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Synthesis of two lipopentasaccharides related to the nodulation factors of the nitrogen-fixing bacterium *Rhizobium* sp. NGR 234

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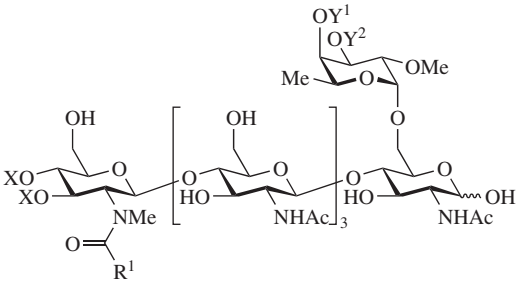
Two lipopentasaccharides related to the Nod factors of *Rhizobium* sp. NGR 234 have been synthesised.

The coordinated interactions of nitrogen-fixing bacteria of the genus *Rhizobium* and legumes become established as a result of mutual exchange with signaling molecules between simbionts.[1,2] So-called nodulation factors (Nod factors) play an important role in the processes, which finally lead to the formation of nodules containing nitrogen-fixing bacteria.[3,4] Chemically, the Nod factors represent lipochitooligosaccharides containing a core constructed from four to five β-(1→4) bonded glucosamine residues. The non-reducing glucosamine residue is N-acylated by a long-chain fatty acid, and all other amino groups are acetylated. A characteristic property of these compounds is an additional modification of both reducing *N*-acetylglucosamine and non-reducing *N*-acylglucosamine. These structural features control the specificity of interactions between rhizobia and their host plants.

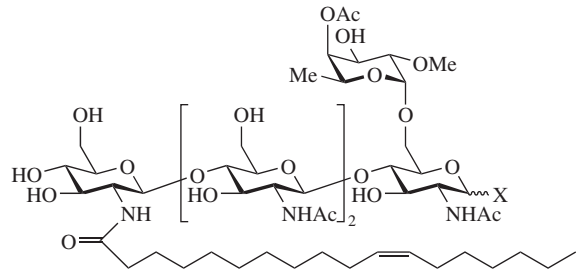
Here we describe an approach that allows synthesising lipooligosaccharides related to Nod factors from *Rhizobium* sp. NGR 234, which enters into symbiosis with more than 110 genera of legumes.[5]



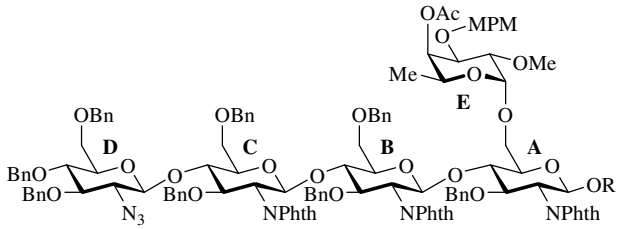
Y^1 = H or Ac, Y^2 = H or SO_3H, X = H or $CONH_2$
R^1 = $(CH_2)_9CH{=}CH(CH_2)_5Me$ or $(CH_2)_{14}Me$

This study deals with the synthesis of *Rhizobium* sp. NGR 234 Nod factors analogues, namely, pentasaccharide **1** and its methyl glycoside **2** with a fixed anomeric configuration at the reducing end.

Structural analysis of the target molecules demonstrated that the amino group of the non-reducing glucosamine residue should be acylated independently of other amino groups. One of the main tasks is the careful selection of protecting groups for the amino groups of glucosamine residues, which will allow making a chemical differentiation of the two types of amino groups in the molecule. To solve the problem, we decided to utilise the orthogonality of azido and phthalimido protecting groups together with mild deprotecting conditions of all the other protecting groups. Thus, protected pentasaccharide derivatives **3** and **4**, the precursors of target compounds **1** and **2**, should contain the following set of protecting groups: hydroxyl groups of chitooligosaccharide core are protected with benzyl groups, the amino group of the non-reducing glucosamine residue is masked as an azido group, the amino groups of three glucosamine residues are protected as phthalimido groups, the α-L-fucose residue bears a methyl group at O-2, acetate at O-4 and a *p*-methoxybenzyl group at O-3.



