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ORIGINAL ARTICLE

Synthesis of Knoevenagel Adducts Under Microwave Irradiation and Biocatalytic Ene-Reduction by the Marine-Derived Fungus *Cladosporium* sp. CBMAI 1237 for the Production of 2-Cyano-3-Phenylpropanamide Derivatives

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Abstract

The organic synthesis has been driven by the need of sustainable processes, which also requires efficiency and cost-effectiveness. In this work, we described the synthesis of nine Knoevenagel adducts between cyanoacetamide and aromatic aldehydes ((*E*)-2-cyano-3-(phenyl)acrylamide derivatives), employing triethylamine as catalyst under microwave irradiation in 30 min with excellent yields (93–99% yield). Then, these adducts were employed in the C–C double bond bioreduction by the marine-derived fungus *Cladosporium* sp. CBMAI 1237 for obtention of 2-cyano-3-phenylpropanamide derivatives in mild conditions and short reaction time for a whole-cells reduction (phosphate buffer pH 7.0, 32 °C, 130 rpm, 8 h) with good yields (48–90%). It is important to emphasize that the experimental conditions, especially the reaction time, should be carefully evaluated for the obtention of high yields. Since a biodegradation process consumed the obtained product in extended periods, probably due to the use of the substrate as carbon and nitrogen source. This approach showed that the use of coupled and greener catalysis methods such as microwave irradiation and biocatalytic reduction, which employs unique biocatalysts like marine-derived fungi, can be an interesting tool for the obtention of organic molecules.

Keywords Biotransformation · Biocatalysis · Bioreduction · Green chemistry · Knoevenagel condensation

Introduction

The use of enzymes for reductive processes has been driven by the need of green and sustainable processes, which also requires efficiency and cost-effectiveness (González-Martínez et al. 2019; Toogood and Scrutton 2014). In this context, reductases have been employed for reduction of different substrates, including ketones, aldehydes, carboxylic acid derivatives, nitriles, and nitro compounds (Winkler et al. 2018). Moreover, whole-cells biocatalysis also presented interesting results, employing fungi, bacteria, plant, and algae for reduction reactions (Birolli et al. 2015; Garzon-Posse et al. 2018; Lin and Tao 2017).

Marine-derived fungi have been successfully employed for reduction of ketones (Liu et al. 2018) and alkenes (de Matos et al. 2019). For example, free and immobilized cells of *Penicillium citrinum* CBMAI 1186 reduced C–C double bonds of α,β-, di-α,β-, and mono-α,β,γ,δ-unsaturated ketones with good yields and selectivities (Ferreira et al. 2015; Ferreira et al. 2014), and also reduced in high yields

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benzylidene-malononitrile derivatives, which were produced by Knoevenagel condensation reactions (Jimenez et al. 2016).

The Knoevenagel condensation is a synthetic tool very useful for the increase of carbon chains, as well as, for functionalization (Khare et al. 2019; Nokami et al. 2001), being applied for the synthesis of polymers (Kwak and Fujiki 2004), intermediates (Zhang et al. 2014), and different bioactive organic compounds (Coulibaly et al. 2015) such as α-glucosidase inhibitors (Kashtoh et al. 2016), antidepressants (Mishra and Hajra 2015), cancer therapy (Ramesh and Lalitha 2015), vasodilators (Girgis et al. 2011), and antimicrobial substances (Al-Mousawi et al. 2016).

Microwave irradiation has been employed in the synthesis of Knoevenagel adducts in mild conditions, short times, easy handling, and using solvents with low toxicity (Zanin et al. 2018). The use of different methods that promote advances towards the green chemistry principles, as biocatalysis and low intensity irradiation, is an interesting approach for new methodologies (Erythropel et al. 2018).

Therefore, the aim of this work was the obtention of the Knoevenagel adducts from aromatic aldehydes and cyanoacetamide under microwave irradiation followed by a C–C double bond reduction by mycelia of marine-derived fungus.

Material and Methods

Reagents and Solvents

The 4-methoxybenzaldehyde (98%) **1a**, 4-nitrobenzaldehyde (98%) **1b**, 4-fluorobenzaldehyde (98%) **1c**, 4-chlorobenzaldehyde (97%) **1d**, 4-bromobenzaldehyde (99%) **1e**, vanillin (99%) **1f**, 3,4,5-trimetoxibenzaldehyde (99%) **1 g**, syringaldehyde (98%) **1 h**, 2-thiophenecarboxaldehyde (98%) **1i**, cyanoacetamide (99%), and sodium borohydride (≥ 98%) were obtained from Sigma-Aldrich.

Anthracene (99%) was acquired from ACROS Organics. The salts employed for production of artificial seawater were purchased from Vetec and Synth. Deuterated chloroform ($CDCl_3$, 99.9%), methanol (MeOD, 99.9%), acetone-d_6 (99.9%), and DMSO-d_6 (99.9%) were acquired from the Cambridge Isotope Laboratories. The high-pressure liquid chromatography (HPLC) grade solvents; hexane, isopropanol and ethyl acetate were obtained from Panreac. The extraction and purification solvents, hexane and ethyl acetate, were obtained from Synth.

Knoevenagel Condensation of Cyanoacetamide and Aromatic Aldehydes 1a-i under Microwave Irradiation

In a round bottom flask of 25 mL was added cyanoacetamide (1.0 mmol), aromatic aldehyde **1a-i** (1.0 mmol), H_2O (4 mL) and triethylamine (10 mol%). Then, the reaction was performed in a CEM Discover reflux open vessel of 300 W with infrared temperature control in 55 W at 85 °C for 30 min. The reaction progress was monitored by Thin Layer Chromatography (TLC) and revealed with iodine-impregnated silica gel.

Subsequently, the reaction was filtered and extracted three times with 10 mL of ethyl acetate. The obtained organic phases were combined, dried with anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure. The products were purified in column chromatography with silica flash using hexane and ethyl acetate (7:3). All the obtained adducts **2a-I** were characterized by melting point (m.p.), Fourier-Transform Infrared Spectroscopy (FTIR), Nuclear Magnetic Resonance (NMR), and Mass Spectrometry (MS).

The m.p. was obtained employing a melting point apparatus Fisatom 431. The IR spectra were acquired from 400 to 400 cm^{-1} with a Shimadzu IRAffinity spectrometer preparing the samples in KBr disks. The MS spectra were obtained in a Shimaduzu MS2010 spectrometer (70 eV). The NMR analyses were carried out on an Agilent 400/54 or Agilent 500/54 premium-shielded spectrometer operated at frequencies of 500 and 400 MHz (^{1}H NMR) or 125 and 100 MHz (^{13}C NMR), respectively. All performed analyzes can be verified in the Electronic Supplementary Material-1 (ESM-1).

(*E*)-2-Cyano-3-(4-Methoxyphenyl)Acrylamide (2a)

$C_{11}H_{10}N_2O_2$, 202.21 g.mol^{-1}; (99% yield); white crystal; m.p. = 117–118 °C; FTIR ν_{max} (cm^{-1}) = 3445, 3305, 3175, 2209, 1698, 1582, 1508; ^{1}H NMR (500 MHz, $CDCl_3$) δ (ppm) = 8.3 (s, 1H), 8.0 (m, 2H), 7.0 (m, 2H), 3.9 (s, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ (ppm) = 164, 163, 153, 133, 124, 115, 99, 56; MS (70 eV) *m/z* = 202 (100%); 201 (100%); 158 (33%); 89 (28%); 186 (24%); 77 (18%); 115 (18%); 114 (18%).

(*E*)-2-Cyano-3-(4-Nitrophenyl)Acrylamide (2b)

$C_{12}H_7N_3O_3$, 217.18 g.mol^{-1}; (95% yield); red solid; m.p. = 110–112 °C; FTIR ν_{max} (cm^{-1}) = 3435, 3344, 2223, 1691, 1601, 1516, 1345; ^{1}H NMR (500 MHz, DMSO-d_6) δ (ppm) = 8.4 (m, 2H), 8.3 (s, 1H), 8.1 (d, *J* = 8.8 Hz, 2H), 8.1 (s, 1H), 7.9 (s, 1H); ^{13}C NMR (126 MHz, DMSO-d_6) δ (ppm) = 163, 149, 148, 138, 131, 131, 125, 124, 116, 111; MS (70 eV) *m/z* = 216 (100%); 200 (92%); 170 (81%); 217 (63%); 89 (49%); 44 (46%); 101 (42%); 75 (41%).

