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CYTOKININ ANTAGONISTS: SYNTHESIS AND PHYSIOLOGICAL EFFECTS OF 7-SUBSTITUTED 3-METHYLPYRAZOLO[4,3-*d*]PYRIMIDINES*

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Key Word Index—*Nicotiana tabacum*; Solanaceae; cytokin in antagonists; pyrazolopyrimidines; callus bioassay.

Abstract—A series of sixteen pyrazolo[4,3-*d*]pyrimidine derivatives has been synthesized and tested for cytokinin antagonist activity in the tobacco bioassay, and effects of structural modifications on the 7-substituent of 7-substituted-3-methylpyrazolo[4,3-*d*]pyrimidines have been studied. In general the structural features of the 7-substituent required for high antagonist activity are similar to those of the corresponding 6-substituents of highly cytokinin-active purine derivatives. Most striking differences are the 3-fold increase in antagonist activity associated with change from the isopentyl to the normal pentyl derivative; the removal of antagonist activity by the formal cyclization of the hexyl chain or by the introduction of two 7-substituents, as in 7-(di-*n*-butylamino)-3-methylpyrazolo[4,3-*d*]pyrimidine; and the drastic shift from a potent antagonist to a weak cytokinin brought about by the presence of a double bond in 7-(3-methyl-2-butenylamino)-3-methylpyrazolo[4,3-*d*]pyrimidine. The active 3-methylpyrazolo[4,3-*d*]pyrimidines appear to be highly specific cytokinin antagonists (as compared to abscisic acid) but in high concentrations they may interfere more generally in purine metabolism. It is suggested that the pyrazolo[4,3-*d*]pyrimidine derivatives may be useful tools in studies of the regulation of growth and the mechanism of cytokinin action in this process.

INTRODUCTION

CYTOKININ antagonists are of potential value in studies of the mechanism of cytokinin action and other physiological studies of growth in various systems (including intact plants and tissues) which do not require an exogenous source of cytokinin for normal development.

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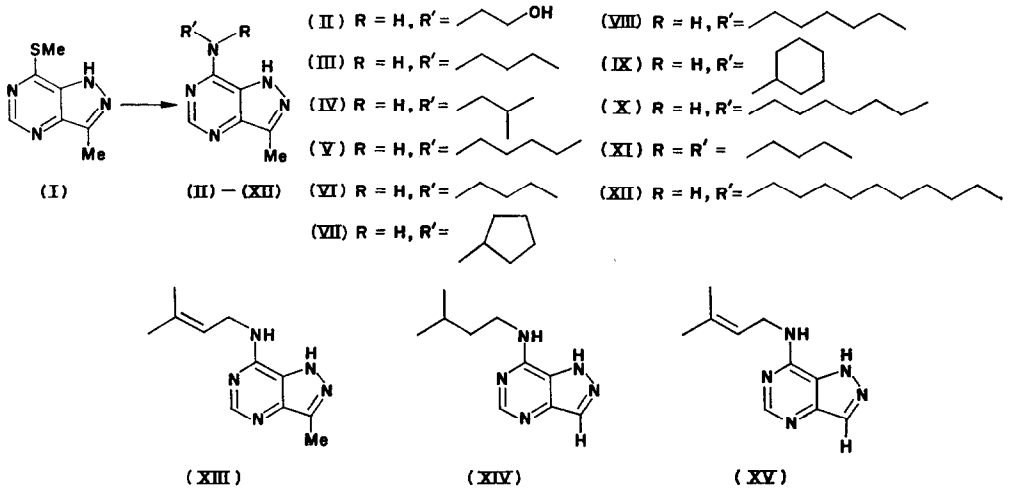
Recognition of this has led to studies of the interaction between cytokinins and synthetic growth inhibitors such as chloramphenicol,[1] (2-chloroethyl)-trimethylammonium chloride (CCC)[2,3] and 1-(2-methylcyclohexyl)-3-phenylurea (siduron).[4] The effects of these materials are not limited to cytokinin-mediated processes and they can often be more effectively counteracted by other factors such as gibberellic acid. More specific cytokinin antagonists have been sought among purine analogs, including 6-methylaminopurine, 2,6-diaminopurine, 8-azaguanine, 8-azaadenine and even adenine itself,[5–8] but with these compounds counteraction of the inhibition by cytokinins is slight.

In the present studies a systematic search for compounds which would specifically oppose the action of cytokinins resulted in the systhesis of a class of cytokinin antagonists which has been discussed in terms of one member of that class, 3-methyl-7-(3-methylbutylamino)pyrazolo[4,3-*d*]pyrimidine.[9,10] We now report on the synthesis and tests for cytokinin antagonist activity of sixteen cytokinin analogs structurally related to 6-(3-methyl-2-butenylamino)purine. These analogs represent systematic variations of the 7-substituent of pyrazolo[4,3-*d*]pyrimidine, and the testing results define the nature of those substituents associated with the most potent anticytokinin activity as determined in the tobacco bioassay. Preliminary tests have also been performed with other plant materials.



