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Phytotoxicity of Quassinoids: Physiological Responses and Structural Requirements

F. E. Dayan,*[,1] S. B. Watson,* J. C. G. Galindo,† A. Hernández,‡ J. Dou,§[,2] J. D. McChesney,§[,2] and S. O. Duke*

**Natural Products Utilization Research Unit, United States Department of Agriculture, Agricultural Research Service, P.O. Box 8048, University, Mississippi 38677; †Department of Organic Chemistry, Faculty of Sciences, University of Cádiz, República Saharaui s/n, Apartado 40, 11520–Puerto Real, Cádiz, Spain; ‡Facultad de Ciencias, Departamento Biologia Vegetal y Ecología, Universidad del Pais Vasco, Apartado 644, 48080–Bilbao, Spain; and §Department of Pharmacognosy, School of Pharmacy, University of Mississippi, University, Mississippi 38677*

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Quassinoids are naturally occurring compounds with phytotoxic and allelopathic activities isolated from several plant species of the Simaroubaceae family. There is relatively little information about the structural characteristics imparting biological activity to these diterpene lactones or their effects on plants. We studied the effects of the oxymethylene ring substitution on the biological activity and molecular conformation of several quassinoids. The presence of this functional group had a great effect on the three-dimensional conformation and biological activity of these natural products. In the absence of the oxymethylene ring, the quassinoids were more planar and had little phytotoxicity. In addition, this bridging function introduced a new reactive center that caused the terpene backbone to bend. Molecules with such conformation were highly phytotoxic, reducing root growth of lettuce (*Lactuca sativa*) and affecting all stages of mitosis in onion (*Allium cepa*) root tips.

Key Words: allelopathy; mode of action; structure–activity relationships; phytotoxicity; SAR; computer modeling; natural products; quassinoids; herbicide.

INTRODUCTION

Agrochemicals used to control weeds target a relatively limited number of molecular sites (1). Evolution of weed resistance to many commercial herbicides is becoming increasingly problematic because resistance to one herbicide may preclude the use of other classes of chemicals targeting the same site of action. In addition, several commercial herbicides have been or will soon be removed from the agrochemical market because of their impact on the environment or the cost of re-registration. As a result, the number of chemical tools available to manage weeds is becoming limited.

Natural products with novel modes of action may be useful for weed management, used directly either as herbicides or as leads for synthetic compounds. Duke *et al.* (1) reported that there is little overlap between the known molecular sites affected by synthetic and natural phytotoxins. Thus, plant-derived secondary compounds may provide a source of environmentally safer herbicides with novel molecular sites of action. This approach has been successfully used to develop the herbicide cinmethylin from a natural monoterpene (2). The reported allelopathic activity of certain quassinoids (3, 4), and their potential herbicidal activity (5) led us to this investigation of the structural and molecular features of herbicidal quassinoids.

Quassinoids are naturally occurring compounds isolated from *Eurycoma longifolia* (6–9), *Brucea* spp. (10), *Quassia indica* (11), *Castela* spp. (12), and *Ailanthus* spp. (5, 13).

[1] To whom correspondence should be addressed: fdayan@ag.gov.

[2] Current address: Natural Products Chemistry, NaPro BioTherapeutics, Inc., 6304 A Spine Road, Boulder, CO 80301.

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Many biological activities, such as anticancer (5), antimalarial (14), anti-leukemic (6), antitubercular (15), antiviral (16), insecticidal (17), fungicidal (18), and herbicidal (3, 5), have been reported for quassinoids.

Recent reports indicate that the mode of action of quassinoids is associated with inhibition of the plasma membrane NADH oxidase (19), but the few structure–activity relationship studies available are limited to their potential pharmaceutical applications (15, 20). There is, however, no information about the structural requirement for biologically active quassinoids as related to their effects on plants. In this paper, we report that the phytotoxic effects of active analogues was accompanied with inhibition of mitosis and that the presence of an oxymethylene bridge between C8 and C11 of the diterpene backbone is a requirement for the herbicidal activity of quassinoids used in this study.

