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Induction of Choline Acetyltransferase Activity in Cholinergic Neurons by Stolonidiol: Structure−Activity Relationship

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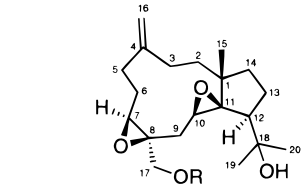
The effect of stolonidiol (**1**), a bioactive marine diterpenoid from the Japanese soft coral *Clavularia* sp., on choline acetyltransferase (ChAT) activity was examined using cultured cholinergic neurons. Stolonidiol (**1**) showed potent ChAT inducible activity in primary cultured basal forebrain cells and clonal septal SN49 cells, suggesting that it may act as a potent neurotrophic factor-like agent on the cholinergic nervous system. Further expansion of the structure−activity relationship to include stolonidiol (**1**) and its derivatives demonstrated that the exo-methylene group and the epoxide group are essential for ChAT-inducing activity. Stolonidiol (**1**) showed the highest activity among the test samples.

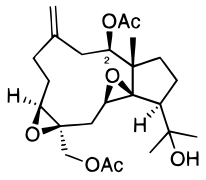
Cholinergic neurons in the basal forebrain innervate the cortex and hippocampus, and their function may be closely related to cognitive function and memory. The degeneration of neuronal cells in this area is considered to be responsible for several types of dementia including Alzheimer's disease. One of the neurotransmitters, acetylcholine, is synthesized from acetyl coenzyme A and choline by the action of choline acetyltransferase (ChAT; EC2.3.1.3). Therefore, induction of ChAT activity in cholinergic neurons may improve the cognitive function in diseases exhibiting cholinergic deficits such as Alzheimer's disease.[1−4]

During the course of our investigation on bioactive substances from the Japanese soft coral, we reported the isolation and structure elucidation of the bioactive marine diterpenoid stolonidiol (**1**) from the *Clavularia* sp.[5] This compound showed remarkable cytotoxic activity against P388 leukemia cells in vitro and ichthyotoxic activity toward a killifish *Oryzias latipes*.[5] However, the effects of stolonidiol (**1**) on other cells or tissues are still unkown.

The present paper deals with the effect of stolonidiol (**1**) on cultured basal forebrain cells and cholinergic-derived SN49 cells.



stolonidiol (**1**) : R = H
2 : R = Ac
3 : R = C(O)$(CH_2)_2CH_3$
4 : R = C(O)$(CH_2)_4CH_3$
5 : R = C(O)$(CH_2)_{10}CH_3$
6 : R = C(O)$(CH_2)_{12}CH_3$
7 : R = Bz



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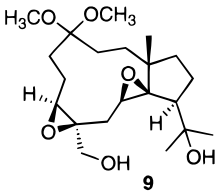

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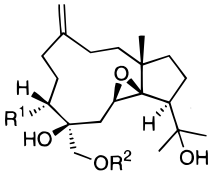
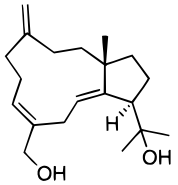


10 : R^1 = OCH_3, R^2 = H
11 : R^1 = Cl, R^2 = *p*-Br-Bz



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Results and Discussion

When basal forebrain cells, which were precultured for 7 days, were cultured for 3 days in the presence of

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Table 1. Effects of Stolonidiol (**1**) on ChAT Activity in Cultured Cells

stolonidiol (**1**) (μg/mL)	ChAT activity (% of control)[a]	
	basal forebrain cells	SN49 cells
0	100.0 ± 5.9	100.0 ± 14.4
0.01	130.2 ± 12.7[b]	146.2 ± 1.4[b]
0.1	177.5 ± 15.6[b]	141.8 ± 12.1[b]
1	138.6 ± 12.6[b]	144.7 ± 11.5[b]
10	38.2 ± 9.8[b]	92.7 ± 12.7

[a] Values represent means (n = 3) ± SD. [b] Data statistically significant with $P < 0.05$ by data comparison with control.

