Polyacetylenes from Sardinian *Oenanthe fistulosa*: A Molecular Clue to *risus sardonicus*

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Received December 4, 2008

An investigation of *Oenanthe fistulosa* from Sardinia afforded oenanthotoxin (1a) and dihydrooenanthotoxin (1b) from the roots and the diacetylenic epoxydiol 2 from the seeds. The absolute configuration of 1a and 1b was established as R by the modified Mosher’s method, and the structure of 2 by chemical correlation with (+)-(3R,8S)-falcarindiol. Oenanthotoxin (1a) and dihydrooenanthotoxin (1b) were found to potently block GABAergic responses, providing a molecular rationale for the symptoms of poisoning from water-dropwort (*Oenanthe crocata*) and related plants. These observations bear relevance for a series of historical and ethnopharmacological observations on the identification of the Sardinian herb and the molecular details of the facial muscular contraction caused by its ingestion (*risus sardonicus*). Plants from the genus *Oenanthe* (Umbelliferae family) are among the most poisonous species of the European flora.1 They bear a general resemblance to parsnip and carrot and are still a cause of often fatal human poisonings.1 Sardinian *Oenanthe* species have a special ethnopharmacological relevance, being considered the most likely candidate for the (in)famous sardonic herb, a neurotoxic plant used in pre–Roman Sardinia for the ritual killing of elderly people.2 According to the ancient historians, elderly people unable to support themselves were intoxicated with the sardonic herb and then killed by dropping from a high rock or by beating to death.2 The facial muscular contraction induced by the sardonic herb mimicked a smile, and the expression *risus sardonicus* (sardonic smile) to indicate a sinister smile is well documented in the Latin and Greek literature and in most modern European languages. It even found its way into the mainstream medical lingo as the hallmark of lockjaw (*trismus*), the spasm of the muscles of mastication.3

The poisonous constituents of *Oenanthe* species are a series of polyacetylenic alcohols exemplified by oenanthotoxin (1a).4 Surprisingly, the absolute configuration at the stereogenic carbon (C-14) of oenanthotoxin is still unknown, and little information on the molecular details of its neurotoxic activity has been reported. As part of a study on poisonous Sardinian plants,5 we have investigated *O. fistulosa* L. (tubular water-dropwort), a species that, despite its very broad distribution in Europe, had so far been overlooked in terms of phytochemical studies. Large amounts of the diacetylenic epoxydiol 2 were obtained from the seeds, while the roots gave, along with the widespread acetylenic falcarindiol (3), oenanthotoxin (1a) and dihydrooenanthotoxin (1b). The absolute configuration of 1a and 1b was clarified by application of the modified Mosher method, while the activity of oenanthotoxin was profiled against GABA receptors, a target involved in the action of many convulsant toxins of plant origin.6

An acetone extract from the underground parts of *O. fistulosa* was fractionated by gravity column chromatography to afford large amounts (ca. 0.80% of dried plant material) of (+)-(3R,8S)-falcarindiol (3)7 and a mixture of oenanthotoxin (1a) and its dihydroderivative (1b) (0.070%).4 Compounds 1a and 1b were easily separated by preparative HPLC and identified by comparison with authentic samples of (+)-oenanthotoxin and (+)-dihydrooenanthotoxin obtained, in over 10-fold higher yield, from the roots of *O. crocata*, a more common species in Sardinia (see Experimental Section). A complete assignment of the 1H NMR resonances of both 1a and 1b is reported in the Experimental Section.

Oenanthotoxin (1a) is isomeric with cicutoxin (4) from water hemlock (*Cicuta virosa* L.), with the only difference between these C-17 acetylenic toxins being the position of a single double bond, located next to the secondary oxyxmethine in cicutoxin and adjacent to the primary hydroxyl in oenanthotoxin.4b The absolute configuration at C-14 of cicutoxin was established as R by application of a CD exciton chirality method on its p-methoxybenzoate, taking advantage of the allylic nature of the stereogenic center, and was confirmed by application of the Mosher method.8 Since the stereogenic center of oenanthotoxin is not allylic, the Mosher method9 was used to assign its absolute configuration.

