rangements of **2** to **6** are governed by electrostatic interactions between cyclopropylcarbinyl cations and PP_i, with PP_i acting as a template for the rearrangement. We also suggested premature capture of **3** is thwarted by binding NADPH near C-1′ in an orientation where a stereoelectronic barrier prevents attack at that position prior to rearrangement to **4**. If the cofactor is moved to a position where hydrogen transfer to C-3′ is possible, the stereoelectronic barrier to nucleophilic attack is removed, and reduction of **3** can occur with concomitant rupture of the C-1′–C-2 bond. Since the absolute stereochemistry at C-3′ in **5** is R, a relative minor displacement of the cofactor by approximately 2.5 Å along the bottom face of the substrate as shown in Chart I suffices to explain the regioselective formation of either product.

Acknowledgment. We thank Dr. Fred Wolf of Amoco Corporation for his generous assistance in establishing cultures of *B. braunii* in our laboratory. This project was supported by NIH Grant GM 21328.

Molecular Recognition in Cytochrome P-450: Alteration of Regioselective Alkane Hydroxylation via Protein Engineering

William M. Atkins and Stephen G. Sligar*

Departments of Biochemistry and Chemistry
University of Illinois, Urbana, Illinois 61801

Received November 17, 1988

The utility of synthetic catalysts in the oxidation of unactivated alkanes and alkenes has been the focus of intense interest.[1-5] In particular, the oxidative capability of the cytochrome P-450 monooxygenases has been modelled through the synthesis of shape-selective, regiospecific metalloporphyrins for use as potential synthetic tools.[6-10] Protein engineering now offers a viable alternative for the design of oxidative catalysts with the predictable regio- and stereospecificity inherent in biological systems. Here we report the manipulation of the regiospecificity of hydroxylation of several bicyclic substrates by the site-directed mutagenesis of cytochrome $P\text{-}450_{cam}$.[11]

Characterization of the gene encoding the soluble, bacterial cytochrome $P\text{-}450_{cam}$[12] and the X-ray crystal structures of several forms of the protein have been published.[13-15,31] The high resolution crystal structures of $P\text{-}450_{cam}$ indicate the existence of several specific active site residues which may differentially affect the energetically favorable binding orientations of various organic substrates. Collectively, these protein–substrate interactions serve to juxtapose the 5-position of the camphor skeleton directly above the heme iron,[17] for efficient hydrogen abstraction followed by stereospecific oxygen rebound[18,19] to afford the 5-*exo*-hydroxycamphor. Three active site residues, Val-295, Val-247, and Tyr-96, have been mutated and the present study undertaken to determine the resulting regiospecificity of hydroxylation for a series of structurally related bicyclic alkanes when these active site residues of cytochrome $P\text{-}450_{cam}$ are altered. Camphor (**1**), 1-CH_3-norcamphor (**2**), and norcamphor (**3**), were examined in order to assess the recognition of substrate methyl groups by the enzyme. An active site hydrogen bond between the substrate camphor and Tyr-96 is clearly indicated in the X-ray structure of $P\text{-}450_{cam}$ and was found to be important in the control of substrate affinity and

