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Lipo-Chitooligosaccharidic Nodulation Factors: Synthesis and Agricultural Perspectives

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Abstract: Lipo-chitooligosaccharidic nodulation (Nod) factors produced by rhizobia are a class of signalling molecules that induce a symbiotic association between legumes and soil bacteria rhizobia leading to the formation of the nitrogen-fixing root nodule. They consist of a chitin oligomeric backbone *N*-acylated at the non-reducing unit and are equipped with a variety of substituents at both ends of the oligosaccharide. This brief account focuses on the different approaches developed for their synthesis with particular emphasis on glycosylation methods. Current use of these Nod factors or analogs as additives in agricultural applications has shown to be very promising for sustainable agriculture.

Keywords: Chitooligosaccharides · Glycosylation · Nitrogen fixation · Nodulation factors · Sustainable agriculture

1. Introduction: Structural Features and Biological Activity

Legume plants are endowed with the exceptional ability to establish root nodule symbiosis with nitrogen-fixing soil bacteria called rhizobia. From the early phase of the infection process up to the final root nodule formation, several molecular components play important roles. Crucial are signal molecules secreted by these bacteria, called nodulation factors (Nod factors). They are lipo-chitooligosaccharides composed of a β-1,4-linked *N*-acetyl-D-glucosamine backbone of which the non-reducing sugar moiety is substituted at nitrogen with an acyl chain (Fig. 1).[1,2]

Most of these nodulation factors possess three to five 2-acylamino-2-deoxy-D-glucopyranosyl sugar units and, depending on the rhizobial species, the remaining hydroxyl groups of the reducing and non-reducing sugar unit can be substituted by additional groups. The type of substitutions, the length of the chitooligosaccharide backbone and the structure of the lipid moiety are characteristic for a rhizobial species, determining the biological activity of the Nod factors for a given plant host. Taking the particular example of the first discovered molecules,[1] the most active Nod factor produced by *Sinorhizobium meliloti*, which is the microsymbiont of the Medicago species, is a tetramer sulfated at O(6) of the reducing sugar, acetylated at O(6) of the non-reducing sugar, and acylated by a specific C16:2Δ1,9 lipid moiety (n = 2, p = 1, q = 1, R^4 = Ac, R^5 = SO_3^- in Fig. 1). This pattern is required to trigger efficiently the formation of infection structures in Medicago at concentrations as low as 10^{-9} to 10^{-12} M.[3] The same structure without the sulphate substituent loses its ability to infect and nodulate alfalfa, but is active on vetch, a non-host legume.[4] The structure-activity relationship with some other legumes is not so strict but the presence of a lipid chain is essential for symbiotic activity as non-acylated chitin oligomers are inactive.[5] Interestingly, rhizobia that nodulate tropical legumes (*e.g.* soybean, peanut) produce Nod factors that are *N*-acylated with fatty acids of the general lipid metabolism such as the palmitic (C16:0) and vaccenic (C18:1) acids.[2] In contrast, in Nod factors of rhizobia that nodulate temperate legumes (*e.g.* alfalfa, pea), the acyl chain is singular in that it contains one or several carbon-carbon *E*-double bonds conjugated with the carbonyl group of the amide.

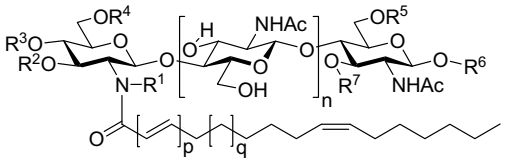

Fig. 1. General structure of most of the Nod factors with the substitution pattern produced by different rhizobial species. The common variations in the lipid chain are also noted.

