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Articles

Determination of the Disulfide Structure of Sillucin, a Highly Knotted, Cysteine-Rich Peptide, by Cyanylation/Cleavage Mass Mapping†

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ABSTRACT: The disulfide structure of sillucin, a highly knotted, cysteine-rich, antimicrobial peptide, isolated from *Rhizomucor pusillus*, has been determined to be Cys2−Cys7, Cys12−Cys24, Cys13−Cys30, and Cys14−Cys21 by disulfide mass mapping based on partial reduction and CN-induced cleavage enabled by cyanylation. The denatured 30-residue peptide was subjected to partial reduction by tris(2-carboxyethyl)-phosphine hydrochloride at pH 3 to produce a mixture of partially reduced sillucin species; the nascent sulfhydryl groups were immediately cyanylated by 1-cyano-4-(dimethylamino)pyridinium tetrafluoroborate. The cyanylated species, separated and collected during reversed phase high-performance liquid chromatography, were treated with aqueous ammonia, which cleaved the peptide chain on the N-terminal side of cyanylated cysteine residues. The CN-induced cleavage mixture was analyzed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry before and after complete reduction of residual disulfide bonds in partially reduced and cyanylated species to mass map the truncated peptides to the sequence. Because the masses of the CN-induced cleavage fragments of both singly and doubly reduced and cyanylated sillucin are related to the linkages of the disulfide bonds in the original molecule, the presence of certain truncated peptide(s) can be used to positively identify the linkage of a specific disulfide bond or exclude the presence of other possible linkages.

The presence of antimicrobial peptides in growth media of *Rhizomucor pusillus* (formerly *Mucor pusillus*) and *Rhizomucor miehei* (formerly *Mucor miehei*) was first observed more than thirty years ago (*1*). Purification and compositional analysis of bioactive peptides isolated from different strains established that *R. pusillus* synthesized a 30-residue peptide termed sillucin, whereas *R. miehei* strains produced the antimicrobial peptides termed mieheins that ranged in size from 56 to 74 amino acids (*2*). The range of antimicrobial activities of these cationic peptides was found to be limited to Gram-positive microorganisms.

A common feature of the antimicrobial peptides from *Rhizomucor* species is the high cysteine content varying from 8 residues in sillucin up to 16 residues in mieheins (*2*). Negative analytical tests for reactive −SH groups in sillucin and mieheins indicated that, most likely, all cysteine residues in these peptides were involved in disulfide bonds, resulting

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in a highly complex tertiary structure. Although the primary structure of sillucin was determined (*3*) to be A***C***LPNS***C***VSK G***CCC***GBSGYW ***C***RQ***C***GIKYT***C*** (where B represents either N or D; in our work, B is determined to be D by accurate mass measurement, as described below), the specific disulfide bond pattern between its cysteine residues has remained unknown.

Antimicrobial peptides from eukaryotes have become the subject of intensive research since they may serve as potential sources of new antibiotics for therapeutic applications (*4*, *5*). Because minor variations in peptide structure can influence antimicrobial activity, precise knowledge of the location of disulfide bonds is a prerequisite for future structure−activity studies of these bioactive peptides.

The conventional methodology for determining disulfide linkages involves enzymatically or chemically cleaving the peptide backbone between the cysteine residues and identifying the peptide fragments that contain disulfide bonds or are linked by them. Analysis of proteins containing adjacent cysteines is very difficult, if not possible, with the conventional methodology as there is no adequate method for cleaving between the adjacent cysteine residues (*6*, *7*). In special cases such as the one described by Poerio et al. (*8*), proteolytic digests can be analyzed by a combination of Edman degradation and fast atom bombardment mass spectrometry (FAB-MS) to determine the linkage of two disulfide bonds involving adjacent cysteines. However, success with this approach is only possible when the residual string of residues on the N-terminal side of the pair of adjacent cysteines in one peptide of the three-peptide proteolytic fragment (which is linked by two disulfide bonds) is shorter than the residual string of residues on the N-terminal side of the cysteine residue in each of the other two peptides (*8*, *9*). A similar fortunate occurrence took place during the analysis of insulin (*9*) and huwentoxin-I (*10*), where 17 and 6 Edman degradation steps, respectively, on the intact molecule were required to provide evidence for the disulfide bond pattern involving two adjacent cysteines.

In another case, fast atom bombardment mass spectrometry/mass spectrometry (FAB-MS/MS) was used to confirm the Cys6−Cys48/Cys47−Cys52 prediction in insulin-like growth factors (IGFs) (*11*). Recently, matrix-assisted laser desorption ionization (MALDI)[1] postsource decay (PSD) was found to successfully fragment the peptide bonds between two adjacent cysteines in a three-peptide proteolytic fragment linked by two disulfide bonds; however, the generality of this procedure remains to be tested (*12*).

In some cases, if enough sample and good crystals can be obtained, high-resolution X-ray crystallography might be used to determine the disulfide structure involving adjacent cysteines (*13*). In similar situations, two-dimensional nuclear magnetic resonance (2D-NMR) could be applied to this analytical problem, if a sufficient sample is available (*14*). In general, efficient chemical methods are still needed for quality control of recombinant disulfide proteins.

[1] Abbreviations: MALDI-MS, matrix-assisted laser desorption and ionization mass spectrometry; TCEP, tris(2-carboxyethyl)phosphine; CDAP, 1-cyano-4-(dimethylamino)pyridinium; TFA, trifluoroacetic acid; HPLC, high-performance liquid chromatography; itz, 2-iminothiazolidine-4-carboxyl, resulting from the CN-induced cleavage by aqueous ammonia on the N-terminal side of the cyanylated cysteines; MH^+, protonated molecule; *m*/*z*, mass-to-charge ratio; β, β-elimination.

Partial reduction by the water-soluble tris(2-carboxyethyl)-phosphine (TCEP) hydrochloride to generate an array of partially reduced protein species containing disulfides and thiols has been widely used to develop new strategies for assigning disulfide bonds (*15*−*18*). Such strategies have been especially useful in determining the disulfide structure of peptides and proteins with a cysteine-knotted core or those with adjacent cysteines, which are very difficult to analyze with traditional strategies based on enzymatic digestions. For most of these strategies, the nascent free cysteine residues produced during partial reduction were alkylated and detected by Edman sequencing, as the basis for deducing the disulfide linkages. However, as alkylation is achieved under basic conditions, artifacts due to disulfide scrambling could be a problem. Another shortcoming of these strategies is that only small peptides can be considered because the resulting alkylated peptides finally must be analyzed by Edman sequencing.

