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Biphasic Enantioselective Fluorescent Recognition of Amino Acids by a Fluorophilic Probe

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Abstract. A fluorophilic fluorescent probe based on a perfluoroalkyl substituted bisbinaphthyl compound is designed and synthesized, which displayed highly enantioselective fluorescence response toward structurally diverse amino acids in a biphasic fluorous/aqueous system with *ef* (enantiomeric fluorescent enhancement ratios, $\Delta I_D/\Delta I_L$) values up to 45.2 (histidine). It can be used to determine the enantiomeric compositions of amino acids and also allows the amino acid enantiomers to be visually discriminated. NMR and mass spectroscopic investigations have provided insights into the observed high enantioselectivity. This biphasic fluorescent recognition has been used to determine the enantiomeric composition of the crude phenylalanine products generated by an enzyme-catalyzed asymmetric hydrolysis under various reaction conditions. The fluorous phase-based fluorescence measurement under the biphasic conditions can minimize the interference of other reaction components and shows potential in asymmetric reaction screening.

Introduction

Enantioselective fluorescent recognition of chiral organic compounds are potentially useful for high throughput assay of asymmetric reactions.[1-3] One challenge for this application is the possible interferences of various reaction components on the fluorescence measurement when a fluorescent probe is used to determine the enantiomeric composition of the desired product in a reaction mixture. In order to minimize such interferences, we have proposed a strategy to develop the fluorous phase-based fluorescent probes by utilizing the lipophobic, hydrophobic and fluorophilic properties of the highly fluorinated materials. Although the fluorous phase-based techniques have been extensively applied to chemical reactions and separations,[4-6] investigation of their use in fluorescent sensing has only just started.[7]

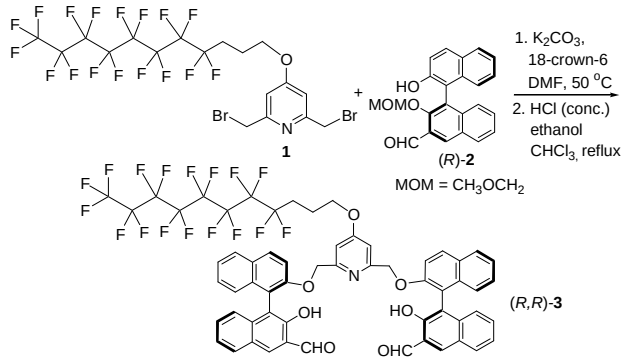
Recently, we found that a bisbinaphthyl-based fluorescent probe can carry out highly enantioselective fluorescent recognition of amino acids in acetonitrile solution.[8,9] On the basis of this work, we have synthesized a fluorophilic probe by incorporating a highly fluorinated alkyl chain. We have demonstrated that in a biphasic aqueous/fluorous system, the probe can carry out highly enantioselective fluorescent detection of amino acids by selectively extracting the substrates from the aqueous phase into the fluorous phase. This is the first example to conduct enantioselective discrimination of amino acids by using a fluorous phase-based separation process. It has been used to determine the enantiomeric composition of crude chiral amino acid samples generated from an enzyme-catalyzed asymmetric reaction. Herein, these results are reported.

Results and Discussion

1. Synthesis of a Highly Fluorinated Bisbinaphthyl Probe

We prepared compound **1**, a 2,6-dibromomethyl pyridine with a highly fluorinated alkyl chain, as a precursor to a fluorophilic probe (Scheme S1). Reaction of **1** with the 1,1'-binaphthyl-based aldehyde (*R*)-**2**[8] in the presence of base followed by acidic deprotection gave the bisbinaphthyl compound (*R,R*)-**3**. This compound was found to be soluble in a fluorous solvent 1H,1H,2H,2H-perfluoro-1-octanol (PFOH) and showed no fluorescence in this solvent.

Scheme 1. Synthesis of the Fluorophilic Probe (*R,R*)-**3**.

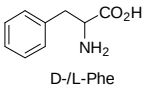


2. Fluorescence Response of (*R,R*)-3 toward D- and L-Phenylalanine (Phe)

We studied the fluorescence response of (*R,R*)-**3** toward an amino acid D-/L-Phe in a biphasic system of H_2O/PFOH (2 mL/ 2 mL). When a solution of (*R,R*)-**3** (4.0×10^{-5} M, 2.0 mL) in PFOH was treated with $Zn(OAc)_2$ (1.0 equiv, 4.0 mM in H_2O, 20 μL), no change in fluorescence was observed (Figure 1a). Mixing this solution with a solution of D-Phe (14.0 equiv, 2.0 mL) in water also did not cause any fluorescence response. However, the fluorescence of (*R,R*)-**3**+Zn(II) in PFOH at λ = 533 nm was turned on when it was mixed with the solution of D-Phe in aqueous tetrabutylammonium hydroxide (TBAH) (2 mM) (Figure 1a).

Chemistry - A European Journal 10.1002/chem.201900880

When L-Phe was used, under the same conditions, the fluorescence enhancement was much smaller (Figure 1b). In these experiments, the fluorescence of the bottom fluorous solution of the two phases in a quartz cuvette was measured directly with no need to remove the aqueous phase. Thus, (*R,R*)-**3** can discriminate the two enantiomers of D- and L-Phe in the biphasic solution system in the presence of Zn(II) and TBAH.



