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PII: S0040-4020(16)30077-1

DOI: 10.1016/j.tet.2016.02.014

Reference: TET 27484

To appear in: *Tetrahedron*

Received Date: 14 October 2015

Revised Date: 26 January 2016

Accepted Date: 4 February 2016

Please cite this article as: Jimenez DEQ, Ferreira IM, Birolli WG, Fonseca LP, Porto ALM, Synthesis and biocatalytic ene-reduction of Knoevenagel condensation compounds by the marine-derived fungus *Penicillium citrinum* CBMAI 1186, *Tetrahedron* (2016), doi: 10.1016/j.tet.2016.02.014.

Graphical Abstract

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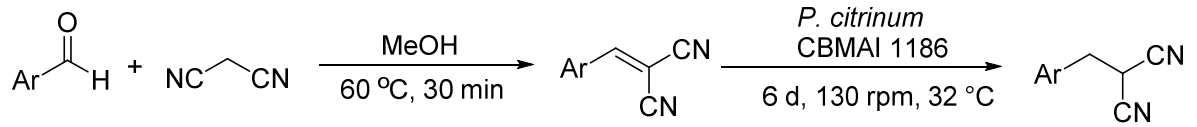

Ar =Ph, 4-F-Ph, 4-Cl-Ph, 4-Br-Ph, 4-OH-Ph, 4-OMe-Ph, 4-OH-3-OMe-Ph, 4-NO_2-Ph, py, tiophene, furane

up to >98%

up to >98%

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Synthesis and biocatalytic ene-reduction of Knoevenagel condensation compounds by the marine-derived fungus *Penicillium citrinum* CBMAI 1186

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ARTICLE INFO

Article history:
Received
Received in revised form
Accepted
Available online

Keywords:
Ene-reductase
Nitrile hidratase
Bioreduction
Biocatalysis
Marine Fungi

ABSTRACT

The chemoselective bioreduction of α,β-unsaturated compounds is an important synthetic tool that can have applications in the synthesis of many fine chemicals and pharmaceutical molecules. The synthesis of aromatic malononitrile derivatives through Knoevenagel condensation by microwave radiation under green chemistry conditions using methanol like solvent, free base and free catalyst is here reported. In addiction the biocatalytic reduction of the C-C double bond of aromatic malononitrile derivatives by whole cells of the marine-derived fungal *Penicillium citrinum* CBMAI 1186 was also tested. The products catalyzed by the fungus ene-reductase were obtained in very good yields (up to >98%).

1. Introduction

The chemoselective bioreduction of α,β-unsaturated compounds is an important synthetic tool that can have applications in the synthesis of many fine chemicals and pharmaceutical molecules.[1,2]

Chemoselective reduction of benzylidene malononitrile have attracted much attention in the last decade because the reduction product that is very important intermediates in organic synthesis mainly on the production of medicinal active compounds[3,±]. However, numerous methods developed for this organic synthesis use $NaBH_4$, $InCl_3$-$NaBH_4$, and other expensive catalysts such as rhodium complexes, and additional harsh experimental conditions also associated with environmental problems.[1,4]

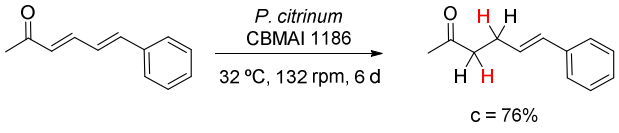
The biotransformation process is only one reaction or a set of simultaneous reactions in cascade performed by enzymes or in the whole cells of microorganisms that can be used complementarily to chemical synthetic methods.[5,4]

Many of those enzymes responsible by chemoselective bioreduction of C-C double bond α,β-unsaturated group are named of ene-reductases (ERs).

Although these catalysts (isolated microorganisms and enzymes) are known since a long time, it has more recently been used as chemical reagents in organic synthesis.

However, the synthetic potential of these enzymes started to be explored in recent decades mainly in the semi-synthesis of natural products, leading to interesting biologically active molecules, drugs or intermediate key compounds.[2,6] The catalytic process by these enzymes occur on the reduction of C-C double bonds activated by electron-withdrawing group, such as, carbonyl, nitroolefin, cianoolefin, and imines.[7,8]

In addition, whole cells of the fungus filamentous marine-derived *Penicillium citrinum* CBMAI 1186 has been also successfully used on the chemoselective bioreduction of chalcones and enones α,β-, α,β,γ,δ- or di-α,β-unsaturated as it was recently shown in our research group (Scheme 1).[9]



Scheme 1. Chemoselective bioreduction with whole cells of the fungus *P. citrinum* CBMAI 1186

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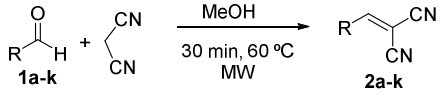
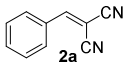
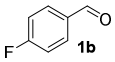
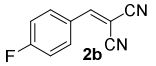
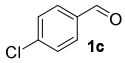
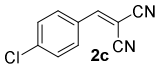
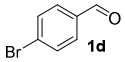
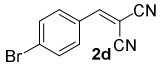
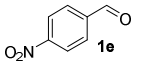
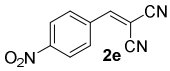
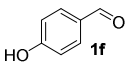
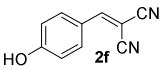
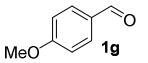
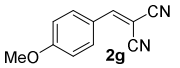
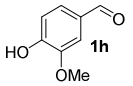
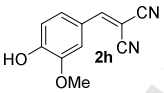
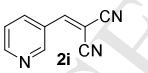
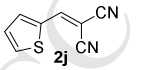
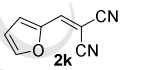
2. Results and discussion

Due to the versatility on the chemoselective biohydrogenation of enones by fungus *P. citrinum* CBMAI 1186,[9] in this work we extend its application to perform the reduction of different aromatic malononitriles, commonly prepared by Knoevenagel condensation.

