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A New Feeding Attractant, Dimethyl-β-propiothetin, for Freshwater Fish

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We attempted here to detect new sulfur-containing feeding attractants using a method in which freshwater fish record their own strike response on a Kimographyone instrument. Among viarious sulfur-containing organic compounds, dimethyl-β-propiothetin (DMPT), and to a lesser extent, dimethylthetin, dipropyl (di)sulfide, dimethylsulfoxide and dimethylsulfone, strongly promoted the striking behavior in goldfish *Carassius auratus*, carp *Cyprinus carpio* and crucian carp *Craccius auratus cuvieri* when included in a synthetic diet (cellulose alone), a semi-natural diet and a natural diet. DMPT had a much greater stimulatory effect than glutamine at various concentrations. Moreover, DMPT induced much stronger neural activity in the olfactory tract of carp than L-glutamine. Thus, our results indicate that DMPT is an effective new attractant for striking of freshwater fish.

The feeding pattern, whereby prefered foods are searched for and ingested, is one of the most important behavior patterns exhibited by fish, and indeed by any living organism. Both electrophysiological and behavioral experiments investigating the taste or the olfactory nerves in fish have provided evidence that several amino acids, betaine and nucleotide 5′-monophosphates, singly or in combination, stimulate fish feeding.[1,2] In addition, similar effects of these compounds have been reported in the culture of the Japanese eel *Anguilla japonica*.[3,4] Here, we have attempted to discover other efficient fish feeding attractants by testing substances contributing to the smell of sea algeae, including dimethylsulfide[5,6] and related compounds.

Materials and Methods

The following sulfur-containing compounds, dimethyl-β-propiothetin (DMPT) (carboxyethyldimethyl sulfonium bromide), 2-mercaptoacetic acid, methyl 3-(methylthio)-propanoate, 3-methylthiopropanoic acid, 3-mercaptopropanoic acid, 3-methylpropanal, 3-methylpropylamine, and 3-methylpropanol were the kind gifts of Dr. Tetsuo Kawai of the Shiono Koryo Co. Ltd., Japan. Dimethyl-, diethyl-, dipropyl-, dibutyl-, dimethyl-4-butylo-, dimethyl-5-pentylo- diethyl-β-propio-, methyl ethyl-, dimethyl-2-methyl-, methyl-thetin and DMPT were synthesized by the reactions of the corresponding dialkyl sufides to the corresponding bromo carboxylic acids at around 40°C during the reflux periods of 3 to 24 h, respectively, and purified by washing them with excess amounts of cold dry ether. Dimethyl-, diethyl-, dimethyl-2-methyl-thetin and DMPT were further crystalized from their methanol solutions. Cellulose powder for animal diets was purchased from the Oriental Yeast Industry Co. Ltd., Japan. All the other compounds used were supplied by Wako Pure Chemicals Co. Ltd., Japan.

Goldfish (*Crassius auratus*; average weight 9.8 g, total length 8.4 cm), carp (*Cyprinus carpio*; average weight 8.0 g, total length 8.8 cm) and crucian carp (*Carassius auratus cuvieri*; average weight 5.7 g, total length 7.8 cm) were reared in separate acryl containers (21×35×26 cm high) containing 15.4 liters of tap water at 20°C. Water was aerated and filtered through glass wool.

A kimograph instrument (Kimographyone, Shimano Seisakusho Co. Ltd., Japan) was used to determine the strike response of the test fish.[7] This apparatus consists of a drum (18×15.5 cm) turning at a constant rate, the surface of which is preliminarily smoked, with a horizontally mounted recording pen. The tapered end of the pen was placed in contact with the drum surface and the other end was connected by a thin cotton thread (24 cm long) to a small magnet attached to the

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bottom of the vessel. Thus, the thread extended vertically from the bottom of the vessel to the recording pen.