MATERIALS AND METHODS

Origin of Quassinoids Used in This Study

The quassinoids used in this study were isolated from *Castella texana* (T. &. G.) Rose (syn. *C. tortuosa* Liebm, *C. nichoslonii* Hook. *B. texana* T. & G.) collected in July 1992, in Terrell County, Texas, by Dou *et al.* (21). A voucher specimen is deposited at the Institute for Botanical Exploration, Starkville, Mississippi.

Effects of the Quassinoid Analogues on Plant Growth

Dose–response curves for each quassinoid were obtained for both lettuce (*Lactuca sativa* cv. Iceberg) and bentgrass (*Agrostis stolonifera* cv. penncross) using a 24-well plate bioassay (22). Each quassinoid was dissolved in acetone to make a 10 mM stock which was diluted with distilled water to obtain final concentrations of 3, 10, 33, and 100 μM in the wells. All wells, including control treatments, received the same amount of acetone (1% v/v). Plates were incubated at 25 $\pm$ 2°C under fluorescent lights with a 16-h photoperiod at 400 μmol m^{-2} s^{-1}. Plants were rated visually on a scale of 0 to 5 (no effect to 100% inhibition of growth) after a 7-day incubation.

The three active quassinoid analogues identified in the previous experiment, chaparrinone (4), glaucarubolone (5), and holacanthone (6), were tested at 10 μM on lettuce seeds in 100 $\times$ 15-mm petri dishes. Test solutions of 10 μM quassinoids (obtained by adding 9 μl of a 10 mM stock solution dissolved in acetone to 9 ml of dH_2O) were applied in 3-ml volumes unto three petri dishes lined with 90-mm-diameter No. 1 Whatman filter paper. Control plates received the same amount of acetone.

Lettuce seeds (0.6 g) were scattered on top of the wet filter paper and the dishes were wrapped with parafilm. Plates were maintained in total darkness for 24 h, exposed to 800 μmol m^{-2} s^{-1} light for 1 h to induce germination, and returned to darkness until germination started. At that point, the plates were incubated at 25 $\pm$ 2°C under fluorescent lights with a 16-h photoperiod at 400 μmol m^{-2} s^{-1}.

Plant responses to the test compounds were determined 8 days after treatment by measurement of root and hypocotyl lengths and chlorophyll contents. Chlorophyll was extracted overnight at room temperature from 15 pairs of cotyledons per treatment in 3 ml dimethyl sulfoxide (23) and chlorophyll concentrations determined spectrophotometrically according to Arnon (24).

Effect of Quassinoids on Onion Root Cell Division (Mitotic Index)

Onion (*Allium cepa* L. cv. Evergreen bunching) seeds were germinated as previously described (22) in the presence of 10 μM each of the analogues at 25°C under a 14-h photoperiod. Root tips were prepared according to Armbruster *et al.* (25) and mitotic analysis was performed on 1000 cells per slide (3000 cells per treatment).

Effect of Quassinoids on Membrane Integrity

The method of Duke and Kenyon (26) was used to measure cellular leakage in order to assess membrane integrity. Fifty 4-mm cotyledon discs of 7- to 10-day-old cucumbers were placed on a 1% sucrose/1mM Mes–NaOH buffer (pH 6.8) containing 0 or 100 μM of test compounds. Plates were incubated in darkness

for 20 h prior to exposure to light (400 μmol $m^{-2}\,s^{-1}$) for an additional 8 h. Electrical conductivity of the bathing solutions was measured at regular intervals before and after exposure to light. The experiment consisted of three replicates and the experiment was repeated.

Computer Modeling of Quassinoids

The six quassinoid analogues used in this study were built using fragments from the fragment library provided in Sybyl 6.3 (Tripos associates, St. Louis, MO) on a Silicon Graphics O_2 250-MHz R10000. Each molecule was assigned appropriate stereochemistry, charges were calculated using the method of Gasteiger-Hückel, and structures were minimized using the Tripos force field to obtain a low energy conformers. Minimization was initiated with Simplex (a non-derivative-based procedure) for 100 iterations, followed by Powell (a first-derivative-based method) for 1000 iterations, until convergence criteria of 0.005 were met.

