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Solid-Phase Synthesis: A Paradigm Shift

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Abstract: A personal review by the first graduate student of Professor R. Bruce Merrifield of the evolution of solid-phase synthesis and its acceptance by various subsets of the chemistry community. Solid-phase synthesis, as currently practised in the synthesis of biopolymers, combinatorial solid-phase organic chemistry, synthesis of natural products, catalyst selection, chemical ligation and materials development, has proven a paradigm shift for the chemistry community. Copyright © 2003 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: solid-phase peptide chemistry; SPPS; solid-phase organic chemistry; SPOC; R. Bruce Merrifield; John M. Stewart; truncation; deletion sequences; combinatorial chemistry

INTRODUCTION

It is rare to be an observer/participant in the development of a technology that has had as much impact on scientific capabilities as has the solid-phase method. While the potential was certainly apparent in 1959 to its inventor, Professor R. Bruce Merrifield (Figure 1) of Rockefeller University, and others as they became exposed to the idea, no one could have predicted its dominance in combinatorial chemistry, a field not yet conceived. It is also interesting to reflect on the severe resistance that the concept generated by the synthetic organic chemistry community whom it would best serve, but this, I expect, is a common phenomena for any paradigm shift that threatens the status quo [1]. What follows is a personal view of solid-phase synthesis, its early development and acceptance from the perspective of someone who was fortunate to be Professor Merrifield's first graduate student at a time when the basic concept was being developed from a prototype into a reliable methodology.

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FROM 1959 TO 1963

A Merrifield notebook entry of 5/26/59 — 'A New Approach to the Continuous, Stepwise Synthesis of Peptides' records the first formal expression of solid-phase synthesis (SPS). In his autobiography written at the request of the American Chemical Society, Bruce outlined his scientific background and experiences at the bench with the solution synthesis of peptides that led him to this revolutionary concept, so trivial once expressed, and so powerful once reduced to practice. With Professor D. W. Wooley's blessing as head of the laboratory, Bruce started the search for the right support, linker, amino protecting group and cleavage procedure. Except for his autobiography, 'Life During a Golden Age of Peptide Chemistry' published by the ACS in 1993, very little has been revealed about the multiple possibilities explored by Merrifield during this period to realize solid-phase synthesis. A number of polymeric supports, linkers, protecting groups, cleavage reagents, etc. were examined and

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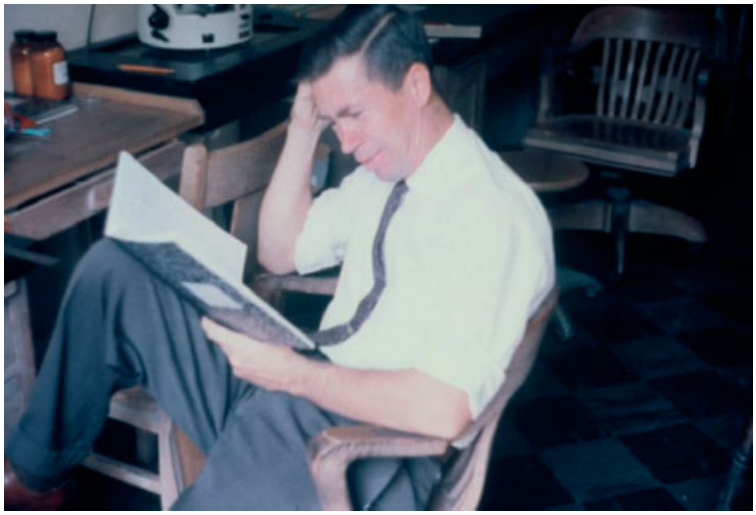

Figure 1 R. Bruce Merrifield at Rockefeller University with laboratory notebook in early 1960s. (Photo courtesy of Prof. John M. Stewart).

found wanting, at least with the technology available at the time. The problem was solved when Bruce obtained a sample of the polymer, polystyrene cross-linked with divinylbenzene (Figure 2), used to make ion-exchange resins for column chromatography that had to be functionalized (Figure 3) to generate linkage sites. His efforts with other supports including cellulose and multiple combinations of protecting groups, linkers and cleavage conditions during this development period were only revealed in any detail recently in this autobiography with direct quotations from his laboratory books.

By the end of 1962, Bruce had demonstrated the feasibility of solid-phase synthesis by preparing a tetrapeptide utilizing the carbobenzoxy group for amino protection and HBr/HOAc for deprotecting the amino group of the growing peptide chain (Merrifield, *JACS* 1963; **85**:2149,). The strongly acidic conditions required for Z removal mandated a more resistant linkage to the polymeric support, so Bruce nitrated the polystyrene resin to generate, in effect, a nitrobenzyl carboxy-protecting group stable to HBr/HOAc that could be cleaved by saponification as shown in Figure 4. The ion-exchange chromatogram shown in Figure 5 of the cleaved product of the synthesis of Leu-Ala-Gly-Val already foreshadowed many of the problems that would have to be overcome to make SPS a reliable procedure. For example, the presence of Gly-Val and Ala-Gly-Val implies that chain elongation was incomplete leading to these truncated sequences due either to incomplete deprotection or

