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Lignans from the roots of *Acorus tatarinowii* Schott ameliorate β amyloid-induced toxicity in transgenic *Caenorhabditis elegans*

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ABSTRACT

A novel tetralignan, tatarinan T (**1**) with the rare C8-C7′ linkage pattern, along with a known monolignan (**2**) were isolated from the roots of *Acorus tatarinowii* Schott. Their chemical structures were elucidated on the basis of NMR and X-ray diffraction analysis. We evaluated the protective effects of two rare lignans against β-amyloid toxicity by using CL4176 transgenic *C. elegans* model for the first time, and found that they significantly delayed paralysis of worms at the concentration of 100 μM. Compound **2** exhibited the more potential protective effect against β-amyloid toxicity, its value of PT_{50} extended up to 62.3% at 100 μM compared with control, especially, it still has 30.8% extension at 10 μM.

1. Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder, and it is the most common cause of dementia in the aged population. Abnormal accumulation of β-amyloid peptide (Aβ) in the brain is regarded to be important in this disease. Thus, many efforts have been made to develop strategies targeting Aβ for the prevention and treatment of AD [1,2].

The roots of *Acorus tatarinowii* Schott (Araceae) are a well-known traditional Chinese medicine used in the improvement of memory and cognition [3]. It has reported that extract of *A. tatarinowii* can protect PC12 cells from amyloid-β induced neurotoxicity [4]. β-asarone, the major ingredient of *A. tatarinowii* Schott, has neuroprotective effects *in vitro* and *in vivo* [5,6,7].

In order to further study the effective material basis of title plant with anti-AD, we used the CL4176 transgenic *Caenorhabditis elegans* (*C. elegans*) as a model organism to examine the protective effects of isolated compounds *via* the potential reduction of Aβ toxicity. The CL4176 transgenic *C. elegans* strain was engineered to inducibly express human $Aβ_{1-42}$ peptide in muscle, and the expression and subsequent aggregation of Aβ in the muscle lead to progressive paralysis when temperature is raised [8,9]. This strain has already been employed as *in vivo* models of AD and used to demonstrate the effect of *Ginkgo biloba* extract EGb 761 [10], Liuwei Dihuang [11], and tetracycline [12], in counteracting the Aβ toxicity.

During our research periods, tatarinan T (**1**), a novel tetralignan with the rare C8-C7′ linkage pattern, along with a known monolignan (**2**) [13] were isolated. We individually investigated their anti-Aβ potential using CL4176 transgenic *C. elegans*, and found that these two compounds showed potential protective effect by delaying paralysis of worms. The present paper reported the isolation, structural elucidation, and anti-Aβ activity of these two lignans.

2. Experimental

2.1. General

Optical rotations were measured with a Rudolph autopol VI polarimeter. The IR spectra were recorded on a Nicolet Magna IR spectrophotometer. The NMR spectra were run on a Bruker AM-400 spectrometer with TMS as internal standard. HR-EI-MS spectra were carried out on a Bruker Apex IV FT-MS spectrometer. Column chromatographic separations were carried out on silica gel H-60 (Qingdao Haiyang Chemical Group Corporation, Qingdao, People's Republic of China), and LiChroprep RP-18 (40–63 μm, Merck). TLC was carried out on silica gel HSGF254 plates (Yantai Chemical Industrial Institute, Yantai, People's Republic of China), and spots were visualized by spraying with concentrated sulfuric acid-vanillin solution followed by heating.

2.2. Plant material

The roots of *A. tatarinowii* Schott, in dried form, were purchased from Kangqiao Pharmaceutical Co., Ltd., Shanghai, China, in 2010. A reference sample was deposited in the Tongji Herbarium.

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Table 1
^1H (400 MHz) and ^{13}C (100 MHz) NMR data for **1** in $CDCl_3$ (δ in ppm, *J* in Hz).

