Synthesis of monosaccharide and nucleoside conjugates of estrone derivatives

The theses of Ph. D. dissertation

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1. Introduction and aims

Steroids are widely used by living organisms for transmitting information in a chemical way. These compounds of different structures with hormonal action have a very important role in regulating the essential processes of cells, therefore any molecule involved in the production, dissimilation, modification, transport of these compounds or can bind to them can be used as potential agents in medications. Nowadays conjugation of an effective steroid with a biomolecule common in nature, *e.g.* a carbohydrate or a nucleoside unit, becomes more and more popular method for the enhancement of selectivity. This bioconjugation approach is already used by Nature; and it has been found that the biological potency of these new hybrids may exceed that of the parent compounds.

Estrogen is responsible for the development of secondary female character, plays a role in the thickening of the endometrium and in the regulation of the menstrual cycle. The oxygen atoms in the molecule are at a well-defined distance which plays an important role in the estrogen and estradiol hormone activity. Chemical modifications of estrone may lead to compounds lacking hormonal behavior which is a key aspect of the development of steroid based anticancer agents. Substitution at C-2, opening of ring D or inversion at C-13 of the estrane skeleton usually leads to the loss of estrogenic activity. We know from initial results that secosteroid derivatives and the 13α isomers are ideal for incorporating further modifications and for forming conjugates.

In my doctoral work, I planned to synthesize carbohydrate- and nucleoside-containing bioconjugates of these steroids with already established antiproliferative properties and investigate how the conjugation affects the strength of the anticancer activity and selectivity.

The purpose of our work was to synthesize estrone–monosaccharide and estron–nucleoside conjugates, in which the two components are linked in a click reaction. In the case of monosaccharides, development of the azide functional group was designed to replace the glycosidic or primary, while for nucleosides the 5'-hydroxy group. The azide derivatives produced this way were expected to react with the estrone derivatives (D-secoestrone, 13α and β-estrone) containing the propargyl functional group at positions 3 or 15, previously obtained by the Steroid Chemistry Research Group, in the presence of a copper(I) catalyst.

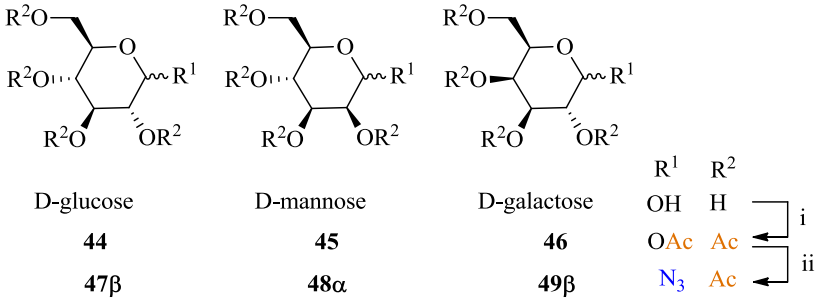
The new conjugates were planned to be evaluated *in vitro* by means of MTT assays for antiproliferative activity; and their potential inhibitory activity on human 17β-HSD1 enzyme was anticipated to be investigated.

2. Experimental methods

All solvents were distilled prior to use. Reagents and materials were obtained from commercial suppliers and were used without purification. Melting points (mp) were determined on a Kofler block and Electrothermal IA8103 apparatus and are uncorrected. The reactions were monitored by TLC on Kieselgel-G (Merck Si 254 F) layers (0.25 mm thick). The conjugates were detected by spraying with 5% phosphomolybdic acid in 50% aqueous phosphoric acid. For the identification of azides the TLC was placed in a 10% solution of triphenylphosphane in CH_2Cl_2 for 2 minutes, dried and then sprayed with ninhydrine solution (0.5% ninhydrine and 5 mM NaOH in ethanol/water 3:1 v/v). The R_f values were determined for the spots observed by illumination at 254 nm. Flash chromatography: Merck silica gel 60, 40-63 μm. NMR spectra were obtained at room temperature with a Bruker DRX 500 instrument. Mass spectra were recorded on a Finnigan MAT TSQ 7000 instrument equipped with an electrospray ion source.

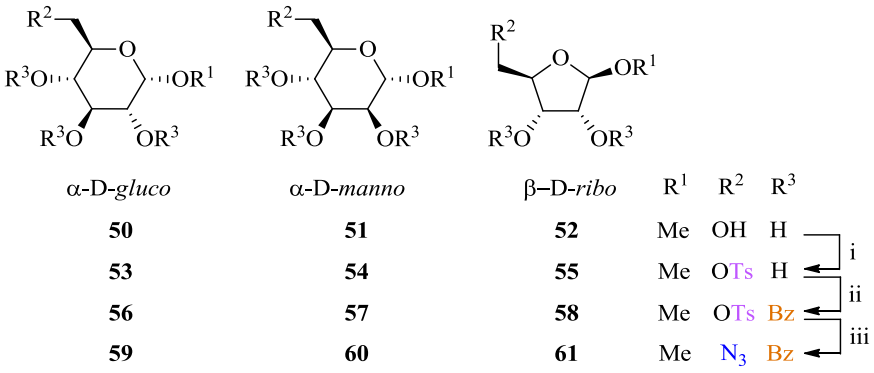
3. Summary of the results*

3.1. The glycosidic *O*-acetyl groups (**44**–**46,** Scheme 1) were replaced with the azide group using tin tetrachloride as a Lewis acid catalyst and trimethylsilyl azide as the source of the nucleophilic azide ion to afford compounds **47**–**49**. For D-glucose and D-galactose the β-anomer, for D-mannose the α-anomer formed in 72–79% yields, respectively.



Scheme 1. Synthesis of protected glycosyl azides (**47**–**49**). Reagents: (i) Ac_2O, NaOAc; (ii) $SnCl_4$, Me_3SiN_3, CH_2Cl_2.