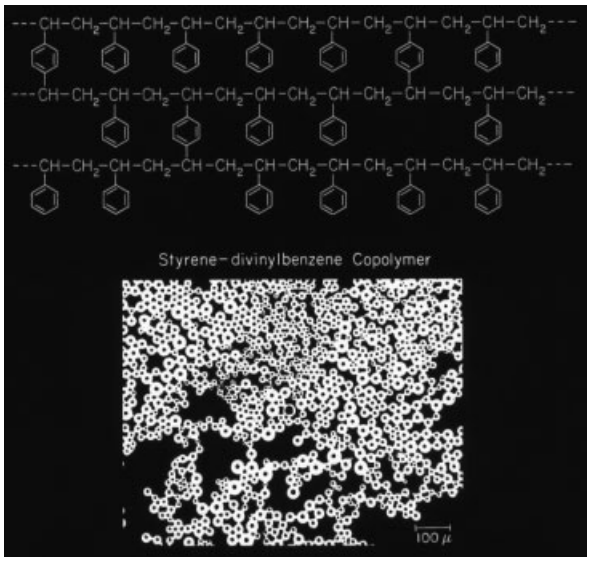

Figure 2 Formula and micrograph of polystyrene beads used for SPS. Slide was used in PhD thesis presentation (1966).

coupling. The presence of the deletion sequences Leu-Ala-Val and Leu-Val that have omitted Gly and Ala-Gly, respectively, imply that incomplete deprotection/coupling was only temporary, and growing chains could resume participation in chain elongation. The tetrapeptide with D-Val at the *C*-terminus implies racemization, either in coupling to the resin or upon saponification. The presence of acetylated peptides implies incomplete washing with retention

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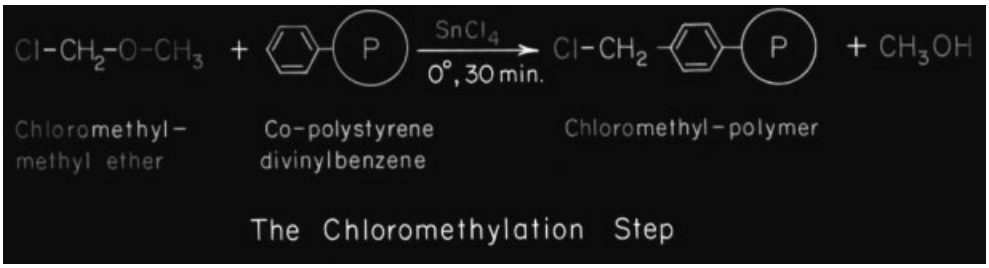

Figure 3 The polystyrene beads had to be functionalized by Friedel-craft alkylation to provide a site for benzyl ester formation with the *C*-terminal amino acid before beginning the repetitive stepwise addition of protected amino acids. The procedure was abandoned when the carcinogenic properties of chloromethylmethyl ether became known (another slide from thesis presentation).

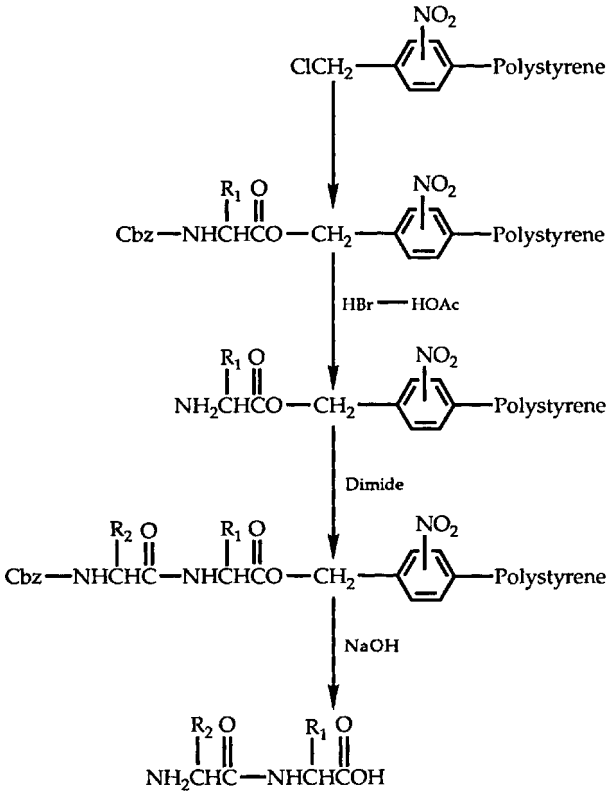

Figure 4 Original SPS protocol published in 1963. Use of Cbz for α-amino protection with strong acidic condition for removal required more stable nitrobenzyl linkage to polymeric support.

of acetic acid into the activation and coupling procedure. Nevertheless, despite these deficiencies, the major peak corresponding to the desired product Leu-Ala-Gly-Val; the ease of synthesis compared with the labour and time to produce a tetrapeptide by conventional solution approaches was sufficient to provoke both intense interest in SPS and a backlash by the solution peptide chemistry community. One of the referee's critiques of the first paper suggested that this SPS should be shunned as it violated the basic principles of synthetic organic chemistry, i.e. isolation and characterization of intermediates. This was not the last time that this criticism would be heard.

