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Carbon-13 Nuclear Magnetic Resonance Spectroscopy of Naturally Occurring Substances. XXXIX. Apramycin—an Application of Amine Protonation Parameters[1]

Ernest Wenkert*[2] and Edward W. Hagaman

Department of Chemistry, Indiana University, Bloomington, Indiana 47401

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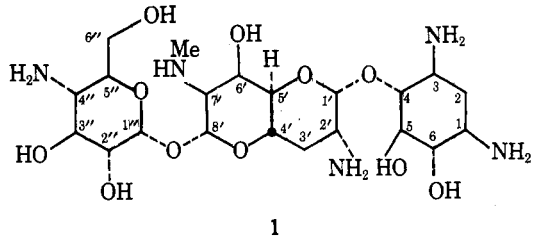
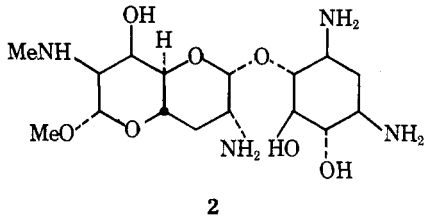
As part of a study of the structures of the nebramycin factors produced by *Streptomyces tenebrarius*[3] the ^{13}C NMR analysis of the kanamycin-like antibiotics has been investigated.[4] Alongside a structure determination of apramycin (**1**), the most complex of the nebramycin factors, by other physical and chemical means[5] the ^{13}C NMR analysis of this antibiotic was undertaken. In this connection recourse was taken to carbon shift perturbations induced by amine protonation, previously found to be an indicator of the substitution pattern in the proximity of the amino carbon.[4]



1

The ^{13}C NMR spectra of the antibiotic and two of its fragments, methyl β-aprosaminide (**2**)[5] and methyl 4-amino-4-deoxy-α-D-glucopyranoside (**3**), were run in aqueous solutions of pH less than 1 and more than 11 and the shift data of trehalose (**4**)[6] and of various compounds from earlier studies were used for the interpretation of the spectra. All chemical shifts are listed in Table I.

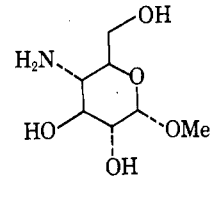


2

Table I
Carbon Chemical Shifts[a]

	1		2		3		
	>pH 11	<pH 1	>pH 11	<pH 1	>pH 11	<pH 1	4
C(1)	50.2	49.8	50.2	49.8			
C(2)	35.5	28.2	35.5	28.2			
C(3)	49.3	48.6	49.3	48.6			
C(4)	86.7	77.7	86.9	78.0			
C(5)	75.7	74.8	75.7	74.8			
C(6)	77.3	72.3	77.2	72.4			
C(1′)	100.4	94.9	100.5	95.1			
C(2′)	48.7	48.0	48.8	48.0			
C(3′)	32.0	26.8	32.1	26.9			
C(4′)	66.7[b]	65.7	66.3[c]	65.3			
C(5′)	69.8[b]	69.2	70.0[c]	69.4			
C(6′)	65.2	62.5	64.8	62.6			
C(7′)	61.2	59.3	61.8	60.2			
C(8′)	95.2	92.4	101.8	98.3			
C(1″)	94.1	94.1			99.1	98.9	92.9
C(2″)	70.7	70.2			72.0	71.2	71.9
C(3″)	73.0	68.9			72.9	68.9	72.5
C(4″)	52.3	52.4			52.4	52.8	69.6
C(5″)	72.4	68.4			71.4	67.4	70.9
C(6″)	60.7	60.3			60.8	60.4	60.4
NMe	32.0	30.4	32.1	30.5			
OMe			56.9	57.4	54.7	55.2	

[a] In parts per million downfield of Me_4Si; 1:1 D_2O–H_2O solutions with dioxane as internal reference. [b,c] The signals may be reversed. If so, they must be reversed at both pH values.



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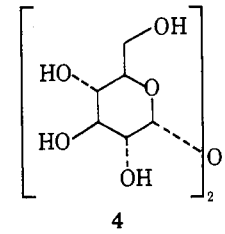
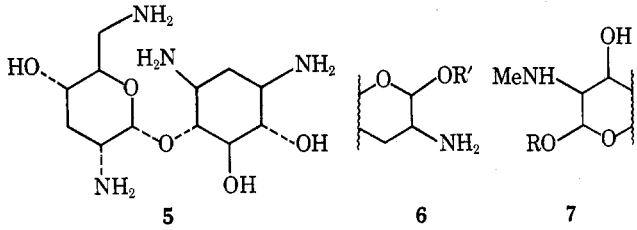


