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Diethyl 2-((aryl(alkyl)amino)methylene)malonates: Unreported Mycelial Growth Inhibitors against *Fusarium oxysporum*

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Abstract: This paper presents the discovery and development of antifungal agents against *Fusarium oxysporum* (*Fox*), a devastating plant pathogen. Diethyl 2-((arylamino)methylene)malonates (DAMMs) were formed as side-products during the synthesis of polysubstituted-2-pyridones through a three-component domino reaction and seemed to have antifungal activity against *Fox*. DAMMs are typically employed as intermediates or precursors to produce further bioactive compounds, but they have never been examined as antifungals. To confirm this latter characteristic, we employed a single-step procedure (i.e., the first step of the Gould-Jacobs reaction) to prepare five DAMMs (74–96% yields) which were subsequently evaluated against *Fox* in terms of their abilities to inhibit mycelial growth. The antifungal outcome was promising (0.013 μM < IC_{50} < 35 μM), involving fungistatic or fungicide effects. This small group of active compounds showed differences in antifungal activity, constituting the basis of further studies to expand the DAMM chemical space and look for improved antifungal activity.

Keywords: enamine esters; microwave; antifungals; *Fusarium oxysporum*

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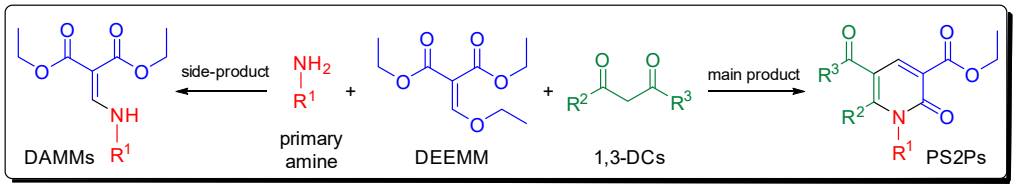
1. Introduction

Plant pathogens are a relevant problem in commercial crops, mainly related to emergence resistance; therefore, discovering and developing new antifungal agents is currently needed [1]. Among problematic phytopathogens, *Fusarium oxysporum* (*Fox*) is a case of interest as an opportunistic microorganism and devastating plant pathogen [2]. As part of our research on compounds against fungal phytopathogens, during the synthesis of polysubstituted-2-pyridones (PS2Ps) using a three-component domino reaction between diethyl ethoxymethylenemalonate (DEEMM), primary amines, and 1,3-dicarbonyl compounds (1,3-DCs) [3], diethyl 2-((ary(alkyl)lamino)methylene)malonates (DAMMs) were formed as side-products by varying the electrophilic nature of the 1,3-DC; this phenomenon was previously unreported for this domino reaction. Notably, amines first reacted with DEEMM instead of the low-electrophilic 1,3-DC (Scheme 1).



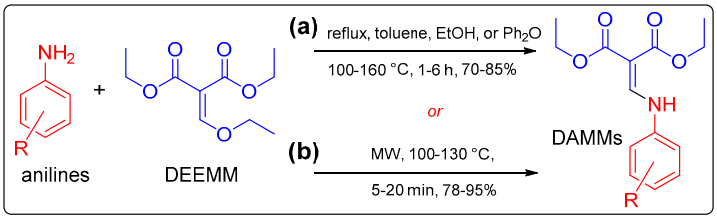
Scheme 1. Three-component domino reaction between diethyl ethoxymethylenemalonate (DEEMM), primary amines, and 1,3-dicarbonyl compounds (1,3-DCs) [3] to produce polysubstituted-2-pyridones (PS2Ps) (main product) and diethyl 2-((arylamino)methylene)malonates (DAMMs) (side-product).

Apart from the moderate scope involved in forming these side-products, they caught our attention, since partially depurated target compounds contaminated with DAMMs

exhibited higher antifungal activity against *Fox* than entirely-purified PS2Ps. This fact promoted our interest in deepening the antifungal activity of DAMMs as bioactive compounds against *Fox*.

