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SYNTHESIS OF 2-METHYLTHIO-*CIS*- AND *TRANS*-RIBOSYLZEATIN AND THEIR ISOLATION FROM *PISUM* *t*RNA

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Key Word Index—*Pisum sativum*, Leguminosae, pea, biological activity, cytokinins, 6-(4-hydroxy-3-methyl-2-butenylamino)-2-methylthio-9-β-D-ribofuranosylpurine, 2-methylthio-9-β-D-ribofuranosyl-*cis*- and *trans*-zeatin

Abstract—The *cis* isomer of 6-(4-hydroxy-3-methyl-2-butenylamino)-2-methylthiopurine and its 9-β- and 9-α-D-ribofuranosyl derivatives have been synthesized and their physical and spectroscopic properties are described The biological activities of these compounds have been determined in the tobacco bioassay and are compared with those of 6-(4-hydroxy-3-methyl-*trans*-2-butenylamino)-2-methylthiopurine and its β-ribofuranoside The 6-(4-hydroxy-3-methyl-2-butenylamino)-2-methylthio-9-β-D-ribofuranosylpurine (ms-ribosylzeatin) isolated from a *Pisum* *t*RNA preparation was shown to consist of both isomers which were separated by TLC and identified by comparisons of UV and MS with those of the synthetic compounds

INTRODUCTION

AMONG the cytokinin-active ribonucleosides isolated from *t*RNA[1] are ribosylzeatin [6-(4-hydroxy-3-methyl-2-butenylamino)-9-β-D-ribofuranosylpurine][2–5] and ms-ribosylzeatin [6-(4-hydroxy-3-methyl-2-butenylamino)-2-methylthio-9-β-D-ribofuranosylpurine],[2,3,6] both of which exhibit *cis–trans* isomerism in the N^6-side chain The synthesis of the *trans* isomer in each case[3,7] has aided greatly in the identification of these compounds from natural sources,[1,3–5,8] but to answer the question of the stereochemistry of the side chain double bond, the *cis* isomer had to be synthesized as well This has been done in the case of ribosylzeatin, and the presence of both geometric isomers in plant *t*RNA has been established by comparisons with the synthetic *cis* and *trans* isomers of ribosylzeatin [2,4,5,9] We now report the syntheses and biological activities of ms-*cis*-zeatin and its ribosides (α and β anomers) and the use of the β anomer, together with synthetic ms-ribosyl-*trans*-zeatin,[3] to demonstrate the presence of both the *trans* and *cis* isomers in a *t*RNA preparation obtained from pea shoots (*Pisum sativum* L) [2]

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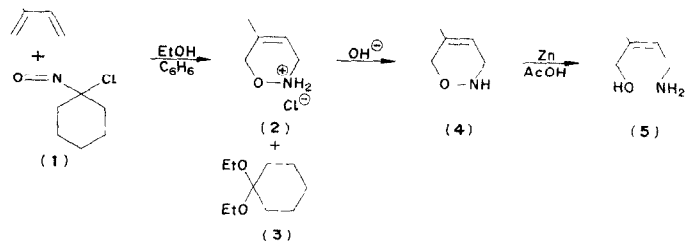
RESULTS AND DISCUSSION

Syntheses and MS

The synthesis of 6-(4-hydroxy-3-methyl-*trans*-2-butenylamino)-2-methyl-thio-9-β-D-ribofuranosylpurine (**8**) has been reported previously [3] The synthesis (**1** → **4**) of 4-hydroxy-3-methyl-*cis*-2-butenylamine (**5**), used earlier in the preparation of *cis*-zeatin[10 12] and its riboside (**6**),[9] is described here in detail Also described is the synthesis of 6-(4-hydroxy-3-methyl-*cis*-2-butenylamino)-2-methylthiopurine prepared by condensation of the *cis*-hydroxyamine **5** with 6-chloro-2-methylthiopurine



(1) (2) (3) (4) (5)

