ORGANIC LETTERS

2008
Vol. 10, No. 10
2047−2050

Synthesis and Stereochemistry-Activity Relationship of *small* Bacteriocin, an Autoinducer of the Symbiotic Nitrogen-Fixing Bacterium *Rhizobium leguminosarum*

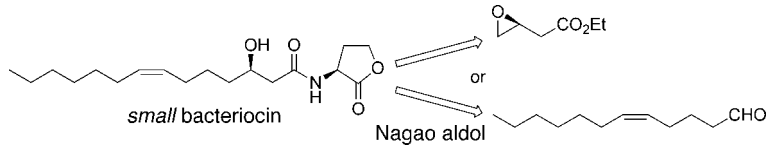
Arata Yajima,*,† Anton A. N. van Brussel,‡ Jan Schripsema,§,¶ Tomoo Nukada,† and Goro Yabuta†

Department of Fermentation Science, Faculty of Applied Biological Science, Tokyo University of Agriculture (NODAI), Sakuragaoka 1-1-1, Setagaya-ku, Tokyo 156-8502, Japan, Institute of Biology, Leiden University, Wassenaarseweg 64, 2333AL Leiden, The Netherlands, Analytical Biotechnology, Department of Biotechnology, Delft University of Technology, Julianalaan 67, 2628 BC Delft, The Netherlands, and Grupo Metabolômica, Laboratório de Ciências Quimicas, Universidade Estadual do Norte Fluminense, Av. Alberto Lamego, 2000, 28015-620 Campos dos Goytacazes, RJ, Brazil

ayaji@nodai.ac.jp

Received March 7, 2008

ABSTRACT



The four stereoisomers of *small* bacteriocin, an autoinducer of the symbiotic nitrogen-fixing bacterium *Rhizobium leguminosarum*, were synthesized via a versatile methodology for 3′-hydroxyacyl homoserine lactones based on the Nagao asymmetric aldol reaction. The synthetic isomers were much less effective at inhibiting the growth of *R. leguminosarum* RBL5523 than the natural isomer, showing the importance of stereochemistry for activity.

N-acyl homoserine lactone (AHL) molecules play an important role in intercellular communication of Gram-negative bacteria as inducers of genes implicated in cellular functions required at higher cell densities (Figure 1).[1] They are often called autoinducers because, in addition to the genes mentioned, they also induce genes encoding enzymes involved in their own synthesis. AHL molecules move out and in the cells and only activate genes when a certain threshold concentration is obtained, which occurs when a higher bacteria cell density (a quorum) is reached in, for example, a colony. Hence this process is called "quorum sensing".[1] *Rhizobium leguminosarum* is a gram negative bacterium living in symbiosis with leguminous plants in which it induces nitrogen-fixing root nodules.[2] The main autoinducer of this bacterium, encoded by the *cinI* gene,

† Tokyo University of Agriculture.
‡ Leiden University.
§ Delft University of Technology.
¶ Universidade Estadual do Norte Fluminense.

(1) Fuqua, W. C.; Winans, S. C.; Greenberg, E. P. *J. Bacteriol.* **1994**, *176*, 269–275.

(2) (a) Kijne, J. W. *Biological Nitrogen Fixation*; Stacey, G., Burris, R. H., Evans, H. J., Eds.; Chapman and Hall: London, UK, **1992**; pp 349−398. (b) van Rhijn, P.; Vanderleyden, J. *Microbiol. Rev.* **1995**, *59*, 124–142. (c) Oke, V.; Long, S. R. *Curr. Opin. Microbiol.* **1999**, *2*, 1299. (d) Perret, X.; Staechelin, C.; Broughton, W. J. *Microbiol. Mol. Biol. Rev.* **2000**, *64*, 180–201.

