New Pulvinic Acid and Phenylalaninol Derivatives from the Mushrooms *Retiboletus griseus* and *R. nigerrimus*

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Dedicated to Professor Heinrich Nöth on the occasion of his 85th birthday

The close genetic relationship of the mushrooms *Retiboletus griseus* and *R. nigerrimus* is supported by the occurrence of the orange-yellow retiboletic acid, resp. its methyl ester, in both species. The structures of these highly substituted pulvinic acid derivatives have been elucidated by spectroscopic investigations. *R. nigerrimus* also contains two *N*-protocatechuyl-L-phenylalaninol derivatives, nigerrimin A and B, the first reported occurrence of such compounds in nature. Their configuration was established by the synthesis of nigerrimin B.

Key words: Pulvinic Acids, *N*-(Protocatechuyl)phenylalaninols, Fungi, Taxonomy, Synthesis

Introduction

Based on DNA sequence data and morphological criteria, Binder and Bresinsky [1] proposed the new bolete genus *Retiboletus*, which includes *R. retipes*, *R. ornatipes*, *R. flavoniger*, *R. griseus*, and *R. nigerrimus*. Whereas the first three species are characterized by the occurrence of retipolides [2], the metabolites of *R. griseus* and *R. nigerrimus* remained unknown. We have now carried out a chemotaxonomic survey of the latter two species, which resulted in the detection of a new pulvinic acid in these fungi, accompanied by two phenylalaninol derivatives in *R. nigerrimus*.

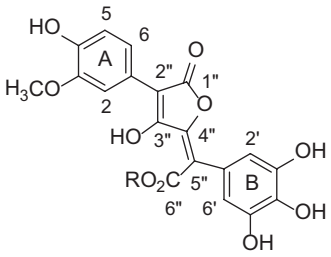
Results and Discussion

*Isolation and structure elucidation of retiboletic acid (**1**) and methyl retiboletate (**2**)*

Retiboletus griseus (Frost) Manfr. Binder & Bresinsky is a fairly common mushroom of oak-hickory forests in eastern North America. It is easily recognized by its grey appearance and the reticulate stem with a yellowish to brown pattern that darkens when the fruit body is handled. In an early investigation of this species, none of the known pulvinic acids could be detected [3]. We now report on the isolation and structural elucidation of a penta oxygen-substituted pulvinic acid, named retiboletic acid (**1**), from the acetone extract of dried fruit bodies. The acid is accompanied by its methyl ester **2**.

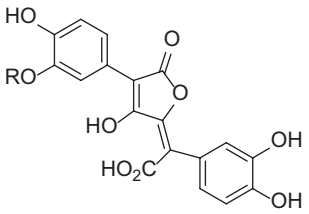
The air-dried mushrooms were powdered and extracted with acetone-water. The crude extract was then distributed between dilute HCl and EtOAc, and the dried and concentrated organic phase was investigated by reversed-phase HPLC. This revealed the presence of two orange-yellow pigments, which could be obtained analytically pure by preparative HPLC. Their absorption spectra strongly resembled those of hydroxypulvinic acids, the lead pigments of *Boletus* species [4]. On TLC comparison, the more polar pigment exhibited nearly the same R_f values as variegatic acid (**3**); however, on oxidation with aqueous $NaHCO_3/K_3[(CN)_6]$, no blue color developed. This excluded a catechol moiety as ring A [5].

The (–)-ESI mass spectrum of retiboletic acid showed a molecular ion at $m/z = 401$ $[M–H]^-$ corresponding to the formula $C_{19}H_{13}O_{10}$. Thus, the new



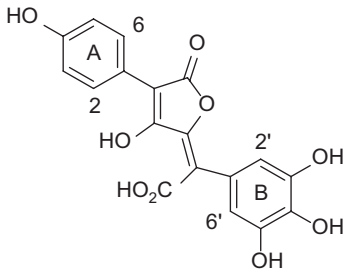
1, R = H, retiboletic acid

2, R = CH_3, methyl retiboletate

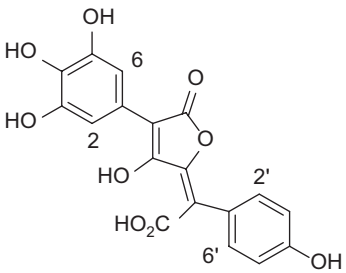


3, R = H, variegatic acid

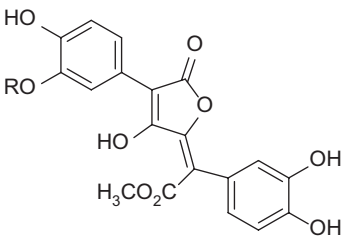
4, R = CH_3, 3-*O*-methylvariegatic acid



5, isogomphidic acid



6, gomphidic acid



7, R = H

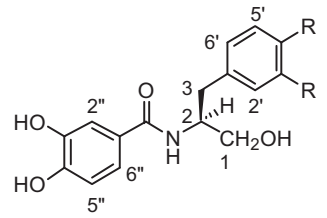
8, R = CH_3

compound contained one oxygen atom more than variegatic acid (**3**) and, in addition, a phenolic *O*-methyl group, which appeared as a 3H singlet in the ^{1}H NMR spectrum (δ_H = 3.84). Four signals in the aromatic proton region were visible, which could be assigned to a 4-hydroxy-3-methoxyphenyl group [ABX system: δ_H = 6.80 (d, J = 8.3 Hz, 5-H), 7.79 (dd, J = 8.3, 1.9 Hz, 6-H), 8.00 (d, J = 1.9 Hz, 2-H)] and a 3,4,5-trihydroxyphenyl residue: δ_H = 6.38 (s, 2′/6′-H). The position of the *O*-methyl group followed from its correlation with 2-H in a NOESY experiment and an HMBC correlation with C-3 (δ_C = 147.2).