3.2. In order to introduce the azide group to positions 6 (hexopyranoses) and 5 (pentofuranose), first the methyl glycosides of D-glucose, D-mannose and D-ribose (**50**–**52,** Scheme 2) were selectively tosylated in pyridine on their primary hydroxy groups without the protection of the secondary hydroxy groups. Unfortunately, the direct replacement of the tosyl group with azide in compounds **53**–**55** was not successful probably due to solubility reasons, therefore the secondary hydroxy groups were benzoylated first and then a tosyl–azide exchange has successfully occurred in all the fully protected monosaccharides (**56**–**58**) and resulted in the fully protected 6-azido-6-deoxy- and 5-azido-5-deoxymonosaccharides (**59**–**61**), respectively.

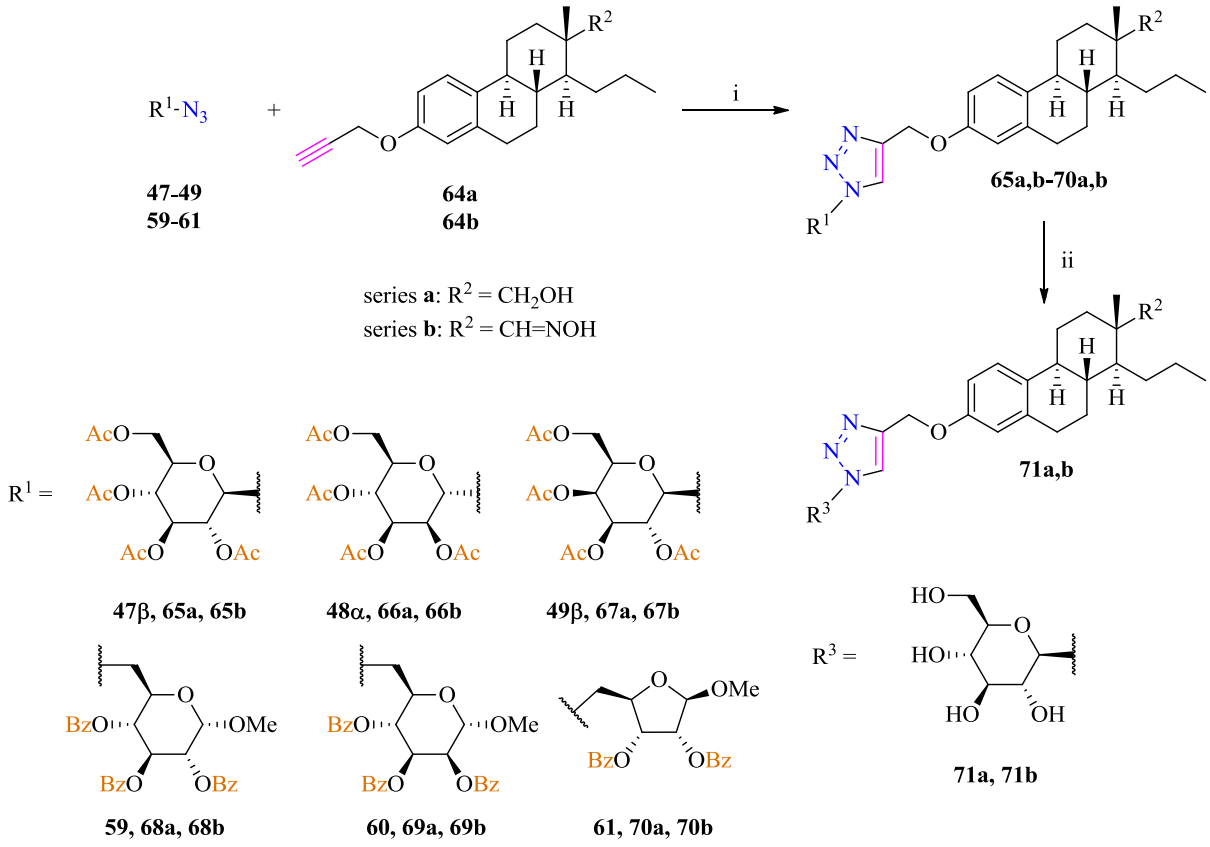


Scheme 2. Synthesis of 6-azido-6-deoxy-D-hexopyranose (**59, 60**) and 5-azido-5-deoxy-D-ribofuranose (**61**) derivatives. Reagents: (i) TsCl, pyridine; (ii) BzCl, pyridine; (iii) NaN_3, LiBr, DMF.

*Numbering of compounds is the same as the one applied in the thesis.

3.3. The azide group-containing monosaccharides (**47–49** and **59–61**) were coupled to the alkyne-containing D-secoalcohol (**64a**) and D-secooxime (**64b**) applying copper catalyzed alkyne-azide click conditions (CuAAC) as follows: copper(I) iodide, triphenylphosphane and DIPEA as a base with a slight access of propargyl-D-secosteroid in toluene at boiling temperature until TLC showed quantitative conversions (Scheme 3).

