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Carlactone-type strigolactones and their synthetic analogues as inducers of hyphal branching in arbuscular mycorrhizal fungi

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ABSTRACT

Hyphal branching in the vicinity of host roots is a host recognition response of arbuscular mycorrhizal fungi. This morphological event is elicited by strigolactones. Strigolactones are carotenoid-derived terpenoids that are synthesized from carlactone and its oxidized derivatives. To test the possibility that carlactone and its oxidized derivatives might act as host-derived precolonization signals in arbuscular mycorrhizal symbiosis, carlactone, carlactonoic acid, and methyl carlactonoate as well as monohydroxycarlactones, 4-, 18-, and 19-hydroxycarlactones, were synthesized chemically and evaluated for hyphal branching-inducing activity in germinating spores of the arbuscular mycorrhizal fungus *Gigaspora margarita*. Hyphal branching activity was found to correlate with the degree of oxidation at C-19 methyl. Carlactone was only weakly active (100 ng/disc), whereas carlactonoic acid showed comparable activity to the natural canonical strigolactones such as strigol and sorgomol (100 pg/disc). Hydroxylation at either C-4 or C-18 did not significantly affect the activity. A series of carlactone analogues, named AD ester and AA'D diester, was synthesized by reacting formyl Meldrum's acid with benzyl, cyclohexylmethyl, and cyclogeranyl alcohols (the A-ring part), followed by coupling of the potassium enolates of the resulting formylacetic esters with the D-ring butenolide. AD ester analogues exhibited moderate activity (1 ng −100 pg/disc), while AA'D diester analogues having cyclohexylmethyl and cyclogeranyl groups were highly active on the AM fungus (10 pg/disc). These results indicate that the oxidation of methyl to carboxyl at C-19 in carlactone is a prerequisite but BC-ring formation is not essential to show hyphal branching activity comparable to that of canonical strigolactones.

1. Introduction

Arbuscular mycorrhizal (AM) fungi belonging to the phylum Glomeromycota form symbiotic association with the roots of more than 80% of land plants (Smith and Read, 2008). AM fungi are ancient, obligate symbionts that rely on carbon provided by their hosts to complete their life cycle and, in return, supply inorganic nutrients, especially phosphate, to the host plants. Root colonization by AM fungi requires mutual recognition of diffusible signals released from the two partners during preinfection stages (Gutjahr and Parniske, 2013). Strigolactones (SLs) exuded from plant roots are host-derived precolonization signals that elicit physiological and morphological responses in AM fungi before physical interaction (Akiyama et al., 2005; Besserer et al., 2006). Originally identified as seed germination stimulants of root parasitic weeds (Cook et al., 1966), SLs have now been shown to be a class of plant hormones regulating several developmental processes that adapt plant architecture to nutrient availability (Gomez-Roldan et al., 2008; Umehara et al., 2008; Xie et al., 2010). Canonical SLs consists of a methylbutenolide ring (D ring) connected to a tricyclic lactone (ABC ring) via an enol ether bond (Fig. 1) (Al-Babili and Bouwmeester, 2015). To date, at least 22 naturally occurring canonical SLs have been characterized from root exudates of various plant species, and a number of structural analogues have been synthesized (Tokunaga et al., 2015; Lopez-Obando et al., 2015). An extensive structure-activity relationship study of SLs for induction of hyphal branching in AM fungi has demonstrated that the C-D part and the intact ABC ring are essential for strong hyphal branching activity (Akiyama et al., 2010).

Canonical SLs are biosynthesized from carotenoids via

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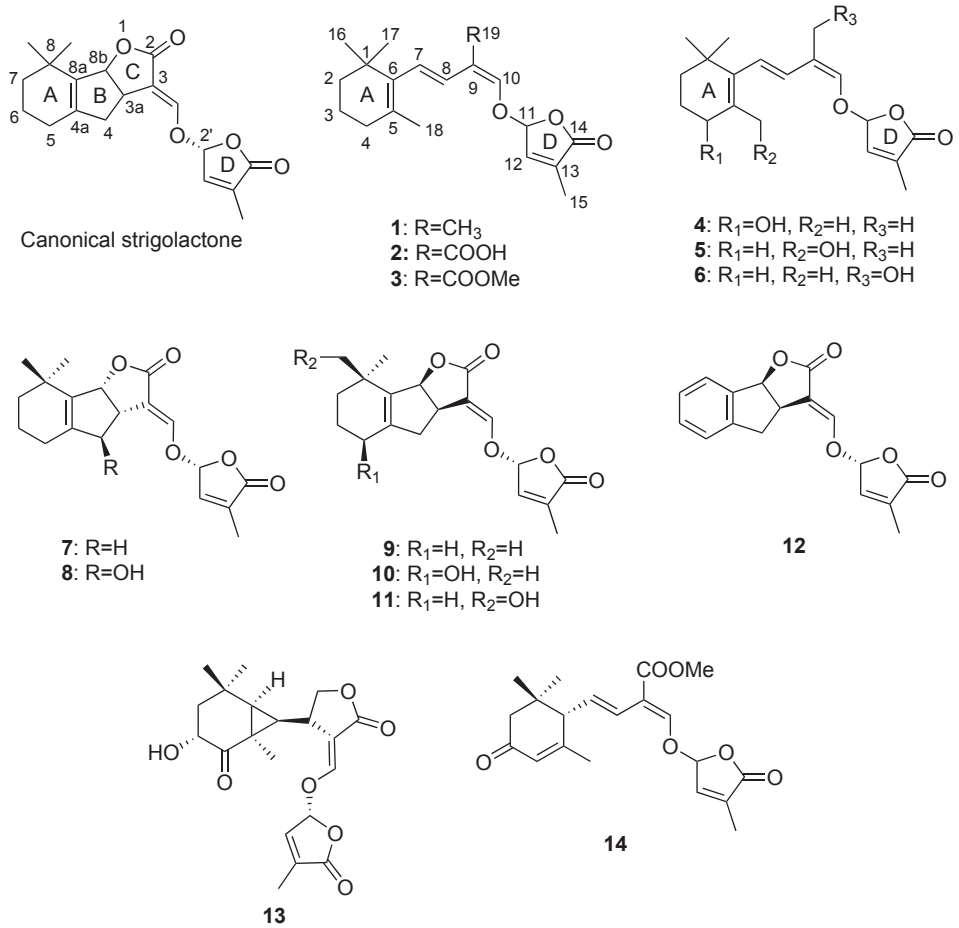

Fig. 1. Structures of canonical and carlactone-type strigolactones. Carlactone (**1**); carlactonoic acid (**2**); methyl carlactonoate (**3**); 4-hydroxycarlactone (**4**); 18-hydroxycarlactone (**5**); 19-hydroxycarlactone (**6**); (−)-4-deoxyorobanchol (**7**); (−)-orobanchol (**8**); (+)-5-deoxystrigol (**9**); (+)-strigol (**10**); (+)-sorgomol (**11**); (+)-GR24 (**12**); avenaol (**13**); heliolactone (**14**).

carlactone (CL, **1**) (Fig. 1) (Al-Babili and Bouwmeester, 2015). CL (**1**), a SL that contains the A and D rings and the enol ether bridge but lacks the B and C rings was first identified as a product of three SL biosynthetic enzymes, a β-carotene isomerase DWARF27, CAROTENOID CLEAVAGE DIOXYGENASE 7 (CCD7), and CCD8, *in vitro* (Alder et al., 2012), and was later shown to be an endogenous biosynthetic precursor for canonical SLs (Seto et al., 2014). In Arabidopsis, CL undergoes three step oxidation at C-19 by the cytosolic cytochrome P450 MAX1 to carlactonoic acid (CLA, **2**), which is further converted to methyl carlactonoate (MeCLA, **3**) which had been tentatively identified as SL-LIKE 1 (Seto et al., 2014; Abe et al., 2014). CL (**1**) and CLA (**2**) have also been identified in the roots of rice, where the both compounds are converted to the canonical SLs, 4-deoxyorobanchol (**7**) and orobanchol (**8**) (Seto et al., 2014; Abe et al., 2014).

According to the proposal by Al-Babili and Bouwmeester (2015), CL-type SLs can be defined as SLs with a CL-type structure lacking the canonical tricyclic ABC ring system. Three CL-type SLs, CL (**1**), CLA (**2**), and MeCLA (**3**), although less active than a synthetic SL analogue, GR24 (**12**), induce seed germination of the root parasitic weeds *Striga hermonthica* and *Orobanche minor* in a concentration-dependent manner (Alder et al., 2012; Abe et al., 2014). Two other CL-type SLs, avenaol (**13**) and heliolactone (**14**), have been isolated as a germination stimulant from root exudates of black oat (*Avena strigosa* Schreb.) and sunflower (*Helianthus annuus* L.), respectively, which are hosts of AM fungi (Kim et al., 2014; Ueno et al., 2014). None of the known canonical SLs was detected in the root exudates of the both plants, suggesting that avenaol and heliolactone are involved in rhizosphere communication with AM fungi. So far, however, CL-type SLs have not been examined for hyphal branching-inducing activity in AM fungi.

To test the possibility that CL-type SLs might act as host-derived precolonization signals in AM symbiosis, natural CL (**1**), CLA (**2**), and MeCLA (**3**), and monohydroxy-CLs, 4-, 18-, and 19-hydroxy-CLs (**4**, **5**, **6**) as well as synthetic CL analogues (**32**–**37**) were chemically synthesized, and tested for hyphal branching-inducing activity in germinating spores of the AM fungus *Gigaspora margarita* in this study. It was found that the oxidation of methyl to carboxyl at C-19 in CL is a prerequisite, but BC-ring formation is not essential for strong hyphal branching activity on the AM fungus.

