

Solid-Phase Synthesis of the Cys-rich Peptide Linaclootide

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INTRODUCTION

Linaclootide is a 14-residue peptide currently undergoing phase II clinical trials for the treatment of gastrointestinal diseases such as chronic constipation (CC). Linaclootide, which can be administered orally, is an agonist of the guanylate cyclase type-C receptor found in the intestine. From a structural point of view, this small peptide presents a constrained structure with the presence of three disulfide bridges between Cys1-Cys6, Cys2-Cys10, and Cys5-Cys13.

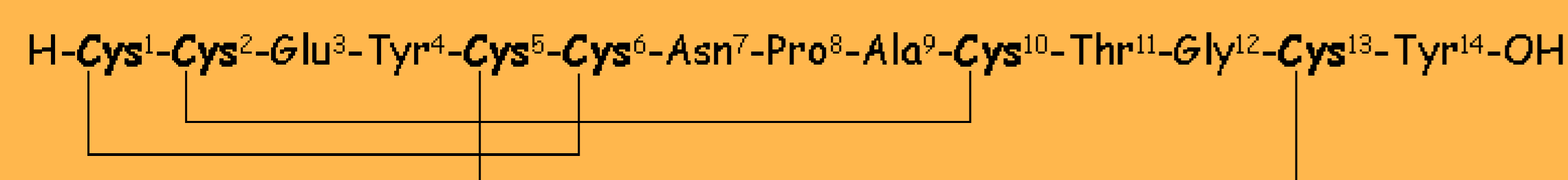


Figure 1. Structure of Linaclootide

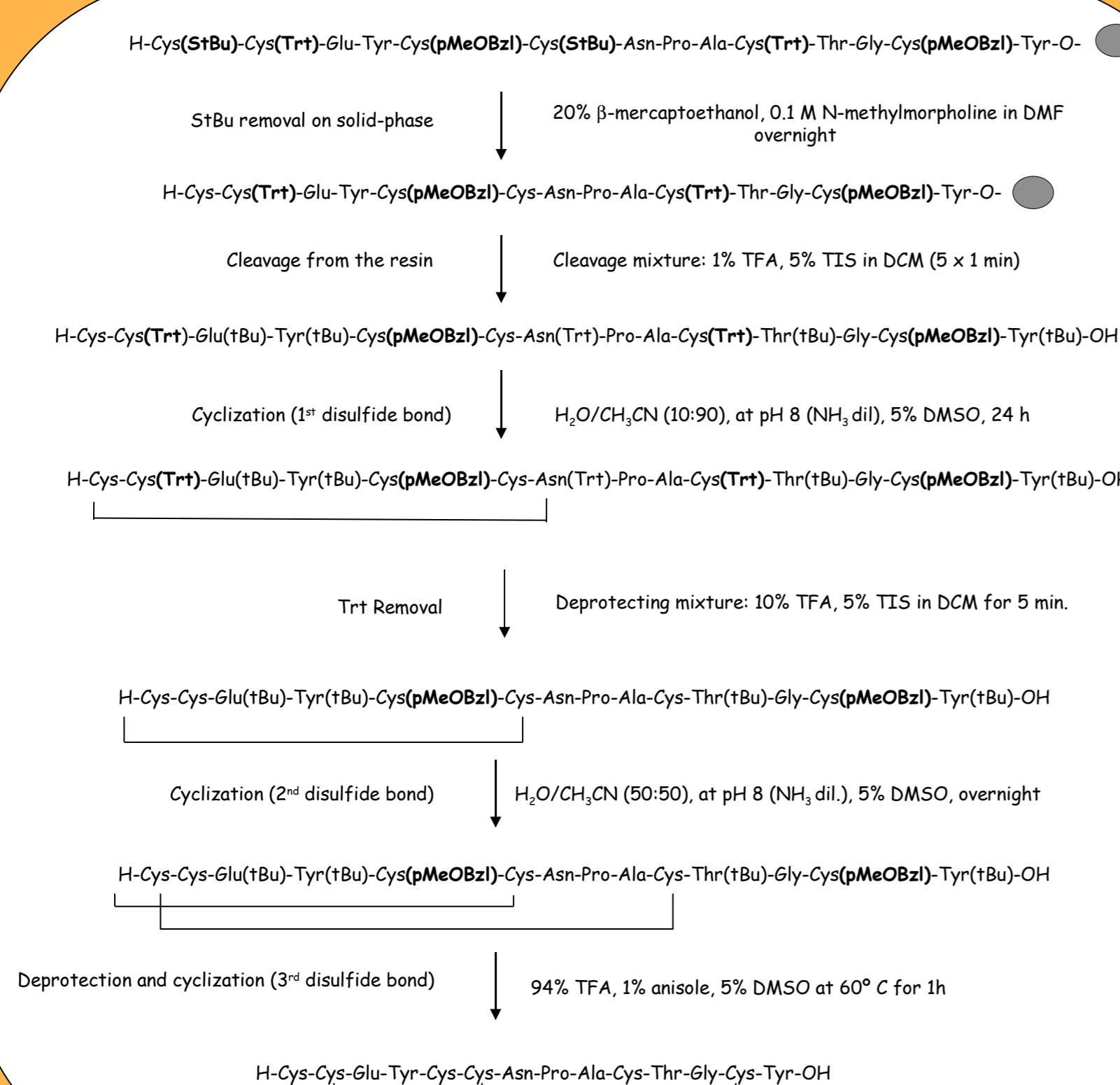
OBJECTIVES

In order to achieve the large amounts required for a marketed peptide, an efficient synthesis needs to be attained. To optimize the synthesis, its fundamental limitations need to be determined and addressed. In the case of Linaclootide, the key points are related to the numerous Cys residues (some of them consecutive) present in the peptide, for two reasons: the potential risk of racemization upon assembling the linear chain, and the misfolding of the three disulfide bridges. To address these points, the concourse of distinct protecting groups and folding conditions as well as the analysis of the disulfide bridges in the final folded peptide has been studied and will be discussed in this presentation.