To achieve this aim, two aliquots of oenanthotoxin (1a) were dissolved in dry pyridine and allowed to react overnight with DMAP and (R)- or (S)-MTPA chloride, respectively, affording the (S)- and (R)-MTPA diesters 5a and 5b, respectively. Analysis of the Δδ(S–R) values of the protons neighboring the oxygenated methine according...
to the Mosher model (Figure 1) allowed the assignment of the R configuration at C-14 of 1a. The same methodology was applied to dihydrooenanthotoxin (1b), which, by analysis of the Δδ(S-R) values between its (S)- and (R)-MTPA diesters 6a and 6b (Figure 1), turned out to have the same R configuration at C-14. In both cases, concomitant MTPA esterification at C-1 did not exert any influence on the anisotropic effect of the phenyl group of the MTPA moiety bound to the C-14, located 13 carbons away from the primary hydroxyl.

The seeds of O. fistulosa were totally devoid of oenanthotoxin-like polyacetylenes and contained rather large amounts of the diacetylenic epoxidol 2. The structure elucidation of this compound (C17H20O4, HRMS) was helped greatly by comparison with falcarnidol (3), since the two compounds showed similar spectroscopic features. Thus, 2 differed from 3 only in the epoxidation of the disubstituted double bond, as evident from the replacement of the two mutually coupled olefin protons of 3 with two oxymethylene signals [δ 3.06 (dd, J = 7.1, 4.0 Hz, H-9) and 2.80 (dt, J = 5.9, 4.0 Hz, H-10)]. Corresponding changes were observed in the 13 C NMR spectrum (upfield shift of the C-9 and C-10 methines, now resonating at δ 57.9 and 57.4, respectively). Full assignment of 1H and 13C NMR resonances of 2 was achieved through analysis of 2D NMR (COSY, HSQC, and gHMBC) data, which confirmed that 2 is an epoxidized derivative of falcarnidol. To further confirm this assignment and establish the configuration of the epoxide ring, the reaction of (+)-(3R,8S)-falcarnidol (3) with peracids was investigated. Enones can be chemoselectively epoxidized, and treatment of falcarnidol with meta-chloroperoxybenzoic acid (MCPBA) under controlled conditions (monitoring of the conversion by 1H NMR analysis, see Experimental Section) gave the epoxide 2 in satisfactory (66%) yield and as the only reaction product. This was identical in terms of both spectroscopic properties and optical rotation to the natural product isolated from O. fistulosa. Prolonged reaction times or the addition of NaHCO3 to buffer out to have the same Δδ(S-R) values for the epoxydiol from O. fistulosa (6a) and dihydrooenanthotoxin (6b).

Figure 1. Application of the modified Mosher’s method for secondary alcohols on the MTPA esters of oenanthotoxin (5a and 5b) and dihydrooenanthotoxin (6a and 6b). Δδ(δ3−δ8) values are given in ppm.

