Chem. Senses 29: 425–430, 2004 DOI: 10.1093/chemse/bjh044

# Natural Ligands of Hamster Aphrodisin

**Loïc Briand[1], Florence Blon[1], Didier Trotier[2] and Jean-Claude Pernollet[2]**

[1]Biochimie de l'Olfaction et de la Gustation, Neurobiologie de l'Olfaction et de la Prise Alimentaire, bâtiment 526, INRA, Domaine de Vilvert, F-78352 Jouy-en-Josas Cedex, France and [2]Laboratoire de Neurobiologie Sensorielle, Ecole Pratique des Hautes Etudes, CNRS, 1 avenue des Olympiades, F-91305 Massy, France

*Correspondence to be send to: J.-C. Pernollet, Neurobiologie de l'Olfaction et de la Prise Alimentaire, bâtiment 526, Domaine de Vilvert, F-78352, Jouy-en-Josas Cedex, France, Tel: 33 1 34 65 27 50; Fax: 33 1 34 65 27 65; E-mail: pernolle@jouy.inra.fr*

## Abstract

The chemical nature of vertebrate pheromones remains largely to be deciphered. Hamster aphrodisin is a rare instance of mammal proteinaceous sexual pheromone. This protein, found in vaginal secretions, facilitates the mounting behaviour of males via activation of a specialized sensory structure named the vomeronasal organ, which activates the accessory olfactory bulb. Since it might carry small pheromonal ligands due to its lipocalin structure, we analysed organic extracts from natural aphrodisin. We identified five predominant compounds specifically bound onto natural aphrodisin as 1-hexadecanol (44.7%), 1-octadecanol (19.5%), *Z*-9-octadecen-1-ol (18.2%), *E*-9-octadecen-1-ol (15.4%) and hexadecanoic acid (2.2%). Interestingly, these compounds are also described as part of insect pheromone blends, disclosing the continuing story of amazing coincidences of chemical communication shared by mammals and insects.

**Key words**: aphrodisin, hamster, pheromone, vaginal secretion, vomeronasal organ

## Introduction

Animals emit chemical information, called pheromones, into their environment via diverse secretion fluids for inter-individual communication among the same species, which favour reproduction and social life organization. In many species, in contrast with the large number of well-documented sex attractants in insects, mammal pheromones are still poorly identified chemical cues (Dulac and Torello, 2003; Schaal *et al.*, 2003; Brennan and Keverne, 2004). Female hamster vaginal secretions contain aphrodisiac substances that facilitate the mating behaviour of young males. The aphrodisiac effect is related to the presence of a protein, named aphrodisin. Aphrodisin is a 17 kDa soluble glycosylated protein originally isolated from hamster vaginal discharges, in which it occurs in relatively high concentrations (5 µg/mg of discharge) (Singer *et al.*, 1986; Henzel *et al.*, 1988). The aphrodisin gene is expressed in vagina, uterus, Bartholin's glands and also in female hamster parotid glands (Kruhøffer *et al.*, 1997; Mägert *et al.*, 1999; Thavathiru *et al.*, 1999). Aphrodisin stimulates the male copulatory behavior via the vomeronasal organ (Clancy *et al.*, 1984; Kroner *et al.*, 1996; Jang *et al.*, 2001), but occurs in vaginal discharges before females reach fertility, suggesting another unknown function for aphrodisin (Mägert *et al.*, 1999).

