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E.I. AND C.I. MASS-SPECTRAL IDENTIFICATION OF SOME DERIVATIVES OF 7-*O*-(2-AMINO-2-DEOXY-α-D-GLUCOPYRANOSYL)-L-*glycero*-D-*manno*-HEPTOSE, OBTAINED FROM LIPOPOLYSACCHARIDES REPRESENTATIVE OF THE *Vibrionaceae* FAMILY

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ABSTRACT

The electron-impact (e.i.) and chemical-ionization (c.i.) mass spectra of the 2-di-*N*-methyl (**2**), 2-*N*-acetyl (**3**), and 2-(*N*-acetyl)-*N*-methyl (**4**) derivatives of 1,5-di-*O*-acetyl-7-*O*-(2-amino-2-deoxy-3,4,6-tri-*O*-methyl-α-D-glucopyranosyl)-2,3,4,6-tetra-*O*-methyl-L-*glycero*-D-*manno*-heptitol, obtained from the methylation analysis of the core oligosaccharides of *Aeromonas hydrophila* Chemotypes I and II, are described and their fragmentation patterns proposed. The e.i.-mass spectrum of the 2-dimethylamino derivative **2** showed the primary fragment ion A_1, characteristic of the glycosyl group of this glycosylalditol, whereas the mass spectra of the 2-acetamido (**3**) and 2-(*N*-methylacetamido) derivatives (**4**) gave the respective primary ions, A_1-fragments, and ald^+-fragments diagnostic of the glycosyl group and the alditol residue of the respective glycosylalditol. The c.i.-mass spectra of the glycosylalditol derivatives **2**, **3**, and **4** gave, as major peaks, the protonated molecular ion $[MH]^+$, together with the primary fragment ion A_1 characteristic of the glycosyl group of the respective derivatives. The e.i.- and c.i.-mass spectra and fragmentation pattern of methyl 7-*O*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-glucopyranosyl)-2,3,4,6-tetra-*O*-acetyl-L-*glycero*-α-D-*manno*-heptopyranoside (**6**) are also reported.

INTRODUCTION

The various chemotypes of *Aeromonas hydrophila* are Gram-negative bacteria belonging to the family *Vibrionaceae,* and are common inhabitants of freshwater lakes and streams[1,2]. They are regarded as opportunistic pathogens causing disease in stressed fish or as secondary invaders in injured ones[3].

Interest in the structure and immunological properties of the cell-surface polysaccharides of the different species of Gram-negative bacteria of the genus

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Aeromonas has increased, in a quest to unravel the basis of the antigen–antibody immunological phenomenon, and to gain a better understanding of the different specificities of the antigenic determinants of the respective lipopolysaccharides[4–6].

In the course of structural investigations on the precise molecular structure of the lipopolysaccharides of the Gram-negative bacteria *Aeromonas hydrophila* Chemotypes I and II, we isolated[6] the disaccharide 7-*O*-(2-amino-2-deoxy-α-D-glucopyranosyl)-L-*glycero*-D-*manno*-heptose (**1**), following the hydrolysis of the respective core-oligosaccharides with 2M hydrochloric acid for 1 h at 100°. The same aminoglycosylheptose **1** had previously been identified in the core region of the lipopolysaccharides of *Escherichia coli* 0111 (ref. 7) and *Shigella flexneri* serotype 6 (ref. 8), and as a constituent of the endotoxin of *Bordetella pertussis*[9].

We now report the e.i.- and c.i.-mass spectra and fragmentation patterns of some derivatives of **1** that were obtained during the methylation analysis and the methanolysis of the core oligosaccharides of *Aeromonas hydrophila* Chemotypes I and II.

RESULTS AND DISCUSSION

During the methylation analysis of the core oligosaccharide of *Aeromonas hydrophila* Chemotype I and *Aeromonas hydrophila* Chemotype II by the Hakomori method, followed by hydrolysis with 2M trifluoroacetic acid, reduction, acetylation, and identification by g.l.c.–m.s., we noticed that not all of the partially

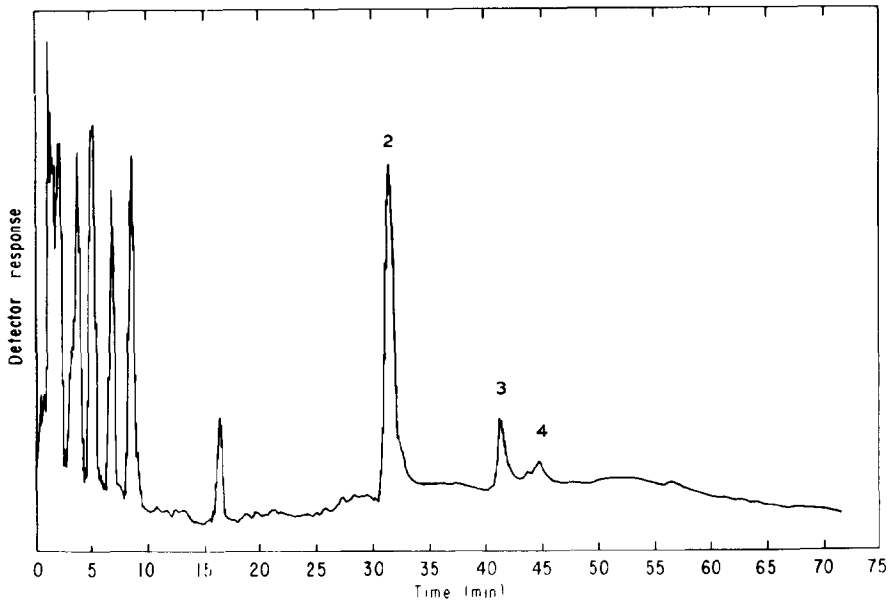


Fig. 1. G.l.c. separation of the partially methylated alditol acetates obtained[16] from hydrolysis of the methylated, semi-rough oligosaccharide of *Aeromonas hydrophila* Chemotype I.

methylated alditol acetates expected were formed. It was clear from the stoichiometric composition of the hydrolysis products of the respective, per-methylated core-oligosaccharides that the partially methylated alditol acetates originating from the aminoglucosylheptose **1** were missing[6]. This observation led us to conclude that the glycosidic linkage of that disaccharide is extremely resistant to the hydrolysis conditions used.

