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Dimeric Sesquiterpene Thioalkaloids with Potent Immunosuppressive Activity from the Rhizome of *Nuphar pumilum*: Structural Requirements of Nuphar Alkaloids for Immunosuppressive Activity

Hisashi Matsuda, Hiroshi Shimoda and Masayuki Yoshikawa*

Kyoto Pharmaceutical University, Misasagi, Yamashina-ku, Kyoto 607-8412, Japan

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Abstract—Potent immunosuppressive dimeric sesquiterpene thioalkaloids, 6-hydroxythiobinupharidine, 6,6′-dihydroxythiobinupharidine, 6-hydroxythionuphlutine B and 6′-hydroxythionuphlutine B, were isolated from the rhizome of *Nuphar pumilum* together with five inactive quinolizidine alkaloids, neothiobinupharidine, nupharidine, deoxynupharidine, 7-epideoxynupharidine and nupharolutine. These dimeric sesquiterpene thioalkaloids were found to significantly inhibit anti-sheep erythrocyte plaque forming cell formation in mouse splenocytes at 1 μM. At this concentration, 6-hydroxythiobinupharidine, 6-hydroxythionuphlutine B and 6′-hydroxythionuphlutine B did not show cytotoxic effects to mouse splenocytes, and 6,6′-dihydroxythiobinupharidine also showed only minor or minimal cytotoxicity. By comparison of the inhibitory activity of several Nuphar alkaloids on anti-sheep erythrocyte plaque forming cell formation, some structural requirements of Nuphar alkaloids for immunosuppressive activity were obtained. Namely, the 6- or 6′-hydroxyl group at the quinolizidine ring of dimeric sesquiterpene thioalkaloids is essential for the immunosuppressive effect. The number of hydroxyl groups appears to be related to the cytotoxicity, and the influence on splenocytes is greater with increasing numbers of hydroxyl groups.

Introduction

Nupharis Rhizoma, the dried rhizome 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 in several studies and a number of sesquiterpene alkaloids such as nupharidine (**9**) and deoxynupharidine (**10**) have been identified from *N. japonicum*.[1] In the course of our studies of natural medicines from aquatic plants,[2] we isolated thiohemiaminal type dimeric sesquiterpene thioalkaloids such as 6-hydroxythiobinupharidine (**2**), 6,6′-dihydroxythiobinupharidine (**3**), 6-hydroxythionuphlutine B (**5**), and 6′-hydroxythionuphlutine B (**6**) from the rhizome of Russian *Nuphar pumilum* and found a new rearrangement reaction of the thiaspiran ring in thiohemiaminal type alkaloids with the 6-hydroxyl group.[3] In addition, new thiaspiran sulfoxide type dimeric sesquiterpene alkaloids named nupharpumilamines A–D were characterized.[4] However, in the pharmacological study of Nuphar alkaloids, only the central paralysis effect of **10** has been reported so far.[5] To find new bioactive function of Nupharis Rhizoma, we investigated the effects of the methanolic (MeOH) extract and alkaloid fraction from *N. pumilum* on the immune system using plaque forming cell (PFC) assay.[6] As a result, both extract and alkaloid fraction were found to inhibit antibody formation from sheep erythrocyte (SRBC)-immunized splenocytes. Through bioassay-guided separation, we isolated five dimeric sesquiterpene thioalkaloids (6-hydroxythiobinupharidine (**2**),[3,7] 6,6′-dihydroxythiobinupharidine (**3**),[3,8] 6-hydroxythionuphlutine B (**5**),[3,9] 6′-hydroxythionuphlutine B (**6**),[3,9] and neothiobinupharidine (**8**)[10]) and four monomeric sesquiterpene alkaloids (**9**,[1] **10**,[1] 7-epideoxynupharidine (**11**)[11] and nupharolutine (**12**)[12]). Moreover, to determine the

*Corresponding author. Tel.: +81-75-595-4633; fax: +81-75-595-4768; e-mail: shoyaku@mb.kyoto-phu.ac.jp

