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HETEROCYCLIC COMPOUNDS AS RELEASERS OF THE FRIGHT REACTION IN THE GIANT DANIO *Danio malabaricus* (JERDON) (CYPRINIDAE, OSTARIOPHYSI, PISCES)

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Abstract—Fifty-nine pteridine, purine, and pyrimidine derivatives were tested with schools of the giant danio *Danio malabaricus* (Jerdon). The fright reaction was elicited by three pteridine derivatives: 2,6-diamino-4-oxodihydropteridine, isoxanthopterin, and 6-acetonylisoxanthopterin. A minor effect could not be excluded for three purine derivatives: I-5-MP, IDP, and ITP.

Key Words—Fish, *Danio malabaricus* (Jerdon), fright reaction, pheromone, heterocyclic compounds.

INTRODUCTION

The fright reaction of the European minnow *Phoxinus phoxinus* (L.) and other Cypriniformes is elicited by an alarm substance deriving from the fish's skin (von Frisch, 1941; recent reviews: Pfeiffer, 1974, 1977). Experiments with isoxanthopterin had shown that this substance may elicit the fright reaction in the giant danio *Danio malabaricus* (Jerdon), although it is not identical with the genuine alarm substance (Pfeiffer and Lemke, 1973; Pfeiffer, 1975). During the investigation on the isolation of the alarm substance from fish skin several heterocyclic compounds were tested with respect to their biological activity.

METHODS AND MATERIALS

Schools of the giant danio *Danio malabaricus* (Jerdon) were used, and

consisted of 6–8 juveniles, each measuring 30–50 mm. The fish were raised at 24°C and daily fed with dry food (Tetramin, Tetra-Werke, Melle, West Germany). Light period lasted from 7 AM to 6 PM. The health of the fish was daily controlled, and only healthy fish were tested. The fish were conditioned to the experimental aquarium prior to testing until they no longer fled when a person approached the tank but remained near the feeding corner at the surface in expectation of food. Such conditioning time was usually about one to two weeks. Since fish may rapidly adapt to substances that elicit the fright reaction, most schools were tested only once with an effective substance. If fish were tested repeatedly, a minimum interval of four weeks elapsed between two successive experiments. The experiments were performed in 150 aquaria ($40 \times 20 \times 20$ cm) containing 20 liters of water. All aquaria were filtered (Billi-filters, Tetra-Werke), and heated with 25-W thermostat heaters (Fa. Jäger, West Germany). Each tank contained moss and rods of *Monstera* or *Syndapsus*, plants which eliminated nitrate and nitrite from the water and also served as hiding places for the fish. After each test with a fright reaction the aquarium was carefully cleaned and filled with tap water from Lake Constance. Both the tanks and the substances tested were selected at random.

The behavior of the fish was observed for 5 min after the substance under investigation had been introduced into the tank. An active test substance induced a fright reaction in 5–30 sec. Fish which had assembled at the feeding corner close to the water surface seemed terrified and fled towards the bottom in confusion; they then retreated. Confidence returned after intervals ranging from minutes to several hours. The intensity of the fright reaction varied and three different situations were distinguished. Such stages were evaluated arbitrarily, and ranged from the most intense reaction where all fish were suddenly frightened and hastily fled, to a scarcely visible intimidation.

Positive Reaction. Most intense reaction with sudden fright and rapid swimming towards the bottom. The fish may rapidly swim around the tank or stay motionless close to the bottom.

Questionable Reaction. Only slightly frightened or somewhat confused; swimming to the middle depth of the aquarium where the school may crowd, but no retreating to the bottom and usually soon quieting down. Sometimes intimidated and less confident at the feeding corner with some crowding together, but the reaction soon disappears.

Negative Reaction. No reaction whatever.

One compound was tested simultaneously on many tanks. With each substance generally 5–12 tests were performed with as many schools. Isoxanthopterin, xanthopterin, inosine, and cAMP were tested for 2–4 days in order to confirm their effects; different schools were used. If the fish did not

react to a compound, their ability to react was tested the following day using skin extract from the European minnow, *Phoxinus phoxinus* (L.). Schools that did not react to the skin extract were thereby eliminated. Solutions were prepared from 1 mg substance and 100 ml solvent (0.01 N HCl or 0.01 N NaOH). Five ml of solution equivalent to 0.05 mg of substance were introduced into the aquarium within a period of 10–15 sec. Experiments with the solvent or with extracts from minnow skin were performed as controls. All the experiments were made by one person only without prior knowledge of the substance under investigation.

RESULTS

Positive results were obtained by a number of substances. However, whereas some substances elicited the fright reaction regularly, others induced it only exceptionally; three groups of compounds were distinguished:

Highly Effective Substances. A compound was considered highly effective if more than 50% of the tests performed produced a positive reaction, and less than 20% a negative reaction.

Ineffective Substances. A compound was considered ineffective if less than 20% of the tests performed produced a positive reaction and more than 50% a negative reaction.