2,6-bis-(Methylthio)purine failed to react readily with 4-hydroxy-3-methyl-*cis*-2-butenylamine (**5**), whereas 6-chloro-2-methylthiopurine and **5** yielded 6-(4-hydroxy-3-methyl-*cis*-2-butenylamino)-2-methylthiopurine (ms-*cis*-zeatin) (**7a**) At the riboside level, 2,6-bis-methylthio-9-β-D-ribofuranosylpurine[13] was even less reactive than the free base, and while 6-chloro-2-methylthio-9-β-D-ribofuranosylpurine, with a better leaving group at the 6-position, is known,[14] it is rather inconvenient to prepare Accordingly, we turned to an alternate synthetic pathway that involved incorporation of the methylthio group in the final step 2,6-Dichloro-9-(2 3 5-tri-*O*-acetyl-β-D-ribofuranosyl)purine was first prepared by the fusion method of Sato[15] and was then caused to react with the hydroxyamine **5** in the usual manner, followed by treatment with methanolic ammonia to insure complete removal of the acetyl protecting groups The intermediate 2-chloro derivative of ribosyl-*cis*-zeatin was purified by chromatography on Sephadex LH 20 and treated with sodium methyl mercaptide [16] The resulting product was purified by chromatography on Sephadex LH 20 two components were isolated and these were identified as the β and γ anomers (**7**, **9**) on the basis of the NMR signals of the anomeric protons The chemical shift for this proton in one of the anomers was practically identical with that of the 1 proton in 6-(4-hydroxy-3-methyl-*cis*-2-butenylamino)-9-β-D-ribofuranosylpurine (**6**) and hence assigned to the β anomer The initial fusion reaction leading to 2 6-dichloro-9-(2 3 5-tri-*O*-acetyl-β-D-ribofuranosyl)purine was suspected as the cause of the final anomeric mixture and examination of the 220 MHz NMR spectrum of the fusion product revealed two complete sets of signals Further investigation showed that the degree of anomerization was related in part to the efficiency with which the acetic acid by-product of the fusion was removed by vacuum

Spectroscopic data were used at every stage for unambiguous structure assignments It was especially useful to be able to compare the data for **7** and **8** directly as in the

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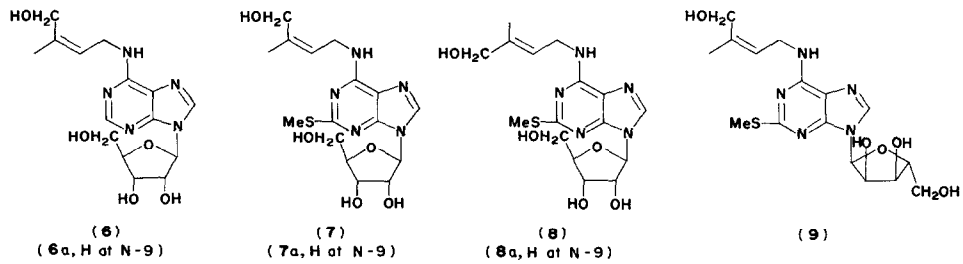
[14] SATO T (1968) *Synthetic Procedures in Nucleic Acid Chemistry* (ZORBACH W W and TIPSON R S eds), Vol 1, p 264, Wiley-Interscience New York
[15] Ref 14, p 266
[16] MAGUIRE M H NOBBS D M EINSTEIN R and MIDDLETON J C (1971) *J Med Chem* **14**, 415

cases of *cis*- and *trans*-zeatin and their ribosides [5,8–12] The mass spectra of the synthetic *cis* (**7**) and *trans* (**8**) isomers of ms-ribosylzeatin were very similar [3,11] For the two naturally occurring samples described below, the MS were determined consecutively on the same instrument at 70 eV using only slightly different probe temperatures, 200° vs 190° and were also quite similar (see below) The MS for the *cis* sample showed relatively higher intensities for the larger fragment ions (*m/e*) while that for the *trans* isomer showed higher intensities for the smaller fragment ions Since exact reproducibility of spectra taken at different times on different instruments and with slight variation in conditions may be uncertain, for distinguishing between *cis* and *trans* zeatin isomers and their ribosides, either unsubstituted or substituted, we place greater reliance on TLC on silica plates in chloroform–methanol (9 1) [9–11] Using this system, ms-ribosyl-*cis*-zeatin (**7**) moves faster, with an R_f value of 0 20 vs 0 16 for ms-ribosyl-*trans*-zeatin (**8**) The same system is useful for separation by column chromatography and isolation of the pure isomers Another TLC system in which the *cis* isomer migrates appreciably faster than the *trans* isomer (e g 0 51 vs 0 37) is on silica plates in $CHCl_3$–HOAc (4 1)



