

Anticytokinin activity of substituted pyrrolo[2,3-*d*]pyrimidines

(cytokinin antagonists/tobacco cultures/growth inhibition/bud formation)

FOLKE SKOOG*, RUTH Y. SCHMITZ*, SIDNEY M. HECHT†, AND R. BRUCE FRYE†

* Institute of Plant Development, University of Wisconsin, Madison, Wisc. 53706; † Department of Chemistry, Massachusetts Institute of Technology, Cambridge, Mass. 02139

Contributed by Folke Skoog, May 12, 1975

ABSTRACT Ten substituted pyrrolo[2,3-*d*]pyrimidines were tested as cytokinins and anticytokinins in the tobacco bioassay. Eight new anticytokinins were identified and two were found to be highly active. The most potent species were 4-cyclohexylamino- and 4-cyclopentylamino-2-methylthiopyrrolo[2,3-*d*]pyrimidine, of which 0.05 and 0.009 μM concentrations, respectively, were required to produce detectable inhibition of the growth of tobacco callus cultured on a medium containing 0.003 μM 6-(3-methyl-2-butenylamino)purine. The inhibition of growth by moderate ($\leq 6.6 \mu\text{M}$) concentrations of these compounds was reversible by equal or higher concentrations of 6-(3-methyl-2-butenylamino)purine, but not by indole-3-acetic acid or gibberellic acid. These substituted pyrrolo[2,3-*d*]pyrimidines were also found to enhance bud formation at high cytokinin concentrations, suggesting that a cytokinin may act at more than one cellular site in exerting its growth-promoting and morphogenetic effects.

Cytokinins are a class of hormones which promote cell division and participate in the regulation of growth and morphogenesis in plants (1). Although all plants are thought to require cytokinins for growth, intact plants generally grow without exogenous cytokinins, presumably because they make their own. In fact, several highly active cytokinins have been found to occur naturally as N^6 -substituted adenine derivatives at the purine, ribonucleoside, and ribonucleotide levels (1). Cytokinins are also present in the transfer RNA's of most forms of life, although their presence in tRNA has not been shown to be related to their regulation of growth (1, 2).

Cytokinins have been found to influence the behavior of mammalian cells. They have been reported, for example, to inhibit platelet aggregation (3) and the growth of several mammalian cell lines, including cancerous lines (4-6 and references in 5). N^6 -(3-Methyl-2-butenyl)adenosine ($i^6\text{Ado}$) has been reported to be an immunosuppressive agent (7) and to induce hematological remissions in leukemia patients (8, 9). Several cytokinins, including $i^6\text{Ado}$, have also been shown to be capable of regulating the growth of phytohemagglutinin-transformed human lymphocytes (10 and references therein), presumably by involvement in cyclic AMP metabolism, and $i^6\text{Ado}$ was shown to act as a competitive inhibitor of beef heart cyclic AMP phosphodiesterase (11).

To facilitate the study of the mechanism of cytokinin action and provide potential chemotherapeutic agents, we designed and synthesized a class of structural analogs of cytokinins which behave as anticytokinins in plant bioassays (12, 13). Proof that these "anticytokinins" act at the same cellular sites as the cytokinins is not presently accessible, since cytokinin-anticytokinin interaction is not monitored at the molecular level, but this interpretation has been proposed on

the basis of the observed biological activities of the analogs in several assays and on the close structural relationship between cytokinins and anticytokinins (14). The successful preparation of a class of antagonists suggested that additional heterocyclic series of anticytokinins should exist. Several potentially inhibitory analogs have been prepared (15-17), but none of these fulfilled the criteria for anticytokinin activity to nearly the same extent as the original class of anticytokinins (14). The present report describes a new class of potential cytokinin antagonists, the activity of which is consistent with the criteria specified for anticytokinins.

MATERIALS AND METHODS

Synthesis of Test Substances. The syntheses of 1 and 2 have been reported (14). Satisfactory elemental analyses were obtained for all new compounds.

