A New and Expedient Total Synthesis of Ochratoxin A and d_5-Ochratoxin A

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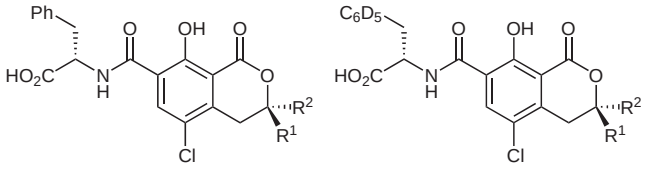
Abstract: A new total synthesis of the mycotoxin ochratoxin A (OTA) is presented, in which it is prepared in 9% overall yield from commercially available substrates. The key step consists of the condensation reaction between protected L-phenylalanine and 5-chloro-8-hydroxy-3-methyl-1-oxoisochromane-7-carboxylic acid (ochratoxin α, OTα). The same strategy could be successfully applied to L-d_5-phenylalanine, leading to the first total synthesis of d_5-OTA, a molecular tracer for the detection and analytical quantification of the natural mycotoxin in food samples by means of stable isotope dilution assay (SIDA).

Key words: heterocycles, natural products, ochratoxin A, stable isotope dilution assay, total synthesis

Ochratoxin A (OTA, **1a**; Figure 1) is a ubiquitous mycotoxin produced by some fungi of the *Aspergillus* and *Penicillium* species (such as *Aspergillus ochraceus* and *Penicillium verrucosum*), and is found in raw and improperly stored food products.[1,2] OTA has been shown to be nephrotoxic, mutagenic, genotoxic, teratogenic, hepatotoxic, neurotoxic, and immunotoxic, in both animals and humans,[3] and in 1993 was classified as a possible carcinogen to humans (Group 2B) by the International Agency for Research on Cancer (IARC).[4]



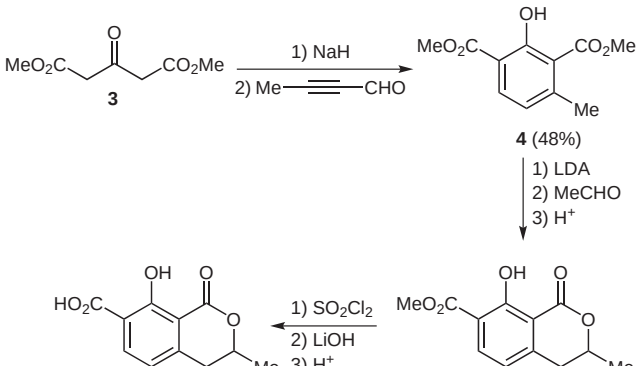
1a R^1 = H, R^2 = Me
1b R^1 = Me, R^2 = H

2a R^1 = H, R^2 = Me
2b R^1 = Me, R^2 = H

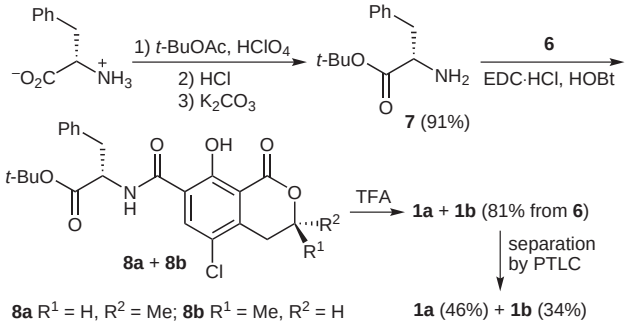
Figure 1 Structures of (–)-ochratoxin A (OTA, **1a**), (–)-d_5-ochratoxin A (d_5-OTA, **2a**), and their (3*S*)-diastereomers **1b** and **2b**, respectively

OTA contamination occurs in a wide range of foods and beverages, including cereals, beans, dried vine fruits, coffee, wine, beer, grape juice, pork, poultry, spices, and chocolate.[1,2] The development of sensitive and accurate methods for the analytical determination of OTA in food products has therefore become increasingly important.[5] Stable isotope dilution assay (SIDA) is currently one of the most promising methods for the highly sensitive quantitative determination of microcomponents in food.[6] Thus, OTA can be detected and quantified in food samples by use of SIDA if, for example, a deuterated derivative, such as d_5-OTA (**2a**; Figure 1), is used as an internal standard.[5a]

In this paper, we report a new and efficient method for the total synthesis of OTA (**1a**) and d_5-OTA (**2a**), together with their (3*S*)-diastereomers **1b** and **2b**, respectively, starting from but-2-ynal and dimethyl 3-oxopentanedioate (**3**) (Schemes 1– 3). The key step of the synthetic strategy consists of the condensation reaction between protected L-phenylalanine **7** (Scheme 2) or protected L-d_5-phenylalanine **9** (Scheme 3) and 5-chloro-8-hydroxy-3-methyl-1-oxoisochromane-7-carboxylic acid (ochratoxin α, OTα), which was prepared by a modification of the method originally proposed by Kraus (Scheme 1).[7]



