Michael-Type Addition of Illudin S, a Toxic Substance from *Lampteromyces japonicus*, with Cysteine and Cysteine-Containing Peptides *in Vitro*

Ken TANAKA,[a] Takako INOUE,*[,a] Yasuhiro TEZUKA,[b] and Tohru KIKUCHI[b]

National Research Institute of Police Science,[a] 6, Sanban-cho, Chiyoda-ku, Tokyo 102, Japan and Research Institute for Wakan-Yaku (Oriental Medicines), Toyama Medical Pharmaceutical University,[b] 2630, Sugitani, Toyama 930-01, Japan. Received August 8, 1995; accepted September 8, 1995

Reactions of illudin S with cysteine derivatives (cysteine methyl ester, glutathione and a peptide, Cys–Asp–Pro–Gly–Tyr–Ile–Gly–Ser–Arg) were investigated. In the reaction with cysteine methyl ester, four products (P1, P2, P3, P4) were obtained and their structures were determined, on the basis of MS and NMR data, to be adducts of the mercapto group of cysteine methyl ester with the α,β-unsaturated carbonyl group of illudin S. In the reactions with glutathione and the peptide, two addition products in each case were identified by liquid chromatography-tandem mass spectrometry (LC-MS/MS) and NMR analyses. The structures of these adducts also indicated that the α,β-unsaturated carbonyl group in illudin S behaves as a Michael acceptor for the mercapto group in cysteine.

Key words illudin S; *Lampteromyces japonicus*; cysteine methyl ester; γ-glutamylcysteinylglycine; liquid chromatography-tandem mass spectrometry

The basidiomycetes *Lampteromyces japonicus* (Japanese name: Tsukiyo-take) is one of the most notorious poisonous mushrooms in Japan. Nakai[1] reported that administration of the methanol extract of this mushroom caused liver damage and hemorrhagic changes in the lung, kidney and digestive organs. Administration of illudin S (**1**), a major constituent of the mushroom, to rats caused complete necrosis of the tips of the villi with capillary circulation disturbance in the duodenum.[2] In addition, inhibitory activity of illudin S (**1**) on RNA synthesis was reported by several groups.[3]

In previous papers,[4] we reported on the biotransformation of illudin S (**1**) into cyclopropane ring-cleaved metabolites (M1, M2) in rat liver (Fig. 1) and on the enzyme systems involved in the process. However, the total yield of the metabolites M1 and M2 accounted for only about 30% of illudin S consumed. We assumed that addition of the mercapto group in endogenous compounds such as cysteine, glutathione (γ-glutamylcysteinylglycine, GSH), thiol enzymes, *etc.* to the α,β-unsaturated carbonyl group in illudin S might also occur.[5] So, we examined the reaction of illudin S with three mercapto group-containing compounds, cysteine methyl ester, GSH and a peptide, Cys–Asp–Pro–Gly–Tyr–Ile–Gly–Ser–Arg. The results are

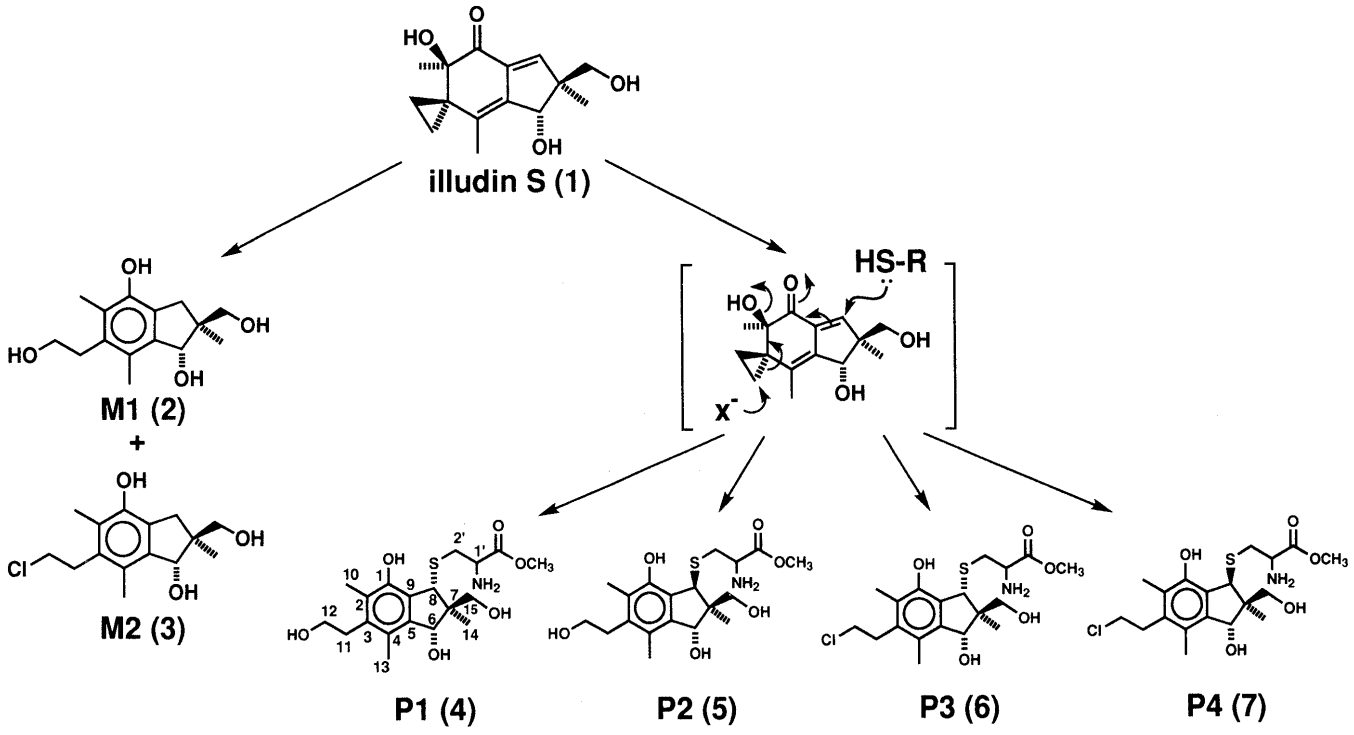

Fig. 1. Metabolites of Illudin S (**1**) in Rat Liver and Reaction Products of Illudin S with Cysteine Methyl Ester
X^- is OH^- or Cl^-.

