The synthesis and analysis of higher ordered structure forming purine analogues

Doctoral thesis

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1. Introduction

Higher-ordered structures, based on self-recognition and self assembly of simpler molecules, are of potential interest in a variety of fields ranging from structural biology, to medicinal chemistry, supramolecular chemistry, nanotechnology and 20 molecular-scale devices. The G-quartet, a hydrogen-bonded macrocycle formed by cation-templated assembly of guanosine, was first identified in 1962 as the basis for the aggregation of 5'-guanosine monophosphate. In 1990 Guschlbauer, Chantot, and Thiele published the review article "Four-Stranded Nucleic Acid Structures 25 Years Later: From Guanosine Gels to Telomer DNA." In that paper they pointed out the emerging importance of the G-quartet, a hydrogen-bonded ionophore first identified in 1962. Renewed attention to the G-quartet in the late 1980s was generated by intriguing proposals that the motif, when formed in DNA, might be biologically relevant. Today, interest in G-quartet structures remains unabated. Thousands of reports on some aspect of G-quartet structure or function have since appeared, including some excellent reviews. Since the discovery of the G tetrad, several other quadruplex structures have been reported, based on experimental results or theoretical considerations. These new structures cover a wide range of changes from small modification in the guanine base to the complete substitution of the whole monomeric building block. It was also shown that in most of the cases cations play a stabilizing role in the formation of quadruplex strands by intercalating into the stacking tetrads. In these situations, quadruplex structures were always neutral, and according to our knowledge an intercalating anion has been reported only in one case. Charged quadruplexes can be important not just because of the intercalation of ions but they also provide new possibilities in the design of novel higher ordered structures (e.g. nano-wires).

It was also pointed out that the G quartet is one of the most suitable structures for stacking since guanine quartets are strongly bound (there are two H-bonds between neighbouring molecules) and already posses a planar equilibrium structure. Other purine-based tetramers (e.g. adenine or inosine) have also been investigated, however these structures have been found less stable and they tend to adopt non-planar geometries. This non-planarity is understandable since the nucleobases in these tetramers are connected to each other by only one H-bond which yields a flexible structure.

Three decades after its identification in 5'-GMP gels, the G-quartet really gained visibility because of the biological implications of G-quadruplex DNA. Consequently, structural characterization of nucleic acid G-quadruplexes has accelerated over the past

decade. In 1992 the Protein Data Bank showed just two entries for G-quadruplexes. Ten years later, there are more than 35 deposited G-quadruplex structures, including 9 crystal structures. This data provides a wealth of information about the noncovalent interactions that drive self-assembly of G-rich nucleic acids.

2. Aims

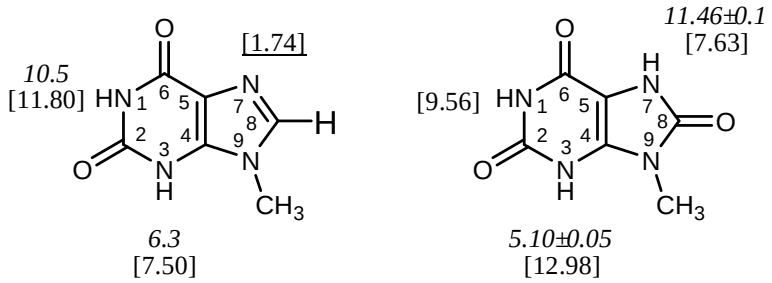
Xanthine derivatives play a decisive role in a variety of intracellular metabolic pathways as substrates and/or intermediates of numerous enzymes or enzyme systems. To date no study has been devoted to investigate the properties of 9- and 3-substituted xanthine derivatives in higher ordered structures. Theorethical molecular modelling and density functional theory (DFT) calculations showed that 3-methylxanthine has the ability to form higher ordered structures with extra hidrogen bonds in it due to the 7*H*-tautomeric properties (similarly to guanine H-bond forming properties), and increasing the stability of the formed assembly. To examine the predicted interactions and self assembly properties, our aim was to synthesize 3-methylxanthine for MS and NMR characteritation.

Based on the promising theoretical molecular modelling calculation results of 3-methyl xanthine, we decided to synthesize new 3-substituted xanthine containing DNA and PNA monomers and their oligomers. Furthermore our aim was the MS and NMR characterization of the prepared 3-substituted xanthine containing DNA and PNA oligomers to examine the predicted interactions and the formation of higher ordered structures.

