Glycoconjugate Journal 15, 555–562 (1998)


# Studies on the reductive amination of 3-deoxy-D-*manno*-octulosonic acid (Kdo)

H. Dieter Grimmecke and Helmut Brade

*Forschungszentrum Borstel, Zentrum für Medizin und Biowissenschaften, Parkallee 22, 23845 Borstel, Germany*

**Reductive amination of 3-deoxy-D-*manno*-octulosonic acid (Kdo) with allylamine (AllN) or 2-(4-aminophenyl)ethylamine (APEA) yields epimer pairs of 2-*N*-allylamino and 2-*N*-[2-(4-aminophenyl)ethylamino]-2,3-dideoxy-D-*glycero*-D-*galacto*- and-2,3-dideoxy-D-*glycero*-D-*talo*-octonic acid. The yields were 50–60% due to reduction of Kdo to the respective polyols as side reaction products. Mass spectrometric analyses proved the amination derivatives to be the expected glycamines. Nuclear magnetic resonance (NMR) studies were performed on 2-*N*-allylamino-2,3-dideoxyoctonic acid which represents the chain terminus of allylaminated oligosaccharides derived from bacterial lipopolysaccharides (LPS) after acid hydrolysis and reductive allylamination.**

***Keywords*: 3-deoxy-D-*manno*-octulosonic acid, Kdo, reductive amination, allylamine, 2-(4-aminophenyl)ethylamine, glycamine, spacer arm, neoglycoconjugate**

## Introduction

Bacterial lipopolysaccharides (LPS) are common constituents of the outer membrane of Gram-negative bacteria and play an important role in pathogenesis [1]. LPS modulates virulence of bacteria [2], and the host immune system recognizes LPS as antigen [3] resulting in the production of antibodies against different parts (epitopes) of the LPS. These epitopes consist of different carbohydrate assemblies and of structures formed from carbohydrates substituted by phosphate, amines, amino acids, pyruvate, and other non-carbohydrate substituents [4]. Chemical syntheses of LPS core part structures and neoglycoconjugates thereof built up of 3-deoxy–D-*manno*-octulosonic acid (Kdo) [5], L-*glycero*-D-*manno*-heptose (LD-Hep) [6], and those containing LD-Hep and Glc [6, 7] have been reported earlier. However, general methods for the preparation of artificial glycoconjugates containing larger carbohydrate haptens such as the complete carbohydrate backbone of rough LPS, are not available. For this reason we have established a method for neoglycoprotein preparation for such oligosaccharides resulting from alkaline degradation of LPS [Grimmecke HD, Brade H, unpublished results]. In continuation of this work we are studying neoglycoprotein preparation from those part structures containing alkali labile substituents. In order to obtain these fragments from LPS, the lipid A moiety has to be split off by mild acetic acid or acetate buffer hydrolysis. The resulting products usually have Kdo as the reducing terminus [8, 9].

Kdo is the acid labile ketose located in the so-called inner core region of the LPS between heptose and lipid A. This location and its acid lability are the basis of preparation of fatty acid free polysaccharide preparations from bacterial LPS. However, during acid degradation Kdo may undergo lactonization, formation of anhydro derivatives, and other not yet characterized artifacts [10, 11]. The extent of such side reactions depends on the nature and concentration of the acid, temperature, and duration of treatment [10, 11]. Likewise, substituents and the salt form of the LPS may influence hydrolysis kinetics, and, hence, the formation of artifacts. For structural research, heterogeneity concerning the last sugar unit of the oligomeric chain results in complex nuclear magnetic resonance (NMR) spectra due to signal splitting for those sugars located near the modified end group [12].

The preparation of neoglycoconjugates representing carbohydrate epitopes of bacterial LPS has been performed using synthetic oligosaccharides containing tailor-made preformed spacer arm carrying constructs [13] for coupling to protein (conjugation), or using oligosaccharides prepared enzymatically [14] by *endo*-glycosidases or chemically [15] from a native origin. Usually the reducing end is stable under those conditions used for its generation and there are numerous examples of the preparation of immunoconjugates containing oligosaccharides with aldose as the reducing end originating from both LPS O-antigens and capsular polysaccharides.

Immunogenic oligosaccharide-protein conjugates made from acid degraded R-LPS have been described by several groups which used reductive amination with cyanoborohydride and 2-(4-aminophenyl)ethylamine (APEA) as the linker unit, described earlier [14] for *Salmonella* O-antigen part structures. In none of those papers known to us are details given concerning the content of unmodified, reducing Kdo on the terminus of the oligosaccharides available for reductive amination, nor was described the degree of incorporation of APEA onto the terminus of the sugar chain. Furthermore, the spacer construction on the Kdo moiety should take into account that the immune response also generates antibodies against the spacer arm [16, 17], particularly against the highly immunogenic aryl linkers [16, 18]. Thus, spacer constructs of low antigenicity should be selected [17]. As entry for efficient neoglycoconjugates with low antigenic spacer arms, 3-(2-aminoethyl-thio)propyl glycoside [5, 13, 19] and *N*-[3-(2-aminoethylthio) propyl] glycamine [Grimmecke HD, Brade H, unpublished results] derived sugars were synthesized from *O*-allyl glycosides or *N*-allyl glycamines, respectively. Following these strategies, this paper concerns products formed by the reductive amination of Kdo using allylamine (AllN) and APEA for the generation of reactive spacer constructs for the preparation of lipopolysaccharide derived neoglycoconjugates.

