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Action of a Pentacyclic Triterpenoid, Maslinic Acid, against *Toxoplasma gondii*

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The action of maslinic acid (2α,3β-dihydroxyolean-12-en-28-oic acid) (**1**), a pentacyclic derivative present in the pressed fruits of the olive (*Olea europaea*), has been studied against the tachyzoites of *Toxoplasma gondii*. The capability of tachyzoites to infect Vero cells treated with **1** was affected. The LD_{50} values were 58.2 μM for the isolated tachyzoites and 236 μM for the noninfected Vero cells. Zymograms of the *T. gondii* proteases incubated with **1** showed a dosage-dependent inhibition of some of the proteases. The parasites treated with **1** showed gliding motility and ultrastructural alterations. The present findings suggest that protease activity of the parasite required for cell invasion is the action target for maslinic acid (**1**).

Toxoplasma gondii is an intracellular protozoan, which, like other protozoa of the phylum Apicomplexa, has a subcellular structure called the apical complex involved in cell invasion.[1] Toxoplasmosis is a zoonosis affecting a third of humankind.[2,3] The prevalence of this infection reaches 16−40% in the U.S. and the U.K., while in Central and South America as well as continental Europe the rate is 50−80%, depending on eating habits. In the U.S., toxoplasmosis is considered the main cause of food infection in humans.[1,4] Furthermore, it is one of the main opportunistic pathogens in patients with HIV and also causes serious congenital infections resulting from an acute primoinfection during pregnancy.[1,5−8]

The intracellular tachyzoite form is capable of invading practically all nucleated cells of an organism.[5,9] The invasion process is divided into two main stages: (1) cell recognition and union and (2) vacuole formation during the entry.[9,10] The penetration into the nonphagocytic cell is an active substrate-dependent locomotive process called "gliding motility", creating a cell−parasite molecular complex called the "glideosome".[3,5,11]

The tachyzoite forms of *T. gondii* possess a battery of proteins that are secreted, and these play a fundamental role in the invasion process. The invasion involves the sequential release of proteins from the typical protozoan organelles: micronemes, rhoptries, and dense granules. Ample data demonstrate the importance of proteases in the parasite−host cell interaction, survival, and pathogenesis of parasitic diseases.[12] These processes include the degradation of hemoglobin and other blood proteins, evasion of the immune response, or the activation of inflammatory processes.[12] The proteins of the micronemes and of the rhoptries are subjected to different stages of proteolytic processing: proteolytic maturity, surface trimming, and surface shedding.[11] Diverse proteases have been described in *T. gondii*, such as proteases TgSUB 1 and 2, analogues in the PfSUB of *Plasmodium falciparum.* The TgSUB2 of the rhoptries is key in the proteolysis of other proteins, as these proteins co-precipitate with the proteins ROP1 and ROP2[13,14] and a TgSUB1, located in the micronemes, and excreted−secreted in a calcium-dependent process.[13,15] The genome sequencing of the different Apicomplexa has expanded knowledge of the micronemal MIC-protein repertory. These proteins are released as complexes and undergo active proteolytic processing.[10,11] Within this class of proteins, the one best described is TgMIC2, which is processed post-exocytosis by the activities of microneme protein protease (MPP1 and MPP2)[11,13,16] and other proteins such as TgROM4, which require the activity of the protein MPP1 in order to mature. Recently, another microneme protein, namely, protease 3 (MPP3), was discovered, with its activity being a prerequisite for the activation of MPP2.[11] The rhomboid family of intramembrane serine proteases, TgROM4 and TgROM5, the keys to MIC shedding, is also significant.[17] TgAMA1 is another protein released from the micronemes, processed proteolytically and co-precipitating with the proteins RON2 and RON4.[16] Moreover, other proteases involved in invasion by *Toxoplasma* are cysteine proteases (toxopaines 1 and 2) and aspartyl proteases (toxomepsins 1, 2, and 3).[10]

After the examination of the inhibition profiles by catalytic-type specific protease inhibitors, serine and cysteine proteases were determined to be the proteases most actively involved in MIC processing.[18,19] Cysteine and serine proteases have been described as clear targets in the cell-invasion process, intracellular development, and the secretory machinery of proteins in *T. gondii* and in other Apicomplexa protozoa, such as *Eimeria tenella*.[6,20−23]

