

# Discovery of new phenylacetone monooxygenase variants for the development of substituted indigoids through biocatalysis

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1. Production of indigo and indirubin in recombinant *E. coli*.

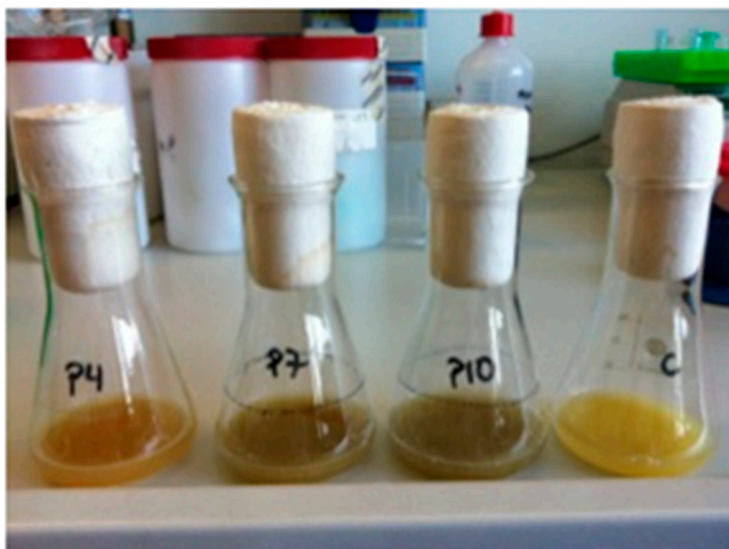
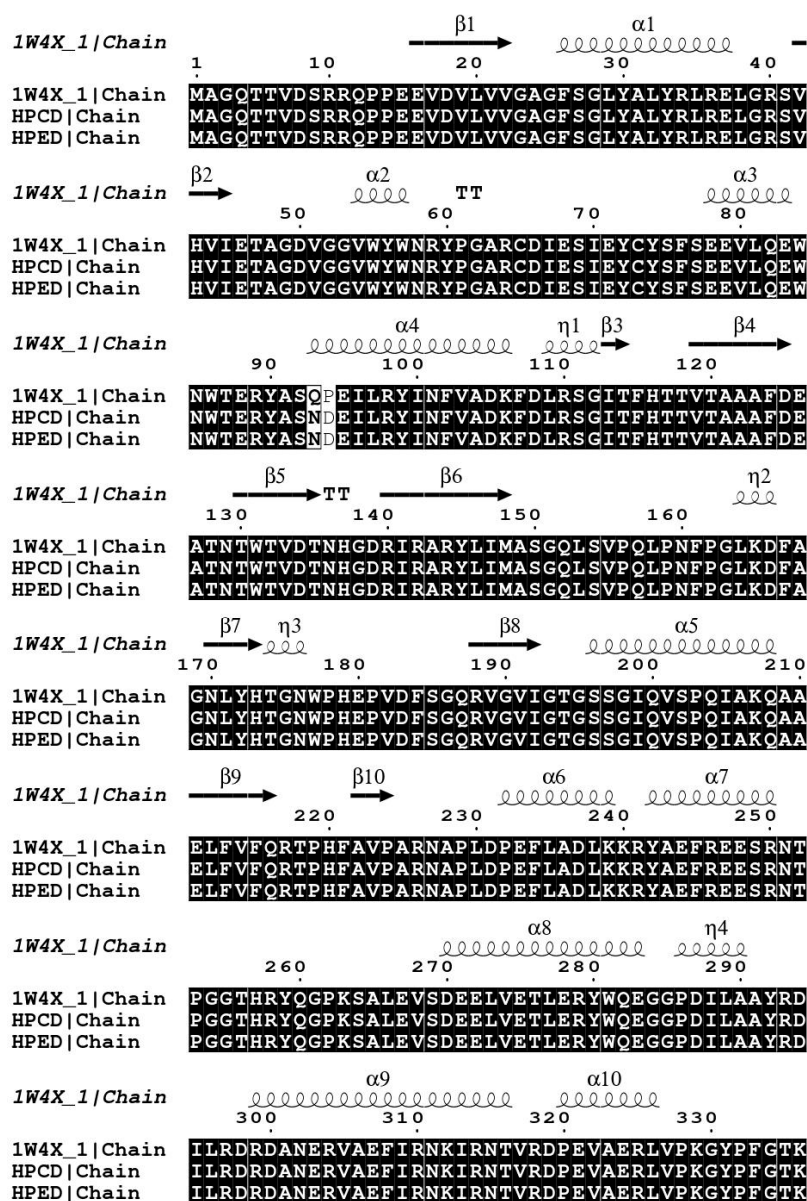


**Figure S1.** Production of indigo and indirubin in recombinant *E. coli* overexpressing PAMO<sup>HPCD</sup> or PAMO<sup>HPED</sup>. The formation of these pigments can be seen by comparing the C flask (*E. coli* overexpressing PAMO<sup>WT</sup>) with *E. coli* overexpressing PAMO<sup>HPCD</sup> in flask P7 and P10, and PAMO<sup>HPED</sup> in flask P4.

