15. The topology-dependent cladistic permutation tail probability (T-PTP) test determines whether the difference in length between the shortest tree supporting the monophyly of this clade (997 steps) and the shortest tree not supporting monophyly (1003 steps) is significantly different from the difference in length expected from randomized data. If the difference in length between the monophyly and nonmonophyly trees is outside 95% of the distribution based on randomized data, it can be concluded that the data significantly support monophyly of the clade [D. P. Faith, *Syst. Zool.* **40**, 366 (1991); J. W. O. Ballard *et al.*, *Science* **258**, 1345 (1992)]. We used 200 randomized data sets that were created with the program Shuffle (J. P. Huelsenbeck, University of Texas at Austin) and were analyzed by means of the parsimony criterion.
16. K. de Queiroz and J. Gauthier, *Syst. Zool.* **39**, 307 (1990).
17. A. Williams and J. M. Hurst, in *Patterns of Evolution as Illustrated by the Fossil Record*, A. Hallam, Ed. (Elsevier, Amsterdam, 1977), pp. 79–121; A. J. Rowell, *Lethaia* **15**, 299 (1982).
18. C. Nielsen, in *Biology of Bryozoans*, R. M. Woolacott and R. L. Zimmer, Eds. (Academic Press, New York, 1977), pp. 519–534; G. Jagersten, *Evolution of the Metazoan Life Cycle* (Academic Press, New York, 1972).
19. R. M. Gustus and R. A. Cloney, *Acta Zool. Stockholm* **53**, 229 (1972).
20. H. Wada and N. Satoh, *Proc. Natl. Acad. Sci. U.S.A.* **91**, 1801 (1994); M. J. Telford and P. W. H. Holland, *Mol. Biol. Evol.* **10**, 660 (1993).
21. L. H. Hyman, *Biol. Bull.* **114**, 106 (1958); K. M. Rudall and W. Kenchington, *Biol. Rev. Camb. Philos. Soc.* **49**, 597 (1973).
22. We thank C. Marshall, G. Freeman, J. Morin, J. Bull, M. Kirkpatrick, and two anonymous reviewers for helpful comments and insights, M. Badgett for laboratory assistance, and the Friday Harbor Labs for providing facilities for some of the work of K.M.H. This research was supported by NSF grants to D.M.H. and to J.A.L.

2 August 1994; accepted 5 January 1995

Sodefrin: A Female-Attracting Peptide Pheromone in Newt Cloacal Glands

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A decapeptide called sodefrin was isolated from the abdominal gland of the cloaca of the male red-bellied newt, *Cynops pyrrhogaster*. The native peptide, as well as the synthetic one, had a female-attracting activity. Sodefrin was found in the apical portion of the epithelial cells of the abdominal gland. Sodefrin is apparently species specific because it did not attract females of *Cynops ensicauda*. This is the first amphibian pheromone to be identified and the first peptide pheromone identified in a vertebrate.

In urodeles, chemical stimuli contribute to sex recognition and courtship behavior (*1*). It has been suggested that males emit olfactory attractants or pheromones to lure females (*2*). The abdominal gland of the cloaca has been thought to be the site where these substances are produced (*3*). We have found that the water in which sexually active male newts (*Cynops pyrrhogaster*) were kept attracted conspecific females (*4*). The attractant pheromone was secreted by or through the abdominal gland of the cloaca because the water in which abdominal gland–ablated males had been kept did not attract females (*4*).

We report here the isolation and characterization of the female-attracting pheromone from the abdominal glands of male newts. Female-attracting pheromone activity was monitored by a preference test (*5*). An aqueous extract of the abdominal glands had a female-attracting pheromone activity. The minimum effective amount of extract in a sponge block that attracted a female placed in a container filled with 3000 ml of water was the equivalent of 0.1% of the abdominal gland content (Fig. 1). The active substance in the abdominal gland was soluble in water but not in organic solvent. When the water-soluble fraction was subjected to gel-filtration column chromatography, the female-attracting pheromone activity emerged in a fraction with a relative molecular mass below 5000. Pronase digestion eliminated the activity, indicating that the active substance is a peptide.

