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ISOLATION AND SYNTHESIS OF 2″-*N*-FORMIMIDOYLISTAMYCINS A AND B, NEW ISTAMYCIN COMPONENTS*

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ABSTRACT

Two new istamycin components have been isolated from culture filtrates of *Streptomyces tenjimariensis*. Their structures were suggested to be 2″-*N*-formimidoylistamycins A and B by spectral analysis, and confirmed by synthesis starting from istamycins A_0 and B_0, respectively.

INTRODUCTION

Two 1,4-diaminocyclitol-containing aminoglycoside antibiotics, istamycins A and B, which exhibit strong inhibition against Gram-positive and -negative bacteria, have been discovered in culture filtrates of *Streptomyces tenjimariensis* nov. sp.[1]. As reported in the preceding paper[2], eight minor components, istamycins C, A_0, B_0, C_0, A_1, B_1, C_1, and A_2 were also isolated and their structures determined. We synthesized istamycin A and its demethyl derivatives from 3′,4′-dideoxyneamine[3,4], and 3-*O*-demethyl and 2″-*N*-formimidoyl derivatives of istamycin B (ref. 5). 2″-*N*-Formimidoylfortimicin A produced by *Dactylosporangium matsuzakiense* was reported by Inouye *et al.*[6] and named dactimicin[7].

We have found that 2″-*N*-formimidoylistamycins A and B accumulate in culture filtrates of *Streptomyces tenjimariensis* as the major components. In this report, the isolation and synthesis of 2″-*N*-formimidoylistamycins A and B are described.

RESULTS AND DISCUSSION

2″-*N*-Formimidoylistamycins A (**1**) and B (**2**) were carefully isolated from the cultured broth by adsorption onto Amberlite IRC-50 (70% Na^+) and elution with hydrochloric acid. By addition of sodium *p*-toluenesulfonate[8] as the organic counterion, these antibiotics in the eluate containing sodium chloride were adsorbed onto

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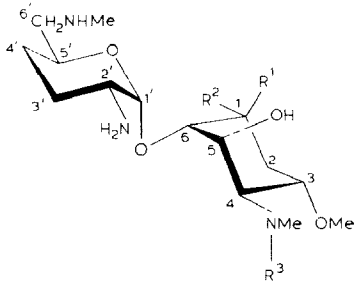
Diaion HP-20 (a macroreticular resin, Mitsubishi Chemical Industries), and separated by elution with 10μM hydrochloric acid containing methanol. Purification of **1** or **2** in each eluate was achieved by chromatography on Amberlite CG-50 followed by charcoal-column chromatography. Pure **1** and **2** were obtained as the disulfates by passing their solutions through a column of Amberlite IRA-400 (SO_4^{2-}).



	R^1	R^2	R^3 (1″2″)
Istamycin A	NH_2	H	$COCH_2NH_2$
Istamycin B	H	NH_2	$COCH_2NH_2$
Istamycin A_0 (in acid)	NH_2	H	H
Istamycin B_0	H	NH_2	H
2″-*N*-Formimidoylistamycin A (1)	NH_2	H	$COCH_2NHCH{=}NH$
2″-*N*-Formimidoylistamycin B (2)	H	NH_2	$COCH_2NHCH{=}NH$

Compounds **1** and **2** have strong antimicrobial activity similar to that of istamycin A and B. They show characteristic i.r. absorption at 1710 cm^{-1} (amidine group) and are unstable in alkaline solution. Treatment of **1** and **2** with 28% aqueous ammonia at room temperature for 30 min afforded istamycins A and B, respectively,

TABLE I

^{13}C-N.M.R. SPECTRA OF 2″-*N*-FORMIMIDOYLISTAMYCIN A (**1**) AND B (**2**) SULFATES[a]