Convulsant site of GABAA receptors and causing death by inducing convulsions and respiratory paralysis.1,5 In addition, oenanthotoxin has also been reported to dramatically affect cationic (calcium and sodium) currents in excitable membranes.14 We found that both oenanthotoxin and dihydrooenanthotoxin could potentially block the GABAergic responses in neuronal cell cultures, with an EC50 value in the low micromolar range for both compounds. Currents elicited by exogenous GABA were recorded in the whole-cell configuration by rapid application of 3 µM GABA at the membrane voltage of ~−40 mV, and a typical GABA-evoked response, recorded in these conditions, is presented in Figure 2 (left). The effect of 1a and 1b on current amplitude was assessed by dividing the current amplitude measured in the presence of this drug by the amplitude value in control conditions measured from the same cell. Oenanthotoxin (1a) clearly inhibited the GABAergic responses in a dose-dependent manner (Figure 2A). A tentative fitting of the logistic equation (y = I/(1+(c/EC50))h) to the amplitude dose-dependence for oenanthotoxin shown in Figure 2B yielded an EC50 value of 1.39 µM (h = 0.59). Dihydrooenanthotoxin (1b) exhibited a similar inhibitory activity on currents elicited by 3 µM GABA, but was slightly more potent (EC50 = 0.835 µM, h = 0.58). The effect of both test compounds was reversible, although at least 10 min of wash was required to restore the control responses. Compound 1b can be viewed both as 2,3-dihydrooenanthotoxin and as 12,13-dihydroicucitoxin (cf. 1a and 4), showing that, in polyacetylenic toxis, the presence of allylic double bonds is redundant for activity and that the conjugated system required for the activity is shorter than previously postulated,13 encompassing four, and not five, unsaturations.

Unlike other plant toxis, the convulsant polyacetylenes from water dropwort and related plant do not evoke unpleasant taste (bitter) or chemesthetic (burning) sensations, and the roots of O. crocata, an exceedingly poisonous plant, have a paradoxical sweetish and pleasant taste and odor. The large concentration of falcarnidol, a bitter compound,16 and the lower contents of polyclaytenyl toxis in O. fistulosa when compared to O. crocata could presumably underlie the observation that the former species has not yet been associated with human or animal poisoning. The name Oenanthe signifies “wine flower”, because the plant produces a state of stupfaction similar to drunkenness.1,2 This, as well as locked jaws (risus sardonicus), has been documented in human poisoning from O. crocata,1,2 and there is little doubt that herba sardonica of the ancient medical literature should be identified with O. crocata,2 a plant that, within the Mediterranean area, is common only in Sardinia.2 The results of our investigation provide a further rationale for this identification, proposing a molecular mechanism for the risus sardonicus described by the ancient authors.

Polyacetylene neurotoxins have emerged as powerful neurochemical tools,17,18 and the scarce attention given so far to the C-17 neurotoxic aceteylenes can best be explained by their chemical instability, especially in the highly purified form required for biological studies. Thus, oenanthotoxin (1a) and dihydrooenanthotoxin (1b), just like many other polyacetylenes, easily turned into highly colored and insoluble polymeric materials upon storage, even at low temperature (~18 °C). To overcome this problem, we have developed a fast chromatography protocol to separate oenanthotoxin (1a) and dihydrooenanthotoxin (1b) from a crude extract of O. crocata and found that these compounds, while being highly unstable in the solid state, can be conveniently stored for at least 6 months in benzene or DMSO solution at 4 °C (see Experimental Section). This might foster interest in this fascinating class of compounds, with its unique capacity of dramatically perturbing calcium and sodium ionic currents in excitable membranes14 and specifically interacting with ligand-gated ion channels like the GABAA receptor.

Experimental Section

General Experimental Procedures. Optical rotations (CHCl3) were measured at 589 nm on a Perkin-Elmer 192 polarimeter equipped with a sodium lamp (λ = 589 nm) and a 10 cm microcell. 1H (500 MHz)
Figure 2. Oenanthonotoxin (1a) and dihydrooenanthotoxin (1b) inhibit GABA-evoked responses in a dose-dependent manner. (A) Typical current traces evoked by rapid application of 3 μM GABA at −40 mV in control conditions (left), in the presence of 10 μM 1a (middle), and in the presence of 10 μM 1b (right). (B) Dose-dependent effect of 1a on GABA-evoked current amplitude relative value. (C) Dose-dependent effect of 1b. Each data point in B and C was calculated from at least 3 values. Asterisks above the bars indicate statistically significant difference.

