Mortality range in the treated group was statistically compared to that in the control group according to the nonparametric Wilcoxon's test.[30] When the statistical test was significant ($p < 0.05$) and I greater than 125, the compound was considered active at the given dose. In many cases, it was necessary to do new assays, with untreated and reference control groups. For the mitomycin-treated group, the oncostatic index varied from one experiment to the other. Therefore, in order that the results from the tested compounds obtained in these experiments might be compared, the following ratio, median survival time of treated group × 100/median survival time of reference group = treated $I \times 100$/ref I, was calculated.

Wilcoxon's test was used between reference and treated groups.

Registry No. **1a**, 117918-56-6; **1b**, 117918-57-7; **1c**, 117918-58-8; **1d**, 117918-59-9; **2a**, 117918-60-2; **2b**, 117918-61-3; **2c**, 117918-62-4; **2d**, 117918-63-5; **3a**, 117918-64-6; **3b**, 117918-65-7; **3c**, 117918-66-8; **3d**, 117918-67-9; **4a**, 117918-68-0; **4b**, 117918-69-1; **4c**, 117918-70-4; **4d**, 117918-71-5; **5a**, 117918-72-6; **5b**, 117918-73-7; **5c**, 117918-74-8; **5d**, 117918-75-9; **6b**, 117918-76-0; **6c**, 117918-77-1; **7b**, 117940-40-6; **7c**, 117918-78-2; **8b**, 117918-79-3; **8c**, 117918-80-6; **9b**, 117918-81-7; **9c**, 117918-82-8; **10b**, 117918-83-9; **10c**, 117918-84-0; DMAD, 762-42-5; mitomycin C, 50-07-7; 2-furoic acid, 88-14-2; 3-thienoic acid, 88-13-1; 2-thienoic acid, 527-72-0; L-proline, 147-85-3; L-thiazolidine-4-carboxylic acid, 34592-47-7; dimethyl 5-[1-(4-acetoxy-2,3-dicarbomethoxy-7-oxabicyclo[2.2.1]-2,5-heptadienyl)]-2,3-dihydro-1*H*-pyrrolizine-6,7-dicarboxylate, 117918-85-1.

1-(2,3-Dideoxy-β-D-*glycero*-pent-2-enofuranosyl)thymine.[1] A Highly Potent and Selective Anti-HIV Agent

Muzammil M. Mansuri,*,† John E. Starrett, Jr.,† Ismail Ghazzouli,‡ Michael J. M. Hitchcock,‡ Roman Z. Sterzycki,† Vera Brankovan,§ Tai-Shun Lin,∥ E. Michael August,∥ William H. Prusoff,∥ Jean-Pierre Sommadossi,⊥,[2] and John C. Martin†

Bristol-Myers Pharmaceutical Research and Development Division, 5 Research Parkway, P.O. Box 5100, Wallingford, Connecticut 06492-7660. *Received May 3, 1988*

The nucleoside analogue 1-(2,3-dideoxy-β-D-*glycero*-pent-2-enofuranosyl)thymine (d4T, **1**) was prepared by ring opening of the 3′,5′-anhydro compound **5**. This method has been refined such that it can be used to prepare d4T (**1**) on a large scale. The triphosphate of d4T (**8**) was also synthesized from **1** in order to examine the mode of action. The in vitro inhibitory activity of d4T was found to be comparable to that of AZT in HIV-infected CEM cells. The triphosphate of d4T (**8**) and that of AZT inhibited the HIV reverse transcriptase with poly(rA):oligo(dT) as the template:primer with K_i values of 0.032 and 0.007 μM, respectively. The in vitro toxicity of d4T against normal human hematopoietic progenitor cells (CFU-GM) was measured in comparison to AZT. While d4T reduces colony-forming units by 50% at a concentration of 100 μM, it takes only 1 μM AZT to have a similar toxic effect. With erythrocyte burst forming units (BFU-E) the in vitro toxicities for d4T and AZT have comparable ID_{50} values of 10 and 6.7 μM, respectively.

Acquired immunodeficiency syndrome (AIDS) is the result of an infection by the human immunodeficiency virus (HIV).[3] This retrovirus shows a specific tropism for the helper/inducer T cells,[4] leading to their depletion. The resultant profound immunosuppression predisposes patients to life-threatening opportunistic infections. Although at present there is no cure for AIDS, 3′-azido-3′-deoxythymidine (zidovudine, AZT) has already proved to be an efficacious treatment in clinical trials and has been approved for use in patients with AIDS.[5] A number of other chemical agents have been reported to have biological activity against the HIV.[6-16] In addition to the aforementioned chemotherapeutic agents, a number of biological response modifiers have also been evaluated.[17]

Traditional research in the antiviral area has concentrated on nucleoside analogues for new therapies against RNA and DNA viral infections. Research for active drugs against HIV has also found nucleoside analogues to be most efficacious. One common feature among the nucleoside derivatives that have shown good in vitro activity is the lack a 3′-OH group on the sugar part of the molecule, thereby enabling these substances to act as possible chain terminators of DNA synthesis.[18] The mode of action of these compounds is believed to require the nucleoside to be metabolized to its 5′-triphosphate form by cellular

† Department of Antiinfective Chemistry.

‡ Department of Virology.

§ Oncogen, Seattle, WA 98121.

∥ Department of Pharmacology, Yale University School of Medicine, New Haven, CT 06510.

⊥ Department of Pharmacology, Division of Clinical Pharmacology, University of Alabama at Birmingham, Birmingham, AL 35294.

(1) The abbreviation d4T used in this paper is derived from the non-IUPAC name 2′,3′-didehydro-2′,3′-dideoxythymidine. This compound has also been referred to as 2′,3′-dideoxythymidinene (ddeThd).

(2) J.P.S. is the recipient of a Junior Faculty Research Award from the American Cancer Society.

(3) (a) Barre-Sinoussi, F.; Chermann, J. C.; Rey, R.; Nugeyre, M. T.; Chamaret, S.; Gruest, C.; Dauguet, C.; Axler-Blin, C.; Rouzioux, C.; Rozenbaum, W.; Montagnier, L. *Science (Washington, D.C.)* **1983**, *220*, 868. (b) Broder, S.; Gallo, R. C. *N. Engl. J. Med.* **1984**, *311*, 1292. (c) Broder, S.; Gallo, R. C. *Annu. Rev. Immunol.* **1985**, *3*, 321.

(4) Popovic, M.; Sarngadharan, M. G.; Read, E.; Gallo, R. C. *Science (Washington, D.C.)* **1984**, *224*, 497. (b) Gallo, R. C.; Sarngadharan, M. G.; Popovic, M.; Shaw, G. M.; Hahn, B.; Wong-Stahl, F.; Robert-Guroff, M.; Salahuddin, Z., Markham, P. D. *Prog. Allergy* **1986**, *37*, 1.

