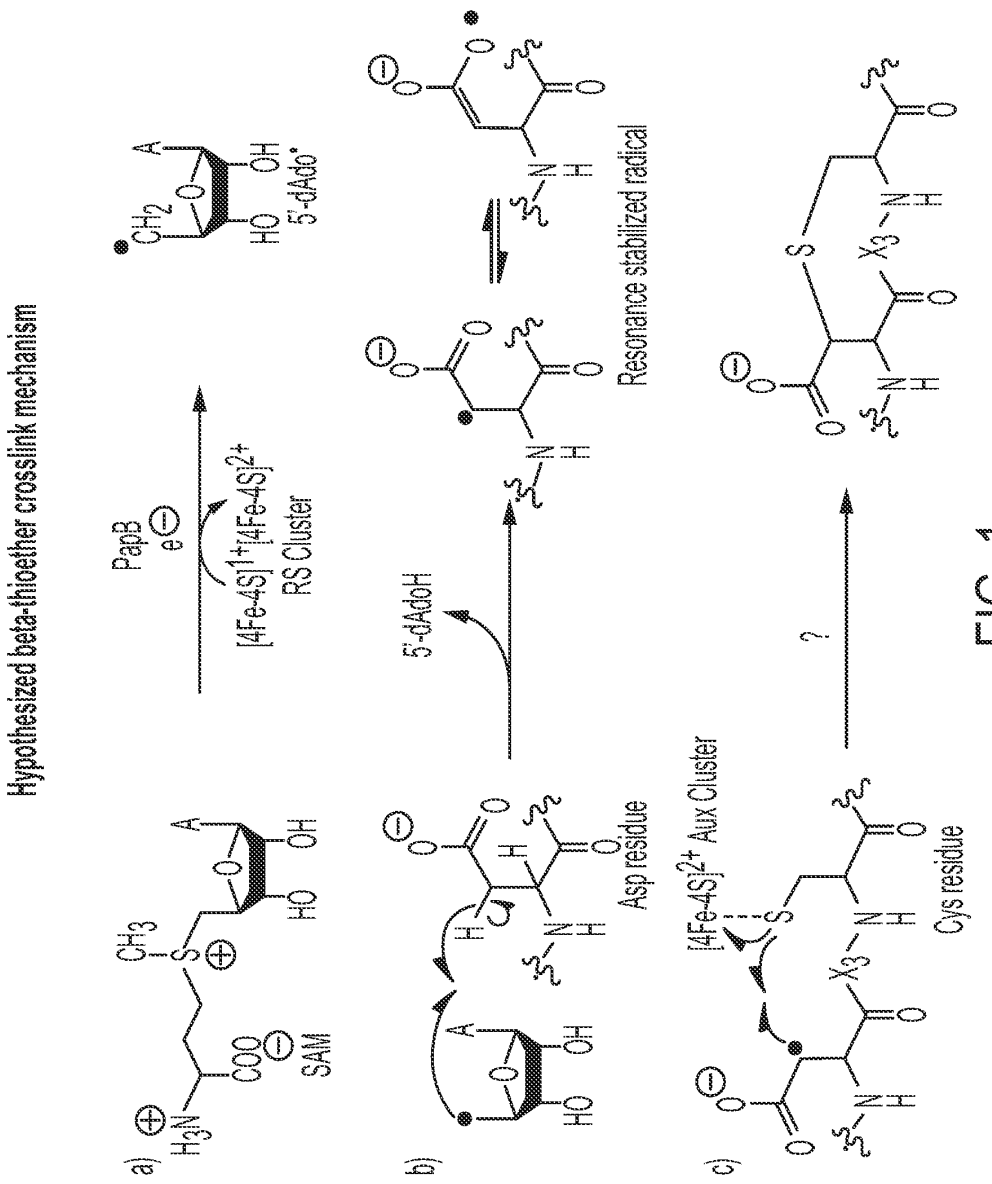

Hypothesized beta-thioether crosslink mechanism
a)
SAM
PapB
e⊖
[4Fe-4S]1+/[4Fe-4S]2+
RS Cluster
5'-dAdo•
b)
Asp residue
5'-dAdoH
Resonance stabilized radical
c)
[4Fe-4S]2+ Aux Cluster
Cys residue
?

FIG. 1

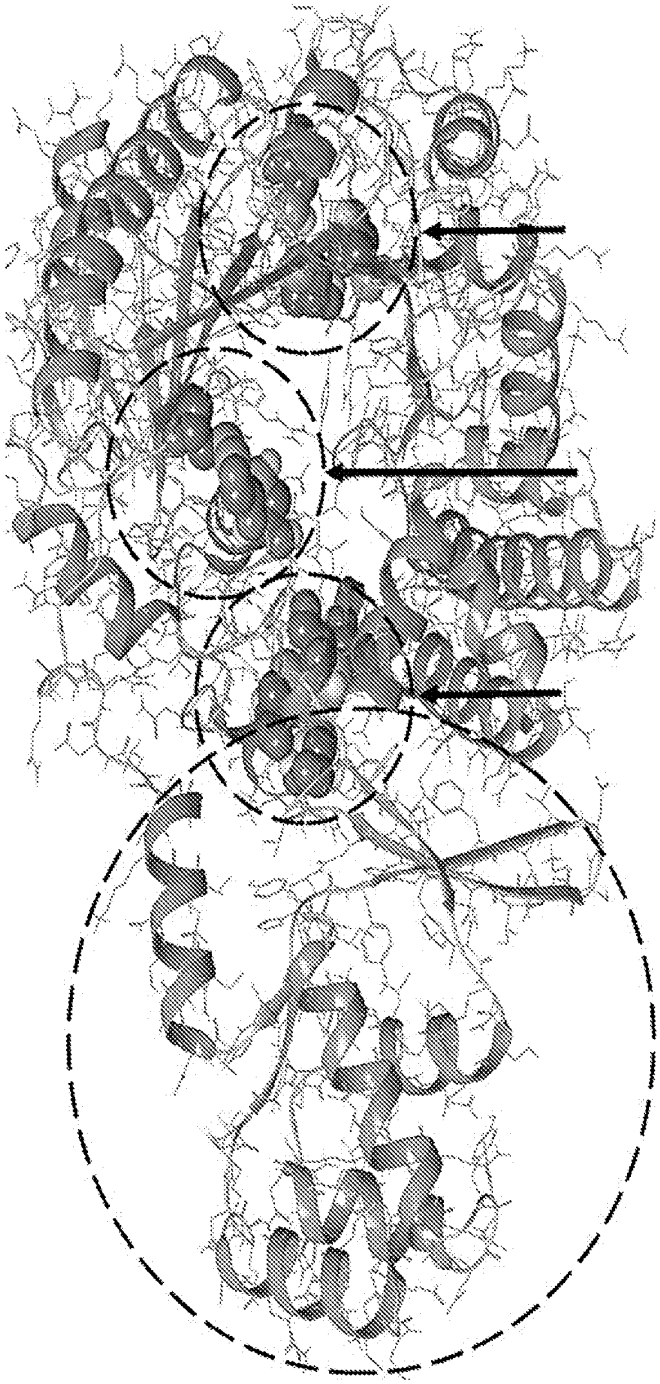


FIG. 2

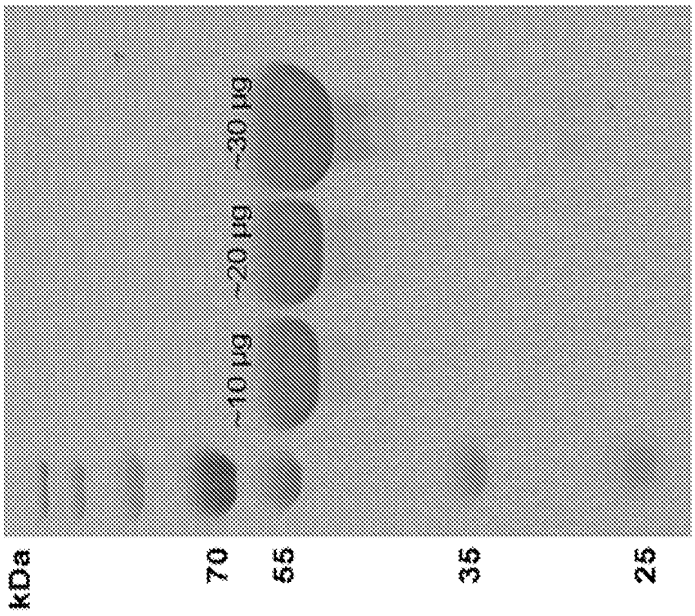


FIG. 3

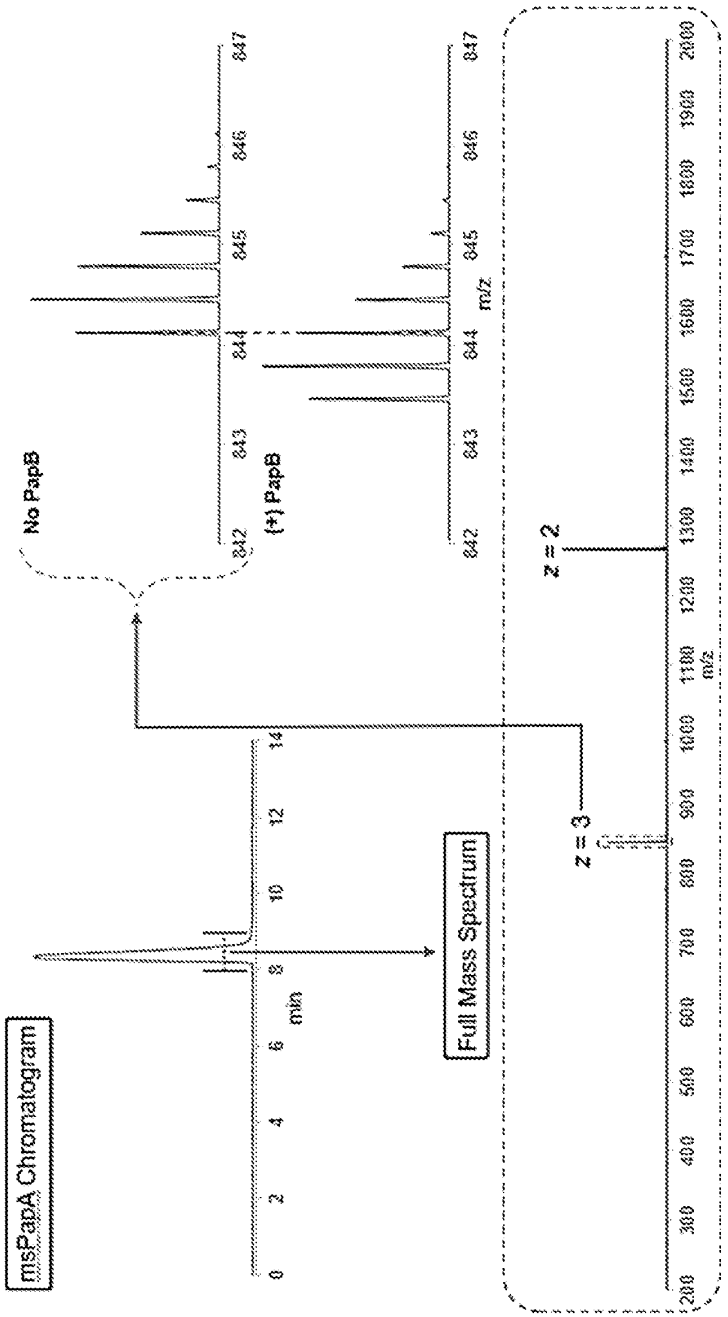

msPapA Chromatogram
min
Full Mass Spectrum
No PapB
(+) PapB
m/z
z = 2
z = 3

FIG. 4A

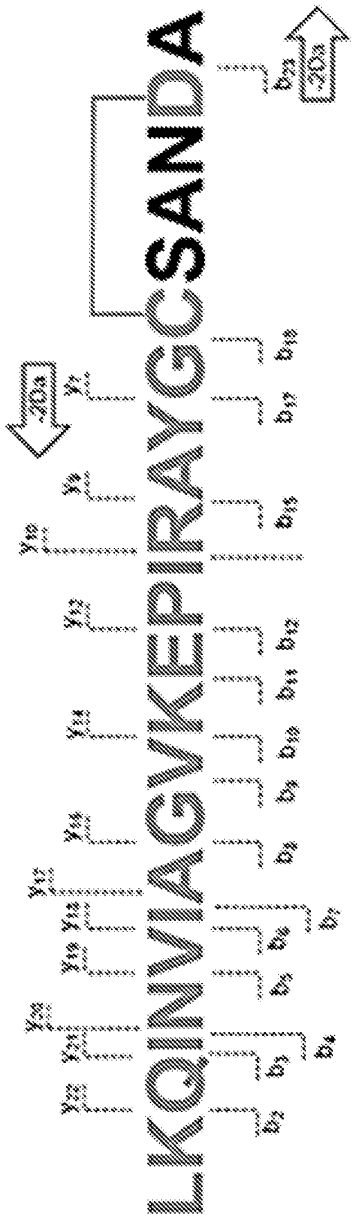


FIG. 4B

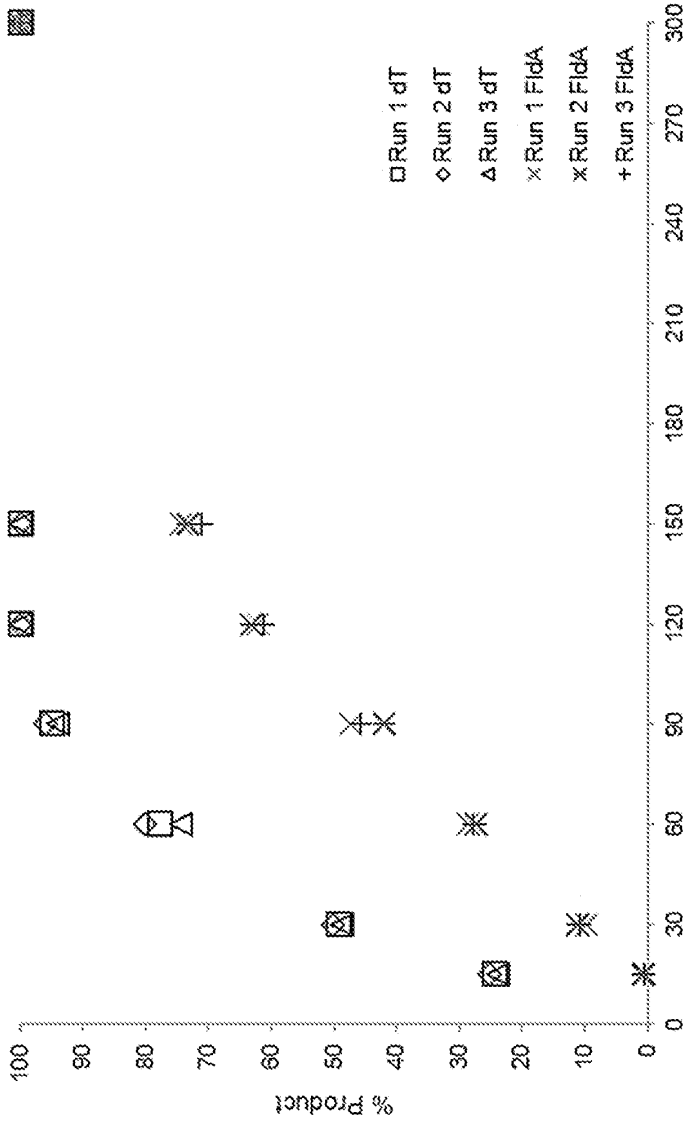

% Product
Time (s)
Run 1 dT
Run 2 dT
Run 3 dT
Run 1 FldA
Run 2 FldA
Run 3 FldA

FIG. 5

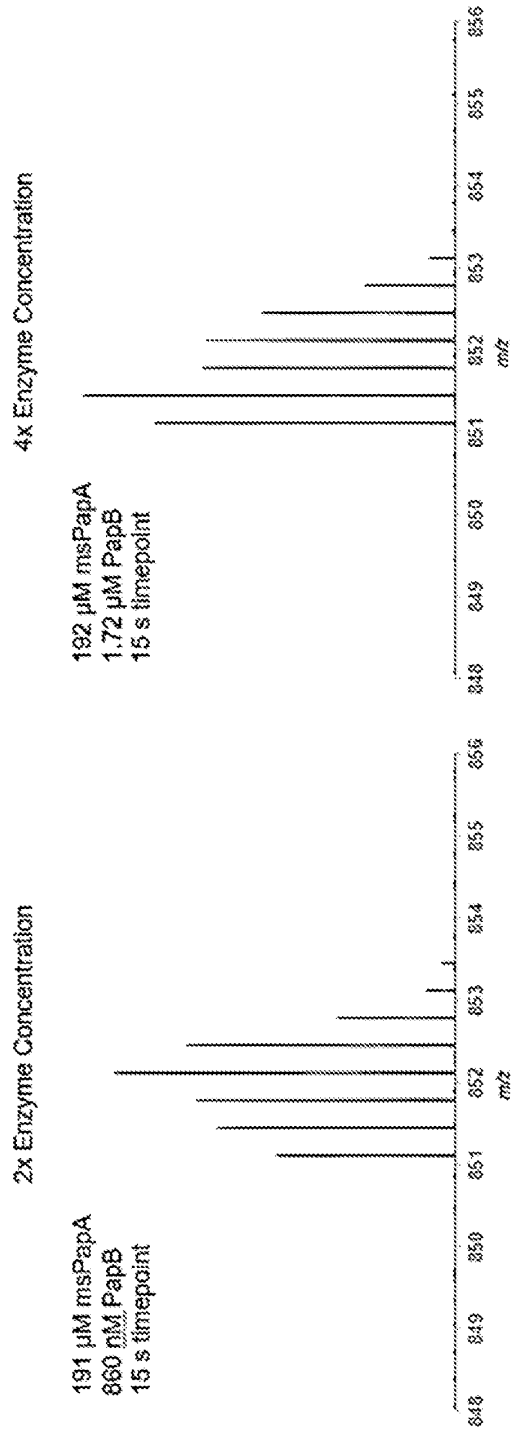

2x Enzyme Concentration
191 µM msPapA
860 nM PapB
15 s timepoint
4x Enzyme Concentration
192 µM msPapA
1.72 µM PapB
15 s timepoint
m/z
848
849
850
851
852
853
854
855
856

FIG. 6

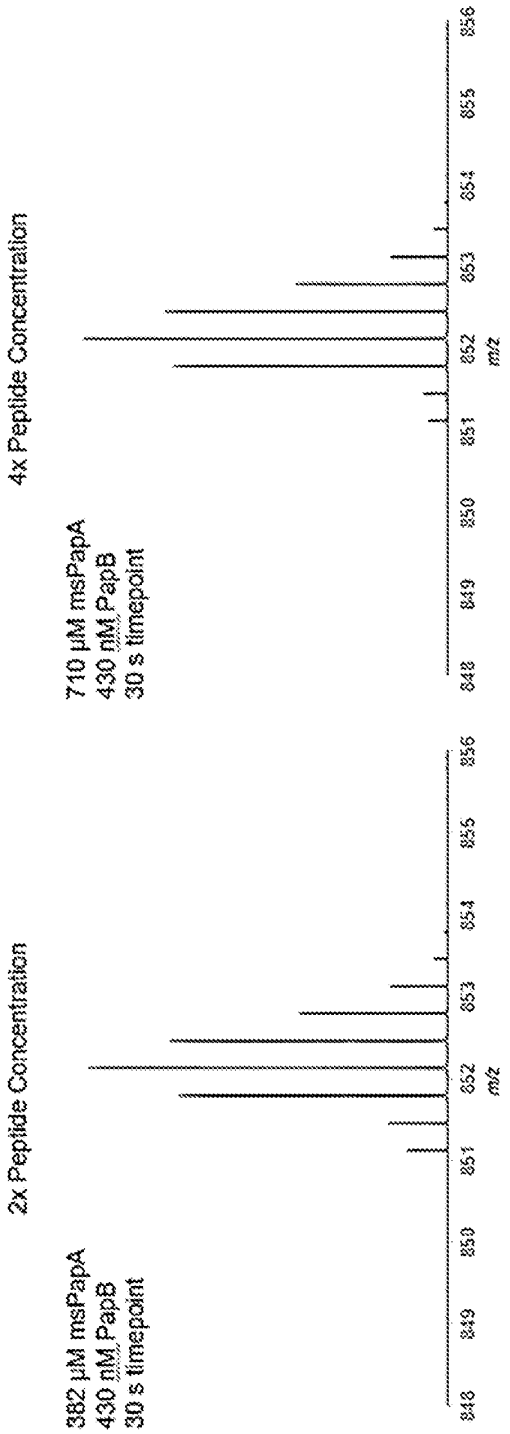

2x Peptide Concentration
382 µM msPapA
430 nM PapB
30 s timepoint
4x Peptide Concentration
710 µM msPapA
430 nM PapB
30 s timepoint
m/z
848
849
850
851
852
853
854
855
856

FIG. 7

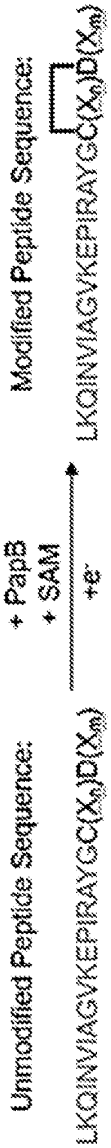

Unmodified Peptide Sequence:
LKQINVIAGVKEPIRAYGC(Xn)D(Xm)
+ PapB
+ SAM
+e-
Modified Peptide Sequence:
LKQINVIAGVKEPIRAYGC(Xn)D(Xm)

FIG. 8A

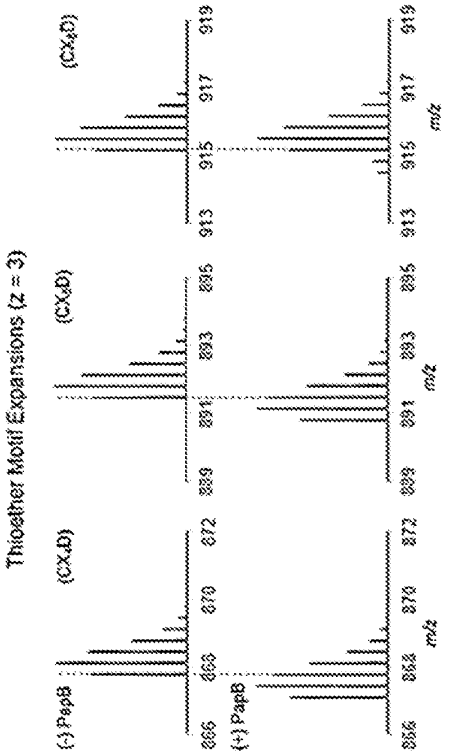

Thioether Motif Expansions (z = 3)
(-) PapB
(+) PapB
(CX4D)
(CX5D)
(CX6D)
m/z

FIG. 8C

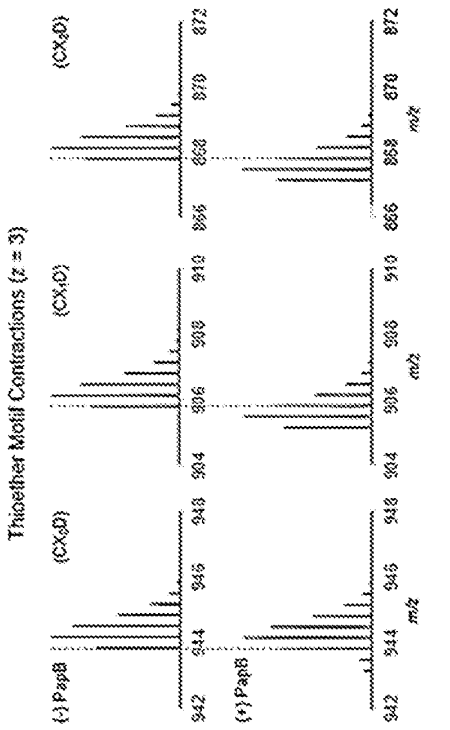

Thioether Motif Contractions (z = 3)
(-) PapB
(+) PapB
(CX3D)
(CX4D)
(CX5D)
m/z

FIG. 8B

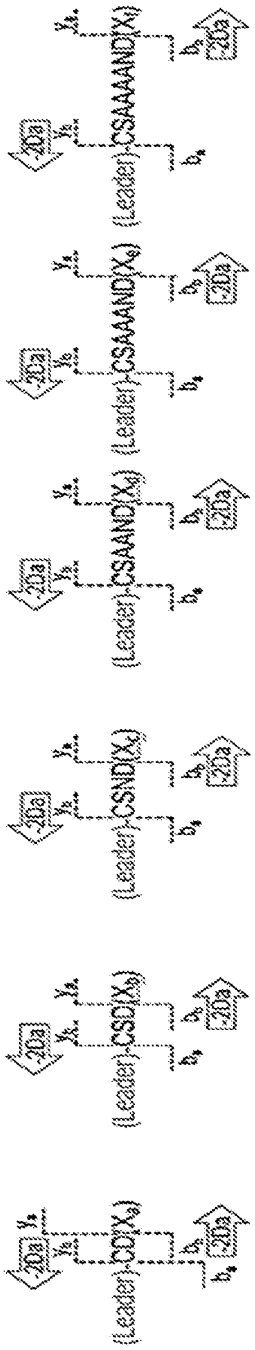
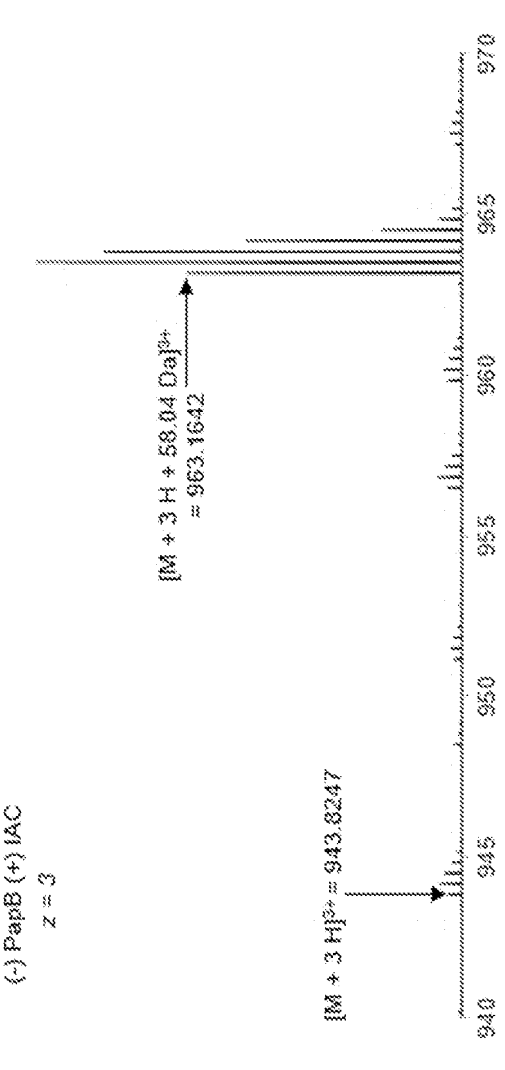
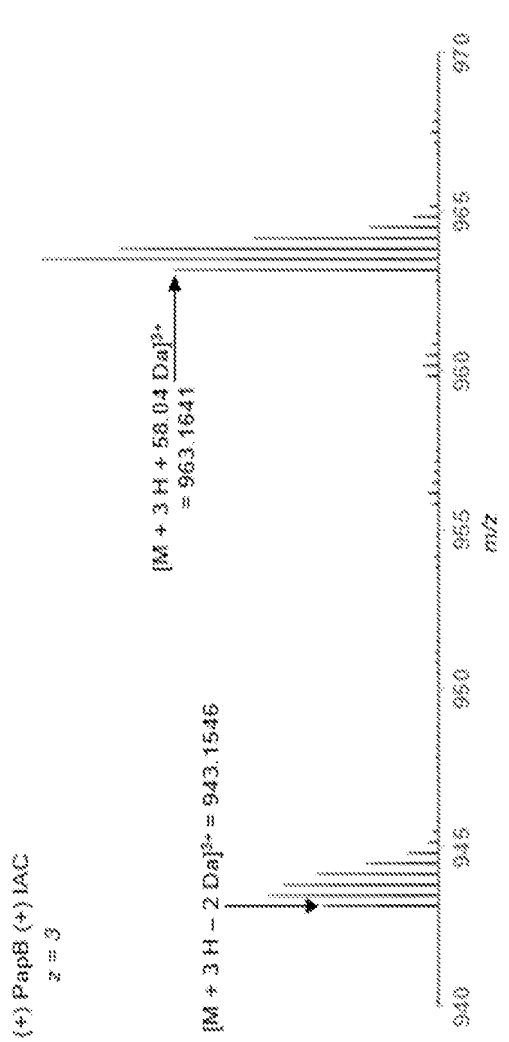

20a
(Leader)-CSD(X9)
(Leader)-CSND(X5)
(Leader)-CSAAND(X9)
(Leader)-CSAAAND(X6)
(Leader)-CSAAAAND(X4)

FIG. 8D



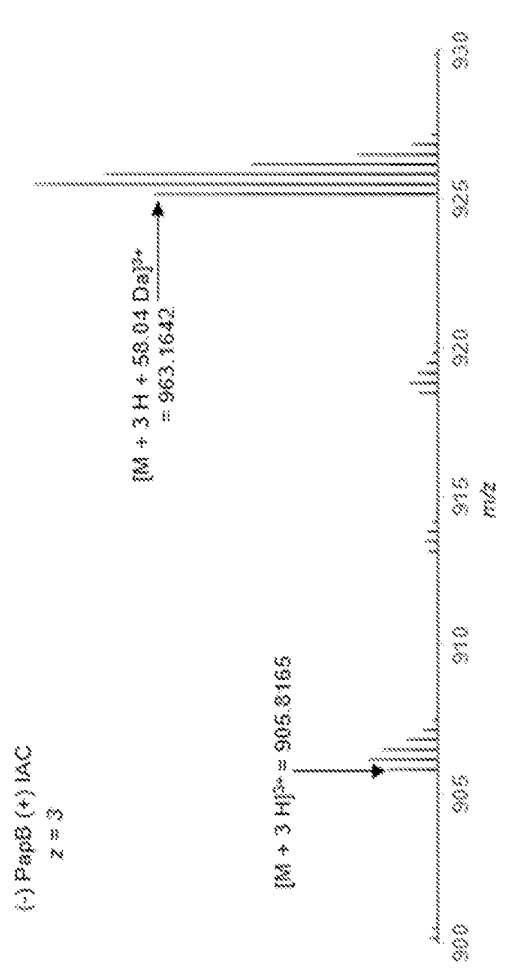
Sequence	Expected Monoisotopic Mass	Observed Monoisotopic Mass	Ppm Error
Leader-CDSNNAANA (CX_6D)	(-) PapB: 943.8247	943.8229	-1.90
Leader-CDSNNAANA (CX_6D) + S-carboxymethyl	(-) PapB + (58.04 Da): 963.1625	963.1642	1.77

FIG. 9A



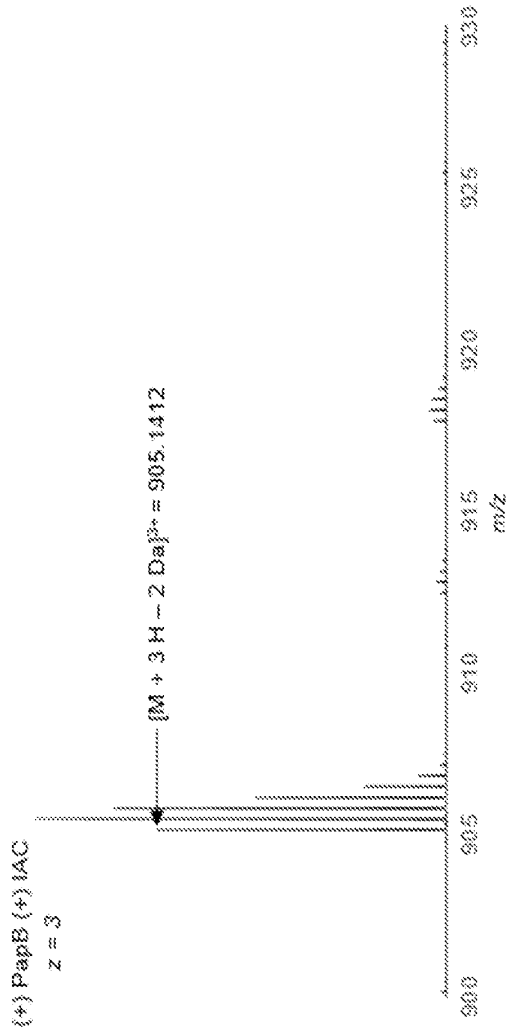
Sequence	Expected Monoisotopic Mass	Observed Monoisotopic Mass	Ppm Error
Leader-CDSNNAANA (CX_3D)	(+) PapB - (2 Da): 943.1555	943.1546	-0.95
Leader-CDSNNAANA (CX_3D) + S-carboxymethyl	(-) PapB + (58.04 Da): 963.1625	963.1641	1.66

FIG. 9B



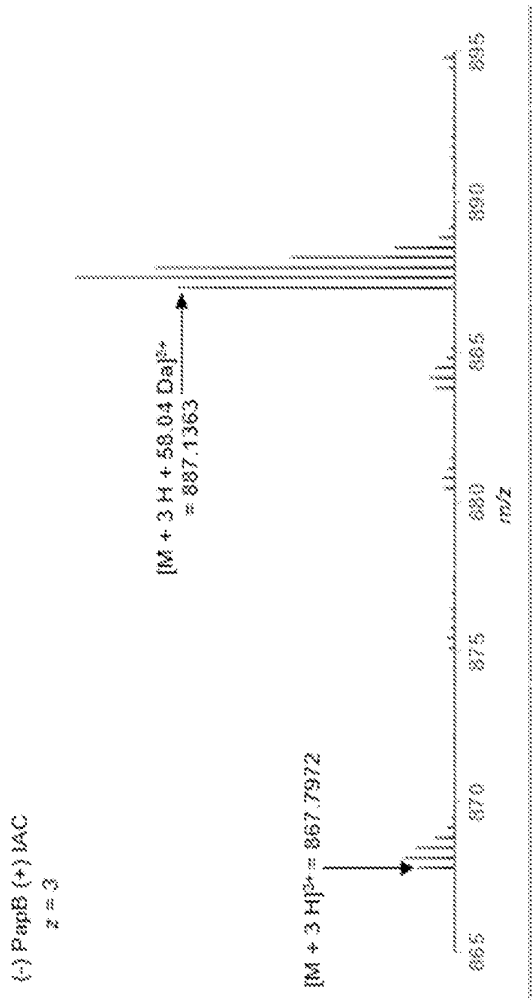
Sequence	Expected Monoisotopic Mass	Observed Monoisotopic Mass	Ppm Error
Leader-CSDNNAAA (CX.D)	(-) PapB: 905.8131	905.8165	3.75
Leader-CSDNNAAA (CX.D) + S-carboxymethyl	(-) PapB + (58.04 Da): 925.1482	925.1488	0.65

FIG. 10A



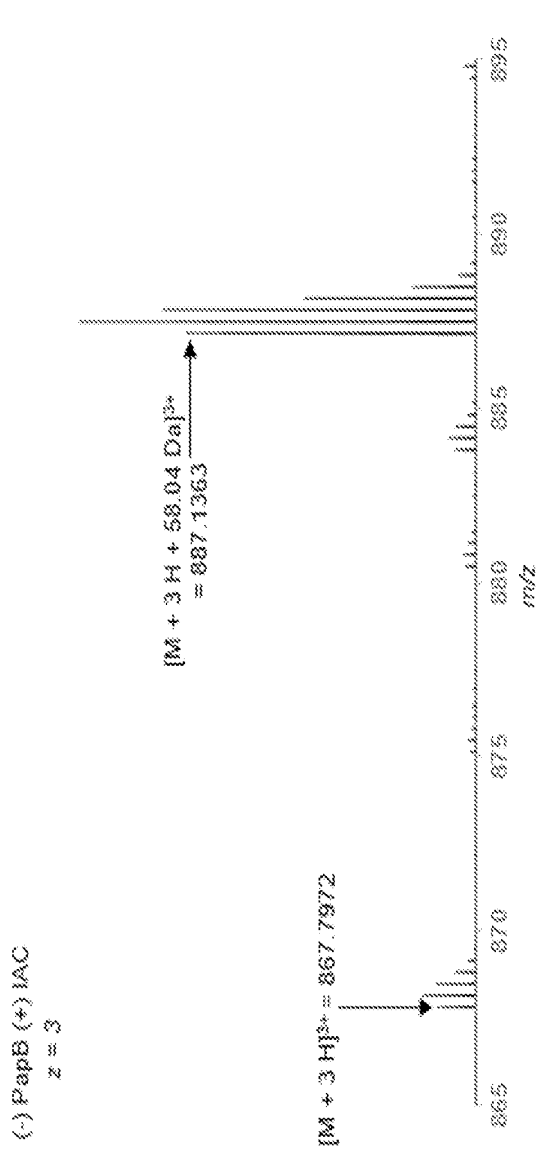
Sequence	Expected Monoisotopic Mass	Observed Monoisotopic Mass	Ppm Error
Leader-CSDNNAAA (CX,D)	(+) PapB - (2 Da) 905.1412	905.1418	0.66
Leader-CSDNNAAA (CX,D) + S-carboxymethyl	(-) PapB + (58.04 Da) 963.1625	Not Found	N/A

