Metabolic Cleavage of Frangufoline in Rodents: *In Vitro* and *in Vivo* Study

Dae-Yeon Suh, Yong Chul Kim, Young-Hwa Kang, Yong Nam Han, and Byung Hoon Han*

Natural Products Research Institute, Seoul National University, 28, Yeongun-dong, Jongro-gu, Seoul 110-460, Korea

Received September 25, 1996[⊗]

Frangufoline, a sedative 14-membered frangulanine-type cyclopeptide alkaloid, was found to be rapidly converted, via enzymatic process, *in vitro* and *in vivo* in rodents to M1 ((*S*)-(*N*,*N*-dimethylphenylalanyl)-(2*S*,3*S*)-3-[(*p*-formylphenoxy)leucyl]-(*S*)-leucine), which is a substituted linear tripeptide. The reaction did not require low molecular weight cofactors, and mammalian serum failed to catalyze the reaction. Structure−reactivity study of cyclopeptide alkaloid analogs suggested that the enamide bond is the site being cleaved, and the reaction was inhibited by organophosphorus esters such as BPNP and by eserine at higher concentrations but not by eserine at lower concentrations or by EDTA and PCMB. On the basis of these results, a possible mechanism for metabolic conversion of frangufoline to M1 was proposed, in which oxidation of the vinyl group and enzyme-catalyzed hydrolysis of the adjacent amide bond, possibly by B-esterase-like enzyme, proceed in a concerted manner.

Several sedative cyclopeptide alkaloids have been isolated from the seeds of *Zizyphus vulgaris* Lamark var. *spinosus* Bunge (Rhamnaceae), which have been used to treat insomnia in Oriental medicine.[1,2] Frangufoline (sanjoinine A), the major active principle, is a frangulanine-type cyclopeptide alkaloid, which is characterized by a strained 14-membered ring system and an aryl ether resulting from *p*-hydroxystyrylamine and *β*-hydroxyleucine (reviewed in ref 3). SAR study of a series of cyclopeptide alkaloids isolated from *Zizyphus* species revealed that the styrylamine unit is important for the *in vivo* sedative activity in mice and that reduction or hydration of the double bond resulted in significant loss in the activity.[2] Cyclopeptide alkaloids were reported to have antibiotic and several other biological properties.[4] In particular, frangufoline was shown to possess ionophore[5] and calmodulin binding activities.[6] However, few studies on the metabolism of 14-membered cyclopeptide alkaloids have been reported. Recently, frangufoline was found to be cleaved to a linear compound via unusual enamide degradation under acidic conditions (2 N HCl, 55 °C, 10 h).[7] In this study, a metabolic study on frangufoline was undertaken to show that it is rapidly converted in rodents *in vivo* and *in vitro* to a substituted linear tripeptide, which is identical to the degradation product formed by acid treatment.

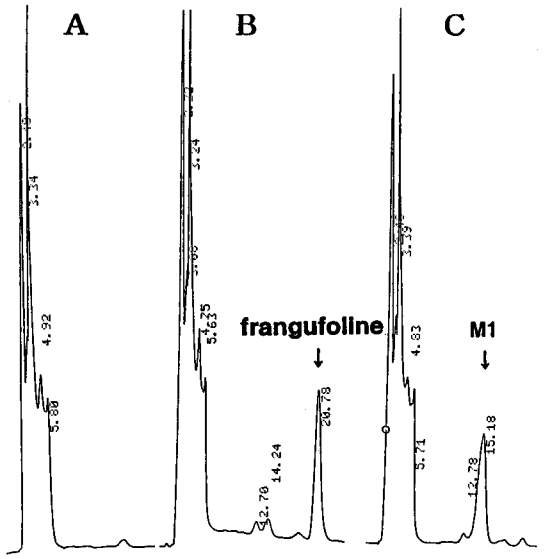


Figure 1. *In vitro* conversion of frangufoline in rat serum. Frangufoline·HCl (0.25 mg/mL) in rat serum was incubated at 37 °C for 0 min (B) or 5 min (C). The reaction was stopped by addition of ethanol, and following centrifugation, a portion of the supernatant was applied onto a reversed-phase column and the sample was eluted in isocratic conditions with acetonitrile in water (1:1.6, pH 3.0 with H_3PO_4) at a flow rate of 1.0 mL/min. Detection was made at 230 nm. Blank serum (A) was also analyzed in the same way.