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[9] S. M. Hecht, R. M. Bock, R. Y. Schmitz, F. Skoog and N. J. Leonard, *Proc. Nat. Acad. Sci. U.S.* **68**, 2608 (1971).
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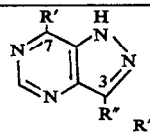
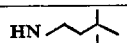
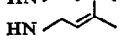
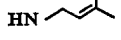
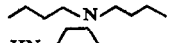
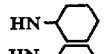
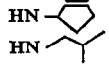
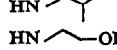
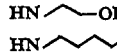
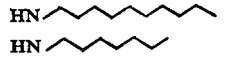
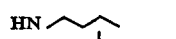
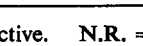
RESULTS

Chemistry of Test Substances

Syntheses have been reported previously for 3-methyl-7-methylthiopyrazolo[4,3-*d*]-pyrimidine (I),[10] 3-methyl-7-(3-methylbutylamino)pyrazolo[4,3-*d*]pyrimidine (VI),[10] 3-methyl-7-(3-methyl-2-butenylamino)pyrazolo[4,3-*d*]pyrimidine (XIII),[10] 7-(3-methylbutylamino)pyrazolo[4,3-*d*]pyrimidine (XIV)[10] and 7-(3-methyl-2-butenylamino)pyrazolo-[4,3-*d*]pyrimidine (XV).[10] The following compounds were synthesized by treatment of 3-methyl-7-methylthiopyrazolo[4,3-*d*]pyrimidine (I) with the appropriate amine at reflux: 7-(2-hydroxyethylamino)-3-methylpyrazolo[4,3-*d*]pyrimidine (II), 7-*n*-butylamino-3-methylpyrazolo[4,3-*d*]pyrimidine (III), 3-methyl-7-(2-methylpropylamino)pyrazolo[4,3-*d*]pyrimidine (IV), 3-methyl-7-*n*-pentylaminopyrazolo[4,3-*d*]pyrimidine (V), 7-cyclopentylamino-3-methylpyrazolo[4,3-*d*]pyrimidine (VII), 7-*n*-hexylamino-3-methylpyrazolo[4,3-*d*]pyrimidine (VIII), 7-cyclohexylamino-3-methylpyrazolo[4,3-*d*]pyrimidine (IX), 7-*n*-heptylamino-3-methylpyrazolo[4,3-*d*]pyrimidine (X), 7-(di-*n*-butylamino)-3-methylpyrazolo[4,3-*d*]pyrimidine (XI) and7-*n*-decylamino-3-methylpyrazolo[4,3-*d*]pyrimidine (XII). The products were purified by chromatography on silica gel or Sephadex LH-20 followed by crystallization from a suitable solvent. 7-Hydroxy-3-methylpyrazolo[4,3-*d*]pyrimidine (XVI) was synthesized by the method of Robins *et al.*[11]

TABLE 1. RELATION OF THE STRUCTURE OF 7-SUBSTUENTS TO BIOLOGICAL ACTIVITY OF PYRAZOLO[4,3-*d*]PYRIMIDINE DERIVATIVES

Compound No.	R″ =	 R′ =	No. of C atoms in R′	Range* tested (μM)	Cytokinin activity Min. conc. (μM) for Detection	Cytokinin activity Min. conc. (μM) for Maximum growth	Antagonist activity against 0·003 μM 2iP Min. conc. (μM) for Detection	Antagonist activity against 0·003 μM 2iP Min. conc. (μM) for Lethal dosage
XIV	H		5	0·009–20	1·0	20	N.A.	—
XV	H		5	0·001–20(3)	0·08	1·0	N.A.	—
XVI	Me	HO	0	0·08–20	N.A.	—	N.A.	—
I	Me		1	0·24–20	N.A.	—	N.A.	—
XIII	Me		5	0·08–20(4)	0·24	6·6	N.A.	—
XI	Me		2 × 4	0·24–20	N.A.	—	N.A.	—
IX	Me		6	0·73–20	N.A.	—	N.A.	—
VII	Me		5	0·73–20	N.A.	—	6·6	N.R.
IV	Me		4	0·24–20	2·2	7†	6·6	N.R.
II	Me		2	0·73–20	N.A.	—	2·2	N.R.
XII	Me		10	0·24–20	N.A.	—	3·0	N.R.
X	Me		7	0·03–6·6	N.A.	—	0·2	2·2
III	Me		4	0·03–6·6	N.A.	—	0·1	0·73
VI	Me		5	0·009–20(4)	N.A.	—	0·1	0·73
VIII	Me		6	0·009–20	N.A.	—	0·03	0·5
V	Me		5	0·009–20(3)	N.A.	—	0·03	0·2

N.A. = Not Active. N.R. = Not Reached.

* All values averages of two tests except as indicated by numbers in brackets. Testing done between April 1970 and December 1971.

† Only slight growth stimulation.

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Structure/Activity Relationships of Cytokinin Antagonists

Development of cytokinin antagonist activity. As shown in Fig. 1, the first highly active cytokinin antagonist, 3-methyl-7-(3-methylbutylamino)pyrazolo[4,3-*d*]pyrimidine (VI), was obtained by three formal modifications in the structure of 6-(3-methyl-2-butenylamino)-purine (2iP), which is itself a highly active cytokinin. Although each of these modifications alone and in combinations of two led to a marked decrease in growth promoting activity, a combination of all three was essential for the development of antagonist activity.

Effect of the 7-substituent on antagonist activity. The structure of the substituents in the 7-position greatly affected the anticytokinin activity of the pyrazolo[4,3-*d*]pyrimidine derivatives. In general an N^7-substituent four to seven carbon atoms in length was required for high activity, as found for compounds III, V, VI, VIII and X in Table 1 and Fig. 2, although the compounds containing the *n*-decyl or *i*-butyl substituent (XII and IV) were also active. In general linear substituents conferred more antagonist activity than did the corresponding branched isomers; compare the activity of the *n*-pentyl with the *i*-pentyl derivative (V and VI) and the corresponding *n*- and *i*-butyl derivatives (III and IV). It is also of interest that the presence of the second butyl group in the dibutyl derivative (XI) resulted in complete loss of antagonist activity.

Formal cyclization of the side chain, as in the cyclopentyl and cyclohexyl derivatives (VII and IX), markedly reduced or eliminated antagonist activity throughout the tested concentration range. The hydroxyethylamino derivative (II) had barely detectable antagonist activity, and the hydroxyl and methylthio derivatives (XVI and I) were inactive. All these results suggest a highly specific spatial requirement for antagonist action, best met by the *n*-pentyl group.

