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Phytogrowth- and photosynthesis-inhibiting properties of nostoclide analogues

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Abstract: Six nostoclide analogues were synthesised from 3-benzyl-2(*5H*)-furanone in one step, with yields ranging from 10 to 71%, and subjected to several biological assays. The two most active of these, 5d and 5e, were shown to be phytogrowth inhibitors of the radicle of *Lolium multiflorum* Lam, while enhancing the root growth of *Physalis ixocarpa* Brot. Both compounds inhibited electron flow (basal, phosphorylating and uncoupled) from water to methylviologen (MV); both acted as Hill reaction inhibitors, since the synthesis of ATP was prevented. The uncoupled electron transport from photosystem II (PSII) (water to 2,6-dichlorophenol-indophenol (DPIP)) and photosystem I (PSI) (2,6-dichlorophenol-indophenol reduced (DPIPred) to MV) was inhibited with 500 μM of 5d by 22 and 14% respectively. In addition, 400 μM of 5d inhibited PSI (from tetramethyl-*p*-benzohydroquinone ($TMQH_2$) to MV) by 40%. Thus 5d inhibited electron transport at the b_6f complex. Finally, 500 μM of 5e inhibited electron flow (basal and phosphorylating) by 25%, and 300 μM of 5e enhanced light-activated membrane-bound Mg^{2+}-ATPase by 66%. Thus 5e behaved as a weak Hill reaction inhibitor and an uncoupler. In general, the phytotoxicity of the synthetic lactones was only weakly related to inhibition of photosynthesis.

Keywords: γ-lactones; photosynthesis inhibitors; nostoclide; plant growth inhibitor

1 INTRODUCTION

In modern agriculture there is a constant need for the development of new herbicides to protect crop plants. This is due in great part to resistance problems caused by severe selection pressure imposed by continuous application of products with the same mechanism of action.[1,2] Among the strategies used by the agrochemical industry to discover new products with new modes of action, the use of natural products continues to be of great importance, mainly as a source of new lead structures for chemical synthesis.[3–6] In the allelopathy field there is increasing interest in understanding plant–plant and plant–micro-organism interactions.[2]

Among many phytotoxic natural products of microbial origin is cyanobacterin (Fig. 1), a lactone isolated from the blue–green alga *Scytonema hofmanni*.[7] This compound is toxic to most cyanobacteria at a concentration of 5 μM and also inhibits the growth of most eukaryotic algae and several monocotyledonous and dicotyledonous angiosperms.[8,9] Cyanobacterin acts by inhibiting photosynthetic electron transport in isolated chloroplasts in a similar way to the herbicide 3-(3,4-dichlorophenyl)-1,1-dimethylurea,[10] leading to a cascade of events which result in the disruption of the thylakoid membrane.[11]

Although several studies have been carried out on the mode of action of cyanobacterin, the biological activity of the structurally similar natural lactones **2a** and **2b** (Fig. 1), known as nostoclides I and II, isolated from the lichen *Peltigera canina* Willd has not yet been fully investigated. Nostoclides I and II have been shown to have moderate cytotoxicity against the mouse neuroblastoma cell lines Neuro-2a CCL and KB CCL17.[12] Owing to the structural similarity of nostoclides and cyanobacterin and the fact that *P. canina* cultures are usually not contaminated with micro-organisms, it has been suggested that these compounds may be allelopathic agents.

Owing to the potential utility of nostoclides as herbicides and our interest in using natural products as a model to prepare new agrochemicals,[13–17] we report the first preparation of several nostoclide analogues and their effect on the germination and growth activity of *Physalis ixocarpa* Brot (dicotyledonous) and *Lolium multiflorum* Lam (monocotyledonous). The effect of

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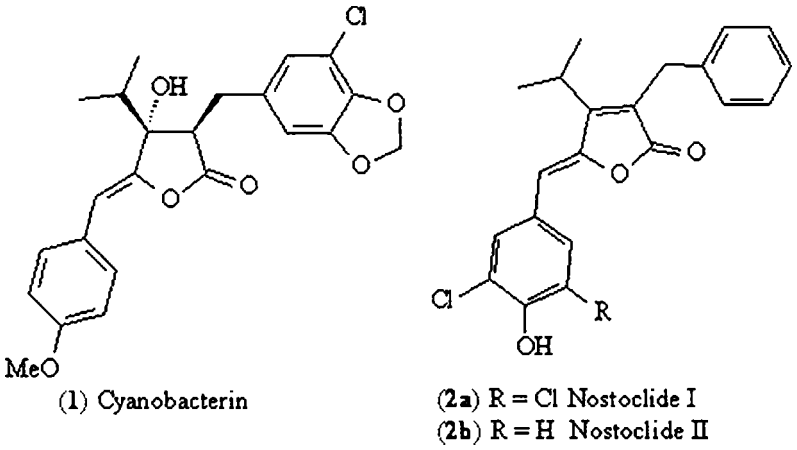

Figure 1. Structures of cyanobacterin and nostoclides.