Possibly Weakly Effective Substances. For one group of compounds closely related to one another a minor effectiveness could not be excluded since approx. 25% of the tests produced a positive reaction, and only 30–50% a negative reaction. A relatively high percentage of the tests with these substances induced questionable reactions.

Three of the pteridine derivatives tested elicited the fright reaction: 2,6-diamino-4-oxodihydropteridine (58% positive, 33% questionable tests), isoxanthopterin (56% positive, 28% questionable tests), and 6-acetonyl-isoxanthopterin (100% positive tests). In contrast, xanthopterin and 7-acetonylxanthopterin were ineffective, as were the other pteridines tested (Table 1).

A minor effect of purine derivatives could not be excluded for I-5-MP, IDP, and ITP: 23–26% of the experiments with these substances elicited the fright reaction, and 21–48% of the tests gave questionable reactions in addition. The other purine derivatives tested were ineffective, as were all the pyrimidine derivatives. All ribonucleoside 2′,3′-cyclic phosphates and 3′,5′-cyclic phosphates were ineffective, including A-3,5-MP and I-3,5-MP (Table 2).

Some substances elicited the fright reaction in less than 20% of the tests, and included pterorhodin, ekapterin, C-3,5-MP, hypoxanthine, and inosine (Tables 1, 2). These compounds were considered ineffective.

TABLE 1. RESULTS OF FRIGHT REACTION TESTS WITH PTERIDINE DERIVATIVES AND CONTROLS

Substance	Positive[a]	Questionable[a]	Negative[a]	Date
4-Hydroxypteridine	0	0	9	7/27/73
4-Hydroxy-6,7-dimethylpteridine	0	0	7	7/26/73
Lumazine (2,4-dihydroxypteridine)	0	0	9	7/27/73
4-Hydroxy-2-mercaptopteridine	0	0	9	7/27/73
8-Ribityl-6,7-dimethyl-lumazine	0	0	9	7/27/73
Pterin (2-amino-4-hydroxypteridine)	0	0	7	7/26/73
2,6-Diamino-4-oxodihydropteridine	7	4	1	1/22/73
L-Monapterin	0	0	7	1/22/73
Pterin-6-carboxylic acid	0	0	7	7/26/73
Pterin-6-acetic acid	0	0	5	1/3/73
Biopterin	0	1	10	10/11/73
D-Neopterin	0	0	5	1/5/73
6,7-Dimethylpterin	0	0	5	1/15/73
7-Methylpterin	0	0	6	1/15/73
Pterin-7-carboxylic acid	0	0	7	7/26/73
Leucopterin	0	0	5	1/3/73
Folic acid	0	0	10	5/30/73
Xanthopterin (6-hydroxypterin)	0	0	12	1/3/73 and 12/12/73

7,8-Dihydroxyxanthopterin	0	0	5	1/15/73
Chrysopterin	0	0	5	1/15/73
7-Acetonylxanthopterin	0	2	8	1/22/73
Ekapterin	1	1	4	1/5/73
Erythropterin	0	0	5	1/5/73
Lepidopterin	0	1	5	1/5/73
Pterorhodin	3	1	13	10/11/73
Isoxanthopterin (7-hydroxypterin)				
1	12	6	0	6/15/72
2	4	0	0	7/14/72
3	2	1	4	11/3/72
4	4	4	2	1/3/73
total	22	11	6	
Isoxanthopterin-6-carboxylic acid	0	0	5	1/3/73
6-Acetonylisoxanthopterin	9	0	0	1/22/73
Controls				
Solvent (0.01 N NaOH or 0.01 N HCl)	0	0	25	3/5/15 and 1/22/73
Skin extract from *Phoxinus*	110	6	4	

[a] See methods section for criteria of positive reaction.

TABLE 2. RESULTS OF FRIGHT REACTION TESTS WITH PURINE AND PYRIMIDINE DERIVATIVES

Substance	Positive[a]	Questionable[a]	Negative[a]	Date
Adenine	0	0	8	5/24/73
Guanine	0	0	8	5/24/73
Hypoxanthine	3	9	23	5/12/75
Adenosine	0	0	10	5/4/73
Guanosine	0	0	6	5/4/73
Inosine				
1	0	0	6	5/4/73
2	1	3	16	5/12/73
Total	1	3	22	
Uridine	0	0	6	5/4/73
Cytidine	0	0	6	5/4/73
2′-Deoxythymidine	0	0	7	5/4/73
Adenosine-5′-monophosphate (A-5-MP)	0	0	8	5/24/73
Adenosine-5′-triphosphate (ATP)	0	0	8	5/24/73
Guanosine-5′-triphosphate (GTP)	0	3	7	5/30/73
Inosine-5′-monophosphate (I-5-MP)	7	14	8	5/27/75
Inosine-5′-diphosphate (IDP)	6	6	14	5/27/75
Inosine-5′-triphosphate (ITP)	10	8	20	5/27/75
Cytidine-5′-monophosphate (C-5-MP)	0	0	8	5/24/73