Scheme 1 Synthesis of ochratoxin α (OTα, **6**)



Scheme 2 Synthesis of (–)-ochratoxin A (OTA, **1a**) and its (3*S*)-diastereomer **1b**

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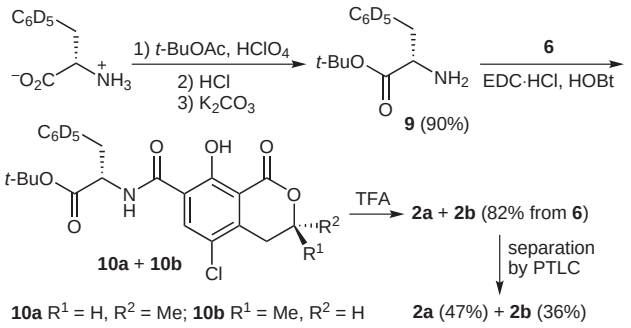

Scheme 3 Synthesis of d_5-(–)-ochratoxin A (d_5-OTA, **2a**) and its (3*S*)-diastereomer **2b**

Thus, following the method recently disclosed by Covarrubias-Zúñiga,[8] crude but-2-ynal (readily available by quantitative oxidation of but-2-ynol with MnO_2 in CH_2Cl_2)[9] was reacted with the sodium salt of commercially available dimethyl 3-oxopentanedioate (**3**) at –10 °C in tetrahydrofuran, to give dimethyl 2-hydroxy-4-methylbenzene-1,3-dicarboxylate (**4**) in 48% yield (Scheme 1). Kraus's method[7] was followed to provide lactone derivative **5**: the methyl group of **4** was deprotonated with lithium diisopropylamide at –78 °C in tetrahydrofuran; addition of acetaldehyde and acidic workup followed; **5** was obtained in 70% yield. Chlorination of **5** with sulfuryl chloride in dichloromethane at room temperature, followed by hydrolysis of the ester group with lithium hydroxide in methanol finally gave OTα (**6**) in 69% yield. The overall yield of **6** was therefore 23% over three steps starting from commercially available **3**, which is higher than that previously obtained by Kraus (17% over 4 steps starting from acetone and ethyl formate),[7] Snieckus and coworkers (6% over 5 steps starting from 4-chlorophenol),[10] and Donner and Gill [who synthesized (*R*)-OTα starting from (*R*)-propylene oxide over 9 steps with an overall yield of 10%].[11]

A condensation reaction between OTα (**6**) and protected L-phenylalanine **7** was then performed in chloroform as the solvent at 25 °C for 20 hours in the presence of *N*-ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide hydrochloride and *N*-hydroxybenzotriazole (BtOH) as coupling agents, to give a mixture of protected diastereomers **8a**,**b** in ca. 86% total yield, based on **6** (Scheme 2). Deprotection of the crude mixture **8a**,**b** with trifluoroacetic acid in dichloromethane at room temperature for six hours led to a mixture of OTA (**1a**) and its (3*S*)-diastereomer **1b** in 81% total yield based on starting OTα (**6**).[12] Separation of the two diastereomers by preparative TLC (PTLC) afforded pure **1a** and **1b** in 46% and 34% isolated yield, respectively. Both diastereomers were fully characterized by IR, ^{1}H NMR and ^{13}C NMR spectroscopy, HRMS, and specific rotation.

Our strategy could be successfully applied to the first total synthesis of d_5-OTA (**2a**) and its (3*S*)-diastereomer **2b** (Figure 1), by use of L-d_5-phenylalanine as deuterated starting material, with essentially the same overall yields (Scheme 3). Thus, condensation of OTα (**6**) with protected L-d_5-phenylalanine **9**, followed by deprotection and separation of the two resulting diastereomers **2a** and **2b** by preparative TLC led to pure **2a** and **2b** in 47% and 36% isolated yield, respectively (Scheme 3). To our knowledge, this is the first example of the total synthesis of both d_5-OTA and its (3*S*)-diastereomer reported in the literature.[13] Both deuterated diastereomers **2a**,**b** were fully characterized by IR, ^{1}H NMR and ^{13}C NMR spectroscopy, HRMS, and specific rotation.

In conclusion, we have developed a novel and convenient total synthesis of ochratoxin A (**1a**) and its (3*S*)-diastereomer **1b**, with overall yields of ca. 9% and 6%, respectively, over only six steps, starting from commercially available starting materials. Our strategy has also allowed the first total synthesis of d_5-OTA (**2a**), which can be used as an internal standard for the quantitative determination of the wild-type ochratoxin in foodstuff.

