Eng, L. F.; Noble, E. P. *Lipids* **1968**, *3*, 157.
Fennema, O. "Principles of Food Science, Part I, Food Chemistry"; Marcel Dekker: New York, 1976; pp 347-381.
Frank, R.; Mickelsen, P. *Am. J. Clin. Nutr.* **1969**, *22* (4), 464.
Haddy, F. J. *Am. J. Med.* **1980**, *69*, 746.
Harris, R.; Von Loesecke, H., Eds. "Nutritional Evaluation of Food Processing"; Avi Publishing Co.: Westport, CT, 1960; pp 109-126.
Jones, C. *Proc. Nutr. Soc.* **1958**, *17* (1), 7.
Linkswiler, H.; Zemel, M. *Contemp. Nutr.* **1979**, *4* (5).
Love, R. *J. Sci. Food Agric.* **1961**, *12*, 439.
Meneely, G. *Qual. Plant.—Plant Foods Hum. Nutr.* **1973**, *23* (1/3), 3.
Meneely, G. R.; Battarbee, H. D. *Am. J. Cardiol.* **1976**, *38*, 768.
National Academy of Sciences "Recommended Dietary Allowances", 8th ed.; NAS: Washington, DC, 1974.
Noller, B.; Bloom H. *Food Technol. Aust.* **1978**, *30*, 11.
"Perkin-Elmer Model 21 Coleman Flame Photometer Operating Directions"; Coleman Instruments Division: Oak Brook, IL 60521, Jan 1979.
Snedecor, G. W.; Cochran, W. G. "Statistical Methods"; Iowa State University Press: Ames, IA, 1967.
Watt, B.; Merrill, A. *U.S., Dep. Agric., Agric. Handb.* **1963**, *No. 8*.
Weinsier, R. *Prev. Med.* **1976**, *5*, 7.

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Properties of Two Toxins Newly Isolated from Oysters

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Two toxins were newly isolated from the hepatopancreas of toxic oysters. The structures of two of these were deduced to be carbamoyl-*N*-sulfo-11α-hydroxysaxitoxin sulfate (1) and the 11β epimer (2), based on elemental analysis, electrophoresis, TLC, ^{1}H and ^{13}C NMR, and conversion experiments. 1 revealed little toxicity on intraperitoneal injection into mice. 2 gave a specific toxicity of 300 ± 15 mouse units/mg. Upon mild acid hydrolysis, 1 and 2 were converted to highly toxic compounds: 11α-hydroxysaxitoxin sulfate and its epimer.

We have already reported on some properties of the two toxins newly isolated from the oyster *Crassostrea gigas* cultured in Senzaki Bay, Yamaguchi, Japan (Onoue et al., 1981). Both toxins revealed an extremely low toxicity in mice as well as quite different chromatographic and electrophoretic behaviors from the previously known toxins.

Meanwhile, two analogous toxins have been isolated from cultures of *Protogonyaulax* sp. (Hall et al., 1980) and *Gonyaulax tamarensis* (Kobayashi and Shimizu, 1981). The structures of two of these were then determined by X-ray crystallography as carbamoyl-*N*-sulfo-11α-hydroxysaxitoxin sulfate and the 11β epimer (Wichmann et al., 1981).

Our continuous efforts were made to characterizing the two isolated oyster toxins, since characterization or identification of them may provide evidence for the involvement of the above two species of dinoflagellates in the infestation to shellfish.

MATERIALS AND METHODS

Toxic Oysters. Toxic specimens of the oyster *C. gigas* cultured in Senzaki Bay, Yamaguchi, Japan, were collected in Jan 1979 and 1980. The oysters, after being shucked, were kept frozen below -20 °C for 3-10 months. The hepatopancreas was removed from the partially thawed oysters and used for the extraction and purification of toxins. The toxicity of organ was determined by using the standard mouse bioassay for paralytic shellfish poison (AOAC, 1975).

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Extraction Procedure. One kilogram of hepatopancreas (240 ± 12 mouse units (MU)/g) was homogenized for 3-5 min with 2000 mL of 80% ethanol adjusted to pH 2 with 1 N HCl. The homogenate was centrifuged at 5000*g* for 20 min. These steps were repeated twice for the residue. The combined supernatant was concentrated in vacuo to 500 mL and washed 5 times with 300 mL of chloroform. The aqueous layer (220 000 MU) from which the residual chloroform was removed by evaporation was adjusted to pH 5.5 with 1 N NaOH.

