with phenylhydrazine gives the known 4-acetyl-5-methyl-1-phenyl pyrazole in good yield. Mannich condensation of the above pyrazole with N-(o-tolyl)-piperazine dihydrochloride and paraformaldehyde in the presence of catalytic amounts of hydrochloric acid affords Go. 1002 as colourless, tasteless leaflets after recrystallization from methanol. It melts with decomposition at 235–237 °C; calculated for $C_{24}H_{28}N_4O.HCl$: C, 67.81, H, 6.88, N, 13.18. Found: C, 68.05, H, 6.89, N, 13.00. Its UV-spectrum shows λ_{max} at 250 nm (log ε 4.34) in ethanolic solution. Its IR-spectrum exhibits a strong band at 1658 cm^{-1} in nujol mull.

Zusammenfassung. 1-(5-Methyl-1-phenyl-4-pyrazolyl)-3-[4-(o-tolyl)-piperazinyl]-1-propanone (I; CIBA 1002-Go), senkt an normotonischen und hypertonischen Tieren den Blutdruck. Die Drucksenkung kann auf eine periphere Vasodilatation und eine Hemmung hypothalamischer oder medullärer Vasomotorenzentren bezogen werden. Ausserdem wirkt CIBA 1002-Go adrenolytisch.

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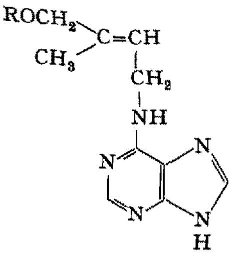
CIBA Research Centre, Goregaon East, Bombay 63, (India), 17th March 1967.

[8] Contribution No. 96 from CIBA Research Centre.

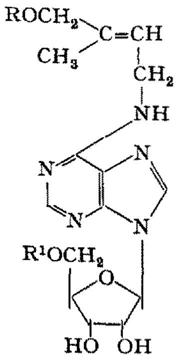
Phosphorylated Derivatives of the Cytokinins, Zeatin and its 9-β-D-ribofuranoside, Naturally Occurring Adenine and Adenosine Derivatives with Plant Cell-Division Promoting Activity

Compounds which induce plant cell division in the presence of indole acetic acid have been named cytokinins[1], and activity of this type is shown in varying degrees by a wide variety of synthetic 6-alkylamino purines related to kinetin (6-furfurylaminopurine), a degradation product of deoxyadenosine[2]. Naturally occurring substances with kinetin-like properties have been detected in a wide variety of excised plant tissues[3–7] and in addition to their cell division promoting activity are implicated in many physiological processes[8–12].

The first naturally occurring cytokinin was isolated from immature sweet corn (*Zea Mays*) kernels and named zeatin[13] (for a review see [14]). Its structure (Ia) was based on a combination of chemical, UV, proton magnetic resonance, mass spectral, and enzymic evidence, and finally confirmed by comparison with synthetic material prepared by our unambiguous route[15].



(Ia) R = H
(Ib) R = $-COCH_3$
(Ic) R = $-SO_2\cdot C_6H_4\cdot CH_3$
(Id) R = $-SO_2\cdot CH_3$



(IIa) R = H, R^1 = H
(IIb) R = H, $R^1 = -PO\cdot(OH)_2$
(IIIa) $R = -P{:}O\cdot(OH)_2$, R^1 = H
(IIIb) $R = R^1 = -P{:}O\cdot(OH)_2$

Most workers report 3 major active cytokinins by bioassay of chromatogram zones in plant extracts, one of which appears generally to be zeatin. The other substances may be the corresponding 9-β-D-ribofuranoside of zeatin (IIa) and its 5′-phosphate (IIb)[16,17]. In one case[16] the structure of the material regarded as the nucleoside (IIa) was fully established by direct comparison with synthetic material[15]. A compound believed to be the nucleotide (IIb) was detected by Miller[17] in maize extracts and a pure crystalline product was isolated from the same source by Letham[18]. The probable structure assigned to the 5′-phosphate was based on its hydrolysis with 5′-nucleotidases to a riboside, presumably 9-β-D-ribofuranosylzeatin (IIa) and the further conversion of this to

