mp 207–210 °C dec; FAB-MS (glycerol) m/z 479 (MH$^+$); $C_{25}H_{18}O_{10}$; UV (50% MeOH) λ_{max} 237 nm (ϵ 29 600), 283 (20 100), 471 (9600); (0.01 N HCl–50% MeOH) precipitation; (0.01 N NaOH–50% MeOH) λ_{max} 224 (26 900), 274 (23 000), 320 (10 400), 509 (11 600); IR (KBr) 3400, 1600, 1440, 1380, 1290, 1255, 1185, 1160, 1120 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 2.58 (3 H, s), 3.89 (3 H, s), 4.17 (1 H, dd, J = 3.9 and 11.1), 4.26 (1 H, dd, J = 3.4 and 11.1), 5.69 (1 H, br s), 5.91 (1 H, br s), 6.67 (1 H, br d, J = 2.1), 6.86 (1 H, s), 7.05 (1 H, br d, J = 2.1), 8.02 (1 H, s), 13.05 (1 H, br s), 14.01 (1 H, s); ^{13}C NMR (DMSO-d_6) δ 186.3, 181.0, 170.1, 165.7, 165.7 164.5, 160.0, 145.9, 142.5, 137.5, 137.5, 132.1, 130.7, 125.9, 119.5, 117.8, 115.9, 113.2, 110.5, 105.9, 105.0, 72.2, 71.7, 55.9, 22.9.

Zinc Dust Distillation of 3. A mixture of **3** (50 mg) and zinc dust (500 mg) was placed in the bulb of a long glass tube (7 × 500 mm), which was then sealed. The bulb was heated over a burner to a red glow and kept for 30 s. After cooling, the tube was broken above the bulb and the upper piece containing the distillate was rinsed with diethyl ether. The ether extract was evaporated to dryness, which was developed on a preparative TLC plate (SiO_2, hexane–benzene, 9:1). The yellow band (R_f 0.41) was cut off and eluted from the silica gel with CH_2Cl_2. Evaporation of the solvent yielded a yellow liquid of **7**: UV λ_{max} (n-heptane) nm 220, 252, 258, 292, 302, 316, 355, 374, 398, 422, 449; EI-MS m/z 292 (M$^+$). These data were consistent with a methyl benzo[a]naphthacene.

Isolation of Amino Sugar 6. Pradimicin A (600 mg) was treated with Ac_2O (6 mL) in MeOH (130 mL) at room temperature overnight. Concentration of the mixture in vacuo afforded a red solid of N-acetyl **1a** (558 mg). This solid (407 mg), without further purification, was hydrolyzed with 5.2 N HCl–MeOH (90 mL) under reflux temperature for 2.5 h. The reaction mixture was neutralized with 6 N NaOH and concentrated to an aqueous solution, which was loaded on a column of Diaion HP-20 (100 mL). The column was eluted with water, and the ninhydrin-positive eluate was evaporated. The residue was chromatographed on Amberlite CG-50 (H$^+$, 60 mL) with elution of 0.01 N HCl. The ninhydrin-positive fractions were pooled, concentrated to dryness (21.2 mg), charged on a column of Sephadex LH-20 (80 mL), and developed with 50% MeOH. Evaporation of eluate containing the sugar afforded a pale-yellow solid (**6α** and **6β**, 7.5 mg): $[\alpha]^{26}_D$ +87.5° (c 0.3, H_2O); EI-MS m/z 191 (M$^+$), 160 (M – OCH_3)$^+$.

All protons of **6α** and **6β** was unequivocally assigned by the ^{1}H–^{1}H COSY experiment. Thus, **6** was identified as an anomeric mixture of methyl 4,6-dideoxy-4-(methylamino)-D-galactopyranoside. Pradimicin C was hydrolyzed in the same way as **1a** and yielded a pale-yellow solid of methyl 4,6-dideoxy-4-amino-D-galactoside mixture (6.2 mg, α:β = 78:22): $[\alpha]^{26}_D$ +89.8° (c 0.29, H_2O); SI-MS (glycerol) m/z 178 (MH$^+$), 200 (M + Na)$^+$.