When gas–liquid chromatography of the partially methylated alditol acetates was conducted under programmed temperature-conditions, three slow-moving components were observed (see Fig. 1), whose identities were established by mass spectrometry as the 2-di-*N*-methyl (**2**), 2-*N*-acetyl (**3**), and 2-(*N*-acetyl-*N*-methyl) (**4**) derivatives of 1,5-di-*O*-acetyl-7-*O*-(2-amino-2-deoxy-3,4,6-tri-*O*-methyl-α-D-glucopyranosyl)-2,3,4,6-tetra-*O*-methyl-L-*glycero*-D-*manno*-heptitol.

The presence of the glycosylalditol derivatives **2**–**4** in the methylation analysis of the aforementioned, core oligosaccharides could be accounted to the fact that they contained the 2-amino-2-deoxy-D-glucosyl group. During the Hakomori methylation, the free amino group of the aminoglucosylheptose unit of the respective core-oligosaccharides initially formed a 2-dimethylamino derivative which was extremely resistant to acid hydrolysis. After hydrolysis and reduction, the 2-di-

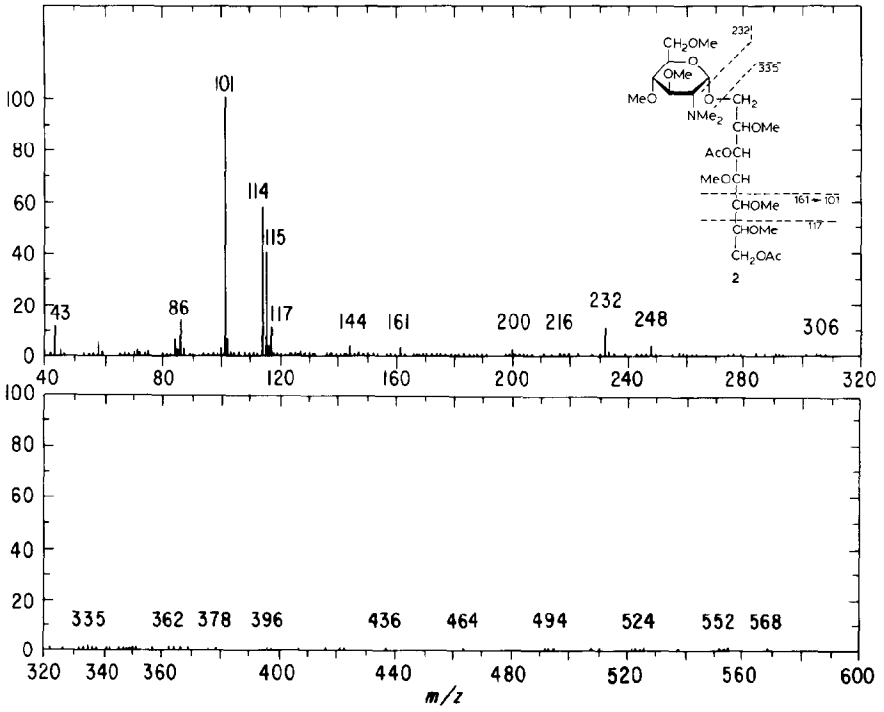


Fig. 2. E.i.-mass spectrum of 1,5-di-*O*-acetyl-7-*O*-[2-deoxy-2-(dimethylamino)-3,4,6-tri-*O*-methyl-α-D-glucopyranosyl]-2,3,4,6-tetra-*O*-methyl-L-*glycero*-D-*manno*-heptitol (**2**).

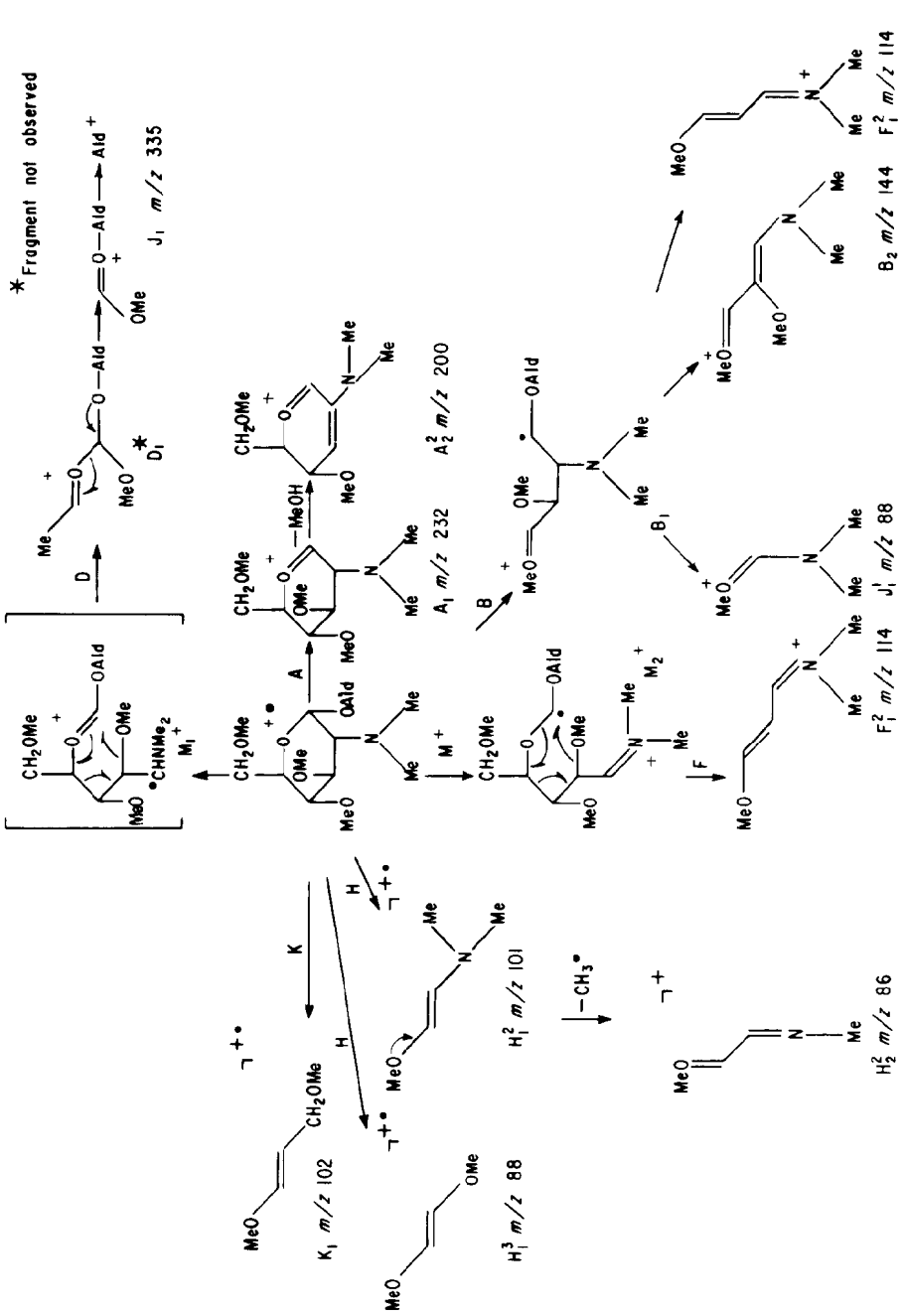
methylamino group of the acid-resistant, methylated aminoglucosylheptose was partially *N*-demethylated to form glycosylalditol derivatives containing methylamino and free amino groups. Finally, after acetylation of the methylation-analysis products, we obtained, as a major part, 1,5-di-*O*-acetyl-7-*O*-[2-deoxy-2-(dimethylamino)-3,4,6-tri-*O*-methyl-α-D-glucopyranosyl]-2,3,4,6-tetra-*O*-methyl-L-*glycero*-D-*manno*-heptitol (**2**), together with lower proportions of the 2-acetamido- and 2-(*N*-methylacetamido)-glycosylalditol derivatives **3** and **4** (see Fig. 1), which were characterized by electron-impact and chemical-ionization (methane), mass spectrometry.