Maslinic acid (2α,3β-dihydroxyolean-12-en-28-oic acid, **1**) is a triterpenoid compound related to oleanolic acid, which is present in numerous plants[24−27] and occurs in considerable amounts in the fruit and leaves of *Olea europaea* L. (Oleaceae).[28,29] Compound **1** is obtained from solid waste during olive-oil production through a commercially viable extraction technique that produces large quantities of this chemically pure product.[30] The *in vitro* biological activity of maslinic acid includes antiviral,[40] cytotoxic,[32,33] and antioxidant.[34]

The present work provides a description of the activity of **1** against *T. gondii*. In addition, we have studied the *in vitro* action of **1** in infectivity and motility of the parasite. Ultrastructural alterations induced by this compound, as well as possible parasite protease inhibitory effects, were also studied.

Results and Discussion

Similar to other protease inhibitors described in the literature as inhibitors of growth and intracellular replication of *T. gondii*,[20,22] maslinic acid (**1**) is capable of blocking the entry of the parasite into the cell. This inhibition is dosage-dependent, with the ID_{50} at 24 h of treatment of the tachyzoites found to be 46 μM (Figure 1A), while at 48 h the ID_{50} is 8 μM (Figure 1B).

The inhibition exerted by **1** on the proteases present in the excreted−secreted products (ESP) fraction also showed a dosage-dependent inhibition similar to that achieved by classical inhibitors of serine proteases, such as PMSF (phenylmethanesulfonyl fluoride) (Figure S1, Supporting Information). As reflected in Figure S2

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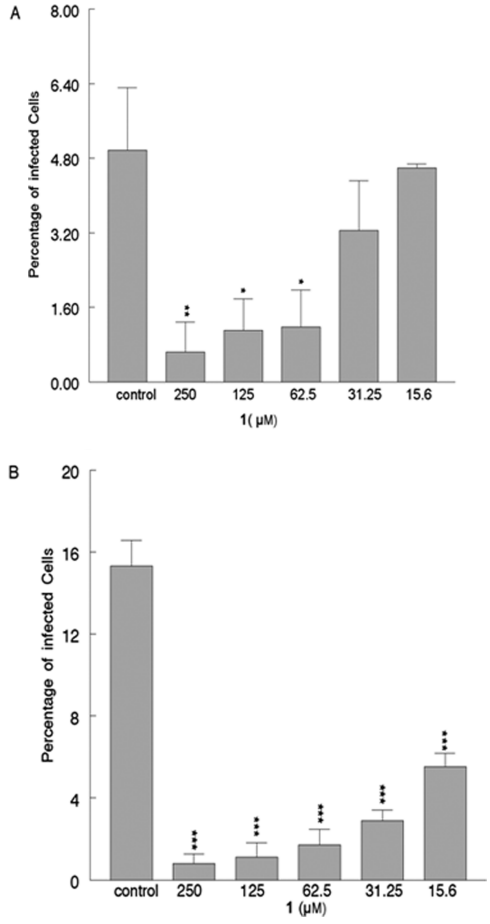

Figure 1. Inhibition of the penetration of *T. gondii* in Vero cells at 24 and 48 h after infection. A suspension containing 2.5 × 10 5 tachyzoites purified from *T. gondii* was pretreated with different concentrations of maslinic acid (**1**) (250−15.6 μM). These tachyzoites (in a proportion of 1:10) were used to infect Vero cells. The cultures were killed and stained at 24 h (A) and 48 h (B) postinfection. A minimum of 500 cells were studied. Each experiment was repeated at least three times, representing the mean of the resulting values ± SEM. A dosage-dependent effect was found, this effect intensifying with the passage of time. *$p < 0.05$, **$p \leq 0.001$, ***$p \leq 0.0001$.

(Supporting Information), the zymograms show that several proteins were released into the medium in the ESP by the micronemal fraction, indicating that the treatment with **1** induces a dosage-dependent inhibition of several of the gelatinolytic-activity bands. This gelatinolytic activity was found only in the induced micronemal secretion, but not in the zymograms registering basal secretion (data not shown).

