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Cytokinins: Development of a Potent Antagonist

(tobacco callus/adenine analogues)

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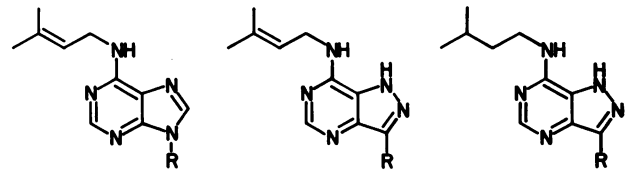
ABSTRACT **A systematic search has resulted in the synthesis of a class of cytokinin antimetabolites. The development and biological properties of the anticytokinins are discussed in terms of one member of the class, 3-methyl-7-(3-methylbutylamino)pyrazolo[4,3-*d*]-pyrimidine.**

The distribution of cytokinins (factors promoting cell division and growth) in natural systems is widespread (1, 2) and includes compounds at the purine, ribonucleoside, ribonucleotide, and tRNA levels (3, 4). Plants presumably require cytokinins for growth, because growth of excised plant tissues is often dependent on an exogenous cytokinin supply. One cytokinin, 6-(3-methyl-2-butenylamino)purine (**1**) has been shown to be the active factor in the plant pathogen *Corynebacterium fascians* (5). In addition, phytohemagglutinin-stimulated human lymphocyte cells are both stimulated and inhibited by 6-(3-methyl-2-butenylamino)-9-β-D-ribofuranosylpurine (**2**) with respect to DNA synthesis, transformation, and mitosis (6). The effect depended upon the stage of the cell cycle and the concentration of **2** employed. Compound **2** has antitumor activity in experimental animals (7, 8) and in preliminary clinical trials (9). In spite of this broad range of physiological activity and the occurrence of several different cytokinins in nature (1), including four as components of tRNA (10), as well as hundreds of synthetic cytokinins (1, 2), only one example of cytokinin antagonism has been reported in a compound that bears an obvious structural relationship to the known cytokinins (5). Although the structural relationship between this compound (6-methylaminopurine) and the cytokinins suggests its potential utility in studies of the mechanism of cytokinin action, it would probably not be suitable, since it has a very slight activity as an antagonist. To date, no systematic search for compounds that would oppose the action of the cytokinins has been reported.

The synthesis of a potent cytokinin antagonist is of considerable interest, since it could extend the study of cytokinins to biological systems that do not require any exogenously added cytokinins, presumably because they produce their own. The antagonist that blocks the action of the endogenous cytokinins in these systems would thus make the tissue cytokinin-dependent. The antagonist would be of greatest utility, of course, if it acted in a reversible manner on the same pathway through which the cytokinin itself exerts its effect. We wish to report the discovery of a series of such compounds, the activity of which is exemplified here by one member of that series, 3-methyl-7-(3-methylbutylamino)pyrazolo[4,3-*d*]pyrimidine (**6**).



MATERIALS AND METHODS

The syntheses of the four compounds, **3–6**, in the substituted-pyrazolo[4,3-*d*]pyrimidine series have been reported (11). The cytokinin activities of these compounds were determined in the tobacco bioassay (12). In order to detect anticytokinin activity, the growth of tobacco callus was observed on a standard medium to which either 6-(3-methyl-2-butenylamino)purine or 6-benzylaminopurine had been added (over a range of concentrations) and to which the other compounds (**3–6**) were also added in different concentrations.

RESULTS AND DISCUSSION

The combined effects of cytokinin and antagonist are illustrated in Figs. 1 and 2. The cytokinin antagonist was designed with the same approximation inherent in the design of any competitive inhibitor, namely, that the modified compound be sufficiently similar to the normal metabolite (cytokinin) in structure to allow participation in the same type of "receptor complexes", but that the modification render it ineffective as a cytokinin. Empirically, then, such a compound would be structurally related to the most active cytokinins, such as **1**, but would be inactive as a cytokinin itself. It was reported (1, 12) that modifications in the heterocyclic (adenine) moiety of cytokinins drastically lower cytokinin activity, which suggests that alkylated hetero-cycle structurally related to **1** might be useful as inhibitors of cytokinin activity. In support of this approach, it has also been reported that heterocyclic modifications of adenine created inhibitors of adenine utilization in other senses. For example, 4-hydroxypyrazolo[4,3-*d*]pyrimi-