A novel analytical strategy developed by Wu and Watson (*19*) uses partial reduction by TCEP of a multi-disulfide protein to produce singly reduced isoforms, which are then cyanylated by 1-cyano-4-(dimethylamino)pyridinium (CDAP) tetrafluoroborate, chemically cleaved by aqueous ammonia on the N-terminal side of the cyanylated cysteine residues, and mass mapped to the sequence for assignment of disulfide bond linkages in proteins. The feasibility of analyzing disulfide structures involving adjacent cysteines by the cyanylation/CN-induced cleavage procedure was proven by Yang et al. (*20*).

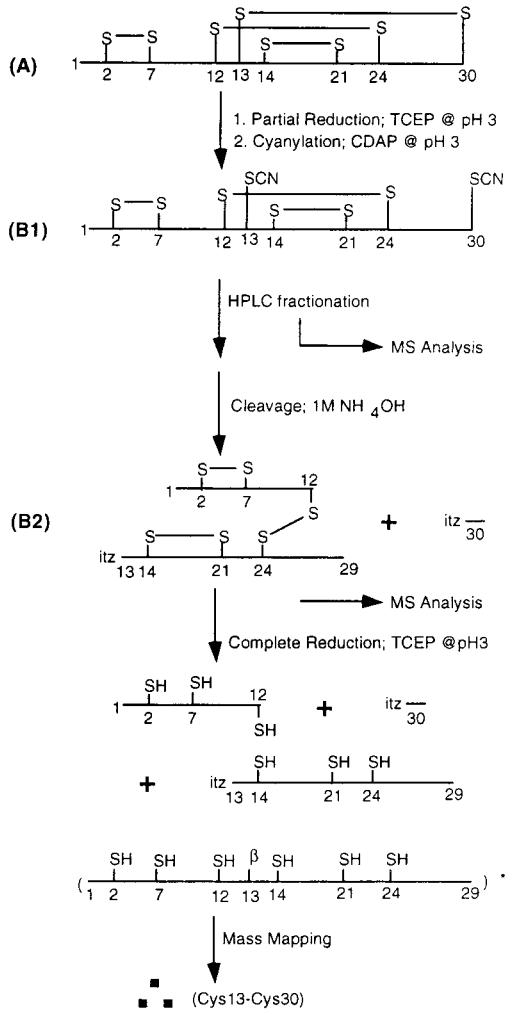
Sillucin is resistant to various proteases, in part because of its three adjacent cysteines and its tightly folded structure, and attempts to determine its disulfide structure using conventional methods based on enzymatic digestion have not been successful. However, by mass mapping the CN-induced cleavage products from both singly reduced and cyanylated and doubly reduced and cyanylated isoforms, we have been able to determine the disulfide structure of sillucin to be Cys2−Cys7, Cys12−Cys24, Cys13−Cys30, and Cys14−Cys21. The chemical processes and array of CN-induced cleavage products used in deducing the disulfide connectivity in sillucin are illustrated in Schemes 1 and 2.

MATERIALS AND METHODS

Chemicals. Sillucin was isolated and purified according to methods previously described (*1*, *2*). Tris(2-carboxyethyl)-phosphine (TCEP) hydrochloride was purchased from Pierce Chemical Co. (Rockford, IL). Guanidine hydrochloride was a product of Boehringer-Mannheim Biochemicals (Indianapolis, IN). 1-Cyano-4-(dimethylamino)pyridinium (CDAP) tetrafluoroborate was purchased from Sigma. Water, acetonitrile, and trifluoroacetic acid (TFA) were HPLC grade. TCEP and CDAP solutions were freshly prepared in 0.1 M citrate buffer (pH 3.0) prior to use. Because of the instability of CDAP in aqueous solution, solid CDAP was dissolved in pH 3 buffer just before use.

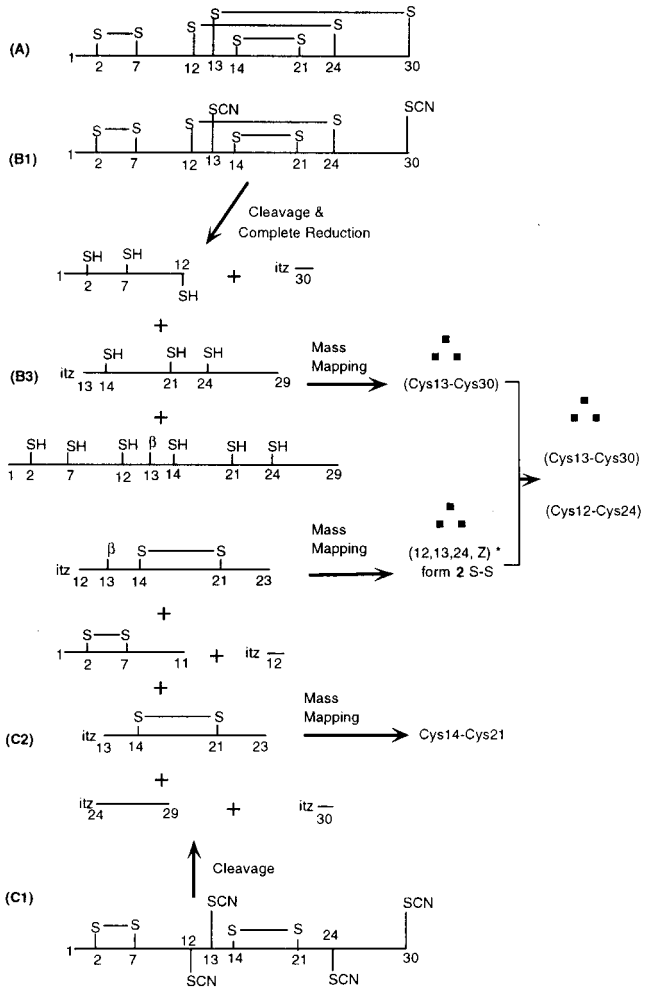
Partial Reduction and Cyanylation. A solution containing 20 nmol of sillucin was prepared in 10 μL of 0.1 M citrate buffer (pH 3.0) containing 6 M guanidine hydrochloride. Partial reduction of sillucin was carried out by adding 1600 nmol of TCEP (16 μL of a 0.1 M TCEP solution), followed by incubation at 50 °C for 10 min. Other trials of partial