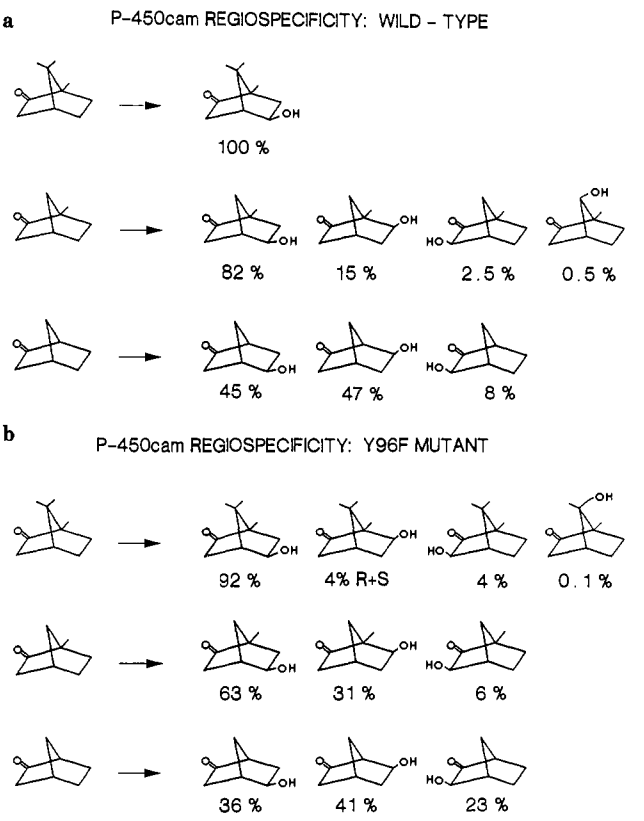

Figure 1. Comparison of regiospecificity of hydroxylation of bicyclic substrates. When the hydrogen bond which normally orients these substrates is removed by site-directed mutagenesis (Y96F), a new metabolite profile is obtained with increased specificity for the 3-position of the substrate. Enzymatic reactions were performed with 2–3 μM $P\text{-}450_{cam}$, 10 μM putidaredoxin, 2 μM putidaredoxin reductase, 200 mM KCl, 500 μM substrate, and 500 μM NADH in Tris buffer, pH 7.4. After reactions had reached completion, the mixtures were diluted to 1 mL with water and extracted twice with equal volumes of $CHCl_3$. The concentrated organic extract was analyzed by gas chromatographic mass spectrometry and compared to authentic standards, with a Hewlett Packard 3700 gas chromatograph equipped with a 30 m capillary DB-5 column in line with a Hewlett Packard 7070E mass spectrometer. Spectra were obtained in the electron impact mode, with an ionizing potential of 70 eV. Relative product yields were obtained by integration of peak areas of the GC traces. GC conditions varied according to the substrate used. Typically, a temperature ramp from 70 °C to 200 °C at 3 °C/min after an isothermal period of 3–5 min.

(1) Cook, B. R.; Reinhart, T. S.; Suslick, K. S. *J. Am. Chem. Soc.* **1986**, *108*, 7281–7286.
(2) Suslick, K. S. In *Activation and Functionalization of Alkanes*; Hill, C. L., Ed.; Wiley and Sons: New York.
(3) Suslick, K. S.; Cook, B. R. *J. Chem. Soc., Chem. Commun.* **1987**, 200–202.
(4) Groves, J. T.; Nemo, T. E. *J. Am. Chem. Soc.* **1983**, *105*, 5786–5790.
(5) Mansuy, D.; Bartoli, J. F.; Momenteau, M. *Tetrahedron Lett.* **1982**, *23*, 2781–2783.
(6) Groves, J. T.; Kruper, W. J.; Nemo, T. E.; Meyers, S. R. *J. Mol. Catal.* **1980**, *7*, 167–177.
(7) Groves, J. T.; Watanabe, Y. *J. Am. Chem. Soc.* **1986**, *108*, 7834–7836.
(8) Traylor, T. G.; Marsters, J. C.; Nakano, T.; Dunlop, B. E. *J. Am. Chem. Soc.* **1985**, *107*, 5537–5538.
(9) Castellino, A. J.; Bruice, T. C. *J. Am. Chem. Soc.* **1988**, *110*, 158–163.
(10) Collman, J. P.; Brauman, J. T.; Meunier, B.; Raybuck, S. A.; Kodadek, T. *Proc. Natl. Acad. Sci. U.S.A.* **1984**, *81*, 3245–3249.
(11) Murray, R. I.; Sligar, S. G. In *Cytochrome P-450: Structure, Mechanism, and Biochemistry*; Ortiz de Montellano, P., Ed.; Plenum Press: New York, 1985; pp 443–483.
(12) Unger, B. P.; Gunsalus, I. C.; Sligar, S. G. *J. Biol. Chem.* **1986**, *261*, 1158–1163.
(13) Poulos, T. P.; Finzel, B. C.; Gunsalus, I. C.; Wagner, G. C.; Kraut, J. *J. Biol. Chem.* **1985**, *260*, 16122–16128.
(14) Poulos, T. P.; Finzel, B. C.; Howard, A. J. *Biochemistry* **1986**, *25*, 5314–5319.
(15) Poulos, T. P.; Howard, A. J. *Biochemistry* **1987**, *26*, 8165–8174.
(16) Atkins, W. M.; Sligar, S. G. *J. Biol. Chem.* **1988**, *263*, 18842–18849.
(17) Poulos, T. P.; Finzel, B. C.; Howard, A. J. *J. Mol. Biol.* **1987**, *195*, 687–700.
(18) Groves, J. T.; McCluskey, G. A.; White, R. E.; Coon, M. J. *Biochem. Biophys. Res. Commun.* **1978**, *81*, 154–160.
(19) Groves, J. T.; McCluskey, G. A. *J. Am. Chem. Soc.* **1976**, *98*, 859–861.