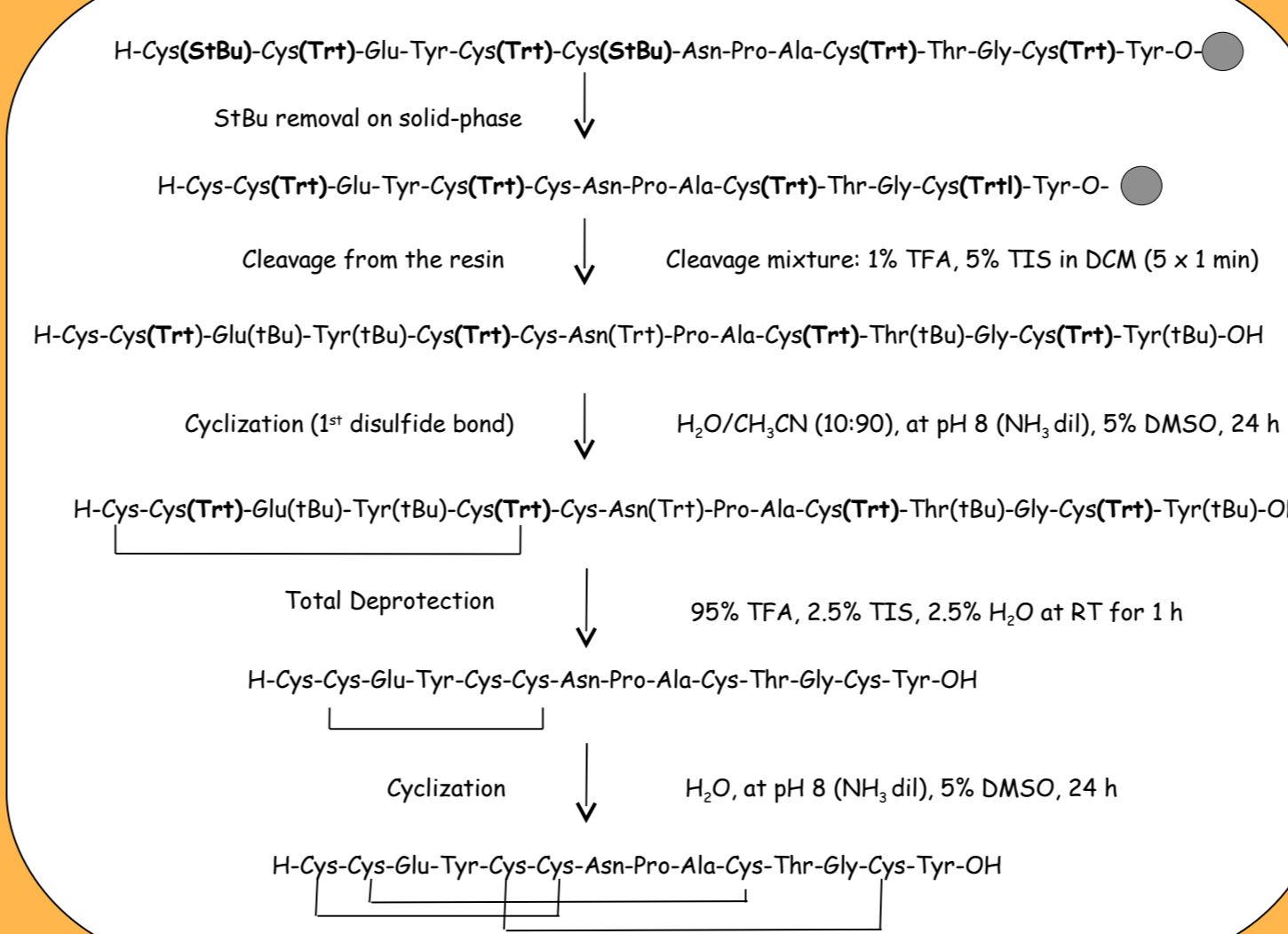
STRATEGIES

Several strategies have been examined using distinct Cys-protecting groups (StBu, Acm, Trt, Mmt, pMeOBzl) and different resins (Wang and CTC), in addition to performing disulfide formation in solid-phase and solution. The Acm group is not compatible with Linaclootide and disulfide bridges cannot be formed in a solid-phase mode. First, a total regioselective method (2+2+2) and semi-regioselective method (2+4) were tried. In parallel, a random/thermodynamic strategy was also investigated (6 Trt). Lineal peptides were synthesized manually using DIPCDI and HOBt in DMF for 1 h at RT to incorporate all the Cys residues. Other amino acids were coupled using HCTU and DIEA in DMF for 1 h at RT.

