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Article

Stilbenoids as Antifungals to Counteract Rice Blast Pathogen *Pyricularia oryzae*

Andrea Kunova,[†] Cecilia Pinna,[†] Sharmila Ghosh, Denise Dozio, Cristina Pizzatti, Salvatore Princiotto, Paolo Cortesi, Sabrina Dallavalle,* and Andrea Pinto

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ABSTRACT: Fungi are among the greatest biotic threats to agricultural and food security. Intensive monoculture cropping and resistance to single-site fungicides in plant pathogens urge the discovery and development of novel compounds that possibly interfere with essential cellular processes in multiple ways. In this article, we describe our recent efforts addressed to the identification of natural compounds as multitarget biofungicides. A set of natural monomeric and dimeric compounds belonging to the class of stilbenoids were synthesized and tested against wild-type (*WT*) and strobilurin-resistant (*RES*) strains of *Pyricularia oryzae*, one of the most dangerous fungal phytopathogens. Monomers deoxyrhapontigenin, pinostilbene, and DMHS showed inhibitory activity higher than 40%, with deoxyrhapontigenin having the highest activity on mycelial growth (60−80% inhibition) on both *WT* and *RES* *P. oryzae* strains. Furthermore, we designed and synthesized a set of molecules having a nature-derived stilbene fragment merged with the pharmacophoric portion of strobilurins, namely, a *β*-methoxyacrylate moiety. We identified two molecules with activity comparable to the reference commercial fungicide azoxystrobin. However, low mycelium growth inhibition of resistant strains indicates that these compounds most likely retain the strobilurin-like mechanism of action. Overall, the results suggest that natural stilbenoids might be used as environmentally friendly biofungicides in rice blast management.

KEYWORDS: *stilbenoids, antifungals, biopesticides, Pyricularia oryzae, rice blast pathogen, natural compounds*

1. INTRODUCTION

Pyricularia oryzae is one of the most dangerous fungal phytopathogens. It is the cause of rice blast, a disease inflicting devastating crop losses in world rice production.[1,2] The current major strategies for containing rice blasts are the breeding of resistant varieties and the application of fungicides. Strobilurin-based compounds are among the most effective fungicides in *P. oryzae* management and have played a big role in successful rice cultivation for decades.[3,4]

Strobilurins are a very successful example of fungicides derived from natural compounds. They belong to the group of QoI (quinone outside inhibitors), which act on complex III (cyt *bc1* complex) in the mitochondrial respiration chain. Strobilurin A (Figure 1), isolated from *Strobilurus tenacellus*,[5] was the first compound of this family, followed by strobilurin B and other natural analogues. Strobilurins share a very similar structure characterized by the *β*-methoxyacrylate pharmacophoric portion, an unsaturated bridge, and a side chain. Despite their high potency, natural strobilurins' use was hampered by their low stability, mainly due to a significant photolability.[6]

Extensive chemical modifications of the strobilurin scaffold were carried out to improve the physical characteristics of the parent compounds. The first stable synthetic strobilurin, featuring a benzene ring in place of the central double bond of the original triene system, was methoxyacrylate stilbene (MOAS), which immediately became a lead synthetic strobilurin because of its stability and increased potency.[7,8]

Successive studies led to the introduction into the market of various synthetic analogs, featuring broad structural variations on the side chain as well as bioisosteres of the (*E*)-methoxyacrylate group (e.g., azoxystrobin, Figure 1).

Notwithstanding their enormous potential, strobilurins' single-site mode of action makes them prone to the development of resistance in fungal pathogen populations. Strobilurin resistance, most often caused by a single nucleotide mutation G143A (substitution of glycine at position 143 for alanine), is nowadays widespread and has been described in more than 50 different fungal plant pathogens, among which also *P. oryzae*.[9]

This evidence poses a threat to the global rice growing sector, as widespread distribution of such resistant strains would seriously compromise rice production.

Often, especially in Europe, there is a lack of valid registered alternatives to strobilurins, leaving farmers without adequate means for rice blast management.

The current situation urges the discovery and development of novel, highly effective compounds to control both wild-type and QoI-resistant populations of *P. oryzae*.

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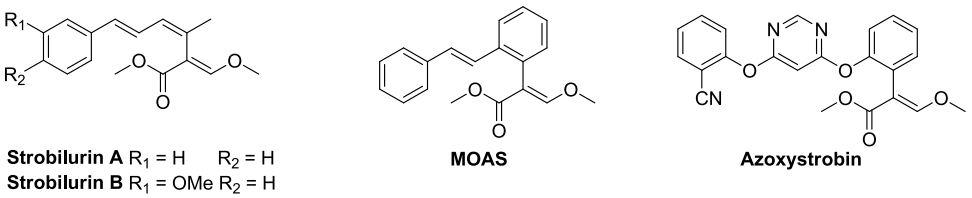

Figure 1. Chemical structures of the representative natural and synthetic strobilurins.

