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Activity of some 3-formylchromone derivatives on the induction of chloroplast-free mutants in *Euglena gracilis*

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Abstract

The hereditary bleaching test on *Euglena gracilis* was used for detecting extranuclear mutations. The highest bleaching activity (induction of the chloroplast-free mutants) was shown by the 6-*R*-3-formylchromones. On the other hand, bleaching-inactive 6-*R*-3-formylchromone acylhydrazones (derived from gallic and salicylic acids), added at sufficient concentrations in the case of chloroplast mutagenesis in *E. gracilis*, act as a potent antimutagen. This effect appeared to be a unique feature of chromone derivatives, but was dependent on the type of mutagen. These substances were very effective against the bleaching activity of acridine orange, and were less effective against *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. The genotoxic effects of these mutagens was reduced, especially during the first stages of induction of this specific cytoplasmic mutation. The experimental study of mutagenicity and antimutagenicty of 3-formylchromone hydrazones was reinforced by data obtained by the semi-empirical AM1 method and lipophilicity values.

Keywords: 3-Formylchromone-*N*-aroyl hydrazones; *Euglena gracilis*; Bleaching; Antimutagenicty; QSAR; AM1

1. Introduction

Chromone derivatives offer interesting possibilities from the point of view of both organic synthesis and assays of their biological activities.

Chromones possess a wide variety of biological activities [1–3]. This paper is a continuation of the previous work [4] and describes the effects of 6-*R*-3-formylchromones and their hydrazone derivatives on *Euglena gracilis*.

Genotoxic properties of chemical compounds and antimutagenicity profiles for some model compounds have been mostly described using bacterial systems [5–7]. Results of recently published work [8–11] show that *E. gracilis* may be used as a eukaryotic organism for studying mutagenesis and antimutagenesis.

Many genotoxic agents exert a mutagenic effect on the unicellular flagellate *E. gracilis* [12]. Green autotrophic cells of *E. gracilis* affected by mutagens are converted to heterotrophic cells due to the irreversible loss of chloroplasts, also called 'hereditary bleaching'. This genotoxic effect of mutagens in *E. gracilis* was reduced by standard antimutagens [9,13,14] and by some commercial salicylates [15]. Inhibitors that reduce the frequency of spontaneous or induced mutation, regardless of the mechanism involved, are called antimutagens. They can be further classified as 'des-mutagens' and 'bio-antimutagens' [16].

The examination and evaluation of the induction of the chloroplast-free mutants by 14 chromones, and the inhibitory effect of these substances on bleaching — induced by the standard mutagens (acridine orange (Ao), *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG)) — is part of this work. Chemical formulae of studied chromones are shown in Scheme 1.

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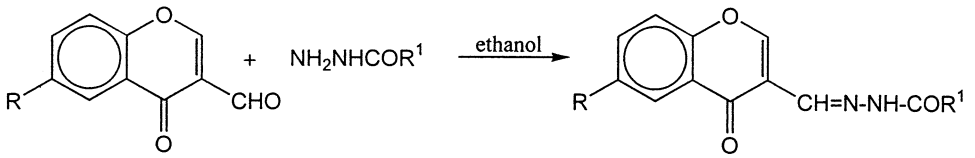
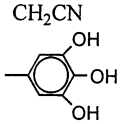
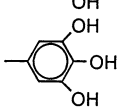
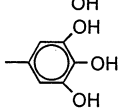
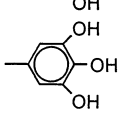


1a - 1d 2 3a - 3k

	R
1a	H
1b	6-CH_3
1c	6-Cl
1d	6-NO_2

	R	R^1
3a	H	
3b	6-CH_3	
3c	H	
3d	6-NO_2	
3e	H	CH_2CN
3f	6-NO_2	CH_2CN
3g	H	
3h	6-CH_3	
3i	6-Cl	
3k	6-OH	

Scheme 1.

2. Experimental

2.1. Chemistry

2.1.1. Synthesis of compounds *3a–3k*

3-Formylchromones were prepared by the Vilsmayer–Haack synthesis. 3-Formylchromone-*N*-acylhydrazones **3a–3k** were prepared by a condensation reaction of 6-*R*-3-formylchromones **1** with hydrazide derivatives **2** in ethanol with *p*-toluensulfonic acid as a catalyst. The method has been previously described in detail [4].

