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NEW SUGARS FROM ANTIGENIC LIPOPOLYSACCHARIDES OF BACTERIA: IDENTIFICATION AND SYNTHESIS OF 3-*O*-[(*R*)-1-CARBOXYETHYL]-L-RHAMNOSE, AN ACIDIC COMPONENT OF *Shigella dysenteriae* TYPE 5 LIPOPOLYSACCHARIDE

NIKOLAI K. KOCHETKOV, BORIS A. DMITRIEV, AND LEON V. BACKINOWSKY

N. D. Zelinsky Institute of Organic Chemistry, Academy of Sciences of the U.S.S.R., Moscow (U.S.S.R.)

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ABSTRACT

A new acidic sugar, 3-*O*-[(*R*)-1-carboxyethyl]-L-rhamnose (**1**), has been identified as a constituent of the O-antigenic lipopolysaccharide of *Sh. dysenteriae* type 5. The structure of **1** has been established by physico-chemical methods and by synthesis. Alkylation of methyl 2,5-di-*O*-benzyl-α-L-rhamnofuranoside (**6**) with (*S*)- or (*R*)-2-chloropropionic acids, followed by removal of the protecting groups, afforded 3-*O*-[(*R*)-1-carboxyethyl]-L-rhamnose (**9**) and 3-*O*-[(*S*)-1-carboxyethyl]-L-rhamnose (**10**), respectively. The properties of **1** coincide with those of **9**.

INTRODUCTION

In a preliminary communication[1], we have reported on the detection of two new acidic monosaccharides, namely, 3-*O*-(1-carboxyethyl)rhamnose and 4-*O*-(1-carboxyethyl)-D-glucose in the O-antigenic lipopolysaccharides of *Sh. dysenteriae*. These monosaccharides can be regarded as analogues of muramic acid, which is a necessary component of a rigid layer of bacterial cell-wall[2], and its *manno* isomer isolated from *Micrococcus lysodeikticus* cell-wall peptidoglycan[3]. These lactic acid-containing sugars are representatives of a new class of carbohydrate and seem to be biogenetically related to pyruvic acid acetals of sugars, which are rather common components of capsular-polysaccharide antigens of Gram-negative bacteria, *e.g.*, of the *Klebsiella* genus[4].

We now report on the identification and synthesis of 3-*O*-[(*R*)-1-carboxyethyl]-L-rhamnose (**1**); data on the 4-*O*-(1-carboxyethyl)-D-glucose will be published elsewhere.

RESULTS AND DISCUSSION

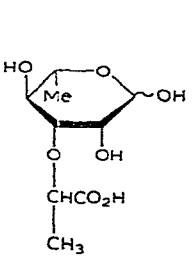
In our studies on the monosaccharide composition of *Sh. dysenteriae* lipopolysaccharides[5], we have used a combination of colorimetric and chromatographic methods of analysis. The lipopolysaccharide isolated from *Sh. dysenteriae* type 5 gave

a positive Dische reaction for 6-deoxyhexoses[6], whereas these sugars were not detected in the hydrolysate of the lipopolysaccharide. Further experiments were therefore carried out with the polysaccharide that was obtained from the O-specific lipopolysaccharide by mild hydrolysis with acid.

This polysaccharide was acidic, and its hydrolysis gave mannose and 2-amino-2-deoxyglucose, in the ratio 2:1, and an acidic monosaccharide which could be detected, after paper electrophoresis, by aniline hydrogen phthalate and periodate–benzidine reagents but not by alkaline silver nitrate. This acidic sugar, which was isolated by ion-exchange chromatography, gave a positive Dische reaction for 6-deoxyhexose and did not contain phosphate or sulphate groups.

The p.m.r. spectrum of the polysaccharide methyl ester contained signals for one *O*-acetyl (δ 2.28), one *N*-acetyl (δ 2.11), and one methyl ester group (δ 3.90 p.p.m.), and a pair of partly overlapping doublets (δ ~1.5 p.p.m.) which could correspond to two CH-linked methyl groups. These data suggest that the new carbohydrate component of the polysaccharide is a 6-deoxyhexose bearing an acidic function, with both *C*-methyl signals belonging to the same monosaccharide. Due to the absence of other high-field signals in the p.m.r. spectrum, this acidic function was assumed to be a lactic acid residue etherifying the 6-deoxyhexose.

