On the mechanism of action of 3',5'-cyclic adenosine monophosphate: a study of four- and five-coordinated phosphorus compounds, modelling the activated state of cAMP

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ON THE MECHANISM OF ACTION OF 3', 5'-CYCLIC ADENOSINE MONOPHOSPHATE

A Study of Four- and Five-coordinated Phosphorus Compounds, Modelling the Activated State of cAMP

N.L.H.L. BROEDERS
ON THE MECHANISM OF ACTION OF 3',5'-CYCLIC ADENOSINE MONOPHOSPHATE

A Study of Four- and Five-coordinated Phosphorus Compounds, Modelling the Activated State of cAMP

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ter verkrijging van de graad van doctor aan de Technische Universiteit Eindhoven, op gezag van de Rector Magnificus, prof. dr. J.H. van Lint, voor een commissie aangewezen door het College van Dekanen in het openbaar te verdedigen op woensdag 30 juni 1993 om 14.00 uur

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1 General Introduction

1.1 Introduction

The study of phosphorus compounds started in 1669, as Hennig Brand, a German alchemist from Hamburg, tried to make gold out of putrefied urine. Brand discovered a substance that is known as white phosphorus: a white, crystalline solid that glows in the dark and ignites spontaneously in air just above room temperature. Later on, white phosphorus attracted considerable attention because of its utility in warfare. White phosphorus burns rapidly in air to form phosphoric oxide ($P_4O_{10}$). In the atmosphere, $P_4O_{10}$ appears as a dense white smoke, and it was discovered during World War I that the obscuring power of this smoke per kg of phosphorus was greater than of any smoke-generating chemical then known. The number of applications of phosphorus compounds has increased enormously since the days of World War I. For example, the discovery of Schrader and others that some organic phosphates display marked toxic and insecticidal properties, has created a whole new industry.1

Today, we encounter phosphorus compounds almost anywhere in our everyday life. A few examples may help to illustrate this. Phosphates occur in our cleaning agents and detergents, phosphates are used in water treatment, and phosphates are abundant in our food (for example, a cola drink is actually a flavoured, carbonated, sweetened, diluted solution of phosphoric acid). Other more exotic phosphorus compounds occur in fertilizers, pharmaceuticals, and as "flame retardants" in...
polymeric materials.

This thesis deals with aspects of the role of phosphorus compounds in the functioning of living cells. A living cell functions through the action of many phosphorus containing compounds: the biophosphates. The most important members of this class are DNA (deoxyribonucleic acid) and RNA (ribonucleic acid), both carriers of the genetic code. In DNA and RNA, phosphate groups are found in the backbone of the well-known spiral-staircase structure. The essential building blocks, called the nucleotides, each consist of a nitrogen-containing base, a five-carbon sugar (for DNA, deoxyribose) and phosphoric acid. Four different nitrogen-containing bases are present in a DNA molecule: the two purines adenine (A) and guanine (G), and the two pyrimidines thymine (T) and cytosine (C). In the double helix, A is coupled to T via two hydrogen bonds and G is coupled to C via three hydrogen bonds. The phosphate groups, which are located on the periphery of the double helix, are of essential importance in DNA-enzyme interactions. Besides, the phosphates play a key role in salt-induced conformational changes of DNA, such as: a righthanded B-DNA helix changing towards a lefthanded Z-DNA helix occurring in d(CG·CG)₂ duplexes.

Other essential biophosphates are ATP (adenosine triphosphate), which serves as an energy carrier, and 3',5'-cyclic adenosine monophosphate (cAMP) which mediates the action of many hormones and regulates important activities in almost every living cell. Specifically, this thesis concentrates on the role of phosphorus in the mechanism of action of cAMP.
The energy carrier ATP serves as a major link between energy-yielding and energy-requiring chemical reactions in the living cell. When one ATP molecule is hydrolyzed at neutral pH at 25 °C to form ADP (adenosine diphosphate) and \( P_i \) (inorganic phosphate), 7.3 kcal/mol free energy is liberated.\(^2\) This free energy of hydrolysis is used to perform biological work. ATP is formed from ADP during the anaerobic oxidation of glucose to form 2 molecules of pyruvate (glycolysis). It should be mentioned that ATP is not the only energy carrying phosphate in the living cell, nor is the free energy of hydrolysis of ATP exceptionally large. Some other energy carrying phosphates are: phosphoenolpyruvate (free energy of hydrolysis: \(-14.8\) kcal/mol), creatine phosphate (\(-10.3\) kcal/mol) and fructose-6-phosphate (\(-3.3\) kcal/mol).\(^2\)

Except being an energy-carrier, ATP is also the precursor of cAMP. The enzyme that converts ATP in cAMP is adenylate cyclase: the 1971 Nobel Prize for physiology and medicine was awarded to E.W. Sutherland for the discovery of cAMP and adenylate cyclase. This investigation led to the second messenger concept, which states that in eukaryotic cells, cAMP acts as a second messenger for many hormones to control several biochemical processes. This concept can best be described using the well-studied biochemical process of the breakdown of glycogen into blood glucose (glycogenolysis) in liver cells, which is controlled by cAMP and visualized in Figure 1.1.\(^2\) The hormone adrenalin (epinephrine; first messenger), secreted in sympathetic nerve endings and transported by the blood stream, binds to specific receptors on the cell membrane without entering the cell. However, the enzyme adenylate cyclase, converting adenosine triphosphate (ATP) into cAMP, is not directly activated by the hormonal stimulus, but it is mediated by the so-called G-protein. This G-protein binds guanyl nucleotides and exists in two forms: (i) the guanosine triphosphate (GTP) complex of the G-protein, which activates adenylate cyclase, and (ii) the guanosine diphosphate (GDP) complex, which does not activate the adenylate cyclase enzyme. The G-protein is converted from the inactive GDP form into the active GTP form by exchange of GDP, bound to the G-protein, by
Figure 1.1 Degradation of glycogen into D-glucose.
1.1 Introduction

GTP. This GTP-GDP exchange is exclusively catalyzed by the receptor-adrenalin complex. The activated G protein then initiates the formation of cAMP (second messenger) from ATP by adenylate cyclase. Because cAMP is such a potent regulator of cellular functions its concentration must be controlled by the cell. Therefore the production of cAMP by adenylate cyclase should be in balance with the degradation of cAMP (hydrolysis of cAMP to 5'-adenosine monophosphate (5'-AMP)), which is regulated by a specific 3',5'-nucleotide phosphodiesterase (Figure 1.2). Binding of cAMP to the regulatory subunits of protein kinase leads to

\[ \text{ATP} \xrightarrow{\text{Adenylate Cyclase}} \text{cAMP} \xrightarrow{\text{Phosphodiesterase}} 5'\text{AMP} \]

*Figure 1.2 Synthesis and degradation of cAMP.*

dissociation of this enzyme into two active catalytic (C) units and a dimer of regulatory (R) subunits. The catalytic subunits activate phosphorylase kinase by phosphorylation. The activated phosphorylase kinase catalyzes the activation of phosphohydrolase b, leading to phosphohydrolase a. Ultimately glycogen is degraded into glucose units, which are released to the blood stream. However, the precise mechanistic details of the biochemical reactions in the cascade, mentioned above, has not yet been elucidated.
1.2 cAMP Analogues

The mechanism of action of cAMP in regulating cellular metabolism has been the subject of numerous studies. A substantial part of this work was carried out with cAMP analogues. A diversity of cAMP analogues have been synthesized and their interactions with many enzymes (phosphodiesterases, protein kinases) has been studied with the aim to formulate structure-activity relationships. Based on this massive amount of work, it is now generally accepted that hydrolysis of cAMP by phosphodiesterases proceeds via a nucleophilic attack on the phosphorus atom of cAMP, either by a water molecule, or by a nucleophilic site of the enzyme. The reaction occurs in an enzyme-cAMP complex; such a complex has been characterized for the hydrolysis of cAMP by bovine intestine 5'-nucleotide phosphodiesterase. An important piece of information concerning the mechanism of cAMP hydrolysis by phosphodiesterases was inferred from stereochemical studies. For instance, hydrolysis of the $S_p$ and $R_p$ stereoisomers of 3',5'-cyclic adenosine monophosphorothioate (cAMPS) by bovine heart cyclic nucleotide phosphodiesterase and baker's yeast cyclic nucleotide phosphodiesterase both proceed with complete inversion of configuration at phosphorus. Also, hydrolysis of $R_p$ 2'-deoxyadenosine 3',5'-cyclic $[^{17}O, ^{18}O]$monophosphate ($R_p ~[^{17}O, ^{18}O]$cdAMP) by bovine heart cyclic nucleotide phosphodiesterase occurs with complete inversion of configuration.

![Diagram of cAMPS and Rp[^{17}O,^{18}O]cdAMP]
Investigations concerning the activation of protein kinases revealed that binding of cAMP is heavily influenced by chemical modifications of the ribose ring; modification of the base (adenine) generally has a smaller impact on cAMP binding. Furthermore, it was found that the negatively charged phosphodiester group of cAMP is usually connected to enzymatic groups via one or more hydrogen bonds.4d

1.3 Role of Phosphorus in the Mechanism of Action of cAMP

According to the current views, the phosphorus atom of cAMP plays a vital role in the mechanism of action. The premise has been set forth that a five-coordinated phosphorus (P^v) intermediate can be formed via nucleophilic attack of an appropriate functionality within the active site of the enzyme (phosphodiesterase, protein kinase, CAP dimer, etc.).8 This idea posed two fundamental questions concerning the exact nature of the P^v intermediate: (i) how is the six-membered 3',5'-dioxaphosphorinane ring oriented with respect to the P^v centre? The P^v atom, having a trigonal bipyramidal (TBP) structure, features two axial and three equatorial binding sites. This means that the 3',5'-dioxaphosphorinane ring can adopt either an equatorial-axial (e,a), or a diequatorial (e,e) orientation; and (ii) what conformation is adopted by the 3',5'-dioxaphosphorinane ring in the P^v intermediate? While it is well-known that the six-membered ring resides in a standard chair conformation in cAMP (evidence from X-ray crystallographic studies9, as well as from NMR studies10), it is by no means trivial that the chair geometry is retained for either the (e,a), or (e,e) orientation in the P^v trigonal bipyramid.

Ad (i): Until recently, virtually no structural information on P^v systems containing a six-membered ring was available. This point was specifically addressed in recent years. For instance, Holmes et al. studied the X-ray crystal structures of a series of pentaoxy phosphoranes which incorporate ring systems ranging from five- to eight-membered.11 In all cases but two, (e,a) orientation of the ring system was
encountered. It was reported by Bentrude et al. that compound 1 features (e,e) orientation of the six-membered ring. Surprisingly, the dioxaphosphorinane ring in 1 adopts a chair conformation. It must be realized, however, that the (e,e) orientation in 1 is not really surprising, since both axial sites are occupied via the four-membered and five-membered ring fragments which are exclusively found in (e,a) orientation. It was found by Holmes et al. that compound 2, incorporating an eight-membered ring, actually showed an (e,e) orientation. It should be mentioned that 3 which is virtually identical to 2, was already studied by Denney et al. in 1985. Their NMR spectroscopic data already led to the conclusion that the eight-membered ring resides in an (e,e) orientation.

Ad (ii): The conformational preference of six-membered rings which are part of a $^3\text{P}^\text{V}$ trigonal bipyramid has been extensively studied by Bentrude et al. In 1987, they published a conformational study of the cis- and trans-diastereoisomers of thymidine 3',5'-cyclic phenyl monophosphate (cis and trans: orientation of the exocyclic substituent bound to phosphorus related to the base), which was regarded as a model for cAMP. The cis-diastereoisomer showed a chair conformation of the 3',5'-dioxaphosphorinane ring, whereas the trans counterpart showed a chair $\neq$ twist conformational equilibrium with an associated free energy difference of only 2.2 kcal/mol. A similar effect was observed by Hermans et al. in studying the conformation of the six-membered phosphate ring of a number of phosphorus-derivatized 3',5'-cyclic nucleotide model compounds. It was postulated that this free energy could be readily supplied by favourable binding interactions in the
1.4 A Closer Look at Five-coordinated Phosphorus

The questions of the exact orientation of the 3',5'-dioxaphosphorinane ring in the cAMP-derived \( \text{P}^\text{V} \) intermediates (i.e. (e,a) or (e,e)), and the corresponding conformation of the 3',5'-dioxaphosphorinane ring, are specifically addressed in this thesis. Therefore, it is worthwhile to review the most important aspects of \( \text{P}^\text{V} \) systems in advance.

Five-coordinated phosphorus structures have been studied extensively in the last decades. Most of this work was stimulated by Westheimer's original discovery of markedly accelerated hydrolysis of cyclic phosphate in comparison with the acyclic
counterparts.\textsuperscript{20} The elegant explanation of this difference, in which a $P^V$ intermediate plays a key role, is one of the milestones in contemporary phosphorus chemistry. Commonly, $P^V$ structures have the geometry of a trigonal bipyramid, although substantial distortions towards a square-pyramidal geometry can occur.\textsuperscript{21} In the trigonal bipyramid, two axial and three equatorial sites can be readily distinguished. Interestingly, the axial and equatorial sites display a different chemical behaviour. The axial bonds are slightly longer and weaker than the equatorial ones. This is sometimes explained in terms of a simple hybridization model which states that phosphorus has $p$-hybridization in the axis, and $sp^2$-hybridization in the equatorial plane. Consequently, and this was one of the key elements in Westheimer's theory, nucleophiles attacking a phosphate substrate initially occupy an axial site in the trigonal bipyramid. Analogously it is always an axial ligand that is expelled when the $P^V$ system relaxes back to a phosphate-type structure in the final stage of the reaction.

Another characteristic property of $P^V$ systems is their fluxional behaviour, usually designated as pseudorotation.\textsuperscript{22} This means that the five ligands are rapidly distributed over the five sites in the trigonal bipyramid. A classical example is $PF_5$,\textsuperscript{23} its $^{19}$F NMR spectrum shows a sharp single doublet at ambient temperature (rapid pseudorotation) but two doublets in the ratio 2:3 at lowered temperature (retarded pseudorotation). Pseudorotation occurs in most of the known stable $P^V$ compounds at ambient temperature. Variable temperature NMR techniques have proved very useful in determining thermodynamic activation parameters of the pseudorotation process.\textsuperscript{24} The mechanism of pseudorotation has been subject of considerable debate. Usually, pseudorotation is explained in terms of the Berry mechanism (Berry pseudorotation: BPR).\textsuperscript{21,22} According to Berry the ligand exchange process can be illustrated as shown in Figure 1.3. In this process two equatorial and two axial ligands interchange their positions via an intermediate $SP$ structure, while one equatorial ligand (the pivot) retains its position. An alternative mechanism, shown in Figure 1.4, is the so-called turnstile rotation (TR), described
by Ramirez and Ugi.\textsuperscript{21,25} In this alternative mechanism, two equatorial ligands move towards each other in the equatorial plane until the angle has become about 90°. One axial ligand and a third equatorial ligand are moved by about 9° from their original positions, followed by an internal rotation of the ligands 3 and 4 and of the three ligands 1, 2 and 5. The results of the TR is exactly the same as the BPR. However there is a fundamental difference. The BPR does not involve internal rotations around an axis through the central phosphorus atom, and calculations show that the transition state for TR is about 2-6 times higher in energy compared to BPR.\textsuperscript{21b} Therefore, BPR is the most plausible ligand exchange mechanism in acyclic phosphoranes, whereas in 'caged' systems, like 7, only the TR mechanism can be regarded as a plausible alternative to Berry pseudorotation.
A number of recent studies on a set of \( \text{P}^\text{V} \) structures such as 8 and 9 have shown the relevance of the typical \( \text{P}^\text{V} \) bonding properties with respect to the conformation of the ligands. In compounds 8 and 9 the axially located \( \text{P}-\text{O}-\text{C}-\text{C}-\text{O} \) fragment preferentially adopts the \( \text{O}-\text{O} \) trans conformation, whereas their four-coordinated counterparts 10 and 11, respectively, prefer \( \text{O}-\text{O} \) gauche conformation.\(^{26}\) This change in the conformational preferences of \( \text{P}-\text{O}-\text{C}-\text{C}-\text{O} \) ligands can be caused by changing the phosphorus coordination from four (\( \text{P}^\text{IV} \)) to five (\( \text{P}^\text{V} \)). This effect is called conformational transmission. The driving force for the conformational change in \( \text{P}^\text{V} \) systems containing a \( \text{P}-\text{O}-\text{C}-\text{C}-\text{O} \) fragment stems from the the intrinsic bonding properties of a \( \text{P}^\text{V} \)-TBP, resulting in electron attraction from the substituents in the equatorial plane, along with release of electron density towards the axial substituents.\(^{27}\) The latter effect leads to an increased electron density on \( \text{O}_5 \) (in compound 8a) or \( \text{O}_a \) (in compound 9a), in comparison with their four-coordinated counterparts 10 and 11, respectively. This will result in an electrostatic repulsion between \( \text{O}_5 \) and \( \text{O}_4 \) (in compound 8a) or between \( \text{O}_a \) and \( \text{O}_b \) (in compound 9a) leading to a preferred \( \text{O}-\text{O} \) trans conformation. Thus electron transfer results in a conformational change in the molecule. This conformational transmission effect has been confirmed in several \( ^1\text{H} \) NMR studies.\(^{28}\) From 300 MHz \( ^1\text{H} \) NMR studies on compounds 8\(^{28a}\) and 9\(^{28e}\) it was concluded that in the compounds 8a and 9a the conformational transmission effect becomes operative. This is in line with the conclusion that no conformational transmission effect is observed in compounds 8b and 9b, since no \( \text{O}-\text{O} \) repulsion
1.5 Aim and Outline of the Thesis

The main goal of this thesis is to get a better insight in the interaction of cAMP with enzymes, like protein kinases and phosphodiesterases. From the past our group has specialized in organophosphorus chemistry. Using this point of view the study has been concentrated on the role of phosphorus in cAMP during its interaction with enzymes. In this thesis, the objectives are concentrated on the study of five-coordinated phosphorus ($P^V$) model systems, with a trigonal bipyramidal geometry (TBP), which are supposed to be intermediates formed during the interaction between cAMP and enzymes, like protein kinases and phosphodiesterases. During this process the coordination of phosphorus increases from four ($P^{IV}$) to five ($P^V$). This phosphorus coordination enhancing process, and the resulting structural properties of the intermediates are studied as the main subjects of this thesis.

Chapter 2 deals with a set of stable $P^V$ model systems, simulating the interaction between cAMP and an enzyme (protein kinase, phosphodiesterase, or CAP-dimer), which are studied by $^1$H NMR spectroscopy. In the beginning of this research no crystal structure was available of a $P^V$-TBP system with a diequatorially located $3',5'$-dioxaphosphorinane ring. In order to address to the diequatorial orientation of the six-membered ring, two of the model systems contain a ligand in which the conformational transmission effect can be operative. This approach is
based on the observation that the conformational transmission effect only occurs in axial ligands of the P\textsuperscript{V}-TBP. Using this approach, the presence of a diequatorially located 3',5'-dioxaphosphorinane ring is indicated. This is in contrast with the equatorial-axial location of the six-membered ring in other model systems containing no fragments in which conformational transmission can occur.

Chapter 3 presents a \textsuperscript{31}P NMR study on the alkaline hydrolysis of a set of \textsuperscript{18}O-labelled cis-nucleotide 3',5'-cyclic aryl monophosphates. The interaction between cAMP and an enzyme (protein kinase, phosphodiesterase, or CAP-dimer) is simulated by these model compounds. The aryl group models the shielding of the negative charge of an exocyclic oxygen atom in cAMP by the enzyme. Introducing an \textsuperscript{18}O-label on phosphorus offers the possibility to determine the stereochmical features of the products formed during hydrolysis. Hydrolysis of P\textsuperscript{IV} compounds takes place via P\textsuperscript{V}-TBP intermediates, therefore determination of the configuration of the products offers the possibility to determine the reaction routes which were passed during hydrolysis. Therefore, using this approach the P\textsuperscript{V}-TBPs can be studied indirectly.

Chapter 4 deals with a novel cAMP model compound, i.e. 2'-O-methyl-cis-adenosine 3',5'-cyclic monophosphate. This compound must be regarded as a unique cAMP model, since of the ribose ring bears a methoxy group; the X-ray crystal structure of 2'-O-methyl-cis-adenosine 3',5'-cyclic methyl monophosphate is described, as well as a detailed study of its conformational properties in methanol. Furthermore, the hydrolysis of 2'-O-methyl-cis-adenosine 3',5'-cyclic methyl monophosphate was studied by means of \textsuperscript{31}P NMR spectroscopy. The combined results on 2'-O-methyl-cis-adenosine 3',5'-cyclic methyl monophosphate substantiate the conclusions derived from other model structures (chapters 2 and 3).
References and Notes


General Introduction


26. (a) In solution the tetrahydrofurfuryl ligand of compound 10 shows a rapid interconversion between three staggered conformations (γ⁺, γ⁻, and γ⁻). The γ⁺ and γ⁻ conformations in compound 10 are favoured over the γ⁻ conformation.²⁸a

(b) The conformation around the Cₐ–C₇ bond of compound 11 is also an equilibrium between staggered rotamers, but as two of these rotamers are mirror images and have identical populations, a two-state description with a gauche and a trans state is used. In compound 11 the O–O gauche conformation is favoured over O–O trans.²⁸e


A 400- and 600-MHz $^1$H NMR Study on the Orientation and Conformation of the 3',5'-Dioxaphosphorinane Ring in Nucleoside 3',5'-Cyclic P$^V$-TBP Systems* 

Abstract

The nucleoside 3',5'-cyclic P$^V$-TBP compounds 2 and 3 were studied as models for the proposed activated state of adenosine 3',5'-cyclic monophosphate (cAMP). Compound 2 features equatorial-axial (e,a) orientation of the 3',5'-dioxaphosphorinane ring. The design of compound 3, which incorporates OCH$_2$CH$_2$OMe as a conformational probe, was based on previous work in our group on conformational transmission in P$^V$-TBP compounds. $^1$H NMR analysis of compound 3 indicate that the conformational properties observed in the OCH$_2$CH$_2$OMe ligand can be explained in terms of conformational transmission. This means that the molecular structure with diequatorial (e,e) location of the 3',5'-ring, and axial location of OCH$_2$CH$_2$OMe contributes significantly to the pseudorotational equilibrium. The conformational transmission effect in 3 has been compared with the P$^V$-TBP compounds 10, in which O-nBu replaces the OCH$_2$CH$_2$OMe group, 11, in which a trans-fused cyclopentane ring replaces the furanose ring of thymidine, and 12, in which the OCH$_2$CH$_2$OMe group is locked in an equatorial position of the P$^V$-TBP. The detailed conformational properties of 2 and 3 were investigated further on the basis of MNDO calculations on the models 15-17. Structural data as obtained from $^1$H NMR were used in the start of the optimizations. The calculations showed that (e,a) orientation of the 3',5'-dioxaphosphorinane ring is favoured by 3-4 kcal/mol over diequatorial orientation. The optimized structures show a twist conformation of the (e,a)-oriented 3',5'-dioxaphosphorinane ring, whereas the (e,e) orientation corresponds with a half-chair geometry.

*This chapter is based on:
2.1 Introduction

The importance of 3',5'-cyclic adenosine monophosphate (cAMP, 1) as second messenger in the regulation of cell metabolism has been described in chapter 1.

Several years ago, the idea was put forward that cAMP may react via an activated state in which phosphorus is in a five-coordinated (P^V) state with a trigonal bipyramidal (TBP) geometry.¹ This P^V-TBP intermediate can be generated via attack of a nucleophile on phosphorus, thereby forcing the 3',5'-dioxaphosphorinane ring into diequatorial (e,e) or equatorial-axial (e,a) orientation. It was proposed by van Ool and Buck that an (e,e) P^V-TBP intermediate is involved in triggering of protein kinases, whereas an (e,a) P^V-TBP controls the hydrolysis of cAMP into 5'-AMP.¹ This dynamic model of the mechanism of action of cAMP has reinforced the interest in the structural properties of stable P^V-TBP compounds with a 1,3,2-dioxaphosphorinane ring, or a related six-membered ring fragment. The present structural knowledge of these systems is mainly based on X-ray crystallographic and NMR studies. The available X-ray data reveal that a six-membered ring favours (e,a) orientation in the P^V-TBP, thereby accommodating a non-chair conformation.² These structural features also prevail in solution, as is apparent from numerous NMR studies.

Bentrude et al. have used NMR techniques to study a number of P^V-TBPs with an 1,3,2-dioxo- or 1,3,2-oxazaphosphorinane ring.³ These systems show a non-chair conformation of the six-membered ring, implying that the formation of P^V-TBP activated cAMP is associated with a substantial conformational change of the 3',5'-ring.
In this chapter, the results are reported of a $^{1}$H- and $^{31}$P-NMR structural study on the $^{PV}$-TBPs 2 and 3, which may be regarded as representative models for cAMP in its activated state. In the case of 2, the (e,a) orientation of the $3',5'$-dioxaphosphorinane ring was assessed via the vicinal proton-phosphorus coupling constant $^{3}J_{POMe}$. In order to determine the orientation of the $3',5'$-dioxaphosphorinane ring in $^{PV}$-TBP 3, the use of conformational transmission was investigated as an indicator for the location of the six-membered ring. Therefore we designed $^{PV}$-TBP 3 containing an O-CH$_2$-CH$_2$-OMe group in which the conformational transmission effect, described in chapter 1, can be operative. In compound 8 the conformational transmission effect is visualized: the axial and equatorial ligands adopt O-O trans and O-O gauche conformation, respectively. Due to the increased electron density on the axial oxygen atom (O$_a$) of the $^{PV}$-TBP 8 in...
comparison with the tetracoordinated counterpart, the contribution of the trans orientation of $O_a$ and $O_b$ is increased significantly. *Mutatis mutandis*, the equatorial ligands display gauche orientation of $O_{c(e^\prime)}$ and $O_{d(d^\prime)}$.

The conformational analysis of the $O-CH_2-CH_2-OMe$ in compound 3 leads to the conclusion that a substantial amount of $O_a-O_b$ trans orientation is present. Using the reversed reasoning of the conformational transmission effect, the $O-CH_2-CH_2-OMe$ group with the $O_a-O_b$ trans orientation will partially adopt an axial location in the $P^V$-TBP system 3. Combining this with the preference of the tetrachloro-1,2-benzoquinone fragment for (e,a) orientation$^5$, it follows that the $3',5'$-ring has a partial diequatorial orientation. Our present results on 2 and 3 indicate that both intermediates (i.e., an (e,a)- and an (e,e)-$P^V$-TBP) can be formed upon activation of cAMP.

2.2 Results and Discussion

2.2.1 Preparation of Compounds 2-9

The synthesis of the cis-phosphites 4 and 5 differs from the two-step method reported by Nelson et al.$^6$ for the preparation of cis-thymidine 3',5'-cyclic phenyl phosphite (overall yield 14%). Compounds 4 and 5 are directly prepared in a 1H-tetrazole-catalyzed reaction of thymidine and bis-(N,N-diisopropylamino)
methoxyphosphine\(^7\) (leading to 4) or bis-(N,N-diisopropylamino)(2-methoxyethoxy)phosphine (leading to 5) (Scheme 2.1). \(^{31}\)P NMR showed formation of 4 and

![Scheme 2.1 Preparation of \(P^\text{V}\)-TBPs (2 and 3) and phosphates (6 and 7).](image)

its trans isomer in the ratio 2:3; 5 and its trans isomer were formed in a 1:1 ratio. Subsequent chromatographic purification exclusively yielded 4 and 5 as pure white solids (yields 23\% and 33\%, respectively). Oxidation of 4 and 5 into the cis-phosphates 6 and 7 respectively, was accomplished through reaction with \(\text{NO}_2/\text{N}_2\text{O}_4\), which is known to proceed with retention of configuration.\(^8\) The \(P^\text{V}\)-TBP target compounds 2 and 3 were prepared through reaction with 1 equiv of tetrachloro-1,2-benzoquinone at -80 °C in a 5-mm NMR tube (solvent CD\(_2\)Cl\(_2\)).
2.2.2 Conformational Analysis

2.2.2.1 Location of the 3',5'-Dioxaphosphorinane Ring in P\textsuperscript{V}-TBP 2 and 3

For compound 2, the location of the 3',5'-ring was determined qualitatively on the basis of the NMR coupling constant between phosphorus and the methoxy protons (\(3^1J_{POMe}\)).\textsuperscript{9} Compound 9 was used as a reference system with respect to compound 2. It should be noted that the experimental \(3^1J_{POMe}(9)\) represents a time-averaged value as a result of pseudorotation around the P\textsuperscript{V} centre, i.e., the three methoxy groups are rapidly exchanged over one axial and two equatorial sites in the TBP. Thus, \(3^1J_{POMe}(9) = \frac{1}{3}[3^1J_{POMe}(axial) + 2\cdot3^1J_{POMe}(equatorial)]\). The hybridization of phosphorus in a TBP results in an enlarged s-character for the equatorial bonds in comparison with the axial ones,\textsuperscript{10} i.e., \(3^1J_{POMe}(equatorial) > 3^1J_{POMe}(axial)\). We found that \(3^1J_{POMe}(2) = 14.3\ Hz\), and \(3^1J_{POMe}(9) = 13.6\ Hz\) (400 MHz \(^1\text{H} \)NMR at 20 °C; solvent CD\(_2\)Cl\(_2\)), i.e., the methoxy group in 2 is predominantly in equatorial location. Combination of this result with the well-known ring strain rule, which states that five-membered rings have strong preference for (e,a) location in the TBP,\textsuperscript{5} reveals that the 3',5'-ring preferentially adopts an (e,a) location in 2. It should be noted that our experimental data on 2 are not conclusive with respect to pseudorotation around the P\textsuperscript{V}-TBP, i.e., no discrimination can be made between the possibilities of either rapid pseudorotation (on the NMR time-scale), or complete absence of pseudorotation. Clearly, if pseudorotation occurs according to the well-known Berry mechanism,\textsuperscript{11} it is the methoxy group which acts as the pivot. In this case, the methoxy group remains equatorial during...
the dynamic exchange of the other four ligands over the axial and equatorial sites in the TBP.

