Carbohydrates

DOI: 10.1002/anie.201106548

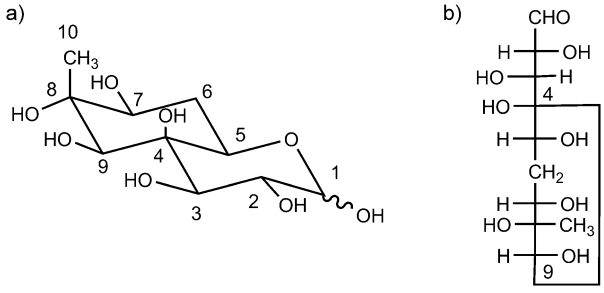
A Unique Bicyclic Monosaccharide from the *Bradyrhizobium* Lipopolysaccharide and Its Role in the Molecular Interaction with Plants

*Alba Silipo, Maria R. Leone, Gitte Erbs, Rosa Lanzetta, Michelangelo Parrilli, Woo-Suk Chang, Mari-Anne Newman, and Antonio Molinaro**

Rhizobia, well-known bacterial actors of symbiosis, are Gram-negative soil bacteria that fix nitrogen into ammonia after becoming established inside root or stem nodules of legumes. A key step for the correct establishment of the symbiotic process is the molecular recognition between the host plant and the microbe occurring in the early stage of the association.[1] The universally accepted "classic" mechanism of recognition relies on the detection of flavonoids by bacteria. The flavonoids are secreted by a host legume plant and trigger the expression of bacterial *nod* genes, the gene products of which (Nod factors) are in turn recognized by the host plant.[1] This recognition can lead to such early processes as root hair deformation, cell wall degradation, and infection thread formation.[1] Recently, it has been demonstrated that not all symbiotic processes rely on this mechanism; for example, the nitrogen-fixing symbiosis between *Aeschynomene indica* and *Bradyrhizobium* sp. BTAi1 does not require Nod factors to establish their symbiotic relationship and thus relies on a still unknown mechanism.[2] Because it has been established that lipopolysaccharides (LPSs) play an important role in the molecular recognition between bacteria and host plants,[3,4] we investigated if LPSs could be a key factor in the early stage of the symbiotic process in the *A. indica* and *Bradyrhizobium* Nod-factor-independent system. As ubiquitous and vital components of the cell surface of Gram-negative bacteria, LPSs have multiple key roles in plant–microbe interactions.[3,4] Defects in the LPS structure cause impairment of infection and nodule development on various legume plants; this phenomenon has been attributed to LPS-dependent promotion of bacterial attachment to plant surfaces.[3–5] The perception of LPS by plant cells can either lead to the direct triggering of defense responses or can prime the plant for a more rapid response.[3–5] The ability of a host organism to recognize LPSs in the plant–microbe interaction is currently the subject of considerable interest. From a chemical point of view, LPSs consist of a hydrophilic heteropolysaccharide (formed by an O-antigen polysaccharide and a core oligosaccharide) that is covalently linked to a lipophilic moiety termed lipid A, which is embedded in the outer leaflet of the membrane.[6]

We demonstrated that a synthetic LPS O-antigen could play a key role in the molecular recognition of a bacterium by its plant host;[7] in particular, the O-antigen of *Xanthomonas campestris* pv. *campesteris* (*Xcc*) is a strong elicitor of *Arabidopsis thaliana* innate immunity, whereas the LPS O-antigen from *Burkholderia rhizoxinica* is crucial for its endosymbiosis with the fungus *Rhizopus microspores*.[8]

Herein, we have identified a unique carbocyclic monosaccharide as the only component of the O-antigen of the *Bradyrhizobium* LPS and established its role as one of the key molecular determinants of the microbe–plant interaction. This sugar has been isolated and identified for the first time and has a new and unique structure with no analogues in nature. It has been named bradyrhizose (Scheme 1 a, b) and



Scheme 1. a) The bicyclic structure and b) the Fischer projection of bradyrhizose.

consists of a methylated polyhydroxy cyclohexane ring that is fused to a six-membered monosaccharide by a *trans*-decaline junction to form a ten-carbon-atom bicyclic sugar. The bradyrhizose homopolymer has been characterized by a combination of chemical analyses, mass spectrometry, and 2D NMR spectroscopy. The ^{1}H NMR spectrum showed a very simple set of signals (Figure 1) that suggested a homopolymeric structure. This conclusion was also supported by the ^{13}C NMR spectrum, which showed only ten signals (see the

[*] Prof. Dr. A. Silipo, Dr. M. R. Leone, Prof. R. Lanzetta, Prof. M. Parrilli, Prof. A. Molinaro
Department of Organic Chemistry and Biochemistry
University of Naples "Federico II"
Via Cinthia 4, 80126 Napoli (Italy)
E-mail: molinaro@unina.it
Dr. G. Erbs, Prof. M.-A. Newman
Department of Plant Biology and Biotechnology
Faculty of Life Sciences, University of Copenhagen
Thorvaldsensvej 40, 1871 Frederiksberg C (Denmark)
Prof. W.-S. Chang
Department of Biology, University of Texas-Arlington
Arlington, TX 76019 (U.S.A.)