Due to the strong deshielding effect of the coplanar hydroxybutenolide ring, protons 2 and 6 at ring A in pulvinic acids generally appear at distinctly lower field than their counterparts 2′-H and 6′-H in ring B, which is twisted by steric interactions with the neighboring carboxyl group [6]. This effect allowed the allocation of the two phenyl rings in retiboletic acid as given in structure **1**. Comparison with the corresponding chemical shifts for 3-*O*-methylvariegatic acid (**4**) (2-H: δ_H = 7.84, 6-H: 7.72) [7] and isogomphidic acid (**5**) (2′/6′-H: δ_H = 6.41) [8, 9] showed close agreement, whereas the corresponding signal for gomphidic acid (**6**) occurs at δ_H = 7.35 [6, 8]. Structure **1** was confirmed by the ^{13}C NMR spectrum and HMBC experiments.

The second pigment closely resembled retiboletic acid (**1**) in its ^{1}H and ^{13}C NMR data, with the exception of signals for an additional *O*-methyl group at δ_H = 3.90 (δ_C = 54.1) which exhibited an HMBC correlation to the carbonyl group at δ_C = 172.3. Acetylation (Ac_2O/cat. DMAP) yielded a tetra acetate; thus, the second pigment is methyl retiboletate (**2**), which is supported by the molecular ion at m/z = 415 [M–H]$^-$ observed in the (–)-ESI mass spectrum. Since work-up and purification were carried out in the absence of methanol, the pigment must be a genuine metabolite.

Retiboletus nigerrimus (R. Heim) M. Binder & Bresinsky [= *Tylopilus nigerrimus* (R. Heim) Hongo & M. Endo] is a blackish bolete with a purple tint and reticulated stipe, which occurs in evergreen, broad-leaved forests in Papua New Guinea, Malaysia, Thailand, and East Asia. Extracts of dried specimens from Japan contained methyl retiboletate (**2**) in admixture with methyl variegatate (**7**) [7, 10] and methyl 3-*O*-



9, R = H, nigerrimin A
10, R = OH, nigerrimin B

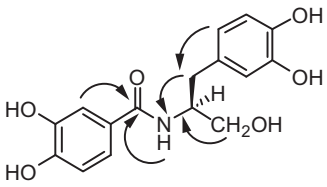


Fig. 1. Selected HMBC correlations of **10**.

methylvariegatate (**8**), a known metabolite from *Hygrophoropsis aurantiaca* [7].

The isolation of the highly substituted retiboletic acid (**1**) and its methyl ester (**2**) from *Retiboletus griseus* and ester **2** from *R. nigerrimus* strongly supports their transfer from *Tylopilus* to another genus within the Boletales. The supposed position within *Retiboletus* on behalf of molecular data [1] remains to be proved. During our investigations of these two species, we were unable to detect any retipolides, the characteristic metabolites of *R. retipes*/*ornatipes* and *R. flavoniger* [2].

Isolation and structure elucidation of nigerrimins A and B

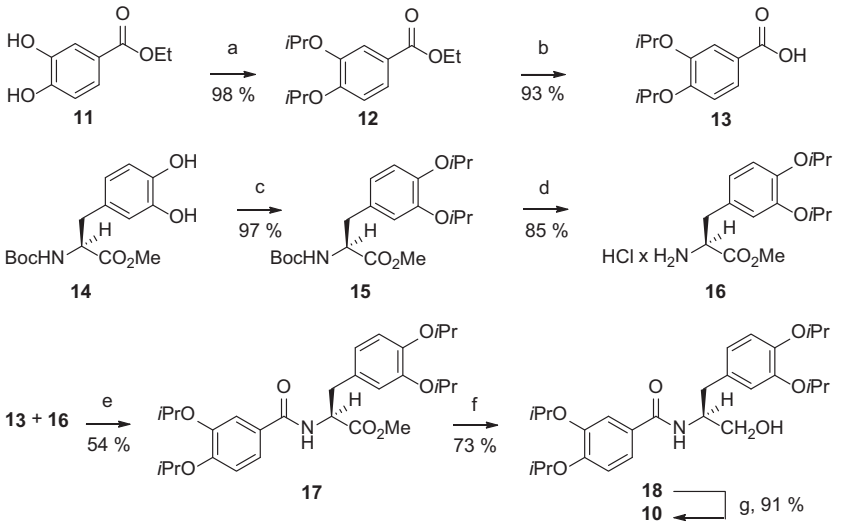
In addition to the pulvinic acids, two colorless metabolites, nigerrimins A and B, were isolated from *R. nigerrimus*. Nigerrimin A exhibited a molecular peak at $m/z = 287$ in the EI mass spectrum, corresponding to the formula $C_{16}H_{17}NO_4$, whereas nigerrimin B ($C_{16}H_{17}NO_6$) contained two additional oxygen atoms. Both compounds showed the base peak at $m/z = 137$ ($C_7H_5O_3$) and an ion at $m/z = 109$ ($C_6H_5O_2$), indicating the presence of a dihydroxybenzoyl residue. In the ^{1}H NMR spectrum of nigerrimin B, signals for the 3,4-dihydroxybenzoyl and an additional 3,4-dihydroxyphenyl residue were visible. The carbonyl group of the former ($\delta_C = 166.0$) is part of an amide moiety ($\delta_{NH} = 7.67$, d, $J = 8.2$ Hz), which is connected to a methine proton ($\delta_H = 3.98$, m). ^{1}H,^{1}H-COSY experiments revealed the coupling of this proton to a neighboring CH_2OH residue ($\delta_H = 3.32/3.40$, each dd, $J = 10.8/6.1$ and $10.8/5.4$ Hz, $\delta_C = 62.9$) and, in addition, a second methylene group ($\delta_H = 2.57/2.69$, each dd, $J = 13.9/8.2$ and $13.9/5.9$ Hz, $\delta_C = 36.1$) which connects to the 3,4-dihydroxyphenyl ring. From this evidence, structure **10** could be proposed for nigerrimin B. It was supported by the ^{13}C NMR data and HMBC experiments (Fig. 1).