4-Amino-2-methylthiopyrrolo[2,3-*d*]pyrimidine (3). To 20 ml of ethanolic ammonia was added 1.0 g (5.0 mmol) of 4-chloro-2-methylthiopyrrolo[2,3-*d*]pyrimidine (18). The mixture was heated in an autoclave at 135° for 20 hr and then concentrated. The ethanol-soluble portion of the residue was purified by chromatography on silica gel, elution with ethyl acetate, and then by preparative thin-layer chromatography on silica gel, development with ethanol, to afford 3 as a white solid, yield 400 mg (40%). The melting point (mp) and ultraviolet spectrum were consistent with the literature values (19).











4-(3-Methyl-2-butenylamino)-2-methylthiopyrrolo[2,3-*d*]pyrimidine (4). To 400 mg (2.0 mmol) of 4-chloro-2-methylthiopyrrolo[2,3-*d*]pyrimidine (18) was added 2 ml of isopentylamine. The solution was heated at reflux under N_2 for 2 hr. The product was purified by chromatography on a 30-g column of Sephadex LH-20, elution with H_2O , and then with increasing concentrations of ethanol. The appropriate fractions were combined and evaporated to dryness and the solid residue was crystallized from ethanol-water to afford white crystals of 4, yield 131 mg (26%), mp 155.0-155.5°; $\lambda_{\text{max}}^{\text{EtOH}}$ (pH 1) 298 nm (ϵ 12,900) and 243 (17,600), λ_{min} 269 (8400); $\lambda_{\text{max}}^{\text{EtOH}}$ (pH 7) 286 (14,200) and 233 (25,800), λ_{min} 254 (4700); $\lambda_{\text{max}}^{\text{EtOH}}$ (pH 10) 286 (14,800) and 234 (25,800), λ_{min} 256 (5200).

4-(3-Methylbutylamino)-2-methylthiopyrrolo[2,3-*d*]pyrimidine (5). Compound 5 was prepared from 3 and isopentylamine in 38% yield by analogy with the preparation of 4, mp 157-158°; $\lambda_{\text{max}}^{\text{EtOH}}$ (pH 1) 297 nm (ϵ 14,000) and 242 (19,000), λ_{min} 268 (8500); $\lambda_{\text{max}}^{\text{EtOH}}$ (pH 7) 286 (15,800) and 233 (27,700), λ_{min} 254 (4800); $\lambda_{\text{max}}^{\text{EtOH}}$ (pH 10) 285 (16,000) and 232 (28,400), λ_{min} 254 (5000).

2-Methylthio-4-(*n*-pentylamino)pyrrolo[2,3-*d*]pyrimidine (6). Compound 6 was prepared from 3 and *n*-pentylamine as white crystals in 57% yield by analogy with the preparation of 4, mp 134-137°; $\lambda_{\text{max}}^{\text{EtOH}}$ (pH 1) 287 nm (ϵ 12,400) and

Abbreviations: $i^6\text{Ade}$, N^6 -(3-methyl-2-butenyl)adenine; $i^6\text{Ado}$, N^6 -(3-methyl-2-butenyl)adenosine; IAA, indole-3-acetic acid; mp, melting point; m/e , mass-to-charge ratio.

Table 1. Biological activity of substituted 4-aminopyrrolo[2,3-*d*]pyrimidines

COMPOUND NO.	R=	R ^I =	R ^{II} =	R ^{III} =	RANGE OF CONC. TESTED	CYTOKININ ACTIVITY, MIN. CONC. (μM) FOR		GROWTH INHIBITION, MIN. CONC. (μM) FOR	
						DETECTION	MAXIMUM GROWTH	DETECTION	* COMPLETE INHIBITION
1.		H	H	H	0.24-20	0.62	6.6	N.A.	---
2.		H	H	H	0.24-20	5.8	>20	N.A.	---
3.	H	SCH ₃	H	H	0.24-20	N.A.	---	N.A.	---
4.		SCH ₃	H	H	0.009-20	N.A.	---	0.24	2.2
5.		SCH ₃	H	H	0.027-20	N.A.	---	0.40	2.0
6.		SCH ₃	H	H	0.08-20	N.A.	---	0.1	1
7.		SCH ₃	H	H	0.009-20	N.A.	---	0.009	0.05
8.		SCH ₃	H	H	0.24-20	N.A.	---	6.6	>20
9.		SCH ₃	H	H	0.003-20	N.A.	---	0.05	0.60
10.		SCH ₃	H	H	0.24-20	N.A.	---	10	>20
11.		H	CN	SCH ₃	0.24-20	N.A.	---	20	N.R.