10.1021/ol8005198 CCC: $40.75
Published on Web 04/11/2008

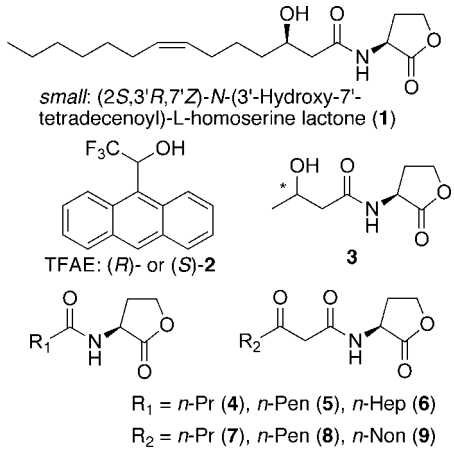


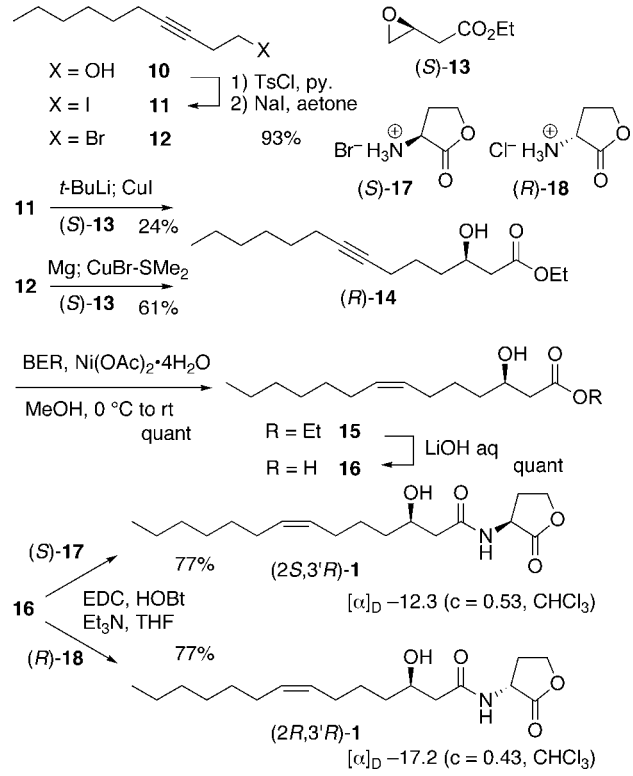
Figure 1. Structures of *small* bacteriocin (**1**) and acyl homoserine lactones from Gram-negative bacteria.

induces the so-called *rhi* (rhizosphere) genes and conjugation of the Sym(biosis) plasmid which harbors the genes involved in symbiotic functions.[3,4] A peculiar property of this AHL molecule is growth inhibition of *Rhizobium* bacteria which harbor the conjugative Sym plasmid pRL1JI.[5] Owing to this property and its small size, this AHL molecule was called *small* bacteriocin by Hirsch.[5] Due to its small size, it diffuses rapidly in an agar plate containing the sensitive bacteria and causes large inhibition zones. This is in contrast to another, bigger, slower diffusing, growth inhibiting molecule (*medium* bacteriocin), which causes smaller, medium size inhibition zones.

The complete chemical structure of this compound was identified by Schripsema and co-workers[6] by NMR, and its absolute configuration was proposed after comparison of the effects of Pirkle's chiral solvating agent, 2,2,2-trifluoro-1-(9-anthryl)ethanol[7] (TFAE, **2**) on the ^{1}H NMR spectra of the natural product and the analogs (2*S*,3′*R*)- and (2*S*,3′*S*)-*N*-(3′-hydroxybutyryl)-L-homoserine lactone (HBHL, **3**). Various acyl homoserine lactones (acyl HSLs) have been recognized as autoinducers from many Gram-negative bacteria (**3**−**9**, Figure 1).[8] Although 3′-hydroxyacyl HSLs (**1** and **3**) have two stereogenic centers, no information was available regarding the stereochemistry-activity relationship of **1**. Because stereochemistry is important in insect pheromone chemistry,[9] we became interested in clarifying the stereochemistry-activity relationship of this quorum-sensing pheromone (quormone).

Optically active **1** was synthesized from optically active starting material with known absolute configuration (Scheme 1).[10] Commercially available epoxide (*S*)-**13** was coupled

Scheme 1. Synthesis of (2*S*,3′*R*)- and (2*R*,3′*R*)-**1**



with the cuprate prepared from **11** by treatment with *t*-BuLi and CuI to give hydroxy ester (*R*)-**14** in low yield (24%).[11] On the other hand, the coupling of (*S*)-**13** with Grignard reagent prepared from known bromide **12**[12] in the presence of CuBr·SMe$_2$ as catalyst gave (*R*)-**14** in moderate yield (61%). *Z*-selective semihydrogenation with borohydride exchange resin-nickel boride (BER-Ni$_2$B)[13] as catalyst gave **15** (*Z*:*E* = >20:1). Hydroxy ester **15** was saponified to give