(*E*)-2-Cyano-3-(4-Fluorophenyl)Acrylamide (2c)

$C_{10}H_7N_2OF$, 190.18 g.mol^{-1}; (94% yield); white crystal; m.p. = 89–91 °C; FTIR ν_{max} (cm^{-1}) = 3336, 3192, 2924,

2211, 1691, 1613, 1588, 1487; ^{1}H NMR (500 MHz, DMSO-d_6) δ (ppm) = 8.2 (s, 1H), 8.0 (m, 2H), 7.8 (d, J = 8.0 Hz, 2H), 7.4 (m, 2H); ^{13}C NMR (126 MHz, DMSO-d_6) δ (ppm) = 165 (d, J = 252 Hz, 1C), 163, 163, 150, 133 (d, J = 9 Hz, 1C), 129 (d, J = 3 Hz, 1C), 117 (d, J = 22 Hz, 1C), 117,107; MS (70 eV) m/z = 189 (100%); 120 (60%); 190 (59%); 146 (41%); 126 (35%); 44 (30%); 75 (28%); 147 (21%).

(*E*)-2-Cyano-3-(4-Chlorophenyl)Acrylamide (2d)

$C_{10}H_7N_2OCl$, 206.63 g.mol^{-1}; (92% yield); pale yellow solid; m.p. = 91–93 °C; FTIR ν_{max} (cm^{-1}) = 3336, 3192, 2215, 1681, 1597, 1380; ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm) = 8.2 (s, 1H), 8.0 (m, 3H), 7.8 (s, 1H), 7.7 (m, 2H); ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm) = 166, 163, 150, 137, 132, 132, 131, 131, 130, 129, 117, 108; MS (70 eV) m/z = 205 (100.00%); 206 (62%); 207 (39%); 136 (33%); 127 (28%); 75 (26%); 162 (25%); 208 (22%).

(*E*)-2-Cyano-3-(4-Bromophenyl)Acrylamide (2e)

$C_{10}H_7N_2OBr$, 249.08 g.mol^{-1}; (91% yield); yellow solid; m.p. = 95–97 °C; FTIR ν_{max} (cm^{-1}) = 3336, 3192, 2217, 1683, 1597, 1465, 1370; ^{1}H NMR (500 MHz, DMSO-d_6) δ (ppm) = δ 8.2 (s, 1H), 7.9 (m, 2H), 7.8 (m, 3H); ^{13}C NMR (126 MHz, DMSO-d_6) δ (ppm) = 163, 150, 133, 132, 132, 126, 117, 108; MS (70 eV) m/z = 251 (100%); 249 (99%); 127 (79%); 250 (62%); 252 (61%); 75 (42%); 128 (40%); 171 (39%).

(*E*)-2-Cyano-3-(4-Hydroxy-3-Methoxyphenyl)Acrylamide (2f)

$C_{11}H_{10}N_2O_3$, 218.21 g.mol^{-1}; (96% yield); black solid; m.p. = 134–135 °C; FTIR ν_{max}(cm^{-1}) = 3335, 3198, 2924, 2213, 1667, 1570, 1514, 1290; ^{1}H NMR (500 MHz, MeOD) δ (ppm) = 8.1 (s, 1H), 7.8 (d, J = 2.1 Hz, 1H), 7.5 (d, J = 2.3 Hz, 1H), 7.4 (d, J = 2.3 Hz, 1H), 6.9 (d, J = 8.3 Hz, 1H), 3.9 (s, 3H); ^{13}C NMR (126 MHz, MeOD) δ (ppm) = 165, 152, 151, 148, 137, 127, 124, 117, 115, 114, 112, 100, 55; MS (70 eV) m/z = 218 (100%); 217 (82%); 44 (59%); 77 (42%); 76 (38%); 137 (35%); 51 (29%); 114 (29%).

(*E*)-2-Cyano-3-(3,4,5-Trimethoxyphenyl)Acrylamide (2g)

$C_{13}H_{14}N_2O_4$, 262.27 g mol^{-1}; (95% yield); yellow crystal; m.p. = 125–127 °C; FTIR ν_{max}(cm^{-1}) = 3346, 3208, 2924, 2213, 1633, 1588, 1510, 1320; ^{1}H NMR (500 MHz, DMSO-d_6) δ (ppm) = 8.1 (s, 1H), 7.8 (m, 2H), 7.4 (s, 2H), 3.8 (s, 6H), 3.7 (s, 3H); ^{13}C NMR (126 MHz, DMSO-d_6) δ (ppm) = 163, 153, 151, 141, 127, 117, 108, 105, 61, 56; MS (70 eV) m/z = 262 (100%); 247 (50%); 202 (27%); 188 (25%); 187 (24%); 44 (19%); 161 (18%); 144 (17%).

(E)-2-Cyano-3-(4-Hydroxy-3,5-Dimethoxyphenyl)Acrylamide (2h)

$C_{12}H_{12}N_2O_4$, 248.24 g.mol^{-1}; (93% yield); yellow solid; m.p. = 123–124 °C; FTIR ν_{max} (cm^{-1}) = 3346, 3204, 2930, 2211, 1675, 1576, 1510, 1314; ^{1}H NMR (400 MHz, MeOD) δ (ppm) = 8.1 (s, 1H), 7.4 (s, 2H), 3.9 (s, 6H).; ^{13}C NMR (100 MHz, MeOD) δ (ppm) = 165, 152, 148, 141, 122, 117, 108, 106, 100, 56, 55; MS (70 eV) m/z = 248 (100%); 247 (54%); 217 (24%); 55 (22%); 73 (21%); 44 (20%); 43 (19%); 117 (18%).

(*E*)-2-Cyano-3-(Thiophen-2-Yl)Acrylamide (2i)

$C_8H_6N_2OS$, 178.21 g.mol^{-1}; (94% yield); brown crystal; m.p. = 115–117 °C; FTIR ν_{max} (cm^{-1}) = 3334, 3194, 2211, 1675, 1578, 1386; ^{1}H NMR (500 MHz, DMSO-d_6) δ (ppm) 8.4 (s, 1H), 8.1 (dt, J = 5.0 and 0.9 Hz, 1H), 7.8 (dd, J = 3.7 and 0.7 Hz, 1H), 7.3 (dd, J = 5.0 and 3.7 Hz, 1H); ^{13}C NMR (126 MHz, DMSO-d_6) δ (ppm) = 163, 144, 138, 136, 135, 129, 117, 102; MS (70 eV) m/z = 178 (100%); 177 (67%); 134 (43%); 108 (41%); 162 (39%); 90 (22%); 44 (22%); 135 (21%).

General Procedure for Reducing the Knoevenagel Adducts 2a-i Employing $NaBH_4$

The Knoevenagel adduct **2a-i** (0.2 mmol) was placed into a 25-mL round flask and dissolved with 5 mL of MeOH. After that, the solution was cooled to 0 °C, and sodium borohydride ($NaBH_4$, 0.2 mmol) was added. The reaction was carried out for 20 min and monitored by TLC (in a mixture containing hexane and ethyl acetate 7:3) and revealed with sublimated iodine impregnated on silica gel. The reaction was extracted with ethyl acetate (3 × 10 mL), and the organic phase was combined, dried with anhydrous Na_2SO_4, filtered and concentrated under reduced pressure. The obtained product was purified by column chromatography with silica flash using hexane and ethyl acetate (7:3). All the obtained adducts **3a-i** were characterized by m.p., FTIR, NMR and MS, as described in the previous section. All performed analyzes can be verified in the ESM-2.

2-Cyano-3-(4-Methoxyphenyl)Propanamide (3a)

$C_{11}H_{12}N_2O_2$, 204.09 g mol^{-1} (70% yield); white solid; m.p. = 169 °C; FTIR ν_{max} (cm^{-1}) = 3407, 3308, 3208, 2949, 2251, 1663, 1613, 1514, 1404, 1315, 1244, 1165, 1035, 826, 597; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.2 (d, J = 8.7 Hz, 2H), 6.9 (d, J = 8.7 Hz, 2H), 3.8 (s, 3H), 3.6 (dd, J = 7.8 and 5.2 Hz, 1H), 3.2 (m, 2H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 166, 159, 130, 127, 118, 114, 55, 41, 35; MS (70 eV) m/z = 121 (100%); 77

(10%); 204 (10%); 122 (9%); 91 (7%); 78 (6%); 89 (4%); 44 (3.1%).

2-Cyano-3-(4-Nitrophenyl)Propanamide (3b)

$C_{12}H_9N_3O_3$, 219.06 g.mol^{-1} (79% yield); yellow solid; m.p. = 157 °C; FTIR ν_{max} (cm^{-1}) = 3412, 3311, 3200, 2929, 2241, 1604, 1514, 1345, 1214, 1115, 846, 706, 617; ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.2 (d, J = 8.8 Hz, 2H), 7.6 (d, J = 8.8 Hz, 2H), 4.1 (dd, J = 8.6, 6.8 Hz, 1H), 3.2 (m, 2H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 166, 147, 145, 131, 124, 118, 39, 35; MS (70 eV) m/z = 175 (100%); 129 (72%); 77 (50%); 89 (44%); 44 (41%); 78 (39%); 106 (38%); 128 (37%).

