ROLE OF THE 1-AMINO GROUP IN AMINOCYCLITOL ANTIBIOTICS: SYNTHESIS OF 1-DEAMINOGENTAMICIN C_2*

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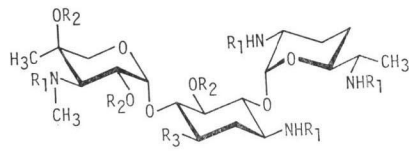
The synthesis of 1-deaminogentamicin C_2 described here, uses 3,2′,6′,3″-tetrakis-*N*-*tert*-butoxycarbonylgentamicin C_2 (**2**) as intermediate. *N*-Formylation of **2** followed by per-*O*-acetylation and dehydration furnished the isocyanide **5**. Radical-induced deamination of the latter using tri-*n*-butylstannane and removal of the protecting groups afforded the target 1-deaminogentamicin C_2 (**7**). Its *in vitro* antibacterial activity is less than that of the parent gentamicin C_2. The behaviour of **7** towards aminoglycoside-inactivating enzymes was also examined; interestingly, it was found to be neither substrate nor inhibitor for such enzymes. These results strongly suggest that the substitution pattern of the 1-position determines the biological properties of the aminoglycoside antibiotics.

The wide variety of naturally occurring aminoglycoside antibiotics and the ready accessibility of chemically modified derivatives have permitted development of a reasonably detailed picture of structure-activity relationships within this important family of antibiotics. It appears from comparison of antibacterial properties of, for instance, kanamycins A, B and C, that the number and location of amino groups in the sugar portions attached to the deoxystreptamine nucleus, have a profound effect on the potency of these drugs[1]. Concerning the amino group of the aglycone it is well established that the 3-amino function is essential for antimicrobial activity and may neither be functionalized nor removed[2]. In contrast, it is also known that the 1-amino group can be acylated or alkylated to yield compounds with an improved spectrum of activity particularly against resistant strains[3~7]. In continuation of our work on the synthesis of the pseudo-disaccharide 1-deaminotobramine, which is devoid of antibacterial activity[8], we decided to extend this interesting biological observation to a genuine aminoglycoside.

In this paper we report the synthesis of 1-deaminogentamicin C_2 (**7**) and its antibacterial properties in comparison with its parent gentamicin C_2. Our synthetic approach was based on a radical induced deamination described by BARTON *et al.*[9]. Due to the low regioselectivity already observed for this reaction with unprotected aminoglycosides[10], we required a fully *N*-protected gentamicin C_2 intermediate **2** having only the 1-amino group free for the projected reactions' scheme.

The intermediate **2** was synthesized from the readily available 3,2′,6′-tris-*N*-*tert*-butoxycarbonylgentamicin C_2[11] in a one-pot reaction *via* selective benzyloxycarbonylation at *N*-1, followed by *tert*-

* Dedicated to Professor EDGAR LEDERER on the occasion of his 75th birthday.



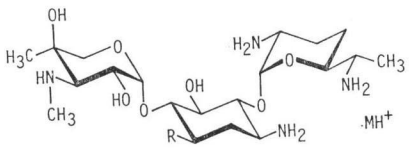
	R_1	R_2	R_3
1	H	H	NH_2
2	Boc	H	NH_2
3	Boc	H	NHCHO
4	Boc	Ac	NHCHO
5	Boc	Ac	NC
6	Boc	Ac	H
7	H	H	H

Boc=$CO_2C(CH_3)_3$

butoxycarbonylation at *N*-3″ and subsequent hydrogenolytic cleavage of the benzyloxycarbonyl-group[7]. *N*-Formylation of **2** was readily achieved with *p*-nitrophenyl formate. All hydroxyl groups in **3** were protected using acetic anhydride in pyridine, in the presence of a catalytic amount of 4-dimethylaminopyridine. Dehydration of the formamide function in **4** to the isocyanide derivative **5** was proved to be more efficient using *p*-toluenesulfonyl chloride in pyridine[12] than phosphorus oxychloride and triethylamine. Tri-*n*-butylstannane reduction of the isocyanide **5** to the corresponding 1-deamino derivative **6** was performed in the presence of azobisisobutyronitrile (AIBN) in refluxing benzene under nitrogen. After *O*-deacetylation and *N*-deprotection, the target molecule **7** was isolated at its hydrochloride salt.

