Electronic Supplementary Information

Colorimetric quantitative detection of steroid hormones using an indicator displacement assay-based chemosensor array

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Contents

1. General

Regents and materials

Commercially available reagents were used to prepare chemosensors, identification, and chemical sensing without further purification. The target steroid hormones and indicators obtained from Tokyo Chemical Industry Co., Ltd. were cortisol (H1), testosterone (H2), corticosterone (H3), cholic acid (H4), dehydroepiandrosterone (H5), estrone (H6), 17β-estradiol (H7), 17α-ethinylestradiol (H8), megestrol acetate (H9), nandrolone (H10), pancuronium bromide (H11), prednisolone (H12), progesterone (H13), drospirenone (H14), vecuronium bromide (H15), 4-amino-4'-dimethylaminoazobenzene (**1**), 4-hydroxy-4'-dimethylaminoazobenzene (**2**), and 4-(4-diethylaminophenylazo)pyridine (**3**). Hydrogen chloride (HCl) and dimethylsulfoxide (DMSO) were purchased from KANTO CHEMICAL CO., INC. The solvent and reagents obtained from Sigma-Aldrich Co., LLC. were D_2O, deuterium chloride (DCl) solution, cucurbit[8]uril hydrate (CB[8]), 2,5-dihydroxybenzoic acid and *α*-cyano-4-hydroxycinnamic acid. Dimethylsulfoxide-d_6 was purchased from Cambridge Isotope Laboratories, Inc.

Apparatuses and methods

NMR analysis**:** A JEOL ECZ-600 spectrometer was used for NMR measurements. Solvent peaks were used for internal standards. The pD of the solutions was adjusted using DCl, which was determined using a LAQUAtwin pH-22B pH meter.

MALDI-TOF MS analysis: Matrix Assisted Laser Desorption/Ionization time of flight (MALDI-TOF) MS measurements were performed using a JEOL JMS-S3000 Spiral-TOF mass spectrometer. *For the complex of indicator and CB[8]*: The mixture of CB[8] (5 mg), **2** (1 mg), and 2,5-dihydroxybenzoic acid (60 mg) was ground using an agate mortar. The obtained sample was dispersed in an aqueous DMSO solution (DMSO:water = 1:9, v/v) and dispensed on a target plate. *For the complex of CB[8] and testosterone*: The mixture of CB[8] (1 mg/mL) and testosterone (2 mg/mL) was dissolved in an aqueous DMSO solution (DMSO:water = 1:9, v/v). The sample was dropcasted on the target plate. Next, a matrix, *α*-cyano-4-hydroxycinnamic acid (10 mg/mL) in an acetonitrile solution (acetonitrile:water = 4:6, v/v) was dropcasted on the target plate.

UV-vis titration: A Shimadzu UV-2600 spectrophotometer was used to record UV-vis absorption spectra within the wavelength range from 350 to 750 nm for **1**, 350 to 700 nm for **2**, and 375 to 650 nm for **3** at 25 °C. All UV-vis titrations were performed in an aqueous DMSO solution (DMSO:water=1:9, v/v) at pH 3.2 at 25 °C. The pH of the solutions was adjusted using HCl. Each concentration of indicators (10 μM) and CB[8] (20 μM) for titrations of steroid hormones was defined based on host titrations. The complexation rates for indicators (10 μM) and CB[8] (20 μM) were estimated to be 78% for **1**, 87% for **2**, and 92% for **3**, respectively.

Array experiments: For a qualitative assay, a mixture solution (100 μL) containing an indicator (**1**, **2**, or **3**) (10 μM), CB[8] (20 μM), and a target steroid hormone (30 μM) was injected into each well of 384-well microtiter plastic plates (Thermo Scientific™, White 384-Well Immuno Plates, No. 460372) using a Pipetman (Gilson, P200M). The colorimetric changes in the chemosensors were recorded using a Biotek SYNERGY H1M microplate reader. Each experiment was replicated 24 times for validation. The obtained data matrix was pre-treated using the Student's *t*-test to exclude 4 outliers from 24 repetitions. Python 3.12 utilizing the NumPy package was employed for the date-processing. Linear discriminant analysis (LDA) was performed using SYSTAT 13 without further treatment. For a semi-quantitative assay, the selected main targets (testosterone (H2) and 17β-estradiol (H7)) were mixed at different molar ratios (100:0, 80:20, 60:40, 40:60, 20:80, and 0:100) in chemosensor solutions. The mixture concentration was set to be 25 μM. For a quantitative assay, the selected main targets (dehydroepiandrosterone (H5), 17*β*-estradiol (H7), and progesterone (H13)) were mixed in

chemosensor solutions. The calibration lines for each steroid hormone were built using a support vector machine (SVM). In this assay, two unknown concentrations for each analyte were predicted using a support vector machine (SVM) with Solo 9.0. (Figure S45).

Progesterone detection in human saliva: The research using human saliva samples was conducted according to The ARRIVE guidelines 2.0. Human saliva samples were provided by a healthy volunteer according to the authorization of the Ethics Committee of the University of Tokyo (ethics authorization code: 24–385). Complete written informed consent was obtained from the volunteer. Before the saliva sample was collected, the subject was required to avoid eating or drinking for at least 30 min and process oral rinsing for 5 min. The collected saliva samples were first pretreated through centrifuging at 3000 rpm for 10 min. After this period, the supernatant (2 mL) was mixed with an aqueous DMSO solution (DMSO:water = 1:9, v/v) at pH 3.2 at 25 °C (18 mL). The analyte solution (progesterone, 10 µL) was added to the above solutions (90 µL) ([Progesterone] = 0−28 ppm). The SVM was performed to build a calibration line using 7 concentration points, followed by the prediction of 2 concentration points.

DFT calculation: All calculations were performed using the B3LYP-D3(BJ) function of the Gaussian 16 package (Revision C.01).[S1] The B3LYP function was appended with DFT-D3 correction using the Becke-Johnson damping function. The geometry optimizations were performed using 6-311+G(d) basis set. The possible starting structures were manually constructed based on single crystal structures[S2] and a previous report,[S3] including the different plausible rotamers and hydrogen bonding patterns. Noncovalent interactions based on Hirshfield partition of molecular density (IGMH) analysis for the optimized geometry structures were obtained by wave function analysis by Multiwfn[S4,S5] and rendered by Visual Molecular Dynamics (VMD) program.[S6] All calculations were carried out under vacuum environment conditions.

2. ^{1}H NMR analysis

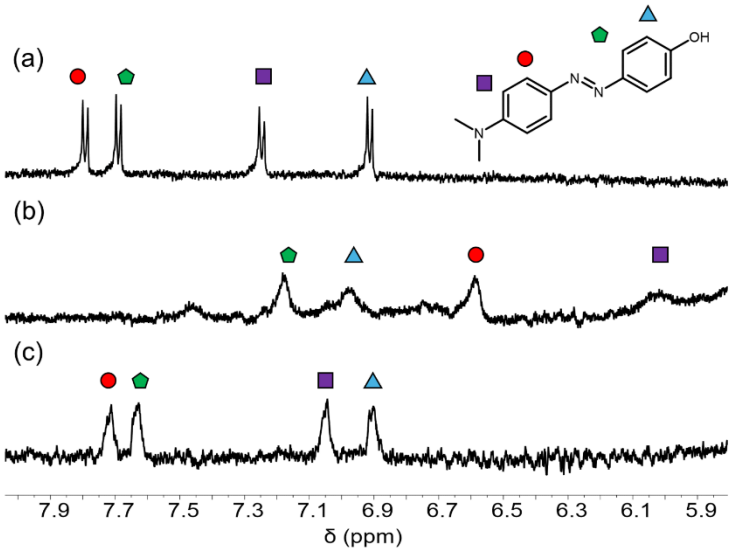

Fig. S1 ^{1}H NMR spectra (600 MHz) of (a) **2** (100 µM), (b) **2**·CB[8] (**2**:CB[8] = 1:2, mol/mol), (c) the mixture of **2**, CB[8], and testosterone (**2**:CB[8]:testosterone = 1:1:10, mol/mol/mol) in DMSO-d_6 : D_2O (1 : 9, v/v) at pD 3.

3. DFT calculations

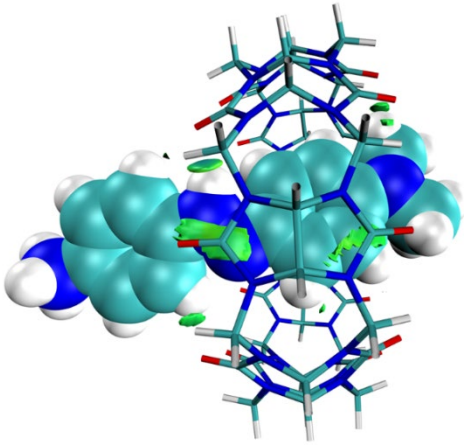

Fig. S2 The isosurface IGMH figure of the optimized complex with **1** and CB[8] at 1 to 1 binding stoichiometry. The green areas indicate the intermolecular noncovalent interactions.

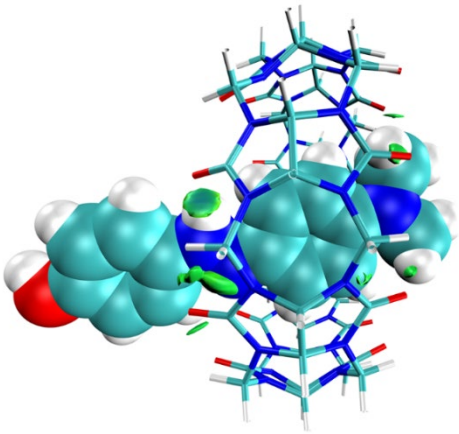

Fig. S3 The isosurface IGMH figure of the optimized complex with **2** and CB[8] at 1 to 1 binding stoichiometry. The green areas indicate the intermolecular noncovalent interactions.

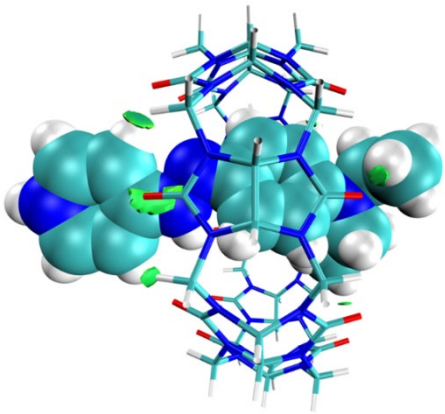

Fig. S4 The isosurface IGMH figure of the optimized complex with **3** and CB[8] at 1 to 1 binding stoichiometry. The green areas indicate the intermolecular noncovalent interactions.

4. MALDI-TOF MS analysis

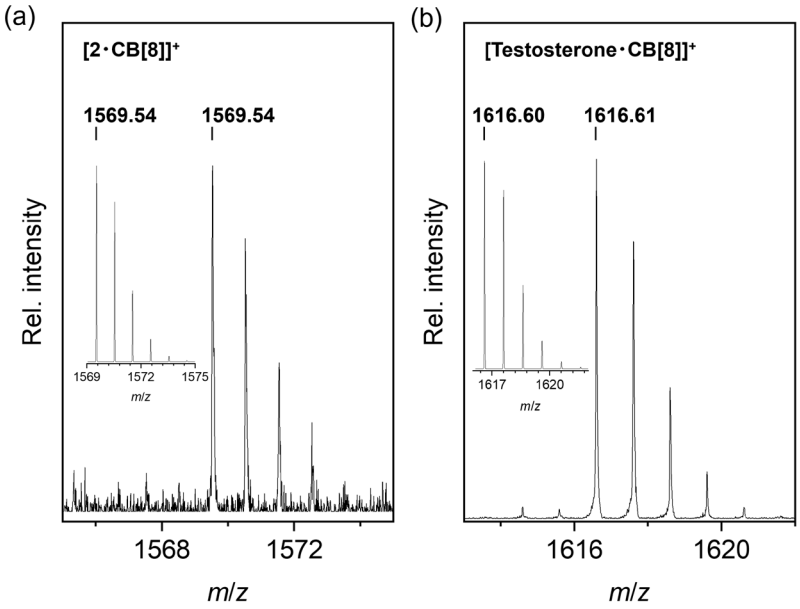

Fig. S5 MALDI-TOF mass spectra of the complexes of (a) **2** and CB[8] and (b) CB[8] and testosterone. The insets show calculation isotope patterns for (a) $C_{62}H_{63}N_{35}O_{17}$ and (b) $C_{67}H_{76}N_{32}O_{18}$.