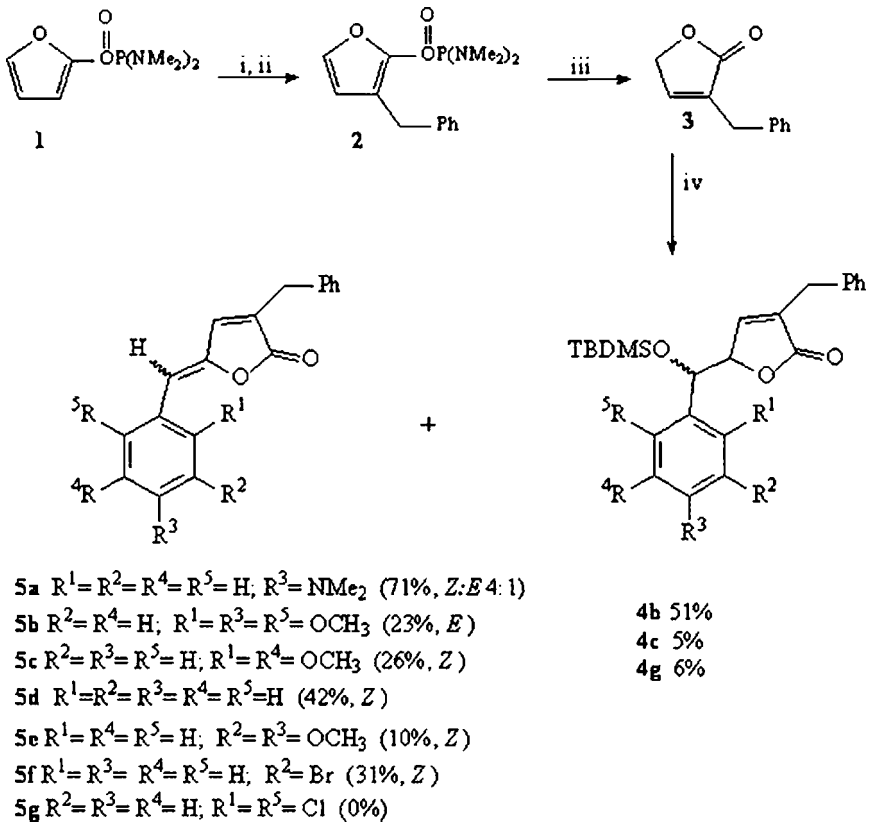

Figure 2. Reaction conditions for the preparation of lactones **5a–5g**: (i) *n*-BuLi, THF, −78 °C; (ii) $PhCH_2Br$, −78 → 0 °C (58%); (iii) HCO_2H, room temperature (RT) (90%); (iv) ArCHO, TBDMSOTf (1.2 equiv.), Et_3N (2.9 equiv.), DCM, RT, 1 h; DBU (2 equiv.), RT, 3 h (yields shown on figure).

the synthetic compounds on various photosynthetic activities was also investigated to determine their sites of action.

2 MATERIALS AND METHODS

The melting points were obtained with an MQAPF301 digital apparatus (Microquímica Equipamentos, Palhoça, Santa Catarina, Brazil) and values are uncorrected. Infrared spectra were registered on a Perkin Elmer Paragon 1000 FTIR spectrophotometer using a potassium bromide disc and scanning from 625 to 4000 cm^{-1} (Perkin Elmer Beaconsfield, Bucks, UK). ^1H and ^{13}C NMR spectra were recorded on a Varian MERCURY 300 instrument at 300 and 75 MHz respectively using deuterated chloroform as solvent and tetramethylsilane (TMS) as reference ($\delta = 0$) (Varian, Palo Alto, CA). The coupling constants are given in Hz. Mass spectra were recorded under electron impact (70 eV) in a VG Analytical ZAB-IF high-resolution spectrometer (Ringoes, NJ). Chromatographic purifications were carried out using silica gel (63–230 μm). Reactions were monitored by thin layer chromatography (TLC) using plates coated with $60GF_{254}$ silica gel. Solvents were purified as described by Perrin and Armarego.[18] Lactone **3** (Fig. 2) was prepared from the commercially available furane **1** (Fig. 2) according

to the procedure described in the literature.[19] The required 2,5-dimethoxybenzaldehyde (65%), 3-bromobenzaldehyde (80%) and 3,4-dimethoxybenzaldehyde (80%) were prepared by Swern oxidation[20] from the corresponding commercially available alcohols.

2.1 Procedure for the preparation of arylmethylenelactone 5a

A solution of compound **3** (107 mg, 0.61 mmol) in dichloromethane (4 ml) was placed in a 25 ml two-necked round-bottom flask and the system was kept under a nitrogen atmosphere at room temperature. To this solution, *tert*-butyldimethylsilyl trifluoromethanesulfonate (TBDMSOTf, 0.17 ml, 0.74 mmol) and 4-(*N*,*N*-dimethylamino)benzaldehyde (110 mg, 0.74 mmol) dissolved in dried dichloromethane (DCM, 3 ml) and triethylamine (TEA, 250 µl, 1.8 mmol) were added. The resultant reaction mixture was stirred at 25 °C for 1 h before addition of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU, 185 µl, 1.25 mmol) and stirring at room temperature for a further 3 h. The reaction mixture was diluted with DCM (80 ml) and washed with hydrochloric acid (3 M, 2 × 40 ml) followed by brine (4 × 40 ml). The organic phase was dried with magnesium sulfate and concentrated under reduced pressure to produce an orange residue. This residue was purified by silica gel column chromatography (column 1.5 cm × 30 cm) eluting with hexane/diethyl ether (4:1 v/v), resulting in the required compound **5a** as an orange solid in 71% yield (132 mg, 0.43 mmol), as a mixture of isomers *Z* and *E* in approximately 4:1 ratio. Further flash column chromatography separation using silica gel and eluting with hexane/diethyl ether (6:1 v/v) led to the isolation of a pure sample of the major *Z* isomer, which was submitted to biological tests.

Compounds **5b**–**5g** were prepared using a similar procedure to that described for compound **5a**, and yields are presented in Fig. 2. Structures of the synthesised compounds were supported by the following spectroscopic data.