DAMMs are chemically characterized as symmetric diesters containing an aromatic-substituted enamine group. They are part of a relevant group of organic compounds that have been used as intermediates or precursors for the synthesis of biologically-active compounds such as quinolones [4], β-oxocarboxylic acids [5], 2,2-*bis*(ethoxycarbonyl)vinylamine derivatives [6], 4-oxoquinoline-3-carboxamide derivatives [7], amido-esters [8], enamino esters [9], functionalized malonic acid half ester [10], 3-formyl-4(1*H*)-pyridones [11], and pyrimidinones [12].

Various protocols have been developed to synthesize DAMMs. They are primarily based on the reaction of anilines with in situ or previously generated electron-deficient olefins, comprising moderate-to-good yields (>50%). For instance, a multicomponent reaction between substituted anilines, diethyl malonate, ethyl orthoformate, acetic anhydride, and catalytic amounts of $ZnCl_2$ or $FeCl_3$ was reported in the 1980s to afford DAMMs (50–85% yield) (Scheme A1a) [13,14]. The base-catalyzed trichloromethyl elimination from diethyl 2-(2,2,2-trifluoro-1-(phenylamino)ethyl)malonate, previously prepared from a two-step procedure, was also reported to produce DAMMs with good three-step overall yields (>70%) (Scheme A1b) [9]. However, the first-reported strategy to prepare DAMMs is the most widely studied procedure, based on the first step of the Gould-Jacobs reaction (reported in the 1930s) and initially performed to obtain quinolines and 4-hydroxyquinoline derivatives [15]. This Gould-Jacobs-based first step involves the thermal reaction of anilines with alkoxy methylene malonic esters or acyl malonic esters [16]. DEEMM is commercially available and is a commonly used electron-deficient olefin. It was used for this reaction with anilines and other nucleophiles since it is a versatile Michael addition acceptor [17]. This thermal addition-elimination over DEEMM is conventionally performed by reflux in ethanol, diphenyl ether, or toluene for 1–6 h at 100–160 °C, achieving to good yields (70–85%) (Scheme 2a) [18–20]. However, metal-catalyzed [5,21] reactions could provide better yields (>95%). Solvent-free microwave (MW)-assisted synthesis has also been developed and reported as an improved reaction and environmentally friendly approach to synthesizing DAMSs [22,23] (Scheme 2b), whose main advantages are related to the MW-mediated reaction rate acceleration owing to the selective heating of more polar reactants by MW irradiation [24]. Thus, MW-assisted synthesis of DAMMs can offer short reaction times, excellent compatibility of various substituents, the absence of solvents and catalysts, low energy consumption, and high yields (>78%).



Scheme 2. Synthetic versions for the synthesis of diethyl 2-((arylamino)methylene)malonates (DAMMs) using the first step of the Gould-Jacobs reaction. (**a**) reflux-based protocol; (**b**) MW-assisted protocol. MW = microwave irradiation; DEEMM = diethyl ethoxymethylenemalonate.

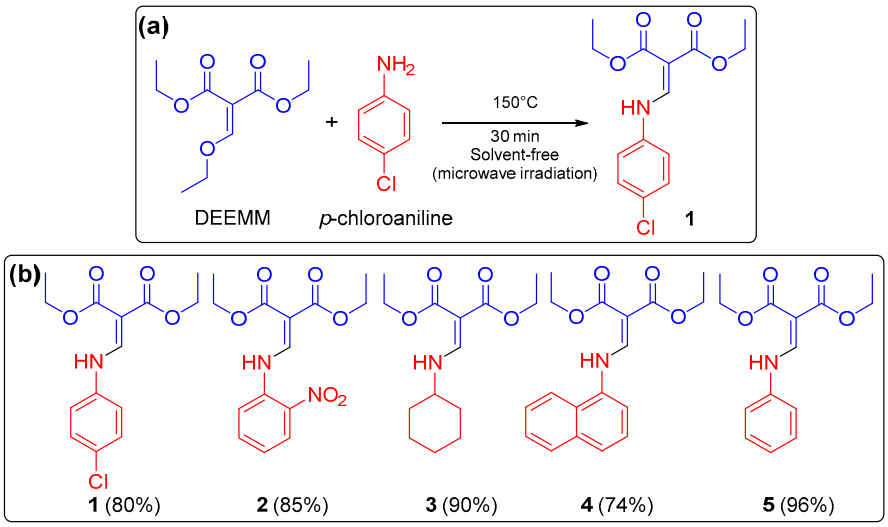
Although DAMMs have mainly been employed as valuable intermediates for synthesizing biologically active compounds, and they are commercially available, their biological properties have not been deeply studied, even though they have shown bioactivity in certain studies [19]. In addition, they contain an enamine ester moiety which has been recognized to exhibit attractive antifungal activity in different enamine-containing compounds [25,26]. With observations of activity as evidenced by the above-mentioned DAMM-contaminated PS2Ps in mind, these structural features led us to hypothesize that DAMMs exhibit antifun-

