Chapter 6

P450 Alkane Hydroxylation Using Terminal Oxidants

A. Abstract

We investigated five P450s for the ability of their active heme-ferryl oxidant to hydroxylate the 104.9 kcal/mol C-H bond of methane within their native active site configuration, using terminal oxidants to circumvent the substrate binding activation of dioxygen in the P450 catalytic cycle. We found that the soluble cytochrome P450 from *Mycobacterium* sp. HXN-1500, CYP153A6, hydroxylates methane with 0.05 turnovers using iodosylbenzene as the oxidant. The methanol product of the iodosylbenzene reaction was validated by isotope labeling using $^{13}CH_4$ and $H_2{}^{18}O$. Attempts to demonstrate this activity under turnover conditions ($NADH/O_2$) in reactions utilizing reconstituted reductase proteins were unsuccessful. We attribute this to the low methane binding affinity of CYP153A6, which does not exhibit a spin-shift in the presence of methane. In contrast, CYP153A6 was found to support both ethane hydroxylation and iodomethane dehalogenation with product formation rates of 61 min^{-1} and 58 min^{-1}, respectively.

B. Introduction

Selective methane conversion to a liquid fuel such as methanol remains one of the great challenges in hydrocarbon chemistry (*1*). Thus far, only a few catalyst systems utilizing transition metals, such as platinum or gold dissolved in specialized environments such as concentrated acid solvents, are capable of this transformation (*2 – 3*). For a variety of reasons including catalyst cost, reaction medium toxicity, catalyst poisoning by oxidized products, and low activity, none of these catalysts are practical (*4*). Methane monooxygenases (MMOs) found in methanotrophic bacteria (*5*) appear to be ideal catalysts for this reaction, as they convert methane to methanol at rates up to 220 min^{-1} using oxygen at ambient conditions (*6*). However, despite decades of research, these enzymes have yet to be functionally expressed in heterologous hosts (*7 – 8*). For these reasons, we and others have been engineering another class of enzymes, cytochrome P450s (P450s), which shares a similar C-H bond activation mechanism as MMOs and utilizes similar high-valent iron oxygen species as the active oxidant, for small alkane hydroxylation with the ultimate goal of achieving methane oxidation (*9 – 13*). In contrast to MMOs, which are only found in methanotropic bacteria, P450s are ubiquitous across all kingdoms of life. Of the more than 11,500 known P450s (data source: http://drnelson.utmem.edu/CytochromeP450.html as published in August 2009), none has been shown to naturally hydroxylate methane.

Using both directed evolution and rational design, propane and ethane hydroxylation activities have been successfully engineered with two different P450s, CYP102A1 (BM3) (*14*) and CYP101 ($P450_{cam}$) (*13*). However, activity for methane remained elusive. Methane presents several challenges as a P450 substrate: its small molecular size presents challenges for both (1)

the initiation of the P450 catalytic cycle, which normally occurs upon the displacement of a distal water ligand as the result of substrate binding, and (2) the formation of the active radical $[(Porp)^{+\cdot}Fe^{IV}=O]^{+}$, known as Compound I (CMP I), which requires substrate-induced water expulsion from the active site. These challenges are faced for any poorly fitting non-natural substrate, albeit magnified by the small size and apolar nature of methane. The strength of the methane C-H bond, however, presents a unique challenge to the oxidizing potential of CMP I.

From density functional theory (DFT) calculations, the transition state for the H-atom abstraction presents the largest barrier in the oxygen insertion reaction of CMP I (*15*). For methane, this barrier height is 26.7 kcal/mol, which is significantly higher than the ca. 19 kcal/mol barrier for substrates such as camphor or propane's secondary C-H bond, or even the 21.6 – 21.8 kcal/mol barriers for ethane and the terminal propane C-H bond (*16* – *19*). As a comparison, this transition state barrier has been calculated to be as low as 13.8 kcal/mol for sMMO acting on methane (*20*).