Aphrodisin consists of 151 amino acids with two disulfide bonds and a blocked *N*-terminus (pyroglutamic acid). Each *N*-glycosylation site (N41 and N69) is linked with only one *N*-acetylglucosamine residue (Singer *et al.*, 1986). Aphrodisin belongs to the lipocalin superfamily, whose β-barrel structure is well adapted to bind small hydrophobic molecules, as evidenced with the recombinant protein (Briand *et al.*, 2000a). Moreover, crystallographic studies of recombinant aphrodisin suggest the presence of a serendipitous artifactual ligand in the binding pocket (Vincent *et al.*, 2001). Among lipocalins, aphrodisin is highly homologous (40% sequence identity) with rat odorant-binding proteins OBP-1 and 1F, which are thought to carry volatile odorants towards olfactory receptors through the nasal mucus (Briand *et al.*, 2000b). In insects, another class of pheromone-carrier proteins with roles analogous to vertebrate OBPs has been described. Insect pheromone-binding proteins (PBPs), like general OBPs, are structurally different from lipocalins without evolutionary relationships (Steinbrecht, 1998). In contrast to aphrodisin, which exhibits a classical lipocalin fold made of an eight-stranded anti-parallel β-barrel, insect

PBPs are all-helical disulfide-rich proteins (Sandler *et al.*, 2000; Lartigue *et al.*, 2004; Mohanty *et al.*, 2004).

In spite of numerous studies on the pheromonal properties of aphrodisin, it has still not been definitely determined if aphrodisin has pheromonal activity by itself or needs the combination with low molecular mass ligands to be active (Singer *et al.*, 1984; Singer and Macrides, 1990; Briand *et al.*, 2004). Its homology with OBP suggests a role as a lipophilic compound carrier, but it could also intrinsically act as a pheromone by direct activation of neuron receptors of the vomeronasal organ. Aphrodisin can therefore be compared to other rodent lipocalins, the major urinary proteins (MUPs), occurring at high level in mouse urine, which bind small airborne chemicals, endowed with a pheromonal activity. However, when deprived of ligands, MUPs play an intrinsic pheromonal role in individual recognition (Hurst *et al.*, 2001). Consequently, the question of whether the aphrodisiac properties of aphrodisin are solely borne by the protein or need the presence of bound small pheromones is still to be resolved.

To identify aphrodisin natural ligands, we prepared crude natural aphrodisin extracted from vaginal secretions, avoiding any contact with plastic, which may contaminate carrier protein (Lartigue *et al.*, 2004). We extracted the protein and identified five non-covalent compounds, specifically bound onto natural aphrodisin.

## Materials and Methods

### Collection of hamster vaginal secretions

To complete natural aphrodisin extraction, two separate collections of Syrian golden hamster (*Mesocricetus auratus*) vaginal secretions were made from five mature animals, three times a week over a period of 4 months. Hamsters (120–160 g) were housed individually in glass cages with fresh bedding so they had no contact with plastic. All experiments followed the rules of French Ministries (Agriculture; Research and Technology). Vaginal discharges were obtained by tactile genital stimulation of slightly anesthetized females using a cotton swab impregnated with MilliQ $H_2O$. Cotton swabs obtained from five females were then placed in a 5 ml glass tube with 1 ml MilliQ and frozen immediately at –20°C until used.

### Preparation of crude aphrodisin from vaginal secretions

Immediately prior to use, diluted vaginal secretions corresponding to 115 samples (~35 ml) were defrosted and pooled. All procedures were carried out at 4°C. Cotton swabs were removed and the resulting solution clarified by centrifugation at 10 000 × *g* for 20 min. After filtration (0.22 µm), diluted vaginal secretions were concentrated to 1 ml using a YM-10 ultrafiltration membrane (Amicon). The experiments were duplicated separately on the two independent vaginal secretion collections.

### Electrophoresis, *N*-terminal sequencing and peptide mass finger printing

SDS/PAGE (16% acrylamide) was performed using a Mini-Protean II system (Bio-Rad, France). The molecular mass calibration kits low range and polypeptides (Bio-Rad) were used and the proteins stained with Serva blue G. The broad electrophoretic band corresponding to aphrodisin was excised and submitted to *N*-terminal sequencing using a Procise Applied Biosystems sequencer after passive transfer onto PVDF membrane. The excised electrophoretic band was also subjected to trypsinolysis for peptide mass fingerprinting using a Perkin Elmer Voyager DE-STR MALDI-TOF spectrometer. This approach confirmed that the crude preparation contained essentially aphrodisin.

