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PAPER

A facile transformation of amino acids to functionalized coumarins†

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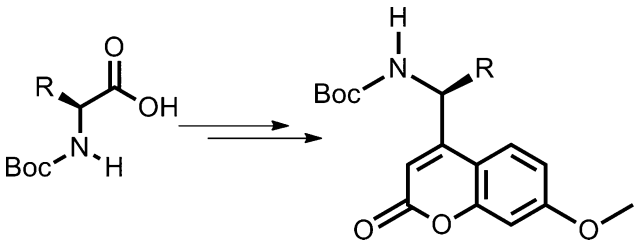
The synthesis of novel chiral coumarins functionalized with proteinogenic amino acid side chains *via* *N*-protected γ-amino-β-keto esters and their incorporation into the cell permeable HIV-1 TAT peptide through the modified solid phase peptide synthesis are described.

Introduction

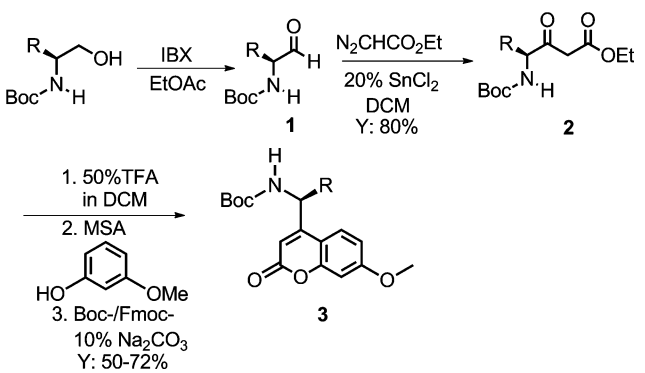
Coumarins are a widely occurring class of compounds isolated from a variety of plant sources.[1] Natural and synthetic derivatives of coumarins have been shown to possess a remarkable array of pharmacological and biomedical properties, including anticancer, anticoagulant, antimicrobial, anti-inflammatory and antioxidant activities.[2] The wide pharmacological properties of coumarins and their ability to interact with more than one target is a significant source of inspiration for synthetic chemists to design structural analogues with improved or entirely different pharmacological properties.[1,3] Functionalized coumarins also find applications as dyes in laser technology,[4] in the perfumery industry,[5] and more importantly as fluorescent tags.[6] Due to the broad spectrum of properties in both biology and material science, unsurprisingly, considerable effort has been made towards the synthesis of coumarins and functionalized coumarins using traditional Pechmann, Perkin, Reformatsky, Knoevenagel, Wittig, Claisen and other methods.[6,7] However, so far there has been no report regarding the synthesis of coumarins functionalized with proteinogenic amino acid side chains in the literature. These proteinogenic amino acid side chain functionalized coumarins may be more relevant from the pharmacological and biomedical perspective. In this regard, we sought to investigate whether α-amino acids can be directly transformed into chiral coumarins (Scheme 1). Herein, we are reporting a facile synthesis of coumarins functionalized with proteinogenic amino acid side chains starting from *N*-protected amino acids and their incorporation into peptides using solid phase synthesis. Further, the structures of Boc-alanylcoumarin and Boc-prolylcoumarin were studied as crystals. A remarkable organic framework is observed in the crystal structure of Boc-prolylcoumarin.



Scheme 1 The transformation of amino acid to 4-substituted coumarin.

Results and discussion

We chose the simplest and widely used Pechmann reaction for the synthesis of functionalized coumarins. It involves phenol condensation with a β-keto ester in the presence of a variety of Brønsted and Lewis acids.[8] Numerous methods exist for the synthesis of proteinogenic amino acid side chains containing γ-amino-β-keto esters.[9] However, we used a Lewis acid-catalyzed protocol for the transformation of α-amino acids to γ-amino-β-keto esters.[10] The ethyl esters of *N*-protected β-keto-γ-amino acids were synthesized *via* semipinacol-type rearrangement starting from the *N*-protected amino aldehydes and the ethyl diazoacetate catalyzed by tin(II) chloride (Scheme 2).[10] The protected amino aldehydes were synthesized from the oxidation of amino alcohols using IBX.[11] *N*-Protected amino alcohols were readily obtained from the mild $NaBH_4$ reduction of corresponding protected amino



Scheme 2 Synthesis of 4-substituted chiral coumarins.

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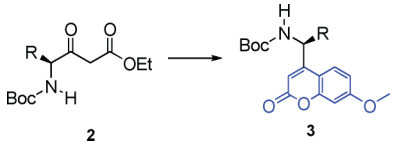
† Electronic supplementary information (ESI) available: Experimental details, NMR spectra, Mass spectra and X-ray crystallography (for compounds **3a** and **3f**). CCDC reference numbers 819624 and 819625. For ESI and crystallographic data in CIF or other electronic format see DOI: 10.1039/c1ob05815k