D-/L-Phe

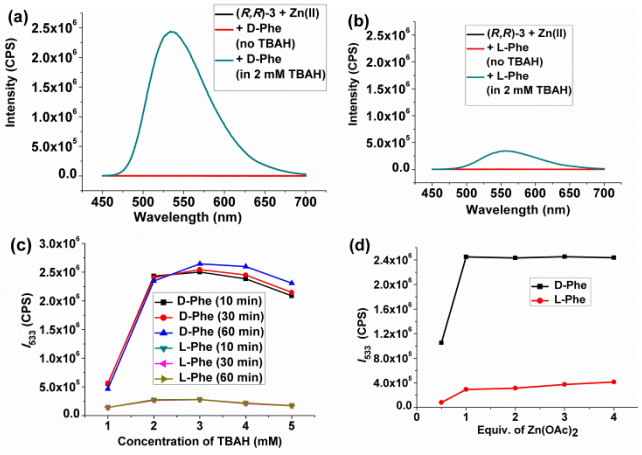


Figure 1. Fluorescence response of (*R,R*)-**3** (1.0 equiv, 2.0 mL, 0.040 mM in PFOH) with (a) D- and (b) L-Phe (14.0 equiv, 2.0 mL, in 0 or 2 mM TBAH aqueous solution) in the presence of $Zn(OAc)_2$ (1.0 equiv, 20 μL, 4 mM in H_2O). (c) Fluorescence intensity at 533 nm (I_{533}) versus the concentration of TBAH. (d) I_{533} versus the equiv of $Zn(OAc)_2$ (20 μL, 2~16 mM in H_2O) with 2.0 mM TBAH. All the biphasic mixtures were stirred at rt for 4 h. For (a), (b) and (d), the fluorescence was measured after the mixing was stopped for 10 min. For (c), the fluorescence was recorded after the mixing was stopped for 10, 30, 60 min respectively. (λ_{exc} = 435 nm. Slit: 2/2 nm)

We studied the fluorescence responses by varying the concentration of TBAH as well as the time after the two solution phases were separated while the mixing was stopped. As shown in Figure 1c, the fluorescence enhancement of (*R,R*)-**3**+Zn at λ = 533 nm in the presence of D-Phe greatly increased as the concentration of TBAH increased from 1 mM to 2 mM which then slightly increased at 3 mM TBAH. After that, the fluorescence started to decrease slightly with increasing TBAH concentration. It also shows that the time after the two phase mixing was stopped had little influence on the fluorescence response. We further studied the effect of the equivalency of Zn(II) on the fluorescence response. As shown in Figure 1d, at $Zn(OAc)_2$ (1.0 equiv) the fluorescence enhancement of (*R,R*)-**3** by D-Phe (14.0 equiv, in 2 mM TBAH) in the biphasic system reached the maximum and no further increase was observed at greater amount of $Zn(OAc)_2$.

Fluorescence response of (*R,R*)-**3** toward D- or L-Phe at various concentrations in the presence of 2 mM TBAH and 1 equiv $Zn(OAc)_2$ in the biphasic system was then investigated. As shown in Figure 2a, the fluorescence intensity of (*R,R*)-**3** at 533 nm increased continuously when the concentration of D-Phe increased from 2 to 20 equiv, but when the concentration of L-Phe increased the fluorescence intensity remained low. In Figure 2b, it shows that at 14.0 equiv of Phe, the enantioselective fluorescence enhancement ratio [*ef* = $(I_D-I_0)/(I_L-I_0)$ = $\Delta I_D/\Delta I_L$. I_D, I_L: fluorescence intensity in the presence of D- or L-amino acid. I_0: fluorescence intensity of (*R,R*)-**3**] was 11.4, representing a high enantioselectivity.

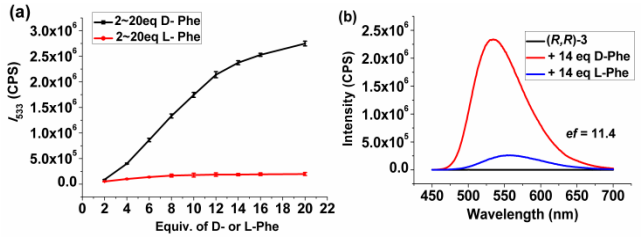


Figure 2. (a) Fluorescence intensity at 533 nm (I_{533}) of (*R,R*)-**3** (1.0 equiv, 2.0 mL, 0.040 mM in PFOH) with (a) D- and (b) L-Phe (0 – 20 equiv, 2.0 mL, in 2 mM TBAH aqueous solution) in the presence of $Zn(OAc)_2$ (1.0 equiv, 20 μL, 4.0 mM in H_2O). The error bars were obtained from three independent experiments. (b) Fluorescence spectra of (*R,R*)-**3** with D- and L-Phe (14.0 equiv) in the presence of TBAH (2.0 mM) and Zn(II) (1.0 equiv). All the biphasic mixtures were stirred at rt for 4 h, and fluorescence was measured 10 min after the mixing was stopped (λ_{exc} = 435 nm. Slit: 2/2 nm).