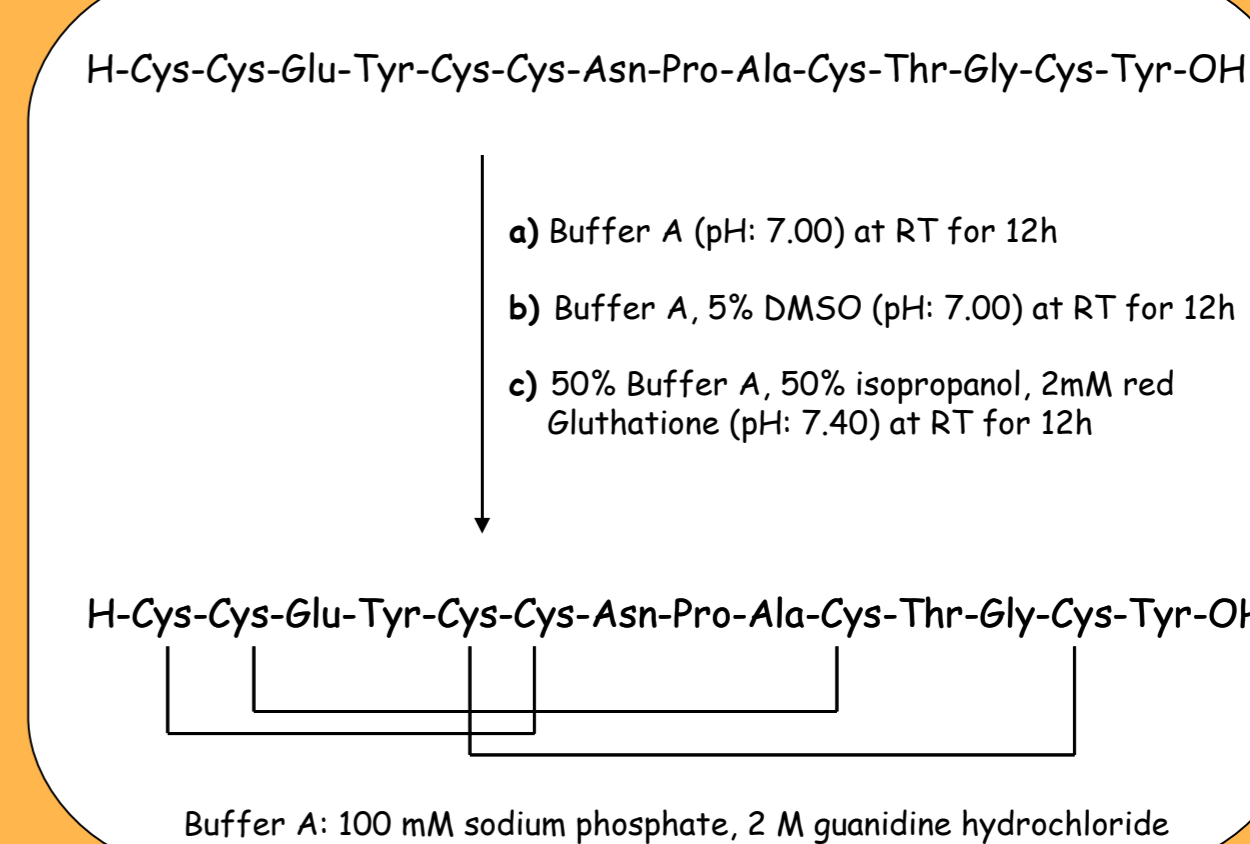
2 StBu + 2 Trt + 2 pMeOBzl Strategy



2 StBu + 4Trt Strategy



6 Trt Strategy



In the 2 +2 +2 Strategy, the Cys5-Cys13 disulfide bond was not easy to obtain in the first step and one of the StBu groups was difficult to remove when it was in the Cys2-Cys10 positions. The best results were achieved with Cys1-Cys6; StBu groups were removed without difficulty and the disulfide bond was the easiest to obtain in the first step.

