Affinities of Nutty and Green-smelling Pyrazines and Thiazoles to Odorant-binding Proteins, in Relation with their Lipophilicity

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Abstract

Thirty-two green- and/or nutty-smelling compounds, most of them pyrazine and thiazole derivatives, were tested in ligand binding assays with purified 19 kDa bovine and 22 kDa porcine olfactive binding proteins (OBPs). Unlike the nutty ones, all the green odorants were found to be good ligands for both proteins. Lipophilicity appears as an interesting discriminating physicochemical parameter, predictive of the affinity for OBPs and of the related odour profile. Chem. Senses 20: 601–608, 1995.

Introduction

The importance of the Maillard reactions in food quality, especially taste and aroma, has been recognized for many years (Vernin et al., 1992). Among the volatiles produced, pyrazines and thiazoles have received much attention due to their pleasant flavours and extremely low odour thresholds (sub-ppb). In particular, they have been identified as the compounds which directly contribute to the roasted or toasted flavours of cooked foods like bread, coffee, meats and to the malt-like flavours of beer (Narziss et al., 1986; Jayalekshmy et al., 1987; Humbert and Sandra, 1988; Blank et al., 1992; Mermet et al., 1992). Such heterocycles have also been found in raw products where they usually exhibit a green odour (Heymann et al., 1986; Harris et al., 1987; Allen et al., 1991; Lacey et al., 1991). Among them, 2-isobutyl-3-methoxypyrazine, the characteristic odorant of green bell-pepper, is used as a probe in this work.

A great many authors have identified such flavouring heterocycles in various food products (Maga, 1975, 1982; Vernin and Vernin, 1982), but a few studies have dealt with understanding their aroma profiles in relation to their chemical structures (Pittet and Hruza, 1974; Pelosi et al., 1983; Mihara and Masuda, 1988; Mihara et al., 1991). This work aims at acquiring better structural and biochemical information on pyrazines and thiazoles whose odours fall into the nutty or green classes. The former, generally referred to as toasted, roasted, burnt, is exhibited by short-chain polyalkylheterocycles, as well as by acetyl- and methoxy-substituted analogues (Fors, 1984; Shibamoto, 1986). The green odour is characteristic of pyrazine and thiazole derivatives bearing longer hydrocarbon chains; it becomes very strong when a methoxy group is also present in the pyrazine derivatives (Koehler et al., 1971; Buttery et al., 1976; Pelosi et al., 1983; Shibamoto, 1986).

As a contribution, we have used purified samples of

Table 1 Odour descriptions and Kovats index (I_K) of pyrazine and thiazole derivatives

No.	Name	I_K	Odour perception by the sniffing method (0.2 μg)	Other references
1	Pyrazine	787	Nutty, cocoa, caramel	Nutty, sweet, pungent[a]
2	2-Methylpyrazine	820	Cocoa, chocolate	Nutty[a,b], chocolate[a], roasted[b]
3	2,3-Dimethylpyrazine	879	Chocolate	Nutty[a,b], caramel, coffee, chocolate[a]
4	2,5-Dimethylpyrazine	873	Nutty, chocolate, caramel	Nutty, roasted[a]
5	2,6-Dimethylpyrazine	873	Nutty, chocolate	Nutty, cocoa[a]
6	2-Ethylpyrazine	877	Nutty, roasted, caramel	Nutty, roasted[a,b]
7	2,3,5,6-Tetramethylpyrazine	1067	Cocoa	Cocoa[a]
8	2,3-Diethylpyrazine	1065	Chocolate	Sweet, caramel, cake[j]
9	2-Methyl-3-propylpyrazine	1074	Nutty	Sweet, maple-like[j]
10	2-Butyl-3-methylpyrazine	1182	Vegetable	
11	2-(3-Methylpentyl)pyrazine	1229	Vegetable	
12	2-Acetylpyrazine	970	Nutty	Crust-like[j], popcorn, cocoa, peanuts[k]
13	2-Acetyl-3-methylpyrazine	1055	Caramel, hay	Nutty, cereal, roasted[a]
14	2-Methoxypyrazine	872	Nutty, caramel	Roasted, nutty, sweet[j]
15	2-Isopropyl-3-methoxypyrazine	1089	Green peas	Vegetable[h], green peas[k]
16	2-Isobutyl-3-methoxypyrazine	1179	Bell pepper	Vegetable[h], bell pepper[c,k]
17	Thiazole	788	Nutty	Nutty[a], roasted peanuts[i]
18	4-Methylthiazole	818	Bread, pungent, nutty ·	Nutty[a], roasted peanuts[i]
19	2-Ethyl-4-methylthiazole	927	Nutty, vegetable	Nutty, green, earthy[i]
20	4-Methyl-5-vinylthiazole	985	Nutty, chocolate	Cocoa, roasted filberts[i]
21	2-Isopropyl-3-methylthiazole	980	Floral, nutty, vegetable	
22	2-Ethoxythiazole	912	Coffee	Nutty, burnt, phenolic[a,b,i]
23	2-Acetylthiazole	965	Nutty, chocolate, caramel	Nutty, cereal, popcorn[a,b,i]
24	5-(2-Hydroxyethyl)-4-methylthiazole	1217	No odor at 0.2 μg*	
25	1,2-Dipyrazinylethane	1637	No odor at 0.2 μg**	
26	N,N'-(4-butyl-5-propyl-2-thiazolyl)-Decanedioic amide	2638	No odor at 0.2 μg***	
27	3-Acetylpyridine	1065	Chocolate	Roasted filberts[a]
28	2-Acetyl-5-methylfurane	987	Nutty	
29	2-Methyl-3-methoxypyrazine	930	Nutty	Nutty, roasted[c,j]
30	2-Isopentylpyrazine	1171	Vegetable	Green[d]
31	4-Butyl-5-propylthiazole	1340	Bell pepper	Bell pepper[e,g]
32	2-Trifluoromethyl-4-butyl-5-propylthiazole			Bell pepper[f]

