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Design, synthesis and evaluation of novel P450 fluorescent probes bearing α-cyanoether

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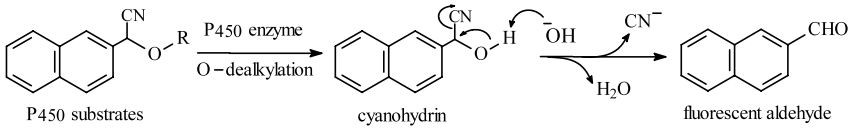
Abstract—Four α-cyano-containing ethers based on 2-alkoxy-2-naphthylacetonitriles have been designed as a novel structural class of cytochrome P450 fluorescent probes. Their syntheses, fluorescence properties and evaluation in the fluorogenic assay of cytochrome P450 monooxygenase are reported. After P450 enzymatic *O*-dealkylation, the cyanohydrin metabolite of the four new substrates rearranges to a fluorescent aromatic aldehyde with a larger Stokes shift, and the new substrates exhibit higher specific activities than that of the commercial substrate 7-ethoxyresorufin (ER). © 2003 Elsevier Science Ltd. All rights reserved.

The cytochromes P450 are involved in regulating hormones, steroids, and fatty acids, and in metabolism of xenobiotics such as drugs, carcinogens, pesticides, and diverse pollutants.[1] Many laboratories in both academia and industry routinely screen novel compounds for inhibition of cytochromes P450 to guard against drug–drug interactions.[2] Numerous types of fluorescent substrates have been designed and synthesized as diagnostic probes of cytochromes P450, including alkoxyresorufins, alkoxyquinolines, alkoxycoumarins and their modified analogues.[3–5] The common chemical structural model is that they are heterocyclic aryl-ether derivatives. After enzymatic *O*-dealkylation, they yield the corresponding phenolic metabolites, which have fluorescence properties for detection.

Over the last few years, we have been interested in developing novel fluorescent enzyme substrates in an effort to expand our knowledge of these enzymes and provide sensitive and easily used assays.[6] A series of α-cyano-containing ester substrates had been developed and demonstrated as novel fluorogenic esterase reporters.[7,8] The concept is that an esterase substrate containing an α-cyano group could be designed with little or no fluorescence background yet be transformed to a strongly fluorescing aldehyde upon ester hydrolysis. Herein, a similar strategy was utilized to design α-cyano-containing ether derivatives based on 2-alkoxy-2-naphthylacetonitriles as a novel structural class of fluorescent substrates for cytochrome P450 monooxygenase (Scheme 1). The reported probes possess one α-cyano group and have very low fluorescence background, undergo *O*-dealkylation by P450 monooxygenase to form cyanohydrins, which spontaneously convert to 2-naphthaldehyde generating larger red shift and obvious higher fluorescence emission intensity for detection. Four new fluorescent probes with methyl, ethyl, pentyl and benzyl substitutents have been synthesized. These are α-cyanodialkyl rather than the commonly used aryl-alkyl ethers and thus provide structural diversity among spectraphotometric sub-



Scheme 1. Metabolism of α-cyanoether substrate to its fluorescent metabolite by P450s.

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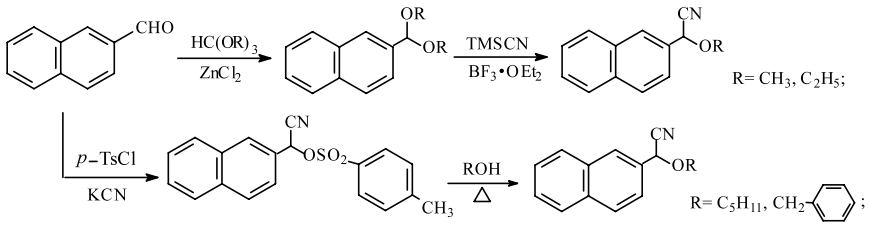

Scheme 2. Synthetic routes to 2-alkoxy-2-naphthylacetonitriles.

strates. The fluorescence properties of these substrates and their fluorescent product 2-naphthaldehyde were compared with the commonly used P450 substrate 7-ethoxyresorufin (ER) and its product resorufin, and the parent compounds were compared as substrates in rat microsomes fortified with NADPH. The significant advantages found indicate that this and related series of α-cyano-containing ethers will complement existing spectral substrates used for the measurement of cytochrome P450 activities.