Supporting information (including experimental details) for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201106548.

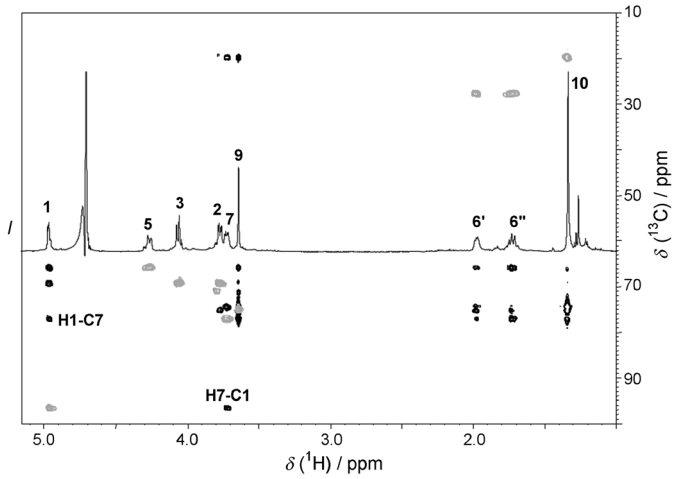


Figure 1. ^{1}H NMR spectrum (black solid trace, left axis) of the bradyrhizose homopolymer O-antigen, in which all the spin systems are shown (for numbering scheme, see Scheme 1). Furthermore, the HSQC spectrum (gray, right axis) is shown, in which all the proton signals are correlated to their respective carbon signals. In the HMBC spectrum (black, right axis), overlapped to the HSQC, all the main important and inter-residual scalar correlations are present; those of the glycosidic linkage are marked.

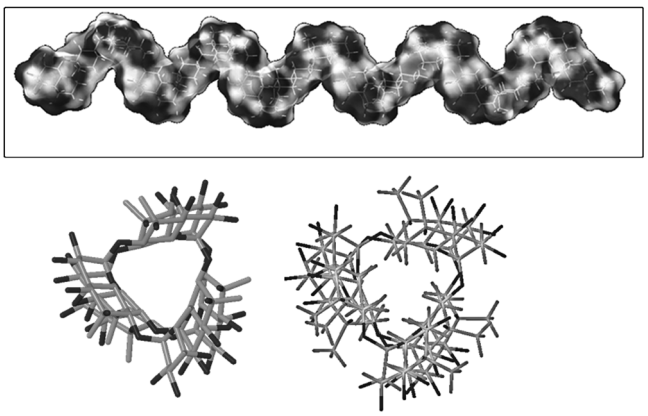


Figure 2. Top: Connelly surface of bradyrhizose eicohexasaccharide in a significant conformation. Bottom: two different sketches of the hydrophobic tunnel inside the helix in the bradyrhizose octasaccharide with methyl groups pointing outwards.

Supporting Information for complete chemical and spectroscopic analyses and full structural discussion, Figures S1–S6, Table S1).

The pyranose ring closure was shown to be due to the reaction of the aldehyde at C-1 with the hydroxy group at C-5. The alternative ring formation with the methyne group at C-9 was ruled out on the basis of the long-range correlation in the HMBC NMR spectrum between H-1 and a carbon signal at 65.8 ppm that belongs to a spin system adjacent to the methylene group at C-6; thus the carbon signal was attributed to C-5. The position of the methyl group at C-8 was suggested to be axial because of an observed NOE contact with the H-6_{ax} proton. The absolute configuration of bradyrhizose (Scheme 1) was determined by circular dichroism spectroscopy using the exciton chiral coupling method (see the Supporting Information for complete chemical derivatization and circular dichroism spectroscopic analyses).[9]

To assess the conformational behavior of the bradyrhizose polymer, a molecular mechanics and dynamics calculation analysis was carried out (the complete discussion and experimental details are given in the Supporting Information).[10] The conformational analysis showed that the polymer tends to adopt a compact two-fold right-handed helicoidal structure, in which all methyne groups are directed inward to create a hydrophobic tunnel inside the helix, whereas all hydroxy and methyl groups are pointing away from the helix and are exposed to the external environment (Figure 2 and Figure S13 in the Supporting Information). These data show that the bradyrhizose polysaccharide is characterized by a specific supramolecular architecture. Further experiments are needed to understand the biological significance of such an interesting three-dimensional arrangement.

