STUDIES ON THE SYNTHESIS OF PHOSPHORYLATED AND ALANYLATED CYTOKININS

To Anne Heleen

Promotor: Prof. Dr. H.C. van der Plas

STUDIES ON THE SYNTHESIS OF PHOSPHORYLATED AND ALANYLATED CYTOKININS

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR IN DE LANDBOUWWETENSCHAPPEN OP GEZAG VAN DE RECTOR MAGNIFICUS, PROF. DR. H.C. VAN DER PLAS, HOOGLERAAR IN DE FYSISCH-ORGANISCHE CHEMIE, IN HET OPENBAAR TE VERDEDIGEN OP WOENSDAG 2 MEI 1990 DES NAMIDDAGS TE VIER UUR IN DE AULA VAN DE LANDBOUWUNIVERSITEIT TE WAGENINGEN.

DOOR

BELAL SHADID

CONTENTS

NN08201, 1352

STELLINGEN

1) Gezien de recente resultaten van Magliozzo *et al.* valt het te betwijfelen of de antitumor werking van bleomyciine het resultaat is van uitsluitend DNA destructie.
   • R.S. Magliozzo, J. Peisach and M.R. Ciriolo, *Mol. Pharmacol.* 1989, *35*, 428.

2) Bij het onderzoek naar de praktische toepassing van witlofwortelextracten als bron van bitterstoffen moet meer aandacht besteed worden aan de onderlinge omzettingen van de bittere Sesquiterpeen lactonen.

3) In de verklaring die Schneider *et al.* geven voor de fragmentatie van bepaalde monogetosyleerde steroidale 1,3-diolen wordt ten onrechte voorbij gegaan aan 'through-bond' interacties, die kunnen optreden na deprotonering van de hydroxyl groep.
   • G. Schneider, S. Bottka, L. Hackler, J. Wölfling and P. Sohár, *Liebigs Ann. Chem.* 1989, 263.

4) De argumenten waarop Aoyama *et al.* de struktuurtoekenning van de atroopisomeren 5,15-bis(8-chinolyl)octaethylporphyrinen baseren, zijn zwak.
   • Y. Aoyama, T. Kamohara, A. Yamagishi, H. Toi and H. Ogoshi, *Tetrahedron Letters*, 1987, *28*, 2143.

5) Het bijprodukt, dat ontstaat bij 'flash vacuum thermolysis' van 3-phenyl-4,5-dihydroisoxazole-5-spirocyclobutane is niet 1-benzoyl-2-methylenepyrollidine, zoals Goti *et al.* beweren, maar (1-phenylethenyl)-2-pyrollidine.
   • A. Goti, A. Brandi, F. De Sarlo and A. Guarna, *Tetrahedron Letters*, 1986, *27*, 5271.

6) De wijze waarop Goldsby en Burke tot de struktuurtoekenning van een uit *Ambrosia peruviana* geïsoleerde sesquiterpeen diol komen, is aanvechtbaar.
   • G. Goldsby and B.A. Burke, *Phytochemistry*, 1987, *26*, 1059.

7) Het rookverbod in openbare ruimten van rijksgebouwen is zonder controle net zo gedoemd te mislukken als het verbod om harder dan 120 km per uur te rijden op rijkswegen.

8) Het is opvallend dat de aanduiding 'mw' voor een vrouw in officiële stukken vaker wordt gebruikt dan het equivalente 'dhr' voor een man. Hieruit zou men kunnen concluderen dat de schrijvers niet echt begaan zijn met het bereiken van gelijkheid tussen mannen en vrouwen.

Wageningen, 2 mei 1990

Belal Shadid

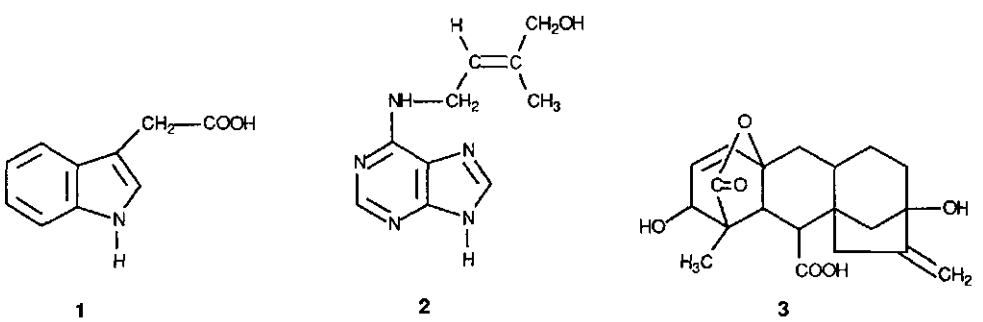
CHAPTER I

GENERAL INTRODUCTION

Plants normally grow and develop in an orderly, organized way. This is one of the many characteristics they share with all living organisms. Such orderly growth and development may be affected in many ways by the environment, which in some cases plays a very important part in controlling or triggering off various patterns of development. For example, some plants only produce flowers if the daily light period exceeds a certain critical length, and some will only do so if they have been exposed to cold at an early stage in their development.
In a general way however, it is obvious that the essential features of growth and development are built into the genetic constitution of the plant and are thus controlled from within. The internal control of growth is achieved in many and complex ways.

One of the important growth-controlling systems in plants is provided by the so-called plant growth substances or plant hormones. Plant hormones differ from animal hormones in a number of ways. Perhaps the most important difference is that animal hormones are produced in specific organs or glands and often have very highly specific effects. Plants hormones, on the other hand, though they may be produced in fairly restricted regions of plants, are manufactured by unspecialized cells and frequently have many different effects upon the plant, depending upon other circumstances. A plant hormone is an organic substance which is produced within a plant. At low concentrations it will promote, inhibit or qualitatively modify growth, usually at a site other than its place of origin. Its effect does not depend upon its calorific value or its content of essential elements[1].

Studies of the chemistry and the physiological activities of naturally occurring plant growth hormones have revealed that three major classes of growth-promoting hormones exist in plants. These are called auxins, cytokinins and gibberellins (see fig. I.1). This thesis deals with the chemistry of cytokinins.



1 2 3

Fig.I.1: Examples of each of three classes of plant growth hormones are: **1**, indolacetic acid (an auxin);**2**, zeatin (a cytokinin): **3**,gibberillin A_3 (a gibberillin).

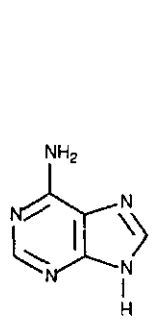
CYTOKININS

The cytokinins, a group of naturally occuring compounds which induce plant cell division[2,3] are N^6-substituted derivatives of adenine (**4**, Fig. I.2). The modern era of research on the cytokinins began in 1955 with the isolation of kinetin from old DNA of herring sperm, by Miller et al[4,5], and was found to be a highly potent growth factor. This group, working at the University of Wisconsin named this product kinetin, because of its promotion of cytokinesis (cell division) in tobacco callus tissue and characterized[6] it as 6-furfurylaminopurine (**5**, fig. I.2). As the synthetic 6-benzylaminopurine (**6**, fig. I.2) and many other N^6-substituted adenine derivatives also produce this result, the class name cytokinin was proposed and applied to the group as a whole[7].

Since the isolation of kinetin in 1955, there have been many reports of the diverse effects of cytokinins on plants. Evidence has accumulated for their role in the following[8-10]:

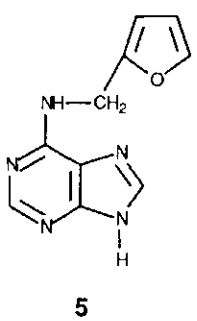
1. induction, promotion, or regulation of DNA, RNA, protein, and thiamine biosynthesis.
2. regulation of organ formation, apical dominance, and branching.
3. enhancement of flowering and of seed germination.
4. regulation of phloem transport and mobilization of metabolites.
5. preservation of flowers, fruits, vegetables, and leaves through prevention of senescence.

Certain cytokinins have also been shown to effect the behavior of mammalian cells. They have been reported to regulate the growth of mammalian cells[11-13], to inhibit platelet aggregration[14], and to have immunosuppressive activity[15]. Cytokinins have also been employed as potential anticancer agents in clinical trials[16-19].



4

adenine (6-aminopurine)



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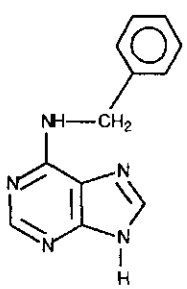
kinetin (6-furfurylaminopurine)



6

6-benzyladenine (6-benzylaminopurine)

Fig. I.2

A. OCCURRENCE OF CYTOKININS IN NATURE

Kinetin (**6**, fig. I.2) was the first compound to be discovered as a stimulator of cell division in plants. Because of the biological effect of kinetin on the growth of plants, it was suggested that a compound closely resembling to kinetin was present in plants. In 1964 Letham[20] and his associates characterized such an active factor for plant growth from young sweetcorn kernels and named it zeatin (from the generic name for corn, zeamays). They characterized it as 6-(4-hydroxy-3-methyl-E-but-2-enylamino)purine (**2**, fig. I.1).

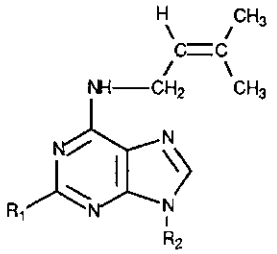
Since that time a large number of cytokinins has been isolated in free form or as a component of tRNA from bacteria, yeast, plants and animals[3,21-25] (see fig. I.3). All these compounds contain the skeleton of 6-(3-methyl-2-butenylamino)purine (**7**, fig. I.3) but feature one of the following modifications:

1. a hydroxy group on the methyl group of the side chain (cis/trans)
2. a saturated instead of a Δ^2 double bond
3. a substituent at N^9 (ribosyl, ribosyl-5'-phosphate, glucosyl or alanyl)
4. replacement of the hydrogen by a thiomethyl group at C_2' position in the purine ring .

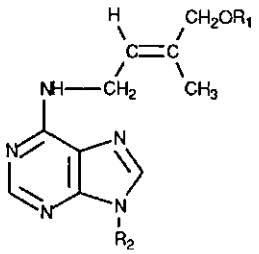
B. CYTOKININS AND NUCLEIC ACID

Unique among the plant growth substances is the well documented localization of cytokinins in nucleic acid. Different laboratories with very different directions of research came upon this phenomenon. Until recently, each nucleic acid (fig. I.4) was thought to be composed of four nucleoside structures. This view has changed markedly in the last years and at present about 10 deoxyribonucleosides and about 40 ribonucleosides have been identified[26]. Modified nucleosides occur in DNA, rRNA and tRNA. Most progress has been made with studies on tRNA. Because of their structural variety, it is impossible to treat the modified nucleosides as a single class of nucleic acid components.

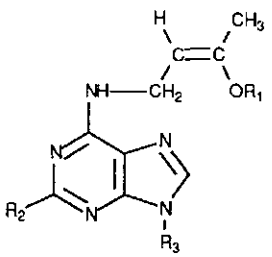
Some of these modified nucleosides which occur in t RNA of most if not all organisms[27-31], are identified as the cytokinins N^6-(Δ^2-isopentenyl)adenosine i.e. 6-(3-methyl-2-butenylamino)-9-



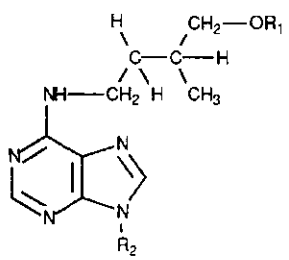
	R_1	R_2
7.	H	H
8.	H	ribosyl
9.	SCH_3	ribosyl
10.	H	riboside



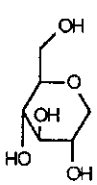
	R_1	R_2
3.	H	H
11.	H	ribosyl
12.	H	riboside
13.	H	glucosyl
14.	glucosyl	H
15.	H	alanine
16.	glucosyl	ribosyl



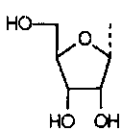
	R_1	R_2	R_3
17.	H	H	H
18.	H	H	ribosyl
19.	H	SCH_3	ribosyl



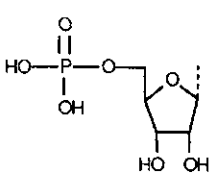
	R_1	R_2
20.	H	H
21.	H	ribosyl
22.	H	riboside
23.	glucosyl	H
24.	glucosyl	ribosyl



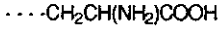
glucosyl



ribosyl



riboside

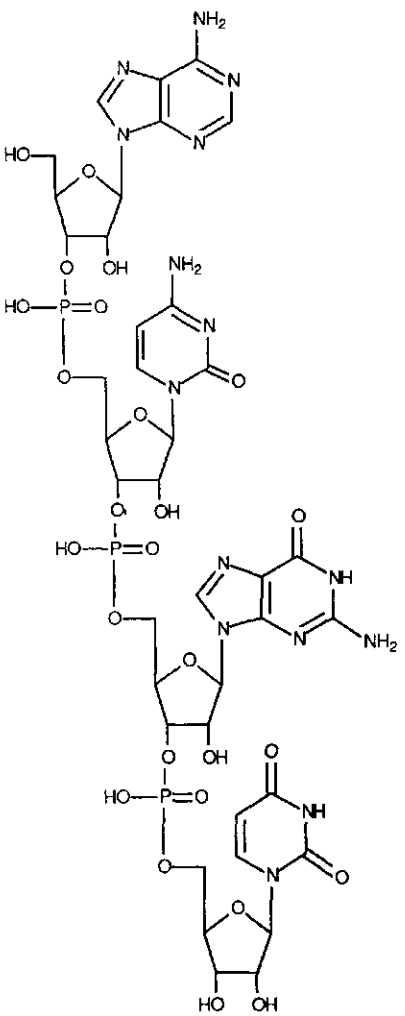


alanine

Fig. I.3

(β-D-ribofuranosyl)purine (**8**, fig.I.3), 6-(4-hydroxy-3-methyl-Z-but-2-enylamino)-9-(β-D-ribofuranosyl)purine (**18**, fig. I.3) and 6-(4-hydroxy-3-methyl-Z-but-2-enylamino)-2-thiomethyl-9-(β-D-ribofuranosyl)purine (**19**, fig. I.3).

Sequencing of several tRNA's has shown cytokinin-active nucleosides to be present exclusively in the position adjacent to the 3' end of the anticodon triplet[32-43] (the sequence of bases, which carries the matching code for the messenger RNA (mRNA), which in turn specifies the proper place for the amino acid in the protein. Many workers in this field suggested that the presence of the cytokinins in some tRNA's is essential for the function of this nucleic acid[3,26,44-47].

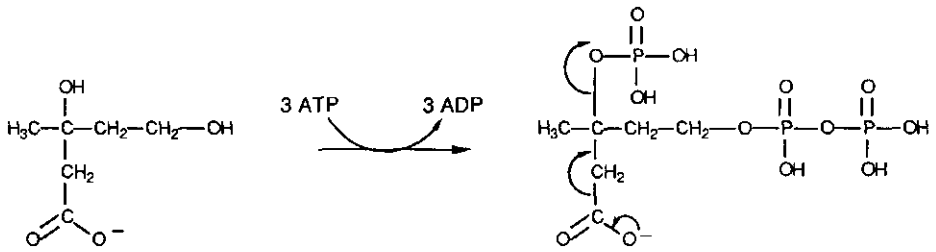


primary structure of RNA

Fig. I.4

C. THE BIOLOGICAL SYNTHESIS OF CYTOKININS IN PLANTS

Since the discovery that naturally occurring cytokinins are typically N^6-substituted adenine derivatives, that several highly active species have been isolated at the purine, ribonucleoside, ribonucleotide, glucoside and alanylated levels (free cytokinins) and that cytokinins are present



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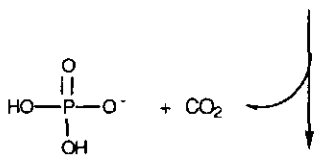

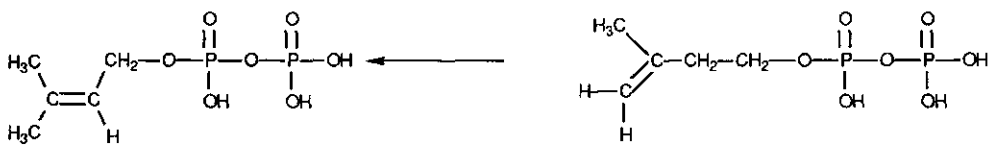

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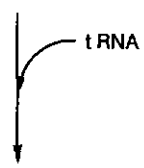
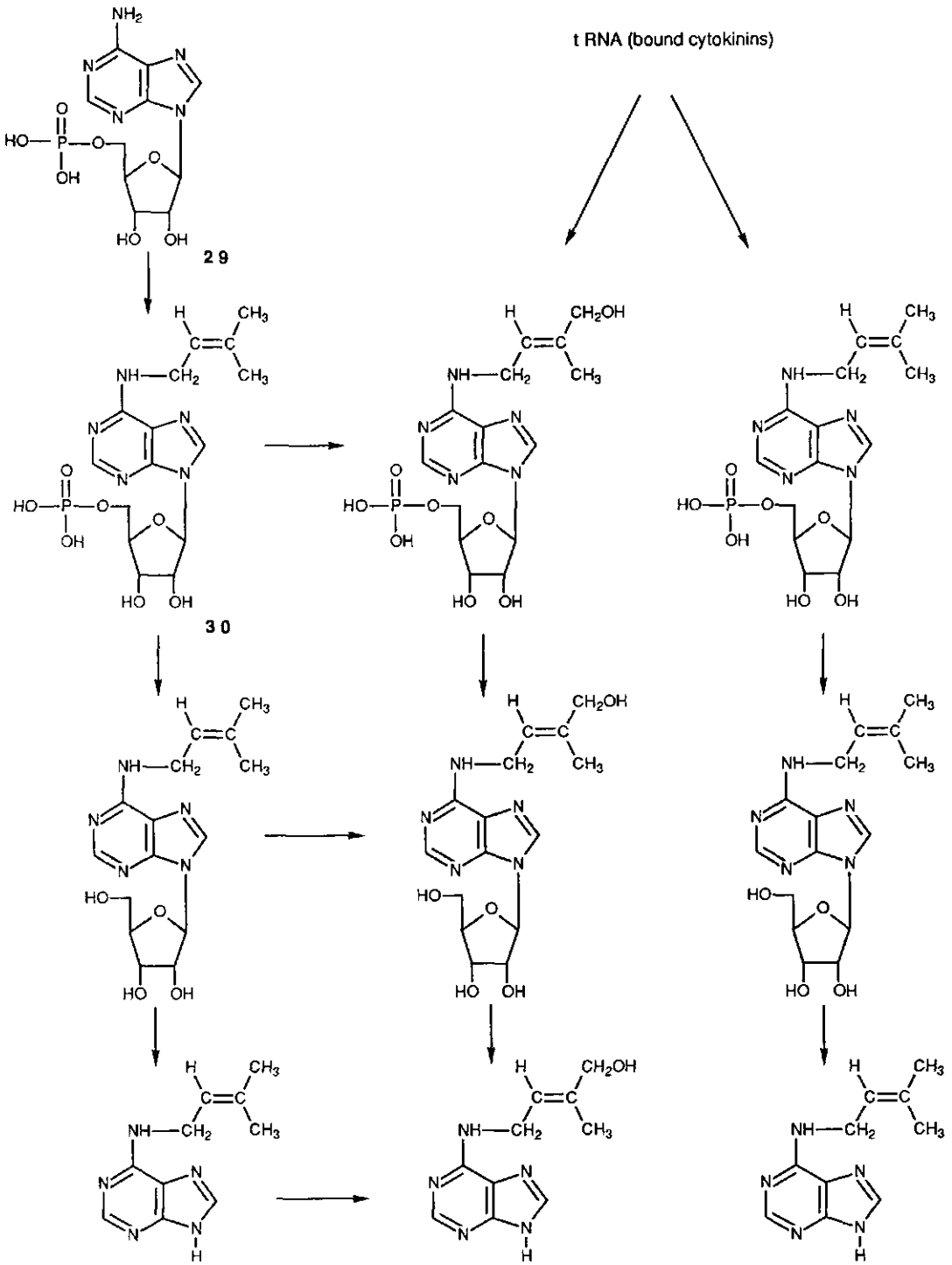


t RNA-i

t RNA-i : represents a N^6-(Δ^2-isopentenyl)adenosine residue in t RNA

Scheme I.5



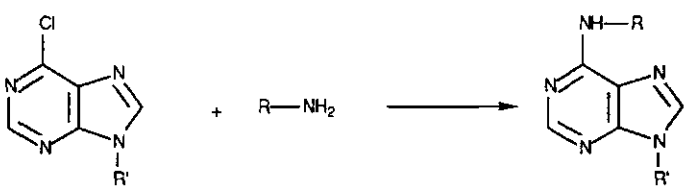
Scheme I.6

as a component of tRNA (tRNA bound cytokinins), there is continued discussion about the biosynthesis of cytokinins. At this moment success is realized in the understanding of the biosynthesis of tRNA bound cytokinins[26,45,47-55]. The isoprenoid nature of the substituent at the N^6-position of the naturally occurring cytokinins suggests that the biosynthesis of these compounds may parallel, to some extent, the biosynthesis of sterols and other terpenoid compounds. A central compound in this pathway is mevalonic acid (**25**, scheme I.5), which functions as precurser of Δ^2-isopentenyl pyrophosphate (**28**, scheme I.5). With the enzym tRNA-Δ^2-isopentenyltransferase as biocatalyst, **28** reacts with the amino group of the adenine moiety in tRNA. Only those adenosine residues in tRNA, which do not contain an isopentenyl group and are adjacent to the 3' end of the anticodon-triplet, can accept te isopentenyl group. Further modification, such as hydroxylation of the allylic methyl group, or introduction of the thiomethyl group at the C_2' position on the purine, gives the RNA bound cytokinins.

The biosynthesis of free cytokinins in plants is not clear and is subject to discussion. At this moment there are three hypotheses for the biological synthesis of the free cytokinins (see scheme I.6). The first hypothesis[62-64] is the synthesis of the free cytokinins by the breakdown of tRNA, which contains cytokinins to yield the free cytokinins. The second hypothesis[56-61] is the synthesis of free cytokinins by the reaction of adenosine 5'-phosphate (5'AMP) (**29**, scheme I.6) with Δ^2-isopentenyl pyrophosphate (**28**) to give Δ^2-isopentenyladenosine 5'-phosphate (**30**). This reaction is catalyzed by the enzym 5'-AMP-Δ^2-isopentenyltransferase. The third hypothesis is a combination of the first and the second hypothesis[65]. Convincing experimental data to confirm any of these hypotheses are lacking[66].

D. THE CHEMICAL SYNTHESIS OF CYTOKININS

To a large extent it was the already advanced knowledge in purine chemistry which permitted the characterization of the naturally occurring cytokinins. The characterization has relied very heavily on application of physical methods, particulary mass spectrometry and ultraviolet spectroscopy.



31

Scheme I.7: The common synthetic method for cytokinins. The reactions with an amine and 6-chloropurine derivative may proceed with R' equal to hydrogen or some other substituent.

The important final step in the identification of the natural product was the synthesis of the compound with the proposed structure and direct comparison with the natural product.

Therefore, syntheses of the known naturally occurring cytokinins are described in the literature. From the study of this literature, we came to the conclusion that in some cases the synthetic methods described in the literature could be improved.

It is not the object of this thesis to provide a complete survey of the various methods employed in the synthesis of cytokinins. The general strategy[67-88] for the synthesis of cytokinins is illustrated in scheme I.7. It involves the reaction of 6-chloropurine derivative **31** with the appropriate amine. In this thesis our attention is focussed on the synthesis of phosphorylated, and alanylated cytokinins, which shall be discussed in chapter II.

This synthetic study was part of a program of cooperation between our laboratory and the Centre of Agricultural and Biological Research at Wageningen, which Centre is directed to study the mechanism of action, metabolism, translocation, uptake, the biological synthesis and the physiological effects of cytokinins in plants[89-93].

This study is directed towards the synthesis of two different classes of cytokinins:

A. the synthesis of phosphorylated cytokinins:
   1. the synthesis of phosphorylated derivatives of zeatin
   2. the synthesis of phosphorylated derivatives of zeatin ribofuranoside
   3. the synthesis of phosphorylated isopentenyl adenine

B. the synthesis of alanylated cytokinins:
   the synthesis of the optically active N^9-alanyl zeatin (D and L lupinic acid).

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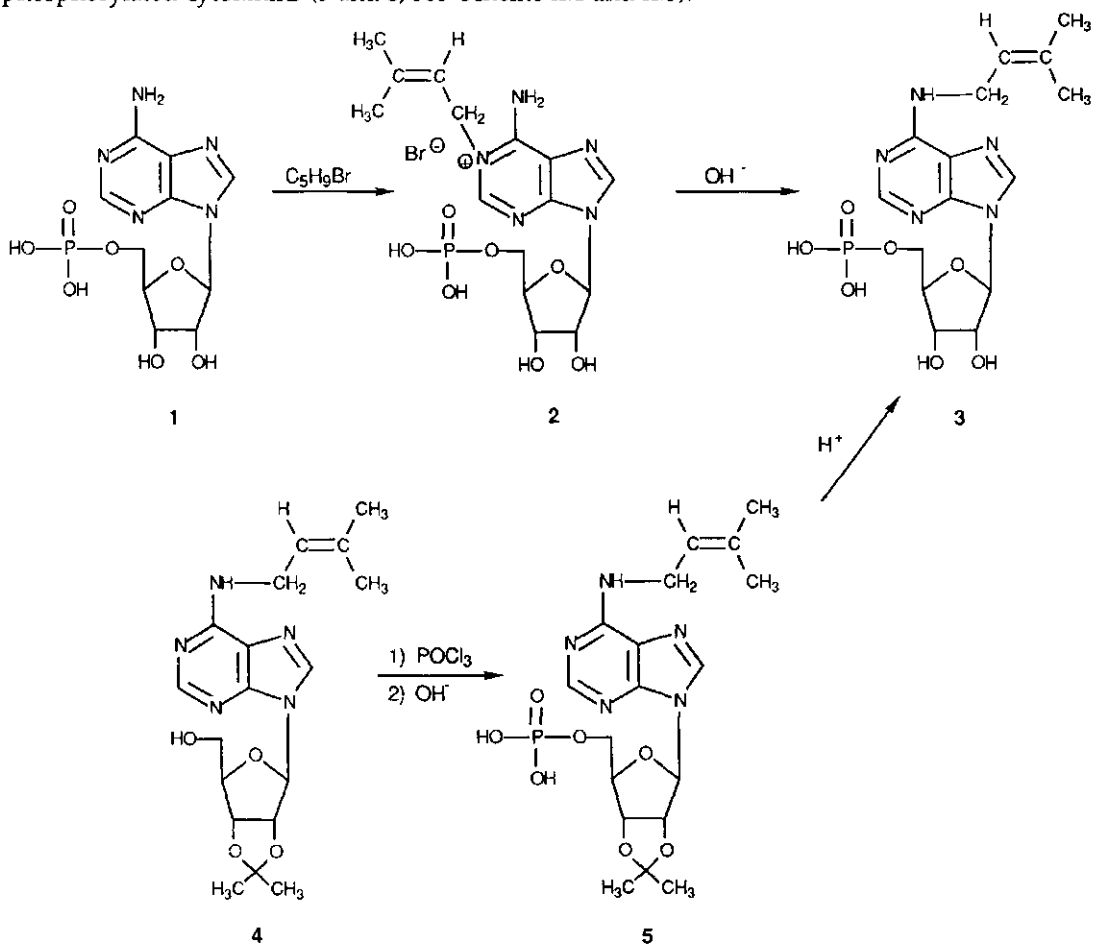
CHAPTER II

INTRODUCTION TO THE SYNTHESIS OF PHOSPHORYLATED AND ALANYLATED CYTOKININS

A. PHOSPORYLATED CYTOKININS

As indicated in chapter I, the introduction of the phosphate group and its modification in naturally occurring cytokinins is the major subject of this thesis. So far a number of chemical procedures have been reported which have been proven to be suitable for the synthesis of 5'-phosphorylated cytokinins (3 and 8, see scheme II.1 and II.3).



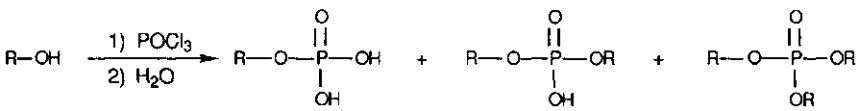
Scheme II.1

One of the first reported syntheses of the phosphorylated cytokinin N^6-(Δ^2-isopentenyl)-adenosine 5'-phosphate (3, scheme II.1) has been achieved by two different routes[1,2]. The first

route[1] involves alkylation of adenosine 5'-phosphate **1** with Δ^2-isopentenylbromide to give N^1-isopentenyl adenosinium salt **2**, and a subsequent Dimroth rearrangement of **2** into **3** by treatment with NaOH. This method, however, has two disadvantages. First, the over-all yield of compound **3** is low (19%), because the yield of the alkylation step(**1** → **2**) is only 28% and secondly, the presence of unreacted adenosine 5'-phosphate **1** makes the purification of the product difficult.

The second route[2] for the synthesis of **3** (see scheme II.1) is based on the phosphorylation of the properly protected N^6-(Δ^2-isopentenyl)adenosine derivative **4** with $POCl_3$, followed by the cleavage of the protective isopropylidene group under acidic conditions to give **3** in 40% yield. The disadvantage of this method is the use of the powerful phosphorylating agent $POCl_3$, which leads to the formation of side products as illustrated in scheme II.2

The formation of side products makes the purification procedure difficult and leads to a low yield of the desired product.

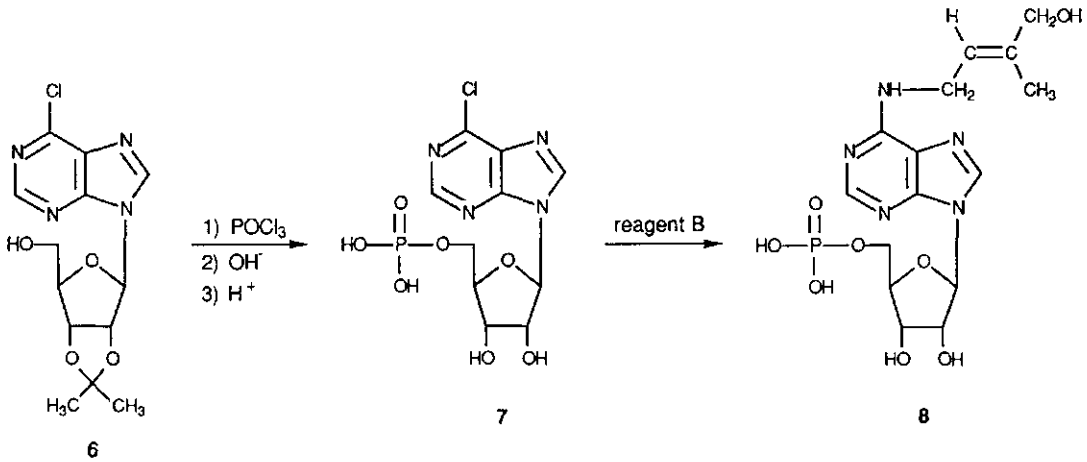


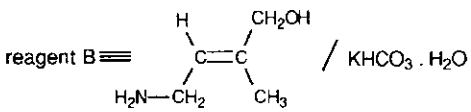
Scheme II.2

The synthesis of another phosphorylated cytokinin i.e. 6-(4-hydroxy-3-methyl-2-butenylamino)-9-β-D-ribofuranosylpurine 5'-phosphate (**8**, scheme II.3) was achieved by the phosphorylation of 6-chloro-9-((2',3'-O-isopropylidene)-β-D-ribofuranosyl)purine **6** with $POCl_3$ followed by the cleavage of the protective isopropylidene group under acidic conditions to afford 6-chloro-9-(β-D-ribofuranosyl)purine 5'-phosphate **7** (yield 30%)[3]. The coupling of **7** with *trans* 4-amino-2-methyl-2-buten-1-ol (reagent B) gives **8** in 16% yield besides an unidentified phosphorylated cytokinin.

This method also has two disadvantages. First, the use of $POCl_3$ leads to the formation of side products resulting in a low yield of the desired product and making the purification of the procedure difficult. Secondly, the coupling of the aminoalcohol (reagent B) with **7** in basic conditions leads to a shift of the phosphate group at the 5'-position to the 3'-position at the ribofuranosyl side.

Show et al[3,4] attempted the synthesis of the allylic phosphates of cytokinins **9** and **10** (fig. II, 1). A first attempt[4] (see scheme II.4) to the synthesis of **9** i.e. by treatment of 6-(4-hydroxy-3-methyl-E-but-2-enylamino)-9-(2',3'-O-isopropylidene-β-D-ribofuranosyl)purine **11** with $POCl_3$, and





Scheme II.3

subsequent removal of the isopropylidene group under acidic conditions, did not result in the formation of diphosphate (**9**, fig. II.1). Only the monophosphate **12** (scheme II.4), was obtained, suggesting that under this condition the allylic phosphate if formed is readily hydrolyzed.