The inhibition of the protease activity through compound **1** leads to the blockage of a number of mechanisms involved in the penetration of the tachyzoites into the host cell or of mechanisms necessary for this penetration, such as gliding motility.[3,5,11] Moreover, treatment with protease inhibitors caused the appearance of cytopathological effects on intra- as well as extracellular forms of the inhibitor-treated parasite.

The inhibition of the internalization capacity could be considered an indirect effect, given the action of **1** on the gliding motility of the parasite. Molecules of recognized inhibitory action on serine proteases, 3,4-DCI (3,4-dichloroisocoumarin), or on cysteine proteases, such as LHVS (morpholinourea-leucyl-homophenolalaninyl-phenyl-vinyl-sulfone) and ZL3VS (*N*-benzoxycarbonyl-(leucyl)$_3$-phenyl-vinyl-sulfone),[20,23] are capable of diminishing or blocking the mobility of the parasite measured as a function of the number of trails associated with the parasite or of the length of those trails.

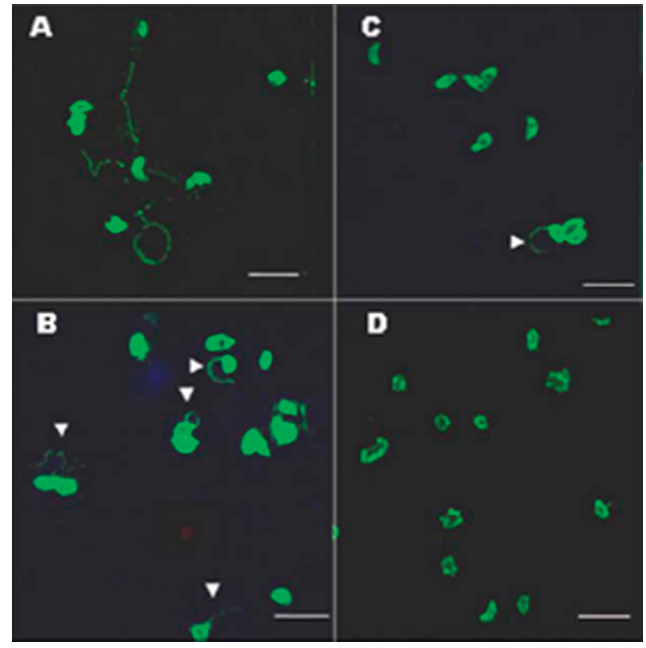

Figure 2. Blockage of gliding motility of *T. gondii* tachyzoites by maslinic acid (**1**). The parasites were treated with DMSO as control (A) or **1** at concentrations of 10 μM (B), 25 μM (C), and 50 μM (D). The tachyzoites decreased the length of their trails (arrows heads) after treatment, as shown in the image, in a dosage-dependent way. Bar: 10 μm.

The effect that protease inhibitors exert on the mobility may be a consequence of the interaction of these inhibitors with the micronemal secretion products,[20,23] as well as other unknown effects, including a decrease of the turnover of the surface glycoproteins, which would not reach maturity in the Golgi apparatus and would accumulate in it. As reflected in our results, maslinic acid (**1**) at a concentration of 50 μM inhibited gliding motility up to 100% (Figure 2 and Table S1 (Supporting Information)). This mechanism is possibly among those responsible for impeding host-cell penetration, an effect that molecule **1** exerts on the tachyzoites in their interaction with the host cell.

An ultrastructural study of the parasite−cell interaction in the presence of **1** revealed a major effect exercised on the tachyzoites (both intra- and extracellular), while the changes induced on the unaffected cells were less notable. These differences might be due to the different level of synthesis as well as the protein turnover between the tachyzoites of *T. gondii* and the potential host cells.

