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RECOGNITION OF NUCLEIC ACIDS BY METAL ION COMPLEXES

Oleg Golubev

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ABSTRACT

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Metal-ion-mediated base-pairing of nucleic acids has attracted considerable attention during the past decade, since it offers means to expand the genetic code by artificial base-pairs, to create predesigned molecular architecture by metal-ion-mediated inter- or intra-strand cross-links, or to convert double stranded DNA to a nano-scale wire. Such applications largely depend on the presence of a modified nucleobase in both strands engaged in the duplex formation. Hybridization of metal-ion-binding oligonucleotide analogs with natural nucleic acid sequences has received much less attention in spite of obvious applications. While the natural oligonucleotides hybridize with high selectivity, their affinity for complementary sequences is inadequate for a number of applications. In the case of DNA, for example, more than 10 consecutive Watson-Crick base pairs are required for a stable duplex at room temperature, making targeting of sequences shorter than this challenging. For example, many types of cancer exhibit distinctive profiles of oncogenic miRNA, the diagnostics of which is, however, difficult owing to the presence of only short single stranded loop structures. Metallo-oligonucleotides, with their superior affinity towards their natural complements, would offer a way to overcome the low stability of short duplexes. In this study a number of metal-ion-binding surrogate nucleosides were prepared and their interaction with nucleoside 5′-monophosphates (NMPs) has been investigated by ^{1}H NMR spectroscopy. To find metal ion complexes that could discriminate between natural nucleobases upon double helix formation, glycol nucleic acid (GNA) sequences carrying a Pd^{II} ion with vacant coordination sites at a predetermined position were synthesized and their affinity to complementary as well as mismatched counterparts quantified by UV-melting measurements.

Key words: non-coding RNA, microRNA, modified oligonucleotide, hybridization, metal-ion-mediated base pair

TIIVISTELMÄ

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Golubev, Oleg: Nukleiinihappojen tunnistus metalli-ionikomplekseilla

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Metalli-ionien välittämä nukleiinihappojen emäspariutuminen on herättänyt huomattavaa kiinnostusta viimeisen vuosikymmenen aikana, koska näin voitaisiin laajentaa geneettistä koodia keinotekoisin emäsparein, valmistaa juosteiden välisiin tai –sisäisiin metallisiltoihin perustuvia nanorakenteita tai muuttaa kaksoiskierteinen DNA nanomittakaavan johtimeksi. Tyypillisesti tällaisissa sovelluksissa kaksoiskierteen molemmissa juosteissa on modifioitu nukleiinihappoemäs. Metalli-ioneja sitovien oligonukleotidien hybridisaatiota luonnollisten nukleiinihappojen kanssa on ilmeisistä sovelluskohteista huolimatta tutkittu huomattavasti vähemmän. Luonnolliset nukleiinihapot hybridisoituvat hyvin selektiivisesti, mutta monien sovellusten kannalta liian heikosti. Esimerkiksi DNA:n tapauksessa huoneen lämpötilassa pysyvään kaksoiskierteeseen tarvitaan yli kymmenen perättäistä Watson-Crick-emäsparia, joten oligonukleotidin kohdentaminen tätä lyhyempiin sekvensseihin on haastavaa. Esimerkiksi monilla syöpätyypeillä on tunnusomainen miRNA-profiili, jonka diagnostiikkaa kuitenkin vaikeuttaa se, että miRNA:ssa on vain lyhyitä yksinauhaisia osia. Luonnollisiin vastinnauhoihinsa voimakkaasti sitoutuvat metallinukleotidit voisivat tarjota ratkaisun tähän ongelmaan. Tässä väitöstutkimuksessa on valmistettu useita metalli-ioneja sitovia nukleosidianalogeja ja tutkittu niiden vuorovaikutuksia nukleosidi-5´-monofosfaattien kanssa ^{1}H NMR-spektrometrisesti. Sellaisten kompleksien löytämiseksi, jotka voisivat tunnistaa halutun vastinemäksen kaksoiskierteessä, valmistettiin glykolinukleiinihappo (GNA) –oligonukleotideja, joihin oli sidottu haluttuun kohtaan Pd^{II}-ioni, jolla oli vapaa koordinaatiopaikka. Näiden oligonukleotidien hybridisaatioteho komplementaaristen sekä virheellisten vastinnauhojen kanssa määritettiin UV-sulamislämpötilamittauksin.

Asiasanat: ei-koodaava RNA, microRNA, modifioitu oligonukleotidi, hybridisaatio, metalli-ioni-välitteinen emäspari

PREFACE

This thesis is based on experimental work carried out in the Laboratory of Organic Chemistry and Chemical Biology at the Department of Chemistry, University of Turku during the years 2011-2016. The financial support of Graduate School of Organic Chemistry and Chemical Biology and Department of Chemistry are gratefully acknowledged.

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Turku, March 2016

Oleg Golubev

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

I. Golubev, O.; Lönnberg, T.; Lönnberg, H. "Metal-ion-binding analogs of ribonucleosides: Preparation and formation of ternary Pd^{2+} and Hg^{2+} complexes with natural pyrimidine nucleosides". *Helv. Chim. Acta*, **2013**, *96*, 1658-1669.

II. Golubev, O.; Lönnberg, T.; Lönnberg, H. "Interaction of Pd^{2+} complexes of 2,6-disubstituted pyridines with nucleoside 5′-monophosphates". *J. Inorg. Biochem.,* **2014**, *139*, 21-29.

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IV. Golubev, O.; Lönnberg, T.; Lönnberg, H. "Formation of mixed ligand Pd^{2+} complexes between nucleoside 5′-monophosphates and some metal-ion-binding nucleoside surrogates". *Molecules,* **2014**, *19*, 16976-16986

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ABBREVIATIONS

A	adenosine
Ac	acetyl
Ac_2O	acetic anhydride
AcOH	acetic acid
AU	absorption unit
AMP	adenosine 5′-monophosphate
C	cytidine
CD	circular dichroism
CMP	cytidine 5′-monophosphate
COSY	correlation spectroscopy
CPG	controlled pore glass
dA	2′-deoxyadenosine
dC	2′-deoxycytidine
dG	2′-deoxyguanosine
DCA	dichloroacetic acid
DCM	dichloromethane
DIPEA	*N*,*N*-diisopropylethylamine
DMAP	4-dimethylaminopyridine
DMF	*N*,*N*-dimethylformamide
DMTr	4,4′-dimethoxytrityl (4,4′-dimethoxytriphenylmethyl)
DMTrCl	4,4′-dimethoxytrityl chloride
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
ESI-MS	electrospray ionization mass spectrometry
G	guanosine
GMP	guanosine 5′-monophosphate
GNA	glycol nucleic acid
HBTU	*O*-(benzotriazol-1-yl)-*N*,*N*,*N*,*N*′-tetramethyluronium hexafluoro phosphate
HMBC	heteronuclear multiple bond correlation
HPLC	high performance liquid chromatography
HSQC	heteronuclear single quantum coherence
IMP	inosine 5′-monophosphate
LCAA-CPG	long chain alkylamine-functionalized controlled pore glass
MeCN	acetonitrile
MeOH	methanol
MeONa	sodium methoxide
$MeNHNH_2$	methylhydrazine
MS	mass spectrometry
NMR	nuclear magnetic resonance
NeMP	nebularine 5′-monophosphate
NMP	nucleoside 5′-monophosphate
ON	oligonucleotide
$POCl_3$	phosphorous oxychloride
Py	pyridine
RNA	ribonucleic acid
RP	reversed phase
T	thymidine
T_m	melting temperature

TBDMS	*tert*-butyldimethylsilyl
TEA	triethylamine
TEAA	triethylammonium acetate
TFA	trifluoroacetic acid
THF	tetrahydrofuran
Tr	trityl
U	uridine
UMP	uridine 5′-monophosphate
UV	ultraviolet

1. INTRODUCTION

1.1 Structure and biological functions of nucleic acids

In nature, DNA stores genetic information in the sequence of four deoxyribonucleosides (dA, T, dG, and dC). RNA, consisting of four ribonucleosides (A, U, G and C) is, in turn, involved in a number of biological processes such as information transfer and protein synthesis. The primary structure of DNA and RNA is formed by phosphodiester bonds between sugar moieties, resulting in a long polymeric backbone to which nucleic acid bases are attached through *N*-glycosidic bonds (Figure 1). The difference between DNA and RNA resides in the sugar structures and in one nucleobase. DNA has 2´-deoxyribose as sugar moiety and nucleobase thymine (T), whereas RNA contains ribose sugar and uracil base (U). The information storage, transfer and implementation into biochemical processes originate from the specific nucleobase sequence. Hydrogen bonding between sequences of two separate strands leads to formation of a double helix called secondary structure. Discrimination between nucleobases is achieved through selective complementary hydrogen bonding called Watson-Crick base-pairing and depicted in Figure 1.[1,2]

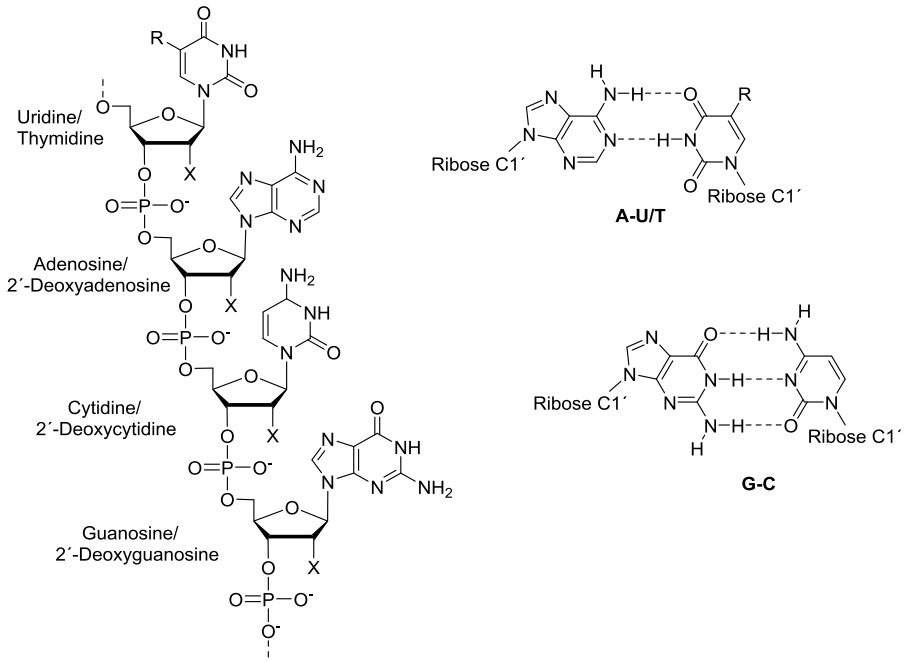


Figure 1. The structures of RNA [R = H (U), X = OH] and DNA [R = Me (T), X = H] and Watson-Crick base-pairing upon double helix formation.

Base pairing between two purines is sterically unfavorable. There is not enough space to fit within the double helix, leading to nucleobase repulsions. For two opposite pyrimidines, the distance inside of double helical structure is too long to allow hydrogen bonding. The selectivity is achieved by selective hydrogen bonding between the bases on opposite strands in base pairs. According to Watson-Crick base-pairing, a purine base always pairs with a pyrimidine base: guanine (G) pairs with cytosine (C) and adenine (A) pairs with thymine (T) or uracil (U).[3]

DNA exists as a double helical polymer fully composed of complementary base pairs, while RNA´s predominant structure is combination of short helical tracts stabilized by Watson-Crick base pairing and sequences of unpaired bases stacked within or outside the helical structure. The helix bends when unpaired bases are stacked within the structure.[4] When the unpaired base sequences are stacked outside, the helix forms loops available for interactions with other nucleic acids, proteins or drugs (Figure 2).

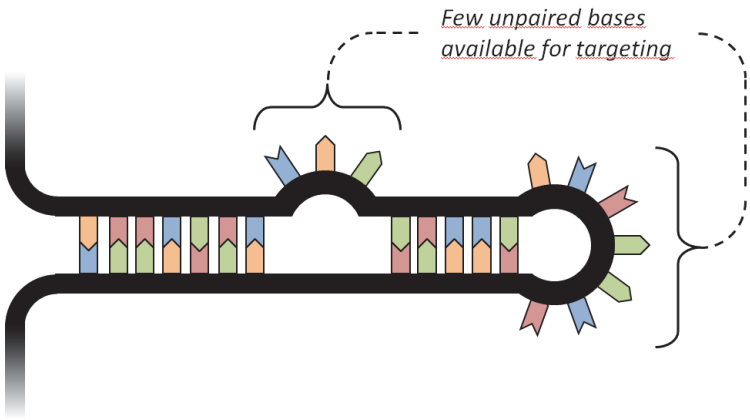


Figure 2. The secondary structure of RNA with external loops and budges.

Selective targeting of various coding or non-coding RNAs leads to a wide spectrum of potential therapeutic applications.[5-11] Some oligonucleotide based drugs have already been approved for clinical use.[12,13] Since the discovery of RNA interference, short regulatory RNA molecules have emerged as an attractive target for chemotherapy. For example, many types of cancer exhibit distinctive profiles of microRNA (miRNA), short hairpin like structures containing usually one loop and bulge that interrupts the otherwise double helical stem. Recognition of such oncogenic miRNA offers a potential approach for cancer diagnostics. One of the serious obstacles on the way to oligonucleotides as therapeutic agents is low affinity to short target sequences. While the natural nucleosides hybridize with high selectivity, the affinity to their

complementary sequences is inadequate for a number of applications.[14] In the case of DNA, for example, more than 10 consecutive Watson-Crick base pairs are required for a stable duplex at room temperature, making targeting of sequences shorter than this challenging. The unpaired nucleobases of the loop region of miRNA have, in fact, been successfully targeted by short oligonucleotides but the oligonucleotide concentration required has been high.[15] There is, hence, a demand for oligonucleotides with enhanced hybridization properties. One of the approaches is structural modification of the nucleobases, aimed at increasing affinity without losing selectivity of hybridization.[16-18] The duplex stability depends on the joint effect of several factors including hydrogen-bonding between complementary bases, hydrogen-bonding between bases and water molecules and vertical stacking interactions between adjacent base pairs. Accordingly, the structure and hydrogen bond donor/acceptor properties of nucleobases determine the duplex stability. Discrimination between nucleobases is achieved through hydrogen-bonding (Figure 1), but stacking of base pairs still accounts for most of the duplex stability.[19] Owing to resonance, the amino groups of the amides and amidines are more acidic than aliphatic amino groups and the amide oxygens are more basic than keto oxygens, which enables formation of rather strong hydrogen bonds.[20]

1.2 Artificial base pairing

Some non-canonical bases are frequently exploited in molecular biology research. The presence of additional hydrogen bond donors or acceptors in the base moiety can significantly influence on duplex stability. One example is offered by 2-aminoadenine-T/U base pair (Figure 3).

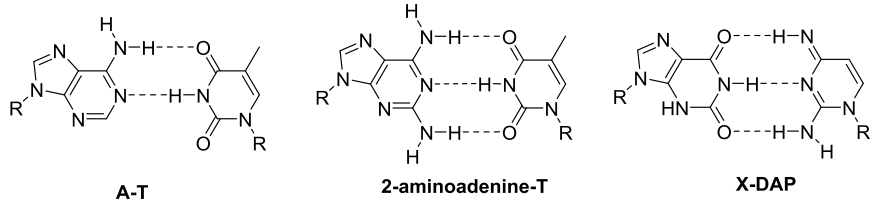


Figure 3. A-T-canonical base pair; 2-aminoadenine-T base pair; xanthine (X)-2,4-diaminopyrimidine (DAP) base pair.

2-Aminoadenine is otherwise identical to adenine, but bears an additional amino group at C2, providing an extra hydrogen bond with thymine oxygen located at C2.[21,22] Stabilization can

also take place by efficient base-pairing of two non-canonical nucleobases. Xanthine, for example, binds tightly to 2,4-diaminopyrimidine by three hydrogen bonds.[23] A large number of artificial base analogues with enhanced stability towards natural bases have been developed and some of them were successfully introduced into oligonucleotide sequence. 9-(2-Aminoethoxy)phenoxazine (G-Clamp), a cytosine analogue that binds to guanine (Figure 4) by four hydrogen bonds, is a well-known example.[24]

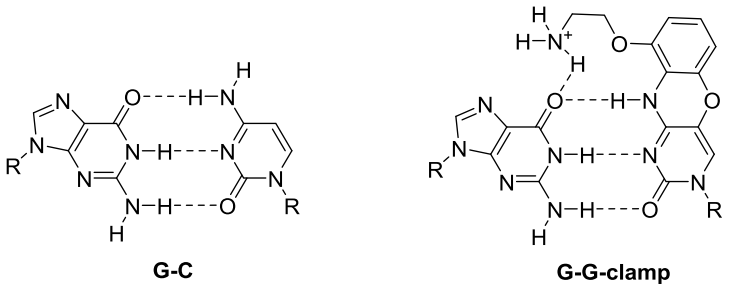


Figure 4. G-G-clamp base pair increases the stability of DNA duplex up to 18 °C relative to canonical G-C base pair

Another approach to stabilize double helical structure is introduction of a metal ion between opposite bases. In this kind of metal-ion-mediated base-pairing, one or several of the Watson-Crick hydrogen bonds are replaced by interaction of a metal ion with both nucleobases (Figure 5). Metal-ion-complexing has been shown to take place between natural mismatched nucleobases,[25,26] between structural derivatives of canonical nucleobases[27] and between artificial base analogues.[28]

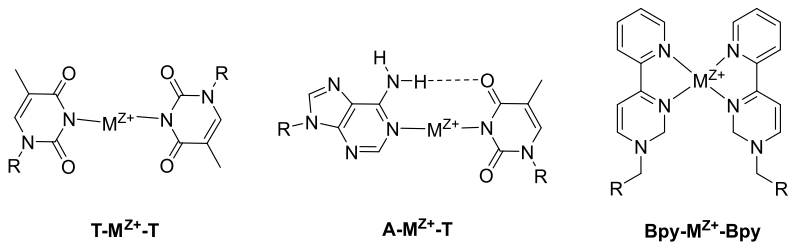


Figure 5. T-M^{Z+}-T: metal ion bridging two thymines; A-M^{Z+}-T: pairing of adenine and thymine mediated by a hydrogen bond and metal ion; Bpy-M^{Z+}Bpy: base pair between two artificial base analogues.

1.2.1 Metal-ion-mediated base pairs of unmodified nucleobases

It was found in the middle of last century that interaction of various inorganic salts with anionic DNA in solution resulted in decrease in viscosity.[29] The initial conclusions were based on the assumption that mercury binds to the phosphate groups forming intramolecular cross-links. Subsequent UV-spectrophotometric investigations, however, proved coordination of mercury ion to the nucleic acid bases.[30] It was suggested that a slippage in DNA double strands brings thymine bases of opposite strands together and this allows bridging of two deprotonated thymine bases by a mercury ion (Figure 6).[25]

Additional studies showed that the strength of the Hg^{II} induced stabilization correlated with the AT content in the helical structure.[31] The crystal structure of the complex formed by 1-methylthymine and Hg^{II} supported the idea of a metal-ion-mediated base pair.[32] The formation of T-Hg^{II}-T base pairs in double strands containing TT mismatches was later confirmed by UV, CD and NMR spectroscopy[33,34] and the data was supplemented by melting curve experiments and ESI mass spectra of mercury containing duplexes.[35,36] Introduction of mercury ions into RNA double helix increased the thermal stability by formation of U-Hg^{II}-U base pairs.[37]

Another example of metal mediated base pair formation between natural nucleobases is offered by C-Ag^{I}-C pairs. Cytosine base is highly selective to Ag^{I} ions. Ag^{I} is the only metal ion that has been observed to stabilize oligonucleotide sequences containing a single CC mismatch pair.[38,39] Two types of structure for Ag^{I} mediated base pair C-Ag^{I}-C, cisoid and transoid, exists (Figure 6).

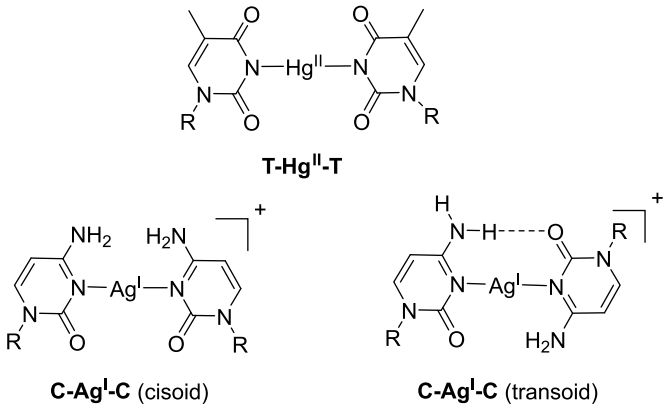


Figure 6. Chemical structure of T-Hg^{II}-T base pair and Ag^{I} mediated base pairs C-Ag^{I}-C (cisoid and transoid) (R refers to DNA backbone).

Formation of cisoid C-Ag^{I}-C base pair is preferred upon formation of Watson-Crick duplexes incorporating a single CC mismatch. The transoid base pair is stabilized by a hydrogen bond, but its formation is preferred only if the strand orientation is not predetermined by neighboring nucleobases.[40,41]

Altering the electron-withdrawing properties of the pyrimidine base moiety by substituent at position C5 (Me, Br, F, CN) affects binding properties and selectivity towards metal ion. Incorporation of such a mismatched base pair into a short oligonucleotide duplex exhibited significant differences in the affinity to Hg^{II} and Ag^{I} in pH range 5.5-9.0.[42]

Hg^{II} apart, only few reports on metal-ion-mediated base-pairing between canonical bases exist. NMR titration analysis based on characterization of imino- proton signals provided data that partial substitution of the hydrogen bonding in natural GC and AT base pairs by Zn^{II}, Co^{II} and Ni^{II} ions takes place at high pH values, consistent with several structural models.[27,36]

1.2.2 Metal-ion-mediated artificial base pairs

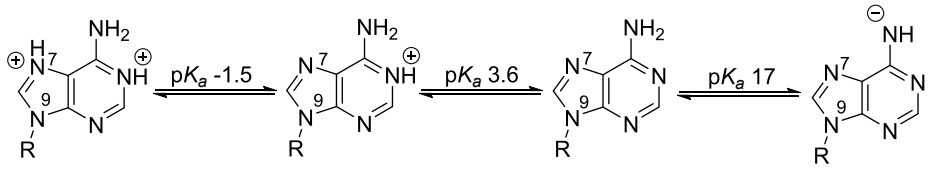
The [3+1] base pair formed by pyridine-2,6-dicarboxylate and pyridine strongly destabilized duplex but addition of $Cu(NO_3)_2$ resulted in a marked increase of the duplex thermal stability. Pyridine-2,6-dicarboxamide was even more strongly stabilizing, while N^2,N^6-dimethylpyridine-2,6-dicarboxamide, did not allow metal mediated base-pairing. No stabilization by other metal ions was observed.[43] 2,6-Bis(methylthiomethyl)pyridine/pyridine [3+1] base pair showed selective stabilization by of Ag^{I} ion.[44] Terpyridine-like artificial bases also form Ag^{I}- mediated [3+1] base pairs with pyridine.[45,46] Ag^{I} ion bridged pyridine[47] or imidazole[48] base pairs constitute a major class of fully artificial [1+1] base pairs. Three consecutive Im-Ag^{I}-Im base pairs have been incorporated into a DNA duplex.[49,50] A number of artificial base pairs, including 1,3-didezaadenine-thyminate,[51] 1,N^6 -ethenoadenine,[52] phenylpyrrolopyrimidine (phPyrdC-phPyrdC) and phenylimidazolopyrimidine (phImidC-phImidC), have been shown to be bridged by two Ag^{I} ions.[53] Ag^{I} mediated base pair between a 4-(1,2,4-triazol-1-ylmethyl)-1,2,3-triazole base and thymine offers an example of unusual [2 + 1] coordination.[54] The other examples of artificial metal ion mediated base pairs include Cu^{II} mediated hydroxypyridone,[55,56] Pd^{II}-mediated mercaptopyridinone,[57] 8-hydroxyquinoline,[58] and various 2,2-bipyridine type [28,59,60,61] pairs. The metal-salen base pair is a model of two salicylaldehyde bases involved in a cooperative stabilization process. Addition of ethylene diamine forms a cross-link between opposite salicylaldehyde bases. Coordination by Cu^{II} or

Mn^{II} ion prevents the hydrolysis of resulting imines in water, providing an exceptionally great stabilization effect.[62]

1.3 Acid-base equilibria, micro acidity constants of nucleosides

The acid base behavior of N9-substituted purines and N1-substituted pyrimidines though it is a bit "loose", determines charge, tautomeric structures and the ability to donate or accept hydrogen bonds, which is the key element in selective base-pairing and metal ion coordination.[63,64]

Adenosine is the 6-amino derivative of purine riboside (nebularine), having in solution two tautomeric forms; amino- and imino-, the proportion of the latter being less than 0.01%.[65,66] The N1 site of the major amino tautomer is the most basic site (Scheme 1). Predominance of N1 protonation has been verified by ^{15}N NMR[67] spectrometry. Adenosine is more basic (pK_a 3.6) than nebularine, owing to electron donation by the amino group at C6.[68,69] In highly acidic media, a second protonation takes place at N7. The macroscopic pK_a value for the dication is -1.5.[68,70] The amino group at C6 is extremely weak acid, ($pK_a \sim 17$) and cannot be protonated at all.[71]

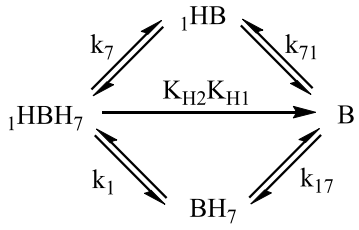


Scheme 1. Acid-base equilibria of adenosine; R refers to ribose.

Neutral forms of inosine and guanosine bear a dissociable proton at N1, the pK_a value of which falls in range 8.8-9.0 and 9.0-9.7, respectively.[72] Both molecules are protonated at N7 with formation of a monocation.[73] The pK_a value for the guanosine monocation is 1.9 and for inosine 2.3 (I = 0-0.1 mol L^{-1}). The primary amino group of guanosine is not a potential site for protonation but, as with adenosine, it is rather acidic than basic (pK_a 15**)**,[71]

The acidity constants of N9 substituted purines are macro constants which describe the overall process. The intrinsic acid-base properties of N1 and N7 as binding sites may be described in terms of micro acidity constants (Scheme 2). Micro acidity constants are useful parameters which allow quantifying the intrinsic acid-base properties of given binding sites which cannot be directly measured. This data allows calculation the ratio of monoprotonated purine

derivatives (N7-H vs N1-H). Micro acidity constants show that the impact of metal ion coordination on the overall acidity of a nucleobase is independent on the coordination site. For example, metal ion coordinated to N7 of a purine nucleoside acidifies N1 to the same extent as N1 coordination would acidify N7.[68,74]



Scheme 2. Micro acidity constant of 9-substituted purines.

Among pyrimidine nucleosides, cytidine has two tautomeric forms. The predominant one is the oxo/amino tautomer.[65] The preferred site for protonation is N3, the pK_a value being 4.5.[73] The second protonation of cytidine takes place at O^2, the macroscopic pK_a value being -6.4.[75] As with purines, the primary amino NH_2 group is not a site for protonation but is acidic (pK_a 15.5).[71]

The dioxo form predominates with uridine and thymidine.[65,76] The pK_a values for N3H of uridine and thymidine fall in the range 9.1-9.3 and 9.6-9.8, respectively.[77] 4-Oxo substituted pyrimidine nucleosides are protonated at O^4, the pK_a values falling in the range from -2.2 to -3.0 for uridine and from -3.0 to -5.2 for thymidine. The protonation of O^2 is even more difficult, the estimated pK_a values are -7.3 and -6.8 for uridine and thymidine respectively.[75,78]

1.4 Metal ion complexes of nucleosides

1.4.1 Metal ion binding sites in neutral nucleobases

Studies on metal-nucleic acid base interactions have become relevant after transition metal complexes having found applications in chemotherapy and molecular biology research. Nucleobases are N and O donor ligands forming reasonably stable complexes with divalent cations. While geometry is largely determined by the coordination nature of the cation, the binding strength depends on the ligand type. Hard Lewis acids (alkali metals, alkaline earth metals, lanthanides) prefer coordination with hard Lewis bases, such as oxygen, while soft Lewis acids (Ag^I, Au^I, Cd^{II}, Hg^{II}, Pd^{II}, Pt^{II}, Ru^{II}) prefer coordination with soft Lewis bases

(sulfur, nitrogen). Intermediate metal ions (Mn^{II}, Fe^{II}, Co^{II}, Ni^{II}, Cu^{II}, Zn^{II}) may coordinate to both hard and soft beses.[79] Natural nucleobases offer several binding sites of varying binding strength. Different cations may prefer different binding sites. Endocyclic N atoms and exocyclic carbonyl O atoms are the potential metal ion binding sites. These are N7, N3, O^6 for guanosine, N3, O^2 for cytidine; N7, N1, N3 for adenosine; O^4, O^2 for thymidine and uridine (Figure 7). These potential binding sites may participate in a unidentate complex formation, chelation or formation of metal ion bridged dimers.[80]

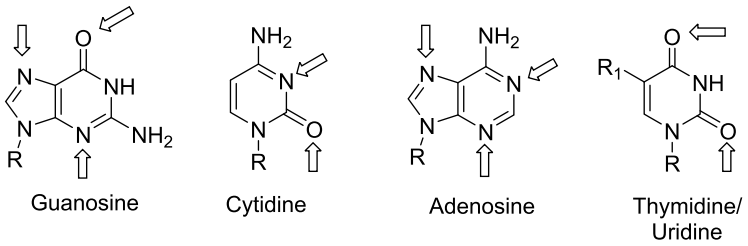
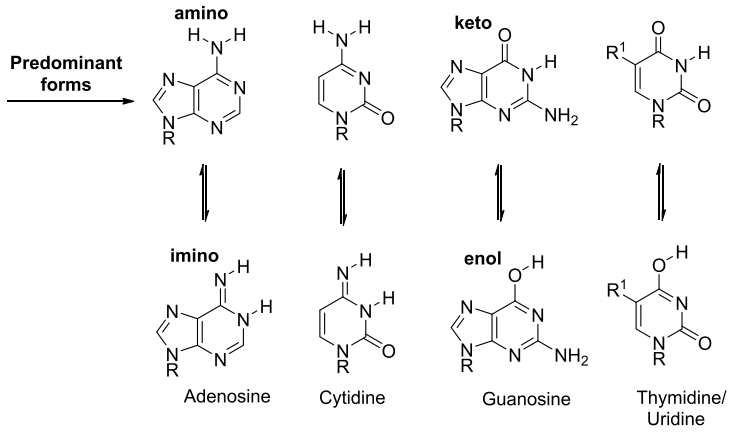


Figure 7. Potential metal-ion binding sites for nucleosides; R_1 = H, CH_3 for uracil and thymine respectively.