2.1.2. Mutagens

Ao was purchased from Loba-Chemie-Wien-Fischamend, MNNG from Fluka (Switzerland). Mutagens and chromone derivatives were dissolved in dimethylsulfoxide (DMSO). The concentration of DMSO in the culture medium never exceeded 0.4%.

2.2. Microbiology

2.2.1. Microorganisms

E. gracilis (strain Z) was maintained on a Cramer–Myers medium [17] supplemented with sodium acetate (0.5%) under static conditions at 26 ± 2°C and with permanent lighting.

2.2.2. Mutagenicity assay (bleaching activity)

This effect was monitored in a Cramer–Myers medium containing appropriate concentrations (10–400 μg/ml) of the tested compounds. Inoculum (10^4 cells/ml) was taken from the exponential growth phase, and culture was performed for 4 days under permanent illumination at 26°C. The cells were then washed, diluted and spread on agar plates with heterotrophic medium [18]. Green and white colonies were analyzed after 10–14 days of culture in the light at 26°C. The degree of toxicity for *E. gracilis* was expressed by the

ED_{50} value corresponding to 50% of surviving cells after 4 days of treatment (at a given concentration of a given mutagen) relative to the untreated control samples.

2.2.3. Antimutagenicity assay

Experiments were carried out by a 48 h treatment of the cells with mutagens (Ao, MNNG) together with chromones (in molar concentrations). Antimutagenicity was expressed (after 10–14 days of culture) as a percentage of inhibition of bleaching activity:

$$\% \text{ inhibition} = 1 - 100(x - b)/(y - b)$$

where x is the number of white mutants (colonies) in the presence of mutagens (Ao, MNNG) and antimutagens (chromone derivatives); y is the number of white mutants in the presence of mutagen (positive control); and b is the number of the spontaneous white mutants in the absence of mutagen (negative control).

The results were statistically evaluated and expressed as means ± standard deviations (SD). Student's t-test was used to determine the statistical significance of experimental values.

3. Results and discussion

The hereditary bleaching test on *E. gracilis* used for detecting extranuclear mutation showed positive results for substances **1a**–**1d** and **3c**–**3f** (Table 1). The highest bleaching activity has been shown by the 6-*R*-3-formylchromones (**1a**–**1d**). The maximum frequencies of mutations (MF_{max}) induced by sublethal concentrations are 14.0–81.0%.

On the other hand, some bleaching-inactive 3-formylchromone derivatives tested have an important inhibitory effect on bleaching — induced by some standard mutagens (Ao, MNNG). Ao and MNNG induced a high number of irreversible white mutants of *E. gracilis*. At the highest concentrations of Ao and MNNG tested, their percentage reached 100.

In the further experiments, the decrease in genotoxicity of Ao and MNNG after application of 6-*R*-3-formylchromone acylhydrazones has been studied. We used 30 μmol/l Ao and 30 μmol/l MNNG. These concentrations had low cell toxicity and produced a high frequency of white mutant cells (70.19% Ao; 77.55% MNNG). On the other hand, 6-*R*-3-formylchromone acylhydrazones — derived from gallic (**3g**–**3k**) and salicylic acids (**3a**, **3b**) — are not mutagenic for *E. gracilis* (mutated cells were not observed, Table 1).

The dose-dependent inhibitory effects of chromones tested on the mutagenicity of Ao (30 μmol/l) and MNNG (30 μmol/l) are shown in Tables 2 and 3. Chromones were applied in amounts (75–300 μmol/l) that are non-toxic concentrations for *E. gracilis*. Table 2 shows that 3-formylchromone hydrazones significantly reduced the proportion of the white mutants of *E. gracilis*, induced by 30 μmol/l Ao (inhibition 1.2 up to 87.2%). The greatest decrease in the mutagenic activity of Ao (35.8 up to 87.2%) was found in the presence of 75–300 μmol/l of am3 - formylchromone - *N* - (3,4,5 - trihydroxyphenylcarbonyl) hydrazone (**3g**). Student's t-test showed mostly significant values. Minimal values of P were found for chromone **3g** ($P < 0.001$). With respect to the proportion of white mutants induced by MNNG (Table 3), compound **3g** was clearly less effective (inhibition 2.7 up to 4.3%). Results were mostly not significant ($P > 0.05$). Thus, the degree of inhibition depends on the type of mutagen as well as on the concentration of competent chromone derivatives.

