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Piperitone-Derived Saturated Lactones: Synthesis and Aphid Behavior-Modifying Activity

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Supporting Information

ABSTRACT: Two racemic and two enantiomeric pairs of new saturated lactones with the *p*-menthane system were obtained. The lactones were synthesized from racemic and enantiomerically enriched *cis*- and *trans*-piperitols, which were obtained from piperitone. The structures of the compounds were confirmed by spectroscopic data. The antifeedant activity of piperitone to *Myzus persicae* was studied, and the biological consequences of structural modifications of piperitone, that is, lactonization and chiral center configuration, were examined as well. The behavioral responses of *M. persicae* to piperitone and piperitone-derived saturated lactones were investigated to reveal the biological background of their deterrent activity. Piperitone appeared rather neutral or weakly deterrent to aphids. The introduction of a lactone moiety into a piperitone molecule dramatically changed its biological activity. All piperitone-derived lactones evoked negative aphid responses. However, the deterrent activity of individual compounds varied in potency, the time of expression, and the duration of the effect, depending on the spatial structure of the lactone. Lactones (1*R*,3*S*,6*R*)-3-isopropyl-6-methyl-9-oxabicyclo[4.3.0]nonan-8-one and *trans*-3-isopropyl-6-methyl-9-oxabicyclo-[4.3.0]nonan-8-one showed the broadest ranges and the highest potencies and durabilities of deterrent activity to *M. persicae*: they acted immediately after application, caused a cessation of probing before aphids reached phloem elements, and decreased the quality of phloem sap.

KEYWORDS: *piperitone, p-menthane lactones, antifeedants, Myzus persicae (Sulz.)*

INTRODUCTION

The annual world crop damage caused by aphids is estimated as at least 2% of all losses attributed to insect feeding.[1] Apart from removing vital fluids from plant sieve elements, aphids are very effective vectors of virus diseases: approximately 60% of all plant viruses are spread by these insects. The indirect damage caused by aphids due to virus transmission exceeds their direct impact on crops.[2] According to Blackman and Eastop,[3] the peach-potato aphid *Myzus persicae* (Sulz.) can infest plants of over 40 different families including many economically important ones worldwide, and it is able to transmit over 100 plant viruses. *M. persicae* is among a group of 20 aphid species that developed clones resistant to one or more insecticides. At the same time, in *M. persicae*, the resistance mechanisms are the most frequent and diverse.[2] Therefore, an alternative method of aphid control is needed and the use of targeted chemicals that would repell aphids or deter their probing and feeding is one of the most promising approaches. For example, antifeedants can be a "push" in the "push−pull" strategy (also known as a stimulo-deterrent diversionary strategy).[4]

Basically, the antifeedants are behavior-modifying substances of natural or synthetic origin that deter feeding through a direct action on peripheral sensilla (= taste organs) in insects.[5] The search for yet undiscovered natural compounds and especially the synthesis of natural antifeedants' analogues of known activities are probably the most promising methods leading to their practical use in insect pest population control. The sesquiterpenoid polygodial was successfully applied in the field against bird cherry-oat aphid *Rhopalosiphum padi* L., giving results similar to those obtained with cypermethrin.[6] However, the application of natural antifeedants for the protection of plants and stored products against insect pests is limited, mainly due to the low content in natural sources. Moreover, the complicated structures of natural deterrents make their synthesis difficult and very expensive. At the same time, it must be kept in mind that the activity of antifeedants is highly species specific and even developmental stage specific.[7] Therefore, careful studies considering various aspects of antifeedant activity are needed for proper interpretation and evaluation of the results. In the case of aphid control, especially with respect to epidemiological issues, the physiological and behavioral aspects of activity are the major constraints that limit the application of antifeedants, apart from the practical and economical reasons. Aphids regulate the amount of ingested food by regulating the duration of phloem sap ingestion periods.[8] The acquisition and inoculation of nonpersistent viruses occur during brief cell punctures that are a key part of pathway probing,[9] whereas those of persistent viruses occur during the phloem phase.[10] The elimination or at least the

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reduction of penetration of plant tissues by aphids may save plants from pathogen infection.[9] Unfortunately, considering the mechanism of virus acquisition and inoculation by aphids, very few natural or synthetic antifeedants that reduce aphid feeding meet also the requirements of virus transmission protective agents. For example, Nisbet et al.[11] found that the application of azadirachtin decreased the duration of phloem sap ingestion while causing an increase in the percentage of time devoted to stylet pathway patterns, the number of probes initiated, and the numbers of sieve tube penetrations in *M. persicae*. The former effect is the typical antifeedant activity, but the latter trends are positively correlated with virus transmission effectiveness.[10]

The most well-known antifeedants come from natural sources, and the most widespread have isoprenoid structure and lactone moiety, especially the sesquiterpenoid lactones.[12,13] Aphids are responsive to many plant monoterpenoids. For example, the repellent properties of linalool and α-terpineol to *M. persicae* were reported by Hori.[14,15] In our former studies, the biological activity of a number of natural monoterpenoids and their analogues has been examined. For example, aphid probing was impeded at the level of peripheral tissues after the application of (*R*)-pulegone and (*R*)-pulegone-derived lactones.[16] (*S*)-Limonene restrained phloem sap ingestion and had other negative effects on the behavior of the peach-potato aphid;[17] citral, linalool, (*S*)-limonene, α-ionone, and camphene reduced the total and mean probing time of aphids and their settling on the leaves.[18] At the same time, the antifeedant activity of synthetic analogues, including terpenoid lactones, was enantiospecific.[16,17,19,20]

In the present work, the antifeedant activity of piperitone to *M. persicae* was studied, and the biological consequences of structural modifications of piperitone, that is, the introduction of a lactone moiety into the molecule and chiral center configuration, were examined as well. The biological study included various aspects of aphid host plant selection and acceptance processes. The behavioral responses of *M. persicae* to piperitone and piperitone-derived saturated lactones were investigated to reveal the biological background of their deterrent activity, and the outcomes are discussed with respect to epidemiological issues.

MATERIALS AND METHODS

Reagents. Tributyltin hydride was purchased from Fluka (Poznań, Poland).

General Procedures. *Analytical TLC* was performed on Fluka Kieselgel 60 F_{254} plates with a mixture of hexane and diethyl ether in various ratios. Compounds were detected by spraying the plates with 1% $Ce(SO_4)_2$ and 2% $H_3[P(Mo_3O_{10})_4]$ in 10% H_2SO_4, followed by heating to 120−200 °C.

Column chromatography was performed on silica gel (Kieselgel 60, 230−400 mesh ASTM, Merck) with a mixture of hexane and diethyl ether (in various ratio) as eluents.

