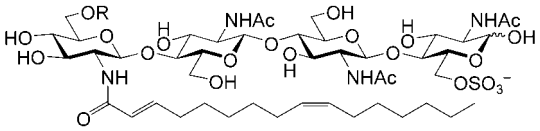
Glycolipid Mimics

Simple Synthesis of Nodulation-Factor Analogues Exhibiting High Affinity towards a Specific Binding Protein**

*Nathalie Grenouillat, Boris Vauzeilles, Jean-Jacques Bono, Eric Samain, and Jean-Marie Beau**

Rhizobial glycolipids (lipochitooligosaccharides (LCOs), also called nodulation (Nod) factors) are important signaling molecules involved in the establishment of symbiosis with legumes.[1] This association results in the formation of rhizobia-infected nodules on plant roots in which atmospheric nitrogen is reduced to ammonia for the benefit of the plant. These nodulation factors represent a large family of lipo-oligosaccharides consisting of a chitin fragment (three–five *N*-acetyl-D-glucosamine units) with an *N*-acyl chain attached to the nonreducing unit and a variety of additional substituents.[1a] The variations are characteristic of each bacterial strain and define the high degree of specificity in the legume–rhizobia interaction. One of the most studied models is the symbiosis of *Sinorhizobium meliloti* and alfalfa (*Medicago sativa*) in which the major bacterial signal molecule **1** (Scheme 1) contains four D-glucosamine residues with a 6-



1 R = Ac; NodSm-IV (Ac, S)
2 R = H; NodSm-IV (S)

Scheme 1. Structures of the major nodulation factors produced by *Sinorhizobium meliloti*.

O-acetyl group and an *N*-(2*E*,9*Z*)-hexadecadienoyl side chain at the nonreducing residue and an essential 6-*O*-sulfate group at the reducing unit which is the main determinant of the host specificity in *M. sativa*.[2]

Although highly active, signal molecule **2** is approximately ten times less active than the acetylated derivative **1** in morphogenic activity. However, since it is more stable, **2** is more suitable for biological studies. Structure–function relationship studies have shown that the correct nature of the lipid chain, in totally synthetic molecules,[3] is essential for triggering an optimal morphogenic activity in *M. sativa* roots.[4] Moreover, the length and the structure of the lipid chain play a role in the high-affinity binding of LCOs to a Nod factor binding site (NFBS2) characterized in cell suspension cultures of legumes.[5–7]

As a step forward in the search for modified molecules for the study of the Nod factor perception mechanisms by the plant, we now report a simple synthesis of analogues by using metabolically engineered bacterial cells, a method that avoids the complexity of total synthetic approaches from *N*-acetyl-D-glucosamine monomers.[3,8] We further demonstrate that the incorporation of a substituted benzamide (see **B** in Scheme 2) provides new analogues able to interact with a putative LCO receptor at nanomolar concentrations.

Scheme 2. An alkoxybenzamide (X = O) or benzylamine (X = H_2) structure **B** can mimic the natural α,β-unsaturated amide **A**.

The aromatic motif was selected because a) it leads to benzamides that are potentially more stable chemically and metabolically than the natural conjugated amides, b) it makes it possible to introduce a photoactivatable group at a strategic position, and c) it is readily prepared from commercial precursors. Although replacement of unsaturated bonds in lipids by aromatic derivatives is frequently used to produce molecular probes and agonists or antagonists of bioactive molecules, this substitution combining an aromatic motif and