*Nutty, beefy-like, **Slightly carrot-like, ***Sulphurous-like by smelling a 1000 ppm solution
[a]Vernin and Vernin, 1982; [b]Pittet and Hruza, 1974; [c]Seifert *et al.*, 1970; [d]Dal Monte *et al.*, 1993; [e]Pelosi *et al.*, 1983; [f]Napolitano and Pelosi, 1992, [g]Buttery *et al.*, 1976; [h]Allen *et al.*, 1991; [i]Maga, 1975; [j]Maga, 1992, [k]Maga and Sizer, 1973.

bovine and porcine olfactive binding proteins (OBPs) to measure the OBP-binding affinities of 32 nutty- and green-smelling compounds (see Table 1), most of them pyrazine and thiazole derivatives. OBPs are soluble proteins present in high concentration in the nasal mucus. They bind several classes of odorants with dissociation constants in the microm-

olar range and are believed to carry hydrophobic odour molecules across the aqueous mucus (Pelosi *et al.*, 1982; Bignetti *et al.*, 1985; Pevsner *et al.*, 1985, 1988, 1990; Topazzini *et al.*, 1985; Baldaccini *et al.*, 1986; Pelosi and Tirindelli, 1989; Pelosi and Maida, 1990; Dal Monte *et al.*, 1993; Pelosi, 1994). Although several members of this

protein family have been purified and characterized, their specific physiological function is not understood. In particular, it remains questionable whether they perform a discriminating function for the different classes of odours. To address this point it appears necessary to have access to a great amount of binding data, obtained with odorants exhibiting different structures and with different OBPs. Such data, besides the light they may shed on the physiological function of odorant-binding proteins, can be of practical use in designing biosensors for odours (e.g. green flavour), based on immobilised OBPs (Felicioli *et al.*, 1994).

In vitro measurements were compared with odour description (*in vivo* data) which was obtained by GC-splitless injection and sniffing detection.

Structural properties of the 32 pyrazines, thiazoles and related compounds were experimentally determined by RP-HPLC. The capacity factors obtained in this way, representative of lipophilicity (El Tayar *et al.*, 1985b), include hydrophobic and enthalpic information, as previously demonstrated by Testa and coworkers (El Tayar *et al.*, 1985a). We have investigated relationships between this lipophilicity index, the binding data for both OBPs, and olfactive notes. The influence of alkoxy, acetyl and alkyl chains on heterocycle flavour is discussed.

Materials and methods

Materials

Binding experiments

2-(3-methylpentyl)pyrazine, 1,2-dipyrazinylethane, N,N'-(4-butyl-5-propyl-2-thiazolyl) decandioic amide, 4-butyl-5-propylthiazole and 2-trifluoromethyl-4-butyl-5-propylthiazole were synthesized and purified along common synthetic routes (P. Pelosi *et al.*, unpublished; Napolitano and Pelosi, 1992). All other ligands were commercially available (Aldrich). Odorant stock solutions (10 mM) were prepared in ethyl alcohol. Working solutions were prepared from the ethanol stock solutions by diluting them with ultrapure water (Milli-Q water purification system, Millipore, Bedford, MA).

