DOI: 10.1002/chem.201102293

Water-Soluble Prodrug of Antimicrotubule Agent Plinabulin: Effective Strategy with Click Chemistry

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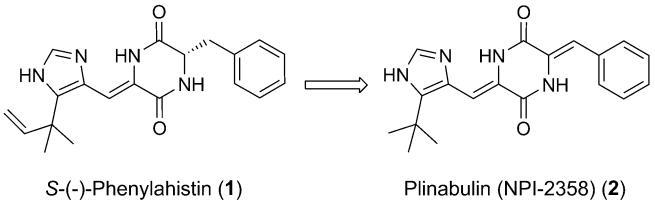
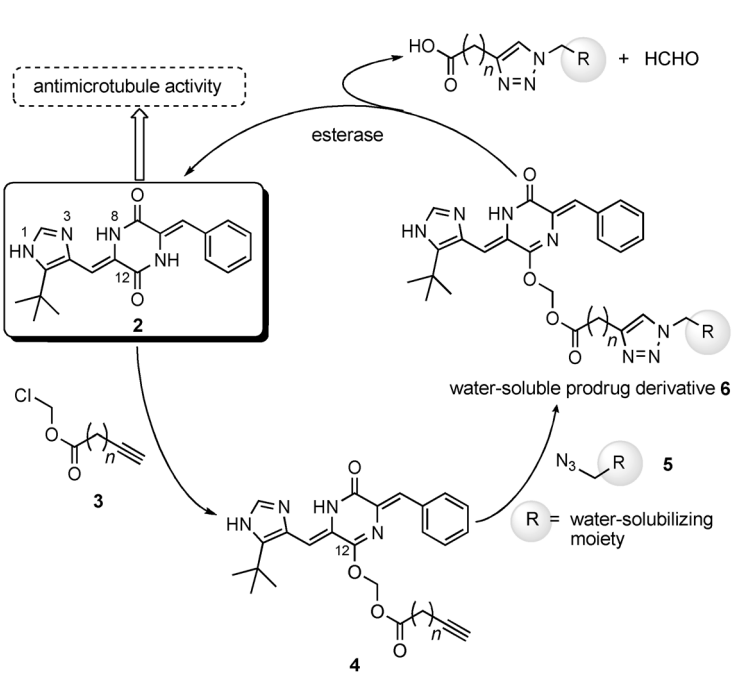
The diketopiperazine (DKP)-containing natural product phenylahistin (**1**) was isolated from *Aspergillus ustus* in 1997 and found to have potential anti-microtubule activity (cytotoxicity: $IC_{50}=394$ nM in HT-29 cells).[1] The total synthesis of **1** was achieved by our group,[2a] Couladouros and co-workers,[2b,c] as well as Joullié and co-workers.[2d] The dedicated structure-activity relationship studies of **1** led us to the discovery of plinabulin[3] (**2**; NPI-2358, cytotoxicity: $IC_{50}=$ 15 nM in HT-29 cells) and phase II clinical trials with intravenous (i.v.) injection were undertaken in the US[4] as a promising vascular disrupting agent (VDA).[5]



Although **2** exhibited potential antitumor activity, its solubility was not compatible with the requirements of a drug ($<0.1\ \mu g\,mL^{-1}$). Compound **2** has been injected with a solubilizing agent, but long-term administration may have the possibility of unexpected safety issues or problems in the quality of life of the patient. Hence, we attempted to improve the aqueous solubility of **2** to increase its pharmaceutical value. Here, we report a novel monolactim prodrug form of plinabulin.

Two methods are generally used to improve the solubility of a drug in water. First, the mother skeleton may be transformed into a soluble structure, and second, aqueous substituents may be attached to the original skeleton.[6] In our case, the latter was selected to prevent complications associated with reconstructing **2**, and at the same time, maintain the attractive activity of the compound.

To produce the desired candidates efficiently by using a short-step synthesis, complicated transformations should be avoided and an aqueous substituent had to be introduced directly without the use of protective polar functional groups. As a result of repeated examination, we planned the effective strategy by using esterase hydrolysis and click chemistry.[7,8] The Huisgen reaction, in particular, was adequate for the purpose of high chemoselectivity, high yield, and mild conditions with unprotected substituents.[9] Our strategy is shown in Scheme 1.



Scheme 1. The strategy for preparing the water-soluble prodrug of plinabulin (**2**) using click chemistry.

