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Synthesis and Identification of the Monocation $Tc(CPI)_6^+$ in $Tc(CNC(CH_3)_2COOCH_3)_6Cl$ and Its Hydrolysis Products

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The complex $Tc(CNC(CH_3)_2COOCH_3)_6Cl$ (cation designated as $Tc(CPI)_6^+$) is one of a series of isonitrile-coordinated technetium complexes being investigated as a prototype radiopharmaceutical to evaluate myrocardial perfusion. It has been prepared from the pertechnetate ion by aqueous $Na_2S_2O_4$ reduction in the presence of the functionalized isocyanide ligand. The octahedral compound with its relatively reactive ester substituents is stable under aqueous aerobic conditions within the pH range 5–7. At high pH, however, the ester moieties undergo random, base-catalyzed hydrolysis while the six ligands remain coordinated in a fixed geometry to the central metal. The nine predicted carboxylic acid containing species were separated and identified by RP-HPLC, FAB-MS, IR, and ^{99}Tc NMR methods. Specific k' values obtained from the RP-HPLC of these characterized compounds were used to identify technetium containing metabolites of the radiopharmaceutical. Varying rates of in vitro enzymatic hydrolysis at the terminal ester moieties of the coordinated ligands were shown to occur when this compound was incubated with blood serum from different animal species.

Introduction

The widespread use of the radionuclide ^{99m}Tc for diagnostic nuclear medicine imaging has prompted the search for radiopharmaceuticals of this metal that exhibit specific biological properties.[2] In particular, much effort has been directed at finding a technetium complex that would be accumulated in normal heart muscle, thus providing a method for detecting coronary artery blockage and for monitoring myocardial perfusion.[3] Empirical screening for successful heart-imaging agents has been complicated by interspecies differences in the biodistribution of various technetium complexes.[4] Recently, several members of a class of compounds in which six isonitrile ligands are octahedrally coordinated to a technetium(I) center have demonstrated accumulation in human myocardial tissue.[5]

Extraction by a target organ, however, is not the only criterion for an optimal radiopharmaceutical. Rapid elimination of a labeled drug from the bloodstream and low retention in the surrounding tissue are also important in obtaining good signal to noise ratios for imaging. An understanding of the biological disposition of these compounds relative to their structure is necessary for designing improved agents. The accessibility of the technetium(I) state and the wide variety of derivatives possible for the alkyl portion of the isonitrile ligand provide numerous options for modifying biodistribution or pharmacokinetics.[6]

In an effort to produce a complex that exhibited myocardial localization as well as rapid clearance from blood, lungs, and liver, a series of functionalized isocyanide ligands and their technetium complexes were synthesized.[7] The strategy was to incorporate reactive organic functional groups on the ligands that might be recognized as substrates by the compartmentalized enzymatic activity of the body. Metabolism of the surface groups on the radiopharmaceutical in nontarget organs would aid in its elimination, thus reducing the background activity and the radiation dose to the patient. In this paper, we report the synthesis and characterization of a technetium(I) hexakis(isonitrile)[8] complex containing functionalized alkyl isocyanide ligands possessing a terminal methyl ester group. The hydrolysis products of this compound have also been characterized and correlated with the enzymatic metabolites of the parent radiopharmaceutical.

Classical synthesis and characterization were performed with the relatively long-lived isotope ^{99}Tc. The identified compounds were then used to corroborate those obtained in radiopharmaceutical preparations with the short-lived, high-specific-activity ^{99m}Tc isotope by dual detection (radiometric and UV) analysis following chromatographic separations. The chromatographic analysis for ^{99m}Tc-containing species, in samples of biological fluids or tissues, also permits the quantification of metabolites at concentrations of the radiopharmaceutical that are relative to those used in clinical nuclear medicine.

Experimental Section

Syntheses. *Caution!* Technetium-99 is a weak β^- emitter (E = 0.292 MeV; $t_{1/2} = 2.12 \times 10^5$ years). All manipulations were carried out in laboratories licensed by the Nuclear Regulatory Commission for low-level radioactive materials. Necessary precautions for its safe handling have been detailed elsewhere.[9]

Materials. The starting materials for the ligand, 2-aminoisobutyric acid, formic acid (puriss grade, >98%), and trichloromethyl chloroformate "diphosgene" (pract grade, ~97%), were obtained from Fluka Chemie AG. Spectroscopic grade methanol (Omnisolv, EM Science, Gibbston, NJ) was kept dry over molecular sieves. Technetium-99, as NH_4TcO_4 in aqueous solution, was obtained as a gift from DuPont/Biomedical Products, Billerica, MA. The "carrier-free" metastable radionuclide ^{99m}Tc, as sodium pertechnetate (30–150 mCi/mL), was obtained from a commercial ($^{99}Mo/^{99m}Tc$) generator in aqueous NaCl (0.15 M) (DuPont/Biomedical Products, Billerica, MA).[10] All other compounds were reagent grade and used without further purification.

Purification of micromole quantities of the technetium complexes for RP-HPLC or mass spectral analysis was performed by using a prewet SEP-PAK C_{18} cartridge (Waters Associates, Milford MA) prepared by

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(10) ^{99m}Tc decays primarily by isotopic transition to ^{99}Tc with the emission of a single γ-photon (E = 140 keV; $t_{1/2}$ = 6.02 h). All manipulations with this high specific activity isotope were performed in shielded areas approved for intermediate levels of radioactivity in accordance with NRC guidelines.

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first injecting via a syringe absolute ethanol (5 mL) through the cartridge, followed by distilled water (5 mL), and finally air (10 mL).

Spectroscopic Measurements. Fourier-transform ^1H NMR spectra were obtained on a Bruker WM 250-MHz or Varian 300-MHz instrument with $CDCl_3$ as solvent and TMS as the internal standard. ^{13}C and ^{99}Tc NMR spectra were obtained on a Varian 400-MHz instrument using a broad-band adjustable probe and $CDCl_3$ as the solvent/standard or TcO_4^- as the external reference, respectively, as described elsewhere.[11] Fast-atom-bombardment (FABMS) and field-desorption mass spectra (FDMS) were obtained in a positive ion mode on a Varian MAT 731 mass spectrometer from a glycerol matrix or carbon deposition probe.[12]