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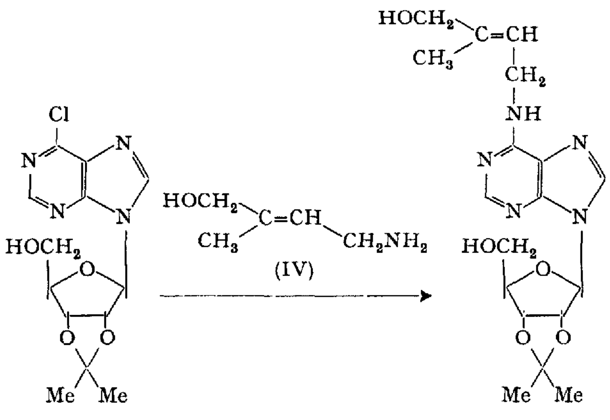
zeatin with periodate and cyclohexylamine. The activity of the nucleotide was less than that of zeatin[18]. Thus at concentrations of $5 \cdot 10^{-8}$, $5 \cdot 10^{-9}$ and $5 \cdot 10^{-10} M$, zeatin induced increments in explant weight of 30, 21 and 6 mg respectively whereas the nucleotide caused increments of 29, 11 and 2 mg. The total activity of sweet corn extracts is largely due to the nucleotide which occurs in considerably greater amount than does the aglycone or the riboside[18].

Cytokinin activity has also been detected in alkaline hydrolysates of sheep and rat liver RNA[19]. Compounds of similar structure to zeatin and its ribosyl derivative have been isolated from yeast s-RNA. One of these has been shown to be 6-γ,γ-dimethylallylaminopurine[20]. In other investigations the same dimethylallyl adenine residue has been identified as a base in both serine specific and alanine specific yeast s-RNAs[21]. 6-Dimethylallylaminopurine is also produced by liquid cultures of the plant pathogen, *Corynebacterium fascians*[22], but has not as yet been detected in higher plants.

The realization that, in addition to zeatin and its riboside which we have already synthesized, the 5′-monophosphate (IIb) is of widespread occurrence and possibly implicated in the mechanism of action of the cytokinins, induced us to investigate its synthesis and that of the related allylic phosphate (IIIa) and the diphosphate (IIIb). The 5′-phosphate (IIb) was readily obtained by reaction of *trans*-4-amino-2-methylbut-2-en-1-ol (IV)[15] with the potassium salt of 6-chloropurine ribotide in aqueous 3% potassium hydrogen carbonate at 38 °C for 70 h, and purified by ion-exchange chromatography and preparative paper chromatography. The same compound was also obtained by reaction of the isopropylidene derivative of 6-chloropurine riboside (V) with the hydroxyallylamine (IV), phosphorylation of the product, presumably (VI), with pyrophosphoryl chloride at −25 °C, and removal of the isopropylidene group by mild acid hydrolysis.

Under these conditions no diphosphate (IIIb) was detected and this suggested that such a compound, which most likely was formed under the powerful phosphorylating conditions used, was readily hydrolysed at the allylic group only, during the subsequent isolation procedure. The structure assigned to the synthetic 5′-phosphate was confirmed by the identity on paper chromatograms of materials prepared by both methods (Table I) by its UV-absorption spectra which at various pH values was almost identical with that of the riboside (Table III), by its behaviour on high voltage electrophoresis (Table II) and by its degradation with an alkaline phosphatase to zeatin riboside (IIa)[15].



(V) (VI)

Table I. Paper chromatography of zeatin and derivatives[a] Rf in solvent system

Compound	A	B	C
Ia	0.78	0.78	0.79
IIb	0.19	0.33	0.03
IIa[b]	0.69	0.86	0.54
IIa	0.69	0.88	0.57
VIIa	0.12	0.14	0.0
VIIb	0.65	0.74	0.17
VIII	0.40	0.65	0.12
VIII[c]	0.39	0.65	0.12
VIII[d]	0.39	0.65	0.12
VIII[e]	0.39	0.67	0.12

[a] Descending technique on Whatman No. 1 paper with the solvent systems (A) *n*-butanol, acetic acid water (12:3:5), (B) *n*-propanol, water (7:3) with an ammonia atmosphere and (C) *n*-butanol saturated with water; ratios in volume proportions. [b] Product from IIb and an alkaline phosphatase. [c] Product from VIIa and *N*-HCl at 100 °C for 20 min. [d] Product (unchanged) from VIII and *N*-HCl at 100 °C for 20 min. [e] Product from reaction of zeatin (Ia) with 2-cyanoethyl phosphate and dicyclohexycarbodiimide in pyridine.

