Investigation of α-thioaryl galactopyranosides to improve affinity towards Galectin-9N

Sofi Gummeson

LUNDS UNIVERSITET

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Supervisor: Ulf Nilsson & Mukul Mahanti
Centre for Analysis and Synthesis
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1 Abstract

Galectins are a family of carbohydrate recognition proteins involved in modulation of cell signaling and cell adhesion. Galectin-9 especially is involved in regulation of the immune response. In this work, galectin-9N inhibitors have been designed, synthesized and evaluated. The aim was to synthesize compounds with higher affinity than currently known compounds. Three compounds were synthesized with different substituents at the alpha position of the anomeric carbon. Substituent patterns employed in this study were phenyl, methyl phenyl and fluoro phenyl. Affinities of compounds were evaluated in a fluorescence polarization assay. The compound with best affinity was the galectin-9N inhibitor with fluoro phenyl as the substituent, with affinity of $5\mu M$. The polar interaction between the binding pocket and the inhibitor is believed to be the reason for the good affinity.

2 Acknowledgements

I would like to thank my supervisor, Ulf Nilsson, for letting me do this project and his group for making me feel welcome from the start.

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3 Abbreviations

CRD	**-**	**Carbohydrate-Recognition Domain**
HPLC	**-**	**High-Performance Liquid Chromatography**
NMR	**-**	**Nuclear Magnetic Resonance Spectroscopy**
FP	**-**	**Fluorescence Polarization Assay**
rt	**-**	**Room Temperature**
NB	**-**	**Non-Binding**

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4 Aim

Aim of the project was to synthesis galactoside inhibitors, mainly for human galectin-9N (N-terminal), with different aromatic thiol groups in the anomeric position and to investigate the effect of the different substituents.

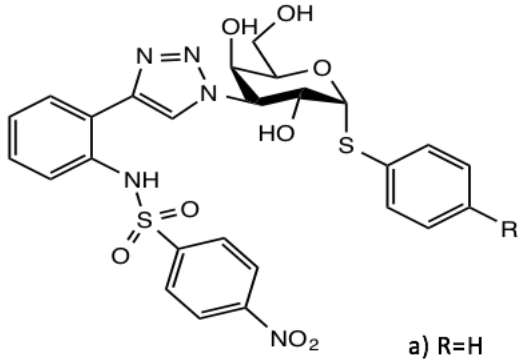


Figure 1. Substituent patterns investigated to increase affinity and selectivity towards galectin-9N.

5 Introduction

Carbohydrates are molecules consisting of carbon, hydrogen and oxygen[1]. Besides acting as an energy source carbohydrates on the cell membrane can communicate and interact with other cells. An example of this is when carbohydrates recognize invading bacteria by immune system as "foreign", which it the first step before marking, killing and removing the bacteria. This works in both ways with bacteria recognizing the carbohydrates on the cells in some infections.[2]

Galectins are glycan-binding proteins, with mediate multiple biological functions. The proteins have either one or two carbohydrate-recognition domains (CRD) and are for example linked to development, progression and metastasis of cancer. Their other functions include damp T-cell mediated immune response and inflammation. There are 15 different types of galectins in mammals and 11 in humans. The proteins can be divided in to three groups, which are based on their structure: 1. prototype galectins that contain one CRD that can form homodimers; 2. tandem repeat-type galectins containing two CRDs and are connected by flexible linker; 3. cimeric-type galectin that contains an N-terminal extension which can form

[1] Maton, D., Hopkins, J., McLaughlin, C. W., Johnson, S., Warner, M. Q., LaHart, D., ... & Deep, V. K. (1997). Human Biology and Health. Englewood Cliffs, New Jersey, US. *Prentice Hall. ISBN 0-13-981176-1. OCLC*, *32308337*, 1993.

[2] Werz, D. B., Ranzinger, R., Herget, S., Adibekian, A., von der Lieth, C. W., & Seeberger, P. H. (2007). Exploring the structural diversity of mammalian carbohydrates ("glycospace") by statistical databank analysis. *ACS chemical biology*, *2*(10), 685-691.

oligomers to increase their binding avidity and a CRD domain.[3] Due to various functions of galectins, they are interesting drug targets for anti-cancer and anti-inflammatory compounds.[4]

Galectin-9N, on which is the focus of this thesis, belongs to group 2. Galectin-9 is found to be important in regulation of the immune response. It has multiple functions that include apoptosis, chemotaxis and cell aggregation. The protein can be found in the cell membrane, nucleus and cytoplasm.[5]