Comparison of biological activities of trans *and* cis *isomers*

In the tobacco bioassays illustrated in Fig 1a, zeatin (*trans* isomer) is *ca* 40 times more active than *cis*-zeatin (**6a**) This value compares with the average value based on a large number of recent experiments, but is somewhat smaller than the value of > 50 times, first reported [10] Of the 2-methylthio-substituted pair, ms-*trans*-zeatin (**8a**) is also considerably

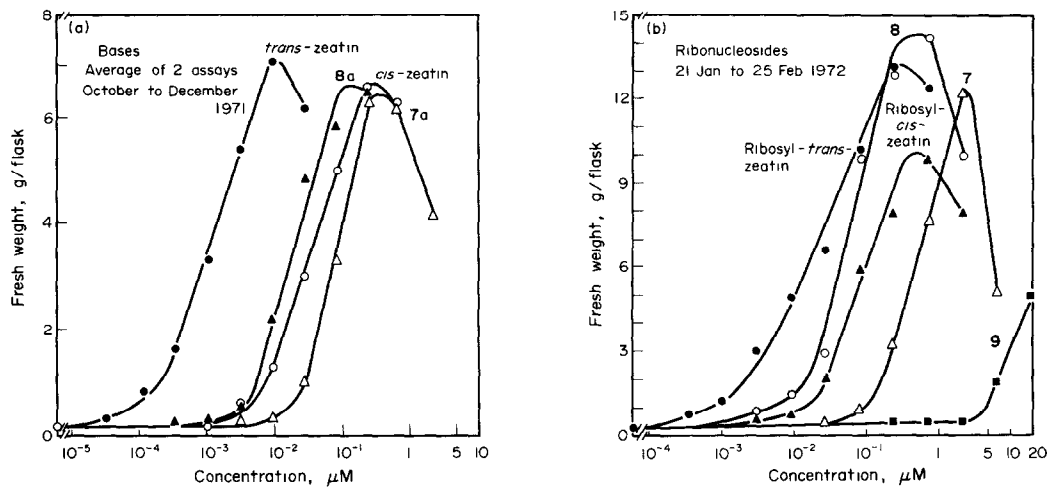


FIG 1 ACTIVITIES OF THE *cis* AND *trans* ISOMERS OF ZEATIN AND METHYLTHIOZEATIN (a) AND THEIR RIBOSIDES (b) IN THE TOBACCO BIOASSAY

more active than the *cis* isomer (7a) but the *trans*-*cis* activity ratio of 7 is based on only two experiments As shown in Fig 1b, the ribosyl derivatives are less active than the corresponding free bases and the *trans* isomer in each case is the more active *Trans*–*cis* activity ratios of *ca* 5 for the ribosylzeatin isomers and *ca* 10 for the ms-ribosylzeatin isomers (**8, 7**) are estimated from the available data It is of interest that the γ anomer of ms-ribosyl-*cis*-zeatin (**9**) is only about one twenty-fifth as active as the β anomer (**7**)

Isolation and identification of ms-ribosyl-cis and trans-zeatin from Pisum *tRNA*

