Biotransformation of 1,4-Dihydro-2,6-dimethyl-4-(2-trifluoromethylphenyl)-3,5-pyridinedicarboxylic Acid Diethyl Ester

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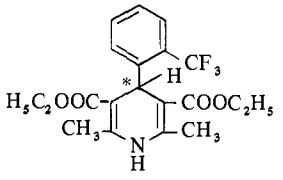
The biotransformation in the dog of 1,4-dihydro-2,6-dimethyl-4-(2-trifluoromethylphenyl)-3,5-pyridinedicarboxylic acid diethyl ester (I) has been studied. Principal urinary metabolites isolated and identified were 2,6-dimethyl-4-(2-trifluoromethylphenyl)-3,5-pyridinedicarboxylic acid (IV), its monoethyl ester (III), 6-hydroxymethyl-2-methyl-4-(2-trifluoromethylphenyl)-3,5-pyridinedicarboxylic acid γ lactone (V), and 2,6-dimethyl-4-(2-trifluoromethylphenyl)-3,5-pyridinedicarboxylic acid mono-2-hydroxyethyl ester (VI). Final proof of structure was by comparison to authentic samples. The synthesis of V and VI is described.

The compound, I, 1,4-dihydro-2,6-dimethyl-4-(2-trifluoromethylphenyl)-3,5-pyridinedicarboxylic acid diethyl ester has been shown in our laboratories to have hypotensive activity.[1,2] The biotransformation of this compound in the dog is reported in this communication; its absorption and excretion in various species have been studied and will be reported in detail elsewhere.[3]



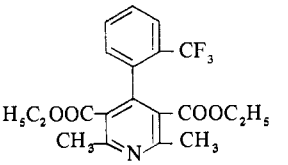
I

*Position of ^{14}C label

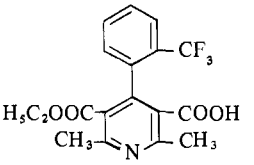
Possible biotransformations of I include aromatization,[4] ester hydrolysis, and hydroxylation. Aromatization was observed but not without partial or complete hydrolysis of the ester groups (III and IV). Two unexpected products were found, a lactone [6-hydroxymethyl-2-methyl-4-(2-trifluoromethylphenyl)-3,5-pyridinedicarboxylic acid γ lactone (V)] and a β-hydroxy ethyl ester [2,6-dimethyl-4-(2-trifluoromethylphenyl)-3,5-pyridinedicarboxylic acid mono-2-hydroxyethyl ester (VI)]. Compounds resulting from hydroxylation of the aromatic ring were not found among the major products.

Results and Discussion

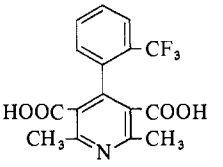
Isolation and Identification of Metabolites. Urine was collected from a dog dosed orally with I labeled with ^{14}C at the 4 position on the pyridine ring.† Other studies[3] have shown that after oral administration of the drug in 50% aqueous polyethylene glycol solution about 31% of the dose was excreted in the urine and about 43% of the dose in the feces in 24 hr; the major fecal component was unchanged I. Preliminary thin-layer chromatography on silica gel in systems A and C (Table I) showed that the urine contained four main metabolites, none of which was the starting drug I or the aromatized drug II.‡



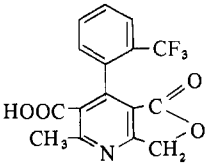
II



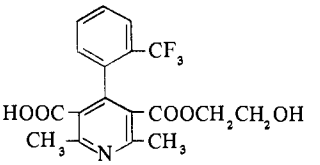
III



IV



V



VI

In another preliminary experiment, aliquots of urine adjusted to pH values from 1 to 12 at unit intervals were extracted with chloroform, ethyl ether, and hexane, respectively, and the extracts were counted. The per cent of radioactivity extracted at each pH was calculated and the results are shown in Figure 1. It is clear that some radioactivity which extracts with chloroform at pH 5 does not extract with ether. Also, some radioactivity which extracts with ether at pH 1 does not extract with chloroform. This