In the case of nigerrimin A, the NMR data indicated the same basic structure, however, the signals of the dihydroxyphenyl group were replaced by those of a simple phenyl residue. Nigerrimin A is therefore *N*-(protocatechuyl)phenylalaninol (**9**). In order to establish the absolute configuration of the nigerrimins, the synthesis of L-nigerrimin B was undertaken.

Synthesis of nigerrimin B

The advanced synthesis commenced from 3,4-diisopropoxybenzoic acid (**13**) and *O*,*O*-diisopropyl-protected L-DOPA methyl ester (**16**), which were prepared in high yield from ethyl 3,4-dihydroxybenzoate (**11**) and *N*-Boc-L-DOPA methyl ester (**14**), respectively (Scheme 1). Coupling of compounds **13** and **16** using TBTU [11] yielded amide **17**, which was reduced with $NaBH_4$ in THF-MeOH [12, 13] to the corresponding alcohol **18**. Treatment of the latter with $AlCl_3$ afforded tetraphenol **10**, in every respect identical with L-nigerrimin B. The synthetic compound showed a similar negative optical rotation ($[\alpha]_D^{25} = -53.6$) as the natural product ($[\alpha]_D^{25} = -52.2$), and there was close agreement of their CD spectra.

The nigerrimins **9** and **10** are new compounds. In contrast to L-phenylalaninol, there are no reports that 3-(3,4-dihydroxyphenyl)alaninol and its derivatives have been detected in nature before. Simple *N*-benzoyl-L-phenylalaninol has been isolated from cultures of *Aspergillus flavipes* [14] and *Penicillium brevicompactum* [15], as well as from higher plants. Since aqueous solutions of the nigerrimins readily darken on exposure to air, the blackish appearance of *R. nigerrimus* may be caused by the presence of these compounds or of L-DOPA in the fruit bodies. The biological activity of the polyphenolic compounds **9** and **10** is unknown and remains to be investigated. Interestingly, phenylalanine and 3,4-dihydroxybenzoic



Scheme 1. Synthesis of L-nigerrimin B (**10**). Reagents and conditions: a) *i*PrBr, K_2CO_3, DMF, 60 °C, 3 h; then 20 °C, 12 h; b) NaOH, EtOH-H_2O, 95 °C, 2 h; c) *i*PrBr , K_2CO_3,DMF, 50 °C, 12 h; d) 2 N HCl in EtOAc, 20 °C, 16 h; e) TBTU, NEt_3, MeCN, 20 °C, 36 h; f) $NaBH_4$, THF, MeOH, 20 °C, 48 h; g) $AlCl_3$, CH_2Cl_2, 20 °C, 48 h.

acid could be detected by GC-MS after trimethylsilylation of the mushroom extract with *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA). This is in accord with the known biosynthesis of L-phenylalaninol from L-phenylalanine in cultures of *Penicillium brevicompactum* [15].

In summary, our investigation of *Retiboletus griseus* and *R. nigerrimus* has added four new members to the list of unique metabolites from this genus [2], which underlines the special taxonomic position of *Retiboletus* in Boletaceae [1].

Experimental Section

General

Melting points (uncorrected): Reichert Thermovar hot-stage apparatus. Optical rotations: Perkin-Elmer 241 polarimeter. IR: Perkin-Elmer 1420 ratio recording infrared spectrometer or Bruker FTIR IFS 45 spectrophotometer. Intensity of the bands: ss (very strong), s (strong), m (medium), w (weak), sh (shoulder). UV/Vis spectra: Hewlett-Packard 8452A diode array spectrophotometer. CD spectra: Jobin Yvon CD-6-Dichrograph. NMR: Bruker AMX 600, ARX 300, and AC 200 instruments; spectra were recorded in $CDCl_3$, [D_6]acetone, or [D_6]DMSO, with the solvent peak as internal standard. MS: Finnigan MAT 90 and 95 Q spectrometers (direct inlet, 70 eV). All solvents were distilled before use. Evaporation of the solvents was performed under reduced pressure using a rotary evaporator. Analytical TLC: silica gel 60 F_{254} aluminum foils (Merck); solvent system A (v/v): toluene-HCO_2Et-HCO_2H (10 : 5 : 3); B: toluene-HCO_2Et-HCO_2H (10 : 10 : 3); C: hexanes-EtOAc (1 : 2). Column chromatography: silica gel 60, 40 – 63 μm (Merck), Sephadex LH-20 (Pharmacia). Analytical and semipreparative HPLC: Waters 510 pump and autosampler 717+ with photodiode array detector 996, column heating compartment Techlab K5 and Millenium chromatography software; Waters 600 E pump and system controller with photodiode array detector 990+. Preparative HPLC separations: Waters-Millipore with gradient controller M 680, two M 590 EF pumps, and U 6 K injector equipped with a Knauer variable-wavelength monitor with a super-preparative flow cell; prefiltration of the solutions through Sep-Pak RP-18 cartridges (Waters). Nucleosil 100 C18 prepacked HPLC columns (Macherey-Nagel) and gradient systems with MeCN-H_2O mixtures with up to 0.5 % TFA were used. TBTU [*O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate] was purchased from Sigma-Aldrich.

Mushrooms

R. griseus was collected in September 1998 in the Lincoln Town Forrest, Lincoln/Maynard, and in the Breakhart Reservation, Saugus/Wakefield, both MA (USA) (*leg. et det.* N. Arnold); *R. nigerrimus* was collected in July and September 1996 near Takarazuka/Nakayama, Hyogo (Japan) (*leg. et det.* R. Marumoto).

