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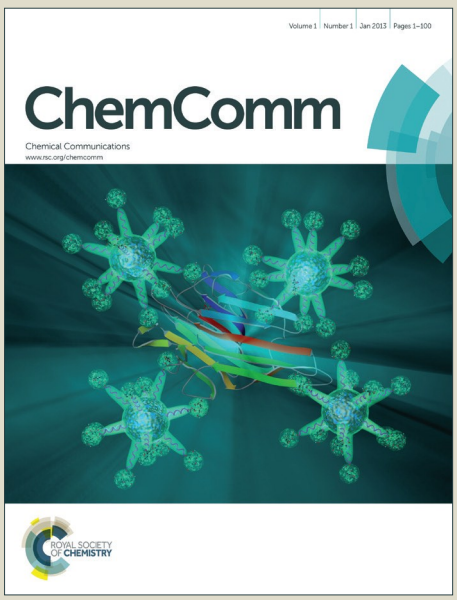

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Synthesis of Bradyrhizose, a Unique Inositol-fused Monosaccharide Relevant to a Nod-factor Independent Nitrogen Fixation

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The symbiosis of *Bradyrhizobium* sp. BTAi1 with its host plant *Aeschynomene indica* relies on a Nod-factor independent mechanism, wherein the *Bradyrhizobium O*-antigen is regarded as a key factor. This *O*-antigen polysaccharide is composed by a unique C10 monosaccharide, namely bradyrhizose, which has a galactose-inositol *trans*-fused scaffold, via a homogeneous α-(1→7)-linkage. Herein, we report the first synthesis of bradyrhizose. The scalable synthesis requires 26 steps in a high overall yield of 9%, with the inositol scaffold being constructed effectively via a Ferrier II rearrangement from a fully functionalized C2 and C4 branched pyranose derivative.

Introduction

Lipochitooligosaccharide Nod factors play a key role in the initiation of the symbiotic associations between legume plants and nitrogen-fixing bacteria.[1] Expressed by bacteria and recognized by plants, Nod factors can cause a series of biological consequences in legume root hairs to result eventually in the so-called nodules that are responsible for the fixation of atmosphere N_2.[2,3] In 2007, Giraud et al. discovered that *Bradyrhizobium* sp. BTAi1, a Gram-negative soil bacterium, was Nod-factor independent in its nitrogen-fixing symbiosis with legume *Aeschynomene indica*.[4] This finding disclosed a different yet unknown nitrogen fixation mechanism.[5,6] Lipopolysaccharides (LPSs), also known as endotoxin, covering 80% of the cell surface of Gram-negative bacteria, are believed to be a key factor in multiple plant-microbe interactions, particularly in the initial recognition.[7,8] Extensive studies have confirmed the necessity of LPSs in either nitrogen fixation or innate defense response.[9-11] LPSs are composed of an *O*-antigen polysaccharide, a core oligosaccharide, and a lipid moiety.[8] The variation of their structures is able to influence the microbe-plant interaction significantly.[12] A structural analysis of *Bradyrhizobium* LPSs revealed an unprecedented *O*-antigen polysaccharide (Figure 1),[13] which consists of a structurally unique bicyclic monosaccharide named bradyrhizose (**1**). This monosaccharide has an unprecedented scaffold of a galactose-like monosaccharide being *trans*-fused to a polyhydroxy inositol, wherein as many as eight oxygen-modified chiral centers are condensed into the ten-carbon skeleton. Connected via an α-(1→7)-glycosidic linkage, the linear *O*-antigen polymer might form a compact helix, which is stabilized by the hydrophilic hydroxyl residues and the hydrophobic carbon backbone.[13] Biological studies showed that this *Bradyrhizobium O*-antigen or LPS could not trigger the innate immunity in different plant families,[13] including its host plant *A. indica*. This is the only unambiguous example so far that an LPS molecule doesn't induce the defense response in plants. Thus, it is hypothesized that the peculiar structure of the *O*-antigen of *Bradyrhizobium* sp. BTAi1 might silence the perception for defense but activate the symbiosis in legume *A. indica*.[14]