Five fish were placed in a polystyrene test vessel (13×20×14 cm high) filled with two liters of freshwater at 20°C. Each solution (0.7 m*l*) to be tested was mixed to a paste with a total of 0.5 g of the following powders in a mortar for several minutes: cellulose powder (0.5 g) along for the synthetic diet; cellulose powder (0.25 g) and commercial fish diet powder (0.25 g) for the semi-natural diet; or commercial diet powder (0.5 g) alone for the natural diet. The commercial fish diet used, "Swimmy mini", was purchased from Nippon Pet Food Co. Ltd., Japan, and is composed of 37% crude protein, 2.5% crude fat, 3.0% crude fiber and 11.0% crude ash.

In a given trial, a ball of the test paste was attached to the thread 3 cm from the bottom of the vessel. When the fish struck the food suspended in the water, this moved the recording pen against the revolving drum, creating peaks and thus recording the striking behaviours. Each experiment was continued for 170 s, one revolution of the drum, after which the number of the peaks occurring in this period was summed. The control trials was conducted at the start and the end of an experiment and their average values were employed. The trials with tested compounds were done in a random sequence between the control ones. After every trial, the vessel, thread and fish used were washed with freshwater, and the water in the vessel was changed. The experiments were repeated six times using different fish, and the number of peaks was totaled and expressed as the striking frequency for the test compound.

For crude measurement of electrical responses from the carp olfactory tract, the fish (*Cyprinus carpio*; average weight 318 g, total length 34 cm) were anesthetized by intraventricular injection of a solution of galamine triethiodide (2 mg/kg body weight), then were fixed to a flexible lead plate. A portion (about 1.2×1.7 cm) of the upper skull of the sedated carp was excised using a drill (Leutor Mini Ace 200, Nihon Seimitsukikai Kousaku Co. Ltd., Japan), and the inner layers of lipid and connective tissue were gently swabbed off with pieces of cotton wool. In this manner, olfactory tract was exposed to air allowing the insertion of two insulated (except at the tips) bipolar stainless steel electrodes (diameter 0.2 mm) about 1 mm apart. Another electrode was introduced into the muscle above the eye as a gorund. The gills

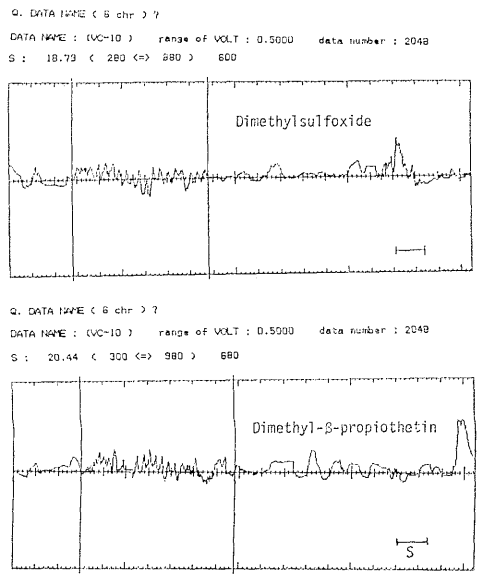

Fig. 1. Computer determination of the areas under traces of brain waves enhanced by dimethylsulfoxide and dimethyl-β-propiothetin.

The reagion in which brain waves are enhanced is delimited by the vertical lines drawn at the start and end points.

The symbol *S* represents the estimated area (μV·s) under the traced brain waves.

The notation "(260<=>880) 600" indicates that there were 600 dots analyzed from the start of the enhanced region at 280 dots to the end point at 880 dots. The entire region analyzed covered 2048 dots ("data number").

For experimental conditions see details in the Materials and Methods section.

of the fish were perfused with tap water at a flow rate of about 15 m*l*/s throughout the experiment. A microdispensor (Drumond Scientific Co. Ltd., U.S.A.) was used to deliver 50 μ*l* aliquots of the test solutions into the continuous water flow (about 8 m*l*/min) which was introduced into the nasal cavity of the fish through a special glass inlet. The test solution arriving at the olfactory sac was diluted about 29-fold.