2. Syntheses

2.1. *Total Syntheses from the Monomer D-Glucosamine*

The crucial role of the Nod factors on an agronomically and ecologically impor-

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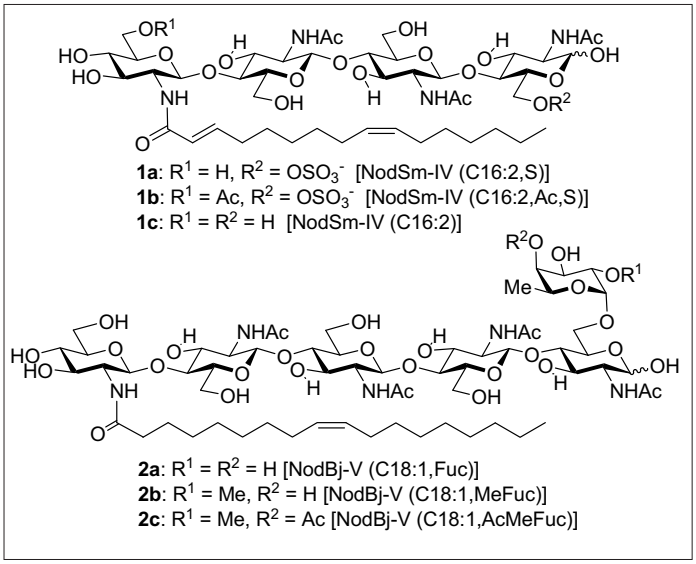


Fig. 2. The Nod factors NodSm-IV from *Sinorhizobium meliloti* and NodBj-V from *Bradyrhizobium japonicum* or *rhizobium* sp. NGR234, targets in the total syntheses from the monomer D-glucosamine.

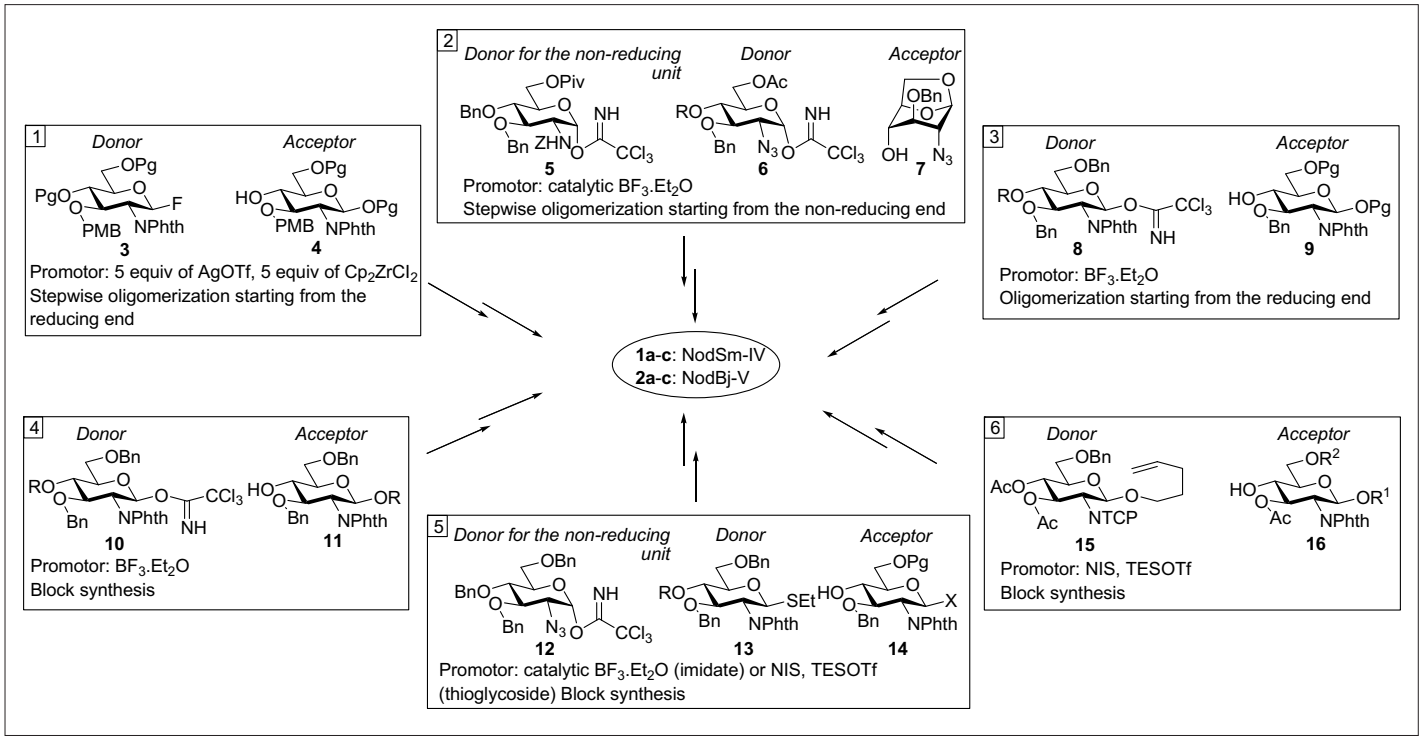
tant symbiosis stimulated synthetic interest soon after their discovery in 1990. Because these molecules are produced by rhizobia as mixtures in only minute quantities, synthetic access also greatly facilitated the biochemical and physiological studies on the sensing mechanisms involved in Nod factors signalling.[6]