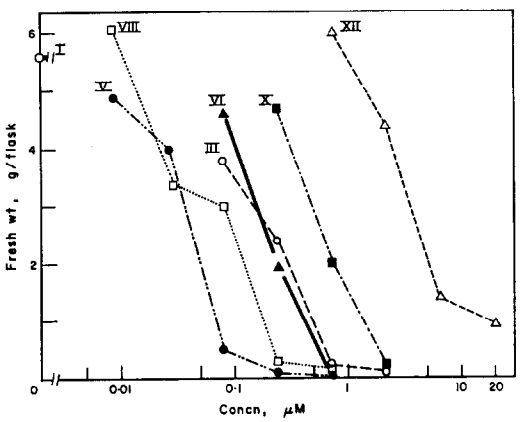

FIG. 2. INHIBITION OF GROWTH BY SERIAL CONCENTRATIONS OF EACH OF THE SIX MOST ACTIVE PYRAZOLO-[4,3-*d*]PYRIMIDINES. YIELDS OF TISSUE CULTURED ON MEDIUM CONTAINING 0·003 μM 2iP.
The compounds are numbered with roman numerals as in Table 1. Average values obtained from two to four tests, conducted between February and December 1971.

In the tested pyrazolo[4,3-*d*]pyrimidine derivatives in which the 3-position was unsubstituted, antagonist activity was absent. If, in addition to the formal removal of the methyl group from the 3-position, a suitable side chain was present in the 7-position, such as *N*-*i*-pentyl or *N*-*i*-pentenyl (XIV and XV), cytokinin activity was exhibited. If this group was

lacking, as in the thio-derivative (not shown), cytokinin activity as well as antagonist activity was lost. It should be noted that even in the presence of the 3-methyl group the double bond of the isopentenyl moiety was sufficient not only to remove antagonist activity, but also to confer weak cytokinin activity (see XIII in Fig. 1 and Table 1).

An Effect of Adenine on Resistance to the Antagonist

In general, tissues grown on medium with sufficiently low levels of the antagonists, or with derivatives lacking antagonist activity, grew as well as the controls and could be transferred at the end of the five week growth period without loss in viability. Tissues which exhibited marked reduction in growth and intense browning or blackening from exposure to high concentrations of antagonists did not recover when transplanted to fresh medium containing 2iP in concentrations adequate for growth. Tissues exposed to intermediate concentrations of antagonists grew irregularly, as if certain cells were more resistant to the inhibitor. These latter pieces developed into loose masses of watery cells tinged brown and displayed varying degrees of recovery when transplanted to medium without an antagonist.

Regardless of the cytokinin concentration, within the wide range used in these experiments, compound VI in concentrations of 1 μM or more led to a sharp decrease in growth accompanied by intense darkening of the tissue. The highest cytokinin concentrations might have permitted some increase in yield, but normal coloration was not obtained. These results suggest that high pyrazolo[4,3-*d*]pyrimidine concentrations may interfere in a system requiring adenine in some form other than cytokinin. This interpretation is supported by results of experiments which showed that exogenously supplied adenine at least doubled the range over which 2iP would effectively counteract VI (Fig. 3), but had no significant effect on the growth of callus cultured without VI or with low concentrations of VI.

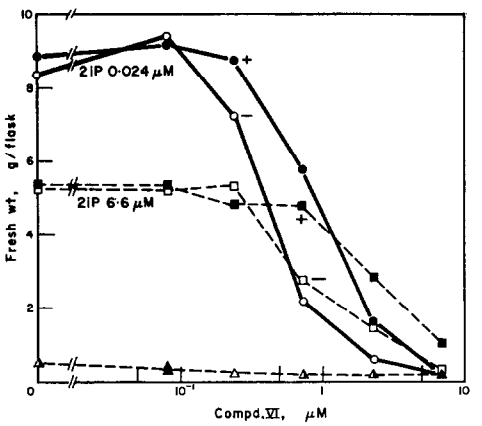

FIG. 3. YIELDS OF TOBACCO TISSUE CULTURED ON MEDIUM CONTAINING SERIAL CONCENTRATIONS OF VI IN THE ABSENCE (open symbols) AND THE PRESENCE (solid symbols) OF 20 mg/l. ADENINE; LACKING 2iP (lowest dashed line) AND WITH TWO CONCENTRATIONS OF 2iP AS INDICATED. Data from Exp. C 141, 29 April–2 June 1971 and C 147, 10 June–7 July 1971.

Antagonism Between VI and Different Cytokinins

The growth inhibitory effect of VI was counteracted not only by 2iP but also by 6-benzylaminopurine (BAP) and, surprisingly, by *N*,*N*′-diphenylurea (DPU) if used in high concen-

trations (Fig. 4, a, b and c). Using the 0·24 μM concentration of VI as a reference, it may be seen that about 12 μM DPU was required to counteract the inhibition of growth, as compared with concentrations of only 0·02–0·03 μM 2iP and BAP. Based on the average of all tests, BAP was about 1/3 and DPU about 1/500 as effective as 2iP. Thus the relative effectiveness of these compounds in counteracting the antagonist roughly parallels their effectiveness as cytokinins in promoting growth of the callus tissue in the absence of the antagonist.