N.A. = Not active. N.R. = Not reached.

* In presence of 0.003 μM i⁶Ade.

238 (20,700), λ_{min} 256 (6100); λ_{max}^{EtOH} (pH 7) 282 (14,000) and 234 (24,700), λ_{min} 253 (5600); λ_{max}^{EtOH} (pH 10) 283 (12,700) and 234 (24,400), λ_{min} 253 (4600).

4-(Cyclopentylamino)-2-methylthiopyrrolo[2,3-*d*]pyrimidine (7). This compound was obtained as tan crystals by treatment of 3 with cyclopentylamine, as discussed above for the synthesis of 4. Yield 40%, mp 190–191°, λ_{max}^{EtOH} (pH 1) 298 nm (ε 13,700) and 242 (18,100), λ_{min} 267 (9800); λ_{max}^{EtOH} (pH 7) 286 (16,000) and 234 (27,600), λ_{min} 256 (6300); λ_{max}^{EtOH} (pH 10) 286 (15,300) and 233 (26,900), λ_{min} 256 (5500).

4-(*n*-Hexylamino)-2-methylthiopyrrolo[2,3-*d*]pyrimidine (8). Species 8 was obtained as white crystals in 60% yield from 3 and *n*-hexylamine, mp 117.5–118.5°; λ_{max}^{EtOH} (pH 1) 297 nm (ε 13,700) and 242 (18,700), λ_{min} 266 (8900); λ_{max}^{EtOH} (pH 7) 286 (15,400) and 235 (27,200), λ_{min} 254 (4600); λ_{max}^{EtOH} (pH 10) 285 (15,800) and 233 (28,500), λ_{min} 254 (5300).

4-Cyclohexylamino-2-methylthiopyrrolo[2,3-*d*]pyrimidine (9). Compound 9 was obtained as white crystals in 51% yield by analogy with the synthesis of 4, mp 189–190°; λ_{max}^{EtOH} (pH 1) 295 nm (ε 12,400) and 239 (20,600), λ_{min} 260 (6500); λ_{max}^{EtOH} (pH 7) 283 (13,200) and 234 (25,100), λ_{min} 254 (4200); λ_{max}^{EtOH} (pH 10) 284 (13,700) and 233 (26,000), λ_{min} 254 (4500); mass spectrum: mass-to-charge ratio (*m/e*) 262 (M⁺), 247, 233, 229, 219, 215, 207, 205, 193, 180, 149, 147, and 134.

4-Benzylamino-2-methylthiopyrrolo[2,3-*d*]pyrimidine (10). Compound 10 was obtained as off-white crystals by treatment of 3 with benzylamine, by analogy with the synthesis of 4. Yield 100 mg (36%), mp 179–180°; λ_{max}^{EtOH} (pH 1) 301 nm (ε 14,800) and 246 (22,900), λ_{min} 272 (9100) and 222 (13,500); λ_{max}^{EtOH} (pH 7) 286 (16,600) and 233

(28,300), λ_{min} 256 (6200) and 218 (15,600); λ_{max}^{EtOH} (pH 10) 287 (17,200) and 233 (29,400), λ_{min} 256 (6800) and 218 (16,100).

4-Cyclopentylamino-6-methylthiopyrrolo[2,3-*d*]pyrimidine-5-carbonitrile (11). To 533 mg (2.61 mmol) of the presumed methoxymethylenamino derivative of 2-amino-3,4-dicyano-5-thiopyrrole (20) was added 3 ml of cyclopentylamine and the mixture was heated at reflux for 2 hr under N₂. The product was purified by chromatography on 30 g of Sephadex LH-20, elution with H₂O and then with increasing concentrations of ethanol. The appropriate fractions were pooled and evaporated to dryness and the solid residue was crystallized from EtOH to afford off-white crystals of 11, yield 112 mg (16%), mp 310–311° (decomposition); λ_{max}^{EtOH} (pH 1) 302 nm (ε 15,900) and 232 (18,700), λ_{min} 261 (3500) and 217 (16,700); λ_{max}^{EtOH} (pH 7) 318 (9500) and 266 (23,700), λ_{min} 282 (4500) and 238 (9100); λ_{max}^{EtOH} (pH 10) 308 (21,600), 261 (19,900), and 256 (19,900), λ_{min} 277 (7000) and 234 (15,200).