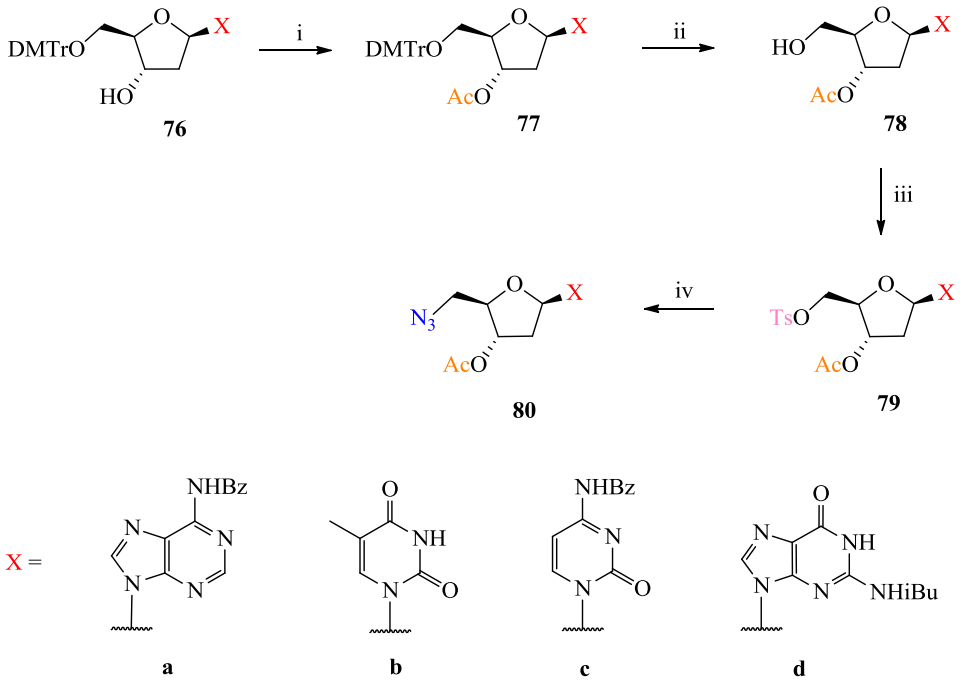
3.4. In case of two glucose-containing bioconjugates (**65a,b**), which showed the best biological activities, the acetyl protecting groups were removed by Zemplén's method using sodium methylate in methanol to obtain their unprotected derivatives (**71a,b,** Scheme 3).



Scheme 3. The synthesis of monosaccharide-D-secoestrone bioconjugates (**65–71**). Reagents: (i) CuI, $(Ph)_3P$, DIPEA, toluene, reflux; (ii) NaOMe, MeOH.

3.5. For the preparation of 5'-azido-2',5'-dideoxynucleosides (**80a–c**, Scheme 4), first we have followed the tosyl–azide replacement method based on the literature, but the isolated yields were significantly lower in our hands owing to the formation of by-products compared to those described in the literature therefore we protected the 3'-hydroxy groups. We have chosen acetyl protection of the 3'-hydroxy group because it was considered to be compatible with the final, basic deprotection of the steroid–nucleoside conjugates. The acetylated 5'-*O*-DMTr-containing nucleosides (**77a–d**)

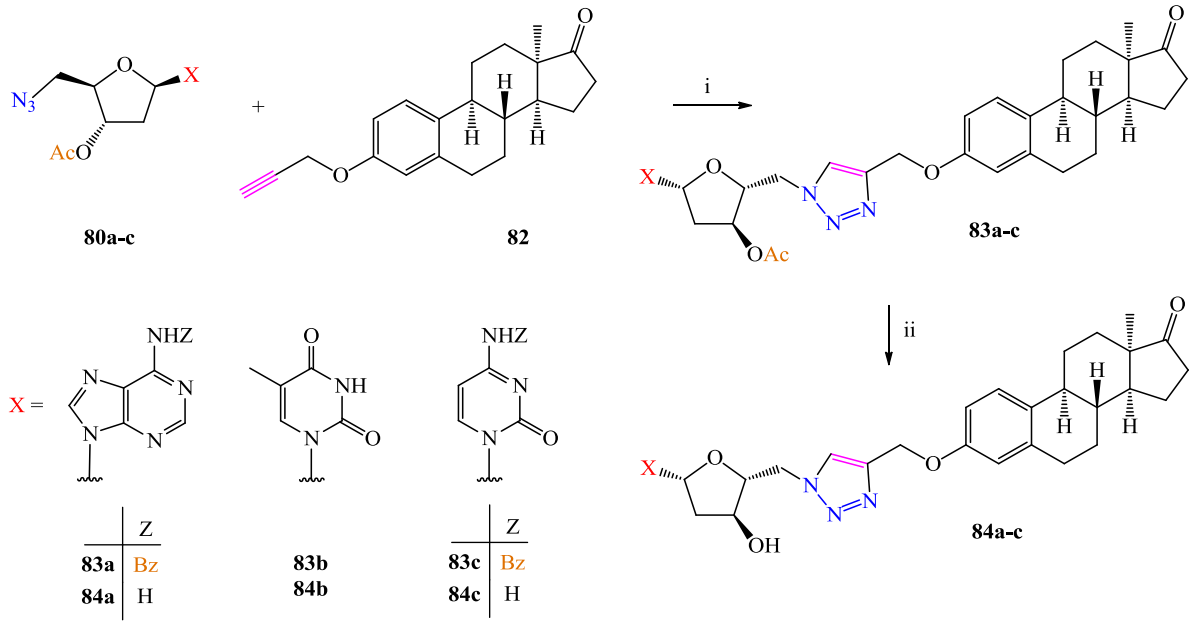
were ditritylated by the Lewis acid boron trifluoride in a 1,1,1,3,3,3-hexafluoroisopropanol–nitromethane solution. Using the 5'-OH containing, 3'-*O*-acetyl-protected nucleosides (**78a–d**) we have carried out the 5'-*O*-tosylation in pyridine at room temperature and after purification a tosyl–azide exchange reaction took place in DMF at 50 °C.



Scheme 4. Synthesis of 5'-azido-2',5'-dideoxynucleosides (**80a–c**). Reagents: (i) Ac_2O, pyridine; (ii) $BF_3{\cdot}Et_2O$, $CF_3CH(OH)CF_3$, CH_3NO_2; (iii) TsCl, pyridine; (iv) NaN_3, LiBr, DMF.

