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Antifungal Polyamides of Hydroxycinnamic Acids from Sunflower Bee Pollen

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Supporting Information

ABSTRACT: The aim of the bioassay-guided fractionation was the selection of the most potent group of compounds responsible for the protection of sunflower bee pollen grains. Synthesis of prospective antifungal polyamides of hydroxycinnamic acids was based on previous structural elucidation of ethanol soluble fraction by $^{1}H,^{1}H$-PFG-COSY, $^{1}H,^{13}C$-HSQC, FT-IR, FT-Raman, and LC−MS experiments. The main compounds found were tri-*p*-coumaroylspermidines accompanied by other HCAA of spermidine and putrescine. Several model HCAA derivatives were prepared to test their antifungal activity against widespread spoilage fungi (*A. niger* 42 CCM 8189, *F. culmorum* DMF 0103, and *P. verrucosum* DMF 0023). *A. niger* CCM 8189 and *F. culmorum* DMF 0103 exhibited higher resistance to the antifungal effects of hydroxycinnamic acid amides, whereas *P. verrucosum* DMF 0023 was the most sensitive strain. It has been discovered the effect of HCAA polarity on the role of secondary metabolites in the microbial protection of pollen grains. The combination of bioassay-guided fractionation, structural elucidation, selection of prospective compounds, and their synthesis to determine their antifungal properties could be considered as an original approach.

KEYWORDS: *hydroxycinnamic acid amides, sunflower bee pollen, Helianthus annuus L., structural elucidation, antifungal activity*

INTRODUCTION

Pollen is the male reproductive cells produced in the anthers of spermatophytes and plays an essential role in the life cycle of flowering plants.[1,2] Pollen grains are excellent sources of carbohydrates, proteins, lipids, vitamins, and minerals necessary for the plant growth, development, and fusion with a female gamete.[2,3] Moreover, nature has equipped pollen with several secondary metabolites to protect them against biotic and abiotic stress. Phytochemical analysis has confirmed the presence of carotenoids, phytosterols, flavonoids, and phenolic acid derivatives.[1,3,4] Plant pollens are gathered by worker honeybees (*Apis mellifera*) and compacted into granules by plant nectar and saliva enzymes.[2,5] After that, bee pollen is transported to bee hive and used for the production of bee bread or royal jelly to feed the larvae and drone bees.[5,6]

Apicultural products including honey, royal jelly, beeswax, and bee pollen have been used in the human diet and personal care for many thousands of years. Current growing demand for bee pollen is connected with the consumption of natural food supplements with antioxidant, hepatoprotective, or anti-inflamatory benefits.[1,6,7] However, bee pollen should be also seen as nutrient-rich medium for the growth of fungi affecting the shelf life and safety of food as the main quality criterions. *Aspergillus* spp., *Fusarium* spp., *Penicillium* spp., *Mucor* spp., *Alternaria* spp., and *Cladosporium* spp. were the most frequent isolates of molds identified in several bee pollen samples. They are common saprophytes, which can be found either in the gut of worker honeybees or in brood combs.[5,7,8] Special attention should be paid to *Aspergillus*, *Fusarium*, and *Penicillium* species producing aflatoxins, ochratoxins, trichothecenes, and many other mycotoxins under optimal growth conditions.[5,7] Microbiological safety of bee pollen is dependent on the preservation either by common microflora or by natural compounds derived from plant pollen. Preservative phytochemicals are mainly expected to be responsible for the pollen protection against fungal spoilage.[8]

Suitable phytochemicals have to be located at the surface of pollen grains, hydrophobic and widespread in the plant kingdom. Such requirements meet hydroxycinnamic acid amides (HCAA), specific metabolites of pollen outer exine. Their accumulation in the floral parts of plants affects flower development, sexual differentiation, fertilization, and senescence.[9,10] Water-soluble aliphatic and aromatic amides of hydroxycinnamic acids are widespread constituents of seeds, roots, flowers, and female reproductive cells. Physiological significance of less substituted amides is plant adaptation to water stress, heat shock, UV radiation, sulfur starvation, and deficiencies of K, Ca, Mg, and phosphorus.[9,11] Infection of *Nicotiana tabacum* leaves by tobacco mosaic virus (TMV) induces accumulation of feruloyl and *p*-coumaroylputrescine in necrotic lesions leading to limited multiplication of TMV virus.[12] Similarly, accumulation of feruloyl and *p*-coumaroyltyramine upon wounding of potatoes is linked to the protection against *Phytophthora infestans* and *Streptomyces scabies*.[9,13] More substituted HCAAs of spermidine and putrescine are

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specific metabolites of pollen coat, where they might protect pollen grains against plant diseases caused by microorganisms and other results of damage, such as UV radiation and oxidative stress.[9−11]

There are a number of publications focusing on the biological activities of bee pollens and their extracts, even though they contained thousands of bioactive compounds. The present study reports the structural analysis of sunflower (*Helianthus annuus* L.) bee pollen fractions focusing on close-up composition of present hydroxycinnamic acid amides. The aim of this work was to synthesize pure HCAAs of putrescine and spermidine and to test their activity against widespread spoilage fungi. Our conclusions may help to understand the role of secondary metabolites in the microbial protection of pollen grains.

MATERIALS AND METHODS

Reagents and Instrumentation. Ferulic acid, *p*-coumaric acid, putrescine, spermidine, *N*-Boc-1,4-butanediamine, *N*,*N*′-dicyclohexylcarbodiimide, tetrahydrofuran, trifluoroacetic acid, and hydrochloric acid were purchased from Sigma-Aldrich Co. All other reagents and solvents were of analytical grade. FTIR spectra (spectral region 4000−400 cm^{-1}, 64 scans, resolution 2 cm^{-1}) of the fractions were recorded in the form of KBr tablets on Nicolet 6700 FTIR spectrometer (Thermo Scientific, Waltham, MA) using Omnic 8.0 software. FT Raman spectra (spectral region 4000−100 cm^{-1}, 1000 scans, resolution 4 cm^{-1}) of the fractions were recorded with FT Raman module of FTIR Nicolet iS50 spectrometer (ThermoScientific, Waltham, MA), equipped with a Nd:YAG laser (excitation line 1064 nm, laser power 100 mW), CaF_2 beam splitter, and InGaAs detector. Obtained spectra were exported to Origin 6.0 (Microcal Origin, U.S.) software in CSV or TXT format, where they were 5-point filtered and baseline corrected. The second derivative algorithm was used for analysis of overlapped bands. ^{1}H NMR and ^{13}C NMR APT spectra of the compounds or fractions dissolved in deuterated solvents were recorded on Bruker Avance 600 and Bruker Avance 500 (Bruker, Inc., Billerica, MA) spectrometers. Working frequencies were 600.1 and 499.8 MHz for ^{1}H, and 150.9 and 125.7 MHz for ^{13}C, respectively. Correlation spectroscopic ^{1}H,^{1}H-PFG-COSY and ^{1}H,^{13}C-HSQC experiments were applied for resolution and assignment of resonance signals. ESI−MS and APCI−MS spectra were measured with LC−MS LTQ-Orbitrap Velos (ThermoScientific, Waltham, MA) and LC−MS TSQ Quantum Access Max Triple Quadrupole mass spectrometers (Thermo Scientific, Waltham, MA). Ultraviolet (UV) spectra were recorded using a Cary 50 UV−vis spectrophotometer (Varian, Inc., Palo Alto, CA). Merck precoated silica gel F_{254} plates were used for thin-layer chromatography (TLC). Spots were detected by heating after spraying with 5% phosphomolybdic acid in EtOH. Melting points were measured on a Boetius hot-stage microscope and were not corrected.