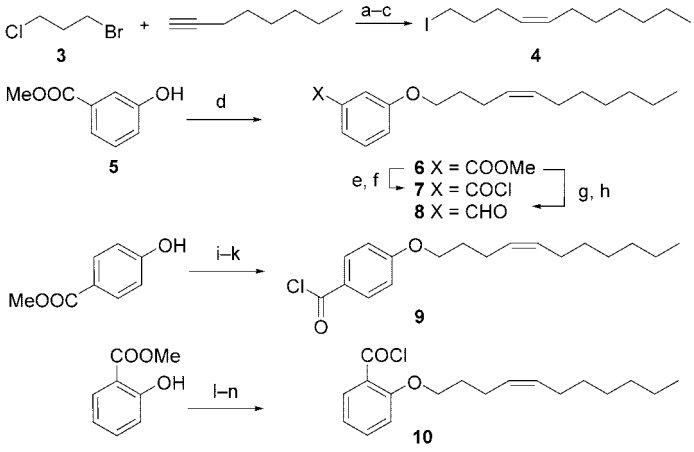
[*] N. Grenouillat, Dr. B. Vauzeilles, Prof. J.-M. Beau
Université Paris-Sud
Laboratoire de Synthèse de Biomolécules
associé au CNRS, Institut de Chimie Moléculaire et des Matériaux
91405 Orsay Cedex (France)
Fax: (+33) 1-6985-3715
E-mail: jmbeau@icmo.u-psud.fr
Dr. J.-J. Bono
Pôle de Biotechnologie Végétale
UMR 5546 CNRS–Université Paul Sabatier
24, Chemin de Borde Rouge, BP 17 Auzeville
31326 Castanet-Tolosan (France)
Dr. E. Samain
Centre de Recherches sur les Macromolécules Végétales
UPR CNRS 5301, BP 53
38041 Grenoble Cedex 9 (France)

[**] We are grateful to Rhone-Poulenc SA (contract no. 041313.00), Aventis CropScience SA, and Bayer CropScience SA for the financial support of this study.

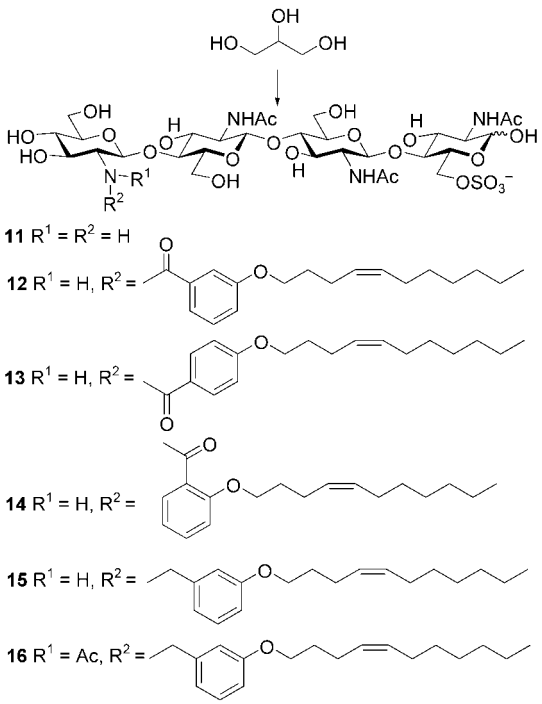
  DOI: 10.1002/ange.200460275

a heteroatom to mimic a carbon–carbon double bond *and* a methylene group in the acyclic system has, to our knowledge, never been described.[9]

We started our synthesis by preparing the unsaturated fatty acid analogues **6**, **9**, and **10** as outlined in Scheme 3.



Scheme 3. Synthesis of the side-chain precursors. a) BuLi, HMPA (1 equiv), THF, −78 °C then reflux, 4 h, 80%; b) Lindlar catalyst, quinoline, cyclohexane, H_2, 25 °C, 7 h, 94%; c) NaI (3 equiv), acetone, reflux, 24 h, 89%; d) **4** (1.1 equiv), K_2CO_3 (1.1 equiv), DMF, 90 °C, 4 h, 76%; e) NaOH (2 equiv), MeOH, reflux, overnight, 96%; f) oxalyl chloride, cat. DMF, CH_2Cl_2, 25 °C, 2 h, 99%; g) $LiAlH_4$ (2 equiv), Et_2O, RT, 1.5 h, 99%; h) PCC (2 equiv), CH_2Cl_2, reflux, 1 h, 99%; i) as (d), 7 h, 79%; j) as (e), 98%; k) as (f), 99%; l) as (d), 8 h, 66%; m) as (e), 24 h, 99%; n) as (f), 99%. DMF = *N*,*N*-dimethylformamide, HMPA = hexamethylphosphoramide, PCC = pyridinium chlorochromate.



