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Short communication

Characterization of eptifibatide during drug formulation stability assays

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Abstract

The objective of the study was to determine the identity of a new impurity detected in HPLC chromatograms of research samples of eptifibatide manufactured by a new process and formulated into drug product. The identification of the unknown impurity was required in order to understand the mechanism of its formation. The analysis was performed by using tandem mass spectrometers coupled with a reversed-phase gradient HPLC system. The unknown compound was then structurally elucidated by matrix-assisted laser desorption ionization (MALDI) tandem mass spectrometry. The mass spectrometric results showed that the protonated molecular ion of the unknown compound was *m/z* 862.3347 with molecular formula: $C_{36}H_{52}N_{11}O_{10}S_2$. The unknown compound was a linear peptide and was related to Asp-clipped eptifibatide. It was formed from Asp-clipped eptifibatide by the reaction of the amino group of tryptophan moiety with formaldehyde followed by electrophilic attack on the nitrogen of indole.
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Keywords: Integrilin; Eptifibatide; Drug manufacture control; Impurity; MALDI-TOF mass spectrometry

1. Introduction

Eptifibatide (Integrilin™) is a cyclic heptapeptide containing six amino acids and one mercaptopropionyl (des-amino cysteinyl) residue, as shown in Fig. 1. An interchain disulfide bridge is formed between the cysteine amide and the mercaptopropionyl moieties. Chemically it is N^6-(aminoiminomethyl)-N^2-(3-mercapto-1-oxopropyl-L-lysylglycyl-L-α-aspartyl-L-tryptophyl-L-prolyl-L-cysteinamide, cyclic disulfide. Eptifibatide is compounded to produce INTEGRILIN® Injection, which is used for the treatment of patients with coronary syndrome [1].

It is well known that platelets play a pivotal role in acute coronary syndromes [2–4]. Plaque rupture exposes highly thrombogenic components that induce platelet activation and initiate coagulation. Platelet activation involves a conformational change in the membrane glycoprotein receptors, and eptifibatide works by inhibition of platelet glycoprotein IIb/IIIa receptors. INTEGRILIN® Injection is a clear, colorless, sterile, non-pyro-

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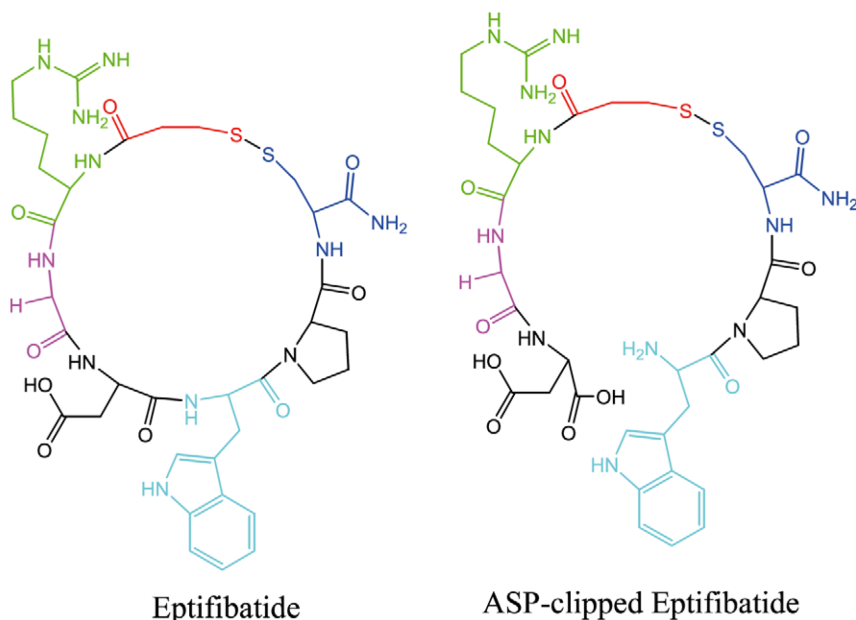


Fig. 1. Structures of eptibibatide and Asp-clipped eptifibatide.

genic solution for intravenous (IV) use. Each 100-ml vial contains 0.75 mg/ml of eptifibatide and 5.25 mg/ml citric acid and sodium hydroxide to adjust the pH to 5.25. The eptifibatide peptide is produced by solution-phase peptide synthesis, and is purified by preparative reverse-phase liquid chromatography and lyophilized. The control of impurity levels throughout product manufacturing development is critical and new, unqualified impurities must be removed and/or prevented from being formed.