### Determination of aphrodisin natural ligands

#### *Ligand extraction*

As controls for putative contaminants, 1 mg of bovine α-lactalbumin (Sigma) and of recombinant aphrodisin were prepared in 35 ml of 25 mM ammonium acetate buffer pH 8.0, treated and concentrated like crude aphrodisin. For ligand extraction, crude aphrodisin, recombinant aphrodisin and α-lactalbumin were concentrated to 50 µl using a YM-3 Microcon centrifugal unit (Amicon). As estimated by SDS-PAGE, ~2.5 mg of natural aphrodisin and control proteins (50 µl) were first denatured with 100 µl of 9 M GuCl by 21-h incubation in a sealed tube at 35°C. Proteins were then digested with 750 µl of 30% (wt/wt) proteinase K (Sigma) in 50 mM phosphate buffer, pH 8.0 by overnight incubation at 35°C. The reaction mixtures were then placed on ice and extracted with 50 µl of $CHCl_3$. After centrifugation at 10 000 × *g* for 30 min at 4°C, the extracts were analysed by GC/MS. An aliquot of ammonium acetate buffer (50 µl) was treated with the same denaturation, proteolysis and extraction procedures and the chloroform extract was analysed by GC/MS as a blank.

#### *GC/MS identification*

Analyses were conducted on a gas chromatograph (6890N; Agilent Technologies Inc.) coupled to a mass spectrometer detector (5973N; Agilent Technologies Inc.). Samples (2 µl) were injected, via a purged-splitless injection system, on a non-polar capillary column (HP-5MS, 30 m × 0.25 mm, 0.25 µm film thickness; Agilent Technologies Inc.). The oven temperature gradient applied was 25°C for 5 min, then 5°C/min to 240°C and held there for 10 min. The carrier gas was helium at 1 ml/min. The column was directly connected to the mass-sensitive detector by an interface heated at 280°C. The electron impact energy was set at 70 eV and mass spectra were recorded in the range of 20–800 atomic mass units. Pheromones were identified by comparison of their retention time in gas chromatography and fragmentation in mass spectroscopy with mass spectra of NIST98 library. Candidate ligands were further confirmed by matching the

retention times and mass spectra with those of commercially available synthetic material purchased from Sigma. The relative abundance of each ligand was determined from their integrated peak areas. Similar results were obtained with crude aphrodisin from the second vaginal secretion collection.

## Results

To identify putative molecules bound onto natural aphrodisin, we collected vaginal secretions from mature Syrian golden hamsters (*M. auratus*), avoiding any contact with plastic, which may contaminate carrier proteins (Lartigue *et al.*, 2004). As already described, hamster vaginal secretions mainly contain aphrodisin, with some other proteins and small peptides in minute amounts (Briand *et al.*, 2000a). Thus, in order to avoid ligand release in the course of purification, we limited purification to a crude aphrodisin preparation. SDS–PAGE analysis of the preparation (Figure 1) revealed a broad electrophoretic band migrating around 17 kDa. The excised electrophoretic band was submitted to trypsinolysis for peptide mass fingerprinting. This approach confirmed that the crude preparation contained essentially aphrodisin. *N*-Terminal sequencing comfirmed the presence of aphrodisin, partially *N*-terminal unblocked, without protein contaminant in appreciable amount. So, this preparation, devoid of free small molecules, did not contain proteins in significant amount other than aphrodisin and was therefore called 'crude aphrodisin'. Using a calibrated solution of recombinant aphrodisin produced by the yeast *Pichia pastoris* (Briand *et al.*, 2000a), the amount of prepared crude aphrodisin was estimated to be ~2.5 mg.

