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SOLANAPYRONES E-G, ANTIALGAL METABOLITES PRODUCED BY A MARINE FUNGUS

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Key Word Index—solanapyrones; antialgal; marine fungus; *Halimeda monile*; phytotoxins.

Abstract—Three new α-pyrones, solanapyrones E (**1**), F (**2**), and G (**3**), in addition to the known phytotoxin solanapyrone C (**4**), were produced by an unidentified filamentous marine fungus isolated from the surface of the green alga *Halimeda monile*. The new solanapyrones were characterized by one and two-dimensional NMR spectroscopic techniques, and the absolute configuration of **2** was assigned by the Mosher method. The solanapyrones showed toxicity to the marine unicellular alga *Dunaliella* sp. at concentrations as low as 100 $\mu g\,ml^{-1}$. The possible role of these compounds in phytotoxic associations between marine fungi and algae is discussed. © 1998 Elsevier Science Ltd. All rights reserved.

INTRODUCTION

Pathogenicity in the marine environment has gained increased interest in the last decade with the discovery of a number of microbial diseases attacking marine organisms [1–6], including marine macroalgae [7]. These plants are an important part of the marine coastal environment from both an ecological and economic standpoint [8] and are host to a variety of microbial pathogens which include bacteria, viruses, and marine fungi [9, 10]. Fungal pathogens of marine algae include zoosporic fungi [11] which are known to infect and destroy a variety of marine plants and filamentous fungi which have been implicated in a number of diseases in marine macroalgae [10, 12].

Thus, while there are an increasing number of studies which have identified fungi as important agents in the pathology of marine plants, very little is known about the mechanism of pathogenesis. Unlike terrestrial systems in which phytopathogenic fungi have been shown to produce phytotoxic secondary metabolites which are important in the diseases of terrestrial plants [13], very little is known about the nature or role of fungal metabolites in the pathology of marine algae. While the number of reports of secondary metabolites from marine fungi is steadily increasing (see reviews [14–16]), only a select few studies have examined fungi isolated from algal surfaces [17, 18]. More importantly, most of this research has been directed toward the examination of fungal metabolites for biomedically relevant activity, largely ignoring the ecological roles of fungal secondary metabolites in the marine ecosystem.

As part of a program directed at studying the antialgal effects of fungal metabolites, we isolated a marine fungus from the surface of the calcareous green alga *Halimeda monile* which produced three new compounds, solanapyrones E (**1**), F (**2**) and G (**3**), in addition to the known phytotoxic compound solanapyrone C (**4**). These compounds exhibited substantial antialgal activity in assays against the unicellular marine alga *Dunaliella* sp.

RESULTS

An unidentified marine fungus designated CNC-159 was isolated from the surface of the calcareous green alga *Halimeda monile* and cultured in a marine medium for chemical study. The ethyl acetate extract of the culture broth was subjected to preparative silica TLC followed by reversed-phase C18 HPLC which led to the isolation of three related new molecules, solanapyrones E-G (**1–3**), and the known compound solanapyrone C (**4**).

The known compound solanapyrone C was identified by interpretation of high resolution mass spectrometry which indicated a molecular formula of $C_{19}H_{25}NO_4$ and by comparison of the ^{13}C NMR data to published results [19, 20]. Solanapyrone C is a potent phytotoxin that has been isolated from *Asco-*

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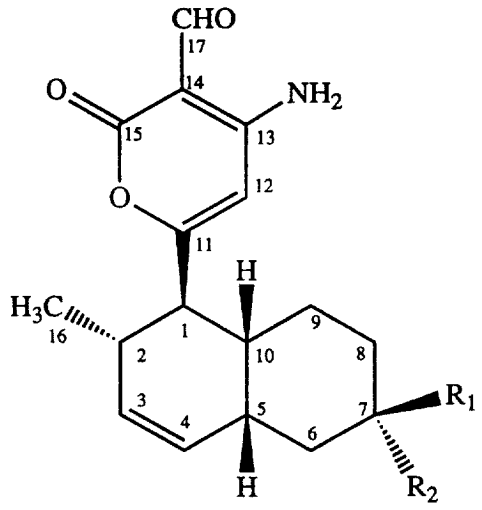

