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R. Bruce Merrifield and Solid-Phase Peptide Synthesis: A Historical Assessment*

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*Dedicated to David Rockefeller, banker, philanthropist, world statesman and friend of Bruce Merrifield. David Rockefeller's wise and generous support of The Rockefeller University, founded in 1901 by his grandfather, John D. Rockefeller, made possible the scientific career of Bruce Merrifield.

Introduction

This paper answers a request from the editors of the Merrifield memorial issue to place Bruce Merrifield and solid-phase peptide synthesis (SPPS) into historical context by considering the following questions. How did such a mild-mannered and relatively unknown biochemist in the mid twentieth century evolve to a global chemical icon[1-5] by the end of the century? What were the challenges and how were they met? Was there a unique scientific life-style that exemplified Merrifield's approach to research and what is his legacy? While I did spend several memorable years in the Merrifield laboratory I do not claim unique insights.[6] This paper derives from a talk given at the Merrifield Memorial Symposium at the Rockefeller University on November 13, 2006. It compliments contributions by Art Felix, Maurice Manning, Arnold Marglin, Garland Marshall, Arthur Robinson, Noah Robinson and John Stewart in this issue and hopefully adds to our understanding and appreciation of Bruce Merrifield.

Historical Sources

R. Bruce Merrifield (1921-2006)

Bruce Merrifield's scientific biography, "Life During a Golden Age of Peptide Chemistry: The Concept and Development of Solid-Phase Peptide Synthesis," provides a history of SPPS from 1959 to 1993.[7] While many readers will be familiar with SPPS literature after 1963, the inclusion of unpublished material from Merrifield's early laboratory notebooks opens a revealing window on the development of SPPS from the formulation of concept in 1959 (p. 56, ref. 7) to the synthesis of a tetrapeptide four years later.[8] Garland Marshall, Bruce's first graduate student (1963-1966), as well as later colleagues, were essentially unaware of the many highways, byways and non-productive routes that Bruce had explored in the early years.[1] Bruce had also

provided a very personal recollection of the discovery and development of SPPS that was incorporated into a video, "Peptide and Protein Synthesis: Origin And Development," and shown at the 17th American Peptide Symposium/2nd International Peptide Symposium (June 2001, San Diego, CA) honoring his 80th birthday.[9]

Joseph S. Fruton (1912-2007)

Joseph Fruton received his Ph.D. (1934, biological chemistry) from Columbia University and then worked in the laboratory of Max Bergmann at the Rockefeller Institute for Medical Research until 1945. He used the benzyloxycarbonyl (Z) group invented by Bergmann and Zervas[10] to prepare synthetic peptides for the study of specificity and mechanisms of catalytic action of proteolytic enzymes such as pepsin. He was until recently Professor Emeritus of Biochemistry and Professor Emeritus of the History of Medicine at Yale University and has an extensive list of journal publications (more than 330 scientific papers, reviews and monographs) on research in his laboratory and studies on the history of science[11,12] and the interactions between chemistry and biology.[13,14] He has also written a candid autobiography documenting the first 80 years of his life.[15]

Peptide Synthesis Circa 1959

It is instructive to survey the world of peptide synthesis that would have been known to Merrifield prior to 26 May 1959, the day he recorded the concept of SPPS in his laboratory notebook. Three major chemists merit discussion.

Emil Fischer (1852-1919)

Theodor Curtius had synthesized the first known peptide derivative (benzoylglycylglycine) in 1882. Emil Fischer, however, beginning with the synthesis of a free dipeptide (glycylglycine) in 1901, is credited with the first systematic attack on a field of natural substances that had been previously avoided by chemists. Fischer, considered by some to be the greatest chemist in the 19th century, had conducted landmark studies in purine and carbohydrate chemistry prior to initiating studies in peptide and protein chemistry. The 1902 Nobel Prize in Chemistry was awarded to Emil Fischer ″in recognition of the extraordinary services he has rendered by his work on sugar and purine syntheses.″[16] Fischer, in his Nobel lecture, compared the challenges of carbohydrate and protein chemistry:

> "Nevertheless, the chemical enigma of Life will not be solved until organic chemistry has mastered another, even more difficult subject, the proteins, in the same way as it has mastered the carbohydrates...***the total amount of work that has to be done here is so enormous that in contrast the elucidation of the carbohydrates seems child's play***."

