An Improved Fluorogenic NAD(P)$^+$ Detection Method Using 2-Acetylbenzofuran: Its Origin and Application

Supporting Information

Takashi Moriya, Ayano Kawamata, Yusuke Takahashi, Yoshiharu Iwabuchi, Naoki Kanoh

Graduate School of Pharmaceutical Sciences, Tohoku University
6-3 Aza-aoba, Aramaki, Aoba-ku, Sendai 980-8578 (Japan)

E-mail: nkanoh@m.tohoku.ac.jp

Table of Contents

1. General……..…1

2. Screening of ketones……..…...2

3. Synthesis of compounds……..…….…….3

4. Comparing the reactivity of ketones……..……...6

5. Comparison of NAD(P)$^+$ detection methods...…….……6

6. Comparison of NAD(P)$^+$ detection limit and linearity…...…….…….……..7

7. Protein purification and expression…….……..8

8. Detection of substrate for Cytochrome P450……...……..8

9. References……...……...9

Experimental Section

1. General

All reactions were carried out under an argon atmosphere with dehydrated solvents under anhydrous conditions, unless otherwise noted. Dehydrated THF and CH_2Cl_2 were purchased from Kanto Chemical Co., Inc. Other solvents were dehydrated and distilled according to standard protocols. Reagents were obtained from commercial suppliers and used without further purification, unless otherwise noted. Reactions were monitored by thin-layer chromatography (TLC) carried out on silica gel plates (Merck Kieselgel 60 F_{254}) or NH_2-coated silica gel plates (Fuji Silysia Chemical Co., Ltd.; TLC Plates NH). Column chromatography was performed on Silica gel 60N (Kanto Chemical Co., Inc.; spherical, neutral, 63-210 μm) or Chromatorex NH (Fuji Silysia Chemical Co., Ltd.; NH_2-coated silica gel, 100-200 mesh). All melting points were determined with Yazawa Micro Melting Point BY-2 and are uncorrected. IR spectra were recorded on a JASCO *FT/IR*-410 Fourier Transform Infrared Spectrophotometer. ^{1}H-NMR (400, and 600 MHz) and ^{13}C-NMR spectra (100, and 150 MHz) were recorded on JEOL JNM-AL-400 and JEOL JNM-ECA-600 spectrometers, respectively. For ^{1}H-NMR spectra, chemical shifts (δ) are given from TMS (0.00 ppm) or $CHCl_3$ (7.26 ppm) in $CDCl_3$, and CHD_2OD (3.30 ppm) in CD_3OD as internal standards. For ^{13}C-NMR spectra, chemical shifts (δ) are given from $CDCl_3$ (77.0 ppm) or CD_3OD (49.0 ppm) as internal standards. The following abbreviations were used to explain the multiplicities: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, sept = septet, br = broad. Mass spectra were recorded on JEOL JMS-DX303, JEOL JNM-AL500, JEOL JMS-700, and Thermo Scientific Exactive Mass Spectrometers. UV-Vis spectra were recorded on a JASCO V-630BIO UV-Vis Spectrometer or BioTek Synergy Mx Monochromator-Based Multi-Mode Microplate Reader. Fluorescent spectra were recorded on a BioTek Synergy Mx Monochromator-Based Multi-Mode Microplate Reader.