Scheme 4. Synthesis of nodulation factor analogues (for conditions, see text.)

Coupling of lithiated 1-octyne with commercially available 1-bromo-3-chloropropane (**3**), followed by a Lindlar reduction and a Cl/I exchange gave the expected unsaturated iodide **4**. Alkylation of methyl 3-hydroxybenzoate (**5**) with iodide **4** provided ester **6**, which could be transformed into acid chloride **7** or aldehyde **8**. The *p*- and *o*-isomeric acid chlorides **9** and **10** were also prepared by following the same synthetic sequence from the corresponding *p*- and *o*-substituted phenols.

The carbohydrate segment, the sulfated tetramer **11** (Scheme 4), was easily prepared by cultivating, at high cell density, recombinant *Escherichia coli* strains coexpressing the *nodBC* genes (encoding the chitooligosaccharide synthase and the chitooligosaccharide *N*-deacetylase) and the *nodH* gene (encoding the chitooligosaccharide sulfotransferase) from *Sinorhizobium meliloti*.[10] Glycerol was provided as the carbon source.

The oligosaccharide recovered from the cell extracts by using the published procedure[10b] contained about 25% of the pentamer and was further purified by column chromatography on silica gel. *N*-Acylation of tetrasaccharide **11** with acid chlorides **7**, **9**, and **10** was performed in DMF containing water and provided the corresponding benzamides **12**–**14** in 33, 40, and 48% yields, respectively (60% conversion). Benzyl amine **15** and its *N*-acetyl derivative **16** were also prepared by reductive alkylation of amine **11** with aldehyde **8**.

The synthetic analogues **12**–**15** were tested, and compared with the NodSm-IV(S) molecule (**2**), for their ability to interact with the binding protein NFBS2 associated with a membrane preparation of *M. varia*. The results (Table 1) show that, in comparison with the reference compound **2**, the affinity for NFBS2 of analogues **12**, **13** and **15** was high and in the same 20–30 nM range as the natural signal molecule.

Table 1: Affinity of analogues **12**–**15** for binding site NFBS2, as measured by competitive inhibition of [^{35}S]-NodSm-IV(S) binding, and the chromatographic behavior of the analogues.

Compound	K_i [nM][a]	t_R [min][b]
2	20 (±5%)	17.2
12	35 (±18%)	16.5
13	28 (±7%)	17.9
14	77 (±19%)	13.7
15	22 (±13%)	20.5

[a] K_i = inhibition constant. This is a measure of the affinity for NFBS2. [b] t_R = Retention times on a C18 HPLC column.

The slightly lower affinity exhibited for the *ortho*-substituted benzamide **14** (K_i = 77 nM) probably reflects a very different local geometry close to the nonreducing-end sugar, a geometry that positions the side chain in a different space location. Moreover, compound **14** appeared less hydrophobic than the other analogues, as assumed from its shorter retention time when analyzed by reversed-phase chromatography on a C18 column (Table 1). This observation is in agreement with previous studies on the selectivity of NFBS2, which showed that the overall hydrophobicity of the LCOs,

mainly due to the structure of the fatty acid chain, was important for high-affinity binding.[6] The morphogenic activity of these benzamide analogues is currently being evaluated for potential agricultural applications, and biological details will be reported in the near future.[11]

