Carbohydrate Research, 81 (1980) 173–176

Note

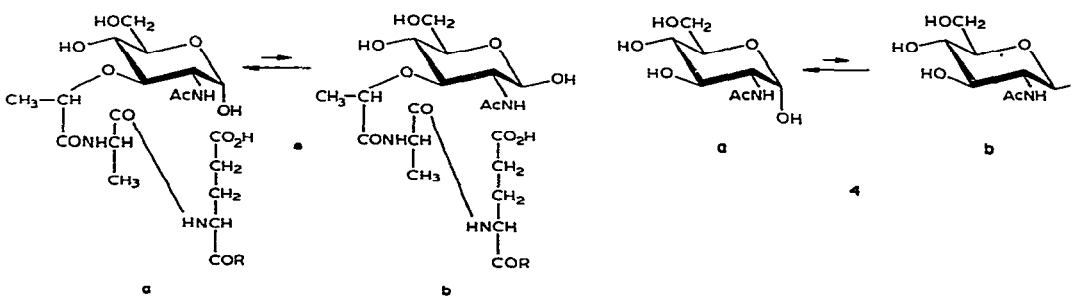
The anomeric configuration of the immunostimulant *N*-acetylmuramoyl-dipeptide and some of its derivatives

TIMOTHY D. J. HALLS, MUPPALA S. RAJU, ERNEST WENKERT,
Department of Chemistry, Rice University, Houston, Texas 77001 (U.S.A.)

MARTIN ZUBER, PIERRE LEFRANCIER,
Institut Choay, 92120 Montrouge (France)

AND EDGAR LEDERER
Laboratoire de Biochimie, C.N.R.S., 91190 Gif-sur-Yvette (France)
(Received April 4th, 1979; accepted for publication in revised form, September 17th, 1979)

The immunoadjuvant *N*-[2-acetamido-3-*O*-(D-ethyl-1-carbonyl)-2-deoxy-D-glucose]-L-alanyl-D-isoglutamine (**1**, *N*-acetylmuramoyl-L-alanyl-D-isoglutamine) first described[1] in 1974, has been the object of many synthetic[2–6] and biological studies (for reviews, see refs. 7–10). Numerous patents on analogs and derivatives of **1** have been filed by French, American, Japanese, and Swiss firms. Methyl α- and β-D-glycosides of **1** have been prepared[4] and a *p*-aminophenyl β-D-glycoside has been synthesized for crosslinking with glutaraldehyde[11]. However, the anomeric equilibrium of synthetic **1** and its derivatives seems not yet to have been defined. We now describe a ^{13}C-n.m.r. study showing a 2.1 : 1 ratio of α- to β-D anomer. The results of this study are in agreement with those obtained by h.p.l.c.



1 R = NH_2
2 R = OH
3 R = OMe

TABLE I

^{13}C-N.M.R. DATA OF COMPOUNDS 1–4[a]

Assignment	*Compound*							
	1a	**1b**	**2a**	**2b**	**3a**	**3b**	**4a**	**4b**
Sugar residue								
C-1	91.0	95.0	91.0	95.0	91.0	95.0	91.0	95.3
C-2	53.8	56.3	53.8	56.3	53.8	56.3	54.6	57.2
C-3	79.7	82.6	79.5	82.4	79.5	82.5	71.1	74.3
C-4	69.1	68.9	69.2	68.9	69.1	68.9	70.5	70.3
C-5	71.6	75.8	71.6	75.7	71.6	75.8	71.8	76.2
C-6	60.7	60.7	60.7	60.9	60.7	60.8	61.0	61.0
*C*H$_3$CO	22.2	22.4	22.2	22.4	22.2	22.4	22.4	22.7
CH$_3$*C*O	174.9	175.6	174.6	175.2	174.7	175.3	174.5	174.8
D-1-Carboxyethyl residue								
C-α	77.7	78.0	77.7	78.0	77.8	78.0		
C-β	18.8	18.8	18.7	18.7	18.7	18.7		
CO	173.8	174.1	173.8	174.1	173.8	174.0		
L-Alanyl residue								
C-α	49.9	49.9	49.7	49.7	49.7	49.7		
C-β	16.8	16.8	17.0	17.0	16.9	16.9		
CO	175.8	175.8	175.5	175.5	175.5	175.5		
D-Glutamine residue								
C-α	52.8	52.8	52.1	52.1	52.2	52.2		
C-β	26.3	26.3	26.0	26.0	26.4	26.4		
C-γ	30.3	30.3	30.1	30.1	31.2	31.2		
C-δ	176.8	176.8	176.8	176.8	177.5	177.5		
CO	174.9	174.9	174.6	174.6	173.1	173.1		

[a]Values δ from tetramethylsilane signal; $\delta(Me_4Si) = \delta$(1,4-dioxane) + 66.6.