Activated Charcoal Treatment. Water-washed activated charcoal (Wako Pure Chemical Industries), 750-800 mL, was added under agitation to the toxin extract and filtered through a Büchner funnel. The charcoal on the funnel was thoroughly washed with water and eluted with 3000 mL of 20% ethanol containing 1% acetic acid. The toxic eluate (152 000 MU) was concentrated in vacuo to 300 mL and lyophilized.

Chromatography on Bio-Gel P-2. The lyophilized toxins were dissolved in 350 mL of water and applied to a Bio-Gel P-2 (Bio-Rad Laboratories) column (6.5 × 50 cm). After half of the bed volume (800 mL) of water was passed through the column at a flow rate of 3 mL/min, the toxins were eluted with 2000 mL of 0.15 M acetic acid. The toxic eluate (150 000 MU) was concentrated and lyophilized.

Chromatography on Amberlite CG-50 II. The gel-treated toxins were dissolved in 5 mL of water, placed on an Amberlite CG-50 II (Rohm and Haas Co.) column (H^+ form, 1.5 × 95 cm) and fractionated with 100 mL of water and then 350 mL each of 0.1 and 0.5 M acetic acid. The water eluate (50 000 MU) was lyophilized and purified by

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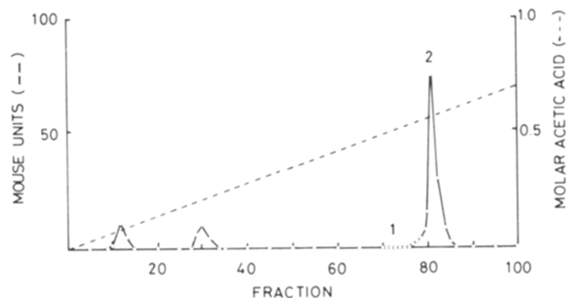


Figure 1. Elution diagram of compounds **1** (fractions 72–76) and **2** (fractions 82–83) from a Dowex WGR column.

chromatography as given below.

Chromatography on Dowex WGR. A portion (10 000 MU) of the toxins was dissolved in 3 mL of water and subjected to a Dowex WGR (Dow Chemical Co.) column (OH^- form, 0.8 × 95 cm). A linear gradient elution of acetic acid from 0 to 1 M was applied at a flow rate of 0.65 mL/min. Fractions of 4 mL were collected. The toxins isolated were immediately lyophilized and analyzed.

Thin-Layer Chromatography (TLC). One microliter of the toxins (50 μg/mL) was chromatographed on a 5 × 20 cm silica gel 60 precoated plate (E. Merck Laboratories) with a solvent system of *tert*-butyl alcohol–acetic acid–water (2:1:1 v/v). Detection of the toxins was made under UV light (365 nm) after spraying the plate with 1% H_2O_2 and heating at 110 °C for 5 min.

Electrophoresis. A portion (1 μL) of the toxins prepared for TLC was applied to a 12 × 12 cm cellulose acetate strip (Chemetron) with 0.08 M Tris buffer (pH 8.7) and electrophoresed at 0.8 mA/cm for 30 min. The strip was treated as above for detection of the toxins.

IR Analysis. IR spectra were obtained on a JASCO IR-S spectrophotometer in KBr pellets.

^{1}H and ^{13}C NMR Analyses. ^{1}H NMR spectra were recorded on a 400-MHz JEOL JNM FX-400 spectrometer in D_2O with tetramethylsilane (Me_4Si) as the external standard. ^{13}C NMR analysis was conducted at 100 MHz on the same spectrometer.

Bioassay. Since the isolated toxins have been found susceptible to low pH, redistilled water was used for dissolution or dilution of them. For preparation of a dose–death time curve 1 mg of **2** or saxitoxin (**6**) equivalent to 300 MU (Schantz et al., 1958) was dissolved in 60 mL of water and diluted 1-, 1.5-, 2-, 2.5-, 3-, 3.5-, 4-, 5-, 5.5-, and 6-fold. Each dilution (1 mL) was intraperitoneally injected into 9–11 male mice (ddY) weighing approximately 20 g, and the time to death was noted. The dose–death time curve of the toxin was obtained from the median death times. One mouse unit is defined as the amount of toxin that kills a 20-g mouse in 15 min.

