Stereoselective Conversion of Anhydrovinblastine into Vinblastine Utilizing an Anti-vinblastine Monoclonal Antibody as a Chiral Mould

Tatsuya Shirahama,[a] Takeyuki Kohno,[c] Tomohiro Kaijima,[a] Yasuo Nagaoka,[a,b] Daisuke Morimoto,[a] Kazumasa Hirata,[d] and Shinichi Uesato*,[a,b]

[a] Department of Biotechnology, Faculty of Engineering, Kansai University; [b] High Technology Research Center, Kansai University; Suita, Osaka 564–8680, Japan: [c] Faculty of Pharmaceutical Sciences, Setsunan University; Hirakata, Osaka 573–0101, Japan: and [d] Environmental Biotechnology Laboratory, Graduate School of Pharmaceutical Sciences, Osaka University; 1–6 Yamadaoka, Suita, Osaka 565–0871, Japan.

Dimeric indole alkaloid, anhydrovinblastine, which can be obtained from catharanthine and vindoline in a high yield, was converted stereoselectively into vinblastine through alternating oxidation–reduction with oxygen and NaBH$_3$CN in the presence of anti-vinblastine monoclonal antibody.
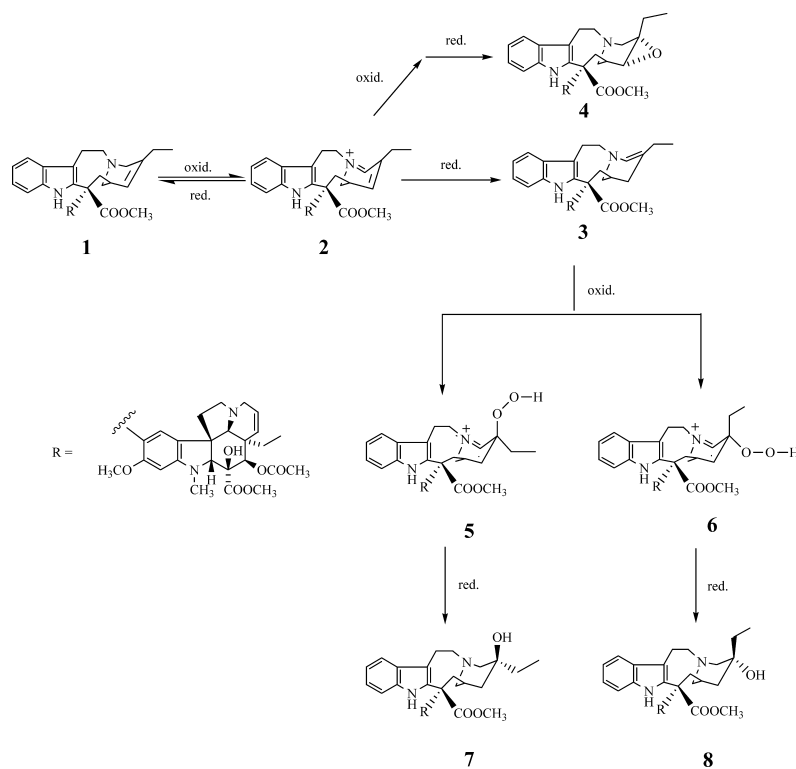
The antibodies (abzymes) which recognize the transition state analogue of a given reaction to catalyze the reaction by lowering an activation free energy in its transition state were first designed by both groups of Lerner[1] and Schultz.[2] The catalytic antibody is well known as the application of monoclonal antibodies for organic reactions. Our group has so far investigated an application of a monoclonal antibody for the synthesis of valuable natural compounds with poly-functional groups. In the present work, we tried to examine the feasibility of the monoclonal antibody raised against a target compound-protein conjugate for a synthesis of a complicated natural compound. It is expected that such an antibody could regulate both regio- and stereo-chemistry for the conversion of an intermediate into the targeted product. We thus performed the synthesis of vinblastine (**7**) using an anti-VLB monoclonal antibody as a chiral mould. Vinblastine (**7**), a dimeric alkaloid occurring in the leaves of *Catharanthus roseus* (L.) G. Don., is an important antitumor agent for the clinical treatments of various carcinomas. However, it's very low occurrence in the plant makes it difficult to provide the agent at a low cost. Thus, synthesis of **7** has fascinated many organic chemists, and diverse synthetic pathways have hitherto been published.[3—7] Many organic chemists have paid attention toward preparation of **7** from monomeric vindoline and catharanthine, both occurring as more plentiful constituents in the plant.[8—14] Langlois and Potier[15] found out that a dimeric alkaloid anhydrovinblastine (**1**) was transformed to **7** and its C-20′ isomer, leurosidine (**8**), together with other diverse dimeric indole alkaloids including leurosine (**4**), though each in a 1—2% yield, by simple agitation of its organic solution under an air atmosphere. Based on the structural profile of the products, they proposed the synthetic pathway; the conjugated iminium intermediate **2** formed by air oxidation of **1** leads, through 1,4-addition of hydride or hydrogen atom from certain unoxidized alkaloid, to the enamine **3** which is the precursor of **7** and **8** (Chart 1). Afterwards, Kutney *et al.* succeeded in the preferential 1,4-reduction of **2** with dihydronicotinamide derivatives to **3**, which was in turn subjected to a sequential oxidation–reduction with air/FeCl$_3$ and NaBH$_4$, giving rise to **7** and **8** (yield, 14 and 10%) *via* the perhydroxylated iminium intermediates **5**