The C12 amide part of **2** was O-alkylated[10] with alkynoate **3**, then the alkyne part of **4** was reacted with the aqueous substituent **5** by using the Huisgen reaction. The ester moiety of **6** was hydrolyzed by esterase to produce the parent compound **2**. By this process, formaldehyde was generated, but prompt metabolic activity produced carbon dioxide that could be exhaled, as reported.[11]

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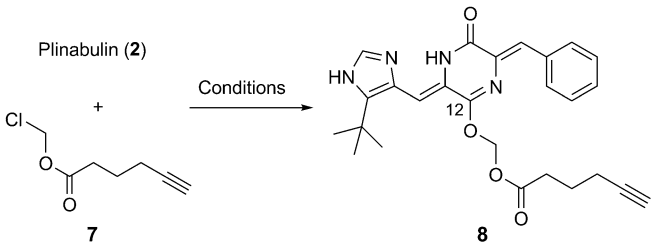
Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/chem.201102293.

Table 1. Synthesis of the alkyne-containing monolactim **8**.

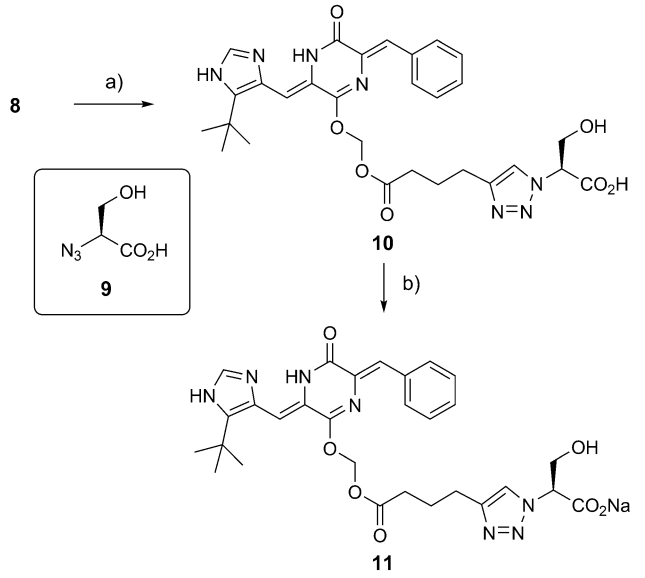


Entry	**7** [equiv]	Base	Solvent	*T* [°C]	*t* [h]	Yield [%]
1	5.0	DBU[a]	DMF	50	8	18
2	2.5	*t*BuOK	THF	RT	8	15
3	1.2	Cs_2CO_3	DMF	RT	8	29
4	2.0	Cs_2CO_3	DMF	50[b]	0.25	54
5	2.0	Cs_2CO_3[c]	DMF	50[b]	0.25	44
6	2.0	Cs_2CO_3	DMSO	50[b]	0.25	51
7	2.0	Cs_2CO_3[c]	DMSO	50[b]	0.25	52

[a] Tetrabutylammonium hydrogen sulfate was added. [b] Microwave irradiation. [c] Tetrabutylammonium iodide was added.

According to Scheme 1, plinabulin (**2**) was O-alkylated with the linker **7** under basic conditions (Table 1). The alkynoate **7** was synthesized from the readily commercially available 5-hexynoic acid and chloromethyl chlorosulfate.[12] Under the conditions, in the presence of 1,8-diazabicyclo-[5.4.0]undec-7-ene (DBU), most of the starting material **2** was recovered, even though the phase transfer catalyst was co-present (Table 1, entry 1). *t*BuOK gave the desired monolactim **8** in only 15%, because the product tended to undergo decomposition (Table 1, entry 2). When other bases (NaH, lithium diisopropylamide (LDA), and lithium hexamethyldisilazide (LHMDS)) were examined, O or N-alkylation did not proceed significantly and the starting material was recovered. Fortunately, Cs_2CO_3 provided the desired compound **8** in a moderate yield (Table 1, entry 3–7). In particular, microwave irradiation provided the monolactim **8** in 54% yield (Table 1, entry 4) and the starting material was also recovered. The phase transfer catalyst and solvent were examined, but much higher yield was not obtained (Table 1, entry 5–7). The effects of the reaction temperature were also measured; temperatures exceeding 50°C led to decomposition of the product. In addition, iodine or bromide alkynoates were tested, however, the yield of **8** was not improved. The tautomerization of the C12 amide and the vulnerable ester moiety made this O-alkylation challenging, but **8** was obtained preferentially. The structure of **8** was determined by X-ray crystallography.[13]

With the alkyne-containing lactim **8** in hand, we turned our attention to the synthesis of the water-soluble prodrug derivatives. The serine derivative **9** (Scheme 2) was prepared to introduce an aqueous substituent, which was obtainable through a diazotransfer reaction in three steps as reported by Tirrell.[14] Amino acid derivatives are commonly used to produce water-soluble prodrugs[15] because of their high solubility and low toxicity. In particular, a serine residue with a