Firstly, different Knoevenagel adducts under microwave radiation in methanol were synthesized. All products were obtained in goods yields (Table 1).

Table 1. Knoevenagel synthesis of aromatic and heteroaromatic malononitriles under MW.[a]



Entry	Aldehydes (**1**)	Knoevenagel adducts (**2**)	Isolated yields[b] (%)
1			98
2			98
3			95
4			93
5			95
6			93
7			99
8			98
9			90
10			85
11			95

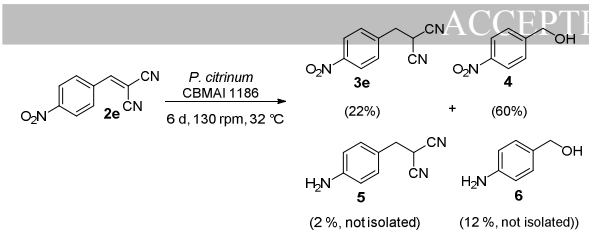
[a]General reaction conditions: benzaldehyde (1 mmol), malononitrile (1.1 mmol), MW power: 20 W, 60 ºC, 30 min. The reactions were monitored by TLC. The crystals were filter out, wash them with water (3 x 5 mL) and recrystallized in a mixture of hexane-dichloromethane (1:1). [b]Isolated yields obtained after purification.

The results from all biotransformations of aromatic and heteroaromatic malononitriles with *P. citrinum* CBMAI 1186 are summarized in Table 2. The reaction of biotransformation of the compound **2a** by fungus marine-derived *P. citrinum* CBMAI 1186 gave exclusively to the formation of the compound **3a** with 97% yield (Entry 1, Table 2), resulting from the action of the ERs on the C-C double bond in the **2a**. The mechanism of reduction of the C-C double bond by ERs is known by the nucleophilic attack of a hydride transfer from the flavin cofactor onto a β-carbon atom of the C-C double bond in the presence of an electron-withdrawing substituent. Then, a tyrosine residue delivers a proton to the α-carbon atom, typically on the alkene opposite face, affording *anti*-hydrogen addition.[8,10]

Based on this observation we tried to investigate and confront this enzyme catalytic mechanism by examining the action of whole cells of the fungus *P. citrinum* CBMAI 1186 in the reduction the C-C double bond of different aromatic malononitriles with substituent electron withdrawing and electron donating.

The bioreduction of benzylidine malononitriles bearing electron withdrawing substituents such as 2-(4-fluorobenzylidene)malononitrile (**2b**), 2-(4-chlorobenzylidene)malononitrile (**2c**), and 2-(4-bromobenzylidene)malononitrile (**2d**) promoted the corresponding reduced products in the C-C double bond with excellent yields (98, 95 and 93%, respectively). The reactions were carried out for 6 days of the inoculation with whole cells of fungus marine-derived *P. citrunm* CBMAI 1186 (Entries 2-4, Table 2). However, the compound **2e,** which also has an electron withdrawing substituent ($-NO_2$) followed a completely different catalytic pathway as its biotransformation led to yielding a mixture of products (Scheme 2). At the end of the biotransformation it was possible to isolate the 2-(4-nitrobenzyl)malononitrile (**3e**) with yield 22%, and the compound (4-nitrophenyl)methanol (**4**) with yield 60% (Supplementary Material). The minority compounds 2-(4-aminobenzyl)malononitrile (**5**) and (4-aminophenyl)methanol (**6**) not were isolated. The compound **6** was confirmed by GC-MS and was compared with the similar structure in the NIST05 library database (Mass Spectral Library NIST/EPA/NIH). The compound **4** can be obtained from the reduction of *p*-nitrobenzaldehyde (*p*NB) by a ketoreductase. The formation of *p*NB possibly occurred via retro-Knoevenagel of **2e**. The nitro group is a stronger deactivator of the aromatic ring, and favoured the nucleophilic attack of water at the benzylic position of compound **2e**. Then, the retro-Knoevenagel occurred, i.e., yielding the *p*-nitrobenzaldehyde and malononitrile. The reduction of *p*-nitrobenzaldehyde was confirmed in the presence of the fungal cells of *P. citrinum* CBMAI 1186 and produced the benzyl alcohol **4** in total conversion. The compounds **5** and **6** were obtained as minor products. The compound **5** was obtained via the action of an ene-reductase. The compound **6** was probably formed from the reduction of the nitro group of **4** by the action of a nitroreductase.

The *p*-nitrobenzaldehyde was subjected to the fungus *P. citrinum* CBMAI 1186 and yielded the benzyl alcohol **4** and the amino compound **6**. By kinetic reaction occurred firstly the reduction of the group benzaldehyde and thereafter reduction of the nitro group by the actions of ketoreductase and nitroreductase, respectively (Supplementary Material).



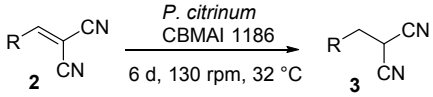
Scheme 2. Biotransformation of the **2e** by *P. citrinum* CBMAI 1186.