Gas chromatography analysis was carried out on a 6890N GC instrument (Agilent Technologies, Palo Alto, CA, USA) equipped with a flame ionization detector (FID) using H_2 as the carrier gas and a capillary column Trace TR-5, 30 m × 0.32 mm × 1.0 μm (Thermo Electron Corp., Runcorn, UK). *Chiral gas chromatography* was carried out on a Supelco BETA DEX 120 (30 m × 0.25 mm × 0.25 μm, Supelco Park, Bellefonte, PA, USA) column for **3a−c** and on a Supelco BETA DEX 325 (30 m × 0.25 mm × 0.25 μm, Supelco Park, Bellefonte, PA, USA) column for **4a−c**. Enantiomeric excesses were determined with the following temperature programs: for **3a−c**, 90 °C, 160 °C (1 °C/min), 200 °C (20 °C/min) (2 min), total run time = 75.00 min, t_R**3b** = 58.36 min, t_R**3a** = 59.04 min; for **4a−c**, 100 °C, 160 °C (1.36 °C/min), 200 °C (20 °C/min) (3 min), total run time = 49.12 min, t_R**4b** = 35.33 min, t_R**4a** = 35.84 min.

^{1}H NMR, ^{13}C NMR, DEPT 135 (distortionless enhancement by polarization transfer), ^{1}H−^{1}H COSY (correlation spectroscopy) and HSQC (heteronuclear single quantum correlation) spectra were recorded for $CDCl_3$ solution on a BrukerAvance II 600 MHz spectrometer (Bruker, Karlsruhe, Germany). Chemical shifts were referenced to the residual solvent signal (δ_H 7.26, δ_C 77.0). Abbreviations for ^{13}C NMR: p, primary; s, secondary; t, tertiary; q, quaternary carbon atom as determined by DEPT experiment.

IR spectra were recorded for liquid films on a Thermo-Nicolet IR300 FT-IR spectrometer (Madison, WI, USA).

Melting points (uncorrected) were determined on a Boetius apparatus.

Optical rotations were determined on an Autopol IV automatic polarimeter (Rudolph, Flanders, NJ, USA) in chloroform solutions with concentrations denoted in g/100 mL.

CH elemental analyses were performed on an EA-1110 elemental analyzer (CE Instruments, Milan, Italy).

X-ray data were collected on a Kuma KM4CCD diffractometer (Kuma Diffraction, Wroclaw, Poland; MoKα radiation; λ = 0.71073 Å) at 100 K using an Oxford Cryosystem device. Data reduction and analysis were carried out with the CrysAlice 'RED' program.[21] The space group was determined using the XPREP program. Structure was solved by direct methods using the XS program and refined using all F^2 data, as implemented by the XL program.[22] Non-hydrogen atoms were refined with anisotropic displacement parameters. All H atoms were found in $\Delta\rho$ maps or placed at calculated positions. Before the last cycle of refinement, all H atoms were fixed and were allowed to ride on their parent atoms. The Friedel pairs were merged before the final refinement.

Synthesis of Compounds. Lactones **3a−c** and **4a−c** were obtained by reduction of δ-iodo-γ-lactones (**1a−c** and **2a−c**, respectively) with tributyltin hydride (n-Bu_3SnH) according to the following general procedure.

To a solution of δ-iodo-γ-lactone (3 mmol) in anhydrous benzene (20 mL) was added tributyltin hydride (6 mmol), and the mixture was heated under reflux for 2 h. Then benzene was evaporated, and the crude product was purified by column chromatography (silica gel, hexane/diethyl ether, gradient from 9:1 to 4:1). The yields of the reactions and physical and spectral data of products obtained are given below.

*(±)-t-4-Isopropyl-r-1-methyl-7-oxa-cis-bicyclo[4.3.0]nonan-8-one ((±)-**3c**).* Lactone (±)-**3c** (yellow oily liquid, 0.69 g, yield 84%) was obtained from δ-iodo-γ-lakton (±)-**1c** (1.35 g, 4.20 mmol): ^{1}H NMR ($CDCl_3$) δ 0.86 and 0.87 (two d, J = 6.8 Hz, 6H, $(CH_3)_2$CH−), 1.00−1.13 (m, 3H, H-4, H-5, axial, and H-3, axial), 1.15 (s, 3H, CH_3-1), 1.36 (ddd, J = 14.5, 12.4, and 4.3 Hz, 1H, H-2, axial), 1.52 (septet d, J = 6.8 and 1.8 Hz, 1H, $(CH_3)_2CH$−), 1.59 (m, 1H, H-3, equatorial), 1.84 (dt, J = 14.5 and 3.5 Hz, 1H, H-2, equatorial), 1.98 and 2.60 (two d, J = 17.0 Hz, 2H, CH_2-9), 2.09 (m, 1H, H-5, equatorial), 4.11 (dd, J = 9.8 and 6.4 Hz, 1H, H-6); ^{13}C NMR ($CDCl_3$) δ 19.59 and 19.60 (p, $(CH_3)_2$CH−), 24.56 (s, C-3), 28.11 (p, CH_3-1), 31.88 (t, $(CH_3)_2$CH−), 33.21 (s, C-5), 33.34 (s, C-2), 38.25 (s, C-9), 38.48 (q, C-1), 40.41 (t, C-4), 85.53 (t, C-6), 176.87 (q, C-8); IR (film, cm^{-1}) 2957 (s), 1779 (s), 1464 (m), 1386 (w), 1370 (w), 1163 (m) 1008 (m). Elemental analysis calcd (%) for $C_{12}H_{20}O_2$ (196.29): C, 73.43; H, 10.27. Found: C, 73.62; H, 10.95.

*(+)-(1S,4S,6S)-4-Isopropyl-1-methyl-7-oxabicyclo[4.3.0]nonan-8-one ((+)-**3a**).* Lactone (+)-**3a** (yellow oily liquid, 0.32 g, yield 78%, ee = 95%) was obtained from iodolactone (+)-**1a** (0.68 g, 2.11 mmol): $[\alpha]_D^{30}$ +29.5° (c 0.95, $CHCl_3$). Its IR and NMR spectra were identical with those of (±)-**3c**.

*(−)-(1R,4R,6R)-4-Isopropyl-1-methyl-7-oxabicyclo[4.3.0]nonan-8-one ((−)-**3b**).* Lactone(−)-**3b** (yellow oily liquid, 0.18 g, yield 82%, ee = 99%) was obtained from iodolactone (−)-**1b** (0.36 g, 1.11 mmol): $[\alpha]_D^{30}$ −30.2° (c 0.60, $CHCl_3$). Its IR and NMR spectra were identical with those of (±)-**3c**.

