Iridoids As Allelochemicals and DNA Polymerase Inhibitors[⊥]

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Growth inhibitory activities and nutritional indices of catalpol (**1**), 8-O-acetylharpagide (**2**), and harpagide (**3**) were determinated in larvae and adults of *Tribolium castaneum*, respectively. Compound **1** produced a series of allelochemical effects probably related with the DNA synthesis. This iridoid possessed the highest inhibitory activity against DNA polymerase. Molecular orbital calculations suggest that a $\pi-\pi$ charge transfer recognition model could explain the action of iridioids toward nucleic acid synthesis.

Iridoid (cyclopentane-[*c*]-pyranomonoterpenoids) glycosides occur in about 57 families of plants and form a collection with almost 600 structures with an important role in chemotaxonomy.[1,2] Several biological activities for this kind of compound have been reported, as well as for iridoid-containing plants.[3−5]

Like many natural products, iridoids have been identified as key molecules in plant/insect and insect/insect-predators interactions. Some of them have been reported as deterrents to a variety of generalist insects; in this sense it has been proposed that these compounds may act as a plant defense against herbivore insects.[6] Several insect species are able to feed on iridioid-containing plants in order to sequester and concentrate these monoterpenoids glycosides for their own defense.[7,8] Studies on *Euphydryas chalcedona* Doubleday & Hewitson (Lepidoptera: Nymphalidae) showed that this insect consumes and sequestrates catalpol (**1**) from its food plant *Castilleja integra* A. Gray.[6] As a plant defense strategy, the phenolic *seco*-iridoid glycoside oleuropein is activated by β-glucosidase to give a glutaraldehyde-like structure with high protein-cross-linker activity.[9] Finally, by means of electrophysiological experiments, taste cells that respond to catalpol (**1**) have been described in *Grammia geneura* (Lepidoptera: Arctiidae).[10]

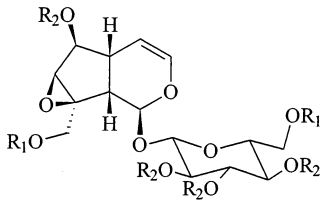
As part of a program aimed to study the biological activities of natural products from plants that grow in the semiarid central-western area of Argentina,[11,12] we report the bioactivities of the iridoids catalpol (**1**), 8-*O*-acetylharpagide (**2**), and harpagide (**3**) toward *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae) larvae development, as well as the antifeedant activity on adult insects. In an attempt to explain the molecular mechanism of the observed *in vivo* bioactivities, DNA polymerase inhibition assays were carried out, and some structure−activity relationships supported by theoretical calculations were performed. Compounds **1** and **2** were isolated from *Buddleja cordobensis* Griseb and *Ajuga reptans* L., respectively (see Experimental Section).[13] Compound **3** was prepared from the derivative **2**.

T. castaneum is one of the most economically important insect pests with a worldwide distribution, and studies on the effects of plant secondary metabolites on it have been reported.[14] Considering the debate that has been presented in the last years on the ecdysteroid agonist activity of 8-*O*-acetylharpagide (**2**) isolated from *Ajuga reptans* L. or by the contaminant ecdysteroids, bioassays aimed at measuring the effects of compounds **1**−**3** on the normal development of this insect were performed.[13,15]

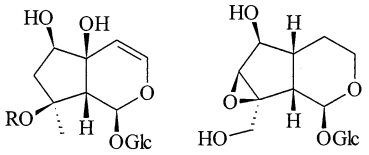
Table 1. Response of *T. castaneum* Larvae in Topical Bioassay

compound[a]	duration of pupal stage (days ± SE)[b]	percent of mortality ± SE[c]
1	8.2 ± 1.6	73.3 ± 11.5[c]
2	11.3 ± 1.9	56.6 ± 5.7[b]
3	12.1 ± 1.4	60.0 ± 10.0[b,c]
control	7.6 ± 1.1	3.3 ± 5.1[a]

[a] Dose: 60 μg/μL, topically applied. [b] Not significantly ($p > 0.05$) different. [c] Means with the same superscripted letters are significantly ($p < 0.05$) different (ANOVA and LSD test).



