Synthesis and Evaluation of Two Coumarin-Type Derivatization Reagents for Fluorescence Detection of Chiral Amines and Chiral Carboxylic Acids

MATTHIAS D. MERTENS AND MICHAEL GÜTSCHOW*
Pharmaceutical Institute, Pharmaceutical Chemistry I, University of Bonn, Bonn, Germany

ABSTRACT The synthesis of two fluorescent coumarin-type chiral derivatization agents (**4** and **11**) is reported. A chiral side chain was introduced at position 7 of the coumarin via Mitsunobu reaction. The two coumarins bear in this side chain either a free amino group or a carboxyl group, making them useful for further transformations. Conjugates of chiral prototype drugs with **4** or **11** were prepared by amide coupling of the analyte's carboxyl group to the reagent's amine group, or vice versa. The separation of seven diastereomeric conjugates through achiral high-performance liquid chromatography (HPLC) on a common C18 column is demonstrated. *Chirality 25:957–964, 2013*.

KEY WORDS: reversed-phase high-performance liquid chromatography; diastereomer separation; chiral coumarins; fluorescence detection; precolumn derivatization; Mitsunobu reaction

Chirality is strikingly important for biological recognition, including drug–target interactions. It is well known that the two enantiomers of a drug can differ in their pharmacological activity, side effects, stability, and pharmacokinetics.[1] Numerous drugs on the market have a chiral center, such as β-blockers, 2-arylpropionic acid-type nonsteroidal antiinflammatory drugs (NSAIDs), antidepressants, e.g., fluoxetine and citalopram, and proton pump inhibitors, e.g., omeprazole. Although some of them are still used as racemates, the number of enantiomeric pure drugs is continuously increasing.[2] Hence, monitoring of the enantiopurity of drugs is crucial for quality control in the production of pharmaceuticals. Sensitive methods for the determination of the optical purity are required and numerous publications report on the analytical separation of drugs.[3,4] Chiral resolution by high-performance liquid chromatography (HPLC) can be employed to determine the enantiomeric purity. While the use of a chiral stationary phase (CSP) represents a direct and accurate method, the formation of diastereomers by means of a chiral derivatization agent (CDA) is an indirect approach, which can be performed on an achiral stationary phase. Both methods possess advantages and disadvantages.[5]

Derivatization reagents are well established tools for analytical chemistry. Among them, the fluorescent derivatization reagents are valued for their detection, which is far more sensitive than the corresponding UV detection. Accordingly, the precolumn fluorescence derivatization is a common method to improve the limit of detection. For chiral derivatization, several reagents have been developed; some of them make use of their fluorescent properties.[6–10] Moreover, a couple of fluorescence derivatization reagents were reported as bearing a coumarin moiety as a fluorophore, one of the most sensitive, generally accepted, and commercially available class of fluorophors.[11–17] Coumarin represents the parent substance of a broad class of naturally occurring benzopyranes; many of its derivatives are highly fluorescent. The structure–fluorescence relationships are well understood and depend on the coumarin's substitution pattern. The excitation wavelengths range from the near-UV to the blue spectral region and are often characterized by an acceptable large Stokes shift.[18] Besides their use as derivatization reagents, coumarins have also been applied to label bioactive molecules, e.g., as inhibitors of disease-related proteins or internally quenched substrates.[19–24]

Coumarin derivatives with an ester moiety or an aryl group in position 3 are known to be highly fluorescent. The fluorescence of the former compounds emerges especially in polar solvents such as water and methanol, and 3-chloroformyl-7-methoxycoumarin (MC3C), by forming the corresponding esters, was useful for the determination of 17-oxosteroids in urine[11] or platelet-activating factors in lysates of human polymorphonuclear leukocytes.[13] Among the 3-aryl coumarins,[25] 7-diethylamino substituted derivatives such as 7-diethylamino-3-[(4-(iodoacetyl)amino)phenyl]-4-methylcoumarin (DCIA)[12] or 3-(4-bromomethylphenyl)-7-diethylaminocoumarin (MPAC-Br)[15] are applicable to separate fatty or bile acids. However, none of these coumarin-type reagents bear a chiral atom, which would make them suitable for chiral derivatization.

In this work, the preparation and characterization of two coumarin molecules featuring a chiral carbon atom for derivatization as well as the resolution of representative drug-coumarin conjugates is presented. The separation efficiency of the derivatization reagents was evaluated in terms of the parameters α and R_S.

MATERIALS AND METHODS

Chemicals and Instruments

Thin-layer chromatography was carried out on Merck (Darmstadt, Germany) aluminum sheets, silica gel 60 F_{254}. Detection was performed with UV light at 254 nm. Preparative column chromatography was performed on Merck silica gel 60 (70–230 mesh). Melting points were determined by a Boëtius melting point apparatus from VEB Wägetechnik Rapido PHMK and are uncorrected. ^{1}H NMR (500 MHz) and ^{13}C NMR

*Correspondence to: Michael Gütschow, Pharmaceutical Institute, Pharmaceutical Chemistry I, University of Bonn, An der Immenburg 4, D-53121 Bonn, Germany. E-mail: guetschow@uni-bonn.de
Received for publication 18 July 2013; Accepted 6 August 2013
DOI: 10.1002/chir.22240
Published online 23 October 2013 in Wiley Online Library (wileyonlinelibrary.com).

spectra (125 MHz) were recorded on a Bruker Avance (Billerica, MA) DRX 500. Chemical shifts δ are given in ppm referring to the signal center using the solvent peaks for reference: $CDCl_3$ 7.26 / 77.0 ppm and DMSO-d_6 2.49 / 39.7 ppm. Optical rotations were determined on a Perkin-Elmer (Norwalk, CT) 241 Polarimeter. LC-DAD chromatograms and ESI-MS spectra were recorded on an Agilent (Palo Alto, CA) 1100 HPLC system with Applied Biosystems (Foster City, CA) API-2000 mass spectrometer. Solvents and reagents were obtained from Acros (Geel, Belgium), Alfa Aesar (Karlsruhe, Germany), or Sigma-Aldrich (Steinheim, Germany). HPLC water was obtained from a Milli-Q Water Purification System from Millipore (Darmstadt, Germany) and HPLC acetonitrile was obtained from VWR BDH Prolabo (Darmstadt, Germany).

*Synthesis of Methyl 7-Hydroxy-2-oxo-2*H*-chromene-3-carboxylate (2)*

2,4-Dihydroxybenzaldehyde (**1**, 5.52 g, 40.0 mmol) was dissolved in MeOH (60 mL). Dimethyl malonate (5.81 g, 5.0 mL, 44.0 mmol, 1.1 eq.) and piperidine (426 mg, 0.50 mL, 5.0 mmol) were added. The solution was refluxed for 2 h. The stirred reaction mixture was cooled to room temperature and kept on an ice bath for 30 min. The product was filtered off and washed with cold MeOH (20 mL) to yield a white solid (5.74 g, 26.1 mmol, 65%): mp 264-265 °C (lit.[26] mp 264-267 °C); ^{1}H NMR (500 MHz, DMSO-d_6, 30 °C) δ 3.78 (s, 3H, CH_3), 6.71 (d, 1H, ^4J = 2.5 Hz, 8-H), 6.82 (dd, 1H, ^3J = 8.5 Hz, ^4J = 2.3 Hz, 8-H), 7.73 (d, 1H, ^3J = 8.8 Hz, 5-H), 8.67 (s, 1H, 4-H), 11.04 (br s, 1H, OH); ^{13}C NMR (125 MHz, $CDCl_3$, 25 °C) δ 52.2, 101.9, 110.6, 112.0, 114.2, 132.3, 149.8, 156.5, 157.3, 163.6, 164.3. MS ESI+: *m/z* 221 ($[C_{11}H_9O_5]^+$); HPLC purity: 99.7% (λ = 220–450 nm).