To release non-covalently bound ligands, the protein was denatured and thoroughly proteolyzed. The same procedure was applied to recombinant aphrodisin produced by *P. pastoris*, which is similar to the natural aphrodisin (Briand *et al.*, 2000a), but did not make contact with the vaginal secretions and to α-lactalbumin, a protein that does not bind hydrophobic molecules. Chloroform extracts of digested proteins were analysed by gas chromatography coupled with mass spectrometry. We identified five predominant compounds specifically present in natural aphrodisin, but not in control proteins, as 1-hexadecanol (44.7%), 1-octadecanol (19.5%), *Z*-9-octadecen-1-ol (18.2%), *E*-9-octadecen-1-ol (15.4%) and hexadecanoic acid (2.2%) (Figure 2). These ligands were further confirmed by matching the retention times and mass spectra with those of commercially available synthetic material (Figure 3). The relative abundance of each compound revealed that ~45% aphrodisin was bound with ligands. Dibutylphthalate, a contaminant originating from unavoidable plasticware, was also found in all protein extracts in equivalent trace amounts.

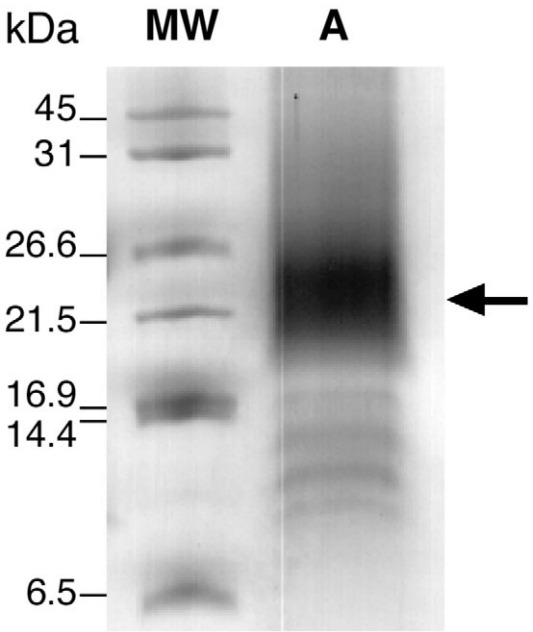


**Figure 1.** SDS–PAGE of crude aphrodisin preparation. MW, mol. wt standards (Bio-Rad low range and polypeptide standards); A, crude aphrodisin preparation; aphrodisin is indicated by an arrow. Gel was stained using Serva blue G.

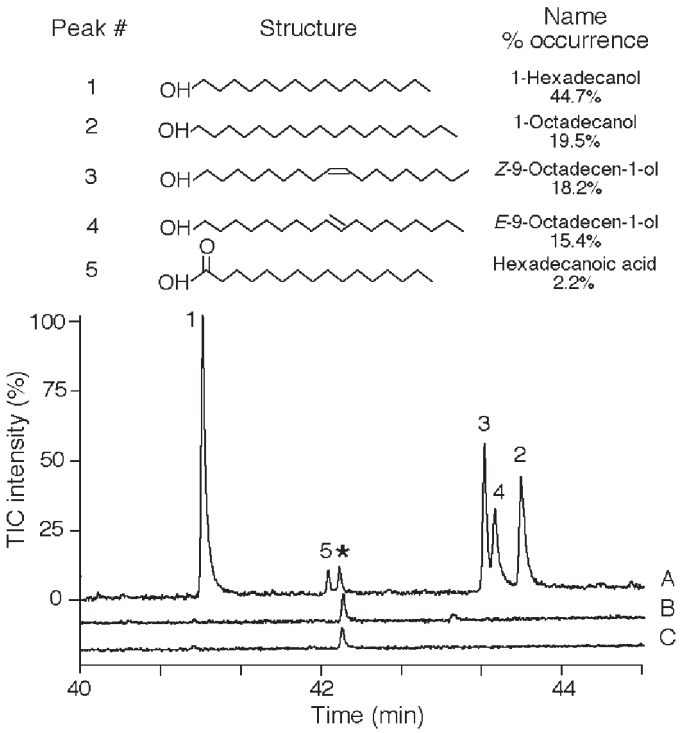


**Figure 2.** Gas chromatograms of the chloroform extract of natural aphrodisin **(A)**, recombinant aphrodisin **(B)** and α-lactalbumin **(C)**. Compounds were identified from their retention time and mass spectra coinciding with data from authentic commercial samples. Compound chemical structures are shown with their percentage occurrence. Contaminating dibutylphthalate is indicated by a star.