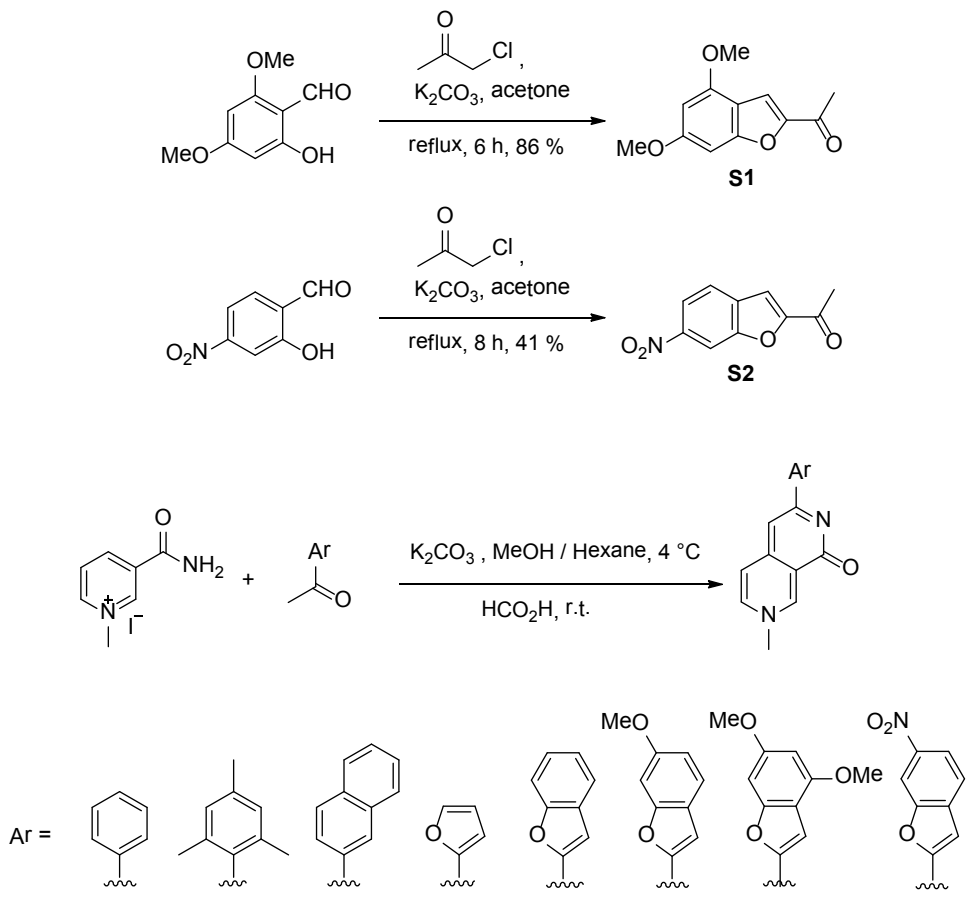
2. Screening of ketones

N-methylnicotinamide (50 μM in H_2O, 50 μL each) was dispensed in the wells of 96-well Nunc round-bottomed fluorescence plates. Then a solution of enolizable ketone in EtOH (25 mM, 20 μL) and aqueous KOH (0.5 M, 20 μL) were added to each well. After incubating at 4 °C for 20 min, 90% formic acid (90 μL) was added to each well, and the mixtures were incubated at RT for 20 min. The fluorescence intensity of each well was recorded at the optimum excitation and emission wavelengths.

Table S1. List of ketones used for screening

2-Acetylbenzofuran	*p*-Acetylphenylboronic acid
Acetylpyrazine	3-Acetylbenzofuran
3',4'-Difluoroacetophenone	Acetoin
2-Indanone	1,1,1-Trifluoroacetone
α-Bromoacetophenone	*m*-Hydroxyacetophenone
2',5'-Difluoroacetophenone	1,1-Diphenylacetone
1-Indanone	1-Phenyl-1,2-propanedione
Acetophenone	2',4',6'-Trimethylacetophenone
2-Acetyl-3-bromothiophene	*N*-Methyl-2-pyrrolidone
2-Acetylfuran	*N*-Vinyl-2-pyrrolidone
α-Tetralone	Acetylacetone
4-Acetylpyridine	1,3-Indandione
3-Acetylthiophene	β-Ionone
3-Acetylpyridine	α-Ionone
3-Acetyl-2,5-dichlorothiophene	2-Acetylpyrrole
5-Chloro-2-pentanone	3-Acetylindole
3',4'-Dimethoxyacetophenone	Phenoxy-2-propanone
4'-Methoxyacetophenone	2-Acetylboronic acid
p-Aminoacetophenone	2-Acetyl-5-methylfuran
5-Methoxy-1-tetralone	2-Thenoyltrifluoroacetone
2-Acetyl-5-bromothiophene	3-Acetylcoumarin
2-Acetylthiophene	2-Acetylfluorene
5-Chloro-2-hydroxyacetophenone	2-Acetylnaphthalene
β-Tetralone	2-Acetyl-6-methoxybenzofuran
2',5'-Dihydroxyacetophenone	2-Acetyl-4,6-dimethoxybenzofuran
2-(Phenylsulfonyl)acetone	2-Acetyl-6-hydroxybenzofuran
6-Methoxy-1-tetralone	2-Acetyl-4-methoxy-6-hydroxybenzofuran
2-Ethoxy-2'-phenylacetophenone	2-Acetylindole
Naringenin	2-Acetylbenzoxazole
2',6'-Difluoroacetophenone	2-Acetylbenzothiazole
3'-Nitroacetophenone	Acetylferrocene
o-Hydroxyacetophenone	