Electron microscopy of the tachyzoites (both extra- and intracellular) treated with maslinic acid showed a number of alterations attributable to the blockage of proteases. The inhibitors of cysteine and serine proteases cathepsin inhibitor III, TCPK (tosyl phenylalanyl chloromethyl ketone), and subtilisin inhibitor III can cause morphological damage and alterations in the endomembrane systems of the parasite[22] similar to those found in our assays. These effects can be attributed to the hampering of a great variety of cell processes in which proteases are involved. Maslinic acid (**1**) penetrates the interior of the parasite (both in its intra- and extracellular form) and blocks the enzymatic activity of the extracellular proteases, such as protein degradation. As evidenced by electron microscopy (Figures S3 and S4, Supporting Information), the treated tachyzoites contained a great quantity of apparently empty spaces that might correspond to the collapse of the Golgi apparatus. The appearance of numerous vacuoles with different electrodense materials, such as polysaccharidic granules, whorls containing cytoplasmic material and ribosomes, was evident in treated extracellular tachyzoites (Figure S3, Supporting Information). Another type of vacuole, apparently empty, related with a

Table 1. In Vitro Activity of Maslinic Acid (**1**), PMSF, and AEBSF against Tachyzoites of *T. gondii* and Vero Cells[a]

compound	*T. gondii* LD 50 (μM)	Vero cells LD 50 (μM)
maslinic acid (**1**)	53.81 ± 1.4	236.61 ± 6.4
PMSF[b]	27.98 ± 0.8	132.77 ± 1.3
AEBSF[c]	19.79 ± 1.4	36.88 ± 3.3

[a] Results are given as the mean ± SEM. [b] PMSF, phenylmethane sulfonyl fluoride, was used as a positive control. [c] AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride, was used as a positive control.

possible collapse of the Golgi apparatus, was induced by **1** (Figure S3, Supporting Information). These effects resemble those described for other protease inhibitors.[41]

Partial ruptures of the plasma membrane with an irregular morphology and the nucleus presenting an undulating and swollen nuclear membrane were seen (Figure S4, Supporting Information). Intracellular parasites (Figure S4, Supporting Information) showed numerous alterations at the level of the surrounding double membrane with ruptures and fusions of the internal membrane, broken in places, forcing the extrusion of the cytoplasmic content. The nuclear membrane of these intracellular tachyzoites underwent swelling (Figure S4, Supporting Information), leaving a space with little content. The effects visible both in the external membrane and in the nuclear membrane could be due to the general blockage of protein turnover, impeding the functionality of the proteins necessary for structural maintenance of the double nuclear membrane.

The nonparasitized cells, treated with **1** at concentrations and times similar to those used for infected cultures, did not result in alterations (Figure S4, Supporting Information). It is noteworthy that treatment with concentrations and treatment times similar to those used in the present experiments did not cause irreversible alterations in the cells, given that after the elimination of **1** the culture developed at the same rate of multiplication as the control cultures. The different ultrastructural effects appearing between *T. gondii* tachyzoites and the host cell treated with **1** could be due, as indicated above, to the different degree of biosynthetic and general metabolic activity.

The cytotoxicity induced by a series of known protease inhibitors, such as PMSF (phenylmethanesulfonyl fluoride) or AEBSF (4-(2-aminoethyl)benzenesulfonyl fluoride), against the tachyzoites of *T. gondii* and Vero cells is shown in Table 1. Therefore, for maslinic acid (**1**), the LD_{50} for the protozoan was less than that for the Vero cells, and the cytotoxic effects observed are appreciably greater than those found in the experiments of inhibiting invasiveness at 48 h. These differences in terms of the dosage between the inhibition of the invasiveness levels and those of cytotoxicity can be explained more by the different accessibility to the proteases involved in the MIC shedding by the inhibitor rather than by the accessibility to the proteases responsible for the cytotoxic damage in the tachyzoites as well as in the treated cells. Therefore, maslinic acid (**1**), a natural compound present in olive oil,[29] found in the diet of many areas of the world for millennia, can be considered a molecule of potential to use to prevent human and animal protozoan parasitosis where the infectivity is mediated by proteases, e.g., toxoplasma, as well as other diseases caused by Apicomplexa protozoa.