3. Results and discussion

In the present thesis, we point out that if one takes xanthine instead of hypoxanthine (which was used previously to model inosine nucleoside), two H-bonds can be formed between neighbouring xanthines by low-barrier hydrogen bond (LBHB). Moreover, naturally occurring uric acid can be also involved in tetramer formation with or without xanthine.

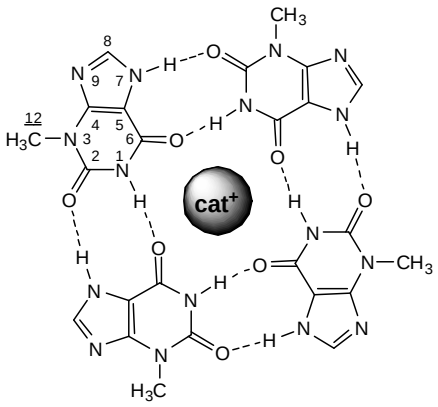
Consequently, the system will have an additional positive charge and this provides a very strong interaction. Xanthine and uric acid are naturally occurring purines. In the present work, methylxanthine and 9-methyluric acid (Scheme. 1) were considered to simulate a possible connection point to any backbone chain (e.g. sugar-phosphate 10 chain or PNA). However, the presence of this backbone chain is not necessary for self-assembly. Moreover, the N(7) protonated form of Xa (XaH^+) will have an important role in our systems.



Xa **Ua**

Scheme 1. 9-Methylxanthine (Xa) and 9-methyluric acid (Ua) with pKa values. Experimental pKa data are shown in *italics*, and pKa value for protonated Xa with underlined characters

Furthermore, we propose 3-substituted xanthines as potential tetrad and quadruplex forming purine derivatives. The dominant 7*H* tautomeric form of a 3-substituted xanthine can bind to each neighboring xanthine moiety with two H-bonds in a tetrad, similarly to guanine. Therefore, we have anticipated that these assemblies should yield strong interactions and a planar geometry in gas phase. As the simplest model, 3-methylxanthine (3MX, Scheme. 2) was chosen for our studies with or without additional cation. We examine herewith the tetrad and octad forming capacity of a 3-substituted xanthine starting from theoretical studies prior to experimental realization and detection.



Scheme 2. Tetrads composed of 7*H*-3-methylxanthine (3MX) and different cations [cat^+ = none, Na^+, K^+, NH_4^+;

3.1. Computational studies

Herein, based on theoretical considerations we propose new quartet systems possessing positive or zero charge, which are composed of xanthine (9-methylxanthine, 3-methylxanthine) and uric acid derivatives. According to our expectation, these systems are

capable to bind anions naturally. Moreover, since the monomers in the tetrad are connected through two H-bonds, they can form strongly bonded, planar structures. To our knowledge, this is the first example of a quartet in which the positive charge is supported by the tetramer structure itself, and not by an additional intercalating ion. This opens new possibilities in the design of novel nanostructures.

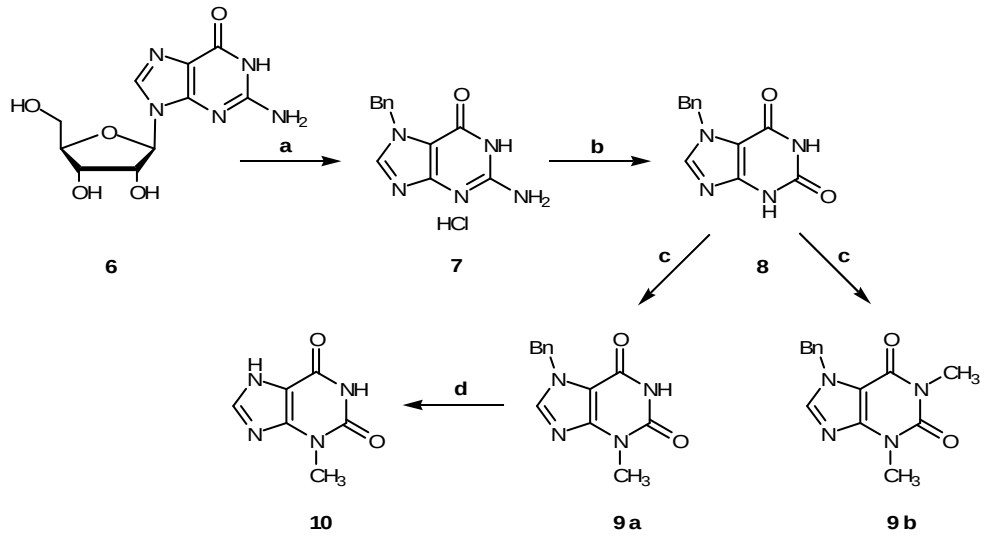
First, computational investigations have been performed for 3MX, as the simplest representative of the family of 3-substituted xanthines, at BLYP-D/TZ2P level of dispersion-corrected Density Functional Theory with ADF and QUILD programs. The calculated hydrogen bond energy of the four 3MX monomer in the tetrad amounted to -66.1 kcal/mol.