2. Results and discussion

2.1. Synthesis of carlactone and its oxidized derivatives

Naturally occurring CL (**1**), CLA (**2**), and MeCLA (**3**) were synthesized as a racemate from β-ionone according to the method reported previously (Seto et al., 2014; Abe et al., 2014). A monohydroxy derivative of CL, 19-hydroxy-CL (**6**), was also prepared as described (Abe et al., 2014). To examine the effect of a hydroxyl group at the corresponding position of strigol (C-5, **10**) and orobanchol (C-4, **8**) on hyphal branching activity, 4- and 18-hydroxy-

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CL (**4** and **5**) were synthesized for the first time in this study.

4-Hydroxy-CL (**4**) was synthesized as shown in Scheme 1 by starting from 4-hydroxy-β-ionone (**15**) that was obtained in two steps from α-ionone as described previously (Ye et al., 2009). After protection of the hydroxyl group by an isopropyldimethylsilyl (IPDMS) group, the resultant silyl-protected ionone **16** was converted to IPDMSO-C_{14}-aldehyde **18** via the Corey–Chaykovsky epoxidation with dimethylsulfonium methylide, followed by methylaluminium bis(4-bromo-2,6-di-*tert*-butylphenoxide) (MABR)-promoted rearrangement of epoxide to aldehyde. The IPDMS group of **18** was partially removed during the purification process with C_{18} reversed-phase column chromatography, yielding the desired hydroxy-aldehyde **19**. Finally, the aldehyde **19** was coupled with bromobutenolide **20** in the presence of 18-crown-6-ether to yield a mixture of 4-hydroxy-CL (**4**) (together with its (9*E*) geometric isomer in a ratio 1:2). Purification of semi-preparative C_{18} reversed-phase HPLC afforded 4-hydroxy-CL (**4**) as a mixture of two epimers at C-11 that was not separable under the conditions used.

To synthesize 18-hydroxy-CL (**5**) (Scheme 2), cyclohexanecarboxylic acid methyl ester **21** (Wada et al., 1994) was converted to the triflate **22**, which was subjected to the Heck reaction with methyl vinyl ketone to afford the β-ionone derivative **23** having a carbomethoxy group at C-13. After protection of the keto group of methyl ester **23** by ketalization with ethylene glycol, the resultant ketal ester **24** was reduced to the alcohol **25** with lithium aluminum hydride followed by TBDMS protection to give the silylated ketal **26**. Deprotection of ketal catalyzed by molecular iodine in acetone gave 13-TBDMSO-β-ionone **27**. The silylated ketone **27** was converted to TBDMSO-C_{14}-aldehyde **29** via the Corey–Chaykovsky epoxidation, followed by MABR-promoted rearrangement as above. *O*-Alkylation of the potassium enolate of aldehyde **29** with bromobutenolide **20** in the presence of 18-crown-6-ether furnished the silylated 18-hydroxy-CL **30** and its (9*E*) geometric isomer **31** in a ratio 1:7. After purification by preparative HPLC, 19-TBDMSO-CL (**30**) was deprotected with aqueous acetic acid to afford 18-hydroxy-CL (**5**).

2.2. Synthesis of carlactone analogues

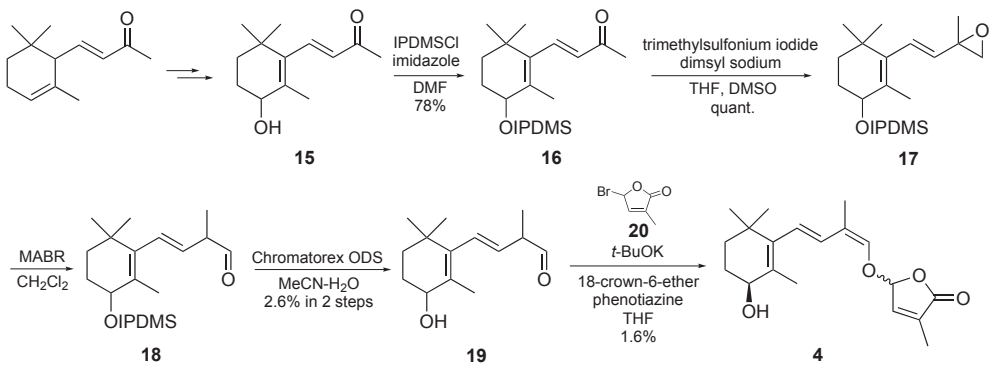
New CL analogues **32**, **34**, and **36** named AD esters were designed by truncating the BC-rings from GR24 (**12**) and 4-deoxyorobanchol (**7**) as a lead structure (Fig. 1). The AD esters consist of a hydroxymethyl-substituted A-ring ester with formylacetic acid connecting to a methylbutenolide (the D-ring) via an enol ether bridge. To synthesize AD esters (Scheme 3), formyl Meldrum's acid (**38**) was reacted with benzyl (**39**), cyclohexylmethyl (**40**), and cyclogeranyl (**41**) alcohols (the A-ring part) to give formylacetic esters **42** (Sato et al., 1986). *O*-Alkylation of the potassium enolates of formylacetates **42** with bromobutenolide **20** furnished AD esters **32**, **34**, and **36** in very low yields probably due to notable instability of formylacetates (Sato et al., 1986) and low *O*-alkylation efficiency. The (*E*)-geometry of the enol ether double bond in AD esters was determined on the basis of the coupling constant ($J = 12.4$ Hz for **32**, **34**, and **36**). Although both (*E*)- and (*Z*)-isomers of methyl 3-(4-methyl-5-oxo-2,5-dihydrofuran-2-yloxy) acrylate were obtained previously in a ratio of about 2:3 (Umehara et al., 2015), the (*Z*)-isomer was not found in the respective reaction mixtures.

Other members of new CL analogues **33**, **35**, and **37** named AA'D diesters were obtained serendipitously in the reaction mixture during the synthesis of AD esters. AA'D diester analogues consist of two A-ring esters and an enol ether bridged D-ring. These analogues can be formed through self aldol condensation of formylacetate **42** to (*E*)-4-formylpent-2-enedioic acid diester followed by *O*-alkylation with bromobutenolide **20**.

2.3. Hyphal branching-inducing activity

Naturally occurring CL-type SLs **1**–**3** and three monohydroxy-CLs **4**–**6** were tested for hyphal branching-inducing activity in germinating spores of the arbuscular mycorrhizal fungus *Gigaspora margarita* by paper disc assay (Table 1). The activity was evaluated by determining the minimum effective concentration using serial dilutions of the test compounds. CL (**1**) showed weak activity (100 ng per disc). CLA (**2**) was the most potent among the three naturally occurring CL-type SLs, inducing hyphal branching at 100 pg per disc. The activity of CLA (**2**) was comparable to that of the natural SLs such as strigol (**10**) and sorgomol (**11**) (Akiyama et al., 2010). MeCLA (**3**) was 10-fold less active (1 ng per disc) than CLA (**2**) presumably due to its relative instability. 19-Hydroxy-CL (**6**) showed intermediate activity between CL (**1**) and CLA (**2**) (10 ng per disc), whereas the activities of 4- and 18-hydroxy-CL (**4** and **5**) were less than or comparable to that of CL (**1**) (1 μg and 100 ng per disc). The 19-oxidized derivatives of CL (**1**), CLA (**2**), MeCLA (**3**) and 19-hydroxy-CL (**6**) induced the formation of low order branches, mainly consisting of long tertiary hyphae, as observed for the natural SLs such as 5-deoxystrigol (**9**) and strigol (**10**), whereas CL, 4- and 18-hydroxy-CLs only induced short tertiary from the treated secondary hyphae as observed for GR24 (**12**) (Akiyama et al., 2010).

To gain the insight into the structural requirements of CL-type SLs for hyphal branching activity, CL analogues, AD esters (**32**, **34**, and **36**) and AA'D diesters (**33**, **35**, and **37**), were tested for hyphal branching activity in *G. margarita* (Table 1). AD ester **32** showed moderate activity, inducing hyphal branching at 1 ng per disc.



Scheme 1. Synthesis of 4-hydroxycarlactone (**4**).

