P. M. Rothe, W. Weigand, R. Wünsch, S. Dick, R. Nesper, M. Wörle, V. Gramlich, *Bull. Soc. Chim. Fr.* **1997**, *134*, 315 – 331; b) D. Seebach, A. Pichota, A. K. Beck, A. B. Pinkerton, T. Litz, J. Karjalainen, V. Gramlich, *Org. Lett.* **1999**, *1*, 55 – 58.

[7] The enantiopurity of the catalyst was obtained by mixing appropriate amounts of the ligand with *R*,*R* and *S*,*S* configuration.

[8] Nonlinear effects in asymmetric catalysis have been described in detail: a) D. Guillaneux, S.-H. Zhao, O. Samuel, D. Rainford, H. B. Kagan, *J. Am. Chem. Soc.* **1994**, *116*, 9430 – 9439; b) M. Reggelin, *Nachr. Chem. Tech. Lab.* **1997**, *45*, 392 – 396; c) C. Girard, H. B. Kagan, *Angew. Chem.* **1998**, *110*, 3088 – 3127; *Angew. Chem. Int. Ed.* **1998**, *37*, 2923 – 2959.

[9] D. M. Knotter, G. van Koten, H. L. van Maanen, D. M. Grove, A. L. Spek, *Angew. Chem.* **1989**, *101*, 351 – 352; *Angew. Chem. Int. Ed. Engl.* **1989**, *28*, 341 – 342.

[10] Selected examples of polynuclear Cu complexes with thiolate ligands: a) W. Clegg, C. D. Garner, J. R. Nicholson, P. R. Raithby, *Acta Crystallogr. Sect. C* **1983**, *39*, 1007 – 1009; b) D. M. Knotter, A. L. Spek, G. van Koten, *J. Chem. Soc. Chem. Commun.* **1989**, 1738 – 1740; c) B. Becker, W. Wojnowski, K. Peters, E. M. Peters, H. G. von Schnering, *Polyhedron* **1990**, *9*, 1659 – 1666; d) D. M. Knotter, H. L. van Maanen, D. M. Grove, A. L. Spek, G. van Koten, *Inorg. Chem.* **1991**, *30*, 3309 – 3317; e) D. M. Knotter, D. M. Grove, W. J. J. Smeets, A. L. Spek, G. van Koten, *J. Am. Chem. Soc.* **1992**, *114*, 3400 – 3410; f) Review article on $Cu^{I}$ compounds: C. E. Holloway, M. Melnik, *Rev. Inorg. Chem.* **1995**, *15*, 147 – 387.

[11] P. Stilbs, *Prog. Nucl. Magn. Reson.* **1987**, *19*, 1 – 45.

[12] All the diffusion measurements were carried out at 400 MHz by dissolving the sample in [$D_8$]THF at 20 °C and using the standard Stejskal – Tanner sequence.[18] The shape for the gradients was rectangular and their strength was varied in the course of the experiment. The pulse length was 5 ms and the time between the two gradients ($\Delta$) was 75 ms.

[13] The aggregation state of organocopper compounds in solution has been determined by others methods: a) R. G. Pearson, C. D. Gregory, *J. Am. Chem. Soc.* **1976**, *98*, 4098 – 4104; b) E. C. Ashby, J. J. Watkins *J. Am. Chem. Soc.* **1977**, *99*, 5312 – 5317; c) A. Gerold, J. T. B. H. Jastrzebski, C. M. P. Kronenburg, N. Krause, G. van Koten, *Angew. Chem.* **1997**, *109*, 778 – 780; *Angew. Chem. Int. Ed. Engl.* **1997**, *36*, 755 – 757; d) H. Huang, C. H. Liang, J. E. Penner-Hahn, *Angew. Chem.* **1998**, *110*, 1628 – 1630; *Angew. Chem. Int. Ed.* **1998**, *37*, 1564 – 1566; e) B. H. Lipshutz, J. Keith, D. J. Buzard, *Organometallics* **1999**, *18*, 1571 – 1574.

[14] Applications in transition metal complexes: a) P. S. Pregosin, R. Salzmann, *Coord. Chem. Rev.* **1996**, *155*, 35 – 68; b) P. S. Pregosin, G. Trabesinger, *J. Chem. Soc. Dalton Trans.* **1998**, 727 – 734.

[15] It is likely that OH deprotonation and subsequent $Mg^{II}$ complexation will prove to be important in determining the stereochemical course of the reaction.

[16] SAINT, Version 4, Siemens Analytical X-ray Systems, Inc., Madison, Wisconsin, USA.

[17] a) SADABS, G. Sheldrick, Göttingen, **1997**; b) SHELXTL program package, Version 5.1, Bruker AXS, Inc., Madison, Wisconsin, USA.

