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BASIC STRUCTURE OF THE OLIGOSACCHARIDE REPEATING-UNIT OF THE *Shigella flexneri* O-ANTIGENS*

LENNART KENNE, BENGT LINDBERG, KURT PETERSSON,

Department of Organic Chemistry, Arrhenius Laboratory, University of Stockholm, S-106 91 Stockholm (Sweden)

AND ELZBIETA ROMANOWSKA

Ludwik Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, 53-114 Wroclaw (Poland)

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ABSTRACT

The *Shigella flexneri* O-antigens are composed of residues of L-rhamnose and 2-acetamido-2-deoxy-D-glucose (3:1), and their basic structure has been investigated. The O-antigen from *Sh. flexneri* variant Y was chosen for this study. Methylation analysis, n.m.r. spectroscopy, and analysis of the product of *N*-deacetylation–deamination were the principle methods used. These studies demonstrate that the O-antigen is composed of repeating units having the following structure:

→3)-β-D-GlcNAc*p*-(1→2)-α-L-Rha*p*-(1→2)-α-L-Rha*p*-(1→3)-α-L-Rha*p*-(1→

INTRODUCTION

The O-specific side-chains of the *Shigella flexneri* lipopolysaccharides are composed of 2-acetamido-2-deoxy-D-glucose and L-rhamnose residues. Two different basic structures, one (**1**) for serotypes 1a, 2a, and variant Y, and another (**2**) for serotypes 3a, 4a, 5, and variant X, have been proposed for the oligosaccharide repeating-unit[1]. Structural variation is further provided by adding terminal α-D-glucopyranosyl groups and *O*-acetyl groups to different positions in these structures.

→6)-D-GlcNAc*p*-(1→2)-L-Rha*p*-(1→4)-L-Rha*p*-(1→

1

→4)-D-GlcNAc*p*-(1→3)-L-Rha*p*-(1→4)-L-Rha*p*-(1→

2

We have recently demonstrated that these structures should be revised and have proposed[2,3] that all *Sh. flexneri* O-antigens contain the same basic repeating-unit (**3**).

→3)-D-GlcNAc*p*-(1→2)-L-Rha*p*-(1→2 or 3)-L-Rha*p*-(1→3 or 2)-L-Rha*p*-(1→

3

*Structural Studies of the *Shigella flexneri* O-Antigens: Part I.

The O-antigen from serogroup 6, which should probably not be classified as a *Sh. flexneri*, has a different structure[4].

Seltmann and Beer (Ref. 5 and preceding papers) have also proposed revised structures for the *Sh. flexneri* O-antigens, based upon the common structure **4**.

—→6)-D-GlcNAc*p*-(1→2)-L-Rha*p*-(1→3)-L-Rha*p*-(1—

4

Structures **3** and **4** are incompatible and also incomplete, as the anomeric configurations of the sugar residues have not been determined. We now report further studies on the structure of the basic oligosaccharide repeating-unit of the *Sh. flexneri* O-antigens; the O-antigen from variant Y was examined, as it does not contain D-glucose residues.

RESULTS AND DISCUSSION

The lipopolysaccharide (LPS) from *Sh. flexneri*, variant Y, was isolated from the bacteria by extraction with phenol-water[6], and the polysaccharide (PS), containing the O-specific side-chains and the core, was prepared from the LPS by mild hydrolysis with acid[7]. In addition to L-rhamnose and 2-acetamido-2-deoxy-D-glucose, D-galactose, D-glucose, and L-*glycero*-D-*manno*-heptose were obtained on acid hydrolysis of the polysaccharide. The last three sugars, which derive from the core region, were present in low percentages only, indicating that the O-specific side-chains constituted the major part of the polysaccharide preparation.

