Cyclopaldic Acid, Seiridin, and Sphaeropsidin A as Fungal Phytotoxins, and Larvicidal and Biting Deterrents against *Aedes aegypti* (Diptera: Culicidae): Structure–Activity Relationships

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Aedes aegypti L. is the major vector of the arboviruses responsible for dengue fever, one of the most devastating human diseases. From a preliminary screening of fungal phytotoxins, cyclopaldic acid (**1**), seiridin (**2**), sphaeropsidin A (**4**), and papyracillic acid (**5**) were evaluated for their biting deterrent and larvicidal activities against *Ae. aegypti* L. Because compounds **1**, **2**, **4**, and **5** exhibited mosquito biting deterrent activities and **1** and **4** demonstrated larvicidal activities, further structure–activity relationship studies were initiated on these toxins. In biting-deterrence bioassays, **1**, **2**, **4**, and **5**, 3,8-didansylhydrazone of cyclopaldic acid, **1F**, 5-azidopentanoate of cyclopaldic acid A, **1G**, the reduced derivative of cyclopaldic acid, **1H**, isoseiridin (**3**), 2′-*O*-acetylseiridin (**2A**), 2′-oxoseiridin (**2C**), 6-*O*-acetylsphaeropsidin A (**4A**), 8,14-methylensphaeropsidin A methyl ester (**4B**), and sphaeropsidin B (**4C**) showed activities higher than the solvent control. Sphaeropsidin B (**4C**) was the most active compound followed by **2A**, while the other compounds were less active. Biting-deterrence activity of compound **4C** was statistically similar to DEET. In the larvicidal screening bioassays, only compounds **1** and **4** demonstrated larvicidal activities. Based on LD_{50} values, compound **4** (LD_{50} 36.8 ppm) was significantly more active than compound **1** (LD_{50} 58.2 ppm). However, the activity of these compounds was significantly lower than permethrin.

Introduction. – *Aedes aegypti* L. (Diptera: Culicidae) is the major vector of dengue fever virus, which can attain epidemic levels resulting in high rates of human mortality. Currently, the only effective way of reducing the incidence of dengue fever is to control the vector mosquito, mainly by application of insecticides to its breeding place [1]. Repellents applied to skin or clothing are recommended as a means of personal protection against biting arthropods [2]. Toxicants can reduce the densities of mosquito and the risk of disease transmission. The synthetic repellent DEET (= *N*,*N*-diethyl-3-methylbenzamide) has been used widely since the 1950s to protect human beings against mosquitoes. Synthetic and natural insecticides, including pyrethroids, are approved by the EPA (Environmental Protection Agency) to be used as repellents, but only when applied on clothing [3].

However, pyrethroids used to control a wide range of arthropods are considered dangerous for agriculture and human health because of their toxicity [2]. These

problems increased by the resistance developed against pyrethroids or insecticides of any class and prompted the development of alternative control strategies. Therefore, great effort has been expended for the development of pest controls using naturally occurring compounds such as secondary plant and fungal metabolites. These are anticipated to overcome the problem of resistance to synthetic insecticides and are generally regarded as more environmentally friendly than their synthetic counterparts [4].

Many naturally occurring repellents and insecticides have potential for development into useful products with lowered risk to mammals and the environment [2][5–8].

In this respect, we initiated the screening of some fungal phytotoxins, cyclopaldic acid (**1**) [9], seiridin (**2**) [10][11], sphaeropsidin A (**4**) [12], obtained from *Seiridium cupressi* and *Diplodia cupressi*, fungi causing different forms of cankers of cypress (*Cupressus sempervirens* L.) [13], and papyracillic acid (**5**), obtained from *Ascochyta agropyrina* var. *nana*, which was proposed as mycoherbicide for the biocontrol of *Elytrigia repens* [14] (*Fig. 1*). We also carried out structure–activity relationship (SAR) studies on the three fungal phytotoxins **1**, **2**, and **4**, by preparing several known and new derivatives.

