Disulfide-Rich Peptide Production at AmbioPharm, Inc.

Michael W. Pennington and Guoqing Zhang

Supplier of Peptide API
Manufacturing and Services

13 years and growing rapidly, AmbioPharm Inc. achieves cost advantages by leveraging peptide production across US and Chinese facilities

Peptide NCE and Complexity Trends Both Increasing

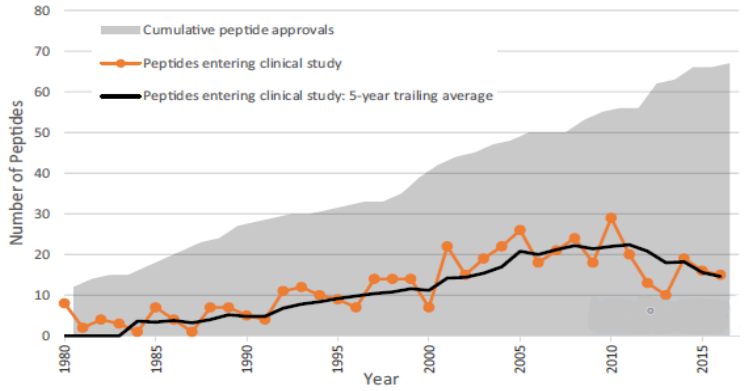


Fig. 2. Cumulative number of peptides approved in major pharmaceutical markets and the number of peptides entering clinical development. Entry into clinical development is defined as the year of the first Phase 1 or pilot human study.

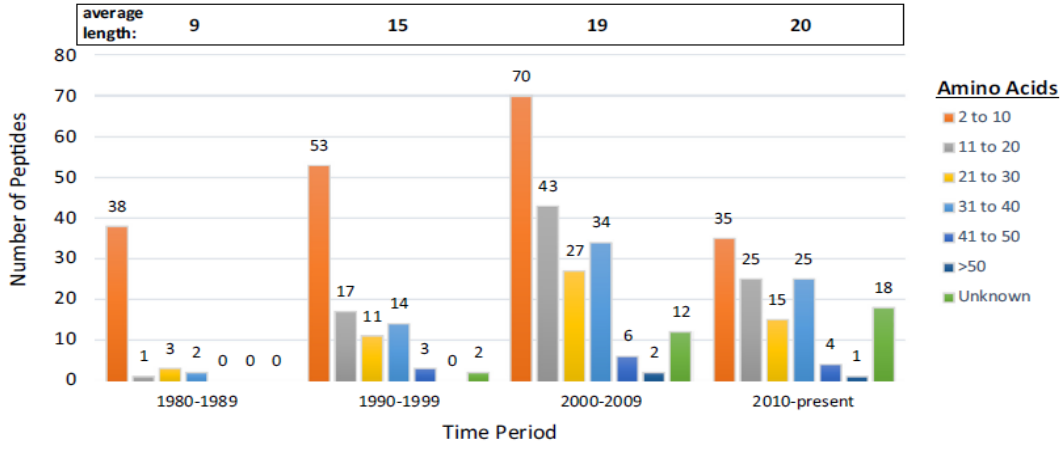


Fig. 3. Length of peptides entering clinical development, by decade. Peptides with unknown length were not included in the average length calculation.

Lau and Dunn (2018) *Bioorg. & Med Chem.* **26**, 2700

Commercial Disulfide Rich Peptide Drugs

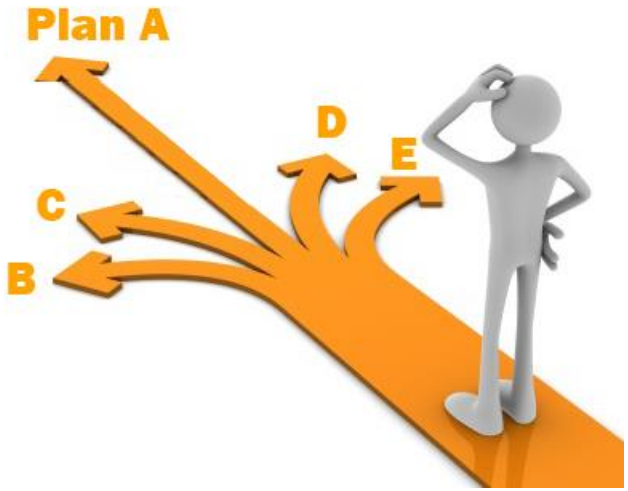
Generic Name	Peptide Name	Peptide Length	Disulfide Bond #	Annual Qty
Ziconotide	ω-Conotoxin MVIIA	25	3	50-100 g
Linaclotide	Heat stable enterotoxin analog	14	3	~80 kg
Plecanatide	Uroguanylin analog	16	2	~100 kg
Aprotinin	BPTI	58	3	~5 kg
Humulin	Rec Insulin	51 (2 chains)	3	~metric tons
Lantus,Toujeo	Rec Insulin Glargine	53 (2 chains)	3	~metric tons

DSR Peptides In or Close to Clinical Trials

Product	Stage	Disease	# of Disulfides	Company
Dalazatide ShK-186	PhIIa	Autoimmune	3	Airmid/Kineta
LJPC-401 hepcidin	PhIIa	B-thalassemia	4	La Jolla Pharm.
BLZ-100 chlorotoxin-dye tozuleristide	PhII-III	Cancer imaging	4	Blaze Biosci.
Iseganan HCl (protegrin)	Ph-III	Antimicrobial	2	Hebrew Univ. (~~Intrabiotics~~)
KCP-400 **α-**conotoxin RGIA4 analog	Pre-clin	Chronic Pain NACh-R	2	Kineta
T20k (cyclotide)	Pre-clin	MS	3 + h-t-t	Cyxone
HsTX-1 R14A	Pre-clin	Autoimmune	4	Monash Univ.
GpTX-1 analog (tarantula)	Pre-clin	Pain Nav1.7	3	Amgen

Where are the Problems?

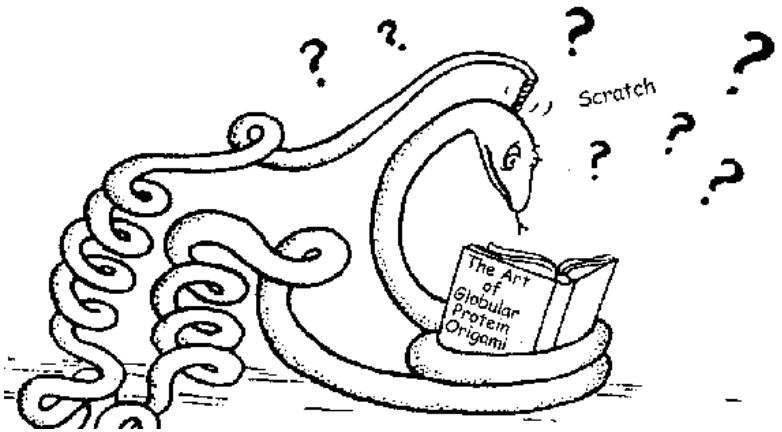
- Solid-Phase Strategy Improvements for longer peptides:
  - Psuedoproline derivatives & dipeptide building blocks
  - Specialty Resins (Peg-resins &/or Low substitution)
- Folding to get correct isomer
  - Decision to use orthogonal protecting group strategy?
  - Random Oxidation (God-Assisted Folding) favoring most favorable thermodynamic isomer?
- Pre-purify the linear peptide or fold the crude linear peptide?