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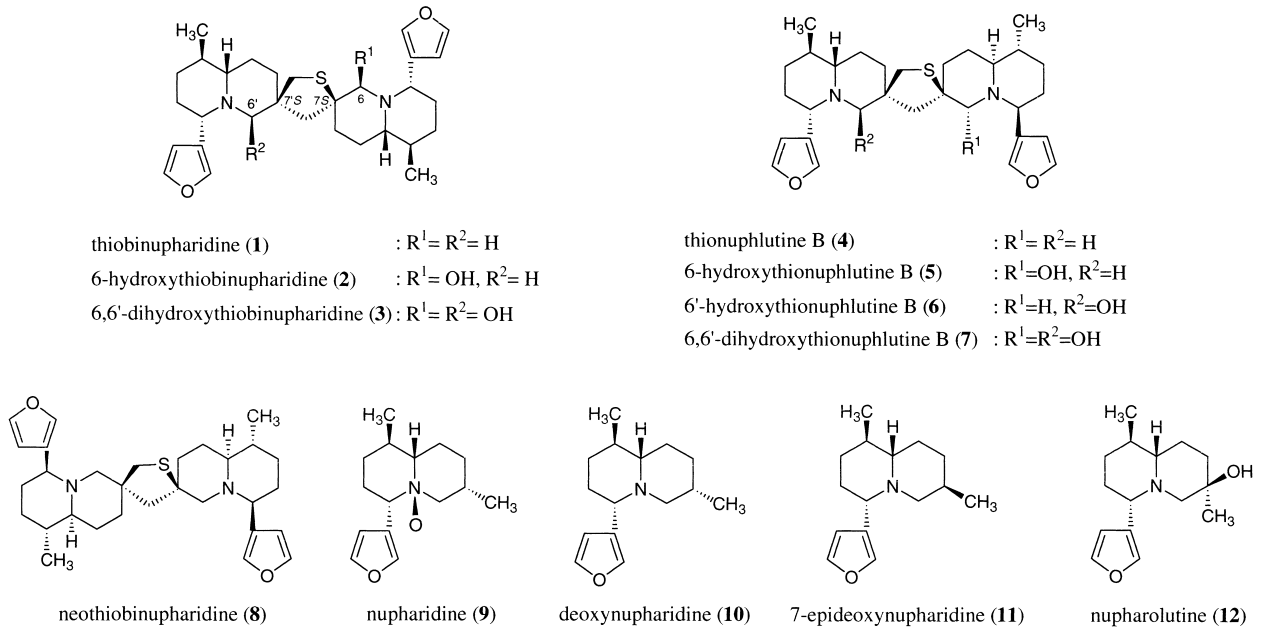

Figure 1. Nuphar alkaloids from the rhizome of *Nuphar pumilum*.

structural requirement of Nuphar alkaloids for immunosuppressive activity, three dimeric sesquiterpene thioalkaloids, thiobinupharidine (**1**),[13] thionuphlutine B (**4**),[7] and 6,6′-dihydroxythionuphlutine B (**7**),[8] were derived from **2**, **5** and **3**, respectively.[3] Here, we describe the immunosuppressive and cytotoxic effects of Nuphar alkaloids and their structural requirement for this activity.

Results and Discussion

Effects of the MeOH extract, alkaloid fraction and Nuphar alkaloids from Nupharis Rhizoma on primary responses in PFC formation

As a result of splenocyte culture with SRBC for 4 days, 80 to 950 counts of PFC were detected in the control group. Figure 2 shows the effects of the MeOH extract and alkaloid fraction from Nupharis Rhizoma on primary responses in PFC formation. The MeOH extract significantly inhibited the primary response in PFC formation from 0.1 to 10 μg/mL, and the alkaloid fraction showed the stronger suppression at 1 and 10 μg/mL. Accordingly, we attempted to isolate the immunosuppressive constituents from the alkaloid fraction and isolated several monomeric (**9**–**12**) and dimeric (**2**, **3**, **5**, **6**, **8**) sesquiterpene alkaloids as shown in Figure 1.

