Enantioselective Synthesis of a Key Intermediate of 20(*S*)-Camptothecin *via* an Enzyme-Catalyzed Resolution

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The key intermediate of a 20(*S*)-camptothecin 1 synthesis was obtained in a highly enantioselective fashion using an enzyme-catalyzed resolution. A commercially available protease was found to exhibit the highest enantioselectivity with moderate activity, and (*S*)-ethyl 2-acetoxy-2-[6-(acetoxymethyl)-1,1-(ethylenedioxy)-5-oxo-1,2,3,5-tetrahydroindolizin-7-yl]butanoate 7c of 98% e.e. was obtained as the remaining substrate.

Key words 20(*S*)-camptothecin; antitumor activity; enzyme-catalyzed resolution; commercially available protease

20(*S*)-Camptothecin **1** continues to be one of the most important lead compounds among natural anticancer products, and was first isolated from a Chinese tree, *Camptotheca acuminata*, by Wall *et al.* in 1966.[1] Only the (*S*)-enantiomer exhibits antitumor activity,[2] and its mechanism was found to inhibit topoisomerase I.[3]

Many approaches to the synthesis of the alkaloid **1** have been reported, but most such syntheses are racemic. Corey *et al.* succeeded in the first complete synthesis of optically active **1** using an optical resolution process.[4]

More recently, Tagawa and his colleagues reported that the chiral synthetic key intermediate, (*S*)-ethyl-6,6-(ethylenedioxy)-7,8-dihydro-1*H*-pyrano[3,4-*f*]indolizin-3,10(4*H*)-dione **2**, was transformed to **1** efficiently (Chart 1).[5] A number of chemical syntheses of the key compound **2** by optical resolution using a chiral auxiliary[5] and by asymmetric dihydroxylation[6] have been reported. We have already reported the synthesis of **2** *via* an asymmetric hydrolysis with commercially available crude papain from *papaya* in a biphase system,[7] and **2** was then converted to 20(*S*)-camptothecin **1**.

In this paper, we describe the asymmetric synthesis of the key intermediates **7a**—**7c** for 20(*S*)-camptothecin synthesis using enzyme-catalyzed enantioselective hydrolysis, and results of a comparison of the substrate specificity of **5a**—**5c** and acetyl-(±)-**2** with enzymes.

Results and Discussion

Preparation of Acetates (5a—5c) for Enzyme Substrates The syntheses of racemic ethyl 2-acetoxy-2-[6-cyano-1,1-(ethylenedioxy)-5-oxo-1,2,3,5-tetrahydroindolizin-7-yl]butanoate **5a**, ethyl 2-acetoxy-2-[6-(acetylaminomethyl)-1,1-(ethylenedioxy)-5-oxo-1,2,3,5-tetrahydroindolizin-7-yl]butanoate **5b**, and ethyl 2-acetoxy-2-[6-(acetoxymethyl)-1,1-(ethylenedioxy)-5-oxo-1,2,3,5-tetrahydroindolizin-7-yl]-butanoate **5c** were achieved by Ejima's route.[8] Compound **5a** was prepared by bromination of the known indolizine **4**[9] followed by acetoxylation and ethylation. Compound **5b** was prepared by hydrogenation of **5a** with Raney-Ni (NDHT-90) in the presence of acetic anhydride, and treatment of **5b** with sodium nitrate followed by rearrangement reaction yielded **5c** (Chart 2). We first used the racemic acetate **5c** as the substrate for the screening of enzymes and attempted resolution by enantioselective hydrolysis.

