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RESEARCH ARTICLE

Chemotactic effect of odorants and tastants on the ciliate *Tetrahymena pyriformis*

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Abstract
Naturally occurring aroma compounds are able to elicit physiological and migratory responses such as chemotaxis even at nano to femtomolar concentrations in organisms at different levels of phylogeny. Despite the amazing chemical variety of these substances the apparatus by which they can be detected i.e. the chemosensory receptors and the signaling pathways seem to be rather uniform and evolutionary well-conserved. The intracellular signaling process is supposed to be mediated by either cAMP or inositol 1,4,5-trisphosphate. The present work aimed to investigate the chemotactic behavior of 11 odorants that occur naturally in foods and are also used by the industry as additives, on the eukaryotic ciliate *Tetrahymena pyriformis*. Intracellular signaling pathways that might be activated by these compounds were also investigated. Activation of the phospholipase C (PLC) was measured by FACS and the stimulation of inositol-1,4,5-trisphosphate 3-kinases (IP3K) was measured using two specific inhibitors, wortmannin and LY294002. The strongest chemoattractant character was observed for isoamyl acetate (10^{-6} M), propyl isobutyrate (10^{-8} M), isobutyl propionate (10^{-6} M). The strongest repellent action was exerted by benzyl acetate (10^{-8} M), furfuryl thioacetate (10^{-12} M). Our results suggest that Tetrahymena responds in a very sensitive way to slight changes in the molecular structure. According to our study, tracer amounts of solvents do not contribute significantly to the chemotactic profile of the respective odorants. No significant activation of PLC or PI3K could be observed following stimulation with attractant odorants which implies that some other pathways may be involved, hence further investigation is needed.

Keywords: Chemotaxis, fruit esters, aromatic aldehydes, essential oils, signaling, *Tetrahymena*

Introduction

The chemosensory monitoring of the ever changing milieu serves to find food, avoid danger, detect mates and offspring and recognize territories (1) and thus is essential for the survival of all organisms ranging from bacteria to mammals. Odorants and tastants constitute a long family of volatile organic compounds with largely varying chemical structure (e.g. esters, alcohols, aldehydes, ketones, terpenoids, etc.) (2) that are present in trace amounts of a few parts per million in foods and beverages (3,4). They can be detected in a wide range of organisms at different levels of the phylogeny ranging from bacteria to mammals by their specific class of receptors, the chemosensory receptors.

Acting as potent chemical stimuli these compounds are able to elicit physiological and behavioral responses such as chemotaxis or other migratory responses even at low, nano to femtomolar concentrations and thus are important semiochemicals. Biological functions of plant produced odorants are primarily attracting pollinators and seed dispersers or repelling pests (5). A well-known attractant is for example isoamyl acetate (IAA) that elicited positive migratory responses in *Caenorhabditis elegans* and (6) *Drosophila melanogaster* (7). This ester is also in the focus of studies searching for novel lures to trap phytophagous insects (8). On the contrary, other odorants are able to induce avoidance reactions. Several acetate esters (like methyl, ethyl and amyl acetate) act as repellent for

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Escherichia coli at the 10^{-1} M concentration (9). Much higher sensitivity could be observed in the avoidance reactions of Tetrahymena induced by eugenol, carvacrol and menthol stimuli at low, nano to micromolar concentrations (10). Nevertheless, the action of these compounds at the receptor level is still not known. Rodgers et al. suggest that some of these compounds may act as non-physiological agonists and activate receptors that evolved to recognize some other endogenous ligands. In humans these volatile compounds have important role in the perception of food, and can influence our behavior related to food. An interesting example is the changing of the suckling habits (longer breast attachment, more suckling and altered intake) of breast-fed human babies following ingestion of diverse flavors by their mother (11).

Recognition of different chemical stimuli is based on the action of G-protein coupled chemosensory receptors (1). Despite the surprising chemical variety of the components that can be detected the intracellular signaling process itself seems to be rather uniform. Two alternative pathways are hypothesized by the literature with activation of two different second messengers cAMP and inositol 1,4,5-trisphosphate (InsP3) (12); though the presumption of InsP3 activation become less supported nowadays (13,14). The cAMP pathway involves in the vertebrate olfactory neurons the action of adenyl cyclase III, the opening of cyclic nucleotide gated ion channels (CNGC), highly permeable to calcium and the opening of the calcium activated chloride channels. By contrast, the major steps of InsP3 pathway are the hydrolyzation of phosphatidylinositol-4,5,bisphosphate by phospholipase C (PLC) into 1,2-diacylglycerol (DAG) and $InsP_3$, stimulation of the type 6 transient receptor potential channels (TRPC6) by the DAG and the release of Ca^{2+} from intracellular stores induced by InsP3 via $InsP_3$R-III (12).

In our work, we studied the migratory responses of the eukaryotic ciliate *Tetrahymena pyriformis* induced by seven esters, four aromatic aldehydes (that naturally occur in foods and are also applied in the food industry as additives) and essential oils derived from two spices. As Rodgers et al. suggest, *Tetrahymena* is a model organism of choice because it may posses some chemosensory pathways similar to those in higher organisms and thus it may allow the high throughput screening of chemical compounds to see if they can elicit threshold depolarization (10).

Our aim was (i) to investigate the chemotactic behavior of the selected odorants. On the one hand (ii) we aimed to settle whether structural isomers among odorants have the same chemotactic characters, and on the other hand to measure the possible contribution of the reagents used during the synthesis of these compounds to the chemotactic profile of the odorants. Moreover, (iii) we tried to gain insight into the intracellular signaling pathways stimulated by these compounds. For this purpose we studied the activation of the phospholipase C (PLC) and the inositol-1,4,5-trisphosphate 3-kinases (IP3K) pathway.