A series of inhibitors based on galactose substituted by sulfonamide in C-3 position was tested towards human Gal-9N. The compounds were found to have affinities around $20\mu M$. To see if the affinity would increase the 3,4-dichlorothiophenol was placed in alpha position on the anomeric carbon[6]. The affinity was excepted to get to nM values, but the original affinity of 20 μM was only increased to about four times. This could be due to the counteractive effect between the sulfonamide part and aryl thiol, with ´bulky´ chlorines, in the anomeric position as can be seen in the figure below. Which did not give as great fit to the protein pocket as expected. In this report, the effect of phenyl rings, unsubstituted and substituted, on affinity were investigated. To find out if these inhibitors can beat previously found inhibitors for human galectin-9N.

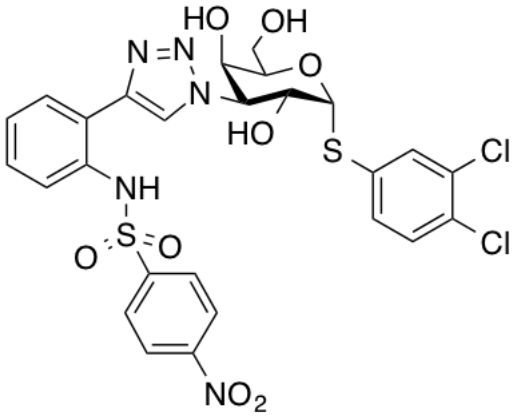


Figure 2. Previously tested galectin inhibitor.

[3] Chou, F. C., Chen, H. Y., Kuo, C. C., & Sytwu, H. K. (2018). Role of galectins in tumors and in clinical immunotherapy. *International journal of molecular sciences*, *19*(2), 430.

[4] Öberg, C. T., Leffler, H., & Nilsson, U. J. (2011). Inhibition of galectins with small molecules. *CHIMIA International Journal for Chemistry*, *65*(1), 18-23.

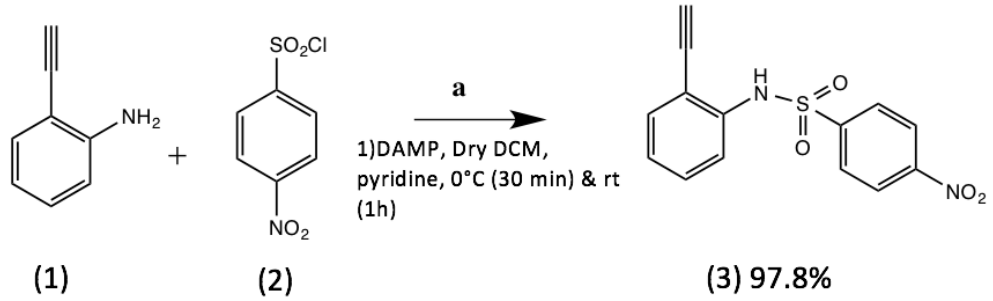
[5] Wu, C., Thalhamer, T., Franca, R. F., Xiao, S., Wang, C., Hotta, C., ... & Kuchroo, V. K. (2014). Galectin-9-CD44 interaction enhances stability and function of adaptive regulatory T cells. *Immunity*, *41*(2), 270-282.

[6] Zetterberg, F. R., Peterson, K., Johnsson, R. E., Brimert, T., Håkansson, M., Logan, D. T., ... & Nilsson, U. J. (2018). Monosaccharide Derivatives with Low-Nanomolar Lectin Affinity and High Selectivity Based on Combined Fluorine–Amide, Phenyl–Arginine, Sulfur–π, and Halogen Bond Interactions. *ChemMedChem*, *13*(2), 133-137.

6 Results and discussion

6.1 Synthesis of Sulfonamide

Sulfonamide **(3)** was synthesized in 98% yield by coupling between amine **(1)** and sulfonyl chlorode **(2)** in presence of pyridine and DMAP at rt for 1 hr.



Scheme 1. Reaction scheme for synthesis of the sulfonamide (3).

