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Side-chain assisted ligation in protein synthesis

K. S. Ajish Kumar, Ziv Harpaz, Mahmood Haj-Yahya, Ashraf Brik *

Department of Chemistry, Ben-Gurion University of the Negev, Beer Sheva 84105, Israel

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ABSTRACT

Chemical ligation methods for the assembly of functional proteins continue to advance our basic understanding of protein structure and function. In this work, we report on our progress towards the full synthesis of HIV-1 Tat utilizing our newly developed ligation method; side-chain assisted ligation. The HIV-1 Tat was assembled from three fragments wherein the two thioester peptides were synthesized efficiently using the side-chain anchoring strategy following Fmoc-SPPS. The side-chain assisted ligation step was efficient and provided the ligation product in good yield. Following this step, native chemical ligation was used to fully assemble the HIV-1 Tat protein. Although the removal of the auxiliary in small peptides was straightforward, in the case of HIV-1 Tat this step was inefficient thus hampering the completion of the synthesis.

Chemical protein synthesis allows the production of functional proteins with full control on the covalent structure wherein the contribution of each atom on the protein structure and activity could in principle be evaluated. Native Chemical Ligation (NCL) continues to lead as the method of choice for assembling the peptide fragments, in aqueous media, to generate the full length polypeptide, which corresponds to folded protein.[1] To facilitate the thiol capture step in NCL, the presence of a Cys residue at the ligation junction is crucial. This limits the use of NCL in the synthesis of a variety of protein systems where a Cys residue either cannot be found or its location is not in a synthetically useful position. As a result, several important developments in the field of chemical ligation were reported aiming to overcome the use of the Cys residue at the ligation junction.[2] Advances in the field include for example, the use of conformationally assisted ligation,[3] removable auxiliaries,[4] Staudinger ligation,[5] thiol alkylation,[6] desulfurization methods,[7] and sugar-assisted ligation (SAL).[8]

Recently, we reported a new method named Side-Chain-Assisted Ligation (SCAL) to assist ligation at AA_2-AA_1-Ser, AA_2-AA_1-Glu, and AA_2-AA_1-Asp junctions (Scheme 1).[9] In this approach, a cyclohexane-based auxiliary linked to Ser, Asp or Glu side chains is used to facilitate the thiol capture step. This in return, increases the effective molarity of the reactive groups (i.e., amine and thioester) promoting an efficient and chemoselective aminoacyl transfer.[8b,9] In SCAL and in analogy to SAL the cyclohexane moiety acts as a template to position the reactive groups in a close proximity. Replacement of the thiol modified cyclohexane with mercaptoacetic acid leads to only 15–20% of the ligation product, despite extending the reaction time to 24 h. This indicates the important role of cyclohexane as a rigid template, which positions the reactive groups in a close proximity.[10]

For any ligation method to be useful in protein synthesis it must be tested in the assembly of large peptide fragments under aqueous media and in a relatively low concentration (1–5 mM) to achieve good solubility of the large peptide fragments. Here, we report that SCAL stood this test and was found to be useful in the ligation of two large peptide fragments corresponds to the C-terminal 59-mer of HIV-1 Tat protein. Subsequently, NCL was used to ligate the resulted peptide to the N-terminal fragment furnishing the full-length HIV-1 Tat polypeptide.

HIV-1 Tat is a regulatory protein containing 86 residues that is involved in the life cycle of the HIV-1 virus.[11] This protein plays an essential role in viral replication and infectivity due to its ability of increasing the HIV gene expression. In its absence, the virus cannot replicate within the host cell despite being able to infect the cell. HIV-1 Tat also has a direct pathogenic role, which penetrates new cells and acts as toxin resulting in apoptosis upon release from the infected cells. Moreover, HIV-1 Tat is known for binding several other proteins such as cellular kinases and the tetramerization domain of the tumor suppressor protein p53.[12] To the best of our knowledge, HIV-1 Tat has never been synthesized using the ligation strategy, although attempts towards direct peptide synthesis of the full length has been tested.[13]

Our strategy for the total synthesis of HIV-1 Tat is shown in Scheme 2. Despite the presence of seven Cys residues in the HIV-1 Tat sequence all of these are located towards the N-terminal, thus limiting the use of NCL for the full assembly of this protein.

* Corresponding author. Fax: +972 8 6472944.
E-mail address: abrik@bgu.ac.il (A. Brik).