Chromatography. Reversed-phase thin-layer chromatography (RP-TLC) was carried out on Whatman MK C-18 plates developed in tetrahydrofuran/methanol/acetonitrile/aqueous buffer (ammonium acetate, 0.5 M) (2:3:3:2 v/v/v/v). Measurement of retention fractions (R_f) for the radiopharmaceutical preparations was obtained on a TLC plot reader by using a collimated NaI crystal interfaced through a Canberra 2007P photo multiplier tube to a Model 2015A single-channel amplifier and Model 14812 rate meter (Canberra Industries Inc., Meriden CT). The printout and integration of this signal were obtained on an HP 3390A integrator (Hewlett Packard). High-performance liquid chromatography (HPLC) was performed on a gradient system employing dual detectors, UV-vis (254 nm) and NaI-based radiometric detection (Waters Associates, Woburn, MA), as described previously.[13]

Analytical HPLC analysis of $Tc(CPI)_6^+$ and its hydrolysis products was carried out in a reversed-phase mode (RP-HPLC). The stationary phase consisted of C-8 bonded spherical silica particles (5 μM) in a 10 × 0.46 cm column (Brownlee OS-MP cartridge, Rainin Instruments, Woburn, MA). The gradient mobile phase was 100% aqueous buffer (ammonium sulfate, 0.05 M, pH 5.5) to 95% methanol in a 5-min linear gradient at 2 mL per min.

Preparation of 2-(Carbomethoxy)-2-methylethyl Isocyanide, $CNC(CH_3)_2CO_2CH_3$ (CPI). The ester isocyanide ligand was synthesized by an adaptation of Ugi's formamide dehydration reaction[14] using the more convenient "diphosgene" Cl_3CO_2Cl in place of "phosgene" as suggested by Efray et al.[15] In brief, the methyl ester of 2-aminoisobutyric acid was obtained by gaseous HCl-induced esterification in dry pure methanol as the solvent/reagent. The product was recrystallized from a mixture of acetone/diethyl ether/hexane and then formylated by reaction with acetic anhydride/formic acid (1:2, v/v) for 48 h.[16] The resulting methyl 2-formamidoisobutyrate was purified by vacuum distillation and converted to the isocyanide as follows. In a 500-mL, four-neck, round-bottom flask, a solution of methyl 2-formamidoisobutyrate, vide supra (5.1 g, 35.2 mmol), in CH_2Cl_2 (100 mL) was purged with argon and cooled to −30 °C in a dry ice/2-propanol bath, and trimethylamine (35 mL) dissolved in CH_2Cl_2 (75 mL) was added. With mechanical stirring and continued cooling, a solution of trichloromethyl chloroformate (4.0 g, 20.0 mmol) in CH_2Cl_2 (50 mL) was added dropwise over 30 min. The reaction was allowed to warm slowly to room temperature and then heated to reflux for 30 min. This mixture was treated with ammonium hydroxide (30%, 100 mL) and the CH_2Cl_2 layer separated. The aqueous layer was washed with CH_2Cl_2 (2 × 30 mL), the extracts were combined and dried over sodium sulfate, and the volume was reduced under vacuum. The colorless product was isolated by vacuum distillation (70–71 °C; 26 mmHg); yield 2.8 g, 22.0 mmol (62%). IR ($CHCl_3$, cm^{-1}): ν_{CN} 2141, ν_{CO} 1752. ^1H NMR (δ, $CDCl_3$): 1.68 (s, 6 H), 3.83 (s, 3 H). $^{13}C\{^1H\}$ NMR (δ, $CDCl_3$): 169.2 (CO), 157.5 (t, CN), 59.0 (t, J = 6 Hz, α-C), 52.9 (OCH_3), 27.0 (β-CH_3). MS (high resolution m/z): 127.14; calcd for $C_6H_9NO_2$ = 127.144.

Preparation of $^{99}Tc(2\text{-(carbomethoxy)-2-methylethyl isocyanide})_6PF_6$, $^{99}Tc(CNC(CH_3)_2COOCH_3)_6PF_6$. To a 100-mL, three-neck flask were added $Tc(SC(NH_2)_2)_6Cl_3$ (0.31 g, 0.47 mmol),[17] a magnetic stir bar, and methanol (50 mL). A water-cooled condenser was attached and the system purged with argon for 15 min prior to addition of 2-(carbomethoxy)-2-methylethyl isocyanide (1.5 mL, 15 mmol). The bright red solution was refluxed for 45 min during which the color faded to pale orange. The volume was reduced to ~15 mL under vacuum, and aqueous NH_4PF_6 (10 mL, 0.3 M) was added. The solution was cooled overnight at 4 °C. The brown precipitate that formed was recrystallized from acetone/diethyl ether/hexane (1:10:1) six times to obtain the slightly off-white product; yield 0.17 g, 0.17 mmol (36% based on Tc). Anal. Calcd for $C_{36}H_{54}N_6O_{12}F_6PTc$: C, 42.95; H, 5.36; N, 8.35. Found: C, 42.77; H, 5.22; N, 8.28. UV/vis [CH_3CN; λ_{max}, nm (ϵ, L M^{-1} cm^{-1})]: 236 (4.9 × 10^4), 263 (sh). IR (KBr, cm^{-1}): ν_{CN} 2093, ν_{CO} 1748. ^1H NMR (δ, $CDCl_3$): 1.65 (s, 6 H, β-CH_3), 3.86 (s, 3 H, OCH_3). $^{13}C\{^1H\}$ NMR (δ, $CDCl_3$): 169.1 (CO), 60.8 (s, α-C), 52.8 (OCH_3), 27.0 (β-CH_3). ^{99}Tc NMR (δ, CH_3CH_2OH): −1925. (+)FABMS (m/z): 861; calcd for $C_{36}H_{54}N_6O_{12}Tc^+$ = 861.8.

Preparation of $^{99}Tc(2\text{-(carbomethoxy)-2-methylethyl isocyanide})_6Cl$, $^{99}Tc(CNC(CH_3)_2COOCH_3)_6Cl$. To a 50-mL, round-bottom flask were added sodium hydrosulfite ($Na_2S_2O_4$) (0.27 g, 1.55 mmol), methanol (5.0 mL), 2-(carbomethoxy)-2-methylethyl isocyanide (1.0 g, 7.87 mmol), and NH_4TcO_4 (0.2 mmol) in saline (10 mL, 0.07 M). The reaction mixture was heated to reflux for 90 min at 60 °C and allowed to cool, and the volume was reduced to ~6 mL under vacuum. An aliquot of this solution (1 mL) was loaded on a prewet SEP-PAK C_{18} cartridge and washed with H_2O (10 mL). The pure product was eluted with methanol/aqueous NaCl (0.15 M) (19:1 v/v) (10 mL) and the solvent removed under reduced pressure; recovered yield 58% based on Tc. IR (KBr, cm^{-1}): ν_{CN} 2093, ν_{CO} 1748. ^{99}Tc NMR (δ, CH_3CH_2OH): −1925. (+)FABMS (m/z): 861; calcd for $C_{36}H_{54}N_6O_{12}Tc^+$ = 861.8. RP-TLC: R_f = 0.7.