Experimental Section

Parasite Strain and Culture. In this study, the RH strain of *Toxoplasma gondii* was used, maintained by injection through the abdominal cavity of Balb/c mice, and 5 to 7 days afterward the parasites were isolated from the ascitic fluid and in Vero cell cultures as described below. The tachyzoites obtained after the host-cell lysis (120 h post-infection of the cell cultures) were collected from the culture medium and purified from the rest of the cells by passage through a 27-gauge hypodermic needle and through a filter of 5 μm pore size (Millipore), as previously described.[35] After filtration, the tachyzoites were concentrated by centrifugation at 1000*g* for 10 min, counted in a Neubauer chamber, adjusted to the concentration needed, and resuspended in DMEM with or without IFCS, according to the requirement of each experiment.

Vero Cell Culture. Vero cells (ECACC no. 84113001) were cultured at 37 °C in a moist atmosphere, enriched with 5% CO_2, in Roux flasks (25 cm^2 surface area), together with Dulbecco's modified Eagle's medium (DMEM) (Sigma), supplemented with 10% heat (56 °C, 30 min)-inactivated fetal calf serum, 2 mM glutamine, and the antibiotics penicillin (1 U/mL) and streptomycin (1 μg/mL) (DMEM-FCS), as previously described.[36]

Test Compounds. Maslinic acid (**1**) was obtained from pressed fruits of olive (*Olea europaea*) by a method previously described.[30] A stock solution of **1** was prepared at a concentration of 10 mM in DMSO. Two inhibitors of serine proteases, phenylmethanesulfonyl fluoride (PMSF) (Sigma) at 10 mM in ethanol and 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF) at 10 mM, were prepared in distilled water. All test compounds were stored frozen at −20 °C until used.

The test compounds were used in the experiments at the following concentrations: 500, 250, 125, 62.5, 31.25, 15.6, 7.8, and 3.9 μM. Also, 50, 25, and 10 μM of **1** were used for the experiments on gliding motility and 25 μM of **1** for ultrastructural studies. As a control, a DMSO solution was used at the maximum concentration of this solvent likely to be present in the protease-inhibitor solutions tested.

Invasiveness Tests. A suspension containing 2.5×10^5 purified tachyzoites was pretreated for 1 h at 37 °C with culture medium containing the different concentrations of **1** and DMSO as a control, as described above. Afterward, these tachyzoites, at a concentration of 10 parasites/cell, were used to infect Vero cells in semiconfluent cultures maintained at 37 °C in a humid atmosphere enriched with 5% CO_2. Then, the medium was removed and fresh medium was added. The slides were removed 24 and 48 h after beginning the experiment, fixed with methanol, and stained with Diff-Quick (Medion Diagnostics, GMBH, CH-3186 Düdingen, Switzerland). After being stained, the cells were studied by light microscopy to determine the percentage of parasitism. In each preparation, 500 cells per slide were examined and the experiment was repeated three times.

Cytotoxicity Assay. The parasites (8×10^4 parasites per well) and Vero cells [semiconfluent cultures (1×10^4 cells per well)] were tested with different concentrations of **1**, PMSF, and AEBSF and were mixed with Alamar Blue (AB) (ABD Serotec, Kidlington, U.K.) following the instructions of the manufacturer, with a final volume of 100 μL per well. Plates were incubated at 37 °C with 5% CO_2 for 24 h. Controls included medium alone, medium plus AB, medium plus AB plus each one of the different drug dilutions, and cells or tachyzoites plus the DMSO dilution mentioned above. After incubation, the percent of reduction of AB was calculated using the manufacturer's formula:

$$\text{percentage reduction} = \frac{(O2 \times A1) - (O1 \times A2)}{(O2 \times P1) - (O1 \times P2)} \times 100$$

where O1 = the molar extinction coefficient (E) of oxidized Alamar Blue at 570 nm; O2 = E of oxidized Alamar Blue at 600 nm; A1 = the absorbance of test wells at 570 nm; A2 = absorbance of test wells at 600 nm; P1 = the absorbance of DMSO growth control well at 570 nm, and P2 = the absorbance of DMSO-growth control well.