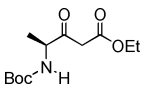
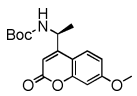
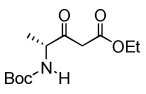
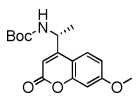
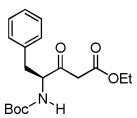
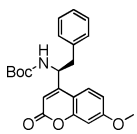
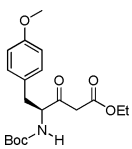
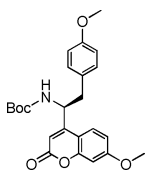
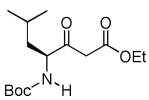
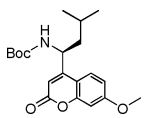
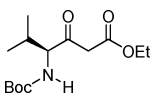
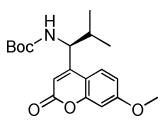
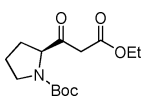
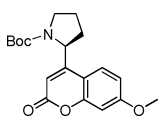
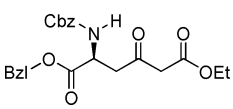
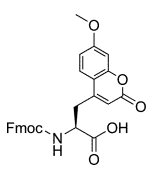
acid mixed anhydrides.[12] The ethyl esters of *N*-protected β-keto-γ-amino acids were isolated in pure form after simple aqueous work-up and column chromatography. In the initial reaction, to verify whether or not these β-keto-γ-amino acids can undergo Pechmann condensation to give functionalized coumarins, we subjected the ethyl ester of Boc-protected-β-keto-γ-alanine (**2a**) to the condensation reaction with 3-methoxyphenol in the presence of methanesulfonic acid (MSA).[13] The schematic representation of the synthesis is shown in Scheme 2. To our surprise, the yield was lower than expected when compared to the simple ethyl acetoacetate (data not shown). We speculate that Boc-group may be responsible for the unexpected impurities in the reaction. To circumvent the formation of impurities, the Boc-group was removed from **2a** prior to the reaction using TFA. The trifluoroacetate salt of free amine was isolated and subjected to the Pechmann condensation. The functionalized free amino coumarin derivative of **3a** was isolated as a red salt after the condensation with 3-methoxyphenol. The free amine of the coumarin was again protected with the Boc-group after neutralizing the methanesulfonate salt with Na_2CO_3. The pure **3a** was isolated in modest yield (72%) after column chromatography. The successful isolation of Boc-alanylcoumarin led us to subject other γ-amino-β-keto esters(**2b–2f**) to the Pechmann condensation. The list of functionalized coumarins (**3b–3f**) synthesized from the Boc-γ-amino-β-keto esters is given in Table 1. Except for **3g**, all compounds were synthesized using the same protocol. To incorporate coumarin as a side chain on an amino acid backbone, we adopted a different strategy. The coumarin amino acid, **3g**, was synthesized starting from the commercially available Cbz-Asp(OH)-OBzl (see the ESI†). The free carboxylic acid was converted to the aldehyde [Cbz-Asp(CHO)-OBzl] through the oxidation of the corresponding alcohol using the protocol described earlier. The aldehyde was transformed to the β-keto ester (**2g**) by reacting with ethyl diazoacetate in the presence of $SnCl_2$ catalyst. The Pechmann condensation of **2g** with 3-methoxyphenol in the presence of MSA led to the formation of the methanesulfonate salt of amino acid coumarin. The free amino group was again protected with Fmoc after neutralizing with Na_2CO_3. The protected amino acid coumarin (**3g**) was isolated in moderate yield (Table 1) after aqueous work-up and column chromatography. The detailed protocol is given in the ESI.† Further, the UV absorption spectra were recorded for all coumarins (**3a–3g**) and the absorption maxima were observed at around 324 nm. The fluorescence spectra of all coumarins were recorded after exciting at 324 nm and are shown in Fig. 1. Further, out of all of the chiral coumarins, we were able to obtain single crystals for the compounds **3a** and **3f** after slow evaporation of their EtOAc/hexane solution. The X-ray structure of **3f** is shown in Fig. 2A and the structure of **3a** is given in the ESI.†

Further, to investigate the racemization during the coumarin synthesis, we synthesized **3a1** and (±)DL-alanylcoumarins starting from Boc-D-Ala and Boc-(±)DL-Ala, respectively. The compounds **3a**, **3a1** and Boc-(±)DL-alanylcoumarins were subjected to chiral HPLC using a Daicel CHIRALPAK-AI column and the chromatograms are shown in Fig. 3. The compounds **3a** and **3a1** gave single peaks with the t_R 11.62 and 7.85, respectively. The Boc-(±)DL-alanylcoumarins gave two peaks with the t_R corresponding to the **3a** and **3a1**. These results indicate that no racemization occurred during the transformation of amino acids to coumarins.

Table 1 List of functionalized coumarins synthesized from ethyl esters of β-keto-γ-amino acids



2 **3**

No.	β-Keto ester (**2**)	Coumarin (**3**)	Yield (%)
a			72
a1			72
b			70
c			68
d			63
e			50
f			71
g			62

Examination of the crystal structure of **3a** reveals that the coumarins are connected by intermolecular H-bonding between the amide NH and CO groups as well as C9H and CO (see the ESI†). Interestingly, a remarkable organic framework is observed in the crystal structure of **3f** (Fig. 2B). Inspection of the crystal structure reveals that the crystal packing is stabilized by CH–O, CH–π and lone pair–π interactions.[14] The hydrogen bond parameters of both the structures (**3a** and **3f**) are given in the ESI.†

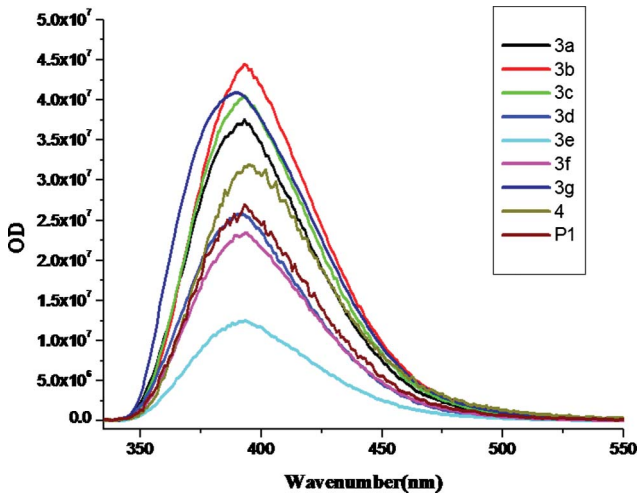


Fig. 1 The fluorescence emission spectra of functionalized coumarins (**3a–3g**) and peptides (**4** and **P1**). All compounds were excited at 324 nm.

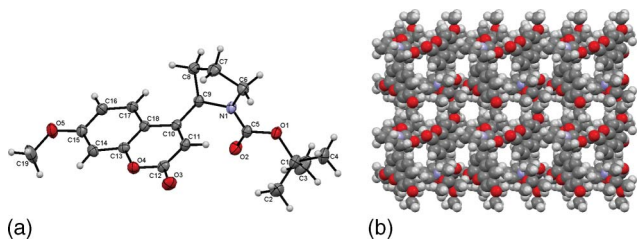


Fig. 2 Crystal structure of Boc-prolylcoumarin. (a) The ORTEP diagram showing the crystal structure of **3f**. The H-atoms are not labelled for clarity. (b) The space filled model depicting the organic framework observed in the crystal packing of **3f**.

