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ON THE SIDE-CHAIN CONFORMATION OF *N*-ACETYLNEURAMINIC ACID AND ITS EPIMERS AT C-7, C-8, AND C-7,8*

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ABSTRACT

The side-chain conformation of *N*-acetylneuraminic acid and analogs has been studied by n.m.r. spectroscopy. The results of the ^{1}H-, ^{13}C-n.m.r.-, and ^{1}H-nuclear-Overhauser-enhancement measurements were used to distinguish between different local-minima conformations suggested by hard-sphere calculations. Attempts were made to correlate the major conformation determined for each compound with the behavior towards activation with *N*-acetylneuraminic acid-CMP-synthetase.

INTRODUCTION

N-Acetylneuraminic acid and numerous of its acyl phosphate, and sulfate derivatives (the sialic acids) are terminal units for various oligosaccharide sequences of many glycoproteins and glycolipids. These compounds may be correlated with a great number of biochemical and biological functions[1,2]. For this reason, many structural transformations of *N*-acetylneuraminic acid (NeuAc) (**1**) have been performed and the corresponding changes of the biochemical features examined[3–5]. In this connection, the inhibitory property of 5-acetamido-2,6-anhydro-3,5-dideoxy-D-*glycero*-D-*galacto*-non-2-enonic acid[6,7], or its C-4 epimer[6], towards sialidases should be mentioned. The shortening of the side chain by one C atom causes a rather small decrease of the activity of the FSH-stimulating hormone[8]. It is worth noting that the replacement of OH-9 of **1** by such various substituents as F, N_3, J. NH_2, and NHAc does not cancel the activation by the CMP-sialate synthetase[9]. On the other hand, a change in configuration at C-4 prevents[9] the activation. Not even the enzymic formation of the 9-azido derivative from pyruvic acid and 2-acetamido-6-azido-2-deoxy-D-mannose was affected[10]. Re-

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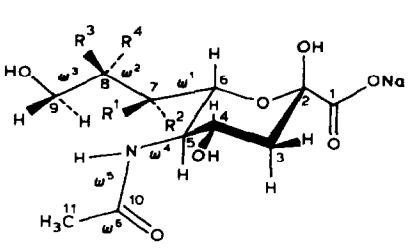
cently, Zbiral and Brandstetter have reported[11] the synthesis of the stereochemically related, side-chain epimers of **1** at C-7 (**2**), C-8 (**3**), and C-7,8 (**4**). A comparison of the enzymic activations showed[11] the relative values of 1 (for **1**), 0.35 (for **2**), 0.5 (for **4**), and no measurable value for **3**. The rather unexpected result, namely that no activation was observed for **3**, made us examine the conformations of the side chains of **2**, **3**, and **4**. We present herein a detailed spectroscopic analysis which, in combination with hard-sphere calculations based on coordinates provided[12] by X-ray analysis of **1**, offers an insight into the conformations of **1** and its epimers in aqueous solution at neutral pH. As will be shown, the comparison of the four side-chain conformations explains the missing activation of **3**.

RESULTS AND DISCUSSION

N-Acetylneuraminic acid (**1**) and its side-chain epimers 5-acetamido-3,5-dideoxy-L-*glycero*-D-*galacto*-2-nonulosonic acid (**2**), 5-acetamido-3,5-dideoxy-D-*glycero*-L-*altro*-2-nonulosonic acid (**3**), and 5-acetamido-3,5-dideoxy-L-*glycero*-L-*altro*-2-nonulosonic acid[11] (**4**) were transformed into the sodium salts and purified by gel chromatography. The conformation of the side chain is determined by the angles ω^1 to ω^3 and, furthermore, by the angles ω^4 to ω^6 of the acetamido group. The proportions of α anomer in **2**–**4** are less then 10% and similar to the values observed[13] for **1**.

