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Synthesis, structural characterization and biological activity of two diastereomeric JA-Ile macrolactones

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Abstract: Jasmonates are phytohormones involved in a wide range of plant processes including growth, development, senescence, and defense. Jasmonoyl-L-isoleucine (JA-Ile, **2**), an amino acid conjugate of jasmonic acid (JA, **1**), has been identified as the bioactive endogenous jasmonate. However, JA-Ile (**2**) analogues trigger different responses in the plant. ω-Hydroxylation of the pentenyl side chain leads to the inactive 12-OH-JA-Ile (**3**) acting as a "stop" signal. On the other hand, a lactone derivative of 12-OH-JA (**5**) (jasmine ketolactone , JKL), occurs in nature, although with no known biological function. Inspired by the chemical structure of JKL (**6**), and in order to further explore the potential biological activities of 12-modified JA-Ile derivatives, we synthesized two macrolactones (JA-Ile-lactones (**4a**) and (**4b**)) derived from 12-OH-JA-Ile (**3**). The biological activity of (**4a**) and (**4b**) was tested for their ability to elicit nicotine production, a well-known jasmonate dependent secondary metabolite. Both macrolactones showed strong biological activity, inducing nicotine accumulation to a similar extent as methyl jasmonate does in *Nicotiana attenuata* leaves. Surprisingly, the highest nicotine contents were found in plants treated with the JA-Ile-lactone (**4b**) which at the cyclopentanone ring has the 3*S* configuration, not present in natural jasmonates. Macrolactone (**4a**) is a valuable standard to explore for its occurrence in nature.

Introduction

Jasmonates (JAs) are a large family of lipid-derived plant metabolites that mediate responses to stress and regulate development.[1,2] These compounds owe their name to the initial isolation and characterization of methyl jasmonate (MeJA, **7**) from jasmine oil from *Jasminum grandiflorum* in the early 60s.[3] Since then, many JAs have been detected and isolated from many different plant species.[4] JAs were first studied because of their properties as odorants, greatly appreciated in perfumery,[5] and later -- and more important -- because of their role as phytohormones.[6-11] JAs occur throughout the plant kingdom (algae, mosses, gymnosperms and angiosperms) and also in fungi. The capacity to produce or transform JAs is extraordinarily high in fungi.[12] JA (**1**) is one of the key players of the JAs family. It is biosynthesized by consecutive enzymatic reactions starting from linolenic acid (Fig. 1).[13] JA (**1**) is produced as the *cis* isomer (with respect to the cyclopentanone ring) (3*R*,7*S*)-JA, but it can readily epimerize at C7 to the more stable *trans* form (3*R*,7*R)*-JA. Both isomers exist in equilibrium in the cell and have different biological activities; generally, the *cis* isomer is more active although it is often found in lower amounts.[14-18] Several enzymes can act on JA (**1**) and transform it into numerous derivatives (Fig. 1). One of these metabolites is 12-OH-JA (**5**) which has been described as a potent tuber-inducing agent.[19,20] The hydroxy acid (**5**) is believed to be the natural precursor of JKL (**6**), a naturally occurring 10-membered ring macrolactone. JKL (**6**) was the first jasmonate reported in literature, interestingly only in its *trans* form and with no biological activity known to date.[21-25]