FIG. 10B



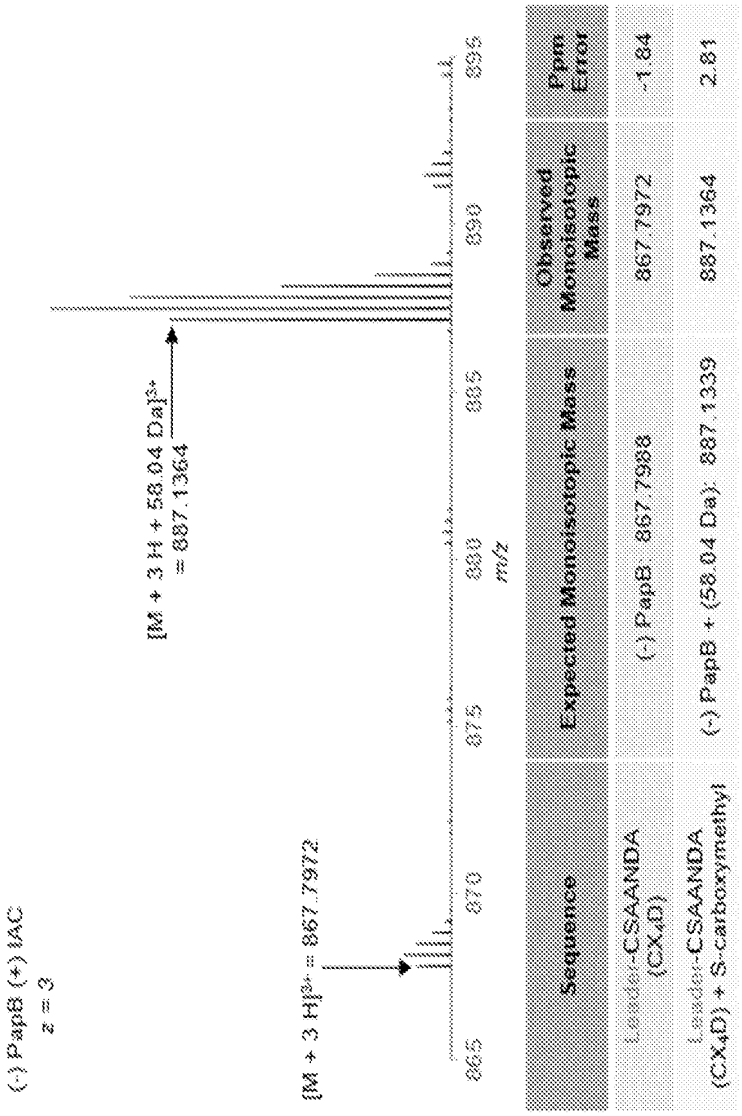
Sequence	Expected Monoisotopic Mass	Observed Monoisotopic Mass	Ppm Error
Leader-CSNDAAA (CX_2D)	(-) PapB: 867.7988	867.7972	-1.84
Leader-CSNDAAA (CX_2D) + S-carboxymethyl	(-) PapB + (58.04 Da): 887.1339	887.1363	2.71

FIG. 11A



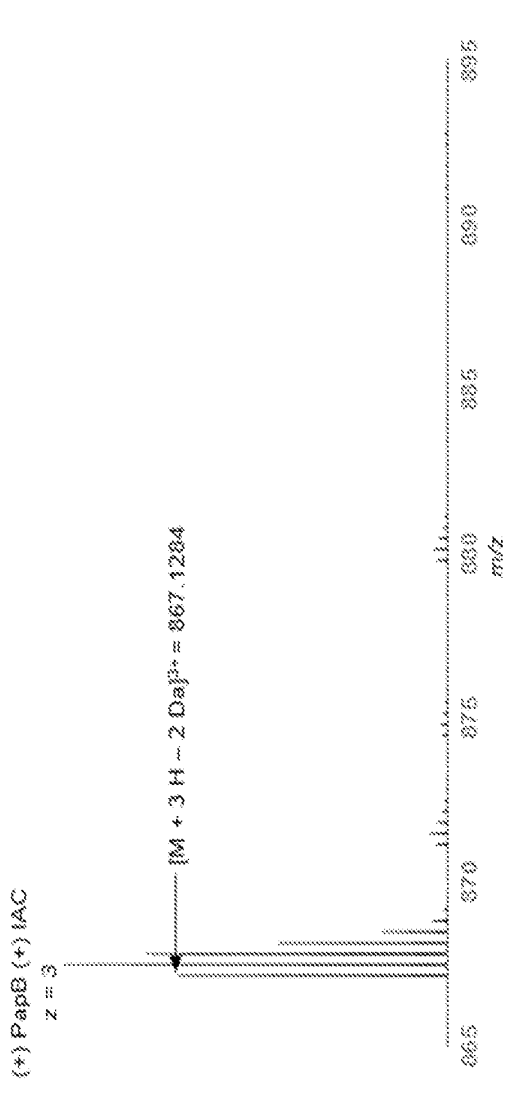
Sequence	Expected Monoisotopic Mass	Observed Monoisotopic Mass	Ppm Error
Leader-CSNDAAA (CX_2D)	(-) PapB: 867.7988	867.7972	-1.84
Leader-CSNDAAA (CX_2D) + S-carboxymethyl	(-) PapB + (58.04 Da): 887.1339	887.1363	2.71

FIG. 11B



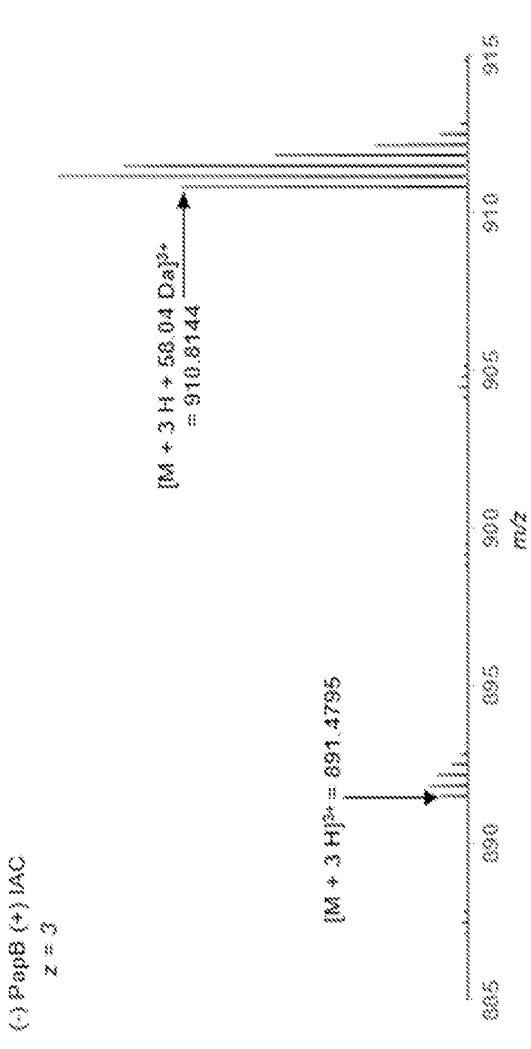
Sequence	Expected Monoisotopic Mass	Observed Monoisotopic Mass	Ppm Error
Leader-CSAANDA (CX4D)	(-) PapB: 867.7988	867.7972	-1.84
Leader-CSAANDA (CX4D) + S-carboxymethyl	(-) PapB + (58.04 Da): 887.1339	887.1364	2.81

FIG. 12A



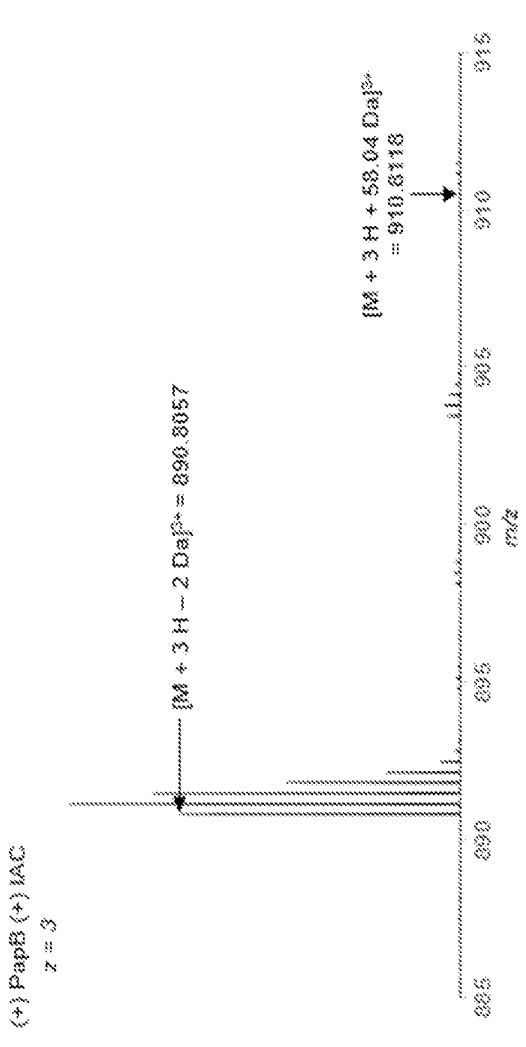
Sequence	Expected Monoisotopic Mass	Observed Monoisotopic Mass	Ppm Error
Leader-CSAANDA (CX4D)	(+) PapB - (2 Da): 867.1269	867.1284	1.73
Leader-CSAANDA (CX4D) + S-carboxymethyl	(-) PapB + (58.04 Da): 887.1339	Not Found	N/A

FIG. 12B



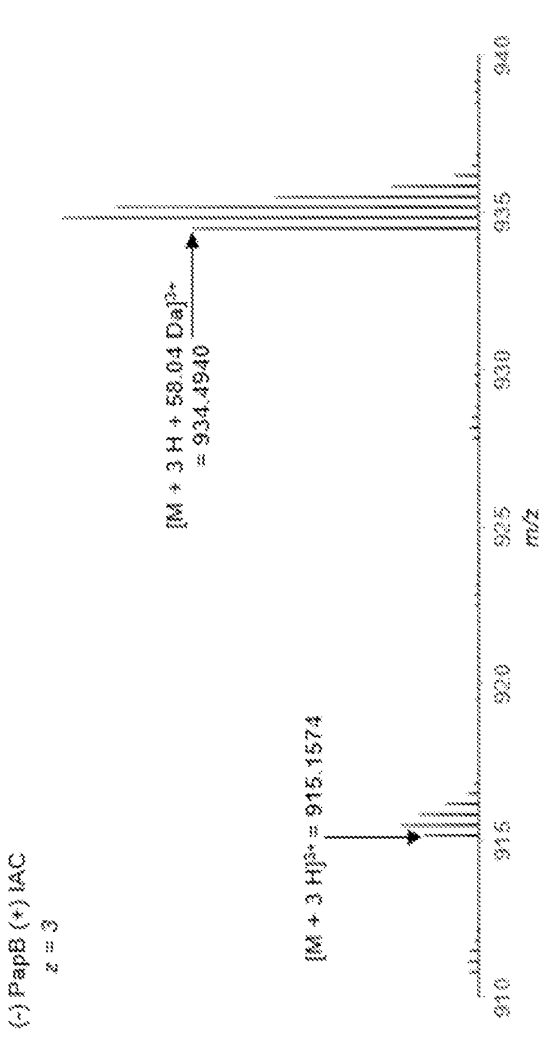
Sequence	Expected Monoisotopic Mass	Observed Monoisotopic Mass	Ppm Error
Leader-CSAAANDA (CX_6D)	(-) PapB 891.4778	891.4795	1.91
Leader-CSAAANDA (CX_6D) + *S*-carboxymethyl	(-) PapB + (58.04 Da): 910.8130	910.8144	1.54

FIG. 13A



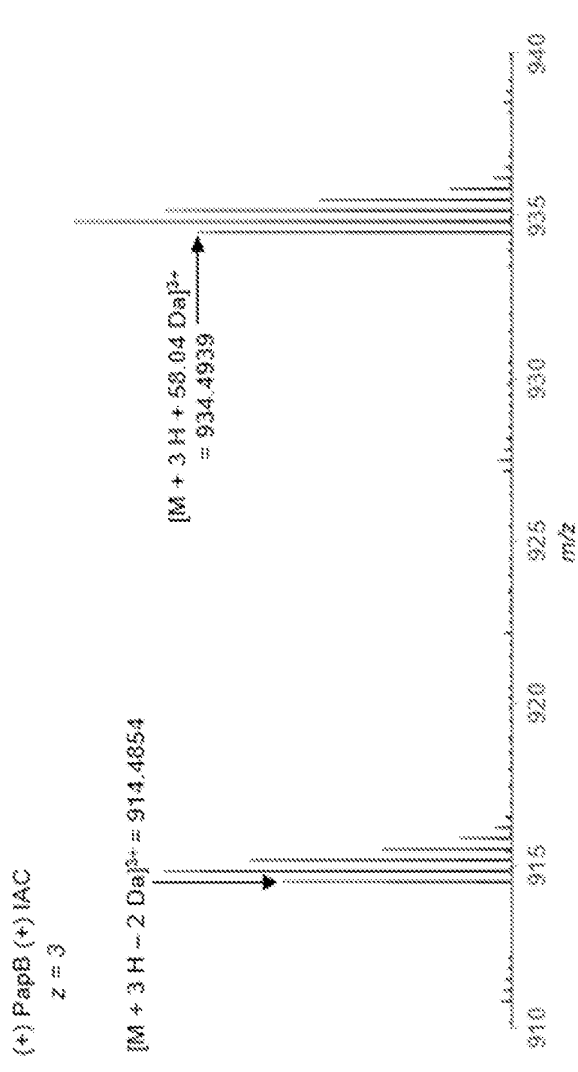
Sequence	Expected Monoisotopic Mass	Observed Monoisotopic Mass	Ppm Error
Leader-CSAAANDA (CX_8D)	(+) PapB - (2 Da) 890.8059	890.8057	-0.22
Leader-CSAAANDA (CX_8D) + S-carboxymethyl	(+) PapB + (58.04 Da) 910.8130	910.8118	-1.32

FIG. 13B



Sequence	Expected Monoisotopic Mass	Observed Monoisotopic Mass	Ppm Error
Leader-CSAAAANDA (CX_6D)	(-) PapB: 915.1568	915.1574	0.66
Leader-CSAAAANDA (CX_6D) + S-carboxymethyl	(-) PapB + (58.04 Da): 934.4920	934.4940	2.14

FIG. 14A



Sequence	Expected Monoisotopic Mass	Observed Monoisotopic Mass	Ppm Error
Leader-CSAAAANDA (CX_6D)	(+) PapB - (2 Da): 914.4850	914.4854	0.44
Leader-CSAAAANDA (CX_6D) + S-carboxymethyl	(-) PapB + (58.04 Da): 934.4920	934.4939	2.03

FIG. 14B

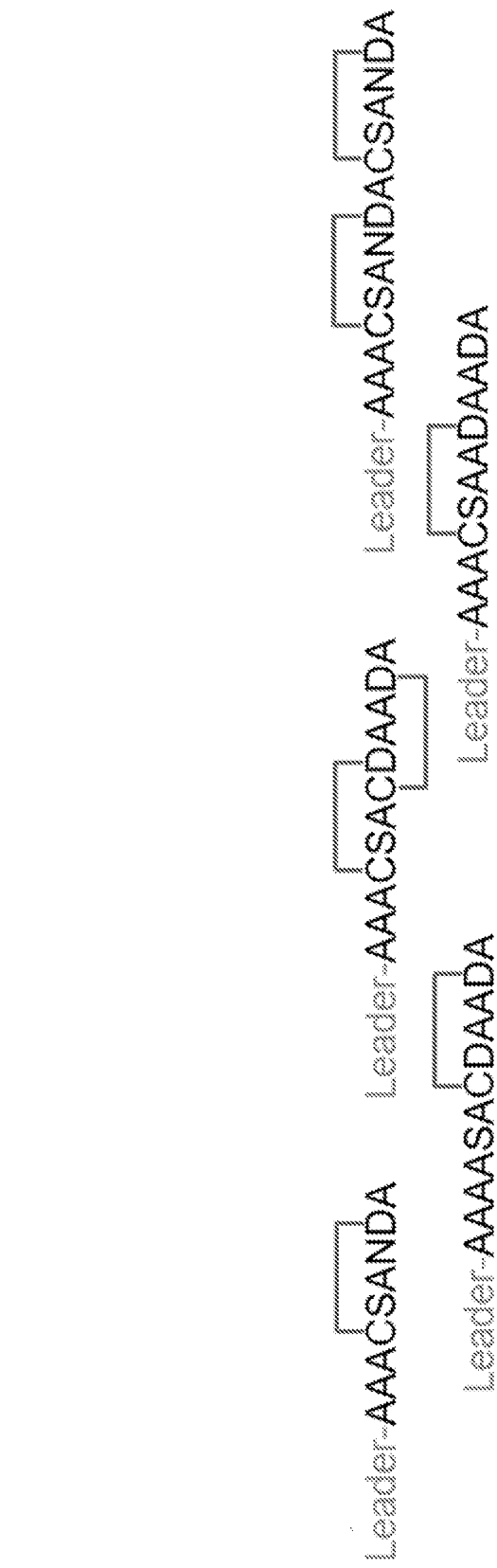

Leader-AAACSANDA
Leader-AAAASACDAADA
Leader-AAACSACDAADA
Leader-AAACSAADAADA
Leader-AAACSANDACSANDA

FIG. 15A

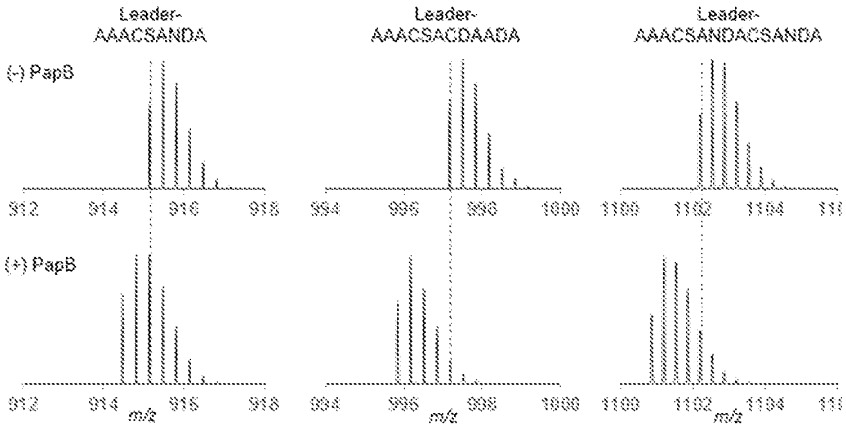

Leader-
AAACSANDA
(-) PapB
(+) PapB
912
914
916
918
m/z
Leader-
AAACSACDAADA
994
996
998
1000
Leader-
AAACSANDACSANDA
1100
1102
1104
Leader-
AAAASACDAADA
(-) PapB
(+) PapB
984
986
988
990
Leader-
AAACSAADAADA

FIG. 15B

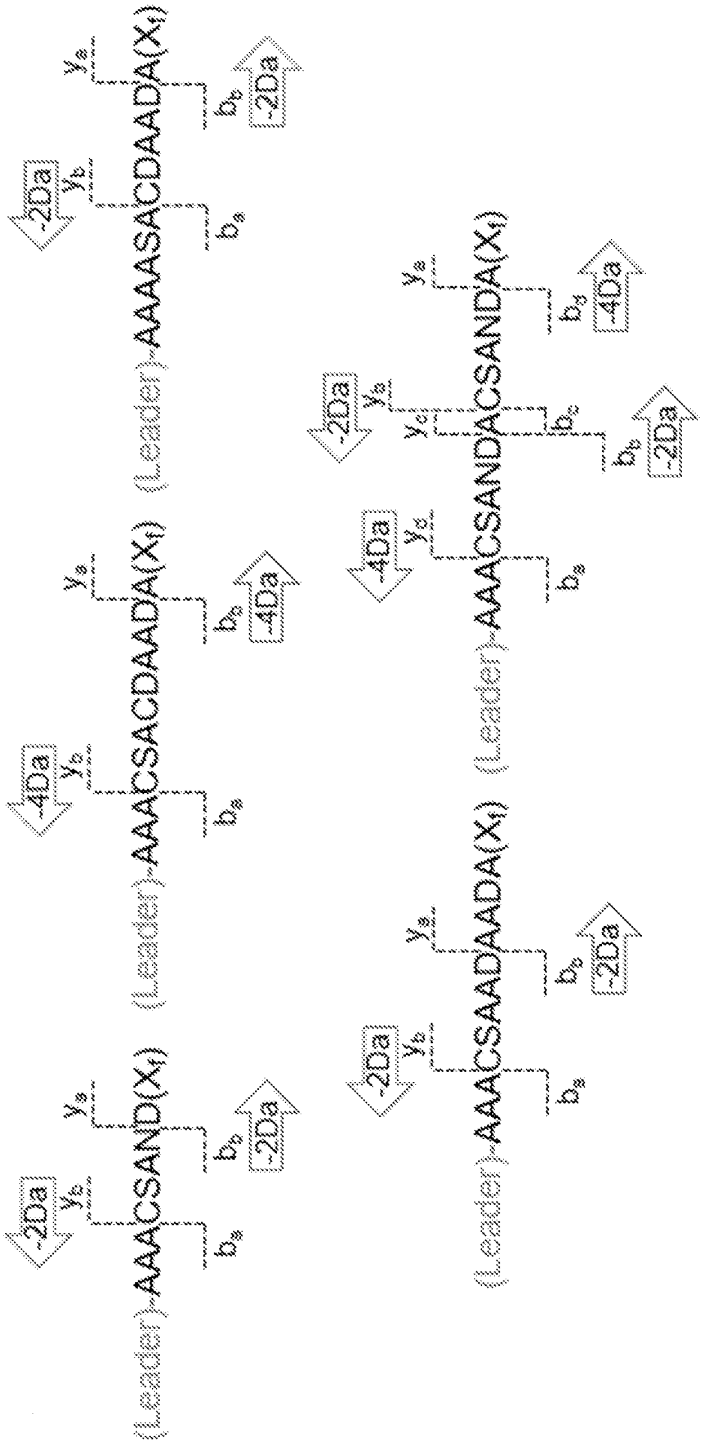
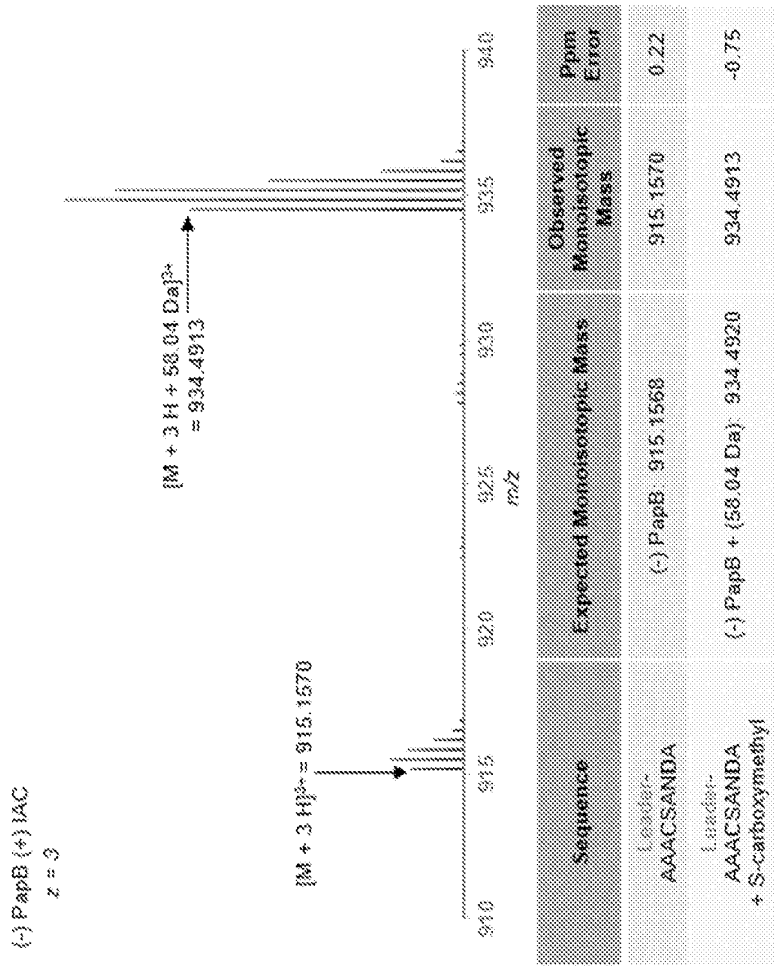
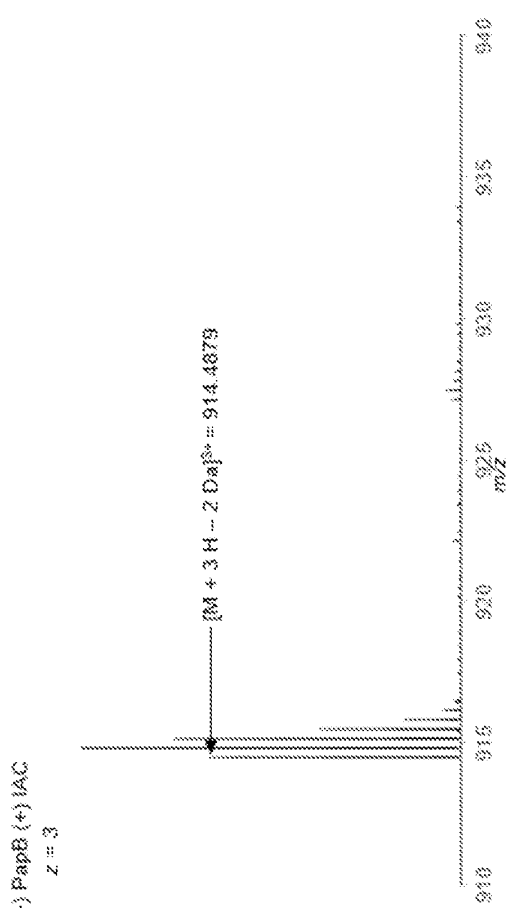

(Leader)-AAACSANDA(X1)
(Leader)-AAACSAADAADA(X1)
(Leader)-AAACSANDACSANDA(X1)
(Leader)-AAACSACDAADA(X1)
(Leader)-AAAASACDAADA(X1)
-2Da
-4Da

FIG. 15C



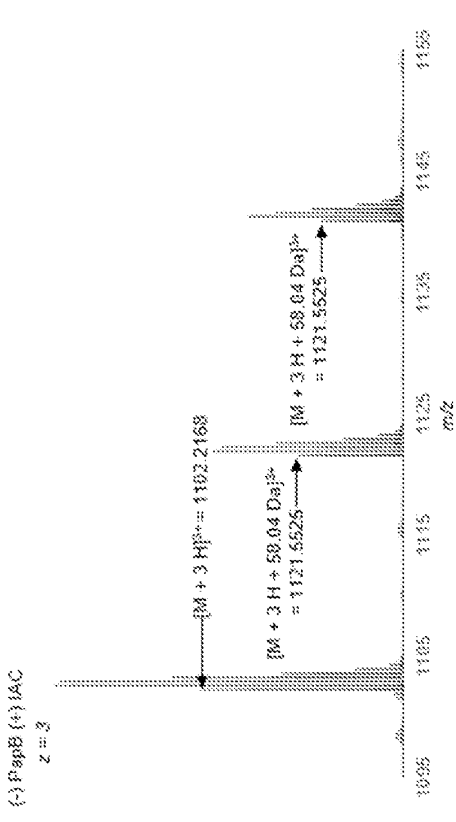
Sequence	Expected Monoisotopic Mass	Observed Monoisotopic Mass	Ppm Error
Linear- AAACSANDA	(-) PapB 915.1568	915.1570	0.22
Linear- AAACSANDA + S-carboxymethyl	(-) PapB + (58.04 Da) 934.4920	934.4913	-0.75

FIG. 16A



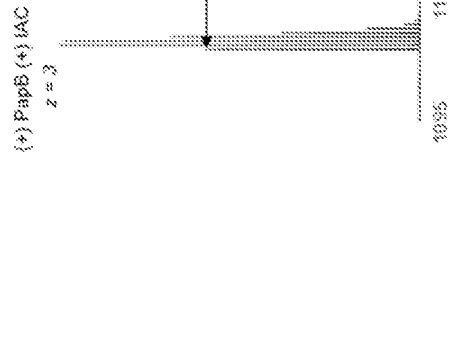
Sequence	Expected Monoisotopic Mass	Observed Monoisotopic Mass	Ppm Error
Lysates - AAACSANDA	(+) PapB - (2 Da): 914.4850	914.4854	0.43
Lysates - AAACSANDA + S-carboxymethyl	(-) PapB + (58.04 Da): 934.4920	Not Found	N/A

FIG. 16B



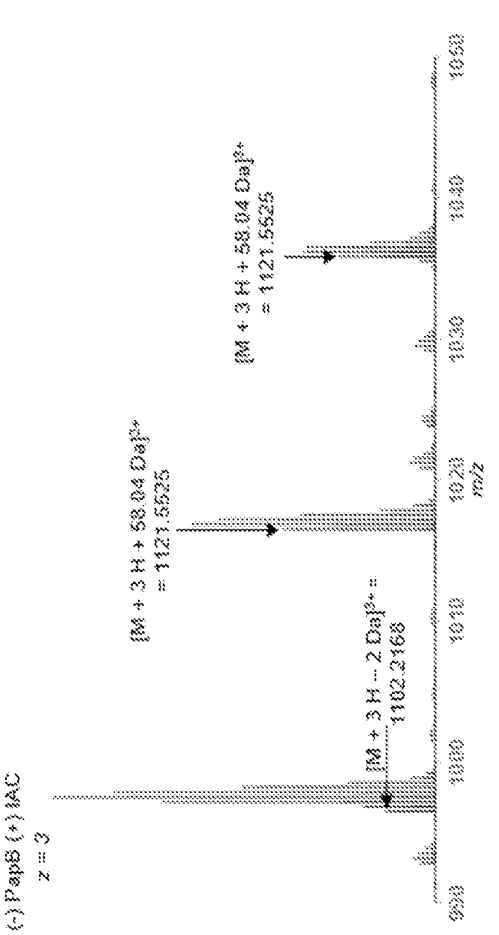
Sequence	Expected Monoisotopic Mass	Observed Monoisotopic Mass	Ppm Error
Linear: AAACSANDACSANDA	(-) PapB 1102.2166	1102.2168	1.63
Linear: AAACSANDACSANDA + S-carboxymethyl	(-) PapB + (58.04 Da) 1121.5538	1121.5525	-1.16
Linear: AAACSANDACSANDA + (2x) S-carboxymethyl	(-) PapB + (58.04 Da) + (58.04 Da) 1140.8889	1140.8914	2.19

FIG. 17A



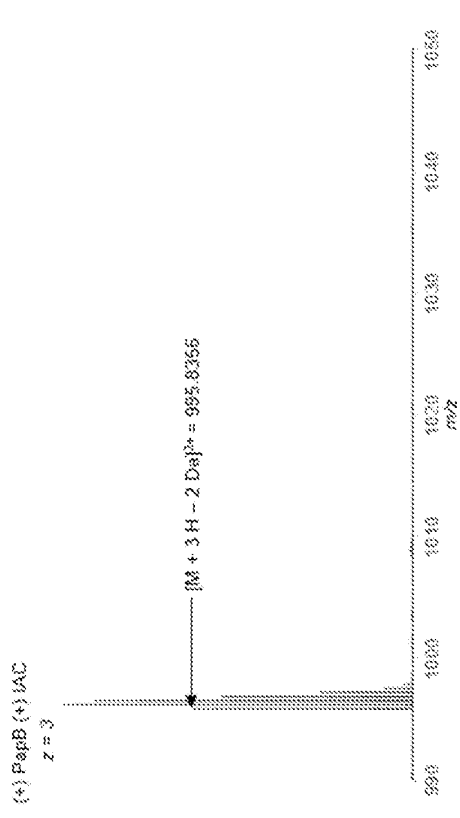
Sequence	Expected Monoisotopic Mass	Observed Monoisotopic Mass	Ppm Error
Location: AAACSAMDACSAMDA	(+) PapB - (4 Da) 1100.8748	1100.8764	1.53
Location: AAACSAMDACSAMDA + S-carboxymethyl	(-) PapB + (58.04 Da) 1121.5536	Not Found	N/A
Location: AAACSAMDACSAMDA + (2x) S-carboxymethyl	(-) PapB + (58.04 Da) + (58.04 Da) 1140.8809	Not Found	N/A

FIG. 17B



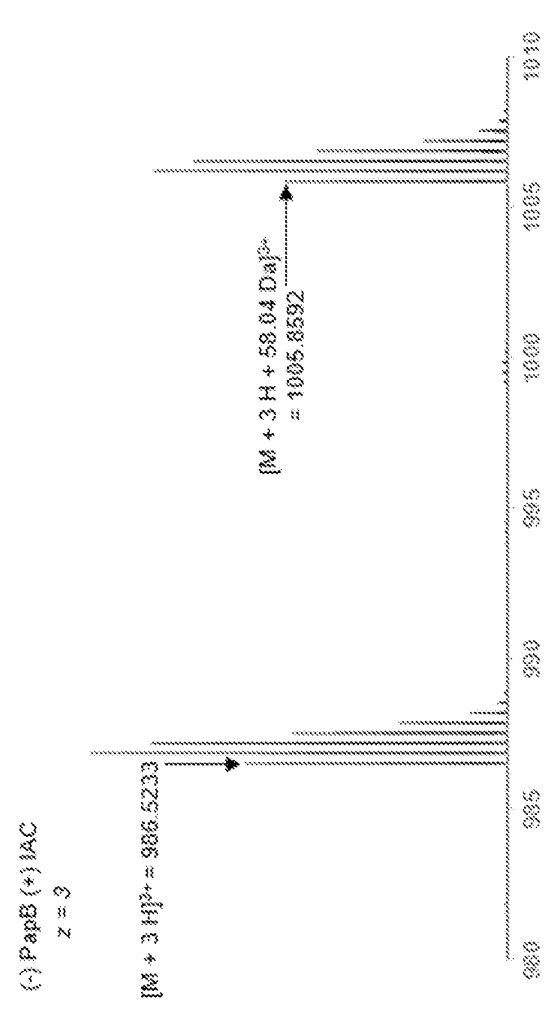
Sequence	Expected Monoisotopic Mass	Observed Monoisotopic Mass	Ppm Error
Leader- AAACSACDAADA	(-) PapB – (2 Da, 1xDisulfide) 996.5074	996.5104	3.01
Leader- AAACSACDAADA + S-carboxymethyl	(-) PapB + (58.04 Da): 1016.5145	1016.5126	-1.87
Leader- AAACSACDAADA + (2x) S-carboxymethyl	(-) PapB + (58.04 Da) + (58.04 Da): 1035.8496	1035.8492	-0.39

FIG. 18A



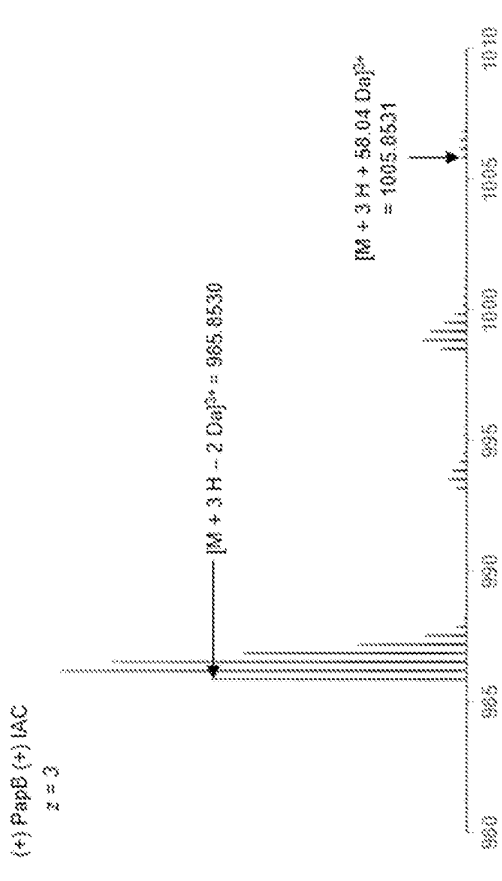
Sequence	Expected Monoisotopic Mass	Observed Monoisotopic Mass	Ppm Error
Leader-AAACSACDAADA	(+) PapB - (4 Da) 995.8355	995.8356	0.10
Leader-AAACSACDAADA + S-carboxymethyl	(-) PapB + (58.04 Da) 1016.5145	Not Found	N/A
Leader-AAACSACDAADA + (2x) S-carboxymethyl	(-) PapB + (58.04 Da) + (58.04 Da) 1035.8496	Not Found	N/A

FIG. 18B



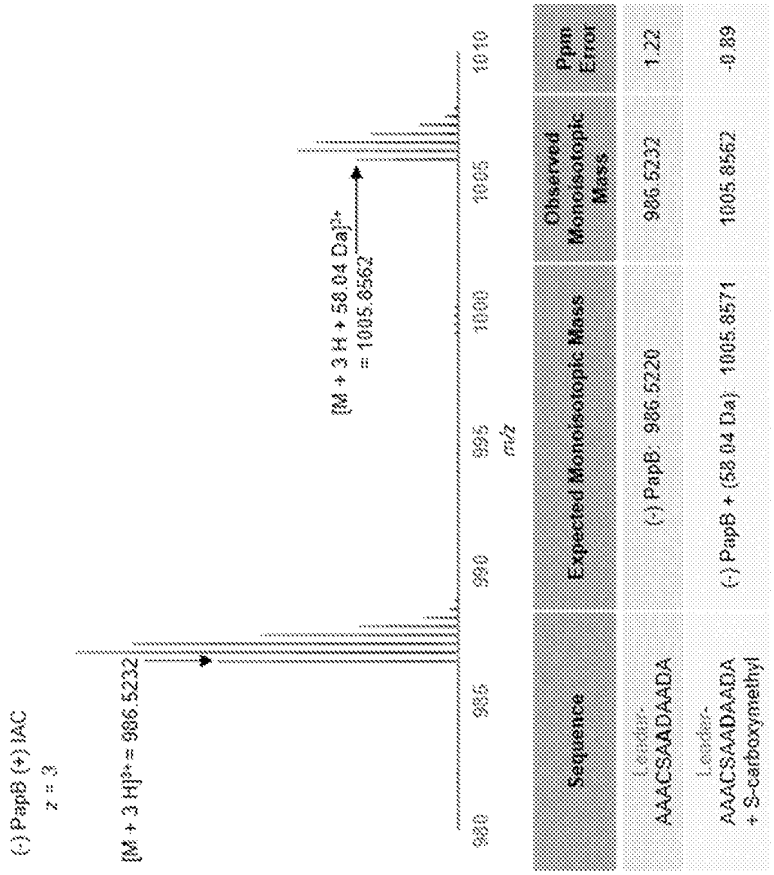
Sequence	Expected Monoisotopic Mass	Observed Monoisotopic Mass	Ppm Error
Linaclotide- AAAASACDAADA	(-) PapB: 986.5220	986.5233	1.32
Linaclotide- AAAASACDAADA + S-carboxymethyl	(-) PapB + (58.04 Da): 1005.8571	1005.8592	2.08