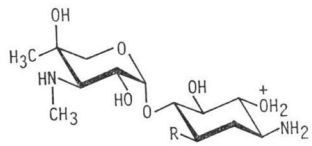
The chemical ionization mass spectrum[13] (Table 1) of **7** as free base (using isobutane as reagent gas) exhibits a parent ion peak MH^+ at *m/z* 449 and a significant peak at *m/z* 307 resulting from the loss of the purpurosaminyl ring; the corresponding ion peaks for the parent gentamicin C_2 (**1**), are observed at *m/z* 464 and *m/z* 322 respectively. These data confirm the absence of the 1-amino group in **7**. The

Table 1. Chemical ionization mass spectrum of gentamicin C_2 (**1**) and 1-deaminogentamicin C_2 (**7**). Intensities (%) are shown in parentheses.



1 R=NH_2 **7** R=H

m/z 464 (100)	*m/z* 449 (100)



m/z 322 (32)	*m/z* 307 (41)

Table 2. ^{13}C NMR data of gentamicin C_2 (**1**) and 1-deaminogentamicin C_2 (**7**) in D_2O at acidic pH with dioxane as an internal reference $\delta_{TMS}=\delta_{Diox.}+67.4$.

	1	**7**		**1**	**7**		**1**	**7**
C-1	50.5[a]	25.9	C-1′	95.8	96.1	C-1″	102	95.2
C-2	28.4	24.6	C-2′	49.5[a]	50.5	C-2″	67.2	67.4 (diox)
C-3	49.5[a]	52.7	C-3′	21.4[b]	21.4	C-3″	64.2	65.0
C-4	77.3	77	C-4′	23.6	25.3	C-4″	69.7	70.8
C-5	75.8	76.8	C-5′	70.7	66.7	C-5″	68.7	69.7
C-6	84.5	78.8	C-6′	49.6[a]	49.7	C-6″	21.8[b]	21.8
			$C_{6'}$–CH_3	13.2	13.5	NH–CH_3	35.4	36.1

[a,b] Assignments may be reversed.

comparison of ^{13}C NMR data of **7** and **1** (Table 2) recorded under acidic conditions, further corroborates the structure of **7**. Significant differences observed for the chemical shifts of C-1, C-2, C-6 and C-1″ are consistent with the removal of the equatorial 1-amino function and are in good agreement with those reported very recently in the literature for 1-deaminogentamicin C_1.[2]

The *in vitro* antimicrobial activity of **7**, in comparison with gentamicin C_2, is shown in Table 3. The ability of some major aminoglycoside inactivating enzymes to modify 1-deaminogentamicin C_2 (**7**) was examined[14,15]. The results shown in Table 4, clearly indicate that **7** is not a substrate for such enzymes. Therefore, it was interesting to test **7** as an eventual inhibitor of enzymatic modifications of gentamicin C_2 or C_{1a}. The data presented in Table 4, show that this is not the case. Furthermore, compound **7** does not interfere with binding of ^3H-6′-*N*-acetyl tobramycin to *Escherichia coli* ribosome[16] even at 10^{-4} M (results not shown). Thus, the 1-amino group seems to be essential for the formation of an enzyme-effector complex and also for the binding to the ribosomes.

Table 3. *In vitro* antimicrobial activity* (MIC μg/ml) of 1-deaminogentamicin C_2 (**7**) and its parent gentamicin C_2 (**1**).

	1	**7**
Staphylococcus aureus ATCC 10832	0.078	0.5
Staphylococcus aureus ATCC 29067	0.625	2.5
Streptococcus faecalis NCIB 776	1.25	12.5
Pseudomonas aeruginosa ATCC 29511	3.12	50
Escherichia coli Δ120	1.56	10
Proteus mirabilis NCIB 60	1.56	10

* Broth dilution test with Trypticase Soy Broth, inoculum 10^6.