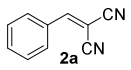
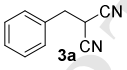
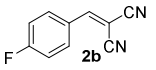
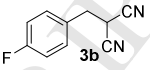
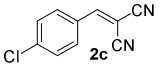
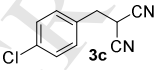
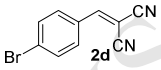
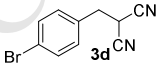
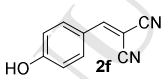
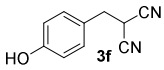
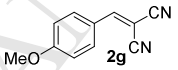
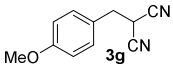
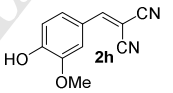
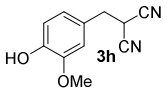
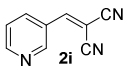
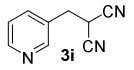
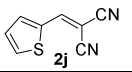
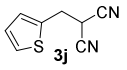
Fungal cells of *P. citrinum* CBMAI 1186 were also employed in the chemoselective reduction of aromatic malononitrile with groups electron donating. On the other hand, the bioreduction of the C-C double bond of these groups of electron donating compounds led to the formation of the desired products in lower yields in comparison to the compounds with substituent electron withdrawing. The chemoselective bioreduction of the C-C double bond of the **2f** produced the corresponding **3f** in a poor yield, 12%. The donor electron effect of OH group disfavors the reaction of biohydrogenation (Entry 5, Table 2). Besides, the reaction of **2h** gave the reduced product **3h** in a moderate yield, 66% (Entry 7, Table 2). By using 2-(4-methoxybenzylidene)-malononitrile (**2g**) was obtained the 2-(4-methoxybenzyl)malononitrile (**3g**) in a 84% yield from whole cells of *P. citrinum* 1186 (Entry 6, Table 2). This group of experiments shows that the success of the reaction of reduction C-C double bond by whole cells of fungus *P. citrinum* CBMAI 1186 depends dramatically on the electronic effects promoted by the substituent attached to the aromatic ring.

In order to examine the scope and limitations of our methodology beyond aromatic derivatives, we carried out also the reaction chemoselective bioreduction of the C-C double bond of heteroaromatic malonitriles **2i-k**.

As can be seen in Table 2, whole cells of *P. citrinum* CBMAI 1186 were also able of reduced the C-C double bond of **2i-j** in the Knoevenagel adducts in good yields. When **2i** was subjected to the bioreduction from mycelium of *P. citrinum* CBMAI 1186 in buffer pH 7 in 6 days, the product 2-(pyridin-3-ylmethyl)malononitrile (**3i**) was isolated in a 93% yield (Entry 8, Table 2). Under the mentioned conditions was obtained the bioreduction of **3j** in a quantitative yield (99%) (Scheme 3).

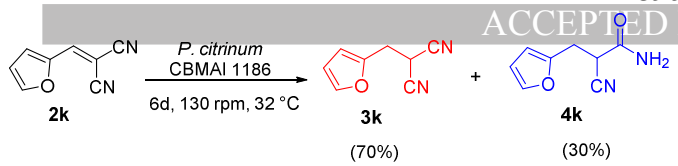
Table 2. Bioreduction of Knoevenagel adducts by mycelium of the marine-derived fungus *P. citrinum* CBMAI 1186.



Entry	Knoevenagel adducts (**2**)	Products (**3**)	Isolated yields (%)
1			97
2			98
3			95
4			93
5			12
6			84
7			66
8			93
9			99

Ultimately, the bioreduction of C-C double bond Knoevenagel adduct **2k** was observed the formation of two products **3k-4k** (Scheme 3). The reduced **3k** was produced in a 70% yield and surprisingly the correspond amide **4k** in 30% yield. The compound **4k** is correspondent product of action of ER and nitrile hydratase from microorganism *P. citrinum* CBMAI 1186. A particular advantage of enzymes bioreduction in comparison with the chemical catalysts is their ability to be conveniently incorporated into reaction cascades. Since all enzymes operate under relatively similar conditions, several of them can be combined in a one-pot reaction.

The reaction of biotransformation of the **2k** by whole cells *P. citrinum* CBMAI 1186 was monitored every 24 h with the withdrawal of 1 mL of sample, extracted with ethyl acetate and analyzed by GC-MS. The product **3k** was majority in first 24 h of the reaction, in a longer incubation time occurred a lowering of the **3k** and increase of the **4k** (Figure 1). The compunds **3k-4k** were identified by NMR (^{1}H and ^{13}C), FTIR and MS (Supplementary Material).



Scheme 3. Biotransformation of the **2k** by fungus *P. citrinum* CBMAI 1186 into **3k** and **4k**.

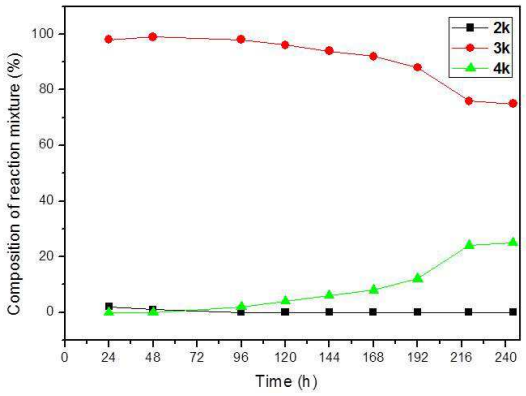


Figure 1. Biotransformation of the **2k** into **3k** and **4k** by fungus *P. citrinum* CBMAI 1186. In Graphical: **2k** (black color), **3k** (red color), and **4k** (green color).