5a: m.p. 121–124 °C; IR (KBr, $\overline{\nu}$) 3086, 3026, 2910, 2816, 1730, 1642, 1609, 1589, 1525, 1362, 1189, 1041, 807, 756, 699 cm^{-1}; isomer *Z*: ^{1}H NMR δ 3.01 (s, 6H, N(CH$_3$)$_3$), 3.71 (s, 2H, H6), 5.81 (s, 1H, H5), 6.66 (d, 2H, $\mathcal{J}$ = 9.0 Hz, H3″, H5″), 6.91 (t, 1H, $\mathcal{J}$ = 1.5 Hz, H3), 7.26–7.36 (m, 5H, H2′, H6′), 7.65 (d, 1H, $\mathcal{J}$ = 9.0 Hz, H2″, H6″); ^{13}C NMR δ 32.08 (C6), 40.57 (N(CH$_3$)$_3$), 112.13 (C3″, C5″), 114.16 (C5), 121.34 (C1″), 126.87 (C4′), 128.87 (C2′, C6′), 129.05 (C3′, C5′), 129.38 (C2), 132.27 (C2″, C6″), 137.93 (C1′), 139.78 (C3), 144.86 (C4), 150.59 (C4″), 171.10 (C1); isomer *E*: ^{1}H NMR δ 2.99 (s, 6H, N(CH$_3$)$_3$), 3.74 (s, 2H, H6), 6.58 (s, 1H, H5), 6.66 (d, 2H, $\mathcal{J}$ = 9.0 Hz, H3″, H5″), 7.20 (d, 1H, $\mathcal{J}$ = 9.0 Hz), 7.26–7.36 (m, 6H, H3, H2′–H6′); ^{13}C NMR δ 32.41 (C6), 40.63 (N(CH$_3$)$_3$), 112.46 (C3″, C5″), 115.80 (C5), 120.99 (C1″), 126.95 (C4′), 128.94 (C2′, C6′), 129.05 (C3′, C5′), 130.50 (C2″, C6″), 133.87 (C2), 134.96 (C3), 137.59 (C1′), 144.86 (C4), 146.60 (C4″), 170.00 (C1); MS, *m*/*z* 305.1415 (C$_{20}$H$_{19}$NO$_2$, [M$^{\cdot+}$], 100), 260 (1), 161 (18), 133 (23), 116 (10), 115 (29), 105 (10), 91 (17), 89 (17), 77 (22), 65 (15), 51 (23).

5b: white solid; m.p. 106–107 °C; IR (KBr, $\overline{\nu}$) 3028, 2968, 2844, 1730, 1606, 1456, 1342, 1047 cm^{-1}; ^{1}H NMR δ 3.68 (s, 2H, H6), 3.72 (s, 6H, 2″-OCH$_3$, 6″-OCH$_3$), 3.82 (s, 3H, 4″-OCH$_3$), 6.09 (s, 2H, H3″, H5″), 6.54 (s, 1H, H5), 7.04 (td, 1H, $\mathcal{J}$ = 0.9, 1.5 Hz, H3), 7.22–7.31 (m, 5H, H2′–H6′); ^{13}C NMR δ 32.29 (C6), 55.82 (4″-OCH$_3$), 55.95 (2″-OCH$_3$, 6″-OCH$_3$), 90.87 (C3″, C5″), 103.88 (C1″), 105.89 (C5), 126.84 (C4′), 128.79 (C2′, C6′), 129.08 (C3′, C5′), 133.01 (C2), 137.81 (C1′), 137.96 (C3), 148.70 (C4), 158.65 (C2″, C6″), 161.84 (C4″), 170.25 (C1); MS, *m*/*z* 352.1311 (C$_{21}$H$_{20}$O$_5$, [M$^{\cdot+}$], 100), 309 (2), 236 (4), 208 (10), 193 (14), 165 (11), 167 (7), 116 (14), 115 (42), 105 (15), 91 (30), 77 (29), 65 (18), 51 (26).

5c: pale yellow solid; m.p. 113.5–115 °C; IR (film, $\overline{\nu}$) 3062, 3028, 3001, 2927, 2851, 2835, 1763, 1646, 1606, 1495, 1427, 1237, 1237, 1046 cm^{-1}; ^{1}H NMR δ 3.72 (s, 2H, H6), 3.80 (s, 3H, 2″-OCH$_3$), 3.82 (s, 3H, 5″-OCH$_3$), 6.39 (s, 1H, H5), 6.77–6.87 (m, 2H, H3″, H5″), 6.95 (t, 1H, $\mathcal{J}$ = 1.5 Hz, H3), 7.24–7.35 (m, 5H, H2′, H6′), 7.72 (d, 1H, $\mathcal{J}$ = 2.7 Hz, H6″); ^{13}C NMR δ 32.17 (C6), 56.29 (2″-OCH$_3$), 56.59 (5″-OCH$_3$), 106.63 (C5), 111.89 (C3″), 115.82 (C4″), 116.61 (C6″), 122.78 (C1″), 127.03 (C4′), 128.95 (C2′, C6′), 129.06 (C3′, C5′), 132.04 (C2), 137.40 (C1′), 140.13 (C3), 153.72 (C2″, C5″), 152.09 (C4), 170.45 (C1); MS, *m*/*z* 322.1206 (C$_{20}$H$_{18}$O$_4$, [M$^{\cdot+}$], 100), 307 (2), 281 (35), 251 (16), 178 (6), 137 (5), 116 (10), 115 (53), 105 (12), 91 (70), 77 (37), 75 (29), 65 (20), 51 (20).

5d: white solid; m.p. 102–103 °C; IR (KBr, $\overline{\nu}$) 3010, 3058, 3026, 2925, 2854, 1760, 1647, 1612, 1602, 1495, 1450, 1358, 759, 695 cm^{-1}; ^{1}H NMR δ 3.73 (s, 2H, H6), 5.87 (s, 1H, H5), 6.95 (t, 1H, $\mathcal{J}$ = 1.5 Hz, H3), 7.25–7.40 (m, 8H, H2′, H3″–H5″, H6′), 7.74 (d, 1H, $\mathcal{J}$ = 6.9 Hz); ^{13}C NMR δ 32.18 (C6), 112.88 (C5), 127.10 (C4′), 128.92 (C2′, C6′), 128.99 (C3′, C5′), 129.04 (C4″), 129.07 (C3″, C5″), 130.56 (C2″, C6″), 132.62 (C2), 133.22 (C1″), 137.26 (C1′), 139.77 (C3), 147.49 (C4), 170.41 (C1); MS, *m*/*z* 262.0992 (C$_{18}$H$_{14}$O$_2$, [M$^{\cdot+}$], 75), 217 (34), 116 (34), 115 (77), 105 (73), 91 (42), 90 (61), 77 (100), 65 (18), 51 (92).