During manufacturing, an eptifibatide degradant, Asp-clipped eptifibatide (Fig. 1), is formed from the peptide-bond hydrolysis between aspartic acid and tryptophan residues. An unknown peak, however, was observed in HPLC chromatograms of research samples of formulated eptifibatide (2 mg/ml eptifibatide in 25 mM citrate, pH 5.35). The levels of the unknown peak from the drug product, with the retention time at about 21 min, start to increase with the decrease of Asp-clipped eptifibatide (Fig. 2) after approximately 2 weeks storage at 25 °C, which suggested that the unknown peak is structurally related to Asp-clipped eptifibatide. After 1 month storage at 25 °C, approximate 60%age of Asp-clipped eptifibatide is degraded into the undesired unknown compound. The unknown peak was fractionally collected and further analyzed by mass spectrometry. The objective of the study was to characterize and identify the unknown by novel tandem mass spectrometry. Recently advances in matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI TOF MS) development revolutionizing bioanalytical chemistry by facilitating very efficient molecular identification have make the technology as a very powerful technique for the analysis of biomolecules [5,6].

2. Experimental

2.1. Materials and methods

HP1100 series controlled by HP Chemstation was used for separation. The HPLC system consists of G1322A Degasser, G1312A Bin pump and G1315A DAD. An isocratic pump from HP (G1310A isopump) was also used for separation. The mass spectrometry analyses were performed by using QSTAR pulsar with ESI- and MALDI ion sources (Applied Biosystems, Inc.).

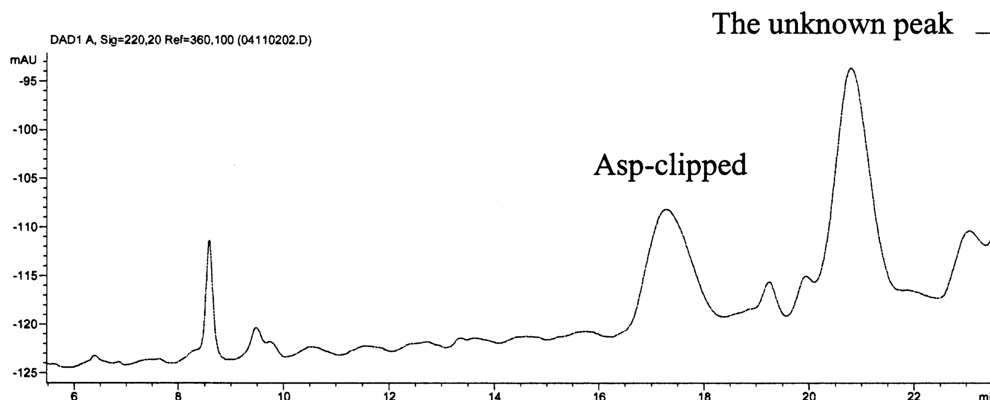


Fig. 2. The HPLC chromatograms of formulated eptibibatide (2 mg/ml eptifibatide in 25 mM citrate, pH 5.35) after 1 month storage at 25 °C.

HPLC grade water and acetonitrile, formic acid (Baker PCS reagent), hydrochloric acid (ULTREX II Ultrapure) and ammonium hydroxide (ACS reagent) were purchased from J.T. Baker. Dithiothreitol (DTT) was from Pierce (Rockford, IL). MALDI-quality Matrix Solution (Alpha-cyano-4-hydroxycinnamic acid) was purchased from Agilent.

