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EPR evidence for hydrogen bond donation to the terminal oxygen of Co-O2 model compounds and cobalt oxymyoglobin

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matography of the crude product was omitted. Instead, the crude product was directly chromatographed on preparative-layer Avicel-F plates (1 mm thick) with 3:1 ethanol/H_2O as the eluent. Recrystallization from H_2O gave **16** (71 mg, 52%) as its monohydrate: mp 216-219 °C [lit.[12] mp (of protio homologue of **16**) 217-220 °C]; ^1H NMR (D_2O) δ 1.85-2.10 (m, 2, CH_2-CH_2-CH), 2.54 (t, J = 7.5 Hz, 2, SCH_2), 2.85-2.95 (m, 2, H-5'), 3.40 (t, J = 6.5 Hz, 1, CH), 4.05 (t, J = 6 Hz, 1, H-4'), 4.72 (d, $J_{1'2'}$ = 6.1 Hz, 1, H-2'), 5.3 (d, $J_{1'2'}$ = 6.1 Hz, 1, H-1'), 8.19 (s, 1, H-8), 8.34 (s, 1, H-2); mass spectrum (DCI; NH_3), m/z (rel intensity) 285 (23.1), 284 (1.5); protio homologue 284 (24.5), 283 (0.8). Anal. ($C_{14}H_{19}{}^2HN_6O_5S\cdot H_2O$) C, H + ^2H, N.

Purification of AdoHcy Hydrolase. Bovine liver was obtained from a local slaughter house and stored at -70 °C. The enzyme was partially purified according to the procedure of Palmer and Abeles.[5] Briefly, 500 g of tissue was homogenized in 10 mM K_2HPO_4, pH 7.2, in a Waring blender at 4 °C. The homogenate was subjected to acid and heat precipitation, ammonium sulfate fractionation, and DEAE-cellulose chromatography. The specific activity of this preparation was 12 μmol of inosine formed/min/mg of protein. One unit of AdoHcy hydrolase activity is defined as 1 μmol of inosine formed/min.

***S*-Adenosyl-L-homocysteine Assays.** The synthetic activity of AdoHcy hydrolase was measured as follows. In a total volume of 1 mL, the reaction mixture contained 250 μM Ado (or Ado-*3'-d*), 1 mM DL-Hcy, 0.1 mM DTT, 1 mM EDTA, 100 μM EHNA, and 150 mM K_2HPO_4, pH 7.6. The reaction was started by the addition of 0.4 units of AdoHcy hydrolase.

To measure the hydrolysis of AdoHcy, the coupled assay[19] utilizing Ado deaminase (to convert Ado formed to inosine) was used. In a total volume of 1 mL, the reaction mixture contained 250 μM AdoHcy (or AdoHcy-*3'-d*), 0.1 mM DTT, 1 mM EDTA, 8 units/mL of Ado deaminase, and 150 mM K_2HPO_4, pH 7.6. The reaction was started by the addition of 1.9 units of AdoHcy hydrolase.

At various times after incubation at 37 °C, 150-μL aliquots were transferred to 0.5-mL tubes containing 5 μL of 4 N $HClO_4$. All samples were stored at -20 °C until HPLC analysis.

HPLC Analysis. The samples were brought to room temperature just before injection and centrifuged in a Eppendorf microcentrifuge (8000 *g*, 1 min) to remove the precipitated material. A 50 μL aliquot of the supernatant was injected into a Perkin-Elmer Series 3 HPLC equipped with a Zorbax C-8 reversed-phase column (25 cm × 4.6 mm) and a Sigma 10B data station. A two-step gradient with acetonitrile and 50 mM NaH_2PO_4, 10 mM heptanesulfonic acid, pH 3.2, was used to elute the samples (0-5 min, 5-15% acetonitrile; 5-15 min, 15-20% acetonitrile). Absorbance at 254 nm was monitored. The retention times for inosine, Ado, and AdoHcy were 5.2, 11.4, and 13.5 min, respectively. The peak areas for AdoHcy (synthetic reaction) and iosine (hydrolytic reaction) were used to calculate the enzyme activity.

