NG-011 AND NG-012, NOVEL POTENTIATORS OF NERVE GROWTH FACTOR

II. THE STRUCTURE DETERMINATION OF NG-011 AND NG-012

MAYUMI ITO, YUKIKO TSUCHIDA, KAZUTOSHI MIZOUE* and KAZUNORI HANADA

Research Center of Taisho Pharmaceutical Co., Ltd.,
1-403, Yoshino-cho, Omiya-shi, Saitama 330, Japan

(Received for publication March 25, 1992)

NG-011 and NG-012, novel potentiators of nerve growth factor (NGF) were isolated from the culture broth of *Penicillium verruculosum* F-4542.

The structures of NG-011 and NG-012 were elucidated by their spectral analysis and degradation experiments as shown in Fig. 1.

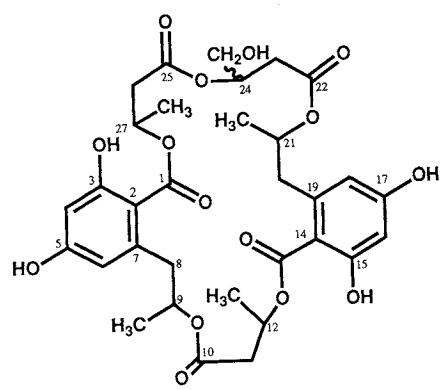
The etiology and pathogenesis of ALZHEIMER's disease and senile dementia of the ALZHEIMER's type is poorly understood, but progressive deterioration of memory and intellectual function is associated with reduction and degeneration of acetylcholine-utilizing neurons in the hippocampus and the cerebral cortex[1]. It was reported that nerve growth factor (NGF) treatment can ameliorate age-related impairment in memory test and prevent lesion-induced loss of septal cholinergic neurons in rats[2~4]. Also it is known that NGF can prevent neuronal loss of the hippocampus in the cerebral ischemia model in the Mongolian gerbil[5]. These findings suggest that NGF can be effective for treatment of the dementia and the cerebral paralysis. However, it is very difficult to use NGF as a medicine since it must be prescribed for a patient *via* intraventricular because of impermeability of its high molecule cross the blood-brain barrier.

To solve this problem, we have explored low molecular compounds in the microbial fermentation broth which can mimic or potentiate NGF by peripheral administration. In the course of our screening program for these compounds, we have discovered novel potentiators NG-011 and NG-012 in the culture broth of *Penicillium verruculosum* F-4542[6]. The present paper deals with the structures of NG-011 and NG-012.

Results and Discussion

NG-011 (**1**) and NG-012 (**2**) had many resemblances in their physico-chemical properties. They gave the identical IR and UV spectra and the same mass fragmentation patterns. The existence of a phenolic alcohol group in both **1** and **2** was indicated by the bathochromic shifts in the UV spectrum and by positive color reaction with $FeCl_3$. And their UV spectra showed characteristic absorptions at 217, 264 and 302 nm, which were similar to that of 3,4-dihydro-6,8-dihydroxy-3-methylisocoumarin[7]. It could be also found in the fermentation broth of *Penicillium verruculosum*

Fig. 1. The structures of NG-011 (**1**) and NG-012 (**2**).



NG-011 (**1**) is an epimer of NG-012 (**2**) at C24

F-4542[6] and obtained as the degradation product (*vide infra*). The IR spectrum showed characteristic absorptions at 1729 and 1734 cm^{-1} due to ester carbonyl groups. The molecular formula of both **1** and **2** was established as $C_{32}H_{38}O_{15}$ by high resolution EI mass measurement of the molecular ions (M^+) at *m/z* 662.2245 of **1** and *m/z* 662.2224 of **2**, respectively, and their elemental analysis. Their molecular formula required 14 unsaturation equivalents. As NG-012 (**2**) was obtained in larger amounts than NG-011 (**1**), **2** was used for structural determination, derivatization and degradation studies. The functionalities of the carbon signals of **2** were established by the INEPT spectrum and all one-bonded carbon lines were assigned by the ^1H-^{13}C COSY spectrum. The ^{13}C NMR spectrum of **2** measured in CD_3OD gave 32 carbon signals, consistent with its molecular formula, which could be classified as $CH_3 \times 4$, $-CH_2- \times 6$, $-CHO- \times 5$, $-CH= \times 4$, $>C= \times 8$ and $>C=O \times 5$ by the INEPT spectrum, indicating that 33 protons are bonded to 19 protonated carbons. The ^1H NMR spectrum of **2** showed 33 proton signals, which indicated a total of five exchangeable protons in the molecule by the consideration of its molecular formula. The ^1H and ^{13}C NMR data are listed in Table 1.