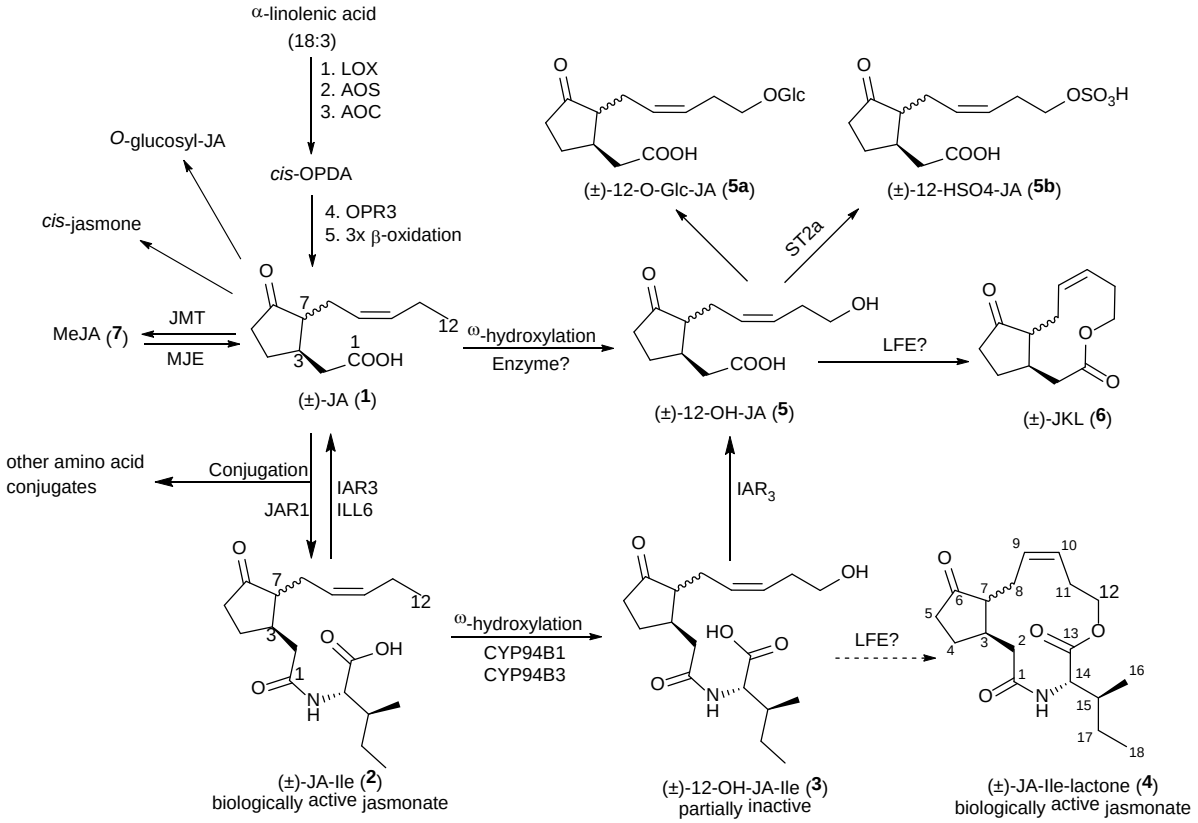

Fig. 1 Simplified scheme of the biosynthesis of jasmonates. JA (**1**) biosynthesis starts with the release of linolenic acid in the plastid. The succession of LOX, AOS, and AOC catalyzes the formation of OPDA, which is transformed by the action of OPR and three rounds of *β*-oxidations into JA (**1**). Hydroxylation of (**1**) produces 12-OH-JA (**5**), which is the precursor of (**5a**), (**5b**) and possibly, JKL (**6**). The activation of JA (**1**) is catalyzed by JAR1 that conjugates it to L-Ile and generates JA-Ile (**2**). Turnover of (**2**) is carried out by members of the CYP94 family producing the hydroxy acid (**3**), a likely precursor of JA-Ile-lactone (**4**). The compounds are shown in the (3,7)-*cis/trans* stereochemistry occurring *in planta*. LOX, 13-lipoxygenase; AOS, 13-allene oxide synthase; AOC, allene oxide cyclase; OPR, 12-oxophytodienoate reductase 3; OPDA, *cis*-(+)-12-oxo-phytodienoic acid; JMT, JA carboxyl methyltransferase; JME, JA methylesterase; JAR1, JA-amino synthetase; ILL6, IAR3: JA-Ile amidohydrolases; CYP94B1/B3, cytochromes P450; LFE, lactone forming enzyme. The dashed arrow indicates a proposed transformation. Nomenclature and numbering commonly used for jasmonates was employed for clarity.

JA-Ile (**2**), an amino acid conjugate of JA (**1**), is the bioactive endogenous jasmonate. This was postulated in 1995 by Krumm et al.[26] and later confirmed by the discovery that in *A. thaliana* JAR1 activates JA by conjugation with L-isoleucine.[18,27] Accumulation of JA-Ile (**2**)

is observed in different plant tissues in response to environmental stresses,[27] but when the activating signal is no longer needed, JA-Ile (**2**) is converted to 12-OH-JA-Ile (**3**) by hydroxylation at C12.[28] The structure of (**3**) possesses a free carboxylic acid group and a hydroxyl group in analogy to the molecule of 12-OH-JA (**5**). Since JKL (**6**) exists in nature, also a macrolactone like JA-Ile-lactone (**4**), derived from 12-OH-JA-Ile (**3**) may exist as well (Fig. 1). Moreover, JA-Ile (**2**) analogues have shown different biological activities. For instance, the phytotoxin coronatine (Fig. S1, Supplementary material), a structural mimic of JA-Ile (**2**), is considerably more active than (**2**) in promoting the interaction of the COI1 (coronatine-insensitive 1) receptor with the JAZ (jasmonate ZIM domain) repressors in vitro.[29] The stronger activity of coronatine compared with JA-Ile (**2**) can be explained by two reasons, i) the larger surface area provided by the cyclohexene ring than the corresponding area of the pentenyl side chain of JA-Ile (**2**) to interact with the COI1 receptor,[30] and ii) the high stability of the *cis*-hydrindanone moiety of coronatine.[31] The methyl oxime derivatives of JA-Ile (**2**) and coronatine (Fig. S1, Supplementary material) act as JA-perception antagonists by binding to the COI1 receptor and hindering the interaction with the JAZ repressors due to the oxime group.[32] As the idea of tailoring jasmonate analogues for specific applications has been discussed[33], these findings suggest a means of manipulating the JA-signaling pathway by chemically modifying the ligand (JA-Ile, **2**).

We hypothesized that a lactone such as the JA-Ile-lactone (**4**) analogous to JKL (**6**) may exist in nature. Furthermore, such a lactone retains the important moieties required for jasmonate perception and therefore may be biologically active.[16] Herein, we present a brief and efficient synthesis to JA-Ile-lactone (**4**). The biological activity of this new synthetic jasmonate was evaluated together with its diastereoisomer (**4b**). Since JKL (**6**) has been only described in the *trans* form, we prepared the (3*R*,7*R*)-isomer of the lactone (**4a**).

Results and discussion

Synthesis of the JA-Ile-lactone (4)

Our synthetic approach is based on two previously reported studies of jasmonates.[34,35] This route allows not only the synthesis of JA-Ile-lactone (**4**), but also the preparation of other 12-modified jasmonates which are of great biological interest (e.g., compounds (**5**), (**5a**), (**5b**) and (**3**)).[36] Furthermore, this synthetic route provides the opportunity to prepare enantiomerically pure amino acid conjugates of JA (**1**) starting from a racemic mixture of commercially available MeJA (**7**).[35] Although most of the bioassays were carried out with commercially available or synthetic JAs consisting of mixtures of isomers,inhibition by the non-natural isomers has not been reported to date.[33,37-40]

