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The Bold Legacy of Emil Fischer‡

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Abstract: A century has passed since Emil Fischer won the Nobel Prize in chemistry. From his first synthesis of glycyl-glycine in 1901 he has been a luminary to peptide chemists over the past 100 years. In this paper, a brief summary of some of the major accomplishments in peptide chemistry will be covered followed by a description of several of our own endeavours in peptide chemistry which arose from the discoveries of the giants of our field. We will include the development of a novel activating agent (DEPBT), the synthesis of a novel building block, α-methyl-D-cysteine, its incorporation into biologically active opioids, and conclude with the synthesis of dendritic collagen mimetics. Copyright © 2003 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: peptide luminaries; DEPBT; peptidomimetics; opioids; collagen; dendrimers

INTRODUCTION

Since Emil Fischer began the field of peptide chemistry with the synthesis of glycyl-glycine in 1901 [1], there have been many advances in the field. Here, we present a brief overview of the major accomplishments in peptide chemistry and identify the scientists responsible for them. We will then present some of our own endeavours in the field of peptide science.

EMIL FISCHER

In 1902, Emil Fischer became a Nobel laureate for his work in the field of carbohydrate chemistry, at this time he also focused his attention on amino acids and proteins. His initial synthesis of glycyl-glycine (Scheme 1) in 1901 by opening of diketopiperazine (piperazine-2,5-dione) represents the first synthesis of a free peptide [1]. He was also responsible for naming the product as a dipeptide thereby defining the terminology of peptide chemistry still in use today. Fischer also carried out the reaction involving aminolysis of optically active α-bromo isovaleric acid to produce the L-leucine residue. He then utilized acid chlorides as a method for peptide bond formation. Employing these methods, Fischer reported the synthesis of an octadecapeptide (Leu-Gly_3-Leu-Gly_3-Leu-Gly_9) in 1907 [2].

THEODOR CURTIUS

The history of peptide chemistry cannot be told without including Theodor Curtius. In his quest for the synthesis of hippuric acid, which he initially synthesized in 1881 [3], Curtius developed the azide reaction. He used this reaction for the synthesis of benzoyl-glycyl-glycine and higher oligomers in 1902

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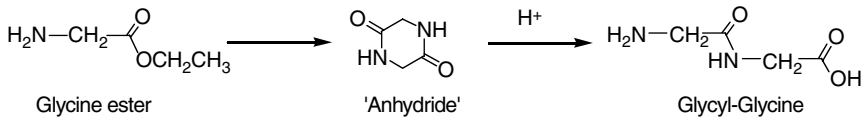
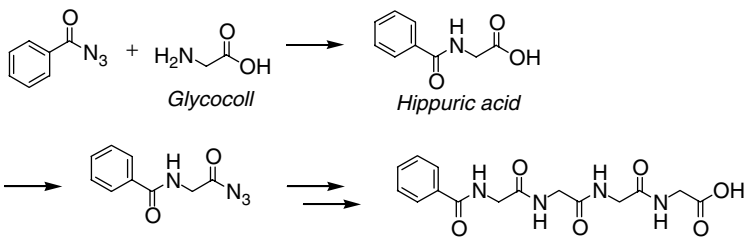
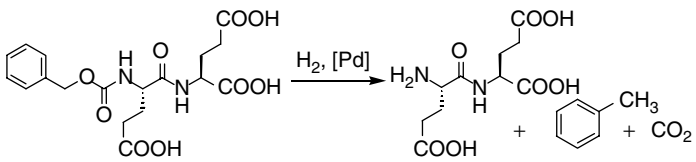
‡A five volume series has just begun to appear. This collection covers a broad range of topics of the synthetic aspects in the field of peptide science. "Houben-Weyl Methods of Organic Chemistry: Synthesis of Peptides and Peptidomimetics" Edited by Murray Goodman, Arthur Felix, Luis Moroder and Claudio Toniolo, Georg ThiemeVerlag Stuttgart, 2001, 2002, and 2003.



Scheme 1 Emil Fischer and his synthesis of glycyl-glycine in 1901 [1].



Scheme 2 Theodor Curtius and his development of the acyl azides [4].



Scheme 3 Bergmann (left) and Zervas (right) develop the benzyloxycarbonyl protecting group.