2.2. Liquid chromatography

Separation of the unknown was done on Nucleosil C18 column (Alltech Associate, Inc., 5 um, 4.6 × 250 mm) with two solvent systems. The system consists of (A) 0.1% formic acid in water/acetonitrile (9:1), and (B) 0.1% formic acid in acetonitrile. The gradient was from 95% A and 5% B to 89% A and 11% B in 30 min at a flow rate of 1 ml/min.

2.3. Mass spectrometry

Samples for MALDI were prepared as the following: About 0.5 ul of MALDI quality matrix solution was spotted on a plate followed immediately by spotting 0.5 ul sample solution on top of the matrix solution. The plate was dried with warm air before MS analysis. Nitrogen laser at 337 nm was used for ionization. MS conditions were: curtain gas setting 25, declustering potential 5 V, focusing potential 175 V, declustering potential (2) 10 V, and collision energy 80 V.

2.4. Cleavage of disulfide bond

DTT was used for cleavage of disulfide bond [7]. Eluent of the unknown peak was collected (HCl solvent system) and then reduced by DTT. The procedures for DTT reduction were: 15 ul HPLC eluent of the unknown peak was mixed with 10 ul NH_4HCO_3, 4 ul NH_2OH (180 mM) and 4 ul DTT (1 M). The mixture was stayed at room temperature for about 10 min before it was analyzed by MALDI-TOF MS.

3. Results and discussions

3.1. Mass spectrometry

The unknown peak collected from HPLC was analyzed by MALDI-TOF MS and MALDI-MS/MS. The MALDI-MS results displayed a single base peak in the mass spectrum from the collected chromatographic unknown-peak fraction. The measured protonated molecular ion of the unknown peak was 862.3347 Da which gives the predictive molecular formula of $C_{36}H_{52}N_{11}O_{10}S_2$ (calculated mass 862.3340, the difference was about 5 mDa or about 6 ppm). The mass of the unknown peak was 30 and 12 Da higher than that of eptifibatide and Asp-clipped eptifibatide, respectively.

The acute coronary syndrome drug, eptifibatide, is cycled through disulfide-bond linkage between