1 R_1=OH R_2=H
2 R_1=H R_2=OH
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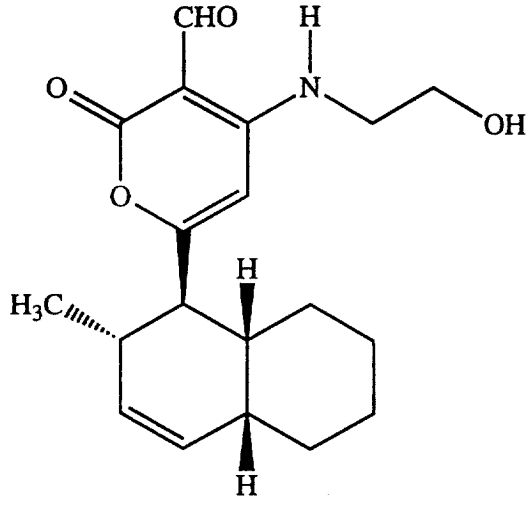

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chyta rabiei, a fungus responsible for the blight of the terrestrial plant *Cicer arietinum* [20], and from *Alternaria solani*, the causal organism of early blight disease in tomato and potato plants [19]. These fungi are common pathogens on various plants and produce a great diversity of phytotoxic metabolites [21].

While the ^{1}H and ^{13}C NMR data for solanapyrone E (**1**) were similar to those of solanapyrone C (**4**), the molecular formula of **1** was determined to be $C_{17}H_{21}NO_4$ by high resolution mass spectrometry. The presence of two exchangeable protons at δ 9.63 and 10.23 due to a primary amine and the loss of two carbons indicated that the ethanolimine moiety in **4** had been replaced by a free amino group in solanapyrone E (**1**). The additional oxygen in the formula could be explained by the presence of a hydroxyl group, as demonstrated by a ^{13}C NMR resonance at 64.9 ppm, and was further supported by an absorption in the IR spectrum at 3337 cm^{-1}. Using ^{1}H and ^{13}C spectroscopic NMR methods, including two dimensional COSY and HMQC methods, the decalin ring system was unambiguously assigned with the alcohol at the C-7 position. Finally, the carbons in the pyrone ring were assigned based on analogy to solanapyrone C (**4**) [20].

The relative stereochemistry of solanapyrone E was assigned based on interpretation of the ^{1}H NMR coupling constants and on results obtained from a 2D NOESY experiment. The key NOE correlations leading to the proposal of a three dimensional structure for **1** are shown in Fig. 1. The ring junction protons at H-5 and H-10 showed a strong correlation thus indicating a *cis* ring fusion. The stereochemistry at C-2 was assigned based on a NOE correlation between H-10 and H-2. The methyl group at C-2 also showed a strong NOE to the proton at position C-1, thus establishing the stereochemistry for the pyrone ring attachment. Finally, the stereochemistry of the alcohol at position C-7 was established by analysis of the ^{1}H NMR coupling constants. The proton at C-5 showed a large axial-axial coupling (12 Hz) to H-6a establishing this proton in an axial position on the ring. In turn, H-6a displayed a small axial-equatorial (2 Hz) coupling to H-7 placing this proton in an equatorial position on the decalin ring and establishing the alcohol as axial. The relative stereochemistry of **1** is identical to that of solanapyrone C (**4**) at C-5, C-10, C-2 and C-1 which was secured by a previously reported X-ray crystal structure [20].

The ^{1}H and ^{13}C NMR data for solanapyrone F (**2**) were nearly identical to those of solanapyrone E (**1**). The high resolution mass spectrum indicated a molecular formula of $C_{17}H_{21}NO_4$, identical to solanapyrone E (**1**), leading to the proposal that **2** was the dias-

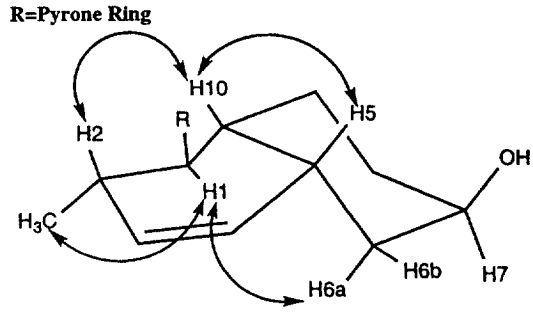

Fig. 1. NOESY analysis of solanapyrone E (**1**), key NOE correlations indicated by arrows. [Pyridine-d_5, 500 MHz, mixing time (τ_m) = 0.9 s, delay value (D1) = 2.7 s].