2-Cyano-3-(4-Fluorophenyl)Propanamide (3c)

$C_{10}H_9N_2OF$, 192.07 g.mol^{-1} (83% yield); white solid; m.p. = 140 °C; FTIR ν_{max} (cm^{-1}) = 3468, 3308, 3188, 2929, 2241, 1683, 1604, 1514, 1404, 1315, 1274, 1205, 1165, 1035, 816, 607; ^{1}H NMR (400 MHz, acetone-d_6) δ 7.4 (ddd, J = 8.5, 5.4 and 2.6 Hz, 2H), 7.1 (m, 2H), 3.9 (dd, J = 8.5 and 6.7 Hz, 1H), 3.2 (m, 2H); ^{13}C NMR (101 MHz, acetone-d_6) δ 167, 163 (d, J = 243.70 Hz, 1C), 134 (d, J = 3.40 Hz, 1C), 132 (d, J = 8.10 Hz, 1C), 118, 116 (d, J = 21.40 Hz, 1C), 40, 36; MS (70 eV) m/z = 109 (100%); 148 (22%); 83 (15%); 192 (13%); 44 (12%); 101 (11%); 122 (9%); 75 (9%).

2-Cyano-3-(4-Chlorophenyl)Propanamide (3d)

$C_{10}H_9N_2OCl$, 208.04 g.mol^{-1}; (89% yield); white solid; m.p. = 169 °C; FTIR ν_{max} (cm^{-1}) = 3428, 3198,, 2909, 2241, 1663, 1604, 1503, 1404, 1096, 1016, 906, 806, 727, 577; ^{1}H NMR (400 MHz, acetone-d_6) δ 7.4 (s, 5H), 4.0 (dd, J = 8.4 and 6.7 Hz, 1H), 3.2 (m, 2H). ^{13}C NMR (101 MHz, acetone-d_6) δ 167, 137, 133, 132, 129, 118, 40, 36; MS (70 eV) m/z = 125 (100%); 127 (34%); 164 (20%); 208 (19%); 89 (14%); 128 (12%); 101 (10%); 75 (10%).

2-Cyano-3-(4-Bromophenyl)Propanamide (3e)

$C_{10}H_9N_2OBr$, 251.99 g.mol^{-1}; (80% yield); white solid; m.p. = 174 °C; FTIR ν_{max} (cm^{-1}) = 3407, 3198, 2939, 2251, 1683, 1604, 1494, 1395, 1075, 995, 906, 786, 607, 477; ^{1}H NMR (400 MHz, acetone-d_6) δ 7.5 (d, J = 8.4 Hz, 2H), 7.3 (d, J = 8.3 Hz, 2H), 4.0 (dd, J = 8.3 and 6.8 Hz, 1H), 3.2 (m, 2H); ^{13}C NMR (101 MHz, acetone-d_6) δ 167, 137, 132, 121, 118, 40, 36, 1; MS (70 eV) m/z = 169 (100%); 171 (95%); 90 (33%); 129 (32%); 89 (26%); 102 (21%); 44 (20%); 252 (19%).

2-Cyano-3-(4-Hydroxy-3-Methoxyphenyl)Propanamide (3f)

$C_{11}H_{12}N_2O_3$, 220.08 g.mol^{-1}; (88% yield); yellow oil; FTIR ν_{max} (cm^{-1}) = 3407, 3308, 3188, 2919, 2251, 1673, 1613, 1524, 1404, 1254, 1214, 1115, 1025, 786, 626; ^{1}H NMR (500 MHz, acetone-d_6) δ 6.9 (d, J = 8.2 Hz, 1H), 6.8 (d, J = 2.2 Hz, 1H), 6.7 (dd, J = 8.2 and 2.2 Hz, 1H), 3.9 (dd, J = 8.6 and 6.6 Hz, 1H), 3.8 (s, 3H), 3.1 (m, 2H). ^{13}C NMR (126 MHz, acetone-d_6) δ 167, 148, 147, 131, 121, 119, 117, 112, 56, 41, 36; MS (70 eV) m/z = 137 (100%) 44 (24%); 77 (18%); 94 (17%); 122 (16%); 40 (13%); 220 (12%); 43 (12%).

2-Cyano-3-(3,4,5-Trimethoxyphenyl)Propanamideacrilamida (3g)

$C_{13}H_{16}N_2O_4$, 264.11 g.mol^{-1}; (83% yield); white oil; FTIR ν_{max} (cm^{-1}) = 3447, 3358, 3219, 2949, 2829 2241, 1713, 1604, 1503, 1463, 1384, 1235, 1115, 995, 796, 527; ^{1}H NMR (500 MHz, acetone-d_6) δ 6.7 (s, 2H), 4.0 (dd, J = 8.5 and 6.7 Hz, 1H), 3.8 (s, 3H), 3.7 (s, 3H), 3.1 (m, 2H); ^{13}C NMR (101 MHz, acetone-d_6) δ 167, 154, 138, 133, 119, 107, 60, 56, 41, 37; MS (70 eV) m/z = 181 (100%); 264 (18%); 148 (13%); 182 (11%); 77 (6%); 91 (5%); 220 (5%); 137 (4%).

2-Cyano-3-(4-Hydroxy-3,5-Dimethoxyphenyl)Propanamide (3h)

$C_{12}H_{14}N_2O_4$, 250.10 g.mol^{-1}; (78% yield); dark yellow solid; m.p. = 173 °C; FTIR ν_{max} (cm^{-1}) = 3392, 3275, 3206, 2950, 2846, 2253, 1683, 1604, 1521, 1452, 1335, 1243, 1115, 894, 801, 661; ^{1}H NMR (400 MHz, DMSO-d_6) δ 6.6 (s, 2H), 3.9 (dd, J = 9.0 and 6.6 Hz, 1H), 3.7 (s, 3H), 3.0 (m, 2H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 167, 148, 135, 127, 119, 106, 56, 40, 35. MS (70 eV) m/z = 167 (100%); 250 (21%); 168 (10%); 123 (7%); 77 (4%); 174 (4%); 106 (4%); 78 (4%).

2-Cyano-3-(Thiophen-2-yl)Propanamide (3i)

$C_8H_8N_2OS$, 180.04 g.mol^{-1} (61% yield); yellow solid; m.p. = 123 °C; FTIR ν_{max} (cm^{-1}) = 3367, 3180, 2919, 2800, 2260, 1673, 1604, 1434, 1404, 1284, 1205, 1137, 859, 661; ^{1}H NMR (400 MHz, acetone-d_6) δ (ppm) 7.3 (dd, J = 5.1 and 1.2 Hz, 1H), 7.0 (dd, J = 3.5 and 1.1 Hz, 1H), 7.0 (dd, J = 5.1 and 3.5 Hz, 1H), 4.0 (dd, J = 8.0 and 6.5 Hz, 1H), 3.4 (m, 2H); ^{13}C NMR (101 MHz, acetone-d_6) δ 167, 139, 128, 127, 126, 118, 41, 31; MS (70 eV) m/z = 97 (100%); 180 (20%); 136 (15%); 45 (10%); 53 (7%); 109 (7%); 98 (6%); 44 (6%).

Isolation and Cultivation of Marine-Derived Fungi

The marine-derived strains employed in this study were collected by Roberto G. S. Berlinck (professor of the Chemistry Institute of São Carlos, University of São

Paulo, IQSC-USP, Brazil) from the South Atlantic Ocean at São Sebastião, São Paulo, Brazil. The marine-derived fungi *Aspergillus sydowii* CBMAI 935 was isolated from the sponge *Chelonaplysilla erecta*. Whereas *Cladosporium* sp. CBMAI 1237, *Microsphaeropsis* sp. CBMAI 1675, *Acremonium* sp. CBMAI 1676, and *Westerdykella* sp. CBMAI 1679 were isolated from *Dragmacidon reticulatum* (Birolli et al. 2016).

The strains *Penicillium citrinum* CBMAI 1186 and *Penicillium oxalicum* CBMAI 1185 were obtained from the marine alga *Caulerpa* sp. (Ferreira et al. 2014; Rocha et al. 2012). Whereas the strain *Trichoderma harzianum* CBMAI 1677 was isolated from the sponge *Didemnum ligulum* (Vacondio 2015). All the strains were deposited and are available at the Brazilian Collection of Environmental and Industrial Microorganisms (CBMAI–http://webdrm.cpqba.unicamp.br/cbmai/, WDCM 823).

