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Research Article

Recovery of the Antibiotic Activity against Resistant Bacterial Strains by Selective Guanidinylation of the 3″-Methylamino Group of Gentamicin

Sandra G Zárate[1,2], Agatha Bastida[1], Andrés G Santana[1*] and Julia Revuelta[1*]

[1]*Departmento de Química Bioorgánica, Instituto de Química Orgánica General (CSIC), Juan de la Cierva, Spain*

[2]*Facultad de Tecnología-Carrera de Ingeniería Química, Universidad Mayor Real y Pontificia de San Francisco Xavier de Chuquisaca, Sucre, Bolivia*

Abstract

Aminoglycoside antibiotics are highly potent, wide-spectrum bactericidals. Unfortunately, their use in clinical practice has been seriously limited as a result of their toxicity and susceptibility to enzymatic inactivation. Herein, we describe the synthesis and biological properties of a new gentamicin derivative (C_{1a} and C_2 gentamicin components). The selective modification of N-3″ in this antibiotic not only maintains an antimicrobial activity similar to the parent aminoglycoside but also against resistant strains expressing several AMEs (AAC (6′)-Ib, APH(2″), ANT(6) and APH(3′)).

Introduction

Ever since their discovery in the mid XX century, aminoglycoside antibiotics have become one of the go to treatments for bacterial infections. The first congener of this family, streptomycin was isolated in 1944 and soon after, several members of this class of compounds were also reported [1]. From a chemical point of view, aminoglycosides are water soluble polycationic pseudo-oligosaccharides constituted by amino sugars attached to a cyclitol by glycosidic linkages. Their potent bactericidal activity relies on binding specifically to the decoding A site of RNA in the bacterial small ribosomal subunit, thus interfering with protein synthesis [2,3]. These compounds are widely used in the treatment of serious infections caused by gram negative/ gram positive bacteria, although their clinical use is associated with dose-limiting nephrotoxicity and ototoxicity [4,5]. Worse yet, many of the aminoglycoside antibiotics originally identified are no longer clinically useful as they are compromised by bacterial resistance mechanisms [6].

Acquired resistance to aminoglycoside antibiotics can occur via three different mechanisms: Mutation of the ribosomal target, reduced permeability for the antibiotics, and enzymatic modification of the drugs, thus leading to inactivation. From a clinical point of view, the most relevant source of resistance is the enzymatic inactivation of the drugs by modification of its amine or hydroxyl groups. Antibiotic Modifying Enzymes (AMEs) can be broadly classified as N-Acetyltransferases (AACs), O-Adenyl Transferases (ANTs) and O-Phosphotransferases (APHs). Each family involves enzymes that catalyse the same reactions but with different regioselectivity and substrate specificity (Figure 1).

With this in mind, and given the urgent demand for the discovery of new antibiotics to overcome the resistance to these compounds, considerable effort has been devoted to designing new semisynthetic aminoglycoside antibiotics that are immune to inactivation [7]. The intrinsic potency of aminoglycosides makes them excellent candidates to explore new ways to avoid bacterial resistance and diminish toxicity. Our group has been actively involved in the design of new antibiotics that are not susceptible to inactivation by AMEs. With this purpose, different design strategies have been employed (Figure 1) [8].

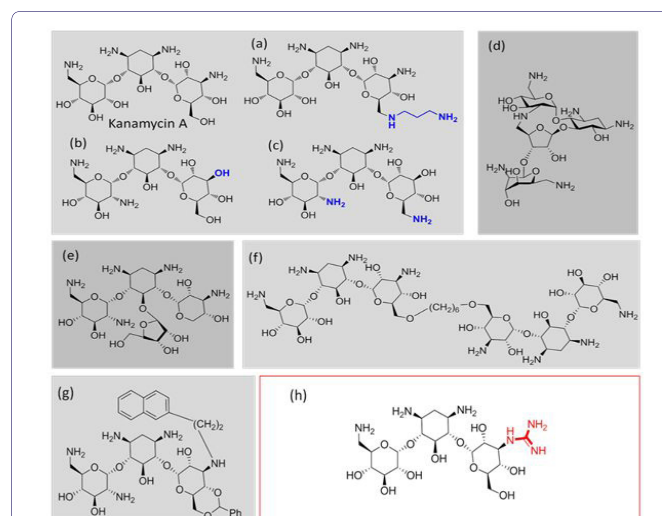

Figure 1: Representation of the different aminoglycoside systems (a-g) designed and synthesized by our group to prevent AME inactivation. Addition of a substituent at the N-3″ position of kanamycin A (strategy highlighted with a red square) is well tolerated by the rRNA and, particularly, guanidinylation of this position prevents the inactivation of the antibiotic by AMEs.