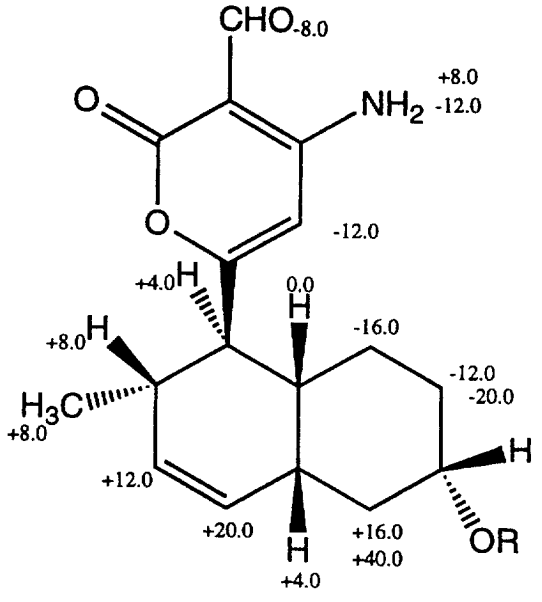

Fig. 2. Analysis of the mosher ester of solanapyrone F (**2**) with $\Delta\delta$H (δS-δR) values expressed in Hz (400 MHz).

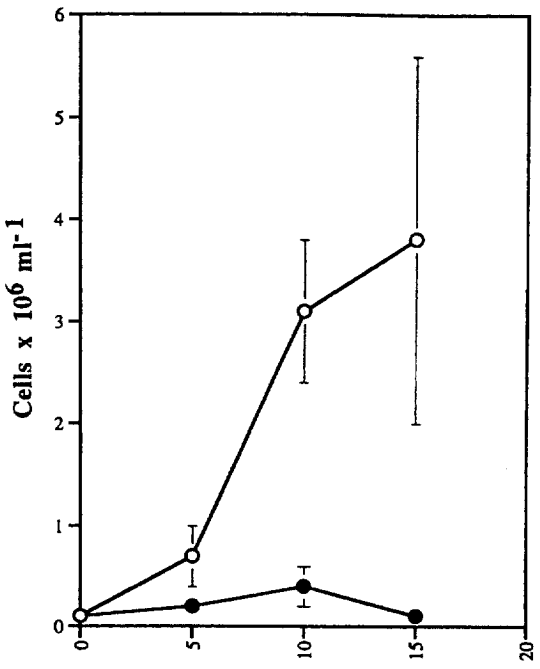

Fig. 3. Growth inhibition of *Dunaliella* sp. in response to solanapyrone C. Values represent the mean for two replicates ± std. dev. Open circles represent ethanol controls, solid circles represent solanapyrone C (100 μg ml^{-1}).

tereomer of **1**. A 4 ppm downfield shift of the C-7 carbon (69.0 ppm) indicated solanapyrone F (**2**) was the epimeric alcohol. This was confirmed by an analysis of the coupling constants for H-7 which showed an axial-axial coupling of 11 Hz and thus indicated the alcohol was in the equatorial position. Solanapyrone F (**2**) was assigned fully based on analogy to **1** and through the use of 2D NMR experiments.

The absolute stereochemistry of solanapyrone F (**2**) was solved by application of Mosher's method [22]. Solanapyrone F (**2**) was selected since it possessed the less hindered equatorial alcohol and was more reactive to esterification. The corresponding MTPA esters were formed and the $\Delta\delta$ values obtained for **2** were determined as shown in Fig. 2. The results of this experiment indicate the *S*-configuration at C-7 and the absolute stereochemistry is thus indicated in Fig. 2.

Finally, the spectral data for solanapyrone G (**3**) were similar to those of both solanapyrones E (**1**) and F (**2**), but the ^{13}C NMR spectrum showed the absence of the alcohol at position C-7. The high resolution mass spectrum confirmed this loss of oxygen and indicated a formula of $C_{17}H_{21}NO_3$. Assignments for solanapyrone G (**3**) by analogy to the other solanapyrones were based upon comprehensive NMR spectroscopic experiments.