Enzymatic Hydrolysis of 5c About one hundred commercially available enzymes including esterases, lipases and proteases from various sources were used for the screening of enzymes towards the enantioselective hydrolysis of **5c**. Many esterases and lipases did not exhibit deacetylating activity due to bulkiness around the hydroxyl group compared to that in primary and secondary alcohol, but they did hydrolyze the ethyl ester. A few proteases from fungi exhibited

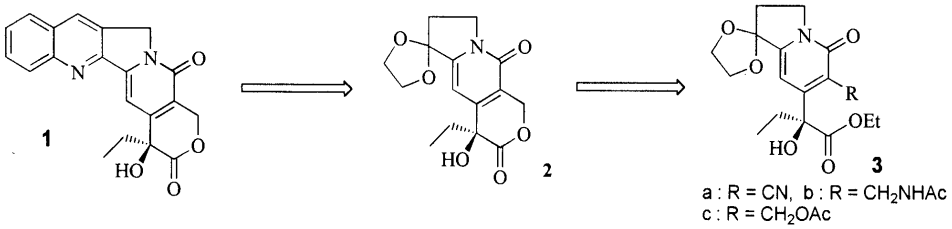

Chart 1

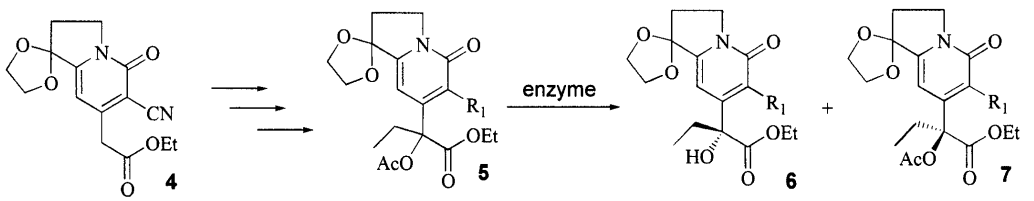

Chart 2

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Table 1. Screening of Enzymes to Catalyze Hydrolysis of Racemate **5c**

Enzyme[a]	Source	Reaction time (h)	*R*-Alcohol **6c**		*S*-Acetate **7c**	
			Yield (%)[b]	e.e. (%)[c]	Yield (%)	e.e. (%)
Protease	*Aspergillus oryzae*	40	20	98	76	28
Protease	*Aspergillus sojae*	48	4	95	94	—
Protease	*Rhizopus* sp.	48	2	95	97	—

a) 1 mg **5c**, 5 mg enzyme, 0.1 M phosphate buffer pH 7.0 (1 ml), 30 °C. *b*) Determined by HPLC analysis with a column of Inertsil ODS-2 (GL-Science) employing 0.05 M phosphate buffer (pH 6.5) : CH_3CN (7 : 3) as the solvent system. *c*) The conditions are described in the Experimental section.

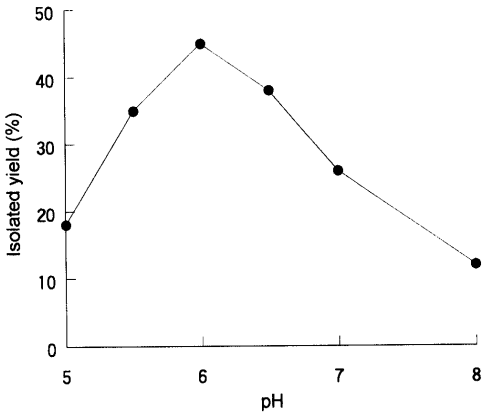

Fig. 1. Relationship between Yield of **6c** and pH

The reaction conditions are described in the Experimental section.

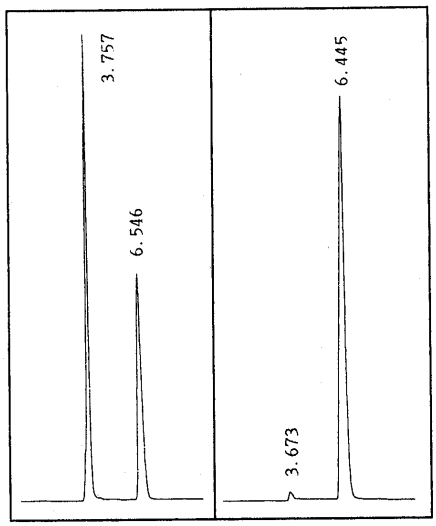

Fig. 2. HPLC of Compound **2**, (±)-Form (Left) and (+)-Form from Enzymatic Resolution (Right)

deacetylating activity without hydrolyzing the ethyl ester and the acetoxymethyl residue at the C-6 position. In addition, the enantiomeric excess of **6c** determined by HPLC was moderately high in general. In particular, a commercially available protease from *Aspergillus oryzae* was found to exhibit high enantioselectivity and the alcohol **6c** was obtained in moderate chemical yield (Table 1).