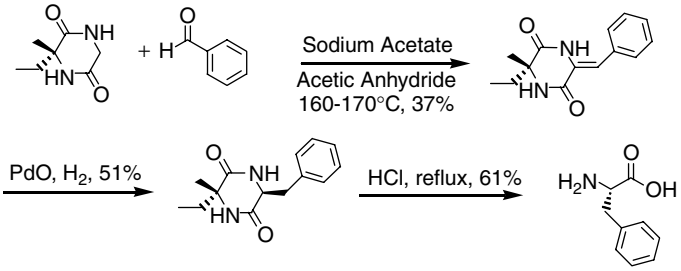
[4] (Scheme 2). The azide reaction became the most utilized activating reagent until the 1960s, but it remains a useful coupling method and the basis for a rearrangement reaction transforming acid chlorides into amines via the Curtius rearrangement [5,6].

MAX BERGMANN AND LEONIDAS ZERVAS

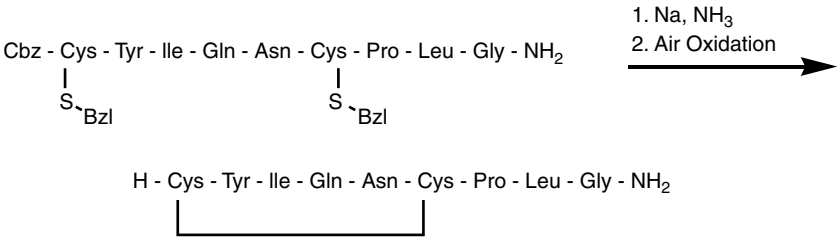
The next milestone in peptide chemistry centered on the development of removable protecting groups by Bergmann and Zervas in 1932 [7]. Together they prepared benzyloxycarbonyl chloride which they allowed to react with the amino functionality of amino acids. The benzyloxycarbonyl (Cbz) amine protecting group, which is easily removed by hydrogenolysis without peptide bond cleavage (Scheme 3), led to the elongation of peptide chains at the amine-terminus. The concept of removable protecting groups revolutionized chemist's thoughts about peptide and organic synthesis in general.

SHIRO AKABORI

Since Emil Fischer's synthesis of L-leucine by aminolysis of optically active α-bromo isovaleric acid, chemists have been aware of the need for synthetic routes to prepare optically active amino acids. One of the first routes to optically active amino acids was achieved by Shiro Akabori in 1951 (Scheme 4) [8]. He went on to develop a



Scheme 4 Shiro Akabori and his synthesis of chiral amino acids [8].



Scheme 5 Vincent du Vigneaud's synthesis of biologically active oxytocin [11].

more general route to chiral amino acid building blocks in 1965 [9]. In a real sense, Akabori's research set the stage for later contributions to enantioselective organic synthesis by the 2001 Nobel Prize winners Sharpless, Noyori and Knowles. Akabori is also known for his pioneering work in peptide sequence determination via hydrazinolysis of peptides resulting in identification of the carboxy terminal amino acid residue of peptides and proteins [10].

VINCENT DU VIGNEAUD

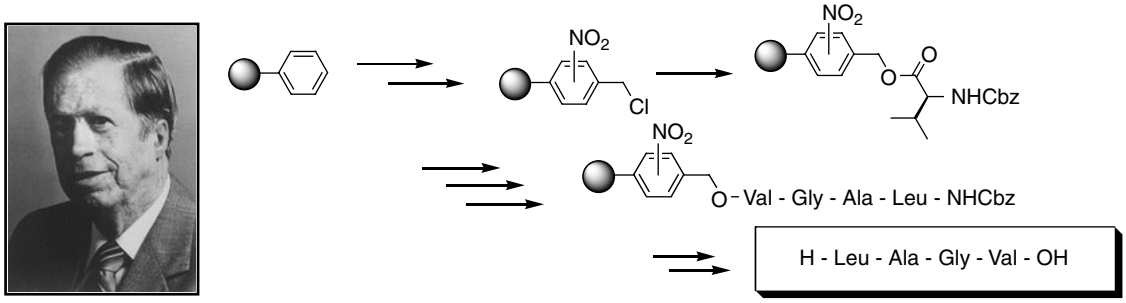
Vincent du Vigneaud is another giant in peptide chemistry. In the early 1950s he determined the structures of oxytocin and vasopressin [12]. He then went on to synthesize oxytocin and found it to be chemically and biologically identical to the natural product (Scheme 5) [11]. This was the first example of such a synthesis in peptide chemistry. Synthetic oxytocin continues to be used for the induction of labour in child birth and other related medical applications. The pioneering work of du Vigneaud initiated the field of synthesis of biologically active peptides which links the fields of biology and chemistry. For his work, he was awarded the Nobel Prize in 1955.