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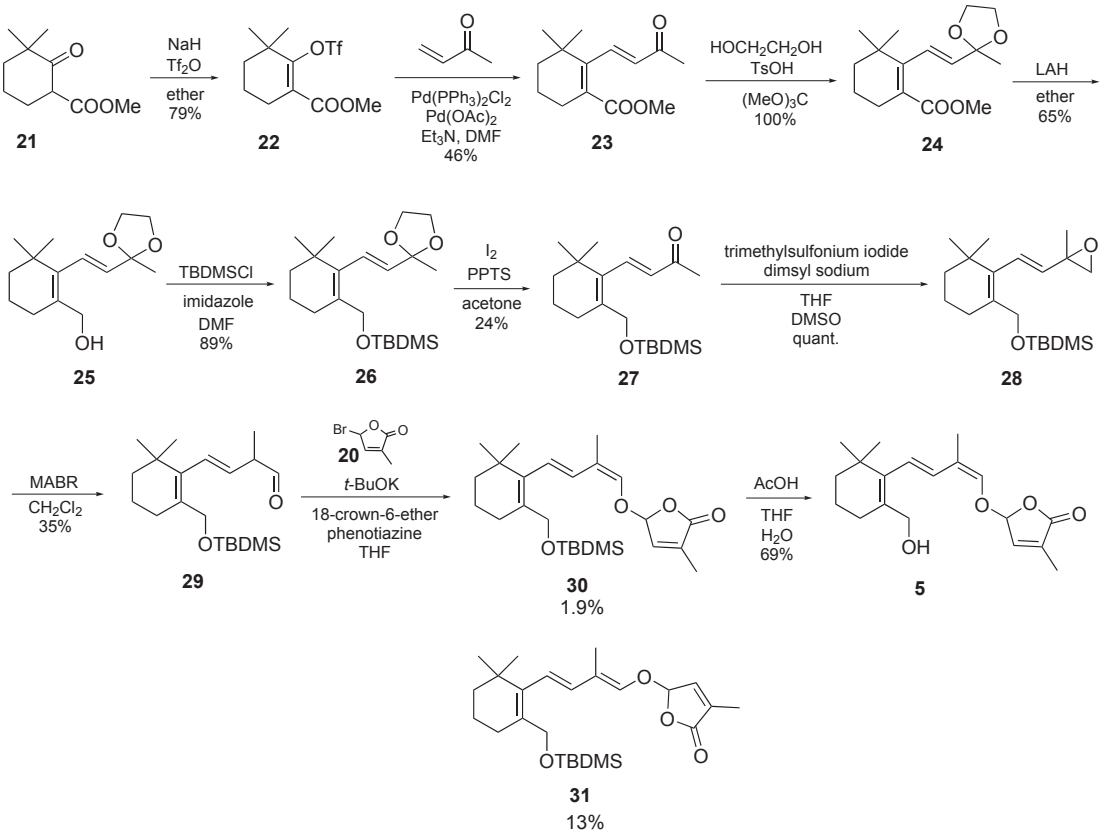
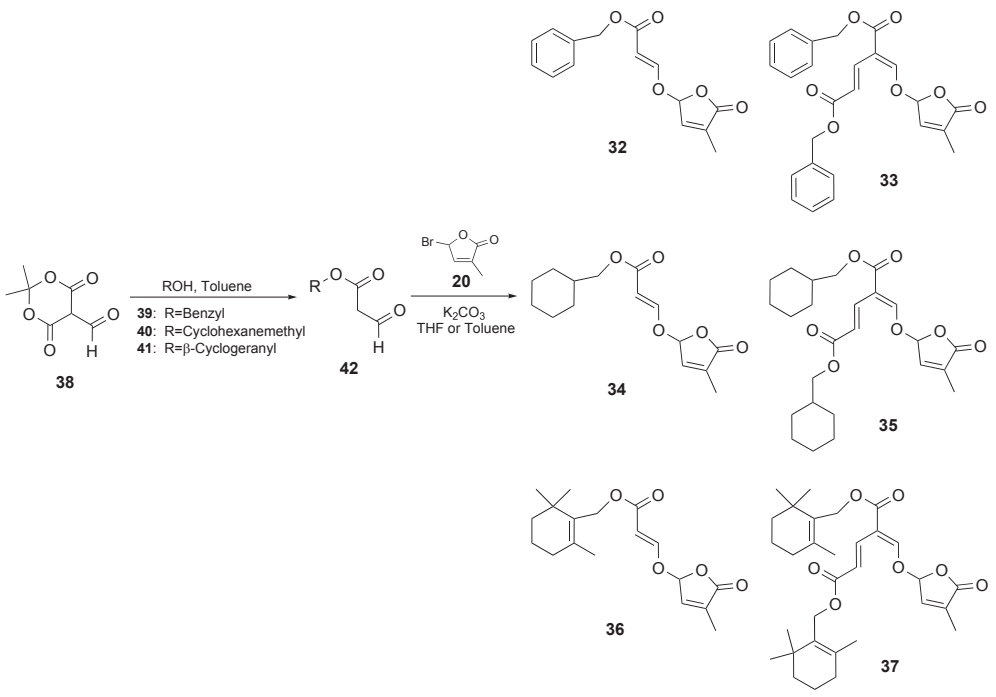


Scheme 2. Synthesis of 18-hydroxycarlactone (**5**).



Scheme 3. Synthesis of carlactone analogues (**32**–**37**).

Replacement of the benzene ring in **32** with cyclohexane as in **34** increased activity by 10-fold (100 pg per disc). Analogue **36** having the β-ionone ring (cyclogeranyl) as in CL (**1**) also showed comparable activity to CLA (**2**) (100 pg per disc). AA'D diester analogue **33** showed activity at 1 ng per disc. Analogues **35** and **37** having cyclohexane and β-ionone ring, respectively, were highly

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Table 1
Minimum effective concentrations (MECs) of carlactone-type strigolactones (**1**–**6**), carlactone analogues (**32**–**37**), and canonical strigolactones (**7**–**12**) for hyphal branching-inducing activity in *Gigaspora margarita*.

Tested compound	MEC (pg/disc)[a]
Carlactone (**1**)	100,000
Carlactonoic acid (**2**)	100
Methyl carlactonoate (**3**)	1000
4-Hydroxycarlactone (**4**)	1,000,000
18-Hydorxycarlactone (**5**)	100,000
19-Hydorxycarlactone (**6**)	10,000
32	1000
33	1000
34	100
35	10
36	100
37	10
(−)-4-Deoxyorobanchol (**7**)	3[b]
(−)-Orobanchol (**8**)	1[b]
(+)-5-Deoxystrigol (**9**)	3[b]
(+)-Strigol (**10**)	100[b]
(+)-Sorgomol (**11**)	100[b]
(+)-GR24 (**12**)	100[b]

[a] Determined by serial 10-fold dilutions.
[b] Tested previously (Akiyama et al., 2010).

active on the AM fungus, showing activity at 10 pg per disc. All analogues tested induced long tertiary hyphae from the treated secondary hyphae as observed for 19-oxidized CLs.

The results obtained for CL-type SLs indicate that hyphal branching activity correlates with the degree of oxidation at C-19 and that the BC ring closure to complete canonical SL structure is not necessary for the activity. Hydroxylation at either C-4 or C-18 in CL did not significantly affect the activity. By contrast, (+)-strigol (**10**) and (−)-orobanchol (**8**), the canonical SLs hydroxylated at the corresponding position, exhibited weaker and stronger activity than their respective parent SLs, (+)-5-deoxystrigol (**9**) and (−)-4-deoxyorobanchol (**7**) (Akiyama et al., 2010). All the CL analogues having the A ring and the enol ether bridged D ring linked to a carboxyl group, but lacked the B and C-rings induced hyphal branching in *G. margarita*. The analogues containing the β-ionone ring were more active than those with benzene ring. Even though an additional bulky A-ring ester is linked to the enol ether double bond, AA'D diester analogues showed activity comparable to or higher than that of their respective AD ester analogues. These facts suggest that the putative SL receptor in the AM fungus has sufficient flexibility to accommodate such a large ligand and that the β-ionone ring rather than benzene ring in the A-ring part is preferred for binding to the receptor. Taken together, the oxidation of methyl to carboxyl at C-19 in CL is a prerequisite but BC-ring formation is not essential to show hyphal branching activity comparable to that of canonical SLs.

3. Concluding remarks

In this study, it was shown that not only canonical SLs, but also CL-type SLs can induce hyphal branching in AM fungi. The naturally occurring 19-oxidized CLs, CLA (**2**) and MeCLA (**3**) are moderate inducers for hyphal branching in *G. margarita*. So far, CLA (**2**) and MeCLA (**3**) have been detected in root extracts, but their exudation from roots has not yet been examined. CLA (**2**) and MeCLA (**3**) could act as a symbiotic signal for AM fungi if they are exuded from AM host roots at sufficient levels to trigger the fungal response. Avenaol (**13**) and heliolactone (**14**), other members of CL-type SLs, discovered from the root exudates of AM host plants fullfil the structural requirements for induction of hyphal branching in AM fungi as revealed by the previous work (Akiyama et al., 2010) and this study, and are expected to act as a host-derived precolonization signal in AM symbiosis. Further studies are needed to show the existence of CLA (**2**) and MeCLA (**3**) in the root exudates of AM host plants and also to investigate the occurrence and distribution of known and novel CL-type SLs in the plant kingdom. These studies could provide new insight into the molecular evolution of CL-type SLs and canonical SLs as signals for AM fungi and root parasitic plants.

4. Experimental

4.1. General procedures

Mass spectra were recorded on a JMS-700 instrument (JEOL), NanoFrontier L (Hitachi High-Technologies), or a GCMS-QP2010 Plus instrument (Shimadzu) in the direct injection mode. ^{1}H and ^{13}C-NMR spectra were obtained with a JNM-AL400 NMR spectrometer (JEOL). Chemical shifts were referenced to tetramethylsilane as an internal standard. Column chromatography (CC) was performed with Wakogel C-200 (Wako Pure Chemical Ind.), Kieselgel 60 (Merck), Chromatorex ODS (Fuji Silysia Chemical), Inertsil ODS-3 (φ 10 × 250 mm, 5 μm; GL Sciences), InertSustain C18 (φ 10 × 250 mm, 5 μm; GL Sciences) and Inertsil SIL-100A (φ 10 × 250 mm, 5 μm; GL Sciences).