Results and Discussion

Frangufoline remained essentially unchanged in gastric juice and in intestinal fluids *in vitro* after 30 min incubation at 37 °C, suggesting that frangufoline was absorbed from the stomach unchanged. On the other hand, frangufoline was readily converted to a metabolite (M1) in rat serum (Figure 1). After 5 min incubation, the frangufoline peak (t_R = 21 min) completely disappeared, and instead, a new peak corresponding to M1 (t_R = 15 min) was observed. The fact that the reaction went at negligible rate in the buffer solution and in the boiled serum indicated that this reaction is an enzymatic process. The reaction also proceeded at a comparable rate with dialyzed rat serum, indicating that low molecular weight cofactor(s) are not involved in the reaction. Mouse serum was capable of converting frangufoline to M1, albeit at a slower rate as compared to rat serum; the relative rate was 1 to 4 in favor of rat serum. On the other hand, both bovine and human sera failed to cleave frangufoline at an appreciable rate under similar reaction conditions.

M1 was found dose-dependently in serum obtained 10 min after iv injection of frangufoline to rats at doses of 5, 10, and 20 mg/kg (Figure 2). Frangufoline was not detected in serum even after higher doses were given (30 min after 50 mg/kg po or 10 min after 25 mg/kg iv).

* To whom correspondence should be addressed. Tel.: 82-2-743−8349. Fax: 82-2-744-4243. E-mail: natural@plaza.snu.ac.kr.

[⊗] Abstract published in *Advance ACS Abstracts,* February 15, 1997.

S0163-3864(96)00661-1 CCC: $14.00

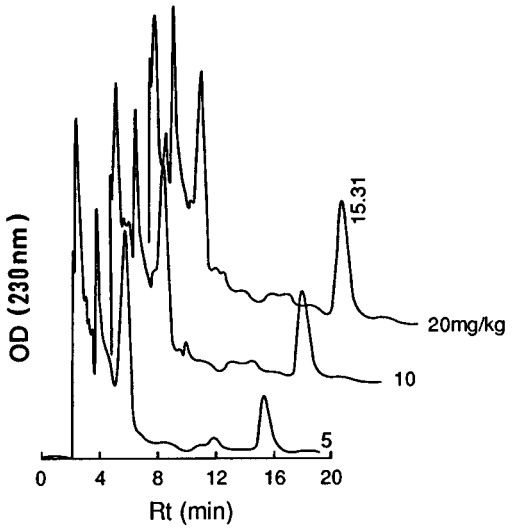


Figure 2. Dose-dependent formation of M1 after iv injection of frangufoline in rats. Frangufoline·HCl in saline was injected into rat tail vein at increasing doses of 5, 10, and 20 mg/kg. After 10 min, serum was obtained and analyzed by HPLC as described in the text.

Table 1. Diagnostic ^{1}H NMR Spectral Data[a] for Frangufoline, M1, and Sanjoinine G_2

	δ_H (*J* Hz)		
H	frangufoline[b]	M1[c]	sanjoinine G_2[d]
ArC*H*=CHN	6.35 d 1H (7.6)		
ArCH=C*H*N	6.67 dd 1H (7.6, 10.2)		
ArCH=CHN*H*	6.45 d 1H (10.2)		5.07, 5.86 s (each 1H, amide NH_2)
O*Ar*−	7.04 dd 1H (2.5, 8.0)	7.04 d 2H (8.7)	7.06 d 2H (8.7)
	7.06 dd 1H (2.5, 8.0)	7.72 d 2H (8.8)	7.80 d 2H (9.0)
	7.13 dd 1H (2.5, 8.0)		
	7.20 dd 1H (2.5, 8.0)		
−CHO		9.72 s 1H	9.86 s 1H

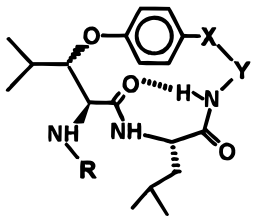
[a] Peaks relevant to this study are shown. For the complete data, refer to the references given below. [b] 360 MHz, $CDCl_3$, TMS as internal standard (taken from ref 9). [c] 500 MHz, CD_3OD, TMS. [d] 300 MHz, $CDCl_3$, TMS, (*S*)-(*N*′,*N*′-dimethylphenylalanyl)-(2*S*,3*S*)-3-[(*p*-formylphenoxy)leucyl]-(*S*)-leucinamide (taken from ref 7).

