HETEROCYCLES, Vol. 68, No. 11, 2006, pp. 2357 - 2364. © The Japan Institute of Heterocyclic Chemistry
Received, 24th August, 2006, Accepted, 5th October, 2006, Published online, 6th October, 2006. COM-06-10868

NANKAKURINE B, A NEW ALKALOID FROM *LYCOPODIUM HAMILTONII* AND REVISED STEREOSTRUCTURE OF NANKAKURINE A

Yusuke Hirasawa,[a] Jun'ichi Kobayashi,[b] Yutaro Obara,[c] Norimichi Nakahata,[c] Nobuo Kawahara,[d] Yukihiro Goda,[d] and Hiroshi Morita[a]*

[a]Faculty of Pharmaceutical Sciences, Hoshi University, Shinagawa-ku, Tokyo 142-8501, Japan; [b]Graduate School of Pharmaceutical Sciences, Hokkaido University, Sapporo 060-0812, Japan; [c]Graduate School of Pharmaceutical Sciences, Tohoku University, Aoba, Aramaki, Aoba-ku, Sendai 980-8578, Japan; [d]National Institute of Health Sciences, Kamiyoga 1-18-1, Setagaya-ku, Tokyo 158-8501, Japan

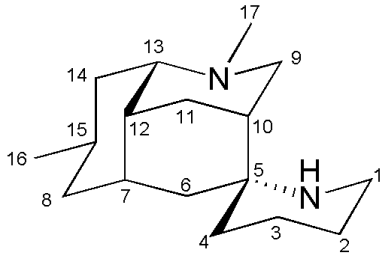
Abstract – A new *Lycopodium* alklaoid, nankakurine B (**2**), has been isolated from the club moss *Lycopodium hamiltonii* together with nankakurine A (**1**). Stereochemistry of **2** was elucidated by combination of NOESY correlations and chemical transformation. Stereostructure of **1** was revised to be the same as that of **2**. Nankakurine A (**1**) induced secretion of neurotrophic factors from human astrocytoma cells.

INTRODUCTION

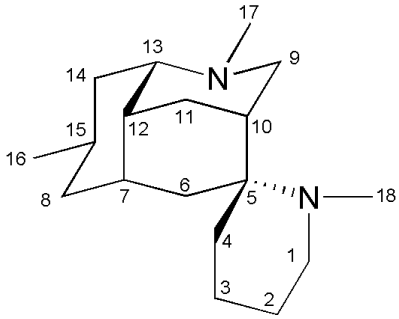
The *Lycopodium* alkaloids represent a large family of plant constituents obtained from the club moss belonging to Lycopodiaceae.[1] These structurally diverse alkaloids often possess unusual skeletons, and many of them continue to be of interest from biogenetic[2] and biological points of view[3] as well as providing challenging targets for total synthesis.[4] In recent ten years, much efforts have been devoted to preparation of structurally simplified analogues and derivatives with the tricyclic skeleton of huperzine A as a promising lead compound of acetylcholinesterase inhibitors.[5]

Our interest has been focused on isolation of structurally interesting *Lycopodium* alkaloids and biosynthetic intermediates to clarify the biogenetic pathway.[5-17] Our previous investigation on extracts of *Lycopodium hamiltonii* (Lycopodiaceae) resulted in the isolation of new alkaloids such as nankakurine A (**1**)[13] and lycoperine A.[17] Further purification of the extract of *L hamiltonii* led to the isolation of a

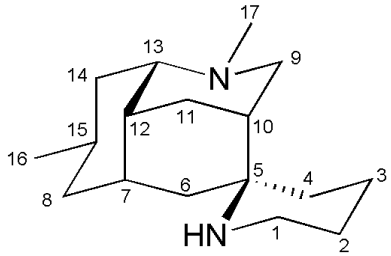
new alkaloid, nankakurine B (**2**). This paper describes the isolation and structure elucidation of **2**, the revised stereostructure of nankakurine A (**1**), and releasing activity of neurotrophic factors from human astrocytoma cells by nankakurine A (**1**).