RESULTS

Extraction and Purification of Oyster Toxins. The 80% ethanol extraction of 1 kg of hepatopancreas followed by defatting with chloroform provided a toxic extract of 220 000 MU. After being treated with activated charcoal, the extract was applied to a Bio-Gel P-2 column. About 30% of the toxicity was lost with the charcoal treatment, but little loss resulted on the gel column. The toxins were then passed through an Amberlite CG-50 II column. Some toxins (50 000 MU) were eluted unadsorbed, whereas other toxins known (80 000 MU) were adsorbed and eluted with an increased molarity of acetic acid from 0.1 to 0.5 M. The unadsorbed toxins were purified by chromatography on a Dowex WGR column. As shown in Figures 1 and 2, two compounds (**1** and **2**), which are interconvertible to each other, were isolated. The yields of **1** and **2** were found to be 60 and 15 mg/kg of organ.

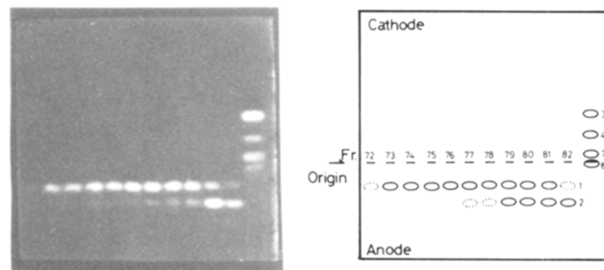


Figure 2. Electrophoretic patterns of **1**- and **2**-containing fractions (72–82) obtained as in Figure 1.

Table I. ^{1}H NMR Data for 1, 2, and 3[a]

proton	1	2	3
H-5	4.64 s	4.67 s	4.59 s
H-6	3.71 dd (10, 5)	3.71 dd (10, 5)	3.62 dd (9.5, 5)
H-10	3.98 d (12.4)	4.01 dd (8, 11.7)	3.94 d (12)
H-10	3.85 dd (5, 12.4)	3.43 dd (7, 11.7)	3.79 dd (4.8, 12)
H-11	4.65 d (5)	4.79 dd (7, 8)	4.61 d (4.8)
H-13	4.18 dd (10, 12.4)	4.22 dd (10, 12.4)	4.05 dd (9.5, 11.8)
H-13	3.94 dd (5, 12.4)	4.00 dd (5, 12.4)	3.82 dd (5, 11.8)

[a] Chemical shifts in ppm relative to Me_4Si. The figures in parentheses are coupling constants in Hz.

Table II. ^{13}C NMR Data for 1, 2, and 3[a]

carbon	1	2	3
C-10	51.3 t	48.0 t	50.2 t
C-6	53.2 d	53.5 d	52.4 d
C-5	58.2 d	57.9 d	57.0 d
C-13	64.3 t	63.6 t	62.5 t
C-11	77.9 d	76.3 d	76.8 d
C-4	81.8 s	82.1 s	80.8 s
C-12	97.8 s	97.8 s	96.7 s
C-2	154.2 s	156.0 s	155.4 s
C-8	156.5 s	158.3 s	157.3 s
C-14	158.3 s	159.3 s	158.1 s

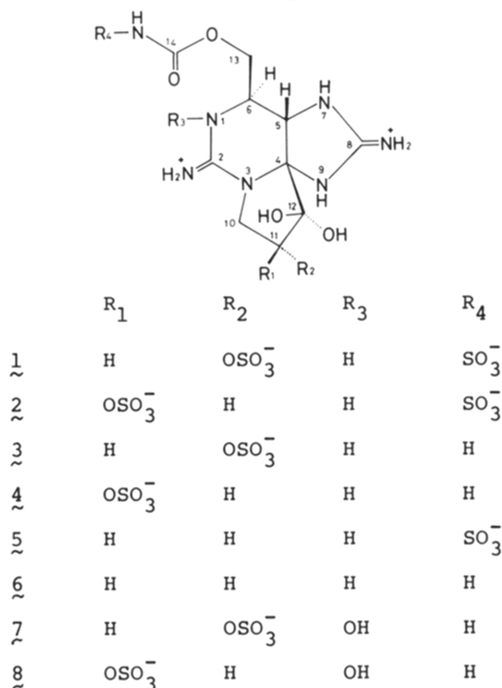
[a] Chemical shifts measured as in Table I. The assignments for carbons 2, 8, and 14 are based on the data of Rogers and Rapoport (1980).

