—Regular Articles—

Attempt to Detect Natural Anticancer Compounds—Protein Binding through Precursor Ion Scan and MS/MS Measurements

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A 2′-succinyltaxol-bovine serum albumin (BSA) conjugate was prepared as an antigen to produce an anti-taxol monoclonal antibody by immunizing mice. Formation of a linkage between hapten and protein is usually confirmed by the UV or fluorescamine method. However, it was difficult to confirm the binding of 2′-succinyltaxol to BSA by these methods owing to the similar UV absorption maxima of 2′-succinyltaxol (273 nm) and BSA (280 nm). In the present study, we therefore conducted a mass spectrometric analysis using the precursor ion scan and MS/MS techniques to confirm the formulation of the 2′-succinyltaxol-BSA conjugate in the following way: The conjugate was subjected to thermal denaturalization, dithiothreitol (DTT)-reduction, iodoacetamide-alkylation and trypsin-digestion, affording a peptide fragment mixture. This was then analyzed by electrospray ionization (ESI)-MS in the positive mode by scanning the peaks containing a mass of 854 corresponding to taxol. The detected peaks were in turn subjected to MS/MS measurements. Among them, a peak at m/z 1247.4 was found to be a peptide fragment containing Lys (ε-2′-succinyltaxol), demonstrating the formulation of the 2′-succinyltaxol-BSA conjugate. In order to confirm the feasibility of this analytical method, the deacetylvinblastine (deacetylVLB)-BSA antigen which produced the anti-VLB monoclonal antibody (MAb-10-A9), was subjected to the same analytical treatment as above, giving a peak at m/z 851.3 originating from a Lys (ε-deacetylVLB). Thus, this new method could serve as an additional tool for confirmation of the formation of hapten-protein conjugates which are difficult to detect by the above spectrophotometric methods.

Key words——taxol, taxol-bovine serum albumin conjugate, vinblastine, deacetylvinblastine-bovine serum albumin conjugate, precursor ion scan, MS/MS

INTRODUCTION

Taxol has marked antitumor activities against refractory solid cancers including breast, ovarian and lung cancers. Our group has been investigating the synthesis of natural anti-tumor compounds, which are present in minute amounts in plants, utilizing monoclonal antibodies. As a monoclonal antibody-catalyzed chemical conversion, the catalytic antibody-mediated reaction[1,2] is well known. This method utilizes a monoclonal antibody raised against the transition state analogue of a certain reaction to accelerate the reaction. Our group has aimed at the synthesis of natural anti-tumor compounds by the use of a monoclonal antibody recognizing them as a reaction mould. We thought that this could be applicable to the synthesis of natural organic compounds possessing complicated structures. We have investigated the conversion of anhydrovinblastine (anhydroVLB) into vinblastine (VLB) using an anti-VLB monoclonal antibody (MAb-10-A9) as a reaction mould, which was obtained through the immunization of mice with a deacetylVLB-bovine serum albumin (BSA) antigen.[3] Although the synthesis of VLB from anhydroVLB has been achieved by some groups, regulation of the C20′ stereochemistry to minimize the yield of its isomeric leurosidine required strict reaction conditions.[4–9] Thus, problems still remain to be overcome. In our study using the antibody MAb-10-A9, the conversion yield of anhydroVLB into VLB was 21.3% at most with a minute amount of leurosidine (Fig. 1).[3]

As part of an investigation of taxol synthesis utilizing the anti-taxol monoclonal antibody, we conducted a mass spectrometric analysis for confirmation of the formation of a bond between 2′-succinyltaxol and BSA as an antigen. The formation of a conjugate between a hapten and a protein is generally examined by

