Identification of isodomoic acid D and two new geometrical isomers of domoic acid in toxic mussels[1]

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Isodomoic acids E and F, two new geometrical isomers of the neurotoxin domoic acid, have been found to occur with domoic acid and isodomoic acid D in extracts of toxic mussels. The entire set of geometrical isomers can be prepared in the laboratory by photolysis.

Key words: amnesic shellfish toxin, domoic acid, isodomoic acid, neurotoxin.

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Dans les extraits de moules toxiques, on a trouvé que les acides neurotoxiques, domoïque et isodomoïque D, sont accompagnés des acides isodomoïques E et F, deux nouveaux isomères géométriques de l'acide domoïque. Faisant appel à la photolyse, il est possible de préparer au laboratoire l'ensemble des isomères géométriques.

Mots clés : toxine amnésique du testacé, acide domoïque, acide isodomoïque, neurotoxine.

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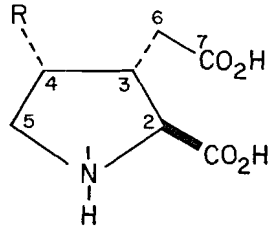
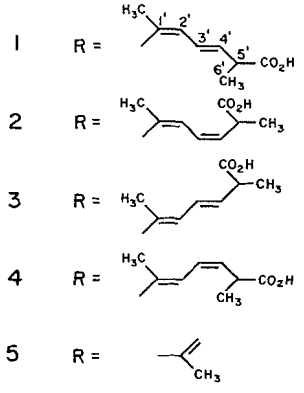
Introduction

We recently isolated and identified the neurotoxin domoic acid **1** as the toxic agent in an outbreak of shellfish poisoning traced to cultured mussels *Mytilus edulis* L. from a localized area of eastern Prince Edward Island, Canada (1). This compound was originally isolated from the red macroalga *Chondria armata* Okamura some 30 years ago (2, 3). Based on its toxic properties and its discovery in shellfish, it has been proposed[3] that **1** be termed an amnesic shellfish poison (ASP). As **1** is the most potent known activator of the kainate receptors in the central nervous system[4] it is of interest from a chemical and neurophysiological standpoint that domoic acid isomers or derivatives be characterized as fully as possible. We report here the isolation of isodomoic acid D **2** (4) and two new geometrical isomers, isodomoic acids E **3** and F **4**, obtained in small quantities during further studies of these toxic mussels.[5]





Results and Discussion

In the original identification of **1** in toxic mussels (1) the chemical and physical properties were identical with published data (5) and the optical rotation of an anhydrous sample of **1** (α_D^{24} −120°) showed that the enantiomeric form was the same as that previously reported from *Chondria armata*. Our NMR experiments (1) confirmed the ^1H assignments and spin–spin coupling constants of ref. 5 and led to minor reassignment of ^{13}C resonances compared to those of ref. 4. Additional homonuclear NOE experiments confirmed the (*Z*, *E*) configuration of double bonds in the side chain and the conformation of the prolyl ring (1).

During subsequent large-scale extraction and purification of domoic acid, additional peaks displaying essentially the same ultraviolet spectra as the toxin were observed. These compounds were obtained pure by repeated reversed-phase chromatography.

The UV spectrum (λ_{max} 244 nm) of the major isomer **2** (MH^+ 312.1448, $C_{15}H_{22}NO_6$, α_D^{25} −72°) indicated the presence of conjugated double bonds, although the IR spectrum showed no absorption in the region 955–980 cm^{-1} characteristic of a *trans*-disubstituted double bond. The NMR data (Tables 1 and 2) show this compound to be isodomoic acid D **2**, previously isolated as a minor component of extracts of *C. armata* (4). Values of δ(H) and *J*(H, H) (Tables 1 and 2) from our spectra are close to the published values, recorded at unspecified pH (4). They indicate that the conformation of the prolyl ring is virtually identical with **1**. Our NOE measurements, showing the proximity of H-2′ and 1′-CH_3, H-2′ and H-5′, H-3′ and H-4′, H-4′ and H_3-6′, confirm the (*Z*, *Z*) configuration of the two conjugated double bonds.

