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Synthesis and Evaluation of Photoaffinity Probes Directed at Lanosterol 14α-Demethylase (P-450$_{14DM}$)

Gerard D. Wright and John F. Honek*

Guelph-Waterloo Center for Graduate Work in Chemistry, Department of Chemistry, University of Waterloo, Waterloo, Ontario, Canada N2L 3G1

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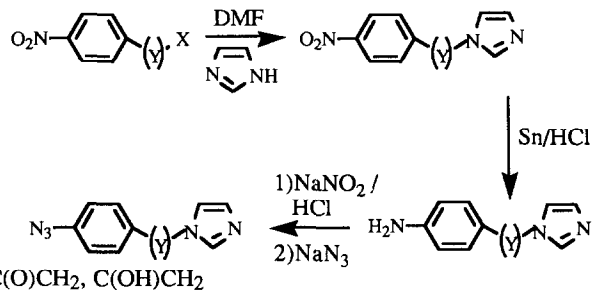
Abstract: A series of aromatic azides which could act as potential photoaffinity probes for the yeast lanosterol 14α-demethylase (P-450$_{14DM}$) were prepared. One of these, 1-[^{3}H]-*p*-azidophenyl-2-(1-imidazolyl)ethanol, has been demonstrated to label purified P-450$_{14DM}$ from *Saccharomyces cerevisiae*. These results provide the framework for future labeling experiments.

Fungal lanosterol 14α-demethylase is the target for the medicinally important imidazole/triazole class of antifungal agents[1]. This enzyme is a member of the cytochrome P-450 monooxygenase family (P-450$_{14DM}$) and effects the demethylation reaction by two regiospecific hydroxylations at the C-32 (14α) methyl group followed by a final O_2-consuming oxidation[2]. The net result of the enzymatic activity is the loss of the methyl group as formic acid and release of the sterol as a $\Delta^{8,9}$-$\Delta^{14,15}$ conjugated diene. The preeminence of this reaction in sterol biosynthesis in both mammals and fungi, although presently only inhibition of the latter has proven medicinally important, has made the elucidation of P-450$_{14DM}$ structure-function relationships paramount in the chemistry of sterol biosynthesis. We report herein the synthesis and evaluation of a series of imidazole-containing aromatic azides as potential photoaffinity labels of purified P-450$_{14DM}$ from the yeast *Saccharomyces cerevisiae*. Such probes could be used to identify active site residues which would be helpful in refining predicted three-dimensional structures of P-450$_{14DM}$ and P-450s in general[3].

Aromatic azides bearing N-linked imidazoles were prepared in the following manner (Scheme 1). First the appropriate parent chloro or bromo *p*-nitrophenyl compounds were reacted with imidazole in DMF at 0 °C to give the N-linked imidazole adduct. These adducts were reduced to the corresponding amines with Sn/HCl, converted to the diazo-compounds and treated with sodium azide to afford the photoaffinity probes 1-(*p*-azidophenyl)imidazole (**1**), 1-(*p*-azidobenzyl)imidazole (**2**) and 1-(*p*-azidophenacyl)imidazole (**3**)[4,5]. The compound, *p*-azidophenyl-2-(1-imidazolyl)ethanol (**4**), was prepared by sodium borohydride reduction of 1-(*p*-aminophenacyl)imidazole (**5**) (the precursor to **3**), followed by diazotization and treatment with azide[5].