In order to investigate the orientation of the 3',5'-dioxaphosphorinane ring in 3, we performed a conformational analysis of the C_a-C_b bond. The four vicinal H-H J-couplings ($J_{H(1)H(3)}$, $J_{H(1)H(4)}$, $J_{H(2)H(3)}$, and $J_{H(2)H(4)}$) were abstracted from the 400 MHz $^1$H NMR spectrum of compound 3 in CD$_2$Cl$_2$ at -41 °C. A standard computer program was used for iterative simulation of the subspectra (see Figure 2.1). The conformation around C_a-C_b can be described in terms one O_a-O_b trans

![Figure 2.1 Computer-simulated (upper trace) and experimental (lower trace) expansion of the H_1/H_2 pattern in the 400 MHz $^1$H NMR spectrum of compound 3 at -41 °C. Note that H_1 and H_2 are diastereotopic, with $\Delta\delta = 0.1$ ppm. The patterns of H_1 and H_2 each consist of 16 lines, which are partly resolved.](image)

and two approximately degenerate O_a-O_b gauche rotamers (+,−) (Figure 2.2). The magnitude of ($J_{H(1)H(3)} + J_{H(1)H(4)} + J_{H(2)H(3)} + J_{H(2)H(4)}$) was calculated as
a function of the $O_a - O_b$ torsion angle, using the empirically parametrized Karplus equation developed by Altona et al. and shown in Figure 2.3.\textsuperscript{12} This revealed that

\begin{align*}
\text{trans} & \quad \text{gauche (+)} & \quad \text{gauche (-)}
\end{align*}

\begin{figure}[h]
\centering
\includegraphics{staggered_conformations}
\caption{Staggered conformations around the C-C bond in the P-O-C-C-O fragment.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics{pp_coupling}
\caption{The sum of the four proton-proton coupling constants ($J_{H(1)H(3)}$, $J_{H(1)H(4)}$, $J_{H(2)H(3)}$, and $J_{H(2)H(4)}$) as a function of the $O_a - O_b$ torsion angle.}
\end{figure}
(J_{H(1)H(3)} + J_{H(1)H(4)} + J_{H(2)H(3)} + J_{H(2)H(4)}) amounts to 30.0 Hz in the trans rotamer and 16.4 for gauche (+) and gauche (−), leading to the formula:

\[
x(O_a-O_b \text{ trans}) = \frac{(J_{H(1)H(3)} + J_{H(1)H(4)} + J_{H(2)H(3)} + J_{H(2)H(4)})_{\text{exp}} - 16.4}{30.0 - 16.4} \times 100\%
\]

The conformational analysis of the C_a–C_b bond in compound 3 resulted in 51% population of the O_a–O_b trans rotamer, and a total of 49% population of the O_a–O_b gauche rotamers. For comparison, we performed an analogous conformational analysis for the cis-phosphite and cis-phosphate counterparts of 3 (i.e., compounds 5 and 7, respectively). These data (Table 2.1) show substantially diminished O_a–O_b trans populations in comparison with 3. Furthermore, compound 3 was compared with three other P^V-TBP systems, i.e. 10-12. Compound 10 represents the absence of conformational transmission (O_b in 3 substituted by C(H_2) in 10). As expected, compound 10 and its cis-phosphate counterpart 10a displayed a highly similar conformational equilibrium around the C_a–C_b linkage^{4a,b} (10:

<table>
<thead>
<tr>
<th>Compd</th>
<th>J_{H(1)H(3)}</th>
<th>J_{H(1)H(4)}</th>
<th>J_{H(2)H(3)}</th>
<th>J_{H(2)H(4)}</th>
<th>ΣJ</th>
<th>% O–O trans</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>2.2</td>
<td>7.1</td>
<td>4.7</td>
<td>9.4</td>
<td>23.4</td>
<td>51</td>
</tr>
<tr>
<td>5</td>
<td>5.7</td>
<td>3.2</td>
<td>3.2</td>
<td>5.7</td>
<td>17.8</td>
<td>10</td>
</tr>
<tr>
<td>7</td>
<td>5.3</td>
<td>3.5</td>
<td>3.5</td>
<td>5.3</td>
<td>17.6</td>
<td>9</td>
</tr>
<tr>
<td>11</td>
<td>3.9</td>
<td>5.9</td>
<td>4.6</td>
<td>11.0</td>
<td>25.4</td>
<td>66</td>
</tr>
<tr>
<td>12</td>
<td>6.5</td>
<td>2.5</td>
<td>2.5</td>
<td>6.5</td>
<td>18.0</td>
<td>12</td>
</tr>
</tbody>
</table>

^4a,b Measured for the conformational transmission probe system in the compounds 3, 5, and 7 at -41 °C in CD_2Cl_2 and 11 and 12 at 20 °C in CD_2Cl_2, along with the calculated percentages of O_a–O_b trans orientation.
$x(O_a-C_c \text{ trans}) = 80\%$; $10a: x(O_a-C_c \text{ trans}) = 79\%$). Conformational analysis of the $C_a-C_b$ bond in 11 showed that $\Sigma J = J_{H(1)H(3)} + J_{H(1)H(4)} + J_{H(2)H(3)} + J_{H(2)H(4)} = 25.4 \text{ Hz}$, i.e., $66\% O_a-O_b \text{ trans}$ orientation exists. Compounds 3 and 11 show virtually analogous conformational characteristics, revealing that substitution of $O_4$ by $C(H_\bullet)$ has no predominant impact on the preferred $(e,e)$ or $(e,a)$ orientation in the present set of model systems. Finally, we compared the data on 3 with those measured for compound 12, in which the $O-CH_2-CH_2-OMe$ probe is in fact locked in an equatorial position in the $P^V$-TBP. Conformational analysis of $C_a-C_b$ in 12 indeed showed a reduced preference for $O_a-O_b \text{ trans} (12\%$, see Table 2.1).

The results on 3, 5, 7, and 10-12 indicate that conformational transmission occurs in 3 and 11. Thus, the dynamic equilibrium of phosphorus pseudorotation in 3 and 11 is such that the $O-CH_2-CH_2-OMe$ group resides most of the time in the axis of the $P^V$-TBP. Combining this with the ring rule (vide supra), it follows that the $3',5'$-ring in 3 and 11 is engaged in an equilibrium between $(e,a)$ and $(e,e)$ orientations in the $P^V$-TBP. The interconversion between $(e,a)$ and $(e,e)$ orientation of the ring can occur via the Berry pseudorotation mechanism, using either $O_3$, or $O_5$, as the pivot. Recently, an X-ray study on a $P^V$-compound (A), containing a diequatorially located 1,3,2-dioxaphosphorinane ring, was reported. However it is not surprising that the six-membered ring adopts a diequatorial orientation, since four- and five-membered rings favour $(e,a)$ locations. In our systems the only way to get access to the orientation of the six-membered $3',5'$-dioxaphosphorinane ring,
2.2 Results and Discussion

A

is by means of the conformational transmission effect. Therefore it is assumed that
the O–O trans conformation of the probe has been related to an axial location in
the P^v-TBP. Since no other methods are available to locate the 3',5'-dioxaphospho-
rinane ring the conformational transmission effect is considered to be an indirect
indicator for the diequatorial orientation of the 3',5'-dioxaphosphorinane ring.

2.2.2.2 Conformation of the 2'-Deoxyribose Ring

Conformational analysis of the sugar ring of 2-9 was performed with the
PSEUROT program.\textsuperscript{16} The sets of vicinal H–H coupling constants ($J_{H(1)H(2')}$, $J_{H(1)H(2')}$, $J_{H(2')H(3)}$, $J_{H(2')H(3)}$, and $J_{H(3)H(4')}$) measured for each compound (see
Table 2.2) were used as input data. PSEUROT calculates the best-fit conformational
parameters of two sugar structures participating in a rapid conformational equilib-
rium, as well as the equilibrium composition. Based on the present data, PSEUROT
rapidly converged towards a single conformation which is characterized by a phase
angle ($P$) of 35.9°, and a maximum puckering amplitude ($\nu_{\text{max}}$) of 39.9°.\textsuperscript{17} As is
well-known, the five endocyclic torsion angles $\nu_0$–$\nu_4$ can be calculated from $P$ and
$\nu_{\text{max}}$ according to the formula $\nu_j = \nu_{\text{max}} \cos(P + (j-2)\cdot144°)$,\textsuperscript{18} i.e.,
$\nu_0[C_4',-O_4'-C_1'-C_2'] = -12.2°; \; \nu_1[O_4'-C_1'-C_2'-C_3_] = -12.4°; \; \nu_2[C_1'-C_2'-C_3'-C_4'] = 32.3°; \; \nu_3[C_2'-C_3'-C_4'-O_4'] = -39.9°; \; \nu_4[C_3'-C_4'-O_4'-C_1'] = 32.2°. \; \text{It follows from these results that the structure of the 2'-deoxyribose ring in 2-9 resides in a twist (T) geometry (vide infra), which is}
Table 2.2 Vicinal $^1H-^1H$ and $^{31}P-^1H$ coupling constants (Hz) measured for compounds 2-7 in CD$_2$Cl$_2$.

<table>
<thead>
<tr>
<th>Compd</th>
<th>$J_{H(1)H(2')}$</th>
<th>$J_{H(1)H(2'')}$</th>
<th>$J_{H(2)H(3')}$</th>
<th>$J_{H(2'')H(3')}$</th>
<th>$J_{H(3')H(4')}$</th>
<th>$J_{H(4')H(5')}$</th>
<th>$J_{PH(5')}$</th>
<th>$J_{PH(5'')}$</th>
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<tr>
<td>2</td>
<td>3.3</td>
<td>8.4</td>
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<td>9.2</td>
<td>7.2</td>
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<td>3</td>
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<td>9.0</td>
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<td>9.1</td>
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<td>7.3</td>
<td>27.4</td>
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<tr>
<td>4</td>
<td>2.5</td>
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<td>8.0</td>
<td>10.9</td>
<td>9.3</td>
<td>10.7</td>
<td>4.4</td>
<td>2.3</td>
</tr>
<tr>
<td>5</td>
<td>2.5</td>
<td>9.0</td>
<td>8.0</td>
<td>11.0</td>
<td>9.2</td>
<td>10.7</td>
<td>4.4</td>
<td>2.3</td>
</tr>
<tr>
<td>6</td>
<td>3.0</td>
<td>8.9</td>
<td>8.4</td>
<td>10.1</td>
<td>9.2</td>
<td>10.7</td>
<td>4.7</td>
<td>1.1</td>
</tr>
<tr>
<td>7</td>
<td>2.6</td>
<td>9.2</td>
<td>8.3</td>
<td>10.4</td>
<td>9.2</td>
<td>10.6</td>
<td>4.7</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

*Spectra of compounds 2, 4, and 6 were recorded at 20 °C; spectra of 3, 5, and 7 were taken at -41 °C.*
2.2 Results and Discussion

in excellent agreement with X-ray crystallographic studies on 3',5'-cyclic phosphites and phosphates.19

2.2.2.3 Conformation of the 3',5'-Dioxaphosphorinane Ring in P^V-TBP 2 and 3

The conformational properties of dioxaphosphorinane rings attached to a P^V-TBP structure have attracted considerable attention, especially since the 3',5'-ring in cAMP is likely to undergo a chair into non-chair (boat and/or twist) conformation prior to, or concerted with, the formation of a P^V-TBP intermediate.3 Our method for conformational analysis of the 3',5'-ring in 2-9 is abstracted from the work of Bentrude et al.20 These studies revealed that cis 3',5'-cyclic nucleotides normally have a chair geometry in which phosphorus and H_5' are antiperiplanar, while phosphorus and H_5, are in a gauche orientation. This geometry results in \( J_{PH(5')} = 2.5 \text{ Hz} \), \( J_{PH(5'')} = 10.7 \text{ Hz} \) for cis-phosphites^{21} and \( J_{PH(5')} = 1 \text{ Hz} \), \( J_{PH(5'')} = 22.5 \text{ Hz} \) for cis-phosphates.6^{22} The vicinal H-P coupling constants fit into classical Karplus type relations with quite different parametrizations for phosphite and phosphate structures.21^{22} Inspection of the data in Table 2.2 shows that \( J_{PH(5')} \) and \( J_{PH(5'')} \) measured for the cis-phosphites 4 and 5 closely resemble the standard values for a chair conformation. Similarly, the \( J_{PH(5')} \), \( J_{PH(5'')} \) data as obtained for the cis-phosphates 6 and 7 also correspond to a chair geometry.
Conformational analysis of the 3',5'-ring in 2 and 3 is based on recent studies of Bentrupe et al. on the P\textsuperscript{V}-TBP systems \textsuperscript{13a} and \textsuperscript{14}; \textsuperscript{3b} compound 14 is closely related to our models 2 and 3. The data on 13 and 14 strongly suggest that a Karplus type equation is valid for \(3\sigma_{\text{PH}}\) scalar couplings in P\textsuperscript{V}-TBP systems.\textsuperscript{3} Antiperiplanar orientation of H and P across H–C–O–P and H–C–N–P coupling paths was found to result in large \(3\sigma_{\text{PH}}\) couplings (26.1 and 25.0 Hz, respectively), while much smaller values of \(3\sigma_{\text{PH}}\) were found for gauche orientations (8.7 and 5.5 Hz for H–C–O–P and H–C–N–P respectively). The large \(3\sigma_{\text{PH}}\) coupling constants (= 27 Hz) observed for 2 and 3 have been interpreted as evidence for antiperiplanar orientation of P and H. Thus, it is concluded from the data in Table 2.2 that phosphorus and H\textsubscript{5'}, are antiperiplanar in 2 and 3, while gauche orientation exists for phosphorus and H\textsubscript{5''}. These results are in close agreement with the data reported for 13 and 14.\textsuperscript{3} Thus, it is clearly demonstrated that 2 and 3 populate a non-chair structure for the 3',5'-dioxaphosphorinane ring.

A second clue for characterization of the 3',5'-ring is provided by the coupling constants \(J_{\text{H(4')H(5')}}\) and \(J_{\text{H(4')H(5'')}}\) from which the conformation around the C\textsubscript{4'}–C\textsubscript{5'}, (\(\gamma\)) bond can be deduced.\textsuperscript{4a-d} Using the generalized Karplus equation of Altona et al.,\textsuperscript{12} we calculated \(J_{\text{H(4')H(5')}}\) and \(J_{\text{H(4')H(5'')}}\) as a function of the torsion angle \([\text{O}_{5'}–\text{C}_{5'}–\text{C}_{4'}–\text{O}_{4'}]\) (see Figure 2.4). This graph indicates that the P\textsuperscript{III} and P\textsuperscript{IV} systems correspond with a torsion angle of approximately 180°. The P\textsuperscript{V}-TBP systems 2 and 3, on the other hand, appear to correspond with a rotation around the C\textsubscript{4'}–C\textsubscript{5'}, bond in such a way that the torsion angle has increased to approximately 200°.

In order to obtain a better insight into the molecular conformation of the P\textsuperscript{V}-TBPs 2 and 3, we performed a set of MNDO semi-empirical calculations\textsuperscript{23} on the isomeric model systems 15, 16 (both (e,a)), and 17 (e,e). The calculations were started using the experimental data concerning the structure of the 2'-deoxyribose ring, the torsion angle \([\text{P–O}_{5'}–\text{C}_{5'}–\text{H}_{5''}]\), and the torsion angle \([\text{O}_{5'}–\text{C}_{5'}–\text{C}_{4'}–\text{O}_{4'}]\) (vide supra) as input values. During the calculations, only the
2.2 Results and Discussion

![Graph showing calculated variation of $3J_{H(4')H(S')}$ and $3J_{H(4')H(S')}$ with the torsion angle $\phi$ with the Karplus equation.](image)

**Figure 2.4** Calculated variation of $3J_{H(4')H(S')}$ and $3J_{H(4')H(S')}$ with the torsion angle $\phi$. The Karplus equation described in ref 12 was used. Filled circles represent data points for the 3',5'-cyclic phosphites 4 and 5, and the 3',5'-cyclic phosphates 6 and 7 (Table 2.2), corresponding with $\phi = 180^\circ$. The data measured for the $P^V$-TBPs 2 and 3 (open circles) point towards a $\phi$ value of $= 200^\circ$, i.e. the $P^V$-TBP structure forces a rotation around the $C_4-C_5$ bond of approximately $20^\circ$, irrespective of (e,a) or (e,e) orientation of the 3',5'-dioxaphosphorinane ring.

The bond angles and torsion angles defining the TBP geometry were fixed. The resulting structures are depicted in Figure 2.5; the calculated heats of formation of 15-17 are -263.7, -262.6, and -259.8 kcal/mol, respectively. Thus, both (e,a) forms are almost equally stable, while the (e,e)-isomer is destabilized by approximately 3-4 kcal/mol.

Inspection of the resulting optimized conformations revealed that the 2'-deoxyribose ring remained almost unchanged in all three calculations. Most probably, the limited structural freedom of the 2'-deoxyribose ring can be attributed to the trans-fusion in our systems. The 3',5'-dioxaphosphorinane ring, on the other hand,
Figure 2.5 MNDO-optimized geometries for the $P^V$-TBP isomeric model systems, 15, 16 (both (e,a)), and 17 (e,e). Structures 15 and 16 have virtually identical heats of formation ($\Delta H_f = -263.7$ and $-262.6$ kcal/mol, respectively). The (e,e) system 17 is only slightly destabilized in comparison with 15 and 16 ($\Delta H_f = -259.8$ kcal/mol).

showed slight structural variations (Figure 2.6). For the (e,a)-isomers 15 and 16, it is found that the ring preferentially adopts a twist structure in which $O_5'$, $C_5'$, $C_4'$, and $O_3$, are approximately in the same plane; the P atom is bent towards the exo-face of this plane, while $C_3'$ is located on the endo-side. The dioxaphosphorinane ring of the (e,e) system 17 is flattened at the phosphorus-end, as is obvious from the low values of the torsion angles [$C_4'\cdot C_5'\cdot O_5\cdot P$] (-5.0° for 17, -31.8° and -27.3° for 15 and 16, respectively), and [$C_5\cdot O_5\cdot P\cdot O_3$] (19.8° for 17, 50.6° and 40.6° for 15 and 16, respectively) (Table 2.3).24 The dioxaphosphorinane ring in 17 can probably be best described as a half-chair (chaise-longue) in which $C_3'$ acts as the back.
2.2 Results and Discussion

Figure 2.6 Detailed view of the conformation of the $3',5'$-dioxaphosphorinane ring in the MNDO-optimized structures 15-17. For the (e,a) systems 15 and 16, a twist conformation is encountered, in which $O_5'$, $C_5'$, $C_4'$, and $O_3'$ are approximately in the same plane; $P$ and $C_3'$ are located on the exo and endo face of this plane, respectively. The (e,e)-isomer 17 shows a half-chair conformation of the $3',5'$-ring; $P$ resides approximately in the plane through $O_5'$, $C_5'$, $C_4'$, and $O_3'$; $C_3'$ is bent towards the endo face of this plane.

Table 2.3 Torsion angles describing the conformation of the $3',5'$-dioxaphosphorinane ring in the MNDO-optimized structures of the model systems 15-17

<table>
<thead>
<tr>
<th>Torsion Angle (°)</th>
<th>15 (e,a)$_1$</th>
<th>16 (e,a)$_2$</th>
<th>17 (e,e)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$[P-0_3'\cdot C_3'-C_4']$</td>
<td>-47.8</td>
<td>-54.2</td>
<td>-50.1</td>
</tr>
<tr>
<td>$[O_3'\cdot C_3'-C_4'-C_5']$</td>
<td>68.3</td>
<td>67.5</td>
<td>67.8</td>
</tr>
<tr>
<td>$[C_3'-C_4'-C_5'-O_5']$</td>
<td>-30.4</td>
<td>-29.5</td>
<td>-35.7</td>
</tr>
<tr>
<td>$[C_4'-C_5'-O_5'-P]$</td>
<td>-31.8</td>
<td>-27.3</td>
<td>-5.0</td>
</tr>
<tr>
<td>$[C_5'-O_5'-P-O_3']$</td>
<td>50.6</td>
<td>40.6</td>
<td>19.8</td>
</tr>
<tr>
<td>$[O_5'-P-O_3'-C_3']$</td>
<td>-3.3</td>
<td>6.4</td>
<td>11.0</td>
</tr>
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*Note the relatively low values of the torsion angles $[C_4'-C_5'-P-O_3']$ for 17 in comparison with 15 and 16 (see text).
2.3 Conclusions

The present experimental and theoretical data clearly show that activation of cAMP via formation of a P\textsuperscript{V}-TBP intermediate will induce a non-chair conformation of the 3',5'-dioxaphosphorinane ring, which is in complete agreement with the results of Bentruce et al.\textsuperscript{3} However an X-ray study on a P\textsuperscript{V}-compound containing a diequatorially located 1,3,2-dioxaphosphorinane ring reveals a chair conformation for the six-membered ring.\textsuperscript{15} Based on this experimental fact, Bentruce assumed that all diequatorially located dioxaphosphorinane rings will adopt a chair conformation, which is premature since an unsubstituted dioxaphosphorinane ring has other conformational preferences than our systems in which the six-membered ring is \textit{trans}-fused to a 2'-deoxyribose ring. Based on the similarity of the coupling constants for H\textsubscript{5'}, H\textsubscript{5''}, and H\textsubscript{4'} of P\textsuperscript{V}-TBP 3 and those of an (e,a) oriented dioxaphosphorinane ring, \textit{trans}-fused to a 2'-deoxyribose ring (like 2) which are definitely in a non-chair conformation, Bentruce concluded that the 3',5'-dioxaphosphorinane ring in P\textsuperscript{V}-TBP system 3 has to adopt an (e,a) orientation. Also this conclusion is premature, since nothing is known about the values of the coupling constants H\textsubscript{5'}, H\textsubscript{5''}, and H\textsubscript{4'} in a definitely diequatorially located dioxaphosphorinane ring which is \textit{trans}-fused to a 2'-deoxyribose ring. Furthermore, our experimental data reveal that formation of a P\textsuperscript{V}-TBP structure may lead to (e,a) or (e,e) orientation of the 3',5'-dioxaphosphorinane ring. Although the MNDO calculations show that an (e,a) orientation is slightly energetically favourable, it appears from the data on 3 that conformational transmission in one of the ligands will help to stabilize the (e,e) isomer.
2.4 Experimental

2.4.1 Material and Methods

The $^1$H NMR spectra were recorded on Bruker AM 600, AM 400 or AC 200 NMR spectrometers. Tetramethylsilane (TMS) was used as the internal standard for NMR samples. $^{31}$P NMR spectra were recorded at 162 or 81 MHz on the AM 400 or AC 200 instruments respectively, and referenced against 85% H$_3$PO$_4$ as external standard. $^{13}$C NMR spectra were recorded at 100.6 or 50.3 MHz on the AM 400 or AC 200 instruments respectively. For all column separations, we used Merck silica gel 60 (particle size 0.063-0.200 mm).

Pyridine was distilled from KOH pellets and dried on 4Å molecular sieves. $^1$H-Tetrazole was purified by sublimation. Reactions were routinely run in an inert atmosphere of dry nitrogen or dry argon. Prior to the cyclization reaction of thymine, the last traces of water were removed from the nucleoside via coevaporation with small portions of dry pyridine. Unless otherwise noted, reactions were run at ambient temperature.

2.4.2 Synthesis

Bis-(N,N-diisopropylamino)(2-methoxy-ethoxy)phosphine. 2-Methoxy-ethanol (79.8 g, 1.05 mol) was added dropwise to phosphorus trichloride (137.5 g, 1.00 mol) over 2 h, while stirring. The inner temperature of the reaction flask was kept between 20 and 30 °C, and the produced hydrochloric acid was absorbed in a gas trap containing a sodium bicarbonate solution. After distillation (bp 67 °C at 20 mm Hg), pure 2-methoxy-ethoxy-dichlorophosphine ($^{31}$P NMR (81 MHz, CDCl$_3$): δ 179.5 ppm) was obtained (101.9 g, 0.58 mol). This compound was added dropwise during 2 h to a solution of N,N-diisopropylamine (348.9 g, 3.45 mol) in 750 mL of dry ether at 0 °C, and the solution was stirred overnight at room temperature. Then, the ammonium salt was removed by filtration, and the solution was concentrated in vacuo. Pure bis-(N,N-diisopropylamino)(2-methoxy-ethoxy)phosphine was
obtained by distillation of the residue at 0.7 mm Hg (bp 105 °C) as a colourless liquid. Yield 126.4 g (41%); $^{31}$P NMR (81 MHz, CDCl$_3$): $\delta$ 127.6; $^1$H NMR (200 MHz, CDCl$_3$): $\delta$ 3.60 (8H, m, 4 H–C–N and 4 H–C–O), 3.38 (3H, s, OCH$_3$), 1.16 (24H, m, CH$_3$).

**Bis-(N,N-diisopropylamino)(1-butoxy)phosphine.** 1-Butanol (74.0 g, 1.00 mol) was added dropwise to phosphorus trichloride (137.5 g, 1.00 mol) over 5 h, while stirring. The inner temperature of the reaction flask was kept between 20 and 30 °C, and the produced hydrochloric acid was absorbed in a gas trap containing aqueous sodium bicarbonate. After distillation (bp 64 °C at 20 mm Hg) pure 1-butoxy-dichlorophosphine ($^{31}$P NMR (162 MHz, CDCl$_3$): $\delta$ 178.4 ppm) was obtained (69.9 g, 0.40 mol). This compound was added dropwise during 3 h to a solution of N,N-diisopropylamine (242.4 g, 2.40 mol) in 600 mL of dry ether at 0 °C and the solution was stirred overnight at room temperature. Then, the ammonium salt was removed by filtration, and the solution was concentrated in vacuo. Pure bis-(N,N-diisopropylamino)(1-butoxy)phosphine was obtained by distillation of the residue at 0.03 mm Hg (bp 80 °C) as a colourless liquid. Yield 25.8 g (9%); $^{31}$P NMR (162 MHz, CDCl$_3$): $\delta$ 127.0; $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 3.55 (4H, m, 4 H–C–N and 2 H–C–O), 1.58 (2H, m, CH$_2$), 1.42 (2H, m, CH$_2$), 1.18 (24H, m, CH$_3$), 0.93 (3H, t, CH$_3$).

**2-(2'-Methoxy-ethoxy)-1,3,2-dioxaphospholane.** Ethylene glycol (31.0 g, 0.50 mol) was added dropwise to phosphorus trichloride (68.8 g, 0.50 mol) over 2 h, while stirring. The inner temperature of the reaction flask was kept between 20 and 30 °C, and the produced hydrochloric acid was absorbed in a gas trap containing a sodium bicarbonate solution. After distillation (bp 60 °C at 30 mm Hg) pure 2-chloro-1,3,2-dioxaphospholane ($^{31}$P NMR (162 MHz, CDCl$_3$): $\delta$ 168.1 ppm) was obtained (30.6 g, 0.24 mol). This compound was added dropwise during 2 h to a solution of 2-methoxy-ethanol (18.4 g, 0.24 mol) and triethylamine (24.4 g, 0.24
2.4 Experimental

mol) in 250 mL of dry ether at 0 °C and the solution was stirred for 1h at room temperature. Then, the ammonium salt was removed by filtration, and the solution was concentrated in vacuo. Pure 2-(2'-methoxy-ethoxy)-1,3,2-dioxaphospholane was obtained by distillation of the residue at 0.6 mm Hg (bp 76 °C) as a colourless liquid. Yield 25.6 g (31%); $^{31}\text{P} \text{NMR (162 MHz, CDCl}_3\text{): } \delta 135.8; ~^{1}\text{H NMR (400 MHz, CDCl}_3\text{): } \delta 4.22 (2\text{H, m, 2H of the 5-ring}), 4.01 (2\text{H, m, 2H of the 5-ring}), 3.91 (2\text{H, m, H}_a), 3.52 (2\text{H, m, H}_b), 3.40 (3\text{H, s, OCH}_3)$. 