Glass containers were used both for the odorant solutions and the incubation mixtures. Tritiated 2-isobutyl-3-methoxypyrazine was prepared at the Radiochemical Centre, Amersham, Bucks, UK, as described (Pelosi *et al.*, 1981). Its specific activity was 1.2 Ci/mmol. Bovine OBP was the 2×19 kDa protein isolated from cow nasal tissue (Bignetti *et al.*, 1985) and porcine OBP was the 22 kDa species obtained from pig nasal tissue (Dal Monte *et al.*, 1991). Both

proteins were purified by anion-exchange chromatography, according to the protocol described for the pig OBP (Dal Monte *et al.*, 1991).

Odour determination

Sniffing analysis (solutions of 100 ppm in dichloromethane, purchased by Romil) were undertaken with a Chrompack 9001 gas chromatograph.

Lipophilicity measurements

Analytical grade methanol was purchased by LabScan; 3-morpholinopropane sulphonic acid (MOPS) and *n*-decylamine were obtained from Sigma.

Methods

Binding experiments

Ligand binding experiments were performed using the filtration assay (Bruns *et al.*, 1983). The protein in 20 mM Tris/HCl buffer was incubated for 1 h at 4°C. The final concentrations of both protein and labelled ligand were equal to the relative dissociation constants (3 μM for the bovine and 0.5 μM for the porcine OBP), while the competitors were used at several concentration values. In a typical experiment, 100 μl of bovine protein solution (15 μM) or 50 μl of porcine protein solution (5 μM) and 150 or 25 μl of a 10 μM radioactive pyrazine solution (for the bovine and the porcine OBPs, respectively) were mixed with the required amount of competing ligand and the volume was adjusted to 500 μl with Tris buffer. After 1 h incubation at 4°C, 150 μl of the mixture was rapidly filtered on glass fibre filters (Whatman GF/B) presoaked in a 1% aqueous solution of polyethylenimine. After washing with 5 ml of ice-cold buffer, the filters were counted in Hydro-Luma (Lumac) scintillation cocktail with a Packard model Tri-Carb 2000 CA counter. All experiments were performed in triplicate; mean values are reported.

Odour determination

Five persons were selected to define pyrazine and thiazole odour descriptors by using a GC-sniffing detector. Chromatographic conditions were: column CP-SIL 5 CB 50 m × 0.32 mm (film thickness: 1.2 μm); carrier gas: He at a flow of 1.5 ml/min; splitless injection time: 0.5 min; oven temperature programmed to rise from 36 to 80°C at 20°C/min, then to 105°C at 0.5°C/min and to 250°C at 5°C/min; injector temperature; 250°C; sniffing detector: 275°C; volume injected: 2 μl.

Lipophilicity measurements

Lipophilicity was measured by RP-HPLC with a chromatograph equipped with a Waters model 510 isocratic pump and a Waters WISP 710 B autosampler. The detector was a Perkin-Elmer LC75 operating at 270 nm for the pyrazine derivatives and at 245 nm for the thiazole derivatives. The Guard-Pak insert was packed with μBondapak™ C18, particle size 10 μM (Waters). The column (25 cm × 4 mm ID) was prepacked with LiChrosorb RP-18, particle size 10 μM (Merck). A digital 380 PC equipped with the Waters 840 acquisition program (version 6.0) was used as an integrator for peak recording and to calculate retention times. The mobile phase was made up volumetrically from various combinations (30–70%) of methanol, and a solution containing MOPS buffer (0.01 M) and *n*-decylamine (0.2% v/v). MOPS buffer was chosen to avoid ion-pair formation; *n*-decylamine was used as a masking agent to eliminate silanophilic interactions (El Tayar *et al.*, 1985a). The pH of the aqueous solution was adjusted beforehand to 7.4 by addition of HCl. The methanol/aqueous solution mixture was filtered with a Millipore HAWP filter (0.45 μM). Retention times (t_r) were measured at room temperature with a 1.5 ml/min flow rate. The column dead time (t_0) was determined with uracil. The capacity factor was defined as $k = (t_r - t_0)/t_0$ (El Tayar *et al.*, 1985a). Log k for 100% water (log k_w) was linearly extrapolated from results obtained for different mobile phase compositions.

