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Note

Evidence for Chitobiose Formation from Peptidoglycan Monomer under Mildly Alkaline Conditions

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Treatment of peptidoglycan monomer (1) from *Brevibacterium divaricatum* with either sodium hydroxide solutions or a strong anion exchanger led to cleavage of the C-3 linkage in the *N*-acetylmuramoyl residue to give the corresponding *D*-lactoylpentapeptide and the saturated disaccharide, chitobiose. Treatment with weakly basic exchangers left 1 unchanged. The results indicate that, provided the pH value of the reaction mixture is high enough, cleavage of 1 proceeds by the same mechanism irrespective of the counter-cation used.

Rosenthal and Shockman¹, and Ghuysen et al.² claimed that cell wall peptidoglycan fragments from *Streptococcus faecalis* eliminate, in aqueous sodium hydroxide, the lactoylpeptide residues with concomitant formation of unsaturated oligosaccharide. Identical β -elimination reaction was observed in aqueous ammonia with the disaccharide-peptide repeating unit from the same microorganism,³ and with *N*-acetylmuramic acid and its C-1 unsubstituted derivatives.⁴

Recently, it was shown⁵ that the immunoadjuvant⁶ repeating peptidoglycan monomer from *Brevibacterium divaricatum* 1, characterized⁷⁻⁹ as [2-acetamido-4-*O*-(2-acetamido-2-deoxy- β -*D*-glucopyranosyl)-2-deoxy-3-*O*-(*D*-ethyl-1-carbonyl)-*D*-glucopyranose]-*L*-alanyl-*D*-isoglutaminyl-[(*L*)-*meso*-diaminopimeloyl-(*D*)-amide-(*L*)-*D*-alanyl-*D*-alanine] {GlcNac- β -(1 \rightarrow 4)-MurNac-*L*-Ala-*D*-iGln-[(*L*)-*meso*-A₂pm-(*D*)-amide-(*L*)-*D*-amide-(*L*)-*D*-Ala-*D*-Ala]; Figure 1} undergoes

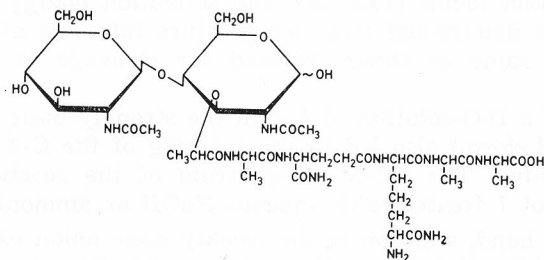


Figure 1. Peptidoglycan monomer from *Brevibacterium divaricatum*.

cleavage by aqueous ammonia at the C-3 ether linkage to give the D-lactoylpentapeptide and the saturated disaccharide GlcNAc- β -(1 \rightarrow 4)-GlcNAc (chitobiose; *N,N'*-diacetylchitobiose).

In order to find out whether the nature of the counter-cation, in particular that incorporated into polymeric matrix, has any influence on the cleavage of the C-3 ether linkage, the reaction of *1* with aqueous sodium hydroxide and with strongly and weakly basic anion exchangers was investigated. In the present paper evidence is presented that *1* is converted by aqueous sodium hydroxide and a strongly basic anion exchanger to D-lactoylpentapeptide and chitobiose. Contrary, to that, *1* is resistant to weakly basic exchangers.

RESULTS AND DISCUSSION

Treatment of *1* with dilute sodium hydroxide (6.53—68.55 mM in D₂O) caused the cleavage of the C-3 ether bond in the *N*-acetylmuramoyl moiety, as evidenced by comparing the NMR spectra of these samples with those of the starting *1*; aqueous-ammonia-treated *1*; the pentapeptide {L-Ala-D-*i*Gln-[(L)-*meso*-A₂pm-(D)-amide-(L)-D-Ala-D-Ala]}; and the disaccharide [GlcNAc- β -(1 \rightarrow 4)-MurNAc]^{5,8,9}. Only the signals of the reducing pyranose ring C-3, and D-lactyl α -C were shifted upfield (by 9.7 and 9.8 p.p.m., respectively), whereas those of the same sugar C-4, as well as of β -C and CO of D-lactyl moiety were shifted downfield (by 3.3; 1.2 and 2.5 p.p.m., respectively). These shifts were identical to those observed for *1* in aqueous ammonia.⁵

In the ¹H-NMR spectrum of the D-lactoylpentapeptide {D-lact-L-Ala-D-*i*Gln[(L)-*meso*-A₂pm-(D)-amide-(L)-D-Ala-D-Ala]} isolated from the NaOH-treated *1* by gel filtration, only the methyl signals of alanines could be assigned precisely, and their values agreed with those of the samples isolated analogously from the ammonia-treated *1*⁸⁻¹⁰. The ¹³C-NMR data of the isolated D-lactoylpentapeptide were identical to those of *1* obtained previously and published for the ammonia-treated *1*⁵.

In the ¹H-NMR spectrum of the disaccharide isolated from NaOH-treated *1*, the methyl signal of the 2-acetamido-2-deoxy-D-glucopyranosyl residue could be determined precisely. The ¹³C-NMR spectrum was fully consistent with the disaccharide structure GlcNAc- β -(1 \rightarrow 4)-GlcNAc.