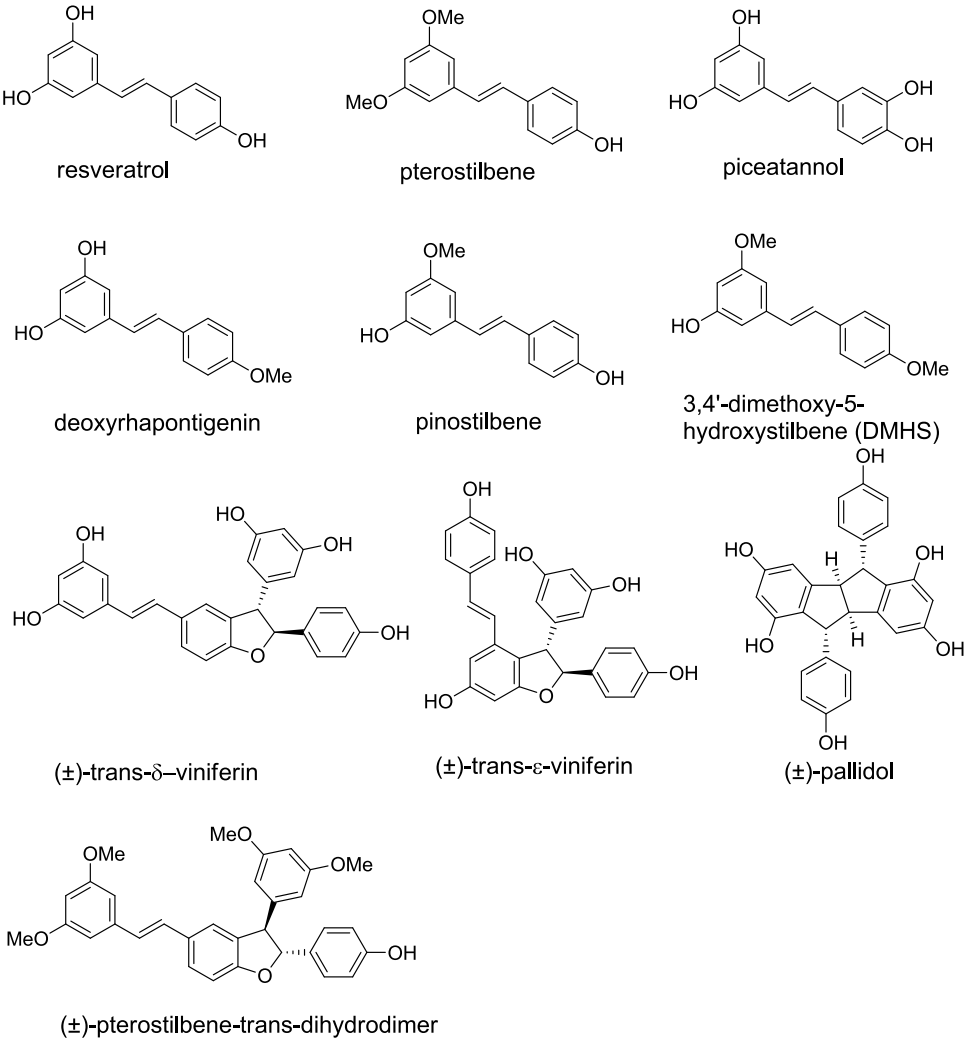

Figure 2. Structures of natural stilbenoids investigated in this study.

In the past decade, the agrochemical industry has refocused its priorities moving toward the use of biological control agents (BCAs), including naturally occurring substances, for an integrated pest management (IPM) program.[10,11]

Recently, the use of natural products from plant extracts as biofungicides has received increasing attention because of their high diversity and versatility. Especially in the context of sustainable agricultural development, research on the transformation of agricultural byproducts into high-value-added extracts or molecules has been intensified. Several active plant byproduct extracts from Vitaceae, Leguminaceae, Gnetaceae, and Dipterocarpaceae contain natural compounds belonging to the class of stilbenoids, a family of nonflavonoid phenylpropanoids structurally characterized by the presence of a 1,2-diphenylethylene unit.[12]

A comprehensive understanding of the mode of action of stilbenoids as antifungals has not yet been completely elucidated. Current evidence suggests multiple mechanisms of action spanning from disruption of the cell wall and membrane to epigenetic effects and interference with fungal mitochondrial functions.[12,13]

These findings hold promise for the development of new multitarget biofungicides containing stilbenoid skeletons to protect plants from fungal phytopathogens. Remarkably, it has been demonstrated that the production of stilbenoids by plants can be activated by adverse conditions, e.g., fungal infections.[14,15] Stark-Lorenzen and co-workers indicated an enhanced resistance to *P. oryzae* of transgenic rice obtained by stable integration and expression of a gene coding for stilbene synthase from grapevine (*Vitis vinifera*).[16] More recently, Niu et al.[17] reported that stilbenoids found in dragon's blood of

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Dracaena cochinchinensis, elicited by fungal species, showed promising activities as natural fungicides. Additionally, Cai et al. demonstrated the inhibitory effects of stilbenes on fungal mycelium growth and spore germination.[18]

In this work, we report the biological activity of a set of natural monomeric and dimeric stilbenoids (Figure 2) against rice blast, both strains sensitive and resistant to strobilurin fungicides. To the best of our knowledge, this is the first study investigating the antifungal activity of a set of plant-derived stilbenoids on *P. oryzae* strains.

Additionally, taking inspiration from the first synthetic MOAS (Figure 1), which featured the stilbene moiety linked to the strobilurin pharmacophore, we designed and synthesized a small series of potential multitarget fungicides in which the stilbenoid skeleton was combined with the strobilurin β-methoxyacrylate moiety. The use of such multitarget fungicides could eventually limit the onset and spread of fungicide resistance, mainly promoted by repeated treatments by agents having a single-site mechanism of action.

The contribution to the biological activity of hydroxy and methoxy groups on the aromatic ring was investigated for both natural and synthetic stilbenoids to rationalize the design of novel hybrid molecules.

2. MATERIALS AND METHODS

2.1. Chemistry: Reagents and Instruments. All reagents and solvents were purchased from commercial suppliers and used without further purification. The ^{1}H NMR and ^{13}C NMR spectra were recorded with a Bruker AV600 (^{1}H, 600 MHz; ^{13}C, 150 MHz) and a Bruker AMX-300 (^{1}H, 300 MHz; ^{13}C 75 MHz) spectrometer. Chemical shifts (δ) are expressed in ppm, and coupling constants (J) are in hertz. All reactions requiring anhydrous conditions were performed under a positive nitrogen flow, and all glassware was oven-dried. Isolation and purification of the compounds were performed by flash column chromatography on silica gel 60 (230−400 mesh). Thin layer chromatography (TLC) analyses were performed by using commercial silica gel 60 F_{254} aluminum sheets. Preparative HPLC purification was performed using a Waters 1525 Binary Pump equipped with a Waters 2489 UV-detector and a column Luna 5 Silica (100 Å), 250 × 21.2 mm^2. HPLC purifications were performed with an isocratic elution (hexane/isopropanol 95:5), flow rate = 15 mL/min, λ = 220 nm.

Hydrophilic lipophilic balance (HLB) and log P were calculated using the ChemAxon calculation method on Marvin Sketch 23.7.[19]

2.2. Chemistry: Synthesis of Target Compounds. Compounds **8**−**10** were prepared as previously reported.[20]

*2.2.1. Methyl (E)-2-(2-((Diethoxyphosphoryl)methyl)phenyl)-3-methoxyacrylate (**11**).* Methyl (*E*)-2-(2-(bromomethyl)phenyl)-3-methoxyacrylate **10** (0.64 mmol, 1 equiv) was dissolved in 0.300 mL of P(OEt)$_3$ (1.72 mmol, 2.7 equiv). The resulting solution was heated to 130 °C and kept under magnetic stirring for 6 h. After reaction completion, P(OEt)$_3$ was evaporated under reduced pressure, and the crude product was purified by column chromatography (cyclohexane/acetone 6:4). Compound **11** was obtained as a yellow oil in a 93% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.58 (s, 1H), 7.52 (d, J = 7.5 Hz, 1H), 7.3 (m, 2H), 7.13 (d, J = 7.5, 1H), 3.96 (m, 2H), 3.83 (s, 3H); 3.70 (s, 3H); 3.10 (m, 2H).

