

# Identification of disulfide bonds in the ninth component (C9) of human complement

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**Abstract** C9 is the most abundant protein of the membrane attack complex of complement. By means of limited proteolysis, different chromatographic techniques, a thiol-specific fluorescence assay, amino acid analysis, and Edman degradation, 9 out of 12 disulfide bridges are definitely assigned (Cys<sup>22</sup>–Cys<sup>57</sup>, Cys<sup>33</sup>–Cys<sup>36</sup>, Cys<sup>67</sup>–Cys<sup>73</sup>, Cys<sup>121</sup>–Cys<sup>160</sup>, Cys<sup>233</sup>–Cys<sup>234</sup>, Cys<sup>359</sup>–Cys<sup>384</sup>, Cys<sup>489</sup>–Cys<sup>505</sup>, Cys<sup>492</sup>–Cys<sup>507</sup>, Cys<sup>509</sup>–Cys<sup>518</sup>). Weaker evidence permits to reduce the number of possible configurations for the remaining 3 cystines (Cys<sup>80</sup>–Cys<sup>91</sup>, Cys<sup>86</sup>–Cys<sup>104</sup>, Cys<sup>98</sup>–Cys<sup>113</sup>, or Cys<sup>80</sup>–Cys<sup>91</sup>, Cys<sup>86</sup>–Cys<sup>113</sup>, Cys<sup>98</sup>–Cys<sup>104</sup>). These findings are discussed in comparison with the strongly related components C6, C7, C8 $\alpha$ , and C8 $\beta$ .

**Key words:** Complement; C9; Disulfide bridge; Terminal complement component

## 1. Introduction

With component C9 playing a key role in its action, the complement system is one of the most important effector mechanisms of vertebrate immune response. After binding to a membrane-bound C5b-8 precursor complex, C9 unfolds and inserts into the lipid bilayer of the target cell [1–3]. Subsequently C9 oligomerization occurs generating a functionally active cytotoxic pore known as membrane attack complex (MAC) [4,5].

Human C9 is a single chain glycoprotein of an apparent molecular mass of about 71 kDa. It contains 538 amino acids including 24 half-cystines [6–8]. These are all engaged in intramolecular disulfide bond formation.

Earlier reports suggest that there are at least five independently folding domains in C9 [9,10]. The first two domains at the N-terminus have been recognized by sequence comparison as thrombospondin type I repeat (TSP I) (Asp<sup>21</sup> to Glu<sup>77</sup>) [11–17] and low density lipoprotein receptor class A module (LDL A) (Asp<sup>78</sup> to Arg<sup>118</sup>) [7]. Near the C-terminus is a motif (Lys<sup>488</sup> to Lys<sup>522</sup>) that is exhibiting partial sequence identity to the low density lipoprotein receptor class B domain (LDL B), also known as epidermal growth factor precursor type domain (EGF) [18]. Each of these modules contains 3 cystines. The

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**Abbreviations:** MAC, membrane attack complex; TSP I, thrombospondin type I repeat; LDL A, low density lipoprotein receptor class A module; LDL B, low density lipoprotein receptor class B module; EGF, epidermal growth factor; RP-HPLC, reversed phase high performance liquid chromatography; SDS-PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate; BNPS-skatole, 2-(2-nitrophenylsulfenyl)-3-methyl-3-bromoindolenine; SBD-F, ammonium 7-fluorobenz-2-oxa-1,3-diazole-4-sulfonate; DTT, dithiothreitol; PTH, phenylthiohydantoin.

remaining 3 disulfides are dispersed across the comparatively heterologous central part of the molecule (Pro<sup>119</sup> to Arg<sup>487</sup>). This area has been reported to contain the functionally important hinge region (Asn<sup>228</sup> to Lys<sup>271</sup>) [9], the membrane-spanning domain (Val<sup>293</sup> to Leu<sup>334</sup>) [19] and the CD59 binding site (Cys<sup>359</sup> to Gly<sup>411</sup>) [20].

Complement C9 is highly similar in structure to the other terminal complement components. Each type of module occurring in C9 is present again in varying number in C6, C7, C8 $\alpha$ , or C8 $\beta$  [21,22]. By sharing some of the substructures C9 is additionally related to several other complement compounds as well as to some proteins that are not associated with the complement system.