0002-7863/89/1511-2715$01.50/0

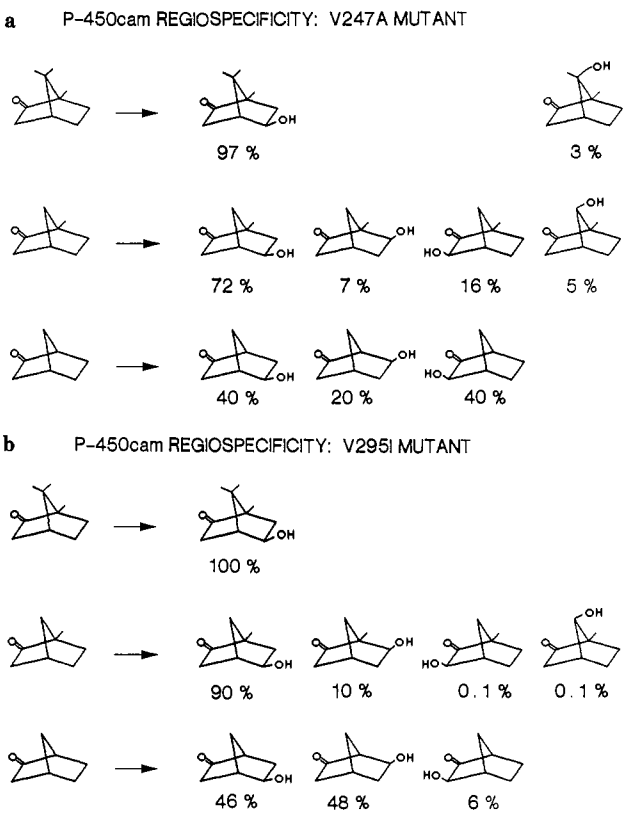

Figure 2. Comparison of the regiospecificity of hydroxylation of bicyclic substrates by mutant proteins with altered hydrophobic residues. The substrate analogues **2** and **3** were metabolized with the V247A and V295I mutants and the products obtained analyzed as described in Figure 1. In the absence of other hydrophobic interactions to aid in substrate orientation, the added steric restraint resulting from substitution at position 295 (V295I) has little effect on the regiospecificity, as indicated with norcamphor. When the 1-methyl group is present on the substrate, the added methyl group at position 295 serves to further orient the substrate with the 5-position near the oxidative intermediate. It is also apparent that this 1-methyl group plays a role in regiospecificity since the 1-methylnorcamphor/V247A complex affords a different product profile than the wild-type complex.

ferric spin state equilibrium of the hemoprotein through site-directed mutagenesis[16] as previously predicted.[29,30] In addition to the hydrogen bonding residue, Tyr-96, the methyl groups present on Val-295 restrict the available binding site topology, and a specific hydrophobic cleft provided by Leu-244 and Val-247 suggests a means for substrate methyl-group recognition.