Using the nomenclature of Kochetkov and Chizov[10], where a glycosyl group is characterized by the fragment ions of pathway A, and the ions of the alditol residue, known as the ald^+ fragments, are produced by the D sequence of fragmentation[11,12], the following rationale is offered for the fragmentation of the foregoing compounds.

It is well known that the e.i.-mass spectral analysis of permethylated *O*-glycosylalditols can be limited to the identification of peaks corresponding to the respective fragmentations of the glycosyl group and the alditol residue[11,12]. The fragments of the alditol chain provide the expected information on the interglycosidic linkages[12,13].

In the e.i.-mass spectrum of compound **2**, the molecular radical ion M^+, formed by expulsion of one electron upon electron impact and by subsequent ionization of the ring-oxygen atom, is not observed, and it undergoes rapid transformation into stable ions (see Fig. 2). The primary fragment ion A_1, at *m/z* 232, is produced by elimination of the aglycon radical from the molecular radical-ion, and is characteristic of the 2-(dimethylamino)hexosyl group. The formation of the secondary fragment ion A_2 at *m/z* 200 results from the elimination of one molecule of methanol from the A_1 fragment. It may be noted that, contrary to the known behavior of a permethylated *O*-glycosylalditol[12,13], there is almost no trace of the ald^+ fragment at *m/z* 335 (very low abundance), which makes the identification of *O*-glycosylalditol derivative **2** practically impossible. Ionization and α-cleavage of the 2- and 3-methoxyl groups in the alditol chain gave the fragment ions at *m/z* 117 and 161. The secondary-fragment ions at *m/z* 114, 101, and 86 were formed during the different fragmentation routes adopted by the molecular radical ion M^+. The mechanism proposed for their formation, based on the recent fragmentation proposals of Zolatorev *et al.*[14], is depicted in Scheme 1.

To confirm that the aforementioned fragments originated from the glycosyl group (and not the alditol chain), we synthesized methyl 2-deoxy-2-(dimethylamino)-3,4,6-tri-*O*-methyl-α-D-glucopyranoside (**5**) by permethylation of methyl 2-amino-2-deoxy-α-D-glucopyranoside[15], and recorded its e.i.-mass spectrum (see Fig. 3). On comparison of the mass spectrum of glycoside **5** with that of the *O*-glycosylalditol derivative **2**, it was evident that the secondary fragment-ions at *m/z* 114, 101, and 86 were, indeed, derived from the ring of the glycosyl group *via* the F, H, and K sequences of fragmentation (see Scheme 1).



Scheme 1. Fragmentation pattern of 1,5-di-*O*-acetyl-7-*O*-[2-deoxy-2-(dimethylamino)-3,4,6-tri-*O*-methyl-α-D-glucopyranosyl]-2,3,4,6-tetra-*O*-methyl-L-*glycero*-D-*manno*-heptitol (**2**).