*(±)-c-4-Isopropyl-r-1-methyl-7-oxa-cis-bicyclo[4.3.0]nonan-8-one ((±)-**4c**).* Lactone (±)-**4c** (yellow oily liquid, 0.87 g, yield 82%)

was obtained from δ-iodo-γ-lakton (±)-**2c** (1.75 g, 5.42 mmol): ^{1}H NMR ($CDCl_3$) δ 0.86 and 0.87 (two d, J = 6.8 Hz, 6H, $(CH_3)_2CH-$), 1.06 (m, 1H, one of CH_2-3), 1.13 (s, 3H, CH_3-1), 1.28−1.37 (m, 2H, H-4 and one CH_2-5), 1.41−1.46 (m, 2H, $(CH_3)_2CH-$ and one CH_2-2), 1.52−1.57 (m, 2H, one CH_2-3 and one CH_2-2), 2.07 (m, 1H, one CH_2-5), 2.25 and 2.38 (two d, J = 16.5 Hz, 2H, CH_2-9, AB system), 4.23 (m, 1H, H-6); ^{13}C NMR($CDCl_3$) δ 19.41 and 19.50 (p, $(CH_3)_2CH-$), 20.86 (p, CH_3-1), 24.20 (s, C-3), 28.42 (s, C-5), 32.02 (t, $(CH_3)_2CH-$), 33.31 (s, C-2), 36.82 (t, C-4), 38.06 (q, C-1), 46.03 (s, C-9), 84.98 (t, C-6), 176.53 (q, C-8); IR (film, cm^{-1}) 2959 (m), 1787 (s), 1462 (w), 1368 (w), 1208 (m). Elemental analysis calcd (%) for $C_{12}H_{20}O_2$ (196.29): C, 73.43; H, 10.27. Found: C, 73.20; H, 10.75.

*(−)-(1S,4R,6S)-4-Isopropyl-1-methyl-7-oxabicyclo[4.3.0]nonan-8-one ((−)-**4a**).* Lactone (−)-**4a** (colorless crystals, recrystallization from hexane, mp 26−27 °C, 0.17 g, yield 78%, ee = 96%) was obtained from iodolactone (+)-**2a** (0.36 g, 1.12 mmol): $[\alpha]_D^{33}$ −53.1° (*c* 1.14, $CHCl_3$). Its IR and NMR spectra were identical with those of (±)-**4c**. Crystal data: $C_{12}H_{20}O_2$, M = 196.28, colorless plate, crystal dimensions 0.32 × 0.26 × 0.10 mm, orthorhombic, space group $P2_12_12_1$, 3*a* = 6.110(2), *b* = 7.173(2), *c* = 26.183(4) Å, V = 1147.5(5) Å^3, Z = 4, *Dc* = 1.136 Mg m-3, μ = 0.075 $mm^{-1}T$ = 100(2) K, R = 0.052, *wR*2 = 0.110 (1081 reflections with $I > 2\sigma(I)$) for 137 variables. Crystallographic data (excluding structure factors) for the structure in this paper have been deposited with the Cambridge Crystallographic Data Centre as supplementary publication CCDC 900439. Copies of the data can be obtained, free of charge, on application to CCDC, 12 Union Road, Cambridge CB2 1EZ, UK [fax, +44(0)-1223-336033; e-mail, deposit@ccdc.cam.ac.uk].

*(+)-(1R,4S,6R)-4-Isopropyl-1-methyl-7-oxabicyclo[4.3.0]nonan-8-one ((+)-**4b**).* Lactone (+)-**4b** (yellow oily liquid, 0.19 g, yield 79%, ee = 98%) was obtained from iodolactone (−)-**2b** (0.39 g, 1.20 mmol): $[\alpha]_D^{33}$ +49.5° (*c* = 1.12, $CHCl_3$). Its IR and NMR spectra were identical with those of (±)-**4c**.

Cultures of Aphids and Plants. Laboratory clone of peach-potato aphid *Myzus persicae* (Sulz.) was maintained on Chinese cabbage *Brassica pekinensis* in the laboratory at 20 °C, 65% relative humidity, and 16:8 h light−dark photoperiod. Apterous females of *M. persicae* (1−7 days old) and 3-week-old plants with 4−5 fully developed leaves were used for experiments. All experiments were carried out under the same conditions of temperature, relative humidity, and photoperiod. The bioassays were started at 10−11 a.m.

Behavioral Responses of Aphids. The effect of piperitone and piperitone-derived saturated lactones was assessed by monitoring aphid settling, probing, and feeding.

Aphid Settling. Aphids settle on a plant only when they accept it as a food source.[23] Therefore, the number of aphids that settle and feed on a given substrate is a good indicator of its suitability. This bioassay allows us to study aphid host preferences under seminatural conditions. Aphids are given free choice between control and treated leaves. The studied lactones were applied by immersing a leaf in 0.1% ethanolic solution of a given compound for 30 s. Control leaves of similar size were immersed in 70% ethanol that was used as a solvent for the studied lactones. Treated and control leaves were placed in a Petri dish and allowed to dry for 1 h to permit the evaporation of the solvent. Subsequently, aphids were placed in the dish along the line that divided the arena into two halves so that aphids could choose between treated (on half of a Petri dish) and control leaves (on the other half of the dish). Aphids that settled, that is, they did not move and the position of their antennae indicated feeding,[24] on each leaf were counted at 1, 2, and 24 h intervals after access to the leaf (8 replicates, 20 viviparous apterous females/replicate). Aphids that were moving or out of any of the leaves were not counted.

The data were analyzed using a Student *t* test. If aphids showed clear preference for the leaf treated with the studied compound (P < 0.05), the compound was described as having **attractant** properties. If aphids settled mainly on the control leaf (P < 0.05), the compound studied in the respective choice test was designated a **deterrent**. From the data thus obtained the relative index of deterrence (DI) was calculated using the equation

$$DI = (C - T)/(C + T)$$

where C is the number of aphids settled on control leaf and T is the number of aphids settled on the leaf that was treated with the studied compound.

The value of DI ranged between +1 (ideal deterrent) and −1 (ideal attractant).