An ^{1}H-n.m.r. spectrum demonstrated that the polysaccharide contained *N*-acetyl (δ 2.06) and *O*-acetyl (δ 2.15) groups in the ratio ~1:2. The *O*-deacetylated polysaccharide gave a better-resolved spectrum which contained, *inter alia*, signals for methyl protons from L-rhamnose residues (δ 1.25–1.32) and from acetamido groups (δ 2.06), and for anomeric protons (δ 4.75–5.15), in the proportions 9:3:4, respectively. The results are therefore in agreement with a repeating unit containing one 2-acetamido-2-deoxy-D-glucose and three L-rhamnose residues. As signals for anomeric protons of α- and β-L-rhamnopyranosyl residues should have low coupling constants, the presence of a signal for one anomeric proton at δ 4.75, with $J_{1,2}$ 7 Hz, is consistent with a 2-acetamido-2-deoxy-β-D-glucopyranosyl residue. The chemical shifts of the other anomeric protons, δ 4.80 and 5.15 (2 H), suggest that at least two of the three L-rhamnopyranose residues are α-linked.

The *O*-acetyl groups present in the O-antigen may be immunologically significant, and will be subject to further studies.

Methylation analysis[8] of the original and *N*-deacetylated PS yielded 3,4-di-*O*-methyl-L-rhamnose and 2,4-di-*O*-methyl-L-rhamnose in the proportions 2:1 and 1:1, respectively (Table I, columns *A* and *B*). On hydrolysis of the product obtained from the *N*-deacetylated PS, the glycosidic linkage of the modified amino sugar is not significantly hydrolysed, and consequently the results demonstrate that the 2-acetamido-2-deoxy-D-glucose is linked to O-2 of an L-rhamnose residue. The 4,6-di-

methyl ether of 2-deoxy-2-*N*-methylacetamido-D-glucose was also obtained, and identified by g.l.c.–m.s. both as its alditol acetate[9,10] and as its acetylated methyl α-glycoside[11].

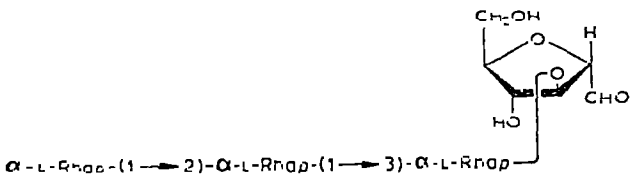
TABLE I

METHYLATION-ANALYSIS DATA FOR THE ORIGINAL AND MODIFIED PS FROM *Sh. flexneri* VARIANT Y

Methylated sugar[a]	T[b]	*Detector response* (%)[c]			
		A	B	C	D
1,2,4,5-Rhamnitol[d]	0.21				26
1,4,6-2,5-Anhydro-Man[d]	0.31			18	
2,3,4-Rha	0.50			17	31
2,4-Rha	0.94	35	45	34	
3,4-Rha	0.87	61	53	31	43
2,3,4,6-Glc	1.00	4	2		

[a]2,3,4,6-Glc = 2,3,4,6-tetra-*O*-methyl-D-glucose, etc. [b]Relative retention times for the corresponding alditol acetates (determined by interpolation between the solvent peak and that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol) on a column of OV-225 S.C.O.T. at 210°. [c]*A*, original PS; *B*, *N*-deacetylated PS; *C*, tetrasaccharide 6; *D*, trisaccharide 7. [d]Parts of this ether and its alditol acetate were probably lost during concentrations.

These results confirm the partial structure **3**, and further demonstrate that the 2-acetamido-2-deoxy-D-glucopyranosyl residue is β-linked.



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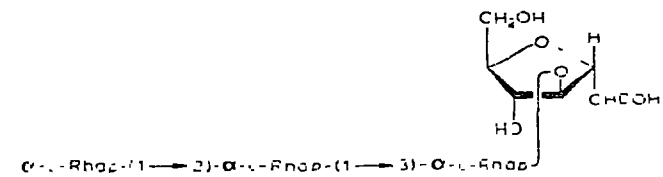

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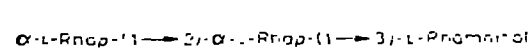

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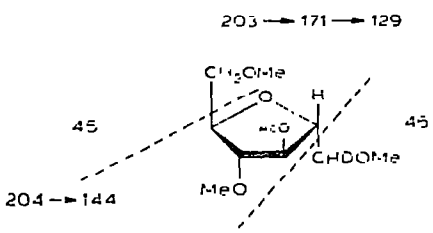
The PS was *N*-deacetylated by treatment with sodium hydroxide–sodium thiophenolate in aqueous methyl sulfoxide[12]. Deamination of this product, by treatment with sodium nitrite in aqueous acetic acid, yielded a tetrasaccharide (**5**).