[18] E. O. Stejskal, J. E. Tanner, *J. Chem. Phys.* **1965**, *42*, 288 – 292.

# Acetyl Substitution of the O-Specific Caryan from the Lipopolysaccharide of *Pseudomonas (Burkholderia) caryophylli* Leads to a Block Pattern**

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Most of the O-specific polysaccharides of lipopolysaccharides (LPS) from Gram-negative bacteria represent heteropolysaccharides furnished with repeating units that comprise between two and eight monosaccharidic residues.[1] A large variety of sugars has been identified in such O-specific polysaccharides, which additionally may be phosphorylated, methylated, substituted by amino acids, or acetylated. Especially the last decoration has been frequently identified; it is always present in nonstoichiometric amounts. The acetylation takes place in later steps of O-specific polysaccharide biosynthesis and has been discussed as a microheterogenic property of LPS. In some cases, homopolysaccharides have been identified as O-specific polysaccharides which are mainly furnished from (amino-)deoxy sugars that may be N- and/or O-acylated, thus resulting in rather hydrophobic molecules.[2, 3] However, the influence of O-acetyl groups on the conformation of O-specific polysaccharides in general and on the establishment of O-antigenic conformative epitopes has not been investigated.

Few bacteria possess LPS that contain two different O-specific polysaccharides. In particular, this phenomenon is typical for *Burkholderia*,[3] a genus that comprises species which are pathogenic either to humans or plants. *Pseudomonas* (*Burkholderia) caryophylli* is a phytopatogenic bacterium responsible for the wilting of carnation.[4] Its LPS contain two linear homopolysaccharides as O-specific polysaccharides that are built up by two novel and rather peculiar monosaccharides. The major LPS portion possesses a polysaccharide termed caryophyllan, consisting of $\alpha$-(1 $\rightarrow$7)-linked caryophyllose (3,6,10-trideoxy-4-*C*-(D-*glycero*-1-hydroxyethyl)-D-*erythro*-D-*gulo*-decose).[5–7] The minor LPS portion contains a polysaccharide named caryan, built up from $\beta$-(1 $\rightarrow$7)-linked caryose (4,8-cyclo-3,9-dideoxy-L-*erythro*-D-*ido*-nonose).[7, 8] These O-specific polysaccharides are thought to be involved

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in bacteria–plant interactions and, thus, to represent important virulence factors. Here we report that the caryan is acetylated in an unusual pattern, which may have an impact on the interaction between the LPS and the plant surface.

The lipid A free caryan (molecular mass 38 kDa) was prepared from LPS by mild acid hydrolysis and purification using gel-permeation chromatography. The 1D $^1$H NMR spectrum of the sample (Figure 1) revealed the presence of O-acetyl groups (signals at $\delta = 2.17$ and 2.18), a feature that had not been detected before. To exclude the possibility of migration and loss of acetyl groups under acidic conditions, milder hydrolysis conditions of LPS were applied, either by reducing the time of hydrolysis in 1% acetic acid to one hour or by hydrolysis in 100 mM sodium acetate buffer containing 1% SDS (pH 4.4, 100 °C, 2 h).[9] The 1D $^1$H NMR spectra revealed in all cases the same acetylation pattern in the caryan. Methylation analysis of the caryan identified exclusively 7-substituted caryose, in accordance with earlier data.[8]

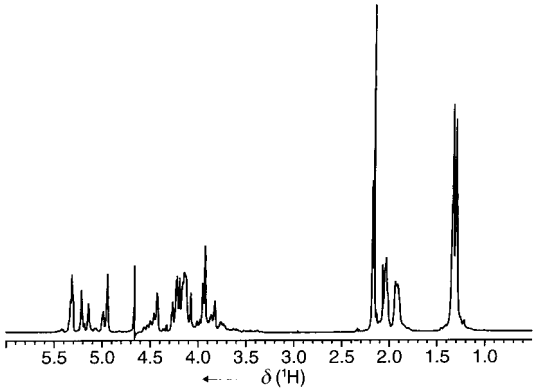


Figure 1. The 1D $^1$H NMR spectrum of the acetylated caryan from LPS of *Pseudomonas* (*Burkholderia) caryophylli*. The spectrum was recorded in $D_2O$ at 750 MHz and 37 °C.

Chemical shifts obtained by NMR spectroscopy were assigned using 2D homo- and heteronuclear experiments at 750 MHz ($^1$H; Table 1). In the $^1$H NMR spectrum of the non-acetylated caryan, the small coupling constant ($^3J_{H1,H2} < 4$ Hz) indicates a synclinal orientation of the protons H1 and H2, that is, the $\beta$ configuration of the glycosidic linkage. The caryose monosaccharide gives rise to two unconnected spin systems, H1/H3 and H5/H7. Each of these spin systems can be assigned using 2D COSY and TOCSY NMR spectra. Furthermore, the protons of the methyl group (H9) are not connected by spin–spin coupling to any of the other protons. However, both separate spin systems can be connected through NOE contacts and long-range $^1$H,$^{13}$C coupling constants. The interpretation of the NOE cross-peaks requires a simple molecular modeling study, as is described in detail below for acetylated polysaccharide.