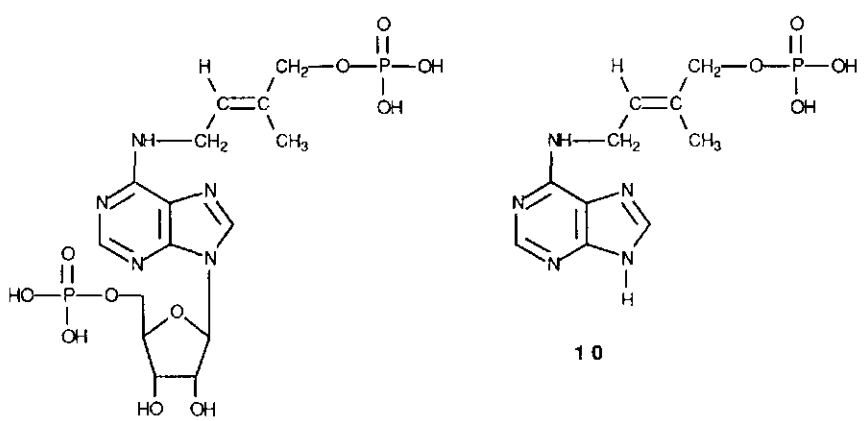
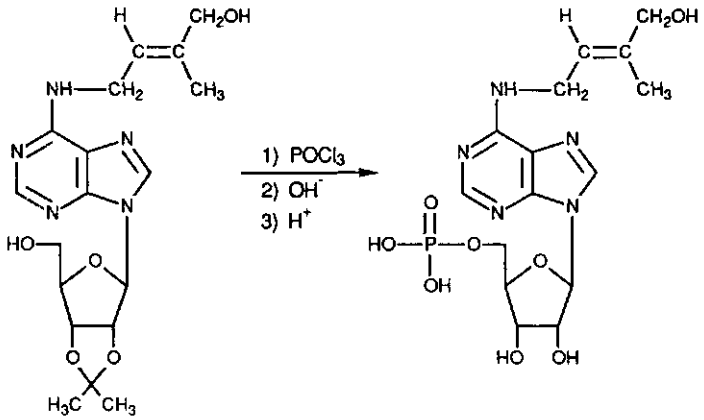
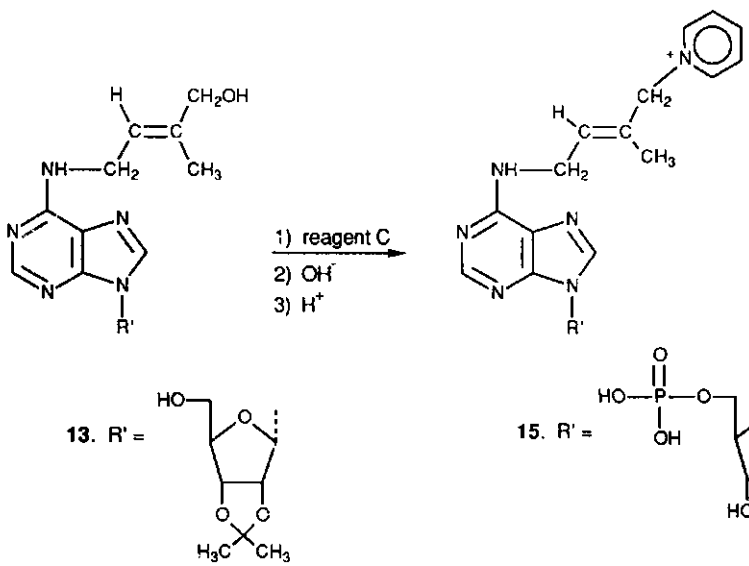


Fig. II.1



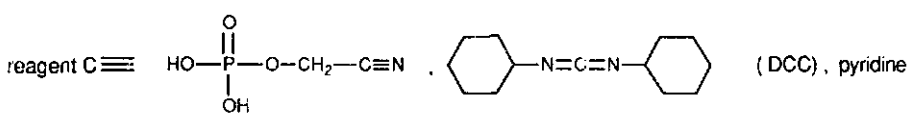
11 12

Scheme II.4



13. R' = 15. R' =

14. R' = H 16. R' = H



Scheme II.5

The second attempt[3] to the synthesis of **9** and **10** by Shaw *et al.* was made by using the phosphorylation of the isopropylidene ribofuranosyl derivative **13**, (scheme II.5) with 2-cyano-ethyl phosphate and dicyclohexylcarbodiimide in pyridine. The formation of the allylic pyridinium derivative **15**, however, implies that during the phosphorylation of the isopropylidene derivative **13** in pyridine both the 5'- and the allylic hydroxy groups are phosphorylated. The allylic phosphate, being a sufficiently powerful leaving group is replaced by the pyridine. Similarly the allylic pyridinium salt **16** was obtained by the phosphorylation of zeatin **14** using the same procedure as described above[3].

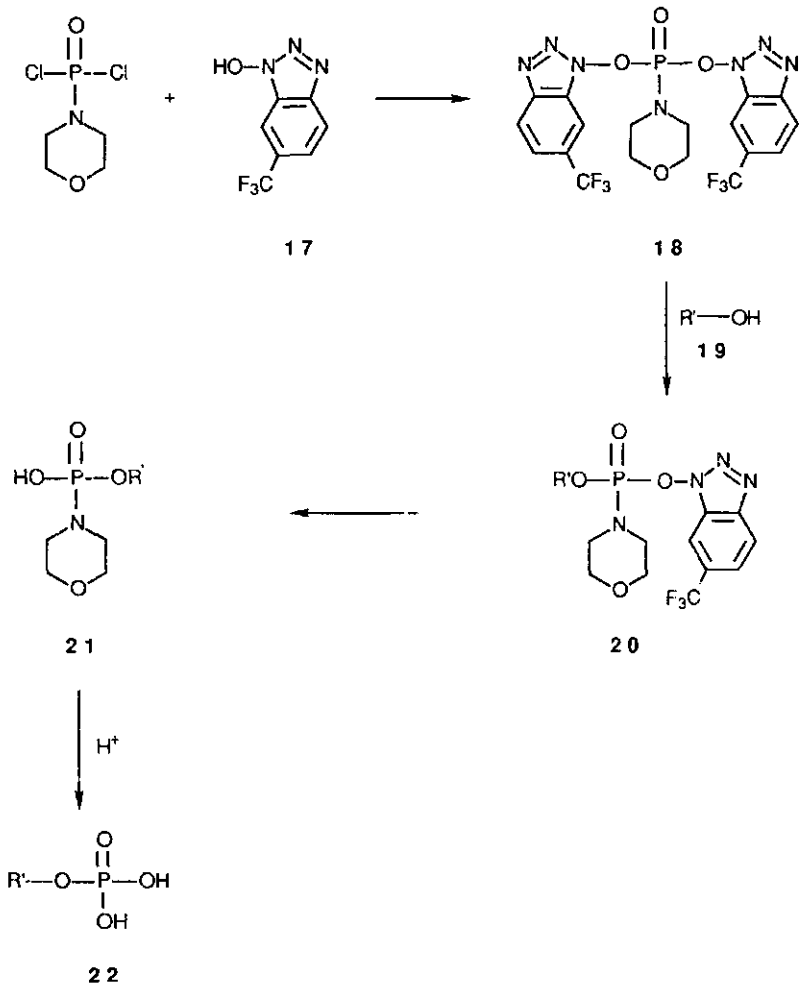
It has now become clear that up till now there was no effective high-yield method for the synthesis of cytokinin phosphates. In this thesis we will describe a succesful approach to synthesize cytokinin phosphates avoiding the problems mentioned above. This succes was realized first by using an efficient way for the preparation of properly protected cytokinins and secondly by developing new methods for the introduction of phosphate groups in the cytokinins. Two approaches, i.e. the phosphatetriester[5,6] (scheme II.6) and the 1-H-phosphonatediester[7,8] (scheme II.7) have been used.

The design of the bifunctional phosphorylating reagent **18**[9], (scheme II.6) obtained by reaction of the morpholinodichlorophosphate with 1-hydroxy(6-trifluoromethyl)benzotriazole **17** was an important breakthrough in the development of the phosphatetriester procedure.

Reagent **18** reacted with the primary hydroxy function (R-OH) to afford intermediate **20**. This was followed by the hydrolysis of the 1-(6-trifluoromethyl)benzotriazolyl and the morpholino groups to give the required phosphate **22**. This phosphatetriester approach was successfully used in the synthesis[10] of **39** and **40** (fig. II, 2).

Despite the broad scope of phosphate triester method[11-20] the preparation of the allylic phosphate **26** appeared to be impossible when applying this methodology. A solution for this problem was found in the 1-H-phosphonatediester approach, which is illustrated in scheme II. 7 The introduction of the easily accessible monofunctional phosphitylating reagent salicyl-chlorophosphite[22] **24** enabled us to prepare the H-phosphonate monoester **26** via intermediate **25**. Reaction of **26** with N,O-bis(trimethylsilyl)acetamide gave intermediate **27**. Treatment of **27** with powdered sulfur gave after hydrolysis the thiophosphate **28**. Reaction of **27** with 2,2'-dipyridyldisulfide gave intermediate **29**, which on hydrolysis with water gave the monophosphate **30**. Methanolysis of **29** with methanol gave methylphosphate **31**, while treatment of **29** with morpholine gave the phosphoroamidate **32**. Compound **32** when reacted with H_3PO_4 gave pyrophosphate **33**.

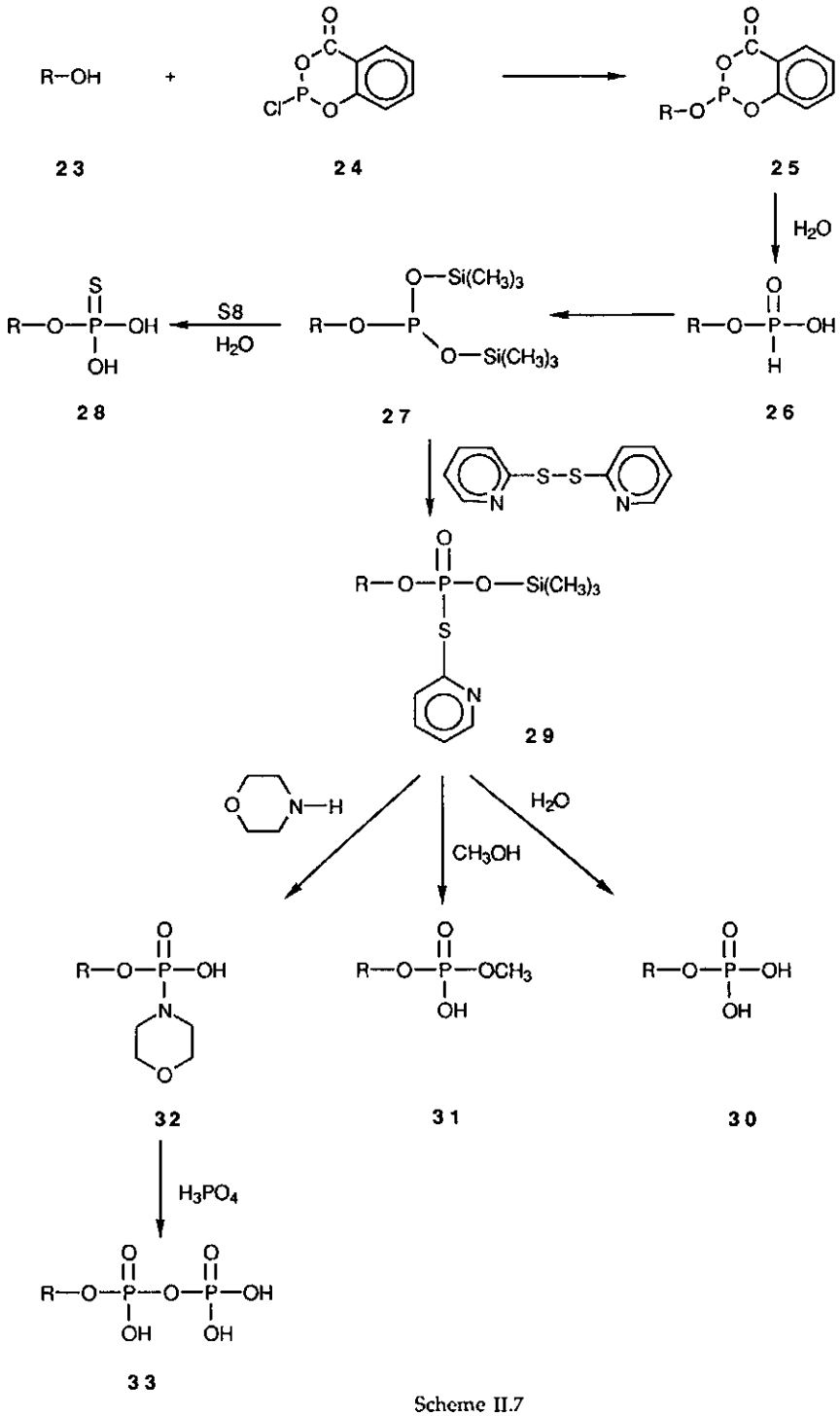
This 1-H-phosphonatediester approach was succesfully used for the synthesis of all cytokininphosphates[23-26], which contain allylic phosphates or pyrophosphate (see fig. II. 2).



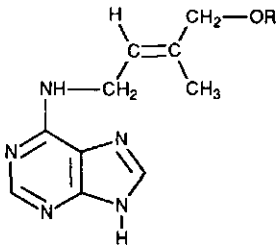
Scheme II.6

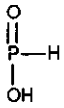
B: THE SYNTHESIS OF OPTICALLY ACTIVE ALANYLATED CYTOKININS

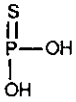
The chemical synthesis of a racemic mixture of D,L-lupinic acid is described in the literature[27]. However, pure L-lupinic acid L-**49** and D-lupinic acid D-**49** (fig. II.3)have never been prepared. Only the enzymatic formation of L-**49** from trans-zeatin **50** and O-acetyl-L-serine, using as enzyme source extracts of Lupinus seedlings has been reported[28]. Attemps[29] to prepare L-**49** by a pyridoxal 5'-phosphate (PLP)-catalyzed reaction between **50** and O-acetyl-L-serine met with little success (yield about 1.5%).

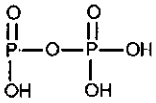


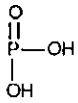
Scheme II.7

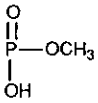


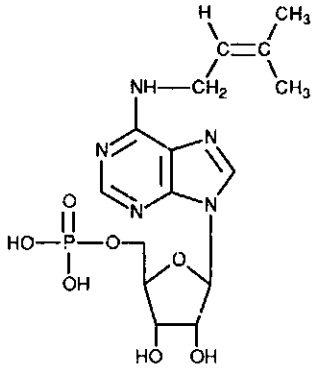
34 . R = 

35 . R = 

36 . R = 

37 . R = 

38 . R = 



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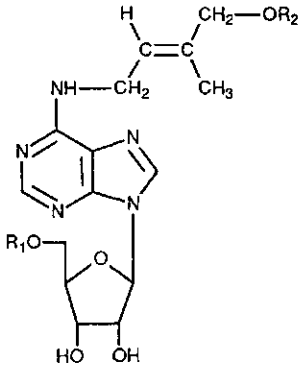

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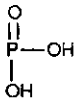
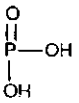
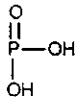
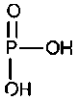
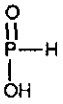
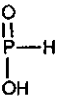
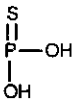
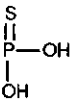
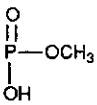
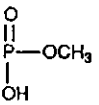
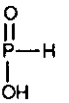
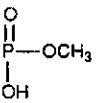
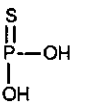
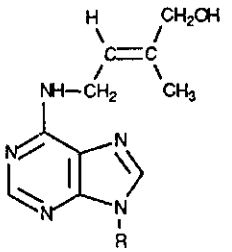
40		H
41		
42	H	
43		
44		
45		
46	H	
47	H	
48	H	

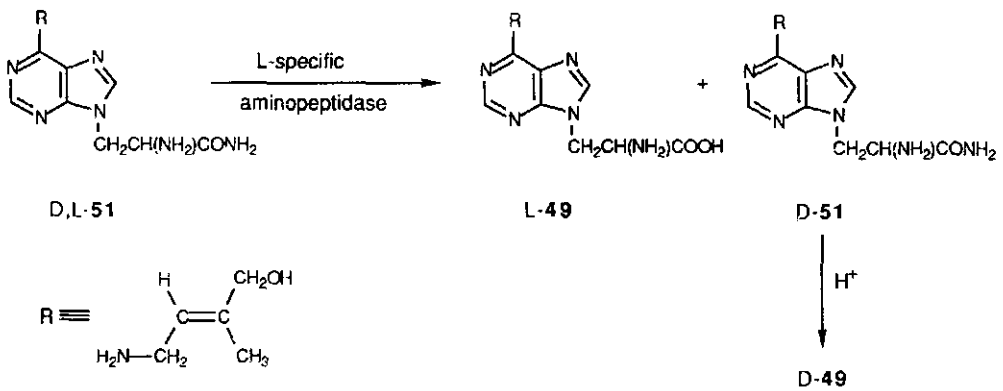
Fig. II.2



49. R = $CH_2CH(NH_2)COOH$
50. R = H

Fig II.3

Our approach (see scheme II 8) for the synthesis of L-lupinic acid L-**49** and D-lupinic acid D-**49** is based on the stereospecific hydrolysis of D,L-lupinic acid amide **51** by using the L-specific aminopeptidase from pseudomonas putida[30,31], to afford L-lupinic acid L-**49** and D-lupinic acid amide D-**51**. Hydrolysis of D-**51** under mild acidic conditions affords D-lupinic acid D-**49**.



Scheme II.8

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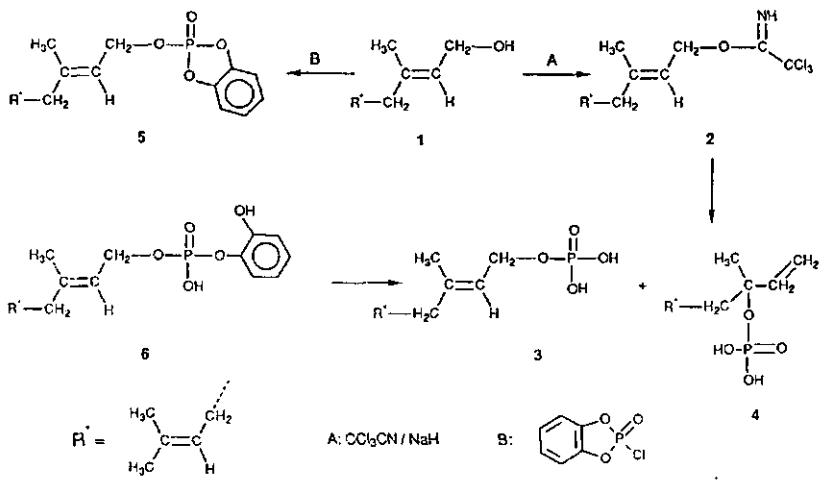
CHAPTER III

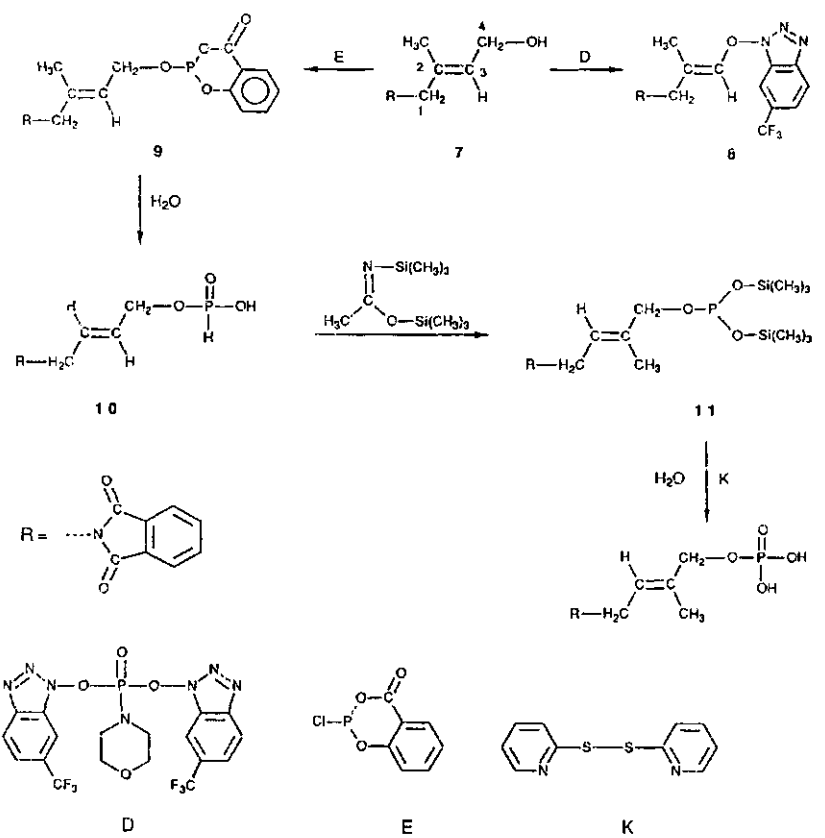
PHOSPHORYLATION OF ALLYLIC HYDROXY GROUPS VIA PHOSPHITE INTERMEDIATES

At present, two distinct approaches have been developed for the chemical phosphorylation of an allylic hydroxy group. In one approach[1] (see scheme III.1), which is based on a procedure originally devised by Cramer *et al.*[2] for the preparation of allylic pyrophosphate esters, the allylic hydroxyl in geraniol **1** was activated with trichloroacetonitrile in the presence of sodium hydride to give intermediate **2**. Displacement of the trichloroacetimidate group by ortho-phosphoric acid then affords the monophosphate **3**. This pathway is unfortunately accompanied by the formation of the rearrangement product **4** which makes the isolation of **3** rather laborious. The other approach[3] (see scheme III.1) consists of a base-assisted (2,6-dimethylpyridine) phosphorylation of **1** with ortho-phenylene phosphorochloridate[4] to give **5**. Hydrolysis of **5**. in the presence of the same base, yields **6** which is then deblocked with lead (IV) acetate to afford **3**. The advantage of this phosphotriester method is the absence of rearrangement product **4** however, the final deblocking step gives rese to the formation of coloured by-products.

The above mentioned problems inherent in the synthesis of allylic monophosphates could be circumvented by using phosphorylating reagents (reagent D and reagent E, see scheme III.2) recently developed for the preparation of nucleic acid derivatives. In order to examine this possibility, 4-hydroxy-3-methyl-E-but-2-enylphthalimide[5] (**7**, 1.0 mmol) in dioxane (5 ml) was treated with the bifunctional phosphorylating reagent (reagent D)[6] (1.2 mmol) in the presence of 4(N,N-dimethylamino) pyridine (1.2 mmol). TLC-analysis (silica gel), after 1h at 20°C, showed the absence of **7** and the presence of a new product with high mobility. Work-up and purification by short-column chromatography[7] gave a homogeneous non-phosphorus containing compound (yield 85%). ^{1}H-, ^{13}C-, ^{19}F-NMR and mass spectroscopic data of this compound were in accord with structure **8**. The exclusive formatio of **8** implies that the intermediate formed triester is, in contrast with the corresponding non-allylic triesters, displaced by the released trifluoromethyl benzotriazoxide. In this respect it is also interesting to note that the formation of **8** is comparible with the rapid quaternisation of an allylic phosphate diester by pyridine[9]. The observation that allylic phosphate di- and tri-esters are still powerful leaving groups urged us to find out whether 1-H-phosphonate mono-esters would be less prone to substitution. To this end, compound **7** (1.0 mmol) in dioxane (5 ml) was phosphitylated with the monofunctional reagent (reagent E)[10] (1.4 mmol) in the presence of N,N-diisopropylethylamine (1.5 mmol). TLC-analysis, after 5 min, showed complete conversion of **7** into phosphite triester **9**. Hydrolysis of the latter intermediate followed by work-up and purification afforded **10**, which was isolated as the Na^{+}-salt (δ_P 5.59, $J_{P\text{-}H}$ 620 Hz), in a yield of 85%. The 1-H-phosphonate ester **10** could easily be converted into the phosphate mono ester **12** by a slight modification of the procedure of Hata *et al.*[11]. Thus silylation of **10** with N,O-*bis* (trimethylsilyl)acetamide gave the intermediate bis (trimethylsilyl) phosphite **11** (δ_P 119.76 ppm). *In situ* oxidation of **11** with 2,2'-dipyridyl disulfide (reagent K) (1.2 mmol) was



Scheme III.1



Scheme III.2

complete within 1 h as evidenced by the formation of an S-pyridyl-O-trimethylsilyl allyl phosphorothioate derivative (δp 9.70 ppm). Hydrolysis of the latter intermediate, subsequent work-up, after 2 h, and purification (DEAE Sephadex A25) yielded homogeneous **12** (yield 80%, Na^+-salt, δp 2.33 ppm).

In conclusion, the results presented in this paper show that salicylchlorophosphite (reagent E) is a promising convenient reagent for the preparation of allylic 1-H-phosphonates which, in turn, can be easily oxidized to give the corresponding mono-phosphates. Further, preliminary experiments indicated that the intermediate obtained after treating **11** with (reagent K) could also be applied towards the synthesis of phosphorothioate and pyrophosphate derivatives of **7**.

EXPERIMENTAL

General procedures.

N,N-diisopropylethylamine and dioxane were dried by refuxing with CaH_2 for 16 h and then distilled. Dioxane was redistilled from $LiAlH_4$ (5g/l). All liqiuds were stored under nitrogen. N,O-bis(trimethylsilyl)acetamide and 2,2'-dipyridyldisulfide were purchased from Janssen Chimica (Belgium). 4-hydroxy-3-methyl-E-but-2-enylphtalimide[5], 2-chloro-4H-1,3,2-benzodioxaphosphorin-4-one[10] and N-morpholino O,O-bis[(6-trifluoromethyl)benzotriazolyl]phosphate[12] were prepared as described previously.

Triethylammonium bicarbonate buffer was prepared by passing a stream of CO_2 gas through a cooled (ice-water bath) 2M solution of triethylamine in deionzed water until solution became neutral. Schleicher and Schüll DC Fertigfolien F 1500 LS 254 were used for TLC. The following solvent system were used. System A (dichloromethane/methanol, 96:4, v,v), system B (dichloromethane/methanol, 80:20, v/v). Short column chromatography was performed on silicagel 60 (230-400 mesh ASTM) suspended in dichloromethane. DEAE Spehadex A25 was purched from Pharmacia (Uppsala, Sweden). Cation-exchange resin (Na^+-form): a solution of NaOH (2M; 100 ml) was passed over a column packed with cation-exchange resin (Dowex 50 Wx-8, 100-200 mesh; Fluka H^+-form, 1.5 x 5 cm) followed by washing of the column with sterile water until pH = 7. ^{1}H-NMR spectra were measured at 200 MHz using a JEOL JNM-FX 200 spectrometer. ^{13}C-NMR spectra weremeasured at 50.4 MHz using a JEOL JNM-FX 200 spectrometer; Proton noise decoupling was used. ^{19}F-NMR spectrum was measured at 282.4 MHz using a Bruker WM-300 spectrometer ^{31}P-NMR spectra were measured at 80.7 MHz, using a JEOL JNM-FX 200 spectrometer. Chemical shifts are in ppm relative to 85% H_3PO_4 as external standard. Mass spectrum of **8** was measured using a AEI MS 902 spetrometer equipped with a VG ZAB console.

Synthesis of 4-trifluoromethylbenzotriazolyl-3-methyl-E-but-2-enylphtalimidide **8**

A solution of phosphorylating agent N-morpholino-O,O-bis[(6-trifluoromethyl)benzotriazolyl]phosphate (reagent D) in dioxane (0.2 M, 6.0 ml, 1.2 mmol) was added to 4-hydroxy-3-methyl-E-but-2-enylphtalimide **7** (0.23 g, 2.0 mmol) which was coevaporated with dioxane (3 x 10 ml) and disolved in dioxane (5 ml), 4(N,N-

dimethylamino)pyridine (0.26 g, 1.2 mmol) was added. After 1 h at 20°C TLC analysis (system A) showed the absence of **7** and the formation of a new compound with Rf = 0.60. The reaction mixture was diluted with dichloromethane (100 ml) and the resulting solution was washed twice with triethylammonium bicarbonate buffer (1M, 50 ml, 0.1M, 50 ml). The organic layers were combined and dried on $MgSO_4$, concentrated to an oil, which was chromatographed on a column of silicagel.Elution with CH_2Cl_2/CH_3OH (100 : 0 → 98 : 2, v/v) gave after evaporation pure **8** as a white solid. Yield 0.35 g (85%), Rf = 0.60 (system A).

Compound **8**; ^{1}H-NMR ($CDCl_3$, 200 MHz, δ-values are given in ppm): 7.80 (m, 7 x ArH), 5.55 (t, J 6.0 Hz, H_2), 4.97 (s, 2 x H_4), 4.23 (d, J 7.0 Hz, 2 x H_1), 2.16 (s, 3x MeH); ^{13}C-NMR ($CDCl_3$, 50.4 MHz, δ-values are given in ppm): 167.4 (2 x C=O), 139.6 (C_3), 133.9, 132.7, 131.6, 127.9, 123.1, 121.0, 107.0 and 66.9 (11 x ArC), 121.1 (C_2), 86.1 (C_4), 34.9 (C_1), 14.5 (Me); ^{19}F-NMR ($CFCl_3$, 282.4 MHz, δ-values are given in ppm): -62.10; Mass spectrum: found m/e 416.1094, calc. for $C_{20}H_{15}F_3N_4O_3$: 416.1096.

Synthesis of 4-(1-H-phosphonate)-3-methyl-E-but-2-enylphtalimide **10**

To a solution of 4-hydroxy-3-methyl-E-but-2-enylphtalimide **7** (0.23 g, 1.0 mmol) and N,N-diisopropylethylamine (0.28 ml, 1.5 mmol) in dioxane 10 ml was added 2-chloro-4H-1,3,2-benzodioxaphosphorin-4-one (reagent E) (0.28 g, 1.4 mmol). After reaction for 5 min. at 20 °C, it was found by TLC analysis (system A) that complete conversion of starting compound into a product with zeromobility has taken place. Water was added (1 ml) and the solution was left for 10 min. at 20 °C. The reaction solution was concentrated to a small volume and triturated with diethylether, the precipitated oil was dissolved in water and applied to a column of DEAE Sephadex A25 (HCO_3^--form) suspended in triethylammonium bicarbonate buffer (0.05M). The column was eluted with a linear gradient of triethylammonium bicarbonate buffer (0.05 → 0.50 M) for 16 h with a flow rate 35 ml/h, and fractions of 10 ml were collected. All UV-positive eluates containing **10**, Rf = 0 (system B), were pooled and concentrated to a small volume, coevaporation with water (4 x 50 ml). Then the solution was applied to a column of Dowex 50 WX-8 cation-exhange resin (Na^+-form, 1.5 x 5 cm).The column was eluted with water and all UV-positieve eluates were collected and concentrated to a small volume. Lyophilization from D_2O gave **10** as white solid yield 0.27 g (85%).

Compound **10**; ^{1}H-NMR (D_2O): 7.58 (m, 4 x ArH), 6.77 (d, J 631.6 Hz, PH), 5.49 (t, J 5.8 Hz,H_2), 4.28 (d, J 9.1 Hz, 2 x H_4), 4.09 (d, J 7.2 Hz, 2 x H_1), 1.83 (s, 3 x MeH); ^{13}C-NMR (D_2O): 171.8 (2 x C=O), 139.6 (d, J 7.3 Hz, C_3), 137.3, 133.4 and 125.8 (6 x ArC), 122.4 (C_2), 70.9 (d, J 4.4 Hz, C_4), 37.8 (C_1), 15.9 (Me); ^{31}P-NMR (D_2O): δp = 5.59 ppm, $J_{p\text{-}H}$ 620 Hz.

Synthesis of 4-phosphate-3-methyl-E-but-2-enylphtalimide **12**

The Na^+-salt of **10** (0.26 g, 0.82 mmol) was repeatedly coevaporated with dioxane (4 x 10 ml). The residue was diluted with dioxane (5 ml) and this mixture was treated with N,N-diisopropylethylamine (0.30 ml, 1,64 mmol) and N,O-bis(trimethylsilyl)acetamide (0.41 ml, 1.64 mmol) after 15 min. ^{31}P-NMR analysis of the solution indicated the complete conversion of **10** into **11**, (δp 5.59 ppm - 119.76 ppm). The reaction solution was further treated with 2,2'-dipyridyl disulfide (0.26 g, 1.2 mmol) for 1 h. After 1 h the ^{31}P-NMR absorption at 119.76 ppm was completely disappeard and ^{31}P-NMR spectrum features a new peak at 9.70 ppm indicating that the conversion of **11** is complete. Then water was added (3 ml) and the solution was applied left

for 2h at 20 °C. The solution was concentrated to an oil which was applied to a column of DEAE-Sephadex in triethylammonium bicarbonate buffer (0.05 M). The column was eluted with a linear gradient of triethylammonium buffer (0.05 → 0.50 M) for 15 h with a flow rate of 35 ml/h and fractions of 10 ml were collected. The UV-positive eluates containing **12**, Rf = 0 (system B) were pooled and concentrated to a small volumn, coevaporated with water (4 x 50 ml). Then the solution was applied to a column of Dowex 50 Wx-8 cation-exhange resin (Na^+-form, 1.5 x 5 cm). The column was eluted with water and all UV positive eluates were collected and concentrated to a small volume lyophilization from D_2O gave **12** as white solid. Yield 0.23 g (80%).
Compound **12** ; ^{1}H-NMR (D_2O): 7.55 (m, 4 x ArH), 5.46 (t, J 5.7 Hz, H_2), 4.22 (d, J 5.8 Hz, 2 x H_4), 4.11 (d, J 8.8 Hz, 2 x H_1), 1.80 (s, 3 x MeH); ^{13}C-NMR (D_2O): 170.4 (2 x C=O), 138.6 (d, J 7.3 Hz, C_3), 135.6, 132.2 and 124.1 (6 x ArC), 119.9 (C_2), 70.0 (d, J 4.4 Hz, C_4), 36.2 (C_1), 14.0 (Me); ^{31}P-NMR (D_2O): δp = 2.33 ppm.

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CHAPTER IV

THE SYNTHESIS OF ALLYLIC PHOSPHATE DERIVATIVES OF *TRANS* ZEATIN

INTRODUCTION

Progress in biochemical research during recent years has clearly pointed out the unique importance of cytokinins in biological systems. The cytokinins, a group of naturally occurring compounds which induce plant cell division, are N-6-substituted derivatives of adenine, which may occur as a free-base, as its ribonucleoside or ribonucleotide.

The first naturally occurring plantgrowth substance with pronounced cell division induction was isolated in pure form from immature sweetcorn kernels[1]. This compound was assigned structure Z_1, based on UV, NMR and Mass spectral data, enzymatic characterisation as well as independent synthesis.

Attempts were made to synthesize the allylic phosphate of zeatin i.e. Z_2, from Z_1 by using the powerful phosphorylating pyrophosphoryl chloride, or by applying a mixture of 2-cyanoethyl phosphate and dicyclohexylcarbodiimide in pyridine. Both attempts failed[2]. It has been reported[3] that phosphorylation of polyprenols, containing an allylic hydroxy group, by a reaction between the active intermediate polyprenol trichloroimidates and anhydrous orthophosphoric acid, was successful, but that in this reaction also rearranged products are obtained.

Recently a new and efficient method for the phosphorylation of an allylic hydroxy group via a phosphite intermediate has been found[4]. This method is based on the phosphitylation of an allylic hydroxy group with salicyl chlorophosphite and oxidation of the allylic phosphonate monoester, being formed to the corresponding phosphate monoester. Application of this phosphitetriester-approach was already demonstrated in the preparation of phosphonate and phosphate monoesters of N-(4-hydroxy-3-methyl-E-but-2-enyl) phthalimide (see chapter III).

We will now report in detail the synthesis of the allylic phosphonate Z_5, the methylphosphate Z_3, and the thiophosphate Z_4 of *trans*-zeatin Z_1.

