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# Anticytokinin Activity of 4-Substituted Triazolo[4,5-d]pyrimidines and 4-Substituted Pyrazolo[3,4-d]pyrimidines

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**Abstract.** The synthesis and physiological activity of some novel 4-substituted triazolo[4,5-d]pyrimidines and 4-substituted pyrazolo[3,4-d]pyrimidines are described. Most of the compounds possessed high anticytokinin activity towards purine (benzyladenine) and phenylurea (4-PU-30) type cytokinins. 1-Benzyl-4-ethoxycarbonylpiperazinyl-1H-1,2,3-triazolo[4,5-d]pyrimidine almost completely removed cytokinin stimulated effects—betacyanin synthesis in *Amaranthus caudatus* cotyledons; growth of radish cotyledons and retention of chlorophyll in leaf explants. Some chemical structure–physiological activity relationships have been established.

Anticytokinins are useful tools in investigations of cytokinin mode of action. In spite of extensive empirical and mathematical studies, the problem of the structural elements required of certain molecules for high physiological activity remains obscure. There are highly active cytokinins and anticytokinins that share the same chemical group (Iwamura et al. 1985, Karanov et al. 1992, Skoog et al. 1975). Moreover, according to Klämbt et al. (1966) (see review of Karanov et al. 1992) some compounds exhibit different regulatory effects depending on the concentration used. Hitherto known cytokinin antagonists can be divided into six groups (Shimizu et al. 1989, Karanov et al. 1992). However, their anticytokinin effects have been studied mostly in relation to the growth of callus tissue. Recently, it was shown that some 4-substituted 1H-pyrazoles and 8-aza analogues of adenine acted as cytokinin agonists or antagonists depending on the test system used (Karanov et al. 1993).

The aim of this article was to describe the synthesis, physiological activity, and structure–activity relationships of some novel 4-substituted triazolo-[4,5-d]pyrimidines and 4-substituted pyrazolo[3,4-d]-pyrimidines.

## Materials and Methods

### *Synthesis of the Compounds*

The synthesis of 4-substituted 1-benzyltriazolo[4,5-d]pyrimidines (Table 1, 1–8) and 4-substituted 1-benzylpyrazolo[3,4-d]-pyrimidines (Table 1, 9–11) was performed by reacting 4-chloro-1-benzyl-1H-1,2,3-triazolo[4,5-d]pyrimidine (Table 1, compound I) (Albert 1969), or 4-chloro-1-benzyl-1H-pyrazolo[3,4-d]-pyrimidine (Table 1, compound II) with polyfunctional nucleophilic reagents (El Hedi Jellali et al. 1975). The reaction of the chloroderivatives with the appropriate nucleophile was carried out at room temperature for 1 h. The compounds 1, 3, 4, 5, 6, 7, 9, 10, 11, and 12 were prepared from the corresponding chloroderivative and amine in 1:2 molar ratio in absolute tetrahydrofurane; 2 was prepared from (I), the corresponding amine hydrochloride and triethylamine in 1:1:2 molar ratio in absolute ethanol, 8 from (II) and a slight molar excess of the corresponding alcohol activated with sodium in absolute tetrahydrofurane. The products obtained were purified by recrystallization.

The plant growth-regulating activity of the known 1-benzyl-4-hydroxy-1H-pyrazolo[3,4-d]pyrimidine (Table 1, compound 12) (El Hedi Jellali et al. 1975) was also studied in order to compare its properties with those of the newly synthesized products.