Experimental Section

Preparation of **12**: Sodium hydrogencarbonate (2 equiv) and a solution of **7** in THF (0.82 M, 1 equiv) were added to a solution of tetrasaccharide **11** (17 μmol) in DMF/water (2.5:1, 350 μL). The reaction mixture was warmed to 60 °C, then more acid chloride solution (5 equiv) and sodium hydrogencarbonate (6 equiv) were added in six portions over a period of 18 h. Flash chromatography with ethyl acetate/methanol/water (7:2:1) as the eluent and deposition of the reaction residue at the top of the column in dichloromethane/methanol (5:1) gave **12** (6.5 mg, 33 %). The purity of the product was checked by elution at 1 mL min^{-1} on a C_{18} HPLC column with a linear gradient of 28→66 % acetonitrile in 10 mM K_2SO_4 (pH 4.6) and detection at 210 nm; ^{1}H NMR (CD_3OD, 250 MHz): $\delta = 7.48$–7.41 (m, 2H; Ar*H*-2, Ar*H*-6), 7.36 (dd, 1H, $J_{5,6} = 7.8$, $J_{5,4} = 8.1$ Hz; Ar*H*-5), 7.07 (ddd, 1H, $J_{4,2} \approx J_{4,6} = 1.4$ Hz; Ar*H*-4), 5.48–5.33 (m, 2H; C*H*=C*H*), 5.03 (d, 0.8H, $J_{1\alpha,2} = 3.2$ Hz; $H_1\alpha^I$), 4.68/4.59/4.50 (3d, 3H, $J_{1,2} = 8.7$ Hz; $H_1^{II,III,IV}$), 4.56 (d, 0.2H, $J_{1\beta,2} = 7.7$ Hz; $H_1\beta^I$), 4.25–3.30 (m, 26H; CH_2–OAr, other carbohydrate H atoms), 2.25 (td, 2H, $J = 6.7$, 6.2 Hz; CH_2–CH=CH–CH_2), 2.08–1.94 (m, 2H; CH_2–CH=CH–CH_2), 2.03/1.99/1.96 (3s, 9H; 3 × CH_3CO), 1.83 (tt, 2H, $J = 6.7$ Hz; $ArOCH_2CH_2CH_2$), 1.35–1.20 (m, 8H; 4 × CH_2), 0.88 ppm (t, 3H, $J = 7.0$ Hz; CH_3); MS (ES): m/z: 1139.4 $[M-Na]^-$.

Binding experiments were performed as previously described.[6] Briefly, microsomal fraction protein (60 μg) was incubated in the presence of 0.8 nM [^{35}S]-NodSm-IV(S) in a binding buffer (total volume 200 μL) with increasing amounts of the different analogues in concentrations ranging between 0.25 nM and 1 μM. The nonspecific binding component was determined in the presence of 2 μM NodSm-IV(S). Incubations were performed for 1 h at 0 °C in 96-well microtiter plates (Nunc), and the steps that followed were as previously described. Binding data were analyzed by the RADLIG software, Version 4 (Biosoft, Cambridge, UK).

Received: April 8, 2004

Keywords: aromatic substitution · glycolipids · lipid analogues · nitrogen fixation · oligosaccharides

[1] a) J. Dénarié, F. Debellé, J.-C. Promé, *Annu. Rev. Biochem.* **1996**, *65*, 503; b) S. R. Long, *Plant Cell* **1996**, *8*, 1885; c) H. P. Spaink, *Annu. Rev. Microbiol.* **1998**, *54*, 257; d) G. E. D. Oldroyd, *Ann. Bot.* **2001**, *87*, 709.

[2] P. Lerouge, P. Roche, C. Faucher, F. Maillet, G. Truchet, J.-C. Promé, J. Dénarié, *Nature* **1990**, *344*, 781; G. Truchet, P. Roche, P. Lerouge, J. Vasse, S. Camut, F. de Billy, J.-C. Promé, J. Dénarié, *Nature* **1991**, *351*, 670; P. Roche, F. Debellé, F. Maillet, P. Lerouge, C. Faucher, G. Truchet, J. Dénarié, J.-C. Promé, *Cell* **1991**, *67*, 1131.

