Disulfide Bond Assignment of ω-Agatoxins IVB and IVC: Discovery of a D-Serine Residue in ω-Agatoxin IVB

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Abstract: A TCEP (tris(2-carboxyethyl)phosphine)-based reduction/cysteine amidoalkylation strategy was utilized to solve the disulfide structures of ω-agatoxins IVB (**1**) and IVC (**2**). These p-type calcium channel antagonists, isolated from the American funnel-web spider *Agelenopsis aperta*, were found to have the same amino acid sequence and disulfide bond motif. The difference between ω-Aga IVB (**1**) and IVC (**2**) resides in the C-termini (Ser_{46}) of both peptides. ω-Aga IVB (**1**) contains a D-serine residue while ω-Aga IVC (**2**) has an L-serine in this position. The existence of D-amino acids in eucaryotic systems is extremely rare. To our knowledge, however, this is the first time that a peptide sequence with an established cystine pattern possesses an amino acid in both D and L configurations.

Cysteine-rich proteins comprise a wide array of biologically important molecules including enzymes, protease inhibitors, plasma proteins, neurotoxins, and hormones.[1] The disulfide bridges of these molecules play a key role in establishing and maintaining their three-dimensional structures.[2] No other molecular interaction imposes such stringent structural requirements on a protein as the disulfide bond (S−S bond length of 2.05 ± 0.03 Å; tortional angle about the S−S bond of ~90°; angle between the disulfide bond and the β-carbon of each Cys residue of ~103°).[3] Disulfide bonds direct the folding of individual peptide chains, while contributing to the conformational and biochemical stability of the protein itself.[1,2]

Many naturally occurring toxins contain a high-density core of cysteine residues. These toxins have greatly enhanced our understanding of the pharmacology of ion channels, which are present in every cell plasma membrane and play a pivotal role in the control of a variety of physiological processes. The specificity of dendrotoxin (*Dendroaspis angusticeps* (mamba snake)),[4] apamin (*Apis mellifera* (honey bee)),[5] and charybdotoxin (*Leiurus quinquestriatus var. hebraeus* (scorpion))[6] for K^+ channel subtypes, α-scorpion toxins (Buthinae subfamily (scorpion))[7] for Na^+ channels, and ω-conotoxin GVIA (*conus geographus* (marine snail))[8] and ω-agatoxin IVA (*Agelenopsis aperta* (funnel-web spider))[9-11] for n- and p-type Ca^{2+} channels, respectively, has been largely responsible for the characterization of these channels. We and others are interested in voltage-sensitive Ca^{2+} channels (VSCC) and the structure/function of ω-agatoxins, which have been isolated from the American funnel-web spider *Agelenopsis aperta*. Our attention has focused on the p-type calcium channel antagonist ω-Aga IVA[9-11] in addition to two biologically similar peptides with diverging potency that we have designated ω-Aga IVB (**1**)[12-15] and IVC (**2**).[15] ω-Aga IVB (**1**) and IVC (**2**) have the same amino acid sequence but are distinguished chromatographically by RP-HPLC, suggesting the possibility of dissimilar disulfide bond patterns.[15a]

Recent 2D NMR experiments[12,13] unambiguously identified the Cys_4−Cys_{20} and the Cys_{27}−Cys_{34} disulfide bonds of ω-Aga IVB (**1**) and suggested the likelihood of Cys_{12}−Cys_{25} and Cys_{19}−Cys_{36} disulfide bonds. Given that the last two disulfide bond assignments were not definitive and sufficient quantities of ω-Aga IVC (**2**) were not available for a similar NMR analysis, we began to pursue alternative methods (requiring <500 μg of native protein) to establish rigorously the disulfide

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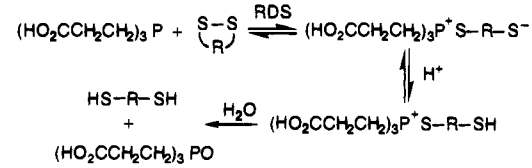
motifs and structures of ω-Aga IVB (**1**) and closely related ω-Aga IVC (**2**).

The unambiguous assignment of the ω-agatoxin disulfide motif(s) is critical for understanding the molecular basis of biological activity and for designing small molecule surrogates. Unfortunately, no general method exists for elucidating disulfide connectivity. Sequential enzymatic and chemical peptide bond cleavages with subsequent analysis of disulfide bond-containing fragments have been employed most often for localization of cystine disulfide bridges in peptides.[16] Success in utilizing this methodology alone, however, is problematic (particularly with highly folded cystine-rich peptides) due to the difficulties encountered in accessing and selectively cleaving individual disulfide-linked portions of the protein.[17] The presence of adjacent cysteines poses a greater challenge, as disulfide mapping, in this case, entails cleaving and subsequently differentiating between both residues.

A number of techniques can be employed in concert with enzymatic digestions and chemical hydrolyses to assist in disulfide bond assignment. Edman degradations[18] have enabled the cleavage of N-terminal peptide bonds; by omitting reductants prior to gas phase sequencing, cystine residues can be identified in the form of phenylthiohydantoin (PTH) derivatives of Cys_2.[18a,b] Mass spectrometry[19] (tandem, FAB, electrospray) has facilitated the characterization of complex enzymatic and chemical digests of native proteins. 2D NMR[12,13,20] and protein crystal structures,[21] as well as solid phase peptide synthesis[22] using orthogonal cysteine protection, have been employed to resolve, confirm, and predict structural assignments. The propensity of disulfides to exchange (scramble)[23,24] in the course of these investigations has contributed to the challenge protein chemists face in unambiguously assigning disulfide bridges.

Scheme 1



In principle, disulfide connectivity could be conveniently assigned if a method were available for sequentially cleaving disulfide bonds and tagging (functionalizing) the disconnected sulfur atoms under conditions that preclude disulfide exchange. In practice, such methodology has inherent liabilities. Selective cleavage (i.e., reduction) of individual disulfide bonds alters protein secondary and tertiary structure and affects the susceptibility of the remaining disulfide bonds to reduction. As a result, the generation of complex mixtures of reduction intermediates (in addition to fully reduced and native protein itself) is apt to occur. Nevertheless, provided that the structural integrity of the remaining intact disulfide bonds was maintained throughout such a procedure, disulfide bond assignment should be feasible.

In our view, trivalent phosphine-mediated disulfide reductions offered the most attractive avenue for exploring sequential and selective disulfide cleavage. Because these reductions are irreversible, theoretically stoichiometric amounts of phosphines can be employed to generate free thiols and substituted phosphine oxides (Scheme 1). The fact that reductions can be carried out at an acidic pH is especially important, given the propensity of disulfide exchange to occur under alkaline conditions. Depending on substitution, most trialkylphosphonium salts have pK_a's in the range of ~3−9.[25] Consequently, phosphine substitution and reaction pH offer two options for attenuating the rate of disulfide reduction and provide a broader window of opportunity for accessing reduction intermediates in these reactions.[26]

A number of trivalent phosphines have been examined for the reduction of disulfide bonds.[24,26,27] Tris(2-carboxyethyl)-phosphine (TCEP)[26] is particularly useful for disulfide reductions because of its water solubility. A TCEP-mediated partial reduction of γ-globulin was reported years ago.[28] Recently, the use of TCEP (estimated pK_a of TCEP HCl of ~7.66)[29] to cleave small organic disulfides under acidic conditions (pH 3−5) was carefully examined by Whitesides et al.[26] Reductions are rapid and complete at pH 4.5 and are kinetically (not thermodynamically) controlled.

A TCEP-based reduction/cysteine alkylation approach for disulfide mapping was reported recently by Gray et al.[24] for a number of disulfide bridged peptides (e.g., conotoxin GI, endothelin, apamin). Reduction under acidic conditions, followed by alkylation of the resultant products, provided a practical methodology for the disulfide mapping of these