Here we report the definite elucidation of 9 of the 12 disulfide bridges occurring in human C9 and present two configurations of which one is likely to apply to the remaining 3 disulfides. We furthermore discuss our findings in relation to the other terminal complement components.

## 2. Materials and methods

Cyanogen bromide was from Merck, BNPS-skatole from Pierce. Pepsin was obtained from Sigma, subtilisin and elastase from Serva. Thermolysin and *Staphylococcus aureus* V8 protease were of sequencing grade supplied by Boehringer Mannheim. SBD-F was from Dojin, Kumamoto, Japan, and tri-*n*-butylphosphine from Aldrich. The reagents used for microsequencing were purchased from Applied Biosystems. The reagents used for RP-HPLC were lichrosolv grade products by Merck. All other chemicals were of analytical grade ordered either from Merck, Sigma, or Fluka.

C9 was isolated from human plasma according to a published procedure [23]. SDS-PAGE was performed as described previously [24].

Cleavage with cyanogen bromide was carried out for 48 h in 70% trifluoroacetic acid in the dark at a reagent/substrate ratio of 5:1 (w/w). BNPS-skatole was applied in a 5 fold excess (w/w) for 72 h in 60% acetic acid, containing 0.2 M phenol. Enzymatic digestions were generally carried out at an enzyme/substrate ratio of 1:50 in the presence of 1 mM iodoacetamide to minimize disulfide interchange. Cleavage of native C9 with elastase occurred in 0.2 M Tris-HCl, pH 8.8, for 72 h. Thermolysinolysis was performed in 10 mM borate buffer, pH 6.5, containing 50 mM NaCl and 2 mM CaCl<sub>2</sub> for 96 h. Subdigestion with V8- protease was done in 50 mM sodium phosphate buffer, pH 7.8 during 74 h. Subdigestion with subtilisin was carried out for 30 h at 37 °C in 0.1 M NH<sub>4</sub>HCO<sub>3</sub>, pH 8.0. Subdigestion with pepsin was performed for 40 h at 37 °C in 10 mM HCl.

Fragments generated by BNPS-skatole and cyanogen bromide were both separated by gel filtration on a Sephadex G-50 superfine column (1.8 × 90 cm) using 0.13 M formic acid as eluant. Enzymatic digests were usually separated first by RP- HPLC on a Bakerbond butyl column (4.6 × 250 mm, wide pore, 5  $\mu$ m; J.T. Baker Chemicals, Deventer, The Netherlands) in a Hewlett-Packard 1090 liquid chromatograph (Hewlett Packard, Waldbronn, Germany). Further purification was achieved applying Aquapore butyl, phenyl or octadecyl columns (2.1 × 100 mm, 7  $\mu$ m) or an Aquapore RP-300 column (1.0 × 100 mm, 7  $\mu$ m). In each case, an acetonitrile gradient in dilute aqueous trifluoroacetic acid was used.

To monitor the cystine content an aliquot of each HPLC fraction was

either reacted with ammonium 7-fluorobenz-2-oxa-1,3-diazole-4-sulfonate (SBD-F) [25] or alternatively subjected to amino acid analysis [26,27].

Disulfide-linked peptides thus identified were finally analyzed in a pulsed-liquid-phase sequenator 477A from Applied Biosystems combined with a model 120A PTH Analyzer using only DTT-free reagents [28]. Di-phenylthiohydantoin-cystine was identified after its release in the corresponding cycle as a double peak [29,30].

### 3. Results

#### 3.1. Cleavage of native C9 with BNPS-skatole

Upon gel filtration the products obtained by BNPS-skatole cleavage yielded two pools, SK1 and SK2. The later eluting SK2 consisted of two fragments in an approximate molar ratio of 2:1. The more prominent fragment ranged from Ala<sup>438</sup> to Lys<sup>538</sup> with the molecular mass of about 12 kDa, whereas the second one apparently extended from Gln<sup>1</sup> to Pro<sup>175</sup>, corresponding to the 21 kDa species. BNPS-skatole generated a new

N-terminus at Ser<sup>31</sup> in the larger fragment. Since C9 is N-terminally blocked, only two sequences were identified in SK2. After the sixth degradation cycle a distinct release of di-PTH-cystine, indicative of a disulfide link between Cys<sup>33</sup> and Cys<sup>36</sup>, was observed (Table 1).