Notwithstanding the massive challenge that peptide and protein synthesis posed to Fischer, he thought that all available tools and resources should be employed (p. 125, ref. 7):

> "Whereas cautious professional colleagues fear that rational study of this class of compounds, because of their complicated structure and their highly inconvenient physical characteristics, would today still uncover insurmountable difficulties, other optimistically endowed observers, among which I would count myself, are inclined to the view that an attempt should at least be made to besiege this virgin fortress with all the expedients of the present; because only through this hazardous affair can the limitations of the ability of our methods be ascertained."

Fischer's optimism was reflected a year earlier (1905) in a letter to Adolph von Baeyer:

> "My entire yearning is directed toward the synthesis of the first enzyme. If its preparation falls into my lap with the synthesis of a natural protein material, I consider my mission fulfilled."

Fischer's progress in peptide synthesis was hampered by the lack of reversible protecting groups. Nevertheless, his use of the haloacyl method yielded an octadecapeptide containing three amino acids.[17]

Max Bergmann (1886-1944)

Max Bergmann completed his doctoral dissertation in Fischer's laboratory (1911) and became an assistant to Fischer until Fischer's death in 1919. He was director of the Kaiser Wilhelm Institute for Leather Research in Dresden from 1922-1933 and during this period Bergmann, with his student Leonidas Zervas, invented the benzyloxycarbonyl (Z) group for the reversible protection of amino groups.[10] The careers of Jewish scientists in Germany virtually ended with the creation of the Nazi state in 1933. Bergmann immigrated to the United States in 1934 where he headed a laboratory at the Rockefeller Institute for Medical Research until his early death from colon cancer in 1944. Zervas had followed Bergmann to the Rockefeller Institute where a talented group of postdoctoral associates that included Joseph Fruton, Klaus Hofmann, Stanford Moore and William Stein was assembled. Fruton, initially tutored by Zervas in peptide synthesis, used the Z group to prepare numerous peptide substrates for specificity studies on a variety of proteolytic enzymes (p. 41, ref. 15). Bruce was very much aware of Bergmann's work performed at the Rockefeller Institute for Medical Research prior to his arrival and felt honored

to work in the same laboratory space that Bergmann had occupied (personal communication, Dr. Ulf Ragnarsson). He was especially proud to have inherited Bergmann's office and desk.

Vincent du Vigneaud (1901-1978)

Vincent du Vigneaud's career spanned nearly 50 years with a focus on sulphur-containing compounds of biochemical importance including insulin, penicillin, and hormones of the posterior pituitary gland (oxytocin, vasopressin). A series of lectures presented at Cornell University, "A Trail of Research in Sulphur Chemistry and Metabolism and Related Fields," describe his meticulously designed and executed research from the late 1920s into the early 1950s.[18] Du Vigneaud's move to Cornell University Medical College as Head of the Biochemistry Department in 1938 placed him next door to the Rockefeller Institute for Medical Research on the upper east side of Manhattan (New York City). This close proximity gave du Vigneaud, son of an inventor and machine designer, early access to research tools developed at Rockefeller that would later prove invaluable in the structure determinations and subsequent syntheses of oxytocin and vasopressin. The invention of countercurrent distribution (CCD) by Lyman Craig (1906-1974) made possible the isolation and purification of antibiotics, peptide hormones (oxytocin, vasopressin, ACTH, MSH, HGH), proteins (TMV protein, ribonuclease, hemoglobin α and β chains) and transfer RNA.[19,20] Stanford Moore (1913-1982) and William Stein (1911-1980) continued the work of the Bergmann laboratory at the Rockefeller Institute for Medical Research and developed the first reliable and sensitive amino acid analysis of peptides and proteins. All of the naturally occurring amino acids from mammalian sources were initially resolved by chromatography on starch columns with a more rapid chromatography later achieved by ion exchange chromatography.[21]