Primary amino groups of C, A and G are not metal ion binding sites. The lone electron pair is delocalized into heterocyclic ring, as reflected by very low basicity of these amino groups. Exocyclic amino groups are metal binding sites only after removal one of the protons by deprotonation or tautomerization.[81]

The abundance of the most stable rare tautomer, *viz*. enol-tautomer of G and T/U or imino-tautomer of A and C, is below 0.01% (Scheme 3).[82]



Scheme 3. For adenosine and cytidine predominant forms are amino; for guanosine, thymine (R^1 = CH_3)/ uridine (R^1 = H) predominant are keto tautomeric forms; R-refers to ribose.

However, a rare tautomer can be generated by coordination of metal ion. Two types of metal-nucleobase interactions are possible. First, coordination of a metal ion to a given donor atom affects electronic structure of the nucleobase causing a shift of the tautomeric equilibrium. The metal ion always remains attached at its initial binding site. For example, $[Pt^{II}(dien)]^{2+}$ binding to A-N7 and G-N7 results in a shift towards an energetically more favorable imino- and enol-tautomer, respectively.[83]

1.4.2 Metal ion binding sites in anionic nucleobases

In the second type of complexes of rare tautomers, the metal ion resides at a site normally occupied by a proton.[84] With A and C this process may occur spontaneously. With A, migration of metal ion from N1 or N7 to N^6 has been observed[85] and with C, metal ion migrates from N3 to N^4.[86] In case of G and U/T, the process is stepwise. Nucleobase is initially deprotonated and the metal ion is then attached to the deprotonated site, followed by reprotonation of the anionic nucleobase at a position different from the original one. Metal ion binding to deprotonated N1 of G and subsequent reprotonation of N7 or O^6 provides alternative metallated forms of G.[87,88,89] Analogously, metal binding to N3 of anionic U or T and reprotonation at N^4 of C provide metallated forms of U/T and C (Figure 8).[90]

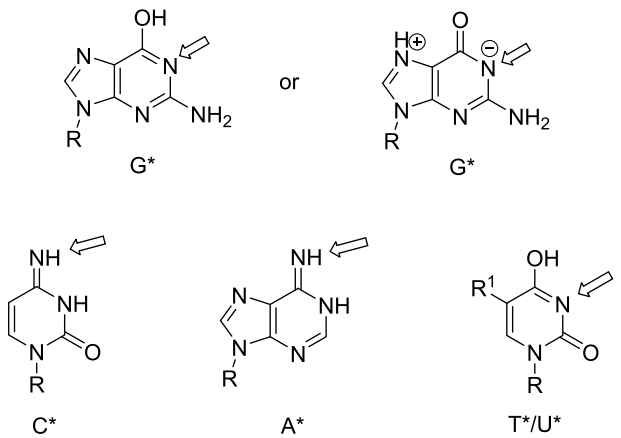
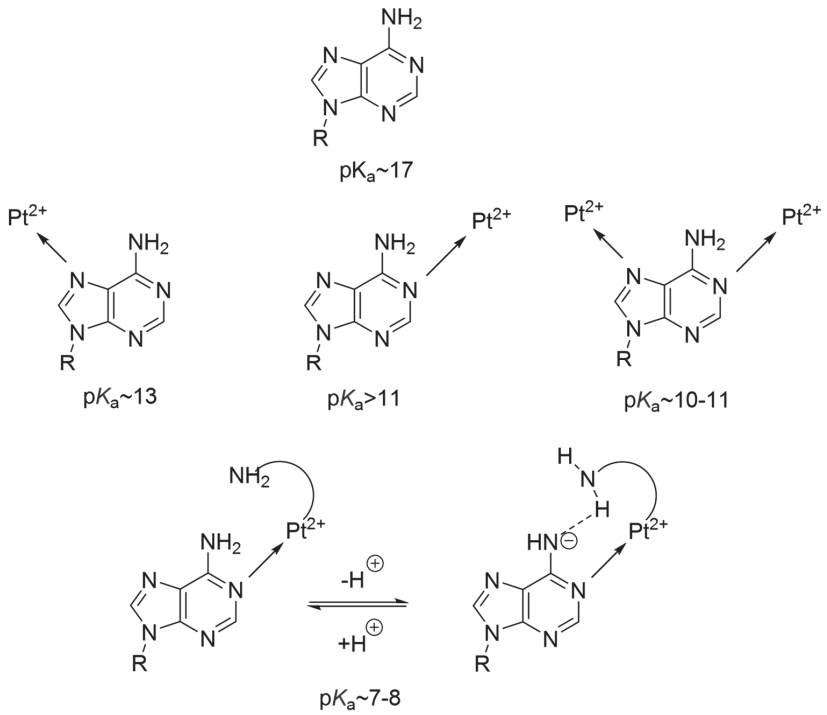


Figure 8. "Metal-ion-stabilized" rare tautomeric forms of nucleosides; R^1 = -H, $-CH_3$ for U* and T* respectively. Arrows show locations of the potential metal ions.

Transition metal ions, Pt^{II}, Pd^{II}, Ru^{II}, Hg^{II} and Zn^{II}, bind deprotonated nucleobases at neutral and even slightly acidic pH. The existence of a deprotonated nucleobase is related to polarizing power of the metal ion bound to the neutral ligand. Protons remote from the metal ion become acidified. Metal oxidation state, the number of coordinated metal ions and presence of a co-ligand of the metal may significantly effect on the pK_a value. The pK_a value of the primary N^6H_2 amino group of 9-methyladenine, for example, is about 17. Attachment of Pt^{II} ion to N1 or N7 decreases the pK_a value of the amino group and in the presence of a hydrogen-bond-donating co-ligand the pK_a value may fall in the range 7-8, *i.e.* close to physiological pH range (Scheme 4).[91]



Scheme 4. Effects of metal coordination on the acidity of the primary amino group of 9-substituted adenine.[91]

Hydrated metal ions undergo deprotonation to a hydroxo complex $[M^{z+}(H_2O)_n(OH)]^{z-1}$. Accordingly, they have their own "base", which is capable of taking part in deprotonation of the nucleobase.[91] An incomplete list of patterns of metal ion complexes containing anionic nucleobases is presented in Figure 9.

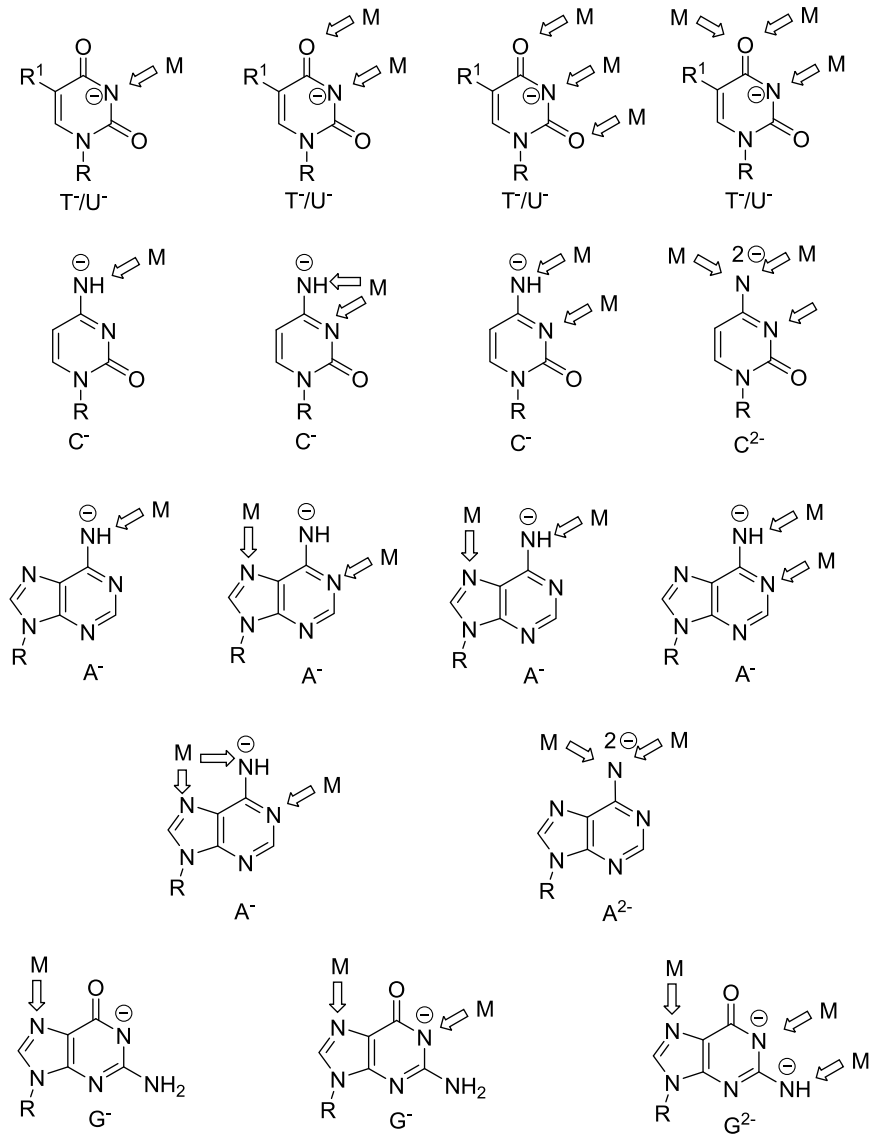


Figure 9. Examples of metal complexes with anionic deprotonated nucleobases; R^1 = -H, -CH_3 for U and T respectively. (The charges of the deprotonated nucleobases have been indicated on the site of deprotonation. Other mesomeric structures are not presented).

1.4.3 Nucleoside complexes of 3d-transition metals and soft- metal ions

While nebularine is protonated at N1, several metal ions bind to both N1 and N7 sites, the N7 site being even slightly favored. According to ^{1}H-NMR relaxation time (T_1) measurements, 3d transition metal ions, such as Cu^{II} and Mn^{II}, coordinate at neutral pH more readily to N7 than N1 of nebularine.[92] The stability of 3d-transition metal ion complexes increases in the order of

Irving-Williams series: $Cu^{II} > Ni^{II} > Co^{II} > Zn^{II}$.[93,94] NMR spectroscopic measurements with Pt^{II} have shown that soft metal ions prefer the N7 binding site. At slight excess of Pt^{II}, an N1, N7-bridged 2:2 (M:L) complex is formed.[68]

The dichotomy between coordination at N1 and N7 sites of adenosine has been intensively studied. The binding mode depends on pH and the nature of the metal ion. In solid state, 3d transition metal ions favor N7 over N1.[95] In aqueous solution, binding to N7 is twice as strong as binding to N1.[96] N3-binding has not been reported. Most probably the presence of the bulky ribosyl substituent at N9 forms a steric obstacle for the binding.[68] Adenosine is more basic than nebularine and in principle forms more stable metal ion complexes. The steric hindrance caused by the amino group seems, however, to overcompensate the stabilizing effect of its electron-donating property. The complexes are less stable than those of nebularine. Evidently, chelate formation between N1 or N7 and the primary amino group does not play a role, either.[94,97] However, indirect chelate formation through hydrogen bonding of the amino group to an aqua ligand of N7-coordinated Cu^{II} and Zn^{II} has been suggested.[98,99] Soft Lewis acids, Ag^{I}, Hg^{II} and Pd^{II}, show enhanced binding to both N1 and N7 of adenosine.[100] **[**$MeHg^{II}]^{+}$ ion binds N1 of adenosine under slightly acidic conditions, and displaces a proton from primary amino group under basic conditions, facilitating the displacement of N^6 protons.[101,102] **[**$MeHg^{II}]^{+}$ species bound to N^6 can disproportionate into a disubstituted species and a free base.[103] Pt^{II} binds to both N7 and N1 sites. Coordination is pH dependent; at acidic pH, dimeric $Ado_2Pt^{II}_2$ and polymeric species are formed.[104] $[Pd^{II}(en)]^{2+}$ and $[Pd^{II}(dien)]^{2+}$ ions bind to both the N1 and N7 sites, binding to N1 being favored.[68,100] Under acidic condition, protonation of N1 competes with metal ion binding. Under basic conditions, the $[Pd^{II}(en)]^{2+}$ ion tends to displace a proton from the primary 6-amino group of one adenosine and at the same time coordinates to the N1 site of the another adenosine. Thus two molecules of adenosine are bridged by two molecules of $[Pd^{II}(en)]^{2+}$.[105]

Neutral inosine and guanosine form approximately as stable complexes as adenosine. N1 of guanosine and inosine remains protonated and the available site for coordination is N7.[101,106,107] The complexes of deprotonated guanosine and inosine are more stable, the metal ions being N1-coordinated.[108] The crossover pH values for guanosine are: Ni^{II} 7.8, Cu^{II} 6.9, Zn^{II} 7.5. For inosine, the values are slightly lower: Ni^{II} 7.1, Cu^{II} 6.1, Zn^{II} 6.7.[68] With **[**$MeHg^{II}]^{+}$ the crossover pH is lower than with Ni^{II}, Cu^{II} and Zn^{II} ions: 4.3 with inosine and 5.6 with guanosine. High concentration of **[**$MeHg^{II}]^{+}$ ion tends to yield N1, N7-dimercurated complex.[109] Under alkaline condition, **[**$MeHg^{II}]^{+}$ ion may displace a proton from the primary amino group providing N1, N^2-dimercurated complexes.[110] Ag^{I} undergoes under slightly acidic conditions N7-

coordination without any interactions with the carbonyl group.[111,112] Both binding modes occur in the pH range close to neutrality, leading to formation of a 4:4 structure.[113] $[Pd^{II}(en)]^{2+}$ ion complexes of 6-oxo purines nucleosides are much more stable than those of 3d transition metal ions. Pt^{II} coordination to 6-oxopurine nucleosides is similar to that of Pd^{II} ions. Around neutrality, Pt^{II} ions bind to both N1 and N7 sites.[104]

Cytidine forms with 3d transition metal ions slightly more stable complexes than adenosine. N3 is the major binding site,[114,115] interaction with O^2 being weak[116] or not existent.[117] The stability is decreased in the order: $Cu^{II} > Ni^{II} > Co^{II} > Zn^{II}$.[118] Hg^{II} ions coordinate to N3. Interaction with O^2 atom is weak.[119] Under alkaline conditions, primary amino protons may be displaced by $[MeHg^{II}]^+$ ions resulting in formation of a trinuclear [N3, N^4, N^4-$(Hg^{II}Me)_3$] complex.[120] $PdCl_2$, $[Pd^{II}(en)]^{2+}$ and $[Pd^{II}(dien)]^{2+}$ also binds to N3.[121] At pH 5, $[Pd^{II}(en)]^{2+}$ binds to N3, and under slightly basic conditions, one of the protons of the primary amino group may additionally be displaced by concomitant formation of dimeric complexes, where two $[Pd^{II}(en)]^{2+}$ ions bridge between N3 of one ligand and the amino group of the other ligand.[122] $[Pd^{II}(dien)]^{2+}$ behaves like $([Pd^{II}(en)]^{2+}$.[123,124]

Under neutral and basic conditions, N3 of uridine and thymine is the most favorable site for coordination. Under acidic conditions, coordination of metal ions to N3H is prevented. The complexes of deprotonated uridine and thymine ligands are approximately as stable as complexes of deprotonated guanosine and inosine.[125,126] Cu^{II} shows interaction with oxygen atoms in addition to N3 coordination.[127] The affinity of Hg^{II} ions to N3 is high, comparable to the proton affinity.[110] Ag^{I} also shows high affinity to deprotonated N3 of the 4-oxopyrimine nucleosides.[128] The affinity of $[Pd^{II}(en)]^{2+}$ and $[Pd^{II}(dien)]^{2+}$ ions is comparable to that of $[MeHg^{II}]^+$ ion.[122] A hydroxo-bridged 2:2 complex may be formed under slightly alkaline conditions.[129]

1.5 Tridentate Pd^{II} chelates with nucleosides

Interaction of a tridentate Pd^{II} complex with a nucleobase leads to formation of a ternary complex where the nucleobase is engaged as a monodentate ligand. The stability of such a ternary complex is strongly affected by non-covalent interactions between non-coordinating functional groups introduced in the ligand structure.[123,130,131,132] The ability of tridentate Pd^{II} complexes to form ternary complexes with nucleobases also largely depends on the type and distribution of the donor atoms in the coordination sphere. Pyridine type ligands allow a reversed electron donation process, so-called back donation of electron density from metal ion

d-orbital to π* orbital of pyridine, which increases electrophilicity of the metal ion and, hence, binding to the entering nucleobase as the first stage of the associative ligand exchange process. Additional electron density from the entering nucleobase is also donated to π*-accepting system which stabilizes the pentacoordinated transition and subsequent intermediate. Accordingly, the reactivity of tridentate Pd^{II} complexes follows the order aaa < apa < aap < pap < app < ppp, where a stands for an aliphatic amino group and p a pyridine-type nitrogen. The aqua complexes are more reactive than the corresponding chlorido complexes. Coordinated water molecule is a better leaving group than chloride. Aqua complexes are susceptible to deprotonation resulting hydroxo complexes and highly reactive dimeric structures containing bridging hydroxo ligand.[130]

Coordination properties of various tridentate nitrogen ligands have been investigated by a set of comparative experiments (Figure 10).

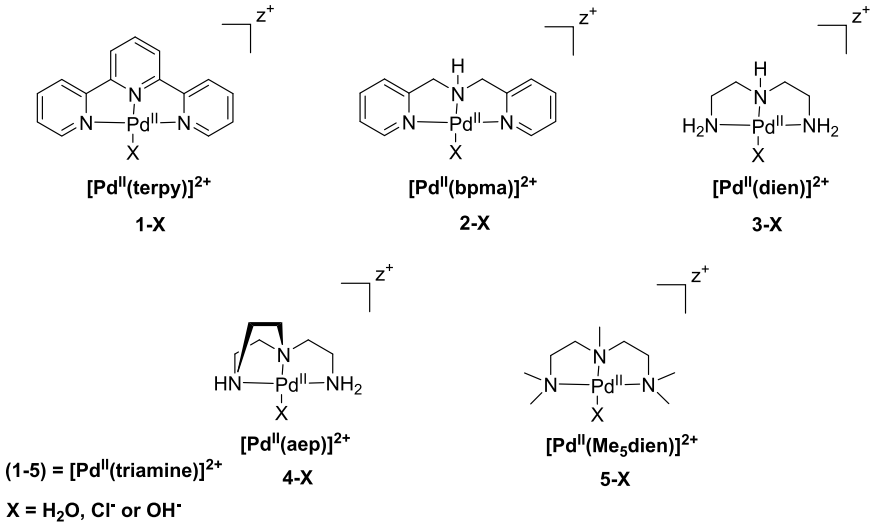


Figure 10. Tridentate Pd^{II} complexes where X- presents the labile monodentate ligands. X = H_2O for aqua complexes; Cl^- for chlorido complexes; OH^- for hydroxo complexes. z^+ is charge: 2^+ for H_2O and 1^+ for Cl^- and OH^- respectively.

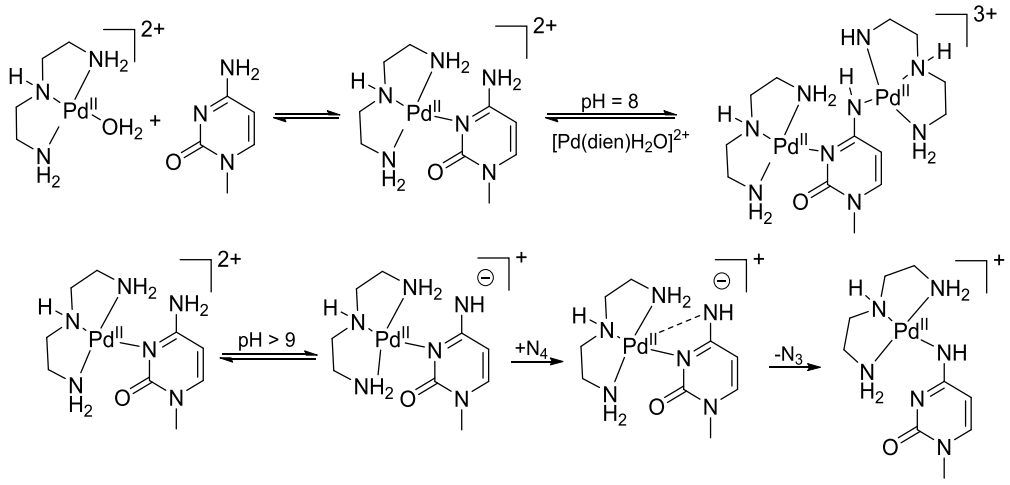
Coordination properties of $[Pd^{II}(bpma)H_2O]^{2+}$ **[2-H_2O]** [bpma = bis(pyridine-2-yl-methyl)amine] are similar to those of $[Pd^{II}(terpy)H_2O]^{2+}$ **[1-H_2O]** [terpy = 2,2′:6′,2″-terpyridine, differing from those of $[Pd^{II}(dien)H_2O]^{2+}$ **[3-H_2O]** [dien = diethylenetriamine]. The formation of hydroxo complexes is preferred in the order: $[Pd^{II}(terpy)H_2O]^{2+}$ > $[Pd^{II}(bpma)H_2O]^{2+}$ > $[Pd^{II}(dien)H_2O]^{2+}$. By contrast, the complexes with all nucleobase derivatives were approximately as stable (Table 1).

Table 1. *Stability constants of mixed ligand complexes of Pd^{II} (T = 298 K, I = 0.2 M)*

Ligand (L)	Species*	$[Pd^{II}(dien)]^{2+}$	$[Pd^{II}(bpma)]^{2+}$	$[Pd^{II}(terpy)]^{2+}$
OH^-	MH_{-1}	-7.50	-7.08	-6.84
Uridine	ML	8.47	8.90	8.29
Cytidine	ML	5.66	5.83	-
MeUH	ML	8.56	9.26	8.42
MeC	ML	5.62	5.84	4.60
	M_2LH_{-1}	-	1.76	3.38
EtGH	MLH	15.74	15.06	15.37
	ML	8.20	8.11	8.44
	M_2L	14.68	14.95	14.56

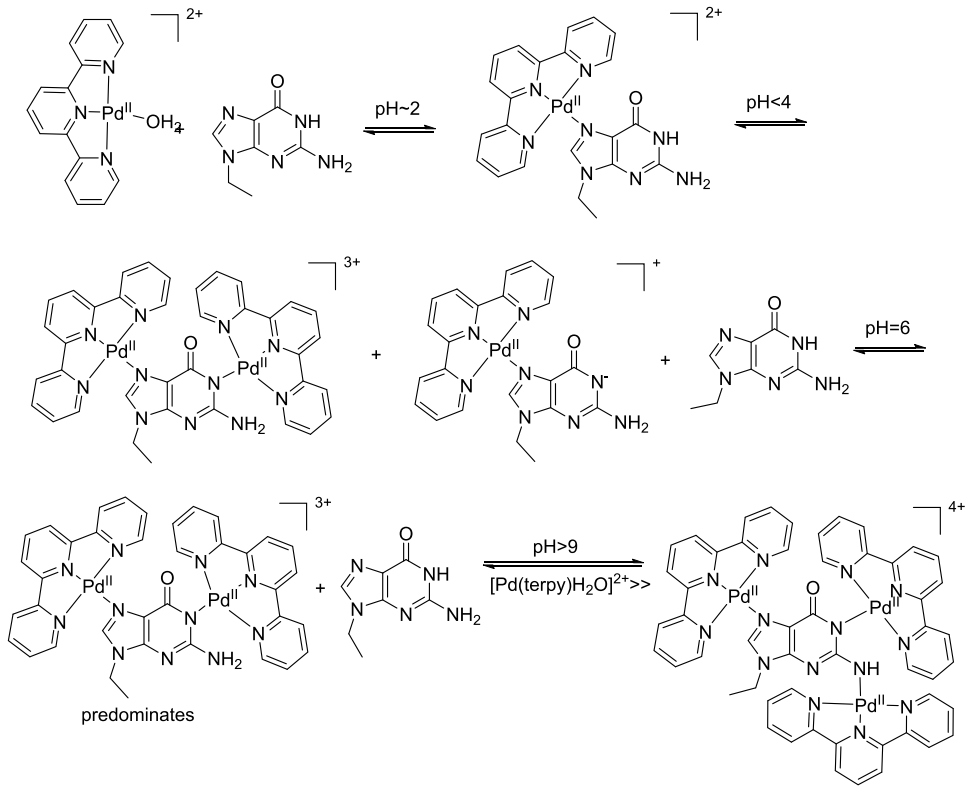
*M stands for the species $[Pd^{II}(dien)]^{2+}$, $[Pd^{II}(bpma)]^{2+}$ and $[Pd^{II}(terpy)]^{2+}$.

