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Benzothiazolium Salts — Relationships between Their Structure, Toxicity and Effect on the Plastid System of *Euglena gracilis*

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ABSTRACT. The effects of 41 benzothiazolium salts on *Euglena gracilis* were characterized with regard to the influence on growth and on chlorophyll synthesis, and to their ability to induce permanent loss of chloroplasts. Some salts induced white mutants of *E. gracilis* (the first benzothiazole derivatives with this activity). The relationship between the biological effect and chemical structure was confirmed and expressed quantitatively by means of Free–Wilson and Fujita–Ban analysis.

The synthesis of benzothiazole derivatives has long been studied in its theoretical aspects and practical application. Some of them have been used in the production of dyes, as adjuncts in photographic emulsions and in the field of catalysis. Many of them are also used in medicine (Negwer 1978). In addition to other advantageous properties benzothiazolium salts are well soluble in water. Many of them were found to exhibit antimicrobial effects (Foltínová *et al.* 1983; Halgaš *et al.* 1983*a*,*b*; Sutoris *et al.* 1983, 1984), stimulatory or inhibitory effects on plant growth (Halgaš *et al.* 1983*a*,*b*; Sutoris *et al.* 1983, 1984, 1985) and the specific effect of increasing the sugar content in sugar-forming plants (Sutoris *et al.* 1982).

Benzothiazolium salts thus represent a group of substances with a significant biological activity. In order to assess the broadest possible spectrum of their effects we used as the test organism *Euglena gracilis* on which several effects can be evaluated simultaneously. It can be used for tentative determination of toxicity of various substances (Vrbovský *et al.* 1975) and for assessment of antiplastid activity (Ebringer 1978) which can also serve as a marker of antibacterial activity. Interaction of chemical substances with chloroplasts can induce various changes, the most dramatic of which is a permanent elimination of chloroplasts. This specific mutation was termed the permanent bleaching activity.

E. gracilis can also be used to determine the effect of chemical substituents on biological activity and to point out the common structure responsible for the biological activity of new derivatives.

MATERIALS AND METHODS

Microorganisms. Euglena gracilis strain Z maintained photoautotrophically on a Cramer-Myers medium (1952) or on a proteose-peptone-tryptone medium (Mego 1964) under permanent illumination at 26 ± 2 °C.

Monitoring of biological effect. I. The effect was studied in a liquid Cramer-Myers medium containing appropriate concentrations of tested substances. The inoculum was taken from the exponential growth phase and cultivation was performed under permanent illumination at 26 ± 2 °C. The following parameters were determined at intervals:

TABLE I. Benzothiazolium salts under study

Compound	R^2	R^3	R^4	R^6	X	M_r	M. p. °C
1	H	CH_3	H	H	I	277.1	213–215
2	H	CH_3	H	H	Br	230.1	218–220
3	H	C_2H_5	H	H	I	291.2	139–142
4	H	C_2H_5	H	H	Br	244.1	210–211
5	H	C_3H_7	H	H	I	305.2	158–160
6	H	C_4H_9	H	H	I	319.2	112–114
7	H	C_4H_9	H	H	Br	272.2	109–111
8	H	$CH_2CH=CH_2$	H	H	I	303.2	158–159
9	H	$CH_2CH=CH_2$	H	H	Br	256.2	147–149
10	H	$CH_2C\equiv CH$	H	H	Br	254.1	165–166
11	CH_3	CH_3	H	H	I	291.2	226–227
12	CH_3	C_2H_5	H	H	I	305.2	197–198
13	CH_3	C_3H_7	H	H	I	319.2	173–174
14	CH_3	C_4H_9	H	H	I	333.2	186–188
15	CH_3	$CH_2CH=CH_2$	H	H	Br	270.2	216–217
16	CH_3	$CH_2C\equiv CH$	H	H	Br	268.2	244–246
17	C_3H_7	CH_3	H	H	I	319.2	247–249
18	C_3H_7	C_2H_5	H	H	I	333.2	176–178
19	C_3H_7	$CH_2CH=CH_2$	H	H	Br	298.2	167–168
20	H	CH_3	Cl	H	I	311.6	261–263
21	H	CH_3	Cl	H	Br	264.6	253–255
22	H	CH_3	H	Cl	Br	264.6	295–298
23	H	$CH_2CH=CH_2$	H	Cl	Br	290.6	210–212
24	H	$CH_2C\equiv CH$	H	Cl	Br	288.6	221 (dec.)
25	H	CH_3	CH_3	H	Br	244.2	209–212
26	H	$CH_2CH=CH_2$	CH_3	H	Br	270.2	198–199
27	H	$CH_2C\equiv CH$	CH_3	H	Br	268.2	245 (dec.)
28	H	CH_3	H	CH_3	I	291.2	212–214
29	H	CH_3	H	CH_3	Br	244.2	205–207
30	H	$CH_2CH=CH_2$	H	CH_3	Br	270.2	191–192
31	H	$CH_2C\equiv CH$	H	CH_3	Br	268.2	215–216
32	H	CH_2COOCH_3	H	H	Br	288.2	152 (dec.)
33	H	$CH_2COOC_2H_5$	H	H	Br	302.2	174 (dec.)
34	H	$CH_2COOC_3H_7$	H	H	Br	316.2	185 (dec.)
35	CH_3	CH_2COOCH_3	H	H	Br	302.2	214–217
36	H	CH_2COOCH_3	H	CH_3	Br	302.2	101–103
37	H	CH_2COOCH_3	H	Cl	Br	322.6	170 (dec.)
38	H	$CH_2C_6H_5$	H	H	Br	306.2	201 (dec.)
39	CH_3	$CH_2C_6H_5$	H	H	Br	320.3	247–249
40	H	$CH_2C_6H_5$	H	CH_3	I	367.3	192–194
41	H	$CH_2C_6H_5$	H	Cl	I	387.7	192–193