Experimental

Melting points were determined on a Reichert hot-plate apparatus and are uncorrected. Optical rotations were measured on a "Quick" Roussel and Jouan polarimeter. IR spectra were recorded on a Perkin-Elmer 297 spectrometer. ^1H NMR spectra were recorded on Varian T60 or EM 360L (60 MHz) instruments and ^{13}C NMR on Bruker WP-60 (15.08 MHz) or HX-90 (22.63 MHz), chemical shifts (δ) are reported with reference to tetramethylsilane. Mass spectra were recorded on either an A. E. I. MS-9 or an A. E. I. MS-50 spectrometer. TLC was performed on Schleicher and Schüll plastic backed

Table 4. Behavior of 1-deaminogentamicin C_2 (**7**) towards some aminoglycoside modifying enzymes.

Organism	Inactivating enzyme[c]	% of inactivation[a]		% of inhibition[b]
		1	**7**	
Escherichia coli R_{135}	AAC (3)	100	10	0
Klebsiella pneumoniae Eclaircy	AAC (3)	100	10	0
Klebsiella pneumoniae R_{176}	AAC (3)	100	10	0
Pseudomonas aeruginosa Stone 130	AAC (3)	100	<5	5
Proteus inconstans PV 164	AAC (2′)	100	15	15
Escherichia coli R_5	AAC (6′)	25	5	0
Escherichia coli Dijon	AAC (6′)	20	10	N.D.
Staphylococcus aureus Palm	AAC (6′)	5	5	0
	APH (2″)	100	5	10

[a] A value of 100% of inactivation was assigned to gentamicin C_{1a}.

[b] The substrates of the enzymatic reactions were gentamicin C_2 with AAC (3), AAC (2′) and APH (2″) and gentamicin C_{1a} with AAC (6′).

[c] AAC: Aminoglycoside acetyltransferase. APH: aminoglycoside phosphotransferase.

silica gel plates (F 1500 S 254); the plates were initially examined under UV light (254 and 366 nm) then developed with appropriate spray reagents. Column chromatography was effected, under low pressure, using Merck Kielselgel (Type 60) and the eluant given in parentheses. Evaporations were carried out at below 40°C using a Büchi rotary evaporator. The microanalyses were performed by the analytical department of the I.C.S.N., Gif-sur-Yvette.

3,2′,6′,3″-Tetrakis-*N*-*tert*-butoxycarbonylgentamicin C_2 (**2**)

To a solution of 3,2′,6′-tris-*N*-*tert*-butoxycarbonylgentamicin C_2[11] (4.34 g) in dichloromethane/methanol (50 ml/2.5 ml) was added dropwise a solution of *N*-benzyloxycarbonyloxyphtalimide (1.77 g) in dichloromethane/methanol (10 ml/1 ml) at room temperature. After stirring for 2 hours the mixture was extracted with aqueous saturated sodium hydrogen carbonate solution until the red color disappeared. To the organic layer was added di-*tert*-butyl dicarbonate (1.8 g) and pyridine (0.5 ml). This solution was stirred at room temperature overnight and then extracted twice with 10% aqueous ammonium hydroxide. The solvent was removed, the residue dissolved in methanol/water (30 ml/3 ml) and ammonium formate (2.3 g) and palladium (10% on charcoal) was added. The mixture was refluxed for 1 hour. Removal of the catalyst and the solvent followed by extraction with dichloromethane - water and chromatography on a silica gel column using dichloromethane - methanol (15: 1) as the eluant yielded **2** as an amorphous solid (3.67 g, 75% yield).