(3) (a) Rodelas, B.; Lithgow, J. K.; Wisniewski-Dyé, F.; Hardman, A.; Wilkinson, A.; Economou, A.; William, P.; Downie, J. A. *J. Bacteriol.* **1999**, *181*, 3816–3823. (b) Thorne, S. H.; Williams, H. D. *J. Bacteriol.* **1999**, *181*, 981–990. (c) Lithgow, J. K.; Willkinson, A.; Hardman, A.; Rodelas, B.; Wisniewski-Dyé, F.; Williams, P.; Downie, J. A. *Mol. Microbiol.* **2000**, *37*, 81–97. (d) Blosser-Middleton, R. S.; Gray, K. M. *J. Bacteriol.* **2001**, *183*, 6771–6777.

(4) Gray, K. M.; Pearson, J. P.; Downie, J. A.; Boboye, B. E. A.; Greenberg, E. P. *J. Bacteriol.* **1996**, *178*, 372–376.

(5) Hirsch, P. R. *J. Gen. Microbiol.* **1979**, *113*, 219–228.

(6) Schripsema, J.; de Rudder, K. E. E.; van Vliet, T. B.; Lankhorst, P. P.; de Vroom, E.; Kijne, J. W.; van Brussel, A. A. N. *J. Bacteriol.* **1996**, *178*, 366–371.

(7) Pirkle, W. H.; Sikkenga, D. L.; Pavlin, M. S. *J. Org. Chem.* **1977**, *42*, 384–387.

(8) (a) Fuqua, C.; Winans, S. C.; Greenberg, E. P. *Annu. Rev. Microbiol.* **1996**, *50*, 727–751. (b) Gray, K. M. *Trends Microbiol.* **1997**, *5*, 184–188. (c) Parsek, M. R.; Greenberg, E. P. *Proc. Nat. Acad. Sci. USA* **2000**, *97*, 8789–8793. (d) Whitehead, N. A.; Barnard, A. M. L.; Slater, H.; Simpson, N. J. L.; Salmond, G. P. C. *FEMS Microbiol. Rev.* **2001**, *25*, 365–404.

(9) (a) Mori, K. In *The Total Synthesis of Natural Product*; ApSimon, J., Ed.; Wiley-Interscience: New York, 1992; Vol. 9. (b) Mori, K. *Bioorg. Med. Chem.* **2007**, *15*, 7505–7523.

(10) Synthesis of 3′-hydroxyacyl HSLs: (a) Cao, J.-G.; Meighen, E. A. *J. Bacteriol.* **1993**, *175*, 3856–3862. (b) Chhabra, S. R.; Stead, P.; Bainton, N. J.; Salmond, G. P. C.; Stewart, G. S. A. B.; Williams, P.; Bycroft, B. W. *J. Antibiot.* **1993**, *46*, 441–454. (c) Chhabra, S. R.; Harty, C.; Hooi, D. S. W.; Daykin, M.; Williams, P.; Telford, G.; Pritcchard, D. I.; Bycroft, B. W. *J. Med. Chem.* **2003**, *46*, 97–104. (d) Janssens, J. C. A.; Metzger, K.; Daniels, R.; Ptacek, D.; Verhoeven, T.; Habel, L. W.; Vanderleyden, J.; De Vos, D. E.; De Keersmaecker, S. C. J. *Appl. Environ. Microbiol.* **2007**, *73*, 535–544.

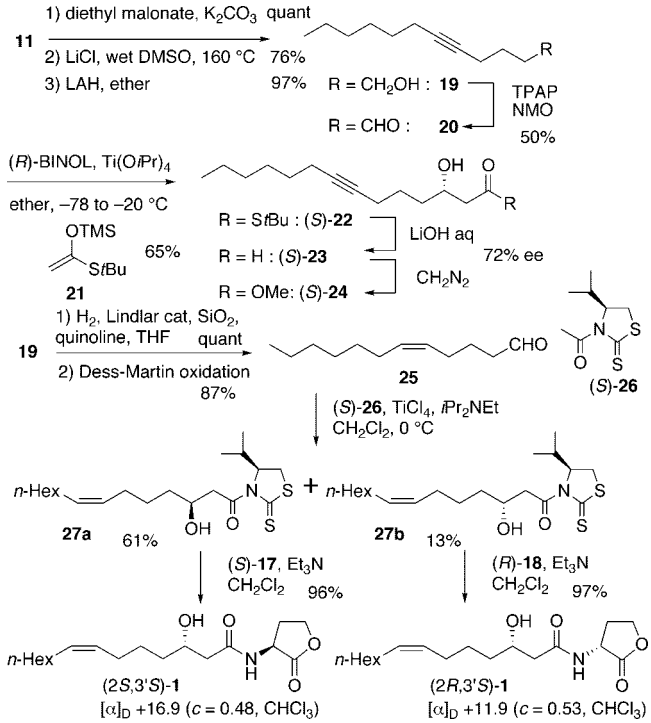
(11) Huang, G.; Hollingsworth, R. I. *Tetrahedron: Asymmetry* **1998**, *9*, 4113–4115.