Identity with methyl 4,6-dideoxy-4-amino-D-galactopyranoside was confirmed by a direct comparison with an authentic synthetic sample.[10]

Supplementary Material Available: ^{1}H NMR chemical shift data of **1a**, **1b**, **1c**, **2**, **3**, and **4** in dimethyl-d_6 sulfoxide and those of **6α**, **6β**, and methyl 4,6-dideoxy-4-amino-D-galactopyranoside in deuteroxide (3 pages). Ordering information is given on any current masthead page.

Biosynthesis of Pradimicin A

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Received December 8, 1988

The biosynthesis of pradimicin A (**1**) has been studied by feeding sodium [1-^{13}C]-, [2-^{13}C]-, and [1,2-$^{13}C_2$]acetates and D- and L-[1-^{13}C]alanines to the producing organism *Actinomadura hibisca* sp. P157-2 (ATCC 53557). ^{13}C NMR spectroscopy established that the aglycon moiety of **1** is derived from 1 alanine unit and 12 acetate units, condensed in the "head-to-tail" fashion typical of polyketide biogenesis. Of particular interest is the efficient incorporation of D-alanine into **1**, suggesting that D-alanine might act as the direct precursor for the D-alanine side chain of **1**.

Introduction

Pradimicin A (**1**), a new antibiotic, has been found in the culture filtrate of *Actinomadura hibisca* sp. P157–2 (ATCC 53557).[1-3] The antibiotic is active in vitro against a wide variety of fungi and yeasts, some Gram-positive bacteria, and viruses. More interestingly, **1** demonstrates in vivo therapeutic activity against systemic fungal infections caused by *Candida albicans* A9540, *Aspergillus fumigatus* IAM2530, and *Cryptococcus neoformans* IAM4514 in mice. The closely related antibiotics benanomicins A (**2**) and B (**3**) have been reported to be produced by an actinomycete, MH193-16F4.[4] Structurally, all of these compounds contain a glycosylated benzo[a]naphthacenequinone that has a D-alanine side chain. As part of our microbial modification program, we initiated a biosynthetic study of pradimicin A by *A. hibisca* sp. P157-2. This paper presents the spectroscopic analysis of ^{13}C-labeled samples of **1**, which established the biosynthesis of the aglycon of **1**.

Results

[1-^{13}C]-, [2-^{13}C]-, and [1,2-$^{13}C_2$]acetates and D- and L-[1-^{13}C]alanines were fed to cultures of *A. hibisca* sp. P157-2 to establish the biosynthetic origin of the aglycon moiety of **1**. The ^{13}C-enriched samples of **1** thus formed were isolated and the positions of the ^{13}C-enriched carbon atoms determined by ^{13}C NMR spectroscopy.

Acetate Connectivity in 1. Accurate chemical shift assignment of each carbon of **1** was essential in determining which pairs of carbons originate from the same molecule of acetate. In the initial ^{13}C NMR experiments chemical shifts of some of the carbons in **1** crossed over or coalesced at certain pH's, which seemed to occur due to the zwitterionic nature of **1**. However, when **1** was isolated as a water-insoluble solid by adjusting an aqueous solution of

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[1] (a) Bristol Myers, USSN 10058, February 2, 1987. (b) Disclosed as BMY-28567 at the 27th ICAAC, New York, NY, October, 1987; Abstr. No. 984.

[2] Oki, T.; Konishi, M.; Tomatsu, K.; Tomita, K.; Saitoh, K.; Tsunakawa, M.; Nishio, M.; Miyaki, T.; Kawaguchi, H. *J. Antibiot.* **1988**, *41*, 1701.

[3] Tsunakawa, M.; Nishio, M.; Ohkuma, H.; Tsuno, T.; Konishi, M.; Naito, T.; Oki, T.; Kawaguchi, H. *J. Org. Chem.*, in press.

[4] (a) Takeuchi, T.; Hara, T.; Naganawa, M.; Okada, M.; Hamada, M.; Umezawa, H.; Gomi, S.; Sezaki, M.; Kondo, S. *J. Antibiot.* **1988**, *41*, 807. (b) Gomi, S.; Sezaki, M.; Kondo, S.; Hara, T.; Naganawa, H.; Takeuchi, T. *J. Antibiot.* **1988**, *41*, 1019.