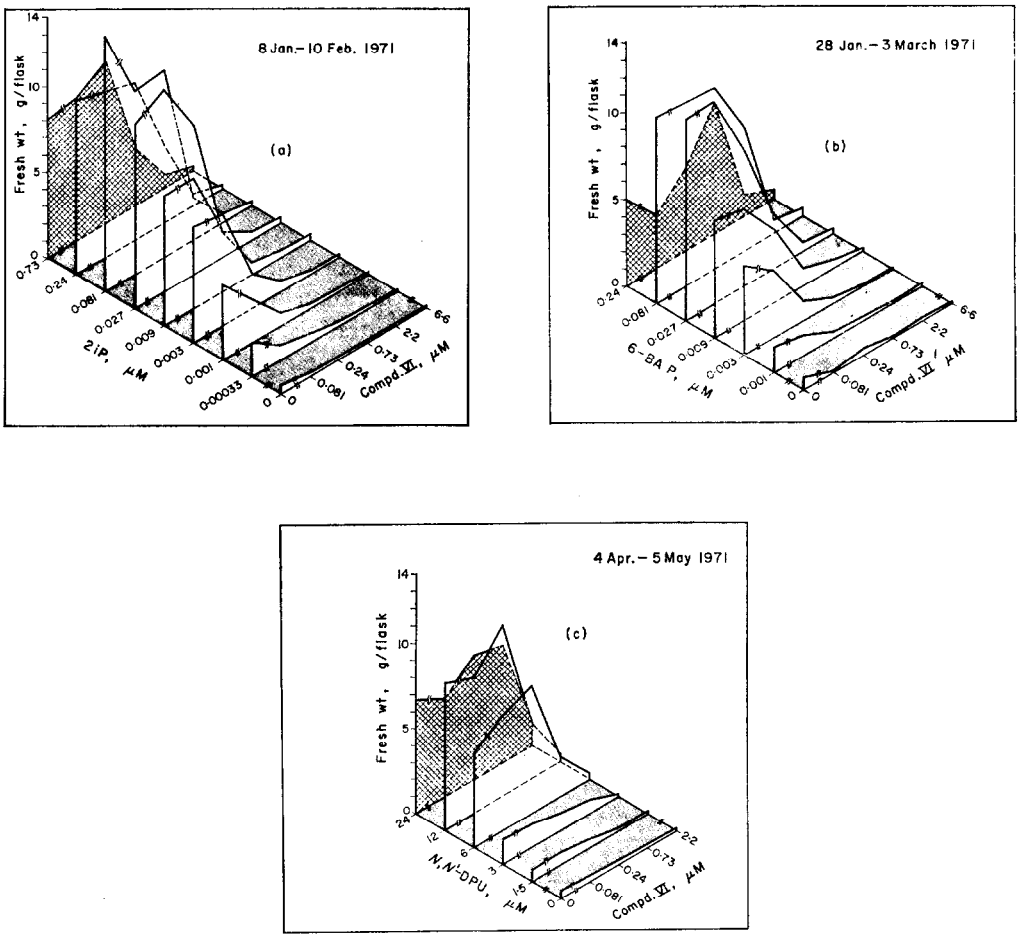

Fig. 4. Yields of tobacco tissue cultured on serial combinations of VI and either 2iP (a), BAP (b), or DPU (c).

Comparisons of Growth Inhibition by VI and Abscisic Acid

The effects of abscisic acid (ABA), a widely distributed, naturally occurring plant growth inhibitor, have been reported to be counteracted by treatment with cytokinins in the processes

of seed germination,[12,13] α-amylase production,[14] senescence,[15] opening and closing of stomata,[16] production of buds in mosses,[17] and the growth of *Lemna minor*.[18] In tests of serial combinations with 2iP, all tested concentrations of ABA (0·08–2·2 μM) affected the growth form of the tissue (as illustrated in Fig. 5 for the treatments with 0·003 μM 2iP), but only the highest concentration significantly lowered the yield of the tissue (see also Table 2, 'O' column). It should be noted that while the treatments with ABA resulted in more compact tissue, they had no influence on the viability and caused no discoloration of the tissue. When ABA was tested in serial combinations with VI in the presence of 0·003 μM 2iP, it neither increased nor counteracted the inhibitory effect of VI (Table 2).

TABLE 2. THE EFFECT OF SERIAL COMBINATIONS OF ABA AND VI ON TOBACCO CALLUS GROWTH*

ABA (μM)	VI (μM) 0	0·081	0·24	0·73	2·2
	Fr. wt (g/flask)				
0	4·3	3·0	1·5	0·09	0·05
0·027	4·3	3·9	2·1	0·07	0·04
0·081	4·4	3·3	2·0	0·11	0·05
0·24	3·0	2·7	1·7	0·10	0·05
0·73	3·8	3·7	1·1	0·25	0·06
2·2	1·7	1·5	1·6	0·14	0·05

* Experiment C 143, 13 May–18 June 1971. The cytokinin supplied in the medium was 0·003 μM 2iP.

These results suggest that the anticytokinin action of the 3-methylpyrazolo[4,3-*d*]-pyrimidines on tobacco tissue is both more drastic and more specific than that of ABA. Furthermore, there was no suggestion of synergism nor interaction between the two inhibitors when both were present (Table 2). The compact callus produced in the presence of ABA suggests that cell expansion rather than cell division was curtailed, while the large watery cells produced in the presence of VI indicates that primarily cell division rather than cell enlargement had become limiting.

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[15] B. I. S. SRIVASTAVA, *Biochim. Biophys. Acta* **169**, 534 (1968).
[16] M. TAL, D. IMBER and C. ITAI, *Plant Physiol.* **46**, 367 (1970).
[17] L. R. G. VALADON and R. S. MUMMERY, *Physiol Plant.* **24**, 232 (1971).
[18] J. VAN OVERBEEK, J. E. LOEFFLER and M. I. R. MASON, in *Biochemistry and Physiology of Plant Growth Substances* (edited by F. WIGHTMAN and G. SETTERFIELD), pp. 1593–1607, Runge Press, Ottawa (1968).