Enzymatic Synthesis of AdoHcy-*3'-d* from Ado-*3'-d*. In a total volume of 2 mL, the reaction mixture contained 150 mM K_2HPO_4, 0.1 mM DTT, 1 mM EDTA, 100 μM EHNA, 50 mM DL-Hcy, 20 mM Ado-*3'-d*, and 15-20 units of partially purified bovine liver AdoHcy hydrolase. After 3 h at 37 °C, the reaction was stopped by immersing the mixture in boiling water. (Less than 2% unreacted Ado-*3'-d* could be detected by HPLC, after the 3-h incubation period.) The precipitate was removed by centrifugation and filtration through Whatman No. 3 filters. AdoHcy-*3'-d*, present in the filtrate, was purified and characterized as described above for the chemical synthesis of AdoHcy-*3'-d*.

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EPR Evidence for Hydrogen Bond Donation to the Terminal Oxygen of Co-O_2 Model Compounds and Cobalt Oxymyoglobin

F. Ann Walker* and James Bowen

Contribution from the Department of Chemistry, San Francisco State University, San Francisco, California 94132. *Received May 29, 1985*

Abstract: EPR investigations of mono-ortho and para acetamide derivatives of cobalt(II) tetraphenylporphyrins, (*o*-$NHCOCH_3)_1$TPPCo and (*p*-$NHCOCH_3)_1$TPPCo, in the presence of *N*-methylimidazole and molecular oxygen, provide information concerning the dynamics of bound dioxygen: When hydrogen bond donation is possible (from the N-H of the ortho acetamide), the EPR spectra of the dioxygen adduct are not motionally averaged, as they are for the para acetamide and all previously studied monomeric Co-O_2 complexes. These results indicate that the motion responsible for the averaging of the EPR signals in fluid solution is the rotation of the dioxygen moiety about the Co-O bond. Investigation of the room-temperature EPR spectrum of cobalt oxymyoglobin indicates that this case as well as internal rotation of bound dioxygen is prevented, presumably by hydrogen bonding of the distal histidine to the terminal oxygen atom.

Synthetic analogues of the dioxygen-carrying proteins myoglobin and hemoglobin have often utilized amide substituents to create a protected "pocket".[1-7] Recent evidence has suggested an important role for hydrogen bond donation from the amide N-H in stabilization of O_2 binding to iron(II) and cobalt(II) model hemes:[3-8] The "bis-pocket" porphyrin,[3,4] which has no amide substituents, has an oxygen affinity much lower than the "picket fence" porphyrin,[1,2] and "basket handle" and "hanging base basket handle" porphyrins having either linkages have much lower oxygen affinities and more rapid oxygen dissociation rates than those having amide linkages.[5-7] Calculations by Jameson and Drago[8]

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have suggested significant enthalpic stabilization due to amide N–H hydrogen bonding to bound O_2.

In the course of an investigation of the effect of amide substituents on the physical and chemical properties of metallotetraphenylporphyrins,[9-13] we discovered that the amide N–H proton is capable of hydrogen bond donation to both displaced anions (for example, to Cl^- in $PFeL_2^+Cl^-$)[11-13] and coordinated ligands (for example, to O_2 in $PCoLO_2$).[12,13] The equilibrium constant for the binding of O_2 to $(o\text{-}NHCOCH_3)_1TPPCoL$, where L = pyridine or *N*-methylimidazole, is ca. five times as large as those for the corresponding meta and para acetamide derivatives, which are similar to the equilibrium constant for O_2 binding to the parent, TPPCoL. These results are discussed in further detail elsewhere.[13] Of importance to this paper, however, is the line shape of the EPR signal observed for the ortho acetamide derivative, because it provides new and important insight into the dynamics of the bound dioxygen moiety in these model compounds, as well as in proteins.