To isolate the active peptide from an aqueous extract of the abdominal glands, we used two purification cycles of reversed-

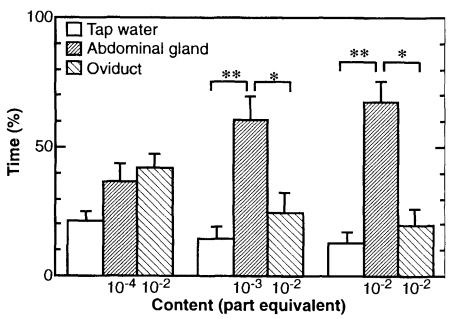

Fig. 1. Female-attracting activity in the abdominal gland of the male newt. Twenty abdominal glands were homogenized in 20 ml of distilled water. The homogenate was centrifuged at 5000*g* for 1 hour at 4°C. The supernatant was lyophilized and was used as the test substance. Oviducal extract was prepared similarly and was used as a control substance. Preference testing was done as described (*5*). Each sponge block contained tap water or extract from the indicated amount of the abdominal glands or oviduct. Results represent mean values (± SE) of eight tests. *Probability of significant difference (P) < 0.05; **P < 0.01.

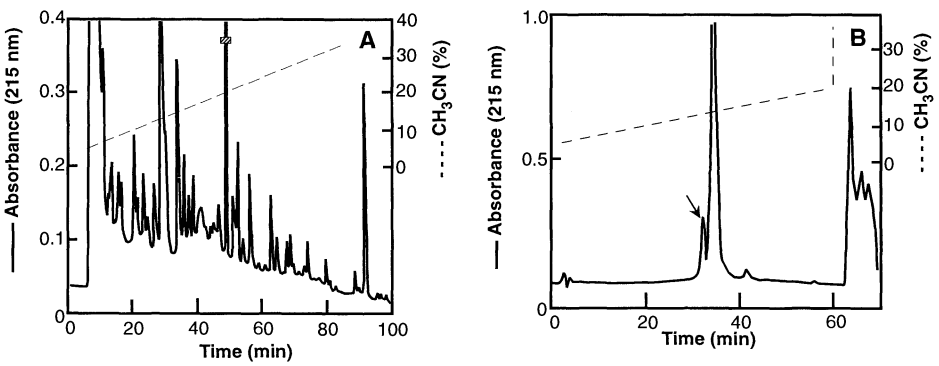

Fig. 2. Purification of female-attracting substance from the abdominal gland of the male newt. (**A**) The first reversed-phase HPLC. Fifty abdominal glands were homogenized in 100 ml of distilled water. After centrifugation at 5000*g* for 1 hour, the supernatant was lyophilized, dissolved in 0.15 M NH_4HCO_3 (pH 8.0), and applied to a Sephadex G-100 column (2.5 cm by 80 cm; Pharmacia) equilibrated with the same solution. Fractions with relative molecular mass below 5000, which had female-attracting activity, were pooled and lyophilized. The lyophilized sample was dissolved in 0.1% trifluoroacetic acid (TFA) and applied to an octadecyl silyl–silica cartridge (C_{18} Sep-Pak; Waters). The adsorbed substances were eluted with acetonitrile containing 0.1% TFA. The lyophilized C_{18}-adsorbed fraction was dissolved in 0.1% TFA and applied to a 4 mm by 125 mm column [Superspher 60 RP-8(e) column; Merck] equilibrated with 0.1% TFA. A gradient of acetonitrile was used at a flow rate of 1 ml min^{-1}. The active fraction, designated by the small hatched bar on the appropriate peak, was collected. (**B**) The second reversed-phase HPLC. The active fraction from (A) was further purified on a 4 mm by 150 mm phenyl column (Inertsil; Gas-Liquid Science, Tokyo) with a gradient of acetonitrile at a flow rate of 1 ml min^{-1} in the presence of 0.1% TFA. Female-attracting activity was detected in the peak fraction designated by an arrow. Detection of female-attracting activity was done as described (*5*). Yield of the final product was 0.6 μg per gland.