Though four proton signals at 6.19, 6.21, 6.22 and 6.24 ppm were attributable to aromatic protons,

Table 1. ^1H and ^{13}C NMR chemical shifts of NG-011 (**1**) and NG-012 (**2**) (in CD_3OD).

Carbon No.	δ_H		δ_C	
	NG-011 (**1**)	NG-012 (**2**)	NG-011 (**1**)	NG-012 (**2**)
1, 13			172[a], 172[a]	172[a], 172[a]
2, 14			107.8, 108.17	107.28, 108.00
3, 15			164.22, 164.31	164.12, 164.30
4, 16	6.19 (d, J=2.4 Hz), 6.20 (d, J=2.4 Hz)	6.19 (d, J=2.4 Hz), 6.21 (d, J=2.4 Hz)	103.47, 103.52	103.51, 103.65
5, 17			165.79, 166.20	165.86, 166.32
6, 18	6.23 (d, J=2.4 Hz), 6.25 (d, J=2.4 Hz)	6.22 (d, J=2.4 Hz), 6.24 (d, J=2.4 Hz)	113.26, 113.41	113.51, 114.23
7, 19			143.90, 143.90	143.88, 144.01
8, 20	2.8, 3.4, 2.9, 3.3	2.98, 3.30, 2.82, 3.41	42.51, 42.62	42.72, 43.37
9, 21	5.04, 5.11	5.05, 5.10	74.36, 74.36	74.18, 74.74
9-CH_3, 21-CH_3	1.17 (d, J=6.3 Hz), 1.20 (d, J=6.3 Hz)	1.11 (d, J=6.2 Hz), 1.16 (d, J=6.2 Hz)	20.57, 20.69	20.34, 20.57
10, 25			172[a], 172[a]	172[a], 172[a]
11, 26	2.78, 2.92, 2.80 (2H)	2.70, 2.88, 2.68, 2.82	41.88, 42.32	41.74, 42.31
12, 27	5.48, 5.57	5.51, 5.51	71.05, 71.16	70.88, 71.05
12-CH_3, 27-CH_3	1.39 (d, J=6.3 Hz), 1.43 (d, J=6.3 Hz)	1.39 (d, J=6.3 Hz), 1.40 (d, J=6.3 Hz)	20.94, 20.94	21.00, 21.00
22			172[a]	172[a]
23	2.46 (2H)	2.66 (2H)	41.15	37.41
24	4.15	5.26 (m)	67.93	73.40
24-CH_2OH	4.08	3.61 (2H)	69.30	64.37

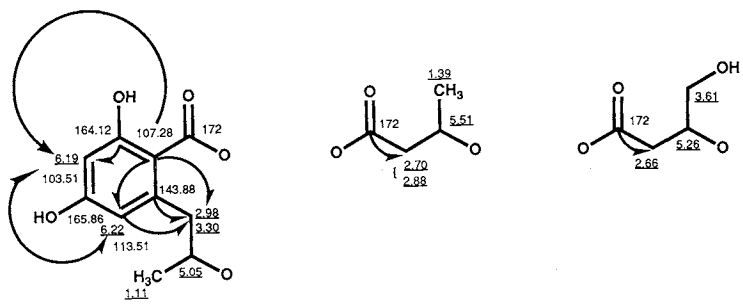
[a] These five signals overlapped at the range of 0.1 ~ 0.2 ppm.