Scheme 2 shows the synthetic routes to the target compounds. The first synthesis was carried out using modified literature procedures.[9,10] Syntheses of dimethyl acetal and diethyl acetal were achieved from readily available starting materials trimethyl orthoformate and triethyl orthoformate, respectively. Then one of the alkoxyl groups of acetals was converted to cyano group using cyanotrimethylsilane under the catalytic action of $BF_3{\cdot}OEt_2$ with high yield. For pentyl and benzyl ethers, the cyanonaphthyl toluenesulfonate alcoholysis reaction was employed for synthesis.[11] Either benzenesulfonyl chloride or *p*-toluenesulfonyl chloride can be used in the reaction with a same yield. The pentyl and benzyl ethers were also prepared according to the same procedure as that of **1** and **2** by refluxing 2-naphthaldehyde with tripentyl orthoformate or benzyl alcohol respectively to give the corresponding diacetal, followed by changing one of the alkoxy group to cyano group with cyanotrimethylsilane and a catalytic amount of $SnCl_2$. All new compounds were characterized via ^1H and ^{13}C NMR, MS and microanalyses.[12]

Fluorescent spectra of compounds **1**–**4** (Fig. 1) were scanned as 10 μM solutions in sodium phosphate buffer (pH 7.6, 0.1 M) at excitation 345 nm. All substrates (quantum yield below 0.03) have a low intensity fluorescence emission peak at 370 nm, while a stronger fluorescence emission is measured at 445 nm with the metabolite 2-naphthaldehyde (quantum yield 0.19). This property helps to minimize possible interference of the assay between aldehyde and substrates. The extinction coefficient of aldehyde in Tris–malate buffer (0.1 M, pH 7.0) at 292 nm was calculated to be 12,700 M^{-1} cm^{-1}, those of substrates were 2,400–10,160 M^{-1} cm^{-1} at their maximum absorbance. The Stokes shift of 2-naphthaldehyde ($\lambda_{ex}=345$ nm, $\lambda_{em}=445$ nm) is also larger than that of resorufin ($\lambda_{ex}=570$ nm, $\lambda_{em}=585$ nm). When the substrates were dissolved by using DMSO as a vehicle, the solubility of the substrates is better than that of commercial substrate ER. These advantages in fluorescent and solubility properties

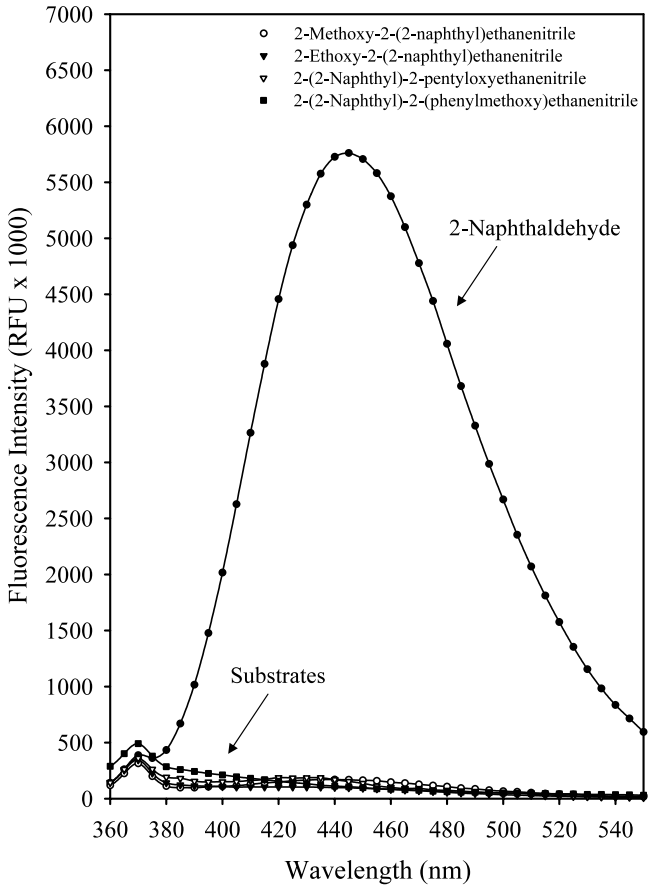

Figure 1. Comparison of new substrates **1**–**4** and 2-naphthaldehyde fluorescent emission. 10 μM solutions in sodium phosphate buffer (pH 7.6, 0.1 M).

provide a sound background for developing α-cyano-ether compounds as novel surrogate substrates for cytochrome P450 *O*-dealkylation reactions.

Enzymatic *O*-dealkylation of the P450 substrates were performed by incubating rat liver microsomes (150 μg) at 37°C in a final volume of 2 mL of 40 mM Tris–HCl buffer (pH 7.8). The substrate was added in 10 uL of DMSO solution to give a final substrate concentration of 50 μM. Reactions were initiated by the addition of 10 μL of 50 mM NADPH in distilled water. After the incubation for 30 min, the reactions were added to tubes containing DEAE Sepharose fast flow (Pharmacia, Uppsala, Sweden) and mixed well to bind the NADPH. Before use, the DEAE ion exchanger was washed five times to equilibrate with the reaction

buffer. The mixed tubes were centrifuged at 3000 rpm for 5 min. The fluorescence intensities were measured in a Fluoromax II spectrofluorometer with excitation at 345 nm and emission at 445 nm. The commonly used commercial substrate ER was also used for a comparison. The results are shown in Figure 2, in which each entry represents an average of three independent tests.