Preparation of $^{99m}Tc(2\text{-carbomethoxy)-2-methylethyl isocyanide})_6Cl$, $^{99m}Tc(CPI)_6Cl$. Sodium hydrosulfite (5.0 mg, 0.029 mmol) was weighed out into a 5-mL glass serum vial. (Note: sodium hydrosulfide was kept under a dry nitrogen atmosphere after opening to prevent its deterioration.) To the serum vial were added ethanol (0.4 mL, 95%) and the neat (CPI) ligand (5 μL, 0.039 mmol). The vial was sealed with a rubber stopper and a $^{99}Mo/^{99m}Tc$ generator eluate containing $^{99m}TcO_4^-$ (0.6 mL, 20–100 mCi, 0.74–3.70 GBq) in aqueous NaCl (0.15 M) was added (specific activity of technetium in the generator eluate was calculated from generator equilibrium equations[18] to be 4.0–6.7 Ci/mol^{-9} and 0.148–2.479 GBq/mol^{-12}). The reaction mixture was shaken for 5 s and then heated for 35 min in a 60 °C water bath. The radiochemical yield and purity were both greater than 97%. Purity was tested by using RP-TLC and RP-HPLC. The $Tc(CPI)_6^+$ cationic complex has R_f = 0.7 in the employed RP-TLC system, with TcO_2 at R_f = 0.0 and TcO_4^- at R_f = 1.0. RP-HPLC analysis demonstrated a capacity factor k' of 4.20.

Preparation of $^{99}Tc(CNC(CH_3)_2COOH)_6Cl$: The Hexahydrolysis Product of $Tc(CPI)_6^+$. Addition of 6.5 equiv of NaOH (2.5 mL, 0.1 M) per $^{99}Tc(CPI)_6Cl$ (0.034 mmol) in methanol/H_2O (5.0 mL) (1:1 v/v) and incubation at 40 °C for 20 min yielded a single peak by both ^{99}Tc NMR and RP-HPLC. This solution was acidified with acetic acid, the volume reduced at low pressure to ~2 mL, and the reaction mixture loaded on a prewet SEP-PAK C_{18} cartridge. The column was washed with H_2O (10 mL) and the product eluted with acetic acid/methanol (5 mL, 0.1 M). This eluate was evaporated to dryness under high vacuum. UV/vis [$CNCH_3$; λ_{max}, nm (ϵ, L M^{-1} cm^{-1})]: 236 (4.7 × 10^4) 265 (sh). IR (cm^{-1}, KBr): ν_{CN} 2093, ν_{CO} 1708, 1643 (vb). ^1H NMR (δ, $CDCl_3$): 1.53 (s, 6 H, β-CH_3). ^{99}Tc NMR (δ, CH_3CH_2OH): −1925 ($Tc(CNC(CH_3)_2COOH)_6^+$), relative to $^{99}TcO_4^-$. (+)FABMS (m/z): 777; calcd for $C_{30}H_{42}N_6O_{12}Tc$ = 777.7.

Preparation of Partially Hydrolyzed $Tc(CPI)_6^+$ Compounds. Mixtures of intermediates in the sequential hydrolysis process were obtained by addition of less than a stoichiometric amount of base to the parent $^{99}Tc(CPI)_6^+$, heating for 15 min at 90 °C, and neutralizing with HCl. These hydrolysis products were analyzed for individual components by RP-HPLC. The protonated form of the hydrolysis products was loaded on to a prewet SEP-PAK C_{18} cartridge; the column was washed with H_2O (to remove excess salt) and eluted with ethanol. The eluate was evaporated under reduced pressure to dryness and analyzed by (+)-FABMS.

The kinetics for random sequential hydrolysis of the technetium hexakis(ester isocyanide) complex were most accurately demonstrated and quantified by RP-HPLC separations of the high-specific-activity $^{99m}Tc(CPI)_6^+$ (10–25 mCi/mL, 0.37–0.93 GBq/mL) preparation buffered at pH 10.0 with 0.5 M phosphate. Samples (0.10 mL) were withdrawn every 10 min, and the reaction was stopped before the RP-HPLC analysis by neutralizing it to pH 6.0. Integration of the digitized signal from the radiometric detector of the chromatographs gave quantitative information for the species present.

Enzymatic in Vitro Hydrolysis of $^{99m}Tc(CPI)_6^+$. Tests for in vitro hydrolysis of the radiolabeled compound were performed by using serum

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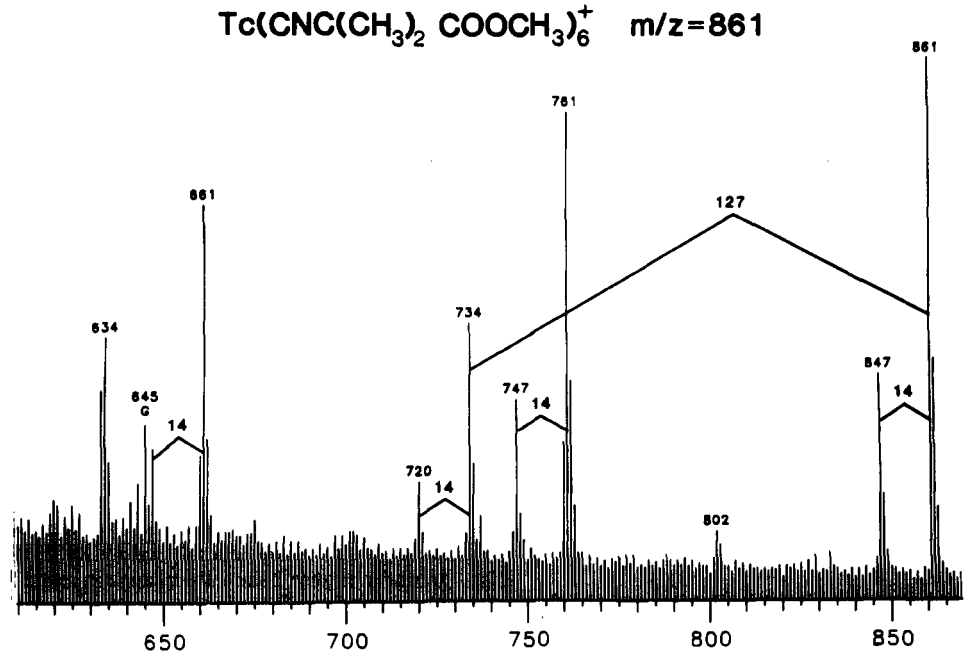