## 2. Sequence analysis of PAMO<sub>WT</sub> and its variants



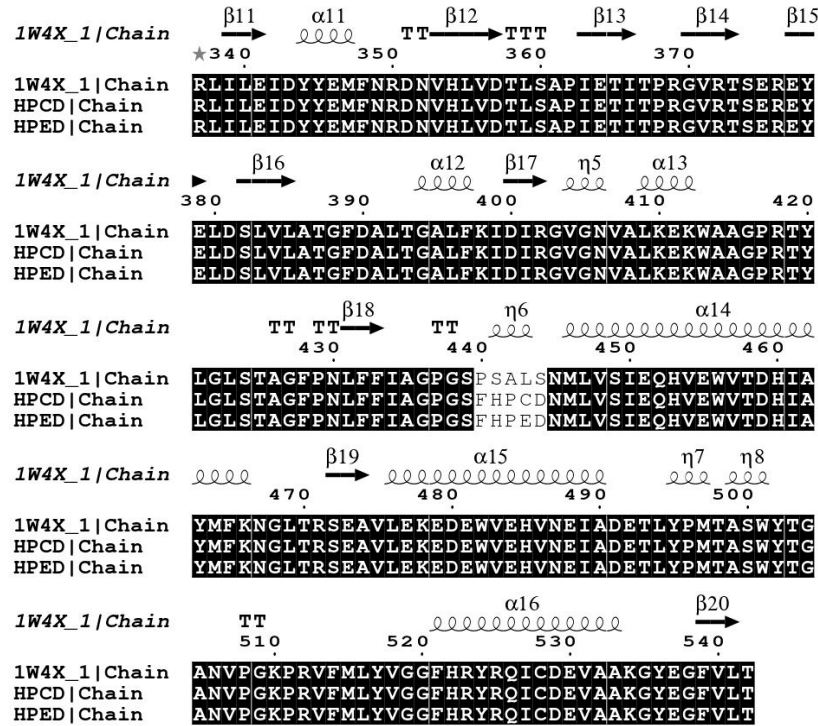


**Figure S2.** Sequence alignment of PAMOWT and its new variants PAMO<sub>HPCD</sub> and PAMO<sub>HPED</sub>. Black boxes correspond to the conserved residues in all sequences, whereas white boxes indicate the residues that have been substituted.

### 3. Electrophoretic mobility analysis of PAMOWT and its variants

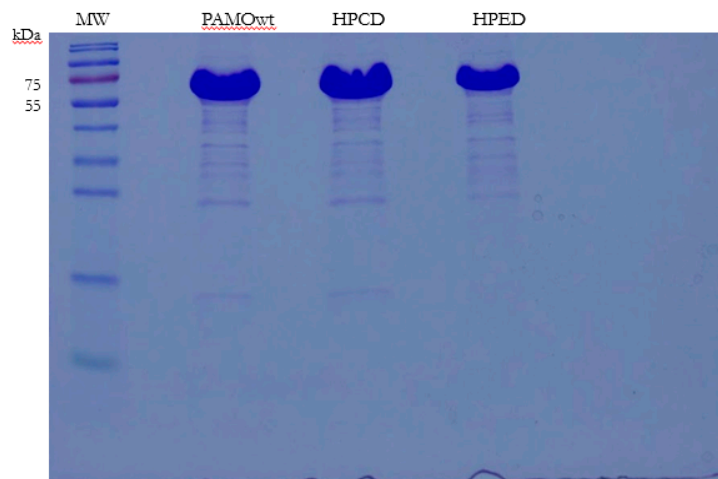


**Figure S3.** SDS-PAGE of the purified enzymes PAMOWT and the new variants PAMO<sub>HPCD</sub> and PAMO<sub>HPED</sub>. The enzymes, which have molecular weight of ~62 kDa, are highly pure and visible in the gap between 55 and 75 kDa of the protein ladder.

#### 4. Thermal stability of PAMO<sub>WT</sub>, PAMO<sub>HPCD</sub> and PAMO<sub>HPED</sub> determined by DSC

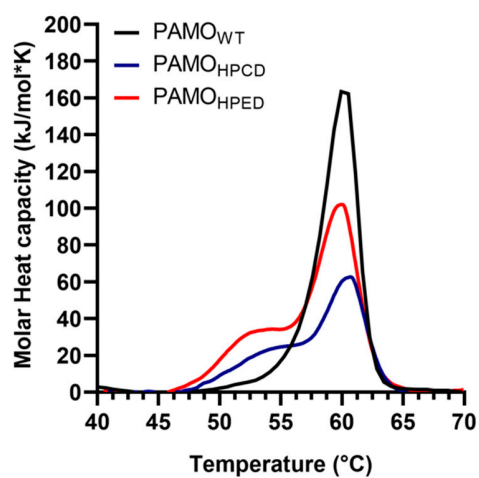


**Figure S4.** Thermal stability of PAMO<sub>WT</sub>, PAMO<sub>HPCD</sub> and PAMO<sub>HPED</sub> determined by DSC. The T<sub>m</sub> of each enzyme corresponds to the temperature in which the molar heat capacity reaches its maximum value.

