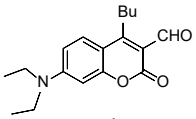
Mechanistic Study on a BINOL-Coumarin-Based Probe for Enantioselective Fluorescent Recognition of Amino Acids

Xuedan Wu[b#], Qin Wang*[a,b#], Diane Dickie[b], Lin Pu*[b]

Abstract. A detailed investigation was conducted on the reaction of a BINOL-coumarin-based fluorescent probe with amino acids. When the probe is excited at λ_1 = 365 nm in a neutral buffer solution, it shows fluorescence enhancement at 465 nm in the presence of an amino acid with only small difference between the two enantiomers. When excited at λ_2 = 467 nm, it shows highly enantioselective fluorescence enhancement at 534 nm. This allows the determination of both concentration and enantiomeric composition of the amino acid. On the basis of the studies including fluorescence spectroscopy, ^{1}H NMR, UV-Vis, mass spectroscopy, single crystal X-ray analysis and molecular modeling, it was found that the distinctively different fluorescent responses of the probe toward the amino acid at the two excitation wavelengths are due to two different reaction pathways that generate different intermediates and products.

Introduction

Because of the importance of chiral α-amino acids in nature as well as in organic synthesis, their enantioselective fluorescent recognition by using molecular probes has been actively investigated in recent decade.[1-3] These probes can potentially provide an analytic method for high throughput analysis or real time imaging of the enantiomers of the amino acids generated from either asymmetric reactions or biological processes. Previously, Feuster and Glass reported the use of an achiral coumarin compound **1** for fluorescent detection of amino acids.[4] It was found that amino acids can enhance the fluorescence of **1** in the absence of a metal ion additive such as Zn^{2+}. This is different from the 1,1'-bi-2-naphthol (BINOL)-based aldehydes, such as (*S*)-**2**, developed previously in our laboratory which requires the addition of Zn^{2+} in order to observe the enantioselective fluorescent enhancement in the presence of amino acids.[5] We have recently linked the chiral BINOL unit with the coumarin aldehyde to make a fluorescent probe for the enantioselective recognition of amino acids in the absence of Zn^{2+}.[6,7] This probe not only shows highly enantioselective fluorescent response toward a number of amino acids, but can also allow the determination of both concentration and enantiomeric composition. It was the first fluorescent probe that can be excited at two different wavelengths to report the two parameters of a chiral substrate. In order to understand the unique fluorescence response of this amino acid probe, we have conducted a detailed study on its reaction with amino acids which has revealed its intriguing reactivity. Herein, these results are reported.



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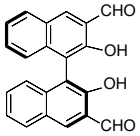


(*S*)-**2**

Results and Discussion

1. Study of the BINOL-Coumarin Conjugates (*S*)- and (*R*)-6.

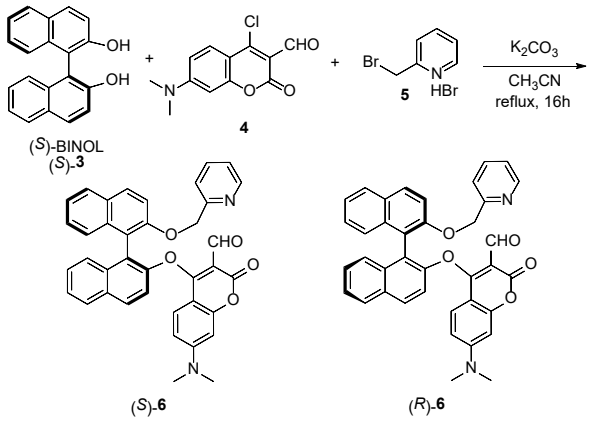
From a one-pot reaction of (*S*)-BINOL, **4**[8] and **5** according to our reported procedure,[6] the BINOL-coumarin conjugate (*S*)-**6** was obtained (Scheme 1). We also prepared compound (*R*)-**6**, the enantiomer of (*S*)-**6**, from (*R*)-BINOL. Previously, we investigated the fluorescence response of (*R*)- and (*S*)-**6** toward the amino acid valine in aqueous buffer solution (HEPES, pH = 7.4).[6] As shown in Figure 1a, in the aqueous solution, (*R*)-**6** gave one broad emission at λ = 554 nm upon excitation at 365 nm, indicating an efficient energy transfer from one naphthalene unit to the conjugated naphthyl-coumarin unit. When treated with valine (100.0 equiv), large fluorescence enhancement at λ = 465 nm was observed upon excitation at 365 nm. Both D- and L-Val generated similar fluorescence enhancement at this wavelength. When the solution of (*R*)-**6** was excited at 467 nm, a broad emission at λ = 558 nm was observed. This emission band was found to exhibit highly enantioselective response upon reaction with the amino acid. While D-Val greatly enhanced the