Table 1. $^1$H and $^{13}$C NMR data for de-O-acetylated (D) and acetylated (A) caryan from the LPS of *Pseudomonas* (*Burkholderia) caryophylli*.[a]

| Unit | Chemical shift $\delta$ ($^1$H/$^{13}$C) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | $3_{ax}$ | $3_{eq}$ | 4 | 5 | 6 | 7 | 8 | 9 |
| D | | | | | | | | | | |
| d-**d**-d | 5.218 | 4.155 | 2.055 | 1.909 | – | 4.271 | 4.219 | 3.953 | – | 1.30 |
| | (96.2) | (64.6) | (30.4) | – | (78.2) | (74.3) | (77.7) | (87.6) | (78.7) | (15.7) |
| t**d** | 5.186 | 4.127 | 2.334 | 2.093 | – | 4.429 | 4.060 | 4.325 | – | 1.30 |
| $\beta$-red | 4.942 | 3.966 | 1.993 | 1.791 | – | – | – | – | – | – |
| $\alpha$-red | 4.559 | 3.854 | 1.838 | 2.111 | – | – | – | – | – | – |
| A | | | | | | | | | | |
| 1, a-**a**-a | 4.944 | 4.127 | 2.036 | 1.933 | – | 4.425 | 5.309 | 3.922 | – | 1.32 |
| | (97.1) | (64.6) | (30.4) | – | (78.6) | (73.7) | (79.4) | (87.0) | (79.3) | (15.7) |
| 2, a-**a**-d | 5.144 | 4.162 | 2.042 | 1.944 | – | 4.496 | 5.324 | 3.928 | – | 1.32 |
| | (97.5) | (64.4) | (30.4) | – | (78.6) | (73.7) | (79.4) | (87.0) | (79.3) | (15.7) |
| 3, d-**a**-a | 4.953 | 4.131 | 2.036 | 1.933 | – | 4.453 | 5.323 | 4.079 | – | 1.30 |
| | (97.1) | (64.6) | (30.4) | – | (78.6) | (73.8) | (79.4) | (85.9) | (79.3) | (15.7) |
| 4, d-**a**-d | 5.144 | 4.162 | 2.042 | 1.944 | – | 4.526 | 5.336 | 4.075 | – | 1.30 |
| | (97.4) | (64.6) | (30.4) | – | (78.6) | (73.8) | (79.4) | (85.9) | (79.3) | (15.7) |
| 5, d-**d**-d | 5.218 | 4.152 | 2.051 | 1.911 | – | 4.272 | 4.225 | 3.954 | – | 1.30 |
| | (96.2) | (64.6) | (30.4) | – | (78.2) | (74.3) | (77.7) | (87.6) | (78.7) | (15.7) |
| 6, d-**d**-a | 4.985 | 4.111 | 2.029 | 1.898 | – | 4.200 | 4.248 | 3.980 | – | 1.30 |
| | (96.4) | (64.6) | (30.4) | – | (78.2) | (74.3) | (77.7) | (87.6) | (78.7) | (15.7) |
| 7, a-**d**-d | 5.207 | 4.148 | 2.044 | 1.905 | – | 4.194 | 4.215 | 3.830 | – | 1.34 |
| | (96.2) | (64.6) | (30.4) | – | (78.2) | (74.2) | (77.7) | (89.1) | (78.7) | (15.7) |
| 8, a-**d**-a | 5.000 | 4.116 | 2.029 | 1.898 | – | 4.194 | 4.191 | 3.822 | – | 1.34 |
| | (96.4) | (64.6) | (30.4) | – | (78.2) | (74.2) | (77.7) | (89.1) | (78.7) | (15.7) |
| t**d**-d | 5.186 | 4.127 | 2.334 | 2.099 | – | 4.432 | 4.064 | 4.330 | – | 1.32 |
| | (96.2) | (64.6) | (30.4) | – | – | (72.1) | (76.8) | (78.1) | – | (15.7) |
| t**a**-d | – | – | – | – | – | 4.356 | 5.417 | 4.009 | – | – |
| t**a**-a | – | – | – | – | – | 4.420 | 5.436 | 4.012 | – | – |
| $\beta$-red | 4.942 | 3.966 | 1.993 | 1.791 | – | – | – | – | – | – |
| $\alpha$-red | 4.466 | 3.824 | 1.794 | 2.079 | – | – | – | – | – | – |