The metabolism of norcamphor by cytochrome P-450$_{cam}$ has been shown to afford several hydroxynorcamphors, and turnover of norcamphor is associated with isotope-dependent metabolic switching,[20,21] suggesting that this substrate analogue may rapidly reorient near the putative $[FeO]^{3+}$ hydroxylating intermediate.[22,30,31] Removal of the potential for hydrogen bonding to Tyr-96 by replacement of this residue by phenylalanine results in a significant increase in the degree of hydroxylation at the 3-position (Figure 1A,B). This suggests a possible directive effect of the Tyr-96/norcamphor hydrogen bond in maintaining the selective, although incomplete, juxtaposition of the opposite end of the molecule near the heme iron. This effect of hydrogen bond removal is also observed with **2**. The contribution of this putative hydrogen bond to the regiospecificity of hydroxylation is less pronounced, perhaps as a result of the complementary fit between the substrate methyl group and the Leu-244–Val-247 cleft which

Table I. Control of P-450$_{cam}$ Spin State and Monooxygenase Stoichiometry

protein/substrate[c]	high spin	stoichiometry[a]	% 5-exo product[b]
wt/(**1**)	95	100	97–100
V247A/(**1**)	95	100	97–100
V295I/(**2**)	49	45	90
wt/(**2**)	37–48	40–45	73–82
V247A/(**2**)	37–48	40–45	73–82
wt/(**3**)	45	12	45
V247A/(**3**)	44	10	21

[a] Total yield hydroxylated product/NADH consumed. [b] Fraction of hydroxylated product which is 5-*exo*-alcohol. [c] wt stands for wild-type.

provides additional binding orientation and keeps the 5- and 6-carbons near the heme iron. The roles of the substrate Tyr-96 hydrogen bond in other aspects of catalysis have been previously discussed.[16]

Hydrophobic interactions between substrates and the protein active site were also explored by constructing V295I and V247A mutants which, respectively, provide greater steric bulk near the Val-295 region by changing it to an isoleucine and reduce the steric bulk near Val-247 by exchanging it for an alanine. These residues are in direct contact with the 8,9-*gem*-dimethyl group and the 6- and 10-carbons of the bound camphor molecule. These mutations have only a minor effect on the regiospecificity of camphor hydroxylation, yet result in predictable changes in the observed specificity of hydroxylation of the bicyclic analogues **2** and **3**, Figure 2A,B. It is clear that in addition to the putative hydrogen bond between Tyr-96 and the ketone group of each of these substrates, the steric bulk of the side chain of Ala-247 affects substrate orientation. The fact that the regiospecificity of hydroxylation of these substrate analogues is affected more than the native substrate camphor emphasizes the importance of the additive contribution of these hydrophobic effects in proper orientation of substrate. Also, there is an increase in the absolute total yield of 5-hydroxy product as specific combinations of hydrophobic contacts are engineered into the active site by alteration of the active site residues or the substrate molecule. P-450 monooxygenases uncouple to varying degrees to produce hydrogen peroxide as well as "excess water" resulting from an apparent oxidase-type activity[23] when the substrate cannot favorably orient for hydrogen abstraction and subsequent hydroxylation.[20,21,23,24] Proper placement of methyl groups within the active site complex results in more efficient monooxygenation of substrate, with diminished hydrogen peroxide and excess water formation, Table I.