$3\beta$-(2'-Methoxy-ethoxy)-trans-2,4-dioxa-3-phosphabicyclo[4.3.0]-nonane. Bis-(N,N-diisopropylamino)(2-methoxy-ethoxy)phosphine (8.71 g, 28.4 mmol) was added to a solution of (1RS, 2SR)-2-hydroxycyclopentanemethanol (prepared as described by Penney and Belleau$^{21b,26}$ (3.00 g, 25.9 mmol) and 1H-tetrazole (3.62 g, 51.7 mmol) in 250 mL of dry pyridine, and the reaction mixture was stirred for about 1 h. Formation of the cyclic phosphate was evident from the $^{31}\text{P} \text{NMR spectrum ((pyridine/pyridine-}d_5\text{ 1:1): } \delta 3(\beta) 122.6 \text{ and } 3(\alpha) 128.4$). The $3(\beta)/3(\alpha)$ ratio was approximately 1:1. The mixture was concentrated in vacuo (at room temperature) and coevaporaled with toluene and dichloromethane. The obtained oil was diluted with 200 mL of ethyl acetate, resulting in sedimentation of 1H-tetrazole and ammonium salts. After filtration, the filtrate was concentrated in vacuo. Distillation of the residue at 0.01 mmHg (bp 80 °C) yielded 2.44 g of 3-(2'-methoxy-ethoxy)-trans-2,4-dioxa-3-phosphabicyclo[4.3.0]-nonane (60% $3(\beta)$ and 40% $3(\alpha)$). Almost pure $3\beta$-(2'-methoxy-ethoxy)-trans-2,4-dioxa-3-phosphabicyclo-[4.3.0]-nonane was obtained as a colourless liquid by chromatography on a silica gel column with ethyl acetate as eluent ($R_f$ 0.62). Yield 1.54 g (26%); $^{31}\text{P} \text{NMR (162 MHz, CDCl}_3\text{): } \delta 123.8 (3(\alpha) 130.0), 3(\beta)/3(\alpha)$ ratio approximately 98:2; $^{1}\text{H NMR (400 MHz, CDCl}_3\text{): } \delta 4.27 (1\text{H, m, H}_5\text{a or H}_5\text{b}), 4.12 (1\text{H, m, H}_1), 4.05 (1\text{H, m, H}_5\text{b or H}_5\text{a}), 3.90 (2\text{H, m, H}_5\text{)}, 3.58 (2\text{H, m, H}_8\text{)}, 3.40 (3\text{H, s, OCH}_3), 2.05-1.10 (7\text{H, m, H}_6\text{, H}_7\text{a, H}_7\text{b, H}_8\text{a, H}_8\text{b, H}_9\text{a, H}_9\text{b})$. $^{21b}$
cis-Thymidine 3',5'-Cyclic Methyl Phosphite (4). Bis-(N,N-diisopropylamino)methoxyphosphine (3.90 g, 14.9 mmol) was added to a suspension of thymidine (3.00 g, 12.4 mmol) and 1H-tetrazole (2.17 g, 31.0 mmol) in 300 mL of dry pyridine, and the reaction mixture was stirred for 2 h. Formation of the cyclic phosphite was evident from the $^{31}$P NMR spectrum ((pyridine/pyridine-$d_5$ 1:1): $\delta$ cis 122.9 and trans 129.0). The cis/trans ratio was approximately 2:3. The mixture was concentrated in vacuo (at room temperature) and coevaporated with toluene and dichloromethane. A white solid was obtained which was suspended in 400 mL of ethyl acetate (3 times). After sedimentation of 1H-tetrazole and ammonium salt, the clear upper layer was separated. The collected layers were concentrated in vacuo and coevaporated with dichloromethane. A white foam appeared which was chromatographed rapidly on a short silica gel column. First, impurities were eluted with dichloromethane, then with a gradient of dichloromethane/ethyl acetate (1:1 v/v $\rightarrow$ 1:4 v/v) as eluent, we obtained 4 as a white solid. Yield: 0.85 g (23%); Mp 127-129 °C; $R_f$ 0.42 (ethyl acetate); $^{31}$P NMR (81 MHz, CD$_2$Cl$_2$): $\delta$ 125.7 (trans 132.5), cis/trans ratio approximately 95:5; $^1$H NMR (600 MHz, CD$_2$Cl$_2$): $\delta$ 9.45 (1H, bs, NH), 7.07 (1H, q, H$_6$ of T), 6.16 (1H, dd, H$_4$), 4.48 (1H, m, H$_3$), 4.42 (1H, m, H$_5$), 4.28 (1H, m, H$_5''$), 3.64 (1H, m, H$_4$), 3.58 (3H, d, OCH$_3$, $^3$JPOCH=12.3 Hz), 2.45 (1H, m, H$_2''$), 2.33 (1H, m, H$_2''$), 1.94 (3H, d, CH$_3$ of T). $^{13}$C NMR (50.32 MHz, CD$_2$Cl$_2$): $\delta$ 164.6 (C$_2$ or C$_4$ of T), 151.0 (C$_4$ or C$_2$ of T), 135.8 (C$_6$ of T), 111.9 (C$_5$ of T), 82.3 (C$_1$), 75.4 (C$_4$'), $^3$JPOCC=7.2 Hz), 68.9 (C$_3$'), 66.2 (C$_5$', $^2$JPOC=3.1 Hz), 50.5 (OCH$_3$, $^2$JPOC=18.9 Hz), 36.3 (C$_2$'), 12.6 (CH$_3$ of T); Anal. Calcd for C$_{11}$H$_{15}$N$_2$O$_6$P: C, 43.71; H, 4.97; N, 9.27; Found: C, 43.78; H, 4.74; N, 9.97.

cis-Thymidine 3',5'-Cyclic 2-Methoxy-ethyl Phosphite (5). Bis-(N,N-diisopropylamino)(2-methoxy-ethoxy)phosphine (2.78 g, 9.1 mmol) was added to a suspension of thymidine (2.00 g, 8.3 mmol) and 1H-tetrazole (1.45 g, 20.7 mmol) in 200 mL of dry pyridine, and the reaction mixture was stirred for about 2 h.
Formation of the cyclic phosphite was evident from the $^{31}$P NMR spectrum ((pyridine/pyridine-$d_5$ 1:1): δ cis 123.1 and trans 129.8). The cis/trans ratio was approximately 1:1. The mixture was concentrated in vacuo (at room temperature) and coevaporated with toluene and dichloromethane. The obtained oil was diluted with 500 mL of ethyl acetate, resulting in sedimentation of 1H-tetrazole and ammonium salt. After filtration, the filtrate was concentrated in vacuo and chromatographed on a silica gel column with ethyl acetate/dichloromethane 1:1 v/v as eluent. A white solid of 5 was obtained in 33%; yield (0.95 g); Mp 51-55 °C; $R_f$ 0.32 (ethyl acetate); $^{31}$P NMR (81 MHz, CD$_2$Cl$_2$): δ 124.3 (trans 131.3), cis/trans ratio approximately 95:5; $^1$H NMR (600 MHz, CD$_2$Cl$_2$): δ 9.89 (1H, bs, NH), 7.11 (1H, q, H$_6$ of T), 6.19 (1H, dd, H$_4$), 4.55 (1H, m, H$_3$), 4.49 (1H, m, H$_5$), 4.28 (1H, m, H$_5$''), 3.97 (2H, m, H$_3$), 3.63 (1H, m, H$_4$), 3.60 (2H, m, H$_b$), 3.40 (3H, s, OCH$_3$), 2.46 (1H, m, H$_2$''), 2.32 (1H, m, H$_2$''), 1.93 (3H, d, CH$_3$ of T); $^{13}$C NMR (50.32 MHz, CD$_2$Cl$_2$): δ 164.6 (C$_2$ or C$_4$ of T), 151.0 (C$_4$ or C$_2$ of T), 135.8 (C$_6$ of T), 111.9 (C$_5$ of T), 82.2 (C$_1$), 75.4 (C$_4$'), $^3$J$_{POCC}$=6.9 Hz), 72.5 (C$_b$, $^3$J$_{POCC}$=5.3 Hz), 68.8 (C$_3$'), 66.3 (C$_5$'), $^2$J$_{POCC}$=3.5 Hz), 62.8 (C$_a$, $^2$J$_{POCC}$=16.6 Hz), 58.9 (OCH$_3$), 36.3 (C$_2$'), 12.6 (CH$_3$ of T); Anal. Calcd for C$_{13}$H$_{19}$N$_2$O$_7$P: C, 45.09; H, 5.49; N, 8.09; Found: C, 43.1; H, 5.8; N, 9.9.

**cis-Thymidine 3',5'-Cyclic Methyl Phosphate (6).**$^{20}$ cis-Thymidine 3',5'-cyclic methyl phosphite (4) (100 mg, 0.33 mmol) was dissolved in dichloromethane (30 mL) at -20 °C. Dichloromethane, saturated with NO$_2$/N$_2$O$_4$, was added until a greenish colour appeared. After complete conversion of compound 4 ($^{31}$P NMR (162 MHz, CH$_2$Cl$_2$/CD$_2$Cl$_2$ 1:1): δ -3.1 ppm) the mixture was evaporated under vacuo. A white foam appeared, which was chromatographed on a silica gel column with methanol/dichloromethane 8:92 v/v as eluent, yielding 57 mg (54%) of compound 6 as a white solid; Mp 97-102 °C; $R_f$ 0.23 (methanol/dichloromethane 8:92 v/v); $^{31}$P NMR (162 MHz, CD$_2$Cl$_2$): δ -3.2; $^1$H NMR (400 MHz, CD$_2$Cl$_2$): δ 9.70 (1H, bs, NH), 7.08 (1H, q, H$_6$ of T), 6.23 (1H, dd, H$_4$), 4.74 (1H, m, H$_3$),
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4.62 (1H, m, H\(_{5'}\)), 4.40 (1H, m, H\(_{5}\)), 3.96 (1H, m, H\(_{4}\)), 3.90 (3H, d, OCH\(_{3}\), \(^{3}J_{\text{POCH}}\)=11.2 Hz), 2.65 (1H, m, H\(_{2}\)), 2.54 (1H, m, H\(_{2}\)), 1.98 (3H, d, CH\(_{3}\) of T); \(^{13}\)C NMR (100.64 MHz, CD\(_{2}\)Cl\(_{2}\)): \(\delta\) 164.3 (C\(_{2}\) or C\(_{4}\) of T), 150.5 (C\(_{4}\) or C\(_{2}\) of T), 136.6 (C\(_{6}\) of T), 112.2 (C\(_{5}\) of T), 86.5 (C\(_{1}\)), 78.4 (C\(_{3}\)), \(^{2}J_{\text{POC}}\)=5.5 Hz), 74.2 (C\(_{4}\)), \(^{3}J_{\text{POCC}}\)=5.6 Hz), 69.9 (C\(_{5}\)), \(^{2}J_{\text{POC}}\)=8.9 Hz), 54.7 (OCH\(_{3}\)), 35.4 (C\(_{2}\)), \(^{3}J_{\text{POCC}}\)=8.3 Hz), 12.5 (CH\(_{3}\) of T); Anal. Calcd for C\(_{11}\)H\(_{15}\)N\(_{2}\)O\(_{7}\)P: C, 41.51; H, 4.72; N, 8.81; Found: C, 40.74; H, 5.02; N, 8.85.

cis-Thymidine 3',5'-Cyclic 2-Methoxy-ethyl Phosphate (7). cis-Thymidine 3',5'-cyclic 2-methoxy-ethyl phosphate (5) (500 mg, 1.45 mmol) was dissolved in dichloromethane (40 mL) at -20 °C. Dichloromethane, saturated with NO\(_{2}\)N\(_{2}\)O\(_{4}\), was added until a greenish colour appeared. After complete conversion of compound 5 (\(^{31}\)P NMR (81 MHz, CH\(_{2}\)Cl\(_{2}\)/CD\(_{2}\)Cl\(_{2}\) 1:1): \(\delta\) -4.4 ppm) the mixture was evaporated under vacuo. A white foam appeared, which was chromatographed on a silica gel column with methanol/dichloromethane 6:94 v/v as eluent, yielding 270 mg (52%) of compound 7 as a white solid; Mp 70-71 °C; R\(_{f}\) 0.22 (methanol/dichloromethane 6:94 v/v); \(^{31}\)P NMR (162 MHz, CD\(_{2}\)Cl\(_{2}\)): \(\delta\) -4.4; \(^{1}\)H NMR (400 MHz, CD\(_{2}\)Cl\(_{2}\)): \(\delta\) 9.45 (1H, bs, NH), 7.04 (1H, q, H\(_{6}\) of T), 6.27 (1H, dd, H\(_{4}\)), 4.68 (1H, m, H\(_{3}\)), 4.59 (1H, m, H\(_{5}\)), 4.46 (1H, m, H\(_{5}\)), 4.28 (2H, m, H\(_{a}\)), 3.93 (1H, m, H\(_{4}\)), 3.68 (2H, m, H\(_{b}\)), 3.41 (3H, s, OCH\(_{3}\)), 2.62 (1H, m, H\(_{2}\)), 2.50 (1H, m, H\(_{2}\)), 1.93 (3H, d, CH\(_{3}\) of T); \(^{13}\)C NMR (100.64 MHz, CD\(_{2}\)Cl\(_{2}\)): \(\delta\) 163.9 (C\(_{2}\) or C\(_{4}\) of T), 150.4 (C\(_{4}\) or C\(_{2}\) of T), 136.2 (C\(_{6}\) of T), 112.3 (C\(_{5}\) of T), 85.9 (C\(_{1}\)), 78.3 (C\(_{3}\)), \(^{2}J_{\text{POC}}\)=5.4 Hz), 74.3 (C\(_{4}\)), \(^{3}J_{\text{POCC}}\)=5.5 Hz), 71.5 (C\(_{b}\)), \(^{3}J_{\text{POCC}}\)=6.0 Hz), 70.0 (C\(_{5}\)), \(^{2}J_{\text{POC}}\)=9.0 Hz), 67.1 (C\(_{a}\)), \(^{2}J_{\text{POC}}\)=5.4 Hz), 59.1 (OCH\(_{3}\)), 35.5 (C\(_{2}\)), \(^{3}J_{\text{POCC}}\)=8.3 Hz), 12.6 (CH\(_{3}\) of T); Anal. Calcd for C\(_{13}\)H\(_{19}\)N\(_{2}\)O\(_{8}\)P: C, 43.09; H, 5.25; N, 7.73; Found: C, 42.2; H, 5.4; N, 9.4.

cis-Thymidine 3',5'-Cyclic 1-Butyl Phosphite. Bis-(N,N-diisopropylamino) (1-butoxy)phosphine (2.77 g, 9.1 mmol) was added to a suspension of thymidine
(2.00 g, 8.3 mmol) and 1H-tetrazole (1.45 g, 20.7 mmol) in 200 mL of dry pyridine, and the reaction mixture was stirred for about 2 h. Formation of the cyclic phosphite was evident from the 31P NMR spectrum ((pyridine/pyridine-d5 1:1): δ cis 122.8 and trans 129.7). The cis/trans ratio was approximately 1:1. The mixture was concentrated in vacuo (at room temperature) and coevaporated with toluene and dichloromethane. The obtained oil was diluted with 450 mL of ethyl acetate, resulting in sedimentation of 1H-tetrazole and ammonium salt. After filtration, the filtrate was concentrated in vacuo and chromatographed on a silica gel column with ethyl acetate/dichloromethane 1:1 v/v as eluent. An oil of cis thymidine 3',5'-cyclic 1-butyl phosphite was obtained in 21% yield (0.59 g); Rf 0.40 (ethylacetate/dichloromethane 1:1 v/v). 31P NMR (162 MHz, CD2Cl2): δ 124.5 (trans 131.6), cis/trans ratio approximately 93:7. 1H NMR (400 MHz, CD2Cl2): δ 9.78 (1H, bs, NH), 7.07 (1H, q, H6 of T), 6.18 (1H, dd, H4), 4.47 (1H, m, H3), 4.43 (1H, m, H5), 4.28 (1H, m, H5,), 3.84 (2H, m, H2), 3.63 (1H, m, H4), 2.43 (1H, m, H2, 2.42 (1H, m, H2), 1.93 (3H, d, CH3 of T), 1.67 (2H, m, H6), 1.43 (2H, m, H3), 0.97 (3H, t, H4); 13C NMR (100.64 MHz, CD2Cl2): δ 164.3 (C2 or C4 of T), 150.8 (C4 or C2 of T), 135.6 (C6 of T), 111.9 (C5 of T), 82.3 (C1), 75.5 (C4, 3JPOCC =6.9 Hz), 68.9 (C3), 66.2 (C5, 2JPOC=3.6 Hz), 63.7 (C6, 2JPOC=18.1 Hz), 36.5 (C2), 33.5(C3, 3JPOCC =5.4 Hz), 19.4 (C7), 13.8 (C8), 12.7 (CH3 of T).

**cis-Thymidine 3',5'-Cyclic 1-Butyl Phosphate (10a).** cis-Thymidine 3',5'-cyclic 1-butyl phosphite (250 mg, 0.73 mmol) was dissolved in dichloromethane (30 mL) at -20 °C. Dichloromethane, saturated with NO2/N2O4, was added until a greenish colour appeared. After complete conversion of cis-thymidine 3',5'-cyclic 1-butyl phosphite (31P NMR (162 MHz, CH2Cl2/CD2Cl2 1:1): δ -4.3 ppm) the mixture was evaporated under vacuo. A white foam appeared, which was chromatographed on a silica gel column with methanol/dichloromethane 5:95 v/v as eluent, yielding 100 mg (38%) of compound 11 as a white solid; Rf 0.23 (methanol/dichloromethane 5:95 v/v); 31P NMR (162 MHz, CD2Cl2): δ -4.3; 1H NMR
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(400 MHz, CD\textsubscript{2}Cl\textsubscript{2}): δ 9.30 (1H, bs, NH), 7.02 (1H, q, H\textsubscript{6} of T), 6.18 (1H, dd, H\textsubscript{4}), 4.68 (1H, m, H\textsubscript{3}), 4.58 (1H, m, H\textsubscript{5}), 4.35 (1H, m, H\textsubscript{5}), 4.16 (2H, m, H\textsubscript{4}), 3.91 (1H, m, H\textsubscript{4}), 2.61 (1H, m, H\textsubscript{2}), 2.54 (1H, m, H\textsubscript{2}), 1.93 (3H, d, CH\textsubscript{3} of T), 1.75 (2H, m, H\textsubscript{6}), 1.47 (2H, m, H\textsubscript{6}), 0.98 (3H, t, H\textsubscript{6}); \textsuperscript{13}C NMR (100.64 MHz, CD\textsubscript{2}Cl\textsubscript{2}): δ 164.3 (C\textsubscript{2} or C\textsubscript{4} of T), 150.5 (C\textsubscript{4} or C\textsubscript{2} of T), 136.6 (C\textsubscript{6} of T), 112.1 (C\textsubscript{5} of T), 86.4 (C\textsubscript{1}), 78.3 (C\textsubscript{3}, \textsuperscript{2}J\text{P\textsubscript{OC}}=5.5 Hz), 74.3 (C\textsubscript{4}, \textsuperscript{3}J\text{P\textsubscript{OC}}=7.7 Hz), 69.8 (C\textsubscript{5}, \textsuperscript{2}J\text{P\textsubscript{OC}}=9.0 Hz), 68.4 (C\textsubscript{6}, \textsuperscript{2}J\text{P\textsubscript{OC}}=5.9 Hz), 35.4 (C\textsubscript{2}, \textsuperscript{3}J\text{P\textsubscript{OC}}=8.4 Hz), 32.5 (C\textsubscript{b}, \textsuperscript{3}J\text{P\textsubscript{OC}}=9.9 Hz), 19.1 (C\textsubscript{2}), 13.7 (C\textsubscript{4}), 12.5 (CH\textsubscript{3} of T).

Pentacoordinated compounds 2, 3, and 10-12. In order to avoid decomposition during handling and purification, we prepared the P\textsuperscript{V} compounds 2, 3 and 10-12 \textit{in situ} in NMR tubes. These syntheses were carried out by addition of one equivalent of tetrachloro-1,2-benzoquinone to the corresponding phosphites, dissolved in CD\textsubscript{2}Cl\textsubscript{2} at -80 °C. The NMR tubes were flushed with dry argon and sealed. After 1 h, the NMR samples were transferred into the NMR instrument, which had been stabilized at constant temperature (20 °C for compounds 2, 11 and 12; -41 °C for compounds 3 and 10), and the \textsuperscript{1}H- and \textsuperscript{31}P-NMR spectra were recorded. The identity of the P\textsuperscript{V}-TBP systems was established on the basis of \textsuperscript{1}H- and \textsuperscript{31}P-NMR spectroscopy, which showed >95% purity in each case.

**Compound 2.** \textsuperscript{31}P NMR (81 MHz, CD\textsubscript{2}Cl\textsubscript{2}, 20 °C): δ -43.3; \textsuperscript{1}H NMR (600 MHz, CD\textsubscript{2}Cl\textsubscript{2}, 20 °C): δ 9.00 (1H, bs, NH), 7.05 (1H, q, H\textsubscript{6} of T), 6.17 (1H, dd, H\textsubscript{4}), 4.76 (1H, m, H\textsubscript{3}), 4.73 (1H, m, H\textsubscript{5}), 4.20 (1H, m, H\textsubscript{5}), 3.98 (1H, m, H\textsubscript{4}), 3.93 (3H, d, OCH\textsubscript{3}, \textsuperscript{3}J\text{P\textsubscript{OC}}=14.3 Hz), 2.48 (1H, m, H\textsubscript{2}), 2.44 (1H, m, H\textsubscript{2}), 1.93 (3H, d, CH\textsubscript{3} of T).

**Compound 3.** \textsuperscript{31}P NMR (162 MHz, CD\textsubscript{2}Cl\textsubscript{2}, -41 °C): δ -44.7; \textsuperscript{1}H NMR (400 MHz, CD\textsubscript{2}Cl\textsubscript{2}, -41 °C): δ 10.29 (1H, bs, NH), 7.12 (1H, q, H\textsubscript{6} of T), 6.31 (1H, dd, H\textsubscript{4}), 4.82 (1H, m, H\textsubscript{3}), 4.75 (1H, m, H\textsubscript{5}), 4.53-4.32 (2H, m, H\textsubscript{4}), 4.23 (1H, m, H\textsubscript{5}), 4.00 (1H, m, H\textsubscript{4}), 3.74-3.55 (2H, m, H\textsubscript{6}), 3.38 (3H, s, OCH\textsubscript{3}), 2.53 (1H, m, H\textsubscript{2}), 2.43 (1H, m, H\textsubscript{2}), 1.96 (3H, d, CH\textsubscript{3} of T).
Compound 10. $^{31}$P NMR (162 MHz, CD$_2$Cl$_2$, -41 °C): δ -44.7; $^1$H NMR (400 MHz, CD$_2$Cl$_2$, -41 °C): δ 10.84 (1H, bs, NH), 7.12 (1H, q, H$_6$ of T), 6.30 (1H, dd, H$_4$), 4.78 (1H, m, H$_3$), 4.74 (1H, m, H$_5$), 4.28 (2H, m, H$_2$), 4.20 (1H, m, H$_5$), 4.03 (1H, m, H$_4$), 2.52 (1H, m, H$_2$), 2.44 (1H, m, H$_2$), 1.97 (3H, d, CH$_3$ of T), 1.67 (2H, m, H$_b$), 1.39 (2H, m, H$_c$), 0.96 (3H, t, H$_d$).

Compound 11. $^{31}$P NMR (162 MHz, CD$_2$Cl$_2$, 20 °C): δ -46.3; $^1$H NMR (400 MHz, CD$_2$Cl$_2$): δ 4.58 (1H, m, H$_5$), 4.30 (3H, m, H$_1$ and H$_6$), 3.96 (1H, m, H$_5$), 3.56 (2H, m, H$_b$), 3.36 (3H, s, OCH$_3$), 2.25-1.20 (7H, m, H$_6$, H$_7$a, H$_7$b, H$_8$a, H$_8$b, H$_9$a, H$_9$b).

Compound 12. $^{31}$P NMR (162 MHz, CD$_2$Cl$_2$, 20 °C): δ -22.6; $^1$H NMR (400 MHz, CD$_2$Cl$_2$, 20 °C): δ 4.20 (2H, m, H$_b$), 4.15 (4H, m, 4H of the 5-ring), 3.52 (2H, m, H$_b$), 3.30 (3H, s, OCH$_3$).

References and Notes


5. Five-membered dioxaphospholene rings have a pronounced preference for (e,a) location in a $P^V$-TBP. The difference in ring strain between (e,a) and (e,e) location amounts to approximately 21 kcal/mol (Holmes, R.R. J. Am. Chem. Soc. 1978, 100, 433; Holmes, R.R. Pentacoordinated Phosphorus; American Chemical Society: Washington, D.C., 1980; Vol I, II; ACS Monograph No. 175, 176). Nevertheless, diequatorial location of the dioxaphospholene ring may occur as intermediate during pseudorotation around pentacoordinated phosphorus (Koole, L.H.; van der Hofstad, W.J.M.; Buck, H.M. J. Org. Chem. 1985, 50, 4381).


13. Both the dioxaphospholene ring and the dioxaphospholane rings in 12 display a marked preference for (e,a) orientation (ref. 5). The O–CH$_2$–CH$_2$–OMe probe acts as the pivot pseudorotation of 12 (compare the methoxy group in 2).

14. Our observation of conformational transmission for 3 and 11 provides indirect evidence for (e,e) location of the 3',5'-dioxaphosphorinane ring. In fact, explicit determination of the molecular conformation of 3 or 11 would require an X-ray diffraction study. Some additional indication concerning the possibility of an (e,e) orientation of the 3',5'-ring follows by comparing the proton-phosphorus
couplings of the probe fragment \( J_{PH(1)} \), \( J_{PH(2)} \) (Figure 2.2)) in 3 with those of compound 12. (3: \( J_{PH(1)} = 6.8 \text{ Hz} \), \( J_{PH(2)} = 2.6 \text{ Hz} \); 12: \( J_{PH(1)} = J_{PH(2)} = 10.7 \text{ Hz} \)). The reduced proton-phosphorus coupling constants of 3 are in agreement with a dynamic equilibrium between an axial and equatorial orientation of the O–CH\(_2\)–CH\(_2\)–OMe fragment.\(^{10}\) From a previous study on conformational transmission in P\(^V\)-TBP compounds is known that the O–CH\(_2\)–CH\(_2\)–OMe fragment in an axial location results in a maximum \( \alpha_{\text{trans}} \) population of approximately 50%, which is close to the value of 51% found for compound 3.\(^9\) Furthermore, we wish to point out that the occurrence of conformational transmission appears to be an exclusive feature of the axial sites in the P\(^V\)-TBP, as based on all P\(^V\) model compounds studied so far.\(^4\),\(^9\) For these reasons, our conclusion that an (e,a) and an (e,e) P\(^V\)-TBP intermediate can be formed during the activation of cAMP, appears to be justified.


24. A chaise-longue conformation of the 3',5'-dioxaphosphorinane ring in the case of (e,e)-orientation in the P^V^-TBP was also predicted by Yu and Bentrude. See ref. 3b.


A $^{31}$P NMR Stereochemical and Kinetic Study of the Alkaline Hydrolysis of cis-Nucleoside 3',5'-Cyclic Aryl [18O]Monophosphates and Unlabelled Analogues*

Abstract

The alkaline hydrolysis of the P-chiral cis-nucleoside 3',5'-cyclic aryl $^{18}$O monophosphates 4a-c and of the unlabelled analogues 3a-e was studied. Hydrolysis of the $^{18}$O-labelled phosphate triesters 4a-c yielded three products: 3',5'-cyclic $^{18}$O phosphat diester, 5'-acyclic aryl $^{18}$O phosphate diester and 3'-acyclic aryl $^{18}$O phosphate diester. The stereochernistry of the formation of the 3',5'-cyclic $^{18}$O phosphat diester was determined by means of methylating the hydrolysis products with methyl iodide. The formation of the 3',5'-cyclic $^{18}$O phosphat diester during hydrolysis of compounds 4a and 4e proceeds with 17% inversion of configuration at phosphorus, whereas 40% inversion is found during hydrolysis of 4b. Inversion of configuration indicates the existence of a P$_v$-T8P with a diequatorial located dioxaphosphorinane ring. Retention of configuration (83% for 4a and 4e and 60% for 4b) can be explained in terms of Berry pseudorotation. The formation of the 5'-acyclic aryl $^{18}$O phosphat diester during hydrolysis of compounds 4a and 4e proceeds with about 50% inversion of configuration at phosphorus whereas formation of the 3'-acyclic aryl $^{18}$O phosphat diester proceeds with an inversion/retention ratio of 88:12 or 12:88 for 4a and 79:21 or 21:79 for 4e. It is clear that Berry pseudorotation takes place during hydrolysis of the 3',5'-cyclic phosphat triesters 4a-c. This is in contrast with earlier hydrolysis studies on 3',5'-cyclic phosphate diesters proceeding without Berry pseudorotation, leading to complete inversion of configuration at phosphorus. Because of the very small amounts of 3'- and 5'-acyclic aryl $^{18}$O phosphat diesters formed during the hydrolysis reaction of compound 4b, the stereochemistry could not be determined. The hydrolysis reactions, which have been studied on the unlabelled compounds 3a-e, obey second order kinetics. Changing the ribose ring to a deoxyribose ring or changing the adenine base to thymine in the 3',5'-cyclic phosphate triester does not dramatically influence the second order reaction rate constant. However, the nature of the P-OR substituent significantly influences the reaction rate. The 3',5'-cyclic phosphate triester with p-nitrophenoxy as substituent hydrolyzes approximately 18 times ($T = 294 K$) faster than the corresponding triester with phenoxy as substituent and yields more 3',5'-cyclic phosphate-diester.