Aphid Probing and Feeding. Aphid probing and feeding (i.e., the phloem sap uptake) by *M. persicae* were monitored using the technique of electronic registration of aphid stylet penetration in plant tissues, known as electrical penetration graph (EPG), which is frequently employed in insect−plant relationship studies.[8] In this experimental setup, aphid and plant are made parts of an electric circuit, which is completed when the aphid inserts its stylets into the plant. Weak voltage is supplied in the circuit, and all changing electric properties are recorded as EPG waveforms that can be correlated with aphid activities and stylet position in plant tissues.[25] The parameters describing aphid behavior during probing and feeding, such as total time of probing, proportion of phloem patterns E1 and E2, and number of probes, are good indicators of plant suitability or interference of probing by chemical or physical factors in individual plant tissues.[26] Lactones were applied to a 3-week-old plant by immersing one leaf in 0.1% ethanolic solution of a given compound for 30 s. Control leaves of similar size were immersed in 70% ethanol that was used as a solvent for the tested lactones. Treated and control leaves were allowed to dry for 1 h before the start of the experiment to permit the evaporation of the solvent. Aphids were attached to a golden wire electrode with conductive silver paint and starved for 1 h prior to the experiment. Probing behavior of 12 apterous females per studied lactone/aphid combination was monitored for 8 h continuously with four-channel DC EPG recording equipment. Each aphid was given access to a freshly prepared leaf. Signals were saved on the computer and analyzed using the PROBE 3.1 software provided by Dr. W. F. Tjallingii (EPG-Systems, Dillenburg 12, 6703 CJ Wageningen, The Netherlands; www.epgsystems.eu). The following aphid behaviors were distinguished: no penetration (waveform np, aphid stylets outside the plant); pathway phase, penetration of nonphloem tissues (waveforms ABC); salivation into sieve elements (waveform E1); ingestion of phloem sap (waveform E2); and ingestion of xylem sap (waveform G). The parameters derived from EPGs were analyzed according to their frequency and duration in configuration related to activities in peripheral and vascular tissues.

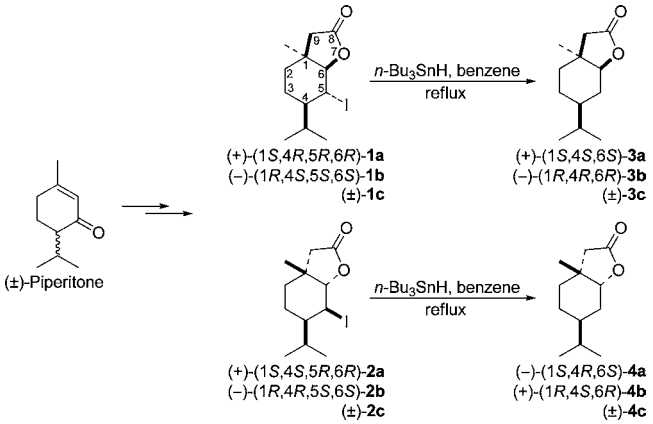
The results were statistically analyzed using the Mann−Whitney *U* test.

RESULTS AND DISCUSSION

Synthesis of Piperitone-Derived Saturated Lactones. Lactones **3a−c** and **4a−c** were synthesized from *cis*- (**1a−c**) and *trans*-iodolactones (**2a−c**) by their reduction with tributyltin hydride (Scheme 1). The racemic iodolactones (**1c** and **2c**) were obtained in a three-step synthesis from respective racemic *cis*- and *trans*-piperitols as described in ref 20. In the same way the enantiomers of *cis*- (**3a,b**) and *trans*-lactones (**4a,b**) were obtained from corresponding optically active iodolactones (**1a,b** and **2a,b**, respectively). Enantiomerically enriched halolactones (**1a,b** and **2a,b**) were synthesized from enantiomers of *cis*- and *trans*-piperitols, which were separated from racemic alcohols via their enzymatic (lipase Amano PS) esterification with vinyl propionate.[27] The optical purity of enantiomers of lactones **3a,b** and **4a,b** was confirmed by GC with application of chiral columns.

Structures of saturated lactones **3a−c** and **4a−c** were confirmed by their spectral data. In the IR spectra of all final products the absorption bands in the range of 1784−1779 cm^{-1} indicate that the γ-lactone ring was not affected. ^{1}H NMR data confirm that the conformation of molecules without iodine is the same as in the case of iodolactones. The coupling constants

Scheme 1. Synthesis of Piperitone-Derived Saturated Lactones 3a−c and 4a−c



J_{H6-H5a} (9.8 Hz) and J_{H6-H5e} (6.4 Hz) found in the spectrum of **3c** indicate the axial orientation of H-6 and the equatorial orientation of the C−O bond of the lactone ring. This means that the γ-lactone ring is still *cis*-situated toward the isopropyl group. The removal of the iodine atom from the molecule of **1c** affects the chemical shift of neighboring protons: the signal of H-6 was shifted from 4.4 ppm (in the spectrum of **1c**) to 4.11 ppm (in the ^{1}H NMR of **3c**), the multiplet of H-4 from 1.48 to 1.12 ppm, and the multiplet of $(CH_3)CH-$ from 2.20 to 1.52 ppm.

The spectral data confirm also the structures of lactones **4a−c**. The small values of coupling constants, difficult to determine from the spectrum, of H-6 with H-5a and H-5e indicate that H-6 in **4c** (as in **2c**) is in equatorial position and the C−O bond of the lactone ring is in axial orientation, which confirms the *trans*-orientation of lactone ring with respect to the isopropyl group.

Such conformation was confirmed by the X-ray structure of lactone **4a** (Figure 1). The torsion angles between bonds C6−H6 and C5−H5a (68.75°) as well as C5−H5e (−48.22°) are in full accordance with coupling constants of H-6 with H-5a and H-5e found in the ^{1}H NMR spectrum.

The lactone with the same summary formula as **3** and **4** was obtained by Witkiewicz and Chabudziński by oxidation of (+)-*p*-ment-1-ene with manganese triacetate.[28] The spectral data of their and our products are entirely different. The data presented by Witkiewicz and Chabudziński indicate that in their product the lactone ring is joined with the cyclohexane ring by bonds which are in *trans*-relation.

Aphid Settling. The monitoring of aphid settling behavior over 24 h after application of piperitone and piperitone-derived saturated lactones showed that significanly fewer aphids were found on the leaves treated with all piperitone-derived saturated lactones than on control leaves (Figure 2). This behavior did not change over the course of time: the freely moving aphids avoided these leaves immediately after the onset and until the end of experiment. The exception was lactone **3a**, which appeared to evoke aphid negative response later than 2 h after application. The relative indices of deterrence were close to the maximum possible value of 1. In contrast, when piperitone was applied, aphids did not show preferences for either control or treated leaves during the initial 2 h. Twenty-four hours after application, more aphids were found on piperitone-treated leaves than on control leaves (Figure 2).