5. UV-vis titrations for CB[8]

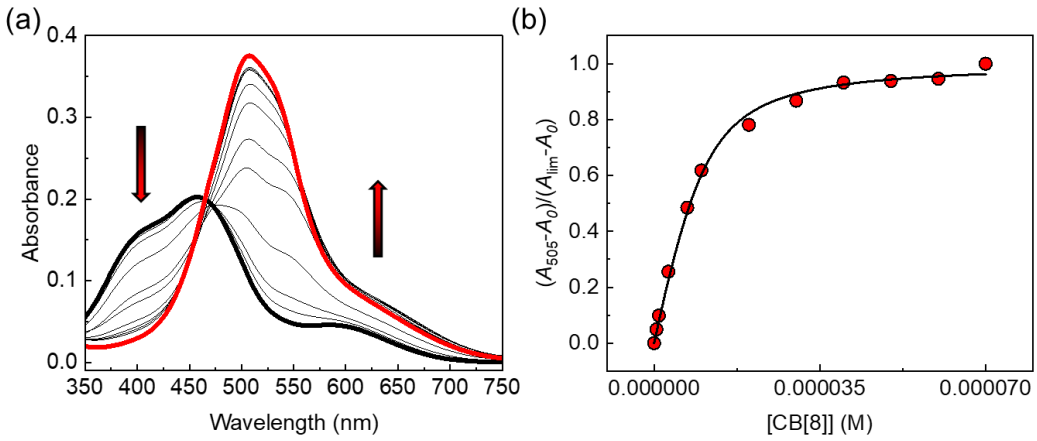

Fig. S6 (a) UV-vis absorption spectra of **1** (10 μM) upon the addition of CB[8] (0−70 μM) in an aqueous DMSO solution (DMSO:water = 1:9, v/v) at pH 3.2 at 25 °C. (b) Titration isotherm for CB[8]. The terms A_0 and A_{505} represent absorbance at a certain wavelength before and after adding CB[8]. The term A_{lim} indicates absorbance at saturated responses.

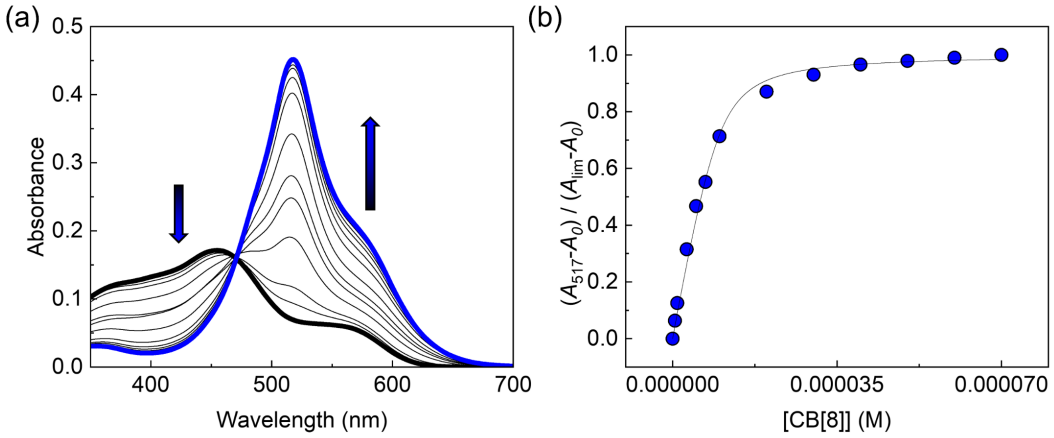

Fig. S7 (a) UV-vis absorption spectra of **2** (10 μM) upon the addition of CB[8] (0–70 μM) in an aqueous DMSO solution (DMSO:water = 1:9, v/v) at pH 3.2 at 25 °C. (b) Titration isotherm for CB[8]. The terms A_0 and A_{517} represent absorbance at a certain wavelength before and after adding CB[8]. The term A_{lim} indicates absorbance at saturated responses.

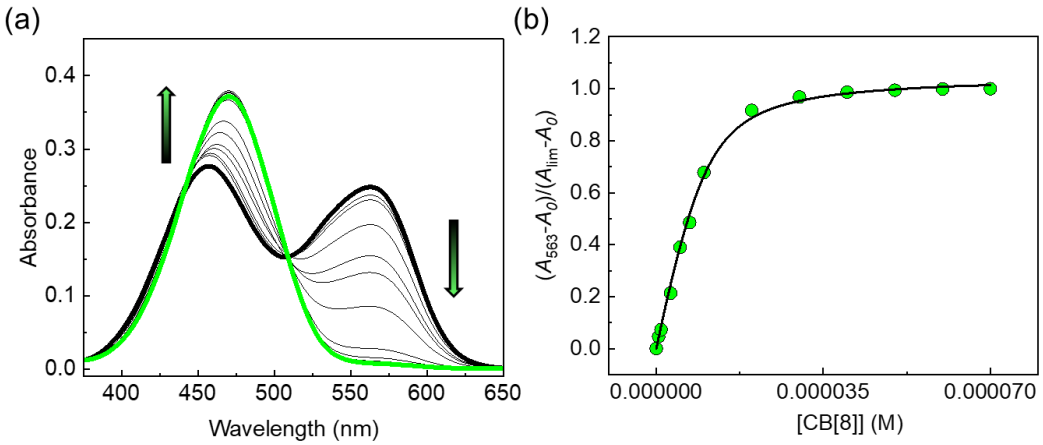

Fig. S8 (a) UV-vis absorption spectra of **3** (10 μM) upon the addition of CB[8] (0–70 μM) in an aqueous DMSO solution (DMSO:water = 1:9, v/v) at pH 3.2 at 25 °C. (b) Titration isotherm for CB[8]. The terms A_0 and A_{563} represent absorbance at a certain wavelength before and after adding CB[8]. The term A_{lim} indicates absorbance at saturated responses.

6. Selected UV-vis titrations for steroid hormones

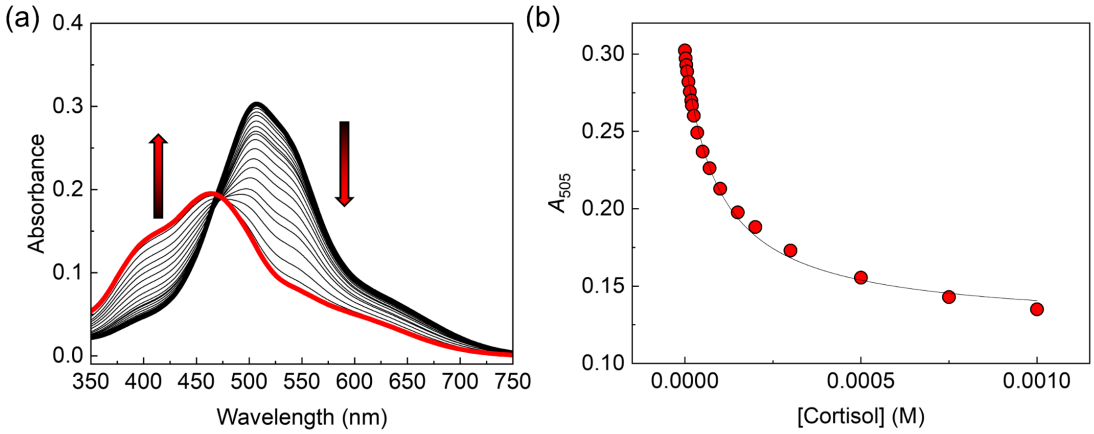

Fig. S9 (a) UV-vis absorption spectra of the complex of **1** (10 μM) and CB[8] (20 μM) upon the addition of cortisol in an aqueous DMSO solution (DMSO:water = 1:9, v/v) at pH 3.2 at 25 °C. (b) Titration isotherm for cortisol. The titration isotherm was obtained by collecting the maximum absorbance at 505 nm. [Cortisol] = 0–1000 μM.

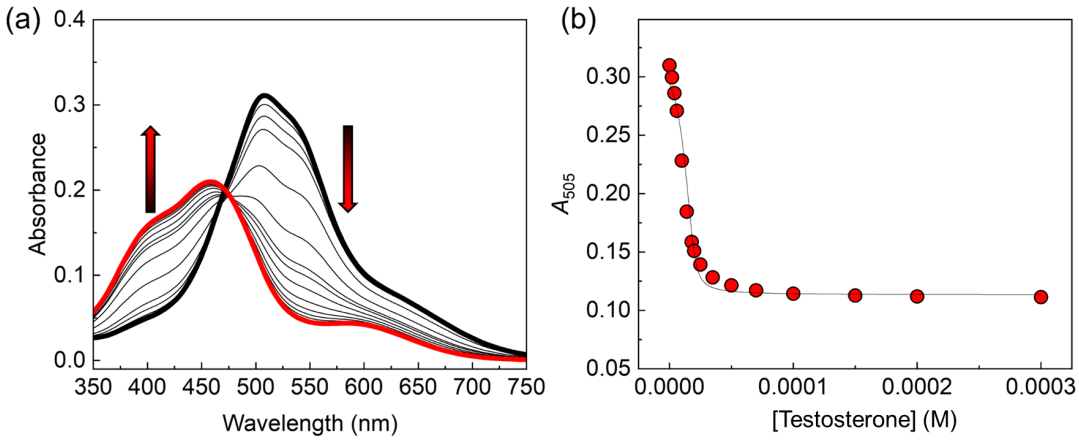

Fig. S10 (a) UV-vis absorption spectra of the complex of **1** (10 μM) and CB[8] (20 μM) upon the addition of testosterone in an aqueous DMSO solution (DMSO:water = 1:9, v/v) at pH 3.2 at 25 °C. (b) Titration isotherm for testosterone. The titration isotherm was obtained by collecting the maximum absorbance at 505 nm. [Testosterone] = 0–300 μM.

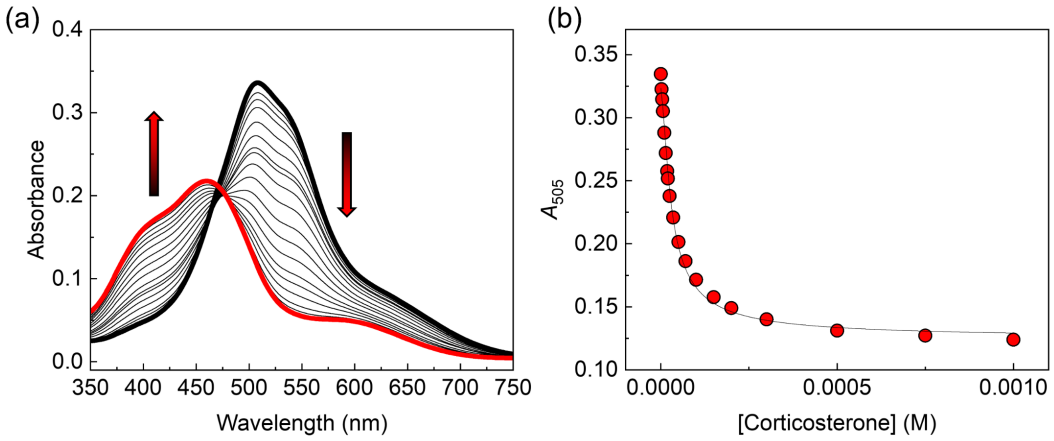

Fig. S11 (a) UV-vis absorption spectra of the complex of **1** (10 μM) and CB[8] (20 μM) upon the addition of corticosterone in an aqueous DMSO solution (DMSO:water = 1:9, v/v) at pH 3.2 at 25 °C. (b) Titration isotherm for corticosterone. The titration isotherm was obtained by collecting the maximum absorbance at 505 nm. [Corticosterone] = 0–1000 μM.

