CHEMBIOCHEM

DOI: 10.1002/cbic.200800317

Tubulin Photoaffinity Labeling with Biotin-Tagged Derivatives of Potent Diketopiperazine Antimicrotubule Agents

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*NPI-2358 (**1**) is a potent antimicrotubule agent that was developed from a natural diketopiperazine, phenylahistin, which is currently in Phase I clinical trials as an anticancer drug. To understand the precise recognition mechanism of tubulin by this agent, we focused on its potent derivative, KPU-244 (**2**), which has been modified with a photoreactive benzophenone structure, and biotin-tagged KPU-244 derivatives (**3** and **4**), which were designed and synthesized for tubulin photoaffinity labeling. Introduction of the biotin structure at the p′-position of the benzophenone ring in **2** exhibited reduced, but significant biological activities with tubulin binding, tubulin depolymerization and cytotoxicity in comparison to the parent KPU-244. Therefore, tubulin photoaffinity labeling studies of biotin-derivatives **3** and **4** were performed by using Western blotting analysis after photoirradiation with 365 nm UV light. The results indicated that tubulin was covalently labeled by these biotin-tagged photoprobes. The labeling of compound **4** was competitively inhibited by the addition of diketopiperazine **1** or colchicine, and weakly inhibited by the addition of vinblastine. The results suggest that photoaffinity probe **4** specifically recognizes tubulin at the same binding site as anticancer drug candidate **1**, and this leads to the disruption of microtubules. Probe **4** serves well as a useful chemical probe for potent antimicrotubule diketopiperazines, much like phenylahistin, and it also competes for the colchicine-binding site.*

Introduction

The introduction of antimicrotubule agents such as taxanes and vinca alkaloids has revolutionized cancer treatment and improved patient survival time.[1] However, tumors can become resistant to these drugs after long-term clinical treatment.[2] Hence, there is a significant need to develop novel antimicrotubule agents. We have focused on a natural diketopiperazine (DKP), phenylahistin, (PLH, halimide) which exhibits colchicine-like antimicrotubule activity,[3] and a highly potent cytotoxic derivative NPI-2358 (**1**, IC_{50} of 15 nM against HT-29 cells), which was developed from structure–activity relationship (SAR) studies. It was also recently shown that NPI-2358 functions as a strong "vascular-disrupting agent" (VDA) and induces tumor-selective vascular collapse.[4] NPI-2358 is now in Phase I clinical trials as a promising anticancer drug in the US.

Although it is known that PLH recognizes the colchicine-binding site on tubulin,[3b] the precise binding mode of NPI-2358 and its microtubule depolymerization mechanism have not been well investigated, and three-dimensional structure studies have failed to favorably superimpose NPI-2358 with colchicine by molecular modeling (Figure S1 in the Supporting Information). In the SAR studies based on compound **1**, a more potent benzophenone derivative KPU-244 (**2**) with a cytotoxic activity of 3 nM (IC_{50}) against HT-29 human colon cancer cells was discovered. Because the benzophenone structure is often used in protein photoaffinity labeling,[5] in the present study, based on the structure of compound **2**, we designed and synthesized biotin-tagged derivatives **3** and **4** that can be detected by an avidin–peroxidase system based on the avidin–biotin catch principle[6] after photoaffinity labeling. Then, the

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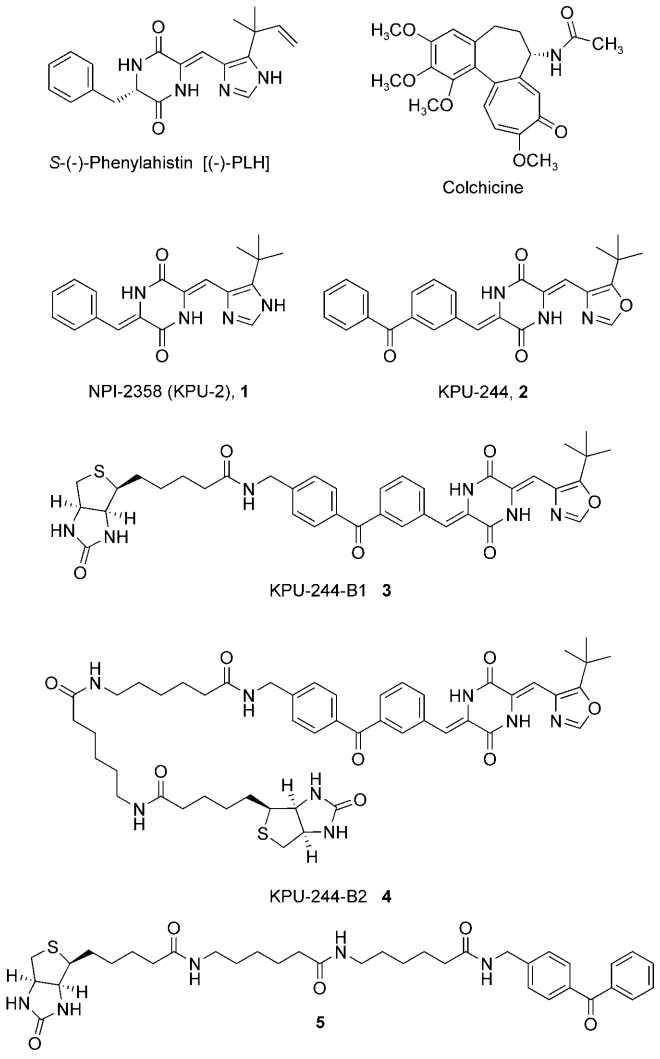
biological activities of these probes were evaluated, and a tubulin photoaffinity labeling study was performed. In the design of compounds **3** and **4**, a biotin tag was introduced to the 4′-position of the benzophenone moiety of **2** through an additional aminomethyl group, and these probes were successfully synthesized. These synthetic compounds exhibited significant biological activities in binding to tubulin in a fluorescence-based binding assay. Hence, a tubulin photoaffinity labeling study was performed with compounds **3** and **4.** The results indicated that tubulin was covalently labeled by both probes (Scheme 1).