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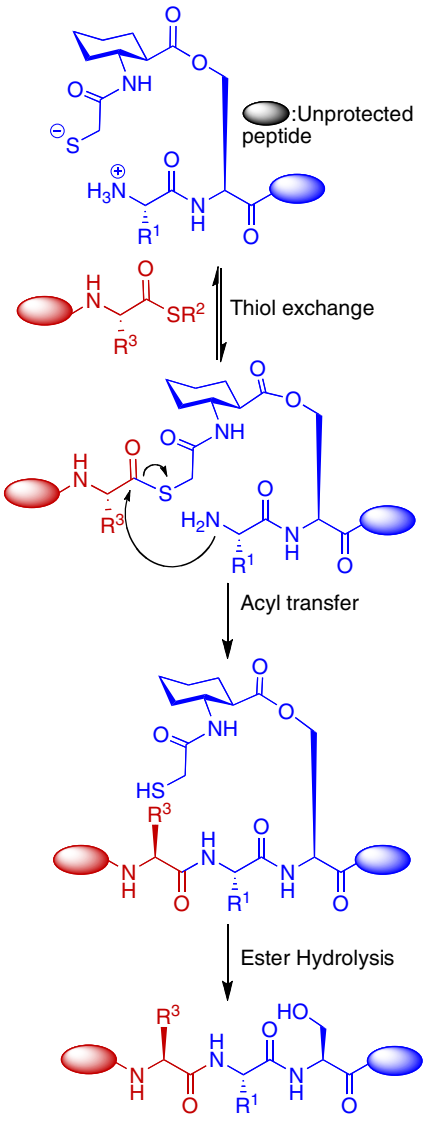
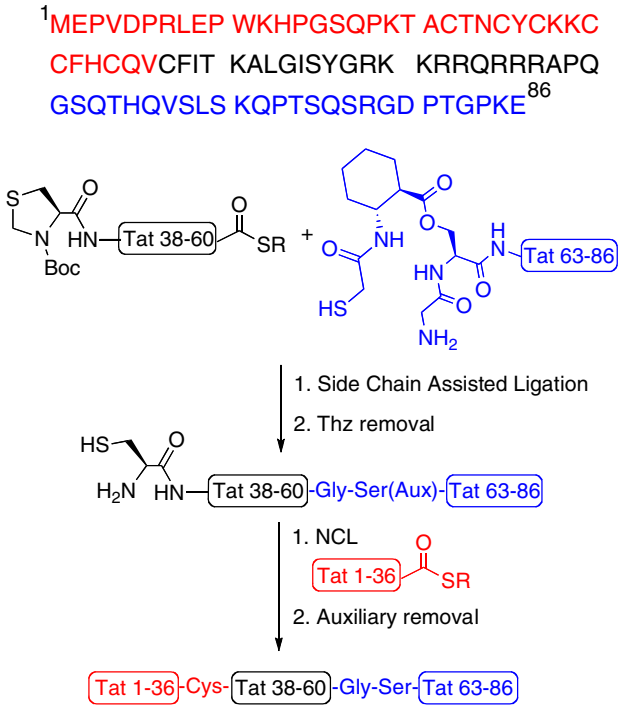


Scheme 1. Proposed mechanism of SCAL.

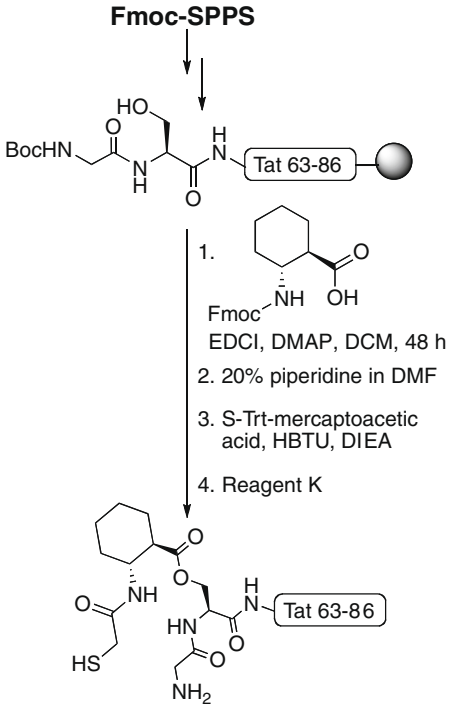
The presence of the Ser residue at position 62 and the Gly-Gln-Ser junction before should allow the use of SCAL to facilitate ligation between the HIV-1 Tat(37–60) and HIV-1 Tat(61–86) peptides. After SCAL step, the N-terminal protecting group will be removed to permit the use of NCL to ligate HIV-1 Tat(37–86) and the N-terminal HIV-1 Tat(1–36) peptides. Following these synthetic sequences, the auxiliary could be removed under basic conditions. Alternatively, this step could be carried out after the completion of the first ligation.