and **6**, respectively.[16—18] They eventually obtained **7** in *ca.* 30% yield by changing the reaction concentration.[18] Furthermore, the group of Tan *et al.* reported the conversion of **1** into **7** and **8** (yield, 10—52% and 6—19.5%, respectively) through sequential treatment with air/FeCl$_3$, HCl and NaBH$_4$ or with air/FeCl$_3$, (NH$_4$)$_2$C$_2$O$_4$, maleic acid, NH$_3$ and NaBH$_4$.[19,20] Anhydrovinblastine (**1**) is easily available in one step reaction from vindoline and catharanthine over 80% yield.[19] This prompted us to investigate the stereoselective conversion of **1** into **7** utilizing the anti-VLB monoclonal antibody as a chiral mould.

Results and Discussion

Deacetylvinblastine (deacetylVLB)–Bovine Serum Albumin (BSA) conjugate was prepared through coupling of deacetylVLB acid azide[21] with the protein according to the procedure by Conrad *et al.*,[22] and was used for antibody binding with ELISA. Furthermore, deacetylVLB–thyroglobulin (TG) conjugate was prepared in the same way and was used for immunizations. These conjugates contained about 3.2 mol deacetylVLB/mol BSA and 8.2 mol deacetylVLB/mol TG, respectively, as estimated by the ultraviolet measurement. Eleven Balb/c mice were immunized i.c. at 2-week intervals each with 100 μg of deacetylVLB–TG conjugate/100 μl phosphate buffer (100 mM, pH 6.2) emulsified in Freund's complete adjuvant. The mice with the high-titer antiserum were selected and their spleen cells were fused with cells from a non-secreting myeloma cells SP2/O-Ag14 using polyethylene glycol. Hybridomas were selected using GIT medium[23] containing HAT in the presence of peritoneal cells[24] and antibody-secreting hybridomas were cloned using S-Clone cloning medium. The hybridoma supernatants were periodically assayed for the presence of anti-VLB monoclonal antibodies. The positive clones were confirmed for deacetylVLB–BSA-binding by ELISA,[25] and four kinds of hybridomas producing an anti-VLB monoclonal antibody were cloned. An anti-VLB monoclonal antibody MAb-10-A9 (in 100 mM citrate–NaOH buffer, pH 6.0) showing the highest-titer for ELISA was used for the synthetic mould of vinblastine (**7**). We chose NaBH$_3$CN and oxygen for oxidation–reduction steps from **1** to **7** in the antibody reaction.