(5) Fischl, M. A.; Richman, D. D.; Grieco, M. H.; Gottlieb, M. S.; Volberding, P. A.; Laskin, O. L.; Leedom, J. M.; Groopman, J. E.; Mildvan, D.; Schooley, R. T.; Jackson, G. G.; Durack, D. T.; King, D. *N. Engl. J. Med.* **1987**, *317*, 185.

(6) Mitsuya, H.; Broder, S. *Proc. Natl. Acad. Sci. U.S.A.* **1986**, *83*, 1911.

(7) (a) Lin, T. S.; Schinazi, R. F.; Chen, M. S.; Kinney-Thomas, E.; Prusoff, W. H. *Biochem. Pharmacol.* **1987**, *36*, 311. (b) Balzarini, J.; Pauwels, R.; Herdewijn, P.; De Clercq, E.; Cooney, D. A.; Kang, G.-J.; Dalal, M.; Johns, D. G.; Broder, S. *Biochem. Biophys. Res. Commun.* **1986**, *140*, 735.

(8) Cheson, B. D.; Levine, A. D.; Mildvan, D.; Kaplan, L. D.; Wolfe, P.; Rios, A.; Groopman, J. E.; Gill, P.; Volberding, P. A.; Poiesz, B. J.; Gottlieb, M. S.; Holden, H.; Volsky, D. J.; Silver, S. S.; Hawkins, M. J. *J. Am. Med. Assoc.* **1987**, *258*, 1347.

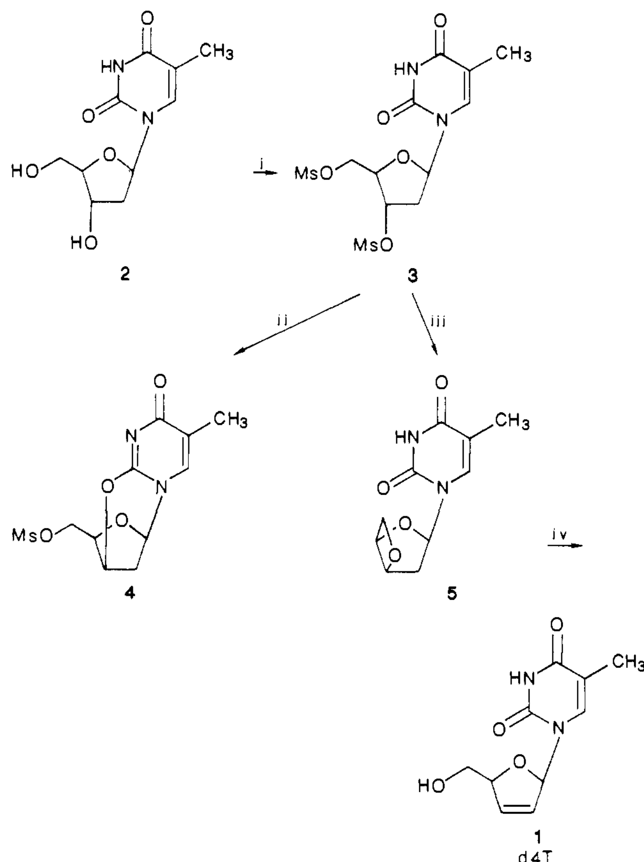
0022-2623/89/1832-0461$01.50/0 © 1989 American Chemical Society

kinases. The triphosphate can then act as an inhibitor of both the reverse transcriptase (RT) of the retrovirus and cellular DNA polymerases and, if incorporated into DNA, will act as a chain terminator. In order to have good selectivity, the triphosphate should be a good inhibitor or substrate for the RT and a poor inhibitor or substrate for the cellular DNA polymerases. With AZT triphosphate, the cellular DNA polymerase α is approximately 100-fold less susceptible than the HIV RT.[19]

It should be noted that while the lack of a 3′-OH group appears to be a prerequisite for a nucleoside analogue to serve as a RT inhibitor or DNA chain terminator, this alone does not guarantee activity against the HIV. One of the reasons for the structure–activity relationship being complex may relate to the stepwise activation required to form the triphosphates, a process that requires recognition by several enzymes. To date, the only compounds with substitutions for the 3′-OH in the nucleoside that are reported to have biological activity are 3′-H,[6] 3′-F,[16] and 3′-N_3[15] and the 2′,3′-olefinic derivatives.[7]

Since there is no animal model that allows one to extrapolate to the human disease, detailed in vitro biological and biochemical assay systems become of paramount importance for predicting the efficacy and toxicity of any potential anti-HIV agent. AZT, the only anti-HIV agent approved by the FDA because of its clinical efficacy, has several undesired side effects, some of which can now be predicted for by in vitro systems. Most noticeable among these side effects is bone marrow toxicity leading to anemia and neutropenia. In human trials the hematopoietic toxicity appears after prolonged treatment with AZT,[20] and

Scheme I[a]



[a] Reagents: (i) mesyl chloride/pyridine; (ii) 1 equiv of NaOH/EtOH; (iii) 3 equiv of NaOH/H_2O; (iv) KOtBu/DMSO.

bone marrow recovery usually occurs following discontinuation of the drug.[21] An in vitro assay that measures the toxicity of AZT to hematopoietic bone marrow cells has recently been described.[22] This now allows other potential anti-HIV agents to be tested for this significant side effect. Biochemical experiments show that AZT is a reasonable substrate for thymidine kinase (TK), and although the corresponding monophosphate has an affinity (K_m) similar to that of thymidine monophosphate (TMP) for thymidylate kinase, it has a very low rate of phosphorylation (V_{max}), being about 0.3% that of TMP.[19] This results in a large buildup of AZT monophosphate (AZT-MP), which in turn has been suggested to be responsible for the inhibition of TMP conversion to thymidine di- and triphosphate (TDP and TTP, respectively) by competitive inhibition. It has been proposed that this reduction in TTP levels may contribute to the anemia side effect by starving the DNA synthesis.[19]

The evaluation of new drug candidates for the treatment of HIV infection needs to address the toxic limitations of AZT. A number of reports have appeared that show that

(9) (a) Balzarini, J.; Mitsuya, H.; De Clercq, E.; Broder, S. *Int. J. Cancer* **1986**, *37*, 451. (b) McCormick, J. B.; Getchell, J. P.; Mitchell, S. W.; Hicks, D. R. *Lancet* **1984**, *ii*, 1367.