Electrical responses from the olfactory tract, enhanced by the administered solutions, were amplified and recorded on the recording paper of an electronencephalograph (Model Me-135D, Nihon Kohden Kogyo Co. Ltd., Japan), and simultaneously on a casette tape recorder (Model RMG-5304, Nihon Kohden Kogyo Co. Ltd., Japan).

To calculate the area under the electrical response patterns traced, data was transfered from

the tape to a dual beam memory oscilloscope VC-10 (Nihon Kohden Kogyo Co. Ltd., Japan) and then loaded into a computer (PC-8801 mkII, Nihon Electric Co. Ltd., Japan). The region of wave enhancement appearing on the oscilloscope was specified by manually positioning two vertical lines. Automatic calculation of the area under the waves and between these lines was then carried out by the computer. Area estimations for the waves enhanced in response to DMPT and dimethylsulfoxide are shown in Fig. 1.

Subsequently, the exposed part of the skull was covered with an uncharged sheet of vinyl resin (Sekisui Kagaku Co. Ltd., Japan) and sealed with a binder, "Suichu Bond" made of epoxy and polyamide resins (Konishi Co. Ltd., Japan). The three electrodes were left in place to monitor electrical responses from the olfactory tract (as described above) of unanesthetized carp during the 2–3 day recovery period.

Results

Effects of Dimethylsulfide and Related Sulfur-containing Compounds on the Strike Response of Goldfish

Effects of dimethylsulfide (DMS), DMPT (a precursor of DMS) and their derivatives on the striking behavior of goldfish were examined using a kimograph. As shown in Tables 1 and 2, DMPT was the most effective stimulant, but dimethylsulfoxide, dimethyl sulfone, dipropylsulfide and dipropyldisulfide also caused significant increases in striking frequency. DMT was further found to be little less effective than DMPT. The longer the carbon chain of the dialkyl portion or the methylene carbon chain on the molecules of the tested compounds was, the less effective their effects on the striking behaviors of goldfish were, when compared to that of DMPT. Furthermore, deletion of one of two alkyl group, replacement of the carboxylic group with aldehyde, amino carbonyl or alcohol group, or combination of methyl group to the methylene carbon also provided lesser striking efficiency.

Similar results were also obtained from carp on the synthetic diet, with DMPT, dimethylsulfoxide, dimethylsulfone, dimethylsulfite, DMS and the control generating striking frequencies of 126, 119, 86, 80, 72, and 54, respectively (data not shown).

Degradation products of DMPT, DMS and acrylic acid, exhibited lower ability to stimulate either singly or in combination, when compared with DMPT.

Effects of Dimethyl-β-propiothetin and Glutamine at Varied Concentrations on the Strike Response of Goldfish

Glutamine has proven to be one of the most effective compounds for promoting fish feeding.[7] Thus, effects of DMPT and glutamine on the striking behavior of goldfish were compared in the concentration range of 10^{-6} to 10^{-1} M. Results are given in Fig. 2. The control to test striking frequency ratio reached a maximum for both compounds at 10^{-3} M, with DMPT being about twice as effective as glutamine at this point but at higher concentrations the ratio decreased somewhat.

Table 1. Effects of DMPT and related compounds on striking behavior of goldfish

Exp.	Compound*	Striking frequency (No.)	Exp.	Compound*	Striking frequency (No.)
I	Control	16	III	Control	32
	DMPT	130		DMPT	136
	Dimethyl sulfide	70		Dimethyl sulfide	64
	Diethyl sulfide	58		Dimethyl sulfoxide	113
	Dipropyl sulfide	109		Dimethyl sulfone	92
	Dibutyl sulfide	81		Dimethyl sulfite	57
II	Control	14	IV	Control	1
	DMPT	230		DMPT	71
	Dimethyl disulfide	109		Dimethyl sulfide	50
	Diethyl disulfide	77		Acrylic acid	18
	Dipropyl disulfide	163		Dimethyl sulfide +Acrylic acid	33
	Dibutyl disulfide	112			

* The compounds were used at a concentrations of 10^{-3} M.
Abbreviations: Exp., Experiment; DMPT, Dimethyl-β-propiothetin (Carboxyethyldimethylsulfonium bromide). Roman numerals show the seperate experiments.
For experimental conditions see details in the Materials and Methods section.