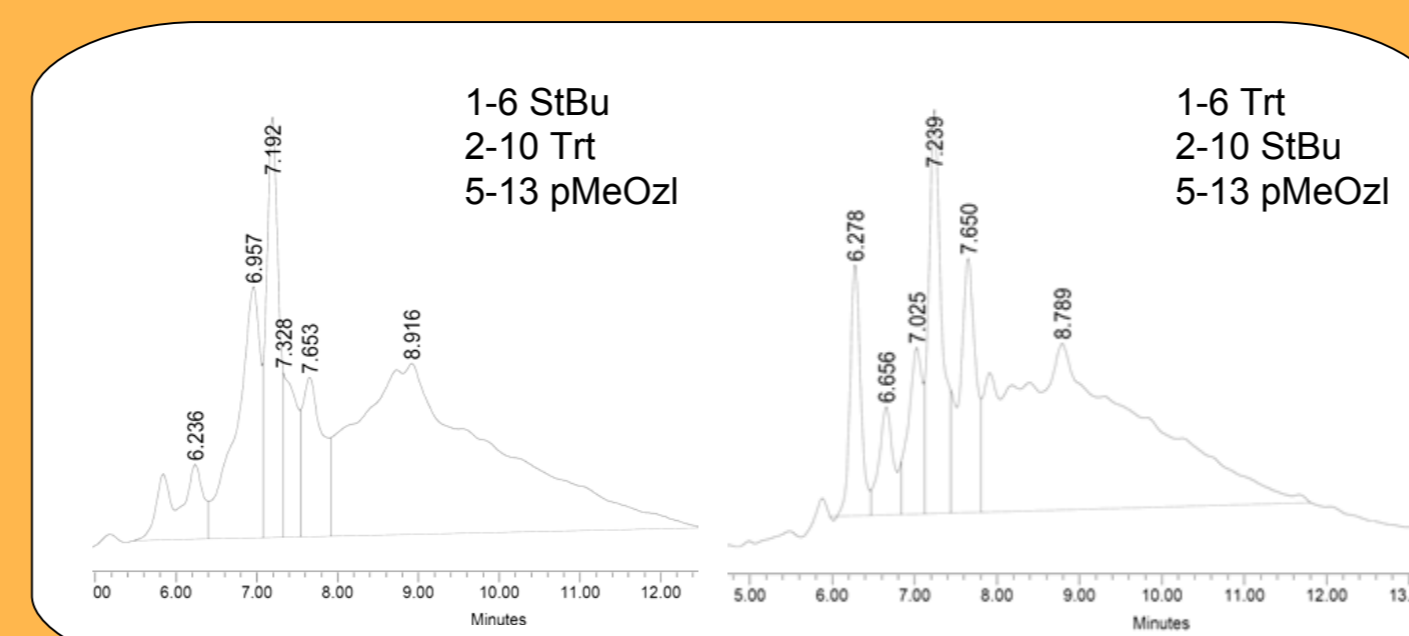


Figure 2. Chromatographic profile of the completely oxidized peptides from 2 StBu + 2 Trt + 2 pMeOBzl Strategy.

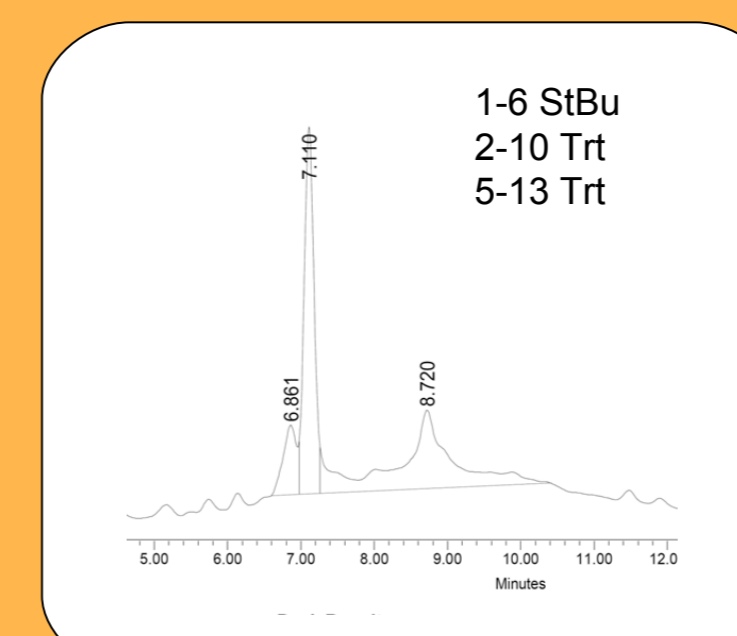


Figure 3. Chromatographic profile of the completely oxidized peptide from 2 StBu + 4 Trt Strategy.