3. Conclusion

In summary, it is reported the first time the ene-reduction of aromatic malononitriles **2a-k** by biotransformation. The products **3a-k** were obtained in very good yields with whole cells marine-derived *P. citrinum* CBMAI 1186. Finally, the biotransformation of compound **2k** showed action of the ene-reductase and the nitrile hydratase and its activity is induced according the substrate used.

4. Experimental

4.1. General methods

All manipulations involving the marine-derived fungus *P. citrinum* CBMAI 1186 were carried out under sterile conditions in a Veco laminar flow hood. A Technal TE-421 orbital shaker was used in the biocatalytic experiments. For gas chromatography–mass spectrometry, a Shimadzu GC2010 Plus gas chromatography system coupled to a mass-selective detector (Shimadzu MS2010 Plus) in electron ionization mode (70 eV) was used. FTIR spectra were recorded on a Shimadzu IRAffinity spectrometer samples were prepared as thin films on KBr disks (solid samples) or liquid film (liquid samples) in the 4000–400 cm^{-1} region. ^{1}H NMR and ^{13}C NMR spectra were recorded on an Agilent Technologies 500/54 Premium Shielded or Agilent Technologies 400/54 Premium Shielded spectrometer, with $CDCl_3$ as the solvent and TMS as the internal standard unless otherwise noted. The chemical shifts are given in ppm and coupling constants (J) in Hz. The microwave radiation experiment was performed using a Discover System from CEM Corporation at a 2.45 GHz frequency, a power output of about 200 W.

4.2. Chemical reagents

Benzaldehyde (99.5%), *p*-anisaldehyde (98%), 4-chlorobenzaldehyde (97%), 4-fluorobenzaldehyde (98%), vanillin (99%), 4-bromobenzaldehyde (99%), 4-hydroxybenzaldehyde (98%), 3-pyridinecarboxaldehyde (98%), 2-thiophenecarboxaldehyde (98%), furfural (99%), malononitrile (99%). All the reagents were purchased of Sigma-Aldrich and were used without further purification. The salts used for preparation of the artificial sea water were purchased from Vetec and Synth (Brazil). Deuterated chloroform was purchased from Cambridge Isotope Laboratories.

4.3. Preparation of the Knoevenagel condensation under microwave irradiation

A mixture of aldehyde (1.0 mmol), malononitrile (1.1 mmol) in methanol (3 mL) was put in the microwave reactor for 30 min at 60 °C, 20 W. The reaction progress was monitored by TLC. At end of the reaction, the solution was filtered out and washed with water (3 x 5 mL). The product was recrystallized in a mixture of hexane-dichloromethane (1:1) to yield the pure compound.

4.3.1. 2-benzylidenemalononitrile (**2a**)[11]: White solid; mp 82-84 °C; yield 151.06 mg (0.98 mmol, 98%); ^{1}H NMR (400 MHz, $CDCl_3$, ppm): δ 7.92 (m, 2H), 7.79 (s, 1H), 7.64 (m, 1H), 7.54 (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$, ppm): δ 159.9, 134.6, 130.8, 129.6, 113.6, 112.5, 82.7; IR (KBr, cm^{-1}): 3011, 2945, 2224, 1605; MS (70 eV): *m/z* 154 (M^{+}, 100%), 127 (88%), 103 (60 %).

4.3.2. 2-(4-fluorobenzylidene)malononitrile (**2b**) [12]: White solid; mp 125-126 °C; yield 167 mg (0.97 mmol, 97%); ^{1}H NMR (400 MHz, $CDCl_3$, ppm): δ 7.98-7.96 (m, 2H), 7.75 (s, H), 7.27-7.23 (m, 2H); ^{13}C NMR (100MHz, $CDCl_3$, ppm): δ 166.11 (d, $^{1}J_{C\text{-}F}$ =207 Hz), 157.98, 133.37, 127.18, 117.18, 113.40, 112.22, 82.05; IR (KBr, cm^{-1}): 3040, 2940, 2235, 1598, 1574, 1503, 1243, 834; MS (70 eV), *m/z* 172 ($M^{+\cdot}$, 100%), 145 (89 %), 121 (54 %).

4.3.3. 2-(4-chlorobenzylidene)malononitrile (**2c**) [12]: White solid; mp 90-91 °C; yield 184.84 mg (0.98 mmol, 98%); ^{1}H NMR (500 MHz, $CDCl_3$, ppm): δ 7.88-7.85 (d, *J*= 8.5 Hz, 2H), 7.74 (s, 1H), 7.55-7.52 (m, 2H); ^{13}C NMR (126 MHz, $CDCl_3$, ppm): δ 158.26, 141.15, 131.83, 130.07, 129.27, 113.42, 112.32, 83.37; IR (KBr, cm^{-1}): 3084, 3047, 2225, 1595, 1527, 1355, 1215, 952. MS (70 eV): *m/z* 153 ($M^{+\cdot}$, 100%), 188 (57 %), 161 (23.2 %).

4.3.4. 2-(4-bromobenzylidene)malononitrile (**2d**) [13]: White solid; mp 165-166 °C; yield 230.73 mg (0.99 mmol, 99%); ^{1}H NMR (400 MHz, $CDCl_3$, ppm): δ 7.76-7.74 (m, 2H), 7.68-7.66 (m, 2H), 7.70 (s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$, ppm): δ 158.38, 133.04, 131.76, 130.01, 129.46, 113.40, 112.22, 83.23; IR (KBr, cm^{-1}): 3014, 2230, 1600, 1570, 1510, 1250, 838. MS (70 eV): *m/z* 232 ($M^{+\cdot}$, 31 %), 234 (M^{+2}, 30%), 153 (100%).