phase high-performance liquid chromatography (HPLC) (Fig. 2). Direct sequencing of the final product with a pulsed gas-liquid phase protein sequencer revealed that it is a decapeptide with amino acid sequence Ser-Ile-Pro-Ser-Lys-Asp-Ala-Leu-Leu-Lys. Its amino acid composition, determined by acid hydrolysis, was Asp, 1.10; Ser, 2.01; Pro, 1.03; Ala, 1.10; Ile, 1.09; Leu, 2.21; and Lys, 2.13. Thus, the amino acid composition data are in agreement with the amino acid residues deduced by peptide sequencing. COOH-terminal analysis by carboxypeptidase-P digestion revealed that the terminus consisted of a free Lys residue. The relative molecular mass of 1071.2, estimated from fast atom bombardment mass spectrometry (6), corresponds with that calculated from the amino acid sequence.

The peptide showed no sequence homology with any known peptide (7). Thus, the peptide was designated sodefrin (derived from the ancient Japanese word "sodefuri," meaning "soliciting"), reflecting its possible function as a sex attractant.

Ten nanograms of native sodefrin absorbed on a block of sponge were enough to attract female newts placed in a container filled with 3000 ml of water. Synthetic sodefrin (8) exhibited female-attracting activity similar to that of the native material. Female newts could recognize a sponge block containing the minimum effective amount (10 ng) of sodefrin even when the container was filled with sodefrin solution diluted to 0.1 pM or less, but not when it was filled with more concentrated sodefrin solutions (1 pM or more). This indicates that the sponge block was releasing enough sodefrin to keep the concentration in the neighboring water above 0.1 pM and below 1 pM and that the minimum effective concentration lies in the range 0.1 to 1.0 pM (Fig. 3).

Frozen sections of abdominal glands stained with antiserum to sodefrin by immunofluorescence (9) showed that the apical region of the epithelial cells was positive for sodefrin (Fig. 4).

In order to observe the effect of sodefrin on a congeneric species of newt, we chose the sword-tailed newt (*Cynops ensicauda*) to use as a model. Although *C. ensicauda* females were insensitive to sodefrin, they were attracted to a water extract of abdominal glands from males of their own species. On the other hand, *C. pyrrhogaster* females responding to sodefrin were not attracted to the water extract of the abdominal glands from *C. ensicauda* males (Fig. 5). These results indicate that the female-attracting substances differ between these two species of the genus *Cynops*.

The existence of pheromones in the class Amphibia has long been postulated. We isolated and characterized a pheromonal peptide with a female-attracting activity in Amphibia. Various vertebrate pheromones have been chemically identified. The majority of the pheromones from terrestrial animals are volatile in nature (*10*), with a few exceptions (*11*). In teleosts, compounds such as prostaglandins (*12*), steroids (*13*), pteridine derivatives (*14*), and lipids (*15*) have been identified as pheromones. In this class of animals, the presence of protein- or peptide-like

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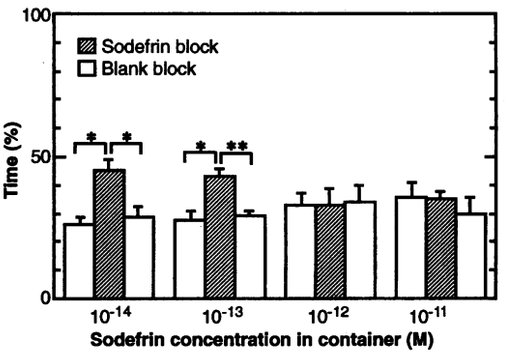
Fig. 3. Determination of the minimum effective concentration of sodefrin. One sponge block with 10 ng of synthetic sodefrin and two blank blocks were placed in a container filled with 3000 ml of the indicated concentrations of sodefrin solution. The minimum effective concentration of sodefrin was estimated from the concentration of sodefrin solution that blocked the attracting activity of sodefrin released from the sponge. Preference tests were done as described (5). Results represent mean values (± SE) of eight tests. $*P < 0.05$; $**P < 0.01$.



