Supporting Information

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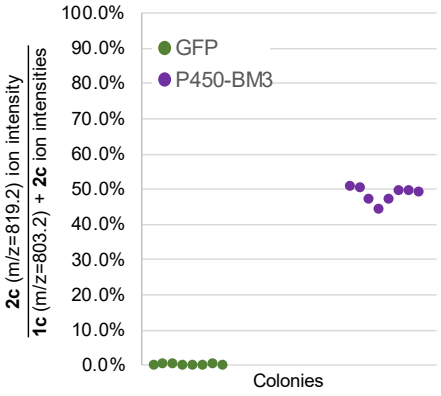
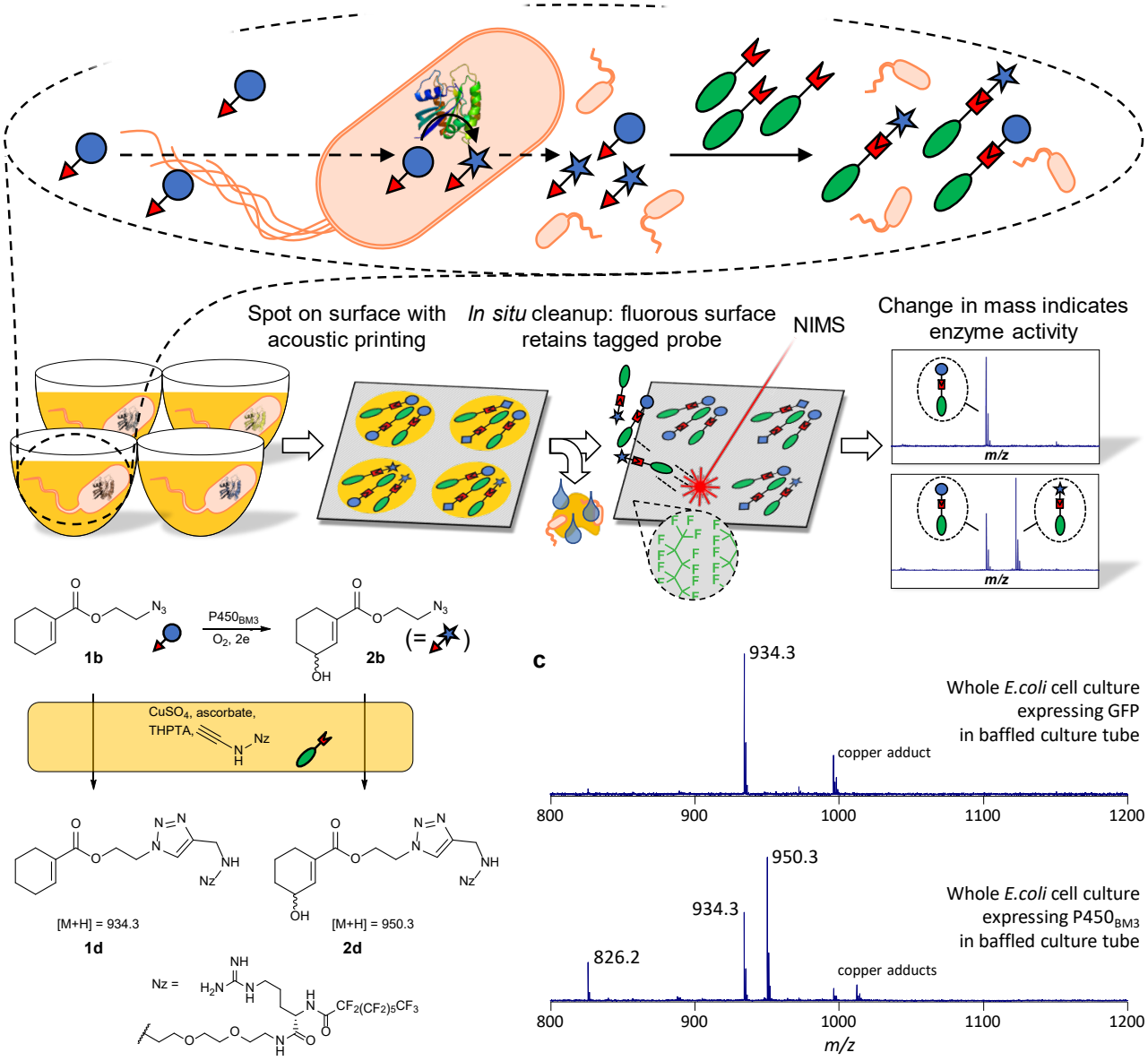
1 SUPPLEMENTARY FIGURES

1.1 Figure S1



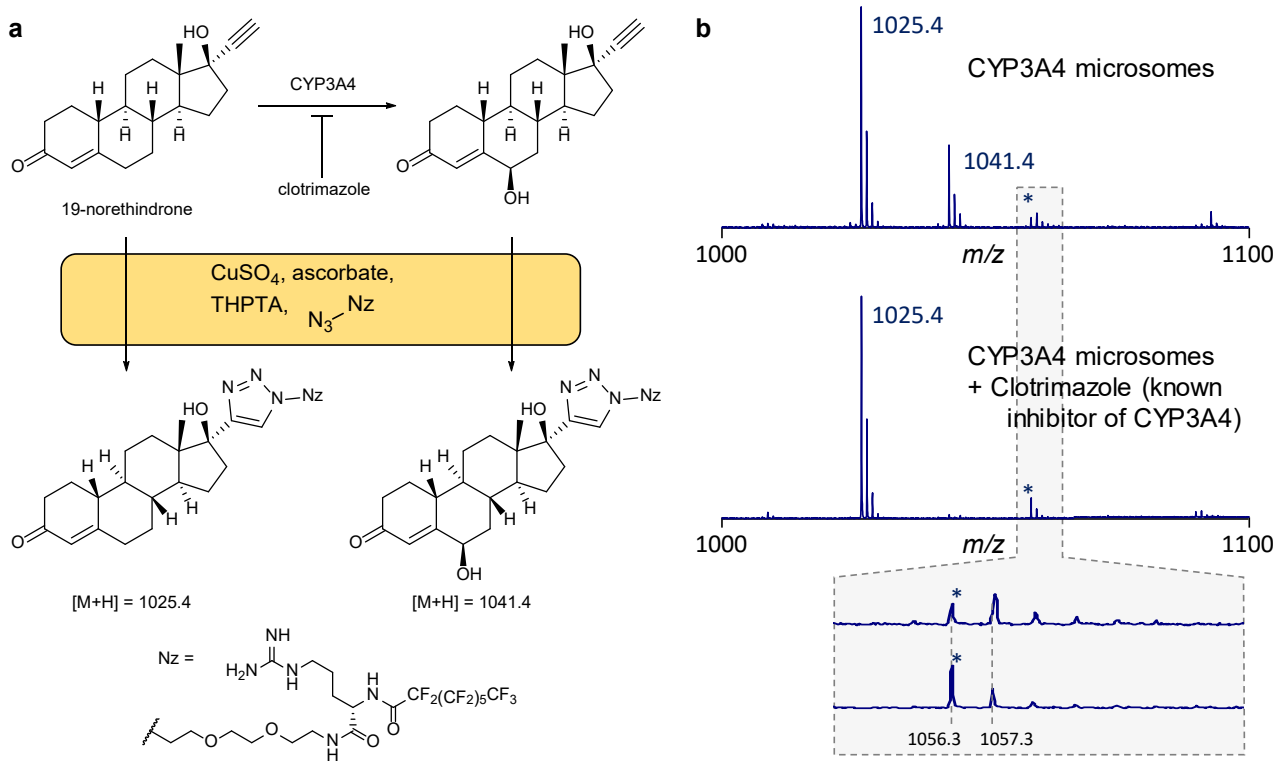
Measurement of $P450_{BM3}$ activity in cell lysate, in 96-deepwell format, using PECAN. 8 colonies expressing GFP (negative control) and 8 colonies expressing $P450_{BM3}$ were grown up, lysed, and fed **1b**. Data presented as percent turnover as calculated from the spots' 803 and 819 ion intensities, summed over +/- 0.5 Da. Data points represent individual colonies. While the turnover in the 96-well plate is not as good as it is in glass vials (~60% conversion vs ~95% in **Figure 2d**; we suspect this may be due to mixing efficiency, oxygen availability, or evaporation), it is sufficiently reproducible to be able to discern the active wells with high likelihood.

1.2 Figure S2



Detection of $P450_{BM3}$ activity in whole *E. coli* cells fed **1b**, using PECAN. a) Overview of the technology. b) The enzymatic reaction being studied here is the same as in Fig. 2, however, for historical reasons, a different PECAN tag was employed in this experiment. c) The resulting mass spectra after a 24-hour bioconversion followed by tagging, spotting and NIMS as usual. The masses are different from those seen in Fig. 2 because of the different tag used. We also observe a mass (m/z=826.2) corresponding to what is likely tagged 2-azidoethanol, the ester hydrolysis product of either **1b** or **2b**. *E. coli* likely harbors a native esterase enzyme that catalyzes the formation of this molecule. Given that the intensity of this ion is much lower in the GFP control than in the strain expressing $P450_{BM3}$, this esterase likely prefers **2b** as a substrate.