## Materials and methods

### Chemicals, reagents and general methods

Allylamine (AllN), 4-(2-aminoethyl)phenylethylamine (APEA), cysteamine hydrochloride (CyN), and ammonium hydrogencarbonate were purchased from Aldrich, Steinheim, Germany, sodium cyanoborohydride from Serva sodium borohydride from Merck, Darmstadt, Germany. Ammonium 3-deoxy-D-*manno*-octulosonate (Kdo) was a gift from P. Kosma, Vienna), and the mixture of 3-deoxy-D-*glycero*-D-*galacto*- and 3-deoxy-D-*glycero*-D-*talo*-octitol was described [20] earlier.

Gel permeation chromatography (GPC) was performed on Sephadex G10 (fine), Pharmacia, columns (1.5 × 68 cm), using 10 mM $NH_4HCO_3$, pH 7.9–8.3 (1 ml $min^{-1}$ and 2.5 ml $min^{-1}$, respectively). Fast liquid chromatography (LC) on Superdex Peptide HR 10/30 (Pharmacia, Freiburg, Germany), 1 ml $min^{-1}$ 10 mM $NH_4HCO_3$, or 10 mM $NH_4HCO_3$, 0.1% trifluoroacetic acid, was used for fine purification of glycamines with a hydrophobic spacer. Both GPC and LC, were monitored using a differential refractometer (Knauer, Berlin, Germany).

Gas-liquid chromatography (GLC) was performed on a Varian model 3700 equipped with a fused silica capillary column (25 m × 0.2 mm) chemically bonded SE-54. Gas-liquid chromatography-mass spectrometry (GLC-MS) was performed on a Hewlett Packard spectrometer (model 5989A) equipped with a chemically bonded fused silica capillary column (SE-54, 25 m × 0.32 mm). Electron-impact mass spectra (El-MS) were recorded at 70 eV, chemical-ionization mass spectra (Cl-MS) were registered using ammonia as the reactant gas.

Samples were analysed by high performance anionic exchange chromatography (HPAEC) on an HPLC system (DIONEX) using a PA-100 column and pulsed amperometric detection. For Kdo derivatives, separation was performed in a gradient of 1 M sodium acetate in 0.1 M NaOH (2–60%) over 20 min.

One-dimensional $^1H$ and $^{13}C$ NMR spectra, $^1H$ homonuclear two-dimensional COSY, RCT-COSY, NOESY, DEPT, and HMQC spectra were recorded on a Bruker AM360L spectrometer. If not stated otherwise, samples were exchanged against $D_2O$ (three times) by evaporation or lyophilization, and chemical shifts were measured relative to acetone ($\delta$2.223), DOH ($\delta$4.750) at 27 °C). $^{13}C$ NMR spectra were obtained using broad-band decoupling and chemical shifts were recorded relative to internal acetone ($\delta$31.45). All experiments were performed with standard Bruker software.

### Reductive amination of Kdo with allylamine (AllN) and cyanoborohydride

3-Deoxy-D-*manno*-octulosonate (13.6 mg, 50 μmol), AllN-$NaBH_3CN$-reagent (250 μl), containing AllN (1 mmol $ml^{-1}$), and $NaBH_3CN$ (1 mmol $ml^{-1}$) in water, adjusted to pH 8 with 10% AcOH, were incubated for 24 h at room temperature (RT) (assay 1) or at 50 °C (assay 2), or 56 h at 65 °C (assay 3). The reactions were monitored by HPAEC. After complete consumption of Kdo, mixtures were desalted on Sephadex G10, and the major peaks containing Kdo derivatives were separated into two peaks (A and B) by (LC). The retention times in the HPAE chromotography were as follows: Kdo standard 6.83 min, peak A, 4.19 to 5.20 min (several partially resolved peaks, 35% all together), peak B, 6.28 min (about 60%, average of all assays), and some minor fractions which were not analyzed. The yield (peak B, obtained after repeated LC steps, and

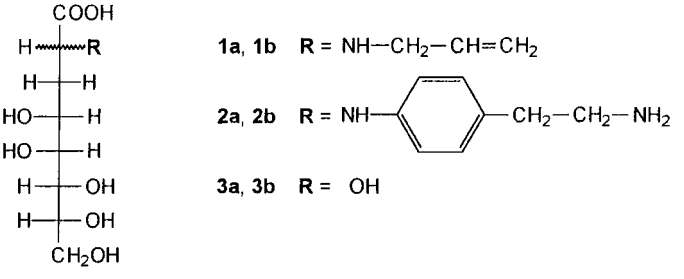


**Figure 1.** Structure of the compounds used in this study. Compound 1 (1a and 1b) and 2 (2a and 2b) were prepared from 3-deoxy–D-*manno*-octulosonate (Kdo, $NH_4^+$-salt) and allylamine (AllN) or 2-(4-aminophenyl)ethylamine (APEA), respectively, by reductive amination, and the structures were investigated as mixtures of the isomers. The mixture of compound 3a and 3b was from [24].