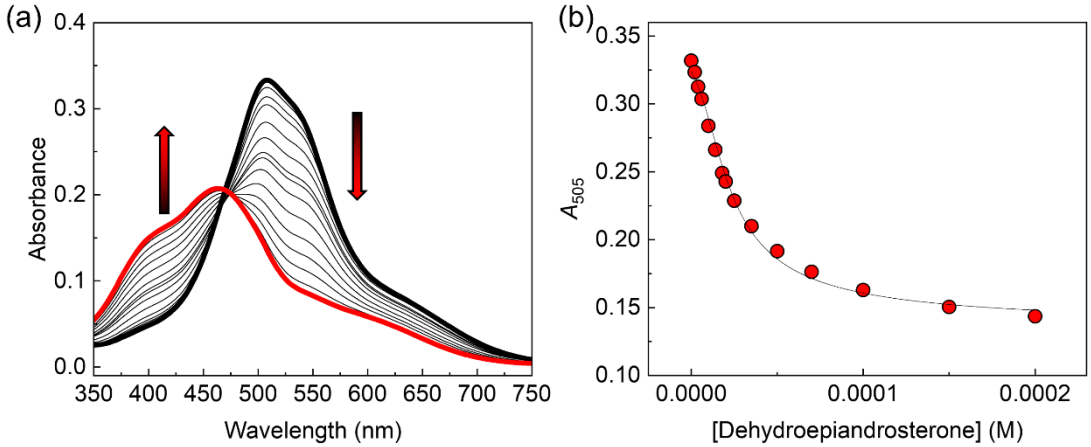

Fig. S12 (a) UV-vis absorption spectra of the complex of **1** (10 μM) and CB[8] (20 μM) upon the addition of dehydroepiandrosterone in an aqueous DMSO solution (DMSO:water = 1:9, v/v) at pH 3.2 at 25 °C. (b) Titration isotherm for dehydroepiandrosterone. The titration isotherm was obtained by collecting the maximum absorbance at 505 nm. [Dehydroepiandrosterone] = 0–200 μM.

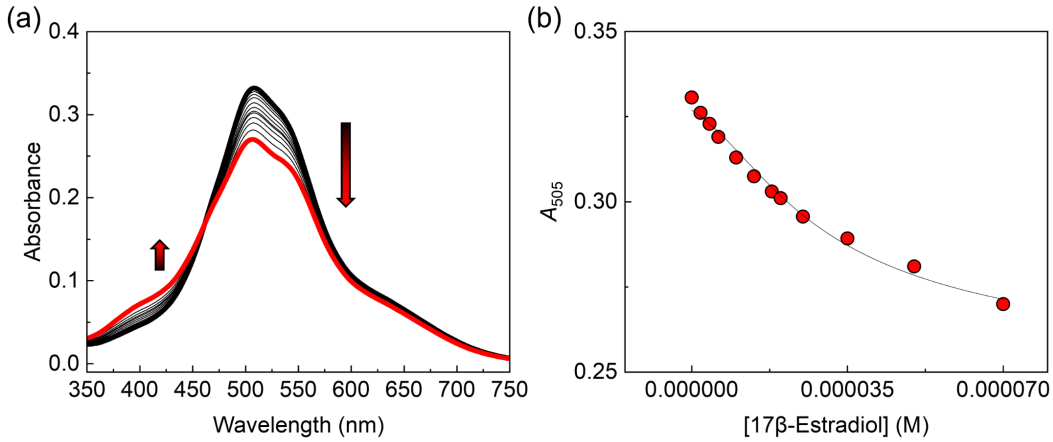

Fig. S13 (a) UV-vis absorption spectra of the complex of **1** (10 μM) and CB[8] (20 μM) upon the addition of 17β-estradiol in an aqueous DMSO solution (DMSO:water = 1:9, v/v) at pH 3.2 at 25 °C. (b) Titration isotherm for β-estradiol. The titration isotherm was obtained by collecting the maximum absorbance at 505 nm. [17β-Estradiol] = 0–70 μM.

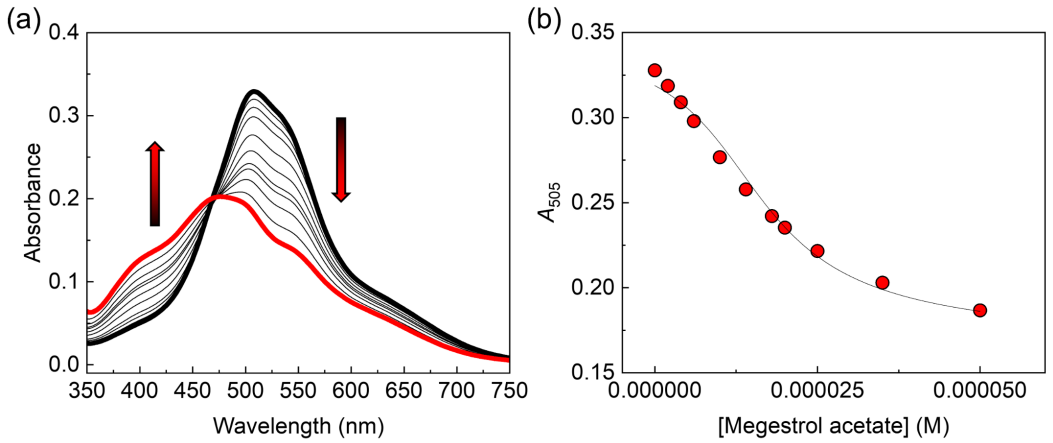

Fig. S14 (a) UV-vis absorption spectra of the complex of **1** (10 μM) and CB[8] (20 μM) upon the addition of megestrol acetate in an aqueous DMSO solution (DMSO:water = 1:9, v/v) at pH 3.2 at 25 °C. (b) Titration isotherm for megestrol acetate. The titration isotherm was obtained by collecting the maximum absorbance at 505 nm. [Megestrol acetate] = 0–50 μM.

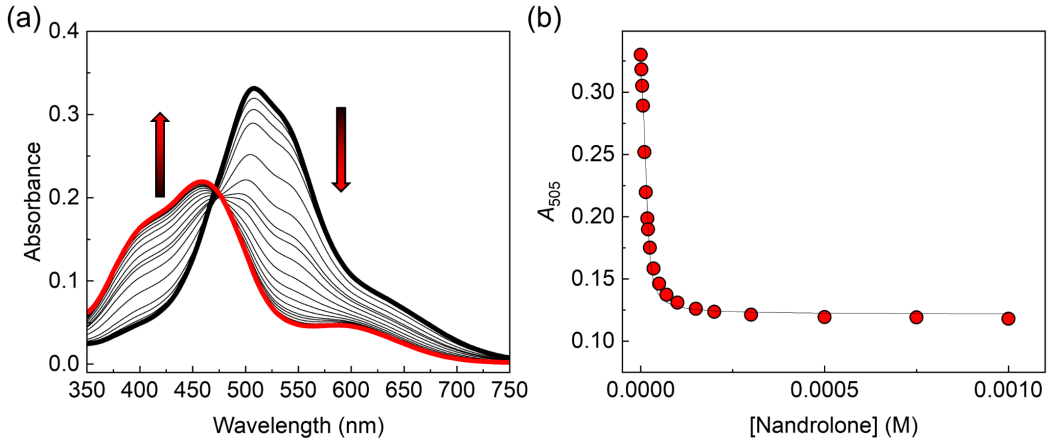

Fig. S15 (a) UV-vis absorption spectra of the complex of **1** (10 μM) and CB[8] (20 μM) upon the addition of nandrolone in an aqueous DMSO solution (DMSO:water = 1:9, v/v) at pH 3.2 at 25 °C. (b) Titration isotherm for nandrolone. The titration isotherm was obtained by collecting the maximum absorbance at 505 nm. [Nandrolone] = 0–1000 μM.

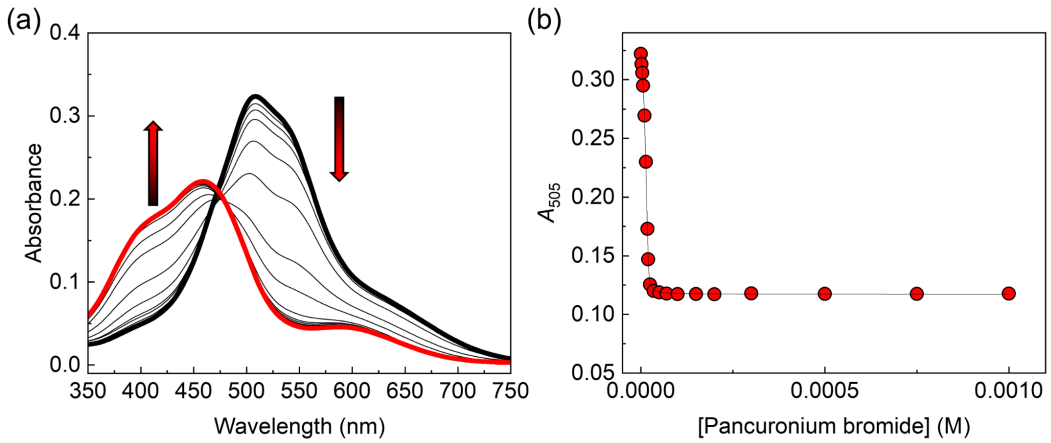

Fig. S16 (a) UV-vis absorption spectra of the complex of **1** (10 μM) and CB[8] (20 μM) upon the addition of pancuronium bromide in an aqueous DMSO solution (DMSO:water = 1:9, v/v) at pH 3.2 at 25 °C. (b) Titration isotherm for pancuronium bromide. The titration isotherm was obtained by collecting the maximum absorbance at 505 nm. [Pancuronium bromide] = 0−1000 μM.

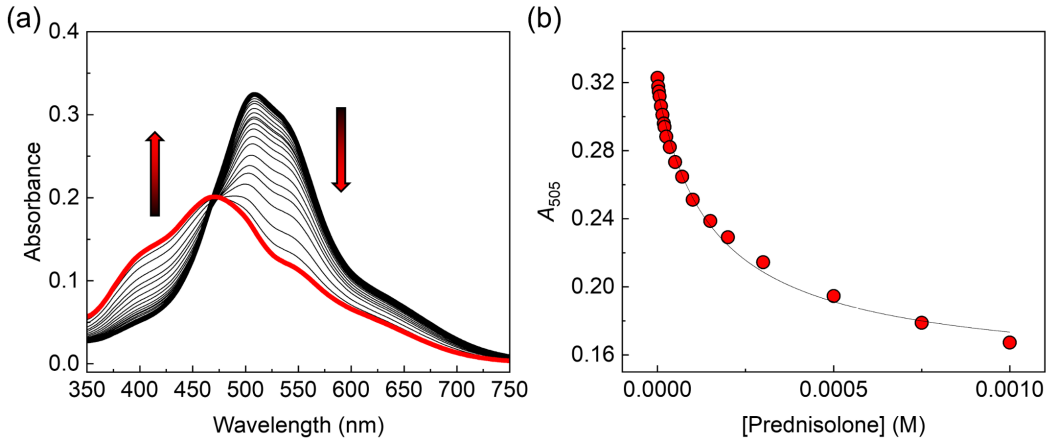

Fig. S17 (a) UV-vis absorption spectra of the complex of **1** (10 μM) and CB[8] (20 μM) upon the addition of prednisolone in an aqueous DMSO solution (DMSO:water = 1:9, v/v) at pH 3.2 at 25 °C. (b) Titration isotherm for prednisolone. The titration isotherm was obtained by collecting the maximum absorbance at 505 nm. [Prednisolone] = 0−1000 μM.

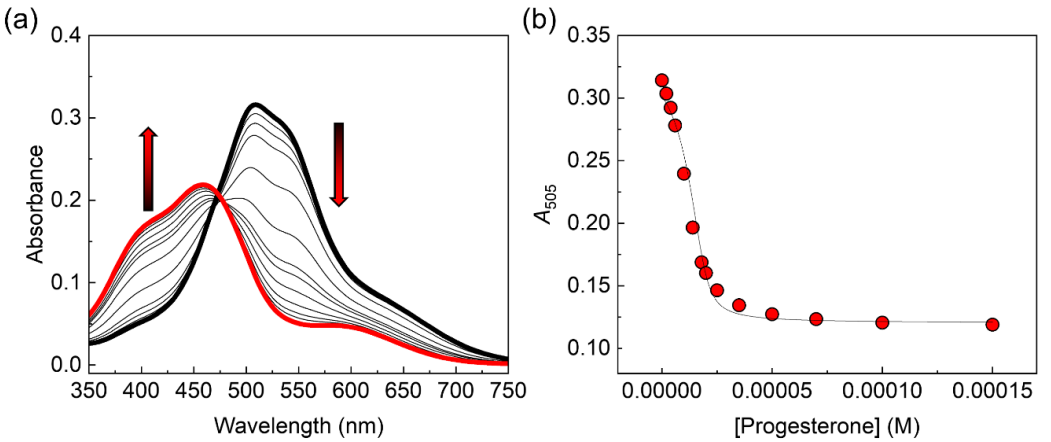

Fig. S18 (a) UV-vis absorption spectra of the complex of **1** (10 μM) and CB[8] (20 μM) upon the addition of progesterone in an aqueous DMSO solution (DMSO:water = 1:9, v/v) at pH 3.2 at 25 °C. (b) Titration isotherm for progesterone. The titration isotherm was obtained by collecting the maximum absorbance at 505 nm. [Progesterone] = 0−150 μM.