Melting points were determined on a Reichert Thermovar apparatus and are uncorrected. Optical rotations were measured on a Jasco DIP-1000 12 polarimeter equipped with a sodium lamp (589 nm) and a 10-cm microcell. ^{1}H NMR (300 MHz) and ^{13}C NMR (75 MHz) spectra of samples dissolved in $CDCl_3$ or DMSO-d_6 were recorded on a Bruker DPX Avance 300 spectrometer at 25 °C; TMS was used as internal standard. IR spectra were recorded on a Perkin-Elmer Paragon 1000 PC FT-IR spectrometer. GC-MS spectra were obtained with a Shimadzu QP-2010 GC-MS apparatus (ionization voltage 70 eV). LC-MS analyses were carried out on a fractionlynx HPLC system composed of a autosampler/collector, a 600E pump working in analytical mode, a 486 UV detector (set to 280 nm) and a ZMD mass spectrometer equipped with an ESI source; a 100 × 3.0 mm ONYX-C_{18} monolithic column was used [flow rate 1.5 mL/min; run time 20 min; gradient elution with 0.5% HCO_2H in H_2O (solvent A) and MeOH (solvent B); solvent run: linear gradient from 95% A to 5% A in 14 min, linear gradient from 5% A to 95% A in 3 min, isocratic 95% A for 3 min]. The MS conditions were the following: capillary voltage 3.15 kV, cone voltage 3 V, extractor 2 V, RF lens 0.2 V, source block and desolvation temperature 120, 250 °C respectively, ion energy 0.5 V, LM resolution 15.0, HM resolution 14.5 and multiplier 650 V. The nebulizer gas was set to 650 L/h. ESI-HRMS experiments were performed on a hybrid Q-Star Pulsar-i Q*q*ToF mass spectrometer equipped with an ion-spray ionization source. All samples were acquired at the optimum ion-spray voltage of 4.8 kV by direct infusion (5 μL/min) of a soln containing the appropriate compound dissolved in MeOH–H_2O (20 μg/mL). The N_2 gas flow was set at 20 psi and the declustering and the focusing potentials were kept at 50 and 220 V relative to ground, respectively. Commercially available flavonoids were used as calibration standard compounds. The accuracy of the measurement was within 5 ppm. MS^2 experiments were performed in the collision cell *q* on the isotopically pure (^{12}C) peak of the selected precursor ions by keeping the first quadrupole analyzer at unit resolution, and scanning the time-of-flight (ToF) analyzer. The collision energy was set to 20 eV, for each compound, while the gas pressure of the collision chamber was regulated at the instrumental parameters CAD 5, which corresponds to a pressure of the chamber of 6.86×10^{-3} Torr and a gas thickness of 9.55×10^{15} molecules/cm^2. All the acquisitions were averaged over 30 scans at a TOF resolving power of 7000. The molecular formula was evaluated by means of Analyst QS software. All reactions were analyzed by TLC on silica gel 60 F_{254} and by GLC on a Shimadzu GC-2010 gas chromatograph and capillary columns with polymethylsilicone with 5% phenylsilicone as the stationary phase. Column chromatography was performed on silica gel 60 (Merck, 70–230 mesh). Preparatory TLC separations

were carried out on Merck silica gel plates (20 × 20 cm, 0.25 mm thickness). MnO_2, but-2-ynol, dimethyl 3-oxopentanedioate (**3**), NaH (95% purity), 2 M LDA in THF–heptane–ethylbenzene, acetaldehyde, SO_2Cl_2, LiOH, L-phenylalanine, *t*-BuOAc, $HClO_4$ (70%), EDC·HCl, BtOH, TFA, and L-d_5-phenylalanine (99% D) were commercially available and were used as received.

Dimethyl 2-Hydroxy-4-methylbenzene-1,3-dicarboxylate (4)

But-2-ynol (5.0 g, 71.3 mmol) was added to a suspension of MnO_2 (74.4 g, 860 mmol) in anhyd CH_2Cl_2 (200 mL) at r.t. under N_2. After stirring at r.t. for 12 h, the mixture was filtered, and the solvent was removed by distillation at atmospheric pressure. The crude but-2-ynal thus obtained (still containing ca. 0.5 mL CH_2Cl_2) was used as such for the next reaction. Dimethyl 3-oxopentanedioate (**3**; 11.5 g, 66.0 mmol) was added dropwise to a stirred suspension of NaH (95% purity; 1.9 g, 75.2 mmol) in anhyd THF (120 mL) at –10 °C under N_2, followed by crude but-2-ynal (obtained as described above). After additional stirring at –10 °C for 6 h, the mixture was poured into dilute aq HCl (250 mL). The organic layer was separated, and the aqueous layer was extracted with EtOAc (3 × 50 mL). The combined organic phases were washed with brine (250 mL) and then dried (Na_2SO_4). After filtration of the mixture and removal of the solvent by rotary evaporation, the residue was purified by column chromatography (silica gel, hexane–EtOAc, 7:3); this gave pure **4**.

Yield: 7.1 g (48%); yellow crystals; mp 43–45 °C (Lit.[8] 44–46 °C).

IR (KBr): 2955 (m), 1730 (s), 1672 (s), 1330 (s), 1298 (m), 1259 (m), 789 (m), 747 (m) cm^{-1}.

^1H NMR (300 MHz, $CDCl_3$): δ = 11.16 (s, 1 H, OH), 7.74 (d, *J* = 8.2 Hz, 1 H, H-6), 6.72 (d, *J* = 8.2 Hz, 1 H, H-5), 3.94 (s, 3 H, CO_2Me), 3.92 (s, 3 H, CO_2Me), 2.33 (s, 3 H, Me at C-4).