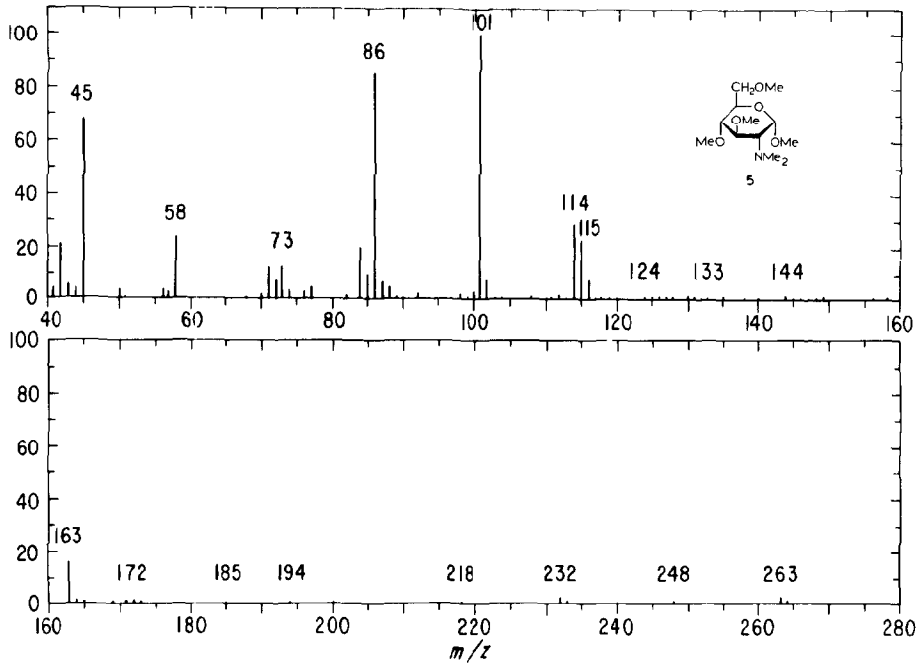


Fig. 3. E.i.-mass spectrum of methyl 2-deoxy-2-(dimethylamino)-3,4,6-tri-*O*-methyl-α-D-glucopyranoside (**5**).

The c.i.-mass spectrum of compound **2** showed a cluster of ions in the high-mass region, having different abundances relative to the protonated molecular-ion $[MH]^+$ at *m/z* 584 (base peak). The molecular ion $[M]^+$ at *m/z* 583 is encountered, as well as the $[M - H]^+$ ion at *m/z* 582 (see Fig. 4). The secondary fragment-ions in the higher-mass region, at *m/z* 552, 524, and 510, are generated by the subsequent loss of a molecule of methanol and acetic acid, respectively, from the protonated molecular-ion $[MH]^+$, and from loss of a (hydroxymethyl)acetyl group from the molecular ion $[MH]^+$. The primary fragment-ion A_1 at *m/z* 232, and its derived, secondary ion A_2 at *m/z* 200, are characteristic of the 2-(dimethylamino)hexosyl group. Like the e.i. spectrum, the c.i.-mass spectrum shows the presence (minute) of the ald^+ fragment at *m/z* 335.

In the e.i.-mass spectrum of 7-*O*-(2-acetamido-2-deoxy-3,4,6-tri-*O*-methyl-α-D-glucopyranosyl)-1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-L-*glycero*-D-*manno*-heptitol (**3**), the molecular radical-ion $M^{\ddagger}$ was absent (see Fig. 5). The primary fragment-ion A_1 at *m/z* 246 was produced by elimination of the aglycon radical from the molecular ion, and is characteristic of the 2-acetamido-2-deoxyhexosyl group. The formation of the secondary fragment-ion A_2, at *m/z* 214, results from the elimination of one molecule of methanol from the A_1 fragment. The ald^+ fragment at *m/z* 335 is diagnostic for the alditol residue of the disaccharide derivative analyzed, and is formed during the D sequence of fragmentation of the molecular

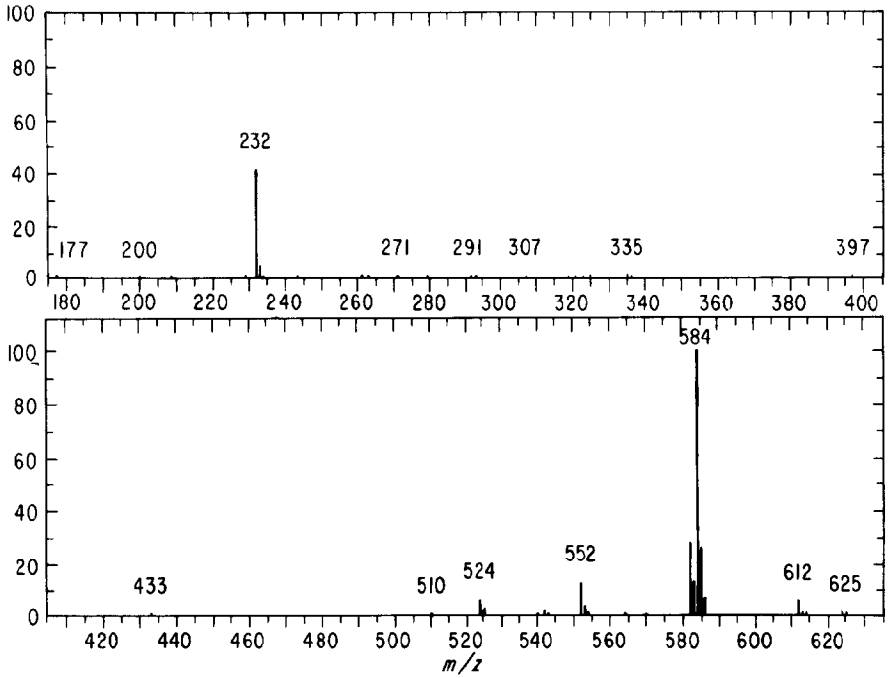


Fig. 4. C.i.-mass spectrum of glycosylalditol derivative 2.