1 X = OH
2 X = β-OMe



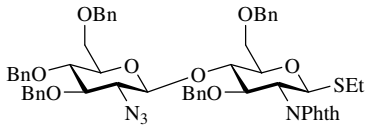
3 R = Bn
4 R = Me

Phth = *o*-$C_6H_4(CO)_2$
MPM = *p*-$MeOC_6H_4CH_2$

While planning the synthesis of protected derivatives **3** and **4**, the second problem emerged: introduction of an α-L-fucose residue at the 6-position of the reducing end of tetrasaccharide. For this purpose, it is necessary to have a selectively removable protecting group at that position. It is well known[6] that fucosylation may lead to a mixture of α- and β-isomers, and their separation could become an unsolvable problem for large molecules. Thus, our decision to perform fucosylation at the di- or trisaccharide step is strategically better justified than the fucosylation of oligosaccharide acceptors with a chain length of more than three sugar residues.

† Deceased.

We chose a convergent synthetic scheme for constructing the carbohydrate backbone of pentasaccharides **3** and **4**, due to the expectation of increasing yield in comparison with the linear scheme. Moreover, the separation of β-L-fucoside, which is formed in the course of α-L-fucosylation in the trisaccharide synthesis, would be much easier than in the case of longer oligosaccharides. Disaccharide glycosyl donor **5** and two trisaccharide acceptors **6** and **7** were chosen for the synthesis.



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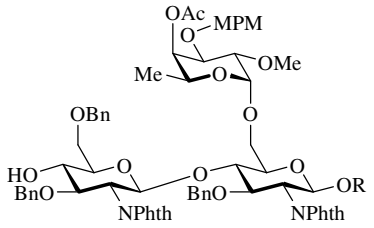
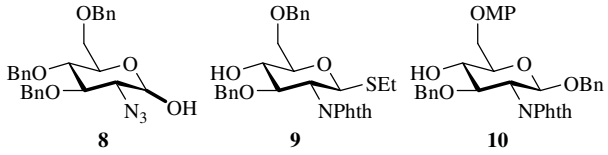
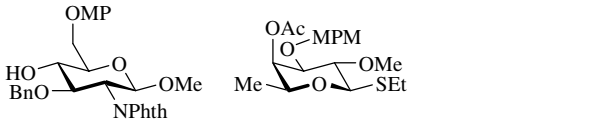


6 R = Bn
7 R = Me

These compounds were synthesised from previously prepared protected monosaccharide derivatives **8**,[7] **9**,[8] **10**, **11**[9] and **12**.[10] Compound **9** was used as a common precursor for the synthesis of disaccharide **5**, as well as for trisaccharide derivatives **6** and **7**.

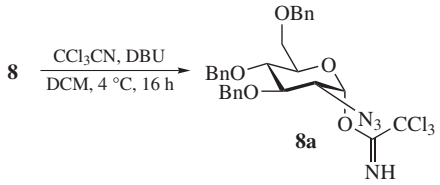


8 **9** **10**



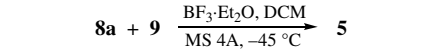
11 **12** MP = *p*-MeOC$_6$H$_4$

Glycosyl donor **8a** was prepared by the reaction of hemiacetal derivative **8** with trichloroacetonitrile in the presence of DBU[7] in 81% yield. According to NMR data, mainly α-trichloroacetimidate **8a** was formed with only traces of the β-isomer.

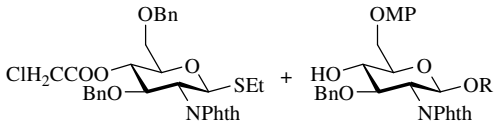


8a

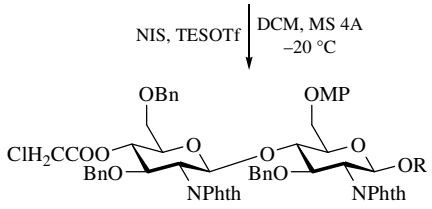
It is well known[11] that, in the course of glycosylation of thioglycoside acceptors by trichloroacetimidate donors, sulfur nucleophilicity leads to side reactions, which may considerably decrease the yield of the target glycoside. However, we succeeded in finding the proper reaction conditions where coupling **8a** with acceptor **9** leads to disaccharide glycosyl donor **5** in 52% yield. The structure of **5** was confirmed by ^{1}H and ^{13}C NMR spectroscopic data. The presence of the spin–spin coupling constant $J_{1',2'}$ 9.8 Hz in its ^{1}H NMR spectrum proved the formation of a 1,2-*trans* glycoside bond.