#### 3.2. Cleavage of native C9 with elastase

Of the elastase fractions collected only E18 showed distinct fluorescence values. Further analysis revealed that it mainly consisted of a single fragment with a molecular mass of about 17 kDa and an N-terminus with the sequence Ser<sup>18</sup>-His-Ile-Asp-Cys-Arg-Met-Ser-Pro-, thus implying that it essentially covered the TSP I and the LDL A region of C9.

#### 3.3. Subdigestion of elastase fraction E18 with V8 protease

E18 was afterwards subjected to V8 protease treatment. Cysteine-containing fraction E18V7 contained two strands detectable with the sequences Phe<sup>84</sup>-Gln-Cys<sup>86</sup>-Ser-Thr-Gly-Arg-Cys<sup>91</sup>

Table 1  
Edman degradation data and identification of disulfide bonds in human C9

Peptide	Fragmentation	Subfragmentation	Structural data	Disulfide bond
SK2	BNPS-Skatole		S <sup>31</sup> QXDPCLR*	33–36
E18V10	Elastase		A <sup>60</sup> VGDRRQXVPTEPTXEDAE	67–73
CB1P16	Cyanogen Bromide	Pepsin	I <sup>356</sup> KRXL N <sup>380</sup> KDDCV*	359–384
CB1V9	Cyanogen Bromide	V8 Protease	S <sup>13</sup> SGSASHIDXR V <sup>48</sup> FGQFNGKRXTD	22–57
CB1V17	Cyanogen Bromide	V8 Protease	F <sup>484</sup> SVRKCHTCQ* D <sup>502</sup> GKX L XAXPF* G <sup>515</sup> IAXE	489–505 492–507 509–518
CB1T8	Cyanogen Bromide	Thermolysin	I <sup>20</sup> DXR F <sup>52</sup> NGKRCTDA	22–57
CB1S11	Cyanogen Bromide	Subtilisin	E <sup>231</sup> Q X C EET	233–234
CB1S16	Cyanogen Bromide	Subtilisin	X <sup>33</sup> DPCL	33–36
CB1S18	Cyanogen Bromide	Subtilisin	S <sup>115</sup> EPRPPC* X <sup>160</sup> NRDRDG*	121–160
CB1S19	Cyanogen Bromide	Subtilisin	S <sup>115</sup> EPRPPCRD G <sup>158</sup> LX	121–160
CB1S24	Cyanogen Bromide	Subtilisin	X <sup>67</sup> VPTEPCED	67–73
CB1S25	Cyanogen Bromide	Subtilisin	X <sup>33</sup> DPCLR	33–36
CB2S16	Cyanogen Bromide	Subtilisin	X X <sup>507</sup> ACPF I <sup>516</sup> AC	509–518

\*Edman degradation data of unreduced peptides; X denotes a Cys residue expected from the known sequence but not observed. C denotes the di-PTH derivative of cystine. Asterisks indicate chain ends that have not been rigorously identified.

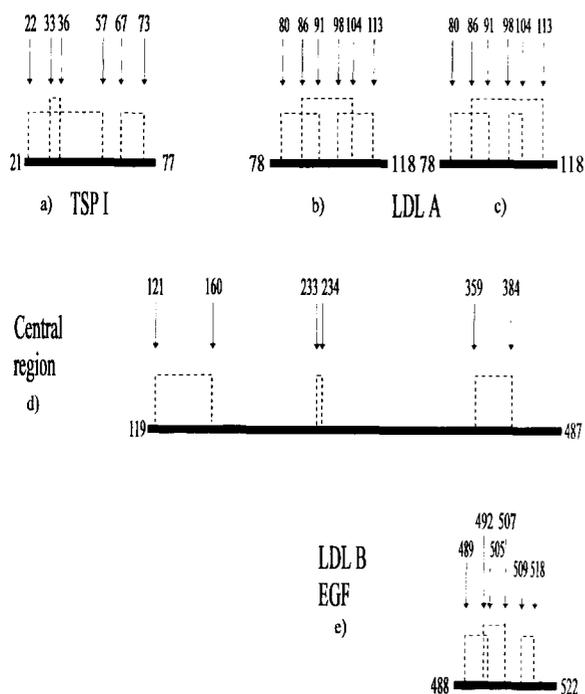


Fig. 1. Schematic representation of the disulfide bonding pattern of component C9 of human complement. Disulfides are indicated by dotted lines and the corresponding cysteine residues by the number in the C9 sequence. b and c represent the two arrangements that can be inferred from the data presented for the LDL A module.