revised stereostructure of nankakurine A (**1**)



nankakurine B (**2**)



proposed stereostructure of nankakurine A

RESULTS AND DISCUSSION

The club moss *L. hamiltonii* was extracted with MeOH, and the extract was partitioned between EtOAc and 3% tartaric acid. Water-soluble materials, which were adjusted to pH 10 with sat. Na_2CO_3, were extracted with $CHCl_3$. $CHCl_3$-soluble materials were subjected to an amino silica gel column (hexane/EtOAc, 1:0 → 0:1, and then $CHCl_3$/MeOH, 1:0 → 0:1), in which a fraction eluted with hexane/EtOAc was purified by a silica gel column ($CHCl_3$/MeOH/TFA, 1:0:0 → 5:5:1) to afford nankakurine B (**2**, 1.1 mg, 0.0002% yield) together with nankakurine A[13] (**1**, 1.6 mg, 0.003% yield).

Nankakurine B {**2**, $[\alpha]_D^{19}$ +12° (*c* 1.0, MeOH)} showed the pseudomolecular ion peak at *m/z* 277 $(M+H)^+$ in the FABMS, and the molecular formula, $C_{18}H_{32}N_2$, was established by HRFABMS [*m/z* 277.2653, $(M+H)^+$, Δ +0.9 mmu]. IR absorptions implied the presence of an amine (3300 cm^{-1}) functionality. ^1H and ^{13}C NMR data (Tables 1 and 2, respectively) revealed eighteen carbon signals due to one sp^3 quaternary carbon, five sp^3 methines, nine sp^3 methylenes, and three methyl groups. Among them, two sp^3 methylene (δ_C 50.4; δ_H 3.13 and 3.62; δ_C 55.8; δ_H 3.35 and 3.80), one sp^3 methine (δ_C 65.5; δ_H 3.40), one sp^3 quaternary carbon (δ_C 67.0), and two sp^3 methyls (δ_C 45.3; δ_H 2.89; δ_C 34.5; δ_H 2.93) were ascribed to those bearing a nitrogen atom. **2** was inferred to possess four rings from four degree of unsaturation.

The gross structure of **2** was deduced from extensive analyses of the 2D NMR data including the ^1H-^1H COSY, HOHAHA, HMQC, and HMBC spectra in CD_3OD (Figure 1). The ^1H-^1H COSY and HOHAHA spectra in CD_3OD revealed connectivities of two partial structures **a** (C-1 ~ C-4) and **b** (C-6 to C-8, C-9 ~

C-16, C-7 to C-12, and C-8 to C-15) like nankakurine A (**1**) as shown in Figure 1. HMBC correlations were observed for H_3-18 to C-1 (δ_C 50.4) and C-5 (δ_C 67.0), suggesting that C-1 and C-5 were connected to each other through a nitrogen atom to form a piperidine ring (ring A) like nankakurine A (**1**).[13] These data suggested that **2** possessed nankakurine A (**1**)[13] skeleton with two *N*-methyl group. Treatment of **1** with formic acid and formaldehyde gave *N*-methyl derivative of nankakurine A (**1**), whose spectral data including optical rotation were identical with nankakurine B (**2**).

Thus, the gross structure of nankakurine B (**2**) was elucidated to be **2** possessing a caged skeleton consisting of a cyclohexane ring (C-7 ~ C-8 and C-12 ~ C-15) with a methyl group at C-15 and a 3-aza-bicyclo[3.3.1]nonane ring (C-5 ~ C-7, C-9 ~ C-13, and N-2) with two *N*-methyl group (C-17 and C-18) connected to a piperidine ring (N-1 and C-1 ~ C-5) through a spiro carbon at C-5.

