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# Biotin labeling of the symbiotically important succinoglycan oligosaccharides of *Rhizobium meliloti* for identification of putative plant receptors

Lai-Xi Wang,[a] Luis G. Melean,[b] Peter H. Seeberger,[b] Graham C. Walker[a,*]

[a]*Department of Biology, Massachusetts Institute of Technology, Building 68-633, 77 Massachusetts Avenue, Cambridge, MA 02139, USA*

[b]*Departments of Chemistry, Massachusetts Institute of Technology, Cambridge, MA 02139, USA*

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**Abstract**

The symbiotically important trimer of the succinoglycan octasaccharide subunit was labeled with a biotin tag through coupling with a 6-biotinamidohexan hydrazide and subsequent reduction with borane. The acetyl and succinyl groups in the molecule were stable to the two-step sequence, while a small percentage of the ketal in the pyruvate groups was reduced to an ether-linked lactic acid moiety attached to either the O-4 or O-6 position of the sugar residue under the reaction conditions. © 2001 Published by Elsevier Science Ltd.

*Keywords:* Biotin labeling; Succinoglycan; Oligosaccharide; Symbiotic signal

## 1. Introduction

The establishment of the nitrogen-fixing symbiosis between *Rhizobium meliloti* and its host plant *Medicago sativa* (alfalfa) is a complex process that involves signal exchanges between the bacterium symbiont and the plant host. Plant flavonoids and the bacterial lipochito-oligosaccharide Nod factors have been characterized as signals involved in the initial stage of the symbiosis.[1–3] In addition, genetic analysis has shown that succinoglycan, an extracellular polysaccharide produced by *R. meliloti* Rm1021, is required for successful nodule invasion by the bacteria, which leads to the formation of large, elongated pink nodules on the roots. Mutants of Rm1021 deficient in the production of succinoglycan form primarily ineffective, empty white nodules.[4–7] Furthermore, small amounts of low molecular weight (LMW) succinoglycan isolated from the bacterial cell culture can partially restore the ability of invasion-deficient bacterial mutants to invade root nodules.[8–10] This suggests that certain species in the LMW succinoglycan may function as a bacterial signal to the host plant for nodule invasion.

Succinoglycan is an acidic polysaccharide consisting of an octasaccharide repeating unit. The octasaccharide subunit is composed of one galactose and seven glucose units bearing acetyl, succinyl, and pyruvate substituents.[11–14] The subunits are polymerized in a fashion that results in a tetrasaccharide with a sequence of β-Glc-(1→4)-β-Glc-(1→4)-β-Glc-(1→3)-β-Gal-(1→4)- in the main chain and a

* Corresponding author. Tel.: +1-617-2536716; fax: +1-617-2532643.

*E-mail address:* gwalker@mit.edu (G.C. Walker).

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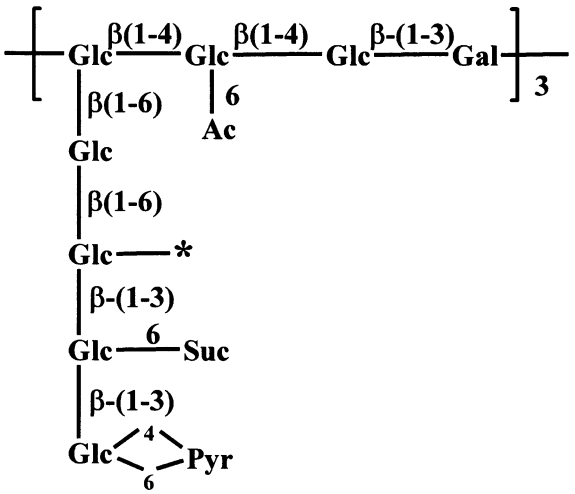



Fig. 1. Structure of the trimer of the succinoglycan octasaccharide subunit (The '*' indicates an additional site for succinylation).