Scheme 1: Overview of the Chemical Reactions Involved in the Disulfide Mass Mapping of a Singly Reduced and Cyanylated Isoform of Sillucin[a]



[a] Recognition of the cleaved fragments by mass spectrometry allows the linkage of the Cys13−Cys30 disulfide bond to be deduced. Itz represents the 2-iminothiazolidine-4-carboxyl-blocked N-terminus resulting from CN-induced cleavage by aqueous ammonia on the N-terminal side of the cyanylated cysteines. The structure in parentheses designates a β-elimination product, which results in this case from loss of HSCN at cyanylated Cys13 (β-elimination) and CN-induced cleavage at cyanylated Cys30.

Scheme 2: Overview of Chemical Reactions Involved in the Disulfide Mass Mapping of a Doubly Reduced and Cyanylated Isoform (C1) of Sillucin (A) as Well as a Singly Reduced and Cyanylated Isoform (B1)[a]



[a] Recognition of the cleaved fragments (C2) from C1 without complete reduction allows the connectivity of the Cys14−Cys21 to be deduced. Recognition of the cleaved fragments (B3) from B2 (shown in Scheme 1) after complete reduction combined with the determination of the Cys14−Cys21 disulfide bond as described above allows the connectivity of the Cys12−Cys24 disulfide bond to be deduced; *Z* could be 2, 7, or 30.

reduction under different conditions of stoichiometry, temperature, and reaction time were conducted similarly to optimize production of the singly or doubly reduced isoforms; such a search for reasonably optimum reaction conditions is typical because of the diverse range of stabilities of disulfide bonds from protein to protein.

To the TCEP reaction mixture containing partially reduced sillucin isoforms was added 4000 nmol of CDAP (20 μL of a 0.2 M CDAP solution; more than 2-fold equivalent to TCEP because residual TCEP reacts with CDAP). Cyanylation of the nascent sulfhydryl groups was accomplished during incubation at room temperature for 15 min.

HPLC Separation of Partially Reduced and Cyanylated Sillucin Species. Mixtures of partially reduced and cyanylated species were separated by reversed phase HPLC using a linear gradient elution of 15 to 40% B over the course of 50 min [B is 90% (v/v) acetonitrile and 0.1% TFA; A is 0.1% TFA in water] with Waters model 6000 pumps controlled by a personal computer. UV detection was carried out at 215 nm. A Vydac C18 (218TP54, 5 μm particle size, 300 Å pores, 4.6 mm × 250 mm) column was used. The predominant HPLC fractions were collected manually, and the masses of the collected species in each fraction (approximately 0.8−1.0 mL) were determined by MALDI-MS.

CN-Induced Cleavage of Partially Reduced and Cyanylated Sillucin Species. A solution of a dried HPLC fraction was reconstituted in 2 μL of 6 M guanidine hydrochloride in 1 M aqueous ammonia to dissolve the partially reduced and cyanylated sillucin species; to this solution was added an additional 8 μL of 1 M aqueous ammonia. CN-induced cleavage of the peptide chain on the N-terminal side of cyanylated cysteine residues was accomplished during reaction at room temperature for 1 h. Excess ammonia was removed in a vacuum system. Truncated peptides, still linked in some cases by remaining disulfide bonds depending on the extent of partial reduction, were dissolved in 10 μL of water; 1 μL of this solution was further diluted with 9 μL of

50% (v/v) acetonitrile and 0.1% TFA, and analyzed by MALDI-MS. The other 9 μL of the solution was dried in a vacuum system for future use.

Complete Reduction of the Remaining Disulfide Bonds. Truncated peptides, from the CN-induced cleavage reaction, some still linked by residual disulfide bonds, were completely reduced by reaction with 200 nmol of TCEP (2 μL of a 0.1 M TCEP solution) at 37 °C for 30 min. Samples were then diluted with 90 μL of a 50% (v/v) aqueous acetonitrile/0.1% TFA solution prior to analysis by MALDI-MS.

MALDI-MS. MALDI mass spectra were obtained on a Voyager DE-STR time-of-flight (TOF) mass spectrometer (Perkin-Elmer Biosystems Inc., Framingham, MA) equipped with a 337 nm nitrogen laser. The accelerating voltage in the ion source was set at 25 kV. Except for the accurate mass measurement of intact sillucin that was carried out in the positive reflectron mode, all other data were acquired in the positive linear mode of operation. Time-to-mass conversion was achieved by internal calibration using standards of bovine bradykinin [monoisotopic mass of the protonated molecule (MH^+), 1060.569 Da; average mass of MH^+, 1061.217 Da], β-laminin (monoisotopic mass of MH^+, 2427.279 Da; average mass of MH^+, 2428.899 Da), and oxidized bovine pancreatic insulin chain B (monoisotopic mass of MH^+, 3494.651 Da; average mass of MH^+, 3496.903 Da) obtained from Sigma Chemical Co. (St. Louis, MO). All experiments were performed using α-cyano-4-hydroxycinnamic acid (Aldrich Chemical Co., Milwaukee, WI) as the matrix. Saturated matrix solutions were prepared in a 50% (v/v) aqueous solution of acetonitrile and 0.3% TFA. Just prior to analysis, 0.5 μL of the matrix solution was applied to the stainless steel sample plate and allowed to air-dry; then 1 μL of the sample solution was added to the top of it, and finally, an additional 1 μL of the matrix solution was added to the same spot. The mixture was allowed to air-dry before being introduced into the mass spectrometer.