These fungi were cultivated in malt 2% medium composed of agar (15 g L^{-1}) for solid medium and malt (20 g L^{-1}) in artificial seawater, which was composed of: $CaCl_2.2H_2O$ (1.30 g L^{-1}), $MgCl_2.6H_2O$ (9.68 g L^{-1}), KCl (0.61 g L^{-1}), NaCl (30.00 g L^{-1}), Na_2HPO_4 (0.014 g L^{-1}), Na_2SO_4 (3.47 g L^{-1}), $NaHCO_3$ (0.17 g L^{-1}), KBr (0.10 g L^{-1}), $SrCl_2.6H_2O$ (0.04 g L^{-1}), and H_3BO_3 (0.03 g L^{-1}) (Vacondio et al. 2015). The pH was adjusted to 7.0 with a KOH aqueous solution (0.1 mol L^{-1}).

Each fungus strain was grown for 5 days in a Petri dish (90 mm × 15 mm) on malt 2% solid medium sterilized in autoclave (20 min, 121 °C, 1.5 kPa). Then, seven small disks with 5-mm diameter from the mycelial colony were transferred to five 250 mL-Erlenmeyer flasks containing 100 mL of 2% malt liquid medium. The flasks were incubated in an orbital shaker (130 rpm, 32 °C) for 5 days and then employed for the reduction reaction (Birolli et al. 2017).

Reduction of Knoevenagel Adducts 2a-i by Marine-Derived Fungi

The bioreduction reaction was carried out employing whole-cells of marine-derived fungi. The mycelial mass was filtered using a Buchner apparatus, and 5.0 g of wet-cells were transferred to a 250-mL Erlenmeyer flask containing 100 mL of phosphate buffer (Na_2HPO_4/KH_2PO_4, pH = 7.0, 0.1 mol L^{-1}) and a cotton plug that enables air exchange.

Then, 50 mg of Knoevenagel adduct **2a-i** was dissolved in 400 μL of DMSO and added to the reactional media. All the manipulations were carried out with sterile instruments in a laminar flow cabinet (Veco), and the reactions were carried out in an orbital shaker (Tecnal TE-421) at 32 °C and 130 rpm for 0.1 to 120 h. These experiments were performed to verify biodegradation products.

Biodegradation of 2-Cyano-3-(4-Methoxyphenyl) Propanamide 3a

The biodegradation of the reduced adduct **3a** was performed as described for the reduction reaction in the "Reduction of Knoevenagel Adducts by Marine-Derived Fungi" section at 32 °C and 130 rpm for 1 to 15 days.

Product Extraction

For extraction of the obtained product by marine-derived fungus, 100 mL of ethyl acetate was added to the reactional flask, and the sample was vigorous stirred magnetically for 30 min for cell lysis and product extraction. The obtained material was filtered using a Buchner apparatus, and a liquid-liquid extraction was carried out with the supernatant. A second step of liquid–liquid extraction was performed with the addition of 50 mL of ethyl acetate to the aqueous phase. Subsequently, the organic phases were combined, dried with anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure.

Determination of Yield and Conversion

The obtained extract was resuspended in a 10-mL volumetric flask, and 800 μL of the sample was placed in a 1-mL volumetric flask with 200 μL of an anthracene solution of 2000 mg L^{-1} employed as internal standard. Resulting in a sample with a maximum concentration of 4000 mg L^{-1} of product **2a** with 400 mg L^{-1} of anthracene, which was employed for GC-MS analyses.

The GC-MS analyses were carried out in a Shimadzu GC2010plus coupled to a mass selective detector (Shimadzu MS2010plus) in electron ionization (EI, 70 eV) mode. The GC-MS (equipped with a 30 m × 0.25 mm × 0.25 μm J&W Scientific DB5 column) conditions were oven temperature started at 90 °C for 2 min, increased to 280 °C at 6 °C min^{-1}, and held for 7.3 min in this temperature. The injector and interface temperature were maintained at 250 °C. The injection volume was 1 μL with split ratio of 5. Helium was used as the carrier gas at a constant column flow of 0.75 mL min^{-1} with a total run time of 41 min. The MS was employed in scan mode of m/z 40–500. This method was modified from a previous work and was also employed for metabolites identification (Birolli et al. 2016).

A calibration curve was obtained for the product **3a** employing solutions of 500, 1500, 2500, 3500, and 4500 mg L^{-1}, $c_{\text{product 3a}} = ((A_{\text{product 3a}}/A_{\text{anthracene}}) - 0.3093)/0.0009)$ with R^2 of 0.99, where c_{product} = product **3a** concentration, $A_{\text{product 3a}}$ = product **3a** area and $A_{\text{anthracene}}$ = area of the internal standard anthracene. The chromatographic yield (y) was determined by the equation $y = (c_{\text{product 3a}}/4040) \times 100$.

A calibration curve was constructed for the reagent **2a** also employing solutions of 500, 1500, 2500, 3500, and 4500 mg L^{-1}, $c_{\text{reagent 2a}} = ((A_{\text{reagent 2a}}/A_{\text{anthracene}}) - 0.5674)/0.0007)$ with R^2 of 0.98, where c_{reagent} = reagent **2a** concentration, $A_{\text{reagent 2a}}$ = reagent **2a** area, and $A_{\text{anthracene}}$ = area of the internal standard anthracene. The chromatographic conversion (conv.) was defined as the reagent consumption determined by conv. = $100 - (c_{\text{reagent 2a}}/4000) \times 100$.

For the isolated yield determination, the products **3a-i** were purified by column chromatography with silica flash employing hexane and ethyl acetate (7:3) as eluent. The obtained product was dried, weighted, and the isolated yield was determined. The remaining reagent was weighted, and the conversion was defined as the consumed percentage of the substrate.

Determination of the Enantiomeric Excess

The enantiomeric excess was determined in a Shimadzu HPLC system composed of: LC-20AT pump, DGU-20A5 degasser, SIL-20AHT sampler, SPD-M20A UV-VIS detector, CTO-20A column oven and CBM-20A controller. A chiralpak AD-H column (25.0 cm × 4.6 mm × 5 μm of particle size) was employed in normal isocratic mode composed of 30% ethanol in hexane at a flow rate of 1 mL min^{-1} with detection at 225 nm. Chromatograms can be verified in ESM-4.

Results and Discussion

Synthesis of the Knoevenagel Adducts 2a-i Under Microwave Irradiation

The synthesis of Knoevenagel adducts **2a-i** was carried out using triethylamine and water under microwave irradiation (Scheme 1). A total of nine adducts were obtained and employed for bioreduction by marine-derived fungi. The obtained yields were high ($y > 93\%$) regardless of the substituent groups present in the aromatic aldehyde employed as reagent. The compound **2a**, which presented the highest yield, was employed as model for maximization of the bioreduction reaction.

Reduction of the Knoevenagel Adducts 3a-l for Obtention of Racemic Standards

A reduction of the Knoevenagel adducts **2a-i** with $NaBH_4$ was carried out for the obtention of racemic standards of **3a-i** for analyses of enantiomeric excess. All the employed adducts resulted in excellent yields, > 70% (Scheme 2).

Fungi Screening for Reduction of the Knoevenagel Adducts 2a-i

An initial screening was performed for selection of an efficient biocatalyst for the reduction of the Knoevenagel adducts. Therefore, 8 strains of marine-derived fungi were screened for the ene-reduction of the compound **2a** (Table 1).

Acremonium sp. CBMAI 1676 presented 14, 27, and 41% yield for 24, 48, and 120 h (Entries 1–3, Table 1), showing that the mycelia remain active for bioreduction for at least 120 h in the employed conditions. In addition, increasing values of conversion (conv.) were also obtained, 16, 30, and 45% for 24, 48, and 120 h, respectively (Entries 1–3, Table 1).

A. sydowii CBMAI 935 presented 56% conv. with 38% yield for 24 h, but for 48 h, 98% conv. was determined (Entries 4 and 5, Table 1). After 120 h, 97% conv. with 44% yield was obtained (Entry 6, Table 1). This conversion of about 100% showed that this yield value cannot be maximized with extended reaction times.