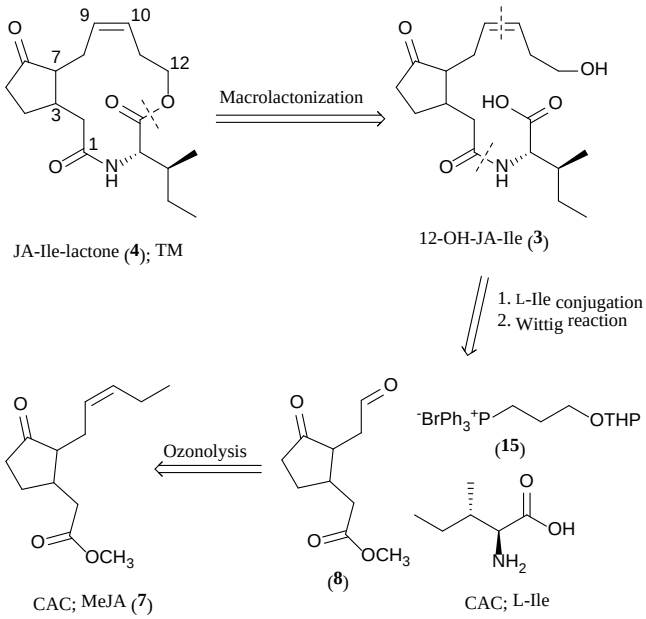

Fig. 2 Retrosynthetic analysis of JA-Ile-lactone (**4**). TM, target molecule; CAC, commercially available compound.

The synthesis of JA-Ile-lactone (**4**) starts from MeJA (**7**) as depicted in the retrosynthetic analysis (Fig. 2). Key steps are the Wittig reaction to generate the *cis* olefin (**9**) (Fig. 3, step b) and the final macrolactonization to JA-Ile-lactone (**4**). Ozonolysis of (**7**) as

previously reported (CH_2Cl_2/MeOH, 1:1),[34] produced a mixture of the aldehyde (**8**) and the dimethyl acetal. To avoid acetal formation, the reaction was conducted in CH_2Cl_2, and the aldehyde (**8**) was obtained in 94% yield after flash chromatography. Regioselective Wittig reaction of the aldehyde (**8**) with the Wittig reagent (**15**)[†] directly afforded the keto ester (**9**). The conditions employed in the reaction produced the *cis* isomer of (**9**) with high selectivity (> 95%, estimated by ^{1}H and ^{13}C NMR, SI) and yield (70%). This is a reliable procedure to obtain 12-modified jasmonates enriched with the *Z*-isomer. Long synthetic routes to these important metabolites are no longer needed.[41] Saponification of (**9**) afforded the free acid (**10**) quantitatively, which was employed in the next step without purification. Conjugation of L-Ile to (**10**) was carried out according to the procedure described for the linolenic acid/L-Ile conjugate.[42] This procedure was more straightforward than the procedure reported by Kramell *et al.*[35] The amino acid conjugate (**11**) (86% crude yield) was directly deprotected with *p*-TsOH in EtOH to afford the seco-acid 12-OH-JA-Ile (**3**) in 90% yield.

Macrolactonization to JA-Ile-lactone (**4**) was the most challenging step of the synthesis. Classical Yamaguchi-Yonemitsu conditions (Et_3N, $Cl_3C_6H_2COCl$, PhMe or PhH, DMAP) did not work at all, but we obtained excellent yields of the macrolactone (**4**) employing the two-step sequential reaction described by Ohba *et al.*[43] Using the ethoxyvinyl-ester (EVE) method to activate the acid group of (**3**), the macrolactone (**4**) was obtained (64% total yield) after flash chromatography. The JA-Ile-lactone (**4**) (mixture of isomers) was chromatographed on silica gel with AcOEt/*n*-hexane (7:3) and afforded two major products, JA-Ile-lactone (**4a**) (11.6 mg, 46%, TLC R_f = 0.26) and JA-Ile-lactone (**4b**) (13.7 mg, 54%, TLC R_f = 0.19). Finally, recrystallization from AcOEt/*n*-hexane and from EtOH/Acetone afforded diastereomerically pure JA-Ile-lactones (**4a**) and (**4b**) respectively, as determined by NMR (SI).

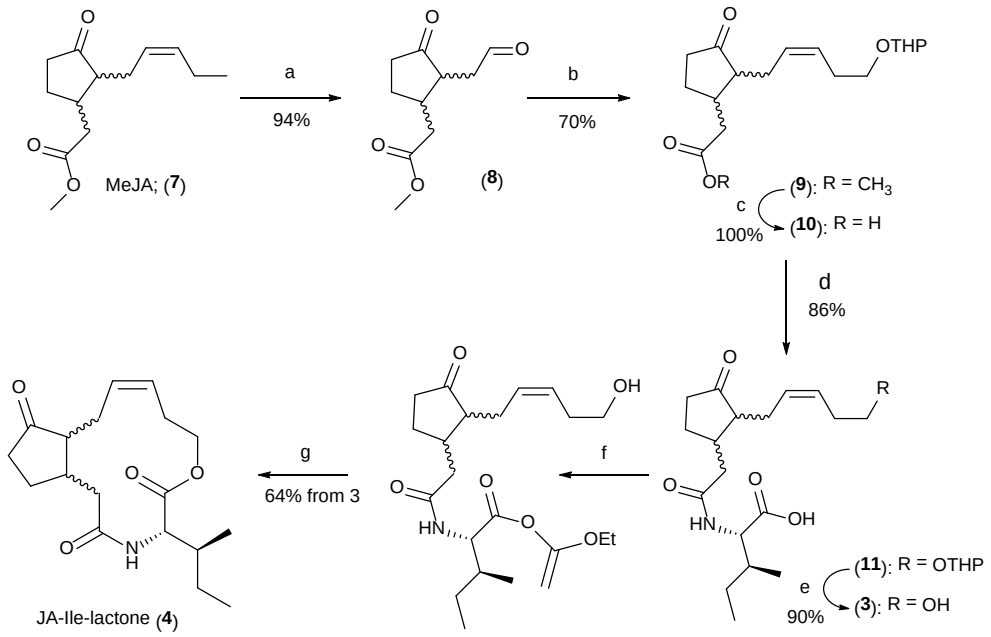

Fig. 3 Synthesis of JA-Ile-lactone (**4**). Reagents and conditions: (a) O_3, CH_2Cl_2, -78 °C, then $(Me)_2S$; (b) **15** (~ 0.2 M), KHMDS (1.2 equiv.), THF, -78 °C, then **8**, -78 °C to room temperature; (c) NaOH (~ 0.3 M), MeOH, 70 °C; (d) ethyl chloroformate ($ClCOOC_2H_5$, 1.1 equiv.), TEA, THF, L-Ile (2 equiv.), 0 °C; (e) pyridinium *p*-toluenesulfonate (10 mol%), EtOH, 55 °C; (f) ethoxyacetylene (4 equiv.), $[Ru(p\text{-cymene})Cl_2]_2$, acetone, 0 °C; (g) *p*-TsOH (0.05 M), 1,2-dichloroethane, high dilution, 50 °C.