Elemental Composition and Molecular Formula. Elemental analysis of **1** showed 26.03% carbon, 4.29% hydrogen, 18.44% nitrogen, 37.19% oxygen, and 12.82% sulfur. As **1** contains two atoms of sulfur, the molecular formula should be $C_{10}H_{17}N_7S_2O_{11}\cdot H_2O$ (calculated: C, 24.34; H, 3.90; N, 19.87; O, 38.90; S, 12.99) with a molecular weight of 493.

Sulfate Content. **1** and **2** produced 2.1 mol each of sulfate when hydrolyzed with 6 N HCl (100 °C, 24 h) and assayed by the benzidine method (Antonopoulos, 1962).

TLC and Electrophoresis. **1** and **2** gave R_f values of 0.41 and 0.33 corresponding to those of the two known toxins, 11α-hydroxysaxitoxin sulfate (gonyautoxin 2, **3**) and the 11β epimer (gonyautoxin 3, **4**). However, on electrophoresis (0.08 M, pH 8.7, Tris buffer), **1** and **2** moved toward the anode—the direction opposite to that of standard toxins (Figure 2).

IR Spectra. **1** and **2** exhibited broad bands (3150–3475 cm^{-1}) attributable to the OH or NH group. The spectra also indicated the presence of the following bonds: C=O or C=N (1600–1700 cm^{-1}); C–O or C–N (1000–1100 cm^{-1}); SO_3H (1130–1170 and 1430–1480 cm^{-1}).



	R_1	R_2	R_3	R_4
1	H	OSO_3^-	H	SO_3^-
2	OSO_3^-	H	H	SO_3^-
3	H	OSO_3^-	H	H
4	OSO_3^-	H	H	H
5	H	H	H	SO_3^-
6	H	H	H	H
7	H	OSO_3^-	OH	H
8	OSO_3^-	H	OH	H

Figure 3. Various forms of paralytic shellfish toxins.

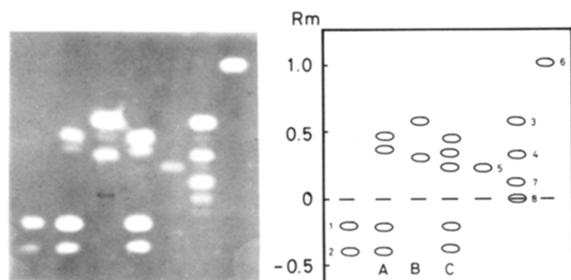


Figure 4. Electrophoretic patterns of products formed from **1** and **2** by heating with water (A), acid (B), or alkali (C).

^{1}H and ^{13}C NMR Spectra. The proton and carbon resonance data for **1** and **2** are summarized in Tables I and II. The chemical shifts (δ) and coupling constants of **1** were very similar to those of **3** (Noguchi et al., 1981), suggesting identity of the carbon skeleton (Figure 3).

The marked differences between the proton chemical shifts of **1** and **2** were seen for H-10 (3.85 vs. 3.43) and H-11 (4.65 vs. 4.79). Some variations were also noted for the other protons except H-6 (3.71). The two compounds bore almost identical chemical shifts for all the carbons excluding C-10 (51.3 vs. 48.0) and C-11 (77.9 vs. 76.3).

Conversions to 3, 4, or Other Compounds by Acid or Alkali Treatment. Figure 4 shows electrophoretic patterns of the products formed during treatments. **1** and **2** were converted to highly toxic compounds **3** and **4** by treating with 0.1 N HCl (100 °C, 20 min). The treatment with 0.1 N NH_4OH (100 °C, 5 min) formed three toxins; one corresponded to carbamoyl-*N*-sulfosaxitoxin (gonyautoxin 5, **5**) in its relative mobility (R_m 0.22) and R_f value (0.24) (Nishio et al., 1982), but the other two (R_m 0.35 and 0.44; R_f 0.40 and 0.33) were unknown. Heating with water (100 °C, 20 min) caused an effect similar to that of alkali treatment. Prolonged exposure to alkali (100 °C, 20 min) resulted in some decomposition of **1** and **2**.