Synthesis of (S)*-(−)-Methyl 7-(2-(*tert*-butoxycarbonylamino)propoxy)-2-oxo-2*H*-chromone-3-carboxylate (3)*

Methyl 7-hydroxy-2-oxo-2*H*-chromene-3-carboxylate (**2**, 440 mg, 2.0 mmol), triphenylphosphine (1.04 g, 4.0 mmol), (*S*)-(−)-*tert*-butyl 1-hydroxypropan-2-yl carbamate (525 mg, 3.0 mmol) were dissolved in THF (30 mL) and cooled to 0 °C. Diisopropyl azodicarboxylate (DIAD) (0.61 g, 0.59 mL, 3.0 mmol) was added dropwise and the solution was stirred overnight at room temperature. After evaporation in vacuo the residue was dissolved in EtOAc (100 mL) and the organic phase was washed with 1 N NaOH (3 × 100 mL) and brine (100 mL). The organic phase was evaporated and the residue was purified by column chromatography using petroleum ether/EtOAc (1/1) as eluent. The product was obtained as pale yellow solid (683 mg, 1.8 mmol, 90%): mp 128–129 °C; $[\alpha]_D^{20}$ = −38.9 (*c* = 0.95, MeOH); UV (H_2O) λ_{max} nm (log ε): 353 (4.41), UV (MeCN) λ_{max} nm (log ε): 347 (4.29), UV (MeOH) λ_{max} nm (log ε): 349 (4.42); ^{1}H NMR (500 MHz, DMSO-d_6, 25 °C) δ 1.12 (d, 3H, ^3J = 7.0 Hz, CHC*H*$_3$), 1.37 (s, 9H, C(C*H*$_3$)$_3$), 3.80 (s, 3H, OC*H*$_3$), 3.82-3.88 (m, 1H, CH), 3.94-3.97 (dd, ^2J = 9.8 Hz, ^3J = 5.7 Hz, 1H, OC*H*H), 4.01-4.04 (dd, 1H, ^2J = 9.8 Hz, ^3J = 6.4 Hz, OCH*H*), 6.89 (d, 1H, ^3J = 7.3 Hz, NH), 6.99 (dd, 1H, ^3J = 8.5 Hz, ^4J = 2.2 Hz, 6-H), 7.01 (d, 1H, ^4J = 2.3 Hz, 8-H), 7.82 (d, 1H, ^3J = 8.5 Hz, 5-H), 8.73 (s, 1H, 4-H); ^{13}C NMR (125 MHz, DMSO-d_6, 25 °C) δ 17.3, 28.3, 45.2, 52.3, 71.5, 77.9, 100.9, 111.6, 113.2, 113.8, 131.8, 149.5, 155.2, 156.3, 157.1, 163.5, 164.2. MS ESI+: *m/z* 378 ($[C_{19}H_{24}NO_7]^+$); HPLC purity: 95.7% (λ = 220–400 nm).

Synthesis of (S)*-(−)-1-(3-(Methoxycarbonyl)-2-oxo-2*H*-chromone-7-yloxy)propan-2-aminium Chloride (4)*

(*S*)-(−)-Methyl 7-(2-(*tert*-butoxycarbonylamino)propoxy)-2-oxo-2*H*-chromone-3-carboxylate (**3**, 1.53 g, 4.0 mmol) was dissolved in EtOAc (10 mL) and cooled to 0 °C. A solution of HCl in EtOAc (1 N, 20 mL, 20.0 mmol, 5.0 eq.) was added via syringe and the solution was stirred for 2 h at room temperature. The precipitate was filtered off, washed with diethyl ether (20 mL) to give the product as a white solid (991 mg, 3.2 mmol, 80%): mp 267–268 °C; $[\alpha]_D^{20}$ = −31.3 (*c* = 1.07, H_2O); UV (H_2O) λ_{max} nm (log ε): 349 (4.38), UV (MeCN) λ_{max} nm (log ε): 342 (4.41), UV (MeOH) λ_{max} nm (log ε): 344 (4.39); ^{1}H NMR (500 MHz, DMSO-d_6, 25 °C) δ 1.30 (d, 3H, ^3J = 6.7 Hz, CHC*H*$_3$), 3.60-3.64 (m, 1H, C*H*NH$_3$), 3.80 (s, 3H, OC*H*$_3$), 4.17 (dd, 1H, ^2J = 10.6 Hz, ^3J = 7.1 Hz, OC*H*H), 4.29 (dd, 1H, ^2J = 10.4 Hz, ^3J = 3.8 Hz, OCH*H*), 7.04 (d, 1H, ^4J = 2.6 Hz, 8-H), 7.07 (s, 1H, 6-H), 7.87 (d, 1H, ^3J = 8.8 Hz, 5-H), 8.36 (br s, 3H, NH_3), 8.75 (s, 1H, 4-H); ^{13}C NMR (125 MHz, DMSO-d_6, 25 °C) δ 15.1, 46.0, 52.4, 69.6, 101.3, 112.1, 113.7, 113.8, 131.9, 149.5, 156.3, 156.9, 163.4, 163.5. MS ESI+: *m/z* 278 ($[C_{14}H_{16}NO_5]^+$); HPLC purity: 99.6% (λ = 220–400 nm).

Synthesis of the Conjugate of Reagent 4 and (S)*-(−)-Ibuprofen (5b)*

(*S*)-(−)-Ibuprofen (62 mg, 0.3 mmol) and **4** (94 mg, 0.3 mmol) were dissolved in DMF (5 mL). DIPEA (125 µl, 0.72 mmol) was added, followed by *O*-(7-azabenzotriazol-1-yl)-*N,N,N,N*-tetramethyluronium hexafluorophosphate (HATU) (137 mg, 0.36 mmol). After 2 h, the mixture was diluted with EtOAc (50 mL) and washed with citric acid solution (2 × 50 mL), saturated $NaHCO_3$ (2 × 50 mL), and brine (50 mL). The product was purified by column chromatography to remove polar byproducts using petroleum ether/EtOAc (1/1) as eluent, which yielded the conjugate **5b** as a white solid (57 mg, 0.12 mmol, 40%): mp 172–174 °C; $[\alpha]_D^{20}$ = −36.8 (*c* = 1.03, MeOH); UV (H_2O) λ_{max} nm (log ε): 355 (4.44), UV (MeCN) λ_{max} nm (log ε): 347 (4.35), UV (MeOH) λ_{max} nm (log ε): 350 (4.40); ^{1}H NMR (500 MHz, DMSO-d_6, 25 °C) δ 0.78 (d, 6H, ^3J = 6.6 Hz, CH(C*H*$_3$)$_2$), 1.16 (d, 3H, ^3J = 6.7 Hz, C*H*$_3$CHAr), 1.28 (d, 3H, ^3J = 6.9 Hz, C*H*$_3$CHNH), 1.71 (sept, 1H, ^3J = 6.7 Hz, C*H*(CH$_3$)$_2$), 2.33 (d, 2H, ^3J = 7.3 Hz, CHC*H*$_2$Ar), 3.56 (q, 1H, ^3J = 6.9 Hz, ArC*H*CH$_3$), 3.80 (s, 3H, OCH$_3$), 3.95-3.96 (m, 2H, CHC*H*$_2$O), 4.14 (app sept, 1H, ^3J = 6.6 Hz, NHC*H*), 6.86 (dd, 1H, ^3J = 8.7 Hz, ^4J = 2.5 Hz, 6-H), 6.91 (d, 1H, ^4J = 2.5 Hz, 8-H), 6.96 (d, 2H, ^3J = 8.9 Hz, 3′-H, 5′-H), 7.15 (d, 2H, ^3J = 8.2 Hz, 2′-H, 6′-H), 7.77 (d, 1H, ^3J = 8.8 Hz, 5-H), 7.97 (d, 1H, ^3J = 7.9 Hz, NH), 8.72 (s, 1H, 4-H); ^{13}C NMR (125 MHz, DMSO-d_6, 25 °C) δ 17.0, 18.6, 22.2, 29.7, 43.7, 44.4, 44.7, 52.3, 71.2, 101.0, 111.6, 113.2, 113.7, 127.0, 128.7, 131.8, 139.1, 139.6, 149.5, 156.3, 157.0, 163.2, 164.2, 173.3. MS ESI+: *m/z* 466 ($[C_{27}H_{32}NO_6]^+$); HPLC purity: 96.9% (λ = 220–400 nm).