gal activity against *Fox*, constituting the main contribution and novelty of our study. In this regard, this paper is intended to report, for the first time, the antifungal activity of a small set of DAMMs, prepared through the reported, advantageous MW-assisted protocol. In this study, the DAMM products contained various representative substitutions, allowing us to explore their influence on antifungal activity preliminarily, through mycelial growth inhibition against *Fox*, and contributing to the discovery of bioactive compounds which may be particularly useful in the plant pathology field.

2. Results and Discussion

2.1. Synthesis of the DAMMs **1**–**5**

The target products were synthesized following the reported methodology (Scheme 3) to prepare DAMMs as intermediates, with some modifications [22]. This methodology has already been reported under the following reaction conditions: 115 °C (300 W) for 10 min. However, in our attempts with the available MW reactor, the reaction time and temperature were found to be different, i.e., 30 min and 150 °C (200 W), respectively. These variations resulted from a testing temperature ramp performed between 90–150 °C using DEEMM and *p*-chloroaniline as model reagents to afford DAMM **1** for comparative purposes (Scheme 3a). Under these conditions, the formation of **1** could be observed at 150 °C, whose TLC band was different from the starting reagents. The reaction was monitored by TLC every 5 min and, after 20 min, the expected product was formed, as revealed with iodine vapor, UV (254–366 nm), and Dragendorff's reagent. After 30 min, the reagents were entirely consumed, and the reaction was quenched. The resulting crude product was then purified by column chromatography (*n*-hexane/ethyl acetate 7:3) to afford isolated **1** (80% yield).



Scheme 3. (**a**). One-step reaction for the free-solvent synthesis of diethyl 2-(((4-chlorophenyl)amino) methylene)malonate (DAMM) **1** by a microwave-assisted procedure as the model reaction. (**b**). Structures of the synthesized DAMMs **1**–**5** and their reaction yield in parenthesis. DEEMM = diethyl ethoxymethylenemalonate.

This reported protocol, experimentally optimized to suit our laboratory conditions, was used to prepare additional DAMMs **2**–**5** by employing several primary amines containing representative moieties in order to examine their influence on the DAMM synthesis, i.e., *o*-nitrophenyl, cyclohexyl, 1-naphthyl, and phenyl, whose structures and yields are shown in Scheme 3b. Although some yield variations were identified between the five products, the outcome indicated good to excellent yields (74–96%). The results led us to briefly describe the influence of some precursor amines on the resulting yields. For instance, product **4**, containing a 1-naphthylamine (bulkier substituent), afforded a 74%

yield. This yield can be compared to that of **5**, which includes a phenylamine moiety as a less bulky substituent and afforded the highest yield (96%). Additionally, it was evidenced that the product prepared from aniline with an electron-withdrawing group (EWG) at the *ortho* position, e.g., **2** (85%), showed a similar yield to the product afforded from aniline with a weak EWG at the *para* position, e.g., **1** (80%). This reaction is also facilitated by using primary amines with an alicyclic substituent, e.g., cyclohexyl amine, which afforded product **3** in good yield (90%). The NMR data of compounds **1**, **2**, and **5** have already been reported in the literature [7,19]; however, in the case of compounds **3** and **4**, only chromatographic data can be found so far and, consequently, the present study provides such data (Supplementary Material).