Chart 1

Thus, **1** (1 eq) was reacted with NaBH$_3$CN (4 eq) and oxygen in the presence of the antibody MAb-10-A9 (1 eq) in the citrate–NaOH buffer solution at 25 °C. Compounds in the reaction mixture were detected at 265 nm on HPLC using MeOH/phosphate–NaOH as a mobile phase, and their retention times (t_Rs) were confirmed by comparison with those of the corresponding authentic samples. In 4 h, **7** (t_R, 21.1 min) was produced in a 16.0% yield with a 80.6% recovery of **1**. The C-20′ isomer of **7**, leurosidine (**8**) (t_R, 20.9 min), was not detected under the condition adopted (see Experimental), whereas **4** (t_R, 20.4 min) was obtained in a 2.1% yield (Fig. 1a). The yield of **7** was increased up to 21.3% in 24 h with significant decrease of the amount of **1** (t_R, 22.6 min) (Fig. 1b). By contrast, in the absence of antibody, the yield of **7** was only 3.9% in 4 h (Fig. 1c). In the same way, the reaction solution containing BSA instead of MAb-10-A9 gave **7** only in a 3.6% yield in 4 h (HPLC chart not shown). Production of **7** was confirmed by co-injection of the reaction mixture in 24 h and an authentic sample of **7** into the HPLC apparatus (Fig. 2). It was also confirmed through HPLC-ESI-MS analysis with an Agilent series HPLC system connected to an API-3000 mass spectrometer in the following way. To ease the monitoring of MS spectrogram, the antibody reaction was carried out at the ten-fold concentration level each for **1**, antibody and NaBH$_3$CN in the reaction solution. After 4 h, the reaction mixture was diluted with CH$_3$CN, sonicated and centrifuged to remove precipitated proteins. The supernatant was injected into the HPLC apparatus, and the HPLC-ESI-MS was performed using CH$_3$CN/NH$_4$OAc as a mobile phase in place of the MeOH/phosphate–NaOH, since a NH$_4$OAc salt, being evaporated off during the ionization process, does not disturb the mass spectrum. Figure 3a exhibited the total ion chromatography (TIC) of the reaction mixture in the scan range from m/z 700 to 900. The t_Rs of **1**,

4, **7** and **8** in TIC were confirmed by comparison with those of the corresponding authentic samples. The occurrence of **7** was also supported from the extract ion current (EIC) chromatogram as shown in Fig. 3b, *i.e.*, an ion peak due to **7** appeared at 24.4 min in the EIC chromatogram which was targeted at m/z 811.5 (M+H$^+$). The EIC peak appearing at 18.7 min was due to leurosidine (**8**) produced in a small amount. Furthermore, it was found out that the EIC peak at 28.3 min was an isotopic ion peak from leurosine (**4**) (molecular weight 808.4) since the EIC chromatogram at m/z 809.5 (M+H$^+$) showed the ion peak at 28.3 min (Fig. 3c).

From the findings mentioned so far, it is most likely that the anti-VLB monoclonal antibody MAb-10-A9 played an important role in stereochemical control during the process from **1** to **7**; presumably it regulated the stereochemistry for an oxidation of the enamine **3** to **5**. Furthermore, the antibody might have contributed to an increment of the yield of **7**, since the reaction deleting the antibody afforded **7** only at a 3.9% yield to a 16% for the reaction containing the antibody.

In conclusion, we have demonstrated the capability of monoclonal antibody MAb-10-A9 recognizing the target compound (**7**)-protein conjugate to control the stereochemistry as a chiral mould. In the present study, the MAb-10-A9 is unable to catalyze the conversion of **1** into **7**. Furthermore, it is unclear why the yields of **7** in the anti-VLB monoclonal antibody reactions were limited to 16.0—21.3% and how the antibody took part in the production of **7**. Further studies are underway to search for new possibility of antibody.