5e: pale yellow solid; m.p. 121.3–122.0 °C; IR (film, $\overline{\nu}$) 3086, 3002, 2954, 2854, 2835, 1759, 1682, 1592, 1515, 1428, 1263, 1066 cm^{-1}; ^{1}H NMR δ 3.73 (s, 2H, H6), 3.82 (s, 3H, OCH$_3$), 3.86 (s, 3H, OCH$_3$), 6.75 (s, 1H, H5), 6.84 (d, 1H, $\mathcal{J}$ = 8.7 Hz, H5″), 6.98 (dd, 1H, $\mathcal{J}_1$ = 8.7 Hz, $\mathcal{J}_2$ = 2.4 Hz, H6″), 7.24–7.55 (m, 6H, H2″, phenyl ring); ^{13}C NMR δ 32.10 (C6), 56.13 (OCH$_3$), 56.21 (OCH$_3$), 110.69 (C5), 111.05 (C2″), 118.61 (C5″), 124.63 (C6″), 127.03 (C4′), 128.79 (C2′, C6′), 129.01 (C1″), 129.05 (C3′, C5′), 132.52

(C2), 146.80 (C3″), 137.30 (C1′), 139.90 (C3), 146.9 (C4″), 150.09 (C4), 170.00 (C1); MS, *m/z* 322.1205 ($C_{20}H_{18}O_4$, [M^+], 100), 307 (2), 251 (16), 178 (9), 137 (4), 115 (63), 105 (10), 91 (78), 77 (44), 75 (30), 65 (29), 51 (30).

5f: white solid; m.p. 136.7–137.4 °C; IR (KBr, $\overline{\nu}$) 3086, 3028, 2924, 2850, 1768, 1650, 1555, 1491, 1453, 1350, 1191, 1075, 790, 750, 670 cm^{-1}; ^1H NMR δ 3.73 (s, 2H, H6), 5.78 (s, 1H, H5), 6.93 (t, 1H, $\mathcal{J} = 1.5$ Hz, H3), 7.20–7.38 (m, 7H, H2′–H6′, H5″), 7.41 (d, 1H, $\mathcal{J} = 9.0$ Hz, H4″), 7.67 (d, 1H, $\mathcal{J} = 8.1$ Hz, H6″), 7.83 (t, 1H, $\mathcal{J} = 1.8$ Hz, H2″); ^{13}C NMR δ 32.22 (C6), 111.00 (C5), 122.97 (C3″), 127.18 (C4′), 128.95 (C6″), 129.03 (C2′, C6′), 129.06 (C3′, C5′), 130.36 (C5″), 131.79 (C4″), 132.92 (C2″), 133.52 (C2), 135.19 (C1″), 137.01 (C1′), 139.49 (C3), 148.22 (C4), 169.99 (C1); MS *m/z* 342.0080 ([$M^+ + 2$], 39), 340.0099 ($C_{18}H_{13}BrO_2$, [M^+], 37), 295 (4), 196 (8), 198 (8), 116 (36), 115 (61), 105 (14), 91 (35), 89 (100), 77 (27), 65 (22), 51 (35).

2.2 Biological tests

2.2.1 Germination and growth assay

Seeds of *P. ixocarpa* and *L. multiflorum* were obtained from Central de Abastos, México DF, Mexico. All undersized and damaged seeds were discarded and the assay seeds were selected for uniformity. Bioassays were carried out in petri dishes of 90 mm diameter, with Whatman No. 1 filter paper as support, as described in the literature.[21] Compounds **5a**–**5f** were evaluated at 50 and 100 μM and all treatments were replicated six times in a completely randomised design. The percentages of root and shoot growth inhibition were calculated in relation to the length of the root and shoot of the water-treated control respectively. The results were analysed by Student's *t*-test at 0.05 probability level.

2.2.2 Isolation of chloroplasts and determination of chlorophyll concentration

Chloroplast thylakoids were isolated from market spinach leaves (*Spinaceae oleraceae* L.) as described earlier[22,23] and suspended in 400 mM sorbitol, 5 mM magnesium chloride and 10 mM potassium chloride buffered with 0.03 M K^+-tricine at pH 8.00. The chlorophyll concentration was measured as described previously.[24]

2.2.3 Measurement of ATP synthesis and electron transport rate

For ATP synthesis and electron transport rate studies the compounds were tested at concentrations of 100, 200, 300, 400 and 500 μM as described in the literature.[25] ATP synthesis was measured as the pH rise from 8.000 to 8.100 using a combination microelectrode connected to a Corning potentiometer with expanded scale. The pH changes were recorded with a Gilson recorder. The reaction medium contained 100 mM sorbitol, 5 mM magnesium chloride, 10 mM potassium chloride and 1 mM K^+-tricine at pH 8.000 in the presence of 1 mM ADP and 3 mM KH_2PO_4.[23] Methylviologen (MV, 0.05 mM) was added as electron acceptor for the Hill reaction. Photosynthetic non-cyclic electron transport activity from water to MV was determined with a Clark-type electrode connected to a YSI model 5300 oxygraph to monitor oxygen evolution (Yellow Spring Instrument Co., Yellow Springs, OH). The reaction medium was the same as in the ATP synthesis assay, except that the tricine concentration was 15 mM and in the presence or absence of 6 mM ammonium chloride.[25] All reaction mixtures were illuminated with actinic light from a projector lamp (GAF 2600-Anscorama, Tucker, CA) passed through a 5 cm aqueous solution of 20 g $litre^{-1}$ cupric sulfate as filter. Photosystem I (PSI) electron transport was determined in a similar way to non-cyclic electron transport.[25] The reagents added were 100 μM 2,6-dichlorophenolindophenol (DPIP), 300 mM Na^+-ascorbate, 10 μM 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) and 6 mM ammonium chloride. Throughout uncoupled photosystem II (PSII) electron flow, 1 μM 2, 5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (DBMIB), 50 μM DPIP/300 mM $K_3[Fe(CN)_6]$ and 6 mM ammonium chloride were added to the basal medium but without MV.

