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Mendeleev Commun., 2019, **29**, 526–528

Mendeleev Communications

Synthesis of allobetulin-based asialoglycoprotein receptor-targeted glycoconjugates

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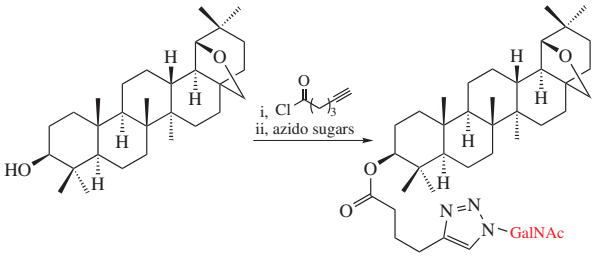
DOI: 10.1016/j.mencom.2019.09.016

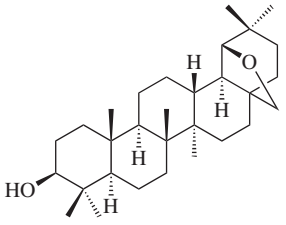
New allobetulin conjugates were obtained through its O-esterification with hex-5-ynoic acid followed by [3+2]-cycloaddition with three azido derivatives of *N*-acetyl-D-galactosamine. The conjugates are non-toxic in micromolar range against hepatocellular carcinoma cell lines and have a high affinity towards the asialoglycoprotein receptor of hepatocytes based on molecular docking and surface plasmon resonance data.



Amphiphilic glycoconjugates are unique objects in medicinal chemistry,[1] and their synthesis is an important task of modern science.[2] Introduction of sugar residues into the structure can significantly change the initial pharmacological profile of the synthesized drugs.[3] Pentacyclic triterpenoids, *e.g.* allobetulin,[4] are highly lipophilic compounds with poor bioavailability. Taking into account their significant initial biological activity, *e.g.* anticancer and antiviral ones,[5] further modification of the pharmacological properties seems topical, in particular, testing of new glycoconjugates for delivery to well-known therapeutic targets.[6] Among them, development of conjugates with *N*-acetyl-D-galactosamine (GalNAc) represents the most progressive method of drug delivery to the liver for the treatment of liver diseases through interaction with the asialoglycoprotein receptor (ASGPR).[7] To the best of our knowledge, conjugates of allobetulin and GalNAc as targeted molecules to the ASGPR were not synthesized.

This paper reports the synthesis of allobetulin conjugates with GalNAc and investigation of their cytotoxic properties and binding affinity to hepatocytes. Our synthetic route is based on a common approach when a parent bioactive compound serves as a starting point for obtaining ASGPR-targeted conjugate. In this case, we used a more convenient and simpler 'tuning' instead of employing branched and bulky multivalent ligands. Allobetulin **1** was selected as a template molecule, and azide–alkyne [3+2]-cycloaddition was applied to conjugate allobetulin with azido sugars **2**–**4** [for their synthesis, see ref. 8(*a*)].

This approach was previously successfully used to create mono- and bivalent ASGPR-conjugates of paclitaxel and doxorubicin.[8] The azide–alkyne [3+2]-cycloaddition[9] results in 1,2,4-triazoles, which can also be responsible for π–π interactions with aromatic rings and hydrogen bonds through the N(2) and N(3) nitrogen atoms thus providing the additional binding with the target protein molecules.[10]



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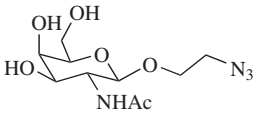

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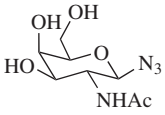

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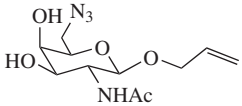

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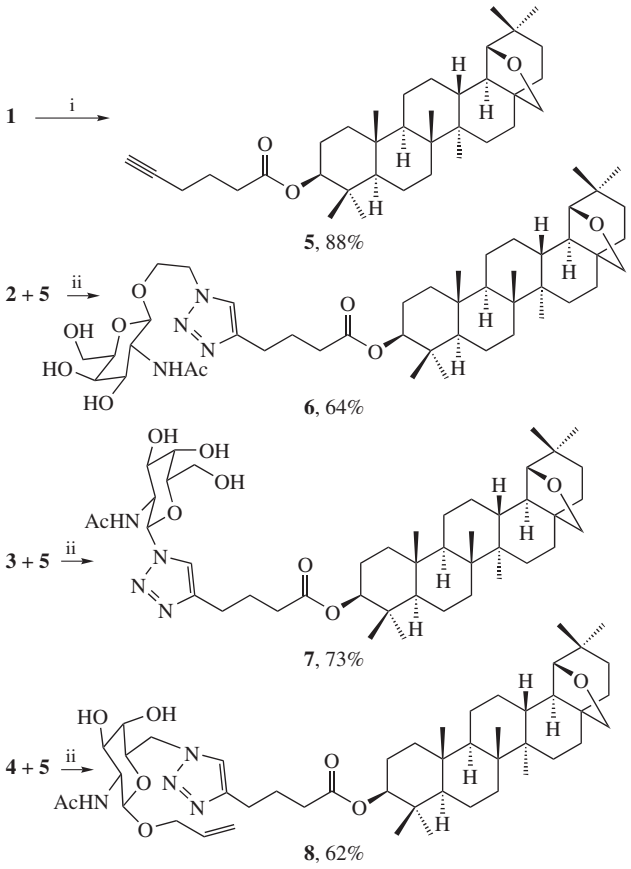
Allobetulin **1** contains a free C(3)-positioned hydroxyl group capable of undergoing acylation. Therefore, as in previous studies,[8] it was acylated with commercially available hex-5-ynoic acid to afford acetylene ester **5** (Scheme 1) whose hex-5-ynoyl moiety served as a linker between GalNAc derivatives **2**–**4** and the natural terpene in prodrug-type precursors of final conjugates.[11]

For C(3)–OH esterification of allobetulin **1**, hex-5-ynoyl chloride in pyridine[12] was used[†] (see Scheme 1). Subsequent CuI-catalyzed conjugation of alkyne **5** with azides **2**–**4** afforded glycotriterpenoids

[†] For procedures and characteristics of the products, see Online Supplementary Materials.