Materials and methods

Cell culturing and chemotaxis assay

T. pyriformis GL ciliate was grown axenically in 1% proteose–peptone, 0.1% yeast-extract (PPY) medium at 28°C. Twenty-four hour old, exponential growth phase cultures were used in modified, two chamber capillary chemotaxis assay (15). Cells placed into the lower chamber were incubated for 20 min with the test substances placed in the upper chamber. A capillary served as a linker between the two chambers. Initial cell density was ~10^5 cells/mL and the number of the parallels tested was eight. The positive responder cells were fixed by PBS containing 4% formaldehyde. The number of cells was determined using Neubauer haemocytometer. *Chemotaxis Index* (Chtx. Ind. [%]) was calculated according to equation (1):

$$chtx.Ind.[\%] = \frac{\sum_{i=1}^{8} N_{x,i}}{\sum_{j=1}^{8} N_{Ctrl,j}} \times 100 \quad (1)$$

where $N_{x,i}$ stands for the number of cells in an upper well "*i*" containing the "*X*" test substance and $N_{Ctrl,j}$ represents the number of cells present in an upper well "*j*" containing the adequate negative control (see the description of the dilution of test substances below).

Test substances

Tested substances were (i) seven esters (that occur naturally in fruits and are also used as food additives) (Table 1), (ii) five organic alcohols and acids applied during the esterification reaction (Table 1), (iii) four aromatic aldehydes (Table 2) and (iv) two essential oils. The structure and the formula of the studied odorants are summarized in Table 3. The chemotactic activity of the organic solvents (benzyl alcohol, amyl alcohol, butyric acid and propionic acid) written in italics in Table 1. was studied in order to see whether they contribute as tracer components to the chemotactic character of the respective esters. The chemotactic behavior was not tested when it was already described (e.g. phenol (16)). All reagents were in analytical purity grade. Aromatic aldehydes (furfuryl mercaptan, methyl furfuryl disulfide, furfuryl pyrrole and furfuryl thioacetate) were obtained from Sigma, St.Louis, USA.

First, stock solutions were prepared from the test substances in absolute alcohol (Molar Chemicals Ltd., Budapest, Hungary). The concentration of the stock solutions was 10^{-3} M except for essential oils where it was 0.1 V/V%. Stock solutions were then further diluted to 10^{-12}–10^{-6} M in PPY medium except for essential oils, for which final concentrations in PPY medium ranged from 10^{-5}–10^{-1} V/V%. Negative controls were prepared for each dilution using fresh PPY medium with the adequate alcohol ratio.

Ester preparation and essential oil extraction

Fruit esters (isobutyl propionate (IBP), propyl isobutyrate (PIB), methyl isobutyrate (MIB), pentyl butyrate (PB), methyl butyrate (MB), IAA and benzyl acetate (BA)) were prepared by us using the classical way of esterification, the Fischer synthesis. The reaction was catalyzed by concentrated sulfuric acid. The yield of the esterification was improved in different ways depending on the ester. In the case of the synthesis of IBP, PIB and PB we removed water by heteroazeotropic distillation with benzene (IBP, PIB) or toluene (PB) using a Dean–Stark apparatus. For the synthesis of IAA, MB and MIB we used the alcohol in large excess as a solvent of the esters. Finally, during the preparation of BA we used acetyl chloride and pyridine as a solvent and organic base to react with the nascent hydrogen chloride. In the following steps we dried the esters on anhydrous magnesium sulfate and purified them by distillation. Identification of products was based on gas chromatography and boiling point (T_B) determination (Table 1).

Essential oils of two spices, anise (*Pimpinella anisum*) and clove (*Syzygium aromaticum*) were obtained by steam distillation followed by extraction with dichloromethane in the presence of sodium chloride. The respective organic phases were dried using anhydrous sodium sulfate and vacuum distilled. Main volatile components were identified using thin layer chromatography. Mixtures of toluene and ethyl acetate or toluene and chloroform were used as solvents for clove and anise extracts respectively. In the essential oil prepared from clove, the predominant volatile compound was eugenol. In the essential oil prepared from anise, the major component was anethole.

Study of the PLC and the PI3K pathway activation

The activation of the PLC pathway was measured by immunostaining coupled to detection by fluorescently activated cell sorter (FACS). Cells were stimulated for 1, 3, 5, 10 or 15 min. with five fruit esters (PB at 10^{-12} M, IAA, IBP, MB, MIB at 10^{-9} M and PIB at 10^{-8} M) that were found chemoattractant in the previously described chemotaxis

Table 1. List of the fruit esters studied with the respective reagents, estimated partitioning coefficient and natural occurrences.