(12) Tamada, S.; Mori, K.; Matsui, M. *Agric. Biol. Chem.* **1978**, *42*, 191–192.

hydroxy acid **16** (quant), and the resulting **16** was condensed with (*S*)-homoserine lactone hydrobromide (**17**) using EDC with HOBt[14] and Et_3N to give the desired (2*S*,3′*R*)-**1**. The ^1H and ^{13}C NMR spectra of (2*S*,3′*R*)-(−)-**1** were, as expected, identical with those of the natural product, for which the absolute configuration of both stereogenic centers had been determined.[6] Unfortunately, the optical rotation of the natural product was not available, thus prohibiting the direct comparison of the absolute configuration of the natural product with the synthetic product. Because agreement of spectroscopic data does not necessarily prove identical stereochemistry,[15] diastereomer (2*R*,3′*R*)-**1** was synthesized from **16** and (*R*)-homoserine lactone hydrochloride (**18**). The ^1H and ^{13}C NMR spectra of the product were clearly different from those of synthetic (2*S*,3′*R*)-(−)-**1** and the natural product, showing that the diastereomers of **1** can be distinguished by their ^1H and ^{13}C NMR spectra.

Although the (3′*S*)-isomers of **1** could be prepared using (*R*)-**13** as the starting material, we investigated a more versatile methodology for the synthesis of 3′-hydroxy HSLs (Scheme 2). Diethyl malonate was alkylated with the iodide **11**, and the product was heated with LiCl in wet DMSO[16]

Scheme 2. Synthesis of **1** via Nagao Aldol Reaction



to give an ester. LAH reduction of the ester gave alcohol **19** (91%), which was oxidized under Ley conditions[17] (50%) to give aldehyde **20**. Keck asymmetric aldol reaction[18] of **20** with **21** gave (*S*)-**22** (65%). The enantiomeric purity of (*S*)-**22** was determined by GC analysis of the corresponding methyl ester to be 72% ee, and its enantiomeric purity could not be improved by simple recrystallization. Thus, to prepare stereochemically pure samples, the Nagao aldol reaction[19] was employed. The *Z*-selective semihydrogenation of **19** with Lindlar catalyst[20] (*Z*:*E* = >20:1) and Dess-Martin oxidation[21] gave **25** (87%). In this step, BER-Ni_2B[12] was not an effective catalyst because a saturated alcohol, caused by undesired overhydrogenation, was produced in 10−15% and could not be separated from the product. According to the modified conditions reported by Smith,[22] Nagao aldol reaction of **25** with (*S*)-**26** gave a diastereomeric mixture (ca. 5:1 by ^1H NMR analysis) which could be easily separated by silica gel column chromatography. The relative stereochemistry of isomers **27a** and **27b** were determined after their conversion to **1**. Major isomer **27a** was treated with (*S*)-**17** and (*R*)-**18** in the presence of Et_3N to give (2*S*,3′*S*)-(+)-**1** and (2*R*,3′*S*)-(+)-**1**, respectively, in good yields, and no signals from the diastereomer were observed. Minor isomer **27b** could also be converted to the two isomers of **1** in the same manner. Since both enantiomers of the Nagao ligand are readily available, all the stereoisomers of **1** can be synthesized using the above methodology.