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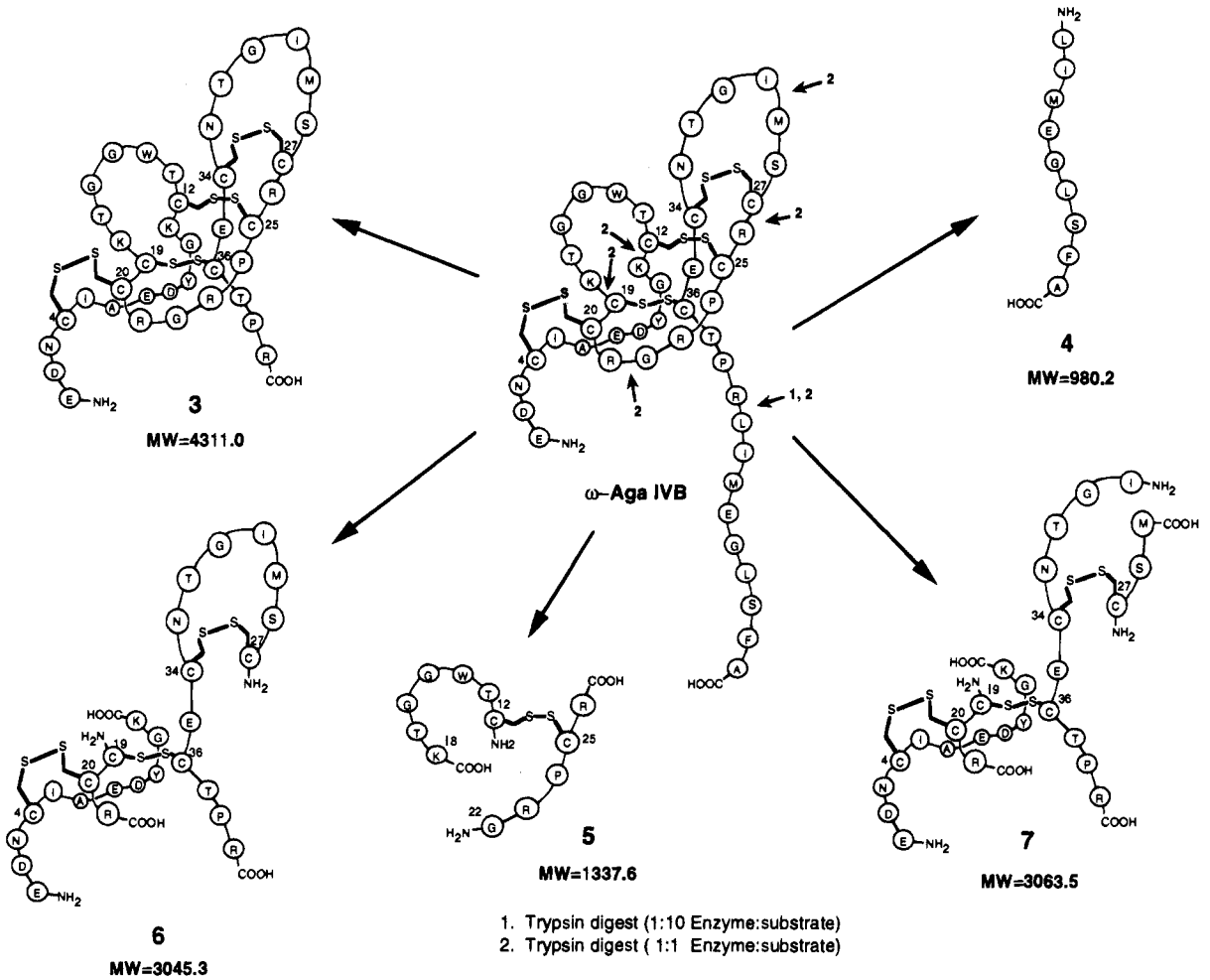

Figure 1. Trypsin digests of ω-Aga IVB (**1**)/IVC (**2**).

peptides. Unfortunately, thiol−disulfide exchange was observed during some of the thiolate alkylations, underscoring the propensity for exchange to occur at alkaline pH.

We also chose to explore a water soluble phosphine[24,26] reduction/thiol alkylation strategy for accessing the disulfide bond connectivities of ω-Aga IVB (**1**) and IVC (**2**). In our approach, acidic conditions would be used both in the reduction and derivatization steps to ensure that thiolate intermediates (which participate in disulfide scrambling) would not be generated. We felt that some configuration of this phosphine reduction/cysteine alkylation strategy[24] would not only be amenable to ω-Aga IVB (**1**) and ω-Aga IVC (**2**) but could be practical and broadly applicable with other disulfide substrates as well.

TCEP treatment of ω-Aga IVB (**1**) under a variety of acidic conditions provided either fully reduced peptide or mixtures of fully reduced and native peptide, demonstrating that, after the initial disulfide cleavage, the three remaining cystine bonds are easily reduced. While the unique molecular framework of ω-Aga IVB (**1**) conspired to undermine this disulfide characterization study, many options for determining disulfide connectivity remained. TCEP disulfide reductions are subject to kinetic control, with preferential reduction of more strained disulfides.[26] As a result, minor structural modifications (e.g., from protolytic digests of ω-Aga IVB (**1**)) should dramatically alter protein tertiary structure and the susceptibility of each of the molecule's disulfide bonds to reduction *even though the amino acid sequence proximal to the disulfide bonds of ω-Aga IVB (1) and a structurally modified ω-Aga IVB would likely be the same.* Consequently, selective peptide bond cleavage within the disulfide core of ω-Aga IVB (**1**) would be explored with the intent of revisiting the reduction/alkylation approach.

Trypsin was a logical choice for initial proteolysis experiments because of the presence of four arginine and two lysine residues in native peptide. Approximately 10−20 μg of ω-Aga IVB (**1**) was used in these enzymatic studies. Reaction products were separated by RP-HPLC and analyzed by electrospray mass spectrometry. A (modified) trypsin digest of ω-Aga IVB (1) (1:10 enzyme:substrate/37 °C/16 h) at a pH of 6.8 (0.05 M NH_4-OAc) resulted in cleavage at Arg_{39} to generate the cystine-bridged portion (**3**) (MW = 4311.0) of native peptide, in addition to the C-terminal fragment H_2N-LIMEGLSFA-OH (**4**) (MW = 980.2) (Figure 1). The inability to cleave within the peptide's highly folded cystine-rich core was not surprising, considering the steric congestion in this region of the molecule.[30]

A (modified) trypsin digest of ω-Aga IVB (**1**) under more forcing conditions (1:1 enzyme:substrate/37 °C/16 h) at a pH of 6.8 (0.05 M NH_4OAc) again provided the "tail" fragment **4** as well as three new fragments **5**−**7** (Figure 1), which were isolated by RP-HPLC and characterized by electrospray mass spectrometry. The pH was crucial to the integrity of this trypsin experiment, as additional fragments indicative of disulfide scrambling were obtained when the digest was carried out under more conventional conditions at a pH of 8.5.

In the pH 6.8 trypsin digest, proteolysis within the disulfide core was observed. A major fragment (MW = 1337.6) from this digest was the disulfide structure

(30) The difficulty in accessing with trypsin the cysteine-rich portion of ω-Aga IVB was also noted by Adams *et al.* (see ref 13).

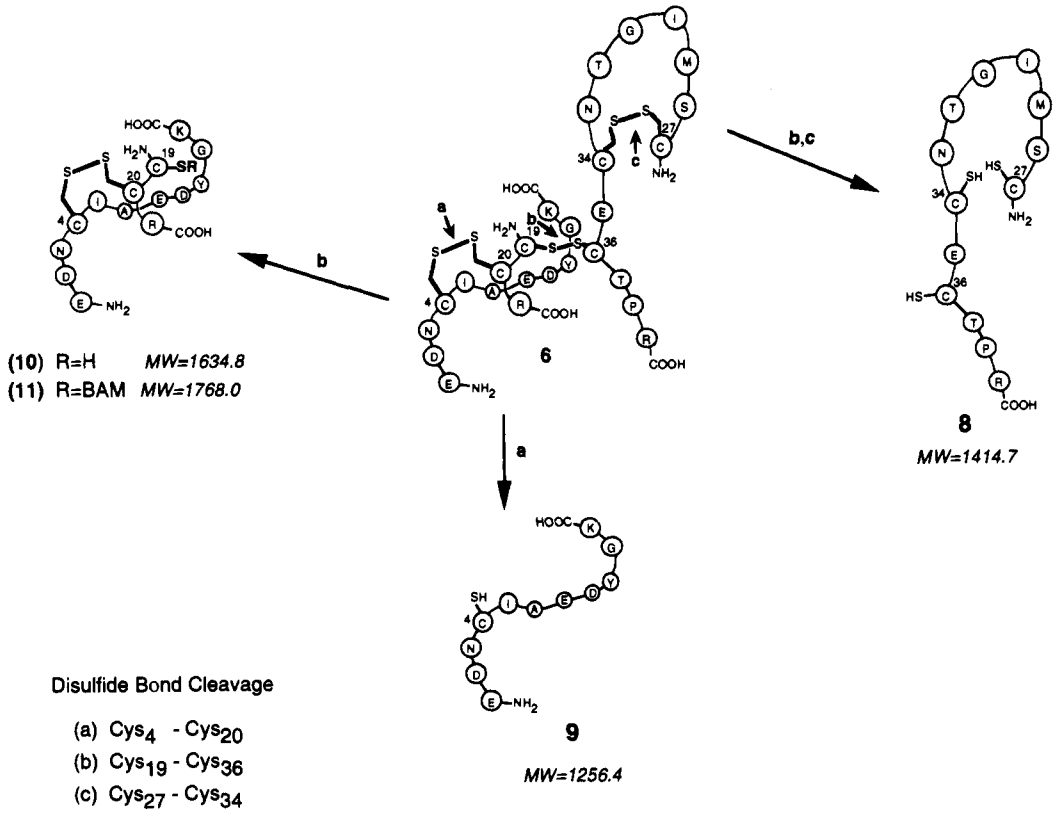

Figure 2. Phosphine reduction of **6**.

$$H_2N\text{-}GRPC_{25}R\text{-}OH\ H_2N\text{-}C_{12}TWGGTK\text{-}OH$$
5

(resulting from C-terminal cleavage at Lys_{11}, Lys_{18}, Arg_{21}, and Arg_{26}). Its structural assignment establishes the Cys_{12}–Cys_{25} disulfide bond connectivity. The two remaining fragments **6** (MW = 3045.3) and **7** (MW = 3063.5) were also isolated by RP-HPLC and contain the molecule's six remaining cysteine residues. Besides cleavage at Lys_{11}, Lys_{18}, Arg_{21}, Arg_{26}, and Arg_{39}, in one of the products (peptide **7**), cleavage at the C-terminus of Met_{29} was observed.