Because of the stereochemical correlations between the side chains of **1** and **2**–**4** and the corresponding hexitols, the ^{13}C-n.m.r. spectra of the latter compounds were recorded too. The assignments of the proton resonances of **1** were taken from Brown *et al.*[14]. The first order interpretation of the ^{1}H-n.m.r.-spectra of **1**, **2**, and **3**



ω^1 H-6-C-6-C-7-H-7
ω^2 H-7-C-7-C-8-H-8
ω^3 H-8-C-8-C-9-O-9
ω^4 H-5-C-5-N-5-H-N
ω^5 H-N-N-5-C-10-O-10
ω^6 O-10-C-10-C-11-H-11

	R^1	R^2	R^3	R^4
1	H	OH	OH	H
2	OH	H	OH	H
3	H	OH	H	OH
4	OH	H	H	OH

Scheme 1. Numbering of atoms, and definition and symbols of angles.

was possible without ambiguity. The values thus obtained were used to refine the parameters in an iterative way by use of a software delivered with the spectrometer. On the other hand, **4** gave even at 500 MHz no spectrum of first order. Although an interpretation was possible, the correct simulation of the parameters failed owing to the small separation of the H-4, -5, and -6 signals within 0.1 p.p.m. (50 Hz), each of them having large coupling constants, and the small separation of the H-7, -8, and -9a signals within 0.1 p.p.m. Attempts to measure the proton–proton shift correlated and the *J*-resolved spectra gave no further improvement. The ^{1}H-n.m.r.-spectra of the alditols were used as published by Hawkes and Lewis[15] (see Tables I, II, and III).

The assignments of the carbon resonances of **1** were obtained as given by Jacques *et al.*[16]. For comparison of the amide carbonyl groups, in the acid and salt forms the two carbonyl group resonances were exchanged. The assignment of C-7 was established by a heteronuclear-decoupling experiment. The assignments for **2** were made in an unambiguous way by means of a carbon–proton, shift-correlated spectrum. C-7 of **3** was assigned by a heteronuclear-decoupling experiment, and C-6 of **4** by means of a deuterium-induced shift[17]. The remaining signals (C-6 and C-8 of **1** and **3**, and C-7 and C-8 of **4**) were assigned empirically to give similar shift-differences between **1, 3, 4**, and the corresponding alditols. The assignments for D-glucitol were as published by Kieboom *et al.*[18]. The assignments for other alditols were used as published by Angyal *et al.*[19]. Because of the small shift-differences for C-4 and C-5 of allitol and D-altritol, and because the temperature at which the spectra were recorded[19] was unknown, the assignment was established for room temperature with the aid of the (1-^{2}H)hexitols.

TABLE I

^{1}H-CHEMICAL SHIFTS (δ)[a] OF COMPOUNDS **1–4** AND RELATED ALDITOLS[b]

Hydrogen atoms[c]	*Compounds*							
	1	D-*Mannitol*	**2**	D-*Altritol*[d]	**3**	D-*Glucitol*[d]	**4**[e]	*Allitol*
3a	1.83		1.87		1.83		1.87	
3e (1a)	2.22		2.23	3.80	2.21	3.83	2.22	
4 (1b)	4.03		3.97	3.67	4.00	3.65	3.99	
5 (2)	3.92		3.80	3.92	3.92	3.77	3.93	
6 (3)	3.99		3.96	3.79	3.82	3.65	4.02	
7 (4)	3.52	3.79	3.70	3.66	3.64	3.85	3.77	3.81
8 (5)	3.76	3.75	4.04	3.94	3.83	3.84	3.75	3.93
9a (6a)	3.85	3.86	3.61	3.66	3.69	3.73	3.82	3.82
9b (6b)	3.62	3.67	3.61	3.66	3.55	3.62	3.60	3.68
Ac	2.06		2.03		2.06		2.06	

[a]Downfield from the signal of Me_4Si at 297° K, and are iterated values (see Experimental section). [b]From ref. 15. [c]Numbering of hexitol atoms is shown in brackets. [d]Numbering of D-altritol and D-glucitol is reversed (from 6a to 1b, corresponding to L-talitol and L-gulitol). [e]The shift value of H-4 could not be precisely determined, but is the most likely one from the simulated spectrum.