Synthesis of (R)*-(+)-Methyl 7-(1-(benzyloxy)-1-oxopropan-2-yloxy)-2-oxo-2*H*-chromene-3-carboxylate (6)*

Methyl 7-hydroxy-2-oxo-2*H*-chromene-3-carboxylate (**2**, 440 mg, 2.0 mmol), triphenylphosphine (1.04 g, 4.0 mmol) and benzyl (*S*)-(−)-lactate (540 mg, 3.0 mmol) were dissolved in THF (30 mL) and cooled to 0 °C. DIAD (0.61 g, 0.59 mL, 3.0 mmol) was added dropwise and the solution was stirred for 2 h at room temperature. After evaporation in vacuo the residue was diluted with EtOAc (50 mL), washed with 1 N NaOH (3 × 50 mL), brine (50 mL), and dried over Na_2SO_4. After evaporation the residue was purified by column chromatography using petroleum ether/EtOAc (1/1) to yield a colorless oil (540 mg, 1.4 mmol, 71%): $[\alpha]_D^{20}$ = +123 (*c* = 1.00, MeOH); ^{1}H NMR (500 MHz, DMSO-d_6, 25 °C) δ 1.57 (d, 3H, ^3J = 6.6 Hz, CHC*H*$_3$), 3.81 (s, 3H, OCH$_3$), 5.16 (d, 1H, ^2J = 12.3 Hz, CH*H*), 5.20 (d, 1H, ^2J = 12.3 Hz, C*H*H), 5.33 (q, 1H, ^3J = 7.0 Hz, C*H*), 6.94 (d, 1H, ^4J = 2.5 Hz, 8-H), 6.99 (dd, 1H, (d, 1H, ^3J = 8.8 Hz, ^4J = 2.5 Hz, 6-H), 7.28-7.35 (m, 5H, arom. H), 7.82 (d, 1H, ^3J = 8.5 Hz, 5-H), 8.73 (s, 1H, 4-H); ^{13}C NMR (125 MHz, DMSO-d_6, 25 °C) δ 18.0, 52.4, 66.6, 72.4, 101.6, 112.1, 113.7, 113.8, 128.0, 128.3, 128.5, 131.9, 135.7, 149.4, 156.2, 156.8, 162.7, 163.4, 170.6. MS ESI+: *m/z* 383 ($[C_{21}H_{19}O_7]^+$); HPLC purity: 96.5% (λ = 220–400 nm).

Hydrogenolysis of Compound 6

(*R*)-(+)-Methyl 7-(1-(benzyloxy)-1-oxopropan-2-yloxy)-2-oxo-2*H*-chromene-3-carboxylate (501 mg, 1.3 mmol) was hydrogenated with Pd/C (50 mg) as catalyst. After 2 h at room temperature, the mixture was filtered through a pad of celite and evaporated in vacuo. The residue was purified by column chromatography using EtOAc + 1% AcOH as eluent. The product, (*R*)-2-(3-(methoxycarbonyl)-2-oxochroman-7yloxy)propanoic acid (**7**), was obtained as a white solid (310 mg, 1.05 mmol, 81%): mp 117-118 °C; ^{1}H NMR (500 MHz, DMSO-d_6, 25 °C) δ 1.47 (d, 3H, ^3J = 6.7 Hz, CHC*H*$_3$), 3.17-3.19 (m, 2H, CH$_2$), 3.65 and 3.66 (s, 3H, OCH$_3$), 4.09-4.13 (m, 1H, CH$_2$C*H*), 4.83 and 4.84 (q, 1H, ^3J = 6.9 Hz, C*H*), 6.60-6.61 (m, 1H, 8-H), 6.65-6.68 (m, 1H, 6-H), 7.20 (d, 1H, ^3J = 8.2 Hz, 5-H), 13.00 (br s, 1H, OH); ^{13}C NMR (125 MHz, DMSO-d_6, 25 °C) δ 18.26 and 18.28, 25.8, 45.73 and 45.75, 52.8, 72.0, 103.0 and 103.2, 111.2 and 111.5, 113.67 and 113.72, 129.2, 151.67 and 151.69, 157.6, 164.77 and 164.79, 168.4, 172.9. MS ESI+: *m/z* 312 ($[C_{14}H_{18}NO_7]^+$), 295 ($[C_{14}H_{15}O_7]^+$); HPLC purity: 96.2% (λ = 220–400 nm).

*Synthesis of 3-(4-Methoxyphenyl)-2-oxo-2*H*-chromen-7-yl Acetate (8)*

2,4-Dihydroxybenzaldehyde (**1**, 2.76 g, 20.0 mmol) and *p*-methoxyphenylacetic acid (3.32 g, 20.0 mmol) were refluxed in a mixture of pyridine (30 mL) and acetic anhydride (30 mL) for 9 h. After cooling to room temperature, the precipitate was filtered off and washed with MeOH (30 mL) to give the product as a white solid (1.92 g, 6.2 mmol, 31%): mp 168–170 °C (lit.[27] mp 181–182 °C); ^{1}H NMR (500 MHz, DMSO-d_6, 25 °C) δ 2.31 (s, 3H, H_3CCO), 3.80 (s, 3H, OCH_3), 7.02 (d, 2H, ^3J = 8.9 Hz, 3′-H, 5′-H), 7.16 (dd, 1H, ^3J = 8.5 Hz, ^4J = 2.2 Hz, 6-H), 7.28 (d, 1H, ^4J = 2.2 Hz, 8-H), 7.69 (d, 2H, ^3J = 8.9 Hz, 2′-H, 6′-H), 7.79 (d, 1H, ^3J = 8.5 Hz, 5-H), 8.19 (s, 1H, 4-H); ^{13}C NMR (125 MHz, DMSO-d_6, 25 °C) δ 21.0, 55.4, 109.7, 113.8, 117.6, 118.8, 125.9, 126.9, 129.3, 129.9, 138.8, 152.5, 153.3, 159.8, 168.9. MS ESI+: *m/z* 311 ($[C_{18}H_{15}O_5]^+$); HPLC purity: 93.8% (λ = 220–400 nm).

*Synthesis of 7-Hydroxy-3-(4-methoxyphenyl)-2*H*-chromen-2-one (9)*

3-(4-Methoxyphenyl)-2-oxo-2*H*-chromen-7-yl acetate (**8**, 1.55 g, 5.0 mmol) and K_2CO_3 (1.38 g, 10.0 mmol) were refluxed for 1 h in MeOH (30 mL). After evaporation in vacuo the residue was dissolved in H_2O (50 mL). The pH was adjusted to 2 with 2 N HCl. The precipitate was filtered off and recrystallized from MeOH (105 mL) to give the product as yellow needles (973 mg, 3.6 mmol, 73%): mp 228 °C (lit.[27] mp 215–216); ^{1}H NMR (500 MHz, DMSO-d_6, 25 °C) δ 3.78 (s, 3H, CH_3), 6.73 (d, 1H, ^4J = 2.3 Hz, 8-H), 6.80 (dd, 1H, ^3J = 8.5 Hz, ^4J = 2.6 Hz, 6-H), 6.98 (d, 2H, ^3J = 8.9 Hz, 3′-H, 5′-H), 7.56 (d, 1H, ^3J = 8.5 Hz, 5-H), 7.64 (d, 2H, ^3J = 8.8 Hz, 2′-H, 6′-H), 8.05 (s, 1H, 4-H), 10.51 (br s, 1H, OH); ^{13}C NMR (125 MHz, DMSO-d_6, 25 °C) δ 55.3, 101.8, 112.2, 113.5, 113.8, 122.0, 127.5, 129.6, 129.8, 139.9, 154.8, 159.3, 160.3, 161.1. MS ESI+: *m/z* 269 ($[C_{16}H_{13}O_4]^+$); HPLC purity: 96.3% (λ = 220–400 nm).

