Isodomoic Acid C, an Unusual Amnesic Shellfish Poisoning Toxin from Pseudo-nitzschia australis †

ARTICLE *in* CHEMICAL RESEARCH IN TOXICOLOGY · JUNE 2005

Impact Factor: 3.53 · DOI: 10.1021/tx0496845 · Source: PubMed

Isodomoic Acid C, an Unusual Amnesic Shellfish Poisoning Toxin from *Pseudo-nitzschia australis*†

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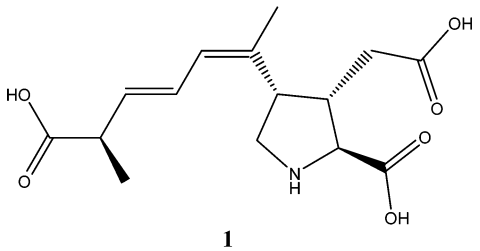
Received November 15, 2004

An unusual isomer of domoic acid (**1**), isodomoic acid C (**2**), has been found in New Zealand shellfish contaminated by amnesic shellfish poisoning (ASP) toxins and was shown to be produced by a local strain of the pennate diatom *Pseudo-nitzschia australis*. A bulk culture of this strain was used to isolate **2**. The structure was determined from spectroscopic data and was shown to correspond to that of **2** from a Japanese red seaweed, the only other reported occurrence of this compound. The affinity of **2** for GluR6 glutamate receptors was 240-fold lower than for **1**, indicating low neurotoxic potential.

Introduction

Amnesic shellfish poisoning (ASP) toxins are neurotoxic nonprotein amino acids that occur naturally as a number of geometrical isomers of domoic acid (**1**) with variations in the stereochemistry and positions of the two olefinic bonds in the branched-C_8 side chain. Domoic acid is the predominant form accumulated in toxic shellfish following ingestion of toxigenic species of the pennate diatom *Pseudo-nitzschia* (*1*−*3*). Domoic acid (**1**), isodomoic acid-C (**2**), and five other isomers were first isolated from the Japanese red seaweed *Chondria armata* (*4*, *5*). Several of these isomeric forms and epidomoic acid, the 5′-diastereoisomer, have also been reported as minor components from shellfish contaminated with **1** (*6*−*8*). **1** and isodomoic acids D, E, and F have conjugated olefinic side chains, while isodomoic acids A, B, and C have unconjugated olefinic bonds. **1** is an extremely potent agonist of kainate type glutamate receptors (*9*), thus acting as an analogue of this excitatory neurotransmitter. This mechanism initiates an NMDA driven (*10*) neurotoxicity and a wide range of adverse effects have been reported for human and marine wildlife from exposures to **1** (*3*, *11*). The isomers have shown neurotoxic potential, although lower than that of **1** (*4*, *11*).

During routine monitoring of shellfish for ASP toxins during 2001−2002 using LC/MS determination, a range of contaminated samples was found to contain significant amounts of a component eluting after **1** with the mass spectral characteristics of an isomer of **1**. In many cases the concentration of this isomer in tissues exceeded 10 mg/kg and was of similar or greater magnitude to that of **1**, which is an unprecedented finding for ASP toxin-contaminated shellfish (*12*). The source of this unusual isomer was shown to be a strain of *Pseudo-nitzschia australis* (*13*). In this report we describe the isolation and structural elucidation of the isomer as isodomoic acid C (**2**) and provide data on glutamate receptor binding.



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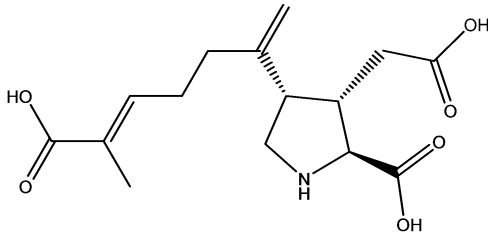

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Materials and Methods

Domoic acid certified reference standard and a mussel certified reference material contaminated with ASP toxins were obtained from the Institute for Marine Biosciences, NRC, (Halifax, Canada) and used to obtain reference spectroscopic data and for calibration of LC-MS analyses. All solvents were LC grade. ^{3}H-kainic acid was obtained from Amersham Biosciences (Buckinghamshire, UK).