Table 2. Effects of Stolonodiol (**1**) and Derivatives on ChAT Activity in Basal Forebrain Cells

compound	ChAT activity (% of control)[a]	
	0.1 μg/mL	1 μg/mL
stolonidiol (**1**)	126.8 ± 3.6[b]	231.1 ± 19.3[b]
2	89.2 ± 5.3	210.2 ± 11.5[b]
3	118.2 ± 5.7[b]	178.7 ± 14.6[b]
4	138.9 ± 7.6[b]	163.5 ± 8.2[b]
5	108.5 ± 6.6	143.1 ± 2.6[b]
6	97.6 ± 7.1	173.9 ± 13.3[b]
7	116.5 ± 8.2	173.4 ± 7.5[b]
8	11.5 ± 6.9	161.2 ± 3.4[b]
9	108.8 ± 5.5	104.5 ± 1.1
10	89.2 ± 5.3	92.3 ± 3.6
11	96.7 ± 4.2	95.3 ± 4.9
12	108.7 ± 2.1	121.9 ± 13.3

[a] Values represent means (n = 3) ± SD. ChAT activity in basal forebrain cells of Tables 1 and 2 are not comparable because different cultures were used. The rate of responsive cells in cholinergic neurons/well is dependent on primary cultures. [b] Data statistically significant with $P < 0.05$ by data comparison with control.

stolonidiol (**1**) at 0.01−10 μg/mL, potent ChAT inducible activity was observed (Table 1). A similar induction was observed using cholinergic-derived clonal SN49 cells (Table 1). Stolonidiol (**1**) at high doses decreased ChAT activity as a result of neuronal cell death, as observed microscopically. To confirm the induction of ChAT by stolonidiol (**1**), the expression of ChAT mRNA was determined using a RT-PCR method. When basal forebrain cells were exposed to 0.1 or 1 μg/mL of stolonidiol (**1**) for 6 h, the level of ChAT mRNA was increased in the cells (data not shown). To examine the structure−activity relationships of stolonidiol (**1**)-induced ChAT activity in basal forebrain cells, the activities of related compounds, stolonidiol acetate **2**,[5] 17-*O*-acyl stolonidiols **3**−**7**,[6] 2-acetoxystolonidiol acetate **8**,[7] acetal **9**, methyl ether **10**,[5] chloride **11**,[5] and triene **12**,[7] were investigated. Stolonidiol acetate **2** or 17-*O*-acyl stolonidiols **3**−**7**, which differ in the chain lengths of acyl groups, also significantly increase ChAT activity in basal forebrain cells at 1 μg/mL, but less so than stolonidiol (**1**) at the same concentration (Table 2). Acylation of the hydroxyl group at C-17 in stolonidiol (**1**) would thus appear not to be essential for activity. 2-Acetoxystolonidiol acetate (**8**), which has two acetyl groups, shows activity similar to other acyl derivatives. With acetal **9**, obtained by ozonolysis of stolonidiol (**1**) in MeOH, activity was abolished (Table 2). For clarification of the effects of epoxide moieties on activity, the methyl ether **10** and chloride **11** derivatives were synthesized. The results obtained with methyl ether **10**, chloride **11**, and triene **12** suggest that the exo-methylene moiety at C-4 and the epoxide moiety at C-7 and C-8 are essential for the induction of ChAT activity.

A significant reduction of ChAT activity has been observed in the central nervous system of people suffering from Alzheimer's disease, which is associated with selective dysfunction of central cholinergic neurons.[1,2] One of neurotrophic factors, nerve growth factor (NGF), has been shown to enhance the ChAT of cultured central cholinergic neurons.[8] Furthermore, NGF injected into the cortex was retrogradely transported to the basal forebrain,[9] and NGF injected into the ventricle increased the ChAT activity of brain cholinergic neurons.[10] Therefore, agents with neurotrophic factor-like activity may have possibilities as therapeutics for dementia. It has been suggested that the administration of NGF improves some of the biological abnormalities that occur in disorders such as Alzheimer's disease. However, oral administration of NGF has no effect on the neurons in the central nervous system because NGF is a high molecular weight protein that cannot pass through the blood−brain barrier and is readily metabolized by enzymes in the digestive organs. Present results show that stolonidiol (**1**) and some stolonidiol derivatives increase the ChAT activity in basal forebrain cells and clonal septal cells. These observations suggest that stolonidiol (**1**) and some stolonidiol derivatives having ChAT inducible activity may serve as neurotrophic factor-like agents or leads for the discovery of more useful agents with potential benefit to the cholinergic nervous system.

Experimental Section

General Experimental Procedures. Optical rotation was measured with a JASCO DIP-360 automatic polarimeter. IR spectra were recorded on a JASCO FT/IR-620 spectrophotometer, and ^{1}H NMR spectra with a Varian Gemini-300 (300 MHz) or a Bruker DPX-400 (400 MHz) spectrometer. For electron ionization MS (EIMS) spectra, a Hitachi M-80 or VG Auto Spec spectrometer was used.