Sunflower Bee Pollen Samples. Monofloral sunflower bee pollen was obtained from the Slovak University of Agriculture in Nitra, Slovak Republic (Assoc. Prof. Jan Brindza). The bee pollen material was taken from the hive storages and stored in the freezer until the time of chemical study. The wet bee pollen contained 15.5% m/m of water, 14.0% m/m of proteins, 6.5% m/m of lipids, 17.0% of carbohydrates, and 2.2% m/m of ash. Botanical origin of the raw material was confirmed by scanning electron microscope (SEM) analyses of the surface of bee pollen granules with a focused beam of electrons (Figure S1).

Extraction and Fractionation of Crude Sunflower Bee Pollen Extract. Monofloral sunflower bee pollen was pulverized. Fine powder (12.684 g, moisture: 9.48%) was subjected to solvent extraction with $CHCl_3$/CH_3OH mixture (2:1, v/v) at room temperature for 6 h and filtered. The filtrate was evaporated under reduced pressure to afford lipophilic extract (1.157 g), which was purified by flash chromatography on silica gel (70−230 mesh) column to yield three fractions: Fr-1 (eluted with petroleum ether/diethyl ether, from 100:0 to 87:13), Fr-2 (eluted with petroleum ether/diethyl ether, from 87:13 to 0:100), and Fr-3 (eluted with acetone and methanol). Each fraction containing compounds of the similar polarity was tested for antifungal activity to identify the most prominent compounds.

Synthesis of *p*-Coumaric and Ferulic Acid Amides of Putrescine. To a stirred solution of selected phenolic acid (9.0 mmol; 1.48 g of *p*-coumaric acid, 1.75 g of ferulic acid, respectively) and *N*-Boc-1,4-butanediamine (8.5 mmol, 1.60 g) in anhydrous CH_2Cl_2 (150 mL) on an ice water bath was added dropwise a solution of *N*,*N*′-dicyclohexylcarbodiimide (DCC, 14.4 mmol, 2.97 g) in 65 mL of CH_2Cl_2. The reaction mixture was allowed to warm to laboratory temperature and stirred for 2 days until the reaction was completed. Precipitated dicyclohexylurea was filtered off. Monoacylated derivatives of *N*-Boc-1,4-butanediamine were purified by column chromatography on silica gel (70−230 mesh). *t*-Butyl carbamate group was deprotected by 30 mL of CF_3COOH in CH_2Cl_2 (150 mL) under an inert atmosphere of argon. After the mixture had been stirred for 40 min at 25 °C, the solvent was evaporated under reduced pressure. The residue was taken up in the mixture of methanol (25 mL) with hydrochloric acid (50 mL, 1 mol/dm^3) and evaporated to dryness. The last step was repeated two times. Finally, dissolution of the resulting phenolamides in abs EtOH and subsequent evaporation to dryness provided the desired products *p*-coumaroyl putrescine (**1a**) and feruloyl putrescine (**1b**) in the form of hydrochloride.[14] Di-*p*-coumaroyl putrescine (**2**) was synthesized in the same manner with the exception that amino groups were not protected by *t*-butyl carbamate group. *p*-Coumaroyl putrescine and feruloyl putrescine were obtained in 93% and 89% yields, respectively.

***p*-Coumaroyl Putrescine Hydrochloride (1a).** The product was obtained in 93% yield as a yellowish-brown solid. Mp = 102−104 °C; R_f = 0.60 (CH_2Cl_2/CH_3OH/27% aq NH_3, 2:2:1); UV (CH_3OH) λ_{max} 308 nm; ^{1}H NMR (500 MHz, CD_3OD) δ 1.47 (4H, m, H-3, 4), 2.72 (2H, t, J = 7.2 Hz, H-2), 3.16 (2H, t, J = 6.5 Hz, H-5), 6.30 (1H, d, J = 15.9 Hz, H-8′), 6.57 (2H, d, J = 8.5 Hz, H-3′, 5′), 7.21 (2H, d, J = 8.5 Hz, H-2′, 6′), 7.32 (1H, d, J = 15.9 Hz, H-7′); ^{13}C NMR (126 MHz, CD_3OD) δ 25.9 (C-4), 27.0 (C-3), 40.4 (C-2), 40.8 (C-5), 115.2 (C-8′), 117.0 (C-3′, 5′), 127.0 (C-1′), 131.3 (C-2′, 6′′), 144.7 (C-7′), 161.5 (C-4′), 168.7 (C-9′); IR (KBr) 3327, 1211 cm^{-1} (OH), 3066, 3036, 1167, 980 cm^{-1} (=CH), 2929, 2851, 1448, 1440, 1371, 736 cm^{-1} (CH_2), 1644, 1537, 1344 cm^{-1} (CONH), 1627, 1601, 1580, 1514 cm^{-1} (C=C), 1132, 828 cm^{-1} (CNC), 1089, 1048 cm^{-1} (CC, CN), 641, 515 cm^{-1} (skeletal); Raman (λ_{ex} 1064 nm) 3328, 1211 cm^{-1} (OH), 3066, 3036, 1168, 979 cm^{-1} (=CH), 2935, 2854, 1444, 1347 cm^{-1} (CH_2), 1706 cm^{-1} (C=O), 1643, 1317 cm^{-1} (CONH), 1627, 1600, 1585, 1517 cm^{-1} (C=C), 1131, 858 cm^{-1} (CNC), 1074, 1050, 1029 cm^{-1} (CC, CN), 644, 536, 455, 414, and 372 cm^{-1} (skeletal); HRESIMS m/z 235.14414 $[M + H]^+$ (calcd for $C_{13}H_{19}N_2O_2^+$ = 235.14410).

