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STRUCTURE OF A RHAMNAN FROM THE SURFACE-LAYER GLYCOPROTEIN OF *Bacillus stearothermophilus* STRAIN NRS 2004/3a*

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ABSTRACT

The structure of a glycan from the surface-layer glycoprotein of *Bacillus stearothermophilus* strain NRS 2004/3a has been studied by ^{1}H- and ^{13}C-n.m.r. spectroscopy. The results indicate the glycan to be a polymer of the trisaccharide repeating-unit

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

INTRODUCTION

The crystalline surface-layer (S-layer) of *Bacillus stearothermophilus* strain NRS 2004/3a consists of identical glycoprotein subunits which are assembled into regular arrays[1]. The S-layer glycoprotein appears to contain two types of glycan chain, one of which is composed of rhamnose residues only[2]. Digestion of the purified glycoprotein with pronase and gel-permeation chromatography of the products on Biogel P-100 gave[2] a peptidorhamnan of apparent molecular weight ~20,000. In view of the small amount (~8 mg) of sample available, an attempt has been made to elucidate the structure of this peptidorhamnan essentially by n.m.r. spectroscopic techniques. The carbohydrate content of the material studied[2] was >95%; hence, signals due to the peptide portion did not interfere with ^{1}H- or ^{13}C-n.m.r. spectroscopy of the rhamnan.

RESULTS AND DISCUSSION

Stereochemistry of the rhamnose residues. — The absolute configuration of the constituent rhamnose was determined by the method of Gerwig *et al.*[3]. The

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derived (−)-2-butyl rhamnopyranoside gave a single g.l.c. peak with (−)-2-butyl-L-rhamnoside and was therefore L, since the mixture with the D-rhamnoside derivative gave two peaks.

Proton-decoupled ^{13}C-n.m.r. spectrum. — This spectrum of the peptidorhamnan contained 15 resolved signals for the ring carbons and three coincident signals for the C-6 of three rhamnose residues. Three signals for anomeric carbons were present at 101.61, 100.65, and 98.95 p.p.m.

All of the signals corresponding to linkage points (80.59, 79.29, and 77.94 p.p.m.) were at low field. Therefore, branched structures having neighbouring linkage-points could be excluded. With the exception of those due to C-6, no signals were present to the high-field side of 70 p.p.m. This finding excluded 4-substituted α-rhamnopyranosyl residues, which would have resulted in an upfield shift of a C-5 signal[4]. Therefore, branched structures containing an α-rhamnopyranosyl residue as branching point can also be excluded.

Conformation of the rhamnose constituents. — Inspection of the ^1H-n.m.r. spectrum revealed the following ^3J values, $J_{1,2} \sim 1$, $J_{2,3} \sim 3.5$, $J_{3,4} \sim 9.5$, and $J_{4,5} \sim 9.5$ Hz, indicating that all three rhamnopyranosyl residues were present in the $^1C_4$ chair form.

Proton-coupled ^{13}C-n.m.r. spectrum. — This spectrum of the peptidorhamnan exhibited features that served to delineate a hypothetical structure. The spacings observed with the three anomeric signals [101.61 (J 170 Hz), 100.65 (J 171 Hz), and 98.95 p.p.m. (J 160 Hz); Table I] indicated the presence of two α- and