Potential Disulfide Isomers

- The number of possible isomers (p) follows this formula where n= # Cys residues: $$p = \frac{n!}{\left(\frac{n}{2}\right)!2^{n/2}}$$

- 2 disulfide bonds: 3 potential isomers
- 3 disulfide bonds: 15 potential isomers
- 4 disulfide bonds: 105 potential isomers
- 5 disulfide bonds: 945 potential isomers



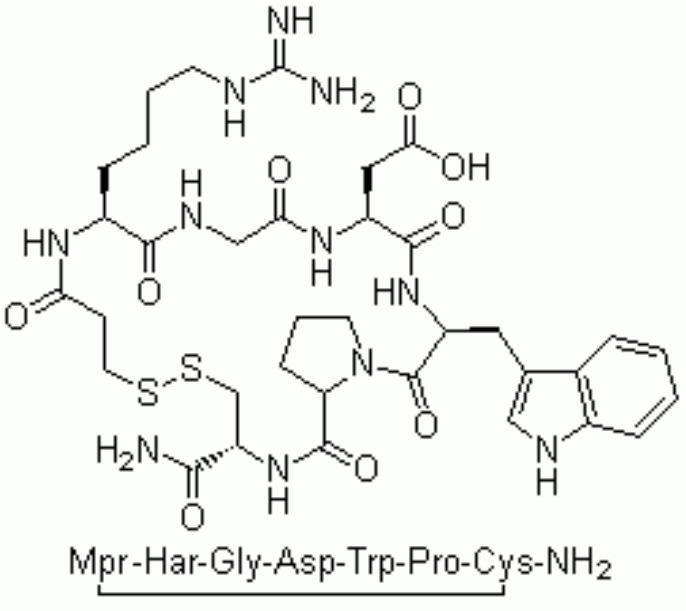
Regioselective choices for Disulfide Introduction

- 1 Disulfide: Cys(Trt)
- 2 Disulfides: Cys(Trt), Cys(Acm)[1]
- 3 Disulfides: Cys(Trt), Cys(Acm), Cys(Mob)[2]
- 4 Disulfides: Cys(Trt), Cys(Acm), Cys(Mob), Cys(Msbh)[3] (4,4'-dimethylsulfinyl benzhydryl)
- 5 Disulfides (?): Cys(*o*Nv) (new photolabile *ortho*-nitroveratryl) [4]

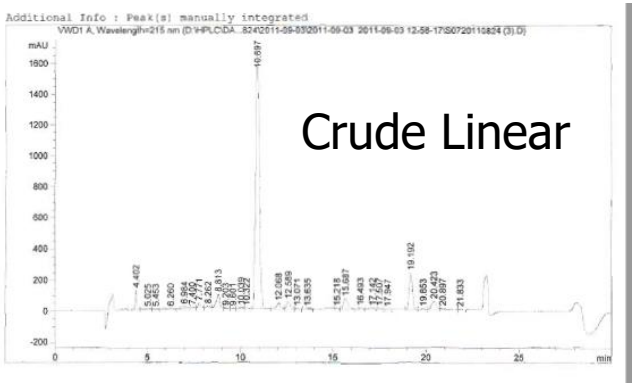
1 Urogaunylin: Klodt et al., (1997) *J. Pep. Res.* **50**, 222
2 hSt. Enterotx: Wolfe et al., (2002) *J. Nucl. Med.* **43**, 392.
3 Hepcidin: Dekan et al., (2014) *Angew. Chemie* **53**, 1-5.
4 Insulin (others) : Patil et al., (2019) *Chem. Eur. J.* 10.1002/chem.201901334

Eptifibatide: Single Disulfide

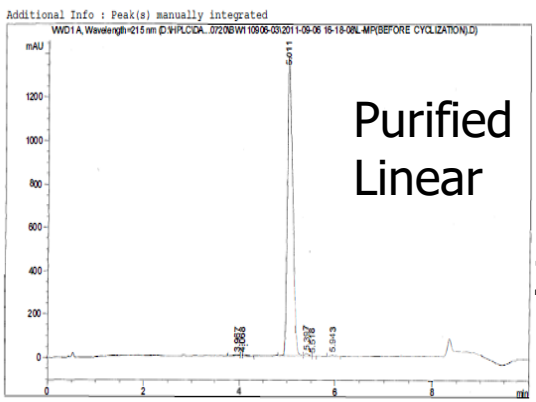
- Generic drug product which binds to platelets at the Glycoprotein IIb-IIIa using “Har-GD” motif. It is used to treat cardiac ischemic events.
- Fmoc-SPPS strategy with unusual derivatives Har (homo-Arg) and Mpa (3-mercaptopropionyl)
- Disulfide bond between Mpa^{1} & Cys^{7} (both sidechain Trt protected)
- Crude linear (reduced) product is pre-purified and subsequently folded using I_2 to facilitate disulfide formation.



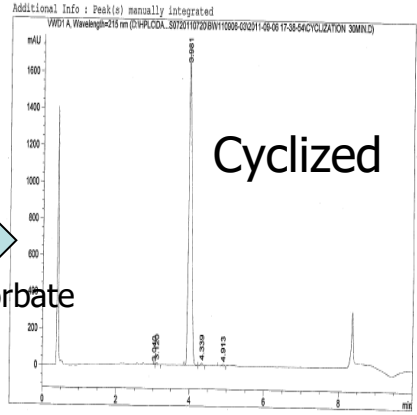
APi1051: Eptifibatide Folding & Final Product Data