Feruloyl Putrescine Hydrochloride (1b). The product was obtained in 89% yield as a yellowish solid. Mp = 113−115 °C; R_f = 0.58 (CH_2Cl_2/CH_3OH/27% aq NH_3, 2:2:1); UV (CH_3OH) λ_{max} 320 nm; ^{1}H NMR (500 MHz, CD_3OD) δ 1.66 (4H, m, H-3, 4), 2.93 (2H, t, J = 7.3 Hz, H-2), 3.33 (2H, t, J = 6.6 Hz, H-5), 3.84 (3H, s, H-10′), 6.46 (1H, d, J = 15.7 Hz, H-8′), 6.76 (1H, d, J = 8.2 Hz, H-5′), 7.00 (1H, dd, J = 8.2 Hz, 2.0 Hz, H-6′), 7.10 (1H, d, J = 1.9 Hz, H-2′), 7.45 (1H, d, J = 15.7 Hz, H-7′); ^{13}C NMR (126 MHz, CD_3OD) δ 24.5 (C-4), 26.0 (C-3), 39.0 (C-2), 39.7 (C-5), 55.0 (C-10′), 110.2 (C-2′), 115.1 (C-5′), 122.3 (C-6′), 126.5 (C-1′), 127.0 (C-1′), 141.8 (C-7′), 148.0 (C-4′), 149.0 (C-4′), 168.4, 169.3, 170.4 (C-9′); IR (KBr) ν = 3381, 1214 cm^{-1} (OH), 3020, 1168, 978 cm^{-1} (=CH), 2932, 2856, 1444, 1363, 737 cm^{-1} (CH_2), 1654, 1539, 1337 cm^{-1} (CONH), 1602, 1582, 1513 cm^{-1} (C = C), 1131, 830 cm^{-1} (CNC), 1108 cm^{-1} (OCH_3), 1014 cm^{-1} (CC, CN), 515 cm^{-1} (skeletal); Raman (λ_{ex} 1064 nm) ν = 3064, 3019, 1168, 973 cm^{-1} (=CH), 2983, 2937, 2867, 2857, 1442 cm^{-1} (CH_2), 1733 cm^{-1} (C=O), 1654, 1315 cm^{-1} (CONH), 1625, 1602, 1583, 1517 cm^{-1} (C=C), 1222 cm^{-1} (OH), 1122, 828 cm^{-1} (CNC), 1081, 1029 cm^{-1} (CC, CN), 644, 524, 449, 416, and 377 cm^{-1} (skeletal); HRESIMS m/z 265.15491 $[M + H]^+$ (calcd for $C_{14}H_{21}N_2O_3^+$ = 265.15467).

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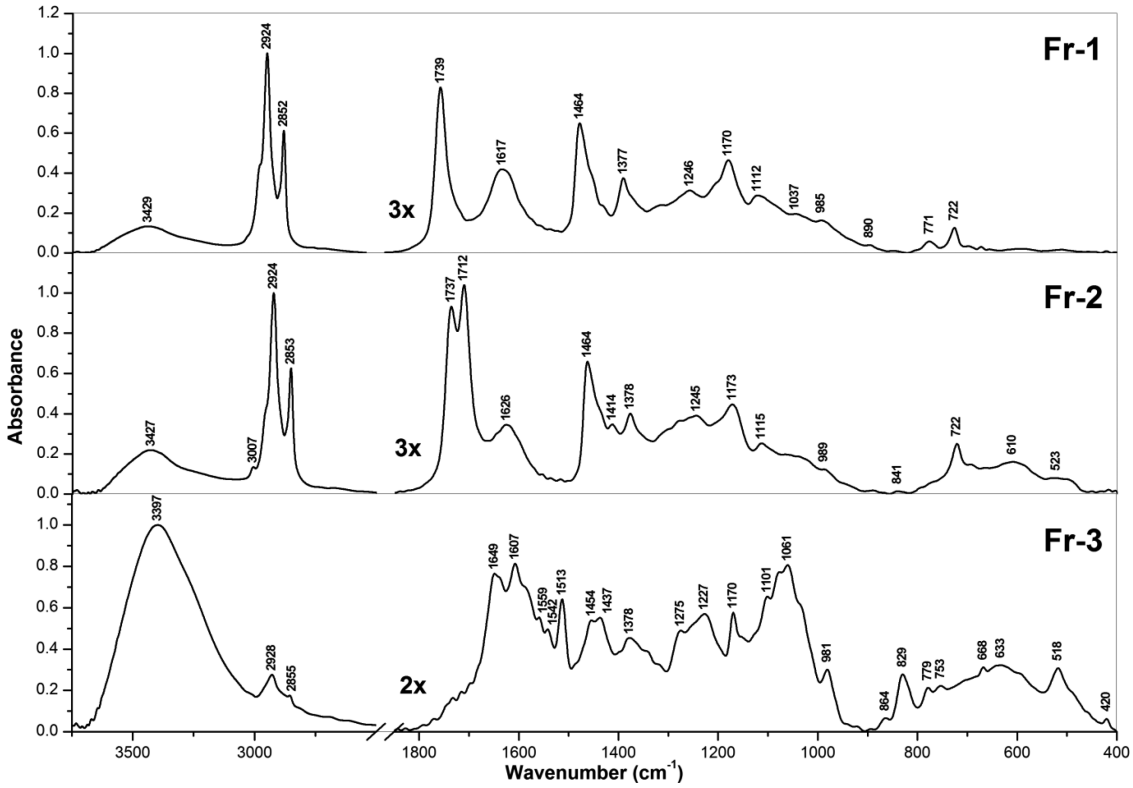

Figure 1. FT-IR spectra of fractions Fr-1−3.

***N,N′*-Di(*p*-coumaroyl) Putrescine (2).** The product was obtained as a pale yellow solid (560 mg). R_f = 0.84 (CH_2Cl_2/CH_3OH/27% aq NH_3, 2:2:1); UV (CH_3OH) λ_{max} 308 nm; ^{1}H NMR (500 MHz, CD_3OD) mixture of conformers, some hydrogen atoms give more than one signal δ 1.66 (4H, m, H-3, 4); 3.36 (4H, overlapped with solvent, H-2, 5); 6.43, 6.55, 6.60 (2H, d, J = 16.0 Hz, H-8′); 6.80, 6.86, 7.22 (4H, d, J = 8.6 Hz, H-3′, 5′); 7.42, 7.56, 7.63 (4H, d, J = 8.6 Hz, H-2′, 6′); 7.47, 7.55, 7.83 (2H, d, J = 16.0 Hz, H-7′); ^{13}C NMR (126 MHz, CD_3OD) mixture of conformers, some carbon atoms give more than one signal δ 26.5, 26.6 (C-3, 4); 38.7, 38.8 (C-2, 5); 112.5, 117.0, 120.6 (C-8′); 115.3, 115.6, 122.0 (C-3′, 5′); 125.5, 126.3, 132.5 (C-1′); 128.5, 129.1, 130.2 (H-2′, 6′); 139.2, 140.3, 147.2 (C-7′); 152.1, 159.1, 160.5 (C-4′); 165.9, 167.1, 167.9 (C-9′); IR (KBr) ν = 3396 cm^{-1} (OH), 3022, 1172, 984 cm^{-1} (=CH), 2955, 2930, 2856, 1442, 1363 cm^{-1} (CH_2), 3202, 1658, 1349 cm^{-1} (CONH), 1606, 1587, 1515 cm^{-1} (C=C), 1134, 833 cm^{-1} (CNC), 1206, 1108 cm^{-1} (OCH_3), 1014 cm^{-1} (CC, CN), 571, 520, and 457 cm^{-1} (skeletal); Raman (λ_{ex} 1064 nm) ν = 3388, 1207 cm^{-1} (OH), 3064, 3019, 1172, 979 cm^{-1} (=CH), 2983, 2935, 1444, 1388, 1361 cm^{-1} (CH_2), 1693 cm^{-1} (C=O), 1656, 1317 cm^{-1} (CONH), 1633, 1604, 1587 cm^{-1} (C=C), 1126, 863 cm^{-1} (CNC), 1023 cm^{-1} (CC, CN), 740, 644, 449, and 416 cm^{-1} (skeletal); APCI-MS m/z 381.18117 $[M + H]^+$ (calcd for $C_{22}H_{25}N_2O_4^+$ = 381.18088).