Trypsin-derived products **6** and **7** contain the three remaining disulfide bonds of Aga IVB (**1**) and provided another opportunity to explore the phosphine-mediated disulfide reduction methodology. In the case of **6**, TCEP (10 mM) treatment (Figure 2) at a pH of 3 (0.5 M citric acid) generated, after 10 min, fully reduced trithiol **8** (MW = 1414.7) containing the Cys_{27}, Cys_{34}, and Cys_{36} residues of ω-Aga IVB along with thiol **9** (MW = 1256.4) containing the peptide's Cys_4 moiety. In addition, a key thiol intermediate **10** (MW = 1634.8) was generated which contained the Cys_4, Cys_{19}, and Cys_{20} residues of the native peptide. Mass spectral data indicated that two of the cysteines in **10** exist as a cystine bridge.

Determination of disulfide connectivity in peptide regions having adjacent cysteine residues is rarely straightforward since the two cysteine residues need to be differentiated in some way and the Cys–Cys peptide bond eventually must be cleaved. Independent solid phase synthesis is commonly employed to overcome this problem.[22a-d] Along with a disulfide bond, **10** contains a free thiol moiety and, as a result, is an ideal substrate for establishing the disulfide connectivity in the Cys_{19}–Cys_{20} region of ω-Aga IVB. Provided that we could alkylate the free thiol substituent of **10** cleanly (without any indication of disulfide scrambling), Edman degradation of the resultant product should indicate which of the adjacent cysteines is disulfide bonded to Cys_4 and which is bonded to another Cys residue in the native peptide.

Protein chemists traditionally rely on pyridylethylation (4-vinylpyridine) or iodoacetic acid alkylation reactions (which utilize conditions that promote disulfide exchange) to derivatize cysteine residues.[31] Our concern over the possibility of disulfide scrambling prompted us to explore acid-catalyzed cysteine alkylations. Of the available options, thiol amidoalkylation chemistry[32] appeared most promising, given that these reactions are rapid, can be carried out at low pH, are high yielding, and have the possibility of providing a UV chromophore to aid in locating functionalized cysteine fragments by HPLC. The benzamidomethyl (BAM)[33] moiety seemed well suited for our purposes and is typically incorporated by (hydroxymethyl)-benzamide treatment of thiols under acidic conditions. By employing various substituted (hydroxymethyl)benzamides for cysteine amidoalkylations, an opportunity theoretically exists for differentiating cysteine pairs in proteins by using the same chemistry to incorporate different BAM "labels" (which can be distinguished by mass or as a PTH derivative generated in Edman sequencing).

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Treatment of thiol **10** with *N*-(hydroxymethyl)benzamide for 45 min in TFA[34] at 23 °C generated a major product **11** (Figure 2), which was purified by RP-HPLC. Mass spectrometric analysis of **11** (MW = 1768.0) indicated that a BAM group was incorporated into this peptide fragment. Longer reaction times resulted in minor incorporation of additional BAM substituents, suggesting that tyrosine amidoalkylation was occurring. To structurally characterize **11**, its remaining disulfide bond was reduced and the free thiols were alkylated with iodoacetic acid to afford two fragments H_2N-C_{19}(BAM)C_{20}-(CM)RCO_2H (**12**) and H_2N-EDNC_4(CM)IAEDYGK-OH (**13**). The presence of the BAM moiety on Cys_{19} and the carboxymethyl (CM) moieties on Cys_4 and Cys_{20} was identified by standard Edman sequencing (Table 1), confirming the Cys_4–Cys_{20} disulfide bond assignment of ω-Aga IVB (**1**).

The published solution NMR of ω-Aga IVB (**1**) left little doubt about the molecule's Cys_{27}–Cys_{34} bond connectivity. Confirmation of this assignment could best be achieved enzymatically by a Glu-C digest of **6** (Figure 3) which (by cleaving at Glu_{35}) would liberate a peptide loop containing the Cys_{27}–Cys_{34} disulfide bridge. Using standard enzymatic conditions (1:10 enzyme:substrate/pH 4.0/37 °C/16 h), no cleavage at Glu_{35} was observed. However, under more forcing conditions (1:1 enzyme:substrate/pH 4.0/37 °C/16 h), a Glu-C digest of **6** provided four major products **14**–**17** (Figure 3). Characterization by mass spectrometry and Edman sequencing (Table 1) identified the anticipated H_2N-C_{27}SMIGTNC_{34}E-OH (**14**) (MW = 955.1), thereby confirming the Cys_{27}–Cys_{34} disulfide bond.

Although the Cys_{19}–Cys_{36} bond connectivity was established by a process of elimination, we wished to confirm this assignment experimentally and, at the same time, probe the generality of the reduction/amidoalkylation methodology by subjecting peptide fragment **15** (obtained in a Glu-C digest of ω-Aga IVC) to tris(2-carboxyethyl)phosphine. Reduction of **15** (Figure 4) with TCEP generated, in addition to fully reduced peptide fragment **19**, a mixture of fully reduced peptide fragment **18** and partially reduced **20** containing both a disulfide and free thiol functionality. Amidoalkylation of the mixture of **18** and **20** provided BAM-containing fragments **21** and **22**, which were separated by RP-HPLC. A standard reduction and alkylation of the remaining disulfide bond of **22** with iodoacetic acid generated two fragments H_2N–C_{19}(BAM)C_{20}(CM)R–OH (**23**) and H_2N–EDNC_4(CM)IAE–OH (**24**), which were identified by standard Edman sequencing, reaffirming the Cys_{19}–Cys_{36} and Cys_4–Cys_{20} bond connectivities.

Having established the disulfide motif of ω-Aga IVB (**1**), the same strategy was employed to map the disulfide bonds of ω-Aga IVC (**2**). We anticipated the difference between ω-Aga IVB (**1**) and IVC (**2**) to reside in disulfide structure and specifically focused on Cys_{19} and Cys_{20} disulfide connectivity. To our surprise, results (Figure 1–4) identical to those of ω-Aga IVB (**1**) were obtained with ω-Aga IVC (**2**), demonstrating that the two peptides have *the same disulfide bond motif.*

We entertained the possibility that ω-Aga IVB (**1**) and IVC (**2**) exist as a pair of topological isomers[35] (with different disulfide cores) generated in protein folding. To verify that ω-Aga IVB (**1**) and IVC (**2**) do, in fact, have structurally-distinct disulfide cores, fragments **3** and **4** from the "standard" trypsin digest of both peptides (Figure 1) were re-examined by RP-HPLC. Instead of observing two distinct Glu_1–Arg_{39} peptide core fragments as anticipated, coinjection studies demonstrated that the Leu_{40}–Ala_{48} "tail" fragments (**4**) of ω-Aga IVB (**1**) and IVC (**2**) were not identical! To pinpoint the difference between these fragments, a standard Glu-C digest of ω-Aga IVB (**1**) and IVC (**2**) (10:1 substrate:enzyme) was performed (Figure

Table 1. Sequence Analysis[a]

Fragment 5 (from ω-Aga IVC)

	Sequence A			Sequence B		
Cycle #	AA	yield	Δ	AA	yield	Δ
1	Cys	ND	ND	Gly	1927	1927
2	Thr	796	787	Arg	2694	2520
3	Trp	1129	1120	Pro	1919	1878
4	Gly	1552	1509	Cys	ND	ND
5	Gly	1603	51	Arg	1499	1209
6	Thr	509	496			
7	Lys	573	563			

Fragment 11 (from ω-Aga IVB)

	Sequence A (13)			Sequence B (12)		
Cycle #	AA	yield	Δ	AA	yield	Δ
1	Glu	360	360	BAM-Cys	NI	NI
2	Asp	292	291	CM-Cys	NI	NI
3	Asn	203	199	Arg	79	75
4	CM-Cys	NI	NI			
5	Ile	114	112			
6	Ala	188	185			
7	Glu	159	156			
8	Asp	175	172			
9	Tyr	225	221			
10	Gly	136	129			
11	Lys	48	48			

Fragment 11 (from ω-Aga IVC)

	Sequence A (13)			Sequence B (12)		
Cycle #	AA	yield	Δ	AA	yield	Δ
1	Glu	87	87	BAM-Cys	NI	NI
2	Asp	56	51	CM-Cys	NI	NI
3	Asn	33	31	Arg	56	53
4	CM-Cys	NI	NI			
5	Ile	19	18			
6	Ala	31	29			
7	Glu	30	28			
8	Asp	32	28			
9	Tyr	25	23			
10	Gly	23	19			
11	Lys	4	4			

Fragment 14 (from ω-Aga IVC)

Cycle #	AA	yield	Δ
1	Cys	ND	ND
2	Ser	168	152
3	Met	925	888
4	Ile	647	641
5	Gly	818	811
6	Thr	281	281
7	Asn	722	719
8	Cys	ND	ND
9	Glu	551	547

Fragment 22 (from ω-Aga IVC)

	Sequence A (24)			Sequence B (23)		
Cycle #	AA	yield	Δ	AA	yield	Δ
1	Glu	34	34	BAM-Cys	NI	NI
2	Asp	19	17	CM-Cys	NI	NI
3	Asn	22	22	Arg	144	140
4	CM-Cys	NI	NI			
5	Ile	18	12			
6	Ala	23	20			
7	Glu	17	15			

[a] For each cycle of sequence analysis, the cycle number, yield (pmol), and Δ, the change in PTH recovery (pmol), are given: ND, result not determined; NI, amino acid assigned peak not integrated.