1 R_1=R_2=H
5 R_1=R_2=Ac
6 R_1= Oleyl, R_2=H
7 R_1= ^{t}BuPh$_2$Si, R_2=Ac



2 R=Ac
3 R= H

4

Results and Discussion

Test solutions (see Experimental Section) were topically applied to the ventral surface of the thoracic segments of the fifth instar larvae with a Hamilton microsyringe (1 μL/larvae), and a single dose of 60 μg/larvae was applied. Controls were treated with solvent alone. Results are shown in Table 1 and indicate that the assayed iridoids did not significantly alter the duration of the pupal stage

[⊥] In memory of Professor Antonio González González.

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Table 2. Nutritional and Feeding Deterrence Indices of *T. castaneum* Adults[a]

compound[b]	RGR ± SE (mg/mg/d)	RCR ± SE (mg/mg/d)	ECI ± SE (%)	FDI (%)
1	−0.010 ± 0.007[d]	0.017 ± 0.007[c]	−66.101 ± 40.774[d]	(−)
2	−0.004 ± 0.002[c]	0.058 ± 0.027[e]	−8.908 ± 6.056[c]	(−)
3	−0.003 ± 0.003[c]	0.019 ± 0.008[c]	−30.729 ± 49.335[c]	(−)
control	0.001 ± 0.003[c]	0.014 ± 0.005[c]	9.904 ± 32.935[c]	(−)

[a] (−) No antifeedant action. (RGR = relative growth rate; RCR = relative consumption rate; ECI = efficiency of conversion of ingested food; FDI = feeding deterrence index). [b] Dose 200 μg/disk. [c,d,e] Means with the same superscripted letters are significantly ($p < 0.05$) different (ANOVA and LSD test).

($p > 0.05$). However, all the compounds were toxic, and compound **1** produced the highest mortality values.

Catalpol (**1**), in topical applications, produced a series of morphological alterations. These can be summarized as insects having severe deficiencies in normal cuticle deposition; first and second pair of wings showing a certain degree of deformation; abnormal disposition of legs and wings, and pupal exuviae attached to the posterior part of the abdomen and to the appendages. Additionally, adultoids were completely enclosed in pupal exuviae, which is usually ruptured along the midline of the pronotum; pupa showed a complete inhibition of ecdysis, with no cuticle deposition. These kinds of results have been found after the treatment of *Tenebrio molitor* L. (Coleoptera: Tenebrionidae), and *T. castaneum* larvae with different natural products, such as 20-hydroxyecdycsone, azadirachtin, benzofurans, and precocene-like compounds.[16,17]

Iridoids are toxic or deterrents to some generalist herbivores,[9] but no negative effects on the feeding and growth have been observed with specialized insects. To evaluate the effects of iridoids on nutrition using a generalist insect, a flour disk bioassay (see Experimental Section) was carried out. A dose of 200 μg/disk of each compound, in aliquots of 5 μL, was spread evenly on the flour disks.[14] Controls were treated with solvent alone. The effects on the nutritional indices of *T. castaneum* produced by catalpol (**1**) (Table 2) indicate that the relative growth rate (RGR) significantly declined ($p < 0.05$) at the evaluated concentration. Compound **1** exhibited post-ingestive toxic effects, showing a reduction in the efficiency of conversion of ingested food (ECI). Our results indicate that catalpol (**1**) did not produce an elongation of the pupal stage (Table 1) or an antifeedant action (RCR, Table 2). Nevertheless, the modification of the normal nutritional behavior caused by compound **1** on *T. castaneum* is clear.

Taking into account the emergence of morphological abnormalities during the topical assays, and considering the high level of morphological changes that occurs during metamorphosis, namely, the active histogenesis, the interaction between iridoids and nucleic acids synthesis was considered.[17]

Considering the chemical structure of the iridoids, it is tempting to suggest a certain resemblance with a nucleoside framework. The bicyclic aglycone moiety, possessing both oxygenated functional groups and one desaturation, could mimic a purine-nucleoside electronic model.