A *t*RNA preparation from *Pisum sativum* L var Alaska was obtained in the usual manner [2] and the cytokinin-active ribonucleosides were isolated as summarized below The purity of the *t*RNA preparation before hydrolysis was 82% (A_{260}/dry wt) Electrophoresis on a 2·4% polyacrylamide gel[17] of a sample indicated that the A_{260} absorbing portion contained 15% high MW material (>25*S*) 21% low MW material (<4*S*) and 4% 4–5*S* RNA Although the 2·4% gel does not resolve 4 and 5*S* RNA experience with other pea preparations fractionated on a 7·5% gel indicated that the 4 5*S* region contains *ca* 90% 4*S* RNA On this basis the *t*RNA preparation is estimated to contain 47% 4*S* RNA From the equivalent of 670 mg of pure 4*S* *t*RNA material, *ca* 90 μg of ms-ribosylzeatin was isolated Of this isolate *ca* 70 μg was separated by TLC into roughly equal amounts of *cis* and *trans* isomers The calculated biological activities of the two samples were 0·23 KE/μg of hydrolyzed presumed *trans* isomer (**8** → **8**a) and 0·048 KE μg of hydrolyzed presumed *cis* isomer (7 → 7a)

The cytokinin-active fraction that had the same R_f in $CHCl_3$ HOAc (4 1) as a sample of synthetic **8** employed as a standard exhibited UV maxima at 243 and 280 nm in methanol, typical of a 2-methylthio-N^6-substituted adenosine and the absorbance corresponded to 62 ± 5 μg of the expected ribonucleoside The low resolution MS at 70 eV, probe temperature 200 gave prominent peaks at *m/e* (%Σ) 397 (0·02) 382 (0·17), 380 (0·04), 308 (0·01), 294 (0·09) 274 (0·01) 265 (0·64), 250 (3·62), 246 (0·87) 245 (1·03), 232 (2·15), 206 (1·01), 194 (0·65) 181 (1·87), 165 (1·20) 151 (1·55) and 135 (2·14) *inter alia*, typical of 6-(4-hydroxy-3-methyl-*cis*- or *trans*-2-butenylamino)-2-methylthio-9-β-D-ribofuranosylpurine and with the R_f information indicative of the *trans* isomer

The cytokinin-active fraction that had the same R_f as a sample of synthetic **7** employed as a standard exhibited UV maxima at 242 and 281 nm in ethanol typical of a 2-methylthio-N^6-substituted adenosine and the absorbance corresponded to 52 ± 5 μg The low resolution MS at 70 eV probe temperature 190 gave prominent peaks at *m/e* (%Σ) 397 (0·14) 382 (0·89), 380 (0·20) 377 (0·05) 313 (0·07) 294 (0·34) 288 (0·03) 274 (0·09), 265 (0·13) 250 (0·85), 246 (0·57) 245 (0·84) 232 (0·78) 206 (0·43) 194 (0·54), 181 (1·29), 165 (0·53), 151 (0·51) and 135 (0·96) *inter alia* satisfactory for 6-(4-hydroxy-3-methyl-*cis*-2-butenylamino)-2-methylthio-9-β-D-ribofuranosylpurine (**7**)

In the case of the ribosylzeatin isomers reported earlier for the same *t*RNA preparation,[2] the visual estimates of the UV absorbtion of the two spots of ribosylzeatin on TLC plates indicated amounts of *cis* and *trans* isomers of the same order However, a contaminant may have contributed to the UV absorbtion in the region of the *trans* isomer, since the estimate based on biological activity was closer to about a 40 1 *cis* *trans* ratio The latter ratio is apparently the more representative as comparable values have been found on the basis of both UV absorbtion (in solution) and biological activity of the isomers isolated from other *Pisum* *t*RNA preparations It may be noted that we have

[17] LOENING U E (1967) *Biochem J* **102**, 251

apparently managed to recover the ms-ribosylzeatins in greater yield, based upon UV absorbtion measurements, in this set of experiments, as compared with those initially reported [2]

TLC of synthetic **7** in $CHCl_3$–HOAc (4 1) or $CHCl_3$–MeOH (9 1) yielded only a single UV absorbing spot Bioassay of the chromatogram developed in $CHCl_3$–HOAc showed that biological activity was associated only with this spot Furthermore, a 10 μM aqueous solution of synthetic ms-ribosyl-*cis*-zeatin stored for 1 yr at 5° yielded only one spot, and its R_f corresponded to the *cis* isomer on thin layer chromatograms developed with the above solvent systems These results indicate that no appreciable conversion of the *cis* to the *trans* isomer occurs under the conditions of the experiments and, therefore, that the isolated ms-ribosyl-*trans*-zeatin is not an artifact due to such conversion