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the UV or fluorescamine method.[10,11] However, we had difficulty confirming the formation of the 2′-succinyltaxol-BSA conjugate (Fig. 1) with these methods owing to the similar UV absorption maxima of 2′-succinyltaxol (273 nm) and BSA (280 nm). We therefore performed a mass spectrometric analysis using the precursor ion scan and MS/MS techniques[12–15] to identify the formulation of the 2′-succinyltaxol-BSA conjugate in the following way: the conjugate was subjected to thermal denaturalization, dithiothreitol (DTT)-reduction, iodoacetamide-alkylation and trypsin-digestion, affording a peptide fragment mixture, which was then analyzed by electrospray ionization (ESI)-MS in the positive mode by scanning the peaks containing m/z 854, the mass of an estimated product ion peak due to taxol. The detected peaks were in turn subjected to MS/MS measurements to pick up fragment ions containing taxol. In the present study, we were able to detect a peptide peak at m/z 1247.4 whose tandem mass spectrum showed a peak at m/z 1051.1 originating from a 2′-succinyltaxol moiety bound to lysine. This result strongly supported the formation of the 2′-succinyltaxol-BSA conjugate prepared in our laboratory.

MATERIALS AND METHODS

Materials Taxol, DTT and iodoacetamide were purchased from Wako Pure Chemical Industries. BSA, *N*-methylpiperazine, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and *N*-hydroxysulfosuccinimide (sulfo-NHS) were obtained from Sigma-Aldrich Japan. Trypsin (V511A 20327804) was supplied by Promega. Centricut was obtained from Kurabo. ESI-MS was performed on an API 3000 mass spectrometer (Applied Biosystems). The ^{1}H-NMR spectrum was recorded on a JEOL EX-400 (400 MHz) using tetramethylsilane as an internal standard.

