Purines, Pyrimidines, and Imidazoles. Part XXVIII.[1] Syntheses of 9-β-D-Ribofuranosylzeatin 5′-Phosphate, a Naturally Occurring Adenylic Acid Derivative with Plant Cell-division Promoting Activity, and a New Synthesis of 6-Chloro-9-β-D-ribofuranosylpurine 5′-Phosphate

By G. Shaw,* B. M. Smallwood, and D. V. Wilson, School of Chemistry, University of Bradford, Bradford 7

9-β-D-Ribofuranosylzeatin 5′-phosphate has been synthesised (*a*) by the reaction of 6-chloro-9-(2′,3′-*O*-isopropylidene-β-D-ribofuranosyl) purine with *trans*-4-amino-2-methylbut-2-en-1-ol and triethylamine, phosphorylation of the resulting isopropylidene zeatin riboside with pyrophosphoryl chloride, and removal of the protecting group by mild acid hydrolysis, and (*b*) by the reaction of 6-chloro-9-β-D-ribofuranosylpurine 5′-phosphate with the *trans*-amino-2-en-1-ol in potassium hydrogen carbonate solution. The identity of the synthetic nucleotide with a naturally occurring zeatin ribonucleotide confirms the structure of the latter. A new and improved synthesis of the 6-chloro-9-β-D-ribofuranosylpurine 5′-phosphate by phosphorylation of 6-chloro-9-2′,3′-*O*-isopropylidene-β-D-ribofuranosylpurine with pyrophosphoryl chloride and removal of protecting groups under mild acid conditions is described.

Part XXIV [2] of this series recorded the synthesis of zeatin (I) and its 9-β-D-ribofuranoside (II), the first naturally occurring plant-growth substances with pronounced cell-division promoting activity (cytokinins) to be isolated in pure form from a plant tissue, in this case sweet corn (*Zea mays*) kernels. However, only a small proportion of the total cell-division promoting activity of crude sweet corn extracts is due to zeatin; [3] moreover, bioassay of chromatogram zones generally reveals the presence of three, or occasionally four,[4] active materials, two of which correspond to the above mentioned compounds (I) and (II). A third compound has been isolated as a crystalline barium salt [10 mg. from sweet corn kernels (60 kg.)] and tentatively identified as the ribosylzeatin phosphate (III).[3] The total activity of the extracts is largely due to the nucleotide, which occurs in much greater quantity than both the aglycone and the riboside, although zeatin (I) is more active than the nucleotide at similar concentrations both in the carrot root tissue assay and in a new assay based on the ability of cytokinins to induce enlargement of excised radish cotyledons.[3] Zeatin, its riboside, and possibly the ribonucleotide have also been isolated from liquid cultures of the puffball fungus *Rhizopogon roseolus*.[5] The related compound 6-(γγ-dimethylallyl)aminopurine was isolated from cultures of the plant pathogen *Corynebacterium fascians*,[6] a bacterium which invades plants and affects their subsequent development. The same compound has also been shown to be a constituent of serine-specific yeast t-RNA.[7]