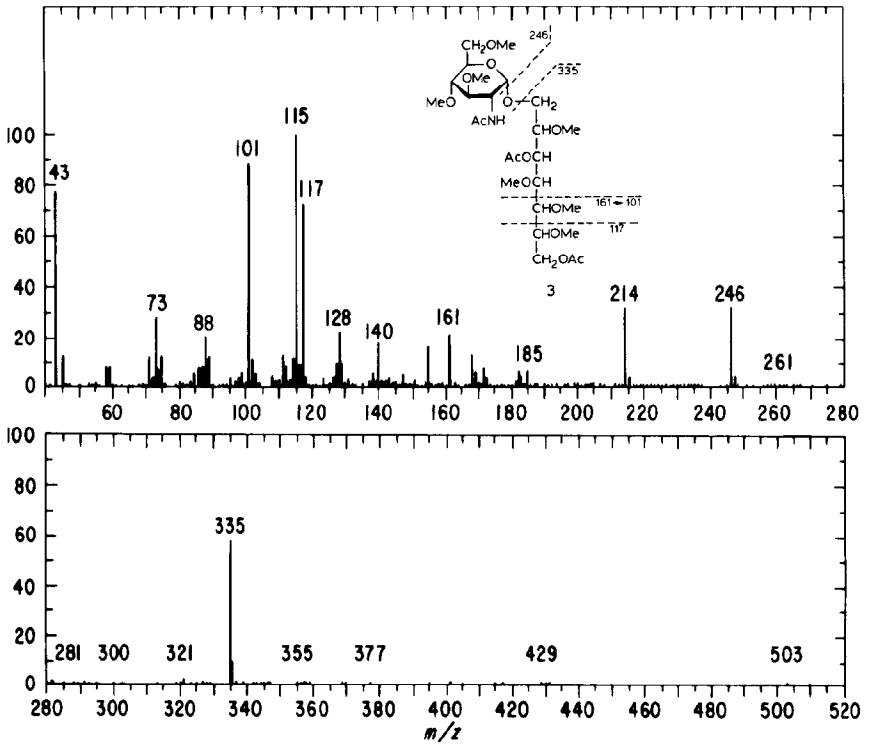


Fig. 5. E.i.-mass spectrum of 7-*O*-(2-acetamido-2-deoxy-3,4,6-tri-*O*-methyl-α-D-glucopyranosyl)-1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-L-*glycero*-D-*manno*-heptitol (3).

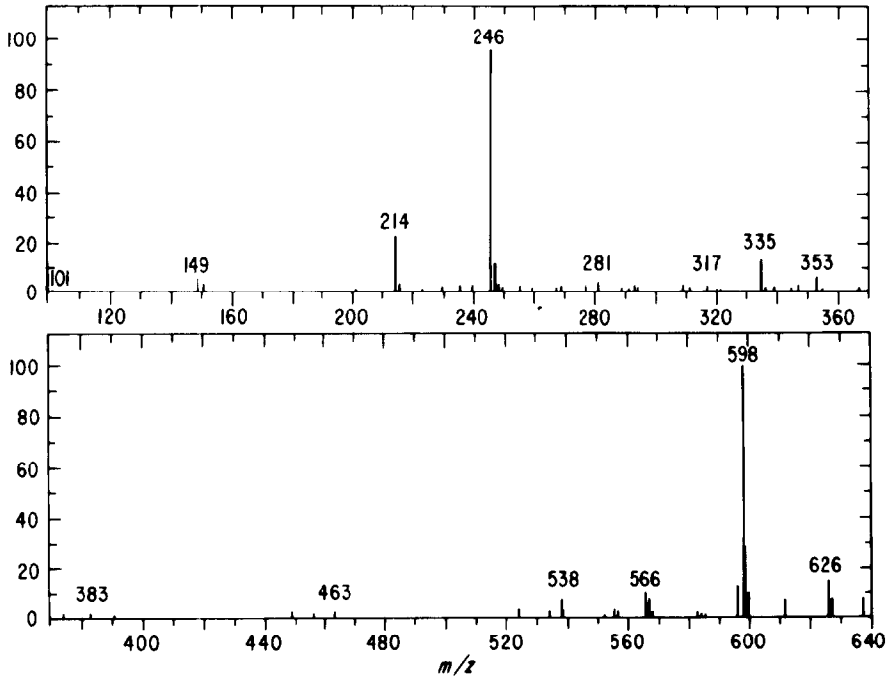


Fig. 6. C.i.-mass spectrum of glycosylalditol derivative **3**.

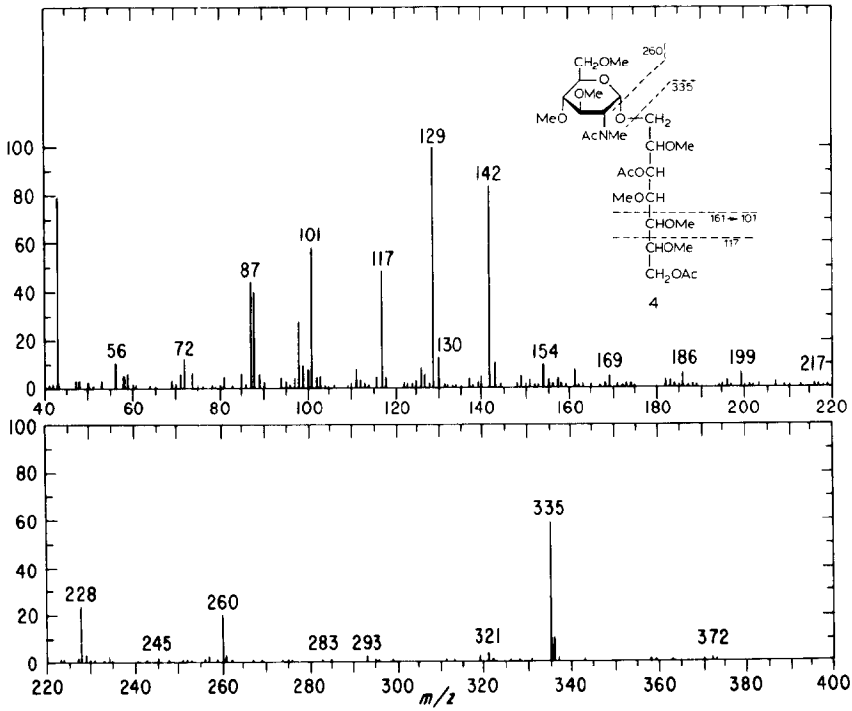


Fig. 7. E.i.-mass spectrum of 1,5-di-*O*-acetyl-7-*O*-[2-deoxy-3,4,6-tri-*O*-methyl-2-(*N*-methylacetamido)-α-D-glucopyranosyl]-2,3,4,6-tetra-*O*-methyl-L-*glycero*-D-*manno*-heptitol (**4**).

ion that proceeds, *via* the D_1 fragment, to the J_1 fragment. The secondary fragment ions at *m/z* 128, 115, and 73, derived from the glycosyl group, are formed during the H, F, and K sequences of fragmentation of the molecular ion. The fragment ions at *m/z* 161 and 117 result from α-cleavage of the 2- and 3-methoxyl groups of the alditol chain, and the secondary fragment ion at *m/z* 101 results from elimination of one molecule of acetic acid from the *m/z* 161 fragment.

