[Chem. Pharm. Bull. 33(9)3821—3825(1985)]

Calmodulin Inhibitors from the Bitter Mushroom *Naematoloma fasciculare* (Fr.) KARST. (Strophariaceae) and Absolute Configuration of Fasciculols

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(Received January 9, 1985)

Calmodulin inhibitors were found in the bitter poisonous mushroom *Naematoloma fasciculare* (Fr.) KARST. (Strophariaceae). The active compounds, fasciculols B, C, and F, were isolated by droplet counter-current chromatography and identified on the basis of various physical and spectral data. Application of the dibenzoate chirality method to a *p*-bromobenzoate derivative of fasciculol C allowed the absolute configurations at the C-2 and C-3 positions to be determined as (*R*) and (*S*), respectively.

Keywords——*Naematoloma fasciculare*; Strophariaceae; calmodulin inhibitor; fasciculol B; fasciculol C; fasciculol F; droplet counter-current chromatography; absolute configuration

The poisonous and bitter tasting mushroom *Naematoloma fasciculare* (Fr.) KARST. (Strophariaceae) has a worldwide distribution. Chemical studies of this mushroom collected in Japan,[1-5] Italy, and Poland[6] have already been reported. However, its biological and pharmacological properties have not yet been investigated except for a plant growth inhibitory activity.[2-4]

In our preliminary screening of a crude methanol extract of the fruiting body of *N. fasciculare* collected in Northern California, we found that it showed calmodulin-inhibitory activity.[7,8] This important activity has been found recently,[9] and only a few compounds possessing such activity have been reported so far.[8,10] Hence, we examined the active principles in this mushroom.

Separation of the methanol extract into ethyl acetate-soluble and water-soluble fractions was carried out, and bioassay indicated that the active components were in the ethyl acetate fraction. The *Rf* values of the main compounds in the ethyl acetate fraction were determined

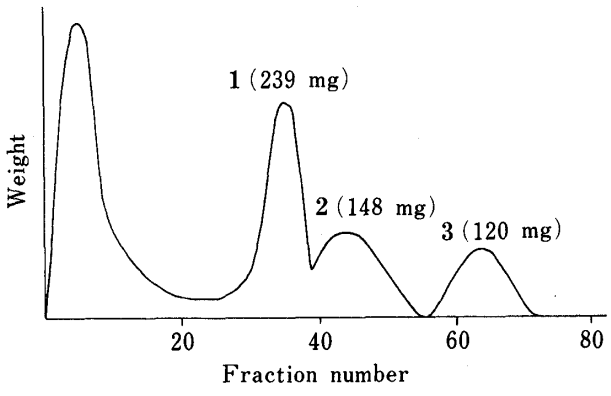


Fig. 1. DCCC of EtOAc Extract (2.4 g) of *N. fasciculare* with C_6H_6–$CHCl_3$–MeOH–H_2O (5:5:7:2, v/v) by the Ascending Method; 5 ml per Fraction