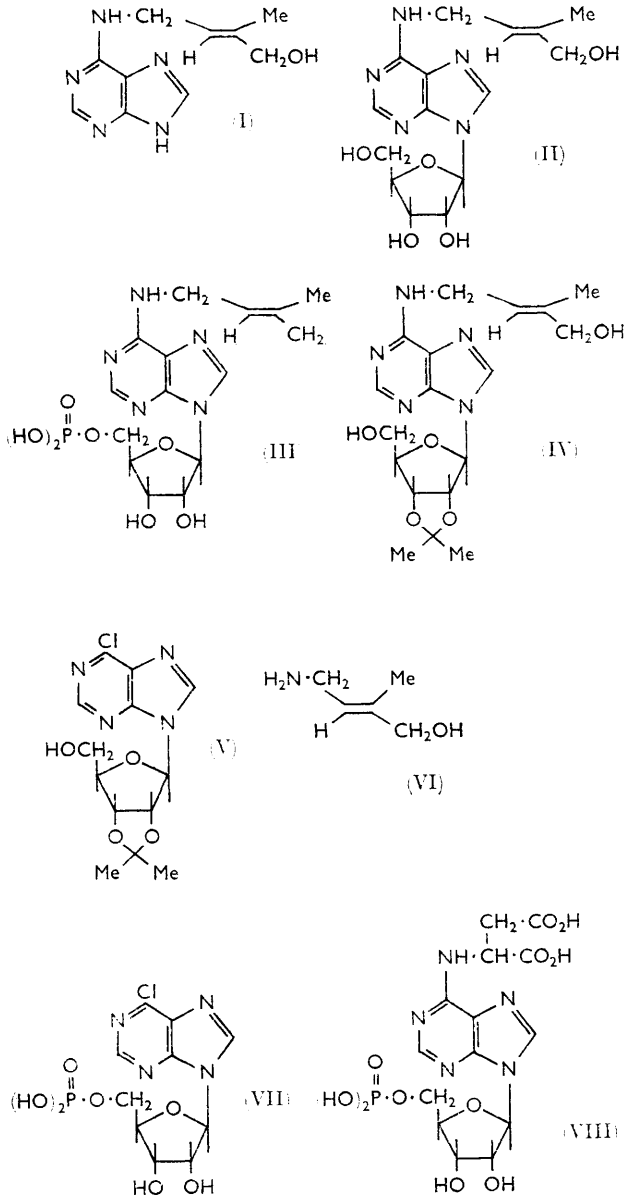
The structure assigned to the nucleotide (III) was based on its u.v. absorption spectra at different pH values, which were very similar to those of the corresponding riboside (II), and its degradation by a highly purified alkaline phosphatase or snake venom 5′-nucleotidase to a compound indistinguishable (t.l.c. and u.v. spectra) from zeatin riboside.[3] In addition, further degradation of the riboside with periodate and cyclohexylamine gave a compound identified as zeatin by its chromatographic behaviour, its u.v. spectra and a characteristic oxidation with permanganate to a mixture of adenine, *N*-(purin-6-yl)glycine and a 6-substituted aminopurine, probably a 1,2-glycol. We now report two independent syntheses of the nucleotide; a preliminary account of some of our initial experiments has been reported.[8]

Reaction of 6-chloro-9-(2′,3′-*O*-isopropylidene-β-D-ribofuranosyl)purine [9] (V) with *trans*-4-amino-2-methylbut-2-en-1-ol [2] (VI) and triethylamine in hot butanol gave the isopropylidene derivative of zeatin riboside (IV) as a crystalline solid. Phosphorylation of the nucleoside with pyrophosphoryl chloride at −30° and removal of the isopropylidene group with dilute acetic acid gave, after ion-exchange chromatography, the nucleotide (III). Under those reaction conditions no evidence was obtained for the formation of a diphosphate, and presumably any phosphorylation of the allylic hydroxy-group which may have occurred was followed by hydrolysis during the isolation procedure. The structure of the nucleotide isolated was confirmed by its u.v. absorption (similar to that of the related riboside),[2,8] its behaviour on electrophoresis,[8] its degradation with alkaline phosphatase to the riboside (II), and its degradation to zeatin by treatment with periodate and cyclohexylamine, or with Dowex 50 (H^+) resin. In addition, analysis of the compound gave the ratio purine : organic phosphate : inorganic phosphate : acid-labile phosphate : pentose 1·00 : 1·06 : 0·09 : 0·05 : 1·00. The nucleotide was also synthesised by reaction of 6-chloropurine ribotide [9] (VII) with the amino-alcohol (VI) in aqueous 3% potassium hydrogen carbonate at 38° for 70 hr. and purification by ion-exchange and pre-

[1] Part XXVII, I. E. Burrows, G. Shaw, and D. V. Wilson, *J. Chem. Soc.* (*C*), 1968, 40.
[2] G. Shaw, B. M. Smallwood, and D. V. Wilson, *J. Chem. Soc.* (*C*), 1966, 921.
[3] D. S. Letham, *Life Sciences*, 1966, **5**, 1999.
[4] C. O. Miller, *Proc. Nat. Acad. Sci. U.S.A.*, 1965, **54**, 1052.
[5] C. O. Miller, *Science*, 1967, **157**, 1055.
[6] J. P. Helgeson and N. J. Leonard, *Proc. Nat. Acad. Sci. U.S.A.*, 1966, **56**, 60; D. Klambt, G. Thies, and F. Skoog, *ibid.*, 1966, **56**, 52.
[7] H. G. Zachau, D. Dutting, and H. Feldmann, *Angew. Chem.*, 1966, **78**, 392.
[8] G. Shaw, B. M. Smallwood, and D. V. Wilson, *Experientia*, 1967, **23**, 515.
[9] A. Hampton and M. H. Maguire, *J. Amer. Chem. Soc.*, 1961, **83**, 150.

parative paper chromatography. The product was identical with the material obtained in the first synthesis.