We first examined the reaction in phosphate buffer (pH 7.0) at room temperature employing protease as a catalyst. Evaluation of optimum pH conditions established that the alcohol **6c** was obtained in 45% yield at pH 6.0 (Fig. 1). Reaction temperatures were also varied, and inactivation of enzyme was found above 40 °C. Therefore, enzymatic hydrolysis using protease from *Asp. oryzae* toward acetate **5c** was carried out in 0.1 M phosphate buffer (pH 6.0) at 30 °C. The pH was maintained at 6.0 by the addition of 10% aqueous sodium hydroxide with an autotitrator. After termination of the reaction, a mixture of alcohol and ester was isolated by extraction, and chromatographed on silica gel to separate the alcohol **6c**, which was hydrolyzed by enzyme, and the ester **7c**, which has a desirable (*S*)-configuration. The enantiomeric excess of **6c** and **7c** was determined by HPLC after derivation to 4-ethyl-6,6-(ethylenedioxy)-7,8-dihydro-1*H*-pyrano-[3,4-*f*]indolizine-3,10(4*H*)-dione **2**.[8] The absolute configuration was determined by comparison of its retention time on HPLC with authentic material.

Comparison of Substrate Specificity of 5a, 5b, 5c and Acetyl-(±)-2 With **5a**, the (*S*)-ester **7a**, which has the desirable configuration, was obtained, but the (*R*)-alcohol **6a** was unstable and not isolated. For **5b**, the (*S*)-ester and (*R*)-alcohol were obtained in good chemical and optical yields.

A comparison of substrate specificity for **5a**—**5c** and acetyl-(±)-**2**, using protease from *Asp. oryzae* and papain from *papaya*, is summarized in Table 2. Protease from *Asp. oryzae* did not hydrolyze acetyl-(±)-**2**, but hydrolyzed **5a**—**5c** which have an ethyl ester moiety. On the other hand, papain from papaya did not hydrolyze **5a**—**5c**, but hydrolyzed acetyl-(±)-**2**, which has a lactone ring. We hypothesized that the enantioselective hydrolysis of **5a**—**5c** and acetyl-(±)-**2** was due to minimal esterase activity of protease or papain. Therefore, we are interested in the substrate specificity for compounds **5a**—**5c** and acetyl-(±)-**2** by purified enzyme.

Conclusion

We have demonstrated a highly enantioselective preparation of compounds **7a**—**7c**, which can be easily converted to 20(*S*)-camptothecin **1**, using an enzyme-catalyzed resolution. Since the protease from *Asp. oryzae* is commercially available, we could examine the enzymatic reaction with good reproducibility of results. Compounds **5a**—**5c** were good substrates for the protease from *Asp. oryzae*, and gave **7a**—**7c** in good chemical and enantiomeric yields. However, acetyl-(±)-**2**, which had a structure similar to those of **5a**—**5c**, had a different substrate specificity against protease from *Asp. oryzae*.

Experimental

General Procedures Melting points were determined on a Yanagimoto apparatus and are uncorrected. Infrared (IR) spectra were recorded on a FT-720 spectrometer (Horiba). ^{1}H-NMR spectra were recorded on a JEOL JNM-EX270 (270 MHz) instrument. Coupling constants are reported in Hertz (Hz) and chemical shift in ppm downfield from internal tetramethylsilane. Mass spectra were recorded on a JEOL JMS-HX110 or JMS-AX505W mass spectrometer. Optical rotations were measured with a SEPA-300 polarimeter (Horiba). Column chromatography was performed on silica gel