Scheme 1. Synthesis of Photoaffinity Labels



X=Cl, Br; Y= -, CH_2, $C(O)CH_2$, $C(OH)CH_2$

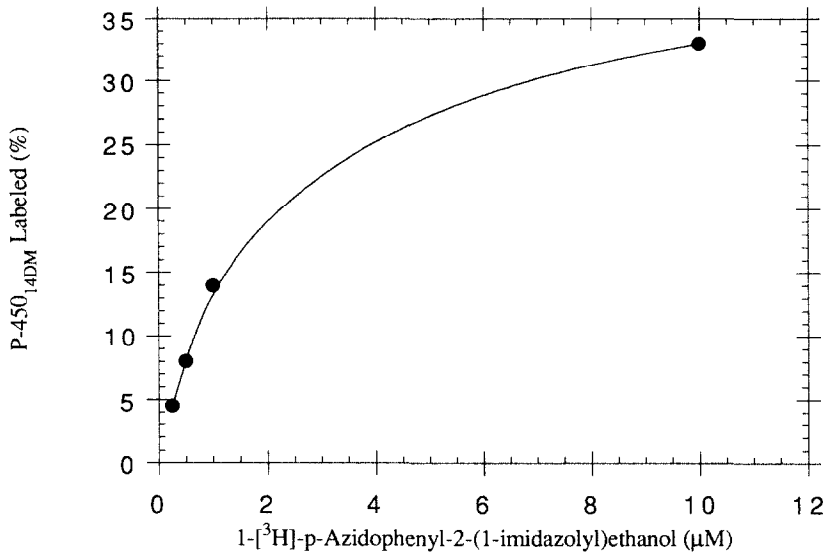
All four azides gave Type II difference spectra with purified P-450$_{14DM}$ indicative of coordination of the imidazole nitrogen with the heme iron (III), and showed high affinity for the enzyme (Table 1)[6,7]. Compound **4** was selected for further study as it demonstrated the highest affinity for P-450$_{14DM}$ and could easily be labeled with ^{3}H by reduction of **5** with NaB[^{3}H]$_4$. The labeled compound, 1-[^{3}H]-*p*-azidophenyl-2-(1-imidazolyl)ethanol (**6**) was obtained in 57% yield from **5** with a specific activity of 26.4 mCi/mmol[10].

Photolysis of **6** was efficient and rapid upon irradiation at 264 nm ($t_{1/2}$ = 2.5 min) in phosphate buffer; however, effective incorporation of **6** into P-450_{14DM} was achieved only upon irradiation at 313 nm ($t_{1/2}$≈45 min in phosphate buffer) for 3 hours[11,12]. Labeling was maximal at 10 fold excess **6** over P-450_{14DM} (Figure 1).

Table 1. Interaction of Aromatic Azides with P-450_{14DM}[6,8]

Compound Number	K_D (μM)
1	**25**
2	**15**
3	**18**
4	**1.4**

Figure 1. Labeling of P-450_{14DM}* with 1-[^{3}H]-p-Azidophenyl-2-(1-imidazolyl)ethanol



*1 μM in 100 mM phosphate, 1 mM EDTA, 0.2% Tergitol 15-S-12, 20% glycerol pH 7.0

Incorporation of ^{3}H into P-450_{14DM} was dependent on irradiation at 313 nm and was substantially decreased upon addition of the imidazole antimycotic, clotrimazole a known active site directed inhibitor of this

enzyme (Table 2). These results, coupled with direct spectrophotometric evidence for a tight P-450_{14DM}-**6** complex (Type II difference spectrum), strongly suggest that the site of labeling is at the enzyme active site. Furthermore, isolation of the porphyrin (as the methyl ester[13]) from tryptic digested P-450_{14DM} revealed ^3H only in the peptide fraction demonstrating that **6** labels the peptide backbone of the enzyme and not the heme prosthetic group.

Table 2. Conditions for Labeling P-450_{14DM} with **6**[12]

Condition*	^3H Associate with P-450_{14DM} (% dpm)
No irradiation	15
Irradiation at 313 nm	100
Irradiation at 313 nm + Clotrimazole (2 mM)	23

*Labeling mixtures consisted of P-450_{14DM} (1 μM) and **6** (10 μM) in 100 mM phosphate, 1 mM EDTA, 0.2% Tergitol 15-S-12, 20% glycerol pH 7.0

This work provides the first description of P-450_{14DM} directed photoaffinity probes and provides the basis for the isolation of labeled active site peptides. Given the low quantities of available enzyme, this will be achieved using compounds incorporating nuclides of higher specific activity (eg ^{125}I). Since many P-450s bind imidazole containing compounds, these photoaffinity labels should be useful as probes for other P-450 monooxygenases.

Acknowledgments

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Notes and References

1. Yoshida, Y. (1988) in *Current Topics in Medical Mycology, Vol. 2*, (McGinnis, M.R. ed) Springer Verlag, New York.

2. Fischer, R.T., Trzaskos, J.M., Magolda, R.L., Ko, S.S., Brosz, C.S. and Larsen, B. 1991, *J. Biol. Chem., 266*, 6124.

3 Three-dimensional structures of mammalian P-450s have been predicted based on the P-450cam crystal structure e.g. Zvelebil, M.J.J.M, Wolf, C.R. and Sternberg, M.J.E. 1991, *Protein Engng., 4*, 271.