4

The δ values of all carbons of the monoglycoside model **3**, except the anomeric carbon shift, are nearly identical with five of the carbons of the monosaccharide unit of the antibiotic, reflecting an α-anomeric attachment of the remainder of the skeleton to this moiety. Two of the three anomeric carbon signals move upfield on lowering of the pH. Since this behavior reflects the vicinality of amino groups to two anomeric carbon sites,[4] the anomeric carbon shift impervious to pH change must be that of the monoglycosyl fragment. The ca. 6 ppm shielding of the latter's anomeric carbon in the face of the normally invariant ca. 100 ppm α-glucopyranosyl anomeric carbon shift[4] implies the presence of a 1-*tert*-alkoxy substituent, e.g., as the fructosyl moiety in sucrose, or of a 1,1 linkage between the glucopyranosyl unit and another glycosyl function, as in trehalose (**4**). The latter molecular array in apramycin followed from further analysis (vide infra).

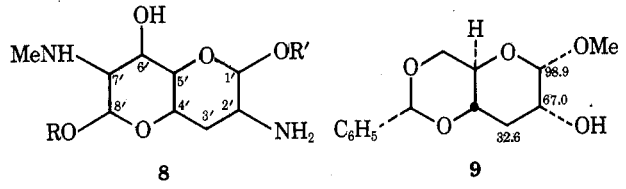
A comparison of the spectra of apramycin (**1**), methyl β-aprosaminide (**2**),[5] and neamine (**5**) reveals their common 2-deoxystreptamine unit. The identity of the six resonances of this ring in the three substances at high and low pH shows the attachment of the inosamine unit to be the same in all cases and hence to involve a C(4) ether linkage. The alternate C(6) oxygen attachment is precluded, since it introduces a different spatial environment around the amino groups of the deoxystreptamine moiety and its

neighboring ring and a consequent difference of conformation-dependent shift perturbation on acidification of the medium.[4] This argument establishes the liaison of the 2-deoxystreptamine unit to the anomeric site of a central saccharide moiety and the latter's α configuration.



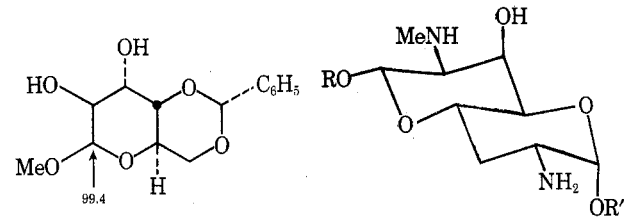
5 6 7

The remaining saccharide unit contains two amino groups, one primary and the other secondary. This difference of N substitution is recognizable easily by a strong shielding alteration of the β carbons ($\Delta\delta^{\beta}$ 4–6 ppm) of primary amines in acid solution and a reduced shielding disturbance ($\Delta\delta^{\beta}$ 2–3 ppm) for secondary amines.[4] Application of this diagnostic test to the N-methylated eight-carbon sugar moiety in apramycin (1) and methyl β-aprosaminide (2) reveals most of its structural detail. An anomeric carbon and the lone methylene group show $\Delta\delta$ values of 5.5 and 5.2 ppm, respectively, while the other anomeric center and an oxymethine exhibit shift differences of 2.8 and 2.7 ppm, respectively. These facts show six of the eight carbons to be contained in structural units 6 and 7 and yield their shift assignments. The remaining oxymethines can weld the two units to each other only in form 8. As a comparison of the anomeric carbon shifts of the antibiotic (1) and its degradation product 2 indicates, the removal of the aminoglucosyl unit leaves one anomeric carbon unfazed. Since the latter must be the site of attachment of the 2-deoxystreptamine moiety and since its $\Delta\delta^{\beta}$ value is 5.5 ppm, the inosamine-substituted anomeric site is vicinal to an amino, and not a methylamino, function. In view of R′ of 8 being the 2-deoxystreptamine unit the substituent R must be the aminoglucosyl residue, thus necessitating the involvement of the latter in a 1,1-disaccharide linkage. Since the ^{1}H NMR coupling characteristics of the anomeric hydrogens revealed the presence of one β and two α configurations in apramycin (1),[5] the aminoglucosyloxy moiety must be β oriented on the central saccharide fragment (8). Even though the 1,1 linkage in the antibiotic (1) differs stereochemically from that in trehalose (4), the anomeric carbon shift perturbation is similar in magnitude in both α–α and α–β relationships.



