Synthesis, structural characterization and biological activity of two diastereomeric JA-Ile macrolactones
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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 a 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 has (3S,7S) configuration at the cyclopentanone not known from 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 Jasminum grandiflorum in the early 1960s.3 Since then, many JAs have been detected and isolated from different plant species.4 JAs were first studied because of their properties as odorants, greatly appreciated in perfumery,5 –10 later – and more important – because of their role as phytohormones.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 JA 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) (3R,7S)-JA, but it can readily epimerize at C7 to the more stable trans form (3R,7R)-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 to be reported in the literature, interestingly only in its trans form and with no biological activity known to date.21–25

JA-Ile (2), an amino acid conjugate of JA (1), is a 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, a macrolactone like JA-Ile-lactone (4), derived from 12-OH-JA-Ile (3), may also exist (Fig. 1). Moreover, JA-Ile (2) analogues have shown different biological activities. For instance, the phytotoxin coronatine (Fig. S1, ESI†), 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 compared with 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, ESI†) 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 (3R,7R)-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.14,35 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.
Macrolactonization to JA-Ile-lactone (4) was the most challenging step of the synthesis. Classical Yamaguchi–Yonemitsu conditions (Et,N, Cl, C,H,COCl, PhMe or PhH, DMAP) did not work at all, but we obtained excellent yields of the macro lactone (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 macro lactone (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 Rf = 0.26) and JA-Ile-lactone (4b) (13.7 mg, 54%, TLC Rf = 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 (ESI†).

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, ESI†). This orientation differs from the packing observed for indanoyl-isoleucine derivatives, which crystalize 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

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

Naturally occurring and synthetic JA analogs may have diverse biological backgrounds and activities.5,7,46–48 These facts have made JAs the target of several synthetic studies that examined the relationship between the molecular structure and their activity.47,49 Inspired by 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 the bioactivity of JAs (e.g., pentenyl side chain on C7, cyclopentanone ring, l-Ile moiety).30 In addition, the macrocycle may confer a certain rigidity to the structure, in analogy to the cyclohexene ring to the molecule of coronatine or the aromatic ring in coronalon (Fig. S1, ESI†).

Nicotine is a typical direct defense stimulated by JAs in tobacco plants.50,51 When MeJA (7) is applied to the leaves it is rapidly converted into bioactive JAs that induce the accumulation of nicotine in N. attenuata plants and served as a 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, this is the first time that a jasmonate having the (3S,7S) configuration (not present in planta) is reported to strongly induce a secondary metabolite.
The precursor of the JA-Ile-lactones, the hydroxy acid 12-OH-JA-Ile (3), is a well-known jasmonate that acts as a stop signal in JA-signaling. 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-step) and efficient synthesis (33% overall yield) of 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. 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. All work-up and purification procedures were carried out with reagent grade solvents. Thin layer chromatography was performed on silica gel 60 F254 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 a split ratio of 10 mL min⁻¹; temperature programme: 60 °C (2 min) at 15 °C min⁻¹ to 280 °C (5 min). Helium at 1.5 mL min⁻¹ served as a 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 Resolution TOF equipment.

NMR spectra were recorded at 300 K either on a Bruker DRX500 spectrometer (operating frequency 500 MHz for 1H NMR and 125 MHz for 13C NMR) or a Bruker Avance 400 NMR spectrometer (operating frequency 400 MHz for 1H NMR and 100 MHz for 13C NMR). 1H NMR chemical shifts were referenced to TMS. Ozone was generated with a Sander Labor-Ozonisator, oxygen flow at 120 L h⁻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 CH₂Cl₂ (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, 3 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.

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 CH₂Cl₂ 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 Et₂O and washed successively with solutions of saturated NaHCO₃ (200 mL), water (200 mL) and brine (200 mL). The aqueous phase was worked-up with fresh Et₂O and the organic phases were combined and dried over Na₂SO₄. 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 Rf = 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 acetone and triphenylphosphine (4.23 g, 16.13 mmol), and K2CO3 (1.01 g, 7.24 mmol) was added and the mixture was refluxed overnight (ca. 14 h). The precipitate was filtered off and the filtrate was poured into 150 mL of Et2O to obtain the solid Wittig salt. After filtration the solid was washed with 100 mL of fresh Et2O. The product (15) (5.81 g, 89%) was dried under vacuum and kept in a dessicator over CaCl2 until use. 1H NMR (DMSO-d6, 400 MHz): 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); 13C NMR (DMSO-d6, 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-yloxy)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 approximately 0.2 M. The mixture was then filtered through a short pad of silica and eluted with AcOEt.

Recrystallization was carried out as described above in the synthesis section for another 6 h and worked up. 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 C18H28NO5, 338.1968); 1H NMR (CDCl3, 500 MHz): 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); 13C NMR (CDCl3, 125 MHz): 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, 25.3, 15.7, 11.7 ppm.