We also prepared (*S,S*)-**3**, the enantiomer of (*R,R*)-**3**, and examined its fluorescence response toward Phe under the same conditions as above. It was found that L-Phe greatly enhanced the fluorescence of (*S,S*)-**3** but not D-Phe (Figure S2). The mirror image relation between the fluorescence responses in Figure 2 and Figure S2 confirmed the observed highly enantioselective fluorescent recognition.

Figure 3 gives the photo images of the biphasic system after (*R,R*)-**3** (1.2 mM) was treated with D- or L-Phe (14.0 equiv, in 16.8 mM TBAH aqueous solution) for 4 h in the presence of Zn(II) (1.0 equiv). It shows that upon UV lamp irradiation, the fluorous phase with D-Phe in the lower layer gave bright yellow emission but the one with L-Phe gave much weaker fluorescence (Figure 3b). Thus, the enantiomers of this amino acid can be visually discriminated.

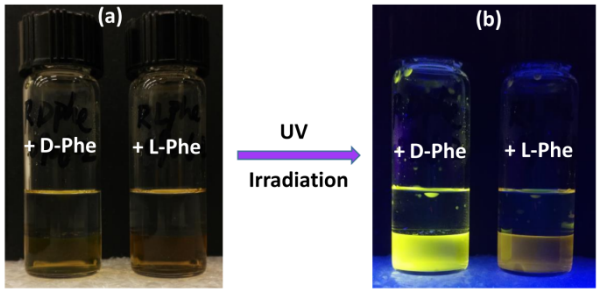


Figure 3. (a) Photos of (*R,R*)-**3** (1.0 mL, 1.2 mM, in PFOH) treated with D- or L-Phe (14.0 equiv, 1.0 mL, in 16.8 mM TBAH aqueous solution) in the presence of Zn(II) (1.0 equiv, 20 μL in H_2O). (b) Under UV lamp irradiation at 365 nm. Upper layer: aqueous phase; lower layer: fluorous phase.

The fluorescence response of the two enantiomers of the probe, (*S,S*)- and (*R,R*)-**3**, toward Phe at various *ee* values [enantiomeric excess = ([D] - [L])/([D] + [L])] was investigated. As shown in Figure 4, the plots of the fluorescence intensity at 533 nm versus the *ee* values of Phe present a mirror-image relation between the two enantiomeric sensors. The linear relations are obtained from the fluorescent response of (*R,R*)-**3** and (*S,S*)-**3** toward Phe with ee's > 0 (D in excess) and < 0 (L in excess), respectively. This plot demonstrates that the enantiomeric composition of the amino acid can be determined by the biphasic enantioselective fluorescent recognition process.

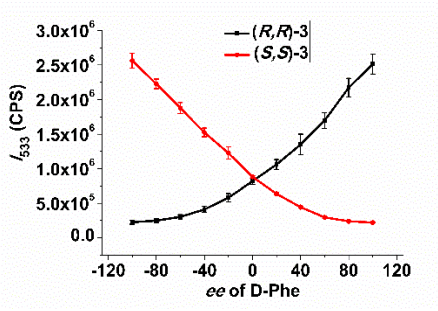


Figure 4. Fluorescence intensity at 533 nm (I_{533}) of (*R,R*)-**3** (black curve) and (*S,S*)-**3** (red curve) (1.0 equiv, 2.0 mL, 0.04 mM in PFOH) with Phe (14 equiv, 0.56 mM in 2 mM TBAH aqueous solution) at various *ee* in the presence of Zn(II) (1.0 equiv, 4.0 mM in H_2O). All the mixtures were stirred at rt for 4 h, and fluorescence was measured 10 min after the mixing was stopped. The error bars were obtained from three independent experiments. λ_{exc} = 435 nm. Slit: 2/2 nm.

3. Fluorescence Response of (*R,R*)-3 Toward Other Amino Acids

We studied the fluorescent response of (*R,R*)-**3** toward 18 common chiral amino acids (including Phe) in the fluorous/aqueous phases. As shown in figure S3, all 17 amino acids could lead to fluorescence enhancement except proline. Among the amino acids enhancing the fluorescence of the probe, 14 of the amino acids generated enantioselective fluorescent response except alanine and tyrosine. The D enantiomers of 9 amino acids including histidine, tryptophan, glutamine, serine, methionine, arginine, cysteine, threonine, and asparagine enhanced the fluorescence of (*R,R*)-**3** greater than their L-enantiomers, whereas the L-enantiomers of 5 amino acids including lysine, glutamic acid, leucine, aspartic acid and valine generated greater fluorescence enhancement than the corresponding D-enantiomers. Figure S4 shows that after the two phases of (*R,R*)-**3** (1.0 equiv, 0.040 mM in 2 mL PFOH), D/L-amino acids (10.0 equiv, 2.0 mL in 3 mM TBAH aqueous solutions), Zn(II) (2.0 equiv, 20 μL in H_2O) were mixed for 3 h, high *ef* values were observed for 6 amino acids: histidine (33.5), tryptophan (5.7), glutamine (4.8), serine (4.6), methionine (4.1) and lysine (6.3, $\Delta I_L/\Delta I_D$).

