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RESEARCH PAPER

Tetramic acid-motif natural products from a marine fungus *Tolypocladium cylindrosporum* FB06 and their anti-Parkinson activities

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Abstract

Tetramic acid-containing natural products are attracting significantly increasing attention from biologists and chemists due to their intriguing structures and biological activities. In the present study, two new tetramic acid alkaloids tolypyridone I (**1**) and tolypyridone J (**2**), together with five known ones (**3**–**7**), were isolated from cultures of a marine fungus *Tolypocladium cylindrosporum* FB06 isolate obtained from a marine sediment in Beaufort sea of North Alaska. Their structures were elucidated using 1D, 2D NMR, and HRESIMS. Their configurations were established on the basis of ^{1}H coupling constants, ROESY correlations and DP4 calculations. Compound **2** was isolated as mixtures of rotational isomers with C-3 to C-7 axis between 4-hydroxy-2-pyridone and 1-ethyl-3,5-dimethylcyclohexane, hindering rotation. In our unbiased screening to discover neuroprotective compounds in an in vitro Parkinson's disease (PD) model, SH-SY5Y dopaminergic cells were treated with isolated compounds followed by treatment with 1-methyl-4-phenylpyridinium (MPP^+), a parkinsonian neurotoxin. Among tested compounds, F-14329 (**7**) significantly protected cells from MPP^+-induced cytotoxicity. MPP^+-mediated cell death is known to be related to the regulation of Bcl-2 family proteins, specifically the down-regulation of anti-apoptotic Bcl-2 and the up-regulation of pro-apoptotic Bax levels. Treatment with 2 mmol/L of MPP^+ for 24 h significantly reduced Bcl-2 levels compared to control treated with vehicle. However, treatment with F-14329 (**7**) attenuated such reduction. This study demonstrates that tetramic acid-motif compounds could be potential lead compounds for treating PD.

Keywords *Tolypocladium cylindrosporum* · Tetramic acid · Tolypyridone · Parkinson's disease

Introduction

Marine microbes offer an incredible amount of biodiversity besides a huge chemical diversity (Xu et al. 2022). They produce a wide range of metabolites with a variety of biological activities, paving the ways for the development of effective new drugs (Hai et al. 2021). Many research teams from pharmaceutical corporations and academic organizations have been focusing on marine fungus as a source of new drug leads during the past 25 years. More than 1000 new natural products have been produced as a result of the recent and thorough chemical characterization of marine fungi from the maritime environment (Rateb and Ebel 2011). Additionally, a number of instances with atypical carbon skeletons and substitution patterns suggest that the marine strains may have unique biosynthetic capacities (Bugni and Ireland 2004; Saleem et al. 2007). *Acremonium chrysogenum*, a fungus found on the Sardinian coast, yielded cephalosporin C, which was later developed as an antibiotic (Abraham 1979). To find effective treatments for neurodegenerative diseases,

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it would be sensible to make use of marine microbes with enormous potential for the discovery of fascinating molecules. Moreover numerous marine fungal strains can produce unique natural chemicals (Wiese and Imhoff 2019). In the present study, we focus on bioactive secondary metabolites produced by a marine fungus *Tolypocladium cylindrosporum* FB06. For many years, *T. cylindrosporum* has been examined in a variety of disciplines. To date polyketides such as cylindromicin, epi-citrinin H1, dicitrinin A, penicitrinone A, and citreorosein and non-ribosomal peptides such as tolypoalbin were reported to be produced by the marine fungus *T. cylindrosporum* (Khan et al. 2021). The strain *T. cylindrosporum* was isolated from polar tundra soils and assessed its ability to degrade cellulose, gallic acid, humic acid, pectin, and starch. The only substance that *T. cylindrosporum* could degrade was starch (Kish et al. 1974). Antagonistic effects of *T. niveum*, *T. cylindrosporum*, and *T. geodes* against 18 species of fungus were investigated and *T. geodes* displayed the most potent antagonistic capacity among the three species, although all three species demonstrated considerable inhibitory effects (Lundgren et al. 1978). It was reported that *Tolypocladium*'s capacity to inhibit growth might be essential for these slow-growing fungi's survival in soil (Bissett 1983). An entomopathogenic member of its genus, *T. cylindrosporum* Gams (Ascomycota: Hypocreales), has been researched as a biological control agent against insects of various orders (Humber 2012). These fungi have been described as having a variety of traits related to their pathogenicity, virulence, and ability to penetrate insect cuticles. Enzyme complexes that can catalyze lipolysis, proteolysis, and chitinolysis might play a role in their penetration. Thus the level of enzyme activity might be used as a diagnostic tool to help select an efficient biological control agent. Secondary metabolic peptides such as destruxins and efrapeptins produced by these entomogenous fungi have been suggested to represent essential virulence components (Scorsetti et al. 2012).

In this study, we carried out chemical investigation of *T. cylindrosporum* FB06 isolated from a glacier silt and investigated neuroprotective activities of compounds isolated from cultures of this strain. Our investigations on FB06 led to the discovery of a series of secondary metabolites, including two new compounds, tolypyridone I (**1**) and tolypyridone J (**2**), and five known compounds (**3**–**7**, Fig. 1). Most of them were 4-hydroxy-2-pyridone-containing compounds biosynthetically derived from tetramic acid. Tetramic acid is a versatile framework that can be altered to create complex, diversified chemical compounds. Natural tetramic acids have received a significant deal of spotlight due to their biosynthesis methods, therapeutic potential, and chemical synthesis because of their complex structures and powerful biological activities (Aoki et al. 2000; Wang et al. 2003). Tetramic acid