tetrasaccharide with a sequence of β-Glc-(1 → 3)-β-Glc-(1 → 3)-β-Glc-(1 → 6)-β-Glc-(1 → 6)- in the side chain (Fig. 1). Our recent structural analysis of succinoglycan indicates that while each octasaccharide subunit contains one pyruvate ketal group between the 4 and 6 hydroxyls of the eighth glucose residue and ~0.7 molar equivalent of acetyl group at the 6-position of the third sugar residue, the number of succinyl groups per subunit can vary from zero to two.[10] Rm1021 produces succinoglycan in two distinct molecular weight forms: the high molecular weight (HMW) succinoglycan consisting of hundreds of the octasaccharide subunits and the low molecular weight (LMW) succinoglycan that consists of monomer, dimer, and trimer of the octasaccharide subunit. Our recent rescue experiments with isolated LMW succinoglycan oligosaccharides indicated that the trimer of the succinoglycan octasaccharide subunit is the most active species that can promote nodule invasion in the establishment of symbiosis.[10] These findings raise the possibility that the succinoglycan trimer may be a bacterial signal for nodule invasion.

To search for any binding proteins that might be putative host receptors for the oligosaccharide signal, we wished to label the biologically active succinoglycan oligosaccharides with a biotin tag. The resulting biotinylated oligosaccharides are intended for use as probes for detecting binding proteins and as affinity adsorbants for protein purification when immobilized on an avidin–agarose column.[15] Several methods have been reported for biotinylation of oligosaccharides, including reaction of reducing sugars with biotin-tagged acyl hydrazide,[16–18] reductive amination of oligosaccharides,[19] and the conjugation via a glycosylamine intermediate.[20] However, none of the existing methodologies for biotin labeling was found to be compatible with the esters and ketal substitutions of the target succinoglycans. We report here a novel method for biotin labeling the succinoglycan trimer, the biologically active species of succinoglycan oligosaccharides.

## 2. Results and discussion

An initial attempt to prepare the corresponding glycosylamine of the succinoglycan trimer by treatment with saturated ammonium hydrogen carbonate at room temperature for 3 days[20] led to complete removal of the acetyl and succinyl groups in the molecule, as indicated by NMR analysis. On the other hand, reaction of the trimer with 2,6-diaminopyridine at elevated temperature[19] resulted in a mixture consisting of partially deacylated trimer and several unidentified products (data not shown). To search for an efficient labeling method with the preservation of the labile ester substituents, we then turned our attention to the coupling of the trimer with 6-biotinamidohexanoyl ('biotinamidocaproyl') hydrazide, which is a weaker base and was recently used for biotin labeling of oligosaccharides.[16–18] High-performance anion-exchange chromatography coupled with pulsed-amperometric detection (HPAEC–PAD) was used to monitor the coupling reactions. Although different degrees of succinylation of the isolated succinoglycan trimer gave six peaks under mild (acetate buffer) HPAEC conditions,[10] the trimer appeared as a single peak ($R_t = 22.3$ min) under the highly alkaline conditions due to in situ deacylation of the oligosaccharides, thus simplifying the analysis (Fig. 2A). The reaction of the trimer with an excess amount of biotinamidocaproyl hydra-