Effects of Nuphar alkaloids (**1**–**12**) and reference compounds (hydrocortisone and cyclosporin A) on primary responses in PFC formation are summarized in Figure 3 as the relative value to each control. Hydrocortisone and cyclosporin A potently inhibited the PFC formation from 0.01 to 1 μM. Monomeric sesquiterpene alkaloids (**9**–**12**) and neothiobinupharidine (**8**), a dimeric sesquiterpene thioalkaloid lacking the hydroxyl group, showed no significant effect. 7-Epideoxynupharidine (**11**, 0.01–1 μM) tended to increase the number of PFC, while nuphalorutine (**12**), a monomeric sesquiterpene possessing the hydroxyl group, tended to suppress the number of PFC. However, the differences were not significant. On the other hand, 6-hydroxythiobinupharidine (**2**), 6,6′-dihydroxythiobinupharidine (**3**), 6-hydroxythionuphlutine B (**5**) and 6′-hydroxythionuphlutine B (**6**), dimeric sesquiterpene thioalkaloids possessing the hydroxyl group in the quinolizidine ring, were found to suppress the PFC formation at 1 μM.

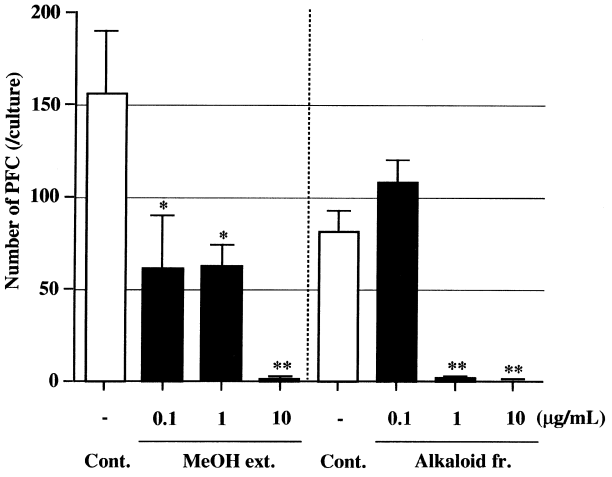

Figure 2. Effects of MeOH extract and alkaloid fraction from *Nuphar pumilum* on primary PFC formation in mouse splenocytes. Splenocytes (8×10^6 cells/mL) were cultured with SRBC (8×10^6 cells/mL) in the presence of 50 μM 2-mercaptoethanol and test samples. Four days later, cells were resuspended in new medium, and diluted guinea pig complements and 30% (v/v) of SRBC were added. The mixture was transferred into a Cunningham's chamber, and incubated at 37 °C for 2 h. After incubation, the number of PFC was counted under a microscope. Each column represents the mean and S.E.M. of five experiments. Asterisks denote significant differences from the control at *: $P<0.05$, **: $P<0.01$.