Results and discussion

From sniffing data (Table 1), it appears that most of pyrazine and thiazole derivatives can be smelled when 0.2 μg arrives in the GC-detector. In order to facilitate the here-below discussion, the proposed descriptors have been classified in three groups: green (vegetable, peas, bell pepper, floral, . . .), nutty (roasted, chocolate, caramel, cocoa, coffee, . . .) and nutty/green odorants.

On this basis, a first 'nutty' class includes structures 1, 2, 3, 4, 5, 6, 7, 8, 9, 12, 13, 14 and 29 (pyrazines), and structures 17, 18, 22, 23 and 24 (thiazoles) bearing alkyl (up to 3 carbon atoms), alkoxy, acetyl, or alcohol substituents. Two more heterocyclic derivatives, 3-acetylpyridine (27) and 2-acetyl-5-methylfuran (28) complete this series of nutty odorants. A second 'green' class includes pyrazines 10, 11, 15, 16 and 30, and thiazoles 31 and 32 bearing hydrocarbon chains of 3–6 carbon atoms, in some cases together with other functional groups. Compounds 19, 20 and 21 present both nutty and green notes. In order to investigate bifunc-

tional ligands, we also included in this study two odourless compounds in which two pyrazine (25) or thiazole (26) rings are linked by an open chain. These latter compounds, odourless because of their reduced volatility, are interesting for investigating OBP binding of bifunctional ligands.

Table 2 summarizes the structural (log k_w) and biochemical p[L]50 data on bovine and pig OBPs for the 32 tested compounds.

Biochemical experiments were performed with both the protein and radioactive 2-isobutyl-3-methoxypyrazine at concentrations equal to the relative dissociation constant, that is concentrations between 0.3 and 100 μM for the former OBP, and between 1 and 100 μM for the latter. As an indication of the binding strength of each odorant, the negative logarithms of the concentrations displacing 50% of the bound radioactive pyrazine from the complex are given in Table 2. These data, however, cannot be directly compared with other published results of this type because they provide a reliable measure of the relative dissociation constants only when the experiments are performed at low protein concentrations. Figure 1 reports the actual competition curves, in order to show experimental facts that cannot be deduced from the data of Table 2. Among all these results, some very important observations can be highlighted:

(i) all nutty odorants (pyrazines, thiazoles, or other heterocycles; group I) fail to show any significant binding to either OBP;

(ii) all the green odorants (group II) displace the radioactive pyrazine from both proteins with dissociation constants similar to that of the reference ligand 2-isobutyl-3-methoxypyrazine;

(iii) the compounds whose odours are described as nutty and green at the same time (group III) show only moderate affinity for both proteins;

(iv) both the bovine and the porcine OBP present similar binding spectra, showing good affinity only for green-smelling odorants; however, some minor, but measurable differences between the two proteins are apparent;

(v) with several derivatives, both pyrazinic and thiazolic, there is a negative competition effect, particularly at the lowest concentrations used. In such cases, the added ligand seems to increase the number of binding sites for radioactive 2-isobutyl-3-methoxypyrazine rather than compete for the same binding site. This fact, for which we have no clear explanation at present, is reproducible and has been observed previously (Dal Monte *et al.*, 1993).

Among the structural data (see Table 2), we can point out

Table 2 Odour descriptions, logarithm of the capacity factor and binding of different odorants to bovine and porcine OBPs. The values of p[L]50 are, for each odorant, the negative logarithm of the molar concentration that displaces 50% of bound labelled 2-isobutyl-3-methoxypyrazine