Effect of VI on Budding

The effect of VI in serial combinations with 2iP was also tested on callus cultures grown on medium with high cytokinin/auxin ratios suitable for the induction of bud formation (see Fig. 6). From this figure and the summary of data plotted in Fig. 7 (a and b), it is clear that

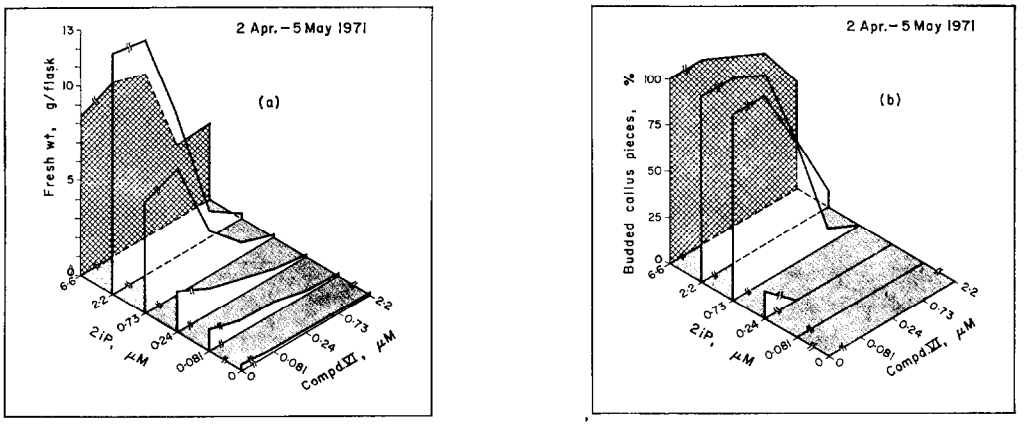

FIG. 7. EFFECT OF SERIAL COMBINATIONS OF VI AND 2iP ON THE YIELD (a) AND ON BUD FORMATION (b) OF TOBACCO TISSUES.
Note that a high cytokinin/auxin ratio was used to promote budding, as explained in legend to Fig. 6.

the influence of the treatments on budding roughly paralleled the effect on growth. The major portion of the fresh wts, however, represents leaf and stem tissue, so that these data do not permit a distinction to be made between the effects of the antagonist on the process of budding and on the growth of the callus tissue.

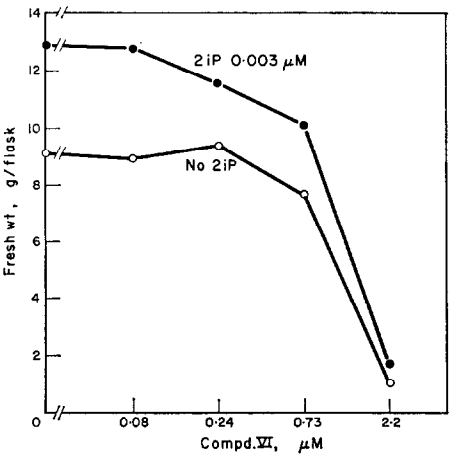

FIG. 8. YIELDS OF A CYTOKININ-AUTONOMOUS STRAIN OF TOBACCO TISSUE CULTURED ON SERIAL CONCENTRATIONS OF VI IN THE PRESENCE AND ABSENCE OF 2iP AS INDICATED. CURVES BASED ON THE AVERAGE VALUES OF FOUR EXPERIMENTS CONDUCTED BETWEEN FEBRUARY AND DECEMBER 1971.

Antagonist Inhibition of Growth in a Cytokinin-Autonomous Strain

The response to VI by a strain of tobacco callus, which has a detectable endogenous cytokinin content (J. W. Einset, unpublished) and is capable of sustained growth without exogenous cytokinin, is shown in Fig. 8. It is clear that the antagonist inhibited growth in the same manner in this strain as in the cytokinin-dependent strain, but as expected in this case somewhat lower added cytokinin concentrations were effective in counteracting the antagonist.

Miscellaneous Tests

A survey has been made of the effects of VI on a variety of plant materials including seeds, seedlings, cuttings and leaf discs. VI did not affect the percentage of germination, but in concentrations of 180 and 360 μM it markedly reduced the initiation and development of roots in wheat and radish seedlings and in *Coleus* cuttings. The same concentrations of 2iP or BAP, however, had comparable effects, so the results cannot be interpreted as due to a specific anticytokinin effect. One difference that was noted between cytokinin and antagonist treatments was in the color of the radish cotyledons, which were yellowish-green in treatments with VI alone, but were dark green in treatments with 2iP or in treatments with VI and 2iP combined. Essentially the same results were obtained in one experiment in which the more active antagonist, V, was used.

Tomato seedlings were highly sensitive to VI and to cytokinins when these were supplied in the nutrient medium in concentrations of from 12 to 120 μM, and the injury (severe wilting followed by death) was not prevented by various combinations of cytokinin and antagonist. Young tomato and tobacco plants sprayed with aqueous solutions of VI in concentrations up to 360 μM generally showed no adverse effects. In one case there was an indication that yellowing of the lower leaves, typical of senescence, was enhanced, but the effect was not striking. The use of carbowax (10%) as a carrier did not increase the effectiveness of VI in these experiments, although it is known to increase the effectiveness of cytokinins applied to intact plants for the release of apical dominance, etc.

Discs excised from mature leaves of sweet corn and various dicotyledonous plants were placed on moist filter paper or floated on solutions of VI alone or of VI in combination with 6–60 μM BAP. The antagonist occasionally enhanced senescence but no consistent effect nor indication of counteraction by BAP was obtained.

DISCUSSION

Two lines of evidence suggest that the active 3-methylpyrazolo[4,3-*d*]pyrimidine derivatives may be specific competitive inhibitors of the cytokinins. Callus growth was dependent on the cytokinin/antagonist ratio over a wide concentration range, and where cytokinin was supra-optimal the addition of antagonist resulted in promotion rather than inhibition of growth. Secondly, the substituents in the 7-position of the most active antagonists and in the corresponding 6-position of the most active cytokinins are similar with regard to size and structure. The only striking difference is that the straight, saturated *n*-pentyl group confers the highest antagonist activity while the branched, unsaturated *i*-pentenyl group confers the highest cytokinin activity.