From a *t*RNA preparation of field-grown, vegetative alfalfa (*Medicago sativa* L var Vernal), we have also identified both ms-ribosyl-*cis*-zeatin (**7**) and ms-ribosyl-*trans*-zeatin (**8**), as shown by chromatography and UV spectra From this source, based on UV spectra, we estimate a ratio of *cis* to *trans* isomers of 9 1

EXPERIMENTAL

M ps are corrected NMR spectra were recorded on Varian Associates 60, 100 and 220 MHz instruments Chemical shifts were measured using tetramethylsilane (TMS) as an internal standard A Varian MAT CH-5 mass spectrometer was employed Gas chromatographic analyses were carried out on a 90 cm × 6 mm SE 30 column (15% on Chromosorb P) using an F & M Model 300 instrument Microanalyses were performed by Mr Joseph Nemeth and associates

1-*Chloro*-1-*nitrosocyclohexane* (**1**) This compound was prepared from cyclohexanone oxime by the method of Muller *et al* [18 19] To avoid an explosion hazard, a final distillation was omitted NMR showed the only impurity at this stage to be a small amount of Et_2O

5-*Methyl*-3,6-*dihydro*-1,2-*oxazine hydrochloride* (**2**) A solution of 72 g (0 49 mol) of I and 2 g of hydroquinone was dissolved in 200 ml 2 5 N isoprene in C_6H_6 and 58 ml EtOH The resulting solution was flushed with N_2 and allowed to stand overnight in a stoppered flask The flask was kept in the dark below 30 The solvents were removed *in vacuo* and the residue was washed with several vols of Et_2O to free it from **3** giving a crude product that was used directly in the next step

5-*Methyl*-3 6-*dihydro*-1,2-*oxazine* (**4**) Crude **2** was dissolved in H_2O and treated with 28 g (0 5 mol) KOH with stirring in an ice bath The product was taken up in Et_2O and the Et_2O soln was dried over KOH A sticky ppt formed, and the dried soln was filtered along with celite to prevent clogging Removal of the Et_2O yielded a dark oil, which GLC showed to have one component that was neither solvent nor polymeric Distillations of this oil did not improve its quality, so it was reduced directly

4-*Hydroxy*-3-*methyl*-cis-2-*butenylamine* (**5**) Liquid **4** was dissolved in 700 ml HOAc and shaken with 80 g (1 2 mol) of freshly activated zinc dust After the mildly exothermic reaction had subsided, shaking was stopped and the mixture was stirred overnight in a stoppered flask The zinc was removed by filtration and washed with AcOH The AcOH was stripped *in vacuo*, and the resulting oil was made basic with conc aq KOH (ice bath) and extracted with $CHCl_3$ The $CHCl_3$ extracts were dried, removal of the $CHCl_3$ yielded 32 g of a dark red–brown oil, which GLC analysis showed to have one major component other than some unreacted **4** Attempted purification effected decomposition *Cis* geometry has also been realized in a synthesis of hydroxygalegine by Desvages and Olomucki,[20] a reference that was inadvertently omitted in describing our stereoselective route to the naturally occurring hydroxygalegine via **1** → **5** [21]

6-(4-*Hydroxy*-3-*methyl*-cis-2-*butenylamino*)-2-*methylthiopurine* (**7a**) 100 mg of 6-chloro-2-methylthiopurine, 500 mg of **5**, and 1 ml EtOH were placed in a sealed tube and heated at 140° overnight The contents of the tube were washed out with MeOH, 1 g silica was added, and the mixture was reduced *in vacuo* to a brown paste, which was applied to a 50-g silica column and eluted with $CHCl_3$–MeOH (9 1) The product-containing fractions were pooled, and removal of solvent gave a brown paste, which on trituration with EtOAc was partially decolorized Recrystallization from EtOH with charcoal decolorization yielded pure material (40 mg), m p 225–230° UV λ_{max}^{EtOH} 279 nm (ϵ = 15 800) 242 (25 000), λ_{min} 257, 220 λ_{max}^{EtOH} (0 1 N HCl) 272 (15 100) 252