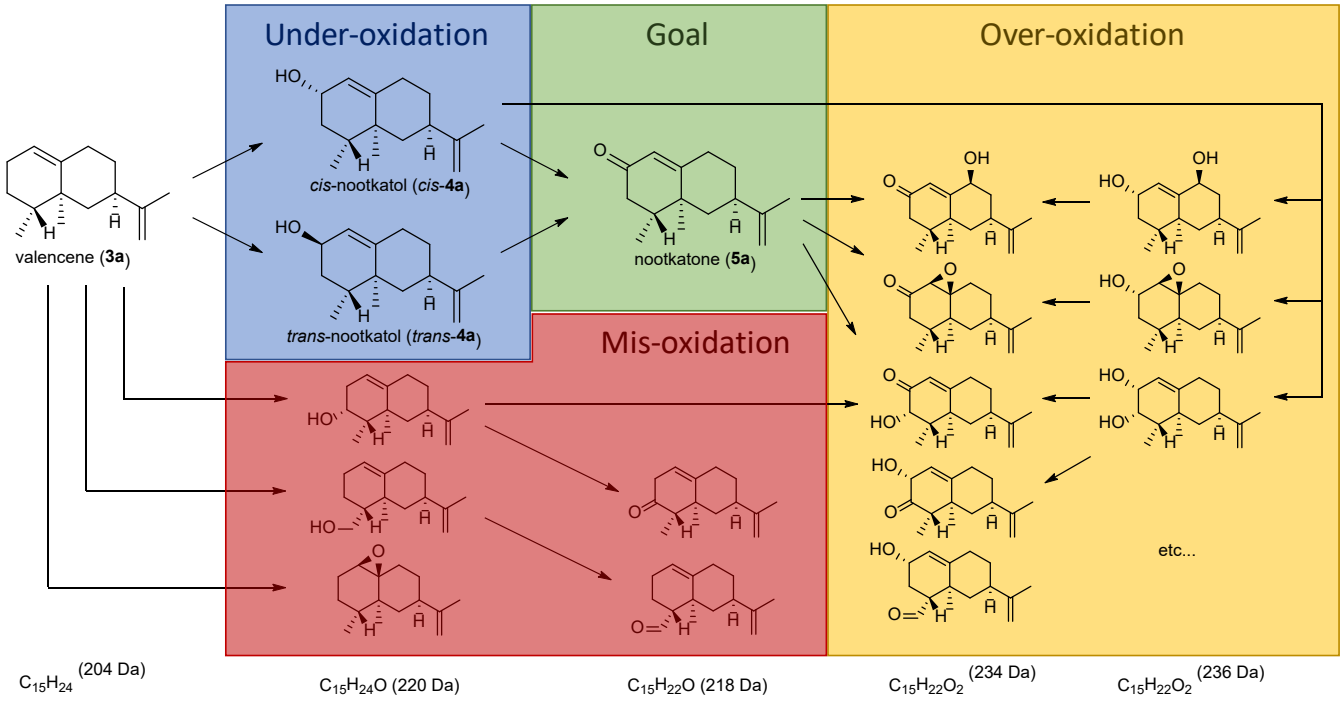
1.3 Figure S3



a) To test whether PECAN is applicable to the measurement of P450 activity in microsomes, a pertinent complex biochemical matrix distinct from cell lysate, we subjected 19-norethindrone – a widely-prescribed hormonal contraceptive harboring a "clickable" alkyne moiety – to the human microsomal P450 CYP3A4. This requires a reversal of the polarity of the tagging "click" reaction compared to **1b**, which contains an azide. This polarity reversal did not noticeably affect the efficiency of the tagging reaction.

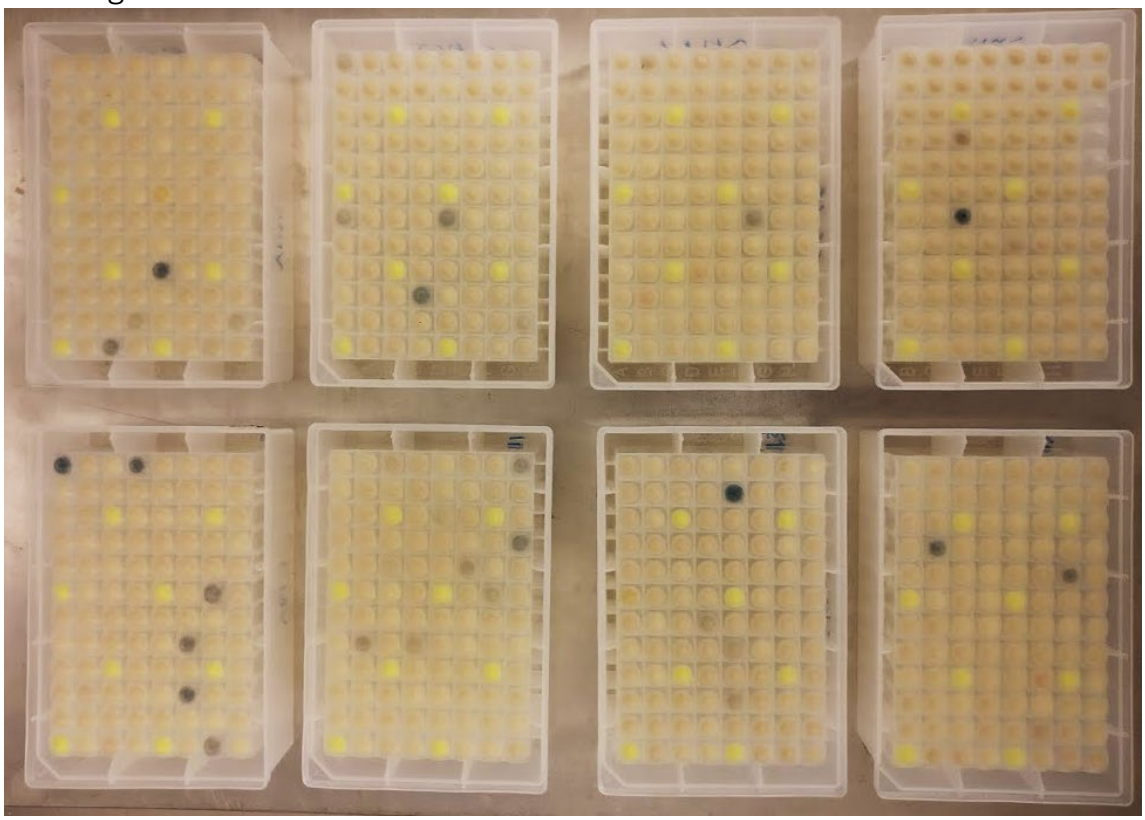
b) In the presence of CYP3A4 microsomes, oxidation products of 19-norethindrone could be detected. CYP3A4 oxidation activity could be abolished in the presence of the known CYP3A4 inhibitor clotrimazole, demonstrating that PECAN may be suitable for testing drug-drug interactions as part of a drug discovery program. The peak marked with an asterisk (*m/z* = 1056.3, see inset bottom-right) is probably an impurity. In the top spectrum (but not in the inhibited reaction), *m/z* = 1057.3 is in fact more intense than *m/z* = 1056.3, which suggests the presence of a twice-oxidized product (1057.3 ≈ 1025.4+16×2).

1.4 Figure S4



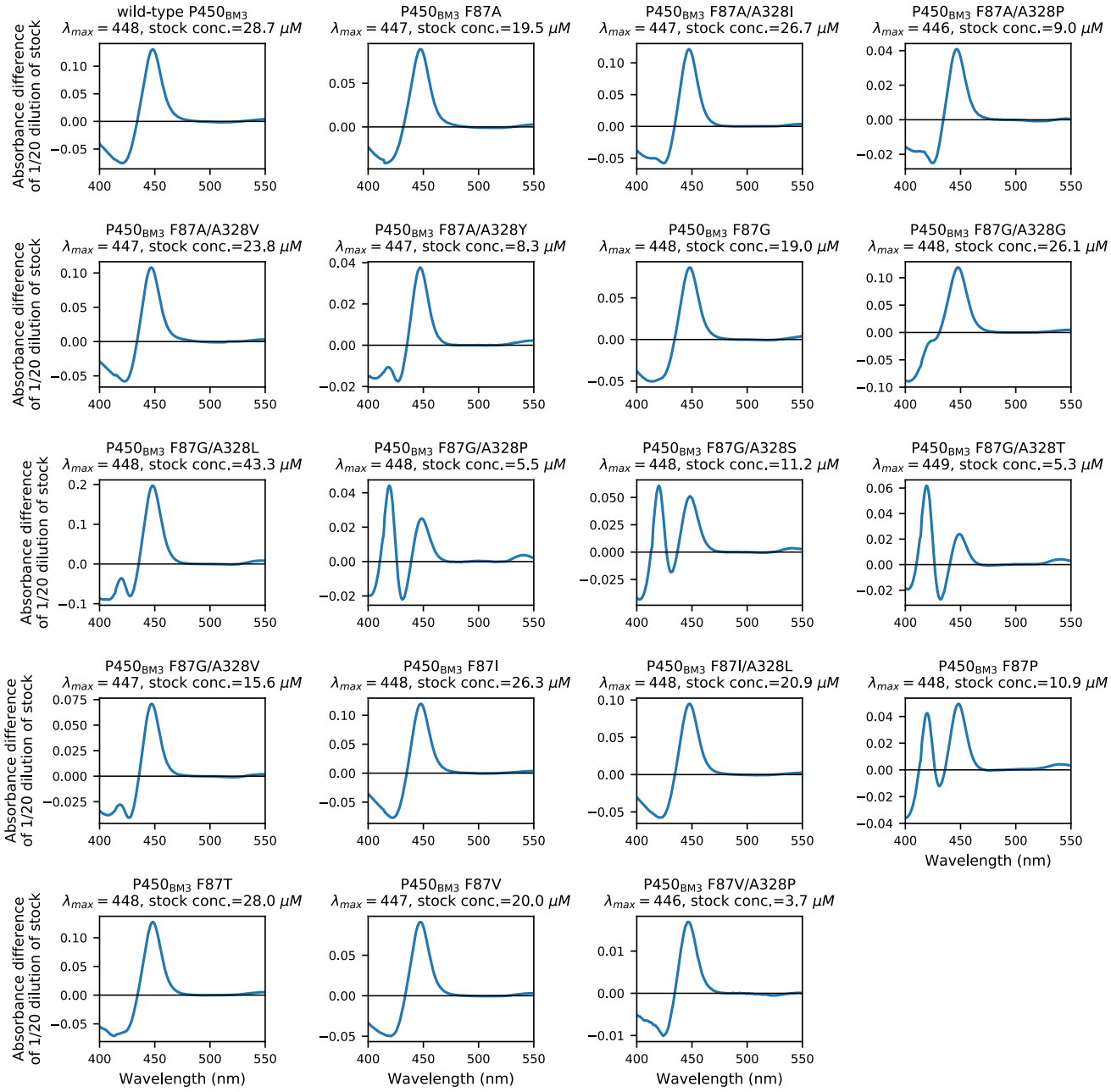
P450-catalyzed oxidation of valencene to produce nootkatols (**4a**), nootkatone (**5a**), and other oxidized isoprenoids. Compounds in the same column have the same elemental composition and nominal mass, as annotated. Mis-oxidized and over-oxidized structures are drawn for sake of illustration; Most of these molecules have not been identified before, though they may reasonably be expected to form.