***Corresponding authors:** Julia Revuelta, Departmento de Química Bioorgánica, Instituto de Química Orgánica General (CSIC) Juan de la Cierva, 3. 28006-Madrid, Spain, Tel: +34 912587487; E-mail: julia.revuelta@iqog.csic.es

Andrés G Santana, Departmento de Química Bioorgánica, Instituto de Química Orgánica General (CSIC) Juan de la Cierva, 3. 28006-Madrid, Spain, Tel: +34 91 2587455; E-mail: andres.g.santana@csic.es

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These derivatives included changes in the distribution of the amino groups (Figure 1a-c) [9], conformational restriction of the drug (Figure 1d) [10], the simultaneous incorporation of kanamycin and ribostamycin fragments within the same antibiotic scaffold (Figure 1e) [11], aminoglycoside dimerisation (Figure 1f) [12] and the introduction of bulky substituent's properly positioned to interfere with drug recognition (Figure 1g) [13]. Unfortunately, despite their structural diversity, these compounds are substrates of the AMEs to some extent and some of them even lost their antibiotic activity. More recently, we have demonstrated that the A site shows a clear tolerance for modification at the N-3″ position of the aminoglycosides [14,15] and that the guanidinylation of this position maintains the antibiotic activity against aminoglycoside 6′-acetyl-transferase and 4′-nucleotidyl-transfersase-expressing strains (Figure 1h) [16,17].

Herein, we propose the synthesis and evaluation of the new gentamicin derivatives 1a and 1b with the aim of expanding the usefulness of our strategy, demonstrating its broad applicability against other resistance enzymes. Gentamicin is a mixture of various congeners: C_1, C_{1a} and C_2 (Figure 2). Structural differences between them are minor, differing only by a methyl or a hydrogen substitution in two R groups (R_1 or R_2) on the purpurosamine residue. This mixture has been the only aminoglycoside antibiotic used to date in clinic and recently it has demonstrated utility for the treatment of sepsis caused by diverse strains of MRSA bacteria [18]. The MIC of gentamicin (2a-c) typically ranges from 6 to 48 mg/ml. However, many strains have acquired aminoglycoside resistance genes that encode various AMEs, which eventually result in very high resistance to gentamicin (MICs usually higher than 200 mg/ml). The most clinically relevant of these genes are aac (6′), aac(2′), aph(2″) and ant(2″).

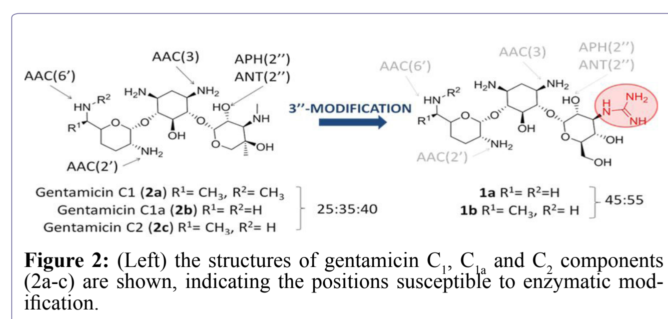


Figure 2: (Left) the structures of gentamicin C_1, C_{1a} and C_2 components (2a-c) are shown, indicating the positions susceptible to enzymatic modification.

Materials and Methods

Gentamicin (2a-c) was purchased from Sigma Aldrich. All the reactions that needed dry conditions were carried out under an argon atmosphere and the solvents were appropriately dried before use by standard techniques. Commercial reagents were used as received. All reactions were magnetically stirred and monitored by TLC in precoated Kiesel gel 60 F254 (Merck). Flash Column chromatography (FC) was carried out on Silica Gel 60 (32-63 µm). Detection was first carried out by UV light (254 nm) and then by charring with a solution of sulfuric acid, ninhydrin or with Mostain. NMR experiments were recorded on a Varian Unity 400 MHz at 313 K. The aminoglycosides 1a-1b was characterized employing a combination of 2D TOCSY, COSY and HSQC experiments. Low and high resolution mass spectra were provided by the Mass Spectrometry Facilities, CSIC and Madrid. The bacterial strains utilized in this study were obtained from the Hospital de Fuenlabrada (Madrid) and from ATCC (American Type Culture Collection) or CECT (Spanish Type Culture Collection).