Although du Vigneaud began receiving nominations for a Nobel Prize as early as 1943 (physiology or medicine) and 1944 (chemistry), it was not until his landmark work on the structure and synthesis of oxytocin reported in 1953 that the Royal Swedish Academy of Sciences decided that the 1955 Nobel Prize in chemistry should be awarded to du Vigneaud.[22] A few points regarding these nonapeptide hormones from the pituitary gland should be noted. The isolation of highly purified oxytocin needed for structural studies was made possible with the CCD apparatus developed earlier by Lyman Craig at the neighboring Rockefeller Institute for Medical Research.[19,20] As structure determinations of peptides in the 1950s precluded the use of modern instrumentation (HPLC, mass spectrometry, high resolution NMR), careful degradative studies, coupled with accurate amino acid analyses, led to a proposed structure for oxytocin which featured a nonapeptide amide containing a disulfide bridge.[23] The synthesis of such a structure in 1953 was formidable task and it was far from obvious how to synthesize a molecule with a disulfide bridge that forms a 20-atom ring containing six of the nine residues present in the molecule. Wieland and Bodanszky have discussed the details of the first and subsequent syntheses of oxytocin.[24] The use of CCD, successfully employed in the isolation of the natural product, proved invaluable in the purification of synthetic oxytocin. The overall yield in the first synthesis of protected nonapeptide by fragment condensations and its conversion to pure, biologically active oxytocin was well under one percent. Subsequent workers improved the overall yields of oxytocin to 5-10% employing fragment condensations. Bodanszky and du Vigneaud employed *p*-nitrophenyl esters of carbobenzoxy-aminio acids to synthesize oxytocin in stepwise manner starting from the C-terminal residue. This new strategy maintained optical purity during the peptide bond-forming reactions which afforded oxytocin in 38% overall yield.[25]

Challenges in the Development and Acceptance of SPPS

We can assume that Merrifield was aware of the work discussed above as well as other publications. The synthetic peptide community and its synthetic literature were not so large as to be unmanageable in the 1950s. Merrifield arrived at the Rockefeller Institute for Medical Research in 1949 after graduate training in biochemistry and microbiology under Max Dunn at UCLA. His work in the laboratory of Dr. D.W. Woolley on the structural characterization of peptide growth factors (strepoginins) eventually led to a need for the chemical synthesis of strepoginin peptides. In describing the solution synthesis of a pentapeptide (Ser-His-Leu-Val-Glu) Bruce remarked that "my overall yield of pentapeptide was 7%, and it took me 11 months. Certainly, an experienced peptide chemist would have done better, but not without considerable effort." (p. 53, ref. 7). Certainly, in Bruce's mind there was a clear need for a more efficient and rapid synthesis of peptides.

Bruce Merrifield had to address three major challenges related to development and acceptance of SPPS. The interrelated challenges were (1) to reduce the concept of peptide synthesis on a insoluble support to practice, (2) overcome the resistance of synthetic chemists to this novel approach and (3) establish that a biochemist had the scientific credentials to effect the proposed revolutionary change in chemical synthesis. How were these challenges met?

Peptide Synthesis on an Insoluble Support (1959 – 1984)

The use of an insoluble polymer covalently linked to a growing peptide chain was without chemical precedent when Bruce began his studies on SPPS in 1959. While a vast literature on the preparation and modification of polymers was known, no one had reported using polymer supports for the synthesis of other classes of compounds. Although Bruce did not recall exactly when he had the idea for SPPS, its genesis was in his words "obviously a result of having recognized a distinct need and of having thought about the general problem for some weeks. I also think it was in large measure a result of having been close to the earlier peptide work in an experimental sense. Because I had done all of the laboratory work myself, I could visualize from direct experience what could be done and what needed to be done (p.55, ref. 7)."

Christian Birr speculates in his monograph, "Aspects of the Merrifield Peptide Synthesis," that Merrifield's idea for SPPS was influenced by the ribosomal biosynthesis of proteins.[26] However, the N- to C- sequential biosynthesis of proteins on ribosomes (Merrifield SPPS proceeds in a C- to N-direction) and was not reported by Howard Dintzis until the early 1960s. On a human interest note, Dintzis recalled taking an advanced biochemistry course at UCLA in the 1940s and remarked that "the laboratory part of that course was excellent, having as teaching assistant a remarkable graduate student: Bruce Merrifield, who later won a Nobel Prize for devising solid state peptide synthesis."[27]

Continuing in a speculative vein, a more obscure precedent for SPPS was recently found in a patent search by this writer, namely "Improvements in and Relating to Polyamides Derived from α–Amino Acid N-Carboxyanhydrides" published in 1957 in Great Britain by Basil Alexander Ripley-Duggan (Patent Specification, Dec. 4, 1957, GB787,344). Briefly stated, a soluble

aminopolystyrene (linear, non-crosslinked) was prepared and reacted in benzene with the N-carboxyanhydride of DL-valine to afford a block copolymer containing 24-9% polystyrene and 76-91% polypeptide. This material dissolved only in *m*-cresol and displayed good thermoplastic properties. Interestingly, many years later, Sarin and Kent used a step-wise approach to prepare peptide-polystyrene supports containing up to 60 amino acid residues (80% peptide content) in a study of synthetic efficiency in SPPS as a function of growing chain length.[28] The observation of high synthetic efficiency at 60 residues and 80% peptide content on a 1% crosslinked polystyrene support demonstrated the lack of intrinsic limitations to stepwise solid-phase synthesis over an extreme range of peptide loading. This was, of course the state of perfection of SPPS in 1984, not 1959.