[a] Dr. Qin Wang
Key Laboratory of Medical Electrophysiology, Ministry of Education
School of Pharmacy of Southwest Medical University
Luzhou 646000, China
E-mail: wq_ring@hotmail.com

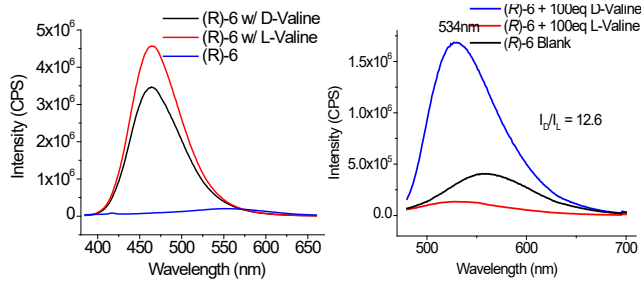
[b] Xuedan Wu, Dr. Diane Dickie, Prof. Dr. L. Pu
Department of Chemistry, University of Virginia
Charlottesville, Virginia 22904-4319 (USA)
E-mail: lp6n@virginia.edu

# These authors contributed equally to this work.

Scheme 1. Synthesis of the BINOL-Pyridine-Coumarin Conjugate (*S*)-**6**.



fluorescence of (*R*)-**6** at λ = 534 nm, L-Val slightly quenched this fluorescence (Figure 1b). The amino acid also caused a small blue shift of the emission band. In these experiments, (*R*)-**6** (1.0 mM in DMSO, 1.0 equiv) was reacted with L- and D-Val (5.0 - 100.0 equiv) in HEPES buffer for 2 h and then diluted to 1.0 x 10^{-5} M with HEPES for fluorescence measurement. The different fluorescence responses of the probe at two excitation wavelengths allowed us to determine the concentration and enantiomeric composition of the amino acid with only one fluorescent probe.[6] Similar fluorescent responses of (*R*)-**6** toward other amino acids were observed which make it useful for the fluorescent recognition of structurally diverse amino acids.



(a) (b)

Figure 1. Fluorescence spectra of (*R*)-**6** (1.0 × 10^{-5} M in HEPES/1% DMSO, pH = 7.4) with L- and D-Val (100 equiv) while excited at (a) λ_{exc} = 365 nm, and (b) λ_{exc} = 467 nm (slits: 3/3 nm)

In order to understand how the probe reacts with the amino acids to give the observed fluorescence responses, we conducted a mass spectroscopic analysis on the reaction of (*S*)-**6** with valine. Because the large amount of HEPES in the reaction mixture interfered with the mass analysis, we acquired the mass spectra for the reaction of (*S*)-**6** (1 mM) in DMSO with D- and L-Val (1 mM) in HEPES (25 mM, pH = 7.4, 120 mM NaCl) by directly diluting it with water instead of HEPES and used the flow injection method to obtain the spectra of the reaction mixture. In the mass spectrum of the product mixture of (*S*)-**6** with L-Val (Figure S18a in SI), we have identified peaks at m/z = 378.1486 for **7** (calcd for **7**+H^+: 378.1494), 333.1453 for compound **8** (calcd for **8**+H^+: 333.1450), 432.2123 for compound **9** (calcd for **9**+H^+: 432.2135), and 692.2758 for intermediate **10** (calcd for **10**+H^+: 692.2761) respectively. Structures of compounds **7** – **10** are shown in Figure 2. Similar mass signals are also found in the product mixture of (*S*)-**6** with D-Val (Figure S18b in SI), but the peak intensity at 692.2748 for intermediate **10** was much weaker than that in the reaction of L-Val.