The detection limit for frangufoline was 0.5 μg/mL under the experimental conditions employed.

Diagnostic ^{1}H-NMR spectral data of M1 are compared to those of frangufoline and sanjoinine G_2 in Table 1. Sanjoinine G_2 is a linear peptide alkaloid isolated from *Z. vulgaris* var. *spinosus* along with other cyclopeptide alkaloids.[8] ^{1}H-NMR data of M1 were in good agreement with those of the acid-catalyzed degradation product of frangufoline,[7] and they were coeluted on HPLC. In particular, a singlet peak at 9.72 ppm and a couple of two proton peaks at 7.04 and 7.72 ppm were indicative of a linear compound with benzaldehyde at one end. The presence of a −COOH at the other end was proved by successful methylation with CH_2N_2 (δ 3.45, s, 3H, $-COOCH_3$). On the basis of these results, M1 was determined to be identical to the acid degradation product of frangufoline, (*S*)-(*N*,*N*-dimethylphenylalanyl)-(*2S*,*3S*)-3-[(*p*-formylphenoxy)leucyl]-(*S*)-leucine. Other spectroscopic data and detailed accounts of structure determination were reported elsewhere.[7]

Among four cyclopeptide alkaloid analogs examined, only sanjoinine Ah_1, which contains the enamide bond, underwent conversion in rat serum, and other cyclopeptide alkaloids remained unchanged after 30 min incubation (Table 2). These results were in agreement with the finding that the enamide bond common in the frangufoline and sanjoinine Ah_1 molecules is the site being cleaved.

Table 2. Structure−Reactivity Relationship



compds	XY	R	*in vitro* conversion in rat serum
frangufoline	CH=CH	L-N(Me)$_2$Phe	yes
sanjoinine Ah_1	CH=CH	D-N(Me)$_2$Phe	yes
dihydrosanjoinine A	CH_2CH_2	L-N(Me)$_2$Phe	no
sanjoinine G_1	$CH(OH)CH_2$	L-N(Me)$_2$Phe	no
sanjoinine D	$CH(OCH_3)CH_2$	L-N(Me)$_2$Phe	no

Table 3. Initial Rates of *in Vitro* Frangufoline Conversion in Rat Serum and Effects of Esterase Inhibitors on the Reaction[a]

inhibitors	initial rate (pmol/min·mg)
no inhibitor	1.44 ± 0.188
PMSF (2 mM)	1.30 ± 0.163
EDTA (2 mM)	0.376 ± 0.148[b]
PCMB (2 mM)	1.34 ± 0.157
BPNP (2 mM)	0.116 ± 0.0137[c]
eserine (2 mM)	0.352 ± 0.143[b]
eserine (10 μM)	1.24 ± 0.161