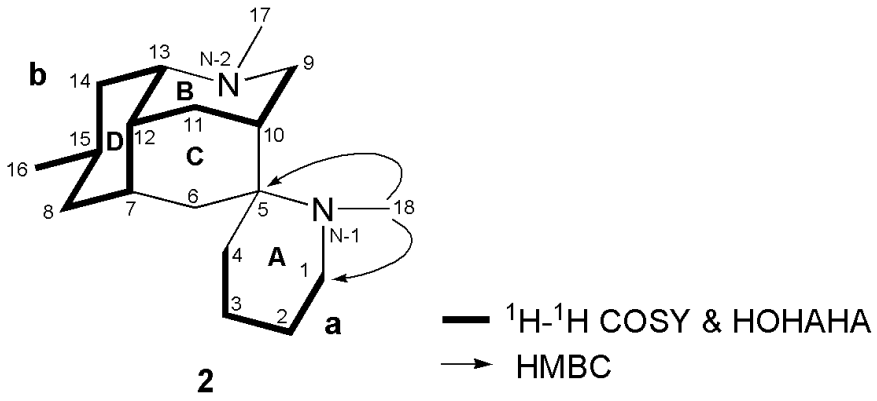


Figure 1. Selected 2D NMR correlations for nankakurine B (**2**).

The relative stereochemistry of **2** was elucidated by NOESY correlations and $^3J_{H\text{-}H}$ couplings as shown in computer-generated 3D drawing (Fig. 2). Conformations of the piperidine ring (N-1, C-1 ~ C-5), the bicyclo[3.3.1]nonane ring (C-5 ~ C-7, C-9 ~ C-13, and N-2), and the cyclohexane ring (C-7, C-8, and C-12 ~ C-15), in which all of the 6-membered rings took chair forms, were deduced from NOESY correlations such as H_3-18/H-2b and H-4a, H-11a/H-9a and H-13, and H-12/H-8a and H-14a as shown in Figure 2. The ^{13}C high field shift at C-2 (δ_C 17.9) by its γ-gauche effect[18] indicated that the *N*-methyl at C-18 took an axial orientation. Stereochemistry of the spiro carbon at C-5 was elucidated to be *R** by the clear NOESY correlations of H-1b/H-6b, and H_3-18/H-9b. Thus, the relative stereochemistry of **2** was assigned as shown in Figure 2. These results were not identical with the relative configuration at C-5 (*S**) deduced in the previous paper[13] in the case of nankakurine A (**1**).

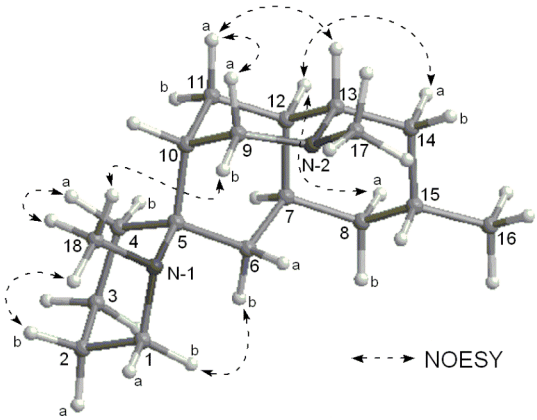


Figure 2. Selective NOESY correlations and relative stereochemistry for nankakurine B (**2**).

There are two possible conformational states for the piperidine ring of **1** and **2** in solution as shown in Figure 3. The conformational space was searched using MMFF force field[19] implemented in the Macromodel program.[20] Each of the lowest energy conformers belonging to two separate clusters are represented as **1a**, **1b**, **2a**, and **2b**. Each conformer possessed a chair conformation in the piperidine part, while the bicyclo[3.3.1]nonane ring and the cyclohexane ring adopted the same chair conformation. In the case of nankakurine B (**2**), **2b** was abundant from the populations calculated for these two clusters (**2a** and **2b**). On the other hand, **1a** and **1b** took a similar energy. Because of its fluctuation of two conformers **1a** and **1b**, uncertain NOESY correlations led to miss assignment of the stereochemistry at C-5 of nankakurine A (**1**). These results of the simulations were consistent with the relative stereochemistry of **2** and the equilibrium to the more stable conformer **2b** in CD_3OD inferred on basis of the NMR data.