*This chapter is based on:
3.1 Introduction

Cyclic adenosine 3',5'-monophosphate (cAMP) is known to be an important molecule which regulates a wide variety of biochemical processes (see chapter 1).

For example, cAMP acts as a mediator of hormone action and as a modulator of enzymatic activity. cAMP is synthesized from adenosine triphosphate by the action of adenylate cyclase. Breakdown of cAMP is catalyzed by phosphodiesterase, which hydrolyzes cAMP into 5'-adenosine monophosphate (5'-AMP). Both processes result in maintaining a steady state intracellular concentration of cAMP. The structural requirements for the binding of cAMP to the regulatory subunit of protein kinases as well as to phosphodiesterases have been investigated in detail.¹

Besides structure-activity studies, numerous studies have been performed in order to investigate the stereochemistry of the enzymatic reactions. It was found that hydrolysis of the \( R_p \) diastereoisomer of 2'-deoxyadenosine 3',5'-cyclic \([^{17}O,^{18}O]\)-monophosphate \( \left( R_p \left[ ^{17}O,^{18}O \right] cdAMP \right) \) by cAMP phosphodiesterases takes place.
3.1 Introduction

with inversion of configuration at phosphorus.² Non-enzymatic hydrolysis of \( R_p \) \([^{17}O,^{18}O]cAMP\) at 100 °C and in a barium hydroxide solution (0.2 M), yields a 4:1 mixture of the \( S_p \) diastereoisomer of 3'-deoxyadenosine \([^{16}O,^{17}O,^{18}O]\) monophosphate \((3'-[^{16}O,^{17}O,^{18}O]dAMP)\) and the \( R_p \) diastereoisomer of 5'-[\(^{16}O,^{17}O,^{18}O]\)dAMP³ Both products are formed with inversion of configuration at phosphorus. Under enzymatic conditions, cAMP is converted exclusively to 5'-AMP by 3',5'-cyclic-nucleotide phosphodiesterase. It has been suggested that during the interaction between the enzyme and cAMP, the electrostatic negative charge of the phosphoryl oxygens of cAMP is shielded by positively charged amino acid residues or bivalent metal ions.⁴ In order to mimic this shielding effect, the non-enzymatic hydrolysis of cyclic aryl phosphate triesters has been studied in \( H_2^{18}O \) (e.g. A and B) or \( H_2^{17}O \) (e.g. C and D). These compounds can be regarded as models for the interaction of cAMP with the enzyme. The main product formed during hydrolysis of the cyclic aryl phosphate triesters A-D is a cyclic phosphate diester, which is formed both with inversion and retention of configuration at phosphorus.⁵ ⁶ However, the nature of the aryl substituent determines the retention/inversion ratio.

In this chapter the results are reported on the alkaline non-enzymatic hydrolysis of the chiral \(^{18}O\)-labelled 3’,5’-cyclic nucleoside phosphate triesters 4a-c and the unlabelled \((^{16}O)\) counterparts 3a-c. These compounds are more realistic model systems for cAMP than compounds A-D. Some structural changes with respect to cAMP are made. In compounds 3a, 4a, 3b, and 4b a thymine base is used, while a
β-D-2'-deoxyribose ring is used instead of the β-D-ribose ring. Compounds 3c and 4c may be regarded as much more realistic models since they contain the normal adenine base, as well as an oxygen substituent on C2'. The 2'-OH group of cAMP is blocked by a methyl group in order to prevent possible side reactions.

3.2 Results and Discussion

3.2.1 Synthesis

Scheme 3.1 illustrates the method of preparation of adequate quantities of 1a,b. The trans epimers (relationship of substituent Me₂N at P and the base (A or B=T X=H
B=A X=OMe

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<th>X</th>
<th>R</th>
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<td>T</td>
<td>H</td>
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</tr>
<tr>
<td>2b</td>
<td>T</td>
<td>H</td>
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<td>3b</td>
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<tr>
<td>3c</td>
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<td>H</td>
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<td>T</td>
<td>H</td>
<td>p-NO₂C₆H₄</td>
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<tr>
<td>4c</td>
<td>A</td>
<td>OMe</td>
<td>C₆H₅</td>
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</table>

Scheme 3.1 Formation of ¹⁸O-labelled phosphate triesters 4a-c.

T) are formed predominantly. Compound 1a was obtained by chromatographic purification of a mixture containing the cis/trans epimers (yield 47% cis/trans ratio 2:98). Compound 1b was only purified by filtration of the reaction mixture. The
filtrate, which is approximately 95% pure, was used without further purification. The cis-3',5'-cyclic phosphate triesters 2a,b were prepared through reaction of 1a with phenol or p-nitrophenol, respectively. After washing with a sodium carbonate solution to remove the excess of phenols, the phosphites 2a,b were isolated as pure compounds (yields 44% and 21%, respectively). Compound 2c was formed in a reaction of 1b with phenol. After washing with a sodium hydroxide solution to remove the phenol, 2c was isolated as a white solid, which was approximately 90% pure (yield 47%).

Compounds 3a-c were obtained through stereospecific oxidation of the cis-3',5'-cyclic phosphate triesters 2a,b with NO2/N2O4 and 2c with tert-butyl hydroperoxide. These oxidations proceed with retention of the configuration at phosphorus. After oxidation, compounds 3a,c are obtained as white solids, whereas 3b is isolated as a light yellow-brown solid. Compound 3a is obtained by recrystallization from ethyl acetate, 3b is isolated by evaporation of dichloromethane and 3c is obtained by means of column chromatography.

Oxidation of 2a-c by H218O/I2, which is also known to proceed with retention of phosphorus configuration, yielded 18O-labelled cis-3',5'-cyclic phosphate triesters 4a-c.

3.2.2 Hydrolysis of 3a-c

To investigate the kinetics and product distribution of the hydrolyzes of the 3',5'-cyclic phosphate triesters, the unlabelled analogues 3a-c are examined first. The alkaline hydrolysis of compounds 3a-c is assumed to follow second order kinetics (first order in the cyclic phosphate triester and OH− concentration). The hydrolysis reactions were carried out by adding an exact amount (in the range of 3-5 equiv) of a sodium hydroxide solution to a solution of the cyclic phosphate triesters 3a-c in D2O/1,4-dioxane-d8 (2:3 or 3:7 v/v). This mixture was chosen to ensure the solubility of the cyclic phosphate triesters 3a-c and the formed phosphate diesters. Upon hydrolysis of the phosphate triesters 3a and 3b, 1 equivalent of OH−
is consumed instantaneously by the relatively acidic H₃ proton of the thymine base (pKₐ = 9.7)₁². Compared with thymine, the adenine of 3c contains no acidic protons. During hydrolysis three products are formed: one 3',5'-cyclic phosphatediester and two acyclic phosphate diesters (5'- and 3'-phosphate diester) (Scheme 3.2). As an example, Figure 3.1 shows the ³¹P NMR spectra at five different reaction times during hydrolysis of 3a. Formation of the 3',5'-cyclic phosphate diester is related to expulsion of phenol (3a and 3c) or p-nitrophenol(3b). Due to the acidic character of the phenols (pKₐ = 10.0 for phenol and 7.2 for p-nitrophenol), an additional equivalent of OH⁻ is consumed in order to form phenoxide or p-nitrophenoxide from the corresponding phenols (Scheme 3.2). During the formation of acyclic 3'- and 5'-phosphate diesters only alkoxides were expelled, without consuming additional amounts of OH⁻. Because of the non-equivalent consumption of OH⁻ for the formation of cyclic and acyclic phosphate diesters, the simple

Scheme 3.2 Hydrolysis of compounds 3a-c.
3.2 Results and Discussion

Figure 3.1 $^{31}$P NMR spectra measured at different reaction times during alkaline hydrolysis of compound 3a at 294 K in $D_2O/1,4$-dioxane-$d_8$ (2:3 v/v). Evidently, three different products are formed (3',5'-cyclic phosphate diester, 5'-phenyl phosphate diester and 3'-phenyl phosphate diester). The reaction times of the spectra 1-5 are: 5, 10, 18, 27, and 60 min, respectively.

second order rate equation has been modified into a more complex equation (see Appendix).

The results on the hydrolysis of 3a-c are visualized in Figure 3.2 and summarized in Table 3.1. The observed straight lines from Figure 3.2 agree with the assumed second order kinetics. The distribution of the reaction products and the overall second order rate constant $k_{obs}$ (=$k_a + k_c$) (Appendix) are slightly dependent on the base (A or T), but clearly dependent on the aryl substituent. The ligand $p$-nitrophenoxy is a better leaving group than phenoxy, resulting in faster hydrolysis of the 3',5'-cyclic phosphate triester and in formation of relatively large amounts of cyclic phosphate diester.

3.2.3 Hydrolysis of $^{18}$O-Labelled 3',5'-Cyclic Phosphate Triesters 4a-c

Hydrolysis reactions of these compounds were studied to determine the stereochemical aspects of the reaction. Alkaline hydrolysis is carried out by adding an exact amount (in the range of 3-5 equiv) of a sodium hydroxide solution to a
Figure 3.2 Visualization of the kinetics of the hydrolysis of compounds 3a (△), 3b (●) and 3c (○). The plots are obtained by using the second order reaction equation (8) described in the appendix. The reaction conditions are described in the Experimental Section. Evidently, the measuring points fit perfectly with the straight lines, indicating that second order kinetics is very plausible (correlation coefficients: 0.995 (△); 0.998 (●); 0.998 (○)).
Table 3.1 Kinetic parameters for the hydrolysis of 3a-3c at 294 K.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Initial concentrations (M)</th>
<th>Rate constants (M⁻¹·s⁻¹)</th>
<th>r²</th>
<th>Product distribution (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10²[P]₀</td>
<td>10²[OH⁻]₀</td>
<td>10³kₐ</td>
<td>10³kₐ</td>
</tr>
<tr>
<td>3aᵃ</td>
<td>5.21</td>
<td>15.6</td>
<td>9</td>
<td>4</td>
</tr>
<tr>
<td>3bᵇ</td>
<td>0.0032</td>
<td>5.05</td>
<td>160</td>
<td>32</td>
</tr>
<tr>
<td>3cᵃ</td>
<td>4.77</td>
<td>14.3</td>
<td>21</td>
<td>10</td>
</tr>
</tbody>
</table>

ᵃKinetics were performed in D₂O/1,4-dioxane-d₈ 3:7 (v/v) and followed by means of ³¹P NMR spectroscopy.ᵇKinetics were performed in D₂O/1,4-dioxane-d₈ 2:3 (v/v) and monitored by means of UV/VIS spectroscopy at 405 nm. ³Estimated errors ± 5%. ⁴Estimated errors ± 20%. ⁵Correlation coefficient. ⁶The product distribution is based on determining the p-nitrophenoxide concentration as a function of time by means of UV/VIS spectroscopy. The p-nitrophenoxide is formed simultaneously with the 3',5'-cyclic phosphate diester. Therefore at any time, the p-nitrophenoxide concentration is always equal to the 3',5'-cyclic phosphate diester. After a long time (approximately 1 h) the concentration of p-nitrophenoxide reaches a constant value. The maximal p-nitrophenoxide concentration, which can be reached is equal to the initial concentration of the cis 3',5'-cyclic p-nitrophenyl phosphate triester (assuming 100% formation of 3',5'-cyclic phosphate diester). The sum of the 3'- and 5'-acyclic phosphate diesters is equal to the difference between the theoretical p-nitrophenoxide concentration, assuming 100% formation of the 3',5'-cyclic phosphate diester, and the observed p-nitrophenoxide concentration, when reaching a constant value. The individual 3'- and 5'-acyclic phosphate diesters are assumed to be formed in equal quantity, which is based on ³¹P NMR spectra. ³¹P NMR spectroscopy revealed a different product distribution (cyclic/3'/5' 90:5:5) compared to UV/VIS spectroscopy.
solution of the cyclic phosphate triesters 4a, 4b, or 4c in D$_2$O/1,4-dioxane-d$_8$ (4a, 2:3 v/v; 4b and 4c, 3:7 v/v). After being mixed the initial concentration of the cyclic phosphate triesters is approximately 0.05 M. The hydrolysis reaction of 4a and 4c is complete within about 0.5-1 h, while complete conversion of 4b has been obtained within 5-10 minutes. Figure 3.3 shows the $^{31}$P NMR spectrum of the products, which have been obtained after complete hydrolysis of 4a (18O/16O 92:8). Obviously, three different products were derived (3',5'-cyclic phosphate diester, acyclic 3'-phosphate diester, and acyclic 5'-phosphate diester), which were assigned by comparing the hydrolysis of unlabelled 3a.

The small peaks represent the unlabelled analogues of the reaction products. The 18O-induced upfield shift was expected from earlier studies.$^{5,14}$ Integration of the [18O]- and [16O]-phosphate diester peaks revealed a ratio of approximately 92:8. This ratio is identical to the 16O/18O ratio in the 3',5'-cyclic phosphate triester. It is therefore concluded that no exchange of the oxygen (16O) from the solvent with the phosphate di(tri)ester oxygen (18O) has occurred under the reaction conditions. Previous studies showed that hydrolysis of cdAMP proceeds with exclusive cleavage of the P-O bond$^3$ without breaking the C-O bond and that introduction of 18O in a cyclic phosphate triester proceeds in a stereospecific and regiospecific way (no
exchange between $^{18}$O and $^{16}$O in the phosphate triester).\textsuperscript{10a} The hydrolysis of 4b results predominantly in formation of the 3',5'-cyclic phosphate diester (90-93%), whereas the hydrolysis of 4c is closely related to that of 4a. Due to the chirality of the 3',5'-cyclic and the 3'- and 5'-acyclic phosphate diesters, introduced by the $^{18}$O-label in the 3',5'-cyclic phosphate triester, each [$^{18}$O]phosphate diester exists as two diastereoisomers. However, in the $^{31}$P NMR spectrum these diastereoisomers are not detected separately, due to the delocalization of the negative charge on the phosphoryl oxygens. Therefore, the sodium counterion of the phosphate diesters, formed during hydrolysis, has been exchanged for potassium, using Dowex-K$^+$,\textsuperscript{15} and the potassium salts have been methylated by methyl iodide in dimethyl sulfoxide-$d_6$ solution and in the presence of 18-crown-6, according to the procedure described by Lowe et al.\textsuperscript{3,10a,16}

3.2.3.1 Stereochemical Analysis of the 3',5'-Cyclic Methyl Phosphate Triesters

Figure 3.4A shows the $^{31}$P NMR spectrum of the methyl phosphate triesters, which have been derived after hydrolysis of 4a and methylation of the hydrolysis products. The two patterns, each consisting of three resonances (peaks 1, 2, and 3 and 10, 11, and 12), located at -3.0 and -4.3 ppm, are assigned to be the trans and cis 3',5'-cyclic methyl phosphate triesters, respectively, since it has been found that cis (OMe pseudoaxial) phosphate triesters of substituted 3',5'-cyclic phosphate triesters resonate at ca. 1-3 ppm upfield relative to the trans (OMe pseudo equatorial) derivatives.\textsuperscript{17,8c} This observation of a large $^{31}$P chemical shift difference is due to the relatively large difference in geometry of the diastereoisomers at the phosphorus centre (OMe cis or trans). Thus, the diastereoisomeric 3',5'-cyclic methyl phosphate triesters do not have to be physically separated. The three low field resonances (peaks 1, 2, and 3) of the 3',5'-cyclic methyl phosphate triesters are due to the $^{18}$O-perturbations on the $^{31}$P NMR resonances. If $^{18}$O is singly bonded to phosphorus, it causes a smaller upfield isotope shift relative to $^{16}$O, than when doubly bonded to phosphorus (ca. 0.02 and 0.04 ppm, respectively).\textsuperscript{5,14}
Figure 3.4 Expansions of the $^{31}$P NMR spectrum of the methylated hydrolysis products of 4a (A), 4b (B), and 4c (C) in DMSO-$d_6$. See text for assignment of the peaks.

Therefore, the $^{31}$P resonance of the trans 3',5'-cyclic methyl phosphate triester 5 trans\(^1\) (compound 5 in a trans configuration and one P–$^{18}$O bond) (peak 2) will be located more downfield than that of the trans 3',5'-cyclic methyl phosphate triester 5 trans\(^2\) (trans configuration and two P–$^{18}$O bonds) (peak 3). The trans 3',5'-cyclic phosphate triester 5 trans\(^0\) (peak 1), containing no $^{18}$O labels, will be located downfield from the $^{18}$O-labelled trans 3',5'-cyclic phosphate triesters (5 trans\(^1\) and 5 trans\(^2\)). Similarly, the cis 3',5'-cyclic methyl phosphate triester 5 cis\(^2\) (peak 12) will resonate upfield from the cis 3',5'-cyclic methyl phosphate triester 5 cis\(^1\) (peak 11) in the $^{31}$P NMR spectrum. The relative intensities of the $[^{18}$O-cis]-triesters (5
3.2 Results and Discussion

trans¹ and 5 cis²) to the [¹⁸O-cis]-triesters (5 trans² and 5 cis¹) in the three low-field and high-field resonances (peaks 1, 2, and 3 and 10, 11, and 12, respectively) of the 3',5'-cyclic phosphate diesters indicate that during the hydrolysis of 4a (¹⁸O-cis) the formation of the 3',5'-cyclic phosphate diester has occurred with 17% inversion and 83% retention of configuration at phosphorus (Table 3.2). Hydrolysis of 4b takes place with 40% inversion and 60% retention, related to the formation of 3',5'-cyclic phosphate diester (Figure 3.4B). During the hydrolysis of 4c the formation of the 3',5'-cyclic phosphate diester occurs with 17% inversion and 83% retention of configuration at phosphorus (Figure 3.4C), which is similar to the hydrolysis of 4a.

3.2.3.2 Stereochemical Analysis of the Acyclic 3'- and 5'-Methyl Aryl Phosphate triesters

Compared with the ⁳¹P NMR chemical shift difference between the trans and cis 3',5'-cyclic methyl phosphate triesters, the chemical shift difference between the acyclic Rₚ and Sₚ 3'- or 5'-methyl aryl phosphate triester is relatively small (ca. 1.3 versus 0.02 ppm), because of the relatively small difference in geometry of the diastereoisomers at the phosphorus centre (Rₚ/Sₚ). The chemical shift difference
Table 3.2 *Product distribution and stereochemistry of the hydrolysis of 4a-c.*

<table>
<thead>
<tr>
<th>Compound</th>
<th>Cyclic&lt;sup&gt;a&lt;/sup&gt;</th>
<th>3′&lt;sup&gt;a&lt;/sup&gt;</th>
<th>5′&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Product distribution (%)</td>
<td>Retention (%)</td>
<td>Inversion (%)</td>
</tr>
<tr>
<td>4a</td>
<td>58</td>
<td>83</td>
<td>17</td>
</tr>
<tr>
<td>4b</td>
<td>90</td>
<td>60</td>
<td>40</td>
</tr>
<tr>
<td>4c</td>
<td>52</td>
<td>83</td>
<td>17</td>
</tr>
</tbody>
</table>

<sup>a</sup>Estimated errors ± 5%. <sup>b</sup>Could not be determined because of the low resolution of the expansions of the 3′- and 5′-phosphate diesters in the 31P NMR spectrum.
between the \( R_p \) and \( S_p \) diastereoisomers\(^{18} \) is in the same range as the \( ^{31}P \) NMR isotope shift of the \( ^{18}O \)-label, related to \( ^{16}O \). Therefore, it is not possible to assign the \( R_p \) and \( S_p \) diastereoisomers of the 3'- or 5'-methyl phenyl phosphates unambiguously. The assignment of the acyclic 3'- and 5'-methyl aryl phosphate triesters in the \( ^{31}P \) NMR spectrum is based on the relative position of the unlabelled 3'- and 5'-aryl phosphate diesters.\(^{13} \)

In the Figures 3.4A and 3.4C, the multiplets of the 5'-methyl phenyl phosphate triesters consist of six \( ^{31}P \) NMR signals, but the intensity distributions are not the same. The two small peaks (4a, peaks 4 and 5; 4c, peaks 7 and 10) were assigned to be the \( R_p \) and \( S_p \) unlabelled 5'-methyl phenyl phosphate triesters (6 \( S_p^0 \) (\( S_p \) configuration, zero \( P-^{18}O \) bonds) and 6 \( R_p^0 \), but these resonances cannot be identified unambiguously at this moment.\(^{18} \) The \( ^{31}P \) NMR multiplets of the 5'-methyl phenyl phosphate triesters, shown in Figure 3.4A and 3.4C, can be divided into two three-line patterns (4a, peaks 4, 6, and 8 and 5, 7, and 9; 4c, peaks 7, 8, and 9 and 10, 11, and 12). The chemical shift difference in the \( ^{31}P \) NMR spectrum between the isotopomers 6 \( S_p^0 \) and 6 \( S_p^1 \) and between 6 \( R_p^0 \) and 6 \( R_p^1 \) is 0.016 ppm (4a: differences between the resonances 4 and 6 and between 5 and 7; 4c:
differences between 7 and 8 and between 10 and 11), whereas a chemical shift difference of 0.041 ppm is observed between the isotopomers $6 \, S_p^0$ and $6 \, S_p^2$ and between $6 \, R_p^0$ and $6 \, R_p^2$ (4a: differences between the resonances 4 and 8 and between 5 and 9; 4c: differences between resonances 7 and 9 and between 10 and 12). These values are in excellent agreement with the values expected for the upfield isotope shift of the $^{31}\text{P}$ resonance (relative to $^{16}\text{O}$), when $^{18}\text{O}$ is singly or doubly bonded to phosphorus (ca. 0.02 or 0.04 ppm, respectively). The $[^{18}\text{O}]-$labelled 5'-methyl phenyl phosphate triesters $6 \, S_p^1$ and $6 \, R_p^2$ are associated with retention of configuration at phosphorus during hydrolysis of 4a and 4c and formation of the 5'-phenyl phosphate diesters, whereas $6 \, S_p^2$ and $6 \, R_p^1$ correspond with inversion of configuration. Since the intensities of the $[^{18}\text{O}]-$labelled 5'-methyl phenyl phosphate triesters ($6 \, S_p^1$, $6 \, S_p^2$, $6 \, R_p^1$ and $6 \, R_p^2$) are almost identical, it is stated that during hydrolysis the formation of acyclic 5'-phenyl phosphate diesters occurs with virtually complete loss of stereochemistry.

The multiplets of the 3'-methyl phenyl phosphate triesters in Figures 3.4A and 3.4C also consist of six $^{31}\text{P}$ NMR signals with an unequal intensity distribution. However, four small and two large $^{31}\text{P}$ NMR peaks have been observed. This means that two unlabelled 3'-methyl phenyl phosphate triesters (4a, peaks 13 and 14; 4c, peaks 13 and 16) and two $[^{18}\text{O}]-$labelled 3'-methyl phenyl phosphate triesters (4a, peaks 15 and 18; 4c, peaks 15 and 17) represent the small peaks and that two $[^{18}\text{O}]-$labelled 3'-methyl phenyl phosphate triesters represent the large peaks. In the Figures 3.4A and 3.4C, the multiplets of 3'-methyl phenyl phosphate triester are composed of two three-line patterns (4a, peaks 13, 15, and 17 and 14, 16, and 18; 4c, peaks 13, 14, and 15 and 16, 17, and 18). The chemical shift difference in both $^{31}\text{P}$ NMR spectra between the isotopomers $7 \, S_p^0$ and $7 \, S_p^1$ and between $7 \, R_p^0$ and $7 \, R_p^1$ is 0.016 ppm (4a, differences between resonances 13 and 15 and between 14 and 16; 4c, differences between 13 and 14 and between 16 and 17). The chemical shift difference between the isotopomers $7 \, S_p^0$ and $7 \, S_p^2$ and between $7 \, R_p^0$ and $7 \, R_p^2$ is 0.041 ppm (4a, difference between resonances 13 and
17 and between 14 and 18; 4c, differences between peaks 13 and 15 and between 16 and 18). These values are equal to those of the 5'-methyl phenyl phosphate triesters. The expected values for the chemical shift difference between isotopomers, indicate that the assigned sequence of the isotopomers \( 7 S_P^0, 7 S_P^1, \) and \( 7 S_P^2 \) and \( 7 R_P^0, 7 R_P^1, \) and \( 7 R_P^2 \) in the \( ^{31}P \) NMR spectra in Figure 3.4A and 3.4C is correct. However, it is not possible to identify the two \( R_P \) and \( S_P \) patterns absolutely. Since, the \([^{18}O]\)-labelled 3'-methyl phenyl phosphate triesters \( 7 S_P^2 \) and \( 7 R_P^1 \) are associated with retention of configuration at phosphorus and \( 7 S_P^1 \) and \( 7 R_P^2 \) are associated with inversion of configuration, no explicit statement can be made about the percentage retention or inversion of configuration.\(^{18}\) From the two three-line patterns of the 3'-methyl phenyl phosphate triester in Figure 3.4A two values can be extracted for inversion or retention of configuration at phosphorus: ca. 12% and 88%. Thus during hydrolysis of 4a, formation of 3'-phenyl phosphate diester proceeds with ca. 12% (or 88%) inversion or retention. From Figure 3.4C, it can be concluded that the formation of acyclic 3'-phenyl phosphate diesters, during hydrolysis of 4c, occurs with ca. 21% (or 79%) inversion or retention of configuration at phosphorus. The hydrolysis of 4b results in formation of approximately 10% acyclic 3'- and 5'-p-nitrophenyl phosphate diesters, which were not clearly detectable in the \( ^{31}P \) NMR spectrum. Due to methylation of the acyclic aryl \([^{18}O]\) phosphate diesters the signal to noise ratio became worse, because of the formation of the four isomers \( (7 S_P^1, 7 S_P^2, 7 R_P^1 \) and \( 7 R_P^2) \). Therefore it was not possible to determine the acyclic 3'- and 5'-methyl p-nitrophenyl phosphate triesters.

3.3 Interpretation and Conclusions

In order to interpret the results, a plausible reaction mechanism has been postulated. Following the original ideas of Westheimer,\(^{19a}\) it is assumed that the nucleophilic attack of an OH\(^-\) ion on the phosphorus centre results in the formation
Scheme 3.3 Hydrolysis pathway.
of a five-coordinated phosphorus (P\textsuperscript{V}) intermediate with a trigonal bipyramidal (TBP) geometry, that the nucleophile enters the P\textsuperscript{V}-TBP at one axial site, and that cleavage also takes place axially.\textsuperscript{19} Furthermore, it is assumed that negatively charged oxygens adopt equatorial sites in the P\textsuperscript{V}-TBP\textsuperscript{19b,20} only, and that Berry pseudorotation\textsuperscript{19a,21} can occur prior to bond breaking. As shown in Scheme 3.3, the initial attack of OH\textsuperscript{-} on phosphorus can take place in three different ways (route 1, 2, and 3). Attack of the OH\textsuperscript{-} on phosphorus opposite the P=O bond is not taken into account because the P\textsuperscript{V}-TBP formed will possess a negatively charged axial oxygen atom, which is energetically unfavourable. Moreover, after protonation of the labelled axial oxygen atom this P\textsuperscript{V}-TBP could lose the oxygen label by cleavage of the axial P=\textsuperscript{18}O bond. This is in contradiction with our results that no exchange of oxygen (\textsuperscript{16}O) from the solvent with the phosphate (di)triester oxygen (\textsuperscript{18}O) can be observed during the hydrolysis reaction.

In route 1, OH\textsuperscript{-} attacks phosphorus opposite to the P=OR bond, resulting in a P\textsuperscript{V}-TBP with an unfavourable diequatorially oriented dioxaphosphorinane ring.\textsuperscript{22} Cleavage of the P=OR bond results in formation of the 3',5'-cyclic phosphate diester 8, with inversion of configuration. Alternatively a simultaneous process can occur in which (i) a proton shifts from the axial oxygen atom to the equatorial oxygen atom and (ii) Berry pseudorotation takes place, leading to two P\textsuperscript{V}-TBPs with axially located \textsuperscript{18}O-labelled hydroxylic groups (Scheme 3.4). After cleavage of this P=\textsuperscript{18}O bond unlabelled 3',5'-cyclic phosphate triesters will be formed. According to our observations no significant loss of the \textsuperscript{18}O-label could be detected. This leads to the conclusion that the alternative process does not take place in a considerable degree and therefore this mechanism will not be taken into account (Scheme 3.4).

In route 2, OH\textsuperscript{-} attacks phosphorus opposite the P=O\textsubscript{2}, bond. Initially, the aryloxy (OR) group of the P\textsuperscript{V}-TBP formed is placed equatorially and cannot be cleaved immediately. Rather, the axial P=O\textsubscript{2}, bond will break resulting in the formation of the acyclic 5'-phosphate diester 9. This process proceeds with inver-
Scheme 3.4 Alternative mechanism leading to unlabelled cyclic phosphate triesters.