RESULTS

Accurate Mass Measurement of Intact Sillucin. The sequence of sillucin, determined previously by Bradley and Somkuti (*3*), is A***C***LPNS***C***VSK G***CCC***GBSGYW ***C***RQ***C***GI-KYT***C*** (where B stands for N or D). As the mass measurement error in the range of 2000−4000 Da using internal standardization in the reflectron mode on a MALDI-TOF mass spectrometer (PE Voyager DE-STR) is less than 30 ppm (data not shown), the uncertainty in the previously determined sequence of sillucin can be resolved by accurate mass measurement. The monoisotopic mass of protonated sillucin (MH^+) is determined to be 3200.23 ± 0.04 Da (3200.23 is mean of seven measurements and 0.04 is the confidence value at the 95% level; mass spectra not shown). The data indicate that sillucin has aspartic acid (D) in position 16 (with an overall calculated monoisotopic mass of 3200.211 Da for MH^+), instead of asparagine (N) (with an overall calculated monoisotopic mass of 3199.227 Da for MH^+). These experimental data also indicate that sillucin has four disulfide bonds because the observed mass of 3200.23 ± 0.04 Da is in agreement with the calculated mass of 3200.211 Da for MH^+, which is calculated assuming that all cysteines are involved in disulfide bonds.

Partially Reduced and Cyanylated Isoforms of Sillucin. Four major components were separated by HPLC after partial reduction and cyanylation of sillucin (Figure 1). Analyses of aliquots of designated fractions by MALDI-MS indicated (data not shown) that the HPLC fraction IP represents the intact peptide [observed MALDI peak at mass-to-charge ratio (*m*/*z*) of 3202.8 represents MH^+, whose calculated average mass is 3202.7 Da]. HPLC fractions 1−3 represent species that are 52, 104, and 208 Da heavier (MALDI peaks at *m*/*z* 3254.9, 3306.6, and 3410.8, respectively), respectively, than the intact peptide. Because two CN groups are added to a singly reduced species upon cyanylation, HPLC fraction 1 corresponds to one of the four possible singly reduced and cyanylated isoforms of sillucin. Similarly, HPLC fraction 2 corresponds to one of the six possible doubly reduced and cyanylated isoforms, and HPLC fraction 3 represents the completely reduced and cyanylated sillucin.

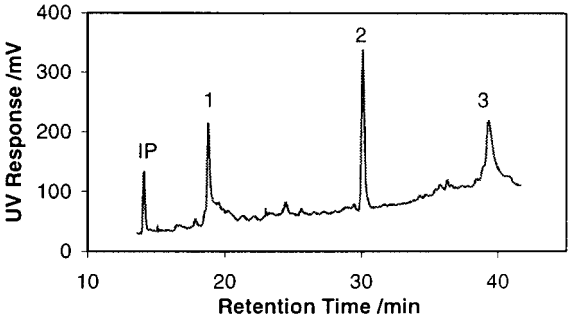


FIGURE 1: HPLC chromatogram of denatured sillucin (IP) and some of its partially reduced and cyanylated isoforms. Separation was carried out on a Vydac C18 column at a flow rate of 1.0 mL/min with a linear gradient of 15 to 40% B in 50 min, where A is 0.1% TFA in water and B is a 90% (v/v) acetonitrile/0.1% TFA mixture. Peak IP, 1, and 2 represent the intact peptide, a singly reduced/cyanylated species, and a doubly reduced/cyanylated species, respectively; peak 3 represents the completely reduced/cyanylated species, as determined from analysis by MALDI-MS.

Singly Reduced and Cyanylated Species. Because there are eight cysteine residues in sillucin, 105 disulfide linkage patterns are possible. Although there are four possible singly reduced species for each of the 105 isomers, the total number of possible singly reduced species is only 28 because some isomers have the same disulfide bonds as the others. The calculated *m*/*z* value for MH^+ of each of the possible CN-induced cleavage fragments resulting from all 28 possible singly reduced and cyanylated species is listed in Table 1.

The MALDI-MS spectrum in Figure 2 was obtained upon analysis of the mixture of products resulting from CN-induced cleavage and following complete reduction of the singly reduced and cyanylated species (HPLC fraction 1); it shows two major peaks at *m*/*z* 1968.1 and 3072.7. As the mass measurement error in the range of 1000−3500 Da using internal standardization in the linear mode on a MALDI-TOF mass spectrometer (PE Voyager DE-STR) is less than 0.02% (data not shown), a certain MALDI peak can be assigned to the CN-induced cleavage product, which has a calculated mass that falls into the range of mass ± (0.02% × mass). According to calculated values in Table 1, the peak at *m*/*z* 1968.1 could correspond to itz-13−29-red or itz-14−30-red ("red" refers to the species in which the remaining disulfide bonds have been reduced during the complete reduction step) because each of their MH^+ ions has a calculated mass of 1968.2 Da. The peak at *m*/*z* 3072.7 can be attributed to 1-(*β@X*)-29-red [calculated mass of MH^+ = 3072.5 Da; *β@X* refers to cyanylation, but subsequent

Table 1: Calculated *m/z* Values for Possible Fragments[e] Resulting from CN-Induced Cleavage of Singly Reduced and Cyanylated Species of Sillucin Isomers with All Possible Disulfide Linkage Patterns