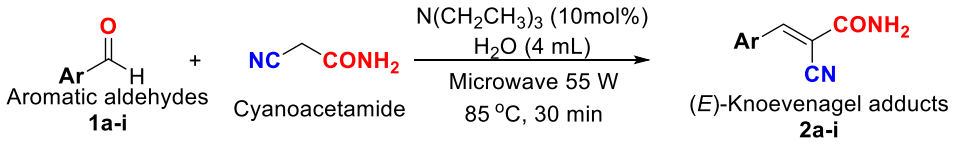
Therefore, a secondary process of biodegradation occurs at the same time of the bioreduction, since about 100% yield was not observed, although about 100% conv. for **3a** was determined. Additionally, the secondary product 3-(4-methoxyphenyl)propanenitrile **4a** considered a biodegradation metabolite was identified by GC-MS analysis (96% of similarity). Additional experiments exploring the process of product biodegradation responsible for the reduced yields for prolonged periods of reaction were described in the "Biodegradation of 2-Cyano-3-(4-Methoxyphenyl)Propanamide **3a**" section.

The same patterns for these bioreduction and biodegradation processes was observed for *Cladosporium* sp. CBMAI 1237, but with a high **3a** yield of 86% with 96% conv. **2a** for 24 h (Entry 7, Table 1), showing that the reaction can achieve about 90% yield in just 1 day. Additionally, 59 and 6% yield with complete conversion (100%) were determined for 48 and 120 h, respectively (Entries 8 and 9, Table 1).

T. harzianum CBMAI 1677 showed a reduced yield for the 24-h reaction, presenting 28% yield **3a** with 35% conv. **2a** (Entry 10, Table 1). Whereas for 48 h, 98% yield with 98% conv. were determined for **3a** (Entry 11, Table 1), showing that the contribution of biodegradation in this reaction was small for this strain in these reactional conditions, since yield and conversion presented the same value. However, for 120 h, a biodegradation process occurred, and a yield of 60% was determined with 100% conversion (Entry 12, Table 1).

Another strain, *Westerdykella* sp. CBMAI 1679 presented a high **3a** yield of 84% with 99% conv. **2a** for only 24 h (Entry 13, Table 1). Whereas for 48 h, a yield of 29% with 99% conv. was determined (Entry 14, Table 1), showing that a greater biodegradation process was observed for longer reaction times. For 120 h, 100% conv. (Entry 15, Table 1) was

Scheme 1 Synthesis of Knoevenagel adducts **2a-i** under microwave irradiation



Ar = 4-$OCH_3C_6H_4$ (**2a**)	y = 99%	Ar = 4-OH,5-$OCH_3C_6H_3$ (**2f**)	y = 98%
Ar = 4-$NO_2C_6H_4$ (**2b**)	y = 97%	Ar = 3-OCH_3,4-OCH_3,5-OCH_3,BrC_6H_2 (**2g**)	y = 96%
Ar = 4-FC_6H_4 (**2c**)	y = 97%	Ar = 3-OCH_3,4-OH,5-OCH_3 (**2h**)	y = 93%
Ar = 4-ClC_6H_4 (**2d**)	y = 94%	Ar = C_4H_3S (**2i**)	y = 95%
Ar = 4-BrC_6H_4 (**2e**)	y = 95%		

observed without detected product, showing that the substrate and product were completely biodegraded.

The same accelerated biodegradation was observed for *P. oxalicum* CBMAI 1996, in which for 24 h, only 16% yield was observed, although 100% conv. was determined for **3a** (Entry 16, Table 1). The about 100% conv. for 48 and 120 h of reaction without yield observation (Entries 17 and 18, Table 1) was due to the fast consumption of both substrate **2a** and product **3a**.

For *P. citrinum* CBMAI1186, 74% yield **3a** with 88% conv. **2a** was determined for 24 h, showing a relatively fast reduction of the substrate (Entry 19, Table 1). However, no yield was observed for 48 and 120 h (Entries 20 and 21, Table 1), showing that the rate of consumption of the reduced product was similar or higher than the rate of reduction of the Knoevenagel adduct, since no product was detected with 100% conv.

The strain *Microsphaeropsis* sp. CBMAI 1675 presented about 80% yield **3a** for 24 and 48 h with about 90% conv. **2a**, showing that for this strain the biodegradation process was slower than for the others evaluated fungi (Entries 22 and 23, Table 1). However, for 120 h, a complete consumption of the substrate and product was observed; therefore, no yield with 100% conv. was determined (Entry 24, Table 1).

The strain *Trichoderma harzianum* CBMAI 1677, which presented 98% yield for 48 h with reduced contribution of the biodegradation process in the reduction reaction, was selected for further studies of experimental conditions aiming the obtention of higher yields with shorter reaction times.

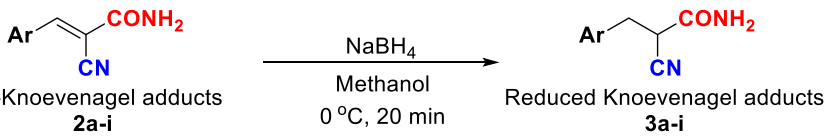
Different reactional media for the bioreduction of **2a** in 16 h were evaluated for the biocatalyst *T. harzianum* CBMAI 1677, including the addition of glucose, the employment of a biphasic system, and the use of different co-solvents as methanol, isopropanol, and DMSO (Table 2).

The reaction in phosphate buffer (0.1 M, pH 7.0) presented 16% yield **3a** with 29% conv. **2a** (Entry 1, Table 2), whereas the addition of 5% (v/v) of methanol to the reaction media aiming increased solubilization of the substrate that promoted an increase in the **3a** yield to 22% with 28% conv. **2a** (Entry 2, Table 2), as observed in the literature (Velankar and Heble 2003). It is notewhorthy that the biodegradation product **4a** was present in this analysis in trace concentration. Since the employment of a co-solvent increased the obtained yield, the use of isopropanol and DMSO as co-solvents was also evaluated.

The reaction medium composed of phosphate buffer (0.1 M pH 7.0) with 5% of isopropanol presented 17% yield **3a** with 33% conv. **2a** (Entry 3, Table 2), a very similar value to the reaction in the absence of a co-solvent (16% yield, Entry 1, Table 2) and inferior to the use of methanol (22% yield, Entry 2, Table 2). The use of DMSO in the reactional medium promoted a yield decrease, 14% yield **3a** with about 30% conv. (Entry 4, Table 2).

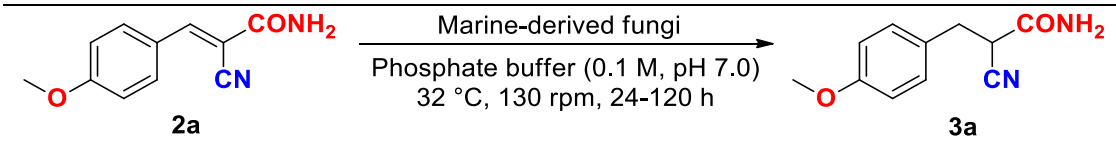
The supplementation of the reactional medium with glucose aiming the acceleration of the cell metabolism for the obtention of higher yields in shorter reaction times was not effective, since the **3a** yield slightly increased to 19% with a conversion of 33% **2a** (Entry 5, Table 2) in comparison with the reaction without an energy source (16% yield, Entry 1, Table 2). The use of both glucose and the co-solvent methanol resulted in 20% yield with 46% conv. (Entry 6, Table 2), showing that these conditions favored the biodegradation

Scheme 2 Reduction of the Knoevenagel adducts with $NaBH_4$



Ar = 4-$OCH_3C_6H_4$ (**3a**)	y = 70%	Ar = 4-OH,5-$OCH_3C_6H_3$ (**3f**)	y = 88%
Ar = 4-$NO_2C_6H_4$ (**3b**)	y = 79%	Ar = 3-OCH_3,4-OCH_3,5-OCH_3,BrC_6H_2 (**3g**)	y = 83%
Ar = 4-FC_6H_4 (**3c**)	y = 83%	Ar = 3-OCH_3,4-OH,5-OCH_3 (**3h**)	y = 78%
Ar = 4-ClC_6H_4 (**3d**)	y = 89%	Ar = C_4H_3S (**3i**)	y = 61%
Ar = 4-BrC_6H_4 (**3e**)	y = 80%		

Table 1 Screening of marine-derived fungi for *ene*-reduction of the Knoevenagel adduct **2a** by marine-derived fungi[a]



2a **3a**

Entry	Biocatalyst	React. time (h)	Yield (%)[c]	Conv. (%)[d]
1	*Acremonium* sp. CBMAI 1676	24	14	16
2		48	27	30
3		120	41	45
4	*Aspergillus sydowii* CBMAI 935	24	38	56
5		48	43	98
6		120	44	97
7	*Cladosporium* sp. CBMAI 1237	24	86	96
8		48	59	100
9		120	6	100
10	*Trichoderma harzianum* CBMAI 1677	24	28	35
11		48	98	98
12		120	60	100
13	*Westerdykella* sp. CBMAI 1679	24	84	99
14		48	29	99
15		120	0	100
16	*Penicillium oxalicum* CBMAI 1996	24	16	91
17		48	2	96
18		120	0	100
19	*Penicillium citrinum* CBMAI 1186	24	74	88
20		48	0	99
21		120	0	100
22	*Microsphaeropsis* sp. CBMAI 1675	24	79	90
23		48	82	93
24		120	0	100

[a] Reactional conditions: 50 mg of **2a**, 5.0 g of humid mycelia of marine-derived fungi, 100 mL of phosphate buffer solution (0.1 M, pH 7.0), 32 °C, 130 rpm, 24–120 h

[b] React. time: reactional time

[c] Yield determined by GC-MS employing analytical curves

[d] *Conv.* Conversion determined by GC-MS employing analytical curves

process, since the difference between yield and conv. increased significantly.