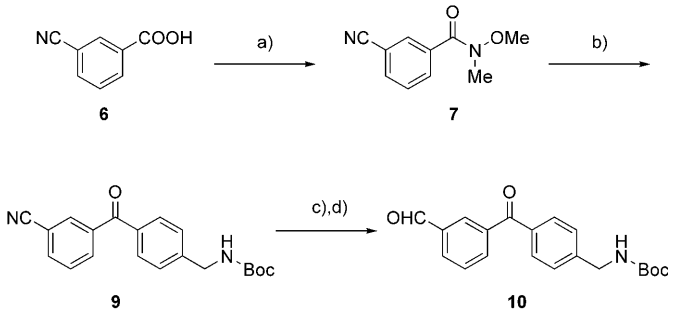


Scheme 1. Structures of antimicrotubule diketopiperazines, colchicine and designed biotin-tagged photoaffinity probes.

Results and Discussion

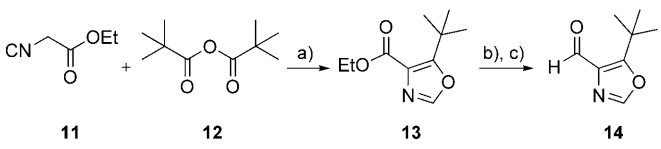
Synthesis of biotin-tagged KPU-244 derivatives 3 and 4

To prepare the biotin-conjugated KPU-244 derivatives **3** and **4**, we first synthesized 3-[*N*-Boc-4-aminomethylbenzoyl]benzaldehyde **10** from 3-cyanobenzoic acid **6** in four steps. After converting compound **6** to the corresponding Weinreb amide (**7**)[7] the anion obtained by a bromo–lithium exchange reaction of protected 4-*N*-Boc-aminomethylbromobenzene **8** (which was prepared from 4-brombenzyl amine and Boc_2O in the presence of Et_3N) with *n*BuLi, was condensed with Weinreb amide **7**.[8] The resultant crude product (**9**) was then purified by silica gel column chromatography. The observed low yield of **9** was probably due to predominant anion production at the amide nitrogen in **8** and subsequent dianion formation by a hindered bromo–lithium exchange reaction. The nitrile and carbonyl groups of the resulting benzophenone derivative **9** were reduced with diisobutylaluminum hydride (DIBALH), then the resulting benzhydryl alcohol was oxidized to the benzophenone with Dess–Martin periodinane[9] to give the desired aldehyde **10** (Scheme 2).



Scheme 2. Synthesis of 3-[*N*-Boc-4-aminomethylbenzoyl]benzaldehyde **10**: a) MeONHMe·HCl, EDC·HCl, Et_3N, DMF, RT, 99%; b) *n*BuLi, 4-*N*-Boc-aminomethylbromobenzene **8**, Et_2O/THF, −78 °C, 17%; c) DIBALH, toluene, −78 °C; d) Dess–Martin periodinane, THF, RT, 86% over two steps.

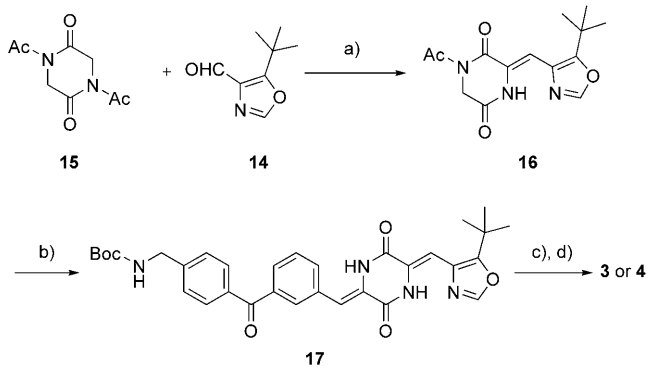
In the synthesis of 5-substituted oxazole-4-carboxaldehyde **14**, we followed a synthetic protocol starting from isonitrile derivative **11**.[10] The synthetic pathway is outlined in Scheme 1. Briefly, ethyl isocyanoacetate and pivalic anhydride **12** were condensed in the presence of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU). The resulting product was purified by silica gel chromatography to afford oxazolecarboxylate **13**, which was reduced with $LiAlH_4$ to yield oxazolyl methanol. This was followed by oxidation with MnO_2 to yield aldehyde **14** (Scheme 3).



Scheme 3. Synthesis of *t*Bu-oxazolealdehyde **14**: a) DBU, THF, RT, 99%; b) $LiAlH_4$, THF, −78 °C, 57%; c) MnO_2, acetone, RT, 67%.

To synthesize biotin-tagged derivatives **3** and **4** as photoaffinity probes, oxazolecarboxaldehyde **14** was condensed to *N*,*N*′-diacethylpiperazine-2,5-dione **15** in the presence of cesium carbonate in degassed *N*,*N*-dimethylformamide (DMF) under an argon atmosphere.[11] The introduction of a second aldehyde by a similar aldol reaction was performed between

resultant mono-dehydro-DKP **16** and substituted benzophenone-carboxaldehyde **10** under the same reaction conditions to give di-dehydro-DKP **17**. After the *tert*-butyloxycarbonyl (Boc) group in **17** was deprotected with 4 M HCl in dioxane, the obtained amine was coupled to D-biotin or long-chained D-biotin derivative **18** by an EDC–HOAt method (EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; HOAt, 1-hydroxy-7-azabenzotriazole) and the crude products were purified by high performance liquid chromatography (HPLC) to afford **3** and **4**. The purity of these compounds was more than 95% by HPLC analysis (Scheme 4). Furthermore, compound **5** was synthesized as a negative control for photoaffinity labeling from benzoic acid in four steps using a similar route as outlined in Scheme 1 (see also Scheme S1).



Scheme 4. Synthesis of biotin-tagged photoaffinity probes **3** and **4**: a) Cs_2CO_3, DMF, RT, 64%; b) **10**, Cs_2CO_3, DMF, RT, 32%; c) 4 M HCl–dioxane, RT; d) D-biotin or D-biotinyl-aminohexanoyl-aminohexanoic acid **18**, EDC·HCl, HOAt, Et_3N, DMF, RT, then HPLC purification, 16% for compound **3** and 31% for compound **4**.