*2.2.2. General Procedure for the Horner−Wadsworth−Emmons (HWE) Olefination of Methyl (E)-2-(2-((Diethoxyphosphoryl)-methyl)phenyl)-3-methoxyacrylate (**11**) with Substituted Aldehydes (**1**−**6**).* A solution of 0.5 M methyl (*E*)-2-(2-((diethoxyphosphoryl)methyl)phenyl)-3-methoxyacrylate **11** (0.22 mmol, 1.1 equiv) and the proper aldehyde (0.20 mmol, 1 equiv) in dry THF was cautiously dropped into a round-bottom flask containing 1 mmol of NaH (5 equiv) in 0.50 mL of dry THF cooled at 0 °C under nitrogen atmosphere. The obtained reaction mixture was stirred at room temperature for 2 h and then quenched with 0.5 M HCl. The aqueous layer was exhaustively extracted with ethyl acetate, the organic phase was dried over Na_2SO_4, filtered, and the solvent was evaporated under reduced pressure. The crude products were purified by column chromatography as described below.

*2.2.3. Methyl (E)-2-(2-((E)-3,4-Bis((tert-butyldimethylsilyl)oxy)-styryl)phenyl)-3-methoxyacrylate (**12**).* 3,4-Dihydroxy benzaldehyde (200 mg, 1.45 mmol) was suspended in dry dichloroethane (14.5 mL) under a nitrogen atmosphere. Imidazole (295 mg, 3 equiv) and *tert*-butyldimethylsilyl chloride (570 mg, 2.6 equiv) were added to the suspension, and the obtained reaction mixture was warmed to 60 °C and stirred for 6 h. After reaction completion, the suspension was cooled to room temperature and washed with brine. The aqueous layer was extracted with dichloromethane, the organic layers were dried over Na_2SO_4, filtered, and the solvent was evaporated. The crude product was purified by column chromatography (cyclohexane/ethyl acetate 95:5), giving the protected aldehyde as a yellow oil in 60% yield. ^{1}H NMR (600 MHz, $CDCl_3$) δ (ppm): 9.80 (s, 1H), 7.35 (m, 2H), 6.91 (m, 1H), 0.97 (m, 18H), 0.21 (m, 12 H). The compound was used for the Horner−Wadsworth−Emmons (HWE) olefination of **11** following the above-reported procedure. The crude product was purified by column chromatography (cyclohexane/acetone 9:1 → 6:4) affording the desired product as a yellow sticky solid in 20% yield. ^{1}H NMR (600 MHz, $CDCl_3$) δ (ppm): 7.69 (d, J = 7.7 Hz, 1H), 7.61 (s, 1H), 7.31 (dt, J = 1.2, 7.5 Hz, 1H), 7.24 (dt, J = 1.2, 7.3 Hz, 1H), 7.15 (dd, J = 1.3, 7.6 Hz, 1H), 6.95 (d, J = 2 Hz, 1H), 6.92 (d, J = 16.3 Hz, 1H), 6.92 (d, J = 2.2 Hz, 1H), 6.80 (d, J = 8.4 Hz, 1H), 3.80 (s, 3H), 3.69 (s, 3H), 1.04 (s, 9H), 1.03 (s, 9H), 0.26 (s, 6H), 0.24 (s, 6H). ^{13}C NMR (300 MHz, $CDCl_3$) δ (ppm): 168.2, 159.9, 146.9, 146.7, 136.7, 131.6, 131.5, 131.1, 129.0, 128.0, 126.9, 125.1, 124.7, 121.1, 120.0, 119.0, 110.6, 61.9, 51.6, 29.6, 26.0 (3C), 25.9 (3C), 18.5, 18.4, −4.0 (4C).

*2.2.4. Methyl (E)-3-Methoxy-2-(2-((E)-styryl)phenyl)acrylate (**1**).* The crude product was purified by flash chromatography (cyclohexane/ethyl acetate 9:1 → 7:3) affording the desired product as a white solid in 15% yield. ^{1}H NMR (600 MHz, $CDCl_3$) δ (ppm): 7.73 (d, J = 7.8 Hz, 1H), 7.64 (s, 1H), 7.46 (d, J = 7.8 Hz, 2H), 7.34 (t, J = 7.5 Hz, 3H), 7.27 (m, 2H), 7.18 (d, J = 7.8 Hz, 1H), 7.08 (d, J = 16.3 Hz, 1H), 7.04 (d, J = 16.3 Hz, 1H), 3.81 (s, 3H), 3.69 (s, 3H). ^{13}C NMR (300 MHz, $CDCl_3$) δ (ppm): 168.5, 160.2, 137.8, 136.6, 131.9, 131.4, 129.6, 128.8 (2C), 128.2, 127.6, 127.4, 127.1, 126.7 (2C), 125.2, 110.6, 62.1, 51.9

*2.2.5. Methyl (E)-2-(2-((E)-4-Hydroxystyryl)phenyl)-3-methoxyacrylate (**2**).* A solution 0.5 M methyl (*E*)-2-(2-((diethoxyphosphoryl)-methyl)phenyl)-3-methoxyacrylate **11** (0.30 mmol, 1.1 equiv) and 4-((*tert*-butyldimethylsilyl)oxy)benzaldehyde (0.27 mmol, 1 equiv) in dry THF was cautiously dropped into a round-bottom flask containing 1.33 mmol of NaH (5 equiv) suspended in 0.63 mL of dry THF cooled at 0 °C under nitrogen atmosphere. The obtained reaction mixture was stirred at room temperature for 3 h and then quenched with $NaHCO_3$ 5%. The aqueous layer was exhaustively extracted with ethyl acetate and the organic phase was dried over Na_2SO_4, filtered, and the solvent was evaporated under reduced pressure. The crude product was purified by column chromatography (cyclohexane/acetone 9:1) affording the desired deprotected product as a yellow sticky solid in 16% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.71 (d, J = 7.7 Hz, 1H), 7.66 (s, 1H), 7.38−7.32 (m, 3H), 7.28 (td, J = 7.4, 1.2 Hz, 1H), 7.19 (dd, J = 7.7, 1.2 Hz, 1H), 7.00 (d, J = 16.2 Hz, 1H), 6.93 (d, J = 16.2 Hz, 1H), 6.83 (d, J = 8.6 Hz, 2H), 3.82 (s, 3H), 3.71 (s, 3H). 13C NMR (400 MHz, $CDCl_3$) δ: 168.8, 160.2, 155.5, 136.8, 131.4, 131.2, 130.5, 129.1, 128.1, 127.9, 126.9, 124.9, 124.7, 115.6, 110.6, 62.0, 51.9.

