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# INHIBITORS OF TWO ENZYMES WHICH METABOLIZE CYTOKININS*

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**Key Word Index**—Cytokinins; metabolism; cytokinin 7-glucosyltransferase; β-(9-cytokinin)alanine synthase; $N^6$-substituted adenines; auxins; indoleacetic acid.

**Abstract**—Compounds which inhibit the natural metabolic inactivation of cytokinins are of considerable physiological significance. In this study, inhibitors have been found for two enzymes which form glucose and alanine conjugates of cytokinin bases, namely, cytokinin 7-glucosyltransferase and β-(9-cytokinin)alanine synthase. The most effective inhibitors found for the former enzyme were the cytokinin analogues 3-methyl-7-*n*-pentylaminopyrazolo[4,3-*d*]pyrimidine, which acted competitively ($K_i$ 22 μM), and the diaminopurine, 6-benzylamino-2-(2-hydroxyethylamino)-9-methylpurine ($K_i$ 3.3 μM). However these compounds were ineffective as inhibitors of the cytokinin-alanine synthase which was inhibited competitively by IAA ($K_i$ 70 μM) and related compounds, especially 5,7-dichloro-IAA ($K_i$ 0.4 μM). Certain urea derivatives were moderately effective inhibitors of the enzymes ($K_i$ *ca* 100 μM).

## INTRODUCTION

One technique for the regulation of the action of plant growth substances is to influence the synthesis or inactivation of the active compounds at the level of enzyme function. At present, four specific enzymes have been studied to some degree in relation to active cytokinin metabolism. The primary enzyme apparently responsible for biosynthesis from AMP and isopentenyl pyrophosphate has not yet been well characterized [1, 2]. The other enzymes are involved in changing biologically active molecules into inactive or weakly active forms. Cytokinin oxidase [3, 4], which cleaves the isoprene side chain from the purine ring of some natural cytokinins, is a good candidate for regulation by specific inhibitors. It was the search for specific inhibitors to block the metabolic modification of active cytokinins, and thus modify hormone action and tissue growth, that led us to further *in vitro* studies of the other two known enzymes, as discussed below.

The enzymes, cytokinin 7-glucosyltransferase (EC 2.4.1.118) and β-(9-cytokinin)alanine synthase (EC 4.2.99.13) have been partly purified from radish cotyledons and developing lupin seed respectively by Entsch *et al.* [5–7]. They have one property in common: the conversion of active cytokinin bases (e.g. zeatin, **1**) to specific conjugates which are almost inactive in some hormone bioassays [8]. The conjugates of zeatin (**2**–**4**) are natural products of metabolism [9, 10]. The glucosyltransferase forms 7- and 9-glucosides of the purine ring, but the former predominates, while the alanine synthase forms strictly 9-alanine conjugates of the purine ring. The alanine conjugate of zeatin (**4**) has been termed lupinic acid.

With very small amounts of enzyme to study, it was decided to concentrate on finding specific inhibitors which might have practical applications. From previous studies of the structural specificity of the active site of cytokinin 7-glucosyl transferase, it was possible to suggest new compounds with specific affinity for the enzyme but without appreciable cytokinin activity. These compounds, 7- or 9-methyl 2,6-diaminopurines, were synthesized and found to be excellent reversible inhibitors of cytokinin 7-glucosyltransferase. However, β-(9-cytokinin)alanine synthase was much more effectively inhibited by close structural analogues of the auxin, indol-3-ylacetic acid (IAA).

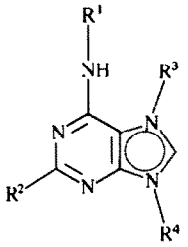
## RESULTS

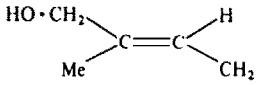
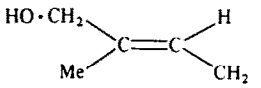
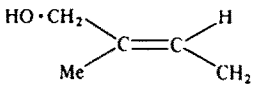
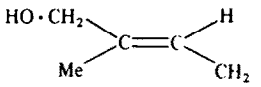
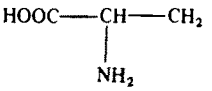
### *Kinetics of inhibition*

Cytokinin 7-glucosyltransferase requires two substrates, a cytokinin and a sugar nucleotide (UDP-glucose). Substrate analogues of the cytokinins were tested with the enzyme. There was no practical value in studying inhibitors of the binding of the sugar nucleotide, since this is a common substrate for many enzymes. All potential inhibitors were tested in assays with zeatin and UDP-glucose as substrates of the enzyme. Zeatin is the most active natural cytokinin, and has been studied extensively as a substrate [6]. Many compounds can replace zeatin in the reaction, but the enzyme is highly specific for the nucleotides, UDP- and TDP-glucose [6].

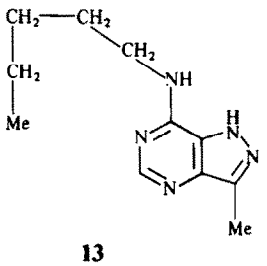
The kinetics of the enzymatic interaction of a close analogue of zeatin, 3-methyl-7-pentylaminopyrazolo[4,3-*d*]pyrimidine (**13**), were examined under standard assay conditions. Reaction rates were measured with a constant, near-saturating concentration of UDP-glucose and varying zeatin concentrations at four inhibitor concentrations. Double reciprocal plots of reaction rates against zeatin

*Part 1 in the series "Inhibitors of Cytokinin Metabolism".