Bioassay Procedures. Cytokinin activity was determined by the tobacco bioassay (21), and growth-inhibiting activity by a modification of this assay. For cytokinin measurement, the media contained the mineral salts specified in Table 6, part A, of ref. 21, to which certain organic substituents were added (14). The test compounds were dissolved in dimethylsulfoxide and added to the cooling, autoclaved medium after suitable dilution (21, 22). Compounds lacking or having very low cytokinin activity were further tested by the addition to the above medium of 0.003 μM N⁶-(3-methyl-2-butenyl)-adenine (i⁶Ade). The enhancing or inhibiting effect on callus growth of the test substances was clearly evident at this sub-optimal cytokinin concentration. An optimal cytokinin concentration, 0.08 μM i⁶Ade, or a series of increasing cytokinin

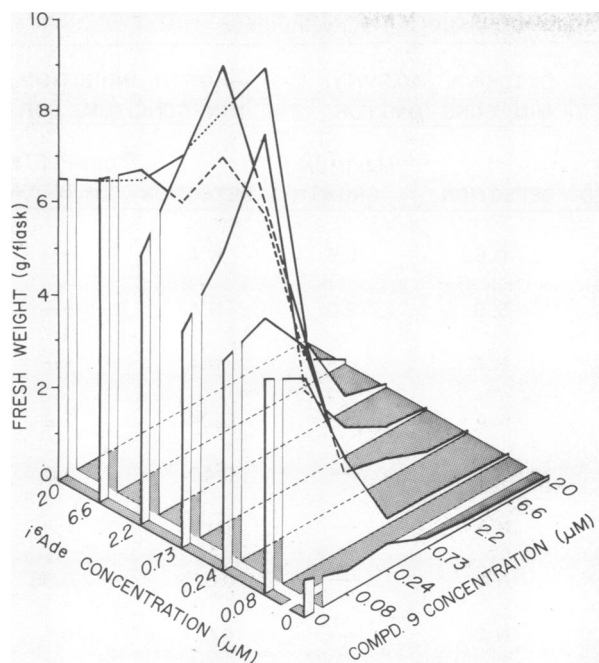


FIG. 1. The effects of serial combinations of 4-cyclohexylamino-2-methylthiopyrrolo[2,3-*d*]pyrimidine (9) and i^6 Ade on the fresh weight yield of tobacco callus. Bud formation occurred in seven treatments as follows: the five treatments with 20 μ M i^6 Ade and 0.08–6.6 μ M 9, and two treatments with 2.2 μ M or 6.6 μ M each of i^6 Ade and 9. Test period: 6 Dec. 1974 to 10 Jan. 1975.

concentrations, was used to evaluate the reversibility of inhibition by the more active antagonists.

RESULTS

Synthesis of substituted pyrrolo[2,3-*d*]pyrimidines

Each of the 4-alkylamino-2-methylthiopyrrolo[2,3-*d*]pyrimidines (4–10) was prepared by heating at reflux a solution of 4-chloro-2-methylthiopyrrolo[2,3-*d*]pyrimidine (19) and the appropriate amine. The purified products were obtained in yields of 25–60% by Sephadex LH-20 chromatography and subsequent crystallization from ethanol–water. The isopentenyl and *n*-hexyl derivatives (4 and 8) were further treated with Raney nickel to give 4-(3-methyl-2-butenylamino)pyrrolo[2,3-*d*]pyrimidine (1) and 4-*n*-hexylamino-2-methylthiopyrrolo[2,3-*d*]pyrimidine (2), respectively (14). 4-Cyclopentylamino-6-methylthiopyrrolo[2,3-*d*]pyrimidine-5-carbonitrile (11) was prepared by heating 2-amino-3,4-dicyano-5-thiopyrrole (20) successively with trimethyl orthoformate and cyclopentylamine.