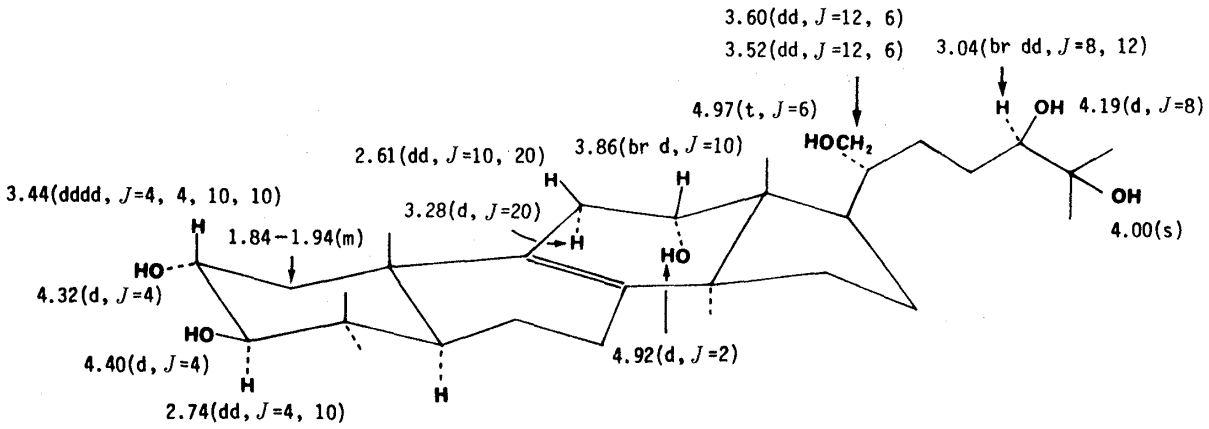
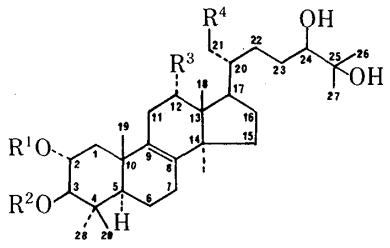


Fig. 2. ^{1}H-NMR Assignments of Fasciculol C (**1**) in DMSO-d_6 (400 MHz)

Decoupling experiments confirmed these assignments. (The chemical shifts are in δ and the coupling constants in Hz.)



1: R^1=H, R^2=H, R^3=OH, R^4=OH
2: R^1=H, R^2=H, R^3=OH, R^4=H
3: R^1=C(=O)–CH_2–C(OH)(CH_3)–CH_2–C(=O)–NH–CH_2–C(=O)–O–CH_3
R^2=H, R^3=OH, R^4=OH
4: R^1=H, R^2=H, R^3=H, R^4=H
5: R^1=*p*-Br-Bz, R^2=*p*-Br-Bz, R^3=OH, R^4=*O*-*p*-Br-Bz
(Bz=benzoyl)

Chart 1

to be about 0.4—0.7 on a silica gel thin-layer chromatography (TLC) plate developed with the organic layer of a benzene–chloroform–methanol–water mixture (5 : 5 : 7 : 2, v/v). Due to the polar nature of the active components, droplet counter-current chromatography (DCCC), which has been previously applied to the separation of many polar mixtures,[11,12] seemed to be appropriate for further separation of the ethyl acetate extract. The DCCC chromatogram shows an almost base-line separation of three pure compounds **1**, **2**, and **3** (Fig. 1).

The major compound **1** showed the molecular ion peak at *m/z* 508 in the mass spectrum (MS). In the proton (^{1}H-) nuclear magnetic resonance (NMR) spectrum of **1** (Fig. 2), seven tertiary methyl signals appeared in the region from δ 0.62 to 1.03. Signals due to the primary and tertiary hydroxy protons were observed at δ 4.97 and 4.00. Furthermore, four secondary hydroxy signals were seen in the region from δ 4.19 to 4.92, and signals assigned to hydroxy methine protons were also observed. From the ^{1}H-NMR, carbon-13 (^{13}C-) NMR, infrared (IR), specific optical rotation ([α]), and ultraviolet (UV) data, **1** was identified as the known triterpene alcohol, fasciculol C.[4,6]

Compounds **2** and **3** were similarly identified as the analogues, fasciculols B[3,6] and F,[4,6] respectively. The content ratio for fasciculols C, B, and F in *N. fasciculare* collected in California was different from that in the same mushroom collected in Japan, in which the major compound was fasciculol A (**4**).[2]

These three fasciculols are responsible for the calmodulin inhibitory activity of the methanol extract (Table I). As a part of our search for new biological activities in natural products, we tested for anti-germination activity[13] and a molluscicidal activity of these fasciculols.[14] However, they did not show any activity at 1000 ppm in the anti-germination

TABLE I. Calmodulin Inhibitory Activity of Fasciculols

Test compounds	IC_{50} (μg/ml)[a]
Fasciculol C (**1**)	400
Fasciculol B (**2**)	21
Fasciculol F (**3**)	70

a) IC_{50} values are concentrations of test compounds at which they showed 50% inhibition of calmodulin-dependent cAMP phosphodiesterase activity in the presence of calmodulin.