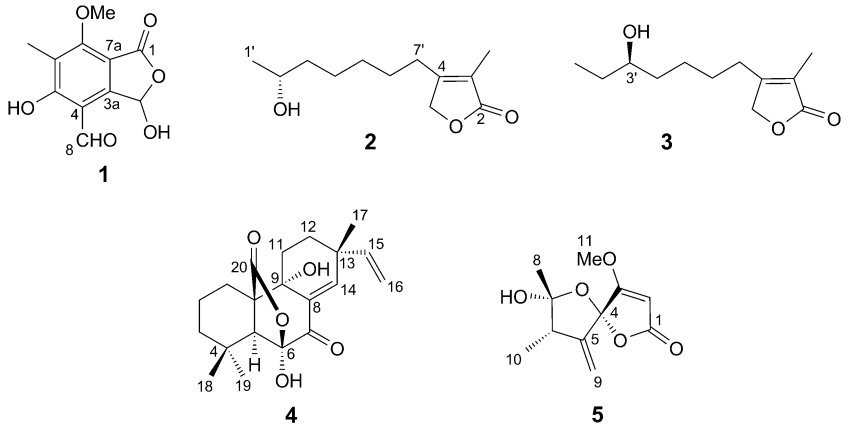


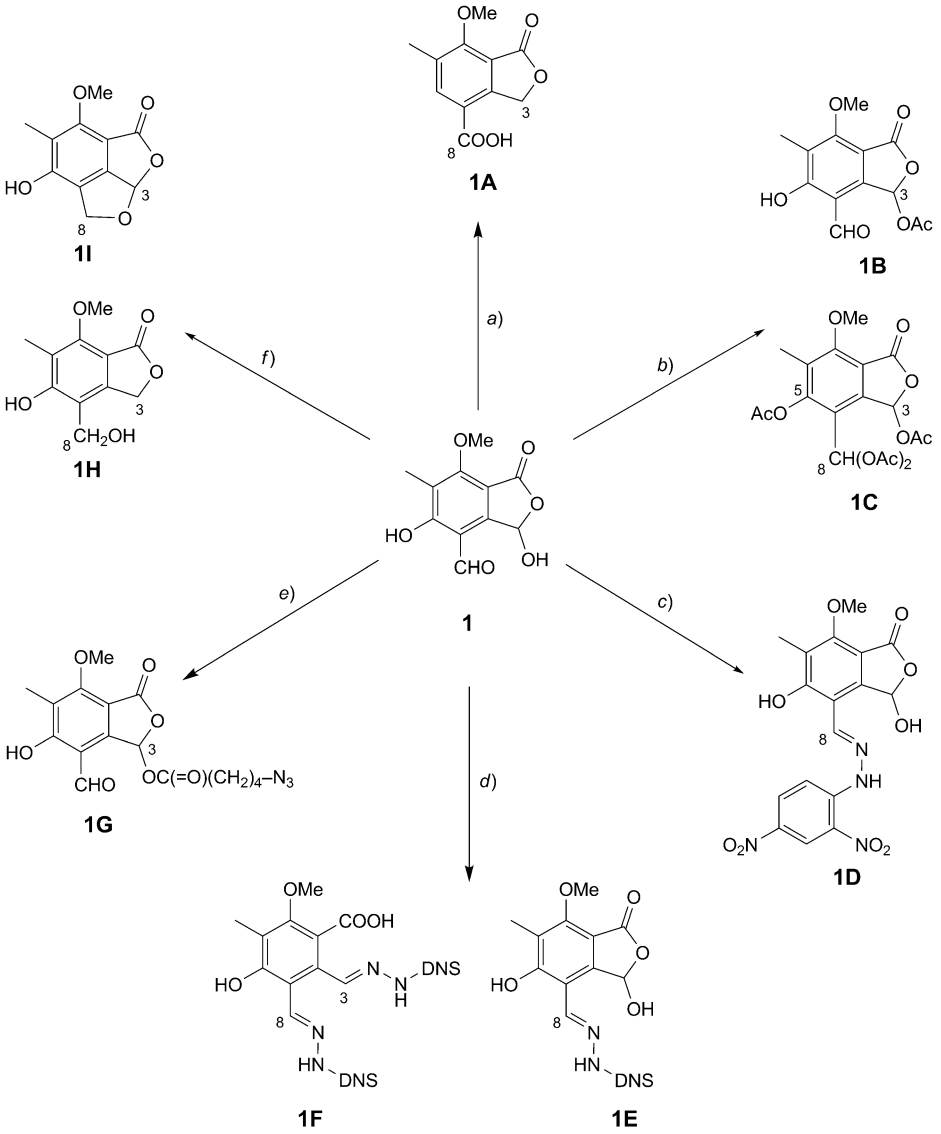
Fig. 1. *Structures of cyclopaldic acid, seiridin, isoseiridin, sphaeropsidin A, and papyracillic acid* (**1**–**5**, resp.)

Results and Discussion. – Cyclopaldic acid (**1**), and seiridin (**2**) and isoseiridin (**3**), and sphaeropsidin A (**4**) were obtained as white crystals, oily compound, and white needles, respectively, from the culture filtrates of *S. cupressi* and *D. cupressi* as described in [9][11][15].

In an effort to discover new natural compounds with the activity against *Ae. aegypti*, several known and new derivatives of **1**, **2**, and **4** were semisynthesized and evaluated for their biting-deterrent and larvicidal activities against *Ae. aegypti*.

Semisynthetic Derivatives of Cyclopaldic Acid (**1**; *Scheme 1*). By treatment with diluted NaOH and according to the well-known *Cannizzaro* reaction, compound **1** was converted into isocyclopaldic acid (**1A**; *Scheme 1*) [16][17], *via* the reduction of the

Scheme 1



a) 2N NaOH, reflux, 15 min. *b*) Ac_2O and anh. AcONa, reflux, 30 min. *c*) 2,4-$(NO_2)_2$–C_6H_3–NH–NH_2, 6N HCl, r.t., 1 h. *d*) Dansyl (= 5-(dimethylamino)naphthalene-1-sulfonyl) hydrazine (DNS–NH–NH_2), 80°, 3 h. *e*) Anh. CH_2Cl_2 and pyridine, *N*,*N*′-dicyclohexylcarbodiimide (DCC), 5-azidopentanoic acid, 0°, 1 h, r.t., 2 h. *f*) $NaBH_4$, 0°,10 min.

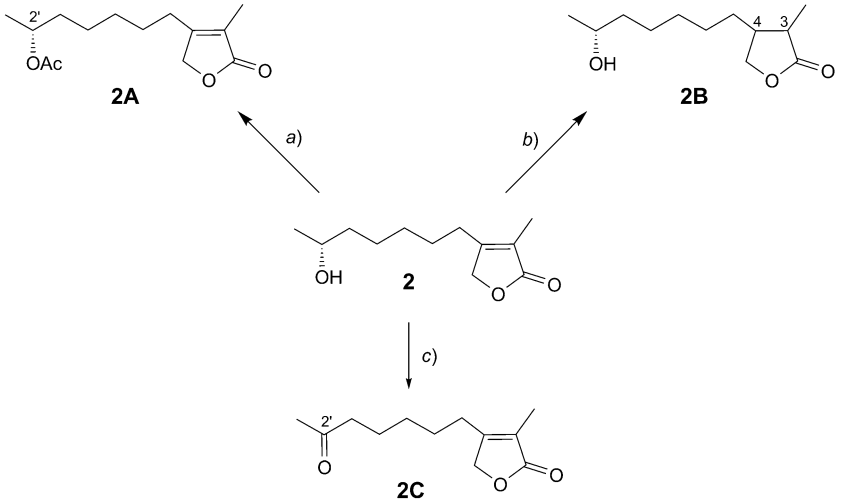
hemiacetal aldehyde at C(3) to a CH_2 group and the oxidation of the aldehyde at C(4) to a COOH group. The acetylation carried out with Ac_2O and anhydrous AcONa allowed the conversion of **1** to the 3-*O*-acetyl and 3,5,8-*O*,*O*′,*O*″-triacetyl-8-acetoxy