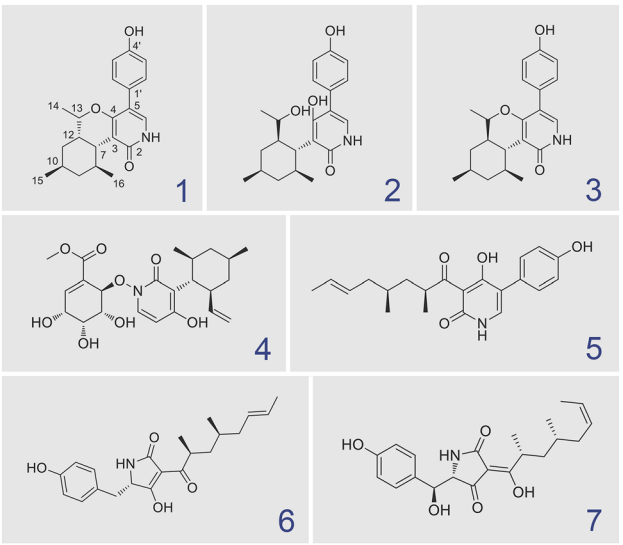

Fig. 1 Structures of compounds isolated from the culture extract of *T. cylindrosporum* FB06

compounds have demonstrated a wide range of bioactivities, such as anticancer and antibiotic activities (Jiang et al. 2020). Based on our investigation, the FB06 fungal strain exhibited anti-Parkinson activity.

Parkinson's disease (PD) is a neurodegenerative disorder that affects millions of people worldwide (Hirsch et al. 2016). It is characterized by the progressive loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc). Degeneration of dopaminergic neurons in SNpc leads to a decreased levels of dopamine in the striatum, which results in a range of motor symptoms such as tremors, rigidity, and bradykinesia (Poewe et al. 2017). Currently there is no cure for PD. Current treatments for PD largely rely on palliative care using supplements of L-DOPA, which can cross the blood–brain barrier and serves as a precursor of dopamine. However, this treatment has common adverse effects including confusion, delusions, agitation, and hallucinations (Friedman and Sienkiewicz 1991). Preventing loss of dopaminergic neurons could represent a significant advancement in the treatment of PD, as approximately 60% of these neurons have already died upon the onset of clinical symptoms (Dauer and Przedborski 2003). Previous studies have shown that natural products such as DA-9805 and WIN-1001X from herbs can attenuate dopaminergic neuronal cell death in both in vitro and in vivo PD models (Jeong et al. 2018; Li et al. 2021). In the present study, we first report that compound **7**, a tetramic acid-motif natural product isolated from a marine fungus *T. cylindrosporum* FB06, can protect dopaminergic neurons from MPP^+ (a parkinsonian neurotoxin)-induced cell death. Mechanistically, we found that compound **7** elevated levels of anti-apoptotic protein, Bcl-2, in MPP^+-treated conditions.

Materials and methods

General experimental procedures

Optical rotation was measured at room temperature on a JASCO P-2000 polarimeter (JASCO, Easton, PA, USA) using a 1 mm cell. ECD spectra were obtained using a Chirascan-plus spectropolarimeter in a 2 mm cell. High-resolution electrospray ionization mass spectrometry (HRESIMS) data were acquired on a Waters QTOF Xevo G2-LC/MS mass spectrometer (Waters Corporation, Milford, MA, USA). One- and two- dimensional (1D and 2D) NMR spectra were obtained at 400 MHz for ^{1}H and 100 MHz for ^{13}C on a JEOL JNM-ECZ400s, 600 MHz for ^{1}H and 150 MHz for ^{13}C on a JEOL JNM-ECA-600, 800 MHz for ^{1}H and 200 MHz for ^{13}C on a Bruker with deuterated methanol (CD_3OD; Cambridge Isotope Laboratories, Inc., Tewksbury, MA, USA). MPLC was carried out on a Biotage® Selekt apparatus (Biotage® Corporation, Uppsala, Sweden) equipped with a UV – vis dual-wavelength detector at 210 and 254 nm and a SNAP Ultra C18 60 g, 120 g, Biotage® Sfär C18 120 g column. High pressure liquid chromatography (HPLC) was performed using a Waters 600 controller (Waters Corporation, Milford, MA, USA) equipped with a Waters 600E PUMP, a Waters 996 Photodiode Array Detector, a preparative column (Phenomenex, Luna C18(2), 5 µm, 250 mm × 21.2 mm), and a semi-preparative column (Phenomenex, Luna C_{18}(2), 5 µm, 250 mm × 10 mm). A conformational search was carried out using MacroModel (version 9.9, Schrödinger LLC) interfaced with Maestro (version 9.9, Schrödinger LLC). MPP^+ iodide was purchased from Sigma-Aldrich (cat # D048, USA).

Fungal materials

Fungal strain FB06 was isolated from marine sediment collected from the Beaufort Sea in North Alaska. This fungal strain was identified as a species of *Tolypocladium cylindrosporum* using internal transcribed spacer(ITS) sequence (GenBank accession number MH861264.1). It was stored in 60% glycerol at – 80 °C.