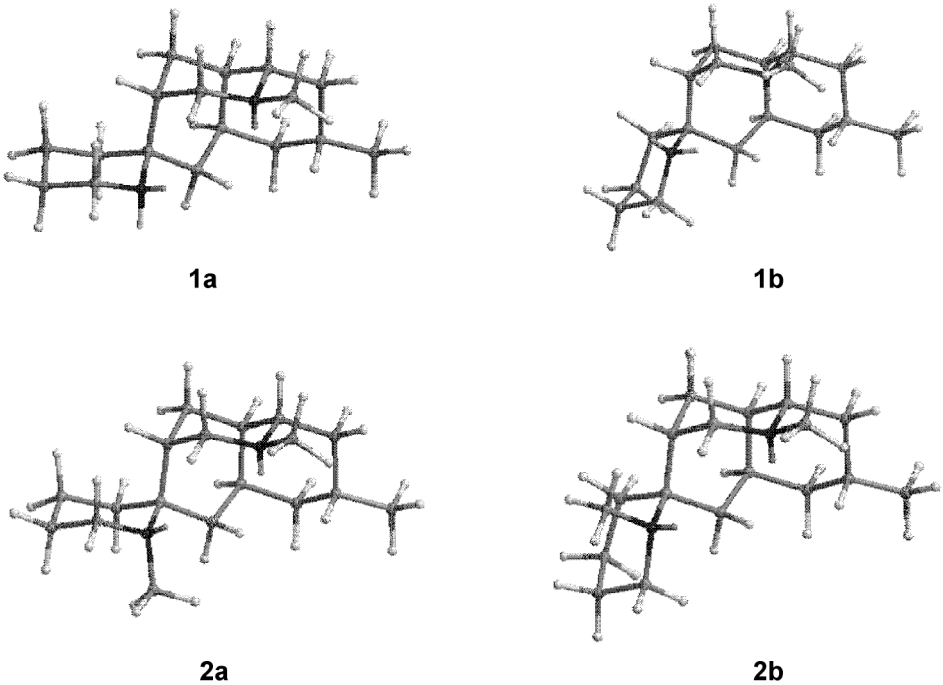


Figure 3. Each two representative stable conformers (**1a** and **1b**; **2a** and **2b**) analyzed by conformational analysis.

Table 1. ^{1}H NMR Data [δ_H (J, Hz)] of Nankakurines A (**1**) and B (**2**) in CD_3OD at 300K

	1[a]	**2**[b]
1a	2.82 (2H, t, 5.1)	3.13 (1H, brd, 13.6)
1b		3.62 (1H, ddd, 13.8, 13.6, 3.2)
2a	1.53 (1H, m)	1.74 (1H, m)
2b	1.58 (1H, m)	1.92 (1H, m)
3a	1.57 (2H, m)	1.74 (1H, m)
3b		2.28 (1H, brd, 14.5)
4a	1.66 (2H, m)	1.73 (1H, m)
4b		1.82 (1H, m)
6a	1.64 (1H, m)	1.93 (1H, m)
6b	2.29 (1H, dd, 12.7, 12.7)	2.58 (1H, brd, 10.8)
7	1.85 (1H, m)	2.17 (1H, m)
8a	1.20 (1H, ddd, 12.6, 12.6, 5.0)	1.38 (1H, m)
8b	1.49 (1H, m)	1.69 (1H, m)
9a	2.14 (1H, dd, 12.1, 2.8)	3.35 (1H, m)
9b	3.00 (1H, ddd, 12.1, 2.4, 2.4)	3.80 (1H, m)
10	1.81 (1H, m)	2.34 (1H, m)
11a	1.53 (1H, m)	1.97 (1H, m)
11b	1.83 (1H, m)	2.15 (1H, m)
12	1.53 (1H, m)	1.99 (1H, m)
13	2.03 (1H, m)	3.40 (1H, m)
14a	0.89 (1H, ddd, 12.1, 12.1, 2.1)	1.38 (1H, m)
14b	2.02 (1H, m)	2.25 (1H, brd, 14.5)
15	1.95 (1H, m)	1.98 (1H, m)
16	0.85 (3H, d, 6.6)	0.99 (3H, d, 6.3)
17	2.12 (3H, s)	2.89 (3H, m)
18		2.93 (3H, s)