In summary, we established a new and unprecedented carbohydrate structure from *Bradyrhizobium* LPS by a combination of chemical and spectroscopic approaches. For the systematic name we suggest 4,9-*cyclo*-6-deoxy-8-*C*-methyl-D-*xylo*-D-*galacto*-nonose and as a common name bradyrhizose. The polysaccharide is arranged in an α-(1→7) homopolymeric repeating unit. The only previous case of a carbocyclic sugar with related structural characteristics, the caryose, was also found in our lab as a LPS O-chain component of *Burkholderia caryophylli*, a pathogenic bacterium interacting with a plant.[11] However, caryose (4,8-*cyclo*-3,9-dideoxy-L-*erythro*-D-*ido*-nonose) is fundamentally different, as it is a 3-deoxy monosaccharide. Although its biosynthesis is unknown, the presence of a 3-deoxy group suggests a biosynthesis in which a six-carbon sugar undergoes typical condensation with a phosphoenol pyruvate C3 carbon chain, in analogy to the biosynthesis of sialic acid and its derivatives.[12] On the other hand, any hypothesis on the biosynthesis of bradyrhizose is impaired by its extremely unusual chemical structure with no parallel in nature.

Previous work on the effects of LPS from bacterial symbionts of plants indicates the occurrence of variations in their LPS structure, which influence the elicitation of host defenses.[13] Therefore, we decided to investigate the role of the bradyrhizose polymer in its interaction with plants.

We tested pure LPS from *Bradyrhizobium* sp. BTAi1 and its bradyrhizose polymer for their ability to activate the innate immune system (by elicitation of the oxidative burst) in two model plants, *Lotus japonicus* and *Arabidopsis thaliana*, as well as in its symbiotic host plant *A. indi*ca. *L. japonicus* was chosen as a model legume representing the symbiotic host plant. As a control, we used the flagellin peptide flg22.[14] The rationale of these experiments was to shed light on the initial stage of this symbiosis at a molecular level and to evaluate the role of the LPS and its bradyrhizose polymer as inducers of plant immunity (see the Supporting Information for complete discussion). None of the three plants recognized *Bradyrhizobium* LPS (Figure 3 a–c) or its bradyrhizose O-antigen (Figure 3 d, e) as a microbe-associated molecular pattern

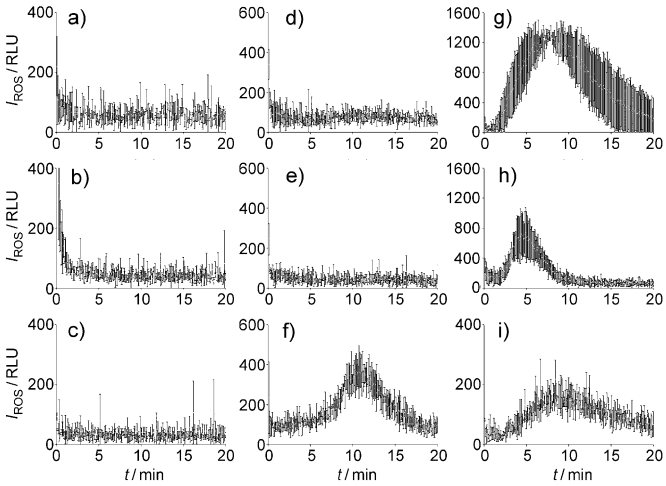


Figure 3. Elicitation of the oxidative burst in *A. thaliana*, *L. japonicus*, or *A. indi*ca. Generation of reactive oxygen species (ROS) was measured as peroxide with a luminol assay and expressed as relative light units (RLU) in leaves of *A. thaliana* (a, d, f, and g), *L. japonicus* (b, e, and h), or *A. indica* (c and i) after exposure to LPS (50 $\mu g\,mL^{-1}$) from either *Bradyrhizobium* (a, b, and c), the O-antigen from *Bradyrhizobium* LPS (50 $\mu g\,mL^{-1}$, d and e), or LPS from the core oligosaccharide from *Xcc* (f), or flg22 (100 nM, g, h, and i). Water did not induce an oxidative burst. Data are the means of at least four replicates. Error bars show the standard deviation.