Table I. Mobility of Standard Compounds

Compd	R_f values in various systems[a]					
	A	B	C	D	E	F
I	0.99	0.98	0.24	0.95	0.98	0.86
II	0.99	0.98	0.46	0.95	0.98	0.90
III	0.65	0.25	0.06	0.75	0.48	0.21
IV	0.32	0.07	0.00	0.46	0.00	0.05
V	0.63	0.21	0.00	0.70	0.42	0.44
VI	0.64	0.24	0.00	0.74	0.44	0.24

[a]The systems used were: A, Merck Silica F_{254}, $CHCl_3$-MeOH (80:20) followed by $CHCl_3$-MeOH-HOAc (50:50:1); B, Merck Silica F_{254}, $CHCl_3$-MeOH (80:20) twice; C, Merck Silica F_{254}, toluene-MeOH (95:5); D, Merck Silica F_{254}, $CHCl_3$-MeOH-HOAc (50:50:5); E, Analtech Avicel, BuOH saturated with 0.3 *N* NH_4OH; F, Merck Silica F_{254}, $CHCl_3$-HOAc (95:5) once followed by $CHCl_3$-EtOH-HOAc (90:10:5) once. All plates were 250 μ.

† We wish to thank Anthony Villani, Dr. Wilford Mendelson, and Dr. Dale Blackburn for preparation of the ^{14}C-labeled drug which was carried out using carbonyl labeled *m*-trifluoromethylbenzaldehyde by the method of ref 1.

‡ Compounds I-IV were available through the courtesy of Dr. Bernard Loev and Mrs. Marjory Goodman who will describe the preparation of compounds not appearing in ref 1 elsewhere.

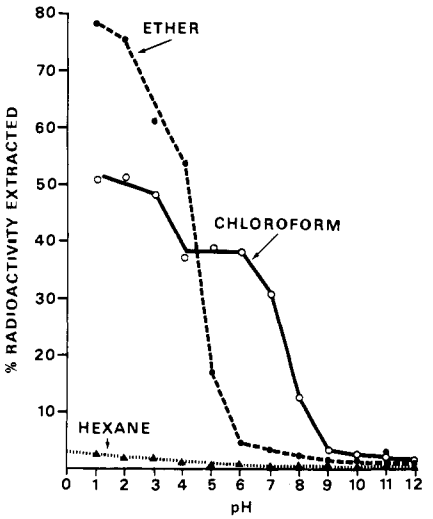


Figure 1. Extraction of radioactivity from urine of dogs treated with ^{14}C-I.

pattern was used to devise a scheme for preliminary separation of metabilites.

Urine was adjusted to pH 1 with HCl and extracted exhaustively with ether. The ether was back-extracted with 5% $NaHCO_3$ which returned most of the material to the aqueous phase. The $NaHCO_3$ solution was adjusted to pH 6 and extracted with $CHCl_3$ to remove weakly acidic components. Finally it was adjusted to pH 1 and extracted with ether to isolate the stronger acids. The latter were purified by tlc using autoradiography to visualize the separation. One of the materials isolated was subjected to mass spectrometry and found to be identical with 2,6-dimethyl-4-(2-trifluoromethylphenyl)-3,5-pyridinedicarboxylic acid monoethyl ester (III). The compound also exhibited the correct R_f values in several tlc systems for III.

For the isolation of the other metabolites identified, urine was adjusted to pH 1 and extracted with ether. Upon extraction with 5% $NaHCO_3$, 76% of the radioactivity was returned to the aqueous phase. The pH of the buffer solution was adjusted stepwise to 6, 4, and 1 and extracted exhaustively at each pH with ether. The ether extract at pH 6 contained 0.4% of the radioactivity, that at pH 4 contained 43.2%, and that at pH 1 contained 21.8%, while 3% was not recovered from the buffer.

Analytical thin-layer chromatography on the pH 4 extract suggested that only one major metabolite was present and that it was the same as III isolated above. This was confirmed by an inverse isotope dilution experiment. The amount of III present in the original urine was calculated to be 48% of the total radioactive metabolites.