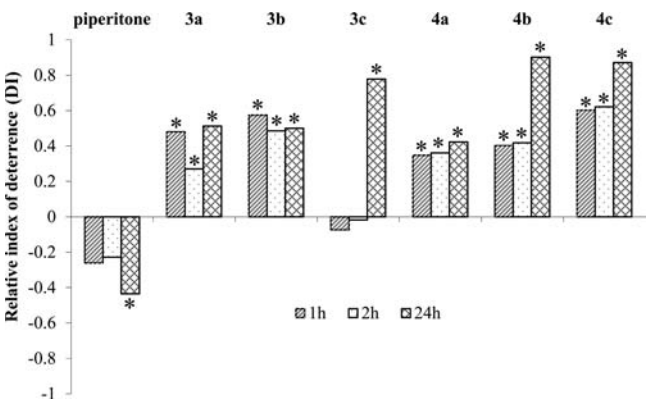


Figure 2. Relative index of deterrence (*DI*) of piperitone and lactones **3a−c** and **4a−c** 1, 2, and 24 h after application. Asterisks (∗) indicate statistically significant biological activity according to Student's *t* test results ($P < 0.05$); $DI < 0$, attractant; $DI > 0$, deterrent.

Aphid Probing and Feeding. The electronic monitoring of aphid probing (EPG) demonstrated that despite individual, aphid-dependent variation, the experimental groups of aphids showed distinct differences in behavior on plants treated with

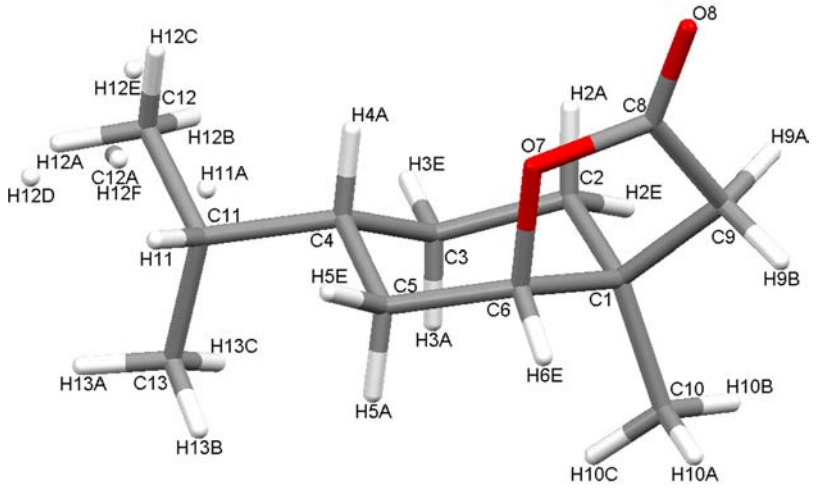


Figure 1. Crystal structure of lactone **4a** (the isopropyl group reveals two-positioned disorder).

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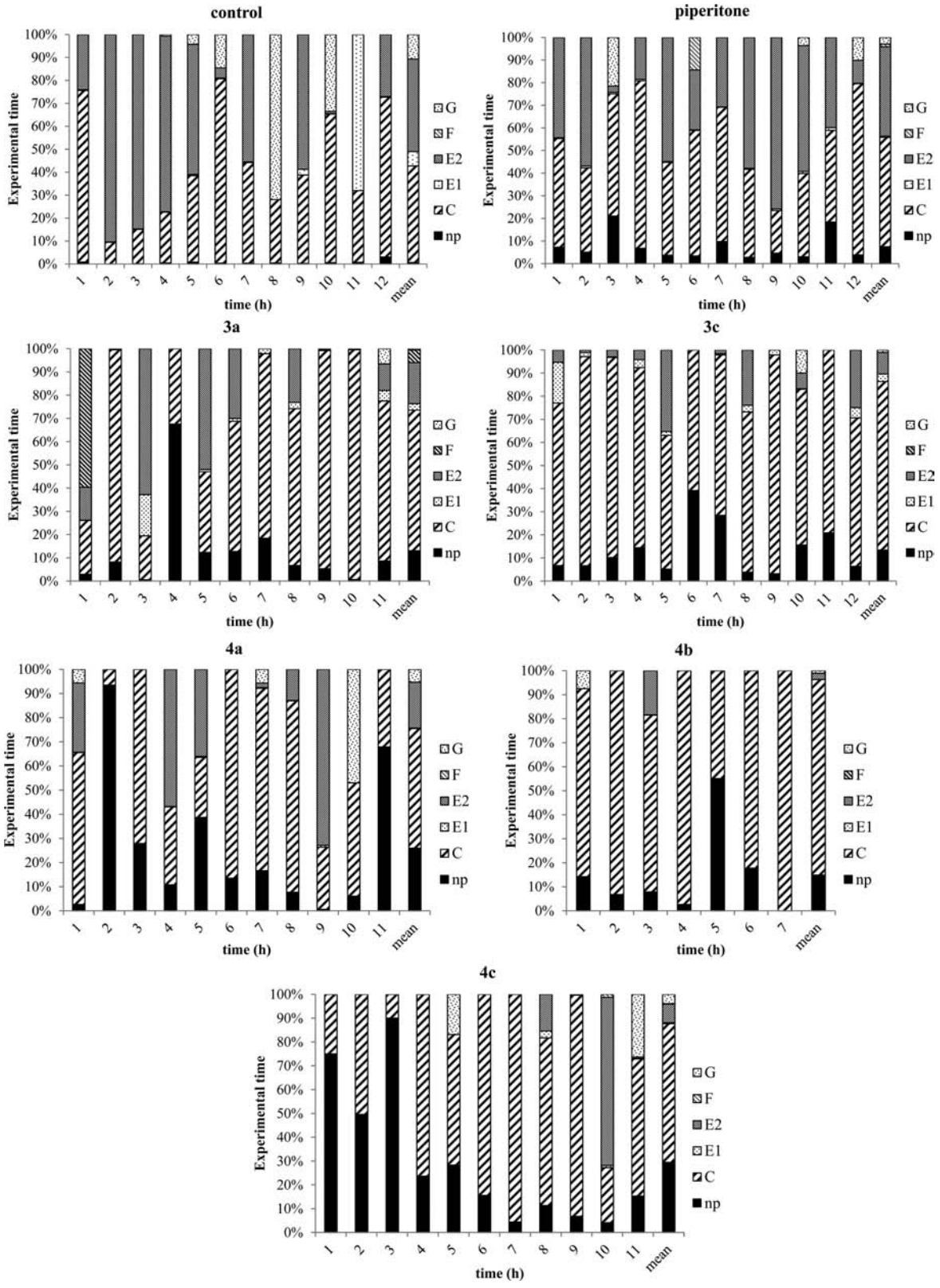

Figure 3. Structure of individual probes after application of piperitone and lactones **3a**,**c** and **4a**−**c**. np, no probing; C, pathway; E1, phloem salivation; E2, phloem sap ingestion; F, derailed stylet activities; G, xylem sap ingestion.