Biological activity of biotin-tagged derivatives 3 and 4

To evaluate whether synthetic biotin-tagged derivatives **3** and **4** can function as antimicrotubule agents as designed, we first performed a binding assay to tubulin. Compounds **1**–**5** were incubated with bovine tubulin in 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer (pH 6.8) at 37 °C and the change in intrinsic fluorescence that is derived from the tryptophan residues in tubulin was determined in comparison to other antimicrotubule DKPs or colchicine. Compounds **1**–**4** quenched this intrinsic fluorescence in a concentration-dependent manner (Figures 1A, and S4–S7). The dissociation constants (K_d) of compounds **3** and **4** with tubulin were calculated to be 7.95 and 7.19 μM from these data, respectively (Figures 1B and S6).[12] Additionally, the binding of biotin derivative **5** to tubulin was so weak that its dissociation constant could not be calculated (Figure S8). These results indicated that the binding affinities of compounds **3** and **4** were lower than those of potent parent antimicrotubule agent **2** and colchicine (Table 1 and Figures S4–S7), this suggests that introduction of the biotin-tag at the 4′-position of the benzophenone ring adversely affected tubulin binding. However, a significant binding ability still remained in both biotin-tagged derivatives for photoaffinity labeling because their K_d values were within the same order of magnitude as the other compounds, and the binding constants ($1/K_d$) of compounds **3** and **4** were only about 2–3 times less than that of colchicine. Additionally, compounds **3** and **4** exhibited mild but significant inhibition of tubulin polymerization at 10 μM (Figures S2 and S3).

Compounds **3** and **4** exhibited low cytotoxicity against HT-29 human colon cancer cell lines with IC_{50} values of 0.91 and 1.1 μM and against HuVEC with IC_{50} values of 130 and 354 nM,

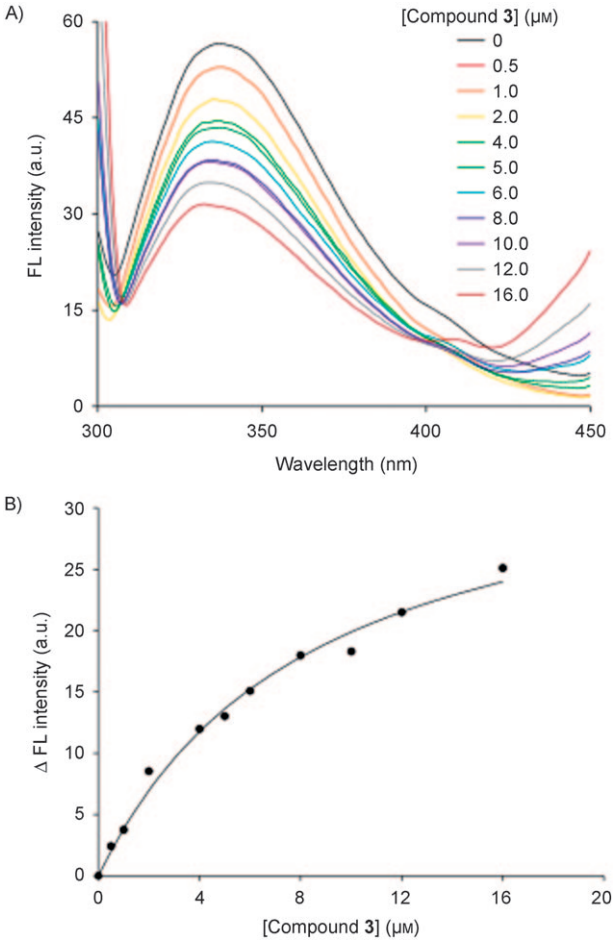


Figure 1. Binding assay based on fluorescence quenching: A) Effects of compound **3** on tubulin fluorescence. B) Increase of tubulin–compound **3** binding complex. Tubulin (0.5 μM) was incubated in the absence or presence of compound **3** at 37 °C for 1 h. After incubation, the samples were excited at 295 nm, and the emission at 300–450 nm was measured. K_d (the dissociation constant) and *n* (the number of binding sites) of compound **3** were 7.95 ± 1.22 μM and 1, respectively.

Table 1. Tubulin binding activity and cytotoxicity of compounds.

Compound	K_d [μM][a]	Cytotoxicity IC_{50} [nM]	
		HT-29	HuVEC
1 NPI-2358	1.46 ± 0.15	14.9 ± 3.8	4.5 ± 1.3
2 KPU-244	0.42 ± 0.11	3.9 ± 1.3	n.d.
3 KPU-244-B1	7.95 ± 1.22	910 ± 380	130 ± 20
4 KPU-244-B2	7.19 ± 1.04	1100 ± 344	354 ± 29
colchicine	3.32 ± 0.31	16.2 ± 3.0	n.d.

[a] Bovine tubulin concentration: 0.5 μM.

respectively. Their higher molecular weights and greater hydrophilicity might contribute to poorer cell uptake. By considering all these findings, we concluded that **3** and **4** functionally act as antimicrotubule agents.

Tubulin photoaffinity labeling using compounds 3 and 4

Having confirmed the tubulin-binding activity of compounds **3** and **4**, bovine tubulin was photoirradiated at 365 nm in the absence or presence of these compounds on ice after incubation at 37 °C in MES buffer (pH 6.8). These samples were then applied to SDS-PAGE by using 7.5 %T polyacrylamide gels and transferred to nitrocellulose membrane,[13] followed by enzymatic detection with an avidin–biotin system (ECL streptavidin–HRP conjugate, GE healthcare) with a luminol reagent as the enzyme substrate. Nonspecific binding was not observed in the non-photoirradiated sample (Figure 2 A, lanes 1, 6, 11).