TABLE II

H,H-COUPLINGS (Hz)[a] OF COMPOUNDS **1–4** AND RELATED ALDITOLS[b]

H atom/H atom[c]		*Compounds*							
		1	D-*Mannitol*	**2**	D-*Altritol*[d]	**3**	D-*Glucitol*[d]	**4**	*Allitol*
3a/3e		−12.9		−13.0		−12.9		−13	
3a/4	(1a/1b)	11.4		11.5	−12.0	10.9	−11.8	12	
3e/4	(1a/2)	4.9		4.5	3.2	4.5	3.0	4	
4/5	(1b/2)	10.3		10.5	7.4	10.2	6.3	8	
5/6	(2/3)	10.3		10.0	5.0	8.3	8.3	10.0	
6/7	(3/4)	1.0	0.0	3.5	8.3	0.5	1.7	1.9	6.5
7/8	(4/5)	8.9	9.0	2.5	1.9	7.2	6.0	7.7	5.8
8/9a	(5/6a)	2.7	3.0	5.9	4.7	3.5	3.6	2.7	3.1
8/9b	(5/6b)	6.5	6.3	6.0	8.0	6.2	6.6	5.4	7.4
9a/9b	(6a/6b)	−11.9	−11.8	−11.9	−11.8	−11.9	−12.0	−11.8	−11.9

[a]Iterated values (see Experimental section). [b]From ref. 15. [c]Numbering of hexitol atoms in brackets. [d]Numbering of D-altritol and D-glucitol atoms is reversed (from 6a to 1b, corresponding to L-talitol and L-gulitol).

TABLE III

^{13}C-CHEMICAL SHIFTS (δ)[a] OF COMPOUNDS **1–4** AND RELATED ALDITOLS

Carbon atoms[b]	*Compounds*							
	1	D-*Mannitol*	**2**	D-*Altritol*[c]	**3**	D-*Glucitol*[c]	**4**	*Allitol*
1	177.55		176.97		177.12		177.08	
2	97.33		96.92		97.19		96.99	
3	40.33		40.09		40.24		40.09	
4 (1)	68.21	64.04	68.12	62.84	67.92	63.62	68.49	63.14
5 (2)	53.24	71.70	54.45	73.37	53.44	71.82	54.05	72.93
6 (3)	71.19[d]	70.13	74.99	72.30	72.49[d]	71.93	73.79	73.09
7 (4)	69.50	70.13	70.69	71.51	69.99	70.46	72.31[d]	73.09
8 (5)	71.31[d]	71.70	71.22	71.23	73.71[d]	73.70	72.43[d]	72.93
9 (6)	64.24	64.04	63.81	63.77	62.84	63.25	63.99	63.14
10	175.71		175.28		175.69		175.38	
11	23.10		23.00		22.93		23.05	

[a]Downfield from the signal of Me_4Si (set at δ 67.4 upfield from the signal of 1,4-dioxane in D_2O at 297° K). [b]Numbering of hexitol atoms in brackets. [c]Numbering of altritol and glucitol atoms is reversed (from 6 to 1, corresponding to L-talitol and L-gulitol). [d]Assignments may be interchanged.

Hardsphere calculations were done as described in the Experimental section. The inclusion of the *gauche* effect[20] gave slightly different potential-energy minima, and changed the location of the minima. The inclusion of electrostatic terms gave no change in the location of the minima, and was therefore omitted, and the inclusion of a term for hydrogen bridges was rejected because each energy contribution of a hydrogen bridge was believed to be easily replaced by a similar energy contribution of the solvent water. The remaining differences between calculated

and observed minima, especially concerning the epimers at C-7 (**2** and **4**) could be explained by the energy contributions of the unfavored 1,3-axial–axial repulsions[18] of >4 kJ. These 1,3-axial–axial repulsions, namely that of carbon–nitrogen parallel to carbon–oxygen, are not properly described by the van der Waals term of the calculation, nor could they be reproduced by the inclusion of an electrostatic term. Instead of adding a single function describing only the additional nitrogen–oxygen repulsion due to 1,3-diaxial effects, the calculations were used only to find the possible locations of the minima and not to reproduce the exact energy relations between global and local minima.