*Isolation of retiboletic acid (**1**) and its methyl ester **2** from R. griseus*

Air-dried, pulverized fruit bodies of *R. griseus* (20.9 g) were extracted with acetone-water (20 : 1, v/v, 4 × 900 mL). The combined extracts were concentrated at 40 °C under reduced pressure, and the brown residue (1.35 g) was dissolved in water with the addition of a few drops of concd. HCl. After extraction of the aqueous solution with EtOAc (4 × 200 mL), the combined extracts were dried (Na_2SO_4) and concentrated. The yellow-brown residue (0.45 g) was dissolved in a small amount of MeCN-water (1 : 1, v/v), the solution was filtered through an RP-18 cartridge, and the pigments were separated by preparative HPLC [Nucleosil 100 C18, 7 μm, 250 × 16 mm; solvent A: H_2O-MeCN (9 : 1) + 0.5% TFA; solvent B: MeCN; gradient: start 90% A + 10% B, 40 min: 50% A + 50% B, 45 min: 100% B, flow rate: 6.8 mL min^{-1}, detection at 265 nm). Retention times: t_R (**1**) = 15.3, t_R (**2**) = 33.4 min. The analytically pure compounds were obtained by semipreparative HPLC [Nucleosil 100 C18, 5 μm, 250 × 10 mm; solvent A: H_2O-MeCN (9 : 1) + 0.5% TFA; solvent B: MeCN; gradient: start 90% A + 10% B, 40 min: 60% A + 40% B, 45 min: 100% B, flow rate: 3.0 mL min^{-1}, detection at 265 nm]. Retention times: t_R (**1**) = 25.4, t_R (**2**) = 24.6 min.

*Retiboletic acid, 3′,4,4′,5′-tetrahydroxy-3-methoxypulvinic acid (**1**)*

3.6 mg (0.017% of dry weight), yellow-brown solid, m. p. 105 – 110 °C. – R_f (TLC) = 0.23 (solvent system A), orange spot, + $K_3Fe[(CN)_6]$ red-brown. – t_R (analytical HPLC) = 21.1 min (Nucleosil 100 C18, 5 μm, 250 × 4 mm; solvent A: H_2O-MeCN (9 : 1) + 0.5% TFA; solvent B: MeCN; gradient: start 100% A, 10 min: 100% A, 50 min: 100% B, 60 min: 100% B, flow rate: 1.0 mL min^{-1}, detection at 265 nm). – UV/Vis (MeOH): λ_{max} (log ε) = 212 (3.97), 263 (3.73), 340 (3.28, sh), 394 (3.37) nm. – ^{1}H NMR (600 MHz, [D$_6$]acetone): δ = 3.84 (s, 3 H), 6.38 (s, 2 H, 2′/6′-H), 6.80 (d, J = 8.3 Hz, 1 H, 5-H), 7.79 (dd, J = 8.3, 1.9 Hz, 1 H, 6-H), 8.00 (d, J = 1.9 Hz, 1 H, 2-H). – ^{13}C NMR (150 MHz, [D$_6$]acetone): δ = 55.7 (CH_3), 97.8 (C-2″), 110.3 (C-2/2′/6′), 114.7 (C-5), 118.3 (C-5″), 119.9 (C-6), 125.0 (C-1), 127.0 (C-1′), 132.5 (C-4′), 144.8 (C-3′/5′), 145.0 (C-4), 147.2 (C-3), 153.3 (C-4″), 167.8 (C-3″), 168.9 (C-1″), 170.0 (C-6″). – MS ((–)-ESI): m/z (%) = 401 (33) [M–H]$^-$. – HRMS ((–)-ESI): m/z = 401.0514 (calcd. 401.0509 for $C_{19}H_{13}O_{10}$, [M–H]$^-$).

*Methyl retiboletate (**2**)*

1.3 mg (0.006% of dry weight), orange solid, m. p. 140 °C (decomp.). – R_f (TLC) = 0.24 (solvent system A), orange spot, + $K_3Fe[(CN)_6]$ red-brown. – t_R (analytical HPLC, same conditions as for **1**) = 26.5 min. – UV/Vis (MeOH): λ_{max} (log ε) = 212 (4.24), 258 (4.02), 337 (3.93), 385 (3.74, sh) nm. – ^{1}H NMR (600 MHz, [D$_6$]acetone): δ = 3.88 (s, 3 H, 3-OMe), 3.90 (s, 3 H, CO_2CH_3), 6.45 (s, 2 H, 2′/6′-H), 6.91 (d, J = 8.3 Hz, 1 H, 5-H), 7.65 (dd, J = 8.3, 1.9 Hz, 1 H, 6-H), 7.76 (d, J = 1.9 Hz, 1 H, 2-H). – ^{13}C NMR (150 MHz, [D$_6$]acetone): δ = 54.1 (CO_2CH_3), 55.7 (3-OCH_3), 104.7 (C-2″), 109.9 (C-2′/6′), 111.4 (C-2), 115.3 (C-5), 116.4 (C-5″), 121.5 (C-6), 121.8 (C-1), 123.7 (C-1′), 133.7 (C-4′), 145.5 (C-3′/5′), 147.3 (C-4), 147.6 (C-3), 153.5 (C-4″), 159.1 (C-3″), 166.4 (C-1″), 172.3 (C-6″). – MS ((–)-ESI): m/z (%) = 415 (49) [M–H]$^-$. – HRMS ((–)-ESI): m/z = 415.0633 (calcd. 415.0665 for $C_{20}H_{15}O_{10}$, [M–H]$^-$).

The tetra acetate was formed by stirring ester **2** (0.8 mg) and a catalytic amount of DMAP in Ac_2O (1.5 mL) overnight at r. t. After addition of a few drops of water, the mixture was extracted with Et_2O, the solvent was removed under reduced pressure, and the tetra acetate was dried *in vacuo*. MS ((–)-ESI): m/z (%) = 583 (79) [M–H]$^-$, 499 (100). – HRMS ((–)-ESI): m/z = 583.1078 (calcd. 583.1088 for $C_{28}H_{23}O_{14}$, [M–H]$^-$).