The cleavage of the C-3 ether bond of *1* was studied at various temperatures and NaOH concentrations. From the data obtained, the pseudo-first-order rate constants (k_{obs}) were calculated (Table I). The k_{obs} depends linearly on the NaOH concentrations within the limits of experimental error, and the second-order rate constant is $1.14 \times 10^{-2} \text{ mol}^{-1} \text{ s}^{-1}$.

The acceleration factor (1.82) and the activation energy (43.3 kJ/mol) of the reaction were determined from temperature intervals of 10 °C and their values were the same as those obtained for cleavage of *1* with aqueous ammonia.⁵

Treatment of a D₂O-solution of *1* with the strongly basic anion exchanger Dowex 2 \times 8 (OH⁻-form) also led to the splitting of the C-3 ether linkage in the MurNAc residue. The ¹³C-NMR spectrum of the reaction mixture was identical to that of *1* treated with aqueous NaOH or ammonia.

On the other hand, addition of the weakly basic anion exchanger Amberlite CG 45 or DEAE-cellulose to a D₂O solution of *1* did not cause any changes in the ¹H- and ¹³C-NMR spectra.

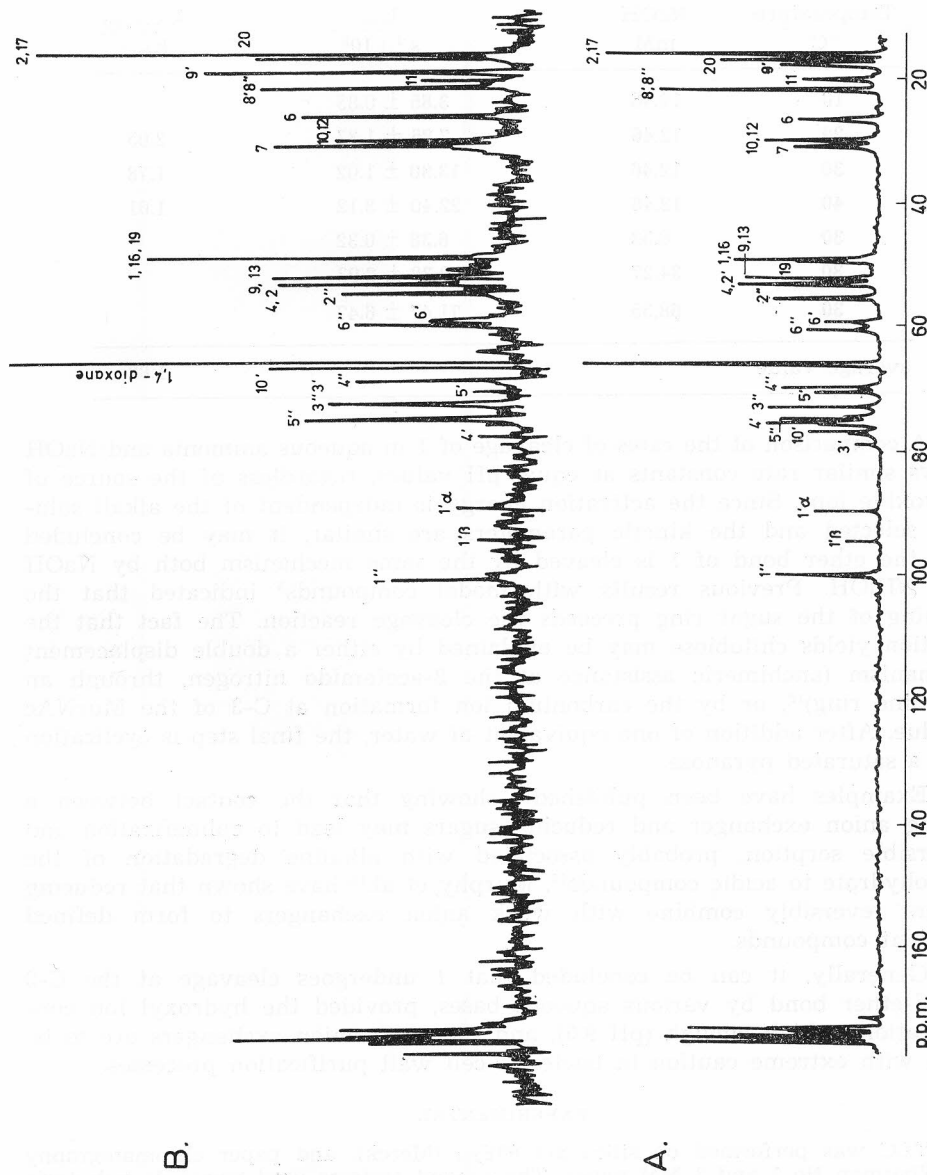


Figure 2. ¹³C-NMR spectrum of: (A) peptidoglycan monomer (I) and (B) the reaction mixture of I and aqueous sodium hydroxide.