Materials. Fetal bovine serum (FBS) was purchased from Bioserum, Australia, and horse serum was purchased from Cell Culture Laboratories, USA. [1-^{14}C]Acetyl CoA (0.15 GBq/mmol) was purchased from NEN, USA. Dulbecco's modified Eagle's medium and Ham's F12 medium were obtained from Gibco (USA).

Acylation of Stolonidiol (1). Typical Procedure. To a solution of stolonidiol (**1**)[5] (5.0 mg) in pyridine (0.4 mL) was added *n*-butyric anhydride (0.05 mL) followed by stirring at room temperature for 14 h. The reaction mixture was diluted with Et_2O, washed with saturated aqueous $CuSO_4$, H_2O, and saturated aqueous NaCl, dried over anhydrous $MgSO_4$, and concentrated under reduced pressure. The residue was purified by silica gel column chromatography (eluted with 5:1 hexane−EtOAc) to give compound **3** (5.9 mg, 97% yield) as a colorless oil. TLC of compound **3** showed one spot (R_f 0.28 (4:1 hexane−EtOAc)); $[\alpha]_D$ −41°(c 0.2, $CHCl_3$); IR ν max (neat) 3503, 2966, 2935, 2862, 1739 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.84 (3H, s, Me-15), 0.95 (3H, t, J = 7.4 Hz, C(O)(CH$_2$)$_2$CH_3), 1.18 (3H, s, Me-19 or 20), 1.28 (3H, s, Me-19 or 20), 1.2−1.4 (2H, m, H-2), 1.5−1.9 (7H, m, H-6, 13a or 13b, 14, C(O)CH$_2$CH_2CH$_3$), 1.96 (2H, m, H-3a or 3b, 13a or 13b), 2.1−2.4 (6H, m, H-3a or 3b, 5a or 5b, 9a or 9b, 12, C(O)CH_2CH$_2$CH$_3$), 2.4−2.6 (2H, m, H-5a or 5b, 9a or 9b), 3.00 (1H, t, J = 6.9 Hz, H-7), 3.15 (1H, d, J = 7.8 Hz, H-10), 3.57 (1H, s, OH), 3.92 (1H, d, J = 12.1 Hz, H-17a or 17b), 4.29 (1H, d, J = 12.1 Hz, H-17a or 17b), 4.71 (1H, s, H-16a or 16b), 4.79 (1H, s, H-16a or 16b); EIMS m/z 406 (M$^+$).

4: colorless oil, TLC of compound **4** showed one spot (R_f 0.29 (4:1 hexane−EtOAc)); $[\alpha]_D$ −39°(c 0.63, $CHCl_3$); IR ν max (neat): 3458, 2962, 2922, 2870, 1746 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.84 (3H, s, Me-15), 0.90 (3H, m, C(O)(CH$_2$)$_4$CH_3), 1.18 (3H, s, Me-19 or 20), 1.28 (3H, s, Me-19 or 20), 1.2−1.4 (6H, m, H-2, C(O)(CH$_2$)$_2$(CH_2)$_2$CH$_3$), 1.5−1.9 (7H, m, H-6, 13a or 13b, 14, C(O)CH$_2$CH_2(CH$_2$)$_2$CH$_3$), 1.95 (2H, m, H-3a or 3b, 13a or 13b), 2.1−2.4 (6H, m, H-3a or 3b, 5a or 5b, 9a or 9b, 12, C(O)CH_2(CH$_2$)$_3$CH$_3$), 2.4−2.6 (2H, m, H-5a or 5b, 9a or 9b), 3.00 (1H, t, J = 7.1 Hz, H-7), 3.15 (1H, dd, J = 7.8, 1.3 Hz, H-10), 3.92 (1H, d, J = 12.1 Hz, H-17a or 17b), 4.29 (1H, d, J = 12.1 Hz, H-17a or 17b), 4.71 (1H, s, H-16a or 16b), 4.79 (1H, s, H-16a or 16b); EIMS m/z 434 (M$^+$).