DNA polymerase activity can be modified by many natural products including diterpenoids,[18] triterpenoids,[19] flavonoids, coumarins, and other related compounds.[20] Using the adequate primer/template DNA and nucleotide analogues, this DNA-replication enzyme has been used as a model to study the corresponding ternary complex.[21] Catalpol (**1**), harpagide (**3**), aucubin, and catalpol derivatives isolated from *Scrophularia nodosa* L., a plant used in folk medicine for cancer healing,[22] and 8-*O*-acetylharpagide (**2**) exhibited a remarkable inhibitory effect on NO-induced skin and hepatic tumors in mouse.[23] Managing their five-membered-ring chirality as an adequate synthon, iridoids have also been used as starting materials to produce nucleoside analogues.[24]

Table 3. Inhibition of *taq* DNA Polymerase Activity

compound	IC_{50} (μM)
1	47.8
2	213.8
3	416.9
4−**7**	no inhibition at 500 μM

Assays based on the PCR method (see Experimental Section) were conducted in a way to determine the bioactivity of compounds **1**−**3** toward *taq* DNA polymerase (Table 3). Catalpol (**1**) was the most active compound, with an IC_{50} of 47.8 μM, and 8-*O*-acetylharpagide (**2**) was weakly active, with an IC_{50} of 213.8 μM. Harpagide (**3**) was an even weaker inhibitor (IC_{50} = 416.9 μM). Considering that the sugar component is identical for the three compounds under discussion, we postulate that the aglycone moiety plays a determining role in the inhibition of the *taq* DNA polymerase activity.

Active compounds (**1**−**3**) displayed a dose-dependent effect. Compounds **1**−**3** have a 2,3-dihydropyrane ring, and it is well known that this kind of heterocycle could act as an alkylation agent from the enol-ether function toward both hydroxyl and thiol groups.[25] When the reduced derivative **4** was assayed, no inhibitory activity was detected. Since the only difference between compounds **1** and **4** was the C-3−C-4 double bond, the loss of activity of compound **4** could be related with the loss of C-3 capability to function as an electrophilic center following interaction of the strongly nucleophilic C-4 center with electrophiles such as proton.

However, when catalpol (**1**) was subjected to reaction using mercaptoethanol and thiophenol as nucleophiles, no reaction was observed even in the presence of Lewis acids. From this result it is possible to deduce that the six-membered ring of the iridoids probably does not act as an alkylating agents toward enzyme hydroxyl or sulfhydryl groups. Hence, the loss of inhibitory activity in the catalytic reaction for compound **4** must be explained by other stereoelectronic factors.

Theoretical descriptions of molecules based on the Frontier Orbital method have been used to explain the interaction between some natural products and biological molecules such as DNA.[26] This prompted us to compare by theoretical methods the reactions of iridoids' aglycone moieties with nucleotide bases and of the iridoids with the nucleosides. A conformational search was made by the Monte Carlo method, using the Merck Molecular Force Field (MMFF94), which appears to be quite well suited for this purpose. The software routine used (see Experimental Section) allows varying several dihedral angles simultaneously, even those corresponding to flexible rings.[27] Figure 1 shows the dihedral angles that were varied for compound **1**, surrounded by a circle. The numbers in boldface indicate the number of steps: "**6**" means that the angle was varied each 60°.

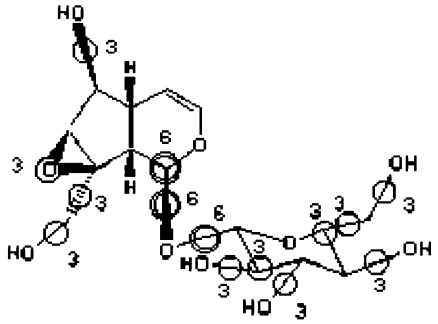

Figure 1. Dihedral angles varied for compound **1** in the conformational search.

Considering the observed dependence of the departure structure with the conformation of the six-membered heterocycle, the initial structure was selected as the lowest energy conformer obtained varying only the bonds and atoms surrounded with a double circle (Figure 1). After obtaining the conformers in gas phase, the aqueous solvation energy was estimated using the SM5.4 procedure previously reported.[27] This energy was added to the gas phase total energy, and the lowest energy conformer was selected to minimize it. The more stable MMFF conformer was minimized using a perturbative Becke−Perdew (BP86) model with a DN* basis set. Isosurfaces were calculated with this model. Figure 2 shows a significant similarity among the HOMO properties of adenosine, guanosine, and compounds **1**−**3**.