The fact that DPU is effective in counteracting the antagonist VI might be viewed as evidence against competitive interaction. However, Australian workers who have investigated

the activity of a series of substituted phenylureas[19] have concluded that these apparently exert their growth promoting effects at the same site as do the N^6-substituted adenine derivatives.[20] Similarities in the overall structure of DPU and the active N^6-adenine derivatives have been noted.[21,22] It has been found that phenylureidopurines, which combine features of both the urea and the purine-type cytokinins, have activities intermediate between the two. On the other hand comparison of the action of DPU and the phenylureidopurines with that of isopentenyladenine and related derivatives on budding of *Funaria* suggests that the mechanism of action of the two types may be somewhat different.[23] Dyson *et al.*[24] report that soybean tissue, unlike tobacco tissue, cannot utilize DPU as a cytokinin. Furthermore, DPU was not metabolized by the soybean tissue, whereas a number of metabolites were extractable from tobacco tissue which had been in contact with media containing DPU. This problem would be resolved if it could be shown that in some manner the active phenylurea derivatives may serve as precursors for active adenine derivatives. Ureidopurines are known to occur in *t*RNA[25–28] but their derivation from urea has not been established, nor have these naturally occurring ureidopurines been shown to act as cytokinins.

The results obtained from a comparison of the effects of the 3-methylpyrazolo[4,3-*d*]-pyrimidines with ABA are further evidence of a highly specific interaction of the former with cytokinins. Preliminary tests with gibberellic acid (GA_3) have shown that under conditions where it promotes growth of the callus tissue it does not counteract the inhibiting effect of VI.

The beneficial effect of adenine in extending the range of concentrations over which the effect of VI was counteracted by cytokinins suggests that at high concentrations the antagonists may interfere more generally in purine metabolism. The greater resistance of the cytokinin autonomous strain to inhibition by VI and the inhibition of budding by VI are also considered as evidence that the 7-substituted-3-methylpyrazolo[4,3-*d*]pyrimidines inhibit processes promoted by cytokinins.

The tobacco bioassay has thus far proved to be the most effective system for the measurement of cytokinin-antagonist interaction. The limited ability of VI to exhibit cytokinin-antagonist action in senescence tests and in tests of germination and seedling growth may be due in part to technical shortcomings in these preliminary experiments.

Among the striking effects of modifications in the side chain in the 7-position of the 3-methylpyrazolo[4,3-*d*]pyrimidines are the three-fold increase in antagonist activity brought about by substituting the *n*-pentyl for the *i*-pentyl moiety and the practically complete removal of activity by cyclization of the chain or from insertion of a second chain (as in the case of the dibutyl derivative). The most spectacular effect of any single structural modifica-

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tion resulted from the formal saturation of the double bond in XIII, which converted it from a substance with definite growth promoting properties (of an apparently essential kind) into a highly lethal agent.

The relationships between structure of the side chain in the 7-position and cytokinin antagonist activity suggest a very specific receptor site for this part of the molecule in some growth regulatory system. On the other hand the simultaneous requirement for the 3-methyl group for any modification in the side chain of the 7-position to be effective in conferring antagonist activity emphasizes the importance of the overall structure. Further study of the structural properties of effective cytokinins and antagonists should provide new insight into this growth regulatory system and its role in plant development.

EXPERIMENTAL

Synthesis of test substances. 3-*Methyl*-7-(3-*methylbutylamino*)*pyrazolo*[4,3-d]*pyrimidine* (VI). This synthesis has already been reported.[10]

7-(2-*Hydroxyethylamino*)-3-*methylpyrazolo*[4,3-d]*pyrimidine* (II). To 83 mg (0·46 mmol) of I was added 5 ml of ethanolamine. The solution was heated at reflux under N_2 for 3 hr and the cooled solution concentrated under diminished pressure. The residue was purified by chromatography on 25 g of silica gel, elution with 3:1 EtOAc–EtOH, which afforded a white solid. Recrystallization from EtOH gave white crystals of II, yield 36 mg (41%), m.p. 232–233°: $C_8H_{11}N_5O$ (M^+ calc. 193·0964; found 193·097); λ_{max}^{EtOH} (pH 1) 310 nm (ϵ 12 000) and 262 (6200), λ_{min} 280 (5100) and 226 (2600); λ_{max}^{EtOH} (pH 7) 319 (sh), 306 (9300), 295 (11 800), and 237 (6000), λ_{min} 304 (9100), 256 (3600) and 223 (3400); λ_{max}^{EtOH} (pH 12) 308 (7300), 264 (sh) and 243 (15 300), λ_{min} 281 (3600) and 228 (8600); MS: *m/e* 193·097 (M^+), 176·091, 174·077, 162·079 and 149·070.

7-(n-*Butylamino*-3-*methylpyrazolo*[4,3-d]*pyrimidine* (III). To 150 mg (0·83 mmol) of I was added 5 ml of *n*-butylamine. The solution was heated at reflux under N_2 for 48 hr. The cooled solution was concentrated under diminished pressure and the residue purified by chromatography on 15 g of Sephadex LH-20, elution with H_2O. The product was crystallized from EtOAc to afford white cyrstals of III, yield 96 mg (56%), m.p. 174·5–175·5°: $C_{10}H_{15}N_5$ (M^+ calc. 205·1312; found 205·132); λ_{max}^{EtOH} (pH 1) 310 nm (ϵ 12 400) and 261 (5900), λ_{min} 280 (4600) and 227 (3100); λ_{max}^{EtOH} (pH 7) 318 (sh), 306 (9400), 295 (11 700) and 236 (5900), λ_{min} 304 (9100), 257 (3300) and 225 (4400); λ_{max}^{EtOH} (pH 12) 308 (7600), 264 (sh) and 244 (14 900), λ_{min} 281 (3600) and 227 (9500); MS: *m/e* 205 (M^+), 189, 176, 162, 149.