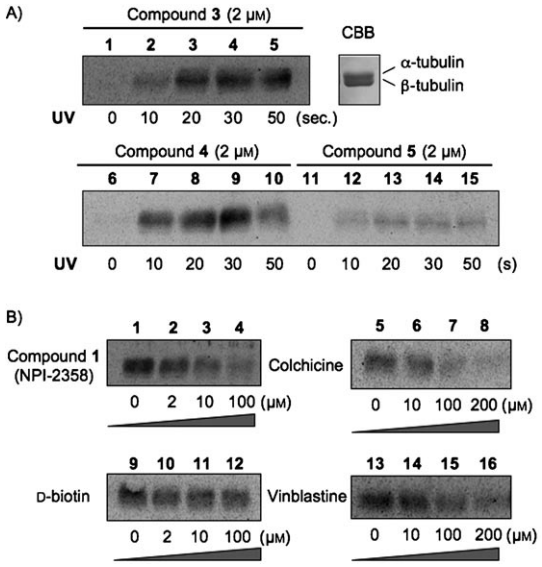


Figure 2. Photoaffinity labeling of tubulin: A) Photoaffinity labeling of tubulin by compounds **3**, **4**, and **5** at different irradiation times. Tubulin (2 μM) and compounds **3**, **4**, and **5** (2 μM, 2% DMSO) were incubated at 37 °C for 30 min in MES buffer. Afterward, samples were irradiated by UV lamp (365 nm, 200 W) for 0–50 s. Photolabeled samples were then resolved by SDS-PAGE by using 7.5 %T polyacrylamide gels. Gels were analyzed by Coomassie Brilliant Blue staining or Western blotting followed by enzymatic detection system by using ECL streptavidin–HRP conjugate. B) Photoaffinity labeling of tubulin with compound **4** in the absence or presence of **1** (NPI-2358), colchicine, vinblastine, or D-biotin. The photoaffinity labeling by compound **4** and Western blotting analysis were performed as described in Figure 2 A.

However, in the photoirradiated samples, a significant irradiation time-dependent labeling was observed (Figure 2 A, lanes 2–5, 7–10). On the other hand, in the case of the samples that were photoirradiated in the presence of photoaffinity negative control **5**, irradiation time-dependent labeling was weakly detected (Figure 2 A, lanes 11–15).

Furthermore, to understand the specificity of photoaffinity binding, a competitive photoaffinity labeling study on compound **4** was carried out in the absence or presence of compound **1** (NPI-2358), colchicine, vinblastine, or D-biotin. Concentration-dependent inhibition of photoaffinity labeling was observed in the presence of compound **1** (NPI-2358) and colchicine (Figure 2 B, lanes 1–8), whereas no competitive inhibition was observed when D-biotin was used as a negative control (Figure 2 B, lanes 9–12). These results seem to be reasonable, because, although the three-dimensional structures of antimicrotubule DKPs are different from that of colchicine, the original phenylahistin [(−)-PLH] is known to recognize the colchicine-binding site by radio-binding assay.[3b] However, the observed weak competitive inhibition with vinblastine (Figure 2 B, lanes 13–16) also suggests more complicated interactions between compound **4** and tubulin. Although the different binding sites of colchicine and vinblastine on tubulin were recently determined by X-ray structural analysis,[14] some examples of long-range effects of these ligands upon each other were also reported.[15] Therefore, some conformational changes of tubulin that are caused by vinblastine binding might influence the photoaffinity labeling of compound **4**. These results also indicate that NPI-2358 and its derivatives can recognize the same binding site on tubulin as compound **4**; this suggests that compound **4** functions as a useful chemical probe for anticancer drug candidate **1**. Further analysis with photoaffinity probes is now underway to understand the binding selectivity to both α and β-tubulin and to determine the actual modified amino acid residues that are affected by the photoaffinity probes. These findings would form a basis for further studies on the precise binding site of NPI-2358 and its microtubule depolymerization mechanism.

Conclusions

We designed and synthesized biotin-tagged derivatives **3** and **4** based on a potent new antimicrotubule agent, KPU-244, which is a photoreactive benzophenone derivative of clinical candidate NPI-2358 and is a anticancer VDA. According to tubulin polymerization and tubulin-binding assays based on fluorescence quenching, we observed that derivatives **3** and **4** recognized tubulin and behaved as antimicrotubule agents. Furthermore, our photoaffinity labeling study revealed an irradiation time-dependent tubulin recognition by these compounds. Also, this labeling by derivative **4** was competitively inhibited by NPI-2358 or colchicine, and weakly by vinblastine. These results suggest that derivative **4** functions as a useful photoaffinity probe with the same recognition site as the anticancer VDA NPI-2358; this means that it is probably near the colchicine-binding site on β-tubulin, which is located at the intradimer space between α- and β-tubulin. However, it is noteworthy that the present results also suggest more complicated binding interactions with tubulin that involve the vinblastine binding site. Further investigations with photoaffinity probe **4** would contribute to a better understanding of the microtubule depolymerization mechanism of anticancer agent NPI-2358, which is currently in a phase I clinical trial.

Experimental Section

Reagents and solvents were purchased from Wako Pure Chemical Ind., Ltd. (Osaka, Japan), Nakalai Tesque (Kyoto, Japan), and Aldrich Chemical Co. Inc. (Milwaukee, WI, USA) and used without further purification. Bovine brain tubulin was purchased from Cytoskeleton, Inc. (Denver, Colorado, USA). All other chemicals were of the highest commercially available purity. Analytical thin-layer chromatography (TLC) was performed on Merck silica gel $60F_{254}$ precoated plates. Preparative HPLC was performed by using a C18 reversed-phase column (19×100 mm; SunFire™ Prep C18 OBD™ 5 μm, Waters, CA, USA) with binary solvent system: linear gradient of CH_3CN in 0.1% aq TFA at a flow rate of 15 mL min^{-1}, detection at UV 230 and 365 nm. The solvents that were used for HPLC were of HPLC grade. All other chemicals were of analytical grade or better. Melting points were measured on a Yanagimoto micro hot-stage apparatus (Yanaco, Kyoto, Japan) and are uncorrected. Proton (^{1}H) and carbon (^{13}C) NMR spectra were recorded on either a JEOL JNM-AL300 spectrometer (Tokyo, Japan) operating at 300 MHz for proton and 75 MHz for carbon, or a Varian UNITY INOVA 400NB spectrometer operating at 400 MHz for proton and 101 MHz for carbon. Chemical shifts were recorded as δ values in parts per million (ppm) downfield from tetramethylsilane (TMS). Low- and high-resolution mass spectra (EI, CI) were recorded on a JEOL JMS-GCmate. Fast atom bombardment mass spectrometry (FAB-MS) was performed on a JEOL JMS-SX102A spectrometer that was equipped with the JMA-DA7000 data system. Elemental analyses were done on a Perkin–Elmer Series CHNS/O Analyser 2400.