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10.1021/tx0496845 CCC: $30.25
Published on Web 04/08/2005

Table 1. ^{1}H (400 MHz) NMR Data of 1 and 2 (D_2O)

	domoic acid **1** δ H (*J*)[a]	isodomoic acid C[b] **2** δ H (*J*)[a]
H-2α	4.16 d (8.1)	4.13 br s
H-3*β*	3.23 dddd (9.0, 8.1, 7.7, 5.8)	3.13 m (overlapped)
H-4*β*	4.01 ddd (7.9, 7.7, 7.4)	3.10 m (overlapped)
H-5α	3.67 dd (12.2, 7.4)	3.51 t (11.7)
H-5*β*	3.89 dd (12.2, 7.9)	3.69 dd (11.7, 7.0)
H-6A	2.68 dd (16.8, 9.0)	2.22 dd (15.5, 8.1)
H-6B	2.94 dd (16.8, 5.8)	2.36 dd (15.5, 6.3)
H-2′(2″)	6.31 d (11.0)	2.3 (2H, m)
H-3′(3″)	6.53 dd (14.9, 11.0)	2.4 (2H, m)
H-4′	5.96 dd (14.9, 7.9)	6.42 br t (7.1)
H-5′	3.47 dq (7.9, 7.0)	
H-6′	1.47 d (7.0)	1.86 br s
H-8′	1.98 s	5.16 s, 4.91 s

[a] Coupling constants (Hz) are given in parentheses. [b] Chemical shifts are reported relative to $(CH_3)_2OD$ at 1.22 ppm.

Culturing of Toxic *Pseudo-nitzschia australis*. Seawater samples were collected from sites in the Malborough Sounds at a depth of 10 m using a van Dorn sampler. Live cells of *Pseudo-nitzschia* species were isolated by micro-pipet into *f*/2 medium and maintained under a 100 $\mu E \cdot m^{-2} \cdot s^{-1}$ photon flux density (14:10 h light:dark) at 19 °C. Whole cell (in situ) hybridization with species-specific large-subunit rRNA-targeted oligonucleotide probes tagged with FITC was used to identify cultured *Pseudo-nitzschia* isolates (*13*, *14*). Clonal isolates were cultured for 5 days under silicate limitation (*f*/2 medium minus $Na_2SiO_3 \cdot 5H_2O$) and then sub-cultured (40% inoculum) into the same medium for 9 days in order to enhance production of domoic acid by applying nutritional stress. Cultures were analyzed for domoic acid and isomers by LC/MS and a strain (CAW−B52; isolated 10 Feb 2003) that produced both **1** and **2** was selected. A mass culture was grown in four 10 L barrels each with an initial inoculum of 100 mL. Stepwise additions of the silicate-limited *f*/2 medium were made, doubling the culture every few days until day 20 of growth (final cell count 5.3×10^6 cell L^{-1}).

Isolation of Domoic Acid and Isomers. The bulk culture (34 L) was acidified to pH 2.5 with HCl and passed through 10 parallel 500 mg Strata-X polymeric SPE columns (Phenomenex, CA) in 200 mL aliquots. After each aliquot the columns were rinsed with water (5 mL) and eluted with 5 mL acetonitrile/water (1+4 v/v). The combined eluants were concentrated to ca. 50 mL by rotary evaporation (35 °C) and applied to a 500 mg SPE anion exchange cartridge (quaternary amine, formate form, Phenomenex, CA). Following a water/acetonitrile (9:1 v/v) rinse, the column was eluted with 1% formic acid through a 500 mg SPE cation exchange cartridge (benzenesulfonic acid, protonated form, Phenomenex, CA). The cation exchange cartridge was eluted with 20 mL of 1% aq. ammonia. This fraction was acidified (pH 2.5) with formic acid, applied to a Strata-X SPE column, rinsed with water (5 mL), and eluted with 5 mL methanol/water (1+4 v/v). **2** was isolated from the concentrated extract and separated from **1** and other minor isomers using preparative LC on a 250 × 4.6 mm 5 μm C18 column (Phenomenex, CA) with a water/methanol/formic acid eluent system (85+15+0.1 v/v). Chelex resin (50 mg minicolumn, H^+ form, methanol rinsed) was used for final desalting with application and elution in water. Based on LC/MS and LC/UV analyses, 80 μg of isodomoic acid C (**2**) was obtained (ca 50% yield, >95% purity): λ_{max} (log ϵ) at pH 3 220 nm (4.08); HRES-MS (ESI-TOF) *m/z* 312.1441 $[M+H]^+$ giving the molecular formula $C_{15}H_{21}NO_6$ (calculated for $[M+H]^+$ 312.1447).