Experimental Section

Specialty chemicals (pyrrole, benzaldehyde, the nitrobenzaldehydes, acetyl chloride, pivaloyl chloride, pyridine, *N*-methylimidazole) were obtained from Aldrich Chemical Co. Solvents (propionic acid, methylene chloride, dimethylformamide, toluene, benzene) were obtained from Spectrum Chemical Co. The acetamide-substituted tetraphenylporphyrins were prepared by first synthesizing the appropriate mixture of nitrophenylporphyrins by our previously published adaptation of the Alder synthesis.[9,14,15] The mono-*o*- or *p*-nitro $TPPH_2$ isomer was separated by gravity chromatography on silica gel (Baker chromatographic grade) utilizing 70:30 benzene:petroleum ether (30–60 °C boiling fraction). The nitro groups were then reduced to amino by means of the $SnCl_2/HCl$ method,[16] followed by chromatography of the product mono-amino-$TPPH_2$. This purified product was then reacted, with stirring, with a twofold excess of acetyl chloride or other acid chloride in dry benzene solution containing enough dry pyridine to maintain the porphyrin in its free base (purple) form. After a half-hour reaction time, the reaction mixture was washed at least five times with dilute sodium hydroxide, followed by water, dried over sodium sulfate, and chromatographed on silica gel with benzene to remove any unreacted aminoporphyrin, then it was eluted with methylene chloride, and the product was evaporated to dryness. Cobalt insertion and purification of the TPPCo derivatives were effected by the same methods as described previously,[17] except that it was usually necessary to shake a methylene chloride solution of the CoTPP derivatives with aqueous sodium dithionite following metal insertion and before column chromatography, since the amide-substituted CoTPP derivatives showed a greater tendency toward autoxidation, not only to Co(III) but an additional step to the π-cation radical. If partially oxidized samples were left in air for long periods of time, the Co(II) state could not be regenerated by shaking with dithionite.

Horse skeletal muscle myoglobin (type I) and cobalt protoporphyrin chloride were obtained from Sigma Chemical Co. Hemin removal and reconstitution with the cobalt-substituted heme were carried out according to the procedures outlined by Yonetani and co-workers.[18] Cobalt myoglobin solutions at pH 6.0 in 10 mM phosphate buffer were utilized immediately following concentration to approximately 10 mM.

EPR studies were carried out on a Varian E-12 equipped with flowing nitrogen temperature controller. The field sweep was calibrated utilizing an NMR gaussmeter, and field-frequency calibration was achieved utilizing a sample of DPPH (g = 2.0036). Temperature calibration was carried out utilizng a small-gauge copper–constantan thermocouple referenced to distilled ice–water. Cobalt myoglobin samples were placed in thin-walled gas-permeable Teflon microtubing (Zeus Industrial Products) which was folded in half and inserted into an open-ended quartz EPR sample tube and thence into the microwave node in the EPR cavity. Flowing oxygen was used in place of nitrogen as the thermostating gas, in order to maintain the myoglobin sample saturated with oxygen throughout the experiment.