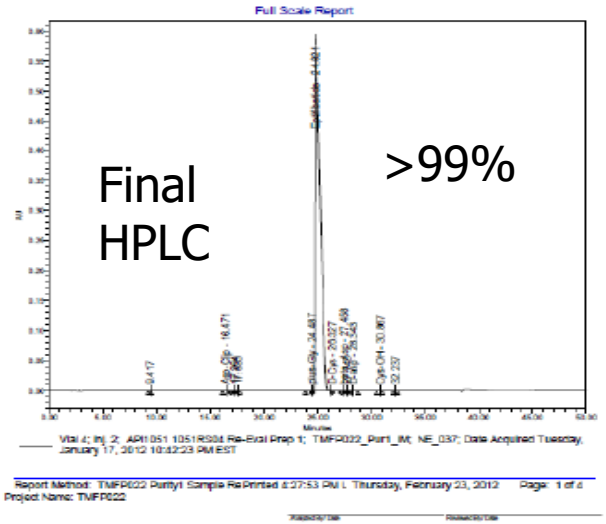


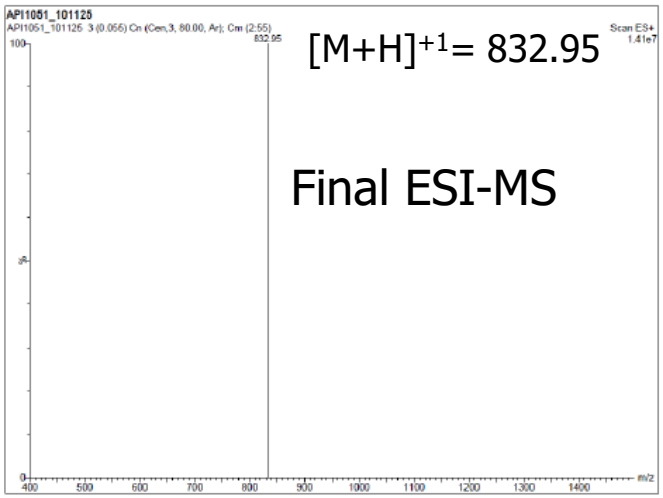
Prep-HPLC

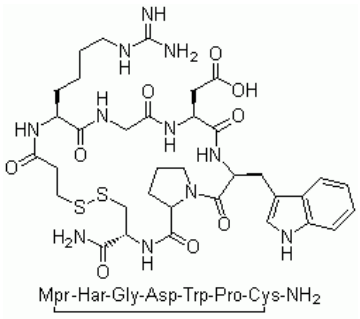


1. I_2
2. Ascorbate

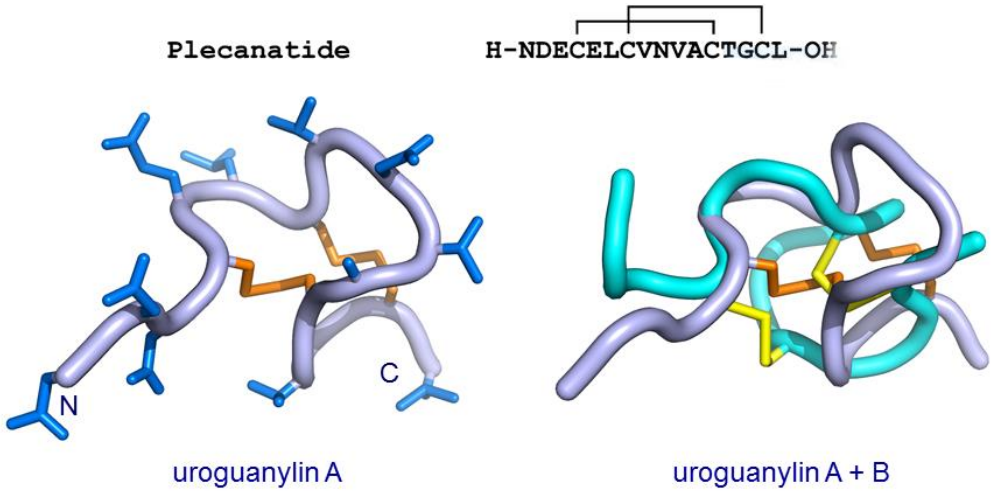






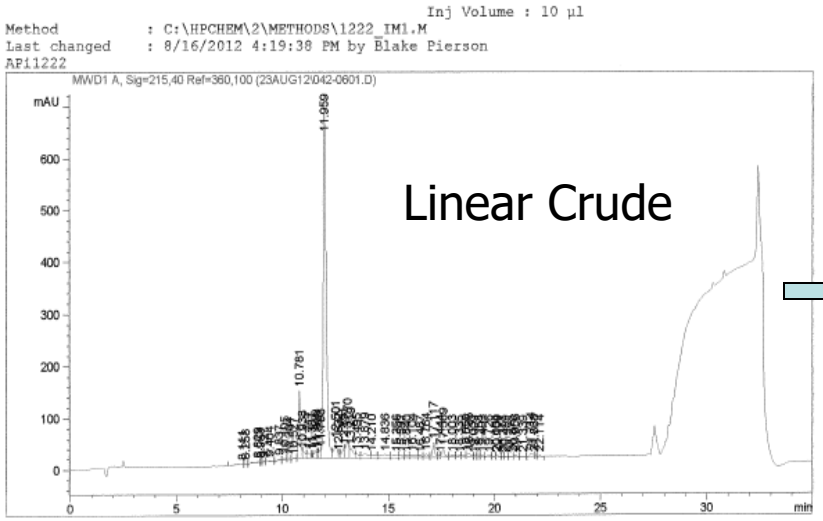


APi1222: Plecanatide: 2 Disulfides



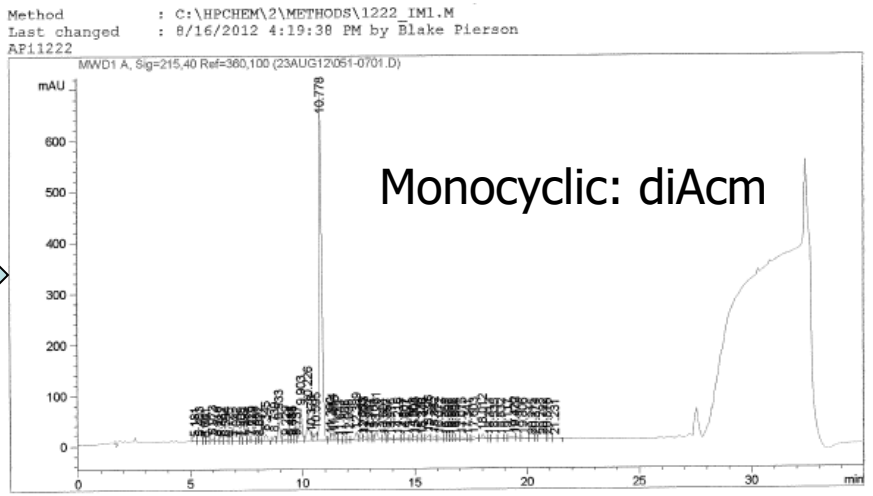
- Glu^{3}-Uroguanylin analog which is a Guanylate Cyclase C agonist (GCC) used to treat chronic constipation via oral administration route.
- Unique non-active conformational topoisomerism presents a serious manufacturing challenge with a degradation impurity.
- Hybrid Fmoc Strategy: 3 fragments assembled for final 16 AA sequence
- Orthogonal Protecting Groups Cys(Trt)^{4,12} and Cys(Acm)^{7,15}