Cytidine-5′-triphosphate (CTP)	0	0	8	5/24/73
2′-Deoxythymidine-5′-monophosphate (dT-5-MP)	0	4	6	5/30/73
2′-Deoxythymidine-5′-diphosphate (dTDP)	0	3	8	5/30/73
Adenosine-3′,5′-monophosphoric acid, cyclic (A-3,5-MP)				
1	0	0	7	10/18/73
2	0	0	7	12/12/73
3	0	3	12	5/24/74
Total	0	3	26	
2′-Deoxyadenosine-3′,5′-monophosphate, cyclic (dA-3,5-MP)	0	0	6	12/11/73
Guanosine-3′,5′-monophosphate, cyclic (G-3,5-MP)	0	0	6	12/11/73
Inosine-3′,5′-monophosphoric acid, cyclic (I-3,5-MP)	0	0	6	12/11/73
Xanthosine-3′,5′-monophosphate, cyclic (X-3,5-MP)	0	0	7	12/12/73
Cytidine-3′,5′-monophosphate, cyclic (C-3,5-MP)	1	2	4	10/18/73
Uridine-3′,5′-monophosphate, cyclic (U-3,5-MP)	0	0	7	10/18/73
2′-Deoxythymidine-3′,5′-monophosphate, cyclic (dT-3,5-MP)	0	1	6	10/18/73
Adenosine-2′,3′-monophosphoric acid, cyclic (A-2,3-MP)	0	0	7	12/12/73
Guanosine-2′,3′-monophosphate, cyclic (G-2,3-MP)	0	0	7	12/12/73
Cytidine-2′,3′-monophosphoric acid, cyclic (C-2,3-MP)	0	0	6	12/11/73
Uridine-2′,3′-monophosphate, cyclic (U-2,3-MP)	0	0	7	12/12/73

[a] See methods section for criteria of positive reaction.

In total, 59 pteridine, purine, and pyrimidine derivatives were tested in some 750 experiments from June 15, 1972 to May 27, 1975 (Tables 1, 2). None of the heterocyclic compounds tested was as effective as the skin extract from *Phoxinus*, with the possible exception of 6-acetonylisoxanthopterin. Whereas skin extract was effective in most experiments, the solvent was always ineffective (Table 1).

DISCUSSION

Whereas isoxanthopterin and especially 6-acetonylisoxanthopterin elicited the fright reaction in the giant danio (*Danio malabaricus*), their isomers xanthopterin and 7-acetonylxanthopterin were ineffective. The following results are in accord with these observations: (1) 6-Acetonylisoxanthopterin in the European minnow (*Phoxinus phoxinus*) produced bradycardia in contrast to 7-acetonylxanthopterin and xanthopterin which did not have this effect (Pfeiffer and Lamour, 1976). (2) Isoxanthopterin and 6-acetonylisoxanthopterin had a strong effect on the central nervous excitation measured quantitatively using the dorsal light response in the unilaterally illuminated black tetra (*Gymnocorymbus ternetzi*). An enhanced optical alertness shown by an increase of the fishes' inclination towards the light was produced with these substances, whereas both xanthopterin and 7-acetonylxanthopterin were ineffective (Pfeiffer and Riegelbauer, 1978).

Both isoxanthopterin and 6-acetonylisoxanthopterin elicit the fright reaction, produce bradycardia, and cause a strong change of the central state in contrast to their biologically ineffective isomers xanthopterin and 7-acetonylxanthopterin.

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REFERENCES

FRISCH, K. VON. 1941. Über einen Schreckstoff in der Fischhaut und seine biologische Bedeutung. *Z. Vergl. Physiol.* 29:46–145.

PFEIFFER, W. 1974. Pheromones in fish and amphibia, pp. 269–296, *in* M.C. Birch (ed.). Pheromones. A. Neuberger and E.L. Tatum (eds.). Frontiers of Biology, vol. 32. North-Holland, Amsterdam.

PFEIFFER, W. 1975. Über fluoreszierende Pterine aus der Haut von Cypriniformes (Pisces) und ihre Beziehung zum Schreckstoff. *Rev. Suisse Zool.* 82:705–711.

PFEIFFER W. 1977. The distribution of fright reaction and alarm substance cells in fishes. *Copeia* 1977:653–665.

PFEIFFER, W., and Lamour, D. 1976. Die Wirkung von Schreckstoff auf die Herzfrequenz von *Phoxinus phoxinus* (L.) (Cyprinidae, Ostariophysi, Pisces). *Rev. Suisse Zool.* 83:861–873.

PFEIFFER, W., and Lemke, J. 1973. Untersuchungen zur Isolierung und Identifizierung des Schreckstoffes aus der Haut der Elritze, *Phoxinus phoxinus* (L.) (Cyprinidae, Ostariophysi, Pisces). *J. Comp. Physiol.* 82:407–410.

PFEIFFER, W., and Riegelbauer, G. 1978. The effect of the alarm substance on the central nervous excitation of the black tetra *Gymnocorymbus ternetzi* (Characidae, Ostariophysi, Pisces) induced by dorsal light response. *J. Comp. Physiol.* 123:281–288.