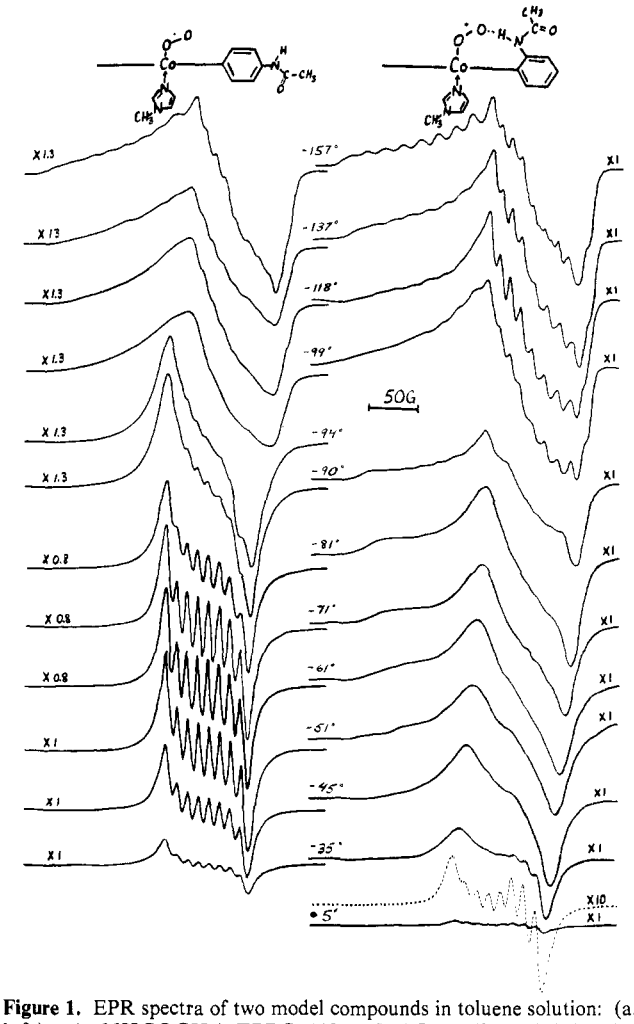

Figure 1. EPR spectra of two model compounds in toluene solution: (a, left) $(p\text{-}NHCOCH_3)_1TPPCo(N\text{-}MeIm)O_2$; (b, right) $(o\text{-}NHCOCH_3)_1TPPCo(N\text{-}MeIm)O_2$.[23] The relative spectrum amplitudes are listed on the outer edges of the spectra. Field calibration was achieved by use of an NMR gaussmeter and frequency calibration by use of a solution of DPPH in toluene (g = 2.0036).

Results and Discussion

In Figure 1 are shown a series of EPR spectra for the dioxygen adducts of the mono-acetamide complexes of TPPCo(*N*-MeIm) in toluene solution as a function of temperature. The para acetamide (Figure 1a) is incapable of H bonding to the bound dioxygen, while for the ortho amide, Figure 1b, CPK molecular models suggest that the N–H proton is at the proper distance for H bond donation to bound dioxygen. The top set of spectra, recorded at −157 °C, are for the immobilized, glassy toluene samples of the para and ortho acetamide complexes. These samples exhibit similar anisotropic EPR parameters to those of symmetrically substituted $TPPCoLO_2$ complexes reported earlier,[19] with g_{zz} = 2.063, g_{yy} = 1.987, and g_{xx} = 1.975, A_{zz} = 17.6 G, A_{yy} = 10.6 G, and A_{xx} = 8.1 G. As the samples are warmed from −157 to −95 °C (the freezing point of toluene), the EPR spectrum of the ortho amide changes very little, while that of the para amide

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begins to lose resolution of the x, y, and z components of the **g** and **A** tensors. At −94 °C the para acetamide complex exhibits an almost completely motionally averaged EPR spectrum, which by −81 °C becomes the typical, eight-line pattern observed for all Co–O_2 complexes above the freezing point of toluene reported previously.[19-21] Over the same temperature range the EPR spectrum of the ortho acetamide complex remains anisotropic, with obvious "parallel" and "perpendicular" branches, although the hyperfine structure is no longer resolved. This anisotropic spectrum slowly collapses toward the motionally averaged signal as the temperature is raised, and begins to show ^{59}Co hyperfine splittings again at about −35 °C, at which point the EPR spectrum of the para acetamide sample, which is still the simple eight-line pattern observed at −81 °C is decreasing in amplitude due to the instability of this dioxygen adduct at higher temperatures. The ortho acetamide complex, stabilized by hydrogen bonding, can be observed up to room temperature, though the rate of autoxidation to the EPR-silent Co(III) complex and then further to the π-cation radical complex[22] precludes quantitative study of the signal. At the highest temperature shown in Figure 1, +5 °C, the eight-line hyperfine pattern of the dioxygen complex of the ortho amide still exhibits variation in line widths due to incomplete motional averaging of the anisotropies in the **g** and **A** tensors.[23]

The rotational correlation time, τ_R, may be calculated from the expression:[24]