**Table 1.** $^{1}$H NMR Data ($\delta$, ppm, $J$, Hz) for the octitols and for the homomorphous polyols

| *Proton* | *3-Deoxyoctitols* | | | *Homomorphous polyol* |
|---|---|---|---|---|
| | *1a* | *3a* | *3b* | *D-glycero-D-galacto-Heptitol (4)* |
| H-1a | | 3.638 (11.8, 3.4) | 3.598 | |
| H-1b | | 3.506 (11.8, 7.0) | 3.482 | |
| H-2 | 3.85 | 3.949 (3.4, 6.3, 7.0, 9.5) | 3.86 | |
| H-3a | 1.97 | 1.967 (2.9, 6.3, 14.5) | 1.882 | |
| H-3b | 1.81 | 1.599 (6.6, 9.5, 14.5) | 1.499 | |
| H-4 | 4.194 | 3.82 (2.9, 6.6, 8.7) | 3.92 | 3.67 (1.5, 9.4) |
| H-5 | 3.831 (1.2, 8.8) | 3.779 (1.3, 8.7) | 3.78 | 3.92 (1.2, 9.4) |
| H-6 | 3.620 (1.2, 9.4) | 3.638 (1.3, 9.5) | 3.64 | 3.81 (1.2, 8.8) |
| H-7 | 3.73 | 3.714 (2.6, 8.5, 9.5) | 3.72 | 3.77 (2.9, 6.4, 8.8) |
| H-8a | 3.82 | 3.823 (2.6, 11.8) | 3.82 | 3.87 (2.9, 11.8) |
| H-8b | 3.64 | 3.635 (8.5, 11.8) | 3.64 | 3.67 (6.4, 11.8) |
| H-1′ | 3.689 | | | |
| H-2′ | 5.90 | | | |
| H-3′ | 5.48 | | | |

[a]Data taken from [25]. The table contains the chemical shifts ($\delta$, ppm) and the coupling constant ($J$, Hz) of the protons H-3 to H-7 positioned in those fields of the isomorphous protons of 3-deoxyoctitols.

**Table 2.** $^{13}$C NMR Data ($\delta$, ppm) for the octitols and for the homomorphous polyols

| *Compound* | *Homomorphous polyol* | *Polyol moiety* | | | | | | | | *Allyl moiety* | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | *C-1* | *C-2* | *C-3* | *C-4* | *C-5* | *C-6* | *C-7* | *C-8* | *C-1′* | *C-2′* | *C-3′* |
| 1a | | 183.07 | 55.07 | 39.26 | 70.61 | 69.02 | 73.39 | 72.17 | 64.32 | 50.00 | 128.55 | 125.21 |
| 1b | | 182.41 | 54.00 | 39.04 | 70.40 | 69.02 | 73.09 | 72.01 | 64.24 | 50.19 | 128.55 | 125.21 |
| 3a | | 66.10 | 71.27 | 37.32 | 70.10 | 70.36 | 73.37 | 72.11 | 64.31 | | | |
| 3b | | 67.27 | 68.56 | 37.38 | 69.64 | 70.60 | 73.53 | 72.17 | 64.31 | | | |
| | Mannitol[a] (C-2 to C-6) | | | | 72.2 | 70.7 | 70.7 | 72.2 | 64.6 | | | |
| 4 | gg-Hep[b] (C-2 to C-7) | | 71.65 | | 70.65 | 69.7 | 77.55 | 72.3 | 64.65 | | | |

[a]Data taken from [27]
[b]D-*glycero*-D-*galacto*-Heptitol. Data taken from [26]

from the two experiments) was 11.7 mg [1(1a and 1b major and minor component, respectively)]. For structures see Figure 1, for NMR data see Tables 1 and 2 and text, and for MS data see Figures 2 and 3 and text.

## Reductive amination of Kdo with allylamine (AllN) and pyridine-borane

3-Deoxy-D-*manno*-octulosonate (13.6 mg, 50 μmol), AllN (250 mmol), and pyridine-borane (250 μmol) were incubated in 25% aqueous MeOH (0.5 ml) for 14 d at 60 °C (procedure according to [21] with minor modification). Isolation of Kdo derivatives was performed as described above.

## Alkaline degradation of (*1*) and saponification of 2-*N*-allylamino-2,3-dideoxy-octonic acid 1,4-lacton penta-acetate

Compound 1 and the products obtained from methanolysis and peracetylation (see below) (2 mg, each) were treated with 0.1 M NaOH (0.5 ml), for 48 h at 60 °C. After neutralization with 0.1 M HCl, the samples were desalted on Sephadex G10 and analysed by HPAEC.

## Reductive amination of Kdo with 2-(4-aminophenyl)ethylamine (APEA)

The method of Kallin [22] was used with minor modifications. Briefly, Kdo (13.6 mg, 50 μmol), APEA (free base, 66 μl, 500 μmol), 1 M $NaBH_3CN$ (500 μl) were mixed and the pH was adjusted to 7.0 with 10% AcOH. The solution was incubated for 72 h at RT. The reaction mixture was applied to Sephadex G10 to isolate a broad peak with several shoulders indicating heterogeneity of Kdo derivatives.