^{13}C NMR (75 MHz, $CDCl_3$): δ = 170.2, 167.6, 159.0, 144.3, 131.0, 123.3, 121.1, 110.8, 52.3, 52.1, 20.1.

GC-MS (EI, 70 eV): *m*/*z* = 224 [M^+] (32), 193 (38), 192 (100), 161 (93), 160 (28), 134 (48), 105 (17), 77 (27).

Methyl 8-Hydroxy-3-methyl-1-oxoisochromane-7-carboxylate (5)

A soln of **4** (6.5 g, 29.0 mmol) in anhyd THF (30 mL) was added dropwise (15 min) to a 2 M soln of LDA in THF–heptane–ethylbenzene (36.3 mL, 72.6 mmol) maintained at –78 °C under N_2. After additional stirring of the mixture at –78 °C for 30 min, acetaldehyde (5.5 g, 124.9 mmol) was added, and the soln was stirred at –78 °C for 15 min and then at 0 °C for a further 15 min. After quenching of the mixture with glacial AcOH (10 mL) at 0 °C, H_2O (30 mL) and Et_2O (30 mL) were added, and the organic layer was separated. The aqueous layer was extracted with Et_2O (2 × 30 mL), and the combined organic layer was dried (Na_2SO_4). After filtration, the solvent was removed by rotary evaporation, and the crude product was crystallized from acetone; this gave pure **5**.

Yield: 4.79 g (70%); yellow solid; mp 108–109 °C (Lit.[7] 108–110 °C).

IR (KBr): 3419 (br m), 1724 (s), 1660 (m), 1619 (m), 1431 (w), 1239 (m), 1218 (m), 1143 (w), 808 (w) cm^{-1}.

^1H NMR (300 MHz, $CDCl_3$): δ = 8.02 (d, *J* = 7.9 Hz, 1 H, H-6), 6.77 (dt, *J* = 7.9, 0.7 Hz, 1 H, H-5), 4.80–4.65 (m, 1 H, C*H*CH_3), 3.93 (s, 3 H, CO_2Me), 3.03–2.90 (m, 2 H, CH_2), 1.53 (d, *J* = 6.4 Hz, 3 H, CHC*H*$_3$). (Note: the OH signal was too broad to be detected.)

^{13}C NMR (75 MHz, $CDCl_3$): δ = 168.2, 166.2, 162.5, 145.2, 137.9, 117.6, 117.2, 110.2, 75.5, 52.1, 35.1, 20.6.

GC-MS (EI, 70 eV): *m*/*z* = 236 [M^+] (59), 205 (52), 204 (18), 192 (28), 160 (100), 104 (24), 91 (6), 77 (30).

5-Chloro-8-hydroxy-3-methyl-1-oxoisochromane-7-carboxylic Acid (Ochratoxin α, OTα, 6)

SO_2Cl_2 (6.9 g, 51 mmol) was added to a stirring soln of **5** (2.4 g, 10.2 mmol) in anhyd CH_2Cl_2 (50 mL) under N_2 at 25 °C. After additional stirring of the mixture at 25 °C for 24 h, the resulting yellow soln was evaporated in vacuo to give methyl 5-chloro-8-hydroxy-3-methyl-1-oxoisochromane-7-carboxylate as a yellow solid, which was suspended in MeOH (50 mL). LiOH (4.46 g, 186 mmol) was added, and the resulting mixture was allowed to reflux for 5 h. During this time a semisolid product separated from the mixture. After removal of the solvent under vacuum, H_2O (25 mL) and Et_2O (25 mL) were added, and the organic layer was separated. The aqueous layer was acidified to pH 2 with 1 N aq HCl, and then extracted with Et_2O (3 × 30 mL); then the combined organic layers were dried (Na_2SO_4). After filtration, the solvent was removed by rotary evaporation, and the crude product was crystallized from acetone; this gave pure ochratoxin α (**6**).

Yield: 1.8 g (69%); colorless solid; mp 245–246 °C (Lit.[7] 246 °C).

IR (KBr): 3266 (br m), 1732 (s), 1700 (s), 1680 (s), 1610 (s), 1440 (s), 1220 (m), 1200 (s), 1149 (s), 1100 (m), 821 (m) cm^{-1}.

^1H NMR (300 MHz, DMSO-d_6): δ = 10.03 (br s, 2 H, CO_2H, OH), 7.98 (s, 1 H, H-6), 4.87–4.60 (m, 1 H, C*H*CH_3), 3.20 (distorted dd, *J* = 17.1, 2.4 Hz, 1 H, C*H*H), 2.87 (distorted dd, *J* = 17.1, 11.6 Hz, 1 H, CH*H*), 1.46 (d, *J* = 6.1 Hz, 3 H, Me).

^{13}C NMR (75 MHz, DMSO-d_6): δ = 167.0, 165.5, 160.4, 143.1, 136.0, 120.6, 118.1, 112.3, 74.4, 32.2, 20.1.