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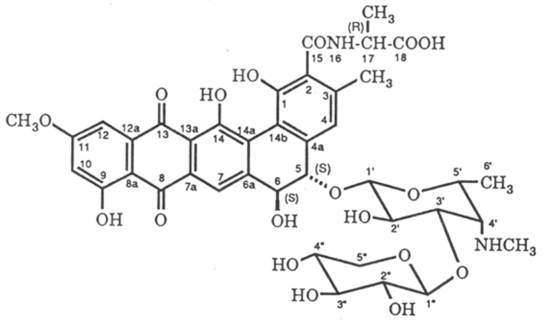
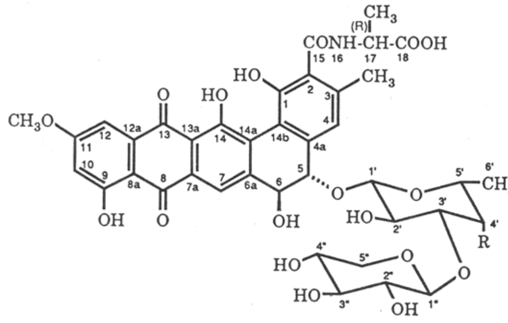
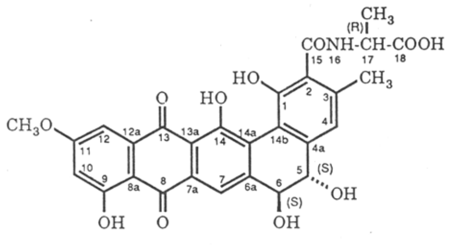


Pradimicin A (1)



Benanomicin A (**2**) R=OH
Benanomicin B (**3**) R=NH2

its hydrochloride to pH 5.5 and dried, all 40 carbon signals consistently appeared in DMSO-d_6 at 60 °C. Assignment of ^{13}C NMR signals of **1** was made with the aid of ^{1}H–^{13}C shift correlation spectroscopy (hetero COSY) and long-range heteronuclear COSY. In addition to these standard carbon assignment techniques, the carbon assignment was ascertained by single-labeling experiments using sodium [1-^{13}C]- and [2-^{13}C]acetates. Feeding of sodium [1-^{13}C]-acetate to growing cultures of *A. hibisca* gave rise to **1**, which was labeled at 12 alternating positions (see Table I for enrichment). A similar experiment with sodium [2-^{13}C]acetate enhanced the ^{13}C NMR signals for 12 carbons adjacent to the first set (see Table I). Acid hydrolysis of these two samples of **1**, derived from the single-labeling experiments, afforded ^{13}C-enriched samples of the aglycon **4**. The data for **4** were consistent with the labeling pattern shown in Table I.



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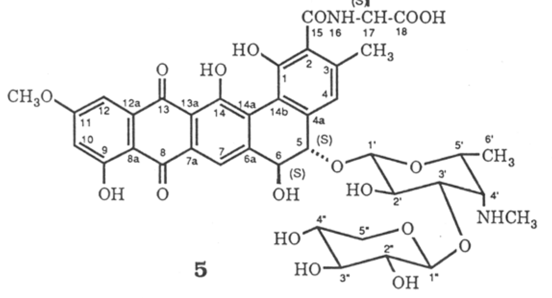

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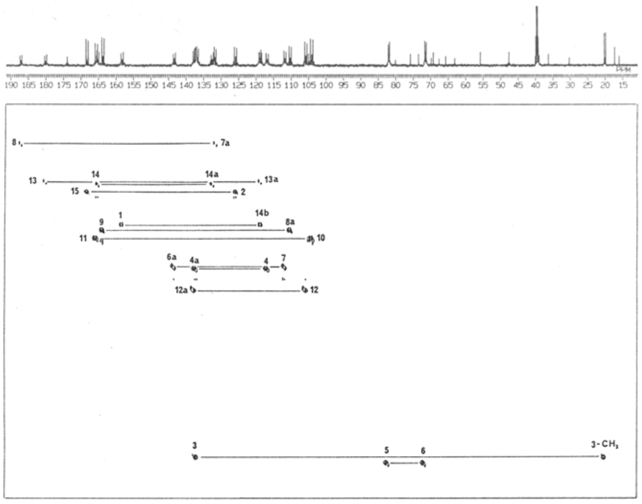

Figure 1. 2D INADEQUATE spectrum of **1** in DMSO-d_6 at 60 °C from cultures supplemented with sodium [1,2-$^{13}C_2$]acetate.