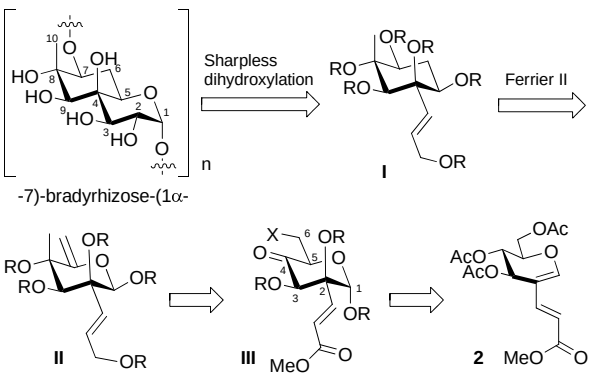
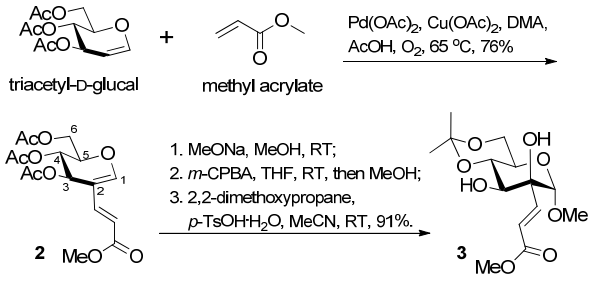


Figure 1. The structure of the *O*-antigen polysaccharide composed of bradyrhizose and the retrosynthetic analysis.

Attracted by the unique and complex structure of bradyrhizose and its significant biological activities relevant to a new mechanism of nitrogen fixation, we embarked on the chemical synthesis and then the biological studies with homogeneous compounds. Here we report an efficient synthesis of bradyrhizose.

Results and discussion

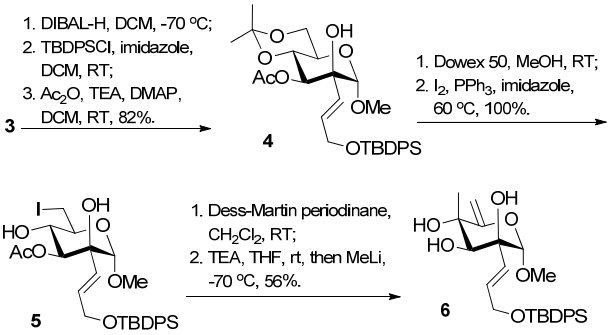
As depicted in Figure 1, a Ferrier II rearrangement on a functionalized pyranoside, such as **II**, would be an effective method to construct the polyhydroxy inositol scaffold.[15,16] The galactose-like moiety could then be elaborated from an allyl branch which had been installed at an early stage.[17] Such a plan would simplify greatly the overall protecting group strategy, with the installation of the galactose 2,3-OH at a later-stage via a Sharpless asymmetric dihydroxylation (on a *trans*-ene derivative, such as **I**) and a selective elaboration of the anomeric aldehyde (and simultaneous formation of the hemiacetal) from a polyol. The Ferrier rearrangement precursor **II** could be prepared from a pyranoside with a carbonyl at C4 (i.e., **III**), which would facilitate the elaboration of the exo-glycal and is also required for the introduction of the methyl branch.[18,19] The *trans*-di-axial hydroxyl groups at C1 and C2 on **III** could be installed by a regio- and stereoselective epoxidation-hydrolysis on the conjugated glycal **2**. To achieve the mostly regio- and stereoselective transformations on the polyhydroxy intermediates, a thoughtful choice of protecting groups is required during the synthesis.



Scheme 1. The synthesis of C2-branched pyranoside **3**. *m*-CPBA = *meta*-chloroperoxybenzoic acid.