they could not be unambiguously assigned because of their overlapping. In the ^1H NMR spectrum of the acetyl derivative (**3**) obtained by treatment of **2** with acetic anhydride in pyridine, these resonances were well separated into two pairs of doublets with $J=2.2$ Hz due to a *meta* coupling, indicating the existence of two tetra-substituted phenol moieties in the molecule. And the presence of a methylene signal, which showed downfield shift from 3.61 ppm to 4.04 and 4.33 ppm by acetylation, in addition to five acetyl methyl lines, demonstrated that one of five exchangeable protons was due to a primary alcohol whereas the others were corresponding to phenolic hydroxyl groups. Eight unsaturations were assigned to two aromatic rings and five to carbonyl substituents, leaving the final unsaturation equivalent to accommodate as a cyclic ring. As all ^1H and ^{13}C signals were doubly observed except for two methylene lines at 2.66 and 3.61 ppm and a methine line at 5.26 ppm and their relevant carbon resonances at 37.41, 64.37 and 73.40 ppm, it was suggested that the molecule of **2** has two pairs of the structurally identical units in a symmetrical portion.

The ^1H-^{13}C *meta* couplings were observed between proton signals (6.19 and 6.22 ppm) and their relevant carbons (113.51, 107.28 and 103.51 ppm) in the tetra-substituted phenol moiety while the cross peaks due to long range couplings were shown from three aromatic carbon signals at 143.88, 107.28 and 113.51 ppm to unequivalent methylene proton signals at 2.98 and 3.30 ppm in the HMBC spectrum, which were coupled to a methine at 5.05 ppm substituted with a methyl doublet at 1.11 ppm as depicted in Fig. 2. Further, the HMBC spectrum of **2** gave a correlation map from a carbon line at 164.12 ppm to a proton line at 6.19 ppm. On the basis of the ^{13}C chemical shift trends, two hydroxyl groups could be located at a *meta* position to each other on the tetra-substituted phenol moiety as shown in Fig. 2. From the UV spectrum of **2**, a carbonyl group was expected to be placed at some position on the phenol moiety[8]. Since long range couplings could not be observed between a carbonyl carbon at 172 ppm, which could not be unambiguously discriminated from the other four lines because of their superimposing at the narrow range of $0.1 \sim 0.2$ ppm, the carbonyl group must be located at a position over three bonds distant from aromatic protons. Therefore, a carbonyl group could be estimated to locate as shown in Fig. 2 and the UV absorptions at 217, 264 and 302 nm were ascribable to a 2,4-dihydroxy-6-(2-hydroxy-*n*-propyl) benzoic moiety[6] (structure A). By repeating the same procedure, another fragment (A′) was deduced.

The partial structure B could be unequivocally assigned by tracing the cross peaks from a methine signal at 5.51 ppm to a methyl doublet at 1.39 ppm and a methylene at 2.70 and 2.88 ppm in the ^1H-^1H

Fig. 2. The partial structures A, B and C.



(A) (B) (C)

⟵ shows proton-carbon long range correlations detected by the HMBC method.

COSY spectrum, and by the observation of long range couplings between a carbonyl carbon at 172 ppm and methylene protons at 2.70 and 2.88 ppm in the HMBC spectrum as shown in Fig. 2. Another partial structure (B') was determined by repeating the same procedure.

The partial structure C could be unambiguously assigned by the observation of correlations from a methine at 5.26 ppm to two methylenes at 2.66 and 3.61 ppm in the ^{1}H-^{1}H COSY spectrum. The location of a free hydroxyl group was confirmed by the downfield shift of methylene signals by *ca.* 0.4~0.7 ppm in the ^{1}H NMR spectrum of a penta acetyl derivative (**3**) (*vide supra*). Thus five partial structures could be established as shown in Fig. 2.

Low field shifts of five methine signals led to the ester linkages of these five partial structures, taking into consideration of the symmetrical location of two pairs of two partial structure, but ambiguity remained to be cleared because of overlapping of five carbonyl resonances at the narrow range of 0.1~0.2 ppm.

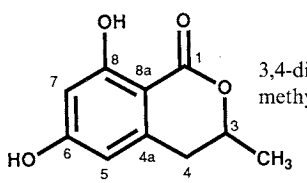
Therefore, degradation experiments were performed. Hydrolysis of **2** with 0.5 N HCl in ethanol gave three products.

Main product (**4**) was isolated and assigned as 3,4-dihydro-6,8-dihydroxy-3-methylisocoumarin[7] by its spectral analysis as depicted in Fig. 3, which confirmed the partial structure A, including the absolute configuration.