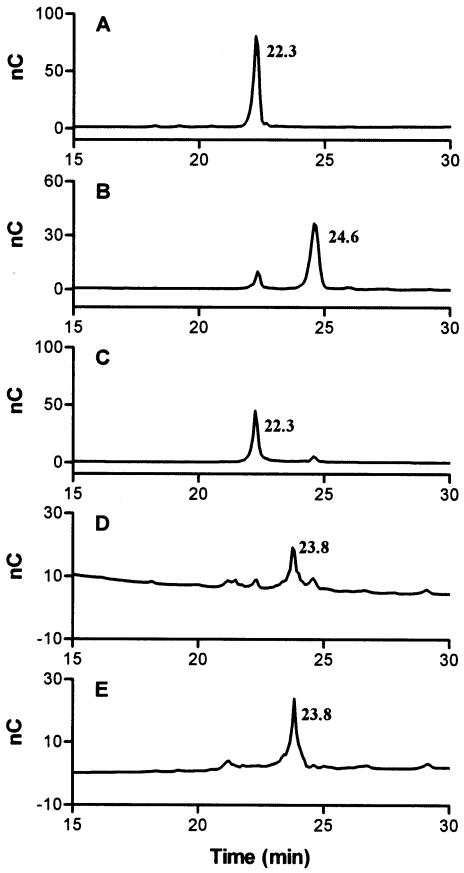
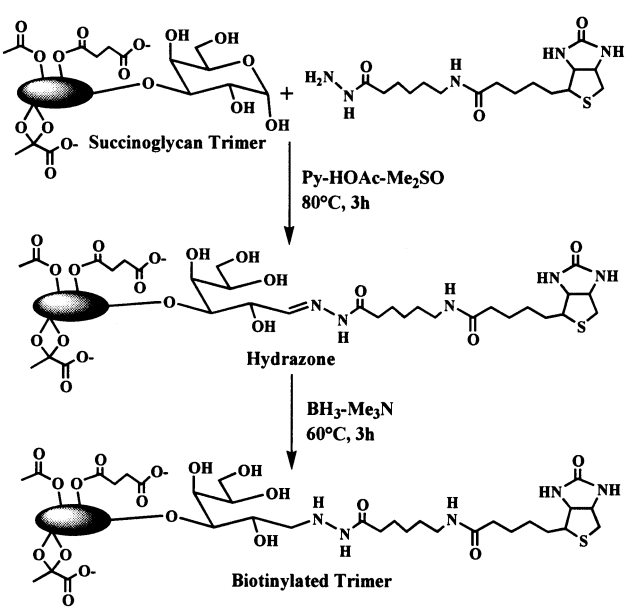



Fig. 2. HPAEC–PAD analysis of the biotin labeling of the succinoglycan trimer. (A) Chromatography of the trimer. (B) Chromatography of the coupling reaction between the trimer and biotinamidocaproyl hydrazide in the solvent at 80 °C for 3 h (the new peak at 24.6 min is the hydrazone intermediate). (C) Chromatography of the intermediate after dialysis against water at 4 °C for 2 days. (D) Chromatography of the reaction mixture after direct reduction of the hydrazone intermediate with borane–trimethylamine complex in the same mixed solvent system at 60 °C for 3 h. (E) Chromatography of the purified, biotin-labeled trimer. Detailed HPAEC–PAD conditions are given in Section 3

zide in a mixed solvent system consisting of (4:2:1, v/v) pyridine–acetic acid–dimethyl sulfoxide at an elevated temperature proceeded smoothly to form an intermediate ($R_t = 24.6$ min), which eluted later than the trimer under the HPAEC–PAD condition (Fig. 2B). This reaction intermediate is likely to be the corresponding hydrazone derivative (Scheme 1), or the corresponding glycosylhydrazide with a closed pyranose ring structure, as suggested in a recent report.[18] However, attempts to purify the intermediate for structural characterization and as a potential probe for binding proteins was not successful, due to its instability in aqueous solution. Dialysis of the reaction mixture that contained ~90% of the intermediate against water at 4 °C for 2 days led to almost complete hydrolysis of the intermediate to the starting trimer (Fig. 2(C)).

In order to prepare a stable biotinylated trimer, the coupling intermediate was subjected to in situ reduction with borane trimethylamine complex in the same mixed solvent system (Scheme 1), resulting in the formation of a new product, presumably the stable biotinylated trimer ($R_t = 23.8$ min) that eluted earlier than the hydrazone intermediate (Fig. 2(D)). After thorough dialysis of the reaction mixture against water to remove excess biotinamidocaproyl hydrazide, a monomeric avidin column was used to purify the biotinylated product.[21] Immobilized monomeric avidin allows reversible binding of biotin species and the biotinylated product is subsequently eluted by a biotin solution. $^1$H NMR analysis of the purified product revealed typical signals for the biotin ring protons at $\delta$ 2.75–3.20, 4.40, and 4.60. The signals for the spacer appeared at $\delta$ 1.35, 1.48–1.70, and 2.22 (Fig. 3). Comparison of the $^1$H NMR spectra of the product with the trimer clearly indicates that the acetyl group ($\delta$ 2.15) and the succinyl groups ($\delta$ 2.52–2.70 for the trimer; $\delta$ 2.43–2.65 for the biotinylated trimer) were completely preserved after the two-step labeling reactions. The signals for the




Scheme 1.