4.3.5. 2-(4-nitrobenzylidene)malononitrile (**2e**)[14]: Orange solid mp149.151 °C; yield 189.16 mg (0.95 mmol, 95%); ^{1}H NMR (400 MHz, $CDCl_3$, ppm): δ 8.38-8.36 (d, *J*= 8.8 Hz, 2H), 8.07-8.04 (d, *J*= 8.8 Hz, 2H), 7.87 (s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$, ppm): δ 156.82, 150.33, 135.76, 131.27, 124.60, 112.58, 111.55, 87.52; IR (KBr, cm^{-1}): 3032, 2227, 1597, 1589, 1579, 1411, 1263, 1024, 692 . MS (70 eV): *m/z* 126 ($M^{+\cdot}$, 100 %), 199 (84 %), 133 (72 %), 141 (68 %), 169 (51 %). See Figures S17-S20. The spectroscopy data are in accordance to the literature.

4.3.6. 2-(4-hydroxybenzylidene)malononitrile (**2f**)[14]: Yellow solid mp187-190 °C; yield 138.23 mg (0.93 mmol, 93%); ^{1}H NMR (500 MHz, $CDCl_3$, ppm): δ 7.93-7.83 (m, 3H), 6.92 (d, *J*= 9.0 Hz, 2H), 4.78 (s, 1H); ^{13}C NMR (125 MHz, $CDCl_3$, ppm): δ 164.0, 159.8, 133.7, 123.1, 116.3, 114.6, 113.6, 76.0. IR (KBr, cm^{-1}): 3352, 3041, 2235, 1610, 1579, 1566, 1444, 1300, 1220, 1174; MS (70 eV): *m/z* 170 ($M^{+\cdot}$, 100 %), 142 (44 %), 119 (38 %), 143 (21 %).

4.3.7. 2-(4-methoxybenzylidene)malononitrile (**2g**)[16] Yellow solid mp 113-114 °C; yield 182.23 mg (0.99 mmol, 99%); ^{1}H NMR (400 MHz, $CDCl_3$, ppm): δ 7.91-7.87 (m, 2H), 7.63 (s, 1H), 7.01-6.97 (m, 2H), 3.89 (s, 3H); ^{13}C NMR (100MHz, $CDCl_3$, ppm): δ 164.72, 158.75, 133.14, 123.24, 114.80, 114.05, 112.84, 78.69, 55.69; IR (KBr, cm^{-1}): 3026, 2986, 2221, 1605. MS (70 eV): *m/z* 184 ($M^{+\cdot}$, 100%), 141 (30%), 114 (49%).

4.3.8. 2-(4-hydroxy-3-methoxybenzylidene)malononitrile (**2h**)[15]: White solid; mp 134-135 °C; yield 196.15 mg (0.98 mmol, 98%); ^{1}H NMR (400 MHz, MeOD, ppm): δ 7.94 (s, 1H), 7.57-7.56 (d, 1H, *J*=2.5 Hz), 7.43 (m, 1H), 7.07 (d, 1H, *J*= 8.5 Hz), 3.95 (s, 3H); ^{13}C NMR (100MHz, $CDCl_3$, ppm): δ 159.76, 153.71, 146.92, 126.05, 124.67, 115.22, 114.45, 113.38, 111.19, 76.87, 55.05; IR (KBr, cm^{-1}): 3400, 3022, 2986, 2226, 1621, 1562, 1507, 1284, 1139. MS (70 eV): *m/z* 170 ($M^{+\cdot}$, 100%), 142 (47%), 119 (42%).

4.3.9. 2-(pyridin-3-ylmethylene)malononitrile (**2i**)[15]: Brown solid; mp 113-114 °C; yield 139.62 mg (0.90 mmol, 90%); ^{1}H NMR (500 MHz, $CDCl_3$, ppm): δ 8.89 (d, *J*=2.3 Hz, 1H), 8.84-8.82 (dd, *J* =4.8, 1.5 Hz, 1H), 8.48-8.47 (d, *J*=8.2 Hz, 1H), 7.83 (s, 1H), 7.54-7.51 (dd, *J*=8.2, 4.8 Hz, 1H); ^{13}C NMR (126 MHz, $CDCl_3$, ppm): δ 156.44, 154.62, 152.37, 135.61, 126.99, 124.29, 112.91, 111.94, 85.65, 77.28, 77.03, 76.7; IR (KBr, cm^{-1}): 3115, 3039, 2231, 1579, 1521, 1344. MS (70 eV): *m/z* 155 ($M^{+\cdot}$, 100 %), 104 (76 %), 128 (53 %), 101 (43 %).

4.3.10. 2-(thiophen-2-ylmethylene)malononitrile (**2j**)[14]: Brown solid; mp 93-95 °C; yield 136.16 mg (0.85 mmol, 85%); ^{1}H NMR (400 MHz, $CDCl_3$, ppm): δ 7.87-7.85 (m, 1H), 7.80-7.78 (dq, *J*=4.3, 0.8 Hz, 1H), 7.26-7.23 (m, 1H); ^{13}C NMR (100 MHz, $CDCl_3$, ppm): δ 151.03, 138.09, 136.84, 135.37, 130.21, 130.05, 129.52, 129.40, 129.00, 128.99, 128.74, 113.74, 112.90, 78.37; IR (KBr, cm^{-1}): 2981, 2935, 2274, 2158, 1645, 1448, 1184. MS (70 eV): *m/z* 160 ($M^{+\cdot}$, 100 %), 133 (56 %), 109 (41 %), 45 (21 %).