As shown in Figure 2, the substrates were rapidly dealkylated in microsomes in the presence of NADPH with the relative rates of *O*-dealkylation in rat liver microsomes of methyl-ether>benzyl-ether>pentyl-ether>ethyl-ether. All the new α-cyanoether substrates exhibited higher specific activities than that of ER in end-point assays. The rapid generation of the fluorescent metabolite from these new substrates supported the general idea of designing additional α-cyanoethers as P450s probes.

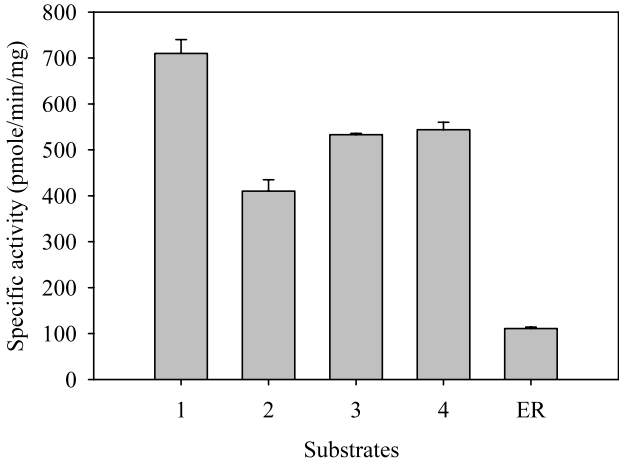

Figure 2. Comparison of specific activities for new substrates **1**–**4** and common P450 substrate ER.

In summary, we have designed, synthesized and evaluated α-cyano-containing ethers based on 2-alkoxy-2-naphthylacetonitriles as novel P450 fluorescent probes. Because of their unique molecular structures representing a new family of fluorescent probes for P450s, their facile synthesis high-turnover and attractive optical properties, they may prove to be useful and attractive tools in pharmacological and biochemical studies. Further studies on their metabolism by recombinant isozymes, synthesis of alternate more red shifted α-cyano-containing ether reporters are currently under investigation.

Acknowledgements

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12. *Preparation of 2-methoxy-2-(2-naphthyl)ethanenitrile* ***1***: A mixture of 1.56 g (10 mmol) 2-naphthaldehyde, 1.74 g (16.4 mmol) freshly distilled trimethyl orthoformate, 4.2 mL of absolute methanol and 10 mg of zinc chloride were refluxed for 6 h under dry conditions. Part of solvent was removed and an additional 0.5 mL of trimethyl orthoformate was added. The reaction mixture continued to reflux overnight and was monitored by TLC. Excess trimethyl orthoformate and solvent was removed under reduced pressure, the residue was treated with aqueous 5% (w/v) $NaHCO_3$ and extracted with diethyl ether. The ether extract was washed with water, dried over $MgSO_4$ and concentrated. The crude dimethyl acetal was used for the next step without further purification. Under nitrogen, 0.1 mL (0.7 mmol) of $BF_3{\cdot}OEt_2$ was added gradually to a mixture of the above-prepared dimethyl acetal and 1.33 mL (0.99 g, 10 mmol) of cyanotrimethylsilane at 0°C. After stirring at room temperature for 2 h, the clear solution turned into a cream colored slurry. TLC indicated that the reaction was complete. The mixture was worked up with dilute aqueous sodium bicarbonate solution, filtered, washed with water and dried in vacuo to give 1.62 g (82.4% yield) of crude product, which was recrystallized from diethyl ether to give a cream colored solid, mp 47–48°C. TLC R_f 0.60 (hexane:EtOAc = 3.5:1, v/v). ^1H NMR ($CDCl_3$): δ 3.56 (s, 3H), 5.39 (s, 1H),

7.50–7.57 (m, 3H), 7.82–8.00 (m, 4H); ^{13}C NMR ($CDCl_3$): δ 57.4 (CH_3), 72.7 (CCN), 117.2 (CN), 124.3, 127.1, 127.2, 127.4, 128.0, 128.5, 129.4, 130.6, 133.1, 133.9; MS (EI, 70 eV): m/z % 197 [M^+, 51], 166 [$(M-OCH_3)^+$, 100], 139 [$(M-OCH_3-HCN)^+$, 21]. ESI-MS calcd for $C_{13}H_{12}NO$ [$(M+H)^+$]: 198.0919. Found: 198.0929.