4. Compound **1** was prepared as described in Swanson, R.A. and Dus, K.M. 1979, *J. Biol. Chem., 254*, 7238.

5. Characteristics of azides: (**2**): mp 26-29 °C, λmax(EtOH) 248 nm, IR($CHCl_3$) 3660, 2100, 1603, 1500 cm^{-1}, ^1H NMR ($CDCl_3$) δ 7.65 (s, 1H), 7.26 (s, 1H), 7.1 (m, 3H), 6.69 (m, 2H), 5.12 (s, 2H), Mass spectrum (CI, NH_3): m/z (relative intensity) 200 (M+1, 8%), 174 (24%), 69 (100%); (**3**): mp 127-129 °C, λmax (EtOH) 280 nm, IR(Nujol mull) 2113, 2246, 1691, 1598, 1569 cm^{-1}, ^1H NMR ($CDCl_3$) δ 8.5 (d, 2H, J = 8 Hz), 7.55 (s, 1H), 7.3 (d, 2H, J = 8 Hz), 7.1 (s, 1H), 6.95 (s, 1H), 5.75 (s, 2H), Mass spectrum (CI, NH_3): m/z (relative intensity) 228 (M+1, 8%), 202 (81%), 136 (100%), 69 (82%); (**4**): mp 162-164 °C, λmax(EtOH) 250 nm, εm 12,030 $M^{-1}cm^{-1}$, IR(Nujol mull) 2126, 1602 cm^{-1}, ^1H NMR($CDCl_3$) δ 7.4 (s, 1H), 7.28 (d, 3H, J = 7 Hz), 7.03 (d, 2H, J = 7 Hz), 6.89 (s, 1H), 4.95 (t, 1H, J = 5 Hz), 4.09 (m, 2H), Mass spectrum (CI, NH_3): m/z (relative intensity) 230 (M+1, 100%), 204 (68%), 202 (18%), 69 (4%).

6. Binding constants (K_D) were determined with purified P-450_{14DM} as described previously[7].

7. Wright, G.D., Parent, T. and Honek, J.F. 1990, *Biochim. Biophys. Acta, 1040*, 95.

8. P-450$_{14DM}$ was purified from *Saccharomyces cerevisiae* JM745/pVK1 by modification of a previously described method[9]; Wright, G.D. Investigation of the Lanosterol 14α-Demthylase (P-450$_{14DM}$) fom *Saccharomyces cerevisiae*, University of Waterloo, 1990. Briefly, the major modifications included substitution of OA Sepharose 4B for AH Sepharose 4B, and the use of Tergitol 15-S-12 rather than Emulgen 911.

9. Yoshida, Y. and Aoyama, Y. 1984 *J. Biol. Chem. 259*, 1655.

10. Compound **6** was prepared as follows: To 2-(1-imidazolyl)-4-aminoacetophenone (6.3 mg) dissolved in anhydrous methanol (2 ml) was added NaB[^{3}H]$_4$ (1.9 mg, specific activity 13.15 mCi/mg) and the mixture stirred at room temperature for 2 hrs under N_2 atmosphere. Anhydrous acetone (2 ml) was added to quench unreacted reducing agent and the solvent was evaporated under a stream of N_2. The residue was applied to a preparative TLC plate and developed with CH_2Cl_2:MeOH (10:1). The [^{3}H] alcohol was dissolved in water (100 ml) and concentrated HCl (47 ml) and cooled to 0 °C. Sodium nitrite (5 mg) dissolved in 50 ml water was added and the mixture stirred at 0 °C for 1 hr followed by the addition of a crystal of urea. A solution of NaN_3 (5 mg) in water (10 ml) was added and the reaction mixture stirred at 0 °C until N_2 evolution ceased. Saturated sodium bicarbonate solution was slowly added dropwise and the reaction mixture extracted with ethyl acetate (5 x 5 ml), the organic fractions were pooled, dried over Na_2SO_4, applied to a preparative TLC plate and developed with CH_2Cl_2:MeOH (10:1). The brown solid (4.1 mg, 57% from starting material) was judged pure by TLC (identical to compound **4**) and found to have a specific activity of 26.4 mCi/mmol.

11. Affinity labeling of P-450$_{cam}$ at 313 nm with **1** has been reported[4].

12. Labeling was carried out in a quartz cuvette at 4 °C with constant stiring in a Rayonet Photochemical Reactor for 3 hrs. The mixture was applied to a Sephadex G-25 column (1x10 cm) equilibrated with 100 mM phosphate pH 7.0 or dialysed against the same buffer to separate bound from free **6**.

13. Stearns, R.A. and Ortiz de Montellano, P.R. 1985, *J. Am. Chem. Soc 107*, 234.