cation	cluster	$E_{iinteraction}$	$E_{deformation}$	$E_{binding}$
Na^+	tetrad + cation	-100.30	3.69	-96.61
	octad + cation	-135.78	10.56	-125.22
K^+	tetrad + cation	-73.09	2.75	-70.34
	octad + cation	-106.27	4.56	-101.71
NH_4^+	tetrad + cation	-75.58	3.75	-71.83
	octad + cation	-103.20	4.39	-98.81
none	four monomers	-73.62	7.52	-66.10
	$tetrad_1$ + $tetrad_2$	-51.84	4.80	-47.04

Table 1. Components of ion binding energies (columns, in kcal/mol) of 3-methylxanthine tetrads and octads (Na^+, K^+, NH_4^+ rows); the components of formation energy of the tetrad and the stacking energy of two tetrads are given in the 'none' rows.

The optimized tetrad structures have been found to be bent even in the ion-free case but cations show similar behavior to guanine tetrads. Namely, sodium ion tends to stay in the plane of the tetrad while potassium and ammonium ions occupy an out-of-plane position. Regarding the octads, the most planar structure was found with the sodium ion, and the structure without any intercalating ion has the most bent optimum. In all cases the $(3MX)_4$ complex turned to be more planar in the octad structure. Cations have been found close to the midpoint between the layers and the rotation of the two layers in octads is ca. 17° in each aggregate. The distances between the layers have also been found to be very similar in each complex, thus neither the metallic ions nor the NH_4^+ affect the optimum stacking distance. Together with the theoretical results for tetrads, this prompted us to investigate the existence of $(3MX)_4$ and $(3MX)_8$ structures with or without intercalating cations experimentally.

3.2. The synthesis of 3-methylxanthine



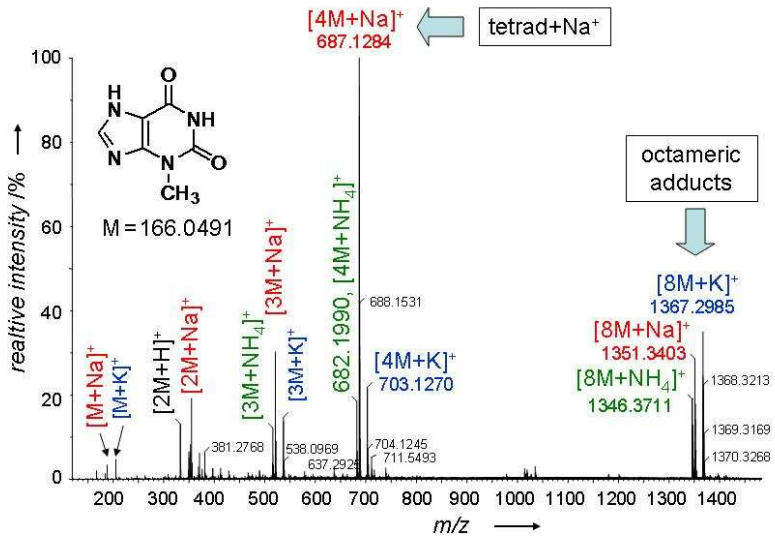
Scheme 3. The synthesis of 3-methylxanthine, reaction conditions: **a**. DMSO, benzyl-bromide, 36% HCl, 24 h, (70-99%), **b**. AcOH, $NaNO_3$, 55 °C, 12 h, (50-90%), **c**. anhydrous DMF, K_2CO_3, CH_3I, 50 °C, (16%), [NaOH, H_2O, CH_3I, rt., (32%)], **d**. AcOH, H-cube®, 75 bar, 90 °C, (46-90%)