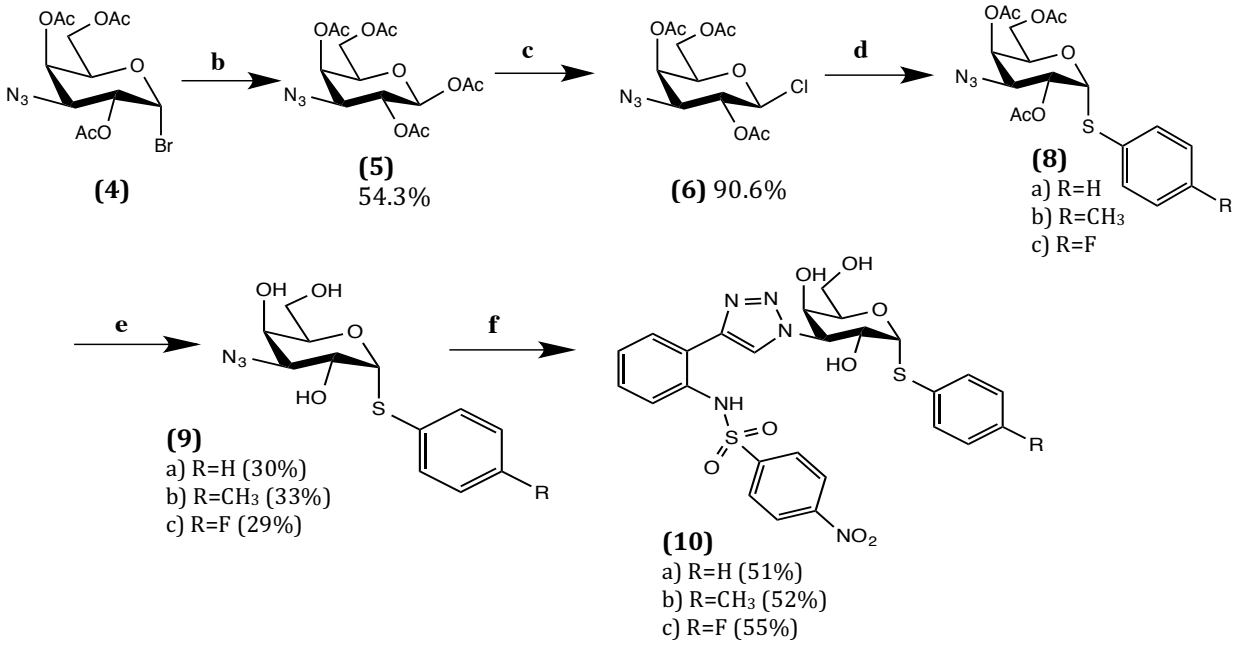
6.2 Synthesis of sulfonamide based galactose inhibitors

From 2,4,6,-tri-O-acetyl-3-deoxy-3-azido- α -D-galactopyranosyl bromide**(4)**, synthesis pathway started by forming 1,2,4,6,-Tetra-O-acetyl-3-deoxy-3-azido- β -d-galactopyranoside **(5)** which was made in bulk. Synthesis pathway for compounds **(10)a-c** can be found in scheme 2.

In step **2b**, scheme 2 step b, 2,4,6,-tri-O-acetyl-3-deoxy-3-azido- α -D-galactopyranosyl bromide **(4)**[7] was acetylated by cesium acetate to form **(5)** in 54.3% yield. The following step, **2c**, **(5)** was chlorinated to compound **(6)** in 90.6% yield. Compound **(8)** was synthesized from azide **(6)** by reacting with sodiated salt of thiophenol **(7a-7c*)** in precens of dry DMF at 50°C. Without further purification these compounds were deacetylated by NaOMe in MeOH to form **(9a)**, **(9b)** and **(9c)** at 30%, 33% and 29% yield respectively from **(6)**. Then there subjected to Cu catalyzed cycloaddition with sulfonamide **(3)** in presence of DIPEA in DCM at rt for overnight to obtain the desired final compounds **(10a-c)** (39-50% yield). They were purified by prep HPLC.

[7] Lemieux, R. U., Szweda, R., Paszkiewicz-Hnatiw, E., & Spohr, U. (1990). The effect of substituting key hydroxyl groups by amino groups on the binding of the Lewis b tetrasaccharide by a lectin and a monoclonal antibody. *Carbohydrate research*, *205*, C12-C17.

** thiophenyl (7a), 4-methylbenzenethiol(7b) or 4-flourothiophenol (7c)*



Scheme 2. Synthesis of sulfonamide based galactose inhibitors.

b= Cesium acetate, DMF, 50°C, **c**= PCl_5, $BF{\cdot}O(C_2H_5)_2$, dry DCM, rt, **d**= NaH, dry DMF, (7; a, b, c), 50°C, **e**= NaOMe, MeOH rt, **f**= CuI, DCM, DIPEA, (3), rt

To conclude, syntheses of the three compounds were successful and the yields correspond to literature.

