Carbohydrate Research, 138 (1985) 65–72
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

TREATMENT OF PEPTIDOGLYCAN MONOMER WITH AQUEOUS AMMONIA: FORMATION OF LACTOYLPEPTIDE AND A SATURATED DISACCHARIDE

BRANIMIR KLAIĆ

Tracer Laboratory, Department of Organic Chemistry and Biochemistry, "Rugjer Bošković" Institute, 41001 Zagreb (Yugoslavia)

(Received July 27th, 1984; accepted for publication, September 6th, 1984)

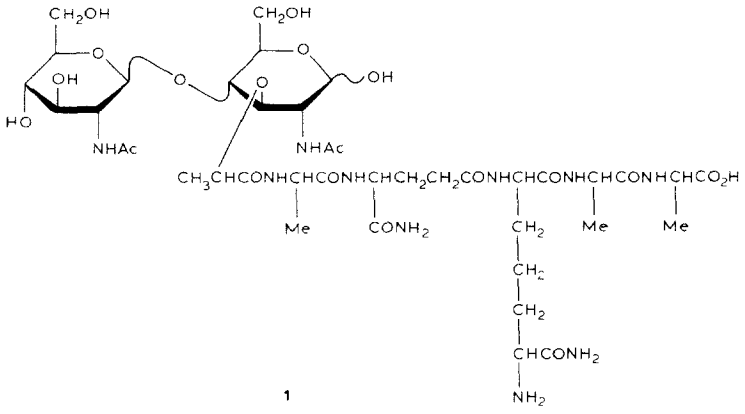
Treatment of peptidoglycan monomer (**1**) from *Brevibacterium divaricatum* with aqueous ammonia led to cleavage of the C-3 ether linkage in the *N*-acetylmuramoyl residue to give the D-lactoylpentapeptide and a saturated disaccharide. By using ^{13}C-n.m.r. spectroscopy, the disaccharide was identified as chitobiosamine. Alkaline treatment of model compounds under similar conditions showed that *N*-acetylmuramoyl derivatives with C-1 unsubstituted undergo cleavage at C-3 to give the corresponding 2-acetamido-2-deoxy-D-glucopyranose derivative. The reaction of **1** with ammonia was monitored by ^{1}H-n.m.r. spectroscopy and, from the data obtained, rate constants and the activation energy were calculated.

INTRODUCTION

In 1960, Perkins reported[1] that, on heating, a triethylamine–water solution (pH 9.5) of the disaccharide-peptide from *Micrococcus lysodeikticus* cell-wall yields a compound which gives a purple colour with the Ehrlich reagent; similar treatment of UDP-*N*-acetylmuramoyl-pentapeptide[2] splits off the lactoylpentapeptide. Ghuysen *et al.*[3] showed that treatment of the peptidoglycan monomer from *Streptococcus faecalis* with alkali results in β-elimination with the formation of lactoylpeptide and an unsaturated disaccharide which gives a purple colour with the Ehrlich reagent. Tipper[4] found that, under mild alkaline conditions, *N*-acetylmuramic acid and its derivatives give a product with a characteristic Morgan–Elson absorption spectrum[5]. Peptidoglycan monomer from *Streptococcus faecalis* was claimed to undergo β-elimination in 0.05M NaOH[6,7] and 4M NH_4OH[6]. After treatment of the peptidoglycan polymer from *Bacillus licheniformis*[8] and the peptidoglycan monomer from *Brevibacterium divaricatum*[9] with ammonium hydroxide, the respective lactoylpentapeptide fragments were detected.

The reaction of the repeating unit of the *Brevibacterium divaricatum* cell-wall in mild alkaline solutions is now reported. The immunoadjuvant[10] repeating-unit was previously fully characterised[9,11,12] as [2-acetamido-4-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-2-deoxy-3-*O*-(D-ethyl-1-carbonyl)-D-glucopyranose]-L-alanyl-

0008-6215/85/$ 03.30



1

D-isoglutaminyl-[(L)-*meso*-diaminopimeloyl-(D)-amide-(L)-D-alanyl-D-alanine] (**1**).

RESULTS AND DISCUSSION

When **1** was treated with aqueous ammonia, the ^{1}H-n.m.r. spectrum of the reaction mixture did not reveal the formation of a glucal structure from the *N*-acetylmuramoyl moiety; the signal at ~6 p.p.m. characteristic[13,14] for H-3 of 2,3-unsaturated 2-amino sugars was absent. However, the intensity of the ^{1}H signal for the *N*-acetyl group of the *N*-acetylmuramoyl residue gradually decreased to zero, whereas that for the *N*-acetyl group of the 2-acetamido-2-deoxy-D-glucopyranosyl residue increased to twice its initial value (Fig. 1); a 2-day reaction sample revealed (^{1}H-n.m.r., t.l.c.) the complete absence of **1**.