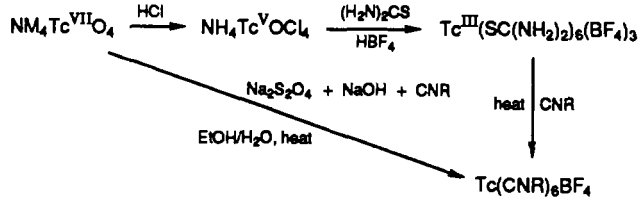
Figure 1. Fast-atom-bombardment mass spectrum (positive ion mode) of $Tc(CNC(CH_3)_2COOCH_3)_6PF_6$.

obtained by centrifuging freshly drawn heparinized blood. Human serum was obtained from fasted (>8 h) normal volunteers as determined by cholesterol, triglyceride, and lipid concentrations. For interspecies comparison, heparinized blood was taken from fasted unanesthetized albino male mice and rats. In each hydrolysis experiment, serum (0.20 mL) was pipetted into a borosilicate culture tube and equilibrated to a specified temperature in a water bath. The $^{99m}Tc(CPI)_6^+$ complex (0.020 mL, 10–50 mCi/mL, 0.37–1.85 GBq/mL) in an ethanol/saline solution (25%, 0.15 M) was added, the contents were shaken, and incubation was continued for various lengths of time. Enzymatic hydrolysis was halted by addition of cold (4 °C) absolute ethanol (1.0 mL) and cooling in an ice bath to precipitate the serum proteins. The samples were centrifuged (15 min, 2500g, 4 °C) to separate the precipitated proteins, and the supernate was analyzed by radiometric quantitation of the RP-HPLC separated products. A quantitative analysis of activity per volume indicated that the ^{99m}Tc complex did not coprecipitate with the proteins. Repeated RP-HPLC analysis on the same sample proved that further hydrolysis did not occur in the ethanolic supernate. Assignment of peaks was made by comparison with chromatographs of the base-catalyzed hydrolysis products.

Results and Discussion

Characterization of $^{99}Tc(CNC(CH_3)_2COOCH_3)_6Cl$, $^{99}Tc(CPI)_6Cl$. The reaction of the pertechnetate ion with sodium hydrosulfite and excess alkyl isocyanide ligands in refluxing solutions of ethanol and aqueous base proceeds quantitatively to the technetium(I) hexakis(alkyl isocyanide) monocation.[19] For the ester derivatives, when macroscopic concentrations of technetium-99 are employed (>1 mM) with stoichiometric amounts of reagents, the increased reaction time necessary to complete the reaction along with the aqueous base necessary to solubilize the sodium hydrosulfite results in hydrolysis of a finite fraction of the ester linkages and a composite of mixed-ligand complexes. The ligand-exchange reaction with a more labile technetium complex in a lower oxidation state, $Tc(thiourea)_6Cl_3$, was employed to synthesize larger quantities of this and other compounds.[20] With the prolonged reaction times required for this ligand exchange and metal reduction reaction to occur, inert atmosphere was maintained to reduce absorption of water, which could lead to hydrolysis.

The ester isocyanide complex was obtained rapidly and directly from the pertechnetate anion when a large excess (>20 times the stoichiometric amount) of reducing agent and ligand were present. These conditions, which approximate the radiopharmaceutical kit, increase the kinetics for complex formation so that quantitative reaction occurs in <1 h. This synthetic route was used to obtain small quantities of the more water-soluble chloride salt of $Tc(CPI)_6^+$.



Technetium(I) hexakis(2-(carbomethoxy)-2-methylethyl isocyanide) is an octahedrally coordinated monocationic complex, which when isolated as the PF_6^- salt, precipitates as a white crystalline solid that is stable in air and slightly soluble in aqueous solutions. In the infrared spectrum of this cationic complex, there is a single intense absorption at 2093 cm^{-1} due to the C≡N stretching mode. This band was 48 cm^{-1} lower in energy than the absorption of the uncomplexed ligand and is consistent with extensive π-donation from the Tc(I) core to the isocyanide ligands. Although the complex is not strictly octahedral, the terminal alkyl functional groups are sufficiently removed from the technetium core to permit virtual O_h symmetry,[21] resulting in the observed single IR stretch rather than the multiple bands expected from S_6 or lower symmetry. Also the frequency of this stretching mode does not change significantly upon hydrolysis of the methyl ester terminus. The presence of the intact ester isonitrile is also confirmed by the ν_{CO} stretch at 1748 cm^{-1}, which was only ~4 wavenumbers removed from the absorption of the free ligand.

Field-desorption mass spectral analysis in the positive ion mode yielding a single peak at m/z 861 provides confirmation of the molecular weight for the parent cationic complex, $Tc(CNC(CH_3)_2COOCH_3)_6^+$. The (+)FAB mass spectrum contained a more complex fragmentation pattern because of the higher energy of this ion-producing technique. Figure 1 shows the presence of the parent M^+ ion as well as prominent peaks at M − 100 ($C_5H_8O_2$) and M − 127 ($C_6H_9O_2N$), corresponding to dealkylation and loss of an intact ligand, respectively. The identity of the species

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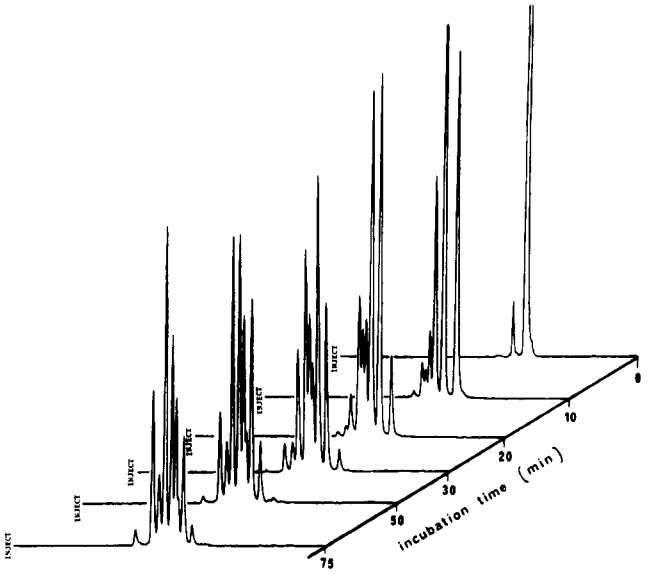

Figure 2. Radiometric detection of technetium-containing species in reversed-phase HPLC separations of the random hydrolysis products generated after incubation of $^{99m}Tc(CNC(CH_3)_2COOCH_3)_6Cl$ in aqueous solution at pH 10.0 and 25 °C as a function of time.

generated in the mass spectrometer, some of which have been observed in other systems,[22] provides insight into the nature of possible impurities, intermediates, metabolites, or degradation products of potential radiopharmaceuticals. Thus, most noticeable in Figure 1 are the overlapping progressive series of peaks separated by 14 mass units, which indicate the occurrence of sequential hydrolysis on the probe.