Synthesis of Compounds 3a-3b

1,3,2′,6′,3″-$(Cbz)_5$-gentamicin C_1 (3a), 1,3,2′,6′,3″-$(Cbz)_5$-gentamicin C_{1a} (3b) and 1,3,2′,6′,3″ -$(Cbz)_5$-gentamicin C_2 (3c).

To a well stirred solution of gentamicin C_1 (2a), C_{1a} (2b) and C_2 (2c) (ratio = 25:35:40) free base (0.3 g, 0.64 mmol) in a saturated aqueous solution of Na_2CO_3 (4 ml) at 0°C was added a solution of CbzCl (benzyl chloroformate) (0.65 g, 3.84 mmol) in acetone (1.5 ml) drop by drop. The mixture was vigorously stirred for 2 hours at this temperature and then 8 hours at room temperature. Subsequently, the solvent was removed under reduced pressure and the residue was pulverized in a mortar. This solid was added to an aqueous solution of HCl (1M) until neutralization and the formed solid product was filtered and dried under vacuum, obtaining the mixture of compounds 3a-c (0.36 g, 50%). MS-API-ES: 1148 $(M+H)^+$ (3a), 1134 $(M+H)^+$ (3b), 1120 $(M+H)^+$ (3c).

1,3,2′,6′,-$(Cbz)_4$-2″,3″-carbamate-gentamicin C_1 (4a)
1,3,2′,6′,-$(Cbz)_4$-2″,3″-carbamate-gentamicin C_{1a} (4b)
1,3,2′,6′,-$(Cbz)_4$-2″,3″-carbamate-gentamicin C_2 (4c)

To a stirred solution of the mixture of compounds 3a-c (0.3g, 0.32 mmol) in a mixture of 1,4-dioxane/H_2O (30 ml/10 ml) was added an aqueous solution of NaOH (2.5M). The mixture was stirred at 50°C for 24 hours, after which was added an aqueous solution of HCl (1M) until pH 10. The mixture was concentrated under reduced pressure and subsequently water was added (25 ml). The obtained suspension was filtered and the solid was dried under vacuum. Finally, the residue was purified through a column chromatography ($CHCl_3$/MeOH/NH_4OH, 8:2:0.2 → 6:3:1), yielding the mixture of products 4a, 4b and 4c as a white solid (60%). MS-API-ES: 1040 $(M+H)^+$ (4a), 1026 $(M+H)^+$ (4b), 1012 $(M+H)^+$ (4c).

1,3,2′,6′-$(Cbz)_4$-gentamicin C_{1a} (5a), 1,3,2′,6′-$(Cbz)_4$-gentamicin C_2 (5b) and 1,3,2′-$(Cbz)_3$-gentamicin C_1 (6).

To a solution of free base gentamicin C_1, C_{1a} and C_2 (2a-c) (ratio = 25:35:40) (0.3 g, 0.64 mmol) in DMSO (4 ml) at 0°C was added N-benzyloxycarbonyl succinimide (0.52 g, 2.11 mmol). The mixture was stirred vigorously for 2 hours at room temperature. Subsequently, Et_2O was added (100 ml), observing the formation of a colorless oil. After solvent decantation the residue was purified by resin chromatography column Amberlite™ IRA-120-H⁺ (5 g), yielding a mixture of compounds 5a and 5b (0.17 g, 37%) when the resin was eluted with a 0.5M solution of NH_4OH in 1,4-dioxane/H_2O (1:1) and compound 6 (73 mg, 13%) when the concentration of NH_4OH was increased to 1M. MS-API-ES: 985 $(M+H)^+$ (5a), 1000 $(M+H)^+$ (5b) and MS-API -ES: 880 $(M+H)^+$ (6).