* To whom correspondence should be addressed.

described in this paper.

Experimental

Materials Illudin S was isolated according to the published method[6] from *Lampteromyces japonicus* collected in Nagano prefecture in October 1992, as described previously.[4] Cysteine methyl ester hydrochloride and GSH (reduced form) were purchased from Nacalai Tesque Inc. (Kyoto, Japan), and a peptide, Cys–Asp–Pro–Gly–Tyr–Ile–Gly–Ser–Arg, was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). A Sep-Pak C_{18} cartridge was obtained from Waters Assoc. (Milford, MA, U.S.A.). All other chemicals and reagents were of the highest grade available.

Analytical Methods TLC was carried out on Merck Kieselgel 60 F_{254} (0.25 mm) plates using methanol–ethyl acetate (1 : 9, v/v) as a developing solvent. Spots were detected under UV light (254 nm) and/or by coloration with ninhydrin reagent. Optical rotations were obtained on a JASCO DIP-4 automatic polarimeter at 25 °C. NMR spectra were measured on a JEOL JNM-GX400 spectrometer in pyridine-d_5 or D_2O using tetramethylsilane as an internal standard. MS were obtained with a Finnigan MAT TSQ700 tandem mass spectrometer (ionization voltage, 70 eV; ionization current, 200 μA; chemical ionization (CI) gas, isobutane at 5000 mtorr; collisionally induced dissociation (CID) gas, argon at 0.5 mtorr; CID energy, −20 eV) using a direct inlet system. High-resolution MS were taken with a JEOL SX-102 mass spectrometer (ionization voltage, 200 eV; ionization current, 300 μA; CI gas, isobutane).

HPLC analyses were carried out on a Shimadzu LC-10A HPLC system equipped with a Shimadzu SPD-10A ultraviolet detector (220 nm). A μ-Bondapack C_{18} column, 2 mm i.d. × 200 mm (Waters Assoc.), was used at a column temperature of 40 °C. The mobile phase consisted of a 0.1% trifluoroacetic acid (TFA) and acetonitrile gradient system. Acetonitrile concentration was increased from 0% to 50% over 50 min for the analysis of GSH adducts, and from 10% to 50% over 80 min for analysis of peptide adducts. The flow rate was 0.3 ml/min.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) measurements were performed on a Finnigan Mat TSQ700 tandem mass spectrometer, equipped with a Shimadzu 10A pump and Finnigan Mat atmospheric pressure ionization (API) system. MS conditions: electrospray ionization (ESI) high voltage, 4.5 kV; capillary temperature, 200 °C; sheath gas, nitrogen at 65 psi; CID gas, argon at 1.25 mtorr; CID energy, −23 eV. HPLC conditions were the same as those described above.

Reaction of Illudin S with Cysteine Methyl Ester A suspension of illudin S (530 mg) in water (10 ml) was added to a solution of cysteine methyl ester hydrochloride (370 mg) in water (40 ml), and the mixture was incubated with shaking for 1 h at 37 °C. After evaporation of the solvent *in vacuo*, four products (P1, P2, P3, P4) were isolated by repeated preparative TLC using methanol–ethyl acetate (1 : 9, v/v) as a developing solvent.

P1 (**4**): Oil (100 mg), $[\alpha]_D$ −32.2° (c = 1.02, CH_3OH). ^{1}H- and ^{13}C-NMR: Tables 1 and 2. CI-MS m/z: 400 ($(M+H)^+$), 382 ($(M+H)^+ - H_2O$), 352 ($(M+H)^+ - HCHO - H_2O$), 298 ($(M+H)^+ - C_4H_8NO_2$), 265 ($(M+H)^+ - C_4H_9NO_2S$), 247 ($(M+H)^+ - C_4H_9NO_2S - H_2O$), 235 ($(M+H)^+ - C_4H_9NO_2S - HCHO$), 217 ($(M+H)^+ - C_4H_9NO_2S - HCHO - H_2O$). CID-MS (from m/z 400) m/z: 382, 364, 352, 298, 265, 247, 235, 217. High-resolution MS m/z: 400.1809 ($(M+H)^+$) (Calcd for $C_{19}H_{30}NO_6S$: 400.1794), 382.1725 (Calcd for $C_{19}H_{28}NO_5S$: 382.1688), 352.1569 (Calcd for $C_{18}H_{26}NO_4S$: 352.1583), 298.1263 (Calcd for $C_{15}H_{22}O_4S$: 298.1238), 265.1439 (Calcd for $C_{15}H_{21}O_4$: 265.1439), 247.1293 (Calcd for $C_{15}H_{19}O_3$: 247.1334), 235.1326 (Calcd for $C_{14}H_{19}O_3$: 235.1334), 217.1230 (Calcd for $C_{14}H_{19}O_2$: 217.1229).