Fermentation, extraction, and isolation

The strain was cultured on solid potato dextrose agar (PDA) medium for 21 days at room temperature. Culture media were cut into small pieces and extracted with ethyl acetate three times. The solvent was then evaporated under pressure at 32 °C. This process was repeated twice to yield two extracts, 1 and 2 (2.2 g and 5.2 g, respectively). Extract 1 (2.2 g) was fractionated by medium pressure column chromatography (MPLC) over normal phase silica with a stepwise gradient of *n*-hexane–acetone (9:1, 4:1, 2:1, 3:2, 1:1, 0:1) and acetone-MeOH (9:1, 1:1, 0:1) to obtain twenty fractions (fraction 1–20). Extract 2 (5.2 g) was fractionated by silica-gel vacuum column chromatography (VLC) to produce six fractions (A–F). Fraction D (2.86 g) from extract 2 was subjected to medium pressure liquid chromatography (MPLC) over C18 resin with MeOH-distilled water (1:9, 3:7, 1:1, 7:3, 9:1, 0:1) to obtain six subfractions (subfraction D1–D6). Subfraction D4 (138.2 mg) was further separated by reversed-phase HPLC (Phenomenex, Luna C18(2), 5 µm, 250 mm × 21.2 mm, isocratic elution 60% MeOH, 5.0 mL/min) to obtain **1** (7.9 mg, t_R = 31.0 min), **3** (12.5 mg, t_R = 34.0 min) and **6** (17.1 mg, t_R = 24.5 min). Subfraction D4-C (6.6 mg) was purified by a reversed-phase column (Phenomenex, Luna C_{18}(2), 5 µm, 250 mm × 10 mm, isocratic elution 37% formic acid 0.1% in CH_3CN, 2.0 mL/min) to yield compound **2** (2.0 mg, t_R = 18.5 min). Fraction 12 (108.7 mg) was separated by reversed-phase preparative column (Phenomenex, Luna C18(2), 5 µm, 250 mm × 21.2 mm, isocratic elution 80% MeOH, 5.0 mL/min) to obtain fraction 12C (21.6 mg). Compound **4** (6.0 mg) was isolated by reversed-phase HPLC (Phenomenex, Phenyl-Hexyl, 5 µm, 250 mm × 4.6 mm, isocratic elution 40% CH_3CN, 2.0 mL/min) from fraction 12C. Fraction D5 (452.5 mg) was subjected to MPLC over normal phase Silica with a stepwise gradient of $CHCl_3$-acetone (1:0, 9:1, 3:1, 1:1, 1:5, 0:1) and acetone-MeOH (9:1, 1:1, 3:7, 0:1) to attain eleven subfractions (from D5-A to D5-K). Fraction D5-D (30.1 mg) was purified by semi-preparative HPLC (Phenomenex, Luna C_{18}(2), 5 µm, 250 mm × 10 mm, isocratic elution 65% CH_3CN, 2.0 mL/min) to obtain compound **5** (1.0 mg). Fraction 13 (272.8 mg) was subjected to MPLC over reversed-phase C18 with CH_3CN-distilled water (1:1, 3:2, 7:3, 4:1, 9:1, 1:0) to obtain thirteen sub-fractions (13A-13M). Fraction 13E (35 mg) was purified by reverse phase HPLC (Phenomenex, Phenyl-Hexyl, 5 µm, 250 mm × 4.6 mm, isocratic elution 50% CH_3CN, 2.0 mL/min) to isolate compound **7** (13.3 mg).

Tolypyridone I (**1**): white solid, $[\alpha]_D$ = – 82.9 (*c* 0.10, MeOH); ECD (c 3.69×10^{-4} M, MeOH) λ_{max} ($\Delta\varepsilon$) 196 (1.17), 209 (– 2.93), 229 (– 0.01), 244 (0.61), 263 (– 1.05) nm; (+)HRESIMS *m*/*z* 340.1906 $[M+H]^+$ (calcd. for $C_{21}H_{25}NO_3$, 339.1834); ^{1}H and ^{13}C-NMR data (in CD_3OD), see Table 1; ^{1}H-^{1}H COSY, H-2′, H-6′ ↔ H-3′, H-5′; H-7 ↔ H-8, H-12; H-8 ↔ H_3-16; H-9a ↔ H-10, H-8; H-10 ↔ H_3-15; H-11a ↔ H-12, H-10; H-13 ↔ H-12, H_3-14; HMBC correlations (CD_3OD, H# → C#) H-2′ → C-1′, C-3′, C-4′, C-5′, C-6′ and C-5; H-3′ → C-1′, C-2′, C-4′, C-5′ and C-6′; H-5′ → C-1′, C-2′, C-3′, C-4′ and C-6′; H-6′ → C-1′, C-2′, C-3′, C-4′, C-5′ and C-5; H-6 → C-1′, C-2, C-4 and C-5; H_3-14 → C-12, C-13; H_3-15 → C-9, C-10 and C-11; H_3-16 → C-7, C-8 and C-9;

Table 1 ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR spectral data for compounds **1** and **2** in CD_3OD

No	1		2 (rotamer A)		2 (rotamer B)	
	δ_C	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)
2	165.5, qC	–	166.1, qC	–	167.9, qC	–
3	112.4, qC	–	114.8, qC	–	115.1, qC	–
4	162.6, qC	–	165.2, qC	–	164.5, qC	–
5	117.4, qC	–	127.1, qC	–	125.6, qC	–
6	131.9, CH	7.12, s	132.2, CH,	7.13, s	132.6, CH,	7.12, s
7	38.7, CH	2.59, dd (11, 3.7)	46.1, CH	2.47, t (10.9)	46.0, CH	2.72, t (11)
8	28.1, CH	1.66, m	33.1, CH	2.64, m	34.0, CH	2.52, m
9a	45.7, CH_2	1.73, m	46.2, CH_2	1.76, m	46.2, CH_2	1.74, m
9b		0.90, m		0.71, m		0.75, m
10	37.8, CH	1.68, m	33.2, CH	1.60, m	33.2, CH	1.53, m
11	37.1, CH_2	1.88, d (11) 1.35, m	33.9, CH_2	1.79, m 0.94, m	33.8, CH_2	1.75, m 1.00, m
12	40.2, CH	1.70, m	44.3, CH	2.15, tt (11.5, 2.8)	46.3, CH	1.91, tt (11.8, 2.5)
13	74.7, CH	4.67, dt (11, 5.8)	69.8, CH	3.67, dq (6.3, 2.2)	69.6, CH	3.65, dq (6.5, 2.1)
14	20.2, CH_3	1.34, d (6.0)	20.8, CH_3	1.12, d (6.3)	20.1, CH_3	1.06, d (6.5)
15	23.3, CH_3	0.95, d (6.4)	23.6, CH_3	0.96, d (6.5)	23.6, CH_3	0.98, d (6.6)
16	21.1, CH_3	0.91, d (6.4)	21.5, CH_3	0.73, d (6.5)	21.4, CH_3	0.77, d (6.5)
1′	126.9, qC	–	117.9, qC	–	118.5, qC	–
2′, 6′	131.5, CH	7.22, d (8.6)	131.7, CH	7.24, d (8.5)	132.1, CH	7.18, d (8.4)
3′, 5′	116.0, CH	6.78, d (8.6)	116.2, CH	6.81, d (8.5)	117.0, CH	6.87, d (8.4)
4′	158.1, qC	–	159.1, qC	–	159.1, qC	–