derivatives **1B** and **1C** (arbitrary atom numbering; *Scheme 1*) [16][17]. Of the derivatives **1B** and **1C**, the first was obtained by the acetylation of the hemiacetale OH group at C(3), and the latter by the acetylation of both the OH groups at C(3) and C(5), followed by acetoxylation of C(8) and finally the acetylation of the resultant hemiacetal OH group at the same C-atom. The reaction of **1** with a acid-saturated solution of (2,4-dinitrophenyl)hydrazine furnished the corresponding hydrazone **1D** (*Scheme 1*) [17], by conversion of the CHO group at C(4) to the corresponding *Schiff*'s base. A similar reaction of **1** with the dansylhydrazine (= [5-(dimethylamino)naphthalene-1-sulfonyl]hydrazine) allowed the preparation of the mono- and didansylhydrazones **1E** and **1F** (*Scheme 1*). These were previously reported, when they were used, together with other derivatives of **1**, to test the specificity of the antibodies produced by rabbit immunization with a conjugate of **1** with bovine serum albumin (BSA) [18]. However, the complete spectroscopic characterization of **1E** and **1F** is reported here for the first time. Derivative **1E**, as **1D**, was formed by the conversion of the CHO at C(4) to the corresponding *Schiff*'s base but the hydrazine N-atom of **1E** was linked to a dansyl group. The didansyl derivative **1F** underwent the same modification as **1E**, but also the opening of the butenolide ring occurred, generating the COO group at C(7a) and the CHO group at C(3a), which was successively converted to the same *Schiff*'s base at C(8). By esterification carried out with the 5-azidopentanoic acid and *N*,*N*′-dicyclohexylcarbodiimide (DCC), **1** was converted to the new derivative **1G** (*Scheme 1*), which, as **1A**, underwent the reversible esterification of the hemiacetal OH group at C(3). Finally by $NaBH_4$ reduction, **1** was converted to the two new reduced derivatives **1H** and **1I** (*Scheme 1*). Derivative **1H** displayed the expected reduction of both the hemiacetalized and lactonized aldehyde at C(3) and the aldehyde at C(4), the latter was converted to a primary OH group. Derivative **1I** was the result of the same reduction of the aldehyde only at C(4), and the subsequent H_2O elimination from the primary OH group at C(8) and that at C(3), generating the new trisubstituted dihydrofuran ring fused to the benzofuranone.

Semisynthetic Derivatives of Seiridin (*Scheme 2*). Seiridin (**2**), as previously reported [11], was converted to the 2′-*O*-acetyl and 3,4-dihydro derivatives **2A** and **2B**, respectively, by usual acetylation with AC_2O and pyridine, and catalytic hydrogenation (*Scheme 2*). The former was formed by the reversible esterification of the OH group at C(2′) (arbitrary atom numbering) of the hydroxyheptyl side chain at C(4) of the butenolide ring, while the reduction of butenolide ring converted **2** to the corresponding butanolide **2B**. The oxidation of **2** with *Jones* reagent [19] furnished the derivative **2C** (*Scheme 2*), which was previously prepared to conjugate seridin to BSA to form a suitable hapten to elicit specific antibodies by a rabbit immunization [20]. Compound **2C** displayed the presence of a C=O group at position 2′ of the heptyl side chain at C(4), which represents, differently from **2A**, a not reversible modification. Another modification of the heptyl side chain was the shift of the secondary OH group from C(2′) to C(3′) in isoseiridin (**3**), the natural isomer of **2** isolated together with seiridin from the culture filtrate of *S. cupressi*.

Semisynthetic Derivatives of Sphaeropsidin A (*Scheme 3*). Sphaeropsidin A (**4**) as described in [21], was converted by usual acetylation and reaction with ethereal CH_2N_2 to the well-known derivatives **4A** and **4B** (*Scheme 3*). Derivative **4A** was formed by the reversible esterification of the hemiketal OH group at C(6), while, in **4B**, this OH group

Scheme 2

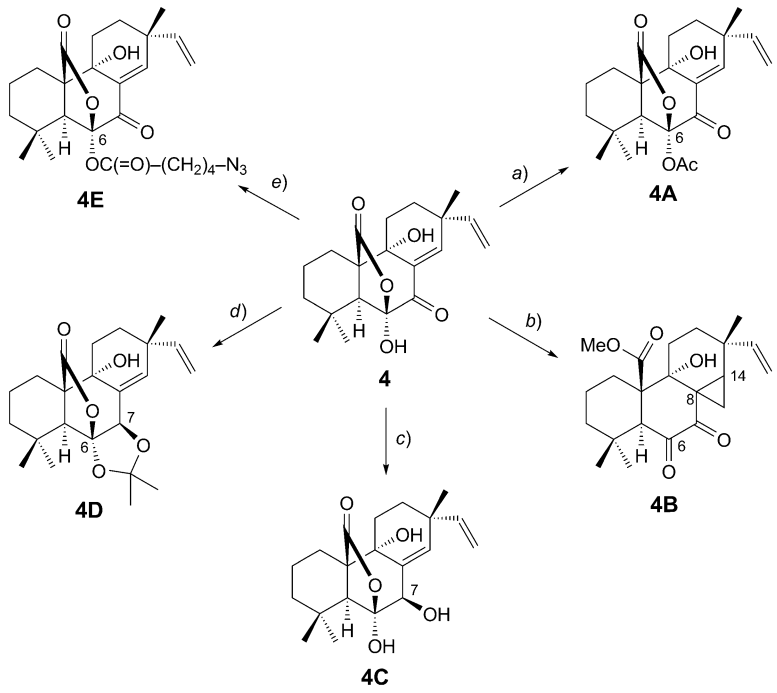


a) Ac_2O, Pyridine, *p*-toluenesulfonic acid (TsOH), r.t., 12 h. *b*) $H_2/PdBaSO_4$, MeOH, r.t., 3 h. *c*) *Jones* reagent, Me_2CO, 0°, 1 min.

was converted to the corresponding C=O group as consequence of the opening of the lactone ring and methyl esterification of the COO group at C(10). The same derivative **4B** was also accessible *via* a methylene insertion by CH_2N_2 on the C(8)=C(14) bond generating the fused oxirane ring. By the well-known enantioselective reduction with $NaBH_4$, as also described in [22], sphaeropsidin A (**4**) was converted into sphaeropsidin B (**4C**) which is another phytotoxin produced together with the other sphaeropsidins C–F and sphaeropsidones by both *Sphaeropsis sapinea* f. sp. *cupressi* (= *D. cupressi*) [15] and *D. mutila* [22]. The synthesized sphaeropsidin B was identified by comparing its ^{1}H-NMR and ESI mass spectra with those reported in [22]. Sphaeropsidin B so obtained was converted to the new isopropylidene derivative **4D** (*Scheme 3*), in which the hemiketal OH group at C(6) and that at C(7) led to the formation of a 2,2-dimethyl-4,5-disubstituted-1,3-dioxolane fused to the pimarane system through C(6) and C(7). The structure of **4D** was confirmed by the significant downfield shift ($\Delta\delta$ 0.97) of H–C(7) resonating as broad *singlet* at δ(H) 5.19, noted when its ^{1}H-NMR spectrum was compared to that of **4C** recorded under the same conditions. The probable alternative isopropylidene derivative that could be formed by acetalization of the tertiary OH group at C(9) was ruled out for mechanistic reasons, using a suitable *Dreiding* model. Similarly, also ketalizations of both OH groups at C(6) and C(9) was ruled out, as sphaeropsidin A (**4**) did not give any ketal by reaction carried out under the same conditions. Finally, the reaction of the hemiketal OH group at C(6) of **4** with the 5-azidopentanoic acid, already described for the conversion of **1** to **1G**, allowed preparation of the new derivative **4E**, which was, as **4A**, formed by esterification of the OH group at C(6) with a different acyl group.