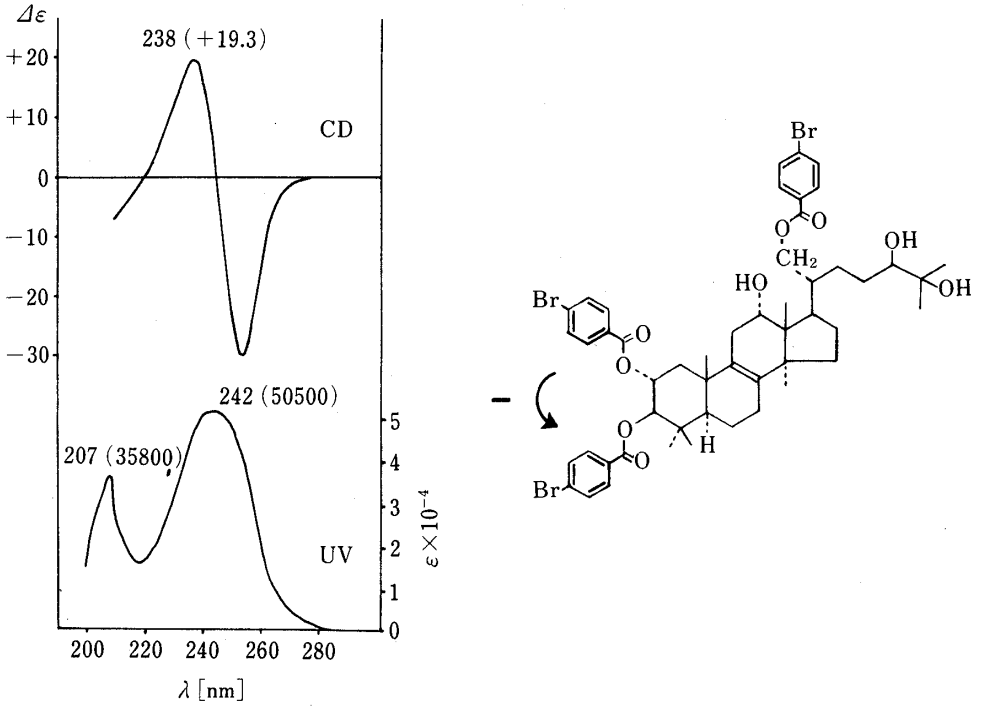


Fig. 3. CD and UV Spectra of Fasciculol C 2,3,21-Tri-*p*-bromobenzoate (**5**) in EtOH

test using lettuce seed, nor did they show molluscicidal activity at 500 ppm against the South American snail *Biomphalaria glabratus.*

The absolute configuration at C-24 of fasciculol A (**4**), isolated by Ikeda *et al.*[2] and having the same carbon skeleton as fasciculols C, B, and F, was determined to be (*R*) based on the lanthanide-induced Cotton effect of fasciculol A 2,3-diacetate as determined by a modification of Nakanishi's method.[5,15] However, the absolute configuration in the ring system was not determined. Hence, we examined the absolute configurations at C-2 and C-3 by application of the dibenzoate chirality method[16] to a *p*-bromobenzoate (**5**) of fasciculol C (**1**). The MS and UV spectra of **5** suggest that **5** is a tribenzoate derivative. In the ^{1}H-NMR spectrum of **5**, two signals due to *p*-bromobenzoate ester methine protons at C-2 and C-3 were observed at δ 5.09 and 5.43, and the presence of *p*-bromobenzoate ester methylene protons at C-21 was confirmed. Therefore, **5** was determined to be fasciculol C 2,3,21-tri-*p*-bromobenzoate.