[3] D. Tailler, J.-C. Jacquinet, J.-M. Beau, *J. Chem. Soc. Chem. Commun.* **1994**, 1827.

[4] N. Demont-Caulet, F. Maillet, D. Tailler, J.-C. Jacquinet, J.-C. Promé, K. C. Nicolaou, G. Truchet, J.-M. Beau, J. Dénarié, *Plant Physiol.* **1999**, *120*, 83.

[5] A. Niebel, J.-J. Bono, R. Ranjeva, J. V. Cullimore, *Mol. Plant-Microbe Interact.* **1997**, *10*, 132.

[6] F. Gressent, S. Drouillard, N. Mantegazza, E. Samain, R. A. Geremia, H. Canut, A. Niebel, H. Driguez, R. Ranjeva, J. Culimore, J.-J. Bono, *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 4704; F. Gressent, N. Mantegazza, J. V. Cullimore, H. Driguez, R. Ranjeva, J.-J. Bono, *Mol. Plant-Microbe Interact.* **2002**, *15*, 834.

[7] By using a genetic approach, two genes involved in the perception of Nod factors and encoding transmembrane-receptor-like serine/threonine kinases with extracellular domains containing modules potentially involved in the oligosaccharide binging (LysM motifs) were recently identified: E. Limpens, C. Franken, P. Smit, J. Willemse, T. Bisseling, R. Geurts, *Science* **2003**, *302*, 630; S. Radutoiu, L. H. Madsen, E. B. Madsen, H. H. Felle, Y. Umehara, M. Gronlund, S. Sato, Y. Nakamura, S. Tabata, N. Sandal, J. Stougaard, *Nature* **2003**, *425*, 585; E. B. Madsen, L. H. Madsen, S. Radutoiu, M. Olbryt, M. Rakwalska, K. Szczyglowski, S. Sato, S. Kaneko, S. Tabata, N. Sandal, J. Stougaard, *Nature* **2003**, *425*, 637.

[8] Synthesis of Nod factors from monomers: K. C. Nicolaou, N. J. Bockovich, D. R. Carcanague, C. W. Hummel, L. F. Even, *J. Am. Chem. Soc.* **1992**, *114*, 8701; S. Ikeshita, A. Sakamoto, Y. Nakahara, Y. Nakahara, T. Ogawa, *Tetrahedron Lett.* **1994**, *35*, 3123; L. X. Wang, C. Li, Q. W. Wang, Y. Z. Hui, *J. Chem. Soc. Perkin Trans. 1* **1994**, 621; J. S. Debenham, R. Rodebaugh, B. Fraser-Reid, *J. Org. Chem.* **1996**, *61*, 6478.

[9] Some examples of aromatic derivatives designed to resemble unsaturated lipids: D. W. Snyder, P. R. Bernstein, *Prostaglandins* **1988**, *35*, 903; R. C. Larock, N. H. Lee, *J. Org. Chem.* **1991**, *56*, 6253; H. Hashizume, H. Ito, K. Yamada, H. Nagashima, M. Kanao, H. Tomoda, T. Sunazuka, H. Kumagai, S. Omura, *Chem. Pharm. Bull.* **1994**, *42*, 512; J. P. F. C. Rossi, J. M. Delfino, A. J. Caride, H. N. Fernández, *Biochemistry* **1995**, *34*, 3802.

[10] a) E. Samain, S. Drouillard, A. Heyraud, H. Driguez, R. A. Geremia, *Carbohydr. Res.* **1997**, *302*, 35; b) E. Samain, V. Chazalet, R. A. Geremia, *J. Biotechnol.* **1999**, *72*, 33.

[11] F. Maillet, J. Dénarié, personal communication.