FIG. 19A



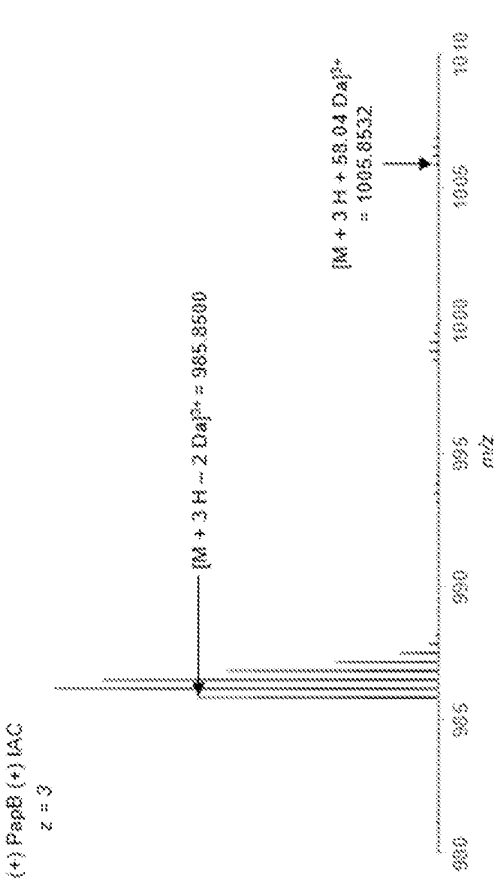
Sequence	Expected Monoisotopic Mass	Observed Monoisotopic Mass	Ppm Error
Lysine-AAAASACDAADA	(+) PapB - (2 Da): 985.8501	985.8530	2.94
Lysine-AAAASACDAADA + S-carboxymethyl	(-) PapB + (58.04 Da): 1005.8571	1005.8531	-3.98

FIG. 19B



Sequence	Expected Monoisotopic Mass	Observed Monoisotopic Mass	Ppm Error
Leader-AAACSAADAADA	(-) PapB: 986.5220	986.5232	1.22
Leader-AAACSAADAADA + S-carboxymethyl	(-) PapB + (58.04 Da): 1005.8571	1005.8562	-0.89

FIG. 20A



Sequence	Expected Monoisotopic Mass	Observed Monoisotopic Mass	Ppm Error
Linear- AACSAADAADA	(+) PapB - (2 Da): 985.8501	985.8500	-0.10
Linear- AAC*SAADAADA + S-carboxymethyl	(+) PapB + (58.04 Da): 1005.8571	1005.8532	-3.87

FIG. 20B

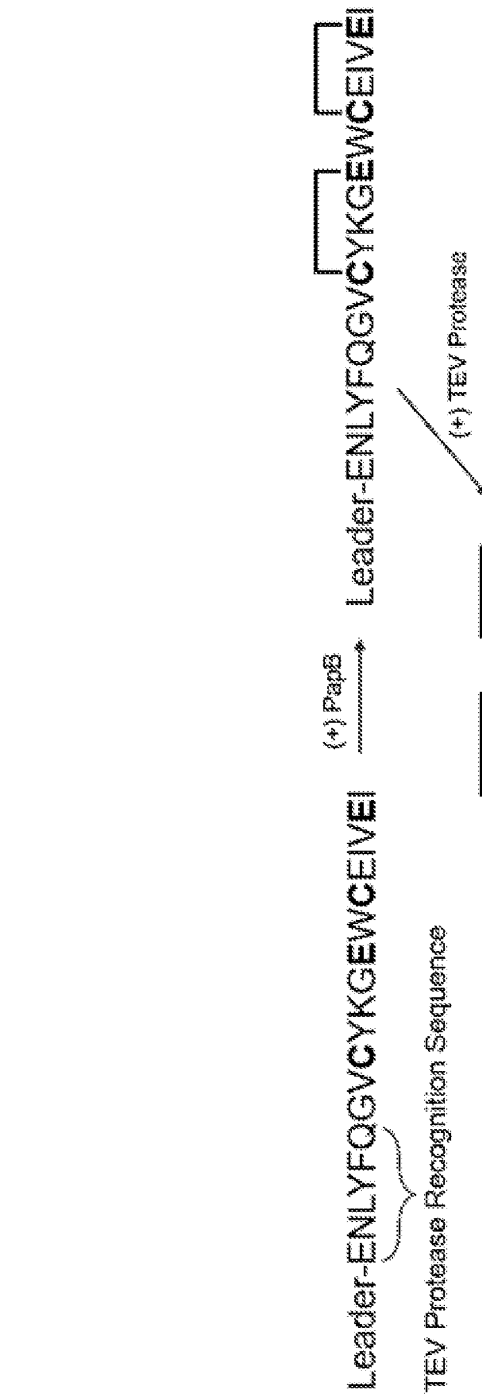

Leader-ENLYFQGVCYKGEWCEIVEI
TEV Protease Recognition Sequence
(+) PapB
Leader-ENLYFQGVCYKGEWCEIVEI
(+) TEV Protease
GVCYKGEWCEIVEI

FIG. 21A

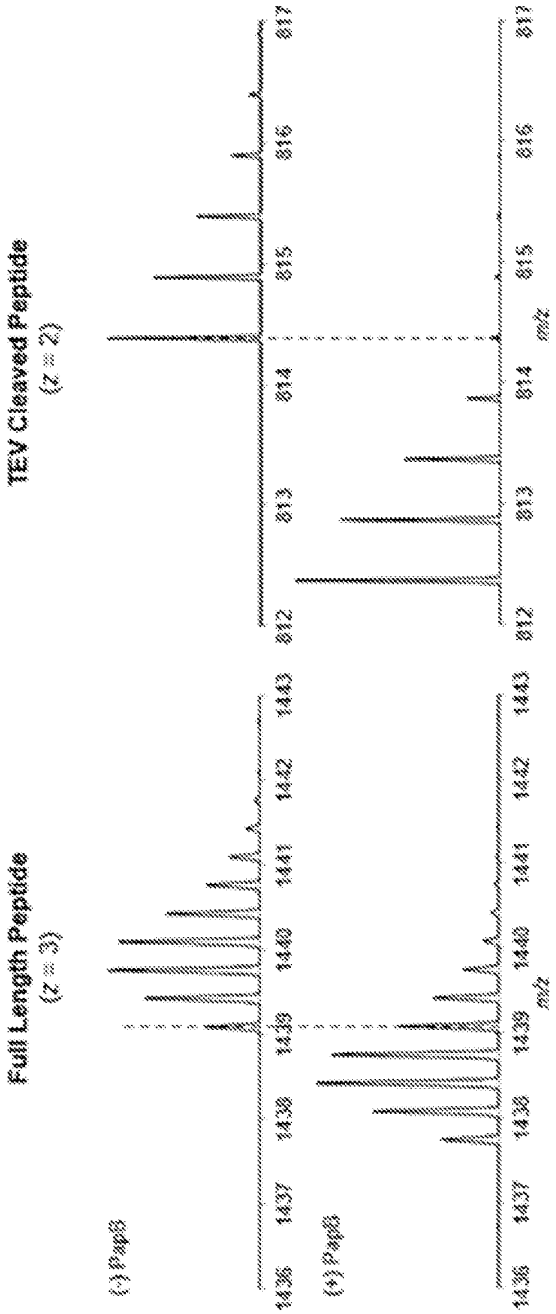

Full Length Peptide
(z = 2)
TEV Cleaved Peptide
(z = 2)
(-) Pep8
(+) Pep8
m/z
1436
1437
1438
1439
1440
1441
1442
1443
812
813
814
815
816
817

FIG. 21B

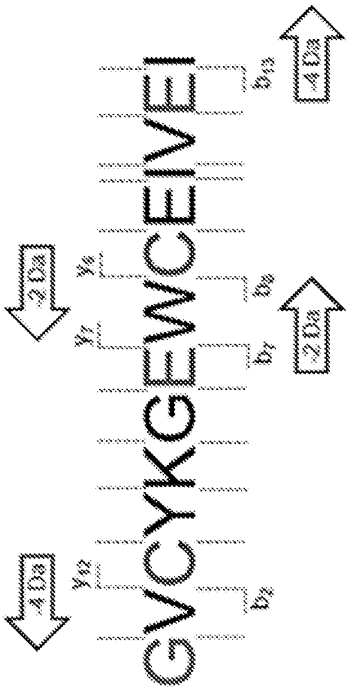


FIG. 21C

FIG. 22A

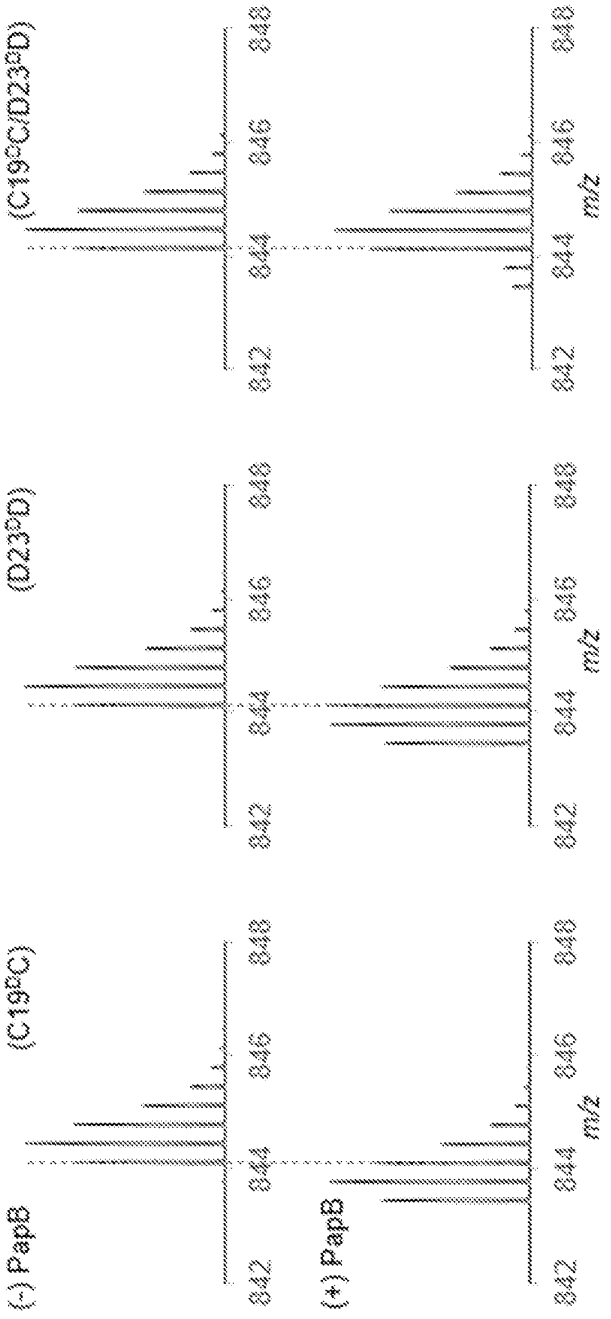


FIG. 22B

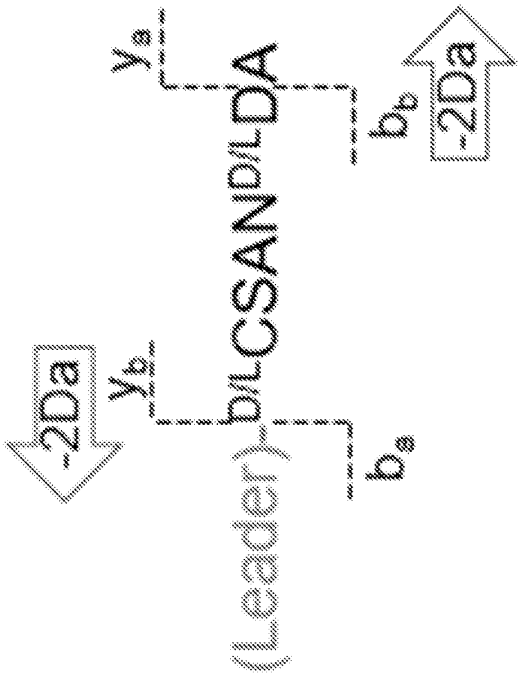
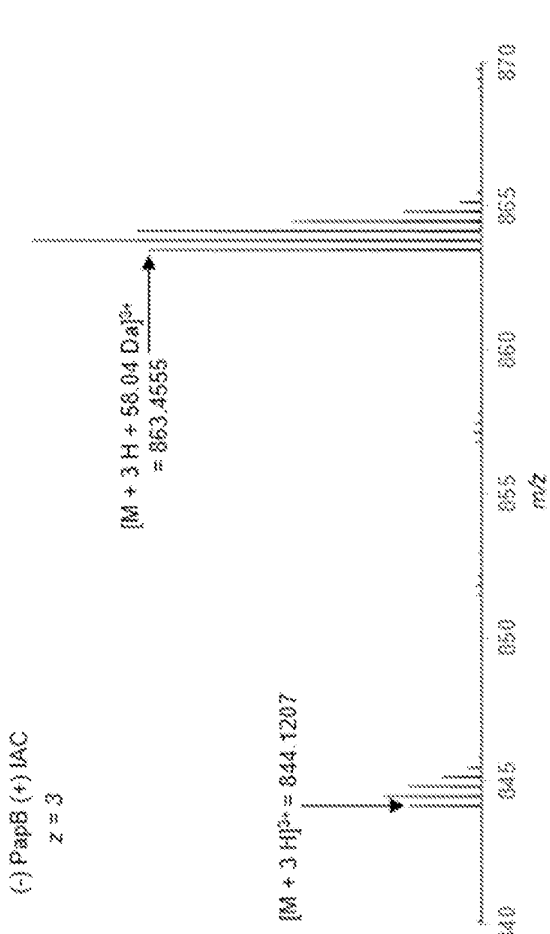
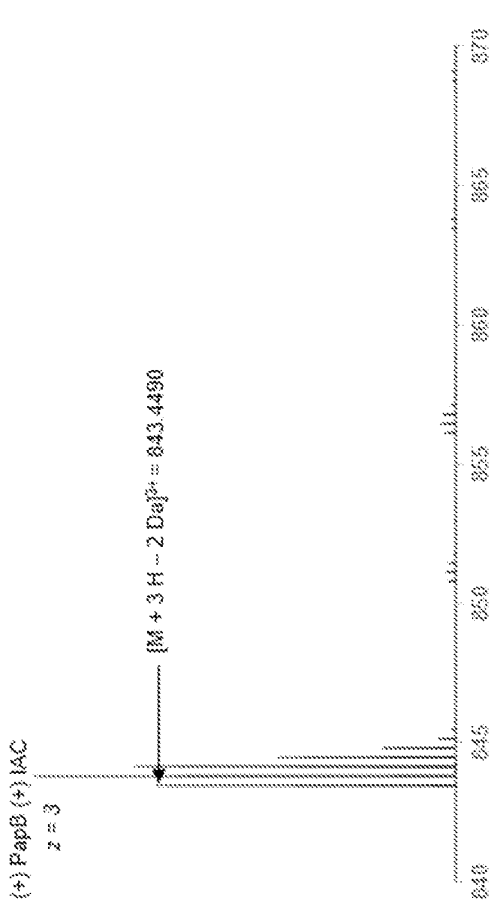


FIG. 22C



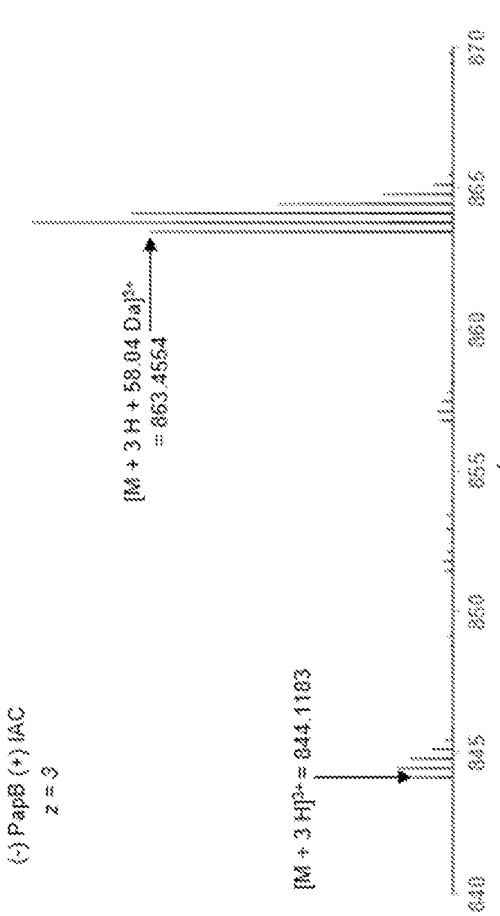
Sequence	Expected Monoisotopic Mass	Observed Monoisotopic Mass	Ppm Error
Leader-^{D}CSANDA	(-) PapB: 844.1197	844.1207	1.18
Leader-^{D}CSANDA + S-carboxymethyl	(-) PapB + (58.04 Da): 863.4549	863.4555	0.69

FIG. 23A



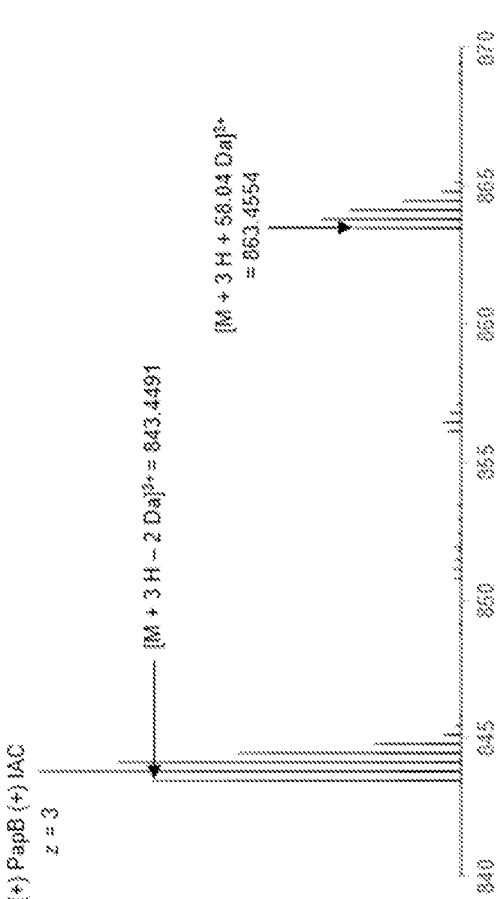
Sequence	Expected Monoisotopic Mass	Observed Monoisotopic Mass	Ppm Error
Leader-βCSANDA	(+) PapB - (2 Da): 843.4478	843.4490	1.42
Leader-βCSANDA + S-carboxymethyl	(-) PapB + (58.04 Da): 863.4549	Not Found	N/A

FIG. 23B



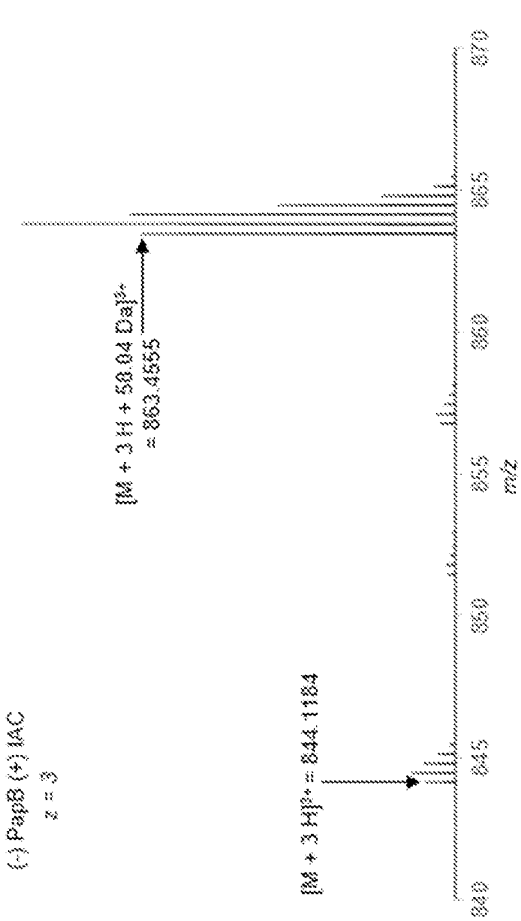
Sequence	Expected Monoisotopic Mass	Observed Monoisotopic Mass	Ppm Error
Leader-CSANFDA	(-) PapB 844.1197	844.1183	-1.66
Leader-CSANFDA + S-carboxymethyl	(-) PapB + (58.04 Da): 863.4549	863.4554	0.58

FIG. 24A



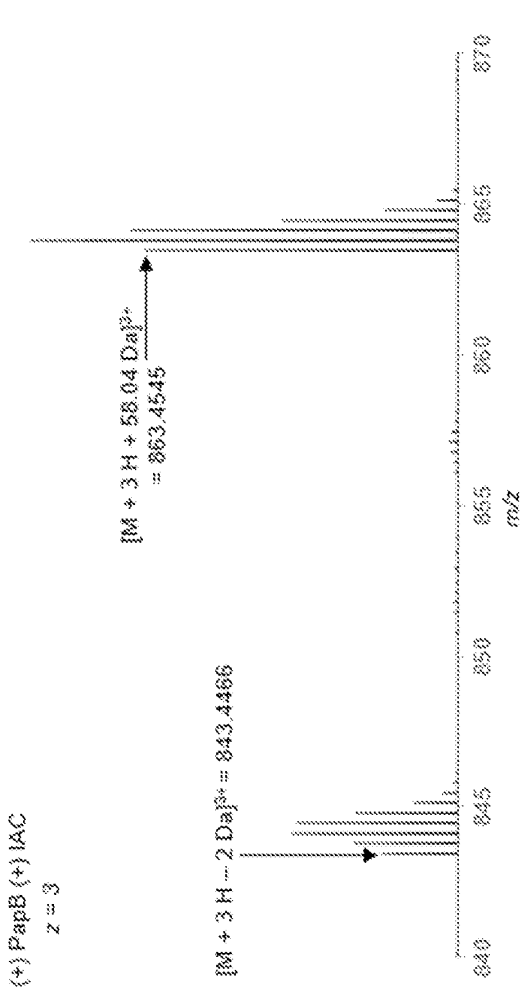
Sequence	Expected Monoisotopic Mass	Observed Monoisotopic Mass	Ppm Error
Leader-CSAN^{P}DA	(+) PapB - (2 Da): 843.4478	843.4491	1.54
Leader-CSAN^{P}DA + S-carboxymethyl	(-) PapB + (58.04 Da): 863.4549	863.4553	0.46

FIG. 24B



Sequence	Expected Monoisotopic Mass	Observed Monoisotopic Mass	Ppm Error
Leu/Ile-^{3CSAN}DA	(-) PapB 844.1197	844.1184	-1.54
Leu/Ile-^{3CSAN}DA + S-carboxymethyl	(-) PapB + (58.04 Da) 863.4549	863.4555	0.69

FIG. 25A



Sequence	Expected Monoisotopic Mass	Observed Monoisotopic Mass	Ppm Error
Leader-3CSAN2DA	(+) PapB - (2 Da): 843.4478	843.4466	-1.42
Leader-3CSAN2DA + S-carboxymethyl	(-) PapB + (58.04 Da): 863.4549	863.4545	-0.46

FIG. 25B

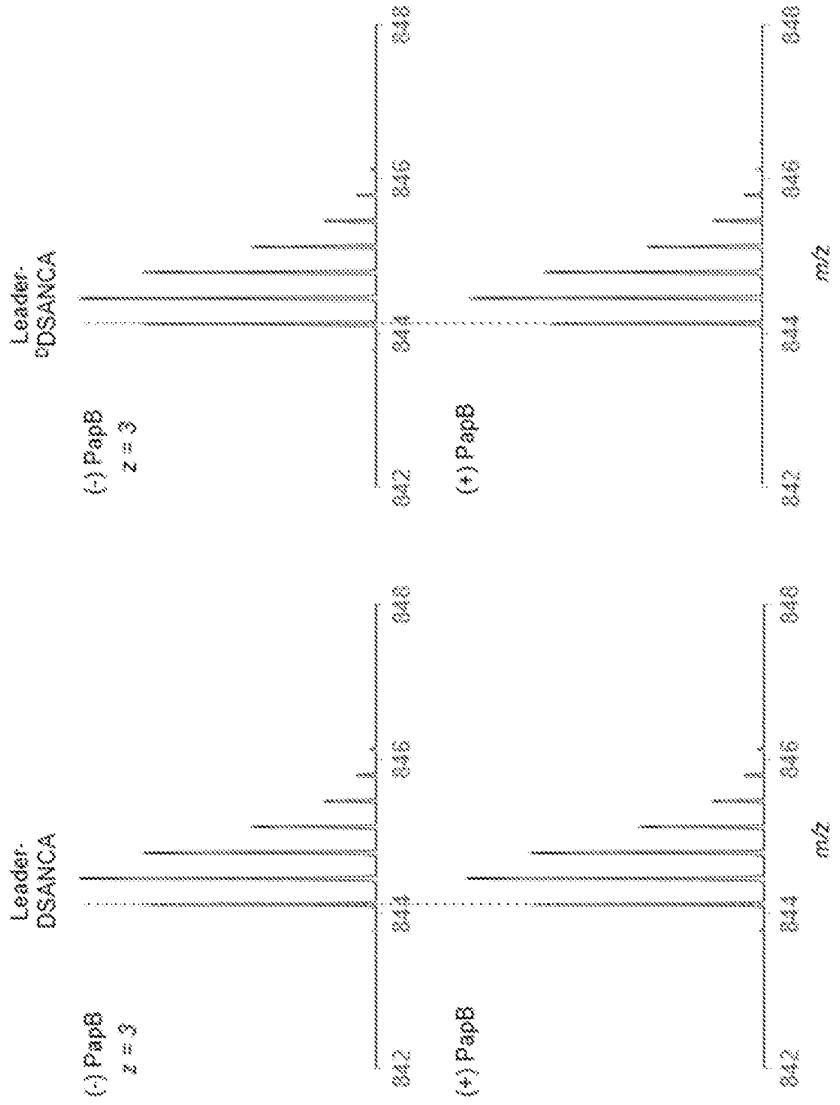

Leader-DSANCA
(-) PapB
z = 3
(+) PapB
Leader-DSANCA
(-) PapB
z = 3
(+) PapB
842
844
846
848
m/z

FIG. 26A

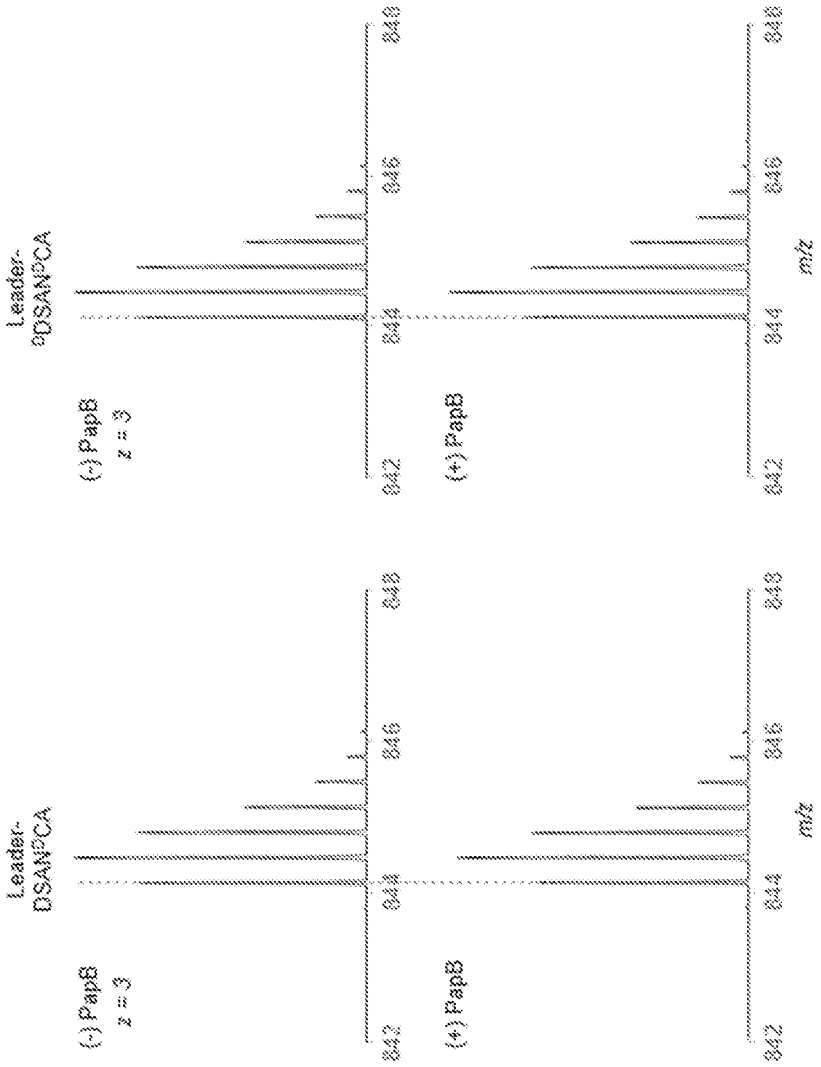


FIG. 26B

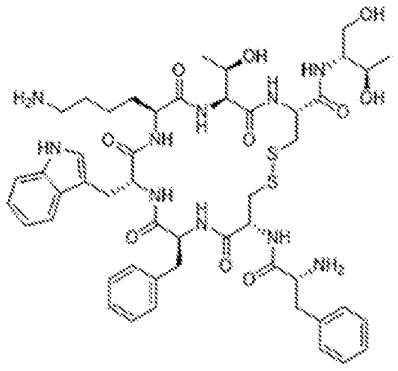


FIG. 27A

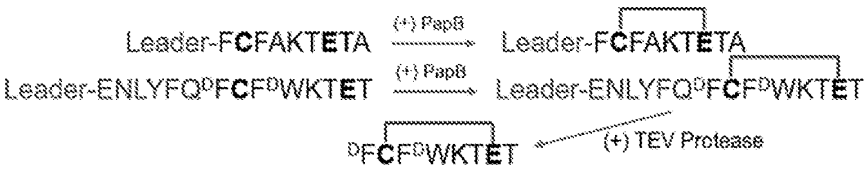


FIG. 27B

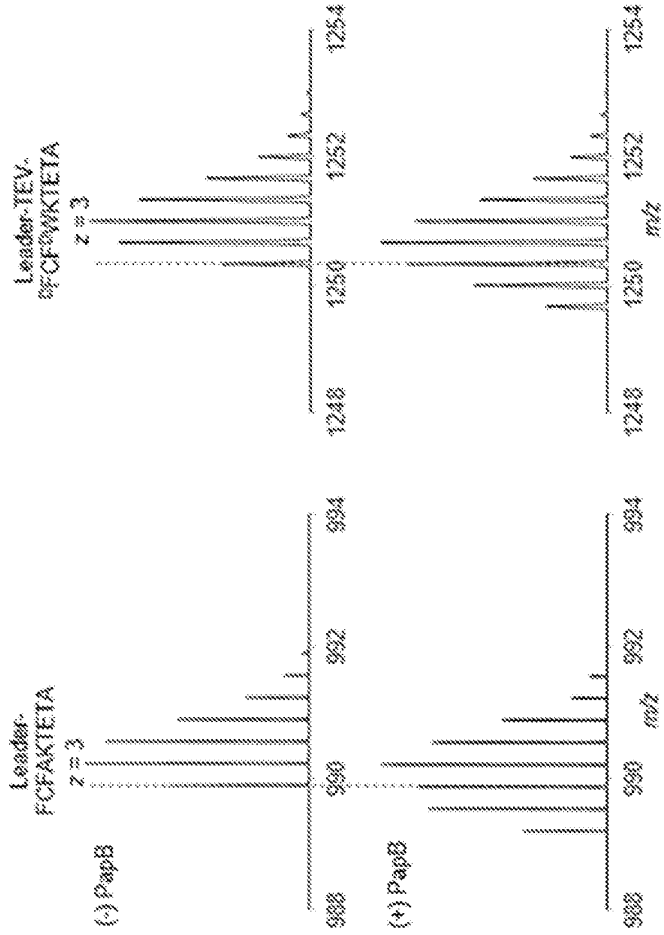

Leader-TEV-
%FCF%WKTETA
z = 3
Leader-
FCFAKTETA
z = 3
(-) PepB
(+) PepB
988
990
992
994
1248
1250
1252
1254
m/z

FIG. 27C

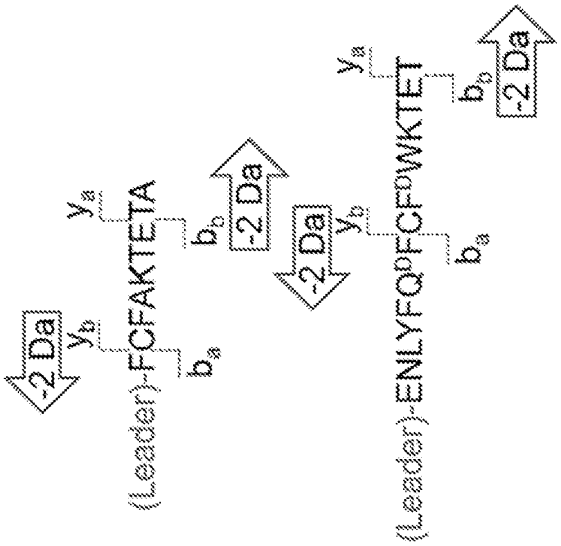


FIG. 27E

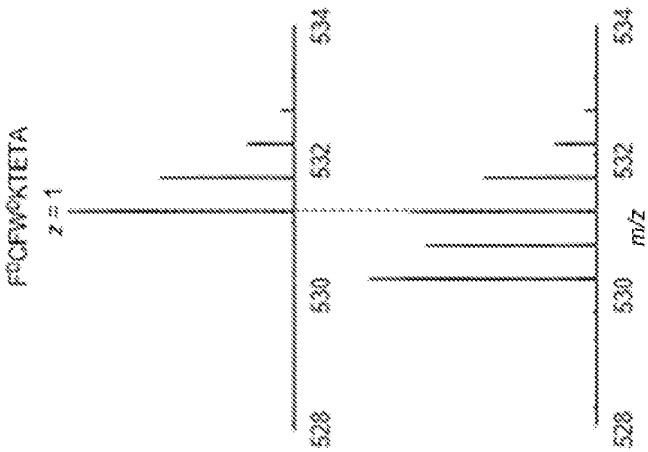


FIG. 27D

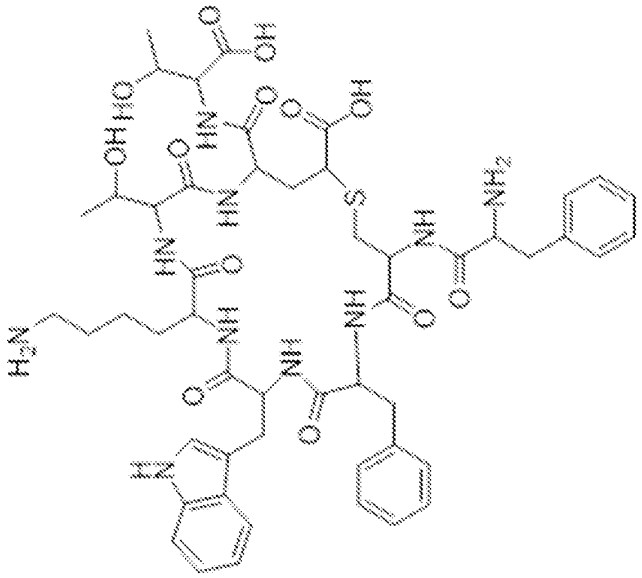


FIG. 28

Successful Crosslink Sequences:	(Bolded residues are not contained within the wild type)
Additional Spacers:	LKQINVIAGVKEPIRAYG**AAA**CSANDA
Independent Nested Crosslinking:	LKQINVIAGVKEPIRAYG**AAA**CSAC**D**A**A**D**A**
Nested Crosslink Delta 1:	LKQINVIAGVKEPIRAYG**AAAA**SAC**D**A**A**D**A**
Nested Crosslink Delta 2:	LKQINVIAGVKEPIRAYG**AAA**CSA**A**C**A**A**D**A
Repeated "CSANDA" with spacer:	LKQINVIAGVKEPIRAYG**AAA**CSANDA**CSANDA**
C20C^{D}:	LKQINVIAGVKEPIRAYGC^{D}SANDA
D24D^{D}:	LKQINVIAGVKEPIRAYGCSAND^{D}A
C20C^{D}/D24D^{D}:	LKQINVIAGVKEPIRAYGC^{D}SAND^{D}A
C20hC (homocysteine):	LKQINVIAGVKEPIRAYG(h**C**)SANDA
D24E:	LKQINVIAGVKEPIRAYGCSAN**E**A
C20hC/D24E:	LKQINVIAGVKEPIRAYG(h**C**)SAN**E**A
D24EneA:	LKQINVIAGVKEPIRAYGCSAN(**EneA**)A (The crosslinked product in a minor product)

FIG. 29

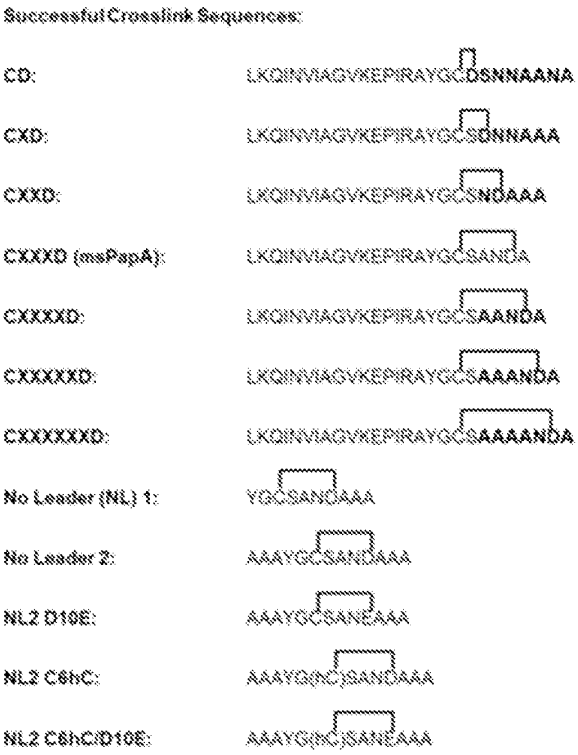


FIG. 29
(Continued)