ESI-MS: *m*/*z* = 279 (100) [M + Na]$^+$, 257 (80) [M + H]$^+$.

ESI-HRMS: *m*/*z* [M + H]$^+$ calcd for $C_{11}H_{10}ClO_5$: 257.0217; found: 257.0199.

MS/MS [M + H]$^+$ (ESI+, 30 eV): *m*/*z* = 239.00 (88.2) [M – H_2O + H]$^+$, 220.99 (100.0) [M – 2H_2O + H]$^+$, 193.00 (59.7) [M – 2H_2O – CO + H]$^+$, 165.00 (15.3) [M – 2H_2O – 2CO + H]$^+$, 137.01 (17.4), 102.04 (10.5).

***tert*-Butyl L-Phenylalaninate (7)**

Concd $HClO_4$ (70%; 1.5 mL, 2.5 g, 17.4 mmol) was slowly added to a suspension of L-phenylalanine (1.8 g, 10.9 mmol) in *t*-BuOAc (27.0 mL, 23.3 g, 200 mmol) under N_2 at 0 °C. After stirring of the mixture at 25 °C for 12 h, H_2O (55 mL) followed by 1 N HCl (30 mL) were added. The mixture was basified to pH 9 by the addition of 10% aq K_2CO_3 soln, and then extracted with CH_2Cl_2 (3 × 25 mL). The combined organic layers were dried (Na_2SO_4). After filtration, the solvent was removed by rotary evaporation, and the crude product was purified by column chromatography (silica gel, hexane–EtOAc, 1:1); this gave pure **7**.

Yield: 2.2 g (91%); colorless oil.

IR (film): 2978 (w), 1729 (s), 1603 (w), 1458 (w), 1368 (m), 1154 (s), 847 (m), 740 (m), 700 (m) cm^{-1}.

^1H NMR (300 MHz, $CDCl_3$): δ = 7.32–7.17 (m, 5 H, Ph), 3.60 (distorted dd, *J* = 7.6, 5.7 Hz, 1 H, C*H*NH_2), 3.02 (distorted dd, *J* = 13.6, 5.7 Hz, 1 H, C*H*H), 2.83 (distorted dd, *J* = 13.6, 7.6 Hz, 1 H, CH*H*), 1.51 (br s, 2 H, NH_2), 1.42 (s, 9 H, *t*-Bu).

^{13}C NMR (75 MHz, $CDCl_3$): δ = 174.3, 137.9, 129.5, 128.4, 126.7, 81.0, 56.5, 41.6, 28.1.

GC-MS (EI, 70 eV): *m*/*z* = 221 [M^+] (absent), 130 (16), 121 (28), 120 (100), 103 (19), 91 (45), 77 (25), 74 (98).

***tert*-Butyl *N*-{[(3*R*/3*S*)-5-Chloro-8-hydroxy-3-methyl-1-oxoisochroman-7-yl]carbonyl}-L-phenylalaninate (8a,b)**

EDC·HCl (208 mg, 1.08 mmol) was added to a stirred soln of **7** (222 mg, 1.0 mmol), **6** (269 mg, 1.05 mmol), and BtOH (142 mg, 1.05 mmol) in anhyd $CHCl_3$ (5 mL) under N_2 at 0 °C. After additional stirring of the mixture at 0 °C for 15 min and at 25 °C for 20 h,

$CHCl_3$ (10 mL) and H_2O (10 mL) were added. The organic layer was separated, washed sequentially with 1 N HCl, H_2O, 5% $NaHCO_3$, and brine (10 mL each), and then dried (Na_2SO_4). After filtration of the mixture, the solvent was removed by rotary evaporation; this gave a crude mixture of diastereomers **8a**,**b**, which was used as such for the next step.

(–)-*N*-{[(3*R*)-5-Chloro-8-hydroxy-3-methyl-1-oxoisochroman-7-yl]carbonyl}-L-phenylalanine (Ochratoxin A, OTA, 1a) and (+)-*N*-{[(3*S*)-5-Chloro-8-hydroxy-3-methyl-1-oxoisochroman-7-yl]carbonyl}-L-phenylalanine (1b)

The crude mixture of diastereomers **8a**,**b**, obtained as described above, was added to a soln of TFA (12.3 g, 108 mmol) in anhyd CH_2Cl_2 (20 mL) at 25 °C under N_2. The reaction mixture was stirred at 25 °C and monitored by TLC. When the reaction was completed (ca. 6 h), the solvent and excess TFA were removed by rotary evaporation. The resulting residual oil was dissolved in CH_2Cl_2, washed with H_2O (3 × 10 mL), and dried (Na_2SO_4). After filtration and removal of the solvent by rotary evaporation, a colorless solid was obtained, which was crystallized from benzene to give a mixture of **1a** and **1b**.

Yield: 345 mg (81% from **6**); colorless solid; mp 168–171 °C (Lit.[9] 169–172 °C).