Preparation of 2-ethoxy-2-(2-naphthyl)ethanenitrile ***2***: By a similar procedure, the target compound was prepared in 70.6% yield, which crystallized as white plates from diethyl ether, mp 53–54°C. TLC R_f 0.52 (hexane:EtOAc = 5:1, v/v). ^{1}H NMR ($CDCl_3$): δ 1.32 (t, J = 6.6 Hz, 3H), 3.65–3.90 (m, 2H), 5.43 (s, 1H), 7.50–7.58 (m, 3H), 7.83–7.98 (m, 4H); ^{13}C NMR ($CDCl_3$): δ 15.2 (CH_3), 66.0 (CH_2), 71.1 (CCN), 117.6 (CN), 124.4, 127.0, 127.1, 127.3, 127.9, 128.5, 129.3, 131.1, 133.1, 133.9; MS (EI, 70 eV): m/z % 211 [M^+, 35], 166 [$(M-OCH_2CH_3)^+$, 100], 139 [$(M-OCH_2CH_3-HCN)^+$, 21]. Anal. calcd for $C_{14}H_{13}NO$: C, 79.59; H, 6.20; N, 6.63. Found: C, 79.66; H, 6.32; N, 6.54.

Preparation of 2-(2-naphthyl)-2-(phenylmethoxy)-ethanenitrile ***3***: A 1.56 g (10 mmol) sample of 2-naphthaldehyde was dissolved in 2.3 mL of tetrahydrofuran, and 1.9 g (10 mmol) of *p*-toluenesulfonyl chloride was added. Then 0.66 g (10 mmol) of potassium cyanide in 2 mL of water was added dropwise with stirring at a rate sufficient to maintain the temperature below 5°C in an ice bath. After the mixture was stirred for 1 h at 0–5°C, the solvent was stripped under reduced pressure washed with ice-cold diethyl ether, dried in vacuo to give 2.52 g of α-cyano-naphthyl toluenesulfonate as a cream colored solid. The above sulfonate was immediately mixed with 3.5 mL of benzyl alcohol and heated to 80°C for 2 h, then filtered and the solution was flash chromatographed on silica gel using (hexane:EtOAc = 3.5:1, v/v) as the eluent to give 2.07 g (75.8% yield) of the target compound as a cream colored solid, mp 36–37°C. TLC R_f 0.68 (hexane:EtOAc = 5:1, v/v). ^{1}H NMR ($CDCl_3$): δ 4.71–4.87 (m, 2H), 5.44 (s, 1H), 7.36–7.44 (m, 5H), 7.54–7.59 (m, 3H), 7.84–8.00 (m, 4H); ^{13}C NMR ($CDCl_3$): δ 69.9 (CCN), 71.9 (CH_2), 117.4 (CN), 124.5, 127.0, 127.3, 127.4, 128.0, 128.5, 128.6 (2C, phenyl), 128.7, 129.0 (2C, phenyl), 129.4, 130.8, 133.1, 133.9, 135.9; MS (EI, 70 eV): m/z % 273 [M^+, 19], 166 [$(M-OCH_2Ph)^+$, 100], 139 [$(M-OCH_2Ph-HCN)^+$, 31]. Anal. calcd for $C_{19}H_{15}NO$: C, 83.49; H, 5.53; N, 5.12. Found: C, 83.20; H, 5.74; N, 5.09.

Preparation of 2-(2-naphthyl)-2-pentyloxyethanenitrile ***4***: The target compound was prepared as described above with a yield of 69.3% as a golden yellow oil. TLC R_f 0.76 (hexane:EtOAc = 3.5:1, v/v). ^{1}H NMR ($CDCl_3$): δ 0.80–0.96 (m, 3H), 1.16–1.42 (m, 4H), 1.55–1.74 (m, 2H), 3.40–3.80 (m, 2H), 5.42 (s, 1H), 7.42–7.62 (m, 3H), 7.74–8.00 (m, 4H); ^{13}C NMR ($CDCl_3$): δ 14.4 (CH_3), 22.8, 28.5, 29.3, 70.5, 71.3 (CCN), 117.7 (CN), 124.4, 126.9, 127.0, 127.5, 128.0, 128.1, 128.5, 129.3, 130.0, 133.2; MS (EI, 70 eV): m/z % 253 [M^+, 28], 166 [$(M-OC_5H_{11})^+$, 100], 139 [$(M-OC_5H_{11}-HCN)^+$, 100]. Anal. calcd for $C_{17}H_{19}NO$: C, 80.60; H, 7.56; N, 5.53. Found: C, 80.21; H, 7.43; N, 5.58.