6.3 Fluorescence Polarization Assay Results

All compounds were tested by FP, results can be found in Table 1. FP is a method used to see if interactions is formed and how strongly. From a strong light source, see figure 3, light waves, with an unlimited number of orientation, travels to a polarization filter which transmits light of one orientation. If the one orientated light excites a fluorophore which is attached to a biomolecule the same orientation of light will be emitted. A fluorophore is a chemical compound that can re-emit light upon light excitation. The emitted light will change orientation because of the small molecules, in solution, are tumbling around.
A second polarization filter is used in which light of the same orientation as the filter completely passes through, while the perpendicular light is blocked. The light which passes the second filter is detected.

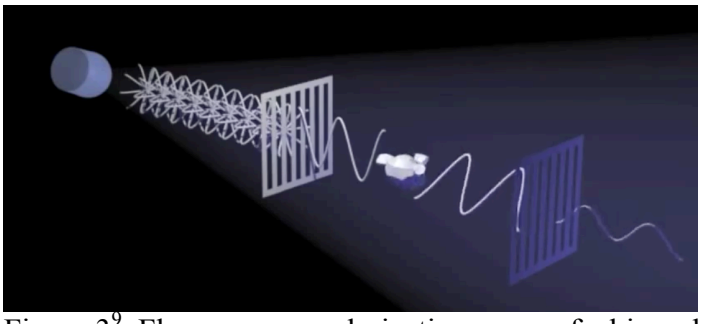


Figure 3[9]. Fluorescence polarization assay of a biomolecule.

The same procedure is done with the biomolecule and the antibody or protein such as a galectin, see figure 4. Because of the higher molecular weight the complex moves more slowly, in a given time, which means polarization of the light is conserved for a longer time giving it a higher polarization signal[8,9]. Higher polarization signal indicates a better interaction.

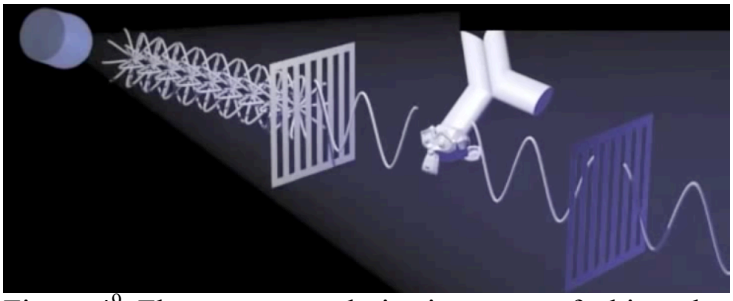


Figure 4[9]. Fluorescence polarization assay of a biomolecule-antibody complex.

From data shown in Table 1 binding of compounds are not as strong as expected. Binding of compounds **(10a)** and **(10b)** to galactin-9N (Gal-9N) was worse (K_d=10.3 and 11.9μM) than when 3,4-dichlorothiophenol was used as substituent on the anomeric carbon ($\sim$6 μM). The best compound is **(10c)** with the 4-fluoro group but the other two groups are close in affinity. The binding pocket may use polar interactions and therefor binds stronger to **(10c)** which has a polar fluorine atom. Maybe the 3-chloro atom at the 3,4-dichlorothiophenol on the anomeric carbon is interacting with the pocket as well. It can therefore be interesting to explore if a fluorines in both position 3 and 4 on the aromatic ring will give better affinity than **(10c)**.

The compounds were tested towards other galectins as well and shown at least two times higher affinity to Gal-9N compared to the rest. The selectivity of the compounds towards Gal-9N is therefore higher than towards the other galectins.

Compound	Gal-9N	Gal-9C	Gal-8N	Gal-8C	Gal-7	Gal-3	Gal-1
(10a)	10.3± 0.9	NA	NB	NB	56±23	NA	NA
(10b)	11.9± 1.5	NA	271±29	NB	43±6	Na	NA
(10c)	5 ± 0.5	25.5±1.7	267±65	NB	28±1	15±1	52±2

Table 1. K_d (μM) of compounds **(10a)**, **(10b)** and **(10c)** in different galectins. NB stands for non-binding and NA for not available.

[8] Lea, W. A., & Simeonov, A. (2011). Fluorescence polarization assays in small molecule screening. *Expert opinion on drug discovery*, *6*(1), 17-32.