8 9

The stereochemistry of the ring juncture of the bicyclic saccharide (8) can be determined by comparison of the C(1′) and C(3′) shifts of methyl β-aprosaminide (2) with the shifts of the related carbons of a model, methyl 4,6-*O*-benzylidene-3-deoxy-α-D-glucopyranoside (9).[7] In view of the identity of the configuration of the C(1′) and C(2′) substituents of 2 with related carbons of 9 and of the β effect of an amino group at high pH with that of a hydroxy function[7] and in the face of the inosamine unit exerting the same effect as a methyl group on the anomeric site of the central ring system the nearly identical shifts of C(1′) and C(3′) of apramycin (1), 2, and the related carbons of model 9 reflect the identity of ring junctions.[8] Only the stereochemistry of the C(6′) and C(7′) substituents remains to be determined in view of the previous establishment of a β-anomeric C(8′) relationship. Two of the four possible configurations can be excluded. One possibility, a β-glucopyranosyl arrangement, is negated by a signal of C(8′) of methyl β-aprosaminide (2) appearing 2.5 ppm upfield of that of anomeric carbons of methyl β-glucopyranosides. While, in principle, the *N*-methyl group could exert a γ effect of such magnitude and field direction, ^{13}C NMR data from work on inositols[9,10] and inosamines[11] show such influence to be possible only in cases of methoxy or methylamino groups, respectively, being attached to carbons adjacent to axially substituted neighbors. The second possibility, a β-altropyranosyl arrangement, is untenable in view of the rigid model methyl 4,6-*O*-benzylidene-β-D-altropyranoside (10)[7] exhibiting an anomeric carbon signal 2.4 ppm upfield of the C(8′) resonance of 2. Thus the C(6′) and C(7′) substituents must have a cis relationship and be part of a β-allopyranosyl or β-mannopyranosyl configurational arrangement. The 8.5-Hz coupling visible in the H(8′) ^{1}H NMR signal and characteristic of a trans H(7′)–H(8′) relationship[5] shows the methylamino group to be equatorial and hence the ring to possess the stereochemistry of a β-allopyranoside as depicted in stereostructure 11 for saccharide 8. The full stereochemical details of apramycin are portrayed in structure 1.



10 11

Experimental Section

The ^{13}C NMR spectra of water solutions with the use of dioxane as internal reference [$\delta(Me_4Si) = \delta(C_4H_8O_2) - 66.3$ ppm] were recorded on Varian DP-60 and XL-100-15 spectrometers operating at 15.1 and 25.2 MHz in the Fourier transform mode, respectively. The chemical shifts in Table I and on formulas 9, 10, i, and ii are in parts per million downfield from Me_4Si.

Registry No.—1, 37321-09-8.

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(2) Address correspondence to Department of Chemistry, Rice University, Houston, Texas 77001.

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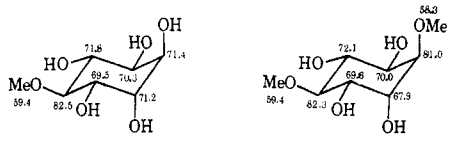
(8) Calculation of the C(2′) shift of 8 from the C(2) shift of 9 by the use of the 19.0 ppm C(2) shift difference between methyl α-D-glucopyranoside and methyl α-D-2-amino-2-deoxyglucopyranoside[4] yields a 48.0-ppm value, in close agreement with the observed shift and in support of identical C(2′) and C(4′) configurations in 8 and 9. Furthermore, the coupling characteristics of the C(3′) hydrogens in a ^{1}H NMR spectrum of 8[5] reveal an axial orientation for H(2′) and H(4′).

(9) D. E. Dorman, S. J. Angyal, and J. D. Roberts, *J. Am. Chem. Soc.*, **92**, 1351 (1970).

(10) Cf. also the shift data on pinitol (i) and pinpollitol (ii) [R. T. Gallagher, *Phytochemistry*, **14**, 755 (1975)]. (The authors acknowledge gratefully a gift of samples of these natural products.)



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Comparative Acidic Cleavage of Methoxybenzyl Protected Amides of Amino Acids[1]

P. G. Pietta,* P. A. Biondi,[2] and Oreste Brenna

Istituto di Chimica Organica dell'Università, 20133 Milano, Italy

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The use of 2,4-dimethoxybenzyl (Dmb) and *p*-methoxybenzyl (pmb) as amido protecting groups for conventional peptide synthesis has been previously reported.[3-5]