and Leu<sup>96</sup>-Arg-Cys<sup>98</sup>-Asn-Gly-Asp-Asn-Asp-Cys<sup>104</sup>-Gly-Asp-Phe-Ser-Asp-Glu- in about equimolar amounts. The obvious absence of di-PTH-cystine after the third degradation step, recorded in repeated experiments, led to the conclusion that Cys<sup>86</sup> is apparently bonded to another half-cystine than Cys<sup>98</sup>. E18V10 comprised a fragment with the disulfide bridge between Cys<sup>67</sup> and Cys<sup>73</sup>.

### 3.4. Cleavage of native C9 with cyanogen bromide

Gel filtration of the cyanogen bromide digest yielded two pools, CB1 and CB2. SDS-PAGE and N-terminal sequence analysis indicated that pool CB1 essentially consisted of fragments derived from the N-terminal cluster of C9 (Gln<sup>1</sup> to Met<sup>272</sup> or Met<sup>287</sup>) with minor impurities originating from the central and C-terminal regions. Pool CB2 mainly contained a chain (Lys<sup>464</sup> to Lys<sup>538</sup>) that comprised the C-terminal EGF-like domain.

### 3.5. Subdigestion of cyanogen bromide fraction CB1 with pepsin

Fraction CB1P16 contained two chains in a roughly equimolar ratio. The corresponding sequences were interpreted as Ile<sup>356</sup>-Lys-Arg-Cys-Leu and Asn<sup>380</sup>-Lys-Asp-Asp-Cys-Val-, thus indicating a disulfide bond between Cys<sup>359</sup> and Cys<sup>384</sup>.

### 3.6. Subdigestion of cyanogen bromide fraction CB1 with V8 protease

CB1V9 comprised the disulfide bond between Cys<sup>22</sup> and Cys<sup>57</sup>. This was derived from compositional and sequence analysis showing the presence of two chains extending from Ser<sup>13</sup> to Arg<sup>23</sup> and from Val<sup>48</sup> to Asp<sup>59</sup>. Fraction CB1V17 comprised

a fragment with three disulfides. According to Edman degradation and amino acid analysis the corresponding strands ranged from Phe<sup>484</sup> beyond Gln<sup>493</sup>, from Asp<sup>502</sup> beyond Phe<sup>511</sup>, and from Gly<sup>515</sup> to Glu<sup>519</sup>, respectively. The liberation of di-PTH-cystine observed after the sixth, the eighth, and to a lesser extent after the ninth degradation step was fully compatible with the known cystine pattern of the epidermal growth factor type domain.

### 3.7. Subdigestion of cyanogen bromide fraction CB1 with thermolysin

N-Terminal sequence and compositional analysis of fluorescent fraction CB1T8 indicated the presence of a chain ranging from Ile<sup>20</sup> to Arg<sup>23</sup>, and a second one extending from Phe<sup>52</sup> to Ala<sup>60</sup>, thus confirming the earlier observed disulfide linkage between Cys<sup>22</sup> and Cys<sup>57</sup>.

### 3.8. Subdigestion of cyanogen bromide fraction CB1 with subtilisin

N-Terminal sequence and compositional analysis of fragment CB1S11 showed a single chain extending from Glu<sup>231</sup> to Thr<sup>237</sup>, with the adjacent Cys<sup>233</sup> and Cys<sup>234</sup> included. Considering the release of di-PTH-cystine distinctly recorded after the fourth degradation cycle, the two half-cystines were consequently assigned to a very unusual vicinal disulfide bridge. Edman degradation of CB1S16 showed a single sequence ranging from Cys<sup>33</sup> to Leu<sup>37</sup>, supporting the earlier observation that Cys<sup>33</sup> forms a disulfide linkage to Cys<sup>36</sup>. Fragment CB1S18 consisted of two peptides with the N-termini Ser<sup>115</sup>-Glu-Pro-Arg-Pro-Pro-Cys<sup>121</sup>- and Cys<sup>160</sup>-Asn-Arg-Asp-Arg-Asp-Gly-. Together with the sequences Ser<sup>115</sup>-Glu-Pro-Arg-Pro-Pro-Cys<sup>121</sup> and Gly<sup>158</sup>-Leu-Cys<sup>160</sup> found in CB1S19, this underlined the occurrence of a disulfide bond between Cys<sup>121</sup> and Cys<sup>160</sup>. The single strand detected in CB1S24 extended from Cys<sup>67</sup> to Asp<sup>75</sup>, emphasizing the presence of a disulfide bond between Cys<sup>67</sup> and Cys<sup>73</sup>. N-Terminal sequence analysis and amino acid composition of CB1S25 revealed a single chain ranging from Cys<sup>33</sup> to Arg<sup>38</sup>. This verified the previously postulated disulfide bond between Cys<sup>33</sup> and Cys<sup>36</sup>.