*Synthesis of (*R*)-(+)-Methyl 2-(3-(4-(4-Methoxyphenyl)-2-oxo-2*H*-chromen-7-yloxy)propanoate (10)*

7-Hydroxy-3-(4-methoxyphenyl)-2*H*-chromen-2-one (**9**, 537 mg, 2.0 mmol), triphenylphosphine (1.04 g, 4.0 mmol) and methyl (*S*)-(−)-lactate (312 mg, 3.0 mmol) were dissolved in THF (30 mL) and cooled to 0 °C. Di-*tert*-butyl azodicarboxylate (690 mg, 3.0 mmol) was added portionwise and the solution was stirred for 2 h at room temperature. After evaporation in vacuo the residue was diluted with EtOAc (50 mL), washed with 1 N NaOH (3 × 50 mL), brine (50 mL), and dried over Na_2SO_4. After evaporation the residue was purified by column chromatography petroleum ether/EtOAc (3/1) to yield a light green solid (545 mg, 1.54 mmol, 77%): mp 172–173 °C; $[\alpha]_D^{20}$ = +74.7 (*c* = 1.07, MeOH); UV (H_2O) λ_{max} nm (log ε): 341 (4.45), UV (MeCN) λ_{max} nm (log ε): 342 (4.54), UV (MeOH) λ_{max} nm (log ε): 345 (4.44); ^{1}H NMR (500 MHz, DMSO-d_6, 25 °C) δ 1.55 (d, 3H, ^3J = 7.0 Hz, CHCH_3), 3.70 (s, 3H, CO_2CH_3), 3.79 (s, 3H, OCH_3), 5.20 (q, 1H, ^3J = 7.0 Hz, CH), 6.94-6.96 (m, 2H, arom. H), 7.00 (d, 2H, ^3J = 8.8 Hz, 3′-H, 5′-H), 7.65-7.68 (m, 3H, arom. H), 8.11 (s, 1H, 4-H); ^{13}C NMR (125 MHz, DMSO-d_6, 25 °C) δ 18.1, 52.4, 55.4, 72.1, 101.5, 113.1, 113.8, 113.9, 123.5, 127.2, 129.70, 129.71, 139.3, 154.4, 159.5, 160.0, 160.1, 171.5. MS ESI+: *m/z* 355 ($[C_{20}H_{19}O_6]^+$); HPLC purity: 99.4% (λ = 220–400 nm).

*Synthesis of (*R*)-(+)-2-(3-(4-methoxyphenyl)-2-oxo-2*H*-chromen-7-yloxy)propanoic Acid (11)*

(*R*)-Methyl 2-(3-(4-(4-methoxyphenyl)-2-oxo-2*H*-chromen-7-yloxy)propanoate (**10**, 469 mg, 1.3 mmol) was dissolved in THF (10 mL) and water (20 mL). LiOH × H_2O (334 mg, 7.8 mmol, 6.0 eq.) was added. The mixture was stirred overnight at room temperature. After filtration, the filtrate was diluted with water (40 mL) and acidified with 2 N HCl to pH ~2. The solution was extracted with CH_2Cl_2 (3 × 60 mL), the combined organic layers were dried over Na_2SO_4. After evaporation the residue was recrystallized from EtOAc and petroleum ether to yield a light gray solid (321 mg, 0.94 mmol, 73%): mp 145–147 °C; $[\alpha]_D^{20}$ = +68.3 (*c* = 1.00, MeOH) UV (H_2O) λ_{max} nm (log ε): 345 (4.40), UV (MeCN) λ_{max} nm (log ε): 342 (4.62), UV (MeOH) λ_{max} nm (log ε): 349 (4.46); ^{1}H NMR (500 MHz, DMSO-d_6, 25 °C) δ 1.54 (d, 3H, ^3J = 6.7 Hz, CHCH_3), 3.79 (s, 3H, OCH_3), 5.04 (q, 1H, ^3J = 6.6 Hz, CH), 6.90 (d, 1H, ^4J = 2.5 Hz, 8-H), 6.93 (dd, 1H, ^3J = 8.7 Hz, ^4J = 2.5 Hz, 6-H), 6.98 (d, 2H, ^3J = 8.9 Hz, 3′-H, 5′-H), 7.64-7.67 (m, 3H, 2′-H, 6′-H, 5-H), 8.11 (s, 1H, 4-H). The CO_2H proton could not be detected. ^{13}C NMR (125 MHz, DMSO-d_6, 25 °C) δ 18.2, 55.4, 72.1, 101.3, 113.1, 113.7, 113.8, 123.3, 127.2, 129.6, 129.7, 139.4, 154.4, 159.5, 160.2, 160.1, 172.6. MS ESI+: *m/z* 341 ($[C_{19}H_{17}O_6]^+$); HPLC purity: 99.5% (λ = 220–400 nm).

*Synthesis of the Conjugate of Reagent 11 and (*S*)-(−)-α-Methylbenzylamine (12e)*

(*S*)-(−)-α-Methylbenzylamine (37 mg, 0.3 mmol) and **11** (106 mg, 0.3 mmol) were dissolved in DMF (5 mL). DIPEA (125 μL, 0.72 mmol) was added, followed by HATU (137 mg, 0.36 mmol). After 2 h, the mixture was diluted with EtOAc (50 mL) and washed with citric acid solution (2 × 50 mL), saturated $NaHCO_3$ (2 × 50 mL), and brine (50 mL). The product was purified by column chromatography to remove polar byproducts using petroleum ether/EtOAc (1/1) as eluent, which yielded the conjugate **12e** as a white solid (99 mg, 0.22 mmol, 74%): mp 152–154 °C; $[\alpha]_D^{20}$ = +71.8 (*c* = 1.04, MeOH); UV (H_2O) λ_{max} nm (log ε): 347 (4.42), UV (MeCN) λ_{max} nm (log ε): 344 (4.49), UV (MeOH) λ_{max} nm (log ε): 346 (4.49); ^{1}H NMR (500 MHz, DMSO-d_6, 25 °C) δ 1.35 (d, 3H, ^3J = 7.0 Hz, CH_3CHAr), 1.49 (d, 3H, ^3J = 6.6 Hz, CH_3CHO), 3.80 (s, 3H, OCH_3), 4.89-4.94 (m, 2H, CH(CH_3)Ar and CH_3CHO), 6.91-6.94 (m, 2H, arom.H), 7.00 (d, 2H, ^3J = 8.9 Hz, 3′-H, 5′-H), 7.20-7.17 (m, 1H, arom.H), 7.28-7.34 (m, 4H, arom.H), 7.63 (d, 1H, ^3J = 8.5 Hz, 5-H), 7.67 (d, 2H, ^3J = 9.2 Hz, 2′-H, 6′-H), 8.11 (s, 1H, 4-H), 8.66 (d, 1H, ^3J = 8.2 Hz, NH); ^{13}C NMR (125 MHz, DMSO-d_6, 25 °C) δ 18.6, 22.3, 48.0, 55.4, 73.9, 101.4, 113.56, 113.63, 113.8, 123.3, 126.0, 126.8, 127.2, 128.3, 129.5, 129.7, 139.4, 144.5, 154.4, 159.5, 160.09, 160.14, 169.6. MS ESI+: *m/z* 444 ($[C_{27}H_{26}NO_5]^+$); HPLC purity: 98.8% (λ = 220–400 nm).

Derivatization Procedure

One equivalent (0.1–0.3 mmol) of the sample (chiral carboxylic acid or chiral amine) and one equivalent of the reagent (**4** or **11**) were dissolved in an appropriate solvent (e.g., DMF or MeCN) and 1.2 equivalents *N*,*N*-diisopropylethylamine (DIPEA) were added, followed by 1.2 equivalents HATU. The reaction mixture was stirred for 2 h at room temperature. The solution was diluted with EtOAc (50 mL) and washed with 10% citric acid solution (2 × 50 mL), saturated $NaHCO_3$ (2 × 50 mL), and brine (50 mL). Products were purified by column chromatography to remove polar byproducts using petroleum ether/EtOAc as eluent. The structures were confirmed by liquid chromatography / mass spectroscopy (LC/MS) and nuclear magnetic resonance (NMR) analytics (see Supporting Material, Table S1).

UV and Fluorescence Spectra

UV spectra were recorded on a Cary 50 Bio device from Varian, fluorescence spectra on a Monaco Safas spectrofluorometer flx. The compounds were solved in DMSO in a concentration of 10 mM, 1.0 mM, and 100 μM. All UV and fluorescence spectra were recorded in water, MeOH, and acetonitrile containing 1% DMSO. The final concentration of the compound was 10 μM for the UV determination and 1.0 μM for the fluorescence spectra. Determination of the extinction coefficients was done according to the Beer Lambert law ($\varepsilon(\lambda) = A / (c \times b)$), where *b* is the cuvette length (1.0 cm), *c* the used concentration (10 μM), and *A* the measured absorption value.