Deprotonation and metal ion binding of the exocyclic amino group of N3-complexed 1-methylcytosine yields dinuclear complexes with $[Pd^{II}(terpy)H_2O]^{2+}$and $[Pd^{II}(bpma)H_2O]^{2+}$ at pH 4 and pH 5, respectively. The high stability of the dinuclear complex $\{[Pd^{II}(terpy)]_2$-(N3, N^4-MeC]$\}^{2+}$ results from strong stacking interaction between the two terpy-ligands. The stability of the $\{[Pd^{II}(bpma)]_2$-(N3, N^4–MeC]$\}^{2+}$ complex is lower, owing to weaker stacking interactions.[131] With $[Pd^{II}(dien)H_2O]^{2+}$, such dinuclear complexes of 1-methylcytosine have been observed only under basic conditions. Increasing pH to > 9 leads to formation of a mononuclear N^4-coordinated $[Pd^{II}(dien)$-(N^4-MeC)$]^+$ as the predominant species (Scheme 5).[123] Complexing with 9-ethylguanine (EtGH) proceds by initial binding to N7 followed by N1 coordination, giving binuclear species with all the tridentate ligands studied.[123]



Scheme 5. Formation of complex of $[Pd^{II}(dien)H_2O]^{2+}$ **[3-H_2O]** with MeC in basic solutions.[123,133]

Similar differences exist between coordination mechanisms of the chlorido complexes, $[Pd^{II}(dien)Cl]Cl$ and $[Pd^{II}(terpy)Cl]Cl$ that are in equilibrium with aqua and hydroxo complexes.[130,133] These chlorido complexes of tridentate Pd^{II} undergo fast ligand exchange, the complex formation with nucleobases being complete in a few minutes. Both complexes bind to N3 of uridine, 1-methyluracil (MeU) and 1-methylthymine (MeTH), binding to 1-methylthymine being slightly favored. Both chlorido complexes form in acidic pH range a binuclear complex with 9-methyladenine (MeA). $[Pd^{II}(terpy)(H_2O)]^{2+}$ forms exceptionally stable dimeric N1-N7 and N(1)-N^6 complexes at pH < 5. Involvement of deprotonated exocyclic amino groups of MeA and MeC was especially favored with $[Pd^{II}(terpy)(H_2O)]^{2+}$. Formation of N7-N1-bridged dimers of inosine and EtGH was characteristic for both $[Pd^{II}(dien)(H_2O)]^{2+}$ and $[Pd^{II}(terpy)(H_2O)]^{2+}$. Excess of $[Pd^{II}(terpy)(H_2O)]^{2+}$ compared to EtGH has at high pH led to formation of a trinuclear species exhibiting N1, N7 and N^2 coordination (Scheme 6). The concentration of each ternary complex is strongly pH dependent and the order of stability at neutral pH is given in order N1/N7(G) > N3(U,T) > N3(C) ≥ N1/N7(A).[133]



Scheme 6. Formation of ternary complex of $[Pd^{II}(terpy)(H_2O)]^{2+}$ **[1-H$_2$O]** with EtGH depending on pH, either mononuclear N7, binuclear N7-N1 or trinuclear N7, N1, N^2H.[133]

The donor atoms in $[Pd^{II}(dien)H_2O]^{2+}$ **[3-H_2O]** and $[Pd^{II}(Me_5dien)H_2O]^{2+}$ **[5-H_2O]** [Me_5dien = *N*,*N*′,*N*″,*N*″-pentamethyldiethylenetriamine] are aliphatic amino nitrogens. $[Pd^{II}(dien)]^{2+}$ can participate in hydrogen bonding interactions via amino groups, while the *N*-Me groups of $[Pd^{II}(Me_5dien)]^{2+}$ only result in steric crowding. Coordination of both of these mono-functional complexes to purine nucleosides shows that the ratio of N7/N1 binding is significantly greater than the basicity ratio N7/N1. $[Pd^{II}(dien)]^{2+}$ does not form a hydrogen bond to O^6 of guanosine or inosine either directly or via a water molecule, but it instead interacts with the 5′-phosphate of the corresponding nucleotides. Both complexes bind to N3 of pyrimidine nucleosides and their 5′-monophosphates. Formation of dimeric structures through exocyclic amino groups has not been observed under the slightly acidic conditions employed. Stability constants of the ternary complexes of $[Pd^{II}(dien)]^{2+}$ with all natural nucleosides are 0.7-1.9 log units higher than the corresponding values for $[Pd^{II}(Me_5dien)]^{2+}$. Due to the *N*-methyl groups in $[Pd^{II}(Me_5dien)]^{2+}$, rotation of purines and pyrimidines around Pd-N3,N1,N7 bonds is restricted. Two rotamers for each type of binding have been assigned on the basis of proton chemical shifts in NMR spectra (Figure 11).[134]

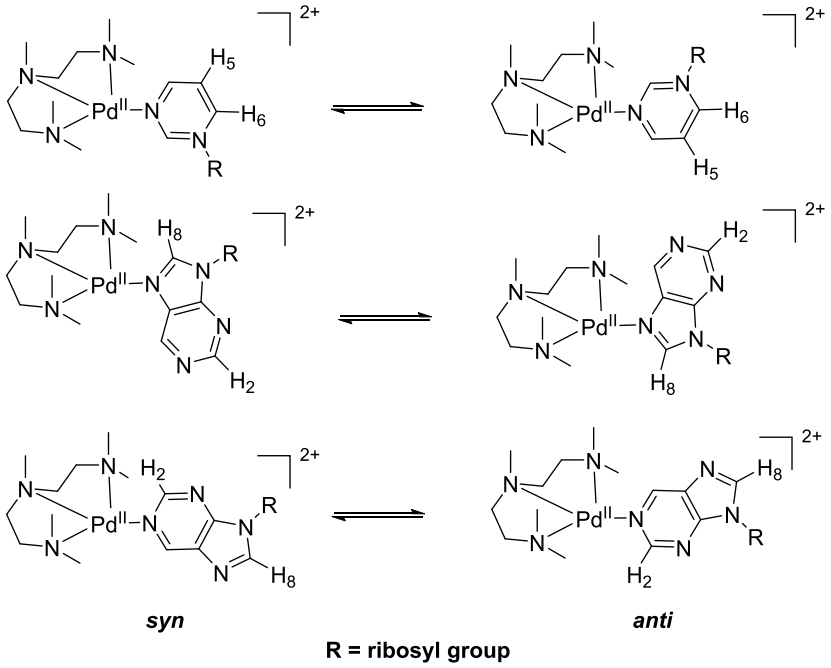


Figure 11. *Syn*- and *anti*- rotamer models of $[Pd^{II}(Me_5dien)]^{2+}$ **(5)** complexes. Pyrimidine model is bound at N3 and purine model at N7and N1.

Interestingly, investigations of substitution reactions of $[Pd^{II}(dien)H_2O]^{2+}$ and $[Pd^{II}(dien)Cl]^{+}$ with nucleobases, nucleosides and nucleotides have shown that the rate-limiting step is not the ligand exchange process between chloride ion and water molecule, but rather subsequent replacement of water molecule by nitrogen ligand.[135,136]

$[Pd^{II}(aep)(H_2O)]^{2+}$ **[4-H_2O]** -[aep = 1-(2-aminoethyl)piperazine] is a cyclic analogue of $[Pd^{II}(dien)(H_2O)]^{2+}$ (Figure 10). While the pK_a value of $[Pd^{II}(dien)H_2O]^{2+}$ is 7.74 at 25 °C and 0.5 M ionic strength,[105] $[Pd^{II}(aep)(H_2O)]^{2+}$ is more acidic, the pK_a being 4.68. Accordingly, the stability of aqua complex is decreased and the electrophilicity of the metal center increased. The main species under physiological conditions is the monohydroxo complex $[Pd^{II}(aep)(OH)]^{+}$. The concentration of the monohydroxo-bridged dimer reaches a maximum of 16% at pH = 4.6. $[Pd^{II}(aep)]^{2+}$ binds to inosine N7 at pH below 2, where N1 remains protonated. Coordination to N7 acidifies N1H. The dimeric species bound to both N1 and N7 starts to be formed at pH > 2 and reaches the maximum concentration 58% at pH 3.8. The N1 coordinated species starts to be formed contemporaneously with the dinuclear complexes and its concentration reaches the maximum level of 92% at pH = 7.6. GMP shows similar behavior but forms slightly more stable complexes, probably due to interactions with the phosphate group. In comparison to $[Pd^{II}(dien)H_2O]^{2+}$, the substitution reactions of $[Pd^{II}(aep)(H_2O)]^{2+}$ occur more slowly.[137]

1.6 Complexing of bidentate Pd^{II} chelates with nucleosides

Interaction of the bidentate Pd^{II} complexes (Figure 12) with nucleic acid bases leads to formation of ternary complexes where the nucleobase is engaged as a monodentate or bidentate ligand.[138-140] The structure and stability of such ternary complexes is strongly affected by non-covalent interactions between non-coordinating functional groups of the ligands. The reactivity of the complex largely depends on the chemical environment in the coordination sphere of metal ion. The reactivity follows the order aa < ap < pp (where a stands for an aliphatic amino group and p for a-pyridine-type nitrogen).[79,130,139,140,141,142] In comparison to tridentate ligands, hydrolytic reactions of bidentate complexes are more complicated. Bidentate Pd^{II} complexes contain two aqua ligands and, hence, in addition to a simple monomeric hydroxo complex, various polynuclear hydroxo-bridged species may be formed.[79,141] Most of the complexes studied (Figure 15) are derivatives of aliphatic amines.

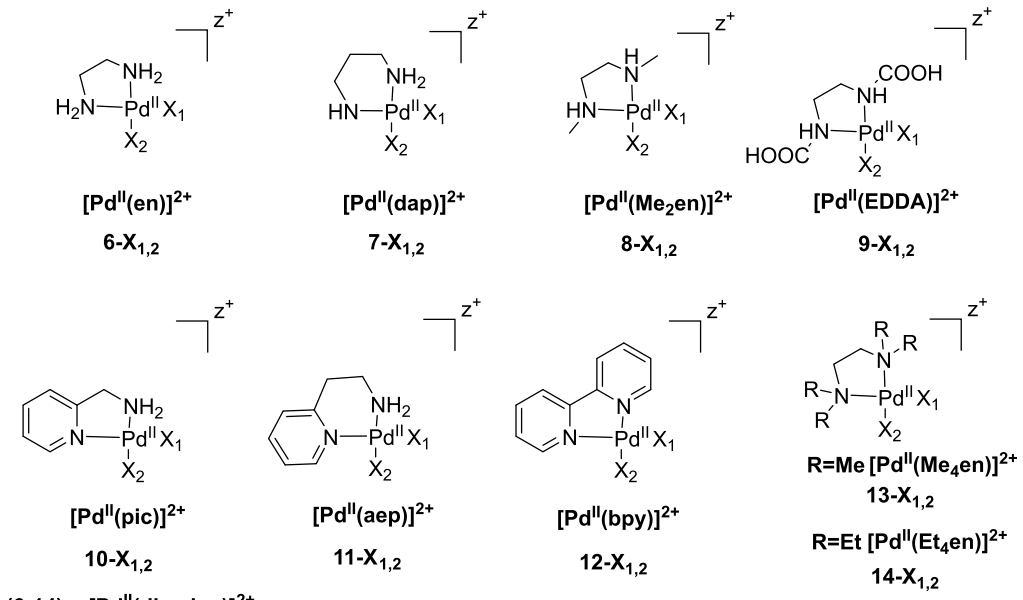


Figure 12. Bidentate Pd^{II} aqua complexes of **6-14** where $X_{1,2}$ stands for two labile monodentate ligands. X = Cl^- for chloride complexes; OH^- for hydroxo complexes. z^+ is charge: 2^+ for H_2O and 1^+ for Cl^- and OH^- respectively and zero charge for $2Cl^-$ and $2OH^-$

These include $[Pd^{II}(en)(H_2O)_2]^{2+}$ [en = ethylenediamine] (**6**-$(H_2O)_2$),[143] $[Pd^{II}(dap)(H_2O)_2]^{2+}$ [dap = 1,3-diaminopropane] **[7-$(H_2O)_2$],**[142] amino substituted $[Pd^{II}(Me_2en)(H_2O)_2]^{2+}$ [Me_2en = *N*,*N*′-dimethyletylenediamine] **[8-$(H_2O)_2$]**,[144] $[Pd^{II}(Me_4en)(H_2O)_2]^{2+}$ [Me_4en = *N*,*N*′,*N*″,*N*″-tetramethylethylenediamine] **[13-$(H_2O)_2$]**,[145] $[Pd^{II}(Et_4en)(H_2O)_2]^{2+}$ [Et_4en = *N*,*N*′,*N*″,*N*″-tetraethylethylenediamine] **[14-$(H_2O)_2$]**[145] and $[Pd^{II}(EDDA)(H2O)_2]^{2+}$ [EDDA= ethylenediamine *N*,*N*-diacetic acid] **[9-$(H_2O)_2$]**.[146] Some of the complexes studied, however, contain one or two heteroaromatic nitrogen bases (pyridine): $[Pd^{II}(pic)(H_2O)_2]^{2+}$ [pic = 2-picolylamine] **[10-$(H_2O)_2$]**,[147] $[Pd^{II}(aep)(H_2O)_2]^{2+}$ [aep = 2-(2-aminoethylpyridine)] **[11-$(H_2O)_2$]**[148] and $[Pd^{II}(bpy)(H_2O)_2]^{2+}$ [bpy = 2, 2′-bipyridine] **[12-$(H_2O)_2$]**.[140] In the following, complexes having special properties are discussed. Effects of bulky substituents and Cl^- ion are considered using *N*,*N*′-disubstituted ethylene diamine palladium complexes as model compounds **[13-$(H_2O)_2$]**, **[14-$(H_2O)_2$]**.

1.6.1 Pd^{II} complexes of aliphatic diamine ligands

Thymidine forms with $[Pd^{II}(en)]^{2+}$-type complexes, somewhat unexpectedly, more stable ternary complexes than uridine, possibly due to the fact that the methyl group at C5 increases π-electron polarizability. Owing to competition with proton for the N3 site, the complex formation takes place above pH 6. Cytidine forms a complex even at slightly acidic pH, often with involvement of the exocyclic amino group. With purines, N1 is protonated at low pH and coordination takes place at N7 binding site, which, in turn, decreases the pK_a value of N1H. The pK_a value of the inosine N7-complex, for example, is 4.11, while that of free inosine is 8.80. Upon increasing pH, deprotonation at N1 takes place with concomitant migration of $[Pd^{II}(diamine)]^{2+}$ to the N1 binding site**.** Some species have been suggested to involve N7-O^6 chelation.[105,145,149] The monophosphates usually afford more stable complexes than nucleosides, and complexes of IMP and GMP are more stable than those of pyrimidine monophosphates.[105,150]

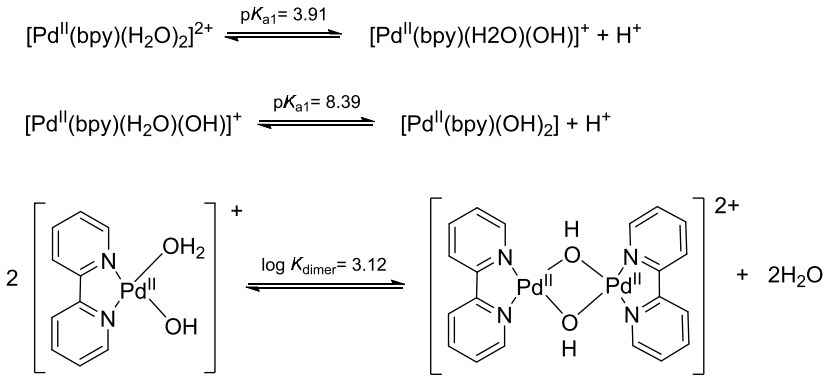
Dinuclear dimeric complexes of the type $[(Pd^{II}(diamine)\text{-}L]_2$ are typical for bidentate Pd^{II} complexes containing sterically unhindered aliphatic ligands. This tendency is less marked for mixed aliphatic/aromatic and fully aromatic systems. Their formation is strongly pH and concentration dependent.[138,139,140] Complexing proceeds in a stepwise manner and the second step is always the slow step.[151] Complexing of adenosine with bidentate Pd^{II} complexes has been studied less extensively, but the basic behavior is similar to the N7/N1 dichotomy of other purines. Under acidic conditions, N7 is the only binding site and upon gradual pH increase, coordination to N1 takes place. At high pH, the exocyclic N^6 undergoes substitution of a proton by the metal ion, leading to formation of various dimers. For aliphatic sterically unhindered complexes, such as $[Pd^{II}(en)]^{2+}$ **(6)**, characteristic coordination involves two bidentate Pd^{II} complexes bridging between N1 of one N^6-deprotonated adenosine and exocyclic N^6 of another.[105]

The pK_{a1} and pK_{a2} values of aromatic $[Pd^{II}(bpy)(H_2O)_2]^{2+}$ **[12-$(H_2O)_2$]** complex (bpy = bipyridine) are lower than those of aliphatic Pd^{II}-diamine complexes,[140] *viz.* 3.91 and 8.39. With $[Pd^{II}(pic)(H_2O)_2]^{2+}$ [pic = 2-picolylamine] **[5-$(H_2O)_2$]**, representing a combination of aromatic and aliphatic nitrogens, the pK_a values fall between those of exclusively aliphatic and aromatic nitrogen donor systems. These differences can be explained by increased positive charge on Pd^{II} ion due to the π-acceptor properties of pyridine ring, which increases the electrophilicity of Pd^{II} ion.[152] The pK_{a1} values for $[Pd^{II}(en)(H_2O)_2]^{2+}$ **[6-$(H_2O)_2$]** and $[Pd^{II}(Me_2en)(H_2O)_2]^{2+}$ [**8-$(H_2O)_2$**] are 5.6 and 5.4, respectively. Most probably the inductive

effect of the methyl substituents provides a slight increase in the electrophilicity of Pd atom, resulting in an increase in the acidity of coordinated water molecule.[122,144,152,]

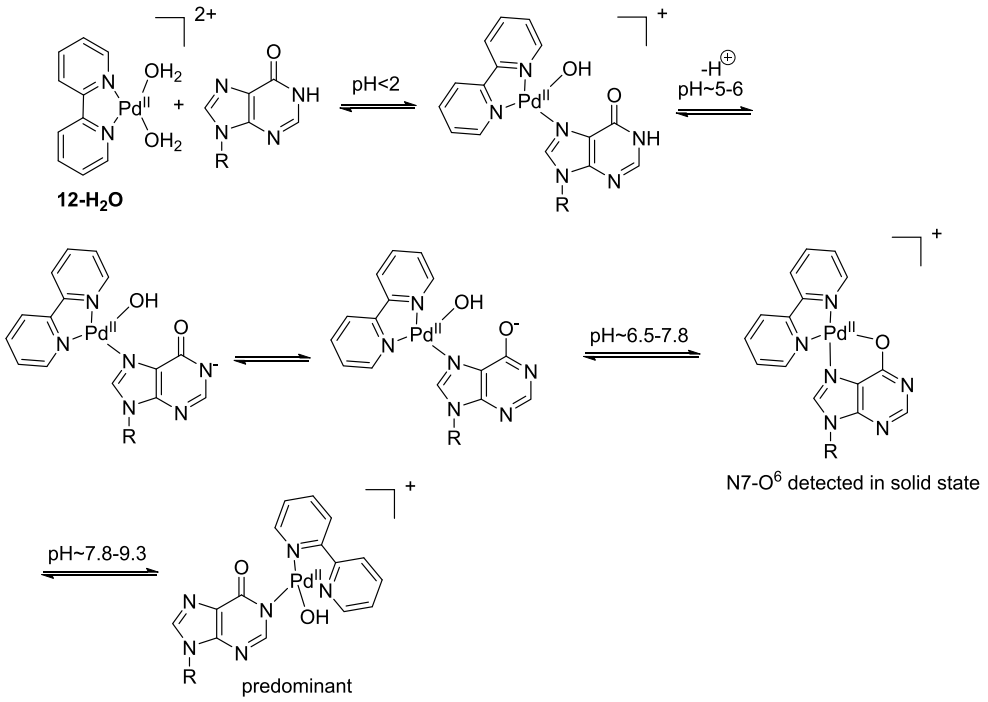
1.6.2 Pd^{II} complexes of aromatic diamine ligands

As mentioned above, $[Pd^{II}(bpy)(H_2O)_2]^{2+}$ [bpy = 2,2`-bipyridine] **[12-$(H_2O)_2$]** is at physiological pH present as a mixture of monohydroxo, $[Pd^{II}(bpy)(H_2O)(OH)]^{+}$, and diaqua, $[Pd^{II}(bpy)(H_2O)_2]^{2+}$ species (Scheme 7).



Scheme 7. $[Pd^{II}(bpy)(H_2O)_2]^{2+}$ **(12-H_2O)** hydrolysis and dimerization pathway.

$[Pd^{II}(bpy)(H_2O)_2]^{2+}$ forms most stable complexes with nucleic acid constituents due to π-π staking interaction between the pyridine rings and purine/pyrimidine rings. Interaction with DNA constituents leads to formation of 1:1 and 1:2 complexes. N7-O^6 chelation occurs but the site of coordination is changed from N7 to N1 upon increasing pH. Complexes with IMP (inosine 5´-monophosphate) are more stable than those with inosine. Proposed coordination process of $[Pd^{II}(bpy)(H_2O)_2]^{2+}$ and 5-IMP is given as an illustrative example in Scheme 8. Stable complexes with deprotonated uridine are formed under basic conditions.[140]



Scheme 8. Interaction of $[Pd^{II}(bpy)(H_2O)_2]^{2+}$ **[12-$(H_2O)_2$]** with IMP; R- refers to ribose 5´-monophosphate.

1.6.3 Comparative studies of mixed aromatic/aliphatic and aliphatic/aliphatic complexes

Coordination modes of two bidentate ligands, $[Pd^{II}(en)(H_2O)_2]^{2+}$ **[6-$(H_2O)_2$]** and $[Pd^{II}(pic)(H_2O)_2]^{2+}$ **[10-$(H_2O)_2$]** (pic = 2-picolylamine), with nucleic acid bases have been investigated by potentiometric method and NMR spectroscopy. $[Pd^{II}(pic)(H_2O)_2]^{2+}$ forms with nitrogen donors more stable complexes than $[Pd^{II}(en)(H_2O)_2]^{2+}$, most probably due to π-π stacking interaction between the pyridine ring and purine/pyrimidine rings. Comparison of the stability constants shows two significant differences between the hydrolytic reactions of $[Pd^{II}(en)(H_2O)_2]^{2+}$ **[6-$(H_2O)_2$]** and $[Pd^{II}(pic)(H_2O)_2]^{2+}$ **[10-$(H_2O)_2$]**. First, the proportion of polynuclear complexes is different. In addition to monohydroxo-, $[(Pd^{II}(en)(H_2O))_2(OH)]^{+}$, (**6a**) and dihydroxo-, $[Pd^{II}(en)(OH)_2]$ **[6-$(OH)_2$]** complexes, formation of dimeric $[(Pd^{II}(en)(OH))_2]^{2+}$ (**6b**) and trimeric $[(Pd^{II}(en)(OH))_3]^{3+}$ (**6c**) hydroxo-bridged species has been detected by NMR spectroscopy (Figure 13).[153]

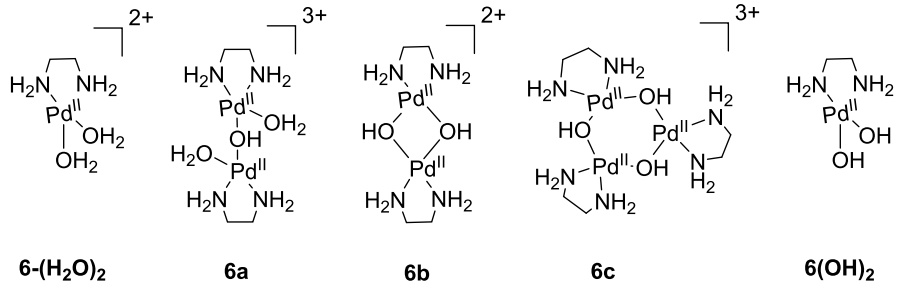


Figure 13. Proposed species for $[Pd^{II}(en)(H_2O)_2]^{2+}$ **[6-(H_2O)$_2$]**.

$[Pd^{II}(pic)(H_2O)_2]^{2+}$ **[10-(H_2O)$_2$]** does not, in turn show any tendency of formation of trinuclear (**6c**) species, but the proportion of the monomer is significant (Figure 14). The decreased polymerization tendency of $[Pd^{II}(pic)]^{2+}$ may result from steric interactions of bulky pyridine moieties. The species $[Pd^{II}(pic)(H_2O)_2]^{2+}$ exists only in acidic media at pH < 2. Both monohydroxo- $[(Pd^{II}(en)(H_2O))_2OH]^{3+}$ (**6a**), $[(Pd^{II}(pic)(H_2O))_2OH]^{3+}$ (**10a**) and dihydroxo-, $[(Pd^{II}(pic)(OH))_2]^{2+}$ (**10b**), $[(Pd^{II}(en)(OH))_2]^{2+}$ (**6b**) bridged dimers are formed in pH-range 3-6, being, hence, the major species under physiological conditions. Under alkaline conditions, formation of dihydroxo species, $[Pd^{II}(en)(OH)_2]$ **[6-(OH)$_2$]** and $[Pd^{II}(pic)(OH)_2]$ **[10-(OH)$_2$]** prevails.

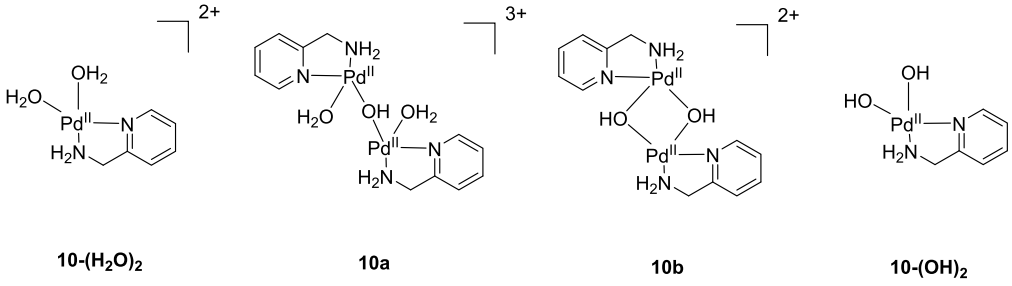
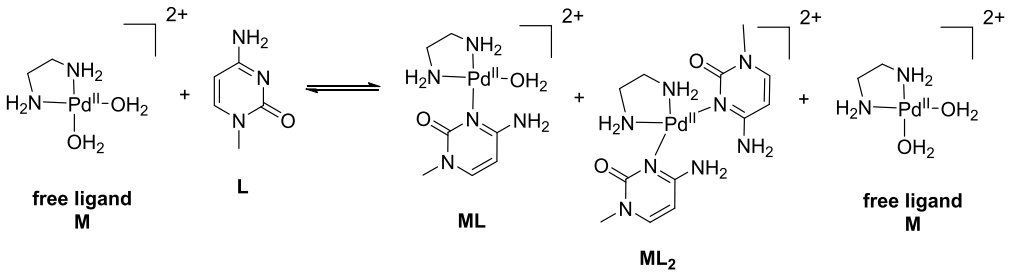


Figure 14. Proposed species for $[Pd^{II}(pic)]^{2+}$ **(10)**.

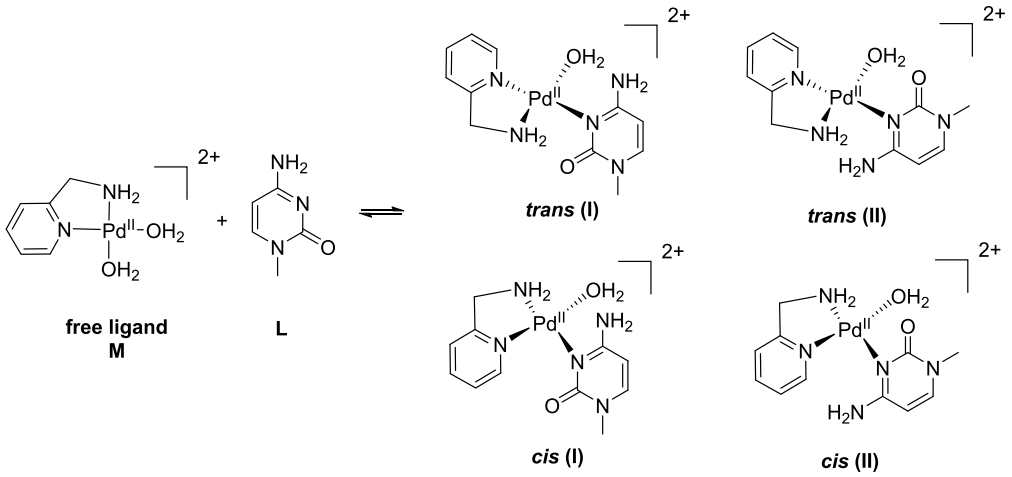
The hydroxo complexes of $[Pd^{II}(pic)]^{2+}$ are more stable than those of $[Pd^{II}(en)]^{2+}$. Due to the presence of aromatic residues, back donation of electron density from Pd d-orbital to the pyridine π^*-orbital stabilizes the system. Both $[Pd^{II}(en)(H_2O)_2]^{2+}$ and $[Pd^{II}(pic)(H_2O)_2]^{2+}$ afford with 1-alkylpyrimidines 1:1 and 1:2 (M:L) complexes. Complexing of 1-methyluracil (MeU) and 1-methylthymine (MeTH) with $[Pd^{II}(en)(H_2O)_2]^{2+}$ and $[Pd^{II}(pic)(H_2O)_2]^{2+}$ yields complexes of comparable strength. Metal binding takes place exclusively through N3. Other species, such as monohydroxo-bridged dimer, $[(Pd^{II}(en)MeU)_2(OH)]^{+}$, and mixed hydroxo complex, $[(Pd^{II}(en)MeU\text{-})(OH)]$, are present at low concentration. Complexing of $[Pd^{II}(en)(H_2O)_2]^{2+}$ and $[Pd^{II}(pic)(H_2O)_2]^{2+}$ with 1-methylcytosine (MeC) is, in turn, different.

NMR studies at equimolar concentration of $[Pd^{II}(en)H_2O]^{2+}$ (M) and MeC (L) show existence of three different species: M, ML and ML_2 (Scheme 9).



Scheme 9. $[Pd^{II}(en)H_2O]^{2+}$ **[6-(H_2O)$_2$]** (M) reaction with methyl cytosine (L) affords mono complex (ML) and bis complex (ML_2).