piperitone and different piperitone-derived lactones (Figures 3 and 4; Table 1). On **control** plants, all aphids spent >95% of experimental time on probing activities, and the probes were rarely interrupted: there were 6.8 probes per aphid on average, and the mean duration of an individual probe exceeded 2 h. More than 90% of aphids reached phloem vessels, and the activities in phloem elements, mainly the passive ingestion of phloem sap, made up to 46% of probing time. The average

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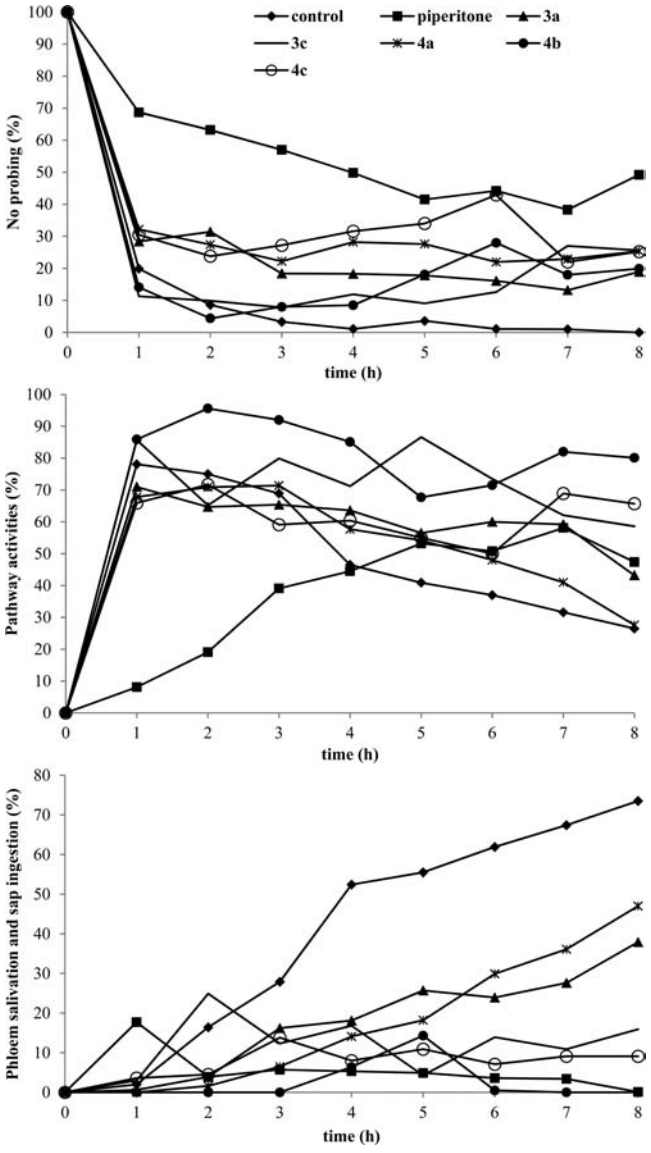

Figure 4. Sequential changes in the peach-potato aphid *Myzus persicae* probing behavior during 8 h after application of piperitone and lactones **3a,c** and **4a−c**.

duration of individual sap ingestion period was nearly 3 h. On **piperitone**-treated plants, probing activities occupied ca. 92% of experimental time and 44% of probing was dedicated to phloem sap ingestion. There were ca. 19 probes per aphid, their mean duration was approximately 0.5 h, and an average period of sap ingestion did not exceed 1 h. All aphids reached phloem elements. On **3a**-treated plants, aphid probing did not exceed 80% of experimental time, and it was divided into ca. 10 probes of average 1.3 h duration. Eighty-three percent of aphids reached phloem elements, sap ingestion was <25% of probing time, and the duration of an average sap ingestion period was ca. 52 min, but almost 40% of aphid activities in sieve elements was watery salivation. On **3c**-treated plants, 86% of experimental time was active probing by aphids, composed of seven probes of 1 h duration, on average. Most of the probing was pathway activity, as phloem sap ingestion occupied <15% of this time, although 83% of aphids reached sieve elements. The average duration of sap ingestion was 35 min, and almost 35% of aphid activities in phloem elements was salivation. On **4a**-treated plants, <75% of experimental time was probing activity (17 probes per aphid, ca. 40 min each), and 22% of that time was spent in the phloem, but only 54% of aphids reached sieve elements. The average sap ingestion period lasted for approximately 2 h, and salivation occupied ca. 2% of aphid activities in sieve elements. On **4b**-treated plants, penetration of plant tissues occupied 85% of the experiment, and the probes were numerous (ca. 22 per aphid) and of relatively long duration (ca. 1.5 h). However, only one of seven aphids reached sieve elements and showed sap ingestion activity. On **4c**-treated plants, probing activities represented 70% of experimental time (15 probes of 1.5 h average duration per aphid), and 23% of these activities was probing in phloem vessels. Thirty-five percent of aphids reached sieve elements, and sap ingestion periods were relatively short (<10 min long, on average). Salivation to sieve elements occupied almost 40% of aphid activities (Figures 3 and 4; Table 1). Ingestion of xylem sap and derailed stylet activities occurred occasionally, irrespective of treatment (Figure 3).

The EPG experiment revealed also sequential changes in aphid behavior during probing in peripheral tissues and in phloem elements. At the beginning of the experiment, all aphids showed similar probing activity; that is, they started probing immediately after having access to the plants. During the initial hour, pathway activities prevailed over all probing activities (Figure 4). On control plants, the proportion of no-probing decreased rapidly during the first hour, and it was replaced by penetration in mesophyll and phloem. The proportion of phloem-related activities increased steadily and reached 75% of aphid probing time after 8 h. The pathway/phloem activities ratio ranged from 4.6 in the second hour to 0.4 at the end of the experiment. On **piperitone**-treated plants, the proportion of no-probing declined initially, but from the third hour onward, it was approximately 40−50% of all aphid activities. The main probing activity was pathway, whereas phloem-related probing was significantly reduced, except for the first hour of the experiment. The pathway/phloem activities ratio increased from 0.5 in the first hour to 17.1 in the eighth hour. On **3a**-plants, probing activities predominated during the whole period of monitoring, and the pathway/phloem activities ratio ranged from 16.6 in the second hour to 1.1 in the eighth hour. On **3c**-treated plants, pathway/phloem ratio changed from 28.6 after the first hour to 2.6 after the second hour, increased to 20.1 in the fifth hour, and then decreased again to 3.6 in the sixth hour. On **4a**-treated plants, the pathway/phloem ratio was 44.3 when the first phloem phase appeared, and at the end of experiment it was 0.6. On **4b**-plants, the first phloem phase appeared in the fourth hour (pathway/phloem activities ratio = 13.3), but 2 h later only pathway acitivities occurred. On **4c**-plants, the pathway/phloem activities ratio was relatively high during the whole experimental period: 18.3 (first hour) and 7.2 (eighth hour) (Figures 4 and 5).