P2 (**5**): Oil (81 mg), $[\alpha]_D$ −2.7° (c = 0.97, CH_3OH). ^{1}H- and ^{13}C-NMR: Tables 1 and 2. CI-MS m/z: 400 ($(M+H)^+$), 382 ($(M+H)^+ - H_2O$), 352 ($(M+H)^+ - HCHO - H_2O$), 298 ($(M+H)^+ - C_4H_8NO_2$), 265 ($(M+H)^+ - C_4H_9NO_2S$), 247 ($(M+H)^+ - C_4H_9NO_2S - H_2O$), 235 ($(M+H)^+ - C_4H_9NO_2S - HCHO$), 217 ($(M+H)^+ - C_4H_9NO_2S - HCHO - H_2O$). CID-MS (from m/z 400) m/z: 382, 364, 352, 298, 265, 247, 235, 217. High-resolution MS m/z: 400.1773 ($(M+H)^+$) (Calcd for $C_{19}H_{30}NO_6S$: 400.1794), 382.1641 (Calcd for $C_{19}H_{28}NO_5S$: 382.1688), 352.1569 (Calcd for $C_{18}H_{26}NO_4S$: 352.1583), 298.1235 (Calcd for $C_{15}H_{22}O_4S$: 298.1238), 265.1437 (Calcd for $C_{15}H_{21}O_4$: 265.1439), 247.1278 (Calcd for $C_{15}H_{19}O_3$: 247.1334), 235.1303 (Calcd for $C_{14}H_{19}O_3$: 235.1334), 217.1230 (Calcd for $C_{14}H_{19}O_2$: 217.1229).

P3 (**6**): Amorphous powder (79 mg), $[\alpha]_D$ −37.6° (c = 0.81, CH_3OH). ^{1}H- and ^{13}C-NMR: Tables 1 and 2. CI-MS m/z: 418 ($(M+H)^+$), 420 (isotope peak), 400 ($(M+H)^+ - H_2O$), 402 (isotope peak), 370 ($(M+H)^+ - HCHO - H_2O$), 372 (isotope peak), 316 ($(M+H)^+ - C_4H_8NO_2$), 283 ($(M+H)^+ - C_4H_9NO_2S$), 285 (isotope peak), 265 ($(M+H)^+ - C_4H_9NO_2S - H_2O$), 235 ($(M+H)^+ - C_4H_9NO_2S - HCHO - H_2O$), 237 (isotope peak). CID-MS (from m/z 418) m/z: 400, 370, 316, 283, 265, 235. High-resolution MS m/z: 418.1481 ($(M+H)^+$) (Calcd for $C_{19}H_{29}ClNO_5S$: 418.1455), 400.1375 (Calcd for $C_{19}H_{27}ClNO_4S$: 400.1350), 370.1266 (Calcd for $C_{18}H_{25}ClNO_3S$: 370.1244), 316.0882 (Calcd for $C_{15}H_{21}ClO_3S$: 316.0900), 283.1089 (Calcd for $C_{15}H_{20}ClO_3$: 283.1101), 265.1007 (Calcd for $C_{15}H_{18}ClO_2$: 265.0996), 235.0861 (Calcd for $C_{14}H_{16}ClO$: 235.0889).

P4 (**7**): Oil (74 mg), $[\alpha]_D$ −6.2° (c = 0.74, CH_3OH). ^{1}H- and ^{13}C-NMR: Tables 1 and 2. CI-MS m/z: 418 ($(M+H)^+$), 420 (isotope peak), 400 ($(M+H)^+ - H_2O$), 402 (isotope peak), 370 ($(M+H)^+ - HCHO - H_2O$), 372 (isotope peak), 316 ($(M+H)^+ - C_4H_8NO_2$), 283 ($(M+H)^+ - C_4H_9NO_2S$), 285 (isotope peak), 265 ($(M+H)^+ - C_4H_9NO_2S - H_2O$), 237 (isotope peak). CID-MS (from m/z 418) m/z: 400, 370, 316, 283, 265, 235. High-resolution MS m/z: 418.1471 ($(M+H)^+$) (Calcd for $C_{19}H_{29}ClNO_5S$: 418.1455), 400.1383 (Calcd for $C_{19}H_{27}ClNO_4S$: 400.1350), 370.1285 (Calcd for $C_{18}H_{25}ClNO_3S$: 370.1244), 316.0893 (Calcd for $C_{15}H_{21}ClO_3S$: 316.0900), 283.1091 (Calcd for $C_{15}H_{20}ClO_3$: 283.1101), 265.1001 (Calcd for $C_{15}H_{18}ClO_2$: 265.0996), 235.0872 (Calcd for $C_{14}H_{16}ClO$: 235.0889).

Reaction of Illudin S with GSH A solution of illudin S (30 mg) in acetonitrile (2 ml) was added to an aqueous solution (5 ml) of GSH (45 mg), and the mixture was stirred at room temperature for 24 h, then washed with ethyl acetate (20 ml) to remove the unreacted starting material. The aqueous layer was lyophilized. The residue was dissolved in 5 ml of water and applied to a Sep-Pak C_{18} cartridge. The cartridge was washed with 10 ml of water, and then eluted with 10 ml of water–acetonitrile (9 : 1, v/v). The eluate was evaporated to dryness *in vacuo* to give the GSH adduct (**8**, *ca.* 6 mg) as a mixture of isomers

Table 1. ^{1}H-NMR Data for the Reaction Products P1 (**4**), P2 (**5**), P3 (**6**) and P4 (**7**)