APi1222: Plecanatide Regioselective Folding

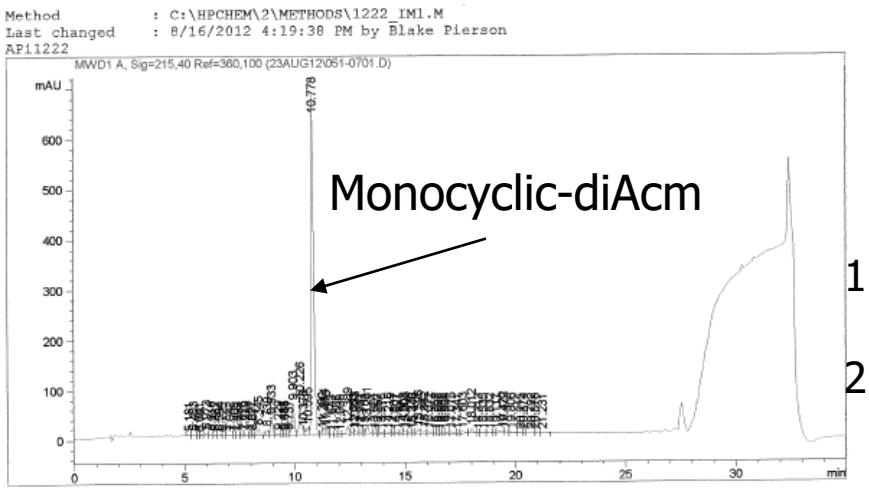


H_2O_2

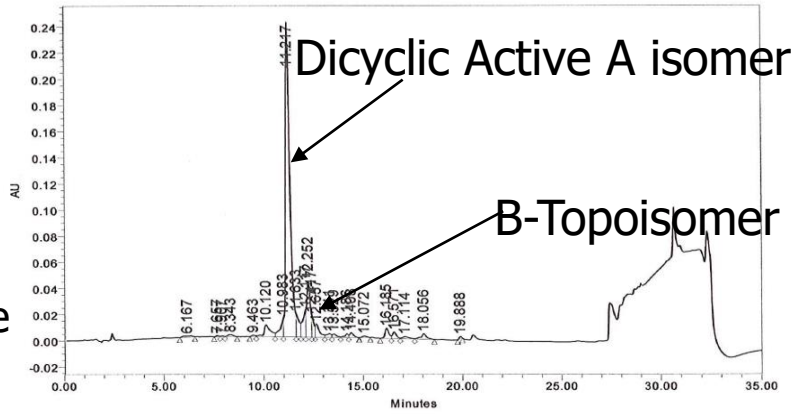
Alk. pH



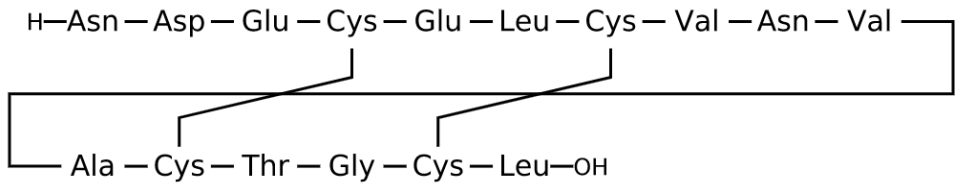
API222: Plecanatide Dicyclization

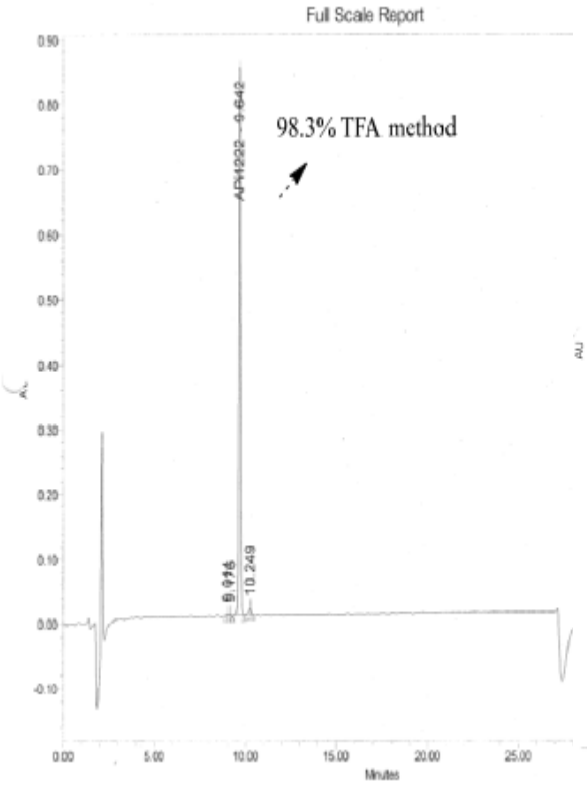


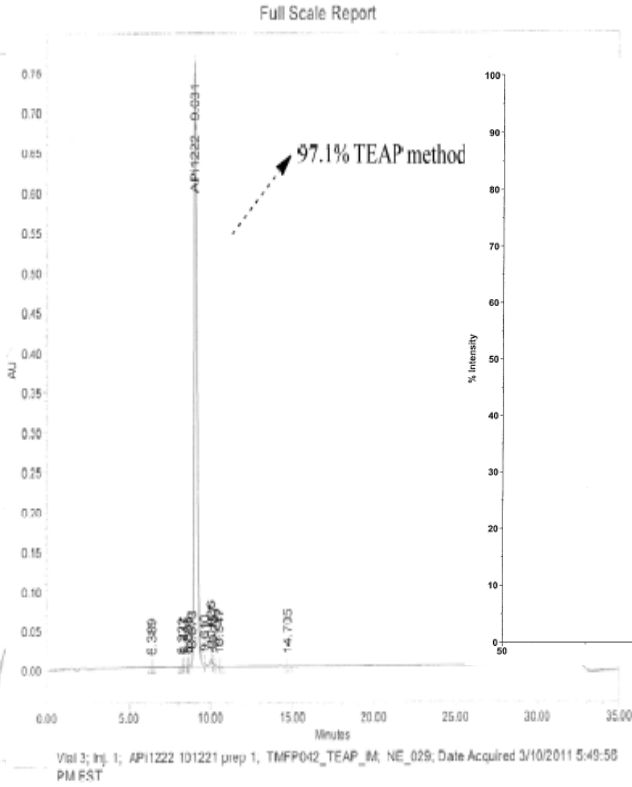
1. I_2, 2hr
2. Ascorbate

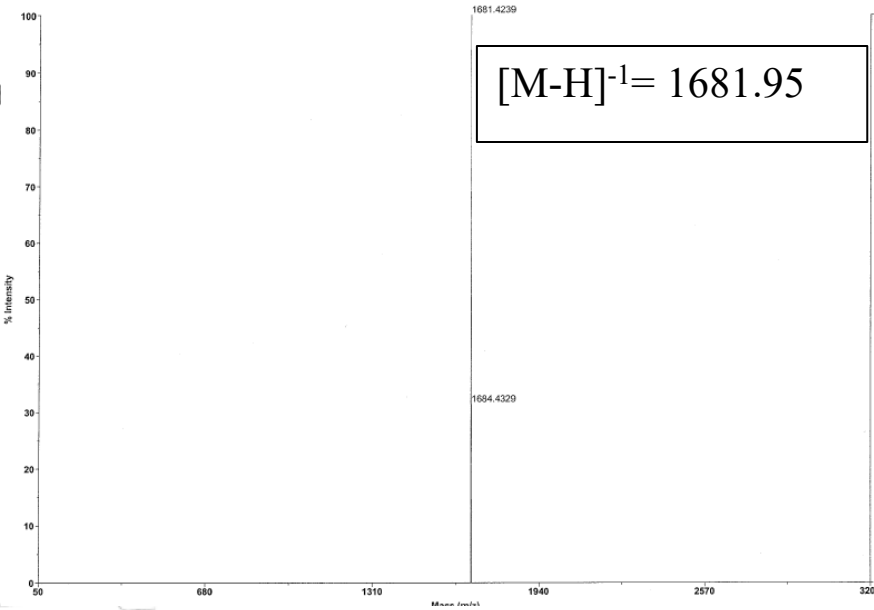


APi1222: Plecanatide Final QC Data





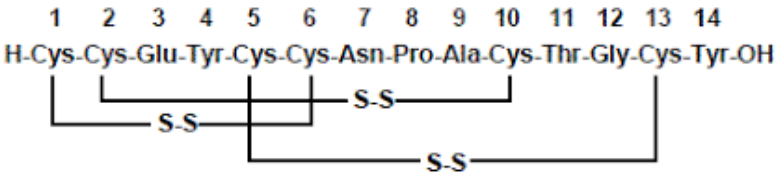


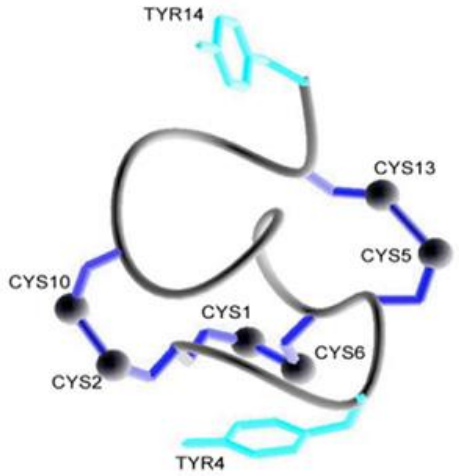


$[M-H]^{-1}$= 1681.95

APi1772: Linaclotide: 3 Disulfides

- 14 residue analog of heat stable enterotoxin which is a GCC agonist used to treat IBS and chronic constipation with an oral administration route.
- Total Fmoc-SPPS synthesis with Cys(Trt) at all six Cys positions
- Prepurify Linear
- God-Assisted folding with Disulfide Shuffling agents (ox/red Cys or Glutathione)