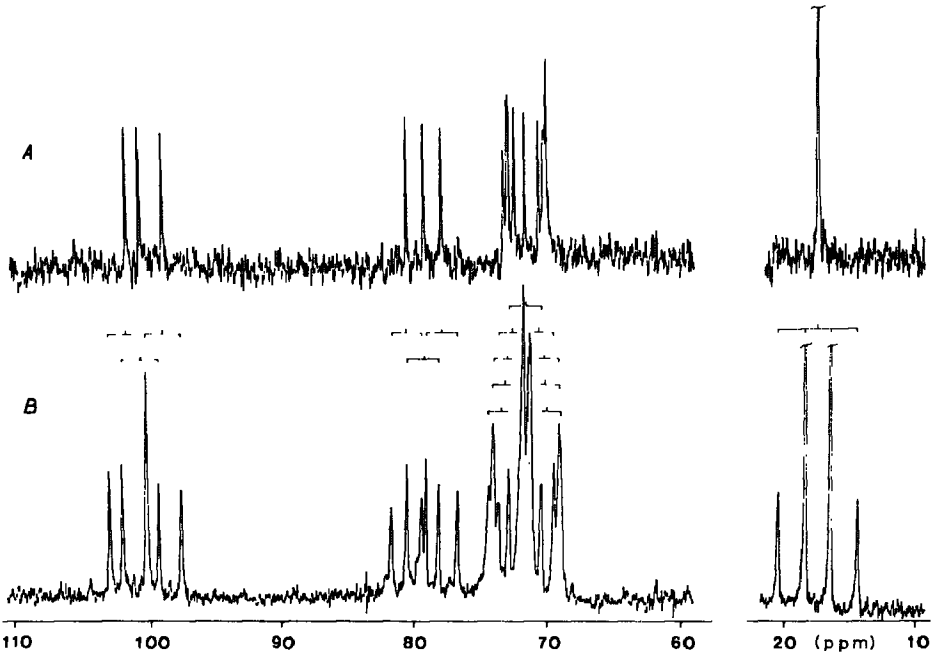

Fig. 1. Proton-decoupled (A) and proton-coupled (B) ^{13}C-n.m.r. spectrum of the peptidorhamnan (see Experimental).

TABLE I

^{13}C-CHEMICAL SHIFTS AND $^{1}J_{C,H}$ VALUES[a] OF THE PEPTIDORHAMNAN AND SELECTED MODEL COMPOUNDS

Rhap residue	*C-1*	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*
(1→2)-α-Rha*p*-(1→2)	100.65	77.94	70.31[b]	72.87[c]	70.05[d]	17.44[e]
	171	152	(142)	(144)	(144)	127
(1→2)-α-Rha*p*-(1→3)	101.61	79.29	70.67[b]	72.98[c]	70.16[d]	17.44[e]
	170	151	(142)	(144)	(144)	127
(1→3)-β-Rha*p*-(1→2)	98.95	71.66	80.59	72.44	73.26[c]	17.44[e]
	160	152	144	(144)	(144)	127
α-Rha*p*[g]	94.70	71.54	70.70	72.91	68.99	17.59[f]
	170	149	(142)	(143)	145	128
β-Rha*p*[g]	94.22	72.06	73.55	72.61	72.78	17.59[f]
	160	148	142	—	(143)	128
α-Rha*p*-OMe[h]	102.0	71.1	71.4	73.1	69.3	17.7
β-Rha*p*-OMe[h]	102.1	71.5	73.8	73.1	73.1	17.6
(1→2)-α-Rha*p*-(1→3)[i]	101.61	78.75	70.67	72.99	70.11	17.49

[a]Shifts are in p.p.m. downfield from the signal of Me_4Si (67.40 p.p.m. upfield from the signal of 1,4-dioxane in D_2O). Couplings are in Hz, and were measured by hand on enlarged 62.9-MHz spectra; the estimated error is ±1 Hz for the non-overlapped signals (couplings determined from overlapped signals are shown in brackets and were determined from the doubled difference between the centre of the signals and one part each of the coupled signals). [b–d]Assignments may be interchanged. [e,f]Signals not resolved. [g]Assignment from ref. 18. [h]Shift values and assignments from ref. 4; 0.3 p.p.m. was subtracted to reference to 1,4-dioxane at 67.4 p.p.m. [i]Shift values and assignments from ref. 11.

one β-rhamnopyranosyl residues per repeating unit[5]. For the carbons involved in the linkages, the $^{1}J_{C,H}$ values were 144 (80.59 p.p.m.), 151 (79.29 p.p.m.), and 152 Hz (77.94 p.p.m.) (Table I). The latter two values are typical of C-2 carrying an axial oxygen, whereas the first value may correspond to C-3 or C-4. These assumptions were based on observations, for the mannose[6] and KDO series[7], that the $^{1}J_{C,H}$ values of pyranoid ring carbons do not change on substitution. Therefore, the C-2 resonance of the third rhamnose residue should occur at 71.66 p.p.m. ($^{1}J_{C,H}$ 152 Hz). The remaining signals, although partially overlapping, had couplings in the range 140–144 Hz and were attributed from a knowledge of the centres of the signals and one part each of the coupled signals.

The inference that two of the rhamnopyranosyl residues were glycosylated at O-2 was substantiated by the β-shifts observed for their C-1 resonances ($\Delta\delta$ −1.4 and −2.4 p.p.m.) by comparison with a non-reducing α-rhamnopyranosyl group[8]. The upfield shift of the C-1 signal attributed to the β-rhamnopyranosyl residue (98.95 p.p.m., $J_{C,H}$ 160 Hz) could be explained by a spatial proton–proton interaction[9] of H-1 of the β-unit with H-2 of the connected α-rhamnopyranosyl residue, and was similar to that reported[10] for C-1 of a β-rhamnopyranosyl group linked at O-2 of an α-rhamnopyranosyl residue (99.7 p.p.m., $^{1}J_{C,H}$ 162 Hz; the difference may have been due to a different mode of standardisation or a different probe-temperature).