(10) (a) Sarin, P. S.; Taguchi, Y.; Sun, D.; Thornton, A.; Gallo, R. C.; Oberg, B. *Biochem. Pharmacol.* **1985**, *34*, 4075. (b) Sandstrom, E. G.; Kaplan, J. C.; Byington, R. E.; Hirsch, M. S. *Lancet* **1985**, *i*, 480.

(11) Lane, H. C.; Fauci, A. S. *Ann. Intern. Med.* **1985**, *103*, 714.

(12) Chandra, P.; Sarin, P. S. *Arzneim.-Forsch./Drug Res.* **1986**, *36*, 184.

(13) Tyms, A. S.; Berrie, E. M.; Ryder, T. A.; Nash, R. J.; Hegarty, M. P.; Taylor, D. L.; Mobberley, M. A.; Davis, J. M.; Bell, E. A.; Jeffries, D. A.; Taylor-Robinson, D.; Fellows, L. E. *Lancet* **1987**, *ii*, 1025.

(14) Faber, V.; Newell, A.; Dalgleish, A. G.; Malkovsky, M. *Lancet* **1987**, *ii*, 827.

(15) (a) Hartmann, H.; Hunsmann, G.; Eckstein, F. *Lancet* **1987**, *i*, 40. (b) Baba, M.; Pauwels, R.; Balzarini, J.; Herdewijn, P.; De Clercq, E. *Biochem. Biophys. Res. Commun.* **1987**, *145*, 1080.

(16) (a) Herdewijn, P.; Balzarini, J.; De Clercq, E.; Pauwels, R.; Baba, M.; Broder, S.; Vanderhaeghe, H. *J. Med. Chem.* **1987**, *30*, 1270. (b) Matthes, E.; Lehmann, C.; Scholz, D.; von Janta-Lipinski, M.; Gaertner, K.; Rosenthal, H. A.; Langen, P. *Biochem. Biophys. Res. Commun.* **1987**, *148*, 78. (c) Polski, B.; Gold, J. M. W.; Hardy, W. D.; Baron, P. A.; Zuckermann, E. E.; Chou, T.-C.; Levine, S. M.; Flomenberg, N.; Wang, L.; Watanabe, K. A.; Fox, J. J.; Armstrong, D. *27th ICAAC* **1987**, Abstract 368, p 161.

(17) (a) Oberg, B. *J. Antimicrob. Chem.* **1986**, *17*, 549. (b) Groopman, J. E.; Mitsuyasu, R. T.; DeLeo, M. J.; Oette, D. H.; Golde, D. W. *N. Engl. J. Med.* **1987**, *317*, 593.

(18) (a) Sanger, F.; Nicklen, S.; Coulson, A. R. *Proc. Natl. Acad. Sci. U.S.A.* **1977**, *74*, 5463. (b) Atkinson, M. R.; Deutscher, M. P.; Kornberg, A.; Russell, A. F.; Moffatt, J. G. *Biochemistry* **1969**, *8*, 4897.

(19) Furman, P. A.; Fyfe, J. A.; St. Clair, M. H.; Weinhold, K.; Rideout, J. L.; Freeman, G. A.; Nusinoff-Lehrman, S.; Bolognesi, D. P.; Broder, S.; Mitsuya, H.; Barry, D. W. *Proc. Natl. Acad. Sci. U.S.A.* **1986**, *83*, 8333.

(20) (a) Yarchoan, R.; Klecker, R. W.; Weinhold, K. J.; Markham, P. D.; Lyerly, H. K.; Durack, D. J.; Gelmann, E.; Lehrmann, S. N.; Blum, R. M.; Barry, D. W.; Shearer, G. M.; Fischl, M. A.; Mitsuya, H.; Gallo, R. C.; Collins, J. M.; Bolognesi, D. P.; Myers, C. E.; Broder, S. *Lancet* **1986**, *i*, 575. (b) Richman, D. D.; Fischl, M. A.; Grieco, M. H.; Gottlieb, M. S.; Volberding, P. A.; Laskin, O. L.; Leedom, J. M.; Groopman, J. E.; Mildvan, D.; Hirsch, M. S.; Jackson, G. G.; Durack, D. T.; Lehrman, S. N. *N. Engl. J. Med.* **1987**, *317*, 192.

(21) Gill, P. S.; Rarick, M.; Brynes, R. K.; Causey, D.; Loureiro, C.; Levine, A. M. *Ann. Int. Med.* **1987**, *107*, 502.

(22) Sommadossi, J. P.; Carlisle, R. *Antimicrob. Agents Chemother.* **1987**, *31*, 452.

1-(2,3-dideoxy-β-D-*glycero*-pent-2-enofuranosyl)thymine (d4T, 1) has in vitro activity against HIV in several cell lines.[23] We now report an improved synthesis of d4T (1) that readily allows for the preparation of multigram quantities. In vitro biological data as well as results of biochemical mode of action studies are presented. In addition, the issues of bone marrow toxicity and cellular DNA and protein synthesis are addressed by in vitro experiments.

Chemistry

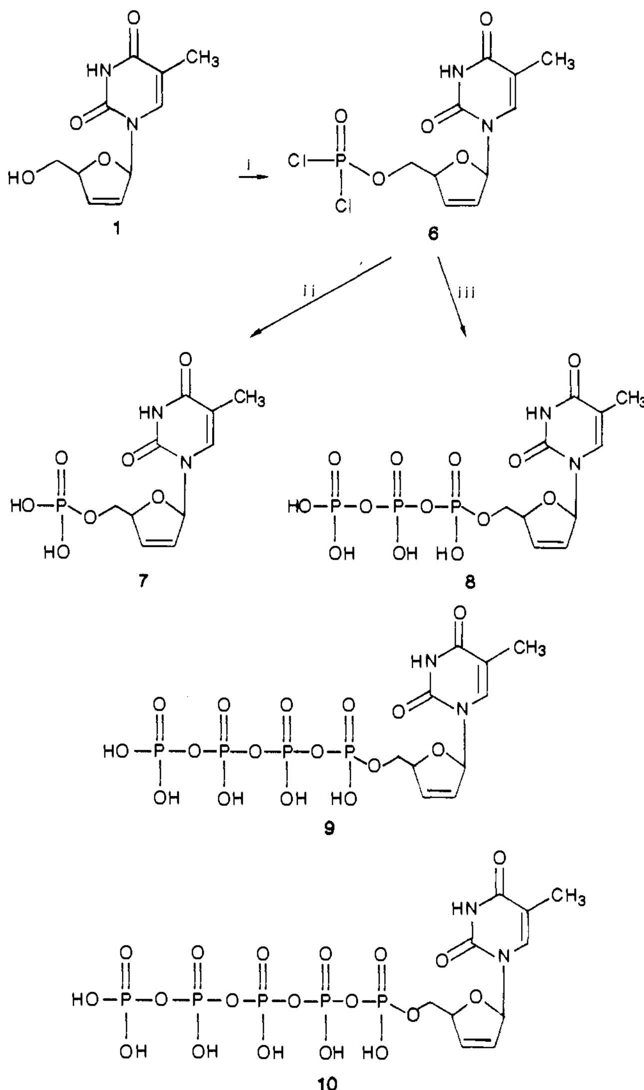
The title compound has been prepared by Horwitz et al. by two different routes.[24,25] We have modified one of these approaches so that it could be used to prepare large quantities of d4T (1) (Scheme I). Thymidine (2) was treated with a slight excess of methanesulfonyl chloride in pyridine to give the bis-mesylate **3** as a white crystalline compound after workup in 98% yield.