4.2. Synthesis of 4-hydroxy-CL (*4*)

4.2.1. (3E)-4-(3-isopropyldimethylsilyloxy-2,6,6-trimethylcyclohex-1-en-1-yl)but-3-en-2-one (*16*)

To a stirred solution of hydroxy-β-ionone **15** (14.7 g, 70.5 mmol) in *N*,*N*-dimethylformamide (DMF, 179 ml) at 0 °C were added imidazole (19.2 g, 282 mmol) and isopropyldimethylchlorosilane (19.3 g, 141 mmol), and the mixture was stirred for 2.5 h at room temperature under Ar. The reaction mixture was quenched by adding 5% (wt/vol) $NaHCO_3$ solution (200 ml), and extracted with Et_2O and *n*-hexane. The organic phase was washed with H_2O, dried over anhydrous Na_2SO_4, and concentrated *in vauo*. Purification by silica gel CC eluted stepwise with *n*-hexane and EtOAc [6% (vol/vol) increments] gave isopropyldimethylsilyloxy-β-ionone **16** (16.9 g, 54.9 mmol, 78%). ^{1}H-NMR ($CDCl_3$, 400 MHz) δ: 0.10 (6H, s, Si-$(CH_3)_2$), 0.81–0.90 (1H, m, Si–CH), 0.97 (3H, d, J = 7.0 Hz, Si–CH–CH_3), 0.98 (3H, d, J = 7.1 Hz, Si–CH–CH_3), 1.02 (3H, s, 6′-CH_3), 1.08 (3H, s, 6′-CH_3), 1.38–1.87 (4H, m, H-4′ and H-5′), 1.76 (3H, s, 2′-CH_3), 2.30 (3H, s, H-1), 4.03 (1H, t, J = 5.6 Hz, H-3′), 6.12 (1H, d, J = 16.5 Hz, H-3), 7.19 (1H, d, J = 16.5 Hz, H-4); ^{13}C-NMR ($CDCl_3$, 100 MHz) δ: −3.8, −3.5, 14.9, 17.0, 18.3, 27.3, 28.3, 29.0, 34.6, 35.3, 70.8, 132.9, 135.4, 138.2, 143.2, 198.6; EI-MS *m/z*: 308 $[M]^+$, 293, 265; HREIMS *m/z*: 308.2164 $[M]^+$ (calcd. for $C_{18}H_{32}O_2Si$, *m/z* 308.2172).

4.2.2. (E)-isopropyldimethyl((2,4,4-trimethyl-3-(2-(2-methyloxiran-2-yl)vinyl)cyclohex-2-en-1-yl)oxy)silane (*17*)

To a solution of trimethylsulfonium iodide (11.1 g, 54.3 mmol) in DMSO (47 mL) was added tetrahydrofuran THF (47 mL) under Ar to yield a finely divided suspension of sulfonium salt. This mixture was then cooled to −5 °C and treated with a solution of dimsyl sodium (4.4 M, 13 mL, 57.2 mmol). The resulting gray colored suspension was treated with a solution of isopropyldimethylsilyloxy-β-ionone **16** (11.2 g, 36.2 mmol) in THF (11.2 mL). After stirring at −5 °C for 45 min, the mixture was warmed to room temperature, quenched by successively adding H_2O (200 mL) and *n*-hexane (200 mL). The organic phase was washed with H_2O, dried over anhydrous Na_2SO_4, and concentrated *in vacuo* to give the epoxide **17** (11.1 g, 36.1 mmol). ^{1}H-NMR ($CDCl_3$, 400 MHz) δ: 0.09 (6H, s, Si-$(CH_3)_2$), 0.80–0.90 (1H, m, Si–CH), 0.95 (3H, d, J = 11.0 Hz, Si–CH–CH_3), 0.96 (3H, s, 4-CH_3), 0.97 (3H, d, J = 7.1 Hz,

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Si–CH–CH_3), 1.02 (3H, s, 4-CH_3), 1.34–1.82 (4H, m, H-5 and H-6), 1.49 (3H, s, 2″-CH_3), 1.69 (3H, s, 2-CH_3), 2.73 (1H, d, J = 5.4 Hz, H-3″), 2.83 (1H, d, J = 5.4 Hz, H-3″), 4.00 (1H, t, J = 5.4 Hz, H-1), 5.28 (1H, d, J = 16.4 Hz, H-2′), 6.15 (1H, d, J = 16.4 Hz, H-1′); ^{13}C-NMR ($CDCl_3$, 100 MHz) δ: −3.7, −3.5, 15.0, 17.0 and 17.1, 18.0 and 18.1, 19.69 and 19.74, 28.2, 29.2, 34.4 and 34.5, 35.08 and 35.13, 55.7 and 55.9, 71.0, 129.7, 130.9, 134.9, 135.0, 139.4 and 139.5; MS data was not obtainable due to instability of the compound.

4.2.3. (E)-4-(3-hydroxy-2,6,6-trimethylcyclohex-1-en-1-yl)-2-methylbut-3-enal (**19**)

To a solution of 2,6-di-*tert*-butyl-4-bromophenol (20.1 g, 72.2 mmol) in CH_2Cl_2 (318 mL) was added at room temperature a 1.4 M hexane solution of trimethylaluminium (Me_3Al, 26 mL, 36.1 mmol), and the solution was stirred at room temperature for 1 h under Ar. To a solution of the MABR (36 mmol) in CH_2Cl_2 was added a solution of epoxide **17** (11.1 g, 36.1 mmol) in CH_2Cl_2 (24 mL) at −78 °C, and the resulting mixture was stirred at −78 °C for 30 min under Ar. The reaction mixture was poured into H_2O, and extracted with *n*-hexane. The organic phase was washed with saturated aqueous $NaHCO_3$, dried over anhydrous Na_2SO_4, and concentrated *in vacuo*. The residue containing IPDMSO-C_{14}-aldehyde **18** was subjected to ODS CC eluted stepwise with 70–100% (vol/vol) CH_3CN in H_2O [5% (vol/vol) increments]; 75–80% CH_3CN eluates were combined, evaporated to H_2O *in vacuo*, extracted with *n*-hexane, and evaporated to give the hydroxy aldehyde **19** (208 mg, 2.6%). ^{1}H-NMR ($CDCl_3$, 400 MHz) δ: 0.98 (3H, s, 6′-CH_3), 1.01 (3H, s, 6′-CH_3), 1.36–1.72 (4H, m, H-4′ and H-5′), 1.80 (3H, br.s, 2′-CH_3), 3.17 (1H, m, H-2), 3.99 (1H, t, J = 4.6 Hz H-3′), 5.39 and 5.40 (1H, dd, J = 7.8, 16.2 Hz, H-3), 6.02 and 6.03 (1H, d, J = 16.2 Hz, H-4), 9.616 and 9.620 (1H, d, J = 1.5 Hz, H-1); ^{13}C-NMR ($CDCl_3$, 100 MHz) δ: 13.6 and 14.2, 18.36 and 18.40, 21.1, 27.19 and 27.21, 28.4 and 28.8, 34.3 and 34.4, 50.6 and 50.7, 60.4, 69.93 and 69.94, 130.0 and 131.1, 130.5, 141.3, 171.2, 201.6; EI-MS *m/z*: 222 $[M]^+$, 207, 166; HREIMS *m/z*: 222.1593 $[M]^+$ (calcd. for $C_{14}H_{22}O_2$, *m/z* 222.1620).

4.2.4. 5-(((1Z,3E)-4-(3-hydroxy-2,6,6-trimethylcyclohex-1-en-1-yl)-2-methylbuta-1,3-dien-1-yl)oxy)-3-methylfuran-2(5H)-one (4-hydroxy-CL (**4**))

To a mixture of 4-HO-C_{14}-aldehyde **19** (104 mg, 0.468 mmol), phenotiazine (1.6 mg), (±)-4-bromo-2-methyl-2-buten-4-olide **20** (52.9 μl, 0.59 mmol), and 18-crown-6-ether (88.4 mg, 0.336 mmol) in THF (3.9 mL), potassium *tert*-butoxide (75.5 mg, 0.620 mmol) was added slowly under Ar, and the reaction mixture was stirred at room temperature under Ar. After stirring for 45 min, the mixture was poured into H_2O (20 mL) and extracted with Et_2O. The organic phase was washed with brine, dried over anhydrous Na_2SO_4, and concentrated *in vacuo*. The residue was subjected to silica gel CC eluted stepwise with *n*-hexane and Et_2O [5% (vol/vol) increments]. The 30–40% Et_2O in hexane eluates containing 4-HO-CL was purified by a semi-preparative Inertsil ODS-3 HPLC (φ 10 × 250 mm, 5 μm; GL Sciences), using isocratic elution with CH_3CN: H_2O (60:40 v/v) at a flow rate of 4.0 mL/min and monitored at 285 nm to give 4-HO-CL **4** [2.43 mg, 7.64 μmol, 1.6%, retention time 16.6 min]. 4-HO-CL (as a mixture with 11-epimer): ^{1}H-NMR (C_6D_6, 400 MHz) δ: 0.96, 0.99, 1.01 and 1.04 (total 6H, s, CH_3-16 or 17), 1.22–1.30 (1H, m, H-2), 1.35 and 1.35 (3H, t, J = 1.5 Hz, CH_3-15), 1.54–1.58 (2H, m, H-3), 1.66–1.73 (1H, m, H-2), 1.587 and 1.591 (3H, s, CH_3-18), 1.90 and 1.91 (3H, br.s, CH_3-19), 3.75 (1H, br.d, H-4), 5.18 (1H, br.s, H-11), 5.77–5.78 (1H, m, H-12), 5.98 (1H, s, H-10), 6.08 (1H, d, J = 16.3 Hz, H-7), 6.90 (1H, d, J = 16.3 Hz, H-8); ^{13}C-NMR (C_6D_6, 100 MHz) δ: 10.2, 14.3, 18.8, 27.62 and 27.67, 28.95 and 29.00, 29.05 and 29.11, 30.4, 34.8 and 34.9, 69.91 and 69.94, 100.0, 116.3 and 116.4, 126.3, 128.6, 130.8, 134.6, 139.41 and 139.42, 141.29 and 141.31, 141.6, 170.5; EI-MS *m/z* (rel. int): 318 $[M]^+$ (11), 300 (2.0), 221 (62), 97 (34), 91 (100); HREIMS *m/z*: 318.1827 $[M]^+$ (calcd. for $C_{19}H_{26}O_4$, *m/z* 318.1831).