*2.2.6. Methyl (E)-3-Methoxy-2-(2-((E)-4-methoxystyryl)phenyl)-acrylate (**4**).* The crude product was purified by preparative TLC (cyclohexane/ethyl acetate 8:2) affording the desired product as a yellow sticky solid in 10% yield. ^{1}H NMR (600 MHz, $CDCl_3$) δ (ppm): 7.70 (d, J = 7.8 Hz, 1H), 7.63 (s, 1H), 7.39 (d, J = 8.6 Hz, 2H), 7.32 (t, J = 7.1 Hz, 1H), 7.25 (t, J = 7.3 Hz, 1H), 7.16 (d, J = 7.8 Hz, 1H), 6.99 (d, J = 16.3 Hz, 1H), 6.93 (d, J = 16.3 Hz, 1H), 6.88 (d, J = 8.6 Hz, 2H), 3.83 (s, 3H), 3.80 (s, 3H), 3,68 (s, 3H). ^{13}C NMR (600 MHz, $CDCl_3$) δ (ppm): 168.4, 160, 159.2, 136.8, 131.5,

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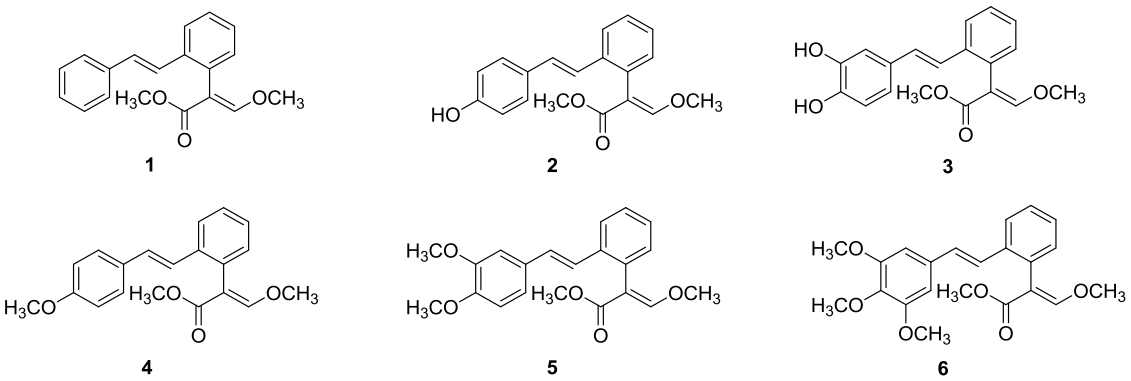
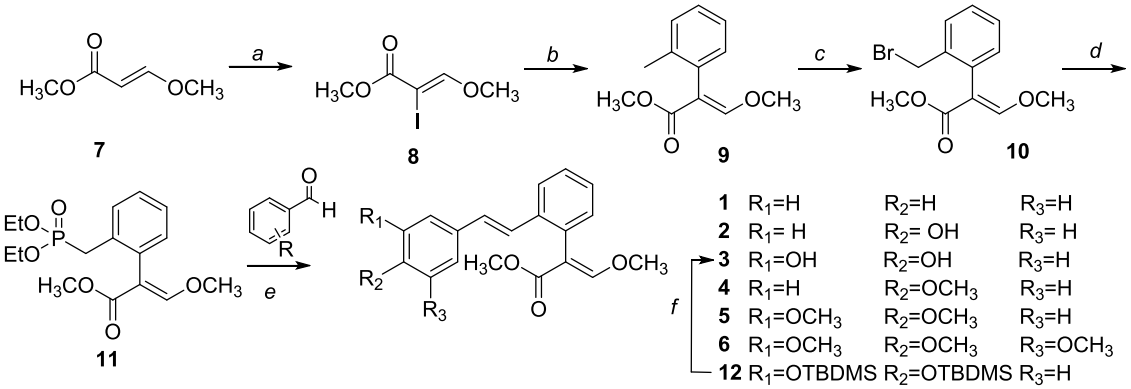


Figure 3. Chemical structures of synthesized methoxyacrylate-stilbene hybrids (MOAS).

Scheme 1. Synthesis of Compounds 1−6



Reagents and conditions: (a) (1) NIS, AcOH, DCM, rt, 24 h; (2) TEA, DCM, rt, 16 h, 62%; (b) 2-tolylboronic acid, $Pd(PPh_3)_4$, K_3PO_4, Dioxane:H_2O 5:1, 90 °C, 6 h, 76%; (c) NBS, AIBN, CCl_4, reflux, 6 h, 69%; (d) $P(OEt)_3$, 130 °C, overnight, N_2, 54%. (e) NaH, THF, N_2, 0 °C → rt, 20%; (f) From **12** to **3**: TBAF, THF 0 °C → rt, 1 h, 25%.

131.2, 130.5, 129.3, 128.9, 128, 127.7, 126.8, 124.9, 124.8, 114.1, 110.6, 61.9, 55.3, 51.7.

*2.2.7. Methyl (E)-2-(2-((E)-3,4-Dimethoxystyryl)phenyl)-3-methoxyacrylate (**5**).* The crude product was purified by flash chromatography (cyclohexane/ethyl acetate 9:1 → 7:3) followed by preparative HPLC affording the desired product as a colorless oil in 10% yield. ^{1}H NMR (600 MHz, $CDCl_3$) δ (ppm): 7.69 (d, J = 7.9 Hz, 1H), 7.62 (s, 1H), 7.33 (t, J = 7.4 Hz, 1H), 7.26 (t, J = 7.9 Hz, 1H), 7.17 (d, J = 7.4 Hz, 1H), 7.09 (d, J = 8.3 Hz, 1H), 6.98 (d, J = 16 Hz, 1H), 6.97 (d, J = 3.6 Hz, 1H), 6.92 (d, J = 16.3 Hz, 1H), 6.85 (d, J = 8.3 Hz 1H), 3.93 (s, 3H), 3.90 (s, 3H), 3.81 (s, 3H), 3.68 (s, 3H). ^{13}C NMR (600 MHz, $CDCl_3$) δ (ppm): 168.4, 160.0, 149.0, 148.8, 136.7, 131.5, 131.2, 130.9, 129.2, 128.0, 126.9, 125.3, 124.9, 119.5, 111.3, 110.6, 109.4, 61.9, 55.9, 51.7.