Reaction of **3** with NaOH in either water or ethanol afforded the anhydronucleoside **4**, which on continued exposure to the basic reaction conditions furnished the oxetane **5** in 74% yield. Although the reaction can be done in either solvent, water is preferable because the reaction mixture is homogeneous, allowing the reaction to go to completion, and the product **5** can be isolated directly. In ethanol, the reaction mixture remained gummy until it reached reflux temperature, leading to problems with stirring the mixture on a larger scale. When the reaction was run in ethanol with 1 equiv of NaOH, the anhydronucleoside **4** was isolated in 95% yield.[24]

The final step required the use of KOtBu (2 equiv) in DMSO.[24] In the original procedure, the workup called for removal of DMSO in vacuo after neutralization. For larger reactions, this proved unfeasible since removal of large volumes of DMSO required prolonged heating which caused cleavage of the glycosidic bond to give substantial amounts of thymine as a byproduct. A shorter reaction time is also desirable since prolonged exposure to base can cleave the glycosidic bond.

Several other solvents were tried as replacements for DMSO in the final step. Although THF was the most convenient on a small scale, the oxetane **5** had only limited solubility. On a larger scale the reaction did not go to completion because of the limited solubility, thereby making the subsequent purification of the final product difficult. If more than 2 equiv of KOtBu in THF were used to drive the reaction to completion, a substantial amount of thymine was formed as a side product. Presumably, the thymine results from deprotonation at the 4'-position by the excess base.[24] The reaction also proceeded in either DMF or DME; however, it is slower than in DMSO. The use of bases other than KOtBu in these various solvents was also tried. No combination was as clean or as efficient as KOtBu in DMSO where the reaction was homogeneous and the elimination to d4T (1) proceeded very quickly.

Scheme II[a]



[a]Reagents: (i) $POCl_3/(CH_3O)_3P(O)$; (ii) $Et_3NH^+HCO_3^-$ (saturated); (iii) $Bu_3N/(Bu_3NH)H_2P_2O_7$, then $Et_3NH^+HCO_3^-$ (saturated).

When the reaction was not homogeneous, competing reactions such as elimination of thymine became a problem since the reaction took much longer to go to completion.

With the conclusion that DMSO was the best solvent, we focused our attention on an alternative workup procedure. This proved more successful. We have found that the salt of d4T (1) formed in the KOtBu reaction precipitates as an oily solid when poured into a large volume of toluene. This material was collected by filtration, dissolved in water, and neutralized with concentrated HCl to pH 7.0 ± 0.1, whereupon d4T (1) began to crystallize from the solution. Additional KCl was added to saturate the solution to maximize the yield of d4T (1). The solid was collected, dissolved in hot acetone, filtered, and evaporated to give pure d4T (1). This method has been used to prepare up to 40 g of **1** in a single batch.

The monophosphate (7) and triphosphate (8) of d4T[26] were prepared for biochemical mode of action studies using a modification of the method of Vrang et al.[27] Reaction of phosphoryl chloride with d4T (1) gave **6**, which was hydrolyzed to the monophosphate **7**. Alternatively, **6** could

(23) (a) Lin, T. S.; Chen, M. S.; McLaren, C.; Gao, Y.-S.; Ghazzouli, I.; Prusoff, W. H. *J. Med. Chem.* **1987**, *30*, 440. (b) Lin, T. S.; Schinazi, R. F.; Prusoff, W. H. *Biochem. Pharmacol.* **1987**, *17*, 2713. (c) Baba, M.; Pauwels, R.; Herdewijn, P.; De Clercq, E.; Desmyter, J.; Vandeputte, M. *Biochem. Biophys. Res. Commun.* **1987**, *142*, 128. (d) Balzarini, J.; Kang, G.-J.; Dalal, M.; Herdewijn, P.; De Clercq, E.; Broder, S.; Johns, D. G. *Mol. Pharmacol.* **1987**, *32*, 162. (e) Hamamoto, Y.; Nakashima, H.; Matsui, T.; Matsuda, A.; Ueda, T.; Yamamoto, N. *Antimicrob. Agents Chemother.* **1987**, *31*, 907.
(24) Horwitz, J.; Chua, J. In *Synthetic Procedures in Nucleic Acid Chemistry*; Zorbach, W. W., Tipson, R. S., Eds.; Interscience: New York, 1968; Vol. 1, p 344.
(25) Horwitz, J.; Chua, J.; Da Rooge, M. A.; Noel, M.; Klundt, I. L. *J. Org. Chem.* **1966**, *31*, 205.
(26) Russell, A. F.; Moffatt, J. G. *Biochemistry* **1969**, *8*, 4889.
(27) Vrang, L.; Bazin, H.; Remaud, G.; Chattopadhyaya, J.; Oberg, B. *Antiviral Res.* **1987**, *7*, 139.