[a] The spectra were recorded at 750 MHz ($^1$H) and 188.6 MHz ($^{13}$C) in $D_2O$ at 37 °C. The $^1$H NMR data are given for each type in the first row, the $^{13}$C NMR data in the second (in parentheses). The coupling constants could not be measured accurately and only the following estimates were possible: $J_{H1,H2}$ small, $J_{H2,H3_{ax}}$ large, $J_{H2,H3_{aq}}$ small, $J_{H5,H6}$ large, $J_{H6,H7}$ medium. The residue observed in the NMR spectra is shown in boldface. A = acetylated caryan; a = 6-O-acetylated monomer; $\alpha$-red = $\alpha$-configured reducing end; $\beta$-red = $\beta$-configured reducing end; D = de-O-acetylated caryan; d = non-acetylated monomer; t = terminal. Signals of the O-acetyl group: $^1$H NMR: $\delta = 2.17$ (a-**a**-a, a-**a**-d), and 2.18 (d-**a**-a, d-**a**-d); $^{13}$C NMR: $\delta = 20.0$ (a-**a**-a, a-**a**-d, d-**a**-a, d-**a**-d).

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The structure of the repeating unit is confirmed by the long-range coupling constants which link the two $^1$H spin systems by connectivities from H9 to C4 and C7 and from $H3_{eq}$ to C4. The glycosidic linkage is clearly proven by the long-range connectivities between H1 and C7 and between C7 and H1. The high quality of the spectra obtained from high-field NMR experiments allows also the identification of the nonreducing terminal residue, which is characterized by chemical shifts at $\delta = 4.060$ (H6) and 4.325 (H7). The comparison with the corresponding shifts for the glycosylated residues ($\delta = 4.219$ and 3.953, respectively) shows a clear glycosylation effect. The signal for the residue at the nonreducing end possesses an relative intensity of approximately 0.5 % relative to that of the residues in the chain. This value is in good agreement with the determined average molecular mass of 38 kDa, corresponding to an average degree of polymerization of approximately 180 monosaccharide units. With regard to the reducing end it is only possible to assign the signals for H1, H2 and the two H3 protons with an $\alpha$:$\beta$ ratio of approximately 2:3.

A more complex problem is posed by the structural elucidation of the acetylated polysaccharide. The NMR data (Table 1) indicate that several different caryose residues occur which differ in their position in the caryan and in their acetyl substitution. As for the non-acetylated caryan, two independent spin systems were identified for each type of residue (H1/H3 and H5/H7). In the 750-MHz $^1$H NMR spectra of the acetylated polysaccharide, eight such spin systems were identified which could all be connected by cross-peaks obtained in a NOESY experiment (Table 2); the cross-peaks result from the close contact between $H3_{ax}$ and H5. Likewise, H9 could be assigned by similar cross-peaks between H7 and H9. These assignments were confirmed by long-range $^{13}$C,$^1$H spin–spin couplings in an HMBC experiment. The spin systems 1–8 (Table 3) identified eight different caryose units, four that are acetylated at O6 (type 1–4) and four that are not (types 5–8). Acetylation at O6 was proven by the down-field chemical shifts observed for H6 and C6, compared to those of unsubstituted caryose, and was confirmed by long range $^{13}$C,$^1$H spin–spin couplings between H6 and the carbonyl carbon atom.

Table 2. NOE contacts observed for the acetylated caryan.[a]

| Type(s) | Monomer(s) | NOE contacts[b] |
|---|---|---|
| *intraresidual* | | |
| 1–4 | -a- | H1–H2 (s), H2–$H3_{eq}$ (s), H2–$H3_{ax}$ (s), $H3_{ax}$–H5 (s), $H3_{ax}$–H9 (s),[c] $H3_{eq}$–H9 (s), H5–H6 (m), H5–H7(m), H6–H7 (m) |
| 5–8 | -d- | H1–H2 (s), H2–$H3_{eq}$ (s), H2–$H3_{ax}$ (s), $H3_{ax}$–H5 (s), $H3_{ax}$–H9 (s),[c] $H3_{eq}$–H9 (s), H5–H7(m), H6–H7 (m) |
| *interresidual towards the reducing end* | | |
| 1+3 | -**a**-a | H1–H6a (s), H1–H7a (m), $H3_{ax}$–H9 (s),[c] H5–H7a (m), H5–H9 (m) |
| 6+8 | -**d**-a | H1–H6a (s), H1–H7a (m), $H3_{ax}$–H9 (s),[c] H5–H7a (m), H5–H9 (m) |
| 2+4 | -**a**-d | H1–H6a (s), H1–H7a (m), $H3_{ax}$–H9 (s),[c] H5–H7a (m), H5–H9 (m) |
| 5+7 | -**d**-d | H1–H6a (s), H1–H7a (m), $H3_{ax}$–H9 (s),[c] H5–H7a (m), H5–H9 (m) |
| *interresidual towards the nonreducing end* | | |
| 1+2 | a-**a**- | H6–H1a (s), H7–H1a (m), H7–H5a (m), H9–$H3_{ax}$a (s),[c] H9–H5a (m) |
| 7+8 | d-**a**- | H6–H1a (s), H7–H1a (m), H7–H5a (m), H9–$H3_{ax}$a (s),[c] H9–H5a (m) |
| 3+4 | a-**d**- | H6–H1a (s), H7–H1a (m), H7–H5a (m), H9–$H3_{ax}$a (s),[c] H9–H5a (m) |
| 5+6 | d-**d**- | H6–H1a (s), H7–H1a (m), H7–H5a (m), H9–$H3_{ax}$a (s),[c] H9–H5a (m) |