Synthesis of *p*-Coumaric Acid Amides of Spermidine. *p*-Coumaric acid (1.09 g, 6.1 mmol) was acetylated in 15 mL of tetrahydrofuran with 1.2 mL of acetyl chloride (16.9 mmol) in the presence of pyridine (1.5 mL) for 12 h at 25 °C. The mixture was worked up by column chromatography on silica gel (70−230 mesh) to yield (*E*)-4-*O*-acetylcoumaric acid (5.44 mmol, 1.34 g, 89.3%). A mixture of (*E*)-4-*O*-acetylcoumaric acid (5.44 mmol, 1.34 g) and thionyl chloride (10 mL) was refluxed at 80 °C for 16 h. The mixture was evaporated to dryness to afford (*E*)-4-*O*-acetylcoumaroyl chloride and applied to the next step without further purification. To a stirred solution of spermidine (1.64 mmol, 0.33 g) and triethylamine (5.5 mmol, 0.56 g) in anhydrous tetrahydrofuran (20 mL) on an ice water bath was added dropwise a solution of (*E*)-4-*O*-acetylcoumaroyl chloride. After the mixture had been stirred for 12 h at 25 °C, the solvent was evaporated under reduced pressure and residue was worked up by column chromatography on silica gel to afford the mixture (*E*)-4-*O*-acetylcoumaroyl amides of spermidine.[15] Acetate esters were deprotected by aqueous solution of NH_3 (27%, v/v, 20 mL) in methanol (55 mL) under an inert atmosphere of argon. After the mixture had been stirred for 5 h at 25 °C, the solvent was evaporated under reduced pressure. The residue was taken up in CH_2Cl_2 and evaporated to dryness to provide the mixture of di-*p*-coumaroyl and tri-*p*-coumaroyl spermidines.[16] Di- and tri-*p*-coumaroyl spermidines were obtained in 18.5% yield.

Di- and Tri-*p*-coumaroyl Spermidines (3). The mixture of di- and tri-*p*-coumaroyl spermidines (307 mg) was obtained as a brownish solid. R_f = 0.76−0.85 (CH_2Cl_2/CH_3OH/27% aq NH_3, 2:2:1); UV (CH_3OH) λ_{max} 308 nm; ^{1}H NMR (500 MHz, CD_3OD) δ 1.59/1.75 (2H, m, H-8), δ 1.64/1.69 (2H, m, H-7), 1.87/1.96 (2H, q, J = 7.0 Hz, H-3), 3.35/3.38 (4H, m, H-2, 9), 3.50/3.58 (4H, m, H-6), 3.54/3.60 (4H, m, H-4), 6.44 (d, J = 15.8 Hz, H-8′), 6.81 (d, J = 8.5 Hz, H-2′, 6′), 6.83/6.87 (d, J = 15.8 Hz, H-8′), 7.43 (d, J = 8.5 Hz, H-3′, 5′), 7.47 (d, J = 15.8 Hz, H-7′), 7.52/7.59 (d, J = 15.8 Hz, H-7′); ^{13}C NMR (126 MHz, CD_3OD) δ 25.0/26.5 (C-7), 26.5/26.6 (C-8), 27.5/29.2 (C-3), 36.5/36.8 (C-2), 38.5/38.7 (C-9), 44.3/45.6 (C-4), 46.2/47.6 (C-6), 113.5/113.7 (C-8′), 115.4 (C-2′, 6′), 116.5 (C-8′), 126.2−126.5 (C-1′, 1′), 129.3 (C-3′, 5′), 141.5 (C-7′), 159.3 (C-4′), 167.8 (C-9′), 168.0 (C-9′); IR (KBr) ν = 3410, 1215 cm^{-1} (OH), 3006, 1277, 983 cm^{-1} (=CH), 2958, 2871, 1450, 1350, 765 cm^{-1} (CH_2), 3201, 1663, 1349 cm^{-1} (CONH), 1606, 1587, 1516 cm^{-1} (C=C), 1127, 849 cm^{-1} (CNC), 1033 cm^{-1} (CC, CN), 569 and 457 cm^{-1} (skeletal); Raman (λ_{ex} 1064 nm) ν = 3388, 1224 cm^{-1} (OH), 3072, 3004, 1162, 979 cm^{-1} (=CH), 2983, 2935, 1452, 1430, 1388, 1363 cm^{-1} (CH_2), 1697 cm^{-1} (C=O), 1658, 1320 cm^{-1} (CONH), 1631, 1600, 1515 cm^{-1} (C=C), 1128, 867 cm^{-1} (CNC), 1089, 1031 cm^{-1} (CC, CN), 651, 570, and 449 cm^{-1} (skeletal); APCI-MS m/z 584.27540 $[M + H]^+$ and m/z 438.23864 $[M + H]^+$ (calcd for $C_{25}H_{32}N_3O_4^+$ = 438.23873).

Antifungal Activity Assay. The mold strain *Aspergillus niger* CCM 8189 was obtained from Czech Collection of Microorganisms (Brno, Czech Rep.); *Fusarium culmorum* DMF 0103 and *Penicillium*

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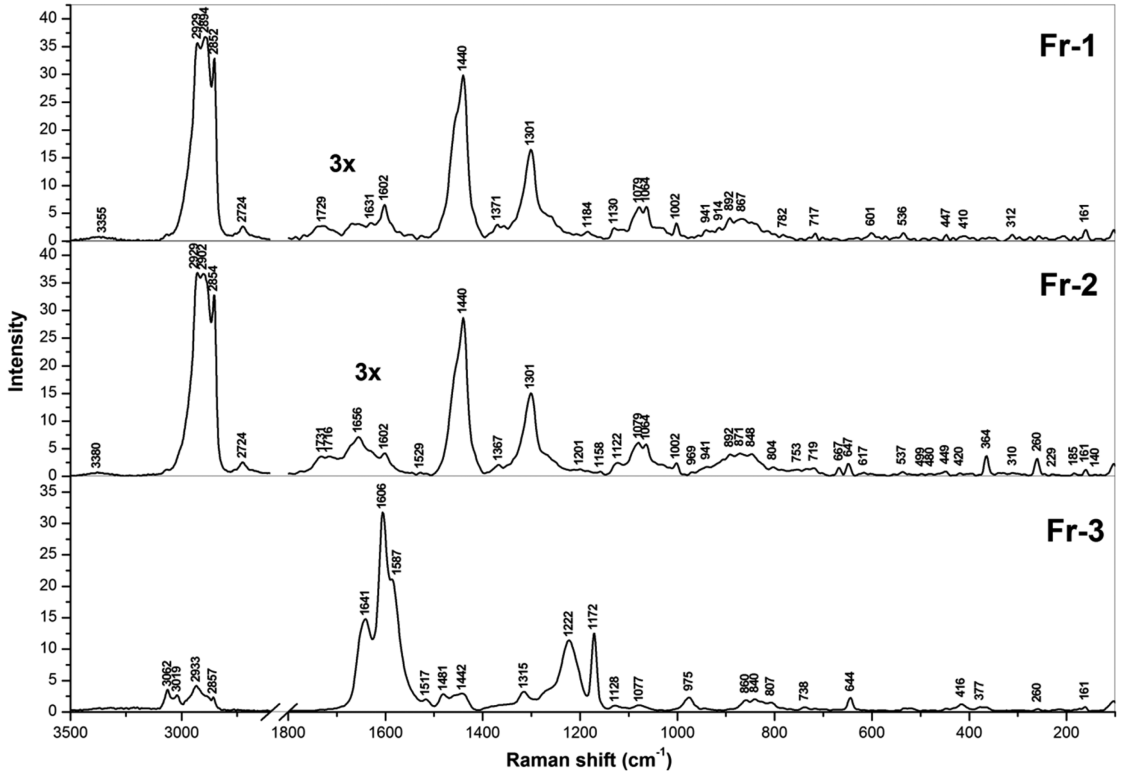