a) cell count on a PICOSCAL instrument;
b) content of chlorophyll according to Arnon (1949) — extraction with 80 % acetone;
c) bleaching effect after spreading of treated *Euglena* cells on a heterotrophic, agar-solidified medium;
d) quantitative appraisal of the toxicity to *Euglena gracilis* by the Free—Wilson (1964) method and the modified method of Fujita and Ban (1971) on a Siemens 4004/15 computer;
e) changes in chloroplast counts in a fluorescence microscope FLUOVAL 2.

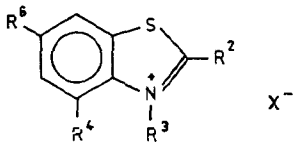
TABLE II. Percentage of *E. gracilis* cells after treatment with benzothiazolium salts[a]

Compound	Concentration, μg/mL						log ED_{50}
	500	200	50	12.5	3.1	0.8	
1	7	58	66	76	100	100.5	2.450
2	8	58	68	76	100	100	2.425
3	5	56	64	79	99	101	2.400
4	6	54	62	84	98	100	2.300
5	11	62	66	71	94	100	2.500
6	13	61	69	86	96	100	2.600
7	14	62	69	84	98	101	2.600
8	0	16	32	68	92	99	1.330
9	0	15	30	70	99	100	1.325
10	0	0	23	50	89	93	1.097
11	48	85	99	101	102	100	2.670
12	42	86	99	101	101	—	2.662
13	52	86	98	100	100	—	2.750
14	52	88	100	101	101	100	2.733
15	10	63	97	100	100	—	2.475
16	0	38	86	90	96	—	2.150
17	48	88	100	100	101	101	2.688
18	46	84	99	100	100	—	2.675
19	13	60	99	100	100	—	2.388
20	1	13	53	66	100	100	1.800
21	1	18	60	70	98	100	1.925
22	2	26	62	71	96	—	1.980
23	0	3	29	63	86	—	1.338
24	0	0	20	50	85	90	1.097
25	0	26	58	71	98	100	1.850
26	0	5	29	54	85	96	1.188
27	0	0	16	41	80	—	0.925
28	0	30	60	70	95	—	1.975
29	0	29	61	70	96	100	1.938
30	0	5	24	52	84	96	1.190
31	0	0	17	43	82	—	0.975
32	13	63	90	99	103	102	2.437
33	5	58	91	100	104	102	—
34	12	63	92	100	103	101	—
35	36	71	92	99	102	100	2.562
36	0	59	76	90	98	—	2.425
37	0	60	82	94	100	100	2.438
38	3	76	90	100	104	101	2.540
39	38	90	100	102	106	—	2.600
40	0	48	63	86	99	—	2.265
41	0	50	64	86	100	—	2.301

[a] After a 5-d treatment; control = 100 %.