^{1}H-^{1}H NOESY, H-7 ↔ H-9a, H-12, H_3-14, H_3-15, H_3-16; H-9a ↔ H_3-14,H_3-15 and H_3-16; H-11a ↔ H-9a, H_3-14, H_3-15, H_3-16; H-11b ↔ H-9b; H-12 ↔ H-7, H_3-14, H_3-15 and H_3-16; H-13 ↔ H-8, H-10, H-11b.

Tolypyridone J (**2**): white solid, ECD (c 1.11×10^{-3} M, MeOH) λ_{max} ($\Delta\varepsilon$) 200 (0.94), 228 (0.38), 240 (0.54), 249 (0.53), 259(0.47), 293 (0.83) nm; (+)HRESIMS *m/z* 358.2018 $[M+H]^+$ (calcd. for $C_{21}H_{27}NO_4$, 357.1945); ^{1}H and ^{13}C-NMR data (in CD_3OD), see Table 1; These NMR spectra indicated that FB06-D4-C4 existed as a mixture of two rotamers. Their ratio was estimated to be approximately 5:4 in CD_3OD by ^{1}H-NMR spectral analysis. For clarity, the NMR spectral analysis was explained with isomer A. ^{1}H-^{1}H COSY, H-2′, H-6′ ↔ H-3′,H-5′; H-7 ↔ H-8, H-12; H-8 ↔ H-9a, H_3-16; H-9a ↔ H-9b, H-10; H-10 ↔ H-11a, H_3-15; H-11a ↔ H-11b, H-12; H-13 ↔ H_3-14; HMBC correlations (CD_3OD, H-# → C-#) H-2′ → C-1′, C-3′, C-4′, C-5′, C-6′ and C-5; H-3′ → C-1′, C-2′, C-4′, C-5′ and C-6′; H-5′ → C-1′, C-2′, C-3′, C-4′ and C-6′; H-6′ → C-1′, C-2′, C-3′, C-4′, C-5′ and C-5; H-6 → C-2, C-3, C-4, C-5 and C-1′; H-7 → C-2, C-3 and C-4; H_3-14 → C-12, C-13; H_3-15 → C-9, C-10, C-11; H_3-16 → C-7,C-8 and C-9. ^{1}H-^{1}H NOESY, H-7 ↔ H-13, H_3-15 and H_3-16; H-9A ↔ H_3-15 and H_3-16; H-11A ↔ H-7; H-12 ↔ H-8, H-10 and H_3-14; H_3-14 ↔ H-10 and H-12.

Conformational search and DP4 analysis

A conformational search was carried out using MacroModel (version 9.9, Schrödinger LLC) interfaced with Maestro (version 9.9, Schrödinger LLC). Using the Merck molecular force field (gas phase) with a 25 kJ/mol upper energy limit as the cutoff afforded 7 conformers for 7*R*, 8*S*, 10*R*, 12*R*, and 13*R* diastereomer, 6 conformers for 7*R*, 8*S*, 10*R*, 12*R*, and 13*S* diastereomer, 5 conformers for 7*S*, 8*S*, 10*R*, 12*S*, and 13*R* diastereomer, 4 conformers for 7*S*, 8*S*, 10*R*, 12*S*, and 13*S* diastereomer, 9 conformers for 7*S*, 8*S*, 10*R*, 12*R*, and 13*S* diastereomer, 9 conformers for 7*S*, 8*S*, 10*R*, 12*R*, and 13*R* diastereomer, 2 conformers for 7*R*, 8*S*, 10*R*, 12*S*, and 13*R* diastereomer and 13 conformers for 7*R,* 8*S,* 10*R,* 12*S* and 13*S* diastereomer. Shielding tensor values were determined by density functional theory (DFT) calculations facilitated by the TurbomoleX 4.3.2 program with the basis set def-SV(P) for all atoms and the functional B3-LYP. Calculated ^{1}H and ^{13}C chemical shift values were averaged using Boltzmann populations.

ECD computational calculation

A conformational search was carried out using MacroModel (version 9.9, Schrödinger LLC) interfaced with Maestro (version 9.9, Schrödinger LLC). Using the Merck molecular

force field (gas phase) with a 25 kJ/mol upper energy limit as the cutoff afforded. It offered 2 conformers for 7*R*, 8*S*, 10*R*, 12*S*, and 13*R* diastereomers and 2 conformers for 7*S*, 8*R*, 10*S*, 12*R*, and 13*S* diastereomers. The energy minimized structures of **1** was conducted using Avogadro 1.2.0 with the MMFF force field. Ground-state geometries were then optimized via TurbomoleX 3.4 by using DFT with def2-SV(P) basis set for all atoms at the DFT level (functional B3-LYP/gridsize m3). Calculated ECD data corresponding to the optimized structures were obtained with TDDFT with the def2-TZVPP basis set for all atoms at the DFT level (functional B3-LYP/gridsize m3). Calculated ECD spectra were simulated by overlapping each transition, where σ was the width of the band at 1/e height.