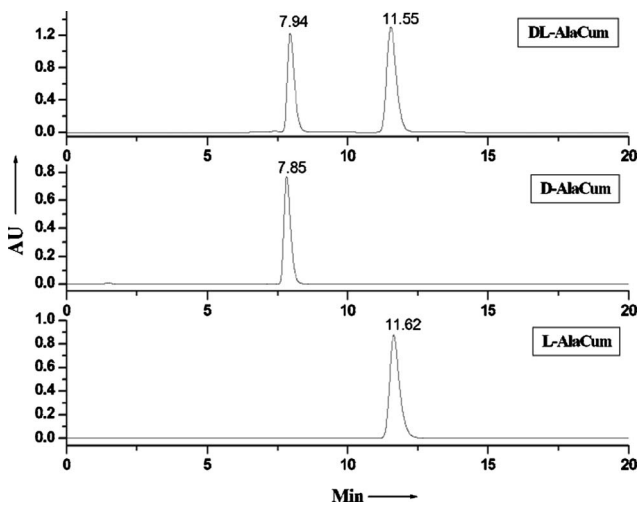
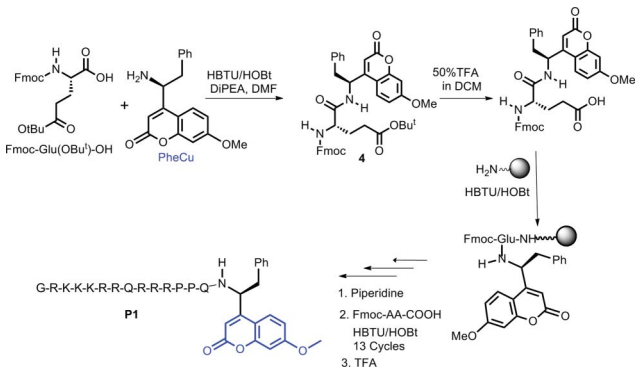


Fig. 3 Chiral HPLC of L-, D- and (±)DL-Boc-AlaCum. The HPLC was performed on a Daicel CHIRALPAK-AI column using 90% isopropanol in n-hexane as the solvent system in isocratic mode with a flow rate of 1 mL min^{-1}: (a) HPLC profile of (±)DL-Boc-AlaCum, (b) HPLC profile of Boc-D-AlaCum and (c) HPLC profile for the Boc-L-AlaCum.

Further, to investigate whether these functionalized chiral fluorescent coumarins could be used in biological studies, we synthesized the cell permeable HIV-1 TAT peptide[15] GRKKKR-RQRRRPPQ (**P1**) by incorporating coumarin **3b** at the C-terminal of the peptide using solid phase synthesis. The advantage is that the coumarins can be directly incorporated into the peptides along with the amino acid side chain without using additional linkers. However, except for **3g**, all of the coumarins lack a carboxylic acid group at the C-terminal for incorporation into the peptides. To insert these coumarins into peptides, we developed a new protocol as shown in Scheme 3. The free carboxylic acid of dipeptide **4** [Fmoc-Glu(OH)-PheCu] was coupled to the Rink amide resin and the peptide synthesis was continued by stepwise addition of Fmoc-amino acids using standard HBTU/HOBt couplings. Finally, the peptide was released from the resin and purified by reverse phase HPLC. The pure peptide was incubated with DLD-1 colon cancer cells and the internalization was visualized by fluorescence laser scanning microscopy. The fluorescence image of the cells is shown in Fig. 4. Overall, we have demonstrated the effective incorporation of amino acid side chain functionalized coumarins into peptides as fluorescent tags.



Scheme 3 Incorporation of chiral coumarin into HIV-1 TAT peptide (**P1**).

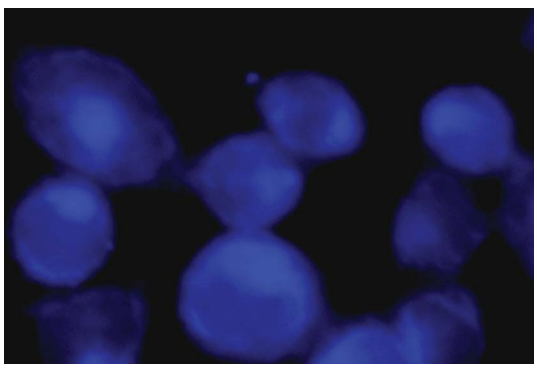


Fig. 4 Fluorescence image of **P1** in DLD-1 colon cancer cells.

Conclusions

In conclusion, we have demonstrated the facile transformation of amino acids into novel coumarins functionalized with amino acid side chains and their incorporation into a peptide using solid phase synthesis. The Boc-prolylcoumarin showed a remarkable organic framework in the crystal structure. The biological properties of these chiral coumarins and their organic framework will be further investigated.

Experimental section

All amino acids, ethyl diazoacetate, LAH, DiPEA, tin(II) chloride, TFA, DMP, Cs_2CO_3, Cbz-Cl were purchased from Aldrich. THF, DCM, DMF, were purchased from Merck. Isobutyl chloroformate, $NaBH_4$, HBTU, HOBt, methanesulfonic acid, di-*tert*-butyl dicarbonate, Fmoc-OSu, 3-methoxyphenol, benzyl bromide were obtained from Spectrochem and used without further purification. THF and DiPEA were dried over sodium and distilled immediately prior to use. Column chromatography was performed on Merck silica gel (120–200 mesh). The ^{1}H spectra were recorded on Jeol 400 MHz (or 100 MHz for ^{13}C) and 500 MHz (or 125 MHz for ^{13}C) Bruker spectrometers using residual solvent signals as an internal reference ($CDCl_3$ δ_H, 7.24 ppm, δ_C 77.0 ppm or [D6] DMSO δ_H = 2.50 ppm, δ_C = 39.5 ppm). The chemical shifts (δ) are reported in ppm and coupling constants in (J) in Hz. Specific rotations were recorded using MeOH as a solvent (Rudolph Analytical Research). UV and fluorescence data were obtained on Thermo Scientific and Fluorolog (Horiba Jobin Yvin) spectrophotometers, respectively. Mass spectra were obtained from MALDI TOF/TOF (Applied Biosciences) and HRMS-ESI (Waters). X-Ray data were collected by using a Bruker APEX DUO.

General procedure for the synthesis of *N*-protected γ-amino-β-keto-esters

All ethyl esters of *N*-protected β-keto-γ-amino acids were synthesized using a previously reported procedure.[10a] In general, the *N*-protected amino aldehyde (10.0 mmol) was dissolved in 15 mL of DCM at room temperature (20–25 °C) and then anhydrous tin(II) chloride (0.796 g, 20 mol%) was added to the reaction followed by ethyl diazoacetate (1.19 g, 10.5 mmol). Immediate gas evolution was observed and it ceased within 30 min. The progress of the reaction was monitored by TLC. After completion (~30 min) of the reaction, it was quenched with 60 mL of 0.5 N HCl and immediately extracted with DCM (80 mL × 3). The combined organic layer was washed with brine (100 mL), dried over anhydrous sodium sulfate and concentrated under reduced pressure to give a greenish oily crude product which was further purified by silica gel column chromatography.