### *Bioassay Procedures*

*Synthesis of Betacyanins in* Amaranthus caudatus *M.* Ten *A. caudatus* explants for each replicate, consisting of the upper portion of the hypocotyl plus the cotyledons, were put in Petri dishes on filter paper, moistened with 2-ml test solutions. The betacyanins were determined by the procedure of Biddington

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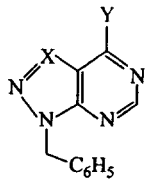
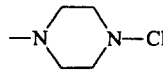
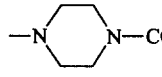
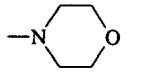
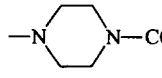
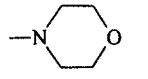
**Table 1.** Compounds, common formula, starting substances (I and II), percent yields, melting points and solvents for recrystalization.



| N: | Compound X= | Y= | % Yield | Melting point (°C) | Solvent |
|---|---|---|---|---|---|
| I. | N | —Cl | | | |
| 1. | N | $-HNCH_2CH_2OH$ | 89 | 167.0–169.0 | Ethylacetate |
| 2. | N | $-HNCH_2CH_2CH_2Cl$ | 85 | 263.0–265.0 | Ethylacetate |
| 3. | N | $-HNCH_2CH_2N(CH_3)_2$ | 86 | 157.5–159.0 | Ethylacetate |
| 4. | N | $-HNCH_2CH_2N(C_2H_5)_2$ | 81 | 92.5–94.0 | Hexane |
| 5. | N |  | 78 | 109.5–111.0 | Hexane |
| 6. | N |  | 70 | 121.0–122.0 | Benzene-hexane |
| 7. | N |  | 81 | 136.0–137.5 | Ethanol |
| 8. | N | $-OCH_2CH_2OCH_3$ | 82 | 165.0–166.0 | Ethylacetate |
| II. | CH | —Cl | | | |
| 9. | CH | $-HNCH_2CH_2OH$ | 72 | 124.0–125.0 | Abs. ethanol–abs. ether |
| 10. | CH |  | 83 | 134.0–135.0 | Ethylacetate |
| 11. | CH |  | 90 | 133.5–134.0 | Ethanol |
| 12. | CH | —OH | | | |

and Thomas (1973). Optical density was measured at 542 nm and 620 nm.

*Growth of Isolated Radish cotyledons.* Cytokinin-stimulated growth of excised radish cotyledons was measured according to Letham (1971). Seeds of radish (*Raphanus sativa* L., cv. Red) were germinated for 72 h in darkness at 25°C on filter paper. Ten detached cotyledons per replicate were placed in Petri dishes on filter paper wetted with 3-ml test solutions. The cotyledons were blotted dry and weighed after 72 h of incubation in a growth chamber (25°C, 12-h photoperiod).

*Cytokinin-Promoted Chlorophyll Retention.* Cytokinin inhibition of chlorophyll degradation was tested with radish (Bruce et al. 1965) and barley (*Horedum vulgare* L.) (Kende 1965) leaf explants incubated on test solutions in darkness (96 h, radish discs, and 72 h, barley segments). Eight radish discs or four barley segments from each replicate were blotted dry and boiled in 10 ml 80% ethanol for 5 min. The optical density of the cooled, decanted solution was measured at 665 nm.

*Preparation of Test Solutions and Application.* The test compounds were dissolved in ethyl alcohol and diluted with distilled water to obtain the concentrations tested. The concentration of the ethyl alcohol was less than 0.1%.

*$N^6$-Benzyladenine(BA) (10 μM) and $N_1$-(2-chloro-4-pyridyl)-$N_2$-phenylurea (4-PU-30) (10 μM) Were Used as Standards.* All the synthesized chemicals were tested in concentration range 100 to 0.1 μM alone or in combination with BA or 4-PU-30.

The data presented are means from three or four experiments, each in four replications. Fisher's procedure was used for statistical calculations (Steel and Torrie 1960).

## Results

The chemical structures, percent yields, melting points (°C) and solvents of the compounds are listed in Table 1.