Proton	Compounds			
	P1 (**4**)	P2 (**5**)	P3 (**6**)	P4 (**7**)
6-H	5.09 s	5.58 s	5.07 s	5.53 s
8-H	4.72 s	4.85 s	4.70 s	4.79 s
10-H_3	2.46 s	2.56 s	2.35 s	2.43 s
11-H_2	3.16 t (8.0)	3.26 t (8.0)	3.12 t (8.3)	3.18 t (8.5)
12-H_2	3.89 t (8.0)	3.98 t (8.0)	3.49—3.54 m	3.58—3.62 m
13-H_3	2.48 s	2.65 s	2.38 t	2.54 t
14-H_3	1.99 s	1.58 s	1.98 s	1.53 s
15-H_2	3.54, 3.73 d (10.0)	4.36, 4.85 d (10.0)	3.52, 3.73 d (10.4)	4.33, 4.81 d (10.0)
1′-H	4.26 dd (5.0, 6.0)	3.88 dd (4.0, 11.0)	4.27 dd (4.6, 5.8)	3.86 dd (3.5, 11.0)
2′-H	3.18 dd (6.0, 15.0)	3.32 dd (4.0, 15.0)	3.16 dd (5.8, 14.3)	3.30 dd (3.5, 15.0)
	3.29 dd (5.0, 15.0)	3.56 dd (11.0, 15.0)	3.28 dd (4.6, 14.3)	3.53 dd (11.0, 15.0)
OCH_3	3.57 s	3.57 s	3.55 s	3.59 s

Spectra were measured in pyridine-d_5 solutions. Values in parentheses are coupling constants (Hz).

(approximate ratio, 3:1), which was subjected to ^{1}H-NMR and LC-MS/MS analyses.

^{1}H-NMR (D_2O) δ: 1.00 (s, minor isomer, 14-H_3), 1.28 (s, major isomer, 14-H_3), 2.15 (s, minor isomer, 10-H_3), 2.17 (s, major isomer, 10-H_3), 2.21 (s, major isomer, 13-H_3), 2.23 (s, minor isomer, 13-H_3). LC-MS/MS: major product, t_R 24.5 min, *m/z* 572 $(M+H)^+$, CID-MS from *m/z* 572, *m/z* 524, 395, 308, 265, 247, 233, 217, 179, 129; minor product, t_R 25.5 min, *m/z* 572 $(M+H)^+$, CID-MS from *m/z* 572, *m/z* 524, 395, 308, 265, 247, 233, 217, 179, 129.

Reaction of Illudin S with Cys–Asp–Pro–Gly–Tyr–Ile–Gly–Ser–Arg A suspension of illudin S (6 mg) in water (1 ml) was added to an aqueous solution (1 ml) of Cys–Asp–Pro–Gly–Tyr–Ile–Gly–Ser–Arg (2 mg), and the mixture was stirred at room temperature for 24 h. The remaining illudin S was extracted with ethyl acetate (20 ml), and the aqueous layer was concentrated to dryness by lyophilization. The residue was dissolved in 5 ml of water and applied to a Sep-Pak C_{18} cartridge. The cartridge was washed with 10 ml of water, and then eluted with 10 ml of water–acetonitrile (85:15, v/v). The eluate was evaporated to dryness *in vacuo* to give the peptide adduct (**9**, *ca.* 2 mg) as a mixture of stereoisomers (approximate ratio of 1.7:1), which was subjected to ^{1}H-NMR and LC-MS/MS analyses.

Table 2. ^{13}C-NMR Data for the Reaction Products P1 (**4**), P2 (**5**), P3 (**6**) and P4 (**7**)

Carbon atom	Compounds			
	P1 (**4**)	P2 (**5**)	P3 (**6**)	P4 (**7**)
1	151.4 s	151.4 s	151.6 s	151.5 s
2	124.1 s	123.2 s	123.9 s	123.2 s
3	137.3 s	137.7 s	135.7 s	136.3 s
4	124.7 s	125.0 s	124.5 s	124.8 s
5	141.7 s	140.0 s	142.1 s	140.3 s
6	77.9 d	78.5 d	77.8 d	78.6 d
7	53.3 s	53.4 s	53.3 s	53.4 s
8	54.6 d	58.7 d	54.5 d	58.6 d
9	126.6 s	125.5 s	127.5 s	126.5 s
10	12.4 q	12.4 q	12.2 q	12.3 q
11	34.5 t	34.3 t	33.9 t	33.8 t
12	61.2 t	61.3 t	42.9 t	42.9 t
13	15.1 q	14.6 q	14.9 q	14.4 q
14	16.9 q	19.1 q	16.9 q	19.0 q
15	69.1 t	66.8 t	69.1 t	66.7 t
1′	54.5 d	58.9 d	54.5 d	58.9 d
2′	36.4 t	37.2 t	36.3 t	37.1 t
CO	174.0 s	175.0 s	173.9 s	174.9 s
OCH_3	51.9 q	51.8 q	51.9 q	51.8 q

Spectra were measured in pyridine-d_5 solutions. The multiplicities of carbon signals are indicated as s (singlet), d (doublet), t (triplet) and q (quartet).

^{1}H-NMR (D_2O) δ: 0.88 (s, minor isomer, 14-H_3), 1.13 (s, major isomer, 14-H_3), 1.97 (s, minor isomer, 10-H_3), 2.06 (s, major isomer, 10-H_3), 2.09 (s, minor isomer, 13-H_3), 2.12 (s, major isomer, 13-H_3). ESI-MS *m/z*: 1231.6 $(M+H)^+$, 967.5, 749.5, 616.3 $(M+2H)^{2+}$. CID-MS (from *m/z* 616.3) *m/z*: 967.4, 749.5 (Y''_7), 651.5 (Y''_6), 482.5 (B_2), 375.4 (Y''^{2+}_7), 265.4, 247.2, 217.1, 201.1.

Results

Reaction of Illudin S with Cysteine Methyl Ester The reaction mixture showed four new spots (*Rf* values: P1, 0.31; P2, 0.42; P3, 0.58; P4, 0.68) along with that of illudin S (*Rf* value: 0.80) on TLC, and the products showed positive coloration with ninhydrin reagent. Each product was purified by repeated preparative TLC, and subjected to NMR and MS analyses.