The c.i.-mass spectrum of the *O*-glycosylalditol derivative **3** had, as its base peak, the protonated molecular ion $[MH]^+$ at *m/z* 598, together with low abundances of the $[M - H]^+$ ion at *m/z* 596, and the diprotonated molecular-ion $[M + 2\ H]^+$ at *m/z* 599 (see Fig. 6). The fragment ions in the higher-mass region, at *m/z* 566 and 538, are generated by the subsequent loss of a molecule of methanol and acetic acid, respectively, from the protonated molecular-ion $[MH^+]$. The primary fragment-ion at *m/z* 246 and its derived, secondary ion at *m/z* 214 correspond to the known A_1 and A_2 oxonium-ion fragments characteristic of the 2-acetamido-2-deoxyhexosylgroup. The ald^+ fragment at *m/z* 335 is visible and is characteristic for the alditol residue of the glycosylalditol derivative **3**.

In the e.i.-mass spectrum of 1,5-di-*O*-acetyl-7-*O*-[2-deoxy-3,4,6-tri-*O*-methyl-2-(*N*-methylacetamido)-α-D-glucopyranosyl]-2,3,4,6-tetra-*O*-methyl-L-*glycero*-D-*manno*-heptitol (**4**), the molecular radical-ion $M^{\ddagger}$ was absent (see Fig. 7). The primary fragment-ion A_1, at *m/z* 260, and its derived, secondary ion A_2 at *m/z* 228, are diagnostic of the 2-(*N*-methylacetamido)-2-deoxyhexosyl group. The ald^+ fragment at *m/z* 335 is characteristic of the alditol residue, and the secondary

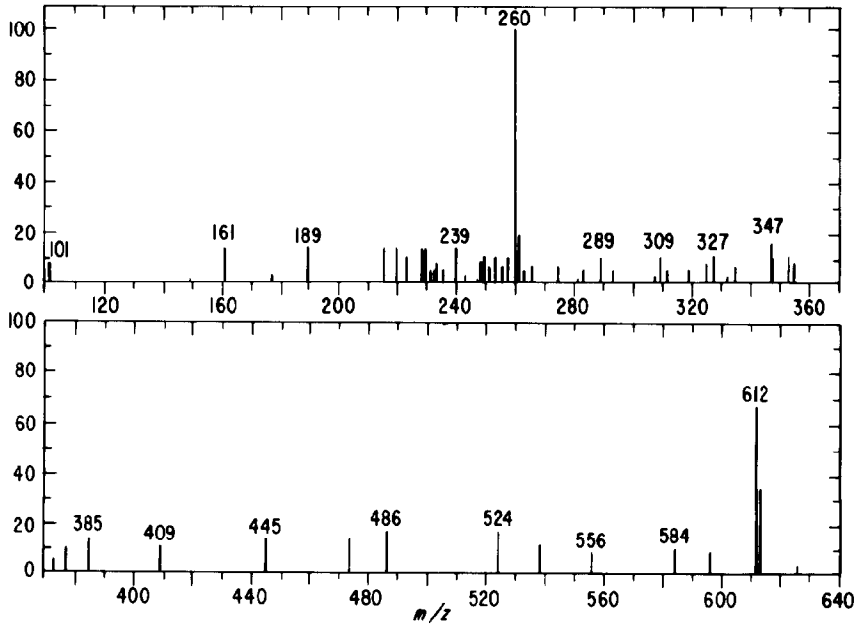


Fig. 8. C.i.-mass spectrum of glycosylalditol derivative **4**.