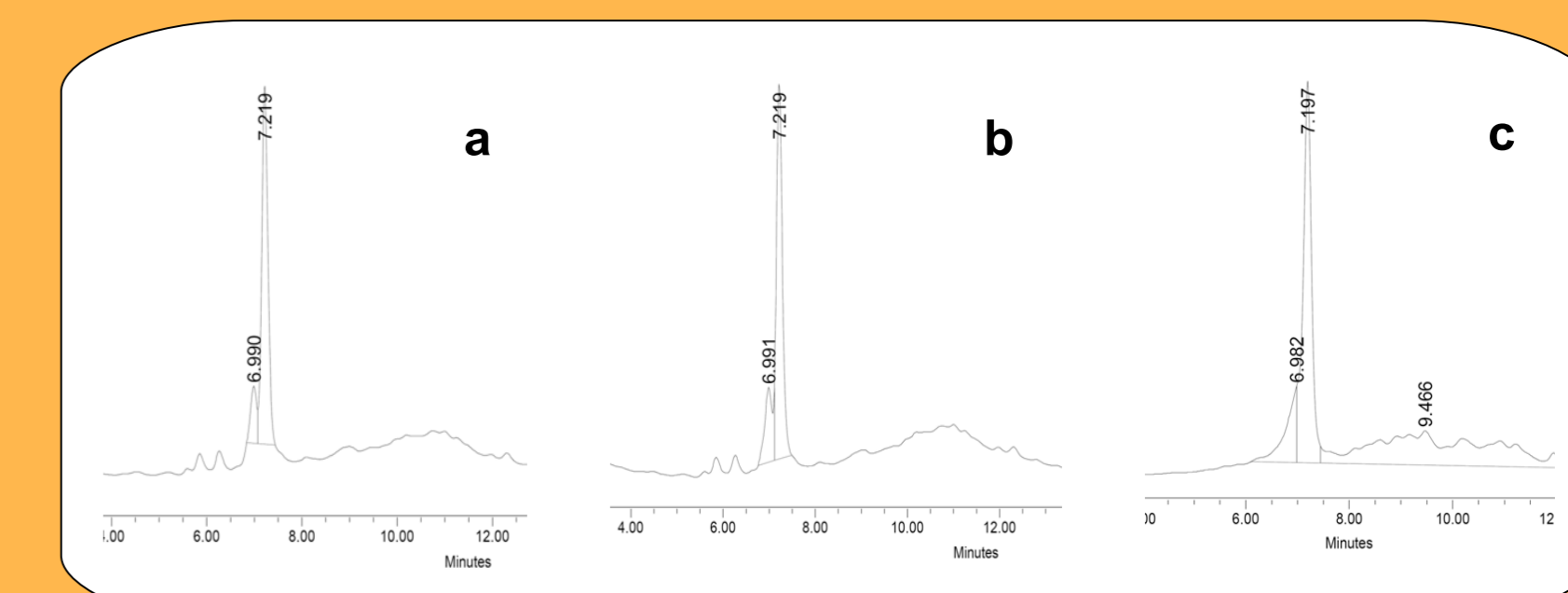


Figure 4. Chromatographic profile of the completely oxidized peptides from 6 Trt Strategy.

DISULFIDE BOND ANALYSIS

Linaclootide contains 14 aa with 6 Cys residues, which can form 15 theoretically possible disulfide structures. In order to confirm that the proper isomer (Cys1-Cys6, Cys2-Cys10, Cys5-Cys13) had been obtained following the 6 Trt Strategy, a disulfide bond analysis was carried out using a modified method of Wu and Watson^[1].

The masses of the resulting peptide fragments are related to the location of the paired cysteines that had undergone reduction, cyanilation, and cleavage. Fragments expected from the Cys1-Cys6 disulfide bond had a mw of 643.16 and 955.30; fragments expected from the Cys2-Cys10 disulfide bond had a mw of 120.04, 570.16 and 925.29; and fragments expected from the Cys5-Cys13 disulfide bond had a mw of 309.08, 515.15 and 791.25.