Biological activities of substituted pyrrolo[2,3-*d*]pyrimidines

1. Growth Promotion and Inhibition. Compounds 1–11 were tested in the tobacco bioassay both as potential cytokinins and anticytokinins. As shown in Table 1, 4-(3-methyl-2-butenylamino)pyrrolo[2,3-*d*]pyrimidine (1) and 4-*n*-hexylamino-2-methylthiopyrrolo[2,3-*d*]pyrimidine (2) were weak cytokinins and were devoid of anticytokinin activity. On the other hand, the 2-methylthio derivatives of 1 and 2 (4 and 8, respectively) were inactive as cytokinins and, at concentrations as low as 0.24 and 6.6 μ M, respectively, inhibited the growth of tobacco callus cultured on a medium containing 0.003 μ M i^6 Ade. Also inhibitory to the growth of tobacco callus were the 4-alkylamino-2-methylthiopyrrolo[2,3-*d*]pyrimidines 5, 6, 7, 9, and 10. Of these compounds, the cyclopentyl and cy-

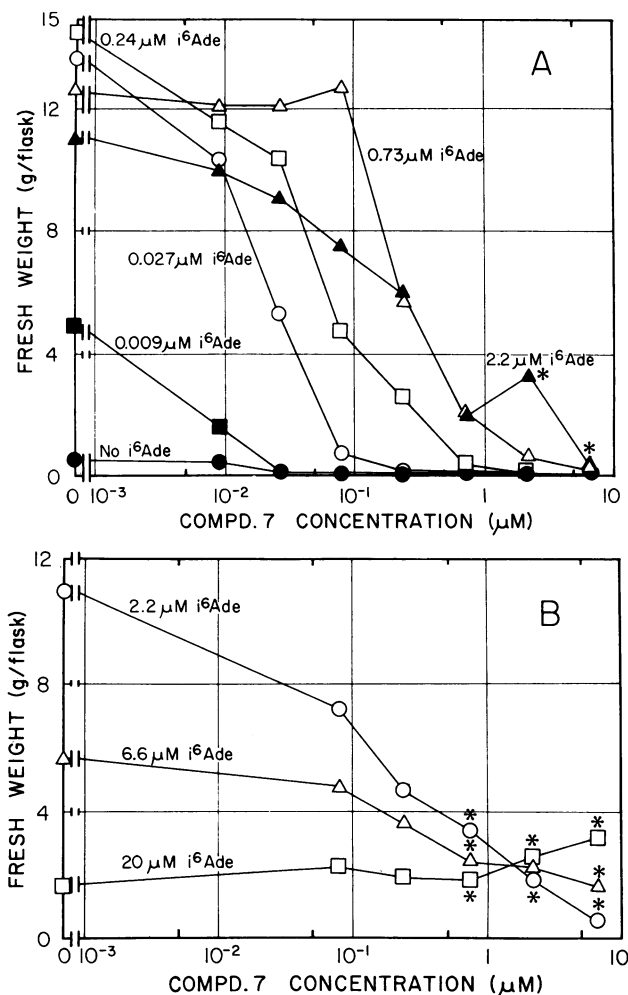


FIG. 2. The effects of serial combinations of i^6 Ade and 4-cyclopentylamino-2-methylthiopyrrolo[2,3-*d*]pyrimidine (7) on the fresh weight yield of tobacco callus. (A) i^6 Ade 0–2.2 μ M. Test period: 6 Dec. 1974 to 10 Jan. 1975. (B) i^6 Ade 2.2–20 μ M. Test period: 5 April to 11 May 1973. * Treatments in which buds formed.

clohexyl derivatives (7 and 9) were especially active as cytokinin antagonists. Compound 7 gave detectable inhibition of cells cultured on 0.003 μ M i^6 Ade at a concentration of 0.009 μ M and complete inhibition at 0.05 μ M. It is thus at least four times as active as the best reported anticytokinin (12, 13). Compound 11, which contained a methylthio group on the pyrrole moiety, was only slightly inhibitory at 20 μ M and did not cause complete inhibition at any tested concentration. 4-Amino-2-methylthiopyrrolo[2,3-*d*]pyrimidine (3), which lacked an N^4 -substituent, had neither cytokinin or anticytokinin activity.