5: colorless oil, TLC of compound **5** showed one spot (R_f 0.35 (4:1 hexane−EtOAc)); $[\alpha]_D$ −15°(*c* 0.1, $CHCl_3$); IR ν max (neat): 3504, 2925, 1740 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 0.83 (3H, s, Me-15), 0.88 (3H, t, J = 7.1 Hz, $C(O)(CH_2)_{10}CH_3$), 1.18 (3H, s, Me-19 or 20), 1.2−1.4 (21H, m, H-2, Me-19 or 20, $C(O)(CH_2)_2(CH_2)_8CH_3$), 1.5−1.9 (7H, m, H-6, 13a or 13b, 14, $C(O)CH_2CH_2(CH_2)_8CH_3$), 1.95 (2H, m, H-3a or 3b, 13a or 13b), 2.1−2.4 (6H, m, H-3a or 3b, 5a or 5b, 9a or 9b, 12, $C(O)CH_2(CH_2)_9CH_3$), 2.4−2.6 (2H, m, H-5a or 5b, 9a or 9b), 3.00 (1H, t, J = 6.8 Hz, H-7), 3.15 (1H, dd, J = 7.6, 1.2 Hz, H-10), 3.92 (1H, d, J = 12.2 Hz, H-17a or 17b), 4.29 (1H, d, J = 12.2 Hz, H-17a or 17b), 4.71 (1H, s, H-16a or 16b), 4.78 (1H, s, H-16a or 16b); EIMS *m/z* 518 (M^+).

6: colorless oil, TLC of compound **6** showed one spot (R_f 0.40 (4:1 hexane−EtOAc)); $[\alpha]_D$ −26° (*c* 0.1, $CHCl_3$); IR ν max (neat): 3505, 2925, 2855, 1741 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 0.83 (3H, s, Me-15), 0.87 (3H, t, J = 7.0 Hz, $C(O)(CH_2)_{12}CH_3$), 1.18 (3H, s, Me-19 or 20), 1.2−1.4 (25H, m, H-2, Me-19 or 20, $C(O)(CH_2)_2(CH_2)_{10}CH_3$), 1.5−1.9 (7H, m, H-6, 13a or 13b, 14, $C(O)CH_2CH_2(CH_2)_{10}CH_3$), 1.95 (2H, m, H-3a or 3b,13a or 13b), 2.1−2.4 (6H, m, H-3a or 3b, 5a or 5b, 9a or 9b, 12, $C(O)CH_2(CH_2)_{11}CH_3$), 2.4−2.6 (2H, m, H-5a or 5b, 9a or 9b), 3.00 (1H, t, J = 6.9 Hz, H-7), 3.14 (1H, dd, J = 7.7, 1.4 Hz, H-10), 3.92 (1H, d, J = 12.3 Hz, H-17a or 17b), 4.29 (1H, d, J = 12.3 Hz, H-17a or 17b), 4.71 (1H, s, H-16a or 16b), 4.78 (1H, s, H-16a or 16b); EIMS *m/z* 546 (M^+).

7: colorless oil, TLC of compound **7** showed one spot (R_f 0.27 (4:1 hexane−EtOAc)); $[\alpha]_D$ -14.4°(*c* 0.54, $CHCl_3$); IR ν max (neat): 3499, 2967, 2935, 1723 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 0.85 (3H, s, Me-15), 1.12 (3H, s, Me-19 or 20), 1.27 (3H, s, Me-19 or 20), 1.2−1.4 (2H, m, H-2), 1.5−1.9 (5H, m, H-6, 13a or 13b, 14), 1.95(2H, m, H-3a or 3b, 13a or 13b), 2.1−2.4 (4H, m, H-3a or 3b, 5a or 5b, 9a or 9b, 12), 2.5 (1H, m, H-5a or 5b), 2.65 (1H, dd, J = 16.2, 8.0 Hz, H-9a or 9b), 3.10 (1H, t, J = 7.2 Hz, H-7), 3.18 (1H, dd, J = 8.0, 1.7 Hz, H-10), 4.20 (1H, d, J = 12.3 Hz, H-17a or 17b), 4.54 (1H, d, J = 12.3 Hz, H-17a or 17b), 4.72 (1H, s, H-16a or 16b), 4.80 (1H, s, H-16a or 16b), 7.45 (2H, s, Ar), 7.57 (1H, m, Ar), 8.06 (2H, m, Ar); EIMS *m/z* 440 (M^+).