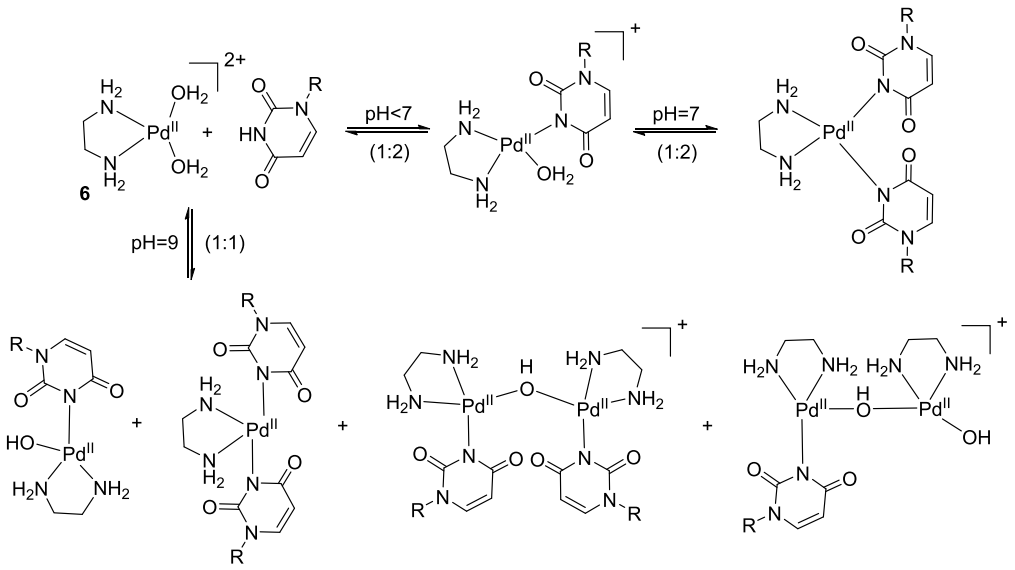
By contrast, free ligand $[Pd^{II}(pic)(H_2O)_2]^{2+}$ and bis complex (ML_2) are not observed at equimolar concentration of $[Pd^{II}(pic)(H_2O)_2]^{2+}$ and MeC. Only the mono complex (ML) is detected. $([Pd^{II}(pic)(H_2O)(MeC)]^{2+}$- is exceptionally stable, owing to hydrogen bond formation between 2-picolyl amine nitrogen and the carbonyl oxygen of coordinated MeC. This suppresses the formation of bis complexes (ML_2). These mononuclear complexes display different sets of peaks in the NMR spectra, which were identified as *cis*- and *trans*- isomers, while the other two isomers origin from hindered rotation of MeC around Pd-N bond and are, hence, rotamers (Scheme 10).



Scheme 10. $[Pd^{II}(pic)(H_2O)_2]^{2+}$ **[10-(H_2O)$_2$]** (M) reaction with methyl cytosine (MeC) affords mono complexes (ML) identified as *cis* and *trans* isomers.

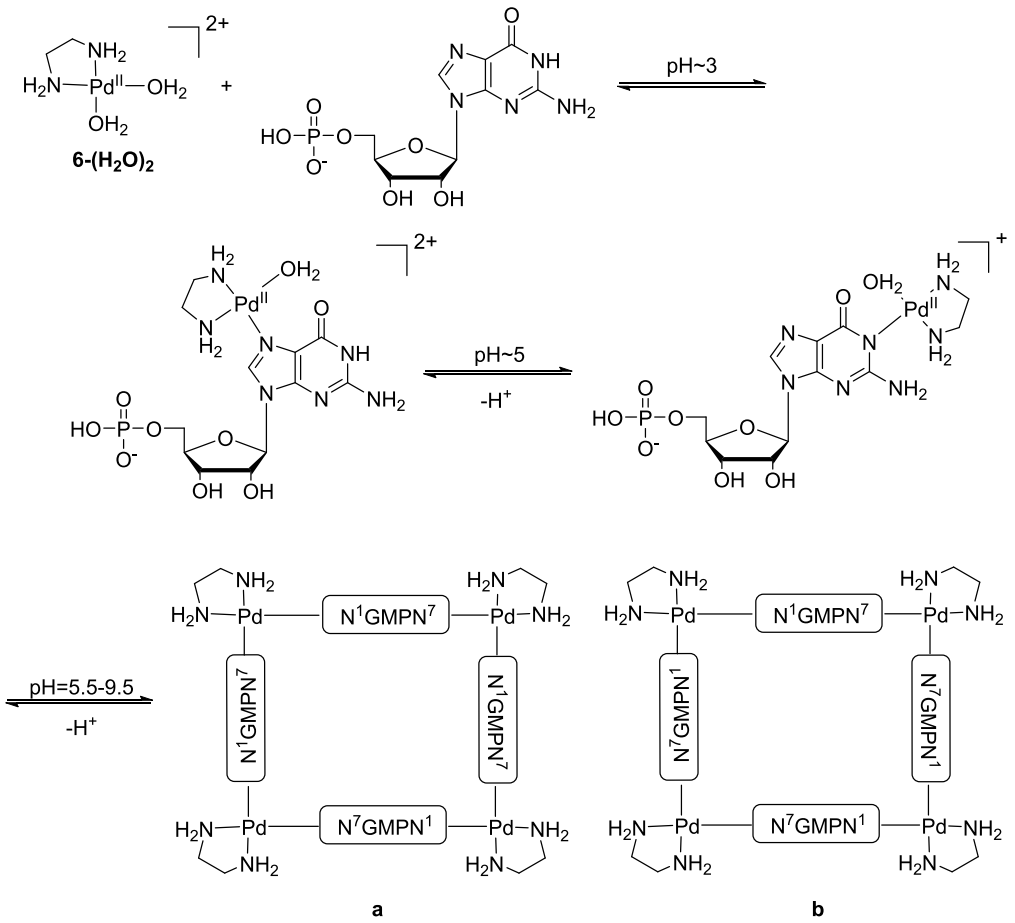
Owing to the symmetric structure and small size of ethylenediamine ligand (en), $([Pd^{II}(en)(H_2O)(MeC)]^{2+}$- is owing to symmetric structure there are not various isomers. The

presence of free starting metal complex and ligand in equimolar system of $[Pd^{II}(en)(H_2O)_2]^{2+}$ and MeC implies that the pyrimidine complexes of $[Pd^{II}(pic)]^{2+}$ are more stable than those of $[Pd^{II}(en)]^{2+}$.[152] Interaction of $[Pd^{II}(en)(H_2O)_2]^{2+}$ with purines and pyrimidines leads to pH-dependent formation of many hydroxo-bridged and other polynuclear Pd^{II} species. Several $[Pd^{II}(en)]^{2+}$ multi nuclear complexes with uridine and cytidine have been detected over a wide pH range and ligand:nucleoside ratios (Scheme 11).[122]



Scheme 11. Complexing of $[Pd^{II}(en)(H_2O)_2]^{2+}$ **[6-$(H_2O)_2$]** with uridine in 1:1 and 1:2 (M/L) mixtures at different pH values; R- stands for ribose. For clarity the charge of Pd (Z = +2) omitted

A number of various complex structures have been reported for guanosine, inosine, adenosine and their 5′-monophosphates.[149] One of the most striking examples of self-assembled cyclic structures is a tetramer formed by guanosine 5'-monophosphate and $[Pd^{II}(en)(H_2O)_2]^{2+}$. Two possible isomers have been proposed for $[Pd^{II}(en)(5'\text{-GMP})]_4^{4+}$ (Scheme 12).[154-156]



Scheme 12. Proposed reaction of $[Pd^{II}(en)(H_2O)_2]^{2+}$ **(6)** with GMP. Possible isomers of $[Pd^{II}(en)(5'\text{-GMP}]_4^{4+}$: (a)-head-to-tail, (b) head-to-head. For clarity in some structures the charge of Pd (Z = +2) omitted

1.6.4 Effect of bulky substituents on coordination equilibria of aliphatic diamino ligands

Binding of the aqua complexes $[Pd^{II}(Me_4en)(H_2O)_2]^{2+}$ **[13-(H$_2$O)$_2$]** [Me_4en = *N*,*N*′,*N*″,*N*″-tetramethylethylenediamine] and $[Pd^{II}(Et_4en)(H_2O)_2]^{2+}$ **[14-(H$_2$O)$_2$]** to nucleobases has been studied with various purines and their monophosphates. Depending on the concentration, complex formation in two consecutive steps, giving 1:1 and 1:2 complexes, has been observed with adenosine, inosine, IMP, AMP and GMP. Among 5-monophosphates, the most stable complex is formed by GMP followed by IMP and AMP. Introduction of the monophosphate group leads to remarkable increase in stability of both 1:1 and 1:2 complexes. Additional steric hindrance markedly decreases the stability of the 1:2 species, offering a method to control the

binding behavior of the Pd^{II} complexes. In comparison to nucleosides, NMPs are more susceptible to steric crowding in the coordination sphere. $[Pd^{II}(Me_4en)(H_2O)_2]^{2+}$ **[13-(H_2O)$_2$]** and $[Pd^{II}(Et_4en)(H_2O)_2]^{2+}$ **[14-(H_2O)$_2$]** [Et_4en = *N*,*N*′,*N*″,*N*″-tetraethylethylenediamine] form only 1:1 species. A set of experiments demonstrates that chloride is a strong competitor for nucleosides. Moderate concentration of Cl^- ion provides a chloro aqua complex of $[Pd^{II}(Me_4en)(Cl)(H_2O)]^+$ **[14-(Cl)(H_2O)]** that readily interacts with nucleosides giving a 1:1 complex. The excess of Cl^- ion terminates any interactions between bidentate Pd^{II} ligands and nucleosides resulting in formation of $[Pd^{II}(Me_4en)Cl_2]$.[145]

1.6.5 Ternary complexes involving CBDCA

Cyclobutane-1,1-dicarboxylic acid (CBDCA) is used as a leaving group in the Pt^{II} drug carboplatin. Instead of two chlorido ligands in cisplatin, CBDCA is bound to the metal ion via its carboxylate groups. This carboxylic acid is widely used in research with the diamine complexes of Pd^{II} in order to develop and study Pd^{II} models that mimic carboplatin analogues.[157]

Purines, 5-IMP and 5-GMP, form more stable complexes with $[Pd^{II}$(diamino)(CBDCA)] than corresponding pyrimidines. $[Pd^{II}$(diamino)(CBDCA)] acts via a ring-opening mechanism where N7 of purine base moiety is bound to $[Pd^{II}(diamino)]^{2+}$ complex, while the fourth coordination site is occupied by one of carboxylate oxygen of CBDCA. The mechanism is feasible under physiological conditions.[158]

2. AIMS OF THE THESIS

Metal-ion-mediated recognition of nucleic acids has attracted considerable attention during the past decade, since it offers means to expand the genetic code by artificial base-pairs, [43,46,159,] to create predesigned molecular architecture by metal-ion-mediated inter- or intra-strand cross-links[28,44,47,55,61,160,161,162] or to convert double-stranded DNA to a nano-scale wire.[35,38] The approaches presented so far largely depend on the presence of a modified nucleobase in both strands engaged in the duplex formation. Hybridization of metal-ion-binding oligonucleotide analogs with natural nucleic acid sequences has received much less attention in spite of obvious applications. While the natural oligonucleotides hybridize with high selectivity, their affinity for complementary sequences is inadequate for a number of applications. The short single-stranded regions of miRNA, for example, are challenging to target with unmodified oligonucleotides.[14,16] Metallo-oligonucleotides, with their superior affinity towards their natural complements, would offer a way to overcome the low stability of short duplexes.

The underlying idea of this PhD thesis is to develop metal-ion-binding surrogate nucleosides, the base moieties of which serve as tri- or bidentate ligands for soft metal ions. The resulting complexes are designed to bind tightly and selectively to natural nucleic acid bases. The ultimate goal is to find metal ion complexes that could discriminate between natural nucleobases upon double helix formation. Special attention has been paid to Pd^{II} complexes. Preparation of oligonucleotides carrying a Pd^{II} ion with vacant coordination sites at a predetermined position has been demonstrated and their affinity to complementary as well as mismatched counterparts has been quantified by HPLC-, ESI-MS and UV-melting measurements.

3. RESULTS AND DISCUSSION

3.1 Synthesis

3.1.1 Synthesis of modified nucleosides

2,6-Bis(3,5-dimethylpyrazol-1-yl)-9-(ß-D-ribofuranosyl)purine

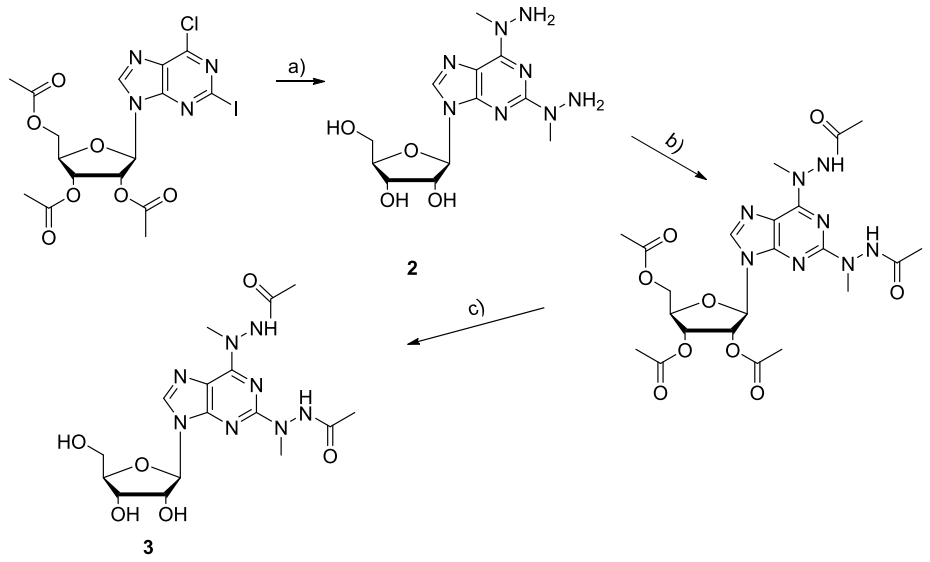
Preparation of 2,6-bis(3,5-dimethylpyrazol-1-yl)-9-(β-D-ribofuranosyl)purine **(1)** used as a reference compound in NMR titrations and as a starting material for oligonucleotide building blocks (Scheme 16) has been described previously.[163]

2,6-Bis(1-methylhydrazinyl)-9-(ß-D-ribofuranosyl)purine and 2,6-bis(2-acetyl-1-methylhydrazinyl)-9-(ß-D-ribofuranosyl)purine.

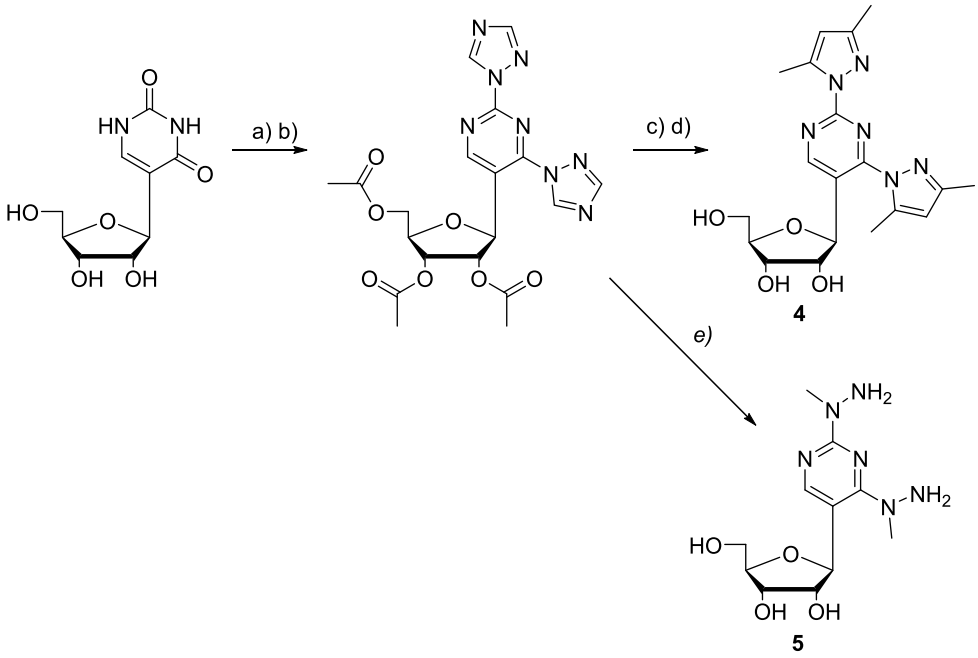
Treatment of commercial 2′,3′,5′-tri-*O*-acetyl-6-chloro-2-iodo-9-(β-D-ribofuranosyl)purine (Scheme 13) with aq. methylhydrazine removed all acetyl groups of the sugar moiety and a nucleophilic displacement of the halogen substituents at positions 6 and 2 of the purine base led to formation of 2,6-bis(1-methylhydrazinyl)-9-(β-D-ribofuranosyl)purine (**2**). Reaction of **2** with acetic anhydride gave the 2,6-bis(2-acetyl-1-methylhydrazinyl derivative as 2',3',5'-tri-*O*-acetate. Removal of the *O*-acetyl groups was accomplished by MeONa-catalyzed transesterification in MeOH giving 2,6-bis(2-acetyl-1-methylhydrazinyl)-9-(β-D-ribofuranosyl)purine (**3**).

2,4-Bis(3,5-dimethyl-1H-pyrazol-1-yl)-5-(ß-D-ribofuranosyl)pyrimidine and 2,4-bis(1-methylhydrazinyl)-5-(ß-D-ribofuranosyl)pyrimidine

Commercial pseudouridine was converted into the 2′,3′,5′-tri-*O*-acetylated analogue, followed by $POCl_3$-promoted replacement of the oxo groups with 1,2,4-triazole (Scheme 14).[164] The 1,2,4-triazolo moieties were substituted by hydrazine groups which then were treated with pentane-2,4-dione[165] affording 2,4-bis(3,5-dimethyl-1*H*-pyrazol-1-yl)-5-(β-D-ribofuranosyl)pyrimidine (**4**). Alternatively, the 1,2,4-triazolo moieties were substituted with methylhydrazine yielding 2,4-bis(1-methylhydrazinyl)-5-(β-D-ribofuranosyl)pyrimidine (**5**).[166]



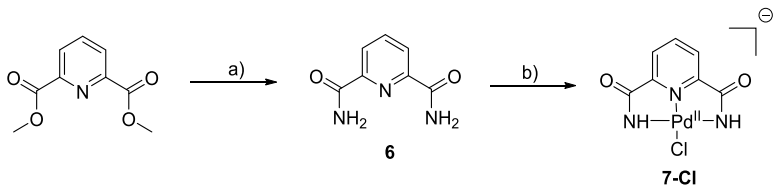
Scheme 13. Preparation of 2,6-bis(1-methylhydrazinyl)-9-(β-D-ribofuranosyl)purine (**2**) and 2,6-bis(2-acetyl-1-methylhydrazinyl)-9-(β-D-ribofuranosyl)purine (**3**). [a]Reagents and condotions: *(a) $MeNHNH_2$ (aq.); (b) Ac_2O, pyridine; (c) MeONa, MeOH.*



Scheme 14. Preparation of 2,4-bis(3,5-dimethyl-1H-pyrazol-1-yl)-5-(β-D-ribofuranosyl)pyrimidine (**4**) and 2,4-bis(1-methylhydrazinyl)-5-(β-D-ribofuranosyl)pyrimidine (**5**). [a]Reagents and condotions: *(a) Ac_2O, pyridine; (b) $POCl_3$, 1,2,4,-triazole, Et_3N, MeCN; (c) $NH_2NH_2{}^oH_2O$; (d) pentane-2,4-dione, TFA, 24h; (e) $MeNHNH_2$.*

3.1.2 Synthesis of pyridine-2,6-carboxamides and their binary Pd^{II} complexes

Commercial dimethyl pyridine-2,6-dicarboxylate was mixed overnight with aq. NH_3 to give pyridine-2,6-dicarboxamide (**6**) (Scheme 15). Potassium chlorido(pyridine-2,6-dicarboxamidato(2-)-κ_3 N_1,N_2,N_6]palladate (1-) (**7-Cl**) was obtained by treating **6** with an equimolar amount of K_2PdCl_4 in water, precipitating the complex at pH 7 and washing repeatedly with water.[166]



Scheme 15. Preparation of pyridine-2,6-dicarboxamide (**6**) and chlorido(pyridine-2,6-dicarboxamidato(2-)-κ_3 N_1,N_2,N_6] palladate (1-), **(7-Cl).** [a]Reagents and condotions: *(a) NH_3ªH_2O; (b) K_2PdCl_4, H_2O, pH = 7.*

The *N*-alkylated derivatives of **6**, *viz.* N^2,N^6-dimethylpyridine-2,6-dicarboxamide and N^2,N^6-diisopropylpyridine-2,6-dicarboxamide were prepared by aminolysis of dimethyl pyridine-2,6-dicarboxylate. The other 2,6-disubstituted pyridines, *viz.* 6-carbamoylpyridine-2-carboxylic acid, 6-aminomethylpyridine-2-carboxamide and 6-(2-aminomethyl)-N^2-methylpyridine-2-carboxamide, were commercial products. The Pd^{II} chlorido complexes of these ligands (**8-12**) (Figure 15) were prepared as described above for **7-Cl** (Scheme 15). According to ESI-MS data, chlorido complexes of **7, 8, 10** and **11** were formed by displacement of two protons of the ligand resulting in negatively charged Pd^{II} binary complexes. Complexes **9** and **12** were obtained by displacement of one ligand proton giving uncharged Pd^{II} binary complexes.[167]

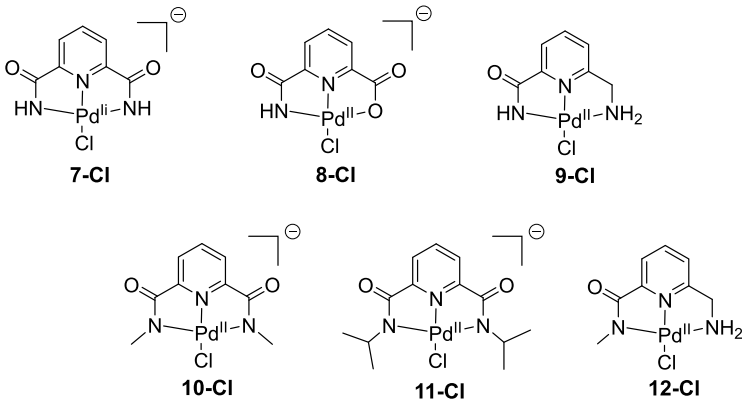
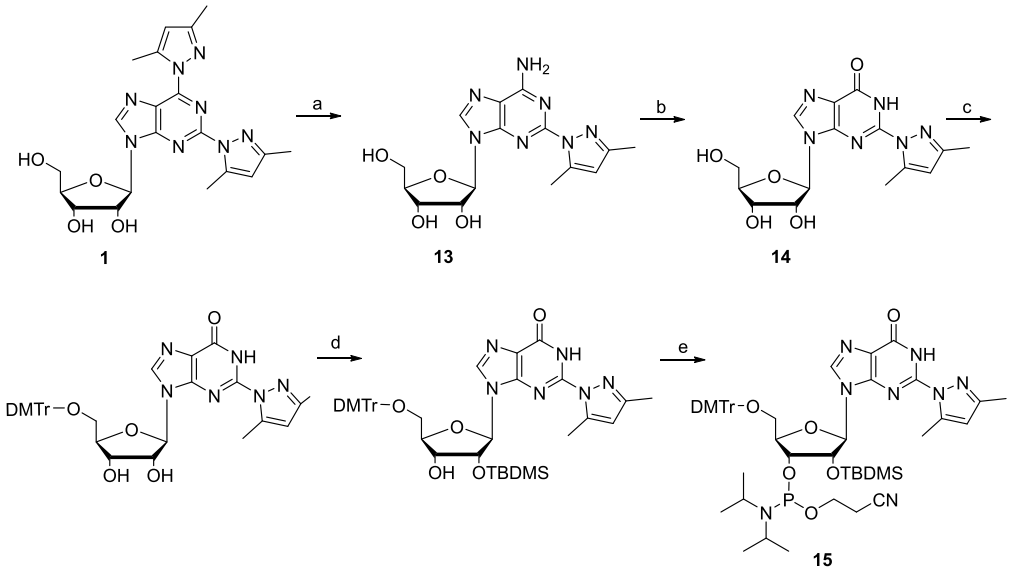


Figure 15. Structures of the binary Pd^{II} complexes used in the present work.

3.1.3 Preparation of modified phosphoramidite building blocks

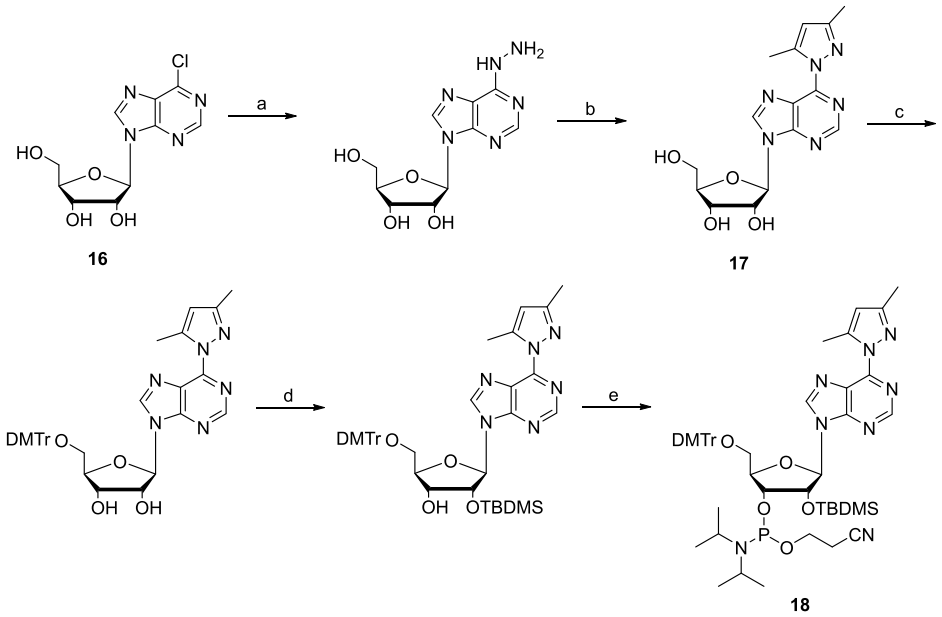
Phosphoramidites of 3,5-dimethylpyrazol-1-yl substituted purine nucleoside

Reaction of 2,6-bis(3,5-dimethylpyrazol-1-yl)-9-(β-D-ribofuranosyl)purine **(1)** with aq. ammonia leads to displacement of the 6-(3,5-dimethylpyrazol-1-yl) substituent by an amino group giving 2-(3,5-dimethylpyrazol-1-yl)adenosine (**13**) (Scheme 16). Treatment of **13** with $NaNO_2$ in aq. acetic acid gives the oxo analog 2-(3,5-dimethylpyrazol-1-yl)inosine (**14**). 4,4′-Dimethoxytrityl chloride (DMTrCl) was used for protection of the 5′-OH group of the artificial nucleoside **14**. Protection of the 2′-OH group with *tert*-butyldimethylsilyl chloride (TBDMSCl) followed by phosphorylation of 3′-OH with 2-cyanoethyl-*N*,*N*-diisopropylaminophosphorochloridite yielded the phosphoramidite building block (**15**).



Scheme 16. Preparation of 2-(3,5-dimethylpyrazol-1-yl)inosine (**14**) and its conversion into a phosphoramidite building block (**15**). [a]Reagents and conditions: *(a) NH_3 H_2O; (b) $NaNO_3$, H_2O, AcOH; (c) DMTrCl, pyridine; (d) TBDMSCl, imidazole, DMF; (e) 2-cyanoethyl-N,N-diisopropylphosphorochloridite, Et_3N, DCM.*

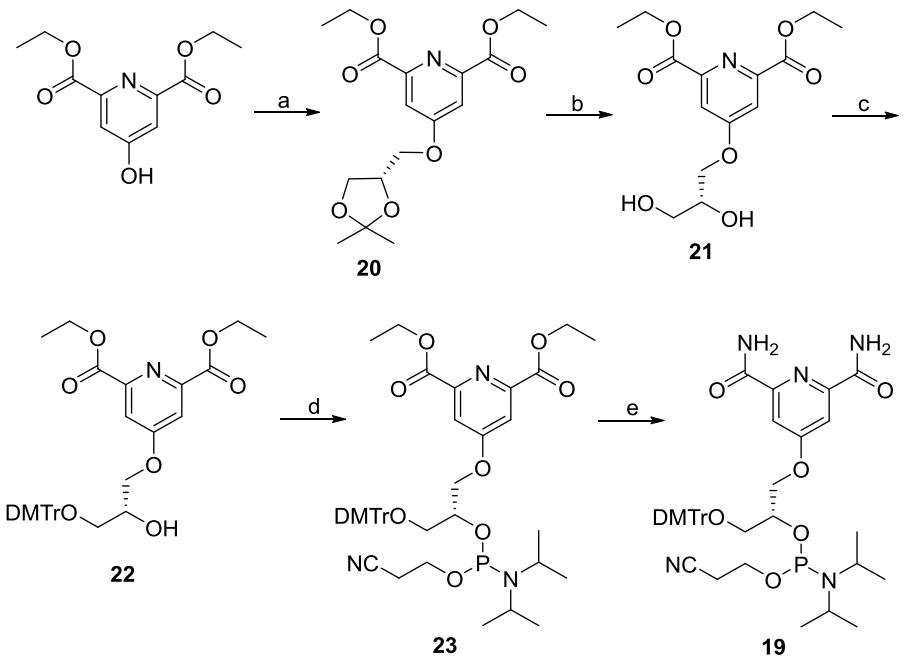
Treatment of commercial 6-chloropurine riboside (**16**) with hydrazine hydrate, followed by reaction of pentane-2,4-dione with the hydrazinyl group afforded 6-(3,5-dimethylpyrazol-1-yl)-9-(β-D-ribofuranosyl)purine (**17**) (Scheme 17). Protection of **17** as a 5′-*O*-(4,4'-dimethoxytrityl) and 2′-*O*-(*tert*-butyldimethylsilyl) ether and subsequent phosphitylation of the 3′-OH yielded the phosphoramidite building block (**18**).[168]



Scheme 17. Preparation of 6-(3,5-dimethylpyrazol-1-yl)-9-(β-D-ribofuranosyl)purine (**17**) and its conversion into phosphoramidite bulding block (**18**). [a]Reagents and conditions: *(a) $NH_2NH_2{\cdot}H_2O$; (b) pentane-2,4-dione, TFA; (c) DMTrCl, pyridine; (d) TBDMSCl, imidazole, DMF; (e) 2-cyanoethyl-N,N-diisopropylchlorophosphoramidite, Et_3N ,DCM.*.