It was suggested that in a $\pi-\pi$ charge transfer recognition model, the most favorable frontier orbital interaction would be between the HOMO of the DNA base (either adenine or guanine) and the LUMO of thymine or cytosine, respectively.[26] Frontier orbital examination of iridoids **1**−**3** suggests that the nodal properties of their HOMOs would complement the nodal properties of the LUMOs for thymidine and cytidine. Figure 2 shows that the HOMO of compound **4** exhibits no similarity with that of compound **1**. Remarkably, the electronic density in the reduced compound's HOMO is located over the sugar part. These changes in the nodal properties could be the cause for the loss of enzyme inhibitory activity.

To determine the role that the sugar moiety could play in the enzyme−iridoid recognition, derivatives **5**−**7** were obtained. Although these compounds showed different patterns of substituents on the sugar hydroxyl groups, no reduction in the enzyme activity was observed with concentrations up to 500 μM for each compound. From these results, it appears that any change in the hydrophilic and/or electronic properties of the sugar part results in a dramatic loss of bioactivity. To support this hypothesis, no significant bioactivities were observed in insect biossays using compounds **5**−**7** (data not shown).

From these results, one can hypothesize that compounds **1**−**3** offer stereoelectronic similarity to the natural substrates of *taq* DNA polymerase, having the ability to adopt a precise electronic profile to compete for the active site of the enzyme. Additionally, hydrogen bonds may form between **1**−**3** and thymidine and cytidine. Figure 3 shows the calculated hydrogen bonding between adenosine and thymidine compared with the same interaction between the most active iridoid, catalpol (**1**), and thyimidine. From this result, it is possible to suggest that the hydrogen bonds could act as an additional recognition factor, as has been previously reported using other nucleoside analogues.[28]

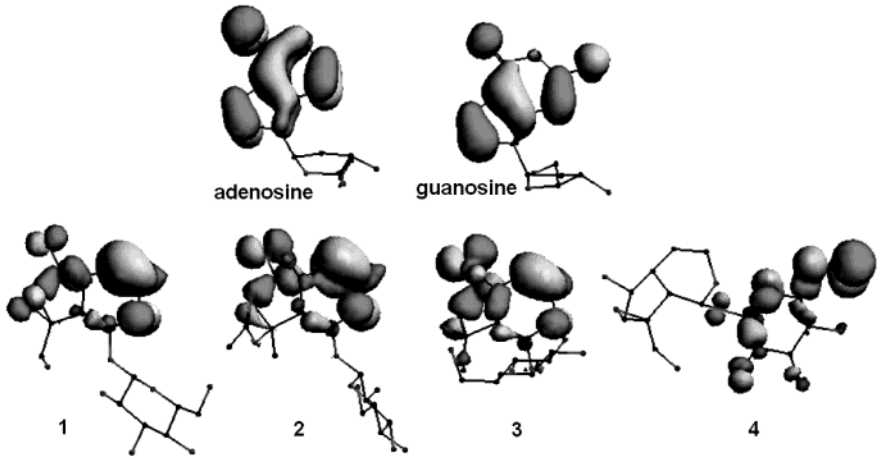

Figure 2. HOMO of adenosine, guanosine, and compounds **1**−**4** calculated with the pBP/DN* method.