*2.2.8. Methyl (E)-3-Methoxy-2-(2-((E)-3,4,5-trimethoxystyryl)-phenyl)acrylate (**6**).* The crude product was purified by flash chromatography (cyclohexane/ethyl acetate 9:1 → 7:3) followed by preparative HPLC affording the desired product as a yellow oil in 10% yield. ^{1}H NMR (600 MHz, $CDCl_3$) δ (ppm): 7.69 (d, J = 7.8 Hz, 1H), 7.63 (s, 1H), 7.33 (dt, J = 7.4, 1.2 Hz, 1H), 7.28 (dt, J = 7.4, 1.1 Hz, 1H), 7.18 (dd, J = 7.5, 1.1 Hz, 1H), 6.95 (s, 2H), 6.67 (s, 2H), 3.90 (s, 6H), 3.86 (s, 3H), 3.82 (s, 3H), 3.69 (s, 3H). ^{13}C NMR (600 MHz, $CDCl_3$) δ (ppm): 168.3, 160.0, 153.3, 137.9, 136.4, 133.5, 131.6, 131.3, 129.5, 128.0, 127.2, 126.7, 125.1, 110.6, 103.8, 62.0, 60.9, 56.2, 53.4, 51.7.

*2.2.9. Methyl (E)-2-(2-((E)-3,4-Dihydroxystyryl)phenyl)-3-methoxyacrylate (**3**).* A 0.05 M solution of **12** in dry THF was prepared under a nitrogen atmosphere and cooled to 0 °C in an iced bath. 2.6 equiv of tetrabutylammonium fluoride (TBAF) was added to the solution, which was warmed up to room temperature and stirred for 1 h. After completion, the reaction was neutralized with water and the aqueous layer was extracted 3 times with ethyl acetate. The organic phase was washed with 0.1 N HCl, dried over Na_2SO_4, and filtered. The solvent was evaporated under reduced pressure. The crude product was purified by column chromatography (dichloromethane/methanol 98:2), affording the desired product as a colorless oil with a 25% yield. ^{1}H NMR (600 MHz, $CDCl_3$) δ (ppm): 7.68 (s, 1H), 7.64 (d, J = 7.9 Hz, 1H), 7.25 (td, J = 1.2, 7.4 Hz, 1H), 7.16 (td, J = 1.2, 7.6 Hz, 1H), 7.05 (dd, J = 1.2, 7.6 Hz, 1H), 6.91 (d, J = 2.2 Hz, 1H), 6.84 (d, J = 16.3 Hz, 1H), 6.80 (d, J = 16.3 Hz, 1H), 6.76 (dd, J = 2.2, 8.2 Hz, 1H), 6.70 (d, J = 8.2 Hz, 1H), 3.80 (s, 3H), 3.66 (s, 3H). ^{13}C NMR (600 MHz, $CDCl_3$) δ (ppm): 169.1, 160.5, 143.9, 143.7, 136.8, 131.5 (2C), 131.3, 129.2, 128.2, 127.1, 125.0 (2C), 120.2, 115.6, 113.1, 110.7, 62.3, 51.9.

2.3. Biology: *In Vitro* Biological Activity of Natural and Synthetic Methoxyacrylate-Stilbene Compounds. *2.3.1. Fungal Strains.* In this study, four strains of *P. oryzae* were used: two strains sensitive to quinone outside inhibitor (QoI) fungicides (*WT*): A252 and TA102, and two strains resistant to QoI (*RES*): PO1312 and PO1336. The strains belong to a vast collection of *P. oryzae* monoconidial isolates maintained at the Laboratory of plant pathology, University of Milan. The strains were maintained as single-spore isolates on malt-agar medium (MA: 20 g/L malt extract, Oxoid, U.K.; 15 g/L agar, Oxoid, U.K.) at 4 °C.

2.3.2. Inhibition of Mycelium Growth of P. oryzae. The *P. oryzae* mycelium inhibition by the tested compounds was evaluated as previously described.[20,21] Briefly, a 0.5 cm mycelium plug obtained from actively growing fungal colonies of *P. oryzae* strains was transferred to MA medium plates supplemented or not with tested compounds in three biological replicates. The natural stilbenoids were tested at the concentration of 200 μM, while the synthesized methoxyacrylate-stilbene hybrids were tested at the concentration of 25 mg/L. Due to the low solubility of the tested molecules in water, they were dissolved in DMSO or acetone. Therefore, multiple

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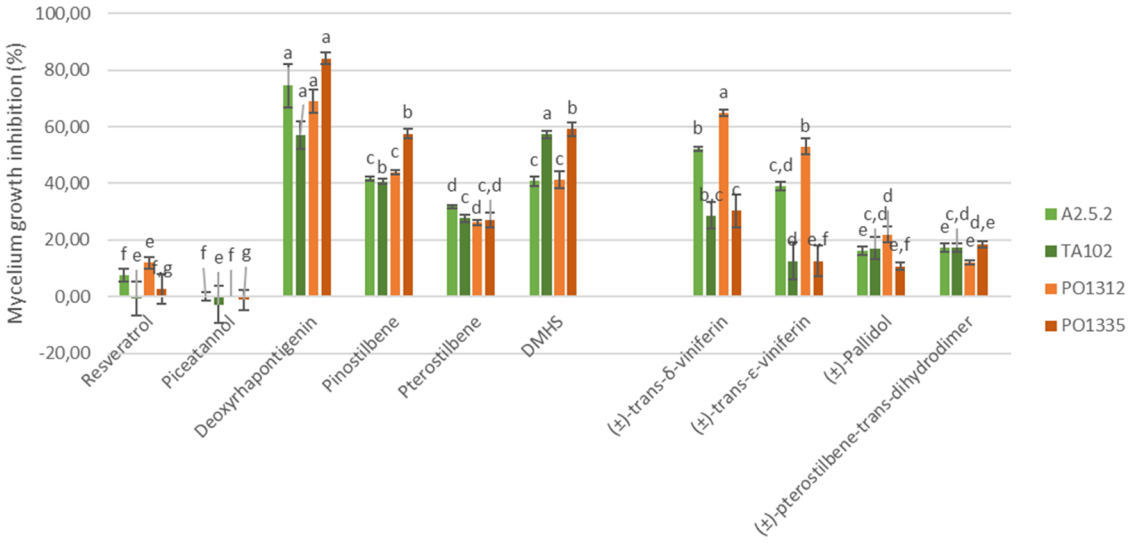