The following three metabolites were isolated by thin-layer chromatography (system F, Table I) from the ether extracted at pH 1. The component at R_f 0.58-0.71 (9.5% of the radioactivity in the original urine) was further purified by rechromatography on cellulose (tlc) in BuOH-EtOH-0.3 *N* NH_4OH (3:1:1) followed by a silica gel tlc in $CHCl_3$-EtOH-HOAc (50:50:5). The mass spectrum indicated that the molecular weight was 337. The infrared spectrum, which showed two carbonyl absorptions at 1780 and 1730 cm^{-1}, suggested a carboxylic acid group which was consistent with its extraction behavior and a lactone. The nmr spectrum had a 3-proton singlet at δ 2.80 assigned to the 2-methyl and a 2-proton singlet at δ 5.41 (clearly a methylene attached to an oxygen) assigned to the lactone and four aromatic protons. This suggested the structure V which was confirmed by synthesis.

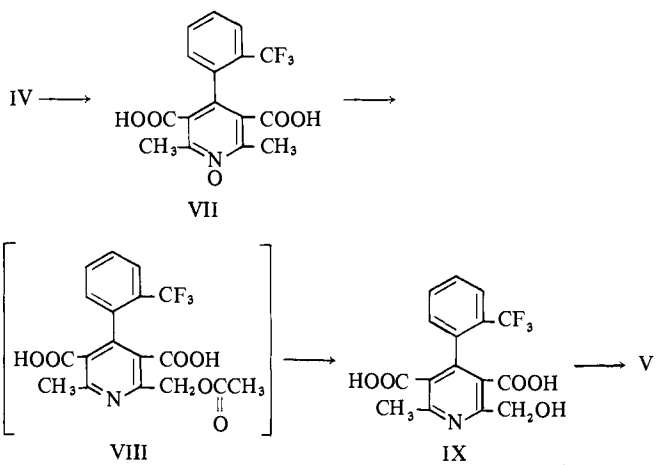
The component R_f 0.23-0.35 (6% of the radioactivity of the original urine) was further purified by rechromatography on silica gel (tlc) in $CHCl_3$-EtOH-HOAc (80:20:5). After the final chromatography, the residue from concentration of the eluent was further purified by dissolving it in $NaHCO_3$ and washing with ether and was recovered by acidification and extraction into ether. The metabolite was identified by mass spectrum, nmr, ir, and finally by synthesis. A molecular weight of 383 as determined by the mass spectrum suggested the addition of one atom of oxygen to the monoethyl ester. The fragmentation pattern indicated that the compound was an ethylene glycol ester.

The nmr spectrum showed two coupled 2-proton triplets at δ 3.97 and 3.43 which suggested the presence of two methylenes next to two different oxygens. A 6-proton singlet at δ 2.62 showed that the 2,6-methyls were intact, and four protons at δ 7.25 and 7.71 showed that the aromatic ring system was not hydroxylated. The structure VI was proposed and was confirmed by synthesis.

The structure of the third metabolite extracted from the buffer at pH 1 having the R_f 0.02-0.18 on the first tlc was suspected of being the dicarboxylic acid IV because of its behavior on extraction and thin-layer chromatography. An aliquot of the radioactive material was subjected to an inverse isotope dilution experiment with authentic IV which confirmed the structural assignment. The radioactive material was calculated to be 85% IV which corresponded to 8.3% of the urinary metabolites.

Synthesis. The lactone V was synthesized according to Scheme I. The pyridinedicarboxylic acid IV was treated

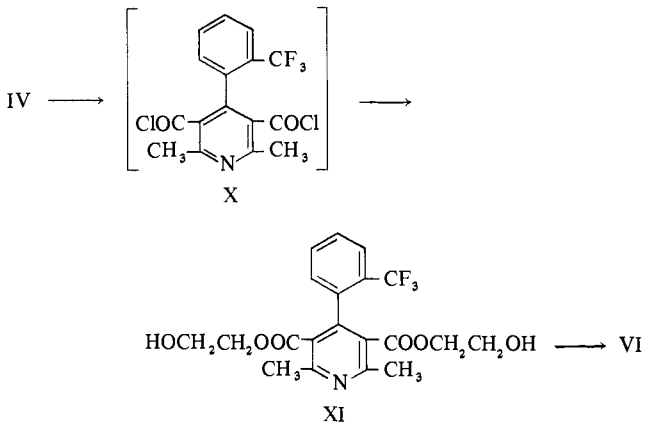
Scheme I



with *m*-chloroperbenzoic acid to give the *N*-oxide VII. The *N*-oxide was treated with acetic anhydride[5] to give the acetate VIII which, after removal of the anhydride under vacuum, without further purification was hydrolyzed with KOH in ethanol. The alcohol IX so obtained was heated with 6 *N* HCl to give the lactone V.