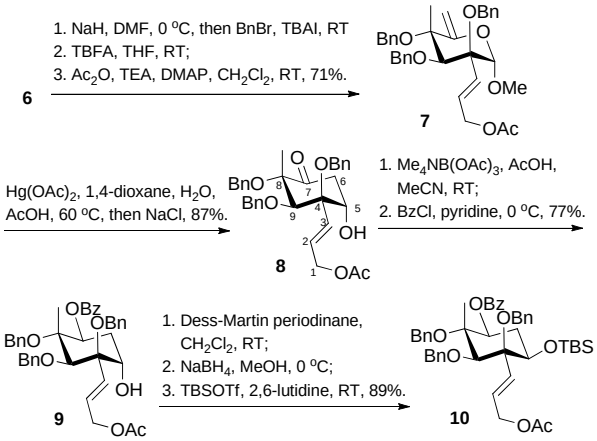
Our synthesis commenced with the preparation of the conjugated glycal **2**, employing modification of a procedure reported recently by Liu et al. (Scheme 1).[17] Thus, replacement of the originally reported $Cu(OTf)_2$ with $Cu(OAc)_2$ and increasing the loading of $Pd(OAc)_2$ to 0.4 equiv. enabled us to obtain **2** in a reproducible ten-gram scale synthesis from the cheap triacetyl glucal and methyl acrylate (76%). We envisioned regio- and stereoselective introduction of the 1,2-diol into diene **2** via the corresponding 1,2-β-epoxide. Previous studies show that the stereoselectivity in epoxidation of a glucal derivative depends on the substituent at C3: a protected hydroxyl group leads to the α-epoxide, while a free hydroxyl group provides the β counterpart.[20,21] Thus, the acetyl groups on **2** were removed (MeONa, MeOH, RT) to give the corresponding triol, which was subjected to epoxidation. After a careful screening of reagents, solvents, and temperatures,[20-24] we were delighted to find that the desired 1,2-β-epoxide could be obtained cleanly with *m*-CPBA in THF at RT; addition of MeOH into the mixture led to the desired C2-branched methyl α-mannoside. THF was proved to be an ideal solvent here: it could dissolve well the polar triol, slow down the epoxidation so as to improve the β-stereoselectivity, and prevent the hydrolysis of the anomeric epoxide until MeOH was added. Further protection of the 4,6-diol with isopropylidene acetal and column purification furnished the α,β-unsaturated ester **3** in an excellent 91% yield (for three steps).

To elaborate an exo-glycal derivative with a methyl branch at C4 (such as **6**), the methyl ester in **3** was reduced into alcohol (with DIBAL-H) which was then selectively protected with a bulky and robust TBDPS group (Scheme 2). Subjection of the resulting 2,3-diol to acetylation led to compound **4**, upon column purification, in a high 82% yield (for three steps). The axial 2-OH was inert toward acetylation, demonstrating the unreactivity of this tertiary hydroxyl group, probably because of steric hindrance.



Scheme 2. The synthesis of the exo-glycal derivative **6**. TBDPS = *tert*-butyldiphenylsilyl.

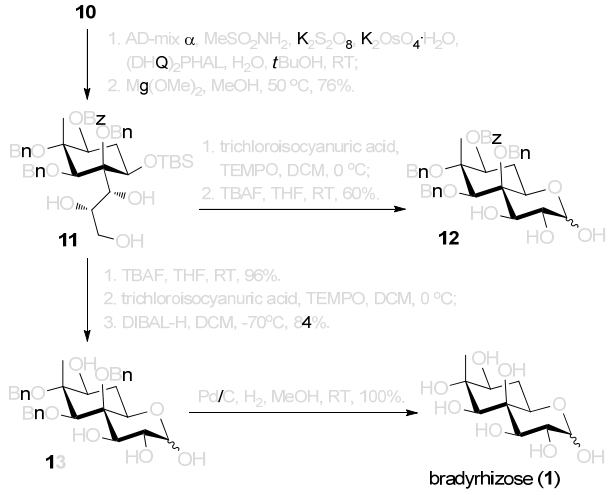
Removal of the 4,6-*O*-isopropylidene acetal in **4** (with acidic resin in MeOH) and subsequent iodination of the resulting primary 6-OH (with I_2/PPh_3) were realized in quantitative yield. Elimination of a C6-iodide to give the corresponding exo-glycal would require a strong base at high temperature and usually result in a modest yield.[16] Thus, we set out to oxidize the 4-OH in **5** into ketone. In fact, in the presence of Dess-Martin periodinane, the desired C4 ketone derivative was readily resulted, which partly underwent elimination to provide the corresponding 5,6-unsaturated 4-ketone. Addition of a weak base (such as Et_3N) at RT, the elimination proceeded smoothly. However, the resultant α,β-unsaturated ketone was unstable upon purification. Thus, the subsequent methylation was conducted in a one-pot fashion, in which MeLi was added at -70 °C once the Et_3N-induced elimination in THF had been complete. Gratifyingly, the desired stereoisomer **6** was obtained in a satisfactory 56% yield (for two steps). It was found that the 3-*O*-acetyl group was removed upon addition of MeLi, the resultant hydroxide then mediated the addition of MeLi onto the C4 ketone from the β-face. The corresponding C4 epimer was not detected at all. In addition, similar addition onto a relevant ketone bearing 3-*O*-benzyl group led to a 1:1 mixture of the diastereoisomer.