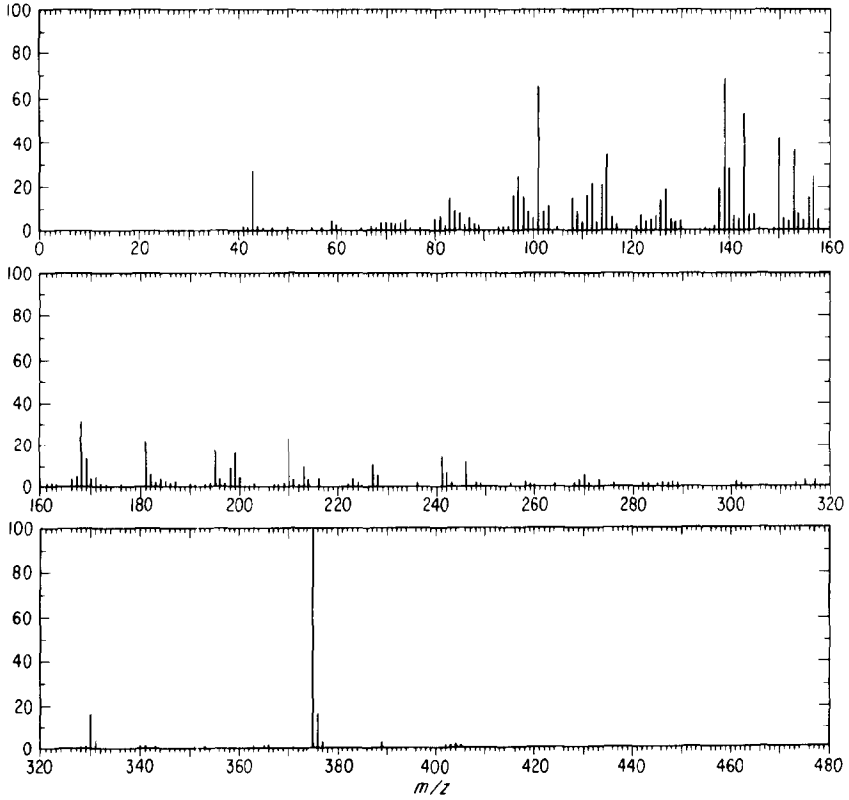
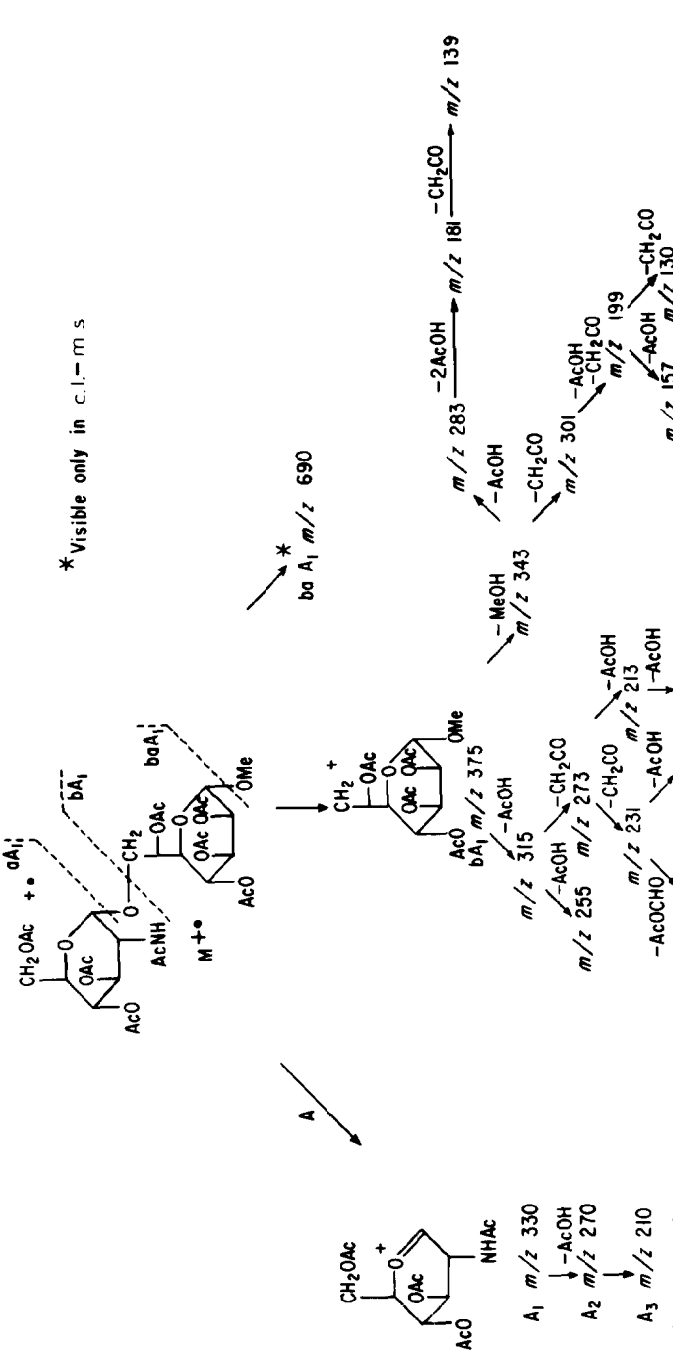


Fig. 9. E.i.-mass spectrum of methyl 7-*O*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-glucopyranosyl)-2,3,4,6-tetra-*O*-acetyl-L-*glycero*-α-D-*manno*-heptopyranoside (**6**).

fragment-ions at *m/z* 142, 129, and 87, originating from the glycosyl group are formed during the fragmentation of the molecular radical-ion M^{+}. The fragment ion at *m/z* 117 results from α-cleavage of the alditol chain, and the secondary ion, at *m/z* 101, from elimination of one molecule of acetic acid from the *m/z* 161 fragment.

The c.i.-mass spectrum of the glycosylalditol derivative **4** gave, as its base peak, the A_1 fragment at *m/z* 260, characteristic of the glycosyl group. In the higher-mass region, at *m/z* 612 and 613, the protonated molecular-ion $[MH]^{+}$, together with a low abundance of the diprotonated molecular-ion [M + 2 H], are seen. The ald^{+} fragment at *m/z* 335, and the A_2 fragment at *m/z* 228, are present in low abundance (see Fig. 8).

We had previously reported the presence, in some lipopolysaccharides representative of the *Vibrionaceae* family, of 3-deoxy-D-*manno*-2-octulosonic acid (KDO), isolated by methanolysis[17] of the core oligosaccharides of *Aeromonas hydrophila* Chemotypes I and II with 2M methanolic HCl. After acetylation, and analysis of the peracetylated methyl glycosides by gas–liquid chromatography



Scheme 2. Fragmentation pattern of methyl glycoside **6**.

under programmed temperature-conditions, we observed a slow-moving component whose identity was established by mass spectrometry as methyl 7-*O*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-glucopyranosyl)-2,3,4,6-tetra-*O*-acetyl-L-*glycero*-α-D-*manno*-heptopyranoside (**6**). This observation suggested that the precursor aminoglucosyl heptose **1**, present in the respective core oligosaccharides, was inert to the methanolysis conditions, as reported by Chaby and Szabó[9].

In the e.i.-mass spectrum of the glycosylglycoside **6**, the molecular radical-ion M^+ was absent (see Fig. 9). The primary fragment A_1, at *m/z* 330, was produced by elimination of the aglycon radical from the molecular ion, and is characteristic for the 2-acetamido-2-deoxyhexosyl group. The formation of the secondary fragment-ions A_2 and A_3 at *m/z* 270 and 210, results from elimination of one or two molecules of acetic acid from the A_1 fragment. The primary fragment, at *m/z* 375, is characteristic of the glycoside methyl residue of that glycosylglycoside A tentative, fragmentation pattern of derivative **6** is proposed in Scheme 2.

