The Supplementary information for
The Biosynthesis of Camptothecin Derivatives by *Camptotheca acuminata*
Seedlings

Yanni Sun, Ning Zhang, Cuiling Wang, Xiao Bai, Yahui Wei, Jianli Liu*

*Key Laboratory of Resource Biology and Biotechnology in Western China, Ministry of Education,
College of Life Science, Northwest University, Xi’an 710069, China*

Correspondence information: Jianli Liu; E-mail address: jlliu@nwu.edu.cn

Tel.: +86 29 88302013; fax: +86 29 88303572.
The Biosynthesis of Camptothecin Derivatives by *Camptotheca acuminata* Seedlings

10-hydroxycamptothecin and 9-methoxycamptothecin, naturally occurring camptothecin derivatives, are reportedly present in *Camptotheca acuminata* with a powerful cytotoxic effect and strong antitumor activity. In this paper, we studied the derivatization reaction of camptothecin catalyzed by *C. acuminata* seedlings. HPLC traced the reaction between exogenous camptothecin and *C. acuminata* seedlings. The results showed that the exogenous camptothecin was converted into 10-hydroxycamptothecin and 9-methoxycamptothecin by the tender roots and stems of *C. acuminata* seedlings, which would be a new method for the synthesis of two camptothecin derivatives.

Keywords: 10-hydroxycamptothecin; 9-methoxycamptothecin; Exogenous camptothecin; Derivatization; *Camptotheca acuminata* Seedlings

1. Experimental section

1.1. Materials and General Methods

*General Methods.* HPLC was performed on a SHIMADZU LC-10A HPLC equipped with online degasser, a SPD-10AM diode array detector (DAD), a SHIMADZU reversed phase-C18 (4.6 mm×150 mm, 5μm) column. The apparatus used for the homogenate was an electric blender (Chijiu, Model D2015W) equipped with a stainless cross-shaped reamer, a 1600 r/min reamer rating and a 150 W rated power, which purchased from Shanghai, China. Distilled water, further purified with a Milli-Q II system (Millipore, Milford, MA), was used for all aqueous solutions. Methanol for HPLC analysis was of chromatographic grade (Fisher, USA). Sodium phosphate salts other reagents used were of analytical grade.

*Experiment Material.* Mature seeds were collected from mature trees (10-15 year-old) of *Camptotheca acuminata* from Northwest University garden, Xi’an, China. The seed was packed into a plate, soaked with water and covered with gauze to keep the seed moist. The water was changed once a day. The seed was starting to sprout about 25 days, and form to tender seeding 10-15 days later (Zu et al. 2002). Removed gauze when seed was starting to
sprout. Seeding percentage was about 40% (Figure S1). The age of seeding in experiment is 15-20 days.

*Preparation of standard solutions.* Camptothecin (CPT) and 10-hydroxycamptothecin (10-OHCPT) standards (98% purity) were purchased from the Aladdin (Shanghai, China) and the standards were dissolved in methanol. Stock solutions of 10-OHCPT and CPT were prepared by dissolving the accurately weighed standard compounds in methanol to give final concentrations of 1mg/mL, respectively. The working standard solution of CPT and 10-OHCPT for calibration curves were obtained by diluting the stock solution with methanol. 

NaH$_2$PO$_4$ and Na$_2$HPO$_4$ (0.2 mol/L) solutions were prepared and sodium phosphate buffers in the pH range 6.0-8.0 were prepared by NaH$_2$PO$_4$ and Na$_2$HPO$_4$ solutions. All the working solutions were kept under 4 °C and brought to room temperature before use.

*HPLC Analysis.* Chromatographic conditions were as follows: water (A) and methanol (B). Gradient elution program: 0-20 min, 40% mobile phase B; 20-25 min, 40-60% mobile phase B; 25-35 min, 60% mobile phase B; 35-40 min, 60-40% mobile phase B; 40-45 min, 40% mobile phase B. The flow rate was 0.8 mL/min, the column temperature was 30 °C, the injection volume was 20 μL and the detection wavelengths were 370 nm for CPT and 380 nm for 10-OHCPT and 9-MCPT. Under these conditions, the three alkaloids were separated at the baseline. CPT and 10-OHCPT were identified by comparing their retention times with corresponding peaks in the standard solution. 9-MCPT is a CPT derivative with similar UV absorption and more lipophilic. In high performance liquid chromatography, 9-MCPT (C$_{21}$H$_{18}$N$_2$O$_5$) was firstly identified by the similar UV absorption of CPT and later retention time, and finally identified by MS data (m/z 379.2 [M+H]$^+$) (Supplementary Figure S2). The relative contents of 9-MCPT was estimated from comparing peak area with CPT.

Corresponding calibration curves for each compound were $Y_{CPT} = 5E+07x + 31438$ (r = 0.997) and $Y_{10-OHCPT} = 6E+07x + 11128$(r = 0.998). Good linearities were found for CPT and 10-OHCPT in the ranges of 1-100 and 0.3-100 μg/mL, respectively.

1.2. General experimental procedures

*Sample Preparation.* Firstly, we have evaluated the contents of CPT, 10-OHCPT and 9-MCPT in various parts of *C. acuminata* seedlings. The *C. acuminata* seedlings were divided into three
parts, roots, stems and leaves. The roots and stems (approx. 10 g) were cut into 0.3-0.5 cm pieces separately. The leaves (approx. 10 g) were cut into 0.3-0.5 cm² pieces. Then, each plant part (2.0 g) was extracted independently with 10 mL methanol. The concentrations of CPT, 10-OHCPT, and 9-MCPT were calculated based on the area of separate peaks using standard calibration curves for roots, stems and leaves of C. acuminata seedings expressed as percentage of wet weight.