Table I. Comparative in Vitro Anti-HIV Efficacy[a] and Cellular Toxicity[b] of AZT and d4T

compd	ID_{50},[c] μM	$TCID_{50}$,[d] μM
AZT	0.10	29
d4T	0.15	90

[a] The antiviral test was performed on HIV (LAV strain) infected CEM cells. [b] The cellular toxicity was measured in CEM cells after 8 days by using a [^{3}H]thymidine uptake assay. [c] The 50% inhibitory dose. [d] The 50% tissue culture inhibitory dose.

be reacted with tributylammonium pyrophosphate in tributylamine to give the triphosphate **8**, after workup, as the tris(triethylammonium) salt (Scheme II). Both **7** and **8** were isolated by preparative HPLC using a C_{18} RP column. The structures of compounds **8–10** were established by ^{1}H and ^{31}P NMR and HPLC. For d4T-TP (**8**) the ^{1}H NMR clearly showed that the nucleoside was intact after the reaction sequence; the downfield shift of the 5′-CH_2 from 3.73 ppm in **1** to 4.15 ppm in **8** indicated that the phosphorylation had occurred at the 5′-position. The integration of the CH_2CH_3 protons of **8** showed that it was the tris(triethylammonium) salt. The ^{31}P NMR gave three peaks of equal intensity at −9.93 (γ-P), −10.77 (α-P) and −22.5 ppm (β-P) relative to phosphoric acid. These were in very close agreement to the values published for AZT-TP.[27] Two other phosphorylated compounds were obtained as byproducts. The HPLC and NMR data indicated that these were the tetraphosphate **9** and pentaphosphate **10**. The ^{31}P NMR of **9** suggested that there are four phosphorus resonances since the integral of the internal phosphorous (−22.52 ppm) was double that of either the α-P or δ-P. Similarly the ^{31}P NMR of **10** suggested five phosphorus resonances, with the internal phosphorous (−22.46 ppm) integrating to 3 times that of either the α-P or ε-P resonances. The ^{1}H NMR of **9** and **10** supported this assignment as the CH_2CH_3 protons integrated for the tetrakis(triethylammonium) and the pentakis(triethylammonium) salts, respectively.

Biological Results and Discussion

The in vitro anti-HIV activity of d4T against HIV (LAV strain) infected CEM cells was compared to AZT. The data in Table I demonstrate the in vitro potency of the compounds as expressed by the 50% inhibitory concentration against HIV. Viral replication (HIV) was measured as the amount of p24 antigen (core protein) in the culture supernatant by using a sandwich ELISA assay.[7a,23a] The cellular toxicity of the compound (tissue culture inhibitory dose, $TCID_{50}$) was also assessed by treating uninfected cells with various concentrations of the compounds. The in vitro antiviral potencies and cellular toxicities of both d4T and AZT appeared comparable in these assay systems. These relative biological activities are in agreement with other published results.[23]

The triphosphate **8** of d4T was prepared (vide supra) in order to examine the mode of action of d4T (**1**). Various concentrations of the triphosphate were incorporated into the reverse transcriptase assay using poly(rA):oligo(dT) as the RNA template/primer pair.[28] Analysis of the data by a Dixon plot[29] gave a K_i for d4T-TP of 0.032 μM (Table II) while AZT-TP gave a K_i of 0.007 μM under the same conditions. Both of these values are comparable to that of ddT-TP. These results are in agreement with the K_i values for AZT-TP published elsewhere.[16b,30]

(28) Chen, M. S.; Oshana, S. C. *Biochem. Pharmacol.* **1987**, *36*, 4361.
(29) Dixon, M.; Webb, E. C. In *Enzymes*, 3rd ed.; Academic Press: New York, 1979; p 350.

Table II. Inhibition[a] of HIV-RT by Nucleoside Triphosphates

compd	template	K_i,[b] μM
ddT-TP	RNA	0.05
	DNA	84.0
AZT-TP	RNA	0.007
	DNA	84.0
d4T-TP	RNA	0.032
	DNA	70.0

[a] Assays were performed as previously described.[28] [d] The K_i values were determined by Dixon plots.[29]

Table III. Cellular Toxicity of AZT and d4T against Normal Human Granulocyte–Monocyte and Erythrocyte Progenitor Cells[a]

	CFU-GM[b]		BFU-E:[c]
compd	ID_{50},[d] μM	ID_{90},[e] μM	ID_{50},[d] μM
AZT	1.0	20.0	6.7
d4T	100.0	>100.0	10.0

[a] The experiments were carried out as described previously.[22] [b] Colony forming units granulocyte–monocyte. [c] Burst forming units erythrocyte. [d] The 50% inhibitory concentration. [e] The 90% inhibitory concentration.

Not only does RT mediate RNA-dependent DNA polymerase but also DNA-dependent DNA polymerase activity. This DNA-dependent DNA polymerase activity suggests that RT may also be responsible for synthesis of the second DNA strand using the first DNA strand as the template primer, after hydrolysis of the RNA in the RNA–DNA pair.[31] Therefore, the inhibition constants were also determined in an assay using a DNA template, in this instance poly(dA):oligo(dT). In contrast to the results for the RNA template, the inhibition constants for the three triphosphates were much higher with the DNA template (Table II). Since HIV-RT uses different templates with different efficiencies,[30,32] the fact that the DNA–DNA step is less sensitive to inhibition by the triphosphates may simply be a reflection of the differences in the ability of the enzyme to efficiently utilize different templates.

As mentioned earlier, AZT has caused bone marrow toxicity and anemia in clinical trials. Recently, an in vitro model was developed that demonstrated that AZT was toxic to normal human granulocyte macrophage and erythrocyte progenitor cells.[22] A comparison of d4T and AZT was performed using this assay; the results in Table III express the bone marrow toxicity of the two test compounds. While d4T reduces colony-forming units (CFU-GM) by 50% at a concentration of 100 μM, AZT shows comparable toxicity at only 1 μM. The 90% inhibitory concentration for AZT is 20 μM while that of d4T is in excess of 100 μM. With the assay for erythrocyte burst forming units (BFU-E), d4T (**1**) is only slightly less toxic than AZT (ID_{50} of 10 μM for d4T as compared to 6.7 μM for AZT).

The effect of both d4T (**1**) and AZT on the metabolism of thymidine in H9 cells was examined. AZT at 50 μM inhibited the uptake of [^{3}H]thymidine into the DNA of H9 cells by approximately 80% during a 24 h continuous exposure period of cells to the drug. At the same concentration d4T showed no inhibition of DNA synthesis. H9 cells were exposed to AZT or d4T at 50 and 100 μM,

(30) St. Clair, M. H.; Richards, C. A.; Spector, T.; Weinhold, K. J.; Miller, W. H.; Langlois, A. J.; Furman, P. A. *Antimicrob. Agents Chemother.* **1987**, *31*, 1972.
(31) (a) Mitsuya, H.; Broder, S. *Nature* **1987**, *325*, 773. (b) Chandra, P.; Vogel, A.; Gerber, T. *Cancer Res.* **1985**, *45*, 4677s.
(32) Eriksson, B.; Vrang, L.; Bazin, H.; Chattopadhyaya, J.; Oberg, B. *Antimicrob. Agents Chemother.* **1987**, *31*, 600.

respectively, for 24 h, followed by a 1 h pulse label with [^{3}H]thymidine and anion-exchange HPLC of the 60% methanol soluble fraction. In the presence of AZT, radiolabeled thymidine conversion to the TDP and TTP forms were decreased whereas the label in the TMP pool increased. D4T, however, caused no significant perturbation in the relative amounts of labeled thymidine metabolites relative to control. Thus, if thymidine triphosphate starvation is important in the expression of the toxic side effects of AZT as previously discussed,[19] then d4T may offer an advantage over AZT since it does not appear to perturb the thymidine triphosphate levels.