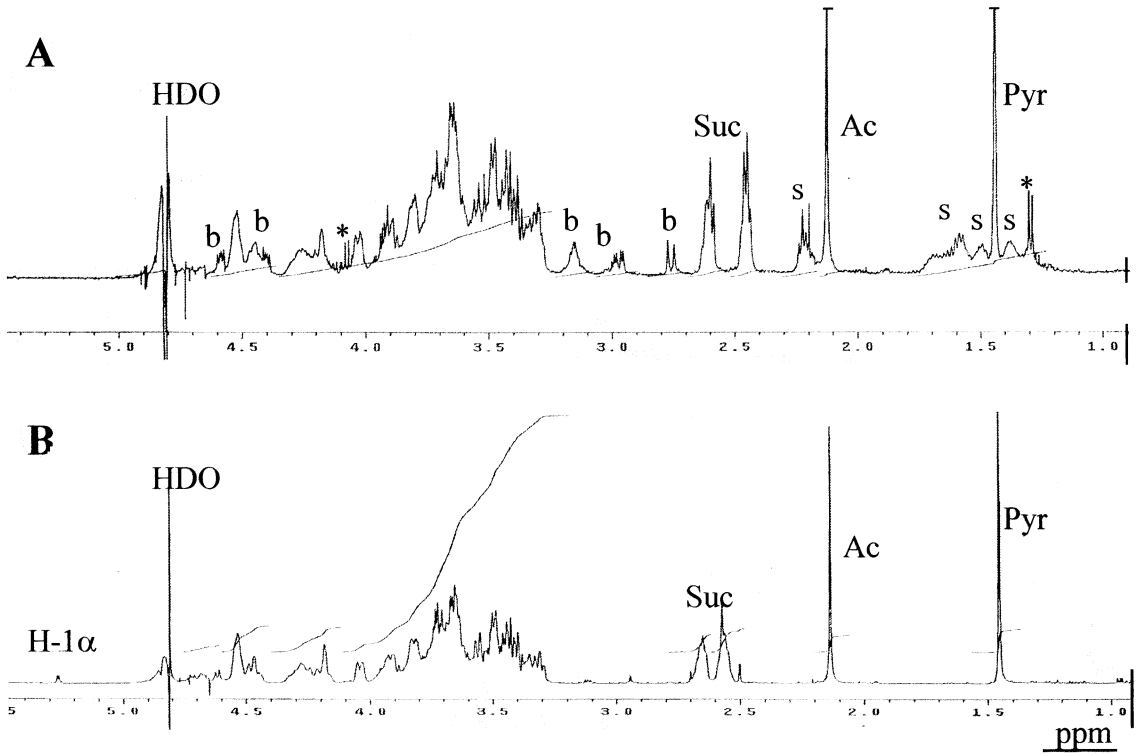



Fig. 3. The $^1$H NMR spectra of the biotinylated trimer (A) and the trimer (B). The signals labeled 'b' are designated to biotin ring protons; the signals labeled 's' are assigned for the protons in the spacer; the signals labeled '*' are assumed to come from the moiety of $CH_3CH(CO_2^-)$–O generated by reductive ring opening of the pyruvate group. Ac, acetyl group; Pyr, pyruvate group; and Suc, succinyl group.

pyruvyl groups in the trimer and biotinylated trimer appeared at $\delta$ 1.43 and 1.45, respectively (Fig. 3). However, a careful comparison of the NMR spectra of the trimer and the biotinylated trimer revealed two small new signals, a doublet at $\delta$ 1.32 ($J = 7.2$ Hz) and a quartet at $\delta$ 4.15 ($J = 7.2$ Hz), that could not be assigned to the biotin and/or the spacer moiety in the molecule (Fig. 3(B)). These signals strongly suggest that there might be partially reductive ring opening of the pyruvate group, forming a $CH_3CH(CO_2^-)$–O– moiety in the molecule that would account for the observed NMR signals. While reduction of the hydrazone intermediate with borane–trimethylamine complex at 60 °C for 3 h resulted in the formation of less than 10% of the ring-opened by-product, prolonged reaction time and/or higher reaction temperature (85 °C) led to a significant formation of the by-product (by NMR analysis), which appeared to overlap with the biotinylated trimer in HPAEC analysis (data not shown). No attempt was made to separate the by-product for further characterization.