Experimental

General Procedure HPLC was carried out on LC-10ADvp (Shimadzu) using a reverse phase column (XTerra^{TM} MSC$_8$, Waters: 5 μm, 2.1 mm ID×150 mm) at 265 nm. The mobile phase was a MeOH/phosphate–NaOH solution (73 mM H$_3$PO$_4$ and 60 mM NaOH); flow rate, 0.4 ml/min with the

following gradient system: 0—10 min, 20—40% MeOH; 10—14 min, 40—50% MeOH; 14—19 min, 50% MeOH; 19—23 min, 50—95% MeOH; 23—23.1 min, 95—100% MeOH; 23.1—30 min, 100% MeOH.[13] HPLC-ESI-MS was carried out using an Agilent 1100 series HPLC system (Yokogawa Analytical Systems) (XTerra™ MSC$_8$, Waters: 5 μm, 2.1 mm ID×150 mm) con-

nected with an API 3000 mass spectrometer (Applied Biosystems) (positive mode). The mobile phase was a CH$_3$CN/10 mM NH$_4$OAc solvent system, and the chromatograms were monitored at 265 nm. Gel-filtration was performed on a Sephadex™ G-25 Medium (Amersham Biosciences) with a Hitachi U-2000 Spectrophotometer at 310 nm. Cell incubation was performed in a CO$_2$ gas incubator (Sanyo Electric). BSA and TG were purchased from Sigma-Aldrich Japan, whereas SP2/O-Ag14 was obtained by ATCC (Catalog No., CRL-1581). Balb/c mice were purchased by Shimizu Laboratory supplies.

Synthesis of Deacetylvinblastine (DeacetylVLB)–BSA Conjugate A solution of deacetylVLB acid azide in 1,4-dioxane (200 μl), which was prepared from vinblastine (**7**) sulfate (10 mg, 11 μmol) according to the procedure by Barnett *et al.*,[21] was added dropwise to a stirred solution of BSA (17.9 mg) in aq. Na$_2$HPO$_4$ (2.0 ml) (0.1 M Na$_2$HPO$_4$ was adjusted to pH 9 by adding a few drops of 0.1 M NaOH). After stirring for 3 h at room temp., the resulting crude conjugate was purified by gel-filtration on a Sephadex G-25 column (2.0 ID×35 cm) using 100 mM phosphate buffer (pH 6.2) as an eluent, yielding the deacetylVLB-BSA conjugate[22] (10 ml, 1.39 mg/ml). This conjugate was found to contain 3.2 mol deacetylVLB/mol BSA by ultraviolet measurement.

Synthesis of DeacetylVLB–Thyroglobulin (TG) Conjugate A solution of deacetylVLB acid azide[21] in 1,4-dioxane (200 μl) was treated with a solution of TG (17.9 mg) in aq. Na$_2$HPO$_4$ (2.0 ml) in the same way as above to give a conjugate solution. Elution with a Sephadex G-25 column with 100 mM phosphate buffer (pH 6.2) afforded the deacetylVLB–TG conjugate (16 ml, 0.60 mg/ml), which contained 8.2 mol deacetylVLB/mol TG by ul-
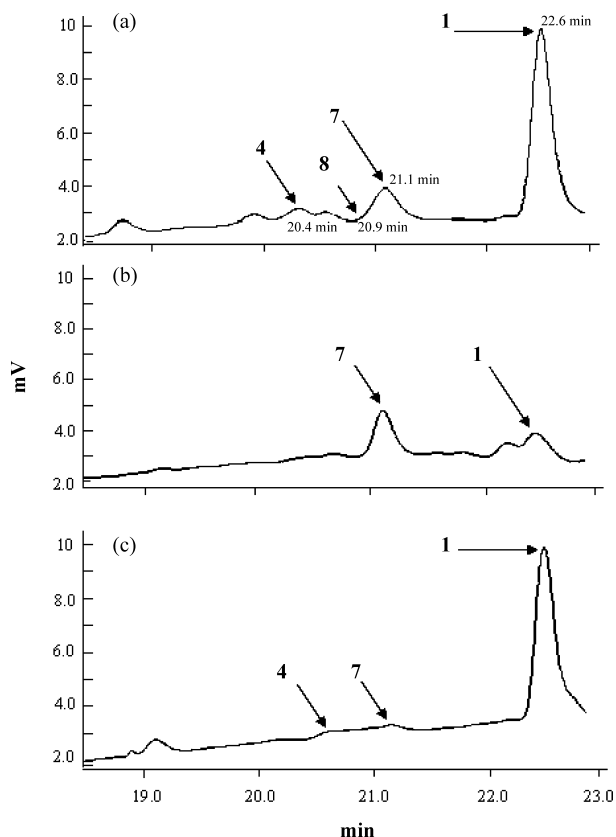