3-Cyano-*N*-methoxy-*N*-methylbenzamide 7: *N*,*O*-dimethylhydroxylamine hydrochloride (3.48 g, 35.68 mmol), Et_3N (5 mL, 35.68 mmol) and 1-ethyl-3-(3-dimethyllaminopropyl)carbodiimide hydrochloride (EDC·HCl, 6.52 g, 35.68 mmol) were added to a solution of 3-cyanobenzoic acid (**5**, 5.0 g, 33.98 mmol) in DMF (110 mL). After the mixture was stirred for 3 h at room temperature, the solvent was removed in vacuo, the residue was dissolved in EtOAc, washed with 10% citric acid, 10% $NaHCO_3$, and sat. NaCl, and dried over Na_2SO_4. The solvent was then removed to give a colorless oil. yield: 7.9 g (99%). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 3.39 (s, 3H), 3.54 (s, 3H), 7.54 (t, J = 7.9 Hz, 1H), 7.75 (d, J = 7.9 Hz, 1H), 7.94 (d, J = 7.9 Hz, 1H), 8.01 ppm (m, 1H); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 33.1, 61.2, 112.3, 118.1, 128.9, 131.9, 132.5, 133.8, 135.1, 167.2 ppm; HRMS (EI): *m/z*: calcd for $C_{10}H_{10}N_2O_2$: 190.1742 $[M]^+$; found: 190.0742.

4-*N*-Boc-aminomethylbromobenzene 8: Di-tert-butyl dicarbonate ($(Boc)_2O$, 1.03 g, 4.71 mmol) and triethylamine (Et_3N, 0.94 mL, 6.74 mmol) were added to a solution of 4-bromobenzylamine (1.0 g, 4.49 mmol) in acetonitrile (10 mL), and the mixture was stirred for 1.5 h at room temperature. After the solvent was removed in vacuo, the residue was dissolved in EtOAc, washed with 10% citric acid, 10% $NaHCO_3$ and sat. NaCl (1×), and dried over Na_2SO_4. The solvent was then removed to give a solid, which was recrystalized from diethyl ether/hexane (1:1) to give a white solid of compound **8**. Yield: 1.4 g (99%); mp 86–88 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.49 (s, 9H), 4.26 (d, J = 5.9 Hz, 2H), 4.84 (br s, 1H), 7.15 (d, J = 8.3 Hz, 2H), 7.44 ppm (d, J = 8.3 Hz, 2H); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 28.3, 44.0, 79.7, 121.1, 129.1, 131.6, 138.0, 155.8 ppm; HRMS (EI): *m/z*: calcd for $C_{12}H_{16}Br\,NO_2$: 285.0364 $[M]^+$; found: 285.0365.

***tert*-Butyl 4-(3-cyanobenzoyl)benzylcarbamate 9**: A 1.58 M solution of *n*BuLi solution in *n*-hexane (1.1 mL, 1.25 mmol) was added dropwise to a solution of compound **8** (200 mg, 0.700 mmol) in anhydrous diethyl ether (2.3 mL) at 0 °C under an argon atmosphere. After the mixture was stirred for 1 h at the same temperature, the mixture was slowly added to a solution of compound **7** (139 mg, 0.735 mmol) in THF (2 mL) at −78 °C under an argon atmosphere. The cooling bath was removed and the mixture was stirred for overnight at room temperature. The solution was poured into ice-cold 1 M HCl (18 mL), neutralized with a powder of $NaHCO_3$, and the organic phase was extracted with EtOAc, washed with brine, and dried over Na_2SO_4. Then the solvent was removed under reduced pressure, and the resulting brown oil was purified by silica gel column chromatography (*n*-hexane /EtOAc, 3:1) to yield a white solid. Yield: 38.8 mg (17%); mp 130–133 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.48 (s, 9H), 4.42 (d, J = 6.1 Hz, 2H), 4.99 (br s, 1H), 7.43 (d, J = 8.1 Hz, 2H), 7.63 (t, J = 7.9 Hz, 1H), 7.75 (d, J = 8.1 Hz, 2H), 7.87 (d, J = 7.9 Hz, 1H), 8.05 (m, 1H), 8.02 ppm (br d, J = 7.9 Hz, 1H); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 28.3, 44.2, 79.9, 112.8, 117.9, 127.3, 129.3, 130.4, 133.3, 133.7, 135.2, 135.3, 138.6, 144.9, 155.9, 193.9 ppm; HRMS (CI): *m/z*: calcd for $C_{20}H_{21}N_2O_3$ $[M+H]^+$: 337.1552; found: 337.1549.

***tert*-Butyl 4-(3-formylbenzoyl)benzylcarbamate 10**: A 1.01 M solution of DIBALH in toluene (0.343 mL, 0.346 mmol) was added dropwise to a solution of compound **9** (38.8 mg, 0.115 mmol) in anhydrous toluene at −76 °C under an argon atomosphere. The cooling bath was then removed and the mixture was stirred for 3 h at 0 °C. The reaction was quenched by the addition of MeOH/AcOH (2:1), diluted with EtOAc, and filtered to remove the produced $Al(OH)_3$. After the resultant organic phase was washed with 5% $NaHCO_3$ and brine, and dried over Na_2SO_4, the solvent was removed under reduced pressure to obtain a white solid. To a solution of this solid in THF (1.2 mL) was added Dess–Martin periodinane (63.92 mg, 0.151 mmol). After the mixture was stirred for 2 h at room temperature, the reaction was quenched by the addition of MeOH (1 mL), then diluted with EtOAc, washed with 10% $NaHCO_3$ and brine, and dried over Na_2SO_4. Then the solvent was removed under reduced pressure to yield a colorless oil; yield: 42 mg (86%). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.48 (s, 9H), 4.43 (d, J = 6.0 Hz, 2H), 5.05 (br s, 1H), 7.43 (d, J = 7.9 Hz, 2H), 7.68 (t, J = 7.6 Hz, 1H), 7.78 (d, J = 8.3 Hz, 2H), 8.03–8.13 (m, 2H), 8.26 (m, 1H), 10.09 ppm (s, 1H); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ = 28.3, 44.3, 127.3, 127.9, 129.2, 130.4, 131.3, 131.6, 132.6, 133.0, 135.4, 135.7, 136.3, 138.5, 141.6, 144.5, 191.4, 195.1 ppm; HRMS (EI): *m/z*: calcd for $C_{20}H_{21}NO_4$: 339.1470 $[M]^+$; found: 339.1473.

