

## Discovery, Synthesis, and Structure–Activity Relationships of Conotoxins

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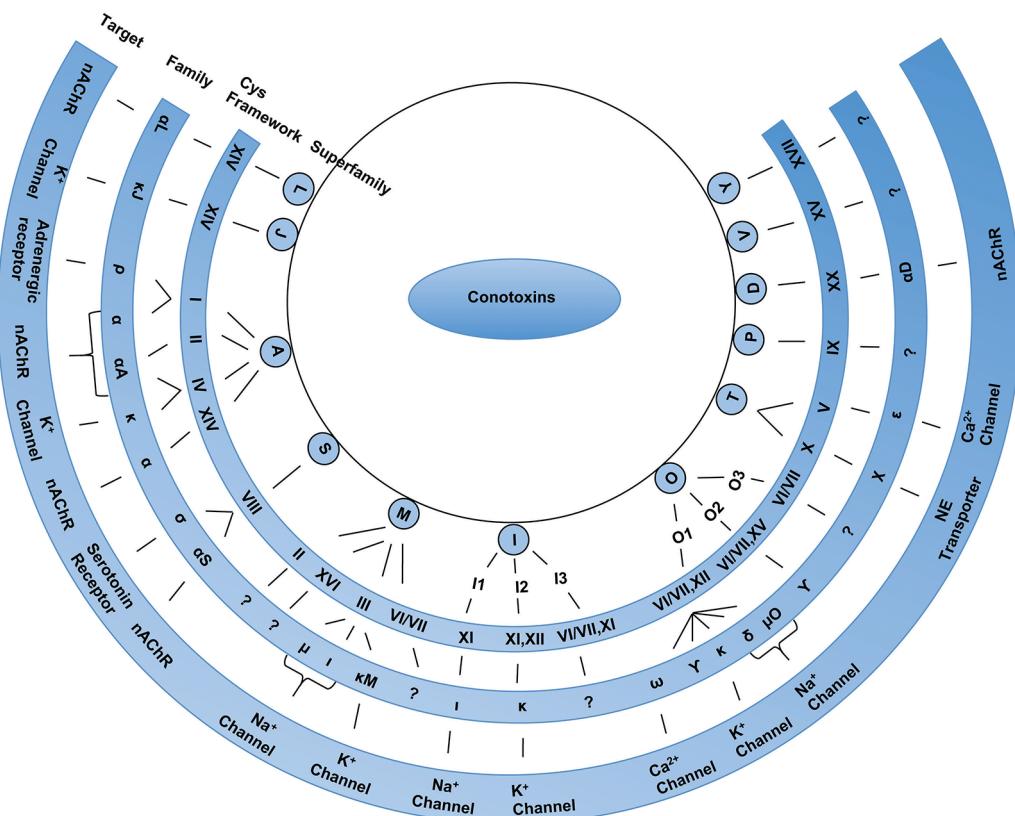
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### 1. INTRODUCTION

Peptide therapeutics are acclaimed as a promising addition to the pharmaceutical arena, and they continue to attract interest due to their high potency, bioavailability, and fewer concerns with toxicity, drug to drug cross-reactions, and tissue accumulation.<sup>1,2</sup> Although poor drug delivery and low *in vivo* stability are still issues to be addressed, peptides look poised to play an important role in the treatment of diseases ranging from Alzheimer's disease to cancer.<sup>2,3</sup> The huge biodiversity offered

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**Figure 1.** Classification of conotoxins into various families and superfamilies. Conotoxins are classified into various superfamilies based on their conserved signal sequence homology. Further classification into families is based on their framework and their target receptor. Target receptors for the conotoxin families, which do not have a specified receptor shown in this figure, are yet to be identified. NE = norepinephrine; nAChR = nicotinic acetylcholine receptor.

by venom peptides, especially conotoxins isolated from the venom of predatory marine snails, holds enormous promise for the development of peptide-based drugs.

## 2. CONE SNAILS

Around 700 species of marine snails of the genus *Conus* are distributed throughout tropical and subtropical waters.<sup>4</sup> As different species preferentially hunt fish, worms, or molluscs they are categorized as piscivorous, vermicivorous, or molluscivorous, respectively, although some cone snail species can feed on more than one prey type. These slow-moving creatures evolved into predators through incorporation of a specialized envenomation apparatus that enables them to quickly subdue their fast-moving prey. Their envenomation apparatus comprises a hollow radular tooth, similar to a harpoon or disposable needle, connected to the venom bulb via a tubular venom duct.<sup>5,6</sup> Cone snail venom first attracted research interest in the 1960s in an attempt to understand the pharmacological basis for human fatalities resulting from cone snail stings. These early studies showed *Conus* venom to be a complex mixture of biologically active components, including a large collection of neuroactive peptides termed conotoxins.<sup>7-9</sup> This cocktail of neurotoxins produced in the venom duct is injected into the prey through the radular tooth, leading to almost instantaneous paralysis. For a long time it was thought that each cone snail species expresses a distinct set of 50–200 peptides that is influenced by environmental and dietary factors.<sup>5,10</sup> From these numbers, which were originally derived from HPLC fractionation and mass spectrometry (MS) analysis, a repertoire of over

70 000 mostly neuroactive peptides was estimated to be produced by the *Conus* species.<sup>4,11</sup> With the advent of more sensitive technology and alternative methods to measure this diversity, it is now clear that this estimate is at the lower end of the scale, with newer estimates going into the hundreds of thousands of distinct neuroactive conopeptides produced by cone snails (see also sections 4.1.2 and 4.1.3).

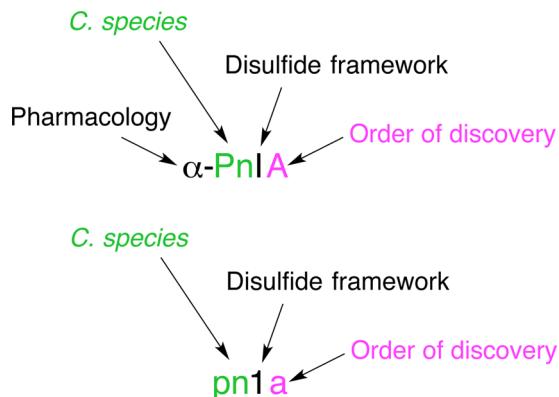
Conopeptides can be broadly classified into two categories. The first group comprises disulfide-poor peptides, which include contulakins,<sup>12</sup> conantokins,<sup>13</sup> conorfamides,<sup>14</sup> conolysins,<sup>15</sup> conophans,<sup>16</sup> conomorphins,<sup>17</sup> contrypphans,<sup>18</sup> cono-map,<sup>19</sup> and conopressins.<sup>20</sup> The second group, termed conotoxins, represents the majority of the venom peptide repertoire and contains multiple disulfide bonds. Conotoxins target a wide range of receptors and ion channels with unparalleled potency and selectivity. They have consequently become the subject of intense research in light of their immense diagnostic and therapeutic potential and are the focus of this review.

### 3. CONOTOXIN CLASSIFICATION AND NOMENCLATURE

Around 1700 mature conotoxin sequences have been identified to date, and this number is rapidly increasing as next-generation gene sequencing and proteomic costs continue to fall. This diverse group of peptides was originally organized into various superfamilies based on two sequence elements, namely, the conserved signal sequence and the characteristic cysteine framework (i.e., cysteine residue arrangement). Historically,

they were further categorized into families based on their receptor target.<sup>21</sup> As the number of sequences expands, it seems that a wide array of conotoxin cysteine frameworks are shared between sequences with highly similar signal sequences.<sup>22</sup> For example, the M superfamily includes nine cysteine frameworks (I, II, III, IV, VI/VII, IX, X, XIV, XVI), and nearly all of them have been found in other superfamilies. Thus, the classification system is undergoing continuous modifications in light of new information becoming available. Figure 1 shows the conotoxin superfamilies, frameworks, and families identified to date. Among these, the  $\alpha$ -conotoxins,  $\mu$ -conotoxins, and  $\omega$ -conotoxins are among the most characterized families so far.

The convention in use for naming conotoxins is based on the NC-IUPHAR (the International Union of Basic and Clinical Pharmacology Committee on Receptor Nomenclature and Drug Classification) system.<sup>23</sup> As illustrated in Figure 2, the



**Figure 2.** Conotoxin nomenclature. Conotoxin naming convention is based on the NC-IUPHAR system. The first letter in Greek indicates the conotoxin's pharmacological target, i.e.,  $\alpha$ -conotoxin targeting the nicotinic acetylcholine receptors. The next one or two uppercase letters represent the species from which it was isolated, in this case *Conus pennaceus*. This is followed by a Roman numeral, I, providing information on the framework (e.g., CC-C-C). Finally, an uppercase letter denotes the order of discovery of the conotoxin within that category. If the mechanism of action of the conotoxin is yet to be determined, the Greek letter is omitted; the species name is in lower case letters, an Arabic numeral is used to designate the cysteine framework, and a small letter is used to specify the peptide variant.

first (Greek) letter indicates the conotoxin's pharmacological target. The next one or two letters (Roman, first letter uppercase) derive from the species name from which the conotoxin was isolated. This letter (or letters in the case of ambiguity) is followed by a Roman numeral, which provides information on the cysteine framework. Finally, an uppercase letter denotes the order of discovery of the conotoxin within that category (species + cysteine framework). If the target receptor of the peptide is yet to be determined then the Greek letter is omitted; lowercase letters are used to indicate the species and Arabic numerals to indicate the framework, and the order of discovery is indicated by a lowercase letter.<sup>24,25</sup>

#### 4. CONOTOXIN DIVERSITY

In the past few years new discoveries on the biology of cone snails and on conotoxin evolution have led to dramatically growing estimates of conotoxin diversity.<sup>11,26,27</sup> The ~1700 toxins for which we have information are now considered to be only a small subset of the available diversity. In this section, conotoxin diversity is described at the sequence and structural

level, providing critical information for chemists wishing to engineer conotoxins. At the sequence level, our current understanding of the natural mechanisms driving conotoxin chemical diversity is described along with artificial chemical modifications that have been introduced to engineer the properties of conotoxins. At the structural level, conotoxin folds and sequence/structure relationships are discussed. ConoServer—a database that curates the sequences and the three-dimensional structures of conotoxins—was the main source of information in this section.<sup>28,29</sup>

##### 4.1. Sequence Diversity

###### 4.1.1. Techniques Used To Study Conotoxin Sequence Diversity.

Conotoxins were initially discovered at the peptide level using a combination of fractionation and liquid chromatography,<sup>30,31</sup> but the advent of molecular biology techniques substantially accelerated the discovery process by accessing information at the nucleic acid level.<sup>25,32–36</sup> More recently, massive amounts of sequence information have been generated using second-generation sequencing technology applied to the transcriptomes or genomes of cone snails.<sup>37–41</sup> Two factors render identification of conotoxins from nucleotide sequences incomplete: first, conotoxins are produced as precursors, and identification of the mature sequence in the precursor is often ambiguous; second, conotoxins often display many types of post-translational modifications (PTMs),<sup>22</sup> most of which cannot be predicted from precursor sequences. In drug discovery and development programs these PTMs (except for disulfide bonds and C-terminal amidation) are often ignored, since it is cheaper and easier to synthesize the unmodified synthetic analogues for initial lead identification. Nevertheless, isolation and characterization of conotoxins at the peptide level is time consuming, and most known conotoxins have been inferred from transcript sequences.<sup>29</sup>

In early studies, conotoxins were sequenced using Edman degradation,<sup>31,42</sup> but mass spectrometry is now the method of choice for the sequencing of mature conotoxins.<sup>43–46</sup> Mass spectrometry is significantly less expensive and allows direct identification of PTMs. Combined proteomic and transcriptomic approaches recently have been employed to explore the venom content of individual *Conus* species with second-generation sequencing, providing an unbiased list of precursor sequences whose mature peptide and PTMs can be identified using modern proteomic techniques.<sup>41,47</sup>

The roles of most conopeptide PTMs remain largely unexplored, but studies of one of the oldest known PTMs, namely, glutamate  $\gamma$ -carboxylation,<sup>48</sup> suggest the possibility of functional or structural roles.<sup>49</sup> However, the importance of this PTM can vary greatly from one conopeptide to another. For example, glutamate  $\gamma$ -carboxylation was shown on one hand to be essential for inhibition of voltage-gated calcium channels by contryphan-M<sup>50</sup> but on the other hand to have little functional influence on inhibition of nicotinic acetylcholine receptors (nAChRs) by  $\alpha$ -Vc1.1,<sup>51</sup>  $\alpha$ -SrIB,<sup>52</sup> or  $\alpha$ -GID.<sup>53</sup> This PTM has also been shown to influence the stability of some conopeptides, probably through the ability of  $\gamma$ -carboxyglutamate to chelate calcium.<sup>54–56</sup> For example, conantokins have a series of 4–5  $\gamma$ -carboxyglutamates that are bridged by calcium ions, resulting in stabilization of  $\alpha$ -helical structures.<sup>55,57</sup> It has further been proposed that  $\gamma$ -carboxyglutamation might facilitate the proper folding of conopeptides within the endoplasmic reticulum, where the concentration of calcium is high.<sup>54</sup> Hydroxylation of proline is a less studied PTM, but

**Table 1.** Conopeptide Gene Superfamilies

gene superfamily	no. of protein precursors <sup>b</sup>	no. of nucleic sequences <sup>b</sup>	cysteine frameworks occurring in superfamily	consensus of signal peptide sequence <sup>c</sup>
A	202	234	I, II, IV, XIV	MGMRRMMFTVFLVVLLATTVVVSXTS
B1	14	5	(disulfide-poor conantokins)	MXLYTYLYLLVPLVTFHILGXGT
B2	1		VIII	MLRLITAALVLSACLA
B3	1	1	XXIV	METLTLWRASSCLLVVLHSLLRLLG
C	4	7	(disulfide-poor contulakins)	MXXAYWVMVMMMVXIXAPLSEG
D	28	14	XV, XX	MPKLEMMLLVLLILPLXYFDAAGG
E	1		XXII	MMTRVFFAMFFLMALTEG
F	1			MQRGAVALLGVVALLVLWPQAGA
G	1	1	XIII	MSGMGVLLLVLLVMPLAA
H	7		VI/VII	MNTAGRLLLCLALGVLFESLG
I1	17	10	VI/VII, XI	MKLXXTFLXLXILPXXXG
I2	57	38	XI, XII	MMFRXTSVXCFLVIXXLNL
I3	7	8	VI/VII, XI	MKLVLALIVXILMLLSLSTGA
J	12	12	XIV	MPSVRSTCCCLLWXMLSXXLVTPGSP
K	4	1	XXIII	MIMRMTLTFLVLLVVMTAASASG
L	13	8	XIV	MXXXVMFXVLXLTMPMLTX
M	361	333	I, II, III, IV, VI/VII, IX, X, XIV, XVI	MMXKXGVXMLXIXLXLFPLXXXQLDA
N	3		XV	MSTLKMMMLLILLLPLXATFDSDG
O1	460	559	I, VI/VII, XII, XIV	MKLTCVIVAVLFLTAXXLXTA
O2	84	73	VI/VII, XIV, XV	MEKLTILLVAVLMSTQALXQS
O3	28	22	VI/VII	MSGLGIMVLTLLLLVFMXTSHQ
P	9	5	IX	MHXXLXXSAVLXLXLLXAXXNFXXVQ
S	13	8	VIII	MMXKGAMFVLLLLFXLSSQQ
T	157	143	I, V, X, XVI	MRCLPVFXILLLIXAPSVDVA
V	2	2	XV	MMPVILLLSSLAIRXXDG
Y	1	1	XVII	MQKATVLLALLLLPLSTA

<sup>a</sup>Conopeptide gene superfamilies are defined by the similarity of the signal peptide regions in conopeptide precursors. The founding reference for each superfamily can be found on the ConoServer Web site (<http://www.conoserver.org>), which also provides regular updates to this table and additional statistics. Definitions of the cysteine frameworks are in Table 2. <sup>b</sup>Number of protein and nucleic precursors catalogued in ConoServer. <sup>c</sup>The consensus signal sequence was obtained by determining amino acids with at least 60% of conservation at each position of the signal sequence in all protein precursors in ConoServer.

again, available data suggest varying roles in different conopeptides. For example, proline hydroxylation has been shown to influence the activity but not the folding of conopeptide  $\mu$ -GIIIA, yet conversely, proline hydroxylation did not affect the biological function of  $\omega$ -MVIIC but did affect folding yields.<sup>58</sup> In terms of structural roles, the backbone conformations of the conopeptides conomarphin and mr3c have been reported to be affected by proline hydroxylation.<sup>59,60</sup> A range of conopeptides has been reported to have O-glycosylated serine or threonine residues, but the biological significance of these PTMs has been reported for only a few examples, including conopeptides  $\kappa$ -SIVA and contulakin-G.<sup>61,62</sup> Conopeptide  $\kappa$ -SIVA is reported to be more active against sodium channels in vivo when one of its serine residues is O-glycosylated.<sup>63</sup> Contulakin-G has an O-glycosylated threonine, which is associated with a 10-fold increase in activity in vivo, but not in vitro, compared to the nonglycosylated form, possibly because glycosylation enhances peptide bioavailability.<sup>12</sup>

One PTM, formation of disulfide-bond cross-links between cysteine residues, has a major impact on protein structure, and determination of disulfide connectivities is therefore an important characterization step for conotoxins. Various techniques have been used to determine conotoxin disulfide connectivities, including proteolytic cleavage and analysis of conotoxin fragments using mass spectrometry,<sup>56,64</sup> chromatographic coelution of native and synthetic peptides displaying selectively formed disulfide bonds,<sup>65,66</sup> or direct structural

determination by X-ray crystallography<sup>67</sup> or NMR NOESY<sup>68</sup>/ROESY experiments.<sup>69</sup>

**4.1.2. Conotoxin Genetic Diversity.** More than 2000 conotoxin nucleotide sequences are cataloged in ConoServer, of which only 15% are genomic sequences. Conotoxin precursor transcript sequences comprise three regions: an endoplasmic reticulum (ER) signal peptide, a mature peptide region, and pre- and/or postpropeptide regions.<sup>32</sup> The sequence of the mature peptide regions is highly variable, and only a handful of conotoxins have been found to be expressed by more than one *Conus* species.<sup>22</sup> In contrast, the ER signal peptide is highly conserved among particular subgroups of conotoxins, and for this reason it is used to cluster conotoxins into gene superfamilies.

The currently known gene superfamilies are summarized in Table 1, which shows the ER signal peptide consensus sequence and the corresponding number of identified peptides for each gene superfamily. The classification into gene superfamilies using the degree of sequence identity between ER signal peptides has been validated using a clustering analysis, available on the ConoServer Web site. A recent phylogenetic analysis also supports the proposed divisions in gene superfamilies.<sup>70</sup> Whereas, originally, only the conotoxins (disulfide-rich conopeptides) were classified into gene superfamilies, this classification has now been extended to some of the disulfide-poor conopeptide families.<sup>70</sup> Several new gene superfamilies have been identified in an early divergent cone

snail clade, and it was suggested that these gene superfamilies might be specific to this clade.<sup>36</sup>

As it is apparent from Table 1, the gene superfamilies, A, I2, M, O1, O2, and T have been extensively sampled, whereas the other gene superfamilies describe fewer than 30 conotoxins in each. This unbalanced number of conotoxins in the gene superfamilies might not completely reflect natural distributions but rather derive from biased sampling methods based on known pharmacology that targets specific gene superfamilies. Recent transcriptomic studies have provided a less biased insight into the relative size of each gene superfamily, confirming that superfamilies O1, M, A, and T are indeed the largest superfamilies found to date.<sup>37–39,41,47</sup> The number of different transcripts recorded in a single cone snail specimen is 50–100 for the most complete studies,<sup>39–41,47</sup> but estimating the number of different conotoxin genes in a single species is problematic due to the large intraspecies variability<sup>11,71,72</sup> and differential expression in particular regions of the venom duct.<sup>73</sup> Furthermore, a reliable estimate of the number of cone snail species still needs to be established,<sup>74,75</sup> and only a lower estimate to the total number of conotoxin genes, around 50 000 genes, can therefore be suggested.

Several mechanisms have been proposed to explain this huge genetic diversity. The conotoxin gene family is thought to be one of the fastest evolving gene families of the animal kingdom<sup>76</sup> due to extensive and continuous gene duplications and positive selection.<sup>76–79</sup> In addition, it has been proposed that allelic variations<sup>71,80</sup> and genetic recombination<sup>72</sup> might also play a significant role in creating conotoxin genetic diversity. Most of our knowledge on conotoxin genetics was gained at the transcript level, but a recent study focusing on genomic sequences from the A gene superfamily revealed the existence of a large number of conotoxin pseudogenes, indicating that conotoxin genes have highly accelerated turnover.<sup>79</sup> On the basis of geographical gene variability for the same species, ecological adaptation to different prey has been suggested as the main driving force of fast conotoxin evolution.<sup>71,72,81</sup> Comparative studies between the conotoxin genes, whose main purpose is to allow the organism to attack prey, and rapidly evolving gene families related to host defense, including immunoglobulins, T-cell receptors, major histocompatibility complex proteins, and defensins, will certainly be a fascinating area for future investigations.