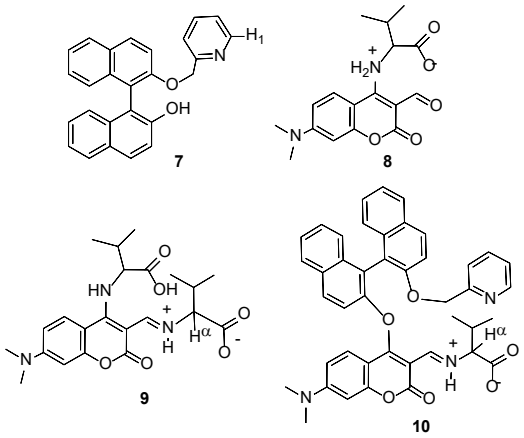
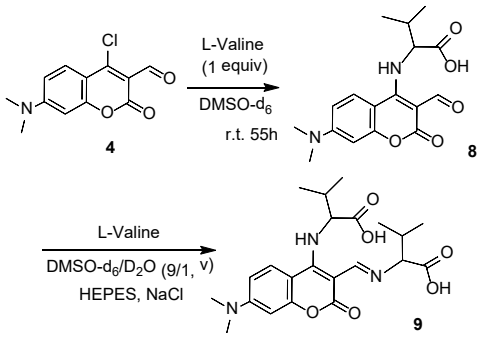


Figure 2. Product and intermediate structures formed from the reaction of **6** with valine.

We further studied the reaction of (*R*)-**6** with valine by ^{1}H NMR spectrometry. As shown in Figure 3, when (*R*)-**6** was treated with D-Val for 2 – 4 h, there was only a small degree of reaction (Figure 3d-f). This suggests that during the fluorescence measurement (2 h reaction time), the extent of the reaction between (*R*)-**6** and the amino acid should be very small. After 36 h, there was about 40% conversion of (*R*)-**6** to the corresponding products (Figure 3i). In Figure 3i, the signals at δ 5.17, 6.88, 7.00 and 8.42 can be attributed to the product **7** by comparing with the ^{1}H NMR spectrum of **7**, prepared from the reaction of **3** with **5**,[9] under the same conditions. The singlet at δ 9.81 is assigned to the aldehyde proton signal of the product **8** and the two signals at δ 4.24 and 4.44 can be assigned to the Hα signals of **9** and **10**.

In order to provide additional characterization for compounds **8** and **9**, we studied the reaction of **4**[8] with L-Val (1.0 equiv) in DMSO-d_6 (Scheme 2). After 55 h at rt, the ^{1}H NMR spectrum of the reaction mixture showed complete consumption of **4** with the formation of compound **8** as the major product (Figure S12 in SI, compound **8** could not be separated from the other minor products).[10] The aldehyde proton signal of **8** was observed at δ 9.74 (in 9:1 DMSO-d_6/D_2O) or 9.72 (in pure DMSO-d_6) very close to that in Figure 4i. When additional amount of L-Val (40 equiv) was added to the solution of **8**, the aldehyde signal of **8** disappeared with the appearance of an imine proton signal at δ 8.32 and the Hα signal at δ 4.35 for the product **9** (Figure S14 in SI). The mass spectra of the reaction mixtures confirmed the formation of compounds **8** and **9** as the major products (Figures S13 and S15 in SI). In the ^{1}H

Scheme 2. Reaction of **4** with L-Val.