Glycosylation of acceptors **10** and **11** with thioglycoside **9a**[8] effected by *N*-iodosuccinimide (NIS) and $Et_3SiOSO_2CF_3$ (TESOTf) at –20 °C led to disaccharide derivatives **13** and **14** in 86% and 95% yields, respectively. Their structures were proved by NMR data. The spin–spin coupling constant $J_{1',2'}$ 8.1 Hz for **13** or 8.0 Hz for **14** confirmed the formation of β-(1→4)-disaccharides.

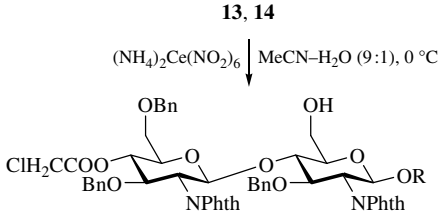


9a **10** R = Bn
11 R = Me



13 R = Bn
14 R = Me

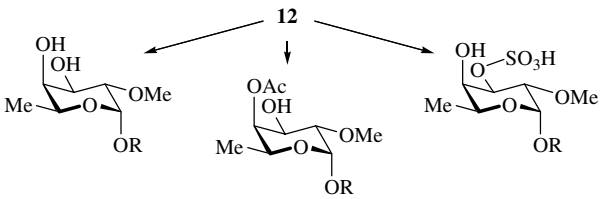
To proceed to fucosylation at the 6-position of the 'reducing' glucosamine residue, it was necessary to remove selectively the methoxyphenyl protecting group from the primary hydroxyl group. This was accomplished by 15 min treatment of a water–acetonitrile solution of **13** at 0 °C with a double excess of ammonium cerium(IV) nitrate followed by column chromatography, which provided derivative **15** in 87% yield. The absence of signals corresponding to the methoxyphenyl protecting group in the NMR spectra proved the structure of **15**.



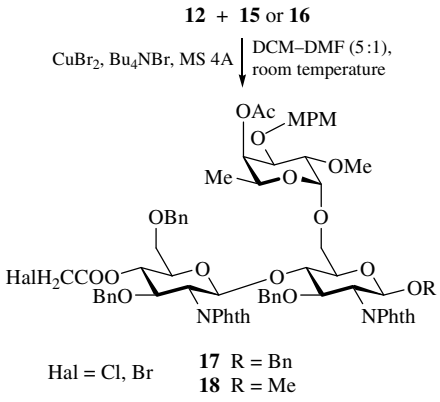
15 R = Bn
16 R = Me

Disaccharide alcohol **16** was obtained analogously in a yield of 93%. Its structure was also proved by NMR spectra.

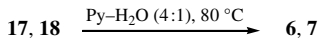
Introduction of an α-L-fucose residue into the 6-position of the reducing glucosamine residue in **15** and **16** was the next step of the synthesis. We used compound **12**[10] as a fucose residue donor, because the combination of protecting groups allows us to get all the possible variants of fucose functionalisation.



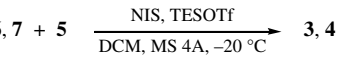
Glycosylation of acceptors **15** and **16** with donor **12** in the presence of $CuBr_2$[6] gave trisaccharide derivatives **17** and **18** in 71 and 77% yields, respectively. The presence of characteristic signals of the fucose methyl group at 1.1 ppm in the ^{1}H NMR spectra of both **17** and **18** proves the trisaccharide formation, and the spin–spin coupling constant $J_{1,2}$ 3.0 Hz for H-1 of fucose clearly shows the formation of α-glycosyl bond.



Heating trisaccharides **17** and **18** in a pyridine–water mixture led to the complete removal of the haloacetyl group. Chromatography of the reaction mixtures gave trisaccharidic glycosyl acceptors **6** and **7** in 98 and 95% yields, respectively.



Glycosylation of trisaccharide derivatives **6** and **7** with a 15% molar excess of disaccharide donor **5** in the presence of NIS and a catalytical amount of TESOTf was accomplished in 15 min according to TLC. After column chromatography of the reaction mixtures, protected pentasaccharides **3** and **4** were isolated in equal yields of 83%.



The presence of all characteristic signals in the ^{1}H and ^{13}C NMR spectra of **3** and **4** together with the spin–spin coupling constants (8.0–8.2 Hz) for H-1 of glucosamine residues proved the structures of the compounds. Additional confirmation of the structures was obtained by two-dimensional ^{1}H–^{1}H ROESY spectroscopy (Figure 1).