Chromatographic Conditions

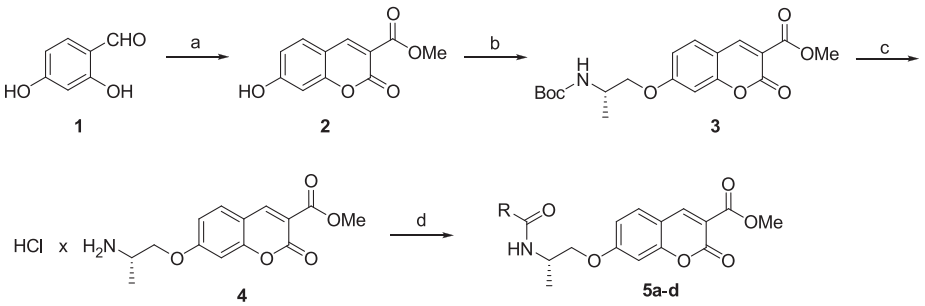
HPLC experiments were performed on a Jasco HPLC 2000 instrument. Chromatograms were obtained using ChromPass (v. 1.8.6.1, Jasco) chromatography software. Chromatographic analyses were performed with a LiChroCART, LiChrosorb column (250–4, RP-18, 7 μm particle size) from Merck. Stock solutions (2.5 mM) of the samples were prepared in acetonitrile and diluted with acetonitrile to 25 μM, 1.0 μM, or 250 nM, respectively, with acetonitrile. A volume of 20 μL was injected into the HPLC device. All mobile phases were degassed with a Jasco DG-2080-53 degasser. A column temperature of 30 °C was applied with a JASCO CO-2060 column oven. UV detection wavelength was fixed at 350 nm using a Jasco UV-2075. For derivatives **5**, the excitation and emission wavelengths were adjusted to 360 nm and 410 nm, respectively, for

derivatives **10** to 350 nm and 450 nm, respectively. Analytical parameters were calculated according to the following equations. Capacity factor k was calculated $k = t_R'/t_M$, where t_R' is the reduced retention time $t_R' = t_R - t_M$ and t_M is the experimental void time. Separation factor α was calculated from the observed $\alpha = {}^2k/{}^1k$, where ${}^2k$ and ${}^1k$ were the calculated capacity factors of the two diastereomers. Resolution R_s was calculated from $R_S = 1.18\ (t_{R2} - t_{R1})/({}^2w_h + {}^1w_h)$, where w_h is the peak width in 50% height. Values t_M were obtained after injection of a thiourea solution (0.1 mg/mL) and UV detection at 240 nm and the respective flow rate (1.0 mL/min or 1.3 mL/min). For the flow rates, the t_M values were 2.59 min and 2.03 min, respectively.

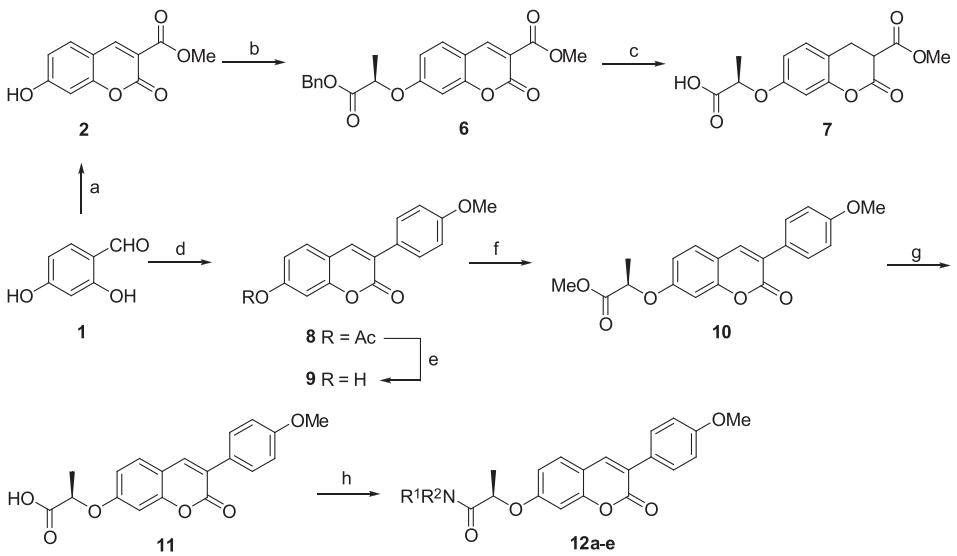
RESULTS AND DISCUSSION

A chiral derivatization reagent has to fulfill the following requirements: (i) a chiral center in close proximity to its reactive functional group, (ii) sufficient reactivity towards the functional moiety of the analyte, (iii) stability of the diastereomeric products, and (iv) the opportunity to exert a sensitive detection technique such as fluorescence.[5,10] We have designed two reagents that meet these requirements, i.e., coumarins **4** (Scheme 1) and **11** (Scheme 2). In both cases, the chiral center is brought as near as possible to the reactive group. To form a stable amide bond, only a coupling additive is needed for the reaction of a carboxylic acid-type analyte with the one, or an amine-type analyte with the other reagent. The resulting conjugates partake in the coumarins' fluorescence, thus allowing for sensitive detection.

The preparation of the reagent for chiral carboxylic acids is shown in Scheme 1. Starting from 2,4-dihydroxybenzaldehyde, Knoevenagel condensation with dimethyl malonate yielded the 7-hydroxy-coumarin derivative **2**. This intermediate represents a fluorophore, and the 7-alkoxy relatives show fluorescence without pH dependency. Mitsunobu reaction of **2** with Boc-(*S*)-(−)-alaninol produced the protected precursor **3**. The Boc group was removed with HCl to yield the derivatization reagent **4** in an overall yield of 47%. The fluorescence properties of coumarin **4** were evaluated in water, acetonitrile, and methanol. As exemplified in Figure 1, it has an absorption maximum at 342 nm and an emission maximum at 402 nm, when the excitation wavelength was 350 nm. Such a notable Stokes shift of 60 nm was similarly observed for **4** in the other solvents (data not shown). These spectroscopic properties are comparable to those of the known MC3C products.[11] An amine functionality for the derivatization has been also used in luminarin 4, a achiral coumarin labeling reagent for carboxylic acids.[14] Compound **4** can be considered as a candidate for further investigation on enantioselective inhibition of cholinesterases.[28,29]



Scheme 1. Synthesis of reagent **4** and reaction with chiral carboxylic acids. Reagents and conditions: (a) dimethyl malonate, piperidine, MeOH; (b) Boc-(S)-alaninol, PPh3, DIAD, THF; (c) HCl, EtOAc; (d) RCO2H, DIPEA, HATU, DMF or MeCN.



Scheme 2. Synthesis of reagent **11** and reaction with chiral amines. Reagents and conditions: (a) dimethyl malonate, piperidine, MeOH; (b) benzyl (S)-(–)-lactate, PPh3, DIAD, THF; (c) H2, Pd/C, MeOH; (d) 4-methoxyphenylacetic acid, Ac2O, pyridine; (e) K2CO3, MeOH; (f) methyl-(S)-(–)-lactate, PPh3, di-tert-butyl azodicarboxylate, THF; (g) LiOH, THF/water; (h) R1R2NH, DIPEA, HATU, DMF or MeCN.

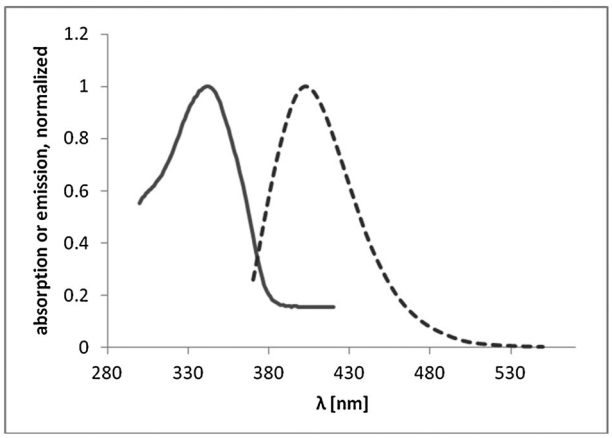


Fig. 1. Absorption (10 μM, solid line) and emission (1.0 μM, dashed line, excitation wavelength 350 nm) spectra of **4** in acetonitrile.