Regardless of what conscious and unconscious forces were at play, when Bruce formally described the concept of SPPS in his laboratory notebook on May 26, 1959, the most trying period in his scientific life was to begin. The search for a suitable support and appropriate chemistry that was originally planned to take 3 months consumed 3 years with Bruce later reflecting, "At the end of the first two years the results were so poor, I wonder what made me think that this approach would ever succeed. I have always been grateful to Dr. Woolley for giving me the freedom in his laboratory to pursue the problem to the end and to the Rockefeller University for supporting me through the long nonproductive period (p. 90, ref.7)". He also noted "When I look back at my old notebooks, I am amazed at how inefficient the early developmental work was. I seemed always to choose the wrong reaction to do first and was not able to identify the most important parameters as the work was progressing. (pp. 89-90, ref.7)." The first successful synthesis of the tetrapeptide Leu-Ala-Gly-Val by SPPS was described at

the meeting of the Federation of American Societies for Experimental Biology in 1962 with a full paper appearing in 1963.[8] The response of non-specialists (scientists employing peptides in biological investigations) was quite enthusiastic while the specialists (synthetic peptide chemists) seemed dismayed. The idea of conducting a multi-step synthesis, no matter what forcing conditions were employed, without isolating, purifying and characterizing intermediates was clearly beyond the pale.

Certainly, no self-respecting peptide chemist was going to abandon classical, solution techniques and adopt SPPS as described in the 1963 paper. However, for Bruce, the diligent optimist, the corner had been turned. The concept of step-wise peptide synthesis on an insoluble matrix had been demonstrated. Now it was time to synthesize larger, more complex peptides using SPPS. Recall that the test tetrapeptide Leu-Ala-Gly-Val was the result of 3 years of slow progress interrupted by numerous setbacks. Now, with more appropriate chemistry becoming available, the pace quickened. Replacement of $N^{\alpha}Z$- with N^{α}–Boc-protecting groups and use of an unsubstituted 2% cross-linked polystyrene resin in place of the originally employed nitropolystyrene support allowed the preparation of fully active and chromatographically pure bradykinin (nonapeptide) in 32% yield in 8 days. A subsequent, improved synthesis, provided bradykinin in 68% yield. Garland Marshall, Bruce's first graduate student, joined the Merrifield group at this time with the task of synthesizing the octapeptide hormone angiotensin II. Garland contrasted this goal with the efforts of Vincent du Vigneaud and a large team of synthetic chemists ten years earlier that resulted in the synthesis of two nonapeptide hormones (oxytocin and vasopressin) and a Nobel Prize for du Vigneaud (vide supra). In Garland's words " Here I was, just ten years later, trying to synthesize an octapeptide hormone, angiotensin II by myself, a

naïve graduate student, with a totally novel approach."[29] Garland, of course, succeeded and a series of increasingly larger, biologically active peptides were prepared over the next three years (1964-1967) in the Merrifield laboratory culminating with bovine insulin.[30] The arrival of Bernd Gutte, Bruce's first postdoctoral fellow, from Germany (1967) provided the opportunity to push existing SPPS methodology to the limit and undertake the synthesis of the 124-residue enzyme RNase A. In early 1969 Bernd Gutte and Bruce Merrifield published the use of SPPS to achieve the total synthesis of an enzyme with RNase A activity.[31] This achievement, coupled with a similar effort by the Merck group using classical solution chemistry[32] attracted global attention in the scientific and popular press.

By the early 1970s it had became apparent that the solid-phase synthesis of RNase A could not be generalized. Consequently, virtually every aspect of SPPS was reexamined and improved during the decade of the 1970s (pp. 151-179, ref. 7). The sensitive detection and elimination of possible side reactions (amino acid insertion, N^{a}-trifluoroacetylation, N^{ae}-alkylation) was examined. An optimization of the HF cleavage reaction based on an understanding of the mechanism was developed. 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. In addition to considerable methodological work on SPPS, parallel synthetic efforts on biologically active peptides such as glucagon, thymosin α_1, epidermal growth factor and antimicrobial peptides were undertaken (pp. 180-195, ref. 7). These and many other studies from the Merrifield laboratory and elsewhere increased the general acceptance of SPPS.