2.2. *Antifungal Activity of DAMMs* **1**–**5**

As mentioned, our aim was based on the discovered antifungal effect of DAMMs when they were formed as side-products during a three-component domino reaction to produce PS2Ps. Thus, once the DAMM antifungal activity was noticed, we employed a known reaction to produce the desired compounds in convenient amounts to validate our previous findings related to the antifungal activity. Hence, DAMMs **1**–**5** were synthesized, isolated to high purity, and evaluated through an in vitro bioassay to assess their capacity to inhibit the mycelial growth of the phytopathogen *Fox* for 72 h. Five concentrations (between 10–0.01 μg/μL) of each test compound were employed for this procedure. Dithane (active ingredient: mancozeb) and Rovral (active ingredient: iprodione) were used as the positive controls, and PDA 0.5% was used as the blank. The antifungal activity results for compounds **1**–**5** are shown in Figure 1.

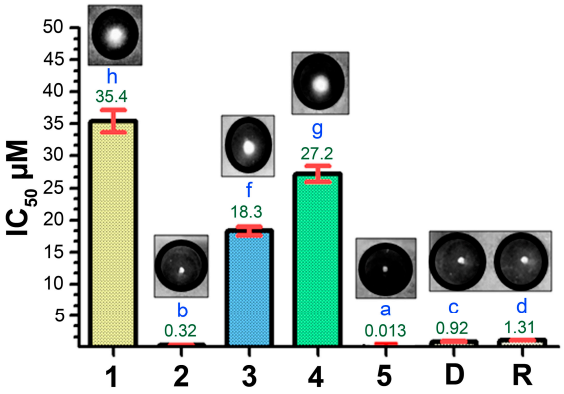


Figure 1. Antifungal activity against *F. oxysporum* (*Fox*) for the diethyl 2-((aryl(alkyl)amino) methylene)malonates **1**–**5** and positive controls (D: Dithane; R: Rovral). Green values over bars correspond to the mean half-maximal inhibitory concentrations (IC_{50} in μM), with red error bars representing the interval confidence. Different blue lowercase letters over bars indicate statistically significant differences according to the Tukey test ($p < 0.05$). Pictures over bars correspond to the observed fungistatic or fungicide effect on *Fox*.

The antifungal activity of DAMMs **1**–**5** followed a dose–response behavior to inhibit the mycelial growth of *Fox*. The best antifungal activity was obtained for **2** and **5** ($IC_{50} < 0.5$ μM), while **1**, **3**, and **4** were less active (18 μM < IC_{50} < 35 μM). The most active DAMM was compound **5**, whose activity was even better than those of the positive controls, constituting a promising antifungal activity against *Fox*. Due to compound **5** not having substitutions in the aromatic ring, and the least active DAMM being **1**, we concluded that the substituent evidenced an unfavorable impact on bioactivity in **1** (e.g., *p*-Cl). However, compound **2** also showed important antifungal activity against *Fusarium oxysporum*, since the IC_{50} was less than 1 μM, suggesting that the *ortho*-substituted nitro group may positively contribute to the inhibition of *Fox*. Likewise, compounds **4** and **3** (having 1-naphthyl and cyclohexyl, respectively, as *N*-substitution) showed low activity, suggesting that a

bulky moiety and an aliphatic ring are plausibly related to the reduced antifungal activity of the tested DAMMs. These observations require further studies, expanding the DAMM chemical space and providing more insights into the structure-dependent variations of the antifungal activity. Additionally, the most-active compounds (**2** and **5**) and positive controls were classified as fungicides (i.e., fungus could not grow in fresh medium after exposure to these treatments), whereas compounds **1**, **3**, and **4** were found to exhibit a fungistatic effect. This fact is also favorable for compound **5** as a promising antifungal, since its effect on *Fox* mycelial growth inhibition was remarkable (IC_{50} = 13 nM), acting as a fungicide [27].