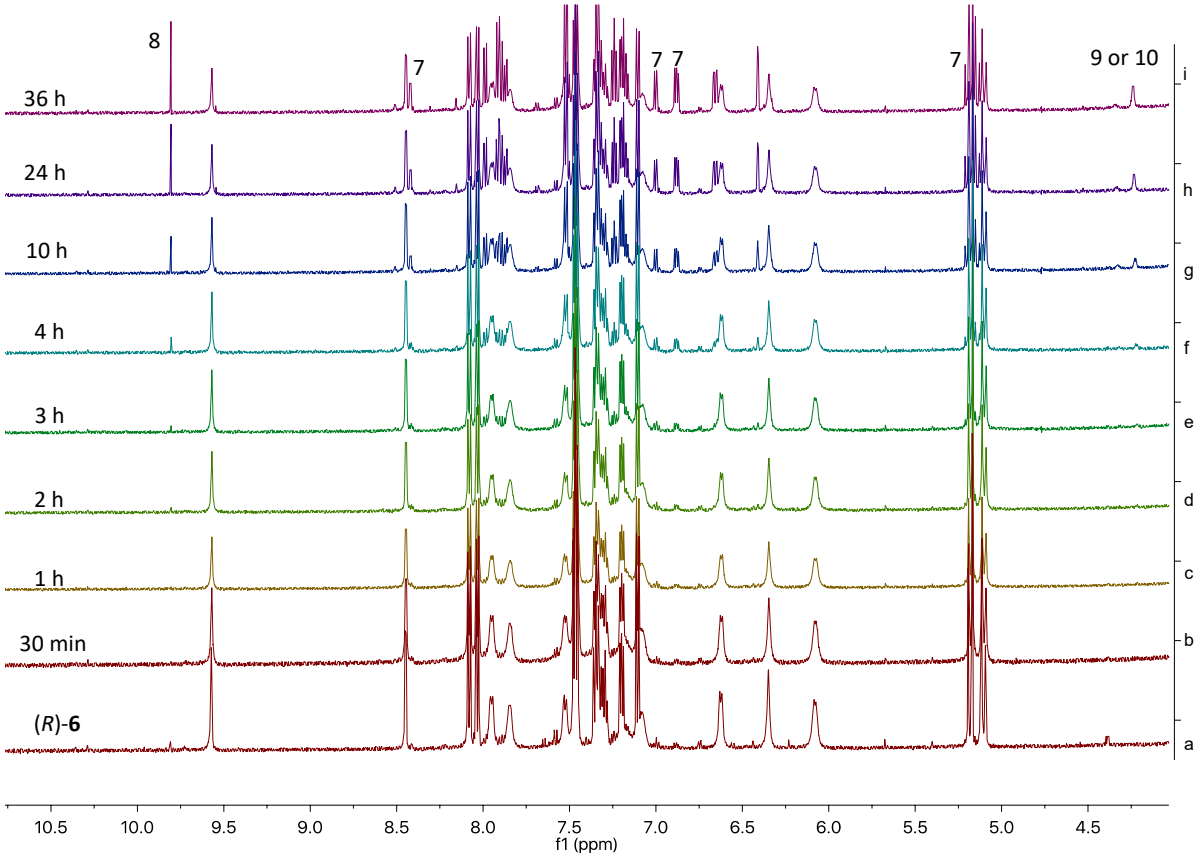
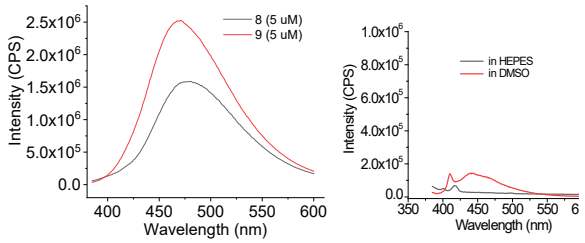


Figure 3. ^{1}H NMR (600 MHz) spectra of (*R*)-**6** (1.0 mM in DMSO-d_6) (a) with D-Val (40 equiv) in HEPES (D_2O, 25 mM HEPES, 120 mM NaCl, pD = 7.4) for 30 min to 36 h (b-i). [DMSO-d_6:HEPES-D_2O = 9:1 (v). The full spectra are given in Figure S10 of SI.]

NMR spectrum of the product mixtures of **8** and **9**, the signals are found to be similar to those in Figure 4i but not identical (Figure S11d in SI). This discrepancy could be attributed to various intermolecular interactions of these highly polar molecules in the HEPES buffer solution of the reaction mixture of (*R*)-**6** with valine.

We measured the fluorescence spectra of the above product mixtures of **8** and **9** after they were diluted with HEPES buffer to ~5 μM. As shown in Figure 4a, both of them show similar fluorescence when excited at 365 nm with emission maxima at 476 and 468 nm for **8** and **9** respectively. When excited at 467 nm, the emissions of **8** and **9** were very weak (Figure S17 in SI). Figure 4b gives the fluorescence spectra of **7** in HEPES buffer solution and pure DMSO which show very weak or no emission when excited at 365 nm. These spectra indicate that the observed enantioselective fluorescence enhancement at 534 nm in the reaction of (*R*)-**6** with D-Val is not contributed by the three products **7**, **8** and **9**.