1.5 Figure S5



Color variation among pellets of a subset of the $P450_{BM3}$ combinatorial 2-codon NNK site saturation library after overnight induction. Image has been flipped, such that despite looking at the bottom of the wellplates, well A1 is on the bottom-left. Color differences due to indole oxidation are visible, as well as the 8 GFP negative controls arrayed in each wellplate. The color variability suggests a variety of mutants were represented in the library.

1.6 Figure S6



CO-difference spectra recoded for the purified $P450_{BM3}$ variants.

2 SUPPLEMENTARY TABLE

2.1 Table S1

In vitro activities of various $P450_{BM3}$ variants isolated in the PECAN screen.

Enzyme variant	JBEI strain registry name	Number of hits in screen	Peak sizes in PECAN re-screening experiment (N = 3) relative to the sum of all peaks within the *m/z* range 869 – 885 (i.e., peaks that derive from the click reaction)…						Products of *in vitro* oxidation (N = 3) of 0.5 mM 3a (valencene) by 1 µM purified $P450_{BM3}$ variant (i.e., 0.2 mol% catalyst loading) and 10 mM NADPH equivalent worth of cofactor generation system, in 1 h, assayed using GC-MS, expressed as % of total GC-MS peak area. Unreacted 3a makes up the remainder. "% Other" columns correspond to the summed peak areas of multiple peaks which could not be definitively identified.						
			…as % (*m/z* = 869 (3c) makes up the remainder)			…as standard deviations over GFP control (serves to subtract out peaks that form spontaneously during click reaction)									
			m/z = 871 (4c)	*m/z* = 869 (5c)	*m/z* = 883 or 885 (over-oxidized)	*m/z* = 871 (4c)	*m/z* = 869 (5c)*	*m/z* = 883 or 885 (over-oxidized)	% cis-nootkatol (cis-4a)	% trans-nootkatol (trans-4a)	% nootkatone (5a)	% other, *m/z* = 220 (mis-oxidized)	% other, *m/z* = 218 (mis-oxidized)	% other, *m/z* = 236 (over-oxidized)	% other, *m/z* = 234 (over-oxidized)
wild-type $P450_{BM3}$	JBEI-17980	0	22.8 ±4.9	10.9 ±3.4	4.6 ±1.4	2.8 ±3.3	-0.2 ±1.5	-0.8 ±0.8	0.05 ±0.04	0.41 ±0.07	ND	0.15 ±0.01	ND	ND	ND
F87A/A238I	JBEI-17981	2	33.9 ±5.1	24.6 ±2.5	5.9 ±1.2	10.2 ±3.4	6.0 ±1.1	-0.0 ±0.7	5.7 ±0.6	1.82 ±0.06	13.7 ±0.2	0.38 ±0.01	0.3 ±0.2	0.6 ±0.1	1.2 ±0.1
F87G/A238V	JBEI-18009	6	25.8 ±1.1	63.2 ±1.3	5.1 ±0.2	4.8 ±0.7	23.6 ±0.6	-0.47 ±0.09	7.7 ±0.6	1.5 ±0.1	11.2 ±0.7	0.34 ±0.03	ND	3.4 ±0.2	3.3 ±0.2
F87P	JBEI-17985	2	30.0 ±2.0	46.5 ±1.3	7.7 ±0.2	7.6 ±1.4	16.0 ±0.6	1.0 ±0.1	7.6 ±0.2	ND	7.3 ±0.1	0.9 ±1.0	10.5 ±1.0	36.0 ±2.1	23.9 ±0.7
F87A	JBEI-17973	1	26.6 ±1.0	52.6 ±5.7	9.3 ±0.2	5.3 ±0.6	18.8 ±2.6	1.90 ±0.09	7.2 ±0.7	ND	5.8 ±0.7	0.39 ±0.06	14.5 ±2.2	34.9 ±3.3	13.9 ±0.4
F87I	JBEI-17988	1	15.3 ±0.5	72.8 ±3.7	3.5 ±0.3	-2.2 ±0.3	28.0 ±1.7	-1.4 ±0.2	5.9 ±0.3	1.6 ±0.2	4.8 ±0.1	2.7 ±0.3	12.1 ±1.3	23.7 ±3.8	19.5 ±2.3
F87G/A238L	JBEI-18004	2	35.0 ±1.6	23.1 ±4.0	4.9 ±0.7	10.9 ±1.1	5.3 ±1.8	-0.6 ±0.4	7.6 ±1.2	0.30 ±0.07	4.2 ±0.6	1.1 ±0.2	0.4 ±0.2	0.6 ±0.1	ND
F87A/A238V	JBEI-18006	1	36.4 ±2.5	29.9 ±6.9	4.7 ±0.7	11.9 ±1.7	8.4 ±3.2	-0.7 ±0.4	5.2 ±0.2	2.0 ±0.2	4.0 ±0.2	0.41 ±0.05	ND	ND	ND
F87G/A238S	JBEI-17995	1	34.8 ±2.9	22.6 ±3.1	5.7 ±0.9	10.8 ±2.0	5.1 ±1.4	-0.2 ±0.5	4.9 ±0.5	0.22 ±0.01	3.8 ±0.3	1.9 ±0.1	0.09 ±0.08	10.4 ±1.1	0.35 ±0.04
F87G/A238N	JBEI-17998	2	34.2 ±2.5	37.3 ±3.2	5.1 ±0.2	10.4 ±1.7	11.8 ±1.4	-0.5 ±0.1	3.9 ±0.7	0.37 ±0.05	3.3 ±0.5	1.2 ±0.2	0.03 ±0.05	2.2 ±0.2	ND
F87V	JBEI-17990	1	21.3 ±0.2	48.2 ±4.3	7.2 ±0.5	1.8 ±0.1	16.8 ±2.0	0.7 ±0.3	1.3 ±0.1	8.3 ±0.5	1.6 ±0.1	3.9 ±0.2	1.7 ±0.3	3.5 ±0.6	0.5 ±0.5
F87T	JBEI-17969	3	24.5 ±1.8	24.5 ±4.0	4.6 ±0.7	3.9 ±1.2	6.0 ±1.8	-0.8 ±0.4	1.3 ±0.1	0.9 ±0.1	1.0 ±0.1	0.39 ±0.04	0.10 ±0.01	0.19 ±0.03	0.07 ±0.06
F87G	JBEI-17975	1	25.0 ±2.2	42.4 ±2.1	24.4 ±1.2	4.3 ±1.5	14.2 ±1.0	10.4 ±0.7	3.68 ±0.07	0.1 ±0.1	0.95 ±0.07	7.0 ±0.2	2.0 ±0.3	31.4 ±0.2	0.6 ±0.1
F87A/A238Y	JBEI-17979	1	37.35 ±0.09	39.2 ±2.4	4.8 ±0.2	12.47 ±0.06	12.7 ±1.1	-0.66 ±0.09	3.7 ±0.8	0.39 ±0.09	0.38 ±0.07	0.03 ±0.05	ND	ND	ND
F87I/A238L	JBEI-17987	1	27.8 ±2.7	26.1 ±4.3	4.5 ±0.3	6.1 ±1.8	6.7 ±2.0	-0.8 ±0.2	3.3 ±0.6	0.15 ±0.06	0.21 ±0.03	0.37 ±0.08	0.05 ±0.08	0.07 ±0.06	ND
F87G/A238P	JBEI-17997	1	28.5 ±0.7	38.1 ±6.8	4.9 ±0.7	6.6 ±0.4	12.2 ±3.1	-0.6 ±0.4	1.3 ±0.5	0.10 ±0.04	0.13 ±0.07	0.12 ±0.02	ND	ND	ND
F87G/A238T	JBEI-17984	6	37.8 ±1.0	39.1 ±1.2	5.6 ±0.5	12.8 ±0.6	12.7 ±0.5	-0.2 ±0.3	1.1 ±0.3	0.06 ±0.01	0.11 ±0.04	0.10 ±0.02	ND	ND	ND
F87G/A238G	JBEI-17968	3	25.3 ±2.5	31.7 ±8.8	5.0 ±1.3	4.5 ±1.6	9.3 ±4.0	-0.6 ±0.7	ND	ND	ND	4.6 ±1.4	3.8 ±0.5	59.7 ±12.2	4.7 ±1.6
F87A/A238P	JBEI-17972	2	33.0 ±1.4	35.5 ±5.9	5.7 ±0.5	9.6 ±0.9	11.0 ±2.7	-0.2 ±0.3	1.3 ±0.2	0.13 ±0.03	ND	ND	ND	ND	ND
F87V/A238P	JBEI-17989	2	24.2 ±1.5	27.8 ±8.4	4.6 ±1.0	3.7 ±1.0	7.5 ±3.8	-0.8 ±0.6	ND	ND	ND	ND	ND	ND	ND
GFP (negative control)	JBEI-12358	0	18.6 ±1.5	11.4 ±2.2	6.0 ±1.8	0.0 ±1.0	0.0 ±1.0	0.0 ±1.0	ND	ND	ND	ND	ND	ND	ND

ND = Not Detected

*Note that in the initial screen, a "hit" was defined as having an *m/z* = 869 signal 10 or more standard deviations above the GFP control. After re-screening in triplicate, not all hits still show such high signal. The hits identified initially were likely statistical outliers and it is likely that if the statistical cutoff for calling a hit had been lowered, many more duplicates of the identified variants – and possibly other variants – would have been identified.