The extent to which inhibition of growth by these analogs can be reversed by cytokinins is illustrated for 9 in Fig. 1 and for 7 in Fig. 2. Concentrations of 9 from 0.73 to 6.6 μ M strongly inhibited cultures grown on low concentrations of i^6 Ade or in the absence of added cytokinin, but not the growth of cultures supplied with high concentrations of i^6 Ade. Particularly striking was the full restoration of growth in cultures with 6.6 μ M 9 and 20 μ M i^6 Ade. However, the inhibition caused by 20 μ M 9 was severe even in the presence of 20 μ M i^6 Ade and is apparently irreversible by cytokinins. Compound 7 also acts as a specific anticytokinin, as judged by the effective cytokinin reversal of inhibition (Fig. 2A).

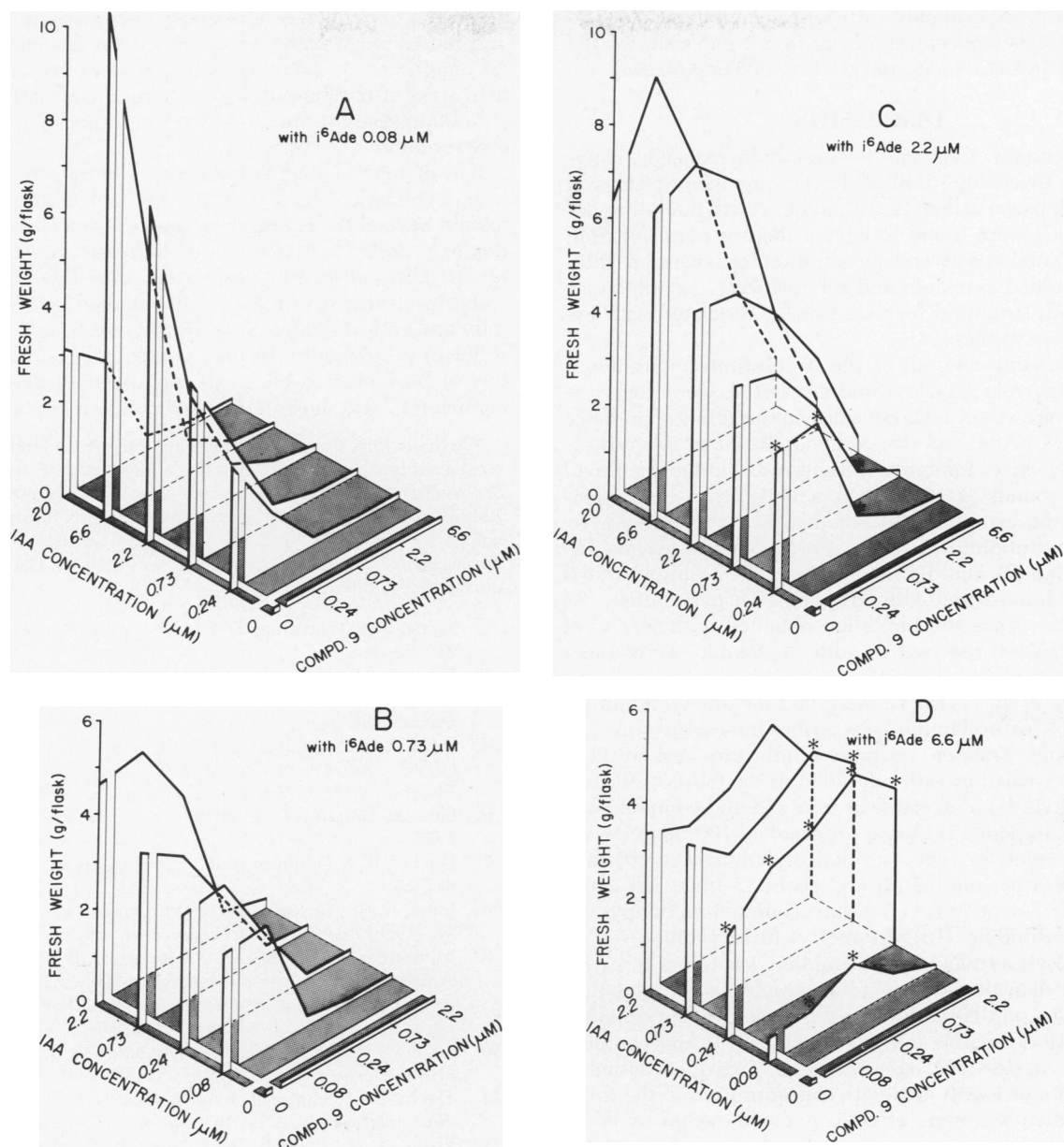


FIG. 3. Effects of serial combinations of 4-cyclohexylamino-2-methylthiopyrrolo[2,3-d]pyrimidine (9), IAA, and i^6 Ade on fresh weight yields of tobacco callus cultures. Concentrations of i^6 Ade: (A) 0.08 μ M (B) 0.73 μ M (c) 2.2 μ M, and (D) 6.6 μ M. Test periods: (A) and (C) (Exp. 294 A and B) 24 Jan. to 27 Feb., 1975; (B) and (D) (Exp. 251 A and B) 25 Jan. to 28 Feb. 1974. * Treatments in which buds formed.

2. Enhancement of Cytokinin-Promoted Budding. Compounds 7 and 9 promoted the formation of buds in some treatments at high cytokinin concentrations, and this may have increased the fresh weight yields somewhat (Figs. 1 and 2). This significant promotion of bud formation by substituted 4-aminopyrrolo[2,3-d]pyrimidines was obtained in the presence of 11.4 μ M indole-3-acetic acid (IAA), as normally employed in cytokinin bioassays. At lower IAA concentrations compounds 7 and 9 had little effect on bud formation.