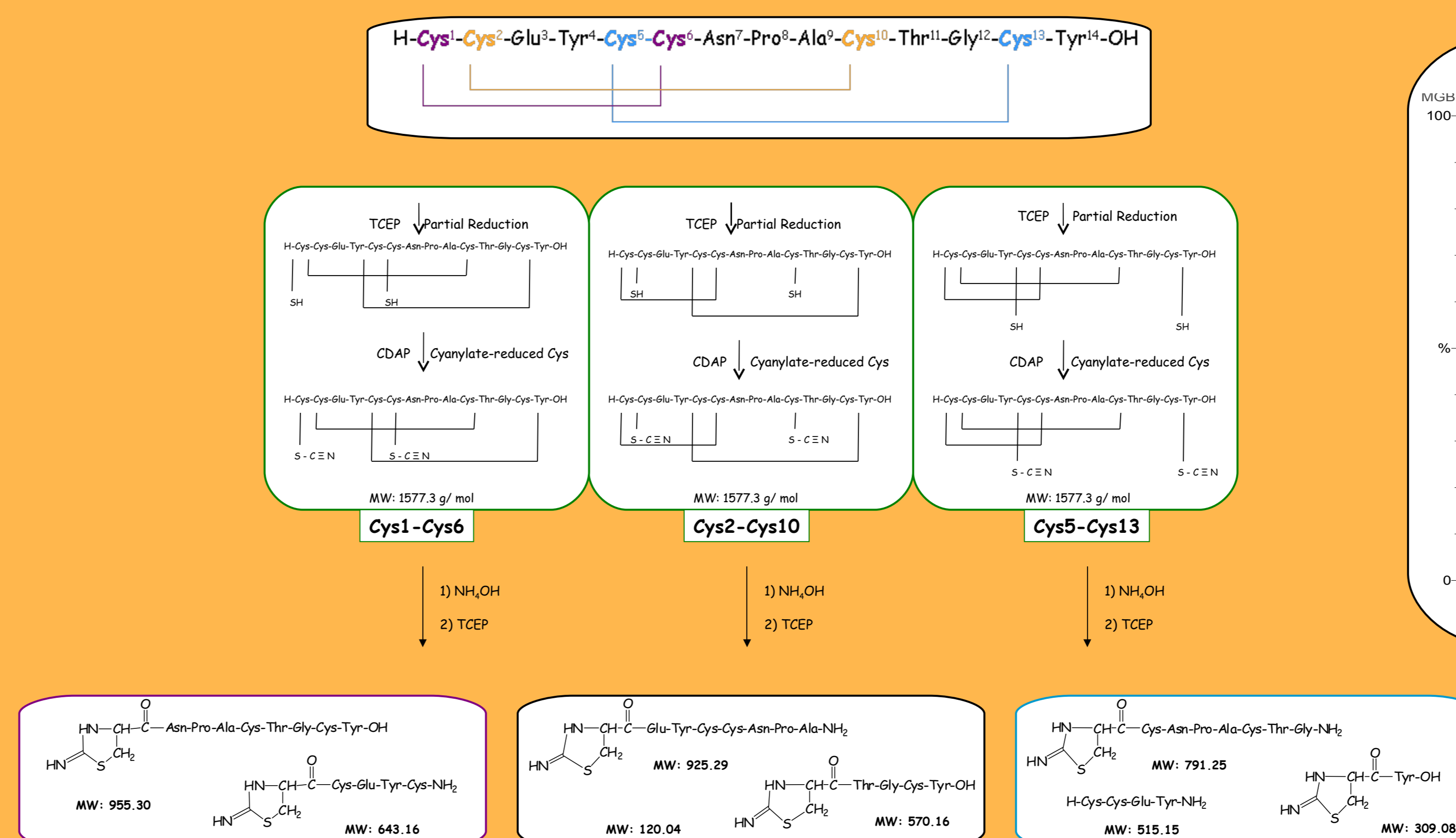


Figure 5. Theoretical analysis (expected masses) of Linaclootide.