3. Materials and Methods

3.1. General

All starting reagents (primary amines and diethyl ethoxymethylenmalonate) were obtained commercially from Merck (Darmstadt, Germany) and Sigma-Aldrich (Burlington, MA, USA). The reactions were monitored by thin-layer chromatography (TLC) using Silica gel 60 F254. The mobile phase was 7:3 ethyl hexane-acetate, and Dragendorff's reagent, iodine vapors, UV-254 nm, and 366 nm were used as developers. For the separation and purification of the reaction crude, column chromatography (CC) was used as the stationary phase using Merck column silica (0.063–0.200 mm) and as the mobile phase a 7:3 *n*-hexane-ethyl acetate mixture. Additionally, preparative chromatography was used for purification using 0.5 mm thick PLC (preparative layer chromatography) glass chromatographic plates (Merck KGaA, Darmstadt, Germany) on which 100 µL of the reaction crude product was seeded and eluted in 50 mL of hexane-ethyl acetate 7:3 mixture as a mobile phase for 1 h. Subsequently, the plate was monitored by UV (254 nm), and the analyte surface to be separated was labeled. Once the silica/target compound mixture was removed from PLC plates, it was vacuum filtered in a Pyrex™ borosilicate glass funnel with a sintered glass disk and eluted with HPLC-grade methanol. Finally, the compound was concentrated in a rotary evaporator, stored in a clean, dry, and weighed container, and monitored by TLC to verify its purity. High-resolution mass spectrometry analyses with electrospray ionization (ESI) were performed using a Bruker micrOTOF–QII mass spectrometer, consisting of two analytical pumps (model LC-20AD) with SIL-20AHT automatic injector, SPD-20A UV/Vis detector, CTO-20A column oven, and CBM-20A controller. Each compound analyzed by this technique was prepared at 1 mg/mL using LCMS grade methanol as solvent. The column used was a Phenomenex Luna PFP (2) (5 µm, 150 × 2 mm). The flow was 0.2 mL/min, and the mobile phase comprised solvents A (0.1% formic acid in H_2O) and B (0.1% formic acid in MeOH). The employed gradient was 0 min, 5% B, maintained for 2 min, from 5 to 30 min until 100% B, and maintained 100% B for 25 min. The oven temperature was 40 °C, and the wavelength was 254 and 280 nm. The ESI interface was operated in a positive mode with 4.5 kV in the capillary and 0.5 kV in the endplate offset. The nebulization gas pressure was 0.4 Bar; the drying gas was maintained at a flow rate of 8 L/min at 200 °C. The collision and the quadrupole energy were set to 12 and 6 eV, respectively. RF1 and RF2 funnels were programmed to 150 and 200 Vpp, respectively. The mass spectra were calibrated using sodium formate. The results were processed in data analysis software to determine the accurate masses. The ^{1}H NMR spectra were recorded at 500 MHz on a spectrometer DRX 500 (Bruker, Billerica, MA, USA) using $CDCl_3$ as a solvent and tetramethylsilane (TMS, 0.05% *v*/*v*) as an internal standard (TMS δ 0.00). Each spectrum resulted from 128 scans with pulse widths (PW) of 8.0 µs (30 °C) and relaxation delays (RD) of 6.0 s. Chemical shifts were expressed in δ (ppm) involving solvent signals at δ_H 7.26 and δ_C 77.16. Multiplicities were abbreviated as follows: singlet (*s*), doublet (*d*), triplet (*t*), quartet (*q*), and multiplet (*m*). The coupling constants (*J*) were expressed in Hz.

3.2. General Procedure for the Microwave-Assisted Synthesis of Diethyl 2-((aryl(alkyl)amino) methylene)malonates **1**–**5**

The general procedure to synthesize DAMMs was adopted from a reported protocol [22] with some modifications. Briefly, diethyl ethoxymethylenemalonate (1.0 mmol)

(Sigma-Aldrich, Burlington, MA, USA) and primary amines (0.5 mmol) were placed into a 5.0 mL high-pressure reaction tube. The tube was closed and stirred for 1 h at room temperature to mix the raw materials well. Subsequently, the reaction mixture was placed in a CEM brand Discover SP microwave synthesizer (CEM, Matthews, NC, USA) for 30 min at 150 °C. Then, the reaction crude product was monitored by TLC using an *n*-hexane-ethyl acetate mobile phase (7:3 ratio), followed by purification by column chromatography and/or preparative plate chromatography. Finally, the products were revealed with Dragendorff's reagent, showing red bands as a positive test for nitrogen-containing compounds. The MS and NMR spectra and spectroscopical data of compounds **1**–**5** are provided in the Supplementary Material.