#### 5. Standardization of HPLC-UV/Vis for indigo and indirubin concentration

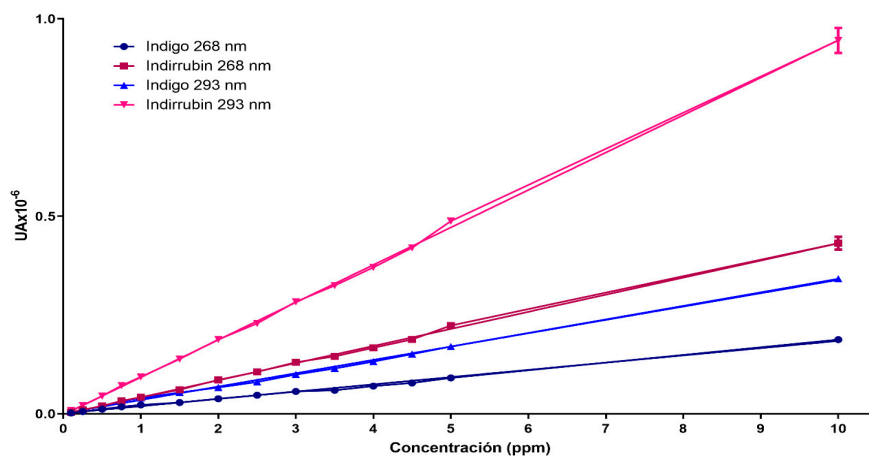


**Figure S5.** Calibration curves for HPLC-UV/Vis.

The calibration curve for HPLC with the standard indigo and indirubin was carried out in a concentration range between 0.1 ppm to 10 ppm with 20  $\mu$ L of injection.

## 6. Pigment patron obtained with PAMO variants

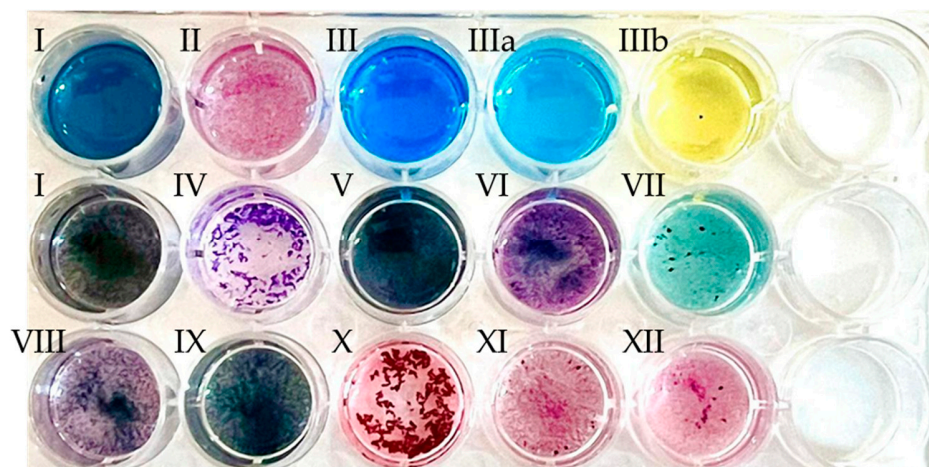


**Figure S6.** Indigoid derivatives obtained by enzyme catalysis of the substrates indicated in Table 2, using clarified crude extracts of *E. coli* cell cultures overexpressing PAMO<sub>HPCD</sub> and supplemented with phosphite dehydrogenase and sodium phosphite for cofactor regeneration. This figure resembles Figure 6 in the main text, with the exception that the indigo derivatives obtained by enzyme catalysis were kept in phosphate buffer, in which most of them are insoluble solids as it is clear from this image. The first row corresponds to commercial indigoid standards. First row: I, indigo (DMSO); II, indirubin (DMSO); III, indigo carmine (water); IIIa, indigo carmine (HCl 0.10 N); IIIb, indigo carmine (NaOH 0.10 M). Second row: I, indigo; IV, 5,5'-dicyanoindigo; V, 5,5'-difluoroindigo; VI, 5,5'-dichloroindigo; VII, 5,5'-hydroxyindigo. Third row: VIII, dimethylindigo; IX, 5,5'-dimethoxyindigo; X, 6,6'-difluoroindigo; XI, 6,6'-dichloroindigo; XII, 6,6'-dibromoindigo.