BRUCE MERRIFIELD

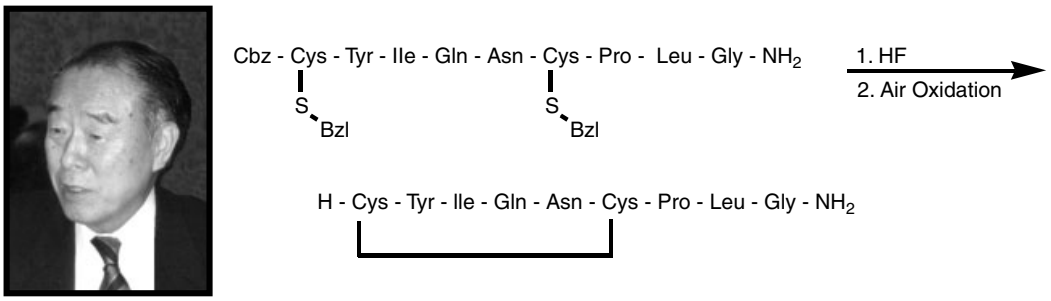
With the development of removable protecting groups, better activating reagents and the desire to synthesize biologically active peptides, the major challenge that persisted in peptide chemistry was the utility of long peptide fragments because of their poor solubility. This problem was addressed by Bruce Merrifield. In 1963, he developed a functionalized resin from styrene-divinylbenzene copolymers to which an amino acid could be anchored [13]. The Merrifield resin attaches the carboxy-terminus of amino acids to the resin to which amino acids with *N*-protecting groups can be readily coupled without racemization (Scheme 6). This method resulted in chirally homogeneous peptides. With the development of solid phase chemistry, long peptides could be synthesized without issues of solubility. The speed of peptide synthesis was enhanced by this method because impurities and unreacted reagents could be removed by simple resin washings and filtrations. For this revolution in both peptide and organic chemistry, Merrifield was awarded the Nobel Prize in 1984.

SHUMPEI SAKAKIBARA

One of the major advances for solid phase chemistry came from Shumpei Sakakibara in 1965. As the



Scheme 6 Bruce Merrifield's development of solid phase synthesis [13].



Scheme 7 Shumpei Sakakibara's development of HF as a deprotection agent [14].

practice of solid phase chemistry was developing so was the need for an efficient reagent to remove protecting groups and simultaneously cleave the peptide from the resin. From his work on thiol protecting groups, Sakakibara discovered that liquid hydrogen fluoride could remove benzyl groups from thiols (Scheme 7). This led him to discover that liquid HF could remove every acid labile protecting group, including benzyl and benzyloxycarbonyl groups [14]. More importantly, it was discovered that liquid HF could be used to cleave peptides from the resin and simultaneously remove all acid stable protecting groups without cleaving peptide bonds. This method has been widely adopted in solid phase peptide synthesis.

FROM THE FISCHER LEGACY TO OUR RESEARCH ENDEAVOURS

Since Emil Fischer first synthesized glycyl-glycine in 1901 [1], researchers have been concerned with developing activating reagents, protecting groups and novel amino acid building blocks for the synthesis of biologically active peptides and peptidomimetics. Our laboratories have focused on the same issues. In this paper, some of the advances we have made in these fields will be discussed.

3-(Diethoxyphosphoryloxy)-1,2,3-benzotriazin-4(3*H*)-one (DEPBT): A Novel Activating Reagent

In the early 1900s, the first activating reagents developed were Fischer's acid chlorides [2] and Curtius' acyl azides [4]. In 1906, α-amino acid *N*-carboxyanhydrides (NCAs) were developed by Leuchs [15]. These effective coupling reagents remained the standards while much of the peptide community was focused on developing readily removable protecting groups. It was not until more complex structures became synthetic targets that more efficient coupling reagents were developed in the 1950s.