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[19] Muller E, Metzger, H and Fries D (1954) *Chem Ber* **87,** 1449

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[21] Leonard N J and Playtis A J (1972) *J C S Chem Commun* 133

(21 600) λ_{min} 277 λ_{max}^{EtOH} (0·1 N NaOH) 286 (14 800) 231 (28 300) λ_{min} 256 NMR [$(CD_3)_2SO$] δ = 1·76 (s, 3) 4·0–4·4 (*m*, 4) 4·7 (*m*, 1) 5·42 (*t*, 1) 7·7 (*m*, 1) 8·00 (s, 1) MS peaks at *m/e* 265, 250, 248, 247, 232, 206, 200, 182, 181, 165, 151, 135, 119 (Found: C, 49·55; H, 5·70. $C_{11}H_{15}N_5OS$ requires: C, 49·79; H, 5·70%)

2,6-*Dichloro*-9-(2,3,5-tri-O-*acetyl*-β-D-*ribofuranosyl*)*purine* The material was prepared from the fusion of 2,6-dichloropurine and 1,2,3,5-tetra-*O*-acetyl-β-D-ribose following the procedure of Sato[15] The crude unrecrystallized mixture of anomers was used directly in the next step when it was found that both anomers could be carried through the entire synthetic sequence and separated cleanly in the final step

2-*Chloro*-6-(4-*hydroxy*-3-*methyl*-*cis*-2-*butenylamino*)-9-β-D-*ribofuranosylpurine* A soln of 800 mg 2,6-dichloro-9-(2,3,5-tri-*O*-acetyl-β-D-ribofuranosyl)purine 4 g of **5** and 16 ml EtOH was heated at reflux for 2·3 hr under N_2 and then stirred at room temp overnight The EtOH was removed *in vacuo* and the residue was dissolved in 200 ml satd methanolic NH_3 After stirring overnight in a sealed flask the solvent was removed and the residue was partitioned between Et_2O and H_2O The H_2O layer was reduced to a small vol and applied to a 100-g Sephadex LH-20 column The column was eluted with H_2O and monitored by UV absorption at 273 nm The product-containing fractions were combined and on removal of the H_2O the product (491 mg, 71%) was obtained as a hard dry glass which was used without further purification in the next step To achieve analytical purity the crude product was dissolved in 98% *n*-BuOH applied to a cellulose column and eluted with the same solvent The product-containing fractions were pooled and reduced *in vacuo* to a colorless glassy solid which was vacuum-dried overnight This was dissolved in hot EtOAc and the soln was filtered and diluted with 2 vols of low-boiling light petrol After the suspension was cooled overnight in the freezer the product was filtered and washed first with EtOAc–light petrol and then with light petrol The wet product was hygroscopic but on overnight vacuum drying at 60° an analytically pure powder with an indefinite m.p. was obtained β isomer UV λ_{max}^{EtOH} 273 nm (ε = 19 700) 204 (24 400) λ_{min} 232 λ_{max}^{EtOH} (0·1 N HCl) 274 (18 900) 205 (22 900) λ_{min} 234 λ_{max}^{EtOH} (0·1 N NaOH) 274 (20 200) λ_{min} 237 NMR [$(CD_3)_2SO$] δ = 1·76 (s, 3) 3·7 (*m*, 2) 4·0–4·3 (*m*, 5) 4·5–4·8 (*m*, 2) 5·0–5·6 (*m*, 5) 5·92 (*d*, 1) 8·38 (*m*, 1) 8·42 (s, 1) MS peaks at *m/e* 387, 384, 370, 368, 354, 296, 253, 237, 236, 235, 222, 220, 200, 193, 172, 170, 169, 114 (Found: C, 46·57; H, 5·48; N, 17·93. $C_{15}H_{20}ClN_5O_5$ requires: C, 46·70; H, 5·23; N, 18·15%)