Figure 4. Inhibitory activity of natural stilbenes on mycelium growth of *P. oryzae* wild-type (green; A2.5.2 and TA102) and strobilurin-resistant (orange; PO1312, PO1335) strains. The error bars represent the standard deviation. Bars of the same color (the same strain) with different letters are statistically different (Tukey's HSD, $p \leq 0.05$).

controls were included: MA medium without any supplement (MA), MA medium supplemented with DMSO or acetone (ACT) at a final concentration of 1% v/v. To assess the activity of the synthesized methoxyacrylate-stilbene hybrids, the commercial fungicide azoxystrobin (AZX) was used as a reference. The plates were incubated at 24 °C in the dark. The mycelium growth was measured at 7 days after inoculation (DAI), and the inhibition of mycelium growth (%) was calculated by comparing the mycelium growth on control (corresponding solvent) and compound-supplemented plates. The inhibition percentage was calculated as $I\% = (C - T)/C \times 100$, where C = mycelium growth in the control medium and T = mycelium growth in the medium added to the tested compound. For AZX, the control was MA medium, while for tested compounds, it was DMSO or ACT. For the quantitative analysis of deoxyrhapontigenin activity, the compound was tested at the following concentrations: 5−10−50−100−200 μM as previously described.

2.3.3. Statistical Analysis of the Mycelium Inhibition Growth Data. The statistical analyses were performed using R software, version 4.2.2.[22] The percentage data of mycelium growth inhibition were square root arcsine transformed and submitted to ANOVA, followed by Tukey's post hoc test for multiple comparison ($P = 0.05$), using the TukeyC package.[23]

For the quantitative study of deoxyrhapontigenin activity, the dose−response curve for mycelium growth was plotted by nonlinear regression analysis using a three-parameter log−logistic model, employing the drc package.[24] The ED_{50} value (the compound concentration causing 50% inhibition of mycelium growth) was estimated by interpolation from the fitted regression curve.

3. RESULTS AND DISCUSSION

3.1. Chemistry: Synthesis of Target Compounds. We prepared a set of natural stilbenoids including monomers (resveratrol, pterostilbene, piceatannol, deoxyrhapontigenin, pinostilbene, and 3,4′-dimethoxy-5-hydroxystilbene) and dimers (δ-viniferin, pterostilbene, ε-viniferin, and pallidol) (Figure 2) to have an overview of the natural compounds' antifungal activity. All of these molecules were synthesized following previously reported procedures.[25,26]

Successively, prompted by our recent interest in the development of multitarget antifungals[20,21] and taking inspiration from the structure of MOAS, we designed and synthesized a set of molecules having the QoI pharmacophore merged with a nature-derived stilbene fragment. The series included the unsubstituted MOAS (**1**) itself and five analogues decorated with phenolic and methoxy groups (Figure 3).

Table 1. Calculated Values of log *P* and HLB for Each Compound in Relation to the Antifungal Activity[a]

compound	*c* log *P*	HLB	mycelium growth inhibition (%)
deoxyrhapontigenin	3.55	8.30	60−80
DMHS	3.69	7.91	40−60
pinostilbene	3.55	8.30	40−60
pterostilbene	3.69	7.91	25−30
resveratrol	3.40	8.66	10−15
piceatannol	3.10	10.55	0
trans-δ-viniferin	5.96	9.35	40−60
trans-ε-viniferin	5.96	9.35	35−55
pallidol	5.31	8.93	20
pterostilbene-trans-dihydrodimer	6.54	7.29	15−20

[a] *c* Log *P* and HLB values were obtained by using the ChemAxon calculation method on Marvin Sketch 23.7.

The synthetic route of compounds **1**−**6** involved the Suzuki coupling of α-iodo-β-methoxyacrylate **8** with 2-tolylboronic acid, to give 2-methyl aryl-BMA **9**, which was converted into the corresponding benzyl bromide by radical bromination in the presence of *N*-bromosuccinimide (NBS).[20] Benzyl bromide underwent a Michaelis−Arbuzov reaction, giving the key phosphonate intermediate **11** that was finally reacted with variously substituted aromatic aldehydes in a Horner−Wadsworth−Emmons (HWE) olefination (Scheme 1). The preparation of compound **3** involved the protection of 3,4-dihydroxy benzaldehyde with *tert*-butyldimethylsilyl chloride in the presence of imidazole and dichloroethane as a solvent to give compound **12** upon reaction with phosphonate **11**. Silyl groups were eventually removed by treatment with tetrabutylammonium fluoride (TBAF) to afford compound **3**.

3.2. Evaluation of the Antifungal Activity of Natural Stilbenoids and Methoxyacrylate-Stilbene Hybrids. The biological activity of the natural stilbenoids and synthesized methoxyacrylate-stilbene hybrids was evaluated by assessing the inhibition of mycelium growth of two strobilurin-sensitive or wild-type (*WT*, A252 and TA102) and two strobilurin-