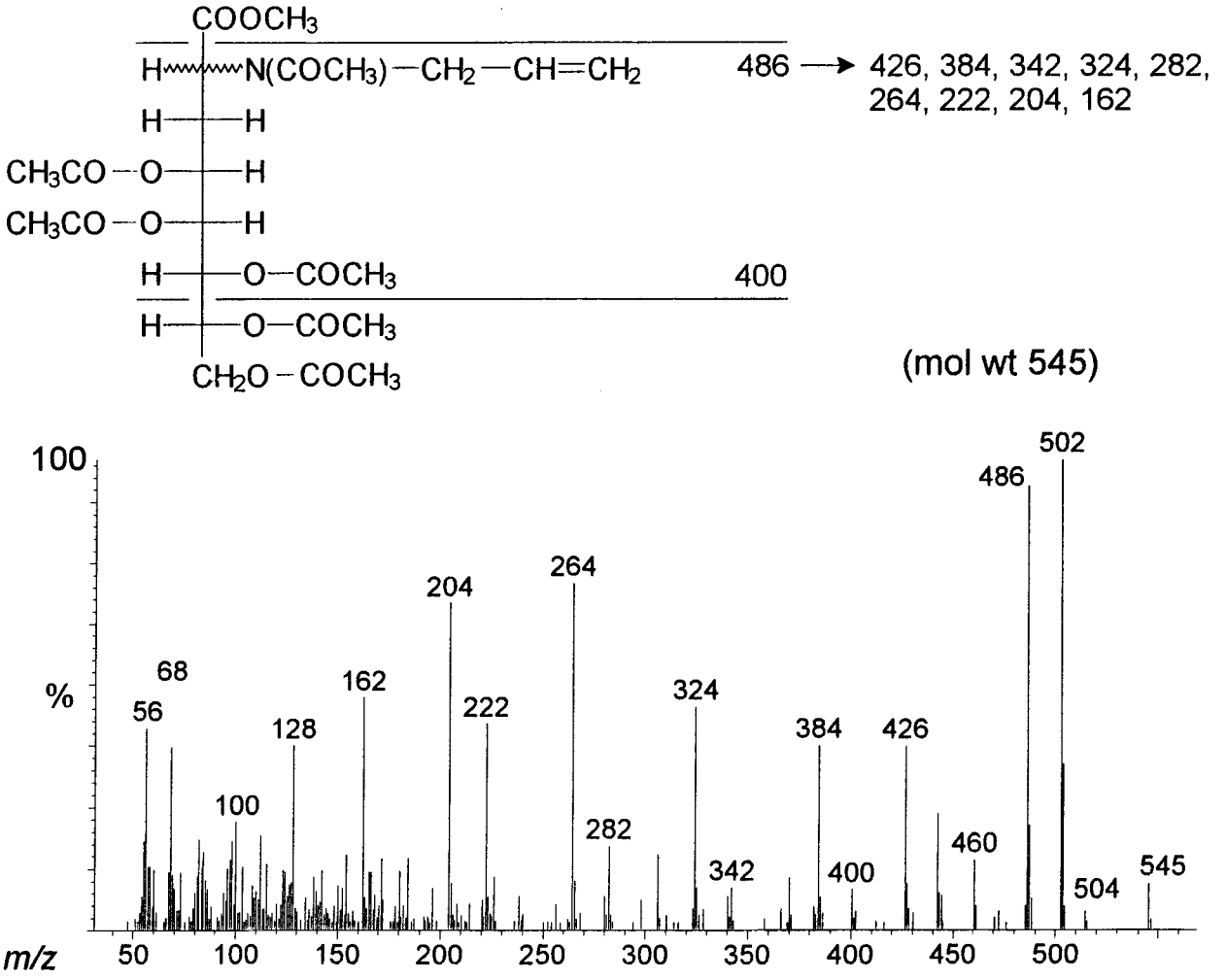


**Figure 2.** Mass spectrum and fragmentation pattern of the peracetylated methyl ester of 2-*N*-allylamino-2,3-dideoxy-D-octonic acids (1a and 1b). The derivatives were obtained from 3-deoxy-D-*manno*-octulosonate (Kdo, $NH_4^+$-salt) and allylamine (AllN) by reductive amination followed by treatment with methanol-hydrochloric acid and peracetylation.

Fractions were tested by HPAE chromatography. Fractions containing Kdo-derivatives were collected and lyophilized. Yield: 14.0 mg [2(2a and 2b)]. For structures see Figure 1, for MS data see Figure 3 and text.

### Derivatization for GLC and GLC-MS

Sample (2 mg) was treated with 0.5 M HCl (0.5 ml) in methanol (45 min, 85 °C) for esterification. Reagents were removed under nitrogen and peracetylation was conducted in pyridine-acetic anhydride (1 : 1, v/v, 100 µl) at 60 °C, for 1 h. Reagents were evaporated under nitrogen, and samples were analyzed by GLC and GLC-MS.

## Results and discussion

Reductive amination on the reducing end of oligosaccharides has found wide application for labeling saccharides and for spacer arm synthesis in neoglycoconjugate preparations because of easy handling, controlled reaction conditions, high reliability and, in general good yields. However, in none of the reports in the literature known to us, were chemical data shown on the behavior of 3-deoxyoctulosonic acids, e.g. Kdo and sialic acid, during reductive amination. For this reason, we performed these reactions with AllN and APEA on synthetic Kdo as a model compound.