Table 1
Structure, toxicity [a] and antiplastid activity [b] of tested compounds

Comp.	Formula	Molecular weight	ED_{50} [a] (μg/ml)	MF [b] (%) maximum	
1a	$C_{10}H_6O_3$	174.16	2.7	81.0	(20.0) [c]
1b	$C_{11}H_8O_3$	186.15	2.3	76.0	(20.0)
1c	$C_{10}H_5ClO_3$	208.50	24.0	25.8	(25.0)
1d	$C_{10}H_5NO_5$	219.5	20.5	14.0	(25.0)
3a	$C_{17}H_{12}N_2O_4$	308.29	283.0	NB [d]	
3b	$C_{18}H_{14}N_2O_4$	322.32	265.0	NB	
3c	$C_{16}H_{11}N_3O_3$	293.28	150.0	12.5	(200.0)
3d	$C_{16}H_{10}N_4O_5$	338.28	145.0	5.5	(200.0)
3e	$C_{13}H_9N_3O_3$	255.23	163.0	11.3	(300.0)
3f	$C_{13}H_8N_4O_5$	300.23	140.0	6.0	(200.0)
3g	$C_{17}H_{12}N_2O_6$	340.29	183.0	NB	
3h	$C_{18}H_{14}N_2O_6$	354.32	205.0	NB	
3I	$C_{17}H_{11}ClN_2O_6$	374.74	210.0	NB	
3k	$C_{17}H_{12}N_2O_7$	356.29	181.0	NB	

[a] Toxicity for *E. gracilis*.
[b] Mutation frequencies (%), frequencies of white mutant colonies-bleaching activity.
[c] In brackets, bleaching active concentrations (μg/ml).
[d] NB, no bleaching-mutated cells were observed.

Table 2
Effect of six chromone derivatives **3** on the bleaching activity of Ao [a]

Concentration				Number of colonies per plate [b]	White colonies		Inhibition (%) [c]
Ao (μmol/l)	+	Chromone (μmol/l)			No.	(%)	
0	+	0		580 ± 20	0	0.0	0.0
30	+	0		510 ± 18	358 ± 6	(70.19)	0.0
40	+	0		240 ± 12	240 ± 12	(100.0)	0.0
30	+	300	**3a**	508 ± 14	310 ± 10	(61.02)	14.0 ± 2.8
30	+	150	**3a**	512 ± 26	340 ± 16	(66.4)	5.0 ± 3.3
30	+	75	**3a**	540 ± 20	350 ± 8	(64.81)	2.3 ± 2.1
30	+	300	**3b**	500 ± 20	324 ± 18	(64.8)	10.0 ± 4.8
30	+	150	**3b**	514 ± 24	340 ± 14	(66.1)	5.0 ± 4.0
30	+	75	**3b**	528 ± 28	354 ± 10	(67.04)	1.2 ± 2.0
30	+	300	**3g**	514 ± 18	46 ± 8	(8.94)	87.2 ± 2.2 [d]
30	+	150	**3g**	520 ± 20	140 ± 14	(26.92)	60.9 ± 4.0 [d]
30	+	75	**3g**	518 ± 22	230 ± 30	(44.40)	35.8 ± 8.4 [d]
30	+	300	**3h**	530 ± 20	110 ± 18	(20.75)	69.3 ± 5.0 [d]
30	+	150	**3h**	538 ± 16	210 ± 20	(39.03)	41.4 ± 5.6 [d]
30	+	75	**3h**	540 ± 16	310 ± 18	(57.40)	13.5 ± 5.1
30	+	300	**3I**	500 ± 16	128 ± 6	(25.6)	64.3 ± 2.1 [d]
30	+	150	**3I**	514 ± 12	212 ± 8	(41.24)	40.8 ± 2.2 [d]
30	+	75	**3I**	520 ± 14	320 ± 20	(61.53)	10.7 ± 5.6
30	+	300	**3k**	520 ± 18	130 ± 12	(25.0)	63.7 ± 3.3 [d]
30	+	150	**3k**	518 ± 10	240 ± 24	(46.33)	33.0 ± 6.7 [d]
30	+	75	**3k**	530 ± 12	324 ± 22	(61.13)	9.5 ± 5.6

[a] Culture for 48 h at 26°C. Ao was applied to *Euglena gracilis* simultaneously with inhibitors. Each value is the average of two separate experiments with two plates each (mean ± SD).
[b] Number of green plus white colonies/plate.
[c] Inhibition of bleaching activity (%) was calculated using the formulae given in Section 2.2.3.
[d] Difference from positive control (30 μmol Ao) is statistically highly significant (*t*-test, $P < 0.01$–0.001).