The ^{13}C-n.m.r. spectra (Table I) of the reaction mixture were compared with those of **1**[11,12] and the pentapeptide[11,12] {L-alanyl-D-isoglytaminyl-[(L)-*meso*-diaminopimeloyl-(D)-amide-(L)-D-alanyl-D-alanine] (**2**)} and the disaccharide[11] {2-acetamido-4-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-3-*O*-[1-(*S*)-carboxyethyl]-2-deoxy-D-glucopyranose (**3**)} obtained by hydrolysis of **1** with *N*-acetylmuramoyl-L-alanine amidase[15]. In the reaction mixture, the signals of the D-lactyl residue and C-3 and C-4 of the *N*-acetylmuramoyl pyranose ring were changed. The ring signal and that of α-C of the D-lactyl residue were shifted to higher fields (by 9.75 and 9.85 p.p.m., respectively), whereas those of C-4 and β-C and CO of the D-lactyl moiety were shifted downfield (by 3.35, 1.20, and 2.50 p.p.m., respectively). These shifts (Table I) indicate that, in the *N*-acetylmuramoyl moiety, the cleavage of the ether bond between C-3 of the pyranose ring and α-C of the D-lactyl residue had been cleaved.

The Me signals in the ^{1}H-n.m.r. spectrum of the D-lactoylpentapeptide **4** {2-hydroxypropionyl-L-alanyl-D-isoglutaminyl-[(L)-*meso*-diaminopimeloyl-(D)-amide-(L)-D-alanyl-D-alanine]}, isolated from the reaction mixture of **1** by column chromatography on Sephadex G-10, were assigned as follows: *C*-terminal D-alanine, 1.38; peptide-bound D-alanine, 1.32; *N*-terminal L-alanine, 1.30; and D-

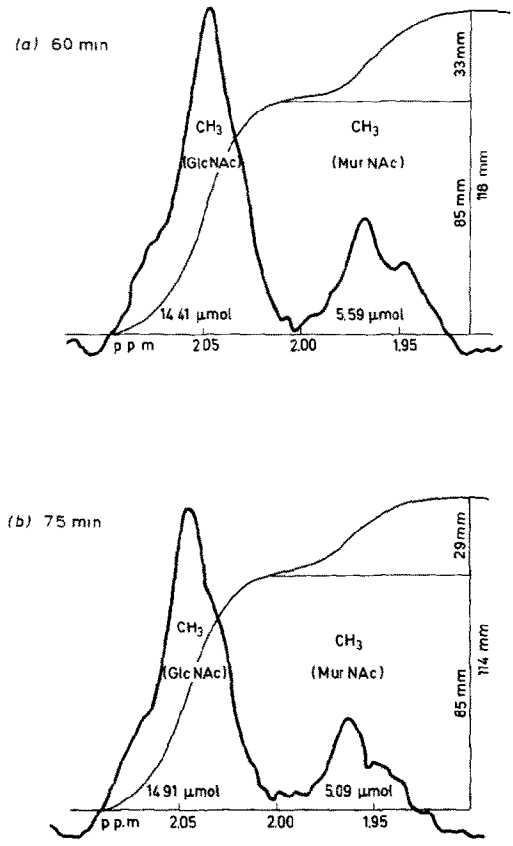


Fig. 1. The cleavage of **1** in NH_4OH–D_2O (1:3): ^{1}H-n.m.r. spectra (303 K) of the methyl signal area of the 2-acetamido groups of the GlcNAc-(1→4)-MurNAc moiety of **1**. Expansion of spectra: 24× (area, 1.9–2.1 p.p.m.).

lactyl residue, 1.32 p.p.m. These values agree with published data[12,16]. In the ^{13}C-n.m.r. spectra, the signal shifts of **4** were identical to those found in the spectra of the reaction mixture of **1** (Table I).