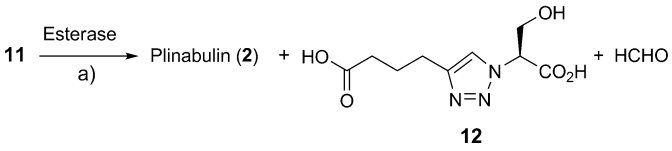


Scheme 2. Synthesis of the water-soluble prodrug candidate **11**. Reagents and conditions: a) Compound **9**, sodium ascorbate, $CuSO_4 \cdot 5H_2O$, H_2O/*t*BuOH/DMF (1:1:1), 50°C, microwave, 10 min then HPLC purification, 67%; b) ion exchange resin DIAION WK11, eluent H_2O/CH_3CN (1:1), 96%.

β-hydroxy carboxylic acid can contribute to the water solubility of a compound and ease of handling.

Compound **8** was combined with **9** by Huisgen reaction with $CuSO_4 \cdot 5H_2O$ and sodium ascorbate in H_2O/*t*BuOH/DMF under microwave irradiation. This reaction proceeded smoothly within 10 min and **10** was obtained as a single isomer (Scheme 2). An aqueous substituent was introduced easily, but contrary to expectations, **10** was not so water-soluble because of an intramolecular hydrogen bond between the carboxylic acid and the triazole moiety, which was apparent in the ^1H and ^{13}C NMR spectra. Therefore, the carboxylic acid of **10** was exchanged to form the sodium salt **11** in the presence of a methacrylic acid ion exchange resin, DIAION WK11. After filtration and lyophilization, a yellow crystalline powder was obtained, and the spectra indicated the presence of the sodium salt **11**. Compound **11** showed a water solubility of 6.38 mg mL^{-1}, which is 64000 times greater than that of **2**.

Next, the utility of **11** as a prodrug was examined. According to our plan, synthesized **11** was solved to the phosphate buffered saline (PBS) solution (pH 7.4) and hydrolyzed with esterase (porcine liver) at 37°C to reproduce **2** (Scheme 3).



Scheme 3. Hydrolysis of **11** with esterase. Reagents and conditions: a) Esterase solution from porcine liver (suspension in 3.2 M $(NH_4)_2SO_4$ solution, pH 8.0), PBS solution (10 mM, pH 7.4), 37°C then DMSO.

The results of the hydrolysis, as monitored by HPLC, are shown in Figure 1 and Figure 2. This reaction proceeded without any side reactions and only the parent drug **2** was produced (Figure 1). The water-soluble auxiliary **12** was detected in the peak of the DMSO area, which was ascertained by distinct HPLC analysis.[16] In addition, esterase hydrolysis was confirmed to occur at the ester moiety, because the absence of esterase in PBS gave only the starting material. The concentration of **11** decreased concurrently with the production of **2** (Figure 2). The half-life ($t_{1/2}$) of **11** was 59.9 min, which is appropriate for water-soluble prodrug systems delivered by i.v. injection without a solubilizing agent. Although hydrolysis was complete, as shown in Figure 2, the detection of **2** reached a maximum at 80% because insoluble **2** precipitated onto the vessel. The high solubility and appropriate $t_{1/2}$ indicates that the designed and synthesized **11** shows potential as a water-soluble prodrug of plinabulin. In addition, the sodium salt **11** was stable over one month at 4°C without decomposition.

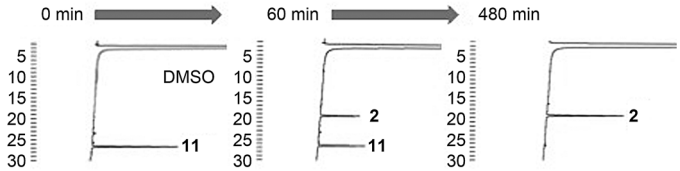


Figure 1. HPLC charts of esterase hydrolysis of prodrug **11** in PBS (pH 7.4, 37°C).

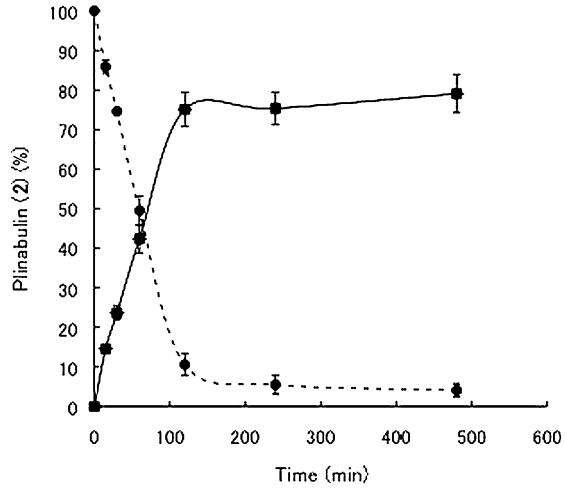


Figure 2. Time course of hydrolysis of prodrug **11** (●) and release of **2** (■) in PBS (10 mM). The percentage was determined by HPLC. The data points are calculated from the average (±standard error) of three assays.