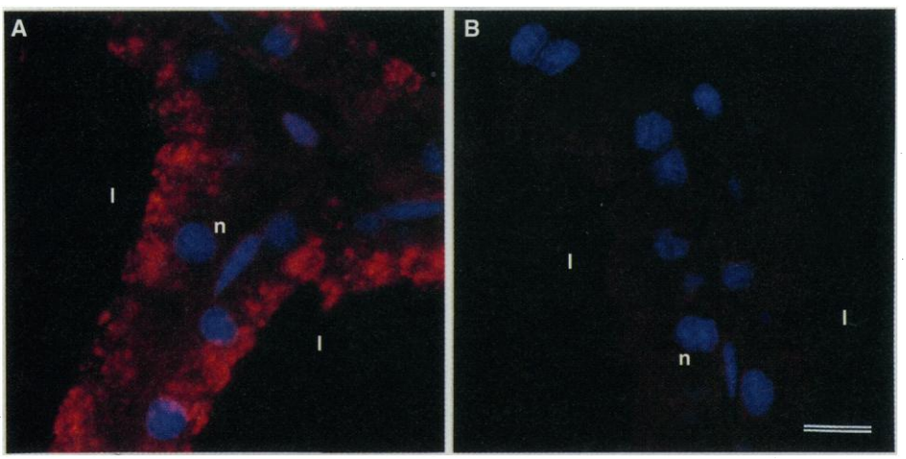


Fig. 4. Localization of sodefrin in the abdominal gland. Immunohistochemical examination of sodefrin localization was done as described (9). (**A**) Abdominal gland stained with antiserum to sodefrin. (**B**) Abdominal gland stained with absorbed antiserum. There is a reduction of the immunofluorescence in (B) as compared with (A). The specimens were examined with a microscope (Olympus BX50) equipped with BX fluorescence attachment. Images were photographed as double exposures. l, lumen; n, nucleus. Bar is 25 μm.

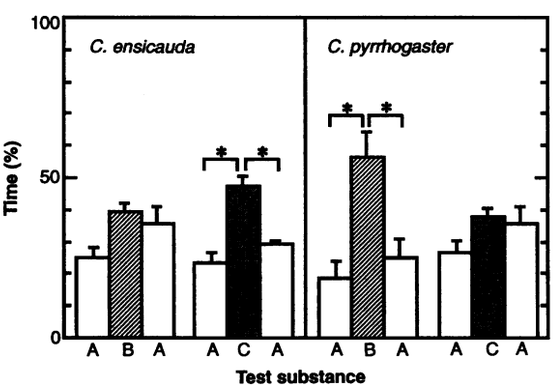


Fig. 5. Attracting effect of sodefrin on females of two different species of the genus *Cynops*. Test females were *C. ensicauda* and *C. pyrrhogaster*. Each sponge block contained tap water (A), 100 ng of sodefrin (B), or a water extract from 1/100th of the *C. ensicauda* abdominal gland (C). Preference tests were done as described (5). Results represent mean values (± SE) of eight tests. $*P < 0.05$.

pheromones has been suggested (*16*), but these putative pheromones have never been isolated. Given the fact that sexually mature newts lead an aquatic life, a nonvolatile, but water-soluble, peptide is a reasonable form to expect as a pheromone in this vertebrate.