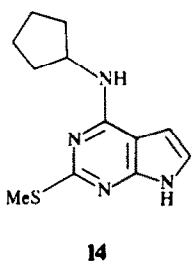


| | $R^1$ | $R^2$ | $R^3$ | $R^4$ |
|---|---|---|---|---|
| **1** |  | H | | H |
| **2** |  | H | β-D-glucopyranosyl | |
| **3** |  | H | | β-D-glucopyranosyl |
| **4** |  | H | |  |
| **5** | $C_6H_5 \cdot CH_2$ | $HO \cdot CH_2 \cdot CH_2 \cdot NH$ | Me | |
| **6** | $C_6H_5 \cdot CH_2$ | $HO \cdot CH_2 \cdot CH_2 \cdot NH$ | | Me |
| **7** | $C_6H_5 \cdot CH_2$ | H | | $HO \cdot CH_2 \cdot CH_2 \cdot CH_2$ |
| **8** | $C_6H_5 \cdot CH_2$ | Cl | Me | |
| **9** | $C_6H_5 \cdot CH_2$ | Cl | | Me |
| **10** | $HO \cdot CH_2 \cdot (CH_2)_3 \cdot CH_2$ | Cl | Me | |
| **11** | $HO \cdot CH_2 \cdot (CH_2)_3 \cdot CH_2$ | Cl | | Me |
| **12** | $HO \cdot CH_2 \cdot (CH_2)_3 \cdot CH_2$ | H | | Me |

concentration gave straight lines which intersected essentially at one point on the velocity axis (Fig. 1). The result was consistent with reversible, competitive inhibition between **13** and zeatin [11]. This was confirmed in the secondary plot by the linear relationship between the slopes of the primary plot and concentrations of **13** (the inhibitor) (Fig. 1). The $K_i$ from this plot was $2.2 \times 10^{-5}$ M (Table 1). A similar study was carried out with constant, near-saturating concentrations of zeatin and a range of concentrations of UDP-glucose at two concentrations of **13**. Reciprocal plots gave parallel lines, which indicated uncompetitive inhibition [11]. Thus, there was no interaction of **13** with the binding of UDP-glucose. Based on these results, it was assumed that other structural analogues of zeatin were also competitive inhibitors of zeatin binding. Inhibition constants were calculated from the equation for competitive inhibition [11] by the use of results from two concentrations of each inhibitor and are recorded in Table 1.



**13**



**14**

The enzyme β-(9-cytokinin)alanine synthase requires

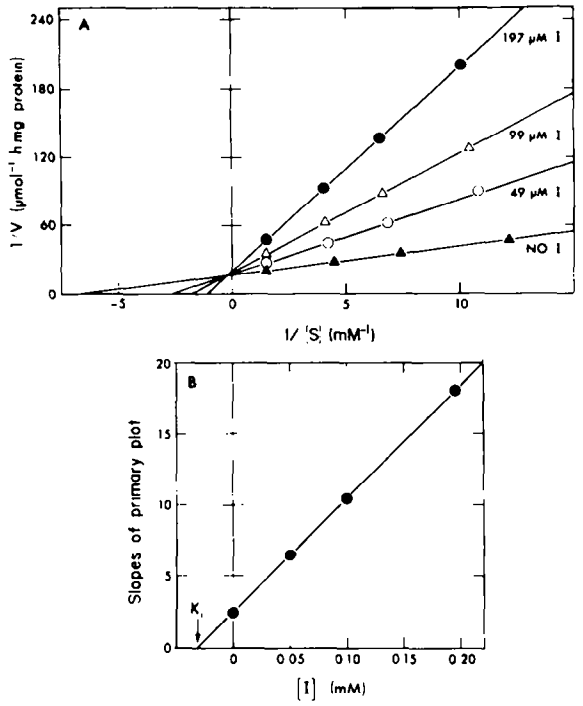



Fig. 1. (A) Lineweaver–Burk plots of data obtained from assays of cytokinin 7-glucosyltransferase in the presence and absence of the inhibitor 3-methyl-7-pentylaminopyrazolo[4,3-*d*]-pyrimidine (**13**). (B) A plot of the slopes of the Lineweaver–Burk plots against inhibitor concentration. The straight line obtained indicates linear competitive inhibition.

two substrates, a cytokinin and *O*-acetylserine. The latter compound is an unusual, unstable metabolite which is involved in crucial metabolic processes, such as cysteine synthesis [12]. Thus, no attention was paid to small molecules which may act enzymatically as direct and stable competitors of this compound. All potential inhibitors were tested with zeatin and *O*-acetylserine as substrates, since zeatin has been studied extensively with the synthase [7].

An unusual feature of β-(9-cytokinin)alanine synthase is its relatively high $K_m$ values for cytokinin analogues [7]. Preliminary studies showed that close analogues of zeatin were not tight-binding inhibitors of the enzyme, and thus were unlikely to have practical value. However, IAA which was not a substrate for the enzyme, was found to be an excellent inhibitor. This observation opened the path to a group of inhibitors. The kinetics of the enzymatic interaction with IAA were examined under standard assay conditions. Reaction rates were measured with constant near-saturating concentrations of *O*-acetylserine and varying concentrations of zeatin, at four concentrations of inhibitor. The results were very similar to those obtained with the glucosyltransferase (Fig. 2). That is, IAA was a reversible, competitive inhibitor of zeatin binding, with a $K_i$ from the secondary plot (Fig. 2) of 70 μM. It should be noted that the maximum practical concentration of zeatin (solubility dependent) is near the $K_m$ for this enzyme. This explains the concentration range reported in the primary reciprocal plots (Fig. 2); the dubious value of any results from a study of the interaction between inhibitors and *O*-acetylserine, which is unstable, is evident. It was assumed that other compounds, which inhibited the enzyme and had some structural analogy to IAA, were also competitive inhibitors of zeatin. Inhibition constants were calculated (as described above) for the tighter binding compounds (Table 2). A more complete kinetic analysis was carried out on the strongest inhibitor, 5,7-dichloroindol-3-ylacetic acid (Table 2). Graphical plots gave the pattern for a reversible, competitive inhibitor.