Fig. 1. HPLC Chromatograms of the Reaction System with Anhydrovinblastine (**1**), NaBH$_3$CN and Oxygen in the Presence of or in the Absence of MAb-10-A9

(a) Chromatogram of the reaction system with **1**, MAb-10-A9 and NaBH$_3$CN (1 : 1 : 4) in 4 h; (b) chromatogram in 24 h; (c) chromatogram of the reaction system with **1** and NaBH$_3$CN (1 : 4) in 4 h, deleting MAb-10-A9. As a mobile phase for HPLC, MeOH/phosphate–NaOH was used. The detailed conditions were described in the Experimental section. The retention times of **1**, **4**, **7** and **8** were confirmed by comparison with those of the corresponding authentic samples.
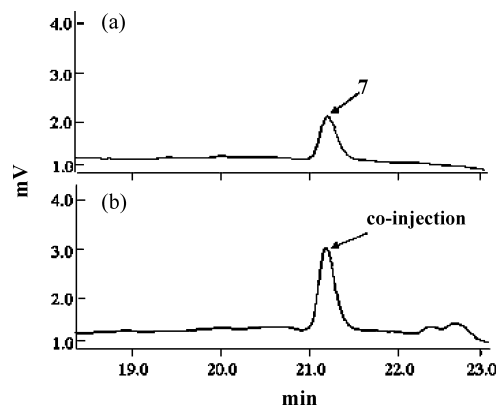

Fig. 2. Co-injection Chromatogram of the Reaction System with **1**, MAb-10-A9 and NaBH$_3$CN (1 : 1 : 4) in 24 h and an Authentic Sample of **7**

(a) Chromatogram of **7** (0.02 nmol, 3.5 μl); (b) co-injection of the reaction mixture (3.5 μl) and **7** (0.02 nmol, 3.5 μl). As a mobile phase for HPLC, MeOH/phosphate–NaOH was used. The detailed conditions were described in the Experimental section.
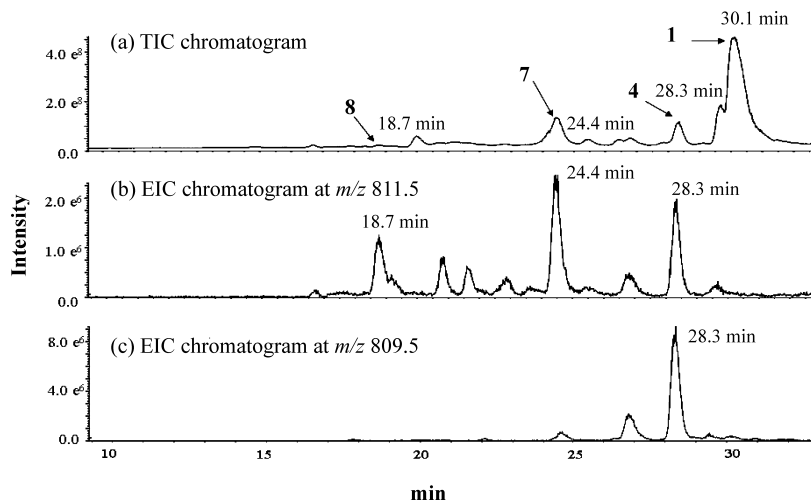