The lines corresponding to the two α-rhamnopyranosyl residues (Table I)

were then empirically assigned using appropriate literature data[8,11], leaving a set of signals which could correspond equally well to a 3- or a 4-substituted β-rhamnosyl residue if glycosidation- and β-shifts are considered. However, substitution of an α-rhamnosyl group at O-4 results in a downfield shift of ~0.5 p.p.m. of the C-6 signal[4]. Given the observation that the shifts of the three C-6 signals coincided in the spectrum of the peptidorhamnan, the β-rhamnopyranosyl residue was assumed to be substituted at O-3.

^{1}H-N.m.r. spectra. — To test the foregoing assumption, an attempt was made to interpret the ^{1}H-n.m.r. spectrum by using selective decoupling experiments and n.O.e. measurements. The signals were assembled into three groups, corresponding to the rhamnosyl residues A, B, and C (the β-unit). Thus, irradiation at the H-6 cluster revealed the positions of the H-5 signals. Irradiation at the H-2 resonance frequencies gave the position of the H-3 adjacent to the single H-2 and the positions of the two H-3's adjacent to the two H-2's whose signals coincide (Table II). The assignment of the H-3 resonances is discussed in the section on n.O.e. The finding that the resonances due to H-3 and H-5 of the β-rhamnosyl residue occur at higher fields is in agreement with data in the literature[12].

N.O.e. experiments. — Saturation at the resonance frequency corresponding to H-1A (5.08 p.p.m.) resulted in n.O.e.'s of the signal due to the adjacent H-2A, and of the signals of H-1B, H-1C, H-2B, and H-2C. Following saturation of the H-1B signal (5.17 p.p.m.), n.O.e.'s are observed for the signals due to H-2B, H-1A, H-1C, and H-3C. With H-1C (4.74 p.p.m., the signal of the β-rhamnosyl residue), n.O.e.'s were observed at the resonance frequencies attributable to H-2C, H-3C, H-5C, H-1A, H-1B, and H-2A. Saturation was also performed at 4.23 p.p.m., the resonance frequency attributed to H-2A; n.O.e.'s were then observed for the signals due to H-1A, H-3A, and H-1C.

In order to assign the H-3 resonances, the following observations had to be taken into account. In a mannose system[13], saturation of H-1 of an α-mannose results in an n.O.e. for H-2, and saturation of H-2 results in n.O.e.'s for H-1,3.

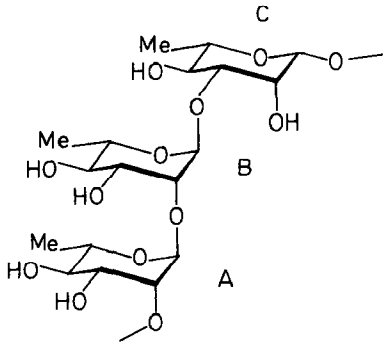

Fig. 2. Proposed structure of the repeating unit of a glycan from the surface-layer glycoprotein of *B. stearothermophilus* NRS 2004/3a.

TABLE II

^{1}H-CHEMICAL SHIFTS AND COUPLINGS[a] OF THE PEPTIDORHAMNAN AND SELECTED MODEL COMPOUNDS

Rhap *residue*	*H-1*	*H-2*	*H-3*	*H-4*	*H-5*	*H-6*
α-Rhap (A)	5.08 (s)[b]	4.23 (3.5)	3.76 (9.5)	3.45–3.50 (3H, dd's)	3.71[c] (6.5)	1.27[c]
α-Rhap (B)	5.17 (s)[b]	4.06 (3.5)	3.94 (9.5)		3.81[c] (6.5)	1.30[c]
β-Rhap (C)	4.74 (s)[b]	4.06 (3.5)	3.61 (9.5)		3.4 (m)	1.24[c]
α-Rhap-OMe[d]	4.62 (1.6)	3.85 (3.5)	3.63 (9.5)	3.36 (9.5)	3.59 (6.2)	1.22
β-Rhap-OMe[d]	4.46 (0.9)	3.90 (3.3)	3.50 (9.2)	3.29 (dd)	3.32 (m)	1.24
(1→2)-α-Rhap-(1→3)[e]	5.21 (1.5)	4.09 (3.1)	3.97 (9.6)	3.53 (9.6)	3.85 (6.2)	1.34