The ^{13}C-n.m.r. spectra* of *N*-acetylmuramoyl-L-alanyl-D-glutamic acid (**2**), methyl *N*-acetylmuramoyl-L-alanyl-D-glutamate (**3**), **1**, and 2-acetamido-2-deoxy-D-glucose (**4**) were recorded and the carbon shifts assigned, as listed in Table I, by comparison of the signals of the *N*-acetylmuramic acid derivatives **1**–**3** with those of **4**, and the use of literature data for monosaccharides[12] and peptides[13–15]. The spectral behavior of the *N*-acetylmuramoyl residue indicated that replacement of OH-2 of the D-glucose residue by an acetamido group results in shielding of C-2, and etherification of OH-3 leads to deshielding of C-3. Owing to the presence of two anomers in aqueous solution, the *N*-acetylmuramoyl residue showed signal twinning. Comparison of the intensities of the nonoverlapping signals of the identical carbon atoms of each anomer with each other after the establishment of anomer equilibrium, indicated an α- to β-D ratio of 2.1:1 for **1**–**3**, and 1.6:1 for **4**. Finally, differentiation of the carbonyl carbon atoms was based on the unique, low-field position of the terminal

*These ^{13}C-n.m.r. analyses constitute Part LXVII of Carbon-13 Nuclear Magnetic Resonance Spectroscopy of Naturally Occurring Substances. For Part LXVI, see ref. 16.

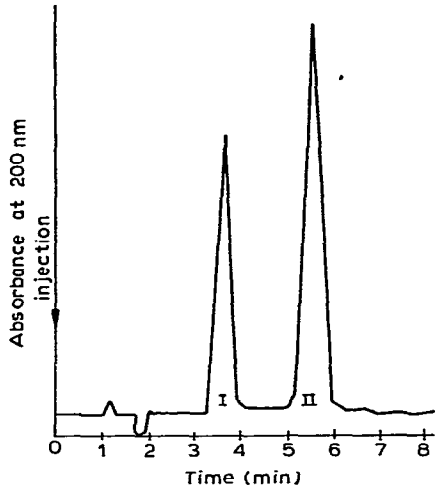


Fig. 1. H.p.l.c. separation of the anomers of **1**: peak I, α anomer; peak II, β anomer.

TABLE II

RATIO OF ANOMERS OF **1** AS DETERMINED BY H.P.L.C.[a]

Time after dissolution (min)	*Ratio of α to β anomer*[b]
3	2.72
15	2.60
30	2.42
60	2.20
120	2.01
180	1.95

[a]See Fig. 1; peak I, α anomer; peak II, β anomer. [b]Ratio of the areas of peaks.

carboxyl group, the *N*-acetyl carbonyl shift of **4**, and the recorded alanine carbonyl shift[15,16].

The high-performance liquid chromatography of **1** on a Spherisorb ODS (5 μm) column with the use of 199:1 (v/v) 5mM ammonium acetate (pH 2.5)–acetonitrile as eluent allowed the separation of the α- and β-D anomers into two peaks showing retention times of 3.5 and 5.5 min (Fig. 1). Because of the rapidity of this analytical process, it was possible to study with fairly good accuracy the ratio of the two anomers and its variation with time as the result of the mutarotation, *i.e.* the optical rotation (*c* 0.5, water) decreasing from $[\alpha]_D^{20}$ +37 to +34° after 4 h.

As seen in Table II, the ratio of α- to β-D anomer decreased slowly, reaching 2:1.

EXPERIMENTAL

^{13}C-N.m.r. spectra. — These spectra were recorded at ambient temperature on solutions of each substance (100 mg) and 1,4-dioxane (0.03 mL) in water (0.4 mL), in 5-mm (o.d.) tubes with a Varian XL-100-15 spectrometer operating at 25.2 MHz in the Fourier-transform mode.

High-performance liquid chromatography. — This was performed with a Spectraphysics 3.500 instrument that included a rotary-valve injector, equipped with a standard, sample-loop (50 μL) injection, and a variable-wavelength u.v. monitor (Model 770). The latter was set at 200 nm and coupled with a two-channel chart recorder (W + W Model 600 Kontron AC, Basel, Switzerland), and with a computing integrator (LTT Model ICAP 10, France). The Spherisorb ODS (5 μm) column (25 × 3 cm) was obtained from Spectra-Physics (Santa Clara, CA 95051, U.S.A.). The solvents were doubly-distilled, de-ionized water and acetonitrile (chromatography grade, Merck, Darmstadt, FRG). The solvents were mixed by volume, filtered through a 0.45-μm filter (type A, Millipore Corp., Bedford, MA 01730, U.S.A.), and allowed to equilibrate at room temperature. No degassing operation was performed, all chromatographic runs were achieved isocratically, and the flow rate was 1 mL/min with a pressure of 170 atm.

An aqueous solution containing 100 μg of **1**/mL was prepared and a 50-μL aliquot was introduced immediately into the column. Identical injections were repeated at various time-intervals for 3 h, and at each time the areas of the two peaks were measured and their ratio calculated.

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