REFERENCES AND NOTES

1. S. N. Salthe and J. S. Mecham, in *Physiology of the Amphibia*, B. Lofts, Ed. (Academic Press, New York, 1974), vol. 2, pp. 309–521; L. D. Houck, in *Chemical Signals in Vertebrates*, D. Duvall, D. Müller-Schwarze, R. M. Silverstein, Eds. (Plenum, New York, 1986), vol. 4, pp. 173–190.
2. L. Cedrini and A. Fasolo, *Monitore Zool. Ital.* **5**, 223 (1971); G. Malacarne and C. Vellano, *Behav. Processes* **7**, 307 (1982).
3. G. Malacarne, L. Bottoni, R. Massa, C. Vellano, *Monitore Zool. Ital.* **18**, 33 (1984).
4. F. Toyoda, S. Tanaka, K. Matsuda, S. Kikuyama, *Physiol. Behav.* **55**, 569 (1994).
5. A plastic container (diameter, 37 cm) was filled with 3000 ml of tap water. Eight test females received daily injections by us of 1 IU of prolactin and 25 IU of human chorionic gonadotropin for 7 to 11 days in order to secure their responsiveness to the sex attractant. On the day after the last injection, each test animal was put in a smaller cylinder of stainless steel mesh that was placed in the center of the plastic container. The container was divided into three sectors into which three sponge blocks with dimensions 5.6 cm by 7.3 cm by 3.4 cm (one per sector) were gently placed. One block contained the test substance dissolved in 100 ml of water, and the others contained control substances. Thirty seconds after the introduction of the sponge blocks, the inner cylinder was removed. The position of the snout of the test animal was observed, and the time spent by the snout in each sector was recorded by video for 10 min. In a series of tests, the test females were not used more than once. The percentage of the period spent by the snout of the test animal in each sector was analyzed statistically with Friedman's two-way analysis of variance, followed by the Wilcoxon matched-pairs signed-ranks test (*4*).
6. H. Rodriguez, B. Nevins, J. Chakel, in *Techniques in Protein Chemistry*, T. E. Hugli, Ed. (Academic Press, San Diego, CA, 1989), pp. 186–194.
7. Homology search of the peptide was done with the National Biomedical Research Foundation Protein Data Base, Release 38: Genetyx (Software Development, Tokyo).
8. Synthetic sodefrin was prepared by solid-phase chemistry (American Peptide, Sunnyvale, CA).
9. We generated an antiserum to sodefrin in a rabbit by injecting sodefrin that was extended on its COOH-terminus with Cys coupled to keyhole limpet hemocyanin (Pierce). Abdominal glands were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 24 hours at 4°C. Frozen sections were cut at a thickness of 6 μm. The sections were incubated with 20% normal goat serum for 30 min before a 2-hour incubation with antiserum to sodefrin diluted at 1:1000 with 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) or with antiserum (1:1000 in 1 ml) absorbed with sodefrin (1 nmol). After the sections were washed with PBS, they were incubated with a mixture of rhodamine-labeled, affinity-purified goat antibody to rabbit immunoglobulin G (Jackson Immunoresearch) diluted with BSA (1%) in PBS and 4′,6-diamidino-2-phenylindole dihydrochloride (2 μg/ml) [K. Takata, T. Kasahara, M. Kasahara, O. Ezaki, H. Hirano, *J. Histochem. Cytochem.* **39**, 287 (1991)].
10. M. Novotony, B. Jemiolo, S. Harvey, in *Chemical Signals in Vertebrates*, D. W. Macdonald, D. Müller-Schwarze, S. E. Natynczuk, Eds. (Oxford Univ. Press, Oxford, 1990), vol. 5, pp. 1–21.
11. R. T. Mason *et al.*, *Science* **245**, 290 (1989).
12. P. W. Sorensen, T. J. Hara, N. E. Stacey, F. W. Goetz, *Biol. Reprod.* **39**, 1039 (1988).
13. L. Colombo, A. Marconato, P. C. Belvedere, C. Friso, *Boll. Zool.* **47**, 355 (1980); N. E. Stacey and P. W. Sorensen, *Can. J. Zool.* **64**, 2412 (1986).
14. W. Pfeiffer, *J. Chem. Ecol.* **4**, 665 (1978).
15. P. Pfuderer, P. Williams, A. A. Francis, *J. Exp. Zool.* **187**, 375 (1974).
16. D. Tucker and N. Suzuki, in *Olfaction and Taste*, D. Schneider, Ed. (Wissenschaftliche Verlagsgesellschaft, Stuttgart, 1972), vol. 4, pp. 121–127; N. Ye. Lebedeva, G. A. Malyukina, A. O. Kasumyan, *J. Ichthyol.* **15**, 472 (1975); H. Okada, K. Sakai, K. Sugiwaka, *Sci. Rep. Hokkaido Fish Hatch.* **33**, 89 (1979).