The synthesis of the hydroxyethyl ester metabolite VI is outlined in Scheme II. The diacid chloride X was prepared by refluxing IV overnight with thionyl chloride in chloroform. After evaporation of the excess thionyl chloride, the otherwise unpurified diacid chloride was treated with ethylene glycol in the presence of sodium carbonate to give the bis(hydroxyethyl) ester XI. Treatment of XI with NaH in ethylene glycol gave the desired VI, presumably by loss of ethylene oxide from the sodium salt of the bis ester.

Scheme II



On the basis of the metabolites identified, several possible pathways for the metabolism of I are possible. Chemically, II requires 20 hr of reflux in ethanol with a sixfold excess of potassium hydroxide for hydrolysis. Even these conditions, however, do not cause hydrolysis of I.[1] Since no aromatized drug II was found as a metabolite, it is believed that although aromatization to II may occur first, hydrolysis is necessary to render the compound polar enough to be excreted in appreciable quantity. However, even III must be excreted relatively slowly since major quantities of products resulting from further hydrolysis and oxidation are found.

Experimental Section

Preparative tlc was carried out using 250–1000-μ Merck Silica F_{254} plates, while analytical tlc was carried out on Merck 250-μ F_{254} plates or Analtech Avicel 250-μ plates. R_f values of authentic samples and unknowns are shown in Table I for the various systems used. Radioactivity was measured using a Packard Tri-Carb liquid scintillation counter with the samples in BBOT Phosphor cocktail. Disintegrations per minute were calculated using external standards in the same phosphor. Samples in organic solvents were evaporated to dryness under nitrogen and redissolved in methanol before counting. Radioactivity on tlc plates was visualized by radioautography using Kodak Royal Blue X-ray film. Standard compounds were visualized using the fluorescence of the plates under short-wave uv light. It was found that 25,000 dpm on a 250-μ silica plate in a streak 2 cm long could be detected after 18 hr of exposure. During the isolation, a Varian Aerograph Model 6000-10 tlc plate radioactivity scanner coupled with a Nuclear Chicago Ratemeter Model 8731 and Digital Integrator Model 8735 became available and this was used instead of radioautography. Where analytical data are indicated only by symbols of the elements, the compounds were analyzed for C, H, and N, and the results obtained were within 0.4% of theory.

Isolation, Purification, and Characterization of Metabolites. Extraction Pattern. Twelve 1.0-ml samples of dog urine (190,000 dpm/ml) were taken and made up to 5.0 ml in volumetric flasks. Then the pH was adjusted to pH 1, 2, 3, . . ., 12 in each of the flasks, respectively. Of these, 0.1 ml of each was taken for counting and three 1.0-ml samples of each pH were pipetted into centrifuge tubes. These were extracted three times with 2.0 ml of hexane, ethyl ether, or chloroform, respectively. Liquid scintillation counting of the combined organic extracts and of the aqueous from each tube was used to determine the per cent extracted. The results are shown in Figure 1.

Isolation of 2,6-Dimethyl-4-(2-trifluoromethylphenyl)-3,5-pyridinedicarboxylic Acid Monoethyl Ester (III) from Dog Urine. Urine (1075 ml) from a dog§ dosed with 5 mg/kg of ^{14}C-labeled drug was adjusted to pH 1 and extracted exhaustively with ether. The ether was extracted with 5% $NaHCO_3$. The aqueous phase was adjusted to pH 6 and extracted with $CHCl_3$ exhaustively. The aqueous phase was then brought to pH 1 and extracted exhaustively with ether. The ether was concentrated and the residue submitted to thin-layer chromatography using $CHCl_3$–MeOH–HOAc (50:50:5). The major radioactive band was isolated, restreaked, and developed with $CHCl_3$–MeOH–HOAc (50:50:5) followed by $CHCl_3$–MeOH (80:20) twice. Again the major radioactive band was isolated and subjected to tlc using $CHCl_3$–MeOH (80:20). The metabolite was eluted from the silica with ethanol and the ethanol was concentrated. Comparison of the mass spectrum of the residue and that of authentic III showed the two were identical.

