POTENT IMMUNOSUPPRESSIVE PRINCIPLES, DIMERIC SESQUITERPENE THIOALKALOIDS, ISOLATED FROM NUPHARIS RHIZOMA, THE RHIZOMA OF *NUPHAR PUMILUM* (NYMPHAEACEAE): STRUCTURE-REQUIREMENT OF NUPHAR-ALKALOID FOR IMMUNOSUPPRESSIVE ACTIVITY

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Potent immunosuppressants, the dimeric sesquiterpene thioalkaloids, 6-hydroxythiobinupharidine (2), 6,6'-dihydroxythiobinupharidine (3), 6-hydroxythionuphlutine B (5) and 6'-hydroxythionuphlutine B (6), were isolated from a natural medicine, Nupharis Rhizoma, the rhizoma of *Nuphar pumilum* (TIMM.) DC., through bioassay-guided separation together with five quinolizidine alkaloids (8, 9, 10, 11, 12). Dimeric sesquiterpene thioalkaloids (2, 3, 5, 6) were found to significantly inhibit anti-sheep erythrocyte plaque forming cell formation in mice spleen cells at 10^{-6} M concentration. At this concentration, 2, 5 and 6 were found to exhibit no cytotoxicity to mice spleen cells, and 3 also showed only a little cytotoxicity. In addition, the inhibitory activity of several Nuphar alkaloids, dimeric sesquiterpene thioalkaloids (1, 4, 7, 8), and monomeric sesquiterpene alkaloids (9, 10, 11, 12) on anti-sheep erythrocyte plaque forming cell formation was examined and some structural requirement of Nuphar alkaloid for immunosuppressive activity was determined.

KEY WORDS immunosuppressive alkaloid; dimeric sesquiterpene thioalkaloid; *Nuphar pumilum*; Nupharis Rhizoma; anti-sheep erythrocyte plaque forming cell formation; 6-hydroxythiobinupharidine

Nupharis Rhizoma, the dried rhizoma of *Nuphar japonicum* DC. and *Nuphar pumilum* (TIMM.) DC., has been prescribed for tonic, hemostatic, and diuretic purposes in Japanese and Chinese traditional preparations. Chemical studies on this natural medicine have been carried out by many investigators and a number of sesquiterpene alkaloids such as nupharidine (9) and deoxynupharidine (10) have been identified from *N. japonicum*.¹⁾ However, in the pharmacological study of Nuphar alkaloid, Only the central paralysis effect of 10 has been reported so far.²⁾ In the course of our studies for bioactive principles of natural medicines,³⁾ we have found that the alkaloid fraction from the

Fig.1. Chemical Structures of Nuphar Alkaloids

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rhizoma of *N. pumilum* showed inhibitory activity on the anti-sheep erythrocyte (SRBC)-plaque forming cell (PFC) formation in mice spleen cells. Through bioassay-guided separation and purification, we have isolated four active dimeric sesquiterpene thioalkaloids, 6-hydroxythiobinupharidine (2),⁴⁾ 6,6'-dihydroxythiobinupharidine (3),⁵⁾ 6-hydroxythionupharidine B (5),⁶⁾ and 6'-hydroxythionupharidine B (6),⁶⁾ together with an inactive dimeric sesquiterpene thioalkaloid, neothiobinupharidine (8),⁷⁾ and four inactive sesquiterpene alkaloids, nupharidine (9),¹⁾ deoxynupharidine (10),¹⁾ 7-epideoxynupharidine (11)⁸⁾ and nupharolutine (12)⁹⁾ from the rhizoma of *N. pumilum*. To determine the structural requirement of Nuphar alkaloids for immunosuppressive activity, three dimeric sesquiterpene thioalkaloids, thiobinupharidine (1),¹⁰⁾ thionupharidine B (4),⁴⁾ and 6,6'-dihydroxythionupharidine B (7)⁵⁾ were derived from 2, 5, and 3, respectively.¹¹⁾ In this communication, we describe the immunosuppressive effect and cytotoxicity of Nuphar alkaloids and their structural requirement for the activity.

The MeOH extract of the rhizoma of *N. pumilum* was partitioned into CHCl₃-aqueous HCl. The aqueous HCl phase was alkalinized with conc. NH₄OH and then extracted with AcOEt. Since the AcOEt soluble portion (the alkaloid fraction) showed immunosuppressive activity, it was subjected to ordinary silica-gel (CHCl₃-MeOH-Et₂NH) and NH-chromatorex (n-hexane:CH₂Cl₂:AcOEt → CHCl₃:MeOH) column chromatography and finally HPLC (Develosil ODS-HG-5, MeOH-H₂O-Et₂NH) to furnish 2 (0.019 % from natural medicine), 3 (0.020 %), 5 (0.002 %), 6 (0.003 %), 8 (0.001 %), 9 (0.0006 %), 10 (0.002 %), 11 (0.004 %), and 12 (0.0008 %). These dimeric sesquiterpene thioalkaloids (2, 3, 5, 6, 8) were first isolated from Nupharis Rhizoma and the absolute structures of 2, 3, 5 and 6 were characterized as shown in Fig. 1.¹¹⁾ Dehydroxyl derivatives (1, 4) were synthesized by NaBH₄ reduction from 2 and 5, respectively, and 7 was derived from 3 by our new transformation method.¹¹⁾