3 METHODS

3.1 Plasmids and strains

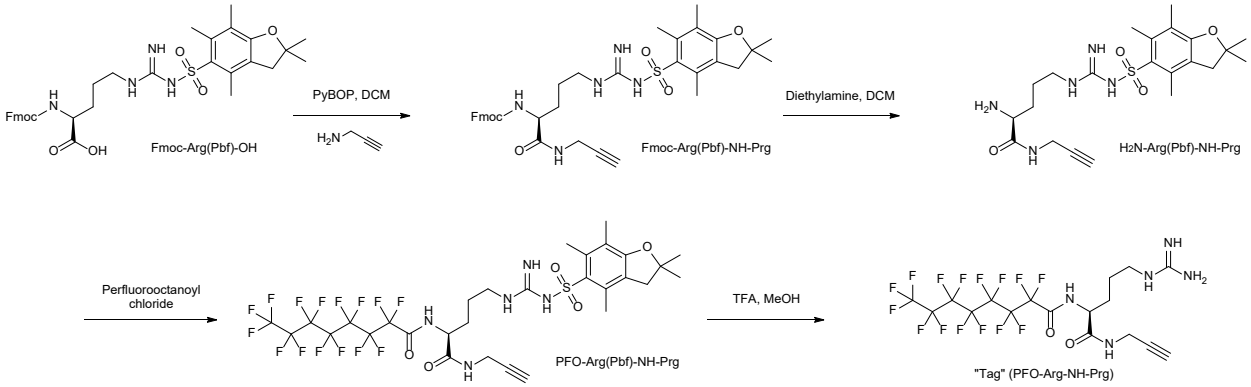
All plasmids described in this manuscript are deposited in the JBEI registry and can be requested via the following URL: https://public-registry.jbei.org/folders/418. Please refer to Table S1 for each plasmid's name in the registry.

pCW_{ori+}-$P450_{BM3}$ (JBEI-4004) was previously described by Dietrich *et al.*[1]. This plasmid was digested with NdeI and HindIII (Thermo Scientific) and a gene for Green Fluorescent Protein was ligated into the backbone to yield pCW_{ori+}-GFP (JBEI-12358).

pCW_{ori+}-$P450_{BM3}$ from Dietrich *et al.*[1] was also used as a starting point for the site-saturation library. Because of the duplicated trc promoter, homology-based cloning (e.g., Gibson assembly and Kunkel mutagenesis) was impractical. An N-terminal 6x-His tag, as well as silent mutations generating XbaI and XhoI restriction sites were introduced 5' and 3' to the DNA encoding F87 and A238 respectively by replacing part of the $P450_{BM3}$ gene by a codon-optimized gBlock (Integrated DNA Technologies) harboring the necessary changes. The resulting plasmid – still encoding wild-type $P450_{BM3}$ – was used as the parent for the subsequent site-saturation mutagenesis.

Mutagenic forward and reverse primers
[5'-acgaaTCTAGAtttgataaaaacctgtctcaggccctgaaattcgtgcgtgatttcgcaggtgacggtctgNNKacttcttggacccacg-3'] and
[5'-acctttCTCGAGcgggtattcaccgcccagaacagtatcttccttcgcatacagagagaacgccggMNNagtcggccacaggcgcaagg-5'] (restriction sites and mutagenized codon in caps) were used to amplify the region from Platinum Taq HiFi (Thermo Scientific). The amplicon was digested with XbaI, XhoI and DpnI (FastDigest from Themo Scientific), and the parent plasmid digested with XbaI and XhoI. Both were gel purified, ligated with T4 ligase (Themo Scientific), and electroporated into ElectroMAX DH10B electrocompetent *E. coli* (Thermo Scientific). The transformed cells were spread on a 12cm × 12cm LB agar plate with 100 mg/L carbenicillin and grown overnight at 37 °C to a confluent lawn, which was scraped into 10mL LB and mini-prepped (Qiagen). the resulting plasmid prep was Sanger sequenced to make sure the F87 and A238 codons were saturated, and transformed into NEB 10-beta chemically competent *E. coli* (New England Biolabs). The cells were plated onto 12cm × 12cm LB agar plates with 100 mg/L carbenicillin at various dilutions. After growth overnight at 37 °C, colonies from the plate showing most distinct colonies, as well as 8 colonies of *E. coli* 10-beta harboring pCW_{ori+}-GFP (into wells A01, A07, E01, E07, C04, C10, G04, and G10), were picked into 200 µL LB with 10% glycerol and 100 mg/L carbenicillin in 96-well plates (Nunc 262146 Clear Polystyrene Round Bottom) using a QPix2 colony picker (Molecular Devices), and grown overnight, shaking at 200 rpm at 37 °C. At this point, the 96-well plates could be stored at -80 °C until needed. 10 wells were picked at random and Sanger sequenced to verify that the library had the appropriate amount of diversity.

3.2 Synthesis of PECAN Tag "*PFO-Arg-NH-Prg*"



Synthetic strategy towards PFO-NH-Prg. Prg = propargyl,
Pbf = Pentamethyldihydrobenzofuransulfonyl, Fmoc = Fluorenylmethyloxycarbonyl, PFO = Perfluorooctanoyl

3.2.1 Fmoc-Arg(Pbf)-NH-Prg

InChI=1S/C37H43N5O6S/c1-7-18-39-34(43)31(41-36(44)47-21-30-27-15-10-8-13-25(27)26-14-9-11-16-28(26)30)17-12-19-40-35(38)42-49(45,46)33-23(3)22(2)32-29(24(33)4)20-37(5,6)48-32/h1,8-11,13-16,30-31H,12,17-21H2,2-6H3,(H,39,43)(H,41,44)(H3,38,40,42)/t31-/m0/s1

Into 20 mL DCM in a round-bottom flask on ice were added, in order, 1.62 g (2.5 mmol) Fmoc-Arg(Pbf)-OH (ChemPep), 1.56g (3 mmol) PyBOP (ChemPep), 412 µL (6 mmol) propargylamine (Sigma-Aldrich), and the reaction was left to warm up to room temperature while stirring overnight. 50 mL aqueous 0.1M HCl was added and the mixture was extracted twice with DCM., dried down and purified over silica gel (hexanes:ethyl acetate, product elutes in 90% ethyl acetate). The pure fractions were evaporated under reduced pressure to afford 980 mg (1.43 mmol, 57% yield) of a white powder.

MS (NIMS): 686.3 (M+H)

3.2.2 H_2N-Arg(Pbf)-NH-Prg

InChI=1S/C22H33N5O4S/c1-7-10-25-20(28)17(23)9-8-11-26-21(24)27-32(29,30)19-14(3)13(2)18-16(15(19)4)12-22(5,6)31-18/h1,17H,8-12,23H2,2-6H3,(H,25,28)(H3,24,26,27)/t17-/m0/s1

686 mg (1 mmol) Fmoc-Arg(Pbf)-NH-Prg was dissolved into 50 mL of 1:1 DCM:diethylamine and stirred at room temperature for 1 h. 10 mL xylenes was added, the mixture evaporated to dryness and chromatographed over silica gel (DCM:methanol, product elutes in 12% methanol). The pure fractions were evaporated under reduced pressure to afford 323 mg (0.50 mmol, 50 % yield) of a very hygroscopic white powder.

^{1}H NMR (500 MHz, Chloroform-*d*) δ 8.05 (s, 1H), 6.69 – 6.56 (m, 1H), 6.48 (s, 2H), 4.03 – 3.88 (m, 2H), 3.58 (d, *J* = 6.9 Hz, 1H), 3.45 (s, 2H), 3.20 (d, *J* = 6.6 Hz, 2H), 2.93 (s, 2H), 2.53 (s, 3H), 2.47 (s, 3H), 2.23 (t, *J* = 2.5 Hz, 1H), 2.06 (s, 3H), 1.81 (q, *J* = 6.5 Hz, 1H), 1.61 (qd, *J* = 15.2, 7.3 Hz, 3H), 1.44 (s, 6H).

^{13}C NMR (126 MHz, CDCl3) δ 174.10, 158.82, 156.55, 138.28, 132.56, 132.20, 124.74, 117.62, 86.50, 79.66, 71.51, 53.99, 43.23, 31.41, 28.96, 28.64, 25.39, 25.35, 19.38, 18.03, 12.54.

MS (NIMS): 464.3 (M+H)

3.2.3 PFO-Arg(Pbf)-NH-Prg

InChI=1S/C30H32F15N5O5S/c1-7-10-47-20(51)17(49-21(52)24(31,32)25(33,34)26(35,36)27(37,38)28(39,40)29(41,42)30(43,44)45)9-8-11-48-22(46)50-56(53,54)19-14(3)13(2)18-16(15(19)4)12-23(5,6)55-18/h1,17H,8-12H2,2-6H3,(H,47,51)(H,49,52)(H3,46,48,50)/t17-/m0/s1