4.3. Synthesis of 18-hydroxy-CL (**5**)

4.3.1. Methyl 3,3-dimethyl-2-(((trifluoromethyl)sulfonyl)oxy)cyclohex-1-ene-1-carboxylate (**22**)

A solution of ester **21** (10.3 g, 55.9 mmol) in Et_2O (90 ml) was added to a suspension of NaH (1.75 g, 72.8 mmol) in Et_2O (120 ml) at 0 °C under Ar. After stirring at 0 °C for 30 min, triflic anhydride (32.5 g, 115 mmol) was added, and the mixture was stirred at the same temperature for 2 h. The reaction mixture was quenched by adding H_2O (150 ml), and extracted with CH_2Cl_2. The organic phase was washed with brine, dried over anhydrous $MgSO_4$, and concentrated *in vacuo*. Purification by silica gel CC eluted with *n*-hexane and EtOAc: *n*-hexane gave the triflate **22** (14.0 g, 44.3 mmol, 79%). ^{1}H-NMR ($CDCl_3$, 400 MHz) δ: 1.22 (6H, s, 3-CH_3 x 2), 1.60–1.78 (4H, m, H-4 and H-5), 2.49 (2H, t, J = 6.2 Hz, H-6), 3.77 (3H, s, OCH_3); ^{13}C-NMR ($CDCl_3$, 100 MHz) δ: 17.9, 26.2, 28.1, 39.6, 52.3, 118.4, 123.0, 155.1, 166.2; EI-MS *m/z*: 316 $[M]^+$, 285, 167; HREIMS *m/z*: 316.0592 $[M]^+$ (calcd. for $C_{11}H_{15}F_3O_5S$, *m/z* 316.0592).

4.3.2. Methyl (E)-3,3-dimethyl-2-(3-oxobut-1-en-1-yl)cyclohex-1-ene-1-carboxylate (**23**)

A mixture of triflate **22** (14.0 g, 44.3 mmol), Et_3N (17.9 g, 177 mmol), methyl vinyl ketone (6.21 g, 88.6 mmol), bis(-triphenylphosphine) palladium(II) dichloride (240 mg, 0.34 mmol), and palladium(II) acetate (480 mg, 2.2 mmol) in DMF (100 mL) was stirred at 75 °C for 24 h under Ar. The reaction mixture was poured into ice-cooled 1 N HCl (100 ml), and extracted with *n*-hexane. The organic phase was washed with brine and H_2O, dried over anhydrous $MgSO_4$, and concentrated *in vacuo*. Purification by silica gel CC eluted stepwise with *n*-hexane and EtOAc [5% (vol/vol) increments] gave the ester **23** (4.8 g, 20.3 mmol, 46%). ^{1}H-NMR ($CDCl_3$, 400 MHz) δ: 1.10 (6H, s, 3-CH_3 x 2), 1.52–1.55 (2H, m, H-4), 1.65–1.73 (2H, m, H-5), 2.29 (3H, s, H-4′), 2.32 (2H, dt, J = 2.1, 6.3 Hz, H-6), 3.65 (3H, s, OCH_3), 6.02 (1H, d, J = 16.2 Hz, H-2′), 7.32 (1H, d, J = 16.2 Hz, H-1′); ^{13}C-NMR ($CDCl_3$, 100 MHz) δ: 18.2, 27.2, 27.5, 28.1, 34.7, 38.4, 51.7, 129.8, 131.0, 143.4, 146.6, 170.1, 198.0; EI-MS *m/z*: 236 $[M]^+$, 193, 179, 177, 163; HREIMS *m/z*: 236.1430 $[M]^+$ (calcd. for $C_{14}H_{20}O_3$, *m/z* 236.1412).

4.3.3. Methyl (E)-3,3-dimethyl-2-(2-(2-methyl-1,3-dioxolan-2-yl)vinyl)cyclohex-1-ene-1-carboxylate (**24**)

A mixture of ester **23** (4.8 g, 20.3 mmol), ethylene glycol (12.6 g, 203 mmol), trimethyl orthoformate (4.3 g, 40.6 mmol), *p*-toluenesulfonic acid monohydrate (58 mg, 0.3 mmol) was stirred overnight at room temperature under Ar. The reaction mixture was quenched by adding saturated aqueous $NaHCO_3$ (50 ml), and extracted with Et_2O. The organic phase was dried over anhydrous $MgSO_4$, and concentrated *in vacuo* to give the ketal **24** (5.7 g, 20.3 mmol, 100%). ^{1}H-NMR ($CDCl_3$, 400 MHz) δ: 1.04 (6H, s, 3-CH_3 x 2), 1.46 (3H, s, 2″-CH_3), 1.47–1.53 (2H, m, H-4), 1.64–1.70 (2H, m, H-5), 2.26 (2H, dt, J = 2.1, 6.3 Hz, H-6), 3.65 (3H, s, OCH_3), 3.87–4.00 (4H, m, H-4″ and H-5″), 5.41 (1H, d, J = 15.7 Hz, H-2′), 6.36 (1H, dt, J = 16.5, 2.1 Hz, H-1′); ^{13}C-NMR ($CDCl_3$, 100 MHz) δ: 18.5, 24.9, 27.5, 28.1, 34.1, 38.2, 51.3, 64.4, 107.0, 115.8, 127.5, 133.3, 146.6, 171.4; EI-MS *m/z*: 280 $[M]^+$, 223, 193, 179, 87; HREIMS *m/z*: 280.1659 $[M]^+$ (calcd. for $C_{16}H_{24}O_4$, *m/z* 280.1674).

4.3.4. (E)-(3,3-Dimethyl-2-(2-(2-methyl-1,3-dioxolan-2-yl)vinyl)cyclohex-1-en-1-yl)methanol (**25**)

A solution of ketal **24** (5.7 g, 20.3 mmol) in Et_2O (25 ml) was added to a suspension of $LiAlH_4$ (1.9 g, 47 mmol) in Et_2O (40 ml) at 0 °C under Ar, and the mixture was stirred at the same temperature

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for 2 h. The reaction mixture was cooled to 0 °C, and quenched by successively adding H_2O (2 ml), 1 N NaOH (2 ml) and H_2O (6 ml). The mixture was extracted with Et_2O. The organic phase was washed with H_2O, dried over anhydrous $MgSO_4$, and concentrated *in vacuo*. Purification by silica gel CC eluted stepwise with *n*-hexane and EtOAc [10% (vol/vol) increments] gave the alcohol **25** (3.85 g, 15.3 mmol, 65%). This compound was readily transformed by intramolecular cyclization into 7,7-dimethyl-1-((2-methyl-1,3-dioxolan-2-yl)methyl)-1,3,4,5,6,7-hexahydroisobenzofuran (see supplement) when dissolved in $CDCl_3$. ^{1}H-NMR ($CDCl_3$, 400 MHz) δ: 1.05 (3H, s, 7-CH_3), 1.09 (3H, s, 7-CH_3), 1.33–1.50 (2H, m, H-6), 1.45 (3H, s, 2′-CH_3), 1.67–1.75 (2H, m, H-5), 1.79 (1H, dd, J = 9.9, 14.5 Hz, 2′-CH_2), 1.88–1.97 (2H, m, H-4), 2.07 (1H, dd, J = 2.2, 14.5 Hz, 2′-CH_2), 3.96–4.00 (4H, m, H-4″ and H-5″), 4.36 (1H, m, H-3), 4.52 (1H, m, H-3), 4.92–5.00 (1H, m, H-1); ^{13}C-NMR ($CDCl_3$, 100 MHz) δ: 19.0, 22.2, 24.2, 27.8, 28.2, 31.1, 40.4, 44.5, 64.37, 64.44, 76.1, 82.5, 109.5, 131.4, 139.9; EI-MS *m/z*: 252 $[M]^+$, 237, 207, 151, 87; HREIMS *m/z*: 252.1741 $[M]^+$ (calcd. for $C_{15}H_{24}O_3$, *m/z* 252.1726).

4.3.5. (E)-tert-Butyl((3,3-dimethyl-2-(2-(2-methyl-1,3-dioxolan-2-yl)vinyl)cyclohex-1-en-1-yl)methoxy)dimethylsilane (**26**)

To a stirred solution of alcohol **25** (3.85 g, 15.3 mmol) in DMF (40 mL) was added imidazole (4.17 g, 61.2 mmol) and *tert*-butyldimethylchlorosilane (4.64 g, 30.8 mmol), and the mixture was stirred for 3 h at room temperature under Ar. The reaction mixture was taken up in 2% (vol/vol) Et_2O in *n*-hexane, washed with H_2O, dried over anhydrous $MgSO_4$, and concentrated *in vauo*. Purification by silica gel CC eluted with *n*-hexane and 10% Et_2O in *n*-hexane gave the silyl protected alcohol **26** (4.99 g, 13.6 mmol, 89%). ^{1}H-NMR ($CDCl_3$, 400 MHz) δ: 0.03 (6H, s, Si-$(CH_3)_2$), 0.89 (9H, s, Si–$C(CH_3)_3$), 0.98 (6H, s, 3-CH_3 x 2), 1.46–1.49 (1H, m, H-4 or H-5), 1.51 (3H, s, 2″-CH_3), 1.54–1.58 (3H, m, H-4′ and H-5′), 2.12 (2H, dt, J = 1.7, 6.3 Hz, H-6), 3.93–4.02 (4H, m, H-4″ and H-5″), 4.12 (2H, s), 5.35 (1H, d, J = 15.9 Hz, H-2′), 6.19 (1H, br.d, J = 15.9 Hz, H-1′); ^{13}C-NMR ($CDCl_3$, 100 MHz) δ: −5.2, 19.0, 22.6, 25.9, 27.3, 28.6, 31.6, 33.9, 39.0, 64.47, 107.4, 127.1, 132.4, 134.4, 138.7; EI-MS *m/z*: 366 $[M]^+$, 351, 309, 265, 251, 87; HREIMS *m/z*: 366.2613 $[M]^+$ (calcd. for $C_{21}H_{38}O_3Si$, *m/z* 366.2590).