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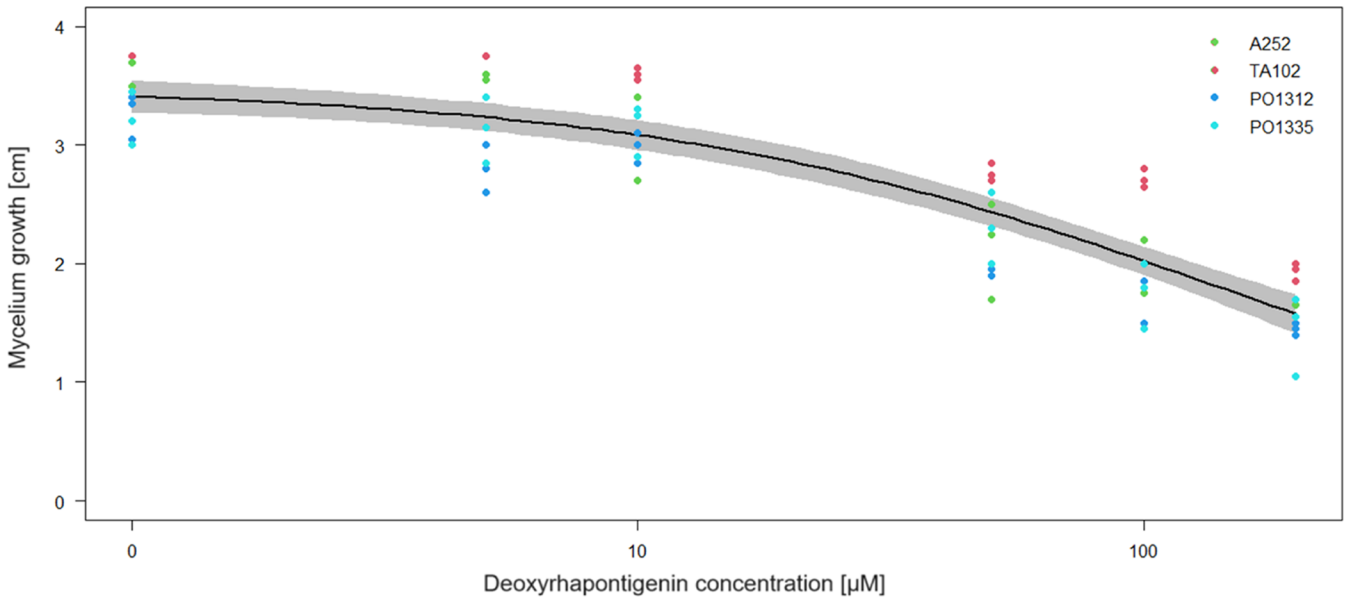

Figure 5. Mycelium growth dose−response curve of *P. oryzae* to increasing concentrations of deoxyrhapontigenin measured after 7 days of growth. The curve (black line) was calculated using the 3-parameter log−logistic model. The 95% confidence interval is depicted by gray shadow. The data of the four strains are indicated by colored dots.

Table 2. ED_{50} Values of Deoxyrhapontigenin (Concentration Causing 50% Mycelium Growth Inhibition) for the Four *P. oryzae* Strains, Calculated Using the 3-Parameter Log-Logistic Model after 7 Days of Growth

strain	ED_{50} [μM] (std. error)
A252	122.3 (±22.1)
TA102	237.9 (±39.4)
PO1312	123.5 (±25.2)
PO1335	132.3 (±18.5)
total	154.7 (±19.6)

resistant (*RES*, PO1312 and PO1335) strains of *P. oryzae*. The compounds were dissolved in DMSO or acetone (ACT) (see the Materials and Methods Section for details). DMSO at a final concentration of 1% in the growth medium slightly inhibited the growth of the used *P. oryzae* strains (11% growth inhibition), while 1% ACT did not have any inhibitory effect on the growth of the strains (Supporting Table S1).

3.2.1. Biological Evaluation of Natural Stilbenoids. The inhibitory activity of natural stilbenoids (Figure 2) against *WT* and *RES* strains of *P. oryzae* was assessed at 200 μM concentration (Figure 4). Specifically, resveratrol showed only minimal inhibition of mycelium growth of all tested strains. Introducing an additional −OH group on C-3 in piceatannol completely abolished its biological activity. Interestingly, replacing a phenolic −OH in resveratrol skeleton with one methoxy group (4′-OCH_3 in deoxyrhapontigenin and 3-OCH_3 in pinostilbene) drastically improved the activity, with 60−80% inhibition by deoxyrhapontigenin even on strobilurin-

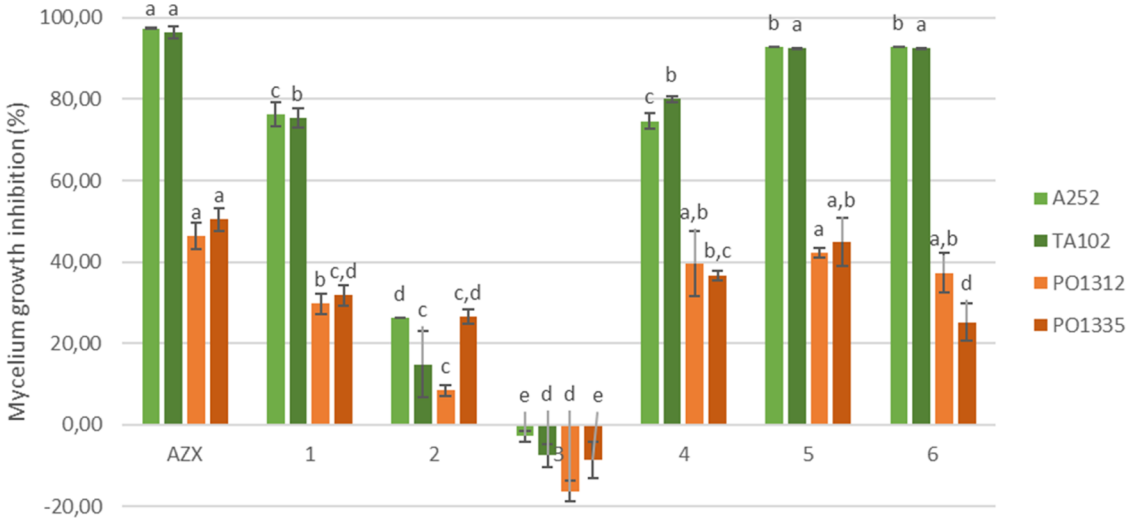

Figure 6. Inhibitory activity of MOAS hybrids **1**−**6** on mycelium growth of *P. oryzae* wild-type (green; A2.5.2, and TA102) and strobilurin-resistant (orange; PO1312, PO1335) strains. Azoxystrobin (AZX) was used as a reference compound. The error bars represent standard deviation. Bars of the same color (the same strain) with different letters are statistically different (Tukey's HSD, $p \leq 0.05$).

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resistant strains. The position of the methoxy group seems to play a major role, as deoxyrhapontigenin shows almost double the activity of pinostilbene. The introduction of two methoxy groups was shown to be detrimental, with a reduction of activity for both DMHS (40−60%) and pterostilbene (25−30%) on *WT* and *RES* strains.