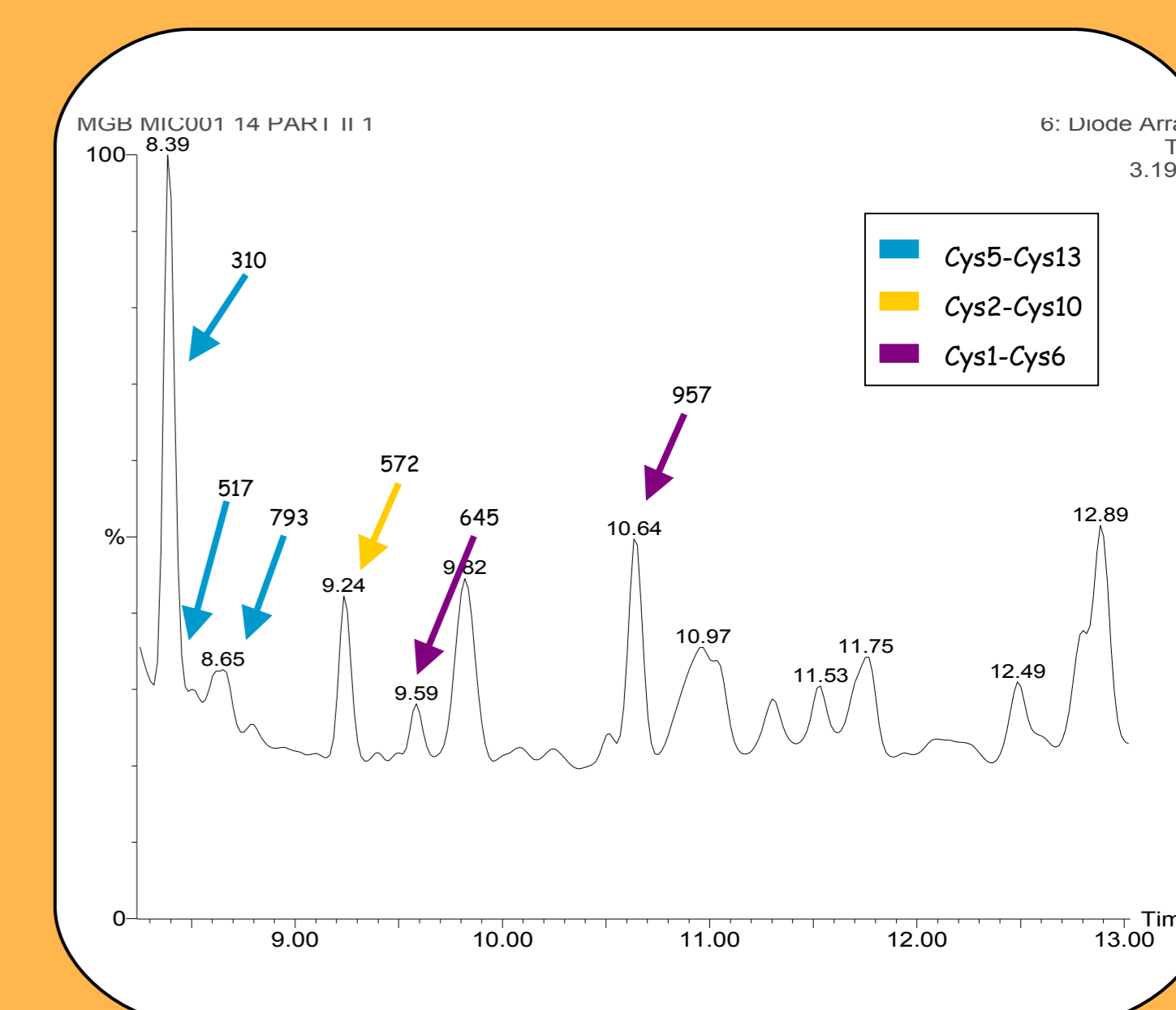


Figure 6. HPLC-ES(+) of the disulfide bond analysis.

CONCLUSIONS

The 6 Trt strategy allowed to obtain the properly and completely oxidized peptide. The disulfide bond analysis confirmed that the suitable isomer (Cys1-Cys6, Cys2-Cys10 and Cys5-Cys13) had been achieved following the random strategy.

The removal of StBu depends not only on its positions in the sequence, but also on the protecting groups of the nearest residues. In the 2 + 4 Strategy the Cys1-Cys6 disulfide bond gives the correct conformation to obtain the properly and completely oxidized peptide.

LITERATURE

[1] Wu, J. & Watson, J.T. *Methods Mol. Biol.* 2002, 194:1-22