The presence of the following cross-peaks, namely, H-1 **C**/H-4 **B**, H-1 **C**/H-6 **B**, H-1 **B**/H-6′ **A** (Figure 1), and H-1 **D**/H-4 **C**, H-1 **D**/H-6′**C** in the ROESY spectrum of **3** unambiguously showed both the presence of a chitotetraose core with β-(1→4) glycoside bonds and α-(1→6) bond between L-fucose and reducing glucosamine residues. Analogous spectral data confirming the structure of **4** were obtained.

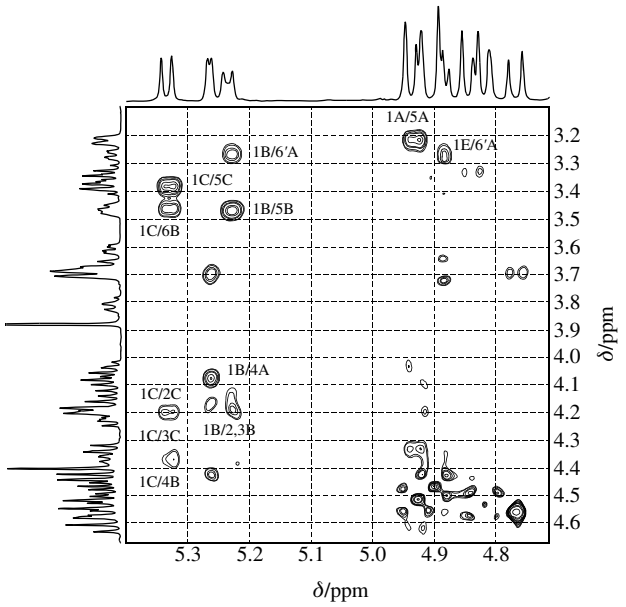
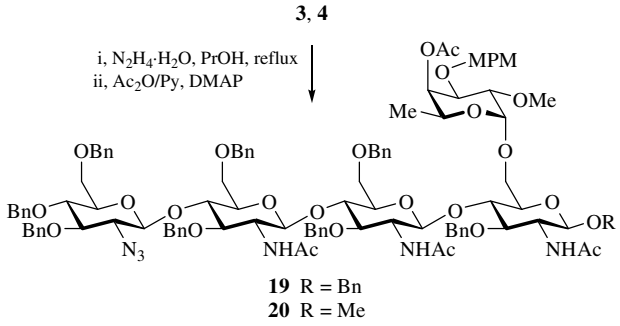


Figure 1 ^{1}H–^{1}H ROESY spectrum of **3**.

For the conversion of pentasaccharide derivatives **3** and **4** into target compounds **1** and **2**, it was necessary to make some manipulations with protecting groups. Primarily, phthalimido groups were replaced with acetamido ones by successive treatment with hydrazine hydrate (without isolation of the corresponding triamine derivative) followed by acetylation in the presence of a catalytic amount of DMAP. Column chromatography of the reaction mixture gave rise to derivatives **19** and **20** in 89 and 87% yields, respectively.

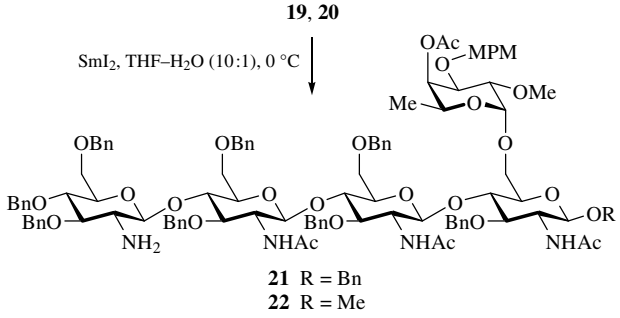


The absence of signals corresponding to phthalimido groups and the presence of three *N*-acetyl signals in the NMR spectra of both **19** and **20** confirmed the completeness of transformation.

Simultaneous reduction of the azido group to the amino one together with the removal of both benzyl and methoxybenzyl groups by hydrogenolysis over a palladium catalyst could be an attractive procedure for the conversion of protected derivatives **19** and **20** into unprotected oligosaccharides with a free amino group at the non-reducing end. It was impossible to carry out this transformation due to incomplete reaction. Probably, it was accounted for by catalyst poisoning with the amine formed because repetitive hydrogenolysis with fresh portions of the catalyst led the reaction to completion.