and 13C (125 MHz) NMR spectra were measured on a Varian INOVA spectrometer. Chemical shifts were referenced to the residual solvent signal (CDCl3: δH 7.26, δC 77.0). Homonuclear 1H connectivities were determined by the COSY experiment. One-bond heteronuclear 1H–13C connectivities were determined with the HSQC experiment. Two- and three-bond 1H–13C connectivities were determined by HMBC experiments optimized for a 2J of 7 Hz. Through-space 1H connectivities were evidenced using a ROESY experiment with a mixing time of 500 ms. Two- and three-bond 1H–13C connectivities were determined by gradient 2D HMBC experiments optimized for a 2J of 9 Hz. Low- and high-resolution EIMS spectra (70 eV) were performed on a VG Prospec (Fisons) mass spectrometer. ESIMS spectra were performed on a LCQ Finnigan MAT mass spectrometer. Silica gel 60 (70–230 mesh) was used for gravity column chromatography. Reactions were monitored by TLC on Merck 60 F254 (0.25 mm) plates, which were visualized by UV inspection and/or staining with 5% H2SO4 in ethanol and heating. Organic phases were dried with Na2SO4 before evaporation.

Plant Material. Oenanthe fistulosa was collected near San Basilio (CA, Sardinia, Italy) on September 14, 2007. The plant was identified by M.B., and a voucher specimen (171007) is held at the University of Cagliari. O. fistulosa (379 g) were extracted with acetone (2 L) to afford 3.1 g of a brownish oil, which was purified with the Biotage SP1 system with a silica gel (10 g) column and a n-hexane–EtOAc gradient of increasing polarities (flow rate 9 mL/min, UV detector 316 nm; fractionation mode: volume 9 mL/fraction). Fractions 29–31 and 34–36 (n-hexane–EtOAc, 6:4) afforded about 88 mg of oenanthonotoxin (1a) and 89 mg of dihydrooenanthotoxin (1b), respectively. These compounds were obtained as colorless foams and could not be stored in this form. However, solutions in hydrocarbon solvents (benzene, toluene) or DMSO could be stored for at least 6 months at 4 °C without any apparent (1H NMR analysis) degradation or development of a reddish color.

Oenanthonotoxin (1a): amorphous foam; [α]D25 +34 (c 0.5, CHCl3); 1H NMR (500 MHz, CDCl3) δ 6.71 (1H, dd, J = 15.5, 11.0 Hz, H-9), 6.39 (1H, dt, J = 15.0, 5.0 Hz, H-2); 6.12 (1H, dd, J = 15.0, 11.0 Hz, H-10), 5.87 (1H, overlapped H-3), 5.86 (1H, overlapped, H-11); 5.57 (1H, d, J = 15.5 Hz, H-8), 4.25 (2H, t, J = 5.0 Hz H-2), 3.60 (1H, m, H-14), 2.10 (3H, m, H=12a, 2.20 (1H, m, H-12b), 1.78 (1H, m, H-13a), 1.50 (1H, overlapped, H-13b), 1.49 (1H, overlapped, H-15a), 1.40 (1H, m, H-15b), 1.30 (2H, m, H2-16), 0.92 (3H, d, J = 7.3, Hz H-17); ESIMS (positive ion) m/z 281 [M + Na]⁺.

Dihydrooenanthotoxin (1b): amorphous foam; 1H NMR (500 MHz, CDCl3) δ 6.65 (1H, dd, J = 15.5, 10.6 Hz, H-9), 6.12 (1H, dd, J = 15.0, 11.0 Hz, H-10), 5.84 (1H, dd, J = 15.0, 5.0 Hz, H-11), 5.50 (1H, d, J = 15.5 Hz, H-8), 3.77 (2H, t, J = 5.0 Hz, H-2), 3.60 (1H, m, H-14), 2.45 (2H, t, J = 5.0 Hz H-3), 2.30 (1H, m, H-12a), 2.21 (1H, m, H-12b), 1.79 (2H, t, J = 5.0 Hz, H-2), 1.75 (1H, m, H-13a), 1.50 (1H, overlapped, H-13b), 1.49 (1H, overlapped, H-15a), 1.40 (1H, m, H-13b), 1.30 (2H, m, H2-16), 0.93 (3H, d, J = 7.3, Hz H-17); ESIMS (positive ion): m/z 283 [M + Na]⁺.