APi1772: Linaclotide

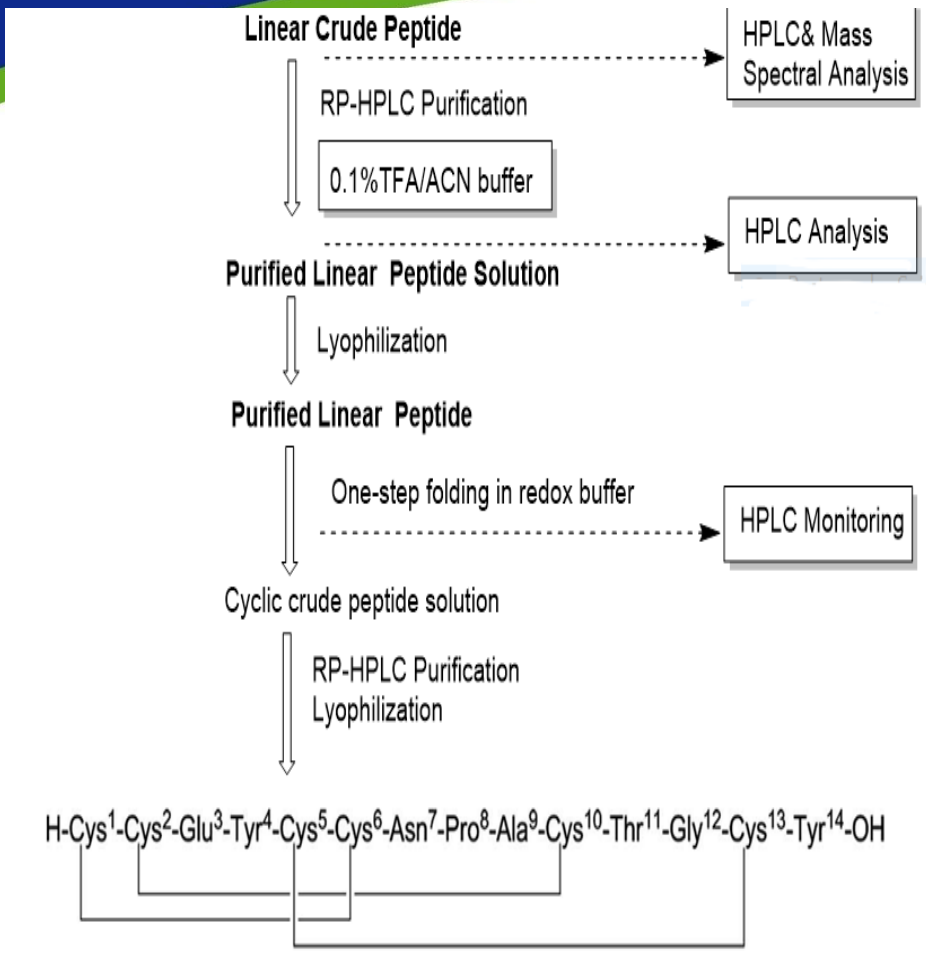
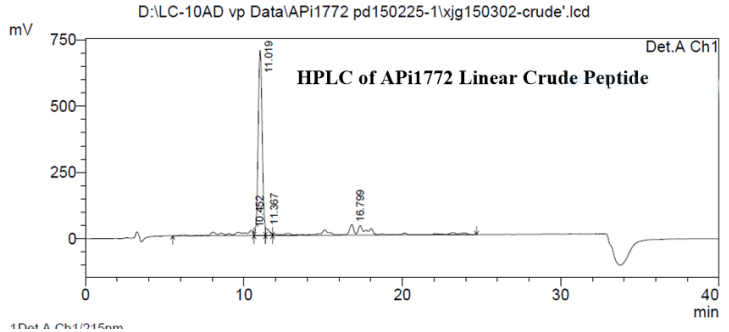
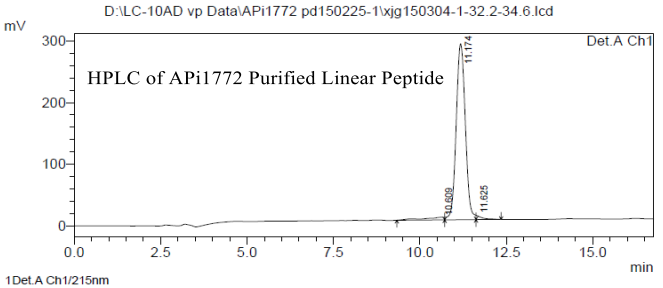


FIG.1. Process Flow Diagram of APi1772(linaclotide)

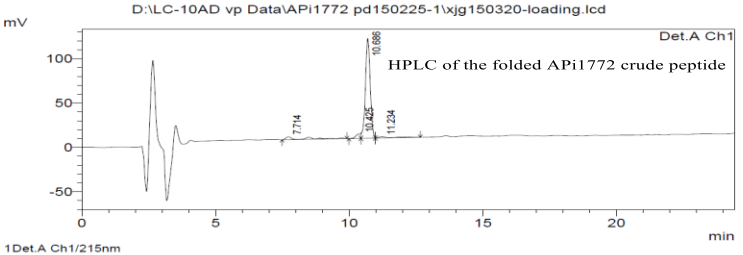




PeakTable

Detector A Ch1 215nm

Peak#	Ret. Time	Area	Height	Area %
1	10.609	188881	4285	3.417
2	11.174	5256537	285496	95.095
3	11.625	82227	6975	1.488
Total		5527646	296756	100.000