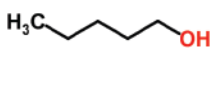
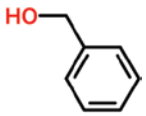
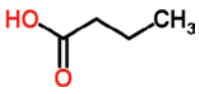
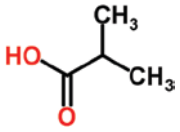
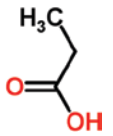
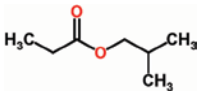
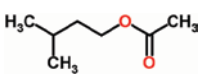
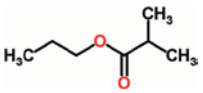
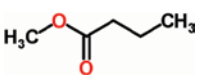
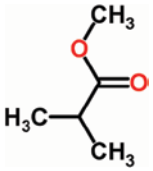
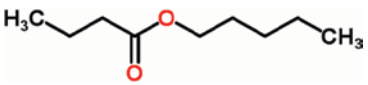
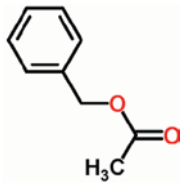
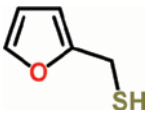
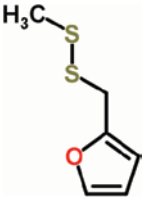
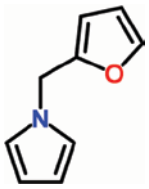
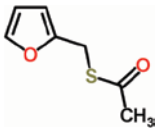
Fruit ester	TB (°C)	logP*	Abbreviation**	Reagents	Natural occurrence	Reference
Isobutyl propionate	136	2.2	IBP	*Propionic acid*, isobutanol, Benzene, sulfuric acid	Apple (*M. domestica*)	(17)
					Passion fruit (*P. edulis*)	(18)
Propyl isobutyrate	134	2.1	PIB	*Isobutyric acid,* propyl alcohol, Benzene, sulfuric acid	Apple	(19)
					Durian (*D. zibethinus)*	(20)
					Muskmelon (*C. melo*)	(18)
Methyl isobutyrate	91	1.2	MIB	*Isobutyric acid*, methanol, Sulfuric acid	Muskmelon (*C. melo*)	(18)
					Tomato *(S. lycopersicum)*	(21)
Pentyl butyrate	186	2.7	PB	*Butyric acid*, *amyl alcohol,* toluene, sulfuric acid	Apple (*M. domestica*)	(22)
					Banana (*M. sapientum*)	(23)
Methyl butyrate	102-3	1.3	MB	*Butyric acid*, methanol, Sulfuric acid	Apple (*M. domestica*)	(22)
					Durian (*D. zibethinus)*	(20)
					Muskmelon (*C. melo*)	(18)
					Murici (*B. crassifolia*)	(24)
					Papaya (*C. papaya)*	(25)
Isoamyl acetate	132	2	IAA	Acetic acid, isoamyl alcohol, Sulfuric acid	Banana (*M. sapientum*)	(23)
					Jackfruit *(A. heterophyllus)*	(26)
Benzyl acetate	206	2	BA	*Benzyl alcohol*, acetyl chloride, Pyridine	Cranberry (*V. macrocarpon*)	(27)
					Muskmelon (*C. melo*)	(18)
					Peach (*P. persica*)	(28)

*Partitioning coefficient (source: pubchem.ncbi.nlm.nih.gov).
**For the sake of simplicity, compounds' name will be abbreviated later by these abbreviations.
***The chemotactic effect of italicized organic reagents was measured to study their potential contribution to the chemotactic profile of the respective esters.

Table 2. Aromatic aldehydes.

Compound	logP	Natural occurrence	Reference
Furfuryl mercaptan	1.3	Coffee	(29)
		Tuna, chocolate, hazelnut, peanut	(30)
Methyl furfuryl disulfide	1.5	Coffee	(31)
		Malt, pork liver, wheat bread, asparagus, potato, roasted turkey, grilled beef, cocoa, cooked rice, mushroms, clams	(32)
Furfuryl pyrrole	1.4	Almond, roasted almond	(33)
		Popcorn	(34)
Furfuryl thioacetate	1.3	Coffee	(35)

Table 3. Molecular structure and formula of the studied compounds.

				
$C_5H_{11}OH$ AA	C_7H_8O BAL	$C_4H_8O_2$ BuA	$C_4H_8O_2$ IbuA	$C_3H_6O_2$ PA
				
$C_7H_{14}O_2$ IBP	$C_7H_{14}O_2$ IAA	$C_7H_{14}O_2$ PIB	$C_5H_{10}O_2$ MB	$C_5H_{10}O_2$ MIB
				
$C_9H_{18}O_2$ PB		$C_9H_{10}O_2$ BA		
				
C_5H_6OS furfuryl mercaptan	$C_6H_8OS_2$ methyl furfuryl disulfide	C_9H_9NO furfuryl pyrrole	$C_7H_8O_2S$ furfuryl thioacetate	

assay. After fixation with 4% paraformaldehyde containing PBS cells were washed twice in PBS containing and were subsequently subjected to 30 min of membrane permeabilization with 0.1% saponine (w/v) (Sigma Ltd. St. Louis, USA) in PBS. Next, cells were incubated with Alexa Fluor® 647 conjugated anti-phospho-PLC-γ1(Y783) monoclonal antibody (BD Biosciences Pharmingen, San Jose, USA), which can recognize phosphotyrosine residue of PLCγ1 (Tyr783), for 30 min at room temperature in the dark. After immunostaining the cells were washed twice with PBS and the samples were stored in 4% formaldehyde containing PBS at 4°C. Finally, intracellular fluorescence intensity was detected by flow cytometry (FACS-Calibur, Becton-Dickinson). The geometric means of the fluorescence intensity were calculated by the in-built software of the machine (CellQest Pro) and were compared to the mean fluorescence of negative controls that were untreated but immunostained cells thus mean fluorescence intensity [control %] was calculated.