Sheehan *et al.* introduced *N*,*N*′-dicyclohexylcarbodiimide (DCC) as a coupling reagent [16,17]. This reagent greatly reduced coupling times, but was shown to be prone to some racemization. Regardless, it was the first step in the development of improved coupling reagents. Since the development of DCC, additional carbodiimides and other coupling reagents have been devised to reduce reaction times and racemization. Our group, together with Ye and her associates in Beijing, have been involved in the development of 3-(diethoxyphosphoryloxy)-1,2,3-benzotriazin-4(3*H*)-one (DEPBT) (Figure 1) [18,19]. DEPBT is a neutral crystalline organophosphorus

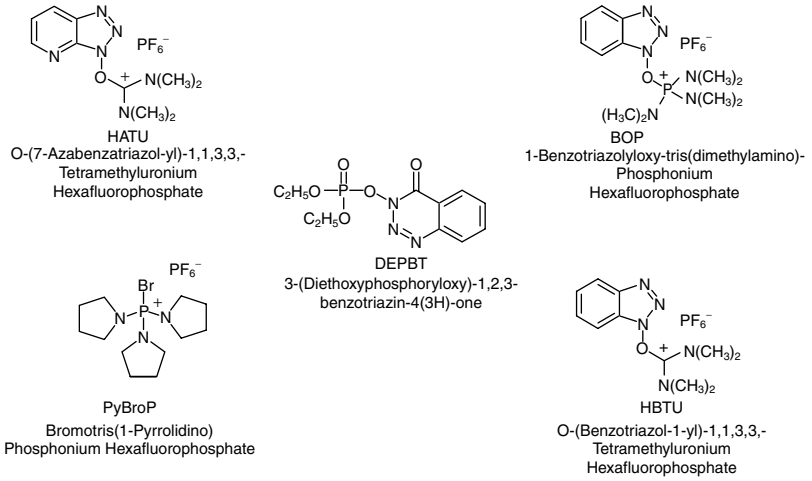
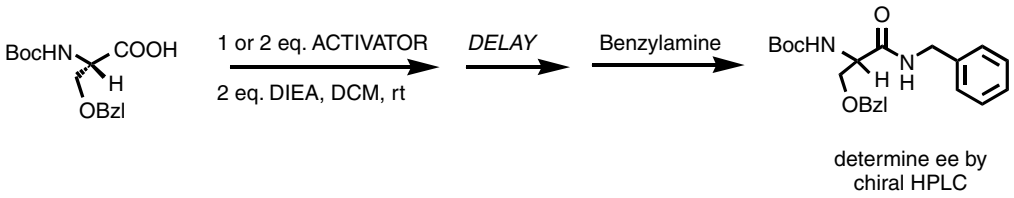


Figure 1 Coupling reagents used in the racemization study.



Scheme 8 Racemization studies of preactivated intermediates for Boc-Ser(Bzl)-OH.

compound that has been proven to supress racemization during amide bond formation.

We compared the characteristics of amide bond forming reactions of DEPBT to those of other highly effective phosphonium and uronium (guanidinium) salt coupling reagents (Figure 1). A residue prone to racemization, such as a serine derivative (Scheme 8), was subjected to the following protocol in order to determine yields and extent of epimerization: The activated carboxyl intermediate was stored in the presence of *N*,*N*-diisopropylethylamine (DIEA) for a specific time period. The activated intermediate was then allowed to react with benzylamine, which quenched the intermediate, and the resulting ratio of enantiomers was determined by chiral HPLC. The results indicated that DEPBT was the coupling reagent which showed the best yield with the lowest extent of racemization (Table 1).

DEPBT has also proven to be extremely efficient in the formation of complex amide bonds including those found in natural products. The utility of DEPBT was demonstrated in the synthesis of (–)tamandarin B by Joullié [20] and in the synthesis of teicoplanin aglycon by Boger [21]. In addition, DEPBT is an extremely efficient reagent for the cyclization of peptides [22]. In our laboratory, DEPBT has been exhaustively employed in the synthesis of biologically active peptidomimetic opioids as demonstrated in the following section.

Table 1 Racemization Studies During *In Situ* Activation

Activation	Delay time (min)	L : D ratio	Yield (%)
PyBroP (1.0 equiv)	4	65 : 35	81
HATU (1.0 equiv)	4	84 : 16	91
HBTU (1.0 equiv)	15	79 : 21	>99
BOP (1.0 equiv)	15	85 : 15	95
DEPBT (1.0 equiv)	60	95 : 5	70
DEPBT (2.0 equiv)	60	96 : 4	>99

The α-Methyl-D-Cysteine Containing Disulfide Enkephalin Analogues: Biologically Active Peptidomimetic Opioids