The IR-spectra of 1,2,3, and 4 exhibited a characteristic NH-band at 3250–3300 $cm^{-1}$. In the $^1H$-NMR-spectra of the compounds studied, the sin-

**Table 2.** Influence of the compounds tested (applied alone and in combination with cytokinin) on the betacyanin synthesis in isolated *Amaranthus caudatus* cotyledons.

| Compounds tested | Optimum conc. [μM] | Without cytokinin | | Comb. with BA 10 μM | | Comb. with 4-PU-30 10 μM | |
|---|---|---|---|---|---|---|---|
| | | $OD_{542-620}$ | % to Control | $OD_{542-620}$ | % to BA | $OD_{542-620}$ | % to 4-PU-30 |
| Control | — | 0.109 | 100 | 0.220 | 100 | 0.192 | 100 |
| 1 | 10 | 0.093 | 85 | 0.183 | 83 | — | — |
| 2 | 1 | 0.120 | 110 | 0.183 | 83 | — | — |
| 3 | 10 | 0.126 | 116 | 0.138 | 63 | 0.130 | 68 |
| 4 | 10 | 0.133 | 122 | 0.150 | 68 | 0.150 | 78 |
| 5 | 10 | 0.123 | 113 | 0.144 | 65 | 0.153 | 80 |
| 6 | 10 | 0.092 | 84 | 0.117 | 53 | 0.132 | 69 |
| 7 | 10 | 0.102 | 94 | 0.106 | 48 | 0.140 | 73 |
| 8 | 100 | 0.076 | 70 | 0.197 | 90 | — | — |
| | 10 | 0.088 | 81 | 0.196 | 89 | — | — |
| 9 | 100 | 0.087 | 80 | 0.195 | 89 | — | — |
| 10 | 10 | 0.081 | 74 | 0.121 | 55 | 0.131 | 68 |
| 11 | 10 | 0.127 | 116 | 0.142 | 64 | 0.155 | 81 |
| 12 | 100 | 0.090 | 82 | 0.279 | 127 | — | — |

LSD 5%: 0.020.
LSD 1%: 0.032.
*Source:* Biddington and Thomas 1973.

glets of $CH_2$ from $-CH_2C_6H_5$ appeared at 5.47–5.83 δ ppm; the 6-H singlet at 8.1–8.65 δ ppm, and the spectra of pyrazolopyrimidines (9–11) exhibited a 3-H singlet at 7.73–7.97 δ ppm. Satisfactory elemental analyses were obtained for all of the products mentioned above.

### *Compounds 1–12 Were Tested Both as Potential Cytokinins and as Anticytokinins*

Cytokinin activity was studied in terms of betacyanin synthesis in *Amaranthus caudatus* M. cotyledons—one of the most specific and sensitive cytokinin assays known (Table 2). Among the triazolo[4,5-d]pyrimidines, compounds 2, 3, 4, and 5 showed a slight cytokinin activity, and 1, 6, 7, and 8 possessed inhibitory effect. Pyrazolo[3,4-d]pyrimidines with ethoxycarbonylpiperazinyl- and aliphatic substituents at the 4th position (10 and 9) as well as compound 12, suppressed betacyanin synthesis.

When tested with simultaneous application of a purine type cytokinin (BA), all the compounds reduced the cytokinin-promoted betacyanin accumulation. Only 1-benzyl-4-hydroxy-1H-pyrazolo[3,4-d]pyrimidine (12) augmented the effect of BA. The highest anticytokinin activity was exhibited by compounds 6 and 10 (carrying a 4-ethoxy-carbonylpiperazinyl moiety at the 4th position) which inhibited the stimulatory action of BA about 50%. The same tendency was observed with the phenylurea cytokinin (4-PU-30), but to a lesser extent.

The compounds from both groups with noncyclic substituents at the 4th position stimulated radish cotyledon growth (Table 3). The effect of 2 at a concentration of 1 μM, was the same as that of BA (155% of the control). 4-Piperazinyl substituted compounds (5, 6, and 10) applied alone did not affect cotyledon expansion.

All of the compounds (except for 12) acted as anticytokinins when applied in combination with BA or 4-PU-30, inhibiting the stimulatory effect of the cytokinins by 13% (compound 11) to 40% (compound 1). The type of cytokinin used did not influence the degree of antagonism observed.