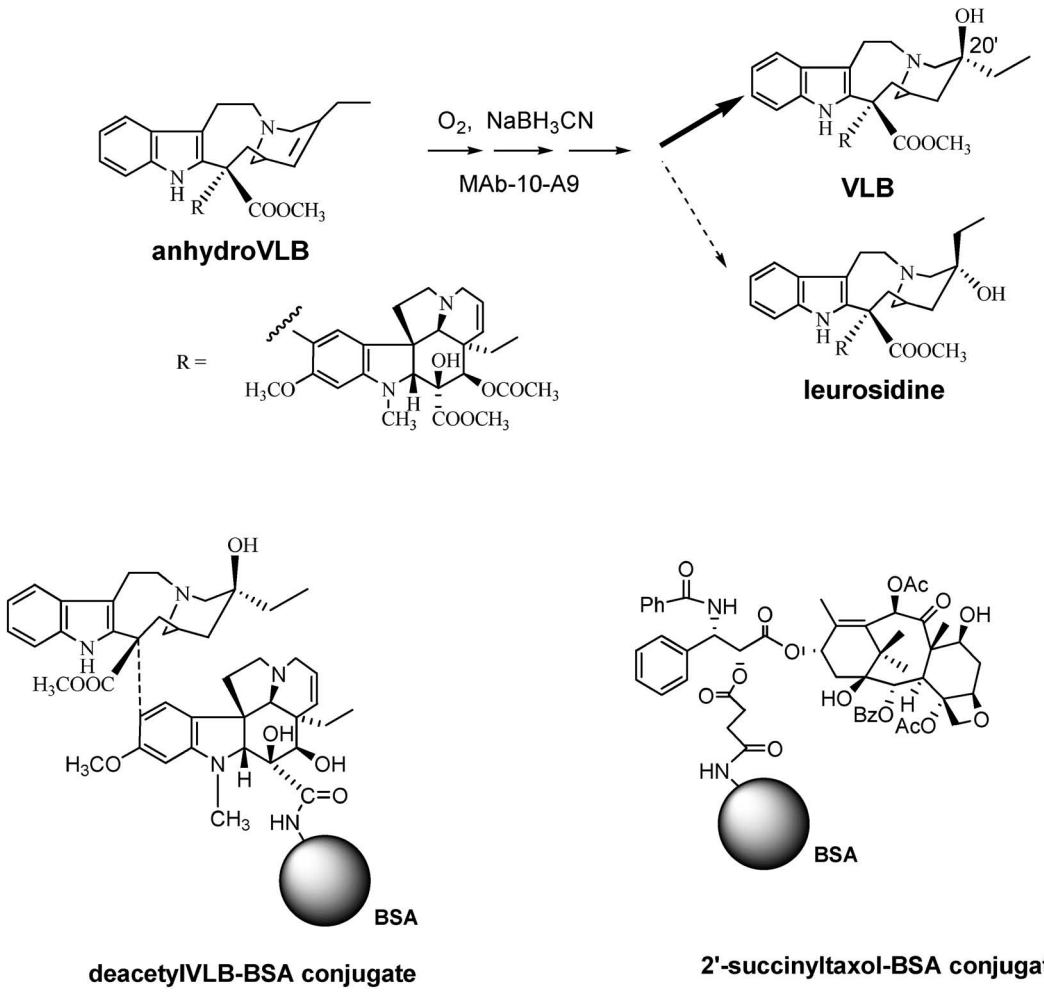

Fig. 1. Structure of VLB, the DeacetylVLB-BSA Conjugate, the 2′-Succinyltaxol-BSA Conjugate, and Related Compounds

2′-Succinyltaxol Taxol (20.0 mg, 23.4 μmol) was treated with succinic anhydride (36.0 mg, 360 μmol) according to the procedure of Leu, J. G., *et al.*,[16] yielding 2′-succinyltaxol (21.0 mg, 22.0 μmol). Production of 2′-succinyltaxol was confirmed from the following physicochemical data.[17]

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^{1}H-NMR ($CDCl_3$) δ: 1.13 (3H, s), 1.22 (3H, s), 1.67 (3H, s), 1.91 (3H, s), 2.21 (3H, s), 2.60 (4H, m), 3.80 (1H, d, J=7.2 Hz), 4.19 (1H, d, J=8.8 Hz), 4.30 (1H, d, J=8.4 Hz), 4.42 (1H, dd, J=4.4, 10.8 Hz), 4.97 (1H, d, J=8.0 Hz), 5.53 (1H, d, J=3.6 Hz), 5.68 (1H, d, J=7.2 Hz), 5.97 (1H, dd, J=3.2, 9.2 Hz), 6.23 (1H, t, J=10.0 Hz), 6.29 (1H, s), 7.02 (1H, d, J=8.8 Hz), 7.38 (8H, m), 7.51 (3H, m), 7.75 (2H, d, J=6.8 Hz), 8.13 (2H, d, J=7.6 Hz).

ESI-MS (positive mode) m/z 953.3 (M^+).

2′-Succinyltaxol-BSA Conjugate The 2′-succinyltaxol thus obtained was converted into the activated ester with EDC (0.46 mg, 2.40 μmol) and sulfo-NHS (2.1 mg, 9.67 μmol), which was in turn reacted with a solution of BSA (30 mg, 0.45 μmol) in 50 mM sodium phosphate buffer (pH 7.0) (1.2 ml) (Fig. 2) according to the procedure established by Grothaus, P. G., *et al.*[11] The reaction solution was concentrated with Centricut to give a residue, which was dissolved in PBS (50 mM sodium phosphate buffer, containing 0.15 M NaCl, pH 7.0), giving rise to the 2′-succinyltaxol-BSA conjugate (4.0 ml, 5.62 mg/ml).

Trypsin Digestion A solution (100 μg/100 μl) of the 2′-succinyltaxol-BSA conjugate (50 mM sodium phosphate buffer, containing 0.15 M NaCl, pH 7.0), BSA (50 mM sodium phosphate buffer, pH 7.0) or deacetylVLB-BSA (100 mM sodium phosphate buffer, pH 6.2) in 50 mM NH_4HCO_3 (pH 7.8) was denatured at 100℃ for 5 min. To this solution was added DTT/50 mM NH_4HCO_3 (pH 7.8) (2 μl, 1.54 mg/ml), and the mixture was incubated at 50℃ for 1 h for the reduction of disulfide bonds in the protein. After treatment with a solution of iodoacetamide/50 mM NH_4HCO_3 (pH 7.8) (5 μl, 18.4 mg/ml) at 37℃ for 30 min for alkylation of the resultant sulfhydryl groups, the solution was digested with trypsin/20 mM HCl (40 μl, 50 μg/ml) at 37℃ overnight to give a peptide fragment mixture.