Retention of configuration at phosphorus. Berry pseudorotation of the \( \text{P}^\text{V}\)-TBP initially formed results in axial location of the OR group. In this situation, breaking of the P—OR bond results in the formation of the 3',5'-cyclic phosphate diester 11 with retention of configuration, whereas cleavage of the axial P—O\( _3\) bond leads to the acyclic 3'-phosphate diester 10, with retention of configuration.

In route 3, OH\(^-\) attacks phosphorus opposite the P—O\( _3\) bond. In the \( \text{P}^\text{V}\)-TBP initially formed the axial P—O\( _3\) bond can be cleaved resulting in the formation of the acyclic 3'-phosphate diester 13. This process proceeds with inversion of configuration at phosphorus. Berry pseudorotation of the \( \text{P}^\text{V}\)-TBP initially formed leads to a \( \text{P}^\text{V}\)-TBP with an axially located OR group. Cleavage of the P—OR bond leads to the 3',5'-cyclic phosphate diester 11 with retention of configuration, whereas cleavage of the axial P—O\( _3\) bond results in the formation of the acyclic 5'-phosphate diester 12, with retention of configuration.
<table>
<thead>
<tr>
<th>Compound</th>
<th>8 (cyclic; inversion) (%)</th>
<th>9 (5'; inversion) (%)</th>
<th>10 (3'; retention) (%)</th>
<th>11 (cyclic; retention) (%)</th>
<th>12 (5'; retention) (%)</th>
<th>13 (3'; inversion) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4a</td>
<td>10</td>
<td>~11</td>
<td>18 or 2</td>
<td>48</td>
<td>~11</td>
<td>2 or 18</td>
</tr>
<tr>
<td>4b</td>
<td>36</td>
<td>b</td>
<td>b</td>
<td>54</td>
<td>b</td>
<td>b</td>
</tr>
<tr>
<td>4c</td>
<td>9</td>
<td>~14.5</td>
<td>15 or 4</td>
<td>43</td>
<td>~14.5</td>
<td>4 or 15</td>
</tr>
</tbody>
</table>

*Based on Table 3.2. *Could not be determined because of the low resolution of the expansions of the 3'- and 5'-phosphate diesters in the $^{31}$P NMR spectrum.
Thus, nucleophilic attack on phosphorus and direct cleavage of the P-OR bond take place with inversion of configuration, whereas breaking the axial bonds after pseudorotation leads to retention of configuration. Taking all these considerations (vide supra) into account, it is believed that the reaction routes in Scheme 3.3 sufficiently describe the hydrolysis of the 3',5'-cyclic phosphate triesters. The total product distribution of the hydrolysis of 3',5'-cyclic phosphate triesters is shown in Table 3.3.

From Table 3.3 it is obvious that during hydrolysis of 4a-c the cyclic product 11 (retention of configuration at phosphorus) is formed as the main product, whereas compound 8 (inversion of configuration) is formed in a small amount. It is believed that the formation of compound 8 is not thermodynamically determined (based on the leaving group character), whereas the formation of compound 11 is based on the pseudorotational equilibrium which is determined by the axiophilicity of the leaving groups (OR) in the $P^V$-TBPs. Compared with the $O_{3^\prime}$- or $O_{5^\prime}$- and OH$^-$-residues, the OR groups are better leaving groups resulting in a preferential formation of product 11.

The formation of the 3',5'-cyclic phosphate diester during hydrolysis of 4a and 4c proceeds with 17% inversion of configuration at phosphorus. These results closely resemble those obtained from $H_2^{17}$O-hydrolysis of monocyclic phenyl phosphate triesters, reported by Gordillo et al.\textsuperscript{6} The formation of the 3',5'-cyclic phosphate diester during hydrolysis of 4b containing p-nitrophenoxy proceeds with 40% inversion, which is slightly less than reported by Gordillo et al.\textsuperscript{6} for monocyclic p-nitrophenoxy analogues (56%) (C and D) but much less than reported by Gorenstein et al.\textsuperscript{5} for a bicyclic system containing an axially located dinitrophenoxy group (83%) (A). Presumably this difference can be attributed to the fact that Gorenstein et al.\textsuperscript{5} used 2,4-dinitrophenoxy as the leaving group, i.e., the $P^V$-TBP intermediate that is formed after nucleophilic attack on phosphorus has a shorter lifetime in Gorenstein's model A\textsuperscript{5} as compared to the models 4a-c and models of Gordillo et al.\textsuperscript{6} The combined experimental data substantiate the conclusion of
Gorenstein et al.\(^5\) that the leaving group character significantly influences the inversion/retention ratio of the 3',5'-cyclic phosphodiesters (Table 3.3).

The formation of the \([^{18}O]\)-labelled 5'-acyclic aryl phosphate diesters is not stereospecific at all (ca. 50% retention), whereas the stereochemical preference (retention or inversion) of the formation of the \([^{18}O]\)-labelled 3'-acyclic aryl phosphate diesters cannot be determined at this moment.

The observed stereospecificity in the 3'-' and 5'-aryl phosphate diesters is not in agreement with the reported non-enzymatic hydrolysis of \(R_p\)-cyclic \([^{17}O,^{18}O]\)dAMP, which proceeds with complete inversion of configuration at phosphorus.\(^3\) The difference in hydrolytic behaviour between our compounds, which hydrolyse both with retention and inversion of configuration at phosphorus, and those of \([^{17}O,^{18}O]\)-cdAMP,\(^3\) proceeding with complete inversion, can be explained in terms of formation of dianions during hydrolysis of the latter. The P\(^V\)-TBPs, initially formed in route 2 or 3 by hydrolysis of 3',5'-cyclic phosphate diesters\(^3\) will adopt dianionic character, because of the deprotonation of the equatorial OH-group (\(R = H\)). Pseudorotation places one of the O\(^-\) ligands in an axial site, which is energetically unfavourable. The only escape for the P\(^V\)-TBPs is to release energy by cleavage of the P-O\(_3\), or P-O\(_5\), bond in route 2 or 3, respectively, resulting in the observed complete inversion of configuration at phosphorus. The P\(^V\)-TBPs, initially formed in route 2 and 3 by hydrolysis of our 3',5'-cyclic phosphate triesters are monooanions with the negative charge on an equatorial site. In this case, the P\(^V\)-TBPs initially formed in route 2 and 3 can 'choose' between two different routes (i) direct cleavage of the P-O\(_3\), or P-O\(_5\), leading to acyclic phosphate diesters (3'(13) or 5'(9)) with inversion of configuration at phosphorus; (ii) pseudorotation leading to P\(^V\)-TBPs with an axially located OR group and possible deprotonation of the equatorially located OH group, leading to cyclic phosphate diester 11 with retention of configuration or to acyclic phosphate diesters (3'(10) or 5'(12)) also with retention of configuration at phosphorus.
In Table 3.3 it is shown that during hydrolysis of 4a-c significant amounts of compound 8 are observed. *Therefore it is concluded that, according to Scheme 3.3, the existence of P^V^-TBPs with a diequatorially located dioxaphosphorinane ring is most likely*. This result is in agreement with the recently reported crystal structures of P^V^-TBPs, containing diequatorially located six- and eight-membered rings.\(^{23a-c}\) Interestingly these results indicate that the P^V^-TBP containing a diequatorially located dioxaphosphorinane ring has comparable stability with respect to all isomeric P^V^-TBPs in which the ring has an equatorial-axial orientation. This observation contrasts with calculational results of Holmes et al.\(^{23c}\) on a model which is perhaps oversimplified. In addition it may be noted that the observed (e,e) orientation of the 3',5'-dioxaphosphorinane ring in P^V^-TBP intermediates perfectly agrees with the conclusion from chapter 2, stating that (e,e) orientation of the six-membered ring is possible.\(^{24}\) *Furthermore, it is clear that during hydrolysis of 3',5'-cyclic phosphate triesters Berry pseudorotation takes place, whereas hydrolysis of 3',5'-cyclic phosphate diesters\(^3\) proceeds without Berry pseudorotation, leading to complete inversion of configuration at phosphorus.*

3.4 Experimental

3.4.1 Materials and Chromatography

For all column separations, Merck silica gel 60 has been used (particle size 0.040-0.063 mm or 0.063-0.200 mm). Dowex-H\(^+\) 50X8-100 was purchased from Janssen Chimica. Dry diethyl ether was obtained by storing diethyl ether, predried on calcium chloride, on sodium wire. Ethyl acetate was refluxed on calcium hydride prior to atmospheric distillation. Pyridine was distilled from KOH pellets and stored on 4Å molecular sieves. Acetonitrile (DNA grade) was purchased from Merck and used as received. Tetrahydrofuran was refluxed on calcium hydride and distilled from lithium aluminium hydride. Tetrahydrofuran and acetonitrile, used as solvents
for $^{18}$O labelling reactions, were deoxygenized by subjecting the solvents to three freeze-pump-thaw cycles. $[^{18}\text{O}]$Water (97 atom%) was obtained from Sigma. Hexamethylphosphorous triamide ($\text{(Me}_2\text{N)}_3\text{P}$) (97%) was purchased from Janssen Chimica and used as received. tert-Butyl hydroperoxide was used as an 80% (8.0 M) solution in di-tert-butyl peroxide, which was obtained from Merck. 1H-Tetrazole was purified through sublimation. $p$-Nitrophenol was recrystallized from a mixture of petroleum ether and ethanol. Reactions were routinely run in an inert atmosphere of dry nitrogen or argon and were run at ambient temperature, unless otherwise noted.

3.4.2 NMR Measurements

$^1\text{H}$ NMR spectra were recorded at 400.13 MHz on a Bruker AM 400 spectrometer. Tetramethylsilane (TMS) was used as the internal standard in $^1\text{H}$ and $^{13}\text{C}$ NMR. $^{13}\text{C}$ and $^{31}\text{P}$ NMR were recorded at 100.62 and 161.98 MHz, respectively, on the same instrument. The $^{31}\text{P}$ NMR spectra were referenced against 85% $\text{H}_3\text{PO}_4$ as the external standard.

3.4.3 UV/Visible Spectroscopy

The hydrolysis of compound 3b was monitored at 405 nm by means of a Hitachi 150-20 spectrophotometer.

3.4.4 Synthesis

General information on synthesis and NMR spectroscopy is described in section 3.3.1 and 3.2.2, respectively.

$\text{trans-}$Thymidine $3',5'$-Cyclic $N,N$-Dimethylphosphoramidite (1a).$^{7,17}$ According to the method described by Bentrude et al.$^{7,17}$ $\text{trans-}$thymidine $3',5'$-cyclic $N,N$-dimethylphosphoramidite is obtained as a white foam in 47% yield: cis/trans ratio 2.98; mp 89 °C; $^{31}\text{P}$ NMR$^{17}$ (CDCl$_3$) δ 146.3 (trans), 140.3 (cis); $^1\text{H}$ NMR$^{17}$
Alkaline Hydrolysis

(CDC\textsubscript{3}) (1a) \(\delta\) 9.51 (1H, bs, NH of T), 7.09 (1H, q, H\textsubscript{6} of T), 6.27 (1H, dd, H\textsubscript{1}), 4.46 (1H, m, H\textsubscript{5}'), 4.22-4.08 (2H, m, H\textsubscript{5} and H\textsubscript{3}), 3.58 (1H, m, H\textsubscript{4'}), 2.72 (6H, d, N(CH\textsubscript{3})\textsubscript{2}, \(J\textsubscript{PNCH}\) = 9.2 Hz), 2.56 (1H, m, H\textsubscript{2}'), 2.32 (1H, m, H\textsubscript{2}'), 1.97 (3H, d, CH\textsubscript{3} of T); \(^{13}\text{C}\) NMR (CDC\textsubscript{3}) (1a) \(\delta\) 163.7 (C\textsubscript{2} or C\textsubscript{4} of T), 150.3 (C\textsubscript{4} or C\textsubscript{2} of T), 134.9 (C\textsubscript{6} of T), 111.7 (C\textsubscript{5} of T), 83.6 (C\textsubscript{1'}), 75.1 (C\textsubscript{4'}, \(J\textsubscript{POC}\) = 12.3 Hz), 74.8 (C\textsubscript{3'}, \(J\textsubscript{POC}\) = 9.4 Hz), 66.8 (C\textsubscript{5'}), 36.5 (C\textsubscript{2'}, \(J\textsubscript{POCC}\) = 5.7 Hz), 34.9 (2C, N(CH\textsubscript{3})\textsubscript{2}, \(J\textsubscript{PNCH}\) = 21.4 Hz), 12.6 (CH\textsubscript{3} of T).

2'-O-Methyl-\textit{trans}-Adenosine 3',5'-Cyclic N,N-Dimethylphosphoramidite (1b). Hexamethylphosphorous triamide ((Me\textsubscript{2}N)\textsubscript{3}P) (0.65 g, 4.00 mmol) was added to a solution of 2'-O-methyl-adenosine\textsuperscript{26} (1.13 g, 4.00 mmol) in 60 mL of dry acetonitrile and the mixture was stirred for 4 h at 50 °C. Afterwards, the \(^{31}\text{P}\) NMR spectrum showed complete conversion of hexamethylphosphorous triamide and the formation of 1b and its epimer (\(\delta\)(CH\textsubscript{3}CN/CD\textsubscript{3}CN): \textit{cis} 137.2; \textit{trans} 147.3; \textit{cis/trans} ratio 2:8). After being stirred for 15 h at room temperature (time is needed for the \textit{cis-\rightarrow trans} epimerization), the mixture was filtered and 3 mL of the filtrate (approximately 5%) was evaporated in vacuo and subjected to \(^{31}\text{P}\) NMR analysis. This indicated additional signals at 19 and 4 ppm, probably due to phosphonates (<5%). The filtrate, predominantly containing compound 1b, was stored at -20 °C in order to prevent degradation. After evaporation of the volatiles of the 3 mL filtrate the \(^{1}\text{H}\) NMR spectrum indicated some dimethylamine (released during cyclization) and some impurities (approximately 5%). Estimated yield 1.1 g (77%): \textit{cis/trans} ratio 1:9; \(^{31}\text{P}\) NMR(CD\textsubscript{3}CN) \(\delta\) 147.3 (\textit{trans}), 137.2 (\textit{cis}); \(^{1}\text{H}\) NMR (CD\textsubscript{3}CN) (1b) \(\delta\) 8.26 (1H, s, H\textsubscript{8} or H\textsubscript{2} of A), 8.01 (1H, s, H\textsubscript{2} or H\textsubscript{8} of A), 6.32 (2H, bs, NH\textsubscript{2} of A), 6.03 (1H, bs, H\textsubscript{1'}), 4.59 (1H, m, H\textsubscript{3}'), 4.41 (1H, m, H\textsubscript{5}'), 4.29 (1H, d, H\textsubscript{2}'), 4.12 (1H, t, H\textsubscript{3}'), 3.95 (1H, m, H\textsubscript{4'}), 3.54 (3H, s, 2'-OMe), 2.69 (6H, d, N(CH\textsubscript{3})\textsubscript{2}, \(J\textsubscript{PNCH}\) = 9.6 Hz).
3.4 Experimental

cis-Thymidine 3',5'-Cyclic Phenyl Monophosphite (2a).\textsuperscript{17} trans-Thymidine 3',5'-cyclic N,N-dimethylphosphoramidite (1a) (1.97 g, 6.25 mmol) was added to a solution of phenol (0.60 g, 6.38 mmol) in a mixture of acetonitrile (25 mL) and dichloromethane (15 mL). During this reaction two 3',5'-cyclic phenyl monophosphates were formed (cis and trans), as revealed by \textsuperscript{31}P NMR ($\delta$(CDCl$_3$) cis (2a): 115.0 ppm; trans (2a): 121.1 ppm). After the mixture was stirred for 5 h at room temperature, the cis/trans ratio was 5:1, whereas complete formation of 2a was achieved after 70 h, which was evident from the \textsuperscript{31}P NMR data ($\delta$(CDCl$_3$): 115.1 ppm). After evaporating the volatiles, the residue was dissolved in 30 mL of dichloromethane, washed five times with 5 mL portions of a sodium carbonate solution (0.5 M) (to remove the phenol and the amine) and the organic layer was dried on magnesium sulfate. After filtration, the solution was concentrated in vacuo, affording a white solid, containing phenol, which was evident from \textsuperscript{1}H NMR data. Addition of dry diethyl ether, afforded a white precipitate of 2a. Isolation of the solid was accomplished by decanting the clear solution, yielding 0.69 g of 2a as a white solid. The decanted clear solution was concentrated in vacuo, and dry diethyl ether was added, resulting in a second crop of 2a as a white precipitate (0.30 g); total yield 0.99 g (44%); mp 140 °C; Anal. Calcd for C$_{16}$H$_{17}$N$_2$O$_6$P: C, 52.7; H, 4.7; N, 7.7. Found: C, 52.1; H, 4.8; N, 7.5. \textsuperscript{31}P NMR\textsuperscript{17} (CDCl$_3$) $\delta$ 114.9; \textsuperscript{1}H NMR\textsuperscript{17} (CDCl$_3$) $\delta$ 8.77 (1H, bs, NH of T), 7.35 (2H, m, 2H of PhO), 7.13 (3H, m, 3H of PhO), 7.07 (1H, q, H$_6$ of T), 6.20 (1H, dd, H$_1$), 4.67 (1H, m, H$_3$), 4.62 (1H, m, H$_5$), 4.44 (1H, m, H$_5$), 3.75 (1H, m, H$_4$), 2.54 (1H, m, H$_2$), 2.42 (1H, m, H$_2$), 1.96 (3H, d, CH$_3$ of T); \textsuperscript{13}C NMR (CDCl$_3$) $\delta$ 163.2 (C$_2$ or C$_4$ of T), 156.6 (C$_{ipso}$ of PhO), 150.0 (C$_4$ or C$_2$ of T), 134.9 (C$_6$ of T), 129.9 (2 meta C of PhO), 124.0 (1 para C of PhO), 119.8 (2 ortho C of PhO, $^3$J$_{POCC} = 7.8$ Hz), 111.9 (C$_5$ of T), 82.1 (C$_1$), 74.9 (C$_4$), $^3$J$_{POCC} = 7.4$ Hz), 69.2 (C$_3$), 66.6 (C$_5$), 36.1 (C$_2$), 12.7 (CH$_3$ of T).
cis-Thymidine 3',5'-Cyclic p-Nitrophenyl Monophosphite (2b). p-Nitrophenol (0.49 g, 3.53 mmol) was dissolved in 25 mL of dichloromethane, and trans-thymidine 3',5'-cyclic N,N-dimethylphosphoramidite (1a) (1.1 g, 3.49 mmol) was added. After being stirred for 70 h, complete formation of 2b was evident from $^{31}$P NMR data ($\delta$ (CDCl$_3$) 114.5 ppm). To remove the p-nitrophenol and the amine, the mixture was washed three times with 8 mL portions of a sodium carbonate solution (0.07 M) and the organic layer was dried on magnesium sulfate. After filtration, the solution was concentrated in vacuo, affording a yellowish solid. The $^1$H NMR spectrum indicated a purity of >98%. Yield 0.3 g (21%); $^{31}$P NMR (CDCl$_3$) $\delta$ 114.5; $^1$H NMR CDCl$_3$ $\delta$ 8.57 (1H, bs, NH of T), 8.25 (2H, m, 2H of p-NO$_2$-PhO), 7.22 (2H, m, 2H of p-NO$_2$-PhO), 7.03 (1H, q, H$_6$ of T), 6.11 (1H, dd, H$_1$), 4.69 (1H, m, H$_3$ of T), 4.61 (1H, m, H$_5$), 4.48 (1H, m, H$_5$ of T), 3.77 (1H, m, H$_4$), 2.60-2.45 (2H, m, H$_2'$ and H$_2''$), 1.95 (3H, d, CH$_3$ of T).

2'-O-Methyl-cis-Adenosine 3',5'-Cyclic Phenyl Monophosphite (2c). Phenol (188 mg, 2.0 mmol) was added to 20 mL of the filtered acetonitrile solution, mainly containing 1b (approximately 1.1 mmol). 1H-Tetrazole (8.5 mg, 0.12 mmol) was added and after 1½ h the $^{31}$P NMR spectrum showed 25% conversion of 1b into the cis- and trans-phenyl phosphite triester ($\delta$(CH$_3$CN/CD$_3$CN) cis 116.1; trans 121.7; cis/trans ratio 1:1). After 60 h, 1b was completely converted into the cis-phenyl phosphite triester (2c) ($^{31}$P NMR $\delta$ 116.1 ppm). The solution was filtered and evaporated until approximately 5 mL of solvent remained. Then, the solution was diluted with diethyl ether and washed two times with a 0.1 M sodium hydroxide solution, in order to remove the phenol and the 1H-tetrazole. After evaporation of all volatiles, a white solid was obtained, with a purity of 90%, according to $^1$H- and $^{31}$P NMR data. Yield: 210 mg (47%). $^{31}$P NMR (CD$_3$CN) $\delta$ 116.2; $^1$H NMR (CD$_3$CN) $\delta$ 8.25 (1H, s, H$_8$ or H$_2$ of A), 8.02 (1H, s, H$_2$ or H$_8$ of A), 7.40 (2H, m, 2H of PhO), 7.20 (3H, m, 3H of PhO), 6.20 (2H, bs, NH$_2$ of A), 5.99 (1H, bs, H$_1$), 5.32 (1H, m, H$_3$), 4.68 (1H, m, H$_5$ of T), 4.47 (1H, m, H$_5$ of T), 4.42 (1H, d, H$_2$),
3.4 Experimental

4.12 (1H, m, H₂⁻), 3.56 (3H, s, 2'-OMe); ¹³C NMR (CD₃CN) δ 157.0 (C₄ or C₆ of A), 154.0 (C₈ of A), 152.9 (Cᵦ (PhO), 150.2 (C₆ or C₄ of A), 140.9 (C₂ of A), 131.0 (2 meta C of PhO), 125.1 (para C of PhO), 121.1 (C₅ of A and 2 ortho C of PhO), 88.5 (C₁⁻), 82.3 (C₂⁻), 72.4 (C₄⁻, ³JPOCC = 7.3 Hz), 71.6 (C₃⁻), 68.0 (C₅⁻), ²JPOC = 4.1 Hz), 59.1 (2'-O-Me).

**cis-Thymidine 3',5'-Cyclic Phenyl Monophosphate (3a).** According to the procedure described by Bentrode et al. cis-thymidine 3',5'-cyclic phenyl monophosphate is obtained. After recrystallization from ethyl acetate, the white solid still contained ethyl acetate (+ 10% w/w), which could not be removed even at 50 °C and under vacuo (20 mmHg). Yield 34%; mp 135-138 °C (lit. mp 134-136 °C). ³¹P NMR (CDCl₃) δ -12.3; ¹H NMR (CDCl₃) δ 8.83 (1H, bs, NH of T), 7.39 (2H, m, 2H of PhO), 7.28 (3H, m, 3H of PhO), 7.01 (1H, q, H₆ of T), 6.05 (1H, dd, H₁⁻), 5.00 (1H, m, H₃⁻), 4.68 (1H, m, H₅⁻), 4.54 (1H, m, H₅⁻), 4.00 (1H, m, H₄⁻), 2.7-2.6 (2H, m, H₂⁻ and H₂⁻), 1.96 (3H, d, CH₃ of T); ¹³C NMR (CDCl₃) δ 163.8 (C₂ or C₄ of T), 150.4 (Cᵦ (PhO), 150.0 (C₄ or C₂ of T), 137.0 (C₆ of T), 130.0 (2 meta C of PhO), 125.5 (1 para C of PhO), 119.6 (2 ortho C of PhO, ³JPOCC = 4.7 Hz), 111.8 (C₅ of T), 87.3 (C₁⁻), 78.4 (C₃⁻), 74.0 (C₄⁻), 69.9 (C₅⁻), ²JPOC = 9.2 Hz), 34.8 (C₂⁻), 12.4 (CH₃ of T).

**cis-Thymidine 3',5'-Cyclic p-Nitrophenyl Monophosphate (3b).** Compound 2b (0.3 g, 0.73 mmol) was dissolved in 10 mL of dichloromethane, and NO₂/N₂O₄ gas was bubbled through the solution until a greenish colour appeared. During this oxidation, a precipitate of 3b was observed. After evaporation of the solvent, a light yellow-brown solid was obtained. Yield 0.27 g (87%); mp 155-158 °C. According to ³¹P and ¹H NMR, the solid showed a purity of >95%; ³¹P NMR (1,4-dioxane-d₈/D₂O 4:1 v/v) δ -11.3; ¹H NMR (1,4-dioxane-d₈/D₂O 4:1 v/v) δ 8.28 (2H, m, 2H of p-NO₂-PhO), 7.51 (2H, m, 2H of p-NO₂-PhO), 7.35 (1H, q, H₆ of T), 6.12 (1H, dd, H₁⁻), 5.09 (1H, m, H₃⁻), 4.71 (1H, m, H₅⁻), 4.60 (1H, m, H₅⁻), 4.02
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(1H, m, H₄), 2.65-2.50 (2H, m, H₂' and H₂''), 1.91 (3H, d, CH₃ of T); ¹³C NMR (1,4-dioxane-d₈/D₂O 4:1 v/v) δ 165.1 (C₂ or C₄ of T), 155.5 (Cipso of p-NO₂-PhO, ²JPOC = 6.3 Hz), 151.1 (C₄ or C₂ of T), 146.0 (para C of p-NO₂-PhO), 138.7 (C₆ of T), 126.7 (2 meta C of p-NO₂-PhO), 121.4 (2 ortho C of p-NO₂-PhO, ³JPOCC = 4.7 Hz), 111.8 (C₅ of T), 87.5 (C₁'), 79.9 (C₃', ²JPOC = 5.7 Hz), 74.1 (C₄', ³JPOCC = 6.2 Hz), 71.7 (C₅', ²JPOC = 9.3 Hz), 34.7 (C₂', ³JPOCC = 9.0 Hz), 12.3 (CH₃ of T).

2'-O-Methyl-cis-Adenosine 3',5'-Cyclic Phenyl Monophosphate (3c). To a stirred solution of 2c (50 mg, 0.12 mmol) in 2 mL of acetonitrile was added tert-butyl hydroperoxide. After 4 h, the solvent was evaporated in vacuo. Pure 3c was obtained as a white solid after column chromatography, using a gradient of methanol (0% → 5% v/v) in acetonitrile as eluent. Yield 40 mg (80%). According to ³¹P and ¹H NMR the solid was pure for more than 95%. Rf 0.24 (methanol/acetonitrile 5:95 v/v); ³¹P NMR (CD₃OD) δ -10.3; ¹H NMR (CD₃OD) δ 8.28 (1H, s, H₈ or H₂ of A), 8.22 (1H, s, H₂ or H₈ of A), 7.50-7.30 (5H, m, 5H of PhO), 6.20 (1H, bs, H₁'), 4.77 (1H, m, H₃'), 4.62 (1H, m, H₅'), 4.58 (1H, d, H₂'), 4.42 (1H, m, H₄'), 3.59 (3H, s, 2'-OMe); ¹³C NMR (CD₃OD) δ 157.5 (C₄ or C₆ of A), 154.1 (C₈ of A), 151.5 (Cipso of PhO), 150.2 (C₆ or C₄ of A), 142.1 (C₂ of A), 131.3 (2 meta C of PhO), 127.0 (para C of PhO), 120.9 (C₅ of A and 2 ortho C of PhO), 92.3 (C₁'), 81.7 (C₂', ³JPOCC = 7.6 Hz), 80.9 (C₃', ²JPOC = 6.1 Hz), 72.1 (C₄' and C₅'), 59.6 (2'-OMe).

cis-Thymidine 3',5'-cyclic Phenyl [¹⁸O]Monophosphate (4a). Compound 2a (106 mg, 0.29 mmol) and pyridine²⁷ (2.4 equiv, 0.70 mmol, 56 μL) were dissolved in 3 mL of dry acetonitrile. To this solution was added slowly 1.75 mL (6 equiv H₂¹⁸O/H₂¹⁶O and 1.2 equiv I₂) of a stock solution²⁸. Complete formation of 4a was realized after 70 h, which was evident from ³¹P NMR data (δ(CD₃CN/CH₃CN) -10.95 (¹⁶O) and -10.99 ppm (¹⁸O), ratio ¹⁸O/¹⁶O 91:9).
Acetonitrile and diethyl ether were evaporated in vacuo. After addition of 120 μL (0.2 equiv) of a 5% sodium bisulfite solution, the solution was extracted with 10 mL of dichloromethane. After evaporation of the dichloromethane, 4a was obtained. 

1H NMR indicated that pyridine (9% w/w) was present; 31P NMR (CDCl3) δ -12.24 (18O) and -12.29 ppm (16O) (Δδ = 6.8 Hz), ratio 18O/16O: 92/8). The 1H- and 13C-NMR spectra were identical to those for 3a.

cis-Thymidine 3',5'-cyclic p-Nitrophenyl [18O]Monophosphate (4b). Compound 2b (312.5 mg, 0.76 mmol) and pyridine27 (2 equiv, 120 μL) were dissolved in 4 mL of dry tetrahydrofuran. To this solution was added slowly a mixture of I2 (271.9 mg, 1.4 equiv) and H218O (2 equiv, 33 μL, 97 atom%) in 1 mL of dry tetrahydrofuran. After 12 h, compound 2b was completely converted into 4b and the excess of I2 was removed in a reaction with 3 mL of 5% sodium bisulfite solution. After addition of 5 mL of chloroform, two layers were formed. The organic layer was evaporated in vacuo, affording a light yellow foam. Yield 0.18 g (55%); 31P NMR (CD3CN) δ -11.85 (16O) and -11.90 ppm (18O) (Δδ = 6.8 Hz), ratio 18O/16O: 93/7). The 1H- and 13C-NMR spectra were identical to those for 3b.