The ^1H NMR spectrum of the complex exhibited only two singlet resonances with chemical shifts similar to those for the free ligand and an integration ratio of 2:1. The absence of multiple peaks for the alkyl region of the ligand is consistent with a symmetrical octahedral coordination permitting free rotation of the terminal ester groups, thus resulting in chemical equivalence of the six coordinated isocyanides in solution. The ^{13}C NMR spectrum contained typical resonances for the ester and alkyl portions of the ligand; however, the isonitrile carbon is not observed due to quadrupolar splitting by the nitrogen and splitting by the spin $^9/_2$ technetium nucleus. The observation of a single ^{99}Tc NMR signal at −1925 ppm, relative to the external standard $^{99}TcO_4^-$, falls within the range of other known technetium(I) complexes and confirms that the product is diamagnetic.[23]

Characterization of $^{99}Tc(CNC(CH_3)_2COOCH_3)_6^+$ ($^{99}Tc(CPI)_6^+$). Reversed-phase high-performance liquid chromatographic analysis for the co-injection of $^{99m}Tc(CPI)_6^+$ and $^{99}Tc(CPI)_6^+$, using radiometric and UV detection, demonstrated coelution of products obtained from the individual syntheses. On the applied-gradient reversed-phase system both technetium-containing species were detected with a retention time of 9.0 min ($k' = 4.20$). This technique was used throughout to confirm the identities of the high-specific-activity ^{99m}Tc-containing compounds. RP-TLC was also performed on ^{99m}Tc preparations primarily to mimic RP-HPLC conditions and to quantitate the presence of any reduced hydrolyzed or polymeric technetium colloid that would not elute from the RP-HPLC column. The $^{99m}Tc(CPI)_6^+$ complex, after separation from reactants and resuspension in saline, is stable for >6 h at pH 4–7.

Characterization of $Tc(CNC(CH_3)_2COOH)_6Cl$. Incubation of the cationic $^{99m}Tc(CPI)_6^+$ complex in aqueous base (NaOH, pH 10.0) at 25 °C produced a series of nine new technetium-containing compounds as analyzed by RP-HPLC. The chromatographs in Figure 2 show the change in reaction products over time with the initial $^{99m}Tc(CPI)_6^+$ species decreasing. If hydrolysis is allowed to continue, subsequent peaks with shorter retention times grow in and subsequently disappear. Obvious from this reversed-phase separation is that all the subsequent species generated are at progressively shorter retention times and are thus more hydrophilic compounds. This is consistent with sequential methyl ester hydrolysis producing complexes of increasing anionic charge. Prolonged incubation of the $^{99}Tc(CPI)_6^+$ complex with excess base yielded a single technetium-containing species by RP-HPLC analysis with a $R_t = 5.6$ min ($k' = 2.24$).

Table I. Ratios of Peak Integrals for Sequential Species in a Reversed-Phase HPLC Separation of Base-Catalyzed Hydrolysis Products of the Cation $Tc(CPI)_6^+$ Where Comparison is Made with Theoretical Ratios for Random Hydrolysis of an Octahedral Complex

peak no.	k'	% of tot.	peak ratios calcd	peak ratios theor	identity
1	2.61	2.4			pentahydrolyzed
2	2.81	2.6			trans tetrahydrolyzed
			4.2:1	4:1	
3	2.94	11.5			cis tetrahydrolyzed
4	3.14	18.8			*mer* trihydrolyzed
			1.4:1	1.5:1	
5	3.25	13.2			*fac* trihydrolyzed
6	3.34	8.2			trans dihydrolyzed
			3.4:1	4:1	
7	3.46	27.7			cis dihydrolyzed
8	3.68	13.5			monohydrolyzed
9	4.20	2.1			$Tc(CPI)_6^+$

The (+)FAB mass spectrum of the end product was obtained after acidification with acetic acid and purification to remove excess Na^+ (Figure 3). The spectrum exhibited a peak at m/z 777 corresponding to the fully protonated species $Tc(CNC(CH_3)_2COOH)_6^+$ as well as six sequential M + 22 peaks arising from substitution of a sodium for each proton. A similar substitution pattern was also superimposed on the fragmentation products observed for the hydrolyzed complex.

The infrared absorption spectrum of the completely hydrolyzed species showed a ν_{CN} stretch at 2093 cm^{-1}, similar to that of the parent ester species but differing in that ν_{CO} stretches were observed at 1708 and 1643 cm^{-1} for the free acid and sodium salt, respectively. The optical spectra for the hydrolyzed product and the parent ester isocyanide complex were nearly identical as would be expected for no change in coordination or oxidation state. A small (+2 nm) shift in the shoulder at 265 nm for the hydrolyzed species was observed, which may be due to an increase in the bond energy of the coordinated anionic isocyanide ligand. ^{99}Tc NMR also demonstrated a single peak at −1925 ppm under acidic conditions, which was the same resonance as the parent ester complex, again confirming the presence of the intact technetium(I) hexacoordinate core.

To ascertain the possible charge of this hydrolyzed species under physiological conditions, the pK_a was determined by dissolving a sample of $Tc(CNC(CH_3)_2COOH)_6Cl$ (6 mg, 0.8×10^{-5} mol) in water and titrating with aqueous NaOH (0.036 M). The curve obtained by using 0.36×10^{-6} mol aliquots exhibited a smooth sinusoidal shape indicating no influence of the molecule's decreasing charge on subsequent protonation. A single equivalent pK_a was graphically determined as 3.50 ± 0.05.