**4.1.3. Natural Conotoxin Chemical Diversity.** Conotoxin precursors are translated in the ER and subsequently undergo several maturation steps, including a range of PTMs that dramatically increase their chemical diversity.<sup>49</sup> Our knowledge of the mature toxins is currently limited to the ~250 conotoxins that have been directly isolated from venoms. The most frequent PTM is formation of disulfide bonds,<sup>82</sup> but 12 other modifications have been identified in wild-type conotoxins. The most common modifications besides disulfide-bond formation are C-terminal amidation, proline hydroxylation, and glutamate  $\gamma$ -carboxylation. A list of PTMs and associated conotoxins is regularly updated on ConoServer.

Size is one measure of diversity. Figure 3 shows the distribution of conotoxin sequence lengths for all the ~1700 currently known peptides either isolated from the venom or predicted from a precursor sequence. Most are small, with a median size of 26 (with the majority ranging from 10 to 45 amino acids), although recently much larger proteins were identified.<sup>83</sup> The pattern of cysteine residues is another measure of diversity, and disulfide-rich conotoxins are categorized into

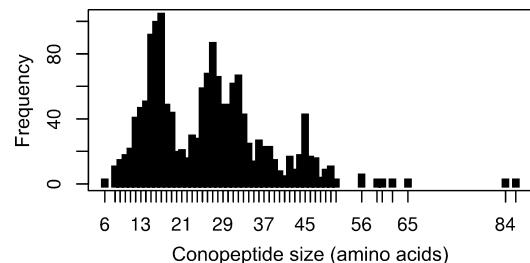


Figure 3. Distribution of mature conopeptide sequence lengths in the ConoServer data set.

cysteine “frameworks” according to the arrangement of cysteines in the mature peptide region of the precursor. Table 2 summarizes the currently recognized frameworks.

Associated with the classic categorization of conotoxins into cysteine frameworks, it has become common practice to refer to the backbone residues between cysteines as “loops”. The simplest and most common use of this terminology occurs in Framework I, with the Cys spacing CC-X<sub>m</sub>-C-X<sub>n</sub>-C defining two loops of size *m* and *n* residues. Table 3 summarizes the various loop length categories for Framework I conotoxins, from which it is apparent that the most common loop subfamilies are the 4/3 and 4/7 subfamilies.

The most optimistic estimates of conotoxin diversity at the peptide level are an order of magnitude higher than at the transcript level, with 1000–9000 peptides per species.<sup>11,47</sup> Considering that only a small number of conotoxins are shared between cone snail venoms from different species,<sup>11,26</sup> it can be estimated that only 0.1% of the total pool of conotoxins has been investigated. The most recent proteomic studies suggest that venoms in different species have vastly different complexity: 419 different peptide masses were found in *C. cossorus*, 455 in *C. novae-hollandiae*,<sup>84</sup> 650 in *C. victoriae*,<sup>84</sup> 845 in *C. imperialis*,<sup>11</sup> 1147 in *C. marmoreus*,<sup>11</sup> and 2428 in *C. textile*.<sup>11</sup> These numbers are lower estimates because large intraspecies variability,<sup>11,84,85</sup> and even intraspecimen variability,<sup>11,86,87</sup> was observed, indicating that further investigations of already well-studied species are likely to reveal additional novel conotoxins.

Several mechanisms contribute to the increased diversity at the protein level compared to the already large genetic diversity of conotoxins. These include differential incorporation of PTMs,<sup>47,85,87,88</sup> conservation of bioactive propeptide regions in the venom,<sup>41,47</sup> biological diversity arising through variable processing of conotoxins<sup>47</sup> and messy transcriptomics at the gene level.<sup>34,89</sup>

**4.1.4. Synthetic Conotoxin Diversity.** Adding to the natural sequence diversity of natural conotoxins, synthetic conotoxins have been engineered to better understand their sequence/structure/function relationships or for pharmaceutical applications.<sup>90</sup> A total of 26 different types of non-natural amino acids have been used in chemically synthesized conotoxins in addition to the post-translationally modified amino acids that occur naturally in conotoxins. Structure/function relationships of  $\omega$ -MVIIA, the only conotoxin approved for the clinic so far,<sup>91</sup> have been probed particularly thoroughly. For example, in one early study, iodotyrosine, norleucine, or oxomethionine was introduced to create subtle chemical variations from the wild-type tyrosine, leucine, and methionine residues.<sup>92</sup> These modifications helped to identify side chains important for  $\omega$ -MVIIA function. The N-terminus of  $\omega$ -MVIIA was also N-acetylated to probe the effects of N-

**Table 2.** Conotoxin Cysteine Frameworks

cysteine framework <sup>a</sup>	cysteine pattern <sup>b</sup>	no. of cysteines	no. of mature proteins	gene superfamilies
I	CC-C-C	4	293	A, M, O1, T
II	CCC-C-C-C	6	3	A, M
III	CC-C-C-CC	6	299	M
IV	CC-C-C-C-C	6	51	A, M
V	CC-CC	4	128	T
VI/VII	C-C-CC-C-C	6	517	H, I1, I3, M, O1, O2, O3
VIII	C-C-C-C-C-C-C-C	10	11	B2, S
IX	C-C-C-C-C-C	6	29	M, P
X	CC-C-[PO]C	4	11	T
XI	C-C-CC-CC-C-C	8	89	I1, I2, I3
XII	C-C-C-C-CC-C-C	8	49	I2, O1
XIII	C-C-C-CC-C-C-C	8	2	G
XIV	C-C-C-C	4	56	A, I2, J, L, M, O1, O2
XV	C-C-CC-C-C-C-C	8	23	D, N, O2, V
XVI	C-C-CC	4	7	M, T
XVII	C-C-CC-C-CC-C	8	1	Y
XVIII	C-C-CC-CC	6	2	
XIX	C-C-C-CCC-C-C-C	10	2	
XX	C-CC-C-CC-C-C-C	10	21	D
XXI	CC-C-C-CC-C-C-C	10	1	
XXII	C-C-C-C-C-C-C-C	8	8	E
XXIII	C-C-C-CC-C	6	6	K
XXIV	C-CC-C	4	1	B3
XXV	C-C-C-C-CC	6	1	
XXVI	C-C-C-C-CC-CC	8	1	

<sup>a</sup>The conotoxin cysteine frameworks are defined according to a specific pattern of cysteine residues in the mature peptide region of conopeptide precursors. The founding reference for each cysteine framework can be found on the ConoServer Web site (<http://www.conoserver.org>), which provides updates to this table and additional statistics. <sup>b</sup>Hyphens separating the cysteine residues (C) stand for a protein segments of one or more amino acids. [PO] indicates a position that could be occupied by a proline (P) or hydroxy-proline (O).

**Table 3.** Loop Classes in Framework I Conotoxins

loop length class	no. of conopeptides	representative conopeptide
3/5	52	$\alpha$ -GI
3/6	4	$\alpha$ -Mn1.1
4/2	1	$\alpha$ -Cl1.1
4/3	63	$\alpha$ -ImI
4/4	14	$\alpha$ -BuIA
4/5	5	$\alpha$ -Ca1.1
4/6	15	$\alpha$ -AuIB
4/7	237	$\alpha$ -Vc1.1
4/8	2	$\alpha$ -Vt1.24
5/2	3	$\alpha$ -Calla
5/8	1	$\alpha$ -Cl1.2

terminal charge.<sup>92</sup> A similar strategy was used with conotoxins  $\alpha$ -AuIB<sup>93</sup> and  $\alpha$ -ImI<sup>94</sup> and with  $\kappa$ -RIIK.<sup>95</sup> An interesting strategy reported by Kasheverov et al. was to substitute some residues of  $\alpha$ -GI with benzoylphenylalanines, which create cross-links with the receptor after photoactivation, unraveling the conotoxin binding site.<sup>96</sup> Non-natural amino acids have also been used to improve the selectivity and affinity for molecular targets<sup>97–99</sup> or to render conotoxins more suitable as drugs by simplifying their structure.<sup>100</sup>

Another important area in conotoxin drug development is the improvement of conotoxin bioavailability, stability, and absorption via chemical re-engineering studies.<sup>101</sup> For example, introduction of lipo-amino acids was employed to improve the oral bioavailability of conotoxin  $\alpha$ -MII,<sup>102</sup> whereas approaches to enhance stability have so far focused on peptide backbone cyclization<sup>93,103–105</sup> or on substitutions of cysteine residues by carba bridges,<sup>106</sup> nonpeptidic backbone spacers,<sup>107</sup> or diselenide bridges.<sup>108,109</sup>

## 4.2. Structural Diversity

**4.2.1. Techniques Used To Explore Conotoxin Structural Diversity.** The majority of structures of conotoxins determined so far have utilized NMR spectroscopy rather than X-ray crystallography.<sup>110</sup> There are two main reasons for this; the first is that conotoxins are difficult to crystallize, and the second is that, being small, they are an ideal size for NMR structure determination and eminently suitable for homonuclear NMR methods, where there is no need for labeling.<sup>111</sup> Thus, it is easy to determine structures for either native peptides or for samples produced by solid-phase chemistry, and the limitation of having to recombinantly express labeled conotoxins is not a consideration.

Thus far there has been no uniform nomenclature to describe the overall folds of conotoxins, and hence, here we propose grouping them into “fold classes” sharing similar shapes and spatial position of disulfide cross-links. The discussion below defines these fold classes (A–L). Table 4 divides conotoxin structures into fold and subfold families, which are illustrated in Figure 4.

**4.2.2. Folds with Four Cysteine Residues.** The most studied class of conotoxins from a structural perspective is the  $\alpha$ -conotoxins, which display cysteine Framework I (CC-C-C).<sup>112,113</sup> Most of these peptides adopt fold A (with the globular disulfide connectivity [1–3, 2–4]). Framework I peptides that have four residues in their first loop form a small helical region (subfold A1), whereas this helical region disappears when the loop is shortened to three residues (subfold A2). Framework X differs from Framework I only by the requirement to have two residues in loop 2, with the second position being occupied by a proline or hydroxy-proline.<sup>114</sup> The three currently known wild-type structures of Framework X peptides, i.e., conotoxins  $\chi$ -MrIA,  $\chi$ -MrIB, and  $\chi$ -MrVIA, display two different disulfide connectivities:  $\chi$ -MrIA and  $\chi$ -MrIB form a ribbon structure (fold D),<sup>104,115</sup> whereas the structure of  $\chi$ -CMrVIA<sup>69</sup> is a mirror of fold A (denoted as fold E in Figure 4b). Interestingly, a synthetic variant of  $\chi$ -CMrVIA, in which the central position of the first loop is mutated to proline, adopts a type A fold (subfold A3),<sup>116</sup> suggesting that the simultaneous presence of a proline in both loops 1 and 2 might “compensate” their effects on the fold.

Ribbon-type structures with four cysteines (fold D, connectivity [1–4, 2–3]) can be divided in two groups depending on the disulfide bond 2–3 adopting a “staple”

**Table 4. Classification of Known Three-Dimensional Structures of Wild-Type and Synthetic Conopeptides into Folds and Subfolds**

name <sup>a</sup>	species <sup>b</sup>	Cys framework <sup>c</sup>	size (aa)	no. of Cys <sup>d</sup>	loop size <sup>e</sup>	gene super family	method <sup>f</sup>	BMRB ID <sup>g</sup>	PDB ID <sup>g</sup>	Cono Server ID <sup>g</sup>
<b>Fold A:</b> four cysteines, globular [connectivity 1–3, 2–4]										
<b>Subfold A1</b> (one turn of helix in first loop)										
$\alpha$ -ImI	<i>C. imperialis</i>	I	12	4	4/3	A	NMR	1G2G, 1IMI, 1CNL, 1IM1	5, 24, 25, 27	
							X-ray	2BYP, 2C9T	34, 35	
$\alpha$ -ImI [DSN]		I	12	4	4/3		NMR	4847	1E76	10
$\alpha$ -ImI [R7L]		I	12	4	4/3		NMR	4846	1E75	9
$\alpha$ -ImI [A9L,W10Y, R11ABA]		I	12	4	4/3		NMR	20107		131
$\alpha$ -ImI [R11E]		I	12	4	4/3		NMR	4845	1E74	8
$\alpha$ -ImI [C2Agl,C8Agl]		I	12	2	4/3		NMR	20033		128
$\alpha$ -ImI [C2U,C8U]		I	12	4	4/3		NMR	6897	2BC7	97
$\alpha$ -ImI [C2U,C3U,C8U, C12U]		I	12	4	4/3		NMR	6896	2BC8	98
$\alpha$ -RgIA	<i>C. regius</i>	I	12	4	4/3	A	NMR	20002, 1543S	2JUT	118, 123
$\alpha$ -RgIA [DSE]		I	12	4	4/3		NMR	15367	2JUR	119
$\alpha$ -RgIA [P6 V]		I	12	4	4/3		NMR	15436	2JUQ	121
$\alpha$ -BuIA	<i>C. bullatus</i>	I	13	4	4/4	A	NMR	15031	2I28	7
$\alpha$ -AuIB	<i>C. aulicus</i>	I	15	4	4/6	A	NMR		1MXN, 1DG2	31, 13
cyclic-AuIB-4 (GGAA)		I	19	4	4/6		NMR			142
cyclic-AuIB-5 (AGAGA)		I	20	4	4/6		NMR			143
cyclic-AuIB-6 (GGAAGG)		I	21	4	4/6		NMR			144
$\alpha$ -EI	<i>C. ermineus</i>	I	18	4	4/7	A	NMR	1K64		18
$\alpha$ -Epi [sTy15 > Y]		I	16	4	4/7		X-ray	1A0M		20
$\alpha$ -GIC	<i>C. geographus</i>	I	16	4	4/7	A	NMR	5985	1UL2	26
$\alpha$ -GID	<i>C. geographus</i>	I	18	4	4/7	A	NMR	5585	1MTQ	15
$\alpha$ -MII	<i>C. magus</i>	I	16	4	4/7	A	NMR		1M2C, 1MII	21, 29
$\alpha$ -MII [E11A]		I	16	4	4/7		NMR			145
cyclic-MII-6		I	22	4	4/7		NMR	6818	2AJW	32
cyclic-MII-7		I	23	4	4/7		NMR	6817	2AK0	33
$\alpha$ -OmIA	<i>C. omaria</i>	I	17	4	4/7	A	NMR	6237	2GCZ	5
$\alpha$ -PeIA	<i>C. pergrandis</i>	I	16	4	4/7	A	NMR			139
$\alpha$ -PIA	<i>C. purpurascens</i>	I	18	4	4/7	A	NMR	6720	1ZLC	36
$\alpha$ -PnI1		I	16	4	4/7		X-ray		1PEN	12
$\alpha$ -PnIA [A10L,D14K, sTy15Y]		I	16	4	4/7		X-ray		2BR8	30
$\alpha$ -PnIB	<i>C. pennaceus</i>	I	16	4	4/7	A	X-ray	1AKG		16
$\rho$ -TIA	<i>C. tulipa</i>	I	19	4	4/7	A	NMR	1IEN		65
$\alpha$ -TxIA	<i>C. textile</i>	I	16	4	4/7	A	X-ray	2UZ6		110
$\alpha$ -Vc1.1	<i>C. victoriae</i>	I	16	4	4/7		NMR	7177	2H8S	4
cyclic-Vc1.1		I	22	4	4/7		NMR			149
$\alpha$ -Vc1.2	<i>C. victoriae</i>	I	16	4	4/7	A	NMR	20126		141
<b>Subfold A2</b> (no turn of helix in first loop)										
$\alpha$ -CnIA	<i>C. consors</i>	I	14	4	3/5		NMR	1B45		53
$\alpha$ -GI	<i>C. geographus</i>	I	13	4	3/5	A	NMR	1XGA		22
							X-ray	1NOT		11
$\alpha$ -GI [N4Benzoyl-phenylalanine]		I	13	4	3/5		NMR	2FRB		3
$\alpha$ -GI [S12Benzoyl-phenylalanine]		I	13	4	3/5		NMR	2FR9		2
$\alpha$ -SI	<i>C. striatus</i>	I	13	4	3/5	A	NMR	4503	1QMW	1
							X-ray	1HJE		17
$\alpha$ -LtXIVA	<i>C. litteratus</i>	XIV	13	4	3/3/2	L	NMR	21014		148
<b>Subfold A3</b> (no turn of helix in first loop, second loop similar to subfold A1)										
$\chi$ -CMrVIA [K6P]		X	11	4	4/2		NMR		2IH6	111
$\chi$ -CMrVIA [K6P] amidated		X	11	4	4/2		NMR		2IH7	112

Table 4. continued

name <sup>a</sup>	species <sup>b</sup>	Cys framework <sup>c</sup>	size (aa)	no. of Cys <sup>d</sup>	loop size <sup>e</sup>	gene super family	method <sup>f</sup>	BMRB ID <sup>g</sup>	PDB ID <sup>g</sup>	Cono Server ID <sup>g</sup>
<b>Fold B:</b> six cysteines, three disulfide bonds not in a knotted arrangement [connectivity 1–4, 2–5, 3–6]										
<b>Subfold B1</b> (one turn of helix in second loop, two turns of helix overall)										
$\mu$ -CnIIIC	<i>C. consors</i>	III	22	6	5/4/5		NMR		2YEN	150
$\mu$ -GIIIA	<i>C. geographus</i>	III	22	6	5/4/4	M	NMR	1664, 1665	1TCG, 1TCJ	82, 84, 134, 135
$\mu$ -GIIIA [R13A]		III	22	6	5/4/4		NMR		1TCH, 1TCK	83, 85
$\mu$ -GIIIB	<i>C. geographus</i>	III	22	6	5/4/4	M	NMR		1GIB	64
$\mu$ -KIIIA	<i>C. kinoshitai</i>	III	16	6	5/4/4	M	NMR	20048		129
$\mu$ -PIIIA	<i>C. purpurascens</i>	III	22	6	5/4/4	M	NMR	6027	1R9I	79
$\mu$ -RIIK [T24A]		III	24	6	6/4/4		NMR			146
$\mu$ -SIIIA	<i>C. striatus</i>	III	20	6	1/4/5	M	NMR	20025		125
$\mu$ -SmIIIA	<i>C. stercusmuscarum</i>	III	22	6	5/4/5	M	NMR	5881	1Q2J	77
$\mu$ -TIIIA	<i>C. tulipa</i>	III	22	6	5/4/4	M	NMR	20024		126
<b>Subfold B2</b> (no turn of helix in second loop, one turn of helix overall)										
$\alpha$ -PIIIE	<i>C. purpurascens</i>	III	24	6	4/5/4	M	NMR	5113	1ASS, 1JLO	51, 68
$\alpha$ -PIIIF	<i>C. purpurascens</i>	III	24	6	4/5/4	M	NMR	5112	1JLP	69
<b>Fold C:</b> six cysteines, three disulfide bonds forming a cystine knot [connectivity 1–4, 2–5, 3–6]										
<b>Subfold C1</b> (six residues in first loop)										
$\delta$ -Am2766	<i>C. amadis</i>	VI/VII	26	6	6/6/3/3	O1	NMR		1YZ2	94
$\omega$ -CVID	<i>C. catus</i>	VI/VII	27	6	6/6/3/6	O1	NMR			138
$\delta$ -EVIA	<i>C. ermineus</i>	VI/VII	32	6	6/9/3/3	O1	NMR		1G1P, 1G1Z	62, 63
$\omega$ -FVIA	<i>C. fulmen</i>	VI/VII	25	6	6/6/3/4		NMR		2KM9	137
$\mu$ -conotoxin-GS	<i>C. geographus</i>	VI/VII	27	6	6/3/4/7	O1	NMR		1AG7	50
$\omega$ -GVIA	<i>C. geographus</i>	VI/VII	27	6	6/6/2/6	O1	NMR		2CCO, 1TTL, 1OMC	72, 89, 100
$\omega$ -GVIA [O10 > K]		VI/VII	27	6	6/6/2/6		NMR		1TR6	86
$\mu$ -MrVIB	<i>C. marmoreus</i>	VI/VII	31	6	6/9/4/4	O1	NMR	6135	1RMK	80
$\omega$ -MVIIA	<i>C. magus</i>	VI/VII	25	6	6/6/3/4	O1	NMR		1DW4, 1DW5, 1MVI, 1OMG, 1TTK	55, 56, 70, 73, 88
$\omega$ -MVIIA with C-terminal Gly		VI/VII	26	6	6/6/3/4		NMR		1FEO	59
$\omega$ -MVIIA [R10 > K]		VI/VII	25	6	6/6/3/4		NMR		1TT3	87
$\omega$ -MVIIIC	<i>C. magus</i>	VI/VII	26	6	6/6/3/5		NMR	4500	1CNN, 1OMN	74
$\omega$ -MVIIIC [S17K, S19R, K25R]		VI/VII	26	6	6/6/3/5		NMR		1V4Q	90
$\kappa$ -PVIIA	<i>C. purpurascens</i>	VI/VII	27	6	6/6/3/5	O1	NMR		1AV3, 1KCP	46
$\omega$ -SO3	<i>C. striatus</i>	VI/VII	25	6	6/6/3/4	O1	NMR		1FYG	61
$\omega$ -SVIB	<i>C. striatus</i>	VI/VII	26	6	6/6/3/5	O1	NMR		1MVJ	71
t7a	<i>C. tulipa</i>	VI/VII	30	6	6/3/4/4	O1	NMR		1EYO	57
$\omega$ -TxVII	<i>C. textile</i>	VI/VII	26	6	6/6/3/3	O1	NMR		1F3K	58
$\delta$ -TxVIA	<i>C. textile</i>	VI/VII	27	6	6/6/3/4	O1	NMR		1FU3	60
$\iota$ -RXIA	<i>C. radiatus</i>	XI	46	8	6/5/2/4	I1	NMR	15175	2P4L, 2JTU	104, 130
$\iota$ -RXIA [BTr33 > W]		XI	46	8	6/5/2/4		NMR	15174	2JRY	105
<b>Subfold C2</b> (three residues in first loop)										
gm9a	<i>C. gloriamaris</i>	IX	27	6	3/5/3/1/4	P	NMR		1IXT	67
<b>Fold D:</b> four cysteines, disulfide bonds with ribbon connectivity [connectivity 1–4, 2–3]										
<b>Subfold D1</b> (disulfide 2–3 in a staple conformation)										
$\chi$ -MrIA	<i>C. marmoreus</i>	X	13	4	4/2	T	NMR	6891	2EW4	102
cyclic-MrIA		X	15	4	4/2		NMR		2J15	49
$\chi$ -MrIB amidated	<i>C. marmoreus</i>	X	13	4	4/2		NMR		1IEO	66
* $\alpha$ -GI ribbon isoform		I	13	4	3/5		NMR		1XGB	23
<b>Subfold D2</b> (disulfide 2–3 in a hook conformation)										
* $\alpha$ -AulB ribbon isoform		I	15	4	4/6		NMR		1MXP	14
* $\alpha$ -BuIA ribbon isoform		I		4	4/4		NMR		2NS3	114
* $\alpha$ -ImI deamidated ribbon isoform		I	12	4	4/3		NMR		2IGU	106
* $\alpha$ -ImI [P6A] ribbon isoform		I	12	4	4/3		NMR		2IFI	108
* $\alpha$ -ImI [P6K] ribbon isoform		I	12	4	4/3		NMR		2IFZ	107
* $\alpha$ -ImI [P6K] ribbon deamidated isoform		I	12	4	4/3		NMR		2IFJ	109