Table 2. Comparison of Substrate Specificity between **5a**—**5c** and Acetyl-(±)-**2**

Run	Substrate	Enzyme	Reaction time (h)	Conv.[a] (%)	Alcohol **6a**—**6c**		Acetate **7a**—**7c**		E[b] value
					e.e. (%)	Config.	e.e. (%)	Config.	
1	**5a**	Papain[c]	40	<1	—	—	—	—	—
2		Protease[d]	72	48	Unstable		98	(*S*)	—
3	**5b**	Papain	40	<1	—	—	—	—	—
4		Protease	40	52	95	(*R*)	98	(*S*)	136
5	**5c**	Papain	40	<1	—	—	—	—	—
6		Protease	48	49	98	(*R*)	98	(*S*)	236
7	Acetyl-(±)-**2**	Papain	40	51	98	(*R*)	99	(*S*)	>400
8		Protease	48	<1	—	—	—	—	—

a) Determination by HPLC analysis (see Table 1). *b*) The enantiomeric ratio E is calculated from the equation: $E=\ln\{(1-c)[1-\text{e.e.(s)}]\}/\ln\{(1-c)[1+\text{e.e.(s)}]\}$.[10] *c*) 0.5 g of substrate, 1.0 g of papain, 0.1 M phosphate buffer pH 6.5/ethyl acetate (4 : 1)–50 ml, 40 °C. *d*) 0.5 g of substrate, 2.0 g of protease (from *Asp. oryzae*), 0.1 M phosphate buffer pH 6.0–200 ml, 30 °C.

(Kieselgel 60, 70—230 mesh, Merck). All chemicals were obtained from commercial sources and were used without further purification. Papain from *papaya* was obtained from Merck . Proteases from *Asp. sojae*, *Asp. oryzae* and *Rhizopus* sp. were obtained from Sigma.

Determination of Enantiomeric Excess of Optically Active Compounds 6b, 6c, 7a, 7b and 7c were converted to 4-ethyl-6,6-(ethylenedioxy)-7,8-dihydro-1*H*-pyrano[3,4-*f*]indolizine-3,10(4*H*)-dione **2**. Determination of enantiomeric excesses of **2** was made by analytical HPLC [ULTRON ES-OVM column (Shinwa Kako) 4.6×150 mm; eluent, 2% ethanol containing 20 mM phosphate buffer (pH 6.0); flow rate, 1.0 ml/min; UV detection 300 nm]. A typical separation is illustrated in Fig. 2.

Comparison of the Rate of Reaction of Enzymatic Hydrolysis of 5c In a parallel treatment of **5c** (100 mg) in 0.1 M phosphate buffer (pH 5.0, 5.5, 6.0, 6.5, 7.0, 8.0) 40 ml, and 200 mg of protease from *Asp. oryzae* was added. The resulting suspension was stirred at 30 °C, and conversion was observed by HPLC analysis [Inertsil ODS-2 column (GL Science) 4.6×150 mm; eluent, 30% acetonitrile containing 50 mM phosphate buffer (pH 6.0); flow rate, 1.0 ml/min; UV detection 300 nm]. The results are shown in Fig. 1.