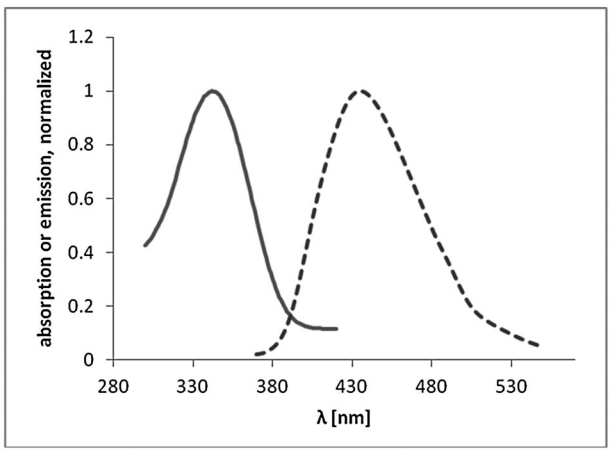
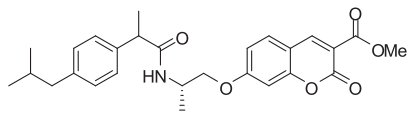
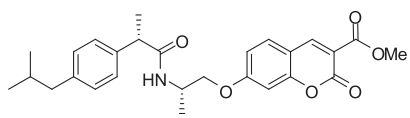
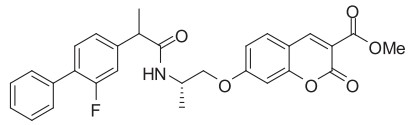
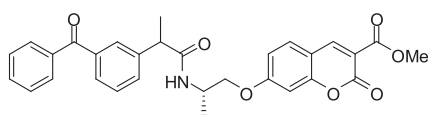


Fig. 2. Absorption (10 μM, solid line) and emission (1.0 μM, dashed line, excitation wavelength 350 nm) spectra of **11** in acetonitrile.

For the design of the other reagent, lactate was chosen as the chiral recognition site to be introduced into the coumarin core. A similar strategy was followed to construct chiral derivatizing agents for ^{1}H NMR inspection.[30] We used benzyl (*S*)-(−)-lactate in a first attempt to obtain a reagent for chiral amines (Scheme 2). After Mitsunobu coupling with **2**, the resulting benzyl ester **6** was removed hydrogenolytically. However, cleavage of the benzyl ester yielded a mixture of nonfluorescent diastereomers (**7**), whose C3-C4 single bonds resulted from a catalytic hydrogenation of the pyran double bond.[31] Therefore, methyl (*S*)-(−)-lactate was employed in an alternative approach which was not compatible with the methyl ester group of **2**. It was exchanged for a 4-methoxyphenyl group as follows. Perkin condensation of 2,4-dihydroxybenzaldehyde and 4-methoxyphenylacetic acid in a mixture of pyridine and acetic anhydride gave derivative **8**, which was deacetylated under basic conditions resulting in **9**. Methyl (*S*)-(−)-lactate was now coupled to **9** using the Mitsunobu protocol to retain the chiral information via Mitsunobu inversion.[32] In contrast to the two aforementioned Mitsunobu reactions, di-*tert*-butyl azodicarboxylate was used, because the previously used DIAD furnished diisopropyl hydrazine-1,2-dicarboxylate, which could not be separated from the product of this reaction. Alkaline hydrolysis of **10** gave the free carboxylic acid **11**, suitable for coupling to different chiral amines after activation. The overall yield for the new 3-phenylcoumarin **11** was 13%. The fluorescence properties of reagent **11** were found to be in accordance with related 3-phenylcoumarins;[25] it shows an absorption maximum at 342 nm and an emission maximum at 436 nm, after excitation at 350 nm. Again, a remarkable Stokes shift of 94 nm was obtained (Fig. 2).

Next, we investigated the suitability of **4** and **11** as chiral derivatizing agents by preparing and analyzing prototype drug conjugates. Both agents need an activation step prior

TABLE 1. Chromatographic parameters for different conjugates of 4 and 2-arylpropionic acids*

Conjugate	2-Arylpropionic acid	Structure	*k*	*α*	R_S
5a	(+/−)-ibuprofen		9.50 and 7.13 (6.92 and 6.63)	1.33 (1.04)	5.26 (2.61)
5b	(−)-ibuprofen		6.73 (6.71)	—	—
5c	(+/−)-flurbiprofen		6.74 and 4.58 (6.49 and 6.14)	1.47 (1.06)	5.69 (2.92)
5d	(+/−)-ketoprofen		3.64 and 2.71 (5.89 and 5.58)	1.34 (1.06)	3.47 (2.52)

*For chromatographic conditions, see Figs. 3, 4, and Supporting Material. Parameters from measurements under isocratic conditions and (in parentheses) gradient elution are given.

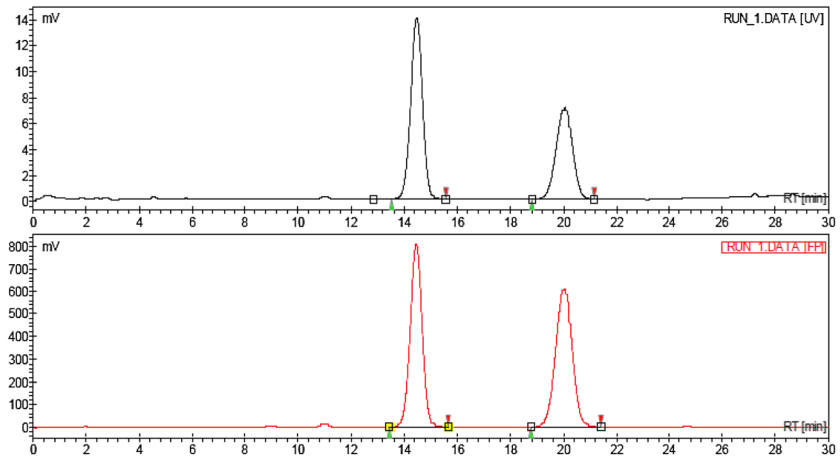


Fig. 3. Representative chromatogram of **5c** (25 μM), indicating the existence of two diastereomers. Top: UV detection (350 nm), 1st peak area 7.5 mV × min (60%), 2nd peak area 5.0 mV × min (40%). Bottom: fluorescence detection (λ_{ex} = 360 nm and λ_{em} = 410 nm), 1st peak area 431.9 mV × min (49.8%), 2nd peak area 436.1 mV × min (50.2%). Conditions: Mobile phase isocratic H_2O (A) : MeCN (B), 0 min/ 50% B, 25 min/ 50% B, 27 min/ 100% B, 28 min/ 50%, 30 min/ 50%; flow rate: 1.0 ml/min.