II. The effect under resting conditions was studied as follows. Green cells in the exponential growth phase in a Cramer-Myers medium were separated by centrifugation. Washed cells were transferred to a resting medium, actually a physiological saline according to Kirk (1961), in which cell division ceased. The medium was supplemented with the tested substances and the bleaching effect was determined.

Tested substances. Benzothiazolium salts were synthesized according to Halgaš *et al.* (1983*a*,*b*) and Sutoris *et al.* (1983, 1984). Forty-one derivatives (**1**—**41**) were studied in all (Table I).



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RESULTS AND DISCUSSION

Under conditions of active reproduction of *E. gracilis* cells benzothiazolium salts affect both the cell division and the chloroplast system. The ultimate effect of this action depends on the chemical structure and the amount of the tested substances (Table II). These salts exhibit a varying degree of growth inhibition, which is naturally associated with their toxicity. Quantitative relationships were expressed in terms of Free—Wilson (1964) analysis and analysis according to Fujita and Ban (1971). The group of 41 synthesized substances (**1**—**41**) included compounds with eight substituents R^3, three R^2, five R^4 and R^6, and two anions X^-. Substances **33** and **34** were left out from the set since their substituent R^3 appears only in one case and cannot thus be satisfactorily appraised.

The quantity characterizing biological activity was taken to be the ED_{50} (log ED_{50}) calculated from data in Table II. These data were also used to calculate the contributions of the substituents in individual positions to the overall activity (Table III).

The z_{jk} values are a quantitative measure of the effect of substituents on the biological activity. Comparison of the Δz_{jk} values (reflecting the differences between the maximum and the minimum contributions z_{jk}) shows that the most marked changes occur in position 3 ($\Delta z_{jk} = 1.20$). However, this effect can be partially affected by the large diversity and abundance of substituents in this position. Substitutions in position 2 ($R^2 = CH_3$, C_3H_7) decrease the overall toxicity, as seen clearly in 3-allyl- and 3-propargylbenzothiazolium bromide with unsubstituted position 2 (compounds **9** and **10**), which exhibit ED_{50} of 1.325—1.097. Their analogues with substituted position 2 (compounds **15**, **16** and **19**) have ED_{50} of 2.475—2.150. Substitution on the benzene ring increases the toxicity; mathematical analysis shows position 4 to be more sensitive than position 6. The smallest changes in biological activity are caused by changes in the anion. Multiple correlation coefficients as well as values of F attest to the high statistical significance of these correlations.

The effects of the tested substances on the chloroplast system of *E. gracilis* are shown in Table IV. The substances affect the synthesis of chlorophyll and some of the cause also permanent changes in the plastid system which may lead to the appearance of white mutants. The induced formation of white mutants is not lethal if the cells are supplied in time with organic energy sources. However, it is permanent since even after a several-fold repeated subcultivation of white colonies on a heterotrophic cultivation medium the cells remained aplastidic and formed only white colonies.

Previsously described benzothiazole derivatives were found to inhibit chlorophyll synthesis but no bleaching activity has so far been detected (Foltínová *et al.* 1980, 1982). The compounds reported in this paper thus form the first group of benzothiazole derivatives with bleaching activity and can be classed among substances inducing hereditary aplastidia in *E. gracilis*. In addition, some structural relationships appear: the strongest effect on chlorophyll synthesis was found in 3-alkylbenzothiazolium salts (compounds

TABLE III. Results of Free-Wilson and Fujita-Ban analysis

Position	Substituent	No. of subst.	Free-Wilson log ED_{50}		Fujita-Ban log ED_{50}
			z_{jk}	Δz_{jk}	z_{jk}[a]
	CH_3	10	0.18		—
	C_2H_5	4	0.19		0.06
	C_3H_7	2	0.35		0.15
	C_4H_9	3	0.43	1.20	0.21
R^3	$CH_2CH=CH_2$	7	−0.51		−0.77
	$CH_2C\equiv CH$	5	−0.77		−1.10
	CH_2COOCH_3	4	0.36		0.21
	$CH_2C_6H_5$	4	0.35		0.15
	H	28	−0.13		
R^2	CH_3	8	0.28	0.55	0.15
	C_3H_7	3	0.42		0.09
	H	23	0.12		—
	4-Cl	2	−0.29		−0.52
$R^{4,6}$	6-Cl	5	−0.08	0.42	−0.23
	$4\text{-}CH_3$	3	−0.30		−0.41
	$6\text{-}CH_3$	6	−0.15		−0.33
X	I				
		15	−0.03		—
	Br	24	0.02		−0.04
Constant μ[b]			2.10		2.40
Multiple correlation coefficient r			0.955		0.962
F-Value			17.790		21.029