The complete absence of methane oxidation activity in numerous BM3 variants evolved for propane and ethane hydroxylation activity led us to question if the P450 CMP I can overcome the 26.7 kcal/mol barrier needed to abstract the hydrogen of the methane C-H bond. In this chapter, we separated the substrate binding problem presented by the small size of methane from the challenge of the higher activation barrier presented by the methane C-H bond by assaying the reactivity of CMP I directly through terminal oxidant-supported P450 reactions. Using iodosylbenzene (PhIO), 3-chloroperoxybenzoic acid (MCPBA), and hydrogen peroxide (H_2O_2), the ability of the CMP I of five P450s, BM3, $P450_{PMO}$ (PMO) (*9*), $P450_{cam}$, CYP153A6(A6), and

CYP153A6 BMO-1(*21*), to hydroxylate alkanes ranging from methane to octane was determined.

BM3 and $P450_{cam}$ were selected because they have been engineered to hydroxylate alkanes as small as ethane (*9, 13*). PMO is a laboratory-evolved BM3 variant, which exhibits wild-type like coupling and catalytic efficiency for propane as its preferred substrate. A6, a natural P450 alkane hydroxylase which prefers medium-chain-length alkanes, and its laboratory-evolved variant CYP153A6 BMO-1 with improved butane hydroxylation activity (*21*) were chosen for their ability to hydroxylate medium-chain-length alkanes at the terminal position. In PhIO-supported reactions, CMP I is formed directly, while a ferric hydroperoxo complex (CMP 0) is formed in reactions with peroxides. The generation of CMP I from this complex requires protonation at the distal oxygen followed by heterolytic O-O bond cleavage (Figure 6.1).

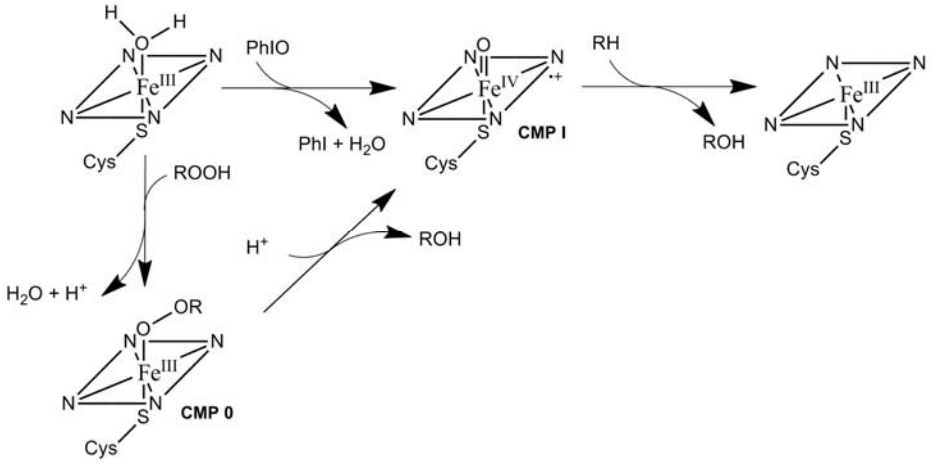

Figure 6.1: Reaction scheme for PhIO and peroxide-supported alkane hydroxylation

From these terminal oxidant-supported P450 reactions, we found A6 and A6 BMO-1, to be able to break the methane C-H bond supporting 0.05 and 0.02 turnovers, respectively, using PhIO as the oxidant. This demonstrates both the feasibility of P450 methane oxidation and the use of terminal oxidant-supported P450 reactions as an assay to investigate the compatibility of

P450 active sites for small alkane oxidation. By chemically generating the active radical, we eliminated the requirement for substrate binding to initiate P450 catalysis, which enabled us to determine the innate substrate range of each P450 active site.

Recently, P450 methane hydroxylation has also been demonstrated with wild-type BM3 through the utilization of perfluoro carboxylic acid additives (*22*). This “chemical tuning” approach apparently resolves the aforementioned challenges of methane as a P450 substrate through the generation of a catalytically active enzyme complex with reduced active site volume using an inert molecule as an external trigger to initiate catalysis. This study also demonstrates that the barrier for P450 methane oxidation is poor activation of the P450 catalytic cycle due to low methane binding affinity rather than the strength of the methane C-H bond.

C. Results and Discussion

C.1. P450s alkane hydroxylation using terminal oxidants

Given the inherent destructive nature of the terminal-oxidant supported reaction for both the enzyme and heme prosthetic group and the likely low efficiency of the oxidation reaction with small alkane substrates, we elected to use a much larger quantity of enzyme (100 μM) in these reactions than previous studies (1 – 2 μM) (*23 – 24*). Only the hydroxylase domain of each P450 was used since terminal oxidant-supported reactions do not require heme iron reduction. Each enzyme was purified through a three-step purification and lyophilized prior to use (see Chapter 8.C for details). The reactions were initiated by the addition of oxidants to pre-incubated mixtures of enzymes and substrates, following previously established protocols (*23 – 25*).