Table I. ^{13}C Chemical Shifts and ^{13}C Enrichments in Pradimicin A (1) Derived from ^{13}C-Labeled Precursors

carbon	^{13}C chem shift,[a] ppm	mult[b]	relative ^{13}C enrichments[c] in 1 derived from [1-^{13}C]-OAc[d]	[2-^{13}C]-OAc[d]	D-[1-^{13}C]-Ala[d]	L-[1-^{13}C]-Ala[d]
1	158.0	s	9.5			
2	126.0	s		9.8		
3	136.6	s	19.3			
4	116.6	d		7.1		
4a	137.4	s	15.8			
5	81.9	d		12.7		
6	71.6	d	18.4			
6a	143.4	s		12.2		
7	111.3	d	11.0			
7a	131.8	s		9.7		
8	187.1	s	13.9			
8a	110.2	s		8.2		
9	163.7	s	11.9			
10	104.0	d		12.5		
11	165.6	s	12.5			
12	105.7	d		15.1		
12a	137.8	s	9.6			
13	180.0	s		13.6		
13a	118.9	s	8.7			
14	165.8	s		17.1		
14a	132.8	s	11.6			
14b	118.7	s		10.2		
15	168.1	s	12.4			
17	47.5	d				
17-CH_3	17.2	q				
18	173.9	s			72.1	23.8
3-CH_3	20.0	q		11.6		
11-OCH_3	55.8	q				
1′	104.0	d				
2′	70.0	d				
3′	80.2	d				
4′	63.1	d				
5'	67.9	d				
4′-NCH_3	36.4	q				
5′-CH_3	16.1	q				
1″	104.9	d				
2″	73.5	d				
3″	75.8	d				
4″	69.2	d				
5″	65.6	t				

[a] 100.4-MHz ^{13}C NMR spectrum in DMSO-d_6 (40 mg/mL) at 60 °C with solvent reference at 39.50 ppm. [b] Multiplicities determined from DEPT spectra. [c] Ratio of carbon signal intensities for enriched and natural abundance samples measured under identical conditions: for normalization the methoxy carbon signal at 55.8 ppm was used as reference. [d] OAc = acetate; Ala = alanine.

Table II. ^{13}C-^{13}C Coupling Constants for $[1,2-^{13}C_2]$Acetate-Enriched 1

coupled carbons	*J*, Hz	coupled carbons	*J*, Hz
C(1)-C(14b)	66.4	C(7a)-C(8)	53.9
C(2)-C(15)	65.3	C(8a)-C(9)	61.6
C(3)-C(3-CH_3)	43.6	C(10)-C(11)	69.3
C(4)-C(4a)	61.6	C(12)-C(12a)	65.3
C(5)-C(6)	40.4	C(13)-C(13a)	58.7
C(6a)-C(7)	60.2	C(14)-C(14a)	61.2

With the spectral assignment complete, the acetate connectivity could be determined. Feeding of sodium $[1,2-^{13}C_2]$acetate gave rise to 12 pairs of coupled signals in the ^{13}C NMR spectrum of 1. The contour plots of the 2D INADEQUATE experiment[5] (Figure 1) uncovered the ^{13}C–^{13}C connectivities listed in Table II.

Origin of the D-Alanine Side Chain in 1. Pradimicin A (1) contains a D-alanine side chain. No L-alanine isomer **5** was present in the culture filtrate when analyzed by HPLC and compared with an authentic sample of **5**.[6]

When either D- or L-[1-^{13}C]alanine was fed to growing cultures of *A. hibisca*, production of 1 remained unaffected and no **5** could be detected in the crude fermentation broth by HPLC. In both cases the ^{13}C NMR signal assignable to the carboxyl carbon atom in the D-alanine side chain of 1 was significantly enriched (see Table I).

Discussion

The ^{13}C spectroscopic data obtained in this study established the biosynthetic origin of the carbon atoms and the C–C bonds transferred intact from acetate in the aglycon moiety of pradimicin A (1) as summarized in Figure 2. The disaccharide moiety of 1 is presumably derived from glucose.