Paper and thin-layer chromatographic comparison of the synthetic and naturally occurring nucleotides suggested that they were identical.[10]



The published preparation of 6-chloropurine ribotide [9] is not generally satisfactory; it involves the use of an enzyme and gives a poor (18%) yield. We now report an alternative method which avoids the use of an enzyme and a column separation and involves isolation under mild conditions, namely by phosphorylation of the isopropylidene 6-chlororiboside (V) with pyrophosphoryl chloride at −25°. The synthesis may be regarded as a first chemical synthesis. Hydrolysis of the product at pH 3 gave the ribotide (VII) at least 80% pure and in a yield of 30—40%. The structure of the chloro-ribotide was confirmed by its u.v. spectra, which were very similar to those recorded, and its behaviour on t.l.c., which revealed only traces of the related riboside and of inosinic acid, both of which are impurities which will not interfere in any subsequent reactions of the compound. In addition, reaction of the nucleotide with L-aspartic acid and preparative paper chromatography of the product gave adenylosuccinic acid (VIII), which acted as a substrate for yeast adenylosuccinase, showing a characteristic reduction in optical density at 280 mμ in a phosphate buffer at pH 7·2.

[10] D. S. Letham, private communication.

EXPERIMENTAL

Evaporations were carried out with a Büchi rotary evaporator, under a water-pump vacuum, with a flask temperature of 40° or less. Thin-layer and/or paper chromatograms were run on glass plates (20 × 20 cm.) coated with cellulose (Whatman CC 41) in the solvent systems (A) butanol–acetic acid–water (12 : 3 : 5), (B) propanol–ammonia (*d* 0·88)–water (6 : 3 : 1), (C) ethanol–M-ammonium acetate (pH 3·8) (7 : 3), (D) butanol saturated with water, (E) water saturated with butanol, (F) ethyl acetate–2-ethoxyethanol–formic acid (2%) (4 : 1 : 2; upper layer), (G) ethyl acetate–propanol–water (4 : 1 : 2; upper layer), (H) propan-2-ol–ammonium sulphate (1%) (2 : 1), (I) butanol–acetic acid–water (10 : 4 : 7), and (J) propanol–water (7 : 3) with an ammonia atmosphere, and spots were detected with an ultraviolet lamp or with the ammonium molybdate reagent. Ion-exchange separations were performed with an apparatus all Teflon or glass equipped with a Buchler micropump and an LKB Uvicord 4701A u.v. absorptiometer with a flow cell of 3 mm. path length for continuous recording of column eluates at 253·7 mμ, or a Vanguard model 1056 double-beam automatic u.v. analyser with continuous recording at a variety of wavelengths between 200 and 400 mμ. All resins used for ion-exchange chromatography were an analytical grade of Dowex from Bio-Rad Laboratories, Richmond, California. Spectra were measured with a Perkin-Elmer 137UV or a Unicam SP 800 spectrophotometer.

6-(4-*Hydroxy*-3-*methylbut*-trans-2-*enylamino*)-9-(2′,3′-O-*isopropylidene*-β-D-*ribofuranosyl*)*purine*. (*Isopropylidene Ribosyl Zeatin*).—A mixture of 6-chloro-9-(2′,3′-*O*-isopropylidene-β-D-ribofuranosyl)purine (0·326 g.), *trans*-4-amino-2-methylbut-2-en-1-ol hemisulphate [2] (0·165 g.), and triethylamine (0·5 ml.) in butanol (4 ml.) was boiled under reflux for 1 hr. The solution was evaporated to a small volume and cooled to give a crystalline precipitate which was collected and washed with a little butanol followed by ether, to give a pale cream solid (0·30 g.). The *isopropylidene ribosyl zeatin* gave prisms, m.p. 117—119° (from benzene) (Found: C, 55·45; H, 6·65; N, 17·55. $C_{18}H_{25}N_5O_5$ requires C, 55·25; H, 6·4; N, 17·9%), $\lambda_{max.}$ 212 (ε 9800) and 265 (ε 14,300) mμ, $\lambda_{min.}$ 231 mμ (in 0·1N-hydrochloric acid); $\lambda_{max.}$ 213 (ε 14,700) and 268 (ε 14,700) mμ, $\lambda_{min.}$ 231 mμ (at pH 7·2); and $\lambda_{max.}$ 222 (ε 4900) and 268 (ε 15,100) mμ, $\lambda_{min.}$ 229 mμ (in 0·1-N-sodium hydroxide).