3. Synthesis of compounds



Scheme S1. Synthesis of 2-acetylbenzofuran derivatives and 2,7-naphthyridin-1(*7H*)-one derivatives

2-Hydroxy-4,6-dimethoxybenzaldehyde,[1] 2-hydroxy-4-nitrobenzaldehyde,[2] 2-acetylbenzofuran,[3] 2-acetyl-4-methoxybenzofuran,[3] and *N*-methylnicotinamide iodide[4] were synthesized according to the reported procedures.

2-Acetyl-4,6-dimethoxybenzofuran (S1)

To a solution of K_2CO_3 (910 mg, 6.59 mmol) in acetone (14 mL) was added 2-hydroxy-4,6-dimethoxybenzaldehyde (1.00 g, 5.49 mmol). Then, chloroacetone (0.51 mL, 6.31 mmol) was added dropwise at RT. The solution was stirred at reflux for 6 h. The resulting solution was allowed to cool to RT and concentrated under reduced pressure. The residue was purified by silica gel column chromatography (AcOEt / hexane = 1 / 2) to afford 2-acetyl-4,6-dimethoxybenzofuran (1.12 g, 5.08 mmol, 92%) as white solid.

2-Acetyl-4,6-dimethoxybenzofuran (**S1**): mp. 115 °C; IR (CH_2Cl_2, cm^{-1}) 1657; ^{1}H-NMR (400 MHz, $CDCl_3$) δ 7.53 (s, 1H), 6.64 (s, 1H), 6.32 (d, *J* = 1.9 Hz, 1H), 3.92 (s, 3H), 3.86 (s, 3H), 2.54 (s, 3H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ 187.2, 162.6, 158.0, 155.2, 151.0, 112.04, 112.01, 95.2, 87.9, 55.8, 55.6, 25.9; HRMS (EI) calcd. for $C_{12}H_{12}O_4$ $[M]^+$ 220.0736, found 220.0728.

2-Acetyl-6-nitrobenzofuran (S2)

To a solution of K_2CO_3 (66.2 mg, 0.479 mmol) in acetone (2 mL) was added 2-hydroxy-4-nitrobenzaldehyde (53.3 mg, 0.319 mmol). Then, chloroacetone (38.5 μL, 0.479 mmol) was added dropwise at RT. The solution was stirred at reflux for 10 h. The resulting solution was allowed to cool to RT and concentrated under reduced pressure. The residue was purified by silica gel column chromatography (AcOEt / hexane = 1 / 2) to afford 2-acetyl-6-nitrobenzofuran (26.8 mg, 0.131 mmol, 41%) as a brown solid.

2-Acetyl-6-nitrobenzofuran (**S2**): IR ($CHCl_3$, cm^{-1}) 1679, 1514; ^{1}H-NMR (400 MHz, $CDCl_3$) δ 8.50 (s, 1H), 8.24 (dd, *J* = 8.7, 1.9 Hz, 1H), 7.86 (d, *J* = 8.7 Hz, 1H), 7.56 (d, *J* = 1.5 Hz, 1H), 2.67 (s, 3H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ 188.4, 156.5, 154.1, 147.5, 132.5, 119.3, 111.4, 108.9, 26.7; HRMS (EI) calcd. for $C_{10}H_7NO_4$ $[M]^+$ 205.0375, found 205.0372.

3-Aryl-7-methyl-2,7-naphthyridin-1(*7H*)-one derivatives: General Procedure

To a solution of *N*-methylnicotinamide iodide (50.0 mg, 0.189 mmol) and methyl/cyclic ketone (5 equiv) in H_2O/MeOH (1:4) (1.25 mL) was added K_2CO_3 (4 equiv). The resulting solution was stirred at 4 °C for 1 h under an O_2 atmosphere. Then, formic acid (1.5 mL) was added, and the solution was stirred at RT for 10 min. The mixture was extracted with hexane. The aqueous layer was supplemented with to 6N aq. KOH, and extracted 5 times with $CHCl_3$. The combined organic extracts were dried over sodium sulfate, filtered, and concentrated under reduced pressure. The residue was purified by silica gel column chromatography over NH_2-coated silica gel (MeOH / $CHCl_3$ = 1 / 20 to 1 / 10) to afford 3-aryl-2,7-naphthyridin-1(*7H*)-one derivative.