4.3.11 2-(furan-2-ylmethylene)malononitrile (**2k**)[14]: Brown solid; mp 70-73 ºC; yield 136,90 mg (0.95 mmol, 95%); ^{1}H NMR (400 MHz, $CDCl_3$, ppm): δ 7.79-7.78 (dt, *J*=1.8, 0.5 Hz, 1H), 7.50-7.49 (d, *J*=0.5 Hz, 1H), 7.35-7.34 (d, *J*=4 Hz, 1H), 6.70-6.69 (ddd, *J*=4, 1.7, 0.4 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$, ppm): δ 149.48, 149.46, 148.05, 148.04, 143.01, 123.35, 114.44, 114.39, 113.74, 112.54. IR (KBr, cm^{-1}): 3124, 3041, 2922, 2231, 1606, 1529, 1456, 1394, 1296; MS (70 eV): *m/z* 144 ($M^{+\cdot}$, 100 %), 115 (44 %), 116 (31 %), 89 (30 %), 117 (21 %).

4.4. Isolation and cultivation of the marine-derived fungus

Marine-derived fungus *P. citrinum* CBMAI 1186 was isolated from the marine alga *Caulerpa* sp., collected by Prof. R. G. S. Berlinck in city of São Sebastião, on the coast of the State of São Paulo, Brazil[9]. The fungus was identified using both conventional and molecular methods at the Chemical, Biological and Agricultural Pluridisciplinary Research Center (CPQBA) at the University of Campinas, SP, Brazil. A type culture of *P. citrinum* CBMAI 1186 was deposited in the Brazilian Collection of Environmental and Industrial Microorganisms (CBMAI – http://webdrm.cpqba.unicamp.br/cbmai/). The microorganism was cultivated on Petri plates containing 2% malt solid culture medium using artificial seawater with the following composition: 20 g L^{-1} malt extract (Acumedia) in artificial sea water. Composition of artificial sea water: $CaCl_2 \cdot 2H_2O$ (1.36 g L^{-1}), $MgCl_2 \cdot 6H_2O$ (9.68 g L^{-1}), KCl (0.61 g L^{-1}), NaCl (30.0 g L^{-1}), Na_2HPO_4 (0.014 mg L^{-1}), Na_2SO_4 (3.47 g L^{-1}), $NaHCO_3$ (0.17 g L^{-1}), KBr (0.1 g L^{-1}), $SrCl_2 \cdot 6H_2O$ (0.040 g L^{-1}), and H_3BO_3 (0.030 g L^{-1}). The pH was adjusted to 8 with KOH solution (0.1 mol L^{-1}).

4.5. Biotransformation of Knoevenagel adducts (2a–k) by the marine-derived fungus *P. citrinum* CBMAI 1186

The mycelia of the fungus *P. citrinum* CBMAI 1186 were harvested via Buchner filtration and 5 g (wet weight) of mycelium suspended in 100 mL of the phosphate buffer solution (Na_2HPO_4/KH_2PO_4, pH = 7, 0.1 mol L^{-1}) contained in a Erlenmeyer flasks (250 mL). The Knoevenagel adduct (50 mg, **2a–k**), previously dissolved in dimethylsulfoxide (400 µL), and then it was added to the culture media. The mixtures were incubated for 6 days in an orbital shaker at 32 °C and 130 rpm. The reactions were monitored using TLC every 24 h. Products in the medium were isolated and identified by spectroscopy analyses (Section 4.6).

4.6. Isolation of products from biotransformation by *P. citrinum* CBMAI 1186

After 6 d of reaction, the mycelium was filtered on a Buchner funnel and the reactional mixture was magnetically stirred for 30 min and filtered again by a Buchner funnel. The filtrate was extracted with EtOAc (3 × 50 mL).[16] The organic phase was dried over anhydrous Na_2SO_4, filtered, evaporated under vacuum and analyzed using GC–MS. The obtained extracts were purified using flash CC over silica gel eluting with *n*-hexane and ethyl acetate (7:3) to yield the pure products. The spectroscopic data of the isolated products were in agreement with those reported in the literature.

4.6.1. 2-benzylmalononitrile (**3a**)[14] White crystal; mp167-169 °C; yield 48.5 mg (0.97 mmol, 97%); ^{1}H NMR (500 MHz, MeOD, ppm): δ 7.34 (m, 5H), 3.28 (sl, 2H); ^{13}C NMR (125 MHz, MeOD, ppm): δ 135.65, 130.42, 130.17, 129.90, 129.71, 129.26, 114.50, 36.96; IR (KBr, cm^{-1}): 3030, 2914, 2256, 1494, 1452, 1074; MS (70 eV): *m/z* 91 ($M^{+\cdot}$, 100 %), 65 (88 %), 156 (13 %).

4.6.2. 2-(4-fluorobenzyl)malononitrile (**3b**)[17]: White solid; mp 107-110 °C; yield 49 mg (0.98 mmol, 98%); ^{1}H NMR (500 MHz, MeOD, ppm): δ 7.47-7.37 (m, 2H), 7.25-7.14 (m, 2H), 3.39 (sl, 2H); ^{13}C NMR (125 MHz, MeOD, ppm): δ 163.52, 161.57, 132.80, 132.76, 131.65, 131.58, 125.79, 125.76, 122.62, 122.50, 116.73, 116.55, 114.26, 114.24, 30.41, 30.39; IR (KBr, cm^{-1}): 2937, 2854, 2256, 1600, 1512, 1222; MS (70 eV): *m/z* 109 ($M^{+\cdot}$, 100%), 83 (15 %), 174 (9 %). See Figures S49-S52. The spectroscopy data are in accordance to the literature.