Uncoupled PSI electron transport from plastoquinol (PQH_2) to MV was measured polarographically; the medium was the same as that from water to MV, except that 200 μM tetramethyl-*p*-benzohydroquinone ($TMQH_2$) was added as electron donor and 6 mM ammonium chloride as uncoupler plus 10 μM DCMU as the inhibitor of electron transfer from quinone A (Q_A) to quinone B (Q_B).[26,27]

2.2.4 Mg^{2+}-ATPase activity

Light-triggered Mg^{2+}-ATPase activity bound to thylakoid membranes was measured as described previously.[28] Released inorganic phosphate was measured as reported previously.[29] For each reaction a blank experiment was performed with the isolated chloroplasts in the reaction medium. All reactions were conducted in triplicate and the data were analysed by analysis of variance (ANOVA). The standard deviation is indicated on each graph. The I_{50} (concentration producing 50% inhibition) values for each activity were estimated from the graph of percentage activity *versus* concentration of the compound under study.

3 RESULTS AND DISCUSSION

3.1 Synthesis

We report the synthesis of six new nostoclide analogues lacking the isopropyl group on the lactone ring (**1**, Fig. 1) and having different substituents at the benzylidene ring. The synthetic procedure used[19] is shown in Fig. 2.

Treatment of the commercially available 2-furyl-*N*,*N*,*N*′,*N*′-tetramethyl-phosphorodiamidate (Fig. 2, **1**) with *n*-butyl lithium followed by addition of benzyl

bromide resulted in the formation of the substituted furan **2**. This compound can be isolated at this step or the crude reaction mixture product can be treated with formic acid[30] to give lactone **3** in 52% overall yield.

The appendage of the arylmethylene groups at carbon C5 on lactone **3** was carried out using the methodology described in the literature for the synthesis of rubrolides C and E.[31] This consisted in the treatment of lactone **3** with TBDMSOTf in the presence of TEA and the required aldehyde. The elimination of the TBDMSO group from the intermediate **4** was achieved by addition of DBU to the crude reaction mixture, resulting in the formation of alquenes **5a**–**5f** in variable yield (Fig. 2). Contrary to results previously published,[30] we isolated some intermediate TBDMS derivatives (**4b**, 51%; **4c**, 5%; **4g**, 6%) during the reaction. However, treatment of pure **4b** with DBU at 40 °C resulted in a further quantity of **5b**, making the total yield for this compound around 49%.

The attempt to prepare the dichloro derivative **5g** was unsuccessful and only a very small amount of the intermediate **4g** (6%) was obtained as an inseparable isomeric mixture.

It was found that direct β-elimination of intermediates **4c**–**4f** resulted in the formation of the corresponding **5c**–**5f***Z* isomers, as observed in the case of rubrolides.[31] However, in the case of compound **5a**, a 4:1 mixture of Z/E isomers was formed. In all cases the geometry of the double bond was confirmed by the observed nuclear Overhauser enhancement (NOE) (approximately 7–10%) at H3 upon irradiation of H5. For the trimethoxy derivative **5b**, irradiation of H3 ($\delta = 7.04$) resulted in NOE at $\delta = 3.68$ (OCH_3) and no effect was observed at $\delta = 6.54$ (H5). These results are in accordance with the E geometry for the double bond in **5b**. We suggest that an interaction between the lactone oxygen lone pair and the methoxy groups at both C2″ and C6″ is responsible for the reverse elimination selectivity.

3.2 Phytotoxicity of lactones 5a–5f

The results of the germination and growth bioassays on pre-emergence plants carried out with γ-lactones **5a**–**5f** on the target species *P. ixocarpa* and *L. multiflorum* are shown in Figs 3 and 4 respectively.

When tested on *P. ixocarpa*, the γ-lactones **5a**–**5e** stimulated root development at 50–100 μM, with **5e** being the most active (113%) followed by **5d** (71%),