The stimulation of the PI3K pathway was studied applying wortmannin and LY294002. The former substance is a metabolite of the fungus *Penicillium funiculosum* and is a specific natural inhibitor of PI3Ks; the later one, in turn, is a widespread synthetic inhibitor. Though both molecules bind to the ATP-binding site of the enzyme, their targets are different. In order to study the eventual activation of the PI3K pathway by the odorants Tetrahymena cells were pretreated for 5 min. with 10^{-5} M wortmannin and 10^{-5} M LY294002. The migratory response of the cells pretreated with the fruit esters was measured in the two chamber capillary chemotaxis assay that was described above. Applied fruit ester concentrations were identical to those where chemoattractant character was observed in chemotaxis assays. The solvent of inhibitors, DMSO was used as the negative control of PI3K assays. Results were given by the inhibition index defined as the ratio of the chemotaxis indexes of the pretreated and the control cell population.

Statistical analysis

Statistical analysis was carried out using Origin®7.0 software. Significance was calculated by one-way ANOVA analysis.

Table 4. Summary of the chemotactic activity of the substances tested. Reagents and solvents used in synthesis are shown in italics. Levels of significance are shown as x: $p<0.05$; y: $p<0.01$; z: $p<0.001$.

Compound	Chemoattractant logC [M]	Chemorepellent logC [M]
Isoamyl acetate (IAA)	–12, –11, –9^{z}, –6^{x}	
Isoamyl alcohol	–12^{x}, –6^{x}	–9, –8, –7
Isobutyl propionate (IBP)	–9^{x}, –7, –6^{x}	–12^{x}
Propionic acid	–8^{x}, –7, –6^{x}	–12, –11
Propyl isobutyrate (PIB)	–12, –10^{x}, –8^{y}, –7^{x}	
Isobutyric acid	–12^{x}, –11, –10, –8^{x}	–7^{y}
Methyl butyrate (MB)	–9^{x}	–12, –10
Butyric acid	–9^{x}, –7^{x}	–11^{x}
Methyl isobutyrate (MIB)	–9^{x}, –8, –6	–10
Isobutyric acid	–12^{x}, –11, –10, –8^{x}	–7^{y}
Pentyl Butyrate (PB)	–12^{x}	
Isoamyl alcohol	–12^{x}, –6^{x}	–9, 8, –7
Butyric acid	–9^{x}, –7^{x}	–11^{x}
Benzyl acetate (BA)	–12, –11	–9, –8^{y}, –7
Benzyl alcohol	–9^{x}, –7^{x}	–12^{x}, 11, –8
Furfuryl mercaptan		–12^{x}, –6^{x}
Furfuryl methyl disulfide	–9^{y}	–12^{x}, –11^{x}, –8, –6^{x}
Furfuryl pyrrole	–11, –9^{x}	
Furfuryl thioacetate	–9^{y}, –8^{y}	–12^{y}
Anise extract*	–5^{z}, –2^{y}	–6, –4^{y}, –1
Clove extract*		–7, –6, –5, –4, –3, –1

*Concentrations are expressed in logV/V.

Results

Chemotaxis

The chemotactic activity of naturally occurring 14 odorant or tastant molecules that may be also used as food additives was studied (Figures 1–4) using two kinds of control: on the one hand the culturing media and on the other hand five of the known reagents applied during the ester synthesis which could be present in the samples in trace amount.

Chemotactic profile of three esters that are structural isomers and share the chemical formula $C_7H_{14}O_2$ is shown on Figure 1. IBP and IAA showed similar significant attractant peaks at 10^{-6} M (262% ± 33 and 252% ± 47) and a smaller one at 10^{-9} M (152% ± 25 and 212% ± 35, respectively). Moreover, IAA had also attractant character at 10^{-12}–10^{-11} M (137% ± 24 and 177% ± 23), while IBP showed strong repellent character at 10^{-12} M (47% ± 5). The third compound, PIB had weak attractant effect at 10^{-10} M (140% ± 21) and a stronger one at 10^{-8}–10^{-7} M (160% ± 24 and 172% ± 19). As to the reagents, chemotactic profiles were different from those of the esters. Three similar peaks were observed between esters and the respective reactants: isoamyl alcohol had a similar but smaller attractant peak at 10^{-6} M (155% ± 22) than did IAA; at 10^{-12} M isoamyl alcohol and IAA had identical, however weak chemoattractant effects; and isobutyric acid had a small attractant peak at 10^{-8} M (127% ± 18) alike PIB. At the lowest concentration tested 10^{-12} M, isobutyric acid was significantly stronger attractant than PIB (154% ± 24), while at the relatively high concentration 10^{-7} M, the reagent was significantly repellent (57% ± 10).