In initial experiments, allylamination reagents containing higher concentrations of AllN and cyanoborohydride (1 and 2 mmol, respectively) were applied to 50 µmol Kdo. Since the yields did not differ from the assay described above we selected a molar ratio 1 : 5 : 5 for reducing sugar, AllN, and cyanoborohydride, respectively.

In order to get rapid information on the progress of the reductive amination, samples (1 µmol) were desalted on Sephadex G10 and analyzed by HPAEC. In all experiments carried out at RT or at 60 °C, the reaction was completed

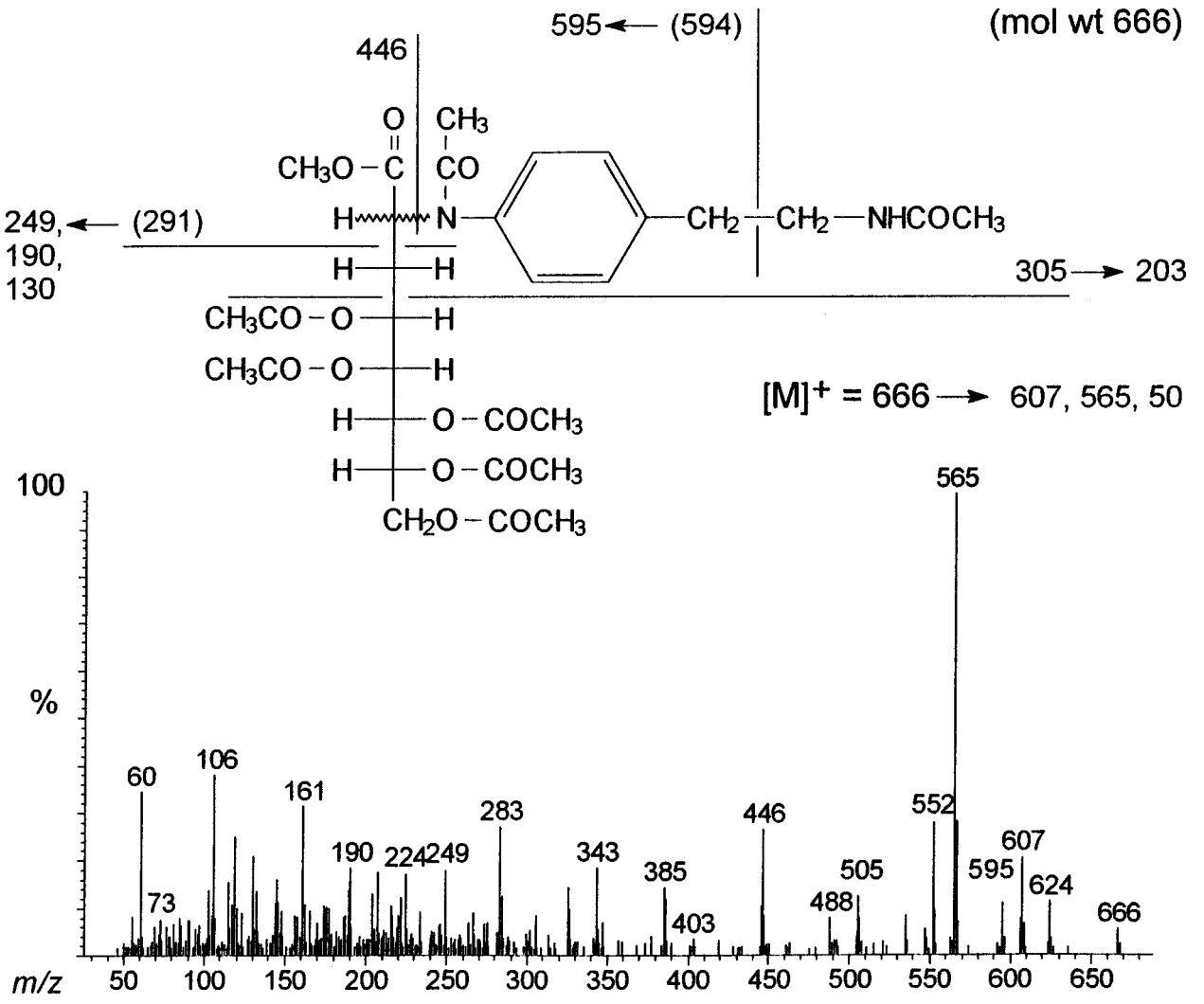


**Figure 3.** Mass spectrum and fragmentation pattern of the peracetylated methyl ester of 2-*N*-[2-(4-aminophenyl)ethylamino]-2,3-dideoxy-D-octonic acids (2a and 2b). The derivatives were obtained from 3-deoxy-D-*manno*-octulosonate (Kdo, $NH_4^+$-salt) and 2-(4-aminophenyl)ethylamine (APEA) by reductive amination followed by treatment with methanol-hydrochloric acid and peracetylation.