Isolation of the pulvinic acid derivatives and nigerrimins from R. nigerrimus

Freeze-dried, pulverized fruit bodies of *R. nigerrimus* (68 g) were defatted with petroleum ether (40–60 °C) and extracted with acetone (2 × 600 mL) under an argon atmosphere. Concentration of the extracts yielded 2.49 g of an orange-brown residue. The mushrooms were then extracted with MeOH (3 × 600 mL), and the combined extracts were concentrated to yield an orange-brown residue (4.91 g), which was equilibrated between EtOAc and acidified water (HCl, pH = 5). The aqueous phase was extracted with EtOAc (5 ×); the dark-brown color of the aqueous phase remained. The organic phases were combined, dried (Na_2SO_4), and concentrated to yield an orange-brown solid (1.55 g), which was indistinguishable from the acetone extract by TLC (solvent system A). The crude extracts were combined, dissolved in a small amount of MeOH, and chromatographed on Sephadex LH-20 with acetone-MeOH (4 : 1). The first fraction contained blue fluorescent compounds, which slowly turned brown on standing on the column, and the second fraction contained orange pigments. The last, dark brown, fraction contained only decomposition products formed during the chromatography. From the first two fractions, the nigerrimins **9** and **10**, methyl 3-*O*-methylvariegatate (**8**), and a 1 : 1-mixture of methyl retiboletate (**2**) and methyl variegatate (**7**) could be isolated in pure form by preparative HPLC (Nucleosil 100 C18, 7 μm, 250 × 16 mm; solvent A: H_2O-MeCN (9 : 1) + 0.1% TFA; solvent B: MeCN + 0.1% TFA; gradient: start 100% A, 3 min: 100% A, 57 min: 100% B, flow rate: 7 mL min^{-1}). Retention times: t_R (**10**) = 17.3, t_R (**9**) = 27.3, t_R (**2**, **7**) = 35.2, t_R (**8**) = 39.1 min.

*Nigerrimin A, (S)-2-(3,4-dihydroxybenzoylamino)-3-phenylpropanol (**9**)*

133 mg (0.2 % of dry weight), colorless solid, m. p. 132 °C (decomp.). – $[\alpha]_D^{25} = -10.4$ ($c = 7.6$, MeOH). – R_f (TLC) = 0.40 (solvent system B), spot turns brown on heating. t_R (analytical HPLC) = 18.8 min (Nucleosil 100 C18, 5 μm, 250 × 4 mm; solvent A: H_2O-MeCN (9 : 1) + 0.1 % TFA; solvent B: MeCN + 0.1 % TFA; gradient: start 100 % A, 60 min: 100 % B, 65 min: 100 % B, flow rate: 1 mL min^{-1}). – UV (MeOH): λ_{max} ($\log\varepsilon$) = 205 (2.94, sh), 256 (2.43), 286 (2.28) nm. – CD (MeOH): λ_{max} ($\Delta\varepsilon$) = 254 (–1.89), 264 (–2.16), 277 (–1.62), 291 (–2.41) nm. – IR (KBr): ν = 3400 (ss, br), 2957 (m), 2930 (m), 1710 (m, sh), 1636 (ss), 1601 (ss), 1546 (s), 1514 (ss), 1453 (s), 1440 (s), 1383 (s), 1360 (s), 1290 (ss), 1251 (s), 1201 (s), 1138 (s), 1114 (s), 1084 (s, sh), 1034 (s), 958 (m), 882 (m, sh), 822 (w), 784 (m), 760 (m), 722 (m), 702 (m) cm^{-1}. – ^{1}H NMR (600 MHz, [D$_6$]DMSO): δ = 2.75 (dd, J = 13.4, 9.0 Hz, 1 H, 3-H$_A$), 2.91 (dd, J = 13.4, 4.9 Hz, 1 H, 3-H$_B$), 3.35 (dd, J = 10.3, 6.8 Hz, 1 H, 1-H$_A$), 3.44 (dd, J = 10.3, 4.9 Hz, 1 H, 1-H$_B$), 4.08 (m, 1 H, 2-H), 6.71 (d, J = 8.0 Hz, 1 H, 5″-H), 7.12 – 7.23 (m, 7 H), 7.79 (d, J = 8.0 Hz, 1 H, NH), 9.04 (br. s, 1 H, OH), 9.39 (br. s, 1 H, OH). – ^{13}C NMR (150 MHz, [D$_6$]DMSO): δ = 36.6 (C-3), 53.2 (C-2), 63.1 (C-1), 114.8 (C-5″), 115.3 (C-2″), 119.1 (C-6″), 125.9 (C-4′), 126.2 (C-1″), 128.2[§] (C-2′/6′), 129.2[§] (C-3′/5′), 139.8 (C-1′), 144.8 (C-3″), 148.3 (C-4″), 166.0 (C=O) ([§] assignments are interchangeable). – MS (EI): m/z (%) = 287 (3) $[M]^+$, 256 (4, $C_{15}H_{14}NO_3$), 210 (1), 196 (54, $C_9H_{10}NO_4$), 178 (19, $C_9H_8NO_3$), 154 (5, $C_7H_8NO_3$), 137 (100, $C_7H_5O_3$), 120 (11, $C_8H_{10}N$), 109 (8), 91 (8), 77 (1), 44 (2). – MS (ESI): m/z (%) = 597 (6) $[2\,M+Na]^+$, 310 (100) $[M+Na]^+$. – HRMS (EI): m/z = 287.1157 (calcd. 287.1157 for $C_{16}H_{17}NO_4$, $[M]^+$).

*Nigerrimin B, (S)-2-(3,4-dihydroxybenzoylamino)-3-(3,4-dihydroxyphenyl)propanol (**10**)*