Figure 3 shows the circular dichroism (CD) spectrum of **5**. It has intense split Cotton effects, and the negative first Cotton effect at 254 nm and positive second Cotton effect lead to the conclusion that the exciton chirality between the transition moment of two *p*-bromobenzoate chromophores is negative. Therefore, the absolute configurations at C-2 and C-3 were determined to be (*R*) and (*S*), respectively.

Experimental

All melting points (mp) were determined on a Sybron Thermolyne Mp-12615 and are uncorrected. $[\alpha]_D$ values were measured with a Perkin–Elmer 241 and 241 MC. UV spectra were determined on a Hitachi 100-80 and IR spectra on a Perkin–Elmer 737B. MS were obtained on a Hitachi RMU 6-MG. CD was measured with a JASCO J-40. ^{1}H-NMR spectra were determined on a JEOL GX-400 instrument for **1** and **5**, and on a JEOL PS-100 for **2** and **3**. ^{13}C-NMR spectra were determined on a JEOL FX-100.Tetramethylsilane was used as an internal reference for NMR measurements.

Materials——Fresh fruit bodies of *N. fasciculare* (400 g) collected at Patricks Point, California, were preserved in MeOH. The MeOH was removed to give 8.2 g of residue, which was separated into an EtOAc soluble fraction (1.0 g) and an H_2O-soluble portion (7.2 g).

DCCC Separation——The separation was carried out on a model DCC-300-G2 apparatus (Tokyo Rikakikai Co.) equipped with 300 glass columns (400 mm length, 2 mm inner diameter). The solvent system of C_6H_6–$CHCl_3$–MeOH–H_2O (5 : 5 : 7 : 2, v/v) was chosen on the basis of a prescreening of the components of the EtOAc extract on a TLC plate (Macherey, Nagel and Co., Duren, G.F.R.; polygram Sil G/UV 254).[11] The upper pahse of this mixture was chosen as the mobile phase. The MeOH extract (2.4 g) was dissolved in a mixture (1 : 1, v/v) of mobile and stationary phases, and injected into the DCCC apparatus using a 10 ml sample chamber. The elutes were collected in fractions of 5 ml/h. The fractions were monitored by TLC, developed with the organic layer of the same solvent system. Visualization of the compounds on the TLC plate was accomplished by UV spectroscopy (Chromato-UVE Cabinet, model CC-60, Ultra Violet Products, Inc.) and with a vanillin–H_2SO_4–EtOH (3 g: 1.5 ml: 100 ml) spray reagent. The DCCC chromatogram is shown in Fig. 1. We obtained 239 mg fasciculol C, 148 mg fasciculol B and 120 mg fasciculol F in this separation.

Fasciculol C (1)——Colorless needles of mp 185—187 °C (recryst. from H_2O–EtOH). $[\alpha]_D^{20}$ +60.0° (c=0.01, EtOH). IR ν_{max}^{Nujol} cm^{-1}: 3350 (OH), 1670 (C=C), 1070 (COC). UV λ_{max}^{EtOH} nm (ε): 200.5 (6350). EI-MS m/z: 508 (M^+), 475, 439, 421, 315, 195, 159. ^{1}H-NMR (DMSO-d_6) δ: shown in Fig. 2. ^{13}C-NMR ($CDCl_3$–C_5D_5N) δ: as reported by Bernardi, *et al.*[6]

Fasciculol B (2)——Colorless needles of mp 230—232 °C (recryst. from H_2O–EtOH). $[\alpha]_D^{20}$ +74.9° (c=0.41, MeOH). IR ν_{max}^{Nujol} cm^{-1}: 3300 (br OH). UV λ_{max}^{EtOH} nm (ε): 210 (5950). ^{1}H-NMR (C_5D_5N) δ: 0.77, 1.14, 1.16, 1.28, 1.46, 1.51, 1.53 (3H each, each s, Me), 1.32 (3H, d, J=8 Hz, 21-Me), 3.36 (1H, d, J=10 Hz, 3-H), 3.80 (1H, m, 2-H). ^{13}C-NMR ($CDCl_3$–C_5D_5N) δ: as reported by Bernardi, *et al.*[6]