Like the stereochemical course of many P-450-mediated reactions,[18,27] P-450$_{cam}$-catalyzed hydroxylation of camphor proceeds with some inversion of configuration at the 5-carbon.[25] Furthermore, an additional stereochemical probe, 5-*exo*-bromocamphor (**4**) is metabolized to afford the *gem*-halohydrin via 5-*endo*-hydrogen abstraction followed by elimination of bromide to form the 5-keto product.[26] We determined the efficiency of converting **4** to the 5-keto product for the mutant proteins described. The relative yield of 5-ketocamphor obtained when the various mutant proteins metabolize **4** reflects the accessibility of the 5-*endo*-hydrogen to the hydroxylating intermediate. Mutants which are characterized by a decrease in regiospecificity of camphor hydroxylation (Y96F and V247A) also demonstrated a 10%–20% increase in total yield of the keto product. Perhaps

(20) Atkins, W. M.; Sligar, S. G. *J. Am. Chem. Soc.* **1987**, *109*, 3754–3760.
(21) Atkins, W. M.; Sligar, S. G. *Biochemistry* **1988**, *27*, 1610–1616.
(22) Ortiz de Montellano, P. In *Cytochrome P-450: Structure, Mechanism, and Biochemistry*; Ortiz de Montellano, P., Ed.; Plenum Press: New York, 1985; pp 217–257.
(23) Gorsky, L. D.; Koop, D. R.; Coon, M. J. *J. Biol. Chem.* **1984**, *259*, 6812–6817.
(24) Pompon, D. *Biochemistry* **1987**, *26*, 6429–6434.
(25) Gelb, M. H.; Heimbrook, D. C.; Malkonen, P.; Sligar, S. G. *Biochemistry* **1982**, *21*, 370–377.
(26) Gould, P.; Gelb, M. H.; Sligar, S. G. *J. Biol. Chem.* **1981**, *256*, 6686–6691.
(27) White, R. E.; Miller, J. P.; Faureau, C. V.; Bhattacharya, A. J. *J. Am. Chem. Soc.* **1986**, *108*, 6024–6029.
(28) Fisher, M. T.; Sligar, S. G. *Biochemistry* **1985**, *24*, 4797–4803.
(29) White, R.; McCarthy, M.; Egeberg, K.; Sligar, S. *Archives Biochemistry Biophysics* **1984**, *228*, 493–502.
(30) Collins, J. R.; Lowe, G. H. *J. Biol. Chem.* **1988**, *263*, 3164–3170.
(31) Raag, R.; Poulos, T. L. *Biochemistry* **1989**, *28*, 917–922.

this reflects increased mobility or alternate binding orientations of **4** in the mutated active site which allow for placement of the 5-*endo*-hydrogen near the oxidative intermediate. Addition of steric bulk (V295I mutant), however, resulted in a 54% decrease in *endo*-hydrogen abstraction from **4**, corresponding to a more restricted binding orientation. The steric bulk of a properly placed amino acid residue may afford greater differential stereoselectivity for the chemically equivalent, epimeric hydrogens at the 5-position of camphor, suggesting the utility of altering regio- and stereospecific oxidative catalysts by engineering the active site of cytochromes P-450s.

Acknowledgment. This work was supported by NIH GM33775 and NIH GM 31756.

Synthesis of a Highly Reactive (Benzyne)ruthenium Complex: C-C, C-H, N-H, and O-H Activation Reactions

John F. Hartwig, Richard A. Andersen,* and Robert G. Bergman*

Department of Chemistry, University of California, and Materials and Chemical Sciences Division Lawrence Berkeley Laboratory Berkeley, California 94720

Received October 31, 1988

We report here the synthesis and chemistry of an exceptionally reactive ruthenium benzyne complex, $(PMe_3)_4Ru(\eta^2\text{-}C_6H_4)$ (**1**). The ruthenium–carbon bond in this molecule reacts with a wide range of organic substrates that are typically inert toward late transition-metal–carbon bonds, including those in benzyne complexes.[1] For example, complex **1** reacts cleanly with arylamine N–H bonds, water O–H bonds, and benzyl and aryl C–H bonds. It reacts with acetophenone to yield an O-bound enolate complex, inserts benzaldehyde cleanly, and cleaves the C–C bond of acetone.