Table 2. Effects of DMPT and closely related compounds on the striking behaviors of goldfish

Exp.	Compound*	Striking frequency	Exp.	Compound*	Striking frequency
		(No.)			(No).
I	Control	19	IV	Control	164
	DMPT	138		DMT	453
	Methyl(3-methythio)propanoate	82		DMPT	575
	2-Mercapto-acetic acid	87		Dimethyl-4-buthylothetin	373
	3-Methylthio-propanoic acid	81		Dimethyl-5-pentylothetin	359
	3-Mercapto-propanoic acid	82			
			V	Control	124
II	Control	44		DMT	386
	DMPT	314		DMPT	503
	3-Methylthio-propanal	109		Diethyl-3-propiothetin	340
	3-Methylthio-propylmaine	101		Methyl ethyl-thetin	323
	3-Methylthio-propanol	129		Dimethyl 2-methyl-thetin	330
III	Control	186	VI	Control	9
	DMT	553		DMPT	143
	Diethyl thetin	419		Dimethyl sulfide	69
	Dipropyl thetin	360		Methyl cystein	56
	Dibutyl thetin	350		Methyl methionine	16

* The compounds were used at a concentration of 10^{-3} M.
Abbreviations: DMT, Dimethyl thetin (Carboxymethyl dimethylsulfonium bromide). Other abbreviationins and illustrations were the same as those in Table 1.
For experimental conditions see details in the Materials and Methods section.

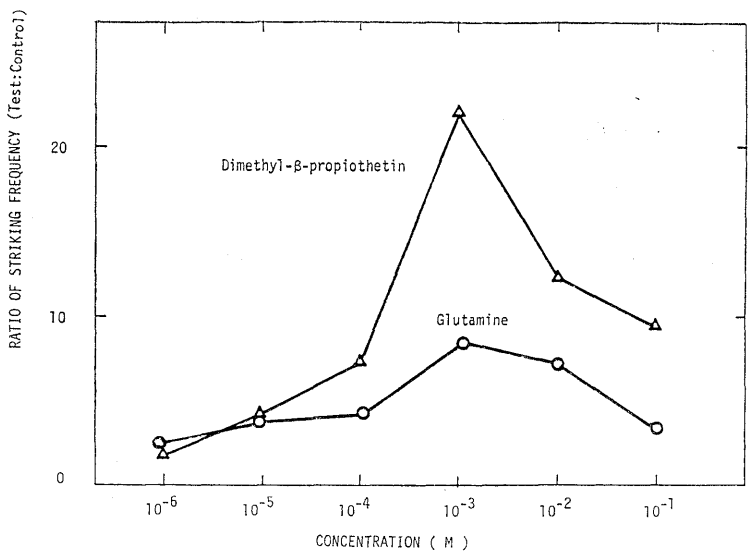

Fig. 2. Effects of dimethyl-β-propiothetin and glutamine at varied concentrations on the response of goldfish.

Two series of experiments, one with dimethyl-β-propiothetin and one with glutamine, were separately conducted using fish on a synthetic diet. Results were expressed as the ratio of the striking frequency in response to the test compund against that of the control diets at each concentration.

For further details see the Materials and Methods section.

Effects of Dimethyl-β-propiothetin on the Strike Response of Some Freshwater Fish with more Nutrient Rich Diets

The same experiments as in Tables 1, 2 and Fig. 2 were performed with goldfish, carp, and crucian carp receiving a smei-natural or natural diet (Talbe 3). In goldfish, DMPT increased the striking frequency by a fator of about 1.4 when these diets were used rather than a completely synthetic regimen. Increases by factors of about 1.3 and 1.5 were seen in crucian carp fed, respectively, a natural and semi, while in carp these were approximately 1.1 and 1.6. Conversely, L-glutamine at a concentration of 1 mM stimulated 0.92 times the number of strikes that the control did in goldfish given a semi-natural ldiet; for carp on a natural diet, the ratio dropped to 0.57 (data not shown).