9-Epoxyfalcarienol (2): colorless oil, [α]D25 +107 (c 0.9, MeOH); IR (liquid film) νmax 3400, 1320, 1451, 1293, 1238, 1164, 983 cm⁻¹; 1H NMR (500 MHz, CD3OD) δ 5.78 (1H, ddd, J = 17.0, 10.2, 5.45 Hz, H-2), 5.37 (1H, brd, J = 17.0 Hz, H-1), 5.03 (1H, brd, J = 10.3 Hz, H-1b), 4.67 (1H, d, J = 5.4 Hz, H-3), 4.20 (1H, d, J = 7.1 Hz, H-8), 3.06 (1H, dd, J = 7.1, 4.0 Hz, H-9), 2.80 (1H, dt, J = 3.0, 4.0 Hz, H-10), 1.53 (2H, brm, H-11a,b), 1.45–1.50 (10H, m, H-12a,b + H-13a,b + H-14a,b + H-15a,b + H-16a,b), 1.01 (3H, t, J = 7.1 Hz, H-17); 13C NMR (125 MHz, CDCl3) δ 136.1 (d, C-2), 116.2 (t, C-1), 79.3 (s, C-7), 79.1 (s, C-4), 70.3 (s, C-5), 70.1 (s, C-6), 63.2 (d, C-3), 61.0 (d, C-8), 57.9 (d, C-9), 57.4 (d, C-10), 31.9 (t, C-15), 27.6 (t, C-11), 29.4, 29.3 (t, C-13 and C-14) 26.6 (t, C-12), 22.8 (t, C-16), 14.1 (q, C-17). HREIMS m/z 276.1737 [M⁺] (calcd for C17H23O5, 276.1725).

MTPA Esters of Oenanthonotoxin and Dihydroenanthonotoxin. Oenanthonotoxin (1a, 2.5 mg) was dissolved in 0.5 mL of dry pyridine and treated with (−)-R-MTPA chloride (20 μL) and N,N-dimethylamino-nopyridine (DMAP, a spatula tip), then maintained at room temperature under stirring overnight. After removal of the solvent, the reaction mixture was purified by HPLC on a SI60 column (eluent n-hexane–EtOAc, 9:1), affording the (S)-MTPA diester 1c in a pure state.
The cells in which series resistance showed a clear tendency to increase during recordings were not considered in the analysis. Recordings in the whole-cell mode were started at least 3 min after establishing the whole-cell mode. This time was sufficient to stabilize the recording conditions. The agonist was applied using the RSC-200 multibarrel rapid perfusion system (Bio-Logic, Grenoble, France). With this system, in the whole-cell configuration, the solution exchange occurred within 30–100 ms. Before each control recording, cells were washed with normal external solution for at least 3 min. In studies aiming at assessment of the impact of considered test compounds [oneantotoxin (1a), dihydrooneantotoxin (1b)] on GABA-elicited responses, these compounds were present both in the washing solution (for at least 3 min before agonist application) and in the agonist-containing saline. For acquisition and data analysis, pClamp 9.2 software was used (Molecular Devices Corporation). To avoid the data scatter due to cell-to-cell variability, the effect of studied drugs was assessed by calculating the relative amplitude values with respect to the controls recorded from the same cell. For the analysis of currents, recorded in the whole-cell configuration, the current signals were low-pass filtered at 3 kHz with a Butterworth filter and sampled at 10 kHz using the analog-to-digital converter Digidata 1322A (Molecular Device Corporation) and stored on a computer hard disk. Data are expressed as mean ± SEM. A paired Student’s t test was used to assess the significance of differences between considered data sets. All experiments were performed at room temperature, 22–24 °C.