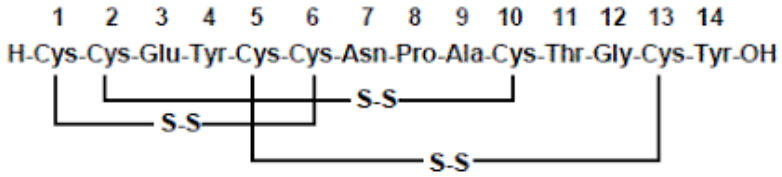


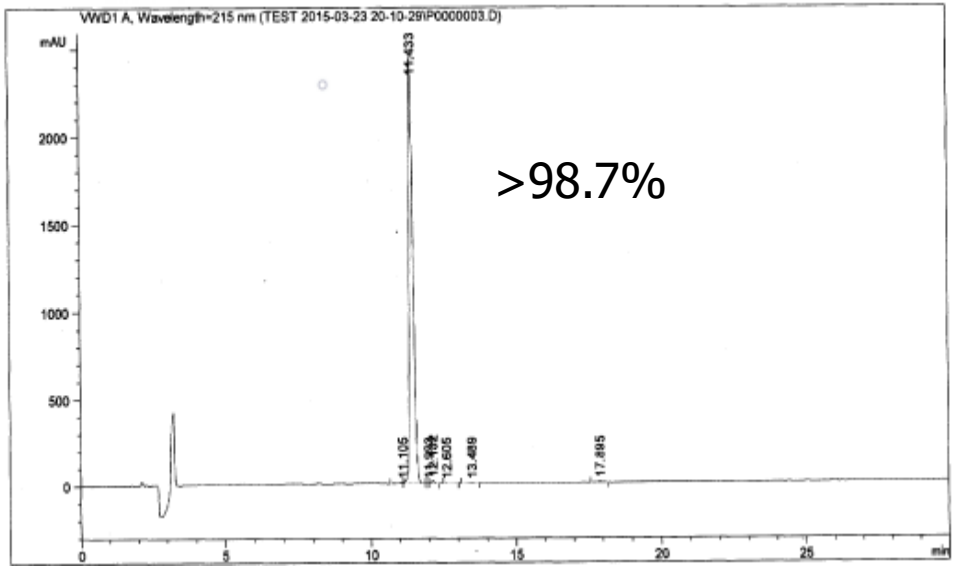
PeakTable

Detector A Ch1 215nm

Peak#	Ret. Time	Area	Height	Area %
1	7.714	122518	3141	7.415
2	10.425	64203	4964	3.885
3	10.686	1405228	112435	85.042
4	11.234	60453	1537	3.658
Total		1652401	122078	100.000

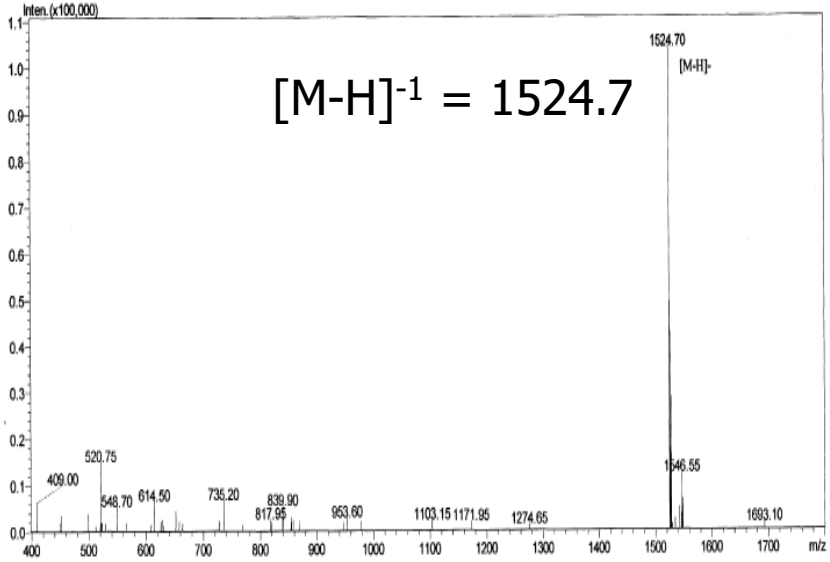
APi1772: Linaclotide: Final Product Data





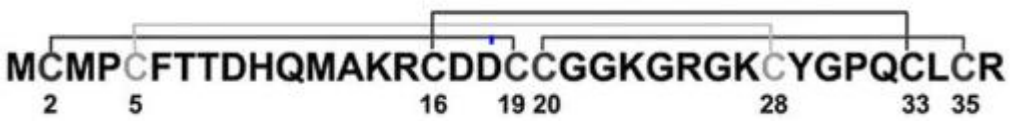
>98.7%

MASS SPECTROMETRY REPORT

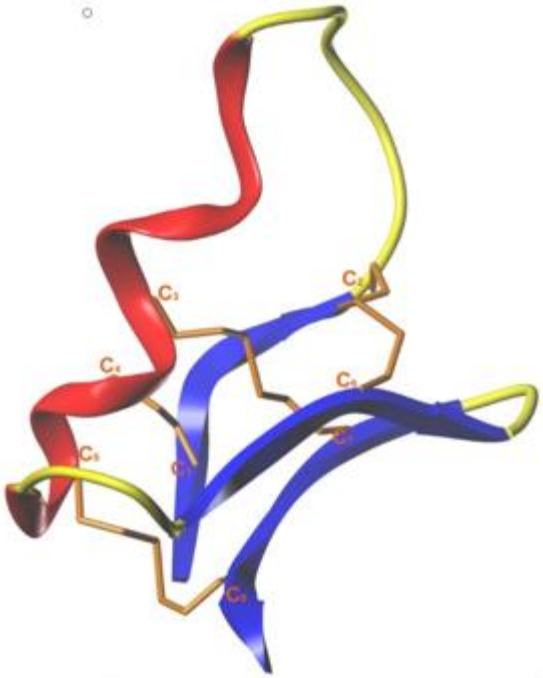


$[M-H]^{-1} = 1524.7$

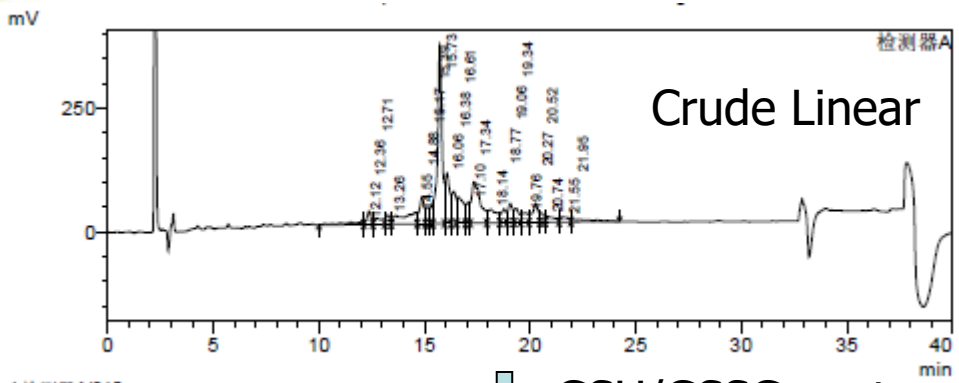
APi2603: Chlorotoxin: 4 Disulfides



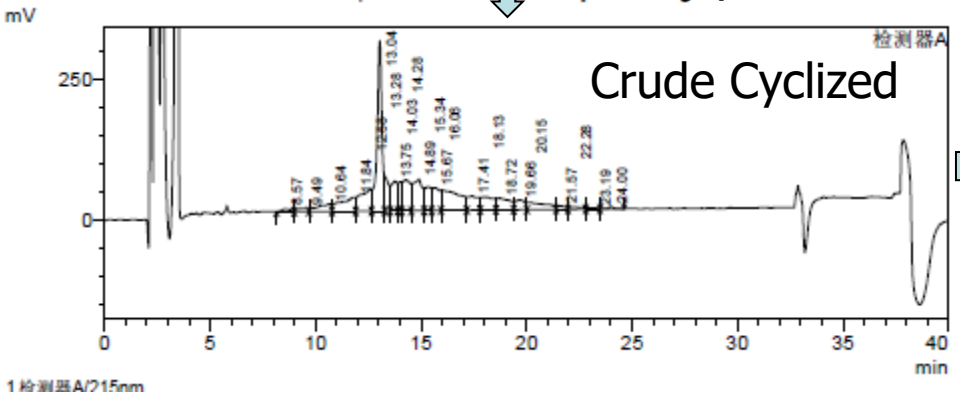
- 36 residue peptide with a C-terminal amide currently in clinical development as an imaging agent for Glioblastoma and other cancers.
- Stepwise Linear Chain build starting with Fmoc-Rink-Amide-resin using Cys(Trt) at all eight Cys positions
- Psuedoproline and dipeptide building blocks were employed at strategic positions.
- Final TFA cleavage also employed an NH_4I with DMS reduction step for Met(O).
- Crude Product dissolved and oxidatively folded at alkaline pH with GSH/GSSG redox pair.