It should be noted that the alanine synthase used for these investigations and that used in the original investigation of enzyme properties [7] were prepared from different harvests of developing lupin seed. In the inhibitor studies, a value of 4.3 mM for the $K_m$ of zeatin was

Table 1. $K_i$ values for inhibitors of cytokinin 7-glucosyltransferase

| Compound | $K_i$ (μM) |
|---|---|
| Analogues of purine cytokinins* | |
| 6-Benzylamino-2-(2-hydroxyethylamino)-7-methylpurine (**5**) | 61 |
| 6-Benzylamino-2-(2-hydroxyethylamino)-9-methylpurine (**6**) | 3.3 |
| 6-Benzylamino-9-(3-hydroxypropyl)purine (**7**) | 200 |
| 2-Chloro-6-(5-hydroxypentylamino)-7-methylpurine (**10**) | 220 |
| 2-Chloro-6-(5-hydroxypentylamino)-9-methylpurine (**11**) | 48 |
| 6-(5-hydroxypentylamino)-9-Methylpurine (**12**) | 230 |
| 3-Methyl-7-pentylaminopyrazolo[4,3-*d*]pyrimidine† (**13**) | 22 |
| 4-Furfurylaminopyrazolo[3,4-*d*]pyrimidine | 280 |
| 4-Cyclopentylamino-2-methylthiopyrrolo[2,3-*d*]pyrimidine (**14**) | 27 |
| Other compounds | |
| Theophylline | 380 |
| *N*-(3-chlorophenyl)-*N'*-Phenylurea | 75 |
| *N*-Benzyl-*N'*-phenylurea | 100 |

*6-Benzylamino-2-chloro-7-methylpurine (**8**) and the 9-methyl isomer (**9**) were also tested but precipitated in the incubation mixture. The apparent $K_i$ values, 66 and 38 μM, respectively, are therefore over estimates.

†The isomeric compound with a 7-(2-methylbutylamino) moiety exhibited a $K_i$ of 28 μM.

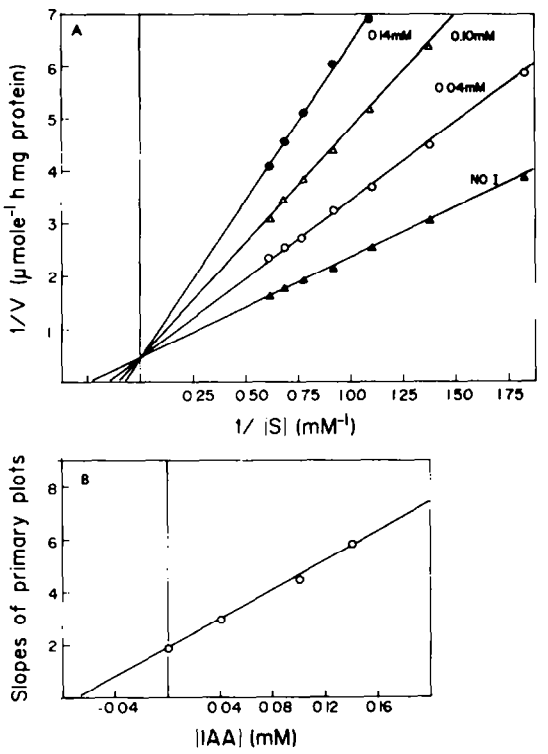



Fig. 2. (A) Lineweaver–Burk plots of data obtained from assays of β-(9-cytokinin)alanine synthase in the presence and absence of the inhibitor indol-3-yl acetic acid (IAA). (B) A plot of the slopes of the Lineweaver–Burk plots against IAA concentration. The straight line obtained indicates linear competitive inhibition.

Table 2. $K_i$ values for inhibitors of β-(9-cytokinin)alanine synthase

| Compound | $K_i$ (μM) |
|---|---|
| Auxins and related compounds | |
| Indol-3-ylacetic acid* (IAA) | 70 |
| 3-(Indol-3-yl)propionic acid* | 2800 |
| 2-(Indol-3-yl)ethanol | 7200 |
| Indole-2-carboxylic acid† | 140 |
| Indole-3-carboxylic acid† | 1100 |
| 4-Chloroindol-3-ylacetic acid* | 2.0 |
| 4,7-Dichloroindol-3-ylacetic acid† | 2.0 |
| 5,7-Dichloroindol-3-ylacetic acid† | 0.4 |
| Phenylacetic acid* | 65 |
| 2,4-Dichlorophenoxyacetic acid* (2,4-D) | 2.0 |
| 2,6-Dichlorophenoxyacetic acid† | 34 |
| Urea derivatives without cytokinin activity | |
| *N*-Benzyl-*N'*-phenylurea‡ | 26 |
| *N*-Benzyl-*N'*-(3-chlorophenyl)urea‡ | 51 |
| *N*-Benzyl-*N'*-(3,4-dichlorophenyl)urea‡ | 23 |
| Urea derivatives with cytokinin activity | |
| *N*-(3-Chlorophenyl)-*N'*-phenylurea | 220 |
| *N*-(3-Nitrophenyl)-*N'*-phenylurea | 91 |

*Compound with moderate or pronounced auxin activity.
†Weakly active or inactive in auxin bioassays.
‡Compound exhibits anticytokinin activity in chlorophyll retention bioassay [16].

found and used in calculations. This value was substantially higher than the earlier estimate of $K_m$ (0.88 mM). It would appear that there was a difference in the enzymes from the batches.