(a) (b)

Figure 4. Fluorescence spectra of (a) the product mixtures of **8** and **9** from the reaction of **4** with valine [diluted with HEPES buffer in H_2O to ~5 μM, λ_{exc} = 365 nm, slits: 3/3 nm]. (b) Compound **7** (1.0 × 10^{-5} M) in HEPES/1%DMSO (pH = 7.4) and DMSO (λ_{exc} = 365 nm, slits: 3/3 nm).

Although no significantly new ^{1}H NMR signal appeared from the reaction of (*R*)-**6** with D-Val in 2 h as shown in Figure 3d, the UV spectrum for the reaction of (*R*)-**6** with D-Val in 2 h gave a new signal at λ = 467 (sh) nm which was missing in the reaction with L-Val (Figure 5a). A close to mirror image relation was observed

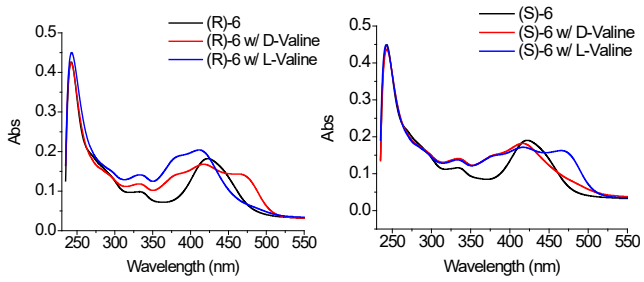


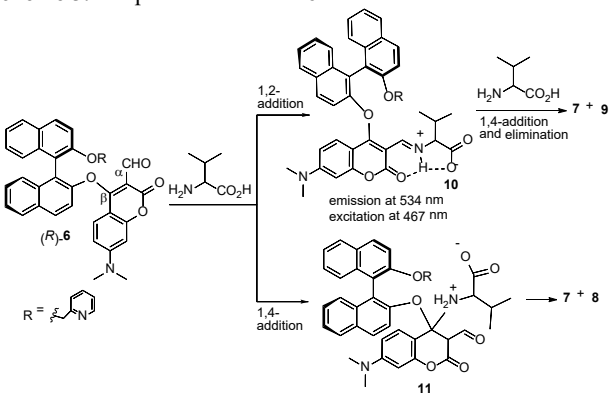
Figure 5. UV-Vis absorption spectra of (*R*)-**6** (1.0 x 10^{-5} M) and its reaction with D- and L-Val [(*R*)-**6** (1.0 mM, DMSO) was mixed with D- or L-Val (100 equiv in HEPES, pH = 7.4) for 2 h and then diluted with HEPES to 1.0 x 10^{-5} M and measured in 30 min.]

for the UV spectra of the enantiomeric probe (*S*)-**6** with L- and D-Val (Figure 5b). The new absorption at 467 nm is correlated with the observed large fluoresce enhancement at 534 nm (λ_{exc} = 467 nm) for the reaction of (*R*)-**6** with D-Val during the same period of reaction time (See the excitation spectra in (Figure S7 in SI). Both **7** and the product mixtures of **8** and **9** from the reaction of **4** with Val do not show this long wavelength absorption at 10 μM (Figure S9b in SI). Therefore, we attribute the observed enantioselective fluorescence enhancement at 534 nm in Figure 1b to the proposed intermediate **10** formed at the beginning of the reaction of (*R*)-**6** with valine, but not the final products **7**, **8** and **9**.

On the basis of the above spectroscopic analyses, a mechanism for the reaction of the probe **6** with valine can be proposed. As

shown in Scheme 3, valine can react with (*R*)-**6** in two different pathways. In one reaction pathway, the 1,2-addition intermediate **10** can be generated,[4] which is supported by the mass spectroscopic analysis of the reaction mixture. This intermediate should give the long wavelength emission at 534 nm due to the conjugation between the coumarin-iminium and the naphthalene ring. The subsequent 1,4-addition of **10** with valine followed by elimination should give the products **7** and **9**. In another reaction pathway, the 1,4-addition[10] intermediate **11** could be generated which is not stable and undergoes elimination to give the products **7** and **8**. Evidence for the formation of the intermediate **11** was obtained when we conducted another high-resolution mass spectroscopic study on the reaction of (*R*)-**6** with the tetrabutylammonium (TBA) salts of D- and L-Val in DMSO. In the mass spectrum of the product mixture for the reaction of (*R*)-**6** with D- and L-Val-TBA (20 equiv) in DMSO (Figure S19 in SI), besides the peaks for **7**, **8**, and **9**, a signal at m/z = 710.2852 could be assigned to **11**-L and that at m/z = 710.2853 to **11**-D (calcd for **11**+H^+: 710.2866).