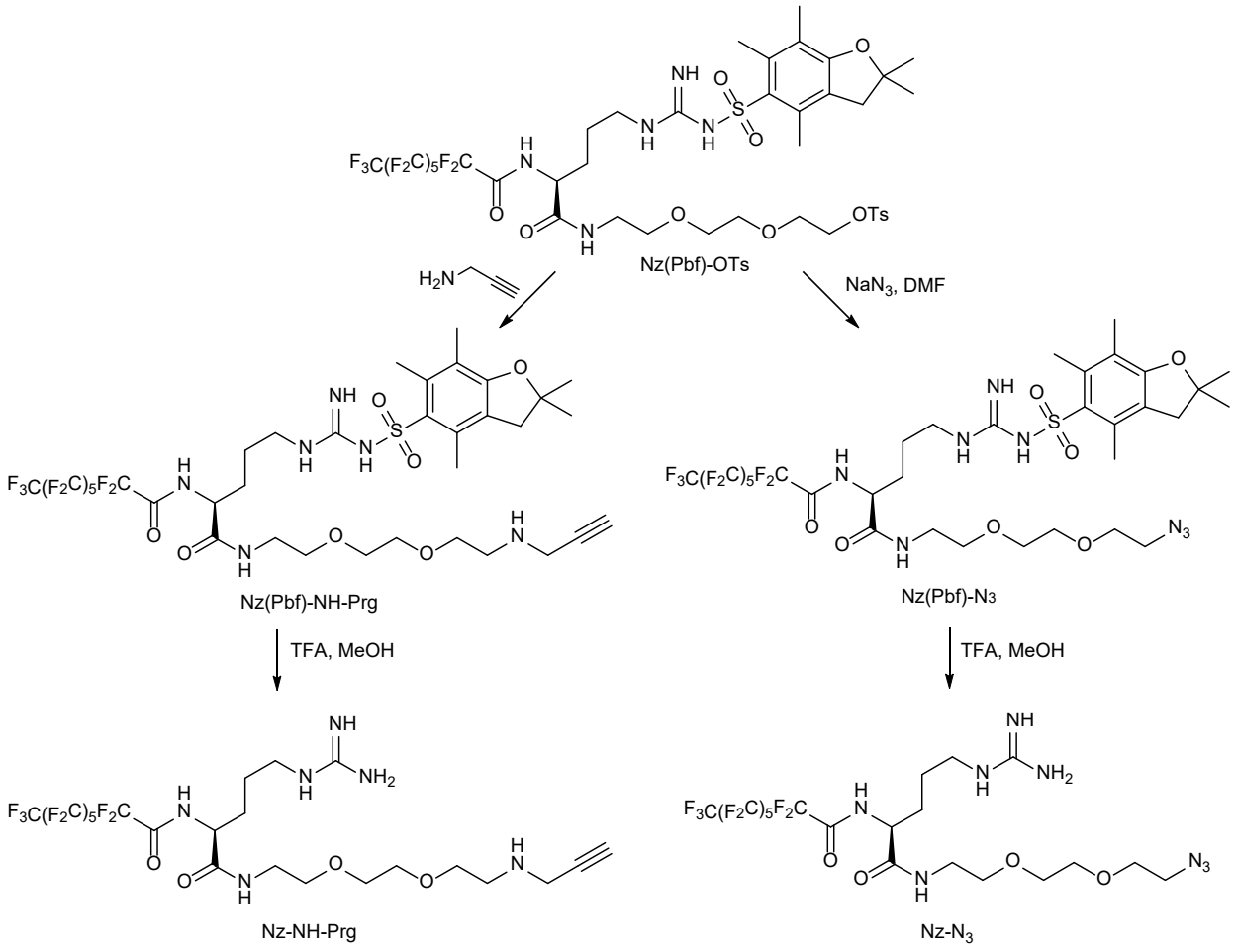
92 mg (0.2 mmol) H2N-Arg(Pbf)-NH-Prg was dissolved in 1mL chloroform. 0.25 mmol (60 µL) perfluorooctanoyl chloride (Sigma) and 1 mL aqueous 0.1 M NaOH was added, and the biphasic reaction vigorously stirred (Schotten-Bauman conditions). Another three 20 µL additions of perfluorooctanoyl chloride were made, spaced out by 30 minutes, all the while ensuring the pH of the aqueous layer remained basic using concentrated aqueous NaOH. The reaction was diluted with 5 mL water and twice extracted with 5 mL chloroform, dried down and passed over silica gel (hexanes:ethyl acetate, product elutes in 70% ethyl acetate). The pure fractions were evaporated under

reduced pressure to afford 74 mg (86 µmol, 43% yield) white powder.
^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.93 – 7.88 (m, 1H), 7.86 (t, *J* = 5.6 Hz, 1H), 6.32 (s, 2H), 6.14 (s, 1H), 4.71 (q, *J* = 7.4, 5.7 Hz, 1H), 3.97 (qdd, *J* = 17.4, 5.5, 2.5 Hz, 2H), 3.39 – 3.30 (m, 1H), 3.26 – 3.16 (m, 1H), 2.94 (s, 2H), 2.55 (s, 3H), 2.48 (s, 3H), 2.18 (t, *J* = 2.5 Hz, 1H), 2.08 (s, 3H), 1.96 (dq, *J* = 12.8, 6.8 Hz, 1H), 1.79 (td, *J* = 13.9, 6.9 Hz, 1H), 1.45 (s, 6H).
^{13}C NMR (126 MHz, $CDCl_3$) δ 159.07, 157.84 (t, $^{2}J_{C-F}$ = 26.60Hz) , 156.49, 138.38, 132.26, 132.07, 124.89, 117.82, 86.63, 79.03, 71.38, 52.84, 50.76, 43.15, 30.04, 29.17, 28.55, 25.22, 23.35, 19.27, 17.93, 12.42. Fluorinated carbons get split into oblivion.
MS (NIMS): 860.2 (M+H)

3.2.4 PFO-Arg-NH-Prg

InChI=1S/C17H16F15N5O2/c1-2-5-35-8(38)7(4-3-6-36-10(33)34)37-9(39)11(18,19)12(20,21)13(22,23)14(24,25)15(26,27)16(28,29)17(30,31)32/h1,7H,3-6H2,(H,35,38)(H,37,39)(H4,33,34,36)/t7-/m0/s1
37 mg (43 µmol) PFO-Arg(Pbf)-NH-Prg was dissolved into 0.5 mL methanol, 4.5 mL trifluoroacetic acid was added and the mixture stirred at room temperature for 48 h. The blue-tinted solution was evaporated under a stream of nitrogen and used as-is, assuming quantitative yield (based on the yield of Nz-NH-Prg under these same conditions, section 3.3.2). At the concentrations and reaction volumes used in our protocol, each tagging reaction requires 1.75 nmol, therefor 43 µmol would be enough for 25000 reactions.
MS (NIMS): 608.1 (M+H)

3.3 Synthesis of "Nz" PECAN Tags "Nz-NH-Prg" and "Nz-N_3"



Synthetic strategy towards Nz-NH-Prg and Nz-N_3. Prg = Propargyl, Pbf = Pentamethyldihydrobenzofuransulfonyl, Fmoc = Fluorenylmethyloxycarbonyl, PFO = Perfluorooctanoyl

3.3.1 Nz(Pbf)-NH-Prg

38.6mg (34.8 µmol) Nz(Pbf)-OTs (synthesis described in Ref. [2]) was stirred in 100 µL (~50 eq.) neat propargylamine (Sigma-Aldrich P50900) at 70 °C overnight. The mixture was evaporated under high vacuum to remove unreacted propargylamine (bp: 83 °C), and the residue was subjected to silica gel chromatography (DCM:methanol, product elutes in 10% methanol). Pure fractions were combined and evaporated to afford 32.5 mg (32.8 µmol, 94% yield) of a white powder.

^{1}H NMR (400 MHz, Methanol-*d*$_4$) δ 4.46 (dd, *J* = 8.9, 5.9 Hz, 1H), 3.62 – 3.58 (m, 6H), 3.54 (t, *J* = 5.5 Hz, 2H), 3.43 (d, *J* = 2.4 Hz, 2H), 3.40 (t, *J* = 5.4 Hz, 1H), 3.38 – 3.34 (m, 1H), 3.20 – 3.12 (m, 1H), 3.00 (s, 2H), 3.00 (s, 1H), 2.86 – 2.81 (m, 2H), 2.62 (t, *J* = 2.5 Hz, 1H), 2.58 (s, 3H), 2.51 (s, 3H), 2.08 (s, 3H), 1.91 – 1.78 (m, 1H), 1.74 (dtd, *J* = 13.9, 9.2, 5.1 Hz, 1H), 1.55 (ddd, *J* = 22.1, 16.1, 9.0 Hz, 2H), 1.46 (s, 6H).

MS (NIMS): 991.3 (M+H)

3.3.2 Nz-NH-Prg

26 mg (26.2 mmol) Nz(Pbf)-NH-Prg was dissolved into 0.5 mL methanol, 4.5 mL trifluoroacetic acid (TFA) was added and the mixture stirred at room temperature for 48 h. The blue-tinted solution was evaporated under a stream of nitrogen, the residue washed with DCM, re-dissolved in methanol, evaporated under reduced pressure and identically washed, redissolved and evaporated twice more to afford 18.3 mg (21.5 µmol, 95% yield, assuming it formed a TFA salt) of product.

^{1}H NMR (400 MHz, Methanol-*d*$_4$) δ 4.48 (dd, *J* = 9.8, 5.2 Hz, 1H), 4.04 – 3.93 (m, 2H), 3.80 (t, *J* = 5.0 Hz, 2H), 3.69 – 3.29 (m, 9H), 3.18 (dd, *J* = 10.0, 4.4 Hz, 3H), 2.03 – 1.87 (m, 1H), 1.80 (dtd, *J* = 14.8, 10.1, 5.1 Hz, 1H), 1.72 – 1.51 (m, 2H).

^{13}C NMR (101 MHz, MeOD) δ 171.66, 79.12, 74.76, 71.34, 66.95, 57.84, 55.00, 49.00, 41.47, 40.53, 37.43, 26.13, 18.47, 15.44, 14.18, 11.87. Carbons part of the perfluorooctanoyl tail get split into oblivion by the fluorines.

MS (NIMS): 739.2 (M+H)

3.3.3 Nz(Pbf)-N_3

5.5 mg (5 µmol) Nz(Pbf)-OTs (synthesis described in Ref. [2]) was dissolved in 1mL DMF. 16.3 mg (250 µmol) NaN_3 was added and the solution stirred overnight at 60 °C. The mixture was diluted in 20 mL water and extracted with ethyl acetate. The organic layer was washed twice with water, evaporated and used immediately in the next reaction.

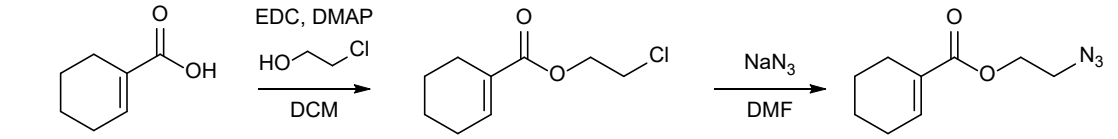
MS (NIMS): 979.3 (M+H)

3.3.4 Nz-N_3

Nz(Pbf)-N_3 from the previous reaction was dissolved into 0.5 mL methanol, 4.5 mL trifluoroacetic acid was added and the mixture stirred at room temperature for 48 h. The blue-tinted solution was evaporated under a stream of nitrogen, and used as-is.