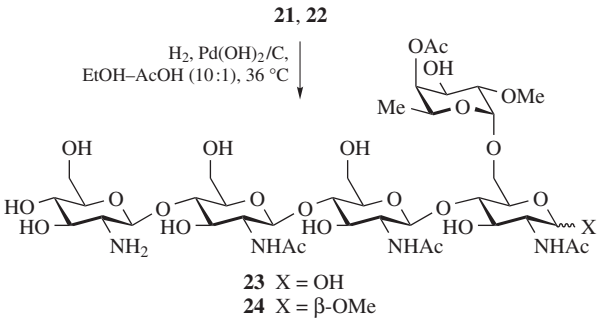
A two-step variation of such conversion was reported,[12] where an azido group was reduced to an amino group with SmI_2 followed by hydrogenolysis of benzyl groups over a palladium catalyst.

After the addition of a THF solution of SmI_2 to solutions of protected **19** and **20** in aqueous THF, TLC of the reaction mixtures showed complete reduction of azido groups into amines, and column chromatography gave pure amino pentasaccharide derivatives **21** and **22** in quantitative yields.



Hydrogenolysis of pentasaccharide amines **21** and **22** in a mixture of EtOH and AcOH with the addition of 0.5% formic acid over 10% $Pd(OH)_2$/C afforded pentasaccharides **23** and **24**, which were isolated by gel filtration on Sephadex G-25 in 85 and 80% yields, respectively. NMR data for **23** and **24** confirmed the complete removal of benzyl and methoxybenzyl groups. High-resolution mass spectra for **23** and **24** comply with the calculated molecular weight (for **23** calculated 990.40, detected 989.39 [M – 1]; for **24** calculated 1004.42, detected 1003.41 [M – 1]). Note that, in the mass spectrum of **23**, a minor signal at 1017.42 was present, which was interpreted as

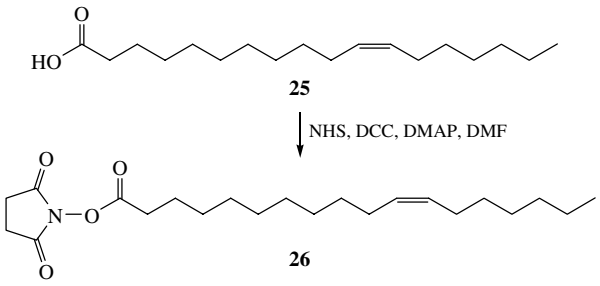
an impurity of a derivative N-ethylated at the non-reducing end. Due to a much lower reaction rate for the acylation of secondary amines in comparison with primary ones, we decided to use substance **23** without further purification.



23 X = OH
24 X = β-OMe

Selective acylation of the amino group in **23** and **24** is the last step in the synthesis of target lipooligosaccharides **1** and **2**. There are many acylation methods, but the most convenient procedure for the acylation of amino groups in the presence of hydroxyls uses the *N*-hydroxysuccinimide esters of corresponding carboxylic acids.

Activated ester **26** was obtained from (*Z*)-11-octadecenoic acid **25**. The ^{1}H and ^{13}C NMR spectra of **26** contained signals at 2.82 and 25.5 ppm, respectively, related to two methylene groups of succinimide; the signals of protons at the double bond remained unchanged,[13] indicating conservation of (*Z*)-configuration of the double bond.



Coupling of **26** with pentasaccharide derivatives **23** and **24** was carried out at room temperature in a DMSO solution in the presence of a catalytic amount of triethylamine. TLC showed complete conversion in 24 h. After column chromatography, compounds **1** and **2** were isolated in 70% yield. At this step, compound **1** was separated from the admixture present in starting compound **23**.

The complete characterisation of target lipooligosaccharides **1** and **2** by NMR spectroscopy was complicated by the impossibility to obtain a solution of sufficient concentration in any of the deuterated solvents or their mixtures. Only general spectra were recorded for **1** and **2**, containing the signals present in the spectra of starting compounds **23** and **24** together with those of vaccenic acid, proving the formation of conjugates. The negative ninhydrin reaction showed the absence of free amino groups. The coincidence of calculated molecular masses for **1** and **2** (1254.65 and 1268.66, respectively) and those recorded by high-resolution mass spectrometry (1253.66 and 1267.67 for [M – 1], respectively) finally proved the structure of the target compounds.

As a conclusion, two lipooligosaccharides **1** and **2** related to Nod factors from *Rhizobium* sp. NGR 234 have been synthesised.

Online Supplementary Materials

Experimental part including both complete synthetic methods and spectral data is presented in Online Supplementary Materials which can be found in the online version at doi:10.1016/j.mencom.2007.01.004.

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