Cell culture

SH-SY5Y cells were generously provided by Dr. Dong-Gyu Jo (Sungkyunkwan University). Cells were cultured in Dulbecco's modified eagle medium (DMEM, Welgene, cat # LM 001-05, South Korea) supplemented with 10% FBS (Gibco, cat # 16000-044, USA) and 1% antibiotic–antimycotic solution (Welgene, cat # LS 203-01, South Korea) and maintained at 37 °C under 5% CO_2.

MTT assay

Cell viability was measured by MTT assay (Sigma-Aldrich, cat # M2128, USA) as described previously (Shin et al. 2019). Briefly, SH-SY5Y cells were seeded in 96-well plates at 2×10^4 cells/well density. Cells were treated with compounds for 24 h in DMEM with 1% FBS and 1% antibiotic–antimycotic solution, followed by incubation with 2 mM MPP^+ for 24 h. Culture media were replaced with serum-free culture media and 10 µL of MTT solution (5 mg/mL in H_2O) was incubated for 3.5 h at 37 °C. Dimethylsulfoxide was added to dissolve the purple formazan. A plate reader (Molecular Devices, cat # M5e, USA) was used to measure optical density at 570 nm.

Western blotting

SH-SY5Y cells were lysed with RIPA buffer (Thermo Fisher Scientific, cat # 89901, USA) containing protease inhibitor (Thermo Fisher Scientific, cat # 78429) and phosphatase inhibitor (Merck, cat # 04 906 837 001). Protein concentrations were quantified with a BCA protein assay kit (Thermo Fisher Scientific, cat # 23225, USA). Protein sample (10 µg each) were separated by 12% polyacrylamide SDS-PAGE and transferred onto PVDF membranes (Merck, cat # IPVH00010). After blocking with 5% skim milk at room temperature for 1 h, membranes were incubated with primary antibodies against Bcl-2 (Santa Cruz Biotechnology, cat # sc-509, 1:500) or β-actin (Cell signaling Technology, cat # 3700S, 1:10,000) at 4 °C overnight. Membranes were washed with TBST (0.05% Tween 20) and incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (1:1000 for Bcl-2, 1:40,000 for β-actin). Immobilon Western Chemiluminescent HRP Substrate (Merck, cat # WBKLS0050) was used to detect the band with a Biomolecular Imaging System (Cytiva, ImageQuant 800 F). Densitometry quantification was conducted using ImageJ software version 1.53 (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis

All statistical analyses were performed using GraphPad Prism, version 9.5.1 (GraphPad Software, Inc.). One-way ANOVA with Dunnett's or Tukey's post hoc test was used to demonstrate statistical differences. Values are presented as mean ± SEM. Significance was determined at a p value below of 0.05, while p values below or equal to 0.05, 0.01, 0.001, and 0.0001 were represented by *, **, *** and ****, respectively.

Results and discussion

Structure elucidation of the isolated compounds

Chemical investigation of *T. cylindrosporum* FB06 afforded isolation of two new compounds (**1** and **2**) and five known ones. Known compounds were identified as tolypyridone A (**3**) (Li et al. 2015), maximiscin (**4**) (Du et al. 2014), tolypyridone C (**5**) (Kassam et al. 2021), tolypoalbin (**6**) (Fukuda et al. 2015), and F-14329 (**7**) (Shang et al. 2015) by comparing their spectroscopic data with previously reported data (Fig. 1).

Compound **1** was isolated as a whitish amorphous solid. Its molecular formula was determined as $C_{21}H_{25}NO_3$ based on (+)HRESIMS accounting for 10 indices of hydrogen deficiency. Compound **1** had almost identical ^1H and ^{13}C resonances and physicochemical data to those reported for tolypyridone A (**3**), which was also isolated in this investigation (Table 1). Its ^1H-NMR data showed a typical A_2B_2 spin coupling system assignable to a *para*-substituted benzene ring at δ_H 6.78 (d, $J = 8.6$ Hz, H-3′,5′) and δ_H 7.22 (d, $J = 8.6$ Hz, H-2′,6′). Analysis of ^1H, ^{13}C-NMR, and HSQC data (Table 1) indicated the presence of three methyl groups (δ_H 1.34, δ_C 20.2; δ_H 0.95, δ_C 23.3; δ_H 0.91, δ_C 21.1), two methylene groups (δ_H 1.73 and 0.90, δ_C 45.7; δ_H 1.88 and 1.35, δ_C 37.1), four sp^3 methine groups (δ_H 2.59, δ_C 38.7; δ_H 1.66, δ_C 28.1; δ_H 1.68, δ_C 37.8; δ_H 1.70, δ_C 40.2), one oxygenated sp^3 methine group (δ_H 4.67, δ_C 74.7), three quaternary aromatic carbons, one of which was oxygenated (δ_C