Crystallography and structural characterization

Lactones (**4a**) and (**4b**) crystallize as orthorhombic colorless prisms. Both are packed in chain-like structures with the proton of the N─H group hydrogen bonding with the keto-amide group of the adjacent molecule (Fig. S2, SI). This orientation differs from the packing observed for indanoyl-isoleucine derivatives, which crystallize as dimers (sandwich-like) with two hydrogen bonds involving the keto group of the cyclopentanone ring and the N─H protons.[44] The absolute configuration of JA-Ile-lactone (**4a**) was assigned by reference to a chiral center of the L-Ile moiety (C14, Fig. 4). The absolute configuration of JA-Ile-lactone (**4b**) was additionally confirmed by anomalous-dispersion effects in diffraction measurements on the crystal using the intensity quotients method.[45]

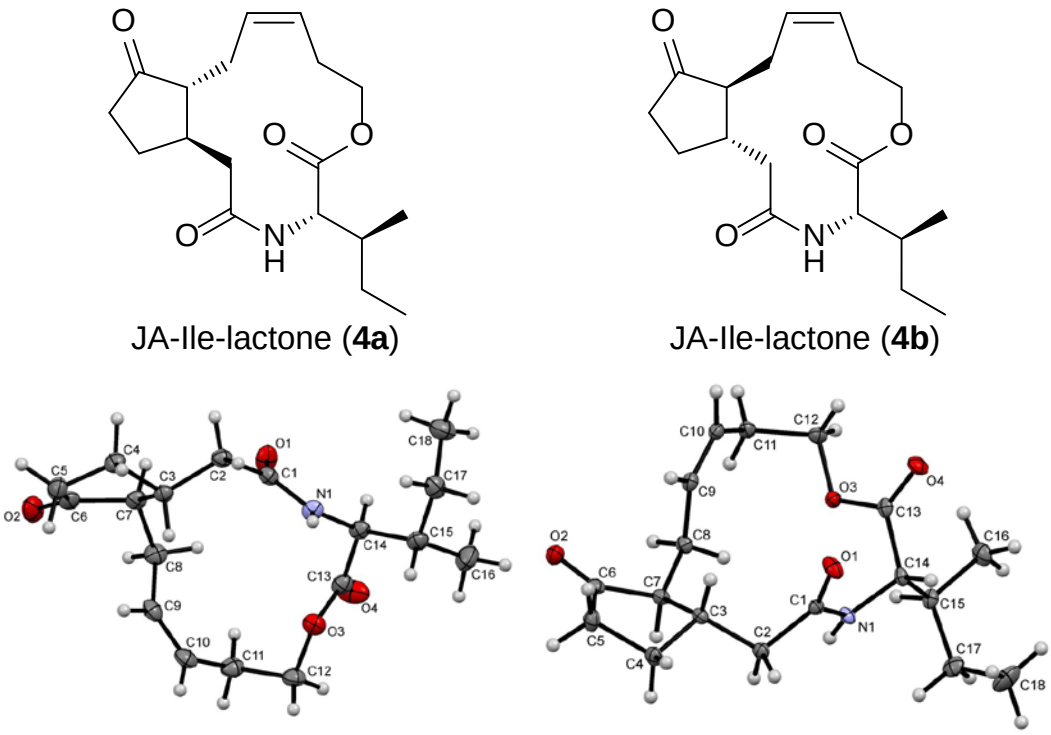

Fig. 4 Absolute configuration and crystal structures of lactones **(4a)** (3*R*,7*R*)-naturally occurring configuration, and **(4b)** (3*S*,7*S*)- not present in nature.

JA-Ile-lactones (4a) and (4b) induce nicotine biosynthesis

Naturally occurring and synthetic JAs have diverse biological backgrounds and activities.[6,7,46-48] These facts have made JAs the target of several synthetic studies that examined the relationship between the molecular structure and their activity.[7,16,49] Inspired in the structure of JKL (**6**) and other JA-Ile analogues, like coronatine, we designed and synthesized JA-Ile-lactones (**4a**) and (**4b**). The structures of these lactones possess all moieties known to be necessary for bioactivity of JAs (e.g., pentenyl side chain on C7, cyclopentanone ring, L-Ile moiety).[30] In addition, the macrocycle may confer certain rigidity to the structure, in analogy to the cyclohexene ring to the molecule of coronatine or the aromatic ring in coronalon (Fig. S1, SI).

Nicotine is a typical direct defense stimulated by JAs in tobacco plants.[50,51] When MeJA (**7**) is applied to the leaves is rapidly converted into bioactive JAs that induce the

accumulation of nicotine in *N. attenuata* plants and was therefore, employed as positive control in our experiments.[52,53] To test the potential biological activity of the JA-Ile-lactones (**4a**) and (**4b**), we determined their ability to induce nicotine production in *N. attenuata* plants.

Both lactones induced nicotine accumulation in *N. attenuata* leaves similarly to MeJA (**7**) (Fig. 5). Strikingly, the JA-Ile-lactone (**4b**) induced the highest nicotine content although this molecule has a non-natural configuration at C3. To our knowledge, it is the first time that a jasmonate having the (3*S*,7*S*) configuration (not present *in planta*) is reported to strongly induce a secondary metabolite.