### 3.9. Subdigestion of cyanogen bromide fraction CB2 with subtilisin

Edman degradation and amino acid composition of fragment CB2S16 exhibited two sequences, one extending from Cys<sup>507</sup> to Phe<sup>511</sup>, and a second one ranging from Ile<sup>516</sup> to Cys<sup>518</sup>. In agreement with the earlier established disulfide bonding pattern of the epidermal growth factor type domain, this indicated that Cys<sup>509</sup> is connected to Cys<sup>518</sup>.

## 4. Discussion

Protease digestion experiments carried out earlier by various authors have indicated that native C9 is particularly resistant towards enzymatic cleavage. This observation was critical to the present study and made necessary the application of a wide range of enzymes and reagents.

First, experiments to establish the so far unidentified disulfides in the N-terminal thrombospondin type I domain (Asp<sup>21</sup> to Glu<sup>77</sup>) involved treatment with BNPS-skatole, thus providing preliminary evidence for the existence of a disulfide bond between Cys<sup>33</sup> and Cys<sup>36</sup>. Data derived from analysis of frac-

tions CB1S16 and CB1S25 later confirmed that assignment. Pools denoted CB1V9 and CB1T8 contained peptides that enclosed the disulfide bond from Cys<sup>22</sup> to Cys<sup>57</sup>. Fractions E18V10 and CB1S24 were shown to comprise the linkage between the half-cystines in positions 67 and 73. These individual results taken together finally exhibit a 1–4, 2–3, 5–6 pattern for the cystine organisation within the TSP I segment of C9 (Fig. 1a).

The following low density lipoprotein receptor class A-homologous domain (Asp<sup>78</sup> to Arg<sup>118</sup>) also contains 6 half-cystines of unknown linking. Previous studies suggest the occurrence of a disulfide bridge between Cys<sup>80</sup> and Cys<sup>91</sup> [31,32]. As analysis of E18, CB1, and SK2 showed, we were able to prepare samples from various digests that essentially contained both N-terminal domains. With sequences ranging from Asp<sup>78</sup> beyond Cys<sup>113</sup>, subsequent application of V8 protease on E18 even yielded, among others, several fragments that were almost fully identical to LDL A. Further subfragmentation of those peptides, however, proved to be extremely difficult. In experiments not further reported here additional attempts were made using such powerful means as 2% formic acid (v/v), 6 M hydrochloric acid, subtilisin, or thermolysin. While hydrochloric acid effected an almost complete degradation even under altered reaction conditions in terms of acid concentration, temperature, and incubation time, neither formic acid nor subtilisin nor thermolysin generated suitable peptides that would have allowed an unambiguous identification of disulfides by the tools at disposal in this study. Therefore only E18V7 gave further accounts of the disulfide structure in that particular region. When that result is combined with the conclusions drawn from the citations above, the number of possible disulfide configurations fulfilling both criteria is significantly reduced to a set of two situations. Assuming that Cys<sup>80</sup> is linked to Cys<sup>91</sup>, Cys<sup>86</sup> is then connected to either Cys<sup>104</sup> or Cys<sup>113</sup>, and Cys<sup>98</sup> reciprocally to Cys<sup>113</sup> or Cys<sup>104</sup>. From a more general view, that actually means that either a 1–3, 2–5, 4–6 or a 1–3, 2–6, 4–5 pattern can be inferred for the LDL A module (Fig. 1b,c).