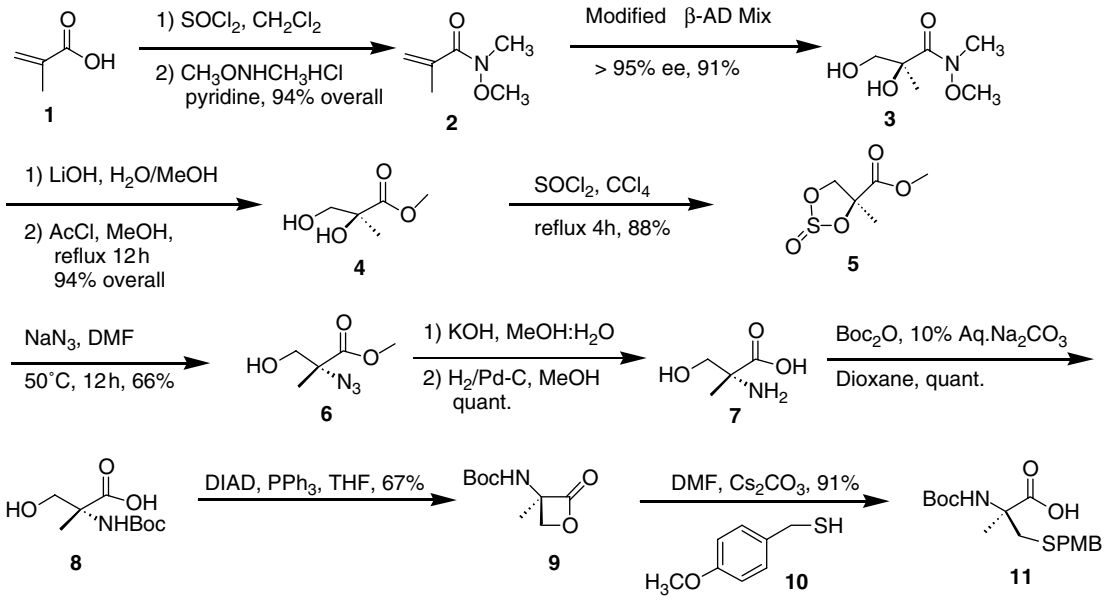
When Vincent Du Vigneaud proved that synthetic peptides have identical biological activities to those

isolated from nature, he opened a whole new area of peptide science. Today we are not only interested in the synthesis of natural products, but also in the preparation of analogues of peptides and peptidomimetics with improved biological properties. In particular, our laboratory has been focused on the synthesis of potent and selective peptidomimetic opioids based on the slightly selective endogenous enkephalins [23]. It is our goal to develop highly potent peptidomimetic opioids which selectively target the δ-receptor. We are interested in the δ-receptor because of previous studies which report diminished side effects of δ-selective ligands, such as reduced respiratory depression, gut motility, and reduced tolerance build up compared with μ-selective drugs such as morphine and fentenyl [24–26].

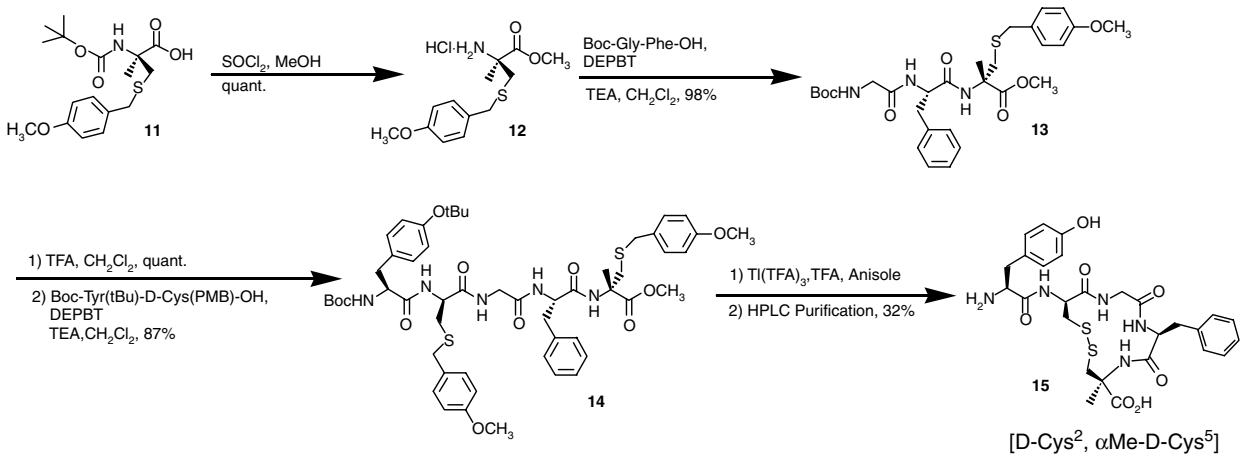
Starting with the pioneering work of Shiro Akabori, synthesizing optically active amino acids has always been a priority in peptide chemistry. We have also been interested in this field especially with the synthesis of novel optically active amino acid building blocks. An example of this is the synthesis of Boc-α-methyl D-cysteine(PMB)-OH **11**, described in Scheme 9 [27]. It is a cysteine analogue with a methyl group in place of the α-proton. The methyl group adds steric hindrance, which restricts the conformational space available to the side chain. When incorporated into cyclic peptidomimetic opioid structures, this building block is anticipated to limit the available conformations of these peptidomimetic opioids. Our aim is to constrain the peptidomimetic opioids into their biologically active conformations which would increase potency and selectivity.