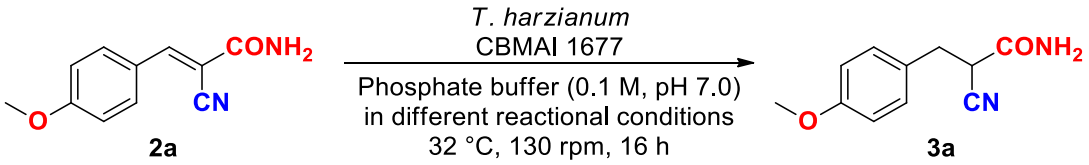
The employment of a biphasic system composed of phosphate buffer (0.1 M, pH 7.0) and hexane (2:1) was also evaluated for reduction of possible inhibition effects of the substrate and product during the reaction (Ou et al. 2019). An excellent reaction yield of 40% with 45% conv. was obtained (Entry 7, Table 2), showing that this approach was interesting for a faster bioreduction with decreased biodegradation.

However, the addition of glucose (5% yield with 49% conv., Entry 8, Table 2), methanol (5% yield with 50% conv., Entry 9, Table 2), and both glucose and methanol (6% yield with 48% conv., Entry 10, Table 2) in the biphasic reactional medium failed in the promotion of synergistic effects that would promote better results.

The reduced yields occurred due to increased biodegradation with the formation of **4a**, 4-methoxybenzaldehyde, 4-methoxyphenol, 1,4-dimethoxybenzene, and 2-cyanoacetamide (Fig. 1). Additionally, the product composition was estimated employing the GC-MS analysis. Mass spectra were presented in ESM-3.

The enantiomeric excess was determined employing liquid chromatography. However, none of the samples presented significant enantioselectivity (see ESM-4). A racemization process for this class of compounds already was described in the literature for reduced Knoevenagel adducts of malononitrile and aromatic aldehydes (Jimenez et al. 2019).

Table 2 Exploration of reactional conditions for the reduction of the Knoevenagel adduct **2a**[a]



Entry	Experimental condition[a]	Yield (%)[b]	Conv. (%)[c]
1	Phosphate buffer 0.1 M pH 7.0	16	29
2	Phosphate buffer 0.1 M pH 7.0 with 5% (v/v) methanol	22	28
3	Phosphate buffer 0.1 M pH 7.0 with 5% (v/v) isopropanol	17	33
4	Phosphate buffer 0.1 M pH 7.0 with 5% (v/v) DMSO	14	31
5	Phosphate buffer 0.1 M pH 7.0 with 0.5% (w/v) glucose	19	33
6	Phosphate buffer 0.1 M pH 7.0 with 5% (v/v) Methanol and 0.5% (w/v) glucose	20	46
7	Biphasic system phosphate buffer 0.1 M pH 7.0 and hexane (1:1)	40	45
8	Biphasic system phosphate buffer 0.1 M pH 7.0 and hexane (1:1) with 0.5% (w/v) glucose	5	49
9	Biphasic system phosphate buffer 0.1 M pH 7.0 and hexane (1:1) with 5% (v/v) methanol	5	50
10	biphasic system phosphate buffer 0.1 M pH 7.0 and hexane (1:1) with 5% (v/v) methanol and 0.5% (w/v) glucose	6	48

[a] Reactional conditions: 50 mg of **2a**, 5.0 g of wet whole-cells of marine-derived fungi, 100 mL of reactional media, 32 °C, 130 rpm, 16 h

[b] Yield determined by GC-MS employing analytical curves

[c] Conv. = Conversion determined by GC-MS employing analytical curves

A tautomeric equilibrium based in the formation of a prototropic ketenimine form was proposed for (±)-2-cyano-3-(furan-2-yl)propanamide, justifying the production of a racemic mixture. This phenomenon is common in compounds containing cyano groups with acidic hydrogens on α-C positions with the nitrile form being the predominant structure (Jimenez et al. 2019; Kasturi et al. 1962; Kasturi et al. 1973).

After the evaluation of different experimental media, a second screening of marine-derived fungi was carried out for 16 h in phosphate buffer and in biphasic system (0.1 M phosphate buffer pH 7.0 and hexane (1:1), 32 °C, 130 rpm, 16 h). The strains *P. oxalicum* CBMAI 1996, *Microsphaeropsis* sp. CBMAI 1675, and *Cladosporium* sp. CBMAI 1237, which presented a faster biotransformation in the screening with about 90% conv. **2a** in 24 h (Entries 16, 22 and 7, Table 1), were reevaluated and compared to *T. harzianum* CBMAI 1677 (Fig. 2).

P. oxalicum CBMAI 1996 presented 20% yield **3a** with 97% conv. **2a** in phosphate buffer for a 16-h reaction, showing that the biodegradation process was also present for a shorter reaction time (Entry 1, Fig. 2). The employment of the biphasic system resulted in 16% yield (Entry 2, Fig. 2), differently from the reduction by *T. harzianum* CBMAI 1677, which presented a yield increase in this reactional condition (from 16 to 40% yield, Entries 7 and 8, Fig. 2).

The same pattern was observed for the fungus *Microsphaeropsis* sp. CBMAI 1675 that presented 45% yield with 84% conv. in phosphate buffer (Entry 3, Fig. 2) and 11% yield with 42% conv. for the biphasic system (Entry 4, Fig. 2), showing that this approach was not successful for this strain either.

The strain *Cladosporium* sp. CBMAI 1237 presented an excellent yield of 95% with 96% conv. for 16 h in phosphate buffer (Entry 5, Fig. 2), whereas in the biphasic system 82% yield with 91% conv. was determined (Entry 6, Fig. 2). The presence of solvents in the reaction medium may promote toxic effects on the employed strains, reducing their performance as biocatalyst, and promoting reduced yields and conversions in the biphasic system.

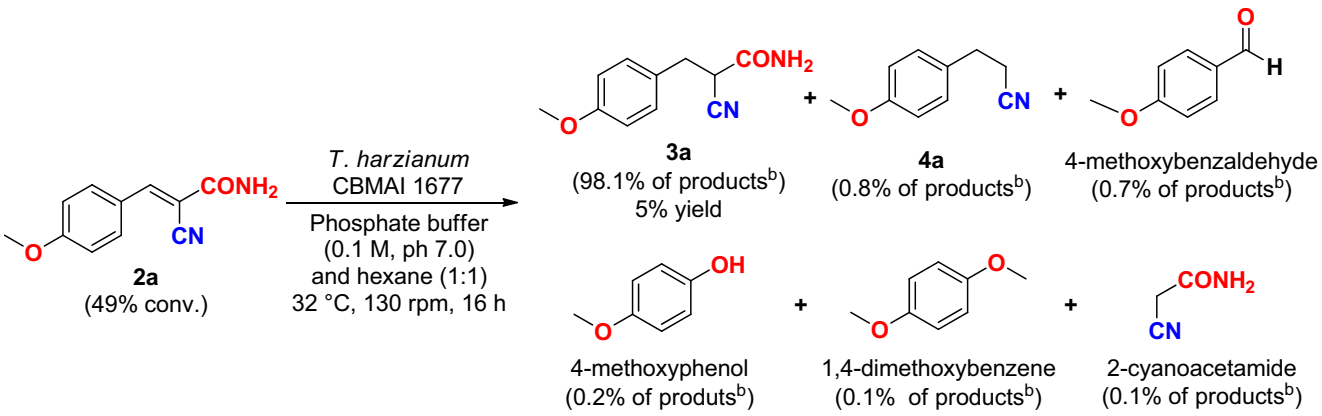


Fig. 1 Biotransformation and biodegradation of the Knoevenagel adduct **2a** by *T. harzianum* CBMAI 1677 in biphasic system (0.1 M phosphate buffer pH 7.0 and hexane (1:1) with 5% (v/v) methanol and 0.5% (w/v) glucose, 32 °C, 130 rpm, 16 h). [a] Conv. = conversion; [b] Percentage of product composition estimated by GC-MS.