[9] Thomas Hengstl, (Thomas Hengstl). (2013, July 16). *Flurescence polarization. https://www.youtube.com/watch?v=OdBNVrPvJMY*

7 Conclusion

All synthesis gave desired products in sufficient amounts to test the affinities in FP. As a result, it was found that the smaller molecules of **(10a)** and **(10b)** did not give better affinity than the compound with 3,4-dichlorothiophenol, their binding was worse. For **(10c)**, however, the study found it to have similar affinity as the compound with 3,4-dichlorothiophenol.

8 Future Prospects

More analogues can be synthesized and tested in fluorescence polarization assay to map the effect of different substituents and whether the affinity can be optimized further. Placing fluorine atoms to both 3 and 4 position of thiophenol as discussed in the results and discussion part of this thesis can be an option. It could also be useful to further investigate the binding pocket to see which interactions are preferred and from this information develop a more efficient inhibitor.

9 Experimental

General Experimental Information

Chemicals were bought from Sigma Aldrich and used without further purification. NMR was done on a 400MHz Bruker Avance NMR spectrometer and chemical shifts were reported on δ −scale in parts per million (ppm). Column chromatography was done on silica and preparative HPLC was preformed on an Aligent 1260 infinity system, column SymmetryPrep-C18, and a 17 mL/min H_2O-MeCN gradient 10-100% 15 min with 0.1% formic acid. Analytical TLC was preformed on silica gel with UV absorption and/or by charring 90:10 (EtOH:Conc H_2SO_4).

Synthesis of Sulfonamide

4-Nitrobenzenesulfonyl chloride (1.093g, 4.93mmol), two beads of 4-dimethylaminopyridine and dry DCM (20ml) under nitrogen atmosphere was placed in ice bath. Pyridine (0.4ml,4.967mmol) was added and reaction was left for 5 minutes. 2-Ethylaniline (0.40ml, 3.51mmol) was added and after 30 minutes the ice bath was taken away. Reaction was left in rt for 1 hour. Reaction was completed and confirmed by TLC (3:1, Heptane:Ethylacetate). Reaction was quenched with HCl (1M, 10ml) and washed with brine. Organic phase was separated and dried with sodium sulfate. The solution was filtrated and evaporated. Column chromatography was done to purify the sulfonamide (1.042g, 3.44mmol, 97.8%). ^{1}H NMR ($CDCl_3$, 400MHz): 8.25 (dt, J=5.2, 22.5Hz, 2H), 7.94 (dt, J=5.2, 22.4 Hz, 2H), 7.62 (dd, J= 2.3, 21.6Hz, 1H), 7.33 (m, 3H), 7.01 (t, J= 17Hz, 1H), 3.34 (s, 1H)

Synthesis of 3-deoxy-3-azido-1,2,4,6-tetra-*O*-acetyl- β –D-galactopyranose(5)

2,4,6,-Tri-O-acetyl-3-deoxy-3-azido- α -D-galactopyranosyl bromide **(4)** (2.684g, 6.528mmol) an cesium acetate (2.5006g, 13.027mmol) were dissolved in DMF (10 ml). Mixture was stirred at 50°C overnight. Compound was washed with ethyl acetate (15ml) and water (15ml). Organic phase was evaporated and column chromatography was done. Compound **(5)** (1.300g, 3.5446mmol, 54.3%) was confirmed by NMR. ^{1}H NMR ($CDCl_3$, MHz): 5.61 (d, J= 49.6Hz, 1H), 5.37(d, J=83Hz, 1H), 5.16(ddt, J=20.6, 26.6, 56.6 Hz, 1H), 4.00 (m, 4H), 3.67 (dd, J=5.0, 2.6 Hz, 1H), 2.09 (s, 3H), 2.04 (d, J=4.7 Hz, 5H), 1.95 (s, 3H)

Synthesis of 3-deoxy-3-azido-2,4,6-tri-*O*-acetyl-β-D-galactopyranosyl chloride(6)

Phosphorus pentachloride (117.37mg, 0.5636mmol), **(5)** (200mg, 0.51237mmol) and dry DCM(10ml) was stirred under nitrogen for (t) min at rt. The mixture was quenched with ice cold $NaHCO_3$ and washed with ice cold water, dried with sodium sulfate, filtrated and evaporated. Yield 90.6-99.3%, (t) 15-45min.

Compound **(6)** (171.5mg, 0.4676mmol, 90.6% yield) was evaporated and dried under vacuum.
Compound **(6)**(187.9mg, 0.51237mmol, 99.3% yield).
Compound **(6)** (187.9mg, 0.51237mmol).