Acknowledgment. P.W., K.S., and J.W.M. were supported by a Wellcome Trust International Senior Research Fellowship in Biomedical Science (grant no. 070231/Z/03/Z).

References and Notes

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(6) Dihydrooneantotoxin-1-[(S)-MTPA diester (1a): amorphous solid; [‘H NMR (500 MHz, CDCl3) δ 7.55 and 7.53 (MTPA phenyl protons), 6.64 (H-9, dd, J = 15.5, 10.6 Hz), 6.09 (H-10, dd, J = 15.0, 10.6 Hz), 5.83 (H-11, dt, J = 15.0, 5.0 Hz), 5.45 (H-8, d, J = 15.5 Hz), 5.20 (H-14, m), 4.39 (H-1, m), 3.58 (MTPA OCH3, s), 2.36 (H3-17, m), 1.95 (H-3, m), 1.60 (H-13a, m), 1.60 (H-13b, m), 1.56 (H-15b, m), 1.58 (H-15a, m), 1.28 (H2-16, m), 0.88 (H-17, d, J = 7.3 Hz); FABMS (gliceryl matrix, positive ions) m/z 691 [M + H]+.

(7) Dihydrooneantotoxin-1-[(R)-MTPA diester (1b): amorphous solid; [‘H NMR (500 MHz, CDCl3) δ 7.45 and 7.35 (MTPA phenyl protons), 6.66 (H-9, dd, J = 15.5, 10.6 Hz), 6.28 (H-2, dt, J = 15.0, 5.0 Hz), 6.09 (H-10, dd, J = 15.0, 10.6 Hz), 5.84 (H-3, overlapped), 5.82 (H-11, overlapped), 5.57 (H-8, d, J = 15.5 Hz), 5.10 (H-14, m), 4.86 (H-1, d, J = 5.0 Hz), 3.55 (MTPA OCH3, s), 2.16 (H-12a, overlapped), 2.12 (H-12b, overlapped), 1.81 (H-13a, m), 1.73 (H-13a, m), 1.65 (H-13b, m), 1.58 (H-15b, m), 1.29 (H2-16, m), 0.88 (H2-17, d, J = 7.3 Hz); FABMS (gliceryl matrix, positive ions) m/z 691 [M + H]+.

(8) Epoxidation of Falcariolid. To a solution of falcariolid (85 mg, 0.36 mmol) in dry CH2Cl2 (3 mL) was added 85% H2O2 (1.1, MeOH).

(9) Cell Culture for Electrophysiological Recordings. Neuronal cell culture was prepared as previously described in detail by Andjus et al. Briefly, P1–P3-day-old Wistar rats were decapitated. The procedure is in accordance with the regulation of the Polish Animal Welfare Act. Hippocampi were removed, manually sliced, treated with trypsin, mechanically dissociated, centrifuged twice at 40 g, plated in Petri dishes, and cultured. Experiments were performed on cells between 10 and 17 days in culture.

(10) Electrophysiological Recordings. Currents were recorded in the whole-cell configuration of the patch-clamp technique using the Axopatch 200B amplifier (Molecular Instruments, Sunnyvale, CA) at a holding potential (VH) of −40 mV. The intrasipet solution contained (in mM) CsCl 137, CaCl2 1, MgCl2 2, 2-bis(2-aminoethoxy)ethane-N,N,N′,N′-tetraacetic acid (BAPTA) 11, ATP 2, and HEPES 10 (pH 7.2 with CsOH). The composition of the standard external solution was (in mM) NaCl 137, KCl 5, CaCl2 2, MgCl2 1, glucose 20, and HEPES 10 (pH 7.2 with NaOH). For the whole-cell recordings, patch pipettes had a resistance of 2.5–4.0 MΩ when filled with internal solution. The whole-cell recordings were included in the statistics when the access resistance was below 10 MΩ.