Scheme 3. The synthesis of inositol derivative **10**. TBAI = tetrabutylammonium iodide.

A range of attempts at Ferrier II rearrangement with enolate triol **6** led unavoidably to complex mixtures. Thus, the three hydroxyl groups in **6** were blocked by benzyl group (BnBr, NaH, TBAI, DMF, RT) (Scheme 3). The bulky TBDPS group was found to retard the later dihydroxylation of the proximal alkene, thus, was replaced by acetyl group to provide the Ferrier rearrangement precursor **7** in a

high 71% yield (for three steps). Upon treatment of exo-glycal **7** with $Hg(OAc)_2$ and NaCl in a mixed solvent of 1,4-dioxane, H_2O, and AcOH at 60 °C, the rearrangement proceeded smoothly to give cyclohexanone **8** in 87% yield, with the nascent chiral center at C5 being completely controlled. Guided by the axial-oriented 5-OH, reduction of the 7-ketone with $Me_4NB(OAc)_3$ led to the desired 5,7-*trans*-diol stereoselectively; the equatorial 7-OH was then selectively protected by benzoyl group to afford **9** (77% yield for two steps), with the 5,7-di-*O*-benzoate being isolated in 16% yield. Epimerization of the 5-OH in **9** was achieved via oxidation with Dess-Martin periodinane and subsequent reduction with $NaBH_4$; the resulting equatorial 5-OH was then protected with TBS (TBSOTf, 2,6-lutidine, RT) to afford **10** (89% for three steps). The structure of **10** was confirmed by COSY and NOESY NMR analysis. In that, the coupling constants of $J_{H5,H6ax}$ and $J_{H7,H6ax}$ at ~12 Hz indicate the axial disposition of H5 and H7, and the NOE correlation between H9, H5, and H7 verifies their *syn*-triaxial orientation.



Scheme 4. The completion of the synthesis of bradyrhizose (**1**). TEMPO = 2,2,6,6-tetramethylpiperidine-1-oxyl, BAIB = iodobenzene diacetate.

AD-mix α would be the reagent of choice to install stereoselectively the desired diol from *trans*-alkene **10**, which has the medium-sized acetoxymethyl residue at one side and the larger inositol residue at the other.[25] Thus, treatment of **10** with a high concentration of AD-mix α, $MeSO_2NH_2$, and co-oxidant $K_2S_2O_8$ in H_2O and *t*BuOH in the presence of an additional $K_2OsO_4{\cdot}H_2O$ (0.03 equiv.) and $(DHQ)_2PHAL$ (0.10 equiv.) at RT led smoothly to the desired diol (Scheme 4).[26] Side products from the migration of acetyl group were resulted, therefore the resultant mixture was subjected to selective removal of the acetyl group with $Mg(OMe)_2$ in MeOH at 50 °C; the expected triol **11** was obtained in 76% yield (for two steps). It is worth noting that the 5-*O*-TBS group was crucial for the desired stereoselectivity in the present dihydroxylation; reaction with a substrate bearing a free 5-OH led to a pair of the diastereoisomers with a ratio of 1:1. In addition, we also tried the dihydroxylation on the relevant substrates with the acetyl group in **10** being replaced by the electron-donating MOM or PMB group, similar outcomes were attained.