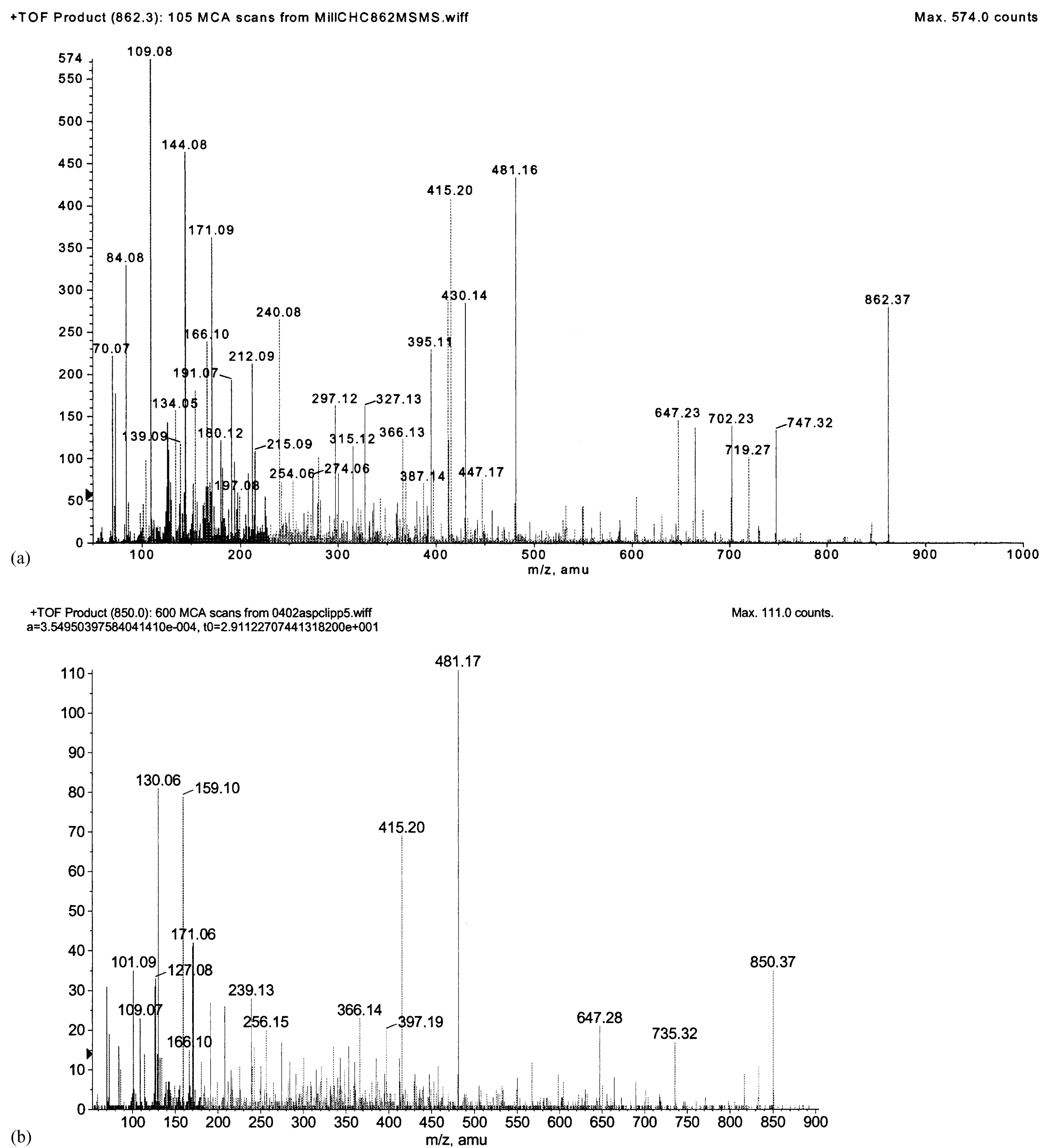


Fig. 3. The MALDI-TOF MS/MS spectrum of the (a) unknown with m/z 862.33, (b) Asp-clipped eptifibatide ($m/z = 850.37$).

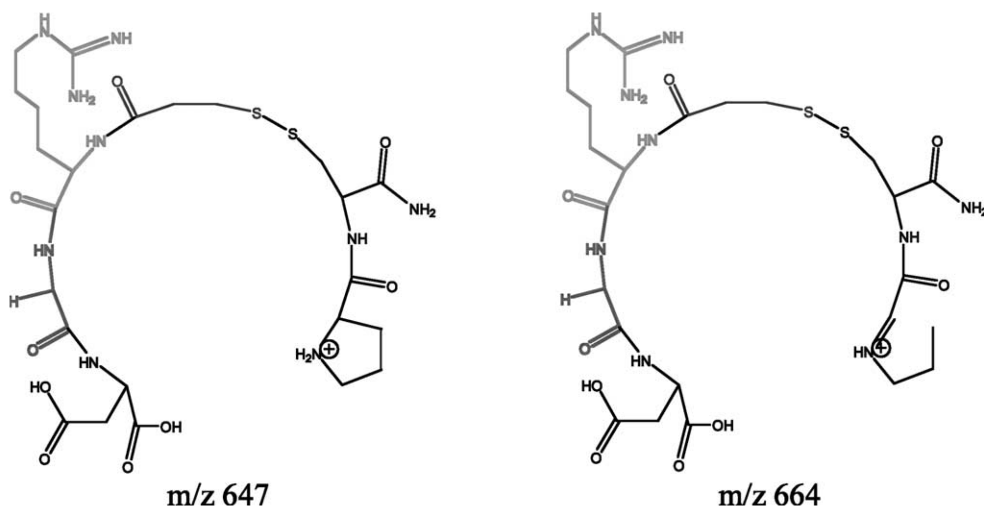
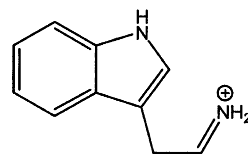
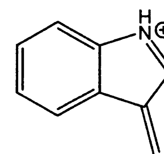