FROM 1963 TO 1966

I was essentially unaware of the many trials of alternative approaches that Bruce had endured when I showed up in his laboratory in the spring of 1963 at the suggestion of my research advisor, the prominent immunologist Henry Kunkel. Professor Kunkel had suggested that I try to develop an immunoassay for angiotensin II, an important octapeptide hormone involved with blood pressure regulation. He suggested that I work with Bruce on coupling the peptide to a carrier protein in order to use it as a hapten and develop antibodies for the immunoassay. The protein chemistry tradition (Max Bergman and Leonid Zervas came from Germany in 1933 just after their publication of the carbobenzoxy (Z) group in 1932) at Rockefeller Institute for Medical Research (later Rockefeller University) was especially strong as was that of immunology (Landsteiner had thoroughly developed the use of haptens conjugated to proteins to generate antibodies that recognized the hapten). At that time, the Merrifield section of the Wooley laboratory consisted of Bruce and his technician Angela Corrigliano. The first paper on solid phase peptide synthesis with Z-amino protection was in press in *JACS* and Bruce was already working on its successor using t-butyloxycarbonyl (Boc) amino protection (Figure 6). Since each protected amino acid had to be generated in the laboratory, each new peptide presented its own set of solution

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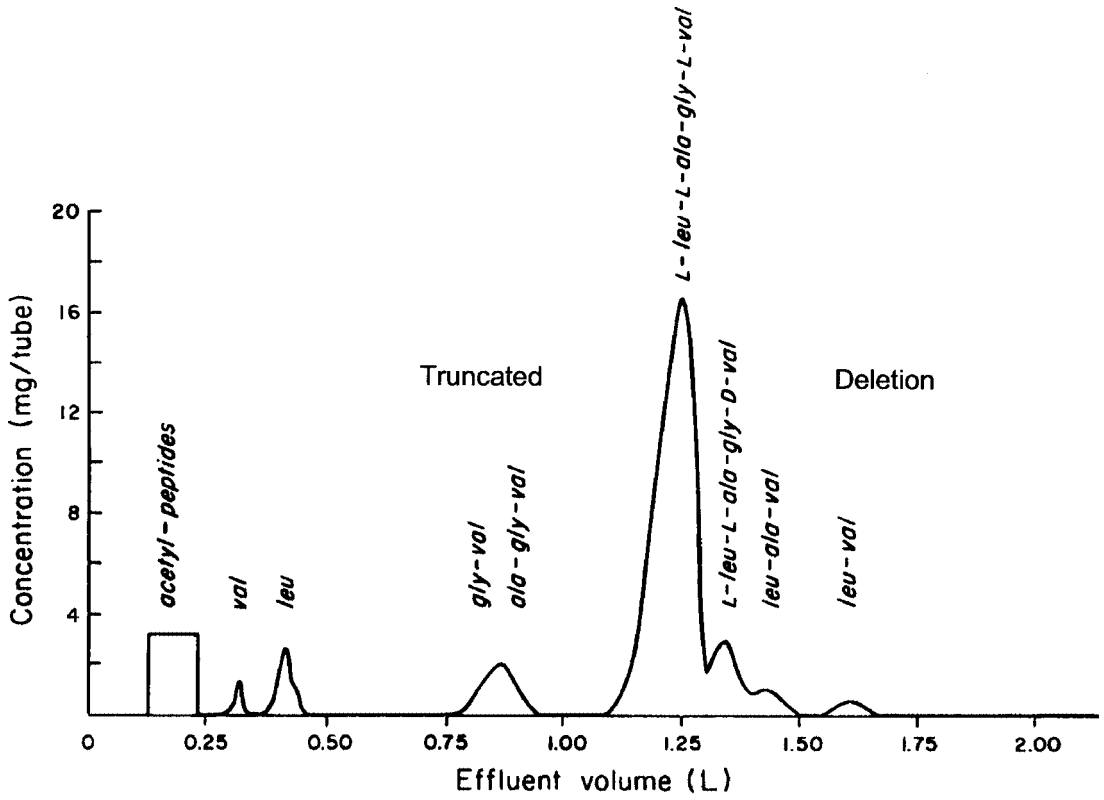

Figure 5 Ion-exchange chromatogram of tetrapeptide product Leu-Ala-Gly-Val with side products from original 1963 JACS publication. Both truncated and deletion side products were detected due to incomplete reactions. Clearly, there was room for improvement, but the crucial feasibility study was complete.

synthetic problems. Bruce and Angela has generated Boc-Arg(NO_2), Boc-Pro, Boc-Phe, Boc-Gly and Boc-Ser(OBzl) for the synthesis of bradykinin, Arg-Pro-Pro-Gly-Ser-Pro-Phe-Arg. It was my initial job to generate the Boc-derivatives of Val and side-chain protected Asp, His and Tyr for the synthesis of angiotensin II, Asp-Arg-Val-Tyr-Val-His-Pro-Phe.