P1 (**4**) and P2 (**5**) showed similar CI-MS and their $(M+H)^+$ ion appeared at *m/z* 400. Their molecular formulae were determined to be $C_{19}H_{29}NO_6S$ from the high-resolution MS, which also confirmed the composition of the fragment ions at *m/z* 298 in the CI-MS of P1 (**4**) and P2 (**5**) to be $C_{15}H_{22}O_4S$, indicating that the cysteine molecule combines with illudin S at the sulfur atom. The CID-MS from the $(M+H)^+$ (*m/z* 400) showed the ion to have been formed by elimination of the cysteine residue at *m/z* 265, along with dehydrated ions at *m/z* 382 and 364 (Fig. 2B). The ions at *m/z* 352 and 217 seemed to be formed by the concerted elimination of formaldehyde and water (reverse Prins reaction[7]) from the $(M+H)^+$ and the ion at *m/z* 265, respectively (Fig. 2). These findings indicated that both P1 (**4**) and P2 (**5**) were adducts of cysteine methyl ester with illudin S and they were stereoisomeric.

The ^{1}H- and ^{13}C-NMR spectra of P1 (**4**) and P2 (**5**) both exhibited signals due to three tertiary methyls, a hydroxy-bearing methylene, a hydroxy-bearing methine, a methine adjacent to a heteroatom and six aromatic carbons, along with signals ascribable to a hydroxyethyl group and cysteine methyl ester moiety, which were analyzed by DEPT (distortionless enhancement by polarization transfer), and ^{1}H–^{1}H and ^{1}H–^{13}C correlation spectroscopy (COSY) (Tables 1, 2, Fig. 4A, D). The significant long-range ^{1}H–^{13}C correlations observed in the long-range ^{1}H–^{13}C COSY spectrum of P1 (**4**) are indicated by arrows in Fig. 3. The long-range ^{1}H–^{13}C COSY

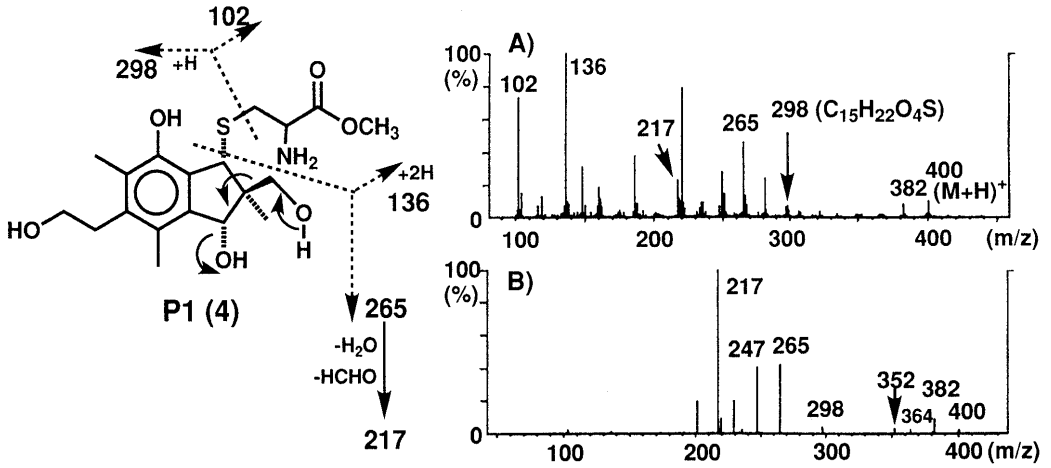

Fig. 2. Mass Spectra of P1 (**4**)

A) CI mass spectrum; B) CID spectrum from the *m/z* 400 ion in A.

