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SHORT COMMUNICATION

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Facile synthesis of the fungus-derived natural products: *N,N′*-dipalmitoleyl urea ($C_{16:1}$) and *N,N′*-dioleyl urea ($C_{18:1}$)

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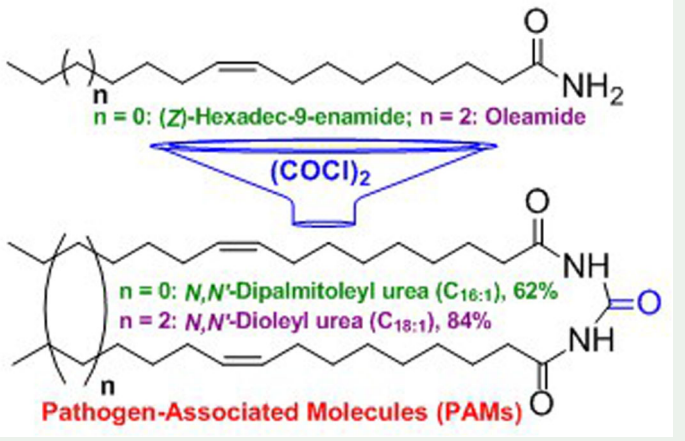
ABSTRACT

Oxalyl chloride is one of the most versatile reagents used in organic synthesis. Oxalyl chloride was employed in the convenient one-pot, two-step synthesis of the fungus-derived naturally occurring lipoids: *N,N′*-dipalmitoleyl urea ($C_{16:1}$) and *N,N′*-dioleyl urea ($C_{18:1}$). The two symmetrical diacyl urea-based natural products were previously identified as fungus-specific pathogen-associated molecules (PAMs), which act as inflammatory mediators during fungal infection. The highly lipophilic natural lipoids were efficiently synthesized from commercially available reagents in yields ranging from good to very good.



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Oxalyl chloride; diacyl urea (DAU); lipoid; pathogen-associated molecules (PAMs); fungal natural products; one-pot reaction

1. Introduction

The synthesis of symmetrical and unsymmetrical diacyl ureas is well established in the literature. The first compound representing this scaffold, the symmetrical diacetyl urea (ureide) was prepared (Schmidt 1872) by the reaction of two equivalents of acetamide with phosgene (Figure 1(a)). On the other hand, condensation of urea with carboxylic

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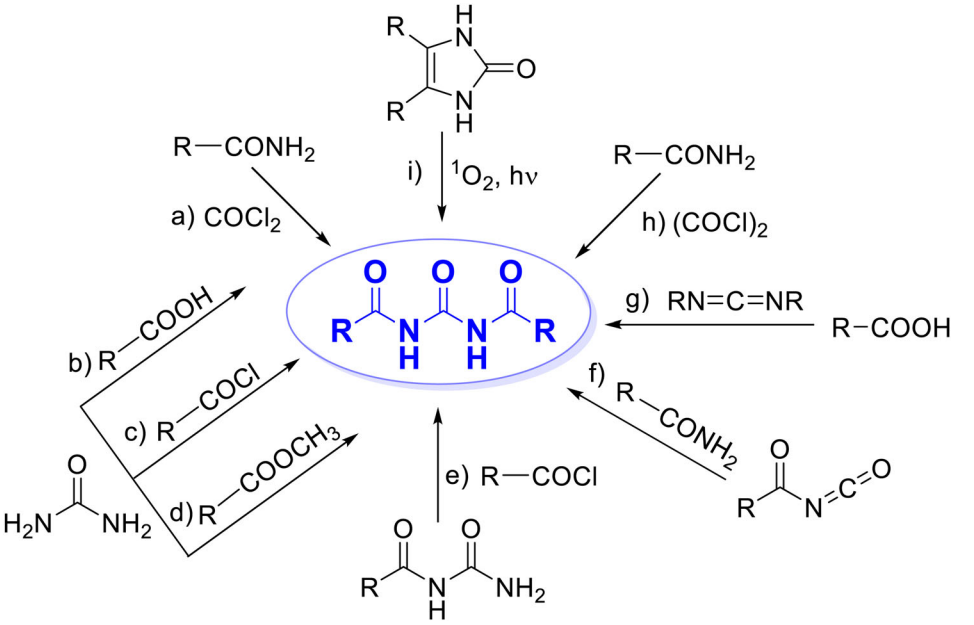