Utilizing the Boc-α-methyl-D-cysteine(PMB)-OH **11** building block, we have synthesized constrained disulfide enkephalin analogues. A representative synthesis of an α-methyl D-cysteine containing disulfide bridged enkephalin is shown in Scheme 10. The Boc group was removed from Boc-α-Me-D-cys(PMB)-OH **11** and the product simultaneously esterified using thionyl chloride and methanol to produce methyl ester **12**. The free amine was then coupled to Boc-Gly-Phe-OH with DEPBT to give tripeptide **13** in a 98% yield. The Boc group was removed with TFA in CH_2Cl_2 and subsequently coupled to Boc-Tyr(tBu)-D-cys(PMB)-OH again with DEPBT to give pentapeptide **14** in a 87% yield. The linear pentapeptide was deprotected and oxidized to the disulfide in one step with thallium trifluoroacetate in TFA [28]. Finally, the cyclic enkephalin analogue was purified by reverse phase HPLC to yield [D-Cys^2, αMe-D-cys^5]E **15**. The enkephalin analogue with the α-methyl D-Cys building block in position 2 ([αMe-D-cys^2, Cys^5]E) was synthesized in a similar fashion.

Both constrained enkephalin analogues were assayed for *in vitro* and *in vivo* activity. The enkephalin analogue with the α-Me-D-cys residue at position 2 [αMe-D-cys^2, Cys^5]E was relatively potent and nonselective at the GPI and MVD which was consistent with the binding assays (Table 2). It is noteworthy that the enkephalin analogue [D-Cys^2,



Scheme 9 Synthesis of Boc-α-methyl-D-cysteine(PMB)-OH.



Scheme 10 Representative synthesis of α-Me-D-Cys containing disulfide bridged enkephalins.

Table 2 Biological Activity and Binding Affinity of α-Me-D-Cys Containing Disulfide Enkephalin Analogues

Compound	GPI IC_{50} [nM]	MVD IC_{50} [nM]	GPI/MVD IC_{50}-ratio	*In vivo* ED_{50} [nM]	K_i (nM)		
					μ	δ	κ
[αMe-D-Cys^2, Cys^5]E	9.4	3.68	2.55	1.59	38	12	>10 000
[D-Cys^2, αMe-D-Cys^5]E	1.25	0.022	57.3	0.042	6.1	1.3	7500
DPDPE	7300	4.10	1800	130	>10 000	2.2	>10 000
Morphine	58.6	644	0.09	15.0	17	150	260

αMe-D-cys^5]E exhibited greater potency at the GPI (1.25 nM) and extremely high potency at the MVD (322 pM). Both compounds were then evaluated for their *in vivo* activity utilizing the rat thermal escape test after intrathecal injection (IT). While [αMe-D-cys^2, Cys^5]E exhibited high potency (1.59 nM), [D-Cys^2, αMe-D-cys^5]E showed extremely high potency in the IT assay with an ED_{50} value of 42 pM. The [D-Cys^2, αMe-D-cys^5]E is among the most potent enkephalin analogues synthesized to date. Research on these interesting peptidomimetic opioids is continuing in our laboratories.