In the ^{1}H-n.m.r. spectra of the disaccharide component (isolated from the reaction mixture of **1** by preparative paper chromatography), only the methyl signal of the 2-acetamido-2-deoxy-D-glucopyranosyl residue could be determined precisely, but the ^{13}C-n.m.r. spectra indicated the compound to be 2-acetamido-4-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-2-deoxy-D-glucopyranose (**5**) and the shifts were in agreement with those published[17]. In the acid hydrolysate of **5**, ~90% of 2-acetamido-2-deoxy-D-glucopyranose was identified by the carbohydrate analyser. Stoffyn–Jeanloz digestion[18] demonstrated the exclusive formation of D-arabinose (paper chromatography in solvents *B* and *C*), which is the degradation product of 2-amino-2-deoxy-D-glucopyranose.

TABLE I

^{13}C-N M R DATA FOR COMPOUNDS **1–5** AND FOR THE PRODUCT MIXTURE FORMED DURING DEGRADATION OF **1** WITH AMMONIUM HYDROXIDE

Assignment	*Compound*					
	1	*Product mixture from* **1**	**2**	**3**	**4**	**5**
*2-Acetamido-2-deoxy-*D*-glucosyl residue*						
C-1	100.4	100 7		100 3		100.7
C-2	56.05	56 15		56.3		55 75
C-3	73.6	73 4		73 6		73.35
C-4	70.4	70.4		70.5		70.4
C-5	75 35	76.0		75.7		76 1
C-6	61.25	60.65		61 4		60 65
CH_3CO	173.5[b]	174.8		174.5		174.7
CH_3CO	22 3	22.2		22 1		22 1
N-*Acetylmuramoyl residue*						
C-1β	95 1	95 25		95.4		94.8
C-1α	90 2	90.2		89 9		89.9
C-2	53.7	53 9		54 7		53 9
C-3	79.55	69 8		78 9		69.8
C-4	76.35	79.7		76.3		79.7
C-5	71.2	71 1		71.3		71.1
C-6	59 9	59 8		59.7		59 8
CH_3CO	174 0	174.8		174 4		174.7
CH_3CO	22 2	22.2		22 1		22.1
CH	77.45	67 6		77.4	67.6	
CH_3	18.4	19 6		18 5	19.7	
CO	174 9[c]	177.4		182.1	177.4	
L-*Alanyl residue*						
CH	49.6	49 55	49 2		49.55	
CH_3	16.8	16.65	16.8		16.65	
CO	174 6[c]	175.1[e]	174 8		175.1[f]	
D-*Isoglutaminyl residue*						
CO	175.2	173 4[d]	173.5		173 35	
CH	53.7	53.9	53.7		53.95	
CH_2	27.15	26.9	27.0		26 9	
CH_2	31.55	31.5	31 3		31.5	
CO	175 7	175.8	175.5		175 75	
D,L-*Diaminopimeloyl residue*						
CO	173.5	173 7[d]	173 5		173.7	
CH	52.75	52 8	52 8		52.85	
CH_2	30 6	30 7	30 4		30.8	
CH_2	20.75	20.95	20 7		20.9	
CH_2	30 7	30 7	30.5		30 8	
CH	52.75	52.8	53 0		52 85	
CO	174 9[c]	175 1[e]	174 8		174 9[f]	
D-*Alanyl residue*						
CH	49 6	49 55	49 2		49 55	
CH_3	16.75	16 65	16 8		16.7	
CO	173 4[b]	173 4[d]	173 5		173 5	
D-*Alanyl residue*						
CH	49.9	49 9	49 5		49 9	
CH_3	17 7	17.6	17.4		17.6	
CO	175 7[c]	175 8[e]	175 5		175.75	

[a]Chemical shifts (δ values) in p.p.m. from tetramethylsilane: δ (Me_4Si) = δ (1,4-dioxane) + 66 6 p p m
[b–f]Assignments may be interchanged.

TABLE II

PSEUDO-FIRST-ORDER RATE CONSTANTS FOR CLEAVAGE OF THE C-3 ETHER BOND OF **1** WITH EXCESS OF NH_4OH

Temperature (degrees)	*[NH_4OH]* (M)	*[NH_4OH]/[D_2O]*	k_{obs} (s^{-1}) × 10^5	$k_{obs}(T+10)/k_{obs}(T)$
10	3.34	1:3	4.1 ±0.3	
20	3.34	1:3	7.5 ±0.8	1.81
30	3.34	1:3	13.9 ±0.3	1.85
40	3.34	1:3	24.2 ±1.1	1.74
30	1.67	1:7	1.6 ±0.3	
30	6.69	1:1	18.9 ±1.5	
Average value				1.80

The C-3 ether cleavage of **1** was further studied at various temperatures and concentrations of ammonia; from the data obtained, the rate constants were calculated (Table II). The reaction rates were measured under pseudo-first-order reaction conditions at 30° with increasing concentrations of ammonia. The dependence of k_{obs} on the concentration of ammonia was linear within the experimental error, with a second-order rate constant of $3.2 \times 10^{-5} mol^{-1}.s^{-1}$.