We have explored various conditions for the biphasic recognition of histidine (His) by (*R,R*)-**3** (Figures S5, S6 and S7). As shown in Figure 5a, under the optimized conditions, the fluorescence of (*R,R*)-**3** in PFOH at λ = 540 nm was greatly enhanced in the presence of Zn(II) (2.0 equiv) by D-His (in 3 mM TBAH) from 1 to 12 equiv but not by L-His. Figure 5b gives the fluorescence spectra of (*R,R*)-**3** upon treatment with 12.0 equiv D- and L-His which gave an *ef* of 45.2, an excellent enantioselectivity.

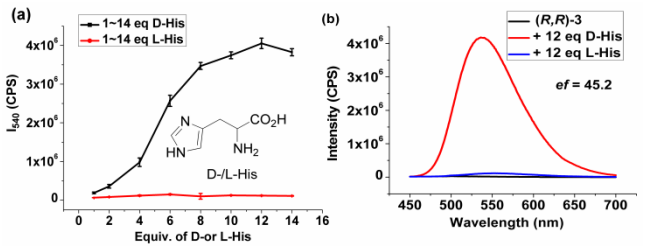


Figure 5. (a) Fluorescence intensity at 540 nm of (*R,R*)-**3** (2.0 mL, 0.040 mM in PFOH, 1.0 equiv.) with. D- and L-His (2.0 mL, in 3.0 mM TBAH aqueous solution, 1.0 – 14.0 equiv) in the presence of $Zn(OAc)_2$ (20 μL, 8 mM in H_2O, 2.0 equiv). (b) Fluorescence spectra of (*R,R*)-**3** with D- and L-His (12.0 equiv) in the presence of TBAH (3.0 mM) and Zn(II) (2.0 equiv). All the mixtures were stirred at rt for 4 h, and fluorescence was measured 10 min after the mixing was stopped. The error bars were obtained from three independent experiments. λ_{exc} = 435 nm. Slit: 4/4 nm.

The high enantioselectivity of (*R,R*)-**3** in the fluorescent recognition of His was further confirmed by the use of the enantiomeric probe (*S,S*)-**3** which gave the expected mirror image relationship (Figure S8). We have used both (*R,R*)- and (*S,S*)-**3** to interact with His (12 equiv) under the biphasic conditions at various enantiomeric compositions. The results in Figure 6 demonstrate that these probes can be used to determine the enantiomeric composition of His. Visual discrimination of the enantiomers of His similar to that shown for Phe in Figure 3 was also observed (Figure S9).

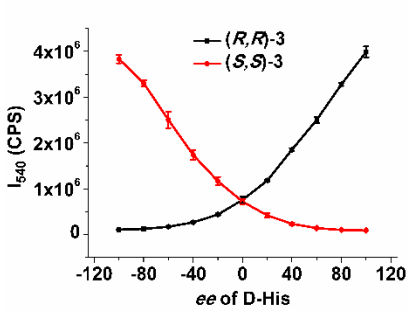


Figure 6. Fluorescence intensity at 540 nm (I_{540}) of (*R,R*)-**3** (black curve) and (*S,S*)-**3** (red curve) (1.0 equiv, 2.0 mL, 0.040 mM in FTOH) toward histidine (12 equiv, 0.48 mM in 3.0 mM TBAH water solution) at various *ee* in the present of Zn(II) (2.0 equiv, 8.0 mM in H_2O). All the mixtures were stirred at rt for 4 h, and fluorescence was measured 10 min after the mixing was stopped. The error bars were obtained from three independent experiments. λ_{exc} = 435 nm. Slit: 4/4 nm.

4. NMR and mass spectroscopic studies for the interaction of (*R,R*)-3 with D-/L-Phe and D-/L-His

In order to gain a better understanding on the fluorescence response of (*R,R*)-**3** toward the amino acids, we have conducted ^{1}H NMR and mass spectroscopic investigations.

The ^{1}H NMR spectra of (*R,R*)-**3** in PFOH after being treated with an aqueous solution of D- or L-Phe was obtained (Figure 7a). A capillary tube filled with acetone-d_6 was added as an external standard. As shown in Figure 7a (the full spectra were displayed in Figure S10), when (*R,R*)-**3** was treated with D- or L-Phe, the aldehyde signal at δ 8.87 of the probe completely disappeared and new and broad peaks appeared between δ 6.6 to 7.2 (Figures $7a_5$ and $7a_6$). The ^{1}H NMR spectra for the reactions of (*R,R*)-**3** with D- and L-Phe in the *homogenous* solution of DMSO-d_6 were also obtained. As shown in Figures $7b_4$ and $7b_5$, both D- and L-Phe reacted with (*R,R*)-**3**+Zn(II) completely to give the corresponding products with well-defined sharp ^{1}H NMR signals. Singlets at δ 8.32 and 8.38 in Figures $7b_4$ and $7b_5$, can be assigned to the imine protons in the proposed products **4** from the condensation of the aldehyde groups of (*R,R*)-**3** with D- and L-Phe respectively in DMSO-d_6 in the presence of $Zn(OAc)_2$.