As illustrated for the determination of the side-chain conformation of **1** (Fig. 1), the global minima and all local minima <16 kJ/mol above the global minima were searched for each compound by means of the ω^1/ω^2 contour diagram and compared with the spectroscopic data. As a first approximation, comparison of the minima with the three-bond, proton–proton-coupling contour diagram was chosen. To avoid the exclusion of minima on the basis of couplings that could result from mixed conformers (this is indeed the case for the relatively free rotating ω three angle[21]), additional parameters were measured as far as possible.

To prove the minima of **1**, the n.O.e. (nuclear Overhauser enhancement) saturating H-7 was measured as an additional parameter. The relative value of the enhancement of H-6 *vs.* the enhancement of H-8 was selected to distinguish between the minima. On saturation of H-7, a ratio of 2.8 for conformer **1a**, of 1.0 for conformer **1b**, and of 0.8 for **1c** was calculated (see Table IV for the corresponding angles). The measured ratio of 2.6 fits the calculated value very well. In addition, calculation for the saturation of H-5 gave n.O.e. values for H-3 and H-7 as reported by Sabesan *et al.*[22].

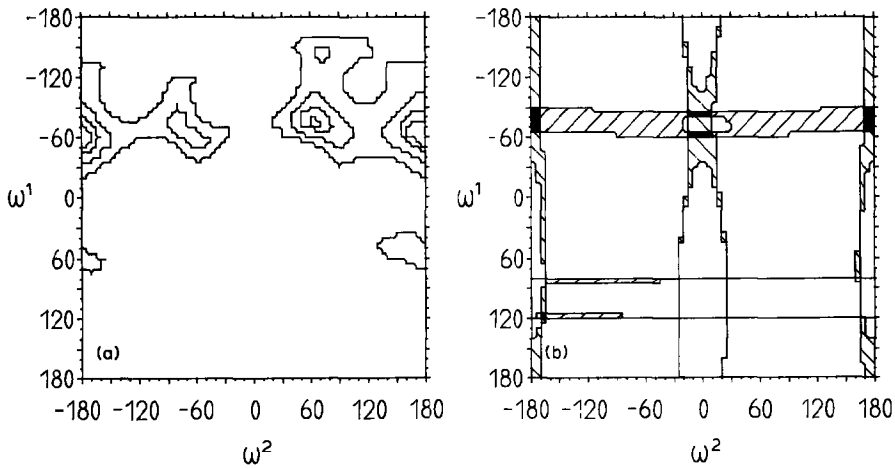


Fig. 1. (a) Isocontour diagram of the energy surface of compound **1**, determined at 4, 8, 16, and 32 kJ/mol above the minimum energy. (b) Isocontour diagram of the calculated H,H couplings for compound **1**, determined at 1.0 ±0.5 Hz for the angle ω^1 and at 8.9 ±0.5 Hz for the angle ω^2. Overlapping parts are shown as black filled areas.

TABLE IV

RESULTS OF CALCULATIONS AND n.O.e. VALUES FOR COMPOUNDS **1–4**

Conformer	*Angle* ω^1/ω^2 [a]	ΔE[b]	$^3J_{H,H}$ *coupling*				*n.O.e. Values*[c]		
			Calc.		*Obs.*		*Calc.*		*Obs.*
			H-6, H-7	*H-7, H-8*	*H-6, H-7*	*H-7, H-8*			
1 a	−70/180	0	1.1	9.0			H-7→H-6	2.8	
b	−70/60	3.3	1.0	3.2	1.0	8.9	H-7→H-8	1.0	2.6
c	−60/−60	9.2	1.4	3.0				0.8	
2 a	−170/−70	0	8.7	1.0	3.5	2.5	H-5→H-8	0.1	3.4
b	−70/−50	2.5	1.9	2.8			H-5→H-3	4.4	
3 a	−80/−170	0	0.9	7.9					
b	−60/70	3.3	1.4	0.7	0.5	7.2			
c	−80/−60	14.2	0.8	3.1					
4 a	−180/−60	0	9.6	4.8					
b	−70/−160	4.2	2.0	7.6	1.9	7.7			
c	−180/80	13.8	9.1	1.2					