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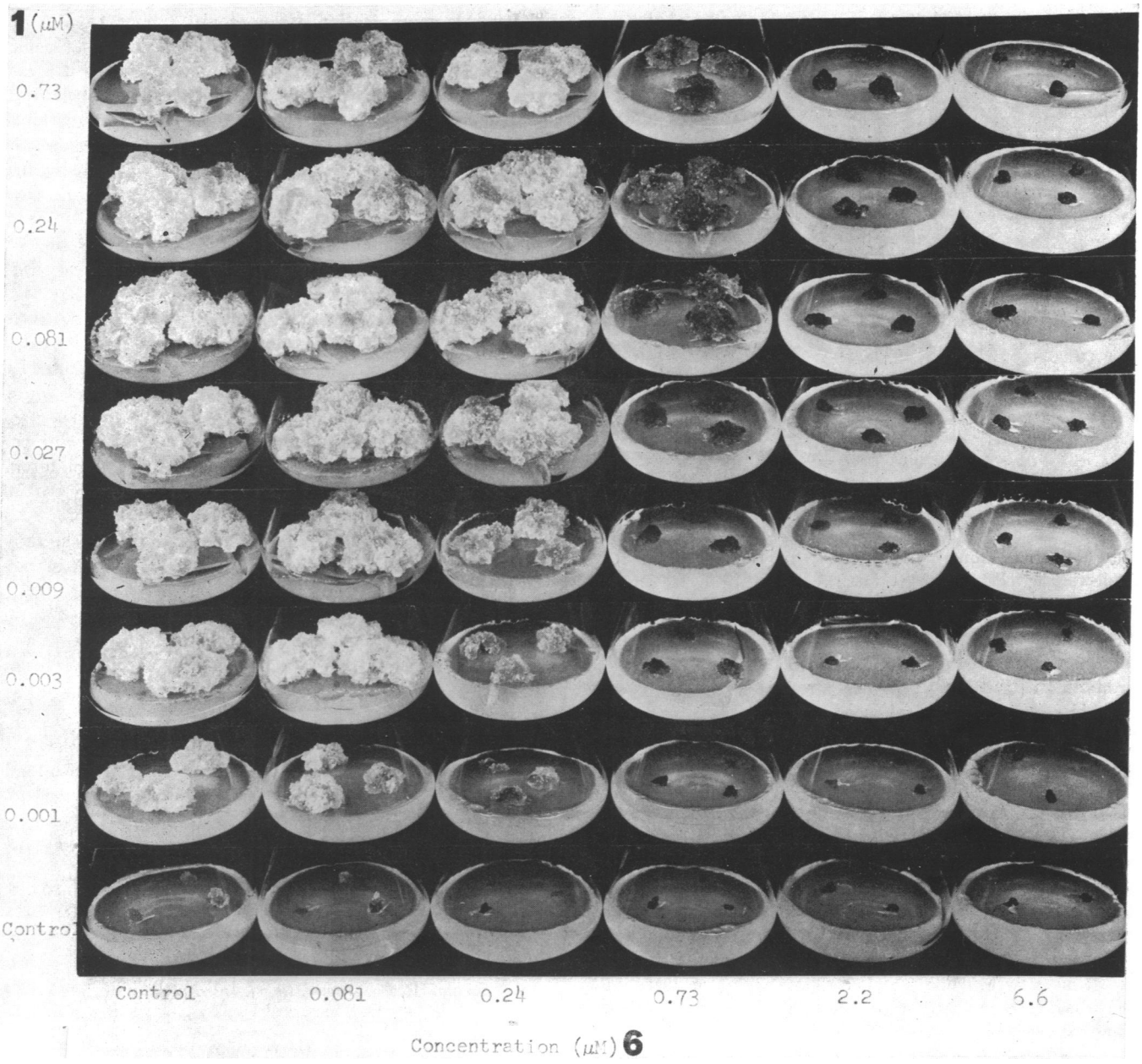

FIG. 1. Effect of various concentrations of cytokinin [6-(3-methyl-2-butenylamino)purine (**1**)] and cytokinin antagonist [3-methyl-7-(3-methylbutylamino)pyrazolo[4,3-*d*]pyrimidine (**6**)] on the growth of tobacco callus.

dine is a potent inhibitor of xanthine oxidase, which utilizes the isomeric hypoxanthine as a substrate (13), and formycin (7-amino-3-β-D-ribofuranosylpyrazolo[4,3-*d*]pyrimidine) triphosphate competes with the isomeric ATP for incorporation into polyribonucleotides by DNA-dependent RNA polymerase (14). Heterocyclic modifications of 6-(3-methyl-2-butenylamino)purine (**1**) were, therefore, considered for potential cytokinin antagonism.

In particular, four compounds in the substituted-pyrazolo-[4,3-*d*]pyrimidine series (**3**–**6**) were synthesized. These compounds were prepared by treatment of the intermediate (3-methyl-)7-methylthiopyrazolo[4,3-*d*]pyrimidine with the appropriate amine at reflux temperature under nitrogen. Details of the syntheses and characterizations were reported (11). It seemed reasonable to expect that if the structure–activity results for the purine series were valid for the pyrazolo[4,3-*d*]pyrimidine series as well, then activity should be considerably lower in those compounds with the isopentyl (**5**, **6**) rather than the isopentenyl (**3**, **4**) side chain, and somewhat lower in those compounds that contained the additional methyl substituent on C-3 (**4**, **6**). The relative order of cytokinin activities was expected to be **3** > **4** > **5** > **6**. We also anticipated that as cytokinin activity diminished, *i.e.*, as the compounds in the series became increasingly poorer in their ability to function as metabolites, the amount of potential antimetabolite activity would increase or decrease in parallel, depending upon whether the diminished cytokinin activity resulted from lowered function of the "active" moiety or from lowered yield of the "active" moiety.