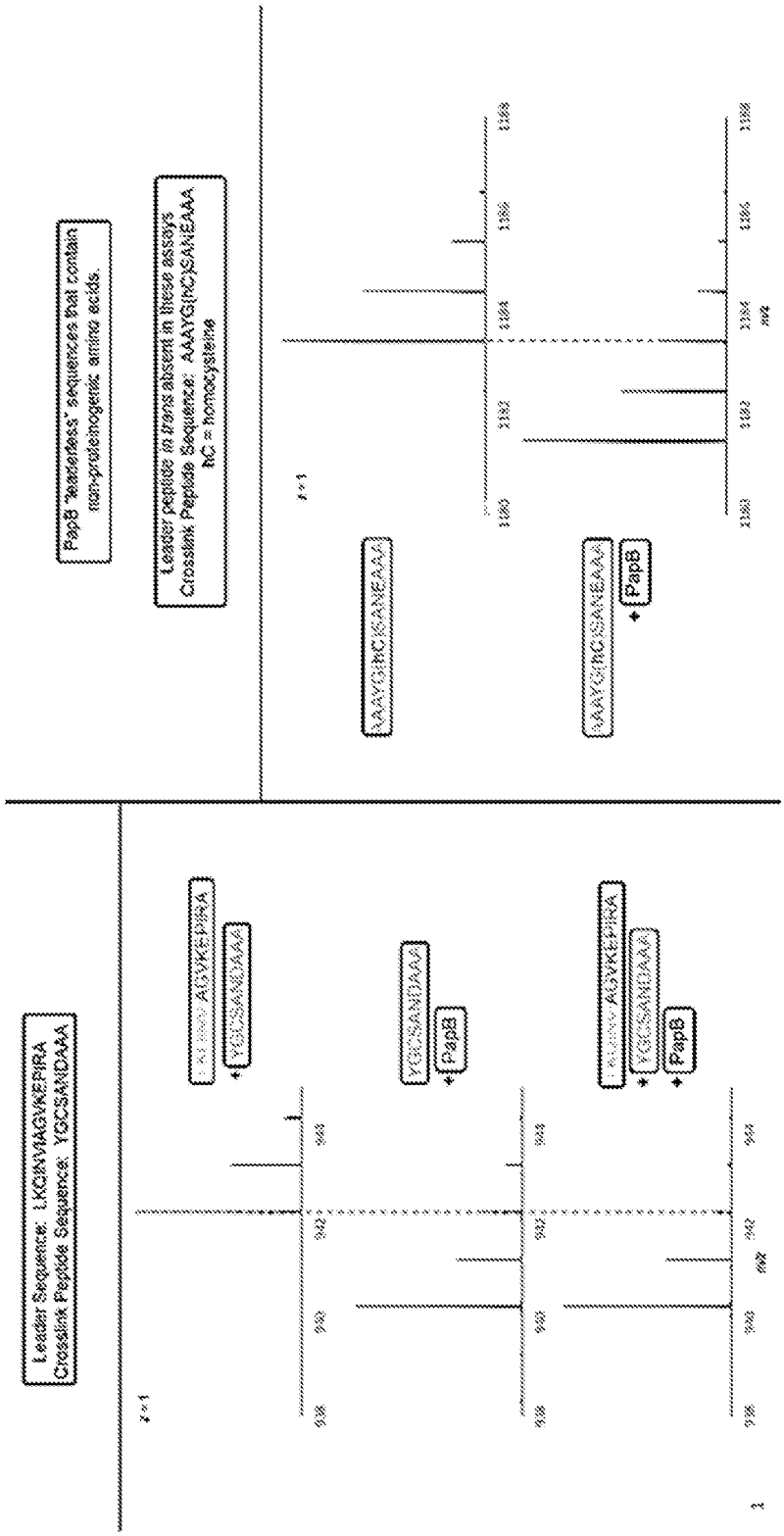

Leader Sequence: LKCIVIAGVKEFIRA
Crosslink Peptide Sequence: YGCSANDAAA
PapB "leaderless" sequences that contain non-proteinogenic amino acids
Leader peptide in trans absent in these assays
Crosslink Peptide Sequence: AAAYG(hC)SANEAAA
hC = homocysteine
YGCSANDAAA
PapB
AAAYG(hC)SANEAAA

FIG. 30

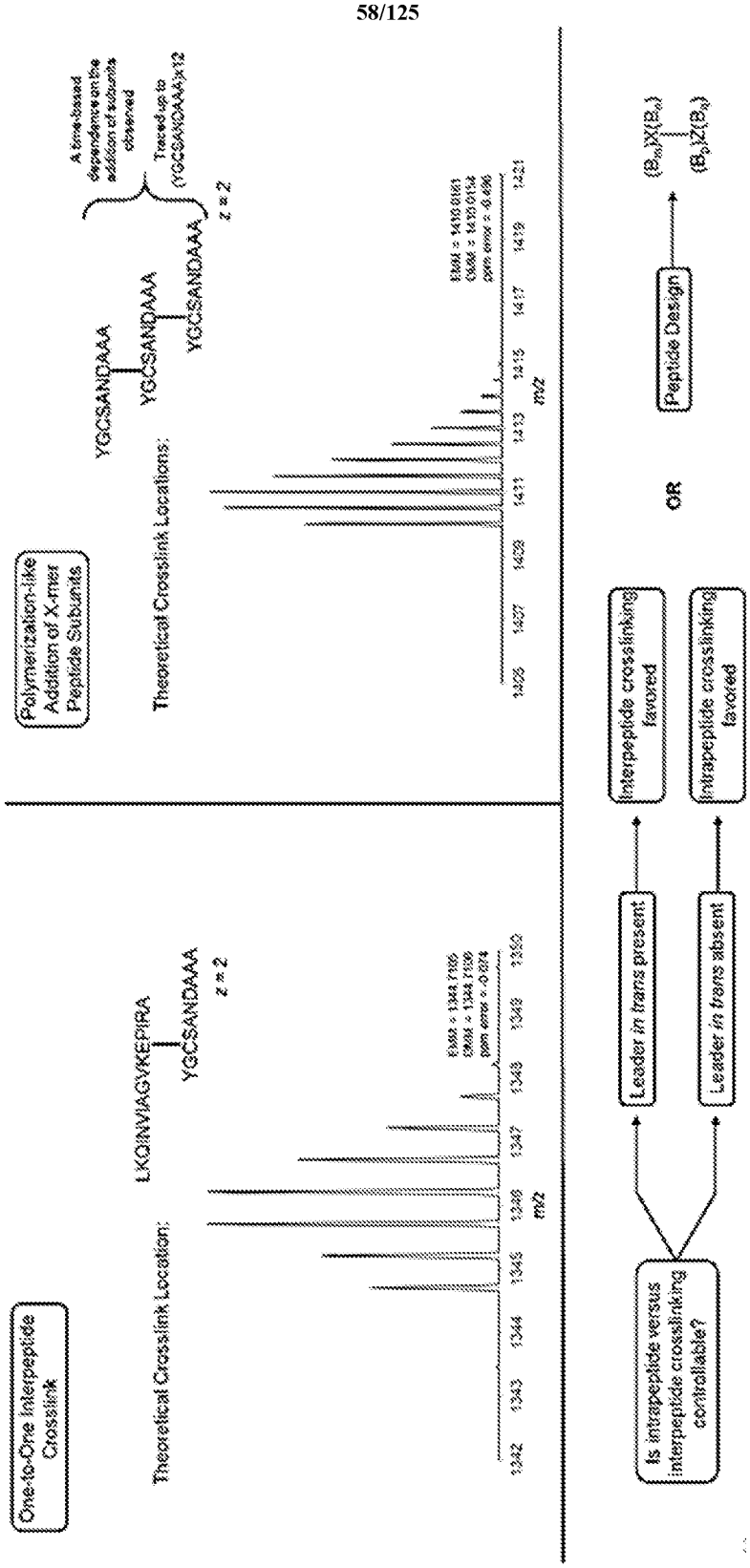

One-to-One Interpeptide Crosslink
Theoretical Crosslink Location:
LKQINVIAGYKEPIRA
YGCSANDAAA
z = 2
m/z
Is intrapeptide versus interpeptide crosslinking controllable?
Leader in trans present
Leader in trans absent
Interpeptide crosslinking favored
Intrapeptide crosslinking favored
OR
Peptide Design
Polymerization-like Addition of X-mer Peptide Subunits
Theoretical Crosslink Locations:
YGCSANDAAA
YGCSANDAAA
YGCSANDAAA
z = 2
A time-based dependence on the addition of subunits observed
Traced up to (YGCSANDAAA)x12
m/z

FIG. 31

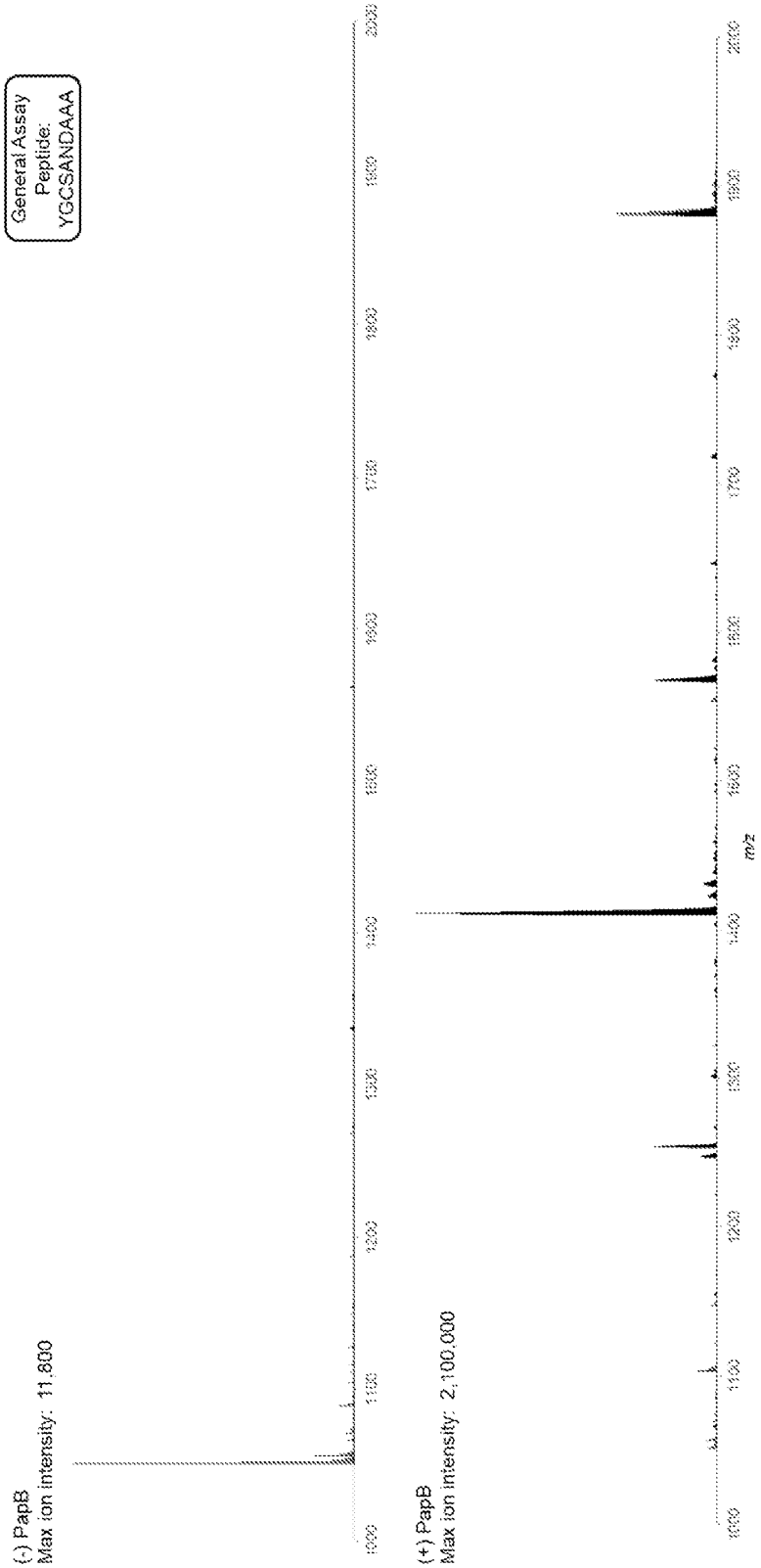

General Assay
Peptide:
YGCSANDAAA
(-) PapB
Max ion intensity: 11,800
(+) PapB
Max ion intensity: 2,100,000
m/z

FIG. 32

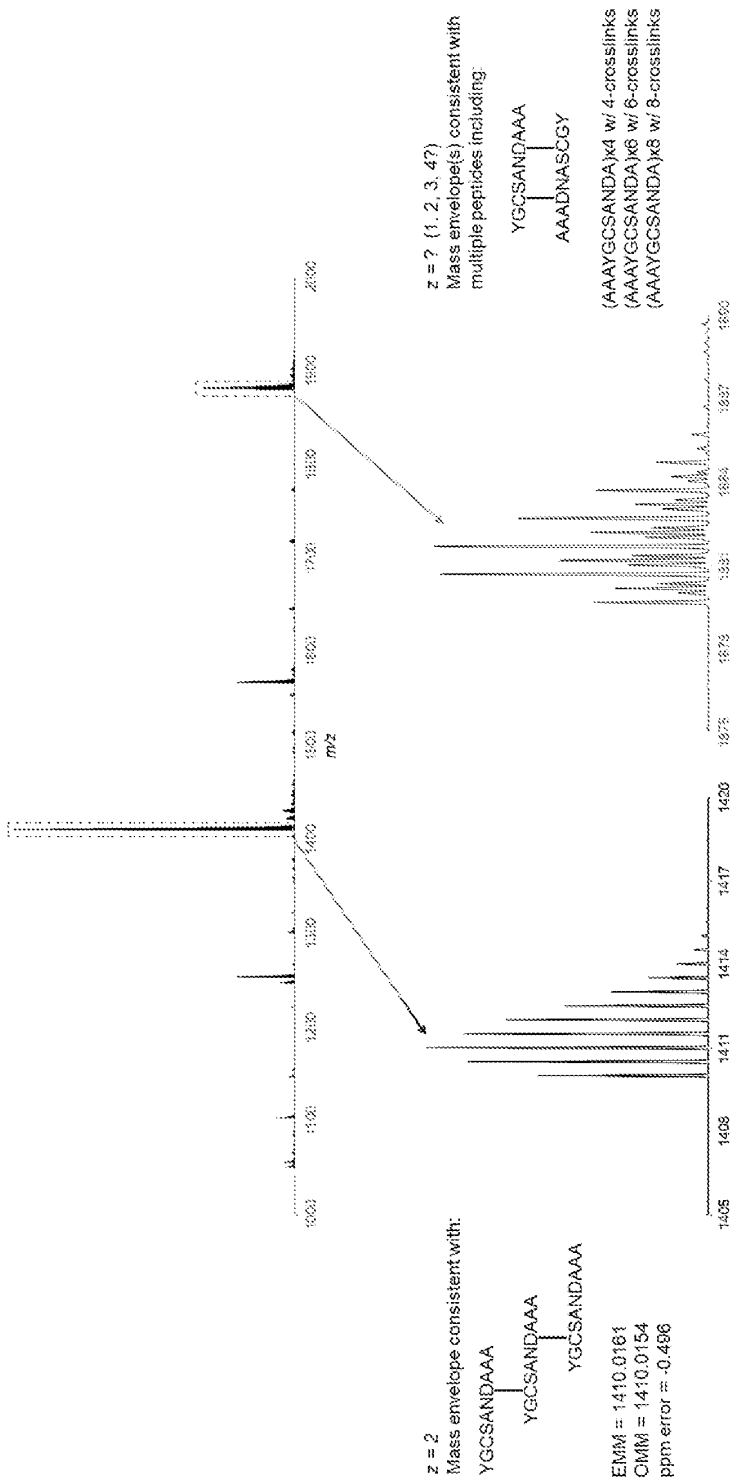

z = 2
Mass envelope consistent with:
YGCSANDAAA
YGCSANDAAA
YGCSANDAAA
EMM = 1410.0161
CMM = 1410.0154
ppm error = -0.496
z = ? (1, 2, 3, 4?)
Mass envelope(s) consistent with
multiple peptides including:
YGCSANDAAA
AAADNASCGY
(AAAYGCSANDA)x4 w/ 4-crosslinks
(AAAYGCSANDA)x6 w/ 6-crosslinks
(AAAYGCSANDA)x8 w/ 8-crosslinks
m/z

FIG. 33

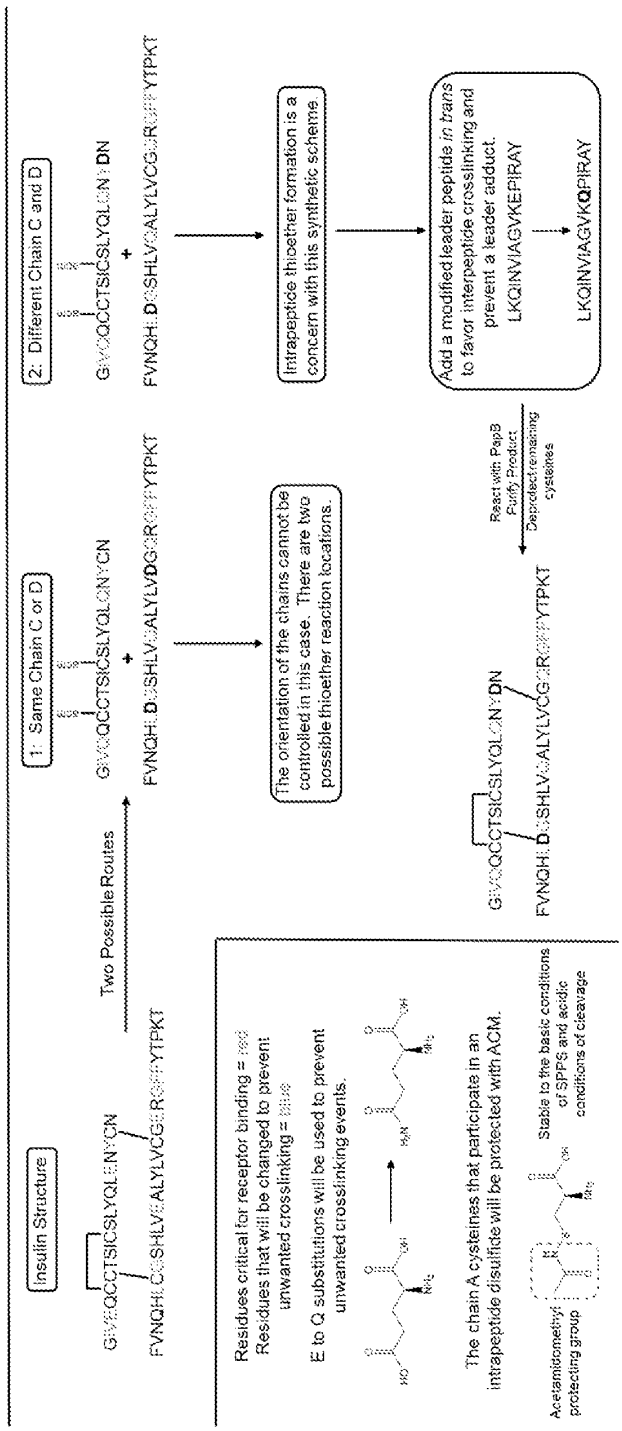

Peptide Design
Insulin Structure
Two Possible Routes
1: Same Chain C or D
2: Different Chain C and D
Thioether Insulin Analog
The orientation of the chains cannot be controlled in this case. There are two possible thioether reaction locations.
Intrapeptide thioether formation is a concern with this synthetic scheme.
Add a modified leader peptide in trans to favor interpeptide crosslinking and prevent a leader adduct.
LKQINVIAGVKEPIRAY
LKQINVIAGVKQPIRAY
React with PapB
Purify Product
Deprotect remaining cysteines
Residues critical for receptor binding = red
Residues that will be changed to prevent unwanted crosslinking = blue
E to Q substitutions will be used to prevent unwanted crosslinking events.
The chain A cysteines that participate in an intrapeptide disulfide will be protected with ACM.
Acetamidomethyl protecting group
Stable to the basic conditions of SPPS and acidic conditions of cleavage

FIG. 34

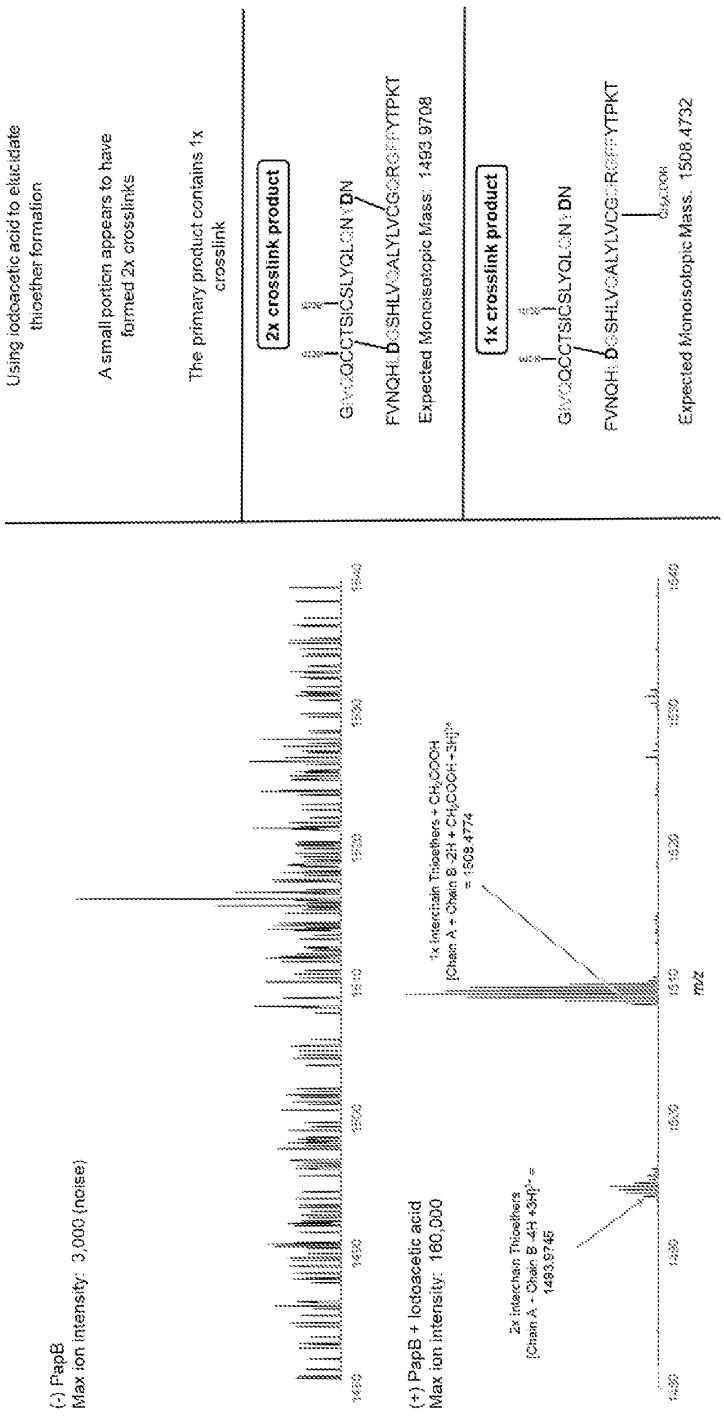

Using iodoacetic acid to elucidate thioether formation
A small portion appears to have formed 2x crosslinks
The primary product contains 1x crosslink
2x crosslink product
1x crosslink product
Expected Monoisotopic Mass: 1493.9708
Expected Monoisotopic Mass: 1508.4732
(-) PapB
Max ion intensity: 3,000 (noise)
(+) PapB + Iodoacetic acid
Max ion intensity: 160,000
2x interchain Thioethers
[Chain A + Chain B -4H +3H]3+ =
1493.9745
1x interchain Thioethers + CH2COOH
[Chain A + Chain B -2H + CH2COOH +3H]3+
= 1508.4774
m/z

FIG. 35

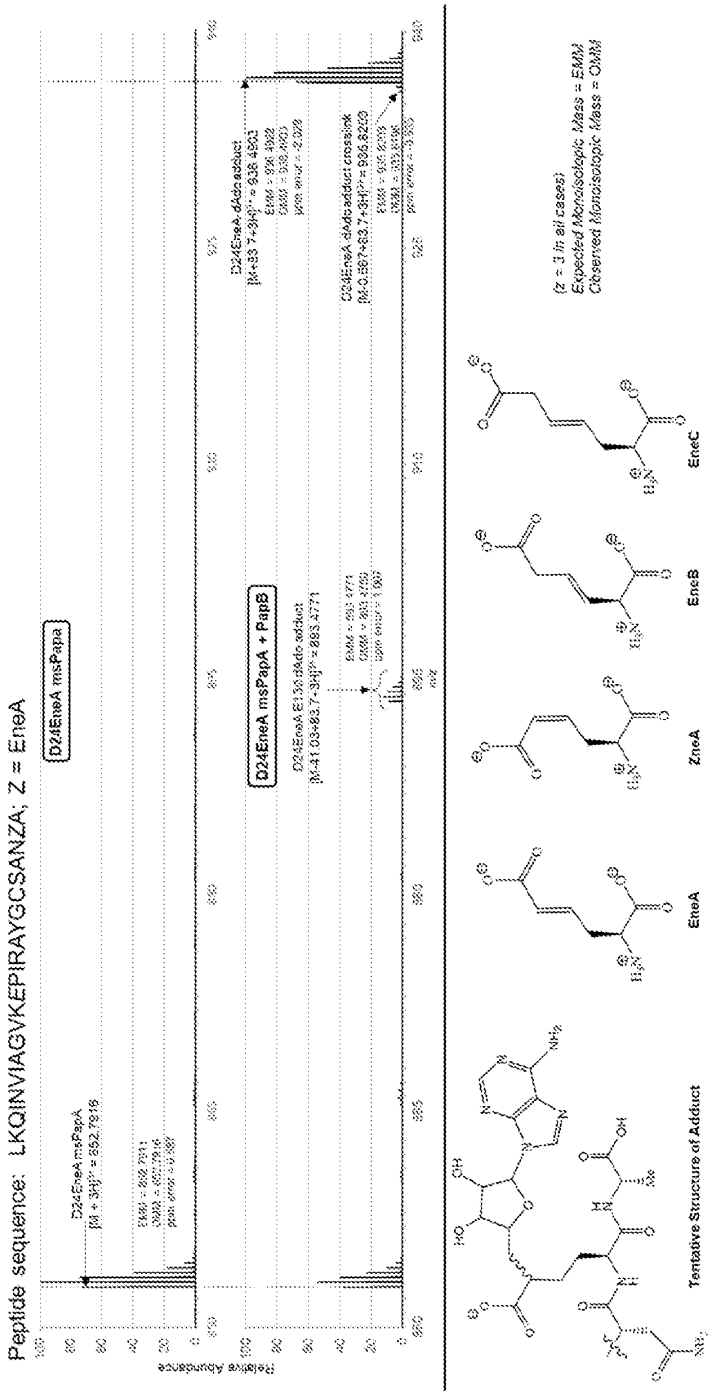

Peptide sequence: LKQINVIAGVKEPIRAYGCSANZA; Z = EneA
D24EneA msPapa
D24EneA msPapA
[M + 3H]3+ = 852.7916
D24EneA msPapA + PapB
D24EneA dado adduct
[M+83.7+3H]3+ = 938.4803
D24EneA dado adduct crosslink
[M-0.987+83.7+3H]3+ = 936.8203
Relative Abundance
Tentative Structure of Adduct
EneA
ZneA
EneB
EneC
(z = 3 in all cases)
Expected Monoisotopic Mass = EMM
Observed Monoisotopic Mass = OMM

FIG. 36

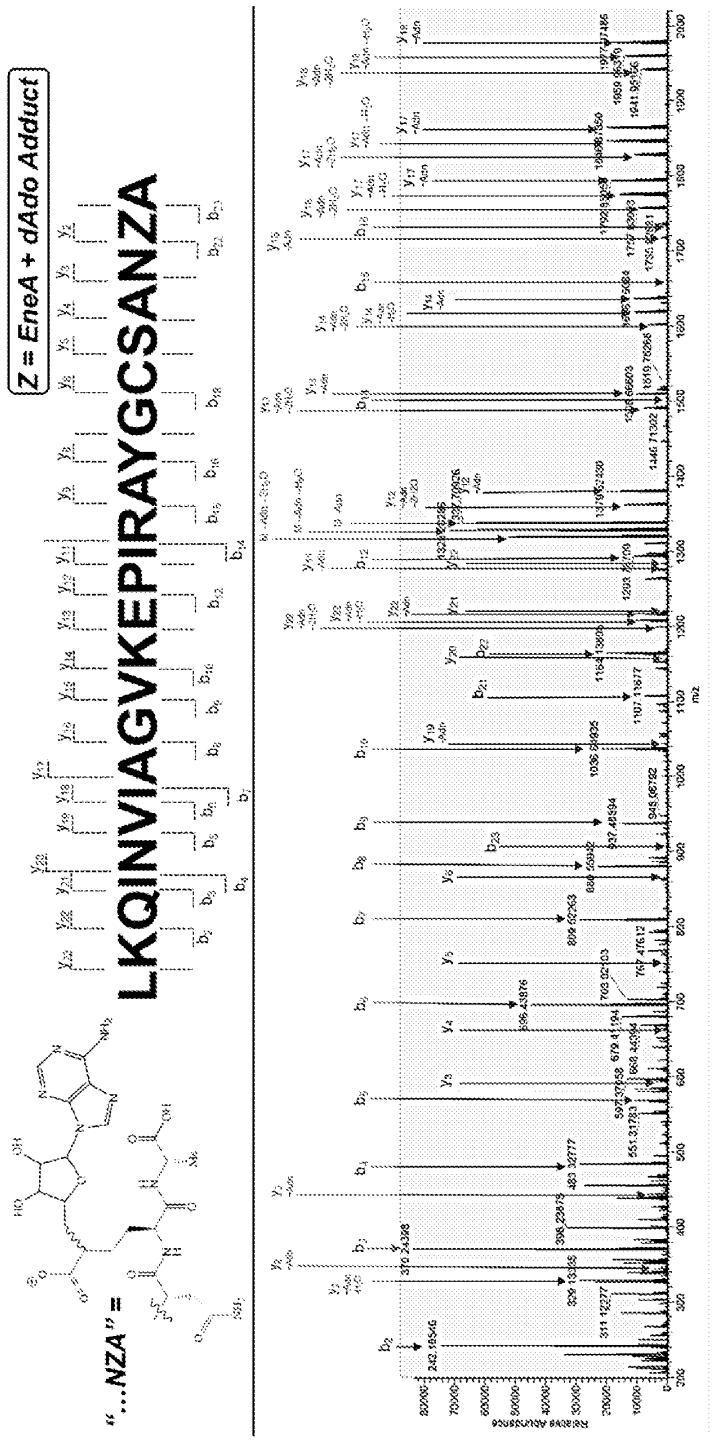


FIG. 37

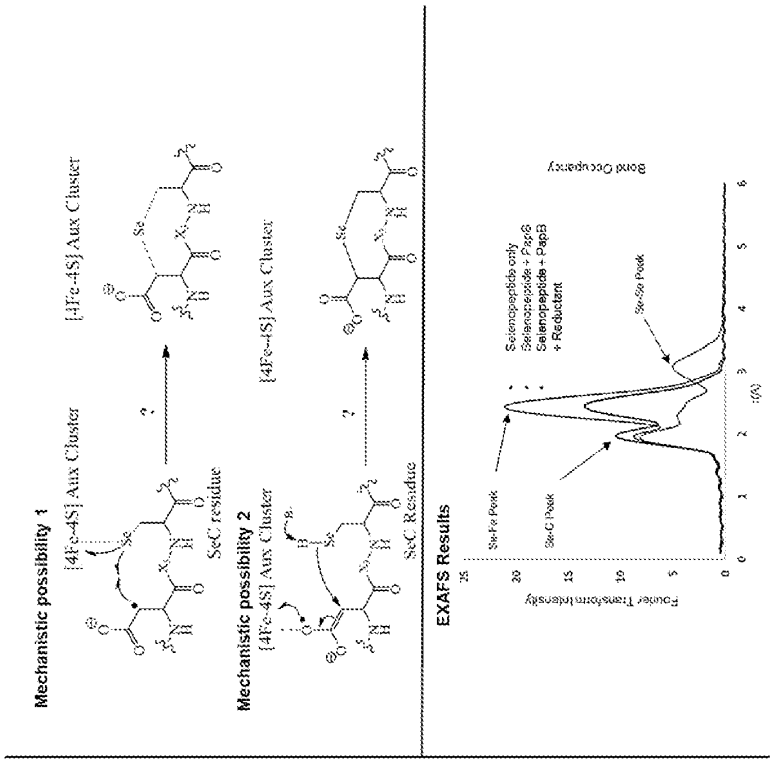
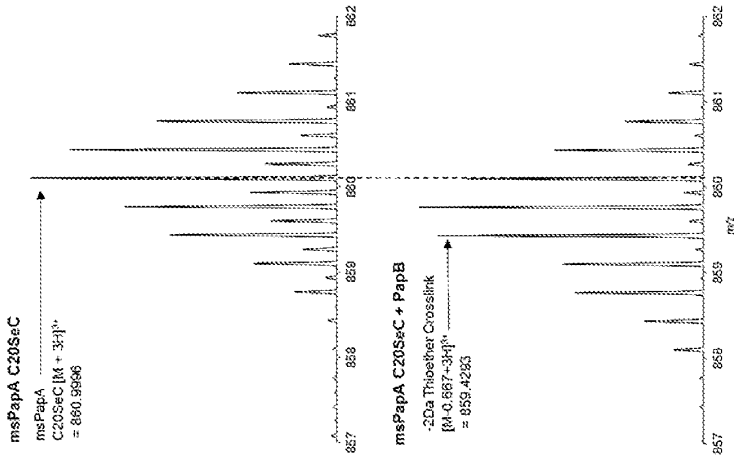

Mechanistic possibility 1
[4Fe-4S] Aux Cluster
SeC residue
[4Fe-4S] Aux Cluster
Mechanistic possibility 2
[4Fe-4S] Aux Cluster
[4Fe-4S] Aux Cluster
SeC Residue
EXAFS Results
Se-Fe Peak
Se-C Peak
Selenopeptide only
Selenopeptide + PapB
Selenopeptide + PapB + Reductant
Se-Se Peak
Fourier Transform Intensity
Bond Decupency

FIG. 38


msPapA C20SeC
msPapA C20SeC [M + 3H]3+ = 860.9996
msPapA C20SeC + PapB
-2Da Thioether Crosslink [M-0.667+3H]3+ = 859.4283
m/z

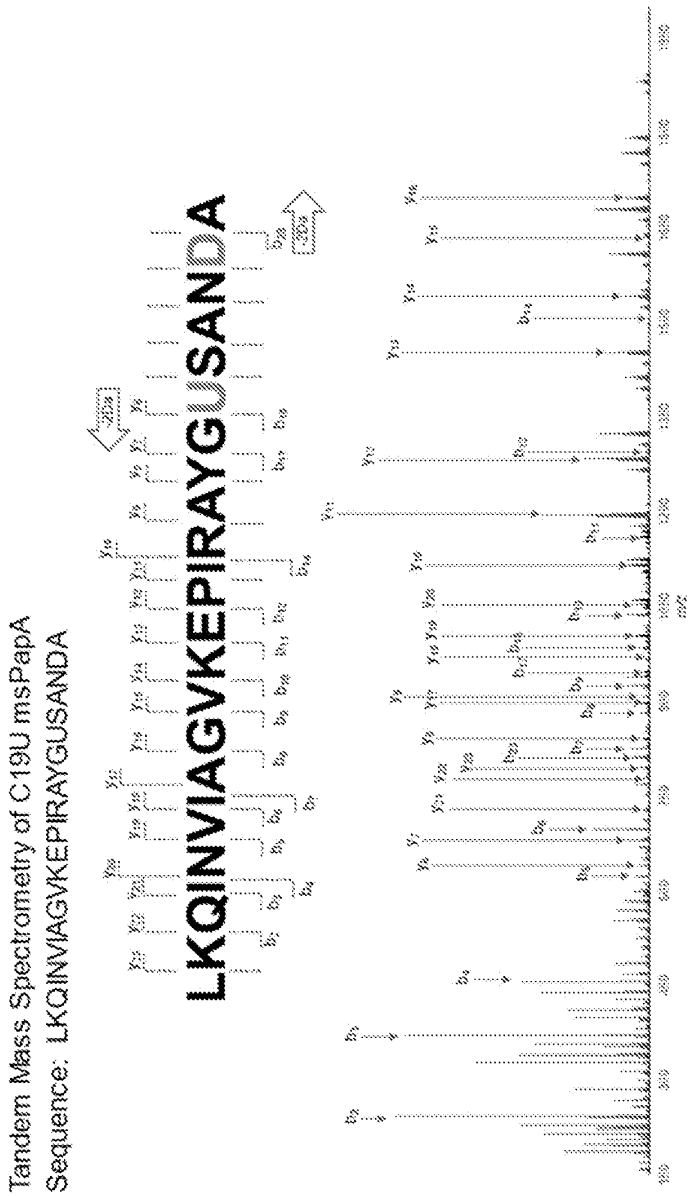

Tandem Mass Spectrometry of C19U msPapA
Sequence: LKQINVIAGVKEPIRAYGUSANDA
LKQINVIAGVKEPIRAYGUSANDA
m/z