Table 3
Effect of four chromones (**3**) on the bleaching activity of *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) [a]

Concentration				Number of colonies per plate [b]	White colonies		Inhibition (%) [c]
Ao (μmol/l)	+	Chromone (μmol/l)			No.	(%)	
0	+	0		530 ± 24	0	0.0	0.0
30	+	0		490 ± 16	380 ± 8	(77.55)	0.0
40	+	0		210 ± 18	210 ± 18	(100.0)	0.0
30	+	300	**3a**	486 ± 18	365 ± 12	(75.10)	4.0 ± 3.4 [d]
30	+	150	**3a**	490 ± 18	370 ± 16	(75.51)	2.7 ± 2.6 [d]
30	+	300	**3g**	488 ± 20	364 ± 10	(74.59)	4.3 ± 2.3 [d]
30	+	150	**3g**	480 ± 14	370 ± 18	(77.08)	2.7 ± 2.4 [d]
30	+	300	**3h**	486 ± 24	368 ± 12	(75.72)	3.2 ± 2.6 [d]
30	+	150	**3h**	488 ± 20	372 ± 10	(76.22)	2.2 ± 2.1 [d]
30	+	300	**3i**	482 ± 18	370 ± 14	(76.76)	2.7 ± 1.0 [d]
30	+	150	**3i**	500 ± 24	372 ± 12	(74.40)	2.2 ± 2.1 [d]

[a] Culture for 48 h at 26°C. MNNG was applied to *E. gracilis* simultaneously with inhibitors. Each value is the average of two separate experiments with two plates each (mean ± SD).
[b] Number of green plus white colonies/plate.
[c] Inhibition of bleaching activity (%) was calculated using the formula given in Section 2.2.3.
[d] Difference from positive control (30 μmol MNNG), $P > 0.05$.

Table 4 summarizes the concentrations of the test substances **3a**, **3b**, **3g**, **3h**, **3i** and **3k** required to inhibit the bleaching activity of Ao and MNNG by 50% (ID_{50}). These concentrations were determined from the dose–response curves (inhibition of bleaching activity–mutagenicity (%) versus tested concentrations of inhibitors). The values (Table 4) confirm that the antibleaching activity of the six 3-formylchromone hydrazones towards Ao was stronger than towards MNNG. A different efficiency of Ao mutagenesis could

Table 4
Doses of chromones tested required to inhibit the bleaching activity (mutagenicity) of Ao (30 μmol/l) and MNNG (30 μmol/l) by 50% (ID_{50})

Comp.	ID_{50} (μmol/l)	
	Ao	MNNG
3a	–[a]	–
3b	–	NT[b]
3g	115	–
3h	175	–
3i	200	–
3k	225	NT

[a] Inhibitory effect does not reach 50%.
[b] Not tested.

be observed when 3-formylchromone hydrazone (**3g**) was added at different time intervals. The highest inhibitory effect on Ao-bleaching activity was achieved (Table 5) when substance **3g** was added 5 h before Ao (pre-treatment, inhibition 39.0 up to 86.6%), and the lowest effect was achieved when **3g** was added 5 h after Ao (post-treatment, inhibition 0.0 up to 1.5%).

E. gracilis is an organism with a multigenomic system containing nuclear, mitochondrial and chloroplast DNA. Organellar genomes are less stable and more sensitive to mutagenesis as compared with the nuclear genome. We assume that a general condition for the response to the bleaching inducing factor is the higher sensitivity of chloroplast DNA compared with that of nuclear DNA. The mutagens (including MNNG or Ao) cause hereditary bleaching through general mutagenic mechanism (alkylation, base substitution, etc) taking place at the level of plastid DNA. Antibiotics induce hereditary bleaching of *E. gracilis* only if acting on dividing cells, mutagens and carcinogens induce such a change when acting on dividing and non-dividing cells [12]. In the case of the chromone derivatives, the hereditary bleaching test showed positive results only when they affect dividing cells, as with bleaching-active antibiotica.