Fig. 3. TIC and EIC Chromatograms of the Reaction System with **1**, MAb-10-A9 and NaBH$_3$CN (1 : 1 : 4) in 4 h

(a) TIC chromatogram of the reaction mixture; (b) EIC chromatogram monitored by targeting at *m/z* 811.5, (c) EIC chromatogram monitored by targeting at *m/z* 809.5. As a mobile phase for HPLC-ESI-MS, CH$_3$CN/NH$_4$OAc was adopted. The detailed conditions were described in the Experimental section. The retention times of **1**, **4**, **7** and **8** in TIC were confirmed by comparison with those of the corresponding authentic samples.

traviolet measurement.

Immunization   Eleven Balb/c mice (aged 7 weeks, female) were immunized i.c. at 2-week intervals each with 100 μg of deacetylVLB–TG conjugate/100 μl of 100 mM phosphate buffer (pH 6.2) emulsified in a Freund's complete adjuvant (FCA). The blood was collected 0, 14, 28 and 42 d after the initial immunization, and the antibody titer of each antiserum was measured by ELISA[25] using the deacetylVLB–BSA conjugate, a blocking agent BSA and (anti-mouse IgG (H+L)) Fab'-peroxidase conjugate.

Fusion and HAT Selection of the Hybridomas   The mice with the high-titer antiserum were selected and their spleen cells were fused in a 5 : 1 ratio with cells from a non-secreting myeloma cells SP2/O-Ag14 using polyethylene glycol (PEG 1500) according to the instructions of Roche Diagnostics (Mannheim). Hybridomas were selected using GIT medium[23] containing HAT in the presence of peritoneal cells[24] collected beforehand.

Cloning of Hybridomas Producing an Anti-VLB Monoclonal Antibody   The anti-VLB monoclonal antibody-secreting hybridomas (positive hybridomas) were cloned using S-Clone cloning medium according to the instructions of Sanko Junyaku. The hybridoma supernatants were periodically assayed for the presence of anti-VLB antibodies. The well number containing positive hybridomas were five for a mouse No. 9, twenty-seven for a mouse No. 10 and twenty for a mouse No. 11. The hybridomas of these wells were further cloned by the limiting dilution method. The positive clones were confirmed for deacetylVLB–BSA-binding by ELISA.[25] Three wells of a mouse No. 9 gave two anti-VLB monoclonal antibodies MAb-9-E7 and MAb-9-D12. Furthermore, nine wells of a mouse No. 10 afforded two anti-VLB monoclonal antibodies, MAb-10-A9 and MAb-10-F10. On the other hand, eleven wells of a mouse No. 11 did not furnished any anti-VLB monoclonal antibody.

A Large Scale Production of the Cloned Hybridoma Producing an Anti-VLB Monoclonal Antibody MAb-10-A9 by Balb/c Nude Mice and Its Purification   The hybridoma cells (2×10^8), which produced the antibody MAb-10-A9 with the highest affinity to the deacetylVLB–BSA conjugate, were administered i.p. to nine Balb/c nude mice (aged 5 weeks, female), giving an abdominal dropsy (8 ml). An aliquot (1 ml) of the dropsy was purified on a Protein A Sepharose 4 Fast Flow column (1 ml) in the conventional way. This purification was repeated five times, yielding anti-VLB monoclonal antibody IgG$_1$ (6.75 mg in 20.6 ml of 100 mM citrate–NaOH buffer, pH 6.0).