Carbon atom	**1**	**2**	*Carbon atom*	**1**	**2**
1	49.4*d	47.1*d	2′	50.7*d	49.4*d
2	29.2 t	29.2 t	3′	21.6 t	21.3 t
3	71.3 d	71.9 d	4′	26.8 t	26.6 t
4	56.5 d	56.5 d	5′	66.3 d	66.5 d
5	69.9 d	68.0 d	6′	52.9 t	52.9 t
6	73.2 d	73.0 d	6′-NCH_3	34.3 q	34.3 q
3-OCH_3	56.7 q	56.5 q	1″	169.0 s	169.0 s
4-NCH_3	31.9 q	31.9 q	2″	44.2 t	44.1 t
1′	95.0 d	92.5 d	CH=NH	155.8 d	155.8 d

[a]Assignments (s, d, t, and q) show off-resonance multiplicities. Asterisks indicate that assignments within any vertical column may be reversed.

in good yield. From the data of ^{13}C-n.m.r. spectra (Table I), the structures of **1** and **2** were suggested[5] to be 2″-*N*-formimidoylistamycins A and B, respectively.

Synthesis of **1** was achieved by acylation of the 4-methylamino group of 1,2′,6′-tri-*N*-benzyloxycarbonylistamycin A_0 with the active ester of N-*tert*-butoxycarbonylglycine, removal of the 2″-N-*tert*-butoxycarbonyl group, and introduction of the 2″-*N*-formimidoyl group with either benzylformimidate hydrochloride or ethylformimidate hydrochloride[9], followed by removal of the *N*-protective groups. The 1,2′,6′-tri-*N*-protected istamycins A_0 and B_0 were synthesized by selective *N*-protection of istamycins A_0 and B_0 by utilizing a nickel or zinc complex between the vicinal, *cis* 4-methylamino and 5-hydroxyl groups[10,11].

EXPERIMENTAL

General methods. — Melting points were determined with a Yamato apparatus and are uncorrected. Optical rotations were measured with a Carl Zeiss LEP A2 polarimeter. I.r. spectra were recorded with a Hitachi Model 260-10 infrared spectrophotometer. The ^{1}H- and ^{13}C-n.m.r. spectra were recorded with a Varian XL-100 spectrometer. The ^{1}H-n.m.r. chemical shifts in D_2O refer to an external standard of tetramethylsilane ($\delta = 0$). The ^{13}C-n.m.r. chemical shifts in D_2O refer Me_4Si as internal standard. T.l.c. for istamycin components was performed on a plate of Silica Gel 60 (E. Merck) developed with 1:8 mixture of 1-propanol and water containing 20% sodium citrate and 5% citric acid; zones were detected by ninhydrin. High-voltage paper electrophoresis (p.e.) was performed on Toyo No. 51 paper with 1:3:36 formic acid–acetic acid–water electrolyte with a Savant Model LT-48A apparatus at 3,000 V for 15 min with cooling below 20°; components were detected by ninhydrin and their mobilities relative to alanine ($R_M = 1.0$) calculated. Bioactivities of antibiotics were determined by the cylinder-plate method against *Bacillus subtilis* PCI219 with pure istamycin B as the assay standard (as 1,000 μg/mg or 100%).

L.c. analysis of istamycin components. — Liquid chromatography was performed with a Waters 206A compact system with a Radial Pak C_{18} (0.5 × 10 cm, 10 μm) column that was developed with 5% aqueous methanol containing 0.3M sodium sulfate and 5mM PIC B7 (Waters Associates, 1-heptanesulfonic acid) as the mobile phase at a flow rate of 1 mL/min. The sample solution was introduced by injection of 5–30 μL and detection was by fluorescence with a reagent consisting of 0.05M borax–6mM *o*-phthalaldehyde in 0.5% aqueous 2-mercaptoethanol (excitation at 340 nm, emission at 425 nm, recorded on a Waters Data module 730). The sample solution was prepared as follows: antibiotics in the culture filtrate (1 mL) were adsorbed onto a column of Amberlite CG-50 (Type I, 70% Na^+, 0.5 mL); the column was washed with water (3 mL) and eluted with 0.25M sulfuric acid (3 mL); the eluate was adjusted to pH 4 and evaporated; and the residue was dissolved in water (1 mL). Retention times were as follows: istamycin A, 6.51; istamycin B, 7.75; formimidoylistamycin A, 8.71, and formimidoylistamycin B, 10.50 min.