6-(4-*Hydroxy*-3-*methyl*-*cis*-2-*butenylamino*)-2-*methylthio*-9-β-D-*ribofuranosylpurine* (**7**) The procedure used is analogous to that employed by Maguire *et al*[16] to prepare 2-alkylthioadenosines About 8 ml MeSH was condensed into a flask, cooled in a dry ice–MeOH bath and equipped with a similar condenser Roughly 460 mg (20 mg-atoms) Na was added and the cooling bath was allowed to warm slowly to room temp The reaction mixture was refluxed at room temp until all the Na had reacted while five 1-ml portions of dimethylformamide were added to help dissolve the sodium methyl mercaptide formed When the reaction was complete 15 ml of dimethylformamide was added and the flask was heated to 85° to drive off the excess methyl mercaptan into a bleach trap To the residual solution was added 490 mg of 2-chloro-6-(4-hydroxy-3-methyl-*cis*-2-butenylamino)-9-D-ribofuranosylpurine in 5 ml of dimethylformamide followed by heating at 85° for 7 hr The mixture was cooled, neutralized with HCl and reduced to dryness *in vacuo* The resulting solid was vacuum-dried overnight over P_2O_5 and extracted with EtOH The EtOH extracts were decolorized with charcoal the EtOH was removed and the residue was redissolved in a little H_2O This solution was applied to a 100-g Sephadex LH-20 column which was eluted with H_2O and monitored by UV absorption at 280 nm Product-containing fractions were pooled and reduced *in vacuo* to a small vol at which point the product crystallized as small needles m.p. 171° yield 92 mg (18%) UV λ_{max}^{EtOH} 283 nm (ε = 18 300) 244 (26 700) λ_{min} 259 224 λ_{max}^{EtOH} (0·1 N HCl) 287 (15 100) 275 (14 900) 254 (17 600) λ_{min} 281 268 232 λ_{max}^{EtOH} (0·1 N NaOH) 283 (18 500) 243 (26 300) λ_{min} 258 NMR [$(CD_3)_2SO$] δ = 1·76 (s, 3) 2·56 (s, 3) 3·7 (*m*, 2) 3·9–4·4 (*m*, 5) 4·7 (*m*, 2) 5·0–5·6 (*m*, 5) 5·93 (*d*, 1) 7·91 (*m*, 1) and 8·26 (s, 1) (Found: C, 48·32; H, 5·77; N, 17·3[illegible]. $C_{16}H_{23}N_5O_5S$ requires: C, 48·35; H, 5·83; N, 17·62%)

6-(4-*Hydroxy*-3-*methyl*-*cis*-2-*butenylamino*)-2-*methylthio*-9-α-D-*ribofuranosylpurine* (**9**) Any α-anomer present will follow **7** off the Sephadex LH-20 column Fractions containing this material were pooled and the solvent was removed The resulting solid was dissolved in the minimum amount of EtOAc and this solution was poured into 3–4 vols of low boiling light petrol The product precipitated as a pure powder with an indefinite m.p. (80–130° dec) yield 58 mg (12%) UV λ_{max}^{EtOH} 281 nm (ε 18 100) 243 (26 000) λ_{min} 257 223 λ_{max}^{EtOH} (0·1 N HCl) 289 (13 900) 274 (13 600), 254 (19 500) λ_{min} 280 269 233 λ_{max}^{EtOH} (0·1 N NaOH) 281 (18 900) 242 (26 500) λ_{min} 257 NMR [$(CD_3)_2SO$] δ = 1·77 (s, 3) 2·55 (s, 3) 3·6 (*m*, 2) 4·05–4·6 (*m*, 7) 4·7 (*m*, 1) 4·9 (*m*, 1) 5·3–5·6 (*m*, 3) 6·33 (*d*, 1), 7·82 (*m*, 1) and 8·24 (s, 1) (Found: C, 48·26; H, 5·75; N, 17·57. $C_{16}H_{23}N_5O_5S$ requires: C, 48·35; H, 5·83; N, 17·62%)

Bioassay procedures Cytokinin activities were determined in the tobacco bioassay and are based on the fr wt yields of cytokinin-dependent callus tissue Synthetic compounds were tested on tissue grown on 50 ml of medium as described[22] To avoid degradation by heat the compounds were dissolved in Me_2SO and added to the autoclaved nutrient agar medium just before it solidified[23] The isolated ribonucleosides were hydrolyzed to free