To achieve our synthetic strategy as depicted in Scheme 2 we started with the synthesis of HIV-1 Tat(61–86) bearing the side chain auxiliary on the Ser side chain (Scheme 3). Fmoc-SPPS strategy was adopted to synthesize this peptide in which unprotected Fmoc-Ser-OH was coupled at position 62 followed by Boc-Gly-OH as the N-terminal amino acid. Esterification of the Fmoc-aminocyclohexyl carboxylic acid with the Ser side chain was accomplished using EDCI, DMAP coupling conditions, followed by Fmoc removal to allow the attachment of the S-Trt-mercaptoacetic acid. Final deprotection and release from solid support using reagent K (Fig. 1), followed by HPLC purification afforded the desired HIV-1 Tat(61–86)-auxiliary peptide in ~30% yield.



Scheme 2. Synthetic strategy for HIV-1 Tat combining SCAL and NCL.

The syntheses of the middle segment and N-terminal peptide thioesters were carried out successfully using Fmoc-SPPS coupled with the side-chain anchoring strategy (Scheme 4).[14] Several methods were reported to prepare Fmoc thioester,[15] most notably, the recent elegant strategy that was reported by Dawson and coworkers based on the formation of C-terminal *N*-acylurea functionality.[16] However, it caught our attention the presence of Gln



Scheme 3. SPPS of HIV-1 Tat(37–60) bearing the side-chain auxiliary.

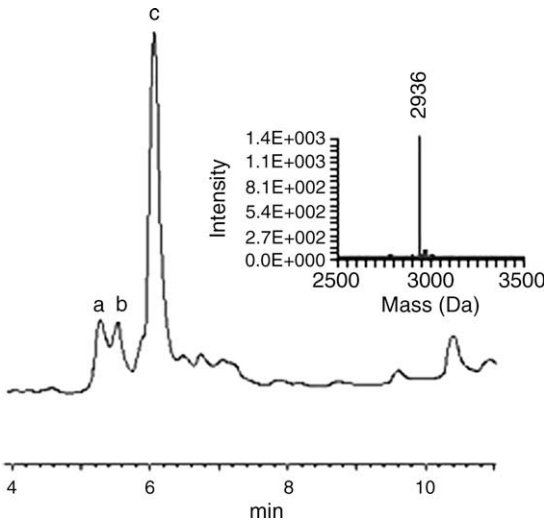
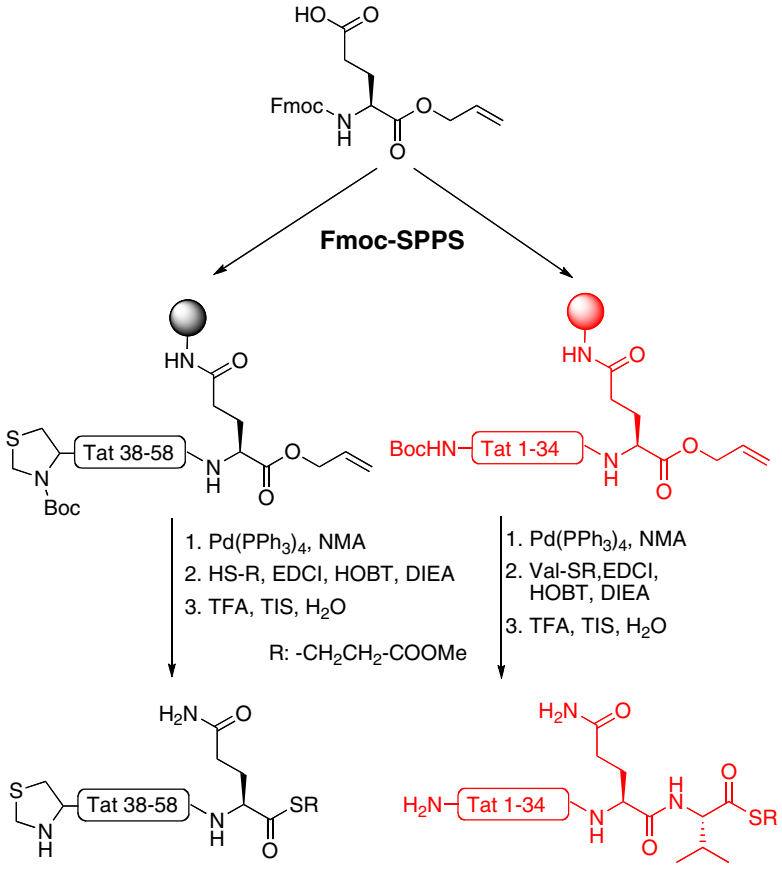