The chemistry we have observed is summarized in Scheme I. The methyl phenyl compound *cis*-$Ru(PMe_3)_4(Ph)(Me)$ (**3**) can be prepared by treatment of *cis*-$Ru(PMe_3)_4(Me)(Cl)$ with PhMgBr. Compound **1** was produced in an NMR tube from the thermolysis of **3** at 110 °C in benzene in 94% yield by ^1H NMR spectroscopy (Cp_2Fe internal standard). Alternatively, the benzyne complex was isolated in gram quantities by the treatment of *trans*-$Ru(PMe_3)_4(Cl)_2$ with 2 equiv of PhMgBr in ether under argon to form the diphenyl compound *cis*-$Ru(PMe_3)_4(Ph)_2$ (**2**) in situ,[2] followed by thermolysis of the reaction solution for 8 h at 65 °C. Crystallization from pentane provided **1** in 46% yield.

Slow crystallization of a pentane solution of **1** gave crystals suitable for X-ray analysis; details of the determination are given in the Supplementary Material, and an ORTEP drawing is included in Scheme I. The length of the C–C bond coordinated to the ruthenium center is 1.355 (3) Å; the other C–C distances in the C_6H_4 ring are noted in Scheme I and range from 1.363 (4) to 1.411 (4) Å with an average value of 1.385 ± 0.015 Å. The two Ru–C distances of 2.072 (2) and 2.111 (2) Å are unequal as are the P(2)RuC(1) and P(4)RuC(2) angles of 101.79 (6)° and 117.06 (6)°; respectively. The Ru–C distances in **1** are ca. 0.07 Å shorter than the equivalent distances in $(PMe_3)_4Ru(\eta^2\text{-}C_2H_4)$[3] which is not unexpected since the hybridization at carbon is different in the two molecules.

The rate of thermolysis of methyl phenyl compound **3** at 110 °C in C_6D_6 solvent was measured in the presence of concentrations of PMe_3 between 1.70×10^{-3} M and 1.87×10^{-2} M. A linear inverse dependence of rate on phosphine concentration was observed. This is consistent with a mechanism requiring initial reversible dissociation of phosphine, leading to an intermediate having a coordinatively unsaturated ruthenium center which can then undergo oxidative addition of the ortho-C–H bond of the attached arene ring. Rapid elimination of methane and recoordination of phosphine would yield **1**. An alternative possibility involves reaction by a four-center mechanism,[4] but given the requirement for initial phosphine dissociation, the oxidative addition pathway appears to be the more reasonable hypothesis.

Despite its thermal stability at moderate temperatures, benzyne complex **1** is reactive toward a wide variety of mild reagents. For example, intermolecular C–H activation of arene solvent (the reverse of the benzyne formation reaction) was observed at 110 °C. Thermolysis of **1** in benzene-d_6 in a sealed, evacuated vessel for 14 h at 110 °C yielded $Ru(PMe_3)_4(\eta^2\text{-}C_6D_4)$, identified by ^1H NMR, ^2H NMR, and mass spectrometry. To distinguish between aryl ring exchange and H/D exchange mechanisms for this process, **1** was thermolyzed in toluene at 110 °C for 5 days. An initial product was observed after 10 h, and this was subsequently transformed thermally to the known orthometalated compound $Ru(PMe_3)_4(\eta^2\text{-}CH_2C_6H_4)$ (**4**).[5] This is consistent with **1** undergoing oxidative addition to benzylic as well as arene C–H bonds.

The high basicity of the metal center in complex **1** also makes it much more reactive than other benzyne complexes or transition-metal alkyls toward weakly acidic hydrogens. Reaction of complex **1** with *tert*-butyl alcohol yielded more than one product, but reaction with water for 1 h at room temperature in benzene yielded the phenyl hydroxide **5** in 67% yield.[6] Even H_2N-*p*-*tert*-Bu-C_6H_4 (pK_a = ca. 27)[7] reacts with **1** at 85 °C in toluene, leading to azametallacycle **7**. Presumably this process proceeds via initial cleavage of the arylamine N–H bond to give the unusual metal (aryl)(amido) complex **6**, followed by ortho-metalation.[8] Initial coordination of the amine to an open site at the metal center created by phosphine dissociation may explain the high reactivity of the amine.