The structures of all the new derivatives of cyclopaldic acid and sphaeropsidin A, **1E**–**1I**, and **4D** and **4E**, respectively, were determined by extensive use of spectroscopic

Scheme 3



a) Ac_2O, Pyridine, TsOH, r.t., 12 h. *b*) Etheral CH_2N_2, r.t., 18 h. *c*) $NaBH_4$, r.t., 2 h. *d*) Anh. acetone and anh. $CuSO_4$, reflux, 24 h. *e*) Anh. CH_2Cl_2 and pyridine, DCC, 5-azidopentanoic acid, 0°, 1 h, r.t, 2 h.

methods as reported in the *Exper. Part*, and also by comparison of their data with those of the parent toxins.

Biting-Deterrent Activity Bioassay. In biting-deterrence bioassays, compounds **1**–**5**, **1F**–**1H**, **2A**, **2C**, and **4A**–**4C** showed activities above that of the solvent control. Biting-deterrence index (*BDI*) values for the active compounds are given in *Fig. 2*. Sphaeropsidin B (**4C**; *BDI* 0.95) was the most active compound, followed by 2′-*O*-acetylseiridin (**2A**; *BDI* 0.73), while for the rest of the compounds the BDI values ranged between 0.3 and 0.69. Biting-deterrence activity of **4C** was similar to that of *N*,*N*-diethyl-3-methylbenzamide (DEET) at 20 nmol/cm^2 concentration.

Structure–Activity Relationships (SARs). The biting-deterrent activity of **1G** was not surprising, as it could be hydrolyzed, according to a lethal metabolism [23], to **1** at physiological pH. This should also occur with **1B**, but they differ in the length of the acyl residue so that, in **1G**, the lipophilicity was increased and membrane crossing was facilitated. The importance of the presence of the CHO group at C(4), which is in agreement with the results of a previous SAR study [17], was revealed by the inactivity of **1C**, **1D** and **1E**, and **1I**. This C=O group in **1G**, which was active, was converted to the corresponding *Schiff*'s base by reaction with dansylhydrazine in **1E**, but this could be reversed to the original functionality by hydrolysis at physiological pH. The same reaction could occur at C(3) of **1F** generating the *ortho*-dicarbaldehyde moiety which

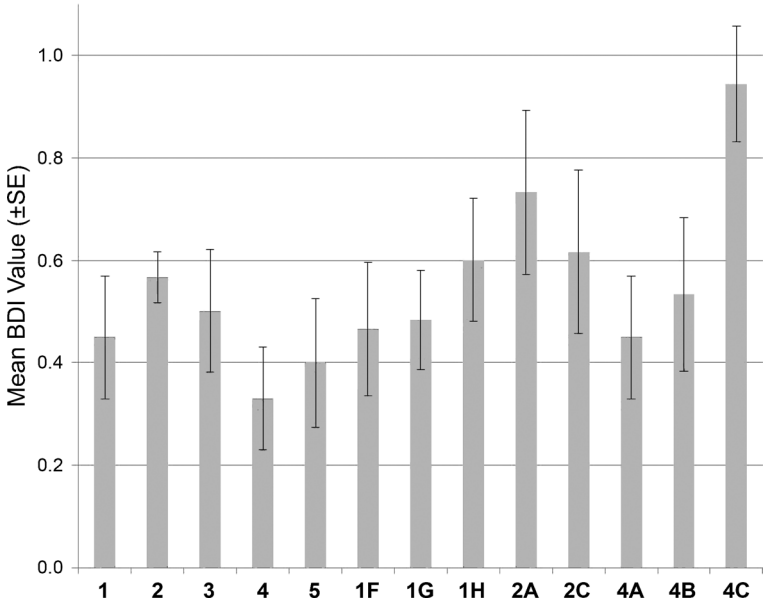


Fig. 2. *Mean biting-deterrence index* (*BDI*) *values* (± SE) *of compounds* **1**–**5** *and selected derivatives thereof against* Aedes aegypti. All the compounds were tested at 25 nmol/cm^2 and evaluated using *N*,*N*-diethyl-3-methylbenzamide (DEET) at 25 nmol/cm^2 as positive control and EtOH as solvent control. A *BDI* value greater than 0 indicates deterrence relative to EtOH, and a *BDI* value not significantly different from 1 shows biting-deterrent effect statistically similar to DEET.

appeared to be a very important structural feature in the above cited SAR study, in which the phytotoxic and the antimicrobial activities of **1** and of some of its several derivatives were assayed [17]. The reduction of the same carbaldehyde at group C(4) to the CH_2OH group, as in **1H**, seems to preserve the activity. This is in contrast to the results of the above mentioned SAR study, in which a derivative obtained from the reduction at C(8) but also at C(1) and C(3) exhibited a significantly decreased activity [17]. However, the lacking of activity of **1I** indicated that the primary OH group at C(8) should be free and not involved in an ether bond as in the 3,4,5-trisubstituted dihydrofuran ring.

The activities of iso-, 2′-oxo- and 2′-*O*-acetylseiridin (**3**, **2C**, and **2A**, resp.) indicated that the presence and the position of the OH group of the hydroxyheptyl side chain at C(4), in contrast to the results of a previous SAR study in which the phytotoxic and the antimicrobial activities of **1** and **3**, and of several of their derivatives were tested [24], are not important features for the activity. On the contrary, in agreement with the mentioned SAR study, the lacking of activity of **2B** showed that the presence of an unaltered butenolide ring, which was converted to a butanolide ring in **2B**, is very important for the activity. The increased activity of 2′-*O*-acetylseiridin (**2A**) with respect to **2**, was probably due to its increased lipohilicity and facilitated membrane crossing. Then according to the lethal metabolism [23] it probably was hydrolyzed at physiological pH.