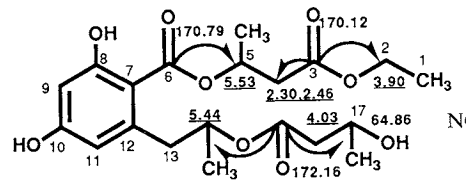
The existence of the partial structure A and two units of the partial structure B in the molecule of the degradation product NG-014 (**5**) was indicated through the ^{1}H-^{1}H COSY spectral analysis for **5** (an ethyl group from ethanol). The ^{13}C NMR spectrum of **5** in benzene-d_6 gave three well-separated carbonyl carbon signals, though five carbonyl carbon lines could not be discriminated from each other in that of **2** in methanol-d_4. The combination of the partial structure A with two units of the partial structure B by two ester linkage as shown in Fig. 3 was confirmed in the HMBC spectrum by the observation of long range couplings between a methine proton at 5.53 ppm (structure B) and a carbonyl carbon at 170.79 ppm (structure A), from two methylene protons at 2.30 and 2.46 ppm (structure B) and at 3.90 ppm on an ethyl substituent to 170.12 ppm (structure B) and from two methine protons at 5.44 ppm (structure A) and at 4.03 ppm (structure B) to a carbonyl carbon line at 172.16 ppm (structure B).

The degradation product NG-015 (**6**) showed a characteristic absorption at 1786 cm^{-1} due to a

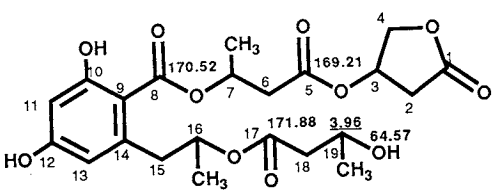
Fig. 3. The structures of degradation products of NG-012 (**2**).



3,4-dihydro-6,8-dihydroxy-3-methylisocoumarin (**4**)



NG-014 (**5**)



NG-015 (**6**)

←— shows proton-carbon long range couplings detected by the HMBC method.

Table 2. ^{1}H and ^{13}C NMR chemical shifts of NG-014 (**5**) and NG-015 (**6**) (in benzene-d_6).

	NG-014 (**5**)			NG-015 (**6**)	
	δ_H	δ_C		δ_H	δ_C
1	0.93	14.04	1		173.43
2	3.90	60.83	2	1.80, 2.05	34.01
			3	4.56	70.49
			4	3.44, 3.79	72.04
3		170.12	5		169.21
4	2.30, 2.46	40.65	6	2.05, 2.30	40.10
5	5.53	69.14	7	5.36	68.57
5-CH_3	1.15	19.61	7-CH_3	1.00	19.58
6		170.79	8		170.52
7		105.16	9		105.16
8		166.43	10		166.66
9	6.63	102.91	11	6.36	103.00
10		162.29	12		162
11	6.42	113.34	13	6.20	113.09
12		143.04	14		142.83
13	2.90, 3.30	42.83	15	2.99, 3.09	42.79
14	5.44	71.82	16	5.36	71.31
14-CH_3	1.27	20.27	16-CH_3	1.17	20.09
15		172.16	17		171.88
16	2.13, 2.30	43.41	18	2.15	43.45
17	4.03	64.86	19	3.96	64.57
17-CH_3	1.00	22.29	19-CH_3	0.92	22.48

γ-lactone. Comparison of the ^{13}C NMR spectrum of **5** with that of **6** revealed the appearance of new five carbon signals in place of two carbon signals corresponding to an ethyl substituent. A 3-dehydro-3-hydroxy-γ-lactone moiety could be assigned by tracing the spin network in the ^{1}H-^{1}H COSY spectrum. The location of a lactone moiety could be determined through comparison of the ^{1}H and ^{13}C NMR spectra of **6** with those of **5** as depicted in Fig. 3. As the lactone was thought to be derived from the partial structure C, it could be estimated to connect with the partial structure A by diester linkages.

The five partial structures could be unambiguously connected by the spectral analysis of the degradation products. The planar structure of **2** was established as shown in Fig. 1.

NG-011 (**1**) has the same molecular formula and weight with **2**[6]. Comparison of ^{1}H and ^{13}C NMR spectra of **2** with those of **1** revealed that the structural differences of **1** and **2** was attributed to the partial structure C as shown in Table 1, and it was concluded that the difference was due to the chirality at C24, indicating that **1** was an epimer at C24 of **2**.