In summary, succinoglycan oligosaccharides can be readily labeled with a biotin hydrazide via hydrazone formation and subsequent reduction with borane in (4:2:1, v/v) pyridine–acetic acid–dimethyl sulfoxide without loss of the labile acetyl and succinyl groups. This reaction sequence should be applicable for labeling other biologically active oligosaccharides containing ester and carboxyl substituents. However, caution must be taken when the oligosaccharides contain pyruvate or other ketal groups, because reductive ring-opening reactions can be competitive. The biotin-labeled succinoglycan trimer was purified by a monomeric avidin column, and is intended for use in screening of succinoglycan binding proteins. Furthermore, the biotinylated trimer was also immobilized on a common avidin column, which will be used for affinity chromatography.

## 3. Experimental

*Materials.*—The succinoglycan trimers of the octasaccharide subunit were prepared from the low molecular weight fraction of succinoglycan by Bio-Gel P6 gel filtration chromatography as described previously.[10] Biotin, biotinamidocaproyl hydrazide, and avidin–agarose resin were purchased from Sigma Chemical Co. (St. Louis, MO). UltraLink™ immobilized monomeric avidin was from Pierce (Rockford, IL). Borane trimethylamine was from Aldrich Chemical Co. (Milwaukee, WI).

*High-performance anion-exchange chromatography coupled with pulsed amperometric detection (HPAEC–PAD).*—The analysis was carried out on a CarboPac PA100 column with a Dionex DX500 chromatography system (Dionex Corporation, Sunnyvale, CA) equipped with a pulsed amperometric detector (ED 40, Dionex Corporation). For gradient elution, eluant A is 100 mM NaOH and eluant B is 500 mM $NaNO_3$ in 100 mM NaOH. The running gradient was: 0–3 min, 0% B; 3–7 min, 0–5% B; and 7–30 min, 5–30% B, with a flow rate of 1 mL $min^{-1}$. The PAD was operated at 0.1 μC sensitivity using the following waveforms (potentials and durations): $E_1 = +0.05$ V ($T_1 = 0$–0.4 s), $E_2 = +0.75$ V ($T_2 = 0.41$–0.6 s), $E_3 = -0.15$ V ($T_3 = 0.61$–1 s). Under these high-pH conditions, all the acetyl and succinyl groups in the trimer molecule were removed in situ and the trimer appeared as a single peak.

*NMR analysis.*—NMR experiments were performed on a Varian Unity 500 spectrometer with a proton frequency of 500.0 MHz. All spectra were acquired in $D_2O$ at ambient temperature. Chemical shifts were referenced relative to the HDO signal (4.77 ppm).

*Coupling of the succinoglycan oligosaccharides with biotinamidocaproyl hydrazide.*—The succinoglycan trimer (2 mg) was dissolved in a mixed solvent of (4:2:1, v/v) pyridine–AcOH–$Me_2SO$ (700 μL), in a screw-capped 1-mL vial, to which was added biotinamidocaproyl hydrazide (3 mg). The mixture was heated at 80 °C with occasional vortexing. During the reaction, the biotinamidocaproyl hydrazide was dissolved gradually. After 3 h at 80 °C, HPAEC analysis showed ~90% of the starting trimer was converted into a new species, supposedly the corresponding hydrazone intermediate. Then borane–trimethylamine (8 mg) was added to the reaction solution, and the mixture was heated for 3 h at 60 °C. The reaction mixture was cooled and added to water (2 mL), which was dialyzed against water (4 L) in a dialysis tube (MWCO, 1000) for 40 h at 4 °C, with 3 exchanges of water. After thorough dialysis, the coupling product was either immobilized or purified as follows.