The activity found for 8,14-methylenesphaeropsidin A methyl ester (**4B**), which is in total disagreement with the results of the previous SAR studies [15][21], indicated that the lactone ring, the hemiketal OH group at C(6), and the C(8)=C(14) bond are not relevant for the activity. The activity of the 6-*O*-acetylsphaeropsidin (**4A**) is in agreement with the lethal metabolism [23] as, after crossing the membrane, it could be hydrolyzed to **4** at physiological pH. This did not occur with another reversible ester, **4E**, which contains the 5-azidopentanoyl residue. The increased activity of sphaeropsidin B (**4C**) suggested that the presence of a secondary OH group at C(7) is more significant compared to the C=O group in **4**. This finding is in total disagreement with the previously SAR study in which a C(7)=O group was reported to be important to impart phytotoxic, antifungal, and antibacterial activity [15][21]. However, the OH at group C(7) must be free, because, if not (as in **4D**), the activity was lost.

Larvicidal Activity Bioassay. In larvicidal bioassays, only sphaeropsidin A (**4**) and cyclopaldic acid (**1**) showed larvicidal activity, while all the other compounds were inactive up to the dose of 100 ppm. The LD_{50} and LD_{90} values for **1** and **4** are compiled in the *Table*. Based on 95% CIs, compound **4** (LD_{50} 36.8 (33.05–41.0)) was significantly more potent than **1** (LD_{50} 58.2 (50.9–66.8)) at 24-h posttreatment, whereas the LD_{50} value of permethrin, used as a positive control, was 0.0031 (0.0016–0.0047) ppm. Although mortality increased, there were no significant differences in the results of 24- and 48-h posttreatment.

Table. *Toxicity of Cyclopaldic Acid* (**1**) *and Sphaeropsidin A* (**4**) *against First-Instar Larvae of* Ae. aegypti *24-h Posttreatment*

Compound	LD_{50}[a]) (95% CI[b])) [ppm]	LD_{90} (95% CI) [ppm]	χ^2	DF
Cyclopaldic acid (**1**)	58.2 (50.9–66.8)	120.1 (110.2–154.5)	88.25	73
Sphaeropsidin A (**4**)	36.8 (33.05–41.0)	75.4 (65.4–90.7)	132.0	73
Permethrin[c])	0.0031			

[a]) LD_{50} and LD_{90} values are (given in ppm) dosages which kill 50 and 90% of the population, respectively. [b]) Confidence interval. [c]) Positive standard, purity 46.1 and 53.2% for *cis* and *trans*, respectively.

Conclusions. – In this work, biting-deterrent activities of the fungal phytotoxins cyclopaldic acid (**1**), papyracillic acid (**5**), sphaeropsidin A (**4**), and seiridin (**2**), as well as larvicidal activities of compounds **1** and **4** were evaluated. A series of derivatives of **1**, **2**, and **4** were semisynthesized and assayed for further structure–activity relationship studies. The bioassay results indicate that structural features responsible for the activity of compounds **1**, **2**, and **4** were the presence of a CHO or CH_2OH group at C(4), an unaltered butenolide ring, and the presence of a C=O or a secondary OH group at C(7). Sphaeropsidin B (**4C**) was the most active biting deterrent, followed by 2′-*O*-acetylseiridin (**2A**) which also showed good larvicidal activity. Compounds **2A** and **4C**, which can also be prepared in only one step in high yield from **2** and **4**, respectively, could be considered as potential natural biting deterrents against *Ae. aegypti.*

The authors thank Dr. *Pierluigi Mazzei* for recording the NMR spectra at the laboratory CERMANU centre, University of Naples Federico II. The authors are grateful to Dr. *John F. Parcher*,

National Center for Natural Products Research, The University of Mississippi, University, MS 38677, USA, for English corrections and suggestions. This was financially supported by the Italian Ministry of University and Research (MIUR), in part by USDA/ARS grant No. 56-6402-1-612, and *Deployed War-Fighter Protection Research Program Grant* funded by the U.S. Department of Defense through the Armed Forces Pest Management Board.

Experimental Part

General. Anal. and prep. TLC: silica gel (*Merck*, *Kieselgel 60*, F_{254}, 0.25 and 0.5 mm, resp.) plates; visualization by exposure to UV light or by spraying with 10% H_2SO_4 in MeOH and then 5% phosphomolybdic acid in EtOH, followed by heating at 110° for 10 min. Column chromatography (CC: silica gel (*Merck*, *Kiesegel 60*, 0.0063–0.200 mm). UV Spectra: in MeOH on a *Perkin–Elmer Lambda 25* UV/VIS spectrophotometer; λ in nm (log ε). IR Spectra: as glassy film on a *Perkin–Elmer Spectrum One* FT-IR spectrometer; $\tilde{\nu}$ in cm^{-1}. ^{1}H-NMR Spectra: at 600 or 400 MHz, resp., in $CDCl_3$, on *Bruker* spectrometers; the same solvent used as internal standard; δ in ppm rel. to solvent peak; *J* in Hz. ESI-MS: *Agilent Technologies* (Milan, Italy) *6120* quadrupole LC/MS instruments; in *m*/*z*.

Fungal strain. D. cupressi was purchased from Centraalbureau voor Schimmelcultures of Baarn (The Netherlands), strain 261.85 CBS, and deposited with the collection of Dipartimento di Protezione delle Piante, Università di Sassari, Italy. *S. cupressi*, (strain No. 1022), isolated from an infected cypress tree at Kos (Greece), was used for toxins production. The isolate was grown in liquid culture under conditions as described in [25]. *Ascochyta agropyrina* var. *nana* was deposited with the culture collection of All-Russian Research Institute of Plant Protection, Pushkin, Saint Petersburg, Russia.

Production, Extraction, and Purification of Cyclopaldic Acid, Seiridin, Isoseiridin, Sphaeropsidin A, and Papyracillic Acid (**1**–**5**, resp.). Cyclopaldic acid (**1**), seiridin and isoseridin (**2** and **3**, resp.) were obtained as white crystals and as oily compound, resp., from the purification of the org. extracts of *S. cupressi* [9][11]. Sphaeropsidin A (**4**) was obtained as white needles from the purification of the org. extract of the culture filtrates of *D. cupressi* as described in [15]. Papyracillic acid (**5**) was obtained as white crystals from *A. agropyrina* var. *nana* solid culture as described in [14].