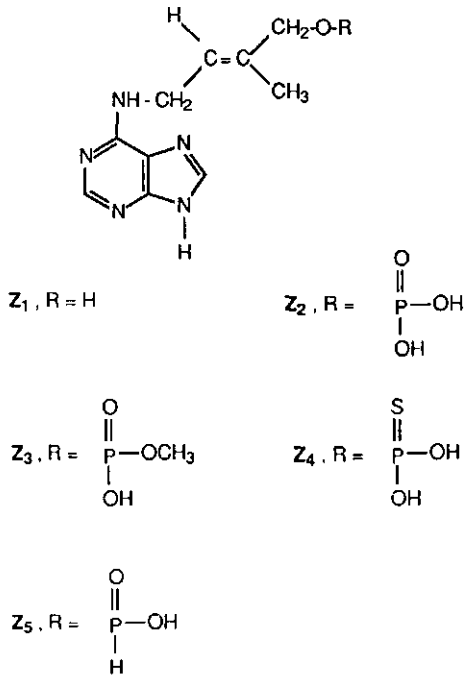
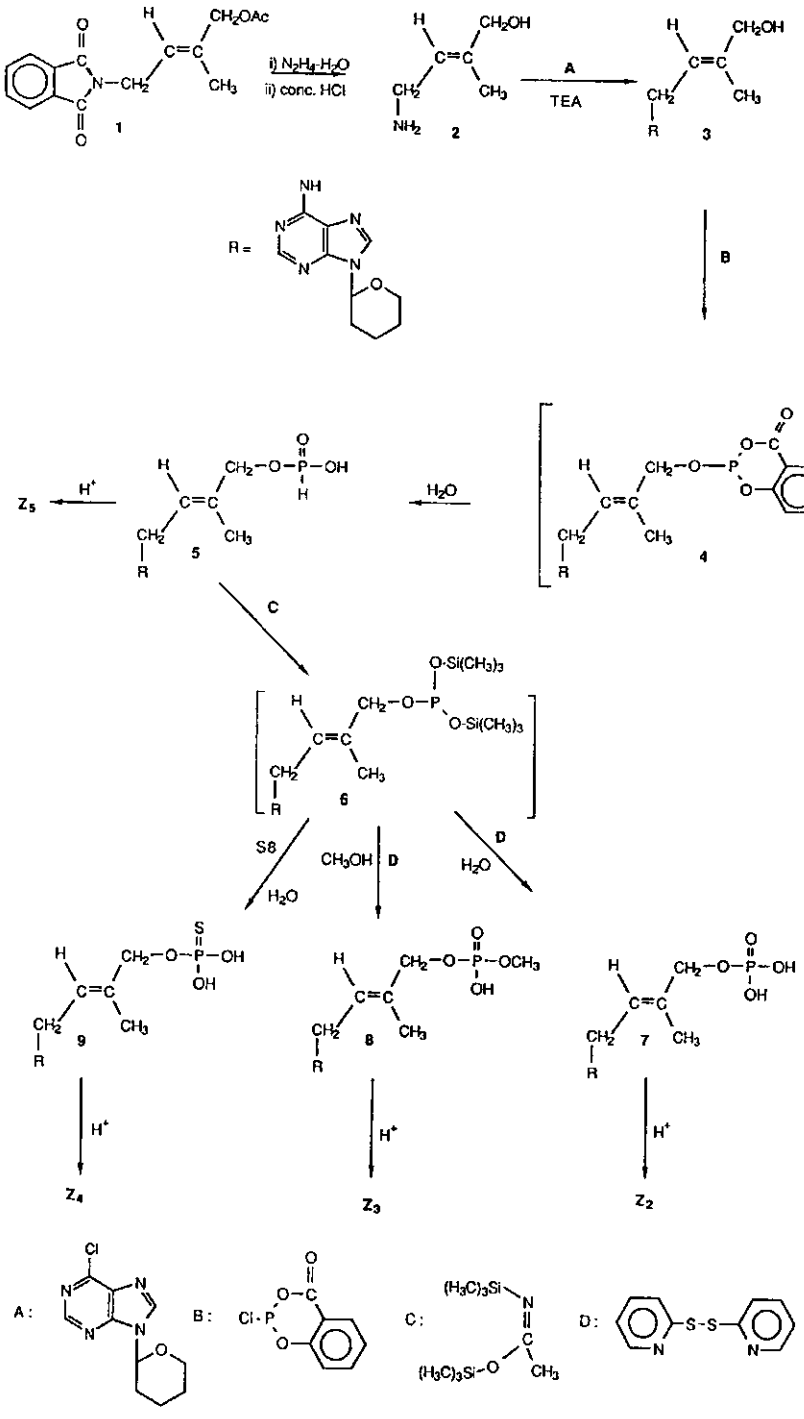


Fig IV.1

RESULTS AND DISCUSSION

The strategy we have adopted for the synthesis of these allylic phosphate derivatives of zeatin consisted of the following steps: i. the synthesis of *trans*-zeatin protected at N-9 with a tetrahydropyranyl (THP)group i.e. **3**; ii. the synthesis of N-9-protected *trans*-zeatin phosphonate **5**; iii. the conversion of **5** into the corresponding phosphate, methylphosphate or thiophosphate **7**, **8** and **9** respectively; iv. the removal of the N-9-protecting group (see scheme IV.1).

Our approach to the synthesis of the N-9-THP *trans*-zeatin **3** is a modification of two syntheses, being already reported. N-(4-acetoxy-3-methyl-E-but-2-enyl) phthalimide **1**, being prepared by a procedure described before[6], was converted in a one-pot reaction into E-4-amino-2-methyl-2-buten-1-ol (**2**) (yield 85%) by treatment with 85% aqueous hydrazine (removal of the phthaloyl group) and subsequent heating with hydrochloric acid (hydrolysis of the acetoxy group). Coupling of **2** with 6-chloro-N-9-THP purine[7] (reagent A) in refluxing butanol in the presence of triethylamine gave N-9-THP *trans*-zeatin **3** (yield 76%). Protection of the N-9-position by the tetrahydropyranyl group was necessary to avoid side-reactions during the phosphitylation reaction. The protective THP group can later be removed under mild acidic conditions[8]. The next step is the synthesis of N-9-THP-*trans*-zeatin phosphonate **5**, being the key intermediate in the synthesis of the allylic phosphate derivatives Z_2, Z_3, Z_4, and Z_5. Treatment of N-9-THP-*trans*-zeatin **3** in dioxane with the monofunctional reagent 2-chloro-4H-1, 3, 2-benzodioxaphosphorin-


i) $N_2H_4 \cdot H_2O$
ii) conc. HCl
A
TEA
B
C
D
H^+
H_2O
S8
CH_3OH
Z_5
Z_4
Z_3
Z_2
A:
B:
C:
D:

Scheme IV.1

4-one (reagent B, 1.4 equiv.) in the presence of N, N-diisopropylethylamine (1.4 equiv.) for 5 min gave phosphite triester **4**, which was hydrolyzed into **5** being isolated as Na^+-salt in a yield of 85%. By ^1H NMR, ^{13}C NMR and ^{31}P NMR data (see experimental part) its structure was proven.

The oxidation of N-9-*trans* zeatin phosphonate **5** into N-9-THP-*trans*-zeatin phosphate **7** was performed according to a slight modification of the procedure of Hata et al[9]. Thus, 1 equiv. of **5** in dioxane was treated with N, O-bis (trimethylsilyl) acetamide (reagent C, 2 equiv.) in the presence of N, N-diisopropylethylamine (2 equiv.) for 15 min. to give intermediate bis (trimethylsilyl) phosphite (**6**). Without further purification **6** was treated with 1.2 equiv. of 2,2'-dipyridyldisulfide (reagent D) for 1h, and the reaction mixture obtained was subsequently treated with water, yielding **7**. Treatment of **6** with 2,2'-dipyridyldisulfide (reagent D) in the presnece of dry methanol (8 equiv.) gave N-9-THP-*trans* zeatin methylphosphate **8**.

Sulfurization of **6** with powdered sulfur for 15 h and subsequent treatment with water gave N-9-THP-*trans*-zeatin phosphorothioate **9**. The cleavage of the ,acid-labile-THP-group in the phosphate derivatives **5, 7, 8** and **9** was effected by acid treatment at pH=2 for 3 h. After work-up *trans*-zeatin phosphonate Z_5, *trans*-zeatin phosphate Z_2, *trans*-zeatin methylphosphate Z_3 and *trans*-zeatin thiophosphate Z_4 were obtained. Over-all yield of these phosphate derivatives fluctuated between 70% and 83% as calculated from N-9-THP *trans*-zeatin **3**.

The identity of *trans*-zeatin allylic phosphate derivatives Z_2, Z_3, Z_4 and Z_5 was ascertained by ^1H NMR, ^{13}C NMR and ^{31}P NMR-spectroscopy (see Experimental Part). The ^1H NMR and ^{13}C NMR data of these compounds in D_2O are almost identical with those of *trans*-zeatin Z_1[10,11] with exception of the ^1H-absorption of CH_2-O which in zeatin is found as singlet at 3.8 ppm., and in the zeatin derivatives Z_2, Z_3, Z_4 and Z_5 as a doublet at about 4.2 ppm., caused by the coupling of ^1H with $^{31}P(^3J_{H\text{-}P})$.

In the ^{13}C NMR-spectra, the difference between zeatin and its phosphate derivatives is obvious in the absorption of CH_2O and $CH_3\underline{C}$=C. The ^{13}C-signal of the CH_2O group appears in zeatin (Z_1) as a singlet at 67 ppm. but in the zeatin phosphate derivatives (Z_2-Z_5) a doublet or a broad singlet at about 70 ppm is observed. The ^{13}C singlet of $CH_3\underline{C}$=C at about 137 ppm. in Z_1 becomes a doublet in the zeatin phosphate derivatives (Z_2-Z_5). The doublets are caused by the coupling of ^{13}C with $^{31}P(^2J_{C\text{-}P}$ and $^3J_{C\text{-}P})$. The range of the coupling depends on the structure of the compound[12]. Proton decoupled ^{31}P NMR spectroscopy revealed the presence of only one resonance absorption which further evidences the structural identity and purity of the compounds.

From these results it can be unequivocally concluded that the method described above is a fast, simple and reliable procedure for the preparation of zeatin phosphate derivatives. These compounds are produced in satisfactory yields without the formation of any side-products.

EXPERIMENTAL

General procedures.

N, N-diisopropylethylamine, dioxane, triethylamine were dried by refluxing with CaH_2 for 16 h and then distilled. Dioxane was redistilled from $LiAlH_4$ (5 g/l). Methanol was dried by refluxing with magnesium methoxide and distilled before use. All liquids were stored under nitrogen. N,O-bis(trimethylsilyl)acetamide and 2,2'-dipyridyldisulfide were purchased from Janssen Chimica (Belgium). N-9-(tetrahydro-2-pyranyl)-6-chloropurine and 2-chloro-4H-1,3,2-benzodioxaphosphorin-4-one were prepared as described previously[7,13]. Triethylammonium bicarbonat buffer was prepared by passing a stream of CO_2 gas through a cooled (ice-water bath) 2M solution of triethylamine in deionized water until solution became neutral. Schleicher and Schüll DC Fertigfclien Fl 1500 LS 254 were used for TLC. The following solvent systems were used: System A (chloroform/methanol, 85:15, v/v), system B (chloroform/methanol, 80:20, v/v), system C (isopropyl alcohol/concentrated ammonium hydroxide/water, 7:1:2, v/v).
Short column chromatography was performed on silicagel 60 (230-400 mesh A STM) suspended in CH_2Cl_2, or on Sephadex LH 20 suspended in CH_2Cl_2/CH_3OH (2:1, v/v), unless otherwise mentioned. DEAE Sephadex A 25 was purchased from Pharmacia (Uppsala, Sweden). Cation-exchange resin (Na^+-form): a solution of NaOH (2M; 100 ml) was passed over a column packed with cation-exchange resin (Dowex 50 Wx-8, 100-200 mesh; Flúka H^+-form, 1.5x5 cm) followed by washing of the column with sterile water until pH=7. Sterile water and glass were used during the whole deblocking and purification processes.
^1H NMR spectra were measured at 300 MHz using a Bruker CXP 300 spectrometer. ^{13}C NMR spectra were measured at 75.460 MHz using a Bruker CXP 300 spectrometer; Proton noise decoupling was used. ^{31}P NMR spectra were measured at 121.470 MHz using a Bruker CXP 300 spectrometer, chemical shifts are in ppm relative to 85% H_3PO_4 as external standard. Mass spectrum of **3** was measured using a AEI MS 902 spectrometer equipped with a VG ZAB console.

Synthesis of (E)-4-amino-2-methyl-2-buten-1-ol **2**

A mixture of N-(4-acetoxy-3-methyl-E-but-2-enyl) phthalimide **1** (3.30 g, 14.12 mmol), 85% aqueous hydrazine hydrate solution (0.85 ml, 14.14 mmol) and methanol (100 ml) was refluxed for 1 h with stirring. TLC analysis (system A) indicated complete conversion of the starting material into a product having Rf=0. After cooling of the solution water (8.7 ml) and conc. hydrochloric acid (8.7 ml) were added and the reaction mixture was heated under reflux for 1 h. After cooling to 0 C the phthalylhydrazide was removed by filtration and the filtrate was evaporated at 30°C in a rotary evaporator. Then the residue was dissolved in water (20 ml) and the insoluble material was removed by filtration. The filtrate was brought to pH=10 with 4N aqueous sodium hydroxide solution. The resulting solution was continuously extracted with chloroform. The organic extract was dried ($MgSO_4$) to give after evaporation the aminoalcohol **2**. Yield 1.21 gram (85%), oil, ^1H NMR-spectrum was identical to that described in the literature[6].

Synthesis of N-9-(tetrahydro-2-pyranyl)-*trans*-zeatin 3

A mixture of aminoalcohol 2 (1.21 g, 12 mmol), N-9-(tetrahydro-2-pyranyl)-6-chloropurine-(2.38 g, 10 mmol), anhydrous triethylamine (1.43 ml) and n-butanol (150 ml) was boiled under reflux for 3 h. TLC analysis (system A) indicated complete conversion of starting material. The reaction solution was concentrated under reduced pressure to an oil, which was chromatographed on a column of silicagel. Elution with CH_2Cl_2/CH_3OH (100:0 → 98:2, v/v) gave after evaporation 2.3 g (76%) of 3, oil, Rf=0.72 (system A).

Compound 3, ^1H NMR ($CDCl_3$): δ = 8.32, s, 1H, H–8; 7.91, s, 1H, H–2; 6.55, t, J = 5.16 Hz, 1H, CH_2-NH; 5.62, m, 2H, CH = C and 1H (THP); 4.22-4.06, m, 4H, CH_2-N, OH and 1H (THP); 3.90, s, 2H, CH_2-O; 3.72, m, 1H, 1H (THP); 2.05 – 1.92, m, 3H, 3H (THP); 1.67 – 1.58, m, 3H, 3H (THP); 1.60, s, 3H, CH_3; ^{13}C NMR: ($CDCl_3$): δ = 154.5, s, C_6; 153.2, s, C_2; 148.1, s, C_4; 139.9, s, C_8; 137.2, s, CH_3-C = C; 120.1, s, CH = C; 119.3, s, C_5; 81.6, 69.7, 67.1, 24.8 and 22.7 5 x s,(THP); 67.1, s, CH_2-OH; 31.8, s, CH_2-N; 13.8, s, CH_3; HRMS, found m/z 303.1703, $C_{15}H_{21}N_5O_2$ requires 303.1695.

Synthesis of N-9-(tetrahydro-2-pyranyl)-*trans*-zeatin phosphonate 5

To a solution of N-9-(tetrahydro-2-pyranyl)-*trans*-zeatin 3 (0.30 g, 1 mmol) and N, N-diisopropylethylamine (0.26 ml, 1.4 mmol) in dioxane (10 ml) was added 2-chloro-4H-1,3,2-benzodioxaphosphorin-4-one (0.28 g, 1.4 mmol). After reaction for 5 min at 20°C, it was found by TLC analysis (system A) that complete conversion of starting compound into a product with zeromobility has taken place. Water was added (1 ml) and the solution was left for 10 min at 20°C. The reaction solution was concentrated to a small volume and triturated with diethylether, the precipitated oil (crude 5) was dissolved in water and applied to a column of DEAE Sephadex A 25 (HCO_3-form) suspended in triethylammonium bicarbonate buffer (0.05 M).

The column was eluted with a linear gradient of triethylammonium bicarbonate buffer (0.05 → 0.70 M) for 16 h with a flow rate of 35 ml/h, and fractions of 6 ml were collected. All UV-positive eluates, containing purine product Rf = 0 (system B), Rf = 0.75 (system C), were pooled and concentrated to a small volume; coevaporation with water (4 x 50 ml) removed most of triethylammonium bicarbonate. Then the solution was applied to a column of Dowex 50 Wx-8 cation-exchange resin (Na^+-form, 1.5 x 5 cm). The column was eluted with water and all UV-positive eluates were collected and concentrated to a small volume. Lyophilization from D_2O gave 5 as white solid. Yield 0.33 g (85%), Rf = 0.73 (system C).

Compound 5, ^1H NMR (D_2O): δ = 7.89, s, 1H, H-8; 7.84, s, 1H, H-2; 6.60, d, $^1J_{H-P}$ = 634.5 Hz, 1H, P-H; 5.44, m, 1H, 1H (THP); 5.27, t, J = 7.2 Hz, 1H, CH = C; 4.12, d, $^3J_{H-P}$ = 7.8 Hz, 2H, CH_2-O-P; 3.84, m, 3H, CH_2-N and 1H (THP); 3.56, m, 2H, 2H (THP); 1.82, s, 3H, CH_3; 1.49, m, 5H, 5H (THP); ^{13}C NMR (D_2O) : δ = 154.7, s, C_6; 153.2, s, C_2; 148.0, s, C_4; 137.3, s, C_8; 136.5, d, $^3J_{C-P}$= 7.3 Hz, CH_3-C = C; 123.4, s, CH = C; 119.4, s, C_5; 82.6, 67.4, 30.5, 25.10 and 23.0 5x s, (THP); 69.5, d, $^2J_{C-P}$ = 7.3 Hz, CH_2-O-P; 39.3, s, CH_2-N; 14.1, s, CH_3; ^{31}P NMR (D_2O); δp = 5.59 ppm, ($^1J_{H-P}$ = 620 Hz).

Synthesis of *trans*-zeatin phosphate Z_2

Crude 5 (synthesized from 1 mmol of 3) was coevaporated with dioxane and dissolved in

dioxane (10 ml). The solution was treated with N, N-diisopropylethylamine (0.35 ml, 2 mmol) and N, O-bis(trimethylsilyl) acetamide (0.5 ml, 2 mmol). After 15 min, ^{31}P NMR analysis of the solution indicated the complete conversion of **5** into **6**, (δp = 6.8 ppm → 118 ppm). The reaction solution was further treated with 2,2'-dipyridyldisulfide (0.26 g, 1.2 mmol) for one hour. After 1 hr the ^{31}P NMR absorption at 118 ppm was completely disappeared and the ^{31}P-spectrum features a new peak at δp = 9.8 ppm indicating that the conversion of **6** is complete (solution A). Then water was added (3 ml) and the solution was applied left for 2 h at 20°C. The solution was concentrated to an oil which was applied to a column of DEAE-Sephadex A 25 (HCO_3-form) suspended in triethylammonium bicarbonate buffer (0.05 M).

The column was eluted with a linear gradient of triethylammonium bicarbonate buffer (0.05 → 0.7 M) for 18 h with a flow rate of 35 ml/h. Fractions of 10 ml were collected, and all UV-positive eluates, containing purine product **7**[14], [Rf = 0 (system B), Rf = 0.51 (system C)], were pooled, concentrated to a small volume and coevaporated with water (4 x 50 ml) to remove most of the triethylammonium bicarbonate and lyophilized from H_2O. The residue was dissolved in water (20 ml) and the pH was adjusted to 2 by the addition of HCl (0.1 N) After 3 h at 20°C TLC analysis (system C) showed that the cleavage of the tetrahydro-2-pyranyl group was completed.

The reaction solution was washed with diethylether (2 x 15 ml). The solution was neutralized with 25% aqueous amonia, concentrated to a small volume and applied to a column of Dowex 50 Wx-8 cation-exchange resin (Na^+-form, 1.5 x 5 cm). The column was eluted with water and all UV-positive eluate, were collected, concentrated to a small volume and lyophilized from D_2O to give Z_2 as white solid. Yield 0.28 g (81%), Rf = 0.42 (system C).

Compound Z_2, ^1H NMR (D_2O): δ = 8.20, s, 1H, H-8; 8.10, s, 1H, H-2; 5.60, t, J = 5.6 Hz, 1H, CH = C; 4.20, d, $^3J_{H-P}$ = 8.3 Hz, 2H, CH_2-O-P; 4.16 d, J = 6.4 Hz, 2H, CH_2-N; 1.75, s, 3H, CH_3; ^{13}C NMR (D_2O): δ = 161.5, s, C_6; 155.9, s, C_2; 152.1, s, C_4; 145.1, s, C_8; 138.9, d, $^3J_{C-P}$ = 9.2 Hz, CH_3-C = C; 122.3, s, CH = C; 118.6, s, C_5; 69.9, br s, CH_2-O-P; 39.8, s, CH_2-N, 14.4, s, CH_3; ^{31}P NMR (D_2O): δp = 2.15 ppm.

Synthesis of *trans*-zeatin methylphosphate Z_3

To solution A (see above) dry methanol (0.32 ml, 8 mmol) was added and this solution was left for 2 h at 20°C. Then solution was concentrated to a small volume and applied to a column of Sephadex LH 20. The column was eluted with (CH_2Cl_2/CH_3OH, 2:1, v/v). The fractions, containing product **8**[15] [Rf = 0 (system B), Rf = 0.67 (system C)] were pooled and concentrated to an oil. After dissolving the oil in water (20 ml), the pH was adjusted to 2 with an aqueous hydrogen chloride solution (0.1 N). After 3 h at 20°C TLC analysis (system C) showed that the cleavage of the tetrahydro-2-pyranyl group was complete. The reaction solution was washed with diethylether (2 x 15 ml). The solution was neutralized with 25% aqueous ammonia, concentrated to a small volume and applied to a column of Dowex 50 Wx-8 cation-exchange resin (Na^+-form. 1.5 x 5 cm).

The column was eluted with water and all UV-positive eluates, were collected, concentrated to small volume and lyophilized from D_2O to give Z_3 as white solid. Yield 0.25 g (75%), Rf = 0.59 (system C).

Compound Z_3, ^1H NMR (D_2O): δ = 8.12, s, 1H, H-8; 8.06, s, 1H, H-2; 5.68, t, J=5.5 Hz, 1H, CH = C;

4.30, d, $^3J_{H-P}$ = 7Hz, 2H, CH_2-O-P; 4.13, d, J = 5.8 Hz, 2H, CH_2-N; 3.55, d, $^3J_{H-P}$ = 10.7 Hz, 3H, CH_3-O-P; 1.78, s, 3H, CH_3; ^{13}C NMR (D_2O): δ = 155.3, s, C_6; 154,2, s, C_2; 152,7, s, C_4; 142.9, s, C_8; 137.8, d, $^3J_{C-P}$ = 5.5 Hz, CH_3-C = C; 123.9, s, CH = C; 118.1, s, C_5; 72.0, br s, CH_2-O-P; 54.6, br s, CH_3O-P; 40.4, s, CH_2-N; 14.0, s, CH_3; ^{31}P NMR (D_2O): δp = 2.10 ppm.

Synthesis of *trans*-zeatin thiophosphate Z_4

Crude **5** (synthesized from 1 mmol of **3**) was coevaporated with dioxane and dissolved in dioxane (10 ml). The solution was treated with N, N-diisopropylethylamine (0.35 mmol, 2 mmol) and N-O-bis (trimethylsilyl) acetamide (0.5 ml, 2 mmol). After 15 min powdered sulfur (1.0 g, 4 mmol) was added. TLC analysis (system C) revealed that it requires 16 h to complete the conversion of phosphonate **5**. Water was added and excess of sulfur was removed by filtration. The resulting solution was evaporated and dissolved in water and applied to a column of DEAE-Sephadex A 25 (HCO_3-form), suspended in triethylammonium bicarbonate buffer (0.05 M).

The column was eluted with a linear gradient of triethylammonium bicarbonate buffer (0.05 → 0.7 M) for 18 h with a flow rate of 35 ml/h. Fractions of 10 ml were collected and all UV-positive eluates, containing product **9**[16], Rf = 0 (system B), Rf = 0.48 (system C), were pooled. They were concentrated to a small volumn, coevaporated with water (4 x 50 ml) to remove most of triethylammonium bicarbonate and lyophilized from H_2O. The residue was dissolved in water (20 ml) and the pH was adjusted to 2 by the addition of aqueous hydrogen chloride (0.1 N). After 3 h at 20°C, TLC analysis (system C) showed that the cleavage of the tetrahydro-2-pyranylgroup was complete.

The reaction solution was washed with diethylether (2 x 15 ml). The solution was neutralized with 25% aqueous ammonia, concentrated to a small volume and applied to a column of Dowex 50 Wx-8 cation -exchange resin (Na^+-form, 1.5 x 5 cm).

The column was eluted with water and all UV-positive eluates were collected, concentrated to a small volume and lyophilized from D_2O to give Z_4 as white solid. Yield 0.3 g (83%), Rf = 0.40 (system C).

Compound Z_4, ^1H NMR (D_2O): δ 8.20, s, 1H, H-8; 8.10, s, 1H, H-2; 5.62, t, J= 6Hz, 1H, CH=C; 4.20, d, $^3J_{H-P}$ = 8Hz, 2H, CH_2-O-P; 4.15, d, J = 6 Hz, 2H, CH_2-N; 1.75, s, 3H, CH_3; ^{13}C NMR (D_2O): δ 162.9, s, C_6; 154.7, s, C_2; 152.5, s, C_4; 147.1, s, C_8; 137.9, d, $^3J_{C-P}$=11.1 Hz, CH_3-C=C; 122.7, s, CH=C; 117.4, s, C_5; 70.0, d, $^2J_{C-P}$ = 11.1 Hz, CH_2-O-P; 39.4, s, CH_2-N; 14.0, s, CH_3; ^{31}P-NMR(D_2O): δp=40.00 ppm.

Synthesis of *trans*-zeatin phosphonate Z_5

Na^+-salt of **5** (0.33 g, 0.84 mmol) was dissolved in water 20 ml and the pH was adjusted to 2 with HCL (0.1 N). After 3 h at 20°C TLC analysis (system C) showed that the cleavage of the tetrahydro-2-pyranyl group was complete.

The reaction solution was washed with diethylether (2 x 15 ml). The solution was neutralized with aqueous ammonia (25%) and concentrated to a small volume, then applied to a column of Dowex 50 Wx-8 cation-exchange resin (Na^+-form, 1.5 x 5 cm). The column was eluted with water and all UV-positive eluates were collected, concentrated to a small volume and lyophilized from D_2O to give Z_5 as white solid. Yield 0.25 g (82%), Rf = 0.7 (system C).

Compound **Z5**, ^{1}H NMR (D_2O): δ 8.28, s, 1H, H-8; 8.19, s, 1H, H-2; 6.76, d, $^{1}J_{H-P}$ = 636 Hz, 1H, P-H; 5.70, t, J = 7.2 Hz, 1H, CH = C; 4.31, d, $^{3}J_{H-P}$ = 9 Hz, 2H, CH_2-O-P; 4.24, d, J = 6.0 Hz, 2H, CH_2-N; 1.8, s, 3H, CH_3; ^{13}C NMR (D_2O): δ 152.2, s, C_6; 149.0, s, C_2; 148.2, s, C_4; 139.4, s, C_8; 137.2, d, $^{3}J_{C-P}$ = 12.94, CH_3-C = C; 121.5, C$\underline{H}$ = C; 119.2, s, C_5; 70.5, br s, CH_2-O-P; 40.4, CH_2-N; 14.0, s, CH_3; ^{31}P NMR (D_2O): δp = 6.70 ppm, ($^{1}J_{H-P}$ = 633 Hz).

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14. ^{1}H NMR of compound **7** (Na^+-salt)(D_2O): δ= 8.00, s, 1H, H-8; 7.90, s, 1H-H-2; 5.50, t, J = 7.20 Hz, 1H, CH = C; 5.30, m, 1H, 1H(THP); 4.2, d, J = 8Hz, 2H, CH_2-O-P; 3.8, m, 3H, CH_2-N and 1H (THP); 3.60, m, 2H, 2H(THP); 2.10 – 1.61, m, 5H, 5H(THP); 1.76, s, 3H, CH_3; ^{31}P NMR (D_2O): δp = 1.80 ppm.
15. ^{1}H NMR of compound **8** (Na^+-salt)(D_2O) : δ = 8.05, s, 1H, H-8; 8.02, s, 1H, H-2; 5.55, t, J = 7.8 Hz, 1H, CH = C; 5.40, m, 1H, 1H(THP); 4.20, d, J = 5.8 Hz, 2H, CH_2-O-P; 4.15, m, 3H, CH_2-N and 1H(THP); 3.70, m, 2H, 2H(THP); 3.50, d, $^{3}J_{H-P}$ = 10 Hz, 3H, OCH_3; 2.00 – 1.40, m, 5H, 5H(THP); 1.7, s, 3H, CH_3; ^{31}P-NMR (D_2O) : δ_p = 2.50 ppm.
16. ^{1}H NMR of compound **9** (Na^+-salt) (D_2O): δ = 8.03, s, 1H, H-8; 8.00, s, 1H, H-2; 5.40, t, J = 6 Hz, 1H, CH = C; 5.20, m, 1H, 1H(THP); 4.20, d, J = 6.2 Hz, 4H, CH_2-N and CH_2-O-P; 4.00 - 3.80, m, 3H, 3H(THP); 2.20 – 1.50, m, 5H, 5H(THP); 1.70, s, 3H, CH_3; ^{31}P NMR (D_2O): δp = 45.00 ppm.

CHAPTER V

A CONVENIENT NEW SYNTHESIS OF THE ALLYLIC PYROPHOSPHATE OF TRANS-ZEATIN

INTRODUCTION

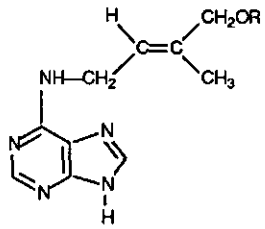
Zeatin, the highly active stimulant of cell division in plant tissue cultures, was first isolated from zeamays, and has the structure 6-(4-hydroxy-3-methyl-E-but-2-enylamino) purine **1a**[1-5]; both E- and Z- isomers have been synthesized [6-10] (see fig.V.1).

In a quite recent paper[11] the unique importance of a new zeatin metabolite i.e. **1b** has been established in plants. The chemical preparation of this metabolite has been reported[12].

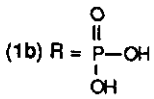
The synthesis of the allylic pyrophosphate of *trans*-zeatin **1c** however, has not been published yet. We now wish to report on the synthesis of this allylic pyrophosphate **1c** (see fig. V.1).

Difficulties encountered in the synthesis of allylic pyrophosphates and subsequent purification can be traced to the fact that the pyrophosphate residues are superb leaving groups, especially when they are protonated[13]. Up to now there are two procedures for the synthesis of allylic pyrophosphate. The first procedure was reported in 1959[14] and has not been altered significantly since then. This one-pot sequence involves treatment of a mixture of an allylic alcohol and inorganic pyrophosphate with trichloroacetonitrile to generate a complex mixture of organic and inorganic mono-, di-, and triphosphates. Yield of the desired products rarely exceeds 30% and further losses are usually encountered during purification. In addition, the procedure becomes difficult to manage if more than 50 mg of the products are desired[15]. The second procedure, which was described by Poulter et al.[15] is based on activation of an allylic alcohol by conversion into an allylic halide. The activated intermediates are treated with the tris-(tetra-n-butylammonium) hydrogen pyrophosphate to obtain the allylic pyrophosphate ester. Yields vary between 34-80%, depending on the structure of the allylic alcohol. In order to examine this possibility for the preparation of the allylic pyrophosphate of *trans*-zeatin attemps were made to halogenate the allylic hydroxy group in *trans*-zeatin **1a** by using either phosphorus tribromide, or N-chlorosuccinimide[15]. Both attempts gave rise to the formation of a lot of coloured products.

It occurred to us that the above-mentioned problems inherent in the synthesis of allylic pyrophosphate of *trans*-zeatin could be circumvented by using the methodology which was originally devised by Khorana et al[16]. for the preparation of nucleoside-5'-pyrophosphate and which is based on the reaction between nucleoside-5'-phosphoromorpholidates and bis-(tri-n-butylammonium) phosphate. In this paper this approach will be described.



(1a) R = H

(1b) R = 

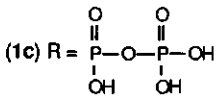
(1c) R = 

Fig. V.1

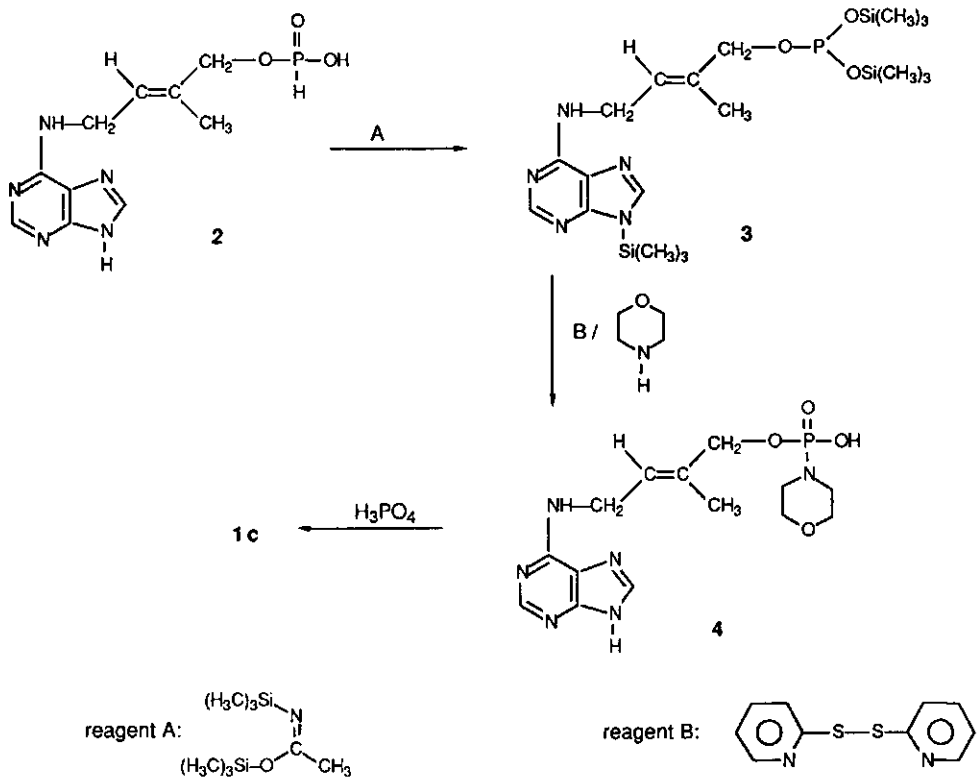
RESULTS AND DISCUSSION

The strategy we have adopted in order to examine this possibility consisted of the following steps (see scheme 1): i. the synthesis of allylic phosphoromorpholidate of *trans*-zeatin i.e. **4**; ii. the conversion of **4** into *trans*-zeatin pyrophosphate **1c** (see scheme V.1).