The synthetic efforts towards 3-substituted xanthines started with 7-benzylxanthine (8) available in a two steps from guanosine according to the method by Bridson *et al*. Methylation of the most acidic NH group in the presence of K_2CO_3 resulted in the formation of 3-methyl (**9a**) and 1,3-dimethyl (**9b**) derivatives. The debenzylation of the former was rather challenging. The usual conditions of hydrogenation failed to give the product (**10**). The application of Pearlman's catalyst [$Pd(OH)_2$/C] in a far larger than catalytic amount at high pressure and elevated temperature eventually furnished 3-methylxanthine (**10**). Alternatively, this deprotection can be achieved in an H-Cube® flow reactor as well under milder conditions and with smaller amount of catalyst.

3.3. Mass spectrometric results

The tetrad formation of 3MX was first examined experimentally by mass spectrometry using a Q-TOF instrument equipped with a nano-ESI ion source. Although only NH_4^+ was intentionally added as an adduct forming ion in aq. methanol, the most intense peak series in the mass spectrum contains Na^+, possibly due to an ion-exchange on the surface of the borosilicate capillary. This is in good accordance with the calculations, where the sodium ion is bound most strongly to the tetrad. Interestingly, the peak intensities

increased along the series of adduct ions of $[3MX+Na]^+$, $[(3MX)_2+Na]^+$, $[(3MX)_3+Na]^+$, and $[(3MX)_4+Na]^+$, in contrast to the "normal" case for compounds not forming tetrads. Furthermore, beyond the $[(3MX)_4+Na]^+$ peak, the next most intense peak series in the upper mass region belongs to the $[(3MX)_8+cat]^+$ peaks suggesting an increased stability of the tetrad constructed by four 3MX molecules.



Scheme 4. Nano-ESI-Q-TOF MS spectrum of 3-methylxanthine (3MX).

3.4. Multinuclear NMR studies

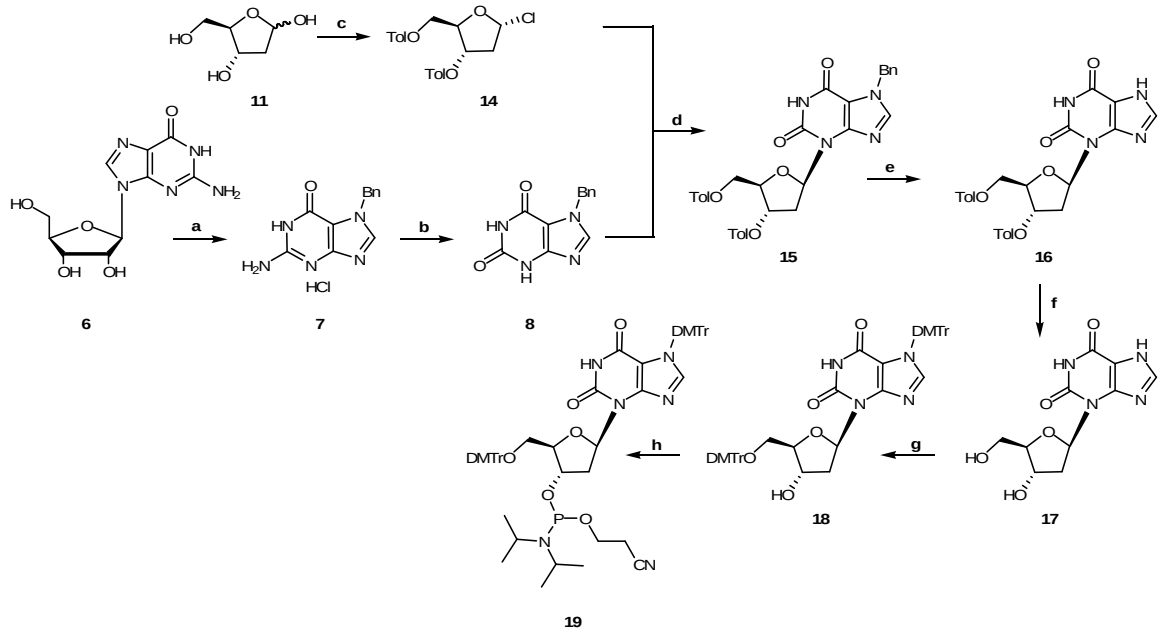
Full ^1H, ^{13}C and ^{15}N NMR assignment of 3MX has been accomplished using standard HMBC techniques in DMSO-d_6 solution (~ 5 mg/500 μL) at 300 K (Tables 3 and 4). These data support the tautomeric form as shown in Fig. 1. Since the MS study suggests $(3MX)_n \bullet cat^+$ aggregates (n = 4, 8; $cat^+ = NH_4^+$, Na^+, K^+), several additional NMR experiments were carried out to disclose these features. Diffusion ordered spectroscopy (DOSY) was used to determine the apparent molecular mass of the aggregates in solution. As an internal mass reference, tetramethylsilane was used because of its inertness and spherical structure. As apparent MW, 920 Da was obtained in DMSO-d_6 solution at 300 K. For a $(3MX)_4$ aggregate MW = 664 Da is expected and the difference between the experimental and theoretical values may be attributed to solvent molecules associated to the clusters of 3MX. To observe the intermolecular H-bonding in