(34) While we were aware of the potential for acid-catalyzed disulfide scrambling (see ref 23a and Benesch, R. E.; Benesch, R. *J. Am. Chem. Soc.* **1958**, *80*, 1666–1669), no products were isolated in the BAM alkylations which were indicative of cystine isomerization. ω-Agatoxins IVB and IVC were both stable to TFA.

(35) Benham, C. J.; Jafri, M. S. *Protein Sci.* **1993**, *2*, 41–54.

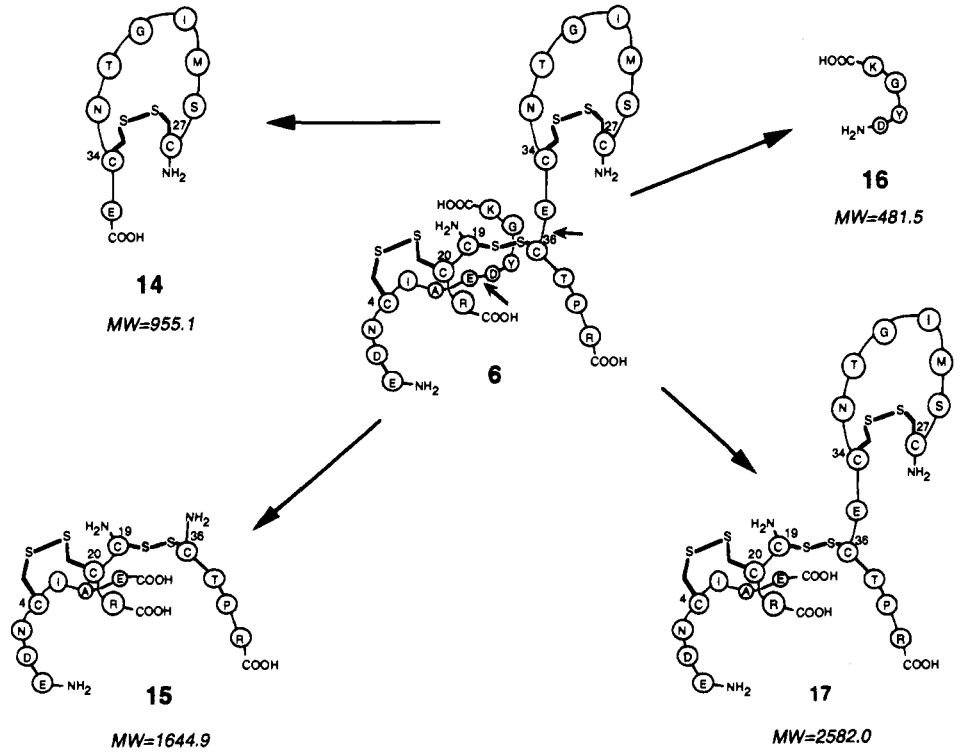


Figure 3. Glu-C digest of **6**.

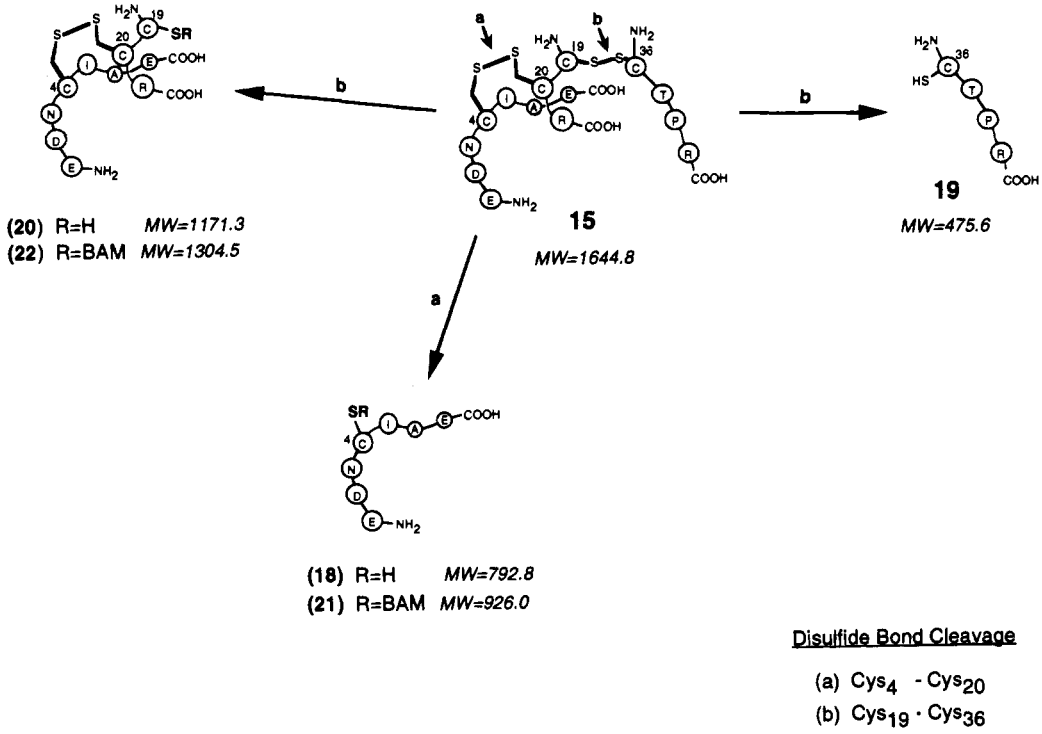


Figure 4. Phosphine reduction of **15**.

5). In addition to a single disulfide core fragment **25**, two pentapeptides H_2N-GLSFA-OH (**26** and **27**) were generated, which again did not coelute on RP-HPLC.

Edman sequencing of **26** and **27**, in concert with electrospray mass spectrometry, verified the integrity of the amino acid assignments and confirmed that both C-termini exist as the free acid. All evidence was consistent with the distinction between ω-Aga IVB (**1**) and IVC (**2**) residing in the absolute configuration of one of the four terminal amino acids of H_2N-GLSFA-OH. To determine which of the two peptides contains the all L-amino acid terminus, pentapeptide **27** was generated by solid phase peptide synthesis. RP-HPLC experiments indicated that **27** coelutes with the C-terminus of ω-Aga IVC (**2**).

The presence of fragment **27** in ω-Aga IVC (**2**) suggested that ω-Aga IVB (**1**) (the more abundant and biologically potent of the two peptides) has at least one D-amino acid residue at its C-terminus. Carboxypeptidase P experiments were consistent with this analysis, as the enzyme sequentially cleaved amino acid residues at the C-terminus of ω-Aga IVC (**2**) but did not cleave beyond the terminal alanine residue of ω-Aga IVB (**1**).

The existence of D-amino acids in eucaryotic systems is extremely rare[36] and would presumably arise from a novel post-

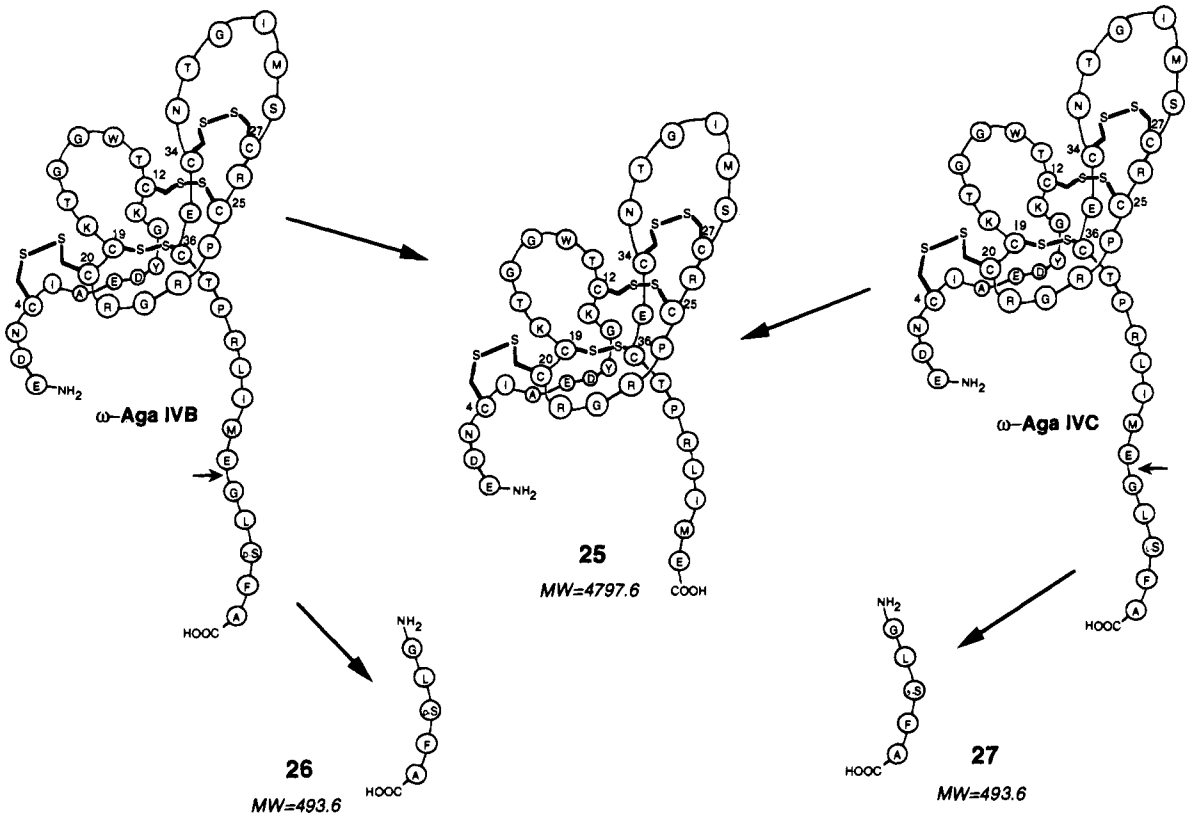


Figure 5. Glu-C digests of ω-Aga IVB(**1**)/IVC (**2**).

translational conversion of an L-amino acid-containing precursor. As a result, isomerization would more than likely involve a single amino acid residue. Given possible mechanistic options, serine seemed most prone to racemization and, therefore, H_2N-GL(D-Ser)FA-OH was targeted for synthesis. A sample was prepared and coeluted on RP-HPLC with **26**, the C-terminus of ω-Aga IVB (**1**).