Of particular interest is the efficient incorporation of both D- and L-alanines into the D-alanine side chain of 1, suggesting that D-alanine might act as the direct precursor for the side chain. In microorganisms D-alanine is an essential component for cell-wall peptidoglycan biosynthesis and is produced from L-alanine by alanine racemase.[7] The apparent difference in ^{13}C incorporation rate between the two alanine isomers, however, may have resulted from the difference in intracellular concentration between D- and L-alanines over the antibiotic production period.

Experimental Section

General Procedures. Sodium [1-^{13}C]acetate (90 atom % ^{13}C) was purchased from ICN Biochemical Inc. Sodium [2-^{13}C]- and $[1,2-^{13}C_2]$acetates (>99.6 atom % ^{13}C) were obtained from Aldrich Chemical Co. D- and L-[1-^{13}C]alanines were purchased from Merck and Co. Ultraviolet (UV) and infrared (IR) spectra were recorded on a JASCO UVIDEC-610C spectrophotometer and a JASCO IR-810 infrared spectrophotometer, respectively. Nuclear magnetic resonance (NMR) spectra were recorded on a JEOL JNM-GX400 instrument with DMSO-d_6 as internal standard for ^{13}C NMR spectra. The 2D INADEQUATE spectrum was recorded at 100.4 MHz with a relaxation delay of 6.0 s. The data were obtained, accumulating 256 scans over a 18.2-KHz sweep width in F2 (4K data points) with a 36.4-KHz spectral width in F1 sampled in 256 increments.

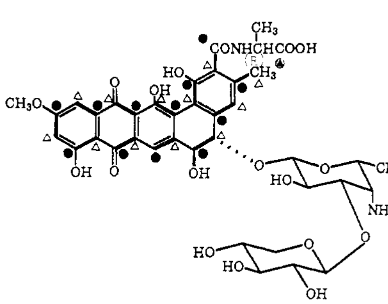

Figure 2. Labeling sites in 1 from [1-^{13}C]acetate (●), [2-^{13}C]-acetate (△), D-[1-^{13}C]alanine (○), and L-[1-^{13}C]alanine (▲); solid bars indicate intact transfer of ^{13}C–^{13}C acetate bonds. Symbols are superimposed for carbon atoms labeled by two precursors in separate experiments.

Fermentation. *A. hibisca* sp. P157-2 (ATCC 53557) was grown at 28 °C for 10 days on medium A (see below). This culture could be stored on medium A at 4 °C for at least 30 days. The seed culture was incubated for 7 days at 28 °C in medium B (see below) in 10-mL volumes in 50-mL Erlenmeyer flasks and aliquots (5 mL) were used as inocula to start all fermentations (medium B). Each feeding experiment was carried out in 100-mL volume in a 500-mL Erlenmeyer flask shaken at 200 rpm for 5 days (120 h) at 28 °C. In all cases ^{13}C-enriched carbon sources were dissolved in distilled water (for singly labeled acetates, 500 mg in 4 mL of water; for doubly labeled acetate, 240 mg in 4 mL of water; for labeled alanines, 300 mg in 3 mL of water). Each solution was sterilized by filtration and a 1-mL aliquot was fed to 100 mL of growing cultures of *A. hibisca*. When labeled acetates were used as ^{13}C sources, additions commenced 48 h after inoculation and continued at 12-h intervals through the 84th hour of the growth period. When labeled alanines were used as ^{13}C sources, additions were made at 48 h, 60 h, and 72 h after inoculation.

The following media were used for this study: Medium A was used for slant culture and consisted of soluble starch (0.5%), glucose (0.5%), fish meat extract (0.1%), yeast extract (0.1%), NZ-case (0.2%), NaCl (0.2%), $CaCO_3$ (0.1%), and agar (1.6%). The pH was adjusted to 7.0 before sterilization. Medium B was used as both the liquid seed medium and the production medium for all the feeding studies and was composed of glucose (3%), soy bean meal (3%), Pharmamedia (0.5%), yeast extract (0.1%), and $CaCO_3$ (0.3%). The pH was adjusted to 7.0 before sterilization.

Isolation of Pradimicin (1). The following procedure is an example of the routine isolation of 1 from 100 mL of fermentation broth. This was also used for preparing samples for analysis by ^{13}C NMR spectroscopy.