[a] free base [b] TFA salt

Table 2. ^{13}C NMR Data (δ_C) of Nankakurines A (**1**) and B (**2**) in CD_3OD at 300K

	1[a]	**2**[b]
1	41.0	50.4
2	26.3	17.9
3	20.9	25.5
4	34.6	17.7
5	56.1	67.0
6	40.0	31.5
7	34.5	32.0
8	41.9	39.0
9	58.5	55.8
10	37.4	35.9
11	32.5	28.4
12	36.9	33.3
13	65.1	65.5
14	40.0	35.3
15	22.0	21.6
16	23.0	22.2
17	43.4	45.3
18		34.5

[a] free base [b] TFA salt

BIOACTIVITY OF NANKAKURINE A (1).

Human astrocytoma cells (glial cell line) were incubated for 2 days with nankakurine A (**1**), and then rat pheochromocytoma (PC-12) cells were cultivated for 2 days in the conditioned 1321N1 culture medium. The culture medium has been shown to contain neurotrophic factors synthesized in 1321N1 cells, which promote the differentiation of PC-12 cells. The culture medium conditioned with 1 μM mankakurine A (**1**) and 100 nM phorbol 12-myristate 13-acetate (PMA), which is an activator of neurotrophic factor biosynthesis, dose-dependently induced neurite extension in PC-12 cells (Fig. 4). These results indicate that nankakurine A (**1**) induced secretion of neurotrophic factors from 1321N1 cells and the released neurotrophic factors promote neuronal differentiation of PC-12 cells.[21] Neurite outgrowth could not be evaluated for nankakurine B (**2**) due to small amount of **2**.

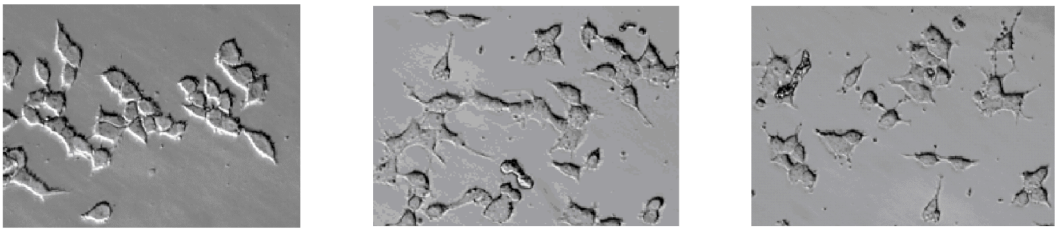


Figure 4. Glial cell-mediated morphological change of PC-12 cells by nankakurine A (**1**).

EXPERIMENTAL

General Experimental Procedures. ^{1}H and 2D NMR spectra were recorded on a 600 MHz and 800 MHz spectrometers at 300K, while ^{13}C NMR spectra were measured on a 150 MHz spectrometer. Each NMR sample of nankakurines A (**1**) and B (**2**) was prepared by dissolving 1.0 mg in 30 μL of CD_3OD in 2.5 mm micro cells (Shigemi Co. Ltd.) or in 700 μL of CD_3OD in 5 mm cells, and chemical shifts were reported using residual CD_3OD (δ_H 3.31 and δ_C 49.0) as an internal standard. Standard pulse sequences were employed for the 2D NMR experiments. ^{1}H-^{1}H COSY, HOHAHA, and NOESY spectra were measured with spectral widths of both dimensions of 4800Hz, and 32 scans with two dummy scans were accumulated into 1K data points for each of 256 t_1 increments. NOESY and HOHAHA spectra in the phase sensitive mode were measured with a mixing time of 800 and 30 ms, respectively. For HMQC spectra in the phase sensitive mode and HMBC spectra, a total of 256 increments of 1K data points were collected. For HMBC spectra with Z-axis PFG, a 50 ms delay time was used for long-range C-H coupling. Zero-filling to 1K for F_1 and multiplication with squared cosine-bell windows shifted in both dimensions were performed prior to 2D Fourier transformation. FABMS was measured by using glycerol as a matrix.