Isolation of formimidoylistamycin A (**1**) *and B* (**2**). — *Streptomyces tenjimariensis*

SS939 on an agar slant was transferred into a 500-mL, baffled Erlenmeyer flask that contained 110 mL of a seed medium (2.0% starch, 0.2% glucose, 2.0% soybean meal, 0.2% sodium palmitate, 0.3% sodium chloride, 0.1% magnesium sulfate heptahydrate, and 0.1% potassium monohydrogenphosphate) and cultured for 24 h at 27° on a rotary shaker (180 r.p.m.). The seed culture (1.5 mL) was inoculated into 110 mL of a production medium containing 6.0% corn meal, 2.0% wheat germ, 0.6% calcium carbonate, and 0.05% magnesium sulfate heptahydrate (adjusted to pH 7.0 with M sodium hydroxide) in each flask and cultured for 7 days at 27° on a rotary shaker (180 r.p.m.).

The cultured broth in 182 flasks was harvested (20 L, bioactivity 113 μg/mL). After two filtrations of the broth at pH 2.5 and pH 7.0, the antibiotics in the filtrate (17 L) were adsorbed onto a column (6.5 × 45 cm) of Amberlite IRC-50 (70% Na^+, 1.5 L) and eluted with M hydrochloric acid. The eluate was collected in 17.5-mL fractions, and active fractions (nos. 47–205) were combined (2900 mL, bioactivity 437 μg/mL). L.c. of the active eluate showed 298 μg/mL for **1** and 153 μg/mL for **2**. After addition of sodium *p*-toluenesulfonate (46 g) and adjustment to pH 5.0 with M hydrochloric acid, the antibiotics in the solution were adsorbed onto a column (6 × 63 cm) of Diaion HP-20 (1.8 L). The column was washed with 3 L of 10μM hydrochloric acid and then eluted stepwise with 7.88, 7.88, 15.75, 13.13, and 10.50 L of 10μM hydrochloric acid in 5, 7.5, 10, 12.5, and 15% methanol, respectively. The eluate was collected in 17.5-mL fractions. Fractions (nos. 2201–2550) containing **1** were combined and concentrated to 1 L (bioactivity 632 μg/mL). Fractions (nos. 2551–3000) containing **2** were combined and concentrated to 1.1 L (bioactivity 284 μg/mL).

Compound **1** in the aforementioned concentrate was adsorbed onto a column (2 × 16 cm) of Amberlite CG-50 (Type I, Na^+, 50 mL) and eluted with 0.25M sulfuric acid (15-mL fractions). The active eluate (fractions 3–26) was concentrated to 180 mL. Compound **1** in a 60-mL portion of the concentrate (adjusted to pH 6.5) was adsorbed onto a column (1.5 × 35 cm) of charcoal (Wako Pure Chemical Industries, 12 g) that was washed with water (180 mL) and then eluted with 5mM sulfuric acid (5-mL fractions). The active eluate (fractions 31–130) was concentrated to 5 mL and made neutral with Amberlite IR-45 (OH^-). The solution was passed through a column (1.2 × 18.5 cm) of Amberlite IRA-400 (SO_4^{2-}, 21 mL) and the column was eluted with water (5-mL fractions). The active eluate (fractions 3–8) was lyophilized to yield a colorless, hygroscopic powder (162 mg) of **1** disulfate trihydrate, m.p. >210° (gradual decomp.), $[\alpha]_D^{19}$ +77° (*c* 1, water); ν_{max}^{KBr} 3400, 3000, 1710, 1640, 1490, and 1110 cm^{-1}; ^{1}H-n.m.r. δ 8.44 (s, 1 H, CH=N), 5.82 (d, 1 H, H-1), 4.87 (bs, 2 H, CH_2N), 3.93 (s, 3 H, 3-OCH_3), 3.64 (s, 3 H, 4-NCH_3), and 3.25 (s, 3 H, 6′-NCH_3); ^{13}C-n.m.r. see Table I; t.l.c. R_F 0.40; p.e. R_M 2.1; and bioactivity 66% of istamycin B.