FIG. 39

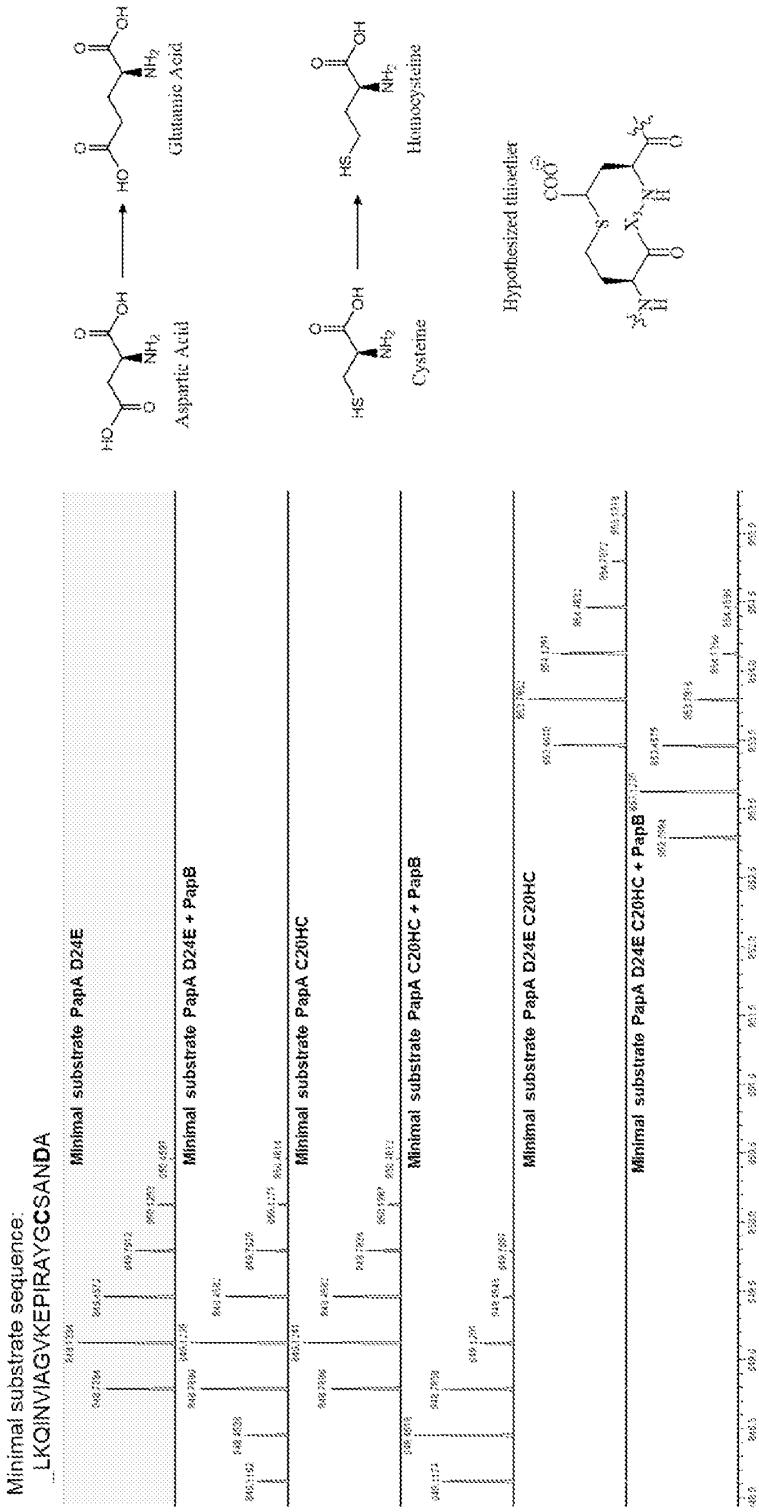

Minimal substrate sequence:
LKQINVIAGVKEPIRAYGCSANDA
Minimal substrate PapA D24E
Minimal substrate PapA D24E + PapB
Minimal substrate PapA C20HC
Minimal substrate PapA C20HC + PapB
Minimal substrate PapA D24E C20HC
Minimal substrate PapA D24E C20HC + PapB
Aspartic Acid
Glutamic Acid
Cysteine
Homocysteine
Hypothesized thioether

FIG. 40

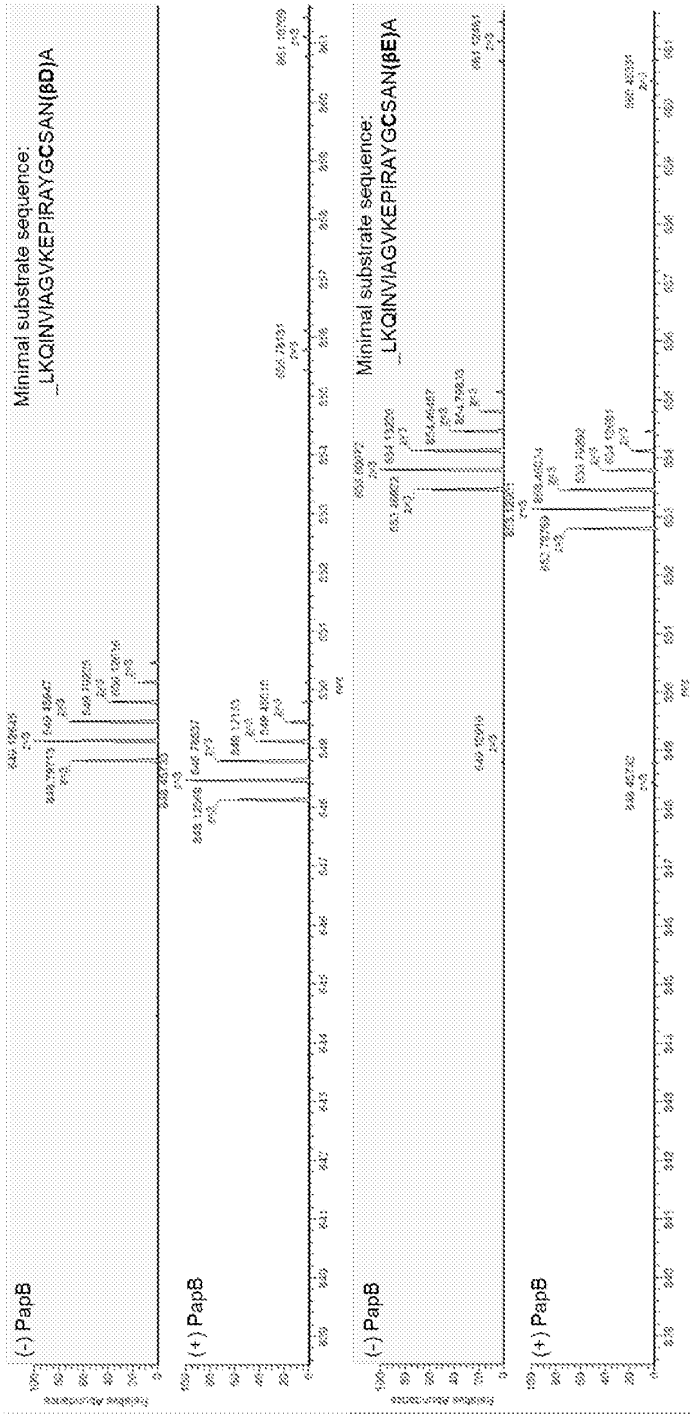

(-) PapB
Minimal substrate sequence:
_LKQINVIAGVKEPIRAYGCSAN(βD)A
(+) PapB
(-) PapB
Minimal substrate sequence:
_LKQINVIAGVKEPIRAYGCSAN(βE)A
(+) PapB

FIG. 41

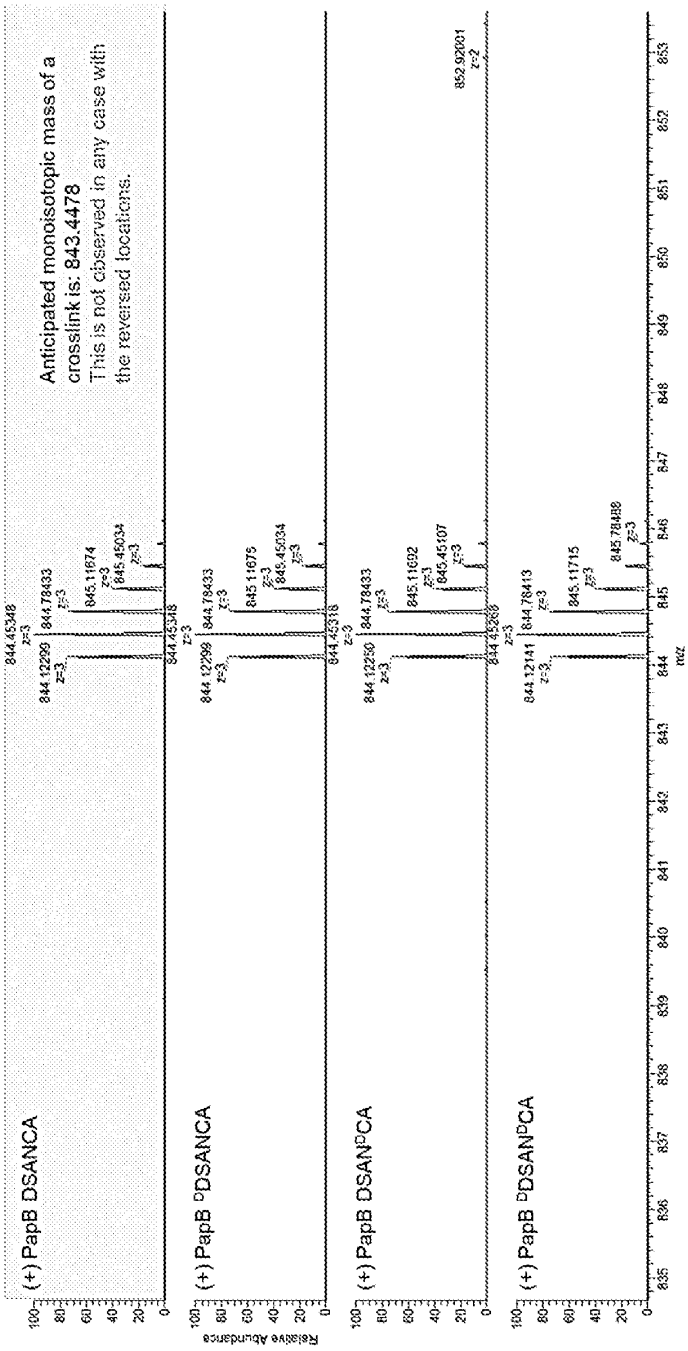

(+) PapB DSANCA
Anticipated monoisotopic mass of a crosslink is: 843.4478
This is not observed in any case with the reversed locations.
(+) PapB DDSANCA
(+) PapB DSANDCA
(+) PapB DDSANDCA
Relative Abundance
m/z

FIG. 42

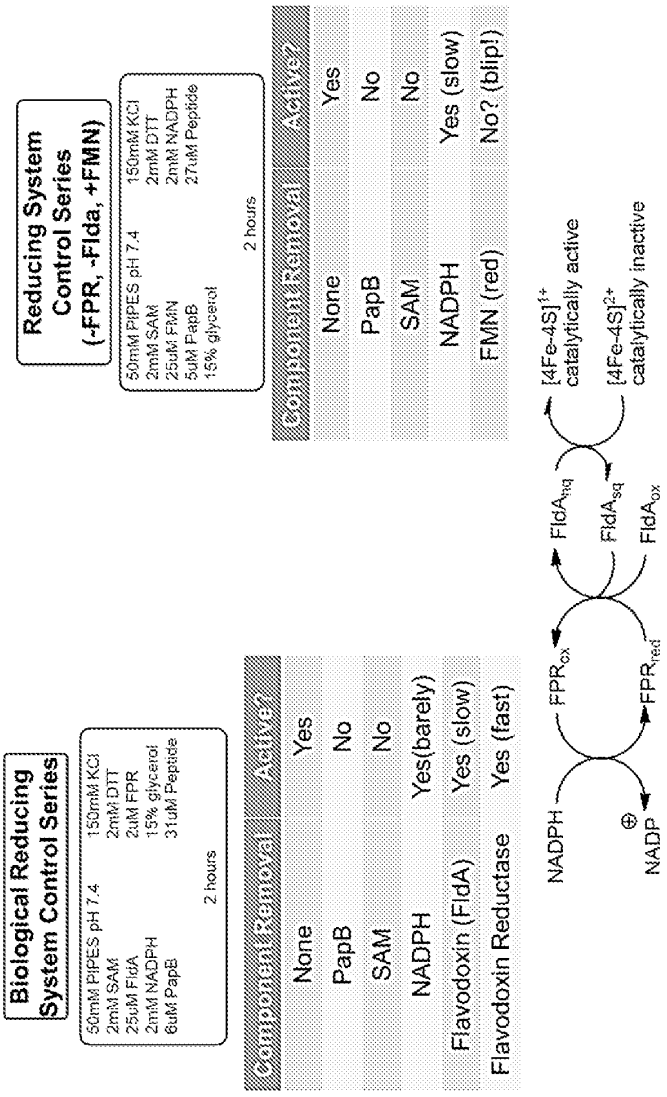

Biological Reducing System Control Series
50mM PIPES pH 7.4
2mM SAM
25uM FldA
2mM NADPH
6uM PapB
150mM KCl
2mM DTT
2uM FPR
15% glycerol
31uM Peptide
2 hours
Component Removed
Active?
None
Yes
PapB
No
SAM
No
NADPH
Yes(barely)
Flavodoxin (FldA)
Yes (slow)
Flavodoxin Reductase
Yes (fast)
Reducing System Control Series (-FPR, -Flda, +FMN)
50mM PIPES pH 7.4
2mM SAM
25uM FMN
5uM PapB
15% glycerol
150mM KCl
2mM DTT
2mM NADPH
27uM Peptide
2 hours
Component Removed
Active?
None
Yes
PapB
No
SAM
No
NADPH
Yes (slow)
FMN (red)
No? (blip!)
NADPH
NADP
FPR$_{ox}$
FPR$_{red}$
FldA$_{hq}$
FldA$_{sq}$
FldA$_{ox}$
[4Fe-4S]$^{1+}$ catalytically active
[4Fe-4S]$^{2+}$ catalytically inactive

FIG. 43

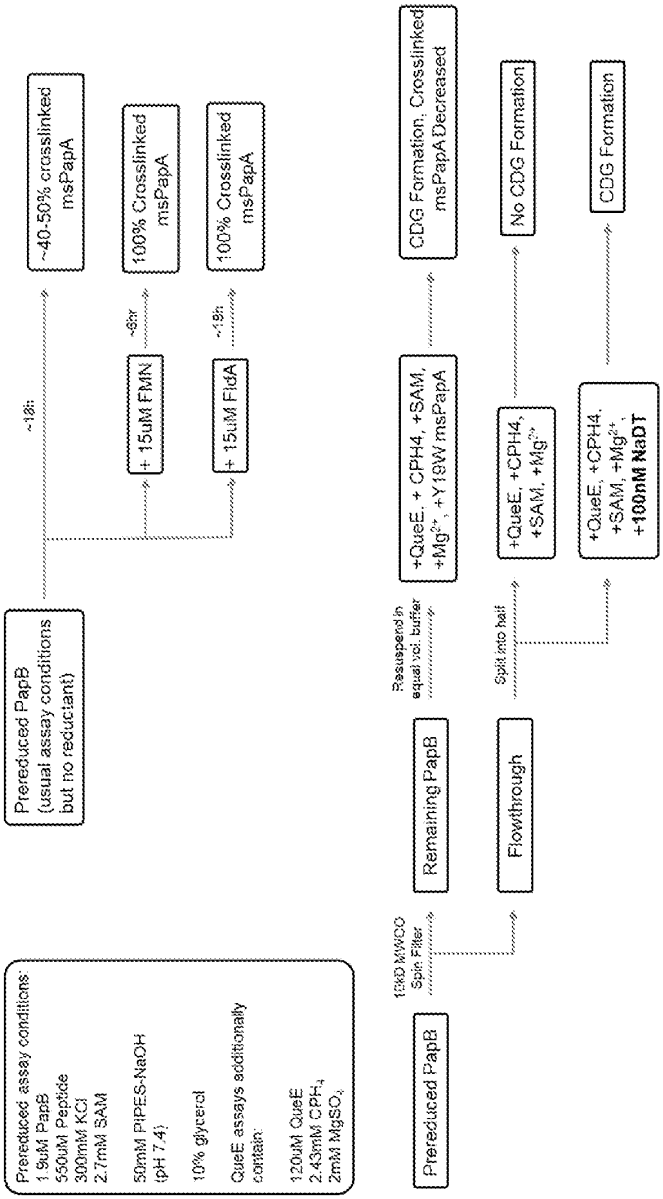

Prereduced assay conditions:
1.9uM PapB
550uM Peptide
300mM KCl
2.7mM SAM
50mM PIPES-NaOH (pH 7.4)
10% glycerol
QueE assays additionally contain:
120uM QueE
2.43mM CPH4
2mM MgSO4
Prereduced PapB (usual assay conditions but no reductant)
~18h
~40-50% crosslinked msPapA
+ 15uM FMN
~8h
100% Crosslinked msPapA
+ 15uM FldA
~18h
100% Crosslinked msPapA
10kD MWCO Spin Filter
Prereduced PapB
Remaining PapB
Resuspend in equal vol. buffer
+QueE, + CPH4, +SAM, +Mg2+, +Y19W msPapA
CDG Formation, Crosslinked msPapA Decreased
Flowthrough
Split into half
+QueE, +CPH4, +SAM, +Mg2+
No CDG Formation
+QueE, +CPH4, +SAM, +Mg2+, +100nM NaDT
CDG Formation

FIG. 44

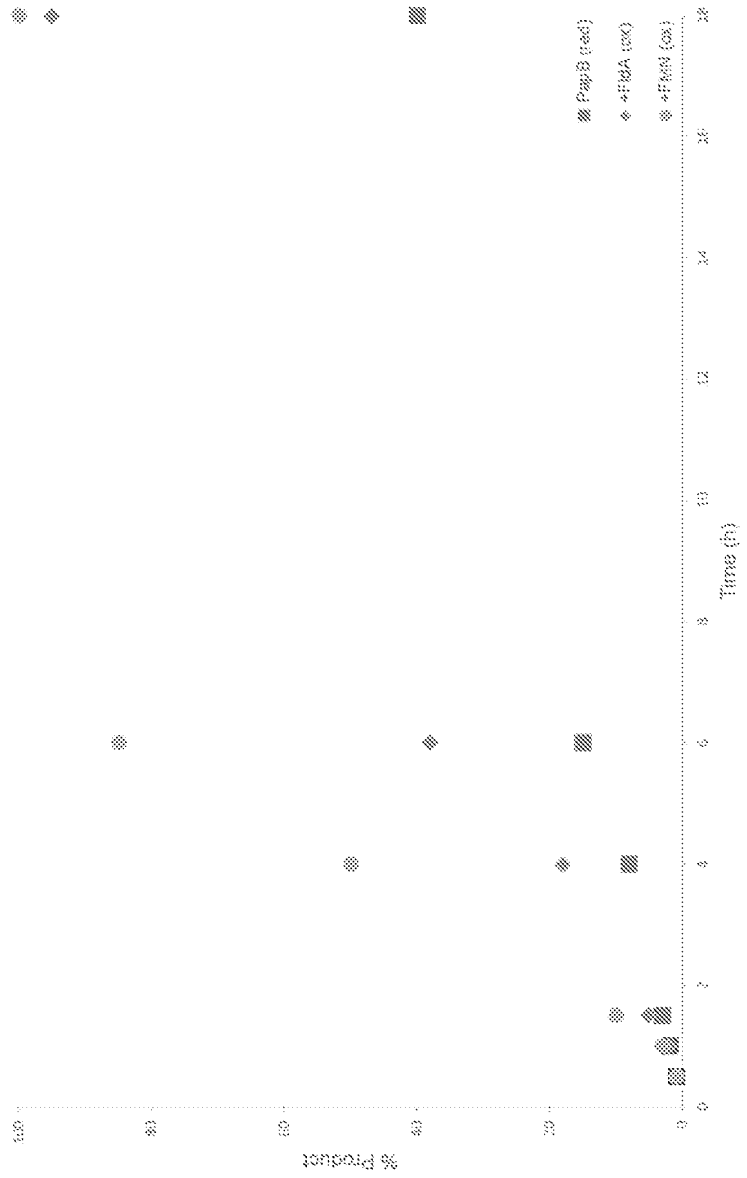


FIG. 45

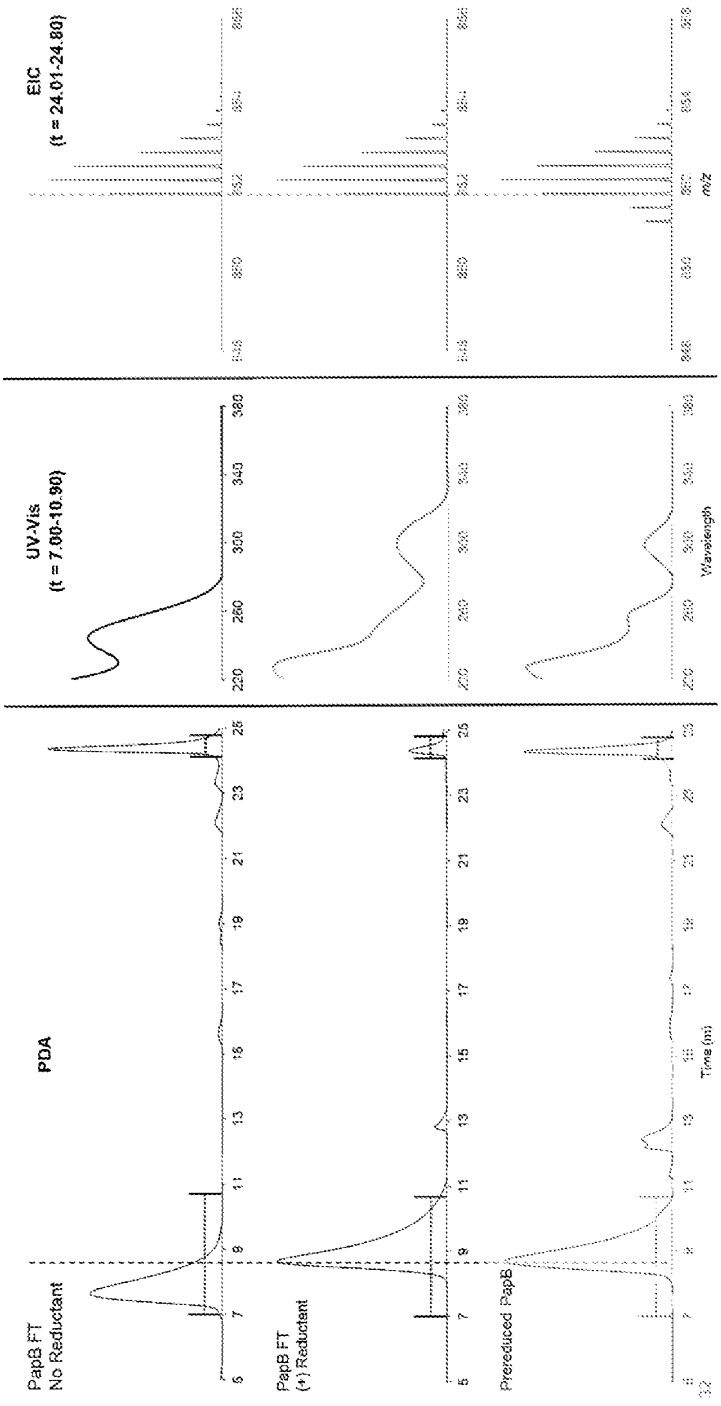

PDA
UV-Vis
(t = 7.00-10.90)
EIC
(t = 24.01-24.80)
PapB FT
No Reductant
PapB FT
(+) Reductant
Prereduced PapB
Time (m)
Wavelength
m/z

FIG. 46

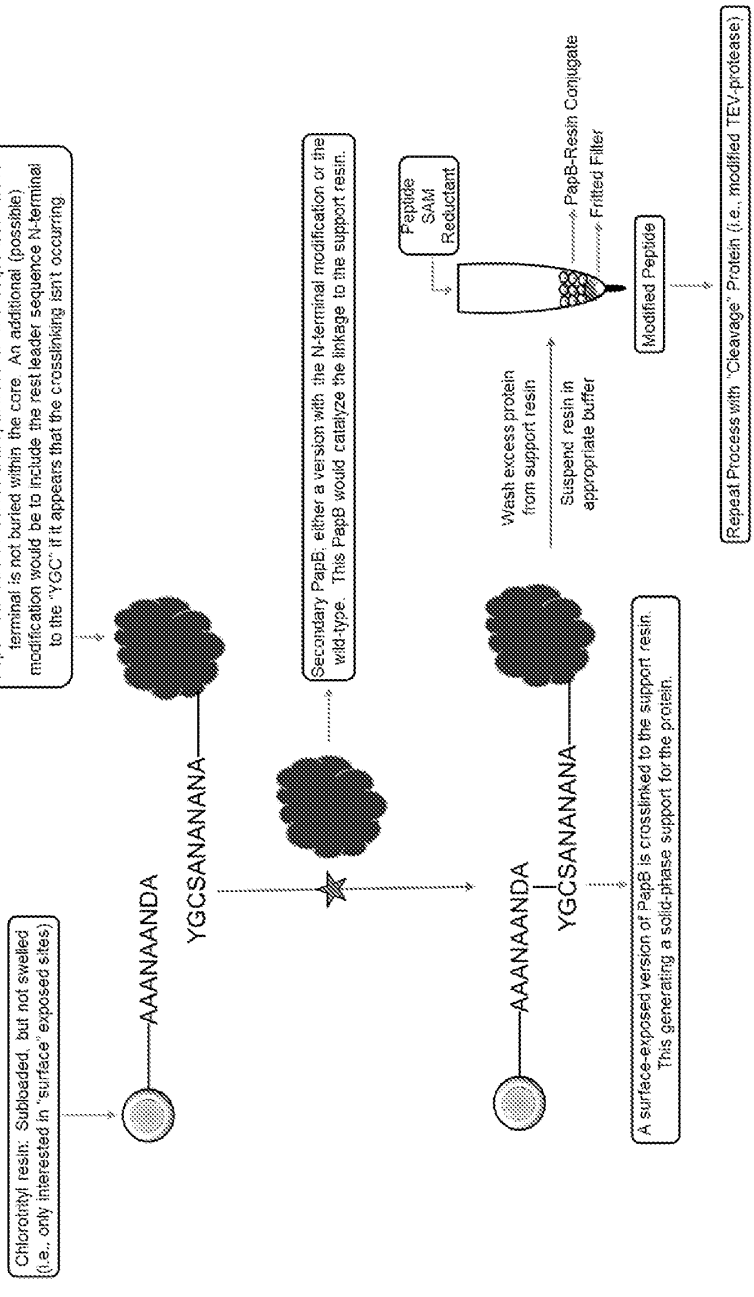

Chlorotrityl resin: Subloaded, but not swelled (i.e., only interested in 'surface' exposed sites)
AAANAANDA
YGCSANANANA
PapB– contains a modified and spaced N-terminal sequence. The N-terminal is not buried within the core. An additional (possible) modification would be to include the rest leader sequence N-terminal to the "YGC" if it appears that the crosslinking isn't occurring
Secondary PapB, either a version with the N-terminal modification or the wild-type. This PapB would catalyze the linkage to the support resin.
A surface-exposed version of PapB is crosslinked to the support resin. This generating a solid-phase support for the protein.
AAANAANDA
YGCSANANANA
Wash excess protein from support resin
Suspend resin in appropriate buffer
Peptide SAM Reductant
PapB-Resin Conjugate
Fritted Filter
Modified Peptide
Repeat Process with "Cleavage" Protein (i.e., modified TEV-protease)

FIG. 47

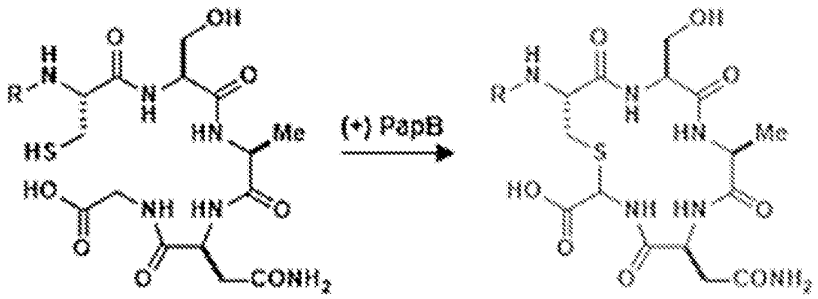


FIG. 48A

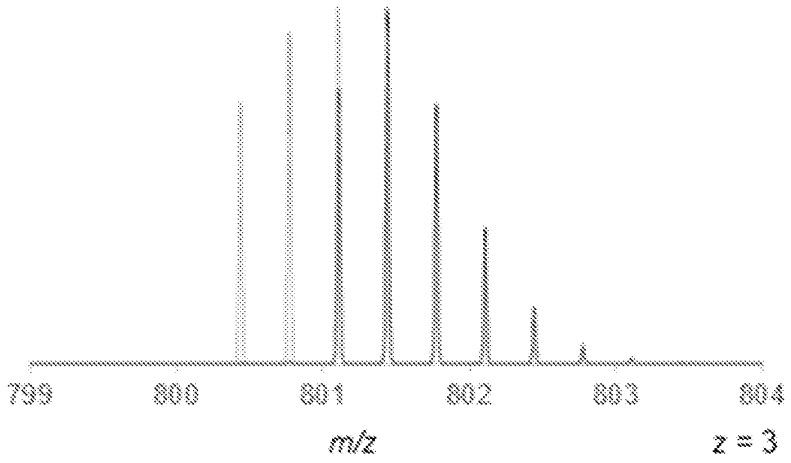


FIG. 48B

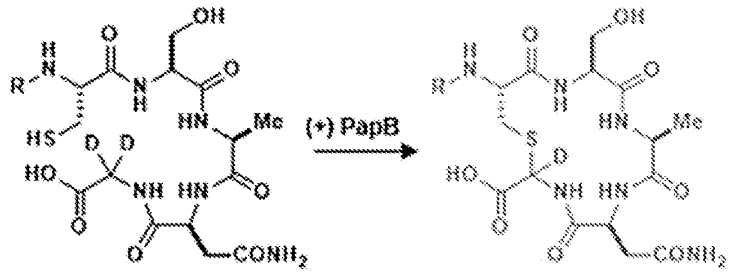


FIG. 49A

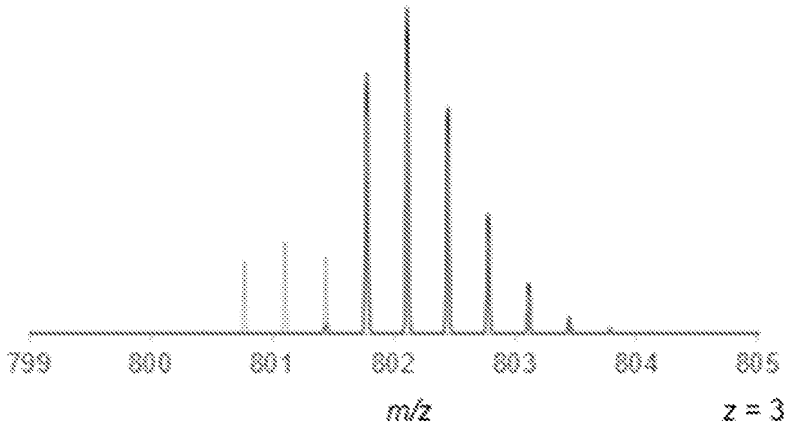


FIG. 49B

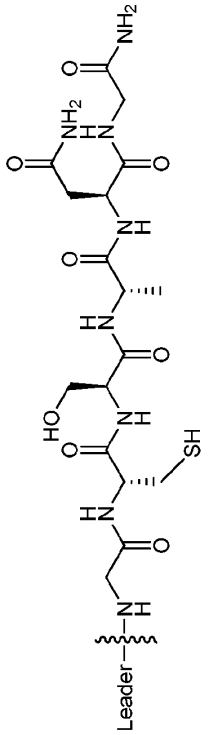


FIG. 50A

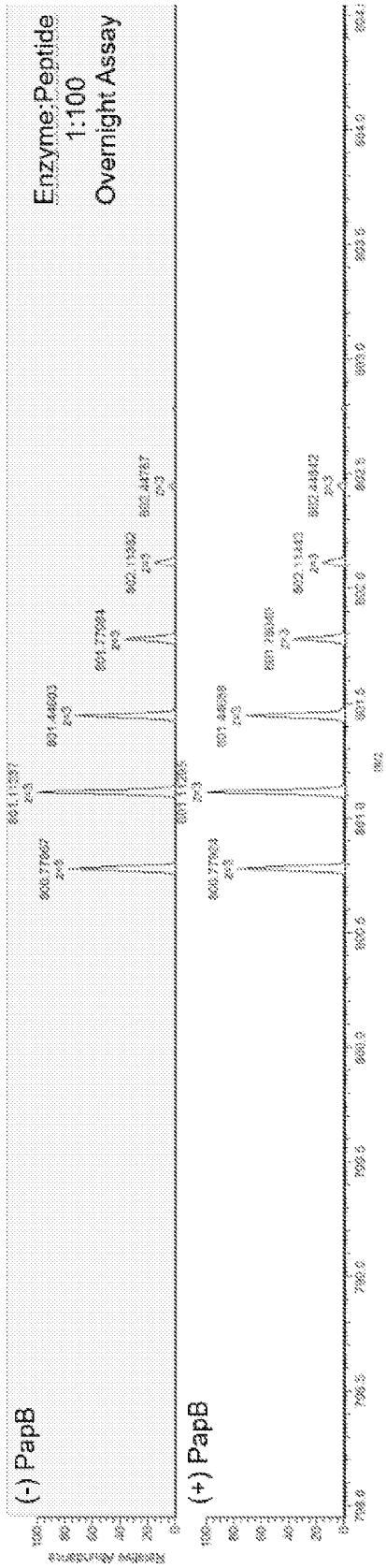


FIG. 50B

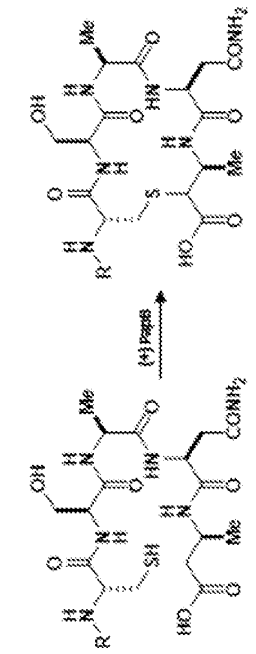


FIG. 51B

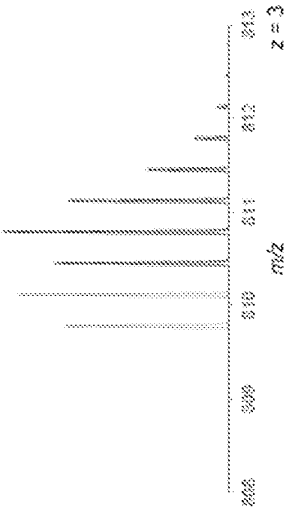


FIG. 51C

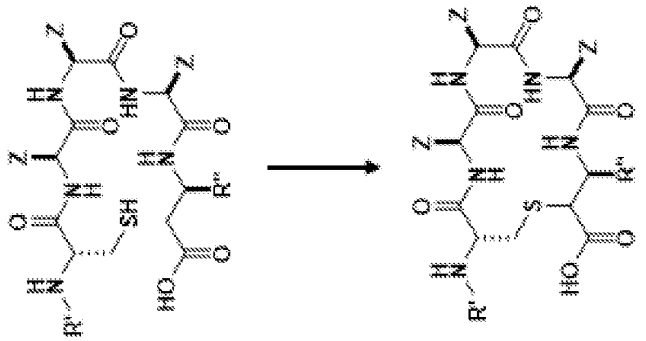


FIG. 51A

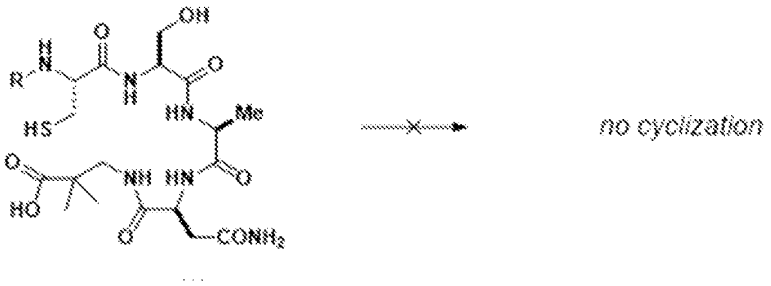


FIG. 52A

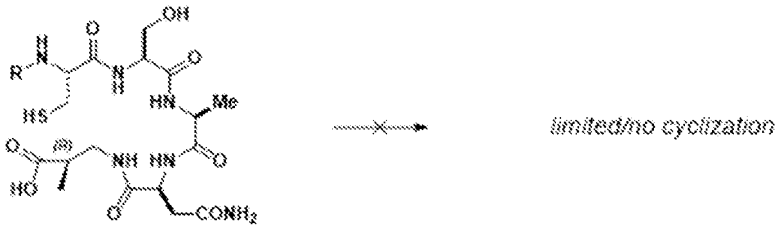


FIG. 52B

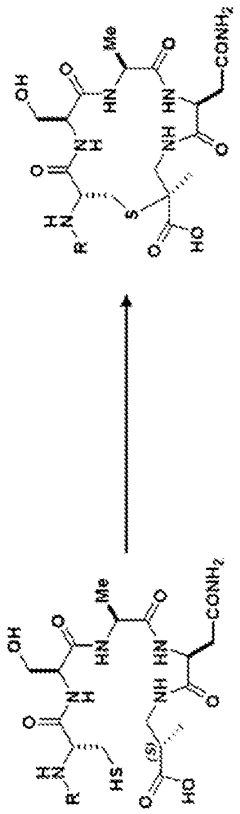


FIG. 52C

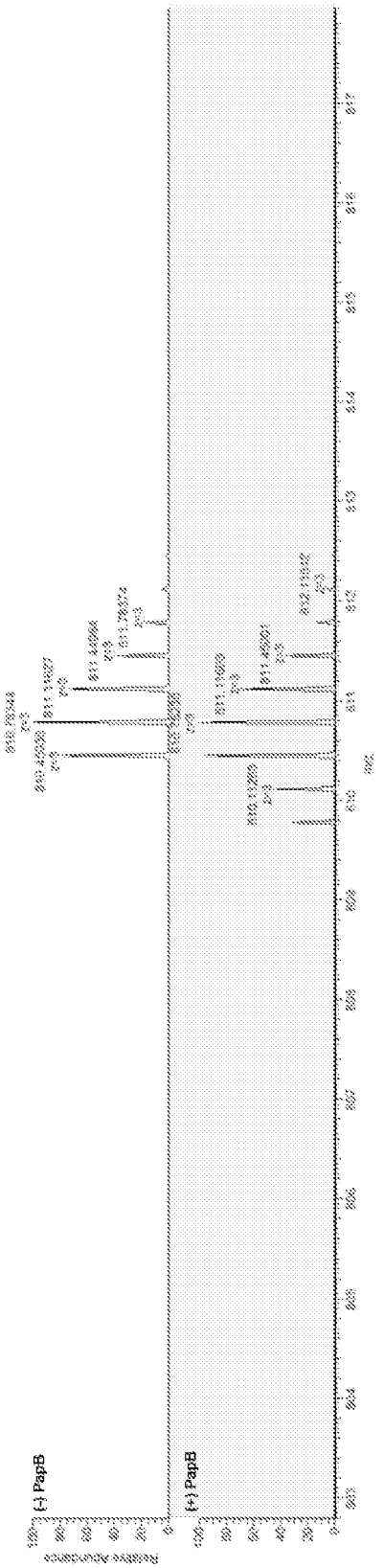


FIG. 52D

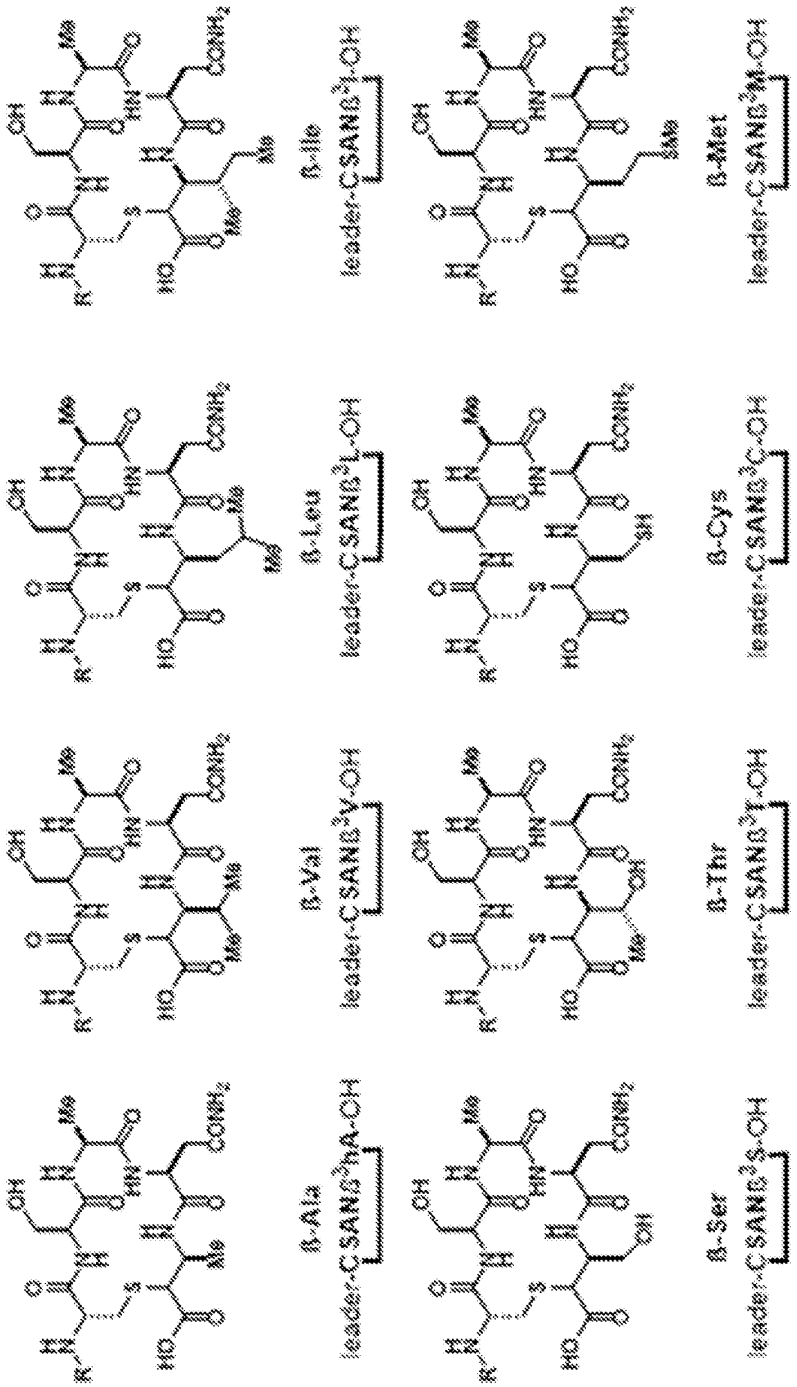

β-Ala
leader-CSANβ3hA-OH
β-Val
leader-CSANβ3V-OH
β-Leu
leader-CSANβ3L-OH
β-Ile
leader-CSANβ3I-OH
β-Ser
leader-CSANβ3S-OH
β-Thr
leader-CSANβ3T-OH
β-Cys
leader-CSANβ3C-OH
β-Met
leader-CSANβ3M-OH