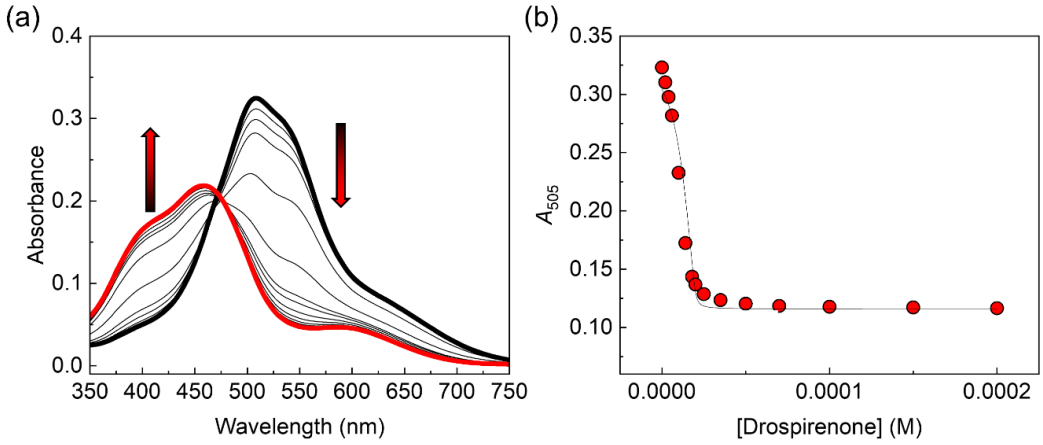

Fig. S19 (a) UV-vis absorption spectra of the complex of **1** (10 μM) and CB[8] (20 μM) upon the addition of drospirenone in an aqueous DMSO solution (DMSO:water = 1:9, v/v) at pH 3.2 at 25 °C. (b) Titration isotherm for drospirenone. The titration isotherm was obtained by collecting the maximum absorbance at 505 nm. [Drospirenone] = 0–200 μM.

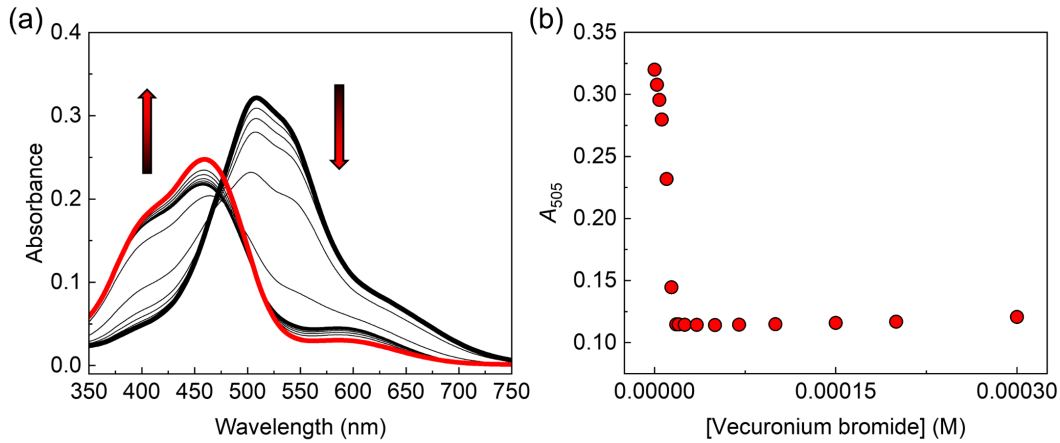

Fig. S20 (a) UV-vis absorption spectra of the complex of **1** (10 μM) and CB[8] (20 μM) upon the addition of vecuronium bromide in an aqueous DMSO solution (DMSO:water = 1:9, v/v) at pH 3.2 at 25 °C. (b) Titration isotherm for vecuronium bromide. The titration isotherm was obtained by collecting the maximum absorbance at 505 nm. [Vecuronium bromide] = 0–300 μM.

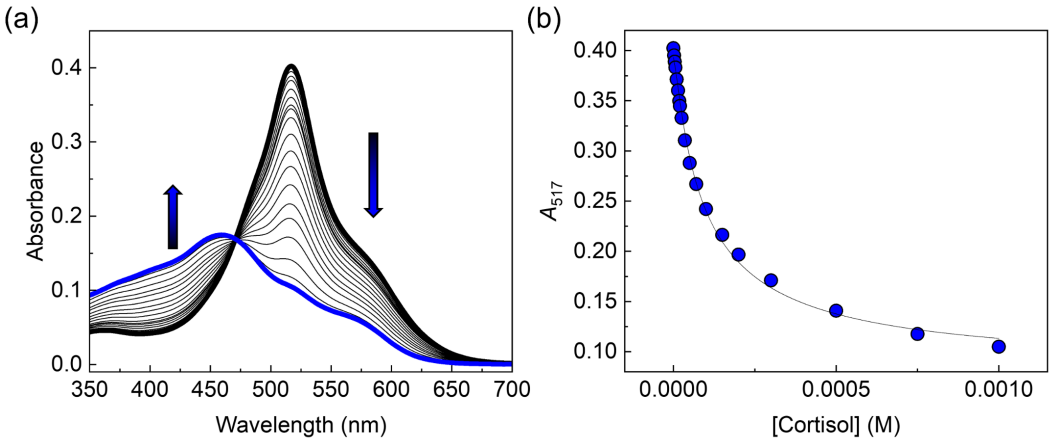

Fig. S21 (a) UV-vis absorption spectra of the complex of **2** (10 μM) and CB[8] (20 μM) upon the addition of cortisol in an aqueous DMSO solution (DMSO:water = 1:9, v/v) at pH 3.2 at 25 °C. (b) Titration isotherm for cortisol. The titration isotherm was obtained by collecting the maximum absorbance at 517 nm. [Cortisol] = 0–1000 μM.

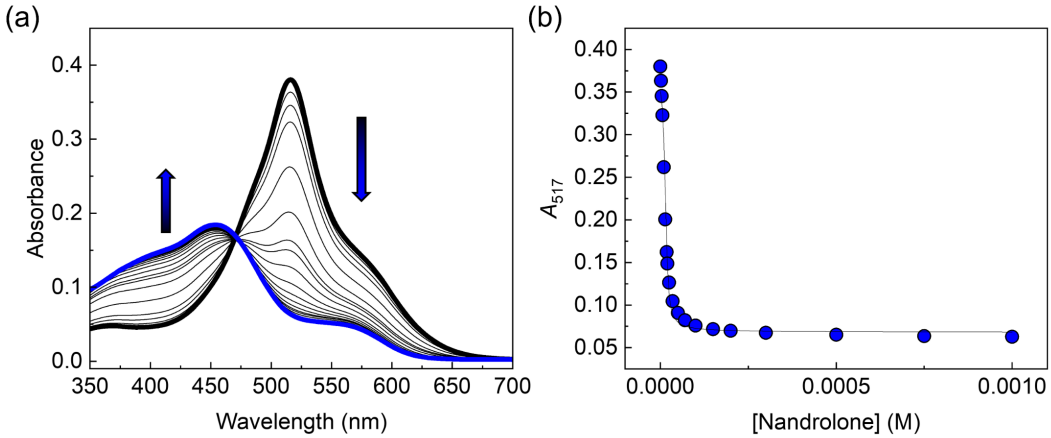

Fig. S22 (a) UV-vis absorption spectra of the complex of **2** (10 μM) and CB[8] (20 μM) upon the addition of nandrolone in an aqueous DMSO solution (DMSO:water = 1:9, v/v) at pH 3.2 at 25 °C. (b) Titration isotherm for nandrolone. The titration isotherm was obtained by collecting the maximum absorbance at 517 nm. [Nandrolone] = 0−1000 μM.

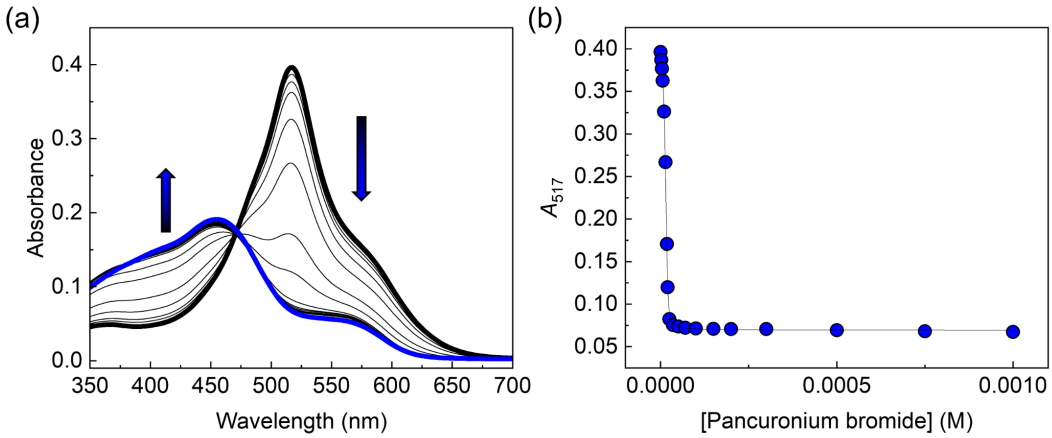

Fig. S23 (a) UV-vis absorption spectra of the complex of **2** (10 μM) and CB[8] (20 μM) upon the addition of pancuronium bromide in an aqueous DMSO solution (DMSO:water = 1:9, v/v) at pH 3.2 at 25 °C. (b) Titration isotherm for pancuronium bromide. The titration isotherm was obtained by collecting the maximum absorbance at 505 nm. [Pancuronium bromide] = 0−1000 μM.

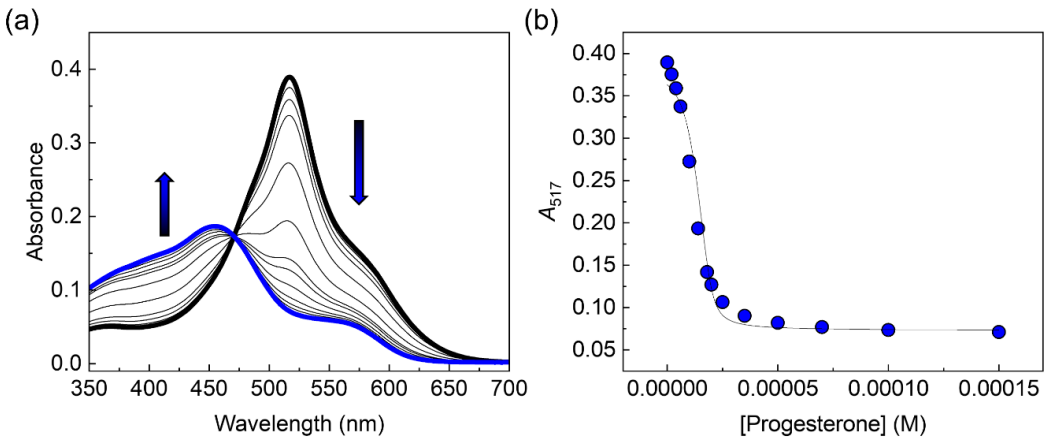

Fig. S24 (a) UV-vis absorption spectra of the complex of **2** (10 μM) and CB[8] (20 μM) upon the addition of progesterone in an aqueous DMSO solution (DMSO:water = 1:9, v/v) at pH 3.2 at 25 °C. (b) Titration isotherm for progesterone. The titration isotherm was obtained by collecting the maximum absorbance at 517 nm. [Progesterone] = 0−150 μM.