Table 4. continued

name <sup>a</sup>	species <sup>b</sup>	Cys framework <sup>c</sup>	size (aa)	no. of Cys <sup>d</sup>	loop size <sup>e</sup>	gene super family	method <sup>f</sup>	BMRB ID <sup>g</sup>	PDB ID <sup>g</sup>	Cono Server ID <sup>g</sup>
<b>Subfold D2</b> (disulfide 2–3 in a hook conformation)										
* $\chi$ -CMrVIA ribbon isoform		X	11	4	4/2		NMR		2B5P	95
* $\chi$ -CMrVIA amidated ribbon isoform		X	11	4	4/2		NMR		2IHA	113
<b>Fold E:</b> four cysteines, mirror of fold A [connectivity 1–3, 2–4]										
$\chi$ CMrVIA	<i>C. marmoreus</i>	X	11	4	4/2		NMR		2B5Q	96
<b>Fold F:</b> four cysteines, disulfide bonds collinear [connectivity 1–3, 2–4]										
$\alpha$ -Pu14a	<i>C. pulicarius</i>	XIV	23	4	10/1/3	A	NMR	21015		147
<b>Fold G:</b> four cysteines, parallel disulfide bonds [connectivity 1–3, 2–4]										
$\kappa$ -PlXIVA	<i>C. litteratus</i>	XIV	25	4	3/10/1	J	NMR	6951	2FQC	103
<b>Kunitz fold:</b> large protein with two disulfide bonds [connectivity 1–4, 2–3]										
Conkunitzin-S1	<i>C. striatus</i>	XIV	60	4	24/20/3		X-ray		1Y62	48
Conkunitzin-S2	<i>C. striatus</i>	XIV	65	4	24/20/3		NMR		2j6d	117
<b>Fold H:</b> six cysteines [connectivity 1–5, 2–4, 3–6]										
mr3e	<i>C. marmoreus</i>	III	16	6	4/3/1	M	NMR	15195	2EFZ	101
<b>Fold I:</b> six cysteines [connectivity 1–5, 2–3, 4–6]										
$\alpha$ -PIVA [Hyp7P, Hyp13P]		IV	25	6	7/2/1/6		NMR		1P1P	75
$\alpha$ -EIVA	<i>C. ermineus</i>	IV	30	6	7/2/1/7		NMR	5869	1PQR	76
<b>Fold J:</b> two cysteines, cystine stabilized turn										
contryphan-R	<i>C. radiatus</i>		8	2	5		NMR		1QFB	47
contryphan-R [ $\Delta$ 1]			7	2	5		NMR		1DG0	45
contryphan-Sm	<i>C. stercusmuscarum</i>		8	2	5		NMR		1DFY, 1DFZ	38, 39
contryphan-Vn	<i>C. ventricosus</i>		9	2	5		NMR		1NXN	43
cyclic-contryphan			8	2	5		NMR		1D7T	37
conopressin-T	<i>C. tulipa</i>		9	2	4		NMR	20007		124
<b>Fold K:</b> no cysteine, fully helical										
conantokin-G	<i>C. geographus</i>		17	0		B	NMR	1AD7, 1AWY, 1ONU	40, 41, 44	
conantokin-T	<i>C. tulipa</i>		21	0			NMR	1ONT		42
<b>Fold L:</b> no cysteine, 3/10 helix and coil										
conomorphin	<i>C. marmoreus</i>		15	0		M	NMR	7397	2YYF	115
conomorphin [d13D]			15	0			NMR		2JQC	116

<sup>a</sup>A brief description of the folds and subfolds is provided in Figure 4a or 4b. The names of non-natural synthetic variants are indented in the case where the fold is the same as the wild-type conopeptide, whereas the name of the variant is preceded by an asterisk in the case where it adopts a different fold from the wild-type. <sup>b</sup>Only wild-type conopeptides are provided with a Conus (*C.*) species. <sup>c</sup>Cysteine frameworks are defined in Table 2.

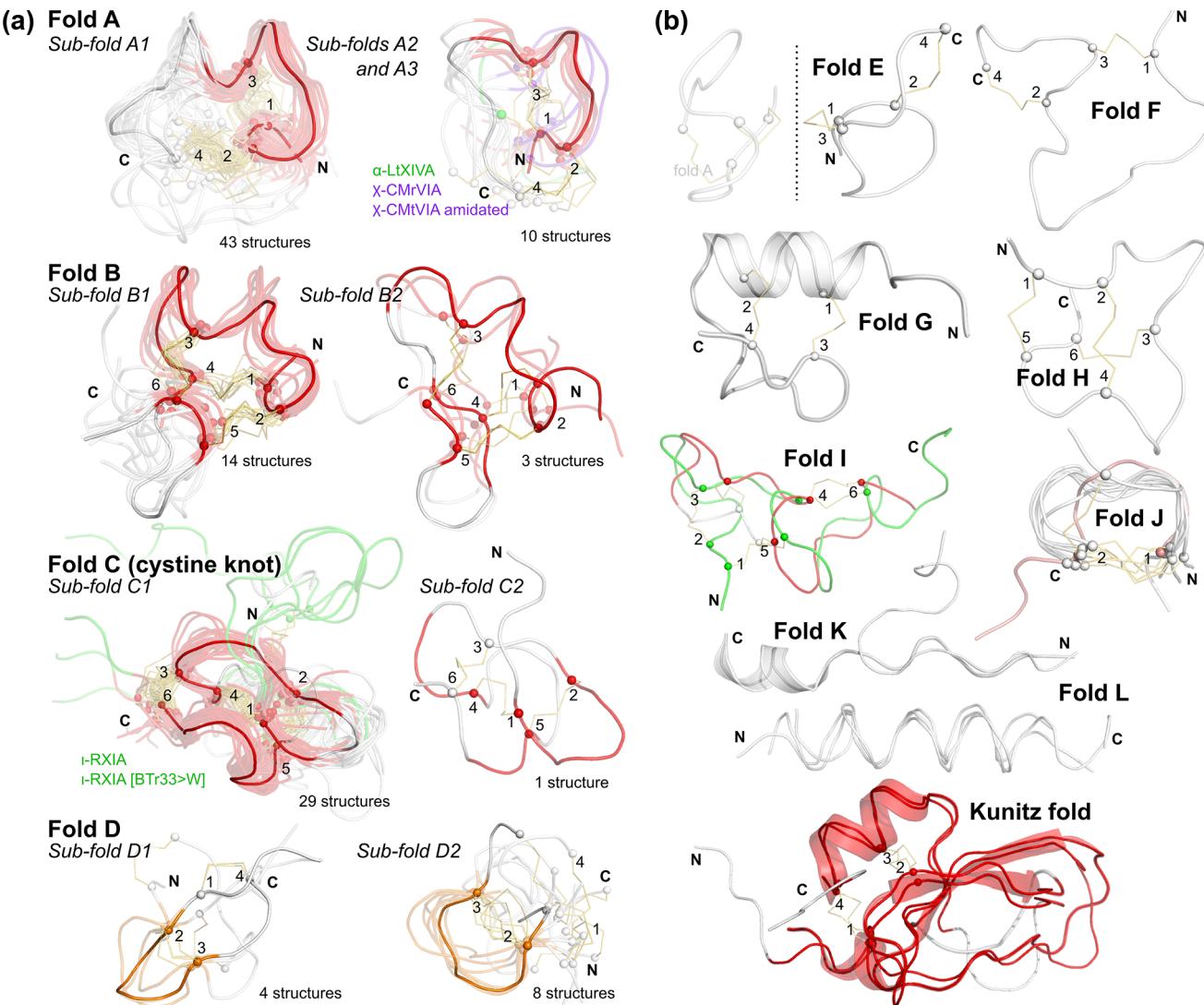
<sup>d</sup>The number of cysteine residues (no. of cysteines) is counted in the sequence of the mature peptide region in the precursor, before modification to cystines. <sup>e</sup>The “loop size” designates the length of the intercysteine segments defined in the cysteine frameworks, whose description is in Table 2.

<sup>f</sup>“Method” refers to the experimental method used to determine the three-dimensional structures. If two different experimental methods were used for the same conopeptide, identifiers are provided on two separate lines. <sup>g</sup>The database identifiers in the Biological Magnetic Resonance dataBank (BMRB), Protein Data Bank (PDB), and ConoServer database are provided. Distinct structural studies are catalogued as different entries in ConoServer, and therefore, each entry in ConoServer can be associated with a BMRB and/or a PDB entry. Some conopeptide three-dimensional structures are only found in ConoServer as they were not deposited by their authors in the PDB or BMRB.

(subfold D1) or a “hook” conformation (subfold D2). Most synthetic Framework I conotoxins synthesized with a ribbon disulfide connectivity adopt a subfold D2, whereas wild-type  $\chi$ -MrIA displays a subfold D1. Interestingly, Framework I conotoxin  $\alpha$ -AuIB has been shown to be also active in its ribbon isoform,<sup>117,118</sup> which was considered as non-natural for Framework I peptides until the recent discovery of minute amounts of a Framework I conotoxin with a ribbon disulfide connectivity in the venom of *C. imperialis*.<sup>119</sup> Framework XIV is the latest framework with four cysteines to have been structurally characterized.<sup>56</sup> Perhaps due to the looser definition of this framework, the corresponding conotoxins show very different shapes, including folds A, F, and G and a Kunitz-type fold.

**4.2.3. Folds with Six Cysteine Residues.** Fold B is characteristic of conotoxins with cysteine Framework III, and

its characteristic shape is strikingly similar to that of fold A, but the spatial arrangement of the disulfide bonds is different. A slight decrease in size of the second loop from 4 to 5 amino acids corresponds to loss of an helical segment (subfolds B1 and B2, respectively), which seems to correlate with a change of pharmacological target, from voltage-gated sodium channels ( $\mu$ -conotoxins) to nAChRs ( $\alpha$ -conotoxins), respectively. Framework III conotoxin mr3e has a different connectivity, i.e., connectivity [1–5, 2–4, 3–6], to the other Framework III conotoxins (connectivities [1–6, 2–4, 3–5] and [1–4, 2–5, 3–6]), and its structure determined by NMR reveals a new fold (fold H).<sup>120</sup> Interestingly, a recent re-evaluation of another Framework III conotoxin,  $\mu$ -KIIIA, indicates that it might use the same connectivity as mr3e and therefore might also adopt fold H.<sup>121</sup>



**Figure 4.** (a) Most commonly studied conopeptide folds. All available three-dimensional structures in ConoServer corresponding to the four folds A–D were overlaid. The peptide backbone of each conopeptide is shown using a ribbon representation. The  $\alpha$  carbon of cysteine residues or equivalent (i.e., selenocysteines or half-carba-bridge) are represented as spheres, and the cross-links are shown using orange sticks. The most structurally conserved regions are highlighted in red or orange. Some structures presenting interesting differences to the fold and discussed in the text are colored in green or blue. The half-cystines have been numbered according to their sequential position in the primary sequence, allowing one to clearly distinguish the cross-link connectivities. A description of all structures is provided in Table 4. This figure was partly drawn using PyMol.<sup>350</sup> (b) Conopeptide folds with only a few representatives. All available three-dimensional structures in ConoServer corresponding to the four folds E–L and Kunitz are overlaid. The peptide backbone structure of each conopeptide is shown using a ribbon representation and also using a cartoon representation for fold G, K, and Kunitz. The  $\alpha$ -carbons of the cysteine residues are represented as spheres, and the cross-links are shown using orange sticks. The most structurally conserved regions are highlighted in red for the Kunitz fold. The half-cystines have been numbered according to their sequential position in the primary sequence to clearly distinguish the cross-link connectivities. A description of all structures is provided in Table 4. The figure was partly drawn using PyMol.<sup>350</sup>

Fold C has the same disulfide-bond connectivity as fold B (connectivity [1–4, 2–5, 3–6]), but here the disulfide bonds are in a knotted arrangement, forming a so-called cystine knot, a stable structural scaffold that has been found in all kingdoms of life.<sup>122</sup> Conotoxins displaying the cystine knot motif target various voltage-gated ion channels corresponding to the  $\omega$ ,  $\kappa$ ,  $\mu$ , and  $\delta$  pharmacological families.<sup>123</sup> It has been hypothesized that the degree of flexibility of certain loops, as monitored by NMR experiments, could play an important role in target specificity.<sup>110,124</sup> The NMR structure of  $\iota$ -conotoxin i-RXIA, which has cysteine Framework XI (eight cysteines, C-C-CC-CC-C-C),<sup>125</sup> forms a cystine knot fold that superimposes very well with subfold C1 structures. The structure of Framework IX

gm9a also displays a cystine knot<sup>126</sup> but is classified as a separate subfold C2 because only two of the disulfide bridges could be overlaid with corresponding disulfide bonds of subfold C1 conotoxins. Subfolds C1 and C2 correspond to classes “conotoxin-1” and “conotoxin-2” in the Knottin database, respectively.<sup>122</sup> Cysteine Framework IV conotoxins so far studied display different disulfide-bond connectivities (connectivity [1–5, 2–3, 4–6]) to the other six cysteine conotoxins. Despite their radically different structures (fold I), these peptides act on nAChRs,<sup>21</sup> similarly to fold A conotoxins.

**4.2.4. Disulfide Poor Conopeptides.** Some disulfide-poor conopeptides, including contulakin-G,<sup>127</sup> have been shown to be intrinsically disordered, whereas others have well-defined

structures. For instance, fold J of contryphans and conopressins includes a turn whose extremities are stabilized by a disulfide bond, creating stable cyclic structures.<sup>128,129</sup> Folds K and L that describe conomorphin and conantokins, respectively, are not stabilized by disulfide bonds but are either partially (fold K) or totally (fold L) helical.<sup>17,130</sup>

## 5. CONOTOXIN SYNTHESIS

The sparse availability of *Conus* venom from natural sources presents a major limitation toward utilization of conotoxins for research and clinical applications. Two approaches are currently employed to produce significant amounts of pure material required to carry out structure–activity relationship (SAR) studies. The first is recombinant expression of conotoxins in heterologous expression systems such as *E. coli* and yeast, an approach that is generally applied for protein production.<sup>131,132</sup> This method is used to produce cDNA libraries for the screening of a wide range of conotoxins, and while one of the advantages is to have rapid access to longer peptides as well as to a diverse range of superfamilies, it also holds several limitations.<sup>133</sup> Incorporation of the vast array of PTMs, characteristically observed in conotoxins, and unnatural amino acids is challenging, and difficulties in isolating the desired products in high purity remain. Many laboratories therefore opt for the viable alternative of chemical synthesis, particularly feasible due to the rather small size of the conotoxins. This approach eliminates undesired (host) protein contamination often faced in recombinant protein production and gives researchers more control and freedom for modifications. Chemical synthesis is rapid, highly automated, and scalable, providing significant quantities of native and modified peptides in high purity. It allows incorporation of unnatural amino acids, a wide array of PTMs, imaging tags, and structural modifications such as backbone cyclization or disulfide-bond replacements that improve stability against enzymes. The first chemical syntheses of conotoxins were accomplished in the early to mid-1980s on  $\alpha$ -conotoxins  $\alpha$ -GI,  $\alpha$ -MI,<sup>134–136</sup> and  $\omega$ -conotoxin  $\omega$ -GVIA.<sup>137,138</sup> Since then, thousands of conotoxin analogues have been synthesized, typically providing multi-milligram amounts for structural or biological studies.

### 5.1. Solid-Phase Peptide Synthesis—Major Chemical Approaches

The method of choice to produce conotoxins in sufficient amounts for biological and structural characterization is solid-phase peptide synthesis (SPPS) utilizing Boc and Fmoc strategies.<sup>139–142</sup> Certain regions in peptides can be difficult to couple during chain assembly or have incomplete  $\text{N}\alpha$  deprotection during assembly due to aggregation or secondary structure formation. The optimized Boc *in situ* neutralization protocol coupled with SPPS significantly improves the synthesis of peptides with such “difficult” sequences.<sup>143</sup> Additional optimization and introduction of rapid synthetic procedures that improve speed and efficiency of SPPS result in powerful syntheses as illustrated with the Boc-SPPS of  $\alpha$ -conotoxin  $\alpha$ -[A10L]-PnIA, which was fully assembled, cleaved, and oxidized within a single working day.<sup>144</sup> Native chemical ligation (NCL) became another important addition to the repertoire of SPPS, which allows synthesis of peptides up to 200 amino acids long.<sup>145,146</sup> In this approach, two unprotected peptide segments, one containing a C-terminal thioester and the other a cysteine residue at its N-terminus, react chemoselectively to give the native amide (peptide) bond.<sup>145</sup> NCL is

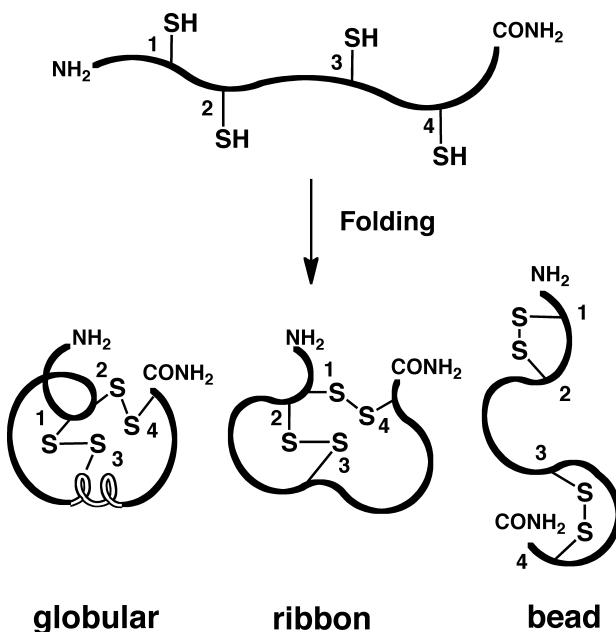
generally not used for standard synthesis since conotoxins fall in the category of small peptides, but NCL plays an important role in combinatorial SAR studies, N–C-terminal backbone cyclization, or in the avoidance of difficult sequences. It is anticipated that the emergence of improved coupling reagents, protecting groups, resins, and purification methods will further facilitate synthesis of longer and more complex peptides, and it is expected that synthesis of high-purity conotoxin libraries will soon be fully automated.

High-throughput synthesis and generation of combinatorial conotoxin libraries are receiving more and more attention due to the importance of efficient SARs in the quest for more potent, selective, and stable conotoxin analogues for drug development. Combinatorial techniques are able to rapidly generate numerous libraries within a fraction of the time it would otherwise take for individual peptide synthesis.<sup>147,148</sup> The applicability of such library design was demonstrated in a recent study where a mixture-based positional scan of  $\alpha$ -ImI was achieved.<sup>98</sup> Analogue mixtures with mutations at positions 9, 10, and 11 were screened for pharmacological activity at  $\alpha 7$  nAChRs, providing candidates for second- and third-generation analogue syntheses. Through this high-throughput procedure 96 analogues of  $\alpha$ -ImI were generated. Whereas the disulfide-rich character and uncontrolled folding are still a major issue in these high-throughput or combinatorial approaches, implementation of novel techniques such as the use of selenocysteine<sup>149</sup> or other folding enhancers<sup>82</sup> will help to create libraries of more complex conotoxin analogues with directed folding in the future.