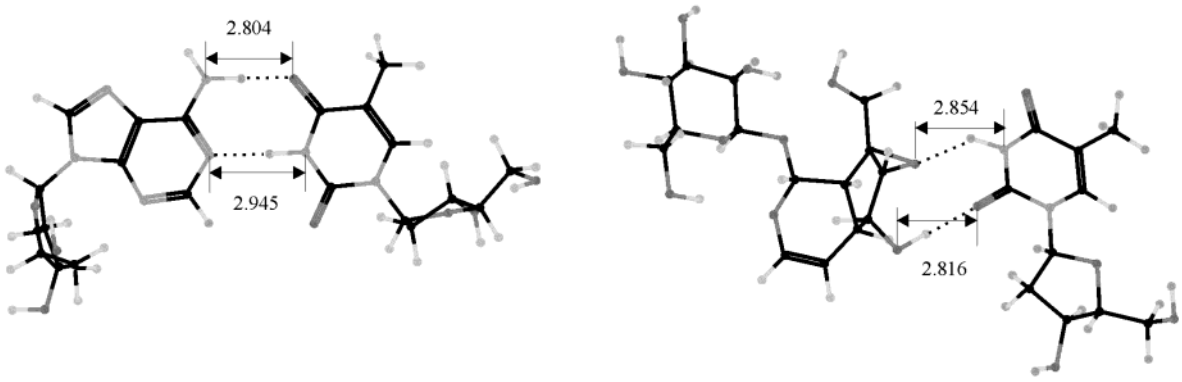

Figure 3. Calculated hydrogen bonding between adenosine and thymidine compared with the same interaction between catalpol (**1**) and thymidine.

The impossibility of the aforementioned additional recognition factor, as well as the steric hindrance produced by the introduced bulky groups in compounds **5** and **6**, should explain their inactivity, although their HOMOs were similar to those of compounds **1**−**3**. For compound **7**, the lack of activity could be attributed to both steric hindrance and the dramatic change in the HOMO properties due to the aromatic rings.

In conclusion, these iridoids are allelochemicals to the generalist insect *T. castaneum*. Taking into account the observed *taq* DNA polymerase inhibition, it is possible to propose a disruption of DNA synthesis as the basis of the observed allelochemical effect. The experimental results are in agreement with the theoretical basis of recognition, and it is possible to propose that the molecular orbital properties of the assayed iridoids are important factors in the recognition process. To our knowledge, it is the first time that iridoids have been reported as DNA polymerase inhibitors.

Experimental Section

General Experimental Procedures. Combustion analyses were performed on an Eager 200 instrument at Instituto Universitario de Bioorgánica Antonio González, La Laguna University, Tenerife, Spain. Optical rotations were obtained on a Perkin-Elmer 341 polarimeter. The ^{1}H NMR spectra were recorded at 200.13 MHz on a Bruker AC 200 with TMS as internal standard. The ^{13}C NMR spectra were obtained with the same instruments at 50.23 MHz. 2D experiments were obtained using standard Bruker microprograms. IR spectra were recorded on an FT-IR Nicolet Protégé 460 spectrometer. PCR experiments were done on a Perkin-Elmer GeneAmp 2400. Column chromatography was performed on Si gel G 70−230 mesh and Kieselgel 60 H; TLC was carried out on Si gel 60 F_{254} (0.2 mm thick plates) using $CHCl_3$−MeOH (70:30) as solvent. The iridoid-containing fractions were detected by anisaldehyde−AcOH−H_2SO_4 (0.5:50:1) spray reagent.

Plant Material. Aerial parts of *Buddleja cordobensis* Griseb. were collected during June 2001, in Cerro de La Cruz, Departamento La Capital, San Luis, Argentina, and a voucher specimen is deposited at the Herbarium of the Universidad Nacional de San Luis: L.A. Del Vitto & E.M. Petenatti-4868 (UNSL).

Extraction and Isolation. The dry aerial parts (3.2 kg) were chopped and macerated twice for 10 day periods with MeOH. The solvent was evaporated under reduced pressure at low temperature, and the residue (177.5 g) taken up in $CHCl_3$ and partitioned against H_2O. The aqueous layer was subjected to lyophylization, and the brown amorphous residue (80.0 g) was purified by Si gel column chromatography using mixtures of $CHCl_3$−MeOH in increasing polarities. After several purifications, compound **1** (3.8 g) was obtained. The structure of **1** was confirmed by comparison of both spectroscopical and physical data with previously published values.[1,29] Compound **2** was kindly supply by Prof. Josep Coll Toledano, Departamento de Química Orgánica Biológica, Institut d'Investigacions Quimiques i Ambientals "Josep Pascual Vila", Barcelona, Spain. This iridioid was obtained from *Ajuga reptans* L.[13]