Plant Material. The club moss *Lycopodium hamiltonii* were collected in Kagoshima in 2004. The botanical identification was made by Mr. N. Yoshida, Health Sciences University of Hokkaido. Each voucher specimen has been deposited in the herbarium of Hokkaido University.

Extraction and Isolation. The club moss *L. hamiltonii* (0.5 kg) was extracted with MeOH, and the extract was partitioned between EtOAc and 3% tartaric acid. Water-soluble materials, which were adjusted to pH 10 with sat. Na_2CO_3, were extracted with $CHCl_3$. $CHCl_3$-soluble materials were subjected to an amino silica gel column (hexane/EtOAc, 1:0 → 0:1, and then $CHCl_3$/MeOH, 1:0 → 0:1), in which a fraction eluted with hexane/EtOAc was purified by a silica gel column ($CHCl_3$/MeOH/TFA, 1:0:0 → 5:5:1) to afford nankakurine B (**2**, 1.1 mg, 0.0002% yield) together with nankakurine A[13] (**1**, 1.6 mg, 0.0003% yield).

Nankakurine A (1): colorless solid; $[\alpha]_D^{21}$ +16° (*c* 0.4, MeOH); IR (neat) ν_{max} 3300, 2920, 2765, 1450, 1340, 1270, 1110, and 1050 cm^{-1}; ^1H and ^{13}C NMR data (Table 1); FABMS *m/z* 263 $(M+H)^+$; HRFABMS *m/z* 263.2497 (M+H; calcd for $C_{17}H_{31}N_2$, 263.2487).

Nankakurine B (2): colorless solid; $[\alpha]_D^{19}$ +12° (*c* 1.0, MeOH); IR (neat) ν_{max} 3300, 2920, 2765, 1450, 1340, 1270, 1110, and 1050 cm^{-1}; ^1H and ^{13}C NMR data (Table 2); FABMS *m/z* 277 $(M+H)^+$; HRFABMS *m/z* 277.2653 (M+H; calcd for $C_{18}H_{33}N_2$, 277.2644).

Chemical transformation of nankakurine A (1) to nankakurine B (2). To a solution of **1** (1.5 mg) in formic acid (41 μl) was added 35% formaldehyde (50 μl). The mixture was allowed to stand at 70°C for 12 hr. After cooling, 10% HCl was added and the solvent was evaporated. The residue was applied to a silica gel column ($CHCl_3$/MeOH, 1:0 → 0:1) to give a compound (0.7 mg), whose spectral data including optical rotation were identical with those of nankakurine B.

Evaluation of Neurite Outgrowth.[23] Human astrocytoma cells (glial cell line) were incubated for 2 days with 1 μM nankakurine A (**1**) and 100 nM phorbol 12-myristate 13-acetate (PMA), and then rat pheochromocytoma (PC-12) cells were cultivated for 2 days in the conditioned 1321N1 culture medium. Cell morphology was assessed under a phase-contrast microscope. Neurite extension from PC-12 cells was regarded as an index of neuronal differentiation.

REFERENCES

1. For reviews of the *Lycopodium* alkaloids, see: (a) J. Kobayashi and H. Morita, In *The Alkaloids*; ed. by G. A. Cordell, Academic Press: New York, 2004; Vol. 61, p1. (b) W. A. Ayer and L. S. Trifonov, In *The Alkaloids*; ed. by G. A. Cordell and A. Brossi, Academic Press: New York, 1994; Vol. 45, p233. (c) W. A. Ayer, *Nat. Prod. Rep.*, 1991, **8**, 455. (d) D. B. MacLean, In *The Alkaloids*; ed. by A. Brossi, Academic Press: New York, 1985; Vol. 26, p241. (e) D. B. MacLean, In *The Alkaloids*; ed. by R. H. F. Manske, Academic Press: New York, 1973; Vol. 14, p348. (f) D. B. MacLean, In *The Alkaloids*; ed. by R. H. F. Manske, Academic Press: New York, 1968; Vol. 10, p. 305.
2. (a) T. Hemscheidt and I. D. Spenser, *J. Am. Chem. Soc.*, 1996, **118**, 1799. (b) T. Hemscheidt and I. D. Spenser, *J. Am. Chem. Soc.*, 1993, **115**, 3020.
3. J. S. Liu, Y. L. Zhu, C. M. Yu, Y. Z. Zhou, Y. Y. Han, F. W. Wu, B. F. Qi, *Can. J. Chem.*, 1986, **64**, 837.
4. (a) C. F. Yen and C. C. Liao, *Angew. Chem. Int. Ed.*, 2002, **41**, 4090. (b) J. Cassayre, F. Gagosz, and S. Z. Zard, *Angew. Chem. Int. Ed.*, 2002, **41**, 1783. (c) C. K. Sha, F. K. Lee, and C. J. Chang, *J. Am. Chem. Soc.*, 1999, **121**, 9875. (d) J. P. Williams, St. D. R. Laurent, D. Friedrich, E. Pinard, B. A. Roden, and L. A. Paquette, *J. Am. Chem. Soc.*, 1994, **116**, 4689. (e) G. C. Hirst, T. O. Johnson, and