3,2′,6′,3″-Tetrakis-*N*-*tert*-butoxycarbonyl-1-*N*-formylgentamicin C_2 (**3**)

A solution of **2** (1 g) and *p*-nitrophenyl formate (970 mg) in dioxane (15 ml) was stirred at room temperature overnight. After evaporation of the solvent *in vacuo*, the residue was chromatographed on a silica gel column using a dichloromethane - methanol - concentrated ammonium hydroxide solution (15: 4: 1, v/v) as the eluant to give **3** as a white solid (825 mg, 80% yield), which was recrystallized from chloroform - ether; mp 160~162°C, $[\alpha]_D^{20}$ +49° (*c* 1, CH_2Cl_2). ^{1}H NMR ($CDCl_3$) δ 7.8 (1H, s, C*H*O).

Anal. Calcd. for $C_{41}H_{73}N_5O_{16}\cdot H_2O$: C 54.11, H 8.30, N 7.69
Found: C 54.28, H 8.38, N 7.62

5,2″,4″-Tri-*O*-acetyl-3,2′,6′,3″-tetrakis-*N*-*tert*-butoxycarbonyl-1-*N*-formylgentamicin C_2 (**4**)

To a cold solution of **3** (1 g) in pyridine (20 ml) containing 4-dimethylaminopyridine (catalytic amount) was added acetic anhydride (1 ml). The mixture was stirred, at room temperature, overnight and then poured into ice-cooled aqueous solution of sodium hydrogen carbonate. The precipitate was filtered off and washed with water and then chromatographed on silica gel column using dichloromethane - ethanol solution (23 : 2) as the eluant. The title compound was recrystallized from chloroform - ether (920 mg, 80% yield); mp 167~169°C, $[\alpha]_D^{20}$ +30° (*c* 1, CH_2Cl_2), IR 1690 cm^{-1} (formamide), 1740 cm^{-1} (ester), ^{1}H NMR ($CDCl_3$) δ 2.05 (3H, s, CO-CH_3).

Anal. Calcd. for $C_{47}H_{79}N_5O_{19}$: C 55.44, H 7.82, N 6.88
Found: C 55.39, H 7.74, N 6.75

5,2″,4″-Tri-*O*-acetyl-3,2′,6′,3″-tetrakis-*N*-*tert*-butoxycarbonyl-1-deamino-1-isocyanogentamicin C_2 (**5**)

To a solution of **4** (1 g) in anhydrous pyridine (20 ml) was added at 0°C, *p*-toluenesulfonyl chloride (570 mg).

The resulting mixture was stirred for 24 hours and then poured into an ice-cooled aqueous solution of sodium hydrogen carbonate. The crude solid was purified by column chromatography on silica gel (dichloromethane - ethanol, 23 : 2). After recrystallization from chloroform - ether, the compound **5** was isolated as white needles (690 mg, 70% yield); mp 157~159°C, $[\alpha]_D^{20}$ +32° (*c* 1, CH_2Cl_2), IR 2140 cm^{-1} (isonitrile), ^{1}H NMR ($CDCl_3$) no signal at δ 7.8.

Anal. Calcd. for $C_{47}H_{77}N_5O_8$: C 53.68, H 7.76, N 7.00
Found: C 53.49, H 7.81, N 7.07

5,2″,4″-Tri-*O*-acetyl-3,2′,6′,3″-tetrakis-*N*-*tert*-butoxycarbonyl-1-deaminogentamicin C_2 (**6**)

A solution of the isocyanide **5** (500 mg) and azobisisobutyronitrile (AIBN) (30 mg) in dry benzene (20 ml) was added dropwise to a refluxing solution of tri-*n*-butylstannane (1 g) in benzene (25 ml) under nitrogen. Heating was maintained for 3 hours. Removal of the solvent and column chromatography on silica gel (dichloromethane - ethanol, 23 : 2) provided **6** which was recrystallized from chloroform -

ether (420 mg, 86% yield); mp 150~152°C, $[\alpha]_D^{20}$ +57° (*c* 1, CH_2Cl_2).