Reaction of Anhydrovinblastine (1) (1 eq) with the Anti-VLB Monoclonal Antibody (1 eq), NaBH$_3$CN (4 eq) in the 100 mM Citrate–NaOH Buffer under an Oxygen Atmosphere   A mixture of **1** (1.13 nmol), anti-VLB monoclonal antibody IgG$_1$ (MAb-10-A9) (1.13 nmol) and NaBH$_3$CN (4.50 nmol) was vigorously stirred at 25 °C in the citrate–NaOH buffer solution (67 μl, 100 mM, pH 6.0, containing 10% CH$_3$CN) using a testing tube (18 mm ID×40 mm) and a stirring bar (10 mm wide×8 mm high, cruciform on both sides) with an oxygen balloon on a top. The reaction was monitored in 1, 2, 3, 4 and 24 h. An aliquot (6.0 μl) of the reaction solution was mixed with CH$_3$CN (3.0 μl) and a phosphate–NaOH solution (73 mM H$_3$PO$_4$ and 60 mM NaOH) (3.0 μl) and sonicated for 3 min. Of the mixture, 10 μl was injected into the HPLC apparatus. Production of vinblastine (**7**) was confirmed through co-injection of the reaction mixture and an authentic sample of **7** into HPLC. The C-20′ isomer of **7**, leurosidine (**8**) (t_R, 20.9 min) was not detected, whereas leurosine (**4**) (t_R, 20.4 min) was obtained in a 2.1% yield; these t_Rs were confirmed by injection of authentic samples of **8** and **4** into HPLC, respectively. Vinblastine (**7**) was produced in a 16.0% yield with a 80.6% recovery of **1** (t_R 22.6 min). Thus, the conversion yield to **7** was 82.2% based on calculation with the calibration curve of **7**. In 24 h, the yield of **7** was approximately 21.3%.

Reaction of Anhydrovinblastine (1) (1 eq) with NaBH$_3$CN (4 eq) in the 100 mM Citrate–NaOH Buffer under an Oxygen Atmosphere   A mixture of **1** (1.13 nmol) and NaBH$_3$CN (4.50 nmol) was vigorously stirred at 25 °C in the citrate–NaOH buffer solution (67 μl, 100 mM, pH 6.0, containing 10% CH$_3$CN) under an oxygen atmosphere in the same way as mentioned above. The yield of **7** was 3.9% in 4 h.

Reaction of Anhydrovinblastine (1) (1 eq) with BSA (1 eq), NaBH$_3$CN (4 eq) in the 100 mM Citrate–NaOH Buffer under an Oxygen Atmosphere   A mixture of **1** (1.13 nmol), BSA (1.13 nmol) and NaBH$_3$CN (4.50 nmol) was vigorously stirred at 25 °C in the citrate–NaOH buffer solution (67 μl, 100 mM, pH 6.0, containing 10% CH$_3$CN) under an oxygen atmosphere in the usual way. The yield of **7** was 3.6% in 4 h.

Reaction of Anhydrovinblastine (1) (1 eq) with the Anti-VLB Monoclonal Antibody (1 eq), NaBH$_3$CN (4 eq) at Their Ten-Fold Concentrations in the 100 mM Citrate–NaOH Buffer under an Oxygen Atmosphere   A mixture of **1** (11.2 nmol), anti-VLB monoclonal antibody IgG$_1$ (MAb-10-A9) (11.2 nmol) and NaBH$_3$CN (44.8 nmol) was vigorously stirred at 25 °C in the citrate–NaOH buffer solution (67 μl, 100 mM, pH 6.0, containing 10% CH$_3$CN) in the usual way except that the concentrations of **1**, the antibody and NaBH$_3$CN were increased up to 10-fold, respectively. After 4 h, the reaction solution was diluted with CH$_3$CN (50 μl), sonicated for 3 min and centrifuged. The supernatant (10 μl) was injected via the autosampler into the HPLC-ESI-MS apparatus. Analysis was performed on a XTerra™ MSC$_8$ using a mobile phase of CH$_3$CN/10 mM NH$_4$OAc with increasing a CH$_3$CN content (0—50 min, 30—75% CH$_3$CN; 50—60 min, 75—100% CH$_3$CN.) The TIC chromatogram of the reaction mixture was recorded in the scan range from m/z 700 to 900, and the EIC chromatograms were monitored by targeting at m/z 811.5 and 809.5, respectively. The t_Rs of **1** (30.1 min), **4** (28.3 min), **7** (24.4 min) and **8** (18.7 min) in the TIC chromatogram were confirmed by comparison with those of their authentic samples, respectively.

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