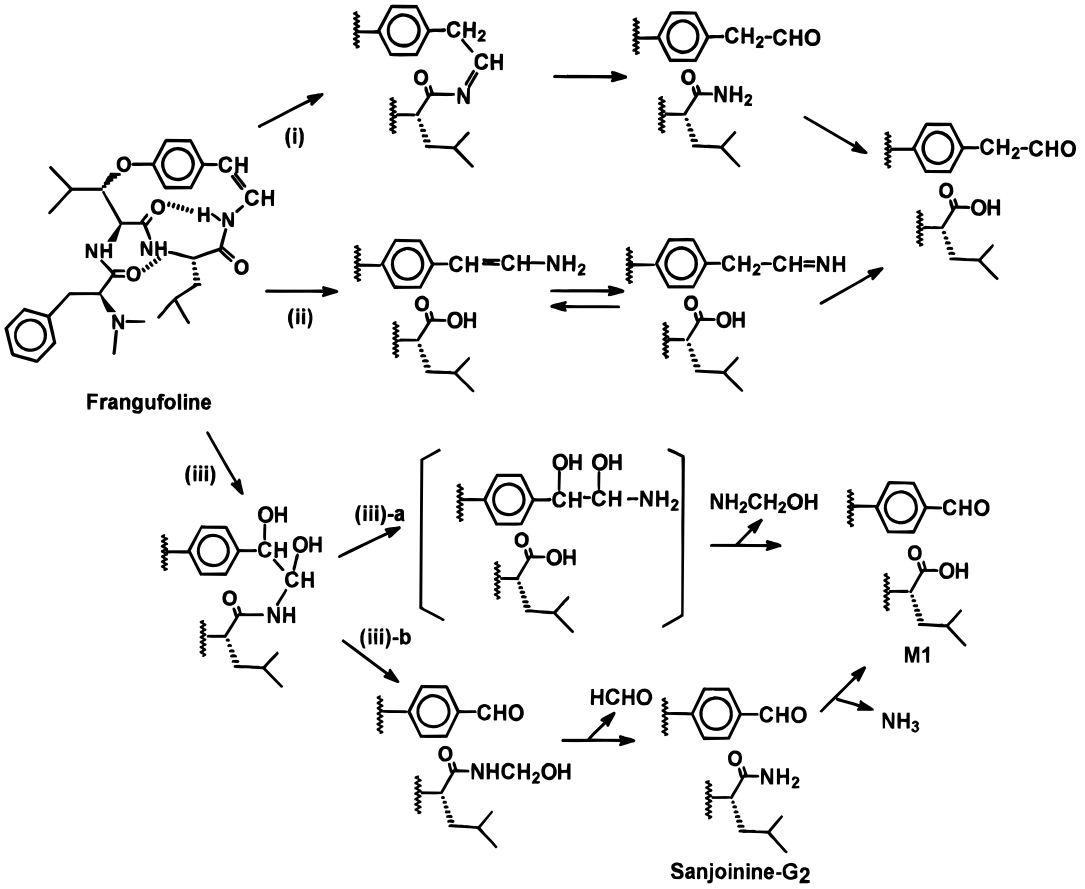
[a] Initial substrate concentration: frangufoline, 0.10 mM. Initial rates are expressed as pmol frangufoline cleaved per min per mg protein. Each value represents the mean ± S.D. of three experiments. Values in parentheses indicate the final concentration of inhibitors added. Significantly different from the control at [b] $p < 0.005$ and [c] $p < 0.001$ (one-way ANOVA).

When added to rat serum, eserine at a concentration of 2 mM but not at 0.01 mM significantly inhibited the metabolic conversion of frangufoline (Table 3). The reaction was also significantly inhibited by bis(*p*-nitrophenyl) phosphate (BPNP) and (phenylmethyl)sulfonyl fluoride (PMSF), but not by EDTA and PCMB.

In the course of pharmacokinetic study on frangufoline, a sedative 14-membered frangulanine-type cyclopeptide alkaloid, we found that it is rapidly cleared from circulation after oral or intravenous administration in mice and rats at pharmacologically active doses. *In vitro* experiments conducted in this study using various rat tissues revealed that frangufoline was rapidly converted to a metabolite (M1) in rat and mouse sera and that it was stable both in gastric juice and in intestinal fluids. Further, M1 was found in serum dose-dependently 10 min after iv administration in rats. These results strongly suggested that frangufoline, once absorbed, rapidly undergoes metabolic conversion to M1 in circulation, although the possibility of presystemic metabolism to other metabolite(s) in the digestive tracts and the liver must be considered.

The results obtained in this study raised a series of intriguing questions on pharmacokinetic and pharmacodynamic aspects of sedative cyclopeptide alkaloids. First, on the basis of the findings that M1 is readily

Scheme 1. Possible Metabolic Degradation of Frangufoline Involving Cleavage at the Enamide Bond[a]



[a] Pathways i and ii illustrate successive enamide−amide hydrolyses and successive amide−enamine hydrolyses, respectively, yielding a degraded product containing a phenylacetaldehyde group. On the other hand, pathway iii illustrates oxidative scission of the enamide to give M1 containing a benzaldehyde group.

formed *in vivo* at pharmacologically active doses and that the most active alkaloids, frangufoline and sanjoinine Ah_1 are the ones having the enamide bond, *i.e.*, the cleavage site (Table 2), it is tempting to suggest that, at least in rodents, M1 may represent the pharmacologically active metabolite. Of course, further metabolism of M1 in rodents and metabolism of frangufoline in other species including human remain to be elucidated. But, being a tripeptide with a bulky substitution at the side chain of the middle amino acid residue, M1 might be fairly resistant to armies of esterases and peptidases. In fact, M1 was resistant to further degradation up to 3 h incubation in rodent serum *in vitro*.