Figure 1. Reported synthetic routes to obtain diacyl ureas.

acids in the presence of a metal catalyst (iron or zinc oxide) (Dean 2002) (Figure 1(b)), or alternatively with the excess of acyl chlorides in a DMAP–pyridine mixture (Kanji Kubo and Mori 2005) (Figure 1(c)) afforded symmetrical diacyl urea derivatives. Reacting urea with various esters in the presence of a base such as sodium ethoxide (Clemmensen and Heitman 1908, 1909) or sodium hydride (Wolfe and Trimitsis 1969) provided access to a diverse set of diacyl ureas (Figure 1(d)). A stepwise approach to unsymmetrical diacyl ureas was developed by reacting either monoacyl ureas with acyl chlorides and sulfuric acid as the catalyst (Werner 1916; Bayer & Co 1919; Stoughton 1938) (Figure 1(e)), or various amides with acyl isocyanates (Scholl 1890; Meyer and Jacobson 1893; Bayer & Co 1913; American Chemical Society 1916; Micich 1982) (Figure 1(f)). Use of aromatic carbodiimides and certain carboxylic acids in pyridine as well provided diacyl ureas (Zetzsche and Lindlar 1938; Kurzer and Douraghi-Zadeh 1967) (Figure 1(g)). The reaction of amides with oxalyl chloride to give symmetrical diaroyl ureas was first reported Bornwater in 1912, and recently this method was extensively elaborated (Garcia Hernandez et al. 2017) to afford a wide range of symmetrical and unsymmetrical diacyl ureas and related compounds in a convenient one-pot protocol (Garcia Hernandez et al. 2017) (Figure 1(h)). The diacyl urea skeleton was also obtained from imidazolin-2-ones *via* dye-induced photooxygenation (Chawla and Pathak 1990) (Figure 1(i)).

Diverse bioactivity of various diacyl ureas was reported in the literature, including hypnotic properties (aliphatic diacyl ureas bearing 8–10 carbon atoms) (Stoughton 1938), plant growth enhancers (*N,N′*-diformylurea) (Dean 2002), inhibitors of fatty acid amide hydrolase (*N*-benzoyl-*N′*-oleyl ureas) (Cramer et al. 2019), and presumably bacteriostatic agents (Micich 1982). Dibenzoyl urea analog of the known insecticide, diflubenzuron was also reported (Garcia Hernandez et al. 2017). More recently, several