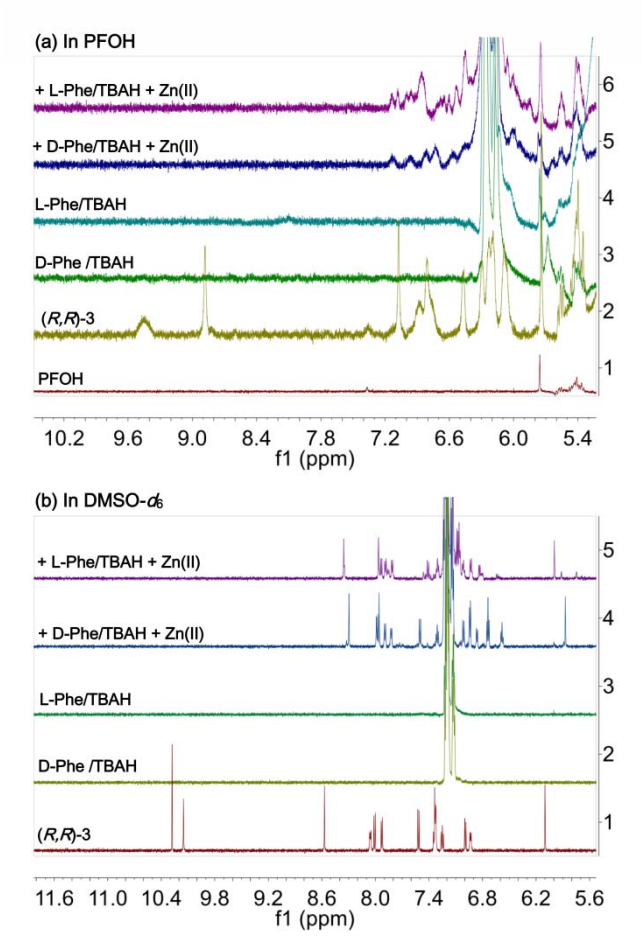
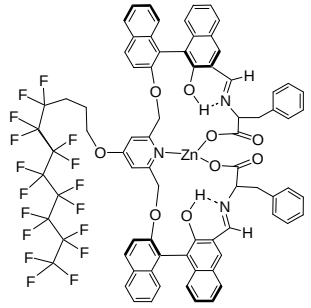


Figure 7. (a) ^{1}H NMR spectra of (*R,R*)-**3** (1.0 equiv, 1.2 mM in PFOH) after being treated with D- and L-Phe (14.0 equiv, 1.0 mL, 16.8 mM, in 16.8 mM TBAH aqueous solution) in the presence of Zn(II) (1.0 equiv, 20 μL, 60 mM in H_2O). (b) ^{1}H NMR spectra of (*R,R*)-**3** (1.0 equiv, 0.40 mM) in DMSO-d_6 after being treated with D- and L-Phe (14.0 equiv, 100 μL, 56.0 mM, in 56.0 mM TBAH D_2O solution) in the presence of Zn(II) (1.0 equiv, 20 μL, 20 mM in D_2O). The mixtures were all stirred at rt for 4 h. The intense signals from the solvent PFOH in the upfield were removed for clarity. The full spectra are given in figure S10.



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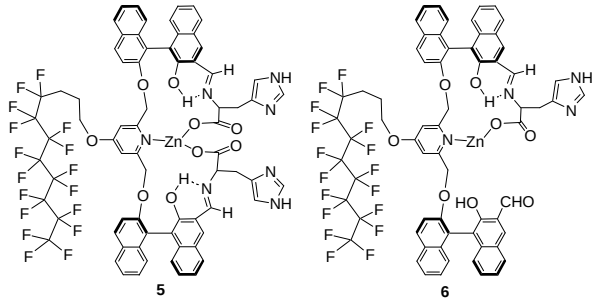
The mass spectra (MALDI-TOF, DCTB negative mode) of the reaction mixtures of (*R,R*)-**3** with D- and L-Phe in PFOH and DMSO were obtained (Figure S11). In PFOH, the reaction of (*R,R*)-**3** with D-Phe gave a peak at m/z 1562.6 as shown in Figure S11a, consistent with the proposed complex **4** (calcd for [M-H]$^-$: 1562.3), but a much weaker peak at m/z 1562.4 was observed for the reaction of (*R,R*)-**3** with L-Phe in PFOH (Figure S11b). In DMSO, the reaction of (*R,R*)-**3** with D-Phe also gave an intense peak at m/z 1562.3 (Figure S11c), but this peak was not observed for the reaction of (*R,R*)-**3** with L-Phe (Figure S11d). Although the ^{1}H NMR spectra in Figures 7b$_4$ and 7b$_5$ show that (*R,R*)-**3** reacted with D- and L-Phe in DMSO to give similar products, their different mass spectra indicate very different stability of these products. The NMR and mass spectroscopic studies suggest that both D- and L-Phe could react with (*R,R*)-**3** to generate the corresponding diastereomeric complexes like **4** and these diastereomers should have different stability and structural rigidity leading to the observed enantioselective fluorescence responses.