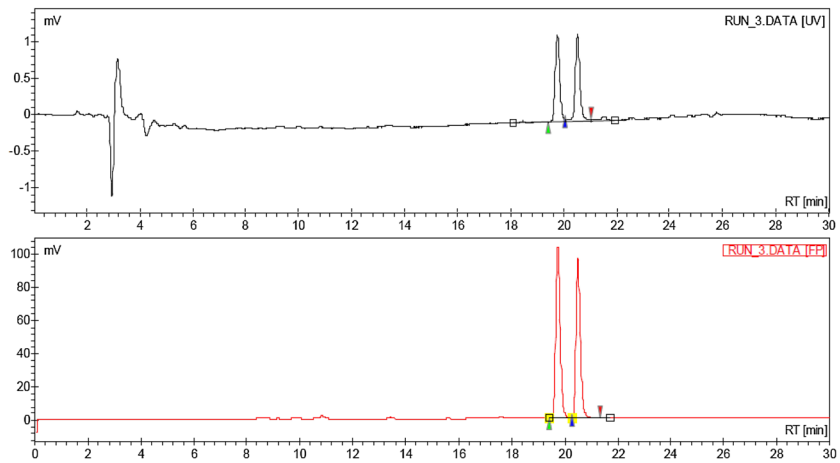


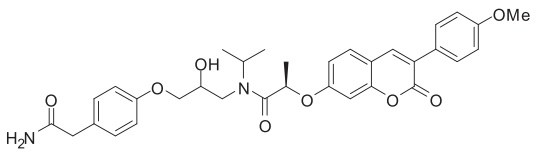
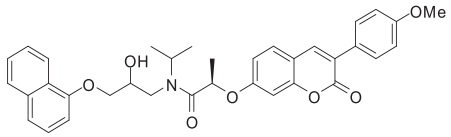
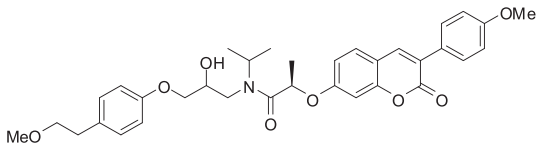
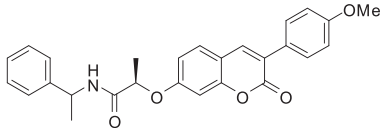
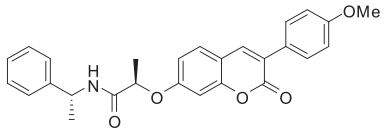
Fig. 4. Representative chromatogram of **5a** (1.0 μM), the conjugate of racemic ibuprofen and **4**, indicating the existence of two diastereomers. Top: UV detection (350 nm), 1st peak area 0.19 mV × min (48%), 2nd peak area 0.21 mV × min (52%). Bottom: fluorescence detection (λ_{ex} = 360 nm and λ_{em} = 410 nm), 1st peak area 19.0 mV × min (51.6%), 2nd peak area 17.8 mV × min (48.4%). Conditions: mobile phase gradient H_2O (A) : MeCN (B), gradient table: 0 min/ 10% B, 25 min/ 100% B, 27 min/ 100% B, 28 min/ 10%, 30 min/ 10%; flow rate: 1.0 ml/min.

to amide coupling. In the case of **4**, the carboxylic acid analyte has to be activated, in the case of **11**, the reagent's carboxyl group. When demonstrating the facile coupling of our reagents, HATU was used for the activation and the reactions proceeded smoothly. We applied HATU in order to minimize racemization. The reaction courses were monitored by thin-layer chromatography (TLC) and found to be completed within 2 h. As a matter of fact, when coupled to a chiral agent, racemic analytes would differ in the reactivity of their enantiomers. This was not further investigated in the course of the product analysis. However, it should be noted that a determined optical purity (of the conjugate) would probably differ from the real optical purity (of the analyte) due to a preferential loss of one diastereomer in the course of coupling and subsequent purification by column chromatography. The spectral properties of the conjugates were similar to those of the reagents, with respect to UV absorption and fluorescence emission (see Supporting Material, Figs. S1 and S2).

As depicted in Scheme 1, agent **4** was coupled to carboxylic acids to produce conjugates **5a-d**. Three 2-arylpropionic acids were selected that are frequently used as NSAIDs for the treatment of inflammation and pain, i.e., ibuprofen, as racemic and enantiopure drug, as well as flurbiprofen and ketoprofen (Table 1). The HPLC analysis was performed under reversed phase conditions using a common achiral C18 column with acetonitrile/water mixtures as eluent. The separation of the 2-arylpropionic acid conjugates was achieved either with isocratic elution (for an example, see Fig. 3) or by means of a simple linear gradient (for an example, see Fig. 4). The separation factor α was greater than 1, indicating the successful separation of the three diastereomeric conjugates **5a**, **5c**, and **5d**, and baseline separation was accomplished under isocratic conditions as the resolution values R_S clearly exceed 1.5 (Table 1). This also holds true for gradient elution, with somewhat weaker, but still sufficient separation (Table 1, values in parentheses). As expected, conjugate **5b** produced a single HPLC peak. A minor impurity was visible under isocratic conditions and fluorescence detection, when a 100 μM solution of **5b** was injected (see Supporting Material, S19). This new fluorescent enantiopure ibuprofen conjugate was fully characterized (see Materials and Methods).

From the pool of chiral amines, three β-blockers and α-methylbenzylamine were chosen as model analytes to be coupled with **11** (Scheme 2, Table 2). The separation of the

TABLE 2. Chromatographic parameters for different conjugates of 11 and chiral amines*

Conjugate	Chiral amine	Structure	*k*	*α*	R_S
12a	(+/−)-atenolol		3.97 and 4.76	1.20	1.62
12b	(+/−)-propanolol		9.22 and 10.0	1.08	1.41
12c	(+/−)-metoprolol		7.70 and 8.76	1.14	2.05
12d	(+/−)-α-methyl-benzylamine		19.68 and 21.02	1.07	1.18
12e	(*S*)-(−)-α-methyl-benzylamine		21.02	—	—

*For chromatographic conditions, see Fig. 5 and Supporting Material. Parameters from measurements under isocratic conditions are given.

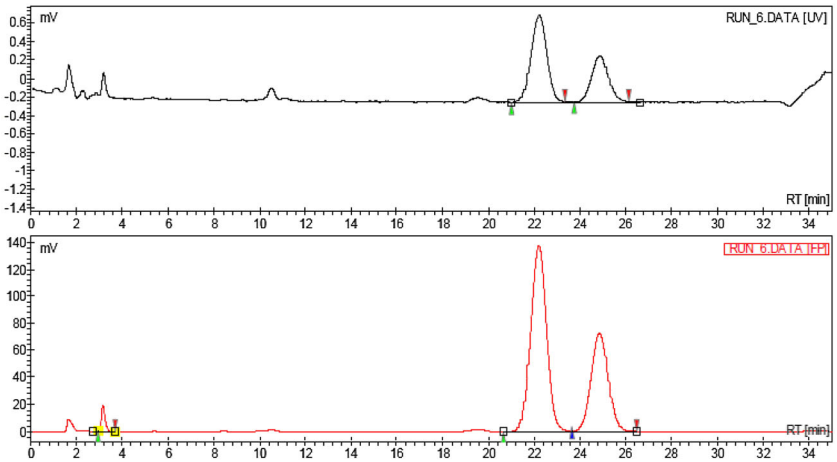


Fig. 5. Representative chromatogram of **12c** (2.5 μM), the conjugate of racemic metoprolol and **11**, indicating the existence of two diastereomers. Top: UV detection (350 nm), 1st peak area 0.8 mV × min (64%), 2nd peak area 0.4 mV × min (36%). Bottom: fluorescence detection (λ_{ex} = 350 nm and λ_{em} = 450 nm), 1st peak area 112.3 mV × min (64.1%), 2nd peak area 62.9 mV × min (35.9%). Conditions: Mobile phase isocratic H_2O (A) : MeCN (B): 0 min/ 50% B, 30 min/ 50% B, 32 min/ 100% B, 33 min/ 50%, 35 min/ 50%; flow rate: 1.0 ml/min.

β-blocker conjugates **12a-c** was carried out by isocratic elution, and for atenolol and metoprolol conjugates (**12a** and **12c**), baseline separation was accomplished with R_S values (1.62 and 2.05) greater than 1.5. The discrimination of the two diastereomeric metoprolol conjugates by HPLC is illustrated in Figure 5. For propanolol and α-methylbenzylamine, an effective enantiomeric separation was attained with somewhat lower resolution. Conjugate **12e**, synthesized from **11** and (*S*)-(−)-α-methylbenzylamine, completes the series of new compounds of this study (see Materials and Methods).

As expected, fluorescence detection resulted in a much better detectability of the conjugates than UV detection. We

obtained usable chromatograms with analyte quantities of 5 pmol when, for example, injecting 20 μL of a 250 nM solution of **5c** or **5d** and monitoring the fluorescence (see Supporting Material S21 and S22). To apply both agents (**4** and **11**) for the determination of optical purity, the reaction conditions and sample work-up should be optimized. As can be seen in Figures 3 and 5, the chromatograms showed different peak areas for both diastereomers, respectively. The resolution of conjugate **5c** reveals this deviation only in the UV channel, obviously a result of the poor UV detectability. Besides their suitability as CDAs for HPLC analysis, the coumarin reagents can be applied as CDAs for NMR spectroscopy. This is exemplarily shown by the duplication of NMR signals assigning conjugates **5a** and **12d** as diastereomers (see Supporting Material S5 and S13).