The mycelia were separated from the whole broth by centrifugation (10 min at 3000 rpm) in a 250-mL polypropylene bottle. The supernatant was adjusted to pH 2.0 with HCl, centrifuged, and filtered through Whatman No. 2 filter paper. The filtrate was adjusted to pH 5.5 with NaOH and placed in a cold room at 5 °C for 0.5 h. The resulting precipitate was collected by centrifugation and then agitated with 1-butanol (30 mL), methanol (10 mL), and water (40 mL) at pH 2.0. The organic layer was collected and the aqueous layer extracted with 1-butanol (30 mL) and methanol (10 mL). The combined organic layers were diluted with water (80 mL) and adjusted to pH 9.5 and the aqueous layer was collected. The organic layer was extracted with 40 mL of water and the combined aqueous layers were concentrated in vacuo to remove traces of the 1-butanol. The aqueous concentrate was adjusted to pH 2.0 and adsorbed on a column of Diaion HP-20 (50 mL). After washing the column with water (500 mL), the antibiotic was eluted with 80% aqueous acetone adjusted to pH 2.5 (100 mL). The eluate was concentrated to 1 mL and lyophilized to give semipure pradimicin A hydrochloride (100–150 mg). Its purity was estimated by comparing visible absorbance at 500 nm in 0.01 N NaOH and HPLC integration reading on a YMC A-301-3 ODS column (3 μm, 4.6 × 100 mm, 254 nm detection) using 20–55:80–45 acetonitrile–0.15% phosphate buffer (pH 3.5) as eluent with an authentic sample of 1 and was usually >85%.

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[5] Mareci, T. H.; Freeman, R. *J. Magn. Reson.* **1982**, *48*, 158.

[6] The authentic sample was prepared by warming *N*-(benzyloxycarbonyl)pradimicin A in formic acetic anhydride followed by hydrogenolysis and chromatographic purification (Konishi, M.; et al, unpublished results).

[7] (a) Wasserman, S. A.; Walsh, C. T.; Botstein, D. *J. Bacterial.* **1983**, *153*, 1439. (b) Wasserman, S. A.; Daub, E.; Grisafi, P.; Botstein, D.; Walsh, C. T. *Biochemistry* **1984**, *23*, 5182.

Column chromatography of the semipure 1 on a YMC ODS A60 (40–63 μm) eluting with 22:78 acetonitrile–0.15% phosphate buffer (pH 3.5) followed by adsorption on a column of HP-20 and elution with 80% aqueous acetone (pH 2.5) afforded pure pradimicin A hydrochloride. Zwitterionic pradimicin A was obtained by adjusting the hydrochloride solution to pH 5.5 with NaOH. The resulting precipitate was collected by centrifugation, washed successively with methanol and acetone, and dried at 60 °C under vacuum for at least 24 h. ^{13}C NMR data are shown in Table I. A 40-mg sample of 1 enriched with [1,2-$^{13}C_2$]acetate was dissolved in 0.6 mL of DMSO-d_6 for the 2D INADEQUATE experiment.

Preparation of the ^{13}C-Labeled Aglycon (4). Acid hydrolysis of the two ^{13}C-labeled samples of 1 enriched with [1-^{13}C]- and [2-^{13}C]acetates was conducted as usual (6 N HCl, 110 °C, 12 h), but before reaction, each sample was diluted with unlabeled 1 (three times by weight). In both cases the products were washed with water, adsorbed on a column of HP-20, and eluted with 80% aqueous acetone. For the unlabeled 4: ^{13}C NMR (100.4-MHz, DMSO-d_6, 40 mg/mL, 60 °C) δ 187.2 (C-8), 180.2 (C-13), 174.2 (C-18), 168.1 (C-15), 166.8 (C-14), 165.5 (C-11), 163.7 (C-9), 157.1 (C-1), 145.3 (C-6a), 140.5 (C-4a), 137.9 (C-12a), 136.2 (C-3), 133.3 (C-14a), 131.8 (C-7a), 125.9 (C-2), 118.6 (C-13a), 118.5 (C-14b), 114.9 (C-4), 110.7 (C-7), 110.2 (C-8a), 105.7 (C-12), 103.9 (C-10), 72.4 (C-6), 71.6 (C-5), 55.7 (CH_3O at C-11), 47.8 (C-17), 19.8 (CH_3 at C-3), 17.4 (CH_3 at C-17). For the ^{13}C-labeled 4 derived from [1-^{13}C]-acetate, ^{13}C NMR spectra were identical except for enrichment of the following 12 carbons: C-8, C-15, C-11, C-9, C-1, C-4a, C-12a, C-3, C-14a, C-13a, C-7, and C-6. For the ^{13}C-labeled 4 derived from [2-^{13}C]acetate, ^{13}C NMR spectra were identical except for enrichment of the following 12 carbons: C-13, C-14, C-6a, C-7a, C-2, C-14b, C-4, C-8a, C-12, C-10, C-5, and CH_3 at C-3.