17. Care of experimental animals was in accordance with institutional guidelines. We thank M. Ohashi and S. Kurono for their help with mass spectrometry. Supported by grants-in-aid from the Ministry of Education, Science, and Culture of Japan (to S.K. and F.T.) and by a research grant from Waseda University (to S.K.).

17 August 1994; accepted 28 December 1994

Epithelial Antibiotics Induced at Sites of Inflammation

Barry S. Schonwetter,* Ethan D. Stolzenberg, Michael A. Zasloff

The role of antimicrobial peptides in epithelial defense is not fully understood. An epithelial β-defensin, lingual antimicrobial peptide (LAP), was isolated from bovine tongue and the corresponding complementary DNA cloned. LAP showed a broad spectrum of antibacterial and antifungal activities. LAP messenger RNA abundance was markedly increased in the epithelium surrounding naturally occurring tongue lesions. This increase coincided with the cellular hallmarks of acute and chronic inflammation in the underlying lamina propria, supporting a role for epithelial antimicrobial peptides as integral components of the inflammatory response.

The epithelia of vertebrates provide the first line of defense between organism and environment (*1*). When this barrier is breached, microorganisms invade and an acute inflammatory response occurs (*2*). The physical barrier is fortified by the secretion of numerous antibacterial agents, including immunoglobulin antibodies, enzymes such as lysozyme, and proteins such as lactoferrin (*3*). Antimicrobial peptides have also been detected in barrier epithelial cells of several mammalian species, including mice, cows, and humans (*4*–*6*). Although the expression of antimicrobial molecules in epithelia suggests that they may participate in host defense, no direct evidence has been obtained to support such a role.

Mammalian tongue contains a dense epithelium that is constantly colonized by the microbial biota of the mouth, which includes bacteria, fungi, and viruses (*7*). Although abrasions to the surface of the tongue occur often, invasive infections in a normal host are rare, remain localized, and heal rapidly. Invasive infections of the tongue would interfere with the processes of chewing, swallowing, taste, and speech (*8*).

Why is this exposed surface free of continuous infection? We approached this prob-

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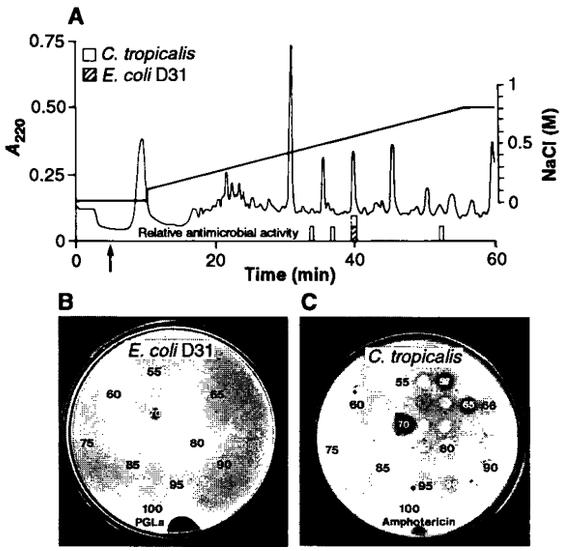

Fig. 1. Purification of lingual antimicrobial peptide (LAP). (**A**) Strong cation exchange chromatography of bovine tongue epithelial extract. (**B**) Antimicrobial assay of fractions against *Escherichia coli* D31. (**C**) Antimicrobial assay of fractions against *Candida tropicalis*. Antimicrobial activity against *E. coli* D31 was detected in fraction 70 (B), corresponding to a peak [in absorbance at 220 nm (A_{220})] eluting at 40 min (A). Antimicrobial activity against *C. tropicalis* was detected in fractions 57, 65, 70, and 96 (C), corresponding to peaks with retention times of 33.5, 37.5, 40, and 53 min (A), respectively. PGLa (5 μg) was used as a control for *E. coli* D31 activity (B), whereas amphotericin B (5 μg) is active against *C. tropicalis* (C).