Anal. Calcd. for $C_{40}H_{72}N_4O_{15}\cdot 2H_2O$: C 54.38, H 8.65, N 6.33
Found: C 54.67, H 8.48, N 6.53

1-Deaminogentamicin C_2 (7)

To a solution of **6** (200 mg) in dry methanol (7 ml) was added a 1 M methanolic sodium methoxide (1 ml). After stirring for 2 hours, the solution was neutralized by addition of Amberlite IRC-50 (H^+) resin. The resin was filtered off and the filtrate concentrated *in vacuo*. The residue (165 mg) was dissolved in trifluoroacetic acid (4 ml). To the stirred solution, after 2 minutes, ether was added to precipitate the salt. The latter was isolated by filtration and deionized with Amberlite IR-45 (OH^-) resin. The aqueous solution containing the free base **7** was lyophilized and then dissolved in methanol. The resulting solution was acidified with 1 M hydrochloric acid (pH 3) and then poured into a ice-cold vigorously stirred ether (40 ml). The precipitated hydrochloride salt was isolated by centrifugation; mp 241~243°C, $[\alpha]_D^{20}$ +62° (*c* 0.7, H_2O).

Anal. Calcd. for $C_{20}H_{40}N_4O_7\cdot 4HCl\cdot 3H_2O$: C 37.07, Cl 22.80, H 8.08, N 8.95
Found: C 36.98, Cl 22.78, H 7.79, N 8.75

Enzyme Preparation

Bacteria were grown in Trypticase Soy Broth (Merieux) in the presence of 20 μg/ml of an aminoglycoside to which there were resistant. The cells were harvested at the late logarithmic phase and washed twice with buffer A: 10 mM tris-HCl pH 7.5, 10 mM $MgCl_2$, 20 mM NH_4Cl, 6 mM β-mercaptoethanol. They were then resuspended in the same buffer (15 ml/g of wet cells) and either submitted to sonication at 4°C (four 30 seconds bursts at 100 W out put, separated by 2 minutes cooling periods) or disrupted by passage through a French pressure cell at 1 kg/cm². In the case of *Staphylococcus aureus*, the washed cells were resuspended in 100 mM NaCl, 20 mM tris-HCl buffer pH 7.5 (25 ml/g). The suspension was stirred gently for 1 hour at 37°C in the presence of lysostaphin (0.5 mg/g of bacteria) and then DNase (4 μg/ml) was added and Mg^{2+} and NH_4^+ concentrations were adjusted to 10 and 20 mM respectively before centrifugation at 20,000×*g* for 20 minutes at +4°C. Cell free extracts were obtained by centrifugation at 200,000×*g* for 2 hours and then submitted to electrofocusing or to affinity chromatography on immobilized aminoglycoside[15,17]. Crude as well as purified enzymes were stored in liquid nitrogen in 500 μl portions.

Enzymatic Assays

Standard phosphocellulose binding assay was used[14]. Each assay contained 5 μl of 2 mM antibiotic solution, 5 μl of labelled donor (^{14}C AcCoA: 2.5 mM, 2 Ci/mole; γ^{32}P GTP: 2 mM, 2.5 Ci/mole), 40 μl of purified enzyme solution in buffer A. The mixture was incubated at 37°C for generally 10 minutes. The inhibition of inactivation of gentamicin C_2 by the 1-deaminogentamicin C_2 (**7**) was done as described above; to the previous mixtures were added either 5 μl of 2 mM solution of **7** or 5 μl of water for the reference experiments.

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References

1) PRICE, K. E.; J. C. GODFREY & H. KAWAGUCHI: Effect of structural modifications on the biological properties of aminoglycoside antibiotics containing 2-deoxystreptamine. *In* Structure-activity Relationships among Semisynthetic Antibiotics. *Ed.* D. PERLMAN, p. 239~395, Academic Press, 1977
2) BOXLER, D. L.; R. BRAMBILA, D. H. DAVIES, A. MALLAMS, S. W. MCCOMBIE, J. B. MORTON, P. REICHERT & H. F. VERNAY: Semisynthetic aminoglycoside antibacterials. 9. Synthesis of novel 1- and 3-substituted and 1- and 3-*epi*-substituted derivatives of sisomicin and gentamicin from the 1- and 3-oxo-derivatives. J. Chem. Soc. Perkin I 1981: 2168~2185, 1981
3) PRICE, K. E.; D. R. CHISHOLM, M. MISIEK, F. LEITNER & Y. H. TSAI: Microbiological evaluation of BB-K8, a new semisynthetic aminoglycoside. J. Antibiotics 25: 709~731, 1972