Effects of Sulfur-containing Compounds on the Olfactory Nerve of Carp

The effectts of several sulfur-containing compounds, all with proven abilities to promote striking behavior of goldfish, on the olfactory tract of carp were examined at a concentration of 10^{-3} M (Table 4). Among these compounds, DMPT most strongly activated the electrical responses from the olfactory tract. DMS, dimethylsulfoxide, dimethylsulfone and L-glutamine all enhanced electrical responses to a similar degree as compared with each other, but dipropyldisulfide had little effect.

Table 3. Effects of dimethyl-β-propiothetin on the striking behavior of goldfish receiving semi-natural and natural diets

Compound	Striking Frequency	
	Semi-natural Diet*1	Natural Diet*2
	(No.)	(No.)
Gold Fish		
Control*3	11	3
Water	113	150
DMPT	157	213
Crucian Carp		
Control*3	17	4
Water	44	45
DMPT	66	60
Carp		
Control*3	0	25
Water	103	346
DMPT	161	389

*1 Mixture of commercial fish food and cellulose powder (1 : 1).
*2 Commercial fish diet alone. *3 Cellulose powder paste mixed with distilled water. DMPT shows dimethyl-β-propiothetin, which was used at 1 mM.

Table 4. Effects of several sulfur-containing compounds on the olfactory nerve of sedated carp

Compound	Olfactory response
DMPT	37.7*1 (135)*2
Diemthylsulfide	26.6 (95)
Dimethylsulfoxide	29.3 (105)
Dimethylsulfone	30.5 (109)
Dipropyldisulfide	14.5 (52)
Glutamine	27.9 (100)

*1 Area of activated brain waves (μV·s.)
*2 Values relative to those of glutamine
The values are the mean of three experiments.
DMPT shows dimethyl-β-propiothetin.
For experimenta, conditions see details in the Materials and Methods section.

Discussion

Many have been found to stimulate fish feeding. They may be classified into those that activate the olfactory nerves, nerves associated with taste or feeding behavior. Among these substances, glutamine, lysine, methionine, arginine, serine, and alanine, to name a few, have proved to be effective olfactory activating agents in carp, catfish, sea bream, and salmon,[2] while alanine, glycine, proline, valine and serine excite the taste nerves and promote feeding behavior in the eel, catfish, puffer, yellowtail, and sea bream.[8] Increased feeding activity has also been reported in the case of puffer, red sea bream and yellowtail given betaine,[1,8] nucleotides or phospholipids.[9] Similar effects have been brought about by fatty acids,[5] alcohols and salts.[2]

Dimethylsulfide (DMS) is known to be the primary agent responsible for the smell we associate with the sea, but which is really produced by sea algae.[5,6] However, there have been no reports detailing the possible effects of this. Thus, we employed a kinographyone to investigate the influence of DMS and related sulfur-containing substances on the strike response of freshwater fish. Kimography is a simple and well established technique which, since it employs similar principles, yields results whose preciseness is believed to be not substantially different from that achieved by recent electronic methods (eg. strain gauges, microswitches).[7,10] On this basis, it was found that feeding activity in goldfish was most efficiently stimulated by dimethyl-β-propiothetin (DMPT) and, to a lesser extent, by dimethylthetin (DMT) and dipropyl (di)sulfide, and by its oxidation products, dimethylsulfoxide and dimethylsulfone. Other compounds, though often structurally

similar compounds to DMPT, proved less attractive to feeding fish.

As DMPT enzymatically and chemically degrades into acrylic acid and DMS,[11-12] the effects of these two agents on the striking behavior of goldfish were examined, singly or in combination. Both gave rise to low striking frequencies, which may suggest that DMPT itself has the most important role as activator.