Scheme 3. Proposed reaction of **6** with Val.



We have conducted an X-ray analysis on a single crystal of (*S*)-**6** which shows that the pyridine ring and the coumarin plane were almost parallel with an angle of 4.50° (Figure 6 and page S50 in SI). A water molecule was found to form hydrogen bonding interaction to link the pyridine nitrogen of one (*S*)-**6** molecule with the aldehyde oxygen of another (*S*)-**6** molecule. In order to

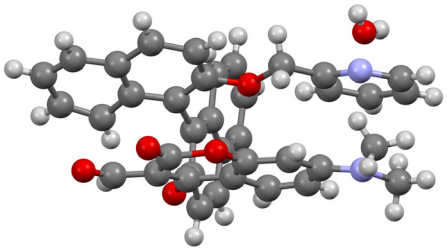
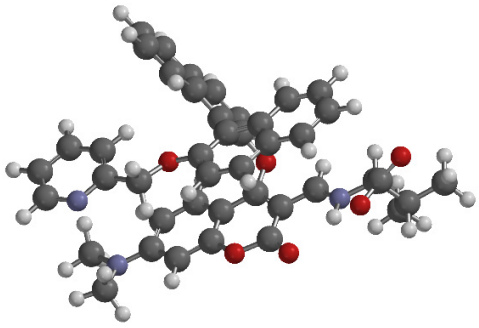
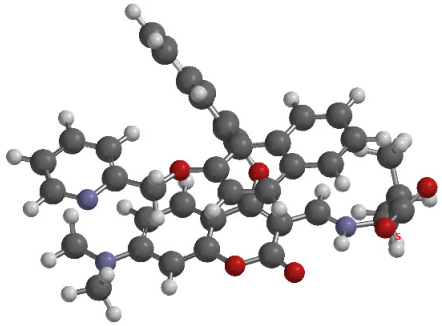


Figure 6. X-ray structure of (*S*)-**6**.

understand the different enantioselective fluorescent responses at the short and long wavelength emissions, we conducted a density functional calculation by using the SPARTAN'18 program (ωB97X-D, 6-31G*). Figure 7 shows the energy minimized structures for the two diastereomeric intermediates **10** formed from the condensation of (*R*)-**6** with D-Val (**10**-D) and L-Val (**10**-L). In these structures, the coumarin ring is parallel with the naphthyl pyridinylmethyl ether unit similar to that observed in the crystal structure of (*R*)-**6** shown in Figure 6. The hydrogen bonding interactions of the iminium proton with the carbonyl group of the coumarin lactone group and with the carboxylate oxygen are present. In **10**-D, the isopropyl group is pointing away from the upper naphthalene ring with little steric interaction. It makes **10**-D more stable than **10**-L in which the isopropyl group is pointing toward the upper naphthalene ring with increased steric interaction. The calculated energy difference of these two diastereomers in water is 7.25 kcal/mole. This stability difference might lead to the observed large difference in the fluorescence response when (*R*)-**6** was treated with D-Val versus L-Val. We calculated the energy difference between the two diastereomers of the intermediate **11** formed from the reaction of (*R*)-**6** with L- and D-Val as well (See Figure S20c,d in SI). It was found that the energy difference of these two diastereomers (**11**-D and **11**-L) is much smaller than that of **10**-D and **10**-L, consistent with the much lower enantioselective fluorescent response at the short wavelength emission.



10-D

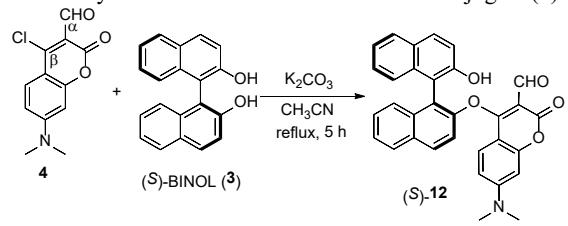


10-L

Figure 7. Molecular modeling structures of the proposed intermediates **10**-D and **10**-L.