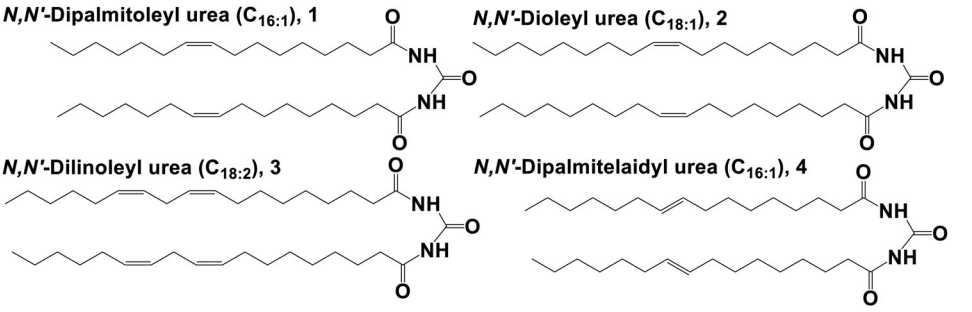
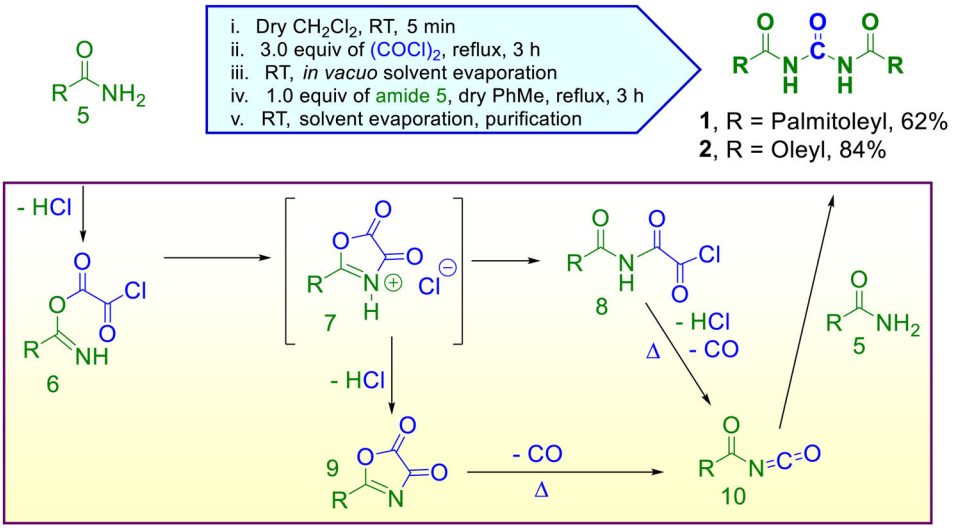


Figure 2. Diacyl urea-based pathogen-associated molecules (PAMs) isolated from fungi.

unusual lipoids possessing the diacyl urea linker and fatty acid chains were identified (Schröder et al. 2002; Schröder and Häsler 2003; Noverr et al. 2003) from fungi (Figure 2). These compounds were characterized as pathogen-associated molecules (PAMs) that display lipid-like leukocyte activator (LILA) properties thus inducing upon infection adherence and degranulation in human neutrophils and activation of leukocytes (Schröder et al. 2002; Schröder and Häsler 2003; Noverr et al. 2003). Compound **1**, the *N,N′*-dipalmitoleyl urea ($C_{16:1}$) turned out to be the most potent molecule, which induced the innate immune responses measured as neutrophil chemotactic activity at $ED_{50} = 140$ nM. Its close analogues, compounds **2** and **3**, were approximately 40-times less potent whereas the *trans* isomer of **1**, compound **4** was inactive thus clearly implying the importance of a *cis* double bond in those PAMs (Schröder et al. 2002). Encouraged with their biological activity and interesting structure, we employed the one-pot, two-step reaction protocol developed previously in our laboratory (Garcia Hernandez et al. 2017) to synthesize *N,N′*-dipalmitoleyl urea ($C_{16:1}$) and *N,N′*-dioleyl urea ($C_{18:1}$) (Figure 2) from commercially available starting materials.

2. Results and discussion

The target compounds **1** and **2** [*N,N′*-dipalmitoleyl urea ($C_{16:1}$) and *N,N′*-dioleyl urea ($C_{18:1}$), respectively] were synthesized from the commercially available starting amides (**5**): (*Z*)-hexadec-9-enamide and oleamide, respectively by following the protocol reported previously (Garcia Hernandez et al. 2017) (Scheme 1). Oxalyl chloride, a highly versatile chemical reagent (Mohammadkhani and Heravi 2019), was used in this transformation as a coupling agent and donor of the carbonyl group according to the well-established reaction mechanism (Speziale and Smith 1962) (Scheme 1). The crude materials were readily isolated followed by standard purification on chromatography column to give the natural products **1** and **2** in the yield of 62% and 84%, respectively. This reaction yield was substantially higher and the one-pot protocol much more convenient when compared to the natural products synthesized by the modified carbodiimide method (Schröder et al. 2002). The final products were easily distinguishable from their respective starting materials by ^{1}H and ^{13}C NMR spectroscopy as they lacked the characteristic signals pertinent to the amide protons (two broad peaks in the range of 5.64–5.39 ppm observed by ^{1}H NMR in $CDCl_3$). The signals pertinent to the imide *N*H in the final products were not visible by ^{1}H NMR when deuterated