Despite my background in biology at Caltech, or perhaps because of it, I instantly recognized the potential impact that SPS would have if developed. With tutoring in synthetic organic chemistry by Professor John M. Stewart, then an assistant professor associated with the Wooley group, I tried not to expose my chemical ignorance during laboratory discussions. I was essentially an apprentice across the laboratory bench from Bruce daily. Despite my naivety, Bruce and other members of the Wooley laboratories never treated me as anything but a colleague, which certainly put pressure on me to learn as quickly as possible. It was an ideal environment in which to mature as a scientist, and my focus on SPS intensified. The method brought many prominent scientists to visit, many of whom claimed to have conceived of SPS, but none other than Bruce had seriously attempted to reduce the concept to practice. I particularly remember the visit of Sir Robert Robinson, Nobel Laureate, to the laboratory in 1964. Bruce was especially excited that such a prominent figure in synthetic chemistry would come to visit him. The Nobel Laureate pulled me aside to tell me how lucky I was to be working with Professor Merrifield; I agreed, and then he said that he intended to nominate Bruce for the Nobel Prize. Every October for 20 years, I awaited the inevitable (at least to me) announcement.

It was obvious to Bruce that the simplicity of the steps involved in SPS could be automated as only introduction of solvent/reagent, shaking and filtering were involved (Figure 7). He had witnessed the automation of amino acid analysis by Professors Moore and Stein at Rockefeller, and saw the dramatic improvement in time savings and reproducibility (we did amino acid analyses manually at the time). Bruce got John Stewart to help with the electronics and drum programmer, and Nils Jernberg to design a novel rotary valve

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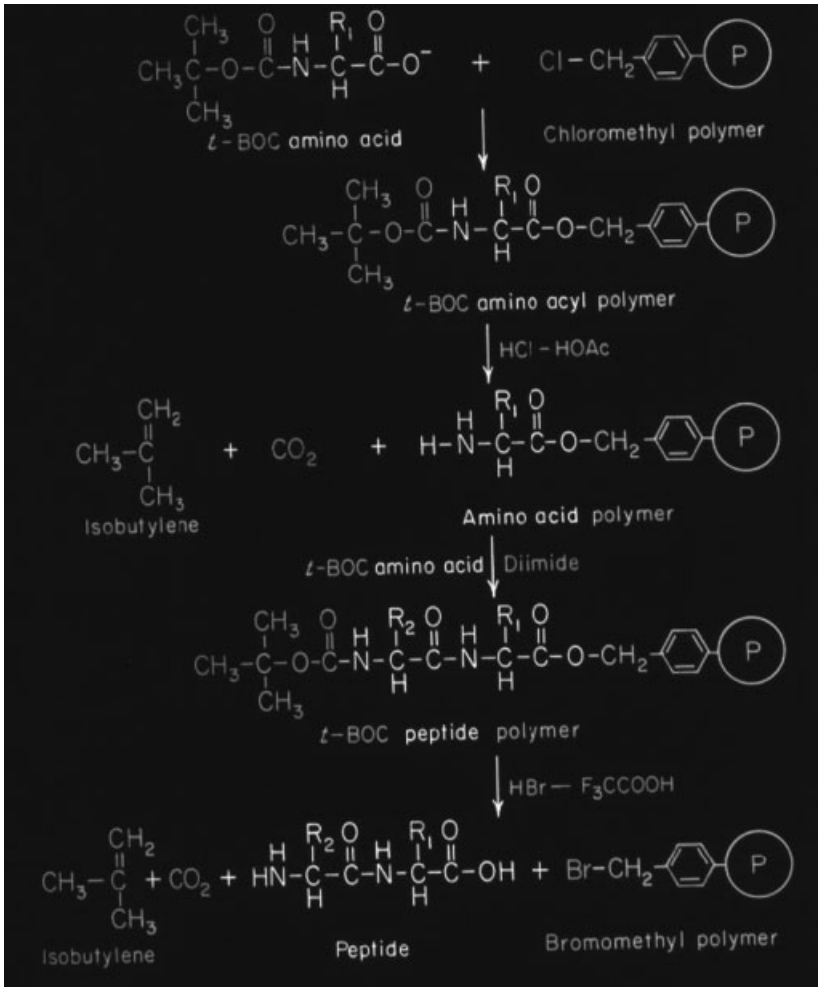

Figure 6 This protocol, based on Boc-protected amino acids, proved very robust (slide from thesis presentation). A significant improvement was the introduction by professor Shumpe Sakakibara at the 7th European Peptide Symposium of HF to replace the HBr/TFA cleavage step of the benzyl ester linker.

to select solvents/reagents while he focused on the plumbing, shaker, etc. In a fairly short time, the prototype [2] (Figure 8) was working 24 hours a day, much more reproducibly than a technician or graduate student. The prototype was eventually retired to the Smithsonian Museum in Washington, DC. I constructed the second automated synthesizer when I moved to Washington University Medical School in 1966. I tried to improve the design with a subroutine for washing built of relays to save space on the drum programmer, but quickly learned that computers built with relays were inherently unreliable, much to the amusement of my computer-experienced colleague, Professor Charles Molnar. Interaction with Professor Molnar eventually led to my involvement with computer-aided molecular design, but that's another story [3,4].