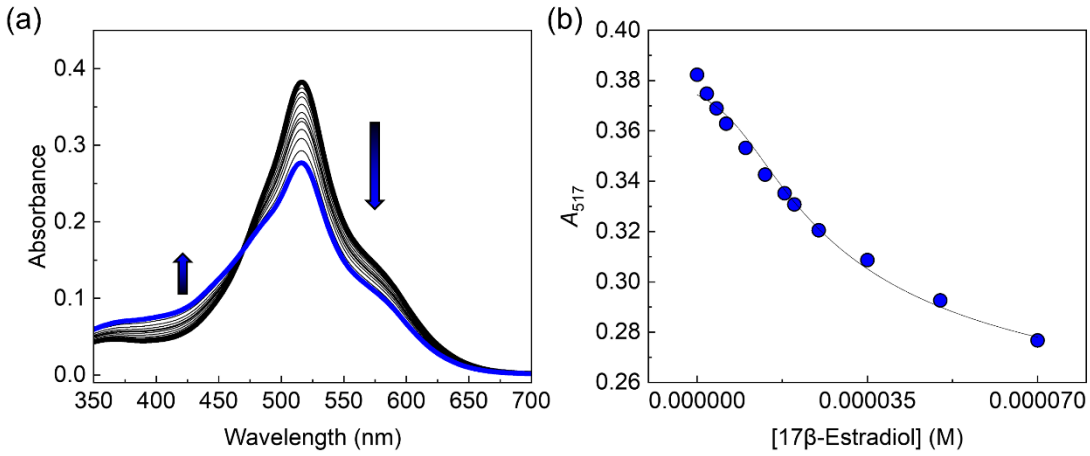

Fig. S25 (a) UV-vis absorption spectra of the complex of **2** (10 μM) and CB[8] (20 μM) upon the addition of 17β-estradiol in an aqueous DMSO solution (DMSO:water = 1:9, v/v) at pH 3.2 at 25 °C. (b) Titration isotherm for 17β-estradiol. The titration isotherm was obtained by collecting the maximum absorbance at 517 nm. [17β-Estradiol] = 0–70 μM.

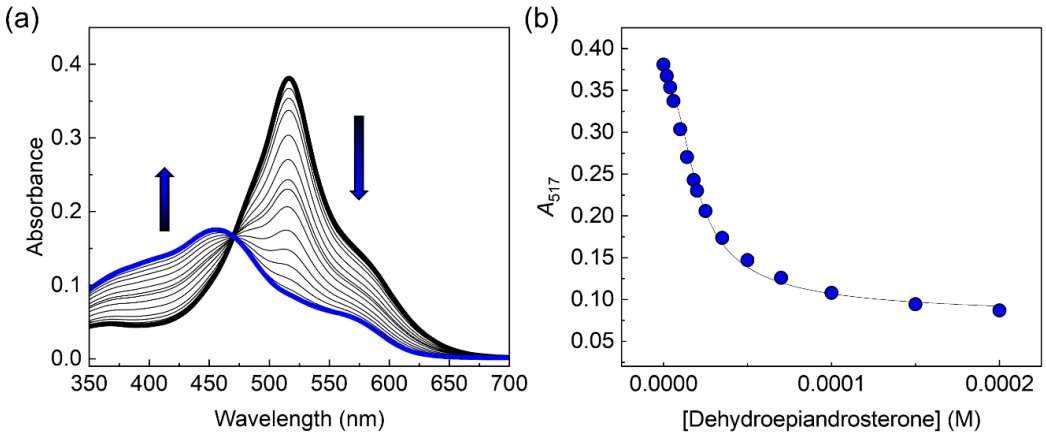

Fig. S26 (a) UV-vis absorption spectra of the complex of **2** (10 μM) and CB[8] (20 μM) upon the addition of dehydroepiandrosterone in an aqueous DMSO solution (DMSO:water = 1:9, v/v) at pH 3.2 at 25 °C. (b) Titration isotherm for dehydroepiandrosterone. The titration isotherm was obtained by collecting the maximum absorbance at 517 nm. [Dehydroepiandrosterone] = 0–200 μM.

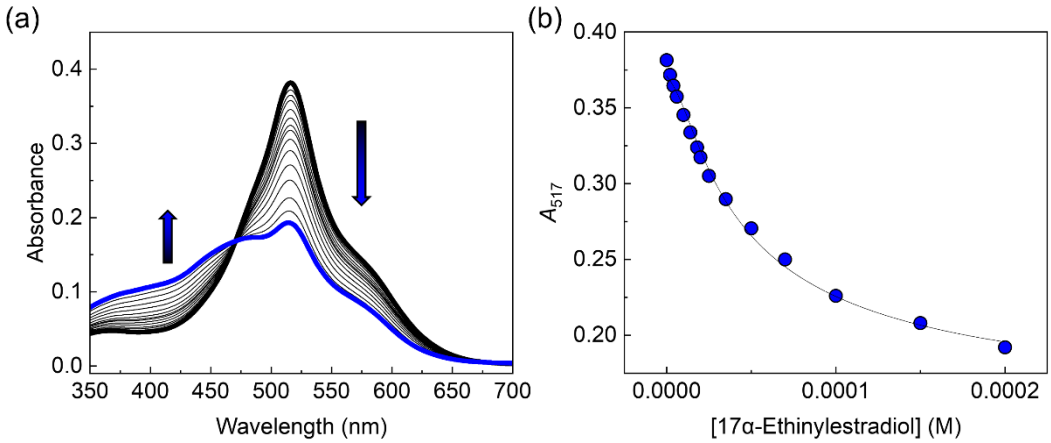

Fig. S27 (a) UV-vis absorption spectra of the complex of **2** (10 μM) and CB[8] (20 μM) upon the addition of 17α-ethinylestradiol in an aqueous DMSO solution (DMSO:water = 1:9, v/v) at pH 3.2 at 25 °C. (b) Titration isotherm for 17α-ethinylestradiol. The titration isotherm was obtained by collecting the maximum absorbance at 517 nm. [17α-Ethinylestradiol] = 0–200 μM.

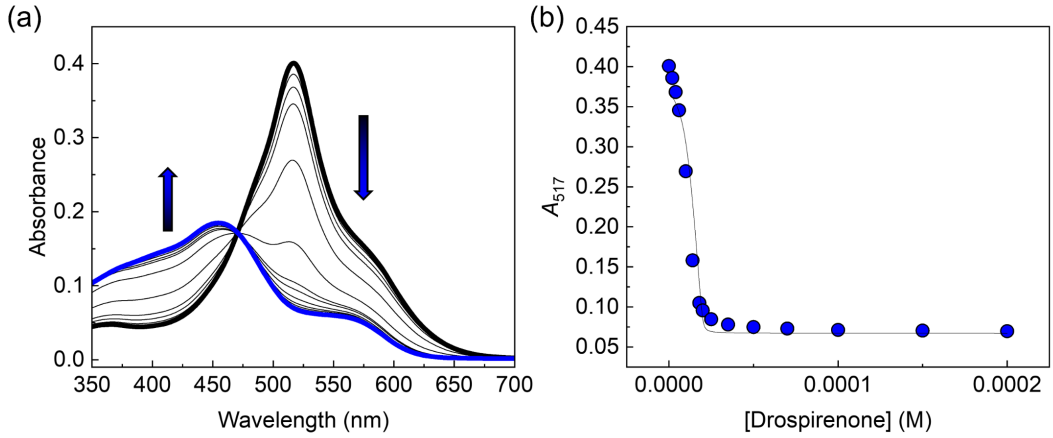

Fig. S28 (a) UV-vis absorption spectra of the complex of **2** (10 μM) and CB[8] (20 μM) upon the addition of drospirenone in an aqueous DMSO solution (DMSO:water = 1:9, v/v) at pH 3.2 at 25 °C. (b) Titration isotherm for drospirenone. The titration isotherm was obtained by collecting the maximum absorbance at 517 nm. [Drospirenone] = 0–200 μM.

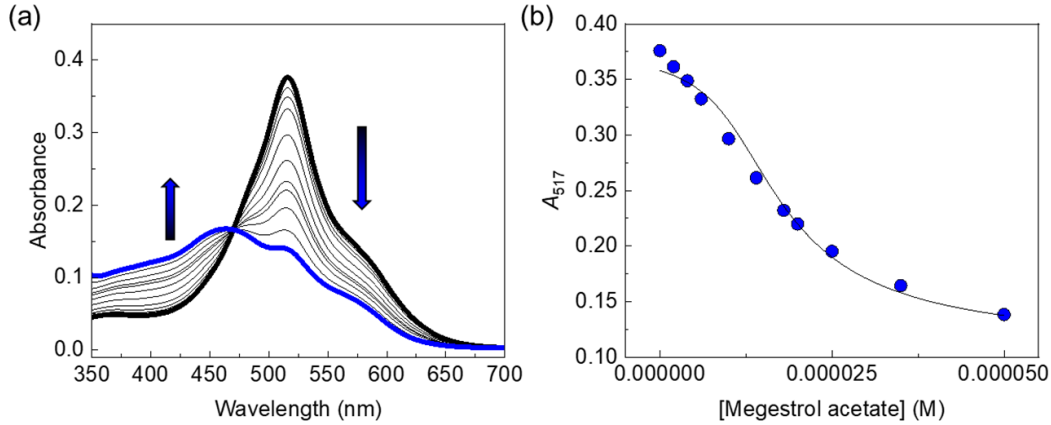

Fig. S29 (a) UV-vis absorption spectra of the complex of **2** (10 μM) and CB[8] (20 μM) upon the addition of megestrol acetate in an aqueous DMSO solution (DMSO:water = 1:9, v/v) at pH 3.2 at 25 °C. (b) Titration isotherm for megestrol acetate. The titration isotherm was obtained by collecting the maximum absorbance at 517 nm. [Megestrol acetate] = 0–50 μM.

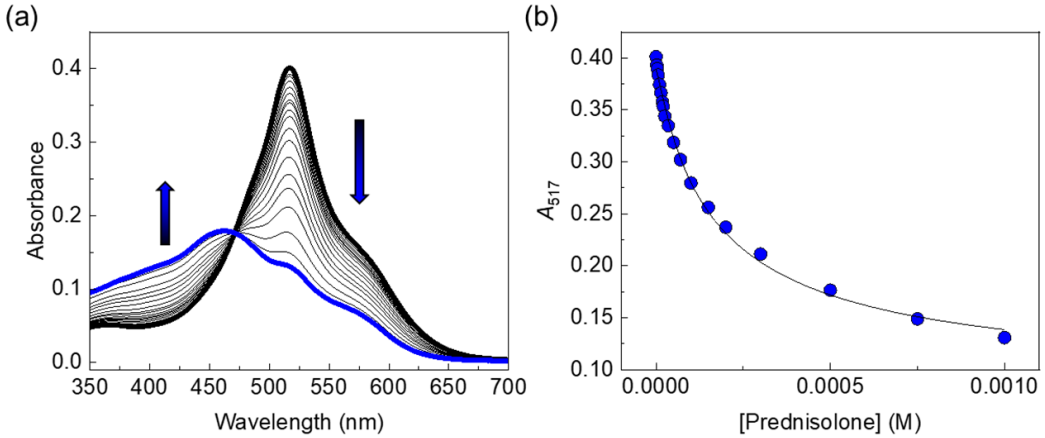

Fig. S30 (a) UV-vis absorption spectra of the complex of **2** (10 μM) and CB[8] (20 μM) upon the addition of prednisolone in an aqueous DMSO solution (DMSO:water = 1:9, v/v) at pH 3.2 at 25 °C. (b) Titration isotherm for prednisolone. The titration isotherm was obtained by collecting the maximum absorbance at 517 nm. [Prednisolone] = 0–1000 μM.

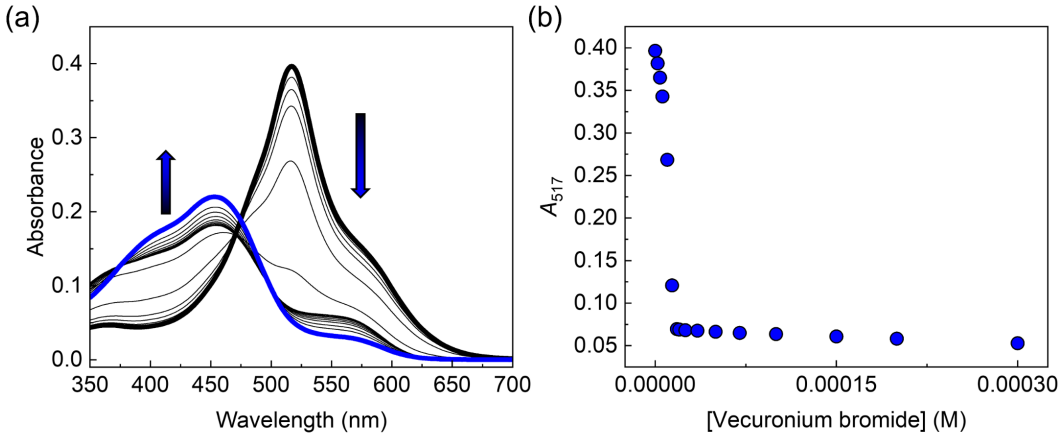

Fig. S31 (a) UV-vis absorption spectra of the complex of **2** (10 μM) and CB[8] (20 μM) upon the addition of vecuronium bromide in an aqueous DMSO solution (DMSO:water = 1:9, v/v) at pH 3.2 at 25 °C. (b) Titration isotherm for vecuronium bromide. The titration isotherm was obtained by collecting the maximum absorbance at 517 nm. [Vecuronium bromide] = 0–300 μM.