3.3. Antifungal Activity

Antifungal activity evaluation of diethyl 2-((aryl(alkyl)amino)methylene)malonates **1**–**5** was performed by measuring the growth halo of the phytopathogen *F. oxysporum* in the presence of the test compounds at different concentrations compared to a blank (PDA 0.5%, without treatment). The culture medium contained 2.4% PDB and 1.5% bacteriological agar in 100 mL of distilled water. First, the medium was homogenized for 2 min in a microwave oven and then sterilized in an autoclave for 1 h at 120 °C. Next, 20 mL of the sterile medium was placed in a sterilized Petri dish. Once it cooled and solidified, a 2-mm plug from a previously prepared monosporic culture was placed onto the central part of the Petri dish and left to grow at 28 °C for 8 days to propagate the fungus.

This antifungal assay involved five concentrations (10, 5, 1, 0.1, and 0.01 μg/μL) of test diethyl 2-((aryl(alkyl)amino)methylene)malonates **1**–**5** as treatments. They were dispersed in a 0.5% supplemented medium [28]. Subsequently, each treatment was randomly placed into a well of a 12-well glass plate (79 × 63 × 4 mm). Finally, a 1-mm plug from the 8-day fungal monosporic culture with a diameter proportional to a 32-mm borosilicate capillary tube was taken and placed onto the central part of each well containing a dispersed treatment into the medium. The plate was placed in a humid chamber for 72 h at 25 °C. The assessment of each concentration per treatment was performed in triplicate. After incubation, a photograph of the 12-well plate was taken and analyzed in ImageJ software. The growth area was then measured. The measured growth areas were employed to calculate the inhibition percentage compared to the blank as follows: % Inhibition = ((blank growth area–target compound growth area)/blank growth area). The calculated inhibition percentages per concentration were used to build a dose–response curve, and the half-maximal inhibitory concentration was calculated by nonlinear regression in Graph Pad Prism 5.0 software.

3.3.1. Fungicidal and Fungistatic Effect

For the fungicidal or fungistatic activity classification procedure, the central plug of the fungus treated with the highest concentration (10 μg/μL) of each test compound **1**–**5** was placed on a fresh, non-amended PDA medium for 72 h. After this, mycelial growth was further monitored. The diethyl 2-((aryl(alkyl)amino)methylene)malonates **1**–**5** were classified as fungistatic or fungicidal if mycelial growth or no mycelial growth, respectively, was observed [29].

3.3.2. Statistical Analysis

A Shapiro-Wilks normality test was performed to assess the normal distribution of the data ($p > 0.05$). Once normal data distribution had been verified, an analysis of variance (ANOVA) was carried out, followed by a post hoc Tukey test to establish significant differences between treatments ($p < 0.05$). These analyses were performed in Infostat statistical software [30].

4. Conclusions

A small set of diethyl 2-((aryl(alkyl)amino)methylene)malonates (**1**–**5**), i.e., side-products of a PS2P-producing domino reaction and prepared by an MW-assisted protocol (>74% yield), showed antifungal activity at different levels. The resulting IC_{50} values of test compounds ranged from 0.013 to 35 μM. The best antifungal outcome was obtained for the test DAMMs with an *ortho*-nitro-substituted or non-substituted aromatic ring (i.e., **2** and **5**, respectively). These two antifungal DAMMs were classified as fungicidal agents, having a promising mycelial growth inhibition effect at the nanomolar scale (320 and 13 nM, respectively). Therefore, they can be considered active candidates for fungicide development to be helpful in plant disease management by controlling the economically relevant phytopathogen, *Fox*.