It was observed that the increased catalysis observed for *T. harzianum* CBMAI 1677 in biphasic system was specific for the enzymes from this fungus strain during the reaction process. But the employment of the strain *Cladosporium* sp. CBMAI 1237 in phosphate buffer constitute a greener methodology, since it avoids the use of hexane and promote increased yields.

Reduction of Knoevenagel Adducts by *Cladosporium* sp. CBMAI 1237

The kinetic of the reduction reaction of **2a** by *Cladosporium* sp. CBMAI 1237 was evaluated aiming the obtention of high yields with reduction of the reactional time. The reaction was evaluated from 0 to 24 h with independent samples, showing that the reaction reaches a maximum yield of 97% after 8 h (Fig. 3).

A reaction kinetic of zero-order ($c = c_{initial} + kt$, where c = concentration (mol L^{-1}), $c_{initial}$ = initial concentration (mol L^{-1}), k = zero-order rate constant (mol L^{-1} h^{-1}), and t = time (h)) was determined for the production of the reduced product, $c = 0.23 + 0.42$ t with $R^2 = 0.98$, showing a productivity from time 0.1 to 5.0 h of 0.42 mol L^{-1} h^{-1}.

This zero-order kinetic is common in enzyme-catalyzed reactions and occurs when a bottleneck is limiting the reaction. Therefore, the observed constant reaction rate can be promoted by reduced contact of the substrate with the enzymes due to limited transportation of the reagent thought the cellular membrane, or by enzyme saturation due to excess of reagent (Atkins and De Paula 2006; Tinoco Jr and Wang 1995).

From 8 to 18 h, the reaction yield and conversion remained constant at about 95% of **3a**, and, after this, a biodegradation process became significant affecting the reaction yield that decreased to 86% from 18 to 24 h. Then the consumption of the product **3a** for energy obtention by the biocatalyst increased and yields of 59% and 6% were determined for 48 h and 120 h (Entries 8 and 9, Table 1).

A first-order kinetic ($c = c_{initial}.e^{-kt}$, where c = concentration (mol L^{-1}), $c_{initial}$ = initial concentration (mol L^{-1}), k = first-order

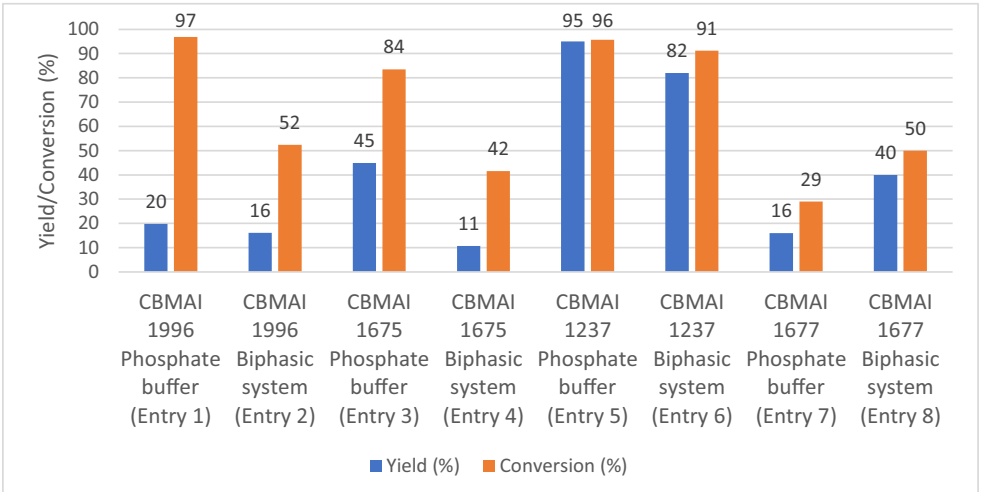


Fig. 2 Screening of marine fungi for the reduction of the Knoevenagel condensation product **2a** in phosphate buffer and in a biphasic system (reactional conditions: 50 mg of **2a**, 5.0 g of humid whole-cells of marine-derived fungi, 100 mL of 0.1 M phosphate buffer pH 7.0 with or without 50 mL of hexane for biphasic system (2:1), 32 °C, 130 rpm, 16 h).

rate constant (h^{-1}), and t = time (h)) of biodegradation was obtained for the reduced product considering the data for 18 (initial time of the biodegradation process, thus 0 h), 21, 24, and 48 h, $c = 2.40.e^{0.0168t}$ with $R^2 = 0.99$, and a half-life of 41.3 h.

The reaction product obtained from the 8-h reaction was purified, and an isolated yield of 88% was obtained for **3a** (Entry 1, Table 3), showing that the reduction reaction of nitrogenated compounds can be performed in high reaction yields. However, it is necessary to emphasize that the reaction time is an important variable of this process, since biodegradation decreases the yield.

Then, different substrates were employed for the evaluation of the reaction scope employing *Cladosporium* sp. CBMAI 1237 as catalyst. The strong electron withdrawing group *p*-NO_2 was employed, and 61% yield was determined for **3b** (Entry 2, Table 3), showing that the employed method can be applied with this type of group in the aromatic ring.

Halogenated substrates were also reduced; the product **3c** with the substituent *p*-F was obtained with 64% yield (Entry 3, Table 3), whereas **3d** with *p*-Cl and **3e** with *p*-Br presented 58% and 65% yield (Entries 4 and 5, Table 3) respectively, showing that electronic effects did not affect significantly the reaction.

Larger aromatic aldehydes were also employed, and the product **3f** with *p*-OH and *m*-OCH_3 was obtained with 48% yield (Entry 6, Table 3); a similar yield of 49% for **3g** with 3,4,5-OCH_3 was also determined (Entry 7, Table 3). However, **3i** with 3-OCH_3, 5-OCH_3, and 4-OH was not obtained; therefore, 0% of yield was determined with a conversion of 79% **2i** (Entry 9, Table 3). Probably, the biodegradation process was faster than the reduction of the substrate, consuming all the obtained product.

Moreover, **3h** was obtained in 90% yield (highest production of this study) in the employed conditions (Entry 8, Table 3), showing that compounds with heteroatoms in the aromatic ring can also be reduced by the marine-derived fungus *Clasporium* sp. CBMAI 1237.

Each compound probably presents an optimal experimental condition for the obtention of a maximum yield. For example, the time in our study strongly influenced the quantity of obtained product; thus, the reaction period should be determined for each employed substrate for obtention of more interesting results. Especially for those that presented 100% of conversion, because shorter reaction times would probably promote increased yields due to reduced biodegradation (Entries 2–5 and 8, Table 3).

The reduction of α,β-unsaturations of malononitrile derivatives was performed by the marine fungus *Penicillium citrinum* CBMAI 1186 in high yields (up to 98%) in reactions of 6 days (32 °C, 130 rpm) without a significant presence of a biodegradation process, as also observed for *E*-2-cyano-3-(furan-2-yl)acrylamide reduction with yields up to 99% for 3 days reactions (32 °C, 130 rpm) by *P. citrinum* CBMAI 1186, *A. sydowii* CBMAI 935, *Trichoderma* sp. CBMAI 932, *Aspergillus* sp. FPZSP152, and *Aspergillus* sp. FPZSP 146 (Jimenez et al. 2019; Jimenez et al. 2016).

Probably, small changes in the substrate structure promoted a faster attack of degradative enzymes, effect that may be enhanced by the employment of nitrogenated compounds that can be used by the strain as carbon and nitrogen source. The biodegradation of substrate in biocatalytic reactions by whole cells already was described for Knoevenagel adducts and isatin, which presented low yields (up to 37%) due to a biodegradation process and moderate enantioselectivities (up to 66%) (Birolli et al. 2017).

The enantiomeric excesses were determined by HPLC. However, all the obtained products were racemic. Chromatograms are available in ESM-4.