within 24 h as indicated by the disappearance of the peak for Kdo. However, HPAE analysis demonstrated formation of complex mixtures. Therefore, samples were derivatized for GLC and GLC-MS to analyze the degree of amination and the side reactions products. The analyses of the products obtained from reductive amination of Kdo for *all* assays investigated in this work did not show any remarkable qualitative or quantitative differences. The chromatograms demonstrated a series of peaks, all together accounting for 70% of the total peak area, or reduced Kdo derivatives [reduced Kdo methyl ester (two minor peaks) and reduced Kdo lactones (two major peaks)] without any labeling of AllN (spectra not shown). Two minor peaks (1.5% and 2.6% peak area) with identical mass spectra were identified as the peracetates of reductively allylaminate Kdo (Figure 2) and two further peaks (15.5% and 10.6% peak area) also with identical mass spectra were the pentaacetate-1,4-lactons of reductively allylaminated Kdo (spectrum not shown). All derivatives were identified by their molecular mass $[M + 1]^+$ and $[M + 18]^+$ ion peaks in Cl-MS (not shown) and primary and secondary fragment ions in El-MS (Figure 3).

The methyl ester hexaacetates of compounds 1a and 1b (Figure 2) gave *m/z* 545 as the molecular ion, *m/z* 502 (base peak) originating most probably from elimination of ketene and addition of a proton, fragment *m/z* 486 as $[M - 59]^+$ from loss of the carboxymethyl group, and *m/z* 400 for the nitrogen-containing fragment resulting from the cleavage of the linkage between C-6 and C-7 of the polyol chain. Other primary fragments originating from C-C cleavages were not assigned because of low intensities. The 2-allylamino-2,3-dideoxyoctonic acid 1,4-lactone pentaacetates were

identified by their molecular ions $m/z$ 472 for $[M + 1]^+$ and $m/z$ 489 for $[M + 18]^+$ in the Cl-MS spectrum, and the ions at $m/z$ 326 and at $m/z$ 182 in the El-MS spectrum. The latter ion resulted from cleavage of C4-C5, and was found to be indicative for the 1,4 lactones (see below) described in this paper.

From these data the question arose whether lactones were formed during reductive amination or methanolysis/peracetylation. In order to identify the reaction products obtained after reductive amination, separation was conducted by GPC and LC. In the different assays, two major peaks (A and B) in the ratios 1 : 1.4 to 1 : 2 by peak area were obtained, derivatized for GLC as described above and characterized by GLC using the analysis data from the GLC experiment conducted on the reaction mixture. Furthermore, retention times in HPAE chromatography were used additionally with Kdo and borohydride reduced Kdo as the standards. Peak A from LC ($T_R = 4.2$–$5.2$ min in the HPAEC), after methyl esterification and peracetylation, was identified as containing a mixture of reduced Kdo, reduced Kdo-1,4-lactone, and minor components most probably Kdo artifacts which were not identified. Peak B ($T_R = 6.28$ min in the HPAEC), after methyl esterification and peracetylation, gave a mixture of allylaminated and reduced Kdo, and allylaminated and reduced Kdo-1,4-lacton as described above. Hence, peak B from LC contained those products formed from Kdo and AllN by reductive amination. Sufficient homogeneity of peak B in both LC and HPAE chromatography allowed the elucidation of the structures of the components (1a and 1b) by NMR analyses.

In order to assign the NMR data of allylaminated and reduced Kdo derivatives, the mixture of synthetic 3-deoxy-D-*glycero*-D-*galacto*- and 3-deoxy-D-*glycero*-D-*talo*-octitol [23] was analyzed by one- and two-dimensional $^1H$ and $^{13}C$ NMR techniques to accomplish complete signal assignment for both isomers (3a and 3b major and minor component, respectively, Figure 1). The $^{13}C$ NMR spectrum showed two series of signals in the ratio of about 1 : 2 demonstrating that chemical synthesis resulted in a nonequimolar formation of the isomers, an effect which was observed also for borohydride reduction of sialic acid [24]. Major and minor signals were extracted from the spectra and assigned according the connectivities in the $^1H$, $^1H$ COSY and HMQC NMR spectra. The data (chemical shifts, and the coupling constants for the major product) are given in Table 1. The $^1H$ NMR data of the 3-deoxy octitols (3a, 3b) and that of the major product of 2-allylamino-2,3-dideoxy octonate (1a) were in good agreement with those of D-*glycero*-D-*galacto*-heptitol (4) [25] in the region H-4 to H-8 and H-3 to H-7, respectively. However, homomorphous structures were demonstrated convincingly by the good agreement between the vicinal coupling constants [25] (Table 1). The $^{13}C$ NMR data clearly demonstrate extensive identities of the homomorphous regions between 3a and 4 but less pronounced for the minor component 3b which showed a high field shift for C-2 to $\delta$68.58, whereas 3a and 4 [26] have C-2 $\delta$71.27 and $\delta$71.65, respectively. Furthermore, the same effects were observed for C-2 of 1a and 1b $\delta$55.07 and $\delta$54.00, respectively. Chemical shifts implicated that 1a was homomorphous to 3a and 1b to 3b. Nevertheless, the NMR data do not allow us to assign the conformations (D-*glycero*-D-*galacto*- or D-*glycero*-D-*talo*) to the conformers 1a and 1b and 3a and 3b.