Obviously, the structural assignments of ω-Aga IVB (**1**) and IVC (**2**) rest primarily on these RP-HPLC coelution studies. To prove that no other D-amino acid-containing peptide isomers would comigrate on RP-HPLC with either peptides **26** or **27**, a library of possible pentapeptides was generated. Theoretically, 16 isomers exist (which ideally would provide eight distinct entities on RP-HPLC). In a single experiment, L-Ala, DL-Phe, DL-Ser, DL-Leu, and Gly residues were sequentially coupled *via* solid phase synthesis to yield a mixture of eight peptides, all of which were seen (peaks 1 and 2 were poorly resolved) by RP-HPLC (Figure 6). The ω-Aga IVB (**1**)-derived peptide **26** coeluted with peaks 1 and 2 (containing D-Ser), while the ω-Aga IVC (**2**)-derived peptide **27** coeluted with peak 4 (containing L-Ser). Furthermore, the possibility that ω-Aga IVB (**1**) contains a D-Ala or D-Phe residue at the C-terminus was ruled out through synthesis of peptides H_2N-GLSF(D-Ala)-OH (**28**) (peak 8) and H_2N-GLS(D-Phe)A-OH (**29**) (peak 7).

Our synthetic studies lead us to conclude that *ω-Aga IVB (1) has a* D-*serine and ω-Aga IVC (2) has an* L-*serine residue near the C-terminus at residue 46* (as shown in Figure 5). Moreover, both peptides have identical disulfide motifs. To our knowledge, this is the first time that two distinct peptides with identical amino acid sequences and disulfide structures have been isolated from an animal source. Our structural assignment of ω-Aga IVB (**1**) and IVC (**2**) has been confirmed by total synthesis[37] and enzymatic studies.[38] The calcium channel activities of synthetic and natural ω-Aga IVB (**1**) and IVC (**2**) are identical.[15b] Biological characterization of these peptides along with the enzymatic conversion of ω-Aga IVC (**2**) to ω-Aga IVB (**1**) by the venom will be reported elsewhere, revealing a fascinating strategy of the *Agelenopsis aperta* spider to respond to evolutionary challenges.[15b]

In the course of establishing ω-Aga IVB (**1**) and IVC (**2**) structure, a practical method for determining the disulfide connectivity has been described. No products indicative of disulfide isomerization (scrambling) were isolated in either the TCEP-mediated disulfide reductions, BAM amidoalkylations, or enzymatic digests employed in these studies. The methodology is compound sparing and flexible and, as a result, could have widespread applicability for the assignment of disulfide connectivity in other cysteine-rich proteins.

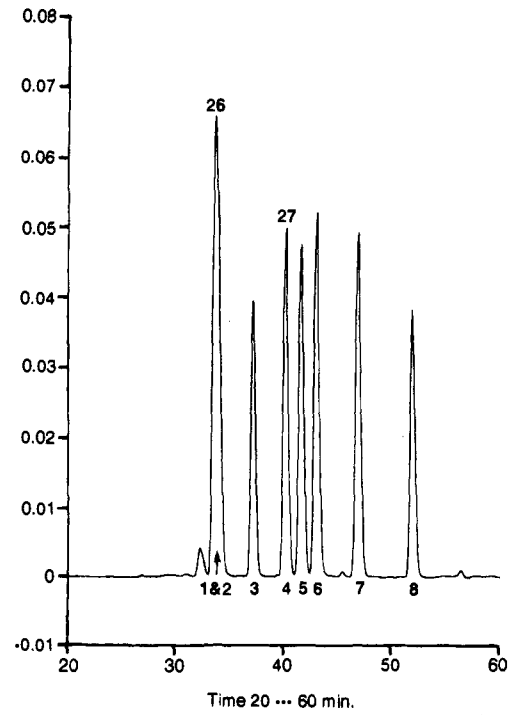
Experimental Procedures

Chemical Materials. Sephadex G-25 superfine was obtained from Pharmacia LKB Biotechnology Inc. and used as described by the supplier. All chromatography columns were obtained from the Nest Group Inc. (Southborough, MA). *N*-(Hydroxymethyl)benzamide (HMBA) was obtained from Lancaster Synthesis Ltd., trifluoroacetic

(36) (a) Richter, K.; Egger, R.; Kreil, G. *Science* **1987**, 200–202. (b) Montecucchi, P. C.; de Castiglione, R.; Piani, S.; Gozzini, L.; Erspamer, V. *Int. J. Peptide Protein Res.* **1981**, *17*, 275–283. (c) Daly, J. W.; Caceres, J.; Moni, R. W.; Gusovsky, F.; Moos, M.; Seamon, K. B.; Milton, K.; Myers, C. W. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 10960–10963. (d) Erspamer, V.; Melchiorri, P.; Falconieri-Erspamer, G.; Negri, L.; Corsi, R.; Severini, C.; Barra, D.; Simmaco, M.; Kreil, G. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 5188–5192. (e) Kreil, G. *J. Biol. Chem.* **1994**, *269*, 10967–10970.

(37) Saccomano, N. A.; Thadeio, P. F.; Phillips, D.; Kelbaugh, P. R.; Guarino, B. C.; Andrews, G. C.; Volkmann, R. A. Manuscript in preparation.

(38) Size exclusion chromatography (Sephadex G-50 superfine) of *Agelenopsis aperta* venom yields a fraction containing isomerase (which converts ω-Aga IVC (**2**) to ω-Aga IVB (**1**)) and endopeptidase (which cleaves H_2N-GLSFA-OH (**27**) to GLS and FA) activities. These results will be described elsewhere.[15b] The endopeptidase activity verified our structural assignments. Pentapeptide **27** {both synthetic and natural (derived from ω-Aga IVC (**2**)} was rapidly cleaved by enzyme; H_2N-GL(D-Ser)-FA-OH (**26**) {both synthetic and natural (derived from ω-Aga IVB (**1**))} was resistant to proteolytic cleavage.



(C-18 Vydac®,4.6 x 250 mm, 5μm, 300 Å, 1ml/min., detection at 220 nm).
Gradient: A=0.1% TFA-H_2O, B= acetonitrile: 10-22% B over 64 min.

Figure 6. RP-HPLC fractionation of the eight H_2N-GLSFA-OH peptides.

acid (TFA) and iodoacetic acid were obtained from Aldrich, and 4-vinylpyridine, Tris base, EDTA, guanidine·HCl, ammonium formate, β-mercaptoethanol, and dithiothreitol (DTT) were obtained from Sigma Chemical Co. and used with no further purification. Tris(2-carboxyethyl)phosphine hydrochloride was prepared from tris(2-cyanoethyl)-phosphine (obtained from Strem Chemicals Inc.) as described in the literature.[26] BOC-D-Ser (Bzl) and Fmoc-D-Phe were purchased from Nova Biochem, Fmoc-D-Ala was purchased from Millipore, BOC-D-Leu was purchased from Peptides International, and BOC-D-Phe was obtained from Penninsula Laboratories. Fmoc-Gly, Fmoc-Leu, Fmoc-Ser(^{t}Bu), Fmoc-Phe, Fmoc-Ala, HMP resin, Fmoc-Ala-HMP resin, BOC-L-Ser (Bzl), BOC-L-Leu, BOC-L-Phe, BOC-Gly, and BOC-L-Ala-PAM resin were purchased from Applied Biosystems. *N*-Methylpyrrolidone (NMP), dichloromethane (DCM), dicyclohexylcarbodiimide (DCC) in NMP, 1-hydroxybenzotriazole (HOBt) in NMP, dimethyl sulfoxide (DMSO) in NMP, and *N,N*-diisopropylethylamine (DIEA) were purchased from Applied Biosystems.

Proteases. Endopeptidase Glu-C (Promega sequencing grade, lot #361,711) and modified trypsin (Promega sequencing grade, lot #314,-305) were obtained from Promega. Carboxypeptidase P (sequencing grade) was obtained from Boehringer Mannheim Biochemica.

Venom Extraction. Both female and juvenile *Agelenopsis aperta* (Aranae, Agelenidae), a common western U.S. funnel-web grass spider, were collected in Utah, Arizona and New Mexico. The spiders were housed individually in mesh-topped plastic containers at room temperature, given daily access to water, and fed weekly. Venom was obtained by electrical stimulation of the cephalothorax of live CO_2-anesthetized spiders.[39] Spiders typically recovered from the procedure and could be milked periodically with ~0.5 μl of venom being obtained per milking. Pooled venom was stored at −80 °C.