Ozonolysis of Stolonidiol (1). A cold (−78 °C) solution of stolonidiol (**1**) (5.1 mg) in CH_2Cl_2 (1.0 mL) and MeOH (1.0 mL) was treated with ozone until a blue color persisted. Excess ozone was removed by an argon flow. The reaction mixture was treated with Me_2S (0.1 mL), allowed to warm slowly to room temperature over 2 h, stirred for 12 h at this temperature, and concentrated under reduced pressure. The residue was diluted with $Et_2O-CHCl_3$ (4:1), washed with H_2O and saturated aqueous NaCl, dried over anhydrous $MgSO_4$, and concentrated under reduced pressure. The residue was purified by preparative TLC (developed with 3:1 hexane−EtOAc) to give methyl acetal **9** (1.7 mg, 29% yield as a colorless oil). TLC of compound **9** showed one spot (R_f 0.15 (1:2 hexane−EtOAc)); IR ν max (neat) 3408, 2968 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 0.81 (3H, s, Me-15), 1.19 (3H, s, Me-19 or 20), 1.27 (3H, s, Me-19 or 20), 2.13 (1H, d, J = 17.0 Hz, H-9a or 9b), 2.28 (1H, d, J = 11.0 Hz, H-12), 2.41 (1H, dd, J = 17.0, 7.0 Hz, H-9a or 9b), 3.12 (1H, d, J = 7.0 Hz, H-10), 3.23 (1H, m, H-7), 3.32 (6H, s, OCH_3), 3.68 (1H, d, J = 12.6 Hz, H-17a or 17b), 3.77 (1H, d, J = 12.6 Hz, H-17a or 17b).

Cell Culture. Preparation of basal forebrain cells was carried out as described previously.[11] In brief, the basal forebrain area (containing the septum and vertical limb of the diagonal band of Broca) of a rat embryo (gestational age: 17−19 days) was removed, dissected into small pieces with scissors under sterile conditions, and then digested at 37 °C for 30 min in a solution containing 180 U of papain, 0.02% L-cystein-HCl, 0.02% bovine serum albumin, and 0.5% glucose in phosphate-buffered saline (PBS, pH 7.4). The digest was centrifuged at 900 rpm for 5 min to obtain the precipitate, which was washed with a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium (DF medium). The washed precipitate was resuspended in DF medium containing 5% fetal bovine serum and 5% horse serum. After gentle drawing through plastic tips, the dissociated cells were plated in the same medium at a density of 1×10^6 cells/well in a 24-well plate.

Mouse clonal septal SN49 cells (hybridoma cell line derived from primary cultured mouse basal forebrain cells and neuroblastoma N18TG2) were maintained in DMEM supplemented with 10% fetal bovine serum. Cells were plated in 24-well plates at a density of 2×10^5 cells/well and incubated at 37 °C for 24 h under 5% CO_2, before adding stolonidiol and its derivatives, and then incubated further for 48 h.

Determination of ChAT Activity. Rat embryo basal forebrain cells and/or SN49 cells were washed with PBS and then solubilized in 210 μL of 50 mM Tris buffer (pH 6.8) containing 1% Triton X-100. The resulting solution was taken for the determination of ChAT activity according to the method of Fonnum.[12] Briefly, the solution was incubated with 90 μL of mixture that contained 25 μM [^{14}C] acetyl CoA (5 mCi/mM), 200 mM NaCl, 50 mM potassium phosphate buffer, 0.1 mM eserin, and 8 mM choline chloride. The mixture was incubated at 37 °C for 1 h and then extracted with 1 mL of acetonitrile solution containing tetraphenylboron (5 mg/ml) and 2 mL of toluene scintillation mixture. After the organic layer containing the [^{14}C]acetylcholine was separated by centrifugation, the radioactivity in the extract was determined with a liquid scintillation counter (Aloka, Tokyo, Japan). Protein content was measured by the method of Bradford[13] using the Bio-Rad protein assay (Bio-Rad, Richmond, CA).

Analysis of PCR Products. Analysis of ChAT mRNA was carried out by the RT-PCR method as described previously.[14] Relative intensities of ethidium-bromide stained PCR products representing transcripts for the ChAT gene in the brain of each rat were analyzed by using the optical scanner and the Image program. Briefly, the image of stained agarose gel was captured using the optical scanner (EPSON GT-6000, Japan) with a resolution of 360 dots per inch by assigning one of 256 gray levels to each dot in the image. The digitized band images were processed using the Image processing program (NIH Image, public domain program, Version 1.47) and normalized against cyclophilin values.

Statistics. Statistical significance of the difference of means was performed using ANOVA followed by Dunnett's post hoc procedure[15] and was calculated using Macintosh software, super ANOVA (Abacus Concepts, Inc., CA).

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(6) 17-*O*-Acyl stolonidiols **3**−**7** were synthesized from stolonidiol (**1**), respectively. See Experimental Section.
(7) 2-Acetoxystolonidiol acetate **8** and triene **12** are new ditepenoids isolated from the Okinawan soft coral *Clavularia* sp. Structural determinations of these compounds will appear in a separate paper.
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