### 5.2. Oxidative Folding Strategies Employed and Challenges

The highly conserved cysteine frameworks in the conotoxin superfamilies have one major function: to form and stabilize the rigid 3D structures that comprise a vast array of secondary structures, including  $\alpha$ -helices,  $\beta$ -sheets, and turns crucial for receptor recognition, potency, and selectivity. In contrast to nondisulfide peptides, which follow Anfinsen’s rules for folding,<sup>150</sup> correct folding of disulfide-rich peptides involves a complex process involving covalent reactions such as oxidation (S–S formation), reduction (S–S breakage), and isomerization or scrambling (S–S rearrangement).<sup>151</sup> Depending on the number of disulfide bonds present, this folding process increases rapidly in complexity due to the increasing number of possible isomers ( $2n!/({2^n}n!)$ , with  $n$  being the number of disulfide bonds formed. Thus, where two, three, or four disulfides are present, 3, 15, or 105 isomers are theoretically possible.<sup>152</sup> Thus far, a maximum of five disulfide bonds have been observed within the conotoxin family. Hence, it is no surprise that correct folding is a significant requirement for chemical synthesis of conotoxins, particularly as generally only one of the isomers corresponds to the bioactive conformation (Figure 5).<sup>153–156</sup>

Cone snails deal with this folding challenge through a combination of post-translational processing, N- and C-terminal propeptides that can act as intramolecular chaperones, folding catalysts, and the intrinsic folding properties within the amino acid sequence itself.<sup>151,157–159</sup> By contrast, *in vitro* folding can be quite inefficient: the conceptually simplest approach involves random oxidation of the fully deprotected peptide to the native isomer. The clear advantage for this approach is that it requires only a single cysteine protecting group during SPPS and a single purification step. However, the



**Figure 5.** Nonselective isomer formation in a two-disulfide-bond-containing peptide.

inherent problem is that formation of disulfide bonds is a nondirected process, and the final yield and disulfide-bond connectivity highly depends on the encoded structural information within the amino acid sequence and the thermodynamic stability of the native conformation versus non-native conformations, which is often only marginally different.<sup>82,160,161</sup> Common folding conditions include slightly alkaline (pH 7.5–8.5) aqueous or aqueous/organic buffers in high dilutions (200–500  $\mu\text{M}$ ) to prevent dimer and oligomer formation. Thiol/disulfide exchange reactions are often induced by addition of GSH/GSSG/peptide ratios (e.g., 100:10:1) to mimic physiological conditions that reshuffle non-native disulfide bonds directing the fold into the thermodynamically favored and assumed native conformation. Denaturants such as 0.5–3.0 M urea or 0.1–1.5 M guanidine hydrochloride are occasionally added to prevent aggregation. Table 5 lists examples of folding conditions that have been used successfully to fold various conotoxin analogues. Once a major isomer is obtained, validation of the native/desired fold is necessary by reductive alkylation, tryptic digest followed by MS/MS, by NMR, or by biological activity determination. Even though this approach has its disadvantages, it is still the most employed for native and unmodified conotoxins based on the belief that the native and bioactive isomer is the thermodynamic most stable fold.

DMSO-promoted oxidation has also found frequent application since it can be applied over an extended pH range from 1 to 8. Generally faster oxidation rates are obtained with DMSO in acidic media, and DMSO is known to disrupt aggregates and assist in dissolving hydrophobic peptides. Oxidation with 10% DMSO or even better 50% DMSO in 1 M HCl leads to disulfide-bond formation in high yields, yet problems may arise in removing DMSO from the reaction mixture. In general, the more reactive the oxidizing agent, the more side reactions are to be expected, particularly at sensitive amino acid residues such as methionine, tryptophan, and tyrosine.

**Table 5. Common Buffer Conditions for Oxidative Folding of Conotoxins<sup>a</sup>**

standard conotoxin folding conditions
0.1 M $\text{NH}_4\text{HCO}_3$ ; pH 7.5–8.5; room temperature
0.1 M $\text{NH}_4\text{HCO}_3$ /30% i-PrOH (or ACN); pH 7.5–8.5; room temperature
0.33 M $\text{NH}_4\text{OAc}/0.5 \text{ M GnHCl}$ , GSH:GSSG:peptide (100:10:1); pH 7.8; 4 °C
30% DMSO/5% acetic acid/65% water; room temperature
30% DMSO/0.1 M $\text{KH}_2\text{PO}_4$ ; pH 6, room temperature

additional conditions
0.33 M $\text{NH}_4\text{OAc}/0.5 \text{ M GnHCl}$ ; pH 7.8; 4 °C
2 M $(\text{NH}_4)_2\text{SO}_4/0.1 \text{ M NH}_4\text{OAc}$ ; pH 7.7; 4 °C
2 M GnHCl/50 mM $\text{NH}_4\text{OAc}$ ; pH 7.7; 4 °C
0.1 M $\text{NH}_4\text{HCO}_3$ ; pH 8; 4 °C
0.05–0.1 M $\text{NH}_4\text{OAc}$ ; pH 7.8–8.4; 4 °C or room temperature
0.1 M $\text{NH}_4\text{HCO}_3$ /30% i-PrOH; pH 8; room temperature
0.1 M $\text{NH}_4\text{HCO}_3$ /15% DMSO/30% TFE; pH 8; room temperature
0.1 M Tris/6 M GnHCl; pH 8.5, 4 °C or room temperature
0.1 M $\text{NH}_4\text{HCO}_3$ /50% TFE; pH 8; room temperature

<sup>a</sup>Recommended peptide concentration = 200–500  $\mu\text{M}$ . GSH/GSSG: reduced/oxidized glutathione. GnHCl: guanidine hydrochloride. TFE: 2,2,2-trifluoroethanol. ACN: acetonitrile. i-PrOH: isopropanol.

Correct conotoxin folding becomes more difficult as soon as *non-native* modifications are embedded in the synthetic design. These modifications can disrupt encoded folding information, switching the thermodynamic equilibrium to favor non-native (and therefore often inactive) disulfide-bond isomers (having the same mass and similar retention times). Given that the main reason to select synthetic chemistry approaches over peptide expression systems is to introduce non-native modifications for such purposes as SAR studies, ligation chemistry, cyclization, fluorescent tagging, PEGylation, etc., it is imperative to have efficient regioselective control over disulfide-bond formation even though this may mean lower yields due to additional purification steps. Currently, this is mainly achieved via orthogonal thiol-protecting groups.<sup>162–164</sup>

### 5.3. Chemically Directed Regioselective Disulfide-Bond Formation

**5.3.1. Chemical Strategies.** The regioselective approach enables directed formation of individual disulfide bonds, thereby ensuring the desired isomer. The general strategy is based on selective deprotection and oxidation of pairwise cysteine residues. Since multiple disulfide bonds are formed step-by-step, reaction conditions are required that prevent breaking or scrambling of the disulfide bond already formed. Therefore, exposure to alkaline conditions, thiols, or other nucleophiles and lengthy reaction times that could allow competitive disulfide-bond disproportionation must be avoided. Cysteine protecting groups fall within four general categories consisting of base labile, acid labile, metal ion labile, and mixed alkyl/aryl disulfide (Table 6). The versatility of the S-Acm group in combination with acid-labile protecting groups makes it one of the most utilized protecting groups in Fmoc- and Boc-SPPS.<sup>165</sup> S-Acm can either be cleaved with mercury salts to obtain free thiols or, as it is mainly the case during conotoxin synthesis, be oxidatively cleaved by iodine or thallium trifluoroacetate to form a disulfide bond with another S-Acm group.<sup>165,166</sup> Reaction conditions for cleavage<sup>167</sup> and deprotection have to be chosen carefully to avoid side reactions such as oxidation of methionine, histidine, tryptophan, or tyrosine

**Table 6. Overview of Commonly Used Cysteine and Selenocysteine Protecting Groups, Their Stability, and Standard Cleavage Conditions<sup>a</sup>**

Chemistry	Protecting Group	Structure	Stability	Removal conditions	Chemistry	Protecting Group	Structure	Stability	Removal conditions
<b>General Synthesis</b>									
<b>Sulfur</b>									
Boc (Fmoc)	4-Methylbenzyl (S-Meb)		Base TFA	HF 5% DMSO/TFA 60°C	Boc	Benzyl (Se-Bz)		Base	Na/NH <sub>3</sub>
Boc (Fmoc)	4-Methoxybenzyl (S-Mob)		Base TFA	HF TFMSA	Boc	4-Methylbenzyl (Se-Meb)		Base TFA	HF
Fmoc	Triphenylmethyl (S-Trt)		Base I <sub>2</sub>	1% TFA Tl(III)	Fmoc	4-Nitro-benzyl (Se-pNB)		Base HF	Zn, then I <sub>2</sub> SnCl <sub>2</sub> , then I <sub>2</sub>
Fmoc	2,4,6-Trimethoxybenzyl (S-Tmob)		Base	7% TFA/scavengers	Fmoc	Acetimidomethyl (Se-Acm)		Base TFA	I <sub>2</sub> HF (partial)
<b>Orthogonal Synthesis</b>									
Boc	9-Fluorenylmethyl (S-Fm)		TFA HF	Base	Fmoc	4-Methoxybenzyl (Se-Mob)		Base	HF TFMSA or TMSBr TFA/DMSO/scavenger DTNP/TFA
Boc	3-Nitro-2-pyridylsulfonyl (S-Npys)		TFA HF	Reducing agents Thiols					
Boc Fmoc	Acetimidomethyl (S-Acm)		Base TFA HF	I <sub>2</sub> Hg(II); Ag(II); Tl(II);					
Boc Fmoc	tert-Butylsulfonyl (S-(tBu))		TFA HF (partial)	Reducing agents Thiols					
Fmoc (Boc)	tert-Butylmercapto (S-tBu)		TFA Base	5% DMSO/TFA 25°C HF (20°C)					
Fmoc	9H-Xanthan-9-yl (S-Xan)		Base	1% TFA/scavengers I <sub>2</sub> Tl(II)					

<sup>a</sup>The most utilized protecting groups are marked in grey. A more complete overview of cysteine and selenocysteine protecting groups and cleavage conditions can be found in the reviews in refs 163, 169, and 170. S/Se in bold is part of the functional group to be protected. The dashed line indicates the cleavage site of the protecting group. S/Se in bold is part of the functional group to be protected. The dashed line indicates the cleavage site of the protecting group.

residues,<sup>165</sup> as well as an S to O Acm shift in peptides serine- and threonine-rich peptides.<sup>168</sup> For more detailed information on existing cysteine protecting groups the reader is referred to the following reviews.<sup>163,169,170</sup> Here we only listed sulfur/selenium protecting groups that have been successfully used in conotoxin synthesis.

#### 5.4. Off-Resin Approaches

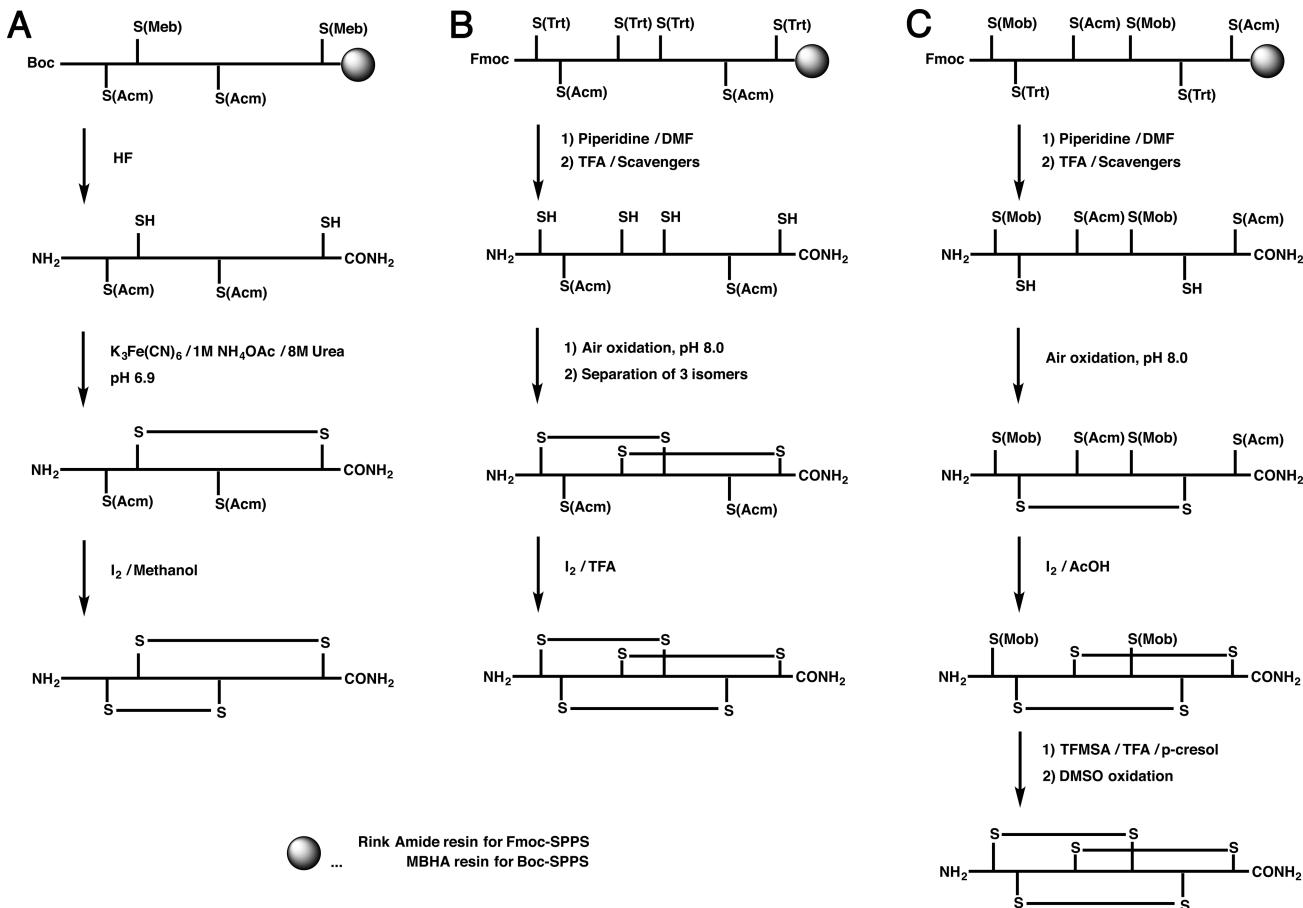
**5.4.1. Syntheses of Conotoxins with 1, 2, 3, and 4 Disulfide Bonds.** Regioselective formation of disulfide bonds can be carried out either while the peptide is still attached to the solid support or upon cleavage in solution. A very common regioselective off-resin approach utilizes the thiol-protecting group acetimidomethyl (S-Acm). Typically, the peptide is cleaved from the solid support together with all protecting groups with the exception of S-Acm. The free cysteine residues are oxidized by mild oxidants to form the first disulfide bridge. The second disulfide bond is generally formed directly by treatment with iodine. This approach was first demonstrated by Nishiuchi and Sakakibara for synthesis of  $\alpha$ -GI and its disulfide isomers using Boc chemistry (Scheme 1A)<sup>136</sup> and has been

applied to the syntheses of many other peptides using both Boc and Fmoc chemistry.<sup>171</sup>

The S-Acm protection strategy can also be applied to the synthesis of conotoxins containing three disulfide bonds by focusing disulfide-bond formation into the desired path. This has been demonstrated by the semidirected synthesis of  $\omega$ -MVIIID (Scheme 1B).<sup>172</sup> Random oxidation of the first two disulfide bonds formed predominantly a single isomer, which was followed by directed formation of the third disulfide bond using iodine-mediated oxidation of two remaining S-Acm protected cysteine residues. In a more selective approach, the S-Mob pair was introduced in addition to the S-Acm and S-Trt protection, which allowed consecutive formation of all three disulfide bonds, illustrated in the synthesis of  $\omega$ -MVIIIA by Fmoc chemistry (Scheme 1C).<sup>173</sup>

The one-pot synthesis of  $\alpha$ -SI employed the combination of the S-Meb and S-tBu protecting groups, where the different temperature dependence of these two groups under DMSO/TFA/anisole oxidation conditions was exploited.<sup>174</sup> Cysteine residues protected with S-tBu were rapidly converted to the corresponding disulfide bond at room temperature, whereas S-

**Scheme 1.** (A) Regioselective Off-Resin Folding Strategy for Synthesis of  $\alpha$ -GI Using S-Acm in Combination with the Acid-Labile S-Meb Protecting Group by Boc Chemistry;<sup>136</sup> (B) Semi-Directed Off-Resin Folding Strategy for Synthesis of  $\omega$ -MVIID Using S-Acm in Combination with the Acid-Labile S-Trt Protecting Group by Fmoc Chemistry;<sup>172</sup> (C) Regioselective Off-Resin Folding Strategy for Synthesis of  $\omega$ -MVIIA Using S-Acm in Combination with an Acid-Labile S-Trt and S-Mob Protecting Groups by Fmoc Chemistry<sup>173</sup>

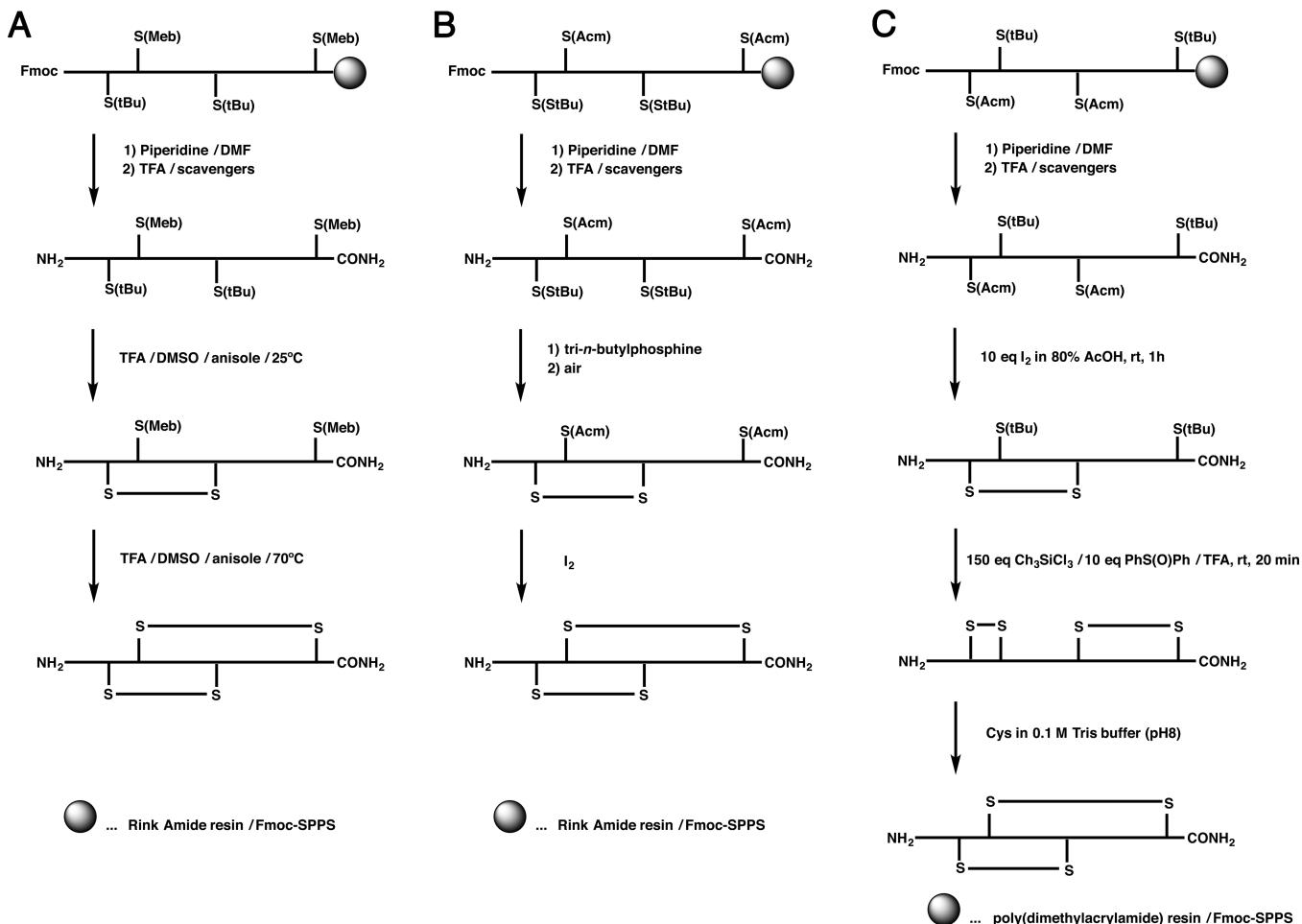


Meb cysteine residues remained largely intact. Subsequent heating of the solution led to deprotection and oxidation of the S-Meb groups, yielding the second disulfide bond (Scheme 2A).<sup>174,175</sup> This approach was successfully combined with the S-Acm/S-Trt combination to allow selective formation of an  $\alpha$ -SI dimer containing four disulfide bonds (Scheme 3).<sup>176</sup> This double one-pot oxidation strategy with appreciable yields was the first example of using four distinct protecting groups to produce conotoxin analogues. Despite the utility of such a procedure, it is restricted by the nature of amino acid residues in the sequence, particularly by tryptophan and methionine, which are known to undergo irreversible oxidation if left unprotected under these conditions.<sup>177</sup> This was evident in the synthesis of  $\alpha$ -ImI in an one-pot procedure, where no detectable amounts of native  $\alpha$ -ImI were recovered.<sup>175</sup> Alternatively, S-(StBu) was used in combination with S-Acm cysteine derivatives for synthesis of  $\alpha$ -GI, where upon reductive cleavage of the S-(StBu) protection the first disulfide bond was formed by air oxidation, which was then followed by iodine oxidation of the S-Acm groups (Scheme 2B).<sup>178</sup> Different stability toward iodine of the S-Acm and the S-tBu protection was explored in the synthesis of  $\alpha$ -GI, where the first disulfide bond was formed by direct oxidation of the S-Acm pair followed by S-tBu oxidation via the chlorosilane/sulfoxide procedure (Scheme 2C).<sup>179</sup> Although the first disulfide was

formed correctly, treatment with chlorosilane/sulfoxide resulted in the non-native and not desired bead isomer and the native isomer could only be recovered by reshuffling with Cys/Tris buffer.