3-*Methyl*-7-(2-*methylpropylamino*)*pyrazolo*[4,3-d]*pyrimidine* (IV). To 150 mg (0·83 mmol) of I was added 5 ml of 3-methyl-2-propylamine. The solution was heated at reflux for 48 hr. The cooled solution was concentrated under diminished pressure and the residue was purified by chromatography on 15 g of Sephadex LH-20, elution with H_2O. The appropriate fractions were pooled and concentrated and the product was crystallized from Et_2O–light petrol. to afford white crystals of IV, yield 71 mg (42%), m.p. 195·5–196·0°; λ_{max}^{EtOH} (pH 1) 311 nm (ϵ 12 500) and 263 (6000), λ_{min} 281 (4700) and 227 (3100); λ_{max}^{EtOH} (pH 7) 318 (sh), 306 (9500), 295 (12 000) and 236 (6000), λ_{min} 303 (9200), 256 (3500) and 225 (4400); λ_{max}^{EtOH} (pH 12) 308 (7500), 264 (sh) and 243 (14 700), λ_{min} 281 (3500) and 228 (8800). (Found: C, 58·50; H, 7·31; N, 34·14. Calc. for $C_{10}H_{15}N_5$: C, 58·52; H, 7·37; N, 34·12%.)

3-*Methyl*-7-n-*pentylaminopyrazolo*[4,3-d]*pyrimidine* (V). To 1·0 g (5·6 mmol) of I was added 10 ml of *n*-pentylamine. The solution was heated at reflux under N_2 for 10 hr and the cooled solution was concentrated under diminished pressure. The residue was purified by chromatography over 30 g of Sephadex LH-20, elution with a H_2O–EtOH gradient. The solid product was recrystallized from Et_2O to afford white crystals of V, yield 808 mg (67%), m.p. 155–156°; $C_{11}H_{17}N_5$ (M^+ calc. 219·1492; found 219·148); λ_{max}^{EtOH} (pH 1) 311 nm (ϵ 13 100) and 263 (6600), λ_{min} 280 (5200) and 226 (2400); λ_{max}^{EtOH} (pH 7) 319 (sh), 307 (10,100), 296 (12 700) and 238 (6200), λ_{min} 304 (9900), 258 (3900) and 224 (3500); λ_{max}^{EtOH} (pH 12) 308 (8100), 265 (sh) and 245 (15 400), λ_{min} 281 (4000) and 229 (9200); MS: *m/e* 219·148 (M^+), 190·110, 176·095, 162·079, 149·073.

7-*Cyclopentylamino*-3-*methylpyrazolo*[4,3-d]pyrimidine (VII). To 150 mg (0·83 mmol) of I was added 5 ml of cyclopentylamine. The solution was heated at reflux under N_2 for 24 hr. The cooled solution was concentrated under diminished pressure and purified by chromatography on 15 g of Sephadex LH-20, elution with H_2O. The product was crystallized from EtOAc to afford white crystals of VII, yield 151 mg (84%), m.p. 201–203°; $C_{11}H_{15}N_5$ (M^+ calc. 217·1327; found 217·133); λ_{max}^{EtOH} (pH 1) 312 nm (14 200) and 263 (6600), λ_{min} 280 (5900) and 227 (2600); λ_{max}^{EtOH} (pH 7) 320 (sh), 308 (10 400), 297 (13 500) and 239 (6100), λ_{min} 305 (10 200), 258 (4100) and 224 (3200); λ_{max}^{EtOH} (pH 12) 310 (8500), 266 (sh) and 246 (15 000), λ_{min} 282 (4200) and 229 (8600); MS: *m/e* 217·133 (M^+), 188·094, 174·078, 149·071.

7-n-*Hexylamino*-3-*methylpyrazolo*[4,3-d]*pyrimidine* (VIII). To 150 mg (0·83 mmol) of I was added 5 ml of *n*-hexylamine. The solution was heated at reflux under N_2 for 6 hr. The cooled solution was concentrated under diminished pressure and the residue was purified by chromatography on 15 g of Sephadex LH-20,

elution with H_2O. The appropriate fractions were combined and evaporated to dryness. The solid product was recrystallized from Et_2O to afford white crystals of VIII, yield 73 mg (38%), m.p. 148–149°; λ_{max}^{EtOH} (pH 1) 310 nm (ε 11 700) and 262 (5500), λ_{min} 280 (4300) and 227 (1900); λ_{max}^{EtOH} (pH 7) 318 (sh), 307 (8900), 295 (11 200) and 237 (5100), λ_{min} 304 (8800), 256 (3000) and 225 (3100); λ_{max}^{EtOH} (pH 12) 308 (7300), 263 (sh) and 244 (13 700), λ_{min} 281 (3600) and 229 (8100). (Found: C, 61·72; H, 8·19. Calc. for $C_{12}H_{19}N_5$: C, 61·78; H, 8·21%.)

7-Cyclohexylamino-3-methylpyrazolo[4,3-d]*pyrimidine* (IX). To 125 mg (0·69 mmol) of I was added 5 ml of cyclohexylamine. The solution was heated at reflux under N_2 for 28 hr and the cooled solution was concentrated under diminished pressure. The residue was purified by chromatography on 15 g of Sephadex LH-20, elution with H_2O. Crystallization from Et_2O–light petrol. afforded white crystals of IX, yield 54 mg (34%), m.p. 195·5–196·5°: $C_{12}H_{17}N_5$ (M^+ calc. 231·1483: found 231·150); λ_{max}^{EtOH} (pH 1) 311 nm (ε 13 000) and 262 (6200), λ_{min} 280 (5400) and 226 (2300); λ_{max}^{EtOH} (pH 7) 319 (sh), 307 (9500), 296 (12 400) and 238 (5700), λ_{min} 304 (9300), 257 (3900) and 224 (3100); λ_{max}^{EtOH} (pH 12) 308 (8000), 264 (sh) and 245 (14 300), λ_{min} 281 (4000) and 228 (8200); MS: *m/e* 231 (M^+), 203, 188, 174, 149.

