Epimerization and Hydrolysis of Etoposide Analogues in Aqueous Solution

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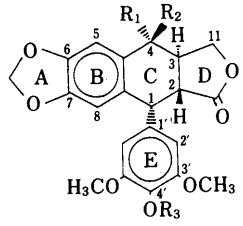
Epimerization and hydrolysis of etoposide and its analogues involving aglycons were examined in alkaline aqueous solution. The *trans*-lactone compounds such as etoposide were epimerized to the *cis*-lactone compounds such as picroetoposide, but not hydrolyzed to *trans*-hydroxy acid derivatives. The *cis*-lactone compounds were susceptible to the hydrolysis of the lactone ring. The epimerization was accelerated by the presence of the sugar substituent at position 4 and of the methoxy group at position 4'. The epimerization was also affected by the configuration of the hydroxyl group at position 4. The hydrolysis rate of the *cis*-lactone was decreased by the sugar substituent at position 4, and increased by the methoxy group at position 4'. The configuration of the hydroxyl group at position 4 had no effect. The nuclear magnetic resonance data suggest that the structure of etoposide is more strained and less stable than that of the aglycon. The acceleration of the epimerization by the sugar substituent may be ascribed to the decrease in the stability of the *trans*-lactone by glycosidation. The decrease of hydrolysis rate owing to glycosidation may be explained in terms of the steric hindrance of the bulky sugar substituent.

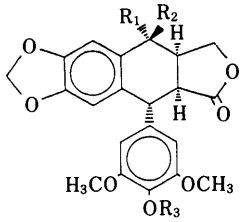
Keywords epimerization; hydrolysis; kinetics; etoposide analogue; NMR

Introduction

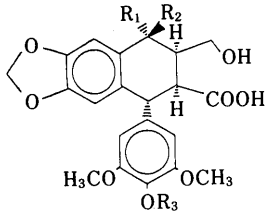
Etoposide, a semisynthetic epipodophyllotoxin analogue, 4'-demethylepipodophyllotoxin 9-[4,6-*O*-(*R*)-ethylidene-β-D-glucopyranoside] (Chart 1) has antineoplastic activity against a variety of malignancies.[1] Beijnen *et al.*[2] have studied the chemical stability of etoposide in aqueous solution, and reported that etoposide is hydrolyzed to its aglycon in acidic solution, but epimerized to picroetoposide at a pH above 6. On the other hand, picroetoposide, which differs from etoposide in the configuration of the hydrogen at position 2, has been reported to be hydrolyzed to its *cis*-hydroxy acid derivative at a pH above 6. The degradation pathway of etoposide in alkaline solution was represented as follows.



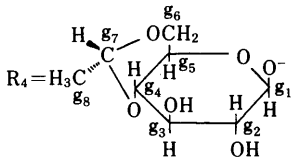
trans-lactone



cis-lactone



cis-hydroxy acid



etoposide: $R_1=H$, $R_2=R_4$, $R_3=H$
DEPT: $R_1=H$, $R_2=OH$, $R_3=H$
DPT: $R_1=OH$, $R_2=H$, $R_3=H$
podophyllotoxin: $R_1=OH$, $R_2=H$, $R_3=CH_3$

picroetoposide: $R_1=H$, $R_2=R_4$, $R_3=H$
DEPP: $R_1=H$, $R_2=OH$, $R_3=H$
DPP: $R_1=OH$, $R_2=H$, $R_3=H$
picropodophylline: $R_1=OH$, $R_2=H$, $R_3=CH_3$

Chart 1. Structures of Etoposide and Its Analogues

$$\text{etoposide} \xrightarrow{k_1} \text{picroetoposide} \xrightarrow{k_2} \textit{cis}\text{-hydroxy acid}$$

Chart 2

They determined the time courses of the concentration of etoposide, picroetoposide and the *cis*-hydroxy acid derivative in the degradation of etoposide, and estimated the rate constants, k_1 and k_2.[2]

The degradation of etoposide is quite different from that of pilocarpine, which has a chiral center next to the lactone carbonyl group, like etoposide. The epimerization of pilocarpine to isopilocarpine is reversible and the lactone rings of pilocarpine and isopilocarpine are hydrolyzed at a similar rate.[3] It is of great interest to know why etoposide is susceptible to epimerization, while picroetoposide is susceptible to hydrolysis.