**HPLC traces the reactions between exogenous CPT and leaves or roots of C. acuminata seedings.** The C. acuminata seedlings were divided into three parts, roots, stems and leaves, in two sets in parallel in each case. One set was used for blank samples and the other set was used for adding exogenous CPT samples. The roots and stems (approx. 20 g) were cut into 0.3-0.5 cm pieces separately. The leaves (approx. 20 g) were cut into 0.3-0.5 cm² pieces. The blank samples (2.0 g) were made to homogenate (Liquid-solid: 1 mL/g; Time: 1min) for shaking 8h in a constant-temperature shaker (SHA-C, China), while the other groups (2.0 g) were added exogenous CPT (0.0050 g) and made to homogenate (Liquid-solid: 1 mL/g; Time: 1min) for shaking 8h in a constant-temperature shaker (SHA-C, China). Then, each plant part was extracted independently with 10 mL methanol. Different parameters affecting the conversion were studied as follows.

**Influence of heating on the conversion.** The roots of C. acuminata seedlings (approx. 30 g) were cut into 0.3-0.5 cm pieces and were mixed, in three sets in parallel. One set was used for blank samples, one set was used for adding exogenous CPT as control group, the last set was treated in an autoclave at 120 °C for 6h before adding exogenous CPT. After shaking 8h, each group was extracted independently with 10 mL methanol.

**Influence of denaturing agents on the conversion.** The roots of C. acuminata seedlings (approx. 30 g) were cut into 0.3-0.5 cm pieces and were mixed, in three sets in parallel in each case. One set was used for blank samples, one set was used for adding exogenous CPT as control group, the last set was denaturing agent group. Denaturing agent was added, such as organic solutes (methanol, chloroform, acetonitrile) and urea. After shaking 8h, each group was extracted independently with 10 mL methanol.

**Influence of exogenous CPT on the conversion.** The roots and stems of C. acuminata seedings (approx. 30 g) were cut into 0.3-0.5 cm pieces and were mixed. In order to study the influence
of the content of exogenous CPT on the conversion, different content of exogenous CPT (0, 0.0010, 0.0020, 0.0050, 0.0100, 0.0150, 0.0200, 0.0300 g) was added to each group (2.0 g). The level of 10-OHCPT and 9-MCPT were studied. After shaking 8h, each group was extracted independently with 10 mL methanol.

Influence of reaction time on the conversion. The roots and stems of C. acuminata seeding (approx. 30 g) were cut into 0.3-0.5 cm pieces and were mixed. Adding a content exogenous CPT (0.0050 g) to each group (2.0 g), the influence of grinding time was studied at shaking for 1 h, 4 h, 8 h, 12 h, 24 h, 36 h, 48 h and 72 h. Blank sample was shaking for 8 h with no exogenous CPT added. Then, each group was extracted independently with 10 mL methanol.

Influence of pH on the conversion. The roots and stems of C. acuminata seedings (approx. 30 g) were cut into 0.3-0.5 cm pieces and were mixed. To investigate the influence of pH on the conversion was selected using sodium phosphate buffer. The sodium phosphate buffers in the pH range 6.0-8.0 were prepared. The operation pH groups were as follows: NaH$_2$PO$_4$, pH 6.0, pH 7.0, pH 8.0, Na$_2$HPO$_4$. The each group (2.0g) was mixed with 0.0050g exogenous CPT and 2 mL of pH buffer solution to make into homogenate for shaking 8h. In the blank group, 2.0 g mixture of roots and stems was mixed with 2 mL of water to make into homogenate for shaking 8h. In the control group, 2 g mixture of roots and stems was mixed with 0.0050 g exogenous CPT and 2 mL of water to make into homogenate for shaking 8 h. Then, each group was extracted independently with 10 mL methanol and stored in the dark at 4 °C until assayed by HPLC.

All tests were run in triplicates and each experiment was repeated three times.

REFERENCES
Figure S1. The *C. acuminata* seedlings

![Image of seedlings]

Figure S2. MS spectra of 9-MCPT.

![Graph of MS spectra][10]

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[10]: The figure shows a mass spectrometry (MS) spectrum with peaks at masses 121.0, 149.1, 219.0, 274.3, 301.2, 318.3, 362.3, and 401.2. The spectrum is labeled as ESI Scan (0.223-0.494 min, 33 scans) with a Fragmentation voltage of 135.0V and a SYN36.d marker. The x-axis represents counts versus mass-to-charge (m/z) ratio, and the y-axis represents intensity on a log scale (10^5).
Figure S3. A, CPT, 10-OHCPT and 9-MCPT in *C. acuminata* seedings roots, stems, leaves; B, The level of 10-OHCPT after adding exogenous CPT; C, The level of 9-MCPT after adding exogenous CPT; D, The influence of the exogenous CPT content on the conversion; E, The influence of the reaction time on the conversion; F, The influence of pH on the conversion.

Figure S4. HPLC traces the extract from the roots of *C. acuminata* seedings of blank group and exogenous CPT group. Column: a SHIMADZU reversed phase-C18 (4.6 mm×150 mm, 5μm); Chromatographic profile at 380 nm; Gradient elution program: water (A) and methanol (B), 0-20 min, 40% B, 20-25 min, 40-60% B, 25-35 min, 60% B, 35-40 min, 60-40% B, 40-45min, 40% B; Flow rate: 0.8 mL/min; Column temperature 30 ℃; Injection: 20μL.

Chromatograms were marked as follows: A, Blank group, the extract from the roots of blank group of *C. acuminata* seedings; B, Exogenous CPT group, the extract from the roots of *C. acuminata* seedings after adding exogenous CPT.

Individual peak was marked as follows: 1, 10-OHCPT; 2, Exogenous CPT; 3, 9-MCPT.