Temperature intervals of 10° were used to determine the acceleration factor of the chemical reaction (Table II). The value of the factor (1.80) changed very little in the temperature range examined, thus indicating that the reaction mechanism remained the same.

The measurements of the reaction rate constants at various temperatures allowed the use of the Arrhenius equation for determining the activation energy:

$$k_{obs} = A.e^{-E_a/RT},$$

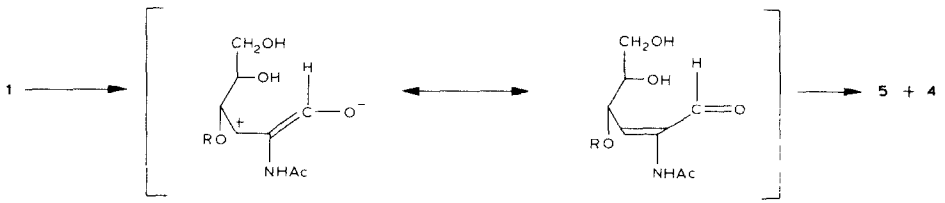
where A is the frequency factor, E_a is the activation energy, R is the universal gas constant, k_{obs} is the reaction rate constant, and T is the absolute temperature. The calculated activation energy of 43.8 kJ/mol can be considered as a relatively small value (Table II).

To obtain some information about the mechanism of the reaction, model compounds were treated under the same conditions as **1**. 2-Acetamido-2-deoxy-3-*O*-[1-(*S*)-carboxyethyl]-D-glucopyranose (*N*-acetylmuramic acid, **6**) underwent almost complete reaction in a few hours; in the ^{13}C-n.m.r. spectra of the mixture, the signals for free lactic acid and 2-acetamido-2-deoxy-D-glucopyranose, together with minimal amounts (5–10%) of its epimers (probably 2-acetamido-2-deoxy-D-allo- and -manno-pyranose), were detectable. As expected, *N*-acetylmuramoyl-L-alanine (**7**) was cleaved to lactoyl-L-alanine and 2-acetamido-2-deoxy-D-glucopyranose. The ^{13}C chemical shifts for *C*-terminal alanine and 2-acetamido-2-deoxy-D-glucopyranose were in agreement with literature data[19–21]. The rate of cleavage of

2-acetamido-2-deoxy-3-*O*-methyl-D-glucopyranose (**8**) was at least five-fold lower than that of **6**, as judged from the ^{13}C-n.m.r. signals of methanol and 2-acetamido-2-deoxy-D-glucopyranose. On the other hand, when *N*-[2-*O*-(benzyl 2-acetamido-2,3-dideoxy-α-D-glucopyranoside-3-yl)-D-lactoyl]-L-alanine methyl ester (**9**) was treated as above, no cleavage of the ether bond could be observed.

The data presented are not sufficient for a mechanistic explanation of the cleavage reaction studied. However, considering the well-known reaction of amino sugars in alkaline media[22,23], some suggestions can be made. The fact that the glycosidic model compound **9** did not undergo the ether cleavage at C-3 indicates that opening of the sugar ring precedes the cleavage reaction.

The high degree of retention of the D-*gluco* configuration in the resulting sugar argues against a simple displacement mechanism, since an inversion of configuration would be expected. One can envisage a scheme including a double-displacement mechanism, *i.e.*, with anchimeric assistance at the amide nitrogen (2-acetamido group)[24,25], through an aziridinium cation if uncatalysed, or an aziridine ring if base-catalysed. However, such an intermediate should be susceptible to nucleophilic attack at C-2 and C-3, giving 2-acetamido-2-deoxy- and 3-acetamido-3-deoxy-D-glucopyranose, respectively, but the formation of the latter compound was not detected. An alternative mechanism involves the formation of a carbonium ion at C-3 of the *N*-acetylmuramoyl residue which, due to its allylic position, should be susceptible to resonance stabilisation (**10**↔**11**). The final step is the return to the keto form of the open sugar and closure to the saturated pyranose ring.



R = 2-acetamido-2-deoxy-D-glucopyranosyl residue

Thus, the results obtained indicate that alkaline treatment of C-1-free *N*-acetylmuramoyl derivatives leads to the cleavage of the C-3 lactyl-ether linkage to give the corresponding 2-acetamido-2-deoxy-D-glucopyranose derivatives.