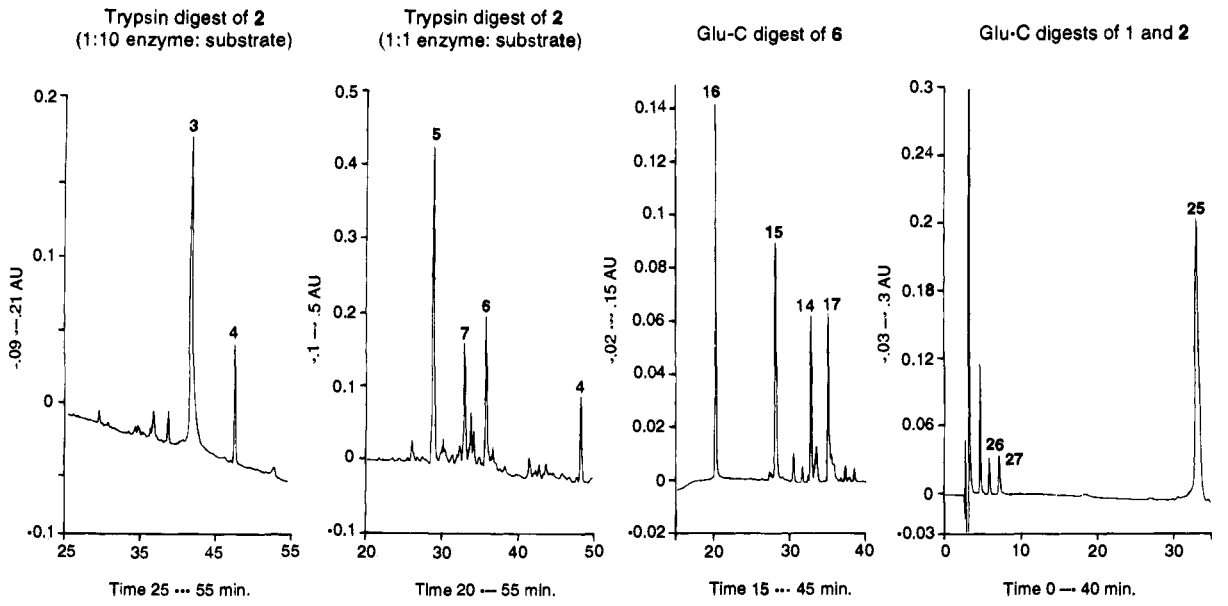
Venom Fractionation and Toxin Purification. Homogeneous and biologically active ω-Aga IVB and IVC were obtained from whole venom by a three-step purification procedure using sequential gel filtration, strong cation exchange chromatography, and reversed-phase (RP) HPLC desalting, similar to that used to isolate native ω-Aga IVA.[10] To obtain ω-Aga IVB and IVC, ~500 μL of whole venom was applied to a Sephadex G-25 superfine column (1.5 × 30 cm) with 100 nM ammonium formate (pH 4.0) as the eluent at a flow rate of 200 μL/min. Fractions were taken every 10 min, and eluent from the column was monitored using UV detection (1/100 dilution) at 220 nm. The column used has V_T = 52.9 cm^3, V_B = 52.0 cm^3, and V_O = 17.3 cm^3. The peptides from *Agelenopsis aperta* venom (~4−10 kD) were eluted from the Sephadex column in fractions 14−20, while the polyamine-containing compounds (<1 kD) were contained in fractions 22−36. Fractions 14−20 were pooled and concentrated by lyophilization. The combined peptide fraction was applied, in thirds, to a sulfoethylaspartamide strong cation exchange column (5 μm, 10 × 200 mm) and eluted using a linear gradient of 0−100% B over 40 min (buffer A: 80% 5 mM phosphoric acid, 20% CH_3CN; buffer B: 80% 5 mM phosphoric acid, 20% CH_3CN/1 M NaCl). The fraction eluting between 20 and 22.5 min contained the desired peptides. Desalting and final purification of peptides ω-Aga IVB and IVC from this ion exchange fraction were accomplished by three identical RP-HPLC separations (C_{18} Vydac, 300 Å, 5 μm, 10 × 250 mm) using a linear gradient of 25−35% B over 60 min (buffer A: 0.1% TFA/H_2O (v/v); buffer B: CH_3CN; 3.5 mL/min flow rate; 220 nM UV detection). ω-Aga IVC and ω-Aga IVB were collected at ~27.5−29.4 and ~29.6−34.0 min, respectively. Using this protocol, ~1.5−2.1 mg of ω-Aga IVB and ~0.75−1.1 mg of ω-Aga IVC were obtained from processing 1000 μL of crude *Agelenopsis aperta* venom. The identity of peptide ω-Aga IVB or IVC for each lot was validated by RP-HPLC, ES-MS, and PTC amino acid analysis. The homogeneity of ω-Aga IVB or IVC was ascertained for each new lot on RP-HPLC (C_{18} Vydac, 300 Å, 5 μm, 4.6 × 250 mm), and peptide concentrations were determined routinely by replicate PTC amino acid analyses. Supplies of natural ω-Aga IVC were supplemented with synthetic peptide.[37]

Amino Acid Analysis. Amino acid analysis was performed with an Applied Biosystems Model 420H hydrolyzer/derivatizer and a Model 130 analyzer. Data were acquired and processed using the PE Nelson data system.

Protein and Peptide Sequence Analysis. Peptide sequencing was performed on a Hewlett-Packard G1005A sequencing system consisting of a G1000A sequencer, an on-line 1090 HPLC unit, and an associated computer system and software.

Reversed-Phase (RP)-HPLC. Synthetic peptides, enzymatic digests, and disulfide reductions were purified on a Waters 600E HPLC system equipped with a 990 photodiode array detector. Samples were injected onto a Vydac C_{18} protein and peptide column (4.6 × 250 mm, 5 μm particle size, 300 Å pore). Mobile phases were buffer A, 0.1%

(39) Bascur, L.; Yevenes, I.; Adrian, H. *Toxicon* **1980**, *18*, 224.



(C-18 Vydac®,4.6 x 250 mm, 5μm, 300 Å, 1ml/min., detection at 220 nm).
Gradient: A=0.1% TFA-H_2O, B= acetonitrile: 25-35% B over 40 min. then 35% B for 10 min.

Figure 7. RP-HPLC Fractionation of trypsin and Glu-C digests of ω-Aga IVC (ω-Aga IVC fragments).

TFA/H_2O (v/v), and buffer B, CH_3CN. Linear gradients were employed at a flow rate of 1.0 mL/min (220 nM UV detection). Unless specifically stated, a linear gradient of 0—30% B over a 35 min period followed by 30—60% B over an additional 25 min period was employed.

Mass Spectral Analysis. Mass spectral information was obtained using ES-MS on a Finnigan TSQ-700 mass spectrometer fitted with an Analytica of Branford (Branford, CT) electrospray ionization source. Samples were dissolved in 0.1% TFA to a concentration of ~10 μM. The flow rate of this solution into the mass spectrometer was 1 μL/min with a sheath liquid of 2-methoxyethanol. The instrument was scanned over the range *m/z* 600—2200 or 400—1800 in the profile mode, and the resulting data were deconvoluted using Finnigan Biotech software. The resolution of the instrument was set such that average mass data (as opposed to monoisotopic data) were collected. For higher MW samples (>4000 Da), accuracy was within 0.01%, while at lower masses, the accuracy was ~0.4 Da. The averaging of multiple molecular ions of different charge states accounts for the improved accuracy at higher mass.

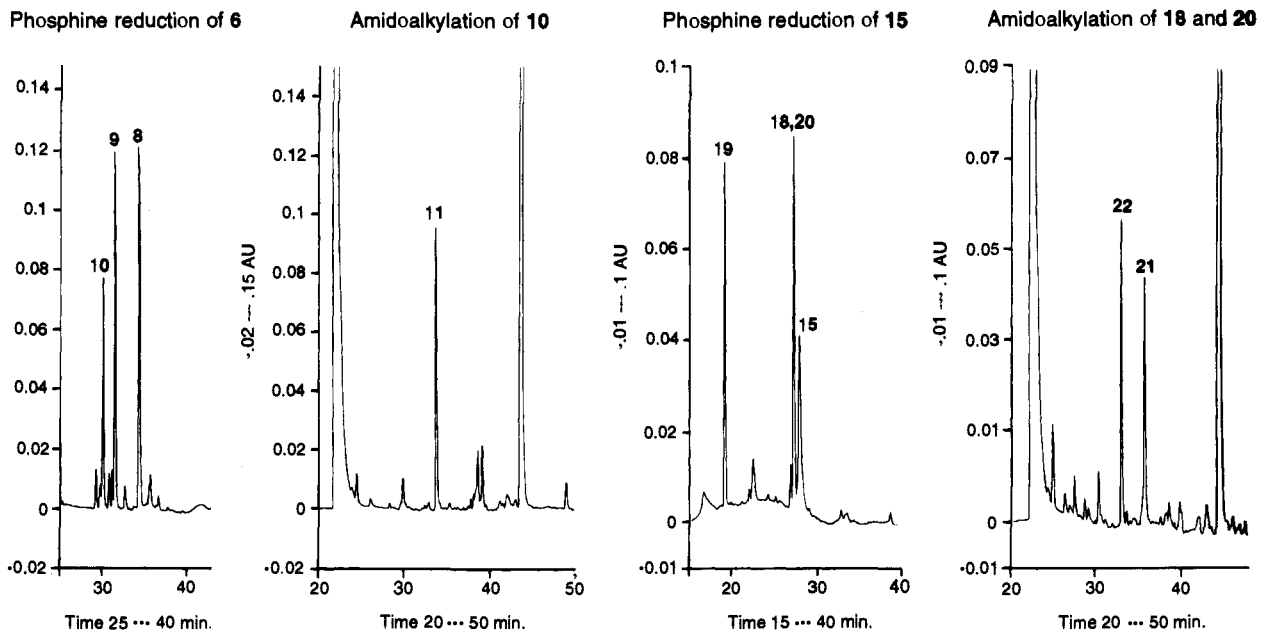
On-line Carboxypeptidase P Digest of ω-Aga IVB and ω-Aga IVC. ω-Aga IVB and ω-Aga IVC (2.5 μg) were individually dissolved in 50 mM ammonium acetate and treated with 0.025 μg of carboxypeptidase P. The reaction was monitored over the course of 50 min by ES-MS. ω-Aga IVC began digesting immediately, with cleavage of the terminal Ala, Phe, and Ser residues occurring by 35 min. After 45 min, only the terminal Ala residue was cleaved in ω-Aga IVB.