Part of **5** was reduced with sodium borodeuteride to give the 2,5-anhydro-D-mannitol-*1-d* derivative **6** which had $[\alpha]_{578}^{25}$ −60°. Another part of **5** was treated with base, causing elimination of the trisaccharide side-chain linked to O-3 of the 2,5-anhydro-D-mannose residue. The trisaccharide was then reduced to its alditol (**7**), $[\alpha]_{578}^{25}$ −44°. Methylation analysis of **6** (Table I, column *C*) confirmed the partial structure **3**, but gave no information on the sequence of the two L-rhamnose residues linked through O-2 and O-3. The substitution pattern of the 3-*O*-acetyl-2,5-anhydro-1,4,6-tri-*O*-methyl-D-mannitol-*1-d* (**8**) was evident from its mass spectrum. The primary fragment *m/e* 203, formed by fission between C-1 and C-2, first loses methanol (*β*-elimination), giving the ion *m/e* 171, and subsequently ketene, giving the ion *m/e* 129. The primary fragment *m/e* 204, formed by fission between C-5 and C-6, however, loses acetic acid, also by *β*-elimination, giving the ion *m/e* 144 The isomer of **8**, namely, 4-*O*-acetyl-2,5-anhydro-1,3,6-tri-*O*-methyl-D-mannitol-*1-d* (**9**), was prepared from heparin. In the mass spectrum of this substance, typical secondary fragments differed by one mass unit from those given by **8**, in agreement with the course of the fragmentation discussed above. Other acetylated methyl ethers of 2,5-anhydrohexitols, monodeuterated at C-1, should also be readily identified from their mass spectra. The combination of deamination and methylation analysis should consequently be of general value in structural studies of complex carbohydrates.



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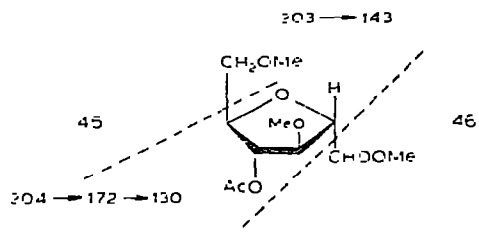

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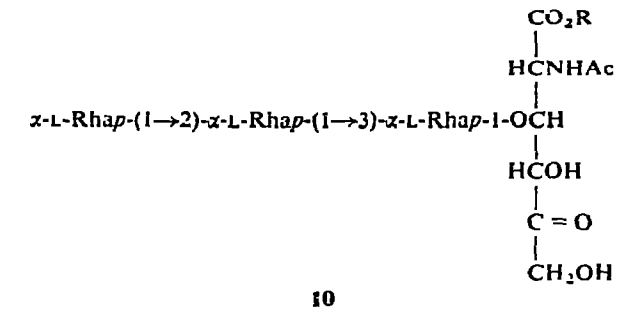
The sequence of the L-rhamnose residues in the PS is evident from the methylation analysis of **7** (Table I, column *D*). The formation of 3,4-di-*O*-methyl-L-rhamnose and 1,2,4,5-tetra-*O*-methyl-L-rhamnitol demonstrates that the L-rhamnose residue linked to the 2-acetamido-2-deoxy-D-glucose residue is also linked through O-3. Methylated **7** gave a single peak on g.l.c., and its mass spectrum was consistent with the postulated structure. The values for the optical rotations of **6** and **7** indicate that all three L-rhamnose residues are α-linked.

Part of the PS was acetylated, and treated with chromium trioxide in acetic acid[13], a procedure known to oxidise *β*-pyranosidic residues to 5-ketoaldonic esters whilst leaving α-pyranosidic residues intact. During *O*-deacetylation of this product with sodium methoxide in methanol, aldonic ester linkages should also be cleaved,



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and a trisaccharide was obtained which was identical (n.m.r.) with the rhamnose trisaccharide obtained on treatment of **5** with base. The trisaccharide is formed by β-elimination of the oxidised product **10**. The results lend further support to the structure **11**, proposed for the repeating unit of the O-antigen.