The results of the alkane hydroxylation reactions supported by terminal oxidants are summarized in Table 6.1. In general, PhIO-supported reactions resulted in higher product yields than peroxide-supported reactions with a few exceptions where yields were similar. This difference highlights uncoupling at CMP 0 as one of the difficulties in the hydroxylation of non-natural substrates. With preferred substrates, substrate binding expels water from the active site such that the protonation of CMP 0 only occurs through the proton transport chain, resulting in CMP I formation (*26*). In contrast, the binding of poorly fitting, non-natural substrates, such as small alkanes, does not fully expel water from the active site, and protonation can occur at the proximal oxygen, resulting in unproductive release of peroxide (*27*).

Table 6.1: Alkane hydroxylation by P450s utilizing terminal oxidants

	product formed, μmol (μmol P450)$^{-1}$ [a]										
Substrate	BM3			PMO	$P450_{cam}$			A6			A6 BMO-1
	PhIO	MCPBA	H_2O_2	PhIO	PhIO	MCPBA	H_2O_2	PhIO	MCPBA	H_2O_2	PhIO
Methane	-[b]	-	-	-	-	-	-	**0.05 (0.02)**	-	-	**0.02 (0.01)**
Ethane	-	-	-	-	-	-	-	2.5	0.34	0.23	2.1
Propane	1.0	0.35	0.30	0.77	0.83	0.78	0.96	3.9	1.6	0.41	3.0
Hexane	1.7	0.35	0.31	0.76	0.53	0.38	0.06	0.48	0.57	0.24	n.d.[c]
Octane	1.4	0.12	0.20	0.29	0.31	0.22	0.07 (0.02)	0.51	0.15	0.30	n.d.

[a] Alkanes (2.5 mM, or saturated at 20 psi) were incubated with P450 (100 μM) and terminal oxidant (5 mM) at 25 ^{o}C for 10 min. The data represent the averages of at least two experiments and do not correct for P450 destruction; standard errors are within 20% of the reported average with exceptions given in parentheses.
[b] Dash indicates a lack of detectable amounts of product.
[c] n.d.–not determined

Wild-type BM3 and $P450_{cam}$ were found to hydroxylate alkanes as small as propane using all three oxidants producing only sub-terminal alcohols. This indicates that alkanes as small as propane can be properly oriented near CMP I in the native active sites for oxidation to occur. In the case of BM3, under turnover conditions ($NADPH/O_2$), alkane hydroxylation has been observed only for hexane and nothing smaller. The lack of activity for the smaller alkanes under turnover conditions is solely due to poor substrate binding, which results in insufficient activation of the catalytic cycle and uncoupling at CMP 0. PMO exhibits the same substrate range as BM3 with similar TON for propane using terminal oxidants. This indicates that the laboratory evolution from BM3-to PMO-enabled propane binding to both activate the catalytic cycle and generate CMP I efficiently without changes in H-atom abstraction reaction, i.e., the reaction between CMP I of BM3 and PMO with propane, remain the same. The readiness of BM3's CMP I to react with propane may also explain the ease with which propane hydroxylation activity was obtained from BM3 through various mutations (Chapter 3).

A6 was found to hydroxylate all alkane substrates, even methane, with PhIO as the oxidant. This demonstrates that direct methane-to-methanol conversion by a P450 heme porphyrin catalyst at ambient conditions is possible and does not necessarily require the use of additional effectors to alter the active site geometry. With PhIO as the oxidant, A6 is able to hydroxylate methane with 0.05 TON. This low TON shows that although methane can be oxidized by A6, it is a poor substrate with minimal reactivity even in the presence of a pre-generated CMP I. The A6 methane TON is 50-fold lower compared to A6 ethane TON, which may reflect poor binding of methane in the active site compared to ethane, since the generation of CMP I is substrate-independent. The higher methane C-H bond strength (104.9 vs. 101.0 kcal/mol) may also be responsible for the decrease in TON. A6 methane reactions with MCPBA