106 mg (0.16 % of dry weight), colorless solid, m. p. 239 °C (decomp.). – $[\alpha]_D^{25} = -52.2$ (c = 10.6, MeOH). – R_f (TLC) = 0.20 (solvent system A), 0.16 (solvent system B), spot turns brown on air exposure. – t_R (analytical HPLC) = 10.3 min (same conditions as for **9**). – UV (MeOH): λ_{max} ($\log\varepsilon$) = 206 (3.15, sh), 256 (2.51), 285 (2.42) nm. – CD (MeOH): λ_{max} ($\Delta\varepsilon$) = 237 (–1.45), 257 (–1.93), 277 (–1.36), 289 (–1.61) nm. – IR (KBr): ν = 3414 (ss, br), 2957 (m), 2928 (m), 1735 (m), 1635 (s, sh), 1603 (s), 1549 (m), 1514 (s), 1443 (m), 1361 (m), 1289 (s), 1253 (s), 1198 (m), 1116 (m), 1085 (w), 1048 (w), 961 (w), 881 (w), 823 (w), 786 (w), 760 (w) cm^{-1}. – ^{1}H NMR (600 MHz, [D$_6$]DMSO): δ = 2.57 (dd, J = 13.9, 8.2 Hz, 1 H, 3-H$_A$), 2.69 (dd, J = 13.9, 5.9 Hz, 1 H, 3-H$_B$), 3.32 (dd, J = 10.8, 6.1 Hz, 1 H, 1-H$_A$), 3.40 (dd, J = 10.8, 5.4 Hz, 1 H, 1-H$_B$), 3.98 (m, 1 H, 2-H), 6.45 (dd, J = 8.0, 1.9 Hz, 1 H, 6′-H), 6.57 (d, J = 8.0 Hz, 1 H, 5′-H), 6.61 (d, J = 1.9 Hz, 1 H, 2′-H), 6.71 (d, J = 8.2 Hz, 1 H, 5″-H), 7.14 (dd, J = 8.2, 2.2 Hz, 1 H, 6″-H), 7.22 (d, J = 2.2 Hz, 1 H, 2″-H), 7.67 (d, J = 8.2 Hz, 1 H, NH). – ^{13}C NMR (150 MHz, [D$_6$]DMSO): δ = 36.1 (C-3), 53.5 (C-2), 62.9 (C-1), 114.8 (C-5″), 115.3 (C-2″), 115.4 (C-5′), 116.6 (C-2′), 119.1 (C-6″), 119.9 (C-6′), 126.3 (C-1″), 130.5 (C-1′), 143.4 (C-4′), 144.9[§] (C-3′), 145.0[§] (C-3″), 148.3 (C-4″), 166.0 (C=O) ([§] assignments are interchangeable). – MS (EI): m/z (%) = 319 (6) $[M]^+$, 196 (8) $C_9H_{10}NO_4$, 178 (11) $C_9H_8NO_3$, 166 (17) $C_9H_{10}O_3$, 154 (26) $C_7H_8NO_3$, 137 (100) $C_7H_5O_3$, 123 (16) $C_7H_7O_2$, 109 (10) $C_6H_5O_2$, 81 (11), 77 (6), 44 (4). – HRMS (EI): m/z = 319.1081 (calcd. 319.1056 for $C_{16}H_{17}NO_6$, $[M]^+$).

*Ethyl 3,4-diisopropoxybenzoate (**12**)*

A stirred mixture of ethyl 3,4-dihydroxybenzoate (**11**; 1 g, 5.5 mmol), anhydrous K_2CO_3 (4.94 g, 35.8 mmol), and isopropyl bromide (3.1 mL, 33.0 mmol) in DMF (30 mL) was heated for 3 h at 60 °C under an argon atmosphere, and then stirred for 12 h at 20 °C. As some starting material was still present [determined by TLC comparison (solvent system A, detection with $FeCl_3$)], the mixture was again treated with isopropyl bromide (1.55 mL, 16.5 mmol) and heated for 4 h at 60 °C. After cooling to r. t., water was added, and the mixture was acidified to pH = 4 with conc. HCl. Extraction with EtOAc (3 ×), drying (Na_2SO_4), and concentration to dryness under reduced pressure yielded **12** as a colorless oil (1.43 g, 98 %). – R_f (TLC) = 0.77 (solvent system A). – ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.13 (d, J = 6.2 Hz, 12 H), 1.17 (t, J = 7.2 Hz, 3 H), 4.14 (q, J = 7.2 Hz, 2 H), 4.33 (m, 2 H), 6.69 (d, J = 8.4 Hz, 1 H), 7.44 (d, J = 2.0 Hz, 1 H), 7.47 (dd, J = 8.4, 2.0 Hz, 1 H). – ^{13}C NMR (75 MHz, $CDCl_3$): δ = 13.8 (CH_3), 21.4 (2 × CH_3), 21.6 (2 × CH_3), 59.9 (CH_2), 70.8 (CH), 71.9 (CH), 114.7 (CH), 118.8 (CH), 122.7 (C), 123.6 (CH), 147.6 (C), 153.0 (C), 165.5 (C=O). – MS (EI): m/z (%) = 266 (39) $[M]^+$, 224 (13), 182 (100), 167 (9), 154 (67), 137 (94), 109 (7), 97 (2), 42 (11). – HRMS (EI): m/z = 266.1499 (calcd. 266.1518 for $C_{15}H_{22}O_4$, $[M]^+$).

*3,4-Diisopropoxybenzoic acid (**13**)*

Ester **12** (766 mg, 2.88 mmol) was heated with 10 % aqueous NaOH (3.8 mL) in 50 % aqueous EtOH (100 mL) for 2 h with stirring at 95 °C. After cooling to r. t., the solution was diluted with water (100 mL) and washed with $CHCl_3$ (100 mL). Then, the aqueous phase was acidified with conc. HCl to pH = 4 and extracted with EtOAc (3 × 100 mL). The combined organic phases were washed with water (150 mL), dried (Na_2SO_4), and concentrated under reduced pressure to yield **13** (637 mg, 93 %) as colorless solid, m. p. 108 °C. – R_f (TLC) = 0.63 (solvent system A). – ^{1}H NMR (200 MHz, $CDCl_3$): δ = 1.36 (d, J = 6.0 Hz, 6 H), 1.38 (d, J = 6.0 Hz,

6 H), 4.51 (m, 1 H), 4.62 (m, 1 H), 6.93 (d, $J = 8.4$ Hz, 1 H), 7.66 (d, $J = 2.0$ Hz, 1 H), 7.74 (dd, $J = 8.4$, 2.0 Hz, 1 H), 12.40 (br. s, 1 H, CO_2H). – MS (EI): m/z (%) = 238 (8) $[M]^+$, 196 (5), 154 (100), 137 (15), 109 (2), 97 (2), 83 (3), 43 (7). – HRMS (EI): $m/z = 238.1199$ (calcd. 238.1205 for $C_{13}H_{18}O_4$, $[M]^+$).