→3)-β-D-GlcNAc*p*-(1→2)-α-L-Rha*p*-(1→2)-α-L-Rha*p*-(1→3)-α-L-Rha*p*-(1→

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Structural studies of O-antigens from different serogroups of *Sh. flexneri*, which presumably derive from this structure by addition of *O*-acetyl and terminal α-D-glucopyranosyl residues, are in progress.

EXPERIMENTAL

General methods. — Concentrations were performed under diminished pressure at bath temperatures not exceeding 40°. For g.l.c., a Perkin–Elmer 990 instrument fitted with a flame-ionisation detector was used. Separations of alditol acetates were performed at 190° on glass columns (180 × 0.15 cm) containing 3% of OV-225 on Gas Chrom Q; for partially methylated alditol acetates, a column (15 m × 0.5 mm) of OV-225 S.C.O.T. was used at 210°. For quantitative evaluation, a Hewlett–Packard 3370B integrator was used. G.l.c.–m.s. was performed with a Varian Mat 311-SS 100 m.s.–computer system. N.m.r. spectra for solutions in D_2O at 85° were recorded on a Varian XL-100 instrument, with sodium 1,1,2,2,3,3-hexadeuterio-4,4-dimethyl-4-silapentane-1-sulfonate as internal standard. Optical rotations were recorded on a Perkin–Elmer 141 instrument. A Waters R-403 differential refractometer was used for monitoring the Sephadex column effluents.

Preparation of polysaccharide. — *Shigella flexneri*, variant Y (–, 3,4) (Dysentery Reference Laboratory, London), was used. The lipopolysaccharide (LPS) was isolated from acetone-dried bacteria by extraction with phenol–water and purified by gel filtration on Sepharose 2-B[14]. Unspecific glucan, present in 7–25%, was removed by filtration through a Concanavalin A–Sepharose 4-B column[15]. LPS (200 mg) was treated with 1% acetic acid (80 ml) for 2 h at 100°, centrifuged, washed with ether (3 × 15 ml), and freeze-dried, yielding the PS (103 mg), $[\alpha]_{578}^{25}$ +1° (*c* 0.5, water). Sugar analysis of an acid hydrolysate of the PS showed L-rhamnose, 2-amino-2-deoxy-

D-glucose, and small proportions of D-glucose, D-galactose, and L-*glycero*-D-*manno*-heptose; the sugars were analysed by g.l.c.–m.s. of their alditol acetates[16]. Methylation analysis (Table I, column *A*) was performed as previously described[8]. The n.m.r. spectrum of the PS was not well-resolved, but showed, *inter alia*, signals for *O*-acetyl and *N*-acetyl groups and for methyl protons in L-rhamnose residues in the proportions 2:1:3.

The PS (28 mg) was *O*-deacetylated by treatment with 0.5M aqueous sodium hydroxide (10 ml) at 25° for 7 h. The solution was neutralised with M hydrochloric acid, and dialysed, and the product (16 mg) was recovered by freeze-drying. The n.m.r. spectrum, which was well-resolved, showed, *inter alia*, signals at δ 1.25–1.32 (9 H), 2.06 (3 H), 4.75 (1 H, $J_{1,2}$ 7 Hz), 4.80 (1 H), and 5.15 (2 H).

N-*Deacetylation*. — A solution of PS (36 mg), sodium hydroxide (720 mg), and thiophenol (150 mg) in water (1.8 ml) and methyl sulfoxide (9 ml) was stirred at 90° for 15 h, neutralised with M hydrochloric acid, dialysed, and freeze-dried. The product (20 mg), $[\alpha]_{578}^{25}$ +8° (*c* 0.6, water), did not contain *N*-acetyl groups, as evident from its n.m.r. spectrum which showed, *inter alia*, signals at δ 1.25–1.32 (9 H), 4.55 (1 H, $J_{1,2}$ 8 Hz), 5.03 (1 H, $J_{1,2}$ 1.5 Hz), 5.17 (1 H), and 5.25 (1 H). The data for the methylation analysis of this product are given in Table I, column *B*.