Synthesis of phenyl 3-deoxy-3-azido-2,4,6-tri-*O*-acetyl-1-thio-α-D-galactopyranosides(8a-c)

NaH(21.2mg, 0.922mmol), **(7a)** (0.105ml, 1.0247mmol) and dry DMF(6ml) was stirred under nitrogen for 30 min in rt. **(6)** (200 mg, 0.572mmol) was dissolved in DMF (10ml) and added to the mixture which was stirred at 50°C for overnight. It was proceeded to the next step with out further purification.

Synthesis of phenyl 3-deoxy-3-azido-1-thio-α-D-galactopyranosides(9a-c)

(8a) was dissolved in MeOH and NaOMe was added until pH value of 12, which was checked with pH paper. Mixture was stirred in rt for over night DOWEX was added, until pH7, and filtrated.

Phenyl 3-deoxy-3-azido-1-thio- α-D-galactopyranoside 9a
t= 15h , 25 mg yield 15%,
4-Methylphenyl-3-deoxy-3-azido-1-thio-α-D-galactopyranoside 9a
t=15h, 30mg, yield 17%
4-Fluorophenyl-3-deoxy-3-azido-1-thio-α-D-galactopyranoside 9a
t=15h, 26 mg, yield 15%

Synthesis of phenyl 3-deoxy-3-(4-phenyl-2(4-nitrobenzenesulfonamide)-1,2,3-triazolyl)-α-D-galactopyranosides(10a-c)

(9), Sulfonamide (32.5mg, 0.10767mmol), CuI(0.68mg, 0.00358mmol), on drop of DIPEA and dry DCM (2ml) was stirred under nitrogen for (t) in rt. Separation by column chromatography and HPLC was done.

Phenyl 3-deoxy-3-(4-phenyl-2(4-nitrobenzenesulfonamide)-1,2,3-triazolyl)-1-thio-α-D-galactopyranoside 10a
Amount 22.9mg, yield 51% & (t) 5 days.
^{1}H NMR (MeOD, 400MHz): 8.32(s,1H), 8.17(dd, J=5.2, 0.017.4 Hz, 2H), 7.79 (dd, J=5.9, 23.4 Hz, 2H), 7.63(m, 5H), 7.26b (m, 4H), 7.21(t, J=16.7 Hz, 1H), 5.77(d, J=13.3Hz, 1H), 4.60(s, 21H), 4.19(s, 1H), 3.72(dq, J=14.6, 290Hz, 3H)

4-Methylphenyl 3-deoxy-3-(4-phenyl-2(4-nitrobenzenesulfonamide)-1,2,3-triazolyl)-1-thio-α-D-galactopyranoside 10b
Amount 29.7mg, yield 52% & 15h
^{1}H NMR (MeOH, 400MHz): 8.32(s,1H), 8.17(dt, J=6, 23.4 Hz, 2H), 7.80(dd, J=5.2, 17.4 Hz, 2H), 7.67(d,J=19 Hz, 1H), 7.60(d,J=3.2 Hz, 1H), 7.51(d, J=20.3 Hz, 2H), 7.34(t, J=18.2 Hz, 1H), 7.20(dd, J=18.8, 45.2 Hz, 3H), 5.69(d, J=13.2Hz, 1H), 4.96(dd, J= 6.8, 28.6 Hz, 3H), 4.61 (s,16H), 4.19(d, J= 4.6 Hz, 1H), 3.72(dq, J= 14.7, 28.6 Hz, 2H)

4-Fluorophenyl 3-deoxy-3-(4-phenyl-2(4-nitrobenzenesulfonamide)-1,2,3-triazolyl)-1-thio-α-D-galactopyranoside 10c
Amount 27.3mg, yield 55% & (t) 15h
^{1}H NMR (MeOD, 400MHz): 8.31(s, 1H), 8.17(dt, J=5, 22Hz, 2H), 7.79(dt, J= 5.9, 18 Hz, 2H), 7.6825-7.6390(m, 3H), 7.59(dd, J= 3.5, 20Hz, 1H), 7.33(ddd, J=3.9, 19, 39 Hz, 1H), 7.22(ddd, J= 3.1, 19, 38 Hz, 1H),7.13-7.07(m, 2H), 5.70(d, J=13Hz, 1H), 4.96(dd, J= 7.1, 29Hz, 1H), 4.84(d, J= 13 Hz, 2H), 4.57(d, J= 16 Hz, 1H), 4.19(d, J= 4.6 Hz, 1H), 3.78-3.65(m, 2H)