(MAMP), whereas flg22 in all three plants induced the innate immune response (Figure 3 g–i). As a positive control, we tested that *A. thaliana* generally responded to LPS from plant pathogens; the O-antigen from the *Xcc* LPS was also tested (Figure 3 f). In agreement with previous data, both of these molecules induced defense responses in *Arabidopsis*.[3,4,7]

Our results show that the LPS, with its corresponding O-antigen, from the symbiotic *Bradyrhizobium* sp. BTAi1 does not trigger the innate immune response in different plant families, likely owing to its particular molecular characteristics, that is the peculiar chemical structure and the three-dimensional arrangement. In fact, we also demonstrate that the putative LPS receptor(s) in *A. thaliana* does not recognize LPS from *Bradyrhizobium* sp. BTAi1. On the other hand, flg22 induced the oxidative burst in all of the three plants, thus indicating an ability of both legume and nonlegume plants to respond to elicitation by MAMPs in general.

To our knowledge, this is the first clear and unequivocal example of an LPS molecule to which plants do not react with a defense response. The recognition of LPSs is an important prerequisite for the successful symbiosis in many plants.[1,3,5,8] Like other legumes, *A. indica* relies heavily on nitrogen fixation by symbiotic bacteria and could either have evolved an ability to suppress its induced innate immune responses or an ability to lose the receptors for important immune elicitors from symbiotic bacteria. Both of these models could play a role in a successful symbiosis between *Bradyrhizobium* sp. BTAi1 and its host *A. indi*ca. We favor the idea that the symbiotic bacterium has evolved into a unique LPS producer by changing the structure of the LPS O-antigen to avoid "harmful" recognition by its symbiotic host.

In summary, *Bradyrhizobium* sp. BTAi1 is coated with a unique LPS that does not induce innate immune responses in its host plant or in different plant families, owing to its peculiar chemical nature.

Received: September 15, 2011
Published online: November 4, 2011

Keywords: carbohydrates · lipopolysaccharides · rhizobia · structure elucidation · symbiosis

[1] W. D'Haeze, M. Holsters, *Glycobiology* **2002**, *12*, 79R – 105R.
[2] 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. Verméglio, C. Médigue, M. Sadowsky, *Science* **2007**, *316*, 1307 – 1312.
[3] M. A. Newman, J. M. Dow, A. Molinaro, M. Parrilli, *J. Endotoxin Res.* **2007**, *13*, 69.
[4] A. Silipo, G. Erbs, T. Shinya, J. M. Dow, M. Parrilli, R. Lanzetta, N. Shibuya, M. A. Newman, A. Molinaro, *Glycobiology* **2010**, *20*, 406.
[5] I. Lerouge, J. Vanderleyden, *FEMS Microbiol. Rev.* **2002**, *26*, 17.
[6] A. Silipo, C. De Castro, R. Lanzetta, M. Parrilli, A. Molinaro in *Prokaryotic Cell Wall Compounds—Structure and Biochemistry* (Eds.: H. König, H. Claus, A. Varma), Springer, Heidelberg, **2010**, p. 133.
[7] E. Bedini, C. De Castro, G. Erbs, L. Mangoni, J. M. Dow, M. A. Newman, M. Parrilli, C. Unverzagt, *J. Am. Chem. Soc.* **2005**, *127*, 2414 – 2416.
[8] M. R. Leone, G. Lackner, A. Silipo, R. Lanzetta, A. Molinaro, C. Hertweck, *Angew. Chem.* **2010**, *122*, 7638 – 7642; *Angew. Chem. Int. Ed.* **2010**, *49*, 7476 – 7480.
[9] N. Harada, K. Nakanishi, *Circular Dichroic Spectroscopy—Exciton Coupling in Organic Stereochemistry*, Oxford University Press, Oxford, **1983**.
[10] T. Ieranò, A. Silipo, P. Cescutti, M. R. Leone, R. Rizzo, R. Lanzetta, M. Parrilli, A. Molinaro, *Chem. Eur. J.* **2009**, *15*, 7156 – 7166.
[11] A. Molinaro, C. De Castro, B. O. Petersen, J. Ø. Duus, M. Parrilli, O. Holst, *Angew. Chem.* **2000**, *112*, 160 – 165; *Angew. Chem. Int. Ed.* **2000**, *39*, 156 – 160.
[12] T. Angata, A. Varki, *Chem. Rev.* **2002**, *102*, 439 – 469.
[13] C. H. R. Raetz, C. Whitfield, *Annu. Rev. Biochem.* **2002**, *71*, 635 – 700.
[14] C. Zipfel, S. Robatzek, L. Navarro, E. J. Oakeley, J. D. Jones, G. Felix, T. Boller, *Nature* **2004**, *428*, 764 – 767.