7. High-resolution mass spectrometry (HRMS) of indigo derivatives. Negative ion spectra.

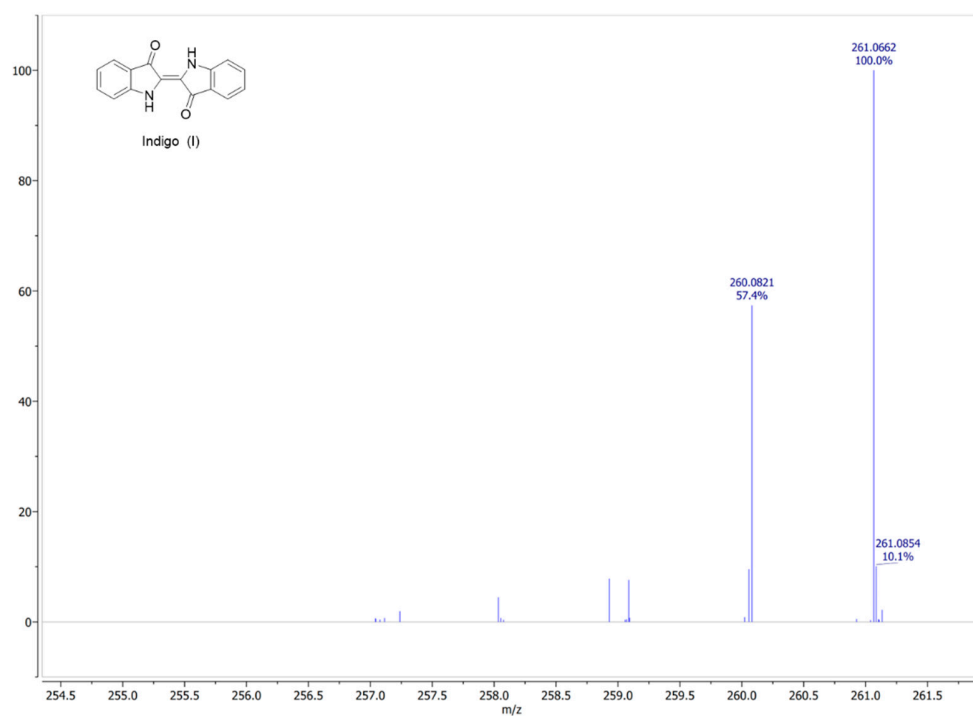


Figure S7. HRMS of indigo (I).  $[M-H]^- = 261.0662$  (100%)

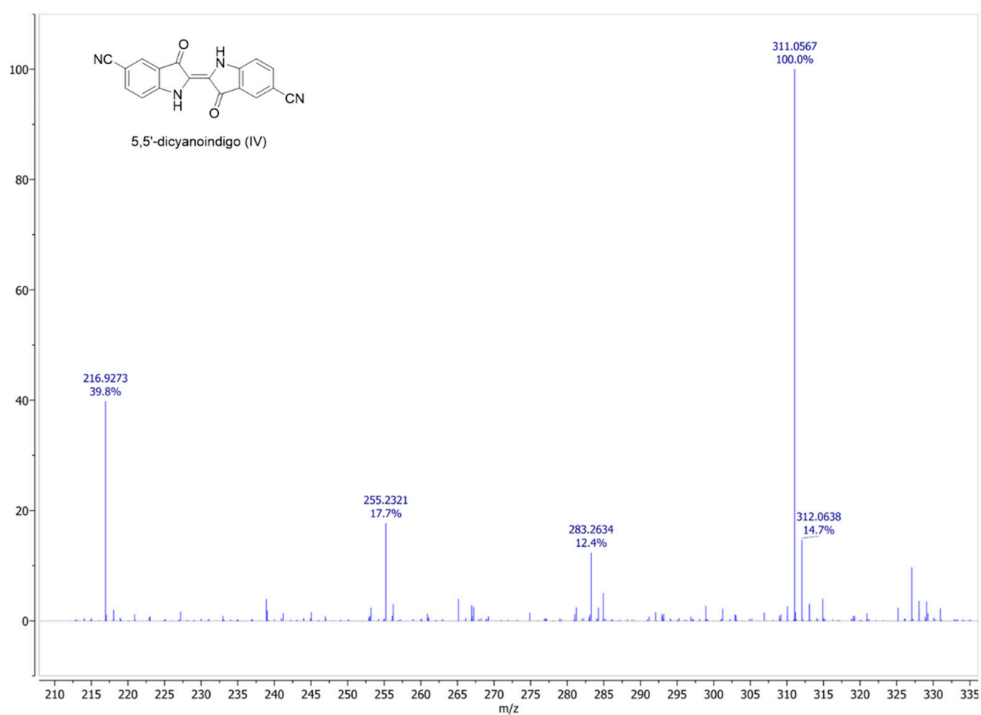


Figure S8. HRMS of 5,5'-dicyanoindigo (IV).  $[M-H]^- = 311.0567$  (100%)