This assumption was confirmed by mass-spectrometric data. The acidic monosaccharide **1** was trimethylsilylated, and treated with lithium aluminium hydride in boiling ether[7] to effect carboxyl-reduction. Hydrolysis of the trimethylsilyl groups, followed by reduction with sodium borohydride and acetylation, afforded the alditol acetate **2**, the most abundant peak in its mass spectrum being *m/e* 101. The position of attachment of the lactic acid residue to the 6-deoxyhexose moiety was elucidated by methylation analysis. The permethylated polysaccharide was treated with lithium aluminium hydride and hydrolysed. The resulting, partially methylated monosaccharides were investigated as the corresponding alditol acetates by g.l.c.–m.s. The methylated alditol acetate **3** was detected, together with a partially methylated mannitol acetate. The mass spectrum of **3** is in accord with the structure proposed, thus proving **1** to be a 3-*O*-(1-carboxyethyl)-6-deoxyhexose.



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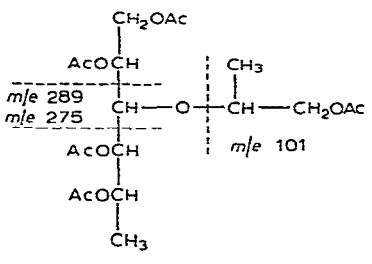

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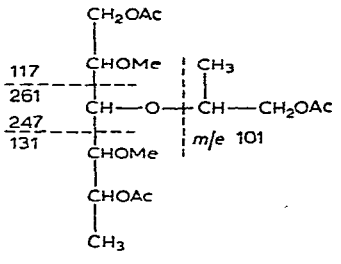

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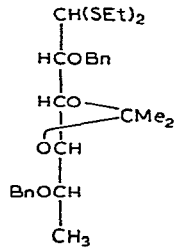
Treatment of **1** with boron trichloride[8] afforded rhamnose, which was not isolated from the reaction mixture but was identified by p.c., t.l.c., and ion-exchange

chromatography; the L-configuration of the rhamnose moiety of **1** was proved by synthesis.

Determination of the absolute configuration of the lactic acid moiety of **1** could not be effected by degradative methods. Elimination of the lactic acid residue did not occur under the mild alkaline conditions successfully used for *N*-acetylmuramic acid[9], and the formation of lactic acid from the 6-deoxyhexose itself could be anticipated by the use of strong alkali[10]. The (*R*) configuration of the lactic acid side-chain was established by a comparison of properties of the natural acid **1** with those of synthetic samples of diastereoisomeric acids.

The synthesis of **1** was accomplished by using methyl 2,5-di-*O*-benzyl-α-L-rhamnofuranoside (**6**) as a starting material. This was obtained by the reaction sequence described by Foster *et al.*[11], with slight modifications: (*a*) benzylation was



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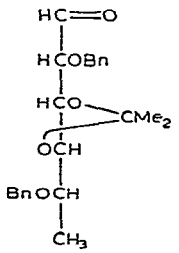

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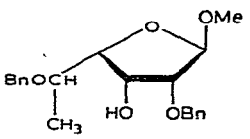

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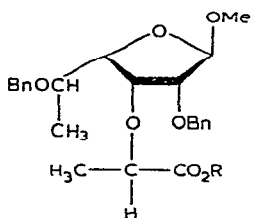
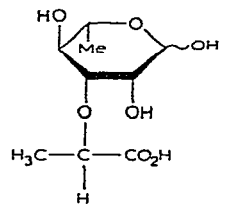


7a R = H
7b R = Me



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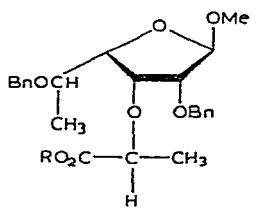
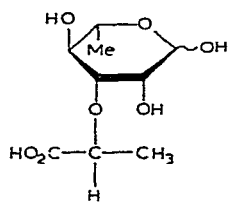


8a R = H
8b R = Me



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carried out by Kuhn's procedure (*N,N*-dimethylformamide in the presence of barium oxide and barium hydroxide), and (*b*) transformation of **4** into **6** was performed by a two-step procedure involving demercaptalation of **4** to give an aldehydo sugar **5** (identified as the crystalline semicarbazone) and subsequent methanolysis of **5** to afford **6**. Attempts to prepare **6** directly from **4** by boiling a methanolic solution in the presence of mercuric chloride[11] led to a complex mixture.