Figure 2. FT-Raman spectra of fractions Fr-1−3.

verrucosum DMF 0023 were from the collection of the Department of Dairy, Fat and Cosmetics (UCT Prague, Czech Rep.). Mold strains were selected to test the growth inhibition activity of sunflower bee pollen fractions, *p*-coumaroyl putrescine hydrochloride (**1a**), feruloyl putrescine hydrochloride (**1b**), *N*,*N*′-di(*p*-coumaroyl) putrescine (**2**), and *p*-coumaroyl spermidines (**3**). The molds were grown on malt extract agar (MEA) plates at 25 °C for 72 h and maintained with periodic subculturing at 4 °C. The spore suspension for further inoculation was obtained by washing the particular plate with 5 mL of physiological saline solution with Tween 80 (0.5%, v/v). The concentration of spores was adjusted to 10^5 spores per mL by proper dilution.[17] Malt extract broth or agar was supplemented with various concentrations (20, 10, 5, 2.5, and 1.25 mmol/dm^3) of the tested compounds and sterilized (121 °C, 15 min, 0.15 MPa) before cultivation. Two different methods were used for antifungal activity determination.

First, growth curves were measured in microtitration plates by spectrophotometric method using an automatic cultivator/reader PowerWave XS (BioTek Instruments, Winooski, U.S.) according to Kosová et al. (2015) with a slight modification: 2 μL of spore suspension (10^5/mL) was inoculated into 200 μL of malt extract broth enriched with different concentrations of tested compounds; pure malt extract broth was used as the control. The optical density was measured at 630 nm.[17] All measurements were performed in triplicate, and means were used for the calculation of minimal inhibitory concentration (MIC) expressed as the lowest concentration of assayed substances inhibiting the visible growth of molds.

Second, the radial growth of molds was monitored on solid MEA supplemented with 10, 5, 2.5, and 1.25 mmol/dm^3 of *p*-coumaroyl putrescine hydrochloride (**1a**) and feruloyl putrescine hydrochloride (**1b**). Plates were inoculated by 5 μL of the spore suspension (10^5/mL) in the middle of the dish and incubated at 25 °C for 10 days; pure malt extract agar was used as the control.[18,19] Antifungal activities were calculated from the following equation:

$$\text{inhibition} = \left(1 - \frac{S_{\text{inhibition}}}{S_{\text{blank}}}\right) \cdot 100\ [\%] \quad (1)$$

where S is the radial growth area (cm^2) of molds on the plates with or without phenolamides.

RESULTS AND DISCUSSION

Extraction and Fractionation of Crude Sunflower Bee Pollen Extract. Specific secondary metabolites of pollen grains were isolated, and the antifungal activity against destructive and mycotoxigenic molds was assayed. The yield of crude extract from sunflower bee pollen was 10.08% by dry weight. TLC results confirmed the complex nature of extract with dozens of potential antimicrobial compounds differing in polarity. Bioassay-guided fractionation was achieved by flash chromatography and resulted in three fractions. In the separation profile, hydrophobic constituents of the fraction Fr-1 (33.7%) were eluted quickly with petroleum ether/diethyl ether from 100:0 to 87:13 followed by medium polar compounds of the fraction Fr-2 (40.4%). A third ninhydrine positive fraction (25.9%) was eluted with acetone and methanol. Most analytes present in the obtained fractions were in general weak chromophores. Therefore, identification of structures was based on MS, NMR, and other spectroscopic data and their comparison with literature. The FT-IR and FT-Raman spectra of the fractions are represented in Figures 1 and 2, respectively.

Structural Elucidation of Fr-1. The FT-IR spectrum of Fr-1 (Figure 1, top) displayed two absorption bands at 1246 cm^{-1} (CO stretching) and 1739 cm^{-1} (C═O stretching), which were assigned to ester groups. The Raman band of esters was found at 1729 cm^{-1} (C═O stretching). The bands at 1667, 1631, and 1602 cm^{-1} arose from stretching vibrations of unsaturated fatty acids and other unsaturated or aromatic molecules or residues. The long alkyl H/C signals of saturated, mono-, and polyunsaturated fatty acids predominates in the ^{1}H and ^{13}C NMR spectra of Fr-1 (Table S1).[20,21] The protons of

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glycerol moiety in TAG were found at δ 5.26 ppm (triplet of triplets), 4.14, and 4.31 ppm (both are doublets of doublets).

The ^{13}C NMR spectrum of Fr-1 showed signals of three enol carbons of β-diketones at δ 194.65, 194.46, and 194.04 (Table S2 and Figure S2).[22] A broad proton signal at δ 15.50 arose from enol hydroxyls. Several triplets at δ 2.26−2.49 and the corresponding positive APT carbon signals at δ 38.36 and 43.73 were assigned to side CH_2 attached to carbonyl in enol form. The proton signals of phenyl ring conjugated with ketone were found at δ 7.88 (*ortho*-position, doublet), δ 7.43 (*meta*-position, triplet), and δ 7.50 (*para*-position, triplet); the corresponding negative APT carbon signals arose at δ 126.93, δ 128.49, and δ 132.11, respectively. The H/C HMQC signals at δ 2.42/38.99 and at δ 2.49/43.75, 43.69 were assigned to the side CH_2 attached to carbonyl in enol form. The H/C HMQC signals of CH_2/=CH group between two carbonyls of aliphatic β-diketones were found at δ 3.54/57.12 (ketone) and at δ 5.47/98.96, 99.03 (enol); corresponding signals of CH_2/=CH group between two carbonyls of aromatic β-diketones were found at δ 4.08/53.92 (ketone) and at δ 6.17/95.96 (enol).[22]

Structural Elucidation of Fr-2. The FT-IR spectrum of the Fr-2 (Figure 1, middle) displayed strong absorption bands of C=O stretching vibrations at 1712 and 1737 cm^{-1} that arose from carboxylic and ester groups, respectively. The Raman bands at 1729 and 1716 cm^{-1} were assigned to C=O stretching in esters and acids, respectively. The bands at 1656 and 1602 cm^{-1} originated from C=C stretching vibrations in unsaturated residues. Like in the case of Fr-1, the ^{1}H and ^{13}C NMR spectra of Fr-2 showed a lot of characteristic signals originated from monoacyglycerols, free fatty acids, and fatty alcohols (Table S1).[20,21]

In addition, the signals of sterols, terpenes, and many other cyclic compounds were also found.[23−25] Allyl moiety characteristic for *ent*-kaurenes (diterpenoids) was found at δ 155.5, 155.7, and 156.7.[23] Carbon signals at δ 64.3, 65.4, 77.2, and 79.0 arose from CH_2OH and CHOH of hydroxylated *ent*-kaurenes; carbonyl carbons at δ 183.7−183.8 indicated the carboxylic group attached at C-4.[24,26] These compounds showed inhibitory effects on activation Epstein−Barr virus. Isolated fractions Fr-1, Fr-2, however, did not show prospective antifungal effects comparable to crude extract and Fr-3.