Secondly, examination of the structure of M1 revealed that frangufoline is converted to M1 in serum via unusual enamide cleavage. There are two amide bonds and an enamide bond in frangufoline. Those two amide bonds were found to be quite inert toward hydrolysis in serum *in vitro*, which is evident from the fact that cyclopeptide alkaloids that do not contain the enamide bond remained essentially unchanged after prolonged incubation in serum (Table 2). In Scheme 1, pathway i represents the enamide hydrolysis leading to a compound having a $-CH_2CHO$ group on the aromatic ring of the β-phenoxyleucyl unit. It involves a proton transfer to form an imine that is subsequently hydrolyzed to yield a phenylacetaldehyde and an amine. However, this may not proceed easily since the enamide proton is shown to be hydrogen-bonded to the carbonyl oxygen of the β-hydroxyleucine unit.[9] In fact, both spectroscopic and chemical evidence indicated that M1 contains a $-CHO$ group, instead.[7] Therefore, pathway ii, which involves successive hydrolyses of amide and enamine groups, and pathway i were ruled out for the possible mechanism of M1 formation.

As mentioned above, M1 was also formed by acid treatment (2 N HCl, 55 °C) of frangufoline.[7] At present, evidence suggests that acid- and enzyme-catalyzed formation of M1 from frangufoline may proceed via different mechanisms. In an acid-catalyzed reaction, frangufoline is likely to be transformed via successive oxidation and a retro-aldol-type rearrangement to sanjoinine G_2, which is then hydrolyzed to M1 (Scheme 1, pathway iii-b), since sanjoinine G_2 was isolated from the reaction mixture and a time-course study of the reaction indicated the intermediate nature of sanjoinine G_2 in the process. Furthermore, the findings that acid-catalyzed conversion of frangufoline to M1 proceeded at negligible rate under N_2 atmosphere and that HCHO was detected by the dimedone method from the reaction products provided strong support for this proposed mechanism.[7]

On the contrary, any evidence for intermediate sanjoinine G_2 or noticeable side reaction yielding a metabolite other than M1 was not found in enzyme-catalyzed reaction in rodent serum. Rather, addition of PMSF, eserine, or BPNP, all of which are inhibitors of serine esterase/protease, resulted in dose-dependent blockage of the cleavage of frangufoline, and any other reaction product was not detected in this study. The inhibition profile (Table 3), in which the reaction was inhibited

by organophosphorus esters such as BPNP and by eserine at higher concentrations but not by eserine at lower concentrations or by EDTA and PCMB, corresponded well to that of B-esterase(s),[10] which are known to be present mainly in rat blood and absent in human plasma.[11] Indeed, the reaction did not require low molecular weight cofactors, and mammalian serum failed to metabolize frangufoline *in vitro*. Taken together, the results obtained in this study suggested a mechanism where oxidation of the vinyl group and enzyme-catalyzed hydrolysis of the adjacent amide bond, possibly by a B-esterase-like enzyme, proceed in a concerted manner (Scheme 1, pathway iii-a). More conclusive results can be obtained when the biochemical nature of the enzyme(s) is uncovered.

Experimental Section

General Experimental Procedures. Male Sprague−Dawley rats (Experimental Animals Facility, NPRI, SNU, Seoul), weighing 250−350 g were used in this study. Animals were fed a commercial pellet diet obtained from Samyang Co. (Wonju, Korea), and drinking water was supplied *ad lib*. Eserine (hemisulfate salt), bis(*p*-nitrophenyl) phosphate (BPNP), *p*-chloromercuribenzoic acid (PCMB), and (phenylmethyl)sulfonyl fluoride (PMSF) were purchased from Sigma (St. Louis, MO). All other chemicals were of analytical or HPLC grade.