Extraction of Major Metabolites from Dog Urine. Urine (7 l.) from dogs dosed orally with 40 mg/kg of cold I and urine from a dog dosed with 5 mg/kg of radioactive I were extracted separately four times at pH 1 with 1 l. of ether. The ether layers were extracted three times with 150 ml of bicarbonate pH 9 buffer. The buffer was adjusted to pH 6, 4, 1 successively and extracted with ether exhaustively at each pH. The ether extracts were concentrated at room temperature under reduced pressure, and the radioactive and cold residues were stored under refrigeration until they were used.

Fractionation and Purification of Metabolites by Tlc. A. pH 1 Extract. The residue from the pH 1 ether extract was enriched with one-third of the corresponding radioactive fraction and then streaked on eight 750-μ 20 × 20 cm Analtech Silica GF tlc plates. These were developed in system F (Table I). Three areas of radioactivity were detected: A, R_f 0.02–0.18 (428,000 dpm); B, R_f 0.23–0.35 (261,000 dpm); C, R_f 0.58–0.71 (425,000 dpm). These were scraped and eluted separately with EtOH. Not all of the radioactivity was recovered from the A band so the A silica was extracted with aqueous NaOH, pH 9.5, and the metabolite was recovered as the free acid by acidification and extraction with ether. This was combined with the fraction A ethanol extract.

(1) Area C Metabolite V (R_f 0.58–0.71, 425,000 dpm). The EtOH extract was concentrated and rechromatographed on four 20 × 20 cm 250-μ cellulose tlc plates using BuOH–EtOH–0.3 *N* NH_4OH (3:1:1). The radioactive band was scraped and eluted with EtOH and rechromatographed on a Merck Silica F_{254} 250-μ tlc plate with $CHCl_3$–EtOH–HOAc (50:50:5). The dark area at R_f 0.57 which was radioactive was scraped and eluted with EtOH. Concentration of this eluent gave a white solid: uv λ_{max}^{EtOH} 290, 276 mμ at pH 1, 290 mμ at pH 14; ir (Nujol mull) 1780 (lactone C=O), 1730 cm^{-1} (acid C=O); nmr (acetone-d_6) δ 2.80 (s, 3 H, 6-CH_3), 5.41 (s, 2 H, CH_2), 7.78 (2, 4 H, aromatic H); mass spectrum, molecular ion at *m/e* 337. This material was identical with synthetic V when compared by tlc ($CHCl_3$–MeOH–HOAc) (50:50:5), ir, nmr, and mass spectrum.

(2) Area B Metabolite VI (R_f 0.23–0.35, 261,000 dpm). The material eluted from area B of the first tlc of the pH 1 extract was dissolved in EtOH. The EtOH was streaked on a 2-mm Merck Silica F_{254} plate and developed in $CHCl_3$–EtOH–HOAc (80:20:5). This gave one radioactive area at R_f 0.47 which was scraped and eluted with EtOH (236,000 dpm). It was again streaked on a tlc plate (Merck Silica F_{254}, 250 μ) and developed in the same system. The single dark blue radioactive band at R_f 0.54 was separated from other contaminants. It was scraped and eluted with EtOH and concentrated to dryness. The residue was dissolved in $NaHCO_3$, washed with ether, brought to pH 1 with HCl, and extracted into ether. Concentration gave a crystalline solid: mass spectrum molecular ion at *m/e* 383; uv λ_{max}^{EtOH} 280, 285 mμ at pH 14, 282 mμ at pH 1; ir (micro KBr pellet) 1720 cm^{-1} (C=O); nmr (acetone-d_6) δ 2.60 (s, 6 H, 2,6-CH_3), 3.43 (t, 2 H, J = 5.25 Hz), 3.97 (t, 2 H, J = 5.25 Hz), 7.25 (broad s, 1 H), 7.71 (d, 3 H, J = 3.75 Hz). This was identical with synthetic VI with regard to tlc ($CHCl_3$–EtOH–HOAc) (50:50:5), nmr, ir, and mass spectrum.