[a]Only the first minimum of the ω^1/ω^2 group distributed in ω^3 is given. For distribution of ω^3, see text. Angles and ω^5 are near to 180° for all minima less than 20 kJ above the global minimum. [b]Energy is given in kJ/mol above the global minimum. [c]Given as ratio with one hydrogen atom as internal standard.

The minima of **2** were proven by n.O.e. saturating H-5, the ratio n.O.e.-H-8 to n.O.e.-H-3 being selected as a scale. On saturation of H-5, values of 0.1 for conformer **2a**, and of 4.4 for conformer **2b** were calculated. The observed value of 3.4 closely corresponds to that for conformer **2b**. The observation that the calculated value for **2a** was the global minimum may be explained by an unfavored 1,3-diaxial interaction of N-5–O-7, which is not properly described by the calculation. In addition, a n.O.e. for H-7 was calculated for conformer **2a**, but not observed. Because, on saturation of H-5, the calculated n.O.e. ratio H-7 to H-3 was 2.4, it was concluded that the proportion of **2a** should be <10%, *i.e.*, unmeasurable.

The measurement of an n.O.e. for **3** failed because the signals were not sufficiently resolved, but comparison of the calculated and the observed couplings seems to be sufficient to prove the validity of the calculation. As an additional parameter, the fact that H-6 of **3** is strongly shielded (Table I) and both O-7 and O-8 of conformer **3a** are distant from H-6 may be helpful.

A measurement of additional parameters for **4** was not possible. From the proton–proton coupling constants, it seems that conformer **4b** was the major one. As for **2a, 4a** could be eliminated owing to a 1,3-axial–axial, N-5–O-7 interaction (the C–N—C–O is −8° in **4a** and −91° in **4b**).

For compounds **1–4**, the couplings H-8 to H-9 were average values. As measured by Czarniecki and Thornton[21], the rotation about the C-8–C-9 axis is not very restricted. The ω^3 angle is ~+65°, −65° distributed (O–O-angle) for **1, 3, 4**, and ~+65, −65, 180° for **2**, to yield the corresponding couplings. The ω^3-angle

distribution for **2** is not only evident from the magnitude of the coupling constants, but H-8 is deshielded, because O-9 is to a larger extent (to 2/3) nearer to H-8 than it is in **1**, **3**, and **4** (to 1/2). This ω^3-angle distribution is very similar to that observed by Hawkes and Lewis[15] for the hydroxymethyl group of the corresponding alditols as deducted from the proton–proton couplings (Table II).

Application of the rules of Jeffrey and Kim[23] to the side-chain carbon atoms of **1**–**4** (Table V) gave values in good agreement with the calculations. In all major conformers, the chain is bent where it is predicted. Only in **2**, the predicted additional rotation was impossible for steric reasons, thus resulting in a 1,3-axial–axial, O-6–O-8 interaction. The observation that the rotation away from the N-5–O-7 interaction occurred led us to believe that the N-5–O-7 interaction is less favored than the O-6–O-8 interaction by at least 4 kJ.

Comparison of ^{13}C shifts of the side-chain carbon atoms with those of the alditols showed that if all rotations predicted to avoid 1,3-axial–axial interactions were made, the ^{13}C-shift differences are very similar to the values expected for replacement of an oxygen by a nitrogen atom, independently whether the rotation occurs at a carbon atom analogous to that of the alditol or not (compound **4** and allitol). Thus, the ^{13}C-chemical shifts were best suited to prove that **4** is free of