(*S*)-Ethyl-4-(*tert*-butoxycarbonylamino)-3-oxopentaanoate (2a). Colorless liquid (0.398 g, 76%); $[\alpha]_D^{25}$ −35.69 (c = 1, MeOH); ^{1}H NMR (500 MHz, $CDCl_3$): δ 12.13 (s, 1H enolic 3.5%), 5.17 (b, s, 1H, NH), 4.43–4.37 (m, 1H, CH), 4.24–4.20 (q, J = 7 Hz, 2H,-OCH_2), 3.62–3.54 (dd, J = 14.5 Hz, J = 10.5 Hz 2H, CH_2, AB coupling), 1.46 (s, 9H, $C(CH_3)_3$, Boc), 1.38–1.36 (d, J = 6.5 Hz, 3H, CH_3), 1.31–1.28 (t, J = 7 Hz, 3H, CH_3); ^{13}C NMR (125 MHz, $CDCl_3$) δ 202.50, 166.96, 155.19, 80.14, 61.57, 55.42, 45.91, 28.32, 17.11, 14.11; HR-MS m/z Calcd. for $C_{12}H_{21}NO_5$ $[M + Na]^+$ 282.1317, observed 282.1317.

(*S*)-Ethyl-4-(*tert*-butoxycarbonylamino)-3-oxo-5-phenylpentanoate (2b). White crystal (0.521 g, 78%); $[\alpha]_D^{25}$ −54.5 (c = 0.6, MeOH); Melting Point = 61.4 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 12.16 (s, 1H, enolic 17%), 7.24 –7.15 (m, 5H, C_6H_5), 5.03–5.01 (d, J = 7.3 Hz, 1H, NH), 4.57–4.52 (q, J = 6.4 Hz, 1H, CH), 4.18–4.12 (q, J = 7.2 Hz, 2H, -OCH_2), 3.51–3.40 (dd, J = 16 Hz, J = 11.4 Hz, 2H, CH_2, AB coupling), 3.15–2.95 (m, 2H, CH_2Ph), 1.38(s, 9H, $C(CH_3)_3$), 1.26–1.22 (t, J = 7.2 Hz, 3H, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$) δ 201.96, 166.86, 155.18, 136.04, 129.24, 128.67, 127.00, 80.21, 61.45, 60.43, 46.86, 36.89, 28.20, 14.02; HR-MS m/z Calcd. for $C_{18}H_{25}NO_5$ $[M + Na]^+$ 358.1630, observed 358.1633.

(*S*)-Ethyl-4-(*tert*-butoxycarbonylamino)-5-(4-methoxyphenyl)-3-oxopentanoate (2c). Semi solid (0.586 g, 80%); $[\alpha]_D^{25}$ = −56.32 (c = 1, MeOH); ^{1}H NMR (400 MHz, $CDCl_3$): δ 12.15 (s, 1H, enolic 10%), 7.07–7.05 (d, J = 8.68, 2H, aromatic), 6.82–7.80 (d, J = 8.65, 2H, aromatic), 5.01–4.99 (d, J = 7.6 Hz, 1H, N*H*), 4.53–4.48 (q, J = 7 Hz, 1H, C*H*), 4.17–4.12(q, J = 7.2 Hz, 2H, -OCH_2), 3.76 (s, 3H, -OCH_3), 3.49–3.38 (dd, J = 16 Hz, J = 11.4 Hz, 2H, CH_2, AB coupling), 3.07–2.88 (m, 2H, CH_2Ph), 1.38 (s, 9H, $C(CH_3)_3$), 1.25–1.22 (t, J = 7.2 Hz, 3H, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ 202.11, 166.85, 158.60, 155.18, 130.26, 127.82, 114.07, 80.17, 61.45, 60.55, 46.93, 36.12, 28.22, 14.04; MALDI TOF/TOF m/z Calcd. for $C_{19}H_{27}NO_6$ $[M + Na]^+$ 388.1736, observed 388.1783.

(*S*)-Ethyl-4-(*tert*-butoxycarbonylamino)-6-methyl-3-oxoheptanoate (2d). Light yellowish liquid (0.476 g, 79%); $[\alpha]_D^{25}$ −53.70 (c = 1, MeOH); ^{1}H NMR (500 MHz, $CDCl_3$) δ 12.10 (s, 1H, enolic 5.5%), 4.96– 4.94 (d, J = 9.5 Hz, 1H, NH), 4.40–4.36 (m, 1H, CH), 4.24–4.20 (q, J = 7 Hz, 2H, -OCH_2), 3.63–3.53 (dd, J = 16.0 Hz, J = 18.5 Hz, 2H, CH_2, AB coupling), 1.74–1.67 (m, 3H, CH_2, CH), 1.46 (s, 9H, $C(CH_3)_3$, Boc-), 1.32–1.29 (t, J = 7 Hz, 3H, CH_3), 0.979 (b, s, 6H, $C(CH_3)_2$); ^{13}C-NMR (125 MHz, $CDCl_3$):δ 203.02, 167.07, 155.55, 80.15, 61.51, 58.19, 46.35, 39.90, 28.31, 24.83, 23.28, 21.59, 14.12; HR-MS m/z Calcd. for $C_{15}H_{27}NO_5$ $[M + Na]^+$ 324.1786 observed 324.1784.

(*S*)-Ethyl-4-(*tert*-butoxycarbonylamino)-5-methyl-3-oxohexanoate (2e). Colorless liquid (0.48 g, 84%);$[\alpha]_D^{25}$ −32.64 (c = 1, MeOH); ^{1}H NMR (500 MHz, $CDCl_3$) δ 12.11 (s, 1H enolic form 6.5%), 5.06 (s, b, 1H, NH), 4.35–4.32 (m, 1H, CH), 4.22– 4.18 (q, J = 7 Hz, 2H, -OCH_2), 3.57–3.50 (dd, J = 15.5 Hz, J = 3 Hz, 2H, CH_2, AB coupling), 2.27–2.23 (m, 1H, $CH(CH_3)_2$), 1.44 (s, 9H, $C(CH_3)_3$, Boc-), 1.29–1.26 (t, J = 7 Hz, 3H, CH_3), 1.029–0.822 (m, 6H, $C(CH_3)_2$); ^{13}C NMR (125 MHz, $CDCl_3$): δ 202.23, 166.75, 155.86, 80.03, 64.38, 61.55, 47.14, 29.56, 28.31, 19.84, 16.67, 14.10; HR-MS m/z Calcd. for $C_{14}H_{25}NO_5$ $[M + Na]^+$ 310.1630, observed 310.1620.