[a] The NOE contacts are divided into strong (s), medium (m), and weak (w). a = 6-O-acetylated monomer; d = non-acetylated monomer. [b] For the interresidual NOEs, the first $^1$H is from the residue in boldface. [c] Sum of intra- and interresidual NOE contacts.

Table 3. Spin systems identified in the acetylated caryan.

| Type | Spin system[a] | Type | Spin system[a] |
|---|---|---|---|
| 1: | a-**a**-a (25 %) | 5: | d-**d**-d (20 %) |
| 2: | a-**a**-d (12 %) | 6: | d-**d**-a (10 %) |
| 3: | d-**a**-a (12 %) | 7: | a-**d**-d (10 %) |
| 4: | d-**a**-d (6 %) | 8: | a-**d**-a (5 %) |

[a] The amounts are given as a percentage, as identified by integration of the NMR signals. a = 6-O-acetylated monomer; d = non-acetylated monomer. The residue observed in the NMR spectrum is shown in boldface.

The linkages between the eight different residues were assigned with data obtained from NMR spectra at 750 MHz. The dipole–dipole correlations observed in a NOESY spectrum identified the sequence of the residues (Figures 2 and 3, Table 2). Important NOE connectivities are those between H1 of the glycosyl donor and H6 and H7 of the glycosylated residue. The NOESY spectrum in Figure 2a shows that a type 1 residue (a-**a**-a, a = acetylated caryose; the residue under consideration is in boldface) is linked to another acetylated caryose (cross-peak between H1 of the residue under consideration and H7 of another acetylated residue). Likewise, the H1 signal of a type 2 caryose (a-**a**-d, d = non-acetylated residue) gave a corresponding NOE signal for a non-acetylated residue. For both type 1 and 2 residues, the residue at the nonreducing end was identified as the acetylated residue, owing to NOE connectivities between H1 of an acetylated residue and H6 and H7 of the glycosylated residue. Similarly, types 3–8 can be assigned. Figure 2b shows that the sequential assignment of acetylated types 1–4 can additionally be obtained by the identification of NOE contacts between H5 of the glycosylating residue and H7 of the glycosylated residue. It should be noted that the NOE signal between H5 and H7 within the same residue is clearly differentiated by a TOCSY spectrum.

The sequence information obtained by NOE contacts was confirmed by long-range $^{13}$C,$^1$H couplings observed in an HMBC experiment. For most of the eight residue types, chemical shifts for C7 are distinct and allow assignment of the long-range connectivities to H1 of the glycosylating residue. Based on the proportion of the eight structure types, as estimated from integration of the 1D $^1$H NMR spectra, the ratio of acetylated to non-acetylated residues is approximately one to one.

The assignment of the residue sequence is only possible since the acetyl group at O6 affects not only the chemical shifts of H6 and the proximal H5 and H7, but also that of H1 in the glycosylating residue (compare the corresponding chemical shifts of, for example, residues of types 1 and 2 (a-**a**-a and