[a] z_{jk} = contribution of a substituent, Δz_{jk} = maximum range of contributions of a substituent in given position.

[b] Biological activity of the basic compound (R^2 = H; R^3 = CH_3; $R^{4,6}$ = H; X = I).

1—**10**) and 4- or 6-substituted 3-alkylbenzothiazolium salts (compounds **20**—**31**). The differences in the amount of chlorophyll after exposure to the highest and the lowest concentration are either significant ($P < 0.05$) or highly significant ($P < 0.01$). These substances induced also the highest percent proportion of white mutants. Analogues of these compounds with substituted position 2 (compounds **11**—**19**) exhibit a substantially lower effect on chlorophyll synthesis and a substantially lower bleaching activity.

TABLE IV. Percentage of chlorophyll and of depigmented colonies of *E. gracilis*[a] induced by benzothiazolium salts[b]

Compound	Concentration, μg/mL					
	500	200	50	12.5	3.1	0.8
1	38/55	54/50	72/40	96/2	100/0	100/0
2	40/55	56/52	78/30	92/1	98/0	100/0
3	39/54	55/50	72/36	86/15	98/1	100/0
4	36/62	51/62	68/35	85/15	98/1	100/0
5	39/53	51/50	65/40	83/15	99/1	100/0
6	50/32	58/26	69/18	80/15	97/1	100/0
7	63/28	70/20	78/12	86/9	100/0	100/0
8	0/0	58/28	69/20	80/15	90/2	99/0
9	0/0	59/29	67/22	70/20	89/5	99/0
10	0/0	0/0	50/8	61/5	86/2	95/0
11	72/4	95/1	99/0	100/0	101/0	101/0
12	73/6	90/2	100/0	101/0	102/0	—
13	80/5	98/1	100/0	101/0	101/0	—
14	78/4	98/1	100/0	100/0	101/0	100/0
15	54/11	90/3	99/1	100/0	100/0	—
16	0/0	65/7	92/3	99/0	100/0	—
17	84/5	93/2	99/0	101/0	101/0	—
18	83/7	93/2	100/0	102/0	101/0	—
19	80/6	94/2	99/0	100/0	100/0	—
20	28/75	51/45	62/5	86/2	98/0	100/0
21	42/50	62/30	73/6	90/0	98/0	100/0
22	38/60	60/36	73/2	96/0	100/0	—
23	0/0	50/16	80/14	90/5	96/0	—
24	0/0	0/0	63/7	69/6	95/0	98/0
25	0/0	62/30	62/4	83/0	99/0	100/0
26	0/0	60/13	83/9	90/0	99/0	—
27	0/0	0/0	31/9	60/5	83/0	—
28	0/0	60/40	65/5	84/1	90/0	—
29	0/0	61/31	60/4	85/0	94/0	100/0
30	0/0	48/16	76/4	84/0	88/0	99/0
31	0/0	0/0	59/9	66/8	89/0	—
32	39/14	73/12	96/1	100/0	106/0	102/0
33	30/16	68/12	93/2	100/0	104/0	102/0
34	38/11	70/6	95/1	100/0	102/0	102/0
35	40/8	79/3	100/0	101/0	101/0	101/0
36	0/0	70/8	86/1	96/0	100/0	—
37	0/0	71/8	87/2	99/0	100/0	—
38	62/5	70/1	99/0	103/0	103/0	102/0
39	84/0	93/0	100/0	101/0	103/0	102/0
40	0/0	76/0	99/0	100/0	100/0	—
41	0/0	70/0	96/0	100/0	100/0	—

[a] Percentage of chlorophyll to percentage of depigmented colonies.
[b] After a 5-d treatment; control = 100 % chlorophyll and 0 % depigmented colonies.