We have detailed a synthetic approach to d4T that allows the preparation of this nucleoside analogue in multigram quantities. In vitro experiments show that d4T, while it has similar potency to AZT, may not have the same toxic side effects as AZT, an observation supported by biochemical experiments. These results show that d4T warrants further evaluation for its potential in the treatment of AIDS.

Experimental Section

Melting points were determined on an Electrothermal capillary apparatus and are uncorrected. TLC was performed on silica gel 60 F-254 plates purchased from E. Merck and Co., and column chromatography was performed on flash silica gel (40-μm particle size, Baker). Elemental analyses were performed by the analytical department, Bristol-Myers, Wallingford. ^{1}H and ^{13}C NMR spectra were recorded on a AM360 Bruker NMR spectrometer using tetramethylsilane as the internal standard; chemical shifts are recorded in parts per million. Analytical HPLC was performed on a Waters C18 reverse-phase column.

1-(2-Deoxy-3,5-bis(methylsulfonyl)-β-D-*erythro*-pentofuranosyl)thymine (3). A 3-L, three-necked, round-bottomed flask was equipped with an overhead stirrer and paddle, a 500-mL dropping funnel, and a Claisen adapter containing an addition funnel and a thermometer. Thymidine (200 g, 0.82 mol, 2) and pyridine (750 mL, 9.65 mol) were added to the flask. The mixture was stirred and then cooled in an ice bath to 0–3 °C and the dropping funnel was charged with methanesulfonyl chloride (207.2 g, 140 mL, 1.8 mol). The methanesulfonyl chloride was then added dropwise over 40 min during which time the temperature rose to 10 °C. The mixture was cooled to 0 °C and stirred for 1 h and then stored at 5 °C for 18 h. The resulting light brown mixture was then poured onto rapidly stirred water (3 L) containing ice (ca. 500 g). The desired product crystallized immediately. After stirring for 0.5 h, the product was collected by filtration and washed with water (3 × 100 mL). The white solid was dried under vacuum overnight (crude weight, 322 g, 98% yield). The product was recrystallized from hot acetone to give 3 (267 g) as a white solid (81% yield): mp 169–171 °C (lit.[24] mp 170–171 °C); ^{1}H NMR (360 MHz, DMSO-d_6) δ 11.40 (s, 1 H, NH), 7.50 (s, 1 H, H-6), 6.21 (t, 1 H, J = 6.5 Hz, H-1′), 5.29 (m, 1 H, H-3′), 4.45 (m, 2 H, H-5′), 4.35 (m, 1 H, H-4′), 3.31 (s, 6 H, SO_2CH_3), 2.50 (m, 2 H, H-2′), 1.78 (s, 3 H, CH_3). Anal. ($C_{12}H_{18}N_2O_9S_2$) C, H, N.

1-(3,5-Anhydro-2-deoxy-β-D-*threo*-pentofuranosyl)thymine (5). 1-(2-Deoxy-3,5-bis(methylsulfonyl)-β-D-*erythro*-pentofuranosyl)thymine (3) (248 g, 0.62 mol) was added in portions to a stirred solution of sodium hydroxide (74.7 g, 1.87 mol) in water (1.6 L) whereupon the reaction mixture became yellow-orange. The stirred solution was then heated to reflux for 45 min. After cooling of the reaction mixture to room temperature, concentrated hydrochloric acid (54 mL) was added. Approximately 1 L of water was removed to give a white slurry, which was left to cool in an ice bath for 2 h. The solid was filtered and washed sparingly with ice water and then vacuum dried and recrystallized from ethanol to give 5 (104 g, 74%): mp 188–190 °C (lit.[24] mp 190–193 °C); ^{1}H NMR (360 MHz, DMSO-d_6) δ 11.35 (s, 1 H, NH), 8.01 (s, 1 H, H-6), 6.49 (t, 1 H, J = 5.6 Hz, H-1′), 5.47 (m, 1 H, H-3′), 4.88 (m, 1 H, H-5′), 4.67 (dd, 1 H, J = 8.1 Hz, H-5′), 3.97 (d, 1 H, J = 8.0 Hz, H-4′), 2.47 (m, 2 H, H-2′), 1.77 (s, 3 H, CH_3); ^{13}C NMR (75 MHz, DMSO-d_6) δ 163.64 (C-4), 151.10 (C-2), 136.57 (C-6), 109.62 (C-5), 88.29, 86.85 (C-1′, C-4′), 79.83 (C-3′), 75.14 (C-5′), 37.17 (C-2′), 12.33 (CH_3). Anal. ($C_{10}H_{12}N_2O_4$) C, H, N.