Harpagide (3). To **2** (30 mg, 0.082 mmol) in MeOH (20 mL) was added NaH (oil dispersion 57−63%). The reaction mixture was stirred at room temperature for 20 min, the volume was reduced to 8 mL in vacuo, and the reaction mixture was washed with *n*-hexane in order to remove the oil. The MeOH layer was concentrated, and the residue was purified by Si gel column cromatography to yield **3** (25 mg, 83%) as colorless crystals. The structure of **3** was confirmed by comparison of both spectral and physical data with previously published values.[1]

Dihydrocatalpol (4). To **1** (200 mg, 0.55 mmol) in MeOH (50 mL) was added Pt/C (5%) (20 mg). The suspension was stirred vigorously under hydrogen (1 atm). When the consumption of H_2 had subsided, the mixture was filtered through a thin layer of Si gel over Celite and concentrated to yield **4** (170 mg, 85%) as an amorphous yellow solid identical in all respects with previously reported data.[1]

Per-*O*-acetylcatalpol (5). Compound **1** (150 mg, 0.41 mmol) was dissolved in dry pyridine (3 mL), acetic anhydride (3 mL) was added, and the mixture was stirred at room temperature for 2 days. The reaction mixture was partitioned between cold water and Et_2O. The organic layer was washed with aqueous $CuSO_4$ (5%) to remove pyridine followed by a solution of $NaHCO_3$ (5%) and water. After drying over Na_2SO_4, the residue was purified by Si gel column chromatography using mixtures of $CHCl_3$−MeOH in increasing polarities to yield compound **5** (120 mg, 80%) as yellow crystals: $[\alpha]_D^{25}$ −54.4 (*c* 0.45, $CHCl_3$); IR (KBr) ν_{max} 2942, 1754, 1369, 1224, 1043, 1014 cm^{-1}; ^{1}H NMR ($CDCl_3$, 200 MHz) δ 6.30 (1H, dd, J = 6.0, 1.9 Hz, H-3), 5.20 (1H, dd, J = 11.0, 7.3 Hz, H-4′), 5.05 (1 H, dd, J = 6.0, 4.0 Hz, H-4), 4.97 (1 H, br s, H-1′), 4.95 (1H, br s, H-2′), 4.82 (1H, d, J = 12.8 Hz, H-10b), 4.75 (1H, d, J = 9.1 Hz, H-1), 4.36 (1H, dd, J = 12.8, 3.65 Hz, H-6′a), 4.11(1H, dd, J = 12.8, 3.65 Hz, H-6b), 3.92 (1H, d, J = 12.8 Hz, H-10 a), 3.69 (1H, m, H-5′), 3.58 (1 H, br s, H-6), 3.56 (1H, br s, H-7), 2.51 (1H, dd, J = 9.1, 7.1, H-9), 2.42 (1H, m, H-5), 2.15−1.95 (18H, OAc); ^{13}C NMR ($CDCl_3$, 50.3 MHz) δ 171.0−169.0 (C, OAc), 140.5 (CH, C-3), 103.3 (CH, C-4), 96.4 (CH, C-1′), 94.2 (CH, C-1), 86.9 (CH, C-6), 72.5 (CH, C-3′), 72.1 (CH, C-5′), 70.5 (CH, C-2′), 68.2 (CH, C-4′), 62.8 (CH_2, C-10), 62.0 (C, C-8), 61.0 (CH_2, C-6′), 57.8 (CH, C-7), 41.4 (CH, C-9), 35.6 (CH, C-5); *anal.* C 52.73%, H 5.70%, calcd for $C_{27}H_{34}O_{16}$, C 52.70%, H 5.54%.