(3) Area A Metabolite IV (R_f 0.02–0.18, 428,000 dpm). Identification by Isotope Dilution. A portion of area A from the first tlc of the pH 1 extract was chromatographed on Merck Silica F_{254} in $CHCl_3$–MeOH–HOAc (50:50:1) and found to have the same R_f (0.42) as synthetic IV. A sample containing 36,766 dpm of this metabolite in ether was mixed with 101.9 mg of synthetic IV and all of the solid dissolved. The solution was taken to dryness and the residue was recrystallized from acetone–petroleum ether (bp 30–60°) four times to constant specific activity (316 dpm/mg). From this, the amount of IV in the fraction was calculated to be 32,200 dpm or 85%.

B. pH 4 Extract. Identification of Metabolite III by Inverse Isotope Dilution. A sample of the pH 4 ether extract containing 1,163,830 dpm was taken and 9.9 mg of authentic III was added. This was dissolved in ethanol and was rechromatographed using $CHCl_3$–EtOH–HOAc (80:20:5). The radioactive area was scraped and eluted from the silica with EtOH. To this (870,500 dpm) an

§ The urinary metabolite pattern for this dog was shown to be very similar both qualitatively and quantitatively to a number of other dogs studied in the absorption and excretion studies.[3]

additional 49.1 mg of III was added. When solution was complete the mixture was evaporated to dryness and the residue was recrystallized once from EtAc–hexane and three times from acetonitrile to constant specific activity (14,606 dpm/mg). From this the amount of III was calculated to be 861,750 dpm or 82% of this fraction.

Synthesis. 2,6-Dimethyl-4-(2-trifluoromethylphenyl)-3,5-pyridinedicarboxylic Acid *N*-Oxide (VII). A solution of 1.02 g (0.003 mol) of IV and 0.65 g (0.00375 mol) of *m*-chloroperbenzoic acid in 10 ml of EtOH was refluxed for 2 hr. The reaction mixture was taken to dryness and water was added. Sufficient 5% NaOH was added to dissolve all the solids. Then the pH was lowered slowly with dilute HCl until the *m*-chlorobenzoic acid crystallized out. It was collected by filtration. On further lowering to pH 1, 1.1 g of product was obtained. Recrystallization from aqueous NH_4OH–HCl gave white crystals, mp 184° dec. *Anal.* ($C_{16}H_{12}NO_5F_3 \cdot H_2O$).

6-Hydroxymethyl-2-methyl-4-(2-trifluoromethylphenyl)-3,5-pyridinedicarboxylic Acid γ Lactone (V). A sample of 0.5 g (0.0014 mol) of VII was treated with 0.18 ml (0.0017 mol) of acetic anhydride at 140° for 5 min, at which time most of the bubbling had stopped. The reaction mixture was evaporated to dryness and partitioned between 5% $NaHCO_3$ (with some concentrated NH_4OH added to keep it basic) and ether. After the aqueous layer had been washed with ether it was brought to pH 1 with HCl. The product was extracted into ether, the ether evaporated to dryness, and the residue dissolved in EtOH and warmed with 0.09 g of KOH (0.0016 mol) and some NH_4OH for a few minutes. This was evaporated to dryness and the residue heated on the steam bath for 2 hr in 10 ml of 6 *N* HCl. Cooling gave a white solid (0.21 g, 45%) which after recrystallization from CH_3CN–H_2O had mp 221.5–224°; uv λ_{max}^{EtOH} 275 mμ (ε 5900). *Anal.* ($C_{16}H_{10}NO_4F_3$).