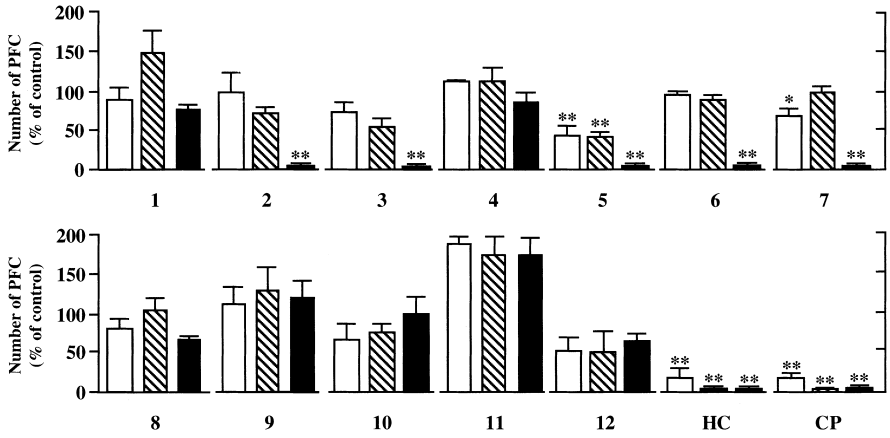

Figure 3. Effects of Nuphar alkaloids (**1**–**12**), hydrocortisone (HC) and cyclosporin A (CP) on the primary anti-SRBC PFC formation in mouse splenocytes. Each column represents the mean and S.E.M. of 3–6 experiments and column pattern shows a concentration of each sample, □: 0.01, ▨: 0.1, ■: 1 μM, respectively. Asterisks denote significant differences from the control at *: $P<0.05$, **: $P<0.01$.

These findings suggest that the hydroxyl group in the dimeric sesquiterpene thioalkaloids is essential for the anti-PFC formation activity. To confirm the necessity of the hydroxyl group for the activity, dehydroxy derivatives (**1**, **4**) and a dihydroxy derivative (**7**) were derived, and their activities were examined. As a result, **1** and **4** did not show inhibitory activity at 1 μM. However, **7** inhibited PFC formation at 0.01 and 1 μM. These findings confirm that the thiohemiaminal structure possessing the 6- or 6′-hydroxyl group is essential for the anti-PFC formation activity.

Effects of Nuphar alkaloids (2, 3, 5–7) on secondary anti-SRBC PFC formation

The dimeric sesquiterpene thioalkaloids (**2**, **3**, **5**–**7**) that inhibited the primary PFC formation were examined for secondary PFC formation. As shown in Figure 4, all compounds completely suppressed the PFC formation at 1 μM. From this observation, the active compounds (**2**, **3**, **5**–**7**) appeared to directly suppress anti-SRBC antibody production in immunized B lymphocytes.

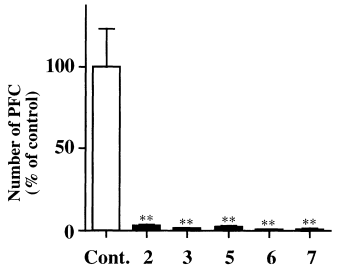

Figure 4. Effects of Nuphar alkaloids (**2**, **3**, **5**, **6**, **7**) on the secondary anti-SRBC PFC formation in mouse splenocytes. Mice were immunized by intraperitoneal injection with SRBC (5×10^8 cells) suspended in saline. Seven days later, the spleen was removed and the splenocytes suspension (5×10^5 cells/mL) was prepared. The cells were cultured with SRBC (5×10^5 cells/mL), 50 μM 2-mercaptoethanol, and 1 μm samples for 4 days. PFC assay was performed according to the method described in the section of primary response in PFC formation. Each column represents the mean and S.E.M. of four experiments. An asterisk denotes significant difference from the control at **: $P<0.01$.

Cytotoxic effects of Nuphar alkaloids against splenocytes

Finally, the trypan blue dye exclusion test and WST-1 assay were performed to clarify whether immunosuppressive activities of dimeric sesquiterpene thioalkaloids depended on their cytotoxic effects. Splenocytes were cultured for 4 days with test compounds. The rate of viable splenocytes in control (vehicle) groups, determined by trypan blue staining, was 73.3±6.5% ($n=18$). When splenocytes were cultured with 1 μM of each test compound for 4 days, compounds **3** and **7** and hydrocortisone slightly decreased the number of viable cells. However, the other compounds did not affect the rate of the viable cells (Fig. 5).