The reactions of (*R,R*)-**3** with D- and L-His in PFOH and DMSO-d_6 gave ^{1}H NMR spectra very similar to those shown in Figures 7a and 7b respectively. In both solvents, the aldehyde signal of (*R,R*)-**3** completely disappeared to give broad signals in PFOH and sharp and well-defined signals in DMSO-d_6 (Figure S12). In the mass spectrum for the reaction of (*R,R*)-**3** with D-His in PFOH, a weak signal at m/z 1542.4 was observed for the proposed double condensation product **5** (calcd for [M-H]$^-$: 1542.3) (Figure S13a). This signal was not observable for the reaction of (*R,R*)-**3** with D-His in DMSO and with L-His in both PFOH and DMSO (Figure S13b-d). A peak at m/z = 1406.3 was observed for the reaction of D-His with (*R,R*)-**3** in both PFOH and DMSO solutions (Figure S13a and S13c) which can be attributed to the mono condensation product **6** (Calcd for [M-H]$^-$: 1406.2). This peak was not observed in the mass spectra for the reaction of (*R,R*)-**3** with L-His in PFOH and DMSO (Figure S13b and S13d). Although the ^{1}H NMR spectra show complete reaction of the aldehyde groups of (*R,R*)-**3** with the amino acid, the corresponding double condensation products might not be stable under the conditions of the mass spectroscopic experiments and give only very weak or nonobservable signal.



5 **6**

5. Determination of the enantiomeric composition of the crude Phe products prepared from an enzyme-catalyzed asymmetric reaction

We have applied the probes (*R,R*)- and (*S,S*)-**3** to evaluate the enantiomeric composition of an amino acid product generated in an enzyme-catalyzed asymmetric hydrolysis of a racemic amino acid ester. Previously, Kise and coworkers reported that racemic amino acid esters such as D-/L-Phe-*O*-Me can undergo highly enantioselective hydrolysis in the presence of the enzyme *α*-chymotrypsin (CT) to generate L-Phe (Scheme 2).[10] We carried out this asymmetric hydrolysis in various solvent systems and used (*R,R*)- and (*S,S*)-**3** to determine the *ee* of the crude product Phe under the biphasic conditions according to Figure 4. On the basis of the fluorescence spectra of the crude products in Figure S14, we estimated the *ee*'s of these products obtained from various conditions as shown in Table 1. All the samples greatly enhanced the fluorescence of (*S,S*)-**3** in PFOH at 533 nm, but caused much weaker fluorescence enhancement on (*R,R*)-**3**. This shows that the hydrolysis product is predominately L-Phe rather than D-Phe. The *ee*'s obtained by the fluorescence measurement under the biphasic recognition are close to those determined by HPLC as shown in Table 1. This demonstrates that the fluorous phase-based fluorescent probe can be used to screen the reaction conditions for the asymmetric synthesis of the amino acid.

Scheme 2. An Enzyme-Catalyzed Enantioselective Hydrolysis for the Asymmetric Preparation of L-Phe.

Chemistry - A European Journal 10.1002/chem.201900880

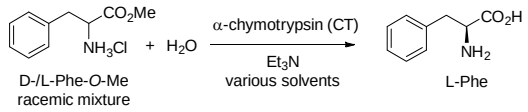


Table 1. Comparison of *ee* values of the crude Phe products[a] from the enzymatic hydrolysis of D/L-Phe-*O*-Me determined by fluorescence (FL) and HPLC analyses

Product	Solvent	Yield /%	ee/% (FL)	ee/% (HPLC)[b]	Deviation /%
Phe-1	Acetonitrile (10% H_2O)	78	90.1	99.8	-9.7
Phe-2	Acetonitrile (20% H_2O)	64	87.4	96.2	-8.8
Phe-3	Acetonitrile (30% H_2O)	75	82.3	94.2	-11.9
Phe-4	2-Propanol (10% H_2O)	34	90.3	99.5	-9.2
Phe-5	1-Propanol (10% H_2O)	50	83.7	94.5	-10.8
Phe-6	Ethanol (10% H_2O)	36	81.8	94.8	-13.0
Phe-7	Acetone (10% H_2O)	78	91.5	99.8	-8.3

[a]DL-phenylalanine methyl ester hydrochloride: 45 mM; Et_3N: 45 mM; CT: 12 μM; Solvent: 20 mL; 25 °C，24 h. *ee* = ([L] - [D])/([L] + [D]. Yield based on the amount of L-Phe formed in theory.

[b]Determined by Daicel Chiral Technologies (China) Co., LTD (Figure S18).

6. Conclusion

We have demonstrated that a fluorophilic probe (*R,R*)-**3** in combination with Zn(II) can carry out biphasic fluorescent recognition of structurally diverse amino acids with fair to excellent enantioselectivity. This study represents the first example for the enantioselective fluorescent recognition of amino acids in the fluorous phase. The ^{1}H NMR and mass analyses of the reaction of (*R,R*)-**3** with D- and L-amino acids indicate the formation of imine-Zn(II) complexes from the condensation of the aldehyde groups of the probe with the amino acid enantiomers and the differences in stability and structural rigidity of these complexes should have contributed to the observed enantioselective fluorescent responses. The biphasic amino acid recognition has been used to determine the enantiomeric composition of the crude products generated by an enzyme-catalyzed asymmetric preparation of a chiral amino acid under various conditions which shows potential of this method in chiral catalyst screening.