Compound **6** was alkylated with (*S*)-2-chloropropionic acid [or the (*R*) isomer] in *p*-dioxane solution in the presence of sodium hydride as described by Sinaÿ *et al.*[12]. The isomeric 2-chloropropionic acids were obtained[12] from L-(and D-)-alanine. The reaction products **7a** (**8a**) were esterified with diazomethane to yield syrupy methyl 2,5-di-*O*-benzyl-3-*O*-[(*R*)-1-(methoxycarbonyl)ethyl]-α-L-rhamnofuranoside (**7b**) and its (*S*) diastereoisomer (**8b**). The p.m.r. spectra of these compounds unambiguously prove their structures.

Hydrogenolysis of **7b** (or **8b**) in the presence of 5% palladium-on-charcoal, followed by acid hydrolysis, afforded two diastereoisomeric acids, **9** and **10**, respectively.

TABLE I

COMPARISON OF DATA FOR 3-*O*-(1-CARBOXYETHYL) DERIVATIVES OF L-RHAMNOSE

Compound	$[\alpha]_D$ (c, *water*) (*degrees*)	R_{MAN}[a]	M_{GALA}[b]	T (*min*)[c]
Natural acid **1**	+12 (1.13)	1.0	0.96	108
9	+20 (1.1)	0.97	0.95	109
10	−14.5 (0.9)	1.10	0.86	60

[a]Paper chromatography. [b]Paper electrophoresis. [c]Analytical ion-exchange chromatography.

Comparison of properties of the natural acid **1** with those of **9** and **10** (Table I) clearly indicates **1** to be 3-*O*-[(*R*)-1-carboxyethyl]-L-rhamnose. Although the chromatographic and electrophoretic properties do not differ markedly, the $[\alpha]_D$ values and ion-exchange chromatography retention-times are in accord with the conclusion made. The striking difference in ion-exchange properties of the diastereoisomeric acids **8** and **10** may be compared with the mobilities of muramic and isomuramic acids upon paper chromatography, where muramic acid migrates almost twice as fast as isomuramic acid[13]. Acid **1** and muramic acid, both having the (*R*) configuration for the lactic acid side-chain, exhibit a more-positive $[\alpha]_D$ value than do acid **10** and isomuramic acid.

EXPERIMENTAL

General methods. — Gel chromatography was performed on a column (65 × 3 cm) of Sephadex G-50 in pyridinium acetate buffer (pH 5.4). P.c. was carried out on Filtrak (GDR) paper FN-11 with 1-butanol–pyridine–water (6:4:3), and paper electrophoresis with 25mM pyridinium acetate buffer (pH 4.5) at 28 V/cm. Reducing

sugars were detected with alkaline silver nitrate and aniline hydrogen phthalate. T.l.c. was performed on Silica Gel KSK with detection by spraying with H_2SO_4 followed by heating. For column chromatography, Silica Gel L 100/160 μm (Lachema, Czechoslovakia) was used. G.l.c. was performed with a Pye series 104 (Model 64) instrument with a dual, heated, hydrogen flame-ionisation detector. Separations were carried out on a steel column (150 × 0.6 cm) packed with 3% of SE-30 on 100–120 mesh Diatomite CQ. G.l.c.–m.s. was performed with a Varian MAT 111 "Gnom" instrument using the same column. Analytical ion-exchange chromatography was performed with the Technicon SC-II system on a column (25 × 0.6 cm) packed with anion-exchange resin DA-x4 (Durrum, U.S.A.) in 0.5M sodium borate buffer (pH 9.0) at 55°. The elution rate was 60 ml/h, and orcinol–sulphuric acid reagent was used to monitor separations. The p.m.r. spectrum of the polysaccharide methyl ester (100 mg/ml, D_2O) was recorded with a Varian XL-100 instrument at 90° with an external standard. Other p.m.r. spectra were recorded with a Varian DA-60-IL instrument for solutions in carbon tetrachloride with tetramethylsilane as internal standard. I.r. spectra (KBr pellets) were measured with a UR-10 spectrometer. Optical rotations were determined with a Perkin–Elmer 141 polarimeter. Solutions were concentrated *in vacuo* at 40°.