and H_2O_2 did not yield detectable methanol product. Considering the low yield of the methane reaction with PhIO and the general trend of peroxide reactions being less efficient, the absence of methanol product in these reactions could be due to the limited ability to detect the product (2.0 μM, corresponding to a signal-to-noise ratio of 3). Surprisingly, reactions with the preferred substrates of A6, hexane and octane, yielded far less product as compared to reactions with ethane and propane. This may be the result of competition between these preferred substrates and PhIO for active site access. Finally, CYP153A6 BMO-1 also exhibited methane oxidation with PhIO, but with only 0.02 TON. Its propane TON also decreased compared to A6 from 3.9 to 3.0, which reflects the diminished activity observed under turnover conditions. Since CYP153A6 BMO-1 was only selected for growth complementation on butane, this loss of activity for smaller alkanes is a consequence of natural drift as these activities were not under direct selection (*28*).

In PhIO-supported reactions of A6 and PMO with hexane and octane, slightly different regioselectivities were observed compared to those obtained under turnover conditions, whereas reactions with BM3 and $P450_{cam}$ displayed similar regioselectivities to those observed under turnover conditions. For A6, 1-hexanol and 1-octanol were obtained with > 95% selectivity under turnover conditions, but in PhIO-mediated reactions, significant sub-terminal products were generated: 34% for hexane and 46% octane. In contrast, PMO produces 2-hexanol and 2-octanol with > 90% selectivity in NADH/O_2 supported reactions, but in PhIO-mediated reactions 24% of 1-hexanol and 30% of 1-octanol were produced. Regioselectivity differences between PhIO-supported and NADPH/O_2 supported reactions have been reported for several substrates (*29*) and suggest there are differences in active site packing between PhIO- and NADH/O_2-supported reactions. These differences could be the result of lingering iodoarene in the active site or the different order in which substrate binding and CMP I formation occurs. Under turnover

conditions, substrate binding precedes CMP I formation, which may allow the substrate to orient in the preferred conformation, whereas in PhIO-supported reactions, the CMP I species is generated in the absence of the substrate. In A6 PhIO-supported reactions with propane, 1-propanol was produced with a similar regioselectivity (35%) as the NADH/O2 supported reaction (41%). Propane hydroxylation may be less sensitive to differences in active site packing between PhIO- and NADH/O_2-supported reactions since it already occurs with low regioselectivity.

^{13}C and ^{18}O labeling experiments were conducted to verify the carbon and oxygen sources of the methanol product generated in PhIO reactions with A6. Reactions with ^{13}C methane produced an m/z 33 ion peak unique to ^{13}C-methanol, which corresponds to a +1 m/z shift of the major ^{12}C-methanol ion of m/z 32 (see Appendix D). This result confirms the carbon source of the methanol product to be the supplied methane gas. Quantification against authentic ^{13}C-methanol standards showed A6 produced 0.035 $\pm$ 0.009 TON with ^{13}C-methane. To confirm the oxygen source of the methanol product, we took advantage of the fact that CMP I generated with PhIO undergoes oxygen exchange with solvent water (*30*), such that solvent oxygen incorporation is a hallmark of the reaction mediated by PhIO. Reactions in the presence of 50% $H_2{}^{18}O$ also produced an m/z 33 ions peak corresponding to a +2 m/z shift of the m/z 31 ion of ^{12}C-methanol, unique to ^{18}O-methanol. Quantification for ^{18}O incorporation was not possible due to the low yield and the presence of a mixture of ^{16}O- and ^{18}O-methanol products. As a general comparison for the PhIO reaction with A6, ethane reactions in the presence of 50% $H_2{}^{18}O$ resulted in a 50% decrease in ^{16}O ethanol, which suggest nearly quantitative ^{18}O incorporation, which is in good accordance with literature values (*25*). These results confirm the obtained methanol product is generated through a PhIO-mediated P450 reaction with methane.

C.2. A6 alkane hydroxylation under turnover conditions

Convinced that the A6 CMP I can hydroxylate methane, we investigated A6 for oxidation of methane and other alkanes under turnover conditions utilizing reconstituted A6 reductase proteins. For the *in vitro* A6 alkane hydroxylation reactions, its reductase components, ferredoxin reductase (fdrA6) and ferredoxin (fdxA6) were purified following literature protocols established for the purification of putidaredoxin and putidaredoxin reductase of $P450_{cam}$ (*31*), which share 44% and 43% sequence identity with the ferredoxin and ferredoxin reductase of A6. The isolated proteins were quantified using known extinction coefficients for their FAD and [Fe_2-S_2] cofactors (*31*) and characterized for electron transfer activity using cytochrome c reduction (*32*).