The structure was then subjected to full geometry optimization via MOPAC (Quantum Chemistry Program Exchange 560, version 6.0, Department of Chemistry, Indiana University, Bloomington, IN) using AM1 (Austin Model) parameterization. Molecules were aligned using the FIT-ATOM function of Sybyl by overlaying the atoms composing the lactone ring common in all analogues. The lowest unoccupied molecular orbital (LUMO) maps were obtained by displaying the individual contribution of each atom onto the electron density surface using the molecular modeling software Spartan (Wavefunction, Inc., Irvine, CA).

RESULTS

Physiological Responses to Quassinoids

The quassinoids quassin (1), neoquassin (2), and picrasin B (3) (Fig. 1) did not affect germination (data not shown) or growth of either bentgrass (*A. stolonifera*) or lettuce (*L. sativa*) at up

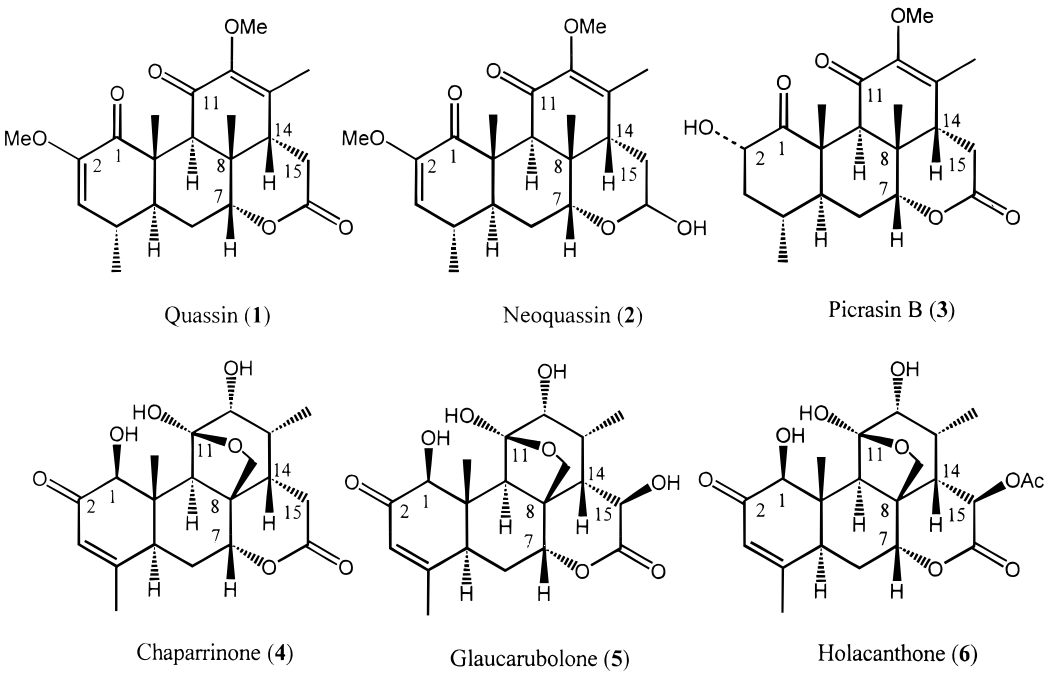

FIG. 1. Structures of the six quassinoids tested. Compounds **1–3** are based on the picrasane backbone and compounds **4–6** are based on the chaparrinone backbone.