In the WST-1 assay, an assay method for evaluation of cell viability, two dimeric sesquiterpene thioalkaloids possessing two hydroxyl groups (**3**, **7**) at 1 μM significantly reduced the formazan formation following WST-1 up-take (Fig. 6). The activities of these alkaloids were weaker than those of hydrocortisone and cyclosporine A. These findings suggest that the direct sup-

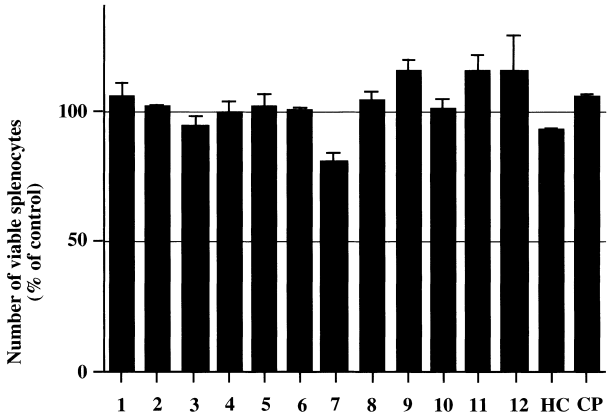

Figure 5. Cytotoxic effects of Nuphar alkaloids, hydrocortisone (HC), and cyclosporin A (CP) on number of viable splenocytes (Trypan blue exclusion test). Splenocytes (8×10^6 cells/mL) were cultured with 1 μM samples. Trypan blue staining was performed on the 4th day. Each value represents the mean and S.E.M. of 3–4 experiments. Differences from the control were not significant.

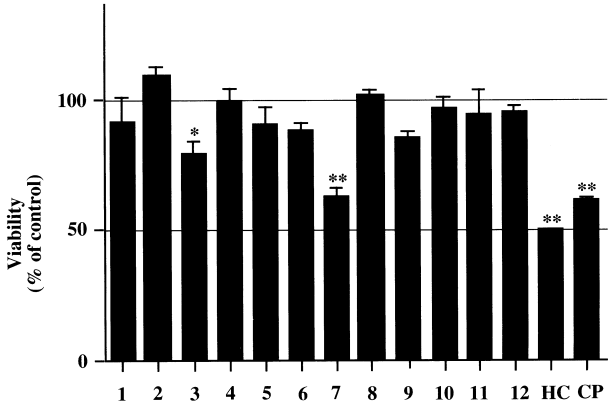

Figure 6. Effects of Nuphar alkaloids, hydrocortisone (HC), and cyclosporin A (CP) on viability of splenocytes (WST-1 assay). Splenocytes (3×10^7 cells/mL) were cultured with 1 μM samples for 2 days. WST-1 assay was performed using the Cell Counting Kit^{TM} and measured absorbance at 405 nm (reference wavelength: 660 nm). Each value represents the mean and S.E.M. of four experiments. Asterisks denote significant differences from the control at *: $P<0.05$, **: $P<0.01$.

pressive effects of the dimeric sesquiterpene thioalkaloids possessing two hydroxyl groups on cell viability might be partly involved in their suppressive activities in antibody production. Furthermore, the number of hydroxyl groups in the quinolizidine ring appears to enhance the cytotoxic effects of the Nuphar alkaloids.

In conclusion, the MeOH extract from the dried rhizome of *N. pumilum* suppressed antibody formation from mouse splenocytes, and four dimeric sesquiterpene thioalkaloids, 6-hydroxythiobinupharidine (**2**), 6,6′-dihydroxythiobinupharidine (**3**), 6-hydroxythionuphlutine B (**5**), and 6′-hydroxythionuphlutine B (**6**), were isolated as active principles. A hydroxyl group in the quinolizidine ring appeared to be essential for the immunosuppressive activity and increase in the hydroxyl groups may enhance their cytotoxic effects.

Experimental

Animals and materials

Female C57BL/6 mice aged 6 to 8 weeks were purchased from Kiwa Laboratory Animals (Wakayama, Japan). They were kept at 22±2 °C in a 12 h light/12 h dark cycle. Standard laboratory chow (MF, Oriental Yeast Co. Ltd., Chiba, Japan) and tap water were given freely.