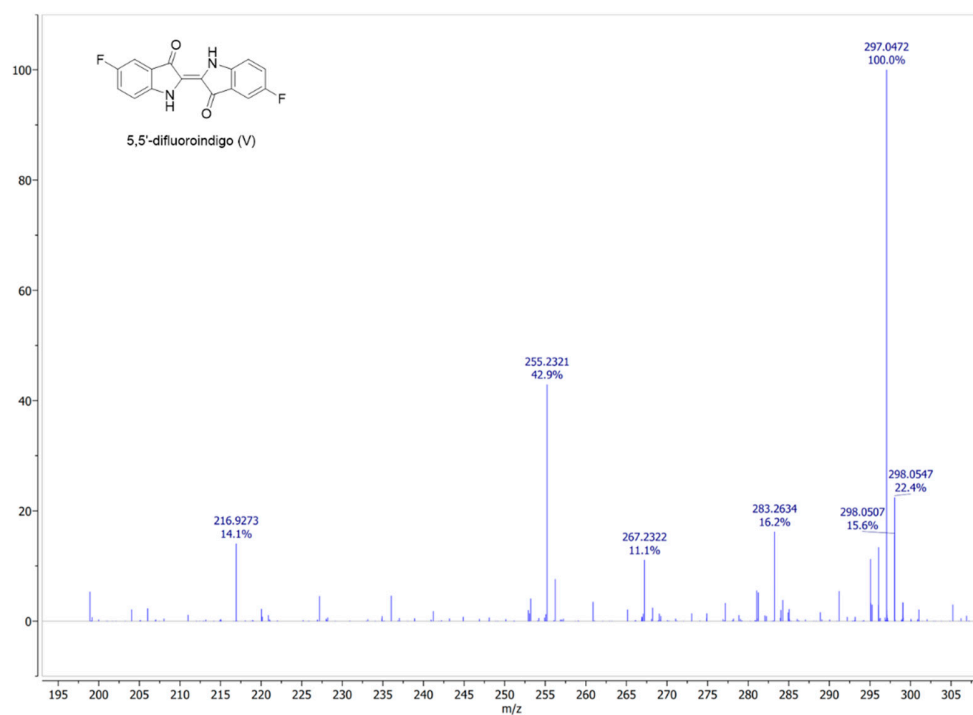


**Figure S9.** HRMS of 5,5'-difluoroindigo (V).  $[M-H]^- = 297.0472$  (100%)

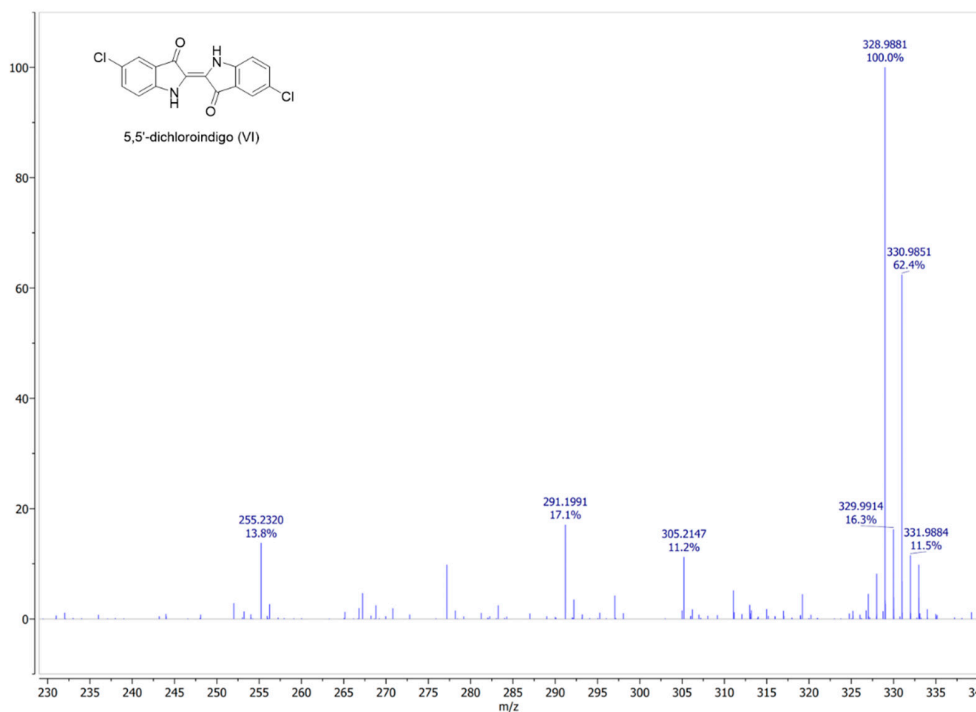


**Figure S10.** HRMS of 5,5'-dichloroindigo (VI).  $[M-H]^- = 328.9881$ ;  $m/z = 329.9914$  (16.3%);  $m/z = 330.9851$  (62.4%);  $m/z = 331.9884$  (11.5%)