*Inhibitors of β-(9-cytokinin)alanine synthase*

The most effective inhibitors found for β-(9-cytokinin)alanine synthase were auxins and closely related compounds (Table 2). Both the natural auxins, IAA and phenylacetic acid were effective inhibitors, but more pronounced inhibition was exhibited by 4-chloro-(a natural auxin), 4,7-dichloro-, and especially 5,7-dichloro ($K_i$ 0.4 μM) IAA. The potent auxin 2,4-dichlorophenoxyacetic acid was also a very effective inhibitor. There is no correlation between $K_i$ and the auxin activity reported for the compounds listed (Table 2). The key structural features needed for effective enzyme inhibition by auxin-like compounds appear to be an aromatic ring and a two-carbon aliphatic side chain with a carboxyl group (which would be ionized at the pH studied). However, a group of less effective inhibitors were urea derivatives (Table 2), which do not have a charged side chain, but rather two aromatic rings with flexible chemical connection. These compounds are more structurally analogous to synthetic cytokinins such as 6-benzylaminopurine and these inhibitors exhibit either cytokinin or anticytokinin activity.

A number of close structural analogues of zeatin were tested as inhibitors of the alanine synthase, including the purines listed in Table 1. Where estimates of $K_i$ were possible, they were, with one exception > 500 μM; 6-benzylamino-9-(3-hydroxypropyl)purine (**7**) exhibited a $K_i$ of 360 μM. Some compounds were probably weak inhibitors, but too insoluble for estimations of $K_i$. Hence, purines which were inhibitors of the glucosyl transferase (see below), including the very effective 9-methyl derivative (**6**) and the anticytokinins **13** and **14**, were very weakly active or inactive as inhibitors of the cytokinin-alanine synthase.

*Synthesis of inhibitors of cytokinin 7-glucosyltransferase*

In previous studies of $N^6$-substituted adenines as substrates of this enzyme [6], benzyl was the optimal substituent at this position. A 2-hydroxyethylamino group at position 2 further enhanced binding to the enzyme [6]. It was likely, therefore, that the 7- or 9-methyl derivatives of 6-benzylamino-2-(2-hydroxyethylamino)purine (**5** and **6**) would be effective inhibitors of the glucosyl transferase. Hence these compounds were synthesized as outlined below.

2,6-Dichloro-7-methylpurine and 2,6-dichloro-9-methyl purine were first prepared; the former was synthesized from 7-methyl xanthine by published methods [13], while 2,6-dichloropurine was reacted with methyl iodide and the products separated by TLC to give the 9-methyl compound. The two chlorines in these methyl purines were replaced sequentially with substituted amino groups by reaction with amines; the 6-chloro was displaced by reaction with benzylamine in *n*-butanol at 100° to yield **8** and **9** while subsequent reaction with 2-aminoethanol at 155° yielded **5** and **6**. Reaction of 7- and 9-methyl 2,6-dichloropurine with 5-amino-1-pentanol yielded the methyl 2-chloro-6-(hydroxypentylamino)-purines, **10** and **11**. Other new hydroxyalkylamino-purines synthesized were **12** and 2-chloro-6-(4-hydroxy-

butylamino)-7-methylpurine. Compound **7**, previously prepared by an inconvenient two-step synthesis from 6-chloropurine [14], was synthesized by lithium aluminium hydride reduction of ethyl 3-(6-benzylaminopurin-9-yl)propionate which was prepared by addition of ethyl acrylate to 6-benzylaminopurine, a Michael-type reaction [8]. 6-Benzylaminopurine was also produced during the reduction, apparently due to reversal of the Michael addition. This type of cleavage was previously noted in the lithium aluminium hydride reduction of β-(6-benzylaminopurin-9-yl)propionitrile when 6-benzylaminopurine was the major product unless a modified reduction reagent was used [8].

*The comparative activity of inhibitors of cytokinin 7-glucosyltransferase*

The most effective inhibitors found for cytokinin 7-glucosyltransferase were close analogues of active cytokinins (Table 1) and **6** was the most active. Compound **6**, a 9-methylpurine derivative, was considerably more effective than the 7-methyl isomer (**5**). Similarly, the 6-(hydroxypentylamino)purine **11** with a methyl at N-9 was a better inhibitor than the 7-methyl isomer (**10**). Hence for binding to the enzyme, an N-9 substituent was preferable to an N-7. Another structural feature which enhances inhibition is a 2-chloro group (cf. $K_i$ for compounds **11** and **12**).