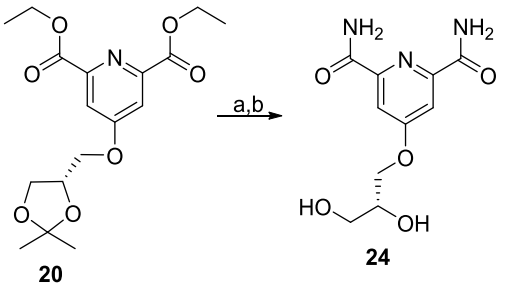
Phosphoramidites for synthesis of glycol nucleic acids (GNA)

(*S*)-2,3-Dihydroxypropyl derivatives of canonical nucleobases, known as glycerol nucleosides, were prepared and converted to phosphoramidite building blocks as described in literature.[169,170,171] A similar phosphoramidite building block of their metal-ion-binding surrogate **19** was synthesized as outlined in Scheme 18. Accordingly, the phenolic OH group of diethyl 4-hydroxypyridine-2,6-dicarboxylate was first etherified by treatment with (*R*)-(2,2-dimethyl-1,3-dioxolan-4-yl)methyl *p*-toluenesulfonate. The isopropylidene protection of the product (**20**) was then removed under acidic conditions affording diol **21**. The primary OH group was protected as a 4,4′-dimethoxytrityl ether to obtain **22** and the secondary OH was then phosphitylated with 2-cyanoethyl-*N*,*N*-diisopropylaminophosphorochloridite. Finally, the ethoxycarbonyl groups of the resulting phosphoamidite (**23**) were subjected by ammonolysis to afford the desired phosphoramidite building block (**19**).



Scheme 18. Synthesis of the modified glycero- nucleoside phosphoramidite building block (**19**). [a]Reagents and conditions: *(a) (R)-(2,2-Dimethyl-1,3-dioxolan-4-yl)methyl p-toluenesulfonate, DMF, K_2CO_3; (b) HCl, MeCN, H_2O; (c) DMTrCl, pyridine; (d) 2-cyanoethyl-N,N-diisopropylaminophosphorochloridite, Et_3N, DCM; (e) NH_3, MeOH.*

(*S*)-4-(2,3-Dihydroxypropoxy)pyridine-2,6-dicarboxamide (**24**) was obtained by removing the isopropylidine protection of the product (**20**) by treatment with dilute hydrochloric acid. All volatiles were removed under reduced pressure and the residue was dried in vacuum. The aminocarbonyl derivative (**24**) was obtained by ammonolysis of the residue (Scheme 19).[172]



Scheme 19. Synthesis of (*S*)-4-(2,3-dihydroxypropoxy)pyridine-2,6-dicarboxamide (**24**). [a]Reagents and conditions: *(a) HCl, MeCN, H_2O; (b) NH_3, MeOH.*

3.1.4 Synthesis of modified oligonucleotides

Synthesis of 2′-O-methyl-RNA oligonucleotides

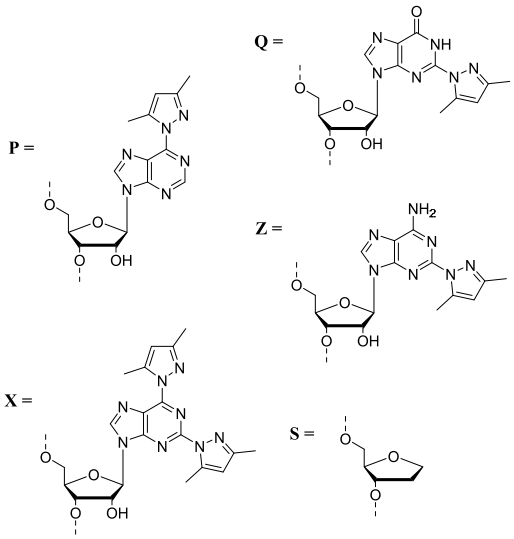
Preparation of the 9-mer 2′-*O*-methyl oligoribonucleotide **ON1x** incorporating the artificial nucleoside analogue **1** has been described previously.[173] The 9-mer 2′-O-methyl oligoribonucleotide **ON1z** bearing artificial unit **14** has been prepared from oligonucleotide **ON1x** by treatment with aqueous ammonia at elevated temperature. Under these conditions the 3,5-dimethylpyrazol-1-yl substituent at position 6 of **1** was displaced by ammonia yielding the modified nucleoside (**14**).[168]

The 9-mer 2′-*O*-methyl oligoribonucleotides (Table 2) **ON1q** and **ON1p** incorporating an artificial nucleoside (**14** or **17**) in an intrachain position were assembled from commercial 2′-*O*-methyl protected building blocks by an automated synthesizer, with the exception of the modified building blocks (**15**, **18**) that were coupled manually.

The 6-mer 2′-*O*-methyl oligoribonucleotide **ON3x** bearing the artificial nucleoside **1** at the 3′-terminus was prepared analogously as described previously.[168] Compound **1** was protected with 5′-*O*-DMTr- and 3′-*O*-TBDMS and then treated with succinic anhydride and linked to the amino-functionalized solid support. The 6-mer 2′-*O*-methyl oligoribonucleotide was assembled on the solid support. Removal of the base and phosphate protection groups and release of the oligonucleotide chain from the solid support was accomplished by ammonolysis at elevated temperature. The TBDMS protecting groups were removed by treatment with triethylamine trihydrofluoride in DMSO. All oligonucleotides were purified by reverse phase HPLC and characterized by electrospray ionization mass spectrometry (MS-ESI).

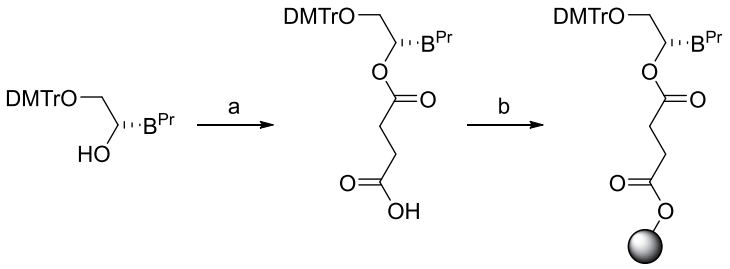
Table 2. *Structures of modified and natural 2′-O-methyl oligoribonucleotides used in present work.*

	Sequence
ON1p	5′-GCGC**P**CCGG-3′
ON1q	5′-GCGC**Q**CCGG-3′
ON1x	5′-GCGC**X**CCGG-3′
ON1z	5′-GCGC**Z**CCGG-3′
ON2s	5′-CCGG**S**GCGC-3′
ON2a	5′-CCGGAGCGC-3′
ON2c	5′-CCGGCGCGC-3′
ON2g	5′-CCGGGGCGC-3′
ON2u	5′-CCGGUGCGC-3′



Preparation of solid support for the synthesis of GNA oligonucleotides

Appropriately protected glycerol nucleosides were immobilized to a solid support as follows. The secondary OH group of the diol moiety was succinylated by treatment with succinic anhydride in anhydrous pyridine (Scheme 20). The crude product was then attached to long chain alkylamine-functionalized controlled pore glass (LCAA-CPG) by conventional HBTU-promoted peptide coupling. Based on the trityl response, loadings of the solid supports thus obtained were found to range from 30 to 50 μmol g^{-1}).[174]

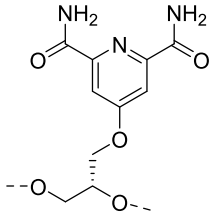


Scheme 20. Preparation of solid supports for the GNA oligonucleotides. [a]Reagents and conditions: *(a) succinic anhydride, DMAP, pyridine; (b) LCAA-CPG, HBTU, DIPEA, DMF*

Synthesis of GNA oligonucleotides

GNA oligonucleotides (Table 3) were assembled on this support by conventional phosphoramidite chemistry on an automated synthesizer.[169,170,171] The building blocks derived from canonical nucleobases were coupled on the synthesizer and the modified building block (**19**) manually. Release from the solid support and deprotection of the base and phosphate moieties was accomplished by treatment with aq. ammonia (for **ON1** and **ON2**) or aq. methylamine (for **ON3**) at elevated temperatures. During the ammonolysis, the 3-terminal hydroxyl group was still kept tritylated to minimize cleavage of the phosphodiester bonds. Removal of the DMTr group with aq. acetic acid at room temperature, hence, was the final step of the oligonucleotide synthesis.

Table 3. *Structures of the GNA oligonucleotides used in present studies.*

	Sequence	
ON1	3′-**Y**CGCGT -3′	**Y** = 
ON2	3′-CGC**Y**GGC-2′	
ON3	3′-GCCTGCG-2′	

The oligonucleotides were purified by reverse phase HPLC and characterized by electrospray ionization mass spectrometry (MS-ESI). Oligonucleotide concentrations were determined by UV-spectrophotometry and the molar absorptivities were predicted by an implementation of the nearest-neighbors method[175-177] assuming the hypochromicities of GNA and DNA oligonucleotides to be equal. For determination of the absorptivity of the modified unit, compound **20** was deprotected and converted into the corresponding dicarboxamide (**24**) (Scheme 19).[172]

3.2 NMR spectrometric titrations

3.2.1 General methodology

The binding affinity and selectivity of modified nucleosides towards natural nucleosides were studied by NMR spectrometric titrations. The NMR spectra were recorded in 0.12 M deuterated phosphate buffer at pH = 7.2. For modified analogues (**1-6**) the titration was performed in several ways.

a) By stepwise increasing the concentration of all the components (uridine, modified nucleoside, and K_2PdCl_4), but keeping the 1:1:1 molar ratio (pH 7.2), the intensity of the new signals was gradually increased compared to the intensity of the signals of uncomplexed (free) species.

b) By gradual dilution of an equimolar mixture of all components in buffer until the signals of uncomplexed natural and modified nucleoside predominated.

c) By gradual addition of K_2PdCl_4 in 0.1 eq. portions to an equimolar mixture of natural and modified nucleosides, resulting in diminution of the signals for uncomplexed nucleosides.

d) By gradual addition of equimolar amounts of a modified nucleoside and K_2PdCl_4 into a 5.0 mM solution of NMP in buffer, keeping the concentration of NMP constant.

In case of Pd^{II} complexes of 2,6-substituted pyridines (**7-12**), NMR titration was performed as follows: To a solution of NMP (5.0 mM) in a phosphate buffer in D_2O (0.12 M, pH 7.2), an equimolar mixture of NMP (5.0 mM) and one of the chlorido complexes **7-12** (5.0 mM) in the same phosphate buffer was gradually added, resulting in mixtures where the concentration of 7–12 were 1.0 - 5.0 mM, while the concentration of NMP always remained constant (5.0 mM).

After each addition, a ^{1}H-NMR spectrum was recorded at 25 °C and the signals appearing/disappearing in the region of aromatic and anomeric protons were integrated. Integrals of two sets of signals (H5, H1´ for pyrimidines and H8, H1´ for purines) of complexed and free nucleosides were used for the calculation of mole fraction of the complex at various concentrations. In order to ensure that the detected signals refer to the mixed-ligand ternary complexes and not to Pd^{II} interactions with natural nucleosides, similar experiments were carried out in the absence of the artificial nucleoside.

3.2.2 Ternary Pd^{II} complexes of pyridine-2,6-dicarboxamide with uridine and cytidine

Ternary Pd^{II} complexes of pyridine-2,6-dicarboxamide (**6**) with natural nucleosides were first investigated as simplified model systems of ternary complexes formation of metal ion binding nucleosides **1-5**. Compound **7-Cl** was prepared and characterized by NMR spectroscopy and mass spectrometry in order to define the binding mode of Pd^{II} with **6**. According to negative mode ESI-MS, Pd^{II} had formed a monochlorido complex with **6** by displacement of two protons. ^{1}H-NMR spectrum showed that two amido protons were displaced, one from each amido group. Formation of the binary Pd^{II} complex (**7-Cl**) was additionally accompanied by changes in aromatic proton signals. Thus the *multiplet* of H-C(3), H-C(4), H-C(5) of compound **6** was split into two separate signals: a *triplet* referring to H-C(4) and a *doublet* referring to H-C(3) and H-C(5) of **7-Cl (**Figure 16**)**.

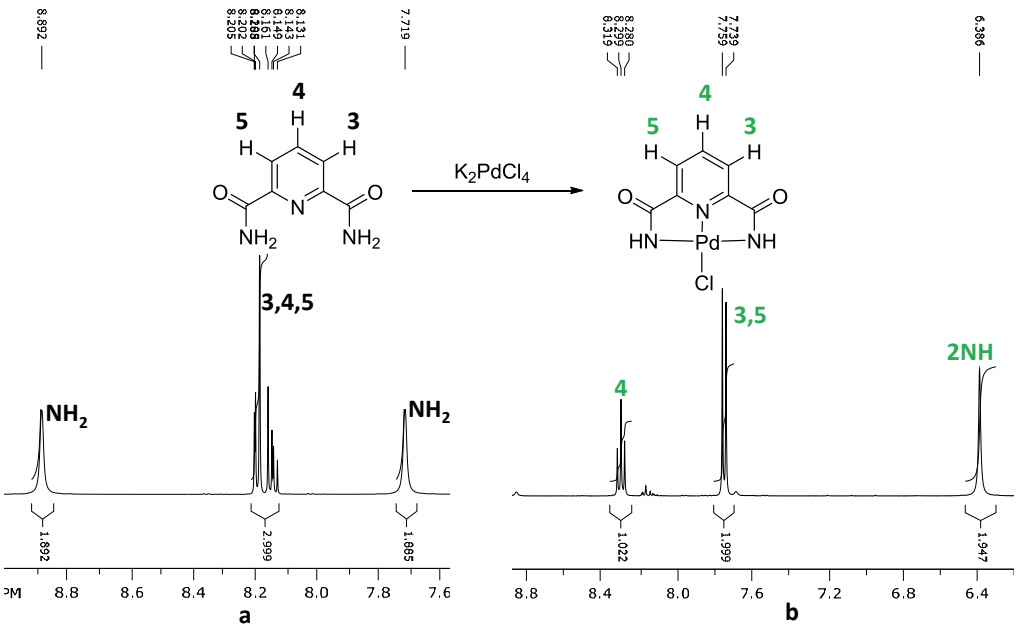


Figure 16. Parts of the ^{1}H-NMR spectrums: a) 2,6-pyridinedicarboxamide **(6)** in d6-DMSO; b) Pd^{II} binary complex of 2,6-pyridinedicarboxamide **(7-Cl)** in d6-DMSO.

Complexing of **6** with K_2PdCl_4 was investigated in deuterated phosphate buffer at pH 7.2. The signals of amido protons were not detected due to rapid deuteration, but the changes of aromatic proton signals were visible. The presence of 1 H *triplet* and 2 H *doublet* evidenced quantitative complexing of **6** with Pd^{II} even at a very low concentration of both species. In other words the equilibrium described by *Eqn.1,*

$$LH_2 + PdCl_4^{2-} \rightleftharpoons PdLCl^- + 3Cl^- + 2H^+ \quad (1)$$

where LH_2 stands for **6** and $PdLCl^-$ for **7-Cl**, was always far on the right hand side. Addition of uridine to the system afforded a ternary complex that gave a new set of signals (Figure 17). These signals differed from those of uncomplexed uridine or **7-Cl**. The ternary complex was formed by replacement of the chlorido ligand of **7** with *N(3)*- deprotonated uridine. This binding mode had previously been reported for binding of uridine to the tridentate $[Pd^{II}(dien)Cl]^+$ and $[Pd^{II}(terpy)Cl]^+$ complexes. [133,134]

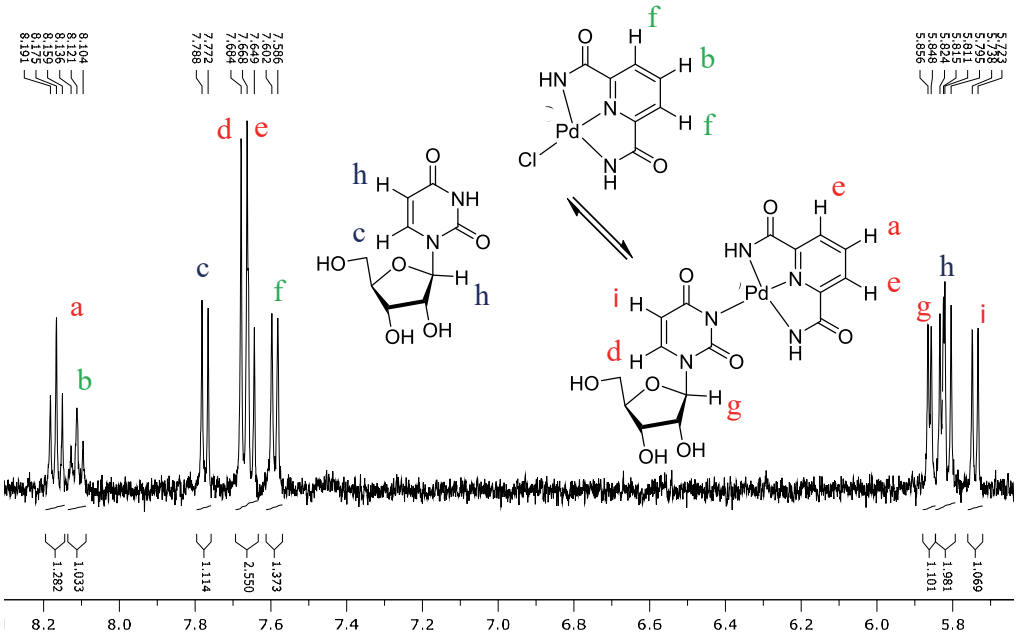


Figure 17. *Part of the ^{1}H-NMR spectrum of the mixture of uridine, pyridine-2,6-dicarboxamide (**6**) and K_2PdCl_4 in D_2O* (pH 7.2, 0.12 M phosphate buffer). The concentration of each component is 0.50 mM. Notation (L^{2-}) refers to the dianion of **6**. (a) H-C(4) of **6** in $LPd^{II}Urd^-$; (b) H-C(4) of **6** in $LPd^{II}Cl^-$; (c) H-C(6) of HUrd; (d) H-C(6) of Urd^- in $LPd^{II}Urd^-$; (e) H-C(3)&H-C(5) of **6** in $LPd^{II}Cl^-$ and $LPd^{II}Urd^-$; (f) H-C(3)&H-C(5) of **6** in $LPd^{II}Cl^-$, (g) H-C(1′) of Urd^- in $LPd^{II}Urd^-$; (h) H-C(1′)&H-C(5) of HUrd; (i) H-C(5) of Urd^- in $LPd^{II}Urd^-$.

The formation of the ternary complex is concentration-dependent. Equal increase in the concentration of all ligands and the metal ion promoted the formation of the ternary complexes at the expense of the uncomplexed ligands and free metal ion. The ratio between free and complexed uridine was equal at 0.3 mM concentration of the starting compounds. The formation of the ternary complex can be described by Eqn. 2, and the equilibrium constant, K_{Urd} for this reaction can be calculated by *Eqn.3*.

$$PdLCl^- + Urd^- \rightleftharpoons PdLUrd^- + Cl^- \quad (2)$$

$$K_{Urd} = [PdLUrd^-][Cl^-](K_a + [H^+]) / \{K_a\,[PdLCl^-][Urd_{free}]\} \quad (3)$$

Free uridine (Urd_{free}) is present in solution both as a neutral molecule (HUrd) and as a deprotonated, but not complexed anion (Urd^-). K_a is the acidity constant of (HUrd). At 0.3 mM concentration of compounds **6**, K_2PdCl_4 and uridine, only 50% of uridine was engaged in the ternary complex. $[PdLUrd^-] = [Urd_{free}] = [PdLCl^-] = 0.15$ mM and $[Cl^-] = 1.05$ mM. Accordingly, $K_{Urd} = 7.0 \times (K_a + [H^+]) \times K_a^{-1} = 1400$ (pK_a value of uridine is 9.5 in D_2O [134]), and $\log K_{Urd} = 3.1$. The $\log K_{Urd}$ of the ternary Pd^{II} complex formed by pyridine -2,6-dicarboxamide and *N(3)*-deprotonated uridine is 3.1.

Formation of the ternary complex with cytidine occurs similarly, but the binding affinity is somewhat lower than that of uridine. Spectral changes of cytidine resonance signals for H-(C5), H-(C6), H-(C1′) and the aromatic protons H-(C3), H-(C5) of **6** were recorded by NMR spectroscopy upon complex formation (Figure 18). 50% of **6** and cytidine were engaged in Pd^{II} ternary complex at 0.5 mM concentration.

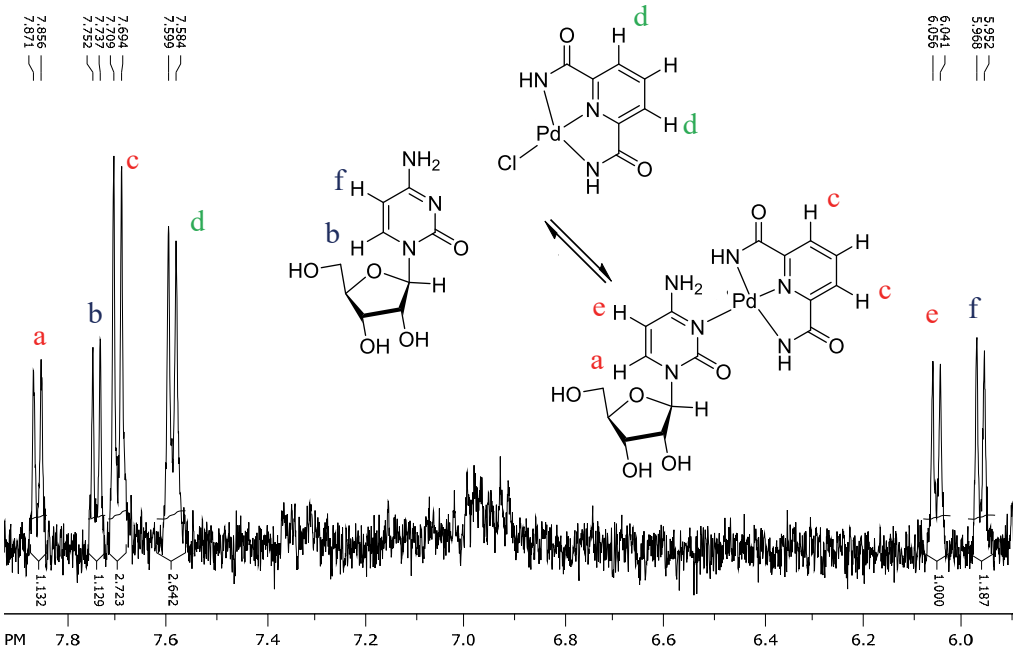


Figure 18. *Part of the ^{1}H-NMR spectrum of the mixture of cytidine, pyridine-2,6-dicarboxamide (**6**) and K_2PdCl_4 in D_2O* (pH 7.2, 0.12 M phosphate buffer). The concentration of each component is 0.5 mM. Notation (L^{2-}) refers to the dianion of **6**. (a) H-C(6) of Ctd in LPd^{II}Ctd; (b) H-C(6) of Ctd; (c) H-C(3)&H-C(5) of **6** in LPd^{II}Ctd; (d) H-C(3)&H-C(5) of **6** in $LPd^{II}Cl^-$; (e) H-C(5) of Ctd in LPd^{II}Ctd; (f) H-C(5) of Ctd.

Higher concentrations resulted in appearance of additional signals which most likely refer to a 2:2:1 complex formed by replacement of cytidine exocyclic amino proton by Pd^{II} complex of **6**. This kind of binding mode has previously been described for $[Pd^{II}(dien)]^{2+}$ binding to 1-methylcytosine.[133]

Uridine forms ternary complexes as a deprotonated monoanion while cytidine is engaged in ternary complex as a neutral molecule. Accordingly, the equilibrium constant K_{Ctd} for the 1:1:1 ternary complex formation was calculated by *Eqns*. 4 and 5, respectively.

$$PdLCl^- + Ctd \rightleftharpoons PdLCtd + Cl^- \quad (4)$$

$$K_{Ctd} = [PdLCtd][Cl^-] / [PdLCl^-][Ctd] \quad (5)$$

Half of cytidine was complexed at 0.5 mM solution of the starting compounds: **6**, K_2PdCl_4 and cytidine. $[PdL^1Ctd] = [Ctd_{free}] = [PdL^1Cl^-] = 0.25$ mM, $[Cl^-] = 1.75$ mM, and $K_{Ctd} = 7$ ($\log K_{Ctd} = 0.8$). Thus, binding affinity of *N(3)*-deprotonated uridine is about 200 times higher compared to neutral cytidine (N3).

3.2.3 Ternary Pd^{II} complexes of metal-ion-binding nucleosides (1-5) with uridine and cytidine

Previous studies have shown that 2,6-bis(3,5-dimethylpyrazol-1-yl)-9-(β-D-ribofuranosyl)purine (**1**) forms a very stable ternary complex with Pd^{II} and uridine.[163] At 0.06 mM concentration of **1**, Urd and K_2PdCl_4 and pH 7, 80% of uridine was engaged in ternary complex. Assuming that formation of binary complex $[Pd^{II}(\mathbf{1})Cl]^+$ is quantitative, as was observed with **6**, the equilibrium concentrations are: $[Pd^{II}(\mathbf{1})Urd^-] = 0.048$ mM, $[Urd_{free}] = [Pd^{II}(\mathbf{1})Cl^-] = 0.012$ mM and $[Cl^-] = 0.228$ mM. The stability constant of the ternary complex $[Pd^{II}(\mathbf{1})Urd]^+$ (*cf*. eqn. 3) is $K(\mathbf{1})_{Urd} = 15\,000$ ($\log K(\mathbf{1})_{Urd} = 4.2$), which is one order of magnitude higher than the value of the corresponding uridine complex with **7**.

The Pd^{II} complex of 2,6-bis(1-methylhydrazino)-9-(β-D-ribofuranosyl)purine (**2**) appears to exhibit lower binding affinity to uridine. Only 48% of uridine was complexed at equimolar 3,2 mM concentration of **2**, K_2PdCl_4 and uridine (Figure 19). Assuming again that formation of binary Pd^{II} complex with **2** is more or less quantitative, the stability constant for the ternary complex (*cf*. eqn. 3) is $K(\mathbf{2})_{Urd} = 1200$, ($\log K(\mathbf{2})_{Urd} = 3.1$) which is comparable to the corresponding complex of **6**.