TABLE V

COMPARISONS OF PREDICTED WITH OBSERVED ROTATIONS[a], AND CORRELATIONS WITH ^{13}C-CHEMICAL SHIFTS

Compounds	*Rotations*			
	Predicted[b]		*Observed*	
1	(LL)DD Extended ↓ ↓		(LL)DD Extended ↓	
2	(LL)LD Bent ↓		(LL)LD Bent ↓	
3	(LL)DL Bent ↓		(LL)DL Bent ↓	
4	(LL)LL Bent		(LL)LL Bent	
	$\Delta\delta\ ^{13}C$[c]			
	C-5–C-2	*C-6–C-3*	*C-7–C-4*	*C-8–C-5*
1 – D-Mannitol	−18.46	1.06	−0.63	−0.39
2 – D-Talitol	−18.92	2.69	−0.82	−0.01
3 – D-Gulitol	−18.38	0.56	−0.47	0.01
4 – Allitol	−18.88	0.70	−0.78	−0.50

[a]According to Jeffrey and Kim[23]. [b]The configurations of C-5–C-8 are given, the brackets indicate that these carbon atoms are part of a ring-system, and the arrows indicate where rotations are predicted or observed. [c]C-5–C-2 means shift of C-5 of **1** to **4**, minus shift of C-2 of the respective alditol.

Although refining the angles to 10° only is a crude approach, the most probable conformation of each side chain could be determined, in conjunction with spectroscopic experiments, to a sufficient precision to explain the biochemical results. As the replacement of O-9 does not inhibit[9] the activation with CMP-NeuAc, it is necessary to search for similar parts of the molecules of **2b** and **4b**, for a lack of similarity of **3a** with a fragment of **1a** in which C-9 has been removed; this would explain the activation of **2** and **4** and the lack of activation of **3** by CMP-NeuAc-synthetase[11]. The absence of activation of **3** may now be considered as a consequence of diminished binding interactions between the hydrophobic and hydrophilic regions of **3** and the activating enzyme, as compared to the other compounds **1, 2,** and **4**. In this connection, similar deductions were made by Lemieux *et al.*[24] for the molecular recognition of the Lewis[6] and Y blood-group determinants by the lectin of *Griffonia simplicifolia*. By observing the molecules in the direction O-2 → C-2 (Fig. 2), the possible-binding side was found to consist of O-2 and one oxygen atom near O-6. Such a model having a polar side located near a rather hydrophobic area would also explain the observation that the epimer at C-4 of **1** having an additional polar group near O-2 is not activated[9].

EXPERIMENTAL

General. — Compounds **2–4** were prepared as described[14]. Compound **1**, D-mannitol, D-glucitol, D-allose, D-altrose, and D-talose were commercial samples. The sodium salts of compounds **1–4** were prepared by neutralization of the corresponding acids with a sodium hydroxide solution in slight excess. The compounds were freed from impurities and the excess of base by passage through a Bio-Gel P-2 (mesh size −400) column. Allitol, D-altritol, and the respective (1-^{2}H)hexitols of allitol, D-altritol, and D-talitol were prepared by sodium boro-hydride or -deuteride reduction of the hexoses at 4° during 2 h. The alditols were freed from salts by gel-permeation chromatography on Bio-Gel P-2.

N.m.r. spectroscopy. — ^{1}H-N.m.r. spectra were recorded with a Bruker WM-250 and a Bruker AM-500 (for compound **4**) instrument at 297°K, with a deuterium lock on the water signal, and an external reference of sodium 4,4-dimethyl-4-silapentanoate in D_2O (δ 0); the spectral width was 2 KHz, and 16 k of memory were used. The spectra were simulated by use of the Bruker Panic program, in two parts for the seven-spin systems of hydrogens 3–8 and hydrogen 4–9.

^{13}C-N.m.r. spectra (62.9 MHz) were recorded with a Bruker WM-250 instrument, equipped with a 5-mm probe head. All spectra were recorded for solutions in D_2O at 297°K, with an external reference of tetramethylsilane (δ 67.40 upfield from the signal of 1,4-dioxane in D_2O),the spectral width was 12 kHz and 32 k of memory were used.