Acknowledgment. We thank Mr. T. Moriyama and Mr. N. Tsunoda for running the ^{13}C NMR spectra.

A Facile, Practical Synthesis of 2,6-Dideoxy-2,6-imino-7-*O*-β-D-glucopyranosyl-D-*glycero*-L-*gulo*-heptitol (MDL 25,637)

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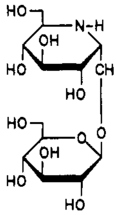
Received July 15, 1988

A facile synthetic route useful for large-scale preparation of the α-glucosidase inhibitor, 2,6-dideoxy-2,6-imino-7-*O*-β-D-glucopyranosyl-D-*glycero*-L-*gulo*-heptitol (1), is described. The protected heptononitrile 5, prepared in three steps from the readily available bisulfite adduct of nojirimycin (2), was stereospecifically converted to carboxylic acid 6 by acid hydrolysis (90% TFA/Hg(TFA)$_2$) and oxidation (N_2O_4). After reduction, the resultant amino alcohol 7 was N-protected and condensed with 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl trichloroacetimidate to provide glucoside 9. Stepwise deprotection of 9 with transfer hydrogenation and base-catalyzed hydrolysis gave compound 1 in 26% overall yield from 2.

Inhibitors of α-glycosidases and glycoprotein trimming enzymes[1] have potential therapeutic uses in diabetes mellitus,[2] tumor metastases,[3] and acquired immunodeficiency syndrome.[4] A potent α-glucosidase inhibitor, 2,6-dideoxy-2,6-imino-7-*O*-β-D-glucopyranosyl-D-*glycero*-L-*gulo*-heptitol (1), has been identified as a drug candidate for antidiabetic therapy.[5] In the course of preparing quantities of 1, an alternative synthesis was developed to eliminate isomeric separations that were required in the original route.[6] Herein we describe a facile synthetic sequence for the preparation of 1 (Scheme I).



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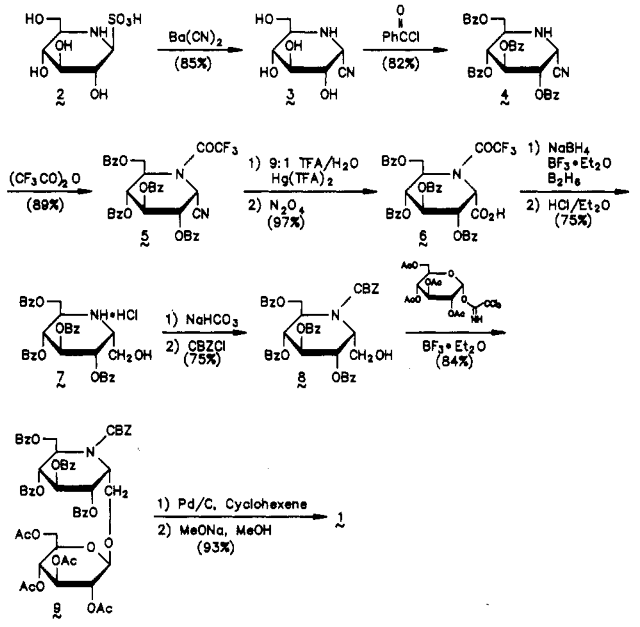
Scheme I



The readily available bisulfite adduct of nojirimycin (2)[7] was selected as a suitable starting material. The additional hydroxymethyl moiety in 1 was appended in a latent form as a nitrile with expectations of its ready conversion to an alcohol. However, even though nitrile 3 was prepared in

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[†] Indianapolis, IN.