Low-valent, electron-rich transition-metal complexes are typically unreactive toward insertion of the strong C–O double bond of ketones and aldehydes.[9,10] However, **1** reacts with benzaldehyde

(1) (a) Gowling, E. W.; Kettle, S. F. A.; Sharples, G. M. *J. Chem. Soc., Chem. Commun.* **1968**, *21*. (b) Bennett, M. A.; Yoshida, T. *J. Am. Chem. Soc.* **1978**, *110*, 1750. (c) Bennett, M. A.; Hambley, T. W.; Roberts, N. K.; Robertson, G. B. *Organometallics* **1985**, *4*, 1992. (d) Arnold, J.; Wilkinson, G.; Hussain, B.; Hursthouse, M. B. *J. Chem. Soc., Chem. Commun.* **1988**, 704.

(2) Jones, R. A.; Wilkinson, G. *J. Chem. Soc., Dalton Trans.* **1979**, 472.

(3) Wong, W.-K.; Chiu, K. W.; Statler, J. A.; Wilkinson, G.; Motevalli, M.; Hursthouse, M. B. *Polyhedron* **1984**, *3*, 1255.

(4) For mechanistic studies of cyclometalations and β-hydride eliminations at the Cp_2Zr^{IV} center, see: Buchwald, S. L.; Nielsen, R. B. *J. Am. Chem. Soc.* **1988**, *110*, 3171 and references therein.

(5) (a) Statler, J. A.; Wilkinson, G.; Thornton-Pett, M.; Hursthouse, M. B. *J. Chem. Soc., Dalton Trans.* **1984**, 1731. (b) Calabrese, J. C.; Colton, M. C.; Herskovitz, T.; Klabunde, U.; Parshall, G. W.; Thorn, D. L.; Tulip, T. H. *Annals. New York Acad. Sci.* **1983**, *415*, 302.

(6) Only dinuclear tetrakis(trimethylphosphine)ruthenium hydroxide complexes have been synthesized: Jones, R. A.; Wilkinson, G.; Colquohoun, I. J.; McFarlane, W.; Galas, A. M. R.; Hursthouse, M. B. *J. Chem. Soc., Dalton Trans.* **1980**, 2480. For monomeric ruthenium triphenylphosphine hydroxide complexes, see: Chaudret, B. N.; Cole-Hamilton, D. J.; Nohr, R. S.; Wilkinson, G. *J. Chem. Soc., Dalton Trans.* **1977**, 1546.

(7) Cox, R. A.; Stewart, R. *J. Am. Chem. Soc.* **1976**, *98*, 488.

(8) Examples of late transition-metal compounds containing both alkyl or aryl and amide substituents include the following: (a) Bryndza, H. E.; Fultz, W. C.; Tam, W. *Organometallics* **1985**, *4*, 939. (b) Bryndza, H. E.; Fong, L. K.; Paciello, R. A.; Tam, W.; Bercaw, J. E. *J. Am. Chem. Soc.* **1987**, *109*, 1444. (c) Klein, D. P.; Hayes, J. C.; Bergman, R. G. *J. Am. Chem. Soc.* **1988**, *110*, 3704.

(9) Yamamoto, A. *Organotransition Metal Chemistry*; Wiley: New York, 1986.

(10) Examples of fluorinated ketone insertions into late transition-metal hydride and alkyl bonds to yield late metal alkoxide complexes are found in the following: Hayashi, Y.; Komiya, S.; Yamamoto, T.; Yamamoto, A. *Chem. Lett.* **1984**, 1363.

0002-7863/89/1511-2717$01.50/0