(*S*)-*tert*-Butyl-2-(3-ethoxy-3-oxopropanoyl)pyrrolidine-1-carboxylate (2f). Colorless liquid (0.445 g, 78%); $[\alpha]_D^{25}$ −64.34 (c = 0.6, MeOH); ^{1}H NMR (400 MHz, DMSO d_6): δ 4.31–4.26 (m, 1H, C*H*), 4.1265–4.06 (m, 2H, -OCH_2), 3.66–3.53 (dd, J = 13.64 Hz, J = 22.4 Hz, 2H, CH_2, AB coupling), 3.34–3.31 (m, 2H, $\delta_C H_2$), 2.07–2.05 (m, 1H, one proton of βCH_2), 1.91–1.68 (m, 3H, γCH_2, and one proton of βCH_2), 1.40, 1.33 (s, 9H, $C(CH_3)_3$), 1.20–1.17 (t, J = 7.2 Hz, 3H, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ 203.49, 167.41, 154.31, 153.31, 79.71, 65.34, 61.14, 47.11, 46.99, 46.07, 45.84, 29.45, 28.51, 28.31, 24.45, 23.60, 14.53; MALDI TOF/TOF m/z Calcd. for $C_{14}H_{23}NO_5$ $[M + Na]^+$ 308.1474, observed 308.1439.

(*S*)-1-Benzyl-6-ethyl 2-(benzylcarbonylamino)-4-oxohexanedioate (2g). White solid (0.589 g, 69%); Melting Point = 68.3 °C; $[\alpha]_D^{25}$ −16.30 (c = 0.3, MeOH); ^{1}H NMR (400 MHz, $CDCl_3$) δ 12.06 (s, 1H, enolic), 7.33–7.28 (m, 10H, aromatic), 5.75–5.72 (d, J = 8.2 Hz, 1H, NH), 5.63–5.59 (m, 1H, CH), 5.14 (s, 2H, -OCH_2Ph), 5.08 (s, 2H, -OCH_2Ph), 4.17–4.11 (q, J = 7 Hz, 2H, -OCH_2Me), 3.39 (s, 2H, CH_2, AB coupling), 3.33–3.10 (m, 2H,

CH_2), 1.24–1.21 (t, J = 7.1 Hz, 3H, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ 200.78, 170.51, 166.42,155.99, 136.02, 135.10, 128.57, 128.50,128.42, 128.21, 128.03, 67.57, 67.08, 61.59, 49.88, 49.07, 44.59, 14.01; MALDI TOF/TOF m/z Calcd. for $C_{23}H_{25}NO_7$ $[M + K]^+$ 466.1268, observed 466.1276.

General procedure for the synthesis of Boc-protected coumarins functionalized with proteinogenic amino acid side chains. Trifluoroacetic acid (4 mL) was added to the cold solution of *N*-Boc-γ-amino-β-keto ester (2.0 mmol) in DCM (4 mL). After 30 min, the reaction mixture was evaporated to dryness to afford brown gummy product, and then 3-methoxyphenol (3.5 mmol, 0.434 g) was added and cooled to 0 °C for 5 min. Further, the methanesulfonic acid (MSA) (50 mmol, 4.8 g) was added slowly to the reaction mixture and stirred for about 3 h. The progress of the reaction was monitored by TLC. After completion of the reaction, it was diluted with diethyl ether (100 mL) and cooled to −15 °C. The ether layer was centrifuged (4000 rpm at 4 °C) and the red precipitate (methanesulfonate salt of coumarin) was separated. The red precipitate was dissolved in a solution of 30% Na_2CO_3 (20 mL) and THF (5 mL) and cooled to 0 °C. A solution of di-*tert*-butyl dicarbonate (0.460 g, 2.1 mmol) in THF (10 mL) was added slowly to the reaction mixture and stirred for about 5 h at room temperature. After completion of the reaction, it was extracted with ethyl acetate (50 mL × 3). The combined organic layer was washed with 5% HCl (50 mL × 2), brine (30 mL), dried over anhydrous sodium sulfate and concentrated under reduced pressure to give reddish gummy crude product which was further purified on silica gel column chromatography.

(*S*)-*tert*-Butyl-1-(7-methoxy-2-oxo-2*H*-chromen-4-yl)ethylcarbamate (3a). White powder (0.460 g, 72%); UV absorption λ_{max}: 323 nm, fluorescence emission λ_{max}: 393 nm; Melting Point = 168 °C; $[\alpha]_D^{20}$ −25.0 (c = 1, MeOH); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.58–7.56(d, J = 8.7, 1H, aromatic), 6.85–6.81(m, 2H, aromatic), 6.26(s, 1H, C═C*H*), 5.12–5.05 (m, 1H, NH-C*H*), 4.84–4.82 (d, J = 6.4, 1H, -N*H*), 3.85(s, 3H, -OCH_3),1.47–1.45 (d, J = 6.9 Hz, 3H, CH_3),1.41(s, 9H, -C(CH_3)$_3$); ^{13}C NMR (100 MHz, $CDCl_3$) δ 162.64, 161.65, 157.76, 155.84, 154.67, 124.90, 112.58, 107.91, 101.14, 80.44,55.75, 28.30, 20.83; MALDI-TOF/TOF m/z Calcd. for $C_{17}H_{21}NO_5$ $[M + Na]^+$ 342.1317, observed 342.1320.