Position	δ_C	δ_H	Position	δ_C	δ_H
1	128.9		7″	40.4	2.58, m
2	152.4		8″	40.8	1.67, m
3	97.6	6.47, s	9″	12.5	0.85, d (6.62)
4	151.7		1‴	125.1	
5	140.4		2‴	151.5	
6	137.5		3‴	96.8	6.45, s
7	52.4	3.95, m	4‴	147.2	
8	42.4	1.25, m	5‴	141.1	
9	24.4	0.90, d (6.43)	6‴	114.4	6.27, s
1′	123.2		7‴	43.1	3.57, m
2′	152.7		8‴	17.8	1.80, m
					1.56, m
3′	98.5	6.41, s	9‴	12.8	0.56, t (7.08)
4′	147.3		OMe	55.5	3.47, s
5′	142.5		OMe	55.6	3.57, s
6′	113.4	6.69, s	OMe	55.7	3.57, s
7′	53.8	3.00, s	OMe	56.1	3.57, s
8′	35.8	2.76, m	OMe	56.1	3.64, s
9′	12.7	0.23, d (7.24)	OMe	56.4	3.80, s
1″	125.3		OMe	56.5	3.82, s
2″	152.4		OMe	56.8	3.82, s
3″	96.0	6.48, s	OMe	56.8	3.85, s
4″	147.1		OMe	57.2	3.85, s
5″	141.8		OMe	57.6	3.87, s
6″	114.8	6.56, s	OMe	60.2	3.92, s

2.3. Extraction and isolation

The air-dried roots of *A. tatarinowii* Schott (5.0 kg) were powdered and then extracted three times with 95% ethanol at room temperature. The combined ethanol extracts were concentrated under reduced pressure to give a residue (700 g), which was then partitioned successively with $CHCl_3$ and *n*-BuOH, respectively. The $CHCl_3$ extract (200 g) was subjected to silica gel column chromatography using a gradient solvent system of petroleum ether–acetone (50:1, 25:1, 15:1, 10:1, 5:1, 2:1, 1:1, v/v) to afford 6 fractions (Fr. 1–Fr. 6). Fr. 2 was subjected to silica gel column chromatography repeatedly and eluted with petroleum ether-EtOAc (15:1, 10:1, 5:1, 2:1, 1:1, v/v) to afford 6 fractions (Fr. 2a–Fr. 2f). Fr. 2a was subjected to repeated silica gel column chromatography with gradient elution with petroleum ether-acetone (20:1, 10:1, 5:1, 2:1, 1:1, v/v) to yield compound **2** (102.0 mg). Fr. 2d was chromatographed repeatedly on a RP-18 column eluting with acetone-H_2O (1:1, 2:1, 3:1, v/v), and a silica gel column eluting with a petroleum ether-EtOAc (5:1, 2:1, 1:1, v/v) to obtain compound **1** (10.6 mg).

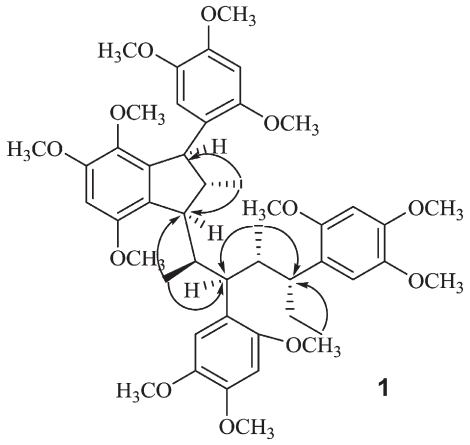

Fig. 2. Key HMBC correlations of compound **1**.

2.4. Spectral data of new compound

Colorless needles; $[\alpha]_D^{25}$-19.0 (c 0.05, $CHCl_3$); IR (KBr) ν_{max} 2963, 2952, 2936, 2833, 1608, 1509, 1465, 1325, 1232, 1204, 1177, 1039, 983, 813 cm^{-1}; ^1H and ^{13}C NMR: see Table 1; HREIMS m/z 855.4282 $[M + Na]^+$ (calcd for $C_{48}H_{64}O_{12}Na$ 855.4290).

2.5. X-ray analysis

Crystal data for compound **1**: formula $C_{48}H_{64}O_{12}$; Mr. = 832.99; monoclinic crystalline system; space group Fdd2; unit cell dimensions a = 61.796 (5) Å, b = 10.2319 (9) Å, c = 30.175 (3) Å; V = 19,080 (3) Å3; Z = 16; Dx = 1.160 mg/m^3; absorption coefficient 0.082 mm^{-1}; F (000) = 7168; R (reflections) = 0.1175 (8867); wR2 (reflections) = 0.1890 (8867). Colorless crystals of **1** were obtained in a mixed solvent of petroleum ether and acetone.

Crystal data for compound **2**: formula $C_{24}H_{32}O_6$; Mr. = 416.49; monoclinic crystalline system; space group P21/n; unit cell dimensions a = 7.054 (2) Å, b = 11.912 (4) Å, c = 27.117 (8) Å; V = 2272.1 (12) Å3; Z = 4; Dx = 1.218 mg/m^3; absorption coefficient 0.086 mm^{-1}; F (000) = 896; R (reflections) = 0.0949 (4230); wR2 (reflections) = 0.2081 (4230). Colorless crystals of **2** were obtained in a mixed solvent of petroleum ether and acetone.