While Bruce recognized the obvious extension of solid phase synthesis to other biologically important heteropolymers (Figure 9), such as nucleic acids and oligosaccharides, the underlying chemistry was less developed and Bruce was loath to claim any potential for SPS not yet realized. As a feasibility experiment, I prepared the first nucleotide, dTT, using SPS in his laboratory in 1965 (Marshall and Merrifield, unpublished), but it was clear that the coupling yields needed to be enhanced for a serious effort in nucleic acid synthesis, and further developments by Letsinger and Caruthers [5,6] were necessary. Certainly, the ability to generate oligonucleotide sequences at will for probes enabled much of modern molecular biology. Bruce finally yielded to laboratory pressure to discuss the generalization of solid phase peptide chemistry to

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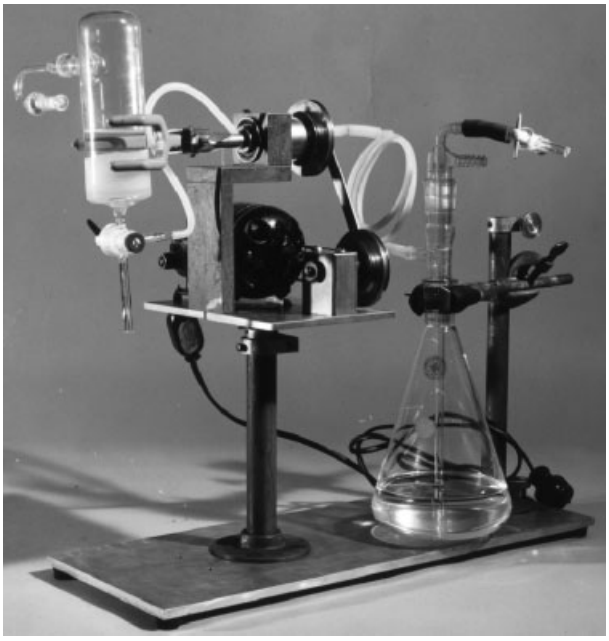

Figure 7 Shaker and glass reaction vessel with fritted glass filter for SPS — to automate, just add graduate student!.

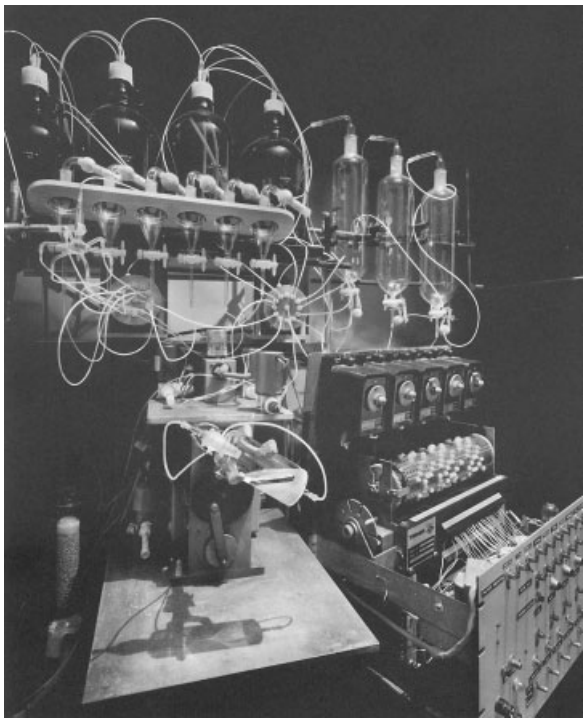

Figure 8 Automated synthesizer with rotary drum programmer, timers and two 12-port rotary Teflon valves for selection of reagents. This device proved much more reliable in the synthesis of longer sequences than the manual approach due to the repetitive nature of SPS.

other heteropolymers in his review in *Science* in 1965 [7]. SPS of oligosaccharides has finally reached an equivalent state of development [8–13]; the delay simply being due to the enhanced complexity of carbohydrates and the necessary developments of appropriate orthogonal protecting groups.