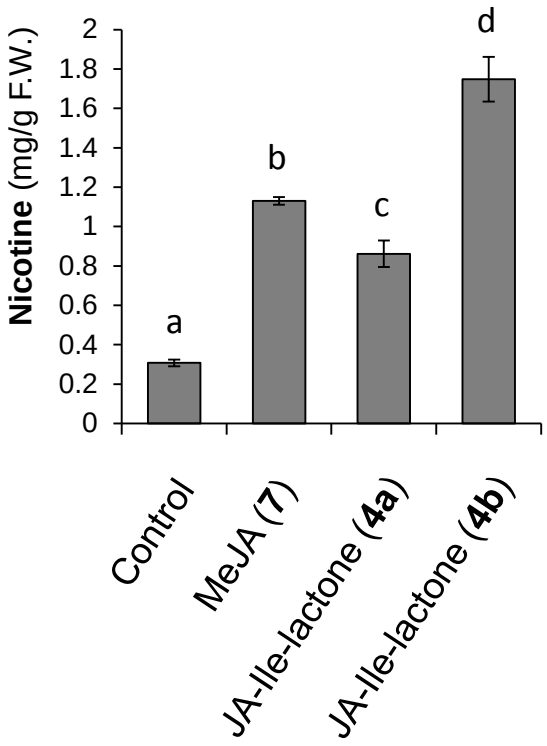

Fig. 5 Nicotine pools in leaves of *N. attenuata* plants after treatment (mean, ± SEM) in milligrams per gram of fresh weight (mg/g F.W.). Control plants where treated with lanolin paste. (n=6) $P \leq 0.001$

The precursor of the JA-Ile-lactones, the hydroxy acid 12-OH-JA-Ile (**3**), is a well-known jasmonate that acts as stop signal in JA-signaling.[8,28,54] However, the lactones (**4a**)

and (**4b**) derived from (**3**) are strongly active at inducing nicotine production. These results suggest that, similarly to JA-Ile (**2**), the JA-Ile-lactones may activate nicotine production via a COI1-dependent mechanism. Further studies are needed to test this hypothesis.

Conclusions

We have developed a short (7-steps) and efficient synthesis (33% overall yield) to JA-Ile-lactones (**4**) from commercially available MeJA (**7**). A mixture of the synthesized JA-Ile-lactone (**4**) can be chromatographically resolved into the diastereomerically pure lactones (**4a**) and (**4b**). Furthermore, enantiomerically enriched C12-modified JA and JA-Ile derivatives (e.g., **5a** and **5b**) can be prepared following our procedure. Both lactones are potent inducers of nicotine accumulation in the leaves of *N. attenuata* plants. The presence of such compounds in nature can now be explored with the synthetic JA-Ile-lactone (**4a**) as a reference. Furthermore, the rigid structure of these lactones makes them valuable molecules (templates) to study their interaction with the jasmonate receptor complex COI1/JAZ.[30,47] Understanding the mechanism of action of these new synthetic jasmonates will shed light on the JA-signaling pathway and therefore on plant-insect herbivore interactions.

Experimental section

General material and methods

All chemicals were obtained from commercial suppliers. If necessary, solvents were purified prior to use.[55] All work-up and purification procedures were carried out with reagent grade solvents. Thin layer chromatography was performed on silica gel 60 F^{254} on aluminum plates (Merck) and visualized with potassium permanganate staining or phosphomolybdic acid in ethanol. Melting points of the lactones were measured in capillary tubes on a Büchi B-540 instrument and are uncorrected. Flash chromatography was performed on silica gel 60 (40-63

μm) from Merck. Proportions of the employed solvents are referred to volume (v/v) otherwise mentioned.

GC-MS spectra were recorded on a ThermoQuest CE Instruments GC 2000 Series coupled to a ThermoQuest Finnigan Trace MS mass spectrometer; GC column HP-5MS capillary column (15 m × 0.25 mm ID with 0.25 μm film thickness, Phenomenex). Injection port: 250 °C; flow, 15 mL min^{-1} with split ratio of 10 mL min^{-1}; temperature program: 60 °C (2 min) at 15 °C min^{-1} to 280 °C (5 min). Helium at 1.5 mL min^{-1} served as carrier gas. The ionization method was electron impact (70 eV) in positive mode (EI^+). HRMS (ESI^+) was performed on a Bruker Daltonics - maXis Ultra High ResolutionTOF equipment.

NMR spectra were recorded at 300K either on a Bruker DRX500 spectrometer (operating frequency 500 MHz for ^1H and 125 MHz for ^{13}C) or a Bruker Avance 400 NMR spectrometer (operating frequency 400 MHz for ^1H and 100 MHz for ^{13}C). ^1H NMR chemical shifts were referenced to TMS. Ozone was generated with a Sander Labor-Ozonisator, oxygen flow at 120 L hr^{-1}, 0.44 A, 90% power at 20 °C.