2,6-Dimethyl-4-(2-trifluoromethylphenyl)-3,5-pyridinedicarboxylic Acid Bis(2-hydroxyethyl) Ester (XI). A sample of IV (6.0 g, 0.018 mol) was refluxed for 18 hr with 15 ml (24.6 g, 0.209 mol) of $SOCl_2$ and 100 ml of $CHCl_3$. The mixture was evaporated to dryness and 20 ml of ethylene glycol and 2.5 g of Na_2CO_3 (0.04 mol) were added. This was heated for 1 hr on a steam bath, diluted to 200 ml with water, and extracted three times with 100 ml of diethyl ether. The ether solution was washed three times with 5% $NaHCO_3$, dried over $MgSO_4$, and concentrated. Recrystallization from toluene gave 3 g (39%) of white crystals, mp 86.0–87.5°. *Anal.* ($C_{20}H_{20}NO_6F_3$).

2,6-Dimethyl-4-(2-trifluoromethylphenyl)-3,5-pyridinedicarboxylic Acid Mono-2-hydroxyethyl Ester (VI). A mixture of 0.83 g (1.95 mmol) of XI was heated with 160 mg of 52% NaH in mineral oil (83 mg of NaH, 3.5 mmol) and 20 ml of ethylene glycol at 100° for 36 hr. It was diluted to 100 ml with water and extracted at this pH (pH ≥8) twice with 100 ml of ether. The pH of the aqueous layer was adjusted to 3.5–4.0 and extracted three times with 100 ml of EtOAc. This was washed twice with 100 ml of water containing 2 drops of 12 *N* HCl. Then it was dried over $MgSO_4$ and evaporated to dryness. This gave 0.41 g (57%) of product which after recrystallization from (1:2) EtOAc–hexane had mp 181–182°; uv λ_{max}^{EtOH} 272 mμ (ε 5040). *Anal.* ($C_{18}H_{16}NO_5F_3$).

Acknowledgments. We wish to thank the staff of our Analytical and Physical Chemistry Section for analytical and spectral analyses, our Biochemistry staff for providing biological samples, and Dr. Sidney Walkenstein for helpful suggestions.

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An Acyclic Puromycin Analog. 6-Dimethylamino-9-[2-hydroxy-3-(*p*-methoxyphenyl-L-alanylamino)propyl]purine†

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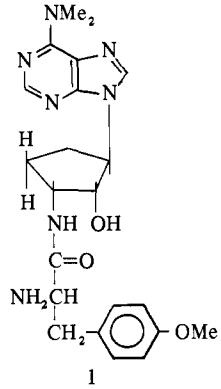
In a continuation of our studies on nonglycosyl puromycin analogs, 6-dimethylamino-9-[2-hydroxy-3-(*p*-methoxyphenyl-L-alanylamino)propyl]purine was synthesized and separated into its two diastereoisomers. The lack of antimicrobial activity of this acyclic puromycin analog when compared with the previously prepared carbocyclic analog is discussed in terms of possible conformational requirements for ribosomal binding.

In a previous report relating to our studies on puromycin analogs, the synthesis and antimicrobial activity of a carbocyclic puromycin analog **1** were described.[1] The antimicrobial activity[1] and the inhibition of *in vitro* protein biosynthesis[2] exhibited by the carbocyclic analog suggest a minimal contribution by the furanosyl oxygen and the hydroxymethyl moiety in the activity of puromycin.

Previous studies with isosteric nucleosides have revealed that rather large changes in the substituent at the 9 position of the purine nucleus can be made without markedly altering the capacity of the compound to bind to a given enzyme.[3] In fact, the replacement of the cyclic moiety at the 9 position of the purine by an acyclic moiety may result in enhanced binding to some enzymes.[4,5] In order to evaluate the antimicrobial activity of a compound with greater conformational freedom than **1**, we decided to prepared the acyclic derivative **2**.



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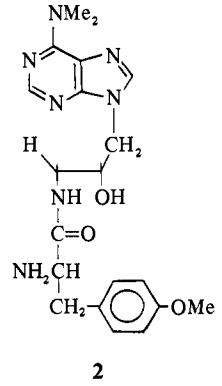

2

The acyclic puromycin analog **2** was synthesized by the route outlined in Scheme I. The condensation of 5-amino-4,6-dichloropyrimidine (**3**) with 2-hydroxy-3-acetamido-

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