The final stage of the synthesis involves a selective oxidation of the primary 1-OH in triol **11**. In the presence of TEMPO/BAIB under dry conditions, the primary hydroxyl could be oxidized into aldehyde,[27,28] however, cleavage of the C-C bonds of the vicinal triol side chain was unavoidable. Replacing the co-oxidant BAIB with the milder TCCA (trichloroisocyanuric acid) avoided the cleavage and furnished the desired aldehyde smoothly.[29,30] Subsequent deprotection of the 5-*O*-TBS group with TBAF in THF gave the pyranose derivative **12** in 60% yield (for two steps), which was structurally confirmed by its benzoylated derivative (**S1**) via COSY, HSQC and NOESY NMR analysis (see Supporting Information for details). Unexpectedly, the corresponding epimer with an axial-OH at C2 was isolated in 20% yield, testifying that an epimerization took place at the α-position of the aldehyde in the presence of the basic TBAF. Cleavage of the TBS group under acidic conditions, such as with HF in pyridine or with TBAF and AcOH could not avoid the epimerization. Thus, we decided to remove the TBS in **11** before the oxidation. As expected, treatment of the resulting tetraol with TEMPO/TCCA at -30 °C afforded the pyranose **12**, however, over-oxidation of the hemiacetal to the corresponding lactone could not be avoided. In fact, the lactone became the major product when the reaction was performed at 0 °C. Subjection of the crude mixture with DIBAL-H in CH_2Cl_2 at -70 °C led to pyranose **13** cleanly (84% for two steps), wherein the 7-*O*-benzoyl group was deprotected at the same time. Finally, the remaining three *O*-benzyl groups were removed by hydrogenolysis over Pd/C, furnishing the target bradyrhizose quantitatively.

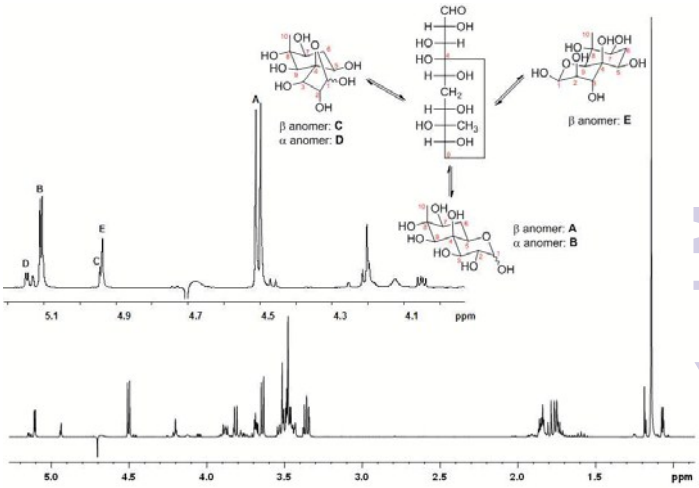


Figure 2. The ^1H NMR spectrum of bradyrhizose. The five different anomeric resonances relative to five different isomers are indicated. The isomeric equilibrium is shown in the inset.

The peculiar structure of the reducing bradyrhizose gives rise in solution to an isomeric equilibrium mixture consisting of two different pyranose forms **A**/**B** (**1**) and **E**, and one furanose form **C**/**D** (Figure 2). The ^1H NMR spectrum in D_2O of the sugar showed anomeric signals ascribable to five spin systems which were identified and completely assigned (Supplementary Table S1 and Figures SA-SB). The main signals (^1H at 4.50 and 5.10 ppm, 55.7% and 27.8% as relative abundance) were attributed to the β and α anomers of pyranose form **A**/**B** (**1**). The long range correlation in the HMBC spectrum of **A**/**B**1 with **A**/**B**5 confirmed the type of ring closure (Supplementary Figure SD). The anomeric configurations of **A** and **B** were supported by the values of $^3J_{H1,H2}$ coupling constants (8.07 and 3.90 Hz) and $^1J_{C,H}$ coupling constants (162.8 and 170.0 Hz, respectively). Within these two spin systems, there were five peaks corresponding to ring protons (H1, 2, 3, 5, 7), a methylene group (H6) and two singlet signals, among which one corresponded to a methyl group (H10) and the other one was identified as an isolated C*H*-OH (H9). Both the anomeric positions correlated with H2 and H3; the multiplicities and the ring coupling constant values confirmed the axial disposition of H2 and H3 protons; the diastereotopic methylene ($H6_{ax}$/$H6_{eq}$) correlated with signals identified as H7 and H5 (Supplementary Figures SB-SD). The relative configuration of bradyrhizose was confirmed by the analysis

of HSQC and HSQC-NOESY (Supplementary Figure SE). In particular, the *intra*-residue NOE contacts between H3 with H5, H7 and H9 indicated their *syn-diaxial* orientation. The *axial* orientation of CH_3 was endorsed by the *intra*-residual NOE with the axial H6.