4.6.3. 2-(4-chlorobenzyl)malononitrile (**3c**)[17]: Colorless crystal; mp 71-73 °C; yield 47.5 mg (0.95 mmol, 95%); ^{1}H NMR (500 MHz, $CDCl_3$,ppm): δ 7.42-7.39 (m, 2H), 7.29 (d, *J*= 8 Hz, 2H), 3.92 (t, *J*=6.8 Hz, 1H), 3.28 (d, *J*=6.8 Hz, 2H); ^{13}C NMR (125 MHz, $CDCl_3$, ppm): δ 131.2, 130.5, 130.0, 129.7, 129.6, 111.8, 36.1, 22.6; IR (KBr, cm^{-1}): 2921, 2854, 2256, 1492, 1382; MS (70 eV): *m/z* 125 ($M^{+\cdot}$, 100%), 127 (33 %), 89 (18 %), 190 (12 %).

4.6.4. 2-(4-bromobenzyl)malononitrile (**3d**)[17]: White solid; mp 165-167 °C; yield 47.5 mg (0.95 mmol, 93%); ^{1}H NMR (500 MHz, $CDCl_3$, ppm): δ 7.56 (d, *J*=8.4 Hz, 2H), 7.22 (d, *J*=8.4 Hz, 2H), 3.93 (t, *J*=6.8 Hz, 1H), 3.26 (d, *J*=6.8 Hz, 2H); ^{13}C NMR (125 MHz, $CDCl_3$; ppm): δ 132.52, 131.75, 130.83, 130.02, 111.86, 36.10, 24.76; IR (KBr, cm^{-1}): 3348, 3224, 2974, 2200,

1712, 1363; MS (70 eV): *m/z* 169 ($M^{+\cdot}$, 100%), 171 (91 %), 90 (33 %), 233 (18 %), 235 (18 %).

4.6.5. 2-(4-nitrobenzyl)malononitrile (**3e**)[17]: Orange solid; mp 153-154 °C; yield 11 mg (0.22 mmol, 22%); ^{1}H NMR (400 MHz, $CDCl_3$, ppm): 8.30 (d, *J*=8 Hz, 2H), 7.55 (d, *J*=8 Hz, 2H), 4.04 (*t*= 6.8 Hz, 1H), 3.41 (d, *J* = 6.8 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$, ppm): 148.49, 139.75, 130.54, 124.65, 111.59, 36.25, 24.56; IR (KBr, cm^{-1}): 3104, 2929, 2256, 1602, 1517, 1346; MS (70 eV): *m*/z 136 ($M^{+\cdot}$, 100%), 106 (70 %), 89 (41 %), 78 (48 %), 89 (36 %), 201 (21 %).

4.6.6. 2-(4-hydroxybenzyl)malononitrile (**3f**)[17]: White solid; mp 173-176 °C; yield 6 mg (0.12 mmol, 12%); ^{1}H NMR (500 MHz, MeOD, ppm): δ 7.19 (d, *J*=8.0 Hz, 2H), 6.81-6.78 (d, *J*=8 Hz, 2H), 3.18 (sl, 1H); ^{13}C NMR (125 MHz, MeOD, ppm): δ 158.54, 131.58, 131.21, 126.25, 116.59, 116.41, 114.62, 36.35 ; IR (KBr, cm^{-1}): 3414, 2920, 2530, 2270, 1608, 1516, 1259; MS (70 eV): *m/z* 121 ($M^{+\cdot}$, 100 %), 122(90 %), 93 (47 %), 65 (41 %).

4.6.7. 2-(4-methoxybenzyl)malononitrile (**3g**)[17]: White solid; mp 80-81 °C; yield 42 mg (0.84 mmol, 84%); ^{1}H NMR (500 MHz, MeOD, ppm): δ 7.30-7.28 (d, *J*=8 Hz, 2H), 6.94-6.92 (d, *J*=8 Hz, 2H), 3.79 (s, 3H). 3.23 (sl, 2H); ^{13}C NMR (125 MHz, MeOD, ppm): δ 161.11, 131.60, 127.51, 115.25, 114.59, 55.73, 36.28; IR (KBr, cm^{-1}): 2933, 2918, 2837, 2256, 1610, 1508, 1249; MS (70 eV): *m/z* 121 ($M^{+\cdot}$, 100 %), 77 (11 %), 186 (9 %).[18]

4.6.8. 2-(4-hydroxy-3-methoxybenzyl)malononitrile (**3h**)[17]: Colorless oil; yield 33 mg (0.66 mmol, 66%); ^{1}H NMR (400 MHz, MeOD, ppm): δ 6.89 (d, *J*=8.1 Hz, 1H), 6.81 (dd, *J*=2.2, 0.4 Hz, 1H), 6.78 (ddt, *J*=8.1, 2.2, 0.5 Hz, 1H), 3.83 (s, 3H), 3.14 (s, 2H); ^{13}C NMR (100 MHz, MeOD, ppm): δ 147.66, 146.40, 126.88, 120.33, 115.79, 113.19, 111.43, 54.97, 35.14. IR (KBr, cm^{-1}): 3394, 2229, 1585, 1564, 1514, 1280; MS (70 eV): *m/z* 137 ($M^{+\cdot}$, 100 %), 202 (19 %), 122 (17 %), 94 (11 %).