MS (NIMS): 727.2 (M+H)

3.4 Synthesis of PECAN probe **1b**



cyclohexenecarboxylic acid

cyclohexenecarboxylic acid chloroethyl ester

cyclohexenecarboxylic acid azidoethyl ester (**1b**)

*Synthetic strategy towards **1b***

3.4.1 Cyclohexanecaboxylic acid chloroethyl ester

Into 5mL dry DCM on ice was added, in order, 1.39g (11 mmol) cyclohexenecarboxylic acid, 670 µL (10 mmol) chloroethanol, 122 mg (1 mmol), N,N-dimethylaminopyridine (DMAP), and 1.86g (12 mmol) 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC). The reaction was stirred overnight, letting it warm up to room temperature, dilute into 20 mL hexanes, and washed twice with 10mL aqueous 1M HCl (to remove coupling agent), twice with aqueous 1M NaOH (to remove any unreacted cyclohexenecarboxylic acid) and once with brine. The organic layer was evaporated under high vacuum for an hour (to remove unreacted chloroethanol, bp: 129 °C) to afford 1.786g (0.95 mmol, 95% yield) of a clear oil.

^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.00 (tt, *J* = 3.8, 1.7 Hz, 1H), 4.32 (dd, *J* = 6.3, 5.2 Hz, 2H), 3.73 – 3.58 (m, 2H), 2.22 (dddd, *J* = 8.3, 6.1, 4.2, 2.3 Hz, 2H), 2.16 (tdd, *J* = 6.1, 3.8, 2.6 Hz, 2H), 1.65 – 1.51 (m, 4H).

^{13}C NMR (101 MHz, $CDCl_3$) δ 166.97, 140.74, 129.77, 63.81, 41.83, 25.82, 24.04, 21.99, 21.38.

*3.4.2 Cyclohexanecaboxylic acid azidoethyl ester (**1b**)*

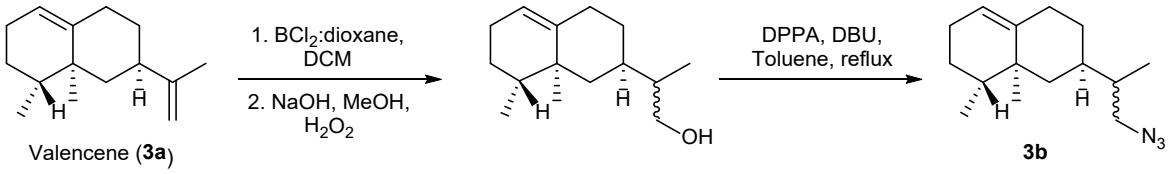
332mg (1.76 mmol) CCEE and 572 mg (8.8 mmol) NaN_3 were dissolved into 1.5 mL DMF, and stirred at 80 °C for 1 h. The reaction was diluted into 30 mL water, extracted with 30mL hexanes, and washed with 30 mL brine. The organic layer was evaporated to afford 293.9 mg (1.51 mmol, 86% yield) of a clear oil.

^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.02 (tt, *J* = 3.8, 1.8 Hz, 1H), 4.31 – 4.23 (m, 2H), 3.48 – 3.44 (m, 2H), 2.24 (tq, *J* = 6.0, 2.0 Hz, 2H), 2.18 (tdd, *J* = 6.2, 3.9, 2.7 Hz, 2H), 1.68 – 1.52 (m, 4H).

^{13}C NMR (101 MHz, $CDCl_3$) δ 167.12, 141.00, 129.76, 63.01, 50.02, 25.89, 24.09, 22.03, 21.39.

MS (NIMS after clicking with Nz-NH-Prg): 934.4 (M(195.1)+Nz-NPrg(738.2)+H(1))

3.5 Synthesis of PECAN probe **3b**



*Synthetic strategy towards **3b***

3.5.1 Valencene 14-hydrate

InChI=1S/C15H26O/c1-11(10-16)13-7-8-14-6-4-5-12(2)15(14,3)9-13/h6,11-13,16H,4-5,7-10H2,1-3H3/t11?,12-,13-,15+/m1/s1

Valencene (Sigma-Aldrich 75056) was purified by passing it over silica gel in hexanes. After this, the valencene was >95% pure by GC-MS.

This hydroboration protocol is based on that of Kanth and Brown[3] and was effective on all terpenes with 1,1-disubsituted alkenes that we have tried it on.

To a dry round-bottom flask on ice was added, in order, 3.5 mL valencene (15.8 mmol), 10 mL dry DCM, and 6.5 mL of 3M dichloroborane:dioxane complex in DCM (1.2 eq, Sigma 555959). The solution was left to stir on ice for 3.5 h, after which was added, very slowly to avoid overheating, in order, 2.5 mL 50% NaOH diluted in 16 mL water, 5 mL methanol, 2.5 mL 30% hydrogen peroxide. The solution was warmed up to room temperature, left to stir for 3h, and stirred for 1h at 40 °C. The mixture was extracted with 3 × 50 mL DCM, dried down, and chromatographed over silica gel (9:1 hexanes:ethyl acetate) and evaporated under reduced pressure to afford 2.83g of a colorless viscous oil (12.7 mmol, 81% yield).

Equimolar mixture of diastereomers. NMR shows “doublets” because the diastereomers have slightly different chemical shifts. For these cases, signals hypothesized to correspond to the same atom in the two diastereomers are grouped using brackets for the ^{13}C NMR listing.

^{1}H NMR (400 MHz, Chloroform-*d*) δ 5.33 – 5.24 (m, 1H), 3.82 – 3.38 (m, 2H), 2.32 – 2.16 (m, 1H), 2.09 – 1.85 (m, 3H), 1.83 – 1.21 (m, 9H), 0.93 – 0.81 (m, 9H).

^{13}C NMR (101 MHz, $CDCl_3$) δ [143.68, 143.65], [119.94, 119.92], [66.55, 66.29], 44.32, 42.26, 41.21, [40.82, 40.79], [37.98, 37.78], [34.87, 34.48], [32.93, 32.77], [32.17, 29.80], [27.29, 27.26], 26.00, 18.64, [15.83, 15.81], [13.78, 13.16].
EIMS: 222 (20), 189 (28), 163 (72), 107 (59), 105 (77), 91 (100), 79 (79), 77 (60), 67 (47).

*3.5.2 Valencene 14-hydroazide (**3b**)*

InChI=1S/C15H25N3/c1-11(10-17-18-16)13-7-8-14-6-4-5-12(2)15(14,3)9-13/h6,11-13H,4-5,7-10H2,1-3H3/t11?,12-,13-,15+/m1/s1
A round-bottom flask with condenser was charged with 20mL toluene, 2.24g valencene 14-hydrate, 6.475mL d iphenylphosphoryl azide (DPPA) (3 eq), and 3mL 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) (For this reaction, it was not necessary to add DEAD or DIAD). The mixture was refluxed for 24 h, quenched with saturated aqueous ammonium chloride, extracted with 2 × 1:1 hexanes:ethyl acetate, dried down and passed over silica in 100% hexanes and evaporated under reduced pressure to afford 2.23g of a colorless oil (9.0 mmol, 90% yield).

Equimolar mixture of diastereomers. NMR shows apparent “doublets” because the diastereomers have slightly different chemical shifts. For these cases, the J-values are marked with an asterisk in the ^{1}H NMR listing (these are not true J-values since the number is affected by the magnetic field the way chemical shift is), and, signals hypothesized to correspond to the same atom in the two diastereomers are grouped using brackets for the ^{13}C NMR listing.
^{1}H NMR (400 MHz, Chloroform-*d*) δ 5.31 (dt, *J* = 4.6*, 2.1 Hz, 1H), 3.32 (ddd, *J* = 12.1*, 5.8, 2.7 Hz, 1H), 3.14 (ddd, *J* = 12.0*, 7.4, 4.3 Hz, 1H), 2.34 – 2.19 (m, 1H), 2.07 (ddd, *J* = 14.0*, 4.3, 2.5 Hz, 1H), 2.00 (tt, *J* = 9.8, 2.6 Hz, 1H), 1.97 – 1.87 (m, 1H), 1.76 (dq, *J* = 12.4*, 2.9 Hz, 1H), 1.72 – 1.52 (m, 4H), 1.40 (dp, *J* = 7.5*, 2.7 Hz, 3H), 0.97 – 0.84 (m, 10H).
^{13}C NMR (101 MHz, $CDCl_3$) δ [143.31, 143.29], [120.20, 120.18], [56.11, 55.89], [44.02, 41.96], 41.20, 38.44, [37.96, 37.78], [35.66, 35.47],[32.79, 32.63], [31.92, 29.77], [27.27, 27.25], 25.99, 18.61, 15.82, [14.82, 14.40].
EIMS: 218 (14, M-HN_2), 204 (47), 190 (24), 187 (76), 176 (30), 162 (63), 159 (30), 145 (42), 131 (36), 119 (48), 107 (58), 105(84), 96 (97), 95 (56), 93 (69), 91 (100), 81(50), 77 (53), 67 (42), 55(61)
MS (NIMS after clicking with): 855.3 (product(247.2)+Nz-N_3(607.1)+H(1))

Each time before using **3b** as a PECAN probe, it was passed over a silica stub in a pipette-filter in hexanes to remove any possible spontaneous breakdown products.

3.6 Recipes

3.6.1 Enzyme reaction buffer

50 mM sodium phosphate, pH 8, 150 mM sodium chloride, 2 mM magnesium chloride

3.6.2 Lysis buffer

50 mM sodium phosphate, pH 8, 150 mM sodium chloride, 2 mM magnesium chloride, 0.1 mg/mL lysozyme (Sigma L6876), 0.01 mg/mL DNAse I (Sigma D4527)

Used to lyse *E. coli* cells. Requires freeze-thaw cycles for thorough lysis.

3.6.3 NADPH cofactor regeneration system (10X)

50 mM sodium phosphate, 150 mM sodium chloride, 2 mM magnesium chloride, 1 unit/mL, 100mM glucose-6-phosphate (G6P, Fisher Scientific), G6P dehydrogenase (Sigma G7877), and 1mM $NADP^+$ (Sigma N5755).