Identification of $^{99m}Tc(CPI)_6^+$ Hydrolysis Products. The irregular pattern of peaks observed in the RP-HPLC chromatographs for the six progressively hydrolyzed species is expected for an octahedral complex with potential geometrical isomers for the di-, tri-, and tetrahydrolyzed products. Comparison of the integrated RP-HPLC peak ratios with ratios predicted for random sequential base-catalyzed hydrolysis allows the assignment of the various isomers. As indicated in Scheme I, continued hydrolysis of the monohydrolyzed species can occur at any of the four ligands adjacent to the carboxylic acid isocyanide or at the single dissimilar

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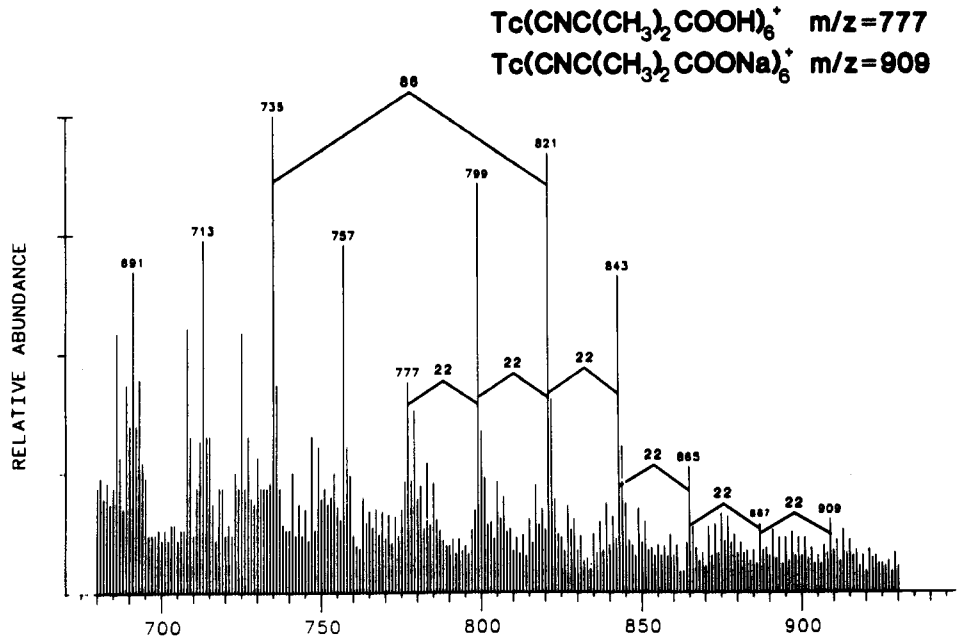


Figure 3. Fast-atom-bombardment mass spectrum (positive ion mode) of $Tc(CNC(CH_3)_2COOH)_6Cl$, showing progressive substitution of sodium for protons.

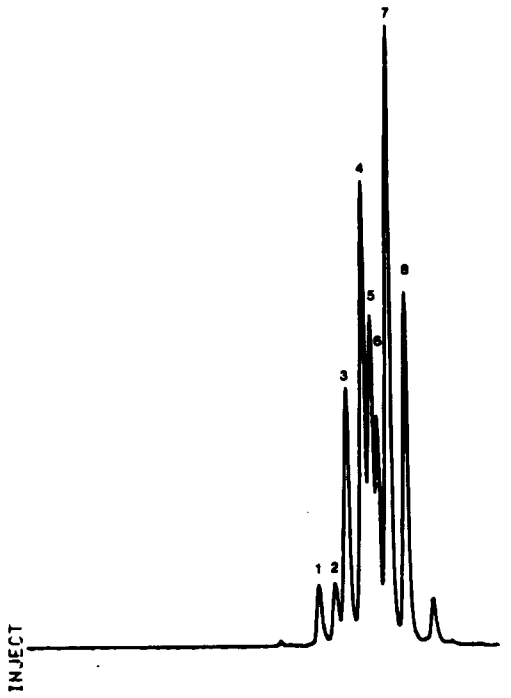


Figure 4. Radiometric detection of ^{99m}Tc-containing compounds in reversed-phase HPLC separations of $^{99m}Tc(CNC(CH_3)_2COOCH_3)_6Cl$ hydrolysis products after 30 min at 25 °C and pH 10.0.

site opposite to it. Thus the theoretically predicted ratio for cis-to-trans isomers of the dihydrolyzed product would be 4:1. A similar argument predicts ratios of 2:3 *fac* to *mer* for the trihydrolyzed and 4:1 cis to trans for the tetrahydrolyzed species. Figure 4 shows a typical chromatogram of the mixture obtained during random base-catalyzed hydrolysis of $^{99m}Tc(CPI)_6Cl$. Integrations for the radiometric detection are presented in Table I along with the theoretically predicted ratios of the sequential peaks. The good correlation for these values is strong evidence for assigning the identities of peaks with specific retention times or k' values. The order of elution from this reversed-phase system was trans isomers preceding cis and *fac* being retained longer than *mer*. This implies that, even with their greater dipole moment, the less symmetrical cis and *fac* complexes are retained better in the nonpolar stationary phase and are thus more "lipophilic". The retention times obtained from these model compounds were used

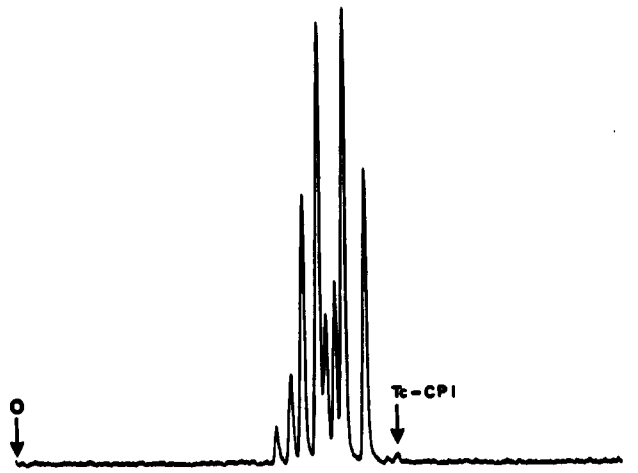


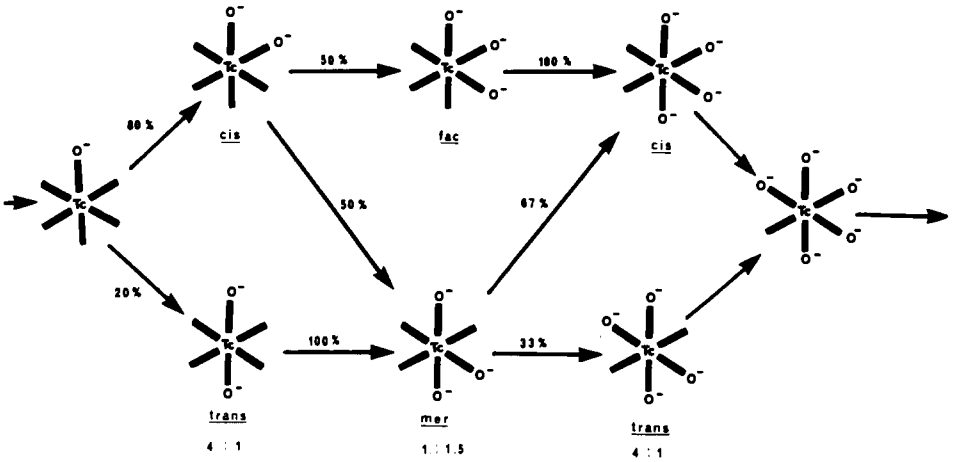
Figure 5. Radiometric detection of technetium-containing species in reversed-phase HPLC separations of products generated after incubation of $^{99m}Tc(CNC(CH_3)_2COOCH_3)_6Cl$ in human serum for 70 min at 37 °C.