Structural Elucidation of Fr-3. Appreciable antifungal activities against *A. niger* CCM 8189, *F. culmorum* DMF 0103, and *P. verrucosum* DMF 0023 were observed only in the case of Fr-3. Minimum inhibition concentrations of ninhydrine positive Fr-3 on all of the tested fungi ranged from 5 to 10 g/dm^3 obtained by microtitration screening method. Crude nonfractionated extract showed a prospective antifungal effect at higher concentrations (>20 g/dm^3). The FT-IR spectrum of the Fr-3 (Figure 1, bottom) displayed strong absorption bands of aromatics and sugars, while the bands of alkyls are relatively weak. A strong broad band with a maximum at 3397 cm^{-1} arose from OH vibrations in water, sugars, and phenolic compounds. Bands at 1607 and 1513 cm^{-1} were assigned to C=C stretching vibrations of phenolic acids and their derivatives (amides, esters).[27] Bands at 1649, 1559, and 1542 cm^{-1} indicated the presence of amide groups. Two bands at 1454 and 1437 cm^{-1} were assigned to CH_2 scissoring vibrations in polyamines.[28] The band at 1378 cm^{-1} is characteristic for symmetric bending vibration of CH_3 in ferulic acid. Bands at 1275 and 981 cm^{-1} were assigned to in-plane and out-of-plane vibrations of =CH in phenolic acids.[27] Highly overlapped bands in the region of 1000−1170 cm^{-1} arose from stretching vibrations of CO, CN, and CC bonds. The band at 829 cm^{-1} was assigned to symmetric CNC stretching in polyamines.[28]

The FT-Raman spectrum of the Fr-3 (Figure 2, bottom) showed strong bands at 1641, 1606, and 1587 cm^{-1} and a smaller one at 1517 cm^{-1} assigned to C=C stretching vibrations in phenolic acids.[27] The bands at 1442, 1481, 2933, and 2857 cm^{-1} were assigned to CH_2 scissoring and stretching vibrations in polyamines.[28] The band at 1222 cm^{-1} was assigned to OH in-plane bending in phenolics.[29] Two bands at 3062 and 3019 cm^{-1} (=CH stretching), 1172 cm^{-1} (=CH in-plane bending), and 975 cm^{-1} (=CH out-of-plane bending) arose from unsaturated and aromatic moieties.

Proton and ^{13}C NMR spectra of Fr-3 showed intense −CH= signals of *para*-substituted phenyls found at δ 6.77 (*ortho*-position, doublet)/δ 116.0, δ 7.37/δ 130.7 (*meta*-position, doublet) (Table S3). APT positive signals of quaternary carbons were observed at δ 159.3 and δ 127.7. The signals of conjugated C=C bond were found at δ 6.38/δ 118.40 and δ 7.49/δ 141.93 and δ 7.52/δ 144.48. Finally, several carbonyl carbon signals were found at δ 169.1−169.4 (amides), δ 170.3−170.6 (esters), and δ 171.8−172.0 (acids). All of these signals confirmed the presence of *para*-substituted phenols with conjugated double C=C bond and carboxylic group that is typical for *p*-coumaric acid and its conjugates (esters, amides).[29] Several methylene proton signals at δ 1.55−1.95 (internal), δ 3.09−3.38 (bound to end amine nitrogen), and δ 3.18−3.58 (bound to internal amine nitrogen) and the corresponding carbon signals at δ 25.5−29.8, 37.8−38.3, and δ 39.7−47.7 confirmed the presence of spermidine molecules conjugated with phenolic acids.[30] Thus, the main compound found in the methanol/ethanol soluble fractions was tri-*p*-coumaroylspermidine[31] accompanied by other HCAA of spermidine and putrescine. LC−MS analyses confirmed exact masses of 584.27540 $[M + H]^+$ and m/z 438.23864 $[M + H]^+$ corresponding to tri- and di-*p*-coumaroylspermidines. These compounds have been previously found in sunflower pollen, and their phagostimulatory activities were described. Further acetone fractionation removed insoluble glyceroglycolipids representing 0.54 g/100 g of dry matter of bee pollen grains. Residue of Fr-3 (2.07 g/100 g) was identified as the mixture of predominating hydroxycinnamic acid amides accompanied by glycerophospholipids based on LC−MS measurements. The results of fractionation were in accordance with literature data.[32] Less intense signals of other phenolic acids were also found. The HMQC signal at δ 3.64/δ 51.99 arose from methoxy groups in ferulic acids. Structural elucidation of Fr-3 resulted in the synthesis of basic and neutral HCAA derivatives.

Synthesis of *p*-Coumaric and Ferulic Acid Amides of Putrescine and Spermidine. A number of scientific publications are devoted to the antimicrobial effects of plant extracts containing HCAA, whether on the growth of bacteria[1,33] or fungi.[34] Most authors, however, deal only with the effect of the crude extracts containing dozens of potential antimicrobial compounds, which may exhibit synergistic antifungal activities as proved for lactic and acetic acids.[35] Therefore, it is important to study pure compounds first to accurately determine their effect.

Hydroxycinnamic acid amides of putrescine and spermidine were difficult to isolate from natural sources such as sunflower

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bee pollen in sufficient yield and required purity. Thus, *p*-coumaroyl putrescine hydrochloride (**1a**), feruloyl putrescine hydrochloride (**1b**), *N*,*N*′-di(*p*-coumaroyl) putrescine (**2**), and *p*-coumaroyl spermidines (**3**) were synthesized (Figure 3 and 4) in good to quantitative yield.

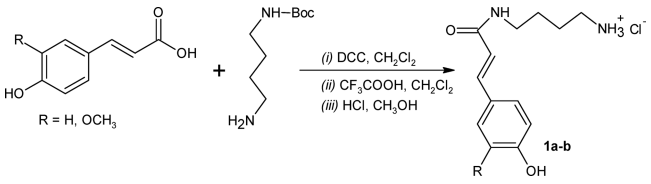

Figure 3. Synthesis of *p*-coumaroyl putrescine hydrochloride (**1a**) and feruloyl putrescine hydrochloride (**1b**).

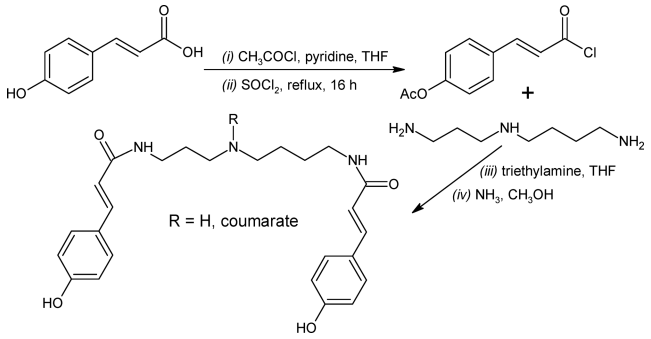

Figure 4. Synthesis of di- and tri-*p*-coumaroyl spermidines (**3**).