Scheme 1. Convenient one-pot, two-step conversion of fatty acid amides into naturally occurring lipoids **1** and **2**.

chloroform ($CDCl_3$) was used as the solvent. However, changing the solvent to deuterated dimethyl sulfoxide ($DMSO_{-d6}$) allowed to discern the imide *N*H by ^{1}H NMR at 10.86 ppm for compound **1**. On the other hand, a characteristic signals were observed at 150.07 and 149.66 ppm by ^{13}C NMR in $CDCl_3$ that were pertinent to the central carbonyl groups present in the final products **1** and **2**, respectively. The exact mass of the final products was confirmed by high resolution mass spectrometry (HRMS). The final products, however, were accompanied with an inseparable impurities (∼3% for **1** and ∼7% for **2**), which were also present in the starting materials, as determined by ^{1}H and ^{13}C NMR. Based on the close proximity and even occasional overlap of the NMR signals associated with the impurity and the desired final product, it could be suspected that the impurity might be the *E*-isomers of the starting amides **5**, which were then converted into the *E*-isomers of the final products **1** and **2**, respectively. It is apparent that the lipoids **3** and **4** (Figure 2) can be readily synthesized by using this synthetic method and the appropriate starting materials [i.e. (9*Z*, 12*Z*)-octadeca-9,12-dienamide and (*E*)-hexadec-9-enamide, respectively], which are also commercially available.

Due to the diacyl urea motif, lipophilic nature, and structural similarity of the natural products to the previously reported oleamide-derived diacyl urea FAAH inhibitors (Cramer et al. 2019), compounds **1** and **2** were tested *in vitro* in search for inhibitory activity of this enzyme. Sadly, neither of those compounds showed any inhibition of the human FAAH enzyme.

3. Experimental

3.1. General information

(*Z*)-Hexadec-9-enamide (95% purity) was purchased from Accel Pharmtech LLC whereas oleamide (95% purity) was purchased from AK Scientific. Oxalyl chloride

(reagent plus grade) and anhydrous toluene ($PhCH_3$) were purchased from Sigma-Aldrich whereas dichloromethane (CH_2Cl_2) was purchased from Fisher Scientific and was dried by distillation over calcium hydride. All other solvents were purchased from Fisher Scientific and used without further purification. ^{1}H NMR and ^{13}C NMR spectra were recorded on Bruker Avance spectrometer at 400 and 100 MHz, respectively. NMR spectra were reprocessed by using the ACD Labs 2019 Spectrus Processor software. Standard abbreviations indicating multiplicity were used as follows: s, singlet; d, doublet; dd, doublet of doublets; t, triplet; q, quadruplet; quin, quintet; m, multiplet; and br, broad. HRMS experiments were performed on an ultra-performance liquid chromatography mass spectrometer (UPLC-MS, Thermo Scientific) with the ultimate 3000 binary pump system. The UPLC-MS system is a Q Exactive Focus Orbitrap. The column used for analysis was a Phenomenex Luna Omega 1.6 μm polar C_{18} 100 × 21 mm column at a flow rate of 0.4 mL/min and column temperature of 40 °C. Heated electrospray ionization (HESI) in the positive mode was utilized. Melting points were measured with a MEL-TEMP II melting point apparatus and are reported uncorrected. TLC was performed on Merck 60 F254 silica gel plates. Traditional flash chromatography was done using silica gel and gradient of ethyl acetate in hexanes as the eluent.