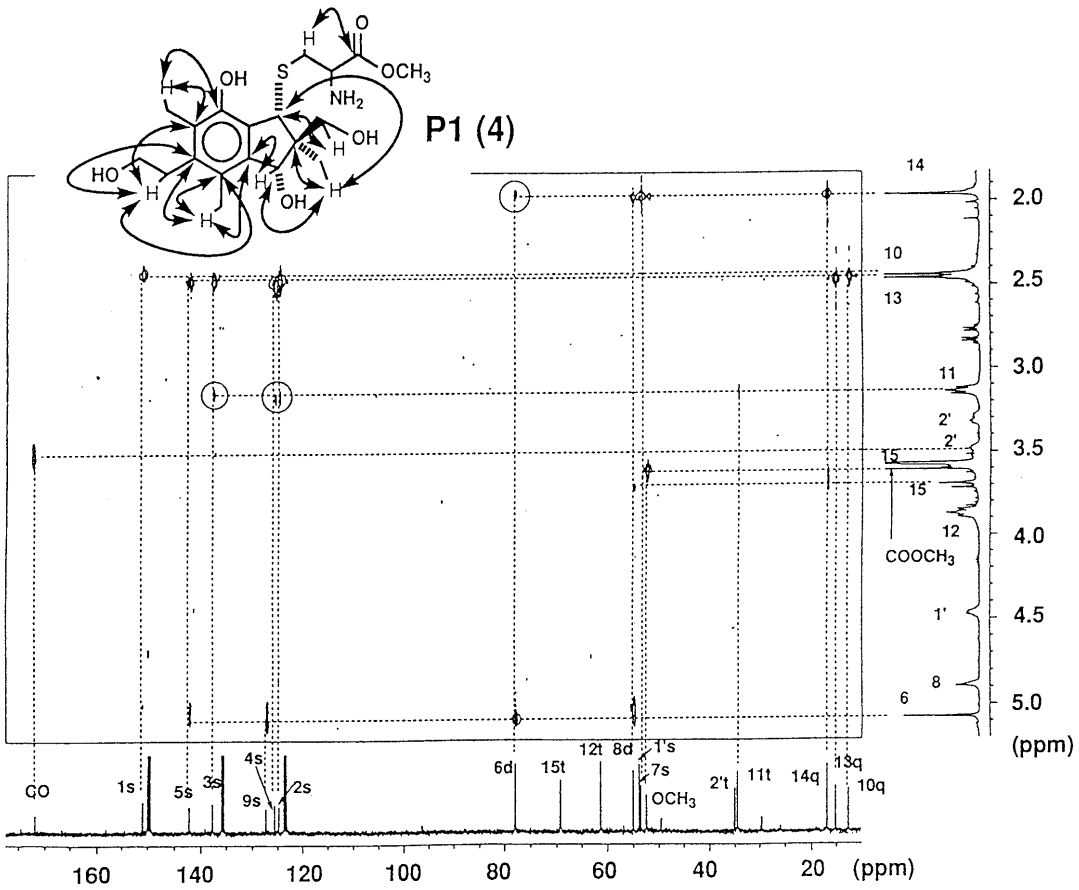

Fig. 3. Long-Range ^{1}H–^{13}C Shift Correlation Spectrum of P1 (**4**) in Pyridine-d_5

spectrum of P2 (**5**) gave similar results to those in the case of P1 (**4**).

From the above spectral data in comparison with those of M1 (**2**),[4] it was suggested that P1 (**4**) and P2 (**5**) contain the M1 (**2**) and cysteine moieties. Because the methylene group at M1 (**2**) is changed to a heteroatom-substituted methine, the cysteine moiety should be located at the C-8 of the M1 moiety.

The configuration at the C-8 position of P1 (**4**) and P2 (**5**) was determined by differential nuclear Overhauser effect (NOE) experiments. As shown in Fig. 4, irradiation of 14-H_3 (δ 1.99) of P1 (**4**) and that (δ 1.58) of P2 (**5**) clearly enhanced the signal intensities of both 6-H and 8-H. In addition, no NOE was observed between 6-H and 8-H in P1 (**4**) or P2 (**5**). These findings indicated that both compounds have the conformation depicted in Fig. 4. The change in the signal intensity of 8-H upon irradiation of 15-H_2 (δ 4.85) of P2 (**5**) was not clarified, because the 8-H signal overlapped with one of the 15-H_2 signals. However, irradiation of 15-H_2 (δ 3.54) of P1 (**4**) clearly enhanced the signal intensities of both 6-H and 8-H. These results suggested the configuration of 8-H in P1 (**4**) and P2 (**5**) to be β and α, respectively.

Both P3 (**6**) and P4 (**7**) exhibited the $(M+H)^+$ ions at m/z 418 and the isotope ion peak at m/z 420 in the intensity ratio of 3 : 1 in the MS, indicating the presence of a chlorine atom in the molecules. The molecular formulae of these compounds were determined to be $C_{19}H_{28}ClNO_5S$ by high-resolution MS and their fragmentation patterns were very similar to each other and to those of P1 (**4**) and P2 (**5**). The ^{1}H- and ^{13}C-NMR spectra of P3 (**6**) and P4 (**7**), analyzed with the aid of ^{1}H–^{13}C COSY and long-range ^{1}H–^{13}C COSY, were also very similar to those of P1 (**4**) and P2 (**5**), respectively, except for slight differences in the chemical shift values of the signals of the 11- and 12-methylenes (Tables 1, 2). Thus, P3 and P4 were considered to have the structures **6** and **7** (Fig. 1), respectively. The stereochemistry at the C-8 position was confirmed by NOE experiments; irradiation of 14-H_3 (δ 1.98) or 15-H_2 (δ 3.73) of P3 (**6**) enhanced the signal intensities of both 6-H and 8-H, and irradiation of 14-H_3 (δ 1.53) of P4 (**7**) increased the signal intensities of 6-H and 8-H.

Reaction of Illudin S with Glutathione The reaction mixture showed two product peaks (intensity ratio, 3 : 1) in the HPLC chromatogram, but due to their instability, they could not be isolated. Thus, their structures were examined by LC-MS/MS and NMR analyses of the mixture after removal of remaining illudin S and GSH using liquid–liquid and solid phase extractions (see Experimental).

The ESI-MS of both product peaks showed the $(M+H)^+$ ions at m/z 572, coincident with the molecular weight of GSH adducts of illudin S, and the low-energy CID spectra from the m/z 572 ions also showed similar fragmentation patterns to each other (Fig. 5). The product ions at m/z 308, 179 and 129 were considered to be derived from the glutathione moiety, and other product ions were interpreted as shown in Fig. 5. From these MS data the reaction products were concluded to be stereoisomers