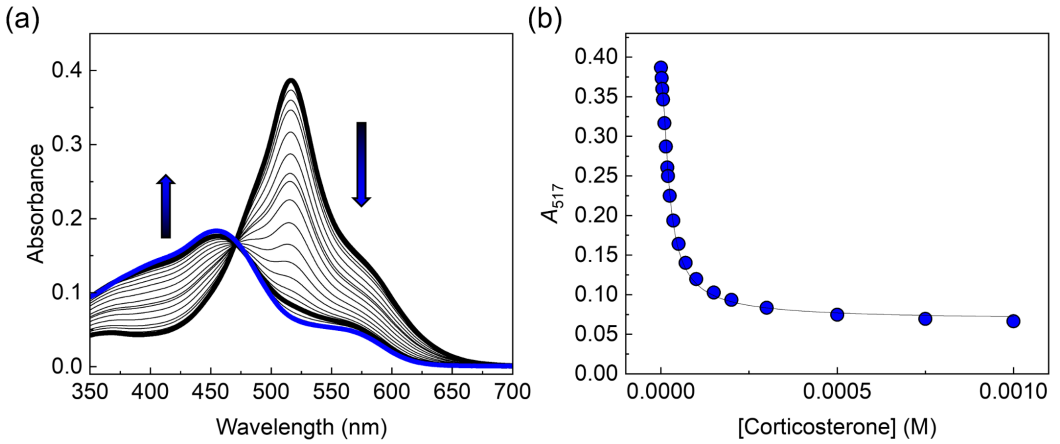

Fig. S32 (a) UV-vis absorption spectra of the complex of **2** (10 μM) and CB[8] (20 μM) upon the addition of corticosterone in an aqueous DMSO solution (DMSO:water = 1:9, v/v) at pH 3.2 at 25 °C. (b) Titration isotherm for corticosterone. The titration isotherm was obtained by collecting the maximum absorbance at 517 nm. [Corticosterone] = 0–1000 μM.

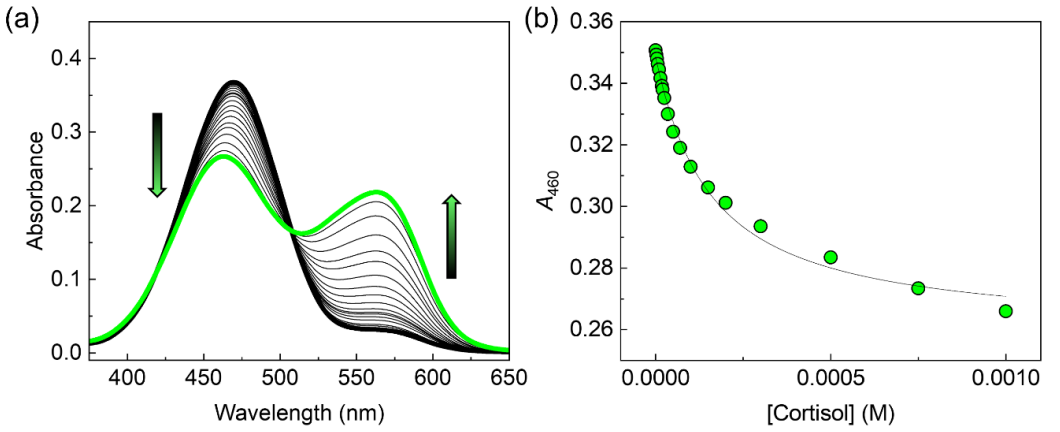

Fig. S33 (a) UV-vis absorption spectra of the complex of **3** (10 μM) and CB[8] (20 μM) upon the addition of cortisol in an aqueous DMSO solution (DMSO:water = 1:9, v/v) at pH 3.2 at 25 °C. (b) Titration isotherm for cortisol. The titration isotherm was obtained by collecting the maximum absorbance at 460 nm. [Cortisol] = 0–1000 μM.

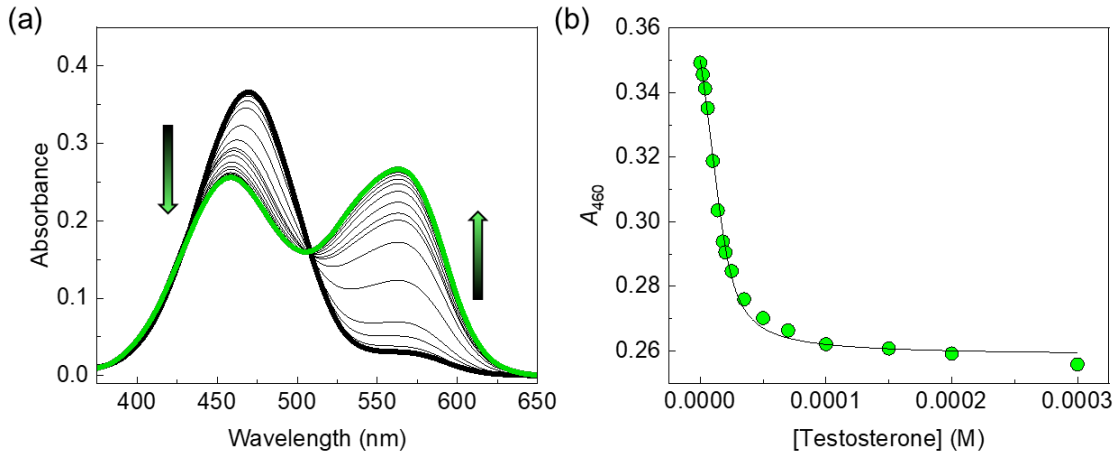

Fig. S34 (a) UV-vis absorption spectra of the complex of **3** (10 μM) and CB[8] (20 μM) upon the addition of testosterone in an aqueous DMSO solution (DMSO:water = 1:9, v/v) at pH 3.2 at 25 °C. (b) Titration isotherm for testosterone. The titration isotherm was obtained by collecting the maximum absorbance at 460 nm. [Testosterone] = 0−300 μM.

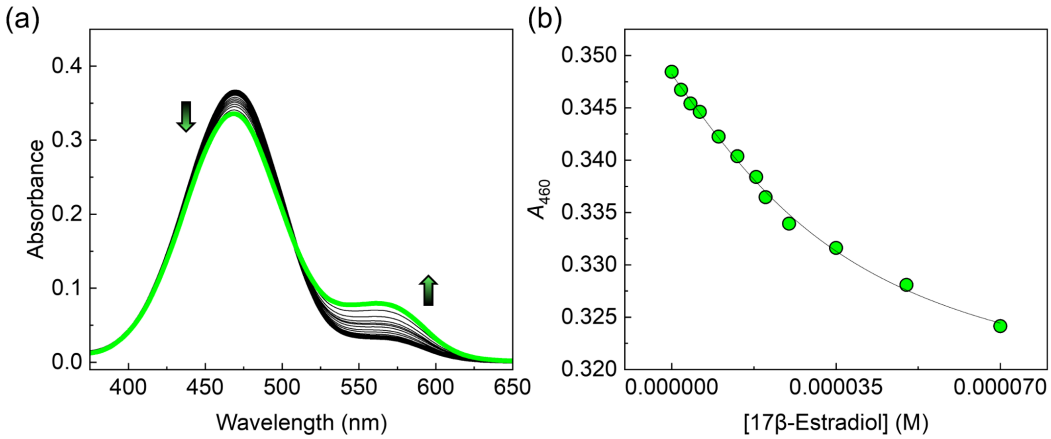

Fig. S35 (a) UV-vis absorption spectra of the complex of **3** (10 μM) and CB[8] (20 μM) upon the addition of 17β-estradiol in an aqueous DMSO solution (DMSO:water = 1:9, v/v) at pH 3.2 at 25 °C. (b) Titration isotherm for 17β-estradiol. The titration isotherm was obtained by collecting the maximum absorbance at 460 nm. [17β-Estradiol] = 0−70 μM.

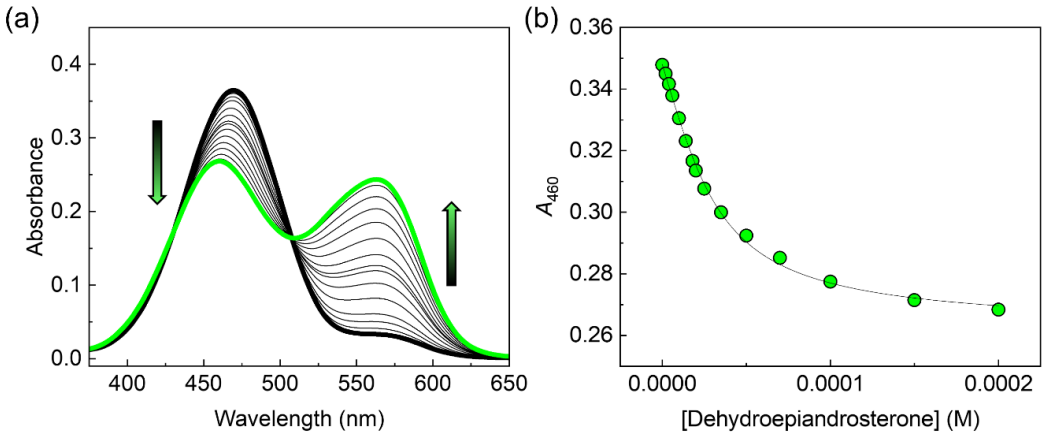

Fig. S36 (a) UV-vis absorption spectra of the complex of **3** (10 μM) and CB[8] (20 μM) upon the addition of dehydroepiandrosterone in an aqueous DMSO solution (DMSO:water = 1:9, v/v) at pH 3.2 at 25 °C. (b) Titration isotherm for dehydroepiandrosterone. The titration isotherm was obtained by collecting the maximum absorbance at 460 nm. [Dehydroepiandrosterone] = 0−200 μM.

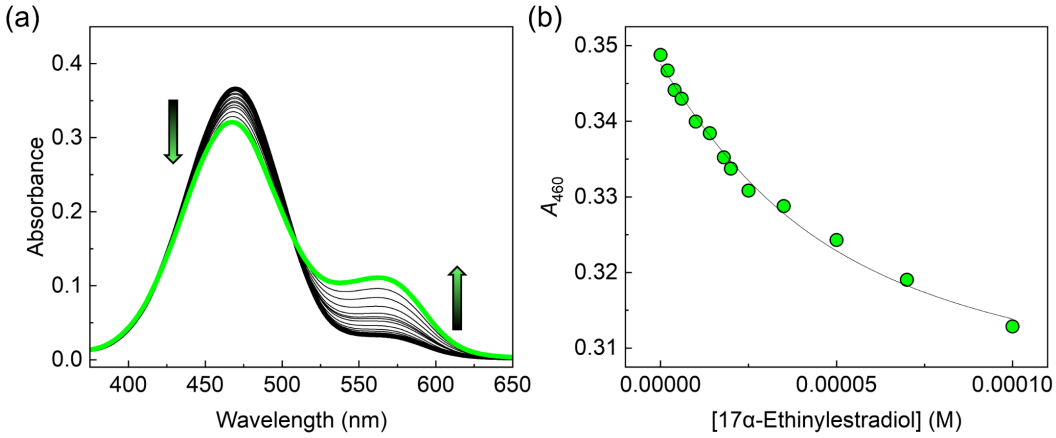

Fig. S37 (a) UV-vis absorption spectra of the complex of **3** (10 μM) and CB[8] (20 μM) upon the addition of 17α-ethinylestradiol in an aqueous DMSO solution (DMSO:water = 1:9, v/v) at pH 3.2 at 25 °C. (b) Titration isotherm for 17α-ethinylestradiol. The titration isotherm was obtained by collecting the maximum absorbance at 460 nm. [17α-Ethinylestradiol] = 0–100 μM.