EXPERIMENTAL

T.l.c. was performed on Kieselgel 60 F_{254} (Merck), and paper chromatography on Whatman No. 1 and 3MM papers. The solvent systems used were: *A*, isobutyric acid–aqueous 25% ammonia–water (66:2:23); *B*, 1-butanol–ethanol–water (4:1:1); and *C*, 1-butanol–pyridine–water (6:4:3). Detection was performed

with ninhydrin, alkaline silver nitrate, and a peptide reagent (1:1 aqueous 1% KI–aqueous 1% soluble starch).

Model compounds were prepared as follows: **7** was obtained by the method of Merser *et al.*[26]; **8**, m.p. 184–186° (from methanol–isopropyl ether), was prepared by debenzylidenation (aqueous 60% AcOH, 95°, 1 h) followed by catalytic hydrogenolysis (10% Pd/C) of benzyl 2-acetamido-4,6-*O*-benzylidene-2-deoxy-3-*O*-methyl-α-D-glucopyranoside[27]; **9**, m.p. 176–177° (from chloroform–light petroleum), was obtained by treatment of *N*-[2-*O*-(benzyl 2-acetamido-4,6-*O*-benzylidene-2,3-dideoxy-α-D-glucopyranoside-3-yl)-D-lactoyl]-L-alanine methyl ester[28] with aqueous 60% AcOH.

N.m.r. spectra were recorded (5-mm o.d. tubes) with a JEOL FX 90 Q Fourier-transform spectrometer operating at 89.55 (^{1}H) and 22.5 (^{13}C) MHz, respectively. The sweep width used for ^{1}H spectra was 1000 Hz, the pulse width was 29 μs, the acquisition time was 2.1 s, and the digital resolution was 0.0027 p.p.m. The sweep width used for ^{13}C spectra was 5200 Hz, the pulse width was 5 μs, the acquisition time was 2 s, and the digital resolution was 0.056 p.p.m. Chemical shifts were measured relative to internal 1,4-dioxane, set at 3.7 (^{1}H) and 66.6 p.p.m. (^{13}C), respectively, downfield of Me_4Si.

The peptidoglycan monomer (**1**; 10 mg, 0.01 mmol) was weighed into an n.m.r. tube and dissolved in NH_4OH–D_2O (0.4 mL; 1:1, 1:3, and 1:7), and 1,4-dioxane (0.01 mL) was added. The desired temperature of the tube (10°, 20°, 30°, or 40°) was achieved in the instrument after a 10-min interval. The ^{1}H spectra were recorded at intervals (5, 10, or 15 min) during 2–3 h. Each set of measurements included 6–12 time-points. Expansion of the range between 1.9 and 2.1 p.p.m. allowed integration of the signals for the 2-acetamido groups of *N*-acetylmuramoyl and 2-acetamido-2-deoxy-D-glucopyranosyl residues. The ratio of the integrals of these groups indicated the degree of conversion of *N*-acetylmuramic acid into 2-acetamido-2-deoxy-D-glucopyranose. The decrease of the *N*-acetylmuramoyl concentration was used to compute the rate constant for the reaction by the least-squares method using non-linear regression. The first computation gave approximate values for the rate constants, and, after removing the data points that deviated strongly from the calculated values, the final values for the rate constants were obtained.

The ammonia-treated samples of **1** were then passed through a column (65 × 1.5 cm) of Sephadex G-10; elution with water gave the D-lactoylpentapeptide[9], whereas the disaccharide was retained on the column and was eluted with 0.1M LiCl. The disaccharide could not be separated from the salt by ultrafiltration through Amicon UM-05 membrane, and was isolated by preparative paper chromatography (solvent *A*) [elution from the paper with ethanol–water (1:1)].

Hydrolysis of a dried sample of disaccharide with 6M HCl (100°, 6 h) gave exclusively 2-acetamido-2-deoxy-D-glucopyranose, identified by using a carbohydrate analyser (Biotronic LC-2000, five-buffer system, detection with copper-*bi*-cinchoninate). D-Arabinose, a degradation product of 2-amino-2-deoxy-D-glucopyranose, was determined according to the Stoffyn–Jeanloz method[18].