Isolation of Alkaloids and Preparation of Frangufoline·HCl. Frangufoline and sanjoinine D and G_1 were isolated from the seeds of *Z. vulgaris* var. *spinosus*, and sanjoinine Ah_1 and dihydrosanjoinine A were prepared as described previously.[8] The HCl salt of frangufoline was obtained by treatment of frangufoline with methanolic HCl (2 equiv) in a quantitative yield.

Metabolic Conversion of Frangufoline *in Vitro*. (1) Reaction with Digestive Fluids. To measure the stability of frangufoline in digestive tracts, aliquots of frangufoline·HCl in saline (5 mg/mL) were added to rat gastric juice or intestinal fluids to a final concentration of 0.5 mg/mL, and the reaction mixture was incubated at 37 °C for 30 min. Following neutralization with NH_4-OH, the gastric juice was then extracted with ethyl acetate, and the organic layer was subsequently analyzed by HPLC (see below). The reaction in the intestinal fluids was similarly analyzed. Rat gastric juice and intestinal fluids were collected 4 h after pylorus ligation from overnight fasted animals.

(2) Reaction with Serum and Identification of the Metabolite (M1). Aliquots of frangufoline·HCl in saline were added to serum obtained either from rat, mouse, bovine, or human at a concentration of 0.25 mg/mL, and the reaction mixture was incubated at 37 °C for 1 h. Proteins were precipitated by addition of two volumes of ethanol, and the resulting supernatant was analyzed by HPLC. In a separate experiment, frangufoline was incubated in rat serum, which had been extensively dialyzed (molecular cutoff: 12 000) against 100 mM Tris·HCl (pH 7.5) at 4 °C for 48 h in order to assess the involvement of low molecular weight cofactor(s) in the metabolic process of frangufoline in serum.

Formation of M1 in Rats *in Vivo*. To determine whether M1, the main metabolite of frangufoline found in rat serum *in vitro*, was also formed in rats *in vivo*, frangufoline·HCl in saline was administered via tail vein to rats at increasing doses of 5, 10, and 20 mg/kg. After 10 min, serum was obtained and analyzed by HPLC as described in the *in vitro* study.

Isolation and Structure Determination of M1. Rat serum (35 mL) containing 10 mg of frangufoline·HCl was incubated at 37 °C for 2 h. Following precipitation by 5 volumes of MeOH, the resulting supernatant was dried under reduced pressure. The butanol-soluble fraction was subjected to semipreparative reversed-phase HPLC using a C18 column (RSIL C18 HL 10 μm, 250 × 10 mm, Alltech, Deerfield, IL). The elution was performed using the following procedure: time 0 (min), 100% H_2O; time 10, 100% H_2O; time 30, 60% H_2O in MeOH; time 70, 20% H_2O in MeOH. Fraction 4 containing crude M1 (t_R = 45 min) was further purified by silica gel column chromatography ($CHCl_3$−MeOH = 7:1 to 5:1) to afford M1 (1 mg): crystalline solid, mp 207−8 °C ($CHCl_3$); ^{1}H NMR (CD_3OD, 500 MHz) δ 0.75, 0.77 (each 3 H, d, J = 5.8 Hz, 2 × Leu-Me), 0.85, 0.89 (each 3 H, d, J = 6.8 Hz, 2 × hyLeu-Me), 1.37 (2 H, m, β-H of Leu), 1.46 (1 H, m, γ-H of Leu), 1.88 (1 H, m, γ-H of hyLeu), 2.27 (6 H, s, *N*,*N*-Me_2), 2.84, 2.96 (each 1 H, dd, J = 5.6, 19 Hz, β-H of *N*,*N*-Me_2Phe), 4.02 (1 H, t, J = 5.0 Hz, α-H of *N*,*N*-Me_2Phe), 4.18 (1 H, m, α-H of Leu), 4.63 (1 H, m, β-H of hyLeu), 4.73 (1 H, m, α-H of hyLeu), 7.04 (2 H, d, J = 8.7 Hz, −O*Ar*), 7.13 (5 H, m, Ar-*N*,*N*-Me_2Phe), 7.52 (1 H, d, J = 5.7 Hz, NH-Leu), 7.62 (1 H, d, J = 5.8 Hz, NH-hyLeu), 7.72 (2 H, d, J = 8.8 Hz, *Ar*CHO), 9.72 (1 H, s, CHO).