3.2. Synthesis of the target compounds 1 and 2

(*Z*)-Hexadec-9-enamide [or oleamide] (1.0 mmol, 1.0 equiv) was placed in a Schlenk Kjeldahl reaction flask and the flask was evacuated/Argon re-filled three times. Subsequently, anhydrous dichloromethane (25 mL) was added and the mixture was stirred at room temperature (RT) for 5 min before dropwise addition of oxalyl chloride (3.0 mmol, 3.0 equiv) followed. The reaction mixture was then stirred at reflux for 3.0 hours before cooling to RT and the solvent was removed under reduced pressure in an air-free manner. Subsequently, (*Z*)-hexadec-9-enamide [or oleamide] (1.0 mmol, 1.0 equiv) was rapidly added *via* regular powder funnel and the flask was again evacuated/Argon re-filled three times before dry toluene (12 mL) was added. The reaction mixture was then stirred at reflux for another 3 h before cooling to RT and concentration on rotary evaporator. The isolated crude material was purified by flash chromatography on silica with gradient of ethyl acetate in hexanes as the eluent.

3.3. Analytical data of the synthesized natural products

Dipalmitoleyl urea ($C_{16:1}$) (1)

Milky-white solid, 332 mg (62%). Melting point: 41–43 °C.

^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 5.41–5.32 (m, 4H, CH = CH), 2.53 (br t, J = 6.8 Hz, 4H), 2.03 (q, J = 6.5 Hz, 8H), 1.69 (quin, J = 7.3 Hz, 4H), 1.38–1.29 (m, 32H), 0.90 (t, J = 6.9 Hz, 6H, CH_3).

^{13}C NMR (100 MHz, $CDCl_3$) δ ppm 174.27 (br s, 2 C, C = O), 150.07 (1 C, C = O), 130.06 (2CH, CH = CH), 129.67 (2CH, CH = CH), 37.68 ($2CH_2$), 31.78 ($2CH_2$), 29.73 ($2CH_2$), 29.68 ($2CH_2$), 29.20 ($2CH_2$), 29.07 ($2CH_2$), 29.01 ($2CH_2$), 28.99 ($2CH_2$), 27.23 ($2CH_2$), 27.16 ($2CH_2$), 24.41 ($2CH_2$), 22.66 ($2CH_2$), 14.10 ($2CH_3$).

^{1}H NMR (400 MHz, DMSO$_{-d6}$) δ ppm 10.86 (br s, 2H, NH), 5.32 (br t, $J = 4.7$ Hz, 4H, CH = CH), 2.49 (br t overlapping with DMSO, $J = 7.3$ Hz, 4H), 1.98 (q, $J = 6.0$ Hz, 8H), 1.52 (quin, $J = 6.8$ Hz, 4H), 1.31–1.25 (m, 32H), 0.85 (t, $J = 6.8$ Hz, 6H, CH_3).

^{13}C NMR (100 MHz, DMSO$_{-d6}$) δ ppm 175.34 (2 C, C = O), 149.95 (1 C, C = O), 130.12 (2CH, CH = CH), 130.07 (2CH, CH = CH), 36.88 ($2CH_2$), 31.60 ($2CH_2$), 29.56 ($2CH_2$), 29.50 ($2CH_2$), 29.01 ($2CH_2$), 28.87 ($2CH_2$), 28.81 ($2CH_2$), 28.74 ($2CH_2$), 27.06 ($2CH_2$), 27.03 ($2CH_2$), 24.36 ($2CH_2$), 22.55 ($2CH_2$), 14.37 ($2CH_3$).

HRMS (HESI+): *m/z* $[M + H]^+$ calculated for $C_{33}H_{61}N_2O_3$: 533.4677; found: 533.4678.

N,N′-dioleyl urea ($C_{18:1}$) (2)

Milky-white solid, 492 mg (84%). Melting point: 76–78 °C.

^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 5.41–5.32 (m, 4H, CH = CH), 2.53 (br t, $J = 6.0$ Hz, 4H), 2.03 (q, $J = 6.0$ Hz, 4H), 1.69 (quin, $J = 7.1$ Hz, 4H), 1.34–1.27 (m, 44H), 0.90 (t, $J = 6.9$ Hz, 6H, CH_3).