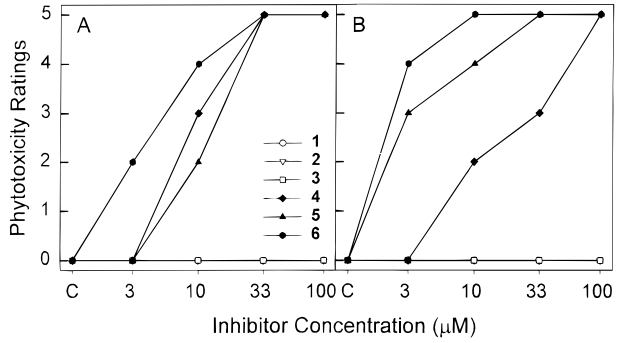

FIG. 2. Effects of quassinoids on development of (A) lettuce (*Lactuca sativa*) and (B) bentgrass (*Agrostis stolonifera*). Ratings ranged from 0 to 5 for no effect to 100% inhibition.

to 100 μM (Fig. 2). In contrast, chaparrinone (4), glaucarubolone (5), and holacanthone (6) (Fig. 1) were highly phytotoxic (Fig. 2), inhibiting plant growth at 10 μM and killing the test species at 100 μM. This level of activity is comparable to many commercial herbicides. Holacanthone had the highest level of activity of the quassinoids tested on bentgrass and lettuce (Fig. 2). In spite of their strong inhibitory activities, these molecules did not reduce the germination rates (data not shown).

Quassinoids **4–6** were selected for further characterization of their physiological effects on plants. Root growth of plants treated with 10 μM of **4**, **5**, and **6** were 80, 66, and 75% shorter than controls, respectively (Table 1). A similar trend was observed in hypocotyl development, with inhibition ranging from 45 to 65%, relative to control. Finally, a dramatic bleaching of the cotyledonary tissues was observed in lettuce seedlings treated with **5** and **6**, with about 90% lower chlorophyll content, relative to the control. Interestingly, **4** did not cause a reduction in chlorophyll concentration in spite of being as strong a growth inhibitor as the other two quassinoids tested (Table 1).

The three quassinoid analogues with no *in vivo* activity (**1–3**) had little observable effects on any phase of mitosis in onion root tips (Fig. 3). Conversely, the three biologically active analogues strongly inhibited mitosis. Holacanthone (**6**), the most toxic of the quassinoids tested, severely inhibited all stages of mitosis. The other two phytotoxic quassinoids (**4** and **5**) were slightly less active and did not affect prophase at the concentration tested.

TABLE 1
Effects of 10 μM Quassinoids on Root and Hypocotyl Length and Chlorophyll Content of 7-Day-Old Lettuce Seedlings

Treatment	Root length (mm)	Hypocotyl length (mm)	Chlorophyll (μg/gFW)
Control	30.7 ± 7.2	10.2 ± 2.1	691 ± 129
4	6.0 ± 1.4	5.6 ± 1.2	528 ± 76
5	10.4 ± 3.2	3.6 ± 0.8	64 ± 16
6	7.6 ± 2.6	4.3 ± 0.9	74 ± 26

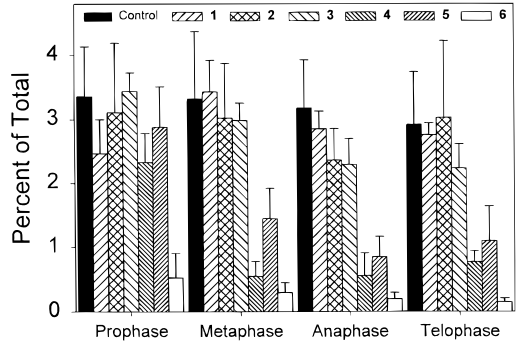

FIG. 3. Distribution of phases of mitotis in onion root tips treated with the quassinoids.

Molecular Characterization of the Quassinoids

All the quassinoids tested possess a lactone ring between C-7 and C-14 in ring A, with the exception of **2** that has a lactol moiety. All except **3** also have an α, β-unsatured ketone system in ring A (Fig. 1). The structural differences are found in the substitution pattern within each group. Picrasane-type quassinoids have a more accessible α,β-unsatured ketone group (double bond at C2–C3) than the chaparrinone- type (double bond at C3–C4). Differences are more evident in ring C, with picrasanes having an additional α, β-unsatured ketone system between C12 and C13 and chaparrinones having an oxymethylene bridge between C11 and C20. Finally, chaparrinone-type compounds have free hydroxyl groups while picrasane-type compounds have mostly methoxyl substituents (Fig. 1).