1-(2,3-Dideoxy-β-D-*glycero*-pent-2-enofuranosyl)thymine (1). A three-necked, 1-L, round-bottomed flask equipped with a mechanical stirrer, thermometer, and nitrogen inlet was charged with dry DMSO (400 mL) and oxetane 5 (90.0 g, 0.402 mol). To this solution was added 97% KOtBu (74.0 g, 0.643 mol) in 1.5-g portions over 25 min.[33] The temperature was maintained between 18 °C and 22 °C by means of an external ice bath. After the addition was complete, the reaction was stirred for a further 1 h. No rise in temperature was observed and TLC indicated that the reaction was approximately 90% complete. The reaction was stirred at 21 °C for 16 h, after which time TLC indicated that the reaction was complete. The viscous solution was poured into cold (4 °C) toluene (3 L), resulting in precipitation of a beige solid. The temperature of the mixture rose to 7 °C upon addition of the DMSO solution. The mixture was occasionally swirled over 20 min and then filtered on a 18.5-cm Buchner funnel.[34] The collected yellowish oily solid was washed twice with cold toluene and allowed to dry under suction for 1 h.[35] The solid was dissolved in 300 mL of water, whereupon two layers formed. The mixture was placed in a separatory funnel, and the upper layer (containing residual toluene) was discarded. The aqueous layer was placed in a 1-L beaker equipped with a pH probe, magnetic stirring bar, and thermometer. The solution was cooled to 10 °C by the use of an external ice bath. Concentrated HCl was added dropwise to the stirred solution at a rate such that the temperature was kept below 15 °C. After the addition of HCl (50.5 mL, 0.61 mol) the pH was 7 ± 0.1 and a precipitate began to form. Potassium chloride (70 g) was added to this thick mixture and stirring was continued at 5 °C for 1 h. The precipitate was collected and left under vacuum for 2 h and then air-dried for 16 h. The solid was crushed, slurried in hot acetone (500 mL), and then filtered. The residue in the filter paper was rinsed with hot acetone (2 × 200 mL), then slurried again with hot acetone (300 mL), filtered, and washed once more with hot acetone (2 × 100 mL). The combined filtrate was concentrated to dryness to give 51.3 g (57%) of d4T (1) as an off-white solid: mp 165–166 °C (lit.[24] mp 164–166 °C); $[\alpha]^{20}_D$ −46.1° (c 0.7, water); ^{1}H NMR (360 MHz, DMSO-d_6) δ 11.29 (s, 1 H, NH), 7.63 (s, 1 H, H-6), 6.80 (d, 1 H, J = 1.2 Hz, H-1′), 6.38 (d, 1 H, J = 5.9 Hz, H-3′), 5.90 (dd, 1 H, J = 1.1, 4.7 Hz, H-2′), 5.01 (m, 1 H, OH), 4.76 (s, 1 H, H-4′), 3.60 (dd, 2 H, J = 4.8, 3.6 Hz, H-5′), 1.71 (d, 3 H, J = 1.2 Hz, CH_3); ^{1}H NMR (360 MHz, D_2O) δ 7.57 (s, 1 H, H-6), 6.88 (d, 1 H, J = 1.2 Hz, H-1′), 6.41 (m, 1 H, H-3′), 5.91 (m, 1 H, H-2′), 4.93 (br s, 1 H, H-4′), 3.73 (q, 2 H, H-5′), 1.79 (s, 3 CH_3);[36] ^{13}C NMR (75 MHz, DMSO-d_6) δ 164.42 (C-4), 151.30 (C-2), 137.23 (C-6), 135.36 (C-3′), 126.35 (C-2′), 109.33 (C-5), 89.15 (C-1′), 87.56 (C-4′), 62.41 (C-5′), 12.15 (CH_3); MS, m/e (methane DCI) (relative intensity) 225 (M + H, 20), 207 (15), 193 (8), 155 (13), 127 (100), 99 (20). IR (cm^{-1}) 3463, 3159, 3033, 1691, 1469, 1116, 1093. Anal. ($C_{10}H_{12}N_2O_4$) C, H, N.

1-(2,3-Dideoxy-5-phospho-β-D-*glycero*-pent-2-enofuranosyl)thymine (7). A solution of 1-(2,3-dideoxy-β-D-*glycero*-pent-2-enofuranosyl)thymine (40 mg, 0.179 mmol) (1) in trimethyl phosphate (0.5 mL) was treated with phosphorus oxychloride (150 mg, 1.61 mmol) at 0 °C and then stirred at room temperature for 2.5 h under argon. The mixture was decomposed by addition to a cold triethylammonium bicarbonate solution (1 M, pH 7.4, 20 mL). The reaction mixture was concentrated in vacuo (bath temperature should be kept below below 30 °C), and the residue was triturated with diethyl ether (3 × 15 mL). The ether solutions were discarded, and the semisolid material was dissolved in a minimum amount of water and purified on a C_{18} HPLC column (Whatman 500 × 18 10%–25% methanol in 0.1 N ammonium formate). Fractions containing the title product

(33) The KOtBu was weighed out in an erlenmeyer flask, and no special precautions were taken to exclude moisture.
(34) A "soft" filter paper was used (Schleicher and Schuell No. 604).
(35) Drying for longer periods (8 h) leads to glycosidic bond cleavage (ca. 10%).
(36) The proton assignment for the sugar protons is H-1′, H-3′, H-2′, H-4′, and H-5′ starting from the most downfield proton. The assignments were made on the basis of long-range and one-bond carbon–proton corrolation NMR experiments. This assignment agrees with the data published by Robins et al.[37]
(37) Robins, M. J.; Hansske, F.; Low, N. H.; Park, J. I. *Tetrahedron Lett.* **1984**, *25*, 367.

as a triethylammonium salt were pooled and lyophilized to afford 24 mg (33%) of **7**: ^{1}H NMR (360 MHz, D_2O) δ 7.56 (s, 1 H, H-6), 6.90 (br s, 1 H, H-1′), 6.42 (br d, 1 H, J = 6.0 Hz, H-3′), 5.89 (br d, 1 H, J = 6.0 Hz, H-2′), 5.03 (br s, 1 H, H-4′), 3.98 (m, 2 H, H-5′), 3.14 (q, 6 H, CH_2 of NEt_3), 1.82 (s, 3 H, CH_3), 1.22 (t, 9 H, CH_3 of NEt_3); ^{31}P NMR (145.8 MHz, D_2O) 0.07 ppm.

1-(2,3-Dideoxy-5-triphospho-β-D-*glycero*-pent-2-enofuranosyl)thymine (8). Tributylamine (0.5 mL, 2.10 mmol) and a solution of tributylammonium pyrophosphate (1.0 g, 2.3 mmol) in dry DMF (4 mL) were added at 0 °C to a solution of phosphodichloridate **6** in trimethyl phosphate (0.6 mL) prepared as above from 1-(2,3-dideoxy-β-D-*glycero*-pent-2-enofuranosyl)thymine (**1**) (25 mg, 0.112 mmol). The reaction mixture was stirred for 3 h and then poured into a cold 1 M solution of triethylammonium bicarbonate (30 mL) and then concentrated in vacuo. The residue was triturated with diethyl ether (3 × 20 mL). The ether solutions were discarded, and the insoluble residue was purified by HPLC on a C_{18} column (Whatman 500 × 18; 0%–20% of methanol in 0.1 M ammonium formate). The fractions were analyzed on an Altech NH_2-bonded column using 2 M ammonium formate in 10% aqueous methanol. Lyophilization yielded the desired product **8** (8.0 mg, 13%) as a tris(triethylammonium) salt: ^{1}H NMR (360 MHz, D_2O) δ 7.57 (s, 1 H, H-6), 6.91 (br s, 1 H, H-1′), 6.49 (br d, 1 H, J = 6 Hz, H-3′), 5.89 (br d, 1 H, J = 6 Hz, H-2′), 5.07 (br s, 1 H, H-4′), 4.15 (m, 2 H, H-5′), 3.14 (q, 18 H, CH_2 of NEt_3), 1.84 (s, 3 H, CH_3), 1.22 (t, 27 H, CH_3 of NEt_3); ^{31}P NMR (145.8 MHz, D_2O) −9.93 (d, J = 20 Hz, γ-P), −10.77 (d, J = 20.3 Hz, α-P), −22.5 (br t, β-P) ppm. Smaller amounts of the corresponding tetra- and pentaphosphates were also isolated.