ACKNOWLEDGMENTS

The author thanks Drs. D. Keglević, V. Šunjić, B. Benko, and M. Sanković for helpful discussions, Mrs. M. Brozinčević and Mrs. B. Metelko for recording the spectra, Dr. Ž. Jeričević for providing the computer programme for the calculation of rate constants, Dr. B. Jeričević for carbohydrate analysis, and Mrs. R. Naumski (PLIVA, Pharmaceutical and Chemical Works) for providing the peptidoglycan samples.

REFERENCES

1 H. R. PERKINS, *Biochem. J.*, 74 (1960) 182–186.
2 H. R. PERKINS, *Biochem. J.*, 102 (1967) 29C–32C.
3 J.-M. GHUYSEN, E. BRICAS, M. LEYH-BOUILLE, M. LACHE, AND G. D. SHOCKMAN, *Biochemistry*, 6 (1967) 2607–2619.
4 D. J. TIPPER, *Biochemistry*, 7 (1968) 1441–1449.
5 W. T. J. MORGAN AND J. A. ELSON, *Biochem. J.*, 28 (1934) 988–995.
6 R. S. ROSENTHAL AND G. D. SHOCKMAN, *J. Bacteriol.*, 124 (1975) 410–418.
7 P. DEZELÉE AND G. D. SHOCKMAN, *J. Biol. Chem.*, 250 (1975) 6808–6816.
8 J. B. WARD AND H. R. PERKINS, *Biochem. J.*, 135 (1973) 721–728.
9 D. KEGLEVIĆ, B. LADEŠIĆ, J. TOMAŠIĆ, Z. VALINGER, AND R. NAUMSKI, *Biochim. Biophys. Acta*, 585 (1979) 273–281.
10 I. HRŠAK, J. TOMAŠIĆ, K. PAVELIĆ, AND Z. VALINGER, *Z. Immunitaetsforsch.*, 155 (1979) 312–318.
11 B. KLAIĆ, *Carbohydr. Res.*, 110 (1982) 325–330.
12 B. KLAIĆ, B. LJUBIĆ, B. METELKO, AND M. PONGRAČIĆ, *Carbohydr. Res.*, 123 (1983) 168–172.
13 N. PRAVDIĆ, B. ŽIDOVEC, AND H. G. FLETCHER, JR., *Croat. Chem. Acta*, 42 (1970) 523–539.
14 A. HASEGAWA, E. TANAHASHI, AND M. KISO, *Carbohydr. Res.*, 79 (1980) 255–264.
15 Z. VALINGER, B. LADEŠIĆ AND J. TOMAŠIĆ, *Biochim. Biophys. Acta*, 701 (1982) 63–71.
16 A. DELBARRE, D. MIGLIORE-SAMOUR, G. H. WERNER, B. P. ROQUES, AND P. JOLLES, *Eur. J. Biochem.*, 118 (1981) 355–361.
17 P. COLSON AND R. L. KING, *Carbohydr. Res.*, 47 (1976) 1–13.
18 P. J. STOFFYN AND R. W. JEANLOZ, *Arch. Biochem. Biophys.*, 52 (1954) 373–379.
19 T. D. J. HALLS, M. S. RAJU, E. WENKERT, M. ZUBER, P. LEFRANCIER, AND E. LEDERER, *Carbohydr. Res.*, 81 (1980) 173–176.
20 S. J. PERKINS, L. N. JOHNSON, D. C. PHILLIPS, AND R. A. DWEK, *Carbohydr. Res.*, 59 (1977) 19–34.
21 C. GRATHWOHL AND K. WUTHRICH, *J. Magn. Reson.*, 13 (1974) 217–225.
22 S. ROSEMAN AND D. G. COMB, *J. Am. Chem. Soc.*, 80 (1958) 3166–3167.
23 B. COXON AND L. HOUGH, *Chem. Ind. (London)*, (1960) 373–375.
24 S. DAYAGI AND Y. DEGANI, in S. PATAI (Ed.), *The Chemistry of the Carbon–Nitrogen Double Bond*, Interscience, London, 1970, pp. 67–68.
25 H. C. VAN DER PLAS, *Ring Transformation of Heterocycles*, Academic Press, London, 1973, pp. 49–105.
26 C. MERSER, P. SINAY, AND A. ADAM, *Biochem. Biophys. Res. Commun.*, 66 (1975) 1316–1322.
27 R. KUHN, H. H. BAER, AND A. SEELINGER, *Justus Liebigs Ann. Chem.*, 611 (1958) 236–241.
28 M. ZAORAL, J. JEZEK, V. KRCHNAK, AND R. STRAKA, *Collect. Czech. Chem. Commun.*, 45 (1980) 1424–1446.