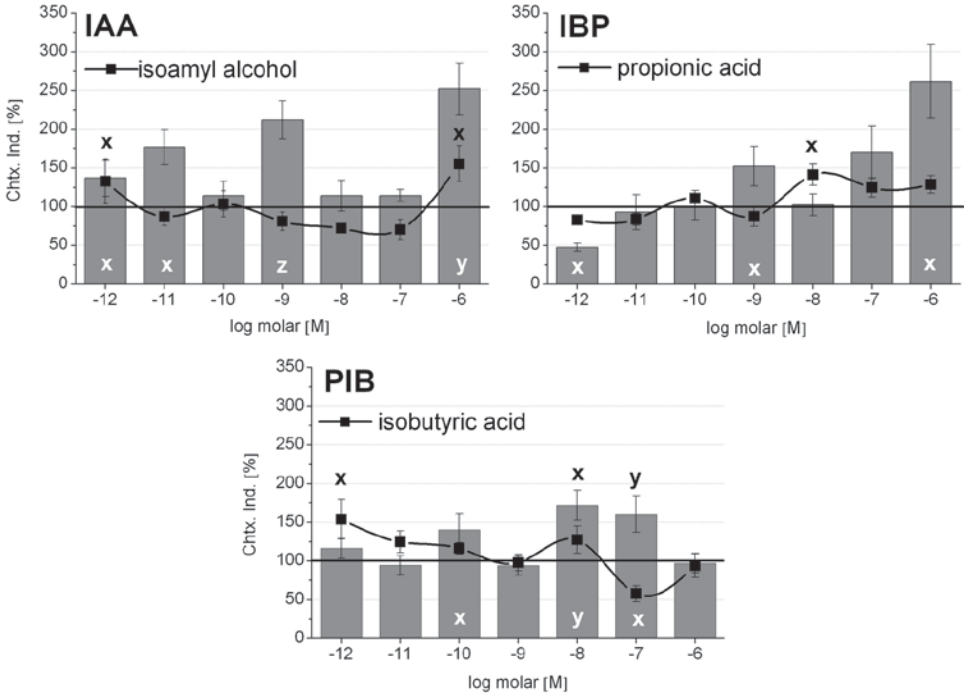


Figure 1. Chemotaxis induced by fruit esters (C7H14O2) in Tetrahymena cells. Tested substances: isoamyl acetate (IAA), isobutyl propionate (IBP), propyl isobutyrate (PIB). Lines represent chemotactic activity of the respective reagents used during the esterification. Significance levels in white correspond to the tested substances, while in black to the reagents (x: $p<0.05$, y: $p<0.01$; z: $p<0.001$).

The migratory responses observed with MB, MIB and the PB are shown on Figure 2. The first two esters had similar profiles, i.e. both of them were rather neutral except at 10^{-9} M, where they acted as chemoattractants (139% ± 9 and 155% ± 6). Our data show that chemoattractant (10^{-12} M) and chemorepellent (10^{-7} M) effects of the reagent isobutyric acid described above were not influencing chemotactic effects even in the case of MIB. The third butyrate ester, the PB was neutral in the entire concentration range as well except for 10^{-12} M, where it showed significant attractant character (148% ± 13). Alike what was described above, the esters did not show similarities in their chemotactic behavior compared to their respective reagents. Butyric acid was attractant at 10^{-9} M and 10^{-7} M (130% ± 12 and 142% ± 15); and was repellent at 10^{-11} M (87 ± 11%). The isoamyl alcohol in turn, was attractant at 10^{-12} M (132% ± 29) as well as at 10^{-6} M (155% ± 23). Only the PB showed some similarity with one of its two solvents (butyric acid and isoamyl alcohol), i.e. both PB and isoamyl alcohol were attractant at 10^{-12} M (148% ± 13 and 133% ± 29, respectively). BA showed on the one hand significant repellent nature at 10^{-8} M (44 ± 9%) and weak but not significant attractant character on the other hand at 10^{-12}–10^{-11} M (128 ± 19% and 140 ± 19%) (Figure 2). The reagent benzyl alcohol in turn, exhibited significant repellent character (59 ± 10%) at 10^{-12} M and acted as chemoattractant at 10^{-9} M and 10^{-7} M (139 ± 18% and 141 ± 20%). Our data show that fruit esters express their identical chemotactic effects in majority of the data points tested and that even in high concentrations of the fruit esters we have no biologically effective remnants of the reagents or solvents used in the synthesis.

In the profiles of the four furfuryl compounds (Figure 3) two similarities were observed. On the one hand, each of them showed attractant character at 10^{-9} M; however this effect was weaker in the case of furfuryl mercaptan (113 ± 14 vs. 130–151% ± 15–25) than in that of the three other molecules. On the other hand, each of the four compounds acted as chemorepellent at 10^{-6} M, yet this effect was significant solely in the case of furfuryl methyl disulfide and furfuryl mercaptan (62–75% ± 10). Moreover, three of the four molecules exhibited repellent character at 10^{-12} M, even if this repellent effect was weaker for furfuryl mercaptan and furfuryl methyl disulfide (75% ± 12) than for furfuryl thioacetate (52% ± 8). Some differences were also observed in the chemotactic behavior of these molecules, furfuryl methyl disulfide proved to be a weak repellent at some concentrations (10^{-12}–10^{-10} M and 10^{-8}–10^{-6} M (75–85% ± 15 and 75–92% ± 10) whereas furfuryl mercaptan and furfuryl thioacetate acted as weak attractants at 10^{-8} M (135% ± 8).

Chemotactic responses obtained with the essential oils of anise and clove are presented in Figure 4. Clove essential oil was strongly repellent in the entire concentration range (41–56% ± 7–12) for each concentration). On the contrary, essential oil from anise acted as attractants at 10^{-2} and 10^{-5} V/V% dilutions (150% ± 24 and 201% ± 23) and were strong repellents at 10^{-4} and 10^{-6} V/V% dilutions (75% ± 9 and 76% ± 9) dilutions.