1,3,2′,6′-$(Cbz)_4$-3″-$(Boc)_2$-guanidine-gentamicin C_{1a} (7a) and 1,3,2′,6′-$(Cbz)_4$-3″-$(Boc)_2$-guanidinio-gentamicina C_2 (7b).

To a stirred solution of compounds 5a and 5b (0.17 g, 0.23 mmol) in 1,4-dioxane (11.7 ml) was added 1,3-$(Boc)_2$-2-(trifluoromethylsulfonyl)guanidine (0.134 g, 0.34 mmol) and Et_3N (95 µ L, 0.69 mmol). The mixture was stirred at room temperature for 5 days. Then, the solvent was removed under reduced pressure and finally the residue was purified in silica gel column chromatography (AcOEt →AcOEt/MeOH 9:1) to yield a mixture of 7a and 7b (0.20 g, 72%) as a white foam. ^1H NMR (DMSO-d6, 400 MHz) (selected signals): 7.60-7.20 (m, 40H), 5.35-5.27 (m, 4H), 5.25-4.95 (m, 10H), 2.10-1.70 (m, 4H), 1.50-1.40 (s, 18H), 1.20 (s, 6H). MS-API-ES: 1228 $(M+H)^+$ (7a), 1244 $(M+H)^+$ (7b).

3″-guanidine-gentamicin C_{1a} (1a) and 3″-guanidine-gentamicin C_2 (1b).

To a solution of compounds 7a and 7b (0.20 g, 0.165 mmol) in MeOH (3.2 ml) was added palladium on carbon (37 mg, 20% w/w) and acetic acid (1.0 ml). The reaction flask was purged three times and the mixture was stirred under a H_2 atmosphere overnight, then filtered over a Celite® pad, washed with methanol and concentrated to dryness. The crude residue was used in the following reaction without further purification. Finally, this material was subjected to deprotection under acidic conditions by dissolving it in DCM/TFA (2.5 ml, 4:1 v/v). The reaction mixture was stirred at r.t. for 5 h, then evaporated to dryness and co-evaporated twice with toluene. The residue thus obtained was dissolved in distilled water, and the clear supernatant was taken and freeze-dried to yield a white fluffy powder 1a and 1b (67 mg, 82% two steps) as their corresponding TFA salts. ^{1}H-NMR (D_2O, 400 MHz): δ 5.19 (d, J = 3.6, 2H), 5.05 (d, J = 3.9 Hz, 2H), 3.83 – 3.79 (m, 2H), 3.79 – 3.61 (m, 6H), 3.47-3.24 (m, 6H), 3.16 (t, J = 5.0 Hz, 2H), 3.02 (t, J = 5.8 Hz, 2H), 2.82 – 2.72 (m, 5H), 2.78 (s, 3H), 2.68 (s, 3H), 2.52 – 2.21 (m, 2H), 1.68-1.48 (m, 4H), 1.48 - 1.39 (m, 2H), 1.19 (d, J = 6.8 Hz, 3H), 1.16 (d, J = 6.9 Hz). ^{13}C-NMR (D_2O, 100 MHz): δ 157.5, 103.1, 90.1, 89.5, 89.3, 77.1, 76.0, 75.1, 72.9, 72.0, 70.4, 67.0, 53.4, 52.5, 52.3, 52.2, 52.1, 51.9, 51.5, 47.5, 38.3, 39.5, 30.1, 29.2, 28.5, 27.7, 25.2, 24.2, 20.3. MS-API-ES (m/z): 492 $(M+H)^+$ (1a), 506 $(M+H)^+$ (1b). HRMS (*m/z*) 461.5631 ($C_{19}H_{39}N_7O_6$, 461.5640) (1a), 475.3115 ($C_{20}H_{41}N_7O_6$, 475.3118) (1b).

MIC Determination

A solution of the selected bacterial strain was grown in 1 ml of Mueller-Hinton broth to an optical density (OD600) of 0.5units. The desired concentrations of guanidino-glycosides 1a-b were added from stock solutions. After incubation at 37°C for 24 h, the OD600 of each sample was read in duplicate. In both cases, the MIC was taken as the lowest antibiotic concentration inhibiting bacterial growth by greater than 90%.

In the case of the resistance strain expressing AAC (6′)-Ib, *E. coli* BL21 (DE3) containing the pET-AAC(6′)-Ib plasmid, enzyme production was induced with IPTG prior to addition of the antibiotics.