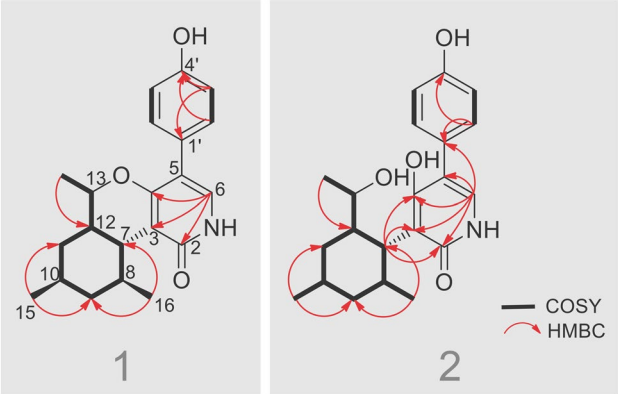

Fig. 2 Key ^{1}H-^{1}H COSY and HMBC correlations for compounds **1** and **2**

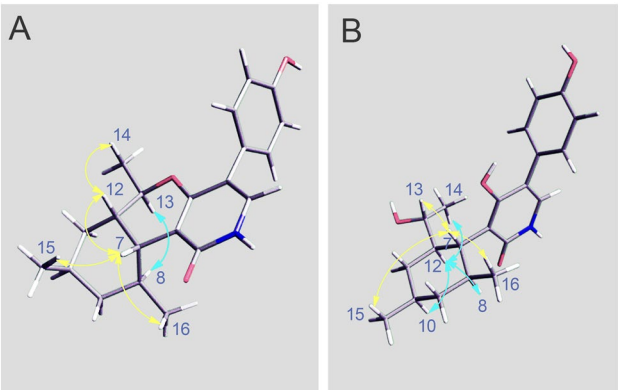

Fig. 3 Key ROESY correlations for compounds **A** and **B**

112.4; δ_C 117.4; δ_C 126.9; δ_C 162.6), one aromatic methine group (δ_H 7.12, δ_C 131.9), and one carboxyl amide carbon (δ_C 165.5). The planar structure of **1**, which was established by analysis of HMBC correlations in addition to ^{1}H and ^{13}C NMR, was identical to that of tolypyridone A (Fig. 2). This implies that compound **1** is a new stereoisomer of tolypyridone A, also backed up by the data below.

There were obvious differences between compound **1** and tolypyridone A (**3**) in the NMR data. The ^{1}H-NMR resonance for H-7 shifted a bit downfield; δ_H 2.19 in **3** and δ_H 2.59 in **1**. Furthermore, the splitting pattern including coupling constants for H-7 differed between compounds **1** and **3**. H-7 in **1** appeared doublet of doublet with $J = 11.0$, 3.7 Hz, while H-7 appeared triplet with $J = 11.0$ Hz in **3**, indicating that they were stereoisomers. The relative configuration of five stereogenic centers (C-7, C-8, C-10, C-12, and C-13) was analyzed by NOESY experiments and DP4 analysis. Strong NOESY correlations between H-13 and H-8 and H-10 arbitrarily assigned 8*S**, 10*R**, and 13*R** (Fig. 3). Further strong NOESY correlations between H-7 and H-12, H_3-14, H_3-15, and H_3-16 indicated that H-7 and H-12 were oriented on the opposite to H-8 and H-10, which was supported by no NOESY correlation between H-7 and H-13. However, the NOESY correlation between H-7 and H-10 was unclear because of overlapped ^{1}H resonances. To verify its stereo-structure, DP4 analysis and ECD calculation were employed. To determine the relative configuration of **1**, NMR chemical shift calculations of eight possible diastereomers (7*R*/8*S*/10*R*/12*R*/13*R*-**1a**, 7*R*/8*S*/10*R*/12*R*/13*S*-**1b**, 7*S*/8*S*/10*R*/12*S*/13R-**1c**, 7S/8*S*/10*R*/12*S*/13*S*-**1d**, 7*S*/8*S*/10*R*/12*R*/13*S*-**1e**, 7*S*/8*S*/10*R*/12*R*/13*R*-**1f**, 7*R*/8*S*/10*R*/12*S*/13*R*-**1g**, and 7*R*/8*S*/10*R*/12*S*/13*S*-**1h**) were performed at B3-LYP/def2-SV(P) level using guage independent atomic orbital (GIAO) method (Supplementary Table S1). Calculation results revealed that **1g** showed the highest DP4 + probability (100%, Fig. 4B) and a linear correlation coefficient ($R^2 = 0.9966$, Fig. 4C), suggesting the 7R^*, 8S^*,10R^*,12S^*,13R^* relative configuration of **1**. Then, the absolute configuration of **1** was confirmed by ECD calculations (Fig. 4D) (Nugroho and Morita 2014). The calculated Cotton effect of **1g** matched experimental ECD data well when comparing data for the enantiomer of **1g** (Fig. 4D). Thus, the absolute configuration of **1** was suggested as 7*R*, 8*S*, 10*R*, 12*S* and 13*R*. Therefore, compound **1** was confirmed to be C-12 epimer of tolypyridone A (**3**) and named tolypyridone I. Although tens of tolypyridone-type compounds where 4-hydroxy-2-pyridone is connected with 1-ethyl-3,5-dimethylcyclohexane have been reported so far (Li et al. 2015; Zhang et al. 2020), to the best of our knowledge, this is the only tolypyridone-type compound with 12*S* configuration.

Compound **2**, named tolypyridone J, was isolated as a whitish amorphous solid. Its molecular formula was established as $C_{21}H_{27}NO_4$ on the basis of its HRESIMS and NMR data, revealing nine degrees of unsaturation. Compound **2** also had similar ^{1}H and ^{13}C resonances and physicochemical data to those reported for tolypyridone A (**3**) (Table 1). However, exactly two sets of signals appeared in its NMR spectra, indicating that it was an inseparable mixture. The ratio of the mixture was approximately 5:4 based on the integrated data of its ^{1}H-NMR. Its 1D and 2D NMR data were analyzed to establish the structure of **2**. Considering that H-13 resonance moved upfield compared with tolypyridone A (**3**) in addition to the difference of eighteen mass unit, the dihydropyran ring in tolypyridone A (**3**) was proposed to open to generate **2**. Its C-13 bears both a methyl group and a hydroxyl group and its C-4 bears a hydroxyl group. Its relative stereochemistry was established by analyzing proton coupling constants and NOESY correlations. The splitting pattern and coupling constant for H-7 in **2** were quite different from those in **1**. H-7 in **2** appeared triplet with 11 Hz of coupling constant while H-7 in **1** was doublet of doublet with 11 and 3.7 Hz