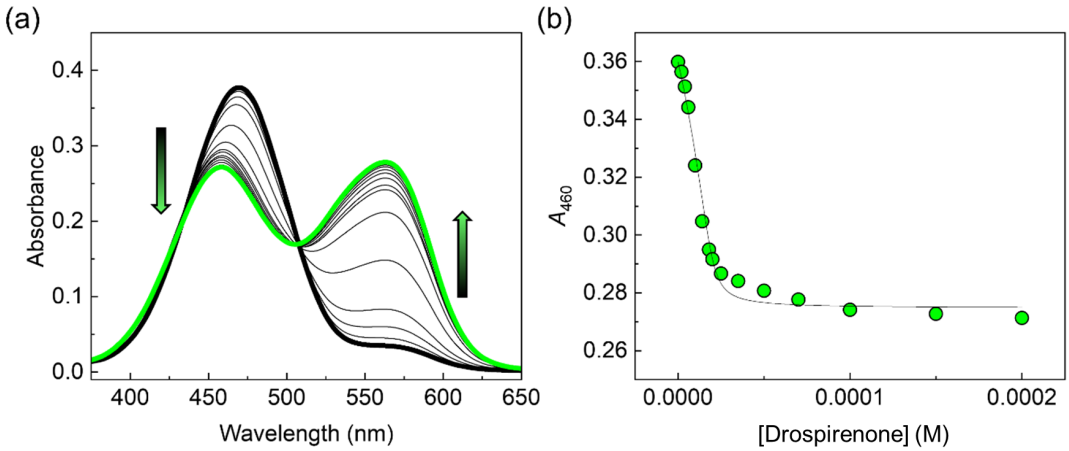

Fig. S38 (a) UV-vis absorption spectra of the complex of **3** (10 μM) and CB[8] (20 μM) upon the addition of drospirenone in an aqueous DMSO solution (DMSO:water = 1:9, v/v) at pH 3.2 at 25 °C. (b) Titration isotherm for drospirenone. The titration isotherm was obtained by collecting the maximum absorbance at 460 nm. [Drospirenone] = 0–200 μM.

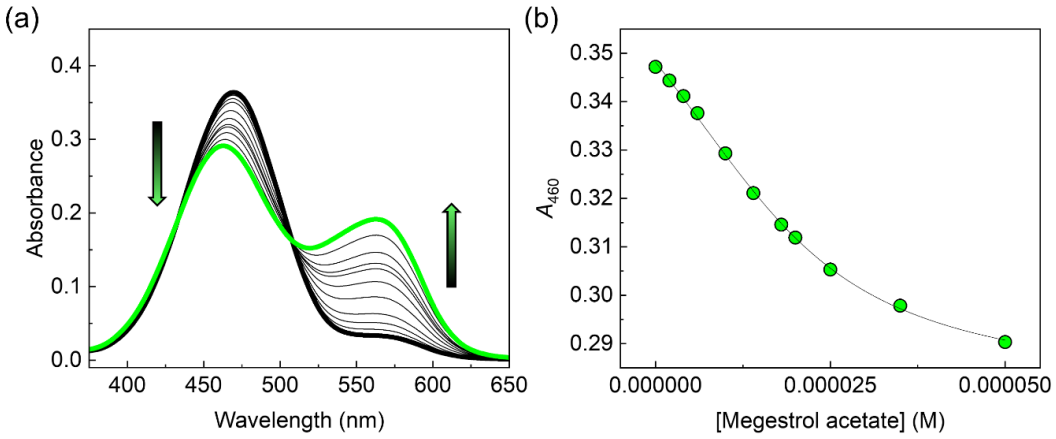

Fig. S39 (a) UV-vis absorption spectra of the complex of **3** (10 μM) and CB[8] (20 μM) upon the addition of megestrol acetate in an aqueous DMSO solution (DMSO:water = 1:9, v/v) at pH 3.2 at 25 °C. (b) Titration isotherm for megestrol acetate. The titration isotherm was obtained by collecting the maximum absorbance at 460 nm. [Megestrol acetate] = 0–50 μM.

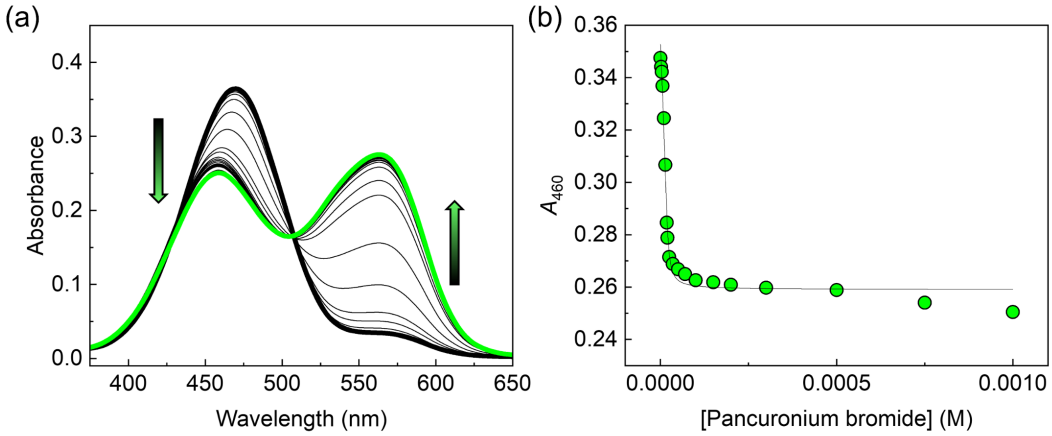

Fig. S40 (a) UV-vis absorption spectra of the complex of **3** (10 μM) and CB[8] (20 μM) upon the addition of pancuronium bromide in an aqueous DMSO solution (DMSO:water = 1:9, v/v) at pH 3.2 at 25 °C. (b) Titration isotherm for pancuronium bromide. The titration isotherm was obtained by collecting the maximum absorbance at 460 nm. [Pancuronium bromide] = 0−1000 μM.

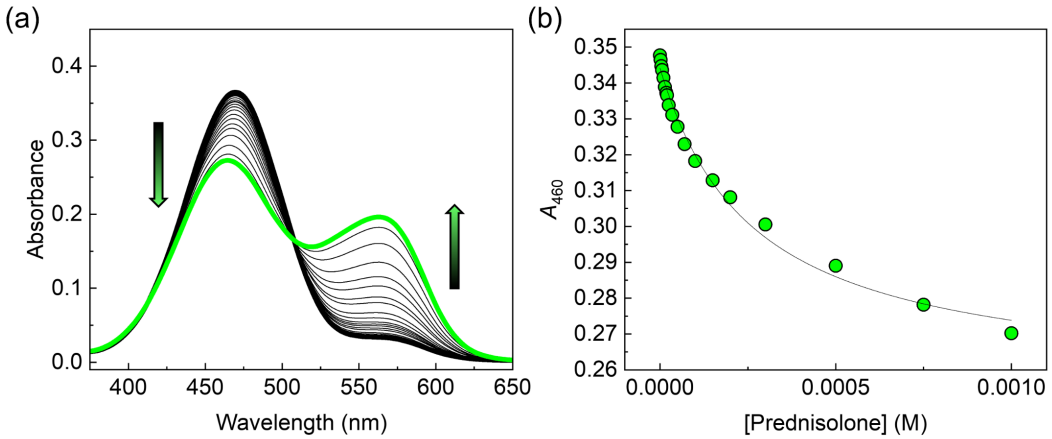

Fig. S41 (a) UV-vis absorption spectra of the complex of **3** (10 μM) and CB[8] (20 μM) upon the addition of prednisolone in an aqueous DMSO solution (DMSO:water = 1:9, v/v) at pH 3.2 at 25 °C. (b) Titration isotherm for prednisolone. The titration isotherm was obtained by collecting the maximum absorbance at 460 nm. [Prednisolone] = 0−1000 μM.

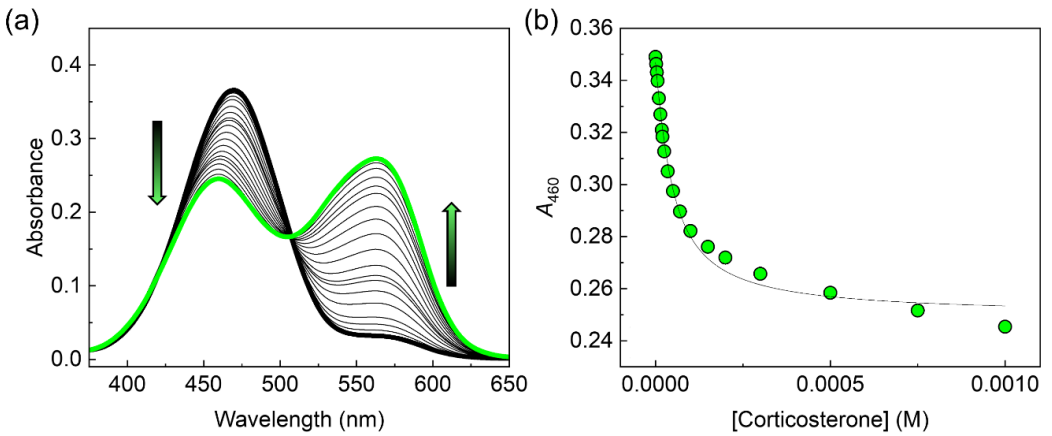

Fig. S42 (a) UV-vis absorption spectra of the complex of **3** (10 μM) and CB[8] (20 μM) upon the addition of corticosterone in an aqueous DMSO solution (DMSO:water = 1:9, v/v) at pH 3.2 at 25 °C. (b) Titration isotherm for corticosterone. The titration isotherm was obtained by collecting the maximum absorbance at 460 nm. [Corticosterone] = 0−1000 μM.

7. Estimation of association constants

The association constants (K_{assoc}) for target steroid hormones and CB[8] were calculated based on the titration isotherms using a nonlinear regression fitting model at 1:1 bindig stoichiometry according to eqs. (1) and (2); [S7]

$$[\mathrm{CB[8]}]_\mathrm{t} = [\mathrm{CB[8]}] + \frac{K_\mathrm{assoc}[\mathrm{CB[8]}]}{1+K_\mathrm{assoc}[\mathrm{CB[8]}]}[\mathrm{G}]_\mathrm{t} + \frac{K_\mathrm{I}[\mathrm{CB[8]}]}{1+K_\mathrm{I}[\mathrm{CB[8]}]}[\mathrm{I}]_\mathrm{t} \quad (1)$$

$$A = \frac{[\mathrm{I}]_\mathrm{t}}{1+K_\mathrm{I}[\mathrm{CB[8]}]}(\varepsilon_\mathrm{I} b + \varepsilon_{\mathrm{CB[8]I}} b K_\mathrm{I}[\mathrm{CB[8]}]) \quad (2)$$

where $[\mathrm{I}]_\mathrm{t}$ is the total concentration of indicator, $[\mathrm{CB[8]}]_\mathrm{t}$ is the total concentration of CB[8], $[\mathrm{G}]_\mathrm{t}$ is the total concentration of guest (*i.e.*, steroid hormone), K_{assoc} and K_I refer to the association constants of the CB[8]–steroid hormone and CB[8]–indicator, respectively. [CB[8]] indicates the unknown concentration of CB[8], which is calculated by K_{assoc} and K_I. In eq. (2), A represents the guest–concentration–dependent absorbance, b donates the cuvette thickness, and ε_I and $\varepsilon_{\mathrm{CB[8]I}}$ mean the molar absorption coefficients of the indicator and the complex of the CB[8]–indicator, respectively.

Table S1. Association constants (K_{assoc}, M^{-1}) for target steroid hormones obtained from UV-vis titrations

	1·CB[8]	**2**·CB[8]	**3**·CB[8]
Cortisol (H1)	(8.3±0.78)×10^5	(1.8±0.14)×10^5	(1.5±0.17)×10^4
Testosterone (H2)	>10^7	>10^7	(6.7±1.0)×10^5
Corticosterone (H3)	(3.9±0.38)×10^5	>10^6	(5.6±0.71)×10^4
Cholic acid (H4)	N.D.[b]	N.D.[b]	N.D.[b]
Dehydroepiandrosterone (H5)	(6.7±0.91)×10^5	>10^6	(1.3±0.097)×10^5
Estrone (H6)	N.D.[a]	N.D.[a]	N.D.[a]
17β-Estradiol (H7)	(3.6±1.3)×10^5	(8.2±2.8)×10^5	(8.9±1.6)×10^4
17α-Ethinylestradiol (H8)	N.D.[b]	(4.1±0.58)×10^5	(5.9±1.3)×10^4
Megestrol acetate (H9)	>10^6	>10^6	(3.0±0.35)×10^5
Nandrolone (H10)	>10^6	>10^7	N.D.[b]
Pancuronium bromide (H11)	>10^7	>10^7	>10^6
Prednisolone (H12)	(4.8±0.57)×10^5	(9.5±0.99)×10^4	(8.4±1.2)×10^3
Progesterone (H13)	>10^7	>10^7	>10^6
Drospirenone (H14)	>10^7	>10^7	>10^6
Vecuronium bromide (H15)	N.D.[b]	N.D.[b]	N.D.[b]

[a.] K_{assoc} could not be calculated due to the small response or precipitate at high concentrations.