Overall molecular parameters, such as Connolly solvent accessible volumes and dipole moments, were similar in all compounds (Table 2). However, the presence of the oxymethylene bridge in **4–6** caused the otherwise planar ring C of **1–3** to bend. The hydroxyl-containing C12 moved downward, the hemiketal carbon (C11) moved upward, and the oxygen of the oxymethylene bridge protruded on the upper surface of the quassinoids (Figs. 4-A and 4B) recovering the chair conformation in ring C.

While there were no significant differences in total energy (TE) and electrostatic potential (EP) between active and inactive compounds (Table 2), the oxygen of the oxymethylene bridge created a highly electronegative region on ring C that was absent in the inactive compounds (Figs. 5A and 5B). Finally, highest occupied molecular orbital (HOMO) and LUMO values were similar for all quassinoids analyzed (Table 2), but molecular mapping of individual LUMO contributions showed different localization on the surface of inactive (Fig. 6A) and active (Fig. 6B) quassinoids.

DISCUSSION

The two sets of quassinoids tested differed greatly in their biological activity. Picrasane-type (**1–3**) were essentially inactive, whereas the chaparrinone-type (**4–6**) were highly phytotoxic (Fig. 2). Holacanthone was the most active of the quassinoids tested against lettuce and bentgrass. Lin *et al.* (5) suggested that chaparrinone was more active than holacanthone, but their conclusion was made by comparing the activity of chaparrinone in their bioassay on several weeds to the activity of holacanthone reported in another study on grape seedlings (18). Hailanthone, another chaparrinone-type quassinoid, was found to have broad weed spectrum activity when applied either as preemergence or postemergence herbicide. Hailanthone provided

TABLE 2
Molecular Properties of the Quassinoids Used in This Study

Parameters	Compounds					
	1	**2**	**3**	**4**	**5**	**6**
MW	388	390	376	378	394	436
$\text{Connolly}_{\text{VOLUME}}$, A^3	389	396	369	363	372	412
Dipole, Debye	4.47	3.15	4.64	3.83	3.28	4.88
Total Energy, eV	1.39	1.10	1.27	1.58	1.9	1.98
Ionization Potential, eV	9.31	9.15	9.40	10.20	10.23	10.20
e_{HOMO}, eV	−9.31	−9.15	−9.40	−10.20	−10.23	−10.19
e_{LUMO}, eV	−0.42	−0.19	−0.45	−0.42	−0.21	−0.41
EP_{MAX}, eV	0.17	1.11	1.09	1.35	1.40	1.51
EP_{MIN}, eV	−1.66	−1.52	−1.47	−2.10	−2.04	−2.06

Note: $\text{Connolly}_{\text{VOLUME}}$, connolly solvent accessible surface volume; e_{HOMO}, energy of highest occupied molecular orbital; e_{LUMO}, energy of lowest unoccupied molecular orbital; EP_{MAX} and EP_{MIN}, maximum and minimum electrostatic potentials.