Deamination. — The *N*-deacetylated PS (19 mg) was dissolved in water (1.3 ml), and 33% aqueous acetic acid (2 ml) and 5% aqueous sodium nitrite (2 ml) were added. The solution was kept at 25° for 40 min, and then passed through a column of Dowex 50 (H^+) resin and freeze-dried.

Half of the product was reduced with sodium borodeuteride (30 mg), and the solution was neutralised with Dowex 50 (H^+) resin, filtered, concentrated, and codistilled with methanol (3 × 3 ml). Fractionation of this reduced material on a column (80 × 1.6 cm) of Sephadex G-25 gave one main fraction (5 mg), $[\alpha]_{578}^{25}$ −60° (*c* 0.5, water), eluted in the tri- to tetra-saccharide region. Some polymeric material (2 mg) was also eluted, and shown by sugar analysis to contain core sugars. Data for the methylation analysis of the reduced oligosaccharide fraction are given in Table I, column *C*. The mass spectrum of 3-*O*-acetyl-2,5-anhydro-1,4,6-tri-*O*-methyl-D-mannitol-*1-d* showed, *inter alia*, peaks at the following *m/e* values: 43 (100), 45 (64), 46 (42), 53 (4), 55 (5), 56 (8), 59 (6), 69 (8), 70 (7), 71 (15), 75 (10), 86 (5), 87 (16), 88 (5), 97 (10), 101 (10), 103 (9), 111 (11), 112 (13), 116 (4), 126 (6), 129 (62), 130 (7), 143 (6), 144 (13), 157 (5), 171 (1), 203 (16), and 204 (4).

The n.m.r. spectrum of the tetrasaccharide **6** showed, *inter alia*, signals at δ 1.23–1.33 (9 H), 4.87 (1 H, $J_{1,2}$ 1.5 Hz), 4.96, (1 H, $J_{1,2}$ 1 Hz), and 5.15 (1 H). On hydrolysis, it yielded 2,5-anhydro-D-mannitol and L-rhamnose in the ratio 1:3.2.

Heparin (20 mg) in water (2 ml) was treated with aqueous nitrous acid (2 ml), prepared by dissolving N_2O_4 in water (pH 1.4). After 30 min at 25°, the solution was freeze-dried, and a solution of the residue in water (4 ml) was reduced with sodium borodeuteride (20 mg) for 2 h. The reaction mixture was neutralised with Dowex 50 (H^+) resin, concentrated, and codistilled with methanol (3.3 ml). Methylation analysis afforded 4-*O*-acetyl-2,5-anhydro-1,3,6-tri-*O*-methyl-D-mannitol-*1-d*, which

gave a mass spectrum having, *inter alia*, peaks with the following *m/e* values: 43 (100), 45 (57), 46 (54), 53 (4), 55 (9), 59 (5), 68 (4), 69 (6), 70 (5), 71 (8), 72 (12), 73 (6), 75 (12), 83 (4), 85 (8), 87 (15), 88 (4), 98 (10), 99 (5), 101 (5), 103 (9), 111 (17), 112 (9), 115 (5), 126 (7), 129 (8), 130 (58), 131 (5), 143 (14), 144 (4), 157 (5), 172 (1), 203 (2), 204 (14), and 205 (2).