*Immobilization and purification of the biotinylated succinoglycan trimer.*—For the immobilization, half of the dialyzed reaction mixture (containing ~1 mg of oligosaccharide) was loaded onto a column of the avidin–agarose resin (2 mL, Sigma) that was pre-equilibrated with phosphate-buffered saline (PBS, pH 7.2). The column was washed with PBS (5 × 2 mL) to remove any unlabeled trimer and other unbound species. The biotinylated trimer was retained in the column, which can serve as an adsorbent for purifying succinoglycan-binding proteins. For the purification of biotinylated trimer, the ultralink™ immobilized monomeric avidin (Pierce) that allows reversible binding of biotinylated species was used. A column of the monomeric avidin resin (3 mL) was pretreated with 20 mM biotin in PBS buffer to block the high affinity, nonreversible binding sites. Then the low affinity, reversible binding sites were revealed by stripping away the loosely bound biotin with a low pH glycine buffer (pH 2.6). The column was equilibrated with PBS, and the above-dialyzed reaction mixture (containing ca. 1 mg of oligosaccharide) was loaded onto the column. The column was washed with PBS (5 × 3 mL) to remove unlabeled trimer, and the biotinylated trimer was eluted with 20 mM biotin in PBS through ligand competition. The fractions containing the biotinylated trimer were pooled, dialyzed against water to remove biotin, and lyophilized to give the product as a white foam.

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## References

1. Fisher, R. F.; Long, S. R. *Nature* **1992**, *357*, 655–660.
2. Long, S. R. *Plant Cell* **1996**, *8*, 1885–1898.
3. Spaink, H. P.; Lugtenberg, B. J. J. *Plant Mol. Biol.* **1994**, *26*, 1413–1422.
4. Leigh, J. A.; Signer, E. R.; Walker, G. C. *Proc. Natl. Acad. Sci. USA* **1985**, *82*, 6231–6235.
5. Long, S.; Reed, J. W.; Himawan, J.; Walker, G. C. *J. Bacteriol.* **1988**, *170*, 4239–4248.
6. Reuber, T. L.; Walker, G. C. *Cell* **1993**, *74*, 269–280.
7. Cheng, H. P.; Walker, G. C. *J. Bacteriol.* **1998**, *180*, 5183–5191.
8. Battisti, L.; Lara, J. C.; Leigh, J. A. *Proc. Natl. Acad. Sci. USA* **1992**, *89*, 5625–5629.
9. Urzainqui, A.; Walker, G. C. *J. Bacteriol.* **1992**, *174*, 3403–3406.
10. Wang, L. X.; Wang, Y.; Pellock, B.; Walker, G. C. *J. Bacteriol.* **1999**, *181*, 6788–6796.
11. Aman, P.; McNeil, M.; Franzen, L. E.; Darvill, A. G.; Albersheim, P. *Carbohydr. Res.* **1981**, *95*, 263–282.
12. Jansson, P. E.; Kenne, L.; Lindberg, B.; Ljunggren, H.; Ruden, U.; Lonngren, J.; Svensson, S. *J. Am. Chem. Soc.* **1977**, *99*, 3812–3815.
13. Chouly, C.; Colquhoun, I. J.; Jodelet, A.; York, G.; Walker, G. C. *Int. J. Biol. Macromol.* **1995**, *17*, 357–363.
14. Reinhold, B. B.; Chan, S. Y.; Reuber, T. L.; Marra, A.; Walker, G. C.; Reinhold, V. N. *J. Bacteriol.* **1994**, *176*, 1997–2002.
15. Wilchek, M.; Bayer, E. A. *Anal. Biochem.* **1988**, *171*, 1–32.
16. Shinohara, Y.; Sota, H.; Kim, F.; Shimizu, M.; Gotoh, M.; Tosu, M.; Hasegawa, Y. *J. Biochem.* **1995**, *117*, 1076–1082.
17. Ridley, B. L.; Spiro, M. D.; Glushka, J.; Albersheim, P.; Darvill, A.; Mohnen, D. *Anal. Biochem.* **1997**, *249*, 10–19.
18. Leteux, C.; Childs, R. A.; Chai, W. G.; Stoll, M. S.; Kogelberg, H.; Feizi, T. *Glycobiology* **1998**, *8*, 227–236.
19. Rothenberg, B. E.; Hayes, B. K.; Toomre, D.; Manzi, A. E.; Varki, A. *Proc. Natl. Acad. Sci. USA* **1993**, *90*, 11939–11943.
20. Manger, I. D.; Rademacher, T. W.; Dwek, R. A. *Biochemistry* **1992**, *31*, 10724–10732.
21. Kohanski, R. A.; Lane, M. D. *Methods Enzymol.* **1990**, *184*, 194–200.