Figure 1. HIV-1 Tat(61–86)-auxiliary HPLC and ESI mass (insert) of *crude* synthetic product. Mass observed of peak c (the desired peptide): [M+H] 2936 Da, (calcd: [M+H] 2936.9 Da). Peaks a and b are deletion peptide products.

amino acid in both peptides at the C-terminal in HIV-1 Tat(37–60) and close to the C-terminal in HIV-1 Tat(1–36). This permitted anchoring of the Fmoc-Glu-OAll through the side chain leaving the C-terminus protected temporarily for further manipulation. Upon completion of the peptide synthesis the allyl protected group was unmasked using $Pd(PPh_3)_4$/TIS conditions, followed by thioesterification with 3-mercapto-methylpropionate to furnish HIV-1 Tat(37–60) peptide thioester after cleavage and purification in 30% yield (Fig. 2A). On the other hand, for the synthesis of HIV-1 Tat(1–36) peptide thioester, following the allyl deprotection, H_2N-Val-SR (R = $-CH_2-CH_2-COOMe$) was coupled to the free C-terminal to give the desired thioester peptide after cleavage and purification in 25% yield (Fig. 2B). These results indicate that this method, when possible, could be an excellent choice for the synthesis of peptide thioester based on the Fmoc-strategy.

Having all desired peptides in hand we then focused on the ligation reactions, bearing in mind that SCAL would be the challenging step in the synthesis. We have previously found that in SCAL the rate-determining step is the aminoacyl transfer and not the trans-thioesterification.[9] This in result, allows us to carry the ligation step with low peptide concentration so a good solubility of the reactants could be achieved. To test the ligation reaction, HIV-1 Tat(37–60)-auxiliary and HIV-1 Tat(61–86)-SR peptides were dissolved at a final concentration of 2–3 mM in 6 M guanidine·HCl, pH 8 (1:1.1 molar ratio of thioester peptide to peptide-auxiliary). The ligation reaction was performed at 37 °C and was monitored using analytical HPLC and ESI-MS (Fig. 3). Our analysis showed that the ligation was completed within 72 h at which all the thioester peptide was consumed and the ligation product appeared as the major peak in the HPLC analysis with a minor by product due to hydrolysis of the thioester peptide (~15%), which elutes with the same retention time of the desired product.

To verify that at this time all the ligation intermediate was converted to the peptidic product, the isolated material was treated with an excess of thiol to convert any remaining thioester interme-



Scheme 4. Side-chain anchoring strategy for the synthesis of HIV-1 Tat(37–60) and Tat(1–36) thioester peptides.