7-Methyl-3-phenyl-2,7-naphthyridin-1(*7H*)-one (**2a**): 40% yield; IR (neat, cm^{-1}) 1646; ^{1}H-NMR (600 MHz, CD_3OD) δ 9.07 (s, 1H), 8.05 (d, *J* = 7.2 Hz, 1H), 8.01 – 8.00 (m, 2 H), 7.71 (d, *J* = 6.6 Hz, 1H), 7.43 – 7.40 (m, 3H), 7.10 (s, 1H), 4.17 (s, 3H); ^{13}C-NMR (150 MHz, CD_3OD) δ 172.3, 166.5, 148.1, 147.3, 140.6, 139.2, 133.2, 129.6, 129.0, 123.5, 118.7, 101.2, 47.1; HRMS (FAB) calcd. for $C_{15}H_{13}N_2O$ $[M+H]^+$ 237.1028, found 237.1051.

7-Methyl-3-(2'-Naphthyl)-2,7-naphthyridin-1(*7H*)-one (**2c**): 25% yield; IR (neat, cm^{-1}) 1649; ^{1}H-NMR (600 MHz, CD_3OD) δ 9.07 (s, 1H), 8.57 (s, 1H), 8.14 (dd, *J* = 8.7, 1.0 Hz, 1H), 8.06 (d, *J* = 6.6 Hz, 1H),

7.98 – 7.97 (m, 1H), 7.93 (d, *J* = 8.4 Hz, 1H), 7.89 – 7.88 (m, 1H), 7.74 (d, *J* = 6.6 Hz, 1H), 7.55 - 7.53 (m, 2H), 7.26 (s, 1H), 4.17 (s, 3H); ^{13}C-NMR (150 MHz, CD_3OD) δ 172.0, 165.8, 147.9, 147.2, 139.3, 137.6, 135.7, 134.7, 130.1, 129.2, 129.0, 128.7, 128.2, 127.5, 126.0, 123.5, 118.8, 101.6, 47.0; HRMS (ESI) calcd. for $C_{19}H_{15}N_2O$ $[M+H]^+$ 287.1179, found 287.1169.

3-(2'-Furanyl)-7-methyl-2,7-naphthyridin-1(*7H*)-one (**2d**): 12% yield; IR (neat, cm^{-1}) 1649; ^{1}H-NMR (400 MHz, CD_3OD) δ 9.12 (s, 1H), 8.18 (d, *J* = 6.8 Hz, 1H), 7.77 – 7.74 (m, 2H), 7.34 (d, *J* = 3.4 Hz, 1H), 7.10 (s, 1H), 6.64 (m, 1H), 4.23 (s, 3H); ^{13}C-NMR (100 MHz, CD_3OD) δ 171.1, 155.5, 154.0, 147.7, 147.6, 146.7, 139.9, 123.4, 119.5, 114.0, 113.6, 98.7, 47.1; HRMS (FAB) calcd. for $C_{13}H_{11}N_2O_2$ $[M+H]^+$ 227.0821, found 227.0823.

3-(2'-Benzofuranyl)-7-methyl-2,7-naphthyridin-1(*7H*)-one (**2e**): 40% yield; IR (neat, cm^{-1}) 1648; ^{1}H-NMR (400 MHz, CD_3OD) δ 8.94 (s, 1H), 7.99 (d, *J* = 6.8 Hz, 1H), 7.64 (d, *J* = 6.8 Hz, 1 H), 7.57 (d, *J* = 8.0 Hz, 1H), 7.53 (s, 1H), 7.44 (d, *J* = 8.4 Hz, 1H), 7.29 (t, *J* = 7.8 Hz, 1H), 7.18 (t, *J* = 7.6 Hz, 1H), 7.15 (s, 1H), 4.08 (s, 3H); ^{13}C-NMR (100 MHz, CD_3OD) δ 188.3, 178.4, 172.7, 150.1, 147.8, 139.7, 130.0, 127.3, 124.5, 123.6, 123.2, 112.4, 109.4, 100.2, 90.8, 88.2, 47.1; HRMS (FAB) calcd. for $C_{17}H_{13}N_2O_2$ $[M+H]^+$ 277.0977, found 277.0983.