Synthetic procedures

Synthesis of methyl 2-(3-oxo-2-(2-oxoethyl)cyclopentyl)-acetate (8): Ozone was bubbled into a solution of methyl jasmonate (MeJA, 3.08 g, 13.7 mmol) in CH_2Cl_2 (150 mL) at -78 °C until the blue color indicated an excess of ozone. A current of nitrogen was passed through the solution to eliminate the excess of ozone, $(Me)_2S$ (5 mL, 68.2 mmol, ca. 5 equiv.) was added and the mixture was stirred overnight and allowed to come to room temperature (RT). The mixture was then concentrated under reduced pressure and chromatographed on silica gel (*n*-hexane/AcOEt, 2:1) to afford (**8**) (2.56 g, 94%). Spectroscopic data were consistent with the literature.[56]

Synthesis of triphenyl(3-((tetrahydro-2H-pyran-2-yl)oxy)-propyl)phosphonium bromide (15): To a solution of 3-bromopropan-1-ol (5.01 g, 36 mmol) and 3,4-dihydro-2H-pyran (12.00 g, 142 mmol) in 140 mL of CH_2Cl_2 at 20 °C was added *p*-TsOH (0.05 g, 0.26 mmol) and the mixture was stirred at RT for 2 h. The solution was then diluted with 200 mL of Et_2O and washed successively with solutions of saturated $NaHCO_3$ (200 mL), water (200 mL) and brine (200 mL). The aqueous phase was worked-up with fresh Et_2O and the organic phases combined and dried over Na_2SO_4. The mixture was concentrated under reduced pressure and chromatographed on silica gel (*n*-hexane/AcOEt, 9:1) to afford the protected alcohol (6.01 g, 75.0%, TLC R_f = 0.26) as a colorless oil. GC-MS (EI^+): *m/z*(%): 41(79), 56(40), 85(100), 120(15), 221(50), 223(53) [M^+]. The alkyl bromide (3.04 g, 13.44 mmol) was dissolved in 15 mL of acetonitrile and triphenylphosphine (4.23 g, 16.13 mmol), and K_2CO_3 (1.01 g, 7.24 mmol) were added and the mixture refluxed overnight (ca. 14 h). The precipitate was filtered off and the filtrate poured into 150 mL of Et_2O to obtain the solid Wittig salt. After filtration the solid was washed with 100 mL of fresh Et_2O. The product (**15**) (5.81 g, 89%) was dried under vacuum and kept in a desiccator over $CaCl_2$ until used. ^{1}H NMR (DMSO-d_6, 400MHz): δ = 7.63-8.12 (m, 15H), 4.53 (br. s., 1H), 3.56-3.78 (m, 4H), 3.49 (dt, *J*=9.9, 6.3 Hz, 1H), 3.43-3.80 (m, 6H), 1.65-1.87 (m, 3H), 1.54-1.64 (m, 1H), 1.34-1.52 ppm (m, 4H) ; ^{13}C NMR (DMSO-d_6, 100 MHz): δ = 135.9, 135.8, 134.2, 134.1, 130.9, 130.8, 119.0, 118.1, 100.5, 64.2, 30.9, 25.3, 20.0 ppm

Synthesis of methyl (Z)-2-(3-oxo-2-(5-((tetrahydro-2H-pyran-2-yl)oxy)pent-2-en-1-yl)cyclopentyl)acetate (9): A 100 mL flask equipped with rubber septum was charged with (**15**) (5.23 g, 10.78 mmol, 1.1 equiv.) and flushed with dry argon. Potassium bis(trimethylsilyl)amide (KHMDS, 17 mL, 0.7 M in toluene, 11.86 mmol, 1.2 equiv.) and THF (35 mL) in that order were added to reach a final concentration of the ylide of approximately 0.2 M. The mixture was stirred at room temperature for 15 min and then

cooled to -78 °C. The aldehyde (**8**) (1.94 g, 9.8 mmol, 1 eq.) in dry THF (15 mL) was added dropwise via syringe and the reaction stirred at -78 °C for an additional hour. The mixture was then filtered through a fritted funnel and *n*-hexane (100 mL) was added, the precipitate was filtered off, and the residual solids were combined and washed twice with *n*-hexane (50 mL portions). The filtrates were combined and concentrated under reduced pressure. Purification by flash chromatography (AcOEt/*n*-hexane, 1:2) provided (**9**) (2.21 g, 70%) as a colorless oil. ^{1}H NMR ($CDCl_3$, 500MHz) mixture of diastereoisomers: δ = 5.44-5.55 (m, 1H), 5.36-5.44 (m, 1H), 4.58 (m, 1H), 3.85 (m, 1H), 3.64-3.77 (m, 1H), 3.69 (s, 3H), 3.46-3.53 (m, 1H), 3.40 (dtd, *J*=9.5, 6.8(x2), 2.5 Hz, 1H), 2.64-2.74 (m, 1H), 2.18-2.43 (m, 7H), 2.11 (ddd, *J*=18.7, 11, 9 Hz 1H), 1.87-1.95 (m, 1H), 1.76-1.86 (m, 1H), 1.63-1.74 (m, 1H), 1.44-1.61 ppm (m, 6H); ^{13}C NMR ($CDCl_3$, 125MHz): δ = 218.9, 172.6, 128.5, 127.8, 99.0, 67.0, 62.5, 54.1, 51.8, 38.9, 38.2, 37.8, 30.8, 28.2, 27.3, 25.9, 25.6, 19.8 ppm

Synthesis of (2-(2-((Z)-5-hydroxypent-2-en-1-yl)-3-oxocyclopentyl)acetyl)-L-isoleucine, 12-OH-JA-Ile (3): Compound (**9**) (0.64 g, 1.73 mmol) was dissolved in 5 mL of MeOH and 12 mL of NaOH (0.3 M) were added. The reaction was heated at 70 °C for 1 h, cooled and diluted with 25 mL of water. A solution of HCl (1 M) was employed to adjust the pH to 3-4. The aqueous phase was then extracted three times with AcOEt (25 mL). The combined organic extracts were washed with brine and dried over anhydrous $MgSO_4$. Evaporation of the solvent under reduced pressure afforded (**10**) (0.45 g, 100%), which was used in the next step without further purification.