Characterisation of the O-specific polysaccharide. — The lipopolysaccharide of *Sh. dysenteriae* type 5 was isolated according to the procedure of Westphal and Jann[14], namely, by extraction of acetone- and ether-dried bacterial cells with hot, aqueous phenol; removal of nucleic acids from the dialysed, aqueous layer by precipitation with Cetavlon (procedure III in Ref. 14; Cetavlon is known not to precipitate carboxyl-containing polysaccharides from solutions in 0.3M NaCl, *cf.* Ref. 4); and, finally, ultracentrifugation at 105,000 *g* to yield ~2.5% of lipopolysaccharide. To isolate the O-specific polysaccharide, the lipopolysaccharide obtained was treated with dilute acetic acid (pH 3.4), and Lipid A was centrifuged-off. The supernatant solution was freeze-dried and the residue was subjected to gel chromatography[15]. Lyophilisation of the fraction of high molecular-weight gave the polysaccharide (yield ~15%), $[\alpha]_D^{23}$ +20° (*c* 0.6, water).

The isolated polysaccharide exhibited acidic properties, and migrated towards the anode during electrophoresis, (M_{GALA}) 0.66. Hydrolysis of the polysaccharide (0.5M HCl, 100°, 16 h) gave neutral, amino, and acidic sugars, as indicated by electrophoresis data. Mannose and 2-amino-2-deoxyglucose were identified by p.c., and also by g.l.c. of the derived alditol acetates[16,17].

The i.r. spectrum of the polysaccharide exhibited bands at 1740 (COOH and/or COOR), 1570 and 1660 (amide), and 1250 cm^{-1} (C–O–C in acetates). When the polysaccharide was treated with saturated, aqueous triethylamine (20°, 16 h), and the solution passed through a column of KU-2(H^+) resin and freeze-dried, the product had no i.r. absorbtion at 1250 cm^{-1} and gave a shoulder at 1725 cm^{-1} (COOH) substituting an intense band at 1740 cm^{-1}.

The action of ethereal diazomethane on a solution of the polysaccharide (45 mg) in distilled methyl sulphoxide (3 ml) for 30 min gave the methyl ester of the

polysaccharide, which was isolated by gel chromatography in a quantitative yield and had $[\alpha]_D^{23}$ +24° (*c* 0.72, water). A solution of the product in deuterium oxide (0.5 ml) was freeze-dried, and this procedure was repeated prior to measurement of the p.m.r. spectrum.

Isolation and characterisation of acid **1**. — The polysaccharide (50 mg) was heated with 0.5M HCl (20 ml) at 100° for 16 h. The hydrolysate was concentrated to dryness and water was distilled from the residue several times. The residue was dissolved in water (10 ml), and the solution was basified with several drops of triethylamine, heated at 60° for 10 min, and then applied to a column (10×2 cm) of Amberlite IRA-401 (CH_3COO^-) resin. The column was washed with water (200 ml) and then with 20% acetic acid (50 ml). The acid eluate was concentrated to dryness, traces of acetic acid were removed by distillation with water, and acid **1** (~10 mg) was obtained as syrup.

The sample of **1** (~0.5 mg) was dried over P_2O_5 at 60°/0.1 mmHg, dissolved in pyridine (1 ml), and treated with hexamethyldisilazane (0.2 ml) and chlorotrimethylsilane (0.1 ml) for 30 min at 20°. The reaction mixture was evaporated to dryness, the residue was extracted with dry ether (2 ml), and the filtered solution was boiled under reflux (anhydrous conditions) with ~20 mg of lithium aluminium hydride for 6 h. The cooled reaction mixture was treated with ethyl acetate followed by water and dilute phosphoric acid, salts were removed by centrifugation, and the aqueous solution was passed through columns of cation- and anion-exchange resins and concentrated to dryness. An aqueous solution of the residue was treated with sodium borohydride and, after conventional work-up, the product was treated with acetic anhydride in pyridine to yield the alditol acetate **2** [*T* 1.25 (relative to mannitol hexa-acetate) at 200°]. The mass spectrum showed, *inter alia*, the following peaks (relative intensities in brackets): *m/e* 43(100), 95(24), 101(100), 113(12), 115(22), 129(17), 155(14), 159(4), 185(4), 187(2.5), 215(8), 275(6), 289(6), and 317(15).