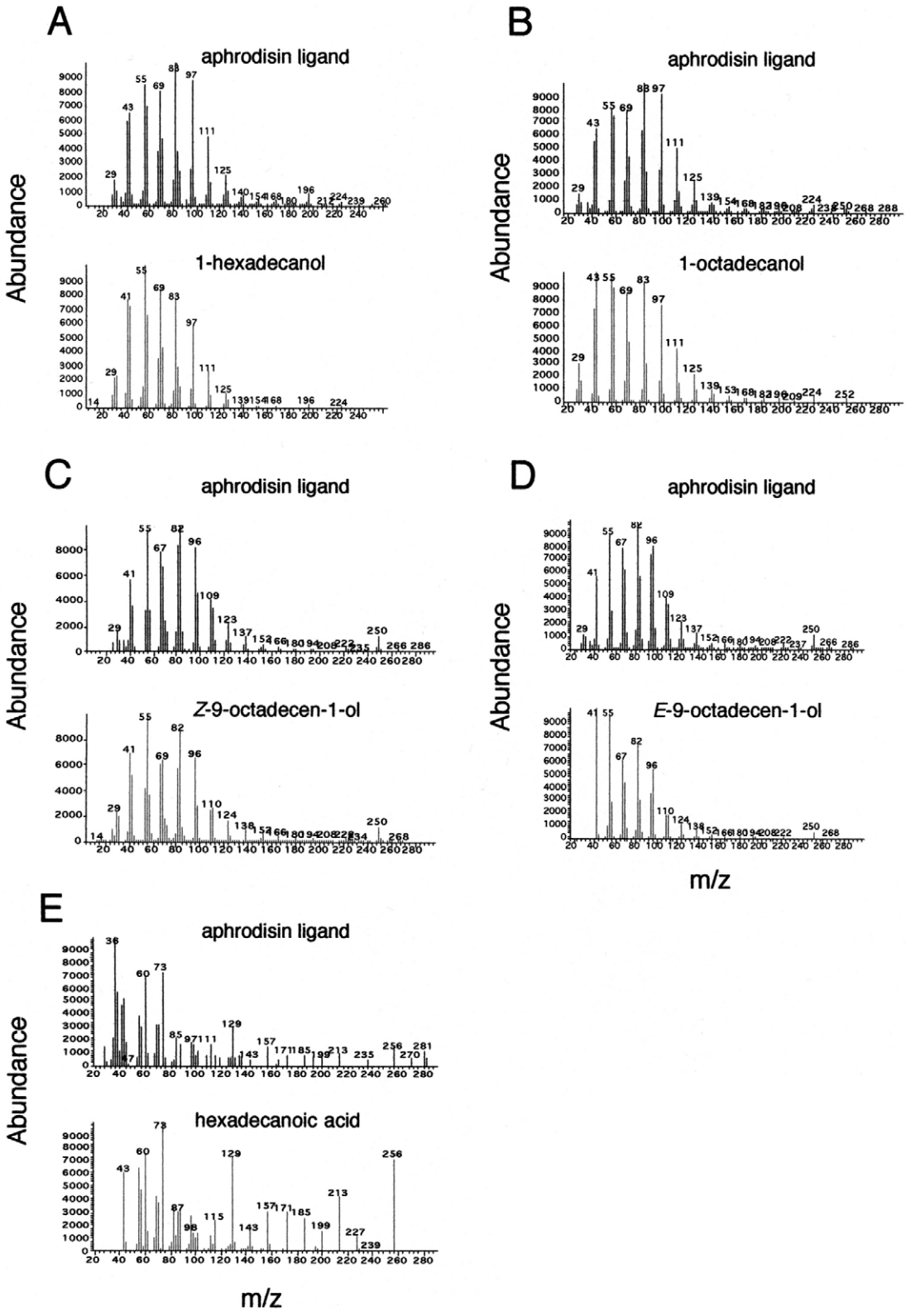


**Figure 3.** Mass spectra of the major compounds extracted from aphrodisin compared with those of pure compounds. Mass spectra of the main peaks identified (top) and of the authentic compounds (bottom): **(A)** 1-hexadecanol; **(B)** 1-octadecanol; **(C)** *Z*-9-octadecen-1-ol; **(D)** *E*-9-octadecen-1-ol; **(E)** hexadecanoic acid.

## Discussion

Up to now, all attempts to identify the endogenous ligand of native aphrodisin have been unsuccessful (Singer *et al.*, 1986; Henzel *et al.*, 1988; Singer, 1991; Petrulis and Johnston, 1995; Ramoni *et al.*, 2001), although identification of lipocalin-bound compounds based on mass spectroscopy and X-ray structure has been achieved in the past, for example in the case of MUPs, in which four pheromonal components were identified as copurified ligands (Böcskei *et al.*, 1992) and bovine OBP, in which a product of rumination, 1-octen-3-ol has been observed (Ramoni *et al.*, 2001). In the present study, owing to the large amounts of collected aphrodisin in vaginal secretions and to our mode of crude aphrodisin purification, we report the identification of five compounds bound into natural aphrodisin. Moreover, the complexes aphrodisin/ligands were well preserved during the preparation, since 45% of aphrodisin was found with bound ligands. Interestingly, these components are also described in insects, especially in the Lepidoptera (for example, 1-hexadecanol in *Chilo zacconius* and 1-octadecanol and hexadecanoic acid in *Myelois cribrella*), as part of female pheromone blends to attract males (Arn *et al.*, 1992). This is not the only case of coincident substrates for sexual chemical communication shared in common by mammals and insects (Kelly, 1996). For instance, two Asian elephant pheromones are also described as insect pheromones. *Z*-7-dodecen-1-yl acetate from the pre-ovulatory urine of female Asian elephants has been identified as a sex attractant for moths (Rasmussen *et al.