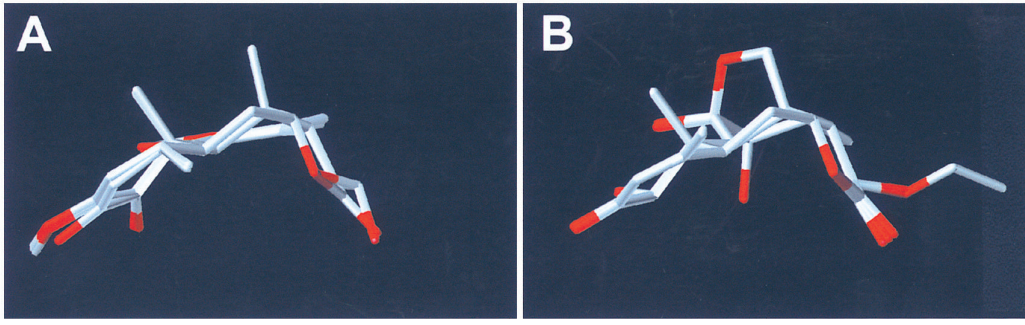

FIG. 4. Three-dimensional conformation of the quassinoids aligned on the lactone substructure: (A) overlay of the biologically inactive picrasane quassinoids (compounds **1±3**) and (B) overlay of the biologically active chaparrinone-type quassinoids (compounds **4±6**). Notice the presence of the oxymethylene bridge between C8 and C11 in the active molecules and the absence of the oxygen bridge in the inactive molecules.

100% weed control of green foxtail (*Setaria viridis*) and sicklepod (*Cassia obtusifolia*) at rates of 0.125 kg/ha).

Reduction of growth is probably associated with the strong inhibition of mitosis caused by quassinoids (Fig. 3). The lack of effect of **4** and **5** on prophase indicates that these quassinoids do not prevent induction of the cell cycle (27). This is strikingly different from the effects observed with phytotoxic sequiterpene endoperoxide lactones such as artemisinin (22). In addition, the strong inhibition of mitosis observed in onion root tips cells treated with the quassinoids **4±6** was not accompanied with aberrant mitotic conformations that were evident in cells treated with several sesquiterpene endoperoxide lactones (22). Our data suggest that sesquiterpene and quassinoid lactones do not have the same mode of action in plants. Moreover, it appears that **6** may have a different mode of action from that of **4** and **5** since this compound strongly inhibited prophase. The morphological effects of hailanthone on *Eragrostis tef*, causing roots to be shorter and swollen, are reportedly similar to those obtained with trifluralin, a herbicide known to disrupt mitotic processes (5, 28).

Quassinoids **4** and **5** caused a reduction in lettuce chlorophyll content similar to that

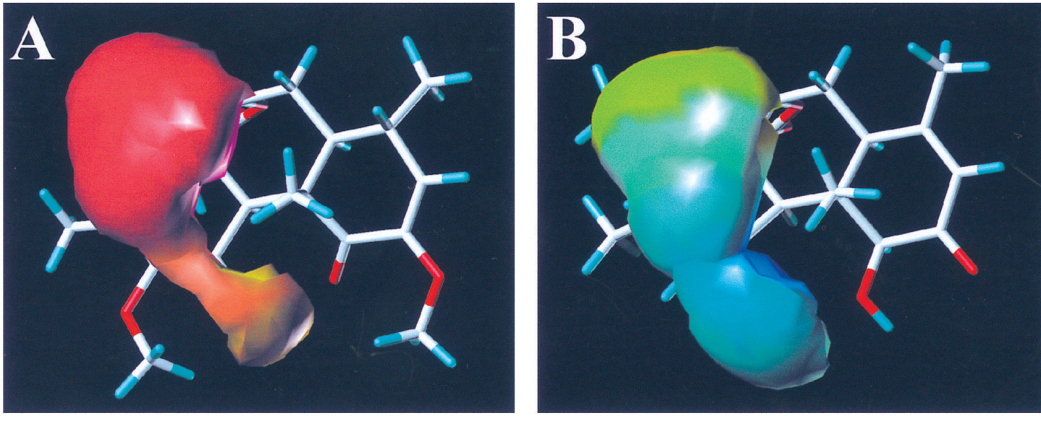

FIG. 5. Electrostatic potential maps of the region of the oxymethylene bridge in (A) the biologically inactive picrasane-type quassinoids (compounds **1±3**) and (B) of the biologically active chaparrinone-type quassinoids (compounds **4±6**). Notice the presence increased electronegativite potential resulting from the presence of the oxymethylene bridge. Electrostatic potentials range from electronegative (purple) to electropositive (red).