Another physiological activity due to cytokinins is the delay of senescence of leaf explants. Some of the more active substances were tested for chlorophyll retention in barley leaf segments (Table 4). Applied by themselves, derivatives 3, 4, and 5 retarded chlorophyll destruction (13–33% compared to the control), while compounds 7 and 10 enhanced chlorophyll degradation. When applied simultaneously with cytokinins of either adenine or phenylurea type, all the compounds exhibited strong anticytokinin activity. They eliminated cytokinin-induced chlorophyll retention in barley segments. The antagonistic effect was expressed to almost the same extent with both types of cytokinins. In contrast, with radish leaf discs, the chlorophyll retention was not affected significantly by the antagonists (data not shown).

In general, the results obtained showed the strong anticytokinin activity of 1-benzyl-4-ethoxycarbonylpiperazinyl-1H-1,2,3-triazolo[4,5-d]-pyrimidine (compound 6) in all bioassays. This led

**Table 3.** Influence of the compounds tested (applied alone and in combination with cytokinin) on the excised radish cotyledon expansion.

| Compounds tested | Optimum conc. [μM] | Without cytokinin | | Comb. with BA 10 μM | | Comb. with 4-PU-30 10 μM | |
|---|---|---|---|---|---|---|---|
| | | Weight [mg/10 Cotyledons] | % to Control | Weight [mg/10 Cotyledons] | % to BA | Weight [mg/10 Cotyledons] | % to 4-PU-30 |
| Control | — | 200 | 100 | 310 | 100 | 390 | 100 |
| 1 | 0.1 | 258 | 129 | 187 | 60 | — | — |
| 2 | 1 | 310 | 155 | 229 | 74 | — | — |
| 3 | 10 | 232 | 116 | 233 | 75 | 302 | 77 |
| 4 | 10 | 233 | 116 | 243 | 78 | 305 | 78 |
| 5 | 10 | 189 | 94 | 242 | 78 | 290 | 74 |
| 6 | 1 | 192 | 96 | 198 | 64 | 206 | 53 |
| 7 | 10 | 221 | 110 | 242 | 78 | 272 | 70 |
| 8 | 100 | 227 | 114 | 238 | 77 | — | — |
| 9 | 100 | 249 | 124 | 207 | 67 | — | — |
| 10 | 10 | 178 | 89 | 237 | 76 | 241 | 62 |
| 11 | 1 | 228 | 114 | 270 | 87 | 310 | 80 |
| 12 | 1 | 234 | 117 | 351 | 113 | — | — |

LSD 5%: 16.
LSD 1%: 21.
*Source:* Letham 1971.

**Table 4.** Influence of the compounds tested (applied alone and in combination with cytokinin) on the senescence of barley leaf explants.

| Compounds tested | Optimum conc. [μM] | Without cytokinin | | Comb. with BA 10 μM | | Comb. with 4-PU-30 10 μM | |
|---|---|---|---|---|---|---|---|
| | | $OD_{665}$ | % to Control | $OD_{665}$ | % to BA | $OD_{665}$ | % to 4-PU-30 |
| Control | — | 0.092 | 100 | 0.252 | 100 | 0.206 | 100 |
| 3 | 10 | 0.104 | 113 | 0.164 | 65 | 0.129 | 63 |
| 4 | 10 | 0.122 | 133 | 0.156 | 62 | 0.129 | 63 |
| 5 | 10 | 0.104 | 113 | 0.139 | 55 | 0.120 | 58 |
| 6 | 10 | 0.092 | 100 | 0.108 | 43 | 0.118 | 57 |
| 7 | 10 | 0.083 | 91 | 0.130 | 52 | 0.119 | 58 |
| 10 | 10 | 0.074 | 81 | 0.114 | 45 | 0.132 | 64 |
| | 1 | 0.087 | 94 | 0.134 | 53 | 0.156 | 76 |
| 11 | 10 | 0.095 | 103 | 0.135 | 54 | 0.123 | 60 |
| | 0.1 | 0.092 | 100 | 0.200 | 79 | 0.185 | 90 |