to monitor the enzyme kinetics for hydrolysis of the parent $^{99m}Tc(CPI)_6Cl$.

Enzymatic in Vitro Hydrolysis of $Tc(CPI)_6^+$. Incubation of the high-specific-activity $^{99m}Tc(CPI)_6Cl$ complex with human serum from normal subjects produced a series of new technetium-containing species. Figure 5 shows the RP-HPLC separation of products obtained after incubation for 70 min at 37 °C at pH 7.4. Nine separated species were observed under these conditions indicating continued sequential hydrolysis. A control chromatograph of the starting material mixed with serum at 0 °C for 5 s confirmed the longest retained peak as the original cationic complex. The retention times for the newly generated species indicate the major products, after 20-min incubation, to be the mono- and dihydrolyzed complexes. The ratio of cis to trans for the dihydrolyzed products was 3.6:1, close to the theoretical value for a random sequential process.

The overall reaction rate in human serum was slow in relation to that in other mammalian species, with only 6% of the monohydrolyzed component present after 10 seconds at 37 °C. When this time point was used to measure the initial hydrolysis rate of $^{99}Tc(CPI)_6^+$ (0.1 mM) in human serum at 37 °C, the concentration of technetium or plasma was varied independently to determine the order of reaction for each component.[24] A plot

Scheme I. Theoretical Prediction of Isomer Ratios for Random Sequential Hydrolysis for the Octahedral Complex $Tc(CNC(CH_3)_2COOCH_3)_6Cl$, Where Each Arm Represents a Functionalized Isonitrile Ligand in a Fixed Geometry



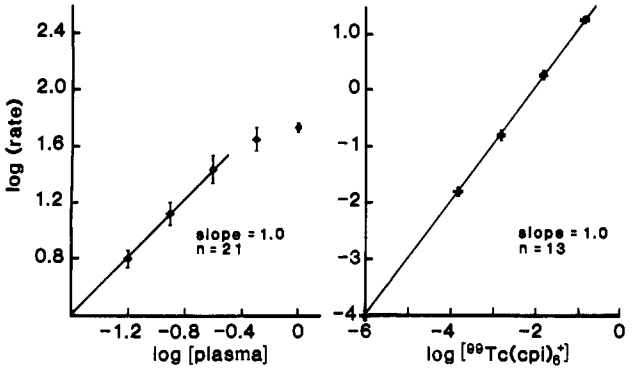


Figure 6. Plots of log [initial rate of $Tc(CPI)_6Cl$ hydrolysis] after 10 s at 37 °C as a function of log [$^{99}Tc(CPI)_6Cl$] and log [serum]. Each point represents the mean of three to seven individual measurements where the SEM was less than 15% of mean value.

of log [initial rate] against log [$Tc(CPI)_6^+$] or log [serum] (Figure 6) yielded straight lines each with a slope of 1. These results confirm an order of 1 with respect to both serum and $Tc(CPI)_6^+$, thus indicating an overall second order enzymatic reaction. By contrast, the process of simple alkaline hydrolysis would have produced a line of slope 0 for the log [rate] versus log [serum] plot and an overall apparent order of reaction equal to 1.

The results obtained after incubation in rat or mouse serum were strikingly different from those obtained with human serum. A comparison of products obtained after 10 s at 37 °C in human, rat, and mouse serum is shown in Figure 7. Two distinct differences in metabolism of the complex are apparent. The rate of hydrolysis in the rodents was more than 1000 times that found in humans, and the products observed were also different. The ratio for cis to trans isomers of the dihydrolyzed products, obtained after incubation in rat serum, was 1:5, i.e., the inverse of the expected 4:1 ratio for a random process (Figure 7). Thus enzymatic hydrolysis of this unnaturally occurring metal complex occurs in individual species by distinctly different enzyme systems.

Summary. Stable six-coordinate octahedral complexes of the man-made element technetium with relatively reactive ester isocyanide ligands can be directly prepared from the pertechnetate anion by reaction with excess ligand and reducing agent in aqueous aerobic conditions. The model compound $Tc(CPI)_6^+$ has been shown to undergo hydrolysis of terminal ester functions while leaving the central core intact. The enzymatic reaction on this unnatural substrate presumably occurs to decrease its lipophilicity and aid in its excretion from the body. Hydrolysis in humans occurs by nonspecific esterases in the blood stream; however, the

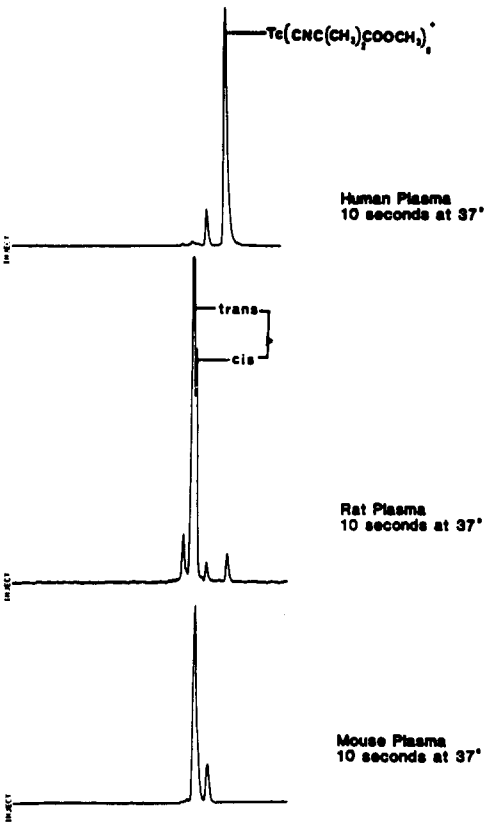


Figure 7. Radiometric detection of technetium-containing species in reversed-phase HPLC separations of products generated after incubation of $^{99m}Tc(CNC(CH_3)_2COOCH_3)_6Cl$ in human, rat, and mouse serum for 10 s at 37 °C.