the self-assemblies we measured the deuteration isotope shifts on the acceptor carbonyls but this was not indicative because of the concurrent two- and three-bond intramolecular effects. In selective transient 1D NOESY experiments we observed significant magnetization transfer between N7H and water due to exchange while in the case of N1H this transfer was negligible. Consequently, we suppose that the „internal" H-bonds (N1···HO6) in the $(3MX)_4$ structures must be stronger than the „external" H-bonds (N7H···O2). This was also the case for the gas phase theoretical results according to which the „internal" H-bonds have always been shorter than the „external" ones. In summary, the DOSY experiments seem to support the presence of $(3MX)_4 \bullet cat^+$ and possibly of $(3MX)_8 \bullet cat^+$ (cat^+ = none or K^+) clusters in DMSO-d_6 solution. Clearly, to achieve stronger interactions in tetrads/quadruplexes composed of 3-substituted xanthines, substituents in position 3 other than methyl should be present to allow for further stabilization.

3.5. The synthesis of 3-substituted xanthine/thymine containing DNA and PNA monomers and their oligomers

Based on the promising theoretical molecular modelling calculation results of 3-methyl xanthine, we decided to synthesize new 3-substituted xanthine containing DNA and PNA monomers and their oligomers. For the synthesis of 3-substituted xanthine containing DNA monomer the phosphoramidite method, and for the preparation of 3-substituted xanthine containing PNA monomers the Fmoc-amino protection strategy were chosen.