The solanapyrones were examined for antialgal activity against the unicellular marine alga *Dunaliella* sp. in liquid media cell growth assays. The known phytotoxic compound solanapyrone C (**4**) showed 97% inhibition after 15 days when tested at 100 μg ml^{-1} (Fig. 3). Solanapyrone E (**1**) and F (**2**) showed only 15% and 25% inhibition at slightly higher concentrations of 200 μg ml^{-1}. Solanapyrone G (**3**) could not be tested due to the very limited quantities available.

DISCUSSION

This work has shown that a marine fungus isolated from the surface of the marine chlorophyte alga *Halimeda monile* produced two new metabolites, solanapyrones E (**1**) and F (**2**), which exhibit moderate antialgal activity against the unicellular marine chlorophyte *Dunaliella* sp. In addition, the known phytotoxic compound solanapyrone C (**4**) was also isolated and exhibited substantial bioactivity in our assay at a concentration of 300 μM. Solanapyrone C (**4**) has previously shown toxicity to terrestrial plants at a concentration of 450 μM [23].

Prior to the work presented here, only one other study has examined the effect of fungal metabolites against marine microalgae [24]. Halymecin A, a metabolite derived from the marine fungi *Fusarium* sp. and *Acremonium* sp., isolated from the surface of a marine alga, has been shown to be toxic to the marine chlorophyte microalga *Brachiomonas submarina* and the marine diatom *Skeletonema costatum* [24]. Unfortunately, there was no discussion in this

work of the ecological relevance of these antialgal effects.

The bioassay organism examined in our study, *Dunaliella* sp., was chosen due to its taxonomic position as a marine chlorophyte and therefore may accurately reflect the activity of antialgal metabolites against other chlorophytes such as *Halimeda monile*. One can infer that the activity seen in the microalgal bioassay is a good indicator of potential activity against macroalgae due to the similarities present in the photosystems of all chlorophyte green algae [25]. In addition, microalgae provide efficient and reliable bioassay systems [26]. The role of the solanapyrones as algal toxins is further supported by the active secretion of these metabolites from the fungal mycelium, suggesting that these metabolites come in contact with and therefore affect algal cells in the associated algae.

However, the activity of the solanapyrones against *Dunaliella* sp. should be more exactly classified as an algistatic effect versus a truly phytotoxic response. This is supported by experiments in which solanapyrones C (**4**), E (**1**) and F(**2**), when applied to the surface of the macroalga *Halimeda monile*, failed to exhibit any of the typical phytotoxic effects (necrosis, tissue blackening and lesions) seen in terrestrial plant systems for solanapyrone C [19, 23]. The compounds may act in a more subtle manner such as affecting the rate of cell division or inhibiting the growth of new cells in the rapidly dividing, young sections of marine algae.

The ability of solanapyrone C (**4**), and to a lesser extent E (**1**) and F (**2**), to inhibit the growth of *Dunaliella* sp. indicates that fungal metabolites may pose a chemical threat to marine algae. Therefore, it is important that we continue to investigate the mechanisms involved in both biological and chemical pathogenicity in marine systems. This study represents an important first step in beginning to understand the chemical mechanisms of fungal pathogenicity in the marine environment and thus provides a parallel study to the extensive knowledge of the role of phytotoxins in terrestrial plant systems.

EXPERIMENTAL

Collection, fermentation, extraction and isolation

The fungus designated CNC-159 was isolated from the surface of the green calcareous marine alga *Halimeda monile* collected subtidally from a mangrove environment in the Bahamas. The fungus was isolated by directly plating the alga on an appropriate nutrient rich medium (YPG; 5 g yeast extract, 5 g peptone, 10 g glucose, 17 g agar, 1 L filtered seawater) containing the antibiotics streptomycin and penicillin to retard bacterial growth.

CNC-159 produced aerial hyphae in culture but failed to sporulate under the conditions examined in this study. Upon repeated inoculations of this fungus from stock cultures, the strain lost viability and thus further taxonomic study could not be undertaken. Therefore, the taxonomic position of this fungus is unclear apart from the description of CNC-159 as an aquatic deuteromycete.