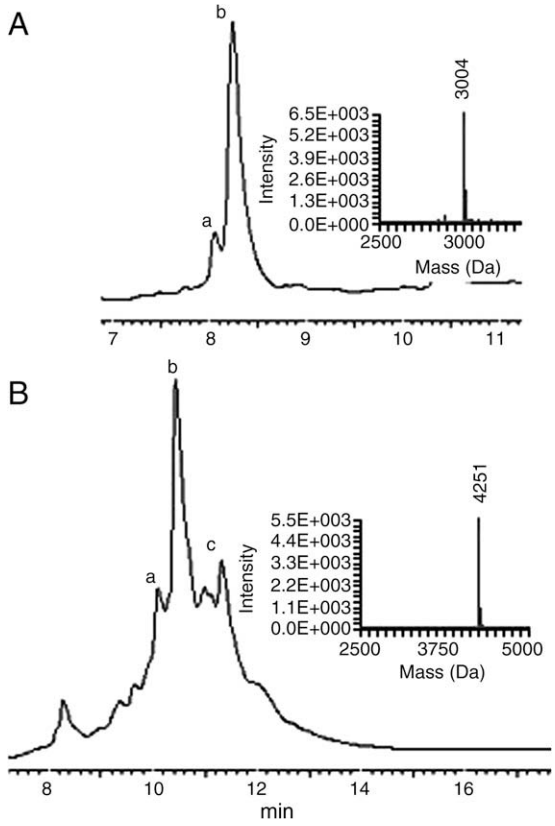


Figure 2. Synthesis of thioester peptides applying the side-chain anchoring strategy. (A) HIV-1 Tat(37–60)-SR (R: $-CH_2-CH_2-COOMe$) HPLC and ESI mass (insert) of *crude* synthetic product. Mass observed of peak b (the desired peptide): [M+H] 3004 Da, (calcd: [M+H] 3004.4 Da). Peak a: the peptide after removing the allyl group. (B) HIV-1 Tat(1–36)-SR HPLC and ESI mass (insert) of *crude* synthetic product. Mass observed of peak b (the desired peptide), [M+H] 4251 Da, (calcd: [M+H] 4250.1 Da). Peak a, the peptide after removing the allyl group while peak c is the +106 Da adduct of the desired product.

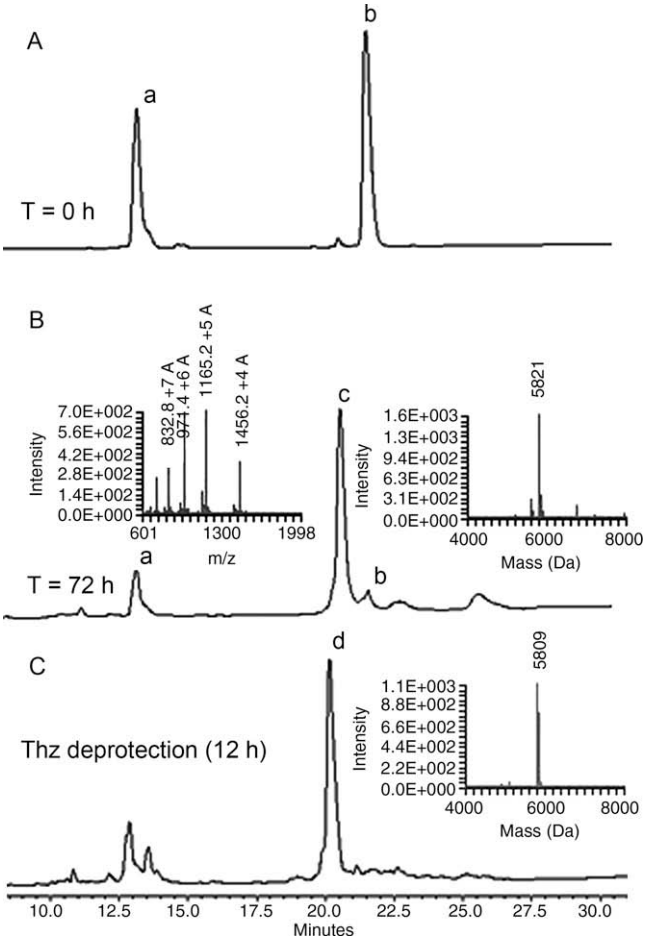


Figure 3. Analytical HPLC trace of ligation reaction: (A) peak a, HIV-1 Tat(37–60)-Auxiliary; peak b, HIV-1 Tat(37–60)-SR. (B) Peak c, ligation product HIV-1 Tat(37–86)-auxiliary with the observed mass of 5821 Da (calcd: [M+H] 5822.3 Da). C) peak d, ligation product after removal of the cys protecting group with the observed mass of 5809 Da (calcd: [M+H] 5809.3 Da).

diate to the corresponding peptide thioester. However, no appearance of any thioester peptide was observed indicating that formation of the peptide bond at the ligation site. Moreover, no hydrolysis of the ligation product was observed when this peptide was treated at pH 10. Finally, during the first hour of ligation, the formation of the thioester intermediate, which was converted completely to the ligation product after 72 h, showed clearly. Following the ligation step (Fig. 2B), methoxylamine was added at a concentration of 0.2 mM to remove the Thz-protecting group of the N-terminal Cys.[17] The ligation mixture was left at 37 °C for 12 h at which full removal was achieved (Fig. 3C). Subsequently, the ligation mixture was purified by preparative HPLC to give the ligation product in 60% yield for both steps. Having succeeded with the SCAL step, the ligation product of HIV-1 Tat(37–86) was reacted with HIV-1 Tat(1–36)-SR using NCL conditions. The reaction was left for 48 h at 37 °C to give, as expected from the NCL step, the full length HIV-1 Tat(1–86)-auxiliary in 70% yield (Fig. 4).