In conclusion, the synthesis of two chiral, coumarin-type derivatives is presented. In both reagents, the functional group and the chiral center were introduced in a side chain, connected via an ether bridge to coumarin C-7. The reagents **4** and **11** differ with respect to their reactive group to allow for the amide coupling of carboxylic acids or amines, respectively. They bear different moieties at 3-position resulting in slightly different spectroscopic properties. The application of **4** and **11** as CDAs was successfully demonstrated by producing representative drug conjugates and analyzing their chromatographic separation under isocratic and gradient conditions.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher's web site.

LITERATURE CITED

1. Caldwell J. Importance of stereospecific bioanalytical monitoring in drug development. J Chromatogr A 1996;719:3–13.
2. Maier NM, Franco P, Lindner W. Separation of enantiomers: Needs, challenges, perspectives. J Chromatogr A 2001;906:3–33.
3. Fukushima T, Santa T, Homma H, Al-kindy SM, Imai K. Enantiomeric separation and detection of 2-arylpropionic acids derivatized with [(N,N-Dimethylamino)sulfonyl]benzofurazan reagents on a modified cellulose stationary phase by high-performance liquid chromatography. Anal Chem 1997;69:1793–1799.
4. Guillarme D, Bonvin G, Badoud F, Schappler J, Rudaz S, Veuthey JL. Fast chiral separation of drugs using columns packed with sub-2 μm particles and ultra-high pressure. Chirality 2010;22:320–330.
5. Ilisz I, Berkecz R, Péter A. Application of chiral derivatizing agents in the high-performance liquid chromatographic separation of amino acid enantiomers: A review. J Pharm Biomed Anal 2008;47:1–15.
6. Martin E, Quinke K, Spahn H, Mutschler E. (−)-(S)-Flunoxaprofen and (−)-(S)-naproxen iscocyanate: Two new fluorescent chiral derivatizing agents for an enantiospecific determination of primary and secondary amines. Chirality 1989;1:223–234.
7. Toyo´oka T, Ishibashi M, Terao T. Fluorescent chiral derivatization reagents for carboxylic acid enantiomers in high-performance liquid chromatography. Analyst 1992;117:727–733.
8. Toyo´oka T, Liu YM. Development of optically active fluorescent edman-type reagents. Analyst 1995;120:385–390.
9. Azuma K, Suzuki S, Uchiyama S, Kajiro T, Santa T, Imai K. A study of the relationship between the chemical structures and the fluorescence quantum yields of coumarins, quinoxalinones and benzoxazinones for the development of sensitive fluorescent derivatization reagents. Photochem Photobiol Sci 2003;2:443–449.
10. Ilisz I, Aranyi A, Pataj Z, Péter A. Recent advances in the direct and indirect liquid chromatographic enantioseparation of amino acids and related compounds: A review. J Pharm Biomed Anal 2012;69:28–41.
11. Hamada C, Iwasaki M, Kuroda N, Ohkura Y. 3-Chloroformyl-7-methoxycoumarin as a fluorescent derivatisation reagent for alcoholic compounds in liquid chromatography and its use for the assay of 17-oxosteroids in urine. J Chromatogr 1985;341:426–431.
12. Grayeski ML, DeVasto JK. Coumarin derivatizing agents for carboxylic acid detection using peroxyoxalate chemiluminescence with liquid chromatography. Anal Chem 1987;59:1203–1206.
13. Mita H, Yasueda H, Hayakawa T, Shida T. Quantification of platelet-activating factor by high-performance liquid chromatography with fluorescent detection. Anal Biochem 1989;180:131–135.
14. Tod M, Prevot M, Chalom J, Farinotti R, Mahuzier G. Luminarin 4 as a labelling reagent for carboxylic acids in liquid chromatography with peroxyoxalate chemiluminscence detection. J Chromatogr 1991;542: 295–306.
15. Kurosawa T, Sato H, Sato M, Takechi H, Machida M, Tohma M. Analysis of stereoisomeric C27-bile acids by high performance liquid chromatography with fluorescence detection. J Pharm Biomed Anal 1997;15:1375–1382.
16. Min JZ, Toyo´oka T, Fukushima T, Kato M. Synthesis and evaluation of new fluorescent derivatization reagents for resolution of chiral amines by RP-HPLC. Anal Chim Acta 2004;515:243–253.
17. Gikas E, Parissi-Poulou M, Kazanis M, Vavagianis A. BrMOZPhC, a novel coumarin type reagent for the fluorescent derivatisation of carboxylic acids. Anal Chim Acta 2005;542:153–163.
18. Lavis LD, Raines RT. Bright ideas for chemical biology. ACS Chem Biol 2008;3:142–155.
19. Becker CF, Hunter CL, Seidel RP, Kent SB, Goody RS, Engelhard M. A sensitive fluorescence monitor for the detection of activated Ras: Total chemical synthesis of site-specifically labeled Ras binding domain of c-Raf1 immobilized on a surface. Chem Biol 2001;8:243–252.
20. Terai T, Nagano T. Fluorescent probes for bioimaging applications. Curr Opin Chem Biol 2008;12:515– 521.
21. Elsinghorst PW, Härting W, Goldhammer S, Grosche J, Gütschow M. A gorge-spanning, high-affinity cholinesterase inhibitor to explore β-amyloid plaques. Org Biomol Chem 2009;7:3940–3946.
22. Edgington LE, Verdoes M, Bogyo M. Functional imaging of proteases: recent advances in the design and application of substrate-based and activity-based probes. Curr Opin Chem Biol 2011;15:798–805.
23. Frizler M, Mertens MD, Gütschow M. Fluorescent nitrile-based inhibitors of cysteine cathepsins. Bioorg Med Chem Lett 2012;44: 1755–1758.
24. Wysocka M, Lesner A, Gruba N, Korkmaz B, Gauthier F, Kitamatsu M, Łgowska A, Rolka K. Three wavelength substrate system of neutrophil serine proteinases. Anal Chem 2012;84:7241–7248.
25. Chen CY, Gompf TE. The absorption and the emission spectra of some substituted 3-phenylcoumarins. Heterocycles 1977;7:933–45.
26. Valizadeh H, Gholipur H, Shockravi A. Microwave assisted synthesis of coumarins via potassium carbonate catalyzed knoevenagel condensation in 1-n-butyl-3-methylimidazolium bromide ionic liquid. J Hetereocyclic Chem 2007;44:867–870.
27. Garazd MM, Garazd YL, Ogorodniichuk AS, Khilya VP. Modified coumarins. 29. Synthesis of structural analogs of natural 6-arylfuro[3,2-g] chromen-7-ones. Chem Nat Compd 2009;45:158–163.
28. Chiou SY, Huang CF, Yeh SJ, Chen IR, Lin G. Synthesis of enantiomers of exo-2-norbornyl-N-n- butylcarbamate and endo-2-norbornyl-N-n-butylcarbamate for stereoselective inhibition of acetylcholinesterase. Chirality 2010;22:267–274.
29. Vanzolini KL, Vieira LC, Corrêa AG, Cardoso CL, Cass QB. Acetylcholinesterase immobilized capillary reactors-tandem mass spectrometry: an on-flow tool for ligand screening. J Med Chem 2013;56:2038–2044.
30. Nagasawa K, Seto N, Ito K. Coumarin-containing amino acids and oxy acids as chiral discriminating agents. Part III. Novel crystalline (R)-(+)- and (S)-(−)-O-coumarinyl lactic acids as chiral derivatizing agents for H-1 NMR inspection of optical purities of alcohols and amines. Heterocycles 1997;46:567– 580.
31. Tang L, Yu J, Leng Y, Feng Y, Yang Y, Ji R. Synthesis and insulin-sensitizing activity of a novel kind of benzopyran derivative. Bioorg Med Chem Lett 2003;13:3437–3440.
32. Rao AVR, Gaitonde AS, Prakash KRC, Rao SP. A concise synthesis of chiral 2-methyl chroman-4-ones: Stereo selective build-up of the chromanol moiety of anti-HIV agent, calanolide A. Tetrahedron Lett 1994;35:6347–6350.