FIG. 53

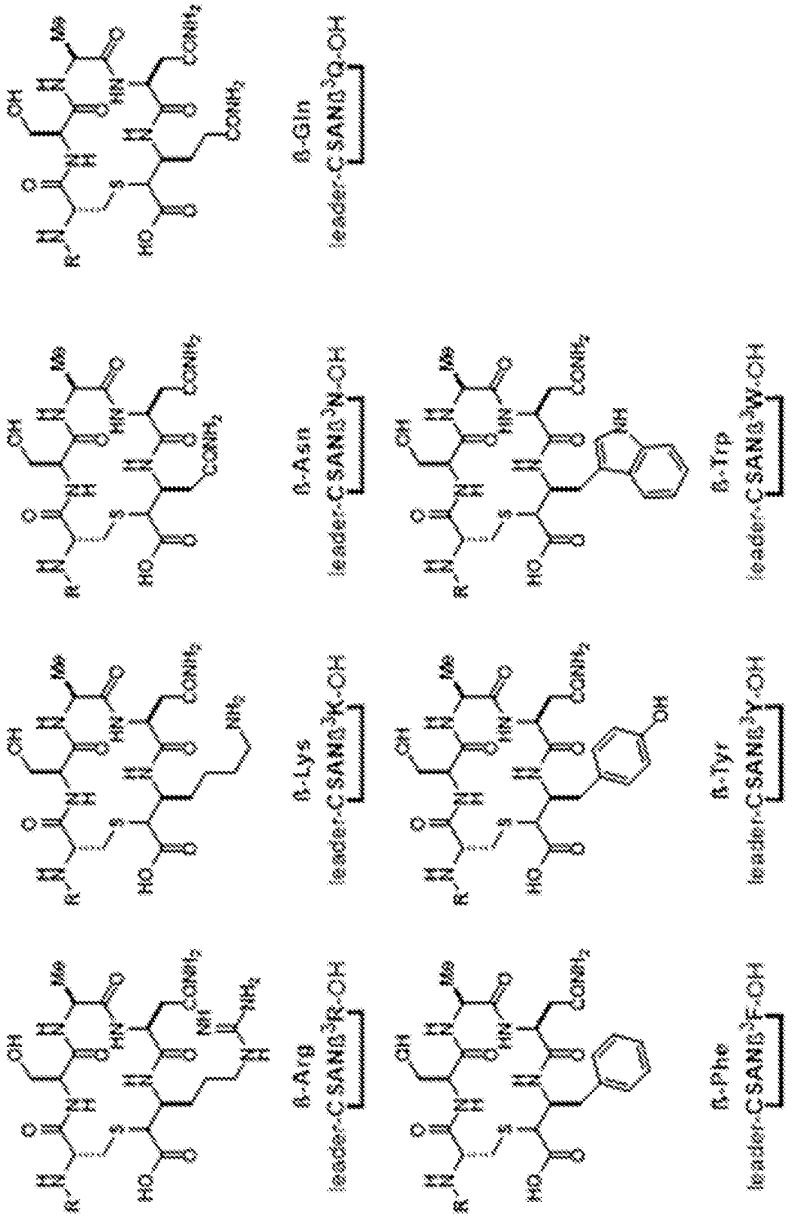


FIG. 53
(Continued)

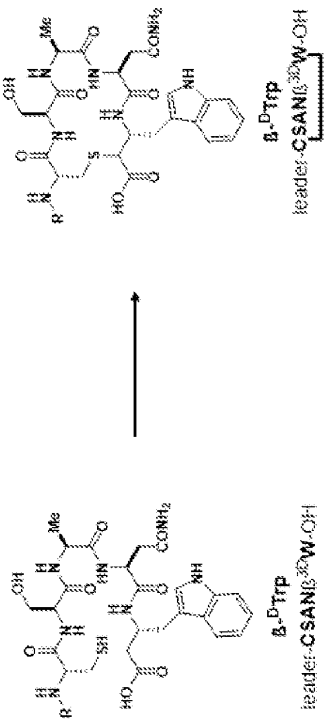


FIG. 54A

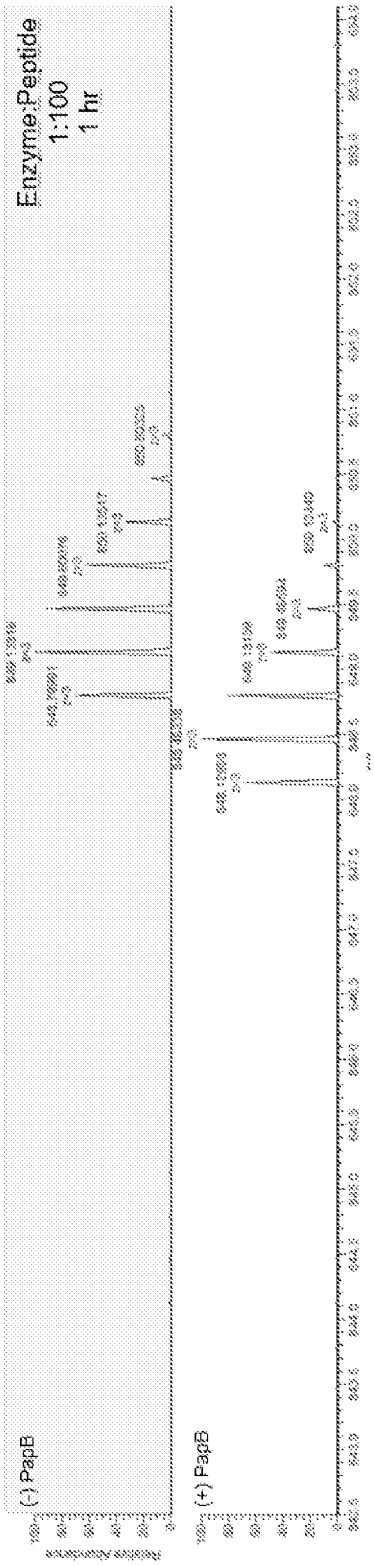


FIG. 54B

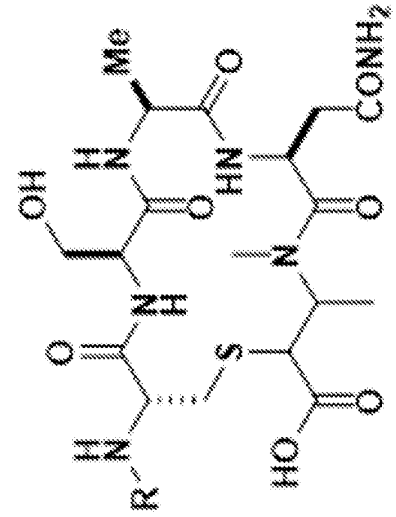


FIG. 55B

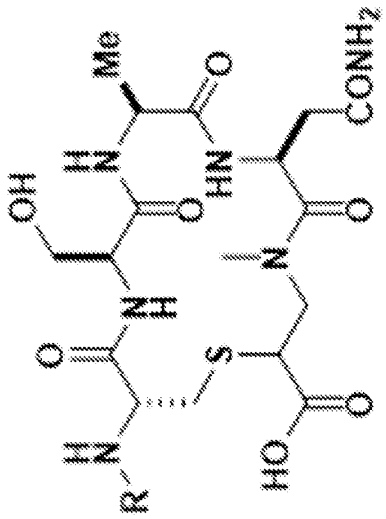


FIG. 55A

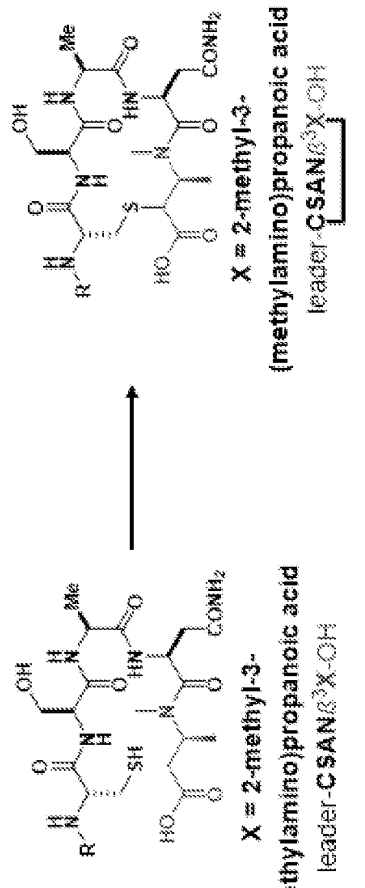


FIG. 55C

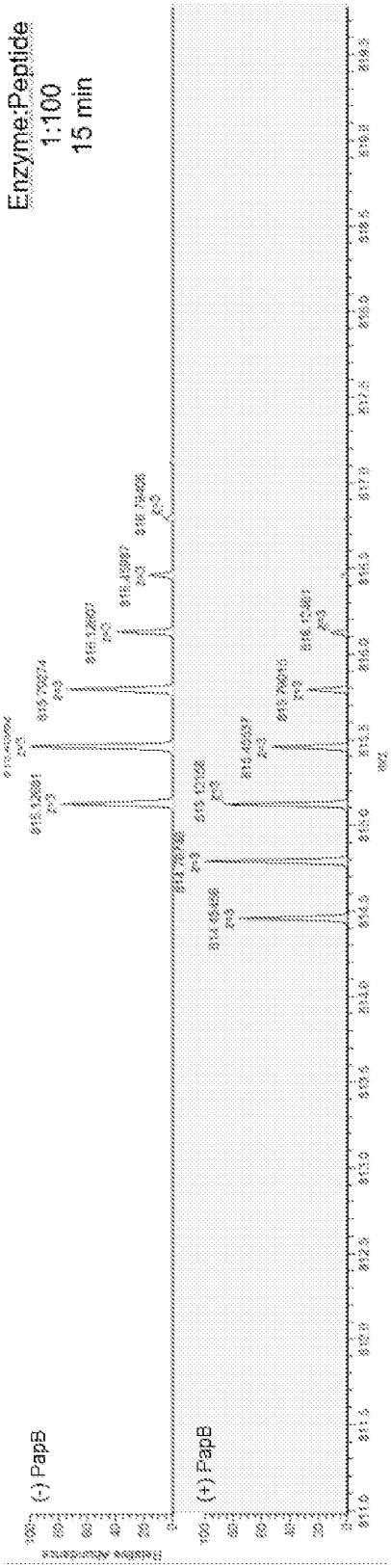


FIG. 55D

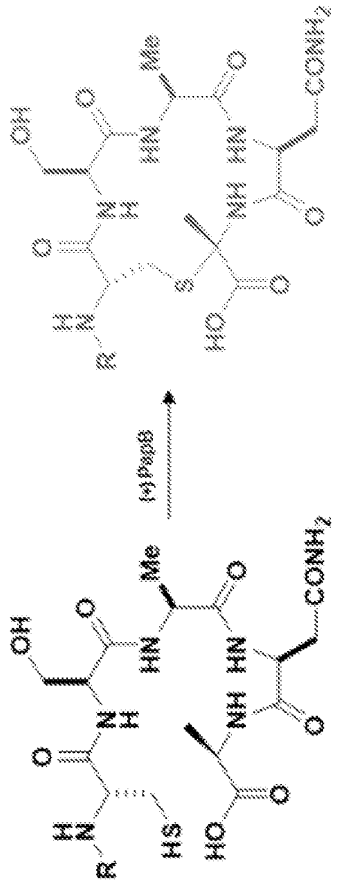


FIG. 56A

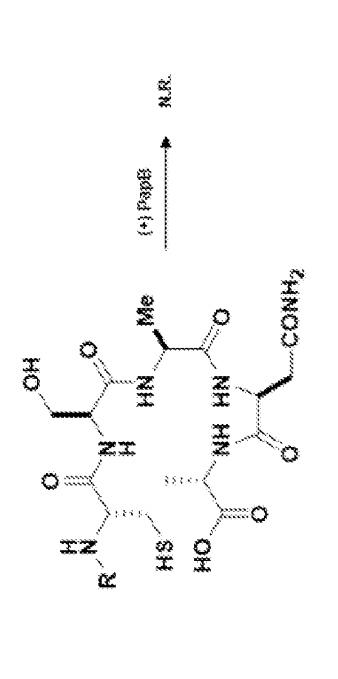


FIG. 56B

FIG. 56C

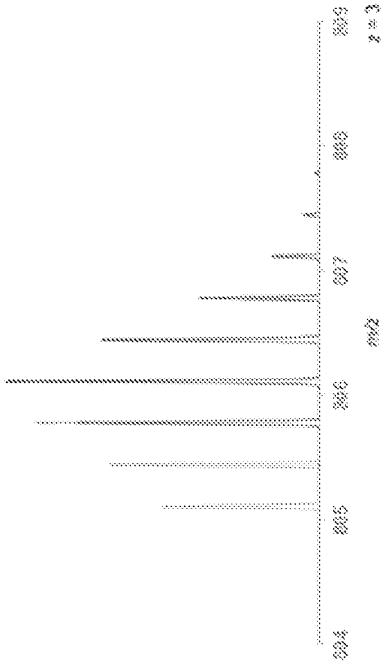


FIG. 56D

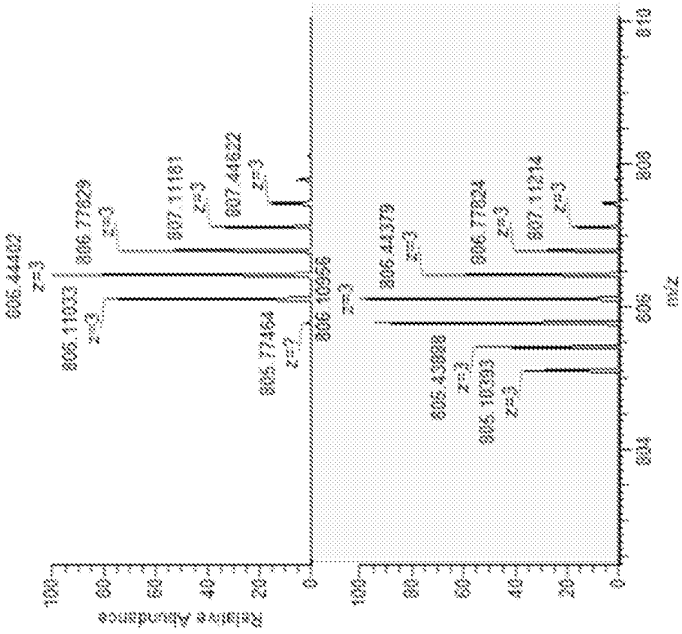


FIG. 57B

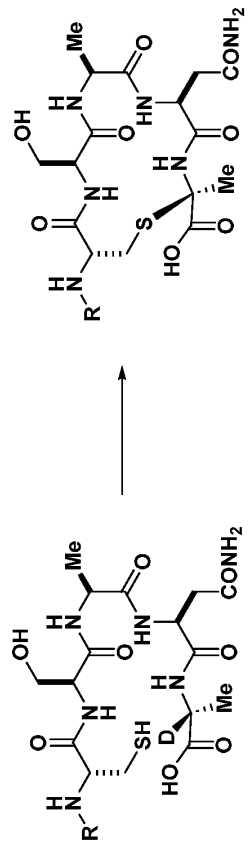


FIG. 57A

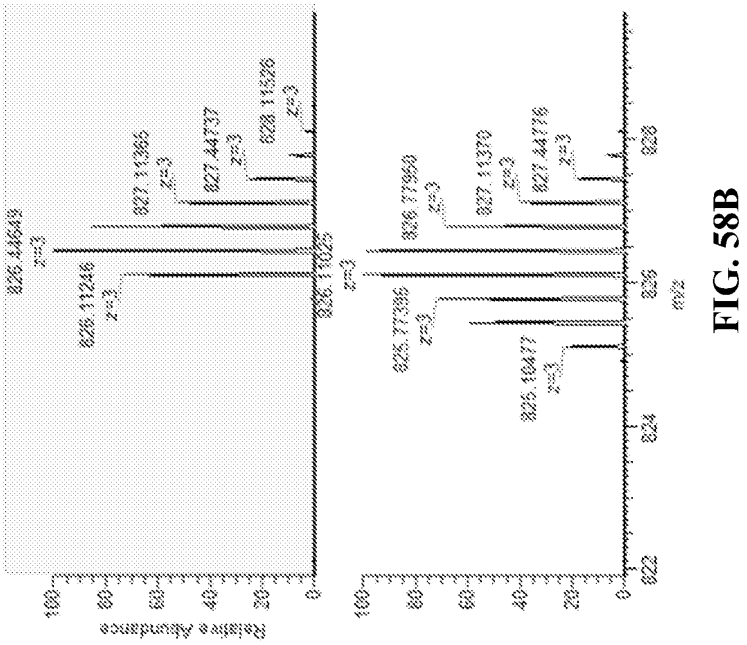


FIG. 58B

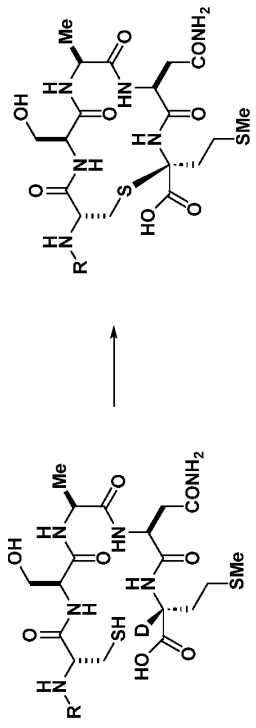


FIG. 58A

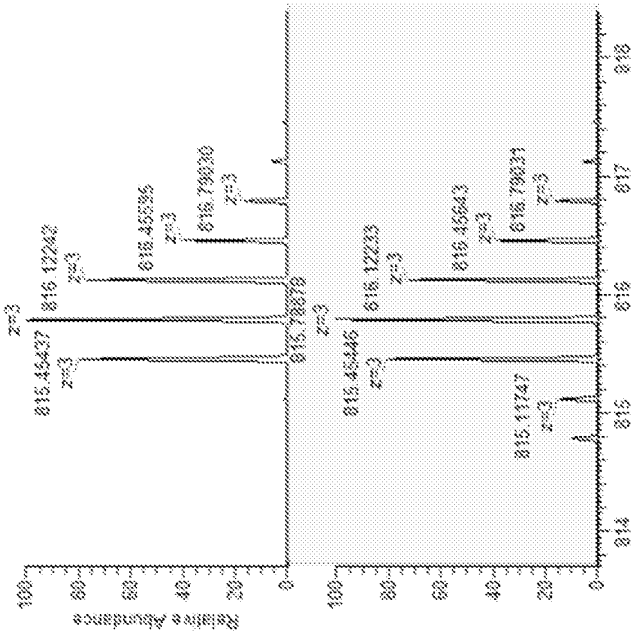


FIG. 59B

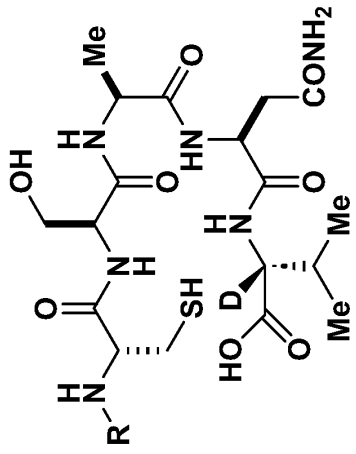


FIG. 59A

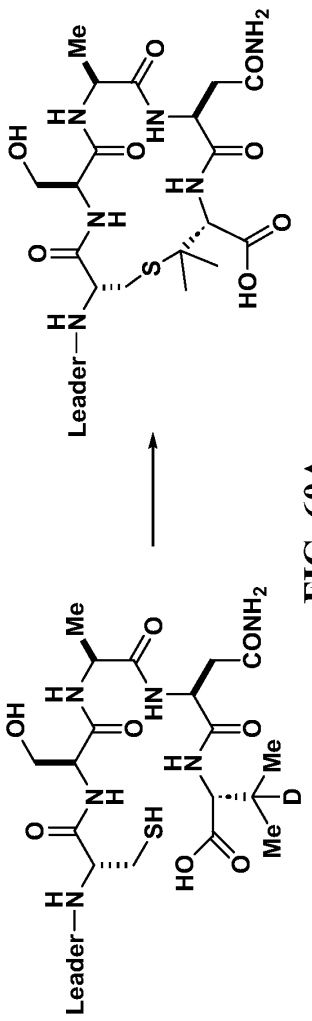


FIG. 60A

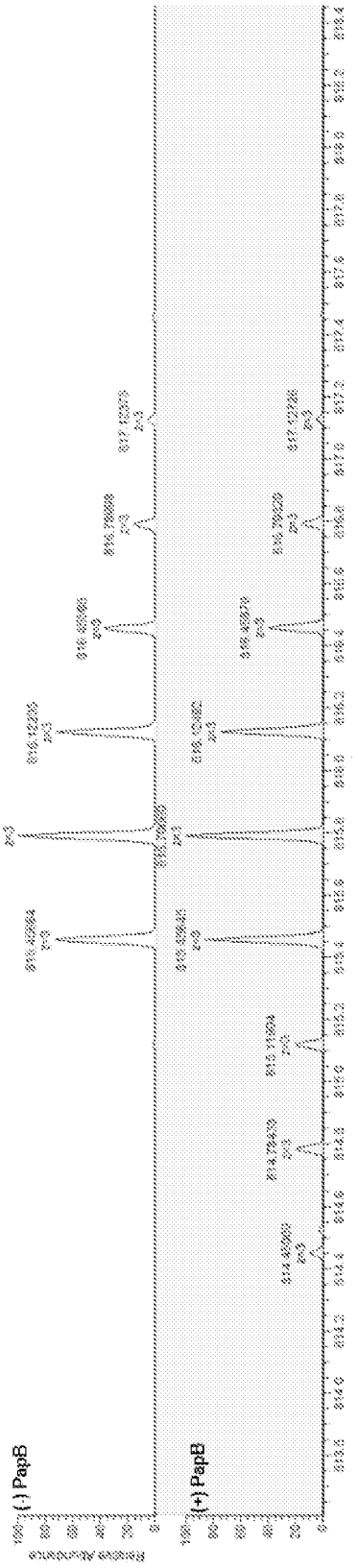


FIG. 60B

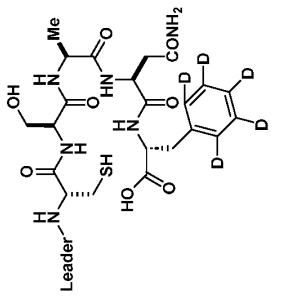


FIG. 61A

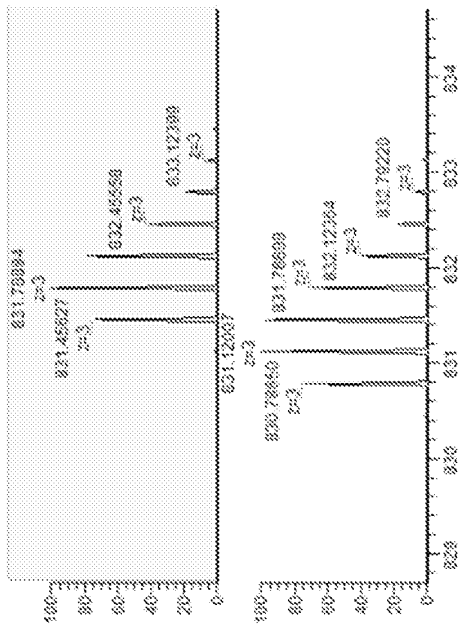


FIG. 61B

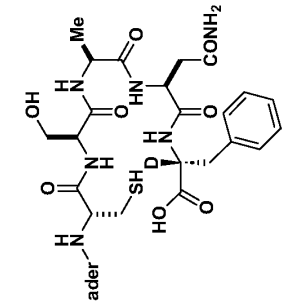


FIG. 61C

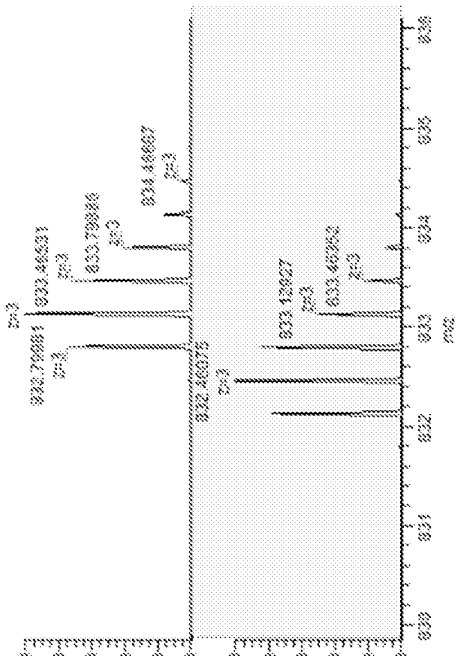


FIG. 61D

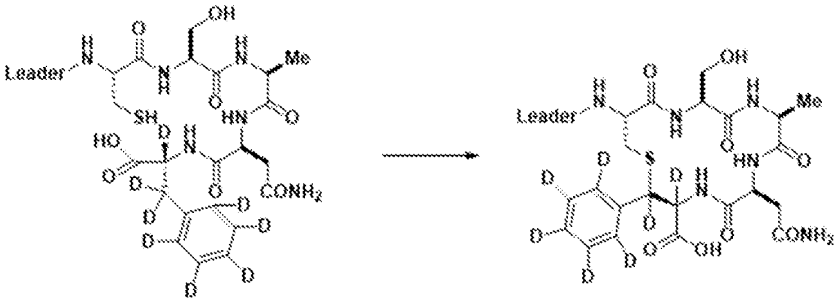


FIG. 62A

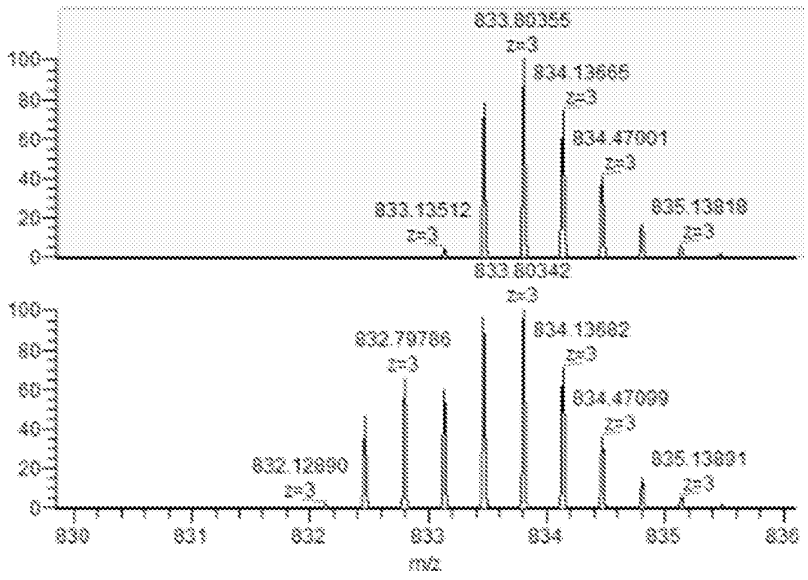


FIG. 62B

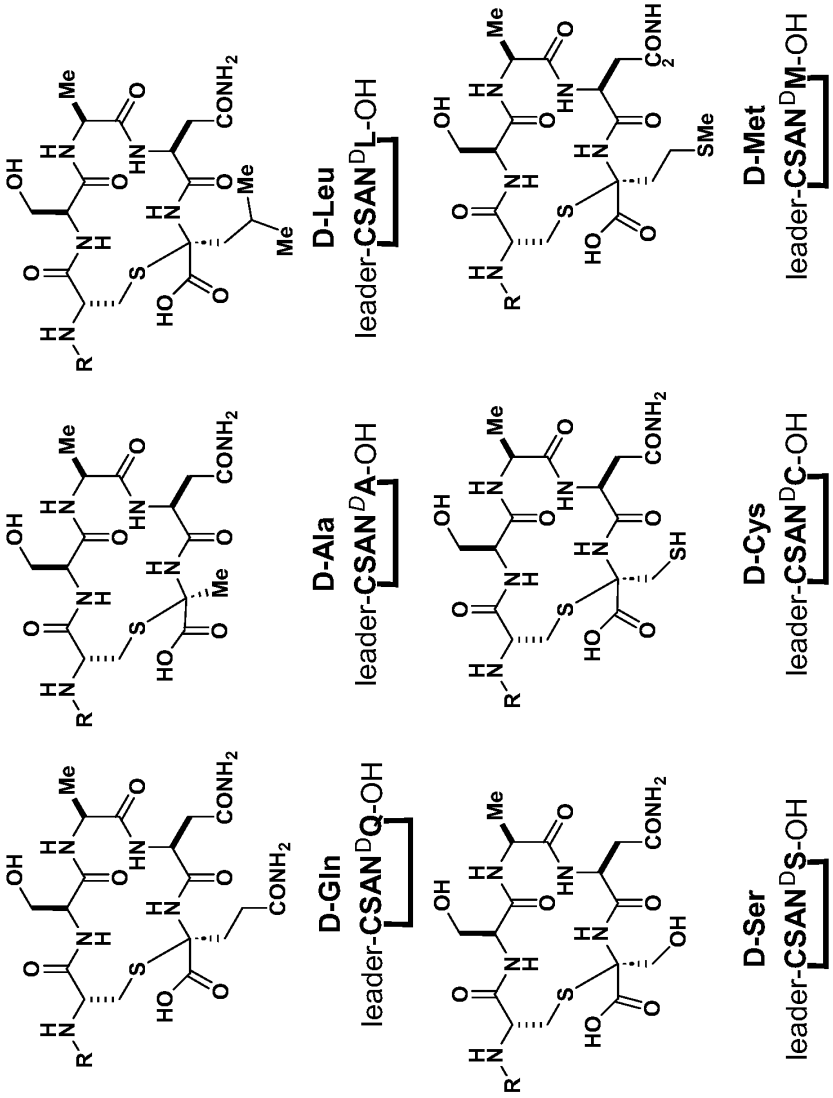

D-Leu
leader-CSAN[D]L-OH
D-Met
leader-CSAN[D]M-OH
D-Ala
leader-CSAN[D]A-OH
D-Cys
leader-CSAN[D]C-OH
D-Gln
leader-CSAN[D]Q-OH
D-Ser
leader-CSAN[D]S-OH

FIG. 63

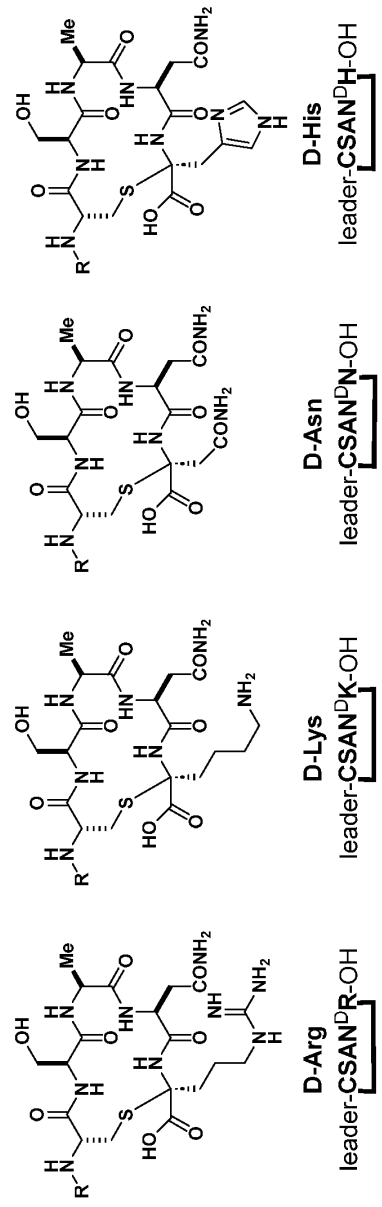


FIG. 63
(Continued)

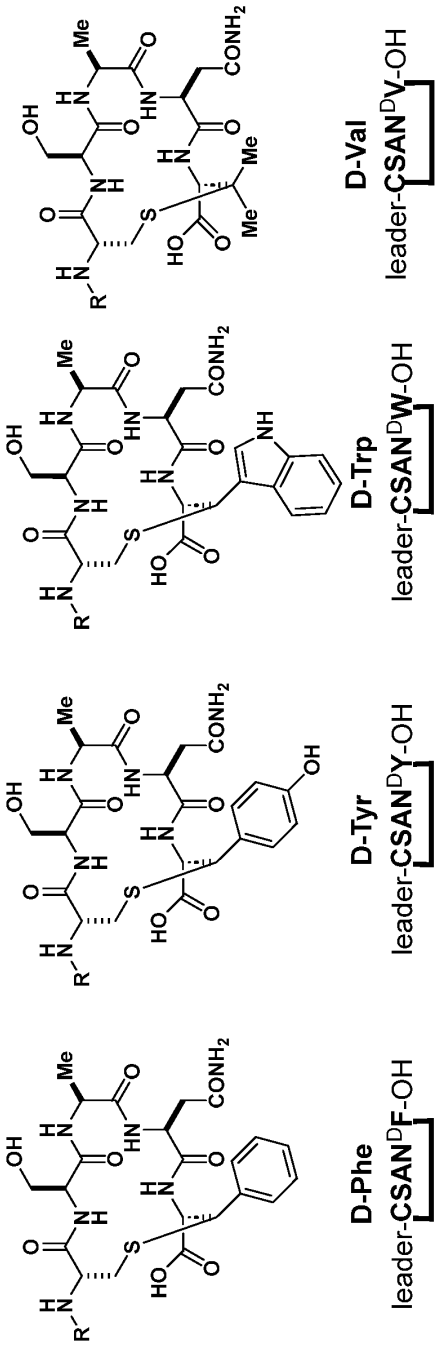

D-Phe
leader-CSAN[D]F-OH
D-Tyr
leader-CSAN[D]Y-OH
D-Trp
leader-CSAN[D]W-OH
D-Val
leader-CSAN[D]V-OH