4.3.6. (E)-4-(2-(((tert-Butyldimethylsilyl)oxy)methyl)-6,6-dimethylcyclohex-1-en-1-yl)but-3-en-2-one (**27**)

A mixture of silyl protected alcohol **26** (2.50 g, 6.8 mmol), pyridinium *p*-toluenesulfonate (170 mg, 0.68 mmol), and iodine (173 mg, 0.68 mmol) in acetone (20 ml) was stirred at room temperature for 1 h under Ar. The reaction mixture was diluted with Et_2O (50 ml), washed with 5% aqueous $Na_2S_2O_3$ and brine, dried over $MgSO_4$, and concentrated *in vacuo*. Purification by silica gel CC eluted stepwise with *n*-hexane and Et_2O [2% (vol/vol) increments] gave the ketone **27** (532 mg 1.65 mmol, 24%). ^{1}H-NMR ($CDCl_3$, 400 MHz) δ: 0.04 (6H, s, Si-$(CH_3)_2$), 0.89 (9H, s, Si–$C(CH_3)_3$), 1.05 (6H, s, 6′-CH_3 x 2), 1.47–1.51 (2H, m, H-5′), 1.55–1.59 (2H, m, H-4′), 2.19 (2H, br.t, J = 6.1 Hz, H-3′), 4.11 (2H, s, OCH_2), 6.10 (1H, d, J = 16.1 Hz, H-3), 7.20 (1H, d, J = 16.1 Hz, H-4); ^{13}C-NMR ($CDCl_3$, 100 MHz) δ: −5.2, 18.3, 18.7, 25.9, 27.6, 28.2, 28.7, 34.2, 39.1, 64.2, 132.5, 137.1, 138.2, 142.2, 198.2; EI-MS *m/z*: $[M]^+$ (*m/z* 322) was not observed, 307, 279, 265.

4.3.7. (E)-tert-Butyl((3,3-dimethyl-2-(2-(2-methyloxiran-2-yl)vinyl)cyclohex-1-en-1-yl)methoxy)dimethylsilane (**28**)

To a solution of trimethylsulfonium iodide (458 mg, 2.3 mmol) in DMSO (2 ml) was added THF (2 ml) under argon to yield a finely divided suspension of sulfonium salt. This mixture was then cooled to 0 °C and treated with a solution of dimsyl sodium (4.4 M, 0.63 ml, 2.7 mmol). The resulting gray colored suspension was treated with a solution of ketone **27** (500 mg, 1.55 mmol) in THF (0.26 ml). After stirring at 0 °C for 1 h, the mixture was warmed to room temperature, quenched by successively adding H_2O (10 mL) and *n*-hexane (30 mL). The organic phase was washed with H_2O, dried over anhydrous $MgSO_4$, and concentrated *in vacuo* to give the epoxide **28** (522 mg, 1.55 mmol, 100%). ^{1}H-NMR ($CDCl_3$, 400 MHz) δ: 0.00 (6H, s, Si-$(CH_3)_2$), 0.86 (9H, s, Si–$C(CH_3)_3$), 0.959 (3H, s, 3-CH_3), 0.961 (3H, s, 3-CH_3), 1.40–1.45 (2H, m, H-4), 1.46 (3H, s, 2″-CH_3), 1.55–1.60 (2H, m, H-5), 2.07–2.13 (2H, m, H-6), 2.69 (1H, dd, J = 0.5, 5.4 Hz, H-3″), 2.80 (1H, d, J = 5.4 Hz, H-3″), 4.08 (1H, d, J = 11.5 Hz, OCH_2), 4.12 (1H, d, J = 11.5 Hz, OCH_2), 5.24 (1H, d, J = 16.1 Hz, H-2′), 6.13 (1H, dt, J = 16.5, 1.9 Hz, H-1′); ^{13}C-NMR ($CDCl_3$, 100 MHz) δ: −5.2, 18.4, 19.0, 19.9, 26.0, 27.5, 28.6, 34.0, 39.0, 55.6, 55.9, 64.5, 128.8, 132.7, 134.9, 139.1; EI-MS *m/z*: 336 $[M]^+$, 321, 305, 279; HREIMS *m/z*: 336.2504 $[M]^+$ (calcd. for $C_{20}H_{36}O_2Si$, *m/z* 336.2484).

4.3.8. (E)-4-(2-(((tert-Butyldimethylsilyl)oxy)methyl)-6,6-dimethylcyclohex-1-en-1-yl)-2-methylbut-3-enal (**29**)

To a solution of 2,6-di-*tert*-butyl-4-bromophenol (183 mg, 0.64 mmol) in CH_2Cl_2 (12 ml) was added at room temperature a 1.4 M hexane solution of trimethylaluminium (Me_3Al, 0.23 ml, 0.32 mmol), and the solution was stirred at room temperature for 1 h under argon. To a solution of the MABR (0.32 mmol) in CH_2Cl_2 was added a solution of epoxide **28** (522 mg, 1.55 mmol) in CH_2Cl_2 (1 ml) at −78 °C, and the resulting mixture was stirred at −78 °C for 30 min under Ar. The reaction mixture was poured into H_2O, and extracted with *n*-hexane. The organic phase was washed with saturated aqueous $NaHCO_3$, dried over anhydrous $MgSO_4$, and concentrated *in vacuo*. The residue was subjected to ODS CC eluted stepwise with 70–100% (vol/vol) CH_3CN in H_2O [5% (vol/vol) increments]; 85–90% CH_3CN eluates were combined, evaporated to H_2O *in vacuo*, extracted with *n*-hexane and Et_2O, and evaporated to give the aldehyde **29** (188 mg, 0.56 mmol, 35%). ^{1}H-NMR ($CDCl_3$, 400 MHz) δ: −0.002 (6H, s, Si-$(CH_3)_2$), 0.86 (9H, s, Si–$C(CH_3)_3$), 0.96 (6H, s, 6′-CH_3 x 2), 1.22 (3H, d, J = 7.1 Hz, 2-CH_3), 1.45–1.48 (2H, m, H-5′), 1.53–1.57 (2H, m, H-4′), 2.11 (2H, dt, J = 1.8, 6.2 Hz, H-3′), 2.45 (1H, m, H-2), 5.32 (1H, dd, J = 7.8, 15.4 Hz, H-3), 6.00 (1H, br.d, J = 15.4 Hz, H-4), 9.59 (1H, d, J = 1.7 Hz, H-1); ^{13}C-NMR ($CDCl_3$, 100 MHz) δ: −5.2, 13.6, 18.4, 18.9, 26.0, 27.5, 28.5, 33.9, 39.0, 50.6, 64.6, 130.1, 130.8, 132.9, 139.4, 201.5; EI-MS *m/z*: 336 $[M]^+$, 321, 305, 279; HREIMS *m/z*: 336.2466 $[M]^+$ (calcd. for $C_{20}H_{36}O_2Si$, *m/z* 336.2484).

4.3.9. 5-(((1Z,3E)-4-(2-(((Tert-Butyldimethylsilyl)oxy)methyl)-6,6-dimethylcyclohex-1-en-1-yl)-2-methylbuta-1,3-dien-1-yl)oxy)-3-methylfuran-2(5H)-one (18-TBDMSO-CL **30** and 18-TBDMSO-(9E)-CL **31**)

To a mixture of TBDMSO-C_{14}-aldehyde **29** (178 mg, 0.53 mmol)), phenotiazine (2.7 mg), (±)-4-bromo-2-methyl-2-buten-4-olide **20** (94 μL, 0.96 mmol), and 18-crown-6-ether (155 mg, 0.59 mmol) in THF (6.8 ml) was added slowly potassium *tert*-butoxide (120 mg, 1.07 mmol) under Ar, and the reaction mixture was stirred at room temperature under Ar. After stirring for 1 h, the mixture was poured into H_2O (20 mL) and extracted with Et_2O. The organic phase was washed with H_2O, dried over anhydrous $MgSO_4$, and concentrated *in vacuo*. The residue was subjected to silica gel CC eluted stepwise with *n*-hexane and Et_2O [3% (vol/vol) increments]. The 9%, 12% and 15% Et_2O eluates containing TBDMSO-CL was purified by a semi-preparative InertSustain C18 HPLC (φ 10 × 250 mm, 5 μm; GL Sciences), using isocratic elution with 90% CH_3CN in H_2O at a flow rate of 4.0 mL/min and monitored at 300 nm to give 18-TBDMSO-CL **30** (4.4 mg, 0.010 mmol, 1.9%, retention time 23.4 min) and 18-TBDMSO-(9*E*)-CL **31** (29.7 mg, 0.069 mmol, 13.0%, retention time 24.3 min). 18-TBDMSO-CL **30**; ^{1}H-NMR (C_6D_6, 400 MHz) δ: 0.07 (6H, s, Si-$(CH_3)_2$), 0.98 (9H, s, Si–$C(CH_3)_3$), 1.03 (3H, s, CH_3-16 or 17), 1.04 (3H, s, CH_3-16 or 17), 1.41 (3H, t, J = 1.6 Hz, CH_3-15), 1.40–1.45 (2H,