C_x-C_y[a]	1-($x-1$)[b]	itz-*x*-($y-1$)	itz-*y*-30	1-(β@*x*)-($y-1$)[c]	itz-*x*-(β@*y*)-30
C_2-C_7	89.1	557.6	2649.1	569.6	3130.6
C_7-C_{12}	603.7	517.6	2174.5	1044.2	2615.0
C_2-C_{12}	89.1	1032.2	2174.5	1044.2	3130.6
$C_{12}-C_{13}$	1078.3	146.2	2071.4	1147.3	2140.4
C_7-C_{13}	603.7	620.7	2071.4	1147.3	2615.0
C_2-C_{13}	89.1	1135.3	2071.4	1147.3	3130.6
$C_{13}-C_{14}$	1181.4	146.2	**1968.2**[d]	1250.4	2037.3
$C_{12}-C_{14}$	1078.3	249.3	**1968.2**	1250.4	2140.4
C_7-C_{14}	603.7	723.9	**1968.2**	1250.4	2615.0
C_2-C_{14}	89.1	1238.5	**1968.2**	1250.4	3130.6
$C_{14}-C_{21}$	1284.5	811.8	1199.4	2019.2	1909.2
$C_{13}-C_{21}$	1181.4	915.0	1199.4	2019.2	2037.3
$C_{12}-C_{21}$	1078.3	1018.1	1199.4	2019.2	2140.4
C_7-C_{21}	603.7	1492.7	1199.4	2019.2	2615.0
C_2-C_{21}	89.1	2007.3	1199.4	2019.2	3130.6
$C_{21}-C_{24}$	2053.3	430.5	812.0	2406.7	1165.3
$C_{14}-C_{24}$	1284.5	1199.4	812.0	2406.7	1934.1
$C_{13}-C_{24}$	1181.4	1302.4	812.0	2406.7	2037.3
$C_{12}-C_{24}$	1078.3	1405.6	812.0	2406.7	2140.4
C_7-C_{24}	603.7	1880.1	812.0	2406.7	2615.0
C_2-C_{24}	89.1	2394.7	812.0	2406.7	3130.6
$C_{24}-C_{30}$	2440.8	708.8	146.2	**3072.5**	777.9
$C_{21}-C_{30}$	2053.3	1096.3	146.2	**3072.5**	1141.3
$C_{14}-C_{30}$	1284.5	1865.1	146.2	**3072.5**	1934.1
$C_{13}-C_{30}$	1181.4	**1968.2**	146.2	**3072.5**	2037.3
$C_{12}-C_{30}$	1078.3	2071.4	146.2	**3072.5**	2140.4
C_7-C_{30}	603.7	2545.9	146.2	**3072.5**	2615.0
C_2-C_{30}	89.1	3060.5	146.2	**3072.5**	3130.6

[a] C_x-C_y designates the disulfide bond that is reduced in the singly reduced and cyanylated species. [b] 1-(*x*−1), itz-*x*-(*y*−1), and itz-*y*-30 designate the CN-induced cleavage fragments. [c] 1-(β@*x*)-(*y*−1) and itz-(β@*y*)-30 designate the β-elimination products. [d] Bold *m/z* values indicate matches with experimental data. [e] Residual disulfides reduced.

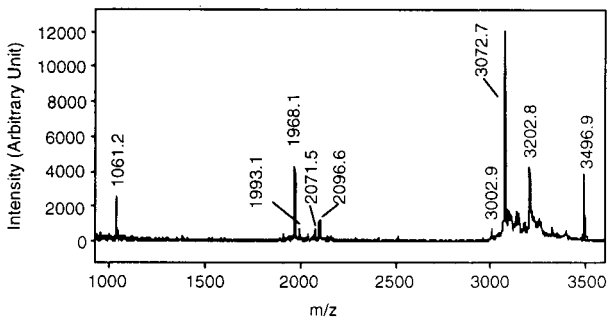
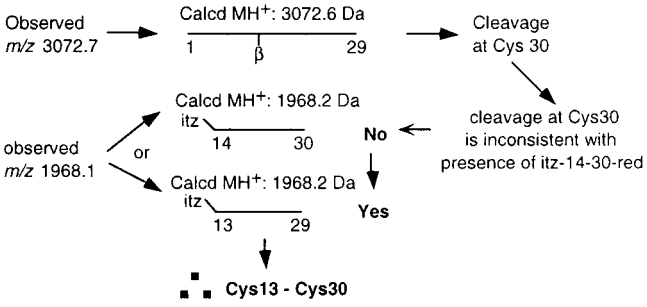


FIGURE 2: MALDI-MS spectrum of the peptide mixture resulting from CN-induced cleavage and complete reduction of the singly reduced and cyanylated sillucin species represented by HPLC peak 1 in Figure 1.

β-elimination (no CN-induced cleavage at an unspecified cyanylated cysteine residue)] which results from CN-induced cleavage on the N-terminal side of cysteine 30 and β-elimination at one of the other cysteines: Cys2, -7, -12, -13, -14, -21, or -24. The presence of 1-(β@*X*)-29-red shows that the disulfide bond being reduced to form the singly reduced and cyanylated species (HPLC fraction 1) is Cys30−Cys*X*, where Cys*X* could be any one of the remaining cysteines: Cys2, -7, -12, -13, -14, -21, or -24. Let us assume that the MALDI peak at *m/z* 1968.1 corresponds to itz-14−30-red, which would infer that the disulfide bond being reduced corresponds to Cys14−Cys*Y*, where Cys*Y* could be either Cys2, -7, -12, or -13. However, the assumption that the MALDI peak at *m/z* 1968.1 corresponds to itz-14−30-red, though arithmetically possible, would be inconsistent with the detected

Scheme 3: Rationale for Assignment of the Cys13−Cys30 Disulfide



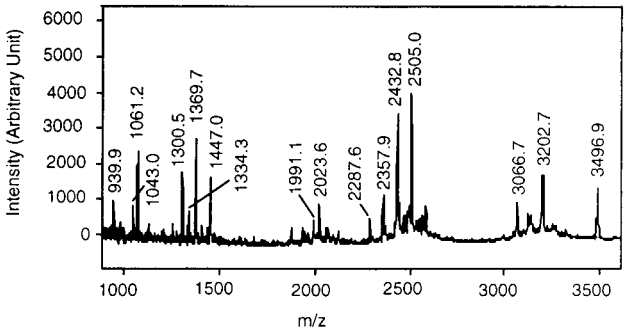


FIGURE 3: MALDI-MS spectrum of the peptide mixture resulting from CN-induced cleavage of the doubly reduced and cyanylated sillucin species represented by HPLC peak 2 in Figure 1.

presence of 1-(β@*X*)-29-red. Consequently, the MALDI peak at *m/z* 1968.1 is better attributed to itz-13−29-red, which infers CN-induced cleavage at Cys13 and Cys30, and thus, the disulfide bond between Cys13 and Cys30 must have been reduced during partial reduction to form the singly reduced and cyanylated species (HPLC fraction 1). Assignment of this disulfide bond, Cys13−Cys30, is illustrated by Scheme 3. Another expected fragment of residues 1−12-red (calculated mass of $MH^+ = 1181.4$ Da) was not detected because of signal suppression in MALDI. It was detected in MALDI after it was separated from other species in the mixture by HPLC (data not shown.) Fragment itz-30 (calculated mass of $MH^+ = 146.2$ Da) falls into the matrix peaks region and cannot be detected.