The immunosuppressive effects of these compounds were determined by *in vitro* PFC assay¹²⁾ using mouse spleen cells. The cervix of female C57BL/6 mice aged 6 to 8 weeks was dislocated and the spleen removed. Spleens were mashed and filtered through a stainless steel mesh in RPMI-1640 medium. The single cell suspension was washed once and resuspended at 8×10⁶ cells/ml in RPMI-1640 containing 10 % fetal calf serum, 100 units of Penicillin G, 100 μg/ml streptomycin and 1mM sodium pyruvate, and seeded in a 24-well culture plate. Then, 8×10⁶ cells/ml SRBC, 5×10⁻⁵ M 2-mercaptoethanol and test compounds dissolved in DMSO were added, and cultured at 37 °C in 5 % CO₂ atmosphere. The final concentration of DMSO was less than 0.01 %, and this concentration had no effect on the PFC response. Four days later, cells were resuspended in the new medium, and diluted guinea pig complements and 30 % (v/v) of SRBC added. The mixture was transferred into a Cunningham chamber,¹³⁾ and incubated at 37 °C for 2h. After incubation, PFC were counted under a microscope. The cytotoxicity to mouse spleen cells was investigated by trypan blue staining and 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium-Na (WST-1) assay.¹⁴⁾ Trypan

Fig.2 Effects of Nuphar Alkaloids, Hydrocortisone (HC) and Cyclosporin A (CP) on the Primary Anti-SRBC PFC Formation in Mouse Spleen Cells.

Each column represents the mean with the S.E. of 3-6 tests, and column pattern shows a concentration of a sample, □: 10⁻⁶, ▨: 10⁻⁷, ■: 10⁻⁸M, respectively. Asterisks denote significant differences from the control at **:p<0.01, *:p<0.05, respectively.

blue staining was performed in accordance with the common method after 4 days culture of spleen cells with 10⁻⁶ M test compounds. WST-1 assay was performed by the following procedure: 100 μl of spleen cell suspensions (3×10⁷ cells/ml) was cultured with 10 μl of test compounds (final conc. 10⁻⁶ M) in a 96-well culture plate for 2 days. WST-1 assay was performed using a cell counting kit^R (DOJINDO

Fig.3 Cytotoxicities of Nuphar Alkaloids, Hydrocortisone (HC) and Cyclosporin A (CP) against mice spleen cells.

Each column value represents mean with the S.E. of 3-4 tests, and each column pattern shows □: absorbance at 405-630 nm(WST-1 assay) and ■: ratio of viable cells(Trypan blue staining), respectively.

Laboratories). Statistical evaluation was performed using Dunnett's multiple test.

Figure 2 shows the effects of Nuphar alkaloids on the primary PFC formation. As a result of spleen cell culture with SRBC for 4 days, 93 to 950 of PFC were detected in the control groups. The dimeric sesquiterpene thioalkaloids possessing OH group in the quinolizidine ring, 2, 3, 5, 6 and 7, significantly inhibited the PFC formation at 10^{-6} M. Hydrocortisone and cyclosporine A also significantly inhibited PFC formation from 10^{-8} to 10^{-6} M. However, dimeric sesquiterpene thioalkaloids (1, 4, 8) lacking the OH group in the quinolizidine ring showed no significant suppression. Figure 3 shows the cytotoxicity of Nuphar alkaloids affecting mice spleen cells. In the investigation of spleen cell viabilities by trypan blue staining, 3, 7 and hydrocortisone slightly decreased the number of existing cells at 10^{-6} M. The other alkaloids had

no suppressive effect. In the investigation of spleen cell proliferation by WST-1 assay, 3, 7, cyclosporin A and hydrocortisone inhibited the color reactions based on the formazan dye production in mitochondria following WST-1 up taking.

In conclusion, we found that dimeric sesquiterpene thioalkaloids (2, 3, 5, 6, 7), possessing an OH group in the quinolizidine ring had potent suppressive effect against *in vitro* antibody formation in mice spleen cells, while, dimeric sesquiterpene thioalkaloids (1, 4, 8) and monomeric sesquiterpene alkaloids (9, 10, 11, 12) lacked this effect. In cytotoxicity studies, 3 and 7 possessing two OH groups showed similar or slightly potent influence on spleen cells compared with the same concentration (10^{-6} M) of hydrocortisone. These results suggest that an OH group at the 6 position in the quinolizidine ring of dimeric sesquiterpene thioalkaloids is essential for expression of the immunosuppressive effect. Moreover, the number of OH groups seems to be related with cytotoxicity, and influence on spleen cells is greater with increase in the number of these groups.

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