The success of SPPS dramatically influenced the chemical synthesis of DNA.[33] The chemical synthesis of DNA had been extremely laborious and time-consuming prior to the development of solid-phase syntheses of DNA. For example, the preparation of a *lac* operator (a 21 base paired DNA duplex) required the equivalent of four years of highly skilled and intense effort. When the appropriate chemistry (phosphoramidite method of DNA synthesis) and support were discovered, the rapid preparation ($\leq$ 1 day) of deoxyoligonucleotides the size of a *lac* operator became possible. Use of automated DNA synthesis machines now leads to very high yields of relatively pure polynucleotides having 100 or more mononucleotides.[33] In retrospect, it seemed likely that Bruce would receive validation from Stockholm. The decisions of the Nobel Committee are not obvious, however.[22] To the great delight of friends and colleagues the call came on October 17, 1984 with the decision to award the 1984 Nobel Prize in Chemistry to Bruce "for his development of methodology for chemical synthesis on a solid matrix."[16]

Resistance and Acceptance of Chemical Synthesis on a Solid Matrix

Merrifield first described the synthesis of the tetrapeptide Leu-Ala-Gly-Val by SPPS at the annual meeting of the Federation of the American Societies for Experimental Biology (FASEB) in the spring of 1962. He recalled that two "peptide experts" were sitting in the front row of the audience. One of the experts was Professor Joseph Fruton, certainly the doyen of American peptide chemistry at that time (*vide supra*). Fruton expressed his displeasure by stating "this is not the way to synthesize peptides."[34] A full paper describing the concept of SPPS and synthesis of the tetrapeptide was submitted in early 1963.[8] One of the reviewers characterized this new approach as a "travestry, …not chemistry at all, a concept which should be suppressed by the community."[29] Garland Marshall recalled the early "vehement and vitriolic" critics in his

discussion of SPPS as a paradigm shift.[1] He also recalled collecting "a fair bit of psychological scar tissue, as did Bruce, in spreading the gospel of this paradigm shift in chemistry." Max Brenner essentially summarized the early criticism of SPPS in 1973 by dyspeptically stating: *"The invention of the solid-phase method looked like an ingenious trick to overcome some of the unpleasant features of the classical methods. As we know today, the ingenuity of the trick remains, but only a large investment of heavy real effort will eventually, if ever, work it into a real progress over the classical approach*."[35]

The early negative receptions from peers, eminent and otherwise, would have dissuaded the weak-kneed among us but not one with the constitution of Bruce Merrifield.[36] Even in his darkest hours, he did not waver and it bears repeating: "At the end of the first two years the results were so poor, I wonder what made me think that this approach would ever succeed. But from the outset I had a strong conviction that this was a good idea, and I am glad that I stayed with it long enough". (p.90, ref.7).

The bradykinin syntheses[37,38] provided the demarcation between 3+ years of developmental work in SPPS and the ability to produce other biologically active peptides of similar size and complexity. However, for SPPS to be accepted, it was essential to have validation from scientists grounded in synthetic organic chemistry. Fortunately, John Stewart joined the Woolley group in 1952 after completing his doctoral research in synthetic organic chemistry with Roger Adams at the University of Illinois. Bruce and John and their families became good friends. In "Remembering Bruce: The Early Years "[39] John recalls during the developmental work on SPPS: "Bruce and I had spent long hours discussing the problems, failures, and possible improvements.

Since the problems were a specialized form of organic chemistry and I was a trained organic chemist, I was able to make useful suggestions. When yet another experiment failed, Bruce would declare that he was going to quit and open a gasoline station on a road in the Arizona desert; Bruce had a great love for the southwest American desert. Fortunately he never carried through on that threat!" In research with Dr. Woolley starting in 1962 John began synthesizing analogs of bradykinin in a search for antagonists. The syntheses employed standard solution peptide synthesis methods and in one year he synthesized three bradykinin analogs. Bruce's success in preparing bradykinin by SPPS led John to adopt SPPS and in the next year he was able to synthesize nearly fifty analogs. This fully convinced Stewart that SPPS would be an incredible improvement for the general synthesis of peptides.[39]