**5.4.2. Polymer-Supported Oxidation.** A variety of polymer-supported oxidants have been explored including hydrocarbon-based resins,<sup>180</sup> agarose-bound chaperones,<sup>181</sup> PEG-PS (polyethylene glycol-polystyrene), Sephadex, controlled pore glass,<sup>182</sup> and, more recently, cross-linked ethoxylate acrylate resin (Clear) preloaded with Ellman's reagent (ClearOx).<sup>183</sup> Commercially available ClearOx resin has been continuously used in conotoxin synthesis, where folding occurs upon immobilization of the reduced peptide with the on-resin disulfide, generating a peptide–resin disulfide intermediate that is released again upon its intramolecular disulfide-bond formation.<sup>183</sup> The low loading of the resin takes advantage of the pseudodilution effect, favoring intramolecular disulfide-bond formation. This makes it an attractive alternative for scale-up and high-throughput folding since the amount of solvent is significantly reduced. ClearOx resin has been successfully applied to fold the  $\alpha$ -SI and  $\alpha$ -GI as well as to the regioselective two-step oxidation procedure forming a non-native isomer using the xanthyl (Xan) and Acm groups.<sup>180,184</sup> More recently, application of ClearOx was successfully extended to three disulfide-bridged conotoxins, namely,  $\mu$ -

**Scheme 2.** (A) Regioselective Off-Resin Folding Strategy for Fmoc Chemistry of  $\alpha$ -SI and  $\alpha$ -GI Using S-tBu and S-Meb with One-Pot Disulfide Formation at Different Temperatures;<sup>174,175</sup> (B) Off-Resin Folding Strategies of  $\alpha$ -GI Using S-(StBu) and S-Acm Groups; (C) Unsuccessful Regioselective Off-Resin Folding Strategy for  $\alpha$ -GI Trying To Exploit the S-tBu Stability to Iodine



SIIIA,  $\mu$ -KIIIA, and  $\omega$ -GVIA, showing improvements compared to standard solution oxidations.<sup>185</sup>

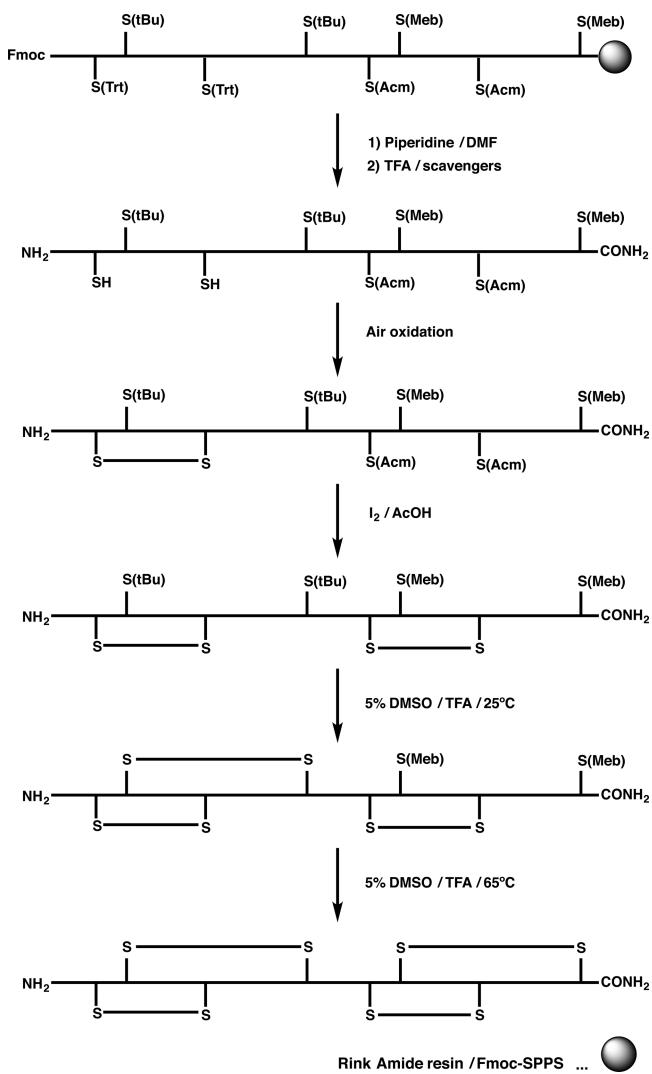
**5.4.3. Native Chemical Ligation.** Considering the disulfide-rich nature of conotoxins it is surprising that NCL has not been exploited more than is currently the case. One reason for this is the small size of conotoxins, yet NCL certainly has the potential to be used in a variety of applications, including incorporation of handles, overcoming difficult sequences, or combinatorial assembly. The latter was demonstrated in the efficient production of chimeras of  $\omega$ -CVID and  $\omega$ -MVIIC to evaluate the contributions of the N- and C-terminal segments to activity.<sup>186</sup> NCL was also employed to synthesize  $\delta$ -PVIA covalently bound to its propeptide to study the involvement of propeptides in the folding process.<sup>187</sup> Last but not least, NCL plays an important role in the synthesis of cyclic versions of conotoxins that possess improved enzymatic stability, a feature that will be discussed in more detail in section 5.6.

### 5.5. On-Resin Approaches

**5.5.1. Recent Successes and Fundamental Problems with This Approach.** The principle of on-resin strategies is to form one or multiple disulfide bridges while the peptide is still anchored to the solid support. On-resin approaches are operationally more convenient, save time-intensive purification

steps, and avoid a large excess of oxidation solvents due to the pseudodilution effect, which simulates high dilution, thus favoring intramolecular disulfide-bond formation. Especially in view of creating conotoxin libraries for SAR or drug discovery purposes, on-resin folding becomes an essential feature. Current limitations to the on-resin approaches are often poor recovery caused by undesired polymerization or disulfide interaction with the resin,<sup>188</sup> in addition to the lack of uniform oxidative folding conditions or strategies to control multiple disulfide-bond formation. For example, in the on-resin synthesis of  $\alpha$ -SI, closure of the smaller loop before the larger loop was important to obtain the desired isomer, while in solution either oxidation order was successful.<sup>188</sup> In addition, disulfide-bond formation on a solid support can often be sluggish due to steric hindrance caused by side chain protecting groups, and recovery yields are often lower than compared with in-solution methods.<sup>188,189</sup> Microwave-assisted on-resin disulfide-bond formation may improve synthesis and folding, and was successfully employed on the synthesis of  $\alpha$ -MII, providing increased yields compared with other on-resin cyclization methods.<sup>190</sup> Scheme 4 depicts three examples of orthogonal on-resin oxidation strategies that have been successfully carried out on conotoxins, yet in all cases, in-solution folding was more efficient in both yields and recovery of the desired isomer.

**Scheme 3. Regioselective Off-Resin Folding Strategy for the Synthesis of an  $\alpha$ -SI Dimer by Fmoc Chemistry Utilizing S(tBu), S(Trt), S(Meb), and S(Acm) for Orthogonal Disulfide-Bond Formation<sup>176</sup>**



The base-labile S-Fm group in combination with the S-Acm group was utilized using Boc chemistry for synthesis of  $\alpha$ -GI (Scheme 4A).<sup>191</sup> In this approach the base-labile S-Fm group was removed on resin using piperidine, followed by S-Acm deprotection by iodine. Although earlier reports suggested that disulfide bonds are unstable under highly acid conditions, careful selection of scavengers for HF cleavage yielded the intact isomer. The Barany lab studied different orthogonal methodologies for synthesis of  $\alpha$ -SI by Fmoc chemistry (Scheme 4B and 4C).<sup>188,189</sup> The initial approach included deprotection of the S-Tmob groups without significant peptide chain loss from the resin, followed by formation of the first disulfide bond via oxidation with CCl<sub>4</sub>—Et<sub>3</sub>N in NMP, with the second cysteine pair still protected with Acm group (Scheme 4B). Oxidation of S-Acm to form the second disulfide bond was carried out with Ti(TFA)<sub>3</sub> in DMF with anisole as the scavenger. Overall yields of the monomeric conotoxin were as high as 14% with additional oligomeric material retained on the solid support. Alternatively, the orthogonal combination of S-Xan and S-Acm can be used, which was illustrated in the

directed on-resin (and in solution) synthesis of all three possible isomers of  $\alpha$ -SI (Scheme 4C).

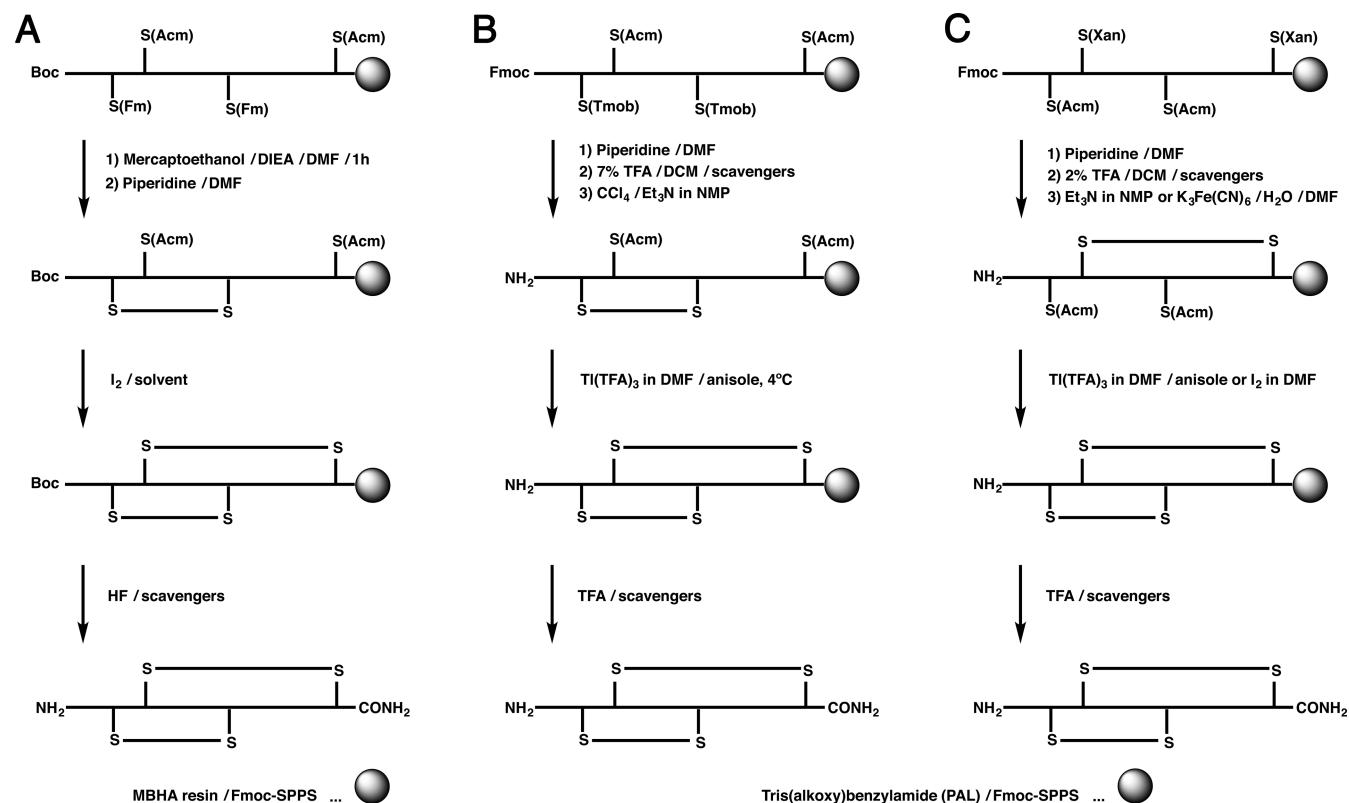
Whereas there is certainly room for improvement for on-resin disulfide-bond formation, one has to remember that when it comes to conotoxin library design, low yields can be sacrificed for higher screening efficiency. Once a hit has been isolated and characterized, in-solution folding might be the better option for scale up and further optimization or SAR studies.

**5.2. High-Throughput Synthesis via Combinatorial Peptide Library Generation.** Cone snails themselves utilize a combinatorial approach to diversify and optimize their venom.<sup>192</sup> Their cell machinery facilitates hypermutations of individual loop residues while maintaining a rigid and highly conserved disulfide-bond framework that gives rise to rigid three-dimensional structures optimized to interact with different receptor classes. Combinatorial chemical synthesis of conotoxin libraries has been more problematic with the main limitation being efficient folding and identification of the correct bioactive isomer. Nevertheless, harvesting non-native disulfide-bond isomers can also lead to active analogues with interesting pharmacological properties. Thus, the non-native ribbon form of  $\alpha$ -AuIB was initially found to be more active than its globular counterpart, and further studies disclosed that these two isomers differ in their inhibitory mechanisms with the ribbon isomer inhibiting only low-sensitivity  $\alpha$ 3 $\beta$ 4 nAChRs competitively, whereas the globular form inhibits  $\alpha$ 3 $\beta$ 4 nAChRs irrespectively of receptor stoichiometry by a noncompetitive mechanism.<sup>113,118</sup> In the case of  $\alpha$ -BuIA, the non-native and inactive ribbon isomer is structurally better defined than the active native form and one might speculate that the ribbon isomer also acts on a target that has yet to be identified.<sup>193</sup>

Drug discovery programs in venom peptide biotechnology companies take advantage of such non-native activities, and a methodology was developed employing a safety catch acid-labile (SCAL) amide linker enabling random DMSO oxidation during linker cleavage that yielded conotoxin disulfide-bond mixtures that can be directly screened against a wide variety of targets.<sup>194</sup> The SCAL linker was also employed in generation of  $\alpha$ -conotoxin libraries where it facilitated on-resin selenocysteine-directed folding, allowing chain assembly, deprotection, and folding to be performed in parallel to yield pure libraries of pure ribbon or globular isomers.<sup>149</sup>

Alternatively, positional scanning synthetic combinatorial libraries (PS-SCL) can be utilized, which are mixture-based libraries providing rapid means to acquire information around each position within a chemical framework. This led to the largest conotoxin library to date, a library generated employing a multistep synthetic combinatorial approach for the purpose of improving the activity and selectivity profile of  $\alpha$ -ImI.<sup>98</sup> In this study, a range of substitution mutants was generated in order to produce  $\alpha$ -ImI analogues with superior potency toward  $\alpha$ 7 nAChR subtypes. A PS-SCL was constructed around three residues important for activity to give a total of 10 648 possible combinations, which was followed up by another two rounds of optimization driven by biological activity. Finally, in the third round, 96 analogues were synthesized of which three analogues were found to display a greater than 10-fold increase in activity toward the  $\alpha$ 7 subtype compared to native  $\alpha$ -ImI. Considering these recent advances in accessing large numbers of correctly folded and modified  $\alpha$ -conotoxin analogues it may be concluded that the initial problems of low-throughput synthesis of conotoxins with one and two disulfide bonds have been

**Scheme 4.** (A) Regioselective On-Resin Folding Strategy Using S-Fm in Combination with S-Acm for Synthesis of  $\alpha$ -GI by Boc-SPPS;<sup>191</sup> (B and C) Regioselective On-Resin Folding Strategy for  $\alpha$ -SI Employing the S(Tmob) and S(Xan) Groups in Combination with the S(Acm) Protecting Group<sup>188,189</sup>



solved, thereby facilitating efficient SAR studies and optimization of potency and selectivity.

### 5.6. Cysteine Isosteres

**5.6.1. Carba, Lactam, Thioether, and Selenocysteine Bridges.** Despite the importance in stabilizing structure, disulfide bonds are inherently unstable under reducing conditions. Reduction or scrambling of disulfide-rich peptide therapeutics by thiol oxidoreductases or other thiol-containing agents, such as serum albumin or glutathione, has the potential to decrease their effectiveness as pharmacological agents *in vivo*.<sup>108</sup> Significant progress has been made in disulfide-bond engineering to improve the stability against such degradation.<sup>108,193,196</sup>

The  $\alpha$ -conotoxins have proven to be a particularly good model system for such disulfide-bond mimetics due to their rigid three-dimensional structure, size, diversity, and ease of synthesis and pharmacological characterization.<sup>113,196</sup> For example, replacement of a disulfide bridge in  $\alpha$ -ImI by an unsaturated dicarba bridge resulted in both *cis* and *trans* isomers, with one displaying no biological activity, while the other retained significantly reduced activity.<sup>106</sup> Reduction of the Cys3 to Cys12 disulfide bond in  $\alpha$ -ImI had no effect on its affinity, and its overall structure was quite similar to the native peptide. However, its rigid conformation was lost to a certain degree with an overall backbone rmsd value of 1.49 Å compared to 0.78 Å for native  $\alpha$ -ImI.<sup>197</sup> Exchange of a single disulfide bond in  $\alpha$ -SI by a lactam bridge resulted in either 60–70-fold or complete loss of activity, depending on which disulfide bond was modified.<sup>198</sup>

More promising was the development of thioether, selenoether, and diselenide mimetics. Initial backbone thioether

replacement of one disulfide bond in  $\alpha$ -GI resulted in a 260–800-fold loss of activity of the two isomers obtained,<sup>199</sup> which can most likely be contributed to the shortening of the loop by deletion of one sulfur atom. Direct isosteric replacement of the disulfide bonds by a CH<sub>2</sub>—S group was introduced in  $\alpha$ -conotoxin  $\alpha$ -ImI, where both disulfide bonds were systematically substituted by redox-stable cystathionine thioethers.<sup>200</sup> Regioselective thioether formation was achieved on resin via substitution of a  $\gamma$ -chloro-homoalanine by an intramolecular cysteine thiol to generate a hybrid thioether/disulfide as well as a dual cystathionine analogue.<sup>200</sup> NMR analysis showed that they had homologous structures to the native peptide. One of the hybrid isomers displayed identical activity to the native peptide, and the other two analogues showed a modest 3-fold decrease in activity.

Similar to the cystathionine analogues, a comprehensive structural and functional study of a wide range of  $\alpha$ -conotoxins containing selenocysteine replacements illustrated that such a modification had no significant impact on torsion angles, activity, or receptor subtype selectivity of this class of peptides.<sup>108,149</sup> X-ray analysis at 1.4 Å resolution of selenoconotoxin  $\alpha$ -PnIA showed that the diselenide bond was 0.3 Å longer than the disulfide bond (2.03 Å) with torsion angles of 93.9° and 83.1°, respectively. In addition, it was shown that the increased hydrophobicity and surface exposure of the diselenide bond had a small beneficial effect on the activity in some of the analogues.<sup>149</sup>

### 5.7. Selenoconotoxins

**5.7.1. Novel Chemical Properties of Selenocysteine.** Selenocysteine (Sec) is referred as the 21st proteinogenic amino acid due to its genetically controlled bioincorporation

and importance in many organisms.<sup>201–203</sup> Sec is often found in enzymatic active sites, where its known function is acting as a nucleophile, a metal ligand, or a redox element.<sup>204,205</sup> Selenium has been investigated as a substitute to sulfur extensively mainly due to its similarity in physicochemical properties. Chemical synthesis and structural and pharmacological analysis of a wide range of seleno analogues of bioactive peptides confirmed its isosteric character to its sulfur homologues. It is particularly interesting for X-ray crystallography since it significantly facilitates the phasing problem, and it avoids the lengthy and problematic heavy-atom screening procedure.<sup>206–208</sup> NMR analysis can also take advantage of the nuclear spin of  $I = 1/2$  of one of its isomers ( $^{77}\text{Se}$ ),<sup>209,210</sup> a feature that has already been exploited in a few studies.<sup>211–213</sup>

Despite selenium and sulfur being neighboring chalcogens, Sec exhibits distinct chemical properties when compared to cysteine. These include higher nucleophilicity,<sup>214–216</sup> better leaving group character,<sup>217</sup> higher susceptibility to nucleophilic attack,<sup>218,219</sup> and higher acidity.<sup>214,220,221</sup> In early  $pK_A$  determination studies selenocysteine exhibited lower  $pK_A$  values than cysteine ( $pK_A(\text{Sec}) = 5.24\text{--}5.63$ ,  $pK_A(\text{Cys}) = 8.25$ ),<sup>214,220,221</sup> suggesting that at physiological pH the Sec residue will be present largely in its reactive anionic form, the selenolate, while the cysteine residue would remain largely protonated. This has been confirmed in a more recent study, where the  $pK_A$  of two selenocysteine residues was determined within the short peptide, vasopressin, to be 3.3 and 4.3, emphasizing that selenoproteins must have a distinct function and activity other than their cysteine homologues.<sup>211</sup> Another feature of diselenide bonds is their lower redox potential ( $E'_0(\text{Sec}) = -386 \text{ mV}$ ;  $E'_0(\text{Cys}) = -223 \text{ mV}$ ),<sup>213,222–224</sup> which not only procures an increased resistance in reducing environments but also allows preferential formation of diselenide over disulfide bonds, a property that has been exploited in the folding of conotoxins.<sup>149,223,225,226</sup>

**5.7.2. Directed Folding of Conotoxin.** The concept of preferred diselenide formation over disulfide-bond formation, originally described by Moroder et al.,<sup>227</sup> was employed for conotoxin synthesis and ultimately shown to be true for a wide range of conotoxins.<sup>149,225</sup> Pairs of complementary cysteine residues were replaced with selenocysteine residues, and diselenide formation occurred rapidly, even under low pH. Once the diselenide bridge was formed, the other disulfide bonds formed accelerated and at lower pH when compared to the all-cysteine homologues.<sup>149</sup> This methodology was also applied on non-native isomer production, where folding was significantly improved; however, complete regioselectivity could not be achieved (observation of some selenylsulfide formation). The overall structures of the selenoconotoxins were comparable with their respective native conotoxins, and functional studies showed that the selenocysteine incorporated peptides exhibited similar or enhanced potency.<sup>108,149,226</sup> This intriguing increase in potency in some of the analogues is proposed to be due to the hydrophobic nature of the diselenide bond. Plasma stability was also enhanced significantly for all the selenocysteine analogues tested.<sup>108,149</sup> This approach can also be used orthogonally with earlier mentioned thiol-protecting groups, enabling folding of more complex peptides containing three or more disulfide bridges.