Among dimers, the most active natural compound was *δ*-viniferin (40−60% mycelial growth inhibition), followed by the other resveratrol-derived dimer *ε*-viniferin (35−55%). Pallidol showed a modest activity (20%) as well as the tetramethoxy derivative pterostilbene dihydrodimer (15−20%).

To investigate the potential correlation between the efficacy of the tested natural compounds and their physicochemical properties, log *P* and hydrophilic−lipophilic balance (HLB) were calculated using the ChemAxon calculation method on Marvin Sketch 23.7.

More specifically, methylated monomers, showing significant inhibition profiles (40−80%), are characterized by *c* log *P* and HLB values in the range 3.55−3.69% and 7.91−8.30, respectively. Conversely, more polar compounds resveratrol and piceatannol are considerably less active in inhibiting mycelium growth (Table 1). Among tested dimers, the most active *δ*- and *ε*-viniferin show *c* log *P* = 5.96 and HLB > 9. Lower activities are observed for pallidol with HLB = 8.93, and pterostilbene-trans-dihydrodimer characterized by a higher degree of methylation, with HLB = 7.29. Importantly, other properties apart from *c* log *P* and HLB, i.e., 3D shape, geometry, and substitution pattern (substituent and position involved), could play a crucial role in the observed biological effect.

In general, the similar behavior of all natural compounds on both *WT* and *RES* strains evidence that they are not sensitive to the G143A mutation present in *RES* strains. Importantly, this result confirms that the promising activity of this class of natural compounds is not related to cyt *bc1* complex inhibition and supports the idea of creating multitarget strobilurin-stilbenoid antifungals.

As deoxyrhapontigenin showed the best mycelium inhibition at a concentration of 200 *μ*M, we studied its activity in a more detailed, quantitative way. The mycelium growth of the four strains was assessed at diverse concentrations ranging from 5 to 200 *μ*M (Figure 5). From the data, the ED_{50}, the compound concentration that causes 50% mycelium growth inhibition, was calculated using the 3-parameter log−logistic model (Table 2). The ED_{50} value ranged from 122.3 to 237.9 *μ*M among the four tested strains.

3.2.2. Biological Evaluation of Synthetic Methoxyacrylate-Stilbene Hybrids. We designed and synthesized a set of molecules having the QoI pharmacophore merged with a nature-derived stilbene fragment. The newly obtained compounds **1**−**6** were screened on the same wild-type (*WT*, A252 and TA102) and strobilurin-resistant (*RES*, PO1312 and PO1335) strains of *P. oryzae* previously used to assess the activity of natural stilbenoids (Figure 6). The synthesized hybrid molecules were tested at a final concentration of 25 mg/L, the same as that of the reference commercial fungicide azoxystrobin (AZX). AZX inhibited >90% of the growth of *WT* strains, while the *RES* strains were inhibited to <50%, confirming the influence of the G143A mutation on the activity of strobilurin fungicides.

As expected, MOAS (**1**) showed a slightly lower activity on *WT* strains (<80%) compared to the commercial fungicide AZX. The introduction of polar phenolic OH groups in compounds **2** and **3** had a deleterious effect. Increasing the number of methoxy groups from one (compound **4**) to two (compound **5**) improved the biological activity against *WT* strains from ca. 70 to >90% inhibition, respectively. An additional methoxy group in compound **6**, however, did not further boost its activity. These results highlight that the presence of methoxy groups and their number should be considered to be a crucial parameter for the activity in *WT* strains.

On the other hand, lower biological activity was observed on resistant strains (20−45%), suggesting that the most active methoxyacrylate-stilbene hybrids still act as strobilurin fungicides, being sensitive to the presence of the G143A mutation in cyt *bc1* complex.

Our efforts allowed us to identify methylated stilbenoids as a promising class of natural fungicides with a low environmental impact. In particular, the monomers deoxyrhapontigenin, pinostilbene, and DMHS showed inhibitory activity higher than 40%, with deoxyrhapontigenin having the highest activity on mycelial growth (60−80% at 200 *μ*M concentration). Remarkably, the comparable inhibition of both *WT* and *RES* strains by the tested natural compounds outlined a mechanism of action different from strobilurins. Overall, this study suggests that natural stilbenoids might be developed into environmentally friendly biofungicides for rice blast management. Current research activities are dedicated to the evaluation of other natural chemotypes to be combined and conjugated with the most promising stilbenoids identified in this work.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsagscitech.3c00275.

Inhibition of mycelium growth of *P. oryzae* on media supplemented with acetone or DMSO at a final concentration 1% (Table S1) (PDF)

■ AUTHOR INFORMATION

Corresponding Author

Sabrina Dallavalle − *Department of Food, Environmental and Nutritional Sciences (DeFENS), University of Milan, 20133 Milan, Italy;* orcid.org/0000-0002-8813-8922; Phone: +39-02-50316818; Email: sabrina.dallavalle@unimi.it

Authors

Andrea Kunova − *Department of Food, Environmental and Nutritional Sciences (DeFENS), University of Milan, 20133 Milan, Italy*

Cecilia Pinna − *Department of Food, Environmental and Nutritional Sciences (DeFENS), University of Milan, 20133 Milan, Italy*

Sharmila Ghosh − *Department of Food, Environmental and Nutritional Sciences (DeFENS), University of Milan, 20133 Milan, Italy*

Denise Dozio − *Department of Food, Environmental and Nutritional Sciences (DeFENS), University of Milan, 20133 Milan, Italy;* orcid.org/0000-0001-7425-1660

Cristina Pizzatti − *Department of Food, Environmental and Nutritional Sciences (DeFENS), University of Milan, 20133 Milan, Italy*

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Salvatore Princiotto − *Department of Food, Environmental and Nutritional Sciences (DeFENS), University of Milan, 20133 Milan, Italy*

Paolo Cortesi − *Department of Food, Environmental and Nutritional Sciences (DeFENS), University of Milan, 20133 Milan, Italy*

Andrea Pinto − *Department of Food, Environmental and Nutritional Sciences (DeFENS), University of Milan, 20133 Milan, Italy;* orcid.org/0000-0002-2501-3348

Complete contact information is available at: https://pubs.acs.org/10.1021/acsagscitech.3c00275

Author Contributions

†A.K. and C.P. contributed equally to this work and should be considered co-first authors.

Notes

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