3.6. 5'-Azido-2',5'-dideoxynucleosides (**80a–c**) were connected to 3-*O*-propargyl-13α-estrone (**82**) in a CuAAC reaction (Scheme 5). The solvent of the click reaction was toluene (thymidine and adenosine) or anhydrous tetrahydrofuran (cytidine) due to solubility problems. Initially, we have followed the commonly used literature methods, using copper(I) iodide catalyst, triphenylphosphane and DIPEA but the reaction did not proceed well even if higher temperature, different solvents and prolonged reaction times (3 days) were applied. The highest reaction temperature was limited to as high as 50 °C, to avoid the potential side-reactions in the nucleoside moieties. We supposed that the reason of the very low yields (<30% according to TLC monitoring) could be the high complex-forming affinity of the *N*-acyl-protected nucleosides which trapped the Cu(I) ion catalyst. Therefore we increased the amount of the Cu(I) catalyst and DIPEA to 1.5 equivalent and eliminated triphenylphosphane from the reaction mixture. With this modified method all conjugation reactions were complete in one day at 50 °C according to TLC and the final isolated yields of protected conjugates (**83a–c**) were acceptable.

3.7. Aqueous ammonia is commonly used for the deprotection of these protecting groups in the nucleic acid chemistry but the protected conjugates were not soluble in the aqueous media therefore this deprotection method failed. We have tested the Zemplén deacetylation protocol using 0.1 M of sodium methylate in methanol but only the acetyl group was removed from the 3'-hydroxy group. Finally, 4 M ammonia solution in methanol was used at 50 °C which removed all the acyl protecting groups of the conjugates (**83a–c**) in 16 hours to yield the conjugates (**84a–c,** Scheme 5).



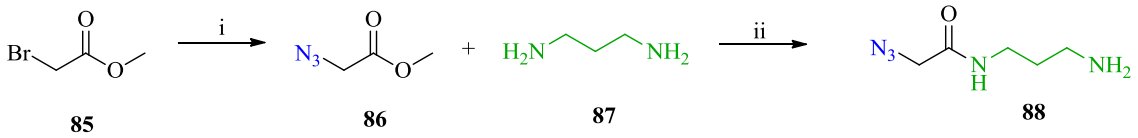
Scheme 5. CuAAC conjugation reaction of 3'-*O*-acetyl-5'-azido-2',5'-dideoxynucleosides (**80a–c**) and 3-*O*-propargyl-13α-estrone (**82**). Reagents: (i) CuI, DIPEA, toulene or THF; (ii) NH_3/MeOH.

3.8. The antiproliferative properties of the new conjugates (**65a,b–71a,b** and **83a–c** and **84a–c**) were characterized *in vitro* on a panel of human adherent cancer cell lines (HeLa, A2780 and MCF-7) by means of MTT assays at the Department of Pharmacodynamics and Biopharmacy, University of Szeged. In the case of monosaccharides conjugates, the most potent compounds (**65b, 66b** and **67b**) exhibited remarkable activities with IC_{50} values in the range 5.3–20.5 μM, exerting their best effects against A2780 cells. The removal of the protecting groups from the most potent (**65b**) compound resulted in a conjugate (**71b**) with decreased antiproliferative properties showing the importance of the nonpolar nature of the moiety attached to the triazole ring for the bioactivity. Comparison of the results for acetylated conjugates formed from the D-secooxime and the D-secoalcohol reveals that the presence of the oxime function generally improves the growth-inhibitory properties of the conjugates. In the case of the nucleoside-conjugates, the protected cytidine conjugate (**83c**) proved to be the most potent with IC_{50} values in the range 9.0–10.4 μM. The removal of the benzoyl and/or the acetyl protecting groups from the nucleoside–13α-estrone

conjugates (**83a–c**) resulted in the corresponding unprotected compounds (**84a–c**) with generally reduced cytostatic properties.

3.9. The newly synthesized nucleoside conjugates (**83a–c** and **84a–c**) of 13α-estrone were tested against the human 17β-HSD1 enzyme *in vitro* at the 1st Department of Internal Medicine, University of Szeged. The results show that only the unprotected thymidine conjugate (**84b**) exerted substantial inhibition, with an IC_{50} value of 19 μM. The decrease in the inhibitory potential of nucleoside-conjugates is probably caused by the steric effect of the larger nucleoside–triazolyl unit on the C-3 position compared to the benzyl derivative synthesized and tested earlier.

3.10. An azido linker molecule was engineered to be bound to the nucleosides with carbamate bonding. Methyl bromoacetate (**85**) was azidated and the methyl ester (**86**) was aminolysed with excess of 1,3-diaminopropane (**87**). Both reaction steps were quick and it was easy to purify the final product with extraction or a simple evaporation to give the linker (**88**) in high yield (Scheme 6).