Crystal data were obtained on a Bruker Smart Apex CCD diffractometer, using graphite-monochromated Mo Kα radiation (λ = 0.71073 Å). Crystallographic data for the structure reported in this paper have been deposited with the Cambridge Crystallographic Data Centre, deposit No. CCDC 1425333 for **1**, and CCDC 1427221 for **2**. Copies of the data can be

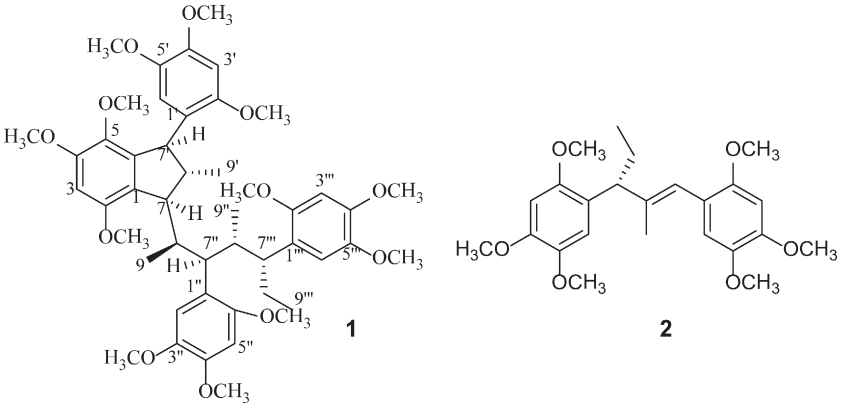

Fig. 1. Chemical structures of compounds **1** and **2**.

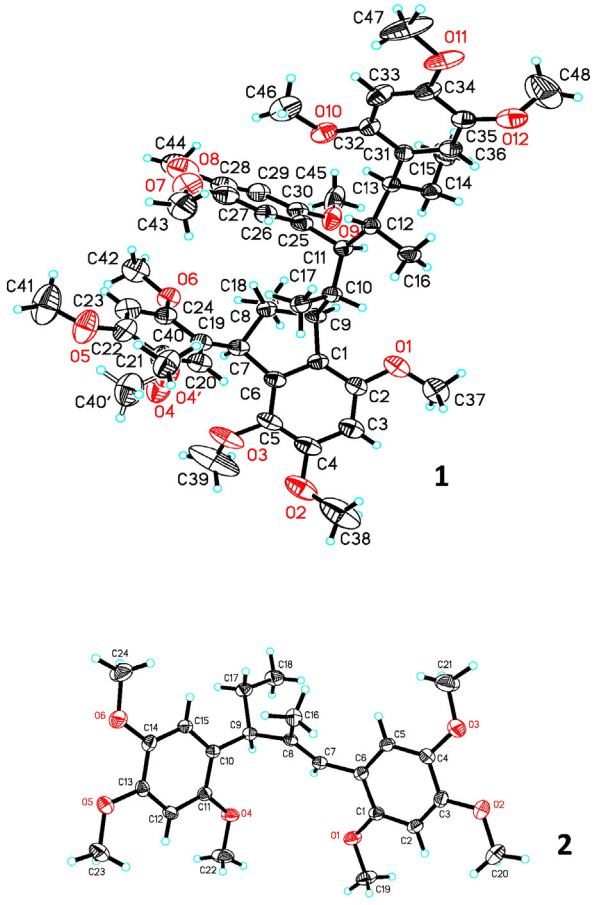

Fig. 3. X-ray crystallographic structure of compounds **1** and **2**.

obtained, free of charge, on application to the Director, CCDC, 12 Union Road, Cambridge CB21EZ, UK [fax: +44-(0)-1223–336,033 or email: deposit@ccdc.cam.ac.uk].

2.6. C. elegans and bacterial strains

The strains of CL4176 transgenic *C. elegans* and *Escherichia coli* OP50 were kindly provided by the Caenorhabditis Genetics Center (CGC), University of Minnesota (Minneapolis, MN, USA). The CL4176 transgenic *C. elegans* were maintained at 16 °C on nematode growth medium (NGM) seeded with *E. coli* OP50 bacteria and was given a temperature upshift at 23 °C for inducing Aβ expression as described [8].