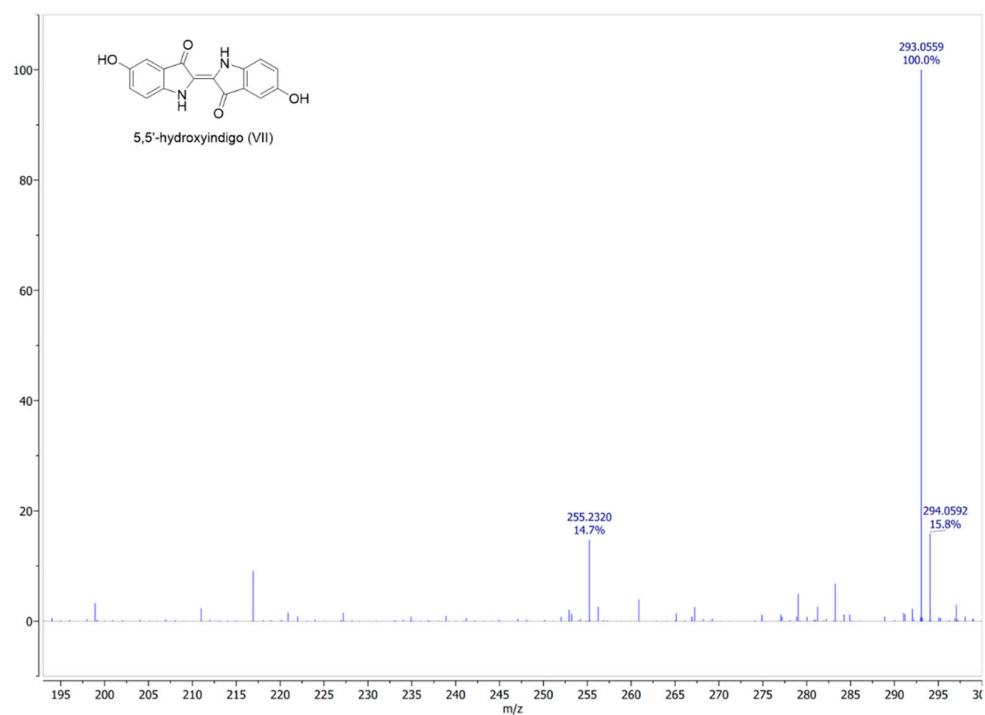


**Figure S11.** HRMS of 5,5'-hydroxyindigo (VII).  $[M-H]^- = 293.0559$  (100%)

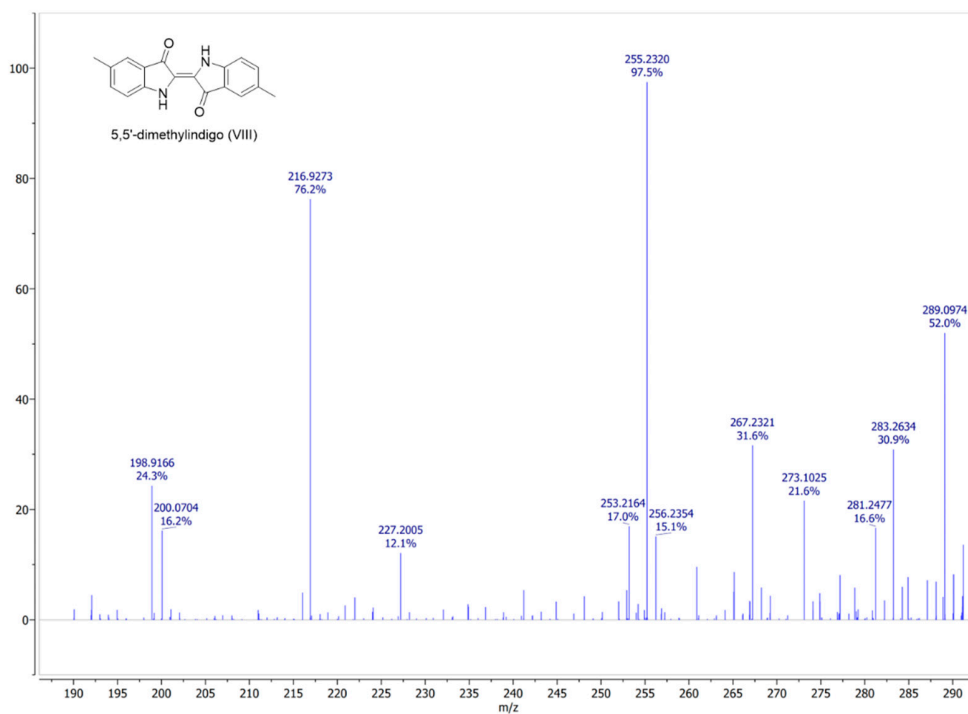


**Figure S12.** HRMS of 5,5'-dimethylindigo (VIII).  $[M-H]^- = 289.0974$  (52%)

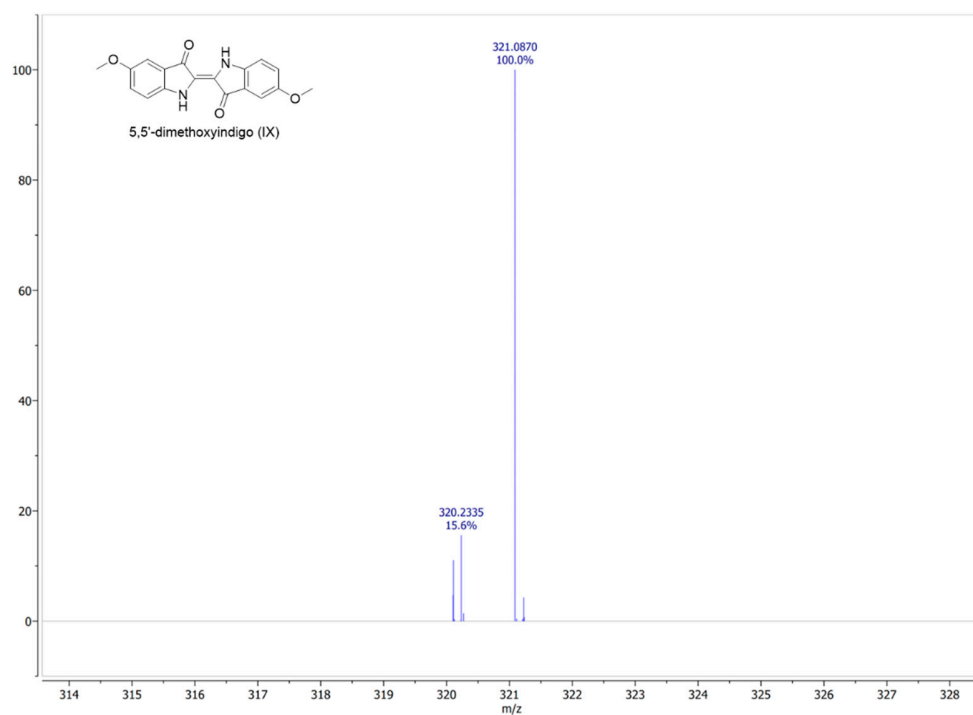