*Methyl (S)-2-(tert-butoxycarbonylamino)-3-(3,4-diisopropoxyphenyl)propanoate (**15**)*

To a stirred solution of *N*-Boc-L-DOPA methyl ester [16–18] (**14**; 2.70 g, 8.67 mmol) in DMF (70 mL) were added, under an argon atmosphere, anhydrous K_2CO_3 (7.79 g, 56.36 mmol) and isopropyl bromide (4.9 mL, 52.0 mmol). The suspension was warmed for 12 h at 50 °C, cooled, and filtered, and the filter was washed with EtOAc. The filtrate was distributed between EtOAc and water; the organic phase was washed with water (3 ×), dried (Na_2SO_4), and concentrated under reduced pressure to yield **15** (3.33 g, 97%) as a colorless oil. – R_f (TLC) = 0.71 (solvent system C). – ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 1.22$ (d, $J = 6.0$ Hz, 6 H), 1.23 (d, $J = 6.0$ Hz, 6 H), 1.32 (s, 9 H), 2.88 (dd, $J = 13.8$, 5.6 Hz, 1 H, 3-H_A), 2.93 (dd, $J = 13.8$, 5.3 Hz, 1 H, 3-H_B), 3.60 (s, 3 H, OCH_3), 4.34 (m, 2 H, 2 × *i*Pr-CH), 4.44 (m, 1 H, 2-H), 4.99 (d, $J = 7.9$ Hz, 1 H, NH), 6.56 (dd, $J = 8.0$, 1.9 Hz, 1 H, 6′-H), 6.60 (d, $J = 1.9$ Hz, 1 H, 2′-H), 6.73 (d, $J = 8.0$ Hz, 1 H, 5′-H). – ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 22.1$ [2 × $CH(CH_3)_2$], 28.1 [$C(CH_3)_3$], 37.5 (C-3), 52.0 (OCH_3), 54.3 (C-2), 72.0 [2 × $CH(CH_3)_2$], 79.6 [$C(CH_3)_3$], 118.0 (C-5′), 119.3 (C-2′), 122.4 (C-6′), 129.2 (C-1′), 148.1 (C-3′), 148.8 (C-4′), 154.9 (CONH), 172.3 (C=O). – EI-MS: m/z (%) = 395 (16) $[M]^+$, 353 (1), 322 (4), 295 (2), 280 (5), 236 (11), 207 (37), 194 (34), 165 (80), 151 (5), 123 (100), 88 (4), 77 (4), 57 (22), 41 (22). – HRMS (EI): $m/z = 395.2313$ (calcd. 395.2307 for $C_{21}H_{33}NO_6$).

*Methyl (S)-2-amino-3-(3,4-diisopropoxyphenyl)propanoate hydrochloride (**16**)*

Compound **15** (186 mg, 0.47 mmol) was treated with 2 N HCl in EtOAc (6 mL), and the solution was stirred for 16 h at 20 °C. Concentration of the solution under reduced pressure yielded **16** (132 mg, 85%) as a colorless solid. – ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 1.24$ (m, 12 H), 3.24 (m, 1 H), 3.33 (m, 1 H), 3.60 (s, 3 H), 4.37 (m, 3 H, 2-H, 2 × *i*Pr-CH), 6.80 (m, 3 H), 8.67 (br. s, 3 H, NH_3^+). – ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 22.1$ (2 × CH_3), 22.2 (2 × CH_3), 35.8 (C-3), 52.8 (OCH_3), 54.4 (C-2), 72.0 (CH), 72.2 (CH), 118.3 (C-5′), 119.5 (C-2′), 122.9 (C-6′), 127.3 (C-1′), 148.5[§] (C-3′), 149.0[§] (C-4′), 169.3 (C-1) ([§] assignments are interchangeable). – MS (EI): m/z (%) = 295 (8) $[M–HCl]^+$, 253 (2), 236 (6), 207 (34), 165 (50), 123 (100), 88 (4), 43 (3). – HRMS (EI): $m/z = 295.1775$ (calcd. 295.1783 for $C_{16}H_{25}NO_4$, $[M–HCl]^+$).

*Methyl (S)-2-(3,4-diisopropoxybenzoylamino)-3-(3,4-diisopropoxyphenyl)propanoate (**17**)*