No.	Name	Odour	Group	log k_w	p[L]50 (bov)	p[L]50 (pig)
1	Pyrazine	Nutty	I	−0.339	<4	<4
2	2-Methylpyrazine	Nutty	I	0.199	<4	<4
3	2,3-Dimethylpyrazine	Nutty	I	0.583	<4	<4
4	2,5-Dimethylpyrazine	Nutty	I	0.640	<4	<4
5	2,6-Dimethylpyrazine	Nutty	I	0.586	<4	<4
6	2-Ethylpyrazine	Nutty	I	0.680	<4	<4
7	2,3,5,6-Tetramethylpyrazine	Nutty	I	1.273	<4	<4
8	2,3-Diethylpyrazine	Nutty	I	1.456	<4	<4
9	2-Methyl-3-propylpyrazine	Nutty	I	1.520	<4	<4
10	2-Butyl-3-methylpyrazine	Green	II	2.011	4.6	4.7
11	2-(3-Methylpentyl)pyrazine	Green	II	2.578	5.5	5.6
12	2-Acetylpyrazine	Nutty	I	0.232	<4	<4
13	2-Acetyl-3-methylpyrazine	Nutty	I	0.763	<4	<4
14	2-Methoxypyrazine	Nutty	I	0.648	<4	<4
15	2-Isopropyl-3-methoxypyrazine	Green	II	2.116	2.9*	4.6
16	2-Isobutyl-3-methoxypyrazine	Green	II	2.507	5.0	5.8
17	Thiazole	Nutty	I	0.373	4	<4
18	4-Methylthiazole	Nutty	I	0.858	4	<4
19	2-Ethyl-4-methylthiazole	Nutty, green	III	1.798	3.8*	0.8*
20	4-Methyl-5-vinylthiazole	Nutty, green	III	1.835 ·	4 3	4.9
21	2-Isopropyl-4-methylthiazole	Nutty, green	III	2.207	5 0	4.5
22	2-Ethoxythiazole	Nutty	I	1.568	<4	<4
23	2-Acetylthiazole	Nutty	I	0.720	<4	<4
24	5-(2-Hydroxyethyl)-4-methylthiazole	Nutty	I	0.387	<4	<4
25	1,2-Dipyrazinylethane	—		0.402	<4	<4
26	N,N'-(4-butyl-5-propyl-2-thiazolyl)-decanedioic amide	—		3.498	4.4	3.9*
27	3-Acetylpyridine	Nutty	I	0.526	<4	<4
28	2-Acetyl-5-methylfurane	Nutty	I	0.925	<4	<4
29	2-Methyl-3-methoxypyrazine	Nutty	I	1.124	<4[a]	<4[a]
30	2-Isopentylpyrazine	Green	II	2.226	5.6[a]	5.7[a]
31	4-Butyl-5-propylthiazole	Green	II	3.721	4.6[b]	5.4[b]
32	2-Trifluoromethyl-4-butyl-5-propylthiazole	Green	II	2.450	5.7[c]	5.8[d]

*See Figure 1: extrapolated value.
[a]Dal Monte *et al*, 1993; [b]Pelosi *et al.*, 1983; [c]Napolitano and Pelosi, 1992; [d]unpublished data.

the higher lipophilicity of the thiazole moiety (17, log k_w = 0.37) as compared to the pyrazine heterocycle (1, log k_w = −0.34), due to the higher hydrophobicity (volume) of the sulphur atom combined with the presence of only one basic nitrogen lone pair in the molecule. In both series, addition of a lipophilic substituent (alkyl chain on pyrazines: 2–11, 30 and on thiazoles: 18, 19, 21, 31; vinyl group in 20) logically induces a log k_w increase. Probably due to a