Preparation of Cyclopaldic Acid Derivatives **1A**–**1D**. Isocyclopaldic, 3-*O*-acetyl-, 3,5,8-*O*,*O*′,*O*″-triacetyl-*8*-acetoxy-, and 8-(2′,4′-dinitro)phenylhydrazone cyclopaldic acid, **1A**–**1D**, resp., were prepared from **1** as described in [9][16][17].

8-Dansylhydrazone and 3,8-Didansylhydrazone of Cyclopaldic Acid (= N′-*[(3,5-Dihydroxy-7-methoxy-6-methyl-1-oxo-1,3-dihydro-2-benzofuran-4-yl)methylidene]-5-(dimethylamino)naphthalene-1-sulfonohydrazide and 3-[(2-{[5-(Dimethylamino)naphthalen-1-yl]sulfonyl}hydrazinylidene)methyl]-2-[(2-{[5-(dimethylamino)naphthalen-1-yl]sulfonyl}hydrazinylidene)methyl]-4-hydroxy-6-methoxy-5-methylbenzoic Acid*; **1E** and **1F**, resp.). To compound **1** (20 mg) dissolved in a CH_2Cl_2/MeOH 1:1 (20 ml) was added 5-(dimethylamino)naphthalene-1-sulfonohydrazide (40 mg). The reaction was carried out at 80° for 3 h. When all starting material was reacted, the solvent was evaporated under reduced pressure. The residue (58 mg) was purified by prep. TLC ($CHCl_3$/^{i}PrOH 9:1) to give **1E** (R_f 0.44; 5 mg) and **1F** (R_f 0.50; 10 mg) as homogeneous yellow amorphous solid.

Data of **1E**. UV: 382 (3.10), 287 (3.36), 255 (3.48). IR: 3175, 1744, 1615, 1572, 1341. ^{1}H-NMR ($CDCl_3$): 10.8 (br. *s*, OH–C(5)); 8.62–7.56 (*m*, 6 H (naphtyl)); 8.38 (br. *s*, HO–C(3)); 8.25 (*s*, CH(8)=O); 7.15 (br. *s*, NH); 6.4 (*s*, H–C(3)); 3.98 (*s*, MeO); 2.90 (br. *s*, Me_2N); 2.03 (*s*, Me–C(6)). ESI-MS: 992 ($[2M+Na]^+$), 508 ($[M+Na]^+$), 486 ($[M+H]^+$).

Data of **1F**. UV: 328 (3.51), 265 (3.83). IR: 3380, 1728, 1620, 1569, 1451. ^{1}H-NMR ($CDCl_3$): 10.8 (br. *s*, OH–C(5)); 8.66–7.08 (*m*, 12 H (naphtyl)); 8.04, 8.03 (2*s*, H–C(3), H–C(8)); 7.18, 6.48 (2 br. *s*, 2 NH); 3.94 (*s*, MeO); 2.88, 2.84 (2*s*, Me_2N); 1.93 (*s*, Me–C(6)). ESI-MS: 771 ($[M+K]^+$), 755 ($[M+Na]^+$), 733 ($[M+H]^+$).

7-Formyl-1,3-dihydro-6-hydroxy-4-methoxy-5-methyl-3-oxo-2-benzofuran-1-yl 6-Azidopentanoate (**1G**). To compound **1** (32 mg) dissolved in anh. CH_2Cl_2 (4.5 ml) and pyridine (100 µl) were added *N*,*N*′-dicyclohexylcarbodiimide (DCC; 70 mg) and 5-azidopentanoic acid (70 µl). The reaction was left at 0° for 1 h and then at r.t. for 2 h. The reaction was stopped by evaporation under N_2. The residue

(100 mg) was purified by prep. TLC ($CHCl_3$/^{i}PrOH 97:3) to yield **1G** (R_f 0.76; 35 mg). Homogeneous amorphous solid. UV: 371 (2.15), 328 (2.29), 261 (2.56). IR: 3300, 2087, 1772, 1640, 1618, 1583, 1489. ^{1}H-NMR ($CDCl_3$): 12.18 (*s*, HO–C(5)); 9.88 (*s*, H–C(8)); 7.64 (*s*, H–C(3)); 4.24 (*s*, MeO); 3.32 (*t*, J = 6.5, CH_2(5′)); 2.48 (*t*, J = 6.3, CH_2(2′)); 2.18 (*s*, Me–C(6)); 1.78–1.75, 1.68–1.65 (2*m*, CH_2(3′), CH_2(4′)). ESI-MS: 749 ($[2M+Na]^+$), 364 ($[M+H]^+$).

*Preparation of Reduced Derivatives of Cyclopaldic Acid: 5-Hydroxy-4-(hydroxymethyl)-7-methoxy-6-methyl-2-benzofuran-1(3*H*)-one* (**1H**) *and 5-Hydroxy-3-methoxy-4-methyl-6,7a-dihydro-2*H*-furo[2,3,4-*cd*][2]benzofuran-2-one* (**1I**). Compound **1** (40 mg) dissolved in MeOH (3 ml) was reduced with $NaBH_4$ (80 mg) under stirring at 0°. The reaction was stopped after 6 min with ice, and the soln. was neutralized with HCl (1N) and extracted with AcOEt (3 × 10 ml). The org. extracts, combined and dried (Na_2SO_4), were purified by TLC ($CHCl_3$/^{i}PrOH 80:20) to give **1H** (R_f 0.71; 11.5 mg) and **1I** (R_f 0.6; 10.5 mg) as homogeneous amorphous solids.

Data of **1H**. UV: 265 (2.88). IR: 3362, 2912, 2857, 1736. ^{1}H-NMR ($CDCl_3$): 5.06 (*s*, CH_2(3)); 4.67 (*s*, CH_2(8)); 3.93 (*s*, MeO); 2.10 (*s*, Me–C(6)). ESI-MS: 225 ($[M+H]^+$), 223 ($[M-H]^-$).

Data of **1I**. UV: 2.59 (3.52). IR: 3351, 2923, 2851, 1722, 1599. ^{1}H-NMR ($CDCl_3$): 6.07 (br. *s*, H–C(3)); 4.85 (*dd*, J = 13.4, 2.6, H_a–C(8); 4.76 (*dd*, J = 13.4, 2.6, H_b–C(8)); 3.95 (*s*, MeO); 2.11 (*s*, Me–C(6)). ESI-MS: 223 ($[M+H]^+$), 221 ($[M-H]^-$).