Mass Spectrometric Analysis of a Peptide Fragment Mixture from 2′-Succinyltaxol-BSA Conjugate Using Precursor Ion Scan and MS/MS Techniques

The peptide fragment mixture obtained from the 2′-succinyltaxol-BSA conjugate was diluted with acetonitrile-distilled water (1 : 1) and injected into the API 3000 mass spectrometer, and a precursor ion scan was performed by scanning the peaks containing a mass of 854 over the mass range of m/z 300–2000 at 30 eV collision energy. The peak at m/z 1247.4 was in turn subjected to MS/MS measurements (mass range m/z 100–2000; collision energy 30 eV).

Mass Spectrometric Analysis of a Peptide Fragment Mixture from BSA Using Precursor Ion Scan and MS/MS Techniques The peptide fragment mixture obtained from BSA was diluted with acetonitrile -distilled water (1 : 1) and injected into the API

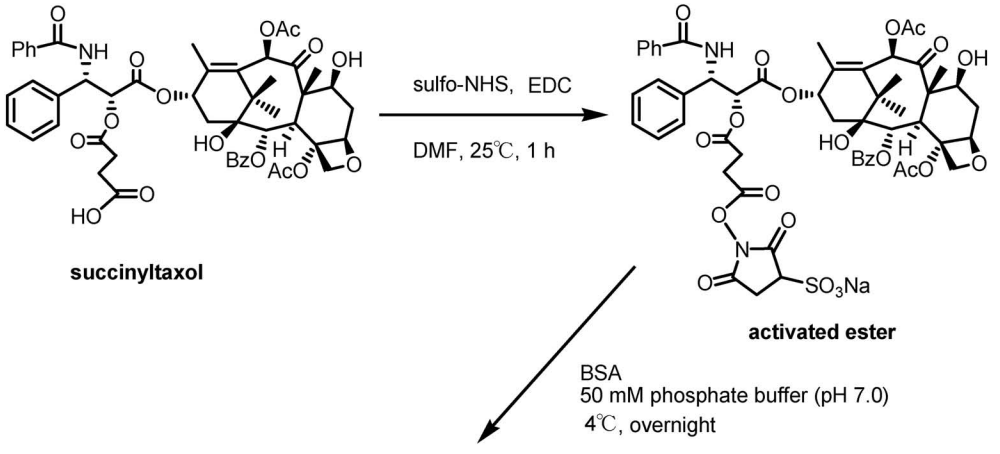

Fig. 2. Preparation of the 2′-Succinyltaxol-BSA Conjugate

3000, and a precursor ion scan was performed by scanning the peaks containing a mass of 854 over the mass range of m/z 300–2000 at 30 eV collision energy. The spectrum showed neither the precursor ion scan peaks detected in the spectrum of the peptide fragment mixture from 2′-succinyltaxol-BSA conjugate nor any other appreciable peaks.

Mass Spectrometric Analysis of a Peptide Fragment Mixture from DeacetylVLB-BSA Conjugate Using Precursor Ion Scan and MS/MS Techniques

A peptide fragment mixture from the deacetylVLB-BSA conjugate[3,18,19] was diluted with acetonitrile-distilled water (1 : 1) and injected into the API 3000, and a precursor ion scan was performed by scanning the peaks containing a mass of 754 over the mass range of m/z 400–1900 at 30 eV collision energy. The peak at m/z 851.3 was in turn subjected to MS/MS measurements (mass range m/z 100–1500; collision energy 30 eV).

RESULTS AND DISCUSSION

Taxol was treated with succinic anhydride,[16] yielding 2′-succinyltaxol, whose production was confirmed from the physicochemical data.[17] The 2′-succinyltaxol thus obtained was converted into the activated ester with EDC and sulfo-NHS and reacted with BSA to