Preingestional, Ingestional, and Postingestional Activity of Piperitone and Piperitone-Derived Lactones. Insect feeding can be constrained at three levels: preingestional (immediate effect associated with host finding and host selection processes involving gustatory receptors), ingestional (related to food transport and production, release, and digestion by salivary enzymes), and postingestional (long-term effects involving various aspects of digestion and absorption of food).[29] In the case of aphids, the preingestional inhibitory effects may be associated with stylet penetration in plant tissues outside the phloem, that is, epidermis and

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Table 1. Effect of Piperitone and Piperitone-Derived Saturated Lactones on Probing Activities of *Myzus persicae* (Sulz.)[a]

EPG parameter	control	piperitone	3a	3c	4a	4b	4c
general aspects of aphid probing behavior[b]							
total duration of nonprobing (min)	23.1 ± 8.1[c]	35.9 ± 506.7[c,d]	97.7 ± 42.8[c,d]	65.1 ± 17.5[c,d]	124.7 ± 43.1[d,e]	71.4 ± 34.2[f]	141.6 ± 42.6[d,e,g]
total duration of pathway (min)	242.7 ± 43.5[c]	251.9 ± 27.8[c]	292.5 ± 44.8[c]	353.3 ± 19.8[c,g]	263.2 ± 44.4[e]	346.0 ± 14.1[d,f,g]	298.8 ± 43.8[e]
total duration of phloem phase (min)	214.2 ± 45.9[c]	192.2 ± 30.7[c]	89.8 ± 35.4[c,d,g]	61.7 ± 20.5[c,d,g]	92.1 ± 38.2[d,e,g]	12.6 ± 12.6[d,f,g]	39.6 ± 31.2[d,e,g]
total duration of phloem sap ingestion phase (min)	185.7 ± 47.8[c]	189.6 ± 30.7[c]	77.6 ± 30.4[c,g]	45.8 ± 17.6[c,d,g]	91.2 ± 37.9[e]	12.6 ± 12.6[d,f,g]	37.8 ± 30.6[d,e,g]
proportion of phloem phase in total probing (%)	46.0 ± 10.0[c]	43.0 ± 7.0[c]	23.0 ± 7.0[c,d,g]	14.0 ± 5.0[c,d,g]	22.0 ± 9.0[e]	11.1 ± 7.7[d,f,g]	8.8 ± 6.8[d,e,g]
no. of probes	6.8 ± 1.6[c]	19.1 ± 3.9[c,d]	9.8 ± 1.9[c]	7.4 ± 0.1[c,g]	17.6 ± 3.5[d,e]	22.1 ± 7.10[f]	14.9 ± 3.25[e]
mean duration of a probe (min)	144.8 ± 160.8[c]	34.4 ± 19.5[c]	81.4 ± 131.3[c,d]	67.7 ± 36.9[c,g]	41.8 ± 56.9[e]	93.3 ± 172.6[f]	43.2 ± 15.5[d,e]
activities in peripheral tissues before phloem phase[h]							
no. of probes before first phloem phase[h]	5.0 ± 1.4[e]	11.2 ± 3.3[c]	4.9 ± 1.6[i]	2.1 ± 0.8[g,i]	20.0 ± 5.9[d,j]	0.0[k]	6.0 ± 0.2[l]
no. of probes <2 min before first phloem phase[h]	1.1 ± 0.6[e]	3.6 ± 1.3[c]	2.0 ± 1.2[i]	0.1 ± 0.1[g,i]	8.3 ± 3.5[d,j]	0.0[k]	2.5 ± 1.5[l]
time from first probe to first phloem phase[m] (min)	187.1 ± 41.1[e]	167.1 ± 34.8[c]	202.1 ± 44.8[e]	195.5 ± 48.5[c]	375.4 ± 38.0[d,e,g]	441.6 ± 38.4[d,f,g]	55.8 ± 57.0[e,g]
activities in sieve elements[h]							
duration of first phloem phase (min)	150.0 ± 54.0[e]	30.0 ± 12.0[c]	48.0 ± 36.0[d,i]	36.0 ± 12.0[i]	96.0 ± 42.0[g,j]	90.0[k]	6.0 ± 0.0[l]
no. of phloem sap ingestion phases	1.8 ± 0.3[i]	4.4 ± 0.8[c]	1.4 ± 0.4[n]	1.6 ± 0.2[o]	1.5 ± 0.3[j]	1.0[k]	1.1 ± 0.7[p]
duration of first phloem sap ingestion phase (min)	138.0 ± 60.0[i]	54.0 ± 30.0[c]	13.6 ± 8.6[n]	30.0 ± 12.0[o]	96.0 ± 42.0[j]	90.0[k]	30.0 ± 24.0[p]
mean duration of phloem sap ingestion phase (min)	165.7 ± 53.0[i]	58.0 ± 43.1[c]	52.01 ± 17.6[n]	33.9 ± 10.2[g,o]	114.1 ± 36.6[j]	88.1[k]	32.6 ± 26.1[d,g,p]
proportion of salivation in phloem phase (%)	11.0 ± 8.0[c]	3.0 ± 1.0[c]	38.5 ± 12.3[i]	34.7 ± 10.7[i]	3.3 ± 2.6[j]	1.0[k]	36.7 ± 22.0[l]

[a]Values represent the mean ± standard error. [b]All aphids were included in analysis, and if an aphid did not show a phloem phase, the value of a given parameter for such individual was zero. [c]$n = 12$. [d]Represents statistically significant differences in relation to control ($P < 0.05$, Mann–Whitney U test). [e]$n = 11$. [f]$n = 7$. [g]Represents statistically significant differences in relation to piperitone ($P < 0.05$, Mann–Whitney U test). [h]Only aphids that showed a phloem phase (E1) or phloem sap ingestion phase (E2) were included in statistical analysis. [i]$n = 10$. [j]$n = 6$. [k]$n = 1$. [l]$n = 4$. [m]Time to reach phloem phase was 8 h − (minus) first no probing (np) period for aphids that did not show this activity until the end of the experiment. [n]$n = 8$. [o]$n = 9$. [p]$n = 4$.