NMR Spectroscopy. NMR spectra were obtained from solutions of **1** (ca 0.5 mg) and **2** (80 μg) in D_2O (99.8+ at. % D; Aldrich, USA) with a Bruker 400 MHz spectrometer. NMR assignments (Table 1) were obtained from examination of ^{1}H and NOESY NMR spectral data. Chemical shifts, determined at 30 °C, are reported relative to 2-propanol where $\delta(CH_3)_2$-CHOD = 1.22 ppm..

LC/MS Analysis of Domoic Acid and Isomers. Quantitative analyses by selected ion recording (SIR; $[MH]^+$ *m/z* 312) or MS/MS (*m/z* 312 > 266) and collisional activation experiments were conducted with a Quattro Ultima instrument (Waters-Micromass, Manchester) using electrospray ionization (source 100 °C, N_2 desolvation gas 400 L/min, capillary 3 kV and cone 50 V). The isocratic LC separation used a 150 × 2 mm Luna 5 μm C18 column (Phenomenex, CA) operated at 0.2 $mL \cdot min^{-1}$ with 30% v/v methanol/water containing 3.6 mM ammonium formate and 46 mM formic acid (*15*). The SIR response factors for **2** and other isomers of **1** were assumed the same as for **1** (from linear calibrations 5−200 ng/mL).

Binding by Glutamate Receptors. Glutamate receptors were produced from cultures of SF9 insect cells transfected with the GluR6 receptor in a baculovirus expression system (*16*). Aliquots of crude cell membrane preparation from lysed cells containing the receptors were incubated with ^{3}H-kainic acid (5 nM in the well) and different concentrations of **1** or **2**. Following filtration to remove unbound ligands, the bound radiolabel was measured by adding liquid scintillant and counting in a Micro-Beta TriLux microplate counter (Wallac, Finland). Curve fitting for the competitive binding data (B/B_o) was carried out using a 4-parameter logistic fit (Prism 4; GraphPad Software, Inc., CA).

Results

Several isolates of *Pseudo-nitzschia* species collected from New Zealand coastal waters were found to produce **1** and **2** and were identified by gene-probes as *P. australis* (*13*). An isolate producing **1** and **2** at ca. 0.9 $pg \cdot cell^{-1}$ each was taken into large scale culture. Approximately 80 μg of **2** was isolated from this culture in high purity (LC/MS; ^{1}H NMR) and gave the molecular formula $C_{15}H_{21}O_6$, consistent with an isomer of **1**. The reversed phase chromatographic retention (C18) for **2** was longer than for **1** and for the minor isomers epidomoic acid and isodomoic acids A, D, and E present in contaminated shellfish certified reference material (*8*). The 220 nm UV absorption maximum for **2** is consistent with a lack of conjugated olefinic bonds (cf. 242 nm for **1**). The collisional activation mass spectra for these two compounds ($[M+H]^+$, collision energy 20 eV) exhibit all the same major peaks, corresponding to successive losses of the elements of formic acid and water, but with different relative intensities: *m/z* (rel. int. **1**/**2**) 312 (19/7%), 266 (100/51%), 248 (32/100%), 220 (21/44%), 161 (34/7%). The base peak for **1** at *m/z* 266 corresponds to loss of HCO_2H, whereas *m/z* 248 (loss of HCO_2H and H_2O) predominated for **2**.

The ^{1}H NMR spectral data reported in Table 1 for **2** (assigned relative to 2-propanol; $(CH_3)_2CHOD$ at 1.22 ppm) are in accord with data reported previously for isodomoic acid C (*4*). The data for **1** is comparable to that previously reported by Garthwaite et al. (*17*). We did not determine the pH(D) of the NMR solutions and therefore some minor pH(D) dependent variations between the NMR data presented in Table 1 and reported elsewhere for **1** (*4*, *6*, *7*) and **2** (*4*) are to be expected. In particular, there is a significantly lower shift for the 4′ proton of **2** from that reported by Maeda et al. (*4*) which we attribute to C7′ carboxylate arising from incomplete desalting and higher pH with this very small sample. The stereochemistry of **2** around the ring is as for **1**, except the larger H-4 coupling constant, which is consistent with a difference in ring conformation (*4*). The NOE data (Figure 1) defined the locations of the side chain double bonds as 1′(8′) and 4′(5′), with a cis-relationship (*Z*-) between the H-3′(3″) methylene and the H-6′ methyl group protons.