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m, H-2), 1.55–1.64 (2H, m, H-3), 1.60 (3H, d, J = 1.2 Hz, CH_3-19), 2.22–2.38 (2H, m, H-4), 4.31 (1H, d, J = 11.1 Hz, H-18α), 4.37 (1H, d, J = 11.1 Hz, H-18β), 5.19 (1H, m, H-12 or 10), 5.85 (1H, m, H-12 or 10), 5.95 (1H, br.d, J = 1.0 Hz, H-11), 6.12 (1H, br d, J = 16.2 Hz, H-7), 6.85 (1H, d, J = 16.6 Hz, H-8); ^{13}C-NMR ($CDCl_3$, 100 MHz) δ: −5.1,: −5.0, 10.2, 14.4, 18.6, 19.4, 26.2, 28.2, 28.85, 28.89, 34.5, 39.5, 65.1, 100.0, 115.9, 125.8, 128.7, 133.0, 134.5, 139.5, 140.8, 141.6, 170.5; EIMS m/z: 432 $[M]^+$, 375, 335, 203, 97; HREIMS m/z: 432.2669 $[M]^+$ (calcd. for $C_{25}H_{40}O_4Si$, m/z 432.2696). 18-TBDMSO-(9*E*)-CL **31**; ^{1}H-NMR (C_6D_6, 400 MHz) δ: 0.08 (6H, s, Si-$(CH_3)_2$), 0.99 (9H, s, Si−C$(CH_3)_3$), 1.07 (3H, s, CH_3-16 or 17), 1.08 (3H, s, CH_3-16 or 17), 1.40 (3H, t, J = 1.5 Hz, CH_3-15), 1.44–1.48 (2H, m, H-2), 1.60–1.68 (2H, m, H-3), 1.84 (3H, d, J = 1.2 Hz, CH_3-19), 2.28–2.36 (2H, m, H-4), 4.27 (1H, d, J = 10.9 Hz, H-18α), 4.33 (1H, d, J = 10.9 Hz, H-18β), 5.22 (1H, m, H-12 or 10), 5.81 (1H, m, H-12 or 10), 6.09 (1H, br d, J = 15.9 Hz, H-7), 6.14 (1H, d, J = 15.9 Hz, H-8), 6.33 (1H, br.d, J = 1.0 Hz, H-11); ^{13}C-NMR (C_6D_6, 100 MHz) δ: −5.0, 9.8, 10.3, 14.2, 18.6, 19.6, 26.2, 28.5, 28.9, 29.0, 34.5, 39.5, 60.0, 65.2, 100.0, 118.5, 123.6, 132.5, 133.1, 134.6, 141.2, 141.6, 142.7, 170.4; EIMS m/z: 432 $[M]^+$, 375, 335, 203, 97; HREIMS m/z: 432.2674 $[M]^+$ (calcd. for $C_{25}H_{40}O_4Si$, m/z 432.2696).

4.3.10. 5-(((1Z,3E)-4-(2-(Hydroxymethyl)-6,6-dimethylcyclohex-1-en-1-yl)-2-methylbuta-1,3-dien-1-yl)oxy)-3-methylfuran-2(5H)-one (18-hydroxy-CL (**5**))

To a solution of 18-TBDMSO-CL **30** (4.4 mg, 0.010 mmol) in THF (0.6 mL) was added at room temperature acetic acid (1.8 mL) and H_2O (0.6 mL), and the mixture was stirred for 9 h. The reaction mixture was poured into H_2O and extracted with Et_2O. The organic phase was washed with H_2O, dried over anhydrous Na_2SO_4, and concentrated *in vacuo*. Purification by silica gel CC eluted stepwise with *n*-hexane and Et_2O [10% (vol/vol) increments] gave 18-hydroxy-CL **5** (2.2 mg, 0.069 μmol, 69%). ^{1}H-NMR (C_6D_6, 400 MHz) δ: 1.01 (3H, s, CH_3-16 or 17), 1.04 (3H, s, CH_3-16 or 17), 1.36 (3H, t, J = 1.6 Hz, CH_3-15), 1.37–1.42 (2H, m, H-2), 1.50–1.57 (2H, m, H-3), 1.57 (3H, d, J = 1.5 Hz, CH_3-19), 2.14 (2H, td, J = 6.3, 1.5 Hz, H-4), 4.09 (2H, s, H-18), 5.18 (1H, dd, J = 1.2, 2.4 Hz, H-12 or 10), 5.81 (1H, m, H-12 or 10), 5.96 (1H, br.d, J = 0.7 Hz, H-11), 6.09 (1H, br d, J = 16.0 Hz, H-7), 6.86 (1H, d, J = 16.0 Hz, H-8); ^{13}C-NMR (C_6D_6, 100 MHz) δ: 10.2, 14.3, 19.4, 28.4, 28.86, 28.91, 34.4, 39.4, 64.6, 100.0, 116.8, 125.8, 128.5, 133.2, 134.5, 139.4, 141.7, 170.6 (one carbon peak was missing due to overlapping with solvent peaks); EIMS m/z (rel. int): 318 $[M]^+$ (22), 221 (46), 203 (73), 97 (100); HREIMS m/z: 318.1836 $[M]^+$ (calcd. for $C_{19}H_{26}O_4$, m/z 318.1831).

4.4. Synthesis of CL analogues

4.4.1. Benzyl (E)-3-((4-methyl-5-oxo-2,5-dihydrofuran-2-yl)oxy) acrylate (**32**) and dibenzyl (2E,4E)-4-(((4-methyl-5-oxo-2,5-dihydrofuran-2-yl)oxy)methylene)pent-2-enedioate (**33**)

To a solution of formyl Meldrum's acid **38** (300 mg, 1.74 mmol) in toluene (3.5 ml) was added benzyl alcohol **39** (208 μl, 2.00 mmol) under Ar. After refluxing for 2 h under argon, the reaction mixture was cooled and concentrated *in vacuo*. The residue was dissolved in THF (2.0 ml). Then, K_2CO_3 (103 mg, 0.75 mmol) and 5-bromo-3-methylfuran-2(5*H*)-one **20** (113 μl, 1.15 mmol) was added at 0 °C under Ar, and the reaction mixture was stirred at the same temperature for 1 h. After stirring overnight at room temperature, the mixture was poured into 0.1 N HCl, and extracted with EtOAc. The organic phase was dried over anhydrous Na_2SO_4, and concentrated *in vacuo*. The residue was subjected to silica gel CC eluted stepwise with *n*-hexane and acetone (5% increments). The 15% acetone eluate was purified by a semi-preparative Inertsil SIL-100A HPLC column (φ10 × 250 mm, 5 μm, GL Scieneces), using isocratic elution with 2% EtOH in *n*-hexane at a flow rate of 3.8 ml min^{-1} and monitored at 236 nm to give benzyl AD analogue **32** (3.3 mg, 0.012 mmol, 0.7% in 2 steps, retention time 14.9 min) and dibenzyl AA'D analogue **33** (5.0 mg, 0.012 mmol, 0.7% in 2 steps, retention time 22.4 min). Benzyl (*E*)-3-((4-methyl-5-oxo-2,5-dihydrofuran-2-yl)oxy)acrylate **32**; ^{1}H-NMR ($CDCl_3$, 400 MHz): δ 2.00 (3H, t, J = 1.5 Hz, 4′-CH_3), 5.18 (2H, s, OCH_2), 5.62 (1H, d, J = 12.4 Hz, H-2), 6.10 (1H, t, J = 1.5 Hz, H-2′), 6.91 (1H, t, J = 1.5 Hz, H-3′), 7.31–7.41 (5H, m, C_6H_5), 7.60 (1H, d, J = 12.4 Hz, H-3); ^{13}C-NMR ($CDCl_3$, 100 MHz): δ 10.7, 66.1, 99.2, 102.3, 128.22, 128.24, 128.6, 135.4, 135.9, 141.0, 158.1, 166.4, 170.4; EIMS m/z (rel. int.): 274 $[M]^+$(0.1), 256 (4.0), 97 (100), 91 (47); HRESIMS m/z 297.0741 $[M+Na]^+$ (calcd for $C_{15}H_{14}O_5Na$, m/z 297.0739). Dibenzyl (2*E*,4*E*)-4-(((4-methyl-5-oxo-2,5-dihydrofuran-2-yl)oxy)methylene)pent-2-enedioate **33**; ^{1}H-NMR ($CDCl_3$, 400 MHz): δ 2.03 (3H, t, J = 1.5 Hz, 4′-CH_3), 5.20 (2H, s, OCH_2), 5.21 (1H, d, J = 12.4 Hz, OCH_2), 5.24 (1H, d, J = 12.4 Hz, OCH_2), 6.18 (1H, t, J = 1.5 Hz, H-2′), 6.72 (1H, d, J = 16.2 Hz, H-2), 6.97 (1H, t, J = 1.5 Hz, H-3′), 7.30–7.41 (10H, m, C_6H_5 x 2), 7.59 (1H, d, J = 16.2 Hz, H-3), 7.80 (1H, s, 4-CH); ^{13}C-NMR ($CDCl_3$, 100 MHz): δ 10.8, 66.3, 66.7, 100.6, 110.5, 121.7, 128.16, 128.20, 128.38, 128.44, 128.5, 128.7, 133.5, 135.6, 136.0, 136.1, 140.8, 157.6, 165.1, 167.3, 170.1; EIMS m/z (rel. int.): $[M]^+$ was not observed, 373 (4.6), 343 (2.2), 283 (2.8), 231 (5.2), 229 (3.8), 181 (7.2), 97 (43), 91 (100); HRESIMS m/z 457.1249 $[M+Na]^+$ (calcd for $C_{25}H_{22}O_7Na$, m/z 457.1263).