Anal. Calc. for $C_{18}H_{36}N_6O_5 \cdot 2H_2SO_4 \cdot 3H_2O$: C, 32.42; H, 6.95; N, 12.60; S, 9.62. Found: C, 32.34; H, 6.52; N, 11.82; S, 9.69.

In similar manner to the purification of **1**, the aforementioned concentrate

(1.1 L, bioactivity 189 μg/mL) containing **2** was treated by chromatography on columns of Amberlite CG-50 (Type I, Na^+, 25 mL), charcoal (6 g), and Amberlite IRA-400 (SO_4^{2-}, 85 mL) to yield a colorless, hygroscopic powder (422 mg) of **2** disulfate tetrahydrate, m.p. >215° (gradual decomp.), $[\alpha]_D^{19}$ +82° (*c* 1, water); ν_{max}^{KBr} 3400, 3000, 1710, 1640, 1490, and 1110 cm^{-1}; ^{1}H-n.m.r. δ 8.39 (s, 1 H, CH=N), 5.85 (d, 1 H, H-1), 4.84 (s, 2 H, CH_2N), 3.90 (s, 3 H, 3-OCH_3), 3.53 (s, 3 H, 4-NCH_3), and 3.16 (s, 3 H, 6′-NCH_3); ^{13}C-n.m.r. see Table I; t.l.c. R_F 0.46; p.e.: R_M 2.1; and bioactivity 73% of istamycin B.

Anal. Calc. for $C_{18}H_{36}N_6O_5 \cdot 2\ H_2SO_4 \cdot 4\ H_2O$: C, 31.58; H, 7.07; N, 12.27; S, 9.37. Found: C, 31.40; H, 6.27; N, 11.58; S, 9.86.

Alkaline hydrolysis of formimidoylistamycin A (**1**) *and B* (**2**). — A solution of **1** (disulfate dihydrate, 80 mg) in 28% aqueous ammonia (4 mL) was kept for 30 min at room temperature and evaporated. L.c. of the residue showed no **1** but only istamycin A (79% yield). The residue was dissolved in water (8 mL) and the solution was passed through a column of Amberlite CG-50 (NH_4^+, 8 mL). The column was washed with water (8 mL), and then eluted with a linear gradient made from 75 mL each of 0.1 and 0.8M ammonium hydroxide (4-mL fractions). Fractions 18–22 were combined and evaporated to yield 35 mg (68%) of istamycin A hemicarbonate[2].

Similarly, alkaline hydrolysis of **2** (disulfate dihydrate, 100 mg) in 28% aqueous ammonia gave 49 mg (69%) of istamycin B carbonate hemihydrate[2].

1,2′,6′-Tri-N-*benzyloxycarbonylistamycin* A_0. — To a solution of istamycin A_0 hemicarbonate (788 mg, 2.17 mmol) in methanol (12 mL) was added nickel diacetate tetrahydrate (1.08 g, 4.34 mmol) with stirring for 3.5 h at room temperature. *N*-Benzyloxycarbonyloxysuccinimide (1.67 g, 6.73 mmol) was added and the mixture was stirred overnight at room temperature. Next was added 28% aqueous ammonia (4 mL) with stirring for 30 min and the resultant precipitate was removed by filtration. The filtrate was evaporated to give a greenish residue that was dissolved in chloroform (80 mL) and the solution washed twice with M ammonium hydroxide (20 mL) and water (20 mL). The organic layer was dried (sodium sulfate) and evaporated to afford a colorless solid that was purified by column chromatography on silica gel (150 g) developed with 10:1 chloroform–methanol to give a colorless powder of 1,2′,6′-tri-*N*-benzyloxycarbonylistamycin A_0 (1.02 g, 64%), m.p. 54–56°, $[\alpha]_D^{16}$ +62° (*c* 1.0, methanol).