With the four stereoisomers of **1** in hand, NMR studies of the synthetic samples with TFAE (**2**) were conducted for verification of the configuration of the asymmetric carbons. ^1H NMR spectra of the four stereoisomers of **1** in the presence of both enantiomers of TFAE (**2**) were recorded. The correlation of the signal displacements of **1** induced by (*S*)- or (*R*)-TFAE (**2**) with those of the corresponding protons in the two reference compounds [(2*S*,3′*R*)- and (2*S*,3′*S*)-HBHL (**3**)][10a] induced by (*S*)-TFAE, yielded four graphs for each stereoisomer of **1** (Figure 2). In each set of graphs, the correct absolute configurations were indicated by the best correlation between the data. In this way for all four stereoisomers, the expected configurations were confirmed in the same way as the absolute configuration of the natural product was confirmed to be 2*S*,3′*R*.[6]

Next, the biological activity of the four stereoisomers of **1** was compared by estimating the minimum amount required to give an inhibition zone on plates with the sensitive bacterium *R. leguminosarum* RBL5523[23] (Figure 3). Quantities of **1** varying from 10 μg to 5 ng were applied on filter paper discs and tested on plates. Three experiments were done with only slightly varying results due to not completely equal culture conditions. Figure 3, representing one of the experiments, gives an impression of the inhibition zones obtained applying concentration series of the four stereo-

(13) Yoon, N. M.; Choi, J. *Tetrahedron Lett.* **1996**, *37*, 1057–1060.
(14) Downing, S. V.; Aguilar, E.; Meyers, A. I. *J. Org. Chem.* **1999**, *64*, 826–831.
(15) (a) Nicolaou, K. C.; Li, J.; Zenke, G. *Helv. Chim. Acta* **2000**, *83*, 1977–2006. (b) Mori, K.; Tashiro, T.; Akasaka, K.; Ohrui, H.; Fattorusso, E. *Tetrahedron Lett.* **2002**, *43*, 3719–3722. (c) Tashiro, T.; Akasaka, K.; Ohrui, H.; Fattorusso, E.; Mori, K. *Eur. J. Org. Chem.* **2002**, 3659–3665.
(16) Krapcho, A. P. *Synthesis* **1982**, 805–822.
(17) Ley, S. V.; Norman, J.; Griffith, W. P.; Marsden, S. P. *Synthesis* **1994**, 639–666.
(18) Keck, G. E.; Krishnamurthy, D. *J. Am. Chem. Soc.* **1995**, *117*, 2363–2364.
(19) (a) Nagao, Y.; Hagiwara, Y.; Kumagai, T.; Ochiai, M.; Inoue, T.; Hashimoto, K.; Fujita, E. *J. Org. Chem.* **1986**, *51*, 2391–2393. (b) Nagao, Y.; Dai, W.-M.; Ochiai, M.; Shiro, M. *J. Org. Chem.* **1989**, *54*, 5211–5217.
(20) Yuasa, H.; Satoda, S.; Aoyama, M. *Jpn. Kokai Tokkyo Koho 2003206254*, **2003.**
(21) Dess, D. B.; Martin, J. C. *J. Org. Chem.* **1983**, *48*, 4155–4156.