Activation of intracellular signaling pathways

The activation of PLC pathway (Figure 5) was studied following stimulation of the cells for 1, 3, 5, 10 or 15 min with

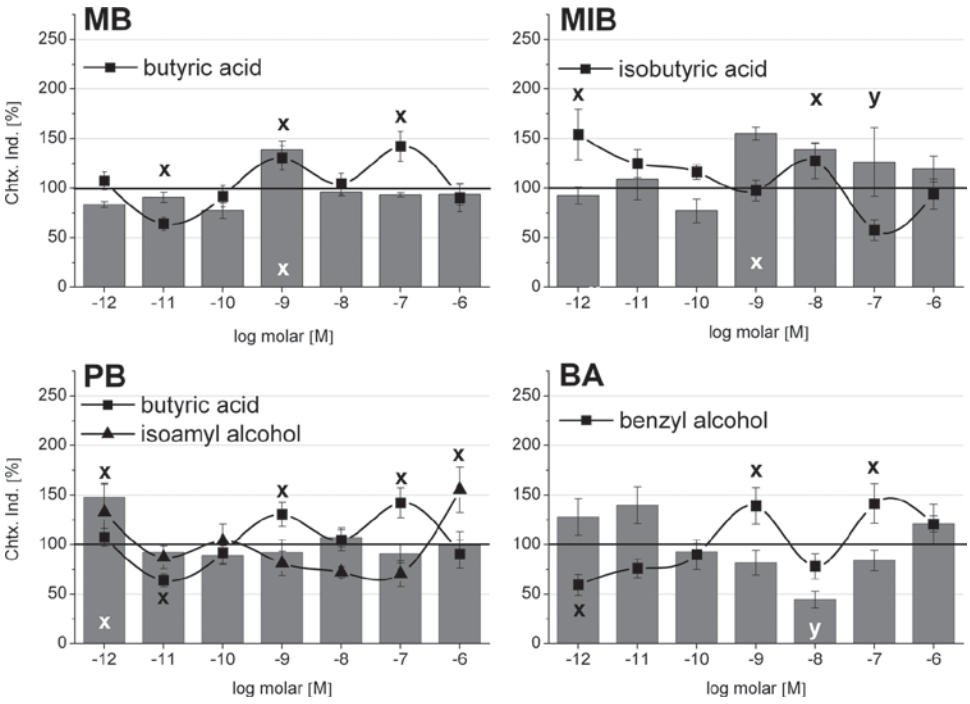


Figure 2. Chemotaxis induced by methyl butyrate (MB), methyl isobutyrate (MIB), pentyl butyrate (PB), benzyl acetate (BA) in Tetrahymena cells. Lines represent the respective reagents used. Significance levels in white correspond to the tested substances, while in black to the reagents (x: $p < 0.05$, y: $p < 0.01$).

the 6 chemoattractant fruit esters: PB at 10^{-12} M, IAA, IBP, MB, MIB at 10^{-9} M and PIB at 10^{-8} M. Data compared to the negative control population (untreated but immunostained cells) showed no PLC phosphorylation except for a slight activation in the case of IBP after 15 min (126.5%), of MB after 1 or 10 min of stimulation (mean fluorescence intensities were 137% and 128%, respectively) and of PB after 3 min (126%).

The stimulation of the PI3K pathway was also investigated using the same five fruit esters. For this purpose we pretreated the cells with a natural or a synthetic PI3K inhibitor, wortmannin and LY294002 respectively. Then we performed the chemotaxis assay. With the wortmannin we could not detect any chemotaxis inhibition compared to the untreated control population. However, LY294002 pretreated cells were slightly less responder to the IBP (inhibition index was 77%) and to the IAA (inhibition index was 84%).

Discussion

Chemotaxis

The study of the chemotactic responses of the *T. pyriformis* elicited by selected volatile odorants confirmed the high sensitivity of this model organism to slight changes in the molecular structure. The observed differences in the migratory behavior in response to compounds with very similar structures (e.g. PIB and IBP) are in good agreement with previous results obtained for the 20 L-amino acids (36) and insulin (37). In studies cited above, Tetrahymena was able to react in different ways to stimuli with the 20 L-amino acids and to distinguish

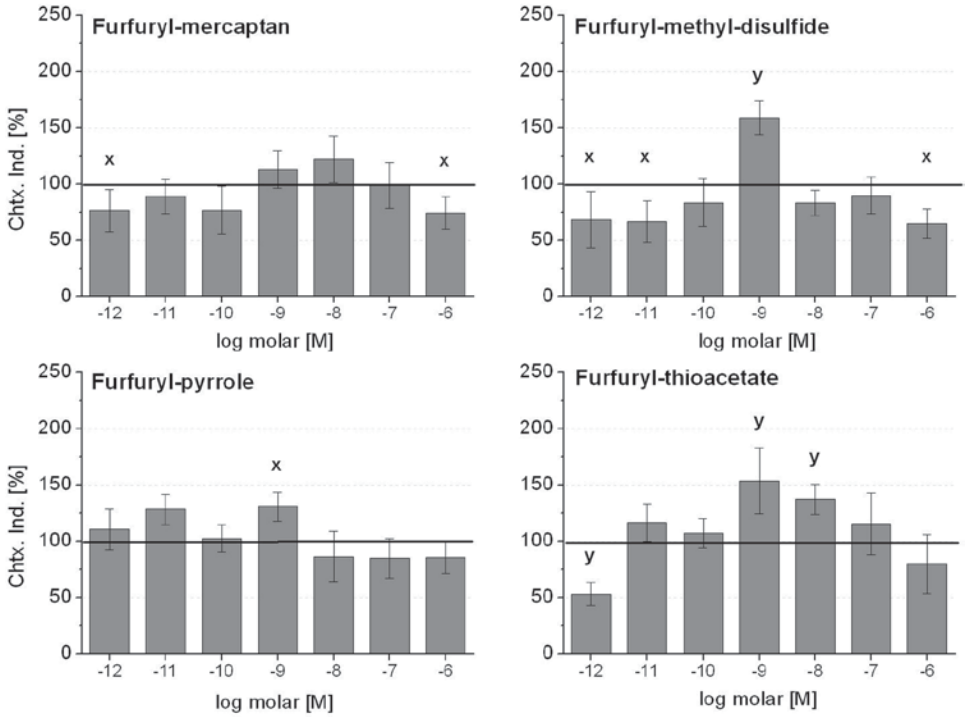


Figure 3. Chemotaxis induced by furfuryl mercaptan, furfuryl methyl disulfide, furfuryl pyrrole and furfuryl thioacetate in Tetrahymena cells. (Significance levels correspond to x: $p < 0.05$, y: $p < 0.01$).