In addition to derivatives with a 6-(substituted amino)purine moiety (**5–12**), two other types of compounds were tested for their ability to inhibit cytokinin 7-glucosyltransferase. One type were urea derivatives of which *N*-(3-chlorophenyl)-*N'*-phenylurea (a compound with cytokinin activity [15] and *N*-benzyl-*N'*-phenylurea (a compound with some anticytokinin activity [16]) exhibited appreciable inhibitory activity (Table 1). The other type were heterocyclic compounds with a ring system (pyrazolo-pyrimidine or pyrrolo-pyrimidine) similar to purines. Of these compounds, **13** (an anticytokinin [17]) was the most effective (Table 1), and this compound has been shown to act competitively (see above). In *Amaranthus* seedlings theophylline, when used as the ethylenediamine complex (aminophylline) at high concentrations (5 mM), has been reported [18] to inhibit conversion of 6-benzylaminopurine into metabolites presumed to be glucosides. However, as an inhibitor of the transferase enzyme, theophylline exhibited only feeble activity (Table 1).

## DISCUSSION

The combined information and techniques developed to prepare and study cytokinin 7-glucosyl transferase and β-(9-cytokinin)alanine synthase [6, 7] have now been utilized for the development of inhibitors of these two enzymes. A rational chemical design was effective in development of inhibitors of the glucosyltransferase, but a selective screening approach was necessary to find good inhibitors of the alanine synthase enzyme. In the process of studying the inhibitors, new information was obtained concerning the specificity of the enzymes and the design of specific inhibitors.

The anticytokinins **13** and **14** [17, 19] were found to be effective competitive inhibitors of the glucosyl transferase. Skoog *et al.* [17] suggested that **13** was a specific competitive inhibitor of cytokinins in induction of growth, a result not confirmed by Helgeson *et al.* [20], but in accord with the more recent findings of Gregorini and Laloue [21]. The inhibition of the glucosyl transferase by **13** reported herein was the first definite evidence that **13** can compete with cytokinin at a site exhibiting specificity for cytokinin-active molecules. In the anticytokinins **13** and **14**, the ring systems are modifications of the purine ring system. Preparation of further inhibitors with these or similar modified rings was not pursued because compounds such as **13** and **14** inhibit cell growth [17, 19, 20]. An alternative approach was to prepare substrate analogues with substituents at the position which accepts the glucosyl moiety. It was found that a methyl group substituted at N-9 of the purine ring had little effect on binding. With enzymatic activity blocked, the rest of the molecule could be designed to maximize binding. The 6-benzylamino group was included, as this was the optimum $N^6$-substituent for binding substrate. A side chain at position 2, a β-hydroxyethylamino moiety, was added, since previous work had shown a positive binding effect from this substituent which also increased solubility. This resulted in compounds **5** and **6** and of these **6** was an excellent inhibitor which probably binds more effectively than substrate. The previously reported ability of the enzyme to accept aromatic groups at either positions 2 or 6 [6] might explain the formation of two products (the 7- and 9-glucosides) with some compounds. The ability of both 2- and 6-benzylaminopurines to serve as substrates suggests that there may be two near-equivalent orientations for binding, such that the 7- or 9-N is positioned for attack in the active site.

Since some substituted ureas (Tables 1 and 2) were inhibitors of both enzymes ($K_i$ values about $10^{-4}$ M), these compounds were capable of interacting with binding sites for cytokinins. This may partly explain the biological activity of such compounds as cytokinins and anticytokinins [15, 16]. Auxins and structurally related compounds were found to be effective inhibitors of β-(β-cytokinin)alanine synthase. Since auxins do not mimic the structural features which confer cytokinin activity on purine derivatives, and which also enhance binding to the synthase [7], a possible explanation for the inhibition of the enzyme by auxins is that they are analogues of the reaction product on the enzyme, or analogues of a transition state in product formation. The original study of β-(9-cytokinin)alanine synthase concluded on the basis of evidence available that a ping pong mechanism was operating in the reaction [7]. This requires two enzyme species binding substrates alternatively. If, as suggested above, auxin structures are analogues of product or a transition state, then such a compound should be a competitive inhibitor of both substrates. Unfortunately, practical considerations did not permit the relationship between IAA and *O*-acetylserine to be determined. Alternatively, inhibition by auxins could also result from a specific, separate capacity of the enzyme to bind auxin molecules for some intended biological function. The site which accepts the auxin would also interact with the site for the cytokinin substrate. The synthase enzyme is inhibited not only by auxins but also by urea derivatives which exhibit cytokinin or anticytokinin activity and which resemble cytokinins such as 6-benzylaminopurine structurally. Thus, it may be that the ultimate inhibitor of the enzyme has yet to be synthesized for it could combine the structural features of auxins and synthetic cytokinins. It would be interesting to know if a compound like 6-

benzylaminopurin-9-ylacetic acid was more effective as an inhibitor than IAA.

Exogenous cytokinins have been reported at different times to elevate auxin levels in tissue, to increase auxin production, to suppress conjugation of auxins to amino acids, and to affect the formation of enzymes which oxidize IAA [22, 23]. Conversely, IAA has been reported to modify cytokinin metabolism in two plant tissues. In *Solanum andigena* stem cuttings, formation of unidentified polar metabolites of 6-benzylaminopurine was induced [24], while in artichoke tuber tissue a rapid metabolism of zeatin nucleotide resulted [25]. In the present study, IAA has been found to inhibit competitively an enzyme involved in cytokinin conjugation. This is the first instance of involvement of auxins in a specific aspect of cytokinin metabolism at the molecular level. In summation, the above observations indicate that cytokinin and auxin interact in a complex manner to mutually control their metabolism.