The synthesis of allylic phosphoromorpholidate **4** was performed according to a slight modification of the procedure of Hata et al.[17]. Thus, allylic phosphonate of *trans*-zeatin **2** (being prepared by a procedure described before)[12] in acetonitrile was treated with N-O-bis-(trimethylsilyl) acetamide in the presence of N,N-diisopropylethylamine to give intermediate bis-(trimethylsilyl) phosphite **3**. Attemps to isolate **3** were unsuccessful. Without further purification **3** was treated with 2,2'-dipyridyldisulfide and the reaction solution was then treated with an excess of dry morpholine to give the allylic phosphoromorpholidate of *trans*-zeation **4** as identified by ^{1}H NMR, ^{13}C NMR and ^{31}P NMR[18].

The conversion of **4** into the allylic pyrophosphate **1c** was succesfully effected by treatment of a solution of **4** in dimethylformamide with mono-(tri-n-butylammonium) phosphate for 18 h at 45° C. After work-up and purification the allylic pyrophosphate **1c** was obtained in good yield (70%). The identity of *trans*-zeatin allylic pyrophosphate was ascertained by ^{1}H NMR, ^{13}C NMR and ^{31}P NMR-spectroscopy (see experimental part).

The ^{1}H NMR and ^{13}C NMR-data of this compound are almost identical with those of *trans*-zeatin **1a** [10,19,21] with exception of the ^{1}H-absorption of CH_2-O, which in zeatin is found as singlet at 3.8 ppm., and in zeatin pyrophosphate **1c** as doublet at about 4.34 ppm., caused by the coupling of ^{1}H with $^{31}P(^{3}J_{H-P})$.



Scheme V.1

In the ^{13}C NMR-spectra, the difference between zeatin **1a** and zeatin pyrophosphate **1c** is obvious in the absorption of $CH_3\underline{C}$=C. The ^{13}C singlet of $CH_3\underline{C}$=C in **1a** becomes a doublet in the zeatin pyrophosphate. The doublet is caused by the coupling of ^{13}C with $^{31}P(^3J_{C\text{-}P})$. Proton decoupled ^{31}P NMR-spectroscopy revealed the presence of two doublets, one doublet at -10.10 ppm., (J=20.2 Hz) and another doublet at -7.00ppm., (J= 20.2 Hz). The doublets are caused by the coupling of ^{31}P with ^{31}P. The ^{31}P chemical shifts and to a lesser extent ^{31}P-^{31}P coupling constants are dependent on pH, counterion and concentration[15].

From these results it can be unequivocally concluded that above mentioned procedure is a fast and simple method for the preparation of allylic pyrophosphate of zeatin. The reaction proceeded smoothly and the pure compound **1c** was isolated in a satisfactory yield.

EXPERIMENTAL

General procedures

N,N-diisopropylethylamine, acetonitrile and toluene were dried by refluxing with CaH_2 for 16 h and then distilled. Dimethylformamide was dried by stirring overnight at room temperature with CaH_2 and then distilled under reduced pressure; morpholine was distilled from sodium. All liquids were stored under nitrogen. N,O-bis-(trimethylsilyl) acetamide and 2,2'-dipyridyldisulfide were purchased form Janssen Chemica (Belgium). Mono-(tri-n-butyl-ammonium) phosphate was prepared as described previously[16]. Triethylammonium bicarbonate buffer was prepared by passing a stream of CO_2 gas through a cooled (ice-water bath) 2M solution of triethylamine in deionized water until the solution became neutral. Schleicher and Schüll DC Fertigfolien F 1500 LS 254 were used for TLC. The following solvent systems were used:
System A (chloroform/methanol, 80:20,v/v) and System B (isopropyl alcohol/concentrated ammonium hydroxide/water, 7:1:2, v/v). Short-column chromatography was performed on silanized silicagel RP 18 (Merck) 70-230 mesh ASTM. The column was eluted with water applying a methanol gradient (0→50%), unless otherwise mentioned. DEAE Sephadex A25 purchased from Pharmacia (Uppsala, Sweden). Cation-exchange resin (Na^+-form): a solution of NaOH (2M; 100 ml) was passed over a column packed with cation-exchange resin (Dowex 50 Wx-8, 100-200 mesh; Fluka H^+- form, 1.5 x 5 cm) followed by washing of the column with sterile water until pH=7. ^1H NMR-spectra were measured at 300 MHz using a Bruker CXP 300 spectrometer. ^{13}C NMR-spectra were measured at 75.460 MHz using a Bruker CXP 300 spectrometer; Proton noise decoupling was used. ^{31}P NMR-spectrometer, chemical shifts are in ppm. relative to 85% H_3PO_4 as external standard.

Synthesis of *trans*-zeatin pyrophosphate **1c**

First the Na^+-salt of *trans*-zeatin phosphonate **2**[12](0.20 g, 0.5 mmol) was repeatedly coevaporated with acetonitrile (4x10 ml), the residue was diluted with acetonitrile (5 ml) and this mixture was treated with N,N-diisopropylethylamine (0.35 ml, 2 mmol) and N,O-bis-(trimethylsilyl) acetamide (0.5 ml, 2 mmol) and reacted for 15 min at 20°C. Then the solution was treated with 2,2'-dipyridyldisulfide (130 mg, 0.6 mmol). After 1 h at 20°C TLC analysis system B showed complete absence of **2**. Then an excess of morpholine was added (1 ml). After 18 h at 20°C TLC (system B) showed the formation of **4**[18] (Rf=0.85). Water was added (1 ml) and the reaction solution was left for 10 min at 20°C. The reaction solution was concentrated to a small volume and coevaporated with toluene (4x10 ml). The latter product was dissolved in dimethylformamide (5 ml) and a solution of mono-(tri-n-butylammonium) phosphate[16] in dimethylformamide (0.5M, 5 ml) was added. The reaction mixture was rendered anhydrous by repeated coevaporation with toluene (3x10 ml) and left at 45°C; after 18 h TLC (system B) showed the complete conversion of **4** into **1c**. The reaction mixture was treated with tri-n-butylamine until the pH had become 8 and concentrated to a small volume (1 ml) and applied to a column of DEAE Sephadex A25 (HCO_3^-

form) suspended in triethylammonium bicarbonate buffer (0.05 M). The column was eluted with the same buffer (linear gradient 0.05→1.0 M) for 18 h with a flow rate of 35 ml/h. Fractions of 10 ml were collected and the UV-positive eluates containing only **1c**, [Rf = 0.3, (system B)] were pooled. They were concentrated to a small volume, coevaporated with water (4x50 ml) and lyophilized from H_2O. The residue was dissolved in water (1 ml) and applied to a column of Dowex 50 Wx-8 cation-exchange resin (Na^+-form, 1.5x5 cm). The column was eluted with water and all UV-positive eluates were collected, concentrated to a small volume and lyophilized from D_2O to give **1c** as white solid. Yield 155 mg (70% based on **2**), Rf=0.3 (system B). Compound **1c** ^1H NMR (D_2O): δ 8.17, s, 1H, H-8; 8.06, s, 1H, H-2; 5.72, t, J=7.2 Hz, 1H, CH=C; 4.34, d, $^3J_{P-H}$=6.9 Hz, 2H, CH_2-O-P; 4.12, d, J=6.4 Hz, 2H, CH_2-N; 1.76, s, 3H,CH_3; ^{13}C NMR (D_2O) : 154.1, s, C_6; 153.2, s, C_2; 150.9, s, C_4; 141.5, s, C_8; 137.3, d, $^3J_{C-P}$=5.5 Hz, CH_3-C=C;122.8, s,CH=C;116.9, s, C_5; 70.5, s, CH_2-O-P; 39.4, s, CH_2-N; 14.0, s, CH_3; ^{31}P NMR (D_2O): δ_P=-10.10, d, J=20.2 Hz, P(1);δ_P=-7.00, d, J=20.2 Hz, P(2).

ACKNOWLEDGEMENT

We wish to thank Mr. A. van Veldhuizen for recording the ^1H NMR, ^{13}C NMR and ^{31}P NMR spectra.

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18. Compound **4** was identified after purification by using RP 18; the purified compound was converted into its sodium salt by passing it over Dowex 50 Wx-8 (Na^+-form) (see General Procedures). Compound **4**, ^1H NMR (D_2O): δ 8.15, s, 1H, H-8; 8.06, s, 1H, H-2; 5.68, t, J=6.8 Hz, 1H, CH=C; 4.22, d, $^3J_{H-P}$=7.4 Hz, 2H, CH_2-O-P; 4.16, d, J=6.0 Hz, 2H, CH_2-N; 3.50, m, 4H, morpholine; 2.85, m, 4H morpholine; 1.77, s, 3H, CH_3; ^{13}C NMR (D_2O): δ 154.7, s, C_6; 153.6, s, C_2, 141.6, s, C_4; 137.3, d, $^3J_{C-P}$=6 Hz, CH_3-C=C; 122.8, s, CH=C; 120.7, s, C_5, 70.2, s, CH_2-O-P; 67.7, d, $^3J_{C-P}$=6.0 Hz, morpholine CH_2-O-CH_2; 45.5, s, morpholine CH_2-N-CH_2; 39.1, s, CH_2-NH; 14.00, s, CH_3; ^{31}P NMR (D_2O): δp=9.2 ppm.
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CHAPTER VI

THE SYNTHESIS OF CYTOKININ PHOSPHATES

INTRODUCTION

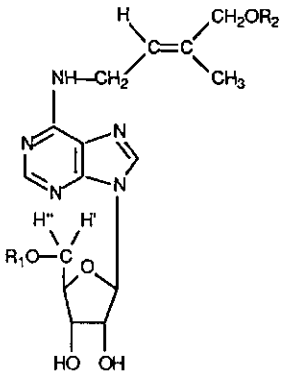
The cytokinins, a group of naturally-occurring compounds which are involved in a variety of aspects of plant development, are established to be N^6-substituted derivatives of adenine, which occur either as a free-base, ribonucleoside or ribonucleotide. One of the first naturally-occurring adenosine derivatives with plant cell division promoting activity was isolated from extracts of sweet corn kernels (zeamays)[1] and was identified as 6-[4-hydroxy-3-methyl-E-but-2-enylamino]-9-(β-D-ribofuranosyl) purine **1a**, usually named ribosyl zeatin. The second adenosine derivative being isolated from extracts of lactobacillus acidophilus, was N^6-(Δ^2-Isopentenyl)adenosine i.e. 6-(3-methyl-2-butenylamino)-9-(β-D-ribofuranosyl)purine **2a** (see fig. VI.1).

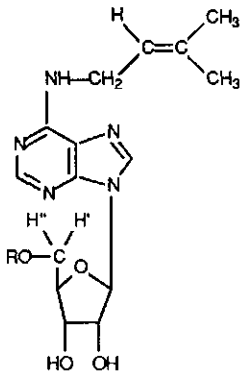
Many workers in this field suggested the presence of phosphates of cytokinin ribonucleosides; up to now only two of these derivatives have been isolated[3,4]. These compounds were assigned the structures of ribosyl zeatin 5'-phosphate **1b** and N^6-(Δ^2-Isopentenyl)-adenosine 5'-phosphate **2b**; these assignments are based on UV spectral data, enzymatic characterisation as well as independent synthesis. A number of chemical procedures has been reported for the synthesis of these ribonucleotides **1b**[6] and **2b**[4,5].

The best approach described for the phosphorylation of the alcoholic group involved the use of the powerful phosphorylating agent pyrophosphoryl chloride[5,6]. The method is simple but requires a difficult purification procedure and gives relatively low yields (≈40%).

Attempts were made to synthesize ribosyl zeatin phosphate **1b**, ribosyl zeatin diphosphate **1c** and ribosyl zeatin allylic phosphate **1d** by using either pyrophosphoryl chloride, or a mixture of 2-cyanoethylphosphate and dicyclohexylcarbodiimide in pyridine. Both attempts failed[7].

We report in detail a new and improved synthesis of **1b** and **2b**, using the phosphotriester approach, and the synthesis of **1c** and **1d**, using the phosphite triester approach (see scheme VI.1).





1 a) $R_1 = R_2 = H$

1 b) $R_1 = P(O)(OH)-OH$, $R_2 = H$

1 c) $R_1 = R_2 = P(O)(OH)-OH$

1 d) $R_1 = H$, $R_2 = P(O)(OH)-OH$

2 a) $R = H$

2 b) $R = P(O)(OH)-OH$

Fig. VI.1

RESULTS AND DISCUSSION

The strategy we have adopted for the synthesis of the ribonucleoside phosphates **1b, 1c , 1d** and **2b** consisted of the following steps: **i**. The preparation of properly protected ribonucleoside derivatives **6, 8, 9** and **10**, (see scheme VI.1); **ii** effective phosphorylation of the free hydroxyl groups in these ribonucleoside derivatives (see scheme 2 and 3); **iii** complete removal of all protective groups.

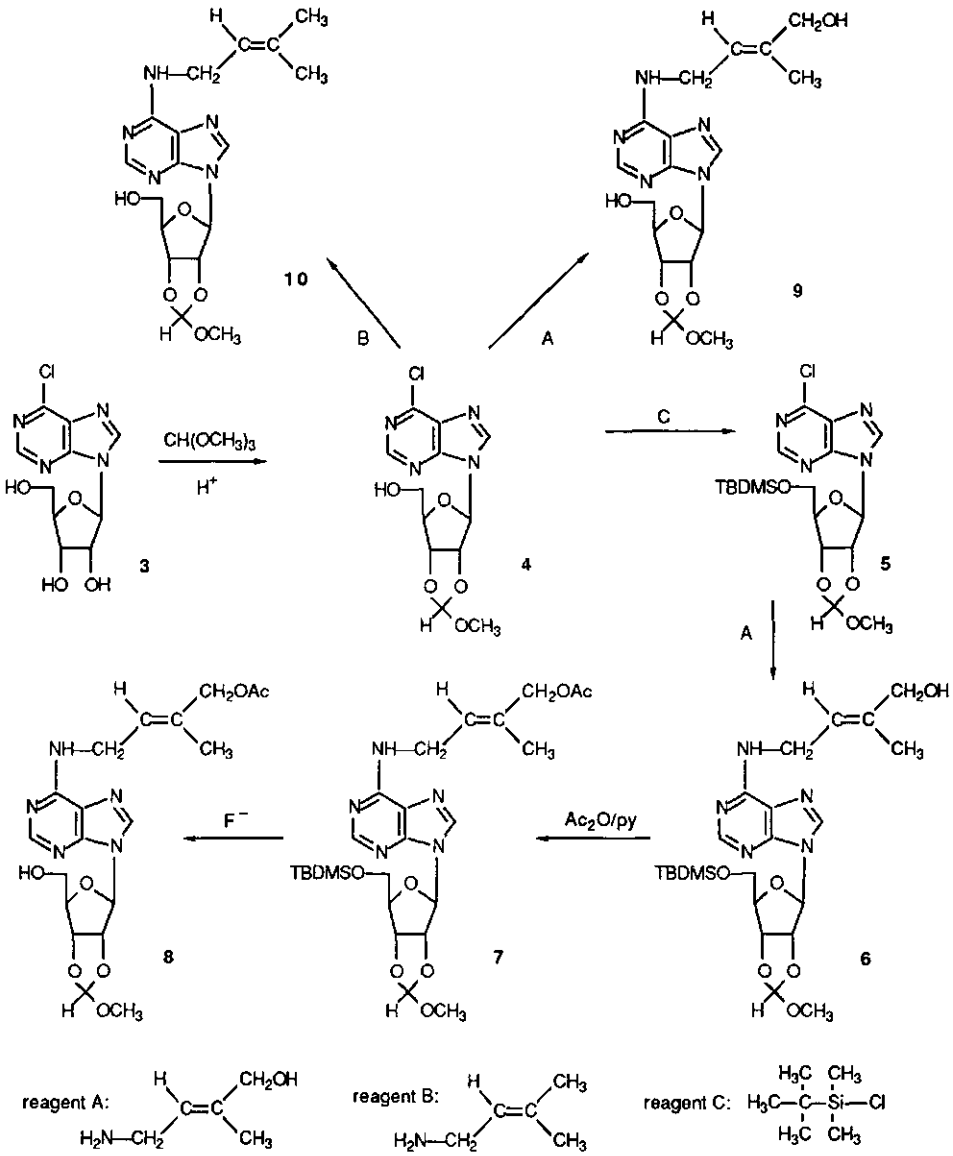
In our strategy dealing with the preparation of properly protected ribonucleoside derivatives we used the base-labile acetyl group for the protection of the allylic hydroxy function, the acid-labile methoxymethylidene group for the protection of the 2'-OH and the 3'-OH function and the acid-base-labile *tert*-butyldimethylsilyl (TBDMS) group for the protection of the 5'-OH function. The TBDMS group was selectively removed in the presence of the other protective groups (methoxymethylidene and acetyl groups) by fluoride ions.

6-Chloro-9-β-ribofuranosyl purine **3**, which was prepared by a procedure described before[8], was converted into 6-chloro-9-(2',3'-O-methoxymethylidene-β-D-ribofuranosyl) purine **4** (yield 70%) by treatment with trimethyl orthoformate in the presence of the monohydrate of toluene-p-sulphonic acid. The TBDMS group was introduced at the 5'-hydroxy function of **4** by treatment with *tert*-butyldimethylsilyl chloride in pyridine, which afforded 6-chloro-9-(2',3'-O-methoxy methylidene-5'-O-*tert*-butyldimethylsilyl-β-D-ribofuranosyl) purine **5** (yield 80%). 6-[4-Hydroxy-3-

methyl-E-but-2-enylamino]-9-(2',3'-O-methoxymethylidene-5'-O-*tert*-butyldimethylsilyl-β-D-ribofuranosyl) purine **6** and 6-[hydroxy-3-methyl-E-but-2-enylamino]-9-(2',3'-O-methoxymethylidene-β-D-ribofuranosyl) purine **9** were produced in yields of 77% and 80% respectively by coupling of 4-hydroxy-3-methyl-E-but-2-enylamine[10] with **5** and **4** respectively in refluxing n-butanol in the presence of triethylamine. Coupling of **4** with 3-methylbut-2-enylamine[11] under the same conditions as described above gave 6-[3-methylbut-2-enylamino]-9-(2',3'-O-methoxymethylidene-β-D-ribofuranosyl) purine **10** (yield 79%).

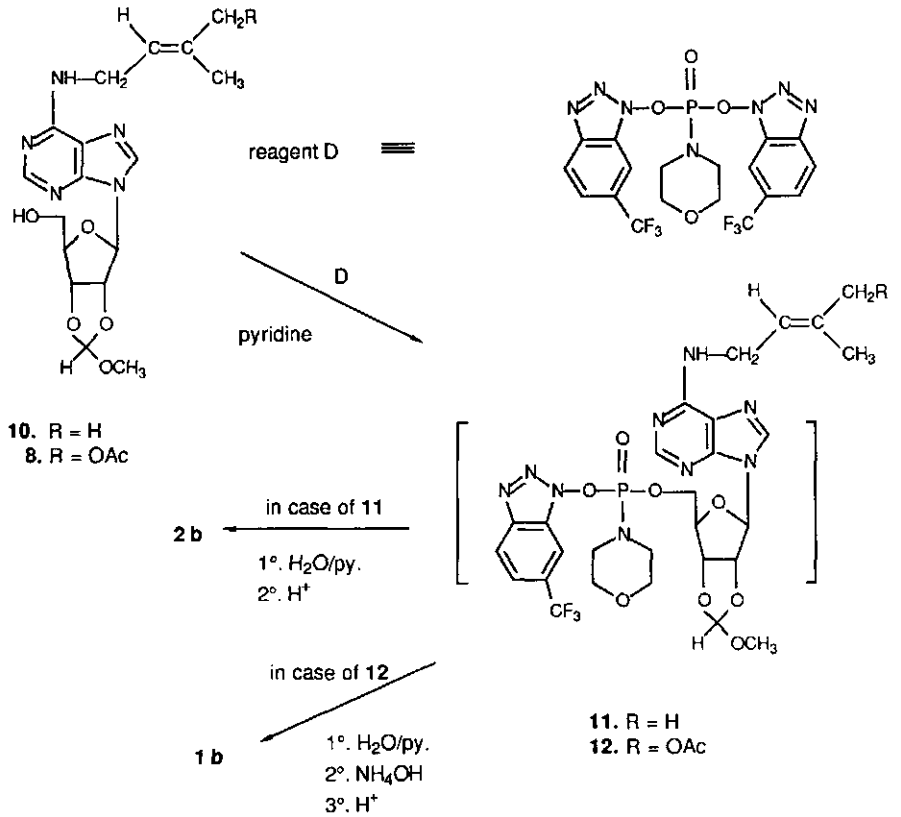
The allylic hydroxy function on **6** was acylated by treatment of **6** with acetic anhydride in pyridine in the presence of 4-dimethylaminopyridine (DAP)[12] to afford the fully protected compound **7** (yield 94%). Selective removal of the TBDMS group in **7** was performed by treatment with tetra-n-butylammonium fluoride (TBAF)[13] in dioxane to give 6-[4-acetoxy-3-methyl-E-but-2-enylamino]-9-(2',3'-O-methoxymethylidene-β-D-ribofuranosyl) purine **8** (yield 82%).

The above described procedures for the synthesis of properly protected ribonucleosides **6, 8, 9** and **10** proved to be relatively fast and gave these partially protected ribonucleosides in good yields. All these reaction steps of above described methodology could be performed on a relatively large scale. The next step in our strategy deals with the effective phosphorylation of the free hydroxy groups on the properly protected ribonucleosides **6, 8, 9** and **10**. So far, the bifunctional phosphorylating reagent N-morpholino O,O-bis[(6-trifluoromethyl)benzotriazolyl] phosphate (reagent D) (see scheme VI.2) was found to be suitable for the preparation of 5'-monophosphates of nucleosides[14]. When we treated a solution of the nucleosides **10** or **8** in pyridine (see scheme VI.2) with reagent D in the presence of N-methylimidazole we succesfully obtained the phosphate triesters **11** and **12** respectively. These fully protected intermediates were not isolated but were hydrolyzed immediately to remove all protective groups. Treatment of intermediate **11** with pyridine/water removed the protective group 6-trifluoromethylbenzotriazolyl and subsequent treatment with acid at pH=2 removed the morpholino as well as the methoxymethylidene group providing **2b** (yield 73% based on **10**). In case of **12** after treatment with pyridine/water the acetyl protective group was removed by treatment with aqueous ammonia and the morpholino as well as the methoxymethylidene function by acid treatment at pH=2 to yield **1b** (yield 68% based on **8**). By ^{1}H NMR, ^{13}C NMR and ^{31}P NMR data (see experimental part) these structures **1b** and **2b** were unequivocally established. The Rf and UV data were identical as described in the literature[4,5,6].The introduction of the phosphate group at the allylic hydroxy group[7,15], leading to the compounds **1c** and **1d** by using the bifunctional phosphorylating reagent D was found to be unsuitable[16]. A new and efficient method for the phosphorylation of an allylic hydroxy group via a phosphite intermediate has been found[16]. This method is based on the initial phosphitylation of the allylic hydroxy group with salicyl chlorophosphite, followed by oxidation of the allylic phosphonate monoester to the corresponding phosphate monoester.



Scheme VI.1

Application of this phosphite triester-approach was already demonstrated in the preparation of the phosphate monoester of N-(4-hydroxy-3-methyl-E-but-2-enyl) phthalimide[16] and further in the preparation of the phosphate monoester of 6-(4-hydroxy-3-methyl-3-E-but-2-enylamino) purine[10].In order to examine this potential route we treated **6** and **9** in dioxane with the monofunctional reagent 2-chloro-4H-1,3,2-benzodioxaphosphorin-4-one (reagent K)[17] (see scheme VI.3) in the presence of N,N-diisopropylethylamine for 5 min. It gave the phosphite triesters **13** and **14** respectively, which were hydrolyzed into **15** and **16** respectively. By ^{1}H NMR,



Scheme VI.2

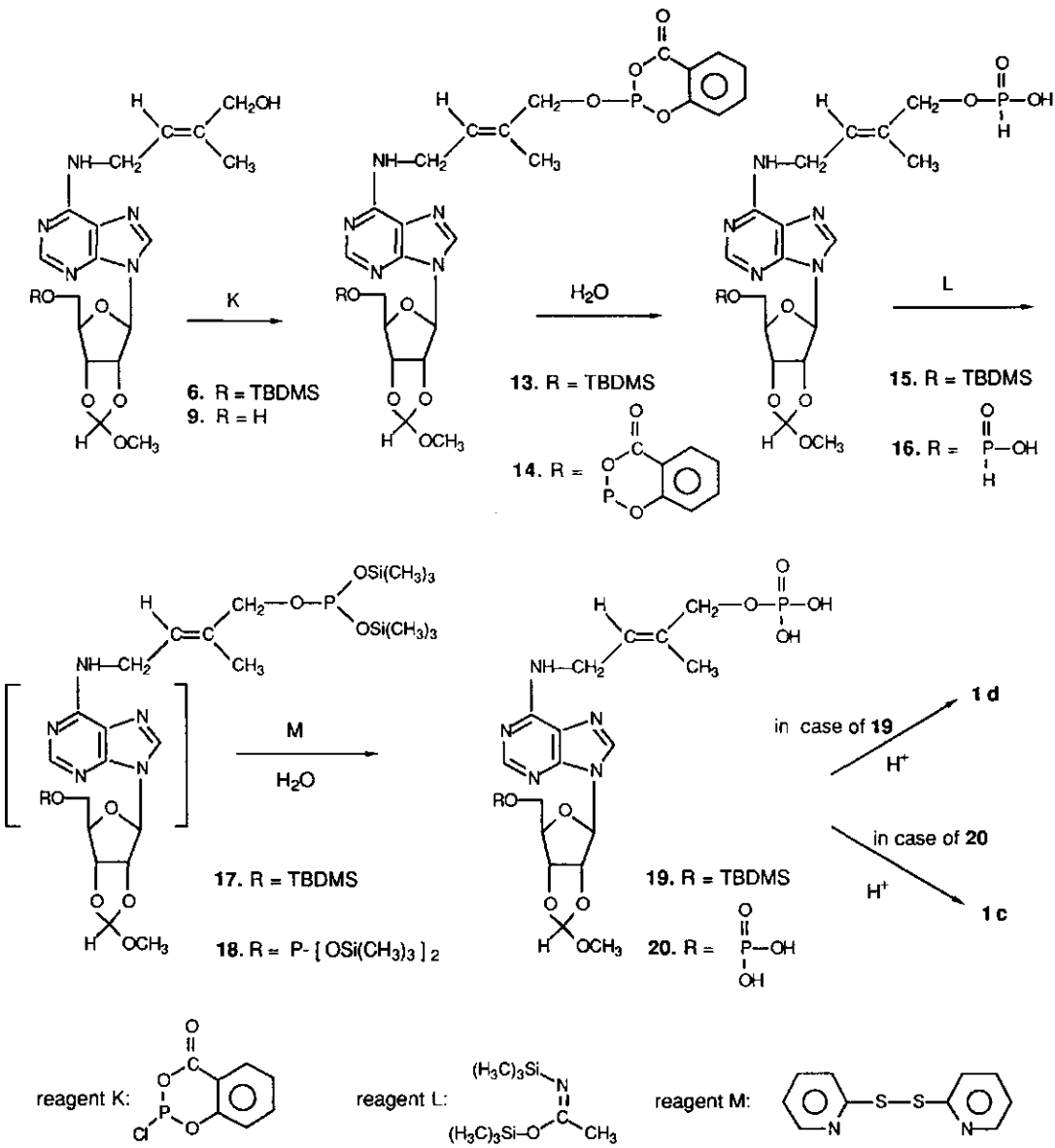
^{13}C NMR and ^{31}P NMR data (see experimental part) these structures were proven.

The oxidation of **15** and **16** was performed according to a slight modification the procedure of Hata et al[18]. A solution of **15** and **16** in acetonitrile when treated with N,O-bis(trimethylsilyl) acetamide (reagent L) in the presence of N,N-diisopropylethylamine for 15 min. gave the intermediates bis (trimethylsilyl phosphite) **17** and **18** respectively. Without further purification **17** and **18** were treated with 2,2'-dipyridyldisulfide (reagent M) for 1h and the reaction mixtures thus obtained were subsequently treated with water affording **19** and **20** respectively. The identity of **19** and **20** was ascertained by ^{1}H NMR and ^{31}P NMR spectroscopy.

Cleavage of the acid-labile group TBDMS and methoxymethylidene group was effected by acid treatment at pH=2 for 3h to give after workup **1d** and **1c** respectively (overall yield of **1d** 76% based on **6**, yield **1c** 70% based on **9**). The identity of these phosphate derivatives **1d** and **1c** was ascertained by ^{1}H NMR, ^{13}C NMR and ^{31}P NMR.

The ^{1}H NMR and ^{13}C NMR data of the ribosyl zeatin derivatives **1b**, **1c** and **1d** are almost identical in comparison with those of ribosyl zeatin **1a**[19]; however we mention some exceptions:

i) The ^{1}H-absorptions of H_5' and H_5'' appear in ribosyl zeatin **1a** as two pairs of double doublets



Scheme VI.3

at 3.75 - 3.88 ppm., due to the fact that H_5' and H_5'' have slightly different chemical shifts and $J(H_5' - H_5'') \neq J(H_5'-H_4') \neq J(H_5''-H_4')$. In **1b** and **1c**, however, the absorption of H_5' and H_5'' takes the form of an undefined multiplet at about 4.10 ppm. No conclusion can be drawn whether ^{31}P coupling occurs with H_5' and H_5''. ii) The ^1H-absorption of C=C-CH_2-O which in ribosyl zeatin **1a** is found as singlet at 3.96 ppm., appears in **1c** and **1d** as a doublet at about 4.20 ppm. The doublets are caused by the coupling of ^1H with ^{31}P ($^3J_{P-H}$). iii) The ^{13}C-signal of $CH_3\underline{C}$=C features in ribosyl

zeatin **1a** as a singlet at about 138 ppm., but in **1c** and **1d** as a doublet. Similarly the ^{13}C-signals of C_4' appear in ribosyl zeatin **1a** as a singlet at about 85 ppm., but becomes in **1b** and **1c** a doublet. The doublets are caused by the coupling of ^{13}C with ^{31}P ($^3J_{C-P}$).

When we compared the ^1H NMR and ^{13}C NMR data of N^6-(Δ^2-Isopentenyl)adenine **2a**[20] with the data of its phosphate **2b**, the same observations were made as decribed above concerning the ^1H-absorption of H_5' and H_5'' and the ^{13}C-absorption of C_4'.

Proton decoupled ^{31}P NMR spectroscopy revealed the presence of the expected number of resonance absorptions, which further proved the structural identity and purity of the compounds. From these results it can be unequivocally concluded that the methods described above are simple and reliable procedures for the preparation of cytokinin phosphates. These compounds are produced in satisfactory yields without the formation of any side-products.

EXPERIMENTAL

General procedures.

Acetonitrile, N,N-diisopropylethylamine, dioxane, N-methylimidazole, pyridine, triethylamine and trimethyl orthoformate were dried by refluxing with CaH_2 for 16h and then distilled. Dioxane was redistilled from $LiAlH_4$ (5 g/l). All liquids were stored under nitrogen. n-Butanol (analyzed grade Baker) was used without further purification. N,O-bis(trimethylsilyl)acetamide and 2,2'-dipyridyldisulfide were purchased from Janssen Chimica (Belgium). 6-Chloro-9-β-D-ribofuranosyl purine[8], 4-hydroxy-3-methyl-E-but-2-enylamine[10], 3-methyl-2-butenylamine[11], tetra-n-butylammonium fluoride (TBAF)[13], 2-chloro-4H-1,3,2-benzodioxaphoshorin-4-one[17] and N-morpholino O,O-bis[(6-trifluoromethyl)benzotriazolyl)] phosphate[14] were prepared as described previously.

Triethylammonium bicarbonate buffer was prepared by passing a stream of CO_2 gas through a cooled (ice-water bath) 2M solution of triethylamine in deionized water until solution became neutral. Schleicher and Schüll DC Fertigfolien F 1500 LS 254 were used for TLC, unless otherwise mentioned. The following solvent systems were used. System A (chloroform/methanol, 96:4, v/v), system B (chloroform/methanol 92:8, v/v), system C (chloroform/methanol, 90:10, v/v), system D (chloroform/methanol, 85:15, v/v), system E (isopropyl alcohol/concentrated ammonium hydroxide/water, 7:1:2, v/v) and system F (n-butanol/acetic acid/water, 12:3:5, v/v, Whatman No. 1 paper was used for TLC). Compound were visualized by UV-light or by spraying with the appropriate reagents. Thus compounds containing ribofuranosyl moieties were visualized by spraying with sulfuric acid/methanol (20:80, v/v). The phosphor containing compounds were visualized by spraying with Zinzadze's reagent. Short column chromatography was performed on silicagel 60 (230-400 mesh ASTM) suspended in chloroform. DEAE-Sephadex A 25 was purched from Pharmacia (Uppsala, Sweden). Cation-exchange resin (Na^+-form): a solution of NaOH (2M; 100 ml) was passed over a column packed with cation-exchange resin (Dowex 50 Wx 8, 100-200 mesh; Fluka H^+-form, 1.5x5 cm) followed by washing of the column

with sterile water until pH=7. Sterile water and glass were used during the whole deblocking and purification processes.

^{1}H NMR spectra were measured at 300 MHz using a Bruker CXP 300 spectrometer; ^{13}C NMR spectra were measured at 75.460 MHz using a Bruker CXP 300 spectrometer; Proton noise decoupling was used. ^{31}P NMR spectra were measured at 121.470 MHz using a Bruker CXP 300 spectrometer, chemical shifts are in ppm. relative to 85% H_3PO_4 as external standard. Mass spectral data were obtained from an AEI MS 902 spectrometer equipped with a VG ZAB console.

Synthesis of 6-chloro-9-(2',3'-O-methoxymethylidene-β-D-ribofuranosyl) purine **4**

A mixture of 6-chloro-9-β-D-ribofuranosyl purine **3** (13.48 g, 47 mmol), toluene-p-sulphonic acid, monohydrate (0.83 g, 4.28 mmol) and dry trimethyl orthoformate (58 ml) was stirred for 16h at 20°C. TLC (system B) indicated complete disappearance of the starting material. The mixture was taken up in chloroform (250 ml) and washed with an aqueous solution of sodium bicarbonate (1M, 2x100 ml) and water (2x100 ml). The organic layer was dried on $MgSO_4$, concentrated to an oil, which was chromatographed on a column of silicagel. Elution with $CHCl_3/CH_3OH$ (100:0→96:4, v/v) gave after evaporation pure **4** as oil. Yield 10.83 g (70%), Rf=0.60 (system B).