3.5.1. The synthesis of 3-substituted xanthine containing DNA monomer and oligomers



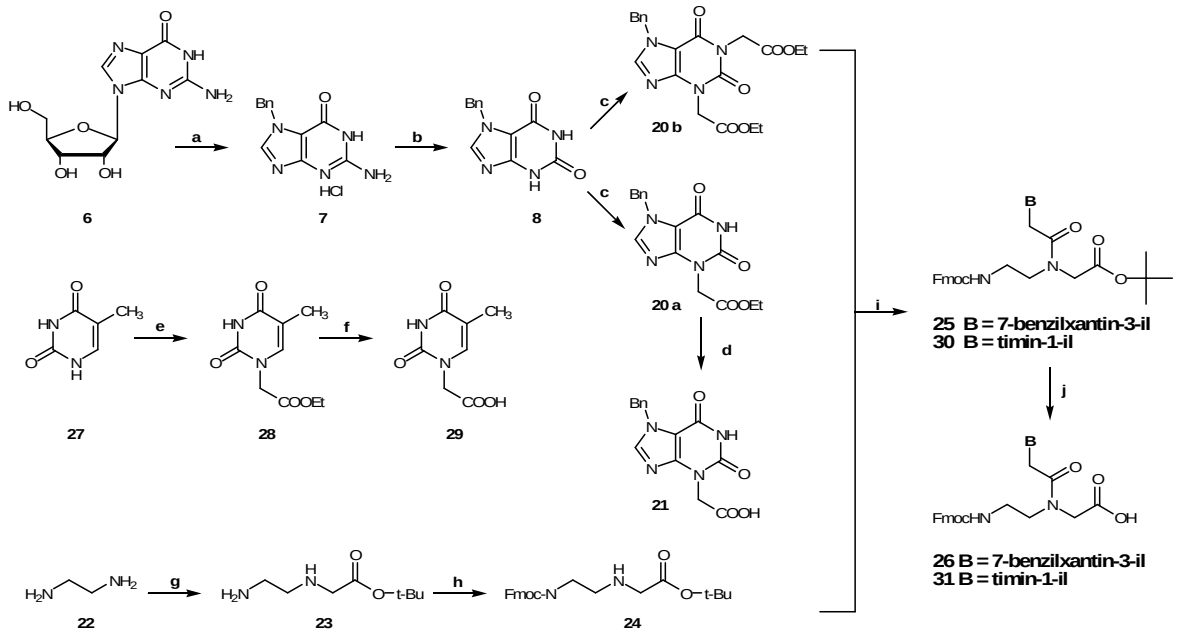
Scheme 5. The synthesis of 3-substituted xanthine containing DNA monomer, reaction conditions: **a**. DMSO, benzyl-bromide, 36% HCl, 24 h, (70-99%), **b**. AcOH, H_2O, $NaNO_3$, 55 °C, 12 h, (50-90%), **c**. 1. MeOH, 1% HCl/MeOH, Ag_2CO_3, 30 min., 2. pyridine, *p*-toluoyl-chloride, 12 h, AcOH, sat. HCl/AcOH, HCl gas, 30 min., **d**. anhydrous dioxane, 55% NaH, Ar atm., 45 °C, 2 h, (26%), **e**. anhydrous dioxane, hydrogen reactor, $Pd(OH)_2$, 100 bar, 90 °C, 24 h, (98%), **f**. 40% $MeNH_2/H_2O$, MeOH, 45 °C, 12 h, (94%), **g**. 4,4'-DMTr-Cl, TEA, pyridine, 3 h, (34%), **h**. 2-cyanoethyl-*N*,*N*,*N*',*N*'-tetraisopropylphosphordiamidite, anhydrous DCM, 1H-tetrazole, Ar atm., 4 h, (93%).

The synthesis of 3-glycosylated xanthine **15** was accomplished according to the sodium salt glycosylation method from 7-benzylxanthine (**8**) and 2-deoxy-3,5-di-*O*-*p*-toluoyl-b-D-ribofuranosyl chloride **14** in dioxane that was superior to acetonitrile (Scheme 5). High-pressure debenzylation was conducted as above to afford glycoside **16** and deacylated to yield nucleoside **17**. Excess 4,4'-dimethoxytrityl chloride under basic conditions gave the 7-*N*,5'-*O*-diprotected derivative **18**. Phosphitylation with 2-cyanoethoxy-bis(*N*,*N*-diisopropylamino)-phosphine eventually afforded the protected phosphoramidite **19**. The introduction of 4,4'-dimethoxytrityl group on N7 allowed the protection of the reactive NH that would interfere with the oligonucleotide synthesis.

The DNA phosphoramidite monomer was applied in preliminary studies to synthesize oligomers incorporating the above xanthine derivative. To this end the sequences T_3XT_2 and TX_4T have been prepared using standard protocols of phosphoramidite chemistry on an Expedite 8909 synthesizer (X denotes the monomer derived from phosphoramidite 10). The

analytical (MS, NMR) analysis of 3-substituted xanthine containing DNA oligomers are in progress.

3.5.2. The synthesis of 3-substituted xanthine/thymine containing PNA monomers and oligomers



Scheme 6. The synthesis of 3-substituted xanthine/thymine containing PNA monomers, reaction conditions: **a**. DMSO, benzyl-bromide, 36% HCl, 24 h, (70-99%), **b**. AcOH, H_2O, $NaNO_2$, 55 °C, 12 h, (50-90%), **c**. anhydrous DMF, K_2CO_3, ethyl bromoacetate, 45 °C, 12 h, (34%), **d**. 5% HCl, THF, 60-80 °C, 24 h, (95%), **e**, **f**. anhydrous. DMF, K_2CO_3, ethyl bromoacetate, rt., 4 h, 2 M NaOH, 90 °C, 20 min, 4 M HCl, rt., 30 perc, (70%), **g**. DCM, *tert*-buthyl bromoacetate, 0 °C, 24 h (66%), **h**. DCM, DIPEA, Fmoc-Osu, rt., 12 h (88%), **i**. anhydrous DMF, DCM, DIPEA, HOBT, HBTU, rt., 48 h, (41%), **j**. DCM, TFA, H_2O, rt., 6 h, (51%).