Used for the *in*-situ generation of NADPH. Since commercial NADPH degrades, it can be difficult to control the number of reducing equivalents added to the reaction. Using this cofactor regeneration system (CRS), the concentration of G6P corresponds to the total amount of reducing power available. 1X CRS contains 10 mM G6P, so for reactions employing 0.5 mM probe, that corresponds to 20 reducing equivalents available for the biotransformation.

3.6.4 Cu(I)-catalyzed click reaction solution (2X)

1:1 methanol:water with 50 mM THPTA (Click Chemistry Tools), 10 mM $CuSO_4$ (Strem Chemicals 93-2959), 50 mM sodium ascorbate (Sigma, from a freshly-made 0.5 M stock)

Used in the tag-clicking reactions, using various tags. Tags harboring an alkyne can be pre-mixed into this solution, being stable for over an hour. Tags harboring an azide degrade fairly quickly under these conditions, therefore, in those cases the click solution was mixed with the probe (often already in a relevant biological matrix) before adding tag in methanol.

3.7 NIMS surface production

3.7.1 Wet-etched NIMS chips

Wet-etched NIMS chips were produced according to the protocol of Hoo *et al.* [4].

3.7.2 Dry-etched NIMS chips

Dry-etched NIMS chips were produced according to the protocol of Gao *et al.* [5]. The chips, thoroughly coated in initiator, were baked at 50 °C in a convection oven for 24 h before they were used. For reasons unknown, this procedure dramatically increases the hydrophobicity of the NIMS surface, preventing deposited analytes from smudging into each other during the aqueous washing step. Sensitivity of these NIMS chips was as good or better than that of unbaked chips. NIMS chips stored under vacuum before baking did not show the same properties as baked chips (suggesting evaporation of the initiator was not the origin of the effect), but after baking, chips could be used under vacuum without a noticeable change in their properties.

3.8 PECAN in *E. coli* cell lysate – technical replicates

5 mL of an overnight culture of E. *coli* DH10b harboring pCW_{ori+}-$P450_{BM3}$, as well as E. *coli* DH10b harboring pCW_{ori+}-GFP to act as a negative control, each grown in LB with 100 mg/L carbenicillin, was diluted into 50 mL Terrific Broth (TB) with 100 mg/L ampicillin and 0.5 mM δ-aminolevulinic acid (δ-ALA) in a glass culture flask. After growth for 3 h at 37 °C, 0.5 mM IPTG was added and the culture left to shake at 200 rpm for 16 h at 30 °C. The cells were pelleted at 3000 × g for 5 min, re-suspended in 10 mL lysis buffer (section 3.6.2), subjected to 3 freeze-thaw cycles between -80 °C and 30 °C and centrifuged at 10,000 × g for 10 min. 450 µL of the supernatant was distributed into 3 glass vials (100 mm × 16 mm diameter) for the $P450_{BM3}$ and GFP negative control each. 5 µL of [50 mM **1b** (synthesis described in section 3.4) in DMSO] was added to each vial, and the reactions were initiated through the addition of 50 µL 10X cofactor regeneration system, such that the final 500 µL reaction mixtures contained 1% DMSO, 0.5 mM **1b** and 1X cofactor regeneration system (i.e., 10 mM G6P, 100 µM NADP+ and 0.1 unit/mL G6P dehydrogenase). The reactions were shaken at 200 rpm for 1 h and quenched with 500 µL methanol. 10 µL of the suspension was mixed with 10 µL 2X tagging solution [2X click solution (described in section 3.6.4), 100 µM Nz-NH-Prg tag (synthesis described in section 3.2)], and the mixture left to react for 1 h at room temperature, after which it was transferred into a 384-well acoustic source plate (Greiner 788986). A wet-etched NIMS chip (production described in section 3.7.1) was taped to a steel MALDI plate and 7 depositions of 1 nL from each well (corresponding to 50 fmol of tagged probe, assuming the click reaction went to completion) were printed onto the NIMS chip using an ATS-100 acoustic transfer system (EDC, Fremont, CA) with a spot pitch (center-to-center distance) of 750 µm. The chip was rinsed with de-ionized water under a running tap for 5 minutes, mounted onto a steel MALDI plate using copper tape, and spectra were acquired on a ABI/Sciex 4800 MALDI TOF/TOF instrument. The resulting MS image was uploaded to OpenMSI[6], which was used to generate the images shown in the manuscript.

3.9 PECAN in *E. coli* cell lysate – biological replicates

8 colonies of *E. coli* DH10b harboring pCW_{ori+}-$P450_{BM3}$, as well as 8 colonies of *E. coli* DH10b harboring pCW_{ori+}-GFP to act as a negative control, were picked into 96-well plates (Nunc 262146 Clear Polystyrene Round Bottom) with 200 µL of LB with 100 mg/L carbenicillin. After growth overnight, 100 µL of the wells were diluted into 1 mL TB

with 100 mg/L ampicillin and 0.5 mM δ-aminolevulinic acid (δ-ALA) in 96-deepwell plates (Corning 3960 Clear Polypropylene Square V-Bottom). The plates were shaken at 300 rpm at 37 °C for 3h, cooled down to room temperature, induced with 0.5 mM IPTG (through the addition of 100 µL TB with 5mM IPTG), and shaken for 18h at 300 rpm at 30 °C, and centrifuged for 5 min at 3,000 × g. The pellets were resuspended in Lysis Buffer (section 3.6.2), shaken at 250 rpm at 30 °C for 1 hour, subjected to 3 freeze-thaw cycles between -80 °C and 30 °C, the last freeze of which occurred overnight. The plates were centrifuged at 3,000 × g for 10 minutes and 110 µL of each of the supernatants was transferred to 96-deepwell plates pre-loaded with 6.25 µL 20X **1b** (10 mM **1b** in DMSO, synthesis described in section 3.4), and the reactions were initiated by adding 12.5 µL 10X cofactor regeneration system, such that the final 125 µL reaction mixtures contained 0.5 mM probe, 5% DMSO, and 1X cofactor regeneration system [i.e., 10 mM G6P, 100 µM NADP+ and 0.1 unit/mL G6P dehydrogenase]. The enzymatic reactions were shaken at 300 rpm for 1 hour and quenched with an equal volume of methanol. Plates were incubated at 4 °C for 30 minutes to expedite protein precipitation, and centrifuged for 10 min at 2,000 × g. 10 µL of each supernatant was transferred to a 384-well PCR plate (Biorad HSP3831 hard-shell skirted) pre-loaded with 10 µL 2X tagging solution [2X click solution (described in section 3.6.3), 100 µM Nz-NH-Prg tag (synthesis described in section 3.2)]. The 384-well PCR plate was sealed and left to react at room temperature for 1 h. 10 µL of the tagging reaction was transferred into a 384-well acoustic source plate (Greiner 788986). A wet- etched NIMS chip (production described in section 3.7.1) was taped to a steel MALDI plate and 1 nL from each well (corresponding to 50 fmol of tagged probe, assuming the click reaction went to completion) was printed onto the chip using an ATS-100 acoustic transfer system (EDC, Fremont, CA) with a spot pitch (center-to-center distance) of 750 µm. The NIMS chip was rinsed with de-ionized water under a running tap for 5 minutes, mounted onto a steel MALDI plate using copper tape, and imaged with a spatial (rastering) resolution of 75 µm on an ABI/Sciex 4800 MALDI TOF/TOF instrument. The resulting MS image was uploaded to OpenMSI[6], which was used to generate the images shown in the manuscript. The data was quantified using the OpenMSI Arrayed Analysis Toolkit[7]

3.10 $P450_{BM3}$ reaction on **1b** in whole *E. coli* cells

0.5 mL of an overnight culture of E. *coli* DH10b harboring pCW_{ori+}-$P450_{BM3}$ in LB with 100 mg/L carbenicillin was diluted into 5 mL TB with 100 mg/L ampicillin and 0.5 mM δ-aminolevulinic acid (δ-ALA) in a glass culture tube. After growth for 3 h at 37 °C, 0.5 mM IPTG was added and the culture grown for 3 h at 30 °C at 200 rpm. 3 mL of the culture was transferred to a baffled culture tube[8] (made by heating a glass culture tube over a Méker-Fisher burner, constantly turning the tube, until the glass starts drooping slightly, at which point a scoopula is forced into the side of the tube, see figure below), 30 µL of [50 mM **1b** (synthesis described in section 3.4) in DMSO] was added and the culture (final concentrations: 1 mM **1b** and 2% DMSO) was left to shake at 200 rpm at 30 °C for 12 h. 3 mL methanol was added and 10 µL of the suspension was mixed with 10 µL 2X tagging solution [2X click solution (described in section 3.6.4), 100 µM Nz-NH-Prg tag (synthesis described in section 3.2)], and the mixture left to react for 1 h at room temperature. The reactions were manually pipetted onto a NIMS chip, the chip was rinsed with de-ionized water under a running tap for 5 minutes and mounted onto a steel MALDI plate using copper tape, and spectra were acquired on a ABI/Sciex 4800 MALDI TOF/TOF instrument.

*Home-made baffled culture tube for in vivo $P450_{BM3}$ activity assays, allowing for thorough aeration (required for P450 activity) while minimizing the total culture volume (and hence the quantity of **1b**). Accommodates ~3mL of culture.*

3.11 Human CYP3A4 reaction in microsomes

5 µL recombinant human CYP3A4 Baculosomes (Thermo Fisher P2377) was diluted into 41.5 µL Enzyme Reaction Buffer (section 3.6.1) in PCR strip tubes. 0.5 µL [20 mM 19-norethindrone (Sigma N4128) in DMSO] was added (final concentration 200 µM 19-norethindrone, 1% DMSO), and for the inhibited sample, 0.5 µL [10 mM clotrimazole (Sigma C6019) in water] was added (final concentration 100 µM clotrimazole). The reactions were initiated using 2.5 µL NADPH Cofactor Regeneration System (section 3.6.3), left for 30 min at room temperature and quenched with 50 µL methanol. 10 µL of the suspension was mixed with 10 µL 2X tagging solution [2X click solution (described in section 3.6.4), 100 µM Nz-NH-N_3 tag (synthesis described in section 3.2)], and the mixture left to react for 1 h at room temperature. The reactions were manually pipetted onto a NIMS chip, after which the chip was rinsed with de-ionized water under a running tap for 5 minutes, mounted onto a steel MALDI plate using copper tape, and spectra were acquired on a ABI/Sciex 4800 MALDI TOF/TOF instrument.