The other half of the deaminated material was treated with 0.05M aqueous sodium hydroxide (1 ml) at 37° for 30 min, neutralised with 0.5M hydrochloric acid, and fractionated on a column (80 × 0.8 cm) of Sephadex G-15. A component was obtained in the trisaccharide region, and its ^{1}H-n.m.r. spectrum showed, *inter alia*, signals at δ 1.24–1.32 (9 H), δ 4.87 (0.5 H, $J_{1,2}$ 1 Hz), 5.01 (1 H, $J_{1,2}$ 1 Hz), 5.10 (0.5 H, $J_{1,2}$ 1.5 Hz), and 5.21 (1 H, $J_{1,2}$ 1 Hz). The trisaccharide alditol (7, 1 mg), obtained by reduction with sodium borohydride, showed $[\alpha]_{578}^{25}$ −44° (*c* 0.1, water) and, after methylation, gave a single peak on g.l.c. using a column containing 3% of OV-1 on Gas Chrom Q. The data for the methylation analysis of 7 are given in Table I, column *D*. The mass spectrum of the fully methylated trisaccharide alditol contained, *inter alia*, the following fragments (relative intensities in brackets): 45 (21), 55 (9), 57 (7), 59 (67), 69 (5), 21 (29), 22 (34), 73 (18), 75 (46), 83 (5), 85 (16), 87 (7), 88 (100), 89 (20), 97 (13), 99 (46), 101 (66), 103 (10), 113 (8), 115 (19), 125 (13), 129 (13), 131 (7), 141 (9), 145 (13), 147 (5), 157 (28), 189 (20), 205 (6), 265 (6), 281 (3), 331 (2), 347 (4), 355 (2), and 363 (1).

Chromium trioxide oxidation. — Fully acetylated PS (35 mg) was treated with chromium trioxide (105 mg) in acetic anhydride for 1 h at 60° in an ultrasonic bath. The reaction mixture was partitioned between chloroform and water, and the chloroform phase was concentrated to dryness. The product was treated with 0.05M methanolic sodium methoxide (20 ml) for 15 h at 25°, and then neutralised with Dowex 50 (H^+) resin. Water (20 ml) was added to the residue, the ion-exchange resin removed by filtration, and the solution concentrated to dryness. Gel filtration of the residue on a column (80 × 0.8 cm) of Sephadex G-25, with water as eluant, gave a trisaccharide (7 mg) which was identical (n.m.r.) with that isolated as the product of sequential deamination and base treatment of *N*-deacetylated PS.

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REFERENCES

1 D. A. R. SIMMONS, *Eur. J. Biochem.*, 11 (1969) 554–575.
2 B. LINDBERG, J. LÖNNGREN, U. RUDÉN, AND D. A. R. SIMMONS, *Eur J. Biochem.*, 32 (1973) 15–18.

3 B. LINDBERG, J. LÖNNGREN, E. ROMANOWSKA, AND U. RUDÉN, *Acta Chem. Scand.*, 26 (1972) 3808–3810.
4 E. KATZENELLENBOGEN, M. MULECYK, AND E. ROMANOWSKA, *Eur. J. Biochem.*, 61 (1976) 191–197.
5 G. SELTMANN AND W. BEER, *Z. Allgem. Mikrobiol.*, 13 (1973) 107–113.
6 O. WESTPHAL, O. LÜDERITZ, AND F. BISTER, *Z. Naturforsch. B*, 7 (1952) 148–155.
7 W. DROGE, O. LUDERITZ, AND O. WESTPHAL, *Eur. J. Biochem.*, 4 (1968) 126–138.
8 H. BJÖRNDAL, C. G. HELLERQVIST, B. LINDBERG, AND S. SVENSSON, *Angew. Chem. Int. Ed. Engl.*, 9 (1970) 610–619.
9 K. STELLNER, H. SAITO, AND S. HAKOMORI, *Arch. Biochem. Biophys.*, 155 (1973) 464–472.
10 G. O. H. SCHWARZMANN AND R. W. JEANLOZ, *Carbohydr. Res.*, 34 (1974) 161–168.
11 S. K. KUNDU, R. W. LEDEEN, AND P. A. J. GORIN, *Carbohydr. Res.*, 39 (1975) 329–334.
12 C. ERBING, K. GRANATH, L. KENNE, AND B. LINDBERG, *Carbohydr. Res.*, 47 (1976) C5–C7.
13 J. HOFFMAN, B. LINDBERG, AND S. SVENSSON, *Acta Chem. Scand.*, 26 (1972) 661–666.
14 E. ROMANOWSKA, *Anal. Biochem.*, 33 (1970) 383–389.
15 E. ROMANOWSKA, unpublished results.
16 J. S. SAWARDEKER, J. H. SLONEKER, AND A. JEANES, *Anal. Chem.*, 37 (1965) 1602–1604.