Much effort has been directed to the synthesis of hormone analogues with enhanced growth promoting activity. However, the present contribution appears to be the first attempt to develop inhibitors which block hormone inactivation. Such inhibitors might potentiate the action of known hormones and their analogues and may eventually contribute to our ability to modify plant development in desirable ways.

## EXPERIMENTAL

*Plant material.* The radish cotyledons were from seedlings of *Raphanus sativus* cv Long Scarlet and the lupin seed from plants of *Lupinus luteus* L., cv Weiko III [6, 7].

*Enzyme preparation.* Cytokinin 7-glucosyltransferase (EC 2.4.1.118) was prepared from radish cotyledons and β-(9-cytokinin)alanine synthase (EC 4.2.99.13) from lupin seed as described in refs [6] and [7], respectively.

*Enzyme assays.* Cytokinin 7-glucosyltransferase was assayed in a vol. of 0.50 ml containing 1.8 mM UDP-glucose, 1.4 mM zeatin, enzyme sample, 0.3 mM EDTA, and 20 mM Pi buffer, pH 7.35. Assays were started by the addition of enzyme and were run for about 120 min at 35°. Complete details are in ref. [6].

β-(9-cytokinin)Alanine synthase was assayed in a vol. of 0.50 ml containing 1.4 mM zeatin, 2.0 mM *O*-acetyl-L-serine, enzyme sample, 25 mM Pi buffer, pH 7.65. Assays were started by the addition of *O*-acetyl-L-serine, and incubated at 25° for about 20 min. Complete details are in ref. [7].

*Testing inhibitors.* Cytokinin 7-glucosyltransferase was examined with modified assay conditions. Zeatin was used in assays at 0.3–0.4 mM (about × 2 $K_m$ for zeatin) and the potential inhibitor was present at 1.5 mM. In most cases, MeOH was required to prepare stock solns of inhibitor. The enzyme activity was not influenced by up to 4% MeOH. Some compounds (e.g. diphenylureas) were extremely insoluble and the final concn of these compounds in assays was about 0.3 mM. $K_m$ values used for zeatin and UDP-glucose were 0.15 and 0.19 mM respectively [6].

Potential inhibitors of β-(9-cytokinin)alanine synthase were assayed at or below a concentration of 0.4 mM. The presence of 4% MeOH (to dissolve inhibitors) in assays resulted in a marked loss of enzyme activity. This problem was overcome when solns of inhibitors were prepared by dissolving the compounds in DMSO followed by the gradual addition of an equal vol. of the assay buffer. The final concn of DMSO in assays and controls was 0.5%. $K_m$ values used for zeatin and *O*-acetyl-L-serine were 4.3 mM and 0.047 mM, respectively [7].

Assays were compared to controls without inhibitor. Products formed were detected and measured by HPLC [6, 7]. Inhibition was established by a relative decrease in the product from zeatin compared to controls.

The most active inhibitors of both enzymes were tested for substrate activity by assaying in the absence of zeatin. Any significant products of such activity would be detected in the HPLC systems used because, being more polar compounds, they would elute before the substrates.

*Chemical syntheses.* Solvents for prep. TLC using Merck silica gel 60 $PF_{254}$ were as follows: A, *n*-BuOH–HOAc–$H_2O$ (12:3:5); B, $CHCl_3$–MeOH (9:1); C, MeCOEt–HOAc–$H_2O$ (16:1:4). Zones containing products were scraped from the plates, packed into a column and eluted by allowing EtOH to flow through the column. The eluate was evaporated and an *n*-BuOH soln of the residue was extracted with an equal vol. of $H_2O$. The *n*-BuOH layer was evaporated to yield the crude product.

EI-MS (probe) were recorded with a Finnigan 4500 instrument at 70 eV. UV spectra were determined in 75% EtOH containing $NH_4OH$ (0.3 N) and in 70% EtOH containing HCl (0.2 N) and these solns are referred to as 'base' and 'acid' solvents. Pronounced and slight shoulders are designated p sh and s sh respectively. Unless stated otherwise, mps were determined with a Kofler apparatus (microscope hot stage).

*6-Benzylamino-2-chloro-7-methylpurine* (**8**). Benzylamine (1.1 ml) and 2,6-dichloro-7-methylpurine (0.40 g; synthesized from 7-methylxanthine according to ref. [13]) were dissolved in *n*-BuOH (4 ml) and the soln was heated at 100° for 5 hr. The resulting soln was evaporated and an EtOAc soln (70 ml) of the residue was shaken first with $H_2O$ (35 ml), then with $H_2O$ (35 ml) containing sufficient HCl to yield an extract with pH 4, and finally with $H_2O$ (35 ml, × 2). The fraction in the washed EtOAc soln was crystallized first from MeOH–$C_6H_6$–petrol and then from MeOH–$H_2O$ to yield **8** (150 mg), mp 199°; $\lambda_{max}$ (acid): 281, 291 (s sh) nm; $\lambda_{max}$ (base): 281.5, 274 (p sh), 291.5 (p sh) nm. MS *m/z* (rel. int.): 275 $[M]^+$ (16), 273 $[M]^+$ (45), 272 (6), 238 (3), 236 (2), 196 (3), 182 (2), 168 (10), 133 (26), 106 (100). (Found: C, 56.8; H, 4.3; N, 25.4; Cl, 13.2. $C_{13}H_{12}N_5Cl$ requires: C, 57.0; H, 4.4; N, 25.6; Cl, 12.95%.)