(*S*)-Ethyl 2-Acetoxy-2-[6-(acetoxymethyl)-1,1-(ethylenedioxy)-5-oxo-1,2,3,5-tetrahydroindolizin-7-yl]butanoate (7c) and (*R*)-Ethyl 2-Hydroxy-2-[6-(acetoxymethyl)-1,1-(ethylenedioxy)-5-oxo-1,2,3,5-tetrahydroindolizin-7-yl]butanoate (6c) The acetate **5c** (4.0 g, 9.1 mmol) was suspended in 0.1 M phosphate buffer (pH 6.0) 800 ml, and protease from *Asp. oryzae* (8 g) was added. The mixture was stirred at 30 °C for 48 h at pH 5.9—6.1 with an autotitrater with 10% aqueous sodium hydroxide. The reaction mixture was filtered through Celite and extracted with dichloromethane (200 ml×3) to obtain the mixture of **6c** and **7c**. The combined organic layers were dried and evaporated *in vacuo*, and the residue was chromatographed on silica. Elution with toluene/ethyl acetate (3/1) gave acetate **7c** (1.68 g, 42.0%, 98% e.e.) as a pale yellow oil. $[\alpha]_D^{25}$ −37.0 (c=0.55, $CHCl_3$); ^{1}H-NMR ($CDCl_3$) δ: 0.82 (t, J=7.6 Hz, 3H, $C\underline{H}_3CH_2$), 1.22 (t, J=6.9 Hz, 3H, $C\underline{H}_3CH_2O$), 2.07 (s, 3H, OAc), 2.13 (s, 3H, OAc), 2.25—2.62 (m, 4H, $CH_3C\underline{H}_2$, $C\underline{H}_2CH_2N$), 4.08—4.30 (m, 8H, $CH_3C\underline{H}_2O$, $CH_2C\underline{H}_2N$, OCH_2CH_2O), 5.34 (s, 2H, $C\underline{H}_2OAc$), 6.37 (s, 1H, Ar-H); MS: m/z 438 (M^++1); IR (neat) 1741, 1657, 1606 cm^{-1}. Elution with toluene/ethyl acetate (2/1) gave alcohol **6c** (1.42 g, 45.0%, 98% e.e.) as a pale yellow oil. $[\alpha]_D^{25}$ +26.9 (c=0.41, $CHCl_3$); ^{1}H-NMR ($CDCl_3$) δ: 0.93 (t, J=7.6 Hz, 3H, $C\underline{H}_3CH_2$), 1.28 (t, J=6.9 Hz, 3H, $C\underline{H}_3CH_2O$), 2.05 (s, 3H, OAc), 2.05—2.40 (m, 4H, $CH_3C\underline{H}_2$, $C\underline{H}_2CH_2N$), 4.06—4.39 (m, 8H, $CH_3C\underline{H}_2O$, $CH_2C\underline{H}_2N$, OCH_2CH_2O), 5.38 (dd, J=11.6 Hz, 2H, $C\underline{H}_2OAc$), 6.42 (s, 1H, Ar-H); MS: m/z 396 (M^++1); IR (neat) 3019, 1733, 1654, 1603 cm^{-1}.

(*S*)-Ethyl 2-Acetoxy-2-[6-cyano-1,1-(ethylenedioxy)-5-oxo-1,2,3,5-tetrahydroindolizin-7-yl]butanoate (7a) Enzymatic hydrolysis of **5a** was performed under the same conditions as for **5c**. Compound **7a** (40.3%, 98% e.e.) was obtained as colorless crystals (recrystallized from 2-propanol). mp 178—180 °C; $[\alpha]_D^{25}$ +68.5 (c=0.52, $CHCl_3$); ^{1}H-NMR ($CDCl_3$) δ: 0.87 (t, J=7.6 Hz, 3H, $C\underline{H}_3CH_2$), 1.25 (t, J=6.9 Hz, 3H, $C\underline{H}_3CH_2O$), 2.29 (s, 3H, OAc), 2.28—2.64 (m, 4H, $CH_3C\underline{H}_2$, $C\underline{H}_2CH_2N$), 4.08—4.32 (m, 8H, $CH_3C\underline{H}_2O$, $CH_2C\underline{H}_2N$, OCH_2CH_2O), 6.48 (s, 1H, Ar-H); MS: m/z 391 (M^++1); IR (KBr) 2220, 1753, 1643, 1612 cm^{-1}. *Anal.* Calcd for $C_{19}H_{22}N_2O_7$: C, 58.46; H, 5.68; N, 7.18. Found: C, 58.60; H, 5.69; N, 7.26.