*, 1996) and frontalin (1,5-dimethyl-6,8-dioxabicyclo[3.2.1]octane), a bicyclic ketal, released from the temporal gland, is a well-studied pheromone in insects (Rasmussen and Greenwood, 2003). The present findings, demonstrating the presence, on natural aphrodisin, of ligands, which are pheromones in unrelated species, raises the question of the intrinsic aphrodisiac activity of unliganded aphrodisin. Thus, aphrodisin would be either a pheromone carrier protein or, like rat MUP, would have a pheromone transport function, in addition to its own pheromonal role (Krieger *et al.*, 1999). Interestingly, the sex pheromone present in the pre-ovulatory urine of female Asian elephants has been demonstrated to interact with a PBP, abundant in the mucus of the trunk, which is homologous to vertebrate OBPs (Lazar *et al.*, 2002). The high homology of aphrodisin with OBPs suggests that at least one function of aphrodisin would be to transport the reported lipophilic ligands through the aqueous mucus to the vomeronasal receptor cells.

We now aim at defining the pheromonal function of the blend or single components extracted from aphrodisin, complexed or not with recombinant aphrodisin. These investigations will provide novels insights into the communication responsible for the fascinating behavioural effects of pheromones in mammals.

## Acknowledgements

The authors are grateful to Claire Schlegel for her precious help during collection of hamster vaginal secretions.

## References

**Arn, H.**, **Toh, M.** and **Priesner, E.** (1992) List of Sex Pheromones of Lepidoptera and Related Attractants. Monfavet, France: Int. Org. Biol. Control.

**Böcskei, Z.**, **Groom, C.R.**, **Flower, D.R.**, **Wright, C.E.**, **Phillips, S.E.**, **Cavaggioni, A.**, **Findlay, J.B.** and **North, A.C.** (1992) *Pheromone binding to two rodent urinary proteins revealed by X-ray crystallography*. Nature, 360, 186–188.