***In Vitro* Metabolism of Other Cyclopeptide Alkaloids: Structure−Reactivity Relationship.** Cyclopeptide alkaloid analogs, sanjoinine D, sanjoinine Ah_1, sanjoinine G_1, and dihydrosanjoinine A were also subjected to metabolic reaction in rat serum. The extent of metabolism of each alkaloid was analyzed by HPLC.

Effect of Protease Inhibitors on *in Vitro* Metabolism. In order to gain insight into the type of enzyme(s) responsible for the cleavage of frangufoline in rat serum, various protease inhibitors were added to the serum and preincubated at 37 °C for 5 min before the addition of frangufoline (0.11 mM). The reaction volume was brought to 200 μL with 100 mM Tris·HCl (pH 7.5). After incubation at 37 °C for 10 min, the reaction was terminated by addition of 0.8 mL of MeOH, and following centrifugation, the amount of frangufoline cleaved was determined by HPLC. The protein concentration in serum was determined by the method of Lowry *et al.*[12] Inhibitors and their concentrations used in the experiments were eserine (final concentration: 2 and 0.01 mM), BPNP (2 mM), PCMB (2 mM), EDTA (2 mM), and PMSF (2 mM).

HPLC Analysis. Using Model SP 8800 HPLC equipped with a Spectra 100 UV detector and SP 4270 integrator (Spectra Physics, San Jose, CA) and a reversed-phase column (SPHERI-5, 220 × 4.6 mm, Spectra Physics) the samples were eluted in isocratic conditions with a mobile phase of acetonitrile in water (1:1.6, v/v), adjusted to pH 3.0 with H_3PO_4, at 1.0 mL/min flow rate. Frangufoline and M1 were detected at 230 nm. A calibration curve was prepared and linearity was found over the investigated concentration range. The initial rate of metabolic cleavage was calculated as pmol frangufoline cleaved/min·mg protein.

Acknowledgment. This study was supported by a grant (G7 project, 4-4-4) from the Ministry of Science and Technology, R.O.K.

References and Notes

(1) Han, B. H.; Park M. H.; Park, J. H. *Pure Appl. Chem*. **1989**, *61*, 443−448.
(2) Han, B. H.; Park M. H.; Han, Y. N. *Yakhak Hoeji* **1993**, *37*, 143−148.
(3) Bhat, K. L.; Joullie, M. M. *J. Chem. Educ.* **1987**, *64*, 21−27.
(4) Tschesche, R.; Kaussmann, E. U. In *The Alkaloids*; Manske, R. H. F., Ed.; Academic Press: New York, 1975; Vol. 15, Chapter 4, pp 165−205.
(5) Park, M. K.; Park, J. H.; Cho, J. H.; Park, J. K.; Han, Y. N.; Han, B. H. *Arch. Pharm. Res*. **1991**, *14*, 103−104.
(6) Han, Y. N.; Kim, G.-Y.; Hwang, K. H.; Han, B. H. *Arch. Pharm. Res*. **1993**, *16*, 289−294.
(7) Suh, D.-Y.; Kim, Y. C.; Han Y. N.; Han, B. H. *Heterocycles* **1996**, *43*, 2347−2351.
(8) Han, B. H.; Park M. H.; Han, Y. N. *Phytochemistry* **1990**, *29*, 3315−3319.
(9) Park, J. H. Studies on the Conformation of Sedative Cyclopeptide Alkaloid of *Zizyphi jujuba* Semen (Sanjoin) and Its Artefact Produced by Heat Treatment. Ph.D. Thesis, Seoul National University, Seoul, Korea, 1986, p 95.
(10) Williams, F. M. *Clin. Pharmacokinet*. **1985**, *10*, 392−403.
(11) Heymann, E. In *Metabolic Basis of Detoxication*; Jakoby, W. B., Bend, J. R., Caldwell, J., Eds.; Academic Press: New York, 1982; pp 229−245.
(12) Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. *J. Biol. Chem*. **1951**, *193*, 265−275.

NP9606613