$$\tau_R = (4/3)\pi r^3(\eta/kT) \quad (1)$$

The value of τ_R for the two complexes should be very similar, since their sizes do not differ appreciably. Calculation of τ_R as a function of temperature indicates that only at and above room temperature is the viscosity of toluene sufficiently low as to produce isotropic spectra through rotation of the entire molecule. Hence, the motionally averaged spectrum exhibited by the para acetamide complex above the freezing point of toluene (Figure 1a) is due to an averaging process which can occur through internal rotation, not involving the entire molecule, i.e., by rotation of the bent dioxygen moiety about the Co–O bond axis. That this is the explanation is supported by the lack of agreement of the observed EPR parameters ($\langle g\rangle$ = 2.004, $\langle a\rangle$ = 10.9 G) with those calculated from the glassy spectrum ($\langle g\rangle$ = 2.008, $\langle a\rangle$ = 12.1 G). The deceptive "isotropic" appearance of the spectra of Figure 1a is probably a result of the principle magnetic axis of the unpaired electron being at approximately the "magic angle" of the axis of the rotation. The ortho acetamide complex is not capable of this internal rotation due to H bonding between the acetamide N–H and the terminal oxygen atom. Other ortho amides vary in their H bonding capability in reverse order to the bulk of the group attached to the carbonyl. For example, the EPR spectrum of the Co–O_2 complex of the mono-ortho pivalamide (trimethylacetamide) derivative in toluene solution at −61 °C is similar in resolution to that of the mono-ortho acetamide at +5 °C. Similarly, in terms of thermodynamic stabilization, the O_2 adduct of the ortho acetamide derivative is a factor of 5 more stable than that of the para acetamide throughout the temperature range −57 to −19 °C,[13] while the factor of 2 stabilization of the Co–O_2 complex of the ortho pivalamide observed at −57 °C is completely lost by −19 °C.[13]

The fact that EPR spectroscopy could act as a probe of immobilization of the internal rotation of bound dioxygen by H bond donation from some nearby residue prompted us to investigate the room-temperature EPR spectrum of the dioxygen complex of cobalt-substituted myoglobin, CoMb. Many scientists have postulated that the distal histidine of myoglobin and hemoglobin

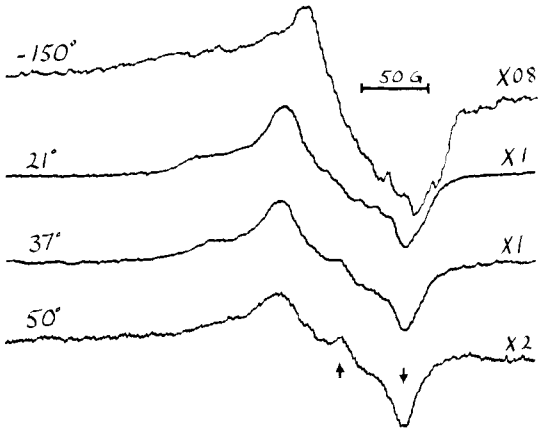

Figure 2. EPR spectra of 10 mM oxy-CoMb in aqueous solution at pH 6.0 in the presence of 1 atm of O_2, recorded as a function of temperature. The position of the maximum and minimum in the EPR spectrum of the π-cation radical, formed by 2-electron autoxidation of some of the CoMb at elevated temperatures, is marked by the two arrows. (see also ref 23.)

is involved in hydrogen bonding to bound dioxygen. Infrared,[25] resonance Raman,[26] and kinetic[27] data appear to support this hypothesis indirectly, while EPR spectra of cobalt-substituted myoglobins and hemoglobins in D_2O[28] and of CoHbA and CoHb Glycera[29] have provided more direct evidence of such H bonding in frozen solutions. Recent X-ray studies of HbO_2[30] and MbO_2,[31] and neutron diffraction studies of MbO_2,[32] have provided strong direct evidence of H bonding between the distal histidine and bound O_2 in the crystalline state. The present study provides additional strong evidence of this H bonding, in this case in homogeneous solution at ambient temperatures.

In Figure 2 is shown the EPR spectrum of $CoMbO_2$, preparbed by the method of Yonetani et al.[18] as a function of temperature. The frozen solution spectrum, recorded at ca. −150 °C, is only slightly different from that obtained at 21 °C in fluid aqueous solution. As the temperature is raised to 37 °C and finally to 50 °C, the spectrum shows significant progress toward motional averaging,[33] even though τ_R for the entire myoglobin molecule in H_2O at 50 °C is approximately the same as that for the model compound (o-NHCOCH$_3$)$_1$TPPCo(N-MeIm)O_2 in toluene at −81 °C. Hence at physiological temperatures the dioxygen adduct of CoMb is subject to the same hindrance in internal rotation of the bent dioxygen moiety as is the model compound of Figure 1b, and only at temperatures elevated significantly above physiological range does the dioxygen moiety begin to rotate. We believe that these results provide direct evidence for stabilization of the dioxygen adduct through H bond donation.