Compound **4**, ^{1}H NMR ($CDCl_3$): δ 8.72, s, 1H, H-8; 8.41, s, 1H, H-2; 6.34, d, J=4.3 Hz, 1H, H_1'; 5.94, s, 1H, CH-OCH_3; 5.28, m, 1H, H_2'; 5.14, m, 1H, H_3', 4.53, m, 1H, H_4'; 3.82-3.74, m, 2H, H_5' and H_5"; 3.30., s, 3H, OCH_3; ^{13}CNMR ($CDCl_3$): δ 151.71, 151.1, 150.4, 144.5 and 117.5, 5xs (purine); 119.14, s, CH-OCH_3; 92.1, s, C_1'; 87.0, s, C_4'; 84.2, s, C_3'; 80.9, s, C_2'; 62.4, s, C_5'; 52.7, s, OCH_3; HRMS, found m/z 328.0571 $C_{12}H_{13}N_4O_5{}^{35}Cl$ requires 328.0574.

Synthesis of 6-chloro-9-(2',3'-O-methoxymethylidene-5'-O-*tert*-butyldimethylsilyl-β-D-ribofuranosyl) purine **5**

To a solution of **4** (4.65 g, 14.25 mmol) in pyridine (20 ml) was added *tert*-butyldimethylsilyl chloride (2.40 g, 16 mmol) and the reaction mixture was left for 16h at 20°C. TLC (system A) indicated complete conversion of the starting compound. After the addition of ice (20 g) the reaction mixture was concentrated to an oil which was dissolved in $CHCl_3$ (200 ml) and washed with an aqueous solution of sodium bicarbonate (1M, 2x100 ml) and water (2x100 ml). The organic layer was dried on $MgSO_4$, concentrated to an oil which was chromatographed on a column of silicagel. Elution with $CHCl_3/CH_3OH$ (100:0→98:2, v/v) gave after evaporation pure **5** as oil. Yield 5.02 g (80%), Rf=0.621 (system A).

Compound **5**, ^{1}H NMR ($CDCl_3$): δ 8.73, s, 1H, H-8; 8.36, s, 1H, H-2; 6.37, d, J=3Hz, 1H, H_1'; 5.95, s, 1H, CH-OCH_3; 5.30 m, 1H, H_2'; 5.01, m, 1H, H_3'; 4.97, m, 1H, H_4'; 3.85-3.74, m, 2H, H_5' and H_5"; 3.30, s, 3H, OCH_3; 0.80, s, 9H (TBDMS); 0.00, s, 6H (TBDMS); ^{13}C NMR ($CDCl_3$): δ 152.1, 151.8, 150.4, 143.7 and 118.2, 5xs (purine); 119.3, s, CH-OCH_3; 91.8, s, C_1'; 87.7, s, C_4'; 84.9, s, C_3'; 81.4, s, C_2'; 63.4, s, C_5', 52.8, s, CH-OCH_3; 25.9, 18.2 and -5.5, 3xs (TBDMS); FD-MS m/z: 442(M^+) $C_{18}H_{27}N_4O_5Si^{35}Cl$; HRMS $(M-CH3)^+$, found m/z 427.1209 $C_{17}H_{24}N_4O_5Si^{35}Cl$ requires 427.1204.

Synthesis of 6-[4-hydroxy-3-methyl-E-but-2-enylamino]-9-(2',3'-O-methoxymethylidene-5'-O-*tert*-butyldimethylsilyl-β-D-ribofuranosyl) purine **6**

A solution of **5** (4.50 g, 10.16 mmol), aminoalcohol (reagent A, 1.50 g, 14.85 mmol), triethylamine (3 ml) in n-butanol (150 ml) was boiled under reflux for 3h. TLC analysis (system A) indicated that the conversion of the starting material was complete. The reaction solution was concentrated under reduced pressure to an oil, which was chromatographed on a column of silicagel. Elution with $CHCl_3/CH_3OH$ (100:0→97:3, v/v) gave after evaporation pure **6** as an oil. Yield 4.00 g (77%), Rf=0.40 (system A).

Compound **6**, ^1H NMR ($CDCl_3$): δ 8.40, s, 1H, H-8; 7.93, s, 1H, H-2; 6.32, t, J=5.35 Hz, 1H, NH-CH_2; 6.25, d, J=2.8 Hz, H_1'; 6.12, s, 1H, CH-OCH_3; 5.65, t, J=6.8 Hz, 1H, CH=C; 5.38, m, 1H, H_2'; 5.26, m, 1H, H_3'; 4.52 m, 1H, H_4'; 4.36, br s, 2H, CH_2-N; 4.04, s, 2H, C=C-CH_2-O; 3.76-3.70, m, 2H, H_5' and H_5''; 3.43, s, 3H, OCH_3; 1.73, s, 3H, CH_3; 0.93, s, 9H, (TBDMS); 0.00, s, 6H, (TBDMS); ^{13}C NMR ($CDCl_3$): δ 154.3, 153.2, 150.0, 142.0 and 119.8, 5xs (purine); 138.8, s, CH_3-C=C; 119.9, s, CH=C; 117.7, s, CH-OCH_3; 91.2, s, C_1'; 87.3, s, C_4', 84.4, s, C_3'; 81.4, s, C_2'; 66.3, s, C=C-CH_2-O; 63.0, s, C_5'; 51.6, s, CH-OCH_3; 38.2, s, CH_2-N; 13.7, s, CH_3; 25.6, 18.2 and -5.6, 3xs (TBDMS); HRMS, found m/z 507.2505 $C_{23}H_{37}N_5O_6Si$ requires 507.2513.

Synthesis of 6-[4-hydroxy-3-methyl-E-but-2-enylamino]-9-(2',3'-O-methoxymethylidene-β-D-ribofuranosyl) purine **9**

A solution of **4** (3.00 g, 9.13 mmol) aminoalcohol (reagent A, 1.00 g, 10 mmol), triethylamine (3 ml) in n-butanol (150 ml) was boiled under reflux for 3h. TLC analysis (system B) indicated that the conversion of the starting material was complete. The reaction solution was concentrated under reduced pressure to an oil, which was chromatographed on a column of silicagel. Elution with $CHCl_3/CH_3OH$ (100:0→95:5, v/v) gave after evaporation pure **9** as an oil. Yield 2.90 (80%), Rf=0.44 (system B).

Compound **9**, ^1H NMR ($CDCl_3$): δ 8.26, 1H, s, H-8; 7.79, s, 1H, H-2; 6.44, t, J=4.97, 1H, NH-CH_2; 6.16, d, J=3.37 Hz, 1H, H_1', 5.93, s, 1H, CH-OCH_3; 5.62, t, J=7.21 Hz, 1H, CH=C; 5.33, m, 1H, H_2'; 5.16, m, 1H, H_3', 4.50, m, 1H, H_4'; 4.20, br s, 2H, CH_2-N; 4.01, s, 2H, C=C-CH_2-O, 3.98-3.93, m, 2H, H_5' and H_5''; 3.66, s, 3H, CH-OCH_3; 1.72, s, 3H, CH_3; ^{13}C NMR ($CDCl_3$): δ 154.7, 152.8, 147.2, 139.1 and 119.4, 5xs (purine); 139.0, s, CH_3-C=C; 120.6, s, CH=C; 117.5, s, CH-OCH_3; 92.7, s, C_1'; 87.4, s, C_4'; 85.9, s, C_3', 81.1, s, C_2'; 67.1, s, C=C-CH_2-O; 62.8, s, C_5'; 52.8, s, CH-OCH_3'; 38.2, s, N-CH_2; 13.8, s, CH_3; HRMS, found m/z 393.1649 requires 393.1648.

Synthesis of 6-[4-acetoxy-3-methyl-E-but-2-enylamino]-9-(2',3'-O-methoxymethylidene-5'-O-*tert*-butyldimethylsilyl-β-D-ribofuranosyl purine **7**

To a solution of **6** (2.00 g, 3.94 mmol) in pyridine (10 ml) was added acetic anhydride (3 ml) and 4-dimethylaminopyridine (150 mg). The reaction solution was stirred for 40h. TLC analysis (system A) indicated that the conversion of the starting material was complete. After the addition of ice (50 g) the reaction mixture was taken up in $CHCl_3$ (150 ml) and washed with an aqueous solution of sodium bicarbonate (1M, 2x50 ml) and water (2x50 ml). The organic layer was dried on $MgSO_4$, concentrated to an oil, coevaporated with toluene (2x100 ml), the oil residue was chromatographed on a column of silicagel. Elution with $CHCl_3/CH_3OH$ (100:0→98:2, v/v) gave after evaporation pure **7** as an oil. Yield 2.03 g (94%), Rf=0.70 (system A).

Compound **7**, ^{1}H NMR ($CDCl_3$): δ 8.36, s, 1H, H-8; 7.92, s, 1H, H-2; 6.24, t, J=6.0 Hz, 1H, NH-CH_2; 6.11, d, J=2.6 Hz, 1H, H_1'; 6.10, s, 1H, CH-OCH_3; 5.65, t, J=6.8 Hz, 1H, CH=C; 5.36, m, 1H, H_2', 5.10, m, 1H, H_3'; 4.40, s, 2H, C=C-CH_2-O; 4.34, m, 1H, H_4'; 4.28, br s, 2H, CH_2-N; 3.75-3.70, m, 2H, H_5' and H_5"; 3.32, s, 3H, CH-OCH_3; 2.05, s, 3H, CH_3-C=O; 1.74, s, 3H, CH_3; 0.88, s, 9H (TBDMS), 0.00, s, 6H, TBDMS; ^{13}C NMR ($CDCl_3$): δ 170.0, s, CH_3-C=O; 154.6, 153,3, 150.4, 138.7 and 119.2, 5xs (purine); 134.2, s, CH_3-C=C; 121.0, s, CH=C; 118.0, s, CH-OCH_3; 90.4, s, C_1'; 86.5, s, C_4'; 83.8, s, C_3', 81.0, C_2'; 68.8, s, C=C-CH_2-O; 63.2, s, C_5'; 51.7, s, CH-OCH_3; 38.3, s, CH_2-N; 20.8, s, CH_3-C=0, 14.1 s, CH_3=C; 25.8, 18.2 and -5.5, 3xs (TBDMS); FD-MS m/z: 549 (M^+) $C_{25}H_{39}N_5O_7Si$; HRMS (M-CH_3CO_2)$^+$, found m/z 490.2483 $C_{23}H_{36}N_5O_5Si$ requires 490.2486.

Synthesis of 6-[4-acetoxy-3-methyl-E-but-2-enylamino]-9-(2',3'-O-methoxymethylidene-β-D-ribofuranosyl) purine **8**

A solution of tetra-n-butylammonium fluoride in dioxane (0.5M, 17 ml) was added to **7** (2.03 g, 3.7 mmol), which was coevaporated with dioxane and dissolved in dioxane (10 ml). The reaction solution was left for 30 min. at 20°C. TLC analysis (system C) indicated complete removal of the silyl group, water was added (2 ml) and the reaction mixture was concentrated to an oil, which was dissolved in $CHCl_3$ (200 ml) and washed with an aqueous solution of sodium bicarbonate (1M, 2x100 ml) and water (2x100 ml). The organic layer was dried on $MgSO_4$, concentrated to an oil, which was chromatographed on a column of silicagel. Elution with $CHCl_3/CH_3OH$ (100:0→96:4, v/v) gave after evaporation pure **8** as an oil. Yield 1.30 g (82%), Rf=0.56 (system C).

Compound **8**, ^{1}H NMR ($CDCl_3$): δ 8.00, s, 1H, H-8; 7.83, s, 1H, H-2; 6.60, t, J=5.5 Hz, 1H, NH-CH_2; 6.03, d, J=3.4 Hz, H_1'; 6.43, t, J=6.9 Hz, CH=C; 5.10, m, 1H, H_2'; 4.90, m, 1H, H_3'; 4.27, s, 2H, C=C-CH_2-O; 4.00, br s, 2H, CH_2-N; 3.66, m, 1H, H_4'; 3.35-3.0, m, 2H, H_5' and H_5"; 1.85, s, 3H, CH_3-C=C; 1.57, s, 3H, CH_3-C=O; ^{13}C NMR ($CDCl_3$): δ 171.0, s, CH_3-C=O; 154.5, 152.6, 141.0, 139.1 and 119.3, 5xs (purine); 133.9, s, CH_3-C=C; 117.5, s, CH-OCH_3; 92.4, s, C_1'; 85.8, s, C_4'; 83.3, s, C_3'; 80.8, s, C_2'; 72.1, s, C=C-CH_2-O; 62.6, s, CH_3-C=O; 13.2, s, CH_3; HRMS, found m/z 435.1742 $C_{19}H_{25}N_5O_7$ requires 435.1754.

Synthesis of 6-[3-methyl-2-butenylamino]-9-(2',3'-O-methoxymethylidene-β-D-ribofuranosyl) purine **10**

A solution of **4** (2.00 g, 6.00 mmol), aminoalcohol (reagent B, 1.02 g, 12 mmol), triethylamine (1.8 ml) in n-butanol (150 ml) was boiled under reflux for 3h. TLC analysis (system B) indicated that the conversion of starting material was complete. The reaction solution was concentrated under reduced pressure to an oil which was chromatographed on a column of silicagel. Elution with $CHCl_3/CH_3OH$ (100:0→96:4, v/v) gave after evaporation pure **10** as an oil. Yield 1.80 g (79%), Rf=0.60 (system B).

Compound **10**, ^{1}H NMR ($CDCl_3$): δ 8.26, s, 1H, H-8; 7.75, s, 1H, H-2; 6.14, m, 2H, NH-CH_2 and H_1'; 5.91, s, 1H, CH-OCH_3; 5.29, t, J=6.88 Hz, 1H, CH=C; 5.19 m, 1H, H_2'; 4.50, m, 1H, H_3'; 4.14, br s, 2H, CH_2-N; 3.90, m, 1H, H_4'; 3.75-3.70, m, 2H, H_5' and H_5"; 3.41, s, 3H, CH-OCH_3; 1.69, s, 3H, CH_3-C=C; 1.67, s, 3H, CH_3-C=C; ^{13}C NMR ($CDCl_3$); δ 154.6, s, 152.6, 147.1, 138.8 and 119.1, 5xs (purine); 136.2, s, $(CH_3)_2$-C=C; 120.6, CH=C; 119.9, CH-OCH_3; 92.54, s, C_1'; 87.3, s, C_4'; 83.74, s, C_3'; 81.05, s, C_2'; 62.6,

s, C_5'; 52.3, s, CH-$\underline{OCH_3}$, 25.3 and 17.5, 2xs $(\underline{CH_3})_2$-C=C; HRMS, found m/z 377.1696 $C_{17}H_{23}N_5O_5$ requires 377.1699.

Synthesis of 6-[4-hydroxy-3-methyl-E-but-2-enylamino]-9-(5'-O-phosphate-β-D-ribofuranosyl) purine **1b** (ribosyl zeatin 5'-phosphate)

A solution of phosphorylating agent N-morpholino O,O-bis[(6-trifluoromethyl)benzotriazolyl] phosphate in dioxane (reagent D, 0.2M, 4.5 ml, 0.90 mmol) was added to **6** (0.22 g, 0.5 mmol) which was coevaporated with pyridine (3x5 ml) and dissolved in pyridine (5 ml). N-methylimidazole (0.07 ml, 0,93 mmol) was then added. After 1h TLC analysis (system B) indicated complete conversion of the purine compound **8** into intermediate **12**, Rf=0.77, water was then added to the reaction mixture (3 ml). After 1h TLC (system B) showed the formation of a new product with zero mobility. The reaction mixture was evaporated and the residue was diluted with 25% aqueous ammonia (10 ml) to remove the acetyl group and the mixture was left for 20h at 20°C. The reaction mixture was concentrated to a small volume. The residue was diluted with water (15 ml) and the pH was adjusted to 2.0 by the addition of HCl (0.1N) to remove methoxymethylidene and morpholino groups. After 15h at 20°C the reaction mixture was extracted with diethyl ether (2x100 ml). Then the pH of the aqueous layer was adjusted to 8.0 with aqueous ammonia 25%, concentrated to a small volume (1 ml) and was applied to a column of DEAE-Sephadex A25 (HCO_3^--form) suspended in triethylammonium bicarbonate buffer (0.05M). The column was eluted with a linear gradient of triethylammonium bicarbonate buffer (0.05→0.7M) for 16h with a flow rate of 35 ml/h. Fractions of 10 ml were collected and all UV-positive eluates, containing purine product **1b**, [Rf=0 (system D), Rf=0.34 (system E)], were pooled, concentrated to a small volume and coevaporated with water (4x50 ml), and lyophilized from H_2O. The residue was dissolved in water (2 ml) and applied to a column of Dowex 50 Wx-8 cation-exchange resin (Na^+-form, 1.5x5 cm).

The column was eluted with water and all UV-positive eluates were collected, concentrated to a small volume and lyophilized from D_2O to give **1b** as a white solid. Yield 160 mg (68% based on **6**), [Rf=0.34 (system E), Rf=0.20 (system F)]. Product identity was confirmed by UV and thin-layer chromatography, which was identical as described in the literature[7], and further with ^{31}P NMR, ^{13}C NMR and ^1H NMR.

Compound **1b**, ^1H NMR (D_2O): δ 8.39, s, 1H, H-8; 8.20, s, 1H, H-2; 6.00, d, J=5.5 Hz, H_1'; 5.59, t, J=6.6 Hz, 1H, CH=C; 4.73, t, J=5.5 Hz, H_2'; 4.48, dd, J(H_3'-H_4')=4.1 Hz, J(H_3'-H_2')=4.8 Hz, 1H; H_3'; 4.38 , m, 1H, H_4'; 4.19, brd, J=4.8 Hz, 2H, CH_2-N; 4.10, m, 2H, H_5' and H_5"; 4.00, s, 2H, C=C-CH_2-O; 1.74, s, 3H, CH_3; ^{13}C NMR (D_2O): δ 155.3, 153.4, 150.1, 140.1 and 119.8, 5xs (purine); 139.7, s, CH_3-$\underline{C}$=C; 88.3, s, C_1'; 85.7, d, $^3J_{C-P}$=3.7 Hz, C_4'; 77.3, s, C_3'; 73.0, s, C_2'; 71.0, s, C=C-$\underline{CH_2}$-O; 68.0, s, C_5'; 40.2, s, CH_2-N, 14.3, s, CH_3; ^{31}P NMR (D_2O): δ_P=1.10 ppm.

Synthesis of 6-[3-methyl-2-but-enylamino]-9-(5'-O-phosphate-β-D-ribofuranosyl) purine **2b** [N^6-(Δ^2-Isopentenyl)adenosine 5'-phosphate]

A solution of phosphorylating agent N-morpholino O,O-bis[(6-trifluoromethyl)benzotriazolyl phosphate (reagent D) in dioxane (0.2M, 4.77 ml), 0.95 mmol) was added to purine compound **10** (0.20 g, 0.53 mmol), which was coevaporated with pyridine (2x10 ml) and dissolved in pyridine (5 ml), N-methylimidazole (0.08 ml, 1 mmol) was then added. After 1h TLC analysis (system B) indicated complete conversion of purine compound **10** into intermediate **11**, Rf=0.82. Water was then added to the reaction mixture (3 ml). After 1h TLC analysis (system B) showed the formation of new product with zeromobility. The reaction mixture was evaporated and the residue was diluted with water (15 ml) and the pH was adjusted to 2.0 by the addition of HCl (0.1N) to remove methoxymethylidene as well as the morpholino groups. After 15h at 20°C the reaction mixture was extracted with diethyl ether (2x100 ml). The pH of the aqueous layer was adjusted to pH=8.0 with aqueous ammonia 25%. The reaction solution was concentrated to a small volume (1 ml) and was applied to a column of DEAE-Sephadex A25 (HCO_3^--form) suspended in triethylammonium bicarbonate buffer (0.05M) to give after work up and purification as described in the synthesis of **1b** the sodium salt of **2b** as a white solid. Yield 177 mg (73% based on **10**), Rf=0.35 (system E), product identity was confirmed by UV and thin-layer chromatography which was identical as described in the literature[4,5] and further with ^{31}P NMR, ^{13}C NMR and ^1H NMR.

Compound **2b**, ^1H NMR (D_2O): δ 8.42, s, 1H, H-8; 8.19, s, 1H, H-2; 6.08, d, J=6 Hz, 1H, H_1'; 5.36, t, J=5.9 Hz, 1H, CH=C; 4.70, t, J=5.8 Hz, 1H, H_2'; 4.45, dd, $J(H_3'-H_4')$=3.9 Hz, $J(H_3'-H_2')$=4.9 Hz, 1H, H_3'; 4.32, m, 1H, H_4'; 4.00, br d, J=6.3 Hz, 2H, CH_2-N; 4.08, m, 2H, H_5' and H_5''; 1.70, s, 3H, CH_3; ^{13}C NMR (D_2O): δ 154.3, 152.9, 148.1, 139.2, 118.8, 5xs (purine); 137.9, s, $(CH_3)_2$-C=C; 87.0, s, C_1'; 84.2, d, $^3J_{C-P}$=8.2 Hz, C_4'; 74.5, s, C_3'; 70.6, s, C_2'; 64.2, s, C_5', 38.9, s, CH_2-N; 24.8 and 17.3, 2xs, $(CH_3)_2$-C=C; ^{31}P NMR (D_2O): δ_P=1.3 ppm.

Synthesis of 6-[4-O-phosphate-3-methyl-E-but-2-enylamino]-9-β-D-ribfuranosyl) purine **1d** (ribosyl zeatin allylic phosphate)

To a solution of **6** (0.20 g, 0.40 mmol) and N,N-diisopropylethylamine (0.10 ml, 0.56 mmol) in dioxane was added 2-chloro-4H-1,3,2-benzodioxaphosphorin-4-one (reagent K, 114 mg, 0.56 mmol). After reaction for 5 min. at 20°C, it was found by TLC analysis (system C) that complete conversion of starting compound into a product with zeromobility has taken place. Water was added (1 ml) and the solution was left for 10 min. at 20°C to give **15** [Rf=0 (system D), Rf=0.67 (system E)]. The reaction solution was concentrated to a small volume and triturated with diethyl ether, the precipitated oil was coevaporated with acetonitrile (4x10 ml) and dissolved in acetonitrile (10 ml). The solution was treated with N,N-diisopropylethylamine (0.15 ml), 0.80 mmol) and N,O-bis(trimethylsilyl) acetamide (0.2 ml, 0.8 mmol). After 15 min. the reaction solution was further treated with 2,2'-dipyridyldisulfide (160 mg, 0.48 mmol) after 1h at 20°C TLC analysis (system E) indicated the complete conversion of **15**.

Then water was added (3 ml) and the reaction solution left for 2h at 20°C to give **19** with Rf=0.40 (system E). The reaction solution was concentrated to an oil and dissolved in water (20 ml). The

pH of the resulting solution was adjusted to pH=2 by the addition of HCl (0.1N). After 3h at 20°C TLC analysis (system E) showed that the cleavage of (TBDMS) as well as methoxymethylidene was complete. The reaction solution was washed with diethyl ether (2x100 ml). The aqueous layer was neutralized to pH=8.0 with aqueous ammonia 25%, concentrated to an oil and applied to a column of DEAE-Sephadex A25 (HCO_3^--form) suspended in triethylammonium bicarbonate buffer (0.05M). The column was eluted with a linear gradient of triethylammonium buffer (0.05→0.70M) for 15h with a flow rate of 35 ml/h and fractions of 8 ml were collected. All UV-positive eluates containing **1d**, [Rf=0 (system D), Rf=0.30 (system E)] were pooled and concentrated to a small volume, coevaporated with water (4x50 ml) and lyophilized from H_2O. The residue was dissolved in water (2 ml) and applied to a column of Dowex 50 Wx-8 cation-exchange resin (Na^+-form, 1.5x5 cm). The column was eluted with water and all UV-positive eluates were collected, concentrated to a small volume and lyophilized from D_2O to give **1d** as a white solid. Yield 145 mg (76% based on **6**), Rf=0.30 (system E).

Compound 1d, ^1H NMR (D_2O): δ 8.19, s, 1H, H-8; 8.12, s, 1H, H-2; 6.00, d, J=6.3 HZ, 1H, H_1'; 5.65, t, J=6.2 HZ, 1H, CH=C; 4.72, t, J=6.2 Hz, 1H, H_2'; 4.38, dd, J(H_3'-H_2')=5.2 Hz, J(H_3'-H_4')=3.3 Hz, 1H, H_3'; 4.24, m, 1H, H_4'; 4.16, d, $^3J_{H-P}$=5.4 Hz, 2H, C=C-$\underline{CH_2}$-O; 4.10, br s, 2H, CH_2-N; 3.88-3.75, 2xdd, J(H_5'-H_5'')=12.8 Hz, J($H_5'$$_5''$-$H_4'$)=2.5 Hz and 3.4 Hz, 2H, H_5' and H_5''; 1.73, s, 3H, CH_3; ^{13}C NMR (D_2O): δ 154.7, 153.1, 148.2, 140.3 and 119.9, 5xs (purine); 138.2, d, $^3J_{C-P}$=7.4 Hz, CH_3-$\underline{C}$=C; 121.0, s, $\underline{CH}$=C, 88.5, s, C_1'; 85.8, s, C_4'; 74.0, s, C_3'; 70.7, s, C_2', 68.9, s, C=C-$\underline{CH_2}$-O, 61.8, s, C_5'; 38.8, s, CH_2N; 13.4, s, CH_3; ^{31}P NMR (D_2O): δ_P=2.12 ppm.; compounds **15** and **19** were identified after purification by using a column of DEAE-Sephadex A 25 (HCO_3^--form); the purified compounds were converted into sodium salts by passing it over Dowex 50 Wx-8 (Na^+-form).

Compound **15**, ^{1} H-NMR (D_2O): δ=8.15, s, 1H, H-8; 8.10, s, 1H, H-2; 6.95, d, $^1J_{P-H}$=632 Hz, 1H, P-H; 6.28, d, J=2.2 Hz, 1H, H_1'; 6.17, s, 1H, $\underline{CH}$-OCH_3; 5.60, t, J=6.5 Hz, 1H, CH=C; 5.40, m, 1H, H_2'; 5.17, m, 1H, H_3', 4.45, m, 1H, H_4'; 4.35, d, $^3J_{H-P}$=8 Hz, 2H, C=C-$\underline{CH_2}$-O; 4.17, br d, J=6.5 Hz, 2H, CH_2-N, 3.88-3.72, m, 2H, H_5' and H_5'', 3.42, s, 3H, CH-$\underline{OCH_3}$; 1.72, s, 3H, CH_3; 0.80, s, 9H, TBDMS; 0.00, s, 6H, TBDMS, ^{13}C NMR (D_2O): δ 155.5, 153.9, 147.2, 141.2 and 119.7, 5xs (purine); 137.1, d, $^3J_{C-P}$=5.4 Hz; CH_3-$\underline{C}$=C; 123.1, s, $\underline{CH}$-OCH_3; 120.1, s, $\underline{CH}$=C; 91.4, s, C_1'; 86.6, s, C_4'; 83.0, s, C_3'; 81.5, s, C_2', 69.8, s, C=C-$\underline{CH_2}$-O; 62,8, s, C_5'; 51.7, s, CH-$\underline{OCH_3}$; 39.7, s, CH_2-N; 26.4, 18.8 and -5.0, 3xs (TBDMS); 14.4, s, CH_3; ^{31}P NMR (D_2O): δ_P=6.77 ppm., $^1J_{P-H}$=633 Hz.

Compound **19**, ^1H NMR (D_2O): δ 8.5, s, 1H, H-8; 8.2, s, 1H, H-2; 6.22, d, J=4 Hz, 1H, H_1'; 6.15, s, 1H, $\underline{CH}$-OCH_3; 5.56, t, J=6.4 Hz; 1H, CH=C; 5.23, m, 1H, H_2'; 4.50, m, 1H, H_3'; 4.28, m, 5H, CH_2-N, H_4' and C=C-$\underline{CH_2}$-O; 3.82-3.75, m, 2H, H_5' and H_5''; 3.75, s, 3H, CH-$\underline{OCH_3}$; 1.74, s, 3H, CH_3; 0.88, s, 9H, TBDMS; 0.00, s, 6H, TBDMS;, ^{31}P NMR (D_2O): δp=2.20 ppm.

Synthesis of 6-[4-O-phosphate-3-methyl-E-but-2-enylamino]-9-(5'-O-phosphate-β-D-ribofuranosyl) purine **1c** (ribosyl zeatin diphosphate)

Ribosyl zeatin diphosphate **1c** was synthesized in a similar procedure as described in the synthesis of ribosyl zeatin allylic phosphate **1d**, thus diphosphonate derivative **16** was obtained from ribofuranosyl derivative **9** (0.20 g, 0.5 mmol), 1,3,2-benzodioxaphosphorin-4-one (reagent K,

0.28 g, 1.4 mmol) and N,N-diisopropylethylamine (0.26 ml, 1.4 mmol). The oxidation of diphosphonate derivative **16**, Rf=0.56 (system E) to diphosphate derivative **20** was realized by using N,O-bis(trimethylsilyl) acetamide (0.5 ml, 2 mmol), N,N-diisopropylethylamine (0.35 ml, 2 mmol) and 2,2'-dipyridylsulfide (260 mg, 1.2 mmol).

The diphosphate derivative **20**, Rf=0.35 (system E) was acidified to pH=2 for 3h to give **1c**, Rf=0.28 (system E), the latter was purified on a column of DEAE-Sephadex A25 (HCO_3^--form) eluted with a linear gradient of triethylammonium buffer (0.05→1M) and was converted into its sodium salt, **1c** was obtained as white solid after lyophylization. Yield 210 mg, (70% based on **9**), Rf=0.28 (system E).

Compound **1c**, ^1H NMR (D_2O): δ 8.40, s, 1H, H-8; 8.14, s, 1H, H-2; 6.05, d, J=5.8 Hz, 1H, H_1'; 5.65, t, J=6.6 Hz, 1H, CH=C; 4.71, t, J=5.5 Hz, 1H, H_2'; 4.45, dd, $J(H_3'-H_4')$=3.7 Hz, $J(H_3'-H_2')$=5.2 Hz, 1H, H_3'; 4.22, m, 1H, H_4'; 4.22, d, $^3J_{H-P}$=6.3 Hz, 2H, C=C-$\underline{CH_2}$-O; 4.15, br d, J=5.8 Hz, 2H, CH_2-N, 4.02, m, 2H, H_5' and H_5''; 1.74, s, 3H, CH_3; ^{13}C NMR (D_2O): δ 154.7, 153.2, 148.5, 139.7 and 119.2, 5xs (purine); 137.1, d, $^3J_{C-P}$=7.8 Hz, CH_3-$\underline{C}$=C; 122.1, s, $\underline{CH}$=C; 87.42, s, C_1'; 84.5, d, $^3J_{C-P}$=5.6 Hz, H_4'; 74.6, s, C_3', 70.7, s, C_2', 69.8, s, C=C-$\underline{CH_2}$-O, 64.28, s, C_5'; 38.8, s, CH_2-N; 13.4, s, CH_3; ^{31}P NMR (D_2O): δ_P=2.55 ppm. and 1.90 ppm.; compounds **16** and **20** were identified after purification by using a column of DEAE-Sephadex A 25 (HCO_3^--form); the purified compounds were converted into sodium salt by passing it over Dowex 50 Wx-8 (Na^+-form).

Compound **16**, ^1H NMR (D_2O): δ 8.11, s, 1H, H-8; 8.00, s, 1H, H-2; 6.15, d, J=2.1 Hz, 1H, H_1'; 6.04, s, 1H, $\underline{CH}$-OCH_3; 6.70, d, $^1J_{H-P}$=635 Hz, 1H, P-H; 5.60, t, J=6.5 Hz, 1H, CH=C; 6.55, d, $^1J_{H-P}$=639 Hz, 1H, P-H; 5.30, m, 1H, H_2', 5.07, m, 1H, H_3'; 4.56, m, 1H, H_4'; 4.23, d, $^3J_{H-P}$=8.6 HZ, 2H, C=C-$\underline{CH_2}$-O; 4.00, m, 4H, CH_2-N, H_5' and H_5''; 3.43, s, 3H, CH-$\underline{OCH_3}$; 1.71, s, 3H, CH_3; ^{13}C NMR (D_2O): δ 154.3, 153.0, 147.8, 135.9 and 118.9, 5xs (purine); 139.5, d, $^3J_{C-P}$=8.3 Hz, CH_3-$\underline{C}$=C; 122.9, s, CH=C; 117.9, s, CH-OCH_3; 90.1, s, C_1'; 85.5, d, $^3J_{C-P}$=7.4 Hz, C_4'; 83.2, s, C_3', 80.5, s, C_2', 68.8, s, C=C-$\underline{CH_2}$-O; 63.4, s, C_5'; 52.7, s, CH-$\underline{OCH_3}$; 38.6, s, CH_2-N, 13.4, s, CH_3; ^{31}P NMR (D_2O): δp=6.73 ppm. ($^1J_{P-H}$=634.7 Hz) and δp=6.67 ppm., ($^1J_{P-H}$=631 Hz).

Compound **20**, ^1H NMR (D_2O): δ 8.38, s, 1H, H-8; 8.15, s, 1H, H-2, 6.20, d, J=2.5 Hz, H_1', 6.06, s, 1H, $\underline{CH}$-OCH_3; 5.50, t, J=6.4 Hz, 1H, CH=C; 5.41, m, 1H, H_2'; 5.14 m, 1H, H_3', 4.30 m, 1H, H_4'; 4.13, br d, J=5.4 Hz, s, 4H, CH_2-N and C=C-$\underline{CH_2}$-O; 3.91, m, 2H, H_5' and H_5''; 3.40, s, 3H, CH-$\underline{OCH_3}$; 1.71, d, 3H, CH_3; ^{31}P NMR (D_2O): δ_P=4.30 ppm. and 4.22 ppm.

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19. Compound **1a**, ^{1}H NMR (D_2O): δ 8.16, s, 1H, H-8; 8.09, s, 1H, H-2; 5.96, d, J=5.9 Hz, 1H, H_1'; 5.54, t, J=6.35 Hz, 1H, CH=C; 4.68, t, J=5.9 Hz, 1H, H_2'; 4.36, dd, J(H_3'-H_2')= 4.9 Hz, J(H_3'-H_4')=3.4 Hz, 1H, H_3'; 4.23, m, 1H, H_4', 4.15, br s, 2H, CH_2-N; 3.96, s, 2H, C=C-CH_2-O; 3.88-3.75; 2xdd, J(H_5'-H_5")=12.8 Hz, J(H_5'$_5$"-H_4')=2.5 Hz and 3.41 Hz; 2H, H_5' and H_5"; 1.69, s, 3H, CH_3; ^{13}C NMR (D_2O): δ 153.8, 152.8, 147.2, 139.7 and 119.0, 5xs (purine); 138.5, s, CH_3-C=C; 120.4, s, CH=C, 88.2, s, C_1', 85.8, s, C_4'; 74.1, s, C_3'; 73.6, s, C_2'; 70.7, s, C=C-CH_2-O; 66.5, s, C_5'; 38.6, s, CH_2-N; 13.6, s, CH_3.
20. Compound **2a**, ^{1}H NMR (D_2O): δ 8.19, s, 1H, H-8; 8.03, s, 1H, H-2; 5.90, d, J=6.12 Hz, 1H, H_1'; 5.26, t, J=6.6 Hz, 1H, CH=C; 4.67, t, J=5.9 Hz, 1H, H_2'; 4.50, dd, J(H_3'-H_2')=5.2 Hz, J(H_3'-H_4')=3 Hz, 1H, H_3'; 4.31, m, 1H, H_4'; 4.20, br d, J=3 Hz, 2H, CH_2-N; 3.86-3.73, 2xdd, J(H_5'-H_5")=12.2 Hz, J(H_5'$_5$"-H_4')=2.5 Hz and 3.4 Hz, 2H, H_5' and H_5"; 1.71, s, 3H, CH_3-C=C; 1.65, s, 3H, CH_3-C=C. ^{13}C NMR (D_2O): δ 153.9, 152.2, 147.1, 140.3, 119.0, 5xs (purine); 137.6, s, CH_3-C=C; 88.4, s, C_1', 85.5, s, C_4', 73.9, s, C_2', C_3', 70.4, s, C_2', 61.2, s, C_5', 38.5,s, CH_2-N, 24.5 and 16.9, 2xs, $(CH_3)_3$-C=C.