Preparation of Seiridin Derivatives **2A**–**2C**. 2′-*O*-Acetyl-, 3,4-dihydro-, and 2′-oxoseiridin (**2A**–**2C**, resp.) were prepared, according to the procedures previously reported [11][20].

Preparation of Sphaeropsidin A Derivatives **4A** *and* **4B**. The 6-*O*-acetylsphaeropsidin A (**4A**) and 8,14-methylensphaeropsidin A methyl ester (**4B**) were prepared as described in [21].

Preparation of Sphaeropsidin B (*=(7β,13α)-6,7,9-Trihydroxy-6,20-epoxypimara-8(14),15-dien-20-one*; **4C**). Fifteen mg of **4** was dissolved in MeOH (2 ml) and reduced with $NaBH_4$ (17.3 mg) under stirring at r.t. The reaction was stopped after 2 h by adding ice and HCl (1N), adjusting the pH to neutrality. The soln. was extracted with AcOEt (3 × 10 ml). The org. extracts, combined and dried (Na_2SO_4), were purified by prep. TLC (hexane/acetone 7:3) to yield **4C**. Homogeneous oily compound (R_f 0.3; 10.3 mg) which was identified by ^{1}H-NMR and ESI-MS data identical to those reported in [22].

Preparation of (6β,13α)-9-Hydroxy-7,20-dioxo-6,20-epoxypimara-8(14),15-dien-6-yl 6-Azidopentanoate (**4E**). To **4** (31 mg) dissolved in anh. CH_2Cl_2 (3 ml) and pyridine (75 µl) were added under stirring DCC (96 mg) and 5-azidopentanoic acid (90 µl). The reaction was left at 0° for 1 h and then for 2 h at r.t. The reaction was stopped by evaporation under N_2. The crude residue (175 mg) was purified by CC ($CHCl_3$/^{i}PrOH 97:3) to yield **4E** (R_f 0.75; 19.3 mg). Homogeneous amorphous solid. UV: 294 (2.05), 247 (3.09). IR: 3483, 2939, 2873, 2093, 1771, 1725, 1632, 1456. ^{1}H-NMR ($CDCl_3$): 6.54 (*d*, J = 1.2, H–C(14)); 5.80 (*dd*, J = 17.5, 10.5, H–C(15)); 5.06 (*d*, J = 17.5, H_a–C(16)); 5.06 (*d*, J = 10.5, H_b–C(16)); 3.33 (*t*, J = 6.5, CH_2(5′)); 2.87 (*s*, H–C(5)); 2.63–2.50 (*m*, CH_2(4′), CH_2(3′)); 2.22 (br. *d*, J = 13.2, H_a–C(1)); 1.90 (*dt*, J = 14.1, 3.4, H_a–C(3); 1.84–1.80 (*m*, CH_2(2′), H_a–C(2), H_a–C(12)); 1.75–1.64 (*m*, H_b–C(1), H_b–C(2), H_b–C(3), H_a–C(11), H_b–C(12)); 1.25 (*m*, H_b–C(11); 1.19 (*s*, Me(18)); 1.10 (*s*, Me(19)); 1.09 (*s*, Me(17)). ESI-MS: 965 ($[2M+Na]^+$), 494 ($[M+Na]^+$), 470 ($[M-H]^-$).

*(7β,13α)-6,7,9-Trihydroxy-6,7-*O,O*-isopropylidene-6,20-epoxypimara-8(14),15-dien-20-one* (**4D**). To compound **4** (20 mg) dissolved in anh. acetone (20 ml) was added under stirring anh. $CuSO_4$ (400 mg). The soln. was heated under reflux. After 24 h, the reaction was stopped by filtration, and the clear soln. was evaporated under reduced pressure. The residue (20 mg) was purified by TLC ($CHCl_3$/^{i}PrOH 95:5) to yield **4D** (R_f 0.54; 3 mg). Homogeneous amorphous solid. UV: 246 (3.42). IR: 3300, 1744, 1709, 1621, 1462, 1454, 1374, 1220. ^{1}H-NMR ($CDCl_3$): 6.86 (*s*, H–C(7)); 5.82 (*dd*, J = 17.4, 11.3, H–C(15)); 5.19 (*s*, H–C(14)); 5.09 (*d*, J = 17.4, H_a–C(16)); 5.07 (*d*, J = 11.3, H_b–C(16)); 2.72 (*s*, H–C(5)); 2.23 (br. *d*, J = 9.7, H_a–C(1)); 1.89 (br. *d*, J = 15.6, H_a–C(3)); 1.83 (*m*, H_b–C(3), H_a–C(12)); 1.60 (*m*, H_b–C(1), CH_2(2), H_b–C(12)); 1.60, 1.25 (2*s*, Me_2C); 1.37 (br. *d*, J = 12.2, H_a–C(11)); 1.29–1.25 (*m*, H_b–C(11)); 1.20 (*s*, Me(18)); 1.19 (*s*, Me(19)); 1.09 (*s*, Me(17)). ESI-MS: 411 ($[M+Na]^+$), 369 ($[M+Na\text{-}C_3H_6]^+$).

Insects. Ae. aegypti used in *K&D* (*=Klun & Debboun*) bioassays were from a laboratory colony maintained using standard procedures [26] since 1952 at the Mosquito and Fly Research Unit at the Center for Medical, Agricultural and Veterinary Entomology, United States Department of Agriculture, Agriculture Research Service, Gainesville, Florida. We received the eggs and stored these in our laboratory (Biological Field Station, The University of Mississippi, Abbeville, MS 38601) until needed.

Mosquitoes were reared to the adult stage by feeding the larvae on a larval diet of 2% slurry of 3 : 2 Beef Liver powder (*Now Foods*, Bloomingdale, Illinois) and Brewer's yeast (*Lewis Laboratories Ltd.*, Westport, CT). The eggs were hatched by placing a piece of a paper towel with eggs in a cup filled with 100 ml of deionized H_2O containing a small quantity of larval diet and maintained under vacuum (*ca.* 1 h). Larvae were removed from vacuum and held overnight in the cup. These larvae were then transferred into 500-ml cups (*ca.* 50–100 larvae per cup) filled with H_2O. Larval diet was added every day until pupation, and the mosquitoes were kept in an environment controlled room. Both the larvae and adults were maintained $27 \pm 2^\circ$ and $70 \pm 5\%$ rel. humidity in a photoperiod regimen of 12 : 12 (L : D) h. The adults were fed on cotton pads moistened with 10% sucrose soln. placed on the top of screens of 4-l cages. Seven- to 14-day-old mated females used in these bioassays were deprived of sucrose for 24 h prior to the test; but they had free access to water-soaked cotton.