It is important to note that biodegradation metabolites were observed for all the employed substrates, except for the

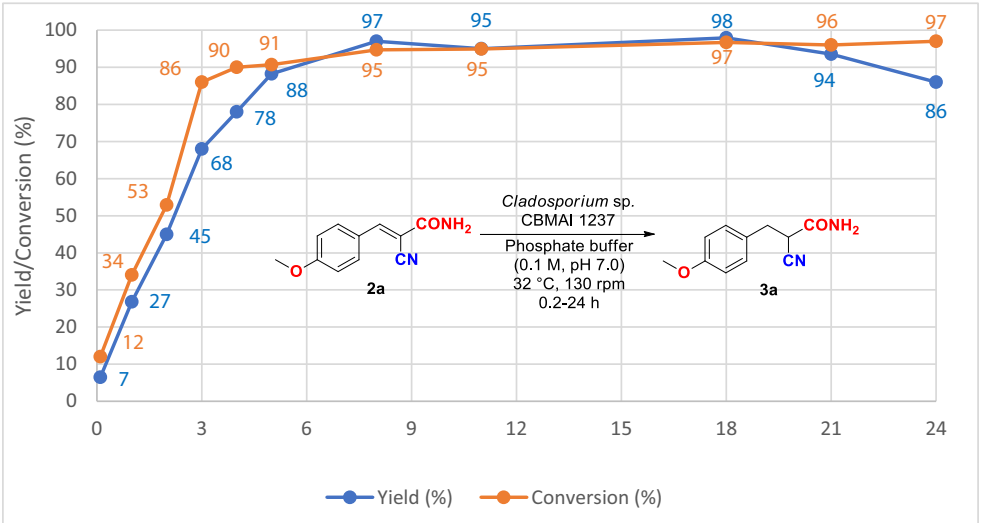
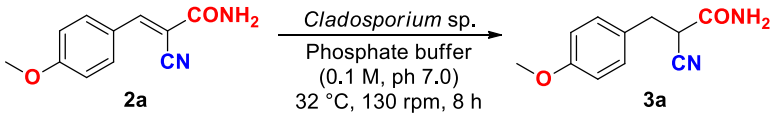
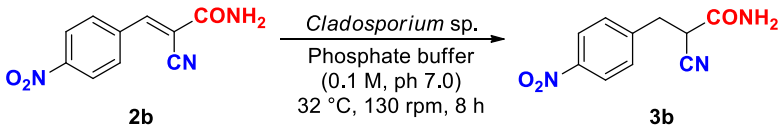
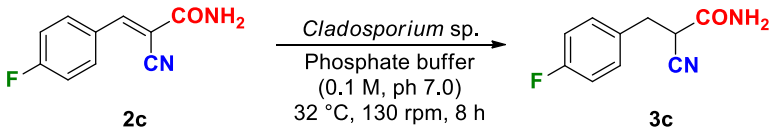
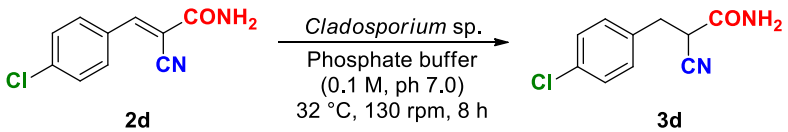
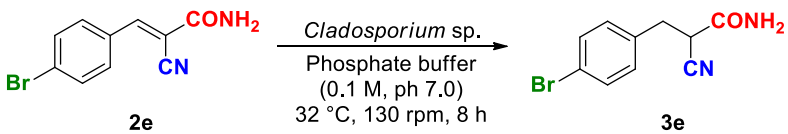
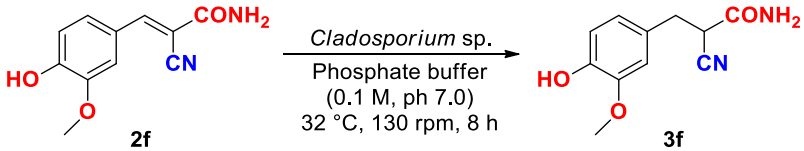
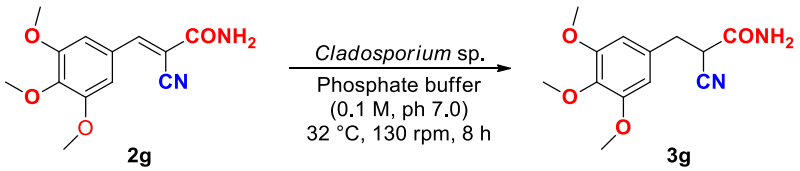
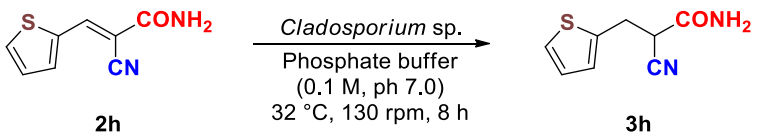
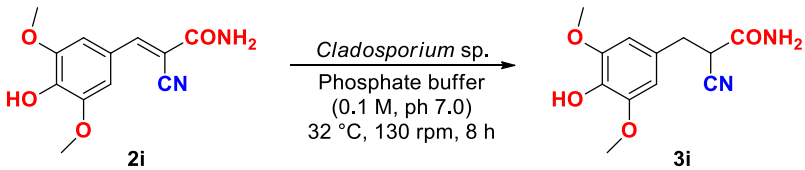


Fig. 3 Reduction of the Knoevenagel adduct **2a** by *Cladosporium* sp. CBMAI 1237 over time. Reactional conditions: 50 mg of adduct **2a**, 5.0 g of humid whole cells of *Cladosporium* sp. 1237, 100 mL of 0.1 M phosphate buffer pH 7.0, 32 °C, 130 rpm, 0.2–24 h.

Table 3 Reduction of different Knoevenagel adducts by *Cladosporium* sp. CBMAI 1237.[a,b,c]

Ent.	Reaction	Isol. yield (%)	Conv. (%)
1		88	93
2		61	100
3		64	100
4		58	100
5		65	100
6		48	95
7		49	76
8		90	100
9		0	79

[a] Reactional conditions: 50 mg of **2a-2i**, 5.0 g of wet whole cells of *Cladosporium* sp. 1237, 100 mL of 0.1 M phosphate buffer pH 7.0, 32 °C, 130 rpm, 8 h

[b] *Ent* = Entry; *Isol. yield* = Isolated yield; *Conv.* = conversion

[c] The *ee* was determined for all products. However, only racemic compounds were obtained in this biocatalytic study

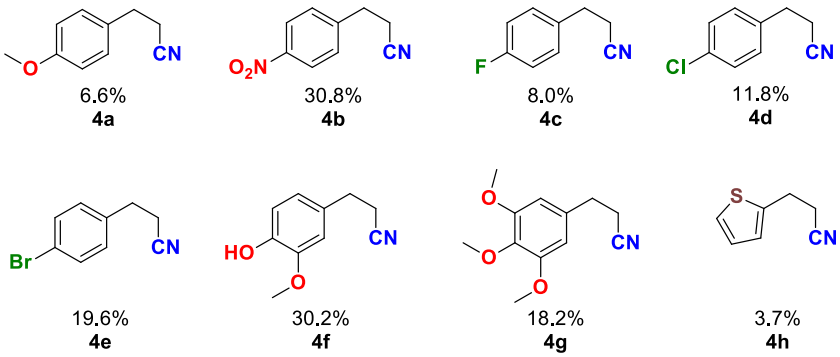


Fig. 4 Biodegradation metabolites identified by GC-MS.

product **3i**. For example, the **2a** reaction had 94% conversion with 88% of **3a** isolated yield, and 6.6% was determined as the biodegradation metabolite **4a** (percentage determined by CG-MS analysis).

Some substrates presented about 10% of biodegradation metabolites such as **4a**, **4c**, **4d**, and **4 h**, whereas some compounds were about 20% of the obtained reaction material, including **4 g** and **4e**. Other compounds presented about 30% in secondary products, **4b** and **4 f**. These findings showed that the biodegradation process is present in the bioreduction of all the employed substrates (Fig. 4). Mass spectra were presented in ESM-3.

Biodegradation of 2-Cyano-3-(4-Methoxyphenyl) Propanamide 3a

The biodegradation of **3a** was carried out for 1, 2, 5, and 14 days, and conversions of 10, 66, 97, and 100% were determined, respectively. The only produced compound was **4a** in small concentrations, thus the substrate **3a** of this reaction was probably employed as carbon and nitrogen source by the biocatalyst *Cladosporium* sp. CBMAI 1237.

Conclusion

A bioreduction methodology of the Knoevenagel adducts **2a-h** between cyanoacetamide and the aromatic aldehydes was carried out in good yields (48–90%) with whole-cells of the marine-derived fungus *Cladosporium* sp. CBMAI 1237, showing the presence of ene-reductases. However, enantioenriched products were not obtained due to the ketenimine equilibrium which racemized the products.

In addition, it is important to emphasize that the experimental conditions, especially the time, should be carefully evaluated for the obtention of high yields. Since a biodegradation process consume the obtained product in extended periods of reaction, probably due to the use of the substrate as carbon and nitrogen source. This study presented a coupled green approach of microwave irradiation and bioreduction for production of organic molecules in a more sustainable process.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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