The *Pseudo-nitzschia australis* variant that produces significant amounts of **2** along with **1** has widespread

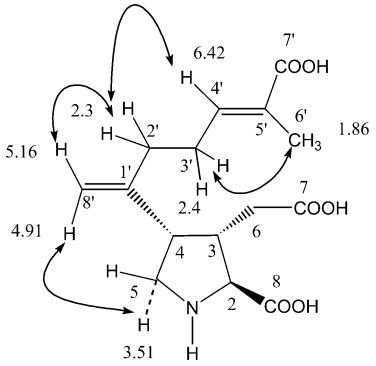

Figure 1. Selected NOESY correlations observed for **2**.

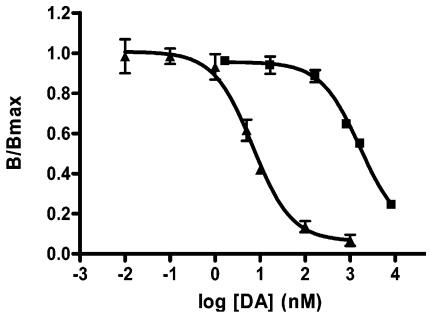

Figure 2. Competition curves for ^{3}H-kainic acid binding to GluR6 receptors with domoic acid (DA, **1**; ▲) and isodomoic acid C **2** (■). Data shown are mean ± SE of three individual analyses.

occurrence in New Zealand coastal waters and leads to toxin accumulation in shellfish (*12*). Hence there is a need for information on the potential risks to human health of **2** from consumption of contaminated shellfish. Due to the limited quantities of **2** available, initial studies were made with receptor binding using cloned GluR6 membranes and ^{3}H-kainic acid. The competitive binding curves for **1** and **2** are given in Figure 2.

The competition curves show that **2** has a considerably lower affinity than **1** for glutamate receptors with a K_i value of 1176 ± 70 nM for **2** versus 4.9 ± 0.29 nM for **1**. This 240-fold lower affinity of **2** to GluR6 was confirmed by measuring dilutions as unknowns against a calibration curve established with **1**, which provided concentrations for **2** ca. 250-fold lower than the gravimetric values in the range 83 to 8333 nM.

Discussion

Although only microgram quantities were produced in the bulk culture, the spectroscopic data on the isolated material enabled a positive identification of the isomer as isodomoic acid C (**2**). This is the first reported finding of significant production of an isomer of domoic acid by a *Pseudo-nitzschia* species and the only finding of isodomoic acid C, other than that reported for the red seaweed *Chondria armata* (*4*). Hampson et al. (*9*) have reported binding data for **1**, isodomoic acid D, E and F, and several synthetic derivatives to both kainate/glutamate and AMPA receptors from frog brain synaptosomes. **1** gave the highest binding affinities (glutamate receptor IC_{50} 4.9 nM) and very low affinities were reported for isomers that lacked a 1′-2′ double bond with the Z configuration. Our glutamate receptor affinity data for **2** supports this structure−activity relationship and, based on these data, it is likely that **2** is considerably less neurotoxic than **1**. Sub-cutaneous injections into cockroaches also indicated that **2** was less toxic than **1** by a factor of 20 (*4*). Further neurotoxicological studies are proceeding to expand on the preliminary receptor binding data reported here that indicates low neurotoxic potential for **2**.

Acknowledgment. Dr Christopher Miles provided useful advice. Janet Adamson gave technical support. The authors are grateful for the financial support of the New Zealand shellfish industry in their research on marine biotoxins. The research was supported in part by the N.Z. Foundation for Research, Science and Technology (Contract No. CAWX0005) and by NOAA/NOS operational funds to G.J.D.

Supporting Information Available: 400 MHz ^{1}H, COSY, and NOESY NMR spectra of isodomoic acid C. This material is available free of charge via the Internet at http://pubs.acs.org.

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TX0496845