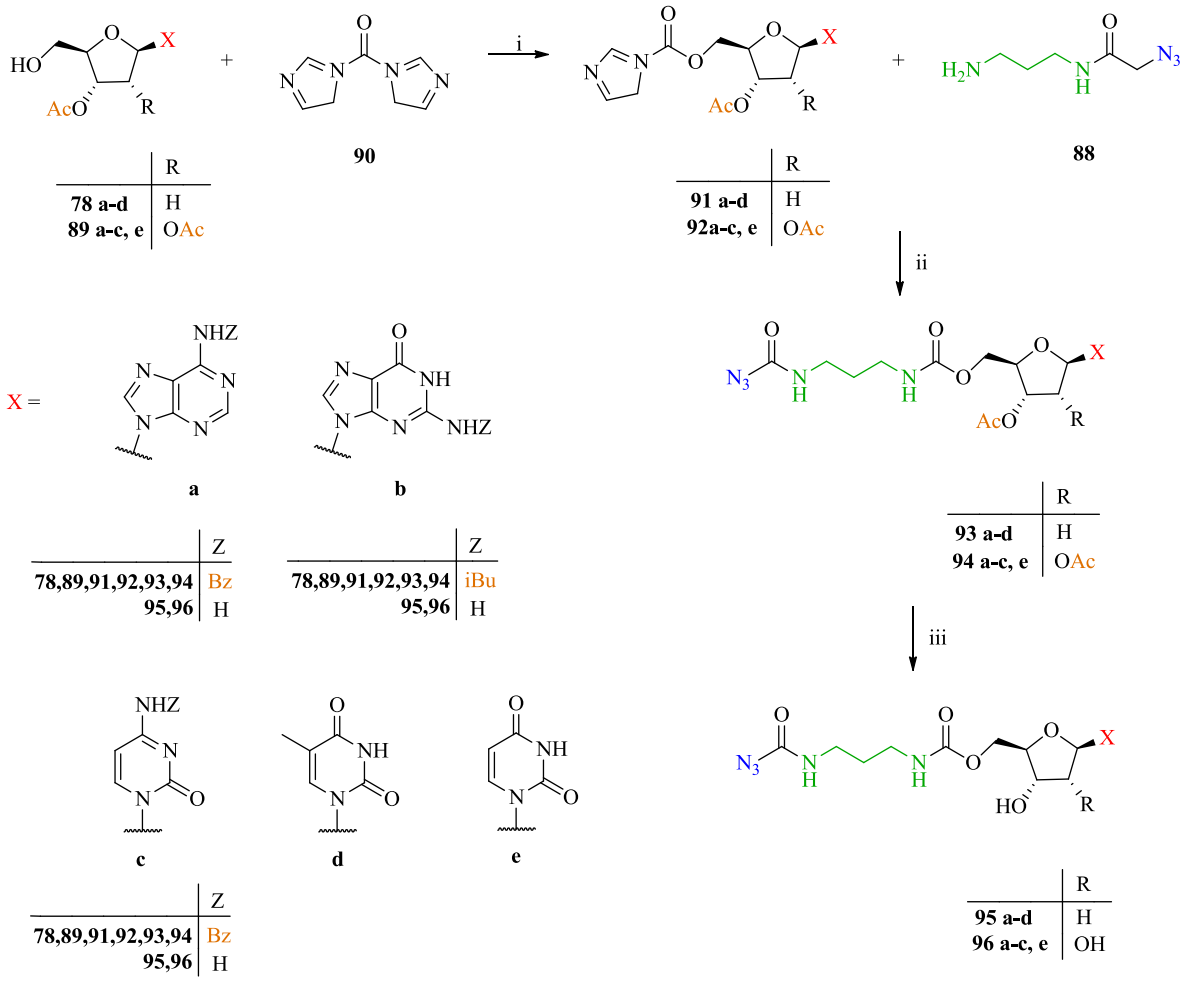


85 **86** **87** **88**

Scheme 6. Synthesis of *N*-(3-aminopropyl)-2-azidoacetamide linker (**88**). Reagents: (i) NaN_3, DMF; (ii) 1,3-diaminopropane (**87**), MeOH.

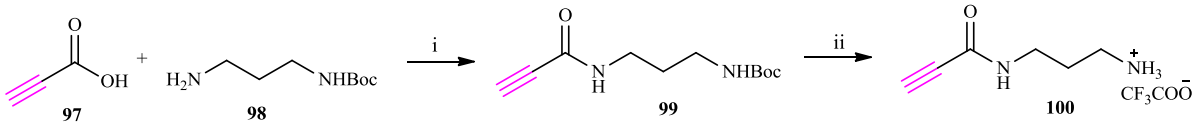
3.11. The coupling reaction of the linker to the nucleoside (Scheme 7) was effected through a urethane intermediate (**91a–d** and **92a–c,e**). First, nucleosides containing the free 5'-hydroxy group (**78a–d** and **89a–c,e**) were reacted with 1,1'-carbonyldiimidazole (**90**) and then excess azidoamine linker (**88**) was added to the reaction mixture. After stirring overnight, the reaction was quenched with an amount of sodium hydrogensulfate equivalent to the amine, evaporated, extracted, and purified by column chromatography. With this method all the eight natural azide derivatives of nucleosides (**93a–d** and **94a–c,e**) could be obtained in very good yields (76–86%) and quite easily.

3.12. 4 M ammonia solution in methanol was used at room temperature which removed all the acyl protecting groups of the protected nucleosides in 16 hours to yield unprotected nucleosides (Scheme 7, **95a–d**, **96a–c,e**).