Table II. Electrophoresis of zeatin and some derivatives and related compounds

Compound	Buffer[a]	Voltage (KV)	Time (min)	Paper[b]	Distance migrated (cm) to anode	to cathode
Ia	A	2	65	Y	–	7.1
Ia	B	2	95	Y	–	1.7
IIb	A	5	60	X	6.7	–
IMP[c]	A	5	60	X	14.2	–
VIIa	A	5	60	X	–	5.3
VIII	B	2	95	Y		14.6
VIIb[d]	B	2	95	Y		14.6
IIa	C	5	60	X	–	9.8
AMP[e]	C	3	80	Y	2.2	–
Adenosine	C	4	60	X	–	8.4
Uridine	C	5	60	X	–	2.4
VIII	A	2	65	Y	–	12.2
VIII[f]	A	2.5	90	Y	–	22.7
VIII[g]	A	2.5	90	Y	–	22.7
VIII[h]	A	2.5	90	Y	–	22.8

[a] Buffers (A) sodium formate-formic acid pH 3.2, (B) ammonium hydrogen carbonate pH 8.1 and (C) ammonium formate pH 3.5. [b] Paper (X) Whatman No. 1 and (Y) Whatman 3MM. [c] Inosine 5′-monophosphate. [d] Product from VIIa and an alkaline phosphatase. [e] Adenosine 5′-monophosphate. [f] Product (unchanged) from VIII and N-HCl at 100 °C for 20 min. [g] Product from VIIa and N-HCl at 100 °C for 20 min. [h] Product from reaction of zeatin (Ia) with 2-cyanoethyl phosphate and dicyclohexylcarbodiimide in pyridine.

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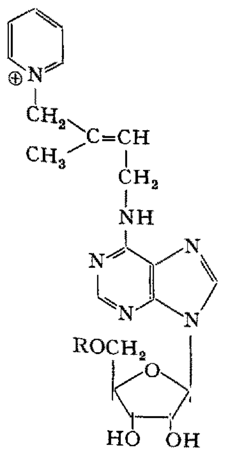
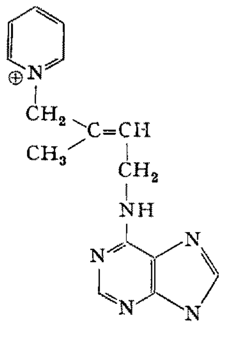
Phosphorylation of the isopropylidene riboside (VI) with 2-cyanoethyl phosphate and dicyclohexylcarbodiimide in pyridine gave an organic phosphate with an UV-absorption spectra very like that of zeatin riboside (IIa) (indicating that the general zeatin structure had been retained) and paper chromatographic behaviour (Table I) similar to that of the 5′-phosphate. The markedly different electrophoretic behaviour (Table II) suggested the presence of a strong basic centre in the molecule and this was confirmed by removal of the phosphate with alkaline phosphatase when a new riboside was obtained which moved much more strongly towards the cathode than zeatin riboside (Table II). Further degradation of this new riboside with acid gave an aglycone with an UV-absorption spectra which differed only slightly from that of zeatin and which also ran more strongly towards the cathode during electrophoresis. These results and other evidence are consistent with formulation of the products as the pyridinium ribotide (VIIa), riboside (VIIb) and aglycone (VIII). These conclusions were confirmed by the preparation of the pyridinium aglycone (VIII) from zeatin and methane sulphonyl chloride in pyridine. The crystalline compound isolated was identical with the material obtained by degradation of the nucleotide on the basis of paper chromatography, electrophoresis, and UV-absorption spectra. The assignment to it of the quaternary structure (VIII) was confirmed by elemental analysis of the base and its picrate, and by NMR-spectra (Table IV) which shows not only the aromatic protons of the pyridinium ring but also the typical downfield shift associated with a CH_2 adjacent to N^+ compared with a normal CH_2–N environment. The NMR-spectra was otherwise similar to that of zeatin and serves to exclude alternate allylic isomeric structures. The results imply that during the phosphorylation of the isopropylidene riboside of zeatin (VI) in pyridine, both the 5′-and the allylic-hydroxyl groups are phosphorylated but the allylic phosphate is a sufficiently powerful leaving group to quaternize the pyridine solvent. This was confirmed by a similar attempt to phosphorylate zeatin with cyanoethylphosphate and dicyclohexylcarbodiimide in pyridine when the same aglycone quaternary salt was obtained and characterized by paper chromatography and electrophoresis and UV-absorption spectra. The ease with which quaternary salts are formed in this series is reminiscent of the cephalosporin C_A compounds where however an allylic hydroxyl is further activated by a carbonyl group but, here, even the allylic acetate is sufficiently active to give quaternary compounds under mild conditions[23]. Neither the acetate (Ib) nor the *p*-toluene-sulphonyl (Ic) derivatives of zeatin reacted with pyridine under mild conditions but the methanesulphonyl derivative (Id) prepared in the absence of pyridine reacted smoothly with pyridine to give (VIII). The ease with which the allylic phosphate derivatives of zeatin and its ribotide react is unusual and would certainly suggest the possibility of the natural occurrence of quaternary derivatives of this type of specific naturally