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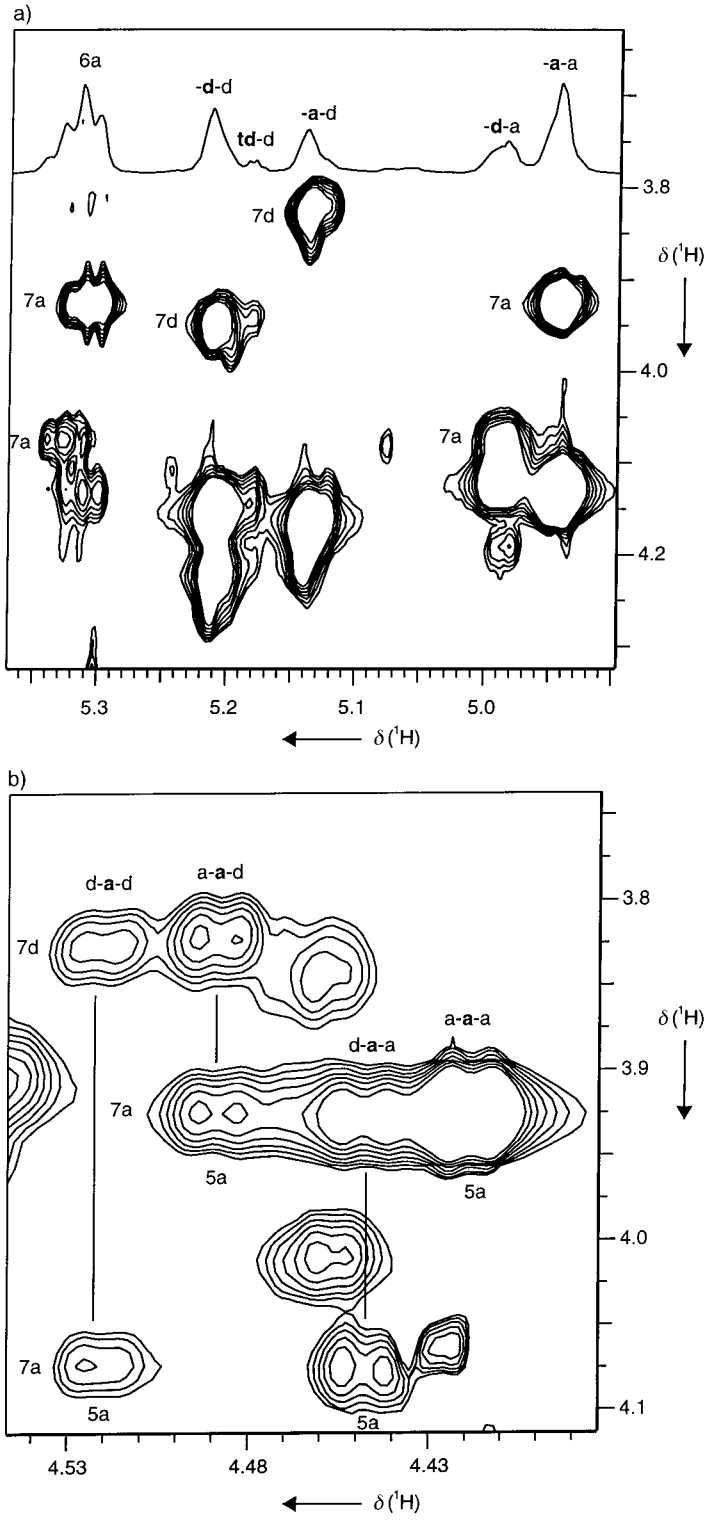


Figure 2. Sections of the NOESY spectrum (40 ms) of the acetylated caryan. a) Regions showing the sequential connectivities between the anomeric H1 and H7 of the adjacent residue; b) region showing the intra- and interresidual NOE connectivities between H5 of acetylated residues and H7 of adjacent residues. The NOE contacts between H1 and H6 are not shown in this section. td = terminal non-acetylated monomer.

a-**a**-d) and 5 and 6 (d-**d**-d and d-**d**-a)). Such substantial effects through space are well described for carbonyl/ester functionalities, which have a large anisotropic effect on chemical shifts. Generally, smaller effects are seen for $^{13}C$ chemical shifts.

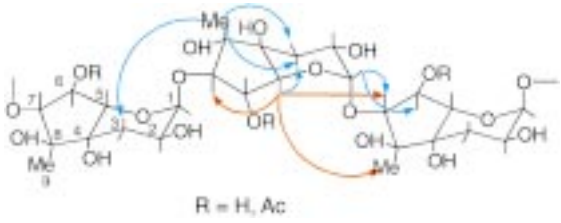


Figure 3. Trisaccharide structures representing structure types 1–8 and important NOE connectivities. Only NOE connectivities for the middle residue are shown; trivial NOEs between vicinal $^1H$ pairs are not included. Important NOE contacts from H5 to other protons are shown in red.

As in the case of the non-acetylated caryan, the NMR data of the terminal residues can be assigned. For the unsubstituted nonreducing end residue that is linked to another non-acetylated residue, a complete assignment was possible. For the other types of terminal residues, only a part of the chemical shifts could be assigned owing to overlapping of signals.