7-n-Heptylamino-3-methylpyrazolo[4,3-d]*pyrimidine* (X). To 150 mg (0·83 mmol) of I was added 5 ml of *n*-heptylamine. The solution was heated at reflux under N_2 for 6 hr. The cooled solution was concentrated under diminished pressure and the residue was purified by chromatography on 15 g of Sephadex LH-20, elution with H_2O. The appropriate fractions were combined and evaporated to dryness and the solid residue was crystallized from EtOAc to afford white crystals of X, yield 63 mg (31%), m.p. 146–147°: $C_{13}H_{21}N_5$ (M^+ calc. 247·1797; found 247·178); λ_{max}^{EtOH} (pH 1) 311 nm (ε 12 900) and 262 (5800), λ_{min} 281 (4500) and 227 (2600); λ_{max}^{EtOH} (pH 7) 318 (sh), 306 (9800), 296 (12 100) and 237 (5800), λ_{min} 303 (9500), 256 (3200) and 226 (4300); λ_{max}^{EtOH} (pH 12) 309 (8500), 263 (sh) and 224 (16 100), λ_{min} 281 (3900) and 228 (10 600); MS: *m/e* 247 (M^+), 231, 218, 204, 190, 176, 162, 149.

7-(Di-n-butylamino)-3-methylpyrazolo[4,3-d]*pyrimidine* (XI). To 150 mg (0·83 mmol) of I was added 5 ml of di-*n*-butylamine. The solution was heated at reflux for 48 hr. The cooled solution was concentrated under diminished pressure and the residue was purified by chromatography on 15 g of Sephadex LH-20, elution with H_2O and then with 25% EtOH. The appropriate fractions were combined and concentrated to afford an off-white solid. This solid was crystallized from ether to afford white crystals of XI, yield 94 mg (44%), m.p. 176·5–178·0°: $C_{14}H_{23}N_5$ (M^+ calc. 261·1962; found 261·195); λ_{max}^{EtOH} (pH 1) 335 nm (sh), 323 (ε 16 200) and 265 (5100), λ_{min} 286 (2800) and 232 (1700); λ_{max}^{EtOH} (pH 7) 330 (10 800), 316 (16 000), 305 (sh) and 245 (3500), λ_{min} 326 (10 100), 269 (1400) and 232 (2100); λ_{max}^{EtOH} (pH 12) 317 (10 000) and 252 (11 000), λ_{min} 388 (3700) and 235 (6800); MS: *m/e* 261 (M^+), 246, 232, 218, 204, 189, 176, 162, 149.

7-n-Decylamino-3-methylpyrazolo[4,3-d]*pyrimidine* (XII). To 150 mg (0·83 mmol) of I was added 5 ml of *n*-decylamine. The solution was heated at reflux under N_2 for 3 hr. The cooled solution was concentrated under diminished pressure and the residue was purified by chromatography on 15 g of Sephadex LH-20, elution with 10% EtOH to afford the amine, and then with 50% EtOH to elute the desired product. The appropriate fractions were concentrated and the solid product was recrystallized from ether to afford white crystals of XII, yield 63 mg (26%), m.p. 113·5–116°; $C_{16}H_{27}N_5$ (M^+ calc. 289·2266; found 289·227); λ_{max}^{EtOH} (pH 1) 310 nm (10 800) and 261 (5500), λ_{min} 280 (4300) and 226 (3100); λ_{max}^{EtOH} (pH 7) 318 nm (sh), 307 (8700), 295 (10 800) and 237 (5500), λ_{min} 304 (8500), 255 (3400) and 225 (4000); λ_{max}^{EtOH} (pH 12) 307 nm (7100), 263 (sh) and 243 (13 300), λ_{min} 280 (4000) and 229 (8200); MS: *m/e* 289·227 (M^+) 260·188, 246·172, 232·156 218·139, 204·125, 190·108, 176·095, 163·086, 162·078, 149·070, 133·051.

Tobacco bioassay. Antagonist activity was measured principally in terms of fresh weight yields of cytokinin-dependent callus tissue derived from *Nicotiana tabacum* var. Wisconsin No. 38 and cultured on medium supplemented with known quantities of cytokinins. The stock tissue consisted of callus originally derived from pith tissue and maintained on medium supplemented with 1·4 μM kinetin. Prior to use in experiments the stock tissue was cultured for two 3-week passages on medium supplemented with only 0·14 μM kinetin.

The medium contained the mineral salts and organic compounds specified by Linsmaier and Skoog[29] (Table 6, parts a and b). 3-Indoleacetic acid was dissolved in H_2O and added to the media before autoclaving, to make the final concentration 11·5 μM. In experiments with a constant (0·003 μM) concentration of 2iP this substance also was added in aq. solution before autoclaving. The compounds to be tested for antagonist activity (and cytokinins in experiments in which serial concentrations were used) were dissolved in dimethylsulfoxide (Me_2SO) in 3-fold serial dilutions and were added to the cooling autoclaved agar medium at the uniform rate of 0·05% (v/v). The final concentration of Me_2SO in the medium thus was 0·05% when the cytokinin concentration was held constant, and 0·1% when it was varied. These concentrations of Me_2SO do not affect the growth of the tissues.[30] This procedure was used to avoid possible degradation of the compounds by heat.

[29] E. M. LINSMAIER and F. SKOOG, *Physiol. Plant.* **18**, 100 (1965).
[30] R. Y. SCHMITZ and F. SKOOG, *Plant Physiol.* **45**, 537 (1970).

For each treatment, twelve pieces of callus (*ca.* 40 mg. each) were planted in lots of three in 125 ml Erlenmeyer flasks containing 50 ml medium, and cultured for a 5-week period at 28°. The tissue received only occasional exposure to light to permit observation during the culture period. In addition to records of fresh wt yields, notes were made on growth form, color, viability, and ability of the callus pieces to form buds under suitable conditions. Miscellaneous tests with other plant materials have been described in connection with the results.