The various synthetic approaches (glycosylation methods and oligomerisation strategies) selected for these syntheses illustrate very well the state of art in the development of improved methods for glycosidic bond formation developed over the years to efficiently construct oligosaccharides. The most targeted Nod factors were the major bacterial signal molecules **1a** and **1b** [NodSm-IV (C16:2,S)] involved in *Sinorhizobium meliloti*/alfalfa (*Medicago sativa*) symbiosis (and the non-sulfated homolog **1c**) and hexasaccharides **2a–c** [NodBj-V(C18:1, Fuc)] from *Bradyrhizobium japonicum*, the microsymbiont of soybean or from *Sinorhizobium* sp. NGR234 (the backbone is a chitotetraose) which enter into symbiosis with more than 110 genera of legumes[7] (Fig. 2).

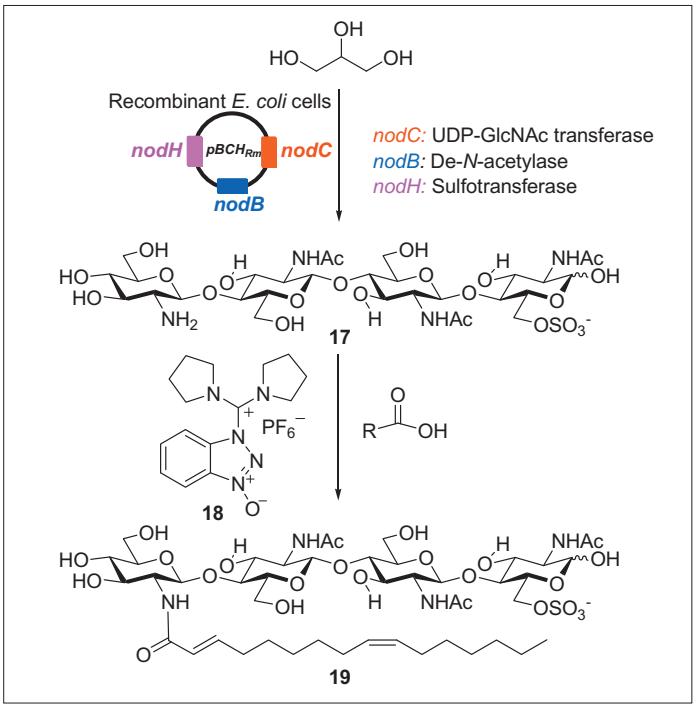
Instead of going through the entire sequence of steps providing the final Nod factors, the focal point of our discussion is based on the glycosylation reactions used in the syntheses because they are central to the oligosaccharide assembly. To achieve the total syntheses from appropriate glucosamine monomers, the synthetic options were mostly associated with the following:

a) selection of a suitable glycosylation procedure able to produce cleanly and efficiently β-glycosidic bonds with the arguably difficult 1,4 junction between two 2-acetamido-2-deoxy-D-glucopyranosyl units,
b) choice of an oligomerisation strategy that would provide the oligosaccharide with the highest efficiency,
c) selective differentiation of identical functional groups, *e.g.* primary or secondary hydroxyl and amino groups present in the chitin fragment. More specifically, the amino group of the non-reducing unit that will be acylated by a fatty acid part must be differentiated from the others. Secondly, the primary hydroxyl groups also need to be distinguished for the introduction at specific positions of the additional functional groups found in the natural Nod Factors (acetate, sulphate, …) or other carbohydrates (L-fucose, 2-*O*-methyl-L-fucose, …). Finally, the presence of C–C double bonds in the lipid part prevents the use of standard deprotection conditions (*e.g.* hydrogenation) in the final stages of the syntheses. These differentiations are controlled by the appropriate choices of protecting group combinations on the monomer.