A second couple of anomeric signals, ^{1}H at 5.14 and 4.93 ppm (both 4.4% as relative abundance), was attributed to the α and β anomers of furanose form **D**/**C**. The long range correlation in the HMBC spectrum of **C**/**D**1 with **C**/**D**4 was a proof of the furanose ring closure (Supplementary Figure SD and Table S1). The fifth spin system with ^{1}H at 4.93 ppm (7.7% as relative abundance) was attributed to the β anomer of pyranose form **E**, formed by the alternative ring closure between positions 1 and 9. The long range correlation in the HMBC spectrum of **E**1 with **E**9 was a proof of the alternative pyranose ring closure (Supplementary Figure SD). It is worth of note that the corresponding α anomer was not detectable by NMR.

Thus, in D_2O there were three different bradyrhizose isomers, of which form **A**/**B** (**1**) accounted for the majority (83.5%) of the species present in solution; less abundant were the furanose forms **C**/**D**, abundance around 8.8%; the alternative pyranose ring closure **E** accounted for 7.7% of the total. We have also assigned and confirmed bradyrhizose isomeric mixture in organic solvents favoring intramolecular hydrogen bond, namely DMSO-d_6 (Supplementary Table S2 and Figures SF-SG) and TFE-d_3 (Figures SH-SI).[31] In both cases, there was no consistent equilibrium shift and the abundance of the bradyrhizose isomers was quite comparable, only a slight decrease of pyranose form **1** (76.7% and 78.6% for DMSO and TFE, respectively) was detected, corresponding to an increase of the abundance of furanose isomer **C**/**D** and the alternative pyranose **E**.

Conclusion

The first synthesis of the polyhydroxy inositol-fused monosaccharide bradyrhizose has been achieved in 26 steps with a high overall yield of 9% from triacetyl glucal, with the inositol scaffold being constructed effectively via a Ferrier II rearrangement from a fully functionalized C2 and C4 branched saccharide derivative. All the reactions were scalable with excellent regio- and stereoselectivities, and only twelve column purifications were required altogether. The judicious choice of protecting groups played an important role in the stereoselective epoxidation, methylation, and dihydroxlation. The synthetic bradyrhizose was fully characterized by NMR spectroscopy, disclosing an isomeric equilibrium consisting of two different pyranose forms **A**/**B** (**1**) and **E**, and one furanose form **C**/**D**.

Rhizobia have received significant attention in agriculture due to their distinctive feature in nitrogen fixation, which could reduce the use of chemical *N*-fertilizer. The remarkable structural diversity in the LPS in rhizobia is deemed as a strategy to modulate plant defence responses, so as to facilitate the establishment of symbiosis. The present availability of the well-equipped bradyrhizose derivatives (i.e., **12**), which is readily convertible into glycosylation donors and acceptors, is the first step toward the synthesis of the homogeneous oligosaccharides relevant to the *Bradyrhizobium O*-antigen for understanding their structure-activity relationship and mechanism of the unique nitrogen-fixing symbiosis.[13,32]