(VIIa) R = –P:O.$(OH)_2$
(VIIb) R = H



(VIII)

Table III. UV-absorption spectra of zeatin and derivatives[a]

Compound	pH[b]	λ_{max_1} (nm)	ε_{max_1}	λ_{max_2} (nm)	ε_{max_2}	λ_{min} (nm)
Ia	A	207	14,500	275	14,650	235
Ia	B	212	17,050	270	16,150	233
Ia	C	220	15,900	276	14,650	242
IIa	A	207–9	19,800	266	18,550	235
IIa	B	210–12	19,300	269–71	17,800	233
IIa	C	215	18,050	269–71	18,300	235
VIII	A	208	18,500	268–73	17,400	235
VIII	B	210	20,250	266–7	17,550	231
VIII	C	221	15,650	267[c], 274	16,800	240
IIb	A	206	–	266	–	235
IIb	B	209	–	270	–	234
IIb	C	218	–	270	–	239
VIIa	A	215	–	266	–	235
VIIa	B	215	–	268	–	234
VIIa	C	216	–	268	–	235

[a] Spectra measured on a Perkin-Elmer model 137 UV recording spectrophotometer. [b] (A) 0.1 N-HCl, (B) M/30 phosphate buffer, pH 7.2 and (C) 0.1 N-NaOH. [c] Shoulder.

Table IV. Nuclear magnetic Resonance Spectra of zeatin and a pyridinium derivative

Compound	τ[a]		No. of protons	Assignment
Ia[b]	8.17 (singlet)		3	$C{:}C \cdot CH_3$
Ia	5.87 (singlet)		2	$:C \cdot CH_2OH$
Ia	5.60 (doublet)	J = 8 c/s	2	$\cdot NHCH_2CH{:}$
Ia	4.35 (triplet)		1	$\cdot CH_2CH{:}C$
Ia	1.26–1.52 (multiplet)		2	C^2-H, C^8-H
VIII[c]	8.10 (singlet)		3	$C{:}CCH_3$
VIII	4.65 (singlet)[d]		2	CH_2N^+
VIII	5.40 (doublet)	J = 8 c/s	2	$NHCH_2CH{:}$
VIII	4.00 (triplet)		1	$NHCH_2CH{:}$
VIII	0.73–1.90 (multiplet)		7	$C_5H_5N^+$, C^2-H, C^8-H

[a] Reference tetramethylsilane. [b] Solution in D_2O-DCl. [c] Solution in D_2O. [d] The downfield shift of methylene hydrogens adjacent to N^+ is in the expected position. Compare the assignments for NCH_2 (τ = 6.91) in 1-*n*-propyl-3-carbamoyl-1,4-dihydropyridine and for N^+CH_2 (τ = 5.26) in 1-*n*-propyl-3-carbamoylpyridinium iodide[24].

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occurring bases. The unusual behaviour of such compounds, for example on basic ion-exchange columns, could lead to their being overlooked, and a search for such compounds should take this into account, especially as they will generally have similar paper chromatographic properties to the related zeatin derivatives. Preliminary tests indicate that the quaternary derivative (VIII) has marked cytokinin activity[25].