*Synthesis of 2″-*N*-formimidoylistamycin A* (**1**). — To a solution of 1,2′,6′-tri-*N*-benzyloxycarbonylistamycin A_0 (931 mg, 1.27 mmol) in 1,4-dioxane (30 mL) were added triethylamine (0.42 mL) and a solution of *N*-hydroxysuccinimide ester of *N-tert*-butoxycarbonylglycine (830 mg, 3.0 mmol) in 1,4-dioxane (5 mL). The mixture was stirred for 4.5 h at 60° and evaporated. The residue was purified by column chromatography on silica gel (150 g) developed with 200:1 dichloromethane–ethanol, followed by a column of Sephadex LH-20 (100 mL) developed with methanol, and 1,2′,6′-tri-*N*-benzyloxycarbonyl-2″-*N-tert*-butoxycarbonylistamycin A (438 mg, 39%) was obtained.

To remove the *N-tert*-butoxycarbonyl group, the *N*-protected istamycin A

(388 mg, 0.44 mmol) was dissolved in 90% aqueous trifluoroacetic acid (5 mL). After 45 min at room temperature, the solution was evaporated and the residue washed with ethyl ether (20 mL) to yield 1,2′,6′-tri-*N*-benzyloxycarbonylistamycin A trifluoroacetate (375 mg). To a solution of the trifluoroacetate in a mixture of methanol (60 mL) and water (8 mL) was slowly (during 15 min) added a solution of benzylformimidate hydrochloride (426 mg, 2.5 mmol) in methanol (10 mL) at pH 8.5 (adjusted with 0.5M potassium hydroxide) under ice cooling. After stirring for 1 h, the solution was adjusted to pH 3.7 with M hydrochloric acid and evaporated. A solution of the residue in chloroform (100 mL) was washed twice with water (30 mL) and evaporated to give a crude powder (411 mg). The powder was purified by column chromatography on silica gel (20 g) developed with 20:1 chloroform–methanol to give 1,2′,6′-tri-*N*-benzyloxycarbonyl-2″-*N*-formimidoylistamycin A hydrochloride (193 mg, 54%).

The hydrochloride (193 mg, 0.24 mmol) was dissolved in 2:1:1 methanol–acetic acid–water (12 mL), and hydrogenated with 5% palladium-on-carbon (100 mg) under a hydrogen stream at atmospheric pressure for 4 h. After removal of the catalyst by filtration, the filtrate was evaporated. A solution of the residue (hydrochloride) in water (1 mL) was passed through a column of Amberlite IRA-400 (SO_4^{2-}, 12 mL) and the column was developed with water (12 mL). The effluent was evaporated to give **1** as the disulfate trihydrate (134 mg, 82%); m.p. >208° (gradual decomp.), $[\alpha]_D^{27}$ +82° (*c* 1.0, water).

1,2′,6′-Tri-N-tert-*butoxycarbonylistamycin* B_0. — To a solution of istamycin B_0 hemicarbonate hemihydrate (500 mg, 1.34 mmol) in methanol (20 mL) was added zinc diacetate dihydrate (500 mg, 2.3 mmol) with stirring for 5 h at room temperature. 2-(*tert*-Butoxycarbonyloxyimino)-2-phenylacetonitrile (BOC-ON, Aldrich, 1.71 g, 6.9 mmol) was added to the solution, which was stirred overnight at room temperature and then evaporated. The residue was purified by column chromatography on silica gel (100 g) developed with 10:1 chloroform–methanol to give a colorless powder of 1,2′,6′-tri-*N*-*tert*-butoxycarbonylistamycin B_0 (412 mg, 49%); m.p. 71–74°, $[\alpha]_D^{22}$ +50° (*c* 1.0, methanol).

*Synthesis of 2″-*N*-formimidoylistamycin B* (**2**). — To a solution of 1,2′,6′-tri-*N*-*tert*-butoxycarbonylistamycin B_0 (200 mg, 0.32 mmol) in 1,4-dioxane (6 mL) were added triethylamine (0.10 mL) and the *N*-hydroxysuccinimide ester of *N*-benzyloxycarbonylglycine (235 mg, 0.76 mmol). The mixture was stirred for 2 h at 60° and then evaporated. The residue was purified by column chromatography on silica gel (30 g) developed with 3:2 ethyl acetate–toluene, followed by a column of Sephadex LH-20 (30 mL) developed with methanol to yield 1,2′,6′-tri-*N*-*tert*-butoxycarbonyl-2″-*N*-benzyloxycarbonylistamycin B (246 mg, 94%), m.p. 104–110° (decomp.), $[\alpha]_D^{23}$ +44° (*c* 1.0, methanol).