*2-Chloro-6-(5-hydroxypentylamino)-7-methylpurine* (**10**). 5-Amino-1-pentanol (1.2 g) was condensed with 2,6-dichloro-7-methylpurine (0.62 g) under the conditions used in the preparation of **8**. The reaction mixture (10 ml) was diluted with *n*-BuOH (40 ml) and EtOAc (50 ml) and was then extracted sequentially with $H_2O$, dil. HCl and $H_2O$ as above. The fraction in the *n*-BuOH-EtOAc phase was subjected to prep. TLC (silica gel, solvent A) and the compound in the principal UV-absorbing zone was crystallized from MeOH–PrOAc–petrol to yield **10** (250 mg), mp 154–155°; $\lambda_{max}$ (acid): 281.5, 291 (s sh) nm; $\lambda_{max}$ (base): 282, 274 (p sh), 292 (p sh) nm. MS *m/z* (rel. int.): 271 $[M]^+$ (7), 269 $[M]^+$ (21), 252 (7), 238 (7), 234 (100), 212 (21), 210 (63), 198 (30), 197 (37), 196 (82), 190 (28), 185 (33), 184 (49), 183 (98), 182 (92), 174 (12), 168 (23), 167 (21), 160 (52), 148 (33), 133 (47), 106 (14). (Found: C, 49.3; H, 6.1; N, 25.7; Cl, 12.8. $C_{11}H_{16}ON_5Cl$ requires: C, 49.0; H, 6.0; N, 26.0; Cl, 13.1%.)

*2-Chloro-6-(4-hydroxybutylamino)-7-methylpurine.* This compound was prepared from 4-amino-1-butanol by the method used for **10** and was crystallized from MeOH–$H_2O$, mp 208–209°, $\lambda_{max}$ as for **10**. MS *m/z* (rel. int.): 257 $[M]^+$ (8), 255 $[M]^+$ (27), 238 (6), 224 (5), 220 (86), 212 (26), 210 (78), 198 (23), 196 (70), 190 (23), 185 (31), 184 (49), 183 (94), 182 (100), 174 (11), 168 (22), 167 (32), 160 (66), 148 (45), 133 (68), 106 (26).

*6-Benzylamino-2-(2-hydroxyethylamino)-7-methylpurine* (**5**). Compound **8** (100 mg) and 2-aminoethanol (1.0 ml) were heated in an evacuated (0.1 mm) sealed tube at 155° for 3 hr. The reaction mixture was diluted with *n*-BuOH (50 ml) and shaken first with

$H_2O$ (50 ml), then with $H_2O$ (50 ml) containing sufficient HCl to yield an extract of pH 7–8, and finally with $H_2O$ (50 ml). The fraction in the BuOH soln was subjected to prep. TLC (silica gel, solvent C) and the compound in the principal UV-absorbing zone was crystallized from $C_6H_6$–MeOH to yield **5** (31 mg), mp 211–212°; $\lambda_{max}$ (acid): 232, 253.5, 292 nm; $\lambda_{max}$ (base): 228.5, 303, 250 (sh) nm. MS *m/z* (rel. int.): 298 $[M]^+$ (32), 279 (12), 268 (84), 267 (88), 254 (100), 238 (6), 189 (4), 177 (9), 175 (11), 163 (11), 149 (31), 148 (15), 133 (9), 122 (14), 106 (23). $^1H$ NMR (DMSO-$d_6$, 200 MHz): $\delta$7.94 (*s*, 1H, purine H-8), 7.21–7.45 (*m*, 5H, aromatic H), 6.48 (*br*, signal absent in presence of $D_2O$, 1H, amino H), 4.69 (*br d*, 2H, benzylic $CH_2$), 3.97 (*s*, 3H, N-7 Me); hydroxymethylene and aminomethylene signals obscured by water. (Found: C, 60.3; H, 6.2; N, 28.1. $C_{15}H_{18}ON_6$ requires: C, 60.4; H, 6.1, N, 28.2%.)

6-(*5-Hydroxypentylamino*)-9-*methylpurine* (**12**). 6-Chloro-9-methylpurine (310 mg), *n*-BuOH (6 ml) and 5-amino-1-pentanol (1.0 g) were heated at 100° for 5 hr. The product was purified by extraction and prep. TLC as described for the preparation of **10**. Crystallization from MeOH–PrOAc–petrol yielded **12** (140 mg), mp 109°; $\lambda_{max}$ (acid): 266 nm; $\lambda_{max}$ (base): 269 nm. MS *m/z* (rel. int.): 235 $[M]^+$ (21), 219 (13), 218 (9), 204 (9), 190 (6), 176 (33), 163 (43), 162 (100), 149 (70), 135 (10), 134 (10), 133 (21), 122 (6).