Zusammenfassung. Zeatin-ribosid-5'-phosphat wurde einerseits durch Umsetzung von 6-Chlorpurin-ribotid mit trans-4-Amino-2-methylbuten-(2)-ol-(1) und andererseits durch Behandlung von Isopropyliden-zeatin-ribosid mit Pyrophosphorylchlorid hergestellt. Die Phosphorylierung des Isopropylidenderivats in Pyridin ergab ein Allylpyridiniumderivat, dessen Struktur spektroskopisch und durch Abbau bewiesen wurde.

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[25] Professor F. C. STEWARD, private communication.

Studies on the Structure of Collagen IV[1]. A C-Terminal Sequence from the Pronase Treated Collagen

In a previous paper[2] the results of a study on the N- and C-terminals of the tropocollagen molecule were reported. From this study it became clear that glycine (gly), arginine (arg), lysine (lys) and alanine (ala) are present as C-terminals in native collagen in the ratio 3:1:1:1 whereas gly, lys and phe were found in pronase treated collagen in a ratio 5:12:1. Pronase treatment was performed according to DRAKE et al.[3]. The increase in the total number of C-terminals and the appearance of phe together with the change in the proportion of individual amino acids indicated a limited action of pronase on the C-end of the tropocollagen molecule. Our further search was directed towards elucidation of the amino acid sequence around the sites where the pronase action is terminated.

Using the technique of partial hydrazinolysis[4], it was possible to separate from both pronase treated soluble and insoluble collagen one peptide (I) (Rf value 0.75 in butanol-pyridine-acetic acid-water 30:20:6:24 – solvent system A) which did not react with the silver nitrate solution suggesting that it had a free carboxyl group and was apparently a C-terminal peptide.

The amino acids present in the peptide (I) (identified by chromatography on amino acids analyzer and as 5-dimethylamino-1-naftalen-sulfonyl- and 2,4-dinitrophenyl-derivatives) were gly, pro, hypro in a ratio 2:1:1. Keeping in mind the possible splitting of some peptide-hydrazides we looked very carefully for the peptide (I) in the mixture obtained after the hydrazinolysis of native soluble collagen. Though the mixtures of peptide hydrazides from pronase-treated and untreated tropocollagen tailed badly in solvent system A and most other solvents, the yellow spot in question is easy to distinguish as only one other spot has a higher Rf value. Following this yellow spot there is a free zone of about the same size as the spot, and then the bulk amount of hydrazides appear. Rechromatography in propanol-water 60:40 resulted in a spot with the Rf value 0.65 with almost no background.

Our search for the same peptide in tropocollagen failed. We conclude that this peptide is a C-terminal peptide of the pronase-treated collagen.

Further work was directed toward elucidating the sequence of peptide (I). It was found that this peptide does not yield the phenylthiohydantoin after reaction with phenylisothiocyanates suggesting the presence of either proline or hydroxyproline on the *N*-end of the molecule. Repeated and prolonged (14 h) hydrazinolysis showed C-terminal glycine in an amount 0.32 mol/mol. Thus one of the following formulae could be assigned to the studied peptide:

(a) (pro.hypro).gly.gly
(b) pro.gly.hypro.gly
(c) hypro.gly.pro.gly

Of the sequence possibilities mentioned the pro.hypro.gly.gly seem most probable considering the frequence of the gly.pro.hypro (gly.pro.X) sequence in the collagen molecule.

The sequence of peptide (I) indicates that pronase attacks at the C-end of the tropocollagen molecule. Moreover it suggests that the action of pronase is terminated by a typically collagenous sequence gly.pro.X. If one takes into account the structure of peptides isolated from the pronase digest of collagen[5], one can see that either these peptides are completely of non-collagenous character or have a collagenous sequence at their *N*-end. The non-

Examples of peptides and/or combined sequences of these peptides isolated by ROSMUS et al.[5] from pronase digest of insoluble calf skin collagen

Gly.Pro.Ala.Gly.Ileu
Gly.Ala.Arg.Gly.Arg
Gly.Pro.Ala.Val.Tyr
Gly.Pro.Ala.(Gly,Leu,Ileu).(Gly,Phe)

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