APi2603: Chlorotoxin Folding & Purification

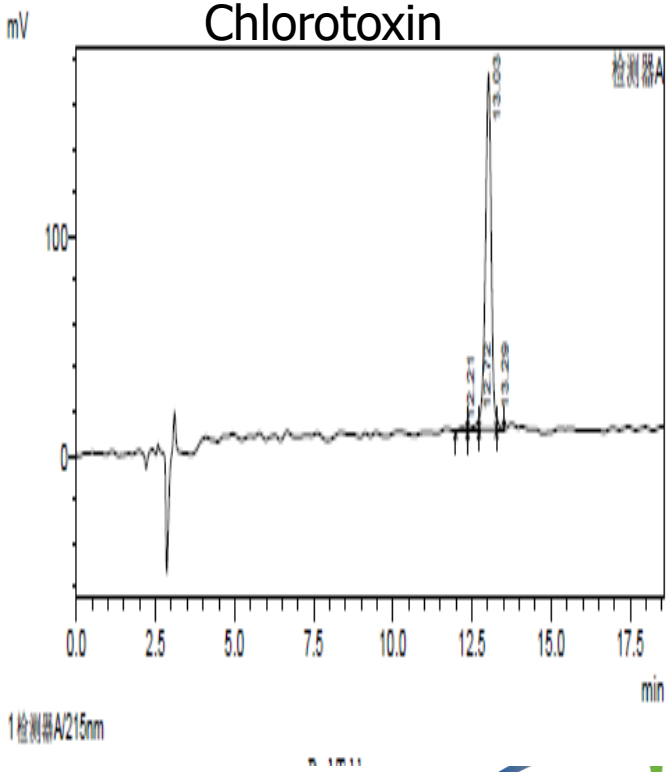


GSH/GSSG ovnt
folding pH 7.8

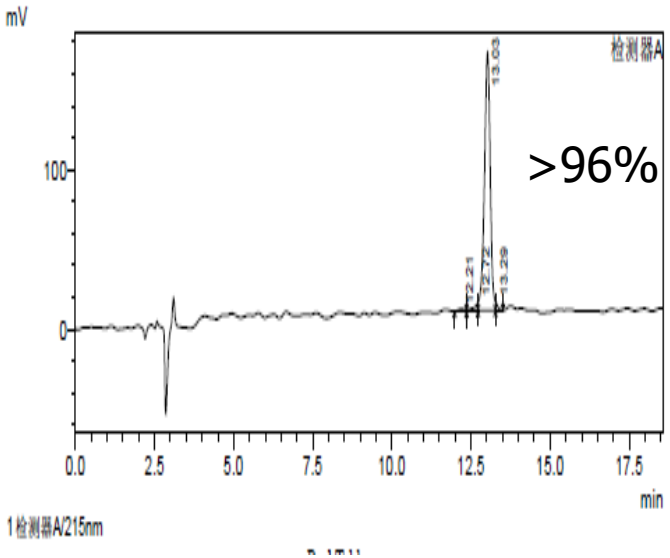


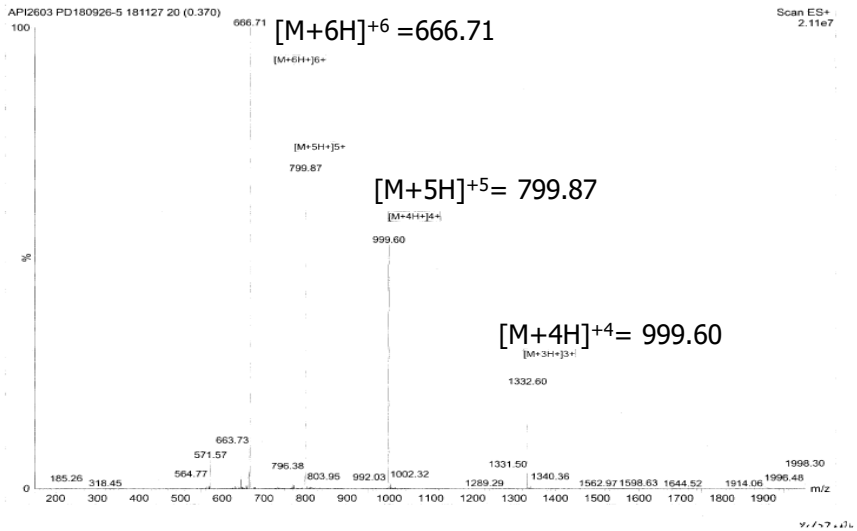
Prep
HPLC

Final Purified
Chlorotoxin



APi2603: Chlorotoxin Data Set





$[M+6H]^{+6}$ =666.71

$[M+5H]^{+5}$= 799.87

$[M+4H]^{+4}$= 999.60

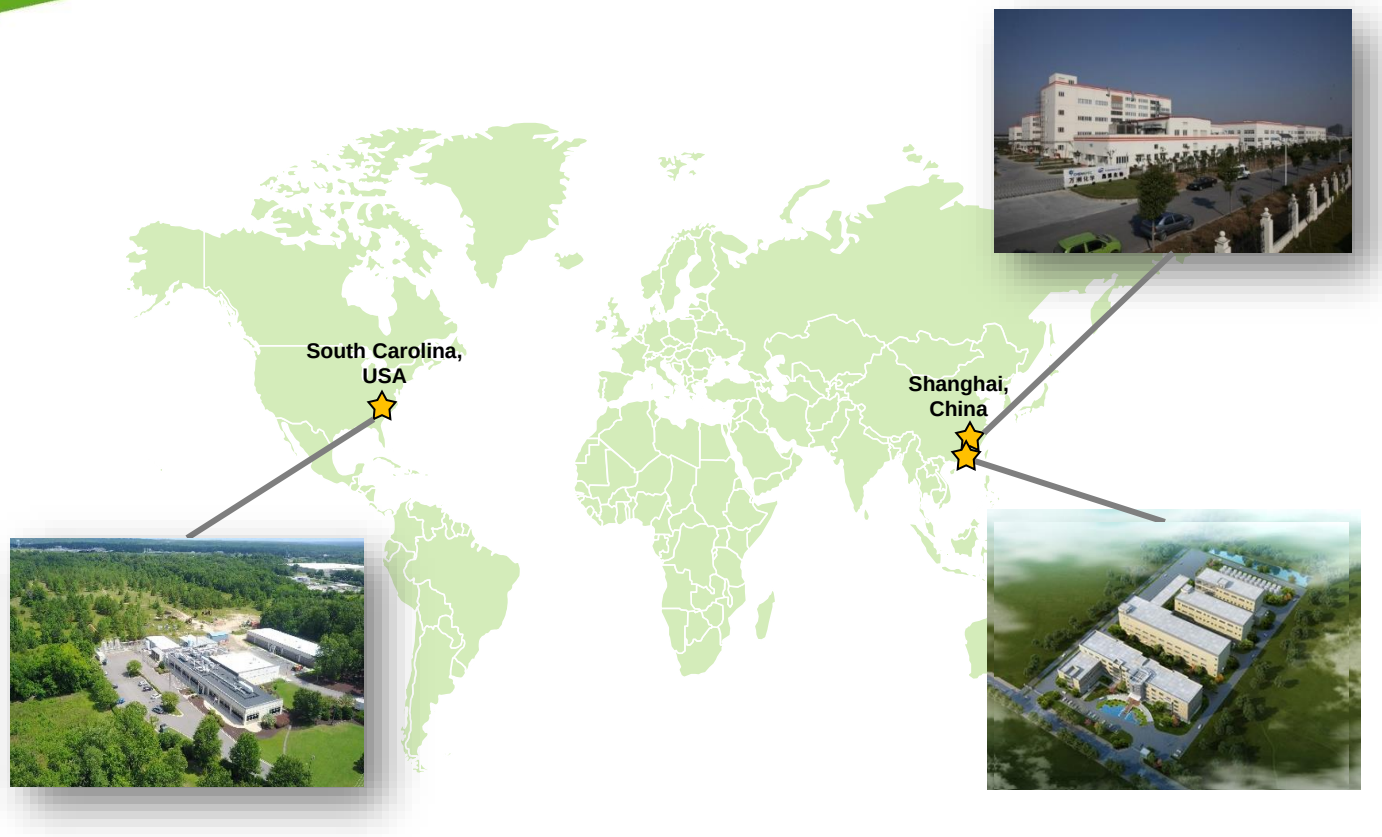
MW = 3994.8

Final Purified Chlorotoxin

Disulfide Rich Peptide Summary

- cGMP synthesis of complex disulfide-rich peptides
- Commercial synthesis of plecanatide at quantities exceeding 100kg/annum
- Innovative approaches to large scale production including SPPS, hybrid and classical synthesis.

AmbioPharm Inc. with 3 sites serving the world



Shanghai expansion to be completed by Q3 2020

"Green Chemistry" : solvent recovery system for main solvents DMF, MTBE and acetonitrile

Waste water treatment|WWTP

Workshop 1: Hydrogenation reactor room & solvent recovery system

Workshop 2:
S to N: synthesis suites(pilot scale);
E to W:1st-3rd floors, synthesis suites(large scale);
4th floor: purification suites;

Workshop3: 1st-3rd floors, synthesis suites (pilot & large scale)