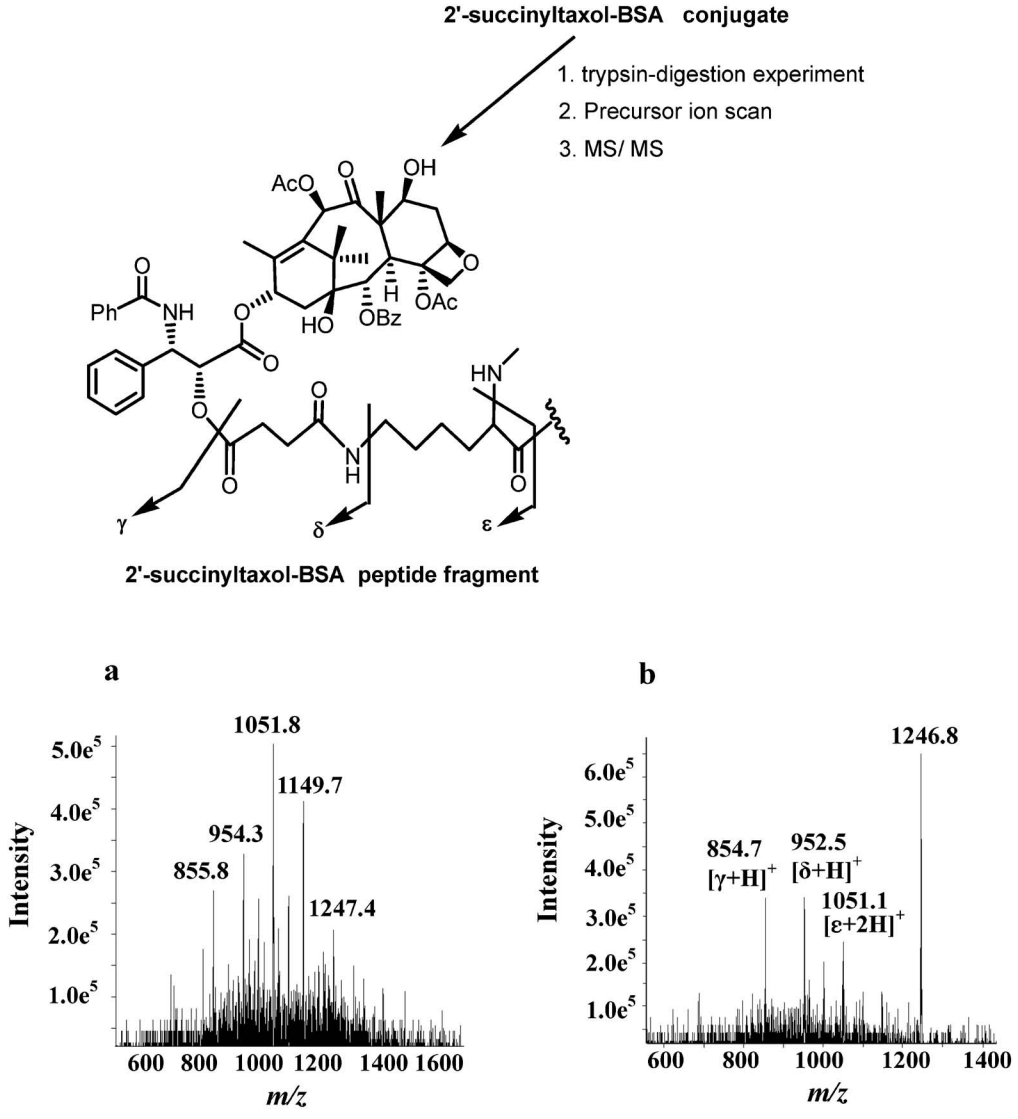

Fig. 3. Precursor Ion Scan and MS/MS Analysis of the Peptide Fragment Mixture Originating from 2′-Succinyltaxol-BSA

(a) Precursor ion spectrum at mass 854. The peptide fragment mixture from 2′-succinyltaxol-BSA was injected into an API 3000 mass spectrometer, and a precursor ion scan was performed by scanning the peaks containing m/z 854. As peaks containing taxol, m/z 855.8, 954.3, 1051.8, 1149.7, 1247.4, *etc.* were detected. (b) MS/MS of the precursor ion with m/z 1247.4. The peak at m/z 1247.4 was subjected to MS/MS measurements, furnishing the fragment ion peaks not only at m/z 854.7 originating from taxol, but also at m/z 952.5 and 1051.1 each originating from a Lys(ε-2′-succinyltaxol).