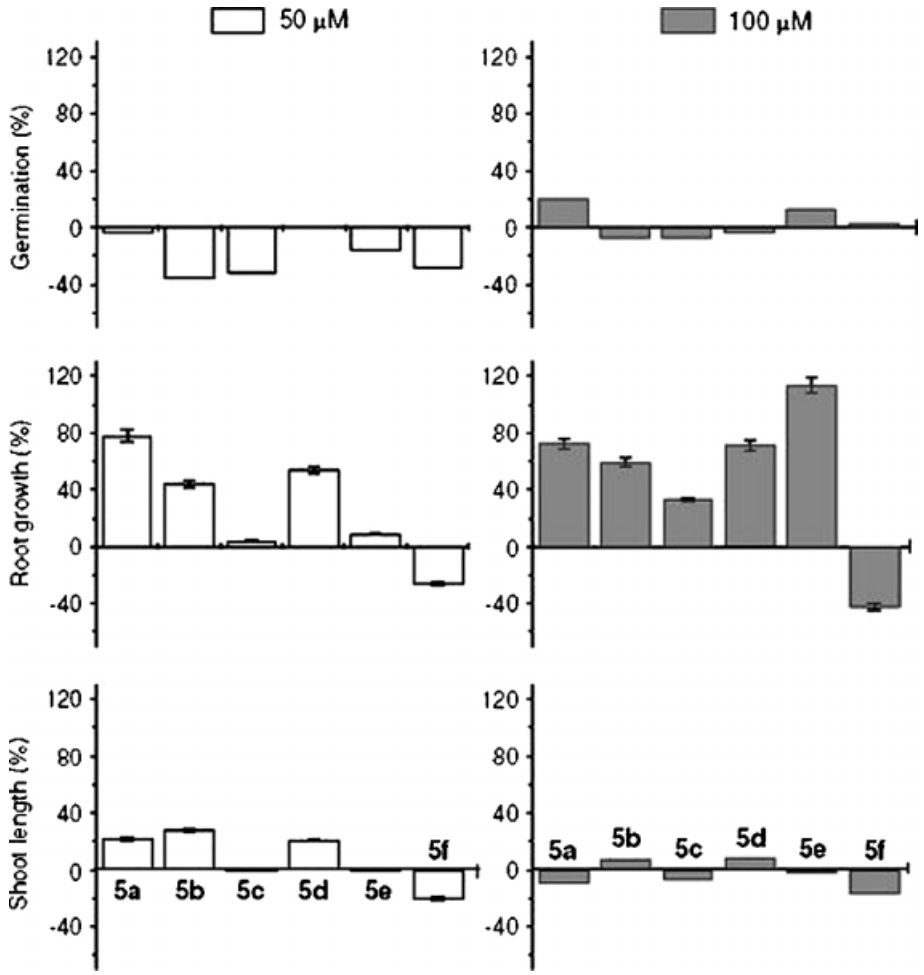

Figure 3. Effects of γ-lactones **5a**–**5f** on the germination and growth (root and shoot lengths) of *Physalis ixocarpa* at 50 and 100 μM. Mean values are presented as % differences from the control (e.g., +11% is 111% compared with the control = 100%, and −11% is 89% compared with the control).

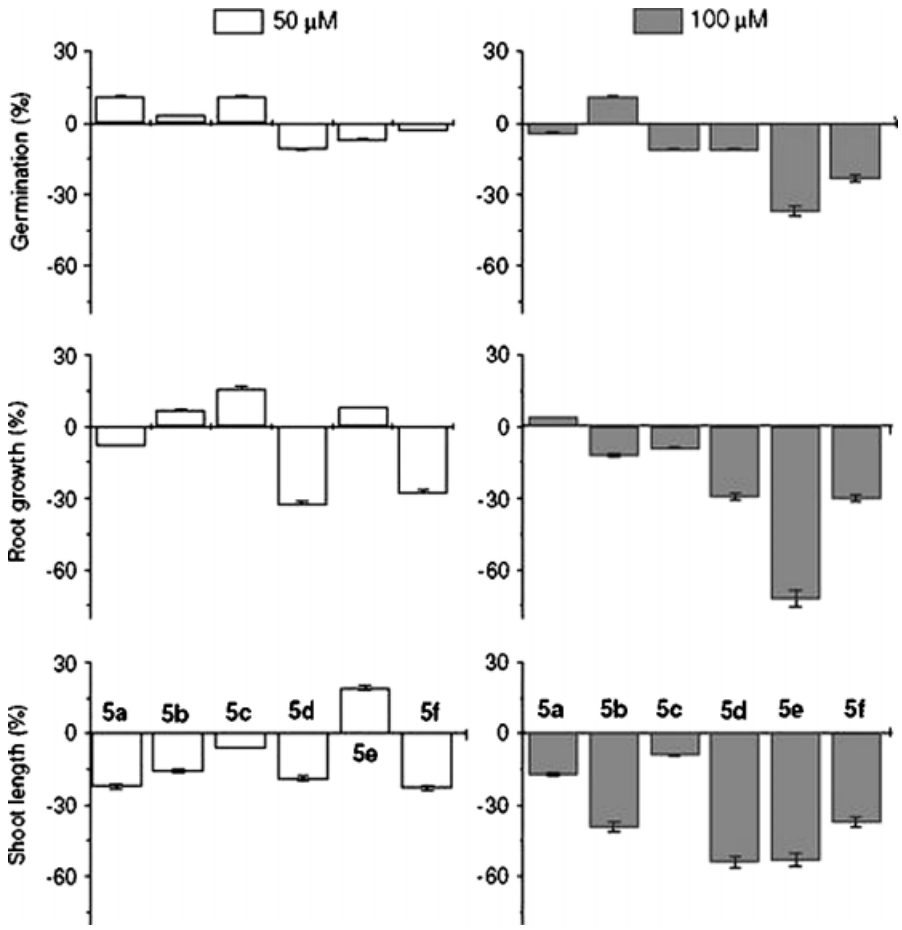

Figure 4. Effects of γ-lactones **5a–5f** on the germination and growth (root and shoot lengths) of *Lolium multiflorum* at 50 and 100 µM. Mean values are presented as % differences from the control (e.g. +11% is 111% compared with the control = 100%, and −11% is 89% compared with the control).

5a (72%), **5b** (59%) and **5c** (33%) at 100 µM (Fig. 3). However, compound **5f** behaved differently, causing inhibition of the root growth (42% at 100 µM). At 50 µM the lactones have the same pattern of effect but to a lesser degree. Shoot length and germination were less sensitive to the γ-lactones. At 100 µM a small reduction in shoot length was observed (**5f** inhibited 17%, **5a** 9% and **5c** 7%) and germination was also slightly reduced in the order **5b** (8%) > **5c** (7%) > **5d** (4%). On the other hand, compounds **5a, 5e** and **5f** slightly stimulated germination at the same concentration (20, 12 and 3% respectively at 100 µM). The results suggest that the electron-withdrawing effect of the Br atom present in lactone **5f** is responsible for the inhibitory activity on root and shoot development of this dicotyledonous species.