During this period (1961 - 1964) Maurice Manning, an organic chemist and Fulbright Travel Grantee from Ireland, was a city block away in the du Vigneaud laboratory (Cornell Medical College) learning to make peptides by solution methods. Manning synthesized three analogs of oxytocin in three years and learned what huge resources, in terms of manpower, equipment and supplies were required to sustain the highly successful research program on the neurohypophysial peptides in the du Vigneaud laboratory. Manning then spent a year learning the recently developed SPPS from Merrifield in the Woolley laboratory at the Rockefeller University (1964 -1965). The remainder of the story as recounted by Manning in "Impact of the Merrifield Solid Phase Method on the Design and Synthesis of Selective Agonists and Antagonists of Oxytocin and Vasopressin: a Historical Perspective," is a magnificent research *tour de force.*[40] Briefly stated, Maurice Manning's synthesis of oxytocin by SPPS opened the way for him to conduct monumental research on the design and synthesis of selective agonists,

antagonists and radioiodinated ligands for oxytocin and vasopressin receptors over a 40 year period without requiring the vast resources and manpower available to large academic and industrial laboratories.

While no one had claimed quantitative (100%) coupling efficiencies in SPPS, the efforts of Merrifeld, Stewart, Manning, Marshall and others demonstrated during the 1960s that SPPS (in combination with purification techniques then available) was fully capable of efficiently producing 10-residue peptides indistinguishable from the corresponding natural products or peptides prepared by solution methods. Nevertheless, the concept of a multi-step synthesis without the isolation and purification of intermediates was an anathema to most synthetic chemists. What then would be the prognosis for the preparation of peptides containing 30 amino acids? 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.[41] The synthetic glucagon was prepared in solution using classical fragment condensation methods by a large, skilled team over a period of several years.[42] Wünsch reviewed the synthesis of naturally occurring polypeptides and reflected on the problems of synthetic peptide research circa 1971.[43] Using the glucagon synthesis as a model, he contended that conventional (solution) synthesis 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. Wünsch did not entertain the prospect of examining structure-function relationships in glucagon using solution synthesis for the production of analogues. The cost, of course, would be astronomically prohibitive in terms of manpower and resources. A large portion of the review is

devoted to SPPS, which 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)."

However, as stated earlier, virtually every aspect of SPPS was reexamined and improved during the decade of the 1970s (pp. 151-179, ref. 7). The first synthesis of mammalian glucagons by SPPS by Svetlana Mojsov, a graduate student in the Merrifield laboratory, was briefly described in 1977 as part of a report on recent developments in SPPS.[44] 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 α-amino protection.[45] The crude synthetic material was purified by gel filtration and ion-exchange chromatography followed by crystallization of the 29-residue hormone from aqueous solution at pH 8.8. The synthetic glucagon was homogeneous and indistinguishable from natural bovine glucagon by gel electrophoresis, ion-exchange 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 Mojsov using a PAM resin with N^{α}-*t*-butoxycarbonyl and benzyl-based side-chain protection for most of the trifunctional amino acids.[46] The newly developed cyclohexyl-protecting group was used for the β-carboxyl of aspartic acid to minimize aspartimide formation.[47] Cleavage of the 29-residue peptide from the resin using an improved HF procedure[48] 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.[45] It is also higher than the yield reported for synthesis by solution methods.[41,42] 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.[49] 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.[50] Again, to belabor the very obvious, a comparable study of structure-function relationships based on the availability of glucagon analogues from solution synthesis[41,42] 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.**[6]

What about the SPPS of peptides larger than glucagon, say 90 to 100 residues? Mitchell and co-workers developed a more acid stable PAM resin support[51-53] and Kent and co-workers improved

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.[54] In addition, several side reactions were examined and eliminated. The improved chemistry and protocols were utilized in the synthesis of the L and D enantiomers of the 99 residue HIV-1 protease (1-99).[55] 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 (NCL) methods using C-terminal peptide-thioesters (obtained from SPPS) that can be coupled together without side-chain protection has provided strategies for the synthesis of larger peptides (> 100 residues).[56,57] Recently, Torbeev and Kent reported the convergent chemical synthesis of a 203 residue "Covalent Dimer" of HIV-1 protease enzyme using NCL methods. The resulting enzyme molecule showed full catalytic activity and a high resolution crystal structure was reported.[58]

Tom Muir has provided an exciting extension of the NCL approach by using recombinant DNA techniques to produce the C-terminal peptide-thioesters employed in NCL. This approach, termed expressed protein ligation (EPL), is an amalgamation of SPPS and ribosomal peptide synthesis.[59] It affords the semi-synthesis of large proteins and allows site-specific introduction of unnatural amino acids and biophysical probes into these proteins. Martin Engelhard recently described the use of EPL for the semi-synthesis of H-Ras protein (166 amino acids). A glutamic acid methylester residue was replaced by glutamine at position 61 to probe the role of Gln61 in the catalysis of GTP hydrolysis reaction by Ras protein.[60]