Crude (**10**) (0.16 g, 0.50 mmol) and TEA (0.06 g, 0.54 mmol, 1.1 equiv.) were dissolved in THF (4 mL) and ethyl chloroformate (0.06 g, 0.55 mmol, 1.1 eq.) was added under stirring at 0 °C. After 5-10 min, L-Ile (0.13 g, 1.00 mmol, 2 eq.) dissolved in NaOH (4 mL, 0.3 M) was added and stirring was continued for 30 min at RT. The reaction mixture was then acidified with HCl (1 M) to pH around 3-4 and extracted with AcOEt. The combined organic extracts

were dried over $MgSO_4$, and removal of solvents afforded (**11**) (0.18 g, 86 %). Crude (**11**) (0.10 g, 0.23 mmol) and pyridinium *p*-toluenesulfonate (6 mg, 0.02 mmol) were dissolved in EtOH (2 mL) and stirred for 2 h at 55 °C. Next, 20 mL of water were added and the reaction extracted three times with AcOEt (20 mL), the combined organic extracts were washed with brine and dried over $MgSO_4$. After evaporation of the solvent and flash chromatography (AcOEt/2-propanol/AcOH, 32:2:1) compound **3** (70 mg, 90%) was obtained as a thick pale yellow oil. HRMS (ESI-TOF): *m/z* = 338.1974 [M-H] (calc. for $C_{18}H_{28}NO_5$, 338.1968); ^{1}H NMR ($CDCl_3$, 500MHz): δ = 6.73 (d, *J*=8.3 Hz, 1H), 6.49 (br. s., 1H), 5.44 (m, 2H), 4.57 (dd, *J*=8.3, 5.0 Hz, 1H), 3.68 (m, 2H), 2.70 (m, 1H), 2.25-2.44 (m, 6H), 2.20 (m, 2H), 2.11 (m, 1H), 1.92 (m, 2H), 1.48 (m, 2H), 1.20 (m, 1H), 0.95 (d, 6 Hz, 3H), 0.92 ppm (t, 7.3 Hz, 3H); ^{13}C NMR ($CDCl_3$, 125MHz): δ = 220.0, 174.9, 172.7, 128.9, 128.6, 62.1, 56.7, 54.2, 41.5, 38.9, 38.1, 37.6, 30.8, 27.4, 25.7, 25.3, 15.7, 11.7 ppm

Synthesis of (4S,Z)-4-((S)-sec-butyl)-3,4,8,11,11a,13,14,14a-octahydro-1H-cyclopenta[g][1]-oxa[4]azacyclotridecine-2,5,12(7H)-trione, JA-Ile-lactone (4): *Activation of the carboxyl group:* Ethoxyacetylene (48 µL, 0.718 g mL^{-1} in hexanes, 0.49 mmol, 4 equiv.) and dichloro(*p*-cymene)ruthenium(II) dimer (1 mg, 0.002 mmol) were dissolved in dry acetone (3 mL) under an atmosphere of argon at 0 °C. 12-OH-JA-Ile (**3**) (42 mg, 0.12 mmol) dissolved in acetone (3 mL) wad added slowly and the mixture was stirred for 1 h at RT. The reaction was then filtered through a short pad of silica and eluted with AcOEt. Evaporation of the solvent afforded the desired EVE derivative. *Macrolactonization: p*-TsOH (230 µL, 0.05 M in EtOH) was diluted in 1,2-dichloroethane (DCE, 20 mL), the solution was warmed up to 50 °C and the previously obtained EVE (in 5 mL of DCE) was injected dropwise for 2 h. The mixture was stirred for another 6 h and worked up. Flash chromatography (AcOEt/*n*-hexane, 7:3) afforded JA-Ile-lactone (**4a**) (11.6 mg) and JA-Ile-

lactone (**4b**) (13.7 mg). The total yield was of 64% (25.3 mg). Recrystallization was carried out as described above in results and discussion.

*JA-Ile-lactone (**4a**):* Silica gel TLC R_f = 0.26; m.p. (from AcOEt/*n*-hexane, uncorrected) 188-189 °C; HRMS (ESI^+-TOF): *m/z* = 344.1846 $[M+Na]^+$ (calc. for $C_{18}H_{27}NNaO_4$, 344.1832); ^1H NMR ($CDCl_3$, 500MHz): δ = 5.71 (d, *J*=8.8 Hz, 1H), 5.43 (m, 1H), 5.18 (m, 1H), 4.57 (ddd, *J*=10.8, 4.3, 3.4 Hz, 1H), 4.49 (dd, *J*=8.9, 6.7 Hz, 1H), 3.90 (td, *J*=11.0, 1.7 Hz, 1H), 2.63 (dd, *J*=12.1, 2.2 Hz, 1H), 2.58-2.38 (m, 4H), 2.36-2.04 (m, 6H), 1.91 (m, 1H), 1.66 (m, 1H), 1.47 (ddq, *J*=13.4, 7.5(x4) Hz, 1H), 1.16 (m, 1H), 0.94 (d, *J*=7.0 Hz, 3H), 0.92 ppm (t, *J*=7.3 Hz, 3H); ^{13}C NMR ($CDCl_3$, 125MHz): δ = 220.0, 170.9, 170.6, 128.9, 128.4, 63.3, 57.3, 53.4, 43.0, 38.3, 37.6, 36.5, 28.6, 27.8, 25.7, 25.1, 15.7, 11.3 ppm

*JA-Ile-lactone (**4b**):* Silica gel TLC R_f = 0.19; m.p. (from EtOH/acetone, uncorrected) 186-187 °C; HRMS (ESI^+-TOF): *m/z* = 344.1838 $[M+Na]^+$ (calc. for $C_{18}H_{27}NNaO_4$, 344.1832); ^1H NMR ($CDCl_3$, 500MHz): δ = 5.62 (d, *J*=8.1 Hz, 1H), 5.45 (m, 2H), 4.41 (m, 2H), 3.95 (ddd, *J*=10.8, 6.4, 2.8 Hz, 1H), 2.60 (m, 2H), 2.48 (m, 1H), 2.37 (m, 3H), 2.25 (m, 3H), 2.06 (dd, *J*=14.2, 10.3 Hz, 1H), 1.96 (m, 2H), 1.60 (m, 1H), 1.46 (m, 1H), 1.19 (m, 1H), 0.96 (d, *J*=6.7 Hz, 3H), 0.93 ppm (t, *J*=7.4 Hz, 3H); ^{13}C NMR ($CDCl_3$, 125MHz): δ = 219.6, 171.4, 171.0, 129.3, 127.8, 64.2, 57.6, 55.9, 42.7, 37.8, 37.2, 36.1, 28.1, 27.1, 25.2, 25.2, 16.0, 11.5 ppm