10,6′-Di-*O*-oleylcatalpol (6). To **1** (100 mg, 0.28 mmol) in CH_2Cl_2 (10 mL) were added oleic acid (160 μL, 0.50 mmol), *N*,*N*-dicyclohexylcarbodiimide (284 mg, 1.38 mmol), and 4-(dimethylamino)pyridine (170 mg, 1.39 mmol).[30] The reaction mixture was stirred at room temperature for 1 day. The mixture was filtered to remove the precipitated dicyclohexylurea. The filtrate was concentrated, and the residue was purified by Si gel column chromatography using mixtures of *n*-hexane−EtOAc in increasing polarities to yield 20 mg (20%) of compound **6** as a colorless oil: $[\alpha]_D^{25}$ −44.7 (*c* 0.15, $CHCl_3$); IR (KBr) ν_{max} 2927, 2856, 1741, 1459, 1247, 1166, 1093 cm^{-1}; ^{1}H NMR ($CDCl_3$, 200 MHz) δ 6.25 (1H, dd, J = 6.0, 1.9 Hz, H-3), 5.32 (4 H, H-9″, H-9‴, H-10″, H-10‴), 1.30 (56 H, −CH_2-oleyl); ^{13}C NMR ($CDCl_3$, 50.3 MHz) δ 174.3 (C, C-1″or C-1‴), 173.4 (C, C-1″or C-1‴), 140.2 (CH, C-3), 130.0 (CH, C-9″ and C-9‴), 129.6 (CH, C-10″ and C-10‴), 103.8 (CH, C-4), 96.0 (CH, C-1′), 93.7 (CH, C-1), 87.0 (CH, C-6), 74.7 (CH, C-3′), 74.1 (CH, C-5′), 70.2 (CH, C-2′), 68.9 (CH, C-4′), 64.8 (C, C-8), 61.8 (CH_2, C-6′), 57.6 (CH_2, C-10), 42.0 (CH, C-9), 35.7 (CH, C-5), 34.2−22.6 (28 carbons, −CH_2-oleyl moiety), 14.1 (CH_3, C-18″ and C-18‴); *anal.* C 69.06%, H 10.02%, calcd for $C_{51}H_{82}O_{12}$, C 69.07%, H 9.25%.

6,2′,3′,4′-Tetra-*O*-acetyl-10,6′-di-*O*-(*tert*-butyldiphenylsilyl)catalpol (7). To **1** (100 mg, 0.28 mmol) dissolved in CH_2Cl_2 (25 mL) were added *tert*-butylchlorodiphenylsilane (200 μL, 0.54 mmmol) and imidazole (200 mg, 2.9 mmol). The mixture was stirred overnight at room temperature and then was partitioned between H_2O and Et_2O. The organic layer was dried and concentrated, and the residue was purified by Si gel column chromatography using mixtures of *n*-hexane−EtOAc in increasing polarities to yield 10,6′-di-*O*-(*tert*-butyldiphenylsilyl)catalpol (65 mg, 65%) as a colorless oil. The silylderivative was peracetylated by standard techniques to yield compound **7** (35 mg, 54%) as an amorphous yellow solid: $[\alpha]_D^{25}$ −22.3 (*c* 0.20, $CHCl_3$); IR (KBr) ν_{max} 3072, 2931, 2858, 1760, 1652, 1473, 1429, 1218, 1114, 823, 701 cm^{-1}; ^{1}H NMR ($CDCl_3$, 200 MHz) δ 7.8−6.9 (20H, aromatic protons), 6.32 (1H, dd, J = 6.0, 1.9 Hz, H-3), 5.15−4.65 (6H, m, H-4′, H-4, H-1′, H-2′, H-10b, H-1), 4.30−4.05 (3H, m, H-6a, H-6b, and H-10a), 3.65−3.30 (3H, m, H-5′, H-6, and H-7), 2.60−2.45 (2H, m, H-9, and H-5), 2.10−1.80 (12 H, s, OAc), 1.15−1.10 (18H, *tert*-butyl protons); *anal.* C 67,49%, H: 6,88%, calcd for $C_{55}H_{66}O_{14}Si_2$, C 67.48%, H 6.74%.

Insects. Experiments were conducted using established colonies of *Tribolium castaneum* Herbst. (Coleoptera: Tenebrionidae). Larvae were reared on a mixture of flour, yeast, and starch (3:3:1) at 25 ± 1 °C, 65% relative humidity, and 16:8 (L:D) photoperiod.