Acid **1** (~1 mg) was dried *in vacuo* over P_2O_5, suspended in dry dichloromethane (2 ml), and treated[8] with boron trichloride (~2 ml). The reaction mixture was concentrated to dryness, and boric acid was removed from the residue by evaporation of methanol (5×2 ml) therefrom. Rhamnose was identified by p.c. and t.l.c. (ethyl acetate–2-propanol–methanol[18], 70:15:15), in comparison with an authentic sample. Analytical ion-exchange chromatography revealed, in addition to rhamnose (*T* 31 min), three peaks having *T*-values 22, 27, and 55 min. Essentially the same pattern was observed when the synthetic acid (**9**) was subjected to the action of boron trichloride.

The polysaccharide (1.5 mg) was methylated according to the Hakomori procedure[19], and the permethylated polysaccharide was isolated by dialysis and freeze-drying. The product was dissolved in dichloromethane–ether, boiled under reflux with lithium aluminium hydride (20 mg) for 16 h, and worked-up as previously described[20]. The reduced polysaccharide was subjected to formolysis, acid hydrolysis, borohydride reduction, and acetylation under conventional conditions[21] to give a mixture of partially methylated alditol acetates, which was analysed by g.l.c.–m.s.

(180–230°, 4°/min). The mass spectrum of **3** (*T* 2.0, relative to 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylmannitol) contains, *inter alia*, the following peaks (relative intensities in brackets): *m/e* 43(100), 85(11), 87(15), 89(16), 101(88), 117(26), 129(7.5), 131(25), 141(5), 145(3), 157(3), 159(2), 187(2.8), 201(1.9), 247(1.8), and 261(1.9).

2,5-Di-O-benzyl-3,4-O-isopropylidene-L-rhamnose diethyl dithioacetal (**4**). — 3,4-*O*-Isopropylidene-L-rhamnose diethyl dithioacetal[22] was treated with benzyl chloride in *N,N*-dimethylformamide in the presence of barium oxide and barium hydroxide octahydrate[23] to yield 70% of syrupy **4**, isolated by column chromatography.

Anal. Calc. for $C_{27}H_{38}O_4S_2$: C, 66.08; H, 7.80; S, 13.07. Found: C, 65.94; H, 7.93; S, 12.84.

2,5-Di-O-benzyl-3,4-O-isopropylidene-aldehydo-L-*rhamnose* (**5**). — A solution of **4** (0.8 g) in acetone (10 ml) and water (1.5 ml) was stirred with yellow mercuric oxide (1.2 g) and mercuric chloride (1.2 g) for 2 h at 20°. The mixture was heated at 50° for 30 min, and t.l.c. (benzene–ether, 85:15) then indicated complete disappearance of starting material. The cooled mixture was filtered through a pad of silicic acid, the filtrate was concentrated to dryness, and the residue was extracted several times with warm chloroform. The combined extracts were washed with M KI and water, dried, and evaporated to dryness to give 630 mg (100%) of syrupy **5**. The semicarbazone of **5**, obtained in the usual way, had m.p. 130–131° (aqueous ethanol), $[\alpha]_D^{25}$ +10° (*c* 1.42, ethanol).

Anal. Calc. for $C_{24}H_{31}N_3O_5$: C, 65.28; H, 7.08; N, 9.51. Found: C, 65.14; H, 7.18; N, 9.49.

Methyl 2,5-di-O-benzyl-α-L-rhamnofuranoside (**6**). — A solution of sugar **5** (600 mg) was boiled under reflux with 10 ml of 0.3M methanolic hydrogen chloride for 2 h, and then evaporated to dryness. The residue was subjected to column chromatography (benzene–ether) to yield **6** as a syrup (300 mg). Approximately the same yield of **6** was obtained when **5** was treated with 0.3M methanolic hydrogen chloride at 20° for 16 h. The *p*-toluenesulphonate of **6** was obtained in the usual way; it had m.p. 65° (from ethanol) and $[\alpha]_D^{25}$ −45.5° (*c* 1.57, chloroform); literature data[11]: m.p. 65°, $[\alpha]_D$ −45° (*c* 1.6, chloroform).