Ethyl 5-(*tert*-butyl)oxazole-4-carboxylate 13: According to the report by Suzuki et al.[16] DBU (34.3 mL, 243 mmol) and pivalic anhydride (49.3 mL, 243 mmol) were added to a solution of ethyl isocyanoacetate (25 g, 221 mmol) in THF (200 mL), and the mixture was stirred overnight at room temperature. After the solvent was removed by evaporation in vacuo, the residue was extracted with EtOAc (200 mL), washed with 10% Na_2CO_3, 10% citric acid, and saturated NaCl (3×), and dried over anhydrous Na_2SO_4. Then the solvent was removed in vacuo. The residual oil was purified by silica gel column chromatography by using hexane/EtOAc (20:1 to 4:1) to give an oil (66.4 g, 99%); ^{1}H NMR (300 MHz $CDCl_3$): δ = 1.41 (t, J = 7.2 Hz, 3H), 1.46 (s, 9H), 4.39 (q, J = 7.2 Hz, 2H), 7.70 ppm (s, 1H); HRMS (EI): *m/z*: calcd for $C_{10}H_{15}NO_3$: 197.1052; found: 197.1050 $[M]^+$.

5-(*tert*-Butyl)oxazole-4-carboxaldehyde 14: $LiAlH_4$ (3.84 g, 101 mmol) was added portionwise to a solution of ethyl 5-(*tert*-butyl)oxazole-4-carboxylate (**13,** 20 g, 101 mmol) in anhydrous THF (250 mL) under an argon atmosphere at −60 °C, and the bath temperature was gradually increased up to −40 °C with stirring for 2 h. (If the temperature was higher than −40 °C, the reduction of the oxazole ring predominantly proceeded.) After the mixture was

quenched with aq sat. NH_4Cl (50 mL) at −60 °C, EtOAc (250 mL) was added, and the resulting precipitate was removed by Celite filtration. The filtrate was washed with H_2O, 10% citric acid and sat. NaCl (3×), dried over anhydrous Na_2SO_4, and concentrated in vacuo to give an oil of the corresponding oxazole alcohol (10.6 g, 67%). This oil was used in the next oxidation without further purification. MnO_2 (27.7 g, 319 mmol) was added to a solution of oxazole alcohol (9.9 g, 64 mmol) in acetone (200 mL), and the mixture was stirred at room temperature overnight. After the mixture was filtered to remove MnO_2, the solvent was removed by evaporation and the residual white powder was purified by silica gel column chromatograpy by using $CHCl_3$ as an eluant to give an oil. Yield: 5.54 g (38% in two steps). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.47 (s, 9H), 7.77 (s, 1H), 10.10 ppm (s, 1H); HRMS (EI): *m/z*: calcd for $C_8H_{11}NO_2$: 153.0790 $[M]^+$; found: 153.0794.

1-Acetyl-3-{(*Z*)-1-[5-(*tert*-butyl)-4-oxazolyl]methylidene}]-2.5-piperazinedione 16: *N*,*N*′-Diacetyl-2,5-diketopiperazinedione (**15,** 2.1 g, 10.6 mmol)[3d] was added to a solution of 5-(*tert*-butyl)oxazole-4-carboxaldehyde **14** (1.0 g, 7.1 mmol) in DMF (10 mL), and the solution was repeatedly evacuated in a short time to remove oxygen and then flushed with argon; Cs_2CO_3 (3.9 g, 12.0 mmol) was added, and the evacuation–flushing process was repeated again. The resultant mixture was stirred for 6 h at 45 °C. After the solvent was removed by evaporation, the residue was purified by column chromatography on silica by using $CHCl_3$ as an eluant to give a pale-yellow solid. Yield: 1.15 g (56%); mp 145–147 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.45 (s, 9H), 2.65 (s, 3H), 4.48 (s, 2H), 7.09 (s, 1H), 7.83 (s, 1H), 11.22 ppm (br s, 1H); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 27.3, 29.5, 33.6, 46.3, 105.6, 126.0, 128.7, 148.1, 159.8, 162.1, 172.6 ppm; HRMS (EI): *m/z*: calcd for $C_{14}H_{17}N_3O_4$: 291.1219 $[M]^+$; found: 291.1217.

3-{(*Z*)-1-[5-(*tert*-Butyl)-4-oxazolyl]methylidene}-6-{(*Z*)-3-[4-*N*-Boc-aminomethylbenzoyl]benzylidene}-2.5-piperazinedione 17: Compound **16** (397 mg, 1.2 mmol) was added to a solution of *tert*-butyl 4-(3-formylbenzoyl) benzylcarbamate (**10,** 324 mg, 1.1 mmol) in DMF (10 mL), and the solution was repeatedly evacuated to remove oxygen and flushed with argon. Cs_2CO_3 (544 mg, 1.7 mmol) was added, and the evacuation–flushing process was repeated again. The resultant mixture was stirred overnight at room temperature. After the solvent was removed by evaporation, the residue was extracted with EtOAc, washed with 10% citric acid, 5% $NaHCO_3$ and brine, and dried over Na_2SO_4. Then the solvent was removed under reduced pressure and the resultant residue was purified by silica gel chromatography ($CHCl_3$/MeOH, 30:1) to yield a pale-yellow solid. Yield: 498 mg (78%); ^{1}H NMR (300 MHz, [D_6]DMSO): δ = 1.40 (s, 9H), 1.40 (s, 9H), 4.23 (d, *J* = 6.4 Hz, 2H), 6.72 (s, 1H), 6.86 (s, 1H), 7.39–7.45 (m, 2H), 7.50 (br t, *J* = 6.4 Hz, 1H), 7.55–7.62 (m, 2H), 7.72–7.83 (m, 4H), 8.60 (s, 1H), 10.57 (br s, 1H), 11.11 ppm (br s, 1H); ^{13}C NMR (75.5 MHz, [D_6]DMSO) δ 28.2, 29.1, 33.0, 43.2, 78.0, 100.7, 114.1, 126.8, 126.9, 127.1, 128.1, 128.8, 129.0, 130.0, 130.1, 133.2, 133.3, 135.3, 137.5, 145.4, 150.1, 155.8, 156.2, 157.0, 157.9, 195.1 ppm; HRMS (EI): *m/z*: calcd for $C_{32}H_{34}N_4O_6$: 570.2478 $[M]^+$; found: 570.2489.