Experiment Section

General Data. All synthetic reactions were carried out under nitrogen atmosphere unless otherwise noted. DL-phenylalanine methyl ester hydrochloride was purchase from Chem-Impex International, Inc. Other chemicals were purchased from Sigma Aldrich Chemical Co. or Alfa Aesar. The fluorous solvent PFOH was purchased from SynQuest Laboratories. Other solvents used in the fluorescent measurement were HPLC grades. Optical rotations were measured on a Jasco P-2000 digital polarimeter. NMR spectra were recorded on a Varian-600 MHz spectrometer. Chemical shifts for ^{1}H NMR spectra were reported in parts per million relative to solvent signals at 7.26 ppm for $CDCl_3$, 2.05 ppm for acetone-d_6, 2.50 ppm for DMSO-d_6. Chemical shifts for ^{13}C NMR were reported relative to the centerline of a triplet at 77.16 ppm for $CDCl_3$. Steady-state fluorescence emission spectra were recorded on Horiba FluoroMax-4 spectrofluorometer. Mass spectra were obtained from the University of Illinois at Urbana-Champaign (UIUC) Mass Spectrometry Facility.

Synthesis and Characterization of (*R,R*)-3. (a) A mixture of **1** (263 mg, 0.355 mmol, 1.0 equiv), (*R*)-2'-hydroxy-2-(methoxymethoxy)-[1,1'-binaphthalene]-3-carbaldehyde [(*R*)-**2**, 318 mg, 0.887 mmol, 2.5 equiv], potassium carbonate (196 mg, 1.42 mmol, 4.0 equiv), 18-crown-6 (9 mg, 0.036 mmol, 0.1 equiv) and *N,N*-dimethylformamide (5.0 mL) was heated at 50 °C for 21 h at which TLC showed the completion of the reaction. The mixture was poured into cold water (30 mL), and then extracted with ethyl acetate (2×25 mL). The combined organic layer was washed with water (1×30 mL) and brine (2×30 mL), and dried with anhydrous Na_2SO_4. After filtration, the filtrate was concentrated under reduced pressure to give a crude product as a yellow oil, which was purified by column chromatography on silica gel by gradient elution with 15-30% ethyl acetate in hexane to afford the dimethoxymethyl protected product diMOM-(*R,R*)-**3** as a light yellow solid (296 mg) in 64% yield. ^{1}H NMR (600 MHz, $CDCl_3$) δ 10.55 (s, 2H), 8.54 (s, 2H), 8.01 (dd, *J* = 8.7, 5.6 Hz, 4H), 7.89 (d, *J* = 8.1 Hz, 2H), 7.44 – 7.19 (m, 16H), 6.01 (s, 2H), 5.10 (d, *J* = 3.8 Hz, 4H), 4.71 (d, *J* = 6.0 Hz, 2H), 4.61 (d, *J* = 6.1 Hz, 2H) , 2.93 (s, 6H), 2.14-2.17 (m, 2H), 1.92-1.95 (m, 2H). ^{13}C NMR (150 MHz, $CDCl_3$) δ 191.06, 166.03, 162.64, 158.31, 154.04, 153.71, 137.08, 133.88, 130.97, 130.68, 130.28, 130.15, 129.35, 129.29, 128.25, 127.31, 127.09, 126.14, 126.09, 126.08, 125.12, 124.30, 118.52, 114.19, 105.64, 100.37, 65.80, 57.20, 36.80-14.23 (m). ^{19}F NMR (564 MHz, $CDCl_3$) δ -80.74 (t, *J* = 9.7 Hz, 3F), -114.25 (m, 2F), -121.58 – -121.59 (m, 2F), -121.78– -121.88 (m, 4F), -122.61 – -122.68 (m, 2F), -123.06 – -123.12 (m, 2F), -126.03 – -126.09 (m, 2F). $[\alpha]_D^{23}$ = +7.8 (c = 1.0, $CHCl_3$).

(b) A mixture of diMOM-(*R,R*)-**3** (239 mg, 0.182 mmol, 1.0 equiv), conc. HCl (2.0 mL), ethanol (1.0 mL) and chloroform (1.0 mL) was heated at reflux for 9 h. The resulting mixture was cooled to rt, and a saturated $NaHCO_3$ solution was added dropwise into the mixture until no gas evolved. The mixture was poured into H_2O (30 mL) and extracted with ethyl acetate (1×30 mL). The organic layer was washed with H_2O (1×20 mL) and brine (1×20 mL), and then dried with anhydrous Na_2SO_4. After filtration, the filtrate was concentrated under reduced pressure to give a crude product as a yellow solid, which was purified by column chromatography on silica gel by gradient elution with 15-25% ethyl acetate in hexane to afford (*R,R*)-**3** as a yellow solid (181 mg) in 83% yield. ^{1}H NMR (600 MHz, $CDCl_3$) δ 10.53 (s, 2H), 10.16 (s, 2H), 8.27 (s, 2H), 7.96 (d, *J* = 9.0 Hz, 4H), 7.90 (d, *J* = 8.4 Hz, 2H), 7.41 (d, *J* = 9.0 Hz, 2H), 7.36-7.39 (m, 6H), 7.30 (t, *J* = 8.4 Hz, 2H), 7.26-7.25 (m, 2H), 7.22 (d, *J* = 8.4 Hz, 2H), 6.21 (s, 2H), 5.15 (m, 4H), 3.37-3.32 (m, 2H), 2.24-2.18 (m, 2H), 1.99-1.94 (m, 2H). ^{13}C NMR (150 MHz, $CDCl_3$) δ 196.72, 165.93, 158.66, 153.70, 153.62, 137.97, 137.61, 133.75, 130.50, 130.39, 129.73, 129.57, 128.36, 127.66, 126.96, 125.57, 124.91, 124.41, 124.11, 122.32, 118.81, 117.80, 114.71, 105.72, 70.95, 65.64, 32.05-14.22 (m). ^{19}F NMR (564 MHz, $CDCl_3$) δ -80.72 (t, *J* = 9.9 Hz, 3F), -114.19 (m, 2F), -121.82 – -121.86 (m, 6F), -122.60 – -122.67 (m, 2H), -123.05 – -123.10 (m, 2H), -126.02 – -126.08 (m, 2H). HRMS Calcd for $C_{60}H_{39}F_{17}NO_7$ (MH^+): 1208.2455, found: 1208.2439. $[\alpha]_D^{23}$ = +143.1 (c = 2.0, $CHCl_3$).