FTIR, FT-Raman, ^{1}H, and ^{13}C APT NMR spectra of synthesized phenolamides **1a**, **1b**, **2**, and **3** confirmed the presence of structural parts of the corresponding phenolic acids and polyamines, that is, phenylpropenoyl and *N*-methylene groups. For example, IR and/or Raman bands at 1625−1633, 1601−1606, 1580−1587, 1513−1517, 1162−1175, and 978−984 cm^{-1} (C═C stretching, ═CH in-plane and out-of-plane bending) are characteristic of phenolic acids.[27] The IR and/or Raman bands of polyamines were found at 2983, 2958−2929, 2851−2871, 1430−1452, 1347−1388, and 736−765 cm^{-1} (vibrations of CH_2).[28] The vibration bands characteristic for amide bonds were found at 3202−3201, 1643−1663, 1537−1539, and 1315−1349 cm^{-1} (vibrations amide A, I, II, and III, respectively) that confirm amidation. Proton and ^{13}C NMR spectra of the products were very complicated because of the presence of several conformers of the polyamine chain. Nevertheless, all of the proton and carbon signals of phenolamides were assigned by using correlation NMR experiments COSY, HMQC, and HMBC. The APT signals of amide carbonyl carbons were found at δ 167.2−168.0 (secondary and tertiary amides) and δ 168.3 (primary amide). The ^{1}H,^{13}C HMBC signals at δ 3.35/167.9, 3.37/168.0, 3.56/167.8, 3.58/167.7, 3.49/167.5, and 3.50/167.5 confirmed the connection between carbonyl carbon of phenolic acids and methylene hydrogens of polyamine residues via amide bonds (Figure 5).

Antifungal Activity of Hydroxycinnamic Acid Amides. Sunflower bee collected pollen is now used as a supplement in the human diet, and therefore the assessment of its microbiological quality is very important. The aim of the antifungal test was to verify if the compounds naturally occurring in bee pollen can reduce the growth of commonly widespread molds. Here, we present the effect of *p*-coumaroyl

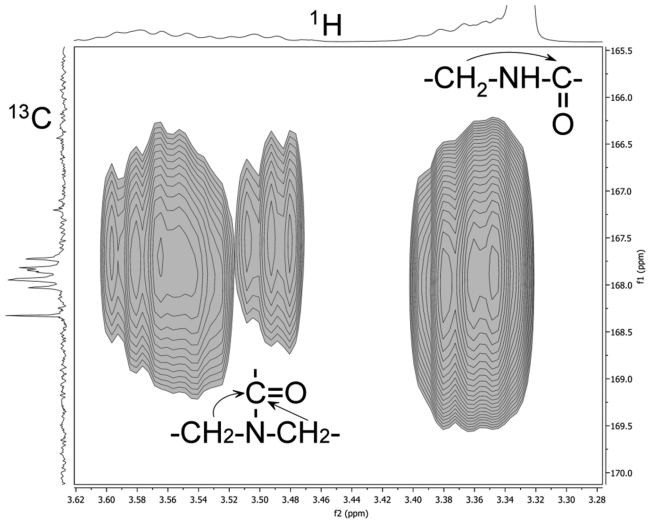

Figure 5. ^{1}H,^{13}C HMBC signals assigned to interactions between methylene protons and carbonyl carbons that confirmed amide linkages in *p*-coumaroyl spermidines and putrescines.

putrescine hydrochloride (**1a**), feruloyl putrescine hydrochloride (**1b**), neutral *N*,*N*′-di(*p*-coumaroyl) putrescine (**2**), and the mixture of *p*-coumaroyl spermidines (**3**) on the growth inhibition of representative bee pollen contaminants *A. niger* CCM 8189, *F. culmorum* DMF 0103, and *P. verrucosum* DMF 0023.[5] Moreover, *Aspergillus* spp., *Fusarium* spp., and *Penicillium* spp. are mycotoxigenic fungi producing aflatoxins, ochratoxins, trichothecenes, and many other mycotoxins under optimal growth conditions.[5,7] The recovery of these molds represents a potential risk for human health.

First, the growth of tested molds in microtitrate plates was used as a screening method to determine the range of required substance concentrations. As can be seen from Table 1,

Table 1. Antifungal Activity of Hydroxycinnamic Acid Amides on the Growth Inhibition of *Aspergillus niger*, *Fusarium culmorum*, and *Penicillium verrucosum* Determined on the Microtitration Plates

tested compound/MIC[a]	*F. culmorum*, mmol/dm^3	*P. verrucosum*, mmol/dm^3	*A. niger*, mmol/dm^3
p-coumaroyl putrescine hydrochloride (**1a**)	5	2.5	10
feruloyl putrescine hydrochloride (**1b**)	5	2.5	20
N,*N*′-di(*p*-coumaroyl) putrescine (**2**)	20	>20	>20
di- and tri-*p*-coumaroyl spermidines (**3**)	20	5	5

[a]MIC showed the minimal inhibitory concentration expressed as the lowest concentration of assayed substances inhibiting the visible growth of molds.

selected phenolic compounds showed significantly different inhibitory effects on the growth and survival of tested molds. *A. niger* CCM 8189 (5−20 mmol/dm^3) and *F. culmorum* DMF 0103 (5−20 mmol/dm^3) species exhibited higher resistance to the antifungal effects of hydroxycinnamic acid amides, whereas *P. verrucosum* DMF 0023 was the most sensitive during the measurement on the microtitration plate (Table 1). Di- and tri-*p*-coumaroyl spermidines (**3**) showed a MIC of 5 mmol/dm^3 for *P. verrucosum* and *A. niger*. Minimum inhibition