3-Alkoxycarbonylbenzothiazolium salts and 4- or 6-substituted 3-alkoxycarbonylbenzothiazolium salts (compounds **32**—**37**) in the lowest concentrations display a strong inhibition of chlorophyll synthesis but the percentage of white mutants is substantially lower than with 3-alkylbenzothiazolium salts. Some of them, especially at lower concentrations, slightly stimulate chlorophyll synthesis. 3-Benzylbenzothiazolium salts (compounds **38**—**41**) inhibit chlorophyll synthesis but exhibit low or no bleaching activity.

In contrast to many antibiotics none of the benzothiazolium salts induced 100 % white mutants. One of the possible reasons may be the negligible difference between their lethal concentration and the highest concentration inducing permanent bleaching; the percentage of white mutants thus cannot be reached simply by increasing the concentration of these agents.

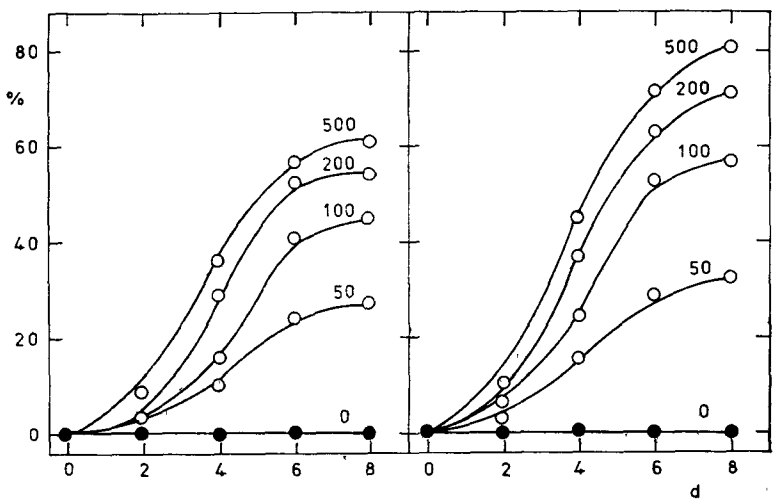

FIG. 1. Percentage of white colonies of *E. gracilis* (%) after treatment with 3-methylbenzothiazolium iodide (**1**; *left*) and 3-ethylbenzothiazolium bromide (**4**; *right*); *numbers at curves* — concentration of compounds (μg/mL).

The bleaching effect is strongly dependent on the length of treatment (Fig. 1); the highest activity is observed after 2—6 d while at later dates the number of mutants increases less sharply. Microscopic analysis showed that in dividing cells benzothiazolium salts with bleaching activity induce non-uniform changes in the number of chloroplasts. Complete elimination of chloroplasts occurs only after a certain time and only in a certain percentage of cells. In a considerable number of cells the number of chloroplasts after a 6-day treatment was merely reduced (Table V). This gradual elimination of chloroplasts from dividing cells points at a certain analogy with the mechanism of the bleaching effect of antibiotics (Ebringer 1978). The highest bleaching activity was found in 3-alkylbenzothiazolium salts and in 4- or 6-substituted 3-alkylbenzothiazolium salts that exhibit at the same time also the highest antibacterial activity (Foltínová *et al.* 1983). This fact can be used also in the interpretation of the results, especially within the framework of the endosymbiotic theory of origin of chloroplasts which postulates

TABLE V. Changes in the number of chloroplasts in *E. gracilis* after treatment with 3-ethylbenzothiazolium bromide (4)[a]

Cultivation period	Compound	Percentage of cells with the given number of chloroplasts											
d	μg/mL	0	1	2	3	4	5	6	7	8	9	10	11[b]
	0	0	0	0	0	0	0	2	12	73	10	2	1
	100	0	0	2	2	4	6	5	12	60	9	0	0
2	200	0	2	2	2	2	6	12	23	42	9	0	0
	400	0	2	2	2	2	5	19	24	36	8	0	0
	0	0	0	0	0	0	0	2	11	75	9	2	1
	100	6	8	7	11	12	16	12	4	24	0	0	0
4	200	15	8	9	10	12	14	12	6	14	0	0	0
	400	18	8	9	10	10	16	9	9	11	0	0	0
	0	0	0	0	0	0	0	2	11	75	8	3	1
	100	18	24	16	12	10	8	12	0	0	0	0	0
6	200	40	19	15	12	7	5	2	0	0	0	0	0
	400	46	21	14	9	6	4	0	0	0	0	0	0

[a] Counted always in 200 cells (100 %).
[b] And more.

an analogy between prokaryotic organisms and semiautonomous cell organelles.