6-(4-*Hydroxy*-3-*methylbut*-trans-2-*enylamino*)-9-β-D-*ribofuranosylpurine* 5′-*Phosphate*.—The isopropylidene derivative (IV) (0·15 g.) was added to pyrophosphoryl chloride (0·62 ml.) at −30° and the mixture was stirred until the solid had dissolved. The dark solution was allowed to

attain room temperature and set aside for 1·5 hr. It was then cooled to −30° and treated with a solution of barium acetate [3 g. in water (9 ml.)]. The mixture was diluted with water and the pH was adjusted to 6·5 with saturated barium hydroxide solution. The precipitate of barium phosphate was coagulated by warming and filtered off. The pH of the combined filtrate and washings was adjusted to 4 with acetic acid, and the mixture was then heated on a water-bath at 100° for 1·5 hr. The solution was evaporated and water (250 ml.) was added to the residue; the pH was adjusted to 7·5 with dilute ammonium hydroxide solution, and the mixture was chromatographed on a column (16 × 1·5 cm.) of analytical grade Dowex 1 × 8 (Br^- form; 200—400 mesh).

A large proportion of the u.v.-absorbing material was eluted when the column was washed with water; this fraction appeared to contain mainly unphosphorylated material. Most of the remaining u.v.-absorbing material was eluted as a single peak in the fractions between 0·45 and 0·66 litres with 0·008 N-hydrobromic acid. The pH of the combined fractions was adjusted to 7 with saturated barium hydroxide solution, and the solution was evaporated to a small volume. After a little solid had been filtered off it was diluted with ethanol (3—4 volumes) to give the *barium salt* of the zeatin riboside 5′-phosphate (20 mg.) as a white solid, $\lambda_{max.}$ 206 (ε 19,250) and 266 (ε 15,950) mμ, $\lambda_{min.}$ 235 mμ (in 0·1N-hydrochloric acid); $\lambda_{max.}$ 212 (ε 18,550) and 270 (ε 18,150) mμ, $\lambda_{min.}$ 241 mμ [at pH 7·2 (M/30-phosphate buffer)]; $\lambda_{max.}$ 218 (ε 14,450) and 271 (ε 16,300) mμ, $\lambda_{min.}$ 237 mμ (in 0·1N-sodium hydroxide); the figures are in close agreement with those recorded earlier for the pure zeatin riboside. The compound migrated as a single discrete u.v.-absorving spot on paper chromatograms, R_F 0·19, 0·33, and 0·03 in solvent systems (A), (J), and (D), respectively, and on t.l.c., R_F 0·52, 0·52, 0·70, 0·03, and 0·99 in solvent systems (A), (B), (C), (D), and (E), respectively; on paper electrophoresis in a formate buffer pH 3·2 at 5 kv during 1 hr. it had migrated 6·7 cm. towards the anode. Inosinic acid moved 14·2 cm. towards the anode under the same conditions.

The barium salt was analysed for inorganic phosphate, acid-labile phosphate (after 7 min. hydrolysis with N-hydrochloric acid), and total phosphate (after preliminary digestion with 60% perchloric acid for 15 min.[11]) with the ammonium molybdate ascorbic acid reagents by measurement of the resulting blue colour at 820 mμ (maximum absorption).[12] Pentose was assayed with the orcinol–ferric chloride reagents by measuring the optical density of the colour at 670 mμ.[13] The results indicated a ratio purine : organic phosphate : inorganic phosphate : acid labile phosphate : ribose of 1·00 : 1·06 : 0·09 : 0·05 : 1·00.