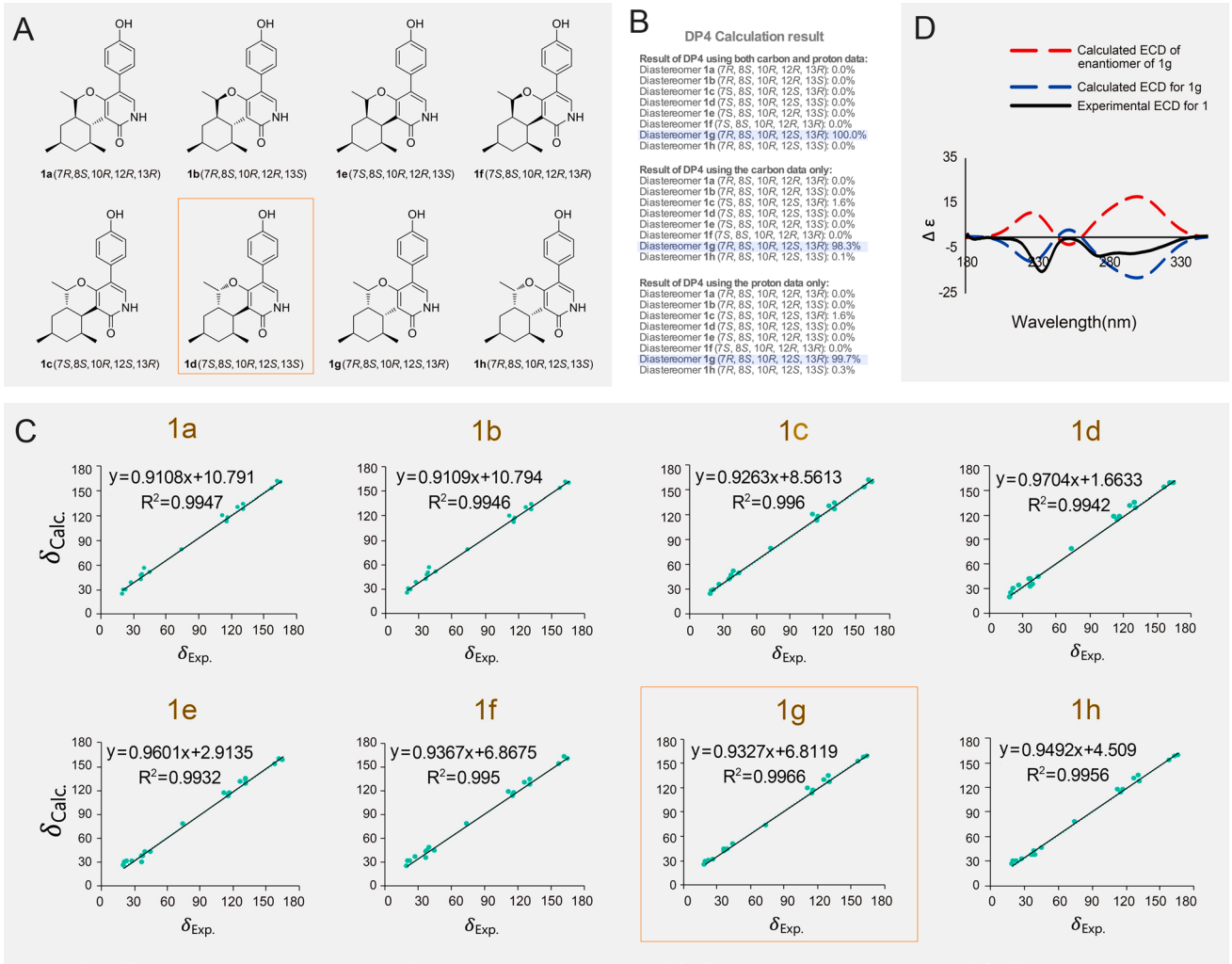

Fig. 4 The result of DP4 analysis for **1**. **A** The simulated models of eight possible diastereomers of **1**. **B** DP4 probability analysis. **C** Linear correlation plots of calculated vs. experimental ^{13}C NMR chemical shift values for **1a/1b/1c/1d/1e/1f/1g/1h** of **1**. **D** Experimental and calculated ECD spectra for compound **1**

of coupling constants, indicating different relative configurations. Analysis of NOESY spectrum also supported the relative configurations proposed by coupling constants (Fig. 3). Strong NOESY correlations between H-7 and H_3-15 and H_3-16 indicated that they were on the same face of the molecule as shown in compound **1**. Strong NOESY correlations between H-12 and H-8/H-10 indicated that H_3-15, H_3-16, and the 1-hydroxyethyl group at C-12 were on the same face of the molecule, which was also supported by coupling constants of H-7 similar to those of tolypyridone A (**3**). In addition, strong NOESY correlation between H-7 and H-13 suggested 13*S** configuration. As mentioned earlier, compound **2** showed two sets of signals in NMR like compound **4**. It was possibly due to rotational isomerism generated because of hindered rotation about a single bond between C-3 and C-7. Compounds **1** and **3** did not cause this rotational isomerism since the ether bond connecting the 4-hydroxy-2-pyridone and the 1-ethyl-3,5-dimethylcyclohexane probably prohibited rotation of the single bond between the 4-hydroxy-2-pyridone and the 1-ethyl-3,5-dimethylcyclohexane moiety. Pyridoxatin, a previously reported tetramic acid derivative, also has two sets of NMR signals, indicating a mixture of rotamers (Teshima et al. 1991). This phenomenon also occurred in a (*P/M*)-1, a mixtures of rotamers for a maximiscin isomer (Du et al. 2014). To investigate the absolute stereochemistry of (*P/M*)-1, its Cu(pyrodoxatin)$_2$ chelates were obtained for crystallization and separation, which established its absolute configuration through X-ray crystallographic analysis (Du et al. 2014). In our study, chelation of compound **2** could not be conducted due to a limited amount of sample.