In the case of *L. multiflorum* it was observed that both root and shoot lengths are generally inhibited by almost all γ-lactones at 100 µM, except for **5a** and root development (Fig. 4). The order of root length inhibition was **5e** (72%) > **5f** (30%) > **5d** (29%) > **5b** (12%) > **5c** (9%) > **5a** (−41%). The order of shoot length inhibition of *L. multiflorum* by the lactones was **5d** (54%) > **5e** (53%) > **5b** = **5f** (37%) > **5a** (17%) > **5c** (9%). In general, root development was more affected than shoot development. Germination was inhibited with 100 µM **5e** and **5f** by 37 and 23% respectively, while only **5b** enhanced germination (11%). The γ-lactones **5e, 5d, 5b,5c** and **5f** have in common the *Z* geometry for the *exo* double bond and this structure is probably required for germination inhibition, as compared with **5b** which has an *E* geometry and enhances germination.

3.3 Behaviour of γ-lactones 5a–5f on isolated spinach chloroplasts

Compounds **5a**–**5f** (Fig. 5) inhibited photophosphorylation from water to MV. Compounds **5e** and **5d** inhibited ATP synthesis in a concentration-dependent manner, reducing the activity by 78 and 65% respectively at 300 µM (Fig. 5). The I_{50} values were 85 and 155 µM respectively. The other compounds were less active. As both **5e** and **5d** have a *Z* geometry, this may be an essential structural requirement for inhibition of photosynthetic activities. It is important to point out that **5d** and **5e** are the active compounds that have the same trend observed for root growth inhibitory activity on *L. multiflorum* and the enhancement of root growth of *P. ixocarpa*. However, these lactones are more active as phytogrowth inhibitors than as ATP synthesis inhibitors *in vitro*.

The light-dependent ATP synthesis by thylakoids is coupled to electron transport through the proton ion gradient across a selectively permeable thylakoid

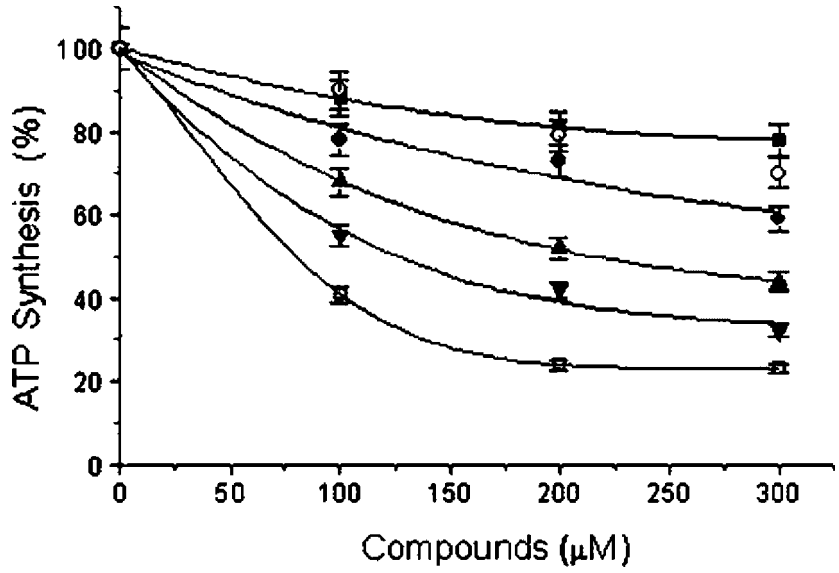

Figure 5. Effect of lactones **5a** (■), **5b** (●), **5c** (▲), **5d** (▼), **5e** (□) and **5f** (○) on ATP synthesis from water to MV. Control rate value was 980 μM ATP mg^{-1} Chl h^{-1}.

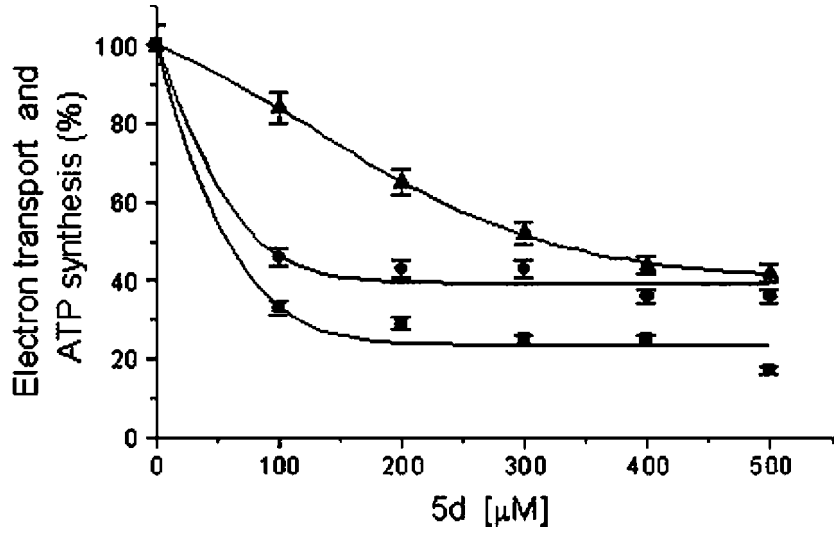

Figure 6. Effect of increased concentrations of lactone **5d** on non-cyclic electron transport, basal (■), phosphorylating (●) and uncoupled (▲), from water to MV. Control rate values for basal, phosphorylating and uncoupled electron transport were 540, 774 and 1074 μequiv. electron mg^{-1} Chl h^{-1} respectively.

membrane, and this proton gradient is generated as a consequence of thylakoid electron transfer.[32] The photophosphorylation may be inhibited by chemicals called energy transfer inhibitors interacting either at F_0 or F_1 or both. ATP synthesis may also be avoided with uncouplers that uncouple electron transport from ATP synthesis. Finally, the photophosphorylation may be avoided by inhibiting thylakoid electron transport carriers at one or more sites, not allowing the formation of a proton gradient; these compounds are called Hill reaction inhibitors. The lactones synthesised in this work could be acting by one of these mechanisms.