FIG. 64

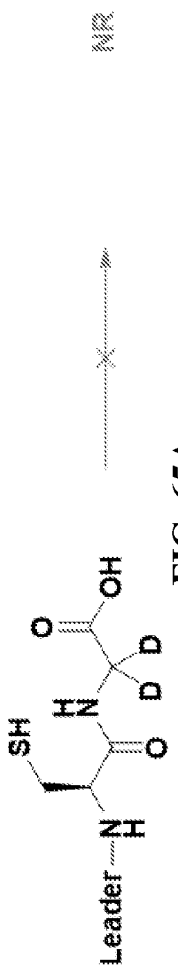


FIG. 65A

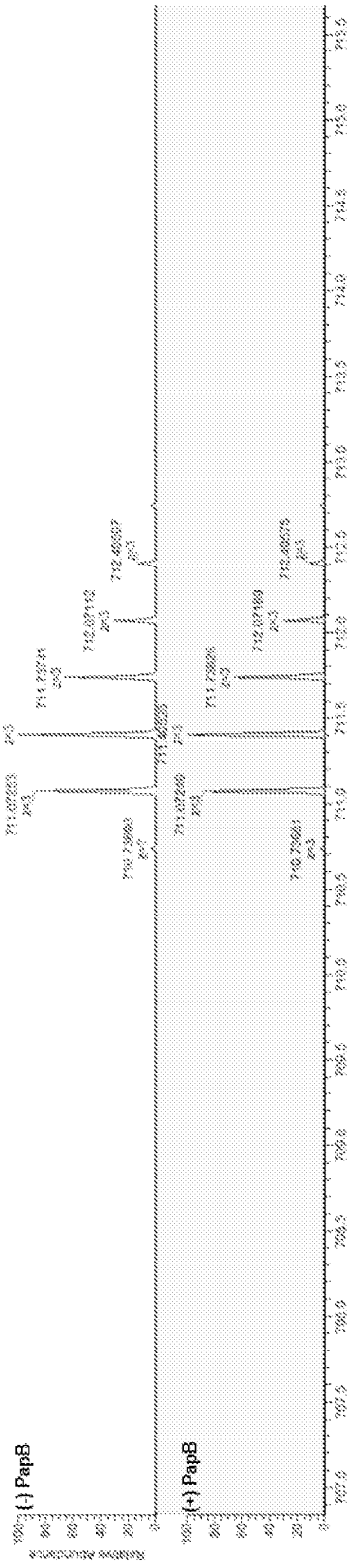


FIG. 65B

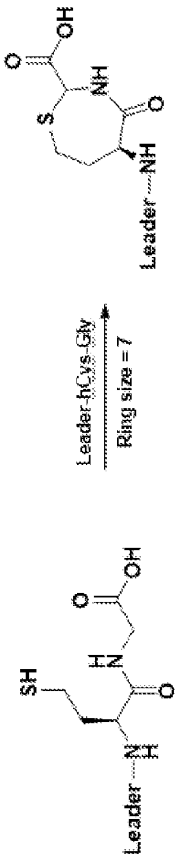


FIG. 66A

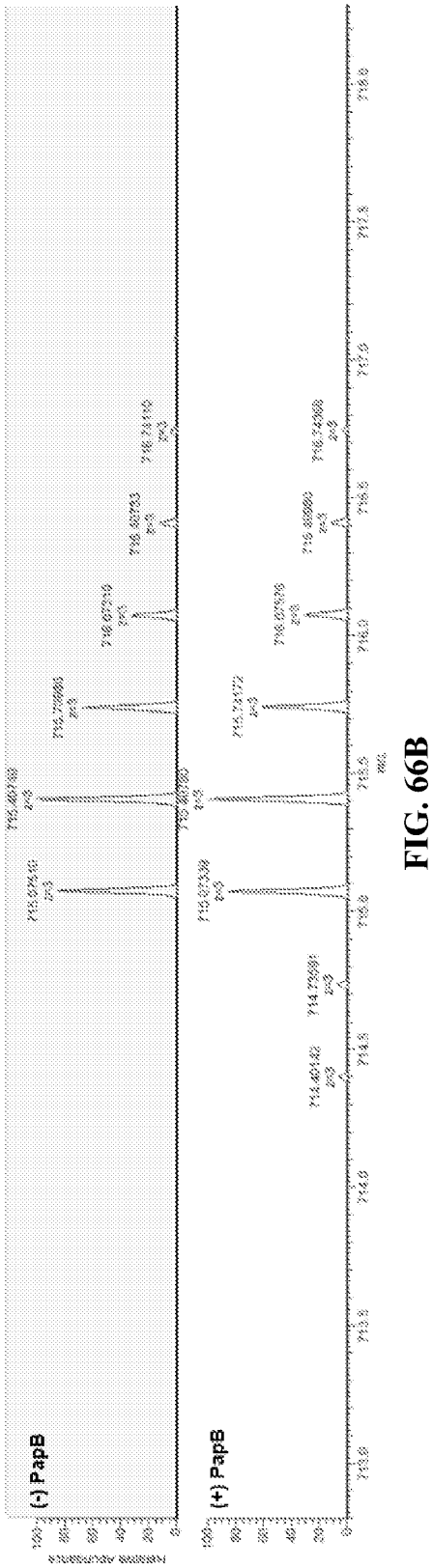


FIG. 66B

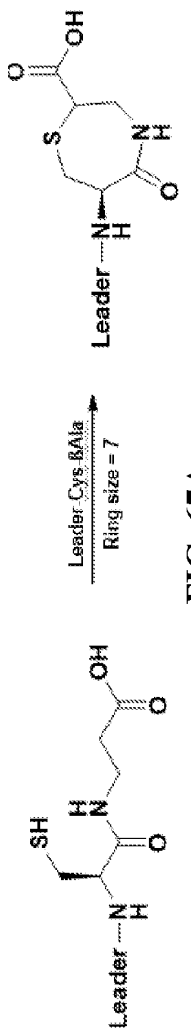


FIG. 67A

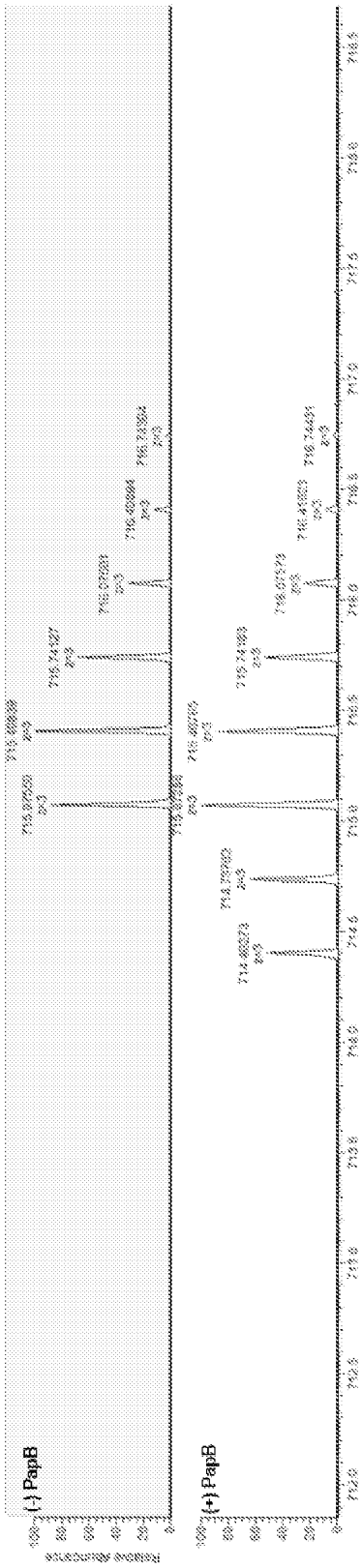


FIG. 67B

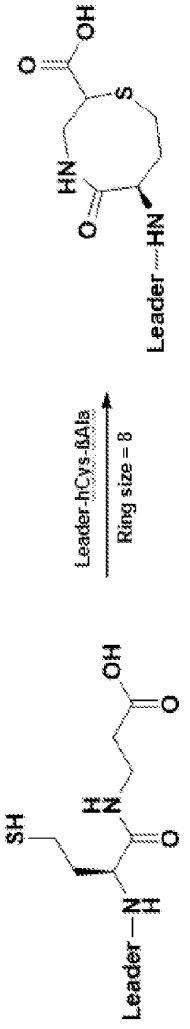


FIG. 68A

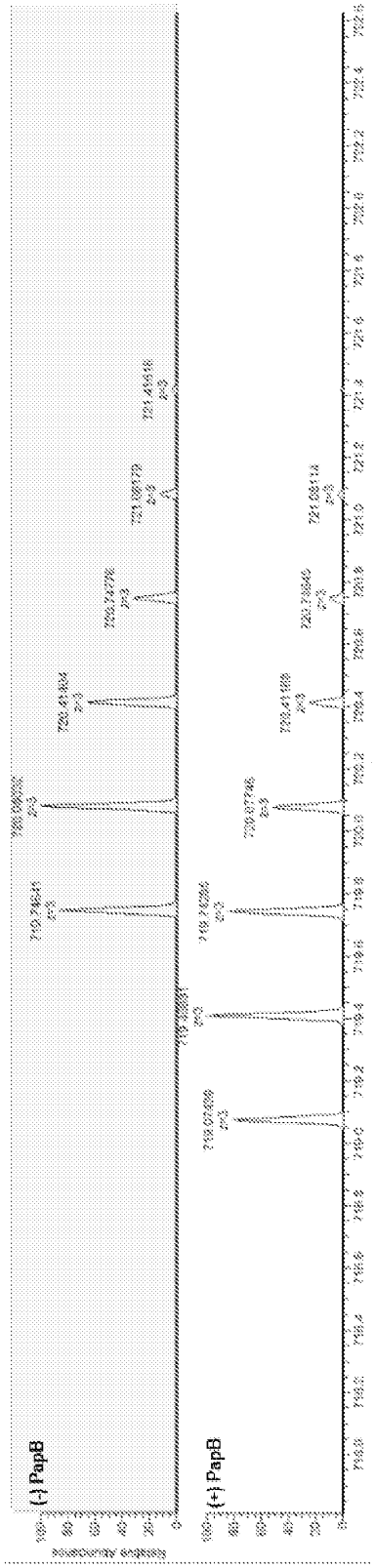


FIG. 68B

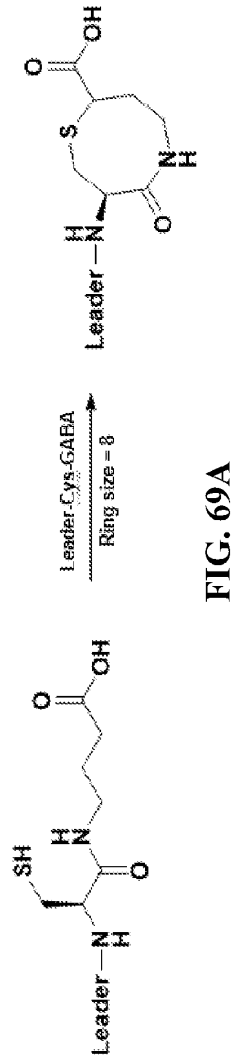


FIG. 69A

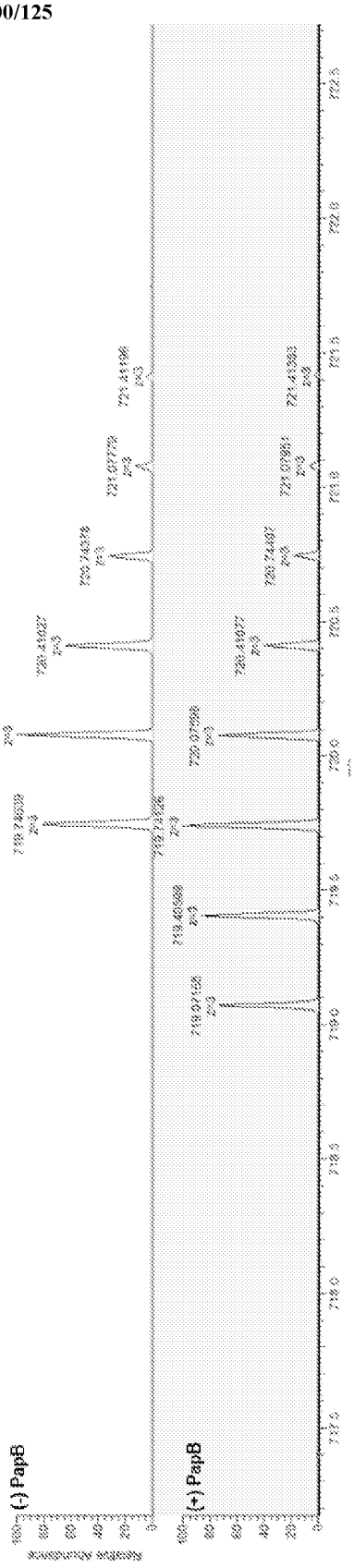


FIG. 69B

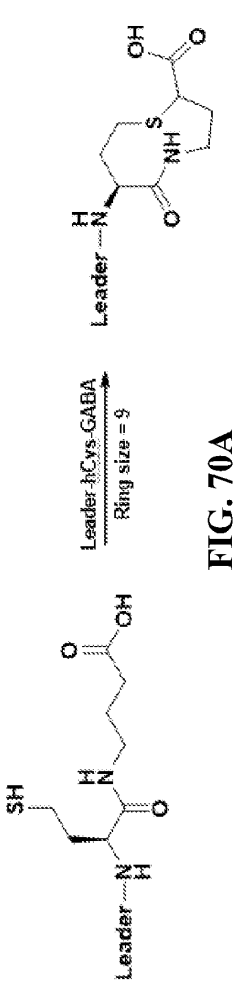


FIG. 70A

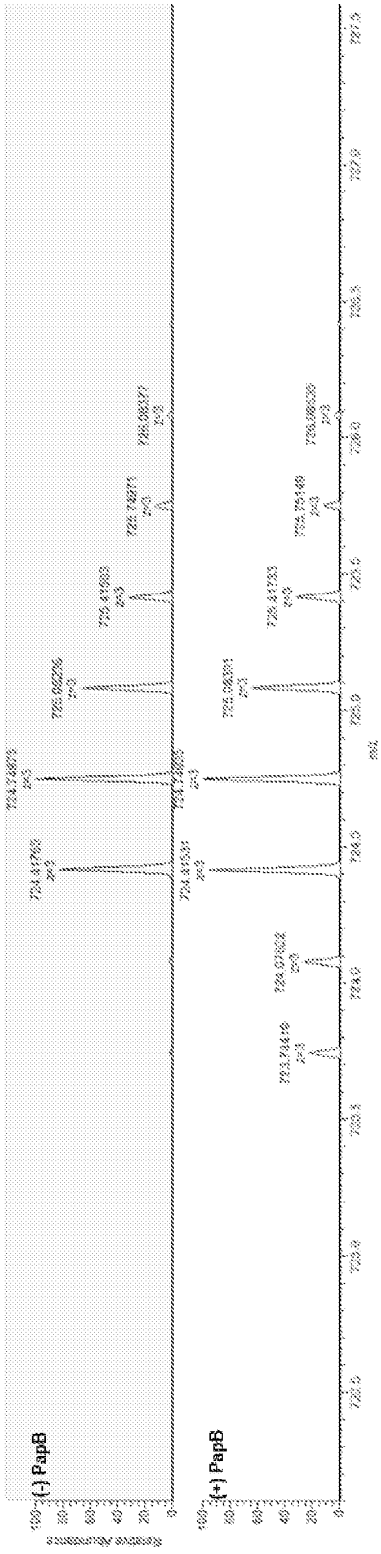


FIG. 70B

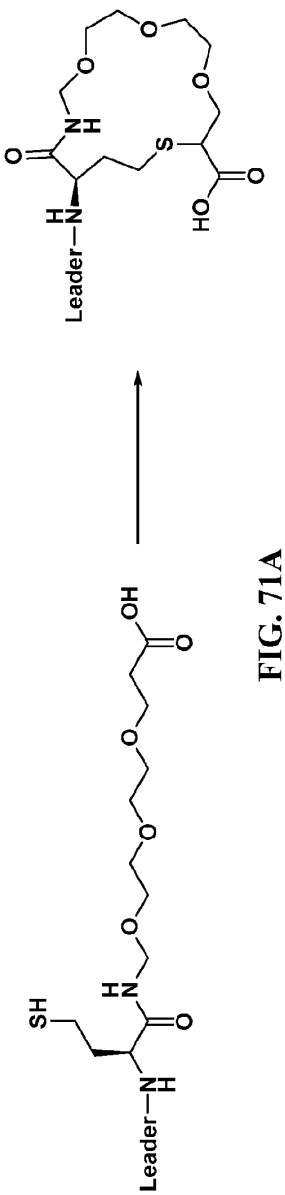


FIG. 71A

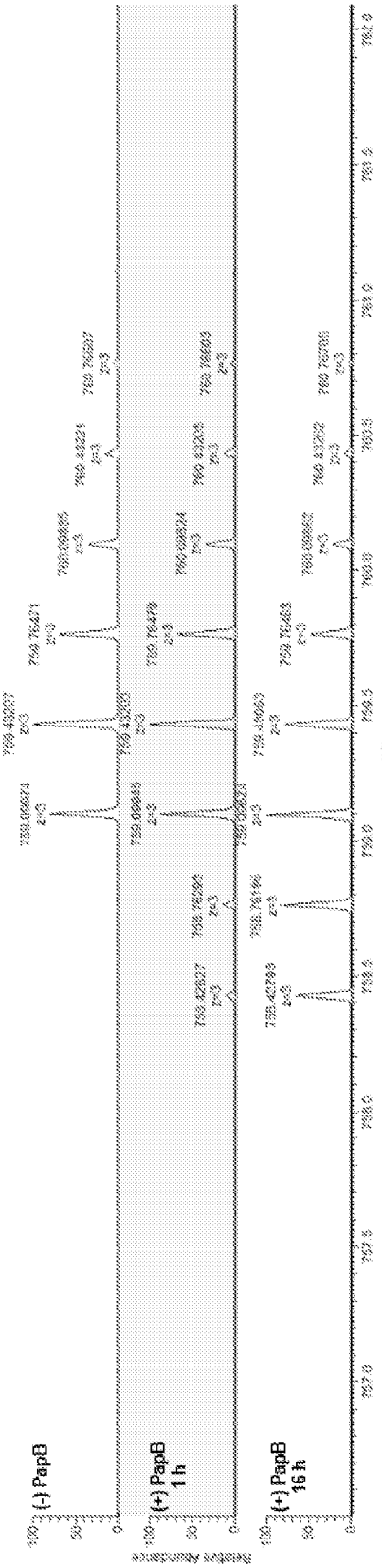


FIG. 71B

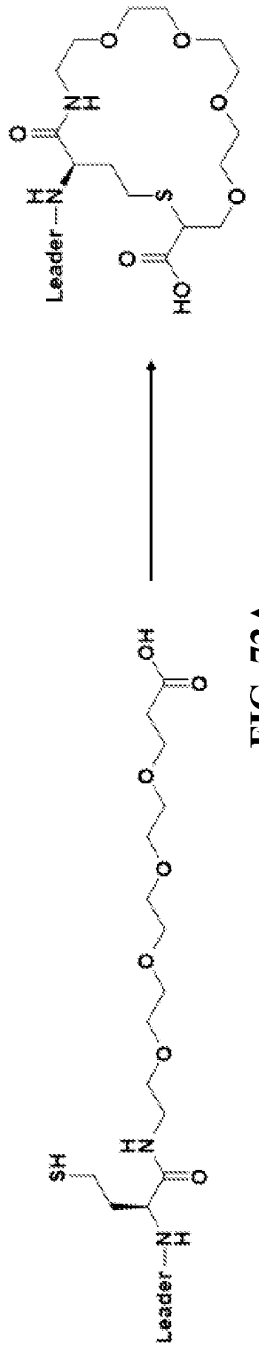


FIG. 72A

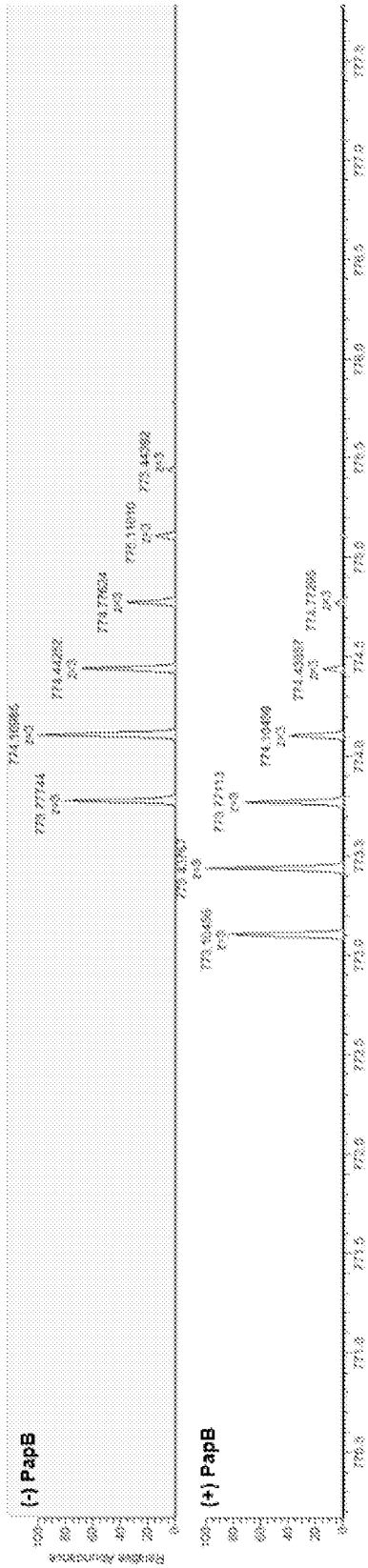


FIG. 72B

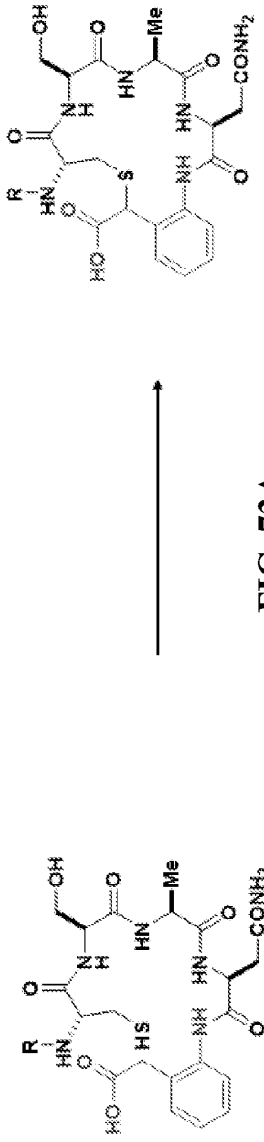


FIG. 73A

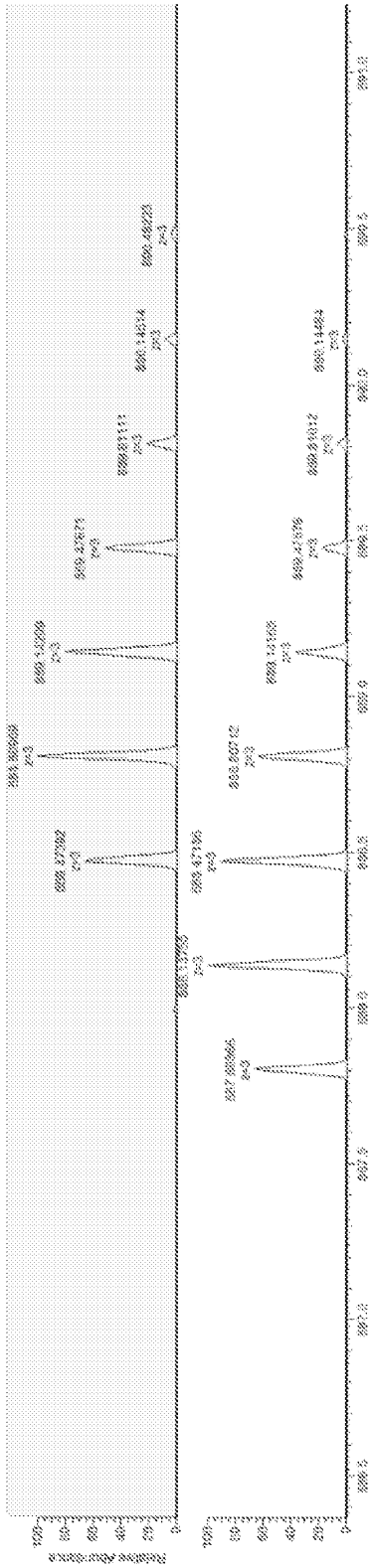


FIG. 73B

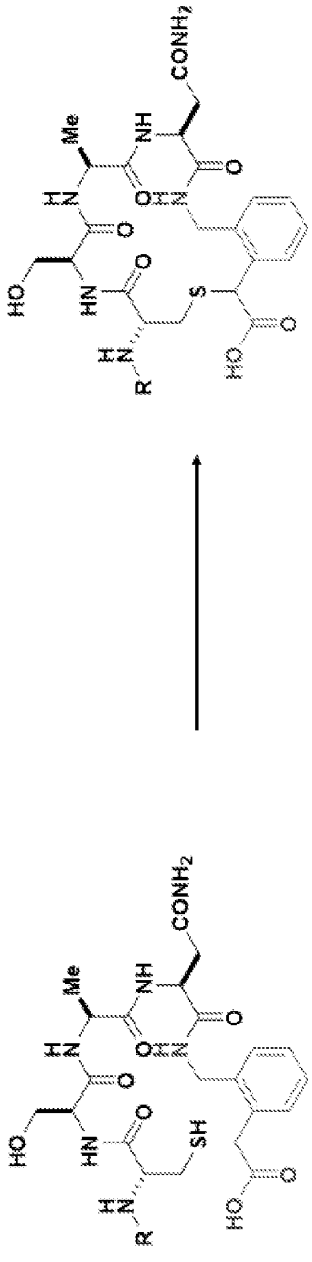


FIG. 74A

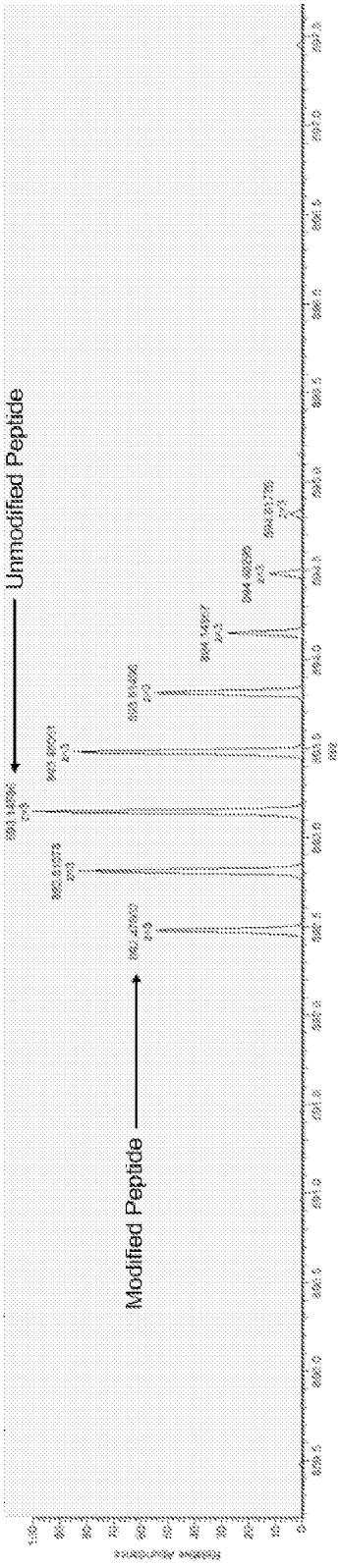


FIG. 74B

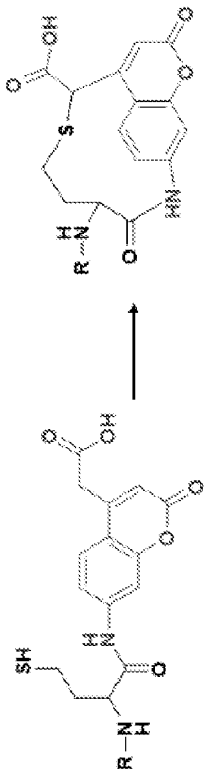


FIG. 75A

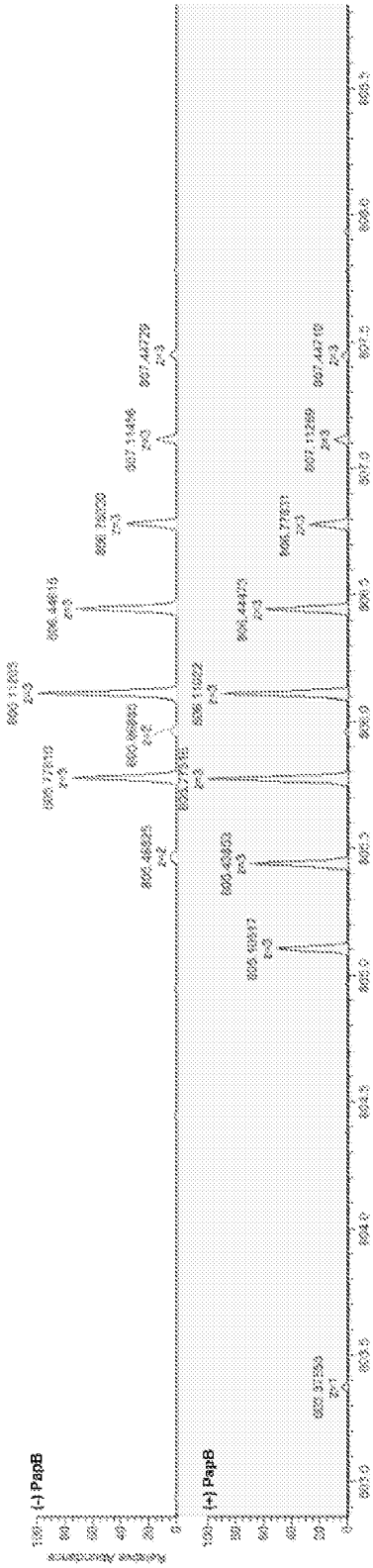


FIG. 75B

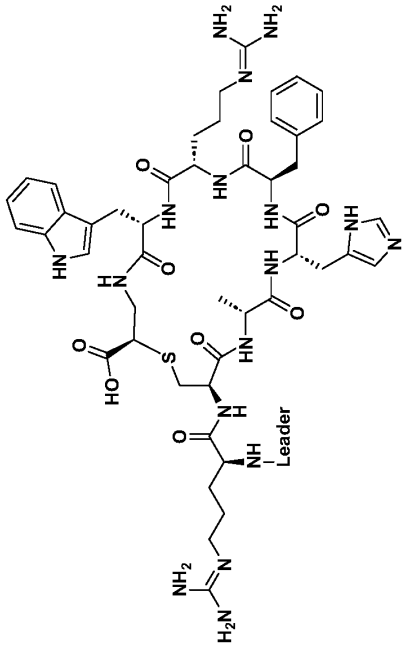


FIG. 76B

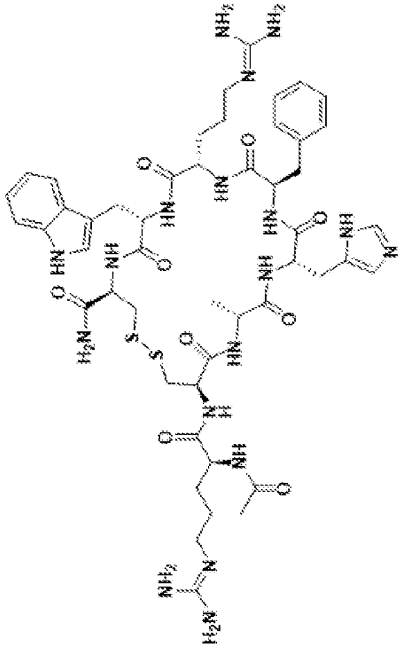


FIG. 76A

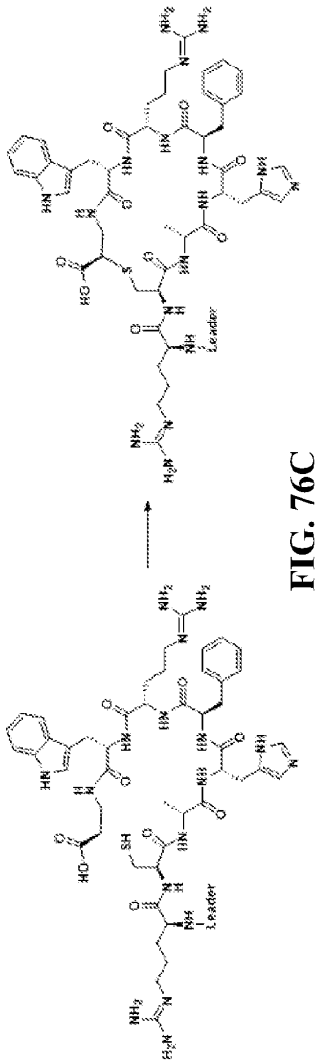


FIG. 76C

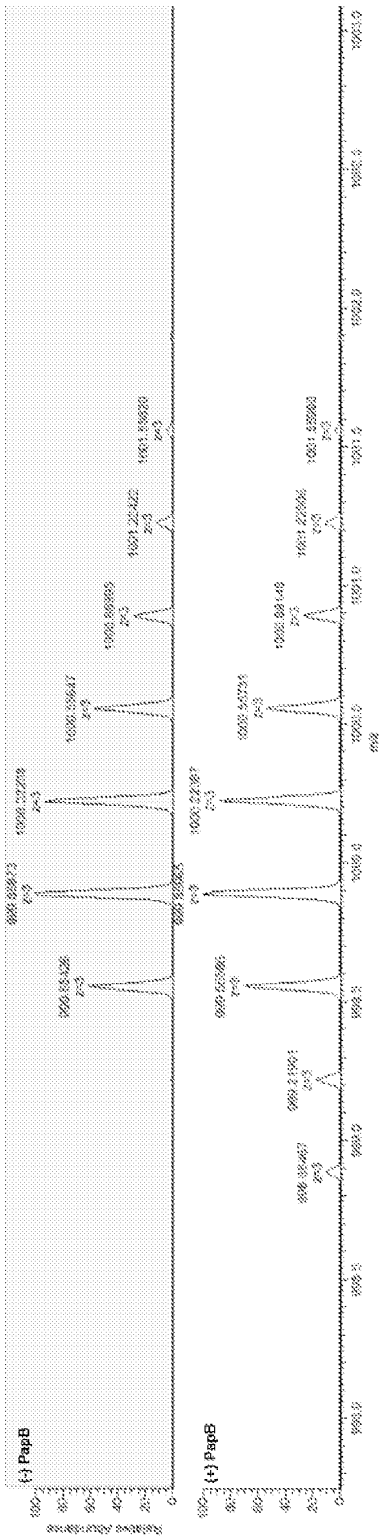


FIG. 76D

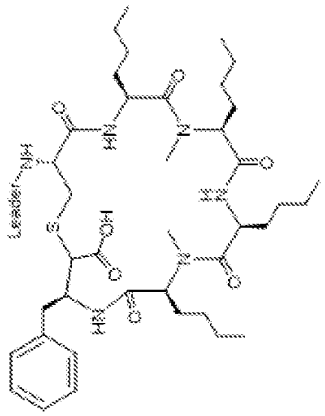


FIG. 77B

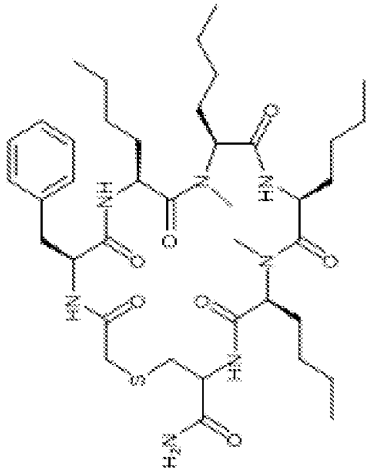


FIG. 77A

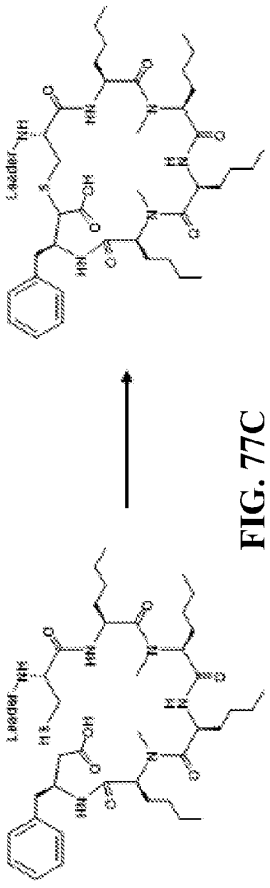


FIG. 77C

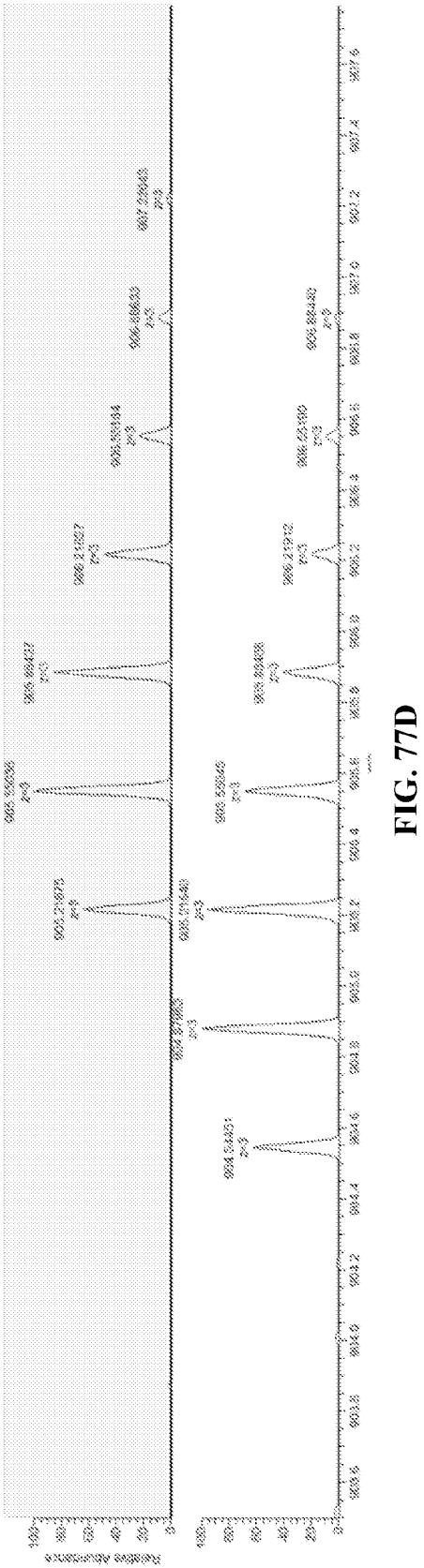


FIG. 77D

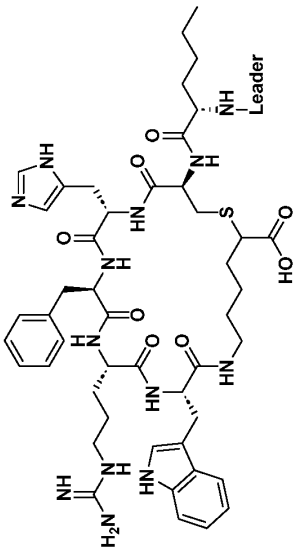


FIG. 78B

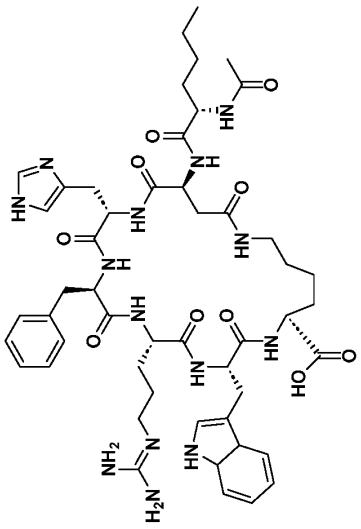


FIG. 78A

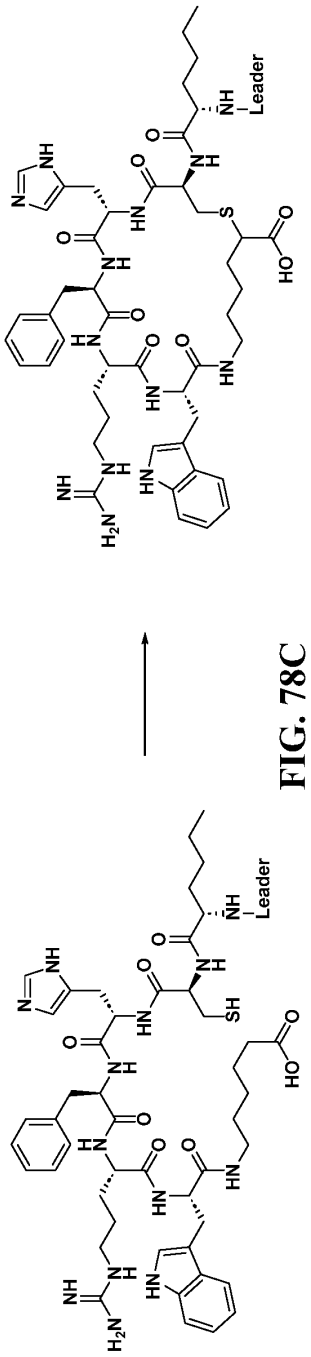


FIG. 78C

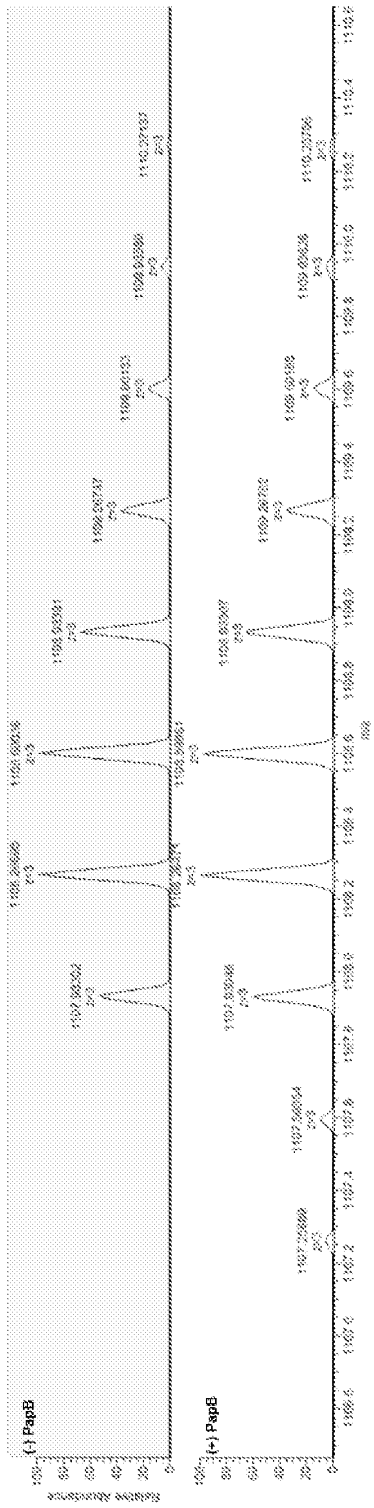


FIG. 78D

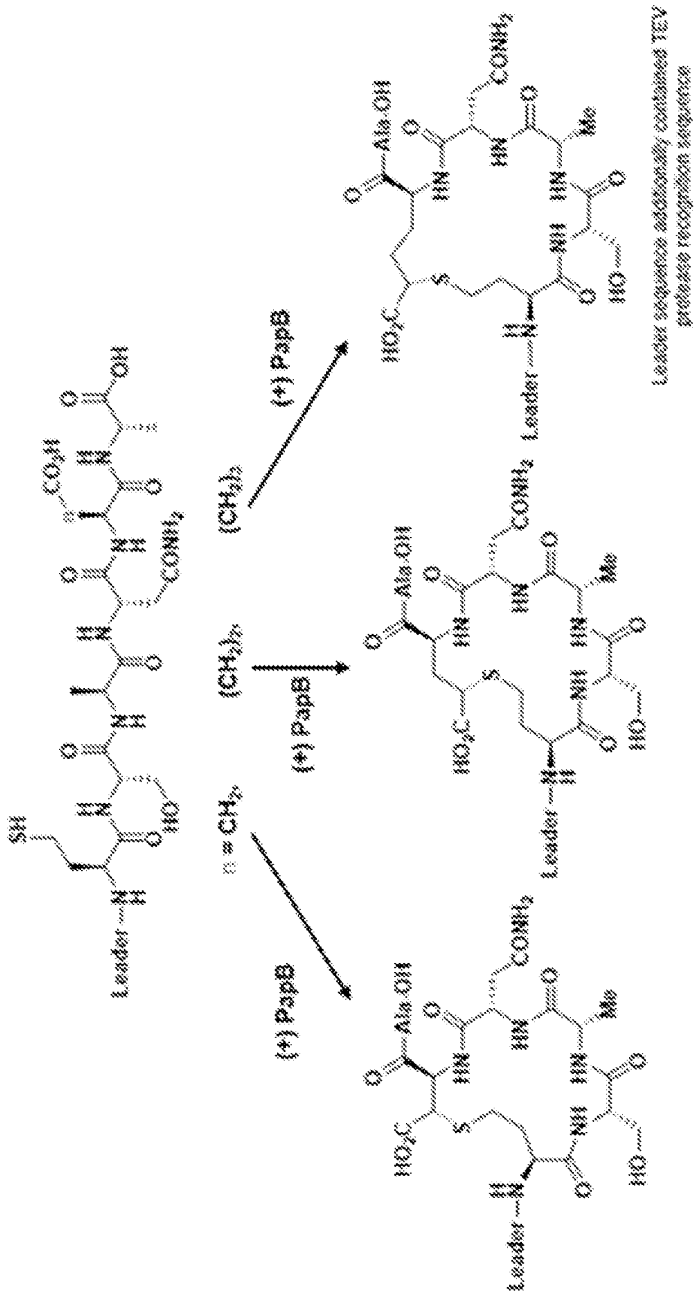

(+) PapB
(+) PapB
(+) PapB
Leader sequence additionally contained TEV protease recognition sequence

FIG. 79A

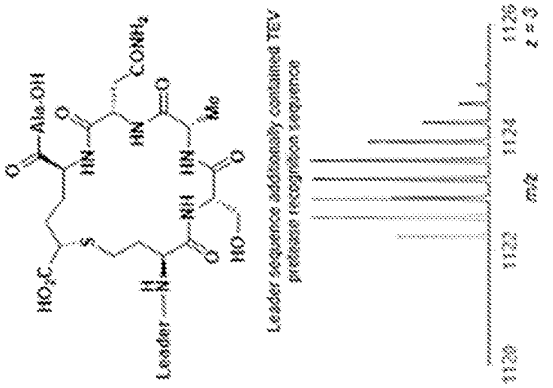


FIG. 79D

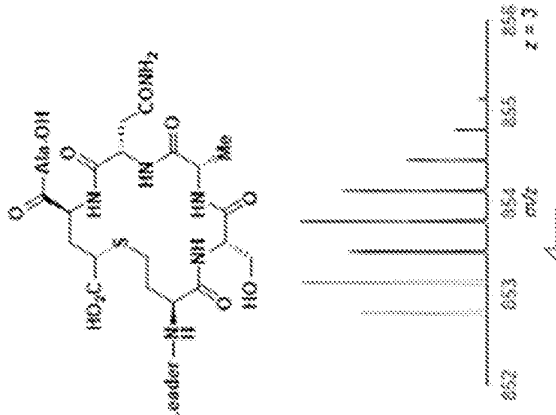


FIG. 79C

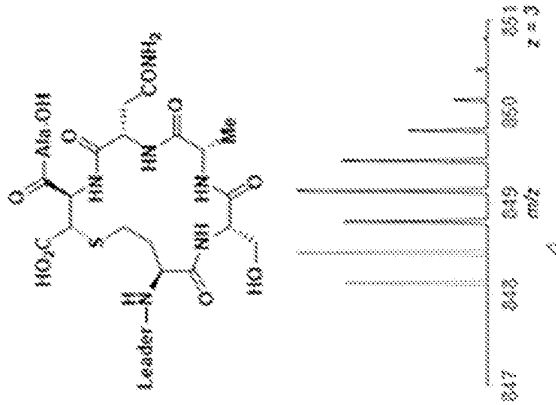


FIG. 79B

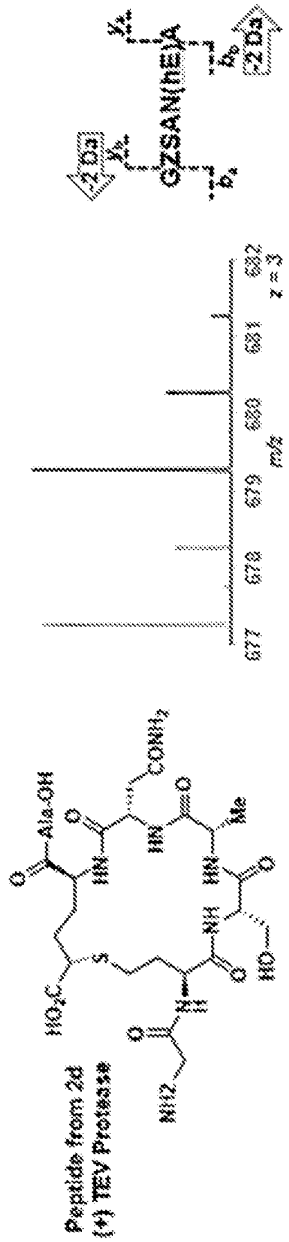

Peptide from 2d
(+) TEV Protease
Ala-OH
Me
CONH2
HO2C
NH2
HO
GZSAN(hE)A
-2 Da
-2 Da
z = 3
677
678
679
680
681
682
m/z

FIG. 79E

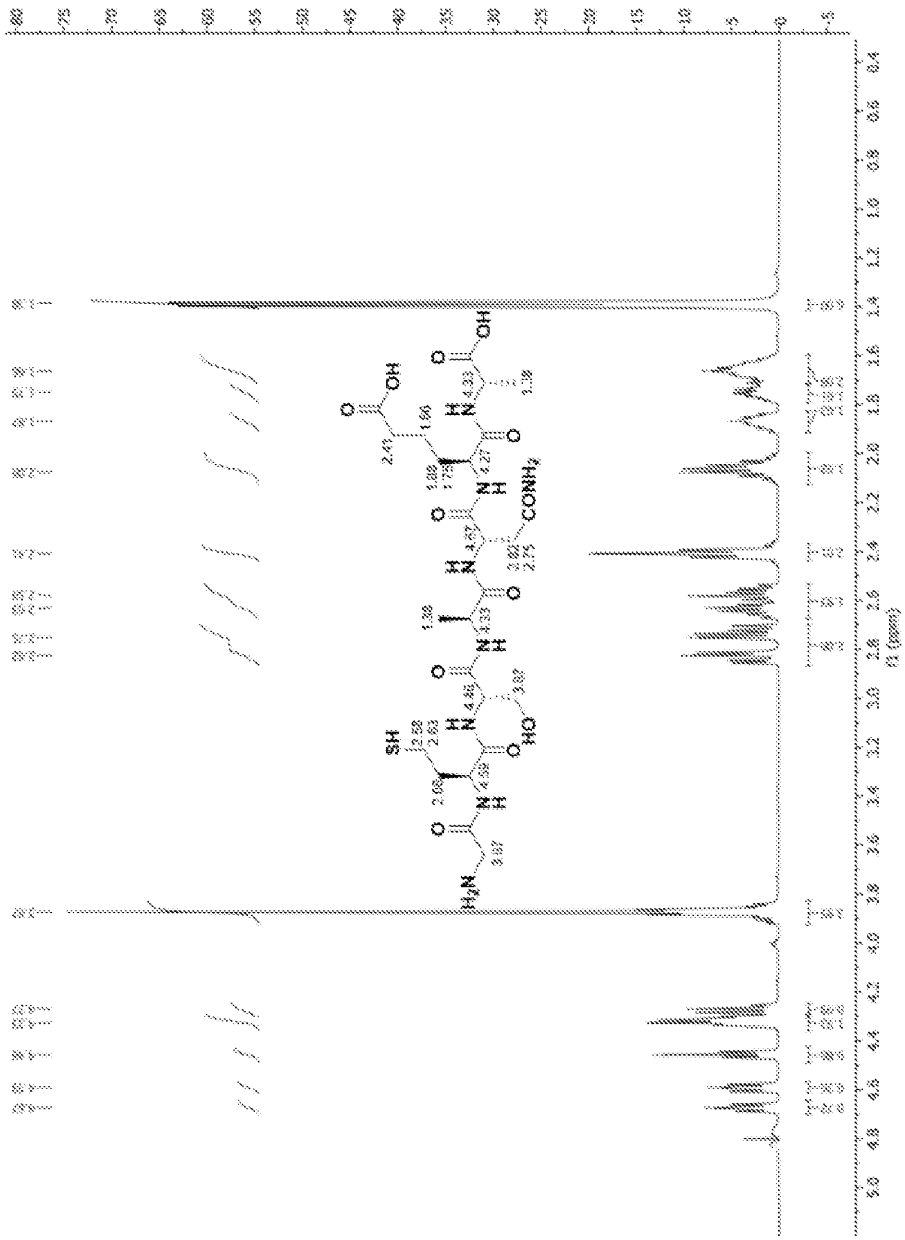


FIG. 80

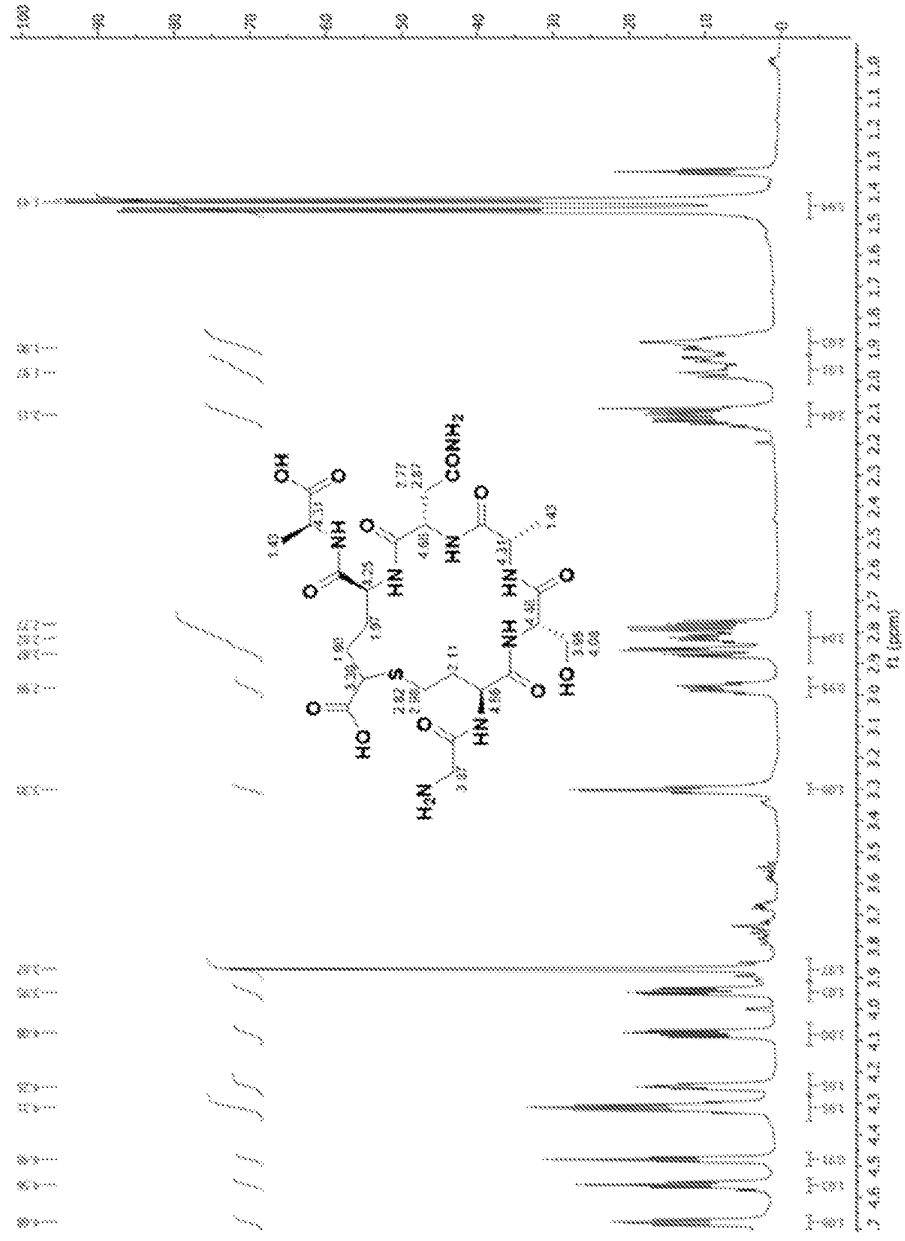


FIG. 81

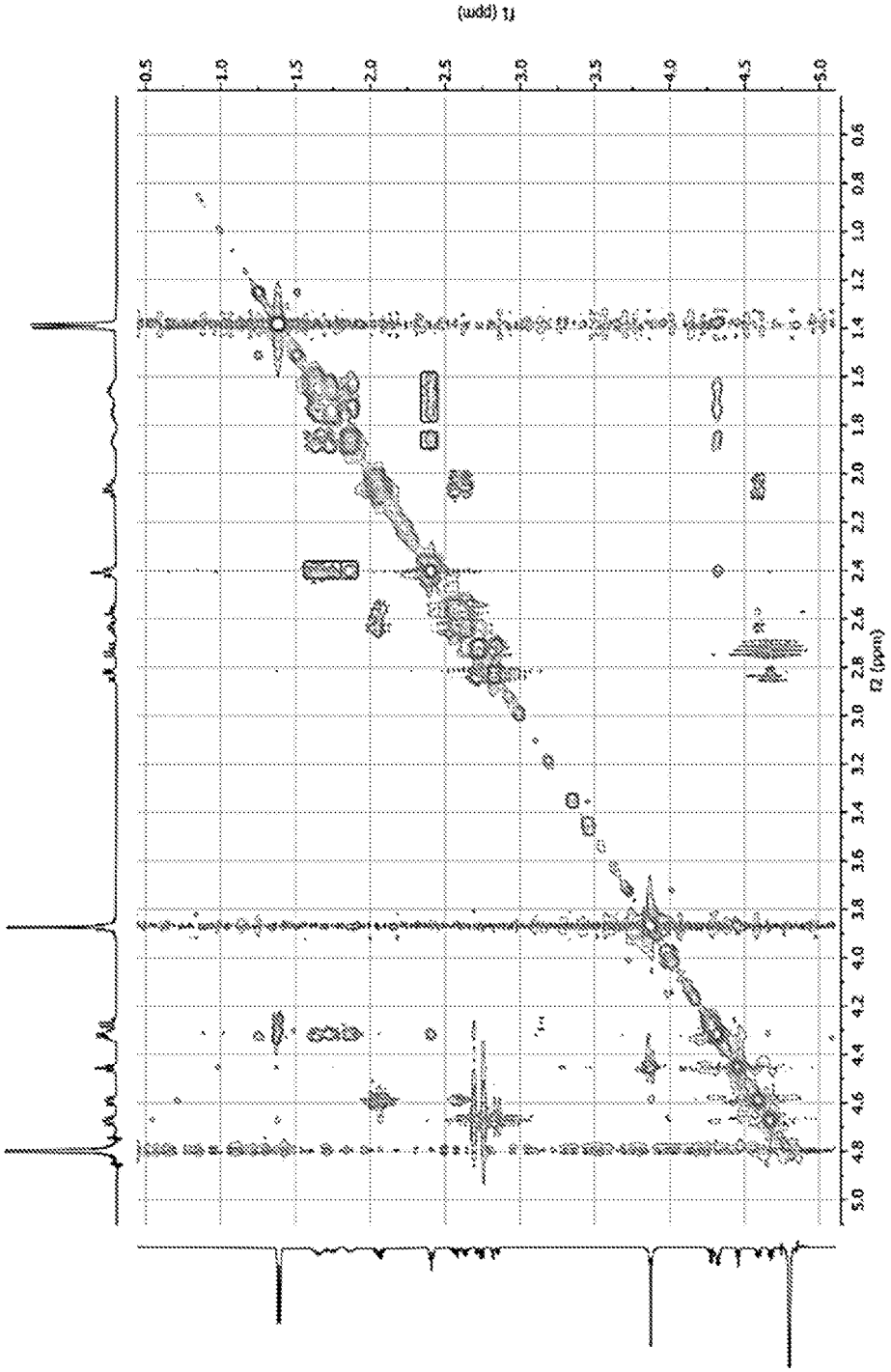


FIG. 82

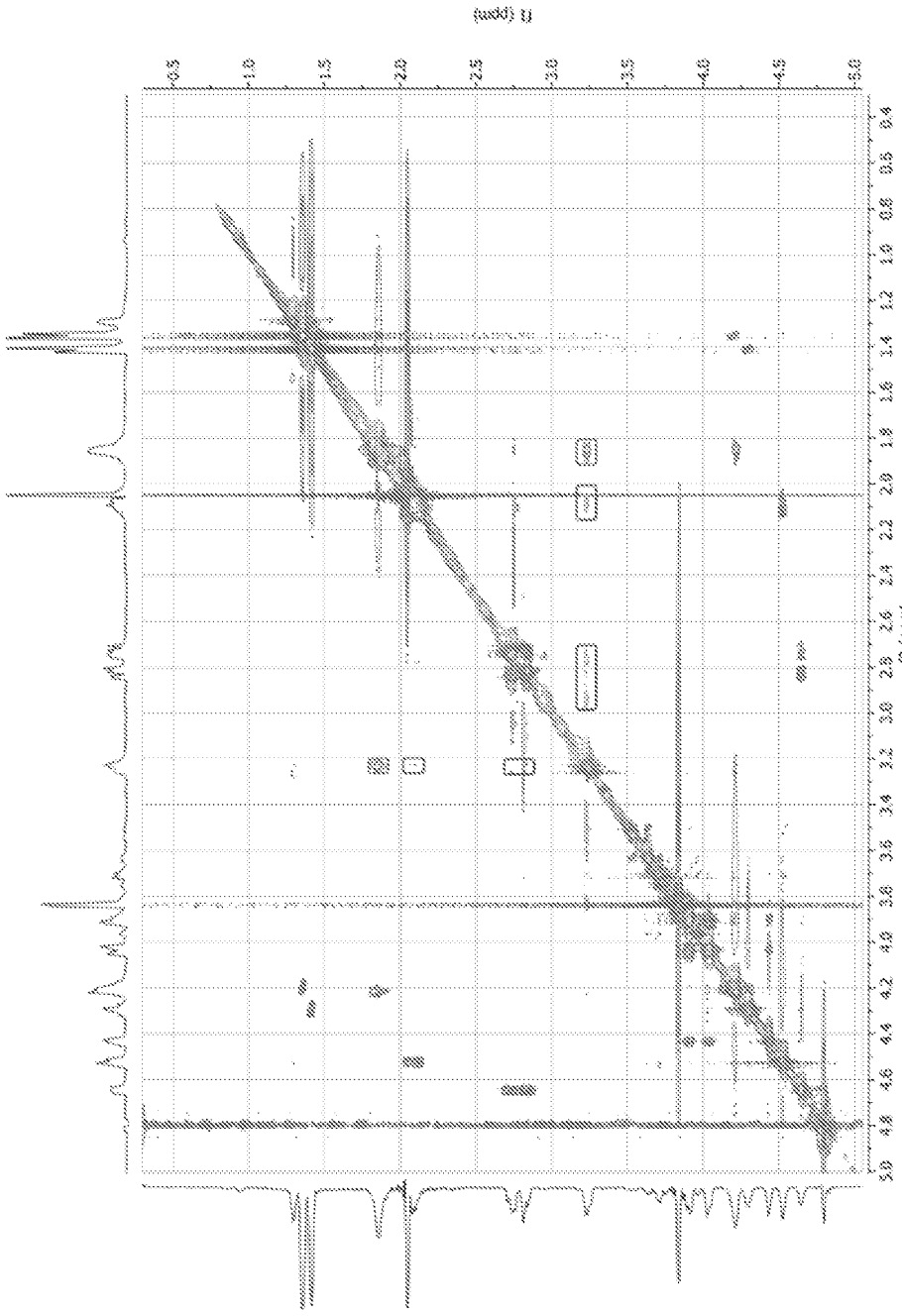


FIG. 83

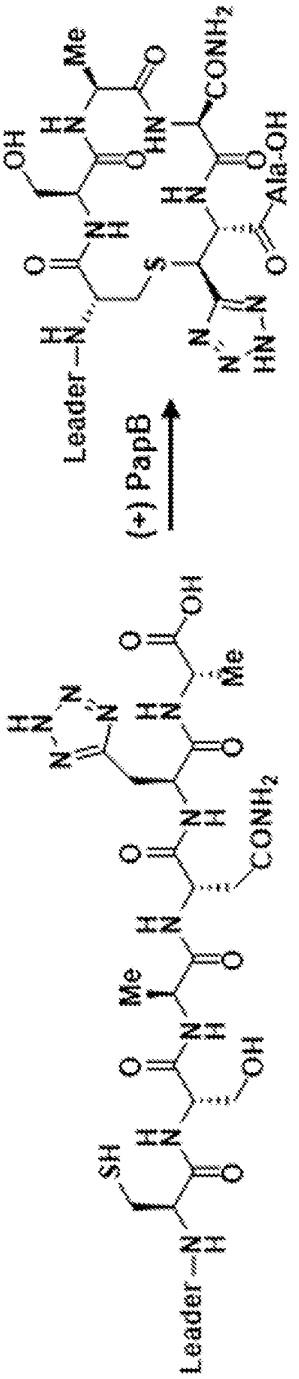


FIG. 84A

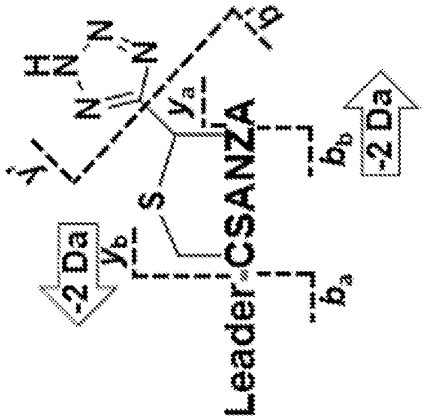


FIG. 84C

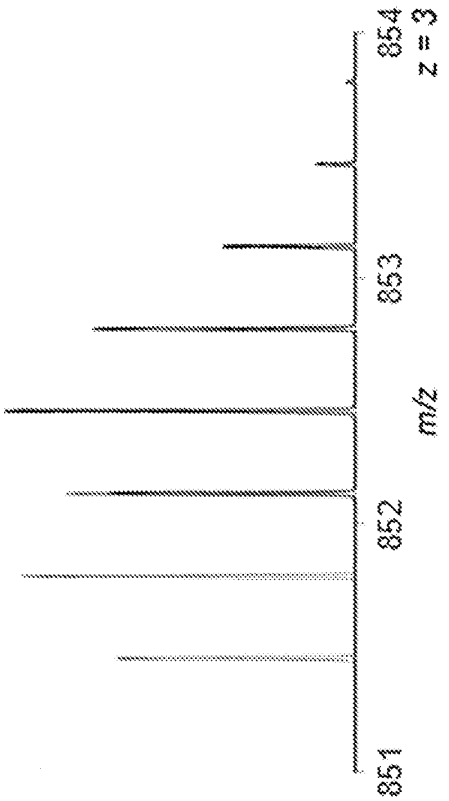


FIG. 84B

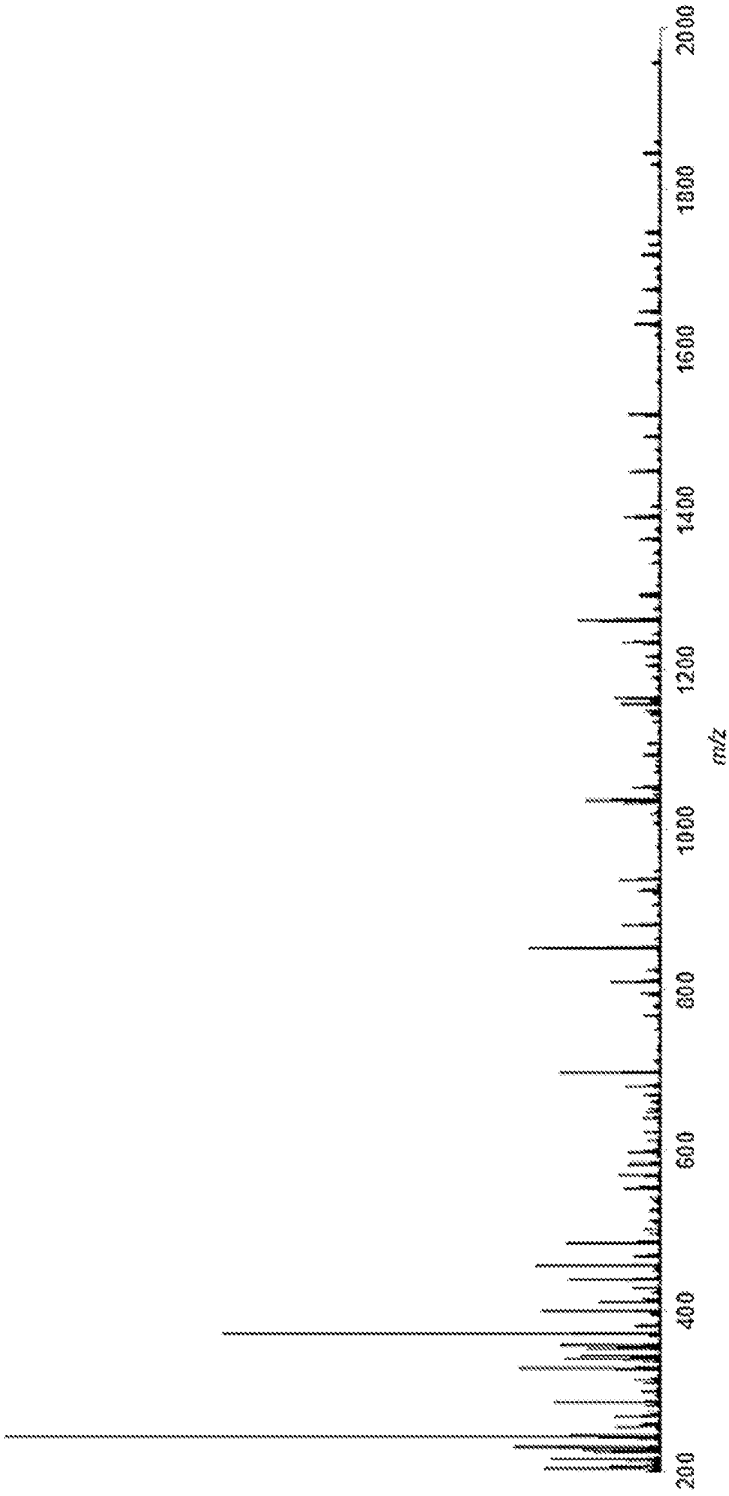


FIG. 85

ion	Sequence	Expected monoisotopic mass	Observed monoisotopic mass	ppm error
b2	LK	242.1863	242.1861	-0.65
b3	LKQ	370.2449	370.2450	0.25
b4	LKQI	483.3289	483.3289	0.00
b5	LKQIN	597.3719	597.3735	2.63
b6	LKQINV	696.4403	696.4382	-3.01
b7	LKQINVI	809.5244	809.5246	0.28
b8	LKQINVIA	880.5615	880.5594	-2.35
b9	LKQINVIAG	937.5829	937.5813	-1.66
b10	LKQINVIAGV	1036.6513	1036.6477	-3.51
b11	LKQINVIAGVK	1164.7463	1164.7490	2.33
b12	LKQINVIAGVKE	1293.7889	1293.7912	1.75
b13	LKQINVIAGVKEP	1390.8417	1390.8402	-1.08
b14	LKQINVIAGVKEPI	1503.9257	1503.9235	-1.43
b15	LKQINVIAGVKEPIR	1660.0268	1660.0295	1.64
b16	LKQINVIAGVKEPIRA	1731.0640	1731.0566	-4.29
b17	LKQINVIAGVKEPIRAY	1894.1273	Not Found	N/A