An optimum ratio of reductase components of 1:1:10 for A6:fdrA6:fdxA6 was determined from octane hydroxylation reactions and used for subsequent experiments (see Appendix D). At this ratio of reductase components, the substrate-free cofactor consumption rate was only 3 – 5 min^{-1}. In the presence of octane, the rate of cofactor consumption reaches ca. 80 min^{-1}, which is significantly lower than that of typical bacterial P450s, which often reaches 1,000s min^{-1} (*33*), but is consistent with the rates of other natural alkane hydroxylases acting on their preferred substrates: sMMO ca. 220 min^{-1} on methane (*6*), AlkB ca. 150 min^{-1} on octane (*34*), and butane monooxygenase ca. 36 min^{-1} on butane (*35*). The rate of octanol formation was found to be 75 min^{-1}, which is slightly higher than the reported value for the same activity determined with A6 in *P. putida* GPo12 cell extracts (*36*). Reactions with ethane at 20 -psi headspace pressure resulted in ethanol formation rates of 32 min^{-1}, but reactions with both ^{12}C- and ^{13}C-methane did not produce detectable amounts of methanol.

The absence of A6 methane hydroxylation activity under turnover conditions can be rationalized by a lack of CMP I formation during the reaction, analogous to the lack of wild-type BM3 propane hydroxylation under turnover conditions. P450 catalysis is initiated by substrate binding, which displaces the distal water ligand inducing a spin-shift from the low spin resting state to a catalytically active high spin state (*37*). For A6, this spin-shift is indicated by UV/Vis difference spectra (*38*) and is observed for alkanes as small as ethane, but is absent for methane (Figure 6.2). This lack of spin-shift in the presence of methane demonstrates the absence of CMP I formation as a barrier for catalysis under turnover conditions.

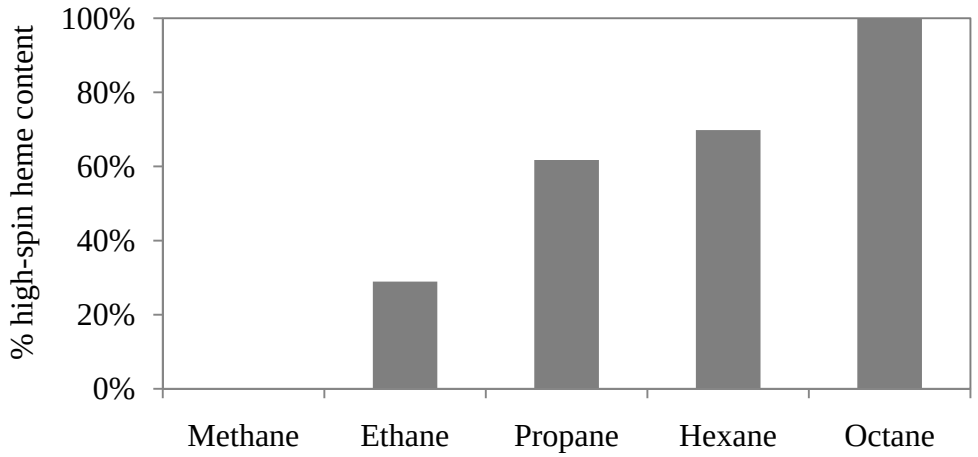

Figure 6.2: Alkane induced spin-shift of A6 as determined at saturation by absorbance difference between A_{392} and A_{418}. The percentage of high-spin content was determined relative to the spin-shift induced by the preferred substrate octane. For gaseous substrates, the spin-shift was determined with 40 -psi head-space pressure. For liquid alkanes, the spin-shift was determined with 1 mM substrate in a 1% ethanol solution.