The fungal isolate was cultured in ten replicate 1 L Fernbach flasks in a marine fungal medium (YPG + C; 5 g yeast extract, 5 g peptone, 10 g glucose, 2 g crab meal, 1 L filtered seawater). The flasks were cultured for 3 weeks under static conditions at room temperature. The culture broth (10 L) was extracted with EtOAc to yield crude extract (500 mg). The entire extract was then subjected to prep. TLC (Si gel, 1000 μm) with 1:1 iso-octane/EtOAc and separated into 10 bands visualized by UV and recovered with EtOAc. The most polar fraction (F10, 53.2 mg) was further purified by semi-preparative reversed phase C18 HPLC (10 × 250 mm, refractive index) in 60% MeOH/H_2O to give solanapyrone E (6.0 mg) and solanapyrone F (5.2 mg). Fraction 8 (22.3 mg) and fraction 6 were also purified by reversed phase HPLC in 80% MeOH/H_2O to yield the known compound solanapyrone C (6.9 mg) and solanapyrone G (3.8 mg).

Bioassay

The purified metabolites were tested for antimicroalgal effects against the unicellular chlorophyte *Dunaliella* sp. in liquid culture growth inhibition assays run in 48 well microtiter plates. To each well was added 1 ml of ES medium [27] with an initial concentration of 1×10^4 cells ml^{-1}. Compounds were dissolved in EtOH and then 10–20 μl of the solution was added to each well to provide either 100 $\mu g\,ml^{-1}$ (solanapyrone C) or 200 $\mu g\,ml^{-1}$ (solanapyrones E and F). The microtiter plates were then placed under constant fluorescent illumination and the cells counted at 5 day intervals by removing 50 μl and fixing the solution with 50 μl of 6% formalin-seawater before counting the number of algal cells with a hemacytometer.

*Solanapyrone E (**1**) white solid, $[\alpha]_D^{25}$ −16.4° (MeOH; c 0.28)*

EIMS, 70 eV, *m/z* (rel. int.): 303 $[M]^+$ (17), 285 (20), 275 (33), 257 (12), 222 (15), 138 (100); HR-CIMS, calculated for $C_{17}H_{21}NO_4$, 303.1471, found 303.1463, 2.5 ppm. UV λ_{max}^{MeOH} nm (log ϵ): 228 (4.01), 267 (3.56), 304 (3.72). IR ν_{max}^{neat} cm^{-1}: 3337, 1701, 1661. ^1H NMR (500 MHz, pyridine-d_5); δ 0.93 (3H, *d*, J = 7.0, H-16), 1.32 (1H, *ddd*, J = 2.0, 12.0, 14.0, H-6a), 1.47 (1H, *ddd*, J = 12.5, H-9a), 1.60 (1H, *m*, J = 13.5, H-8a), 1.72 (1H, *m*, J = 13.5, H-8b), 1.91 (1H, *m*, J = 14.0, H-6b), 2.24 (1H, *m*, H9-b), 2.28 (1H, *m*, H-10), 2.51 (1H, *dd*, J = 11.0, H-1), 2.60 (1H, *m*, H-2), 2.86 (1H, *m*, J = 4.5, 12.0, H-5), 4.20 (1H, *m*, H-7), 5.42 (1H, *dd*, J = 9.5, H-3), 5.63 (1H, *m*, H-4), 6.28 (1H, *s*, H-12), 9.63 (1H, *br s*, H-18a), 10.23 (1H, *br s*, H-18b), 10.58 (1H, *s*, H-

17). ^{13}C NMR (100 MHz, pyridine-d_5); δ 20.4 (C-16), 22.3 (C-9), 28.4 (C-8), 31.3 (C-5), 35.0 (C-2), 36.0 (C-10), 37.0 (C-6), 46.3 (C-1), 64.9 (C-7), 95.8 (C-14), 100.8 (C-12), 131.2 (C-3), 132.3 (C-4), 161.8 (C-15), 164.5 (C-11), 171.3 (C-13), 191.0 (C-17).