4th floor: purification suites;

ADM Building(including R&D centers)

Shanghai expansion to be completed by Q3 2020

October 2019

January 2020

North Augusta Expansion

Largest peptide API manufacturing capacity

1 Meter HPLC column and 1,000 L lyophilizer will be installed by Q2 2020

Synthesis

Pilot Scale:
Solid phase 1L to 200L
Solution phase 10L to 200L

Commercial Scale:
Solid phase up to 3,000L
Solution phase up to 5,000L

Purification

Preparative HPLC Columns:
5, 8, 15 and 20cm
30cm
45cm
60cm
100cm* (in 2020)

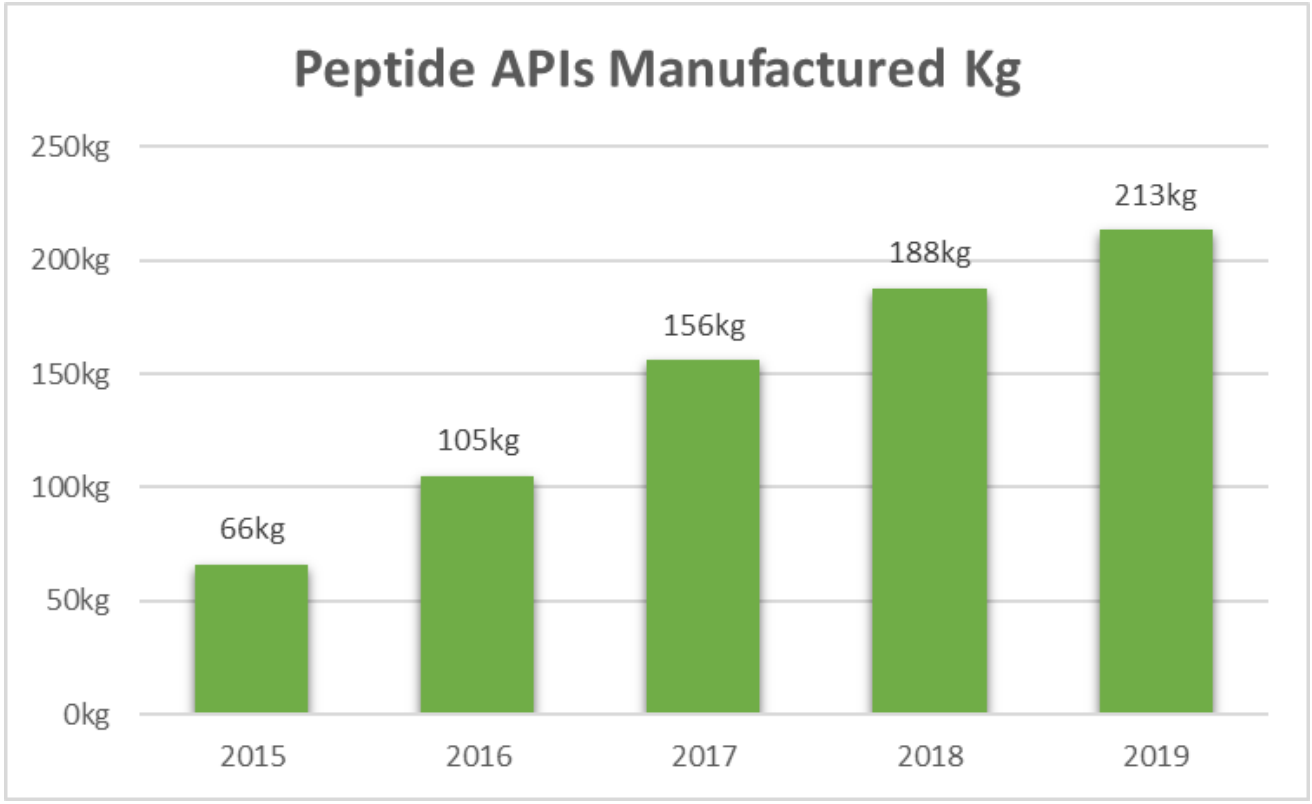
Lyophilization

Multiple Manifold lyophilizers:
Tray lyophilizers: 100L, 200L, 400L, 800L & 1000L* (in 2020)

QC Laboratory

HPLC/UPLC
GC
LC-MS
IC
SEC
KF
Nitrogen Content
Amino Acid Analysis
Endotoxin and Bioburden

Accelerated demand from our customers



AmbioPharm Inc: Complex Peptides are our Forte

- AmbioPharm is a commercial supplier of peptide cGMP-grade APIs.
- We routinely handle very complex disulfide-rich peptides from inception R&D (mg) to commercial launch (>100kg).
- We are uniquely positioned to offer exceptional service for very economical rates due to our business model encompassing both China and USA.