**Brennan, P.A.** and **Keverne, E.B.** (2004) *Something in the air? New insights into mammalian pheromones*. Curr. Biol., 14, R81–R89.

**Briand, L.**, **Huet, J.-C**, **Perez, V.**, **Lenoir, G.**, **Nespoulous, C.**, **Boucher, Y.**, **Trotier, D.** and **Pernollet, J.-C.** (2000a) *Odorant and pheromone binding by aphrodisin, a hamster aphrodisiac protein*. FEBS Lett., 476, 179–185.

**Briand, L.**, **Nespoulous, C.**, **Perez, V.**, **Rémy, J.-J.**, **Huet, J.-C.** and **Pernollet, J.-C.** (2000b) *Ligand-binding properties and structural characterization of a novel rat odorant-binding protein variant*. Eur. J. Biochem., 267, 3079–3089.

**Briand, L.**, **Trotier, D.** and **Pernollet, J.-C.** (2004) *Aphrodisin, an aphrodisiac lipocalin secreted in hamster vaginal secretions.* Peptides, in press.

**Clancy, A.N.**, **Macrides, F.**, **Singer, A.G.** and **Agosta, W.C.** (1984) *Male hamster copulatory responses to a high molecular weight fraction of vaginal discharge: effects of vomeronasal organ removal*. Physiol. Behav., 33, 653–660.

**Dulac, C.** and **Torello, A.T.** (2003) *Molecular detection of pheromone signals in mammals: from genes to behaviour*. Nat. Rev. Neurosci., 4, 551–562.

**Henzel, W.J.**, **Rodriguez, H.**, **Singer, A.G.**, **Stults, J.T.**, **Macrides, F.**, **Agosta, W.C.** and **Niall, H.** (1988) *The primary structure of aphrodisin*. J. Biol. Chem., 263, 16682–16687.

**Hurst, J.L.**, **Payne, C.E.**, **Nevison, C.M.**, **Marie, A.D.**, **Humphries, R.E.**, **Robertson, D.H.**, **Cavaggioni, A.** and **Beynon, R.J.** (2001) *Individual recognition in mice mediated by major urinary proteins*. Nature, 414, 631–634.

**Jang, T.**, **Singer, A.G.** and **O'Connell, R.J.** (2001) *Induction of c-fos in hamster accessory olfactory bulbs by natural and cloned aphrodisin*. Neuroreport, 12, 449–452.

**Kelly, D.R.** (1996) *When is a butterfly like an elephant?* Chem. Biol., 3, 595–602.

**Krieger, J.**, **Schmitt, A.**, **Löbel, D.**, **Gudermann, T.**, **Schultz, G.**, **Breer, H.** and **Boekhoff, I.** (1999) *Selective activation of G protein subtypes in the vomeronasal organ upon stimulation with urine-derived compounds*. J. Biol. Chem., 274, 4655–4662.

**Kroner, C.**, **Breer, H.**, **Singer, A.G.** and **O'Connell, R.J.** (1996) *Pheromone-induced second messenger signaling in the hamster vomeronasal organ*. Neuroreport, 7, 2989–2992.

**Kruhøffer, M.**, **Bub, A.**, **Cieslak, A.**, **Adermann, K.**, **Kunstyr, S.**, **Forssmann, W.-G.** and **Mägert, H.-J.** (1997) *Gene expression of aphrodisin in female hamster genital tract segments*. Cell Tissue Res., 287, 153–160.

**Lartigue, A.**, **Gruez, A.**, **Briand, L.**, **Blon, F.**, **Bezirard, V.**, **Walsh, M.**, **Pernollet, J.-C.**, **Tegoni, M.** and **Cambillau, C.** (2004) *Sulfur-SAD*

*crystal structure of a pheromone binding protein from the honeybee* Apis mellifera *L*. J. Biol. Chem., 279:4459–4464.