Crystal structure determination

The intensity data for the compounds were collected on a Nonius KappaCCD diffractometer using graphite-monochromated Mo-K_α radiation. Data were corrected for Lorentz and polarization effects but not for absorption effects.[57,58] The structures were solved by direct methods (SHELXS[59]) and refined by full-matrix least squares techniques against Fo^2 (SHELXL-97).[59] All hydrogen atoms were located by difference Fourier synthesis and

refined isotropically. All non-hydrogen atoms were refined anisotropically. Mercury 3.5.1 (Cambridge Crystallographic Data Centre, Build RC5) software was used for structure representations.

*Crystal data for JA-Ile-lactone (**4a**):* $C_{18}H_{27}NO_4$, M = 321.41 g mol^{-1}, colourless prism, size 0.075 x 0.054 x 0.048 mm^3, orthorhombic, space group P 2_1 2_1 2, a = 15.6931(3), b = 21.8725(4), c = 5.0383(1) Å, V = 1729.38(6) $Å^3$, T= -140 °C, Z = 4, $\rho_{calcd.}$ = 1.234 gcm^{-3}, μ (Mo-K_α) = 0.86 cm^{-1}, F(000) = 696, 12151 reflections in h(-20/20), k(-28/27), l(-6/6), measured in the range 2.27° ≤ Θ ≤ 27.48°, completeness Θmax = 99.8%, 3985 independent reflections, R_{int} = 0.0599, 3378 reflections with $F_o > 4\sigma(F_o)$, 316 parameters, 0 restraints, $R1_{obs}$ = 0.0466, wR^2_{obs} = 0.0881, $R1_{all}$ = 0.0617, wR^2_{all} = 0.0946, GOOF = 1.164, Flack-parameter -1.4(8), largest difference peak and hole: 0.207 / -0.192 e $Å^{-3}$.

*Crystal data for JA-Ile-lactone (**4b**)*: $C_{18}H_{27}NO_4$, M = 321.41 g mol^{-1}, colourless prism, size 0.06 x 0.06 x 0.04 mm^3, orthorhombic, space group P 2_1 2_1 2_1, a = 5.7917(3), b = 15.4852(7), c = 18.6976(9) Å, V = 1676.91(14) $Å^3$, T= -140 °C, Z = 4, $\rho_{calcd.}$ = 1.273 gcm^{-3}, μ (Mo-K_α) = 0.89 cm^{-1}, F(000) = 696, 27196 reflections in h(-5/7), k(-18/19), l(-23/24), measured in the range 2.85° ≤ Θ ≤ 27.52°, completeness Θmax = 99.7%, 3401 independent reflections, R_{int} = 0.0326, 3299 reflections with $F_o > 4\sigma(F_o)$, 316 parameters, 0 restraints, $R1_{obs}$ = 0.0296, wR^2_{obs} = 0.0749, $R1_{all}$ = 0.0309, wR^2_{all} = 0.0759, GOOF = 1.031, Flack-parameter -0.1(2), largest difference peak and hole: 0.219 / -0.190 e $Å^{-3}$.

Supporting information available: Crystallographic data (excluding structure factors) has been deposited with the Cambridge Crystallographic Data Centre as supplementary publication CCDC-1004515 for JA-Ile-lactone (**4a**), and CCDC-1004516 for JA-Ile-lactone (**4b**). Copies of the data can be obtained free of charge on application to CCDC, 12 Union Road, Cambridge CB2 1EZ, UK [E- mail: deposit@ccdc.cam.ac.uk].

Plant material and planting conditions

In the present study, we used wild type *N. attenuata* Torr. Ex. Watson plants of the 31st inbred generation derived from seeds collected at the Desert Inn Ranch in Utah, UT, USA in 1988. Before planting, the seeds were surface sterilized and germinated on Gamborg's B5 media as described by Krügel, et al. [60] Ten-days old seedlings were transferred to Teku pots for another ten days (Pöppelmann GmbH & Co. KG, Lohne, Germany) before planting them into 1L pots filled with washed sand. Twenty days later, 0.8 µmol of each compound (per plant) dissolved in lanoline paste were applied to the petioles of rosette-stage plants. The treatments were repeated every other day for five days to obtain nine treated leaves in total. Lanolin-treated plants were used as negative control (n=6).[61] The leaves were harvested 24 h after the last treatment, flashed frozen in liquid nitrogen and stored at -80°C until analyzed. Nicotine was quantified as previously described.[62,63] Plants were grown at 45-55% relative humidity and 24-26°C during days and 23-25°C during nights under 16 h of light. Plants were watered twice every day by and automatic irrigation system.

Statistics

The statistical tests were carried out with Sigma Plot 12.0 (Systat Software Inc., San Jose, CA, USA) using analysis of variance. Levene's and Shapiro–Wilk tests were applied to determine error variance and normality. Holm–Sidak *post hoc* test was used for multiple comparisons. To fulfill the assumptions for ANOVA, the data set was root square-transformed prior to analysis.

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[†]The THP moiety was unstable under classical conditions (Ph_3P, toluene, reflux). Acetonitrile as solvent in the presence of K_2CO_3 avoided this problem. This protecting group is crucial to selectively obtain the *Z*-isomer in the Wittig reaction.

Electronic Supplementary Information (ESI) available: [Fig. S1, S2 and copy of NMR spectra of important compounds]. See DOI: 10.1039/b000000x/

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