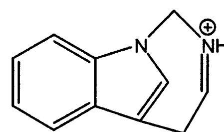
Fig. 4. The m/z 647 and m/z 664 fragment ions of Asp-clipped eptifibatide and unknown peak.

the cysteine amide and the mercaptopropionyl moieties. In order to determine whether the unknown is in cyclic form as eptifibatide or in linear form as Asp-clipped eptifibatide, the unknown was reduced with DTT and then analyzed by MALDI-TOF MS. The cyclic or linear structure of the unknown molecule may be determined if the protonated molecular ion of unknown compound under DTT reduction increases additional 2 Da from m/z 862 (disulfide) to m/z 864 (sulfhydryl) (in cyclic form as eptifibatide) or reduced into two smaller peptides (in linear form as Asp-clipped eptifibatide). The mass spectrum results showed that the base peak at m/z 864 was not presented, evidencing that the unknown was in linear structure as Asp-clipped eptifibatide.

The unknown structure may be further elucidated by MALDI-TOF MS/MS analysis (Fig. 3a) in comparison with the product ion spectrum of Asp-clipped eptifibatide (Fig. 3b). The product ion spectrum of the unknown displayed base peaks (m/z 664 and m/z 647, respectively) corresponding to protonated molecular ion (m/z 862) by a loss of mass 198 and the fragment loss of NH_3 from m/z 664. Both fragments m/z 664 and 647 were observed from the product ion spectra of Asp-clipped eptifibatide and unknown (Fig. 4). The m/z 664 ion is the fragment formed from the protonated Asp-clipped eptifibatide (m/z 850) by







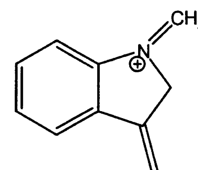


Fig. 5. Fragment ions of Asp-clipped eptifibatide and the unknown.

the loss of tryptophan moiety (186 Da). Therefore, the structure of the unknown possess the same amino acid residues: aspartate (Asp), glycine (Gly), homoarginine (Har), β-mercaptopropionic acid (Mpr), cysteine amide (Cys-NH_2) and proline (Pro) as in Asp-clipped eptifibatide (Fig. 1). The mass spectrum results suggested that difference in structure between the unknown and Asp-clipped eptifibatide resides is the tryptophan moiety. The

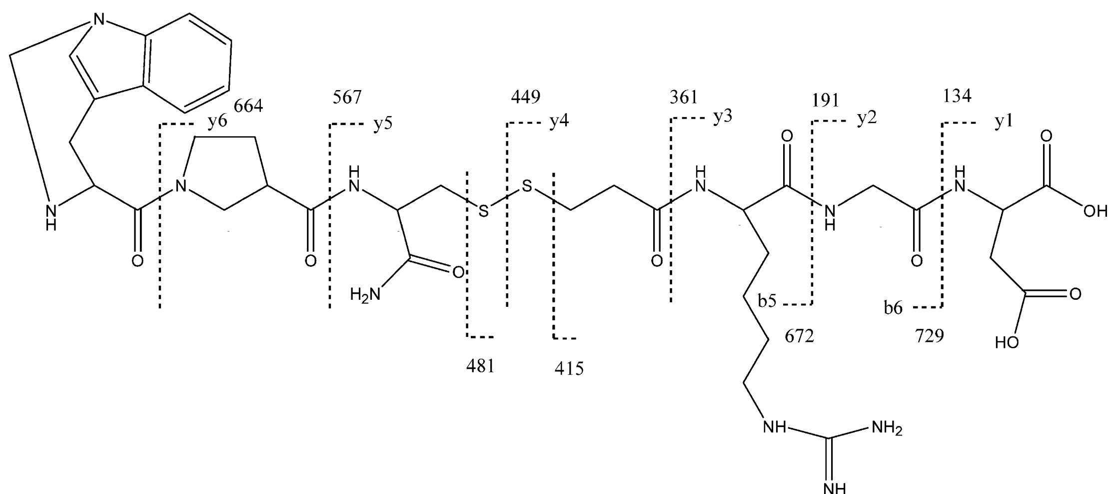


Fig. 6. Structure of the unknown.