(*R*)-*tert*-Butyl-1-(7-methoxy-2-oxo-2*H*-chromen-4-yl)ethylcarbamate (3a1). Colourless crystalline compound (0.460 g, 72%); UV absorption λ_{max}: 323 nm, fluorescence emission λ_{max}: 393 nm; Melting Point = 167 °C; $[\alpha]_D^{20}$ = +25.4 (c = 1, MeOH).^{1}H NMR (400 MHz, $CDCl_3$) δ 7.58–7.56 (d, J = 8.7, 1H, aromatic), 6.85–6.81(m, 2H, aromatic), 6.26(s, 1H, C═C*H*), 5.08 (b, 1H, NH-C*H*), 4.84 (b, J = 6.4, 1H, -N*H*), 3.8469(s, 3H, -OCH_3),1.46–1.44 (d, J = 6.9, 3H, CH_3),1.4109(s, 9H, -C(CH_3)$_3$); ^{13}C NMR (100 MHz, $CDCl_3$) δ 162.63, 161.66, 157.76, 155.79, 154.67, 124.91, 112.55, 111.23, 107.92, 101.14, 80.41, 55.74, 45.74, 28.29, 20.82; MALDI-TOF/TOF m/z Calcd. for $C_{17}H_{21}NO_5$ $[M + Na^+]$ 342.1317, observed 342.1392.

(*S*)-*tert*-Butyl-1-(7-methoxy-2-oxo-2*H*-chromen-4-yl)-2-phenylethylcarbamate (3b). White powder (0.550 g, 70%); UV absorption λ_{max}: 324 nm, fluorescence emission λ_{max}: 393 nm; Melting Point = 175 °C; $[\alpha]_D^{20}$ = 21.67 (c = 1, MeOH); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.63–7.61(d, J = 8.7, 1H, aromatic), 7.29–7.23(m, 3H, Ph-group), 7.13–7.11(d, J = 6.9, 2H, Phe-group), 6.85(s, 1H, aromatic ring of coumarin), 6.83(s, 1H, aromatic ring of coumarin), 6.13 (s, 1H, C═C*H*), 5.34–5.27(q, J = 5.5, 1H, NH-C*H*), 4.85–4.84(d, J = 7.8, 1H, -N*H*), 3.85(s, 3H, -OCH_3), 3.22–2.88 (m, 2H, -CH_2-Ph), 1.34 (s, 9H, -C(CH_3)$_3$); ^{13}C NMR (100 MHz, $CDCl_3$) δ 162.69, 161.39, 156.13, 155.75, 154.78, 135.79, 129.09, 128.79, 127.31, 124.77, 112.64, 111.25, 109.02, 101.25, 80.50, 55.77, 50.58, 40.46, 28.21; MALDI-TOF/TOF m/z Calcd. for $C_{23}H_{25}NO_5$ $[M + Na]^+$ 418.1630, observed 418.1649.

(*S*)-*tert*-Butyl-1-(7-methoxy-2-oxo-2*H*-chromen-4-yl)-2-(4-methoxyphenyl)ethylcarbamate (3c). White powder (0.580 g, 68%); UV absorption λ_{max}: 325 nm, fluorescence emission λ_{max}: 391 nm; Melting Point = 177 °C; $[\alpha]_D^{20}$ = 51.57 (c = 1, MeOH); ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.63–7.60(d, J = 8.7, 1H, aromatic), 7.02–7.00(d, J = 6.9, 2H, Phe-group), 6.86–6.80(m, 4H, Ph-group, aromatic ring of coumarin), 6.09(s, 1H, C═C*H*), 5.27–5.24(q, J = 5.5, 1H, NH-C*H*), 4.83–4.81(d, J = 6.9, 1H, -N*H*), 3.85(s, 3H, -OCH_3), 3.76(s, 3H, -OCH_3), 3.15–2.83 (m, 2H, -CH_2-Ph- OCH_3), 1.35 (s, 9H, -C(CH_3)$_3$);^{13}C NMR (100 MHz, $CDCl_3$): δ 162.65, 161.39, 158.80, 156.16, 155.74, 154.81, 130.16, 127.41, 124.78, 114.17, 112.62, 111.26, 109.01, 101.24, 80.47, 55.76, 55.25, 50.69, 39.49, 28.23; MALDI-TOF/TOF m/z Calcd. for $C_{24}H_{27}NO_6$ $[M + Na]^+$ 448.1736, observed 448.1786.

(*S*)-*tert*-Butyl-1-(7-methoxy-2-oxo-2*H*-chromen-4-yl)-3-methylbutylcarbamate (3d). White powder (0.455 g, 63%); UV absorption λ_{max}: 323 nm, fluorescence emission λ_{max}: 394 nm; Melting Point = 101 °C; $[\alpha]_D^{20}$ −48.33 (c = 1, MeOH); ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.59–7.57(d, J = 8.7, 1H, aromatic), 6.86–6.81(m, 2H, aromatic), 6.22(s, 1H, C═C*H*), 5.07–5.03 (m, 1H, NH-C*H*), 4.81–4.79 (d, J = 7.3, 1H, -N*H*), 3.84(s, 3H, -OCH_3), 1.89–1.78 (m, 2H, -CH-CH_2), 1.62–1.56 (m, 1H, -C*H*-(CH$_3$)$_2$ 1.41 (s, 9H, -C(CH_3)$_3$), 1.07–1.05 (d, J = 6.4, 3H, CH-(CH_3)$_2$), 0.95–0.93 (d, J = 6.4, 3H, CH-(CH_3)$_2$); ^{13}C NMR (100 MHz, $CDCl_3$): δ 162.63, 161.72,157.83, 155.82, 154.99, 124.75, 112.56, 108.12, 101.21, 80.37, 55.74, 48.39, 44.25, 28.29, 25.34, 23.26, 21.53; MALDI-TOF/TOF m/z Calcd. for $C_{20}H_{27}NO_5$ $[M + Na]^+$ 384.1787, observed 384.1706.

(*S*)-*tert*-Butyl-1-(7-methoxy-2-oxo-2*H*-chromen-4-yl)-2-methylpropylcarbamate (3e). White powder (0.361 g, 50%); UV absorption λ_{max}: 323 nm, fluorescence emission λ_{max}: 393 nm; Melting Point = 99 °C; $[\alpha]_D^{20}$ −42.6 (c = 1, MeOH); ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.58–7.55 (d, J = 8.7, 1H, aromatic), 6.85–6.82(m, 2H, aromatic), 6.16(s, 1H, C═C*H*), 4.89 (b, 2H, NH-C*H*, -N*H*), 3.84 (s, 3H, -OCH_3), 2.12–2.04 (m, 1H, -C*H*-(CH$_3$)$_2$ 1.41(s, 9H, -C(CH_3)$_3$), 1.06–1.05 (d, J = 6.4, 3H, CH-(CH_3)$_2$), 0.89–0.87 (d, J = 6.4, 3H, CH-(CH_3)$_2$); ^{13}C NMR (100 MHz, $CDCl_3$): δ 162.72, 161.53, 156.54, 155.74, 155.33, 125.11, 112.55, 111.81, 108.92, 101.18, 80.36, 55.75, 54.95, 31.49, 28.29, 20.30, 16.78; MALDI-TOF/TOF m/z Calcd. for $C_{19}H_{25}NO_5$ $[M + Na]^+$ 370.1630, observed 370.1666.