It was also clear to Merrifield that filterable, polymeric protecting groups were generally applicable to synthetic organic chemistry. He stated in a review in 1969, 'A gold mine awaits discovery by organic chemists', but it was not his objective to generalize the concept beyond biopolymers. Pioneers in this effort were Professor Robert L. Letsinger of Northwestern University and Professor Clifford C. Leznoff of York University in Canada. Letsinger [14] functionalized several different polymers as potential filterable protecting groups. Leznoff used insoluble polymeric supports to overcome a number of synthetic organic problems. For example, monoreactions of symmetrical bifunctional compounds were demonstrated using a functionalized diol to react with a symmetrical dialdehyde. Leznoff also used this approach to synthesize insect sex attractants and carotenoids [15,16]. In his excellent review [16] on 'The Use of Insoluble Polymeric Supports in General Organic Synthesis' published in *Acc. Chem. Res.* in 1978, Leznoff quotes Merrifield's comment on the gold mine awaiting organic chemists with the comment, 'Many gold nuggets have now been mined... and some iron pyrites.' Several other synthetic organic chemists also realized the potential advantages of SPS, and their pioneering efforts need to be recognized considering the cold reception that most of the synthetic chemistry community gave SPS. The Patchornik group in Israel developed a number of insoluble polymeric reagents [17,18] including coupling reagents and demonstrated their utility in the synthesis of peptides. Crowley and Rapoport evaluated the hyperentropic utility of polymeric supports as an alternative to high dilution and concluded, based on experimental work, that co(polystyrene-2% divinylbenzene) did not provide adequate site isolation [19]. Depending on the cross-linking and loading of reactive groups on the polymeric support, intermolecular side reactions including aggregation were readily demonstrated.

FROM 1966 TO 1972

The development of the automated synthesizer led to the synthesis of both chains of insulin and

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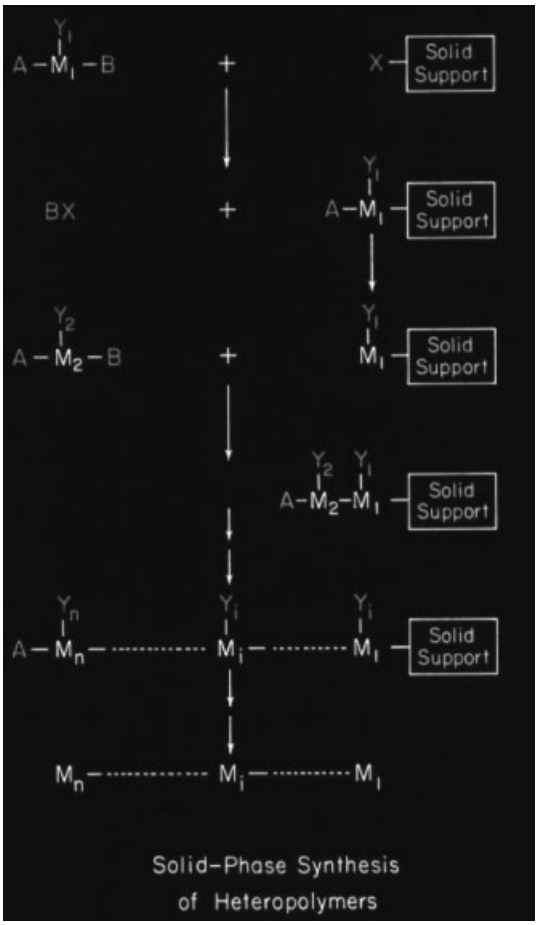

Figure 9 Generalized scheme for heteropolymer SPS (slide from thesis presentation, 1966). Merrifield clearly anticipated expansion of approach to the synthesis of other biopolymers such as nucleic acids (DNA and RNA) and oligosaccharides. The first dinucleotide, dTT, was prepared by SPS in the Merrifield laboratory in 1965 (Marshall and Merrifield, unpublished). It was Letsinger and Caruthers, however, who developed the methodology that revolutionizes the synthesis of oligonucleotides by SPS.

their recombination by Marglin and Merrifield in 1966 [20], ribonuclease (124 residues) by Gutte and Merrifield in 1969 [21,22] and to our own synthesis of the 74-residue acyl carrier protein [23] involved in fatty acid biosynthesis in collaboration with its discoverer, Professor P. Roy Vagelos. It was exciting to receive an invitation from the program committee of the 11th European Peptide Society to present our ACP work [23–25] in Vienna in 1971 [26], but I was not prepared for the warmth of the reception that I received. The established heads of many peptide laboratories, whose work I venerated, were more than generous with their criticisms; the only real defence was that the Vagelos group could not distinguish our synthetic product from the protein isolated from liver using the biochemical and biophysical techniques available at the time. Fortunately, Ralph Hirschmann of Merck and Joseph Rudinger of the Czech Academy of Sciences both befriended me, and reassured me that my treatment was a reaction to the threat of SPS to the status quo of solution peptide chemistry and not to the science that I presented. Thus, it was an exceptional pleasure to have attended the 27th EPS in Sorrento some 30 odd years later, which confirmed that SPS has become the dominant approach in peptide synthesis and combinatorial chemistry.