Acknowledgements

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Notes and references

1. P. Lerouge, P. Roche, C. Faucher, F. Maillet, G. Truchet, J. C. Prome and J. Denarie, *Nature*, 1990, **344**, 781-784.
2. M. Parniske and J. A. Downie, *Nature*, 2003, **425**, 569-570.
3. W. D'Haeze and M. Holsters, *Glycobiology*, 2002, **12**, 79R-105R.
4. E. Giraud, L. Moulin, D. Vallenet, V. Barbe, E. Cytryn, J. C. Avarre, M. Jaubert, D. Simon, F. Cartieaux, Y. Prin, G. Bena, L. Hannibal, J. Fardoux, M. Kojadinovic, L. Vuillet, A. Lajus, S. Cruveiller, Z. Rouy, S. Mangenot, B. Segurens, C. Dossat, W. L. Franck, W. S. Chang, E. Saunders, D. Bruce, P. Richardson, P. Normand, B. Dreyfus, D. Pignol, G. Stacey, D. Emerich, A. Vermeglio, C. Medigue and M. Sadowsky, *Science*, 2007, **316**, 1307-1312.
5. G. E. D. Oldroyd and J. A. Downie, *Annu. Rev. Plant Biol.*, 2008, **59**, 519-546.
6. G. E. D. Oldroyd, *Nat. Rev. Microbiol.*, 2013, **11**, 252-263.
7. C. R. H. Raetz, *Annu. Rev. Biochem*, 1990, **59**, 129-170.
8. M. Caroff and D. Karibian, *Carbohydr. Res.*, 2003, **338**, 2431-2447.
9. A. Silipo, G. Erbs, T. Shinya, J. M. Dow, M. Parrilli, R. Lanzetta, N. Shibuya, M. A. Newman and A. Molinaro, *Glycobiology*, 2010, **20**, 406-419.
10. M. A. Newman, J. M. Dow, A. Molinaro and M. Parrilli, *J. Endotoxin Res.*, 2007, **13**, 69-84.
11. I. Lerouge and J. Vanderleyden, *FEMS Microbiol. Rev.*, 2002, **26**, 17-47.
12. C. R. H. Raetz and C. Whitfield, *Annu. Rev. Biochem.*, 2002, **71**, 635-700.
13. A. Silipo, M. R. Leone, G. Erbs, R. Lanzetta, M. Parrilli, W. S. Chang, M. A. Newman and A. Molinaro, *Angew. Chem. Int. Ed.*, 2011, **50**, 12610-12612.
14. M. A. Newman, T. Sundelin, J. T. Nielsen and G. Erbs, *Front. Plant Sci.*, 2013, **4**, 139.
15. R. J. Ferrier, *J. Chem. Soc., Perkin Trans. 1*, 1979, 1455-1458.
16. R. J. Ferrier and S. Middleton, *Chem. Rev.*, 1993, **93**, 2779-2831.
17. Y. Bai, J. Zeng, S. Cai and X. W. Liu, *Org. Lett.*, 2011, **13**, 4394-4397.
18. M. Miljkovic, M. Gligorijevic, T. Satoh and D. Miljkovic, *J. Org. Chem.*, 1974, **39**, 1379-1384.
19. B. G. Davis, R. J. Nash, A. A. Watson, C. Smith and G. W. J. Fleet, *Tetrahedron*, 1999, **55**, 4501-4520.
20. I. Marín, M. I. Matheu, Y. Díaz and S. Castillón, *Adv. Synth. Catal.*, 2010, **352**, 3407-3418.
21. I. Marin, J. Castilla, M. I. Matheu, Y. Diaz and S. Castillon, *J. Org. Chem.*, 2011, **76**, 9622-9629.
22. J.-Y. Kim, V. D. Bussolo and D. Y. Gin, *Org. Lett.*, 2001, **3**, 303-306.
23. G. Bellucci, G. Catelani, C. Chiappe and F. D'Andrea, *Tetrahedron Lett.*, 1994, **35**, 8433-8436.
24. V. Bilik and S. Kucar, *Carbohydr. Res.*, 1970, **13**, 311-313.
25. B. Meunier, S. P. de Visser and S. Shaik, *Chem. Rev.*, 2004, **104**, 3947-3980.
26. H.-S. Byun, E. R. Kumar and R. Bittman, *J. Org. Chem.*, 1994, **59**, 2630-2633.
27. A. De Mico, R. Margarita, L. Parlanti, A. Vescovi and G. Piancatelli, *J. Org. Chem.*, 1997, **62**, 6974-6977.
28. T. Vogler and A. Studer, *Synthesis*, 2008, **2008**, 1979-1993.
29. L. De Luca, G. Giacomelli and A. Porcheddu, *Org. Lett.*, 2001, **3**, 3041-3043.
30. M. Angelin, M. Hermansson, H. Dong and O. Ramstrom, *Eur. J. Org. Chem.*, 2006, **2006**, 4323-4326.
31. A. Molinaro, C. De Castro, R. Lanzetta, E. Manzo and M. Parrilli, *J. Am. Chem. Soc.*, 2001, **123**, 12605-12610.
32. A. Silipo, G. Vitiello, D. Gully, L. Sturiale, C. Chaintreuil, J. Fardoux, D. Gargani, H. I. Lee, G. Kulkarni, N. Busset, R. Marchetti, A. Palmigiano, H. Moll, R. Engel, R. Lanzetta, L. Paduano, M. Parrilli, W. S. Chang, O. Holst, D. K. Newman, D. Garozzo, G. D'Errico, E. Giraud and A. Molinaro, *Nat. Commun.*, 2014, **5**, 5106.