4.6.9. 2-(pyridin-3-ylmethyl)malononitrile (**3i**)[18]: Red oil; yield 46.5 mg (0.93 mmol, 93%); ^{1}H NMR (400 MHz, MeOD, ppm): δ 8.58 (dd, *J*=2.3, 0.8 Hz, 1H), 8.53 (dd, *J*=4.9, 1.6 Hz, 1H), 7.89 (dt, *J*=7.9, 1.9 Hz, 1H), 7.46 (ddd, *J*=7.9, 4.9, 0.9 Hz, 1H), 3.38 (s, 2H); ^{13}C NMR (100 MHz, MeOD, ppm): δ 150.97, 150.95, 149.94, 139.30, 132.28, 125.43, 125.37, 114.18, 111.96, 33.88; IR (KBr, cm^{-1}): 2264, 2931, 2272, 1712, 1394; MS (70 eV): *m/z* 92 ($M^{+\cdot}$, 100 %), 65 (27 %), 157 (26 %).

4.6.10. 2-(thiophen-2-ylmethyl)malononitrile (**3j**)[19]: Colorless oil; yield 49.5 mg (0.99 mmol, 99%); ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.31 (dd, *J*=5.1, 1.1 Hz, 1H), 7.10 (d, *J*=3.1 Hz, 1H), 7.02 (dd, *J*=5.1, 3.5 Hz, 1H), 3.93 (t, *J*= 6.8 Hz, 1H), 3.53 (d, *J*=6.8 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 128.4, 127.7, 126.5, 114.0, 111.8, 25.5, 22.7; IR (KBr, cm^{-1}): 2954, 2924, 2882, 1735, 1465. MS (70 eV): *m/z* 97 ($M^{+\cdot}$, 100 %), 162 (17 %), 45 (14 %), 53 (11 %).

4.6.11. 2-(furan-2-ylmethyl)malononitrile (**3k**)[18]: Yellowish oil; yield 35 mg (0.70 mmol, 70%); ^{1}H NMR (400 MHz, $CDCl_3$, ppm): δ 7.41 (d, *J*=0.9 Hz, 1H), 6.37 (dd, *J*=4.8, 2.5 Hz, 1H), 4.02 (t, *J*=7.1 Hz, 1H), 3.36 (d, *J*=7.1 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$, ppm): δ 146.2, 143.5, 111.8, 110.9, 109.9, 29.8, 22.6; IR (KBr, cm^{-1}): 3124, 3041, 2231, 1606, 1456, 1394; MS (70 eV): *m/z* 81 ($M^{+\cdot}$, 100 %), 164 (12 %), 53 (21 %), 65 (12 %).

4.6.12. (4-nitrophenyl)methanol (**3e1**)[19]: Yellow crystal; mp 92-95 °C; yield 30 mg (0.60 mmol, 60%); ^{1}H NMR (400 MHz, $CDCl_3$, ppm): δ 7.41 (d, *J* = 0.9 Hz, 1H), 6.37 (dd, *J*=4.8, 2.5 Hz, 1H), 4.02 (t, *J*=7.1 Hz, 1H), 3.36 (d, *J*=7.1 Hz, 1H). ^{13}C NMR (100 MHz, $CDCl_3$, ppm): δ 146.2, 143.5, 111.8, 110.9, 109.9, 29.8, 22.6; IR (KBr, cm^{-1}): 3414, 3025, 1743, 1521, 1356, 1003; MS (70 eV): *m/z* 77 ($M^{+\cdot}$, 100 %), 107 (52 %), 89 (41 %), 78 (36 %), 153 (23 %).

4.6.13. 2-cyano-3-(furan-2-yl)propanamide (**4k**)[19]: Brown oil; yield 15 mg (0.30 mmol, 30%); ^{1}H NMR (500 MHz, $CDCl_3$, ppm): δ 7.38-7.35 (m, 1H), 6.32 (dd, *J*=3.1, 1.9 Hz, 1H), 6.25 (d, *J*=3.1 Hz, 1H), 3.70 (dd, *J*=8.0, 5.4 Hz, 1H), 3.40-3.23 (m, 1H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 165.29, 149.13, 142.57, 117.32, 110.61, 108.47, 37.51, 28.41; IR (KBr, cm^{-1}): 3454, 3361, 3240, 2216, 1647, 1546, 1517 1352, 1174; MS (70 eV): *m/z* 81($M^{+\cdot}$, 100 %), 53 (18 %), 164 (15 %), 65 (11 %), 120 (8 %).

Acknowledgments

DEQJ thanks to Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), IMF and WGB thank to Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for scholarships, respectively. The authors thank to CNPq and Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP) for financial support. ALMP thanks to Núcleo de Pesquisa em Ciência e Tecnologia de BioRecursos (CITECBio) of the University of São Paulo for financial and structural support. ALMP and LJFP thank to CNPq (Programa Ciência sem Fronteiras - Pequisador Visitante Especial (PVE, Proc. 400202/2014-0).

Supplementary Material

Supplementary data (^{1}H NMR, ^{13}C NMR spectra, FTIR, GC-MS) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.tetlet.2014. xx.

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- Fast and efficient synthesis of Knoevenagel adducts under microwave irradiation in methanol;
- Whole cells of *P. citrinum* CBMAI 1186 were able of reduced the C-C double bond in the Knoevenagel adducts in good yields;
- First biotransformation of Knoevenagel addcts using fungal cells.