To obtain further information, the effect of lactones on photosynthetic electron transport was investigated. As shown in Fig. 6, the electron flow (basal, phosphorylating and uncoupled) from water to MV was inhibited in a concentration-dependent manner by **5d**. These results indicate that lactone **5d** behaves as a Hill reaction inhibitor, since it inhibited thylakoid electron transport (Fig. 6) and prevented the photophosphorylation process (Fig. 5).

To localise the site of inhibition of lactone **5d**, partial reactions (PSII and PSI) were measured using artificial electron donors and electron acceptors as well as appropriate inhibitors.[32,33] The uncoupled PSII electron transport from water to DPIP was increased by 22% and the uncoupled PSI electron transport from DPIPred to MV by 14% by compound **5d** at 500 μM (Table 1). On the other hand, compound **5d** inhibited the uncoupled electron transport from $TMQH_2$ to MV (40%, 400 μM). These results indicate that **5d** inhibits electron transport by interacting at the level of complex b_6f. On the other hand, lactone **5e** inhibited ATP synthesis and the uncoupled electron flow. However, compound **5e** at 500 μM caused a 25% inhibition of

Table 1. Effect of compound **5d** at different concentrations on electron transport rate in uncoupled, PSII, PSI and partial reactions. Values are in μequiv. electron mg^{-1} Chl h^{-1}

Conc. (μM)	Water to DPIP	DCPIPred to MV	$TMQH_2$ to MV
0	514	1599	286
100	514	1599	272
200	514	1542	257
300	498	1485	215
400	457	1485	172
500	400	1370	172

the basal and phosphorylating electron flow (Fig. 7). Therefore compound **5e** also acts as a Hill reaction inhibitor. The results also indicate that the target is exposed in uncoupled conditions (non-energised).

3.4 Mg^{2+}-ATPase activity

To explain why **5e** has its major effect on ATP synthesis and yet phosphorylating electron flow is only partially inhibited, the effect of **5e** on Mg^{2+}-ATPase activity was tested. It was found that **5e** enhances light-dependent Mg^{2+}-ATPase activity bound to membranes by 66% at 300 μM. Thus **5e** also acts as an uncoupler. Ammonium chloride was used as a positive control, which is a typical synthetic uncoupler of photosynthesis, i.e. it enhances Mg^{2+}-ATPase activity (Table 2).

Table 2. Effect of compound **5e** at different concentrations and of ammonium chloride on Mg^{2+}-ATPase activity

Compound	Conc. (μM)	μM AMP mg^{-1} Chl	Activity (%)
5e	0	114	100
	100	166	146
	300	189	166
	500	136	119
NH_4Cl	0	114	100
	1	144	126
	3	170	149
	6	245	215

4 CONCLUSIONS

Lactone **5d** inhibits electron flow at complex b6f, while **5e** acts as a mild Hill reaction inhibitor and as an uncoupler by interacting with H^+-ATPase. γ-Lactones have inhibition range concentrations similar to those of other natural compounds, e.g. phenolic allelochemicals have I_{50} values on chloroplasts ranging from 50 to 5000 μM. Both compounds **5d** and **5e** (at 100 μM) act as phytogrowth inhibitors to monocotyledonous weed species (*L. multiflorum*). The selectivity of these compounds needs to be further studied, as lactones **5d** and **5e** also induced root growth. Finally, lactone **5f** brought growth inhibition only to dicotyledonous weed species (*P. ixocarpa*), causing 42% root length inhibition at 100 μM. The potency of inhibition of the new γ-lactones presented in this paper is within the thresholds (100–1000 μM) of several allelopathic compounds that cause seedling growth inhibition.[34] The results show that the synthetic lactones in some way interfere with the plant growth process (root and shoot development) and also with the photosynthesis system.

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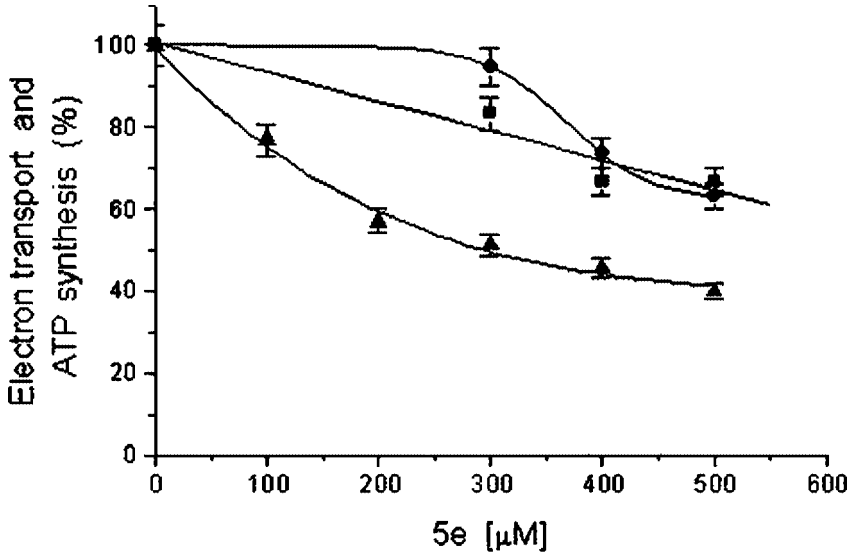

Figure 7. Effect of increased concentrations of lactone **5e** on non-cyclic electron transport, basal (■), phosphorylating (●) and uncoupled (▲), from water to MV. Control rate values for basal, phosphorylating and uncoupled electron transport were 540, 774 and 1074 μequiv. electron mg^{-1} Chl h^{-1} respectively.

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