COLLAGEN MIMETICS: DENDRITIC PEPTIDES

In a paper published by Emil Fischer in 1907 on the synthesis of an octadecapeptide, he made a few statements about peptide chemistry at the time [2]. First he wrote, 'With a molecular weight of 1213, the octadecapeptide exceeds that of most fats (for example tristearine 891)'. His synthesis of an octadecapeptide was quite an accomplishment. He went on to state, 'For other natural products the estimates are higher, i.e. 12 000–15 000. But my opinion is that these estimates are founded on very uncertain premises because of a lack of any guaranty that natural proteins are homogeneous compounds'. Even the great Emil Fischer could be proven wrong with the determination that natural proteins are in fact homogeneous. Finally, Fischer concluded his statements with, 'Temporarily I have to abstain from such experiments however, not only because they are very laborious but also very costly'. After a hundred years these issues still remain true, fortunately these difficulties have not stopped researchers from pursuing the synthesis of large peptide-based molecules.

Our laboratory has been interested in the synthesis of dendritic collagen-like structures. Collagen is the main structural protein in mammals and is composed of Gly-Xaa-Yaa repeats. Natural collagen is triple helical and many synthetic collagen mimetics have been synthesized that exhibit triple helical conformations. We have synthesized an octadecapeptide

which is comprised of six repeats of Gly-Nleu-Pro (where Nleu denotes *N*-isobutyl glycine) (Figure 2) [29]. This Boc protected octadecapeptide, which is terminated with a methyl ester, has a molecular weight of 1759 (larger than Fischer's 1213). Our octadecapeptide was conformationally characterized by optical rotation and circular dichroism and was found to be disordered in aqueous solution (Figure 3).

We then removed the Boc protecting group and three octadecapeptides were attached to a Boc-β-alanyl tris(carboxyethoxymethyl)aminoethane (TRIS) scaffold using DEPBT to yield a collagen mimetic with molecular weight of 5385 (Figure 2). Again this collagen mimetic was assayed for triple helicity and was shown to exhibit a CD spectra indicative of a triple helical conformation with a melting transition of 33 °C in water (Figure 3).

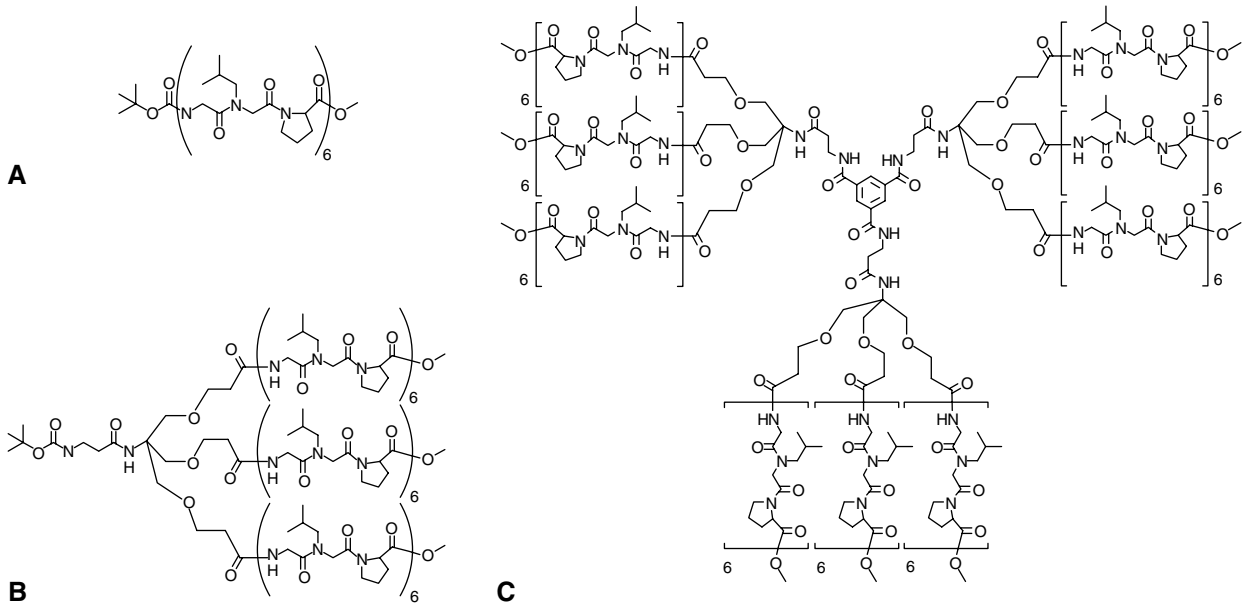


Figure 2 (A) Octadecapeptide with six repeats of (Gly-Nleu-Pro). (B) Three octadecapeptides attached to Boc-β-Ala-TRIS scaffold. (C) Collagen mimetic dendrimer with nine octadecapeptides attached to a trimesic acid (TMA) core.