Finally, the c.i.-mass spectrum of the glycosylglycoside **6** gave the A_1 fragment-ion at *m/z* 330 (base peak) characteristic of the nonreducing 2-acetamido-2-deoxyhexosyl group, and the A_2 fragment-ion, at *m/z* 270, resulting from the loss of one molecule of acetic acid from the A_1 fragment. In the higher-mass region, the protonated molecular-ion $[MH]^+$ occurs at *m/z* 722, together with a lower abundance of the diprotonated molecular-ion $[M + 2\ H]^+$ at *m/z* 723. The fragment ions at *m/z* 690 and 662 are respectively generated by the loss of a molecule of

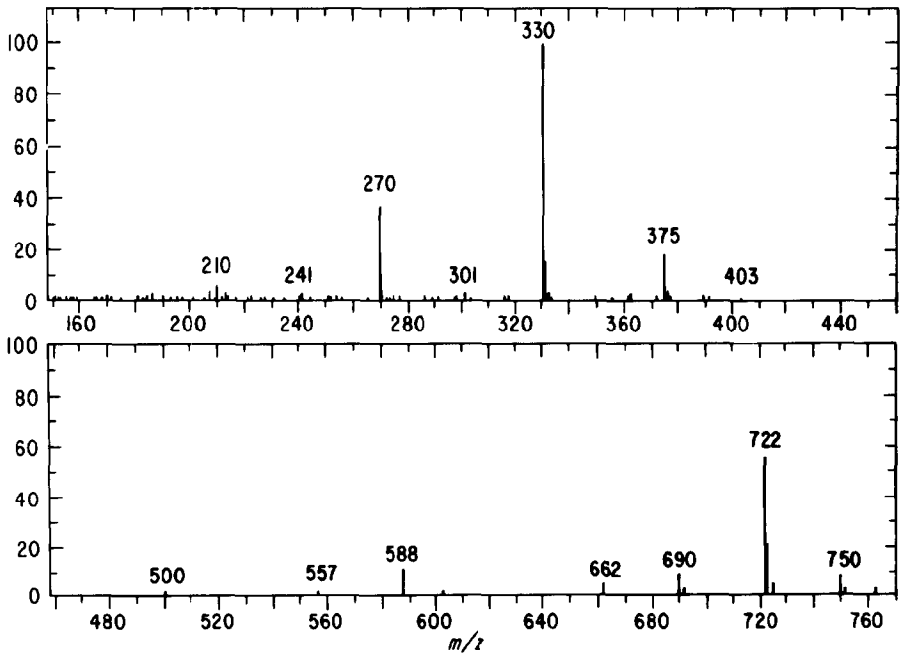


Fig. 10. C.i.-mass spectrum of methyl glycosylglycoside **6**.

methanol and a molecule of acetic acid from the $[MH]^+$ ion. The secondary fragment-ion at *m/z* 589 originates from the loss of a molecule of acetic acid and a molecule of ketene from the fragment ion *m/z* 691. The "aglycon" fragment-ion at *m/z* 375 is present (see Fig. 10).

In contrast to e.i.-mass spectra, the c.i.-mass spectra of derivatives **2**, **4**, **5**, and **6** exhibit few fragmentation pathways by molecular disintegration, because of the mildness of the indirect-ionization procedure. The simple, clear-out spectra show the protonated molecular-ions and fragment ions in the high-mass region. In conclusion, the relevant data obtained from the e.i.- and c.i.-mass spectra provide valuable information for the clear identification of this series of reported derivatives of an aminoglucosylheptose.

EXPERIMENTAL

Bacterial culture. — *Aeromonas hydrophila* Chemotype I, strain No. SJ-55, and Chemotype II, strain No. SJ-26, were obtained from the Northwest Atlantic Fisheries Centre collection.

Extraction of lipopolysaccharide. — All strains were grown in trypticase soy-broth (25 L) (Baltimore Biological Laboratories) for 24 h at 25°, with aeration of 12 L/min, as previously described[4]. Lipopolysaccharide was extracted from the wet cell-cake by the aqueous-phenol method of Westphal and Jann[18]. Production of core oligosaccharide, devoid of O-polysaccharide and lipid A, was by hydrolysis of the lps in 1% aqueous acetic acid for 90 min at 100°, followed by gel chromatography on Sephadex G50, as previously described[4].

Gas–liquid chromatography–mass spectrometry. — Gas–liquid chromatography of the partially methylated alditol acetates and the peracetylated methyl glycosides was performed in packed columns (183 cm × 2 mm i.d.) of 1.5% of Silar 7 CP on Gas Chrom Q (100–120 mesh) in a Perkin–Elmer model 3920 gas chromatograph equipped with a hydrogen-flame detector; the temperature program was started at 180° for 32 min, and then increased to 270° at 8°/min (final temperature held for 64 min). Gas–liquid chromatography was also performed in a 25-m WCOT CP-Sil 5 (0.25-μm film-thickness) capillary column (Chrompack, The Netherlands), using the same temperature-program.

Combined gas–liquid chromatography–electron impact-mass spectrometry was performed in a Hewlett–Packard model 5980A GC/MS instrument controlled by a 5934A data system, with a membrane separator, a source temperature of 160°, and an ionizing voltage of 70 eV, using the same temperature-program. Combined gas–liquid chromatography–chemical ionization (methane)-mass spectrometry was performed in a Hewlett–Packard model 5985 GC/MS/DS instrument equipped with a dual e.i./c.i. source. Spectra were recorded at a source pressure of 120 Pa, using methane as the reagent gas and the carrier, a source temperature of 150°, and an ionizing voltage of 230 eV. The temperature program started at 180° for 32 min, and was then increased to 270° at 8°/min, the final temperature being held for 64

min; a packed glass-column of 2% of OV-101 on Chromosorb W (H.P.) (80–100 mesh) was used.

Methylation analysis. — The core oligosaccharides were methylated by the Hakomori method[19] and then purified on Sephadex LH-20 (Pharmacia, Fine Chemicals). The methylated oligosaccharides were hydrolyzed with 2M trifluoroacetic acid for 12 h at 100°. The resulting, partially methylated sugars were reduced with sodium borohydride, the alditols acetylated, and the acetates analyzed by g.l.c.–m.s.

Methanolysis. — Solutions of the core oligosaccharides in 2M methanolic HCl were heated for 16 h at 85°, cooled, and evaporated to dryness; each residue was dissolved in methanol, and re-evaporated (to remove traces of HCl). The methyl glycosides obtained were acetylated with 1:1 acetic anhydride–pyridine for 1 h at 100°, and the solution was evaporated to dryness.

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