We have traveled almost 50 years from Bruce's formulation of the concept of SPPS to the

chemical synthesis of uniquely labeled proteins using combinations of SPPS, NCL and EPL. This was made possible through the continuing dedication of Bruce Merrifield and colleagues to improve SPPS. Even near the end of his life, with a progressively debilitating illness and invasive medical treatments taking their toll, Bruce in his 80s was designing experiments to analyze and minimize reactions that lead to deletion peptides in SPPS.[61] The early criticisms of SPPS, mostly addressed over the years and now forgotten, reside in the dustbin of peptide history.

Biochemistry and the Legacy of "Tierchemie ist Schmierchemie"

To answer the question of how can a biochemist create a revolutionary change in the approach to chemical syntheses, we need to first consider the interplay between biochemical and chemical research in the 19th century. The famous German chemist, Justus von Liebig (1803 – 1873), made seminal contributions to agricultural, biological and organic chemistry in addition to training a great number of students that influenced the course of organic chemistry in 19th century Germany (pp.16-71, ref. 11). There was great interest in the English translation of Liebig's "Die Organische Chemie in ihrer Anwendung auf Physiologie und Pathologie" under the title "Animal Chemistry or Organic Chemistry in its Application to Physiology and Pathology," both published in 1842 (p. 64, ref.14). The interdisciplinary approach of Liebig in the mid-19th century gave way to a transformation of the theoretical and practical style of organic chemical research by 1900, with an emphasis on the proof of structure through synthesis. This accentuated the separation of the organic or "pure" chemists from physiological chemists or biochemists. As the physiological chemists then lacked the tools to investigate the constitution and dynamics of living systems to match the standards of the new organic chemistry, the widely

quoted saying, "Tierchemie ist Schmierchemie": Animal chemistry is messy chemistry, evolved (p. 57, ref.14). Emil Fischer also shared this disdain. In a letter to a colleague in 1904 Fischer wrote: "Regrettably, biological chemistry is that part of our science in which imprecise and incomplete experiments are often padded with the dazzling ornamentation of so-called ingenious reflections to produce pretentious treatises (p. 64, ref.14)." It should be noted in this context that Otto Warburg (1883-1970), Nobel Laureate (1931, Physiology) and perhaps the most prominent biochemist of the 20th century, received his doctorate from Emil Fischer in 1906 for the first synthesis of optically active peptides using the halogenacyl halide method (pp. 208-210, ref.11). Frank Westheimer (1912 - 2007) was an eminent professor at Harvard University, a key figure in 20th century chemistry and a pioneer in investigating biochemical problems using physical organic principles.[62] He reflected in an essay titled "Musings" that the German dogma stating that "Tierchemie ist Schmierchemie" or "biochemistry is sloppy chemistry" (Westheimer's translation) seemed to be alive and well into the 1960s due to the tight compartmentalization of chemists.[63] Organic chemists did not read the biochemical literature or attend biochemical seminars. A colloquium set up for Westheimer's students and those of another professor in the chemistry department at Harvard quickly divided along an imaginary line between chemists and biochemists.

Max Brenner, in an essay titled "Need for Solid-Phase Thinking in Solid-Phase Synthesis," characterized the solid-phase method as an "ingenious trick" that would never really be able to compete with the classical (solution) approach to peptide synthesis.[35] To directly quote Brenner: "Euphoria seized biochemists and others: disappearance of the solubility problem and of the isolation problem meant disappearance of the yield problem, because quantitative yield now

looked merely like a matter of simple mass action!" In other words this "ingenious trick" to overcome the disadvantages of classical synthesis could only have been conjured up by a biochemist and, of course, only biochemists and their ilk would subscribe to this nonsense – shades of "Tierchemie"?

How is it that a biochemist, and not an organic chemist invented a technique that has broad and growing applications in many areas of synthetic chemistry? Garland Marshall recalls after the publication of Bruce's first paper in 1963 a "steady stream of prominent scientists visited the laboratory. Almost each one mentioned at some point in the visit how he had thought of using a filterable polymeric support as a protecting group (the essence of solid phase synthesis), but, of course, none had spent the years exploring alternative approaches until a practical solution was found."[29] Bruce's problem was analogous to the problems physiological chemists faced almost a century earlier – the study of complex systems but lacking the necessary tools. Bruce, like his predecessors, was undaunted by complexity and worked for three years to prepare a tetrapeptide.[8] However, the concept of a multi-step synthesis without the isolation, purification and characterization of intermediates was akin to the "Schmierchemie" of an earlier era to some synthetic chemists. Bruce, a man modest in demeanor but strong in character, looked beyond the early criticism and ridicule and pushed on. The rest, of course, is history (7). It is the supreme irony that the latest advance in the chemical synthesis of proteins, namely Expressed Protein Ligation[59], is an amalgamation of SPPS (invented by a biochemist) and ribosomal peptide synthesis (discovered by biochemists).