The (*E*, *E*) isomer structure was consistent with the UV absorption (241 nm) and IR spectrum (960 cm^{-1}) of isodomoic acid E **3** (MH^+ 312.1430, $C_{15}H_{22}NO_6$, α_D^{25} −19.5°), which eluted after **2**, and was confirmed by the NOE data showing the proximity of H-3′ to H-5′, H_3-6′ and 1′-CH_3, and of H-4′ to H-5′, H_3-6′ and H-2′. The values of δ(H) and *J*(H, H) (Tables 1 and 2) show similarity of the prolyl ring protons to **1** and **2**, except that a reduction in *J*(2, 3) indicates that the average conformation of the ring reduces the (H-2)—C—C—(H-3) angle, from ca. 143° to ca. 120°, if Karplus-type parameters for proline (6) are applied to this case. Closer proximity of these protons seems to be indicated by NOE between H-2 and H-3, which is not observed in **1** and **2**. Similarly, *J*(4, 5α) shows an increase in the relative angle of the 4-H and 5-αH bonds, also reflected in the occurrence of NOE between H-4 and H-5β.

The last compound to elute (MH^+ 312.1444, $C_{15}H_{22}NO_6$,

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[3]T. M. Perl, L. Bedard, T. Kosatsky, R. S. Remis, E. C. D. Todd, and J. C. Hockin. Submitted for publication.

[4]G. Dobennel, L. Beauchesne, and C. De Montigny. Submitted for publication.

[5]Aspects of this work were reported at a Symposium on Domoic Acid Toxicity in Ottawa, April 10–11, 1989.

TABLE 1. ^{1}H and ^{13}C chemical shifts[a] and nuclear Overhauser enhancements[b] for domoic acid and isodomoic acids from toxic mussels

			Position								
	Isomer	pH	2	3	4	5α	5β	6a	6b	7	2-CO_2H
δ(H) (ppm) and multiplicity	**1**	3.4	3.98d	3.05dddd	3.84ddd	3.49dd	3.71dd	2.76dd	2.50dd		
	2	1.9	4.15d	3.10dddd	3.87ddd	3.52dd	3.72dd	2.75dd	2.53dd		
	3	1.8	4.36d	3.26dddd	3.23ddd	3.58dd	3.72dd	2.57dd	2.53dd		
	4	1.6	4.39d	3.27dddd	3.32ddd	3.63dd	3.77dd	2.63dd	2.53dd		
NOE (%) when H at head of column is irradiated	**1**	3.4	None	H4 4.7	H3 6.4 H3′ 14.9 H5β[e]	H5β 12.5 1′Me 1.0	H5α 11.2 H4[e]	H6b 5.8	H6a 9.0		
	2	1.9	None	H4 2.5	H3 2.3 H3′ 6.3	H5β 4.7 1′Me 0.4	H5α 1.6[c]	H6b 4.4	H6a 1.8		
	3	1.8	H3 1.1 H6a 1.2 H6b 0.9	H2 2.9 H4[c,e] H6a 3.2 H6b 1.5 H2′ 3.6 1′Me 1.9	H3[c,e] H5β 4.3	H5β 8.1 H6a 1.0 H6b 1.0 H2′ 7.4	H5α 5.0	H6b[d,f] H2 2.9 H2′ 0.8 H3 3.0 1′Me 0.3	H6a[d,f]		
	4	1.6	H6a 1.4 H6b 0.8 1′Me 0.4	H2 2.2[c] H6a 4.7 H6b 4.1	H5β 3.5[c] H2′ 3.9 1′Me 1.8	H5β 3.0 H2′ 5.9	H4 2.8[d]	H2 1.1 H3 2.3	H2 1.1 H3 2.9 1′Me 0.7		
δ(C) (ppm)	**1**	3.4	67.1	44.6	42.7	49.1		35.4		177.5	174.9
	1	0.6	65.4	43.9	42.7	49.7		35.1		177.6	172.9
	2	0.3	65.6	43.9	42.8	49.8		35.1		177.6	172.9
	3	0.7	66.1	43.3[h]	49.6[h]	49.2		35.3		177.7	173.3
	4	0.6	66.0	43.3[h]	50.0[h]	49.3		35.3		177.6	173.1