To date, the directed folding approach using selenocysteine has been applied to the  $\alpha$ -,  $\mu$ -, and  $\omega$ -conotoxin classes,<sup>108,149,225,226,228,229</sup> yet the strategy can in principle be extended to all disulfide-bond-containing peptides (and many

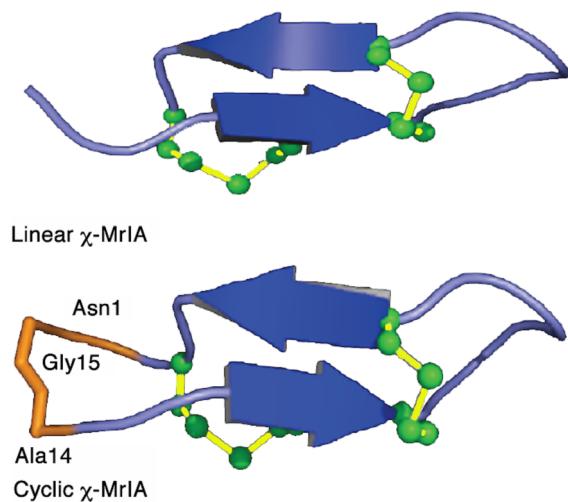
other peptides have been synthesized).<sup>161,230–232</sup> Its compatibility with solid-support chemistry enables generation of libraries, thus accelerating drug discovery efforts as well as SAR studies. During a selectivity optimization project on the three-disulfide-bond conotoxin  $\mu$ -KIIIA incorporation of a diselenide bond in combination with depletion of a disulfide bond simplified its synthesis dramatically without compromising its bioactivity.<sup>229</sup> This facilitated rapid positional scanning, yielding an improved selectivity mutation in blocking  $\text{Na}_v1.2$  over  $\text{Na}_v1.4$  subtype. Additionally, its stability against scrambling makes diselenides attractive for drug development, since thiols present in the body (e.g., serum albumin or glutathione) cannot deactivate such compounds.<sup>108,149</sup> The orthogonal character of diselenide formation adds to the repertoire of controlled regioselective folding, and it is expected that different Sec protecting groups will be developed in the future, further expanding this toolbox.

## 5.8. Cyclic Conotoxins

**5.8.1. N to C Cyclization, Including Regiocontrol of Disulfide Bonds, Enzyme Stability, and Oral Availability.** The short half-life of peptides is a major hurdle in drug development, and numerous studies have investigated means to extend the half-life of conotoxins *in vivo*.<sup>103,104</sup> Although disulfide-rich peptides (in particular, cystine knot motifs) that possess rigid secondary structures display improved stability compared to unstructured peptides, exopeptidases can break down peptides rapidly via C- and N-terminal access. Backbone N–C cyclization is an elegant approach to prevent such degradation and can easily be carried out via NCL owing to the cysteine-rich nature of the conotoxins. In this method, the N- and C-termini of the peptide are joined directly or by addition of short amino acid linkers to cyclize the peptide backbone. The linkers are chosen based on the distances between termini and their orientation so as to ensure that the overall structure of the re-engineered conotoxins is not compromised. Cyclization also stabilizes the structure of peptides by decreasing the conformational energy of the unfolded state<sup>233</sup> and can introduce additional rigidity that can protect against endopeptidases.<sup>234</sup>

Studies exemplifying the advantages of cyclization have been conducted on both  $\alpha$ - and  $\gamma$ -conotoxins.<sup>103,104,235–237</sup> Three cyclic analogues of  $\alpha$ -MII comprising of five, six, or seven residue linker sequences were synthesized. The 6- and 7-residues linker analogues of  $\alpha$ -MII retained the structural and biological features of the native peptide while significantly increasing their plasma stability. By contrast, the five-residue linker analogue displayed structural perturbations, resulting in loss of biological activity.<sup>103</sup> In another study, a cyclic analogue of  $\gamma$ -MrlA, containing a two-residue linker joining the ends of the native peptide sequence, was synthesized (Figure 6), and structural and functional analysis revealed that the cyclic analogue maintained the native peptide structure and equivalent biological activity.<sup>104,238</sup> Furthermore, it had substantially increased resistance to trypsin proteolysis. This was an interesting observation as the trypsin cleavage site was distant from the site of cyclization, confirming that backbone cyclization can also improve stability against endopeptidases.<sup>104</sup>

The linker length can influence the relative abundance of the possible disulfide isomers during random oxidation, in some cases leading to non-native isomers, as demonstrated in a study on cyclic  $\alpha$ -ImI analogues.<sup>236</sup> This minor synthetic drawback was overcome using a selective orthogonal cysteine protecting



**Figure 6.** Structural representations of linear  $\chi$ -MrIA (top) and cyclic  $\chi$ -MrIA (bottom).<sup>104</sup> Both peptides have very similar structures.  $\beta$ -Sheets are in blue; loop and turn regions are in purple. Residues used to link N- and C-termini of  $\chi$ -MrIA are labeled and highlighted in orange. Disulfide bonds in green are shown in a ball-and-stick representation. Structures were visualized using PyMol.<sup>350</sup>

scheme.<sup>236</sup> It would be of interest to see if this isomeric control could also be achieved via the selenocysteine strategy with advantage of the rapid in situ folding and suppression of scrambling. A direct comparison between different disulfide-bond mimetics and N- to C-terminal cyclization showed that backbone cyclization yielded significantly higher stability compared to disulfide-bond mimetics.<sup>195</sup> The cyclization approach also has wider applications as it can be used on any protein or peptide with termini no more than  $\sim 20$  Å apart. For such cases, linkers up to seven amino acids long can be used.<sup>103,239</sup>

To date, the most impressive cyclic conotoxin analogue is a cyclic  $\alpha$ -Vc1.1 analogue that is being developed for treatment of neuropathic pain. Although preclinical development of a linear synthetic version of  $\alpha$ -Vc1.1 was earlier discontinued, the more stable cyclic version displayed analgesic activity in rats when delivered orally.<sup>235</sup> Use of cyclization to engender a peptide with oral activity is an exciting breakthrough that has the potential to overcome a long-standing bioavailability problem generally associated with peptide-based drugs.

## 6. DISSECTING STRUCTURE–ACTIVITY RELATIONSHIPS

### 6.1. Conotoxin Discovery

In the postgenomic era, understanding ligand–protein interactions remains a priority to better understand the molecular basis of biological processes, particularly those associated with human disease. The function of many proteins is modulated by a complex network of interactions with associated proteins and/or endogenous ligands. Mapping these interactions on three-dimensional structures is expected to provide a framework for the design of better drugs. Conotoxins are a unique class of venom peptides that have high affinity and selectivity for many functionally critical membrane proteins, characteristics that make them valuable for target validation and drug development.<sup>240</sup> To expand their potential as templates for peptidomimetic development, a deep understanding of the

specific interactions made with their targets is required to facilitate rational design and associated lead optimization.

The high-affinity interaction of conotoxins with their target receptors is thought to be mainly driven by shape complementarity (e.g., concept of lock-and-key), hydrophobic contacts, and electrostatic potentials.<sup>241</sup> While the final complex toxin–receptor is optimally stabilized by a more extensive network of short-range attractive forces (hydrophobic interactions, hydrogen bonds, aromatic stacking, electrostatic, and van der Waals interactions), the first steps of the formation of this complex are predominantly stabilized by long-range electrostatic interactions between the protein and the ligand.<sup>242</sup> Favorable electrostatic interactions are produced by complementary charge distribution between binding partners, which in turn dictate the overall association rate.<sup>243</sup> Conotoxins often contain ionizable amino acids, whose ionization state influences their physicochemical properties and net electrostatic potential. The distribution of charges throughout the three-dimensional structure of conotoxin thus contributes to the on rate of binding through these long-range electrostatic effects as well as contributing to the selectivity profile of each ligand through shorter range complementary interactions, including salt bridge and cation– $\pi$  interactions.<sup>241</sup>

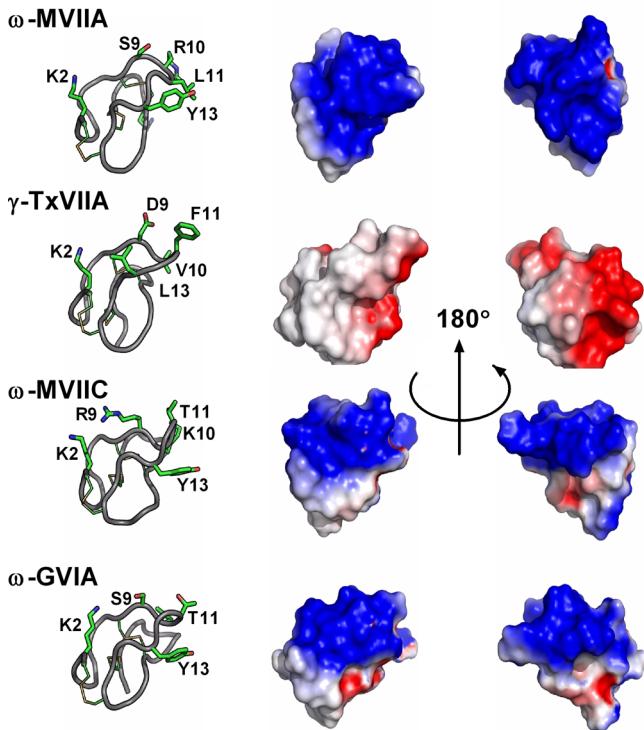
### 6.2. Positional Scanning

SAR studies integrating crystallographic and/or NMR three-dimensional structures (see section 4) with the results from alanine scanning along the conotoxin sequence have revealed the position and nature of critical residues contributing to target binding and subtype selectivity. These residues often involve charged and polar amino acids on the peptide surface, with the disulfide bonds often largely buried in the molecule, suggesting that hydrophobic effects contribute to the folding of disulfide-rich conotoxin.<sup>82</sup> In general, the relative contributions of electrostatic and hydrophobic interactions to the energy of association and dissociation appear to be dependent on the type of interaction. For example, electrostatic interactions appear more important for ion channel “pore blockers”, while conotoxins having a lock-and-key-type interaction, like the  $\alpha$ -conotoxins, rely on a more diverse combination of interactive forces.<sup>3</sup> In the next section, we detail the better-defined examples of conotoxin–receptor interactions, with a particular emphasis on the contribution of charge complementarity and electrostatic potentials to binding.

### 6.3. $\omega$ -Conotoxin SAR

$\omega$ -Conotoxins isolated from the venom of piscivorous cone snails are among the most potent antagonists of voltage-gated calcium channels (VGCCs) known to date.<sup>26</sup> The high potency of  $\omega$ -MVIIA combined with its high selectivity for the mammalian N-type calcium channel (Ca,<sub>2.2</sub>) was instrumental in the identification of this receptor as a spinal pain target.<sup>245</sup> This remarkable property led to development of this  $\omega$ -conotoxin as a novel type of analgesic (Prialt), the first marine drug approved by the FDA.<sup>91</sup> Although highly efficacious, dose-limiting side effects have limited the market for Prialt, possibly due to on-target effects on neuronal pathways outside the ascending pain pathway and uncharacterized off-target effects.<sup>246</sup> Attempts to design small and orally available molecules, nonpeptidic mimics of  $\omega$ -conotoxins, have been unsuccessful so far, most likely due to their reduced size which limits receptor interaction and receptor selectivity, resulting in an increased side effect profile when delivered peripherally.<sup>247</sup>

The three-dimensional structures of  $\omega$ -GVIA,  $\omega$ -MVIIC (both  $\text{Ca}_{v}2.2$  selective), and  $\omega$ -MVIIC ( $\text{Ca}_{v}2.1$  selective) have been determined using NMR spectroscopy.<sup>248–250</sup> Remarkably, all three  $\omega$ -conotoxins display a similar backbone conformation built around an inhibitory cystine knot motif (see section 4), despite divergent primary structures (Figure 7).<sup>251</sup> Since these



**Figure 7.** Structures and electrostatic surfaces of  $\omega$ -conotoxins. Important residues identified through SAR studies are indicated on the left panels.  $\omega$ -MVIIA,  $\omega$ -MVIIC, and  $\omega$ -GVIA target mammalian voltage-gated calcium channels, whereas TxVIIA is a mollusc-selective toxin. Obvious differences in electrostatic potentials likely account for the different pharmacologies.

conotoxins discriminate among closely related targets (VGCCs), it appears that shape complementarity might play an important role in determining subtype specificity. However, TxVIIA isolated from the molluscivorous *Conus textile* also possesses the typical inhibitory cystine knot motif with an overall similar shape to piscivorous  $\omega$ -conotoxins but has no detectable activity at mammalian VGCCs.<sup>252</sup> Mapping the electrostatic potentials on its three-dimensional surface revealed dramatic differences in the distribution of charges compared to  $\omega$ -MVIIA,  $\omega$ -MVIIC, and  $\omega$ -GVIA (Figure 7). TxVIIA is devoid of positively charged residues, which results in a hydrophobic, negatively charged molecule compared to the strongly positively charged surface of piscivorous  $\omega$ -conotoxins. This is consistent with the expected “pore-blocking” effect of  $\omega$ -conotoxins, which presumably have evolved a positively charged electrostatic surface that mimics the  $\text{Ca}^{2+}$  ions transported by this channel. Indeed, the external vestibule of VGCCs with its negatively charged residues has been identified as the likely binding site for  $\omega$ -conotoxins, allowing long-range attractive force to orientate the positively charged  $\omega$ -conotoxins.

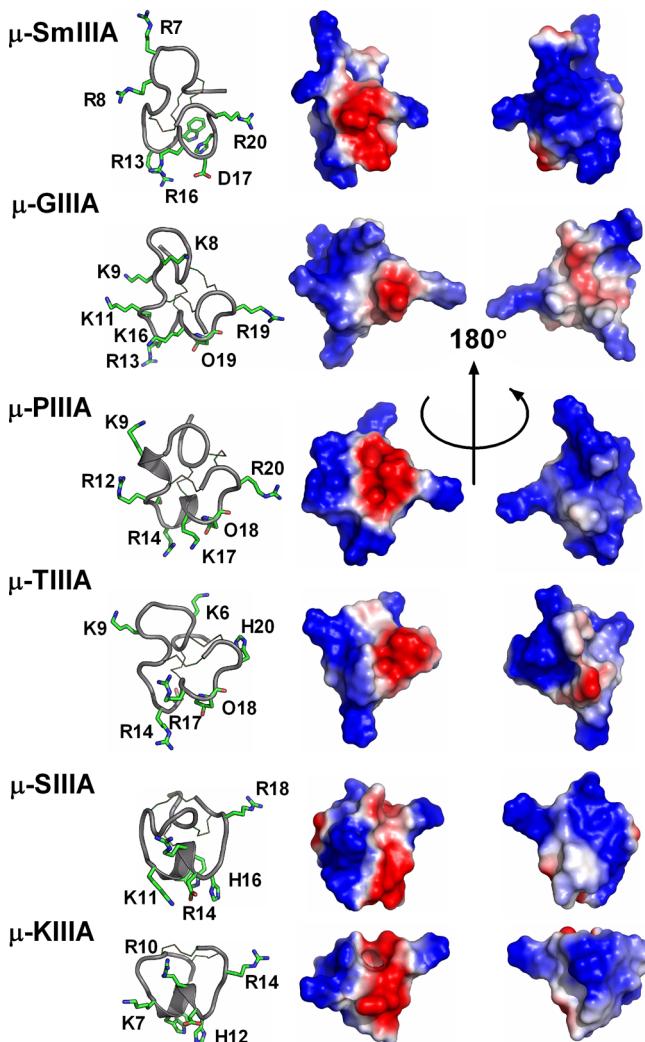
$\omega$ -MVIIA,  $\omega$ -MVIIC, and  $\omega$ -GVIA SAR studies have revealed a highly conserved dyad of critical residues (Tyr13 and Lys2 in

$\omega$ -MVIIA) that has been identified in other animal toxins that target VGCCs.<sup>248,253–255</sup> Although the aromatic moiety, its orientation, and the hydroxyl group of Tyr13 appear critical for high-affinity binding to Cav2.2,<sup>253,254,256</sup> the positive charge of Lys2 is not as essential as originally anticipated.<sup>257</sup> Indeed, Lys2 is involved in intramolecular stabilization of the three-dimensional structure, as alanine replacement shows increased loop flexibility. In support of a nondirect interaction of Lys2 with the receptor, the side chain of this positively charged residue in  $\omega$ -GVIA was shown to be tolerant to lengthening and shortening. A specific interaction with the receptor usually implies an optimum distance between partners, and variations around this length are expected to result in significant decrease in affinity.

Other residues in intercysteine loops 2 and 4 also contribute to  $\omega$ -conotoxin affinity and selectivity.<sup>248,249,258</sup> Affinity appears mainly driven by loop 4 residues R21 in  $\omega$ -MVIIA and R17, Y22, and K24 in  $\omega$ -GVIA, while the residue at position 10 in loop 2 seems to influence subtype selectivity.<sup>249</sup> Indeed, the residue at position 10 is consistently a lysine in  $\omega$ -conotoxins with high affinity for  $\text{Ca}_{v}2.1$  but is replaced with an arginine or hydroxy-proline in  $\text{Ca}_{v}2.2$ -selective peptides.<sup>92,250</sup> Interestingly, an arginine in position 10 reduces recovery from  $\text{Ca}_{v}2.2$  block for both  $\omega$ -MVIIA and  $\omega$ -CVID, suggesting that the effect of this residue on subtype selectivity could be due to altered recovery characteristics.<sup>259,260</sup> Together with Lys2 and Tyr13, this key residue at position 10 forms a highly positively charged face of  $\omega$ -conotoxins (Figure 7). More subtle electrostatic differences are also visible on the surface of  $\omega$ -MVIIA when compared to  $\omega$ -MVIIC and  $\omega$ -GVIA that could contribute to the observed differences in subtype selectivity. Thus, the pharmacophore of  $\omega$ -conotoxins responsible for high-affinity, subtype-selective inhibition of  $\text{Ca}_{v}2$  is becoming increasingly clear. It remains to be seen if our current understanding can be successfully applied to the design of orally active  $\omega$ -conotoxin peptidomimetics that are analgesic, although early attempts have shown some promise.<sup>247,261,262</sup>

#### 6.4. $\mu$ -Conotoxin SAR

As part of their prey–capture strategy, cone snails have also evolved a diverse range of peptides that modulate voltage-gated sodium channels (VGSCs), including the  $\mu$ -,  $\mu\text{O}$ -,  $\delta$ -, and  $\iota$ -conotoxins. In this section, we focus on the best studied and most promising in terms of drug development, the  $\mu$ -conotoxins that target TTX-sensitive VGSCs. Of the nine subtypes of VGSCs ( $\text{Na}_v1.1$ – $\text{Na}_v1.9$ ) found in mammals, several are therapeutically relevant, including  $\text{Na}_v1.7$  and  $\text{Na}_v1.8$  for treatment of acute and chronic pain. While all characterized  $\mu$ -conotoxins show potent inhibition of the skeletal muscle  $\text{Na}_v1.4$  and brain  $\text{Na}_v1.2$  channels, none to date have been found to selectively target therapeutically relevant subtypes. The three-dimensional structures of several  $\mu$ -conotoxins show a conserved central  $\alpha$ -helical motif, which is reminiscent of the structures of neuronal nicotinic antagonists  $\alpha$ -conotoxins (Figure 8). Accordingly, the recently described  $\mu$ -CnIIIC was found to inhibit VGSCs as well as neuronal nAChRs ( $\alpha 3\beta 2 > \alpha 4\beta 2 > \alpha 7$ ).<sup>263</sup> Mapping the electrostatic potentials on the surface of three-dimensional structures of  $\mu$ -conotoxins revealed a strong net positive charge (Figure 8). Although  $\omega$ -conotoxins also display an overall positive charge,  $\mu$ -conotoxins have a different fold and distribution of positive charges, with the arginine and lysine residues distributed in a “ring” protruding out from the core of the molecule. Since  $\mu$ -



**Figure 8.** Structures and electrostatic surfaces of  $\mu$ -conotoxins. Important residues identified through SAR studies are indicated on the left panels.  $\mu$ -SmIII,  $\mu$ -GIII,  $\mu$ -PIII, and  $\mu$ -TIII target  $\text{Na}_v1.4 > \text{Na}_v1.2$  mammalian voltage-gated calcium channels, whereas  $\mu$ -SIII and  $\mu$ -KIII have shorter sequences and display a reverse selectivity ( $\text{Na}_v1.2 > \text{Na}_v1.4$ ).

conotoxins are known pore blockers, such a charge orientation appears well suited to interact with the ring of negatively charged residues associated with the selectivity filter of VGSCs. Recently, the crystal structures of bacterial  $\text{Na}_v\text{Ab}$  and  $\text{Na}_v\text{Rh}$  confirmed that glutamate side chains do indeed line the pore vestibule, creating a strong long-range electrostatic attraction to positively charged molecules including  $\text{Na}^+$  ions and  $\mu$ -conotoxins.<sup>264,265</sup>

The venom of *C. geographus* provided the first  $\mu$ -conotoxins investigated, and  $\mu$ -GIII-C were found to potently target skeletal muscle  $\text{Na}_v1.4$ .<sup>266</sup> Other  $\mu$ -conotoxins have now been isolated from a range of piscivorous species, including *C. tulipa* ( $\mu$ -TIII),<sup>267</sup> *C. striatus* ( $\mu$ -SIII and  $\mu$ -SIIIB),<sup>268,269</sup> *C. kinoshitai* ( $\mu$ -KIII),<sup>268</sup> *C. purpurascens* ( $\mu$ -PIII),<sup>270</sup> and *C. stercusmuscarum* ( $\mu$ -SmIII).<sup>271</sup> The pharmacophore of these  $\mu$ -conotoxins has been mainly mapped to Arg13 and Arg14 in loop 2, where replacement of Arg13 in  $\mu$ -GIII dramatically reduced the affinity for  $\text{Na}_v1.4$ .<sup>272</sup> This particular residue has been demonstrated to interact directly with two glutamate residues in domain I of the channel.<sup>273</sup> Recent simulations of  $\mu$ -

P IIIA binding to the bacterial sodium channel  $\text{Na}_v\text{Ab}$  predict subnanomolar affinity, demonstrating that these bacterial channels will be useful models for studying  $\mu$ -conotoxin–VGSC interactions.<sup>274</sup> Unexpectedly, the simulations demonstrated that docked  $\mu$ -PIIIA could adopt different binding orientations or alternative binding modes in the channel. Although these docking poses need to be confirmed experimentally, this result suggests that the pseudosymmetry of the  $\mu$ -conotoxins might allow different electrostatic complexes to form. With the six basic residues potentially able to plug the pore, the overall net charge of  $\mu$ -conotoxins appears essential for high-affinity binding to VGSCs.