3-(6'-Methoxy-2'-benzofuranyl)-7-methyl-2,7-naphthyridin-1(*7H*)-one (**2f**): 26% yield; IR (neat, cm^{-1}) 1647; ^{1}H-NMR (400 MHz, CD_3OD) δ 9.02 (s, 1H), 8.09 (d, *J* = 6.0 Hz, 1H), 7.70 (d, *J* = 6.4 Hz, 1 H), 7.57 (s, 1H), 7.52 (d, *J* = 8.8 Hz, 1H), 7.14 (s, 1H), 7.09 (s, 1H), 6.91 (dd, *J* = 8.8, 2.0 Hz, 1H), 4.26 (s, 3H), 3.89 (s, 3H); ^{13}C-NMR (100 MHz, CD_3OD) δ 177.7, 158.4, 148.9, 147.2, 137.1, 123.7, 123.0, 114.5, 110.5, 109.9, 96.4, 90.8, 56.3, 30.8; HRMS (FAB) calcd. for $C_{18}H_{15}N_2O_3$ $[M+H]^+$ 307.1083, found 307.1093.

3-(4',6'-Dimethoxy-2'-benzofuranyl)-7-methyl-2,7-naphthyridin-1(*7H*)-one (**2g**): 12% yield; IR (neat, cm^{-1}) 1685; ^{1}H-NMR (400 MHz, CD_3OD) δ 9.19 (s, 1H), 8.26 (d, *J* = 7.1 Hz, 1H), 7.84 (d, *J* = 6.8 Hz, 1 H), 7.73 (s, 1H), 7.25 (s, 1H), 6.78 (s, 1H), 6.42 (s, 1H), 4.26 (s, 3H), 3.93 (s, 3H), 3.85 (s, 3H); ^{13}C-NMR (100 MHz, CD_3OD) δ 182.4, 166.0, 163.1, 159.2, 155.9, 147.5, 128.1, 123.7, 122.4, 114.2, 108.3, 98.9, 89.0, 56.4, 38.8, 30.7; HRMS (FAB) calcd. for $C_{19}H_{17}N_2O_4$ $[M+H]^+$ 337.1188, found 337.1197.

7-Methyl-3-(6'-nitro-2'-benzofuranyl)-2,7-naphthyridin-1(*7H*)-one (**2h**): 15% yield; IR (neat, cm^{-1}) 1679, 1513; ^{1}H-NMR (600 MHz, DMSO) δ 9.12 (s, 1H), 8.56 (s, 1H), 8.22 (d, *J* = 6.0 Hz, 1H), 8.19 (d, *J* = 7.8 Hz, 1H), 7.98 (d, *J* = 8.4 Hz, 1H), 7.82 (d, *J* = 7.2 Hz, 1H), 7.70 (s, 1H), 7.12 (s, 1H), 4.16 (s, 1H); ^{13}C-NMR (150 MHz, DMSO) δ 169.4, 161.4, 154.6, 153.6, 146.7, 146.0, 145.2, 138.8, 134.7, 122.7, 121.7, 119.1, 118.8, 107.9, 106.9, 96.6, 48.8; HRMS (ESI) calcd. for $C_{17}H_{12}N_3O_4$ $[M+H]^+$ 322.0822, found 322.0815.

4. Comparing the reactivity of ketones

To 96 wells of a Nunc 96-well round-bottomed fluorescence plate containing NAD^+ (50 μM each) solution in potassium phosphate buffer (pH 7.4, 50 μL) was added a solution of methyl ketone (acetophenone or 2-acetylbenzofuran) in EtOH (25 mM, 20 μL), and 0.5 M aqueous KOH (0.5 M, 20 μL) was added to each well. After incubating at 4 °C for various amounts of time, 90% formic acid (90 μL) was added to each well, and the plate was incubated at RT for 20 min. The fluorescent intensity of each well was read on a microplate reader with excitation/emission wavelengths set at 372/444 nm or 421/480 nm, respectively. All experiments were performed in triplicate. The amount of fluorescent product generated in each well was calculated using a standard curve of **2a** or **2d**.