CHAPTER VII

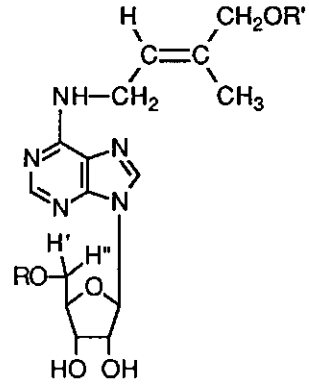
THE SYNTHESIS OF PHOSPHOR DERIVATIVES OF RIBOSYL ZEATIN

INTRODUCTION

Cytokinins are a class of hormones that were first isolated from plant tissues. They regulate cell division and growth in plants and participate in the differentiation process through their interaction with other plant hormones[1]. Certain cytokinins have also been shown to affect the behaviour of mammalian cells. For example, they have been reported to regulate the growth of mammalian cells[2,3,4], to inhibit platelet aggregration[5] and to have immunosuppressive activity[6]. Cytokinins have also been employed as potential anticancer agents in clinical trials[7,8]. Naturally occurring cytokinins are N^6-substituted adenine derivatives and several highly active species have been isolated at the purine, ribonucleoside and ribonucleotide levels[9-12]. One of these adenine derivatives with plant cell division promoting activity was isolated[13] from Zeamays and was identified as 6-(4-hydroxy-3-methyl-E-but-2-enylamino)-9-(β-D-ribofuranosyl)purine i.e. **1a**, usually named ribosyl zeatin.
In an earlier work we reported on the synthesis of ribosyl zeatin phosphates[14]. Now we wish to describe the synthesis of some phosphor derivatives of ribosyl zeatin i.e. **1b-1g** (see fig. VII.1)..

RESULTS AND DISCUSSION

Recently we described a new and efficient method for the synthesis of the phosphate, methyl phosphate and thiophosphate monoester of the allylic hydroxy group in 6-(4-hydroxy-3-methyl-E-but-2-enylamino)purine[15]. This method is based on the phosphitylation of the allylic hydroxy group with salicyl chlorophosphite yielding the allylic phosphonate monoester, and subsequent oxidation or sulphurization.



1a. R' = R = H

1b. R' = $\overset{\overset{O}{\|}}{\underset{\underset{H}{|}}{P}}$—OH , R = H

1c. R' = R = $\overset{\overset{O}{\|}}{\underset{\underset{H}{|}}{P}}$—OH

1d. R' = $\overset{\overset{O}{\|}}{\underset{\underset{OH}{|}}{P}}$—$OCH_3$, R = H

1e. R' = R = $\overset{\overset{O}{\|}}{\underset{\underset{OH}{|}}{P}}$—$OCH_3$

1f. R' = $\overset{\overset{S}{\|}}{\underset{\underset{OH}{|}}{P}}$—OH , R = H

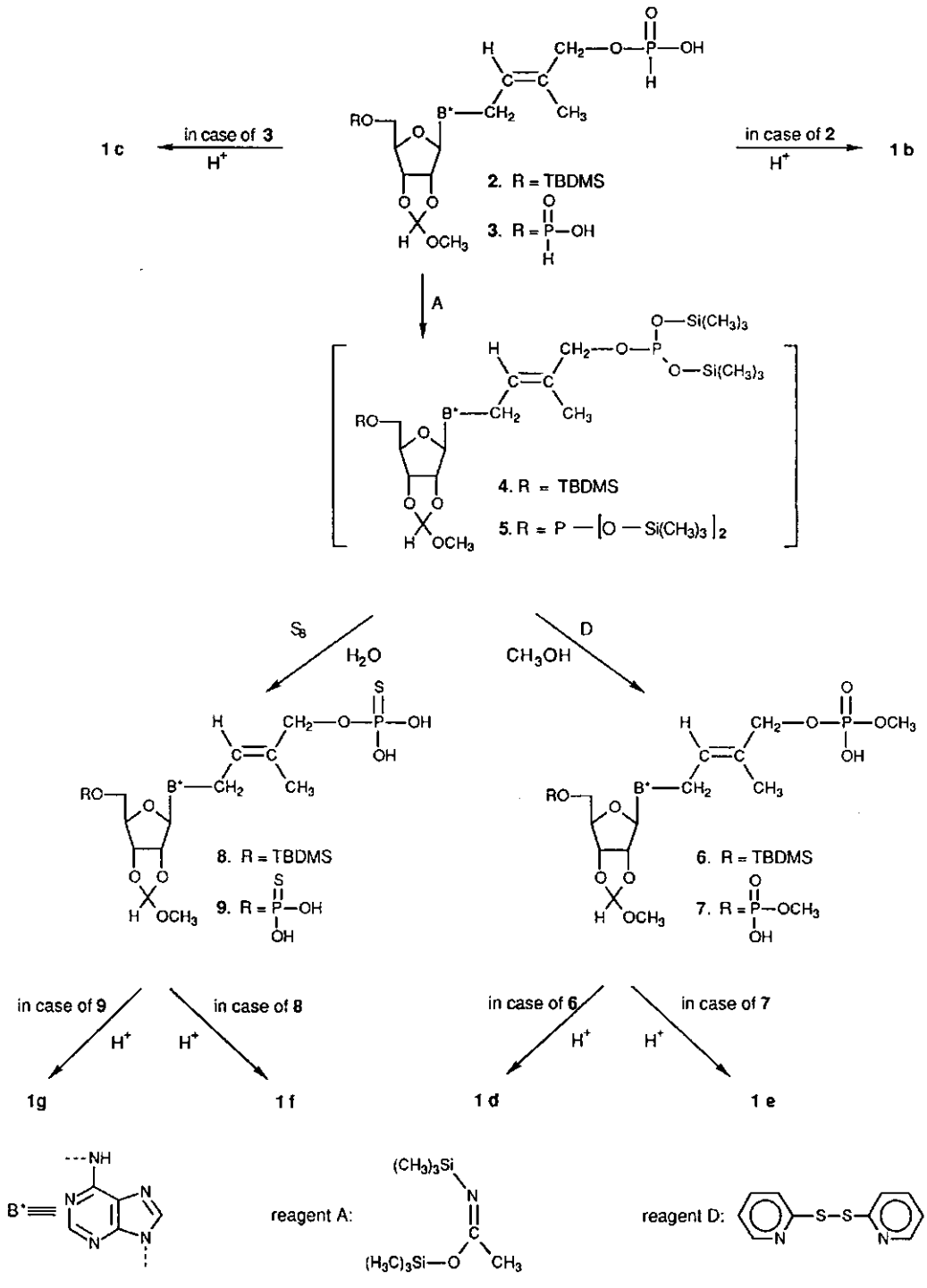
1g. R' = R = $\overset{\overset{S}{\|}}{\underset{\underset{OH}{|}}{P}}$—OH

Fig. VII.1

We wanted to examine whether this methodology is also suitable for the synthesis of some phosphor derivatives of ribosyl zeatin i.e. **1b-1g.**

According to a slight modification of the procedure of Hata et al.[16] ribosyl zeatin allylic phosphonate **2** and ribosyl zeatin bis(phosphonate) **3** (both compounds **2** and **3** being prepared by a procedure described before[14]) were treated with N,O-bis(trimethylsilyl)acetamide (reagent A) in the presence of N,N-diisopropylethylamine for 15 min. to give the intermediary bis(trimethylsilyl)phosphites **4** and **5**, respectively (see scheme VII.1). Without any further purification both **4** and **5** were treated with 2,2'-dipyridyldisulfide (reagent D) and the reaction solution thus obtained was treated with dry methanol affording **6** and **7**, respectively. The structures of **6** and **7** were ascertained by ^{1}H-NMR and ^{31}P-NMR spectroscopy. Treatment of **4** and **5** with powdered sulfur for 15 h and subsequent treatment with water afforded the sulfur compounds **8** and **9**, respectively. The structure of these compounds was ascertained by ^{1}H-NMR and ^{31}P-NMR spectroscopy.

The cleavage of the acid-labile groups tert-butyldimethylsilyl (TBDMS) and methoxymethylidene in the derivatives **2, 3, 6, 7, 8** and **9** was effected by acid treatment at pH=2 for 3 h. The ribosyl zeatin allylic phosphonate **1b**, ribosyl zeatin bis(phosphonate) **1c**, ribosyl zeatin allylic


1 c
in case of 3
H+
in case of 2
H+
1 b
2. R = TBDMS
3. R = P(O)(H)OH
A
4. R = TBDMS
5. R = P(O-Si(CH3)3)2
S8
H2O
D
CH3OH
8. R = TBDMS
9. R = P(S)(OH)OH
6. R = TBDMS
7. R = P(O)(OCH3)OH
in case of 9
in case of 8
in case of 6
in case of 7
H+
1g
1 f
1 d
1 e
reagent A:
reagent D:

Scheme VII.1

thiophosphate **1f**, ribosyl zeatin bis(thiophosphate) **1g**, ribosyl zeatin allylic methyl phosphate **1d** and ribosyl zeatin bis(methyl phosphate) **1e** were obtained in good yields. Over-all yields in the preparation of the compounds **1b-1g** fluctuated between 70% and 90% (calculated from **2** and **3**).

The structure of **1b-1g** was proven by ^{1}H-NMR, ^{13}C-NMR and ^{31}P-NMR data. The ^{1}H-NMR and ^{13}C-NMR data of the ribosyl zeatin derivatives **1b-1g** bear a strong resemblance to those of ribosyl zeatin[14]. However, there are some exceptions: i. The ^{1}H-absorption of H_5' and H_5'' appears in ribosyl zeatin **1a** as two pairs of double doublets at 3.75-3.88 ppm., due to the fact that H_5' and H_5'' have slightly different chemical shifts and $J(H_5'\text{-}H_5'') \neq J(H_5'\text{-}H_4') \neq J(H_5''\text{-}H_4')$. In **1c**, **1e** and **1g** the absorption of H_5' and H_5'' appears as an undefined multiplet at about 4.10 ppm. No conclusion can be drawn whether ^{31}P coupling occurs with H_5' and H_5''. ii. The ^{1}H-absorption of C=C-CH_2-O in ribosyl zeatin **1a** is found as a singlet at 3.96 ppm., but in **1b-1g** this absorption appears as a doublet at about 4.20 ppm. The doublets are caused by the $^{3}J_{H\text{-}P}$ coupling. iii. The ^{13}C-resonance of CH_3-C=C in ribosyl zeatin **1a** is a singlet (at about 138 ppm.), but in **1b-1g** it is a doublet. Similarly, the ^{13}C-signals of C_4' appear in ribosyl zeatin **1a** as a singlet at about 85 ppm., but in **1c**, **1e** and **1g** they appear as doublets, due to coupling of ^{13}C with $^{31}P(^{3}J_{C\text{-}P})$. Proton decoupled ^{31}P-NMR spectroscopy revealed the presence of the expected number of resonance absorptions, which further proves the structural identity and purity of the compounds.

In conclusion, the methodology described in this paper presents an elegant way for the synthesis of phosphate derivatives of ribosyl zeatin. The yields are satisfactory, the reaction proceeds smoothly under mild conditions.

EXPERIMENTAL

General procedures.

N,N-diisopropylethylamine and acetonitrile were dried by refluxing with CaH_2 for 16 h and then distiled. Methanol was dried by refluxing with magnesium methoxide and distiled before use. All liquids were stored under nitrogen. N,O-bis(trimethylsilyl)acetamide and 2,2'-dipyridyldisulfide were purchased from Janssen Chimica (Belgium). Triethylammonium bicarbonate buffer was prepared by passing a stream of CO_2 gas through a cooled (ice-water bath) 2 M solution of triethylamine in deionized water until the solution became neutral. Schleicher and Schüll DC Fertigfolien were used for TLC. The following solvent system was used: system A (isopropyl alcohol /concentrated ammomium hydroxide /water, 7:1:2, v/v). Short column chromatography was performed on Sephadex LH 20 suspended in CH_2Cl_2/CH_3OH (2:1, v/v), unless otherwise mentioned. DEAE-Sephadex A 25 was purchased from Pharmacia (Uppsala, Sweden). Cation-exchange resin (Na^+-form): a solution of NaOH (2 M; 100 ml) was passed over a column packed with cation-exchange resin (Dowex 50 Wx-8, 100-200 mesh; Fluka H^+-form, 1.5x5 cm) followed by washing of the column with sterile water until pH=7. Sterile water and glass were used during the whole deblocking and purification processes. ^{1}H-NMR spectra were measured at 300 MHz

using a Bruker CXP 300 spectrometer. ^{13}C-NMR spectra were measured at 75.460 MHz using a Bruker CXP 300 spectrometer; proton noise decoupling was used. ^{31}P-NMR spectra were measured at 121.470 MHz using a Bruker CXP 300 spectrometer, chemical shifts are in ppm relative to 85% H_3PO_4 as external standard.

6-(4-O-Phosphonate-3-methyl-E-but-2-enylamino)-9-(2',3'-O-methoxymethylidene-5'-O-tert-butyl-dimethylsilyl-β-D-ribofuranosyl) purine **2** and 6-(4-O-phosphonate-3-methyl-E-but-2-enylamino)-9-(2',3'-O-methoxymethylidene-5'-O-phosphonate-β-D-ribofuranosyl)purine **3** were prepared as described previously[14].

Synthesis of 6-(4-O-methylphosphate-3-methyl-E-but-2-enylamino)-9-(β-D-ribofuranosyl)purine **1d** (ribosyl zeatin allylic methylphosphate)

The Na^+-salt of **2** (0.25 g, 0.42 mmol) was repeatedly coevaporated with acetonitrile (4x10 ml). The residue was diluted with acetonitrile (5 ml) and this mixture was treated with N,N-diisopropylethylamine (0.15 ml, 0.84 mmol) and N,O-bis(trimethylsilyl)acetamide (0.21 ml, 0.84 mmol). After 15 min. the solution was reacted with 2,2'-dipyridyldisulfide (108 mg, 0.5 mmol) for 1 h at 20°C. TLC analysis (system A) indicated the complete absence of **2**. After addition of an excess of methanol (3 ml) the solution was left for 15 h at 20°C to give **6**, Rf= 0.52 (system A). The reaction solution was concentrated to an oil and dissolved in water (20 ml). The pH of the resulting solution was adjusted to 2 by the addition of HCl (0.1 N). After 3 h at 20°C, TLC analysis (system A) showed that the cleavage of TBDMS as well as methoxymethylidene was complete. The reaction solution was washed with diethylether (2x100 ml). The aqueous layer was neutralized to pH=8.0 with aqueous ammonia (25%), concentrated to an oil and applied to a column of DEAE-Sephadex A 25 (HCO_3^--form) suspended in triethylammonium bicarbonate buffer (0.05 M). The column was eluted with a linear gradient of triethylammonium buffer (0.05→0.50 M) for 15 h with a flow rate of 35 ml/h and fractions of 10 ml were collected. The UV-positive eluates containing **1d**, Rf=0.37 (system A) were pooled and concentrated to a small volume, coevaporated with water (4x50 ml) and lyophilized from H_2O. The residue was dissolved in water (1 ml) and applied to a column of Dowex 50 Wx-8 cation-exchange resin (Na^+-form, 1.5x5 cm). The column was eluted with water and all UV-positive eluates were collected, concentrated to a small volume and lyophilized from D_2O to give **1d** as a white solid. Yield 144 mg (74% based on **2**).

Compound **1d**; ^{1}H-NMR (D_2O): δ 8.20, s, 1H, H-8; 8.10, s, 1H, H-2; 6.01, d, J=6.0 Hz, 1H, H_1'; 5.62, t, J=6.2 Hz, 1H, CH=C; dd, J(H_3'-H_2')=3.4 Hz, J(H_3'-H_4')=5.4 Hz, 1H, H_3'; 2.28, m, 1H, H_4'; 4.19, d, $^3J_{H\text{-}P}$=6.5 Hz, 2H, C=C-CH_2-O; 3.90, brs, 2H, CH_2-N; 3.86-3.50, 2xdd, J(H_5'-H_5")=12.8 Hz, J(H_5'$_5$"-H_4')=2.6 Hz and 3.4 Hz, 2H, H_5' and H_5"; 3.46, d, $^3J_{H\text{-}P}$=10.8 Hz, 3H, P-OCH_3; 1.70, s, 3H, CH_3; ^{13}C-NMR (D_2O): δ 154.6, 152.8, 150.0, 148.1 and 119.6, 5xs (purine); 136.2, d, $^3J_{C\text{-}P}$=6.3, CH_3-C=C; 122.1, s, CH=C; 88.5, s, C_1'; 85.8, s, C_4'; 74.1, s, C_3'; 70.8, s, C_2'; 70.7, s, C=C-CH_2-O; 61.9, s, C_5'; 53.7, s, P-OCH_3; 39.2, s, CH_2-N; 14.0, s, CH_3; ^{31}P-NMR (D_2O): δ_P=2.17 ppm.

Compound **6** was identified after purification by using a column of DEAE-Sephadex A25 (HCO_3^-); the purified compound was converted into the sodium salt by passing it over Dowex 50 Wx-8 (Na^+-form).

Compound 6; ^{1}H-NMR (D_2O): δ 8.05, s, 1H, H-8; 8.00, s, 1H, H-2; 6.10, d, J=4.1 Hz, 1H, H_1'; 6.00, s, 1H, CH-OCH_3; 5.58, t, J=6.2 Hz, 1H, CH=C; 5.5, m, 1H, H_2'; 4.80, m, 1H, H_3'; 4.60, m, 1H, H_4'; 4.10, d, $^3J_{H-P}$=7.8 Hz, 2H, C=C-CH_2-O; 4.00, m, 2H, CH_2-N; 3.60, m, 2H, H_5' and H_5"; 3.50, d, $^3J_{H-P}$=10.7 Hz, 3H, P-OCH_3; 3.15, s, 3H, CH-OCH_3; 1.70, s, 3H, CH_3; 0.88, s, 9H, (TBDMS); 0.00, s, 6H, (TBDMS); ^{31}P-NMR (D_2O): δ_P=2.19 ppm.

Synthesis of 6-(4-O-methylphoshate-3-methyl-E-but-2-enylamino)-9-(5'-O-methylphosphate-β-D-ribofuranosyl) purine **1e** [ribosyl zeatin bis(methylphosphate)]

Since compound **1e** was synthesized according to a similar procedure as described before in the synthesis of **1d**, we refrain from an extensive description and give here only some notes. The conversion of the Na^+-salt of bis(phosphonate) derivative **3** (0.25 g, 0.44 mmol) into the bis(methylphosphate) derivative **7** was realized by using N,O-bis(trimethylsilyl)acetamide (0.44 ml, 1.76 mmol), N,N-diisopropylethylamine (0.31 ml, 1.76 mmol), 2,2'-dipyridyldisulfide (228 mg, 1.1 mmol) and methanol (4 ml). The bis(methylphosphate) **7**, Rf=0.37 (system A) was acidified to pH=2. After 3 h **1e** was obtained, Rf=0.31 (system A) which was purified on a column of DEAE-Sephadex A 25 (HCO_3^--form). The column was eluted with a linear gradient of triethylammonium buffer (0.05→0.5 M) and was converted into its sodium salt. Compound **1e** was obtained as a white solid. Yield=192 mg (75% based on **3**).

Compound **1e**; ^{1}H-NMR (D_2O): δ 8.36, s, 1H, H-8; 8.14, s, 1H, H; 6.02, d, J=5.6 Hz, 1H, H_1'; 5.62, t, J=6.5 Hz, 1H, CH=C; 4.30, t, J=5.9 Hz, 1H, H_2'; 4.28, m, 1H, H_3; 4.26, m, 1H, H_4'; 4.23, d, $^3J_{H-P}$=6.6 Hz, 2H, C=C-CH_2-O; 4.21, brd, J=6.5, 2H, CH_2-N; 4.12, m, 2H, H_5' and H_5"; 3.56, d, $^3J_{H-P}$=12.6 Hz, 3H, P-OCH_3; 3.52, d, $^3J_{H-P}$=12.6 Hz, 3H, P-OCH_3; 1.70, s, 3H; ^{13}C-NMR (D_2O): δ 151.8, 153.4, 148.4, 139.5, 119.4, 5xs (purine), 136.2, d, $^3J_{C-P}$=5.60 Hz, CH_3-C=C; 123.1, s, CH=C, 87.6, s, C_1', 83.9, d, $^3J_{C-P}$=7.4 Hz, C_4'; 74.3, s, C_3', 70.8, s, C_2', 70.5, s, C=C-CH_2-O; 65.1, s, C_5', 53.2, s, 2x(P-OCH_3); 38.7, s, CH_2-N; 14.0, s, CH_3; ^{31}P-NMR (D_2O): δ_P=1.90 ppm and 2.05 ppm,

Compound **7** was identified after purification by using a column of DEAE-Sephadex A 25 (HCO_3^-); the purified compound was converted into sodium salt by passing it over Dowex 50 Wx-8 (Na^+-form).

Compound **7**; ^{1}H-NMR (D_2O): δ 8.22, s, 1H, H-8; 8.15, s, 1H, H-2; 6.30, d, J=2.6 Hz, 1H, H_1', 6.10, s, 1H, CH-OCH_3; 5.28, t, J=5.7 Hz, 1H, CH=C; 4.79, m, 1H, H_2'; 4.74, m, 1H, H_3'; 4.70, m, 1H, H_4'; 4.26, d, J=7.0 Hz, 2H, C=C-CH_2-O; 4.00, brd, J=4.2 Hz, 2H, CH_2-N; 3.90, m, 2H, H_5' and H_5"; 3.40, d, $^3J_{H-P}$=10.0 Hz, 3H, P-OCH_3; 3.36, d, $^3J_{H-P}$=10.0 Hz, 3H, P-OCH_3; 3.30, s, 3H, CH-OCH_3; 1.76, s, 3H, CH_3; ^{31}P-NMR (D_2O): δ_P=3.80 ppm and 4.21 ppm.

Synthesis of 6-(4-O-thiophosphate-3-methyl-E-but-2-enylamino)-9-(β-D-ribofuranosyl) purine **1f** (ribosyl zeatin allylic thiophosphate)

The Na^+-salt of **2** (0.25 g, 0.42 mmol) was repeatedly coevaporated with acetonitrile (4x10 ml). The residue was diluted with acetonitrile (5 ml) and this mixture was treated with N,N-diisopropylethylamine (0.15 ml, 0.84 mmol) and N,O-bis(trimethylsilyl)acetamide (0.21 ml, 0.84 mmol). After 15 min. the solution was treated with powdered sulfur (1 g) for 16 hrs. TLC analysis (system A) revealed that 16 h is required to complete the conversion of phosphonate **2** into

thiophosphate **8**, Rf=0.38 (system A). After this time water (1 ml) was added and the excess of sulfur was removed by filtration. The resulting solution was concentrated to a small volume and triturated with diethyl ether. The oil formed was dissolved in water (20 ml), the pH of this solution was adjusted to **2** by the addition of HCl (0.1 N). After 3 h at 20°C TLC analysis (system A) showed the complete cleavage of the TBDMS as well as the methoxymethylidene group. The reaction solution was washed with diethyl ether (2x100 ml), the aqueous layer was neutralized to pH=8.0 with aqueous ammonia (25%), concentrated to an oil and applied to a column of Sephadex LH 20. The column was eluted with CH_2Cl_2/CH_3OH (2:1, v/v). The fractions containing purine product **1f**, Rf=0.28 (system A) were pooled, concentrated to an oil and applied to a column of Dowex 50 WX-8 cation-exchange resin (Na^+-form 1.5x5 cm). The column was eluted with water and all UV-positive eluates were collected, concentrated to a small volume and lyophilized from D_2O to give **1f** as a white solid. Yield 167 mg (80% based on **2**).

Compound **1f**; ^{1}H-NMR (D_2O): δ 8.12, s, 1H, H-8, 8.00, s, 1H, H-2; 6.02, d, J=6.21 Hz, 1H, H_1'; 5.67, t=6.5 Hz, 1H, CH=C; 4.60, brs, 1H, H_2'; 4.14, dd, J(H_3'-H_2')=4.3 Hz, J(H_3'-H_4')=3.4, 1H, H_3'; 4.27, m, 1H, H_4'; 4.17, d, $^3J_{H\text{-}P}$=5.2 Hz, 2H, C=C-CH_2-O; 4.25 brs, 2H, CH_2-N; 3.75-3.90, 2xdd, J(H_5'-H_5'')=12.8 Hz, J($H_5'_5''$-H_4')=2.2 Hz and 3.4 Hz, 2H, H_5' and H_5''; 1.72, s, 3H, CH_3; ^{13}C-NMR (D_2O): δ 156.9, 153.1, 148.0, 140.0, 119.5, 5xs (purine); 138.2, d, $^3J_{C\text{-}P}$=7.4 Hz, CH_3-C=C; 121.5, s, CH=C; 89.1, s, C_1'; 86.2, s, C_4'; 74.6, s, C_3'; 71.2, s, C_2'; 67.9, s, C=C-CH_2-O; 62.6, s, C_5'; 39.4, s, CH_2-N; 14.0, s, CH_3; ^{31}P-NMR (D_2O): δp=46.00 ppm;

Compound **8** was identified after purification by using a column of Sephadex LH 20 suspended in CH_2Cl_2/CH_3OH (2:1, v/v); the purified compound was converted into sodium salt by passing it over Dowex 50 Wx-8 (Na^+-form).

Compound **8**; ^{1}H-NMR (D_2O): δ 8.25, s, 1H, H-8; 8.15, s, 1H, H-2, 6.20, d, J=3 Hz, 1H, H_1'; 6.05, s, 1H, CH-OCH_3; 5.60, t, J=6.5 Hz, 1H, CH=C; 4.80, m, 1H, H_2'; 4.72, m, 1H, H_3'; 4.20, m, 5H, CH_2-N, H_4' and C=C-CH_2-O; 3.80, m, 2H, H_5' and H_5''; 3.32, s, 3H, CH-OCH_3; 1.74, s, 3H, CH_3; 0.85, s, 9H, (TBDMS); 0.00, s, 6H, (TBDMS); ^{31}P-NMR (D_2O): δp=43.01 ppm.

Synthesis of 6-(4-O-thiophosphate-3-methyl-E-but-2-enylamino)-9-(5'-O-thiophosphate-β-D-ribofuranosyl) purine **1g** [ribosyl zeatin bis(thiophosphate)]

Compound **1g** was synthesized according to the same procedure as described above in the synthesis of ribosyl zeatin allylic thiophosphate **1f**; therefore only some notes are given. The sulfurization of the Na^+-salt of bis(phosphonate) derivative **3** (0.25 g, 0.44 mmol) was performed by using N,N-diisopropylethylamine (0.31 ml, 1.76 mmol), N,O-bis(trimethylsilyl)acetamide (0.31 ml, 1.76 mmol) and powdered sulfur (1g). The bis(thiophosphate) **9**, R=0.33 (system A) was acidified to pH=2. After 3 h **1g** was obtained, Rf=0.25 (system A), which was purified by using a column of Sephadex LH 20 eluting with CH_2Cl_2/CH_3OH (2:1, v/v). The purified compound was converted into its sodium salt. **1g** was obtained as white solid. Yield 227 mg (82% based on **3**).

Compound **1g**; ^{1}H-NMR (D_2O): δ 8.52, s, 1H, H-8; 8.13, s, 1H, H-2; 6.00, d, J=5.1 Hz, 1H, H_1'; 5.60, t, J=6.2 Hz, 1H, CH=C; 4.54, dd, J(H_3'-H_4')= 5.3 Hz, J(H_3'-H_2')=3.4 Hz, 1H, H_3'; 4.40, m, 1H, H_4'; 4.30, d, $^3J_{H\text{-}P}$=6.5 Hz, 2H, C=C-CH_2-O; 4.10, m, 4H, CH_2-N, H_5' and H_5''; 1.77, s, 3H, CH_3; ^{13}C-NMR (D_2O): δ 155.3, 153.8, 149.1, 140.5 and 119.8, 5xs (purine); 137.8, d, J=7.3 Hz, CH_3-C=C; 122.4, s, CH=C;

88.1, s, C_1'; 85.2, d, J=9.3 Hz; C_4'; 75.4, s, C_3'; 71.6, s, C=C-$\underline{CH_2}$-O; 70.4, s, C_2'; 64.9, s, C_5'; 39.5, s, CH_2-N; 14.0, s, CH_3; ^{31}P-NMR (D_2O): δ_P=49.52 ppm and 48.40 ppm;

Compound 9 was identified after purification by using a column of Sephadex LH 20 suspended in CH_2Cl_2/CH_3OH (2:1, v/v); the purified compound was converted into the sodium salt by passing it over Dowex 50 Wx-8 (Na^+-form).

Compound 9; ^{1}H-NMR (D_2O): δ 8.44, s, 1H, H-8; 8.13, s, 1H, H-2; 6.30, d, J=3.4 Hz, 1H, H_1'; 6.20, s, 1H, $\underline{CH}$-OCH_3; 5.61, t, J=5.6 Hz, 1H, CH=C; 5.43, m, 1H, H_2'; 3.39, m, 1H, H_3'; 4.67, m, 1H, H_4'; 4.21, d, J=6.4 Hz, 2H, C=C-CH_2-O; 4.15, brd, 2H, CH_2-N; 4.07-3.98, m, 2H, H_5' and H_5"; 3.35, s, 3H, CH-$\underline{OCH_3}$; 1.75, s, 3H, CH_3; ^{31}P-NMR (D_2O): δ_P=59.40 ppm.

Synthesis of 6-(4-O-phosphonate-3-methyl-E-but-2-enylamino)-9-(β–D-ribofuranosyl) purine **1b** ribosyl zeatin allylic phosphonate) and of 6-(4-O-phosphonate-3-methyl-E-but-2-enylamino)-9-(5'-O-phosphonate-β-D-ribofuranosyl) purine **1c** [ribosyl zeatin bis(phosphonate)]

The Na^+-salt of **2** (0.25 g, 0.42 mmol) and **3** (0.25 g, 0.44 mmol) respectively were dissolved in water (20 ml), the pH was adjusted to 2 with HCl (0.1 N) and this solution was kept for 3 h at 20°C. TLC analysis (system A) showed that the cleavage of the TBDMS as well as methoxymethylidene group was then complete. The solution was washed with diethyl ether (2x100 ml), neutralized with aqueous ammonia (25%), concentrated to a small volume and then applied to a column of Dowex 50 Wx-8 cation-exchange resin (Na^+-form, 1.5x5 cm). The column was eluted with water and all UV-positive eluates were collected, concentrated to a small volume and lyophilized from D_2O. The amount of **1b** obtained was 166 mg (90% based on **2**) and the yield of **1c** was 200 mg (86% based on **3**) respectively.

Compound **1c**; ^{1}H-NMR(D_2O): δ 8.20, s, 1H, H-8; 8.08, s, 1H, H-2 6.68, d, $^1J_{H-P}$=639.2, 1H, P-H; 6.66, d, $^1J_{H-P}$=639.2 Hz, 1H, P-H; 6.00, d, J=5.4 Hz, 1H, H_1'; 5.60, t, J=6.8 H_2, 1H, CH=C; 4.66, t, J = 5.4, 1H, H_2'; 4.39, dd, J(H_3'-H_4') = 4.4 Hz, J(H_3'-H_2') = 5.0 Hz, 1H, H_3'; 4.29, m, 1H, H_4'; 4.23, d, $^3J_{H-P}$ = 8.8 Hz, 2H, C=C-CH_2-O; 4.07, m, 4H, CH_2-N, H_5' and H_5"; 1.71, s, 3H, CH_3; ^{13}C-NMR (D_2O): δ 155.7, 154.1, 150.3, 141.0, 119.7, 5xs (purine); 136.8, d, $^3J_{C-P}$ = 7.4 Hz, CH_3-$\underline{C}$=C; 123.3, s, $\underline{CH}$=C; 88.1, s, C_1'; 84.7, d, $^3J_{C-P}$ = 5.4 Hz, C_4'; 77.7, s, C_3'; 71.4, s, C_2'; 69.8, s, C=C-$\underline{CH_2}$-O; 64.0, s, C_5'; 39.2, s, CH_2-N; 14.0, s, CH_3; ^{31}P-NMR (D_2O): δ_P=7.03 ppm, $^1J_{H-P}$=634.8 Hz and δ_P=6.65 ppm, $^1J_{H-P}$=632.3 Hz.

Compound **1b**; ^{1}H-NMR(D_2O): δ 8.40, s, 1H, H-8; 7.73, s, 1H, H-2; 6.74, d, $^1J_{H-P}$=636.7 Hz, 1H, P-H; 6.05, d, J=6.3 Hz, 1H, H_1'; 5.60, t, J=6.5 Hz, 1H, CH=C; 4.52, dd, J=5.4 Hz and 3.4 Hz; 1H, H_3'; 4.30, m, 1H, H_4'; 4.28, d, J=5.2 Hz, 2H, C=C-CH_2-O; 4.25, brd, 2H, CH_2-N; 3.90-3.75, 2xdd, J(H_5'5")=12.8 Hz, J(H_5'5'-H_4')=2.2 Hz and 3.4 Hz, 2H, H_5' and H_5"; 1.72, s, 3H, CH_3; ^{13}C-NMR (D_2O): δ 155.5, 153.7, 148.6, 140.2, 119.6, 5xs (purine); 135.9, d, $^3J_{C-P}$=6.0 Hz, CH_3-$\underline{C}$=C; 122.9, s, $\underline{CH}$=C; 88.5, s, C_1'; 86.0, s, C_4'; 73.9, s, C_3'; 70.9, s, C_2'; 69.0, s, C=C-$\underline{CH_2}$-O; 61.7, s, C_5'; 38.7, s, CH_2-N; 14.4, s, CH_3; ^{31}P-NMR (D_2O): δ_P=6.51 ppm, $^1J_{H-P}$=634.7 Hz.