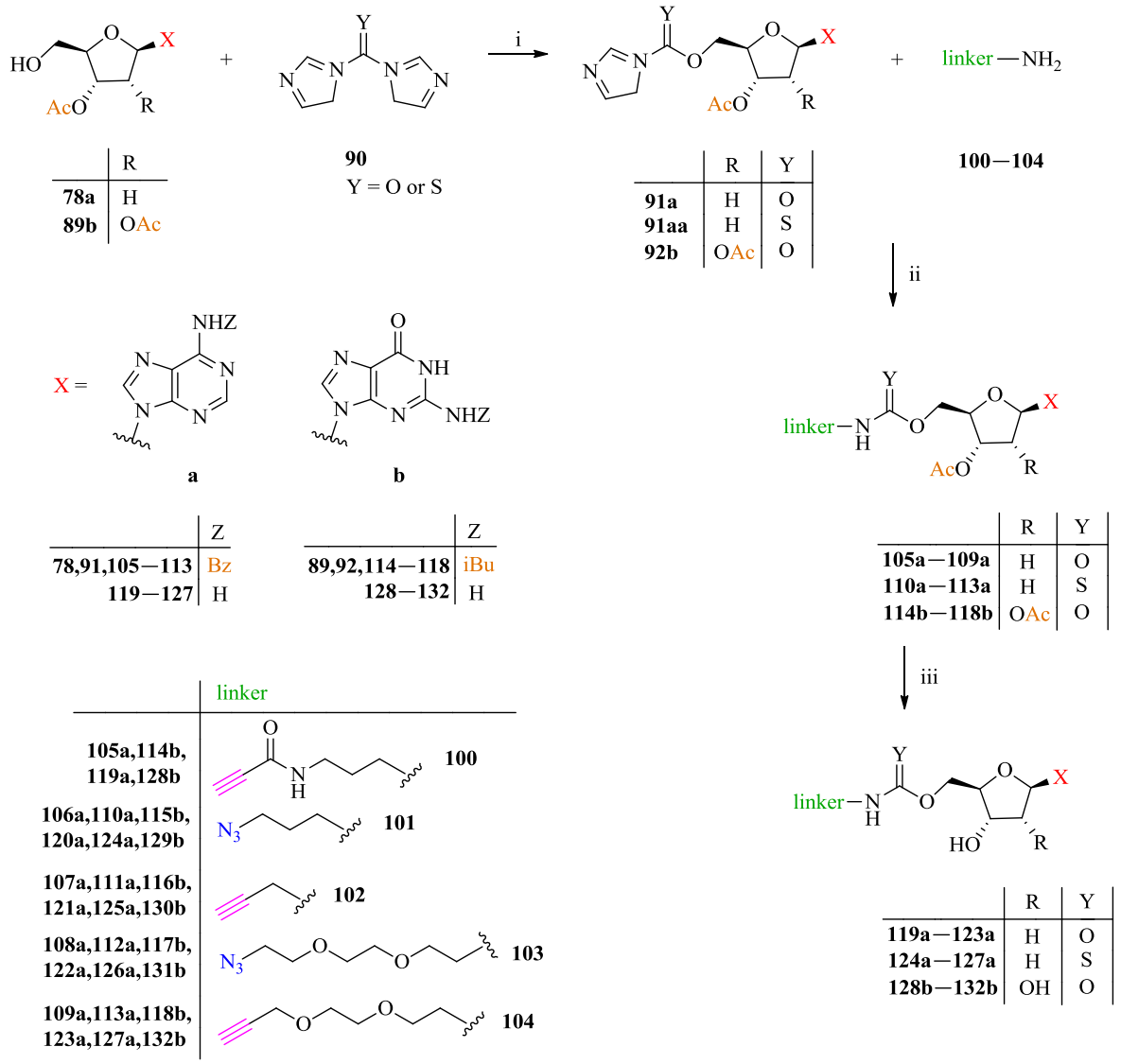
Scheme 7. Synthesis of 5'-linker-containing azidonucleosides (**93a–d, 94a–c,e and 95a–d, 96a–c,e**). Reagents: (i) CDI, DMAP, DMF; (ii) azidoamine linker (**88**); (iii) NH_3/MeOH.

3.13. We prepared a similar alkylamido linker (**100**, Scheme 8) we made previously with azide end, but here we prepared its alkyne-ending analogue from propiolic acid (**97**) by a two-step synthesis (Scheme 8). The trifluoroacetic acid salt of the desired *N*-(3-aminopropyl)-2-propinamide (**100**) linker was used for the synthesis of nucleosides by releasing its free amine with 1.5 equivalents of DIPEA from its salt after adding the linker salt directly to the reaction mixture.



Scheme 8. Synthesis of *N*-(3-aminopropyl)-2-propinamide (**100**). Reagents: (i) DCC, CH_2Cl_2; (ii) CF_3COOH, CH_2Cl_2.

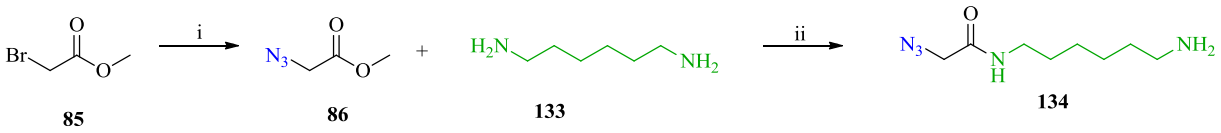
3.14. More nucleoside building blocks have been prepared according to the foregoing methods, which may be suitable for the production of nucleoside containing bioconjugates. The protected forms of the two most sensitive purine-based nucleosides (2'-deoxyadenosine **78a** and guanosine **89b**, Scheme 9) were chosen as starting materials and versatile linkers (**100–104**) were attached to the nitrogen atom of the carbamate/thiocarbamate functional group on the 5'-hydroxy: alkyl amidoalkyl, alkylene and oligoethylene glycol. These linkers were coupled to the nucleosides in the way described above (**105a–113a, 114b–118b**).



Scheme 9. Synthesis of 5'-linker-containing 2'-deoxyadenosine and guanosine derivatives (**105a–113a, 114b–118b** and **119a–127a, 128b–132b**). Reagents: (i) CDI or thiocarbonyldiimidazole (**90**), DMAP, DMF; (ii) linker (**100–104**); (iii) NH_3/MeOH.

3.15. The removal of the protecting groups was performed with 4 M ammonia solution in methanol treatment at room temperature to obtain their unprotected derivatives (**119a–127a, 128b–132b,** Scheme 9).

3.16. We prepared the *N*-(6-aminohexyl)-2-azidoacetamide linker (**134**, Scheme 10). Methyl bromoacetate (**85**) was azidated and the methyl ester (**86**) was aminolysed with excess of 1,6-diaminohexane (**133**). This linker was connected to adenosine (**89a**) as shown in Scheme 7.