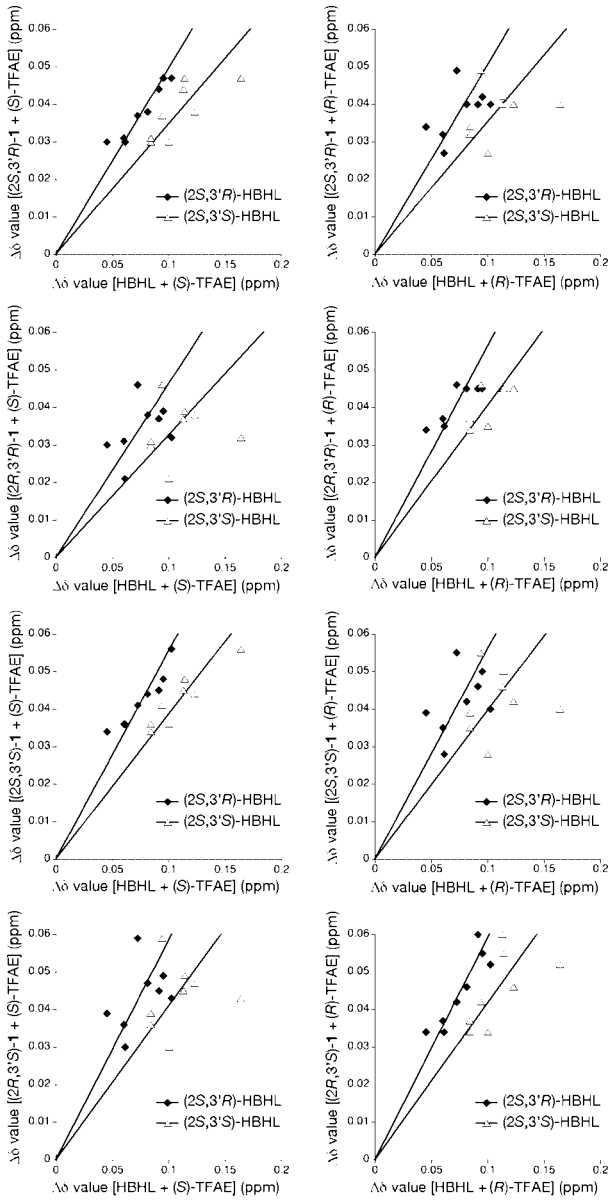


Figure 2. Correlation of the Δδ values of signals of the synthetic *small* bacteriocin stereoisomers with those of the corresponding signals of reference compounds [(2*S*,3′*R*)-**3** (◆) and (2*S*,3′*S*)-**3** (Δ)].

isomers. The biological activity expressed as minimum amount of **1** required for an inhibition zone was as follows: 2*S*,3′*R*: 5−10 ng; 2*R*,3′*R*: 50 ng; 2*S*,3′*S*: 1000 ng; 2*R*,3′*S*: 5000 ng. These results clearly indicate that the stereochemistry of **1** is very important for growth inhibition and the configuration of the side-chain stereogenic center is more

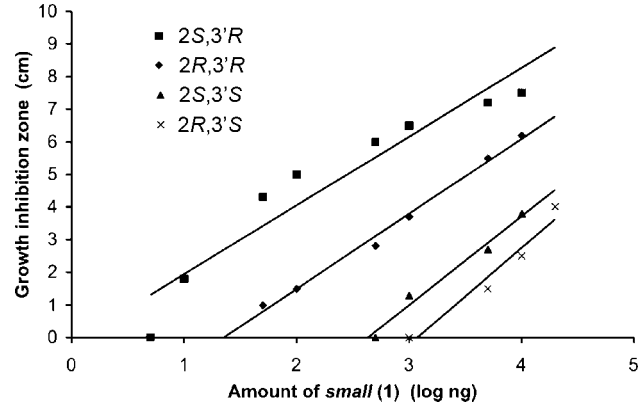


Figure 3. Inhibitory activity of the stereoisomers of **1**. A concentration series of **1** was applied to RBL5523/YMB agar plates, and after 2 days inhibition zones around the application point, visible as clear circular spots in the bacterial mass, were measured (*y*-axis).

important than that of the homoserine-lactone part. The amount of **1** produced in a *R. leguminosarum* LPR5045[24] stationary phase culture (500 mL) was estimated to be ca. 0.2 mg/L by comparing the growth inhibition zones of a $CHCl_3$ extract of this culture with those of synthetic (2*S*,3′*R*)-**1**.

In summary, we achieved the stereoselective synthesis of the four stereoisomers of **1**, an autoinducer of *R. leguminosarum*, using a methodology that will be useful for the synthesis of various 3′-hydroxy AHLs. The natural isomer exhibited the greatest inhibitory activity against the growth of *R. leguminosarum* RBL5523, whereas the other three isomers exhibited 5−500 times weaker activity. The chirality of autoinducers, which are quorum-sensing pheromones, is important for chemical communication among bacteria.

Acknowledgment. This work was supported in part by a Grant for Faculty Research Projects from the NODAI (Tokyo University of Agriculture) Research Institute for A.Y.

Supporting Information Available: Experimental details and spectral data for new compounds. This material is available free of charge via Internet at http://pubs.acs.org.

OL8005198

(22) Smith, T. H.; Djang, M.; Velander, A. J.; Downey, C. W.; Carroll, K. A.; van Alphen, S. *Org. Lett.* **2004**, *6*, 2317–2320.
(23) Laus, M. C.; van Brussel, A. A. N.; Kijne, J. W. *Mol. Plant-Microb. Interact.* **2005**, *18*, 533–538.
(24) Hooykaas, P. J. J.; Schnijdewindt, F. G. M.; Schilperoort, R. A. *Plasmid* **1982**, *8*, 73–82.