2'-O-Methyl-cis-Adenosine 3',5'-cyclic Phenyl [18O]Monophosphate (4c). Compound 2c (54 mg, 0.13 mmol) and pyridine27 (0.37 mmol, 30 μL) were dissolved in 2 mL of anhydrous acetonitrile. To this solution was added slowly 800 μL (6 equiv H218O/H216O and 1.2 equiv I2) of a stock solution28. After being stirred for 15 h the solution was filtered and evaporated in vacuo. The residue was chromatographed on a silica gel column, using a gradient of methanol (0% → 5% v/v) in acetonitrile as eluent. Pure compound 4c was obtained as a white solid. Yield 41 mg (72%). Rf 0.24 (methanol/acetonitrile 5:95 v/v); 31P NMR (CD3OD) δ -10.29 (16O) and -10.33 ppm (18O) (Δδ = 6.6 Hz), ratio 18O/16O: 90/10). The 1H- and 13C-NMR spectra were identical to those for 3c.
3.4.5 Hydrolysis

3.4.5.1 General Procedure for the Hydrolysis of 3a, 3b and 3c

The 3',5'-cyclic phosphate triester (0.026 mmol) was transferred into a 5-mm NMR sample tube and dissolved in a mixture of 300 μL of 1,4-dioxane and 161 μL of D₂O. Addition of 39 μL (3 equiv) of a 2.00 M sodium hydroxide solution initiated the hydrolysis. After vigorous shaking of the NMR tube for 5 s, the reaction was monitored at 21 °C by means of ³¹P NMR: 8 FIDs were accumulated (time domain: 16K; size: 16K; sweep width: 3240 Hz) prior to Fourier transformation at different reaction times, and the start of the fifth scan was chosen as the reaction time. Because of the fast hydrolysis of compound 3b, it was not possible to monitor the reaction by means of ³¹P NMR. Therefore, compound 3b was hydrolyzed in a very dilute solution (3.2 × 10⁻⁵ M, 1,4-dioxane/H₂O 3:2 v/v) and the reaction was monitored by means of UV/Visible spectroscopy at 405 nm.

(3a): ³¹P NMR (1,4-dioxane-d₈/D₂O 3:2 v/v) δ -1.59 (cyclic phosphate diester), -3.89 (5'-phosphate diester), -4.87 (3'-phosphate diester).

(3b): ³¹P NMR (1,4-dioxane-d₈/D₂O 3:2 v/v) δ -1.65 (cyclic phosphate diester), -4.89 (5'-phosphate diester), -5.92 (3'-phosphate diester).

(3c): ³¹P NMR (1,4-dioxane-d₈/D₂O 7:3 v/v) δ -1.63 (cyclic phosphate diester), -4.00 (5'-phosphate diester), -4.84 (3'-phosphate diester).

3.4.5.2 Hydrolysis of cis-Thymidine 3',5'-Cyclic Phenyl [¹⁸O]Monophosphate (4a) and Methylation of the Products.

In a 5-mm NMR tube, cis-thymidine 3',5'-cyclic phenyl [¹⁸O]monophosphate (4a) (11.3 mg, 0.03 mmol) was dissolved in a mixture of 300 μL of 1,4-dioxane-d₈ and 120 μL of D₂O, resulting in a yellowish solution. Then, 80 μL (5.4 equiv, 0.16 mmol) of a 2.00 M sodium hydroxide solution was added, thereby changing the colour from light yellow to pink. After 1 h, the solution adopted a purple colour and 4a was completely hydrolyzed, which was evident from ³¹P NMR data (δ(1,4-
dioxane-$d_8$/D$_2$O 3:2 v/v) -1.66 ([$^{16}$O]-cyclic phosphate diester), -1.69 ([$^{18}$O]-cyclic phosphate diester), -3.86 ([$^{16}$O] 5'-phosphate diester), -3.89 ([$^{18}$O] 5'-phosphate diester), -4.96 ([$^{16}$O] 3'-phosphate diester), -4.99 ([$^{18}$O] 3'-phosphate diester)).

To this solution 500 $\mu$L of H$_2$O was added, and the mixture was placed on a Dowex-H$^+$ column (height: 2.5 cm, diameter: 1 cm), in order to neutralize the excess of sodium hydroxide, and collected in 10 mL of water (solution became colourless). The solution was concentrated to approximately 1-2 mL and placed on a Dowex-K$^+$ column (height 2 cm, diameter 1 cm), in order to exchange H$^+$ by K$^+$, and collected in 15 mL of water. To this solution, 18-crown-6 (20.5 mg, 0.08 mmol) was added and the solvents were evaporated and coevaporated (three times) with 10 mL of acetonitrile. The residue was dissolved in 500 $\mu$L of DMSO-$d_6$ and transferred into a 5-mm NMR tube; $^{31}$P NMR (DMSO-$d_6$) δ -1.96 and -2.00 ($^{16}$O and $^{18}$O cyclic phosphate diester), -3.75 and -3.79 ($^{16}$O and $^{18}$O acyclic 5'-phosphate diester), -4.35 and -4.38 ($^{16}$O and $^{18}$O acyclic 3'-phosphate diester) (ratio cyclic/5'/3' = 58:22:20). To this solution 37 $\mu$L (20 equiv, 0.59 mmol) of methyl iodide was added. After 2-3 days the methylation was complete and the solution became brown.

$^{31}$P NMR (DMSO-$d_6$) δ -2.99, -3.01, -3.03 (trans-thymidine 3',5'-cyclic methyl phosphate triesters), -4.25, -4.26, -4.29 (cis-thymidine 3',5'-cyclic methyl phosphate triesters), -4.07, -4.08 (2×), -4.10, -4.11, -4.12 ($R_p$ and $S_p$ diastereoisomers of the acyclic 5'-methyl phenyl phosphate triesters), -5.13, -5.14, -5.15, -5.16, -5.17, -5.18 ($R_p$ and $S_p$ diastereoisomers of 3'-methyl phenyl phosphate triesters).

3.4.5.3 Hydrolysis of cis-Thymidine 3',5'-Cyclic p-Nitrophenyl [$^{18}$O]Monophosphate (4b) and Methylation of the Products.

In a 5-mm NMR tube, cis-thymidine 3',5'-cyclic p-nitrophenyl [$^{18}$O]monophosphate (4b) (36.7 mg, 0.09 mmol) was dissolved in a mixture of 560 $\mu$L of 1,4-dioxane-$d_8$ and 80 $\mu$L of D$_2$O. To this solution was added 160 $\mu$L (3.7 equiv, 0.32 mmol) of a 2.00 M sodium hydroxide solution. After 1 h complete hydrolysis of 4b
was observed, which was evident from $^{31}$P NMR data ($\delta$(1,4-dioxane-$d_8$/D$_2$O 7:3 v/v) -1.66 ([1$^{16}$O]-cyclic phosphate diester), -1.69 ([1$^{18}$O]-cyclic phosphate diester), -3.89 ([1$^{16}$O] and [1$^{18}$O] 5'-phosphate diester), -4.66 ([1$^{18}$O] and [1$^{18}$O] 3'-phosphate diester)$^{13}$ (ratio cyclic/5'/3' 91:4:5)). To this solution 1-2 mL of H$_2$O was added and in order to neutralize the excess of sodium hydroxide, the mixture was placed on a Dowex-H$^+$ column. The solution was concentrated and placed on a Dowex-K$^+$ column,$^{15}$ and after addition of 18-crown-6 (130 mg, 0.49 mmol), the solvents were evaporated and coevaporated (three times) with 15 mL portions of anhydrous acetonitrile. The residue was dissolved in 1 mL of DMSO-$d_6$ and transferred into a 5-mm NMR tube. To this solution was added 300 µL (0.93 mmol) of methyl iodide. After 2-3 days the methylation was complete and the solution became brown; $^{31}$P NMR (DMSO-$d_6$) $\delta$ -3.04, -3.06, -3.09 (trans-thymidine 3',5'-cyclic methyl phosphate triesters), -4.43, -4.44, -4.47 (cis-thymidine 3',5'-cyclic methyl phosphate triesters). The acyclic phosphate triesters were not clearly identified (two broadened peaks were visible at -4.12 and -4.97 ppm).

3.4.5.4 Hydrolysis of 2'-O-Methyl-cis-Adenosine 3',5'-Cyclic Phenyl [1$^{18}$O]Monophosphate (4c) and Methylation of the Products.

In a 5-mm NMR tube, 2'-O-methyl-cis-adenosine 3',5'-cyclic phenyl [1$^{18}$O] monophosphate (4c) (16 mg, 0.038 mmol) was dissolved in a mixture of 350 µL of 1,4-dioxane-$d_8$ and 93 µL of D$_2$O. To this solution was added 57 µL (3 equiv, 0.11 mmol) of a 2.00 M sodium hydroxide solution. After 1 h complete hydrolysis of 4c was observed, which was evident from $^{31}$P NMR data ($\delta$(1,4-dioxane-$d_8$/D$_2$O 7:3 v/v) -1.62 ([1$^{16}$O]-cyclic phosphate diester), -1.66 ([1$^{18}$O]-cyclic phosphate diester), -4.06 ([1$^{16}$O] 5'-phosphate diester), -4.09 ([1$^{18}$O] 5'-phosphate diester), -4.84 ([1$^{16}$O] 3'-phosphate diester), -4.87 ([1$^{18}$O] 3'-phosphate diester)$^{13}$ (ratio cyclic/5'/3' 52:29:19)). To this solution 1 mL of H$_2$O was added and in order to neutralize the excess of sodium hydroxide, the mixture was placed on a Dowex-H$^+$ column. The solution was concentrated and placed on a Dowex-K$^+$ column,$^{15}$ and after addition
of 18-crown-6 (13 mg, 0.049 mmol), the solvents were evaporated and coevaporated (two times) with 10 mL portions of anhydrous acetonitrile. The residue was dissolved in 500 μL of DMSO-d_{6} and transferred into a 5-mm NMR tube. To this solution was added 28 μL (0.46 mmol) of methyl iodide. After 2-3 days the methylation was complete and the solution became brown; $^{31}$P NMR (DMSO-d_{6}) δ -2.73, -2.75, -2.77 (trans-2'-O-methyl-adenosine 3',5'-cyclic methyl phosphate triesters), -4.11, -4.13, -4.15 (cis-2'-O-methyl-adenosine 3',5'-cyclic methyl phosphate triesters), -4.17, -4.19, -4.21, -4.23, -4.25, -4.27 ($R_{p}$ and $S_{p}$ diastereoisomers of 5'-methyl phenyl phosphate triesters), -4.71, -4.73, -4.75, -4.79, -4.81, -4.83 ($R_{p}$ and $S_{p}$ diastereoisomers of 3'-methyl phenyl phosphate triesters).

Appendix

Second order kinetics:

$$\frac{d[P_{c}]}{dt} = k_{c}[P][OH^-]$$  \hspace{1cm} (1)

$$\frac{d[P_{a}]}{dt} = k_{a}[P][OH^-]$$  \hspace{1cm} (2)

$[P]_o$ = initial concentration of the cyclic phosphate triester. [mol.l^{-1}]

$[P]$ = concentration of the cyclic phosphate triester. [mol.l^{-1}]

$[P_{a}]$ = concentration of the acyclic phosphate diesters (3' and 5'). [mol.l^{-1}]

$[P_{c}]$ = concentration of the cyclic phosphate diester. [mol.l^{-1}]

$[OH^-]_o$ = initial concentration of $OH^-$. [mol.l^{-1}]

$[OH^-]$ = concentration of $OH^-$. [mol.l^{-1}]
\[ k_c = \text{overall reaction rate constant for the formation of cyclic phosphate diester.} \ [\text{l.mol}^{-1} \cdot \text{s}^{-1}] \]

\[ k_a = \text{overall rate constant for the formation of acyclic phosphate diesters (3'- and 5'-phosphate diester).} \ [\text{l.mol}^{-1} \cdot \text{s}^{-1}] \]

\[ t = \text{reaction time.} \ [\text{s}] \]

During the formation of the cyclic phosphate diester from the cyclic phosphate triester 2 equiv of \( \text{OH}^- \) are consumed, while 1 eq of \( \text{OH}^- \) is consumed during the formation of the acyclic phosphate diesters (3' and 5').

\[ [P] = [P]_0 - [P_c] - [P_a] \]  \hspace{1cm} (3)

\[ [\text{OH}^-] = [\text{OH}^-]_0 - 2[P_c] - [P_a] \]  \hspace{1cm} (4)

From (1) and (2):

\[ \frac{[P_a]}{[P_c]} = \frac{k_a}{k_c} = \kappa \]  \hspace{1cm} (5)

Substitution of (3) and (4) in (1), combined with (5) yields:

\[ \frac{d[P_c]}{dt} = ([P]_0 - (1 + \kappa)[P_c]) ([\text{OH}^-]_0 - (2 + \kappa)[P_c])k_c \]  \hspace{1cm} (6)

or

\[ \int_o^{t} \frac{d[P_c]}{([P]_0 - (1 + \kappa)[P_c]) ([\text{OH}^-]_0 - (2 + \kappa)[P_c])} = k_c \int_o^{t} dt \]  \hspace{1cm} (7)
Integration yields:

$$\ln \frac{([P]_o - (1+\kappa)[P_c])}{([OH^-]_o - (2+\kappa)[P_c])} = ((2+\kappa)[P]_o - (1+\kappa)[OH^-]_o)k_f + \ln \frac{[P]_o}{[OH^-]_o} \tag{8}$$

References and Notes


Alkaline Hydrolysis


13. The assignment of the 3'- and 5'-phosphate diester peaks in the $^{31}$P NMR spectrum is based on a 2D $^{31}$P−$^1$H correlation spectrum and on a one dimensional phosphorus-proton coupled spectrum of the hydrolysis products of 3a. The 5'-phosphate diester resonates downfield from the 3'-phosphate diester.


15. Dowex K⁺ column was prepared by percolating a Dowex H⁺ column subsequently with 10 mL of a 1 M potassium chloride solution and 10 mL of a 1 M potassium hydroxide solution. Afterwards the column was washed with water until the eluent became neutral.


18. The sequence rules of Cahn, Ingold and Prelog state that substituents (in this case oxygen substituents) have priority over isotopes (¹⁸O). Therefore, the isotopomers $6 S_p^0$, $6 S_p^1$, $6 S_p^2$ or $7 S_p^0$, $7 S_p^1$, $7 S_p^2$ adopt the same configuration ($S_p$), whereas $6 R_p^0$, $6 R_p^1$, $6 R_p^2$ or $7 R_p^0$, $7 R_p^1$, $7 R_p^2$ have the $R_p$ configuration. See also: (a) Cahn, R.S.; Ingold, S.C.; Prelog, V. *Angew. Chem.* 1966, 78, 413. (b) Prelog, V.; Helmchen, G. *Angew. Chem.* 1982, 94, 614. (c) Cahn, R.S. *J. Chem. Educ.* 1964, 41, 116. (d) Herdering, W.; Seela, F. *J. Org. Chem.* 1985, 50, 5314.


25. The assignment of the carbons, based on a 2D $^1$H–$^{13}$C correlation spectrum is in agreement with Bajwa, G.S.; Bentrude, W.G. *Tetrahedron Lett.* 1978, 5, 421, reporting the $^{13}$C NMR spectrum of 1a except for the carbons of thymine.


27. Pyridine is used in order to neutralize the HI, which is formed during the oxidation reaction.
28. The stock solution was obtained by dissolving as much $\text{H}_2^{18}\text{O}/\text{H}_2^{16}\text{O}$ (ratio $^{18}\text{O}/^{16}\text{O}$: 96/4) and $\text{I}_2$ in a mixture of acetonitrile/diethyl ether (3:1 v/v) until a concentration is reached of $1\text{M} \text{H}_2^{18}\text{O}/\text{H}_2^{16}\text{O}$ and $0.2\text{M} \text{I}_2$. Diethyl ether is used in the stock solution in order to increase the solubility of iodine in acetonitrile.

2'-O-Methyl-cis-Adenosine 3',5'-Cyclic Methyl Monophosphate, a New Model System for cAMP. Aspects of Structure and Reactivity

Abstract

This study is focused on 2'-O-methyl-cis-adenosine 3',5'-cyclic methyl monophosphate (cis-4; cis describes the relationship between OMe (bound to phosphorus) and the adenine base) which is regarded as a model for enzyme-bound adenosine 3',5'-cyclic monophosphate (cAMP). In this complex the negative phosphate-charge is shielded in part via complexation with cationic sites on the enzyme surface. The synthesis, crystal structure, solution conformation (400 MHz $^1$H NMR) and kinetic-mechanistic aspects of the alkaline hydrolysis of cis-4 are reported. The methanol solvate of cis-4 crystallizes in the orthorhombic space group $P2_12_12_1$ and the cell dimensions are $a = 8.170(2)$ Å, $b = 9.249(1)$ Å, $c = 23.669(4)$ Å; $V = 1788.5(6)$ Å$^3$; $Z = 4$ molecules per cell. Least-squares refinement converged at $R = 0.062$ for 2006 observed reflections. The adenine bases are linked via $N_6$-H···$N_1$ and $N_6$-H···$N_7$ hydrogen bonds in such a way that infinite one-dimensional chains are formed. This hydrogen bond scheme is very similar to that observed in the structure of 2'-deoxy-3',5'-di-O-acetyl adenosine. An additional hydrogen bond is formed between the methanol, incorporated in the crystal structure, and the adenine base. The conformational preferences of the cis-4 dissolved in methanol have been determined with 400 MHz $^1$H NMR. It is found that the conformation found in the solid and solution states are practically the same. Hydrolysis of the title compound yields two acyclic phosphate diesters (a 3'- and a 5'-phosphate diester). The intermediates formed during this hydrolysis reaction are most likely five-coordinated phosphorus (P$^V$) compounds with a trigonal bipyramidal geometry and an equatorial-axial located 3',5'-dioxaphosphorinane ring.

*This chapter is based on:
4.1 Introduction

As described in chapter 1, adenosine 3',5'-cyclic monophosphate (cAMP; A) plays an important regulatory role in cells (both eukaryotic and prokaryotic). Our interest is primarily focused on the role of 3',5'-linked phosphodiester in the mechanism of action of cAMP. Most likely, a five-coordinated phosphorus (P^V) species is involved, either as an intermediate or as a transition state, in both the hydrolysis of cAMP to 5'-AMP and the activation of protein kinases by cAMP. These ideas have prompted several groups, including ours, to investigate model systems for the proposed activated state of cAMP. Essentially, these models incorporate a stabilized five-coordinated phosphorus atom (P^V) that is part of a 3',5'-dioxaphosphorinane ring, which is trans-fused with the ribose ring. These investigations support the idea that the 3',5'-dioxaphosphorinane ring has access to both an equatorial-axial (e,a) and a diequatorial (e,e) orientation with respect to the trigonal bipyramidal (TBP) structure of the five-coordinated phosphorus. As an alternative approach, the alkaline hydrolysis of a set of cAMP models which feature a cis-aryl-3',5'-cyclic-phosphotriester structure (B) has been studied. These studies independently support the ideas that (i) hydrolysis proceeds via a five-coordinated phosphorus structure, and (ii) both (e,e) and (e,a) orientations of the 3',5'-dioxaphosphorinane ring are feasible as long as five-coordination is present. In the course of these investigations 2'-O-methyl-cis-adenosine 3',5'-cyclic methyl monophosphate, the key compound of the present study, has been synthesized and character-
4.2 Results and Discussion

4.2.1 Synthesis

The preparation of cis-4 is outlined in Scheme 4.1. 2'-O-Methyl adenosine is brought into reaction with bis(N,N-diisopropylamino)methoxyphosphine and 1H-

![Scheme 4.1 Preparation of cis-4.](image_url)
tetrazole used as catalyst. This results in the formation of 2′-O-methyl-cis-adenosine 3′,5′-cyclic methyl monophosphate (cis-3) and its trans epimer in a ratio of ca. 3:2 (cis/trans). At elevated temperature and in the presence of 1H-tetrazole, the trans diastereoisomer completely inverts into the cis-diastereoisomer. After evaporation of the volatiles of the reaction mixture, the residue was suspended in ethyl acetate and filtered. The filtrate was washed with a sodium bicarbonate solution. Evaporation of ethyl acetate, and suspension of the residue in diethyl ether, afforded cis-3 as a white solid (yield 39%). Oxidation of cis-3, using tert-butyl hydroperoxide as oxidation agent, yielded pure crystalline cis-4 (yield 72%) (see Scheme 4.1).

4.2.2 Crystal Structure

The molecular structure of cis-4 with its atomic numbering is shown in Figure 4.1. The fractional coordinates, along with equivalent isotropic temperature factors are summarized in Table 4.1. The bond distances and bond angles between non-hydrogen atoms are given in Table 4.2 and Table 4.3, respectively. Table 4.4 contains selected torsion angles for non-hydrogen atoms, describing the geometry of the six-membered dioxaphosphorinane ring, the furanose ring as well as the conformation around the glycosidic (C1′-N9) bond. Table 4.5 lists the hydrogen-bond geometries (distances and bond angles).

4.2.2.1 Conformation of the Ribose Ring

The ribose ring is found in the C$_{3′}$-endo puckered form. A more precise description of the ribose ring conformation can be given with the pseudorotation concept, which relates the five endocyclic torsion angles mathematically to a phase angle of pseudorotation (P), which actually indicates which part of the ring is bent, and a puckering amplitude ($\nu_{\text{max}}$), which is an upper limit for the values of the endocyclic torsion angles. From the crystal structure data of compound cis-4, five endocyclic torsion angles ($\nu_0$...$\nu_4$) are derived (Table 4.4): $\nu_0[C_4′-O_4′-C_1′-C_2′] = -23.9°$, $\nu_1[O_4′-C_1′-C_2′-C_3′] = -4.5°$, $\nu_2[C_1′-C_2′-C_3′-C_4′] = 29.4°$, $\nu_3[C_2′-C_3′-C_4′] = 180°$, $\nu_4[C_3′-C_4′] = 0°$. 
4.2 Results and Discussion

Results and Discussion

From these angles, the parameters \( P \) and \( \nu_{\text{max}} \) can be derived, using the formula:

\[
\nu_j = \nu_{\text{max}} \cdot \cos(P + (j-2) \cdot 144)^\circ
\]

A value of 49.1° for \( P \) and a \( \nu_{\text{max}} \)-value of 46.1° is found for the ribose ring of compound \( cis-4 \). It follows from these results that the structure of the ribose ring can best be described as a twist \( (4T^3) \) conformation, which is characteristic for cyclic nucleotides (Table 4.6). This conformation is embedded between two geometries: a twist \( (4T^3) \) and an envelope \( (4E) \) geometry. From Table 4.6, showing the pseudorotational parameters of several cyclic nucleotides, it
Table 4.1 Refined Fractional Coordinates of Non-Hydrogen Atoms, and Equivalent Isotropic Thermal Parameters $U_{eq}$ ($\overline{A}^2$); Estimated Standard Deviations are given in Parentheses.

<table>
<thead>
<tr>
<th></th>
<th>x</th>
<th>y</th>
<th>z</th>
<th>$U_{eq}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>P</td>
<td>0.0720(2)</td>
<td>0.5992(2)</td>
<td>0.20366(7)</td>
<td>0.0182(4)</td>
</tr>
<tr>
<td>O$_2'$</td>
<td>0.3691(4)</td>
<td>0.5267(3)</td>
<td>0.3520(1)</td>
<td>0.027(1)</td>
</tr>
<tr>
<td>O$_3'$</td>
<td>0.1366(5)</td>
<td>0.5706(4)</td>
<td>0.2654(2)</td>
<td>0.019(1)</td>
</tr>
<tr>
<td>O$_4'$</td>
<td>0.4498(5)</td>
<td>0.8356(4)</td>
<td>0.2858(2)</td>
<td>0.020(1)</td>
</tr>
<tr>
<td>O$_5'$</td>
<td>0.2172(5)</td>
<td>0.6712(4)</td>
<td>0.1690(2)</td>
<td>0.020(1)</td>
</tr>
<tr>
<td>O$_6$</td>
<td>-0.0509(5)</td>
<td>0.7293(4)</td>
<td>0.2086(2)</td>
<td>0.024(1)</td>
</tr>
<tr>
<td>O$_7$</td>
<td>0.0100(5)</td>
<td>0.4685(5)</td>
<td>0.1766(2)</td>
<td>0.024(1)</td>
</tr>
<tr>
<td>N$_1$</td>
<td>0.7960(5)</td>
<td>1.0852(6)</td>
<td>0.4744(2)</td>
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</tr>
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<td>N$_3$</td>
<td>0.7328(6)</td>
<td>0.8856(6)</td>
<td>0.4142(2)</td>
<td>0.022(2)</td>
</tr>
<tr>
<td>N$_6$</td>
<td>0.6063(5)</td>
<td>1.2581(6)</td>
<td>0.5017(2)</td>
<td>0.023(2)</td>
</tr>
<tr>
<td>N$_7$</td>
<td>0.3614(6)</td>
<td>1.0865(5)</td>
<td>0.4284(2)</td>
<td>0.019(2)</td>
</tr>
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<td>N$_9$</td>
<td>0.4492(7)</td>
<td>0.8895(5)</td>
<td>0.3817(2)</td>
<td>0.018(1)</td>
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<tr>
<td>C$_2$</td>
<td>0.8292(7)</td>
<td>0.9662(7)</td>
<td>0.4462(3)</td>
<td>0.023(2)</td>
</tr>
<tr>
<td>C$_4$</td>
<td>0.5804(7)</td>
<td>0.9419(6)</td>
<td>0.4119(3)</td>
<td>0.018(2)</td>
</tr>
<tr>
<td>C$_5$</td>
<td>0.5264(6)</td>
<td>1.0626(6)</td>
<td>0.4400(3)</td>
<td>0.018(2)</td>
</tr>
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<td>C$_6$</td>
<td>0.6409(7)</td>
<td>1.1388(6)</td>
<td>0.4726(3)</td>
<td>0.019(2)</td>
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<tr>
<td>C$_8$</td>
<td>0.3233(7)</td>
<td>0.9843(6)</td>
<td>0.3937(3)</td>
<td>0.021(2)</td>
</tr>
<tr>
<td>C$_1'$</td>
<td>0.4502(7)</td>
<td>0.7768(6)</td>
<td>0.3412(3)</td>
<td>0.021(2)</td>
</tr>
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<td>C$_2'$</td>
<td>0.3027(4)</td>
<td>0.6689(3)</td>
<td>0.3453(1)</td>
<td>0.021(2)</td>
</tr>
<tr>
<td>C$_3'$</td>
<td>0.2249(6)</td>
<td>0.6909(6)</td>
<td>0.2892(3)</td>
<td>0.016(2)</td>
</tr>
<tr>
<td>C$_4'$</td>
<td>0.3661(7)</td>
<td>0.7325(7)</td>
<td>0.2518(2)</td>
<td>0.020(2)</td>
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<tr>
<td>C$_5'$</td>
<td>0.3123(7)</td>
<td>0.7881(6)</td>
<td>0.1950(3)</td>
<td>0.023(2)</td>
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<td>C$_6'$</td>
<td>-0.2017(7)</td>
<td>0.7086(7)</td>
<td>0.2411(3)</td>
<td>0.028(2)</td>
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<tr>
<td>C$_2''$</td>
<td>0.2585(4)</td>
<td>0.4319(3)</td>
<td>0.3805(1)</td>
<td>0.033(3)</td>
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<tr>
<td>O$_1$</td>
<td>0.8842(7)</td>
<td>0.6219(5)</td>
<td>0.3781(2)</td>
<td>0.051(2)</td>
</tr>
<tr>
<td>C$_1$</td>
<td>0.8249(9)</td>
<td>0.5274(8)</td>
<td>0.4212(3)</td>
<td>0.059(3)</td>
</tr>
</tbody>
</table>

$U_{eq} = (1/3)$trace of orthogonalized $U_{ij}$ tensor.
Table 4.2 Bond distances (Å).