Results and Discussion

Chemistry

Recently, we have described the synthesis of 3″-guanidino kanamycin A by regioselective 3″-amino deprotection of tetra-Cbz-protected kanamycin A under basic conditions and subsequent guanidinylation [16]. Using a similar approach (Figure 3) 1,3,6′,2′-3″-$(Cbz)_5$-gentamicins (3a-c) were obtained by treatment of gentamicin (2a-c) with benzyl chloroformate in a Na_2CO_3 saturated aqueous solution and acetone mixture. However, when 3a-c were reacted with a 2.5M solution in a 1,4-dioxane and H_2O mixture at 50°C [19], the carbamate of position 3″ cyclized, obtaining a mixture of urethanes 4a-c as expected, but unfortunately these intermediates were too stable and did not undergo ring opening. The use of higher temperature and/or longer times and/or a higher concentration of base and/or a stronger base such as KOH at different concentrations caused side reactions with concomitant chemical decomposition of the starting material.

In view of these results a slightly different approach was employed (Figure 4). Treatment of gentamicin 2a-c with N-(benzyloxycarbonyloxy)succinimide in DMSO afforded a mixture of 6′,2′,1,3-$(Cbz)_4$-gentamicin C_{1a} (5a) and 6′,2′,1,3-$(Cbz)_4$-gentamicin C_2 (5b) in 37% (45:55) which was employed in the next reaction and 2′,1,3-$(Cbz)_3$-gentamicin C_1 (6) in 13% yield that was discarded [20]. Conversion of 3″-methyl amino groups to the corresponding guanidine was achieved by treatment of the mixture (5a-b) with 1,3-di-Boc-2-(trifluoromethylsulfonyl)guanidine and Et_3N [16], obtaining 6′,2′,1,3-$(Cbz)_4$-3″-guanidino$(Boc)_2$ gentamicins C_1 and C_2 (7a-b) in 72% yield. Catalytic hydrogenolysis of the Cbz groups and finally, acidic deprotection of the guanidine Boc groups, afforded a mixture of 3″-guanidino-gentamicins C_1 and C_2 (1a-b) in 82% yield as their TFA salt (Figure 4).

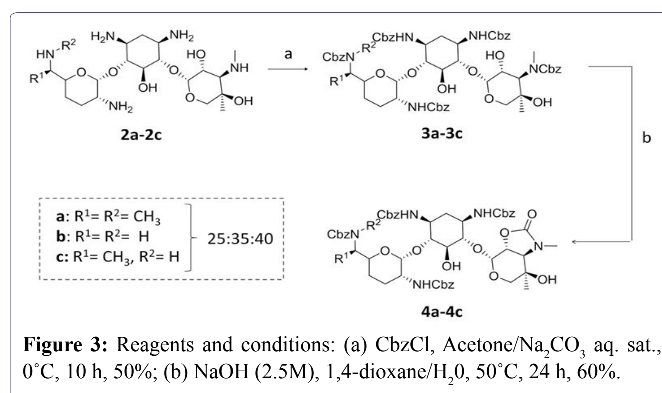


Figure 3: Reagents and conditions: (a) CbzCl, Acetone/Na_2CO_3 aq. sat., 0°C, 10 h, 50%; (b) NaOH (2.5M), 1,4-dioxane/$H_2$0, 50°C, 24 h, 60%.