Light has an important influence on the effect of benzothiazolium salts on *E. gracilis* (Fig. 2). Induction of white mutants during active culture growth takes place only when the cultivation is performed under permanent illumination. When the agents are left to act in the dark no white mutants are found and the inhibition of the culture growth is also lower.

Many natural and synthetic compounds have the ability to induce permanent bleaching in *E. gracilis* (Ebringer 1972, 1978). Antibiotics cause a hereditary elimination of chloroplasts only in actively growing cultures

TABLE VI. Effect of 3-ethylbenzothiazolium bromide (4) on the autotrophic form of *E. gracilis* under growth and resting conditions[a]

μg/mL	Percentage of white colonies	
	Growth conditions	Resting conditions
500	54.0	–[b]
250	38.5	0.3[c]
125	31.8	0
62.5	23.5	0
31.25	7.1	0
15.62	0.5	0
Control	0	0

[a] 3-d incubation with the substance.
[b] No colony growth; the concentration is lethal.
[c] Total number of colonies is about 30 % of control.

whereas mutagens induce permanent aplastidia also when used with non-dividing cells (Ebringer *et al.* 1976). This finding can be used to distinguish between an antibiotic and a mutagenic effect of various substances. For this reason we tested some derivatives exhibiting bleaching activity under growth conditions also with nondividing cells in resting state. Under these conditions none of the tested substances induced white mutants (Table VI).

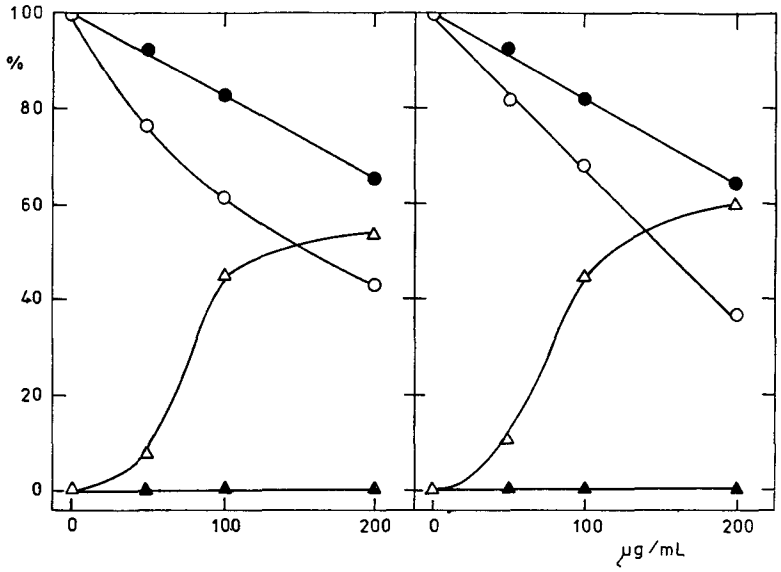

FIG. 2. Percentage of cells (*circles*) and percentage of white colonies (*triangles*) after treatment with 3-methylbenzothiazolium iodide (**1**; *left*) and 3-ethyl-benzothiazolium bromide (*4*; *right*) during cultivation in the light (*open symbols*) and in the dark (*closed symbols*). No spontaneous mutants appeared in the control.

Consequently, the above group of compounds can be characterized as nonmutagenic, even though the designation seems paradoxical in view of their effects on *E. gracilis*. It should be kept in mind, however, that although the ultimate result of action of antibiotics and mutagens (a specific mutation — permanent bleaching of cells) is the same, the mechanisms of production of aplastidic mutants differ. Various symptoms, as well as our previous experience with antibiotics (Ebringer 1978), indicate that benzothiazolium salts may inhibit the synthesis of some enzymes vitally important for the replication of chloroplasts. This inhibition may lead to a decrease in the number, or a complete elimination, of chloroplasts from dividing cells.

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