L. E. Overman, *J. Am. Chem. Soc*., 1993, **115**, 2992. and references therein.

5. H. Morita, M. Arisaka, N. Yoshida, and J. Kobayashi, *J. Org. Chem.,* 2000, **65**, 6241.
6. J. Kobayashi, Y. Hirasawa, N. Yoshida, and H. Morita, *Tetrahedron Lett*., 2000, **41**, 9069.
7. J. Kobayashi, Y. Hirasawa, N. Yoshida, and H. Morita, *J. Org. Chem.,* 2001, **66**, 5901.
8. H. Morita, Y. Hirasawa, N. Yoshida, and J. Kobayashi, *Tetrahedron Lett.,* 2001, **42**, 4199; Y. Hirasawa, H. Morita, and J. Kobayashi, *Tetrahedron,* 2003, **59**, 3567.
9. Y. Hirasawa, H. Morita, and J. Kobayashi, *Tetrahedron,* 2002, **58**, 5483.
10. H. Morita, Y. Hirasawa, and J. Kobayashi, *J. Org. Chem.,* 2003, **68**, 4563.
11. Y. Hirasawa, H. Morita, M. Shiro, and J. Kobayashi, *Org. Lett.,* 2003, **5**, 3991.
12. H. Morita, Y. Hirasawa, T. Shinzato, and J. Kobayashi, *Tetrahedron,* 2004, **60**, 7015.
13. Y. Hirasawa, H. Morita, and J. Kobayashi, *Org. Lett.,* 2004, **6**, 3389.
14. H. Morita, K. Ishiuchi, A. Haganuma, T. Hoshino, T. Hoshino, Y. Obara, N. Nakahata, and J. Kobayashi, *Tetrahedron,* 2005, **61**, 1955.
15. K. Koyama, H. Morita, Y. Hirasawa, M. Yoshinaga, T. Hoshino, Y. Obara, N. Nakahata, and J. Kobayashi, *Tetrahedron,* 2005, **61**, 3681.
16. H. Morita, Y. Hirasawa, and J. Kobayashi, *J. Nat. Prod.,* 2005, **68**, 1809.
17. Y. Hirasawa, J. Kobayashi, and H. Morita, *Org. Lett.,* 2006, **8**, 123.
18. D. Dalling and D. Grant, *J. Am. Chem. Soc*., 1967, **89**, 6612; D. Dalling and D. Grant, *J. Am. Chem. Soc*., 1972, **89**, 5318.
19. T, Halgren, *J. Am. Chem. Soc.,* 1990, **112**, 4710.
20. Monte Carlo simulation and molecular mechanics calculations were conducted by Macromodel program: F, Mohamadi, N. G. J. Richards, W. C. Guida, R. Liskamp, M. Lipton, C. Caufield, G. Chang, T. Hendrickson, and W. C. J. Still, *J. Comput. Chem.,* 1990, **11**, 440.
21. Y. Obara, H. Kobayashi, T. Ohta, Y. Ohizumi, and N. Nakahata, *Mol. Pharmacol.,* 2001, **59**, 1287.