We also prepared the BINOL-coumarin conjugate (*S*)-**12** from the reaction of **4** with (*S*)-BINOL in the presence of K_2CO_3 (Scheme 4). The fluorescence response of (*S*)-**12** toward valine in aqueous solution (HEPES buffer, pH = 7.4) was studied. In these experiments, (*S*)-**12** (1.0 mM) was used

Scheme 4. Synthesis of the BINOL-Coumarin Conjugate (*S*)-**12**.



to react with D- and L-Val for 2 h and then diluted with HEPES to 1.0 x 10^{-5} M before fluorescence measurement. When (*S*)-**12** was excited at λ = 365 nm in the presence of valine (100 equiv), large fluorescence enhancement at the short wavelength (467 nm) was observed with small enantioselectivity (Figure 8a). When (*S*)-**12** was excited at λ = 467 nm, it gave an emission signal at 555 nm which was greatly reduced upon reaction with the amino acid and little enantioselectivity was observed (Figure 8b). Study of (*S*)-**12** demonstrates that the pyridine group of (*S*)- or (*R*)-**6** is

necessary for the observed enantioselective fluorescent response when excited at 467 nm. ^{1}H NMR spectroscopic study was conducted for the reaction of (*S*)-**12** (1.0 mM) with D- and L-Val (20 equiv) in DMSO/HEPES (9:1) solution which showed similar reaction pattern as those observed for (*R*)-**6** (Figure S11a,b,d in SI).

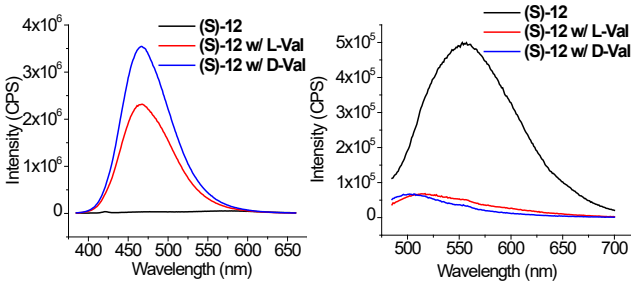


Figure 8. Fluorescence spectra of (*S*)-**12** (1.0 x 10^{-5} M in HEPES/ 1% DMSO, pH = 7.4) with L- and D-Val (100 equiv) while excited at (a) λ_{exc} = 365 nm and (b) λ_{exc} = 467 nm (slits: 3/3 nm).

Conclusion

The BINOL-coumarin-based compound **6** was found to show very different fluorescent response toward the enantiomers of amino acids when excited at two different wavelengths. On the basis of a series of spectroscopic studies, we have identified two different pathways for the reaction of the probe with an amino acid. That is, the α,β-unsaturated aldehyde unit of **6** can undergo 1,4- and 1,2-additions to give different intermediates and products which contribute to the observed very different fluorescent responses. The 1,2-addition of **6** with an amino acid can generate an iminium ion intermediate with extended conjugation which probably gives the highly enantioselective long wavelength emission. The 1,4-addition of **6** with the amino acid disrupts the conjugation between the naphthalene ring and the coumarin unit, leading to products of short wavelength emission. The very different fluorescence responses at the two wavelengths allow the use of this probe to determine both the concentration and enantiomeric composition of amino acids. This is remarkable since normally two independent probes are needed in order to determine both concentration and enantiomeric composition of a chiral substrate except in rare cases.[11] Our study of **6** demonstrates that when two different reaction pathways are incorporated into a fluorescent probe, they may be used to report more information about the substrates than probes with only one type of binding or reactive site. This represents a new strategy to design enantioselective fluorescent probes for chiral recognition.