Scientific Style of the Merrifield Research Group

Joseph Fruton, in his monumental "Contrasts in Scientific Style. Research Groups in the Chemical and Biochemical Sciences," begins with Justus Liebig's group at Giessen.[11] This is followed by analyses of other prominent German chemical (Adolf von Baeyer, Emil Fischer) and biochemical research groups (Felix Hoppe-Seyler, Willy Kiihne, Franz Hofmeister) in the period 1830-1914. Biographical details of each leader's scientific progeny fill seven appendices. Such an analysis of the Merrifield research group is clearly beyond the scope of this paper. Garland Marshall has described the hierarchical system in place at Rockefeller when he entered the Woolley laboratory to work with Merrifield in 1963.[29] Each research group was organized under the leadership of a Professor in a pyramidal/Prussian manner. Prof. D. Wayne Woolley was a founder of the field of antimetabolites and had two junior faculty (Merifield and Stewart) working in his group as well as postdocs, graduate students and technicians. Merrifield became head of the laboratory when Woolley died in 1966 and John Stewart moved to the University of Colorado Medical School in 1968.

The scientists discussed in Fruton's treatise are multi-faceted as in the case of Emil Fischer, for example. On the one hand, Fischer could be dictatorial and demanding:
"With a stern eye he inspected the laboratory workers, who reported to him the progress of their experiments. Fearsome was his *Flügelschlagen* [flapping of wings], without further comment, for the poor wretch if something had gone thoroughly wrong. Only rarely did the chief sit on a stool and conduct a brief private conversation. Then it was even permissible to laugh. However, the slightest attempt at intimacy would terminate the conversation immediately (p. 171, ref.11)"
On the other hand, Fischer treated the foreign visitors in his laboratory more kindly. The American physician Herrick, who worked in Fischer's laboratory in 1905, described him as

follows:

"He was modest, kindly, always the gentleman. Twice a day he made the rounds, moving quietly from desk to desk inspecting the work, always seeming interested, criticizing, helpfully suggesting. He had the faculty of seeing quickly where one's trouble lay. So gentle in manner was he that one scarcely realized he was a good executive commanding officer (p.172, ref.11)."

When Garland Marshall began his thesis research across the bench from Bruce he noted: "Although I had the illusion that I was working independently, Bruce obviously was a dominant influence by his logical approach to all experimental questions, discussed regularly across the bench top. Bruce was a superb experimentalist who designed experiments that unambiguously focused on the question to be resolved."[29]

Bruce was the antithesis of the dictatorial and demanding laboratory head. I would in fact characterize his leadership as minimalist and non-intrusive. 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. The arrivals in Bruce's laboratory after 1963 accepted SPPS, with its early imperfections, as a *fait accompli* that we used and/or improved upon with time.[6] Although Bruce' s early notebooks were available to all in the laboratory, I can't recall discussions on the early difficulties and setbacks as Bruce, always the quintessential optimist and forward looking scientist, was more focused on the future than the past. However, when one had problems even remotely related to any aspect of SPPS, he was very open to discussing the problem, its

significance and possible solutions.

Fruton says little how the personal lives of his subjects influenced their scientific lives. The Merrifield that 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 (pp. 208-227, ref. 7). This genuine, deep-seated outlook and civility enabled us to do the best work we were capable of.

Scientific Legacy of R. Bruce Merrifield

Bruce Merrifield's original intent was simply to make the task of peptide synthesis less onerous. He could not have imagined, especially in the early years, that his work would result in a paradigm shift in synthetic chemistry.[1] 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. Also, the impact of solid-phase synthesis on combinatorial chemistry, a field not yet conceived, could not have been predicted in 1959. Merrifield's influence on his scientific progeny, and their progenies, is also a part of his scientific legacy. His generous acknowledgements of colleagues and their work in his biography should serve as a model for others. Finally, even in the competitive world of science, to quote Garland Marshall one last time, "Bruce serves as a perfect example of the encouraging fact that good guys can finish first."[29]

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