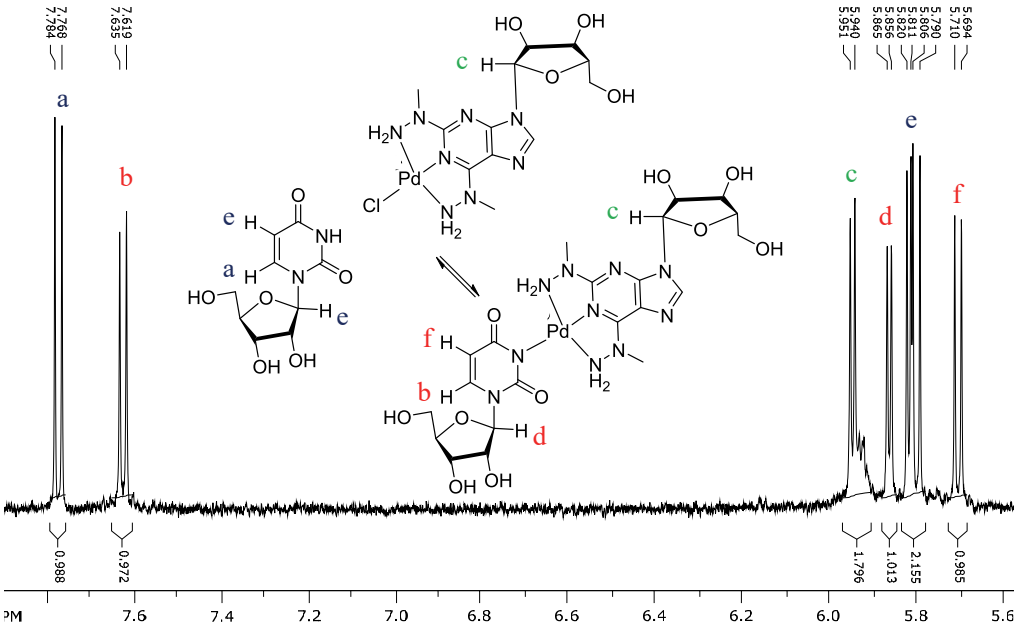


Figure 19. *Part of the ^{1}H-NMR spectrum of the mixture of uridine, 2,6-bis(1-methylhydrazin-1-yl)-9-(β-D-ribofuranosyl)purine (**2**) and K_2PdCl_4 in D_2O* (pH 7.2, 0.12 M phosphate buffer). The concentration of each component is 3.2 mM. (a) H-C(6) of HUrd; (b) H-C(6) of Urd$^-$ in (**2**)Pd^{II}Urd$^+$; (c) H-C(1′) of **2** in (**2**)Pd^{II}Cl$^+$ and (**2**)Pd^{II}Urd$^+$; (d) H-C(1′) of Urd$^-$ in (**2**)Pd^{II}Urd$^+$; (e) H-C(1′) and H-C(5) of HUrd; (f) H-C(5) of Urd$^-$ in (**2**)Pd^{II}Urd$^+$.

The Pd^{II} complex of the N-acetylated analog of **2**, 2,6-bis(2-acetyl-1-methylhydrazinyl)-9-(β-D-ribofuranosyl)-9H-purine (**3**), did not form a detectably stable ternary complex.

Interaction of the pseudouridine derivative 2,4-bis(3,5-dimethylpyrazol-1-yl)-5-(β-D-ribofuranosyl)pyrimidine (**4**) with K_2PdCl_4 and uridine gave a poorly soluble precipitate. An equimolar solution (5mM) of **4** and uridine was prepared in deuterated phosphate buffer and 2.5µl of EtOH was introduced as internal standard. Then K_2PdCl_4 in 0.1 eq. portions was gradually added to the equimolar mixture. This resulted in diminution of the signals for both ligands without any clear signals referring to the ternary complex. The diminution of the signals of **4** and uridine was similar suggesting that the precipitate contained both nucleosides in a 1:1 ratio. Comparison of the signal intensities with the signals of EtOH, used as an internal standard revealed that 25% of **4** and uridine had precipitated (most likely as a ternary complex) when the total concentrations of both nucleosides and K_2PdCl_4 were 3.6, 3.6 and 1,4, respectively. According to these results, formation of ternary complex was significantly weaker than with **1**. With 2,4-bis(1-methylhydrazinyl)-5-(β-D-ribofuranosyl)pyrimidine (**5**), the precipitation was even more quantitative preventing any investigations.[166]

3.2.4 Ternary Hg^{II} complexes of metal-ion-binding nucleosides (1-5) with uridine and cytidine

Formation of ternary complexes between metal ion binding nucleosides **1-5** and uridine was studied in deuterated phosphate buffer using $HgCl_2$ as the source of the central metal ion. In comparison to Pd^{II} complexes, the ligand exchange reactions of Hg^{II} are fast in NMR time scale and, hence, the Hg^{II} ion binding does not provide a set of new peaks, but only shifts the signals of the ligands.

NMR studies revealed that the affinity of $HgCl_2$ to uridine is higher than to 2,6-bis(3,5-dimethylpyrazol-1-yl)purine riboside (**1**). The shift of uridine signals was more marked than the corresponding shift of the signals of **1** when the nucleosides were present in excess compared to $HgCl_2$. At an equimolar concentration of Hg^{II}, uridine and **1**, the signals of both nucleosides exhibited similar shifts, suggesting formation of a 1:1:1 complex, $[Hg^{II}(\mathbf{1})Urd]^+$.

3.2.5 Binary Pd^{II} complexes of modified nucleosides.

Complexing of metal-ion-binding nucleosides (**1**-**5**) with Pd^{II} was studied at various metal ion concentrations by comparing the intensities of aromatic and anomeric protons of the complexed and free nucleosides (Figure 20). Addition of K_2PdCl_4 into aq. solution of 2,6-bis(3,5-dimethylpyrazol-1-yl)purine riboside gave a mononuclear complex with tridentate ligand coordination. This 1:1 complex predominated at the equimolar concentration (1-5 mM) of both components. It displayed only one set of ^{1}H-NMR signals, evidently referring to a chlorido complex: $[Pd^{II}\mathbf{(1)}Cl]^+$. The most probable coordination sites are N1 of the purine base and N(2) atoms of the pyrazolyl moieties. Formation of a 1:2 complex $[Pd^{II}(\mathbf{l})_2]^{2+}$ took place when the concentration of **1** was higher than that of K_2PdCl_4. The chlorido ligand was replaced by another molecule of **1** which potentially can be bound to N1 or N7 of purine ring. Most likely binding to N7 is preferred as this site is sterically less hindered.[167]

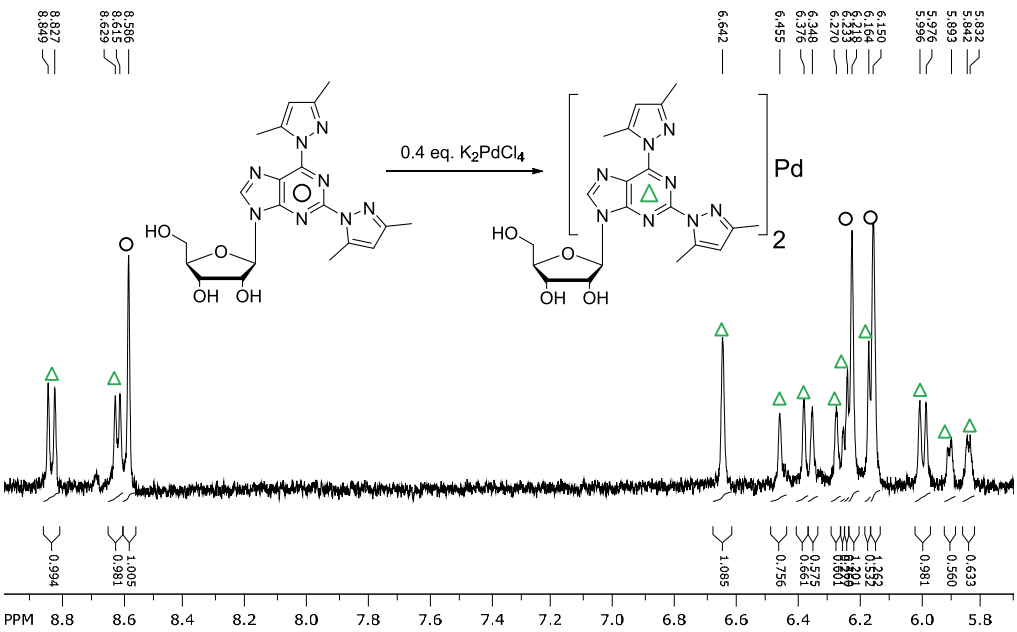


Figure 20. *Aromatic and anomeric proton signals for the mixture of 2,6-bis(3,5-dimethylpyrazol-1-yl)-9-β-D-ribofuranosylpurine (**1**, 5.0 mM) and K_2PdCl_4 (2.0 mM) in D_2O.* (phosphate buffer 0.12 $molL^{-1}$ pH 7.2, 25 ºC). Notation: Open black circles refer to uncomplexed **1** and green triangles to a 1:2 complex (Pd^{II}:$\mathbf{1}_2$)

Replacement of aromatic pyrazolyl groups on **1** with aliphatic 1-methylhydrazinyl groups reduced the binding of Pd^{II}. Only half of 2,6-bis(1-methylhydrazinyl)-9-β-D-ribofuranosylpurine was engaged in tridentate complex at equimolar 5.0 mM concentration of K_2PdCl_4 and **2**.

Interaction of 2,4-bis(3,5-dimethylpyrazol-1-yl)-5-(β-D-ribofuranosyl)-pyrimidine (**4**) with K_2PdCl_4 gave several species instead of a single tridentate complex. Addition of K_2PdCl_4 to a solution of 2,4-bis(1-methylhydrazinyl)-5-(β-D-ribofuranosyl)pyrimidine did not give any signals referring to complex formation. Most likely, the presence of ribofuranosyl group attached next to the one of the hydrazinyl (**5**) or pyrazolyl (**4**) groups sterically retards binding of Pd^{II} ions[167] (Figure 21)

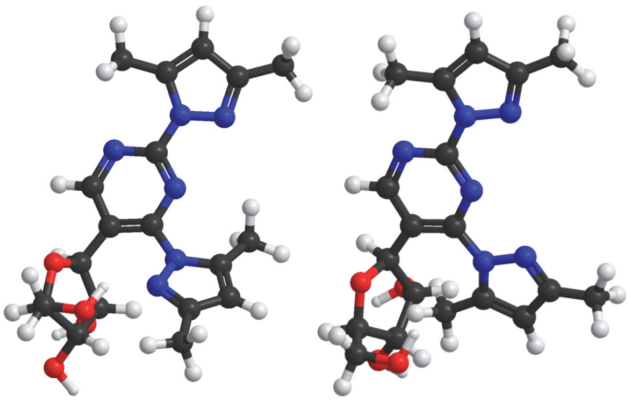


Figure 21. Semi-empirical (PM6) minimized structure for 2,4-bis(3,5-dimethylpyrazol-1-yl)-5-(β-D-ribofuranosyl)pyrimidine **(4)** (left) and the structure allowing tridentate binding to the N2 atoms of the 3,5-dimethylpyrazolyl groups and the intervening N3 of the pyrimidine ring (right). In the latter structure the steric repulsion is much more pronounced than in the former.

3.2.6 Mixed-ligand complexes of Pd^{II} with nucleoside 5´-monophosphates

Formation of mixed ligand complexes between 2,6-bis(3,5-dimethylpyrazol-1-yl)purine riboside **(1)** and NMPs was studied by ^{1}H-NMR spectroscopy. Equal amounts of **1** and K_2PdCl_4 were gradually introduced into 5.0 mM solution of NMP in a deuterated buffer, keeping the concentration of NMP constant. Upon addition of **1** and K_2PdCl_4 into the solution of UMP, the signals of UMP gradually disappeared and a new set of peaks referring to the ternary Pd^{II} complex of **1** and UMP appeared (Figure 22). Evidently, the ternary complex was formed by substitution of the chlorido ligand of tridentate **1** with N(3)-deprotonated uridine. The final concentration of **1**, K_2PdCl_4 and UMP was 4.0, 4.0 and 5.0 mM respectively, 78% of UMP was engaged in the ternary Pd^{II} complex, the theoretical maximum being 80% (Table 4).

Table 4. *Mole fraction of NMPs engaged in a mixed-ligand Pd^{II} complex with modified nucleosides (**1, 2, 4, 5, 17**) when the total concentration of **1**, K_2PdCl_4 and NMP is 4.0, 4.0 and 5.0 mM, respectively.*

	1	**2**	**4**	**5**	**17**
UMP	0.78	0.41	*d*	*b*	*d*
CMP	≈0.2	0.26	*b*	*d*	*d*
GMP	0.45	0.61	*b*	*b*	*d*
IMP	0.57	*c*	*c*	*c*	*c*
AMP	*a*	*d*	*b*	*b*	*b*
NeMP	*b*	*c*	*c*	*c*	*c*

[a]Precipitation occurred. [b]No mixed-ligand complex formed. [c]Not studied. [d]Traces of several species formed in parallel.

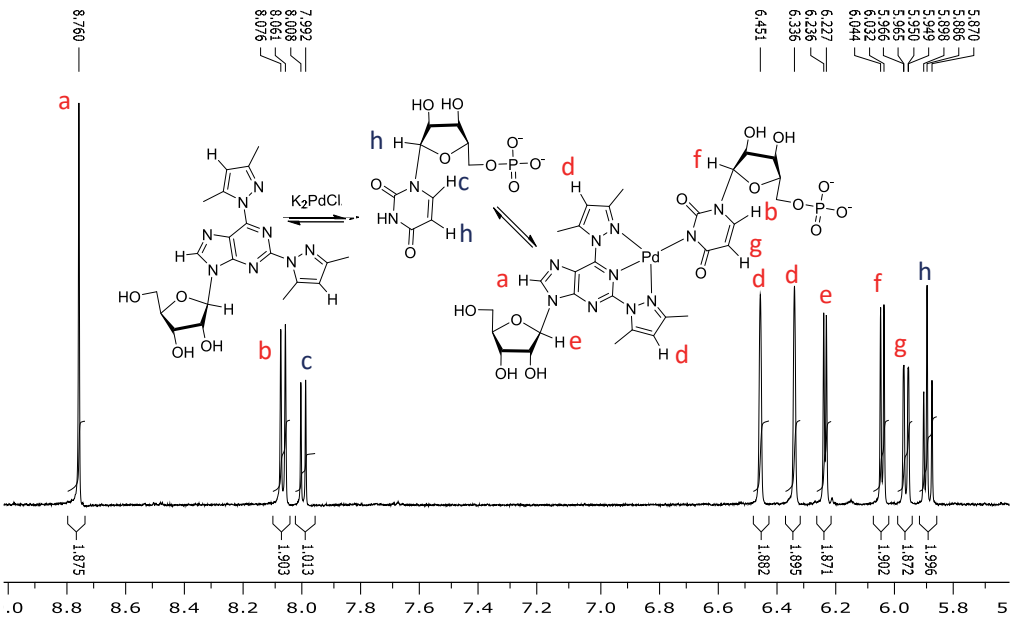


Figure 22. *Part of the ^{1}H-NMR spectra of the mixture of UMP (5.0 mM), **1** (3.4 mM) and K_2PdCl_4 (3.4 mM) in D_2O at pH 7.2* (0.12 M phosphate buffer; 25ºC). (a) H-C(8) of **1** in [Pd^{II}(**1**)UMP]$^+$; (b) H-C(6) of UMP in [Pd^{II}(**1**)UMP]$^+$; (c) H-C(6) of UMP; (d&d) H-C(4&4′)-pyrazol of **1** in [Pd^{II}(**1**)UMP]$^+$; (e) H-C(1′) of **1** in [Pd^{II}(**1**)UMP]$^+$; (f) H-C(1′) of UMP$^-$ in [Pd^{II}(**1**)UMP]$^+$; (g) H-C(5) of UMP$^-$ in [Pd^{II}(**1**)UMP]$^+$; (h) H-C(5&1′) of UMP.

Formation of mixed ligand Pd^{II} complexes of **1** with any other NMP is weaker: 45% of GMP and 57% of IMP were engaged in the Pd^{II} ternary complex. The binding sites could not be assigned due to controversial changes in chemical shifts. Only 20% of CMP was complexed with **1**. Binary and ternary Pd^{II} complexes of AMP formed as precipitate. Interaction with nebularine-5′-monophosphate is very weak; no signs of mixed ligand complexes were detected.

In fact, the Pd^{II} complex of **2** forms a relatively stable complex with GMP. Approximately 61% of GMP was engaged in the mixed Pd^{II} complex under conditions: [K_2PdCl_4] = [**2**] = 4.0 mM, [NMP] = 5.0 mM. With UMP the stability is less than half of that of the corresponding complex of **1**. Analogous ternary complexes with CMP are also less stable than the corresponding complexes of **1**. According to the downfield shift of the H-C(8) resonance, the binding site of GMP is N7. Interaction with adenosine is very weak.

Apparently, 2,6-bis(3,5-dimethylpyrazol-1-yl)purine riboside (**1**) and 2,6-bis(1-methylhydrazinyl)-9-(β-D-ribofuranosyl)purine (**2**) form more stable complexes with NMPs that have a displaceable proton in the base moiety and may, hence, engage in the ternary complex as anionic ligands.

6-(3,5-Dimethylpyrazol-1-yl)purine riboside (**17**) was designed to serve as a bidentate ligand. Interaction of **17** and K_2PdCl_4 with UMP, CMP or GMP provides very complicated ^{1}H-NMR data. Assignment of any single mixed ligand complex was impossible. With AMP, no complexes were observed.

The pyrimidine derivatives 2,4-bis(3,5-dimethylpyrazol-1-yl)-5-(β-D-ribofuranosyl)-pyrimidine **(4)** and 2,4-bis(1-methylhydrazinyl)-5-(β-D-ribofuranosyl)-pyrimidine **(5)** did not form Pd^{II} complexes (Table 4).[167]

3.2.7 Interaction of Pd^{II} complexes of 2,6-disubstituted pyridines with NMPs

To learn more about the underlying principles of metal-ion-mediated recognition of nucleic acid bases, chlorido complexes **7-12** (Figure 15) and their interaction with nucleoside 5′-monophosphates (NMPs) were investigated by ^{1}H-NMR spectroscopy in deuterated phosphate buffer at pH 7.2. As discussed above (see chapter 3.2.2), the aromatic signals of free 2,6-disubstituted pyridines, *viz.* H-C(3), H-C(4) and H-C(5), appear as a *multiplet* in the ^{1}H-NMR spectra. Introduction of Pd^{II} ion changes dramatically the aromatic proton region. H-C(4) appears as a *triplet* and H-C(3) and H-C(5) as a *doublet*.[166] These binary Pd^{II} complexes are very stable and do not tend to dissociate, even at very low concentration. The signals corresponding to free 2,6-disubstituted pyridines were not observed during these studies. The changes in the chemical shifts of the aromatic protons of the complexes formed by binding of **7-12** to canonical NMPs[178] allow some conclusions regarding the binding mode of the complexes. Pd^{II} complexes **7-12** with three chelated N donors bind with UMP by displacement of the H-N(3)[100] which results in an upfield shift of the H-C(6) resonance.[179] UMP forms only mononuclear ternary complexes with all the species **7-12** (Figures 23; 24). With CMP, a downfield shift of the H-C(6) resonance takes place upon complexing with **7-12**. According to previous studies,[122,133,179,] binding to N3 of cytosine results in downfield shift of the H-C(6) resonance, while the displacement of a proton at exocyclic amino group N-C(4) results in an upfield shift. Upon gradual addition of **7-10** to CMP solution, N(3) coordinated 1:1:1 ternary complexes of CMP and **7-10** were formed (Figure 25). When the amount of both ligands was equimolar, a new set of peaks appeared, presumably due to formation of (N3-N^4) dinuclear complexes (Figure 26). Interestingly, formation of binuclear species with sterically demanding **11** was not detected and only traces of binuclear species were observed with **12**.

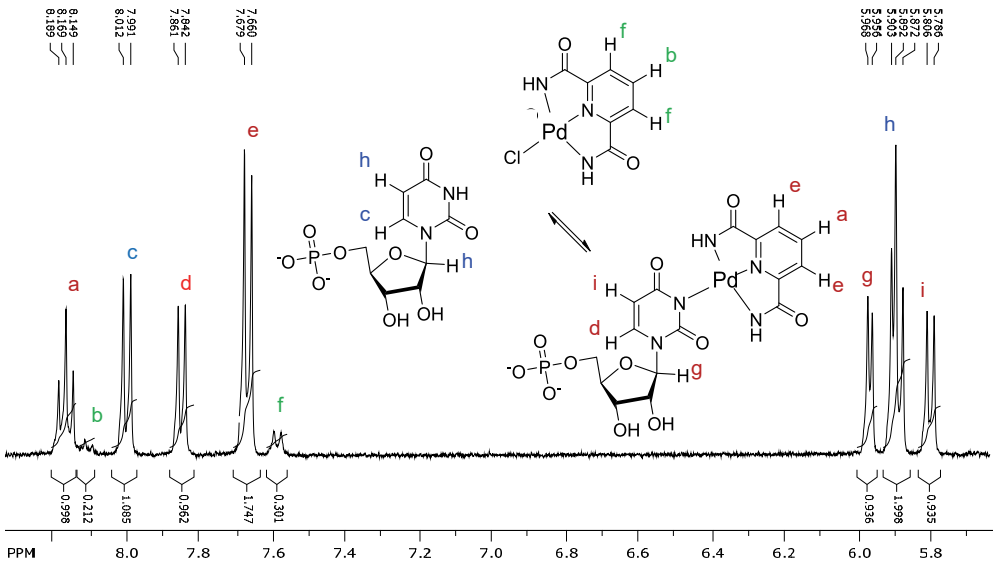


Figure 23. *Part of the [1]H-NMR spectrum of the mixture of UMP (5.0 mM) and* ***7*****-Cl (2.7 mM) in D_2O at pH 7.2** (0.12 M phosphate buffer; 25ºC). (a) H-C(4) of **7** in UMP·**7**; (b) H-C(4) of free **7**; (c) H-C(6) of UMP, (d) H-C(6) of UMP in UMP·**7**; (e) H-C(3&5) of **7** in UMP·**7**; (f) H-C(3&5) of free **7**; (g) H-C(1′) of UMP·**7**; (h) H-C(5&1′) of UMP; (i) H-C(5) of UMP in UMP·**7**.

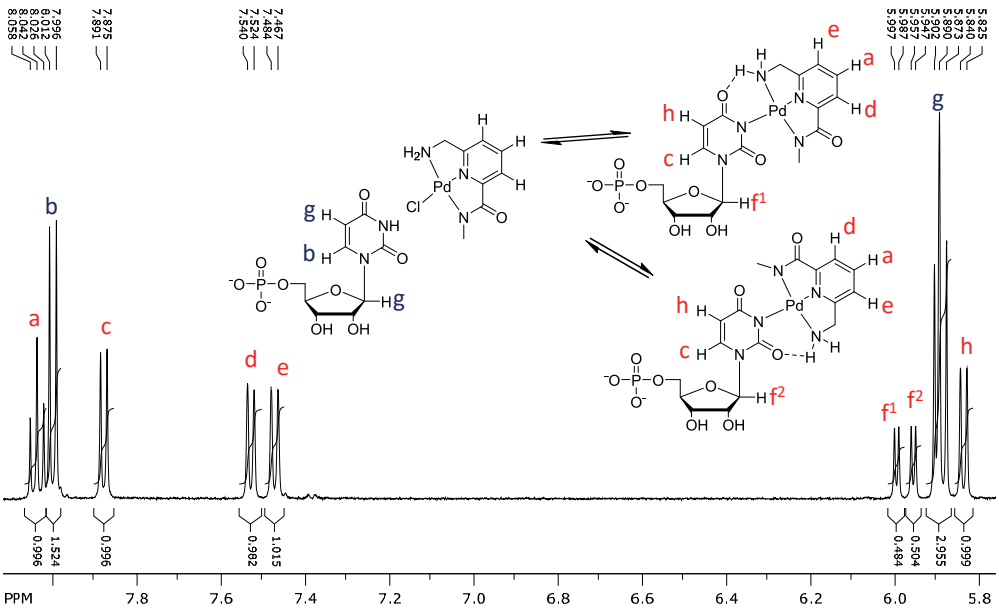


Figure 24. *Part of the [1]H-NMR spectrum of the mixture of UMP (5.0 mM) and* ***12*****-Cl (1.5 mM) in D_2O at pH 7.2** (0.12 M phosphate buffer; 25ºC). (a) H-C(4) of **12** in UMP·**12**; (b) H-C(6) of UMP; (c) H-C(6) of UMP in UMP·**12;** (d) H-C(3) of **12** in UMP·**12**; (e) H-C(5) of **12** in UMP·**12**; (f^1&, f^2) H-C(1′) of UMP·**12**^{1}&UMP·**12**^{2} (syn-&anti-);(g) H-C(5&1′) of UMP; (h) H-C(5) of UMP in UMP·**12**.

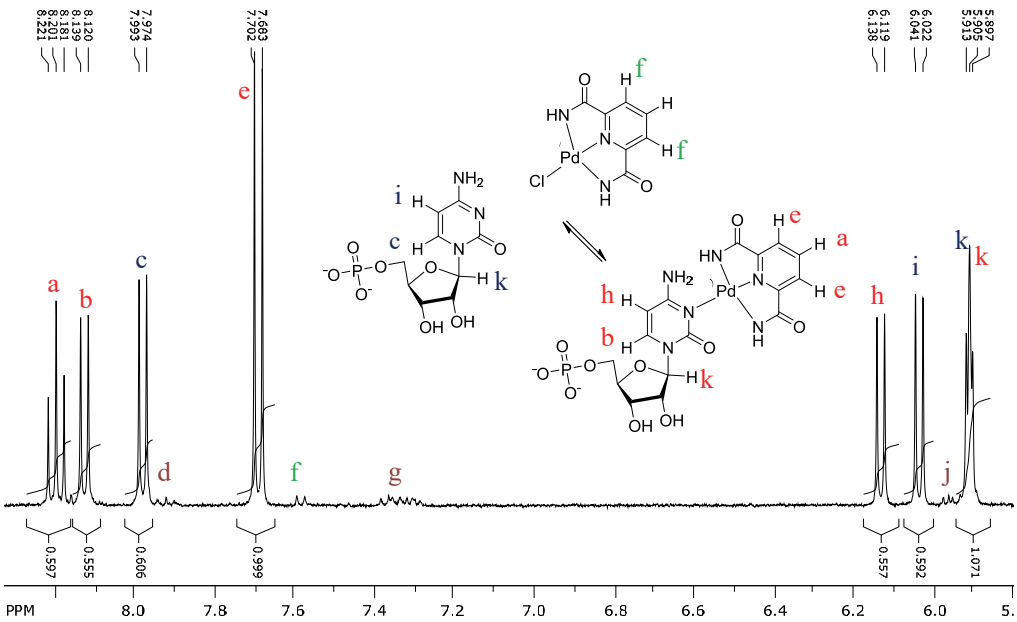


Figure 25. *Part of the ^{1}H-NMR spectrum of the mixture of CMP (5.0 mM) and* **7***-Cl (3.0 mM) in D_2O at pH 7.2* (0.12 M phosphate buffer; 25 °C). (a) H-C(4) of **7** in CMP·**7**; (b) H-C(6) of CMP in CMP·**7**; (c) H-C(6) of CMP; (d) H-C(4) of **7** in CMP·$\mathbf{7}_2$; (e) H-C(3&5) of **7** in CMP·**7**; (f) H-C(3&5) of **7**; (g) H-C(3&5) of **7** in CMP·$\mathbf{7}_2$; (h) H-C(5) of CMP·**7**; (i) H-C(5) of CMP; (j) H-C(5&1′) of CMP in CMP·$\mathbf{7}_2$; (k) H-C(1′) of CMP and CMP·**7**.

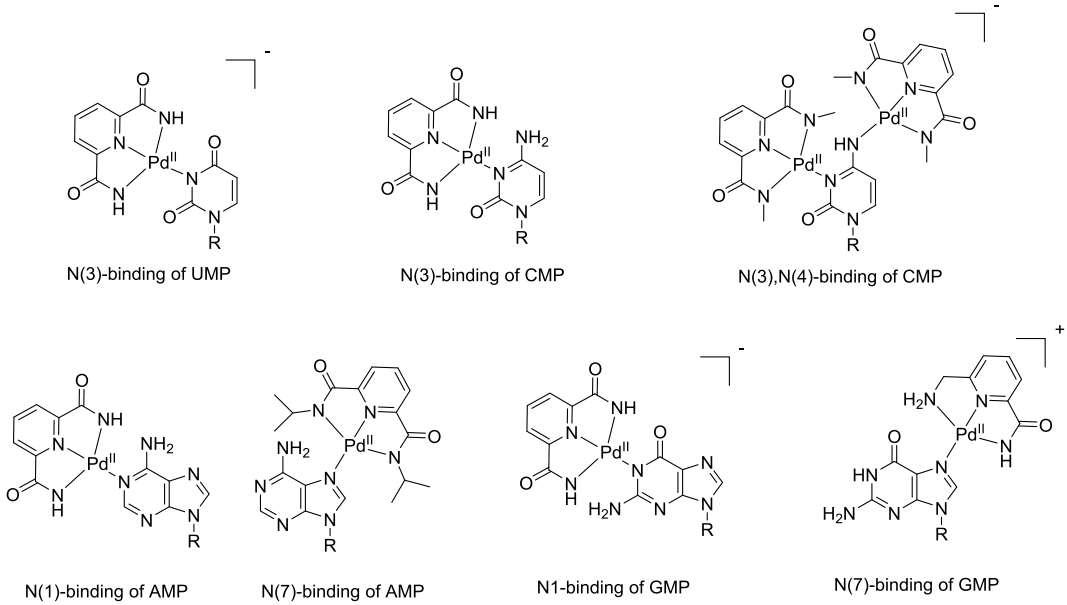


Figure 26. Various binding modes of NMPs.