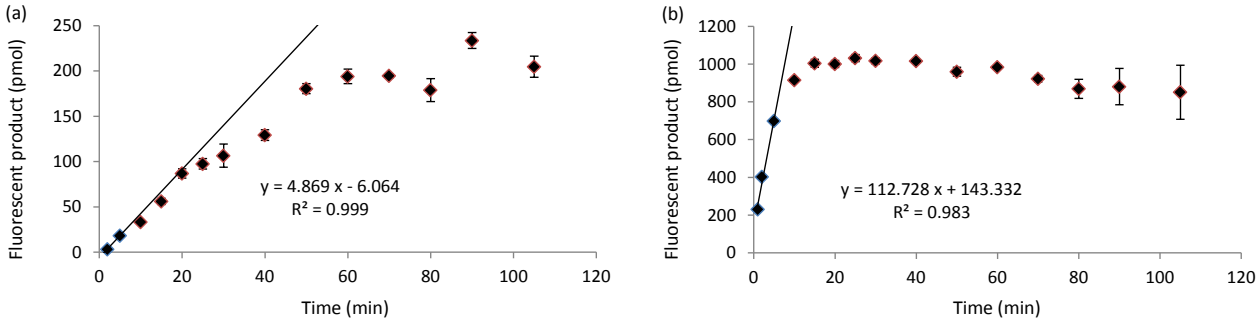


Figure S1. Time-dependent change in the amount of fluorescent product during the reaction of NAD^+ with (a) acetophenone or (b) 2-acetylbenzofuran.

5. Comparison of $NAD(P)^+$ detection methods

Detection of NADH and NAD^+ by NADH absorbance

The wells of a Nunc 96-well round-bottomed fluorescence plate containing an NADH or NAD^+ (50 μM each) solution in H_2O (50 μL) were read on a microplate reader with the absorbance wavelength set at 340 nm.. All experiments were performed in triplicate.

Detection of NADH and NAD^+ by NADH fluorescence

The wells of a Nunc 96-well round-bottomed fluorescence plate containing an NADH or NAD^+ (50 μM each) solution in H_2O (50 μL) were read on a microplate reader with the excitation and emission wavelengths set at 340 nm and 440 nm, respectively. All experiments were performed in triplicate.

Detection of NADH and NAD^+ by the alkali method

To 96 wells of a Nunc 96-well round-bottomed fluorescence plate containing an NADH or NAD^+ (50 μM each) solution in H_2O (50 μL) was added 7 M aqueous NaOH (130 μL), and the plate was incubated at RT. After 1 h, the fluorescent intensity of each well was read on a microplate reader with excitation and emission

wavelengths set at 360 nm and 453 nm, respectively. All experiments were performed in triplicate.

Detection of NADH and NAD^+ by methyl ketone (acetophenone or 2-acetylbenzofuran)

To 96 wells of a Nunc 96-well round-bottomed fluorescence plate containing an NADH or NAD^+ (50 μM each) solution in H_2O (50 μL) was added a solution of methyl ketone (acetophenone or 2-acetylbenzofuran) in EtOH (25 mM, 20 μL), and then 0.5 M aqueous KOH (0.5 M, 20 μL) was added to each well. After incubating at 4 °C for 20 min, 90% formic acid (90 μL) was added to each well, and the plate was incubated at RT for 20 min. The fluorescent intensity of each well was read on a microplate reader with excitation/emission wavelengths set at 372/444 nm, or 421/480 nm, respectively. All experiments were performed in triplicate.

6. Comparison of $NAD(P)^+$ detection limit and the linearity

The relative fluorescence intensities obtained from the two methods were plotted as a function of the amount of NAD^+.