2-*Chloro*-6-(*5-hydroxypentylamino*)-9-*methylpurine* (**11**). To a stirred soln (8 ml) of 2,6-dichloropurine (800 mg; 4.2 mmole) in dry DMF, NaH (127 mg; 5.3 mmole) was added followed by MeI (0.34 ml; 5.5 mmole). The soln was stirred under anhydrous conditions at 23° for 18 hr when crushed ice (40 ml) was added. The mixture was then shaken with 3 equal vols of EtOAc and the extracted fraction was subjected to prep. TLC (silica gel, solvent B). Elution of the principal UV absorbing zone ($R_f$ relative to 2,6-dichloro-7-methylpurine, 1.34) with EtOAc–EtOH (1:1) yielded 2,6-dichloro-9-methylpurine (UV and MS identical to the product prepared according to ref. [26]). 2,6-Dichloro-9-methylpurine was condensed with 5-amino-1-pentanol under the conditions used for the preparation of the 7-methyl isomer **10** and the product purified by the methods used for **10**. The chromatographed product ($R_f$ silica gel solvent A, 0.67; cf. $R_f$ **10**, 0.48) was crystallized from MeOH–EtOAc–petrol to give **11**, mp 153–154°; $\lambda_{max}$ (acid): 274.5 nm; $\lambda_{max}$ (base): 274 nm. MS *m/z* (rel. int.): 271 $[M]^+$ (4), 269 $[M]^+$ (12), 252 (7), 234 (40), 212 (10), 210 (30), 198 (33), 196 (100), 190 (11), 185 (17), 183 (50), 169 (11), 167 (8), 160 (7), 148 (20).

6-*Benzylamino*-2-*chloro*-9-*methylpurine* (**9**). 2,6-Dichloro-9-methylpurine (prepared as above) was reacted with benzylamine under the conditions used for the preparation of **8**. The product was purified by solvent partitioning as for **8** and was then crystallized from MeOH–$H_2O$ to yield **9**, mp 228–229°; $\lambda_{max}$ (acid): 274.5, 266 (s sh), 285 (s sh) nm; $\lambda_{max}$ (base): 273.5 nm. MS *m/z* (rel. int.): 275 $[M]^+$ (24), 273 $[M]^+$ (71), 272 (20), 238 (7), 196 (8), 169 (5), 168 (7), 133 (23), 106 (100). (Found: C, 56.7; H, 4.3; N, 25.3; Cl, 13.1. $C_{13}H_{12}N_5Cl$ requires: C, 57.0; H, 4.4; N, 25.6; Cl, 12.95%.)

6-*Benzylamino*-2-(*2-hydroxyethylamino*)-9-*methylpurine* (**6**). Compound **9** (70 mg) and 2-aminoethanol (0.6 ml) were heated in an evacuated (0.1 mm) sealed tube at 155° for 3 hr. The reaction soln was diluted with *n*-BuOH (20 ml) which was shaken sequentially with $H_2O$ (20 ml), $H_2O$ (20 ml) containing sufficient HCl to yield an extract of pH 7–8 and finally $H_2O$ (20 ml). The fraction in the BuOH solution was subjected to prep. TLC (silica gel, solvent A) and the principal UV-absorbing compound ($R_f$ 0.53) was eluted and crystallized from MeOH–EtOAc–petrol to yield **6** (40 mg), mp 126°; $\lambda_{max}$ (acid): 237.5, 291.5, 245–255 (p sh) nm; $\lambda_{max}$ (base): 231, 290.5, 250–261 (p sh). MS *m/z* (rel. int.): 298 $[M]^+$ (87), 280 (4), 279 (7), 267 (100), 254 (25), 253 (7), 238 (3), 212 (2), 189 (5), 177 (3), 163 (11), 149 (20), 148 (14), 140 (10), 133 (18), 122 (5), 121 (7), 106 (23). (Found *m/z*: $[M]^+$ 298.1544, $C_{15}H_{18}ON_6$ requires 298.1541.) $^1H$ NMR (DMSO-$d_6$, 200 MHz): $\delta$7.80 (*s*, 1H, purine H-8), 7.25–7.48 (*m*, 5H, aromatic H), 6.09 (*br*, signal absent in presence of $D_2O$, 1H, amino H), 4.75 (*br d*, 2H, benzylic $CH_2$), 3.65 (*s*, 3H, N-7 Me); hydroxymethylene and aminomethylene signals obscured by $H_2O$.

6-*Benzylamino*-9-(*3-hydroxypropyl*)*purine* (**7**). To a stirred mixture of $LiAlH_4$ (0.13 g, 3.4 mmole) and dry THF (20 ml), ethyl 3-(6-benzylaminopurin-9-yl)propionate [8] (1.1 g, 3.4 mmole) in THF (5 ml) was slowly added under anhydrous conditions. The mixture was heated at 60° for 18 hr when $H_2O$ (0.35 ml) was added at 2°. The resulting soln was filtered and evaporated to dryness. The residue was stirred with $H_2O$ (pH maintained at 11 by addition of $NH_4OH$) to yield a sparingly soluble fraction (A) and a soln which was passed through a column of Dowex 1 (acetate form, equilibrated to pH 10.9). The column was washed with 0.05 N $NH_4OH$ and then with 70% EtOH (combined washes termed fraction B), prior to elution with 1 N HOAc (fraction C). Crystallization of fractions A and B from EtOH–petrol yielded **7** (major product), capillary mp 127°; $\lambda_{max}$ (acid): 268 nm; $\lambda_{max}$ (base): 270 nm. MS *m/z* (rel. int.): 283 $[M]^+$ (100), 282 (21), 267 (2), 266 (4), 253 (7), 252 (5), 239 (37), 238 (37), 225 (14), 224 (17), 178 (10), 148 (7), 134 (7), 121 (8), 120 (8), 119 (8), 106 (68), 91 (44). (Found: C, 63.6; H, 6.2; N, 24.6. Calc. for $C_{15}H_{17}ON_5$: C, 63.6; H, 6.05; N, 24.7%.)

Crystallization of fraction C from EtOH–$H_2O$ yielded a second product (capillary mp 236–237°) identified as 6-benzylaminopurine (mp, MS, UV, TLC).

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