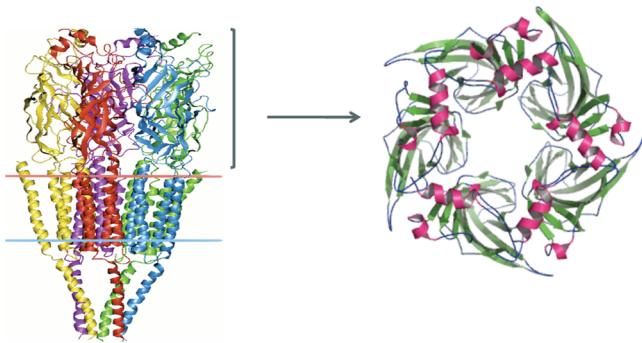
The natural primary structure variations that exist between  $\mu$ -S IIIA and  $\mu$ -S IIIB (Arg14 > Lys14 and Asp15 > Gly15, respectively) offer some insights into the possible engineering of neuronal selective  $\mu$ -conotoxins. Indeed,  $\mu$ -S IIIA preferentially inhibits neuronal sodium channel ( $\text{Na}_v1.2$ ) over the muscle sodium channel subtype ( $\text{Na}_v1.4$ ), whereas  $\mu$ -S IIIB shows the reverse selectivity.<sup>275</sup> However, this appears contradictory to the  $\mu$ -[E15A]-T IIIA analogue having enhanced affinity for the  $\text{Na}_v1.2$  subtype.<sup>267</sup> Interestingly, extensions to the N- and C-termini of  $\mu$ -S IIIA and  $\mu$ -S IIIB can increase selectivity for  $\text{Na}_v1.2$  over  $\text{Na}_v1.4$ , without significantly compromising rat neuronal affinity.<sup>257</sup> The smaller  $\mu$ -conotoxins  $\mu$ -K IIIA and  $\mu$ -S IIIA have a different pharmacophore centered around a helical motif in loops 2 and 3.<sup>275</sup> In contrast to most of the larger  $\mu$ -conotoxins, they show a preference for the  $\text{Na}_v1.2$  over  $\text{Na}_v1.4$  subtype. In particular, much attention has been directed to  $\mu$ -K IIIA since it was shown to be analgesic after systemic administration.<sup>276</sup> The most promising analogue,  $\mu$ -K IIIA-[R14A], preferentially blocks  $\text{Na}_v1.7$  over  $\text{Na}_v1.2$  and  $\text{Na}_v1.4$ .<sup>277</sup> Very recently, different disulfide-bond isomers of  $\mu$ -K IIIA were shown to potently block the  $\text{Na}_v1.4$  subtype, with surprisingly a non-native fold being more potent.<sup>278</sup> This result further supports the net charge of  $\mu$ -conotoxins being the main driver of its binding to VGSCs and offers insight into novel engineering opportunities. While VGSCs comprising only the  $\alpha$  subunit are fully functional, they are often associated with auxiliary subunits in vivo, which modulate the pharmacology of these channels. For instance, coexpression of the  $\text{Na}_v\beta$  subunit with the  $\alpha$  subunit markedly modifies the kinetics of interaction of  $\mu$ -conotoxins with VGSCs, complicating the interpretation of in vitro experimental results.<sup>279</sup>

### 6.5. $\alpha$ -Conotoxin SAR

Nicotinic acetylcholine receptors are divided into two distinct classes: the muscle nAChRs and the neuronal nAChRs.<sup>280</sup> The adult muscle nAChR is made of  $2\alpha$ ,  $\delta$ ,  $\gamma$ , and  $\beta$  subunits and is the major neurotransmitter receptor at the neuromuscular junction, representing a target of choice for many paralyzing toxins.<sup>281</sup> Not surprisingly given their key physiological roles, nAChRs are targeted by a number of venomous animals to facilitate prey capture and/or defensive strategies. Most *Conus* species venom investigated to date contain at least one  $\alpha$ -conotoxin that inhibits these ligand-gated ion channels.<sup>282</sup> In fact,  $\alpha$ -conotoxins represent the largest group of venom peptides isolated from cone snail venoms and appear to have evolved early in the radiation of this genus.<sup>79</sup> As a result of their high affinity and exquisite selectivity, many  $\alpha$ -conotoxins have significantly contributed to the pharmacological characterization of the various subtypes of nAChRs both in vitro and in vivo.<sup>283</sup> The conotoxin-nAChR complex is also one of the best

understood toxin–protein interactions, owing to decades of biochemical and structural studies.<sup>3</sup>

The overall structure (Figure 9) of each nAChR subunit comprises an extracellular N-terminus (the ligand-binding



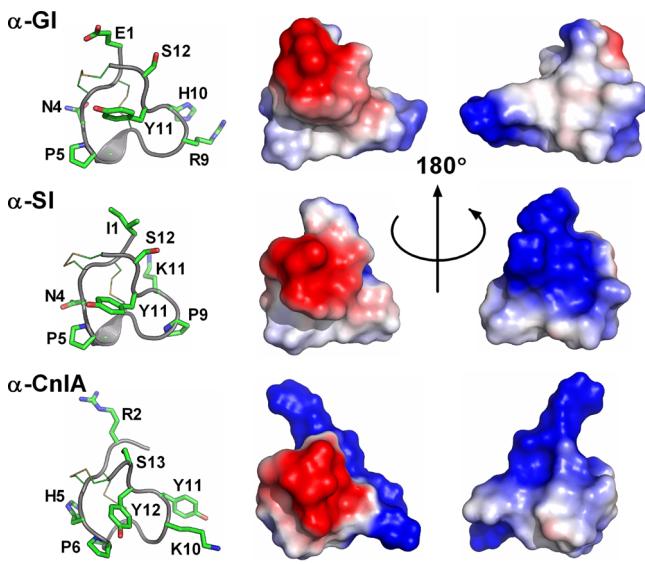
**Figure 9.** (Left) 4 Å resolution structure of torpedo acetylcholine receptor showing the extracellular, transmembrane, and intracellular domains.<sup>351</sup> The receptor is made of five subunits (each subunit is shown in a different color). (Right) 2.2 Å resolution crystal structure of AChBP, which is homologous to the extracellular (ligand binding) nAChR domain.<sup>331</sup> This protein is a pentamer with five identical subunits surrounding the channel pore.  $\beta$ -Sheets are shown in green and  $\alpha$ -helices in pink.

domain), four transmembrane domains, an intracellular loop, and an extracellular C-terminus.<sup>280</sup> A low-resolution (4 Å) structure of the muscle nAChR determined by cryo-electron microscopy<sup>284</sup> and crystallographic structures of bacterial homologues are now available to define the overall structure of these membrane proteins.<sup>285–287</sup> However, the crystal structure of the acetylcholine binding protein (AChBP), a soluble homologue of the ligand-binding domain of nAChRs,<sup>288</sup> provided the first detailed view of the ligand-binding domain. AChBP, first isolated from the mollusc *Lymnaea stagnalis*, is a soluble pentameric protein that binds to all prototypic nAChR ligands.<sup>289</sup> It has become the established model for the ligand-binding domain of ligand-gated ion channels. Since this initial discovery, AChBPs have also been characterized from other snails, including *Aplysia californica*, *Bulinus truncatus*, and recently the annelid worm *Capitella teleta*.<sup>290–292</sup> The structures of these AChBPs solved by crystallography are all superimposable, consistent with a strong conservation of structure and function, despite high primary sequence variation. The binding site for competitive agonists and antagonists is formed in a small pocket lined by aromatic residues at the interface of two  $\alpha$  subunits or at the interface of  $\alpha$  and  $\beta$  subunits.<sup>288</sup> AChBP has also been cocrystallized with several  $\alpha$ -conotoxins, and therefore, the corresponding binding site and molecular determinants for conotoxin-nAChR interactions are well understood.<sup>290,293,294</sup>

$\alpha$ -Conotoxins that bind to muscle nAChR are among the smallest venom peptides known, yet they exhibit high affinity and remarkable selectivity. The first characterized conotoxin was  $\alpha$ -GI isolated from *Conus geographus*, which acts as a potent antagonist of the muscle-type nAChR in both binding assays and animal isolated tissues.<sup>31</sup> An alanine walk along the sequence of  $\alpha$ -GI revealed a crucial role of Arg9 for both high potency and selectivity for the muscle-type nAChRs.<sup>295,296</sup> Remarkably, these  $\alpha$ -conotoxins can also distinguish between nonequivalent binding sites within the same receptor.<sup>297</sup> Indeed,  $\alpha$ -MI isolated from *Conus magus* preferentially binds

to the  $\alpha/\delta$  interface with  $a > 10\,000$ -fold higher affinity over the  $\alpha/\gamma$  interface in the mouse receptor, whereas it displays the opposite selectivity in nAChRs found in the electric organ of the *Torpedo* ray.<sup>298,299</sup> The binding affinity of some  $\alpha$ -conotoxins is even influenced by the fifth subunit, as observed for the binding of  $\alpha$ -AuIB to  $\alpha 3\beta 4\alpha 3\beta 4\alpha 3$  vs  $\alpha 3\beta 4\alpha 3\beta 4\beta 4$ .<sup>118</sup>

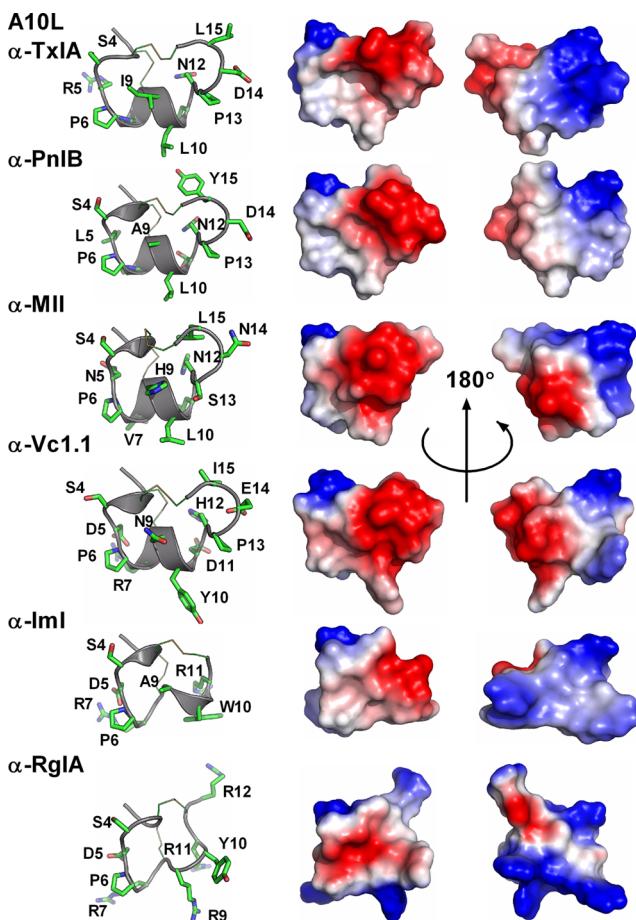
The three-dimensional structures of several muscle-selective  $\alpha$ -conotoxins have been determined by NMR and show a superimposable backbone conformation with the highly conserved Pro and Tyr residues providing key binding determinants (Figure 10). Pairwise interactions have been



**Figure 10.** Structures and electrostatic surfaces of muscle  $\alpha$ -conotoxins. Important residues identified through SAR studies are indicated on the left panels.  $\alpha$ -GI,  $\alpha$ -SI, and  $\alpha$ -CnIA target muscle nicotinic receptors.

identified between  $\alpha$ -conotoxins and the muscle nAChR, indicating that hydrophobic interactions stabilize the complex.<sup>301</sup> However, electrostatic interactions were also shown to play an important role in the binding of  $\alpha$ -conotoxins to muscle nAChR. For example, an arginine residue at position 9 was responsible for  $\alpha$ -GI selectivity at  $\alpha/\delta$  vs  $\alpha/\gamma$  interfaces, while  $\alpha$ -SI from *Conus striatus* has a proline residue at this position and did not discriminate between these two interfaces.<sup>296</sup> Furthermore, addition of a positively charged residue at the C-terminus increases affinity for the muscle nAChR. Mapping the electrostatic potentials to the surface of  $\alpha$ -GI,  $\alpha$ -SI, and  $\alpha$ -CnIA reveals a conserved hydrophobic side (seen as a white and red patch in Figure 10) and complementary side that is largely positively charged. This “two-faced”  $\alpha$ -conotoxin structure fits well with the binding site properties of the muscle nAChR (Figure 11). The principal side ( $\alpha 1$  subunit) of the receptor is mainly hydrophobic, whereas the complementary side is highly negatively charged. Therefore, in contrast to  $\omega$ - and  $\mu$ -conotoxins, which are pore blockers,  $\alpha$ -conotoxins instead fit tightly into their binding site nested between two nAChR subunits. This lock-and-key type of interaction<sup>302</sup> explains the high affinity observed for such small ligands with low contact surface area.

Although a muscle nAChR-selective antagonist is an obvious weapon to paralyze prey, the neuronal active  $\alpha$ -conotoxins have proved most interesting. Indeed, they can distinguish among



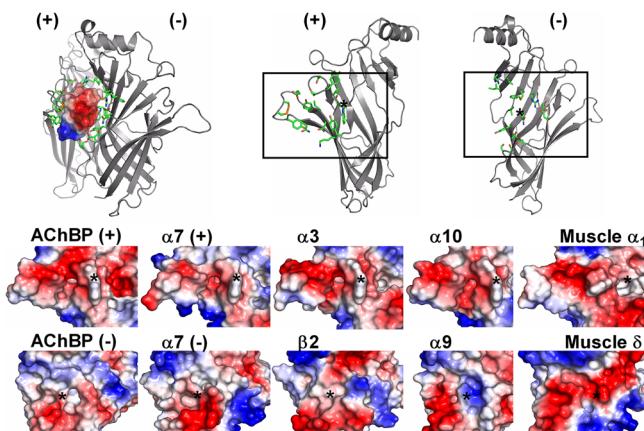
**Figure 11.** Structures and electrostatic surfaces of neuronal  $\alpha$ -conotoxins. Important residues identified through SAR studies are indicated on the left panels; 4/7 Conotoxins  $\alpha$ -A10L-TxIA,  $\alpha$ -PnIB,  $\alpha$ -MII, and  $\alpha$ -Vc1.1 target AChBP,  $\alpha$ 7,  $\alpha$ 3 $\beta$ 2, and  $\alpha$ 9 $\alpha$ 10 nAChRs, whereas 4/3 conotoxins  $\alpha$ -ImI and  $\alpha$ -RgIA target  $\alpha$ 3 $\beta$ 2 and  $\alpha$ 9 $\alpha$ 10 nAChRs, respectively.

different subunit arrangements and therefore represent valuable research tools to study the properties of nAChR subtypes and their distribution in native tissues and may have potential as leads to new therapeutics. From the 12 neuronal subunits known, only a few form functional homopentamers ( $\alpha$ 7 and  $\alpha$ 9), with most native nAChRs having a subunit stoichiometry that comprises combinations of different  $\alpha$ - and  $\beta$ -subunits, e.g.,  $\alpha$ 3 $\beta$ 2 or  $\alpha$ 6 $\alpha$ 4 $\beta$ 2 $\beta$ 3.<sup>283</sup> A number of nAChR subtypes have therapeutic potential. For instance, abnormal functions of  $\alpha$ 6 $\beta$ 2 $\beta$ 3 have been linked to neurodegenerative diseases,<sup>303</sup> and activation of  $\alpha$ 7 nAChR subtype has also been implicated in cancer.<sup>304</sup> The main nAChR subtype present in the brain is composed of  $\alpha$ 4 and  $\beta$ 2 subunits and is responsible for nicotine addiction.<sup>305</sup> Only  $\alpha$ -conotoxin  $\alpha$ -GID and  $\alpha$ -MII show submicromolar affinity for  $\alpha$ 4 $\beta$ 2 nAChR, though these peptides have nanomolar affinity for  $\alpha$ 3 $\beta$ 2 and  $\alpha$ 7 nAChRs.<sup>306,307</sup> Therefore, like several other human nAChRs, this subtype remains orphan of any selective  $\alpha$ -conotoxin. A recent study has shown that only two residues in the  $\alpha$ 4 subunit prevent binding of  $\alpha$ -conotoxins, and in principle, the  $\alpha$ 4 $\beta$ 2 binding pocket can accommodate such ligands, since the double-mutant [R185I/P195Q] $\alpha$ 4 $\beta$ 2 could allow high-affinity binding of  $\alpha$ -TxIA.<sup>308</sup> Interestingly, an alanine scan along the  $\alpha$ -GID sequence revealed that the majority of mutations have 10-fold or

complete loss of activity compared to the native peptide.<sup>309</sup>  $\alpha$ -GID has an unusual 4-residue N-terminal tail, which appears critical for activity at  $\alpha$ 4 $\beta$ 2 but not  $\alpha$ 3 $\beta$ 2 or  $\alpha$ 7. Therefore, engineering  $\alpha$ -conotoxins with N-terminal extensions might provide a new strategy to achieve  $\alpha$ 4 $\beta$ 2 selectivity.