3. Specific Anticytokinin Nature of the Growth Inhibitory Effect. To determine whether the inhibition of growth was in the nature of a specific anticytokinin effect or could also be reversed by auxins, experiments were carried out with serial combinations of 9, IAA, and i^6 Ade. The results of two such experiments are summarized in Fig. 3. In the presence of the lowest concentration of added cytokinin (0.08

μ M i^6 Ade, Fig. 3A) growth increased as a function of the auxin concentration, but the inhibition of growth by 9 (detectable at 0.24 μ M and almost complete at the 0.73 μ M) was not significantly affected by any concentration of IAA. However, as the concentration of cytokinin was increased successively (Fig. 3B, C, and D), the yields of tissue increased as a function of the IAA concentration in the presence of correspondingly higher concentrations of 9. Thus the highest cytokinin concentration (6.6 μ M i^6 Ade, Fig. 3D) completely counteracted the inhibition of growth by the highest tested concentration of 9 (2.2 μ M). These results are in accordance with expectations on the basis of known relationships between cytokinin and auxin concentrations in their effect on yield (23) and the assumption that 9 acts as a specific anticytokinin.

The same conclusion was reached from the fact that gibberellic acid was found not to reverse the growth inhibitory

action of **9** in experiments with serial combinations of the two compounds (concentrations up to 2.2 μM each) in the presence of IAA (11.4 μM) and $i^6\text{Ade}$ (0.003 or 0.08 μM).

DISCUSSION

Although certain 7-alkylamino-3-methylpyrazolo[4,3-*d*]pyrimidines reversibly inhibited the cytokinin-promoted growth of tobacco callus (12, 13), most growth inhibitory analogs (15–17) were found to have a more general function when evaluated at a given concentration for reversal of inhibition by added cytokinin and for correlation of inhibitory activity with structural features usually associated with cytokinin-active species (14).