Methyl 2,5-di-O-benzyl-3-O-[(R)-1-(methoxycarbonyl)ethyl]-α-L-rhamnofuranoside (**7b**). — To a solution of **6** (760 mg) in absolute *p*-dioxane (20 ml, freshly distilled from lithium aluminium hydride), sodium hydride (500 mg of a 50% suspension in mineral oil) was added, and the mixture was stirred at 95° for 1 h with protection from moisture. The mixture was cooled to 65°, and a solution of (*S*)-2-chloropropionic acid {1.28 g, $[\alpha]_D^{25}$ −16.25° (pure liquid, 1-dm path)} in 5 ml of *p*-dioxane was added with vigorous stirring. After 1.5 h, a suspension of sodium hydride (2 g) was added and stirring was continued overnight at 65°. The cooled reaction mixture was carefully treated with water (10 ml), and the upper layer was separated, concentrated, diluted with water, and extracted with chloroform (5 × 5 ml). The main part of the condensation product, together with mineral oil, was extracted at this step, whereas extraction of the acidified aqueous layer with chloroform gave only a

small proportion of the product (t.l.c.; chloroform–acetone 8:2). The first extract, which was free from excess chloropropionic acid, was subjected to column chromatography (chloroform) to yield **7a** as a syrup; **7a** was dissolved in ether and treated with ethereal diazomethane to give, after column chromatography, **7b** as a syrup (620 mg, 66%), $[\alpha]_D^{22}$ +28° (*c* 1.56, chloroform). P.m.r. data: δ 1.23 (d, *J* 6 Hz, CH_3 of rhamnose); 1.33 (d, *J* 6 Hz, CH_3 of lactic acid residue); 3.33 (s, OCH_3); 3.42 (s, $COOCH_3$); 3.68–4.38 (m, H-2,3,3′,4,5); 4.37–4.78 (m, benzylic protons); 4.89 (d, $J_{1,2}$ 4 Hz, H-1); 7.2, 7.26 (2 s, aromatic protons).

Methyl 2,5-di-O-benzyl-3-O-[(S)-1-(methoxycarbonyl)ethyl]-α-L-rhamnofuranoside (**8b**). — By essentially the same procedure, **6** and (*R*)-2-chloropropionic acid {$[\alpha]_D^{22}$ +13.5° (pure liquid, 1-dm path); lit.[12]: $[\alpha]_D$ +15.6°} gave syrupy **8b** (60%), $[\alpha]_D^{20}$ −29° (*c* 1.54, chloroform). P.m.r. data: δ 1.20 (d, *J* 6 Hz, CH_3 of rhamnose); 1.27 (d, *J* 6 Hz, CH_3 of lactic acid residue); 3.3 (s, OCH_3); 3.45 (*s*, $COOCH_3$); 3.67–4.4 (m, H-2,3,3′,4,5); 4.5–4.6 (m, benzylic protons); 4.87 (d, $J_{1,2}$ 4 Hz, H-1); 7.2, 7.25 (2 s, aromatic protons).

3-O-[(R)-1-carboxyethyl]-L-rhamnose (**9**). — A solution of **7b** (540 mg) in methanol (10 ml) was hydrogenated in the presence of 5% palladium-on-charcoal (50 mg) for 2 h at 50°, fresh catalyst was then added, and hydrogenation was continued for 4 h. T.l.c. (chloroform–acetone, 8:2) then indicated complete debenzylation of **7b**. The catalyst was centrifuged off, the solution was taken to dryness, and a solution of the residue (300 mg, ~100%) in 20 ml of 0.5M HCl was heated for 16 h at 100° in a stoppered flask. The hydrolysis product, **9**, possessed R_F, *M*, and *T* values upon p.c., electrophoresis, and ion-exchange chromatography, respectively, very close to those exhibited by the natural 3-*O*-[(*R*)-1-carboxyethyl]-L-rhamnose (Table I).

3-O-[(S)-1-carboxyethyl]-L-rhamnose (**10**). — This was obtained by hydrogenolysis of **8b** at room temperature for 16 h, followed by acid hydrolysis as described above. Its properties are listed in Table I.

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