Assignment of the signals in the NMR spectra of 1 was achieved using 2D-NMR experiments. In the $^1H$, $^1H$ COSY, H-3 showed crossed peaks to H-4 at $\delta$4.194 (dd) and to H-2 at $\delta$3.82 (broad m). The protons H-2 and H-4 were also distinguished by the different multiplicities and by the crossed peak H-2/H-4 of the major component of 1 in the RCT1 spectrum. The HMQC spectrum showed two crossed peaks for H-2 (m, $\delta$3.85) at $\delta$55.07 and $\delta$54.00 (major and minor component, respectively). All other signals in the $^1H$ and $^{13}C$ NMR spectra were assigned using the connectivities in the 2D NMR spectra, chemical shifts of 3a and 3b (Tables 1 and 2), and references data [27, 28]. Similarly, allylic H-2′ at $\delta$5.90 pointed out H-1′ to be at $\delta$3.689, and C-1′ of the allyl moiety was found to be in the expected position at $\delta$50.0 and $\delta$50.2 for the major and the minor component, respectively.

The carboxyl group of 1 showed resonances at $\delta$183.1 and $\delta$182.4 (major and minor component, respectively) downfield shifted in comparison to Kdo as already known for aldonic acids [28]. Kdo-1,4-lactone [29] and 1,4-lactones of aldonic acids [28], however, showed high field shifts due to lactonization. Hence, compounds 1a and 1b cannot be 1,4-lactones as identified in GLC and GLC-MS after treatment with MeOH/HCl and peracetylation. To confirm this conclusion, both 1 and its derivatives from methanolysis and peracetylation were treated with 0.1 M NaOH for saponification of the 1,4-lactone ring. There were no differences in the $^1H$ and $^{13}C$ NMR spectra nor in the HPAE chromatograms for 1 before and after saponification indicating that no carboxyl group was generated in saponified 1. In a control experiment, methanolysis, peracetylation, and GLC and GLC-MS experiments after saponification again resulted in the mixture containing 1,4-lactones as described above. Thus, reductive amination of Kdo with AllN and cyanoborohydride carried out following standard procedures [22, 30] yielded considerable amounts of reduced Kdo, Kdo-artifacts, and about 50–60% of the Kdo in the reaction assay gave the expected 2-allylamino-2,3-dideoxy-octonate. As for synthetic 3-deoxy-D-*glycero*-D-*galacto*- and D-*glycero*-D-*talo*-octonic acid (3) [23], the reductive amination gave both possible isomers but not in equimolar amounts. Hence, one conformer in each of the cases investigated in this paper predominated (compounds 1a and 3a (see Tables 1 and 2). Differences concerning the yields of reduced Kdo derivatives and 2-*N*-allyl-2,3-dideoxy-octonic acids estimated from GLC and HPAEC were caused by the high polarity of the amine components resulting in a lower response in

GLC. Thus, for evaluation of reaction progress and for calculation of yields we prefer HPAEC.

As described for AllN, Kdo was also reductively aminated with 4-(2-aminoethyl)phenylethylamine (APEA) according to a standard method [22] which is equivalent to other procedures [14] published earlier. It is known [22] that APEA acts as a heterobifunctional linker for the reductive amination of aldoses. Because of the different character of the amino groups [22], it couples with both the aliphatic and aromatic amino function depending on the reaction conditions [22]. This feature has not been accepted [31] yet. However, in some groups correct structures were recognized [32–34] early, whereas the existence of Schiff base as an intermediate step remained unproven for this reaction. Those results obtained from reductive amination of Kdo and AllN with cyanoborohydride or pyridine-borane (this paper, see above) clearly demonstrate a lower effectiveness (50–60%) of this reaction for Kdo compared to aldoses which gave high yields for gluco-configurated hexoses [Grimmecke HD, Brade H, unpublished results]. For the evaluation of the reductive amination of Kdo and APEA, GLC and GLC-MS studies were carried out using the carboxymethylation in MeOH/HCl and peracetylation for derivatization. As found for the experiment using AllN, reductive amination of Kdo with APEA using cyanoborohydride gave the same pattern of those derivatives without incorporation of the amine component (see above) and two peaks which were identified from their Cl and El mass spectra to be 2-*N*-[2-(4-aminophenyl)ethyl]amino-2,3-dideoxyoctonic acid methyl ester peracetates (Figure 3) and 2-*N*-[2-(4-aminophenyl)ethyl]amino-2,3-dideoxyoctonic acid-1,4-lacton peracetates (spectrum not shown) indicating the formation of 2-*N*-[2-(4-aminophenyl)ethyl]amino-2,3-dideoxyoctonic acid (2). Thus, APEA and AllN gave analogous reaction products in cyanoborohydride-mediated amination. High polarity of polyol derivatives coupled to APEA caused by the two acetamido groups was the reason for difficulties in reproducing the peak areas in GLC on different columns for quantification. Nevertheless, HPAEC gave ratios of unlabelled Kdo derivatives and compound 4 of 1 : 1.2, similar to AllN labeling of Kdo (see above).