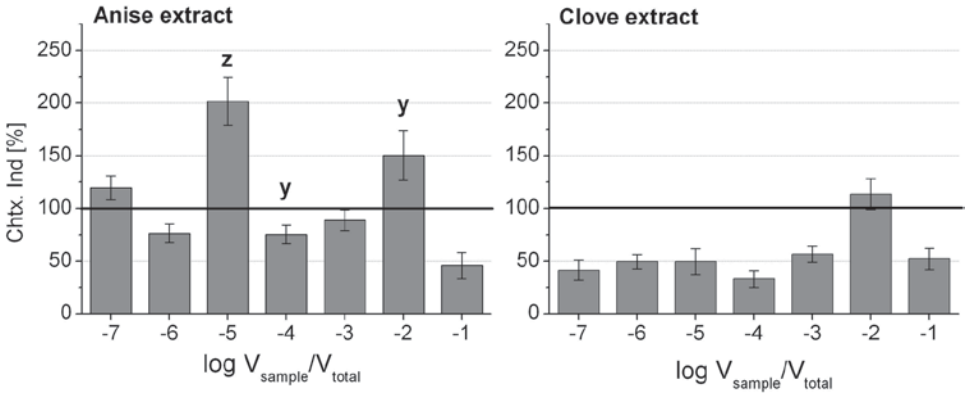


Figure 4. Chemotactic responses of Tetrahymena model cells obtained with essential oils of clove and anise. (Significance levels correspond to x: $p < 0.05$, y: $p < 0.01$).

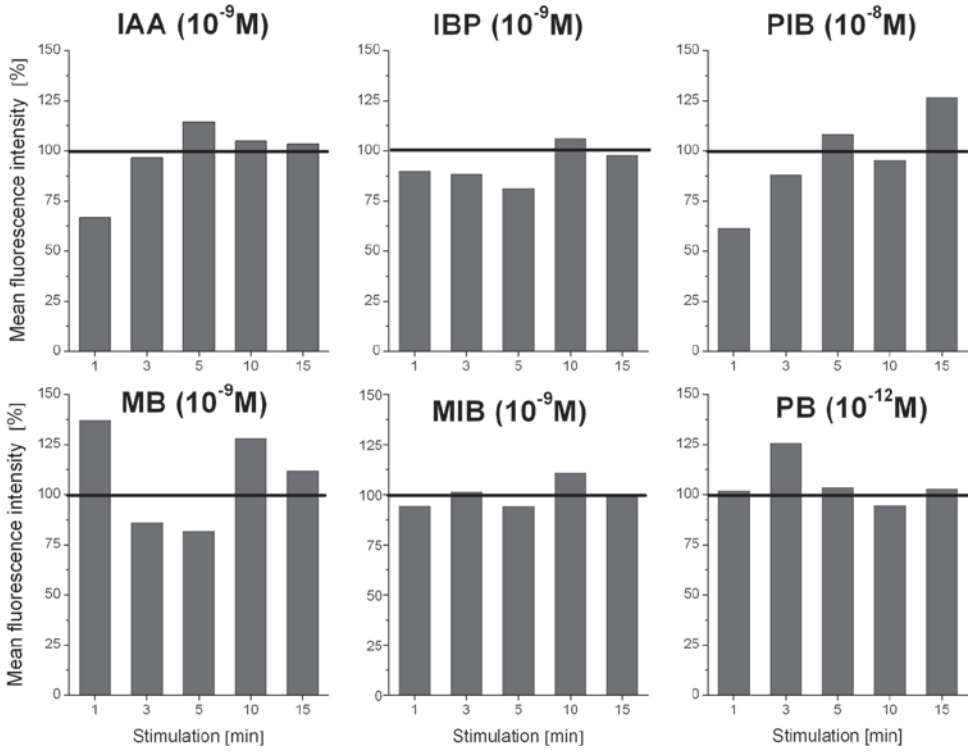


Figure 5. Time dependent phosphorylation of PLC in response to chemoattractant stimuli of fruit esters tested in their lowest, most significant effective concentrations in Tetrahymena cells (pentyl butyrate – PB, isoamyl acetate – IAA, isobutyl propionate – IBP, propyl isobutyrate – PIB, methyl butyrate – MB, methyl isobutyrate – MIB,).