The MeOH extract, alkaloid fraction, and sesquiterpene alkaloids (**2**, **3**, **5**, **6**, **8**–**12**) were prepared from Russian Nupharis Rhizoma as described previously.[3] Briefly, the MeOH extract from the dried rhizome of *N. pumilum* was partitioned into $CHCl_3$–aq HCl. The aq HCl phase was alkalized with concd NH_4OH and then extracted with AcOEt. The AcOEt soluble fraction (the alkaloid fraction) was subjected to ordinary silica gel ($CHCl_3$–MeOH–Et_2NH) and NH-chromatorex (*n*-hexane–CH_2Cl_2–AcOEt→$CHCl_3$–MeOH) and finally HPLC (Develosil ODS-HG-5, MeOH–H_2O–Et_2NH) to furnish **2** (0.019% from the natural medicine), **3** (0.020%), **5** (0.003%), **6** (0.002%), **8** (0.001%), **9** (0.0006%), **10** (0.002%), **11** (0.004%), and **12** (0.0008%). The dehydroxyl derivatives (**1**, **4**) were synthesized by $NaBH_4$ reduction of **2** and **5**, respectively, and **7** was derived from **3** by the rearrangement reaction reported previously.[3]

RPMI-1640 medium, fetal calf serum (FCS), penicillin G and streptomycin were purchased from Gibco BRL, Life Technologies, Inc. (Rockville, MD, USA). Cell Counting Kit^{TM} was purchased from Dojindo Co. Ltd. (Kumamoto, Japan). SRBC and guinea pig complements were purchased from Cosmo Bio Co. Ltd. (Tokyo, Japan). Other reagents were purchased from Wako Pure Chemical Industries Co. Ltd. (Osaka, Japan).

Measurement of primary response in PFC formation

The spleen was removed from each mouse and mashed in RPMI-1640 medium and filtered through a stainless steel mesh (200 mesh). The cell suspension was washed ($350\times g$, 3 min) once and resuspended at 8×10^6 cells/mL in RPMI-1640 containing 10% FCS, 100 units/mL penicillin G, 100 μg/mL streptomycin, and 1 mM sodium pyruvate and seeded in a 24-well culture plate. Then, SRBC (8×10^6 cells/mL), 50 μM 2-mercaptoethanol and test samples dissolved in dimethylsulfoxide (DMSO) were added to the medium and cultured at 37 °C in a 5% CO_2 atmosphere. The final concentration of DMSO was adjusted to less than 0.01% and this concentration had no effect on the PFC response. Four days later, cells were resuspended in 160 μL of new medium, and 20 μL of diluted (×2) guinea pig complements and a similar volume of 30% (v/v) SRBC was added. The whole mixture was transferred into a Cunningham's chamber[14] and incubated at 37 °C for 2 h. After incubation, the number of PFC were counted under a microscope (×40).

Measurement of secondary response in PFC formation

Mice were immunized by intraperitoneal injection with SRBC (5×10^8 cells) suspended in saline. Seven days later, the spleen was removed and a splenocytes suspension (5×10^5 cells/mL) was prepared. The cells were seeded in 24-well plates and cultured with SRBC (5×10^5 cells/mL), 50 μM 2-mercaptoethanol and samples for 4 days. PFC assay was performed according to the method described in the section of primary response in PFC formation.

Cytotoxicity against splenocytes

Measurement of viable cells (trypan blue exclusion test). One-hundred microliters of splenocytes suspension (8×10^6 cells/mL) was cultured with 1 μM test compound in a 96-well culture plate. Trypan blue staining was performed on the 4th day. The viable cells (non-staining cells) were counted under a microscope, and the ratio of viable cells to all cells was calculated.

Measurement of cell activity [(2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium·Na (WST-1) assay[15])]. One-hundred microliters of splenocytes suspension (3×10^7 cells/mL) was cultured with 1 μM test compound in a 96-well culture plate for 2 days. WST-1 assay was performed using the Cell Counting Kit™. The formazan formation, which is an indication of cell viability, was measured for absorbance at 405 nm (reference wavelength: 660 nm).

Statistics

Values are expressed as means ± S.E.M. For statistical analysis, one-way analysis of variance following Dunnett's test was used. Probability (P) values less than 0.05 were considered significant.

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