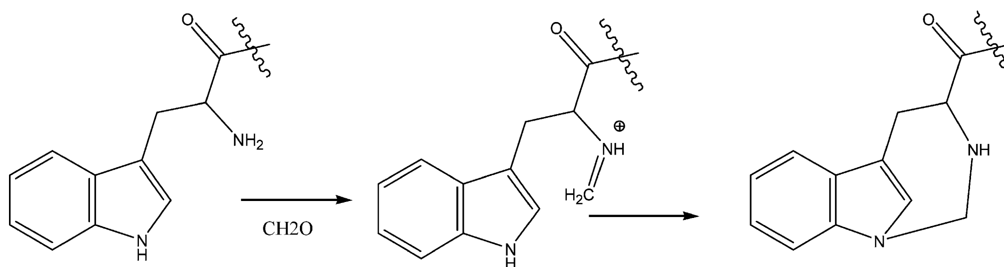


Fig. 7. The chemical formation pathway of the unknown.

mass of the modified tryptophan moiety of the unknown is 12 Da more than that of Asp-clipped eptifibatide.

It is consistent that the product ion spectrum of Asp-clipped eptifibatide showed the intense tryptophan residue ammonium ion at *m*/*z* 159, as shown structurally in Fig. 5. However, due to the modification of the tryptophan moiety, instead of *m*/*z* 159 from Asp-clipped eptifibatide, the fragment ion, *m*/*z* 171 (*m*/*z* 159+12), was detected in the MALDI-TOF MS/MS spectrum of the unknown. The structure of the modified ammonium ion of tryptophan at *m*/*z* 171 was suggested in Fig. 5. By the mass accuracy of the measurement, the calculated mass of the modified tryptophan ammonium ion is 171.0922, which matched the measured mass 171.0949 very closely. In addition, the product ion spectrum of Asp-clipped eptifibatide showed the fragment at *m*/*z* 130 (Fig. 3b) which was formed from the further fragmentation of the ammonium ion of tryptophan at *m*/*z* 159 (Fig. 5). Instead of the *m*/*z* 130 ion, the secondary product ion in the unknown sample at *m*/*z* 144 (Fig. 5) fragmented from *m*/*z* 171 was also observed in the MALDI-TOF MS/MS spectrum.

The fragmentation pattern of peptides depends on the collision energy, the number of charges being carried by the peptide and the amino acid sequence [8]. Generally, peptides were fragmented at the amide bonds and then produce b- and y-ion series [9]. In the MALDI-TOF MS/MS mass spectra, y-ions and b-ions were detected in the product ion spectrum of the unknown peptide (Fig. 6) which confirmed the peptide sequence of the unknown. However, the unknown peptide is not a typical peptide and contains modified amino acids, a carboxylic acid and a disulfide bond. The intense ions at *m*/*z* 481 and 415 were formed from

the cleavage of C–S bond; *m*/*z* 447 was formed by the cleavage of the disulfide bond and *m*/*z* 430 was formed from *m*/*z* 447 by a loss of NH_3; fragments at *m*/*z* 747 was formed by a loss of the side chain of homoarginine (115 Da).

3.2. Formation of the unknown

The formation pathway of the unknown was proposed in Fig. 7. The first step was the reaction of tryptophan residue from Asp-clipped eptifibatide with formaldehyde to form iminium bond which is an effective electrophile. The second step was the electrophilic attack on the indole nitrogen to form the unknown. The chemistry of indoles was well studied and the reaction of indole nitrogen with formaldehyde and amine was recorded in [10,11].

4. Conclusion

The unknown compound from a research sample of eptifibatide was analyzed by high resolution MALDI-TOF MS/MS mass spectrometry. The protonated molecular ion of the new compound was *m*/*z* 862.3347 Da showing the molecular formula of $C_{36}H_{52}N_{11}O_{10}S_2$. The unknown peptide was from the tryptophan moiety in Asp-clipped eptifibatide reacting chemically with formaldehyde.

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