give rise to the 2′-succinyltaxol-BSA conjugate by the reported method.[16]

In the next experiment, the synthesized 2′-succinyltaxol-BSA conjugate was subjected to thermal denaturalization, DTT-reduction, iodoacetamide-alkylation, and finally trypsin-digestion. The resulting peptide fragment mixture was analyzed by ESI-MS in the positive mode utilizing the precursor ion scan[12–15] and MS/MS techniques (a manual for Analyst by Applied Biosystems). The precursor ion scan was performed by scanning the peaks containing 854, the mass of the estimated product ion peak due to taxol. As peaks containing taxol, peaks at *m/z* 855.8, 954.3, 1051.8, 1149.7, 1247.4, *etc.* were detected (Fig. 3). The MS/MS of the peak at *m/z* 1247.4 furnished the fragment ion peaks not only at *m/z* 854.7 originating from taxol, but also at *m/z* 952.5 and 1051.1 each derived from a 2′-succinyltaxol moiety bound to lysine (Fig. 3). Moreover, MS/MS measurements of the peak at *m/z* 1051.8 gave peaks at *m/z* 854.5 and 952.7 (data not shown). It was thus assumed as shown in Fig. 3 that the fragment at *m/z* 1247.4 decayed to 854.7 $[\alpha+H]^+$ and 952.5 $[\beta+H]^+$ *via* 1051.1 $[\gamma+2H]^+$.

Subsequently, in order to establish that the peak at *m/z* 1247.4 originates not from the starting material BSA, but from 2′-succinyltaxol-BSA, BSA was submitted to trypsin digestion in the same way as the 2′-succinyltaxol-BSA conjugate. The peptide fragment was analyzed on the API 3000 by scanning the peaks containing *m/z* 854. However, the spectrum showed neither a peak at *m/z* 1247.4 nor any other appreciable peak.