With AMP, tN1 and N7 are both potential binding sites. Binding mode of $[Pd^{II}(dien)]^{2+}$ to AMP has been studied previously.[179] According to these studies, coordination of Pd^{II} to N1 or N7 results in downfield shifts of both H-C(8) an H-C(2) resonances. With N1-binding, H-C(8) resonance is shifted downfield less than H-C(2) resonance, while with N7-binding the situation is opposite. When both N1 and N7 binding sites are engaged in complex formation, the shifts are approximately equal: δ_H(H-C(8)) ≈ δ_H(H-C(2)).

On these basis, pyridine-2,6-dicarboxamide (**7**) prefers coordination to N1 (Figure 27), while its sterically demanding analogues N^2,N^6-dimethyl-pyridine-2,6-dicarboxamide (**10**) and N^2,N^6-diisopropylpyridine-2,6-dicarboxamide (**11**) as well as 6-aminomethylpyridine-2-carboxamide (**9**) undergo coordination to N7. 6-Carbamoylpyridine-2-carboxylic acid (**8**) and 6-(-aminomethyl)-N^2-methylpyridine-2-carboxamide (**12**) show mixed N1/N7 binding mode, N1 binding being favored with (**8**) and N7 favored with (**12**).

GMP also features two different binding sites, N1 and N7. Chlorido complexes **7** and **8** displace the proton at N1, resulting in H-C(8) upfield resonance shift (Figure 28), while **9**-**12** prefer coordination to nonprotonated N7, resulting in H-C(8) downfield resonance shift (Figure 29). Complexes **9**, **10** and **12** show moderate tendency to form binuclear N1, N7 –complexes. Previous studies of complexation of GMP with $[Pd^{II}(dien)]$ at pH 7, indicates significant preference of N7-binding mode over N1.[113,179] Studies of (Gly-His)Pd^{II} also indicate preference of N7–binding site,[105] while (Gly-Tyr)Pd^{II} show that, depending on the ligand structure, the formation of mononuclear N1-complex may become rather preferred. Hence, the tendency of binary Pd^{II} complexes **7** and **8** to bind N1 site is not unexpected.

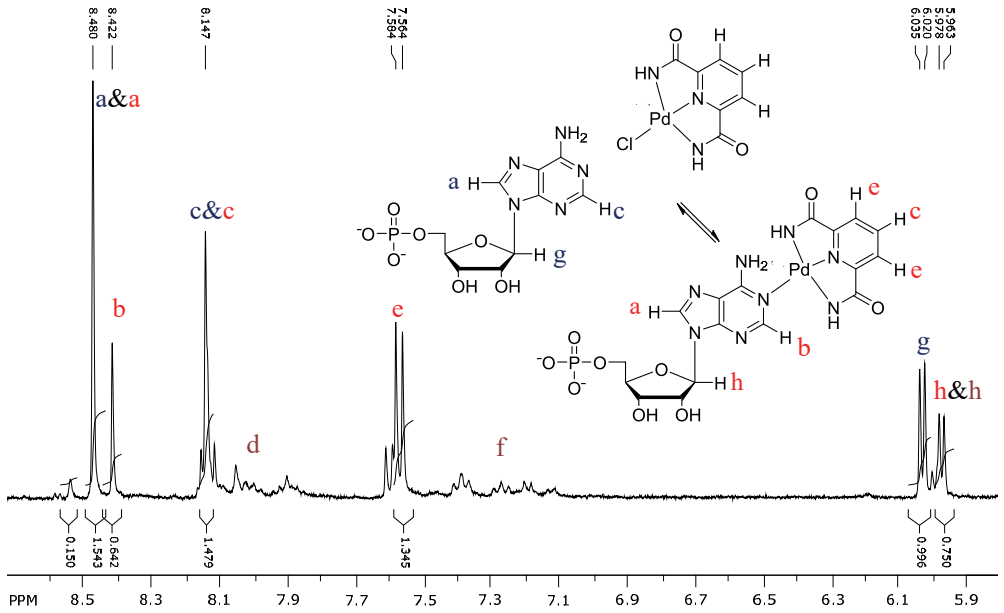


Figure 27. *Part of the ^{1}H-NMR spectrum of the mixture of AMP (5.0 mM) and* ***7-Cl*** *(3.1 mM) in D_2O at pH 7.2* (0.12 M phosphate buffer; 25 °C). (a) H-(C8) of AMP and AMP·**7**; (b) H-C(2) of AMP in AMP·**7**; (c) H-C(2) of AMP and H-C(4) of **7** in AMP·**7**; (d) H-C(4) of **7** and in AMP·$**7**_2$; (e) H-C(3&5) of **7** and in AMP·**7**; (f) H-C(3&5) of **7** in AMP·$**7**_2$.

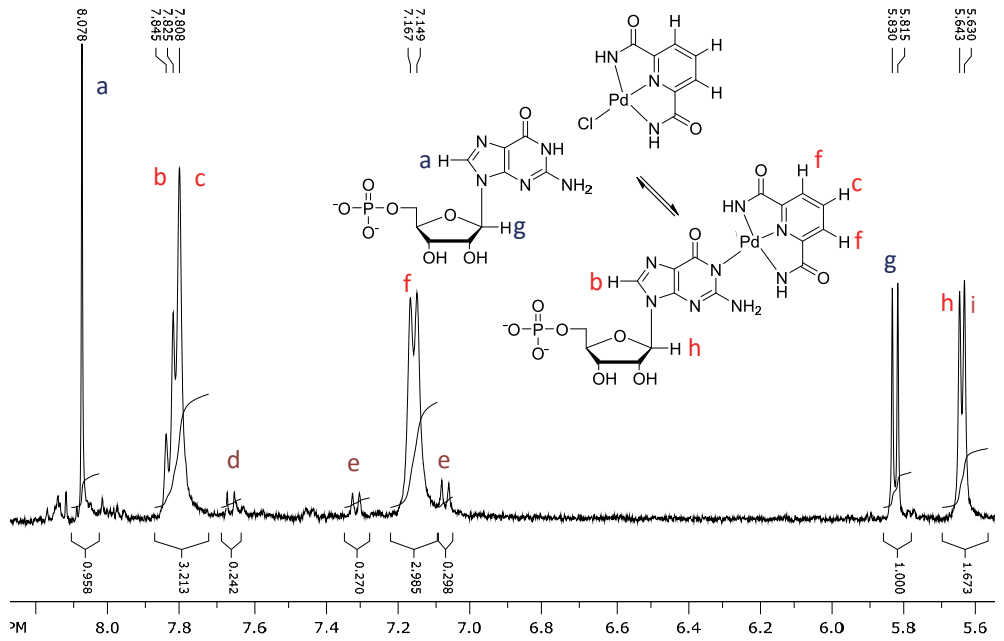


Figure 28. *Part of the ^{1}H-NMR spectrum of the mixture of GMP (5.0 mM) and* **7***-Cl (3.4 mM) in D_2O at pH 7.2* (0.12 M phosphate buffer; 25 °C). (a) H-C(8) of GMP; (b&c) H-C(8) of GMP H-C(4) of **7** in GMP·**7**; (d) H-C(3&5) of **7**; (e) H-C(3&5) of **7** in GMP·$**7**_2$; (f) H-C(3&5) of **7** in GMP·**7**; (g) H-C(1′) of GMP; (h&i) H-C(1′) of GMP·**7** and in GMP·$**7**_2$.

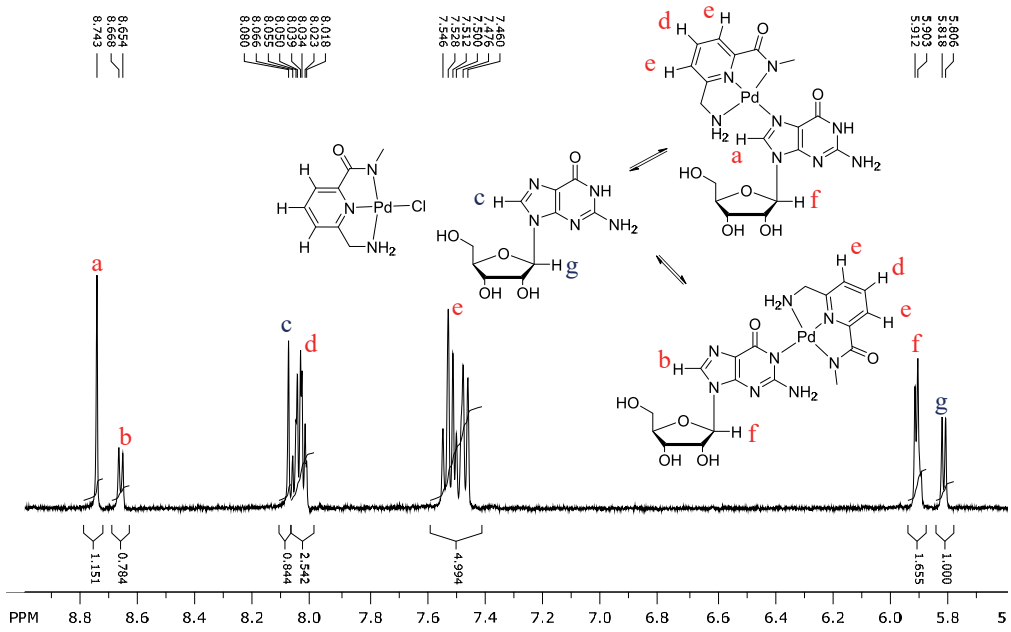


Figure 29: *^{1}H NMR spectrum of the mixture of GMP* (5.0 mM) *and* ***12****-Cl* (3.3 mM) *in D_2O at pH 7.2* (0.12 M phosphate buffer; 25 °C). (a) H-C(8) of GMP(N7)·**12**; (b) H-C(8) of GMP(N1)·**12**; (c) H-C(8) of GMP; (d) H-C(4) of **12** in GMP(N7)·**12** and GMP(N1)·**12**; (e) H-C(3&5) of **12** in GMP(N7)·**12** and GMP(N1)·**12**; (f) H-C(1′) of in GMP(N7)·**12** and GMP(N1)·**12**; (g) H-C(1′) of GMP.

Interaction of the Pd^{II} complexes of 2,6-disubstituded pyridines with NMPs having non-canonical base moieties, *viz.* purine, hypoxanthine and xanthine, was additionally studied. IMP provides a soluble complex with **7**. Ternary complex formation occurs by displacement of the N1 proton by **7**. Complexing of IMP with **8** and **9** took place, but precipitation prevented detailed investigations, since the ^{1}H resonance signal of free IMP gradually disappeared upon stepwise addition of **8** and **9**. XMP showed two different binding modes analogously to GMP. Binding to N1 was preferred with **8** and to N7 with **9**. Interaction of XMP with **7** exhibited both N1 and N7 bindings, N7 is predominating. NeMP formed soluble complex only with **9**. Upon complexation, all purine protons underwent downfield shift and the resonances of the complexed purine could not be assigned. Hence, no conclusion regarding the site of coordination could be elaborated. A single set of new signals refers to either N1 or N7 binding mode.[178]

The NMR data discussed above allows comparison of the affinities of the binary Pd^{II} chloride complexes **7-12** to NMPs. The molar fractions of NMPs engaged in mono- or binuclear-complexes were determined by gradual increase (1.0-5.0 mM) of the chlorido complexes (**7-12**) into the solution of NMPs, the concentration of which was kept constant (5.0 mM). The

proper concentrations of **7-12** were calculated comparing the intensities of aromatic and anomeric protons of NMPs. The values obtained were plotted against to the total concentrations of **7-12** and the mole fraction referring to 3.0 mM concentration of **7-12** was calculated and used as a measure of the complexing ability (Table 5) (Figure 30).

Table 5. *The mole fractions of NMPs engaged in mononuclear (1:1) and dinuclear (1:2) complexes with* ***7-12*** *obtained by mixing NMP (5.0 mM) with the chloro complex of* ***7-12*** *(3.0 mM) at pH 7.2* (0.12 M phosphate buffer; T = 25 ºC).

	UMP	**CMP**	**AMP**	**GMP**	**NeMP**	**IMP**	**XMP**
NMP·**7**	0.49	0.45	0.27[a]	0.56[a]	precipitated	0.23[a]	0.35[c]
NMP·**7**$_2$	-	0.04	0.16	-		0.13	0.08
NMP·**8**	0.51	0.31	0.47[d]	0.54[a]	precipitated	precipitated	0.41
NMP·**8**$_2$	-	0.15	-	-			0.08
NMP·**9**	0.49	0.39	0.36[b]	0.43[b]	0.32	precipitated	0.53[b]
NMP·**9**$_2$	-	0.02	-	0.10	-		-
NMP·**10**	0.56	0.32	0.23[b]	0.28[b]			
NMP·**10**$_2$	-	0.13	0.11	0.08			
NMP·**11**	0.60	0.59	0.58[b]	0.31[b]			
NMP·**12**	0.55	0.49	0.56[e]	0.45[b]			

[a]N1-binding; [b]N7-binding; [c]Ratio of N7/N1-binding 1.5; [d]Ratio of N7/N1-binding 0.3; [e]Ratio of N7/N1-binding 2.2.

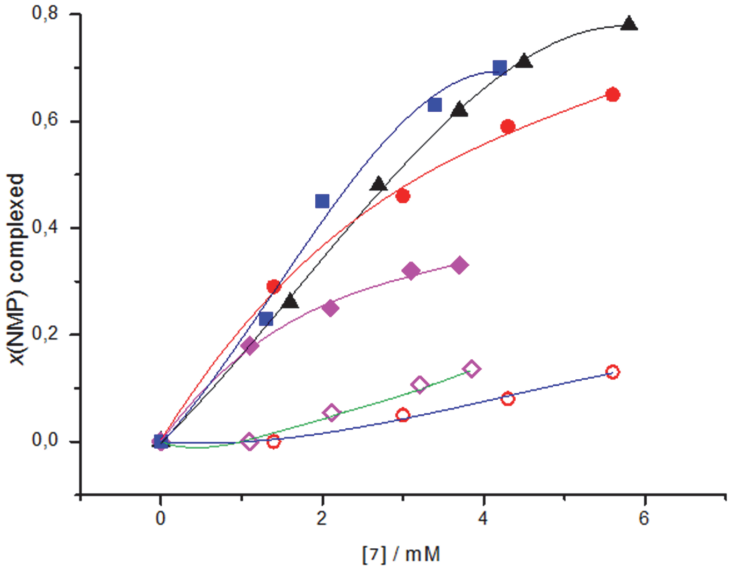


Figure 30. *Mole fraction of NMPs engaged in mono- or dinuclear complexes with 7*: UMP·**7** (black solid triangles), CMP·**7** (red solid circles), CMP·**7**$_2$ (red open circles), AMP·**7** (purple solid diamonds), AMP·**7**$_2$ (purple open diamonds), GMP·**7** (blue solid squares).

Under neutral conditions, the Pd^{II} complex of pyridine-2,6-dicarboxamide (**7**) binds both pyrimidine NMPs with comparable efficiency. It is worth noting that the data in Table 5 and Figure 31 refer to conditions where the total concentration of Pd^{II} complex (**7-12**) and NMP was 3 and 5 mM, respectively. Accordingly, the theoretical maximum for the mole fraction of NMP in the ternary complex is 60%. Surprisingly, sterically more demanding N^2,N^6-diisopropyl analogue **11** exhibited higher affinity than **7** to both NMPs. Evidently, the NH groups of **7** do not stabilize ternary complexes as hydrogen bond donors. The expected negative impact of sterically bulky substituents is presumably eliminated due to the perpendicular orientation of the pyridine and purine rings. The Pd^{II} complex of 6-aminomethylpyridine-2-carboxamide (**9**) contains a primary amino group that can serve as potential hydrogen bond donor. Still this complex exhibits only slightly higher affinity to UMP over CMP. Affinity of the methylated amino derivative **12** is similar to that of **9** towards both pyrimidines.

The Pd^{II} complex of 6-carbamoylpyridine-2-carboxylic acid (**8**) has a carboxylate substituent as a potential hydrogen bond acceptor, but this is not reflected as significant changes on the stability of UMP·**8** compared to UMP·**7**. Formation of a dinuclear complex CMP·$\mathbf{8}_2$ is enhanced, evidently due to formation of a dinuclear species by displacement of the N^4-proton of CMP,[122,133] but this happens at the expense of the mononuclear complex CMP·**8**. N^2,N^6-dimethylamino derivative **10** binds UMP and CMP in a similar manner as **8**. Introduction of sterically bulky isopropyl groups (**11**) completely prevented N^4-binding of CMP, providing highly stable mononuclear complexes of UMP and CMP.

Mononuclear complexes of purine nucleotides show greater variation with respect of the nucleotide structure. AMP forms a stable complex with **8**. Evidently, interaction of the N^6 -amino protons with the carbonyl group of **8** makes binding to N7 more favorable. Highest stability is achieved with **11** and **12**, possibly due to hydrophobic substituents that may cause additional stabilizing interligand interactions.

In comparison to AMP, the binding mode of GMP is opposite. The highest stability is obtained with small and rather hydrophilic binary ligands **7** and **8**. Coordination takes place at N1 and is significantly favored compared to binding of less hydrophobic complexes (**9-12)** that prefer N7 binding. The information about complexing of other 6-oxo-substituted purines like IMP and XMP remains scanty.[167]

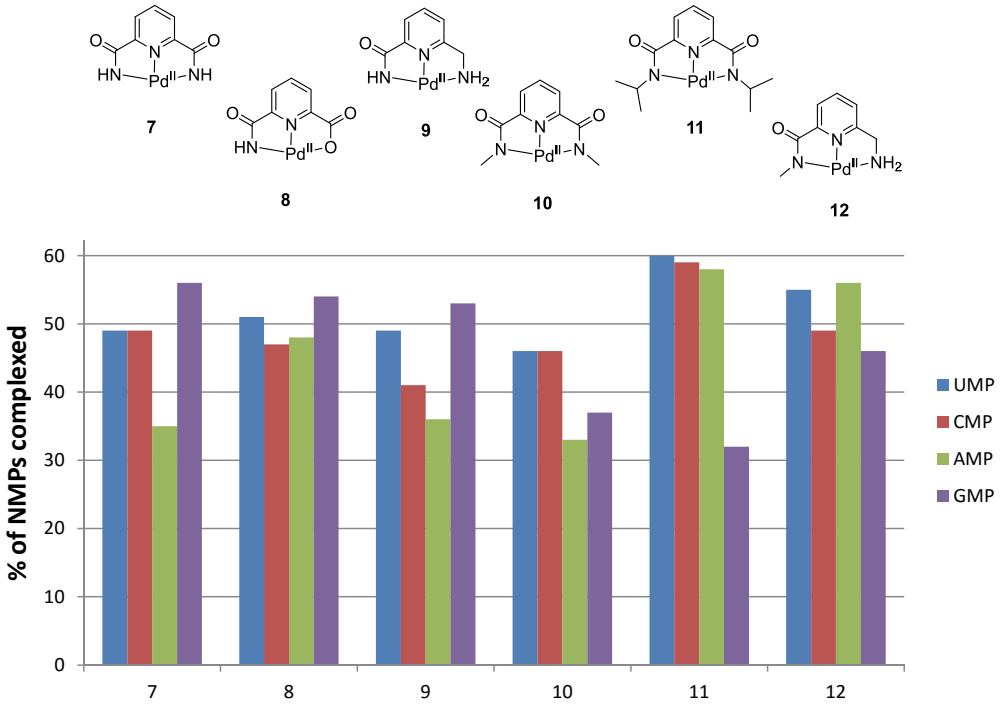


Figure 31. *Percentage of NMPs bound to Pd^{II} complexes **7-12** when the total concentration of NMPs and **7-12** is 5.0 mM and 3.0 mM, respectively.*

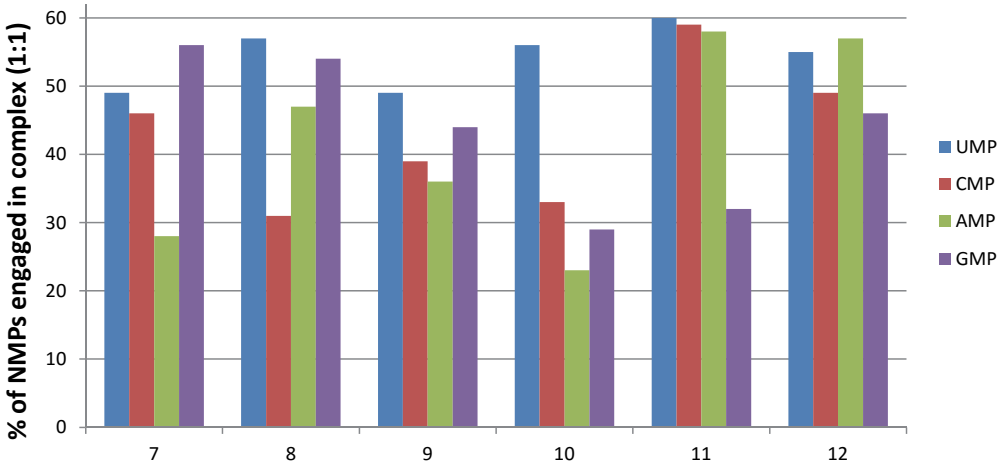
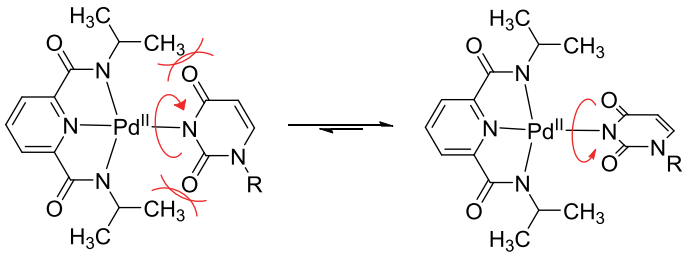


Figure 32. *Percentage of NMPs engaged in mononuclear complexes with **7-12** when the total concentration of NMP and **7-12** is 5.0 mM and 3.0 mM, respectively.*

The ability of complexes **7**-**12** to discriminate between NMPs is illustrated in Figure 31 that displays the percentage mole fraction of NMPs engaged in either mono- or dinuclear complexes. Figure 32, in turn**,** displays similar data for mononuclear complexes. The discrimination obtained by varying the hydrogen bonding properties and the size of the ortho-substituent of Pd^{II} bound pyridine complexes is rather modest. Complex **7** binds less efficiently to AMP than the other canonical NMPs. Methylation of amido groups inhibits binding to GMP and affords dinuclear binding of CMP. Complexation of UMP remains similar for all pyridine derivatives (**7**-**12**). In contrast to expectation, sterically demanding **11** exhibits highest stabilities with UMP, CMP, AMP though not with GMP. Most likely rotation around the Pd-N bond between the nucleobase and the Pd^{II} complex allows alleviation of the steric crowding and, hence, the effects on stability remain small (Scheme 21).[167]



Scheme 21. The rotation around the Pd-N bond between the nucleobase and the Pd^{II} complex.

Within a double helical structure the situation may be different, since stacking with the neighboring base pairs tends to force the nucleobase and the pyridine ligand to take a coplanar orientation.

3.3 Stability of oligoribonucleotides incorporating a metal-mediated-base pair

3.3.1 Melting temperature

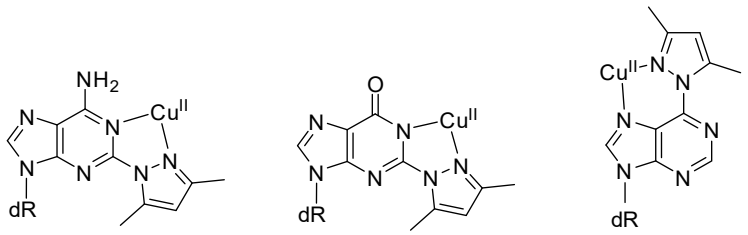
Hybridization efficiency of the modified oligonucleotides **ON1p**, **ON1q**, **ON1z** and **ON1x** with their unmodified complements **ON2a**, **ON2c**, **ON2g** and **ON2u** was studied by T_m measurements in the presence and absence of metal ions. Oligonucleotide **ON2s** containing an abasic site in a central position was used as a reference within which the metal-ion-mediated-base pairing is eliminated, while the stacking interactions with the neighboring base pairs still

exist (Table 2). Oligonucleotide concentrations were determined by UV-spectrophotometry on the basis of molar absorptivities that were obtained by an implementation of the nearest-neighbors method.[176,177]

In the studies with 6-(3,5-dimethylpyrazol-1-yl)purine riboside (**17**) discussed above (see chapter 3.2.6), metal ion mediated interactions of bidentate ligands are complicated, leading to unpredictable ^{1}H-NMR patterns and making interpretation of the data obtained at the monomeric level exceedingly challenging.[167] Within a double-helical structure, stacking with the neighboring base pairs tends to force the nucleobase and the metal-ion-binding base surrogate to adopt a coplanar orientation. Higher order metal-ligand interactions become suppressed, leaving two coordination sites of the metal ion complex for interaction with the natural nucleobase. Divalent metal ions Cu^{II} and Zn^{II} were used for recognition of natural nucleic acid bases by bidentate metal ion binding chelates **13, 14, 17** within oligonucleotide secondary structure.

Stability of the oligonucleotide duplexes containing a bidentate surrogate **13, 14** or **17** in one of the strands were first studied in the absence divalent metal ions. According to these studies, nucleoside **17**, bearing the 3,5-dimethylpyrazol-1-yl substituent at position 6, is destabilizing, whereas the analogues **13** and **14** bearing the same group at position 2 are stabilizing. Previously studied 2,6-bis(3,5-dimethylpyrazol-1-yl)purine riboside[163,173] (**1**) is also stabilizing but much less than **13** or **14**. Consequently, pyrazole ring has a stabilizing effect in position 2 but not in position 6. The stabilizing effect at positon 2 may tentatively be attributed to enhancement of π-stacking interaction with the neighboring nucleobases, which is not observed with the 6-pyrazolyl analogues.

The 3,5-dimethylpyrazolyl substituent on **13, 14** and **17** provides an additional binding site, *viz* the pyridine-like N2 atom having a lone electron pair that is not part of the aromatic system. Most probably the base moieties of compounds **13**, **14** are bound to the central metal ion through purine N1 and pyrazole N2 and the base moiety of **17** through purine N7 and pyrazole N2 (Figure 33).



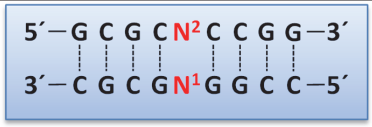
13:Cu^{II} **14**:Cu^{II} **17**:Cu^{II}

Figure 33. Coordination sites of modified nucleosides **13, 14, 17**

Duplexes formed by all of the modified oligonucleotides **ON1p**, **ON1q**, **ON1z** and the previously studied **ON1x**[173] with their unmodified counterparts exhibit the highest stability in the presence of 1 eq. of Cu^{II} ions. Stabilization effect was not observed with duplexes having an abasic unit opposite to the metal-ion-chelating nucleobase. Both facts are consistent with formation of a Cu^{II}-mediated base pair[180] between the artificial nucleobase and the natural one opposite to it (Table 6).

Interesting exception are the Zn^{II}-mediated base pairs between **14** and natural nucleobases. Cu^{II} was replaced by Zn^{II} without loss in thermal stability. Such behavior could be explained for example by high affinity of modified nucleoside **14** for Zn^{II}

Table 6. *Melting temperatures of duplexes formed between modified oligonucleotides **ON1p, ON1q** and **ON1z** with their unmodified complementary strands **ON2a, ON2c, ON2g, ON2u**, and **ON2s.***



		N^1				
M^{II}	**N^2**	**A**	**C**	**G**	**U**	**S**
no metal	**P**	53.1 ± 0.4	50.1 ± 0.5	53.0 ± 0.4	51.9 ± 0.4	58.3 ± 0.2
	Q	66.5 ± 0.8	66.3 ± 0.6	66.1 ± 0.8	67.0 ± 0.6	69.3 ± 0.4
	Z	62.7 ± 0.6	63.4 ± 0.7	63.8 ± 2.1	63.4 ± 0.9	69.6 ± 0.7
Cu^{II}	**P**	54.5 ± 0.5	53.3 ± 0.6	56.3 ± 0.6	58.7 ± 0.8	58.0 ± 0.3
	Q	75 ± 2	73 ± 2	72 ± 1	72.2 ± 0.3	69.6 ± 0.4
	Z	N/A[c]	70.6 ± 1.0	N/A[c]	64.8 ± 1.0	N/A[c]
Zn^{II}	**P**	52.8 ± 0.8	51.3 ± 0.6	54.0 ± 0.8	51.8 ± 0.4	58.4 ± 0.1
	Q	73 ± 2	72 ± 2	73 ± 2	74 ± 2	69.4 ± 0.1
	Z	63.6 ± 0.8	62.5 ± 0.5	65.5 ± 1.1	65.8 ± 1.0	69.5 ± 1.5

[a]Conditions: pH = 7.4 (20 mM cacodylate buffer); [oligonucleotides] = 3.0 μM; [metal ions] = 0 or 3.0 μM; *I*(NaClO4) = 0.10 M, [b] No sigmoidal melting curve was obtained. Structures of modified residues **P**, **Q, Z** and **S** presented in Table 2.[168]

Stability of metallo-base pairs seems to be related to the ability of the nucleobase to serve as an anionic ligand. High T_m values were recorded upon metal ion addition (Cu^{II} or Zn^{II}) to the systems where at least one of the members may be deprotonated. At physiological pH, modified nucleobase **14** within oligonucleotide **ON1q** most probably becomes deprotonated upon metal ion coordination, which seems to result in marked duplex stabilization, almost irrespectively of the opposite canonical base. By contrast, **13** and **17** do not undergo deprotonation under experimental conditions and the oligonucleotides incorporating these modified nucleosides (**ON1z** and **ON1p**, respectively) favor hybridization with complementary oligonucleotides that can provide an anionic ligand (guanine or uracil) to the metal ion mediated base pair: **ON2g** and **ON2u**. Analogous behavior of metal-mediated-base pairing was observed at the monomeric studies with tridentate ligands **1** and **2** and their interaction with nucleoside-5′-monophosphates (NMPs) (Table 4).[167]

Assuming square planar or octahedral coordination geometry for the bridging metal ion, bidentate nucleoside analogues **13, 14, 17** leave two vacant coordination sites for the natural nucleobases. Square-planar or octahedral geometry around Cu^{II} has been observed previously.[55,181,159,182,183] According to previous studies, N1 and N7 of purines[68,69,92,93] and N3 of pyrimidines [98,184, 185,186] are the preferred binding sites for Cu^{II}.[96,115,187,188,] Usually at pH values close to neutral, adenosine coordinates through N7 and guanosine through N1.