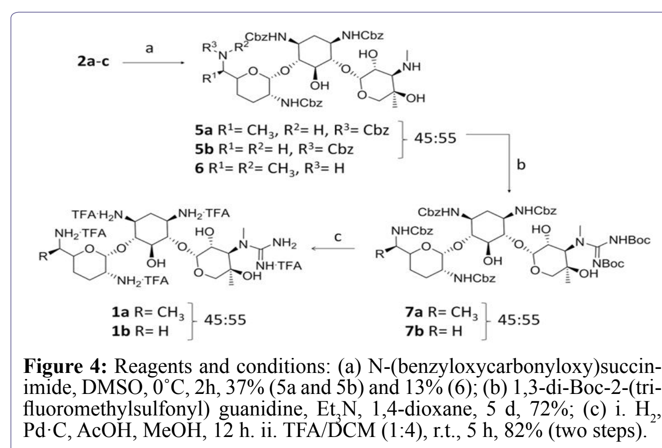
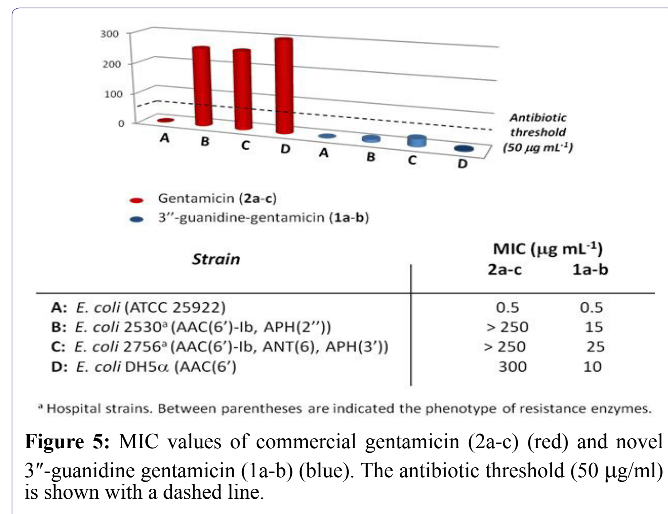


Figure 4: Reagents and conditions: (a) N-(benzyloxycarbonyloxy)succinimide, DMSO, 0°C, 2h, 37% (5a and 5b) and 13% (6); (b) 1,3-di-Boc-2-(trifluoromethylsulfonyl) guanidine, Et_3N, 1,4-dioxane, 5 d, 72%; (c) i. H_2, Pd·C, AcOH, MeOH, 12 h. ii. TFA/DCM (1:4), r.t., 5 h, 82% (two steps).

Antibacterial activities

In order to determine the biological activity of 3″-guanidino-gentamicin (1a-b), Minimum Inhibitory Concentration Experiments (MICs) were carried out against a wide variety of bacterial strains (Figure 5). According to our results, the mixture of gentamicin congeners (2a-c) is a powerful antibiotic against *E. coli* (strain ATCC 25922) (MIC= 0.5 µg/ml), which is in accordance with results previously reported [21]. However, when AAC (6′)-Ib is expressed, alone or in combination with APH (2″) or APH(3′), gentamicins (2a-c) completely lose the antibiotic activity (MIC > 250 µg/ml). To our delight, 3″-guanidino-gentamicins (1a-b) still maintained a significant antibiotic activity against *E.coli* and more importantly, against resistant strains expressing AAC (6′)-Ib, APH (2″) and APH (3′). Finally, we tested the effect of 1a-b against ANT (6), an aminoglycoside resistance enzyme which is only efficient on streptomycin. We chose this particular enzyme since previous (unpublished) results from our group showed that the introduction of guanidine moieties on kanamycin A turns this scaffold into a ligand (not a

substrate) of this enzyme. According to these results, we decided to test the new antibiotic against this enzyme to demonstrate that this modification does not transform gentamicin into a substrate either.



Strain	MIC (µg mL⁻¹) 2a-c	1a-b
A: *E. coli* (ATCC 25922)	0.5	0.5
B: *E. coli* 2530[a] (AAC(6')-Ib, APH(2''))	> 250	15
C: *E. coli* 2756[a] (AAC(6')-Ib, ANT(6), APH(3'))	> 250	25
D: *E. coli* DH5α (AAC(6')	300	10

[a] Hospital strains. Between parentheses are indicated the phenotype of resistance enzymes.

Figure 5: MIC values of commercial gentamicin (2a-c) (red) and novel 3″-guanidine gentamicin (1a-b) (blue). The antibiotic threshold (50 µg/ml) is shown with a dashed line.

Conclusion

In the present study, we have synthesized two new 3″-guanidino gentamicin derivatives. The guanidinylation at the N-3″ position not only maintains the biological activity against a simple *E.coli* strain, but also against a representative set of resistant strains expressing several AMEs (AAC(6′)-Ib, APH(2″), ANT(6′) and APH(3′)). This result provides an excellent starting point for the development of semisynthetic next generation compounds effective in the presence of AMEs and it is a confirmation of our previous results on regioselective modification of position 3″ of aminoglycoside antibiotics.

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