Trypsin Digest of ω-Aga IVB and ω-Aga IVC. Generation of Peptide Fragments 3 and 4. ω-Aga IVB (10 μg, 1.9 nmol) was dissolved in H_2O (29 μL) and combined with modified trypsin (1 μg) which was predissolved in 100 μL of 0.05 M NH_4OAc (pH 6.8). The mixture was kept at 37 °C for 16 h and injected onto a Vydac C_{18} RP-HPLC column using the above conditions. Fragments **3** and **4** eluted after 41.6 and 47.0 min, respectively, and were collected and characterized by electrospray mass spectrometry. ES-MS: **3**, obsd M^+ = 4310.7 (calcd MW = 4311.0, $C_{170}H_{266}N_{56}O_{58}S_9$); **4**, obsd $(M + H)^+$ = 981.1 (calcd MW = 980.2, $C_{45}H_{73}N_9O_{13}S$). Under identical conditions, ω-Aga IVC provided **3** and **4** (Figure 7), which eluted after 41.6 and 47.2 min, respectively, and were collected and characterized by electrospray mass spectrometry. ES-MS: **3**, obsd M^+ = 4310.8 (calcd MW = 4311.0, $C_{170}H_{266}N_{56}O_{58}S_9$); **4**, obsd $(M + H)^+$ = 981.1 (calcd MW = 980.2, $C_{45}H_{73}N_9O_{13}S$).

Generation of Peptide Fragments 4, 5, 6, and 7. ω-Aga IVB (20 μg, 3.8 nmol) was dissolved in H_2O (20 μL) and combined with modified trypsin (20 μg) which was predissolved in 100 μL of 0.05 M NH_4OAc (pH 6.8). The mixture was kept at 37 °C for 20 h and injected on a Vydac C_{18} RP-HPLC column using the protocol described above. Fragments **4**, **5**, **6**, and **7** eluted after 48.2, 29.1, 36.2, and 33.3 min, respectively. Samples of crude eluent were analyzed by electrospray mass spectrometry. ES-MS: **4**, obsd $(M + H)^+$ = 981.0 (calcd MW = 980.2, $C_{45}H_{73}N_9O_{13}S$); **5**, obsd M^+ = 1338.0 (calcd MW = 1337.6, $C_{54}H_{88}N_{20}O_{16}S_2$); **6**, obsd M^+ = 3044.9 (calcd MW = 3045.3, $C_{116}H_{186}N_{36}O_{46}S_7$); **7**, obsd M^+ = 3063.1 (calcd MW = 3063.5, $C_{116}H_{188}N_{36}O_{47}S_7$). Under identical conditions, ω-Aga IVC provided fragments **4**, **5**, **6**, and **7** (Figure 7), which eluted after 48.2, 28.9, 36.0, and 33.0 min, respectively. Samples of crude eluent were analyzed by electrospray mass spectrometry. ES-MS: **4**, obsd $(M + H)^+$ = 981.0 (calcd MW = 980.2, $C_{45}H_{73}N_9O_{13}S$); **5**, obsd M^+ = 1338.0 (calcd MW = 1337.6, $C_{54}H_{88}N_{20}O_{16}S_2$); **6**, obsd M^+ = 3044.9 (calcd MW = 3045.3, $C_{116}H_{186}N_{36}O_{46}S_7$); **7**, obsd M^+ = 3063.1 (calcd MW = 3063.5, $C_{116}H_{188}N_{36}O_{47}S_7$).

Partial Reduction of Peptide Fragment 6 with Tris(2-carboxyethyl)phosphine. Preparation of Peptides Fragments 8, 9, and 10. Approximately 10 μg (3.3 nmol) of **6** (from ω-Aga IVB) was combined with 200 μL of 10 mM tris(2-carboxyethyl)phosphine (2 μmol) in 0.5 M citric acid buffer (pH 3.0) for 10 min. The reaction mixture was immediately injected on a Vydac C_{18} RP-HPLC column using the protocol described above. Three major peptide fragments **8**, **9**, and **10**, which eluted after 34.2, 31.3, and 30.2 min, respectively, were collected and lyophilized to dryness. ES-MS: **8**, obsd M^+ = 1414.6 (calcd MW = 1414.7, $C_{53}H_{91}N_{17}O_{20}S_4$); **9**, obsd M^+ = 1256.2 (calcd MW = 1256.4, $C_{51}H_{77}N_{13}O_{22}S$); **10**, obsd M^+ = 1634.8 (calcd MW = 1634.8, $C_{63}H_{99}N_{19}O_{26}S_3$). Under identical conditions, **6** (from ω-Aga IVC) provided peptide fragments **8**, **9**, and **10** (Figure 8), which eluted after 34.6, 31.3, and 30.2 min, respectively, and were collected and lyophilized to dryness. ES-MS: **8**, obsd M^+ = 1414.3 (calcd MW = 1414.7, $C_{53}H_{91}N_{17}O_{20}S_4$); **9**, obsd M^+ = 1256.5 (calcd MW = 1256.4, $C_{51}H_{77}N_{13}O_{22}S$); **10**, obsd M^+ = 1634.7 (calcd MW = 1634.8, $C_{63}H_{99}N_{19}O_{26}S_3$).

Preparation of BAM-Protected Peptide 11. The lyophilized fraction of **10** (prepared in the reduction of 20 μg (6.6 nmol) of **6** (from ω-Aga IVB)) was reconstituted in neat TFA (40 μL). To this solution at ambient temperature was added 10 μL (66 nmol) of a 6.6 mM (hydroxymethyl)benzamide/TFA solution. The reaction mixture was vortexed, allowed to sit for 45 min, and concentrated *in vacuo*. The residue was reconstituted in H_2O (200 μL) and injected onto a Vydac C_{18} RP-HPLC column using the protocol described above to afford



(C-18 Vydac®,4.6 x 250 mm, 5μm, 300 Å, 1ml/min., detection at 220 nm).
Gradient: A=0.1% TFA-H_2O, B= acetonitrile: 25-35% B over 40 min. then 35% B for 10 min.

Figure 8. RP-HPLC fractionation of phosphine (TCEP) reductions/amidoalkylations (BAM) of ω-Aga IVC fragments.

BAM-protected peptide **11** which eluted after 34.2 min. A sample of crude eluent containing **11** was analyzed by electrospray mass spectrometry. ES-MS: **11**, obsd M^+ = 1768.3 (calcd MW = 1768.0, $C_{71}H_{106}N_{20}O_{27}S_3$). Under identical conditions, **10** (from ω-Aga IVC) provided BAM-protected peptide **11** (Figure 8), which eluted after 34.2 min. A sample of crude eluent containing **11** was analyzed by electrospray mass spectrometry. ES-MS: **11**, obsd M^+ = 1768.6 (calcd MW = 1768.0, $C_{71}H_{106}N_{20}O_{27}S_3$).

Glu-C Digest of Peptide Fragment 6. Generation of Peptide Fragments 14, 15, 16, and 17. Approximately 20 μg (6.6 nmol) of **6** (from ω-Aga IVB)) was reconstituted in a solution (100 μL) of 25 mM NH_4OAc (pH 4.0) containing 20 μg of Glu-C. The mixture was kept at 37 °C for 16 h and injected on a Vydac C_{18} RP-HPLC column using the protocol described above. Fragments **14**, **15**, **16**, and **17** eluted after 32.8, 28.0, 20.2, and 35.0 min, respectively. Samples of crude eluent containing these fragments were analyzed by electrospray mass spectrometry. ES-MS: **14**, obsd $(M + H)^+$ = 955.9 (calcd MW = 955.1, $C_{35}H_{58}N_{10}O_{15}S_3$); **15**, obsd M^+ = 1645.2 (calcd MW = 1644.9, $C_{60}H_{101}N_{21}O_{25}S_4$); **16**, obsd $(M + H)^+$ = 482.7 (calcd MW = 481.5, $C_{21}H_{31}N_5O_8$); **17**, obsd M^+ = 2581.6 (calcd MW = 2582.0, $C_{95}H_{157}N_{31}O_{39}S_7$). Under identical conditions, **6** (from ω-Aga IVC) provided fragments **14**, **15**, **16**, and **17** (Figure 7), which eluted after 32.8, 28.0, 20.2, and 35.0 min, respectively. Samples of crude eluent containing these fragments were analyzed by electrospray mass spectrometry. ES-MS: **14**, obsd $(M + H)^+$ = 955.8 (calcd MW = 955.1, $C_{35}H_{58}N_{10}O_{15}S_3$); **15** obsd M^+ = 1644.8 (calcd MW = 1644.9, $C_{60}H_{101}N_{21}O_{25}S_4$); **16**, obsd $(M + H)^+$ = 482.5 (calcd MW = 481.5, $C_{21}H_{31}N_5O_8$); **17**, obsd M^+ = 2581.7 (calcd MW = 2582.0, $C_{95}H_{157}N_{31}O_{39}S_7$).