[a]Shifts are in p.p.m. downfield from the signal for Me_4Si (2.15 p.p.m. upfield from the signal of acetone-d_6 in D_2O). Couplings are in Hz, and are rounded to the nearest 0.5 Hz for the peptidorhamnan. [b]Due to signal broadening caused by the high molecular weight, $^3J_{H,H}$ was not measurable. The signal due to H-1 of the C-residue (β-rhamnopyranosyl residues) is smaller than the signals due to the H-1 of the A- and B-residues. [c]Assignments may be interchanged. [d]Shift values and couplings from ref. 8; 0.03 p.p.m. was added to reference the spectrum to acetone-d_6 at 2.15 p.p.m. [e]Shift values and couplings attributed to the partial structure of a rhamnan in ref. 11.

TABLE III

OBSERVED AND CALCULATED N.O.E'S[a] FOR THE PEPTIDORHAMNAN

Saturated		*1A*	*1B*	*1C*	*2A*	*2B*	*2C*	*3A*	*3B*	*3C*	*4?*[c]	*5C*
1A	meas.	100	9	10	9		22[b]	3	3	5		8[b]
	calc.	100	6	8	13	17	—	—	—	—	—	—
1B	meas.	7	100	4	3		12[b]	—	2	11		11[b]
	calc.	7	100	—	—	16	—	—	—	15	—	—
1C	meas.	12	5	100	16		12[b]	5	1	11		17[b]
	calc.	6	—	100	10	—	11	—	—	6	—	11
2A	meas.	6	2	9	100		7[b]	14	1	2		11[b]
	calc.	11	—	11	100	7	—	16	—	—	—	—

[a]In % relative to the saturated hydrogen. Due to the high molecular weight, the signs of the saturated and observed values are the same[8]. [b]Observed n.O.e.'s for H-2B and H-2C coincide. Both the H-4? and H-5C are visibly affected, but integration was possible only for the sum of H-4's and H-5C. [c]As well as the observed n.O.e. for H-4?, there are always residual n.O.e.'s to the other H-4's. Integration was only possible over the complete range for H-4's, and H-5C.

Saturation of H-1 of a β-mannose results in n.O.e.'s for H-2,3,5, and saturation of H-2, in n.O.e.'s for H-1,3. The resonance at 3.76 p.p.m. could not be due to H-3B because saturation of the connected H-2 at 4.23 p.p.m. gave no n.O.e. at H-1B. The resonance at 3.61 p.p.m. also cannot be H-3B, because saturation at H-1B resulted in a strong n.O.e. at this frequency. Therefore, by exclusion, the resonance at 3.94 p.p.m. was due to H-3B. The resonance at 3.61 could not be due to H-3A because saturation at the H-1C frequency resulted in equally strong n.O.e. for H-1A and at 3.61 p.p.m. Also, H-1C, if close to H-1A, will be relatively far from H-3A and *vice versa*. Similarly, irradiation at the resonance frequency of H-1B

cannot give rise to n.O.e. of the H-1A and H-3A signals simultaneously. Therefore, the signal at 3.61 p.p.m. was assumed to be due to H-3C, an assignment in good agreement with data in the literature[12].

Thus, the cluster of signals assigned to residue A is in agreement with expectations for a 2-substituted α-rhamnopyranosyl residue linked at O-3 of an α-rhamnopyranosyl residue. Signal cluster B would similarly correspond to a 2-substituted α-rhamnopyranosyl residue linked at O-3 of a β-rhamnopyranosyl residue, and C to a 3-substituted β-rhamnopyranosyl residue linked at O-2 of an α-rhamnopyranosyl residue. In this manner, the interpretation of the ^{1}H-n.m.r. data corroborated the structure assignment made on the basis of the ^{13}C-n.m.r. spectra.

Bock[9] has observed and calculated the n.O.e. of the H-5A resonance upon irradiation at the H-1B resonance in model systems corresponding to the linkage α-Rha*p*-(1→2)-α-Rha*p*, as applied to the Y polysaccharide of *Shigella flexneri*[14]. Surprisingly, an analogous n.O.e. was not observed with the peptidorhamnan studied here. Furthermore, all three H-1 resonances were inter-related by n.O.e.'s, and irradiation at these H-1 frequencies resulted in a slight n.O.e. of one of the H-4 signals (3.49 p.p.m.). These unexpected observations could be reconciled only if the assumption is made that the signs of the $\phi^{H}\psi^{H}$-angles, as expected on the basis of the h.s.e.a. calculations, were reversed for the α-rhamnopyranosyl unit A. It is not surprising that this alternative conformation would be assumed by one of the