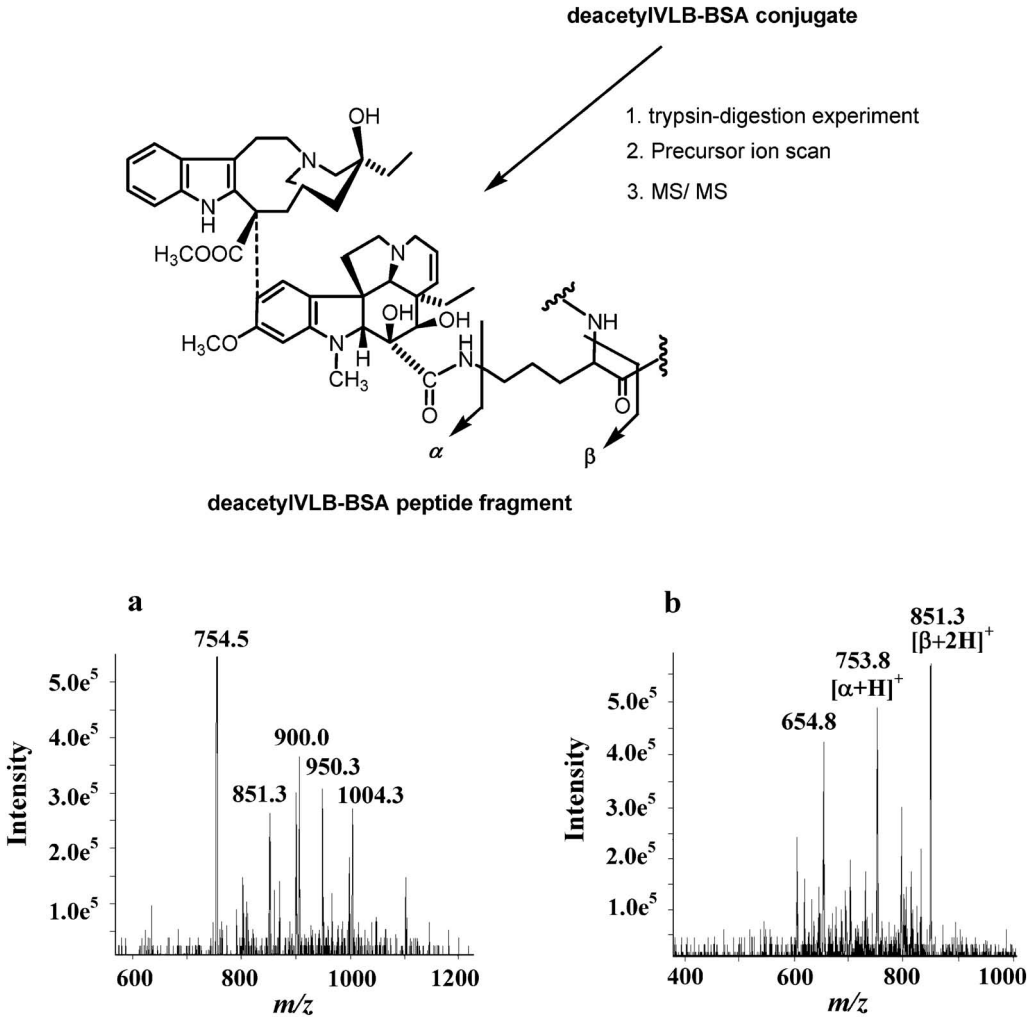

Fig. 4. Precursor Ion Scan and MS/MS Analysis of the Peptide Fragment Mixture Originating from the DeacetylVLB-BSA Conjuate

(a) Precursor ion spectrum at mass 754. The peptide fragment mixture from deacetylVLB-BSA was analyzed on an API 3000 mass spectrometer utilizing the precursor ion scan. Scanning of the peaks containing 754 resulted in detection at *m/z* 851.3, 900.0, 950.3, 1004.3, etc. (b) MS/MS of the precursor ion with *m/z* 851.3. The peaks in Fig 4(a) were then subjected to MS/MS measurements. Among them, the peak at *m/z* 851.3 gave the fragment ion peak *m/z* 753.8 due to deacetylVLB, suggesting that it is the peak originating from a Lys(ε-deacetylVLB).

ble peaks (data not shown). This finding strongly suggested that the peak at m/z 1247.4 is attributable to a taxol-containing peptide fragment and that a linkage between 2′-succinyltaxol and BSA was formed.

We examined the feasibility of this method for confirming the formation of a hapten-protein conjugate by applying it to an established conjugate, DeacetylVLB-BSA,[3] an antigen of anti-VLB monoclonal antibody. This conjugate was synthesized previously in our laboratory according to a reported procedure,[18,19] and the formation of a bond between the deacetylVLB and BSA was confirmed with the UV method since the absorption maxima of deacetylVLB (310 nm) and BSA (280 nm) were well separated. This conjugate in fact produced the anti-VLB monoclonal antibodies including MAb-10-A9. Scanning of the peaks containing 754, the mass corresponding to the estimated product ion [deacetylVLB(-$CONH_2$)+H]$^+$, resulted in the detection of peaks at m/z 851.3, 900.0, 950.3, 1004.3, *etc.* (Fig. 4). These peaks were then subjected to MS/MS measurements. The peak at m/z 851.3 gave the fragment ion peak m/z 753.8 due to [deacetylVLB(-$CONH_2$)+H]$^+$, which is most likely a fragment derived from a lysine-connected deacetylVLB. This finding demonstrated the feasibility of the mass spectrometric method including the precursor ion scan and MS/MS techniques for confirming the formation of the deacetylVLB-BSA conjugate.

The findings obtained so far demonstrated the mass spectrometric method including precursor ion scan and MS/MS techniques to be a useful tool for confirming the formation of hapten-protein conjugates, besides the UV and fluorescamine methods.

Unfortunately, the peak intensities in the present mass spectra were too weak to determine amino aid sequences of the taxol-bound peptide fragments or the number of taxol molecules bound to protein. We are thus planning to separate the tripsin-digested peptides or their *N*-methylpiperazine acetoamide derivatives on LC to enhance their S/N ratios. The *N*-methylpiperazine acetoamide derivatives are expected to show comparatively intense signals attributed to a *N*-methylpiperazine in mass spectra, which should make easier the determination of amino acid sequences and the number of bound taxol molecules.[20,21]

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