^{1}H-n.O.e. experiments were performed in the difference mode for solutions in D_2O at 297° K.

^{1}H–^{13}C-shift-correlated spectra were recorded with the Bruker program.

Computations. — All calculations were done with an NCR-Decision-mate V microcomputer, equipped with a 0.5 megabyte random-access memory and a 8087/3 numerical co-processor: preparation of starting coordinates for hardsphere calculations, calculation of couplings, and nuclear Overhauser enhancements. As starting coordinates for **1**, the X-ray structure determined by Flippen[12] was used, and the hydrogen atoms were located at a distance of 1.1 Å. The hydrogen atoms connected to oxygen atoms were not included. For the preparation of the coordinates of **3**, the side chain (C-8, C-9, and connected atoms) of **1** was inverted, of **4**, the side chain (C-7, C-8, C-9, and connected atoms) of **1** was inverted, and of **2**, the side chain (C-8, C-9, and connected atoms) of **4** was inverted. Bond angles and distances were not modified by this procedure and were kept invariant during all calculations. The potential energy was computed by taking into account the van der Waals and torsional contributions. The van der Waals contributions between nonbonded atoms were calculated by use of the Kitaigorodsky expression[25]. A three-fold torsional potential of 11 kJ/mol was used for rotations ω^1, ω^2, and ω^3; 4 kJ/mol for rotations ω^4 and ω^5; and 2 kJ/mol for the rotation[26] ω^6 (for the naming of the angles, see Scheme 1). For the angles ω^1, ω^2, and ω^3, an additional two-fold torsional potential of 4 kJ/mol was used to correct for the *gauche* effect[20]. Although the angles ω^1 and ω^2 are referred as hydrogen–hydrogen angles, the calculation was done by use of oxygen–oxygen angles. The angles ω^1 and ω^2 were varied in steps of 10°, and for each combination the global energy minimum ω^3–ω^6 was searched. N.O.e. values were calculated, without correction, with hydrogen relaxation rates and $^3J_{H,H}$ couplings were calculated by use of the Karplus equation with the electronegativity correction proposed by Haasnoot *et al.*[27].

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Computations. — All calculations were done with an NCR-Decision-mate V microcomputer, equipped with a 0.5 megabyte random-access memory and a 8087/3 numerical co-processor: preparation of starting coordinates for hardsphere calculations, calculation of couplings, and nuclear Overhauser enhancements. As starting coordinates for **1**, the X-ray structure determined by Flippen[12] was used, and the hydrogen atoms were located at a distance of 1.1 Å. The hydrogen atoms connected to oxygen atoms were not included. For the preparation of the coordinates of **3**, the side chain (C-8, C-9, and connected atoms) of **1** was inverted, of **4**, the side chain (C-7, C-8, C-9, and connected atoms) of **1** was inverted, and of **2**, the side chain (C-8, C-9, and connected atoms) of **4** was inverted. Bond angles and distances were not modified by this procedure and were kept invariant during all calculations. The potential energy was computed by taking into account the van der Waals and torsional contributions. The van der Waals contributions between nonbonded atoms were calculated by use of the Kitaigorodsky expression[25]. A three-fold torsional potential of 11 kJ/mol was used for rotations ω^1, ω^2, and ω^3; 4 kJ/mol for rotations ω^4 and ω^5; and 2 kJ/mol for the rotation[26] ω^6 (for the naming of the angles, see Scheme 1). For the angles ω^1, ω^2, and ω^3, an additional two-fold torsional potential of 4 kJ/mol was used to correct for the *gauche* effect[20]. Although the angles ω^1 and ω^2 are referred as hydrogen–hydrogen angles, the calculation was done by use of oxygen–oxygen angles. The angles ω^1 and ω^2 were varied in steps of 10°, and for each combination the global energy minimum ω^3–ω^6 was searched. N.O.e. values were calculated, without correction, with hydrogen relaxation rates and $^3J_{H,H}$ couplings were calculated by use of the Karplus equation with the electronegativity correction proposed by Haasnoot *et al.*[27].

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