Partial Reduction of Peptide Fragment 15 with Tris(2-carboxyethyl)phosphine. Preparation of Peptide Fragments 18, 19, and 20. Approximately 20 μg (12 nmol) of **15** (from 100 μg, 19.0 nmol, of ω-Aga IVC) was combined with 200 μL of 10 mM tris(2-carboxyethyl)phosphine (2 μmol) in 0.5 M citric acid buffer (pH 3.0) for 10 min. The reaction mixture was immediately injected onto a Vydac C_{18} RP-HPLC column using the protocol described above. In addition to starting **15** (eluted after 27.8 min), three major peptide fragments **18**, **19**, and **20** (Figure 8), which eluted after 27.2, 19.3, and 27.2 min, respectively, were collected and lyophilized to dryness. ES-MS: **18**, obsd $(M + H)^+$ = 793.6 (calcd MW = 792.8, $C_{30}H_{48}N_8O_{15}S$); **19**, obsd $(M + H)^+$ = 476.5 (calcd MW = 475.6, $C_{18}H_{33}N_7O_6S$); **20**, obsd M^+ = 1171.3 (calcd MW = 1171.3, $C_{42}H_{70}N_{14}O_{19}S_3$).

Preparation of BAM-Protected Peptides 21 and 22. The lyophilized fraction containing **18** and **20** (prepared in the reduction of 20 μg (12 nmol) of **15** (from ω-Aga IVC)) was reconstituted in neat TFA (50 μL). To this solution at ambient temperature was added 10 μL (66 nmol) of a 6.6 mM (hydroxymethyl)benzamide/TFA solution. The reaction mixture was vortexed, allowed to sit for 45 min, and concentrated *in vacuo*. The residue was reconstituted in H_2O (200 μL) and injected onto a Vydac C_{18} RP-HPLC column using the protocol described above to afford BAM-protected peptides **21** and **22** (Figure 8), which eluted after 35.3 and 33.0 min, respectively. Samples of crude eluent were analyzed by electrospray mass spectrometry. ES-MS: **21**, obsd $(M + H)^+$ = 926.9 (calcd MW = 926.0, $C_{38}H_{55}N_9O_{16}S$); **22**, obsd M^+ = 1304.4 (calcd MW = 1304.5, $C_{50}H_{77}N_{15}O_{20}S_3$).

Preparation of CM-Cys-Protected Peptides 12, 13, 23, and 24. Approximately 1 μg (0.5 nmol) of **11** (prepared in the HMBA alkylation of **10** (from ω-Aga IVB)) was reconstituted in 100 μL of buffer (6 M guanidine·HCl, 0.5 M Tris base, and 2 mM EDTA, pH 8.3) containing DTT (154 μg, 1 μmol). The mixture was kept at 55 °C for 90 min. Following reduction, 10 μL (10 μmol) of 1.0 M iodoacetic acid/H_2O was added and the resultant mixture was maintained at 55 °C for 30 min. The reaction was quenched by addition of 1 mL of 0.1% TFA/H_2O and immediately loaded onto the hydrophobic portion of a Hewlett Packard dual sequencing column system.[40] The column was rinsed with an additional 1 mL portion of 0.1% TFA/H_2O and reassembled with its hydrophilic counterpart. Edman sequencing identified both CM-Cys-protected peptides **12** and **13**. Under identical conditions, peptide **22** (prepared in the HMBA alkylation of **20** (from ω-Aga IVC)) was reduced and carboxymethylated to afford peptides **23** and **24**, which were characterized by Edman sequencing as above.

Glu-C Digest of ω-Aga IVB and ω-Aga IVC. Generation of Peptide Fragments 25, 26, and 27. ω-Aga IVB (10 μg, 1.9 nmol) was dissolved in H_2O (10 μL) and combined with Glu-C (2 μg), which was predissolved in a solution (100 μL) of 25 mM NH_4OAc (pH 4.0). The mixture was kept at 37 °C for 20 h and injected onto a Vydac C_{18} RP-HPLC column using a linear gradient over 60 min of 20–35% B (buffer A: 0.1% TFA/H_2O (v/v); buffer B: CH_3CN). Fragments **25** and **26** (Figure 7) eluted after 32.5 and 6.0 min, respectively, and were collected and characterized by electrospray mass spectrometry. ES-MS: **25**, obsd M^+ = 4797.2 (calcd MW = 4797.6, $C_{192}H_{304}N_{60}O_{64}S_{10}$); **26**, obsd $(M + H)^+$ = 494.9 (calcd MW = 493.6, $C_{23}H_{35}N_5O_7$). Under identical conditions, ω-Aga IVC provided **25** and **27** (Figure 7), which eluted after 32.5 and 7.5 min, respectively, and were collected and

(40) Hewlett-Packard Technical Note TN 92-3, Hewlett-Packard, Palo Alto, CA, 1992.

characterized by electrospray mass spectrometry. ES-MS: **25**, obsd $M^+ = 4797.2$ (calcd MW = 4797.6, $C_{192}H_{304}N_{60}O_{64}S_{10}$); **27**, obsd $(M + H)^+ = 494.9$ (calcd MW = 493.6, $C_{23}H_{35}N_5O_7$).

Preparation of H_2N-Gly-Leu-(D-Ser)-Phe-Ala-OH (26) and H_2N-Gly-Leu-Ser-Phe-Ala-OH (27). Peptides **26** and **27** were synthesized by the solid phase method on an Applied Biosystems Model 430A peptide synthesizer utilizing a program (version 1.4) for single amino acid coupling. BOC-Ala-PAM resin (0.5 mol) was elongated using BOC-Phe-OH, BOC-L-Ser-OH for **27** or BOC-D-Ser-OH for **26**, BOC-Leu-OH, and BOC-Gly-OH. To remove BOC groups, the resin was treated with TFA in DCM. Single couplings were carried out by preforming amino acid HOBt esters (by treatment of 4 equiv each of DCC, HOBt, and BOC-amino acids) and allowing them to react with the α-amino terminus of the resin-bound amino acid. Unreacted free amine was subsequently capped with acetic anhydride after DIEA neutralization. Once the resin-bound fully protected pentapeptides were prepared, they were treated with TFA to remove the terminal BOC group. The product was washed thoroughly with DCM and dried. The resin was treated with HF in the presence of *p*-cresol at −2 °C for 1 h to remove the protecting groups. After the HF was evaporated *in vacuo*, the liberated peptide was slurried with TFA and filtered through a sintered glass funnel into ether to remove particulates. The precipitated peptides were filtered, washed with additional ether, and dried *in vacuo*. Analytical samples of **26** and **27** were obtained following purification on a Vydac C_{18} RP·HPLC column using a linear gradient over 60 min of 0−60% B (buffer A: 0.1% TFA/H_2O; buffer B: CH_3CN); **26** and **27** eluted after 28.8 and 30.1 min, respectively.

Preparation of H_2N-Gly-(D,L-Leu)-(D,L-Ser)-(D,L-Phe)-(L-Ala)-OH (26−33). H_2N-Ala-PAM (0.5 mol) was coupled separately with BOC-D-Phe and BOC-L-Phe because of solubility problems encountered in the coupling of H_2N−Ala-PAM with BOC-D,L-Phe. The D-Phe residue was predissolved in 1 M HOBt/NMP before coupling to the Ala resin. Resin obtained in each coupling was combined and coupled sequentially with BOC-D,L-Ser, BOC-D,L-Leu, and BOC-Gly as described in the preparation of **26** and **27**. The peptide mixture was removed from the resin as described above and resolved on a Vydac C_{18} RP-HPLC column using a linear gradient over 64 min of 10−22% B (buffer A: 0.1% TFA/H_2O; buffer B: CH_3CN) (Figure 6).

Preparation of H_2N-Gly-Leu-Ser-Phe-(D-Ala)-OH (28) and H_2N-Gly-Leu-Ser-(D-Phe)-Ala-OH (29). FastMoc chemistry on a 0.25 mM scale was employed on an Applied Biosystems Model 433A peptide synthesizer. Fmoc (D-Ala)-OH was loaded onto HMP resin, capped with benzoic anhydride, and sequentially coupled with Fmoc-Phe, Fmoc-Ser(^{t}Bu), Fmoc-Leu, and Fmoc-Gly. The crude resin was treated with 95% TFA (aq) for 1 h and purified by RP-HPLC using standard conditions to afford **28**. Pentapeptide **29** was generated in a similar fashion by using Fmoc-Ala-HMP resin and employing Fmoc(D-Phe) in the coupling sequence.

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