In this paper, the epimerization and hydrolysis of etoposide and picroetoposide were compared with those of their analogues, and the effects of substituents on the epimerization and hydrolysis are discussed in regard to the difference in reactivity between the lactone pair. In particular, the effects of the sugar substituent on the epimerization rate of *trans*-lactone were investigated by proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy.

Experimental

Materials Etoposide, picroetoposide, 4'-demethylepipodophyllotoxin (DEPT), 4'-demethylpodophyllotoxin (DPT) and podophyllotoxin were kindly supplied by Nippon Kayaku Co. (Tokyo, Japan). 4'-Demethylepipicropodophylline (DEPP), 4'-demethylpicropodophylline (DPP) and picropodophylline were prepared from DEPT, DPT and podophyllotoxin, respectively, according to the literature.[4] Standard solutions of *cis*-hydroxy acids of picroetoposide, DEPP, DPP and picropodophylline were

obtained by hydrolysis of picroetoposide, DEPP, DPP and picropodophylline, respectively, in 0.1 M NaOH solution. Other chemicals were of reagent grade.

High-Performance Liquid Chromatography (HPLC) The chromatographic system consisted of a model 655 HPLC system (Hitachi Co., Ltd., Tokyo, Japan) equipped with a type 2725 injection valve (Rheodyne, Berkeley, CA., U.S.A.) and a packed column (μBONDASPHARE Phenyl, 3.9 mm × 15 cm, Nihon Waters Ltd., Tokyo, Japan). The mobile phase was composed of 0.01 M acetate buffer pH 4.0–acetonitrile (4:1) and was delivered at a flow rate of 1.0 ml/min.[5] The eluate was monitored at 290 nm. The degradation products were eluted separately under our conditions and detected without any interference with the peaks of interest.

Kinetic Studies Kinetic studies were carried out in a 0.2 M borate buffer solution, ionic strength 0.2, at pH 10 and 37°C. Sample solutions were prepared by dissolving etoposide, picroetoposide, DEPT, DEPP, DPT, DPP, podophyllotoxine and picropodophylline in a suitable amount of dioxane (the final concentration of dioxane in the reaction mixture was adjusted to be 9%) and added to the buffer solution. The final concentrations of these solutions were 0.6 mg/ml for etoposide and picroetoposide, and 0.3 mg/ml for DEPT, DEPP, DPT, DPP, podophyllotoxin and picropodophylline. Aliquots of the reaction solution were withdrawn at appropriate intervals, and diluted with the mobile phase solvent containing an internal standard (2,6-dichlorophenol 10 μg/ml). Twenty microliters of the solution was injected into the chromatograph.

^{1}H-NMR Spectroscopy ^{1}H-NMR measurements were done on a Bruker AM 400 spectrometer operating at 400 MHz. The etoposide analogues were dissolved in deuteriochloroform (5 mM or less). The chemical shifts and coupling constants for etoposide and picroetoposide shown in Tables II and III agree with those previously reported.[6]

Results and Discussion

Degradation Kinetics of Etoposide Analogues in Alkaline Solution Typical time courses of the degradation of etoposide and picroetoposide are shown in Fig. 1. Etoposide was epimerized to picroetoposide according to pseudo-first order kinetics, and picroetoposide thus formed was successively hydrolyzed to the *cis*-hydroxy acid derivative, as described by Beijnen *et al.*[2] When picroetoposide was used as a starting material, the epimerization of picroetoposide to etoposide was not observed under the present conditions. The *cis*-hydroxy acid was found to be stable in the reaction mixture during the experiment. The experimental data shown in Fig. 1a were fitted to the scheme shown in Chart 2 by the use of a non-linear curve fitting program (MULTI).[7] The estimated values for k_1 and k_2 were 0.0382 and 0.0129 min^{-1}, respectively. The estimated k_2 value agreed well with the rate constant obtained for the hydrolysis of picroetoposide as a starting material, 0.0129 min^{-1} (Fig. 1b). The epimerization of picroetoposide to etoposide and the hydrolysis of etoposide to its *trans*-hydroxy acid derivative were not observed under the present conditions.