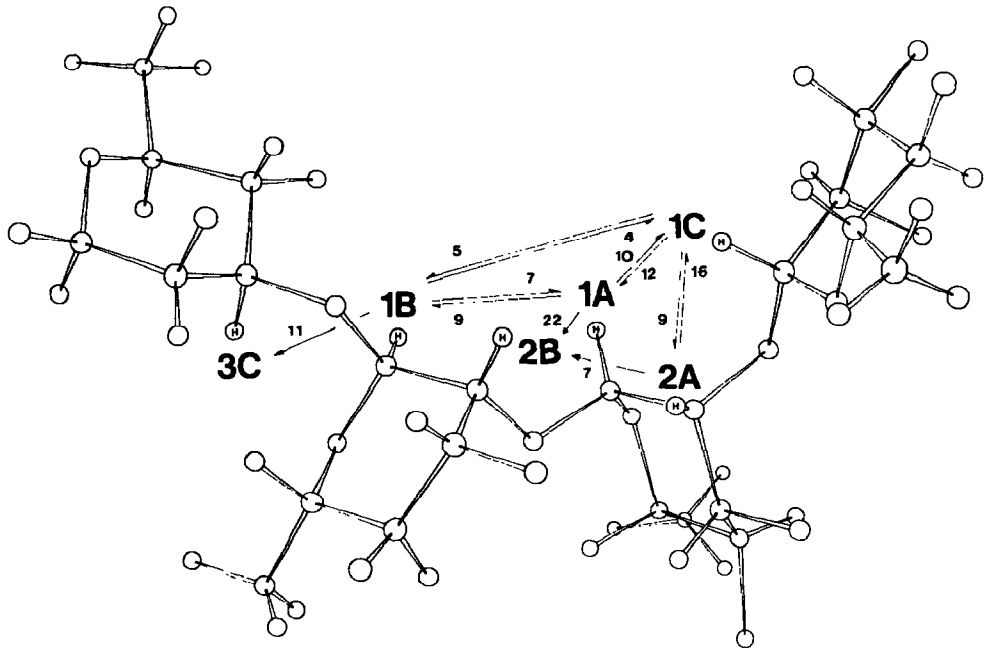

Fig. 3. Stereochemical drawing of the proposed structure of the rhamnan from *B. stearothermophilus* NRS 2004/3a. The arrows indicate inter-residue n.O.e.'s.

α-rhamnopyranosyl residues where the *exo*-anomeric effect plays a less dominant role than with the β-glycosides[15]. As verified by calculation, H-5A is remote from H-1B in the resulting conformer. Moreover, the three H-1 atoms are closely adjacent and each may interact by intersegment n.O.e. with the same H-4 (3.49 p.p.m.).

EXPERIMENTAL

The absolute configuration of the rhamnose was determined by the method of Gerwig *et al.*[3], using a Hewlett–Packard 5840A gas chromatograph equipped with capillary-inlet and an SP2100 fused-silica column (50 m × 0.25 mm). All operations were performed as described[3], using the programme B.

^{13}C-N.m.r. spectra (62.9 MHz) were recorded with a Bruker WM 250 instrument, equipped with a 5-mm probe-head, working in the F.t. mode. All spectra were recorded at 297 K for solutions in D_2O (external Me_4Si; 67.40 p.p.m. upfield from the signal of 1,4-dioxane in D_2O). The spectral width was 7.2 kHz and 16 k of memory were used.

^{1}H-n.m.r.-spectra (250 MHz) were recorded on the same instrument at 313 K, with deuterium-lock on the water signal. The instrument was referenced to acetone-d_6 at 2.15 p.p.m. The spectral width was 2 kHz and 16 k of memory were used.

^{1}H-N.O.e. difference-spectra were measured for solutions in D_2O–acetone-d_6 (4:1) at 313 K, locked on acetone-d_6 to prevent loss of sensitivity due to thermal drifts of the water signal.

Hard-sphere *exo*-anomeric-effect (h.s.e.a.) calculations were performed as described by Lemieux *et al.*[16], using the formulas and values as given by Bock[17], on an NCR-Decision-mate V microcomputer, equipped with a 0.5 Megabyte random-access-memory and a 8087/3 numerical co-processor, for a segment comprising three repeating-units. Due to the large number of computations necessary, refinement of the $\phi^H\psi^H$-values was done to an accuracy of ten degrees only. N.O.e. values were calculated for the middle repeating-unit, and no attempt was made to calculate the intersegment n.O.e.'s.

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