^{13}C NMR (100 MHz, $CDCl_3$) δ ppm 174.22 (1 C, C = O), 174.18 (1 C, C = O), 149.66 (1 C, C = O), 130.07 (2CH, CH = CH), 129.68 (2CH, CH = CH), 37.72 ($2CH_2$), 31.93 (CH_2), 31.92 (CH_2), 29.77 (CH_2), 29.71 ($2CH_2$), 29.69 ($2CH_2$), 29.66 ($2CH_2$), 29.60 (CH_2), 29.53 (CH_2), 29.45 (CH_2), 29.37 (CH_2), 29.34 (CH_2), 29.32 (CH_2), 29.29 (CH_2), 29.20 (CH_2), 29.08 (CH_2), 29.04 (CH_2), 29.01 (CH_2), 27.24 (CH_2), 27.17(CH_2), 24.44 (CH_2), 24.41 (CH_2), 22.69 ($2CH_2$), 14.12 ($2CH_3$).

^{1}H NMR (400 MHz, DMSO$_{-d6}$) δ ppm 10.86 (br s, 2H, NH), 5.38–5.26 (m, 4H, CH = CH), 2.30 (t, $J = 7.6$ Hz, 1H), 2.05–1.91 (m, 7H), 1.58–1.43 (m, 6H), 1.38–1.15 (m, 42H), 0.85 (t, $J = 6.4$ Hz, 6H, CH_3).

^{13}C NMR (100 MHz, DMSO$_{-d6}$) δ ppm 175.38 (1 C, C = O), 175.34 (1 C, C = O), 149.94 (1 C, C = O), 130.13 (2CH, CH = CH), 130.08 (2CH, CH = CH), 36.88 ($2CH_2$), 31.74 ($2CH_2$), 29.56 ($2CH_2$), 29.49 ($2CH_2$), 29.29 ($2CH_2$), 29.15 ($2CH_2$), 29.04 ($2CH_2$), 28.99 ($2CH_2$), 28.86 ($2CH_2$), 28.80 ($2CH_2$), 27.08 ($2CH_2$), 27.03 ($2CH_2$), 24.36 ($2CH_2$), 22.55 ($2CH_2$), 14.40 ($2CH_3$).

HRMS (HESI+): *m/z* $[M + Na]^+$ calculated for $C_{37}H_{68}N_2NaO_3$: 611.5122; found: 611.5121.

3.4. hFAAH enzyme screening assay

Human recombinant FAAH enzyme (Item No. 100101183, Batch No. 0523867) (Item No. 10011669) was obtained from Cayman Chemical. Measurement of FAAH potency was performed using the substrate *N*-(6-methoxypyridin-3-yl) octanamide (OMP) ([S]final = 50 μM) in sodium phosphate buffer (0.1 M, pH = 8, 0.1 mg/mL BSA). Progress of the reaction was measured by fluorescence detection of 6-methoxypyridin-3-amine at an excitation wavelength of 303 nm and an emission wavelength of 394 nm at 37 °C by the use of microplate reader (Molecular Devices, CA, USA). All experiments were run in triplicate, and values reported as average ± SD. The substrate OMP was synthesized following a previously reported synthetic procedure and reaction conditions (Wilt et al. 2020). Quantification of inhibitor potencies were performed following the previously published procedure for FAAH enzyme (Kodani et al. 2016).

4. Conclusions

Herein we report a convenient and high-yielding synthesis of the symmetrical fatty acid-derived diacyl ureas *N,N'*-dipalmitoleyl urea ($C_{16:1}$) and *N,N'*-dioleyl urea ($C_{18:1}$). Those compounds were initially isolated from fungi and defined as PAMs, which act as pro-inflammatory factors. The presented herein quick access to those molecules and their derivatives might spark an interest in investigating further their immunomodulatory function. Additionally, those unusual natural products could be used for detection of fungi in biological samples. The natural products were screened *in vitro* in FAAH enzyme assay and unfortunately, they turned out to be inactive.

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Disclosure statement

The authors have no conflict of interest to declare.

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