A solution of acid **13** (50 mg, 0.21 mmol) in MeCN (4 mL) was stirred with hydrochloride **16** (62 mg, 0.19 mmol), triethylamine (0.12 mL, 0.84 mmol), and TBTU (67.4 mg, 0.21 mmol) for 36 h at 20 °C. Then, the reaction mixture was equilibrated between water (50 mL) and EtOAc (50 mL); the organic phase was extracted with water (2 × 50 mL) and then washed with saturated aqueous $KHSO_4$ (50 mL), aqueous $NaHCO_3$, and water. After drying (Na_2SO_4), the solvent was removed, and the residue was purified by column chromatography on silica gel (hexanes-EtOAc, 4 : 1) to yield **17** (58.0 mg, 54%) as a colorless oil. – R_f (TLC) = 0.77 (solvent system A). – UV (MeCN): λ_{max} ($\log \varepsilon$) = 204 (4.74), 257 (4.06), 285 (3.84) nm. – IR (film): $\nu = 3326$ (m, br), 2978 (ss), 2934 (s), 1746 (s), 1640 (s), 1602 (s), 1578 (s), 1537 (s), 1503 (ss), 1467 (s), 1445 (s), 1426 (m), 1384 (s), 1372 (s), 1334 (s), 1269 (ss), 1215 (ss), 1178 (s), 1136 (ss), 1109 (ss), 1026 (w), 985 (s), 951 (m), 885 (w), 852 (m), 752 (s) cm^{-1}. – ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 1.21–1.33$ (m, 24 H), 3.10 (dd, $J = 14.0$, 5.3 Hz, 1 H, 3-H_A), 3.16 (dd, $J = 14.0$, 5.7 Hz, 1 H, 3-H_B), 3.73 (s, 3 H, OCH_3), 4.37, 4.50 (each, m, 2 H, 2 × *i*Pr-CH), 4.98 (m, 1 H, 2-H), 6.44 (d, $J = 7.5$ Hz, 1 H, NH), 6.62 (dd, $J = 8.0$, 2.0 Hz, 1 H), 6.65 (d, $J = 2.0$ Hz, 1 H), 6.79 (d, $J = 8.0$ Hz, 1 H), 6.84 (d, $J = 8.4$ Hz, 1 H), 7.20 (dd, $J = 8.4$, 2.0 Hz, 1 H), 7.36 (d, $J = 2.0$ Hz, 1 H). – ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 22.1$, 22.2 (each 4 × CH_3), 37.3 (C-3), 52.3 (OCH_3), 53.5 (C-2), 71.9 (*i*Pr-CH), 72.1 (2 × *i*Pr-CH), 72.5 (*i*Pr-CH), 116.1 (C-5″), 117.1 (C-2″), 118.2 (C-5′), 119.4 (C-2′), 120.5 (C-6″), 122.5 (C-6′), 126.8 (C-1″), 129.2 (C-1′), 148.3, 148.7, 149.1 (each C), 152.4 (C-4″), 166.3 (CONH), 172.2 (C=O). – MS (EI): m/z (%) = 515 (16) $[M]^+$, 278 (100), 236 (39), 221 (14), 207 (11), 194 (66), 179 (14), 165 (24), 153 (10), 137 (34), 123 (31), 109 (3). – HRMS (EI): $m/z = 515.2875$ (calcd. 515.2869 for $C_{29}H_{41}NO_7$, $[M]^+$). – $C_{29}H_{41}NO_7$ (515.64): calcd. C 67.55, H 8.01, N 2.72; found C 67.26, H 7.89, N 2.71.

*(S)-2-(3,4-Diisopropoxybenzoylamino)-3-(3,4-diisopropoxyphenyl)propanol (**18**)*

To ester **17** (87 mg, 0.17 mmol) in anhydrous THF (1 mL) were added $NaBH_4$ (13 mg, 0.34 mmol) and MeOH (1 mL), and the mixture was stirred for 48 h at r. t. under an argon atmosphere. Then, the reaction mixture was cooled to 0 °C, acidified with 10% aqueous citric acid to pH = 4, and concentrated under reduced pressure. The residue was dissolved in water (20 mL) and washed with CH_2Cl_2 (3 × 25 mL). Then, the combined organic phase was dried (Na_2SO_4) and concentrated under reduced pressure to yield **18** (60 mg, 73%) as a colorless oil. – R_f (TLC) = 0.37 (solvent system

A). – UV (MeCN): λ_{max} ($\log\varepsilon$) = 208 (5.43), 259 (4.89), 287 (4.67) nm. – IR (film): ν = 3339 (m, br), 2976 (ss), 2930 (s), 1631 (m), 1602 (m), 1574 (m), 1538 (m), 1501 (ss), 1467 (m), 1424 (w), 1383 (m), 1268 (ss), 1221 (m), 1177 (m), 1108 (ss), 986 (w), 951 (w), 761 (w) cm^{-1}. – ^1H NMR (300 MHz, $CDCl_3$): δ = 1.23 – 1.31 (m, 24 H), 2.85 (m, 2 H, 3-H), 3.43 (br. s, 1 H, OH), 3.67 (m, 2 H, 1-H), 4.23 (m, 1 H, 2-H), 4.44 (m, 4 × *i*Pr-CH), 6.40 (d, J = 7.3 Hz, 1 H, NH), 6.73 (dd, J = 8.0, 2.0 Hz, 1 H, 6′-H), 6.82 – 6.78 (m, 3 H, 2′/5′/5″-H), 7.14 (dd, J = 8.4, 2.0 Hz, 1 H, 6″-H), 7.31 (d, J = 2.0 Hz, 1 H, 2″-H). – ^{13}C NMR (75 MHz, $CDCl_3$): δ = 22.1, 22.2 (each 2 × CH_3), 22.3 (4 × CH_3), 36.7 (C-3), 53.6 (C-2), 64.9 (C-1), 72.1, 72.3, 72.4, 72.6 (each *i*Pr-CH), 116.6 (C-5″), 117.4 (C-2″), 118.9 (C-5′), 119.4 (C-2′), 120.4 (C-6″), 122.4 (C-6′), 127.5 (C-1″), 131.1 (C-1′), 148.2, 149.0, 149.6 (each C), 152.5 (C-4″), 167.8 (CONH). – MS (EI): m/z (%) = 487 (8) $[M]^+$, 469 (2), 262 (27), 250 (100), 221 (47), 208 (28), 179 (19), 166 (19), 137 (46), 123 (24), 110 (4), 109 (4), 81 (1), 43 (11). – HRMS (EI): m/z = 487.2933 (calcd. 487.2934 for $C_{28}H_{41}NO_6$, $[M]^+$).

*Nigerrimin B (**10**)*

To a solution of compound **18** (25 mg, 0.05 mmol) in CH_2Cl_2 (4 mL) was added anhydrous $AlCl_3$ (49 mg, 0.37 mmol), and the mixture was stirred for 48 h at r. t. under an argon atmosphere. Then, saturated aqueous ammonium chloride (2 mL) was added, and the reaction mixture was equilibrated between water (5 mL) and EtOAc (10 mL). The aqueous phase was washed with EtOAc (2 × 10 mL), and the combined EtOAc phases were again washed with water. Then, the solution was dried (Na_2SO_4), filtered, and concentrated under reduced pressure to yield nigerrimin B (**10**) (14.9 mg, 91 %) as a colorless solid, m. p. 240 – 243 °C (decomp.), $[\alpha]_D^{25}$ = −53.6 (c = 9.3, MeOH). The synthetic compound was identical with the natural product by HPLC comparison and exhibited closely similar spectroscopic data (UV, IR, ^1H NMR, ^{13}C NMR, MS) and physical properties (m. p., $[\alpha]_D$, CD).

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