**Synthesis of (4S,2S,4S,7S,7aR,14R)-4-(5-(sec-butyl)-3,4,8,11,11a,13,14,14a-octahydro-1H-cyclopenta[g][1]oxa[4]azacyclotridecine-2,5,12,15-tetrahydro-4H-trione, JA-Ile-lactone (4)**. Activation of the carbonyl group: ethoxycetene (48 µL, 0.718 g mL⁻¹ in hexanes, 0.49 mmol, 4 equiv.) and dichloro(p-cymene)ruthenium(II) dimer (1 mg, 0.02 mmol) were dissolved in dry acetonitrile (3 mL) under an atmosphere of argon at 0 °C. 12-OH-JA-Ile (4 mg, 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 MgSO4, 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 was extracted three times with AcOEt (20 mL), and the combined organic extracts were washed with brine and dried over MgSO4. 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 C18H28NO5, 338.1968); 1H NMR (CDCl3, 500 MHz): 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); 13C NMR (CDCl3, 125 MHz): 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, 25.3, 15.7, 11.7 ppm.
Crystal structure determination

The intensity data for the compounds were collected on a Nonius KappaCCD diffractometer using graphite-monochromated Mo-Kα radiation. Data were corrected for Lorentz and polarization effects but not for absorption effects. The structures were solved by direct methods (SHELXS) and refined by full-matrix least-squares techniques against F^2 (SHELXL). 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 determinations.

Crystal data for JA-Ile-lactone (4a). C_{18}H_{27}NO_{4}, M = 321.41 g mol\(^{-1}\), colourless prism, size 0.075 × 0.054 × 0.048 mm\(^3\), orthorhombic space group P2\(_1\).2\(_1\).2\(_1\), a = 15.6931(3) Å, b = 21.8725(4) Å, c = 5.0383(1) Å, V = 1729.38(6) Å\(^3\), T = −140 °C, Z = 4, \(\rho_{calcd} = 1.234\) g cm\(^{-3}\), \(\mu (\text{Mo-Kα}) = 0.86\) cm\(^{-1}\), \(F(000) = 696\), 12,151 reflections in \(h \neq 0\), \(k \neq 0\), \(l \neq 0\), measured in the range 2.27° ≤ Θ ≤ 27.48°, completeness \(\Theta_{max} = 99.8\%\), 3985 independent reflections, \(R_{int} = 0.0599\), 3378 reflections with \(F_o > 4\sigma(F_o)\), 316 parameters, 0 restraints, \(R^2_{obs} = 0.0466\), \(wR^2_{obs} = 0.0881\), \(R^2_{all} = 0.0617\), \(wR^2_{all} = 0.0946\), GOOF = 1.164, Flack parameter = −1.48(4), 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 × 0.06 × 0.04 mm\(^3\), orthorhombic space group P2\(_1\).2\(_1\).2\(_1\), a = 15.7917(3) Å, b = 18.6976(9) Å, c = 18.5485(7) Å, V = 1676.91(14) Å\(^3\), T = −140 °C, Z = 4, \(\rho_{calcd} = 1.273\) g cm\(^{-3}\), \(\mu (\text{Mo-Kα}) = 0.89\) cm\(^{-1}\), \(F(000) = 696\), 27,196 reflections in \(h \neq 0\), \(k \neq 0\), \(l \neq 0\), measured in the range 2.85° ≤ Θ ≤ 27.52°, completeness \(\Theta_{max} = 99.7\%\), 3401 independent reflections, \(R_{int} = 0.0326\), 2399 reflections with \(F_o > 4\sigma(F_o)\), 316 parameters, 0 restraints, \(R^2_{obs} = 0.0296\), \(wR^2_{obs} = 0.0749\), \(R^2_{all} = 0.0309\), \(wR^2_{all} = 0.0759\), GOOF = 1.031, Flack parameter = −0.12(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).

Plant material and planting conditions

In the present study, we used wild-type N. attenuata Torr. Ex. Watson plants of the 31\(^{st}\) 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. Ten-day-old seedlings were transferred to Teku pots for another ten days (Pöppelmann GmbH & Co. KG, Lohne, Germany) before planting them into 1 L pots filled with washed sand. Twenty days later, 0.8 µmol of each compound (per plant) dissolved in lanoline paste was 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 a negative control (n = 6). The leaves were harvested 24 h after the last treatment, flash frozen in liquid nitrogen and stored at −80 °C until analyzed. Nicotine was quantified as previously described. 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 an 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’s tests were applied to determine error variance and normality. The Holm–Sidak post hoc test was used for multiple comparisons. To fulfill the assumptions for ANOVA, the data set was sorted from structure-transformed prior to analysis.

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