4.4.2. Cyclohexylmethyl (E)-3-((4-methyl-5-oxo-2,5-dihydrofuran-2-yl)oxy)acrylate (**34**) and bis(cyclohexylmethyl) (2E,4E)-4-(((4-methyl-5-oxo-2,5-dihydrofuran-2-yl)oxy)methylene)pent-2-enedioate (**35**)

To a solution of formyl Meldrum's acid **38** (215 mg, 1.25 mmol) in toluene (2.5 ml) was added cyclohexanemethanol **40** (184 μl, 1.50 mmol) under Ar. After refluxing for 2 h under Ar, the reaction mixture was cooled and concentrated *in vacuo*. The residue was dissolved in THF (2.0 ml). Then, K_2CO_3 (138 mg, 0.75 mmol) and 5-bromo-3-methylfuran-2(5*H*)-one **20** (150 μl, 1.53 mmol) was added at 0 °C under Ar, and stirred at the same temperature for 1 h. After stirring overnight at room temperature, the mixture was poured into 0.1 N HCl, and extracted with EtOAc. The organic phase was dried over anhydrous Na_2SO_4, and concentrated *in vacuo*. The residue was subjected to silica gel CC eluted stepwise with *n*-hexane and acetone (5% increments). The 15% acetone eluate was purified by a semi-preparative Inertsil SIL-100A HPLC column (φ10 × 250 mm, 5 μm, GL Scieneces), using isocratic elution with 2% EtOH in *n*-hexane at a flow rate of 2.8 ml min^{-1} and monitored at 236 nm to give cyclohexylmethyl AD analogue **34** (1.9 mg, 0.0068 mmol, 0.5% in 2 steps, retention time 23.8 min) and bis(-cyclohexylmethyl) AA'D analogue **35** (7.3 mg, 0.016 mmol, 1.3% in 2 steps, retention time 31.9 min). Cyclohexylmethyl (*E*)-3-((4-methyl-5-oxo-2,5-dihydrofuran-2-yl)oxy)acrylate **34**; ^{1}H-NMR ($CDCl_3$, 400 MHz): δ 0.91–1.05 (2H, m), 1.10–1.33 (4H, m), 1.58–1.80 (5H, m), 2.01 (3H, t, J = 1.5 Hz, 4′-CH_3), 3.94 (2H, d, J = 6.6 Hz, OCH_2), 5.58 (1H, d, J = 12.4 Hz, H-2), 6.11 (1H, t, J = 1.5 Hz, H-2′), 6.92 (1H, t, J = 1.5 Hz, H-3′), 7.56 (1H, d, J = 12.4 Hz, H-3); ^{13}C-NMR ($CDCl_3$, 100 MHz): δ 10.7, 25.6, 26.3, 29.7, 37.1, 69.5, 99.1, 102.6, 135.4, 141.1, 157.6, 166.7, 170.5; EIMS m/z (rel. int.): $[M]^+$ was not observed, 236 (0.4), 185 (5.5), 184 (2.8), 97 (100); HRESIMS m/z 303.1219 $[M+Na]^+$ (calcd for $C_{15}H_{20}O_5Na$, m/z 303.1208). Bis(cyclohexylmethyl) (2*E*,4*E*)-4-(((4-methyl-5-oxo-2,5-dihydrofuran-2-yl)oxy)methylene)pent-2-enedioate **35**; ^{1}H NMR ($CDCl_3$, 400 MHz): δ 0.93–1.07 (4H, m), 1.10–1.34 (6H, m), 1.58–1.80 (12H, m), 2.05 (3H, t, J = 1.5 Hz, 4′-CH_3), 3.97 (2H, d, J = 6.6 Hz,OCH_2), 4.01 (2H, d, J = 6.4 Hz, OCH_2), 6.22 (1H, t, J = 1.5 Hz, H-2′), 6.65 (1H, dd, J = 0.6, 16.2 Hz, H-2), 7.00 (1H, t, J = 1.5 Hz, H-3′), 7.53 (1H, d, J = 16.2 Hz, H-3), 7.76 (1H, d, J = 0.6 Hz, 4-CH); ^{13}C-NMR ($CDCl_3$, 100 MHz): δ 10.7, 25.6, 25.7, 26.3, 26.4, 29.65, 29.73, 37.1, 37.2, 69.6, 70.1, 100.5, 110.8, 122.0, 133.0, 135.9, 140.9, 156.9, 165.4, 167.6, 170.1; EIMS m/z (rel.

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int.): 446 $[M]^+$ (0.7), 349 (0.5), 253 (3.8), 237 (18), 141 (12), 97 (100); HRESIMS *m/z* 469.2212 (calcd for $C_{25}H_{34}O_7Na$, *m/z* 469.2202).

*4.4.3. (2,6,6-Trimethylcyclohex-1-en-1-yl)methyl (E)-3-((4-methyl-5-oxo-2,5-dihydrofuran-2-yl)oxy)acrylate (**36**) and bis((2,6,6-trimethylcyclohex-1-en-1-yl)methyl) (2E,4E)-4-(((4-methyl-5-oxo-2,5-dihydrofuran-2-yl)oxy)methylene)pent-2-enedioate (**37**)*

To a solution of formyl Meldrum's acid **38** (288 mg, 1.67 mmol) in toluene (10 ml) was added β-cyclogeraniol **41** (346 μl, 2.00 mmol) under Ar. After heating under refluxconditions for 2 h under Ar, the reaction mixture was cooled to 0 °C. Then, K_2CO_3 (276 mg, 2.00 mmol) and a solution of 5-bromo-3-methylfuran-2(5*H*)-one **20** (295 μl, 3.00 mmol) in toluene (2 ml) was added under Ar, and stirred at 0 °C for 1 h. After stirring at room temperature for 1 h, the mixture was poured into 0.1 N HCl, and extracted with Et_2O. The organic phase was dried over anhydrous Na_2SO_4, and concentrated *in vacuo*. The residue was subjected to silica gel CC eluted stepwise with *n*-hexane and EtOAc (5% increments). The 25% acetone eluate was purified by a semi-preparative Inertsil SIL-100A HPLC column (φ10 × 250 mm, 5 μm, GL Scieneces), using isocratic elution with 2% EtOH in *n*-hexane at a flow rate of 2.8 ml min^{-1} and monitored at 236 nm to give β-cyclogeranyl AD analogue **36** (1.1 mg, 0.0031 mmol, 0.2% in 2 steps, retention time 23.5 min) and bis(β-cyclogeranyl) AA'D analogue **37** (13.0 mg, 0.025 mmol, 1.5% in 2 steps, retention time 31.1 min). (2,6,6-Trimethylcyclohex-1-en-1-yl)methyl (*E*)-3-((4-methyl-5-oxo-2,5-dihydrofuran-2-yl)oxy)acrylate **36**; ^{1}H-NMR ($CDCl_3$, 400 MHz): δ 1.00 (6H, s, 6″-CH_3 x 2), 1.44–1.49 (2H, m, H-5″), 1.59–1.64 (2H, m, H-4″), 1.67 (3H, s, 2″-CH_3), 1.97–2.04 (2H, m, H-3″), 2.01 (3H, t, J = 1.5 Hz, 4′-CH_3), 4.65 (2H, s, OCH_2), 5.58 (1H, d, J = 12.3 Hz, H-2), 6.11 (1H, t, J = 1.2 Hz, H-2′), 6.91 (1H, t, J = 1.6 Hz, H-3′), 7.54 (1H, d, J = 12.3 Hz, H-3); ^{13}C-NMR could not be measured due to the scarcity of the compound; EIMS *m/z* (rel. int.): 320 $[M]^+$(0.8), 302 (0.9), 250 (0.6), 223 (0.5), 206 (11), 153 (12), 136 (100), 121 (80), 97 (68); HRESIMS *m/z* 343.1536 $[M+Na]^+$ (calcd for $C_{18}H_{24}O_5Na$, *m/z* 343.1521). Bis((2,6,6-trimethylcyclohex-1-en-1-yl)methyl) (2*E*,4*E*)-4-(((4-methyl-5-oxo-2,5-dihydrofuran-2-yl)oxy)methylene)pent-2-enedioate **37**; ^{1}H NMR ($CDCl_3$, 400 MHz): δ 1.00 (12H, s, 6″-CH_3 x 2, 6‴-CH_3 x 2), 1.42–1.49 (4H, m, H-5″ and H-5‴), 1.56–1.64 (4H, m, H-4″ and H-4‴), 1.96–2.04 (4H, m, H-3″ and H-3‴), 2.04 (3H, br.t, 4′-CH_3), 4.68 (2H, s, OCH_2), 4.71 (2H, s, OCH_2), 6.20 (1H, br.t, H-2′), 6.66 (1H, d, J = 16.3 Hz, H-2), 6.98 (1H, t, J = 1.2 Hz, H-3′), 7.51 (1H, d, J = 16.3 Hz, H-3), 7.69 (1H, s, 4-CH); ^{13}C-NMR ($CDCl_3$, 100 MHz): δ 10.7, 19.2, 19.80, 19.84, 28.3, 32.88, 32.90, 33.99, 34.01, 39.2, 60.9, 61.4, 100.4, 111.0, 122.1, 131.8, 132.2, 132.9, 135.9, 136.1, 136.68, 136.74, 141.0, 156.7, 165.6, 167.9, 170.2; EIMS *m/z* (rel. int.): $[M]^+$ was not observed, 389 (0.9), 229 (9.2), 136 (100), 121 (40), 97 (47); HRESIMS *m/z* 549.2811 (calcd for $C_{31}H_{42}O_7Na$, *m/z* 549.2828).

4.5. Hyphal branching assay

Hyphal branching activity on a germinating spores of *Gigaspora marigarita* Becker & Hall (MAFF 520054) was conducted as reported previously (Akiyama et al., 2010). The purity of each tested compound was checked either by analytical silica gel TLC (*n*-hexane-EtOAc/acetone) or normal (*n*-hexane-EtOH)-/reversed (CH_3CN–H_2O)-phase HPLC before assay.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.phytochem.2016.05.012.

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