.; Brenner, M. in "The Chemistry of Polypeptides," Katsoyannis, P. G., Ed.; Plenum Press: New York, 1973, p 389-411.
50. Bogdanov, M. N. J Gen Chem USSR Eng Trans 1958, 28, 1670-1671.
51. Sparrow, J. T.; . J Org Chem 1976, 41, 1350-1353.
52. Mitchell, A. R. in Chemistry and Biology of Peptides," Meienhofer, J., Ed.; Ann Arbor Science: Ann Arbor, MI, 1972, p 98.
53. Erickson, B. W.; Merrifield, R. B. J Am Chem Soc 1973, 95, 3757-.
54. Erickson, W.; Merrifield, R. B. in "The Proteins," Vol.2, 3rd Ed, Neurath, H.; Hill, R. L., Eds.; Academic Press: New York, NY, 1976, p 255-527.
55. Zaugg, H. E.; Martin, W. B. Org Reactions 1965, 14, 52-267.
56. Mitchell, A. R.; Kent, S. B. H.; Erickson, B. W.; Merrifield, R. B. Tetrahedron Lett 1976, 17, 3795-3798.
57. Chauffe, L.; Andrews, L. J.; Keefer, R. M. J Org Chem 1966, 31, 3758-3764.
58. Tam, J. P.; Kent, S. B. H.; Wong, T. W.; Merrifield, R. B. Synthesis 1979, 955-957.
59. Sarin, V. K.; Kent, S. B. H.; Mitchell, A. R.; Merrifield, R. B. J Am Chem Soc 1984, 106, 7845-7850.
60. Wünsch, E.; Jaeger, E.; Scharf, R. Chem Ber 1968, 101, 3664-3667.
61. Wünsch, E. Z Naturforschung 1967, 22b, 1269-1276.
62. Unger, R. H.; Orci, L. Lancet 1975, 1, 14-16.
63. Merrifield, R. B.; Barany, G.; Cosand, W. L.; Engelhard, M.; Mojsov, S. in "Peptides," Goodman, M.; Meienhofer, J., Eds.; Wiley: New York, 1977, p 488-502.
64. Mojsov, S.; Merrifield, R. B. Biochemistry 1981, 20, 2950-2956.
65. Mojsov, S.; Merrifield, R. B. Eur J Biochem 1984, 145, 601-605.
66. Tam, J. P.; Wong, T. W.; Riemen, M. W.; Tjoeng, F. S.; Merrifield, R. B.

Tetrahedron Lett 1979, 4033-4036.
67. Tam, J. P.; Heath, W. F.; Merrifield, R. B. Tetrahedron Lett 1982, 23, 4435-4438.
68. Unson, C. G. Int J Pept Res Therap 2007, 10.1007/s10989-007-9082-3, in press.
69. Personal communication, Dr. Nagarajan Chandramouli, The Rockefeller University.
70. Schnolzer, M.; Alewood, P.; Jones, A.; Alewood, D.; Kent, S. B. H. Int J Prpt Protein Res 1992, 40, 180-193.
71. Milton, R. C. D.; Milton, S. C. F.; Kent, S. B. H. Science 1993, 256, 1445-1448.
72. Dawson, P. E.; Muir, T. W.; Clark-Lewis, I.; Kent, S. B. H. Science 1994, 266, 776-779.
73. Tam, J. P.; Lu, Y. A.; Liu, C. F.; Shao, J. Proc Natl Acad Sci U S A 1995, 92, 12485-12489.
74. Torbeev, V. Y.; Kent, S. B. H. Angew Chem Int Ed 2007, 46, 1667-1670.
75. Gisin, B. F. Helv Chim Acta 1973, 56, 1476-1482.
76. Birr, C. in "Aspects of the Merrifield Peptide Synthesis," Springer: New York, 1978, p 42.
77. Kent, S. B. H.; Mitchell, A. R.; Engelhard, M.; Merrifield, R. B. Proc Natl Acad Sci U S A 1979, 76, 2180-2184.
78. Fujino, M.; Kitada, C.; Yoshida, I. in "Peptide Chemistry 1976," Nakjima, T., Ed.; Protein Research Foundation: Japan, 1976, p 28-32.
79. Kent, S. B. H.; Mitchell, A. R.; Barany, G.; Merrifield, R. B. Anal Chem 1978, 50, 155-159.
80. Mitchell, A. R.; Kent, S. B. H.; Chu, I. C.; Merrifield, R. B. Anal Chem 1978, 50, 637-640.
81. Feinberg, R. S.; Merrifield, R. B. Tetrahedron 1972, 28, 5865-5871.
82. Kashelikar, D. V.; Ressler, C. J Am Chem Soc 1964, 86, 2467-2473.
83. Konig, W.; Geiger, R. Chem Ber 1970, 103, 788-798.
84. Hruby, V. J.; Muscio, F.; Groginsky, C. M.; Gitu, P. M.; Saba, D. J. Med Chem 1973, 16, 624-629.
85. Mojsov, S.; Mitchell, A. R.; Merrifield, R. B. J Org Chem 1980, 45, 555-560.
86. Bissell, E. R.; Mitchell, A. R.; Smith, R. E. J Org Chem 1980, 45, 2283-2287.
87. Bissell, E. R.; Mitchell, A. R.; Pearson, K. W.; Smith, R. E.: US Patent 4,388,233, 1983.
88. Kwon, Y.; Welsh, K.; Mitchell, A. R.; Camarero, J. A. Org Lett 2004, 6, 3801-3804.
89. Camarero, J. A.; Hackel, B. J.; de Yoreo, J. J.; Mitchell, A. R. J Org Chem 2004, 69, 4145-4151.