activity of these enzymes varies remarkably between species. The interspecies differences in metabolism of compounds being tested as possible radiopharmaceuticals complicate the analysis of their biodistribution and must be included as part of any screening protocol.[25]

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Registry No. CPI, 71015-20-8; $H_2NC(CH_3)_2CO_2CH_3$, 13257-67-5; $H_2NC(CH_3)_2CO_2H$, 62-57-7; $OHCNHC(CH_3)_2CO_2CH_3$, 109862-23-9; $^{99}Tc(SC(NH_2)_2)_6Cl_3$, 89172-46-3; $^{99}Tc(CPI)_6PF_6$, 124171-54-6; ^{99}Tc-

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$(CPI)_6Cl$, 133471-53-1; $NH_4{}^{99}TcO_4$, 34035-97-7; $^{99}Tc(CNC(CH_3)_2COOH)_6Cl$, 133471-54-2; $^{99}Tc(CNC(CH_3)_2COOH)_5(CPI)Cl$, 133495-00-8; *trans*-$^{99}Tc(CNC(CH_3)_2COOH)_4(CPI)_2Cl$, 133471-55-3; *cis*-$^{99}Tc(CNC(CH_3)_2COOH)_4(CPI)_2Cl$, 133574-95-5; *mer*-$^{99}Tc(CNC(CH_3)_2COOH)_3(CPI)_3Cl$, 133471-56-4; *fac*-$^{99}Tc(CNC(CH_3)_2COOH)_3(CPI)_3Cl$, 133574-96-6; *trans*-$^{99}Tc(CNC(CH_3)_2COOH)_2(CPI)_4Cl$, 133471-57-5; *cis*-$^{99}Tc(CNC(CH_3)_2COOH)_2(CPI)_4Cl$, 133574-97-7; $^{99}Tc(CNC(CH_3)_2COOH)(CPI)_5Cl$, 133471-58-6; $^{99}Tc(CNC(CH_3)_2COONa)_6{}^+$, 133495-01-9; trichloromethyl chloroformate, 503-38-8.

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Structure and Metal Coordination of the Diphosphane 2,2′-Bis((diphenylphosphino)methyl)-1,1′-biphenyl ("BISBI")

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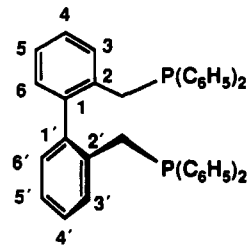
2,2′-Bis((diphenylphosphino)methyl)-1,1′-biphenyl (**1**, BISBI) crystallizes in the orthorhombic space group $Pca2_1$, with a = 22.845 (1) Å, b = 13.3796 (6) Å, c = 19.642 (1) Å, V = 6004 Å^3, and Z = 8. The X-ray crystal structure analysis of **1** revealed two independent molecules in the unit cell. [2,2′-Bis((diphenylphosphino)methyl)-1,1′-biphenyl]tetracarbonyl-molybdenum (**3**), prepared in 47% yield from (norbornadiene)$Mo(CO)_4$ and the ligand BISBI, was also structurally characterized. Crystals of **3**·0.92CH_2Cl_2, grown from methylene chloride at room temperature, are monoclinic, with space group $P2_1/n$, a = 15.444 (1) Å, b = 13.957 (1) Å, c = 18.503 (1) Å, β = 92.55 (1)°, V = 3984 Å^3, and Z = 4. The structure of **3** shows a nearly octahedral coordination of the molybdenum atom, with the diphosphane ligand in cis positions. The P–Mo–P′ "bite angle" is 103.54 (2)°. **3** belongs to the very few known complexes that exhibit nine-membered, metal-containing ring systems of diphosphane ligands. BISBI can adopt to any coordination geometry with small (90°) and large "bite angles" (180°) upon *intra*molecular (chelating) metal attachment while there is no experimental evidence for *inter*molecular complexation.

Introduction

It is well-known that increasing steric bulk of monodentate ligands leads to higher regioselectivity in hydroformylation.[1] Bidentate phosphanes with large P–M–P′ "bite angles" thus should improve the *n*/iso ratio in this reaction. In a recent paper, Casey and coworkers[2] reported on the coordination chemistry of the novel ligand 2,2′-bis((diphenylphosphino)methyl)-1,1′-biphenyl (**1**, "BISBI") in a pentacoordinated iron complex. It was found that this particular diphosphane has a P–M–P′ bite angle greater than 120°. In the context of our ongoing work on hydroformylation,[3,4] we were interested in the molecular structure of the free ligand as compared with its conformation when membered in a penta- or hexacoordinated metal complex. To this end, we prepared an octahedral molybdenum(0) complex. This work is reported in the present paper.



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Results

A. Crystal Structure of the Ligand. BISBI is a relatively new diphosphane that has been described in several patents of Kodak.[5] A new convenient synthetic route is now available from our own laboratory.[4] Single crystals of compound **1** were grown from an ethanol solution at room temperature. The X-ray crystal structure (Figure 1) shows two independent molecules with different conformations in the unit cell. Molecule A has a smaller interplanar angle of the biphenyl moiety (dihedral angle for molecule A is 69.87°, and it is 76.97° for molecule B defined by C51–C56–C66–C61). As a consequence the P···P′ distances are 6.73 Å for molecule A and 6.86 Å for B. While the conformations around P1A and P1B are similar for both molecules, they are quite different at the atoms P2A and P2B as shown in Figure 1. The phenyl groups attached to these phosphorus atoms are twisted in an opposite manner. The coordination geometry around all phosphorus atoms is as expected for sp^3 hybridization including a lone pair. Corresponding bond distances and bond angles of A and B are nearly equal. These data fall in the normal range for P–C(aliphatic) and P–C(aromatic) bonds.

From reflection statistics and systematic absences, the acentric space group $Pca2_1$ clearly was determined, although a refinement in the enantiomorphic setting gave no significant change in the R values. However, both independent molecules have different conformations and cannot be symmetry related.

The NMR data provide information about the structure of the free ligand **1** in solution. The $^{13}C\{^1H\}$ NMR spectrum shows 14 different signals in the typical aromatic shift region due to the axial chirality of the molecule. The two phenyl rings directly attached to the phosphorus atom are *non*equivalent and give rise to separate signals for each carbon atom, indicating that inversion at the phosphorus atom is very slow on the NMR time scale. The

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