The cytotoxic activities of **11** and **12** were verified in vitro with human colon HT-29 cell lines (Table 2).[17] Compound **11** exhibited one-tenth of the IC_{50} of **2** under normal conditions (Table 2, entry 1).[18] This result may be explained by the fact that the monolactim prodrug **11** reacted with small amounts of esterases produced by cells, which was exuded from cells to medium, or the inner part of the cells directly. On the other hand, the water-soluble auxiliary **12** proved to be non-toxic even when the concentration was raised to 2 mM (Table 2, entry 2). From these results, this water-soluble prodrug system would be safe and efficacious in humans.

Table 2. Cytotoxic activity toward human colon HT-29 cells.

Entry	Compound	IC_{50} [nM][a]
1	**11**	101.4±5.7
2	**12**	>2 mM
3	plinabulin (**2**)	13.5±2.2
4	colchicine	16.6±0.9

[a] Concentration of compound that produced 50% inhibition of cell proliferation.

In summary, we have designed and synthesized a water-soluble prodrug of plinabulin (**2**) using click chemistry, which produced a unique monolactim skeleton with high water solubility, and regeneration of the parent compound **2** upon esterase hydrolysis. The safe and stable prodrug **11** was transformed in three steps from the parent compound. In addition, alkyne **8** can be functionalized with various aqueous azide substituents. This provided significant advantages to the novel prodrug system, including facile optimization of the water-soluble substituent. Higher-order functional assessment and investigation of water-solubilizing group are now in progress.

Experimental Section

General procedure for click chemistry: A solution of azide (28.8 mg, 0.22 mmol) in H_2O (0.40 mL), $CuSO_4{\cdot}5H_2O$ (2.7 mg) and sodium ascorbate (65 mg), were added to a solution of alkyne (50 mg, 0.11 mmol) in DMF (0.40 mL) and *t*BuOH (0.40 mL), and the mixture was stirred for 10 min at 50°C under microwave irradiation. Then, the mixture was cooled to RT and the solvent was removed in vacuo. The residue was diluted with AcOEt and water, and extracted with AcOEt. The extract was washed with brine, dried over Na_2SO_4, filtered and concentrated. The residual oil was purified through reverse phase high performance liquid chromatography (SunFire PrepC18 OBD 19×150 mm (5 mm, 12 nm), gradient: milli-Q water (TFA 0.1%)/CH_3CN (TFA 0.1%)=35:65 to milli-Q water (TFA 0.1%)/CH_3CN (TFA 0.1%)=75:25 over 40 min, Flow rate: 9.00 mL min^{-1}, UV: 365 nm and 230 nm) to give the desired product as a yellow solid.

Acknowledgements

This work was supported by grants from MEXT (Ministry of Education, Culture, Sports, Science and Technology), Japan, including the Grant-in-Aid for Scientific Research (B) (to Y.H.; No. 20390036), Grant-in-Aid for Research Activity Start-up (to F.Y.; No. 21890262) and Grant-in-Aid for Young Scientists (B) (to Y.Y.; No. 21790118).

Keywords: antitumor agents · click chemistry · monolactim · peptides · prodrugs

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[10] Our earlier research data indicated that the C12 amide part of **2** was O-alkylated preferentially under the basic conditions. Because N1 possessed steric hindrance due to the *t*Bu substituent, N3 and N8 participated in the intramolecular hydrogen bonding. In addition, **2** contained the α,β-unsaturated diketopiperazine ring, which assisted the generation of O-alkylated compound, monolactim, bearing the large conjugated system. The molecular structure of monolactim: phosphorous oxymethyl derivative (unpublished data), was confirmed by X-ray crystallography (CCDC-829269 contains the supplementary crystallographic data for phosphorous oxymethyl derivative. This data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif).

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[13] ORTEP drawing based on the X-ray structure of **8** is presented in the Supporting Information. CCDC-829268 contains the supplementary crystallographic data for **8**. This data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.

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[16] See the Supporting Information; compound **12** was synthesized in three steps from 5-hexynoic acid for HPLC analysis and the cytotoxicity assay.

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Received: July 26, 2011
Published online: September 29, 2011