For auxiliary removal, the full length HIV-1 Tat(1–86) was treated under basic conditions (pH 10–11) for several minutes as we previously reported with the model systems. Unfortunately, no efficient ester hydrolysis was achieved even after prolonged treatment (20 min). Efforts to remove the auxiliary after the first ligation also led to similar results. Several other conditions were also examined such as the use of thiols as external nucleophiles (pH 10) to induce transthioesterification at the auxiliary part, hydrolysis under acidic condition (pH 1–2, 50 °C), but were unsuccessful. Apparently the size of the polypeptide might be causing hindrance to the incoming nucleophile to ultimately affecting hydrolysis. A possible solution to this issue is to use an auxiliary, which can be detached from the protein after ligation through intramolecular cyclization. This proposal is put forth on the basis of our initial results with model peptides using a thiol-modified proline as an auxiliary, instead of cyclohexane. In this case, we found that the proline-based auxiliary undergoes favorable intramolecular cyclization leading to an indolizidine skeleton, and thereby detached from protein even under our ligation conditions (pH 8).

In summary, the full length HIV-1 Tat(1–86) synthesis was achieved using SCAL combined with NCL indicating that SCAL could be used in protein synthesis. Notably, the SCAL step provided a clean reaction with only one byproduct generated from thioester hydrolysis. Our strategy also showed that the anchoring strategy for the synthesis of peptide thioester via Fmoc-SPPS could be a very useful method for achieving an efficient synthesis of this important building block. Despite the full assembly of HIV-1 Tat(1–86), we were unsuccessful in efficiently removing the auxiliary, however, a possible redesign of the auxiliary could lead to more efficient removal. We are currently working to achieve the final synthesis of unmodified HIV-1 Tat(1–86) and its mutants for biological studies.

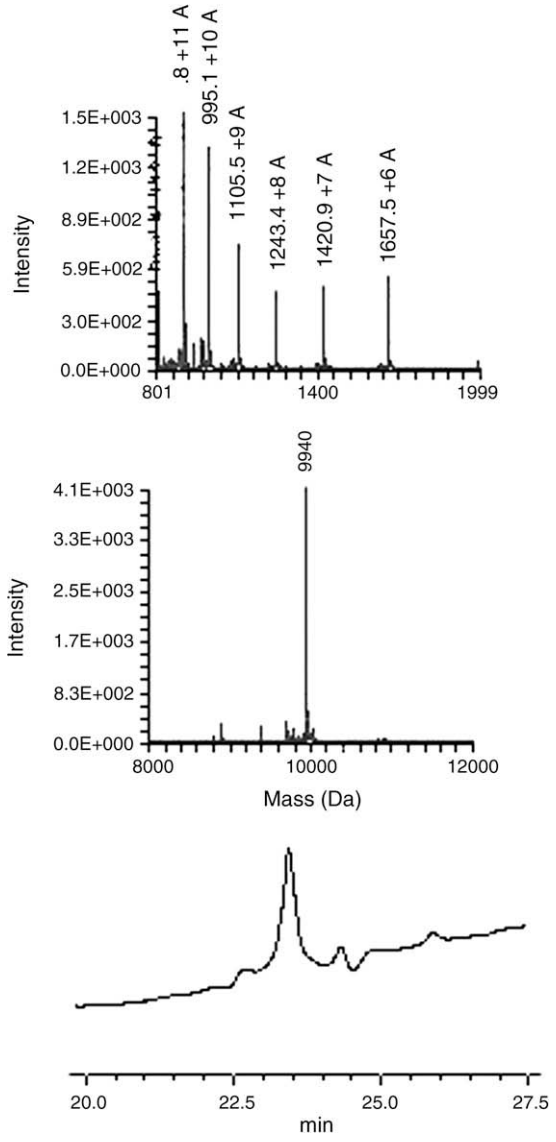


Figure 4. HPLC analysis and ESI-MS of the pure full length HIV-1 Tat(1–86)-auxiliary. Mass observed [M+H] 9940 Da, (calcd: [M+H] 9940.1 Da).

Acknowledgments

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