In the first synthesis of NodSm-IV reported by Nicolaou and colleagues, the assembly of the monosaccharides relied on the use of glycosyl fluorides **3** as donors with a participating group at C(2), integrated in a classical oligomerisation starting from the protected reducing end (Box 1, Scheme 1).[8] The synthesis we reported is an ex-



Scheme 1. Analysis of the chemical syntheses targeting the Nod factors.



Scheme 2. Example of NodSm-IV(C16:2,S) produced by recombinant *Escherichia coli* cells and a chemical *N*-acylation.

ample of a non-conventional construction of a linear oligosaccharide starting from the non-reducing end (Box 2, Scheme 1).[9] The first β-glycosylation reaction was secured by a participating benzyloxycarbonyl carbamate group at C(2) of donor **5**.[10] The non-classical choice of a non-participating azido group at C(2) of donor **6** provided efficiently a 1,2-*trans* glycosylation through a clean S_N2 process. Key to this success was the repeated use of 1,6-anhydro derivative **7** as the glycosyl acceptor with a reactive hydroxyl group at C(4).

The route chosen by Ogawa and colleagues is close to that of Nicolaou. It relies upon the *N*-phtalimido participating group at C(2) with glycosyl trichloroacetimidates **8** and a glycosyl fluoride to finally introduce the non-reducing unit (Box 3, Scheme 1).[11] These authors followed a similar sequence to access NodBj-V.[12] The strategy of Hui and colleagues represents a block synthesis with the preparation of the tetrasaccharide from two preformed disaccharidic moieties.[13] In this route, the non-reducing unit was introduced using a non-participating azido group at C(2), which differentiated this nitrogen from the others, introduced as participating *N*-phthalimido groups (Box 4, Scheme 1).

The Torgov, Shibaev and colleagues approach also utilises the orthogonality of the azido and phthalimido protecting groups to distinguish the non-reducing moiety from the others, in a block synthesis of the Nod factors from *Sinorhizobium* sp. NGR234 (Box 5, Scheme 1).[14] They also utilize thioethyl glycosyl donors **13**. Frazer-Reid and colleagues reported a synthesis of the Nod factor from *Sinorhizobium fredii* USDA257 with a chitotriose skeleton. Amine differentiation is accomplished by the participating tetrachlorophthaloyl (TCP) protected glucosamine **15** in the presence of other phthalimide-protected amino sugars (Box 6, Scheme 1).[15] The oligosaccharide is then assembled *via* coupling of the *n*-pentenyl glycosyl donors.

These lengthy procedures encouraged Samain and Driguez to develop an improved access to these compounds by a biotechnological approach as shown below.

2.2 *Syntheses Using Metabolically Engineered Bacterial Cells*

When naturally occurring oligosaccharides are targeted in a synthesis, the use of enzymatic systems becomes a powerful choice for their preparation. Part of a biosynthetic sequence starting from monomer sugars leading to the oligomer may even be programmed using metabolically engineered bacterial cells.[16] This was cleverly developed 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* with glycerol as the carbon source.[17] This specific construct produces the sulfated tetramer **17** (Scheme 2).

This operates well because the growing bacterial *E. coli* cells are naturally equipped to regenerate the necessary starting sugar nucleotide UDP-GlcNAc and act as 'living nano-reactors' that utilize the intracellular pool of the sugar nucleotide as substrate for the *in vivo* synthesis of 'recombinant' oligosaccharides. Sulfated and acetylated chitooligosaccharide precursors of natural Nod factors have been efficiently produced using this biotechnological approach.[18] The 'recombinant' chitooligosaccharides (*e.g.* **17**) have been acylated at the free amino group of the non-reducing unit with the *in situ* generated guanidinium coupling reagent **18** to give the main Nod factors produced by *S. meliloti* (*e.g.* **19**) or the non-sulphated glycolipid homolog.[19]