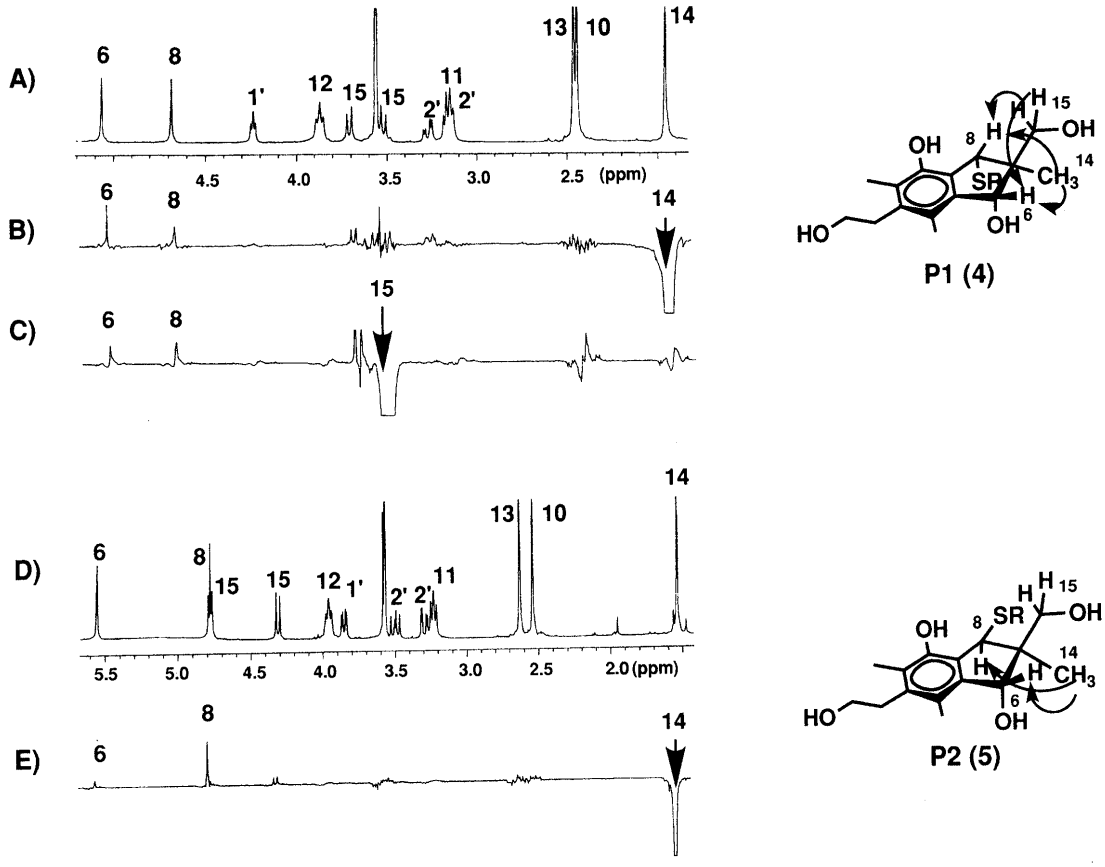

Fig. 4. ^{1}H-NMR Spectra of P1 (**4**) and P2 (**5**)

A) NMR spectrum of P1 (**4**) in pyridine-d_5; B) NOE difference spectrum irradiated at δ 1.99; C) NOE difference spectrum irradiated at δ 3.54; D) NMR spectrum of P2 (**5**) in pyridine-d_5; E) NOE difference spectrum irradiated at δ 1.58.

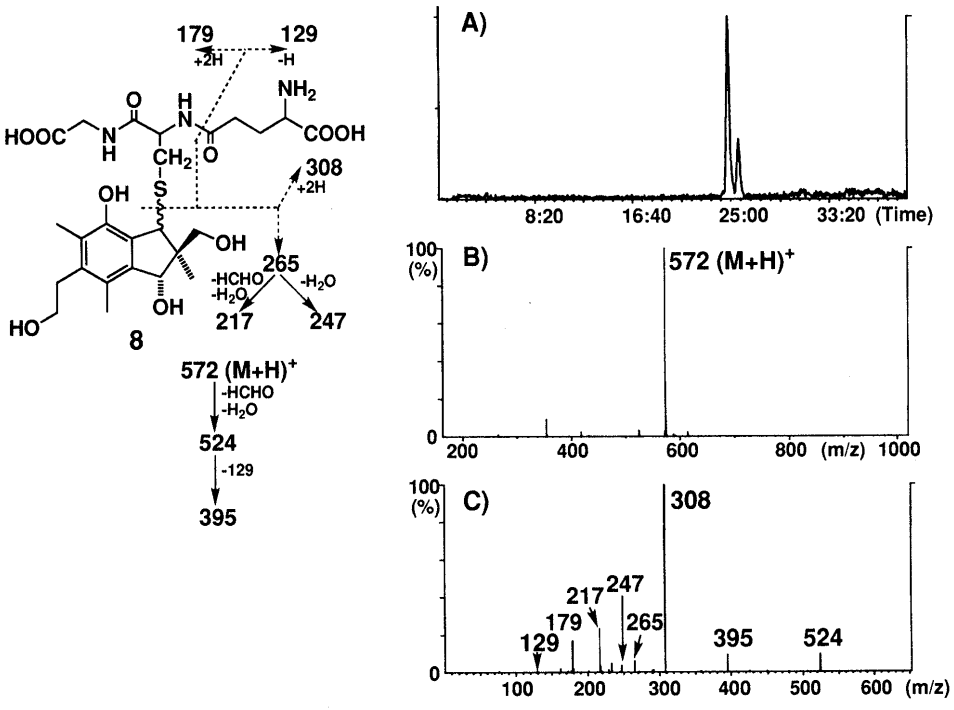

Fig. 5. Mass Spectra of the GSH Adduct (**8**) of Illudin S

A) Total ion chromatogram of reaction product; B) ESI-MS of the major peak in A; C) CID spectrum from the m/z 572 ion in B.