(*S*)-*tert*-Butyl-2-(7-methoxy-2-oxo-2*H*-chromen-4-yl)pyrrolidine-1-carboxylate (3f). White powder (0.486 g, 71%); UV absorption λ_{max}: 322 nm, fluorescence emission λ_{max}: 391 nm; Melting Point = 119 °C; $[\alpha]_D^{20}$ −125.00 (c = 1, MeOH); ^{1}H NMR (400 MHz,

$CDCl_3$): δ 7.48-7.45 (d, J = 8.7, 1H, aromatic), 6.85–6.80 (m, 2H, aromatic), 6.07, 6.05 (s, 1H, C═C*H*), 5.23–5.20, 5.07–5.05 (d, J = 8.2, 1H, NH-C*H*), 3.85–3.84 (s, 3H, -OCH_3), 3.62–3.56, 3.51–3.44 (m, 2H, -CH_2), 2.41–2.30 (m, 1H, -CH_2), 1.93–1.84 (m, 2H, -CH_2), 1.44, 1.24 (s, 9H, -C(CH_3)$_3$); ^{13}C NMR (100 MHz, $CDCl_3$) δ 162.50, 161.67, 156.38, 155.84, 154.30, 124.91, 124.62, 112.44, 112.38, 111.27, 107.79, 101.19, 101.03, 80.32, 80.18, 57.17, 56.84, 55.72, 47.01, 46.70, 32.93, 31.78, 28.42, 28.20, 28.20, 23.62, 23.02; MALDI-TOF/TOF m/z Calcd. for $C_{19}H_{23}NO_5$ [M + Na]$^+$ 368.1473, observed 368.1480.

(*S*)-2-(((9*H*-Fluoren-9-yl)methoxy)carbonylamino)-3-(7-methoxy-2-oxo-2*H*-chromen-4-yl)propanoic acid (3g). Compound **2g** (0.427 g, 1 mmol) was treated with 3-methoxyphenol (0.615 g, 5 mmol) and the mixture was cooled to 0 °C under N_2 atmosphere. After stirring for 5 min, MSA (2.4 g, 25 mmol) was added slowly under constant stirring. The reaction mixture was allowed to warm to room temperature and the stirring was continued for another 2 h. The progress of the reaction was monitored by TLC. After completion of the reaction (~2 h), it was diluted with diethyl ether (100 mL) and cooled to −15 °C. The ether layer was centrifuged (4000 rpm at 4 °C) and the red precipitate (methanesulfonate salt of coumarin) was separated. The red precipitate was dissolved in a solution of 30% Na_2CO_3 (20 mL) and THF (5 mL) and cooled to 0 °C. A solution of Fmoc-OSu (0.370 g, 1.1 mmol) in THF (5 mL) was added to the reaction mixture and allowed to stir overnight to complete the reaction. After completion (monitored by TLC), it was washed with ether (20 mL × 2) and the aqueous layer was acidified with 3 N HCl (up to pH 3) under ice cold conditions. The combined organic layer was washed with 5% HCl (50 mL × 2), brine (30 mL), dried over anhydrous sodium sulfate and concentrated under reduced pressure to give reddish gummy crude product which was further purified on silica gel column chromatography to give pure white powder. Yield: 0.315 g (62%), UV absorption λ_{max}: 324 nm, fluorescence emission λ_{max}: 384 nm; $[\alpha]_D^{20}$ = 14.5 (c = 1, MeOH); ^{1}H NMR (400 MHz, DMSO): δ 13.09 (s, b, 1H, -COO*H*), 7.88–7.86 (d, J = 7.32, 2H, aromatic Fmoc), 7.74–7.63 (d, J = 9.16, 1H, aromatic ring of coumarin), 7.63–7.59 (dd, J = 7.36, J = 3.64 2H, aromatic Fmoc), 7.41–7.37 (t, J = 7.56, 2H, aromatic Fmoc), 7.31–7.25 (dd, J = 7.32, J = 6.88, 2H, aromatic Fmoc) 7.02–6.979 (m, 2H, aromatic ring of coumarin), 6.24 (s, 1H, C═C*H*), 4.33-4.27 (m, 1H, Fmoc-C*H*), 4.23–4.14 (m, 3H, Fmoc-CH_2), 3.84 (s, 3H, -OCH_3), 3.34– 3.04 (m, 2H, CH-CH_2);^{13}C NMR (100 MHz, $CDCl_3$): δ 172.58, 162.38, 159.95, 155.94, 155.04, 152.62, 143.64, 140.66, 128.16, 127.64, 127.04, 125.83, 125.12, 120.12, 112.41, 112.03, 101.13, 65.74, 56.06, 52.76, 46.51, 32.60; MALDI TOF/TOF m/z Calcd. for $C_{28}H_{23}NO_7$ [M + Na]$^+$ 508.1372, observed 508.2198.