<table>
<thead>
<tr>
<th>Bond</th>
<th>Length</th>
<th>Bond</th>
<th>Length</th>
</tr>
</thead>
<tbody>
<tr>
<td>P–O$_6$</td>
<td>1.572(4)</td>
<td>N$_3$–C$_4$</td>
<td>1.351(8)</td>
</tr>
<tr>
<td>P–O$_7$</td>
<td>1.459(5)</td>
<td>N$_6$–C$_6$</td>
<td>1.331(8)</td>
</tr>
<tr>
<td>P–O$_3'$</td>
<td>1.576(5)</td>
<td>N$_7$–C$_5$</td>
<td>1.393(7)</td>
</tr>
<tr>
<td>P–O$_5'$</td>
<td>1.589(5)</td>
<td>N$_7$–C$_8$</td>
<td>1.290(8)</td>
</tr>
<tr>
<td>O$_6$–C$_6'$</td>
<td>1.465(7)</td>
<td>N$_9$–C$_4$</td>
<td>1.377(8)</td>
</tr>
<tr>
<td>O$_4$–C$_1'$</td>
<td>1.420(8)</td>
<td>N$_9$–C$_8$</td>
<td>1.381(7)</td>
</tr>
<tr>
<td>O$_4$–C$_4'$</td>
<td>1.423(7)</td>
<td>N$_9$–C$_1'$</td>
<td>1.416(8)</td>
</tr>
<tr>
<td>O$_2$–C$_2'$</td>
<td>1.432(4)</td>
<td>C$_4$–C$_5$</td>
<td>1.372(8)</td>
</tr>
<tr>
<td>O$_2$–C$_2''$</td>
<td>1.428(4)</td>
<td>C$_5$–C$_6$</td>
<td>1.403(8)</td>
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<tr>
<td>O$_3$–C$_3'$</td>
<td>1.441(7)</td>
<td>C$_1'$–C$_2'$</td>
<td>1.568(6)</td>
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<td>O$_5$–C$_5'$</td>
<td>1.467(7)</td>
<td>C$_2'$–C$_3'$</td>
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<td>N$_1$–C$_6$</td>
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<td>1.323(8)</td>
<td>O$_1$–C$_1$</td>
<td>1.428(9)</td>
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</tbody>
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Table 4.3 Bond angles (°).

<table>
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<th>Bond</th>
<th>Angle</th>
<th>Bond</th>
<th>Angle</th>
</tr>
</thead>
<tbody>
<tr>
<td>$O_6 - P - O_7$</td>
<td>116.4(2)</td>
<td>$N_7 - C_5 - C_4$</td>
<td>110.2(5)</td>
</tr>
<tr>
<td>$O_6 - P - O_3$</td>
<td>105.9(2)</td>
<td>$N_7 - C_5 - C_6$</td>
<td>132.4(5)</td>
</tr>
<tr>
<td>$O_6 - P - O_5$</td>
<td>101.2(2)</td>
<td>$C_4 - C_5 - C_6$</td>
<td>117.5(5)</td>
</tr>
<tr>
<td>$O_7 - P - O_3$</td>
<td>112.6(3)</td>
<td>$N_1 - C_6 - N_6$</td>
<td>118.9(5)</td>
</tr>
<tr>
<td>$O_7 - P - O_5$</td>
<td>112.3(3)</td>
<td>$N_1 - C_6 - C_5$</td>
<td>117.1(5)</td>
</tr>
<tr>
<td>$O_3' - P - O_5'$</td>
<td>107.4(2)</td>
<td>$N_6 - C_6 - C_5$</td>
<td>124.0(5)</td>
</tr>
<tr>
<td>$P - O_6 - C_6$</td>
<td>118.4(4)</td>
<td>$N_7 - C_8 - N_9$</td>
<td>114.6(5)</td>
</tr>
<tr>
<td>$C_1' - O_4' - C_4'$</td>
<td>105.5(4)</td>
<td>$O_4' - C_1' - N_9$</td>
<td>110.1(4)</td>
</tr>
<tr>
<td>$C_2' - O_2' - C_2''$</td>
<td>112.1(3)</td>
<td>$O_4' - C_1' - C_2'$</td>
<td>107.4(4)</td>
</tr>
<tr>
<td>$P - O_3' - C_3'$</td>
<td>113.6(4)</td>
<td>$N_9 - C_1' - C_2'$</td>
<td>115.0(5)</td>
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<td>$P - O_5' - C_5'$</td>
<td>119.2(4)</td>
<td>$O_2' - C_2' - C_1'$</td>
<td>107.5(3)</td>
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<td>$O_2' - C_2' - C_3'$</td>
<td>112.8(3)</td>
</tr>
<tr>
<td>$C_2 - N_3 - C_4$</td>
<td>110.8(5)</td>
<td>$C_1' - C_2' - C_3'$</td>
<td>100.7(4)</td>
</tr>
<tr>
<td>$C_5 - N_7 - C_8$</td>
<td>104.0(5)</td>
<td>$O_3' - C_3' - C_2'$</td>
<td>117.2(4)</td>
</tr>
<tr>
<td>$C_4 - N_9 - C_8$</td>
<td>104.5(5)</td>
<td>$O_3' - C_3' - C_4'$</td>
<td>110.6(5)</td>
</tr>
<tr>
<td>$C_4 - N_9 - C_1'$</td>
<td>127.3(5)</td>
<td>$C_2' - C_3' - C_4'$</td>
<td>103.5(4)</td>
</tr>
<tr>
<td>$C_8 - N_9 - C_1'$</td>
<td>127.6(5)</td>
<td>$O_4' - C_4' - C_3'$</td>
<td>102.0(4)</td>
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<tr>
<td>$N_1 - C_2 - N_3$</td>
<td>129.7(5)</td>
<td>$O_4' - C_4' - C_5'$</td>
<td>114.6(5)</td>
</tr>
<tr>
<td>$N_3 - C_4 - N_9$</td>
<td>127.1(5)</td>
<td>$C_3' - C_4' - C_5'$</td>
<td>112.9(5)</td>
</tr>
<tr>
<td>$N_3 - C_4 - C_5$</td>
<td>126.2(6)</td>
<td>$O_5' - C_5' - C_4'$</td>
<td>106.1(5)</td>
</tr>
<tr>
<td>$N_9 - C_4 - C_5$</td>
<td>106.7(5)</td>
<td></td>
<td></td>
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</tbody>
</table>
4.2 Results and Discussion

Table 4.4 Selected torsion angles (°) for non-hydrogen atoms.

<table>
<thead>
<tr>
<th>Bond</th>
<th>Torsion angle</th>
<th>Bond</th>
<th>Torsion angle</th>
</tr>
</thead>
<tbody>
<tr>
<td>(-O_4,-C_1,-C_2,-C_3, (\nu_0))</td>
<td>-23.9(5)</td>
<td>(-N_9,-C_1,-O_4,)</td>
<td>69.2(7)</td>
</tr>
<tr>
<td>(-O_4,-C_1,-C_2,-O_2, (\nu_1))</td>
<td>-4.5(5)</td>
<td>(-N_9,-C_1,-C_2,)</td>
<td>-52.3(8)</td>
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<tr>
<td>(-C_2,-C_3,-C_4, (\nu_2))</td>
<td>29.4(5)</td>
<td>(-N_9,-C_1,-C_2,)</td>
<td>137.9(5)</td>
</tr>
<tr>
<td>(-C_2,-C_3,-O_4', (\nu_3))</td>
<td>-45.5(5)</td>
<td>(-N_9,-C_1,-C_2,-O_2,)</td>
<td>-123.5(4)</td>
</tr>
<tr>
<td>(-O_4',-C_4',-C_3, (\nu_4))</td>
<td>42.5(5)</td>
<td>(-N_9,-C_1,-C_2,-C_3,)</td>
<td>118.3(5)</td>
</tr>
<tr>
<td>(-C_4,-N_9,-C_1',-O_4,)</td>
<td>164.8(5)</td>
<td>(-C_4',-O_4,-C_1,-N_9)</td>
<td>-149.7(4)</td>
</tr>
<tr>
<td>(-O_2,-C_2,-C_3,-C_4,)</td>
<td>-84.8(4)</td>
<td>(-O_6,-P-O_3,-C_3,)</td>
<td>-62.3(4)</td>
</tr>
<tr>
<td>(-O_2,-C_2,-C_3,-O_3,)</td>
<td>37.1(5)</td>
<td>(-O_5,-P-O_3,-C_3,)</td>
<td>45.2(4)</td>
</tr>
<tr>
<td>(-C_1,-C_2,-C_3,-O_3,)</td>
<td>151.4(4)</td>
<td>(-O_6,-P-O_5,-C_5,)</td>
<td>65.7(4)</td>
</tr>
<tr>
<td>(-O_3,-C_3,-C_4,-O_4,)</td>
<td>-171.8(4)</td>
<td>(-O_7,-P-O_5,-C_5,)</td>
<td>-169.4(4)</td>
</tr>
<tr>
<td>(-C_2,-C_3,-C_4,-C_5,)</td>
<td>-168.9(4)</td>
<td>(-O_7,-P-O_5,-C_5,)</td>
<td>-45.0(4)</td>
</tr>
<tr>
<td>(-O_3,-C_3,-C_4,-C_5,)</td>
<td>64.7(6)</td>
<td>(-P-O_3,-C_3,-C_4,)</td>
<td>-56.6(5)</td>
</tr>
<tr>
<td>(-O_4,-C_4,-C_5,-O_5,)</td>
<td>-175.2(4)</td>
<td>(-P-O_3,-C_3,-C_2,)</td>
<td>-174.8(3)</td>
</tr>
<tr>
<td>(-C_3,-C_4,-C_5,-O_5,)</td>
<td>-59.0(6)</td>
<td>(-P-O_5,-C_5,-C_4,)</td>
<td>51.5(5)</td>
</tr>
</tbody>
</table>
| \(-C_4,-N_9,-C_1','-O_4, (\chi)\) | -100.7(6)   **
Table 4.5 Hydrogen-bond geometries (distances in Å, angles in °).

<table>
<thead>
<tr>
<th>Donor</th>
<th>Acceptor</th>
<th>D···A</th>
<th>D−H</th>
<th>H···A</th>
<th>D−H···A</th>
</tr>
</thead>
<tbody>
<tr>
<td>O1−H3···N3</td>
<td>2.865(7)</td>
<td>0.90</td>
<td>1.965</td>
<td>180</td>
<td></td>
</tr>
<tr>
<td>N6−H1···N1'</td>
<td>2.974(6)</td>
<td>0.90</td>
<td>2.074</td>
<td>180</td>
<td></td>
</tr>
<tr>
<td>N6−H2···N7''</td>
<td>3.024(7)</td>
<td>0.90</td>
<td>2.124</td>
<td>180</td>
<td></td>
</tr>
</tbody>
</table>

The symmetry operation is performed on the acceptor atom A:
(i) $\frac{1}{2} + x, 2\frac{1}{2} - y, 1 - z$; (ii) $\frac{1}{2} + x, 2\frac{1}{2} - y, 1 - z$

can be observed that the values of phase angle and amplitude of pseudorotation ($P$ and $\nu_{\text{max}}$) vary within rather narrow ranges ($P$: 15-51°; $\nu_{\text{max}}$: 43-50°). The pseudorotational parameters of the ribose ring in *cis*-4 fit perfectly in the data ranges as shown in Table 4.6.

4.2.2.2 Conformation of the 3′,5′-Dioxaphosphorinane Ring

The 3′,5′-dioxaphosphorinane ring adopts a distorted-chair conformation. In order to characterize this conformation three planes have been defined: plane 1 is the least-squares plane through the atoms O3', O5', C3, and C5, (-0.411X + 0.286Y - 0.0091Z = 1; $\sigma_{\text{plane}} = 0.016$ Å; X, Y and Z are measured in Angstroms along with the crystallographic a, b and c axis). Plane 2 is the plane through O3', P and O5', (-0.0512X + 0.0147Y + 0.0450Z = 1), and plane 3 is the plane defined by the atoms C3', C4', and C5', (-0.00355X + 0.111Y + 0.0436Z = 1). The dihedral angle between the planes 1 and 2 is 39.4°, whereas the dihedral angle between the planes 1 and 3 is 56.7°. This means that the ring resides in a distorted-chair conformation in which P and C4' are on opposite sides with respect to plane 1. The distances of the atoms P and C4' to plane 1 are 0.59 Å and 0.69 Å, respectively. This leads to the conclusion that the P part is flattened in comparison with
Table 4.6 *Pseudorotational parameters and some conformational properties of the ribose ring in 3',5'-cyclic nucleotides.*  

<table>
<thead>
<tr>
<th></th>
<th>cis-4</th>
<th>A&lt;sup&gt;b&lt;/sup&gt;</th>
<th>B</th>
<th>C&lt;sub&gt;A&lt;/sub&gt;&lt;sup&gt;c&lt;/sup&gt;</th>
<th>C&lt;sub&gt;B&lt;/sub&gt;&lt;sup&gt;c&lt;/sup&gt;</th>
<th>D</th>
<th>E</th>
<th>F</th>
<th>Mean Value&lt;sup&gt;d&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>$P$ (°)</td>
<td>49.1</td>
<td>46.5</td>
<td>27.6</td>
<td>33</td>
<td>15</td>
<td>44.5</td>
<td>51.0</td>
<td>48.8</td>
<td>39(13)</td>
</tr>
<tr>
<td>$\nu_{\text{max}}$ (°)</td>
<td>46.1</td>
<td>46.9</td>
<td>43.4</td>
<td>49</td>
<td>47</td>
<td>49.9</td>
<td>47.3</td>
<td>47.5</td>
<td>47(2)</td>
</tr>
<tr>
<td>Ribose pucker</td>
<td>$^{4}T^{3}$</td>
<td>$^{4}T^{3}$</td>
<td>$^{3}T_{4}$</td>
<td>$^{3}T_{4}$</td>
<td>$^{3}T_{2}$</td>
<td>$^{4}T^{3}$</td>
<td>$^{4}T^{3}$</td>
<td>$^{4}T^{3}$</td>
<td>–</td>
</tr>
</tbody>
</table>

* The estimated standard deviations are given in parentheses.

<sup>b</sup> Compound names and references: A: 5-iodo-2'-cis-deoxyuridine 3',5'-cyclic methyl monophosphate<sup>9a</sup>; B: inosine 3',5'-cyclic monophosphate<sup>9b</sup>; C: adenosine 3',5'-cyclic monophosphate sodium salt<sup>9c</sup> (there are two molecules of the salt in the asymmetric unit (C<sub>A</sub> and C<sub>B</sub>)); D: cis-adenosine 3',5'-cyclic ethyl monophosphate<sup>9d</sup>; E: 8-((2-aminoethyl)amino)adenosine cyclic 3',5'-monophosphate tetrahydrate<sup>9e</sup>; F: 2'-acetyl-cis-uridine 3',5'-cyclic benzyl monophosphate<sup>9f</sup>.

<sup>c</sup> Compound C remains in two different geometries.

<sup>d</sup> The mean values and standard deviations of the tabulated values.
Table 4.7 Torsion angles (°) of the 3',5'-dioxaphosphorinane ring in 3',5'-cyclic nucleotides.\(^a\)

<table>
<thead>
<tr>
<th></th>
<th>cis-4</th>
<th>A(^b)</th>
<th>B</th>
<th>C(_A)(^c)</th>
<th>C(_B)(^d)</th>
<th>D</th>
<th>E</th>
<th>F</th>
<th>Mean Value(^d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[P−O₅−C₅−C₄]</td>
<td>51.5(5)</td>
<td>52.1(8)</td>
<td>59.8(3)</td>
<td>57.2</td>
<td>56.6</td>
<td>50.9</td>
<td>60.5</td>
<td>53.9</td>
<td>55(4)</td>
</tr>
<tr>
<td>[O₅−C₅−C₄−C₃]</td>
<td>-59.0(6)</td>
<td>-66.0(10)</td>
<td>-64.5(3)</td>
<td>-61.1</td>
<td>-60.8</td>
<td>-59.3</td>
<td>-62.9</td>
<td>-58.9</td>
<td>-62(3)</td>
</tr>
<tr>
<td>[C₅−C₄−C₃−O₃]</td>
<td>64.7(6)</td>
<td>69.4(10)</td>
<td>69.1(3)</td>
<td>66.6</td>
<td>68.3</td>
<td>65.9</td>
<td>66.8</td>
<td>67.1</td>
<td>67(2)</td>
</tr>
<tr>
<td>[C₄−C₃−O₃−P]</td>
<td>-56.6(5)</td>
<td>-52.8(8)</td>
<td>-59.7(3)</td>
<td>-58.1</td>
<td>-61.9</td>
<td>-54.1</td>
<td>-60.0</td>
<td>-60.9</td>
<td>-58(3)</td>
</tr>
<tr>
<td>[C₃−O₃−P−O₅]</td>
<td>45.2(4)</td>
<td>36.1(7)</td>
<td>47.8(2)</td>
<td>46.0</td>
<td>48.9</td>
<td>39.7</td>
<td>50.1</td>
<td>49.3</td>
<td>45(5)</td>
</tr>
<tr>
<td>[O₃−P−O₅−C₅]</td>
<td>-45.0(4)</td>
<td>-37.4(9)</td>
<td>-51.0(2)</td>
<td>-49.0</td>
<td>-49.9</td>
<td>-40.3</td>
<td>-53.6</td>
<td>-49.1</td>
<td>-47(6)</td>
</tr>
</tbody>
</table>

\(^a\) The estimated standard deviations are given in parentheses.
\(^b\) Compound names and references: A: 5-ido-2'-cis-deoxyuridine 3',5'-cyclic methyl monophosphate\(^{9a}\); B: inosine 3',5'-cyclic monophosphate\(^{9b}\); C: adenosine 3',5'-cyclic monophosphate sodium salt\(^{9c}\) (there are two molecules of the salt in the asymmetric unit (C\(_A\) and C\(_B\)); D: cis-adenosine 3',5'-cyclic ethyl monophosphate\(^{9d}\); E: 8-[(2-aminoethyl)amino]adenosine cyclic 3',5'-monophosphate tetrahydrate\(^{9e}\); F: 2'-acetyl-cis-uridine 3',5'-cyclic benzyl monophosphate\(^{9f}\).
\(^c\) Compound C remains in two different geometries.
\(^d\) The mean values and standard deviations are reported of the tabulated values.
the C4' part of the 3',5'-dioxaphosphorinane ring. The distortion of the 3',5'-dioxaphosphorinane ring can also be characterized by the significantly different values of the bond angles C3'-O3'-P (113.6°) and C5'-O5'-P (119.2°). The largest endocyclic torsion angle in the dioxaphosphorinane ring is found in the C3'-C4' bond ([C5'-C4'-C3'-O3'] = 64.7°), the trans junction with the ribose ring. The endocyclic P-O bonds show the smallest ring torsion angles ([O3'-P-O5'-C5'] = -45.0°, [C3'-O3'-P-O5'] = 45.2°) (Table 4.7). These values also demonstrate the P part is flattened in comparison with the C4' part of the six-membered ring.

The values of the torsion angles of the 3',5'-dioxaphosphorinane ring in cis-4 shown in Table 4.7 fit perfectly within the data ranges defined by several 3',5'-cyclic nucleotides.9

4.2.2.3 Geometry and Orientation of the Base

The torsion angle \( \chi[O4'-C_1'-N_9-C_4'] \) about the glycosidic bond is -100.7° and corresponds with the anti conformation. The values of the bond lengths and angles for the purine base are similar to those reported for the standard adenine ring.10 Largest deviations were found for the N1-C2 and N7-C8 (bond lengths smaller than the standard values by 0.023 and 0.022 Å, respectively). The least-squares plane through the nine atoms of the purine base is \(-0.255X - 0.559Y + 0.789Z = 1.610; \sigma_{plane} = 0.019 \) Å; where X, Y and Z are measured in Angstroms along with the crystallographic a, b and c axis. The dihedral angle between the planes of the pyrimidine and imidazole ring is 1.7°. The largest torsion angle in the adenine ring is found about the N3-C4 and N7-C8 bond ([C2-N3-C4-C3] = -2.4°, [C5-N7-C8-N9] = 1.6°) resulting in a small deviation from planarity (see Table 4.4).

4.2.2.4 Molecular Packing and Hydrogen Bonding

In Figure 4.2 is illustrated that each NH2 group in cis-4 is hydrogen bonded with two molecules. There is one hydrogen bond between N6 and N7 (\( \frac{1}{4} + x, 2\frac{1}{2} - y, \)
Figure 4.2 Projection of a part of the structure down the c axis, showing the hydrogen-bonded chain in the ab plane. Hydrogen atoms not involved in hydrogen bonds have been omitted for clarity. The cell origin is indicated by the black dot. Molecule A is at 1 - x, -\(\frac{1}{2}\) + y, \(\frac{1}{2}\) - z; molecule B is at \(\frac{1}{2}\) - x, 2 - y, -\(\frac{1}{2}\) + z; and molecule C is at 1\(\frac{1}{2}\) - x, 2 - y, -\(\frac{1}{2}\) + z.

1 - z) and one between N\(_6\) and N\(_1\) (-\(\frac{1}{4}\) + x, 2\(\frac{1}{2}\) - y, 1 - z) (see Table 4.5). The hydrogen bond accepting molecules are one translation apart along the a axis, leading to a one-dimensional chain of hydrogen bonded molecules. The direction of the one-dimensional chain is almost parallel to the a axis. The hydrogen bonds between the bases are very similar to those in 2'-deoxy-3',5'-di-O-acetyl adenosine.\(^{11}\) An additional hydrogen bond is formed between the OH-group of the methanol molecule, which is incorporated in the crystal structure, and the adenine base: O\(_1\) - H\(_3\) \(\cdots\) N\(_3\).
4.2 Results and Discussion

4.2.3 Conformation in Solution

The conformation of cis-4 in methanol-$d_4$, was determined at room temperature with $^1$H NMR at 400.13 MHz. The set of experimental coupling constants are summarized in Table 4.8.

Table 4.8 Proton—proton and proton—phosphorus coupling constants (in Hz) of cis-4, measured in methanol-$d_4$ at 20 °C.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value$^a$</th>
<th>Parameter</th>
<th>Value$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$J_{H(1')H(2')}$</td>
<td>0.8</td>
<td>$J_{H(4')H(5'')}$</td>
<td>4.7</td>
</tr>
<tr>
<td>$J_{H(2')H(3')}$</td>
<td>5.1</td>
<td>$J_{H(5')H(5'')}$</td>
<td>-9.3</td>
</tr>
<tr>
<td>$J_{H(3')H(4')}$</td>
<td>9.8</td>
<td>$J_{H(5')P}$</td>
<td>$-0^b$</td>
</tr>
<tr>
<td>$J_{H(3')P}$</td>
<td>1.4</td>
<td>$J_{H(5'')P}$</td>
<td>22.3</td>
</tr>
<tr>
<td>$J_{H(4')H(5')}$</td>
<td>10.6</td>
<td>$J_{\text{POMe}}$</td>
<td>11.2</td>
</tr>
</tbody>
</table>

$^a$ Estimated error ±0.1 Hz
$^b$ Estimated error ±0.2 Hz

4.2.3.1 Conformation of the Ribose Ring

The conformational analysis of the sugar ring of cis-4 was performed with the PSEUROT program$^{12}$. The sets of vicinal H—H coupling constants ($J_{H(1')H(2')}$, $J_{H(2')H(3')}$, $J_{H(3')H(4')}$) measured for cis-4 (Table 4.8) were used as input data. PSEUROT calculates the best-fit conformational parameters of two sugar structures (a North (C$_{3}$-endo) and a South (C$_{2}$-endo) conformation) participating in a rapid conformational equilibrium as well as the equilibrium composition. PSEUROT converged towards a single conformation (North), characterized by a phase angle ($P$) of 23.7° and a maximum puckering amplitude ($\nu_{\text{max}}$) of 41.4°. The five endocyclic torsion angles ($\nu_0...\nu_4$) can be calculated from $P$ and $\nu_{\text{max}}$ according to the
formula: \[ \nu_j = \nu_{\text{max}} \cdot \cos(P + (j-2) \cdot 144) \],
\[ \nu_1[C_4'\text{--}O_4'\text{--}C_1'\text{--}C_2'] = -4.1^\circ, \]
\[ \nu_2[C_1'\text{--}C_2'\text{--}C_3'\text{--}C_4'] = 37.9^\circ, \]
\[ \nu_3[C_2'\text{--}C_3'\text{--}C_4'\text{--}O_4'] = -40.4^\circ, \]
\[ \nu_4[C_3'\text{--}C_4'\text{--}O_4'\text{--}C_1'] = 27.5^\circ. \]
From these results it can be derived that the structure of the ribose ring in cis-4 resides in a twist (3T\(_4\)) conformation, which is closely related to the 4T\(^3\) conformation in the solid state. This conformation is embedded between two geometries: a twist (3T\(_4\)) and an envelope (3E) geometry.

4.2.3.2 Conformation of the 3',5'-Dioxaphosphorinane Ring
The rigidity of the trans-fused six-membered ring allows us to take only a few conformations into account: (i) a chair, (ii) two boat conformations (one with P and C\(_4\)' as prow and one with O\(_5\)' and C\(_3\)' as prow) or (iii) a twist-boat geometry. Discrimination between these geometries is based on the values of two H--P coupling constants (\(3J_{PH(S')}\) and \(3J_{PH(S'')}\), describing the conformation around the O\(_5\)'--C\(_5\)' bond) and two H--H coupling constants (\(3J_{H(4')H(S')}\) and \(3J_{H(4')H(S'')}\), characterizing the conformation around the C\(_4\)'--C\(_5\)' bond). Generally the H--P coupling constants of phosphates fit into the classical Karplus-type relations. From the work of Bentrode et al.\(^{14}\) on cyclic phosphates a trans orientation of a P--O--C--H fragment corresponds with a \(3J_{POCH}\) value of \(\approx 22.5\) Hz, whereas a gauche conformation is related with a \(3J_{POCH}\) value of \(\approx 1\) Hz. From Table 4.8 is concluded that H\(_5\)' and P adopt a trans orientation ([P--O--C--H\(_5\)'] = \(\pm 60^\circ\)). These results are in agreement with the chair conformation. The boat geometry, in which O\(_3\)', O\(_5\)', C\(_3\)' and C\(_5\)' are approximately in the same plane while P and C\(_4\)' are both located on the exo side of this plane, would yield approximately identical values for \(3J_{PH(S')}\) and \(3J_{PH(S'')}\). Whereas the twist-boat and the boat conformation, in which O\(_5\)' and C\(_3\)' are located on the endo side of the least-squares plane through P, C\(_4\)', C\(_5\)' and O\(_3\)', would correspond with a large value for \(3J_{POCH(S')}\) (\(\approx 20-23\) Hz) and a small value for \(3J_{POCH(S'')}\).

A second clue for characterization of the geometry of the 3',5'-dioxaphospho-
rinane ring is provided by the coupling constants $^{3}J_{H(4')H(5')}$ and $^{3}J_{H(4')H(5'')}$, from which the conformation around the $C_{4'}.C_{5'}$ bond can be deduced. By using the generalized Karplus equation of Altona et al., $^{3}J_{H(4')H(5')}$ and $^{3}J_{H(4')H(5'')}$, are calculated as a function of the torsion angle $[O_{5'}.C_{5'}-C_{4'}-O_{4'}]$ (see Figure 2.4 from Chapter 2). This graph combined with the values of Table 4.8 indicate that the torsion angle adopts a value of approximately $180^\circ$, which is in agreement with the chair conformation of the six-membered ring.

4.2.4 Hydrolysis of Cis-4

In order to obtain information about the mechanistic aspects of the alkaline hydrolysis of cis-4, the kinetics and product distribution have been investigated by means of $^{31}$P NMR. The hydrolysis reaction was assumed to obey second order kinetics: first order in cyclic phosphate triester and first order in hydroxide. Using equivalent amounts of cis-4 and OH$^-$ the kinetic equation becomes:

$$\frac{1}{[P]} = k^{\text{exp}}t + \frac{1}{[P]_0}$$

(1)

With: $[P]$ = Concentration of cis-4 at time $t=t$. [mol.l$^{-1}$]  
$[P]_0$ = Initial concentration of cis-4. [mol.l$^{-1}$]  
$k^{\text{exp}}$ = Experimental second order reaction rate constant. [l.mol$^{-1}$.s$^{-1}$]

The data on the hydrolysis reaction of cis-4 are visualized in Figure 4.3; the linear relationship ($r = 0.999$) between $[P]^{-1}$ and $t$ substantiates the assumption that the hydrolysis proceeds via a second order reaction mechanism. In Table 4.9 the $k^{\text{exp}}$ value is tabulated. Two different acyclic products were formed: a $3'$-phosphate diester and a $5'$-phosphate diester (ratio $3'/5' = 65:35$) (see Scheme 4.2). Comparing the product distribution in the hydrolysis of cis-4 with those of 2'-O-methyl-cis-adenosine 3',5'-cyclic phenyl monophosphate$^4$ (Table 4.9) it is remarkable that during hydrolysis the latter compound affords 52% of 3',5'-cyclic phosphate diester,
whereas hydrolysis of cis-4 yields no cyclic product at all. The reason for this difference may be found in the formation of different $P^V$-TBPs during the hydrolysis reactions. The main difference between the $P^V$-TBPs is the location of the 3',5'-dioxaphosphorinane ring: (e,e) or (e,a). Since phenoxide ($pK_a$ (phenol) = 10.1) is a much better leaving group than methoxide ($pK_a$ (methanol) = 15.5), in $P^V$-TBPs the phenoxy group compared with methoxy is more axiophilic. This means that during hydrolysis of 2'-O-methyl-cis-adenosine 3',5'-cyclic phenyl monophosphate formation of the 3',5'-cyclic phosphate can be expected. Since methoxy has no axiophilic character, hydrolysis of cis-4 yields only acyclic phosphates.