3-{(*Z*)-1-[5-(*tert*-Butyl)-4-oxazolyl]methylidene}-6-{(*Z*)-3-[4-*N*-biotinylaminomethylbenzoyl]benzylidene}-2,5-piperazinedione (KPU-244-B1) 3: For Boc-deprotecton, compound **17** (125 mg, 0.22 mmol) was dissolved in 4 M HCl in dioxane (1.5 mL), and stirred at room temperature for 1 h. After the solvent was removed by evaporation, the residue was washed with Et_2O (3×). HOAt (45 mg, 0.33 mmol), EDC·HCl (63 mg, 0.33 mmol), and Et_3N (46 μL, 0.33 mmol) were added to a solution of this residue and D-biotin (67 mg, 0.33 mmol) in DMF (7 mL), and the mixture was stirred at room temperature for 24 h. After the solvent was removed by evaporation in vacuo, the residue was extracted with EtOAc, washed with 10% citric acid, 5% $NaHCO_3$ and saturated NaCl, and dried over anhydrous Na_2SO_4. Then the solvent was concentrated in vacuo, and the resultant residue was purified by preparative HPLC (with a linear gradient of 40–55% CH_3CN in 0.1% aq TFA over 30 min) to give a pale-yellow powder. Yield: 24.3 mg (16%); ^{1}H NMR (400 MHz, [D_6]DMSO): δ = 1.23–1.67 (m, 6H), 1.40 (s, 9H), 2.18 (t, *J* = 7.4 Hz, 2H), 2.57 (d, *J* = 12.4 Hz, 1H), 2.81 (dd, *J* = 5.0, 12.4 Hz, 1H), 3.06–3.14 (m, 1H), 4.12 (dd, *J* = 4.2, 7.5 Hz, 1H), 4.30 (dd, *J* = 4.8, 7.5 Hz, 1H), 4.37 (d, *J* = 6.0 Hz, 2H), 6.25–6.48 (br s, 1H), 6.40 (br s, 1H), 6.72 (s, 1H), 6.86 (s, 1H), 7.43 (d, *J* = 8.1 Hz, 2H), 7.54–7.66 (m, 2H), 7.71–7.83 (m, 4H), 8.41 (t, *J* = 6.3 Hz, 1H), 8.59 (s, 1H), 10.57 (s, 1H), 11.1 ppm (s, 1H); HRMS (FAB): *m/z*: calcd for $C_{37}H_{41}N_6O_6S$: 697.2808 $[M+H]^+$; found: 697.2800; elemental analysis calcd (%) for $C_{37}H_{40}N_6O_6S{\cdot}2H_2O{\cdot}0.25CF_3COOH$: C 59.16, H 5.86, N 11.04; found: C 59.35, H 5.65, N 10.74.

D-Biotinylaminohexanoylaminohexanoic acid 18: This compound was synthesized by a Fmoc-based solid-phase method on Wang resin (1.1 mmol g^{-1}) by coupling with Fmoc-aminohexanoic acid and D-biotin by using a conventional procedure.[17] After deprotection of the peptide resin with the TFA–thioanisole–*m*-cresol system, the obtained crude product was purified by a reversed-phase HPLC (with a linear gradient of 0–40% CH_3CN in 0.1% aq TFA over 30 min) to give a white powder of compound **18**. Yield: 59 mg (13% from the resin); ^{1}H NMR (400 MHz, [D_6]DMSO): δ = 1.18–1.65 (m, 18H), 2.02 (t, *J* = 6.8 Hz, 2H), 2.04 (t, *J* = 7.2 Hz, 2H), 2.18 (t, *J* = 7.6 Hz, 2H), 2.57 (d, *J* = 6.0 Hz, 1H), 3.04 (t, *J* = 6.8 Hz, 2H), 3.07–3.12 (m, 1H), 4.11–4.14 (m, 1H), 4.30 (dd, *J* = 4.8, 7.6 Hz, 1H), 6.35 (br s, 1H), 6.42 (br s, 1H), 7.69–7.74 ppm (m, 2H); HRMS (FAB): *m/z*: calcd for $C_{22}H_{39}N_4O_5S$: 471.2641 $[M+H]^+$; found: 471.2638.

3-{(*Z*)-1-[5-(*tert*-Butyl)-4-oxazolyl]methylidene}-6-{(*Z*)-3-[4-*N*-(D-biotinylaminohexanoylaminohexanoyl) aminomethylbenzoyl] benzylidene}-2.5-piperazinedione (KPU-244-B2) 4: According to the procedure for the synthesis of compound **3**, HOAt (11.5 mg, 0.084 mmol), EDC·HCl (16.2 mg, 0.084 mmol), Et_3N (20 μL, 0.084 mmol) were added to a solution of deprotected compound **17** (40 mg, 0.071 mmol, Boc-form) and compound **18** (40 mg, 0.084 mmol) in DMF (4 mL), and the mixture was stirred at room temperature overnight. After the solvent was removed by evaporation in vacuo, the residue was extracted with EtOAc, washed with 10% citric acid, 5% $NaHCO_3$, and sat. NaCl, and dried over anhydrous Na_2SO_4. Then the solvent was concentrated in vacuo and the obtained residue was purified by preparative HPLC (with a linear gradient of 35–55% CH_3CN in 0.1% aq TFA over 40 min) to give a pale-yellow powder; yield: 20 mg (31%). ^{1}H NMR (400 MHz, [D_6]DMSO): δ = 1.13–1.65 (m, 18H), 1.40 (s, 9H), 2.02 (t, *J* = 7.2 Hz, 2H), 2.03 (t, *J* = 7.2 Hz, 2H), 2.16 (t, *J* = 7.2 Hz, 2H), 2.57 (d, *J* = 12.5 Hz, 1H), 2.81 (dd, *J* = 5.1, 12.5 Hz, 1H), 3.00 (q, *J* = 6.0 Hz, 4H), 3.06–3.11 (m, 1H), 4.10–4.14 (m, 1H), 4.28–4.31 (m, 1H), 4.37 (d, *J* = 5.5 Hz, 2H), 6.23–6.44 (br s, 1H), 6.42 (br s, 1H), 6.72 (s, 1H), 6.86 (s, 1H), 7.43 (d, *J* = 8.0 Hz, 2H), 7.55–7.68 (m, 2H), 7.70–7.83 (m, 6H), 8.42 (t, *J* = 6.0 Hz, 1H), 8.61 (s, 1H), 10.59 (s, 1H), 11.12 ppm (s, 1H); HRMS (FAB): *m/z*: calcd for $C_{49}H_{63}N_8O_8S$: 923.4490 $[M+H]^+$; found 923.4493.