Scheme 1 *Reagents and conditions:* i, $HC{\equiv}C(CH_2)_3C(O)Cl$, Py, DMAP, reflux, 12 h; ii, CuI, DMF, DIPEA, Ar, room temperature, 24 h.

6–8 with high regioselectivity towards 1,4-substituted 1,2,4-triazoles. Structures of compounds **6–8** were proved by ^1H and ^{13}C NMR spectra showing the signals of 1,2,4-triazole rings and GalNAc moieties. Furthermore, initial azido sugars **2–4** were individual β-anomers, and this configuration retained in the course of conjugation (evidenced by the *J* values of 8–9 Hz of the glycoside protons in their ^1H NMR spectra[13]). High-resolution mass spectrometry (ESI HRMS) data on compounds **6–8** were in full accordance with the obtained results.

As a follow-up study, we have performed *in silico* evaluation of the obtained conjugates **6–8** using molecular docking approach (interaction with the ASGPR binding site). Crystallographic data from Protein Data Bank (PDB ID 5JPV)[14] was used for the development of a 3D computational model of the ASGPR binding site. The developed docking model was validated by redocking of the crystallographic ligand. Then, conjugates **6–8** were docked into the binding site to assess their affinity.‡

The glycosidic fragment of conjugate **7** occupied the corresponding binding site and formed hydrogen bonds with the residues Glu252, Asp241, Asn264 and Gln239, as well as interaction with calcium ion. The ester group of the linker moiety in compound **7** formed hydrogen bonds with Arg236, and the allobetulin moiety formed a hydrophobic contact with Pro237. The glycoside residues of conjugates **6** and **8** did not fit into the corresponding receptor pocket. However, hydrogen bonds were formed between **6** and residues Glu238, Asp241 and Gln239; and between **8** and residues Asn264 and Gln239. Besides, conjugate **8** showed the highest energy score value E_{score} = –5.21 kcal mol^{-1}. As a result of *in silico* studies, the ability to interact with ASGPR was predicted for all the structures docked with similar binding affinities.

‡ For additional data on MTT-test, SPR-assay and molecular docking, see Online Supplementary Materials

Next, affinity of the obtained compounds to a protein molecule of ASGPR was estimated using surface plasmon resonance (SPR) technique by analogy with the procedure described by Stokmaier *et al.*[15] Glycoconjugates **6–8** showed high binding affinity (K_d ~ nM) that considerably outnumbers K_d values for native ASGPR ligands, *viz. N*-acetyl-D-galactosamine and galactose,[16] probably due to the large aliphatic hydrocarbon skeleton. In general, conjugates **6** and **7** demonstrated a weaker binding affinity in comparison to **8**. Compound **8** revealed the best result with K_d 8.3 nM. This study proved the results of molecular docking and thereby demonstrated the potency of allobetulin glycoconjugates to active ASGPR-mediated transport into hepatocytes.

We have also performed MTT-based cytotoxicity assay[17] against the selected hepatocellular carcinoma cells HepG2 and Huh7. As a control, we used prostate cancer cells PC3 and human embryonic kidney cells Hek293. In all cases, compounds **6–8** were non-toxic in the micromolar range and inhibitory concentration has not been achieved below 100 μM. Notably, the low cytotoxicity of allobetulin-based ASGPR-conjugates should promote further investigation of different biological properties against liver disease, such as antiviral, antimalarial, *etc.*, except anticancer activity against hepatocellular carcinoma.

In conclusion, the synthesis of novel monovalent conjugates of allobetulin and *N*-acetyl-D-galactosamine has been described. A subsequent biological evaluation revealed that the resultant compounds were non-toxic against hepatocarcinoma cell lines HepG2 and Huh7 in the micromolar range, as well as against Hek293 cells and prostate cancer cell line PC3. Molecular docking simulations were applied to predict and analyze the binding mode of conjugates to carbohydrate recognition domain of the ASGPR. The excellent binding affinity of the allobetulin conjugates to ASGPR was evaluated using SPR spectroscopy. Finally, obtained results present an attractive example of new triterpenoid-based targeted derivatives for further investigations of natural products against liver diseases.

The study was supported by grants from the Russian Science Foundation (project no. 17-74-10204 – synthesis, cytotoxicity assays and *in silico* studies) and from Skolkovo Institute of Science and Technology (Master Research Agreement no. 182-MRA – binding affinity studies). The authors would like to express their gratitude to Thermo Fisher Scientific Inc., MS Analytica (Moscow, Russia) and personally to Professor Alexander Makarov for providing mass spectrometry equipment for this work.

Online Supplementary Materials

Supplementary data associated with this article can be found in the online version at doi: 10.1016/j.mencom.2019.09.016.

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Received: 11th April 2019; Com. 19/5881