According to the method of MOOR *et al.*[9], we tried to prepare the other degradation products several times, however, we could not obtain lactones derived from the partial structures B and C in hydrolysis. The absolute configurations at C24, C12 and C27, except for a 2,4-dihydroxy-6-(2-hydroxy-*n*-propyl)-benzoic moiety, remained to be determined. Crystals of **1** and **2** suitable for X-ray analysis could not be obtained, and preparation of crystallizable derivatives is under way.

Experimental

General

MP's were determined with a Yanagimoto micro-melting point apparatus and were uncorrected.

Optical rotations were measured on a Jasco DIP-360 polarimeter in 10 cm tube. IR spectra were recorded on a Perkin-Elmer 1760 FT-IR spectrophotometer. UV spectra were measured on a Hitachi 220A spectrophotometer. EI-MS, FAB-MS and HREI-MS spectra were obtained with a Jeol JMX-SX 102 mass spectrometer. NMR spectra were measured on a Jeol JNM-GX 400 spectrometer at ambient temperature at 400 MHz (^{1}H) and 100 MHz (^{13}C) using the solvent peaks as internal references downfield of TMS at 0 ppm.

Low pressure liquid chromatography (LPLC) was run using a ODS (C18) column (a Kusano CIG Prepack column, 22 mm × 30 cm) equipped with a Kusano Kp-6H Micro-pump system developed with 35% acetonitrile solution at a flow rate of 8 ml/minute at room temperature, monitoring absorbance at 260 nm. The effluent was each fractioned at the weight of 10.0 g.

Preparative HPLC separations were performed using a Senshu-Pak ODS column (ODS-4251-N, 10 mm × 25 cm) with a Waters Model 600E system, maintained at 50°C and developed with 30% acetonitrile solution at a flow rate of 5 ml/minute, monitoring the absorbance at 215 nm.

Acetylation of NG-012 (**2**)

NG-012 (51 mg) was stirred with acetic anhydride (1 ml) in dry pyridine (1 ml) for 18 hours at room temperature. The reaction mixture was diluted with cold water (150 ml) and extracted with ethyl acetate (150 ml). The organic layer was washed successively with dil HCl (200 ml) and water (300 ml), and dried over sodium sulfate and concentrated *in vacuo* to give a penta-*O*-acetyl derivative (**3**) (51.5 mg).

Penta-*O*-acetyl NG-012 (**3**)

Colorless sticky solid; FAB-MS *m/z* 873 $(M+H)^+$; ^{1}H NMR (400 MHz, CD_3OD) δ_H 1.00 (3H, d, J=6.1 Hz), 1.29 (3H, d, J=6.1 Hz), 1.35 (3H, d, J=6.3 Hz), 1.36 (3H, d, J=6.3 Hz), 2.02 (3H, s), 2.15 (3H, s), 2.22 (3H, s), 2.26 (3H, s), 2.28 (3H, s), 2.34 (1H, dd, J=8.1 and 13.4 Hz), 2.56~2.76 (7H, m), 2.84 (1H, dd, J=9.3 and 14.2 Hz), 3.15 (1H, dd, J=3.3 and 14.2 Hz), 4.04 (1H, dd, J=5.0 and 12.1 Hz), 4.33 (1H, dd, J=3.9 and 12.0 Hz), 4.82 (1H, m), 5.00 (1H, m), 5.38 (1H, m), 5.50 (2H, m), 6.77 (1H, d, J=2.2 Hz), 6.91 (1H, d, J=2.2 Hz), 6.98 (1H, d, J=2.2 Hz), 7.02 (1H, d, J=2.2 Hz).

Acid Hydrolysis of NG-012 (**2**)

A solution of **2** (1.0 g) in ethanol (50 ml) and 0.5 N HCl (50 ml) was refluxed for 6 hours. The ethanol was removed *in vacuo* and the concentrate was diluted with water (100 ml) and extracted with ethyl acetate (100 ml × 2). The organic layer was washed with water and dried over sodium sulfate followed by crystallization from *n*-hexane to obtain the main product (**4**) as colorless needles (300 mg).