Reactions of the Zeatin Ribonucleotide.—(a) *Hydrolysis with an alkaline phosphatase.* A little of a solution of the barium salt described above was added to a mixture of equal volumes of 0·1N-glycine buffer (pH 8·6) and 0·05M-magnesium chloride solution. One drop of a solution containing an alkaline phosphatase preparation (9 mg./ml.) from calf intestinal mucosa (Koch-Light and Co., Colnbrook) was added, and the mixture was incubated at 37° for 3 hr. T.l.c. in four different solvent systems revealed the presence of zeatin riboside only, with neither starting material nor other substances present.

(b) *Reaction with sodium periodate and cyclohexylamine.* 0·1M-Sodium periodate (0·3 ml.) was added to a solution of the barium salt (0·5 mg.) in water (0·1 ml.). Precipitated barium periodate was removed by centrifugation, and the solution was set aside for 2 hr. at room temperature. Cyclohexylamine (0·05 g.) was then added and the solution was set aside for a further 1 hr. The solution was evaporated to a small volume; t.l.c. in solvent systems (D), (E), (F), and (G) showed the presence of zeatin, unchanged zeatin riboside, and an unidentified compound which tended to run behind zeatin on the thin-layer plates.

(c) *Catalytic hydrolysis with Dowex 50 (H^+) Resin.*—A solution of the barium salt (1 mg.) in water (2 ml.) was heated on a water-bath for 2·5 min. in the presence of Dowex 50 (H^+) resin [aqueous suspension (1 ml.)]. The resin was collected, washed with water, then eluted with 6N-ammonium hydroxide. The ammoniacal solution was evaporated to a small volume; t.l.c. in solvent systems (A), (B), (D), (E), and (G) showed the presence of zeatin, starting material, and an unidentified u.v.-absorbing compound.

6-Chloro-9-β-D-ribofuranosylpurine 5′-Phosphate.—6-Chloro-9-(2′,3′-*O*-isopropylidene-β-D-ribofuranosyl)purine (0·2 g.) was stirred into redistilled pyrophosphoryl chloride (0·675 g.) maintained at −25°; precautions were taken to exclude moisture. The mixture was allowed to attain room temperature then set aside with occasional stirring for 1·5 hr. to give a clear syrup. This was cooled to −25° and added to water (15 ml.) at 0°. Dilute lithium hydroxide solution was added to the stirred mixture at a rate sufficient to keep the pH between 3·0 and 3·2. After about 30 min. the temperature was raised to 50°; hydrolysis then appeared to be complete and the solution pH was constant. The mixture (pH 3·1) was then heated at 100° for 30 min. to remove the isopropylidene group, cooled to 0°, and treated with 2M-barium acetate (8 ml.).

The solution was set aside at 0° for 1 hr. and a precipitate was then collected by centrifugation, washed with small portions of water, and discarded. The combined supernatant and washings when mixed with absolute ethanol (230 ml.) produced a white precipitate which was collected by centrifugation. The solid was extracted twice with water (5 ml. and 3 ml.) and the extract was treated with ethanol (3 vol.) to give the *barium salt* of the 6-chloropurine nucleotide (99 mg.) which was washed successively with ethanol (2 × 5 ml.) and ether (5 ml.) then dried at room temperature *in vacuo* (P_2O_5). It had $\lambda_{max.}$ 263—264 mμ, $\lambda_{min.}$ 230 mμ in 0·2M-sodium acetate–hydrochloric acid buffer (pH 4·95), and optical ratios 0·79 (250 : 260 mμ) and 0·18 (280 : 260 mμ). Hampton *et al.*[9] quote (for the same material) $\lambda_{max.}$ 263 mμ (ε 8400), $\lambda_{min.}$ 226 mμ (ε 2100), and optical density ratios 0·82 and 0·17. Spectroscopic analysis of our material by use of the quoted molecular absorption coefficient indicated that it was at least 80% pure. T.l.c. on CC 41 cellulose in solvents (A) and (H) gave a main component with R_F values 0·39 and 0·29, respectively, and traces of inosinic acid and 6-chloropurine riboside were the only other u.v.-absorbing impurities.

Reaction of 6-Chloro-9-β-D-ribofuranosylpurine 5′-Phosphate with L-Aspartate and trans-4-Amino-2-methylbut-2-en-1-ol.—(a) The barium salt was condensed with L-aspartic acid as described by Hampton *et al.*[9] Preparative paper chromatography gave as the main reaction product, adenylosuccinic acid, which on paper chromatography had

[11] E. J. King, *Biochem. J.*, 1932, **26**, 292.
[12] D. N. Fogg and N. T. Wilkinson, *Analyst*, 1958, **83**, 406.
[13] G. Ashwell, in ' Methods in Enzymology,' Academic Press, London and New York, 1957, vol. 3, p. 73.