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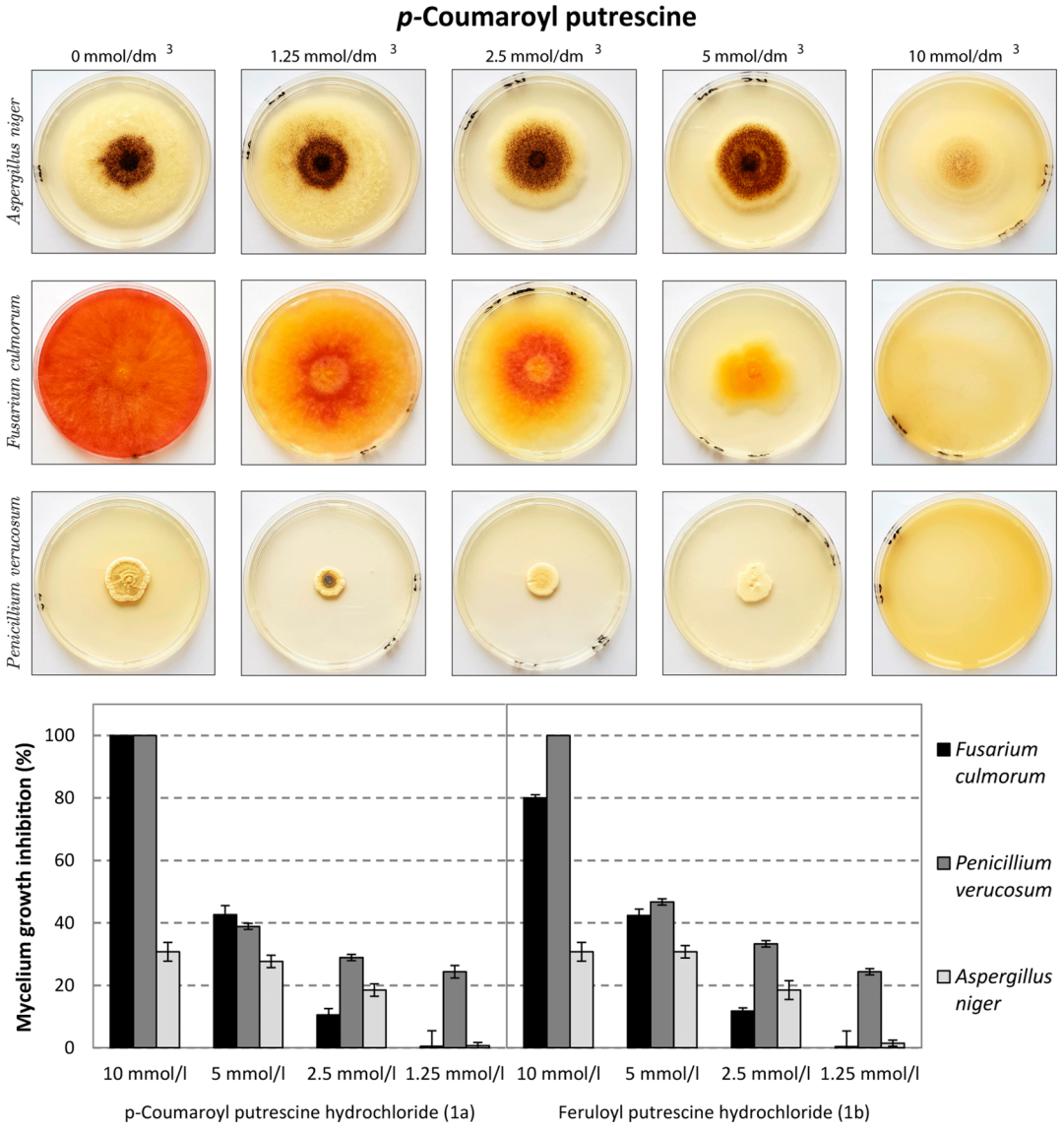

Figure 6. Mycelium growth inhibition of tested molds on malt extract agar with different concentrations (0−10 mmol/dm^3) of *p*-coumaroyl putrescine hydrochloride (**1a**) and feruloyl putrescine hydrochloride (**1b**) after 10 days at 25 °C. The mycelium growth inhibition was calculated through eq 1 (see Material and Methods).

concentrations of *p*-coumaroyl putrescine hydrochloride (**1a**) on all of the tested mycotoxigenic fungi ranged from 2.5 to 10 mmol/dm^3 (Table 1). Lipophilic character of putrescine derivatives was considered as a key factor in determining solubility and antifungal activity. HCAA in the form hydrochloride had the highest inhibitory effects and the lowest lipophilicity based on partition coefficient ($K_{o/w}$). Positively charged compounds (**1a**, **1b**) with protonated amino groups at physiological pH could play a defensive role against fungal spoilage by electrostatic interactions with negatively charged proteins, phospholipids, and nucleic acids, while neutral HCAA could serve as antioxidant and the pool of antifungal agents.[36]

On the basis of the previous results, *p*-coumaroyl putrescine hydrochloride (**1a**) and feruloyl putrescine hydrochloride (**1b**) were used for the determination of fungal radial growth inhibition on a solid media. The results (Figure 6) showed that the MIC values detected for the most sensitive *P. verrucosum* DMF 0023 by spectrophotometric method (2.5 mmol/dm^3) differed from the growth inhibition on agar media (10 mmol/dm^3). The method used may influence the determination of the MIC. The minimum inhibitory concentrations of tested substances **1a** and **1b** found by microplate cultivation were lower than those on solid agar culture. Concentrations of these substances of 2.5 mmol/dm^{-3} for *F. culmorum* and 5 mmol/dm^{-3} for *P. verrucosum* prevented mold growth completely (Table 1). In the case of growth on malt extract agar, only 40% inhibition of growth was observed at these concentrations. Differences between these methods may be due to different growth rates during growth in liquid and solid media. Solid culture media usually allow regular growth and allow the mold to sporulate more easily as compared to liquid media. On the basis of practical experience, the cultivation on solid media can be rather recommended, also due to better access of mold to oxygen.[37]

Antifungal activity of *p*-coumaroyl putrescine hydrochloride (**1a**) was slightly increased in relation to that of feruloyl putrescine hydrochloride (**1b**). *p*-Coumaroyl putrescine hydrochloride could be considered an analogue of benzoic acid and

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its derivatives, fundamental industrial biocide. The presence of the methoxy group in feruloyl putrescine hydrochloride increased a bit the compound polarity and decreased the biocide action on lipophilic outer membrane of molds (Table 1). Tested molds could be classified according to the inhibitory effect of positively charged HCAA on the radial growth in ascending order: *A. niger* CCM 8189 < *Fusarium culmorum* DMF 0103 < *Penicillium verrucosum* DMF 0023.

As can be seen from Figure 6, the addition of assayed compounds did not affect only the radial growth of molds, but also their ability to sporulate. The spore formation of *Fusarium culmorum* was significantly slowed. Microscopically, the occurrence of macroconidia in the randomly selected 15 visual fields of the control and supplemented samples by HCAA in the culture medium was controlled. The number of *F. culmorum* macroconidia was noticeably reduced during the growth on agar with the addition of tested substances (data not shown). Moreover, results demonstrated that positively charged HCAA affected colony morphology.[38] Colonies of *Aspergillus* spp., *Fusarium* spp., and *Penicillium* spp. species with mycelium growth inhibition above 30% were not pigmented or adopted lighter coloration in comparison with control experiments.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.8b03976.

Tables S1−S3 and Figures S1 and S2 (PDF)

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

HCAA, hydroxycinnamic acid amides; TMV, tobacco mosaic virus; FTIR, Fourier-transform infrared spectroscopy; FT, Fourier transformation; NMR, nuclear magnetic resonance; HSQC, heteronuclear single-quantum coherence; COSY, correlation spectroscopy; HMBC, heteronuclear multiple-bond correlation; UV−vis, ultraviolet−visible spectroscopy; TLC, thin-layer chromatography; SEM, scanning electron microscopy; Boc, *tert*-butyloxycarbonyl; DCC, *N*,*N*′-dicyclohexylcarbodiimide; CCM, Czech Collection of Microorganisms; DMF, Dairy, Fat and Cosmetics collection; MEA, malt extract agar; MIC, minimum inhibition concentration; SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; TAG, triacyglycerol; EI−MS, electron impact mass spectrometry; TIC, total ion current

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