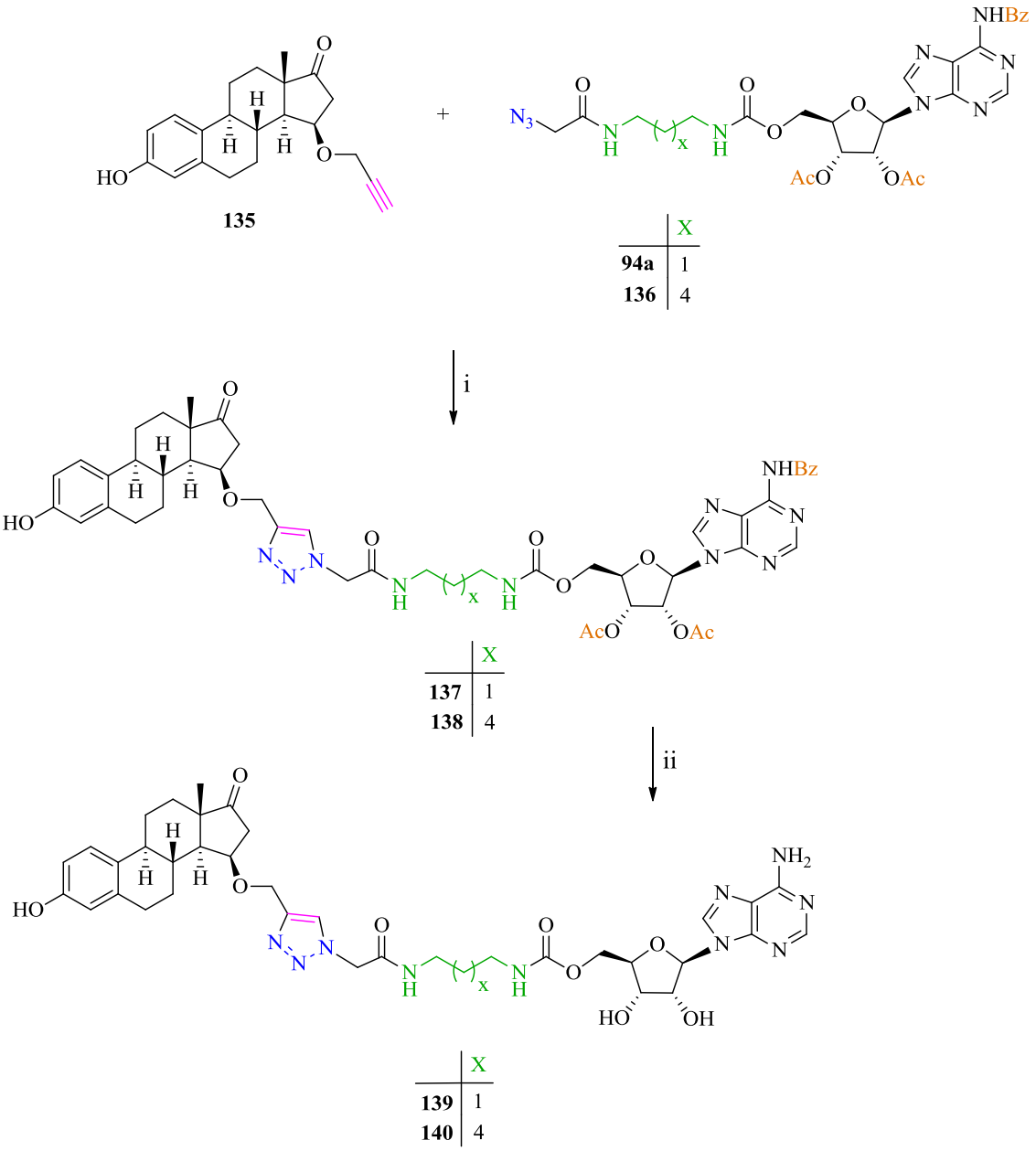


85 **86** **133** **134**

Scheme 10 Synthesis of *N*-(6-aminohexyl)-2-azidoacetamide linker (**134**). Reagents: (i) NaN_3, DMF; (ii) 1,6-diaminohexane (**133**), MeOH.

3.17. Two different length of alkylamido linker containing azido-adenosines (**94a, 136**, Scheme 11) were produced and were coupled to a 13β-estrone containing the propargyl function at position 15 (**135**) with a click reaction. The reaction was completed in abs. THF, at 50 °C overnight with 1 equiv. of copper(I) catalyst.

3.18. 4 M ammonia solution in methanol was used at room temperature which removed all the acyl protecting groups of the conjugates (**137, 138**) in 16 hours to yield conjugates (**139, 140**, Scheme 11).



Scheme 11 CuAAC between azidolinker-containing nucleosides (**94a, 136**) and 15-*O*-propargyl-13β-estrone (**135**). Reagents: (i) CuI, DIPEA, THF; (ii) NH_3/MeOH.

3.19. During my doctoral work, I prepared 81 newly synthesized compounds.

4. Publications related to the thesis:

1. **Bodnár B.,** Mernyák E., Wölfling J., Schneider G., Herman B. E., Szécsi M., Sinka I., Zupkó I., Kupihár Z. and Kovács L. Synthesis and biological evaluation of triazolyl 13α-estrone–nucleoside bioconjugates, *Molecules*, **2016**, *21*, 1212–1228. **IF: 2.861**

2. **Bodnár B.,** Mernyák E., Szabó J., Wölfling J., Schneider G., Zupkó I., Kupihár Z. and Kovács L. Synthesis and *in vitro* investigation of potential antiproliferative monosaccharide–D-secoestrone bioconjugates, *Bioorganic and Medicinal Chemistry Letters*, **2017**, *27*, 1938–1942. **IF: 2.454**

3. Kupihár Z., **Bodnár B.,** Kovács L. 5'-Linker modified nucleosides, Hungarian patent, P1700542, **2017.12.19.** (5'-Linkerrel módosított nukleozidok, magyar szabadalmi bejelentés)

4. **Bodnár B.,** Kovács L., Kupihár Z. An efficient way to synthesize azide-containing nucleosides for click conjugations (in preparation)