Supplementary Materials: The following supporting information can be downloaded online, Physical and spectroscopical data of DAMMs (**1**–**5**); Figure S1: High-resolution mass spectra of compound **1**; Figure S2: Assignments of ^{1}H and ^{13}C NMR chemical shifts of compound **1**; Figure S3: ^{1}H NMR spectrum ($CDCl_3$, 500 MHz) of compound **1**; Figure S4: ^{13}C NMR spectrum ($CDCl_3$, 125 MHz) of compound **1**; Figure S5: High-resolution mass spectra of compound **2**; Figure S6: Assignments of ^{1}H and ^{13}C NMR chemical shifts of compound **2**; Figure S7: ^{1}H NMR spectrum ($CDCl_3$, 500 MHz) of compound **2**; Figure S8: ^{13}C NMR spectrum ($CDCl_3$, 125 MHz) of compound **2**; Figure S9: High-resolution mass spectra of compound **3**; Figure S10: Assignments of ^{1}H and ^{13}C NMR chemical shifts of compound **3**; Figure S11: ^{1}H NMR spectrum ($CDCl_3$, 500 MHz) of compound **3**; Figure S12: ^{13}C NMR spectrum ($CDCl_3$, 125 MHz) of compound **3**; Figure S13: High-resolution mass spectra of compound **4**; Figure S14: Assignments of ^{1}H and ^{13}C NMR chemical shifts of compound **4**; Figure S15: ^{1}H NMR spectrum ($CDCl_3$, 500 MHz) of compound **4**; Figure S16: ^{13}C NMR spectrum ($CDCl_3$, 125 MHz) of compound **4**; Figure S17: High-resolution mass spectra of compound **5**; Figure S18: Assignments of ^{1}H and ^{13}C NMR chemical shifts of compound **5**; Figure S19: ^{1}H NMR spectrum ($CDCl_3$, 500 MHz) of compound **5**; Figure S20: ^{13}C NMR spectrum ($CDCl_3$, 125 MHz) of compound **5**; Scheme S1. Reaction mechanism to produce DAMMs.

Author Contributions: Conceptualization, E.C.-B.; methodology, W.-F.C.-V.; software, W.-F.C.-V. and E.C.-B.; validation, W.-F.C.-V., D.Q. and E.C.-B.; formal analysis, W.-F.C.-V. and E.C.-B.; resources, D.Q. and E.C.-B.; data curation, W.-F.C.-V. and E.C.-B.; writing—original draft preparation, W.-F.C.-V.; writing—review and editing, W.-F.C.-V., D.Q. and E.C.-B.; supervision, E.C.-B.; project administration, E.C.-B.; funding acquisition, D.Q. and E.C.-B. All authors have read and agreed to the published version of the manuscript.

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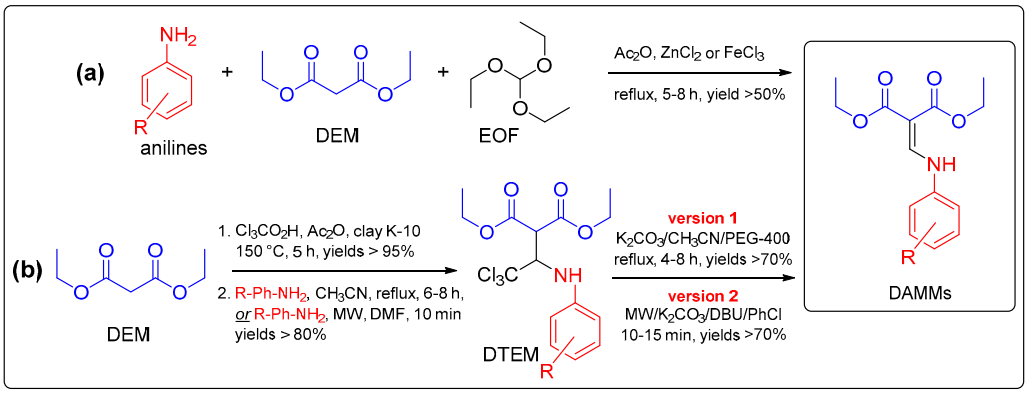
Conflicts of Interest: The authors declare no conflict of interest.

Appendix A



Scheme A1. Synthetic strategies for the synthesis of diethyl 2-((arylamino)methylene)malonates (DAMMs). (**a**) multicomponent strategy; (**b**) three-step strategy. DEM = Diethyl malonate; EOF = ethyl orthoformate; MW = microwave irradiation; DTEM = 2-(2,2,2-trifluoro-1-(phenylamino) ethyl)malonate; DMF = *N*,*N*-dimethylformamide; PEG-400 = polyethylenglycol-400; DBU = 1,8-diazabicyclo(5.4.0)undec-7-ene.

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