Some years ago Lang and Marshall[34,35] noted the temperature dependence of the quadrupole splitting of the Mössbauer signal

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of oxyhemoglobin which suggested the presence of low-lying excited states. They postulated that these excited states were related to a rotation of the O_2 molecule. In a later Mössbauer study of crystals of the picket fence iron porphyrin dioxygen complex,[35,36] similar temperature dependence in ΔE_Q was observed and explained on the basis of rotation of the O–O moiety between the two possible orientations of the Fe–O_2 and Fe–imidazole planes observed in the crystals. However, this interpretation did not appear to be applicable to the protein data.[37] It would be interesting to reconsider the Mössbauer data in light of the possibility of H bonding from a distal residue of the protein or an amide "picket" of the model compound.

While the results presented herein do not rule out the possibility that O_2 rotation is prevented by the steric restrictions imposed by the internal sculpture of the protein pocket, rather than by H bond donation from the distal histidine, the weight of X-ray and neutron diffraction data, along with the present EPR results, argues strongly in favor of the H-bonding explanation. It further provides a new technique for observing this H bonding in solution at room temperature. Further investigation of the room-temperature EPR spectra of $CoMbO_2$ and $CoHbO_2$ is underway.

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Registry No. (*p*-$NHCOCH_3$)TPPCo(*N*-MeIm)O_2, 98859-46-2; (*o*-$NHCOCH_3$)TPPCo(*N*-MeIm)O_2, 98859-47-3; O_2, 7782-44-7.

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X-ray and ^{1}H NMR Analyses of the Structure and Conformation of 2-Azacoformycin, a Potent Inhibitor of Adenosine Deaminase[1]

George I. Birnbaum,*[2a] Jean Robert Brisson,[2a] Steven H. Krawczyk,[2b] and Leroy B. Townsend[2b]

Contribution from the Division of Biological Sciences, National Research Council of Canada, Ottawa, Ontario, Canada K1A 0R6, and the Department of Medicinal Chemistry, College of Pharmacy, University of Michigan, Ann Arbor, Michigan 48109. *Received May 3, 1985*

Abstract: The three-dimensional structure of 2-azacoformycin was determined by X-ray crystallography. The crystals belong to the orthorhombic space group $P2_12_12_1$, and the cell dimensions are a = 7.022 (1) Å, b = 8.491 (1) Å, and c = 20.773 (1) Å. Intensity data were measured on a diffractometer, and the structure was determined by direct methods. Least-squares refinement, which included all hydrogen atoms, converged at R = 0.043 for 1502 observed reflections. The diazepine ring is in a somewhat distorted and flattened sofa conformation, C(7) being displaced from the mean plane. The distribution of electrons in the aglycon can be inferred from its geometry. The glycosyl torsion angle is in the high-anti range (χ_{CN} = 72.4°), the furanose ring has a C(2′) endo/C(3′) exo ($^2T_3$) pucker, and the conformation of the $-CH_2OH$ side chain is gauche$^-$. The solution conformation was determined by high-resolution ^{1}H NMR spectroscopy. The conformation of the diazepine ring in solution is essentially the same as that in the solid state. It is shown that the interpretation of NMR data on the basis of average furanose conformations may lead to inaccurate results.

A number of adenosine analogues are known to possess significant antiviral and antileukemic activities.[3] However, a major problem encountered in the use of such compounds is the facility with which they undergo intracellular deamination by the enzyme adenosine deaminase (ADA), since the products (inosine analogues) exhibit lower or no activity. The inhibition of this enzyme is not only therapeutically attractive,[4,5] but it is also thought to cause immunosuppression which is desirable in tissue transplants.[6] In cases of lymphoblastic leukemia, coformycin, a potent inhibitor of ADA, has been shown to induce a remission when used alone.[7] Biological testing of coformycin has also demonstrated[8] that the inhibitor can be incorporated into cellular DNA and that it has different effects on the cell cycle in normal and virally transformed cells.[9]

Of the known inhibitors of ADA, coformycin (**1a**) and its 2′-deoxy derivative, pentostatin (**1b**), are the most potent. Furthermore, they are noteworthy because they exhibit anomalous binding kinetics characteristic of transition-state analogues.[10] Heterocyclic analogues of these inhibitors had not been reported and therefore a structure–activity study of the relationship of inhibition by this class of transition-state analogues could not be accomplished. This prompted us to apply some previously developed methodology from our laboratory to synthesize[11-14] several

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