The exocyclic oxo-substituents are favored coordination sites over exocyclic amino groups, because the oxygen still has a lone electron pair which is not a part of whole π-electron system part.[189,190]

The potential structures of metal-ion-mediated base pairs formed by modified nucleobases **13**, **14**, **17** and the natural nucleobases presented in Figures 34, 35 and 36.

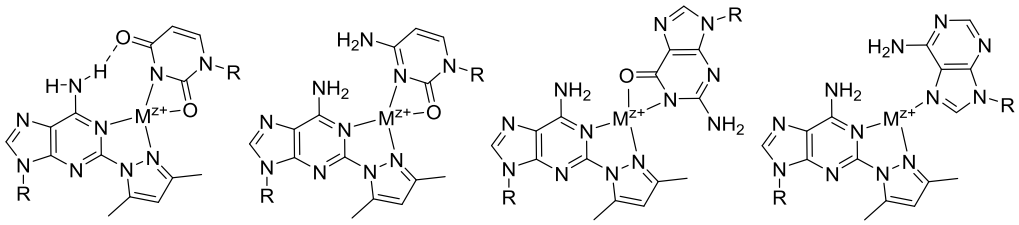


Figure 34. Proposed *metal-ion-mediated base pairs within double helical structures between **13** and natural nucleobases:* **U**-uracil, **C**-cytosine, **G**-guanine and **A**-adenine respectively; **M**$^{z+}$-divalent metal ion (Cu^{II} or Zn^{II})

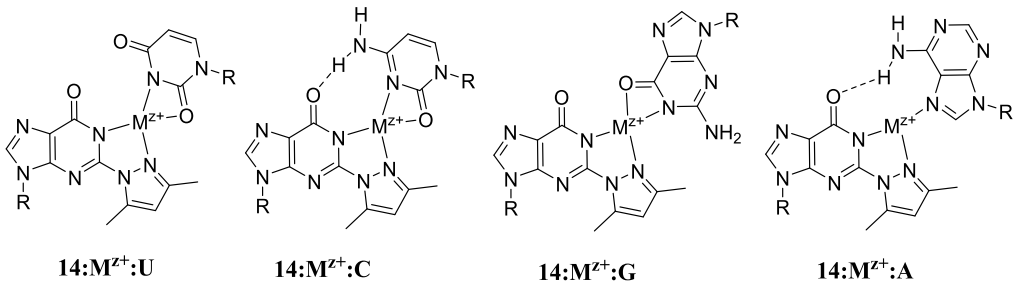


Figure 35. Proposed *metal-ion-mediated base pairs within double helical structures between **14** and natural nucleobases*: **U**-uracil, **C**-cytosine, **G**-guanine and **A**-adenine respectively; **M**$^{z+}$-divalent metal ion (Cu^{II} or Zn^{II})

Besides electronic effects, steric repulsions between the amino and methyl substituents is also evident in the case of the **17**:M^{z+}:A and **17**:M^{z+}:C pairs (Figure 38).

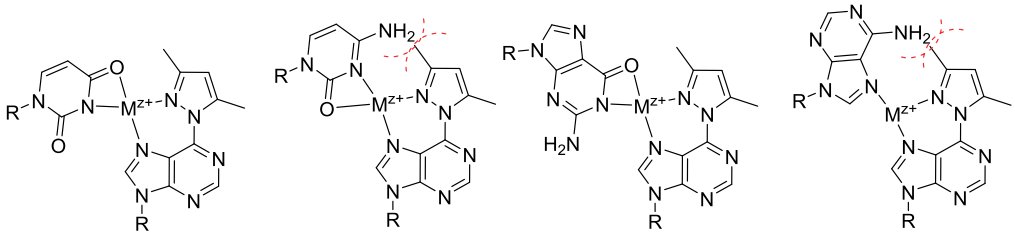


Figure 36. Proposed *metal-ion-mediated base pairs within double helical structures between **17** and natural nucleobases*: **U**-uracil, **C**-cytosine, **G**-guanine and **A**-adenine respectively; **M**$^{z+}$-divalent metal ion (Cu^{II} or Zn^{II})

3.3.2 CD spectrophotometric studies

The secondary structure of all duplexes formed in the presence of divalent metal ions was verified by CD spectroscopy. CD spectra[180] of oligonucleotide duplexes were measured over a wide temperature range (6-94 °C). At low temperatures, CD spectra characteristic of an A-type double helix were observed.[191- 195] In other words, the difference in absorptivity for left and right circularly polarized light is maximal between 260 and 270 nm. Increase in temperature led to gradual decrease of this CD signal with concomitant shift of the maximum towards a longer wavelength. As an illustrative example, temperature-dependent CD spectra of the **ON1z**:**ON2c** duplex in the presence of Cu^{II} is depicted in Figure 37A. As seen from Figure 37B, the UV melting curve and the curve referring to changes in the CD spectrum as a function of temperature exhibit approximately equal inflection points.

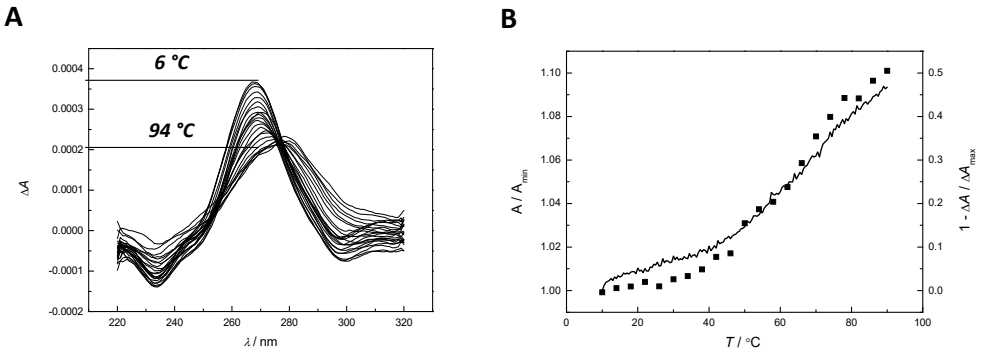


Figure 37. (*A) CD spectra of oligonucleotide duplex **ON1z**:**ON2c** in the presence of Cu^{II}, recorded at 4 °C intervals between 6 and 94 °C. (B) Thermal hyperchromicity at 260 nm (solid line) and loss of ellipticity (■) of the same duplex*; [**ON1z**] = [**ON2c**] = [Cu^{II}] = 3.0 μM; pH = 7.4 (20 mmol cacodylate buffer); *I*($NaClO_4$) = 0.1M.

3.4 Pd^{II}-mediated hybridization of GNA oligonucleotides

3.4.1 Preparation of a terminal GNA-Pd^{II}-complex, **ON1**$_2$:Pd^{II}_2

Oligonucleotide **ON1** bearing (*S*)-4-(2,3-dihydroxypropoxy)pyridine-2,6-dicarboxamide (**24**) in its 3′-terminus was freeze-dried, dissolved in aq. solution of K_2PdCl_4 and kept overnight, after which the mixture was diluted with phosphate buffer (pH 7.2). The mixture of products was fractionated by RP-HPLC eluting with MeCN gradient in a phosphate buffer. The collected fractions were desalted by RP-HPLC eluting with MeCN gradient in water. Based on the HPLC data, **ON1** was converted almost quantitatively to products with lower mobility (Figure 38). The sequence of four canonical nucleobases in **ON1** allows formation of a duplex by four CG base-pairs, and terminal Pd^{II}-mediated base pairs between the pyridine-2,6-dicarboxamide residue **24** and thymine. Formation of the desired structure **ON1**$_2$:Pd^{II}_2 was verified by ESI-MS (Figure 39). Each of Pd^{II} ions had displaced two protons.[172] The analogous binding mode had been observed previously for pyridine-2,6-dicarboxamide at monomeric level.[166]

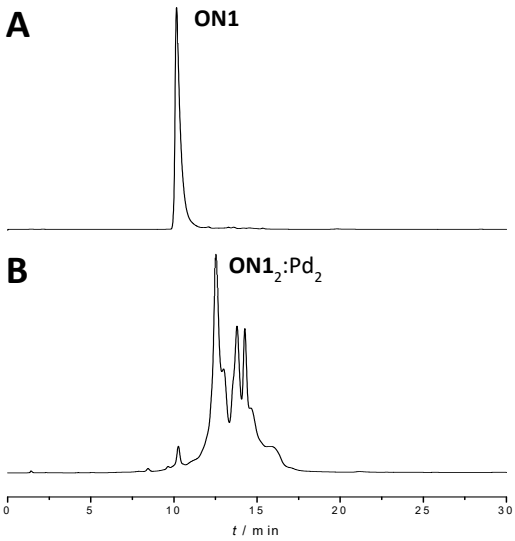


Figure 38. *HPLC traces for (A) purified **ON1** and (B) product mixture of the reaction of **ON1** with 1.5 eq. of K_2PdCl_4;* Thermo Scientific Aquasil C18 column (150 × 4 mm, 5 μm); flow rate = 1.0 mL min^{-1}; linear gradient (0 to 30% over 30 min) of MeCN in 60 mM phosphate buffer (pH = 7.2).

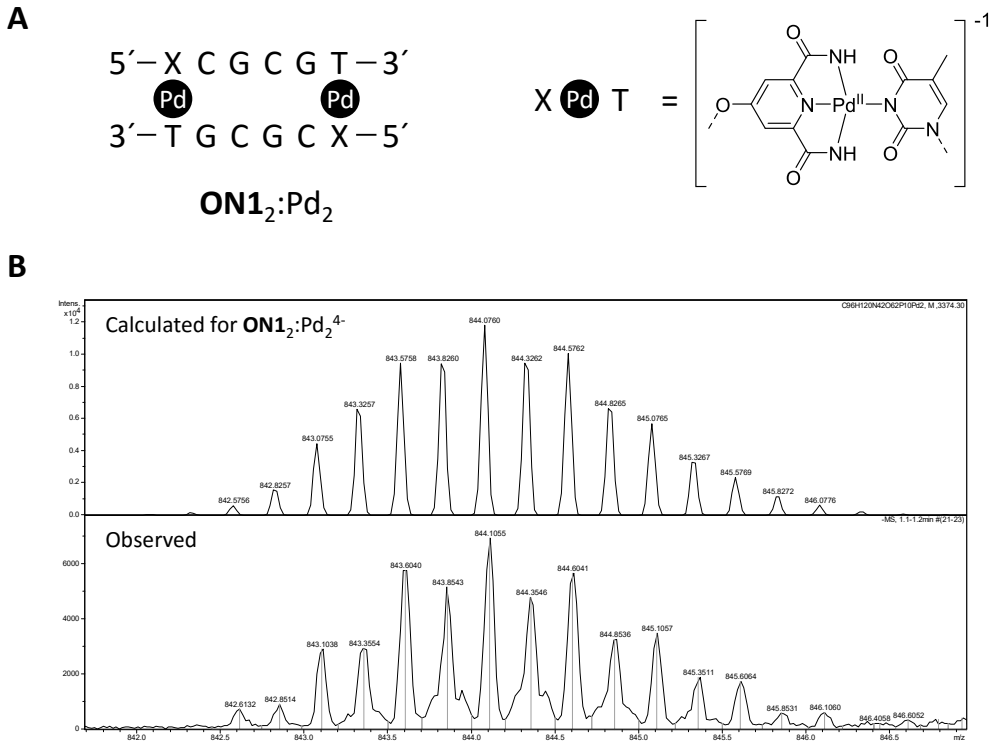


Figure 39. (A) Proposed structure of **ON1**$_2$:Pd^{II}_2 and (B) calculated and observed mass spectra for its tetraanion.

3.4.2 Preparation of a nonterminal GNA Pd^{II}-complex, ON2:Pd^{II}:ON3.

Oligonucleotide **ON2**, incorporating artificial nucleoside analogue **24** in an intrachain position was treated with aq. K_2PdCl_4, kept overnight, after which it was freeze-dried. An equimolar amount of the complementary oligonucleotide **ON3** was then added and the mixture was diluted with a phosphate buffer (pH 7.2). The RP-HPLC chromatogram of the crude product showed two main peaks. The one at the longer retention time was identified as **ON3** by spiking (Figure 40C). The peak at the shorter retention time did not correspond to either of the starting materials. In other words no unreacted oligonucleotide **ON2** was detected. Injection of a small amount of the mixture gave two peaks, the areas of which were almost equal. Upon gradual increase of the injection volume, the faster eluting peak gradually grew at the expense of the slower eluting peak. It appears reasonable to assume that the two peaks referred to an equilibrium mixture of the Pd^{II} containing duplex, **ON2**:Pd^{II}:**ON3** and **ON3**. Increase in the injection volume and, hence, the concentration retards dissociation of **ON2**:Pd^{II}:**ON3** during chromatography. To receive additional support for this conclusion, the faster eluting peak was collected and reinjected. Two separate peaks appeared in the chromatogram and were identified as the reinjected fast eluting material and oligonucleotide **ON3**. However, **ON2** was not detected (Figure 40D). The absence of **ON2** could be explained by the formation of **ON2**:Pd^{II} and assuming that the retention time of this metallo-oligonucleotide is very similar to its heterodimer **ON2**:Pd^{II}:**ON3**. The dissociation of **ON3** suggests that the desired duplex **ON2**:Pd^{II}:**ON3** is not as stable as the homodimer **ON1**$_2$:Pd^{II}_2 and rapidly decomposes upon dilution. Attempts to desalt the product were unsuccessful due to denaturation of the **ON2**:Pd^{II}:**ON3** duplex and loss of the Pd^{II} bridging ion at the low ionic strength. A small sample treated as described above for **ON1**:Pd^{II} was analyzed by ESI-MS. A mixture of oligonucleotides **ON2**, **ON3** and **ON2**:Pd^{II} was detected, suggesting that the coordination of Pd^{II} takes place at the desired binding positions, but the stability of the heterodimer **ON2**:Pd^{II}:**ON3** is much lower than that of the corresponding homodimer **ON1**$_2$:Pd^{II}_2.[172]

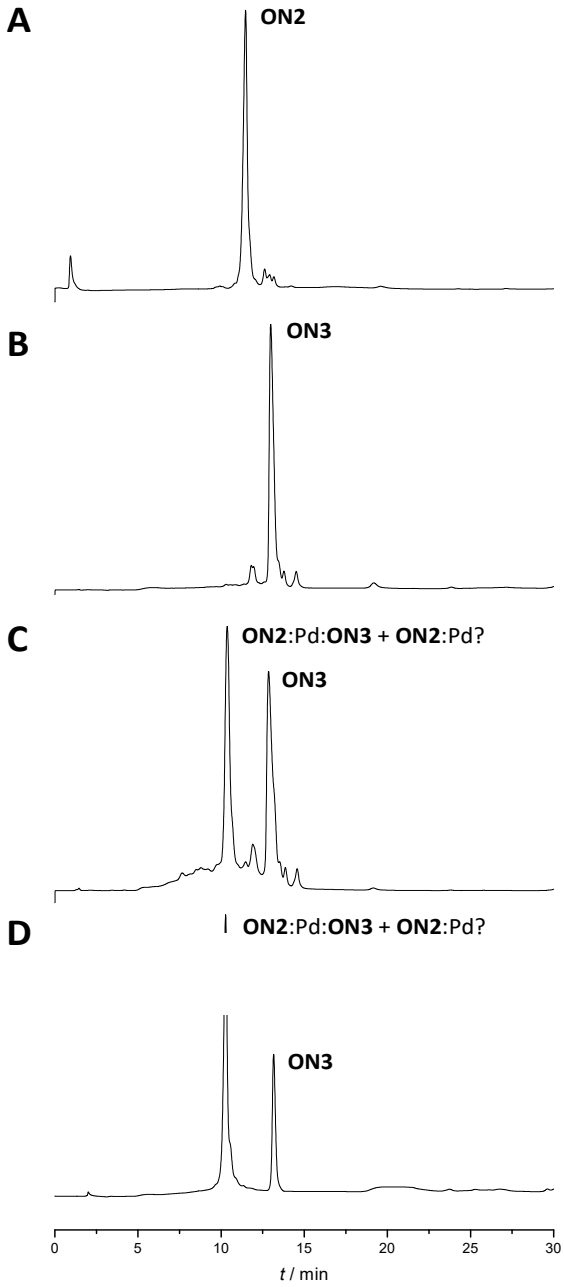


Figure 40. *HPLC traces for (A) purified **ON2**, (B) purified **ON3**, (C) product mixture of the reaction of **ON2** and 1.1 eq. of K_2PdCl_4, followed by 1.0 eq. of **ON3** and (D) reinjection of the faster-eluting peak of chromatogram C.* Thermo Scientific Aquasil C18 column (150×4 mm, 5 µm); flow rate = 1.0 mL min^{-1}; linear gradient (0 to 30% over 30 min) of MeCN in 60 mM phosphate buffer (pH = 7.2).

3.4.3 Melting temperatures of GNA duplexes incorporating a Pd^{II}-mediated base pair

Melting temperature determination of non-metallated oligonucleotides, **ON1**$_2$ and **ON2**:**ON3** (Table 3), gave typical sigmoidal curves (Figure 41) with T_m of 45.9 ºC and 36.3 ºC for **ON1**$_2$ and **ON2**:**ON3**, respectively. Pd^{II} containing duplexes **ON1**$_2$:Pd^{II}_2 and **ON2**:Pd^{II}:**ON3** behaved very differently. Extraordinary increase in stability was observed for **ON1**$_2$:Pd^{II} having the Pd^{II}-binding **24** placed in the terminus of the oligonucleotide. Melting temperature of **ON1**$_2$:Pd^{II}_2 was too high to be determined. By contrast, no stabilization was detected when the Pd^{II} bearing surrogate nucleoside was placed in the middle of oligonucleotide **ON2**:Pd^{II}:**ON3**. Related results have been previously reported for double stranded 2′-*O*-methyl-RNA oligoribonucleotides bearing 2,6-bis(3,5-dimethylpyrazol-1-yl)purine riboside (**1**) as the metal ion chelating residue.[163] Most likely double-stranded oligonucleotides have difficulties in accommodating of Pd^{II}- mediated base pair within the oligonucleotide environment as a result of the deviation from optimal geometry required to fit the Pd^{II} mediated base pair as part of the normal Watson-Crick double helix. At a terminal position, such base pairs may be highly stabilizing even if they are not accommodated in the base stack of the oligonucleotide duplex. It is also worth noting that **ON1**$_2$:Pd^{II}_2 was synthesized and purified before the melting temperature measurements, whereas addition of Pd^{II} into a solution of **ON1**$_2$ in *situ* did not provide the desired duplex as the sole product, resulting in a T_m value lower than the one observed without ^{II} ion. (41.5 and 45.9 ºC respectively). Most likely the typical condition of hybridization studies fail to afford the desired metal-ion-mediated duplex due the relatively slow ligand exchange reactions of Pd^{II}.[172]

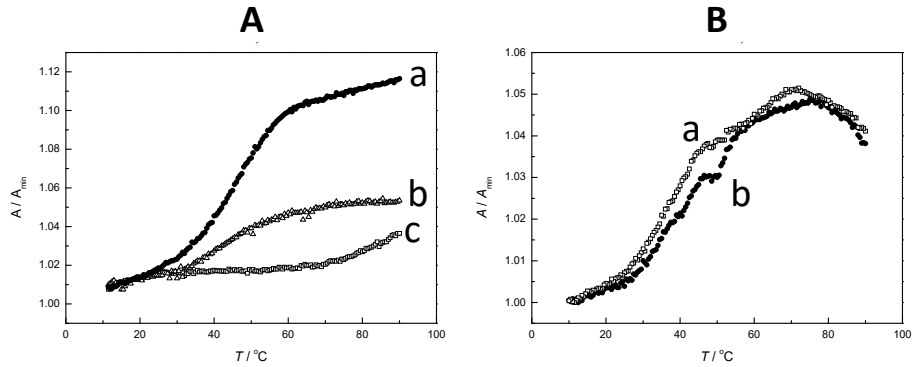


Figure 41. UV melting profiles for (A) **ON1**$_2$ (a); **ON1**$_2$:Pd_2 (c) and **ON1**$_2$ with 2.0 eq. of K_2PdCl_4 added *in situ* (b) and (B) **ON2**:**ON3** (b) and **ON2**:Pd:**ON3** (a); [**ON1**] = 6.0 μM; [**ON2**] = [**ON3**] = 3.0 μM; [Pd^{II}] = 0 / 3.0 / 6.0 μM; I($NaClO_4$) = 0.10 M; pH = 7.4.

3.4.4 CD spectrophotometric studies

Additional information of the helical structure of GNA oligonucleotide duplexes formed in the presence and in the absence of Pd^{II} was obtained by CD spectrophotometric studies. CD spectra of oligonucleotide duplexes[180] were measured over a wide temperature range (6 – 94°C) and the secondary structure of the corresponding oligonucleotides was verified. The curves recorded at low temperatures were consistent with an (*S*)-GNA double helix[196]. Increase in temperature resulted gradual decrease of CD signals with all oligonucleotides. In the case of **ON1**$_2$:Pd^{II}_2, some ellipticity was observed even at 90 °C, which indicates that Pd^{II}-mediated base pairs stabilize the duplex even when the helical secondary structure was thermally disrupted **(**Figure 42).[172]

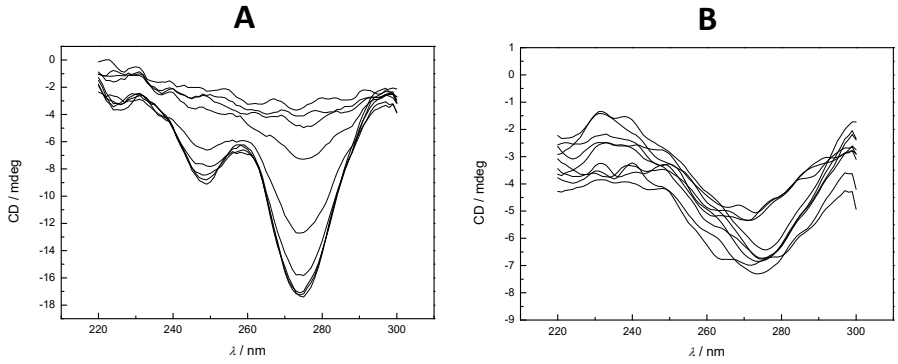


Figure 42. CD spectra of oligonucleotide duplexes (A) **ON1**$_2$ and (B) **ON1**$_2$:Pd_2, recorded at 10 °C intervals between 10 and 90 °C; [**ON1**$_2$] = [**ON1**$_2$:Pd_2] = 3.0 μM; pH = 7.4 (20 mM cacodylate buffer); *I*($NaClO_4$) = 0.10 M.

4 CONCLUSIONS

According to previous reports, the great stability of the Pd^{II}-mediated base pair between 2,6-bis(3,5-dimethylpyrazol-1-yl)purine riboside (**1**) and uridine is very modestly reflected in the thermal stability of double-stranded oligonucleotides.

2,4-Bis(3,5-dimethylpyrazol-1-yl)-5-(β-D-ribofuranosyl)pyrimidine (**4**) was prepared as a sterically less bulky analogue of **1**. Unexpectedly, this pyrimidine analogue formed with uridine a less stable Pd^{II}-mediated complex than **1**. Replacement of pyrazolyl rings by hydrazinyl substituents did not improve binding properties. Neither 2,6-bis(1-methylhydrazinyl)-9-(β-D-ribofuranosyl)purine (**2**) nor 2,4-bis(1-methylhydrazinyl)-5-(β-D-ribofuranosyl)pyrimidine (**5**) formed markedly stable ternary complexes. As regards binary complexes, the modified purine derivatives **1** and **2** bound Pd^{II} much more tightly than their pyrimidine counterparts **4** and **5**. Most probably the presence of bulky ribofuranosyl group next to one of the substituents retards the binding of Pd^{II}.

The purine based analogs **1** and **2** also showed some signs of selectivity. The mixed-ligand ternary complexes are much more stable with NMPs having an acidic proton (UMP, IMP and GMP) and, hence, being able to bind Pd^{II} as anionic ligands.

2,6-Disubstituted pyridines provide stable mixed-ligand Pd^{II} complexes with NMPs, but the selectivity is rather modest. Most likely rotation around the Pd-N bond between the nucleobase and the Pd^{II} complex eliminates the steric hindrances that bulky *ortho*-substituents of Pd^{II}-bound pyridine ligands could be expected to exert in case of coplanar conformation of the pyridine ligand and the nucleobase.

Three 3,5-dimethylpyrazol-1-yl substituted purine nucleosides, *viz.* 2-substituted adenosine (**13**), 2-substituted inosine (**14**) and 6-substituted purine riboside (**17**), were designed as bidentate ligands and introduced into 2′-*O*-methyl oligoribonucleotides. Stability of the metal mediated base pair within oligonucleotide duplexes with natural nucleobases was observed to depend on the location of the pyrazolo substituent and on the ability of the base to participate in the metal-mediated base pairs as an anionic ligand. 2-(3,5-Dimethylpyrazol-1-yl)inosine exerted the greatest stabilizing effect in the presence of Cu^{II} or Zn^{II} .

Glycerol nucleic acid oligomers incorporating (*S*)-4-(2,3-dihydroxypropoxy)pyridine-2,6-dicarboxamide (**24**) as a Pd^{II}-binding unit either in terminal (**ON1**) or nonterminal position (**ON2**) were synthesized. Extraordinary increase in stability was obtained only for **ON1.** High

melting temperatures could not be reproduced by simply adding Pd^{II} into a solution of **ON1,** but only by studying the thermal stability of pre-fabricated dimer (**ON1**)$_2Pd^{II}_2$. Most likely the desired duplex consisting of four interchain CG pairs in addition to terminal **24**:Pd^{II}:T pairs was not formed as the major species under the typical conditions of hybridization studies, possibly due to the relatively slow ligand exchange reactions of Pd^{II}. Evidently, the Pd^{II}-mediated base pairs may be highly stabilizing at a terminal position even if they are not accommodated in the base stack of oligonucleotide duplex. By contrast, studies with **ON2** showed that similar stabilization cannot be achieved by an identical intrachain modification. Possibly, formation of a Pd^{II}-mediated base pair would too severely distort the normal W-C double helical structure.

5 EXPERIMENTAL

5.1 General methods

The detailed synthetic methods of the compounds used in the studies can be found in the original publications. The structures of nucleosides and Pd^{II} binary complexes were characterized by ESI-MS, ^{1}H NMR, ^{13}C NMR and ^{31}P NMR spectrometry. COSY, HSQC and HMBC correlation analyses were applied for assignment of the ^{1}H and ^{13}C signals. The ^{1}H NMR spectra were recorded at 400 or 500 MHz frequency and the chemical shifts are given in ppm. All oligonucleotides were prepared by conventional phosphoramidite method on an Applied Biosystems 3400 DNA/RNA synthesizer and analyzed by HPLC and ESI-MS.

5.2 Melting temperature studies

The UV melting curves (absorbance versus temperature) were measured at 260 nm on a PerkinElmer Lambda 35 UV-vis spectrophotometer equipped with a Peltier temperature controller. The temperature was changed at a rate of 0.5 °C min^{-1} from 10 to 90 °C. The measurements were carried out at pH 7.4 in 20 mM cacodylate buffer with ionic strength of 0.10 M, adjusted with $NaClO_4$. For all measurements the concentration of the oligonucleotides and Cu^{II}, Zn^{II} or Pd^{II} was 3 μM. The T_m values were determined as the maxima of the first derivative of the melting curves.

5.3 CD measurements

The CD spectra were acquired by an Applied Photophysics Chirascan Spectropolarimeter. The temperature range was from 6 to 94°C and scan range was between 220 and 320 nm.

6 REFERENCES

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