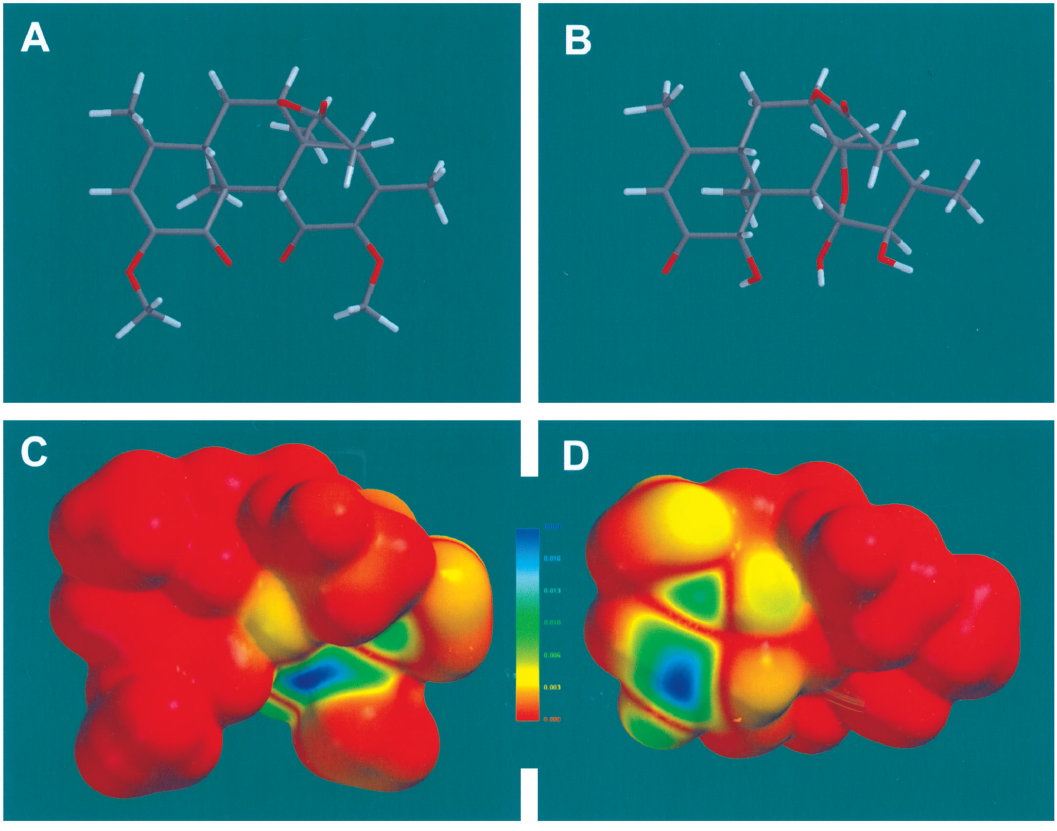

FIG. 6. Three-dimensional conformation of (A) quassin **1** and (B) chaparrinone **4** showing the similarity in carbon skeleton between active and inactive quassinoids, respectively. Lowest unoccupied molecular orbital (LUMO) maps displaying the individual contribution of each atom to the LUMO onto the electron density surface of (C) the biologically inactive quassin **1** and (D) the biologically active chaparrinone **4**. Notice the difference in the location of the LUMO. The color indicates the absolute LUMO value, with red corresponding to a zero value and blue corresponding to a maximum value.

observed with artemisinin and several sesquiterpene analogues (22), but there is no evidence that these compounds inhibited chlorophyll synthesis directly. Bleaching of photosynthetic tissues is often associated with cellular and subcellular membrane degradation (26). However, none of the compounds caused cellular leakage in cucumber cotyledon disks, suggesting that membrane integrity was not disrupted (data not shown). These findings were unexpected since Morre *et al.* (19) reported that one of the quassinoids used in this study (4) was a potent inhibitor of plasma membrane NADH oxidase. If the primary mode of action of quassinoids was associated with destabilization of membranes by inhibiting plasma membrane NADH oxidase, significant electrolyte leakage should have been observed.