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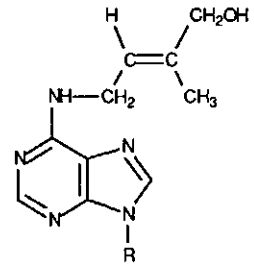
CHAPTER VIII

THE SYNTHESIS OF L (-) AND D (+) LUPINIC ACID

INTRODUCTION

L-amino acids, containing a heterocyclic ring on the β-position of alanine, occur widely in nature and are produced in plants. In these non-protein L-amino acids six-membered heterocyclic rings (trigonelline, mimosine, willardine, and isowillardine), five-membered rings (quisqualic acid, β-(pyrazol-1-yl)alanine, β-(3-isoxazolin-5-on-2-yl)alanine, β-(5-methylisoxazolin-3-on-2-yl)alanine, β-(3-amino-1,2,4-triazol-1-yl)alanine, β-(2-furoyl)alanine, as well as bicyclic heterocycles (lupinic acid, β-(6-benzoylaminopurin-9-yl)alanine, histidinoalanine are identified as β-heterocyclic moieties. There is continuing interest in the synthesis of these naturally-occuring heterocyclic β-substituted alanines[1]. Plant extracts have been isolated, which contain enzyme systems (β-substituted alanine syntheses), which catalyze the syntheses of β-substituted heterocyclic alanines from O-acetyl L-serine and the appropriate heterocycle. It was found that neither serine, nor its O-phospho and its O-sulpho derivative could serve as donor of the alanyl moiety. Attempts are reported to prepare above-mentioned β-substituted alanines biomimetically using a pyridoxal-5'-phosphate (PLP) catalyzed chemical reaction between O-acetyl L-serine and the heterocycle in the presence of metal ions, especially gallium ions. The yields are usually low-to-very low; 30% yields or more are mentioned for β-(pyrazol-1-yl)-alanine (40-45%), β-(5-methylisoxazolin-3-on-2-yl)alanine (0.15%) and β-(3-amino-1,2,4-triazol-1-yl)alanine (30-35%)[1].

Due to our interest in the use of enzymes in synthetic procedures we turned our attention to the preparation of the β-purinyl-L-alanine derivative, called L-lupinic acid **1a**, which is isolated from *lupinus angustifolius* [2] and which is a principal metabolite of the phytohormone *trans*-zeatine (E-**1b**), being one of the most effective natural stimulants of plant cell division (see fig. VIII.1).



1a: R = $CH_2CH(NH_2)CO_2H$

1b: R = H

Fig. VIII.1

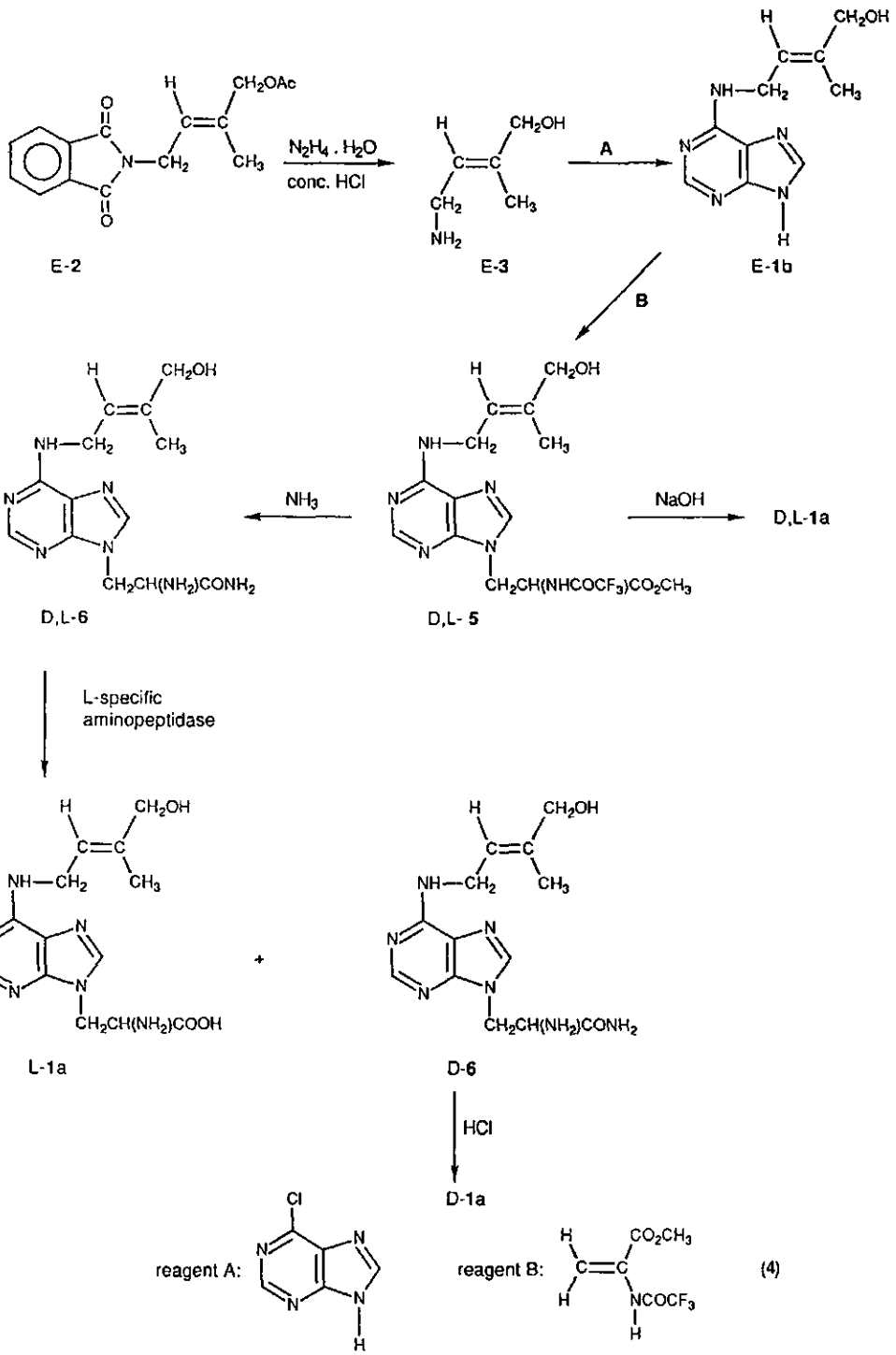
The chemical synthesis of a racemic mixture of D,L-**1a** has been described[2]. However, pure L-lupinic acid (L-**1a**) and D-lupinic acid (D-**1a**) have never been prepared. Only the enzymatic formation of L-**1a** from *trans*-zeatin (E-**1b**) and O-acetyl L-serine, using as enzyme source extracts of Lupinus seedlings has been reported. Attempts to prepare L-**1a** by a PLP-catalyzed reaction between E-**1b** and O-acetyl-L-serine with little succes (yield about 1,5%)[3].

For the production of optically pure D- and L-amino acids enzymatic resolution methods have proven to be powerful tools. The enzymatic resolution process developed by DSM (Dutch State Mines), based on the stereospecific hydrolysis of amino acid amides by using the L-specific aminopeptidase from *Pseudomonas putida* has found wide application[4]. We wanted to study if this enzyme would also be useful for the preparation of L-lupinic acid (together with D-lupinic acid amide) from DL-lupinic acid amide; from D-lupinic acid amide D-lupinic acid could be obtained.

RESULTS AND DISCUSSION

The strategy we have adopted for the synthesis of L-lupinic acid (L-**1a**) and D-lupinic acid (D-**1a**) consists of the following steps i) the synthesis of *trans*-zeatine (E-**1b**) ii) the synthesis of trifluoroacetylaminoacrylate **4**, iii) coupling of **4** with E-**1b** at position 9 of the purine ring into D,L-**5**, iv) removal of the protecting trifluoroacetyl group and conversion of the ester group into the amide function affording D,L-lupinic acid amide (D,L-**6**), v) enzymatic stereospecific hydrolysis of DL-**6** with L-specific aminopeptidase into L-lupinic acid (L-**1a**) and D-lupinic acid amide (D-**6**), vi) the chemical conversion of D-Lupinic acid amide (D-**6**) under mild acid condition into D-Lupinic acid (D-**1a**) (see scheme VIII.1).

Our synthesis of *trans*-zeatin (E-**1b**) is a modification of two syntheses, being already reported[5,6]. In our work N-(4-acetoxy-3-methyl-E-but-2-enyl) phthalimide (E-**2**), being prepared by a procedure described before, was converted in a one-pot reaction into 4-hydroxy-3-methyl-E-but-



Scheme VIII.1

2-enylamine (E-3) by treatment with 85% aqueous hydrazine to remove the phthaloyl group and subsequent heating with hydrochloric acid to hydrolyse the acetate group (yield 85%). Coupling of E-3 with 6-chloropurine (reagent **A**) in refluxing butanol in the presence of triethylamine gave *trans*-zeatine (E-**1b**). The coupling of E-**1b** with methyl trifluoroacetylamino acrylate (reagent **B**: **4**) into β-[6-(4-hydroxy-3-methyl-E-but-2-enylamino)purin-9-yl]-N-trifluoro-acetylalanine methyl ester (D,L-**5**) requires difficult conditions[7]. Optimum yields (about 80%) were obtained when a solution of an equimolar amount of **4** and E-**1b** in dimethyl sulphoxide, containing potassium bicarbonate was stirred for three days at 25°C. Using the stronger base potassium carbonate instead of potassium bicarbonate resulted in lower yields of D,L-**5**. Removal of the protecting N-trifluoroacetylgroup in D,L-**5** and conversion of the ester group into a carboxylic amide function was realized by heating a solution of D,L-**5** in butanol, being saturated with ammonia, for three days at 35°C in a sealed tube. The yield of the racemic mixture of D,L-lupinic acid amide (D,L-**6**) amounted to 85%.

The resolution was succesfully achieved by using L-aminopeptidase from *Pseudomonas putida*, which converts L-carbonamide **6** into the corresponding L-lupinic acid L-**1a**, but does not hydrolyse D-carbonamide **6**. By use of a strong basic ion-exchange column the L-lupinic acid (L-**1a**) could be easily separated from the D-lupinic acid amide (D-**6**). D-Lupinic acid amide (D-**6**) was treated with 6N hydrochloric acid at room temperature to obtain D-Lupinic acid (D-**1a**).

The optical purities of L-**1a** and D-**6** were established by reacting each of these compounds as well as the racemic mixture of D,L-**1a** (obtained by treatment of a solution of D,L-**5** in dioxane-water with aqueous sodium hydroxide at room temperature) with pure S-2-chloropropionyl chloride[8]. This reagent reacts with D,L-α-amino acids into the corresponding diastereoisomeric N-(S-2-chloropropionyl) derivatives. In the ^{1}H-NMR spectra of these D,L derivatives the chemical shift for the methyl doublet of the CH_3-CHCl moiety is found to be different for both diastereoisomers. When this technique is applied to D,L-**1a** we observed two methyl doublets of the CH_3-CHCl part at 1,53 and 1,56 ppm. However, the ^{1}H-NMR spectrum of the N-(S-2-chloropropionyl) derivative of L-**1a** exhibits only a CH_3-CHCl methyl doublet at 1,53 ppm; the N(S-2-chloropropionyl) derivative of D-**6** only gave the methyl doublet at 1,56 ppm. From these results it can be unequivocally concluded that the enzymatic separation was succesful and that L-lupinic acid has been obtained with high optical purity (e.e > 95%). Acid hydrolysis of D-**6** gave optically pure D-lupinic acid (D-**1a**).

EXPERIMENTAL

General methods and materials

Schleicher and Schül DC Fertigfolien F1500 LS 254 were used for TLC, the following solvent systems were used: System A (chloroform/methanol, 90:10, v/v), system B (chloroform/methanol, 86:14, v/v), system C (chloroform/methanol, 80:20, v/v), system D (chloroform/methanol/concentrated ammonium hydroxide, 60:40:20, v/v). ^{1}H-NMR-spectra were measured

using a Hitachi Prekin-Elmer R-248 or a Varian EM 390 spectrometer. ^{13}C-NMR-spectra were measured at 75.460 MHz using a Bruker CXP 300 spectrometer; proton noise decoupling was used. Mass-spectra were recorded on an AEI MS 902 instrument, equiped with a VG/ZAB console. Ultraviolet spectra were determined using a Beckmann Du-7 spectrometer. Melting points were determined on a Kofler hot stage equipped with a microscope and a polarizer. They are uncorrected. Optical rotations were measured at 20° C with a Perkin Elmer 141 polarimeter.

Dimethylsulphoxide was dried by stirring with CaH_2 for 16 h, then distilled under reduced pressure and stored over molecular sieves 4A. Triethylamine was dried by refluxing with CaH_2 for 16 h, then distilled and stored over molecular sieves 4A. n-Butanol (analyzed grade, Baker) was used without further purification. n-Butanolic ammonia was prepared by passing dry (KOH) ammonia gas through butanol at -20°C until saturation.

Synthesis of 4-hydroxy-3-methyl-E-but-2-enylamine (E-3)

A mixture of N-(4-acetoxy-3-methyl-E-but-2-enyl)phthalimide (E-2) (9.46, 40.7 mmol), 85% aqueous hydrazine hydrate solution (2.44 ml, 40.8 mmol) and methanol (100 ml) was refluxed for 1 h with stirring. TLC-analysis (system A) indicated that under these conditions complete conversion of the starting material took place into a product having Rf=0. After cooling of the solution, water (25 ml) and concentrated hydrochloric acid (25 ml) were added and the reaction mixture was heated under reflux for 1 h. After cooling to 0°C phthalylhydrazide precipitated, which was removed by filtration; the filtrate was evaporated at 30°C in a rotary evaporator. The residue was dissolved in water (20 ml) and the insoluble material was removed by filtration; the filtrate was made alkaline untill pH of 10 with 4N aqueous sodium hydroxide solution. This solution was continuously extracted with chloroform. The organic extract was, after drying on $MgSO_4$ evaporated to give the aminoalcohol E-3. Yield 3.5 g (85%), oil. ^{1}H-NMR-spectral data are identical with those reported in the literature[5].

Synthesis of *trans*-zeatin (E-1b)

A mixture of aminoalcohol E-3 (3.5 g, 34.7 mmol), 6-chloropurine (5.02 gram, 32.7 mmol), anhydrous triethylamine (4.7 ml) and n-butanol (150 ml) was boiled under reflux for 3 h. TLC-analysis (system B) indicated that the conversion of the starting material was complete. The solvent was removed and the residue recrystallized twice, first from water, then from ethanol, to give *trans*-zeatin as white crystals. Yield 5.73 g (80%), m.p. 208-209°C, m.p. lit. 209-210°C, ^{1}H-NMR and ^{13}C-NMR spectral data were identical as described[9,10].

Synthesis of D,L-β-[6-(4-hydroxy-3-methyl-E-but-2-enylamino)purin-9-yl]-N-trifluoroacetylalanine methylester (D,L-5)

A mixture of *trans*-zeatin, (E-1b) (5.7 g, 26,2 mmol), methyl 2-trifluoro-acetylaminoacrylate[2] (5.2 g, 26,2 mmol) dry dimethylsulphoxide (25 ml) and $KHCO_3$ (150 mg) was stirred for 3 days at 20°C, TLC-analysis (system C) indicated that then the reaction was complete. The reaction mixture was poured into water (50 ml), extracted with chloroform (3x100 ml) and the combined organic extracts were dried with $MgSO_4$. The organic layer was concentrated to an oil, which was

chromatographed on a column of silica gel (230-400 mesh ASTM). Elution with chloroform/ methanol (100:0 → 98:2, v/v) gave after evaporation pure D,L-5 as an oil. Yield 8.16 g (75%), Rf=0,8 (system C)

^{1}H-NMR ($CDCl_3$): δ 1.70, s, 3H, CH_3; 3.92, s, 3H, $COOCH_3$; 4.10, s, 2H, CH_2O; 4.30, t, J=3.8 Hz, 2H, CH_2N; 4.8-5.2, m, 3H, C_α-H, 2x C_β-H; 5.57, t, J=6.2 Hz, 1H, CH=C; 6.75, t, J=3,8 Hz, 1H, NHCH$_2$; 7.81, s, 1H, H-2; 8.42, s, 1H, H-8; 10.20, m, 1H, $NHCOCF_3$; ^{13}C-NMR ($CDCl_3$): δ 167.73, $COOCH_3$); 157.57, q, J_{C-F}=39 Hz, $COCF_3$; 154.48, C_6; 152.70, C_2; 148.68, C_4; 139.96, C_8; 138.90, CH_3C=C; 119.82, CH=C; 119.15, C_5; 115.40, q, J_{C-F}=287 Hz, CF_3; 66.90, CH_2OH; 52.94, OCH_3; 52.80, C_α; 44.23, $NHCH_2$; 38.3, C_β, 13.52, CH_3; FD/MS: m/z: 416, [M$^+$]; 396, [(M-HF)$^+$]; 385, [(M-CH_3O)$^+$]; 357, [(M-$C_2H_3O_2$)$^+$].

Synthesis of D,L-β-[6-(4-hydroxy-3-methyl-E-but-2-enylamino)purin-9-yl]alanine carbonamide (D,L-6)

The racemic mixture D,L-5 (6.74 g, 16.2 mmol) was dissolved in dry n-butanol (150 ml), which was saturated with NH_3; the solution was heated at 30°C for 3 days in a sealed tube. TLC-analysis (system C) showed the complete absence of D,L-5 and formation of a new compound with Rf=0.23. The solution was evaporated to an oil, which was crystallized from methanol to give D,L-6 as white crystals. Yield 4.2 g (85%). mp. 190-192°C, [Rf=0.23 (system C, Rf=0.86 (system D).

^{1}H-NMR (DMSO-d6): δ 1.73, s, 3H, CH_3; 3.66, dd, J=8.1 Hz and 5.2 Hz, 1H, C_α-H; 3.84, d,J =5.7 Hz, CH_2O; 4.17, m, 3H, NH-CH_2 and C_β-H; 4.45, dd, J=13.9 Hz and 5.2 Hz, 1H, C_β-H; 4.79, t, J=5.7 Hz, 1H, OH; 5.59, t, J=6.1 Hz, 1H, CH=C; 7.22 and 7.55, br s, 2H, $CONH_2$; 7.85, br s, 1H, NHCH$_2$; 8.07, s, 1H, H-2; 8.27, s, 1H, H-8; ^{13}C-NMR (D_2O): δ 176.55, C=O; 154.46, C_6; 152.75, C_2; 148.39, C_4; 141.99, C_8; 138.75, CH_3C=C; 121.11, CH=C; 118.70, C_5; 66.89, CH_2O, 54.19, C_α; 47.18, NH CH_2, 38.63, C_β, 13.28, CH_3; FD/MS: m/z 306, [(M+H)$^+$]; 305 [M$^+$]; 219 [(M-$C_3H_7N_2O$)$^+$].

UV-spectra showed λ_{max} at the pH of 3, of 6 and of 11 at 266.3, 269 and 270 nm, respectively, being characteristic of (N^6,N^9)-disubstituted adenines[11].

Synthesis of D,L-β-[6-(4-hydroxy-3-methyl-E-but-2-enylamino)purin-9-yl]alanine. (D,L-**1a**)

A stirred solution of the ester D,L-5 (140 mg, 0.34 mmol) in dioxane (10 ml) and H_2O (5 ml) was treated with 1 N NaOH for 4h. The pH of the solution changed from 6 to 10, after 4h at 20°C. TLC-analysis (system D) indicated complete conversion of the starting material. The solution was percolated through a column (50 ml) of the anion exchange resin Dowex AC-1-X8 (OH^--form); the column was washed with water (150 ml) and then eluted with 0.1 N HCl. The eluate was passed directly through a column (60 ml) of cation exchange resin Dowex AC-50W-X8 (H^+-form). Then the column was washed with water and eluted with ammonia (1N). This eluate was evaporated to dryness and the residue was crystallized from ethanol/water.

Yield: (±)-Lupinic acid (D,L-**1a**): 128 mg (70%), m.p. 215-216°C; lit.[1]: 216-217°C, [Rf=0 (system C), Rf=0.74 (system D)].

^{1}H-NMR (DMSO-d6): δ 1.72, s, 3H, CH_3; 3.83, s, 2H, CH_2O; 3.88, dd, J=4.3 Hz and 8.2 Hz, 1H, C_α-H; 4.17, br s, 2H, $NHCH_2$; 4.56-4.70, m, 2H, 2x C_β-H; 4.88, br s, 1H, CH_2-OH; 5.57, t, J=5.9 Hz, 1H, CH=C; 7.92, br s, 1H NH-CH_2; 8.17, s, 1H, H-2; 8.27, s, 1H, H-8; ^{13}C-NMR (DMSO-d6): δ 171.08, C=O; 155.69, C_6; 154.10, C_2; 150.16, C_4; 143,19, C_8, 139.99, CH_3C=C; 121.83, CH=C; 120.02, C_5; 67.72, CH_2O; 55.89,

C_α; 45.91, $NHCH_2$; 14.84, CH_3; FD/MS: m/z: 306 [M^+]; 262 [$(M-CO_2)^+$]; 218[$(M-C_3H_6O_2N)^+$].

Synthesis of L-β-[6-(4-hydroxy-3-methyl-E-but-2-enyl amino)purine-9-yl]alanine (L-lupinic acid, L-1a)

The amide of D,L-lupinic acid (D,L-6) (2.0 gram, 4.93 mmol) was dissolved in 40 ml water and treated for 20 h at 40°C at pH=10[12] with aminopeptidase from *Pseudomonas putida* (4) (0.4 g of crude cells containing about 1% of active enzyme). The L-amino acid (L-1a) was separated from the D-amide (D-6) by the use of a strong basic ion-exchange column (see previous sections). Elution with 2N acetic acid gives L-lupinic acid (L-1a) 0.85 g, Rf=0.74 (system D), $[\alpha]_D^{20}$ = -24.5 (c=1, H_2O) and the unreacted D-lupinic acid amide (D-6) 0.78 g, Rf=0.86 (system D),$[\alpha]_D^{20}$ = -99 (c=1, H_2O). ^{1}H-NMR and ^{13}C-NMR and mass spectrometric data of compound L-1a and D-6 are identical with those of D,L-1a and D.L-6, respectively.

Synthesis of D-β-[6-(4-hydroxy-3-methyl-E-but-2-enylamino)purine-9-yl]alanine (D-Lupinic acid D-1a)

A solution of the unreacted D-Lupinic acid amide (D-6) (120 mg, 0.40 mmol) in H_2O (5 ml) was treated with 6N HCl (2 ml). After one week at 20°C, TLC analysis (system D) showed complete conversion op the starting material with Rf=0.86 into the product D-1a, Rf=0.74. The reaction solution was neutralized with NaOH (4N) to pH=7.5. The reaction solution was concentrated to a small volume (1 ml) and applied to silanized silica gel RP18. The column was eluted with water applying a methanol gradient (0→60%), which gave after evaporation D-1a as a white solid. Yield 95 mg (77%), Rf=0.78, $[\alpha]_D^{20}$ = +26.0 (c=1, H_2O). ^{1}H-NMR, ^{13}C-NMR and mass spectrometric data of D-1a are identical with those of D,L-1a.

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12. At this pH a very small amount of D,L-carbonamide is hydrolyzed into the D,L-acid.

CHAPTER IX

GENERAL DISCUSSION

During the investigations described in this thesis considerable progress has been made in the synthetic development of two classes of cytokinins, namely a. the synthesis of phosphorylated cytokinins (1-15) and b. the synthesis of alanylated cytokinins (16) (see fig. IX.1).

A. THE SYNTHESIS OF PHOSPHORYLATED CYTOKININS

The introduction of the phosphate group and modified phosphate groups in naturally occurring cytokinins is the major subject of this thesis.

The strategy we have adopted for the synthesis of these cytokinins is :

1. The preparation of properly protected cytokinins (see fig. IX.2) which serve as starting material for the synthesis of phosphorylated cytokinins.
2. the effective phosphorylation of the free hydroxyl groups in these protected cytokinins
3. the removal of all protective groups.

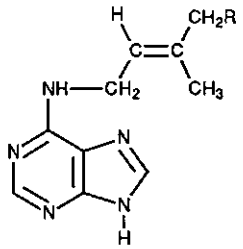
In our strategy dealing with the preparation of properly protected cytokinins, we used the base-labile acetyl group for the protection of the allylic hydroxy function, the acid-labile methoxymethylidene group for the protection of the 2'-OH and 3'-OH function, the acid-base-labile-tert-butyldimethylsilyl (TBDMS) group for the protection of the 5'-OH-function and the acid-labile tetrahydropyranyl (THP) for the protection of N^9-position at the purine.

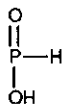
All these protective groups were relatively easy to introduce and stable under all conditions required in the synthesis of the phosphorylated products. Furthermore, the removal of these protective groups caused no instability of the products.

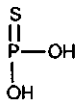
The use of these protective groups was necessary for selective introduction of the phosphate groups at the desired allylic position, and/or at the 5'-position in the ribose ring. Moreover, by the use of protective groups side reactions are avoided during the phosphorylation.

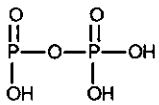
The second step in our strategy deals with the effective phosphorylation of the free hydroxy groups of the properly protected cytokinins.

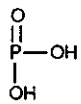
There are many phosphorylating agents described in the literature for the introduction of phosphorus containing groups on primary hydroxy groups present in naturally occurring compounds. A good and concise review of phosphorylating reagents, which covers the literature up to 1980, was presented by Scheit[1]. An effective bifunctional phosphorylating reagent, N-morpholino-O,O-bis[(6-trifluormethyl)-benzotriazolyl]phosphate **22** was found to be suitable for the preparation of 5'-monophosphates of nucleosides [2]. We applied this phosphorylating reagent for the synthesis of cytokinin-5'-monophosphates **6** and **7** (see scheme IX.1). Treatment of

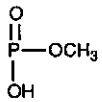


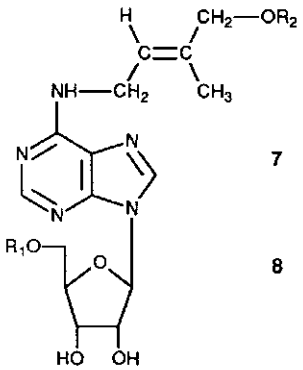
1 . R = 

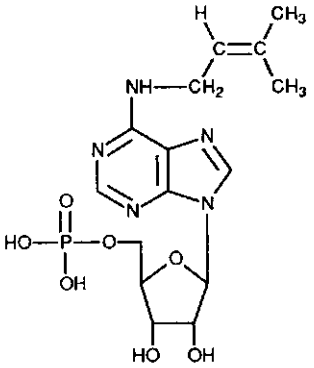
2 . R = 

3 . R = 

4. R = 

5 . R = 





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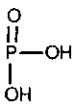
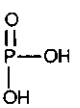
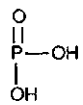
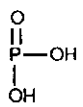
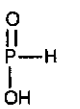
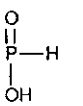
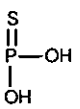
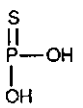
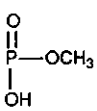
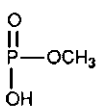
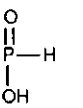
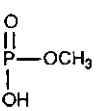
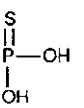
	R_1	R_2
7		H
8		
9	H	
10		
11		
12		
13	H	
14	H	
15	H	

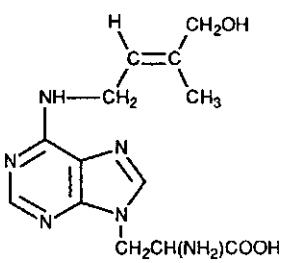


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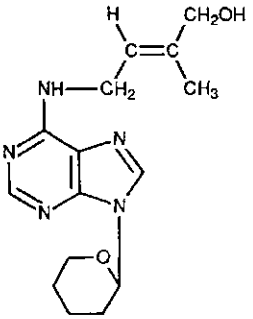
Fig . IX.1



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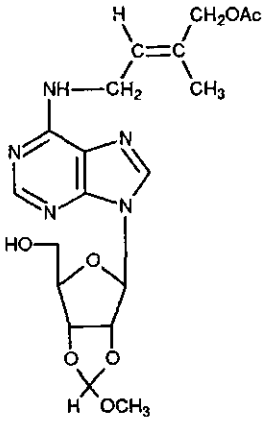

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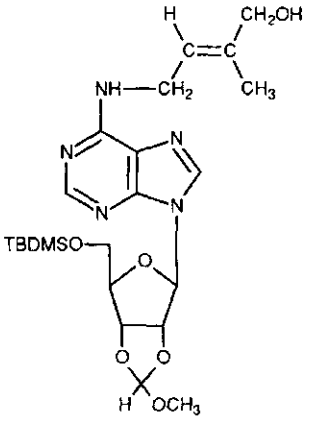

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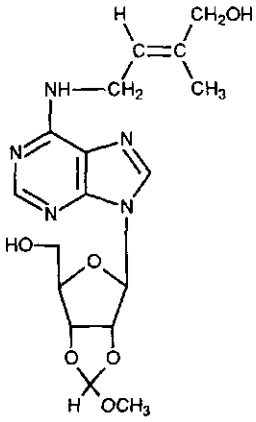

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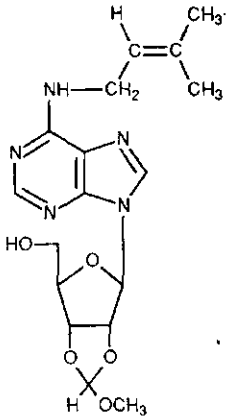
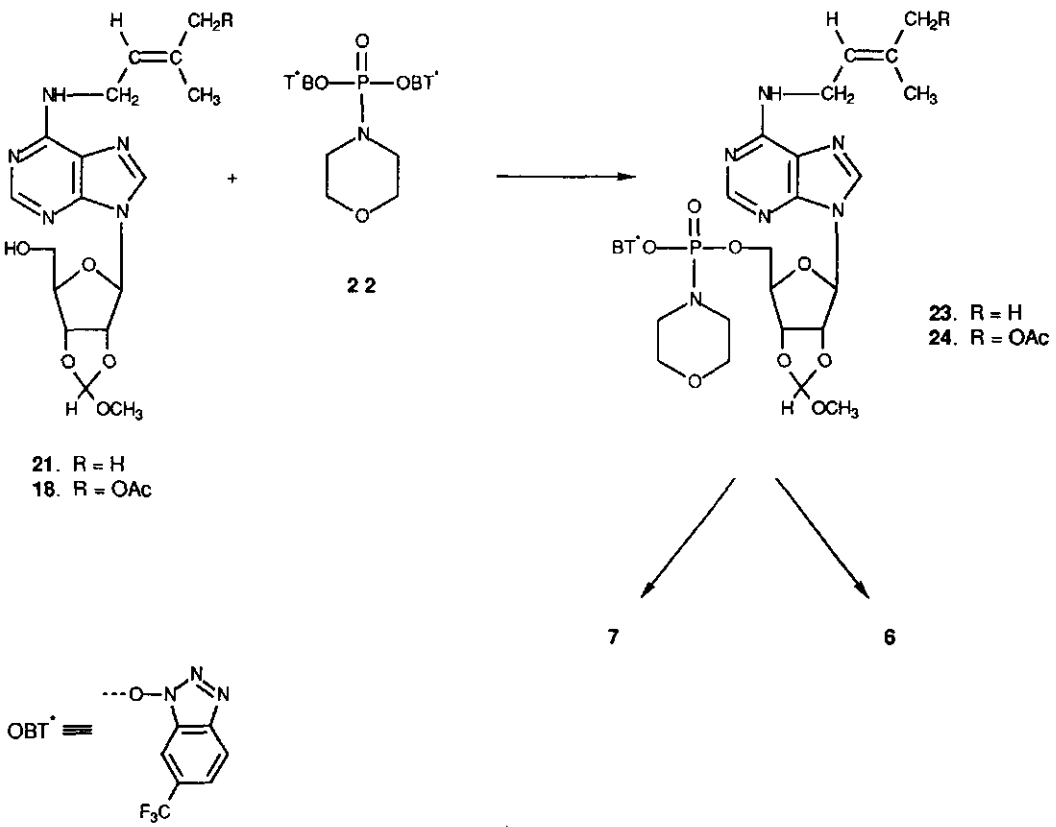


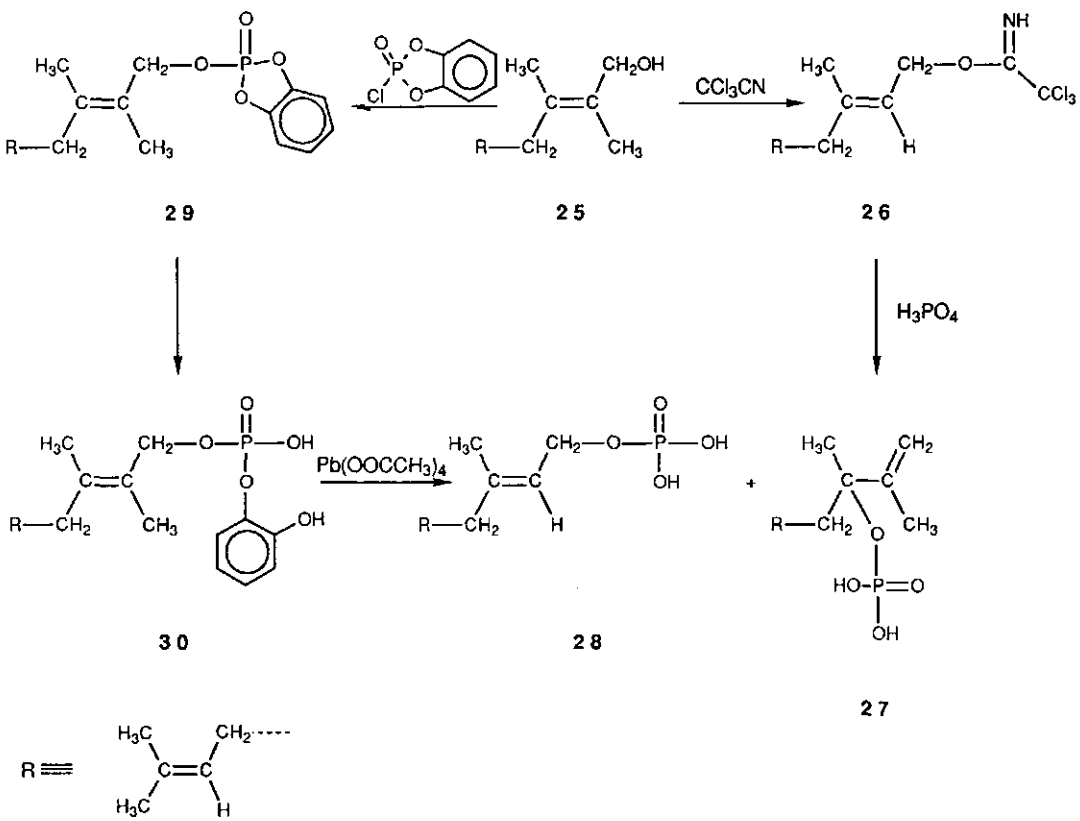
21

Fig. IX.2

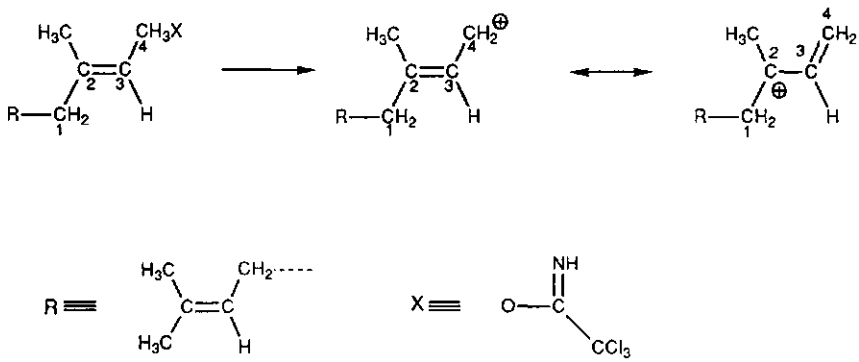
the properly protected cytokinins **21** and **18** with **22** yielding the phosphate triesters **23** and **24**, respectively and cleavage of all protective groups gave the phosphorylated cytokinins **6** and **7**, respectively. The introduction of the phosphate group and its modification at the allylic position is difficult, because allylic phosphates are good leaving groups. There are two methods for the chemical phosphorylation of an allylic hydroxy group. One method[3] (see scheme IX.2) is based on the activation of the allylic hydroxy group on **25** with trichloroacetonitril in the presence of sodium hydride to give intermediate **26**. Replacement of the trichloroacetamide group by ortho-phosphoric acid affords the monophosphates **27** and **28**. The formation of the rearrangement product **27**, however, makes the isolation of **28** rather laborious. The formation of the two products **27** and **28** suggests that the allylic substrate **26** undergoes nucleophilic substitution by an SN_1 mechanism, yielding the resonance stabilized allylic carbocation (see scheme IX.3). C_4 and C_2 each carrying a partial positive charge are attacked by the nucleophilic H_3PO_4 to give the normal product **28** and the rearranged product **27**.