LSD 5%: 0.012.
LSD 1%: 0.021.
Initial state: 5.45 mg chl. a + b/dm$^2$ after 72 h aging 1.20 mg chl. a + b/dm$^2$.
*Source:* Kende 1965.

us to perform additional experiments to characterize in detail its antagonistic properties and determine whether the anticytokinin activity expressed was reversible by subsequent application of cytokinin. Radish cotyledon growth and retention of chlorophyll degradation in barley leaf segments were used for these tests. A 1-μM solution of compound 6 was applied and after a period of incubation (24 or 48 h) it was replaced with 10-μM solution of BA or 4-PU-30.

Compound 6 did not affect cotyledon growth compared to the control (Fig. 1A, and 1B). BA and 4-PU-30 strongly stimulated cotyledon expansion, especially in the first hours after application. Replacement of 6 with BA or 4-PU-30 at 24 h led to a sharp increase in growth. However, cotyledon weight remained less than with BA or 4-PU-30 treatment. Similarly, the inhibitory effect of compound 6 was observed to be overcome after change of solutions at 48 h.

Similar results were obtained with ageing of barley leaf explants (Figs. 2 and 3). During the whole period, there were not significant differences between the chlorophyll content in control barley segments and those incubated on solutions of compound 6. Both cytokinins strongly retarded chloro-

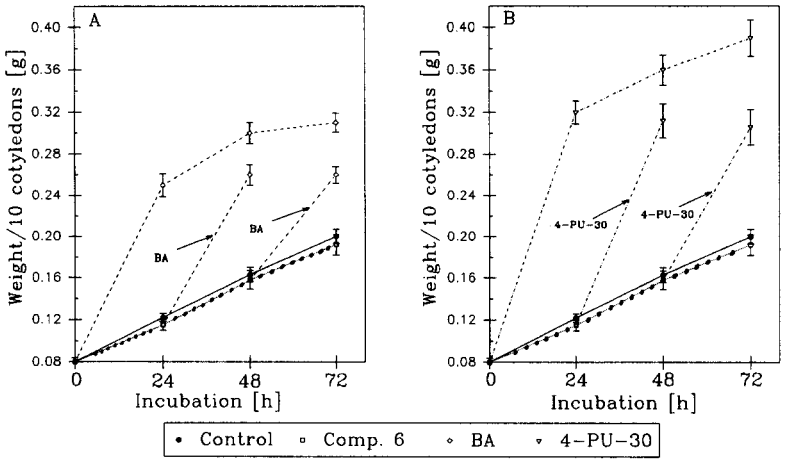



**Fig. 1.** Influence of BA (10 μM), 4-PU-30 (10 μM), and 1-benzyl-4-ethoxycarbonylpiperazinyl-1H-1,2,3-triazolo[4,5-d]pyrimidine (compound 6, 1 μM) on the growth of excised radish cotyledons. *Arrows* indicate the replacement of test solutions. **(A)** Replacement of antagonist with BA (10 μM). **(B)** Replacement of antagonist with 4-PU-30 (10 μM). Scale bars represent ± SE.