b18	LKQINVIAGVKEPIRAYG	1951.1487	1951.1490	0.15
b19	(-1 Da) LKQINVIAGVKEPIRAYGC	1027.0787	Not Found	N/A
b20	(-1 Da) LKQINVIAGVKEPIRAYGCS	1070.5947	Not Found	N/A
b21	(-1 Da) LKQINVIAGVKEPIRAYGCSA	1106.1133	Not Found	N/A
b22	(-1 Da) LKQINVIAGVKEPIRAYGCSAN	1163.1347	Not Found	N/A

Sequence: LKQINVIAGVKEPIRAYGCSANZA
Where Z = (2H-tetrazol-5-yl)propanoic acid
Unhighlighted indicates z = 1 charge state
Light gray highlight indicates z = 2 charge state.
Dark gray highlight indicates z = 3 charge state.

FIG. 85
(Continued)

y23	{-2 Da} LKQINVIAGVKEPIRAYGCSANZ	821.7728	821.7701	-3.29
M	{-2 Da} LKQINVIAGVKEPIRAYGCSANZA	851.4553	851.4530	-2.75
y23	{-2 Da} KQINVIAGVKEPIRAYGCSANZA	813.7606	813.7625	2.28
y22	{-2 Da} QINVIAGVKEPIRAYGCSANZA	771.0623	Not Found	N/A
y21	{-2 Da} INVIAGVKEPIRAYGCSANZA	1092.0606	1092.0640	3.12
y20	{-2 Da} NVIAGVKEPIRAYGCSANZA	1035.5185	1035.5170	-1.48
y19	{-2 Da} VIAGVKEPIRAYGCSANZA	978.4971	978.4948	-2.35
y18	{-2 Da} IAGVKEPIRAYGCSANZA	1856.9184	1856.9253	3.71
y17	{-2 Da} AGVKEPIRAYGCSANZA	1743.8344	1743.8314	-1.71
y16	{-2 Da} GVKEPIRAYGCSANZA	1672.7972	1672.8032	3.59
y15	{-2 Da} VKEPIRAYGCSANZA	1615.7758	1615.7773	0.91
y14	{-2 Da} KEPIRAYGCSANZA	1516.7074	1516.7124	3.32
y13	{-2 Da} EPIRAYGCSANZA	1388.6124	1388.6169	3.26
y12	{-2 Da} PIRAYGCSANZA	1259.5698	1259.5750	4.13
y11	{-2 Da} IRAYGCSANZA	1162.5170	1162.5186	1.35
y10	{-2 Da} RAYGCSANZA	1049.4330	1049.4342	1.10
y9	{-2 Da} AYGCSANZA	893.3319	893.3350	3.49
y8	{-2 Da} YGCSANZA	822.2948	822.2938	-1.17
y7	{-2 Da} GCSANZA	659.2314	659.2297	-2.50
y6	{-2 Da} CSANZA	602.2100	602.2109	1.52
y5	{-1 Da} SANZA	500.2086	Not Found	N/A
y4	{-1 Da} ANZA	413.1766	Not Found	N/A
y3	{-1 Da} NZA	342.1395	Not Found	N/A
y2	{-1 Da} ZA	228.0965	Not Found	N/A
y1	A	90.0550	Not Found	N/A

FIG. 85
(Continued)

Sequence: LKQINVIAGVKEPIRAYGCSANZ'A
Where Z' is (2H-tetrazol-5-yl)propanoic acid with the tetrazole moiety fragmented in the CID c
Unhighlighted indicates z = 1 charge state.
Light gray highlight indicates z = 2 charge state.
Dark gray highlight indicates z = 3 charge state.

Ion	Sequence	Expected monoisotopic mass	Observed monoisotopic mass	ppm error
b23'	(-2 Da) LKQINVIAGVKEPIRAYGCSANZ'	1198.149	Not Found	N/A
M	(-2 Da) LKQINVIAGVKEPIRAYGCSANZ'A	828.7846	828.7836	-1.26
y23'	(-2 Da) KQINVIAGVKEPIRAYGCSANZ'A	1186.131	Not Found	N/A
y22'	(-2 Da) QINVIAGVKEPIRAYGCSANZ'A	1122.064	1122.086	2.06
y21'	(-2 Da) INVIAGVKEPIRAYGCSANZ'A	1058.054	1058.055	0.58
y20'	(-2 Da) NVIAGVKEPIRAYGCSANZ'A	1001.512	1001.513	0.73
y19'	(-2 Da) VIAGVKEPIRAYGCSANZ'A	1887.975	Not Found	N/A
y18'	(-2 Da) IAGVKEPIRAYGCSANZ'A	1788.906	1788.903	-1.48
y17'	(-2 Da) AGVKEPIRAYGCSANZ'A	1675.822	1675.824	1.41
y16'	(-2 Da) GVKEPIRAYGCSANZ'A	1604.786	1604.793	4.69
y15'	(-2 Da) VKEPIRAYGCSANZ'A	1547.764	1547.768	2.68
y14'	(-2 Da) KEPIRAYGCSANZ'A	1448.696	1448.701	4.06
y13'	(-2 Da) EPIRAYGCSANZ'A	1320.6	1320.604	3.07
y12'	(-2 Da) PIRAYGCSANZ'A	1191.558	1191.554	-2.57
y11'	(-2 Da) IRAYGCSANZ'A	1094.505	Not Found	N/A
y10'	(-2 Da) RAYGCSANZ'A	981.4207	Not Found	N/A
y9'	(-2 Da) AYGCSANZ'A	825.3196	825.3211	1.81
y8'	(-2 Da) YGCSANZ'A	754.2825	Not Found	N/A
y7'	(-2 Da) GCSANZ'A	591.2191	591.2202	1.86
y6'	(-2 Da) CSANZ'A	534.1977	534.1995	3.37
y5'	(-1 Da) SANZ'A	432.1963	Not Found	N/A
y4'	(-1 Da) ANZ'A	345.1643	Not Found	N/A
y3'	(-1 Da) NZ'A	274.1272	Not Found	N/A
y2'	(-1 Da) Z'A	160.0842	Not Found	N/A

FIG. 86