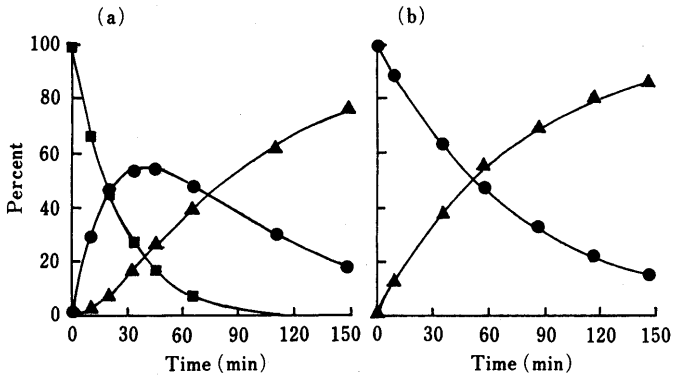


Fig. 1. Typical Time Courses of Epimerization and Hydrolysis of Etoposide (a) and Picroetoposide (b) at pH 10 and 37 °C

■, etoposide; ●, picroetoposide; ▲, *cis*-hydroxy acid derivative of picroetoposide.

It is possible that the reactivity of the lactone pair is affected by the presence of the sugar substituent and the methoxy group at position 4′, or the configuration of the hydroxy group at position 4. In order to clarify the effect of these steric factors on the reactivity of the lactone pair, the degradation of etoposide analogues was studied at pH 10 and 37°C. The degradation rate constants are shown in Table I.

The steric effect of the sugar substituent on the epimerization and hydrolysis was examined with DEPT and DEPP, which are the aglycons of etoposide and picroetoposide, respectively (Chart 1). DEPT was epimerized to DEPP, which was successively hydrolyzed to the *cis*-hydroxy acid derivative. The decrease of DEPT and the formation of DEPP and its *cis*-hydroxy acid derivative could be analyzed in a similar manner to etoposide as a consecutive reaction process. The estimated values for k_1 and k_2 were 0.0141 and 0.0252 min^{-1}, respectively. The estimated k_2 value agreed well with the rate constant obtained for the hydrolysis of DEPP as a starting material, 0.0256 min^{-1}. The reverse epimerization of DEPP to DEPT and the hydrolysis of DEPT to its *trans*-hydroxy acid derivative were not observed. The sugar substituent accelerated the epimerization, but decreased the hydrolysis rate by about one-half, as shown in Table I (compare the rate constants of etoposide and picroetoposide with those of DEPT and DEPP, respectively).

The effect of the configuration the hydroxyl group of ring C on the epimerization and hydrolysis was examined with DPT and DPP, which are C-4 epimers of DEPT and DEPP, respectively. It was found that the configuration of the hydroxy group affects the epimerization rate, but not the hydrolysis rate. DPT was epimerized to DPP, but the hydrolysis of the *trans*-lactone ring of DPT was not observed under the condition studied. On the other hand, DPP was hydrolyzed to the *cis*-hydroxy acid derivative. The degradation of DPT could also be treated in a similar manner to that of etoposide. The epimerization of DPT (α-configuration) was about three times faster than that of DEPT (β-configuration). DPP (α-configuration) and DEPP (β-configuration) were hydrolyzed at a similar rate (Table I).

TABLE I. Epimerization and Hydrolysis Rate Constants of Etoposide and Its Analogues

Compound	Epimerization (min^{-1})	Hydrolysis (min^{-1})
Etoposide	0.0382	Not observed
DEPT	0.0145	Not observed
DPT	0.0480	Not observed
Podophyllotoxin	0.122	Not observed
Picroetoposide	Not observed	0.0129
DEPP	Not observed	0.0256
DPP	Not observed	0.0218
Picropodophylline	Not observed	0.0360

pH 10.0 and 37 °C.