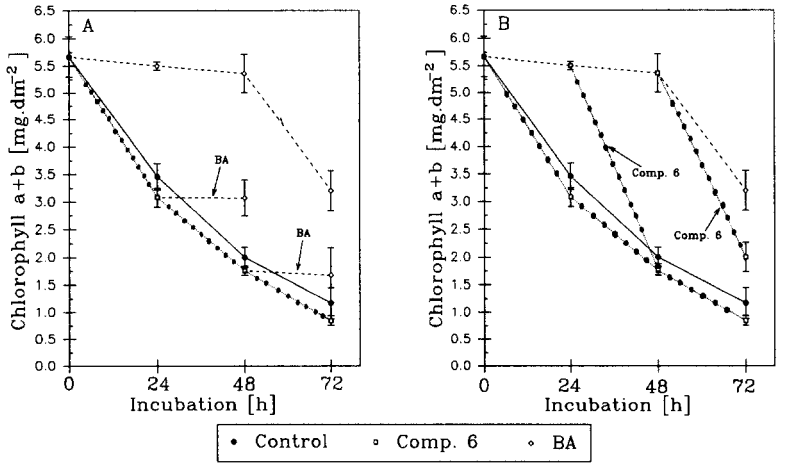



**Fig. 2.** Chlorophyll degradation in barley leaf segments, influenced by BA (10 μM) and 1-benzyl-4-ethoxycarbonylpiperazinyl-1H-1,2,3,-triazolo[4,5-d]pyrimidine (compound 6, 1 μM). *Arrows* indicate the replacement of test solutions. **(A)** Replacement of antagonist with BA (10 μM). **(B)** Replacement of BA with antagonist (1 μM). Scale bars represent ± SE.

phyll degradation. The replacement of antagonists with cytokinin (6 → BA or 6 → 4-PU-30; Figs. 2A and 3A) led to a delay of chlorophyll destruction. At 48 h, the chlorophyll level in control explants had diminished 42% in comparison with the value at 24 h. However, replacing 6 with cytokinins retained the chlorophyll content. The same trend was observed when the solutions were changed at the 48 h.

Cytokinins of both types removed the inhibitory effect of compound 6. Other experiments were carried out to study the influence of the anticytokinin on cytokinin-stimulated chlorophyll retention. Compound 6 completely removed the senescence-delaying action of BA and 4-PU-30 (Figs. 2B and 3B). The change BA → 6 or 4-PU-30 → 6 at 24 h resulted in a strong decrease in chlorophyll. 24 h later it was only 30% of the chlorophyll of BA-treated explants and 47% of 4-PU-30-treated explants. The same replacement at 48 h caused similar but reduced effects (62% for BA-treatment and 82% for 4-PU-30-treatment), that is, anticytokinin activity of compound 6 was better expressed when the antagonists was applied earlier.

## Discussion

Recently, some 8-aza adenines were reported to be strong cytokinin antagonists. Moreover, some of them acted as competitive inhibitors against purine-type cytokinins (Karanov et al. 1993). The success of this class of antagonists led us to hypothesize that additional series of anticytokinins should exist. That is why we designed, synthesized, and tested a series of 4-substituted 1-benzyltriazolo[4,5-d]pyrimidines and 4-substituted 1-benzylpyrazolo-[3,4-d]pyrimidines. Most of the derivatives possessed a chemical function at the side chain that could be modified additionally.

The compounds presented in this article were shown to have high physiological activity as plant growth regulators. Applied alone, some of them ex-