To remove the *N*-benzyloxycarbonyl group, the *N*-protected istamycin B (237 mg, 0.29 mmol) was dissolved in a mixture of methanol (12 mL), acetic acid (0.01 mL), and water (3 mL), and hydrogenated with 5% palladium on carbon (50 mg) under a hydrogen stream at atmospheric pressure for 3 h. The catalyst was

removed by filtration and the filtrate evaporated to give 1,2′,6′-tri-*N*-*tert*-butoxycarbonylistamycin B acetate (237 mg). To a solution of the acetate (200 mg) in methanol (20 mL) was added ethylformimidate hydrochloride[7] (233 mg, 2.1 mmol). The solution was stirred for 4 h at room temperature and evaporated. The residue was purified by column chromatography on silica gel (42 g) developed with 7:1 chloroform–methanol, and 1,2′,6′-tri-*N*-*tert*-butoxycarbonyl-2″-*N*-formimidoylistamycin B hydrochloride (82 mg, 38%) was obtained.

The hydrochloride (54 mg, 0.07 mmol) was dissolved in 90% aqueous trifluoroacetic acid (2 mL). After 2 h at 0–5°, the solution was evaporated. The residue (trifluoroacetate) was dissolved in water (1 mL) and passed through a column of Amberlite IRA-400 (SO_4^{2-}, 4 mL). The column was developed with water (4 mL). The effluent was evaporated to give **2** as the disulfate dihydrate (45 mg, 97%); m.p. >202° (gradual decomp.), $[\alpha]_D^{17}$ +78° (*c* 1.0, water).

REFERENCES

1 Y. Okami, K. Hotta, M. Yoshida, D. Ikeda, S. Kondo, and H. Umezawa, *J. Antibiot.*, 32 (1979) 964–966.
2 D. Ikeda, Y. Horiuchi, M. Yoshida, T. Miyasaka, S. Kondo, and H. Umezawa, *Carbohydr. Res.*, 109 (1982) 33–45.
3 D. Ikeda, T. Miyasaka, M. Yoshida, Y. Horiuchi, S. Kondo, and H. Umezawa, *J. Antibiot.*, 32 (1979) 1365–1366.
4 D. Ikeda, Y. Horiuchi, S. Kondo, and H. Umezawa, *J. Antiobiot.*, 33 (1980) 1281–1288.
5 Y. Horiuchi, D. Ikeda, S. Kondo, and H. Umezawa, *J. Antiobiot.*, 33 (1980) 1577–1580.
6 S. Inouye, K. Ohba, T. Shomura, M. Kojima, T. Tsuruoka, J. Yoshida, N. Kato, M. Ito, S. Amano, S. Omoto, N. Ezaki, T. Ito, and T. Niida, *J. Antiobiot.*, 32 (1979) 1354–1356.
7 K. Ohba, T. Tsuruoka, K. Mizutani, N. Kato, S. Omoto, N. Ezaki, S. Inouye, T. Niida, and K. Watanabe, *J. Antibiot.*, 34 (1981) 1090–1100.
8 T. Kusama, M. Nobe, S. Gomi, Y. Ogawa, and K. Watanabe, unpublished data.
9 R. Ohme, and E. Schmitz, *Angew. Chem. Int. Ed. Engl.*, 6 (1967) 566.
10 T. L. Nagabhushan, A. B. Cooper, W. N. Turner, H. Tsai, S. McCombie, A. K. Mallams, D. Rane, J. J. Wright, P. Reichert, D. L. Boxler, and J. Weinstein, *J. Am. Chem. Soc.*, 100 (1978) 5253–5254.
11 T. Tsuchiya, Y. Takagi, and S. Umezawa, *Tetrahedron Lett.*, (1979) 4951–4954.