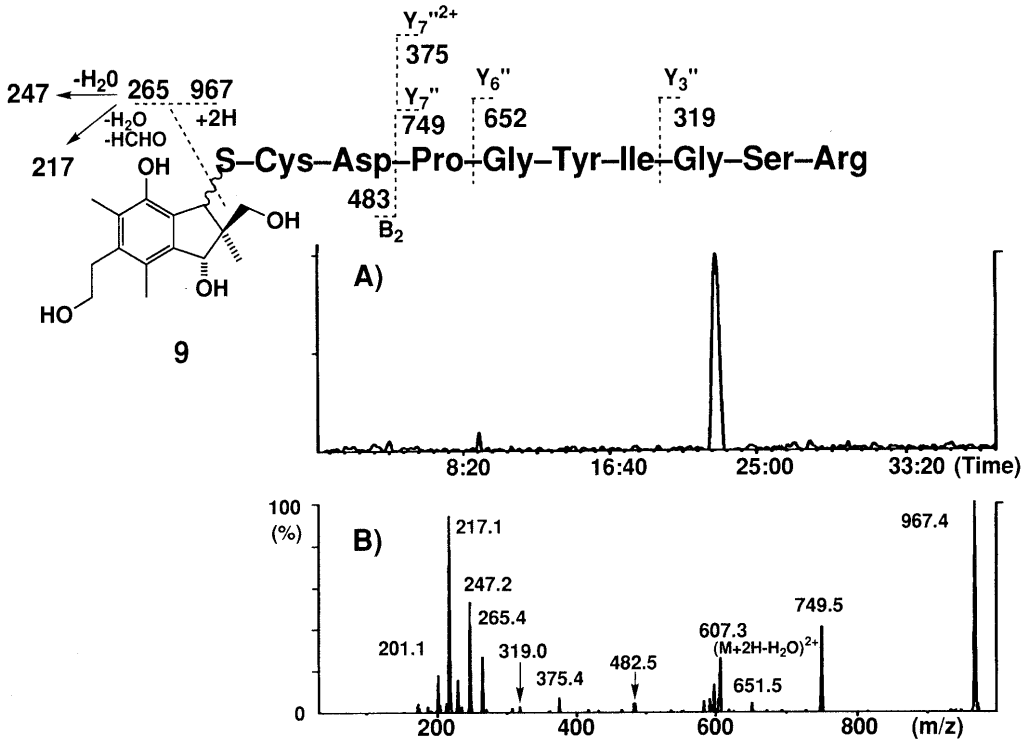

Fig. 6. LC-MS/MS of the Peptide Adduct (**9**) of Illudin S

A) Total ion chromatogram; B) CID spectrum from the $(M+2H)^{2+}$ ion at *m/z* 616.3.

represented by the structure formula **8**.

In the NMR spectrum of the product mixture, the major product showed a higher chemical shift value of 14-H_3 and a smaller difference between the chemical shift values of 10-H_3 and 13-H_3 than those of the minor product. Comparison of these data with those of cysteine methyl ester adducts (P1, P2, P3, P4) indicated that major product has a *cis* relation between the GSH moiety and 6-OH.

Reaction of Illudin S and Cys–Asp–Pro–Gly–Tyr–Ile–Gly–Ser–Arg In LC-MS analysis of the reaction mixture of illudin S and the peptide, one new peak was observed. The ESI-MS of this peak showed the $(M+H)^+$ ion at *m/z* 1231.6 and the $(M+2H)^{2+}$ ion at *m/z* 616.3, corresponding to the molecular weight of the peptide adduct of illudin S.

The CID-MS from the $(M+2H)^{2+}$ ion at *m/z* 616.3 of the product gave the fragment ions at *m/z* 967.4, 749.5, 651.5, 607.3 and 375.4, which were ascribable to ions due to the peptide moiety, Y''_7, Y''_6, $(M+2H-H_2O)^{2+}$ and Y''^{2+}_7, respectively (Fig. 6). In addition, a series of ions at *m/z* 265, 247 and 217 were observed as well as the CID-MS of P1 (**4**) (Fig. 2B) and GSH adducts (**8**) of illudin S (Fig. 5C), indicating the presence of the indanol skeleton. The presence of the ion at *m/z* 482.5, which corresponds to the ion indanol–Cys–Asp, indicated that the indanol moiety is attached to the peptide through the N-terminal cysteine.

NMR analysis of the Sep-Pak C_{18} eluate revealed that the reaction product was a mixture of two compounds (approximate ratio of 1.7:1). From a consideration of the chemical shift values of 14-H_3 and the differences of those of 10-H_3 and 13-H_3, as in the case of the reaction products with glutathione, the major product was assumed to have a *cis* relation between the peptide moiety and the secondary hydroxyl group. Based on these spectroscopic data, the reaction product was concluded to be a mixture of stereoisomers represented by the structure formula **9** in Fig. 6.

Discussion

The present paper describes the reactions of illudin S with cysteine methyl ester, glutathione and a cysteine-containing peptide Cys–Asp–Pro–Gly–Tyr–Ile–Gly–Ser–Arg. Our results show that illudin S reacts with these compounds to form four, two and two adducts, respectively. These adducts all possess the C–S bond between indane C-8 and the mercapto group of cysteine, and include both stereoisomers at indane C-8. The configuration of C-8 of the major products seemed to be *cis* to the secondary hydroxyl group in the five-membered ring. McMorris *et al.*[8,9] reported that the reaction of illudin M, an 15-H_3 analog of illudin S, with GSH gave GSH-adducts as a mixture of isomers (*ca.* 3:1) and that the major product was tentatively assigned *cis* configuration for the new substituent and the secondary hydroxyl group in the five-membered ring. Their results are in accordance with our results presented here as to the configuration of products.

In addition, from the viewpoint of the structure–toxicity relationship, McMorris *et al.*[9,10] suggested that the reactivity of illudin M and its analogs with thiol-containing compounds such as thioglycolate, cysteine and glutathione may be responsible for their extreme toxicity.

High reactivity of illudin S with the thiol group of cysteine, as described in this paper, may imply that the residual illudin S that was lost in the *in vitro* experiment had combined with proteins such as thiol enzymes and that binding of illudin S to vital thiol enzymes might cause

the necrosis in digestive organs.

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