[b.] K_{assoc} could not be fit due to biphasic behaviors.

8. Estimation of the limit of detection

The estimation of the limit of detection (LoD) for target steroid hormones was performed based on 3σ methods (LoD = 3σ/S),[S8] where the σ values were calculated based on the initial absorbance of each titration spectrum for **1**·CB[8], **2**·CB[8], and **3**·CB[8], respectively and S refer to the slope of the fitting line obtained from each titration.

Table S2. Estimated limit of detection for target steroid hormones (ppm) based on the 3σ method

	1·CB[8]	**2**·CB[8]	**3**·CB[8]
Cortisol (H1)	4.21	2.93	7.94
Testosterone (H2)	0.61	0.41	1.09
Corticosterone (H3)	1.68	1.13	2.90
Cholic acid (H4)	13.63	7.29	13.64
Dehydroepiandrosterone (H5)	1.25	0.83	2.14
Estrone (H6)	5.31	3.54	9.57
17β-Estradiol (H7)	3.60	2.41	7.01
17α-Ethinylestradiol (H8)	3.17	2.22	5.97
Megestrol acetate (H9)	1.55	1.07	2.67
Nandrolone (H10)	0.67	0.47	1.17
Pancuronium bromide (H11)	2.18	1.73	3.13
Prednisolone (H12)	5.34	3.72	8.08
Progesterone (H13)	0.72	0.48	1.12
Drospirenone (H14)	0.65	0.45	1.12
Vecuronium bromide (H15)	0.98	0.70	1.61

9. Array experiments

Qualitative assay

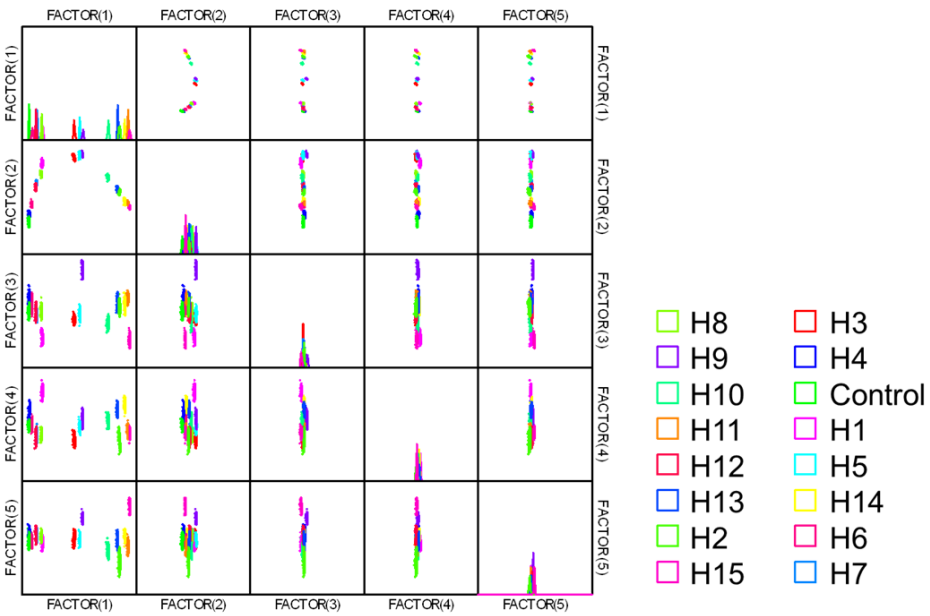

Fig. S43 Canonical score plot for the qualitative assay.

Table S3. The jackknifed classification matrix of the qualitative assay

Jackknifed Classification Matrix																	
	H3	H4	Control	H1	H5	H14	H6	H7	H8	H9	H10	H11	H12	H13	H2	H15	%correct
H3	20	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	100
H4	0	19	1	0	0	0	0	0	0	0	0	0	0	0	0	0	95
Control	0	1	19	0	0	0	0	0	0	0	0	0	0	0	0	0	95
H1	0	0	0	20	0	0	0	0	0	0	0	0	0	0	0	0	100
H5	0	0	0	0	20	0	0	0	0	0	0	0	0	0	0	0	100
H14	0	0	0	0	0	20	0	0	0	0	0	0	0	0	0	0	100
H6	0	1	0	0	0	0	19	0	0	0	0	0	0	0	0	0	95
H7	0	0	0	0	0	0	0	10	0	0	0	0	10	0	0	0	50
H8	0	0	0	0	0	0	0	0	20	0	0	0	0	0	0	0	100
H9	0	0	0	0	0	0	0	0	0	20	0	0	0	0	0	0	100
H10	0	0	0	0	0	0	0	0	0	0	20	0	0	0	0	0	100
H11	0	0	0	0	0	0	0	0	0	0	0	20	0	0	0	0	100
H12	0	0	0	0	0	0	0	9	0	0	0	0	11	0	0	0	55
H13	0	0	0	0	0	0	0	0	0	0	0	0	0	19	1	0	95
H2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	20	0	100
H15	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	20	100
Total	20	21	20	20	20	20	19	19	20	20	20	20	21	19	21	20	93

Semi-quantitative assay

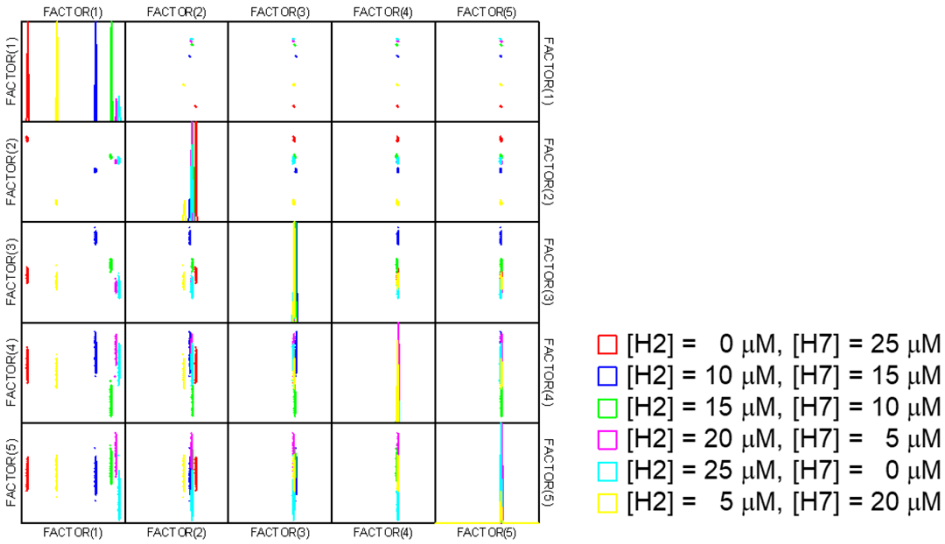

Fig. S44 Canonical score plot for the semi-quantitative assay in the mixtures of H2 (testosterone) and H7 (17β-estradiol).

Table S4. The jackknifed classification matrix of the semi-quantitative assay in the mixtures of H2 and H7

Jackknifed Classification Matrix							
	[H2] = 0 μM, [H7] = 25 μM	[H2] = 10 μM, [H7] = 15 μM	[H2] = 15 μM, [H7] =10 μM	[H2] = 20 μM, [H7] = 5 μM	[H2] = 25 μM, [H7] = 0 μM	[H2] = 5 μM, [H7] = 20 μM	%correct
[H2] = 0 μM, [H7] = 25 μM	20	0	0	0	0	0	100
[H2] = 10 μM, [H7] = 15 μM	0	20	0	0	0	0	100
[H2] = 15 μM, [H7] =10 μM	0	0	20	0	0	0	100
[H2] = 20 μM, [H7] = 5 μM	0	0	0	20	0	0	100
[H2] = 25 μM, [H7] = 0 μM	0	0	0	0	20	0	100
[H2] = 5 μM, [H7] = 20 μM	0	0	0	0	0	20	100
Total	20	20	20	20	20	20	100

Quantitative assay

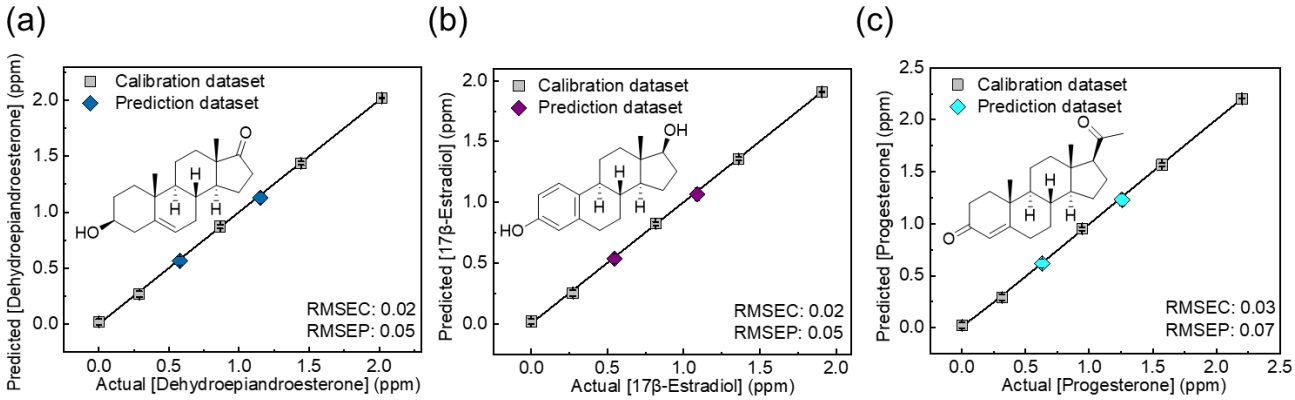

Fig. S45 Regression analysis using SVM for (a) dehydroepiandrosterone (H5), (b) 17*β*-estradiol (H7), and (c) progesterone (H13) in their mixtures. RMSEC and RMSEP represent root-mean-square errors for calibration and prediction, respectively.

References

S1. M. J. Frisch, G. W. Trucks, H. B. Schlegel, G. E. Scuseria, M. A. Robb, J. R. Cheeseman, G. Scalmani, V. Barone, G. A. Petersson, H. Nakatsuji, X. Li, M. Caricato, A. V. Marenich, J. Bloino, B. G. Janesko, R. Gomperts, B. Mennucci, H. P. Hratchian, J. V. Ortiz, A. F. Izmaylov, J. L. Sonnenberg, D. Williams-Young, F. Ding, F. Lipparini, F. Egidi, J. Goings, B. Peng, A. Petrone, T. Henderson, D. Ranasinghe, V. G. Zakrzewski, J. Gao, N. Rega, G. Zheng, W. Liang, M. Hada, M. Ehara, K. Toyota, R. Fukuda, J. Hasegawa, M. Ishida, T. Nakajima, Y. Honda, O. Kitao, H. Nakai, T. Vreven, K. Throssell, J. A. Montgomery Jr., J. E. Peralta, F. Ogliaro, M. J. Bearpark, J. J. Heyd, E. N. Brothers, K. N. Kudin, V. N. Staroverov, T. A. Keith, R. Kobayashi, J. Normand, K. Raghavachari, A. P. Rendell, J. C. Burant, S. S. Iyengar, J. Tomasi, M. Cossi, J. M. Millam, M. Klene, C. Adamo, R. Cammi, J. W. Ochterski, R. L. Martin, K. Morokuma, O. Farkas, J. B. Foresman and D. J. Fox, Gaussian 16 (Revision C.01), Gaussian, Inc., Wallingford CT, 2016.

S2. L. Chen, M. Liu, Y. Zhang, Q. Zhu, J. Liu, B. Zhu and Z. Tao, *Chem. Commun*., 2019, **55**, 14271–14274.

S3. S. Shradha, R. Kommidi and B. D. Smith, *J. Org. Chem.,* 2023, **88**, 8431–8440.

S4. T. Lu, F. Chen, *J. Comput. Chem*., 2012, **33**, 580–592.

S5. T. Lu, Q. Chen, *J. Comput. Chem.,* 2022, **43**, 539–555.

S6. W. Humphrey, A. Dalke, K. V. M. D. Schulten, *J. Mol. Graph.*, 1996, **14**, 33–38.

S7. A. E. Hargrove, Z. Zhong, J. L. Sessler, E. V Anslyn, *New J. Chem.*, 2010, **34**, 348–354.

S8. J. N. Miller, J. C. Miller, *Statistics and Chemometrics for Analytical Chemistry*. Essex: Pearson Prentice Hall; 2005.