**Figure S13.** HRMS of 5,5'-dimethoxyindigo (IX).  $[M-H]^- = 321.0870$  (100%)

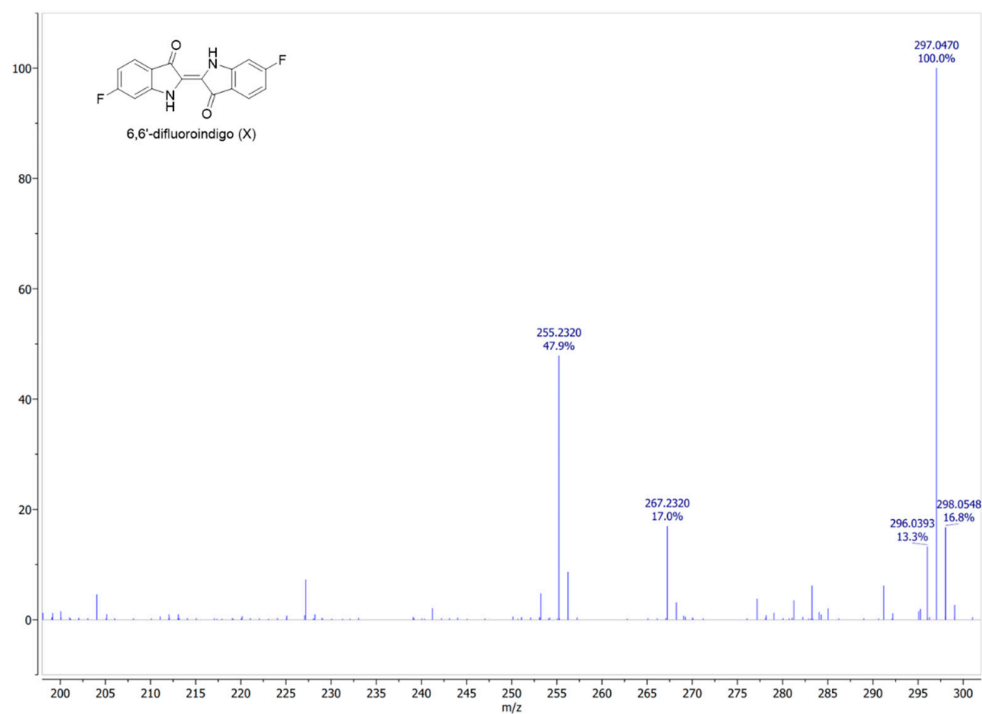


**Figure S14.** HRMS of 6,6'-difluoroindigo (X).  $[M-H]^- = 297.0470$  (100%)

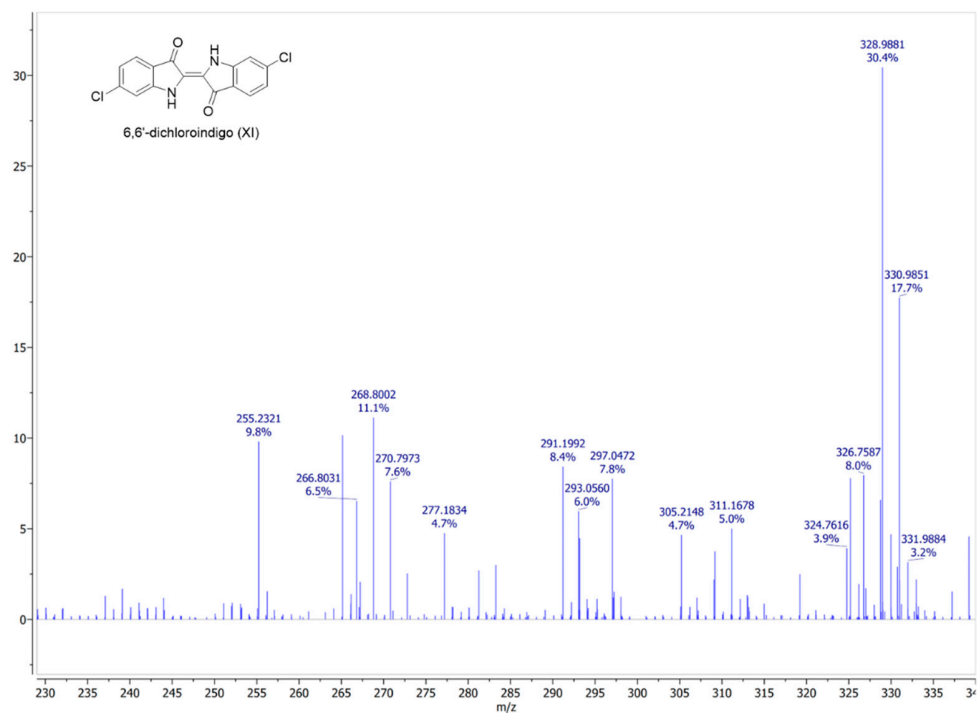