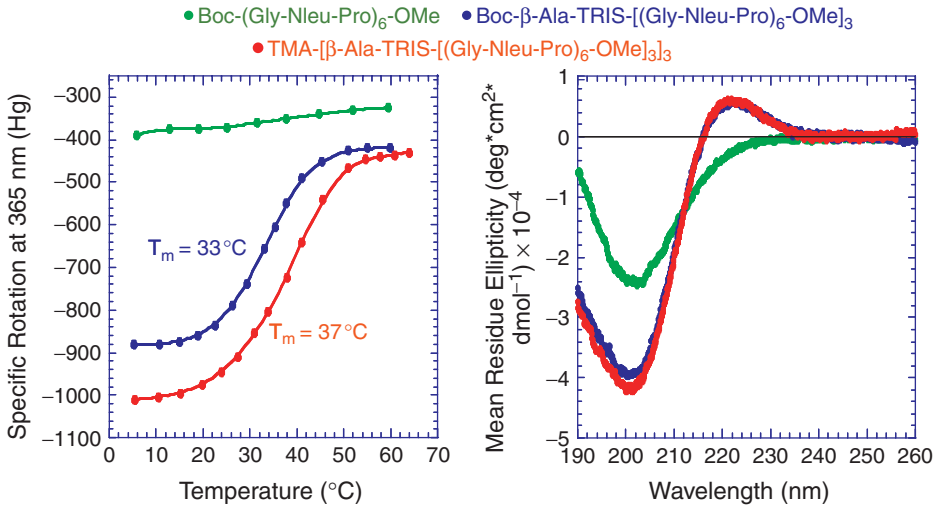


Figure 3 (Left) Thermal denaturations measured by changes in optical rotations carried out in H_2O (0.2 mg/ml). (Right) CD spectra in H_2O at 8 °C.

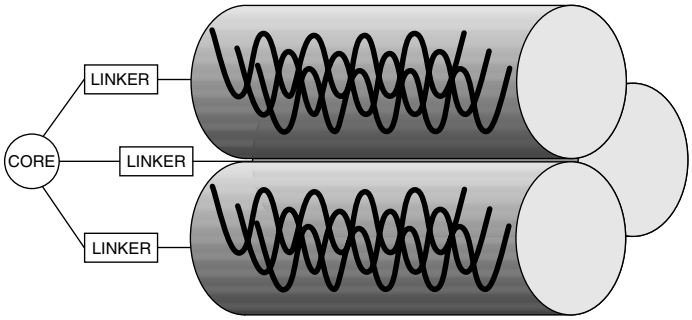


Figure 4 A schematic representation of the triple helix cluster.

A dendritic collagen mimetic structure was then synthesized in which three β-Ala-TRIS assembled (Gly-Nleu-Pro) mimetics were attached to a trimesic acid (TMA) core followed by a series of segment condensations to produce a dendritic structure containing nine octadecapeptides with a molecular weight of 15 965 (Figure 2). In order to determine if the three sets of triple helical bundles would interact with one another, the triple helicity of the dendritic collagen structure was investigated. The circular dichroism showed a triple helical structure for the dendrimer with a melting transition of 37 °C in water which was higher than the scaffold terminated structure with three octadecapeptides (Figure 3) [29]. This indicated that the triple helical bundles interact with each other. There was no concentration effect for triple helical stability when the transitions were measured between 0.05 and 2.0 mg/ml in water. We therefore believe that the stabilizing effects arise from an intramolecular clustering of the triple helical arrays about the core structure (Figure 4). This ensemble excludes solvent from the interior portion of the array which likely stabilizes the triple helical bundle.

CONCLUSIONS

In the beginning of peptide chemistry the focus was on amide bond formation, stereochemistry, protecting groups and purity. Many advances have been made in the field of peptide chemistry over the past 100 years, yet the focus remains the same. There continue to be innovations and discoveries made in the development of activating reagents, enantioselective syntheses of amino acids and peptidomimetics, and purification. We are true disciples of the giants of our field since we continue the search for new reagents and reactions applicable to peptide chemistry. In looking to the future, much remains to be accomplished. The development of genomics and proteomics will continue to link the fields of biology and chemistry demanding greater versatility of design and synthesis of target structures.

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