insulin preparations according to their amorphous or crystalline form and also according to their bovine or porcine origin. In our work most potent chemoattractant odorants were IAA, PIB at 10^{-6} M and PB at 10^{-12} M. These substances are abundant in fruits, mainly in ripening ones, such as banana (23) or apple (19). The strong positive migratory response elicited by the IAA corroborates with literature data obtained in multicellular organisms such as *C. elegans* (6) and *D. melanogaster* (7). However, comparison of sensitivity between species is not possible because of a fundamental difference in the experimental setups, i.e. in these studies IAA was used in the vapor phase. The most important role of short chain aliphatic esters including IAA and PIB is to attract pollinators and guiding other insects to their food. Moreover, they can also act as pheromones and influence motility (38), foraging (39) and sexual behavior (40). Because of their highly attractant character IAA and PIB are also studied as a potential lure for insect traps (8,41). Less strong, but significant attractant behavior was observed using different concentrations of anise essential oil, IBP, MIB and three of the aromatic aldehydes (furfuryl methyl disulfide, furfuryl pyrrole and furfuryl thioacetate). Weak attractant effect could also be observed in some concentrations of MB. Purely chemorepellent odorants were furfuryl mercaptan at 10^{-12} M and 10^{-6} M and the extract of clove in almost the entire concentration range tested. Furfuryl mercaptan is well-known for its ability to activate cAMP dependent olfactory signaling pathway (42) and its antioxidative property (43) but no literature data is available about its chemotactic behavior. On the contrary, the essential oil of clove as well as its main component, the eugenol were described by several authors previously as potent repellents in Tetrahymena migratory assays even at micromolar concentration (10,44). Hence they might be considered as reference repellent substances. Both of them are well-known antibacterial and antioxidant compounds. They can permeabilize nonspecifically the cellular and intracellular membranes, consequently perturb ion flux and interfere with the cellular respiration (45-47). Eugenol was also reported to inhibit *E. coli* and *Listeria monocytogenes* motility at milimolar concentration (46) and this finding was linked to its capacity to disrupt bacterial cell membrane(46). Rodgers et al. in turn rose that the avoidance reaction of Tetrahymena induced by eugenol might be mediated by some primitive receptors of the ciliate (10).

Our results show that some of the tested substances (e.g. BA, furfuryl methyl disulfide, furfuryl thioacetate and anise essential oil) had ambiguous character as they also exhibited both chemoattractant and repellent effect at different concentrations. It is difficult to find a general correlation between molecular structure and chemotactic effects. Nevertheless it is interesting to compare the observed chemotactic behavior to the respective octanol/water partitioning coefficient (logP) (Table 1.) as this parameter can be correlated to the solubility of a given compound in the cytoplasmic membrane (48). Our results show that single substances with logP values higher than two had only attractant

effect, while compounds with smaller logP had either ambiguous or repellent character. This observation seems to contradict to some literature data, like the work of Wu et al. They reported that opiod agents were able to inhibit Tetrahymena motility (i.e. decrease the swimming speed) not because of their specific action on an opioid receptor but because of their membrane toxicity (49). This inhibitor potential increased with the logP value. However, there is a significant difference in the logP values in the two cases: it ranged from 1.3 to 57 in the experiment of Wu and from 0.8 to 2.7 in our work. It is highly probable that because of their hydrophobic nature odorants are able to interact directly with cellular surface membrane but because of the lower concentration and their lower logP values they had no direct membrane toxic effect.

Comparison of the chemotactic behavior of the esters and the organic reagents applied during their preparation showed no significant similarity. The high risk of application substances prepared by organic reagents is that these compounds will modify biological activity of the substances even in low concentrations. Our data showed that there were only few cases (e.g. IAA vs. isoamyl alcohol 10^{-12} M; 10^{-6} M or MB vs. butyric acid 10^{-9} M) where this kind of undesired effects of the reagents we should consider. In general, our work has confirmed that even in high concentrations there are no detectable biological effects of reactants and that fruit esters can elicit their molecule specific chemotactic effects on the model cells.

Signal transduction

Despite the high chemical variety of odorants the activated intracellular signaling cascade is uniform and conserved among phyla. Formerly literature hypothesized two second messenger molecules as mediators during olfaction: cAMP and insP3 (12) and odorants were classified as cAMP and insP3 type odorants based on their second messenger (50,51). Similarly, during the chemotaxis of eukaryotes both cAMP and InsP3 play important role in the intracellular signaling and regulation (52–54). Though in the theory of olfaction the importance of insP3 decreased in the last years, cAMP is still being considered as the only major excitatory second messenger in olfaction (13,14,55). However, Elsaesser et al. reported recently that PLCβ activity could be observed exclusively in a specific subset of olfactory cells that also contained other elements of the InsP3 signaling cascade. They concluded that these cells might represent a novel class of secondary sensory cells and shed light on the role of InsP3 in olfaction (12). Our results obtained by immunostaining of the phosphorilated PLC and inhibiting PI3K showed no significant activation of this signaling pathway. This is in accordance with previous studies which reported that short chain aliphatic esters and aromatic aldehydes (e.g. IAA and furfuryl mercaptan) at the micromolar–milimolar concentration range elicited cAMP mediated intracellular Ca^{2+} signal in olfactory neurons of Xenopus (56), salamander (57) and rat (58). Trinh and Storm also described that adenylcyclase III knock out mice were not able to detect IAA (59). Thus further investigation is needed to study activation of the cAMP dependent cascade during the odorant evoked chemotaxis of Tetrahymena. Moreover, some other alternative pathways may also be concerned mediated by for example the cGMP (60) or the NO (61). Finally, tested odorants may act independently from specific receptors and interact directly with cellular surface membrane as suggested by chemotaxis results. All in all, deeper analysis of the potential signaling events is necessary.

Conclusions

The chemotactic character of different naturally occurring odorants and tastants that may be also used as food additives was tested on the highly sensitive eukaryote ciliate model *T. pyriformis*. It was found that even slight changes in the chemical structure may result in different chemotactic profiles as it was shown in the case of the structural isomer fruit esters. Results also confirm that reagents applied during the synthesis of these compounds do not contribute to the chemotactic character of these substances.

Our results obtained during the study of the PLC-PI3K pathway suggest that it is not the predominant pathway of the intracellular signaling of chemoattraction exerted by fruits esters. Further investigation is needed in order to identify other pathways (cAMP, cGMP and NO) that may be involved in the signaling of these components.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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