4) MILLER, G. H.; G. ARCIERI, M. J. WEINSTEIN & J. A. WAITZ: Biological activity of netilmicin, a broad-spectrum semisynthetic aminoglycoside antibiotic. Antimicrob. Agents Chemother. 10: 827~836, 1976
5) NAGABHUSHAN, T. L.; A. B. COOPER, H. TSAI, P. J. L. DANIELS & G. H. MILLER: The synthesis and biological properties of 1-*N*-(*S*-4-amino-2-hydroxybutyryl) gentamicin B and 1-*N*-(*S*-3-amino-2-hydroxypropionyl) gentamicin B. J. Antibiotics 31: 681~687, 1978
6) MALLAMS, A. K.; J. B. MORTON & P. REICHERT: Semisynthetic aminoglycoside antibacterials. 10. Synthesis of novel 1-*N*-amino-alkoxycarbonyl and 1-*N*-aminoalkylcarboxamido derivatives of sisomicin, gentamicin B, gentamicin C_{1a} and kanamycin A. J. Chem. Soc. Perkin I 1981: 2186~2208, 1981 and references cited there in.
7) STREICHER, W. & H. LOIBNER: Synthesis and structure/activity relationship of 1-*N*-substituted aminoglycoside antibiotics. 1st European Symposium on Carbohydrates and Glycoconjugates. Wien, Sept. 14~17, 1981
8) QUICLET-SIRE, B.: Ph. D. Thesis, University of Paris XI, Orsay, 1980
9) BARTON, D. H. R.; G. BRINGMANN, G. LAMOTTE, W. B. MOTHERWELL, R. S. HAY-MOTHERWELL & A. E. A. PORTER: Reactions of relevance to the chemistry of aminoglycoside antibiotics. 14. A useful radical-deamination reaction. J. Chem. Soc. Perkin I 1980: 2657~2664, 1980
10) BARTON, D. H. R.; G. BRINGMANN & W. B. MOTHERWELL: Reactions of relevance to the chemistry of aminoglycoside antibiotics. 15. The selective modification of neamine by radical-induced deamination. J. Chem. Soc. Perkin I 1980: 2665~2669, 1980
11) NAGABHUSHAN, T. L.; A. COOPER & W. N. TURNER: Selective blocking of aminogroups. U.S. Patent 697,297, June 17, 1976
12) HERTLER, W. R. & E. J. COREY: A novel preparation of isonitriles. J. Org. Chem. 23: 1221~1222, 1958
13) SEPULCHRE, A. M.; P. VARENNE, B. C. DAS, S. D. GERO & H. E. AUDIER: The chemical ionisation mass spectrometry of aminocyclitol-aminoglycoside antibiotics. Nov. J. Chim. 2: 405~409, 1978
14) HAAS, M. J. & J. E. DOWDING: Aminoglycoside modifying enzymes. *In* J. H. HASH, *Ed.* Methods in Enzymology 43: 611~628, 1975
15) LE GOFFIC, F.; N. MOREAU & M. CHEVEREAU: Purification par chromatographie d'affinité d'une acétyltransférase et d'une phosphotransférase inactivant les antibiotiques aminosidiques. Biochimie 55: 1183~1185, 1973
16) LE GOFFIC, F.; M. L. CAPMAU, F. TANGY & E. CAMINADE: Have deoxystreptamine aminoglycoside antibiotics the same binding site on bacterial ribosomes? J. Antibiotics 33: 895~899, 1980
17) MOREAU, N. & F. LE GOFFIC: Unpublished results.