**Figure S15.** HRMS of 6,6'-dichloroindigo (XI).  $[M-H]^- = 328.9881$  (30.4%);  $m/z = 330.9851$  (17.7%);  $m/z = 331.9884$  (3.2%)

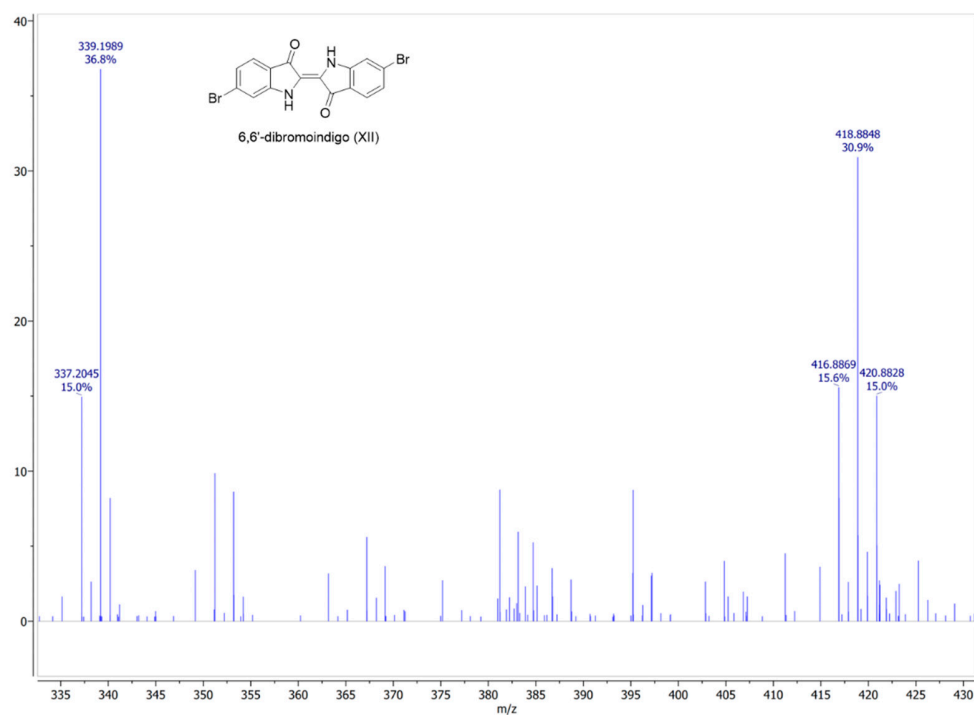


**Figure S16.** HRMS of 6,6'-dibromoindigo (XII).  $[M-H]^- = 416.8869$  (15.6%);  $m/z = 418.8848$  (30.9%);  $m/z = 420.8828$  (15%)