2.7. C. elegans maintenance and treatment

The CL4176 strains were propagated at 16 °C on NGM plates (35 × 10 mm) seeded with *E. coli* OP50 for food. To prepare age-synchronized animals, nematodes were transferred to fresh NGM plates on reaching reproductive maturity at 3 d of age and allowed to lay eggs for 4–6 h. The synchronized eggs of CL4176 strains were cultured on fresh NGM plates at 16 °C. Stock solutions of isolated compounds were made with 100% DMSO. The final concentration of DMSO did not exceed 0.3% in the *E. coli* OP50 food. The isolated compounds for treatment of experimental animals were added directly to the OP50 food source and the working concentrations was equal to 10 and 100 μM for individual compounds. From the L1 stage of above synchronized CL4176 strains, the worms were fed with various concentrations of isolated compounds or with 0.3% DMSO as a solvent control.

2.8. Paralysis assays

The above L1 stage CL4176 strains were transferred to fresh NMG plates (~30 worms/plate), fed with either a vehicle or drug, and continue to maintain at 16 °C for 32 h. Transgene expression was induced by upshifting the temperature form 16 to 23 °C for 24 h. Paralyzed worms were counted at I h intervals from 25th hour after 23 °C. Worms were scored as paralysis if then moved its head only or did not move at all by a repeated touching stimulus with a platinum wire pick [10,14].

2.9. Statistical analysis

The data was analyzed by one way ANOVA tests using Graphpad Prism 5 software. All values were expressed as means ± SD. All the experiments were performed at least three times. Differences between the data were considered significant at $P < 0.05$.

3. Results and discussion

Isolation of the EtOH extract of the roots of *A. tatarinowii* Schott by all kinds of column chromatography methods resulted in one new tetralignan (**1**) and one known monolignan (**2**) (Fig. 1). Interestingly, compounds **1**–**2** are tetramer and dimer of asarone with the rare C8-C7′ linkage pattern, and **2** was naturally obtained for the first time. All compounds were evaluated their protection against Aβ induced toxicity in CL 4176 transgenic *C. elegans*.

Compound **1** was obtained as a colorless crystal. Its molecular formula was determined to be $C_{48}H_{64}O_{12}$ by HREIMS at m/z 855.4282 $[M + Na]^+$ (calcd for $C_{48}H_{64}O_{12}Na$ 855.4290), requiring 17 degrees of unsaturation. The ^1H NMR spectrum of **1** (Table 1) showed seven aromatic proton signals at δ 6.27 (s, H-6‴), 6.41 (s, H-3′), 6.45 (s, H-3‴),

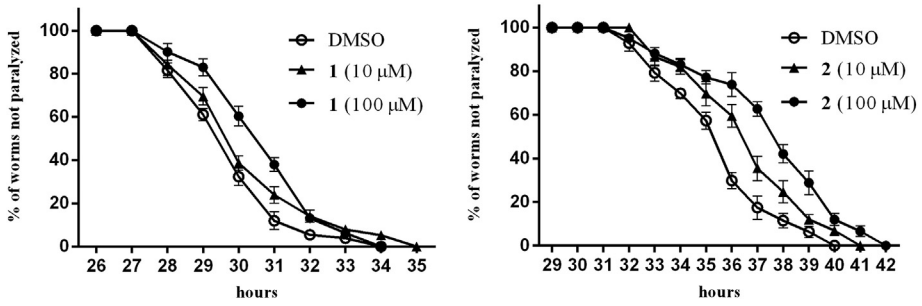

Fig. 4. Effect of compounds **1**–**2** on Aβ-induced paralysis in CL4176 strains. Egg-synchronized CL4176 worms were placed at 16 °C on fresh NMG plates seeded with *E. coli*. The above CL4176 strains (L1 stage) were transferred to fresh NMG plates (~30 worms/plate), fed with either a vehicle or drug, and continue to maintain at 16 °C for 32 h. Transgene expression was induced by upshifting the temperature from 16 to 23 °C for 24 h. Paralyzed worms were counted at I h intervals from 25th hour after 23 °C. The values shown in the graphs are the mean ± SD of percentage of nonparalyzed worms from three independent experiments.

Table 2
Quantitative analysis of paralysis.