To gain more insight into the differences in A6-catalyzed oxidation of a preferred substrate vs. a smaller, non-natural substrate, the kinetic parameters (K_M and k_{cat}) and the kinetic isotope effect (KIE) were determined for the oxidation of hexane, octane, and the dehalogenation of iodomethane (Table 6.2). Attempts to characterize ethane hydroxylation kinetics were unsuccessful, as saturating kinetics were not observed over the pressure range investigated (see Appendix D). Therefore, iodomethane was chosen as a surrogate for the small gaseous alkanes,

because it possesses both a molecular size and a C-H bond strength (102.9 kcal/mol) intermediate of those of methane (104.9 kcal/mol) and ethane (101.0 kcal/mol). The liquid form of iodomethane offers the additional benefit that saturating kinetics can be observed. From the preferred substrate octane to iodomethane, a 50-fold increase in K_M from 0.32 mM to 17.7 mM was observed. Surprisingly, there was only a small difference in the k_{cat} values for these two substrates, 75 min^{-1} for octane vs. 58 min^{-1} for iodomethane. The overall 70-fold decrease in catalytic efficiency from $3.9x10^3$ $M^{-1}s^{-1}$ for octane to 55 $M^{-1}s^{-1}$ for iodomethane is thus largely due to the higher K_M.

The kinetic isotope effect (KIE) of a P450 reaction, determined by comparing the reaction rate of deuterated and non-deuterated substrates, can indicate if the C-H bond activation is the rate-limiting step in catalysis. Since a C-D bond has lower vibrational frequency compared to a C-H bond, it has a lower zero point energy. As a result of this lower ground state energy, the deuterated substrate has a higher activation barrier for reaction, which would produce a slower reaction rate if breaking of the C-H bond is rate-limiting. For the preferred A6 substrates hexane and octane, KIEs of near unity were observed. This indicates the reaction of CMP I with these substrates is not rate-limiting under turnover conditions, which is expected, as the second electron transfer step is generally rate-limiting for P450s acting on their preferred substrates (*39*). In contrast, a KIE of 5.8 was observed for iodomethane dehalogenation, which demonstrates that the H-atom abstraction reaction has become rate-limiting. A KIE of 5.8 falls within the classical limit and indicates an absence of hydrogen atom tunneling, which has been suggested to occur during sMMO oxidation of methane (*40*). As a comparison, a similar KIE of 6.4 has been observed for $P450_{cam}$ hydroxylation of (1R)-5,5-difluorocamphor (*41*). By blocking the preferred hydroxylation site, this study showed that the C-H bond activation step can become rate-limiting

when the oxidation occurs under unfavorable geometries. The higher mobility of iodomethane within the A6 active site may have a similar effect such that the substrate C-H bond is not properly oriented near CMP I for reaction.

Table 6.2: A6 alkane hydroxylation under turnover conditions

	k_{cat} (min^{-1})	K_M (mM)	k_{cat}/K_M (M^{-1} s^{-1})	Coupling (%)	KIE (k_H/k_D)
Methane [a]	0	n.d.[b]	n.d.	n.d.	n.d.
Iodomethane [c]	58 (5.1)	17.7(1.4)	55	42	5.8
Ethane [a]	61 (8.3)	n.d.	n.d.	74	n.d.
Hexane [c]	98 (7.0)	0.78 (0.04)	$2.1x10^3$	96	1.0
Octane [c]	75 (7.2)	0.32 (0.02)	$3.9x10^3$	98	1.0

[a] Reactions contained 0.5 μM A6, 0.5 μM FdrA6, 5 μM FdxA6,1 mM NADH, in 0.1 M phosphate buffer, pH 8.0 under alkane atmosphere with head-space pressure ranging from 20 – 60 psi. Ethane k_{cat} and coupling were determined at 40 psi head-space pressure, corresponding to the maximum rate of ethanol formation observed. [b] n.d.–not determined. [c]Reactions contained 0.5 μM A6, 0.5 μM FdrA6, 5 μM FdxA6,1 mM NADH, the substrate in 2% ethanol and 0.1 M phosphate buffer, pH 8.0. The data represent the averages of three replicates; values in parentheses are the standard errors.

In conclusion, we have demonstrated the use of terminal oxidants for evaluating the innate substrate specificity of P450s, independent of the requirement for substrate binding to initiate catalysis. Using this assay, we were able to show that CMP I of A6 can support methane oxidation, just as the CMP I of BM3 is poised for propane oxidation, despite the fact that neither activity is observed under turnover conditions. This result confirms that the methane C-H bond of 104.9 kcal/mol can be oxidized by a P450 and suggests that A6 can be a good starting point for the engineering of a P450 methane monooxygenase.

D. References

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