### 4.3 Conclusions

The molecular conformation of cis-4 is not significantly different from the conformations of several cAMP-analogues. The ribose ring of cis-4 shows virtually
Table 4.9 Kinetic parameters for the hydrolysis of cis-4 at 294 K.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Initial concentrations [M]</th>
<th>Rate constant [M^{-1}.s^{-1}]</th>
<th>(r^c)</th>
<th>Product distribution [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(10^2[P]_0)</td>
<td>(10^2[OH^-]_0)</td>
<td>(10^3k_{exp})</td>
<td>(3')</td>
</tr>
<tr>
<td>cis-4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.00</td>
<td>1.00</td>
<td>33</td>
<td>0.999</td>
</tr>
<tr>
<td>B&lt;sup&gt;b&lt;/sup&gt; (R=OMe; Base=adenine)</td>
<td>4.77</td>
<td>14.3</td>
<td>21</td>
<td>0.998</td>
</tr>
</tbody>
</table>

<sup>a</sup> Kinetics were performed in D<sub>2</sub>O.

<sup>b</sup> Kinetics were performed in D<sub>2</sub>O/1,4-dioxane-\(d_8\) 3:7 (v/v).

<sup>c</sup> Correlation coefficient.
Scheme 4.2 Hydrolysis of cis-4.

the same configuration in the solid state and in solution. Comparison of the crystal structure of cis-4 with a set of related systems shows that the absence or presence of the substituent on C2 is relatively unimportant.

Due to the trans-fusion of the 3',5'-dioxaphosphorinane ring to the 5-membered ribose ring, the 3',5'-dioxaphosphorinane ring of cis-4 adopts a fixed distorted-chair conformation in the solid state. In this distorted-chair geometry the atoms: O3', O5', C3', and C5', are located in approximately the same plane, whereas P and C4' are situated on opposite sides of this plane. The P side of the six-membered ring is flattened in comparison with the C4' side. This conformation resembles with the distorted-chair conformation found in the set of related systems mentioned above. With respect to the conformation of the 3',5'-dioxaphosphorinane ring of cis-4 in
solution it must be mentioned that the available set of coupling constants do not allow us to discriminate between a chair conformation and a distorted-chair conformation.

In the crystal structure the adenine ring is oriented in the anti conformation and the adenine bases are linked via $N_6\text{-}H_{1\cdots N_1}$ and $N_6\text{-}H_{2\cdots N_7}$ hydrogen bonds, in such a way that infinite one-dimensional chains are formed. This hydrogen bond scheme is very similar to that observed in the structure of 2'-deoxy-3',5'-di-O-acetyl adenosine. An additional hydrogen bond is formed between the OH-group of methanol and the adenine base.

Alkaline hydrolysis of cis-4 yields two acyclic products: only 3'-phosphate diester and a 5'-phosphate diester. Compared to the hydrolysis of 2'-O-methyl-cis-adenosine 3',5'-cyclic phenyl monophosphate no cyclic phosphate diester has been formed. It can be concluded that hydrolysis of cis-4 is "thermodynamically controlled", whereas hydrolysis of 2'-O-methyl-cis-adenosine 3',5'-cyclic phenyl monophosphate is more "kinetically controlled" due to the good leaving group character of phenoxy.

4.4 Experimental

4.4.1 Materials and Chromatography

Merck silica gel 60 (particle size 0.063-0.200 mm) was used for column separations. Dry diethyl ether was obtained by storing diethyl ether, predried on calcium chloride, on sodium wire. Ethyl acetate was refluxed on calcium hydride prior to atmospheric distillation. tert-Butyl hydroperoxide was used as a 80% (8.0 M) solution in di-tert-butyl peroxide. 1H-tetrazole was purified through sublimation. Reactions were routinely run at ambient temperature in an inert atmosphere of dry nitrogen or argon, unless otherwise noted.
2'-O-Methyl-Cis-Adenosine 3',5'-Cyclic Methyl Monophosphate

4.4.2 NMR Spectroscopy

$^1$H NMR spectra were recorded at 400.13 MHz on a Bruker AM 400 spectrometer. Tetramethylsilane (TMS) was used as the internal standard. $^{13}$C- and $^{31}$P-NMR were recorded at 100.62 and 162.98 MHz, respectively, on the same instrument. The $^{31}$P NMR spectra were referenced against 85% H$_3$PO$_4$ as external standard.

4.4.3 Synthesis

2'-O-Methyl-cis-Adenosine 3',5'-Cyclic Methyl Monophosphite (cis-3).

2'-O-Methyladenosine$^5$ (2.25 g, 8.0 mmol) and 1H-tetrazole (1.68 g, 24.0 mmol) were dissolved in 160 mL of acetonitrile. A solution of bis(N,N-diisopropylamino)-methoxyphosphine (2.52 g, 9.6 mmol) in 10 mL of dichloromethane was added and the mixture was stirred for 20 h. $^{31}$P NMR analysis indicated a trans-phosphite/cis-phosphite ratio of ca. 2:3 (δ(CH$_3$CN/CD$_3$CN 1:1 v/v): 129.7 ppm (trans), 123.5 ppm (cis)). In order to convert the trans epimer into the cis epimer, the temperature of the mixture was raised to 40 °C and maintained at this value for 90 h. $^{31}$P NMR indicated complete conversion of the trans isomer. The solution was filtered and evaporated in vacuo. The solid was suspended in 50 mL of ethyl acetate, and after filtering the filtrate was washed twice with a saturated sodium bicarbonate solution. The ethyl acetate layer was dried on magnesium sulfate. After filtering and evaporation of the filtrate, the residue was suspended in diethyl ether. After filtering a white solid is obtained. Yield 0.92 g (39%). $^{31}$P NMR (CD$_3$CN): δ 123.5; $^1$H NMR (CD$_3$CN): δ 8.26 (1H, s, H$_6$), 7.98 (1H, s, H$_2$), 6.11 (2H, bs, NH$_2$), 5.95 (1H, bs, H$_1$), 4.90 (1H, ddd, H$_3$), 4.42 (1H, ddd, H$_5$), 4.34 (1H, bd, H$_2$), 4.28 (1H, ddd, H$_5$), 4.00 (1H, ddd, H$_4$), 3.59 (3H, d, OCH$_3$, $^3$JPOCH = 12.0 Hz), 3.55 (3H, s, 2'-OCH$_3$); $^{13}$C NMR (CD$_3$CN): δ 157.0 (C$_4$ or C$_6$), 154.1 (C$_8$), 150.3 (C$_6$ or C$_4$), 140.6 (C$_2$), 121.0 (C$_5$), 88.5 (C$_1$), 82.7 (C$_2$), 72.8 (C$_4'$, $^3$JPOCC = 7.2 Hz), 71.0 (C$_3$), 67.1 (C$_5'$, $^2$JPOC = 4.0 Hz), 59.5 (2'-OCH$_3$), 50.9 (OCH$_3$, $^2$JPOC = 17.3 Hz).
2'-O-Methyl-cis-Adenosine 3',5'-Cyclic Methyl Monophosphate (cis-4).

tert-Butyl hydroperoxide (0.15 mL, 1.2 mmol) was added to a solution of cis-3 (100 mg, 0.29 mmol) in 3 mL of acetonitrile and the mixture was stirred for 15 h. After filtering, the filtrate was evaporated and dissolved in dichloromethane/methanol 95:5 (v/v). After addition of diethyl ether, a precipitate was formed, which was isolated by means of centrifugation. Afterwards, purification was obtained by means of column chromatography on a silica gel column with a gradient of methanol in acetonitrile (5→10 vol% methanol). Compound cis-4 was obtained as a white solid. Recrystallization from methanol yielded colourless crystals. Yield 75 mg (72%). Rf 0.28 (methanol/acetonitrile 1:9 v/v). $^{31}$P NMR (CD$_3$OD): δ -3.00; $^1$H NMR (CD$_3$OD): δ 8.25 (2H, s, H$_8$ and H$_2$), 6.18 (1H, bs, H$_1$), 5.40 (1H, ddd, H$_3$), 4.66 (1H, ddd, H$_5$), 4.53 (1H, bd, H$_2$), 4.45 (1H, bt, H$_5$), 4.32 (1H, ddd, H$_4$), 3.94 (3H, d, OCH$_3$, $^3$J$_{POCH} = 11.2$ Hz), 3.60 (3H, s, 2'-OCH$_3$); $^{13}$C NMR (CD$_3$CN): δ 157.4 (C$_4$ or C$_6$), 154.2 (C$_8$), 150.2 (C$_6$ or C$_4$), 141.7 (C$_2$), 120.7 (C$_5$), 92.0 (C$_1$), 81.8 (C$_2$, $^3$J$_{POCC} = 7.6$ Hz), 80.6 (C$_3$, $^2$J$_{POC} = 5.9$ Hz), 72.1 (C$_4$, $^3$J$_{POCC} = 5.8$ Hz), 71.3 (C$_5$, $^2$J$_{POC} = 8.9$ Hz), 59.6 (2'-OCH$_3$), 55.1 (OCH$_3$, $^2$J$_{POC} = 6.0$ Hz).

4.4.4 Hydrolysis of Cis-4

Cis-4 (15 mg; 0.038 mmol) was transferred into a 5-mm NMR sample tube and dissolved in 360 µL of D$_2$O. After addition of 19 µL of a 2.00 M sodium hydroxide solution, initiating the hydrolysis, the solution turned yellowish. The reaction was monitored at 21 °C by means of $^{31}$P NMR: 8 FIDs were collected (time domain: 16K; size: 16K; sweep width: 4854 Hz) at distinct time intervals. The start of the 5th scan was chosen as the reaction time. $^{31}$P NMR (D$_2$O) δ 2.19 (5'-phosphate diester), 1.51 (3'-phosphate diester).$^{18}$

4.4.5 Crystallographic Measurements and Structure Resolution

C$_{12}$H$_{16}$N$_5$O$_6$P·CH$_3$OH, fw = 389.30. Orthorhombic, $a = 8.170(2)$ Å, $b =$
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9.249(1) Å, c = 23.669(4) Å, V = 1788.5(6) Å³, Z = 4, Dc = 1.446 g cm⁻³, λ(MoKα) = 0.71073 Å, t = 100 K, μ(MoKα) = 2.1 cm⁻¹, F(000) = 816. Absent reflections: h00, h odd; 0k0, k odd; and 00l, l odd; space group P2₁2₁2₁ (D₂, No. 19).

A colourless crystal with dimensions (mm) 1.1 × 0.5 × 0.2 was used for data collection on an Enraf-Nonius CAD-4 diffractometer. Lattice parameters were determined from the setting angles of 25 reflections in the Θ range 10.66–19.20°. The intensities of 8388 reflections (2358 unique, Rint = 0.049) were collected at liquid-nitrogen temperature (100 K) within the sphere of reflection limited by 2Θ = 55° and −10 ≤ h ≤ 0, −12 ≤ k ≤ 12, and −30 ≤ l ≤ 30, using Zr-filtered MoKα radiation. The ω/2Θ-scan technique was applied with, Δω = (1.74 + 0.35tan Θ)°. The intensities of three standard reflections (024, 203 and 122), measured every hour showed an average deviation of less than 3% during the duration of the data collection. The intensities were corrected for Lorentz-polarization effects, but not for absorption. A total of 2006 reflections that satisfied the criterion I ≥ 2.5σ(I) were retained for the structure determination and refinement. σ(I) was derived from counting statistics. The structure was solved with the direct methods program of SHELXS86, the coordinates of all non-hydrogen atoms being determined from an E-map. The hydrogen atoms were placed at idealized positions (C−H 1.00 Å) and refined riding on their bonded atoms, except those involved in hydrogen bonds. The three donor hydrogen atoms could be located in difference Fourier maps. As these atoms showed appreciable drifts upon refinement, they were fixed on the donor···acceptor vectors at 0.90 Å from the donor atoms, assuming linearity of the hydrogen bond. The overall isotropic thermal parameter of all hydrogen atoms refined to 0.056(5) Å². The absolute configuration was assigned in correspondence with that reported for a number of 3',5'-cyclic nucleoside monophosphates (see ref. 9 and Table 4.6). Anisotropic full-matrix least-squares refinement on F of 230 parameters converged at R = 0.062 and Rw = 0.043 with w = 1/σ²(Fo). The quantity minimized was ΣΔF², the goodness of fit,
\[ s = [\Sigma w \Delta F^2/(m-n)]^{1/2}, \] was 1.57 and the average and maximal shift to error ratios 0.004 and 0.03, respectively. A final \( \Delta F \) map showed maximal fluctuations of +0.65 and −0.42 eÅ⁻³, the positive maxima lying near the phosphorus atom. The scattering factors were those of Cromer and Mann and anomalous-dispersion terms from Cromer and Liberman. The least-squares refinement was performed with the SHELX76 program and the program package EUCLID was used for the calculations of geometries and preparation of illustrations. All calculations were performed on an ULTRIX DECsystem-5000.

References and Notes


In this generalized equation, the standard Karplus relation is extended with a correction term which accounts for the influence of electronegative substituents on $^3J_{HH}$:

$$^3J_{HH} = 13.22 \cos^2 \phi - 0.99 \cos \phi + \Sigma (0.87 - 2.46 \cos^2 (\xi_i \phi + 19.9 |\Delta \chi_i|)) \Delta \chi_i$$

$\phi$ is the proton–proton torsion angle. $\Delta \chi_i$ is the difference in electronegativity between the substituent and hydrogen according to the electronegativity scale of de Leeuw, F.A.A.M.; Altona, C. J. Chem. Soc., Perkin Trans. II 1982, 375.
Huggins, and $\xi$, is a substituent orientation parameter.


18. The assignment of the 3'- and 5'-phosphate diester lines in the $^{31}\text{P}$ NMR spectrum is based on a 2D $^{31}\text{P} - ^1\text{H}$ correlation spectrum.


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Summary

This thesis describes experimental investigations on the structural properties of cAMP-derived five-coordinated phosphorus (P⁵⁺) intermediates. These intermediates, with a trigonal bipyramidal geometry (TBP), are presumed to be formed during the interaction between cAMP and enzymes like phosphodiesterases and protein kinases. This assumption, based on quantum chemical CNDO/2 calculations, was first proposed by van Ool and Buck in 1982. They predicted that the 3',5'-dioxaphosphorinane ring preferred the diequatorial (e,e) location to the equatorial-axial (e,a) orientation in the P⁵⁺-TBP. The results of experimental investigations on P⁵⁺-TBP systems of the groups Holmes et al. and Bentrude et al. show, however, that nearly all P⁵⁺-TBP compounds adopt an (e,a) location of the dioxaphosphorinane ring in a twist (non-chair) conformation. The results of chapters 2, 3, and 4, mainly based on ¹H-, ¹³C-, and ³¹P-NMR spectroscopy, show that both an (e,a) and an (e,e) orientation of the six-membered ring in P⁵⁺-TBPs is possible. This study therefore has contributed to the insight of the dynamics of P⁵⁺-TBPs, modelling the activated state of cAMP.

Chapter 2 describes the ¹H-, ¹³C-, and ³¹P-NMR study of P⁵⁺-TBP model compounds for the activated state of cAMP during its interaction with enzymes (e.g. phosphodiesterases and protein kinases). The results of this study confirm the findings of Holmes et al. and Bentrude et al. However the study on a P⁵⁺-TBP with an O=C=C=O ligand indicate that the six-membered dioxaphosphorinane ring partly adopts an (e,e) location and a twist (non-chair) conformation in the P⁵⁺-TBP. The design of this compound was based on the conformational transmission effect, which states that O=O gauche conformation of the O=C=C=O fragment corresponds with an equatorial position of the O=C=C=O fragment in the P⁵⁺-TBP. Mutatis mutandis, the O=O trans conformation corresponds with an axial location. Thus the conformation of the O=C=C=O fragment can give information about the location of the ligand in the P⁵⁺-TBP. Combining this with the knowledge that
almost all five-membered rings (i.e. the ring formed upon complexation of tetra-chloro-1,2-benzoquinone and phosphorus) are attached (e,a) to phosphorus in the P\textsuperscript{V}-TBP, it is possible to locate the orientation of the dioxaphosphorinane ring (in this case diequatorial). Although an (e,a) orientation is energetically favourable, it appears to be that conformational transmission in one of the ligands can help to stabilize the (e,e) isomer. Recently, Bentrude et al. confirmed the possibility of a diequatorially located dioxaphosphorinane ring in a P\textsuperscript{V}-TBP by means of X-ray crystallography.

In chapter 3, the alkaline hydrolysis of cis-nucleoside 3',5'-cyclic aryl [\textsuperscript{18}O] monophosphates is described. These compounds, containing an aryl group on the axial exocyclic oxygen, can be considered as models for the interaction between cAMP and enzymes, in which the negative charge on one of the exocyclic oxygen atoms is shielded by positively charged amino acid residues or by bivalent metal ions. The stereochemistry of the hydrolysis reaction was determined after methylation of the hydrolysis products, using methyl iodide as methylation agent. Since it is generally accepted that hydrolysis of phosphates proceeds via P\textsuperscript{V}-TBP intermediates, it is possible to determine the hydrolysis pathway via the phosphorus configuration of the hydrolysis products. This study reveals that formation of a 3',5'-cyclic [\textsuperscript{18}O]phosphate diester proceeds partially with inversion of configuration at phosphorus, indicating the existence of a P\textsuperscript{V}-TBP with an (e,e) located dioxaphosphorinane ring. Retention of configuration at phosphorus can be explained in terms of Berry pseudorotation. The formation of the 5'-acyclic aryl [\textsuperscript{18}O]phosphate diester proceeds with about 50% inversion of configuration at phosphorus, whereas formation of the 3'-acyclic aryl [\textsuperscript{18}O]phosphate diester proceeds with an inversion/retention ratio significantly deviating from 1. Since the stereochemistry of 3'-aryl [\textsuperscript{18}O]phosphate diester could not be determined unambiguously no explicit statement can be made concerning the hydrolysis routes. Furthermore it is clear that Berry pseudorotation significantly contributes to the hydrolysis reaction.

The crystal structure and the kinetic-mechanistic aspects of the alkaline hydro-
lysis of 2′-O-methyl-cis-adenosine 3′,5′-cyclic methyl monophosphate are described in chapter 4. This compound is regarded a model for the enzyme-bound 3′,5′-cyclic adenosine monophosphate. In this complex the negatively charged exocyclic oxygen is shielded in part via complexation with positively charged sites of the enzyme. The adenine bases are linked via N6–H···N1 and N6–H···N7 hydrogen bonds in such a way that infinite one-dimensional chains are formed. This hydrogen bond scheme is very similar to that observed in the structure of 2′-deoxy-3′,5′-di-O-acetyl adenosine. An additional hydrogen bond is formed between the methanol, incorporated in the crystal structure, and the adenine base. The conformational preferences of the 2′-O-methyl-cis-adenosine 3′,5′-cyclic methyl monophosphate dissolved in methanol have been determined with 400 MHz 1H NMR. It is found that the X-ray structure and the solution conformation are practically the same. Hydrolysis of the title compound yields two acyclic phosphate diesters (a 3′- and a 5′-phosphate diester). Supposedly due to the thermodynamic control of the reaction no cyclic 3′,5′-phosphate diester is formed during the hydrolysis: the most stable intermediates, $P^V$-TBPs with an equatorial-axial oriented 3′,5′-dioxaphosphorinane ring, will be formed during the reaction.
Samenvatting

Dit proefschrift beschrijft het experimentele onderzoek naar de structurele eigenschappen van cAMP-afgeleide vijf-gecoördineerde fosfor (P⁵) intermediairen. Deze verbindingen, met een trigonaal bipiramidale geometrie (TBP), worden verondersteld op te treden tijdens de interactie tussen cAMP en enzymen, zoals fosfodiësterasen en proteïne kinases. Deze aanname, die gebaseerd is op quantum chemische CNDO/2 berekeningen, werd in 1982 voor het eerst voorgesteld door van Ool en Buck. Ze voorspelden dat de 3′,5′-dioxafosforinaanring een grotere voorkeur had voor een diequatoriële (e,e) locatie dan voor een equatoriaal-axiale (e,a) oriëntatie in de P⁵-TBP. Uit de resultaten van het experimentele onderzoek van Holmes et al. en Bentrude et al. blijkt echter dat in vrijwel alle P⁵-TBP systemen de dioxafosforinaanring een (e,a) locatie aanneemt, waarbij een twist (niet-stoel) conformatie werd waargenomen.

In hoofdstuk 2 wordt een ¹H-, ¹³C-, ³¹P-NMR studie beschreven van P⁵-TBP modelverbindingen van de geactiveerde toestand van cAMP tijdens de interactie met enzymen (bijvoorbeeld fosfodiësterasen en proteïne kinases). De resultaten van deze studie bevestigen de bevindingen van Holmes et al. en Bentrude et al. Echter de studie van een P⁵-TBP met een O—C—C—O ligand toont aan dat de dioxafosforinaanring gedeeltelijk een (e,e) oriëntatie aanneemt in de P⁵-TBP, waarbij een twist (niet-stoel) conformatie wordt aangenomen. Het ontwerp van deze verbinding is gebaseerd op het conformatie transmissie effect, wat inhoudt dat een O—O gauche conformatie van het O—C—C—O fragment correspondeert met een equatoriaal plaatsing van het O—C—C—O fragment in de P⁵-TBP. Mutatis mutandis, een O—O trans conformatie correspondeert met een axiale locatie. De conformatie van het O—C—C—O fragment geeft dus informatie over de positie van het ligand in de P⁵-TBP. Door dit te combineren met de kennis dat vrijwel alle vijfringen (d.w.z. de ring die ontstaat door complexatie van tetrachloro-1,2-benzoquinone met fosfor) een (e,a) oriëntatie aannemen in de P⁵-TBP, is het mogelijk om de oriëntatie van de
Samenvatting

dioxafosforinaanring vast te stellen (in dit geval diequatoriaal). Ofschoon een (e,a) oriëntatie energetisch gunstiger is dan (e,e), blijkt dat conformatie transmissie in een van de liganden kan bijdragen tot stabilisatie van de (e,e) isomeer. Recentelijk heeft Bentrude et al. d.m.v. een Röntgenanalyse bevestigd dat een diequatoriale plaatsing van een dioxafosforinaanring in een $P^V$-TBP mogelijk is.

In hoofdstuk 3 wordt de studie van de alkalische hydrolyse van cis-nucleoside 3',5'-cyclische aryl $[^{18}O]$monofosfaten beschreven. Deze verbindingen, die een arylgroep hebben op de axiale exocyclische zuurstofatomen, kunnen beschouwd worden als modelstappen voor de interactie tussen CAMP en enzymen, waarbij de negatieve lading van een van de exocyclische zuurstofatomen wordt afgeschermd door positief geladen aminozuurresiduen of door bivalente metaalionen. De stereochemie van de hydrolysereactie werd vastgesteld na methylering van de hydrolyseproducten, waarbij methyljodide werd gebruikt als methyleringsreagens. Aangezien algemeen geaccepteerd wordt dat de hydrolyse van fosfaten verloopt door middel van $P^V$-TBP intermediairen, is het mogelijk om na het vaststellen van de fosforconfiguratie van de hydrolyseproducten, het reactiepad van de hydrolyse vast te stellen. Uit deze studie blijkt dat de vorming van een 3',5'-cyclische $[^{18}O]$fosfaatdiëster gedeeltelijk verloopt met inversie van de configuratie op fosfor, wat het bestaan aangeeft van een $P^V$-TBP met een (e,e) georiënteerde dioxafosforinaanring. De retentie van fosforconfiguratie kan uitgelegd worden in termen van Berry pseudorotatie. De vorming van de 5'-acyclische aryl $[^{18}O]$fosfaatdiëster verloopt met ongeveer 50% inversie van de fosforconfiguratie, terwijl de vorming van de 3'-acyclische aryl $[^{18}O]$fosfaatdiësters verloopt met een inversie/retentieverhouding die significant afwijkt van 1. Aangezien de stereochemie van de 3'-acyclische aryl $[^{18}O]$fosfaatdiësters niet eenduidig bepaald kon worden, is het niet mogelijk om een uitspraak te doen over de gevolgde hydrolyserouten. Verder is vastgesteld dat Berry pseudorotatie een duidelijke bijdrage heeft in de hydrolysereactie.

In hoofdstuk 4 wordt de kristalstructuur en de kinetisch-mechanistische aspecten beschreven van de alkalische hydrolyse van 2'-O-methyl-cis-adenosine 3',5'-cyclisch
methyl monofosfaat. Deze verbinding wordt beschouwd als een modelsysteem voor het enzymgebonden 3',5'-cyclische adenosine monofosfaat. In dit complex wordt de negatief geladen exocyclische zuurstofatomen gedeeltelijk afgeschermd door positief geladen sites van het enzym. De adeninebasen zijn zodanig verbonden via $N_6-H\cdots N_1$ en $N_6-H\cdots N_7$ waterstofbruggen, dat oneindig lange ééndimensionale ketens worden gevormd. Dit stelsel van waterstofbruggen komt sterk overeen met dat wat waargenomen is in de structuur van 2'-desoxy-3',5'-di-O-acetyl adenosine. Een extra waterstofbrug wordt gevormd tussen de methanol, aanwezig in het kristalrooster, en de adeninebase. De conformationele voorkeur van 2'-O-methyl-cis-adenosine 3',5'-cyclisch methyl monofosfaat opgelost in methanol is bepaald met 400 MHz $^1$H NMR. Hierbij werd vastgesteld dat Röntgenstructuur praktisch identiek is met de conformatie in oplossing. Tijdens de hydrolyse van 2'-O-methyl-cis-adenosine 3',5'-cyclisch methyl monofosfaat werden twee acyclische fosfaatdiesters (een 3'- en een 5'-fosfaatdiëster) gevormd. Vermoedelijk wordt als gevolg van de thermodynamisch bepaalde hydrolysereactie geen 3',5'-fosfaatdiëster gevormd: dus tijdens de reactie zullen de meest stabiele intermediairen, $P^V$-TBPs met een equatoriaal-axiaal georiënteerde 3',5'-dioxafosforinaanring, gevormd worden.
Curriculum Vitae


Van 1 november 1988 tot 1 februari 1993 was hij als assistent in opleiding (a.i.o.) werkzaam binnen de vakgroep Organische Chemie aan bovengenoemde Universiteit. In deze periode werd het onderzoek, zoals beschreven is in dit proefschrift, uitgevoerd. Aanvankelijk stond het onderzoek onder leiding van prof. dr. H.M. Buck, maar in een later stadium kwam dit onderzoek onder leiding van prof. dr. E.W. Meijer.
Dankwoord

Graag wil ik iedereen hartelijk danken die, op welke wijze dan ook, heeft bijgedragen aan de totstandkoming van dit proefschrift. In het bijzonder wil ik prof. dr. E.W. Meijer en prof. dr. E.M. Meijer danken voor hun bereidheid om als eerste, respectievelijk als tweede promotor op te treden. Ook dr. ir. Leo Koole wil ik speciaal dankzeggen voor zijn voortdurende bereidheid om, zelfs ook op afstand, ondersteuning te verlenen.

Verder wil ik ir. Arthur van der Heiden (a.i.o.-2) en de afstudeerders ir. Imre Peeters en ir. Henk Janssen bedanken voor hun belangrijke bijdragen.

Naast alle medewerkers en ex-medewerkers van de vakgroep Organische Chemie wil ik met name mijn kamergenoot ir. Sjoerd Miesen danken voor de vele zinvolle discussies en Henk Eding voor het vervaardigen van de vele illustraties.

STELLINGEN

1. In tegenstelling tot de proton-fosfor-koppelingsconstante van een P–OCH₃ ligand, aanwezig in vijfgecoördineerde fosforverbindingen met een trigonaal bipiramidale geometrie, kan bij een P–O–CH₂–CH₂–OCH₃ ligand niet eenduidig onderscheid gemaakt worden tussen een equatoriale of axiale oriëntatie van het ligand.


2. Huang, Arif en Bentrude gaan er ten onrechte van uit dat in vijfgecoördineerde fosforverbindingen met een trigonaal bipiramidale geometrie elke diëquatoriaal georiënteerde dioxafosforinaanring een stoelconformatie aanneemt.


3. De door Bajwa en Bentrude in aceton-₅₆ gemeten koolstof-fosfor-koppelingsconstante van 8,9 Hz op C₂, van de 2′-desoxyribose-ring van *cis*-thymidine 3′,5′-cyclische methylfosfiet, kan niet gereproduceerd worden.


4. Het verdient aanbeveling om oude geaccepteerde quantumchemische berekeningen regelmatig te herberekenen met recente betere rekenmethoden.

5. De constatering dat de Lijkwade van Turijn volgens de koolstof-14 methode dateert uit de periode 1260-1390 hoeft niet te betekenen dat de lijkwade niet afkomstig kan zijn geweest van Jezus Christus.

6. Gezien de huidige wetenschappelijke kennis, is het niet zinvol om Röntgenstralen nog langer aan te duiden met de term "X-rays".

7. De algemeen gangbare, onterechte opvatting dat éénkristallen uit één zuivere verbinding bestaan kan leiden tot foutieve interpretatie van de uit een Röntgendiffractie-analyse verkregen structurele parameters.


10. De door Greenpeace gevoerde slogan: "Don’t panic it’s only organic" mag niet van toepassing zijn op vogels die besmeurd zijn met olie afkomstig uit olietankers.

11. Om het veiligheidsgevoel van voetgangers bij het oversteken van een straat te vergroten, is het aan te bevelen om auto’s voortaan ook aan de voorzijde te voorzien van remlichten.

N.L.H.L. Broeders

Eindhoven, 30 juni 1993