The possibility of using Dmb and pmb also in the solid-phase peptide synthesis[6] led us to study the relative stabilities of these groups in the acidic conditions normally associated with removal of the amino protecting groups.[7,8] For this purpose, cleavage of Dmb and pmb at 25° by 50% (v/v) trifluoroacetic acid in methylene chloride, 1 *N* HCl–acetic acid, and trifluoroacetic acid was investigated with the following protected amino acids: *N*-terbutyloxycarbonyl-N^{β}-*p*-methoxybenzyl and N^{β}-2,4-dimethoxybenzyl-L-asparagine [Boc-Asn(pmb)-OH and Boc-Asn(Dmb)-OH], *N*-*tert*-butyloxycarbonyl-N^{γ}-*p*-methoxybenzyl and N^{γ}-2,4-dimethoxybenzyl-L-glutamine [Boc-Gln(pmb)-OH and Boc-Gln(Dmb)-OH], and glycine-*p*-methoxybenzyl and 2,4-dimethoxybenzylamide (H-Gly-NHpmb and H-Gly-NHDmb). The removal of pmb and DMB was followed by thin layer chromatography. Partial cleavage of the amide protective groups was readily detected, since unchanged protected amides were well separated from L-asparagine, L-glutamine, and L-glycine amide resulting from cleavage.

In the case of H-Gly-NHpmb and H-Gly-NHDmb the removal of the protective groups was also checked by gas chromatography. Finally the action of liquid HF at 0° was examined[9] to ascertain whether pmb and Dmb had been removed under conditions where the side-chain protecting groups normally used in solid-phase peptide synthesis are cleaved.

This investigation has resulted in a method which is suitable to the quantitative determination of amino acid amides released during acidolysis of the corresponding *p*-methoxybenzyl or 2,4-dimethoxybenzyl amides. It should be noted that the results obtained by TLC are in good agreement with those obtained by GC (Table I).

The *p*-methoxybenzyl group was slightly affected by 1 *N* HCl–CH_3COOH under any conditions while prolonged exposure to this reagent caused partial cleavage of the 2,4-dimethoxybenzyl group (Table I).

Trifluoroacetic acid cleaved completely the 2,4-dimethoxybenzyl group after prolonged treatment (72 h), whereas the *p*-methoxybenzyl group was attacked partially only after 48 h (Table I).

2,4-Dimethoxybenzyl was completely removed by means of liquid HF; on the other hand, under these conditions the *p*-methoxybenzyl group was removed only partially. Boc-Gln(pmb)-OH, especially, has been shown to be sluggish to cleavage, confirming the work reported by Hruby et al.[10] Prolonged reaction times (12 h) were found necessary to complete removal.

These results demonstrate that 2,4-dimethoxybenzyl is suitable as an amido protecting group for asparagine and glutamine in solid-phase peptide synthesis and also indicate that *p*-methoxybenzyl is less promising in this connection.

Experimental Section

The Dmb derivatives were prepared as previously described.[5] The pmb derivatives were obtained in our laboratory as indicated below and their purity was checked by TLC using the following systems: A, benzene–ethyl acetate–petroleum ether (5:3:2 v/v); B, benzene–ethyl acetate–acetic acid–water (10:10:2:1 v/v); C, chloroform–methanol–acetic acid (15:3:2 v/v).

The removal of pmb and Dmb was followed by running chromatograms on Kieselgel G with 1-butanol–acetic acid–water (4:1:1 v/v); spots were detected with ninhydrin–cadmium acetate (0.2% v/v)[11] and evaluated by densitometry[12] using a chromoscan Zeiss double beam densitometer with thin layer attachment.

The gas chromatographic analysis was carried out using a Fractovap Model G.V. equipped with flame ionization detector. The

Table I
Stability[a] to Acidic Cleavages[b] of Dmb and pmb Amido Protecting Groups

Time, h	TLC/GC[c]		TLC			TLC		
	A	C	A	B	C	A	B	C
	H-Gly-NH-Dmb (35088-22-3)			Boc-Asn (Dmb)-OH (47553-91-3)			Boc-Gln (Dmb)-OH (31874-52-9)	
6	6/5	21/19		23	31		21	27
12	18/18.5	40/37		44	62		41	55
24	20/17.5	63/64	20	63	77	20	60	73
36	28/26	73.5/74	29	80	86	28	80	85
48	35/32	81/80	35	99	99	35	97	98
72	39.5/35	91/90	42	100	100	41	100	100
	H-Gly-NH-pmb (57459-57-1)			Boc-Asn (pmb)-OH (27482-66-2)			Boc-Gln (pmb)-OH (27482-68-4)	
48	3/2.6	26/28			13			15
72	6/5.5	35/35.5	5		20	3		21

[a] The percents of methoxybenzyl amides cleaved for different times are reported (mean of three independent determinations). [b] A, 1 N HCl–CH_3COOH; B, 50% CF_3COOH–CH_2Cl_2 (v/v); C, CF_3COOH. [c] Result of densitometric analysis/result of gas chromatographic analysis.