Merrifield did an interesting experiment that dramatized the unanticipated sequence-dependent problems that occasionally plagued SPS. Since bradykinin and angiotensin had each been successfully synthesized, he decided to synthesize a hybrid continuing the sequence of angiotensin appended to the grown bradykinin sequence on the polymeric support. Figure 10 shows the coupling yields for each residue. In this case truncation of the growing chain could be overcome by modifying the divinylbenzene cross-linking and deprotection reagent. From our work on acyl carrier protein came additional insight into one of the underlying problems of SPS — changes in the physical properties of the polymeric support with chain elongation [27]. We utilized titration of the amine on the polymer with a radioactive isotope of chlorine (^{36}Cl) following the method of Dorman [28]. The *C*-terminus of ACP showed a dramatic decrease in the growing chain that reappeared leading to a deletion sequence (deletion and truncation sequences were clearly defined and their origins discussed by Hancock *et al.* [27]. The titration showed that all the bound chloride could not be removed by simple washing with triethylamine in organic solvent, but additional chloride was rendered accessible by shrinking the polymer with t-butanol and then reswelling in DCM, followed by washing with additional triethylamine in organic solvent. This implied that part of the polymer had become inaccessible to solvent due to the heterogeneity of sites and changes in the ratio of peptide to polystyrene; Steve Kent and Bruce Merrifield later attributed this to formation of β-sheet aggregation of the peptide consistent with the amino acid sequence of the ACP peptide. What was of some surprise was the ability of different laboratories to reproduce the difficulties with this sequence despite

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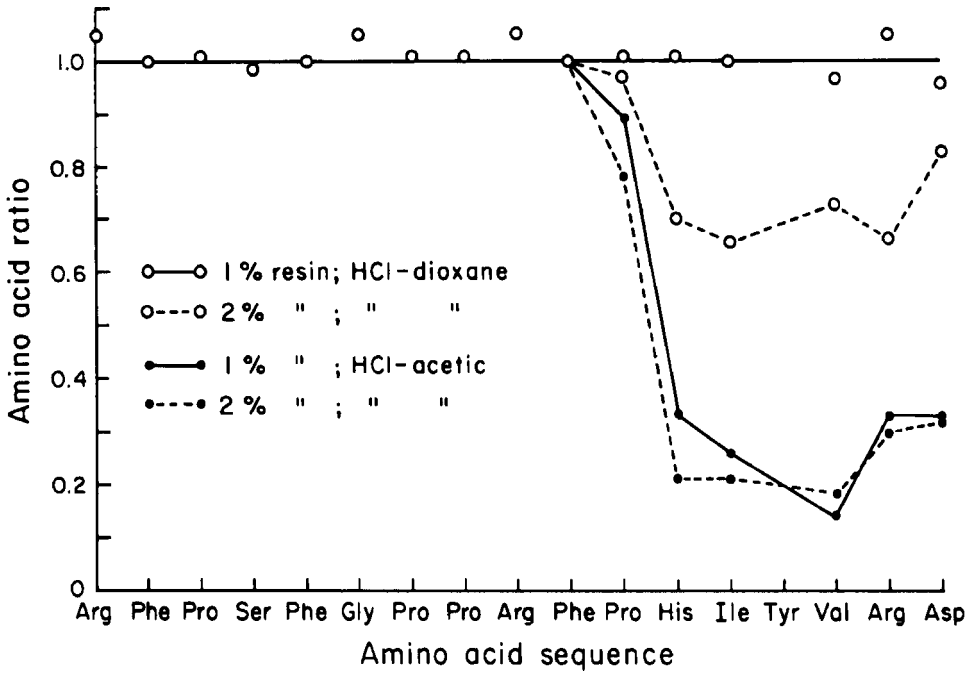

Figure 10 Graph showing dramatic truncation of growing peptide sequence in synthesis of angiotensinylbradykinin and effect of changing cross-linking of support and solvent used for Boc cleavage.

the variations in support, linker and loadings that were used suggesting that aggregation of the growing peptide chain was the most likely explanation.

FROM 1972 TO 1984

This period saw a rapid acceptance of solid phase synthesis for both peptides and nucleic acids. The availability of a number of reliable automated synthesizers with great effort paid to optimization of yields and elimination of side reactions made reliability an achievable goal. There were the occasional 'difficult sequences', but overall chemists were making products in good yield and with acceptable purities. The increasing availability of HPLC and mass spectrometers made purification and characterization more routine. The many chemists who helped optimize solid-phase peptide chemistry are too numerous to name, but there was a heavy concentration of young associates (DiMarchi, Erickson, Gisin, Hodges, Kent, Mitchell, Tam, Wang, etc.) in Bruce's laboratory at Rockefeller University that explored many variations of support, monitoring methods, deprotection schemes, side reactions, etc. during this period. Finally, on 17 October 1984, the announcement that R. Bruce Merrifield had won the Nobel Prize in Chemistry came from Stockholm. The annual October vigil that began with Sir Robert Robinson's comment had finally come to fruition.

FROM 1984 TO THE PRESENT

Vindication of all the effort that had been focused on transforming the idea into a prototype and finally, into the method of choice, was gratifying to all those who worked on solid-phase synthesis. But there were always new horizons and opportunities for expanding the scope of application areas, and the advent of combinatorial chemistry was just such an opportunity. The impetus for combinatorial chemistry was the development of high-throughput *in vitro* binding assays and tissue-culture screens that allowed testing of large numbers of compounds in the pharmaceutical industry. The traditional approach of medicinal chemistry to sequentially synthesize a logically designed set of analogues based on a pharmacological lead was surpassed by the ability to screen whole compound libraries accumulated over years by large pharma.