Mosquito-Biting Bioassays. Bioassays were conducted using a six-celled *in vitro K&D* module bioassay system developed by *Klun et al.* [27] for quant. evaluation of biting-deterrent properties of candidate compounds. Here we use feeding deterrent in the sense of *Dethier et al.* (1960) [28], *i.e.*, a chemical that inhibits feeding when present in a place where the insects feed in its absence. This is contrasted by repellent, which is a chemical that causes insects to make oriented movement away from its source. The *K&D* system consists of a six-well reservoir with each of the 4×3-cm wells containing 6 ml of feeding soln. As described by *Ali et al.* [29], we used CPDA 1 + ATP soln. instead of human blood. CPDA-1 and ATP preparations were freshly made on the day of the test and contained a red dye that allowed for identification of mosquitoes that had fed on the soln. (see below). DEET (97% purity *N*,*N*-diethyl-*meta*-toluamide) was obtained from *Sigma–Aldrich* and used as a positive control. Molecular biology-grade EtOH was obtained from *Fisher Scientific Chemical Co.* (Fairlawn, NJ 07410). All compounds and DEET treatments were prepared in EtOH. All the compounds and the positive control DEET were tested at a concentration of 25 nmol/cm^2 except in dose–response study. The stock solns. were kept in a refrigerator at $3–4^\circ$. Treatments were prepared fresh at the time of bioassay.

During the bioassay, temp. of the soln. in the reservoirs, covered with a collagen membrane, was maintained at 37.5° by circulating water through the reservoir with a temp.-controlled circulatory bath. This CPDA-1 + ATP soln. membrane unit simulated a human host for mosquito feeding. The test compounds and controls were randomly applied to six 4×3-cm marked portions of Nylon organdy strip, which was positioned over the six, membrane-covered wells. A *Teflon* separator was placed between the treated cloth and the six-celled module. A six-celled *K&D* module containing five females per cell was positioned over the six wells, trap doors were opened, and mosquitoes were allowed access at the module for a 3-min period, after which they were collected back into the module. Mosquitoes were squashed and the presence of red dye (or not) in the gut was used as an indicator of feeding. A replicate consisted of six treatments: four test compounds, DEET (a standard bite-deterrent compound), and 95% EtOH as solvent control. Five replicates were conducted per day using new batches of mosquitoes for each. Total 15 replications were conducted for each treatment. Bioassays were conducted between 13 : 00 and 16 : 00 h.

Larval Bioassays. Bioassays were conducted by using the bioassay system described by *Pridgeon et al.* [30] to determine the larvicidal activity of isolated fungal toxins activity against 1-d old *Ae. aegypti* larvae. In brief, the eggs were hatched under vacuum (1 h) by placing a piece of a paper towel with eggs in a cup filled with 100 ml of deionized water containing a small quantity of larval diet. Larvae were removed from vacuum and held overnight in the cup in a temp.-controlled chamber maintained at $27 \pm 2^\circ$ and $70 \pm 5\%$ rel. humidity at a photoperiod regimen of 12 : 12 (light:dark) h. Five 1-d-old first instar *Ae. aegypti* were added to each well of 24-well plates placed on illuminated light box by using a disposable 22.5-cm *Pasteur* pipette with a droplet of water. Fifty µl of larval diet (2% slurry of 3 : 2 Beef Liver powder (*Now Foods*, Bloomingdale, Illinois) and Brewer's yeast (*Lewis Laboratories Ltd.*, Westport, CT)) were added to each well by using a *Finnpipette* stepper (*Thermo Fisher*, FI-Vantaa). All chemicals to be tested were diluted in DMSO. Eleven µl of the test chemical was added to the labeled wells, and, in control treatments, 11 µl of DMSO alone were added. Each well had a total volume of 1.1 ml. After the treatment, the plates were swirled in clockwise and counterclockwise motions, and front and back, and side to side five times to ensure even mixing of the chemicals. Larval mortality was recorded 24-, 48- and 72-h posttreatment. Larvae that showed no movement in the well after manual disturbance of water by a

pipette tip were recorded as dead. A series of dosages was used in each treatment to obtain a range of mortality. Treatments were repeated 15 times in each extract. Permethrin used as positive control was obtained from *Sigma–Aldrich*.

Data Analyses. Proportion not biting (PNB) was calculated using the procedure described by *Ali* and co-workers [29]. Since the *K&D* module bioassay system can handle only four treatments along with negative and positive controls, to make direct comparisons among more than four test compounds and to compensate for variation in overall response among replicates, repellency was quantified as Biting-Deterrence Index (*BDI*). The *BDI* values were calculated using the following formula:

$$[BDI_{i,j,k}] = \left[\frac{PNB_{i,j,k} - PNB_{c,j,k}}{PNB_{d,j,k} - PNB_{c,j,k}}\right]$$

where $PNB_{i,j,k}$ denotes the proportion of females not biting test compound i for replication j and day k ($i=1-4$, $j=1-5$, $k=1-3$), $PNB_{c,j,k}$ denotes the proportion of females not biting the solvent control for replication j and day k ($j=1-5$, $k=1-3$), and $PNB_{d,j,k}$ denotes the proportion of females not biting in response to DEET (positive control) for replication j and day k ($j=1-5$, $k=1-3$). This formula makes an adjustment for inter-day variation in response and incorporates information from the solvent control as well as the positive control. A *BDI* value of 0 indicates an effect similar to EtOH, while a value significantly greater than 0 indicates biting-deterrent effect relative to EtOH. *BDI* Values not significantly different from 1 are statistically similar to DEET. BDI Values were analyzed using SAS Proc ANOVA (*SAS Institute*, 2007), and means were separated using the *Ryan–Einot–Gabriel–Welsch* Multiple Range Test. The LD_{50} values for larvicidal data were calculated by using SAS, Proc Probit. Control mortality was corrected by using *Abbott*'s formula.

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Received October 22, 2012