More strikes were induced by DMPT than by glutamine, which has previosuly been reported to be the strongest known activator of the olfactory nerve[2] and also to be the potent amino acid stimulator of goldfish of striking behavior.[7] This characteristic of DMPT was not only observed in cases of goldfish, carp, crucian carp, and tilapia (*Tilapia nilotica*; unpublished data) on a synthetic diet of cellulose powder but when these fish were given a semi-natural or a natural diet. Glutamine at a concentration of 10^{-3} M, however, did not increase, but rather inhibited the striking activity of goldfish and carp on either a semi-natural or natural diet. Thus, 10^{-3} M DMPT exerted a clear stimulatory effect on strike response even when contained within a nutrient-rich, complete food ball.

There is a brief report that not DMPT but DMT increasese the food intake in Dover sole.[14] However, no comparison of their feeding responses with DMPT, DMT and other sulfur-containing compounds or with Dover sole and other fish species was made in the report. This has showed the positive but equivocal feeding stimulation effect of DMT. Present experiments, however, provided clear evidence that DMPT and, to a lesser extent, DMT exerts the strongest feeding attraction among the closely related sulfur-containing compounds and DMPT exhibits high striking responese in different fish fed on different diets, and further that DMPT is much more effective in various concentrations than glutamine which showed the most strikes among many amino acids tested.[7]

It has been reported that carp have a characteristic receptor on their taste buds for monosodium glutamate.[15] Form our findings, such fish may also have a specific chemoreceptor for DMPT in their gustatory or olfactory organs, since DMPT induced almost the same levels of striking activity with various diets.

DMPT was found to be a strong excitor of the olfactory nerve in experiments conducted on the olfactory tracts of anestheticzed carp. Olfactory response was remeasured 2 to 3 days later using the same, but normally swimming (*i.e.* recovered) carp; responses to DMPT measured relative to those produced by glutamine (100) were 135 during surgery and 112 2 to 3 days later. These findings suggest that DMPT fisrst activates the olfactory sense, then induces feeding motion and striking behavior. Further support for this is provided by the fact that carp under normal conditions but without olfactory tracts could not eat even natural food boluses dropped just before them, while carp which had undergone the same operations but which had two olfactory tracts could immediately consume them (unpublished data). Moreover, the fish in the present trials could actively swallow semi-natural food after striking them, when it conitaned DMPT. For these reasons, DMPT is thought to strongly induce a series of feeding movements; approaching as the olfactory sense is stimulated, striking, tasting, and swallowing DMPT-supplemented food. At this time, stimulation of the olfactory sense may trigger feeding behavior.[16]

The distribution of DMPT in the aquatic environment is world-wide. That is, it occurs, along with its degradation product, DMS (unpublished data), in many fresh and sea water organisms, including unicellular algae,[13] multicellular algae,[5,11,12,17] krills,[18] mollusks,[19,20] and fish.[20-23] However, mollusks and fish are believed to obtain DMPT from their diets, while algae and perhaps krills actually synthesize DMPT and accumulate it at levels as high as 3 to 5×10^{-3} M.[5] This is likely since the DMPT degrading enzyme has not been found in fish[20-23] but is present in algae,[5,19] and DMPT concentrations found in mollusks and fish are similar to those measured in their respective diets.[20-24] DMPT levels appear to decrease in the order of algae (10^{-3} M), mollusks (10^{-4} M) and fish (10^{-5} M),[5,19,20,22,23] indicating that DMPT may play an important role in directing the food chains linking algae, krills, mollusks, small fish and large fish in various aquatic environments. Some support for this has been provided by the finding[20] who found that Alaska pollack which ate large quantities of krill contained elevated amounts of DMPT in their muscles. To substatiate this hypothesis, however, many questions need to be answered concerning the biosynthesis of DMPT in various aquatic organisms, the feeding habits of fish, and the interactions of members of relevant food chains.

Experiments to further examine the effects of DMPT and its derivatives are now in progress.

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