The groundwork for combinatorial chemistry had already been set in peptide chemistry by Geysen with his pin approach [29–31] and Houghten with his 'teabag' approach to epitope mapping [32]. Both

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approaches used physical separation of polymers to control reaction sequences and thus peptide products. Lam [33] and Furka [34] conceived independently of the 'one bead, one product' split-and-mix approach that has been so powerful. Houghten has shown that synthesis of large mixtures followed by screening and deconvolution to identify the active components is a viable and efficient technique [35–39]. In many ways, it is analogous to isolation of active natural products from fermentation broths. Nevertheless, the pressure from the medicinal chemistry community in the pharmaceutical industry has focused on combinatorial synthesis of single compounds, partially due to perceived problems with false-positives in the deconvolution process.

The flood of recognition by the medicinal chemistry community of the advantages of filterable, polymeric protecting groups (advocated in 1971 in a review by Marshall and Merrifield [40] and demonstrated so convincingly by Leznoff [15,16]) was catalysed by a paper in 1992 by Bunin and Ellman [41] on the synthesis of a combinatorial library of benzodiazepines, a privileged class of structures thought to mimic β-turns. The generation of a compound library of direct interest to the pharmaceutical interest because of the many biological activities found with benzodiazepines was a turning point in acceptance of the overall approach. It has become difficult to find a chemical reaction, or class of compound, that has not been adapted to solid phase chemistry. As examples of the pervasiveness of the approach in synthetic organic chemistry, two reviews, one on multiple approaches to traceless supports [42] for SPS and the other on heterocyclic chemistry [43], have recently appeared. Synthesis of complex natural products as diverse as sarcodictylins, chalcones and epothilones [44] utilizing solid-phase organic chemistry are becoming more commonplace as the advantages of a filterable, polymeric protecting group become more widely recognized. The paradigm shift has even extended to the search for metal-binding ligands, catalysts and new materials [45,46].

While a number of small proteins have been successfully assembled by SPS, practical limitations regarding the ability to purify and characterize the mixtures that inevitably result from less than complete reactions and side reactions during deprotection limit most efforts to below 100 residues. The advent of chemical ligation where purified fragments without side-chain protection can be stitched together has provided a viable hybrid strategy for larger proteins. This approach evolved from the thiol-capture approach of Dan Kemp [47] and has been actively developed in the laboratories of Steve Kent [48,49] and James Tam [50]. The most recent paradigm shift has come from the laboratory of Tom Muir where expressed protein fragments are ligated to synthetic peptides to generate hybrids [51,52]. A student in my group, Lori Anderson, has used expressed protein ligation to specifically label the *C*-terminal segment of the α-subunit of a G-protein for both MAS NMR and ESR studies aimed at mapping the interface between the α-subunit and the activated GPCR rhodopsin. This blend of synthetic chemistry and molecular biology has enormous potential as we attempt to understand the dynamics of large complex multi-protein systems found in biology.

CONCLUSIONS

Often, it is the outsider who brings a fresh perspective to a problem that generates the insight necessary for a paradigm shift. Certainly, the invention by Merrifield of SPS with its following automation is a classic example of scientific revolution as discussed by Thomas Kuhn [1]. Traditional synthetic organic chemistry required isolation and characterization of intermediates as concrete evidence supporting the chemical structure of the product. By substituting the use of excess reagents to force chemical reactions to completion (or as close as possible), solid-phase chemistry was anathema to traditional synthetic practice of the time. Resistance to change by synthetic chemists in general, and peptide chemists in particular, was both vehement and vitriolic. In addition, solid-phase chemistry required careful purification and characterization of its product that did not depend on the history of the synthetic process. In reality, this was only possible with a concomitant improvement in both purification techniques and analytical methods of structural characterization. Without modern HPLC, capillary electrophoresis, NMR, mass spectroscopy, etc., solid-phase chemistry would not have been so feasible. The practical advantages in handling and automation offered by a filterable, polymeric protecting group in automation of chemical synthesis far outway the increased needs for more effort in purification and characterization.

I presume that Emil Fischer would be both amused and impressed with the progress made in the past century. The ability to synthesize peptides, small proteins and nucleic acids by

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SPS has enabled much of modern molecular biology. In turn modern molecular biology has provided us with the plethora of therapeutic targets through cloning and expression that has driven both combinatorial chemistry and structural biology. Technology enables us to ask relevant questions; there is little doubt that expression ligation combined with SPS of labelled peptides will enable the dissection of many significant biological systems.

Acknowledgements

From the contents of this personal view of SPS, my deep debt of gratitude to my mentor, colleague and friend, Professor R. Bruce Merrifield, should be obvious. In addition, Professor John M. Stewart of the University of Colorado Medical School exerted a strong positive influence on my scientific development. I would be remiss if I did not acknowledge the support over these many years from NIH that have allowed me to indulge my scientific interests and that supported the development of SPS in so many different laboratories. To the many who have laboured to make SPS reliable and who were not specifically acknowledged in this overview, both my thanks for your efforts and my apologies for the omission.

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