The fragments $[M - 72 + 1]^+$, m/z 595 (Figure 3), and m/z 521, for the methylester and the 1,4-lactone, respectively, resulting from C-C-cleavage in the aliphatic non-linked site of the APEA moiety, are taken as indicators for the identification of the aromatic amino function as the reactive group during reductive amination. This result is in a good agreement with other data [22, 32], and confirms the rule [22] for coupling procedures of APEA and reducing oligosaccharides. Moreover, our data disprove the structures [14, 35] of the pioneering papers concerning the development of LPS-derived neoglycoprotein vaccines, which, unfortunately, have been accepted [31] up to date.

In conclusion, AllN and APEA are suitable reagents for spacer construction on Kdo, and, therefore, also for oligosaccharides having Kdo as the reducing terminus. Although the yields are lower with Kdo than with gluco-configurated aldoses and related oligosaccharides, this procedure is useful for coupling oligo- and polysaccharides of LPS to protein; however, the degradation and the coupling procedures must be analytically controlled.

## Acknowledgments

We are grateful to Mr. H. Moll for recording the E.I. and C.I. mass spectra, and we thank Mr. H-P. Cordes for recording the NMR spectra.

## References

1 Holst O, Ulmer AJ, Brade H, Rietschel ET (1994) In *Immunotherapy of Infections* (Masihi N, ed) pp 281–308. New York: Marcel Dekker, Inc.
2 Paradis S-E, Dubreuil D, Rioux S, Gottschalk M, Jaques M (1994) *Infect Immun* **62**: 3311–19.
3 Poxton IR (1995) *J Immunol Methods* **186**: 1–15.
4 Lindberg B (1990) *Adv Carbohydr Chem Biochem* **48**: 279–318.
5 Holst O, Brade L, Kosma P, Brade H (1991) *J Bacteriol* **173**: 1862–6.
6 Swierzko A, Brade L, Brabetz W, Zych K, Paulsen H, Brade H (1994) *J Endotoxin Res* **1**: 38–44.
7 Nepogodev SA, Pakulski Z, Zamojski A, Holst O, Brade H (1992) *Carbohydr Res* **232**: 33–45.
8 Holst O, Röhrscheidt-Andrzejewski E, Brade H, Charon D (1990) *Carbohydr Res* **204**: 93–102.
9 Aspinall GO, Lynch CM, Pang H, Shaver RT, Moran AP (1995) *Eur J Biochem* **231**: 570–8.
10 McNicolas PA, Batley M, Redmond JW (1987) *Carbohydr Res* **165**: 17–22.
11 Auzanneau F-I, Charon D, Szabo L (1990) *Carbohydr Res* **201**: 337–41.
12 Vinogradov EV, Stuike-Prill R, Bock K, Holst O, Brade H (1993) *Eur J Biochem* **218**: 543–54.
13 Kosma P, Sekljic H, Balint G (1996) *J Carbohydr Chem* **15**: 701–14.
14 Svenson SB, Lindberg AA (1979) *J Immunol Methods* **25**: 323–35.
15 Kleinhammer G, Himmelspach K, Westphal O (1973) *Eur J Immunol* **3**: 834–8.
16 Erlanger BF (1980) *Methods Enzymol* **70**: 85–104.
17 Boeckler C, Frisch B, Muller S, Schuber F (1996) *J Immunol Methods* **191**: 1–10.
18 Stowell CP, Lee YC (1980) *Adv Carbohydr Chem Biochem* **37**: 225–81.
19 Lee RT, Lee YC (1974) *Carbohydr Res* **37**: 193–201.
20 Tobias PS, Soldau K, Ulevitch RJ (1986) *J Exp Med* **164**: 777–93.
21 Yoshida T (1994) *Methods Enzymol* **247**: 55–64.
22 Kallin E, Lönn H, Norberg T (1986) *Glycoconjugate J* **3**: 311–19.
23 Krülle T, Holst O, Brade H, Schmidt RR (1983) *Carbohydr Res* **247**: 145–58.

24 Zbiral E, Kleineidam RG, Schreiner E, Hartmann M, Christian R, Schauer R (1992) *Biochem J* **282**: 511–16.
25 Lewis D (1986) *J Chem Soc Perkin Trans* 2 **1986**: 467–70.
26 Angyal SJ, Fur RL (1984) *Carbohydr Res* **126**: 15–26.
27 Angyal SJ, Fur RL (1980) *Carbohydr Res* **84**: 201–9.
28 Bock K, Pedersen C (1983) *Adv Carbohydr Chem Biochem* **41**: 27–66.
29 Barton DHR, Jaszberenyi JC, Liu WS, Shinada T (1996) *Tetrahedron* **52**: 2717–26.
30 Yoshida T, Lee YC (1994) *Carbohydr Res* **251**: 175–86.
31 Verheul AFM, Snippe H, Poolman JT (1993) *Microbiol Rev* **57**: 34–49.
32 Zopf DA, Smith DF, Drzeniek Z, Tsai C-M, Ginsburg V (1978) *Methods Enzymol* **50**: 171–5.
33 Smith DF, Zopf DA, Ginsburg V (1978) *Methods Enzymol* **50**: 169–71.
34 Semprevivo LH (1988) *Carbohydr Res* **177**: 222–7.
35 Svenson SB, Nurminen M, Lindberg AA (1979) *Infect Immun* **25**: 863–72.

Received 29 November 1996, revised and accepted 7 February 1997