			Position							
	Isomer	pH	1′	2′	3′	4′	5′	6′	1′-Me	5′-CO_2H
δ(H) (ppm) and multiplicity	**1**	3.4		6.13d	6.35dd	5.78dd	3.30dq	1.27d	1.81s	
	2	1.9		6.43d	6.28dd	5.49dd	3.69dq	1.25d	1.85s	
	3	1.8		5.95d	6.55dd	5.87dd	3.38dq	1.33d	1.85s	
	4	1.6		6.19d	6.47dd	5.60dd	3.73dq	1.33d	1.87s	
NOE (%) when H at head of column is irradiated	**1**	3.4		H4′ 13.0 1′Me 1.5	H4 8.5 H5′ 4.5	H2′ 8.0 H5′ 4.7	H3′ 3.5 H4′ 3.0	H4′ 5.2 H5′ 10.7	H5α 4.5 H6b 2.4 H2′ 13.3	
	2	1.9		H5′ 6.2 1′Me 0.6	H4 5.3 H4′ 3.2	H3′ 2.8 H_36' 0.7	H2′ 6.1[c] H_36' 1.2	H4′ 2.8 H5′ 3.6	H5α 3.3 H2′ 3.3	
	3	1.8		H5α 3.6 H3 0.8 H4 0.8 H4′[g]	H5′ 6.0 H_36' 0.3 1′Me 2.0	H5′ 3.4 H_36' 0.5 H2′ 1.6[g]	H3′ 5.4 H4′ 2.8 H_36' 3.0	H3′ 2.2 H4′ 3.1 H5′ 6.5	H3 1.0 H4 1.5 H3′ 8.7	
	4	1.6		H4 3.5 H5α 5.1 H6b 1.3 H5′ 6.3	H4′ 8.5 1′Me 1.9	H3′ 6.7 5′Me 0.5	H2′ 8.2[d] H4′ 1.4 H_36' 1.1	H4′ 4.1 H5′ 6.6	H2 1.1 H3 1.8 H4 1.9 H3′ 7.6	
δ(C) (ppm)	**1**	3.4	133.8	132.8	128.6	135.2	44.9	18.6	23.5	181.9
	1	0.6	133.7	133.5	128.9	135.5	45.0	18.8	23.7	181.9
	2	0.3	136.1	128.5	127.2	133.2	40.7	19.8	24.2	181.9
	3	0.7	134.2	130.0	129.5	135.6	45.0	18.9	18.7	181.9
	4	0.6	136.7	125.2	127.8	133.1	40.7	19.7	18.6	181.8

[a]Reference TSP in concentric tube, temperature 20°C, s = singlet, d = doublet, q = quartet. Data previously reported (1) for domoic acid are included for comparison. Solvent: D_2O.

[b]Error ca ±0.5% for single protons, ca ±0.2% for methyl protons.

[c,d]Resonances too close for separate irradiation.

[e,f,g]Resonances too close for observation of full NOE between them.

[h]Assignments by heterocorrelation might be reversed; ^{1}H peaks nearly overlap.

TABLE 2. ^1H—^1H scalar coupling constants for domoic and isodomoic acids from toxic mussels

		Coupled pair											
	Isomer	2,3	3,4	3,6a	3,6b	4,5α	4,5β	5α,5β	6a,6b	2′,3′	3′,4′	4′,5′	5′,6′
J(H,H)	**1**	8.1	8.4	5.8	9.1	7.3	7.9	−12.2	−16.7	11.1	14.9	7.8	7.1
(Hz)[a]	**2**	8.1	7.9±0.5	6.3	8.3	7.9±0.4	7.7	−12.4	−17.1	11.6	10.0	10.0	7.0
	3	4.0	7.4	6.4	6.4	10.0	7.3	−11.9	−16.9	10.5	15.2	7.9	7.0
	4	4.6	7.2	7.2	7.1	9.5	7.3	−11.9	−17.1	11.0	11.1	10.0	7.0

[a]Error ca. ±0.2 Hz unless stated. Coupling connectivity confirmed by ^1H COSY spectra. Some couplings determined by simulation using Bruker PANIC program. Data previously reported (1) for domoic acid are included for comparison. pH as for ^1H data in Table 1.

α_D^{25} −85°) was identified as isodomoic acid F **4**. The UV (243 nm) spectrum confirms the presence of two conjugated double bonds. The IR spectrum (no band in the region 955–980 cm^{-1}) shows there is no *trans*-disubstituted double bond. Occurrence of NOE between H-3′ and H-4′, as well as between H-2′ and H-5′, and H-3′ and 1′-CH_3, confirms the (*E*, *Z*) configuration of the double bonds. The reduction in *J*(2, 3) and increase in *J*(4, 5α) compared to **1** and **2**, and the occurrence of NOEs between H-2 and H-3, and H-4 and H-5β, indicate that the ring conformation is more similar to **3**. In this regard it is noteworthy that **3** and **4** both possess a (1′*E*) configuration. In all the isomers, the coupling constants (Table 2) and ^{13}C chemical shifts (Table 1) were consistent with the proposed structures. The chirality at C-5′ and the enantiomeric form of each isomer remain to be established.