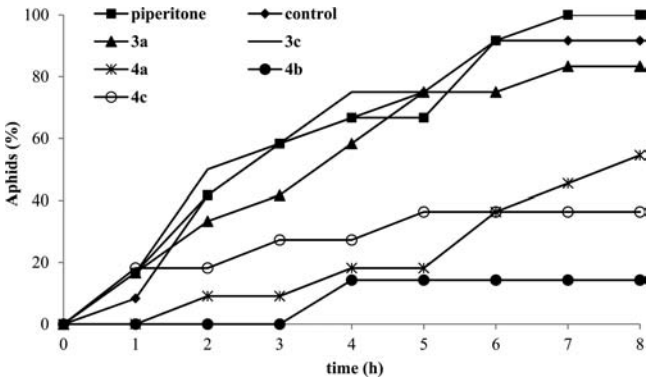

Figure 5. Cumulative proportion of aphids that show probing activities in phloem elements after application of piperitone and lactones **3a,c** and **4a–c**.

mesophyll. The stylet pathway in these tissues is extracellular, but it is marked by frequent brief intracellular punctures.[30] During these short punctures, aphids ingest small samples of cytoplasm that may contain probing-stimulatory or probing-inhibitory cues.[31] Indeed, a plant that does not contain phagostimulants or one that does contain phagodeterrents may be identified by aphids as a nonhost before they start ingestion from the phloem.[32] In EPG experiments, the difficulties in finding and reaching phloem tissue by aphids are manifested in prolonged time preceding the first contact with phloem vessels, greater number of probes not reaching beyond mesophyll before first phloem phase (i.e., <2 min), and an overall failure in finding sieve elements during the 8 h experiment.[16,19] The phloem phase of aphid probing is crucial for the decision on acceptance of a host plant for feeding and settling. On suitable host plants, the sap ingestion periods may last for many hours with no interruption.[31] Therefore, the termination of feeding may be the demonstration of ingestional deterrence by unfavorable changes in the composition of the phloem sap or the presence of deterrent factors. The EPG-registered symptoms of deterrent properties of phloem sap include the decreased duration of ingestion, lower proportion of aphids with sustained sap ingestion, decrease in proportion of phloem phase in all probing activities, and increased proportion of salivation in aphid activities in phloem.[16,19] The egestion of watery saliva is an integral part of the phloem phase,[8] and it supposed to be used by aphids to suppress plant responses induced in sieve elements. Watery saliva contains various enzymes associated with detoxification of plant allelochemicals, such as UPD-glucose transferases, polyphenol oxidases, and peroxidases.[33] Therefore, the long duration or frequent periods of watery salivation that interrupt sustained ingestion may indicate problems in sap acceptance.[31] Finally, if unsuitable phloem sap has been consumed, postingestional aftereffects may occur. In aphids, the suppression of settling on plants despite no apparent suppression of feeding can be a

symptom of delayed negative effects of sap components.[16,17,19] The behavior of aphids on **piperitone**-treated plants is difficult to interpret. On the one hand, the total probing time was reduced and phloem activities were seriously impeded (although all aphids reached phloem vessels, most of them showed sustained phloem sap ingestion during the first probe, and total sap ingestion time and the duration of watery salivation were comparable to those on control plants; the sap ingestion periods were shorter, and time spent on ingestion was a small portion of probing during the successive hours of the experiment). Such behavior of aphids would have indicated the preingestional and ingestional deterrent activity of piperitone. On the other hand, 24 h after application, but not at the beginning of experiment, more aphids were found on piperitone-treated leaves (settling experiment). However, it is difficult to determine whether these aphids on piperitone-treated leaves were probing or not, on the basis of only visual observation. These results may indicate a weak deterrent effect of piperitone that ceased over the course of time. Such changes in stability and nature of behavioral effect of exogenously applied chemicals were reported in earlier studies, and the possible explanation was the alteration of local conditions in plant tissues over the course of time due to plant and aphid metabolism.[16,19,34] In contrast, all **piperitone-derived saturated lactones** caused the deterrence of aphid probing, although the intensity and physiological level of deterrence (preingestional/ingestional) varied depending on the lactone. The strongest symptoms of preingestional deterrence occurred in aphids that were exposed to plants treated with **4b**. On these plants, aphid probes were discontinued after probing within mesophyll. Only one of seven aphids reached phloem elements, and even that aphid showed no sap ingestion during the 3 h that followed the termination of the single ingestion period. Lesser, but also strong, preingestional deterrent effects were caused by **4a** and **4c**. The total probing time was considerably reduced, and the proportion of activities in sieve elements was very low on plants with these lactones. Less than 50% of aphids reached phloem elements. The relatively strong preingestional deterrent effect was found after the application of lactones **3a** and **3c** that caused the decrease in total probing time and the proportion of time spent on activities in the phloem. However, >80% of aphids reached sieve elements and started sap ingestion. Lactones **3a**, **3c**, and **4c** acted also at the ingestional stage of aphid probing, that is, at the phloem level. The quality of sap in plants treated with these lactones must have been seriously affected, as watery salivation made up to 40% of all aphid activities in sieve elements. No postingestional effect was observed after the application of the studied lactones.

Consequences for Plant Virus Transmission by Aphids. The amount of ingested phloem sap by aphids depends on the duration of ingestion from plant sieve elements.[8] The acquisition and inoculation of nonpersistent viruses occur during brief cell punctures that are a key part of pathway probing,[9] whereas those of persistent viruses occur during the phloem phase.[10] In this view, all studied **piperitone-derived saturated lactones** may appear suitable as direct damage-reducing factors: all of them limited the duration of sap ingestion and some of them (**4a**–**c**) prevented aphids from reaching sieve elements and ingesting phloem sap. None of the studied lactones offers a possibility to eliminate indirect effects of nonpersistent virus transmission, because no reduction of probing activities in mesophyll tissue was found after their application. However, **4b** can be considered as a potential protective chemical against the transmission of persistent viruses, as its application provided >95% success in the elimination of the sieve element contact by tethered aphids and an absolute avoidance of treated leaves by the free-moving individuals.

Structure–Activity Aspects. The activity of the natural monoterpene ketone **piperitone** appeared to have weak attractant properties, although certain weak deterrent effects on aphid behavior were observed initially.

The introduction of a lactone moiety into a piperitone molecule dramatically changed its biological activity. All piperitone-derived lactones evoked negative aphid responses. However, the deterrent activity of individual compounds varied in potency, time of expression, and duration of the effect, depending on the spatial structure of the lactone. Lactones (1*R*,3*S*,6*R*)-3-isopropyl-6-methyl-9-oxabicyclo[4.3.0]nonan-8-one (**4b**) and *trans*-3-isopropyl-6-methyl-9-oxabicyclo[4.3.0]-nonan-8-one (**4c**) showed the broadest range, highest potency, and durability of deterrent activity to *M. persicae*: they acted immediately after application, caused a cessation of probing before aphids reached phloem elements, and decreased the quality of phloem sap.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

NMR spectra (^{1}H NMR, ^{13}C NMR) of **3c** and **4c**; crystallographic data for **4a** in CIF format; CGC chromatograms of lactones **3a**–**c** and **4a**–**c**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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