The strong NOE signals observed between $H3_{ax}$ and H9, which are important for the assignment of the configuration of the caryose monosaccharide, could lead to a wrong assignment for the repeating unit of the polysaccharide, as it would indicate a diaxial arrangement. At the same time a strong NOE contact is observed between $H3_{eq}$ and H9, indicating an equatorial orientation of H9. This can be explained by modeling a larger structure with an equatorial orientation of H9, where a fairly short distance is seen between H9 and $H3_{eq}$ of the adjacent residue. Thus, the observed NOE contact between H9 and $H3_{ax}$ reflects the sum of NOE signals between intra- and interresidual protons. Furthermore, it is in agreement with an equatorial orientation of H9 and with the overall configuration of the isolated monomer, as described previously.[8]

The minimum-energy conformation of the acetylated trisaccharide is shown in Figure 4. It can clearly be seen how acetylation of O6 accounts for significant effects on the

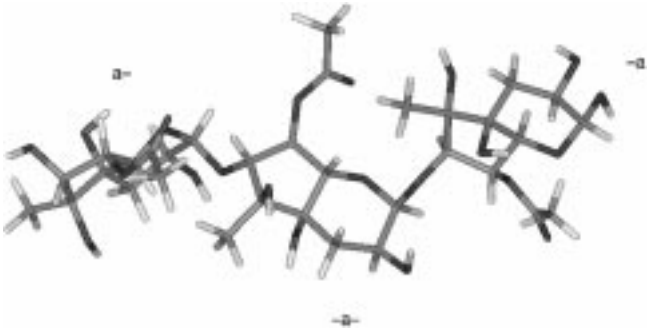

Figure 4. Stick representation of a minimum-energy conformation of the acetylated trisaccharide a-a-a, based on the identified NOE connectivities as described in the text and summarized in Table 2.

chemical shift of H1 of the glycosylating residue. Molecular modeling of larger structures indicated that the linear sequence does not result in any significant long-range interaction. Thus, the overall conformation is determined by local interactions between adjacent residues. Therefore, it

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may not be possible to determine by NMR spectroscopy the length of stretches of structures 1–8. Surprisingly, a comparison of the conformations of the acetylated and non-acetylated stretches (modeled in trisaccharide sequences) gave only small differences, indicating that the two conformations do not differ much.

In conclusion, we have characterized the acetylation pattern of the O-specific caryan of LPS from *Pseudomonas* (*Burkholderia) caryophylli*. Considering the amounts of units 1–8, we propose a model of a repeating unit of the caryan that comprises smaller areas of both acetylated and non-acetylated caryose residues (Figure 5). Thus, acetylation occurs mainly


Figure 5. Proposed structure of the repeating unit of the caryan from *Pseudomonas* (*Burkholderia) caryophylli*. The repeating unit is built up from 19 caryose residues. The acetylation/deacetylation pattern reflects the occurrence of units 1–8, as given in Table 3. For clarity, units 1–8 are depicted using different colors. Acetylated areas (A) are followed by non-acetylated areas (D); in both cases, the chains are not very long and comprise about 4–5 caryose residues. Furthermore, "erraneous" acetylation may occur, leading to units 4 and 8.

blockwise. The biosynthesis of such an acetylation pattern and its impact on the biological properties of the caryan are not yet understood. However, the acetylation should increase the hydrophobicity of the polysaccharide, which may be important for the interaction between the bacteria and plant cell surface molecules.

## *Experimental Section*

General: The content of caryose and caryophyllose was identified by GC and GC-MS of the corresponding alditol acetates or acetylated methyl glycosides. The caryan was methylated according to the procedure of Hakomori.[10] Methylation analysis was achieved using GC-MS (Hewlett-Packard 5890, SPB-5 capillary column (0.25 mm × 30 m, Supelco), temperature program: 150 °C for 5 min, then 5 °C min$^{-1}$ to 300 °C) after hydrolysis of the methylated caryan, reduction with $NaBD_4$, and acetylation with acetic anhydride in pyridine at 120 °C for 20 min.

Isolation of acetylated caryan: A portion (100 mg) of the LPS mixture[7] was placed on a Sephadex G-100 column (3 × 100 cm, Pharmacia), eluted with 0.2 M NaCl, 1 mM EDTA, 10 mM Tris-HCl, 0.25 % (v/v) sodium deoxycholate, and 0.02 % (w/v) $NaN_3$ in water. Three fractions were detected using a differential refractometer (Knauer) and were dialyzed first against 0.2 M NaCl, 1 mM EDTA, 10 mM Tris-HCl, and 0.02 % (w/v) $NaN_3$ in water (3 d), then against 0.2 M NaCl in water (3 d), and finally against water (3 d). After lyophilization, the fractions were analyzed by sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE, 10 % gel): The first fraction (4 mg, 4 %) contained a polysaccharide which was not detectable by SDS-PAGE and which is currently being investigated. The other two fractions 2 (63 mg, 63 % of LPS) and 3 (27 mg, 27 % of LPS) contained different proportions of the two LPSs, that is, one possessing the caryophyllose and the second possessing the caryose O-specific polysaccharide. Owing to the acid lability of the glycosidic linkage of caryophyllose, fractions 2 and 3 (90 mg) were hydrolyzed (2 mL of 1 % aqueous acetic acid, 100 °C, 2 h). The lipid A (sediment) and the mixture of caryan and hydrolysis products (supernatant) were collected by ultracentrifugation (100.000 × *g*, 4 °C, 1 h). The last fraction was lyophilized (yield: 50 mg, 50 % of crude LPS) and separated on a Sephadex G-50 column (3 × 50 cm, Pharmacia) with pyridine/acetic acid/water (4/10/1000 v/v/v; pH 4.5) as eluent; the separation was monitored with a differential refractometer. The fraction eluting in the void volume contained pure caryan (10 mg, 10 % of crude LPS), whereas fractions that eluted later contained mainly oligomers of caryophyllose.