Experiments	PT_{50} (h)	Extension (%)	*p* value
Control	2.45 ± 0.4		
1 (10 μM)	2.65 ± 0.3	8.16	0.45
1 (100 μM)	3.40 ± 0.4	38.78	0.01
Control	4.16 ± 0.6		
2 (10 μM)	5.44 ± 0.7	30.77	0.03
2 (100 μM)	6.75 ± 0.5	62.26	0.0006

6.47 (s, H-3), 6.48 (s, H-3″), 6.56 (s, H-6″) and 6.69 (s, H-6′), four tertiary methyl proton signals at δ 0.23 (d, J = 7.24 Hz, H_3-9′), 0.56 (t, J = 7.08 Hz, H_3-9‴), 0.85 (d, J = 6.62 Hz, H_3-9″), 0.90 (d, J = 6.43 Hz, H_3-9), one methylene proton signal at δ 1.80 (m, H_a-8‴), 1.56 (m, H_b-8‴), seven methine proton signals at δ 1.25 (m, H-8), 1.67 (m, H-8″), 2.58 (m, H-7″), 2.76 (m, H-8′), 3.00 (d, H-7′), 3.57 (m, H-7‴), 3.95 (m, H-7), and twelve methoxy proton signals at δ 3.47 (s, 3H), 3.57 (s, 9H), 3.64 (s, 3H), 3.80 (s, 3H), 3.82 (s, 6H), 3.85 (s, 6H), 3.87 (s, 3H), 3.92 (s, 3H). The ^{13}C NMR spectrum of **1** (Table 1) exhibited 48 carbons including 17 aromatic quaternary carbons, 14 methines (seven olefinic), one methylene, 16 methyls (12 oxygenated). The above-mentioned evidence are basically similar to those of 2,3-Dihydro-4,5,7-trimethoxy-3-(2,4,5-trimethoxyphenyl)-1-(3-(2,4,5-trimethoxyphenyl)pentan-2-yl)-2-methyl-1H-indene, a trimer of asarone obtained by synthesis [15], except for the presence of another asarone unit. Calculation of the degrees of unsaturation of **1** confirmed the presence of fourth asarone unit. From the HMBC spectrum (Fig. 2), correlations between H-9′ and C-8′, C-7′, C-7, H-9 and C-8, C-7, C-7″, H-9″ and C-7″, C-7‴, H-9‴ and C-8‴, C-7‴ were observed. Analysis of the ^1H-^1H COZY, HMBC, and HMQC spectra permitted full assignments of the ^1H and ^{13}C NMR data. The proposed relative structure of **1** was confirmed by X-ray crystallography analysis (Fig. 3). Thus, **1** was determined as tetralignan, a new natural product named as tatarinan T.

The known compound **2**, named as tatarinan S, was identified by comparing its spectral data with literature [13], and its relative structure was confirmed by X-ray crystallography analysis (Fig. 3).

These structural types of lignan including monlignan, sesquinlignan, and tetralignan from the roots of *A. tatarinowii* Schott have been reported by Yu's group, and these metabolites displayed potent and selective *in vitro* GK activity [16,17]. However, a possible anti-Aβ potential of these kinds of lignan from *A. tatarinowii* has not been reported. In the present study, we firstly used the CL4176 transgenic *C. elegans* strain to assess the protective effects of compounds **1**–**2** against β-amyloid toxicity in a *C. elegans in vivo*. The L1 stage CL4176 strains were transferred to fresh NMG plates, fed with above isolated compounds (10, 100 μM) or vehicle (0.3% DMSO) for 32 h at 16 °C, followed by temperature upshift from 16 to 23 °C to induce transgene expression. Worms that did not move or only moved the head, under a gentle touch with a platinum loop were scored as paralyzed. Fig. 4 represent paralysis in *C. elegans* CL4176 fed with compounds **1**–**2** or vehicle respectively. Apparently, Aβ-induced paralysis was delayed in worms fed with compounds **1**–**2** respectively, especially at concentration of 100 μM.

For quantitative analysis, we define PT_{50} as time duration at which 50% worms were paralyzed from the onset of paralysis (Table 2). A significant delay of Aβ-induced paralysis was observed in the worms fed with compounds **1**–**2** at concentration of 100 μM. Between both tested compounds, compound **2**, a known monolignan, exhibited the more potential protecive effect against β-amyloid toxicity, its value of PT_{50} extended up to 62.3% at 100 μM compared with control, especially, it still have 30.8% extension at the concentration of 10 μM.

In the present experiments, we evaluated the protective effects of two rare lignans against β-amyloid toxicity by using CL4176 transgenic *C. elegans* model for the first time, and found that monolignan, a dimer of asarone, has more potential to protect transgenic *C. elegans* strains from the paralysis phenotype. These results suggest that this type of lignan might offer aselective therapeutic strategy for Alzheimer's disease.

Conflict of interest statement

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the position presented in, or the review of, the manuscript entitled "Lignans from the roots of *Acorus tatarinowii* Schott ameliorate β amyloid-induced toxicity in transgenic *Caenorhabditis elegans*"

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