5. **Bodnár B.,** Kovács L., Kupihár Z. Synthesis of versatile 5'-linker containing purine nucleosides as clickable building blocks for efficient synthesis of bisubstrate inhibitors (in preparation)

5. Other publications

1. Mernyák E., Fiser G., Szabó J., **Bodnár B.,** Schneider G., Kovács I., Ocsovszki I., Zupkó I., Wölfling J. Synthesis and *in vitro* antiproliferative evaluation of D-secooxime derivatives of 13β- and 13α-estrone, *Steroids*, **2014**, *89*, 47–55. **IF: 2.639**

2. Paragi G., **Bodnár B.,** Szolomájer J., Batta G., Bényei A., Kele Z., Kupihár Z., Fonseca Guerra C., Bickelhaupt M. F., Ciesielski A., Haar S., Samori P.,Masiero S., Spada P. G., Kovács L. *Szupramolekuláris rendszerek purinokból,* Értékteremetés és értékközvetítés: Válogatás a Bolyai Ösztöndíj 15 éves évfordulója alkalmából tartott ünnepélyes tudományos ülés előadásaiból, **2015**, 179–185.

6. Lectures and posters related to the thesis

1. **Bodnár B.,** Kovács L., Kupihár Z., *Synthesis of 5'-azidonucleosides as building blocks for the preparation of biologically active bioconjugates*, 19th International Conference on Organic Chemistry, 28-29 August, **2017**, Paris, France

2. **Bodnár B.,** Kupihár Z., *Synthesis of 5'-azidonucleosides*, MTA Szénhidrát, nukleinsav és antibiotikum munkabizottsági ülés, **2017.**május 31.-június 2. Mátraháza

3. **Bodnár B.,** Kovács L., Kupihár Z., *5'-Azidonukleozidok előállítása*, XX. Tavaszi Szél Konferencia, **2017**. március 31.-április 2. Miskolc

4. **Bodnár B.,** Rakk D., Kovács L., Kupihár Z., *5'-Azidonukleozidok előállítása*, XXII. Nemzetközi Vegyészkonferencia, **2016**. november 3.-november 6. Temesvár, Románia

5. **Bodnár B.,**Mernyák E., Wölfling J., Zupkó I., Kupihár Z., *Synthesis of nucleoside–estrone bioconjugates applying copper catalyzed alkyne–azide click reaction*, 8th Central European Conference "Chemistry towards Biology", August 28-September 1, **2016**, Brno, Czech Republic

6. Tétényi E., **Bodnár B.,** Mernyák E., Kupihár Z., *Azido-nukleozidok előállítása és kapcsolása szteroidokhoz réz-katalizált alkin-azid klikk reakcióval*, XXXVIII. Kémiai Előadói Napok, **2015**. október 26-28. Szeged

7. Szabó J., **Bodnár B.,** Wölfling J., Schneider G., Zupkó I., Mernyák E., *Preparation of novel cytostatic D-secoestrone conjugates*, 20th International Conference on Organic Chemistry, June 29-July 4, **2014**, Budapest

7. Lectures and posters not related to the dissertation

1. Ferenc G., Gombos M., Rakk D., **Bodnár B.**, Kupihár Z., Györgyey J., Dudits D., *Oligonukleotidok lipid konjugátumainak felvetetési próbái növényi sejtekkel*, XXII. Nemzetközi Vegyészkonferencia, **2016**. november3.-november 6. Temesvár, Románia

2. Rakk D., **Bodnár B.,** Kincses F., Ferenc G., Nyerges Á., Mészáros M., Veszelka Sz., Deli M., Dudits D., Kovács L., Kupihár Z., *Lipid derivatives of oligonucleotides – preliminary results*, EuroTides Conference in Berlin, November 16-18, **2015**, Berlin, Germany

3. **Bodnár B.,** Kovács L., Kupihár Z., *Toward sthesynthesis of short, double stranded cyclic oligonucleotides*, MTA Szénhidrát, nukleinsav és antibiotikum munkabizottsági ülés, **2015**. május 27-29. Mátraháza

4. Jozilan H., Horváth P., Kósa J. P , Lakatos P., Németh D., Wölfling J., Kovács D., **Bodnár B.,** Mátyus P., Horváth E., Kovalszky I., Szalay F., *Increased anti-tumor effect of vitamin D after*

CYP24A1 inhibition on HCC cell lines, 50th Annual Meeting of The European Association for the Study of the Liver, April 22-26, **2015**, Vienna, Austria

5. **Bodnár B.,** Kovács L., Kupihár Z., *Synthesis of short circular DNAs for biological applications*, MTA Szénhidrát, nukleinsav és antibiotikum munkabizottsági ülés, **2014**.május 21-23. Mátraháza

6. **Bodnár B.,** Kupihár Z., Gyurcsik B., *Kémiailag módosított nukleinsav próbák szintézise fehérje-nukleinsav kölcsönhatás vizsgálatokhoz*, XXXVI. Kémiai Előadói Napok, **2013**. október 28-30. Szeged

7. **Bodnár B.,** Kupihár Z., Kovács L., Ferenc G., *Synthesis of short, cyclic double-stranded oligonucleotides for biological applications*, Symposium on Biomolecular Synthesis and Nanotechnology, June 3-4, **2013**, Odense, Denmark

8. **Bodnár B.,** Szolomájer J., Paragi G., Kupihár Z., Kele Z., Ciesielski A., Haar S., Samori P., Masiero S., Spada P. G., Fonseca Guerra C., Bickelhaupt M. F., Kovács L., *3-Alkyl- and 3-glycosylxanthines: syntheses and behavior in gas and liquid states and at a solid-liquid interface*, MTA Szénhidrát, nukleinsav és antibiotikum munkabizottsági ülés, **2013**. május 22-24. Mátrafüred