Identification of neuroprotective compounds for treating Parkinson's disease

To identify neuroprotective compounds in an in vitro Parkinson's disease (PD) model, we used 1-methyl-4-phenylpyridinium (MPP^+) neurotoxin known to induce dopaminergic neuronal cell death (Chun et al. 2001). As shown in Fig. 5A, treatment with MPP^+ dose-dependently decreased cell viability of SH-SY5Y dopaminergic neurons. Next, we tested neuroprotective effects of three of the seven isolated compounds using an in vitro PD model because four isolated ones showed cellular toxicity (data not shown). Interestingly, pretreatment with 10 µmol/L of compound **7** for 24 h protected SH-SY5Y cells against MPP^+-induced cytotoxicity (Fig. 5B). To confirm the screening, we treated SH-SY5Y cells with compound **7** for 24 h, followed by treatment with 2 mmol/L of MPP^+ for 24 h. Consistent with Fig. 5B, compound **7** significantly increased cell viability compared to MPP^+-treated cells (Fig. 5C). Previous reports have suggested that MPP^+-mediated cell death is related to the regulation of Bcl-2 family proteins (O'Malley et al. 2003; Ahn et al. 2009). We thus examined levels of Bcl-2, an anti-apoptotic protein. Treatment with 2 mmol/L of MPP^+ for 24 h significantly reduced Bcl-2 levels compared to control treated with vehicle. However, pretreatment with compound **7** attenuated such effect (Fig. 5D, E).

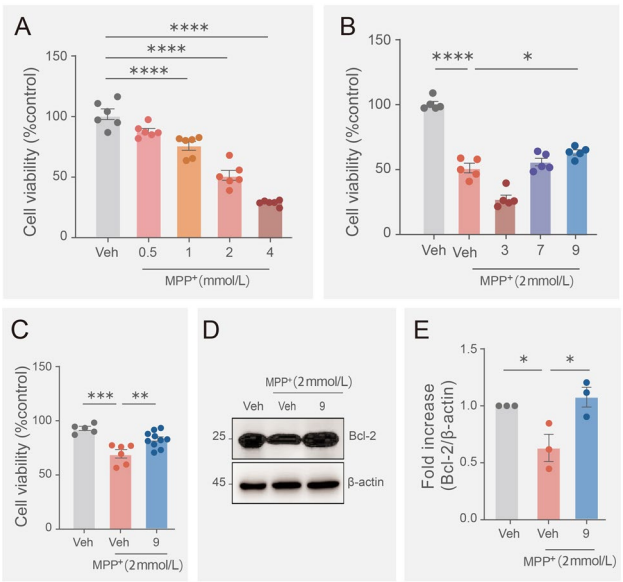

Fig. 5 Compound **7** protects dopaminergic neurons from MPP^+-induced cell death through regulation of anti-apoptotic protein, Bcl-2. **A** MPP^+ treatment for 24 h dose-dependently decreased cell viability (****$p<0.0001$, One-way ANOVA with Dunnett's post hoc test). **B** Identification of compound **7** as a neuroprotective molecule in an in vitro PD model (*$p<0.05$, ****$p<0.0001$, One-way ANOVA with Tukey's post hoc test). **C** Pretreatment with compound **7** protects cells from MPP^+-induced cytotoxicity (**$p<0.01$, ***$p<0.001$, One-way ANOVA with Tukey's post hoc test). **D**, **E** Western blot and its quantification show that treatment with compound **7** can rescue levels of anti-apoptotic protein, Bcl-2, in MPP.$^+$-treated conditions (*$p<0.05$, One-way ANOVA with Tukey's post hoc test)

Conclusions

In conclusion, seven tetramic acid alkaloids including two new ones, tolypyridone I (**1**) and tolypyridone J (**2**), were isolated from cultures of a marine fungus FB06 identified as *T. cylindrosporum*. By analyzing 1D and 2D NMR spectra with DP4 analysis and ECD calculations, **1** was determined to be a C-12 epimer of tolypyridone A (**3**). To the best of our knowledge, this is the only tolypyridone-type compound with 12*S* configuration. Compound **2** was generated by ring opening of the dihydropyran ring in tolypyridone A (**3**). Compound **2** was isolated an inseparable mixture, showing two sets of NMR signals with similar resonances. Based on 1D and 2D NMR spectra, **2** was established as rotational isomers, which were also found in compounds without dihydropyran ring between 4-hydroxy-2-pyridone and cyclohexane ring such as maximiscin and pyridoxatin. Among metabolites of FB06, **7** significantly protected SH-SY5Y dopaminergic neurons against MPP^+-induced cytotoxicity. Mechanistically, compound **7** prevented cell death by restoring the down-regulated anti-apoptotic Bcl-2 protein levels. Given that intracellular iron plays a role in MPP^+-induced cell death (Kalivendi et al. 2003), it is possible that the neuroprotective effect of compound **7** is due to its ability to chelate metal ions including iron (Shang et al. 2015). Anti-Parkinson capabilities of fungus *Tolypocladium cylindrosporum* have not been described yet, making this study important. This research suggests that marine-derived microorganisms could be a valuable resource to develop novel medications for treating Parkinson's disease.

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Author contributions YJ and CK conducted experiment implementation and prepared the original draft manuscript. JK conducted the computational work. JWL, M-KS, and SHS provided the experimental ideas, helped data analysis, and revised the manuscript. All authors have read and approved the final manuscript.

Data availability The data that support the findings of this study are included in the supplementary information file.

Declarations

Conflict of interest The authors they have no conflicts of interest relevant to this study to disclose.

Animal and human rights statement This article does not contain any studies with human participants or animals performed by the authors.

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