**Lazar, J.**, **Greenwood, D.R., Rasmussen, L.E.** and **Prestwich, G.D.** (2002) *Molecular and functional characterization of an odorant binding protein of the Asian elephant,* Elephas maximus*: implications for the role of lipocalins in mammalian olfaction*. Biochemistry, 41, 11786–11794.

**Mägert, H.-J.**, **Cieslak, A., Alkan, O., Lüscher, B., Kauffels, W.** and **Forssmann, W.-G.** (1999) *The golden hamster aphrodisin gene.* Structure, *expression in parotid glands of female animals, and comparison with a similar murine gene*. J. Biol. Chem., 274, 444–450.

**Mohanty, S.**, **Zubkov, S.** and **Gronenborn, A.M.** (2004) *The solution NMR structure of* Antheraea polyphemus *PBP provides new insight into pheromone recognition by pheromone-binding proteins*. J. Mol. Biol., 337, 443–451.

**Petrulis, A.** and **Johnston, R.E.** (1995) *A reevaluation of dimethyl disulfide as a sex attractant in golden hamsters*. Physiol. Behav., 57, 779–784.

**Ramoni, R.**, **Vincent, F., Grolli, S., Conti, V., Malosse, C., Boyer, F.D., Nagnan-Le Meillour, P., Spinelli, S., Cambillau, C.** and **Tegoni, M.** (2001) *The insect attractant 1-octen-3-ol is the natural ligand of bovine odorant-binding protein*. J. Biol. Chem., 276, 7150–7155.

**Rasmussen, L.E.** and **Greenwood, D.R.** (2003) *Frontalin: a chemical message of musth in Asian elephants (*Elephas maximus*).* Chem. Senses, 28, 433–446.

**Rasmussen, L.E.**, **Lee, T.D., Roelofs, W.L., Zhang, A.** and **Daves, G.D. Jr** (1996) *Insect pheromone in elephants*. Nature, 379, 684.

**Sandler, B.H.**, **Nikonova, L., Leal, W.S.** and **Clardy, J.** (2000) *Sexual attraction in the silkworm moth: structure of the pheromone-binding-protein–bombykol complex.* Chem. Biol., 7, 143–151.

**Schaal, B.**, **Coureaud, G., Langlois, D., Ginies, C., Semon, E.** and **Perrier, G.** (2003) *Chemical and behavioural characterization of the rabbit mammary pheromone*. Nature, 424, 68–72.

**Singer, A.G.** (1991) *A chemistry of mammalian pheromones*. J. Steroid Biochem. Mol. Biol., 39, 627–32

**Singer, A.G.** and **Macrides, F.** (1990) *Aphrodisin: pheromone or transducer?* Chem. Senses, 15, 199–203.

**Singer, A.G.**, **Clancy, A.N., Macrides, F.** and **Agosta, W.C.** (1984) *Chemical studies of hamster vaginal discharge: male behavioral responses to a high molecular weight fraction require physical contact*. Physiol. Behav., 33, 645–651.

**Singer, A.G.**, **Macrides, F., Clancy, A.N.** and **Agosta, W.C.** (1986) *Purification and analysis of a proteinaceous aphrodisiac pheromone from hamster vaginal discharge*. J. Biol. Chem., 261, 13323–13326.

**Steinbrecht, R.A.** (1998) *Odorant-binding proteins: expression and function*. Ann. N. Y. Acad. Sci., 855, 323–332.

**Thavathiru, E.**, **Jana, N.R.** and **De, P.K.** (1999) *Abundant secretory lipocalins displaying male and lactation-specific expression in adult hamster submandibular gland. cDNA cloning and sex hormone-regulated repression*. Eur. J. Biochem., 266, 467–476.

**Vincent, F.**, **Lobel, D., Brown, K., Spinelli, S., Grote, P., Breer, H., Cambillau, C.** and **Tegoni, M.** (2001) *Crystal structure of aphrodisin, a sex pheromone from female hamster.* J. Mol. Biol., 305, 459–469.

*Accepted April 6, 2004*