Synthesis and Characterization of (*S,S*)-3. Compound (*S,S*)-**3**, the enantiomer of (*R,R*)-**3**, was obtained as a yellow solid in a two-step yield of 75% by using the same procedure described above but starting with (*S*)-**2**. The ^{1}H/^{13}C/^{19}F NMR data of (*S,S*)-**3** were consistent with those of (*R,R*)-**3**. $[\alpha]_D^{23}$ = – 142.3 (c = 2.0, $CHCl_3$).

Enzymatic Hydrolysis of DL-Phenylalanine Methyl Ester Hydrochloride. Typically, DL-phenylalanine methyl ester hydrochloride (194 mg, 0.9 mmol, 1.0 equiv) was dispersed in an

organic solvent (18 mL), and then an equimolar amount of triethylamine (125 μL, 0.9 mmol, 1.0 equiv) and an aqueous solution (2.0 mL) of enzyme CT (6.0 mg) were successively added to the organic suspension. The final concentration of DL-phenylalanine methyl ester hydrochloride was 45 mM, and the total volume of solvent was 20 mL, containing 10% (in volume) H_2O. For the conditions with the use of acetonitrile containing 20% and 30% H_2O, the ester salt (194 mg) was first dispersed in 16 and 14 mL acetonitrile respectively, and then an equimolar amount of triethylamine and an aqueous solution (4.0 and 6.0 mL) of the enzyme CT (6.0 mg) were successively added to the organic suspension. The reaction mixture was stirred at rt for 24 h. At the end of the 3 reactions in acetonitrile (10% H_2O), 2-propanol (10% H_2O) and acetone (10% H_2O), precipitates were collected by filtration and dried under vacuum to give crude products Phe-1 (58.1 mg, 78% yield), Phe-4 (25.5 mg, 34% yield), Phe-7 (57.9 mg, 78% yield). The reactions in the other 4 solvents including acetonitrile (20% H_2O), acetonitrile (30% H_2O), 1-propanol (10% H_2O) and ethanol (10% H_2O) gave only small amount of precipitate because of better solubility of Phe in these solvents. The solvents were first removed under vacuum, and then 9.0 mL acetonitrile and 1.0 mL H_2O were added to the mixture. The precipitates were collected by filtration and dried under vacuum to give crude products Phe-2 (47.7 mg, 64% yield), Phe-3 (55.7 mg, 75% yield), Phe-5 (37.3 mg, 50% yield) and Phe-6 (26.9 mg, 36% yield). The crude products Phe-1 ~ Phe-7 were directly used for *ee* measurement by using the fluorescence probe and HPLC without further purification. Phe-1: ^{1}H NMR (600 MHz, D_2O) δ 7.45 – 7.33 (m, 5H), 4.00 (dd, J = 8.0, 5.2 Hz, 1H), 3.30 (dd, J = 14.6, 5.2 Hz, 1H), 3.13 (dd, J = 14.5, 8.0 Hz, 1H). HRMS Calcd for $C_9H_{12}NO_2$ $(MH)^+$: 166.0868, found: 166.0873

Preparation of Samples for Fluorescence Measurement. Stock solutions of 1.2 mM (*R,R*)-**3** or (*S,S*)-**3** in PFOH, 2.0 or 3.0 M TBAH aqueous solutions and 4.0 or 8.0 M $Zn(OAc)_2$ aqueous solutions were freshly prepared for each measurement. 1.2 mM (*R,R*)-**3** or (*S,S*)-**3** solutions were diluted to 0.040 mM with PFOH prior to being treated with amino acids. Amino acids were first dissolved in 2.0 or 3.0 M TBAH aqueous solutions to achieve the desired concentrations, and then these aqueous solutions (2.0 mL) were mixed with the probe-containing fluorous solutions (2.0 mL 0.040 mM). Finally, $Zn(OAc)_2$ aqueous solutions (20 μL) were added to the mixtures. For the crude products of Phe formed from the enzymatic hydrolysis, when they were added to TBAH (2.0 mM) aqueous solutions, no clear solution could be obtained even after ultrasonication due to reaction impurities. These mixtures were directly stirred with the probe-containing fluorous solutions without filtration at rt for 4 h (unless otherwise noted) without nitrogen protection. Then, each of these mixtures was transferred to a 4 mL quartz cuvette (1 cm × 1 cm) for fluorescence measurement after being allowed to stand at rt for 10 min for two-phase separation. The two light paths in the spectrofluorometer only went through the lower fluorous phase.

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Supplementary Materials Available: Additional experimental description and spectroscopic data.

Keywords: Fluorous phase, enantioselective recognition, fluorescent probe, amino acid, BINOL

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