Separation of 1a and 1b

A mixture of the two diastereomers **1a** and **1b** (100 mg) dissolved in $CHCl_3$ was then subjected to preparative TLC on plates coated with silica gel (20 plates, 20 × 20 cm, 0.25 mm thickness, benzene–acetone–HCO_2H, 79:20:1); this gave pure **1a** (R_f = 0.47) and pure **1b** (R_f = 0.43).

1a

Yield: 46 mg (46%); colorless solid; mp 110–112 °C; $[\alpha]_D^{25}$ –31.5 (*c* 5 mg/mL, $CHCl_3$).

IR (KBr): 3029 (m), 2985 (m), 2928 (m), 1742 (m), 1674 (s), 1614 (m), 1534 (s), 1427 (m), 1391 (w), 1214 (s), 1171 (m), 1139 (m), 809 (w), 758 (w), 702 (w) cm^{-1}.

^1H NMR (300 MHz, $CDCl_3$): δ = 12.74 (br s, 1 H, OH), 8.50 (br d, *J* = 6.8 Hz, 1 H, NH), 8.42 (s, 1 H, H-6), 7.34–7.20 (m, 6 H, Ph, OH), 5.07–4.98 (m, 1 H, C*H*NH), 4.81–4.68 (m, 1 H, C*H*CH_3), 3.40–3.15 (m, 3 H, C*H*$_2$Ph, C*H*HCHCH$_3$), 2.84 (dd, *J* = 17.6, 11.7 Hz, 1 H, CH*H*CHCH$_3$), 1.59 (d, *J* = 6.3 Hz, 3 H, Me).

^{13}C NMR (75 MHz, $CDCl_3$): δ = 174.6, 169.7, 163.3, 159.1, 141.0, 139.0, 135.8, 129.3, 128.7, 127.3, 123.2, 120.2, 110.1, 75.9, 54.4, 37.3, 32.3, 20.7.

ESI-MS: *m*/*z* = 426 (68) $[M + Na]^+$, 404 (100) $[M + H]^+$.

ESI-HRMS: *m*/*z* $[M + H]^+$ calcd for $C_{20}H_{19}ClNO_6$: 404.0901; found: 404.0891.

MS/MS $[M + H]^+$ (ESI+, 20 eV): *m*/*z* = 404.08 (34.8) $[M + H]^+$, 386.07 (6.4) $[M – H_2O + H]^+$, 358.08 (79.1) $[M – H_2O – CO + H]^+$, 341.05 (17.6) $[M – H_2O – CO – NH_3 + H]^+$, 239.00 (100.0) $[M – C_9H_{11}NO_2]^+$, 220.99 (7.2) $[M – C_9H_{13}NO_3]^+$, 120.08 (6.2) $[C_8H_{10}N]^+$.

1b

Yield: 34 mg (34%); colorless solid; mp 182–183 °C; $[\alpha]_D^{25}$ +66.7 (*c* 3 mg/mL, $CHCl_3$).

IR (KBr): 3022 (w), 2925 (s), 2851 (m), 1741 (s), 1673 (s), 1615 (w) 1541 (m), 1427 (m), 1214 (s), 1380 (m), 1170 (m), 1139 (m), 809 (m), 742 (w), 705 (w) cm^{-1}.

^1H NMR (300 MHz, $CDCl_3$): δ = 12.71 (br s, 1 H, OH), 8.50 (br d, *J* = 7.2 Hz, 1 H, NH), 8.40 (s, 1 H, H-6), 7.34–7.18 (m, 5 H, Ph), 7.08 (br s, 1 H, OH), 5.05 (distorted dd, *J* = 12.6, 6.8 Hz, 1 H, C*H*NH), 4.80–4.66 (m, 1 H, C*H*CH_3), 3.39–3.15 (m, 3 H, C*H*$_2$Ph, C*H*HCHCH$_3$), 2.89–2.76 (m, 1 H, CH*H*CHCH$_3$), 1.58 (d, *J* = 6.3 Hz, 3 H, Me).

^{13}C NMR (75 MHz, $CDCl_3$): δ = 174.8, 169.7, 163.1, 159.2, 141.0, 139.1, 136.0, 129.4, 128.7, 127.3, 123.3, 120.6, 110.2, 75.9, 54.5, 37.6, 32.4, 20.7.

ESI-MS: *m*/*z* = 426 (100) $[M + Na]^+$, 404 (87) $[M + H]^+$.

ESI-HRMS: *m*/*z* $[M + H]^+$ calcd for $C_{20}H_{19}ClNO_6$: 404.0901; found: 404.0886.

MS/MS $[M + H]^+$ (ESI+, 20 eV): *m*/*z* = 404.09 (33.7) $[M + H]^+$, 386.08 (5.9) $[M – H_2O + H]^+$, 358.08 (81.4) $[M – H_2O – CO + H]^+$, 341.06 (17.9) $[M – H_2O – CO – NH_3 + H]^+$, 239.01 (100.0) $[M – C_9H_{11}NO_2]^+$, 221.00 (8.6) $[M – C_9H_{13}NO_3]^+$, 120.08 (8.1) $[C_8H_{10}N]^+$.