Structural similarities within each set of quassinoids were evident when the molecules were aligned along their lactone ring substructure (Figs. 4A and 4B). While the overall arched conformation of the quassinoids, with the lactone ring D bending down, was common to all

the molecules, presence or absence of the oxymethylene bridge greatly affected the conformation of ring C. Indeed, the α,β-unsatured ketone system found in **1–3** rendered ring C planar (Fig. 4A), whereas the oxymethylene bridge found in **4–6** caused the ring to assume a more twisted conformation, close to the typical cyclohexane chair conformation (Fig. 4B).

Differences in activity between quassinoids does not seem to be associated with accessibility to the target site, since overall molecular properties were similar among the six quassinoids (Table 2). TE of the two sets, which can be compared without major concern because of their structural similarities, were also similar. Nominally higher TE of **4–6**, relative to **1–3**, may be associated either with the loss of the π-conjugated system in the upper ring and/or with a slightly higher reactivity (Table 2).

Natural products containing α,β-unsatured carbonyl systems may interact with other biological molecules via Michael additions occurring at the β-carbon of the olefin bond (29–32). This mechanism of action does not seem to be operative with these quassinoids since both active and inactive molecules possess α,β-unsatured carbonyl systems. Moreover, the biologically inactive **1–3** have an additional and readily accessible α,β-unsatured ketone system in ring C.

Charge distribution at C-11, where major structural differences associated with the presence of the hemiketal functionalization, was essentially the same between the two sets of quassinoids (data not shown). Thus, nucleophilic attacks at this very hindered point do not seem to be a key factor for the difference in activity between the two classes of quassinoids tested.

While there was no large difference in the EP of the whole molecules, the two sets of quassinoids differed dramatically in the region of the oxymethylene bridge (i.e., C-8, C-9, C-11, and C-21 and the oxygens of the hemiketal group). The biologically inactive picrasane-type compounds have a relatively positive EP domain (Fig. 5A), whereas this region is more electronegative in the active chaparrinone-type quassinoids (Fig. 5B).

According to perturbation molecular orbital theory (PMO), the course of a chemical reaction can be predicted by analyzing how the approaching reactant molecules mutually perturb the molecular orbitals of the reaction partner. PMO theory also states that the LUMO of electron-accepting molecules interact with the HOMO of the electron-donating molecule (33–35). While there is essentially no difference between the LUMOs of the active and inactive compounds (Table 2), the individual LUMO contribution of each atom differed between the two sets of quassinoids. The LUMO region was associated with the α,β-unsatured ketone system of ring C in the inactive compounds (Fig. 6C), whereas this region was associated with the α,β-unsatured ketone system of ring A of the active compounds (Fig. 6D). In fact, analysis of the individual LUMO coefficients indicates that the three carbon involved in the enones on ring C for the picrase-type and ring A for the chaparrinone-type quassinoids contributed 73 and 77% of the total LUMO, respectively. As expected, the highest LUMO contribution was associated to the β-carbon of the enones.

Finally, the higher number of hydroxyl groups in chaparrinone-type compounds may also influence the solubility of these compounds. Thus, differences in activity between the two sets of compounds seem to be due to the greater negative EP that the oxymethylene bridge and hemiketal functionalization introduces.

In summary, we have demonstrated that some quassinsoids containing an oxymethylene bridge are highly phytotoxic, resulting in strong inhibition of growth and mitosis and plant death. Quassinoids without the bridging function had no detectable activity in our assays. Computational chemistry demonstrates that the oxymethylene bridge strongly influences the three-dimensional structure of the molecule, resulting in a less planar structure and introducing a new reactive center. Although an oxymethylene bridge-containing quassinoid was shown to strongly inhibit plasma membrane NADH oxidase in animal cells, our results suggest a different molecular target site in plants. Considering the high

level of phytotoxicity of these compounds, further research to determine their molecular site of action is warranted.

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