Insect Bioassays. Topical Applications. These were performed using fifth instar larvae of *T. castaneum* randomly selected. Test solutions were topically applied to the ventral surface of the thoracic segments with a Hamilton microsyringe (1 μL/larvae; equivalent to 60 μg of the assayed compound).[12] Controls were treated with the solvent alone. There were three replicates of 10 larvae for each individual compound tested. After treatment, insects were placed into plastic vials (10 cm diameter × 7 cm high) containing food and held at 25 ± 1 °C with a 16:8 (L:D) photoperiod. The duration of the pupal stage (in days) and larval mortality were recorded every 24 h for 30 days (end-point of the experiment). The appearance of morphological alterations was documented. Results are shown in Table 1.

Flour Disk Bioassay. Flour disks were prepared using 200 μL of a stirred suspension of wheat flour in water (20 g in 50 mL).[14] Using water as solvent, solutions of each compound (**1**−**3**) (200 μg/disk in aliquots of 5 μL) were applied. Controls were treated using the solvent alone. The solvent was allowed to evaporate for 24 h, and two disks of the same treatment were placed in each plastic vial (diameter 3 cm, height 2 cm). The disks were weighed, and a group of 10 weighed unsexed adults of *T. castaneum* was added to each vial. Five replicates were set up for each compound and control. After 5 days, the flour disks and live insects were weighed again, and survival of the insects was recorded. Nutritional indices were calculated as previously described:[14] relative growth rate (RGR) = $(A - B)/(B \times \text{day})$, where A = weight of live insects on the fifth day (mg)/number of live insects. B = original weight of insects (mg)/number of insects at the begining of bioassay. Relative consumption rate (RCR) = $D/(B \times \text{day})$, where D is biomass ingested (mg)/number of live insects on the fifth day. Efficiency of conversion of ingested food = (ECI) (%) = (RGR/RCR) × 100. Feeding deterrence index (FDI) (%) = $[(C - T)/C] \times 100$, where C = consumption of control disk, and T = the consumption of treated disks. Results are shown in Table 2.

Statistical Analysis. Data of the topical bioassay and flour disks were treated by an ANOVA test. Differences between treatments were checked by using a least significant difference test (LSD) at the $p < 0.05$ level.

PCR Assays. The assayed compounds were dissolved in DMSO. The PCR master mixture consisted of 40 mM Tris-acetate pH 8.3 containing 1 mM EDTA, 25 mM $MgCl_2$, 4.0 U of *taq* DNA polymerase (Promega Co., Madison, WI), 20 mM each oligonucleotide primer, and 2.5 mM each desoxynucleotide triphosphate (dNTP). Inhibition studies were carried out with varying compound concentrations. All PCRs were done in 20 μL reaction volumes. The sequence of the sense primer was 5-TTG TTT GGT GTA TGG CTT GT-3, and the antisense primer, 5-CTT AGA GAA ATG GAC ACC TT-3. Thermocycling conditions consisted of 35 cycles of denaturation at 95 °C for 1 min followed by primer annealing at 56 °C and primer extension at 72 °C for 2 min.

Analysis of PCR Products. Relative intensities of ethidium bromide stained PCR products were analyzed by using the optical scanner and the image program. The image of stained agarose gel was captured using a Polaroid photography camera and then was scanned (Hewlett-Packard 3200 C). The digitized band images were processed using the Image processing program (Scion Image, public domain program); IC_{50} values were determined by the GraphPad Prism program. Results are shown in Table 3.

Theoretical Calculations. All theoretical calculations were performed using PC Spartan Pro software on a PC with an AMD Athlon(tm) processor. The Spartan Pro routine for conformational search was based on the Monte Carlo method, and the Merck molecular force field (MMFF94) was used to perform the search.[27] The more stable MMFF conformer was minimized using a density functional method: perturbative Becke−Perdew (pBP), with a numerical polarization basis set (DN*). HOMO and LUMO were calculated at 0.032 as isovalue, the electron density was estimated at 0.002 electrons/au^3 as isovalue, and the molecular electrostatic potential was calculated at ±20 kcal/mol as isovalue. Detailed information on the computational procedures is obtainable from the authors.

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