masking effect of the nitrogen lone pair (repulsion between the exocyclic oxygen lone pairs and the pyrazine or thiazole nitrogen lone pair), the alkoxy groups increase the log k_w value much more than expected from the π (Hansh and Fujita, 1964), or f (Rekker and de Kort, 1979) hydrophobic increments (see Table 2: 14 versus 1; 22 versus 17, 29 versus 2). In the same way, the acetyl function significantly increases the log k_w values (12 versus 1; 13 versus 2; 23
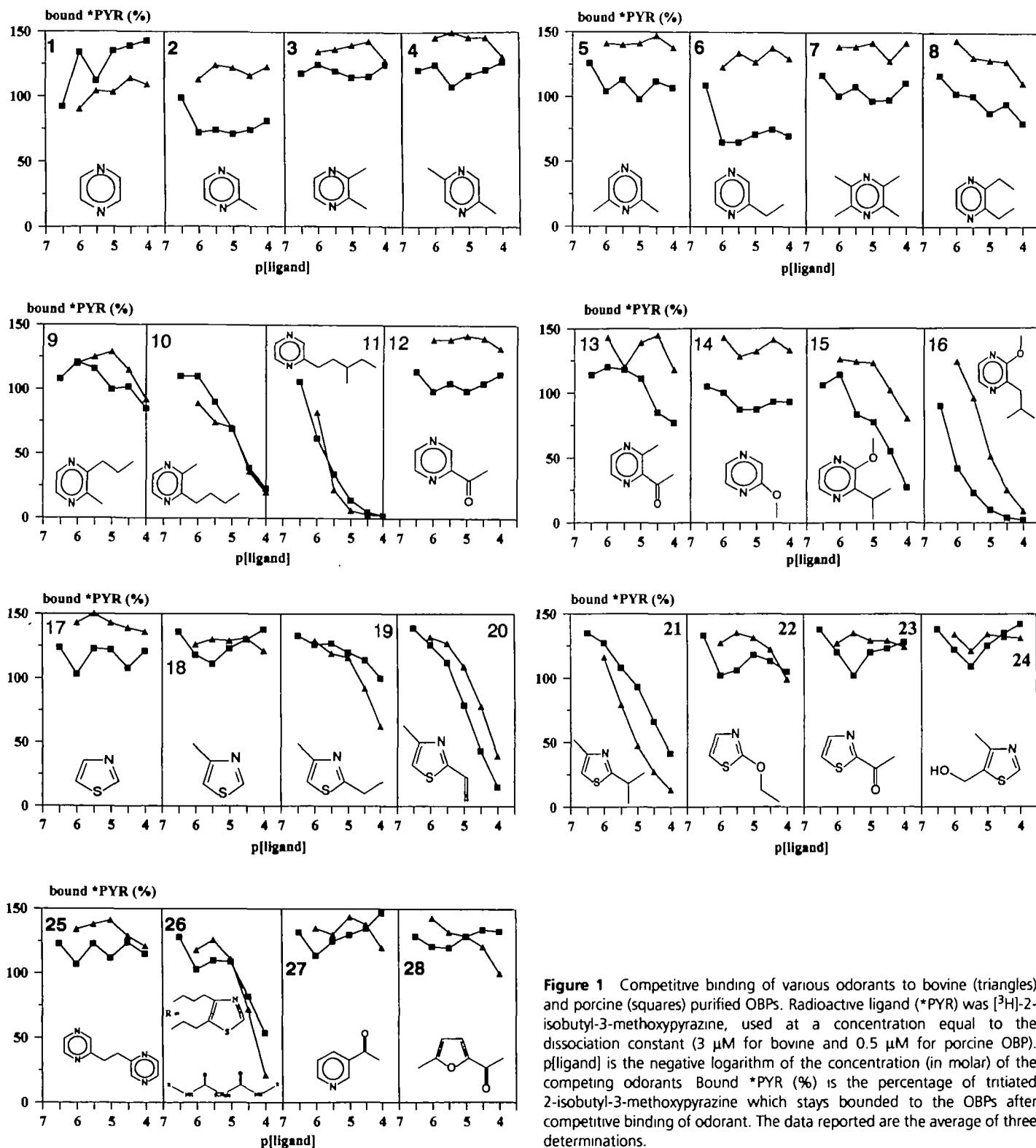
Figure 1 Competitive binding of various odorants to bovine (triangles) and porcine (squares) purified OBPs. Radioactive ligand (*PYR) was [^{3}H]-2-isobutyl-3-methoxypyrazine, used at a concentration equal to the dissociation constant (3 μM for bovine and 0.5 μM for porcine OBP). p[ligand] is the negative logarithm of the concentration (in molar) of the competing odorants Bound *PYR (%) is the percentage of tritiated 2-isobutyl-3-methoxypyrazine which stays bounded to the OBPs after competitive binding of odorant. The data reported are the average of three determinations.

versus 17). Surprisingly, the addition of CF$_3$ group (32) on the 4-butyl-5-propylthiazole (31) leads to a significant lipophilicity decrease, despite the positive *f* increment of 1331 (Rekker and de Kort, 1979).

When structural data are compared with the olfactive information, it appears that all the green-smelling compounds (group II) are characterized by a higher lipophilicity value (log k_w > 2.00). Hydrophobic compounds such as 11 and

16 are the best ligands for the pig OBP. Our results are confirmed by the lipophilicity values of compounds 30–32, whose biochemical affinities have been previously determined (Dal Monte *et al.* 1993; Napolitano and Pelosi, 1992). In order to confirm our first conclusions, it would be interesting to synthesize new, extremely lipophilic pyrazines and thiazoles, and determine their olfactive notes and affinities for OBPs.

Among the three nutty/green-smelling compounds (group

III), structures 20 and 21 which bind both OBPs are also the most lipophilic (log $k_w > 1.83$). In conclusion, lipophilicity appears as a very interesting discriminating physicochemical parameter, predictive of the affinity for OBPs. As shown by compound 26 (very high lipophilicity without GC-sniffing perception at 0.2 µg), however, the three-dimensional structure (steric hindrance) constitutes an other determinant property which modulates the interaction with the protein active site.

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