Experimental Section

General Data. Nitrogen atmosphere was applied to all the synthetic reactions unless otherwise noted and the commercially available compounds were from Sigma Aldrich Chemical Co. or Alfa Aesar. All solvents used in the fluorescence measurement were HPLC or spectroscopic grades. Optical rotation measurements were conducted on a Jasco P-2000 digital polarimeter. NMR spectra were recorded on a Varian-600 MHz spectrometer and a Bruker-600 MHz spectrometer. Chemical shifts for ^{1}H NMR spectra were recorded in parts per million relative to solvent signals at 7.26 ppm for $CDCl_3$, and 2.50 ppm for DMSO-d_6. Chemical shifts for $^{13}C\{^1H\}$ NMR were recorded relative to the centerline of a triplet at 77.16 ppm for $CDCl_3$. Mass spectroscopic analyses were conducted by the University of Illinois at Urbana-Champaign Mass Spectrometry Facility and the Agilent LC-Q-TOF Mass Spectrometer in the Department of Chemistry of University of Virginia. Steady-state fluorescence spectra were recorded with a Horiba FluoroMax-4 spectrofluorometer. UV-Vis spectra were measured by Shimadzu UV-2600 UV-Vis spectrometer. Single crystal X-ray diffraction data were collected on a Bruker Kappa APEXII Duo diffractometer running the APEX3 software suite using the Mo Kα fine-focus sealed tube (λ = 0.71073 Å) for (*S*)-**6**. The structures were solved and refined using the Bruker SHELXTL Software Package within OLEX2. Non-hydrogen atoms were refined anisotropically. Hydrogen atoms were placed in geometrically calculated positions with Uiso = 1.2Uequiv of the parent atom (Uiso = 1.5Uequiv for methyl).

Synthesis and characterization of (*S*)-12. (*S*)-BINOL (0.8576 g, 3.0 mmol) and **4** (0.7512 g, 3.0 mmol) were stirred in refluxing CH_3CN (20 mL) in the presence of K_2CO_3 (2.0732 g, 15 mmol) for 5 h. After filtration to remove the remaining K_2CO_3 and roto-evaporation to remove the solvent, the residue was purified by column chromatography on silica gel (gradient elution with hexanes/ethyl acetate from 1:5 to 1:1) to give the product (*S*)-**12** as a yellow powder in 66% yield (0.9916 g). ^{1}H NMR (600 MHz, $CDCl_3$) δ 9.90 (s, 1 H), 7.86 (m, 4 H), 7.57 (s, 1 H), 7.43 (t, J = 18.0 Hz, 1 H), 7.33 (m, 3 H), 7.27 (m, 3 H), 7.13 (m, 2 H), 6.37 (m, 1 H), 6.35 (s, 1 H), 3.03 (s, 6 H). $^{13}C\{^1H\}$ NMR (151 MHz, $CDCl_3$) δ 187.4, 166.1, 157.6, 157.6, 155.7, 152.8, 152.6, 134.3, 133.9, 130.7, 130.6, 130.2, 130.2, 128.9, 128.2, 128.2, 127.6, 127.5, 126.4, 125.8, 125.8, 125.4, 125.4, 124.4, 123.1, 119.2, 113.9, 110.3, 105.1, 97.1, 40.2, 40.2. HRMS (ESI) m/z: [M + H]+ calcd for $C_{32}H_{24}NO_5$: 502.1654, found 502.1657. $[\alpha]_D^{23}$ = –230.50 (c = 0.05, DMSO).

Sample Preparation for Fluorescence Measurement. All HEPES buffers used were prepared with 25 mM HEPES and 120 mM NaCl in water (HPLC grade) (pH = 7.4). Stock solutions of (*S*)-**6** or (*R*)-**6** (1 mM in DMSO), and 1 – 100 mM amino acids (in HEPES buffer) were all freshly prepared for each measurement. The reaction mixtures (typically 25 μL stock solution of **6** in DMSO and 25 μL amino acid solution in HEPES buffer) were all allowed to stand at rt for 2 h (unless otherwise noted) without nitrogen protection. Then, they were diluted to 1.0 × 10^{-5} M with the HEPES buffer. Fluorescence measurements were conducted after 1 h, and finished within 30 min.

AUTHOR INFORMATION

Corresponding Author
lp6n@virginia.edu (Lin Pu)
wq_ring@hotmail.com (Qin Wang)

ORCID
Lin Pu: 0000-0001-8698-3228
Qin Wang: 0000-0001-9852-5347
Xuedan Wu: 0000-0001-5875-4233
Diane Dickie: 0000-0003-0939-3309

Notes
The authors declare no competing financial interest.

Supplementary Materials Available: Additional experimental description, fluorescence and UV spectra, molecular modeling data and X-ray analysis experiment. CCDC 1976654 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/structures.

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