Doubly Reduced and Cyanylated Isoform. The MALDI-MS spectrum in Figure 3 corresponds to the analysis of the products resulting from CN-induced cleavage (before complete reduction) of the doubly reduced and cyanylated isoform (HPLC fraction 2). The MALDI peak at *m/z* 1300.5 can be attributed to itz-13−23-ox (calculated mass of $MH^+ = 1300.4$ Da; "ox" indicates that fragment contains residual disulfide bonds). As this analysis by MALDI-MS was done before complete reduction of the sample, the presence of itz-13−23-ox implies the existence of a disulfide bond between Cys14 and Cys21 (Scheme 4). With the knowledge of the Cys14−Cys21 disulfide bond, the MALDI peak at *m/z* 1369.7 can be inferred as representing itz-12-(β@13)-23-ox (calculated mass of $MH^+ = 1369.5$ Da); thus, Cys12, Cys13, Cys24, and Cys*Z* form two disulfide bonds since two disulfide bonds had to be reduced to form the doubly reduced and cyanylated species (HPLC fraction 2). Together with the knowledge of the Cys13−Cys30 disulfide bond described above, Cys*Z* must be Cys30. Thus, the existence of another disulfide bond, Cys12−Cys24, can be deduced (Scheme 4).

Scheme 4: Rationale for Assignment of the Cys14−Cys21, Cys12−Cys24, and Cys2−Cys7 Disulfide Bonds

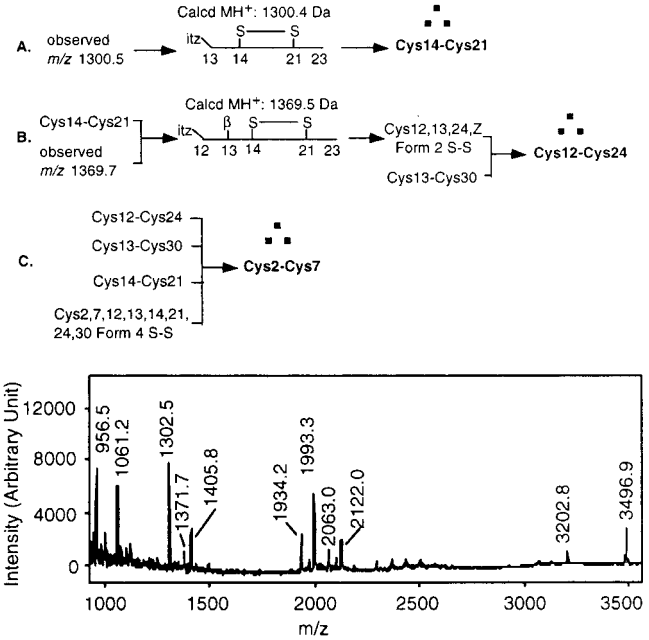


FIGURE 4: MALDI-MS spectrum of the peptide mixture resulting from CN-induced cleavage and complete reduction of the doubly reduced and cyanylated sillucin species represented by HPLC peak 2 in Figure 1.

Analysis by MALDI-MS (data not shown) shows that denatured intact sillucin does not react with CDAP, the cyanylating reagent. This infers that there are no free sulfhydryls in the sillucin molecule and that all eight cysteine residues are engaged in forming four disulfide bonds. This observation is in agreement with the accurate mass measurement described above and literature results (*3*). With the three disulfide bonds, Cys12−Cys24, Cys13−Cys30, and Cys14−Cys21, having been deduced as described above, the fourth disulfide bond (Cys2−Cys7) can be deduced by default. These assignments are summarized in Scheme 4.

The MALDI-MS spectrum in Figure 4 was obtained from analysis of the products from CN-induced cleavage and complete reduction of the doubly reduced and cyanylated species (HPLC fraction 2). The MALDI peak at *m*/*z* 1302.5 (Figure 4) is 2 mass units higher than the corresponding peak at *m*/*z* 1300.5 (Figure 3), which represents the oxidized form itz-13−23-ox (calculated mass of $MH^+ = 1300.4$ Da); the peak at *m*/*z* 1302.5 can be attributed to itz-13−23-red (calculated mass of $MH^+ = 1302.4$ Da), in which the Cys14−Cys21 disulfide bond had been reduced by the subsequent complete reduction step. This mass spectrometric evidence of a shift of 2 Da upon reduction of itz-13−23-ox to itz-13−23-red confirms the existence of the Cys14−Cys21 disulfide bond. Similarly, the MALDI peak at *m*/*z* 1371.7 can be assigned to itz-12-(*β*@13)-23-red (calculated mass of $MH^+ = 1371.5$ Da), which results from reduction of the Cys14−Cys21 disulfide bond in itz-12-(*β*@13)-23-ox (observed at *m*/*z* 1369.7; calculated mass of $MH^+ = 1369.5$ Da).

In summary, the linkage of four disulfide bonds in sillucin is identified as Cys2−Cys7, Cys12−Cys24, Cys13−Cys30, and Cys14−Cys21, by partial reduction, cyanylation, and CN-induced cleavage with or without complete reduction, followed by mass mapping.

DISCUSSION

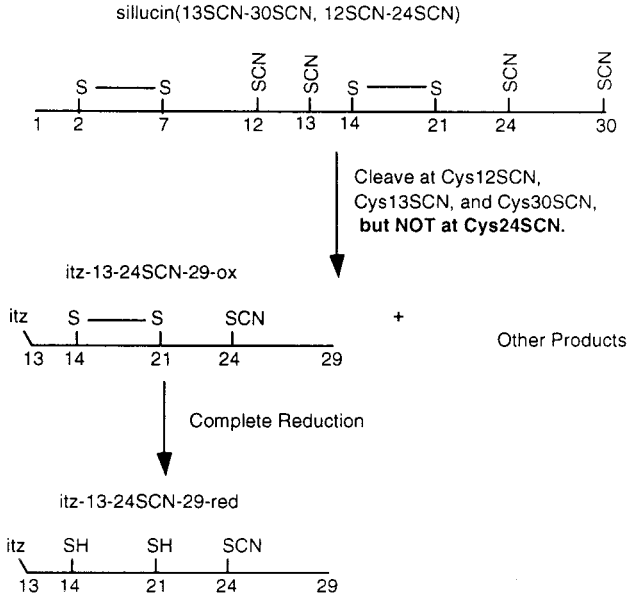
Partial Reduction. Obtaining several partially reduced species of a multi-disulfide peptide is essential for successful determination of the disulfide linkages. All the disulfide linkage information is lost by generating the completely reduced peptide species. The rationale for optimizing partial reduction conditions is to generate a reasonable yield of partially reduced peptides with at least one disulfide bond opened, while at least one remains closed (*21*). For cysteine-rich, knotted peptides, it is best to use a large excess of reducing reagent (TCEP) and control the extent of reduction by varying the time and temperature (*21*). Partial reduction can be accomplished in a relatively short time (in the range of 2−15 min) and stopped by removing the TCEP by HPLC when the reaction mixture is injected while still under kinetic control. As a result, a fair amount of the starting material will be left untouched, while a useful amount of partially reduced peptide species is obtained. The general guidelines for optimization of partial reduction have been established by Gray (*21*) and Wu (*19*); usually, only a few experiments are needed (*21*).