The conjecture concerning the diminution of cytokinin activity was correct. Although all of the compounds were much less active than the corresponding adenine derivatives, they did show the same serial order of activity among themselves, with **3** being the most active and **4** and **5** being suc-

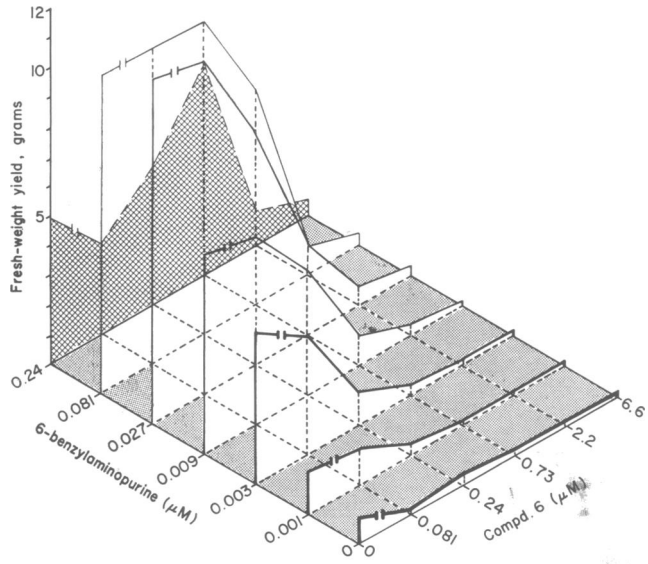

FIG. 2. Effect of various concentrations of cytokinin (6-benzylaminopurine) (BAP) and cytokinin antagonist [3-methyl-7-(3-methylbutylamino)pyrazolo[4,3-*d*]pyrimidine (**6**)] on the fresh-weight yield of tobacco callus.

cessively less active (11). Compound **6**, which was anticipated to be the least active cytokinin, was in fact without growth-promoting activity and exhibited strong activity as an anti-cytokinin in appropriate tobacco bioassays. Thus, compound **6** caused complete inhibition of growth of tobacco callus supplied with optimal concentrations of either 6-(3-methyl-2-butenylamino)purine or 6-benzylaminopurine, when it was added to the tissue in 100- to 200-fold excess over the cytokinins. Fig. 1 depicts cultures growing on media containing various concentrations of cytokinin [6-(3-methyl-2-butenylamino)purine (**1**)] and antagonist (compound **6**). It is clear that at any given concentration at which the antagonist has an effect intermediate between no inhibition and total inhibition, a higher concentration of cytokinin has a restorative effect on growth, while a higher concentration of antagonist causes a further reduction in growth. This implies a competition between the cytokinin and antagonist for the "receptor sites" associated with the promotion of cell division and growth.

An additional effect is apparent from the graph of fresh-weight yields corresponding to the cultures (Fig. 2). At the highest concentration of 6-benzylaminopurine (0.24 μM), where the cytokinin itself is becoming inhibitory, the addition of antagonist offsets the excess cytokinin and enhances growth, as though a smaller, "effective" amount of cytokinin was being used. Thus, the antagonist is not merely toxic to the plant tissue, but competes with the cytokinin in a reversible manner.

The interaction is more complicated than this, however, as one may observe from the contour map in Fig. 2, which indicates that while there is a very definite competition between the cytokinin and inhibitor at moderate concentrations of each, higher concentrations of cytokinin can only offset the effect of increased antagonist concentration to a certain point. Above this concentration of antagonist (about 1 μM), no amount of cytokinin will entirely prevent inhibition of tissue growth. This effect is also apparent in Fig. 1, where it is associated with discoloration of the callus at high concentrations of antagonist. A strain of tobacco callus that grows without exogenously added cytokinin and has cytokinin activity (J. Einset, University of Wisconsin) was also inhibited by the antagonist at close to 1 μM. Strong evidence is thus provided that this tissue uses endogenous cytokinin. As in the case of the cytokinin-dependent tissue, when the concentration of the antagonist **6** required for effective competition is exceeded, the toxicity effect becomes apparent.

The effect of compound **6** on intact plants and seedlings has also been tested. The germination and growth in the early seedling stages of tomato, wheat, and radish were inhibited by the cytokinin antagonist **6**, but *Coleus* cuttings and young tomato and tobacco plants were affected only by very high concentrations. The addition of exogenous cytokinin with the antagonist in the seedling tests possibly offset the inhibitory action of the antagonist to some extent, in the early part of the growing period, but it did not reverse the inhibition.

3-Methyl-7-(3-methylbutylamino)pyrazolo[4,3-*d*]pyrimidine (**6**) is the first recognized example of a close structural analog of known cytokinins that is itself a potent anticytokinin. A description of other cytokinin antagonists and the structure–activity relationships relevant to synthesis of more potent examples of this type of compound will be reported elsewhere.

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