In the present case, all of the *N*-substituted 4-amino-2-methylthiopyrrolo[2,3-*d*]pyrimidines tested were found to inhibit the growth of tobacco callus cultured in the presence of 0.003 μM $i^6\text{Ade}$, and two compounds (**7** and **9**) were exceptionally active. Inhibitory activity was limited exclusively to compounds (4–10) having a close structural resemblance to the cytokinins 4-(3-methyl-2-butenylamino)pyrrolo[2,3-*d*]pyrimidine (1) and 4-*n*-hexylaminopyrrolo[2,3-*d*]pyrimidine (2), and did not extend to the *N*-unsubstituted analog 4-amino-2-methylthiopyrrolo[2,3-*d*]pyrimidine (3). Furthermore, since the inhibition obtained with several of these analogs was reversed by added cytokinin, we consider these compounds to be specific anticytokinins.

Iwamura *et al.* (17) have described the anticytokinin activity of 4-furfurylamino-7-(β -D-ribofuranosyl)pyrrolo[2,3-*d*]pyrimidine. Tests on a sample kindly provided by Prof. Iwamura revealed growth inhibition in the tobacco bioassay (0.003 μM $i^6\text{Ade}$), detectable at 0.73 μM and complete at 5 μM . This compound is thus approximately 100 times less active as an inhibitor than 4-cyclopentylamino-2-methylthiopyrrolo[2,3-*d*]pyrimidine (**7**) and about 25 times less active than either 7-*n*-pentyl- or 7-*n*-hexylamino-3-methylpyrazolo[4,3-*d*]pyrimidine (13). In fact, 4-furfurylamino-7-(β -D-ribofuranosyl)pyrrolo[2,3-*d*]pyrimidine was found to be less inhibitory than the corresponding unsubstituted compound (4-amino-7-(β -D-ribofuranosyl)pyrrolo[2,3-*d*]pyrimidine) which gave detectable inhibition at 0.36 μM and complete inhibition at 0.88 μM . However, the partial inhibition of growth obtained with moderate concentrations of the furfuryl derivative was more effectively counteracted by added cytokinin.

The range of concentrations over which cytokinins promote growth of tobacco callus varies with the auxin concentration and *vice versa*. The maximum yield obtainable depends on both the concentrations and the proportions of the two hormones. High fresh weight yields are normally obtained with low cytokinin–auxin ratios, and high cytokinin concentrations must be matched by even higher auxin concentrations for high yields to be obtained. However, low auxin–cytokinin ratios may lead to bud formation, which in turn may increase fresh weight yield. Therefore, the finding (Fig. 3) that **9** was equally inhibitory over the entire range of IAA concentrations in the presence of low cytokinin concentrations, and that concentrations of **9** up to 2.2 μM exerted no inhibitory effect in the presence of high cytokinin concentrations, was consistent with the behavior to be expected if **9** were a specific anticytokinin.

The finding that both **7** and **9** augmented the effect of $i^6\text{Ade}$ in promoting bud formation while counteracting its growth-promoting effect on the callus can be consistent with

the action of cytokinins and antagonists at common sites, if differences in cytokinin requirements for the two functions are considered. It should be noted, however, that some augmentation of budding by high cytokinin concentrations was also obtained with 4-amino-7-(β -D-ribofuranosyl)pyrrolo[2,3-*d*]pyrimidine.

It is of interest that at high concentrations of antagonist (e.g., 0.08–2.2 μM for **7**; Fig. 2), the further addition of cytokinin beyond the concentration which reversed the inhibition (e.g., 0.73 μM $i^6\text{Ade}$) tended to inhibit growth. This effect parallels, at least in a superficial way, the inhibitory effects on cultured mammalian cells obtained both with cytokinins and anticytokinins (S. M. Hecht, and R. B. Frye, manuscript in preparation). In fact, compounds 4–10 are inhibitors of beef heart cyclic AMP phosphodiesterase (11) and compound **7** was shown to be a competitive inhibitor.

We thank Miss Virginia Lightner and Mrs. Anna Hilden for technical assistance. This investigation was supported at the University of Wisconsin by National Science Foundation Research Grant BMS72-02226 (GB-35260X) and at Massachusetts Institute of Technology by Public Health Service Research Grant CA-14896 from the National Cancer Institute. R.B.F. was a Union Carbide Fellow during the summer of 1973.

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