Overall, it seems that many  $\alpha$ -conotoxins have naturally evolved to selectively target  $\alpha$ 7 and  $\alpha$ 3 $\beta$ 2 nAChRs, suggesting that these subtypes or close homologues of these have critical roles in their prey physiology.<sup>309,310</sup> More surprisingly,  $\alpha$ -conotoxins that are selective for the unusual  $\alpha$ 9 $\alpha$ 10 nAChR have also been discovered, revealing a potential role of this subtype in pain.<sup>311</sup> Of particular interest,  $\alpha$ -conotoxins  $\alpha$ -PnIB,  $\alpha$ -MII,  $\alpha$ -Vc1.1,  $\alpha$ -A10L-TxIA,  $\alpha$ -ImI, and  $\alpha$ -RgIA have been the subject of extensive structure–activity studies,<sup>94,293,308,312–319</sup> and their mode of interaction with nAChR provides the best experimental data for understanding  $\alpha$ -conotoxin subtype selectivity (Figure 11).  $\alpha$ -PnIB together with  $\alpha$ -PnIA have been isolated from the venom of *C. pennaceus*<sup>320</sup> and were shown to have an unusual sulfotyrosine at position 15.<sup>321</sup> Interestingly,  $\alpha$ -PnIA and  $\alpha$ -PnIB differ only at two positions (10 and 11), but their nAChR selectivity is shifted dramatically from  $\alpha$ 3 $\beta$ 2 to  $\alpha$ 7, respectively. A hydrophobic side chain (Leu) at position 10 was found critical for the  $\alpha$ 7 preference.<sup>315,316</sup> Analysis of molecular surface electrostatics of  $\alpha$ 3 $\beta$ 2 and  $\alpha$ 7 nAChR homology models reveals that this lengthy hydrophobic side chain can be accommodated in a unique hydrophobic cavity on the complementary (–) face of  $\alpha$ 7, which is smaller in  $\alpha$ 3 $\beta$ 2 mainly because of the presence of the bulky side chain of  $\beta$ 2-Phe117 (Figure 11).<sup>302</sup> An analogue of  $\alpha$ -PnIA cocrystallized with AChBP confirmed the predominant hydrophobic interaction between the two partners.<sup>290</sup> AChBP has also been used as a bait to screen cone snail venoms, and all *Conus* species investigated showed nicotinic activity.<sup>293</sup>  $\alpha$ -TxIA was pulled out of *C. textile* crude venom using this strategy, despite being expressed at very low levels.<sup>293</sup>  $\alpha$ -TxIA has very high affinity for AChBP (1 nM) and  $\alpha$ 3 $\beta$ 2 (2 nM) but is at least 200-fold less active at  $\alpha$ 7 (400 nM) nAChRs. An  $\alpha$ -A10L-TxIA analogue showed improved affinity at  $\alpha$ 7 (10-fold) while retaining the same binding properties at AChBP and  $\alpha$ 3 $\beta$ 2. This more potent analogue was cocrystallized with AChBP and showed an overall similar binding mode compared to the  $\alpha$ -PnIA variant except for a 20° rotation around Pro7. This tilt in the orientation of  $\alpha$ -A10L-TxIA was due to a specific and critical salt bridge between R5 and D195. Interestingly, this ionic bond was also important for the interaction with  $\alpha$ 7 but not  $\alpha$ 3 $\beta$ 2 nAChRs, providing some molecular basis for nAChR selectivity.

$\alpha$ -MII from *C. magus* has the highest reported affinity for the  $\alpha$ 6\*-containing nAChR subtypes (e.g.,  $\alpha$ 6 $\alpha$ 3 $\beta$ 2).<sup>306,317</sup> This peptide was used to show that  $\alpha$ 6\* nAChRs are down-regulated following long-term nicotine exposure in rats.<sup>322,323</sup> Radio-labeled and fluorescent  $\alpha$ -MII also helped to identify that  $\alpha$ 6\* nAChRs are involved in dopamine release in the striatum and down-regulation in brain regions is affected by Parkinson's disease.<sup>324</sup> Similarly to  $\alpha$ -A10L-TxIA and  $\alpha$ -PnIB,  $\alpha$ -MII has a hydrophobic patch on its surface (made of P6, V7, and L10) (Figure 12). However, the critical hydrophobic residue present at position 9 of  $\alpha$ 7-selective  $\alpha$ -conotoxins is replaced with a His residue in  $\alpha$ -MII, which likely explains its pH dependence and selectivity for  $\alpha$ 3- and/or  $\alpha$ 6-containing nAChRs.<sup>293</sup> Interestingly,  $\alpha$ -Vc1.1, an analgesic  $\alpha$ -conotoxin isolated from *C. victoriae*, uses Ser4 and Asn9 to bind with high affinity to  $\alpha$ 9 $\alpha$ 10.<sup>313</sup> This is consistent with the rather hydrophilic nature



**Figure 12.** Structures and electrostatic surfaces of nAChR subtypes. (Top left) “Lock-and-key” mechanism of interaction as seen in the  $\alpha$ -A10L-TxIA/AChBP complex. (Top right) Principal and complementary subunits between which conotoxins need to fit. (Bottom) Comparison of the molecular surfaces of the binding site in AChBP,  $\alpha$ 7,  $\alpha$ 3 $\beta$ 2,  $\alpha$ 9 $\alpha$ 10, and muscle nAChRs.

of the  $\alpha$ 9 $\alpha$ 10 nAChR binding site (Figure 11) and recent identification of Thr59 as a key determinant for the high potency of  $\alpha$ -Vc1.1 in rat.<sup>325</sup>  $\alpha$ -Vc1.1 has an order of magnitude lower affinity for the human receptor, which has a hydrophobic Ile at this position.<sup>325</sup> This structure–activity data is expected to help in the design of a ligand selective for the human  $\alpha$ 9 $\alpha$ 10 subtype, which is involved in immune responses and pain.<sup>311</sup> Design of an N–C cyclized form of  $\alpha$ -Vc1.1 that retained analgesic properties and was orally bioavailable showed that  $\alpha$ -conotoxins can provide a suitable template for novel therapeutic development.<sup>235</sup> However, since GABA<sub>B</sub> has also been proposed to be the pain target of  $\alpha$ -Vc1.1, the exact mechanism through which  $\alpha$ -Vc1.1 achieves analgesia is still a matter of conjecture.<sup>326</sup>

The smaller  $\alpha$ -conotoxins  $\alpha$ -ImI and  $\alpha$ -RgIA also target neuronal nAChRs.  $\alpha$ -ImI was isolated from the venom of the vermicivorous *C. imperialis* and initially found to be  $\alpha$ 7 selective.<sup>327</sup> However,  $\alpha$ -ImI was later shown to inhibit  $\alpha$ 3 $\beta$ 2 with a 10-fold higher potency compared to  $\alpha$ 7 nAChR.<sup>328</sup> To date, all SAR studies have been performed on the  $\alpha$ 7 subtype where residues Asp5, Pro6, and Arg 7 in the first loop and Trp10 in the second loop were shown to interact with the  $\alpha$ 7 nAChR.<sup>94</sup> Thermodynamic mutant cycle analysis was used to determine several pairwise interactions, including a major contribution between Arg7 and Tyr195.<sup>318</sup> On the basis of these distance constraints, docking solutions using nAChR homology models and an NMR structure of  $\alpha$ -ImI were obtained that satisfied most experimental data.<sup>329,330</sup> The precise interaction of  $\alpha$ -ImI within the ACh binding pocket was eventually solved with the crystal structure of the  $\alpha$ -ImI-AChBP complex.<sup>294,331</sup> The structure of  $\alpha$ -ImI bound to AChBP was found to align well with that of the  $\alpha$ -PnIA variant bound to AChBP, suggesting that this particular orientation allows favorable interactions between the ligand and the receptor. Recently, a molecular dynamics simulations study concluded that the affinity of  $\alpha$ -ImI for  $\alpha$ 7 was mostly driven by van der Waals and nonpolar desolvation energies as well as confirming that electrostatic interactions were critical for selectivity.<sup>332</sup>

$\alpha$ -RgIA was originally cloned from *C. regius*, another worm-hunting species, and shown to be the most selective  $\alpha$ 9 $\alpha$ 10 ligand to date.<sup>333</sup> The composition of the first loop of  $\alpha$ -RgIA is

identical to  $\alpha$ -ImI, but the second loop is highly divergent. As determined for  $\alpha$ -ImI, residues Asp5, Pro6, and Arg7 in the first loop are important for binding to both  $\alpha$ 9 $\alpha$ 10 and  $\alpha$ 7, while the analogue  $\alpha$ -Y10W-RgIA had no effect.<sup>312</sup> In contrast to  $\alpha$ -ImI, replacement of the residue in position 9 by an alanine in  $\alpha$ -RgIA demonstrated the critical role of Arg9 for specific binding to  $\alpha$ 9 $\alpha$ 10. Similar to  $\alpha$ -Vc1.1,  $\alpha$ -RgIA was also shown to be analgesic in a rat model of neuropathic pain,<sup>311</sup> and a N–C cyclic version of  $\alpha$ -RgIA retained analgesic activity and had increased stability in human plasma,<sup>334</sup> again highlighting the potential of  $\alpha$ -conotoxin templates for future drug development.

In conclusion, the interaction of  $\alpha$ -conotoxins with the various nAChR subtypes relies on a lock-and-key binding mechanism in contrast to the pore blockers  $\mu$ - and  $\omega$ -conotoxins. Hydrophobic contacts anchor  $\alpha$ -conotoxins deep into the ACh binding site, with complementary interactions (H bonds and salt bridges) tuning the selectivity. The accumulated wealth of structural and pharmacological information, together with future additional cocrystal structures, homology modeling, and docking simulations, are expected to provide exciting new opportunities for development of subtype selective nAChR inhibitors.

## 6.6. Conotoxin Mimetics

Differences in conotoxin loop sizes result in remarkable changes in target receptor selectivity and potency. Several loop size truncations are observed in nature among  $\alpha$ -conotoxins. This family has thus been further classified into subfamilies based on the number of residues in loops 1 and 2 (Table 3).  $\alpha$ -Conotoxins that belong to the 3/5 subfamily, isolated from fish-hunting cone snails, are found to exclusively block the muscle type nAChRs. This subfamily has a generic sequence—CCXPACG(K/R)XYSC.<sup>335</sup> The 4/3, 4/4, 4/6, and 4/7 subfamilies have 4 residues in loop 1 and 3–7 residues in loop 2. These peptides generally have conserved Ser and Pro residues in loop 1 as mentioned earlier. They predominantly antagonize various neuronal nAChRs subtypes and are found in fish-, worm-, and mollusc-hunting cone snails. The novel  $\alpha$ 4/5 subfamily conotoxin ca1.1 was discovered and successfully synthesized; it did not antagonize any of the  $\alpha$ 7,  $\alpha$ 3 $\beta$ 2,  $\alpha$ 3 $\beta$ 4,  $\alpha$ 4 $\beta$ 2,  $\alpha$ 9 $\alpha$ 10, and muscle nAChR subtypes tested, and its target receptor remains to be identified. As the peptide was also quite flexible in solution, a high-resolution NMR structure could not be obtained.<sup>336</sup>

Aside from natural loop size variations, truncation mutagenesis studies have been carried out on a number of conotoxins.<sup>100,154,338</sup> These have been undertaken to define the relationship between loop size and conotoxin structure and function. Among these, a novel “molecular pruning” approach was employed to evaluate affects of truncation on  $\alpha$ 4/7-conotoxin  $\alpha$ -[A10L]PnIA.<sup>337</sup> Loop 2 of this peptide was progressively truncated to give  $\alpha$ -4/4[A10L]PnIA and  $\alpha$ -4/3[A10L]PnIA analogs. Interestingly, the 4/4 truncation mutant retained activity and did not show any significant structural changes, whereas the 4/3 truncated loop analogue lost both activity and structure. This sequential shortening of loop 2 seems to have caused conformational instability as demonstrated by more facile disulfide-bond scrambling.<sup>337</sup>

Disulfide bonds play a very important role in conotoxin structure and function, and several disulfide-bond deletion studies have revealed their significance. Removal of individual disulfide bridges in  $\omega$ -MVIIA reduced binding affinity by 68–

5200-fold at the VGCCs, and CD analysis showed significant differences in secondary structure compared to the native fold.<sup>338</sup> Similar sequential replacement of cysteine pairs with serine residues in  $\omega$ -GVIA disrupted its structure and rendered the analogues inactive.<sup>155</sup> Hence, for the  $\omega$ -conotoxins studied, the approach of minimizing conotoxin structures has yet to lead to high-potency molecules. In  $\alpha$ -conotoxins, however, removal of one of the two disulfide bonds in  $\alpha$ -GI eliminated both structural integrity and bioactivity,<sup>156</sup> while the single disulfide-bond-deficient analogues of  $\alpha$ -ImI retained complete activity.<sup>197</sup> Thus, the results of disulfide-bond deletion seem to be very much dependent on the properties of the individual conotoxin.

As the number of disulfide bonds increases, folding of the synthetic peptide to the native conformation continues to be a significant challenge (see section 5). Hence, identification of the minimum structure required to maintain activity would be an immense advantage.<sup>339</sup> This strategy was employed on  $\mu$ -KIIIA, which induces potent analgesia in mice on systemic administration by acting on  $\text{Na}_v1.2$  and  $\text{Na}_v1.4$  voltage-gated sodium channels (VGSCs).<sup>276</sup> Disulfide-bond-deficient analogues with one pair of the native disulfide-bond connectivities between C1–C9, C2–C15, or C4–C16 removed one at a time were synthesized. The first disulfide bridge deletion analogues with respective Cys residues 1 and 9 replaced by alanine residues showed similar structure to native  $\mu$ -KIIIA without loss of biological activity. The on rate for these analogues increased at both  $\text{Na}_v1.2$  and  $\text{Na}_v1.4$  subtypes. The off rates, however, were significantly larger when compared to the native peptide.<sup>341</sup> The order of preference for the various VGSC subtypes remained unchanged. The second disulfide bridge deletion analogue showed a substantial decrease in activity, while the third disulfide bridge deletion mutant essentially lost all activity.<sup>341</sup> Structural analysis showed that removal of the first disulfide bond did not affect the integrity of the helical region. However, removal of the second or third disulfide bond disrupted the  $\alpha$ -helix, indicating that this motif is important for both conotoxin structure and function. The study also showed that these two disulfide bonds are essentially enough to maintain native characteristics. Thus, the active “core” structure of  $\mu$ -KIIIA is a loop 1 truncated analogue with only two disulfide bonds. While it maintains activity akin to the native peptide, it is also more readily synthesized.<sup>341</sup> Further, peptidomimetics were also employed in this study, where nonessential N-terminal serine residues were substituted with backbone spacers to give [desC1]KIIIA[S3/4Aopn, C9A] analogue (Aopn standing for 5-amino-3-oxo-pentanoic acid).<sup>340</sup> It is important to note that this peptidomimetic analogue of  $\mu$ -KIIIA could still block VGSCs and retained analgesic activity in the inflammatory pain mouse model. Thus, this approach successfully minimized the bioactive conformation of a three-disulfide-bridged  $\mu$ -conotoxin  $\mu$ -KIIIA.<sup>342</sup> On the basis of these observations, future SAR studies could be conducted by grafting the  $\mu$ -KIIIA pharmacophore onto helical peptidomimetic scaffolds such as peptoids,  $\beta$ -,  $\gamma$ -, or  $\beta/\gamma$ -peptides, lactam bridge helix stabilization, or other synthetic nonpeptide mimetics. Similar studies on adding backbone spacers to disulfide-deficient  $\omega$ -GVIA diminished its potency.<sup>343,344</sup> Thus, in general, conotoxin peptidomimetic studies have only had limited success in maintaining both potency and selectivity.

## 7. CONOTOXINS AS DRUG CANDIDATES

Although less than 0.1% of the venom repertoire has been pharmacologically characterized so far, a few *Conus* peptides have reached preclinical/clinical development.<sup>90,345</sup> Prialt, a synthetic version of the  $\omega$ -conotoxin  $\omega$ -MVIIA, was the first conotoxin to gain FDA approval in 2004 for treatment of chronic pain.<sup>6,346</sup> Its introduction onto the market not only demonstrated the therapeutic potential of conotoxins but also stimulated more interest from biotechnology companies into conotoxin research. Some of the conotoxins currently in clinical/preclinical trials include an analog of the  $\chi$ -conotoxin  $\chi$ -MrIA (Xen2174), which noncompetitively inhibits noradrenaline transporter and is undergoing phase II clinical trials as a treatment for neuropathic pain.<sup>347</sup> Other  $\omega$ -conotoxins in the pipeline included  $\omega$ -CVID (also called leconotide, AM336, and CNSB004) and  $\omega$ -GVIA (SNX-124), which successfully completed preclinical studies, yet due to the high cytotoxic effects of these peptides observed during phase I/IIa trials, they did not proceed further.<sup>6,86</sup>  $\alpha$ -Vc1.1 was also initially considered to be an attractive candidate for clinical trials due to its analgesic activity; however, a number of challenges were encountered that ultimately led to it being discontinued: low stability and an inability to clearly characterize the systemic processing of the peptide were some of them, but more importantly, changes in pharmacological characteristics, i.e., switch from competitive in rat to noncompetitive binding in human nAChR subtypes were a major concern.<sup>349</sup> Thus, species diversity among different nAChR subtypes can itself present challenges in the pursuit of conotoxin drug therapeutic development.

Despite promising advances of conotoxins through various stages of drug development, the inability of these peptides to cross the blood–brain barrier necessitates intrathecal administration.<sup>90,349</sup> This remains a major limiting factor to be addressed from a clinical perspective for widespread application of conotoxins as therapeutics. Improving in vivo stability and absorption will also greatly enhance their clinical success, and chemical modifications such as disulfide-bond re-engineering and cyclization as described herein offer promise that this will be achieved.

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### Notes

The authors declare no competing financial interest.

## Biographies



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Sébastien Dutertre was born in France, where he obtained his M.Sc. degree from the University of Paris XII, School of Industrial Chemistry of Paris, and National Museum of Nature History of Paris in 2001. He received his Ph.D. degree in Molecular Bioscience in 2006 from The University of Queensland, Australia. He was then awarded an EMBO postdoctoral fellowship to join the laboratory of Professor Heinrich Betz at the Max-Planck-Institute for Brain Research, Frankfurt, Germany. Next, he joined Atheris laboratories (Switzerland) in 2008 to work on an innovative and ambitious postgenomic project dedicated to the discovery and development of novel biopharmaceuticals generated by the broad biodiversity of animal venoms. In 2010, he was awarded a University of Queensland postdoctoral fellowship to join the laboratory of Professor Richard J. Lewis and develop an integrated approach to accelerate the discovery of novel peptides from cone snail venoms using second-generation sequencing technologies and proteomic methods. His research interests encompass the discovery of venom peptides, their precise mode of interaction with targeted receptors, and their associated therapeutic potential.



Markus Muttenthaler was born in Austria, where he obtained his B.Sc. and M.Sc. degrees in Applied Synthetic Chemistry from the University of Technology, Vienna. From 2002 to 2003 he spent 1 year at the City College in New York to specialize in Organic Synthesis. He received his Ph.D. degree in Biological and Medicinal Chemistry in 2009 from the University of Queensland, Brisbane, Australia, where he also conducted two-year postdoctoral work in the laboratory of Professor Alewood at the Institute for Molecular Bioscience. He was then awarded a Marie Curie Fellowship, which allowed him to join the laboratory of Associate Professor Philip Dawson at the Scripps Research Institute in La Jolla, CA, and the laboratory of Professor Fernando Albericio at the Institute for Research in Biomedicine Barcelona. His research areas cover peptide chemistry, natural product drug discovery (particularly venom peptides), design and development of molecular probes to dissect neuropathic pain, therapeutic peptide dendrimers, and oxytocin/vasopressin research.



Quentin Kaas was born in France, where he obtained his Chemical Engineering degree in 2001 from the Ecole Normale Supérieure de Chimie de Montpellier (ENSCM) and his Ph.D. degree in Bioinformatics in 2005 from the University of Montpellier II. He conducted 1 year of postdoctoral research in the laboratory of Immunoinformatics of Professor Marie-Paule Lefranc in Montpellier, studying antigen/receptor interactions. He was then awarded an Australian Postdoctoral fellowship by the Australian Research Council to undertake 3 years of postdoctoral research on plant cyclic peptide structure–activity relationships in the laboratory of Professor David Craik at the Institute for Molecular Bioscience, The University of Queensland, Australia. He is currently working in the laboratory of Professor David Craik, and the focuses of his research are structural bioinformatics and computational modeling studies of toxins extracted from plants and animals. He has developed and currently maintains

the only database specialized on sequences and structures of cone snail toxins, conoserver (<http://www.conoserver.org>).



David J. Craik is a NHMRC Senior Principal Research Fellow at the Institute for Molecular Bioscience, The University of Queensland. He obtained his Ph.D. degree in Organic Chemistry from La Trobe University in Melbourne, Australia (1981), and undertook postdoctoral studies at Florida State and Syracuse Universities before taking up a lectureship at the Victorian College of Pharmacy in 1983. He was appointed Professor of Medicinal Chemistry and Head of School in 1988. He moved to University of Queensland in 1995 to set up a new biomolecular NMR laboratory. His research focuses on application of NMR in drug design and on toxins, including conotoxins. His group has a particular focus on structural studies of disulfide-rich proteins and on the discovery and applications of circular proteins and novel protein topologies. He has trained more than 60 Ph.D. students and is the author of 500 scientific publications.



Richard J. Lewis was born in Australia, where he obtained his B.Sc. degree from James Cook University of North Queensland in 1977. He received his Ph.D. degree in Zoology in 1985 from The University of Queensland, before taking a Fisheries Biologist position with the Queensland Department of Primary Industries to continue his Ph.D. research on the origin, detection, structure, and pharmacology of ciguatoxins. In 1995 he moved back to The University of Queensland to research venom peptides, especially conotoxins with therapeutic potential. He cofounded Xenome Ltd. in 2000 and CSO from 2005 to 2009, helping take Xen2174 into the clinic. In 2009 he was promoted to Professor at the University of Queensland to continue researching venom peptides and ciguatoxins.



Paul F. Alewood was born in Sydney Australia, where he received his B.Sc. (Hons) degree from the University of New South Wales in 1969. He received his Ph.D. degree at the University of Calgary, Canada, in 1974 before studying in Switzerland and London. He moved to the University of Melbourne in 1977 before joining the Victorian College of Pharmacy in 1983 as a lecturer where he began a life-long interest in peptide drug development. In 1998 he was a foundation member of Bond University, Australia, before moving to the University of Queensland in 1991 as Deputy Director and Professorial Fellow of the Centre for Drug Design and Development. He was a foundation member of the Institute of Molecular Bioscience at the University of Queensland in 2000, where his group is focused on peptide drug development from Australia's venomous creatures.

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