R_F 0·38 [solvent system (I)]. It had $\lambda_{max.}$ 268 mμ in a phosphate buffer (pH 7·2) (lit., $\lambda_{max.}$ 267 mμ at pH 7·0) and showed a characteristic reduction in absorbance at 280 mμ when incubated with yeast adenylosuccinase in 0·1M-phosphate buffer (pH 7·2).

(b) *With* trans-4-*amino*-2-*methylbut*-2-*en*-1-*ol*. Sufficient AG50W × 8 (K^+) resin was added to a suspension of the barium salt (105 mg.) in water (2 ml.) to give a clear solution; this was passed through a short column of the same resin, the column was washed with water, and the combined eluate was evaporated to a small volume (*ca.* 2·5 ml.). The *trans*-amine hemisulphate (104 mg.) and potassium hydrogen carbonate (0·16 g.) were added, and the mixture was heated at 35°. The progress of the reaction was followed by t.l.c. on CC 41 cellulose with solvent system (A) or (B). After 68 hr. the reaction appeared to be complete. The solution was acidified with hydrochloric acid, the excess of carbon dioxide was removed by partial evaporation, and then the pH was adjusted to 7 with aqueous sodium hydroxide. The solution was placed on a column of AG1 × 8 (200—400 mesh) (Br^-) resin (18·5 × 1·5 cm.) and the column was washed with water (2 l.) then eluted with a non-linear gradient of hydrobromic acid generated by means of a Buchler Varigrad, initially charged with the solutions shown in the Table. Water was

Chamber	1	2	3	4	5	6	7	8	9
M-Hydrobromic acid (ml.)	0	1	1	3	3	5	20	100	150

added to each chamber to give a solution weight of 307 g. Fractions (10 ml.) were collected. Material with significant u.v. absorption appeared in tubes 64—124 (Fraction B) and 167—183 (Fraction D).

Fraction B contained about 50% of the optical density units applied originally to the column. It was neutralised with barium hydroxide solution, evaporated to a small volume (*ca.* 4 ml.) and clarified by centrifugation. The solution, with absolute ethanol (50 ml.) gave a flocculent precipitate. The solution was stored in the refrigerator overnight and the precipitate (28 mg.) was then collected by centrifugation, washed at the centrifuge with absolute ethanol (2·5 ml.) and ether (5 ml.), and dried in air. More material (15 mg.) was recovered from the supernatant solution by evaporation and addition of ethanol. The two fractions (43 mg.) were combined, dissolved in water (8 drops), converted into ammonium salts with AG5OW × 8 (100—200 mesh) (NH_4^+) resin and further purified by preparative paper chromatography on Whatman 3 MM paper in solvent system (B). Three separate u.v.-absorbing components were detected, R_F 0·08 (corresponding to inosinic acid, R_F 0·09), 0·42 (S), and 0·61 (F). Components F and S both gave positive tests for phosphate with the molybdate spray reagents. They were eluted from the paper with water and the extracts were freeze-dried to give F [solid (12 mg.), $\lambda_{max.}$ 266 mμ, $\lambda_{min.}$ 240 mμ (at pH 2); $\lambda_{max.}$ 270 mμ, $\lambda_{min.}$ 240 mμ (in water); and $\lambda_{max.}$ 270 mμ, $\lambda_{min.}$ 242 mμ (a pH 10·5)] and S [16·9 mg., $\lambda_{max.}$ 266 mμ, $\lambda_{min.}$ 237 mμ (at pH 2); $\lambda_{max.}$ 271 mμ, $\lambda_{min.}$ 235 mμ (in water); and $\lambda_{max.}$ 270 mμ, $\lambda_{min.}$ 235 mμ (at pH 10·5)].

Compound S was identical (t.l.c., paper chromatography, electrophoresis) with the zeatin ribonucleotide prepared before and was similarly converted into zeatin riboside with alkaline phosphatase. The riboside had the same chromatographic properties as an authentic specimen. Compound F was also degraded by alkaline phosphatase to give a non-phosphorus-containing material which tended, however, to run ahead of zeatin riboside on chromatograms.

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