Toxicity in Mice. **1** remained little or nontoxic until epimerized to **2** which, in turn, became neutralized upon epimerization to **1**. The dose–death time curve of **2** is illustrated in Figure 5, in comparison with that of **6**. The specific toxicities of **1** and **2** were estimated to be 30 ± 2 and 300 ± 15 MU/mg by use of this curve.

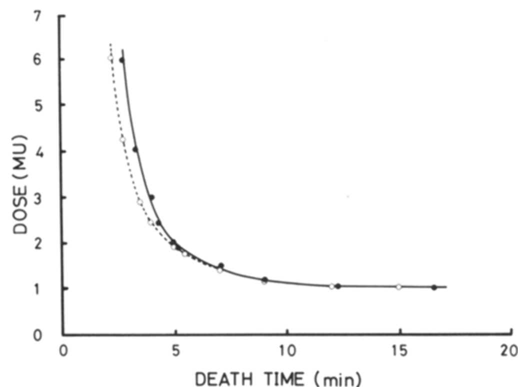


Figure 5. Dose–death time curves of **2** (●) and **6** (○).

DISCUSSION

Approximately 60 mg of **1** (1800 MU) and 15 mg of **2** (4500 MU) were isolated from 1 kg of oyster hepatopancreas. **1** and **2** readily epimerized each other and formed a 3:1 mixture in aqueous solution, as determined by ^{1}H NMR.

The close resemblance between the ^{1}H and ^{13}C NMR spectra of **1** and **3** bestowed the identical carbon skeleton to both toxins. However, **1** and **2** had a net negative charge at pH 8.7; **3** and **4** had an opposite charge at the same pH. The former toxins were adsorbed on a Dowex WGR anion exchanger but not on cation exchangers such as Amberlite CG-50 II and Bio-Rex 70 (Bio-Rad Laboratories) on which the latter toxins were retained.

Elemental and colorimetric analyses disclosed that **1** and **2** contained 2 mol each of sulfate in their molecules. One of the sulfate-binding sites was confirmed to be C-11 on the basis of conversion experiments. The other binding site was speculated to be the carbamate amino group from a comparison of the proton and carbon chemical shifts of **1** and **3**; the largest downfield shifts ($\Delta\delta$) of **1** occurred on the two H-13 protons (+0.12 and +0.13) and the C-13 carbon (+1.8). It is therefore reasonable to assign carbamoyl-*N*-sulfo-11α-hydroxysaxitoxin sulfate and the 11β epimer to **1** and **2** as their structures. These two compounds have previously been found in toxic dinoflagellates (Wichmann et al., 1981; Kobayashi and Shimizu, 1981).

Three different toxins were derived from heating **1** and **2** with alkali. One of them agreed with **5** in its electrophoretic and chromatographic behaviors. It is likely to be further converted to **6** with a release of another sulfate. The structures of the other two remain to be elucidated.

The dose–death time curve of **2** was incompatible with that of **6** which has been widely accepted for the mouse bioassay of paralytic shellfish poison. The doses for both toxins were very close to each other at death times exceeding 7 min, but the correlation in doses decreased at death times shorter than 5 min.

1 and **2** were also extractable from cultures of *Protogonyaulax catenella* collected in oyster farming areas of Senzaki Bay (Onoue et al., 1981). It suggests that this dinoflagellate might have been involved in the toxification of oysters.

Thus, the structures of **1** and **2** appear to be identical with those of two toxins from dinoflagellates. These two toxins are extremely hazardous to humans because their toxicity rises precipitously through conversion to **3** or **4** in acidic media. The establishment of a pertinent measure for monitoring such noxious compounds of cryptic nature is urgently needed.

Note Added in Proof Compounds 1 and 2 exhibit the same electrophoretic and thin-layer chromatographic behaviors as *Protogonyaulax* toxins C1 and C2, both of which were cordially provided to us in crystalline forms by Dr. S. Hall, Woods Hole Oceanographic Institution, Woods Hole, MA.

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Registry No. 1, 80173-30-4; 2, 80226-62-6; 3, 60508-89-6; 4, 60537-65-7; 5, 64296-25-9.