7-Benzylxanthin-3-ylacetic acid (**21**) was also obtained from compound (**8**) according to Bridson *et al.* Coupling of Fmoc-protected PNA backbone **24** with acid **21** afforded ester **25** (Scheme 6). The acid hydrolysis of **25** resulted the formation of the unprotected 3-substituted xanthine containing PNA monomer (**26).** The synthesis strategy of thymine containing PNA monomer (31) was similar with the above mentioned. The preparation of xanthine and thymine containing PNA oligomers from compounds **26, 31** and their analytical analysis (MS, NMR) are in progress.

Publications related to the doctoral thesis

I. G. Ferenc, P. Pádár, **J. Szolomájer**, L. Kovács (2009): *N*-Alkylated guanine derivatives. *Curr. Org. Chem.*, **13**, 1085-1135, IF = 3,184 (2008).

II. G. Ferenc, P. Pádár, **J. Szolomájer**, N. M. Howarth, L. Kovács (2011): Transition metal ion complexes of *N*-alkylguanines. *Curr. Org. Chem.*, **14,** IF = 3,184 (2008), (accepted).

III. G. Paragi, L. Kovács, Z. Kupihár, **J. Szolomájer**, B. Penke, C. Fonseca Guerra, F. M. Bickelhaupt (2010): Neutral or positively charged new purine base tetramer structures: a computational study of xanthine and uric acid derivatives. *New J. Chem.*, **34**, IF = 3,006 (2009), (in press), DOI: 10.1039/c0nj00613k.

IV. **J. Szolomájer**, G. Paragi, G. Batta, C. Fonseca Guerra, F. M. Bickelhaupt, Z. Kele, P. Pádár, Z. Kupihár and L. Kovács (2010): 3-Substituted xanthines as promising candidates for quadruplex formation: computational, synthetic and analytical studies. *New J. Chem.*, **34,** IF = 3,006 (2009), (submitted).

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Posters and presentations

1. J. Szolomájer (2006): Xantin-tartalmú nukleinsavanalógok elöállítása. *29. Kémiai Elöadói Napok. Szerves, Gyógyszer- és Biokémiai Szimpózium*. (Szeged, okt. 30-31, 2006), (presentation).
2. P. Pádár, J. Szolomájer, G. Ferenc, Z. Kupihár, E. Szájli, L. Kovács (2006): Preparation and examination of xanthine-containing DNA and PNA oligomers. *17th International Round Table Nucleosides, Nucleotides and Nucleic Acids* , PO203. (Bern, September 3-7, 2006), (poster).
3. J. Szolomájer, P. Pádár, G. Ferenc, Z. Kupihár, E. Szájli, L. Kovács (2008): Xantintartalmú DNS és PNS monomerek és oligomerek előállítása és karakterizálása. *14th International Conference on Chemistry* , 316-320. (Kolozsvár/Cluj, nov. 13-15, 2008), (poster).
4. Paragi Gábor, Kovács Lajos, Kupihár Zoltán, Penke Botond, Celia Fonseca Guerra, F. Matthias Bickelhaupt: Neutral or positively charged new purine base tetramer structures: a computational study of xanthine and uric acid derivatives. *Symposium on Nucleic Acid Chemisty, Structure and Interaction*, Slovenian NMR Centre, Nova Gorica, Slovenia, May 29-31, 2010, p. 61, (poster).
5. Szolomájer János, Paragi Gábor, Kele Zoltán, Pádár Petra, Kupihár Zoltán, Kovács Lajos: 3-Substituted xanthines as promising candidates for tetrad formation: synthetic, analytical and computational studies. *Symposium on Nucleic Acid Chemisty, Structure and Interaction*, Slovenian NMR Centre, Nova Gorica, Slovenia, May 29-31, 2010, p. 62, (poster).
6. Paragi Gábor, Szolomájer János, Batta Gyula, Kovács Lajos, Kele Zoltán, Kupihár Zoltán, Pádár Petra, Celia Fonseca Guerra, F. Matthias Bickelhaupt: Higher-ordered structures based on purines: towards expanding the alphabet – computational, synthetic and analytical studies. *Guanosines and quadruplexes. COST Action MP0802 Annual Meeting*, 14^{th}-17^{th} September, 2010, London, p. 11, (presentation).

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