The isomers are only available in small amounts following tedious and repetitive chromatography. However, more material was required for toxicological studies so alternative methods for their production were investigated. Exposure of dilute aqueous solutions of domoic acid to ultraviolet light (250 nm) resulted in the rapid conversion to the geometrical isomers. Maximum yield of the isomers **2**–**4** was reached after 9–12 min, at which time the proportions of **1**–**4** were respectively 0.28:0.12:0.27:0.13 relative to an initial unit amount of **1**.

It is now well established that domoic acid is produced by the diatom *Nitzschia pungens* (7, 8). Traces of the isomers **2**, **3**, and **4** have been observed in laboratory cultures of *N. pungens* using sensitive analytical techniques (M. Quilliam, personal communication), but these isomers are much more obvious in extracts of various shellfish, suggesting that further conversion of domoic acid occurs within the digestive system of the mollusc. Optical rotations for the isomers produced by photolysis were identical within error (±2°) to those for the corresponding isomers from mussels.

As the isomers have been observed in essentially the same proportions in both steamed and uncooked mussels, it appears that the steaming process does not contribute significantly to isomer production. Another popular recipe is to heat mussels in a solution of vinegar and white wine for several minutes. When domoic acid was warmed (80°C) in aqueous solutions containing acetic acid (3%; 15%; 60%) or trifluoracetic acid (10%) no trace of the isomers was observed by HPLC–DAD analysis, even after prolonged periods (up to 3 h).

Domoic acid is the most potent member of a family of glutamate agonists that includes kainic acid **5**. A portion of these molecules (C2-CO_2H, C-2, C-3, C-6, and C-7) can mimic glutamic acid and bind to glutamate receptors in the brain (9–14). The nature of the side chain has obvious implications with respect to the efficacy of binding to these receptors located in the membrane, and to the potency of the compound as a neuro-excitatory amino acid. Work on the relationship between biological activity and chemical structure of the four geometrical isomers is in progress.

Experimental

HPLC–DAD analysis and all NMR, MS, IR, and UV spectra were obtained with the instruments and conditions previously described (1).

Isolation of isomers

The aqueous methanol extract of blended steamed mussel tissue was concentrated and partially purified by flash chromatography using a C_{18} reversed-phase support (55–105 μm; Waters Prep Pak) and elution with acetonitrile–water mixtures containing 1% acetic acid. The fraction (20% acetonitrile/water) containing domoic acid and the isomers was further purified by Lobar chromatography (C_8 reversed-phase support) and elution with the same mixtures. Finally, fractions enriched in domoic acid and the isomers were separated into various components by repeated reversed-phase HPLC (10 μm; Vydac 201TP 1010; 8% acetonitrile–water containing 0.1% trifluoracetic acid) runs using diode-array detection. Domoic acid **1** was the major component (>90% of the mixture) and the isomers eluted at relative retention times (RRT) **2** (0.84), **3**(0.90), and **4**(1.34) (Vydac 201TP52, 250 mm × 2 mm). The isomers were obtained as oils in the proportions indicated: **2** (ca. 5%), **3** (ca. 2%), **4** (ca. 1%).

The physical and spectroscopic properties of the isomers are described in Results and discussion and in Tables 1 and 2.

Photolysis of domoic acid

Photochemical reactions were carried out at 30°C in a quartz tube (35 × 5 cm diameter) using an ultraviolet photoreactor (Southern New England Ultraviolet Company, Middletown, Conn.) equipped with eight ultraviolet tubes (253.7 nm Rayonet photochemical reactor lamp). Dilute aqueous solutions (500 mL) of domoic acid (100 μg/mL) were degassed by filtration followed by purging with argon. Initially, to set up optimum reaction conditions, the isomerisation was followed by HPLC–DAD analysis. In a typical production run the photolysis reaction was halted after 15 min, the solution concentrated *in vacuo*, and the isomers collected by repeated reversed-phase HPLC runs as described above. The ratio of isomers **1**–**4** was 1:0.42:0.96:0.46. Isomers prepared in this way had identical spectroscopic properties (UV, IR, NMR, $[\alpha_D]$) to those obtained from cooked mussels.

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