3,4-Dihydro-6,8-dihydroxy-3-methylisocoumarin (**4**)

MP 211~214°C; $[a]_D^{26}$ −49° (*c* 1.0, MeOH); UV (MeOH) 216, 268, 301 nm; IR ν_{max} cm^{-1} (KBr) 3211, 1655, 1634; ^{1}H NMR (400 MHz, acetone-d_6) δ 1.44 (3H, d, J=6.1 Hz, 3-CH_3), 2.84 (1H, dd, J=11 and 16 Hz, 4-Ha), 2.94 (1H, dd, J=3.4 and 16 Hz, 4-Hb), 4.69 (1H, m, 3-H), 6.25 (1H, d, J=2.3 Hz, 7-H), 6.29 (1H, d, J=2.3 Hz, 5-H), 9.48 (1H, br s, 6-OH), 11.28 (1H, br s, 8-OH); ^{13}C NMR (100 MHz, acetone-d_6) δ 20.80 (q, 3-CH_3), 35.05 (t, C-4), 76.31 (d, C-3), 101.64 (s, C-8a), 101.93 (d, C-7), 107.44 (d, C-5), 143.19 (s, C-4a), 165.00 (s, C-8), 165.23 (s, C-6), 170.68 (s, C-1).

Mother liquid was concentrated and purified by LPLC. The fractions eluting between No. 48 and No. 58 were combined and concentrated to give **5** (49 mg).

NG-014 (**5**)

Colorless oil; EI-MS *m/z* 412 (M^+); IR ν_{max} cm^{-1} (neat) 3392, 2981, 1733, 1651, 1622; ^{1}H NMR and ^{13}C NMR data are reported in Table 2.

The fractions eluting between No. 23 and No. 30 were collected and further purified by preparative HPLC. The fraction from 14.0 to 15.5 minutes of retention times were combined and concentrated to give **6** (1 mg).

NG-015 (**6**)

White powders; EI-MS *m/z* 468 (M^+); IR ν_{max} cm^{-1} (neat) 3369, 2980, 1786, 1651, 1621; ^{1}H NMR

and ^{13}C NMR data are exhibited in Table 2.

References

1) KATZMAN, R. & T. SAITO.: Advances in Alzheimer's disease. FASEB J. 5: 278~286, 1991
2) FISCHER, W.; K. WICTORIN, A. BJÖRKLUND, L. R. WILLIAMS, S. VARON & F. H. GAGE: Amelioration of cholinergic neuron atrophy and spatial memory impairment in aged rats by nerve growth factor. Nature 329: 65~68, 1987
3) WILLIAMS, L. R.; S. VARON, G. M. PETERSON, K. WICTORIN, W. FISCHER, A. BJÖRKLUND & F. H. GAGE: Continuous infusion of nerve growth factor prevents basal forebrain neuronal death after fimbria fornix transection. Proc. Natl. Acad. Sci. U.S.A. 83: 9231~9235, 1986
4) KROMER, L. F.: Nerve growth factor treatment after brain injury prevents neuronal death. Science 235: 214~216, 1987
5) SHIGENO, T.; T. MIMA, R. TOKIWA, K. TAKAKURA, S. FURUKAWA & D. I. GRAHAM: Intraventricular administration of nerve growth factor prevents the development of delayed neuronal death in the hippocampus. Igaku no Ayumi 145: 579~580, 1988
6) ITO, M.; M. MARUHASHI, N. SAKAI, K. MIZOUE & K. HANADA: NG-011 and NG-012, novel potentiators of nerve growth factor. I. Taxonomy, isolation, and physico-chemical and biological properties. J. Antibiotics 45: 1559~1565, 1992
7) KENDALL, J. K. & T. H. FISHER: An improved synthesis of 6,8-dimethoxy-3-methylisocoumarin, a fungal metabolite precursor. J. Org. Chem. 54: 4218~4220, 1989
8) SCOTT, A. I. (*Ed.*): Interpretation of the Ultra-violet Spectra of Natural Products. pp. 68~123, Pergamon Press, 1964
9) MOOR, R. E.; A. J. BLACKMAN, C. E. CHUCK, J. S. MYNDERSE, G. K. MATSUMOTO, J. CLARDY, R. W. WOODARD & J. C. CRAIG: Absolute stereochemistries of the aplysiatoxins and oscellatoxin A. J. Org. Chem. 49: 2484~2489, 1984