On the other hand, the bleaching-inactive 6-*R*-3-formylchromone hydrazones (derived from gallic and salicylic acids) have an important inhibitory effect on bleaching induced by classic mutagens (Ao, MNNG); they then act as potent antimutagens in the case of chloroplast mutagenesis in *E. gracilis*. The ultimate effect of this action depended, among others, on the type of mutagen. The results of the present study showed that 6-*R*-3-formylchromone hydrazones were very effective against Ao, and less effective towards *N*-methyl-*N*-nitro-*N*-nitrosoguanidine. This genotoxic effect of mutagens is reduced, especially during the first stages of induction of this specific cytoplasmic mutation. Preincubation increased the antimutagenic potential of 6-*R*-3-formylchromone hydrazones. Substance **3g** reduced Ao-mutagenesis by either pre-treatment or simultaneous treatment, but not by post-treatment. The results suggest that substance **3g** probably interacted with intact Ao or its active form. The mechanism(s) of induction (or inhibition) of mutagenic activity by some chromones cannot be determined from the present results, but will be studied in the near future.

The experimental study of mutagenicity and antimutagenicity of 3-formylchromone hydrazones was updated with a calculated study of the optimal structures, atomic charges and the dipole moments of hydrazones by the semiempirical AM1 method [19] with standard parameterization (keyword PRECISE). The theoretical values of $\log P$ were obtained by Crippen's method [20]. The above data were correlated with the toxicity for *E. gracilis*. A low correlation coefficient (0.394) was obtained, which means that lipophilicity probably did not influence the studied toxicity of the 3-formylchromones. Correlations of the toxicity for *E. gracilis* with the charge on the carbon atoms at positions 3 and 9 (aldehyde group) obtained by the quantum chemical method (Table 6) have given statistically significant correlations.

$$\log(1/\mathrm{ED}_{50}) = (-10.307 \pm 0.289)Q(C_3) + 1.459$$

$$R = 0.921,\ s = 0.289,\ F = 67.1,\ n = 14$$

Table 5
Inhibitory effect (%) [a] of 3-formylchromone hydrazone (**3g**) added at different time intervals on bleaching activity of Ao

Ao	+	Chromone	Chromone **3g** added		
(μmol/l)		**3g** (μmol/l)	5 h before	Simultaneously with	5 h after
30 [b]	+	0			
30	+	300	86.6	82.5	1.5
30	+	150	67.0	63.0	0.2
30	+	75	39.0	36.0	0.0

[a] Inhibition of bleaching activity (%) was calculated using the formula given in Section 2.2.3. Each value (%) is the average of two separate experiments with two plates each.
[b] Ao (30 μmol/l) without chromone **3g** induced 405 mutants/plate (positive control).

Table 6
Theoretical values of log P and charge densities of compounds **1** and **3**

Comp.	Log P	$Q(C_3)$	$Q(C_9)$ [a]
1a	1.22	−0.359	0.242
1b	1.69	−0.360	0.243
1c	1.74	−0.357	0.242
1d	1.17	−0.346	0.241
3a	2.12	−0.224	−0.111
3b	2.59	−0.225	−0.110
3c	1.09	−0.224	−0.112
3d	1.05	−0.204	−0.123
3e	1.21	−0.227	−0.107
3f	1.16	−0.208	−0.113
3g	1.55	−0.222	−0.115
3h	2.02	−0.224	−0.114
3I	2.07	−0.218	−0.118
3k	1.27	−0.226	−0.115

[a] Aldehyde group.

$$\log(1/ED_{50}) = (3.933 \pm 0.483)Q(C_9) + 4.176$$

$R = 0.920$, $s = 0.290$, $F = 66.4$, $n = 14$

It has been concluded from the values of the F-test that the statistical significance of all regression equations is higher than 99.5%. Acceptable results were obtained for correlations with two and three independent variables ($\log P$, $Q(C_3)$ and $Q(C_9)$), but single linear correlations were more statistically significant. The results show that the toxicity of 3-formylchromones for *E. gracilis* depends on the atomic charges.

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