Tubulin binding assays based on the fluorescence quenching: Tubulin (800 μL, 0.5 μM) in MES buffer (0.1 M MES, 0.5 mM $MgCl_2$, 1 mM EGTA, 1 mM GTP, pH 6.8) was incubated with different concentrations of the test compounds (0–20 μM, 1% DMSO) at 37 °C for 1 h. After incubation, the fluorescence of each solution was measured (excitation at 295 nm, emission at 300–450 nm) by using

an FP-750 spectrofluorometer (JASCO, Tokyo, Japan). The fluorescence of all samples were corrected for inner filter effects. The concentrations of tubulin–compound complex were estimated from the decreased fluorescence intensity at 335 nm, and the dissociation constant between tubulin and compounds were calculated by using the following binding equations (1), (2) of the Langmuir model.

$$\Delta FL = \theta C_b \quad (1)$$

$$C_b = 0.5\,[(C_t + n\,P_t + K_d) - \{(C_t - n\,P_t - K_d)^2 + 4\,K_d\,C_t\}^{1/2}] \quad (2)$$

ΔFL: the decreased fluorescence intensity of tubulin (arbitrary unit/cm), C_b: the concentration of tubulin–compound complex (μM), θ: the molar fluorescence intensity of tubulin (arbitrary unit/μM cm^{-1}), C_t: the total concentration of compounds (μM), P_t: the total concentration of tubulin (μM), n: the number of binding sites for compounds with tubulin, K_d: the dissociation constant of compounds with tubulin (μM).

Tubulin polymerization assay: Polymerization of tubulin was monitored by an increase in turbidity at 37 °C. Microtubule associated protein (MAP)-rich tubulin (2 mg mL^{-1}) was mixed with compounds **3**, **4**, **5** (10 μM, 1% DMSO), or NPI-2358 (5 μM, 1% DMSO) on ice in PIPES buffer (80 mM PIPES, 2 mM $MgCl_2$, 0.5 mM EGTA, 1 mM GTP, 5% glycerol, pH 6.9) that had been incubated on ice for 10 min. Then, the samples were incubated at 37 °C to initiate polymerization by a temperature shift. Turbidity was measured with a thermocontrolled spectrophotometer (model 680XR microplate reader™, BIO-RAD Laboratories, CA, USA) at 340 nm every 30 s for 15 min.

In vitro cytotoxicity assay: The cytotoxicity assays were performed essentially as described previously.[4a] Briefly, human colon adenocarcinoma (HT-29) cells and human umbilical vein endothelial cells (HuVEC) were plated in 96-well flat-bottomed plates and allowed to attach for 24 h at 37 °C. Serially diluted compounds were added in triplicate to cells at final concentrations that ranged from 2 pM to 20 μM. Cells were treated with a final concentration of 0.25% (*v*/*v*); DMSO served as the vehicle control. Cell viability was assessed 48 h later by measuring the reduction of resazurin with a fluorimeter (Perkin–Elmer, Torrance, California, USA). The IC_{50} values (the drug concentration at which 50% of the maximal observed cytotoxicity is established) were calculated in Prism (GraphPad, San Diego, California, USA) or XLFit 3.0 (ID Business Solutions, Emeryville, California, USA) by using a sigmoidal dose–response model.

Photoaffinity labeling: A solution of compounds **3** or **4** in DMSO (final concentration; 2 μM) was added to a tubulin solution (2 μM) in MES buffer (0.1 M MES, 0.5 mM $MgCl_2$, 1 mM EGTA, 1 mM GTP, 2% DMSO, pH 6.8), and the solution was incubated at 37 °C for 30 min, followed by UV irradiation at 365 nm at a distance of 10 cm on ice for appropriate time by using a UV irradiator (model L2859–01; Hamamatsu Photonics, Hamamatsu, Japan).

SDS-PAGE, Western blotting: Photolabeled tubulin was separated by SDS-PAGE in 7.5%T polyacrylamide gels and transferred to nitrocellulose membrane. The membrane was incubated with a blocking solution containing 5% (*w*/*v*) skim milk in PBS-T buffer (137 mM NaCl, 8.10 mM Na_2HPO_4, 2.68 mM KCl, 1.47 mM KH_2PO_4, 0.1% Tween 20, pH 7.4) at room temperature for 1 h and washed with PBS-T (1×20 min and 2×10 min). For the detection of photolabeled proteins, the membrane was incubated with streptavidin–horseradish peroxidase conjugate (GE Healthcare) for 1 h at room temperature, and washed again with PBS-T as the same manner mentioned above. The membrane was treated with ECL Western Blotting detection reagents (GE Healthcare), and the emission was detected by using an imaging system (LAS-1000plus, FUJIFILM).

Competitive assay with NPI-2358, colchicine, vinblastine and D-biotin: Tubulin (2 μM) was preincubated with a different concentrations of NPI-2358, colchicine, vinblastine or D-biotin in MES buffer for 30 min at room temperature, followed by addition of compound **4** (2 μM in DMSO). After incubation at 37 °C for 30 min, samples were UV irradiated at 365 nm at a distance of 10 cm on ice for 25 s, and separated by SDS-PAGE. Photolabeled tubulin was detected by streptavidin–HRP/ECL system.

Acknowledgements

This research was supported by various grants from MEXT (Ministry of Education, Culture, Sports, Science and Technology, Japan), including the 21st Century COE Program. We are grateful to Prof. T. Koide, Waseda University, for photoirradiation, and K. Oda and I. Takao for mass spectra measurements. We thank Dr. J.-T. Nguyen for his manuscript revision.

Keywords: alkaloids · anticancer agents · photoaffinity labeling · tubulin · vascular-disrupting agent

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Received: May 10, 2008
Published online on November 14, 2008