Synthesis of dipeptide Fmoc-Glu(OBu^t)-Phe-coumarin (4). Trifluoroacetic acid (5 mL) was added to the cold solution of Boc-Phe-coumarin (0.396 g, 1 mmol) in DCM (5 mL), and the reaction mixture was stirred for about 30 min in an ice bath. After completion of the deprotection, TFA was removed under reduced pressure and the residue was dissolved in water (50 mL). The pH of the solution was adjusted to ~10 by the addition of solid Na_2CO_3. The free coumaryl amine was extracted with ethyl acetate (30 mL × 3). The combined organic layer was washed with brine and concentrated to ~2–3 mL and immediately subjected to the coupling reaction. The Fmoc-Glu(OBu^t)-OH (0.425 g, 1 mmol) was dissolved in DMF (4 mL) and NH_2-Phe-coumarin (in ~2–3 mL of EtOAc, obtained from the above step) was added to the solution followed by HBTU (0.416 g, 1.1 mmol) and HOBt (0.148 g, 1.1 mmol). The reaction mixture was cooled to 0 °C. After stirring for 5 min, DiEPA (0.35 mL, 2 mmol) was added to the reaction mixture and it was allowed to warm to the room temperature. The progress of the reaction was monitored by TLC. After completion of the reaction (~5 h), the mixture was diluted with 100 mL of ethyl acetate, washed with 5% HCl (2 × 30 mL), 10% Na_2CO_3 (2 × 40 mL) followed by brine (30 mL). The organic layer was dried over anhydrous sodium sulphate and evaporated under reduced pressure to give gummy yellowish product, which was further purified by column chromatography. The pure peptide was isolated as a solid white powder. Yield:0.5 g (78%); UV absorption λ_{max}: 324 nm, fluorescence emission λ_{max}: 384 nm; $[\alpha]_D^{20}$ −23.33 (c = 1, MeOH); ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.75–7.74 (d, J = 7.3, 2H, aromatic Fmoc), 7.64–7.62(d, J = 8.5, 1H, aromatic ring of coumarin), 7.56–7.54(d, J = 6.9, 2H, aromatic Fmoc), 7.38–6.80 (m, 10H, aromatic ring of Phenyl, aromatic Fmoc, -N*H*), 6.17 (s, 1H, C═C*H*), 5.65–5.63 (d, J = 7.3,1H, -N*H*), 5.58–5.52 (q, J = 7.3, 1H, NH-C*H*), 4.38–4.31(m, 2H, Fmoc-C*H*, NH-C*H*), 4.14–4.07 (m, 2H, Fmoc-CH_2), 3.83(s, 3H, -OCH_3), 3.22–2.94 (m, 2H, CH_2-Ph), 2.24–2.17 (m, 2H, -CH_2), 1.97–1.77 (m, 2H, -CH_2), 1.12 (s, 9H, -C(CH_3)$_3$); ^{13}C NMR (100 MHz, $CDCl_3$): δ 170.97, 162.76, 161.23, 158.16, 155.72, 155.39, 143.66, 141.27, 135.76, 128.99, 128.70, 127.76, 127.07, 125.09, 125.04, 120.00, 112.70, 111.12, 109.36, 101.24, 100.60, 81.44, 67.08, 55.96, 49.43, 47.03, 40.24, 31.89, 29.75, 28.03; MALDI-TOF/TOF m/z Calcd. for $C_{42}H_{42}N2O_8$ [M + Na]$^+$ 725.2838, observed 725.2869.

SPPS synthesis of H_2N-Gly-Arg-Lys-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-Pro-Pro-Gln-FCuM (P1)

Synthesis of Fmoc-Glu(OH)-Phe-coumarin (**5**): Trifluoroacetic acid (5 mL) was added to the cold solution of Fmoc-Glu(OBu^t)-Phe-coumarin (0.420 g, 0.6 mmol) in DCM (5 mL) and the mixture was stirred for 30 min in an ice-bath. After the deprotection (~30 min), TFA was removed from the reaction mixture under reduced pressure and the residue was diluted with EtOAc (100 mL), washed with water and brine, and dried over anhydrous sodium sulfate. The organic layer was concentrated under reduced pressure to give gummy product which was further recrystallized using EtOAc/hexane to obtain a white solid powder (**5**) which was used for the synthesis of **P1** using SPPS (solid phase peptide synthesis) without further purification. Peptide synthesis was carried out by a manual stepwise solid phase method using standard Fmoc/Boc chemistry at 0.1 mmol scale on Rink amide resin. In the initial step, the dipeptide acid **5** was coupled to the resin using standard coupling agents, HBTU/HOBt. After the coupling, the Fmoc deprotection was carried out using 25% piperidine in DMF. The peptide elongation was continued by successive addition of Fmoc-amino acids followed by Fmoc deprotection. The final peptide (**P1**) was released from the resin using a TFA/H_2O (19 : 1) (10 mL) mixture. Further, the peptide was purified through reverse phase HPLC using a C_{18} column with acetonitrile/H_2O gradient. Homogeneity of the peptide was confirmed by analytical reverse phase HPLC and MALDI TOF/TOF m/z Calcd. for $C_{99}H_{167}N_{33}O_{19}$ [M^{4+} + H^+] 2124.32 Da, observed 2124.79 Da.

Peptide visualization in DLD-1 colon cancer cells

DLD-1 cells were plated onto four-chamber culture slides and grown overnight in Dulbecco's minimum essential medium complemented with 10% fetal bovine serum and 100 units/ml penicillin/streptomycin in a 5% CO_2 atmosphere at 37 °C. Cells were treated with 10 μM **P1** for 3 h. Then the culture slides were washed twice with phosphate-buffered saline and the cells were fixed with 3% paraformaldehyde at room temperature (30 min). The fixed cells were washed repeatedly with phosphate-buffered saline. Culture slides were mounted using anti-FAD fluorescent mounting medium and the fluorescence image recorded. Excitation was provided with a mercury ion laser set at 405 nm, and the emitted light was filtered with an appropriate long pass filter.

Crystal structure Boc-Pro-coumarin (3f). Crystals were grown by slow evaporation from a solution of EtOAc/hexane. A single crystal (0.38× 0.25 × 0.15 mm) was mounted on a loop with a small amount of paraffin oil. The X-ray data were collected at 200 K on a Bruker APEX DUO CCD diffractometer using Mo-Kα radiation (λ = 0.71073 Å), ω-scans (2θ = 56.56), for a total of 3198 independent reflections. Space group $C2$, a = 16.779(3), b = 10.0638(17), c = 11.0022(19), β = 103.905(4), V = 1803.4(5) Å^3, Monoclinic, Z = 4 for chemical formula $C_{19}H_{23}NO_5$, with one molecule in asymmetric unit; ρ calcd. = 1.272 g cm^{-3}, μ = 0.092 mm^{-1}, $F(000)$ = 736, R_{int} = 0.0216. The final R value was 0.0355 (wR_2 = 0.0896) 2882 observed reflections ($F_0 \geq 4\sigma$ ($|F_0|$)) and 230 variables, S = 1.052. The structure was obtained by direct methods using SHELXS-97.[16] All non-hydrogen atoms were refined anisotropically. The hydrogen atoms were fixed geometrically in idealized positions and refined in the final cycle of refinement as riding over the atoms to which they are bonded. The largest difference peak and hole were 0.169 and –0.202 e Å^3, respectively.

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