(*S*)-Ethyl 2-Acetoxy-2-[6-(acetoxyaminomethyl)-1,1-(ethylenedioxy)-5-oxo-1,2,3,5-tetrahydroindolizin-7-yl]butanoate (7b) and (*R*)-Ethyl 2-Hydroxy-2-[6-(acetoxyamino-methyl)-1,1-(ethylenedioxy)-5-oxo-1,2,3,5-tetrahydroindolizin-7-yl]butanoate (6b) Enzymatic hydrolysis of **5b** was performed under the same conditions as for **5c**. Compound **7b** (40.8%, 98% e.e.) was obtained as a pale yellow oil. $[\alpha]_D^{25}$ −30.3 (c=0.40, $CHCl_3$); ^{1}H-NMR ($CDCl_3$) δ: 0.85 (t, J=7.6 Hz, 3H, $C\underline{H}_3CH_2$), 1.26 (t, J=6.9 Hz, 3H, $C\underline{H}_3CH_2O$), 1.94 (s, 3H, OAc), 2.21 (s, 3H, OAc), 2.42—2.65 (m, 4H, $CH_3C\underline{H}_2$, $C\underline{H}_2CH_2N$), 3.71 (s, 2H, $C\underline{H}_2NHAc$), 4.13—4.36 (m, 8H, $CH_3C\underline{H}_2O$, $CH_2C\underline{H}_2N$, OCH_2CH_2O), 6.43 (s, 1H, Ar-H); MS: m/z 437 (M^++1); IR (neat) 1741, 1658, 1598, 1513 cm^{-1}. Compound **6b** (39.2%, 95% e.e.) was obtained as a pale yellow oil. $[\alpha]_D^{25}$ +21.5 (c=0.42, $CHCl_3$); ^{1}H-NMR ($CDCl_3$) δ: 0.91 (t, J=7.6 Hz, 3H, $C\underline{H}_3CH_2$), 1.27 (t, J=6.9 Hz, 3H, $C\underline{H}_3CH_2O$), 2.00 (s, 3H, OAc), 2.09—2.40 (m, 4H, $CH_3C\underline{H}_2$, $C\underline{H}_2CH_2N$), 4.08—4.39 (m, 8H, $CH_3C\underline{H}_2O$, $CH_2C\underline{H}_2N$, OCH_2CH_2O), 5.42 (dd, J=10.4 Hz, 2H, $C\underline{H}_2NHAc$), 6.40 (s, 1H, Ar-H); MS: m/z 395 (M^++1); IR (neat) 3190, 1749, 1650, 1529 cm^{-1}.

(*S*)-4-Ethyl-6,6-(ethylenedioxy)-7,8-dihydro-1*H*-pyrano[3,4-*f*]indolizine-3,10(4*H*)-dione (2) Compound **7c** (1.42 g, 3.3 mmol) was dissolved in 50% aqueous methanol (20 ml), and potassium carbonate (1.60 g, 12 mmol) was added, and the mixture was left at room temperature (3 h). The reaction mixture was evaporated by a half volume, adjusted to pH 2 with 10% hydrochloric acid, and extracted with dichloromethane (10 ml×3). The organic layers were evaporated *in vacuo*, and the residue was crystallized from ethyl acetate to obtain colorless crystals of **2** (0.88 g, 88.2%, >99% e.e.). mp 168—169 °C; $[\alpha]_D^{25}$ +103.2 (c=0.50, $CHCl_3$); ^{1}H-NMR ($CDCl_3$) δ: 0.98 (t, J=7.3 Hz, 3H, $C\underline{H}_3CH_2$), 1.62 (m, 2H, $CH_3C\underline{H}_2$), 2.42 (t, J=6.9 Hz, 2H, C_7-H), 4.13 (m, 6H, C_8-H, OCH_2CH_2O), 5.16, 5.61 (ABq, J=16.4 Hz, 2H, C_1-H), 6.57 (s, 1H, C_5-H); MS: m/z 307 (M^+); IR (KBr) 3311, 1755, 1658, 1590 cm^{-1}; *Anal.* Calcd for $C_{15}H_{17}NO_6$: C, 58.63; H, 5.58; N, 4.56. Found: C, 58.69; H, 5.59; N, 4.62.

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