***tert*-Butyl L-d_5-Phenylalaninate (9)**

Concd $HClO_4$ (70%) (200 μL, 336 mg, 2.34 mmol) was added slowly to a suspension of L-d_5-phenylalanine (99% D; 255 mg, 1.5 mmol) in *t*-BuOAc (3.6 mL, 3.1 g, 26.7 mmol) under N_2 at 0 °C. After stirring of the mixture at r.t. for 12 h, H_2O (15 mL) followed by 1 N aq HCl (10 mL) were added. The mixture was basified to pH 9 by the addition of 10% K_2CO_3, and then extracted with CH_2Cl_2 (3 × 15 mL). The combined organic layers were dried (Na_2SO_4). After filtration, the solvent was removed by rotary evaporation, and the crude product was purified by column chromatography (silica gel, hexane–EtOAc, 1:1); this gave pure **9**.

Yield: 307.0 mg (90%); colorless oil.

IR (film): 3381 (br s), 1735 (m), 1621 (s), 1212 (m), 1154 (m) cm^{-1}.

^1H NMR (300 MHz, $CDCl_3$): δ = 3.61 (distorted dd, *J* = 7.6, 5.8 Hz, 1 H, C*H*NH_2), 3.04 (distorted dd, *J* = 13.6, 5.8 Hz, 1 H, C*H*H), 2.84 (distorted dd, *J* = 13.6, 7.6 Hz, 1 H, CH*H*), 1.53 (br s, 2 H, NH_2), 1.42 (s, 9 H, *t*-Bu).

GC-MS (EI, 70 eV): *m*/*z* = 227 $[M^+]$ (absent), 125 (100), 126 (10), 107 (5), 106 (5), 96 (10), 74 (37).

ESI-HRMS: *m*/*z* $[M + H]^+$ calcd for $C_{13}H_{15}D_5NO_2$: 227.1803; found: 227.1795.

MS/MS $[M + H]^+$ (ESI+, 20 eV): *m*/*z* = 171.11 (55.7) $[M – t\text{-Bu} + H]^+$, 125.11 (100.0), 124.10 (16.0).

***tert*-Butyl *N*-{[(3*R*/3*S*)-5-Chloro-8-hydroxy-3-methyl-1-oxoisochroman-7-yl]carbonyl}-L-d_5-phenylalaninate (10a,b)**

EDC·HCl (208 mg, 1.08 mmol) was added to a stirred soln of **9** (227 mg, 1.0 mmol), **6** (269 mg, 1.05 mmol), and BtOH (142 mg, 1.05 mmol) in anhyd $CHCl_3$ (5 mL) under N_2 at 0 °C. After additional stirring of the mixture at 0 °C for 15 min and at r.t. for 20 h, $CHCl_3$ (10 mL) and H_2O (10 mL) were added. The organic layer was separated, washed sequentially with 1 N aq HCl, H_2O, 5% $NaHCO_3$, and brine (10 mL each), and then dried (Na_2SO_4). After filtration of the mixture, the solvent was removed by rotary evaporation; this gave a crude mixture of diastereomers **10a**,**b**, which was used as such for the next step.

(–)-*N*-{[(3*R*)-5-Chloro-8-hydroxy-3-methyl-1-oxoisochroman-7-yl]carbonyl}-L-d_5-phenylalanine (d_5-Ochratoxin A, d_5-OTA, 2a) and (+)-*N*-{[(3*S*)-5-Chloro-8-hydroxy-3-methyl-1-oxoisochroman-7-yl]carbonyl}-L-d_5-phenylalanine (2b)

The crude mixture of diastereomers **10a**,**b**, obtained as described above, was added to a soln of TFA (12.3 g, 108 mmol) in anhyd CH_2Cl_2 (20 mL) at r.t. under N_2. The reaction mixture was stirred at r.t. and monitored by TLC. When the reaction was completed (ca. 6 h), the solvent and the excess TFA were removed by rotary evaporation. The resulting residual oil was dissolved in CH_2Cl_2, washed with H_2O (3 × 10 mL), and dried (Na_2SO_4). After filtration and removal of the solvent by rotary evaporation, a colorless solid was ob-

tained, which was crystallized from benzene; this gave a mixture of **2a** and **2b**.

Yield: 350 mg (82% from **6**); colorless solid; mp 93–95 °C.

Separation of 2a and 2b

A mixture of the two diastereomers **2a** and **2b** (100 mg) dissolved in $CHCl_3$ was then subjected to preparative TLC on plates coated with silica gel (20 plates, 20 × 20 cm, 0.25 mm thickness, benzene–acetone–HCO_2H, 79:20:1); this gave pure **2a** (R_f = 0.47) and pure **2b** (R_f = 0.43).

2a

Yield: 47 mg (47%); colorless solid; mp 112–113 °C; $[\alpha]_D^{25}$ –32.7 (*c* 5 mg/mL, $CHCl_3$).