The effect of the methylation of the hydroxyl group at position 4′ on the epimerization and hydrolysis was examined with podophyllotoxin and picropodophylline which are methylated derivatives of DPT and DPP, respectively, at the hydroxyl group at position 4′. The methylation of the hydroxyl group was observed to accelerate both epimerization and hydrolysis. Podophyllotoxin was epimerized to picropodophylline, and picropodophylline thus formed was hydrolyzed to the *cis*-hydroxy acid derivative. The degradation of podophyllotoxin could be represented by the consecutive reaction model. The epimerization rate of podophyllotoxin was about twice that of DPT, and the hydrolysis rate of picropodophylline was about twice that of DPP as shown in Table I.

^{1}H-NMR Measurement of Etoposide, Picroetoposide and Their Aglycons The effect of the sugar substituent on the epimerization and hydrolysis was interpreted by taking into account the structural difference of etoposide, picroetoposide and their aglycones. Tables II and III show typical proton chemical shifts and coupling constants. The differences in the chemical shifts and coupling constants between etoposide and its aglycone, DEPT, were smaller than those between picroetoposide and its aglycon, DEPP, especially for the protons at positions 1, 2 and 4. This indicates that the conformations of the *trans*-lactones are not changed by glycosidation because of their rigid structure,[6a] but the conformation of DEPP is much more changed than that of DEPT by the glycosidation. We therefore suggest that picroetoposide accommodates the bulky sugar substituent more easily without a significant increase in the energy of the molecule. This was confirmed by the nuclear Overhauser effect (NOE) experiments: the signals of protons at position 4 and position g_1 (proton at the anomeric carbon of glucose) in etoposide were enhanced when the proton at position 5 was irradiated, whereas only the signal of the proton at position 4 was enahanced in picroetoposide. The rotation of the sugar substituent seems to be hindered in the case of etoposide to a larger extent than in the case of the picrocoformer.

This must affect the reactivity of these compounds. Although the above NMR data were obtained in deuteriochloroform solution, the discussion regarding the structure of etoposide analogues may also hold true in aqueous solution. Etoposide could be less stable than DEPT because of its more strained structures owing to glycosidation, and thus the epimerization rate of the *trans*-lactone may be increased by introduction of the sugar substituent. On the other hand, the bulky sugar substituents of picroetoposide may inhibit the attack of hydroxide ion and/or water molecule. The decrease in the hydrolysis rate following the glycosidation of DEPP may thus be explained by steric hindrance of the bulky sugar substituent.

TABLE II. Chemical Shifts of Etoposide and Its Analogues

H	Chemical shift (ppm)			
	Etoposide	DEPT	Picroetoposide	DEPP
1	4.58	4.59	4.20	4.43
2	3.23	3.24	3.15	3.41
4	4.89	4.85	4.92	4.81

TABLE III. Coupling Constants of Etoposide and Its Analogues

H	Coupling constant (Hz)			
	Etoposide	DEPT	Picroetoposide	DEPP
1	5.17	5.08	4.87	3.31
2	14.27	14.09	*ca.* 10	10.53
	5.22	5.04	4.9	3.52
4	3.44	Small	3.02	*ca.* 4.3

Conclusion

(1) It was shown that degradation of etoposide and its analogues examined could be considered as a consecutive reaction: *trans*-lactone→*cis*-lactone→*cis*-hydroxy acid

(2) Epimerization of the *trans*-lactone to the *cis*-lactone was accelerated by the introduction of the sugar substituent at position 4 and the methoxy group at position 4′ of ring E, and was affected by the configuration of the hydroxyl group at position 4 of ring C.

(3) Hydrolysis of the *cis*-lactone was accelerated by the methoxy group at position 4′ and retarded by the sugar substituent at position 4. The configuration of the hydroxyl group at position 4 had no effect.

(4) The NMR data suggest that the structure of etoposide is more strained than that of DEPT. The acceleration of epimerization by the sugar substituent may be explained on the basis of the decrease in the stability of *trans*-lactone by glycosidation. The decrease in the hydrolysis rate by the glycosidation of DEPP may thus be explained by steric hindrance of the bulky sugar substituent.

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