3.12 High-throughput PECAN Screen

Except where noted, all liquid-handling was performed on a Beckman Coulter Biomex FX robot with a 96-well pipetting head.

The double site-saturated $P450_{BM3}$ library (construction described in section 3.1) with 8 GFP negative control wells per 96-well plate was grown overnight in 100 µL LB with 100 mg/L carbenicillin and diluted into 1 mL TB with 100 mg/L ampicillin and 0.5 mM δ-aminolevulinic acid (δ-ALA) in 96-deepwell plates (Corning 3960 Clear Polypropylene Square V-Bottom). The plates were shaken at 300 rpm at 37 °C for 3h, cooled down to room temperature, induced with 0.5 mM IPTG (through addition of 100 µL TB with 5mM IPTG), shaken for 18h at 300 rpm at 30 °C, and centrifuged for 5 min at 3,000 × g. The pellets were resuspended in Lysis Buffer (section 3.6.2), shaken at 250 rpm at 30 °C for 1 hour, subjected to 3 freeze-thaw cycles between -80 °C and 30 °C, the last freeze of which occurred overnight. The plates were centrifuged at 3,000 × g for 10 minutes and 110 µL of each of the supernatants was transferred to glass-covered 96-deepwell plates ("Plate +" from Thermo Scientific, 60180-P308. Glass-covered plates decrease adsorption of the hydrophobic **3b** to the plate. Plates could be re-used by rinsing them with water until visually clean and then soaking them in isopropanol overnight) pre-loaded with 6.25 µL 20X **3b** (10 mM **3b** in DMSO, synthesis described in section 3.5, dispensed using a Tecan Freedom EVO liquid-handling robot equipped with metal fixed tips), and the reactions were initiated by adding 12.5 µL 10X cofactor regeneration system, such that the final 125µL reaction mixtures contained 0.5 mM probe, 5% DMSO (the high DMSO concentration helps keep the **3b** sufficiently in solution), and 1X cofactor regeneration system [i.e., 10 mM G6P, 100 µM NADP+ and 0.1 unit/mL G6P dehydrogenase]. The enzymatic reactions were shaken at 300 rpm with a throw of 5mm (the small volume and high shaking speed helps aeration. A 800 rpm with a throw of 1mm had similar results) at 30 °C for 1 hour (longer incubations led to significant evaporation of **3b**) and quenched with an equal volume of isopropanol. Plates were incubated at 4 °C for 30 minutes to expedite protein precipitation, and centrifuged for 10 min at 2,000 × g (The "Plate +" plates would crack if centrifuged harder than that). 17.5 µL of each supernatant was transferred to a 384-well PCR plate (Biorad HSP3831 hard-shell skirted) (combining 4 96-well plates worth of enzymatic reactions) pre-loaded with 17.5 µL 2X tagging solution [2X click solution (described in section 3.6.4), 100 µM PFO-Arg-NH-Prg tag (synthesis described in section 3.2)], dispensed using a Tecan Freedom EVO liquid-handling robot]. The 384-well PCR plate was hermetically sealed with aluminum foil using a heat sealer and left to react at room temperature for 24 h. To prevent the formation of bubbles (which impair the ability of the acoustic printer to print from that well) while pipetting, 2 µL methanol was pre-dispensed into a 384-well plate (Greiner 788986) before transferring 10 µL of the tagging reaction into it. A dry-etched NIMS chip (production described in 3.7.2) was taped to a steel MALDI plate, and 10 nL from each well (corresponding to 0.5 pmol of tagged probe, assuming the click reaction went to completion) was printed onto the NIMS chip using a ATS-100 acoustic transfer system (EDC, Fremont, CA) with a spot pitch (center-to-center distance) of 750 µm, in a pattern preserving the screening campaign's 96-well plates. The NIMS chip was rinsed with de-ionized water under a running tap for 5 minutes, and gently shaken in a bath of de-ionized water overnight. The NIMS chip was mounted onto a steel MALDI plate using copper tape, and imaged with a spatial (rastering) resolution of 75 µm on an ABI/Sciex 5800 MALDI TOF/TOF instrument. The data presented in this manuscript is the result of 3 separate imaging experiments corresponding to 2, 4 and 8 96-well plates respectively. The resulting MS image was uploaded to OpenMSI[6], which was used to

generate the images shown in the manuscript. The data was quantified using the OpenMSI Arrayed Analysis Toolkit[7]

The raw MS image data acquired for this experiment can be accessed at the following three URLs:
https://openmsi.nersc.gov/openmsi/openmsi/client/viewer/?file=%2Fproject%2Fprojectdirs%2Fopenmsi%2Fomsi_data_private%2Fjiangao%2F06162017_Tristan_BlackSilicon.h5&dataIndex=0&expIndex=0&channel1Value=869&channel1RangeValue=0.5&channel2Value=855&channel2RangeValue=0.5&channel3Value=869&channel3RangeValue=0.5&rangeValue=0.5&cursorCol1=462&cursorRow1=296&cursorCol2=456&cursorRow2=358&enableClientCache=false,
https://openmsi.nersc.gov/openmsi/openmsi/client/viewer/?file=%2Fproject%2Fprojectdirs%2Fopenmsi%2Fomsi_data_private%2Fraad0102%2F20170530MdR_5800_Black_NIMS_Tristan_75um.h5&dataIndex=0&expIndex=0&channel1Value=869.3&channel1RangeValue=0.5&channel2Value=855.3&channel2RangeValue=0.5&channel3Value=869.3&channel3RangeValue=0.5&rangeValue=0.5&cursorCol1=19&cursorRow1=289&cursorCol2=238&cursorRow2=337&enableClientCache=false,
https://openmsi.nersc.gov/openmsi/openmsi/client/viewer/?file=%2Fproject%2Fprojectdirs%2Fopenmsi%2Fomsi_data_private%2Fraad0102%2F20170315MdR_5800_NIMS_black_Tristan_screen_1.h5&dataIndex=0&expIndex=0&channel1Value=869&channel1RangeValue=0.5&channel2Value=855&channel2RangeValue=0.5&channel3Value=869&channel3RangeValue=0.5&rangeValue=0.5&cursorCol1=105&cursorRow1=10&cursorCol2=148&cursorRow2=9&enableClientCache=false

3.13 Analysis of valencene (3a) oxidation products by GC-MS

The *E. coli* DH10b $P450_{BM3}$ mutant cultures considered a "hit" in the PECAN screen were grown overnight in LB with 100 mg/L carbenicillin. 12.5 mL of this culture was diluted into 250 mL TB with 100 mg/L ampicillin and 0.5 mM δ-ALA in 1 L glass flasks and shaken at 200 rpm at 37 °C for 3.5 h. the cultures were cooled to 30 °C, induced with 0.5 mM IPTG, and shaken at 200 rpm at 30 °C for 18 h. The cultures were harvested by centrifugation at 3000 × g, resuspended in 15 mL lysis buffer (see section 3.6.2), shaken at 200 rpm at 30 °C for 1 hour, and subjected to 3 freeze-thaw cycles between -80 °C and 30 °C, the last freeze of which occurred overnight. The lysates were clarified by centrifugation at 10,000 × g for 15 min at 4°C, and the clarified lysates gently shaken with 1 mL of Ni-NTA Agarose beads (Qiagen 30210) in the presence of 10 mM imidazole, at 4 °C for 1 h. The beads were subjected to 3 × 5 mL washes of enzyme buffer (see section 3.6.1) + 10 mM imidazole, gently shaken for 30 min at 4 °C, and eluted in 4 mL enzyme buffer + 200 mM imidazole. The eluents were concentrated in 3 kDa MWCO filters to ~500 µL final volume.

P450 concentrations were measured by CO-difference assays[9], performed as follows: 100 µL of concentrated protein was diluted into 2 mL (20-fold dilution) and pipetted into two plastic cuvettes. CO was bubbled through one of the cuvettes for 30 s. 10 µL 0.1 M sodium dithionite was added to both of the cuvettes, both were mixed by inversion, left to react for 30 s, and the cuvette without CO added used as blank for the measurement of the spectrum of the cuvette with CO added in a UV-Vis spectrometer between 400 and 550 nm. The spectra were normalized by setting the absorbance at 490 nm to 0, and the maximum absorbance between 440 nm and 460 nm was used to determine P450 concentration, using 91 $mM^{-1}\,cm^{-1}$ as extinction coefficient.

In vitro assays (500 µL total, in 100 mm × 16 mm glass vials, in triplicate) contained: 1 µM enzyme, 500 µM valencene (freshly passed over a silica stab), 5% DMSO, and enzyme reaction buffer (see section 3.6.1) up to 450 µL total volume, and were initiated by the addition of 50 µL cofactor regeneration system (3.6.3). The reactions were shaken at 200 rpm at room temperature for 1 h, and quenched by the addition of 500 µL ethyl acetate and thorough vortexing. The organic layer was collected and 1 µL of it analyzed by GC-MS using an Agilent 7890A GC with 5975C inert XL MS. Column: HP5-ms (30 m × .25 mm) (Agilent 19091S-433), Helium flow rate: 1mL/min; Inlet temperature: 250 °C; MS transfer line temperature: 280 °C. Oven temperatures: Hold at 80 °C for 1 min, ramp to 160 °C at 20 °C/min, ramp to 235 °C at 7.5 °C/min, ramp to 300 °C at 40 °C/min, hold at 300 °C for 2.75 min. MS was scanned from 50 to 350 Da, the fragmentation patterns used to identify peaks by comparison to literature spectra[10,11], and the total ion chromatogram used for quantitation.

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