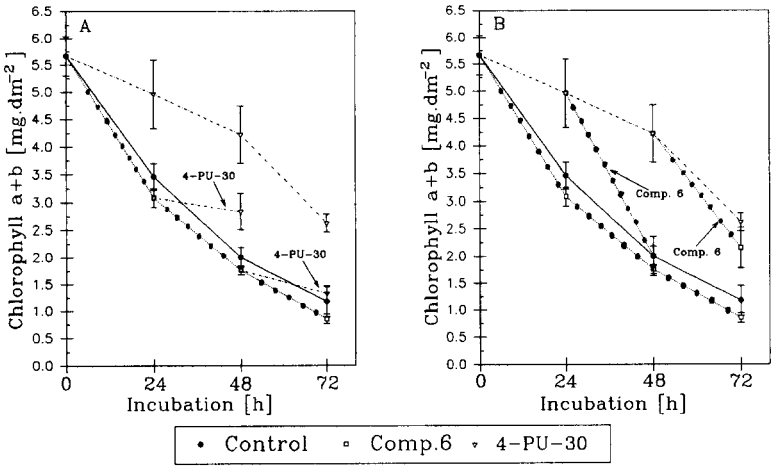



**Fig. 3.** Chlorophyll degradation in barley leaf segments, influenced by 4-PU-30 (10 μM) and 1-benzyl-4-ethoxycarbonylpiperazinyl-1H-1,2,3-triazolo[4,5-d]pyrimidine (compound 6, 1 μM). *Arrows* indicate the replacement of test solutions. **(A)** Replacement of antagonist with 4-PU-30 (10 μM. **(B)** Replacement of 4-PU-30 with antagonist (1 μM). Scale bars represent ± SE.

hibited slight cytokinin-like effects (stimulation of betacyanin synthesis and growth of radish cotyledons; retention of chlorophyll). The compounds were evaluated for antagonism of typical cytokinin effects. In most cases, the antagonistic properties of the six structural classes anticytokinins have been studied in relation to purine-type cytokinins (kinetin or BA). Only a few reports on interaction between anticytokinins and phenylurea cytokinins are available. However, such investigations would give additional information on the problem of cytokinin mode of action.

The combined application of antagonist with benzyladenine reduced to a significant extent cytokinin effects on the retention of chlorophyll in leaf explants; the stimulation of betacyanin synthesis, and the promotion of radish cotyledon growth (Tables 2, 3, and 4). The same tendency was observed in tests when the antagonists were applied in combination with 4-PU-30. It should be remarked that, even though active against both types of cytokinins, the compounds tested expressed slightly stronger anticytokinin activity towards the purine derivative, presumably due to their close structural resemblance.

The comparison of both series (1–8, 9–12) showed that the substitution N → C at the 3rd position of the ring, led to a slight reduction in anticytokinin activity, that is, the antagonistic action of triazolopyrimidines exceeded those of pyrazolopyrimidines.

On the other hand, compounds carrying a heterocyclic substituent at the 4th position on the ring had higher anticytokinin activity than did those with a noncyclic side chain. All of the known anticytokinins possess a secondary nitrogen, that is probably responsible for the linkage with the electronic interaction site or basic group of the receptor (Shimizu et al. 1989). On the basis of previous investigations (Karanov et al. 1993) and the above results, it could be noted that the HN-moiety at 4th position of pyrazolopyrimidines and triazolopyrimidines is an *essential, important, but not compulsory,* structural element for anticytokinin activity. The compounds with a tertiary nitrogen at the 4th place on pyrimidine ring also exhibit strong antagonism to cytokinins and in some cases their antagonistic effects exceeded those of adenine analogues with primary and secondary nitrogen at 4th position (Karanov et al. 1993). However, the anticytokinin activity observed, like any type of inhibition, may be nonspecific because not only structural analogues of purines and phenylurea act as antagonists. Some unrelated compounds, phenethylamines (Christou and Barton 1989), 4-substituted pyrazoles (Karanov et al. 1993) etc., were found to possess anticytokinin activity. Thus, biological response may not always be associated with specific chemical structure.

Although the chemical structure is a primary determinant of biological activity, the anticytokinin activity observed was expressed to a different degree in relation to the test system used (growth of radish cotyledons < betacyanin synthesis in *Amaranthus caudatus* M. < senescence of barley leaf explants).

Among the derivatives studied that were inactive or weakly active as cytokinins and tested as antagonists, compound 6 exhibited strong anticytokinin effects in all bioassays and in the whole concentration range. The results shown in Figs. 2 and 3 demonstrate the antagonism by compound 6 of cytokinin-induced chlorophyll retention in barley leaf segments as well as the reversibility of the action of 6. Similar results were observed using the radish cotyledon growth assay (Fig. 1). The antagonism appears independent of the chemical nature of the cytokinin used. This is in agreement with the hypothesis which suggests a common mode of action of

both purine and phenylurea cytokinins (Karanov et al. 1992, Kurosaki et at. 1981, Yamaguchi and Shudo 1991).

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