1-(2,3-Dideoxy-5-tetraphospho-β-D-*glycero*-pent-2-enofuranosyl)thymine (9): ^{1}H NMR (360 MHz, D_2O) δ 7.58 (s, 1 H, H-6), 6.91 (m, 1 H, H-1′), 6.51 (dm, 1 H, J = 6.3 Hz, H-3′), 5.88 (dm, 1 H, J = 5.9 Hz, H-2′), 5.07 (br s, 1 H, H-4′), 4.14 (m, 2 H, H-5′), 3.13 (q, 24 H, CH_2 of NEt_3), 1.84 (s, 3 H, CH_3), 1.23 (t, 36 H, CH_3 of NEt_3); ^{31}P NMR (145.8 MHz, D_2O) −10.17 (d, J = 17.5 Hz, δ-P), −10.91 (d, J = 16.2 Hz, α-P), −22.52 (br t, internal P) ppm.

1-(2,3-Dideoxy-5-pentaphospho-β-D-*glycero*-pent-2-enofuranosyl)thymine (10): ^{1}H NMR (360 MHz, D_2O) δ 7.58 (s, 1 H, H-6), 6.91 (br s, 1 H, H-1′), 6.52 (br d, 1 H, J = 6.0 Hz, H-3′), 5.88 (br d, 1 H, J = 6.0 Hz, H-2′), 5.08 (br s, 1 H, H-4′), 4.16 (m, 2 H, H-5′), 3.14 (q, 30 H, CH_2 of NEt_3), 1.84 (s, 3 H, CH_3), 1.22 (t, 45 H, CH_3 of NEt_3); ^{31}P NMR (145.8 MHz, D_2O) −10.15 (br s, ε-P), −10.87 (br s, α-P), −22.46 (br s, internal P) ppm.

Antiviral Assays. The anti-HIV/LAV activity was measured in cultures of CEM-F cells. The CEM cells were infected with approximately 30 $TCID_{50}$ (50% tissue culture infectious dose) of HIV (LAV strain). The cells were then incubated for 45 min 37 °C. The test compounds, in culture medium, were added at various concentrations to the infected cells and then incubated for a further 8 days. After 8 days the antiviral activity was evaluated in the culture media supernatant for p-24 gag protein by an enzyme capture assay (ELISA). The antiviral activity was expressed as the dose that inhibits 50% of the virus expression (ID_{50} in μM) as detected by the assay described.

The culture assay for granulocyte–macrophage CFU (GM-CFU) was performed by using the assay previously described.[22]

Acknowledgment. This research was supported in part by United States Health Service Grants CA-05262 and CA-44094-01 (WHP) and CA-25784 and CA-13148 (JPS) from the National Cancer Institute.

Registry No. **1**, 3056-17-5; **2**, 50-89-5; **3**, 56822-33-4; **5**, 7481-90-5; **6**, 117269-77-9; **7**·NEt_3, 117556-48-6; **8**·3 NEt_3, 117404-75-8; **9**, 117269-78-0; **10**, 117269-79-1.

Potent Angiotensin II Antagonists with Non-β-Branched Amino Acids in Position 5

J. Samanen,*[,†] D. Narindray,[†] T. Cash,[†] E. Brandeis,[†] W. Adams, Jr.,[†] T. Yellin,[†] D. Eggleston,[‡] C. DeBrosse,[§] and D. Regoli[∥]

Peptide Chemistry Department, Structural/Physical Chemistry Department, Analytical Chemistry Department, Smith Kline & French Laboratories, King of Prussia, Pennsylvania 19406-0939, and Department of Pharmacology, University of Sherbrooke, Sherbrooke, Quebec J1H 5N4, Canada. *Received May 12, 1988*

Amino acids with lipophilic side chains that contain more than one functional group on the β-carbon, i.e. a β-branched hydrocarbon moiety, are required in position 5 of angiotensin II (AII) analogue with potent agonist activity. This requirement for agonist activity does not follow for AII analogues with potent antagonist activity. Straight-chain amino acids may be substituted into position 5 of [Sar^1,X^5,Ile^8]AII with retention or enhancement of antagonist activity, e.g. (X^5, pA_2 rabbit aorta) Phe, 9.15; Tyr, 9.6; His, 9.0; Arg, 9.0; Glu, 9.0; Nle, 8.85, compared to Ile, 9.1. β-Branched side chains can still enhance the antagonist activities of [Sar^1,X^5,Ile^8]AII analogues, e.g. X^5 = (βMe)Phe, pA_2 = 9.3. An X-ray crystal structure of the Boc-(βMe)Phe DCHA salt, prepared for the synthesis of [Sar^1,(βMe)Phe^5,Ile^8]AII, revealed an *S,S* configuration of α- and β-carbon atoms. Contrary to previous literature reports, chemical nonequivalence of the δ-protons of Pro was observed in the ^{1}H NMR spectra of [Sar^1,X^5,Ile^8]AII analogues bearing both β-branched X^5 side chains (X^5 = Ile) and non-β-branched X^5 side chains (X^5 = Ala, His).

In recent years, potent antagonists to angiotensin II have been developed through a variety of alterations in position 1 (aspartic acid)[1] and eight (phenylalanine).[2] Several of these analogues have been shown to lower blood pressure in humans with high plasma renin levels, including [Sar^1,Ala^8]AII (saralasin)[3] and [Sar^1,Ile^8]AII.[4]

* Address correspondence to: Dr. James Samanen, Peptide Chemistry Department, Smith Kline & French Laboratories, P.O. 1539, King of Prussia, PA 19406-0939.
† Peptide Chemistry Department.
‡ Structural/Physical Chemistry Department.
§ Analytical Chemistry Department.
∥ Department of Pharmacology.

(1) The abbreviations for natural amino acids and nomenclature for peptide structures follow the recommendations of the IUPAC–IUB Commission on Biochemical Nomenclature (*J. Biol. Chem.* **1971**, *247*, 977. Unnatural amino acids used in this study have been given the following abbreviations: Cpg, L-cyclopentylglycine; (βMe)Phe, β-methylphenylalanine; Boc-ON, 2-[(*tert*-butoxycarbonyl)oxy]imino]-2-phenylacetonitrile; Chg, L-cyclohexylglycine; (SMe)Pen, *S*-methyl-L-penicillamine; Phg, L-phenylglycine; (pNO$_2$)Phe, L-*p*-nitrophenylalanine; Peg, L-phenylethylglycine, (OMe)Thr, *O*-methyl-L-threonine.

0022-2623/89/1832-0466$01.50/0 © 1989 American Chemical Society