IR (KBr): 3020 (m), 2985 (br m), 2932 (m), 1728 (s), 1677 (s), 1611 (w), 1531 (s), 1426 (m), 1219 (m), 1138 (w), 810 (w), 770 (w) cm^{-1}.

^1H NMR (300 MHz, $CDCl_3$): δ = 12.72 (br s, 1 H, OH), 8.50 (br d, *J* = 7.2 Hz, 1 H, NH), 8.41 (s, 1 H, H-6), 7.73 (br s, 1 H, OH), 5.10–5.01 (m, 1 H, C*H*NH), 4.82–4.67 (m, 1 H, C*H*CH_3), 3.40–3.16 (m, 3 H, CH_2Ph, C*H*HCHCH_3), 2.84 (distorted dd, *J* = 17.6, 11.7 Hz, 1 H, CH*H*CHCH_3), 1.58 (d, *J* = 6.3 Hz, 3 H, Me).

^{13}C NMR (75 MHz, $CDCl_3$): δ = 174.8, 169.7, 163.2, 159.2, 140.9, 139.1, 135.8, 129.0 (t, *J* = 23.8 Hz), 128.2 (t, *J* = 23.8 Hz), 127.3–126.3 (m), 123.3, 120.6, 110.2, 75.9, 54.4, 37.5, 32.4, 20.7.

ESI-MS: *m*/*z* = 409 (100) $[M + H]^+$.

ESI-HRMS: *m*/*z* $[M + H]^+$ calcd for $C_{20}H_{14}D_5ClNO_6$: 409.1210; found: 409.1199.

MS/MS $[M + H]^+$ (ESI+, 20 eV): *m*/*z* = 409.11 (43.03) $[M + H]^+$, 391.10 (7.7) $[M – H_2O + H]^+$, 363.11 (86.8) $[M – H_2O – CO + H]^+$, 346.08 (17.6) $[M – H_2O – CO – NH_3 + H]^+$, 239.00 (100.0) $[M – C_9H_6D_5NO_2]^+$, 220.99 (8.7) $[M – C_9H_8D_5NO_3]^+$, 125.11 (6.6) $[C_8H_5D_5N]^+$.

2b

Yield: 36 mg (36%); colorless solid; mp 183–185 °C; $[\alpha]_D^{25}$ +60.2 (*c* 3 mg/mL, $CHCl_3$).

IR (KBr): 3072 (w), 2927 (br m), 2855 (w), 1740 (s), 1672 (s), 1626 (m), 1544 (s), 1427 (m), 1380 (w), 1214 (m), 1139 (w), 810 (w), 769 (w) cm^{-1}.

^1H NMR (300 MHz, $CDCl_3$): δ = 12.70 (br s, 1 H, OH), 8.49 (br d, *J* = 7.2 Hz, 1 H, NH), 8.40 (s, 1 H, H-6), 7.16 (br s, 1 H, OH), 5.05 (distorted dd, *J* = 12.2, 5.9 Hz, 1 H, C*H*NH), 4.80–4.67 (m, 1 H, C*H*CH_3), 3.39–3.16 (m, 3 H, CH_2Ph, C*H*HCHCH_3), 2.89–2.76 (m, 1 H, CH*H*CHCH_3), 1.58 (d, *J* = 6.3 Hz, 3 H, Me).

^{13}C NMR (75 MHz, $CDCl_3$): δ = 174.8, 169.7, 163.1, 159.2, 140.9, 139.1, 135.8, 129.0 (t, *J* = 23.8 Hz), 128.2 (t, *J* = 23.8 Hz), 127.2–126.3 (m), 123.3, 120.6, 110.2, 75.9, 54.5, 37.5, 32.4, 20.7.

ESI-MS: *m*/*z* = 409 (100) $[M + H]^+$.

ESI-HRMS: *m*/*z* $[M + H]^+$ calcd for $C_{20}H_{14}D_5ClNO_6$: 409.1210; found: 409.1192.

MS/MS $[M + H]^+$ (ESI+, 20 eV): *m*/*z* = 409.12 (41.20) $[M + H]^+$, 391.11 (7.4) $[M – H_2O + H]^+$, 363.11 (84.5) $[M – H_2O – CO + H]^+$, 346.08 (18.7) $[M – H_2O – CO – NH_3 + H]^+$, 239.01 (100.0) $[M – C_9H_6D_5NO_2]^+$, 221.00 (9.2) $[M – C_9H_8D_5NO_3]^+$, 125.11 (8.0) $[C_8H_5D_5N]^+$.

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(13) The only method reported in the literature so far for the synthesis of d_5-OTA (**2a**) was based on a semi-synthetic sequence involving: (a) acid hydrolysis of OTA to give (*R*)-OTα; (b) conversion of (*R*)-OTα into the corresponding acyl chloride by reaction with $SOCl_2$; (c) reaction between the acyl chloride and L-d_5-phenylalanine methyl ester to give methyl-protected d_5-OTA; (d) deprotection of the latter with NaOH in MeOH to give d_5-OTA (**2a**). The overall yield based on starting OTA was 5% over 4 steps. See ref. 5a.