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

bases in 0·1 N HCl for 45 min at 100° to increase the activity level and were tested on tissue grown on 20 ml medium to minimize the quantities needed for the assays.[2]

Isolation of ms-ribosylzeatin from tRNA, separation and identification of the cis- *and* trans-*isomers* The tRNA was prepared from 13·2 kg of frozen, 7-day-old pea shoots (*Pisum sativum* L. var. Alaska).[2] The tissue was extracted with Tris-buffered, aq. sodium dodecyl sulfate. Protein and rRNA were removed with phenol and 3 M NaCl respectively. DNA and the major portion of the carbohydrate material were removed by two successive fractionations of the preparation on DEAE-cellulose columns. The material eluted with 1 M NaCl was dialyzed against water for 16 hr to remove residual salt and other low MW material. The tRNA (24 600 A_{260}) was hydrolyzed with *Crotalus adamanteus* venom and calf intestinal mucosa alkaline phosphatase. Lipophilic ribonucleosides were extracted with EtOAc and fractionated on a Sephadex LH-20 column with 33% EtOH. The cytokinin-active fraction, with an elution vol. corresponding to that of synthetic ms-ribosylzeatin, was further fractionated on a second LH-20 column with H_2O as eluent, and finally chromatographed on Whatman No. 1 paper with 19% (v/v) EtOH. From the observed absorbance at λ_{max} (276 nm) of the purified product and the extinction coefficient (17 200) of the synthetic compound,[3] the quantity of ms-ribosylzeatin was calculated to be 90 ± 5 μg. The isolated product had a UV spectrum characteristic of 2-methylthio-substituted N^6-isopentenyladenosines,[3,25] and it was identified as 6-(4-hydroxy-3-methyl-2-butenylamino)-2-methylthio-9-β-D-ribofuranosylpurine by comparison of the MS with that of a sample of synthetic ms-ribosylzeatin.

A year later, when a method had been worked out for separating *cis* and *trans* isomers, the residual sample (*ca.* 70 μg) that had been stored dry at −20° was dissolved in a small amount of MeOH, streaked on four TLC plates (10 × 10 cm, 6060 silica gel with fluorescent indicator, Eastman Chromagram Sheet), and developed 20 min with $CHCl_3$–HOAc (4:1). The sample separated into two UV absorbing bands with R_fs of 0·47 and 0·32, respectively. As judged by visual inspection, the former absorbed *ca.* half as much UV light as the latter. A mixture of synthetic *cis* (**7**) and *trans* (**8**) isomers of ms-ribosylzeatin chromatographed on the same plates gave R_f of 0·51 and 0·37, respectively. The two bands from the natural isolate were eluted separately with 95% EtOH and rechromatographed separately with $CHCl_3$–HOAc (4:1) along with a reference mixture. The presumed *cis* isomer moved as a single UV-absorbing band and showed no detectable UV absorption at the R_f of the *trans* isomer, but the chromatogram with the rechromatographed presumed *trans* isomer showed a faint second UV-absorbing band at the R_f of the *cis* isomer. The chromatograms were dried and developed once more in the same solvent to improve the separation of the two bands. Again, each unknown and the corresponding synthetic compound moved at the same rate. The presumed *trans* isomer on one chromatogram was eluted with 95% EtOH, concentrated, transferred to Whatman No. 1 paper and developed with 19% EtOH for 6 hr. It moved as a single UV absorbing band which had an R_f of 0·64, as compared with 0·70 for **8**, the synthetic *trans* isomer. The two synthetic isomers move at the same rate in this system (19% EtOH on paper). The lower R_f of the isolated compared with the synthetic isomers could be due to impurities in the former. The presumed *cis* isomer, pooled from the two chromatograms, was similarly re-chromatographed. It gave a single UV absorbing band at R_f 0·59 as compared with 0·70 for **7** the synthetic *cis* isomer. The bands were eluted separately with 95% EtOH and, after removal of 5% aliquots for bioassay, were dried in air. The aliquots for bioassays were hydrolyzed to the respective bases and then incorporated into the bioassay medium.[2]

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