Table 2  
Prediction of the disulfides in C6, C7, C8 $\alpha$ , and C8 $\beta$ , as can actually be inferred from the investigations made of C9

	C9	C6	C7	C8 $\alpha$	C8 $\beta$
Tsp I	22–57 33–36 67–73	61–96 72–75 106–112	6–41 17–20 51–57	9–44 20–23 54–60	11–46 22–25 56–62
LDL A	80–91 86–104 98–113	119–130 125–143 137–152	63–74 69–87 81–97	66–78 72–91 85–100	68–79 73–92 86–101
or	80–91 86–113 98–104	119–130 125–152 137–143	63–74 69–97 81–87	66–78 72–100 85–91	68–79 73–101 86–92
Central region	121–160 233–234 359–384	159–197 378–399	106–143 315–331	110–147 345–369	108–146 324–349
LDL B (EGF)	489–505 492–507 509–518	502–518 505–520 522–531	435–451 438–453 455–464	469–485 472–487 489–498	451–467 454–469 471–480

The half-cystines indicated are numbered proceeding from the N-terminus of the mature proteins [13,14,16,21,22,38]. The individual sequences were compared with the use of the program package CLUSTAL [37].

LDL A is followed by a long central stretch of amino acids (Pro<sup>119</sup> to Arg<sup>487</sup>) that is lacking distinct modular features. It contains again 6 half-cystines. Their adjoinment has partially been postulated by means of limited proteolysis soon after the determination of the C9 sequence [7]. It has to be mentioned, however, that there have been uncertainties especially as far as the linking of the adjacent Cys<sup>233</sup> and Cys<sup>234</sup> is concerned [9].

The present study provides evidence that the disulfides in that particular region are in fact arranged as previously suggested. Characterization of fractions CB1S18 and CB1S19 confirmed the connection of Cys<sup>121</sup> with Cys<sup>160</sup>. CB1P16 contained a fragment that included the disulfide bond between Cys<sup>359</sup> and Cys<sup>384</sup>. Upon analysis of CB1S11 we further demonstrated that Cys<sup>233</sup> is really bonded to Cys<sup>234</sup> in a vicinal linkage (Fig. 1d).

Vicinal disulfide bridges have only rarely been encountered so far. Since the residues involved have to attain a highly strained conformation, such arrangements are extremely unstable. In the context of C9, it is of special interest that Cys<sup>233</sup> and Cys<sup>234</sup> lie within a wide heterologous sequence insertion of pronounced structural flexibility [9]. Accordingly, that region is considered to act as hinge when C9 unfolds upon interaction with the membrane-bound C5b-8 receptor complex. With regard to the vicinal disulfide pairing proved in this study, it is therefore attractive to relate the shape change of the molecule from a globular into an extended state after activation with a dissociation of the very cystine. The liberated sulfhydryl groups might subsequently form intermolecular cross-links with neighbouring C9 units, which are known to occur in high concentration at the sites of MAC formation. The final consequences of such events would be imaged by the extremely resistant C9 tubule. The notion of a local disulfide exchange is supported by studies carried out previously [33,34].

The 6 disulfides within the adjoining low density lipoprotein receptor class B module (Lys<sup>488</sup> to Lys<sup>522</sup>), synonymously known as epidermal growth factor domain, were shown to follow the general pattern for EGF [35,36]. CB2S16 contained a fragment comprising the disulfide-bridge between Cys<sup>509</sup> and Cys<sup>518</sup>. Upon analysis of CB1V17 we were furthermore able to observe the disulfides between Cys<sup>489</sup> and Cys<sup>505</sup>, between Cys<sup>492</sup> and Cys<sup>507</sup>, and to a lesser extent again the link between Cys<sup>509</sup> and Cys<sup>518</sup>. The well-known 1–3, 2–4, 5–6 disulfide arrangement is thus deduced (Fig. 1e).

The entire length of the C9 sequence is highly conserved in all other terminal complement proteins. With the exception of two adjacent residues almost in the very center of the polypeptide strand, all the half-cystines occurring in C9 appear in comparable positions in C6, C7, C8 $\alpha$ , and C8 $\beta$ . The disulfides assigned in this study can therefore be superimposed on the sequences of those proteins. Such a sequence comparison was carried out by means of the sequence alignment program package CLUSTAL (PC/GENE) [37]. The data obtained are summarized as a final result in Table 2.

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