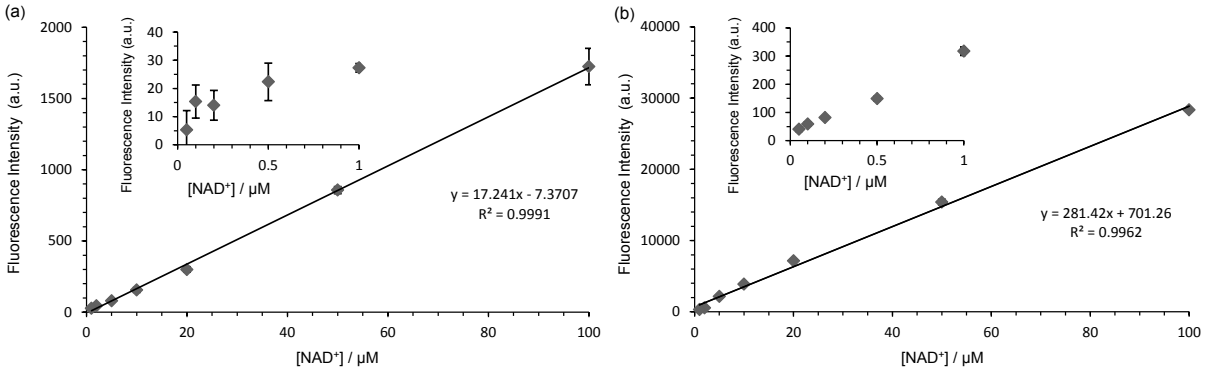


Fig. S2 Various amounts of NAD^+ were detected by use of (a) acetophenone or (b) 2-acetylbenzofuran. Reagents and conditions: NAD^+ (various amounts), ketone (5.6 mM), aq. KOH (111 mM), H_2O/EtOH (1:4, 90 μL), 4°C, 20 min; then 90% HCO_2H (90 μL), RT, 20 min. Relative fluorescence intensities of the resulting reaction mixtures were measured at the optimum excitation- and emission wavelengths, respectively.

7. Protein purification and expression

7-1. P450cam, Pdx, PdR

P450cam, Pdx, and PdR were expressed and purified according to our previous report. [5]

7-2. P450BM3

P450BM3 was expressed and purified with reference to a previously reported method. [6]

For the expression of recombinant P450BM3, *E. coli* BL21(DE3)pLysS cells containing the construct were grown in TB medium containing ampicillin (100 mg/L) with shaking at 37 °C until the OD_{600} was approx. 0.2. The cells were induced with IPTG (0.5 mM), and then 5-aminolevulinic acid (100 mg/L) was added and the solution was shaken at 23 °C for 6 h. The cells were collected by centrifugation, re-suspended in 20 mM potassium phosphate buffer (pH 7.4), and subjected to lysis by lysozyme (5 mg/mL) at 4 °C for 1 h. The lysate was sonicated and centrifuged at 12,000 rpm for 20 min at 4 °C. The supernatant was loaded on a DE-52 column (Whatman), which had been equilibrated with 20 mM Tris-HCl buffer (pH 7.4). The protein was eluted with a linear gradient of KCl (0.05 – 0.4 M in the equilibration buffer). The red fraction was concentrated by using an Amicon Ultra centrifugal filter 10K device (Millipore). The concentrated sample was loaded on a P-100 Gel column (Bio-Rad). The protein was eluted with 20 mM Tris-HCl buffer (pH 7.4). The red fractions containing P450BM3 enzyme were concentrated by using an Amicon Ultra centrifugal filter 10K device. The protein concentration was determined by a CO difference spectrum.[7]

8. Detection of substrate for Cytochrome P450

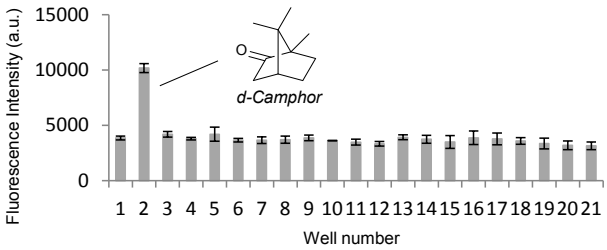


Fig. S3 Detection of substrate for purified P450cam. Reagents and conditions: P450cam (0.25 μM), Pdx (1.0 μM), PdR (0.25 μM), test compound (20 μM), and NADPH (50 μM) were incubated in potassium phosphate buffer, pH 7.4, RT, 4 min; then accumulated NAD^+ was detected by the 2-acetylbenzofuran method.

Table S2. List of compounds used for P450 substrate candidates

Well number	Compounds	Well number	Compounds
1	Dimethyl sulfoxide	11	Cyclohexane
2	*d*-Camphor	12	Decaline
3	Adamantane	13	*p*-Methylanisole
4	Myristic acid	14	Benzophenone
5	Laulic acid	15	Menthol
6	Testosterone	16	Colesterol
7	Oleanolic acid	17	Dimethylaminohexanol
8	7-Ethoxycoumarin	18	Caffeine
9	Naringenin	19	Cyclosporin A
10	Hexane	20	Imidazole
		21	2-Aminoadamantane

9. References

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