TABLE I

Pseudofirst-order Constants for the C-3 Ether Bond Cleavage of 1 with Diluted Aqueous Sodium Hydroxide Solutions

Temperature °C	NaOH mM	k_{obs} $\text{s}^{-1} \cdot 10^5$	$\frac{k_{\text{obs}(T+10)}}{k_{\text{obs}(T)}}$
10	12.46	3.85 ± 0.85	
20	12.46	7.85 ± 1.37	2.05
30	12.46	13.90 ± 1.02	1.78
40	12.46	22.40 ± 3.12	1.61
30	6.53	6.38 ± 0.32	
30	34.27	49.38 ± 2.93	
30	68.55	71.47 ± 6.47	
average value			1.82

A comparison of the rates of cleavage of *1* in aqueous ammonia and NaOH shows similar rate constants at equal pH values, regardless of the source of hydroxide ions. Since the activation energy is independent of the alkali solution selected and the kinetic parameters are similar, it may be concluded that the ether bond of *1* is cleaved by the same mechanism both by NaOH and NH_4OH . Previous results with model compounds⁵ indicated that the opening of the sugar ring precedes the cleavage reaction. The fact that the reaction yields chitobiose may be explained by either a double displacement mechanism (anchimeric assistance at the 2-acetamido nitrogen, through an aziridine ring)¹², or by the carbonium ion formation at C-3 of the MurNAc residue. After addition of one equivalent of water, the final step is cyclization into a saturated pyranose.

Examples have been published¹³ showing that the contact between a strong anion exchanger and reducing sugars may lead to epimerization and reversible sorption, probably associated with alkaline degradation of the carbohydrate to acidic compounds¹⁴. Murphy et al.¹⁵ have shown that reducing sugars reversibly combine with weak anion exchangers to form defined covalent compounds.

Generally, it can be concluded that *1* undergoes cleavage at the C-3 lactyl-ether bond by various aqueous bases, provided the hydroxyl ion concentration is high enough (pH 9.5), and therefore anion exchangers are to be used with extreme caution in bacterial cell wall purification processes.

EXPERIMENTAL

TLC was performed on silica gel 60F₂₅₄ (Merck), and paper chromatography on Whatman No 1 and 3 MM paper. The solvent systems used were: A: isobutyric acid-25% aq. ammonia-water (66:2:23), B, butanol-ethanol-water (4:1:1) and C, butanol-pyridine-water (6:4:3). Chromatograms were sprayed with ninhydrin, alkaline silver nitrate or peptide reagent (1% aq. sol. KI- 1% aq. sol. starch (1:1).

¹H- and ¹³C-NMR spectra were recorded on a JEOL FX 90 Q Fourier-transform spectrometer operating at 89.55 and 22.5 MHz, respectively. The sweep width used for ¹H spectra was 1000 Hz, pulse width 29 μs (90°), acquisition time 2.1 s, and

the digital resolution 0.0027 p.p.m. The sweep width used for ^{13}C spectra was 5200 Hz, pulse width 5 μs (90°), acquisition time 2 s, and the digital resolution 0.056 p.p.m. Chemical shifts were measured relative to internal 1,4-dioxane, set at 3.7 (^1H) and 66.6 p.p.m. (^{13}C) downfield of Me_4Si , respectively.

Peptidoglycan monomer (*I*, 10 mg, 0.01 mmole) was weighed into an NMR tube and dissolved in a D_2O solution of NaOH (0.4 mL; 6.53, 12.46, 34.27 and 68.55 mM) containing 1,4-dioxane (0.1 mL). The recording conditions were previously reported⁵.

The NaOH solutions of *I* were then passed through a column of Sephadex G-10 (65×1.5 cm). Elution with water gave the D-lactoylpentapeptide, whereas the disaccharide was retained on the column and subsequently eluted with 0.1 M LiCl. Isolation of the disaccharide was achieved by preparative paper chromatography (solvent A; elution: EtOH— H_2O , 1:1).

To a solution of *I* (50 mg, 0.05 mmole) in D_2O (0.4 mL) and 1,4-dioxane (0.1 mL) a moist basic anion exchanger (Dowex 2×8 , Fluka AG; Amberlite CG 45, Fluka AG; or DEAE-cellulose, Serva; 100 mg; OH⁻-form) was added. The reaction mixture was kept 1.5 days at room temperature, neutralized with 1 M HCl, and the ^{13}C spectrum was recorded.

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SAŽETAK

Dokaz o nastajanju hitobiozamina iz peptidogliškanskog monomera u blago alkalnim uvjetima

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Djelovanje razrijeđene vodene otopine natrij-hidroksida ili jakog anionskog izmjenjivača na peptidoglikan-monomer (*I*) iz bakterije *Brevibacterium divaricatum* uzrokuje cijepanje C-3 eterske veze u N-acetilmuramoilnom ostatku i daje odgovarajući D-laktolipentapeptid i zasićeni disaharid, hitobiozamin.

Dobiveni rezultati upućuju na to da se cijepanjem *I* uz isti reakcijski mehanizam dobiva zasićeni disaharid, bez obzira na upotrijebljeni protukation, ako je pH-vrijednost reakcijske smjese dovoljno visoka.