Deacetylation of the caryan: The caryan (5 mg) was dissolved in 10 mM NaOH (1 mL) and left at 20–22 °C for 16 h. The solution was neutralized with Dowex 50 WX 8 ($H^+$) and lyophilized (yield: 4.1 mg).

Size determination: The caryan (1 mg) was dissolved in 50 mM $NH_4HCO_3$ (0.5 mL) and separated by chromatography on a Sephacryl S 300 HR column (1 × 30 cm, Pharmacia) with 50 mM $NH_4HCO_3$ as eluent (0.4 mL min$^{-1}$) and monitored with a differential refractometer. The column was calibrated using dextran standards (molecular masses: 670, 150, 80, 12, and 5 kDa).

NMR spectroscopy: NMR spectroscopy was carried out at 500 and 750 MHz using Varian UNITY INOVA 500 and 750 spectrometers. Samples were prepared in $D_2O$ (0.6 mL), and spectra were obtained at 37 °C. The chemical shifts are given relative to those of acetone ($\delta = 2.225$ for $^1H$ and 31.4 for $^{13}C$). Homo- and heteronuclear experiments were carried out as described previously,[11] with the exception that NOESY data were obtained with mixing times of 40 ms.

Molecular modeling: Molecular modeling was carried out using the CVFF force field in the Discover program.[12] The caryose residue was constructed using standard bond lengths and angles in the Insight II program (MSI, San Diego). Molecular dynamics simulations were performed for the trisaccharide fragments in a water box with side length of 40 Å for up to 400 ps and with a step length of 1 fs. Full coordinates were saved every 2 ps.

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[1] U. Mamat, U. Seydel, D. Grimmecke, O. Holst, E. T. Rietschel in *Comprehensive Natural Products Chemistry*, *Vol. 3* (Eds.: D. Barton, K. Nakanishi, O. Meth-Cohn, B. M. Pinto), Elsevier, Oxford, **1998**, pp. 179.
[2] Y. Y. Knirel, N. K. Kochetkov, *Biochemistry (Engl. Transl.)* **1994**, *59*, 1325–1383.
[3] P.-E. Jansson in *Endotoxin in Health and Disease* (Eds.: H. Brade, D. C. Morrison, S. Opal, S. Vogel), Marcel Dekker, New York, **1999**, p. 155.
[4] L. K. Jones, *Phytopathology* **1941**, *31*, 199.
[5] M. Adinolfi, M. M. Corsaro, C. De Castro, R. Lanzetta, M. Parrilli, A. Evidente, P. Lavermicocca, *Carbohydr. Res.* **1995**, *267*, 307–311.
[6] M. Adinolfi, M. M. Corsaro, C. De Castro, A. Evidente, R. Lanzetta, L. Mangoni, M. Parrilli, *Carbohydr. Res.* **1995**, *274*, 223–232.
[7] C. De Castro, A. Evidente, R. Lanzetta, P. Lavermicocca, E. Manzo, A. Molinaro, M. Parrilli, *Carbohydr. Res.* **1998**, *307*, 162–172.
[8] M. Adinolfi, M. M. Corsaro, C. De Castro, A. Evidente, R. Lanzetta, A. Molinaro, M. Parrilli, *Carbohydr. Res.* **1996**, *284*, 111–118.
[9] M. Caroff, A. Tacken, L. Szabo, *Carbohydr. Res.* **1988**, *175*, 273–282.
[10] S. Hakomori, *J. Biochem*. **1964**, *51*, 205–208.
[11] E. V. Vinogradov, B. O. Petersen, J. E. Thomas-Oates, J. Ø. Duus, H. Brade, O. Holst, *J. Biol. Chem*. **1998**, *273*, 28122–28131.
[12] A. T. Hagler, S. Lifson, R. Dauber, *J. Am. Chem. Soc*. **1979**, *101*, 5122–5130.

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