Because 8 of the 30 amino acid residues in sillucin are cysteine, including three adjacent cysteines, the peptide appears to be in a knotted arrangement, which offers great resistance to chemical reduction. For this reason, a large excess (10-fold equivalent excess over total disulfide content) was used in the initial trial for optimizing the partial reduction reaction. For the same reason, the temperature of the partial reduction reaction was increased from room temperature to 40 °C, and eventually to 50 °C. A good yield (≥25%) of doubly reduced sillucin was produced during the third attempt to optimize the partial reduction reaction by increasing the temperature to 50 °C while using a 20-fold equivalent excess over total disulfide content for 10 min; the relative distribution of products in this reaction mixture is represented by the HPLC chromatogram shown in Figure 1. The reaction mixture from the first two optimization trials gave similar chromatograms (data not shown), except with considerably smaller peaks for the singly and doubly reduced species.

Information leading to the identification of one disulfide bond (Cys13−Cys30) can be gleaned from mass mapping analysis of the reaction mixture following CN-induced cleavage of a singly reduced isoform of sillucin before and after complete reduction of residual disulfide bonds; the key reactions and data are shown in Scheme 1. Ideally, one would hope to isolate a singly reduced isoform of the intact peptide that corresponds to each of the disulfide bonds; this was the case for ribonuclease A (consisting of eight cysteines in the form of four disulfides), where nearly equal amounts of four singly reduced isoforms were available for mass mapping (*19*). However, in the case of sillucin, its knotted conformation apparently prevents equal access of the chemical reducing agent to each of the disulfide bonds, and thus, only one of the four possible singly reduced isoforms can be readily generated.

In situations where an insufficient number of singly reduced isoforms of the analyte is available, attempts to produce and isolate doubly reduced isoforms of the analyte may solve the problem. Such is the case with sillucin, where as indicated in the HPLC chromatogram shown in Figure 1,

Scheme 5: Formation of Itz-13−24SCN-29-ox and Itz-13−24SCN-29-red Due to Incomplete CN-Induced Cleavage (at 24SCN)

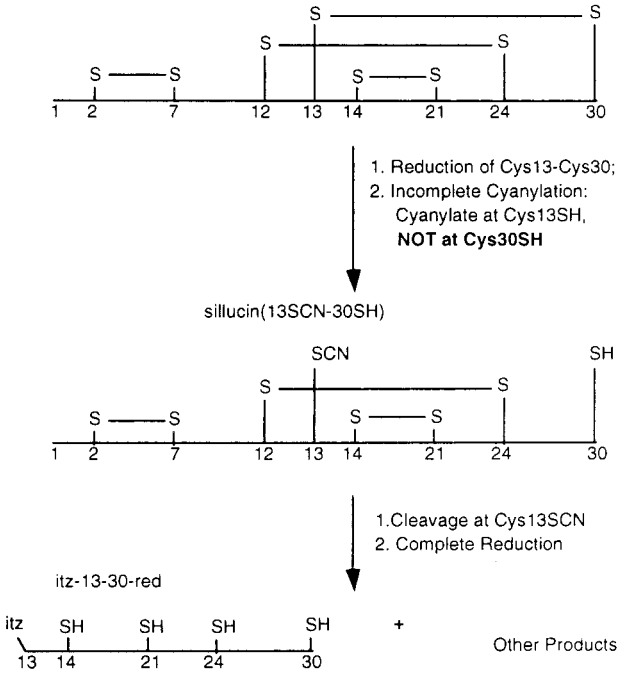


a substantial amount of one of the six possible doubly reduced isoforms is available for analysis. As summarized in Scheme 2, mass mapping analysis of the reaction mixture resulting from CN-induced cleavage before and after subsequent complete reduction to reduce the residual two disulfide bonds allows the connectivity of two more disulfide bonds (Cys14−Cys21 and Cys12−Cys24) to be deduced. The connectivity of the fourth disulfide is available by default.

Incomplete Cyanylation, Incomplete CN-Induced cleavage, and Disulfide Scrambling. Although the efficiency of the cyanylation reaction is usually ≥95% (*22*), there are various imperfections associated with the cyanylation and CN-induced cleavage reactions, especially when there are three adjacent cysteines to offer extreme steric hindrance for the cyanylation and cleavage reagents to access the middle cysteine residue. In Scheme 1, we show a product from a competing side reaction, β-elimination of HSCN from cyanylated cysteine residues; depending on the sequence, variable amounts of this β-elimination product are generated in competition with the desired CN-induced cleavage on the N-terminal side of cyanylated cysteine residues. Other side reactions include incomplete cyanylation and incomplete CN-induced cleavage at cysteine residues. Schemes 5 and 6 show some examples of incomplete cyanylation and incomplete CN-induced cleavage reactions. These side reactions, including β-elimination, incomplete cyanylation, and incomplete CN-induced cleavage, can occur in combination. Furthermore, these side reactions contribute additional peaks to the MALDI spectra of the CN-induced cleavage reaction mixture. Sometimes these peaks are minor, indicating little side reaction; in other cases, these peaks dominate as a result of the supersensitivity of some of these minor products in the MALDI process.

Another complication is the fact that the partially reduced and cyanylated isoforms of the peptide are sometimes similar, and carryover or coelution of such species with others is possible during HPLC separation. This aspect can also

Scheme 6: Formation of Itz-13−30 from Incomplete Cyanylation[a]



[a] Cys30SH is used to designate the free cysteine residue.