LITERATURE CITED

Antonopoulos, C. A. *Acta Chem. Scand.* **1962**, *16*, 1521–1522.
AOAC "Methods of Analysis", 12th ed.; Association of Official Analytical Chemists: Washington, DC, 1975; pp 319–321.
Hall, S.; Reichardt, P. B.; Neve, R. A. *Biochem. Biophys. Res. Commun.* **1980**, *97*, 649–653.
Kobayashi, M.; Shimizu, Y. *J. Chem. Soc., Chem. Commun.* **1981**, 827–828.
Nishio, S.; Noguchi, T.; Onoue, Y.; Maruyama, J.; Hashimoto, K.; Seto, H. *Bull. Jpn. Soc. Sci. Fish.* **1982**, *48*, 959–965.
Noguchi, T.; Kono, M.; Ueda, Y.; Hashimoto, K. *J. Chem. Soc. Jpn.* **1981**, 652–658.
Onoue, Y.; Noguchi, T.; Maruyama, J.; Hashimoto, K.; Ikeda, T. *Bull. Jpn. Soc. Sci. Fish.* **1981**, *47*, 1643.
Rogers, R. S.; Rapoport, H. *J. Am. Chem. Soc.* **1980**, *102*, 7335–7339.
Schantz, E. J.; MacFarren, E. F.; Schafer, M. L.; Lewis, K. H. *J. Assoc. Off. Agric. Chem.* **1958**, *4*, 160–177.
Wichmann, C. F.; Niemczura, W. P.; Schnoes, H. K.; Hall, S.; Reichardt, P. B.; Darling, S. D. *J. Am. Chem. Soc.* **1981**, *103*, 6977–6978.

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Germination Responses of Several Species of Rust Spores to 5-Methyl-2-hexanone, Isomers of Ionone, and Other Structurally Related Flavor Compounds

Richard C. French

Uredospores of *Puccinia punctiformis*, *Puccinia chondrillina*, *Puccinia iridis*, and *Uromyces trifolii-repentis* were tested for germination responses to β-ionone, α-ionone, 5-methyl-2-hexanone, and other related compounds. Maximum germination by any treatment ranged from 80 to 90% for the four species. Germination of uredospores of *P. punctiformis* and *P. chondrillina* was stimulated most by 5-methyl-2-hexanone and 2-heptanone. *P. iridis* was stimulated most by β-ionone. *P. punctiformis* was stimulated by α-ionone but not by β-ionone. *P. chondrillina* and *P. iridis* were stimulated by β-ionone but not α-ionone. Both *P. punctiformis* and *P. chondrillina* could be chemically stimulated over a temperature range of 10–25 °C. Uredospores of *U. trifolii-repentis* were stimulated most effectively by β-ionone, followed by α-ionone and octyl cyanide. 5-Methyl-2-hexanone is the third compound (after nonanol and β-ionone) found to be the most effective chemical germination stimulator for certain groups of rust species.

Differences in response of uredospores of various species of rusts to certain flavor chemicals have been reported previously (French and Gallimore, 1971; French et al., 1975a,b, 1977; French and Wilson, 1981). Some of the chemicals studied also have been found in uredospores of several species (French and Weintraub, 1957; Rines et al., 1974). For example, nonanal and 6-methyl-5-hepten-2-one have been identified in rusts, and they also occur naturally as components of flavors and fragrances in a variety of natural products, including citrus peel oils, and other food items (Furia and Bellanca, 1975). These two compounds also have been identified as insect pheromones (Blum, 1969). β-Ionone and 6-methyl-5-hepten-2-one have been found in volatiles from algal cultures, and β-ionone has been reported to inhibit growth of several algal species (Jüttner, 1979). Virtually all of the compounds used in this research have been reported to occur in various natural products. This study of the effect of chemical structure on stimulator activity was conducted to determine the most efficient compound for stimulating spores of several rust species, including two which are pathogens of weeds. This information may be useful in providing effective tools to solve practical problems in uredospore germination, such as obtaining maximum germination in uredospores applied to weed pests in biocontrol operations or causing germination of pathogenic spores at an opportune time for disease control. Determining the structural requirements for chemical stimulation also should provide a starting point for research on mechanism of action.

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MATERIALS AND METHODS

Since rust fungi are obligate parasites, uredospores were produced on the appropriate hosts, usually in the greenhouse. Uredospores or aeciospores of *Puccinia punctiformis* (Strauss) Roehl. were collected in the field from plants of Canada thistle, *Cirsium arvense* (L.) Scop.

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