Fasciculol F (3)——Amorphous product of mp 100—102 °C. $[\alpha]_D^{20}$ +20.4° (c=0.27, MeOH). IR ν_{max}^{Nujol} cm^{-1}: 3350 (br OH and NH), 1710 (br OCO), 1650 (CONH), 1030 (COC). UV λ_{max}^{EtOH} nm (ε): 205 (5050). EI-MS m/z: 550 (M^+ − 173), 538, 517, 508, 475, 457, 539, 421. ^{1}H-NMR (C_5D_5N) δ: 0.76, 1.18, 1.21, 1.29, 1.45, 1.55, 1.58, 1.78 (3H each, each s, Me), 3.07 (2H, d, J=8 Hz, 21-H_{ab}), 3.52 (1H, d, J=10 Hz, 3-H), 3.62 (3H, s, 9′-Me), 4.32 (2H, d, J=6 Hz, 7′-H_{ab}), 5.50 (1H, br dd, J=10, 10 Hz, 2-H), 6.84 (1H, br s, NH). ^{13}C-NMR (C_5D_5N) δ: as reported by Bernardi, *et al.*[6]

Assay for Calmodulin Inhibitory Activity——Test compounds were considered to have inhibitory activity when IC_{50} (concentration of a compound at which it showed 50% inhibition of calmodulin-dependent cyclic adenosin monophosphate (cAMP) phosphodiesterase activity) in the presence of calmodulin was less than IC_{50} in the absence of calmodulin. The enzyme activity was measured by the procedure of Teo and Wang,[7] using 5′-nucleotidase from *Crotulus adamanteus* venom (Sigma), calmodulin isolated from beef brain (Amano), and calmodulin dependent cAMP phosphodiesterase purified from beef heart according to the method of Ho *et al.*[17]

2,3,21-Tri-*p*-bromobenzoate Derivative of Fasciculol C (5)——*p*-Bromobenzoyl chloride (117 mg) and **1** (30 mg) were dissolved in dry C_5D_5N (1 ml), and the mixture was stirred at room temperature for 2 d. The reaction mixture was applied to a silica gel column and a major product was eluted with petroleum Et_2O–EtOAc, then crystallized from Et_2O–*n*-hexane to give 18 mg of colorless needles of mp 150—151 °C. $[\alpha]_D^{24}$ −67.1° (c=0.14, $CHCl_3$). IR ν_{max}^{Nujol} cm^{-1}: 3440 (OH), 1720, 1270 (OCO), 1590, 1460 (arom.). UV λ_{max}^{EtOH} nm (ε): 242 (50500), 207 (35800). CD (c=0.0065, EtOH) $\Delta\varepsilon^{25}$: −31.9 (254) (negative max.), +19.3 (238) (positive max.). EI-MS m/z: 1022, 1020, 1007, 1005, 803. ^{1}H-NMR (DMSO-d_6) δ: 0.69, 0.94, 1.04, 1.05, 1.08, 1.09, 1.14 (3H each, each s, Me), 3.78 (1H, dd-like, 12-H), 4.54 (1H, dd, J=3, 12 Hz, 21-H_a), 4.85 (1H, br d, J=12 Hz, 21-H_b), 5.09 (1H, d, J=10 Hz, 3-H), 5.43 (1H, dt, J=10, 5 Hz, 2-H), 7.65—7.85 (12H, m, arom. H).

Acknowledgement We thank Professor T. Kamikawa and Professor A. S. Kende for NMR measurements, Professor N. Harada for a CD measurement, and Dr. K. Tsujimoto for MS measurements. Thanks are also due to Dr. Y. Fukuyama for the assay of calmodulin inhibitory activity and Dr. D. Largent for identification of the mushroom. Work at the University of California was supported in part by a U.S. Public Health Service Biomedical Grant to I. K. and work at Humboldt State University was supported by a grant from the Dawel Nielson Institute.

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