Scheme IX.1

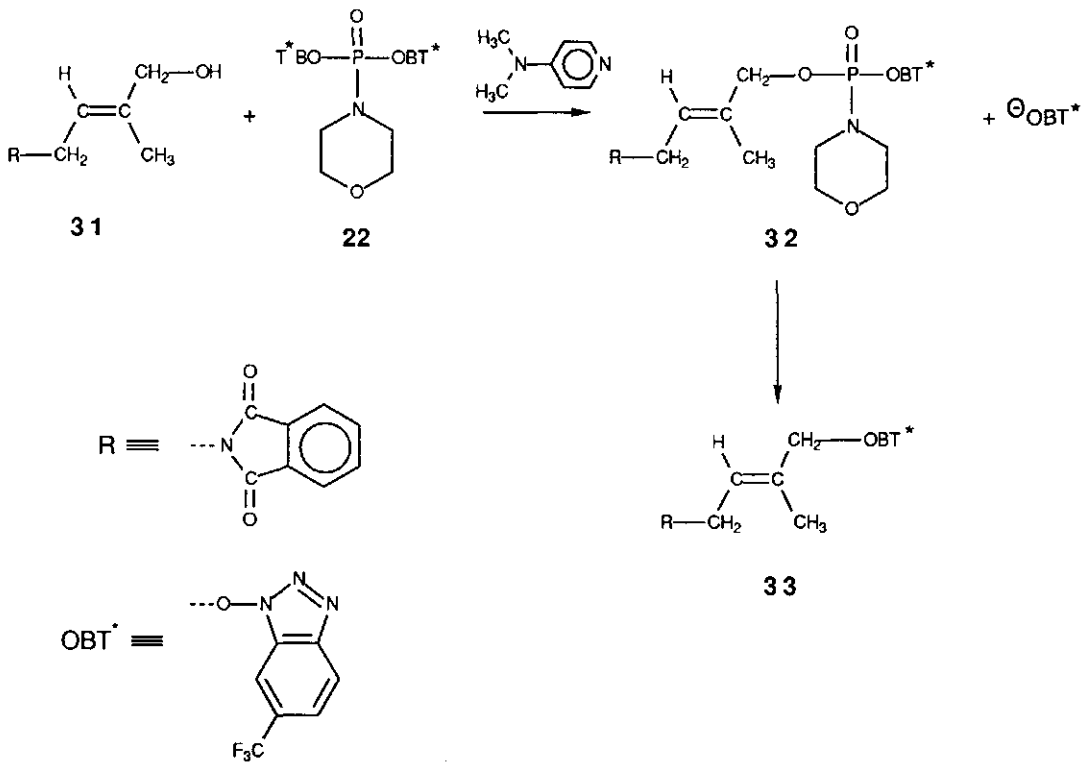


Scheme IX.2



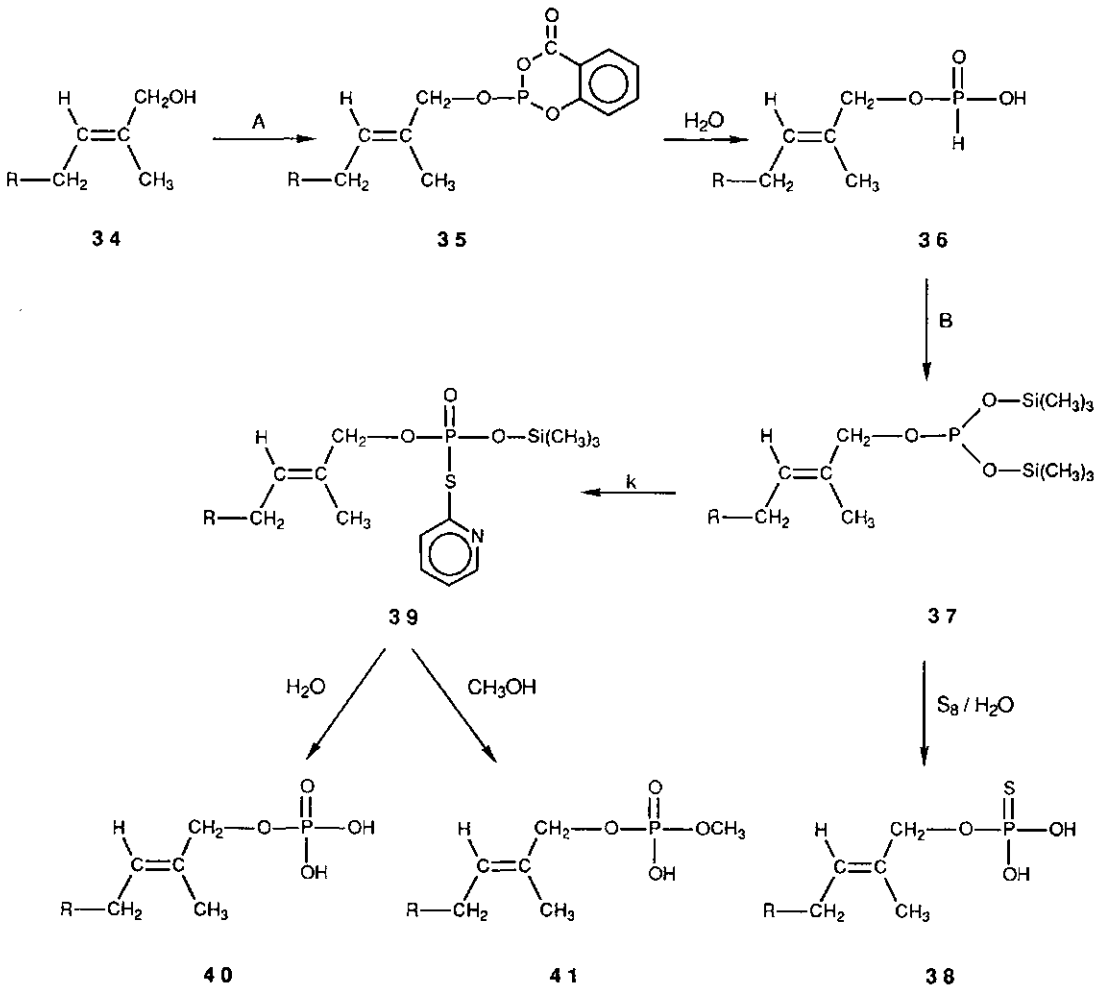
Scheme IX.3

The second method[4] for the synthesis of allylic phosphates, which is illustrated in scheme IX.2, is based on conversion of the allylic hydroxy group of geraniol **25** with O-phenylphosphorochloridate into **29**, which after hydrolysis yields **30**. However, deblocking of the intermediate phosphodiester **30** with lead(IV)acetate, gives rise to the formation of many coloured by-products.



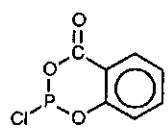
Scheme IX.4

The above-mentioned problems induced us to look for new phosphorylating agents in order to achieve a succesful synthesis of cytokinins, which contain allylic phosphates. Preliminary experiments to introduce a phosphate group in the allylic position of alcohol **31** (see scheme IX.4) by treatment with the bifunctional phosphorylating reagent **22** in the presence of a base gave however the non-phosphorus containing compound **33**. This suggested that after the allylic alcohol of **31** was phosphorylated, this group is easily replaced due to the good leaving character of the allylic phosphate triester group in **32**, by the nucleophile trifluoromethylbenzotriazolyl anion, present in the solution. This problem was circumvented by using the phosphitetriester approach, which is illustrated in scheme IX.5. This methodology was not only suitable for the introduction of the phosphate group on the allylic alcohol, but was also suitable for the



see for R: structure 17-21

A:



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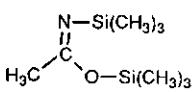
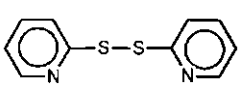
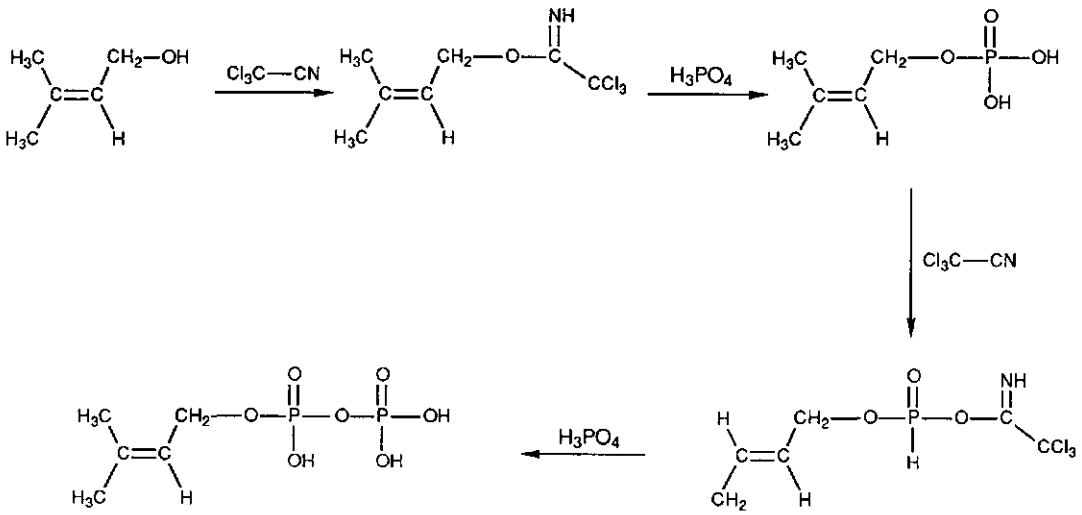


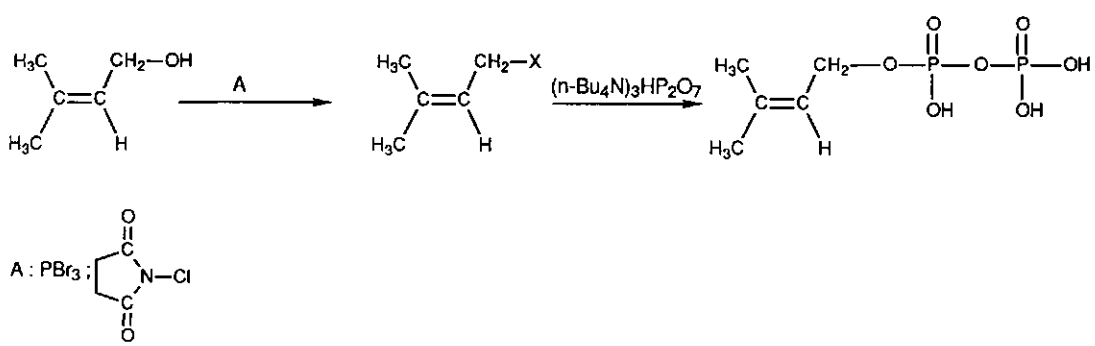
K:



Scheme IX.5



Scheme IX.6



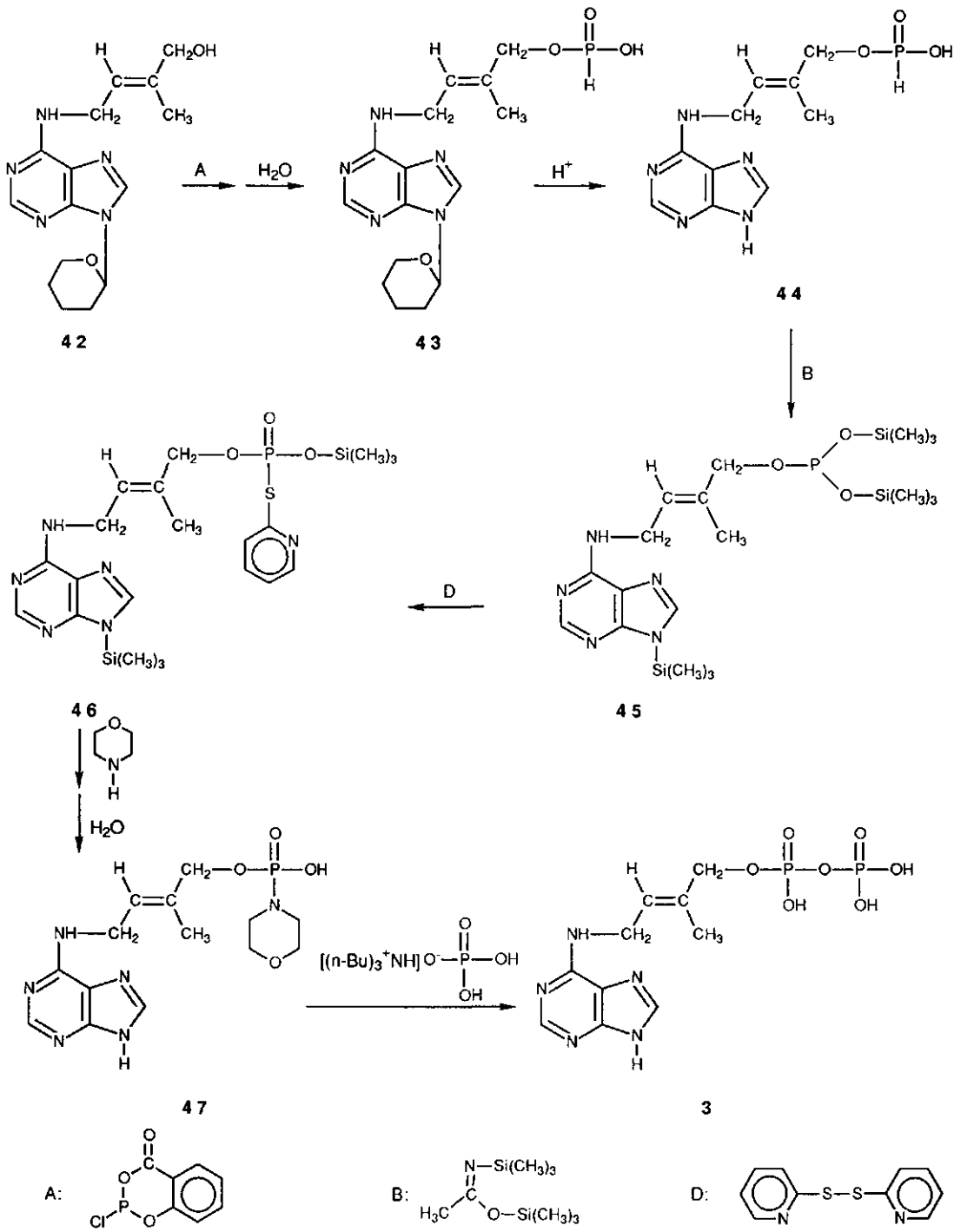
Scheme IX.7

introduction of methylphosphate and thiophosphate. This method consists of treatment of the appropriately protected allylic alcohol with the phoshitylating reagent salicyl chlorophosphite, yielding intermediate **35**, which is easily hydrolysed into the phosphonate monoester **36**. The phosphonate monoester **36** was silylated with N,O-bis(trimethylsilyl)-acetamide to give intermediate **37**. The silylation step is necessary[5] to transform the phosphate esters into the trivalent form of bis(silyl)phosphites **37**, which are more susceptible to oxidation or sulfurization than the tetracoordinated species of type **36**. Reaction of **37** with powdered sulfur gave after hydrolysis the thiophosphate **38**. Reaction of **37** with 2,2'-dipyridyldisulfide gave intermediate **39**, which on hydrolysis with water gave the monophosphate **40**. Methanolysis of **39** with dry methanol gave methylphosphate **41**. This above mentioned phosphitetriester approach appeared to be very succesful in the synthesis of cytokinin derivatives with allylic phosphor containing groups. Thus, the phosphitylation of the free hydroxy groups in the properly protected cytokinins **17**, **19** and **20** (see fig. IX.2) with salicyl chlorophosphite gave the corresponding phosphate monoester. This phosphate monoester was converted into the corresponding phosphate, methylphosphate, and thiophosphate; after cleavage of the protective groups THP, methoxymethylidene and TBDMS under mild acidic conditions the phosphorus derivatives of cytokinins were formed[6,7,8]

Finally, we will discuss the synthesis of the allylic pyrophosphate of *trans*-zeatin. Two methods are discribed in the literature for the synthesis of the allylic pyrophosphate. The first procedure[9] (see scheme IX.6) is based on treatment of the allylic alcohol with trichloroacetonitril and orthomonophosphate. It gives, however, a mixture of organic and inorganic mono-, di- and triphosphate. The yield of the desired product rarely exceeds 30% and further losses are encountered during purification. In addition, the procedure becomes difficult to manage if more than 50 mg of the product is desired.

The second procedure[10] described in the literature is based on activation of an allylic alcohol by conversion into an allylic halide (see scheme IX.7). The activated intermediates are treated with tris-(tetra-n-butylammonium)hydrogenpyrophosphate to obtain the allylic pyrophosphate ester. In order to examine this above mentioned possibility for the preparation of the allylic pyrophosphate of *trans*-zeatin **3**, we made attempts to halogenate the allylic hydroxy group in trans zeatin by using either phosphorus tribromide or N-chlorosuccinimide. Both attempts were unsuccessful since they gave rise to the formation of a lot of coloured and unidentified products.

A succesful synthesis of allylic pyrophosphate **3** was realized by the phosphitylation of the allylic hydroxy group in **42** with salicyl chlorophosphite to give the allylic phosphonate **43** (scheme IX.8). Cleavage of the tetrahydropyranyl groups under mild acidic conditions gave **44**. The allylic phosphonate **44** was converted into the corresponding phosphoromorpholidate **47**, which was treated with mono-(tri-n-butylammonium)phosphate to give the allylic pyrophosphate **3**.

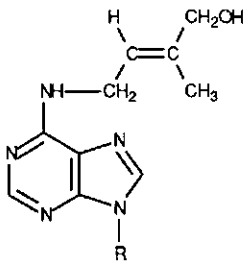


Scheme IX.8

B. THE SYNTHESIS OF ALANYLATED CYTOKININS.

the synthesis of the optically active N9-alanylzeatin
(D and L lupinic acid)

The chemical synthesis of a racemic mixture of D,L-lupinic acid is described in the literature[11]. Pure L-lupinic acid and D-lupinic acid have never been prepared; only enzymatic formation of L-**16** from trans zeatin **49** (see fig. IX.3) and O-acetyl-L-serine, using as enzym source extracts of lupinus seedling has been reported[12]. Attempts[13] to prepare L-**16** (see fig. IX.3) by a pyridoxal 5'-phosphate (PLP)-catalyzed reaction between **49** and O-acetyl-L-serine have met with little success (yield 1.5%).



16, R = $CH_2CH(NH_2)COOH$
49, R = H

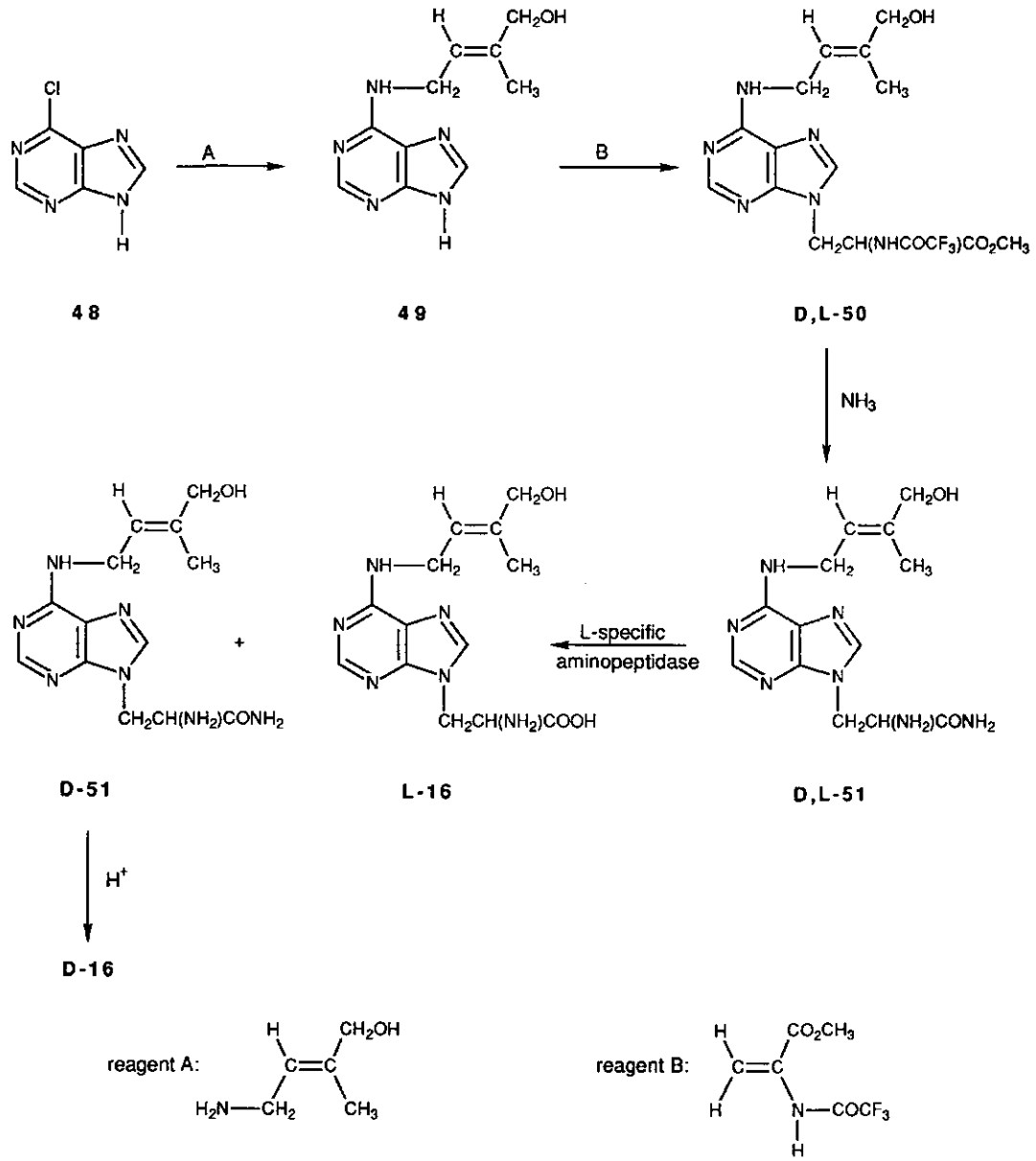
Fig IX.3

For the production of optically pure D- and L-amino acids, enzymatic resolution methods have proven to be powerful tools. The enzymatic resolution process developed by DSM[14], based on the stereospecific hydrolysis of aminoacid amides by using the L-specific aminopeptidase from Pseudomonas Putida, has found wide application.

In order to examine the above described methodology as being suitable for the synthesis of L-lupinic acid L-**16** and D-lupinic acide D-**16** we developed the following strategy (see scheme IX.9):

1. the synthesis of racemic D,L-amino acid amide D,L-**51**.
2. the stereospecific hydrolysis of D,L-**51** by using the L-specific aminopeptidase to afford L-acid L-**16** and D-amide D-**51**.
3. the chemical conversion of the D-**51** into D-acid D-**16**.

The synthesis of D,L-lupinic aminoacid amide D,L-51 (see scheme IX.9) was achieved by i) coupling of the aminoalcohol reagent A with 6-chloropurine **48** ii) coupling of **49** with methyltrifluoracetylaminoacrylate (reagent B) to afford D,L-**50** iii) treatment of D,L-**50** with NH_3.


A
B
48
49
D,L-50
NH3
L-specific
aminopeptidase
D-51
L-16
D,L-51
H+
D-16
reagent A:
reagent B:

Scheme IX.9

The second part of our strategy dealing with the enzymatic stereospecific hydrolysis of the racemate D,L-**51** with L-aminopeptidase from Pseudomonas Putida, gave L-lupinic acid **L-52** the amide **D-51**, both in an optical purity of more than 95%. The optical purity of both enantiomeric compounds was determined by means of ^{1}H-NMR spectroscopy using the chiral shift reagent S-2-chloropropionylchloride (see chapter VIII). The unreacted **D-51** was hydrolyzed under mild acid conditions to afford the optically pure D-Lupinic acid **D-16**.

CONCLUSION

In this thesis we described the synthesis of sixteen phosphorylated cytokinins and two alanylated cytokinins.

The succesful synthesis of phosphorylated derivatives was realized by using an efficient way of preparing properly protected cytokinins and new phosphorylation methods. This combination of methods leads to a fast and reliable procedure for the preparation of phosphorylated cytokinins in satisfactory yields.

The results of the synthesis of optically pure alanylated cytokinins lead unequivocally to the conclusion that the enzymatic separation by Pseudomonas Putida was succesful.

In this way we introduced a new, efficient method for the synthesis of optically active D- and L-lupinic acid. Furthermore, we believe that this method is also suitable for the synthesis of many optically active aminoacids with a heterocyclic ring on the β-position. As we have mentioned previously, this synthetic study was part of a program between our laboratory and the Centre of Agricultural and Biological Research at Wageningen. This centre uses the produced phosphorylated and alanylated compounds for biological research. Some of these results have been published in an article: "Formation of zeatin allylic phosphate by the microsomal fractions of bulb disks of Iris x hollandica Tub. and tubers of Helianthus tuberosus L". The phosphorylated cytokinins also attracted the attention of the National Cancer Institute in the U.S.A. for clinical research. We reported above (see chapter I, page 2) that cytokinins have been employed as potential anticancer agents in clinical trials. One of the phosphorylated compounds is being tested at this moment in the anticancer screening program of this institute.

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SUMMARY

New approaches are described in this thesis towards the syntheses of phosphorylated and alanylated cytokinins.

In chapter I a general picture of the stucture of cytokinins, their occurence in nature, their biological synthesis, their effects on plants and their chemical synthesis is described.

A literature survey of the synthesis of phosphorylated and alanylated cytokinins is given in chapter II. Furthermore, this chapter contains a short introduction of our approach towards the synthesis of phosphorylated and alanylated cytokinins.

In chapter III the phosphorylation of allylic hydroxy groups via phosphite intermediates is described. A new method is introduced for the introduction of a phosphate group at the allylic position. This phosphite triester approach is based on the phosphitylation of the allylic alcohol with salicyl chlorophosphite, followed by the oxidation of the allylic phosphonate monoester to the corresponding phosphate monoester.

The synthesis of allylic phosphor derivatives of *trans*-zeatin is described in chapter IV. We showed in this chapter that the phosphite triester approach is not only suitable for the synthesis of the allylic phosphate of the cytokinin *trans*-zeatin, but is also suitable for the synthesis of the allylic thiophosphate and the methylphosphate of *trans*-zeatin.

Chapter V deals with a convenient new synthesis of the allylic pyrophosphate of *trans*-zeatin. This method is based on the conversion of the allylic phosphonate of *trans*-zeatin into the corresponding allylic phosphoromorpholidate, which after treatment with the mono(tri-n-butylammonium) phosphate gave the allylic pyrophosphate of *trans*-zeatin.

In chapter VI the synthesis of the phosphate derivatives of the ribosyl zeatin and N^6-(Δ^2-isopentenyl)adenosine is described.

Two approaches were used for the synthesis of these cytokinin phosphates. A phosphor triester approach which is based on the use of the bifunctional phosphorylating reagent N-morpholino-O,O-bis[(6-trifluormethyl)benzotriazolyl]phosphate, for the preparation of ribosyl zeatin 5'-phosphate and N^6-(isopentenyl)adenosine 5'-phosphate, whereas phosphite triester approach, which is based on the use of the monofunctional phosphitylation reagent salicylchlorophosphite, was applied to the synthesis of ribosyl zeatin allylic phosphate and ribosyl zeatin diphosphate.

In chapter VII the syntheses of phosphor derivatives of ribosyl zeatin are described. The phosphite triester approach was also used here. Phosphitylation of the properly protected zeatin ribosyl derivatives by using salicylchlorophosphite gave the corresponding phosphonates which could be converted into the corresponding phosphate, methylphosphate and thiophosphate. After cleavage of the protective groups the phosphorderivatives of ribosyl zeatin are obtained.

In chapter VIII the synthesis of alanylated cytokinins is described, namely the synthesis of optically active L-lupinic acid and D-lupinic acid. These were obtained in an optical purity of more than 95%, by enantioselective hydrolysis of a racemic mixture of the amide of D,L-lupinic acid with aminopeptidase from Pseudomonas Putida. Hydrolysis of D-lupinic acid amide gave D-lupinic acid.

SAMENVATTING

In dit proefschrift zijn nieuwe methodes voor de synthese van gefosforyleerde en gealanyleerde cytokininen beschreven.

In hoofdstuk I wordt de struktuur van cytokininen, hun voorkomen in de natuur, biologische synthese, effekten in planten en hun chemische synthese in het algemeen beschreven. Een overzicht van de synthese van gefosforyleerde en gealanyleerde cytokininen, beschreven in de literatuur, wordt gegeven in hoofdstuk II. Dit hoofdstuk bevat verder een korte inleiding tot onze methode voor de synthese van gefosforyleerde en gealanyleerde cytokininen.

In hoofdstuk III wordt de fosforylering van allylische hydroxy groepen via fosfiet-intermediairen beschreven. Een nieuwe methode werd geintroduceerd voor de invoering van de fosfaat groep op het allylisch alcohol. Deze fosfiet triester methode is gebaseerd op de fosfietylering van de allylische alcohol met salicylchlorofosfiet, gevolgd door oxidatie van de allylische fosfonaat monoester tot de overeenkomstige fosfaat monoester.

De synthese van allylische fosfor derivaten van *trans*-zeatin wordt beschreven in hoofdstuk IV. In dit hoofdstuk laten we zien dat de bovengenoemde fosfiet triester methode niet alleen geschikt is voor de synthese van het allylisch fosfaat van *trans*-zeatin, maar ook voor de synthese van het allylisch thiofosfaat en het methylfosfaat van *trans*-zeatin.

In hoofdstuk V wordt een geschikte nieuwe methode voor de synthese van het allylische pyrofosfaat van *trans*-zeatin behandeld. Deze methode is gebaseerd op de omzetting van het allylisch fosfonaat van *trans*-zeatin in het overeenkomstig allylisch fosformorfolidate, hetgeen na behandeling met mono(tri-n-butylammonium)fosfaat het allylisch pyrofosfaat van *trans*-zeatin geeft. In hoofdstuk VI beschrijven we de synthese van de fosfaat derivaten van ribosyl zeatine en N^6-(Δ^2-isopentenyl)adenosine. Twee methodes worden gebruikt voor de synthese van deze cytokinine fosfaten. De fosfaat triester methode die gebaseerd is op het gebruik van het bifunctionele fosforylerende reagens N-morfolino-O,O-bis[(6-trifluormethyl)benzotriazolyl]-fosfaat, werd toegepast voor de synthese van het ribosyl zeatine 5'-fosfaat en het N^6-(Δ^2-isopentenyl)adenosine 5'-fosfaat, terwijl de fosfor triester methode, gebaseerd op het gebruik van het monofunctionele fosfietylerende reagens salicylchlorofosfiet, toegepast werd voor de synthese van het ribosyl zeatine difosfaat.

In hoofdstuk VII wordt de synthese van fosforderivaten van het cytokinine ribosyl zeatine beschreven. De fosfor triester methode werd ook hier gebruikt. Fosfietylering van de op de juiste manier beschermde ribosyl zeatine derivaten met salicylchlorofosfiet gaf de overeenkomstige fosfonaten, die konden worden omgezet in het overeenkomstige fosfaat, methylfosfaat en thiofosfaat. Na afsplitsing van de beschermende groepen werden de fosforderivaten van ribosyl zeatine gevormd.

In hoofdstuk VIII wordt de synthese van gealanyleerde cytokinine beschreven; namelijk die van optisch aktief L-lupine zuur en D-lupine zuur. Beiden werden verkregen met een optische zuiverheid van meer dan 95%, door enantioselectieve hydrolyse van het racemische D,L-lupine zuuramide met aminopeptidase van Pseudomonas Putida. Hydrolyse van D-lupine zuur amide gaf D-lupine zuur.

CURRICULUM VITAE

De schrijver van dit proefschrift werd geboren op 31 januari 1957 te Illar (Jordanië). Het einddiploma Scientific Stream behaalde hij in 1975 aan de Attil Secondary Boys' School te Attil (Jordanië). In september 1976 begon hij met de studie scheikunde aan de Rijksuniversiteit Leiden. Het kandidaatsexamen (S1) werd in juni 1982 afgelegd. De studie voor het doctoraalexamen omvatte het hoofdvak organische chemie (Prof. Dr. J.H. van Boom), het bijvak chemische technologie (Prof. B. Scarlett M.Sc. - Technische Universiteit Delft) en de derde richting bio-anorganische chemie (Prof. Dr. J. Reedijk). Het doctoraalexamen werd op 28 juni 1985 afgelegd.

Vanaf februari 1986 was hij als wetenschappelijk assistent verbonden aan het Laboratorium voor Organische Chemie van de Landbouwuniversiteit Wageningen, alwaar hij onder leiding van Prof. Dr. H.C. van der Plas het in dit proefschrift beschreven onderzoek verrichtte. Vanaf februari 1990 is hij werkzaam bij Duphar B.V. op de afdeling Chemische Ontwikkeling te Weesp.

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