*Solanapyrone F (**2**) white solid, $[\alpha]_D^{25}$ −53.2° (MeOH; c 0.30)*

EIMS, 70 eV, *m/z* (rel. int.): 303 $[M]^+$ (27), 285 (16), 275 (23), 257 (12), 222 (9), 138 (100); HR-CIMS, calculated for $C_{17}H_{21}NO_4$, 303.1471, found 303.1473, 0.8 ppm. UV λ_{max}^{MeOH} nm (log ϵ): 228 (4.01), 266 (3.62), 304 (3.71). IR ν_{max}^{neat} cm^{-1}: 3342, 1698, 1658. ^{1}H NMR (500 MHz, pyridine-d_5); δ 0.84 (3H, *d*, J=6.0, H-16), 1.34 (1H, *m*, H-6a), 1.43 (1H, *m*, H-9a), 1.54 (1H, *m*, J=14.0, H-8a), 1.65 (1H, *m*, H-9b), 1.89 (1H, *m*, H-8b), 2.06 (1H, *m*, H-6b), 2.09 (1H, *m*, H-5), 2.10 (1H, *m*, H-10), 2.53 (1H, *dd*, J=9.0, H-1), 2.57 (1H, *m*, H-2), 3.73 (1H, *m*, J=4.0, 11.0, H-7), 5.35 (1H, *d*, J=10.0, H-3), 5.56 (1H, *dd*, J=10.0, H-4), 6.22 (1H, *s*, H-12), 10.19 (1H, *br s*, H-18a), 10.57 (1H, *s*, H-17), 10.59 (1H, *br s*, H-18b). ^{13}C NMR (125 MHz, pyridine-d_5); δ 19.2 (C-16), 25.9 (C-9), 30.3 (C-8), 33.9 (C-10), 34.0 (C-2), 35.5 (C-5), 38.8 (C-6), 45.5 (C-1), 69.0 (C-7), 94.8 (C-14), 100.2 (C-12), 130.2 (C-3), 130.2 (C-4), 160.8 (C-15), 163.6 (C-11), 170.1 (C-13), 190.1 (C-17).

*Solanapyrone G (**3**) white solid*

EIMS, 70 eV, *m/z* (rel. int.): 287 $[M]^+$ (57), 259 (62), 138 (100); HR-CIMS, calculated for $C_{17}H_{22}NO_3$, 288.1600, found 288.1565, 12.0 ppm. UV λ_{max}^{MeOH} nm (log ϵ): 236 (3.94), 266 (3.69), 312 (3.83). IR ν_{max}^{neat} cm^{-1}: 1696, 1658, 1520. ^{1}H NMR (500 MHz, $CDCl_3$); δ 0.95 (3H, *d*, J=7.5, H-16), 1.15 (1H, *m*, H-6a), 1.19 (1H, *m*, H-8a), 1.27 (1H, *m*, H-7a), 1.47 (1H, *m*, H-8b), 1.52-1.58 (2H, *m*, H-9), 1.71 (1H, *m*, H-6b), 1.72 (1H, *m*, H-7b), 2.15 (1H, *m*, J=2, H-5), 2.30 (1H, *m*, J=2, H-10), 2.35 (1H, *dd*, J=11.5, 10.0, H-1), 2.60 (1H, *m*, H-2), 5.44 (1H, *m*, J=11.5, 10.0, H-3), 5.57 (1H, *br s*, H-18b), 5.65 (1H, *m*, H-4), 5.68 (1H, *s*, H-12), 9.86 (1H, *br s*, H-18a), 10.05 (1H, *s*, H-17). ^{13}C NMR (100 MHz, $CDCl_3$); δ 20.3 (C-16), 21.1 (C-8), 26.0 (C-7), 28.4 (C-9), 29.8 (C-6), 34.6 (C-2), 35.4 (C-10), 36.7 (C-5), 46.9 (C-1), 95.5 (C-14), 100.2 (C-12), 130.3 (C-3), 131.5 (C-4), 160.4 (C-15), 163.9 (C-11), 172.0 (C-13), 191.5 (C-17).

*Preparation of (R)- and (S)-MTPA esters of solanapyrone F (**2**).*

To solutions of solanapyrone F (0.9 mg, 0.7 mg) in pyridine (400 μl) were added either (*R*) or (*S*)-MTPA chlorides (10 μl) with DMAP (1 mg) and the mixtures allowed to stir at room temperature for 3 hours. The reactions were stopped by adding 1:1 Et_2O/$NaHCO_3$ (6 ml, saturated), which was then allowed to stir vigorously for 30 min. The organic layers were removed and the remaining aqueous layer extracted twice with Et_2O. The combined organic layers were dried under vacuum and subjected to Si HPLC (10 × 250 mm, 30% EtOAc/hexane) to afford the pure MTPA-esters **2a,b**.

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