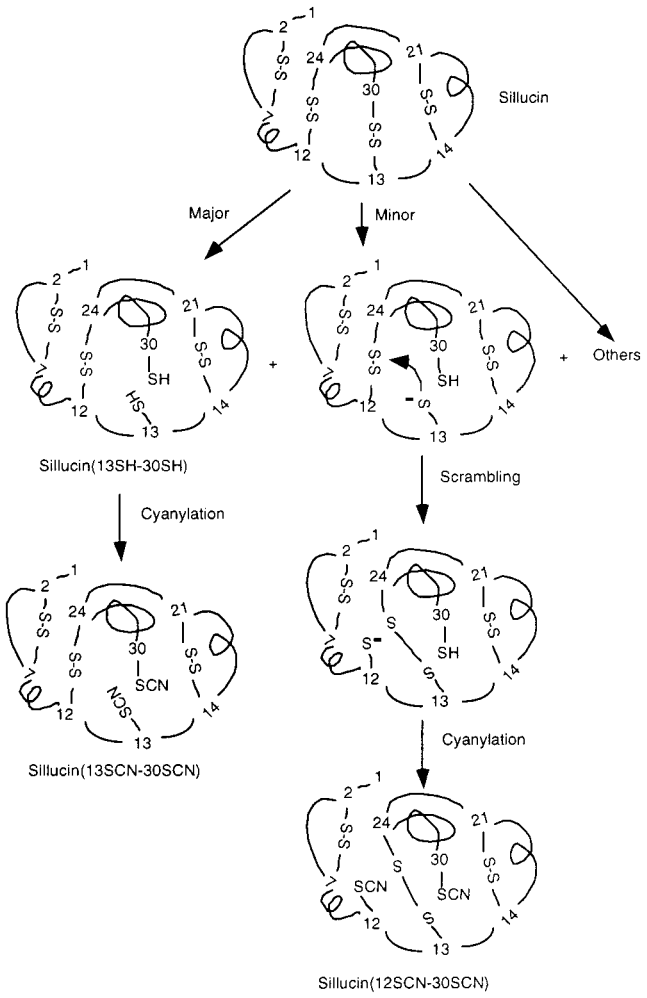
contribute to additional MALDI peaks that are not related to the CN-induced cleavage products of a specific partially reduced and cyanylated species.

Most of the MALDI peaks in Figures 2−4, beyond those described for determination of the disulfide structure of sillucin, can be attributed to the side reactions discussed above. Because these "extra" peaks can be accounted for, they do not preclude correct data interpretation, but simply complicate it. Some representative MALDI peaks related to side reactions are described below.

Some of the MALDI peaks in Figure 2, other than those identified in the Results, can be interpreted as follows. The peak at *m*/*z* 1993.1 corresponds to itz-13−24SCN-29-red (calculated mass of MH^+ = 1993.2 Da; 24SCN is used to designate a cyanylated cysteine residue at position 24). Itz-13−24SCN-29-red could result from incomplete CN-induced cleavage (CN-induced cleavages occur at Cys13SCN and Cys30SCN, but not at Cys24SCN) and complete reduction of minor "carryover" of the doubly reduced and cyanylated species [sillucin(13SCN-30SCN, 12SCN-24SCN)] in which Cys13−Cys30 and Cys12−Cys24 disulfide bonds have been reduced and cyanylated (another partially reduced and cyanylated species is designated similarly in the following discussions) as represented by HPLC peak 2 in Figure 1. Formation of itz-13−24SCN-29-red is illustrated in Scheme 5.

The peak at *m*/*z* 2071.5 could be attributed to itz-13−30-red or itz-12−29-red (calculated mass of both MH^+ ions = 2071.4 Da). Itz-13−30-red could result from CN-induced cleavage and complete reduction of the singly reduced and incompletely cyanylated species [sillucin(13SCN-30SH)] ("SH" designates a free sulfhydryl at Cys30), which is a minor component in collected HPLC fraction 1. Also, [sillucin(13SCN-30SH)] (calculated mass of MH^+ = 3229.7 Da; 27 Da heavier than that for intact sillucin) is represented by a minor MALDI peak at *m*/*z* 3229.7 (data not shown). Scheme 6 illustrates the formation of itz-13−30-red.

Scheme 7: Didactic Illustration of Possible Scrambling in Singly Reduced Sillucin(13S−-30SH), Illustrated for the Case in Which the Cys13−Cys30 Disulfide Has Been Reduced, and a Trace Amount Has Dissociated To Form the Thiolate Ion [Sillucin(13S−-30SH)], Which Scrambles to [Sillucin(12S−-30SH)] before Cyanylation Is Accomplished



Itz-12−29-red can result from CN-induced cleavage and complete reduction of scrambled and cyanylated species [sillucin(12SCN-30SCN)], which results from scrambling between Cys12 and Cys13 (Scheme 7).

Further experiments showed that the MALDI peak at *m*/*z* 2071.5 disappeared after additional HPLC separation removed the minor component [sillucin(13SCN-30SH)] from HPLC fraction 1; the purified species [sillucin(13SCN-30SCN)] was cleaved, and the remaining disulfide bonds were completely reduced (data not shown). This infers that no scrambling occurred; the peak at *m*/*z* 2071.5 represents itz-13−30-red, which is a CN-induced cleavage product of the singly reduced and incompletely cyanylated species [sillucin(13SCN-30SH)]. Disulfide scrambling does not occur despite the existence of three adjacent cysteines, probably due to the fact that partial reduction and cyanylation are carried out at pH 3, and thus, the concentration of thiolate anion is minimal.

In summary, the cysteine-rich and apparently highly knotted antimicrobial peptide sillucin, which is resistant to the proteolytic mapping approach to disulfide mapping, is amenable to partial reduction, cyanylation, and CN-induced cleavage mass mapping methodology. One of four possible singly reduced isoforms and one of six possible doubly reduced isoforms of sillucin generated sufficient information from mass mapping of the cyanylation and CN-induced cleavage products to assign the connectivity (Cys2−Cys7, Cys12−Cys24, Cys13−Cys30, and Cys14−Cys21) and rule out the other 104 isomeric possibilities.

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