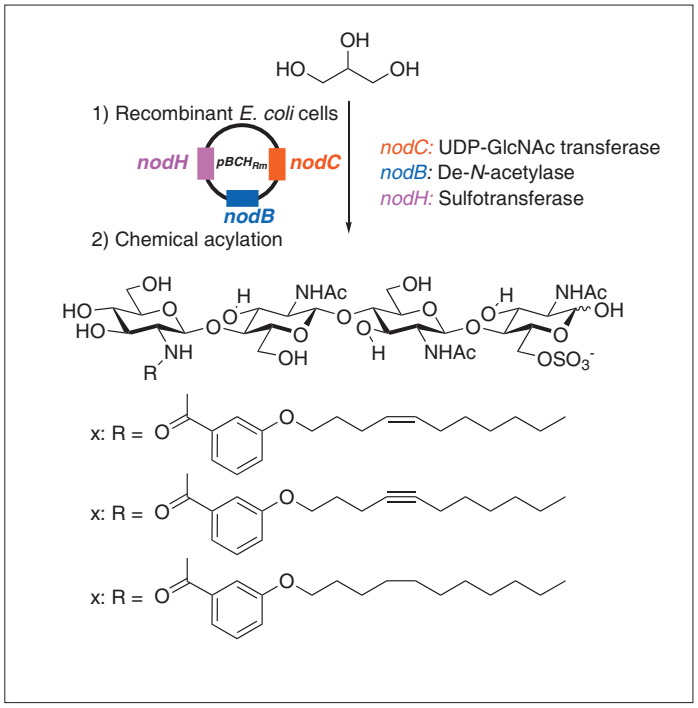
3. Agricultural Applications

The rhizobial symbiotic associations are credited for fixing an estimated average of the equivalent of 30–40 kg of nitrogen for every tonne of dry matter generated by crop legumes. An approximate calculation would then be that biological nitrogen fixation represents around 20 million tonnes of nitrogen every year.[20] Factors that influence legume growth will directly determine the amount of nitrogen fixed by the plant. Therefore, any means of favouring the colonization of legumes by rhizobia would favour nitrogen fixation.

One way to enhance plant growth is by inoculating legume crops with selected rhizobia. The use of commercial rhizobial inoculants is a common agricultural practice and represents one of the oldest commercial crop inputs. They are usually solid inoculants made of a carrier (peat or a range of recyclable agro-industrial wastes) containing a high number of bacteria (ideally over 2×10^9 of live rhizobium cells/g of the inoculant product) and are commercialized as a powder or in granular form.[21]

Seed treatment with the Nod factors or with rhizobium inoculants containing Nod factors is an additional way to ensure optimal nodulation for efficient nitrogen fixation. It was initially shown in field experiments that soybean, peanut and alfalfa seed treatments with rhizobial inoculants enriched with the appropriate Nod factors could significantly increase crop yields. This is due to their attractive ability to stimulate not only nodule formation, but also mycorrhiza formation and root system development.[22] Nod factors are now used on an industrial scale and this technology is commercialized for improved root and shoot development for most crop legumes at very low concentrations.[23]

To further improve the efficiency of legume seeds Nod factors treatments, we have explored the possibility of using Nod factor analogs that could combine interesting features such as the simplicity of synthesis and high stability in field tests while maintaining high biological activity. Thus, using the cellular approach reported above, we have synthesized a series of diverse mimics of the Nod factor of *S. meliloti,* the microsymbiont of alfalfa (Scheme 3).[24]



Scheme 3. Synthesis of a few benzamides analogs of the Nod factors highly active on alfalfa.

Our study has revealed that some benzamide mimics of *S. meliloti* Nod factors, with an aromatic ring motif replacing the important conjugated unsaturation, was able to interact with a putative Nod factor receptor at low nanomolar concentrations. This opens new ways for the simple construction of fluorescent or photo-activatable molecular probes for the in-depth investigation of this important symbiotic system. Most interestingly, some of the substituted benzamides retained high morphogenic activity that warrant potential agricultural applications.[25] These aromatic analogs, readily prepared from commercial precursors which are potentially more chemically and metabolically stable than the natural Nod factors with the conjugated amides, are currently being evaluated in field tests with various crop legumes using very low concentrations.

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