Gliding Motility. A motility study was carried out using the method described previously,[37] placing the tachyzoites on borosilicate glass slides at 5 mm diameter/well and pretreating for 30 min with a solution of 0.1% poly-L-lysine (Sigma) in 0.015 M phosphate saline buffer at pH 7.2 (PBS). The tachyzoites were pretreated with **1** for 15 min at 37 °C, with each of the test compound concentrations. The gliding control was the maximum concentration of DMSO used as solvent. After treatment, the slides were washed in PBS at least three times and fixed for 20 min at room temperature in a solution of 4% formaldehyde and 0.02% glutaraldehyde in PBS. Then, the slides were washed in PBS and blocked for 30 min in PBS supplemented with 10% ICFS. Next, the slides were incubated 1 h at 37 °C at a dilution of 1/50 of mouse monoclonal antibody (Mab) anti-P30 G-II 9 (Argene, Verniolle, France), antibody directed against an epitope of the surface glycoprotein of *Toxoplasma gondii* SAG-1. The slides were washed at least three times in PBS and incubated in a moist chamber for 40 min at 37 °C with a 1/100 solution of an anti-IgG antibody labeled with fluorescein isothiocyanate (FITC) (Sigma). Finally, the slides were washed once again in PBS and treated with mounting medium (Prolong Antifade Kit, Molecular Probes, Invitrogen, Barcelona, Spain). The number of

trails and the lengths were studied and recorded under a Leika DMI 6000 confocal microscope.

Preparation of the Excretion−Secretion Products (ESP). For the screening and quantification of the micronemal ESP, the tachyzoites were resuspended in DMEM medium without ICFS (6×10^7 parasites in a volume of 100 μL) after pretreatment with **1** at different concentrations for 15 min at room temperature. Secretion was induced using the method described previously,[38] in which the parasites were incubated in a water bath at 37 °C plus 200 mM ethanol (EtOH), and afterward the parasites were centrifuged at 1000*g* for 10 min and the bottom was placed at 0 °C on ice to stop micronemal secretion.

The ESP fraction was mixed 1:1 with SDS-PAGE electrophoresis nonreducing buffer sample (10 mM Tris/HCl; 1 mM EDTA; 2.5% SDS, pH 8.0) to obtain the zymogram of the proteases released. The samples were stored at −20 °C.

SDS-PAGE and Zymograms. SDS-PAGE electrophoresis,[39] at 12% polyacrylamide and 0.1% gelatin, was used to analyze the zymograms of proteases in the ESP fraction. Electrophoresis was carried out at a constant voltage of 90 V at 4 °C. After electrophoretic separation, the gel was submerged in a Triton X-100 solution at 2.5% (w/v) for 60 min and incubated overnight at 37 °C in a developer buffer (50 mM Tris HCl, 10 mM $CaCl_2$ at pH 6.8). Finally, the gel was stained with Comassie brilliant blue. The gelatin digestion areas were visualized as unstained regions of the gel.

Azocasein Measurements. The protease activity of the micronemal ESP fraction exposed to maslinic acid (**1**) and PMSF was determined using azocasein (Sigma) as a colorimetric substrate with the method described previously.[40] Hence, incubation was carried out for 1 h at 37 °C with 100 μL of ESP and 200 μL of azocasein (1 mg/mL) dissolved in PBS. After incubation, the reaction was stopped with the addition of 700 μL of TCA (10%) at 4 °C and then centrifuged at 1500*g* for 15 min. The supernatant was removed, 1 mL of 1 M NaOH was added, and the absorbance was measured at 440 nm in a Hitachi F-2000 spectrofluorometer. The relative enzymatic activity of each of the inhibitors was calculated with respect to the positive control ESP without prior incubation with the inhibitors. All the measurements and samples were made in triplicate.

Electron Microscopy. To study the ultrastructural effects induced by maslinic acid (**1**), the cells infected for 24 h were treated with 25 μM **1** for 8 h and also the purified tachyzoites for 4 and 8 h at 25 μM. After treatment, the cultures were fixed in 2.5% (v/v) glutaraldehyde in cacodylate buffer, scraped with a rubber policemen, postfixed in 1% (v/v) buffered OsO_4 for 30 min, and then dehydrated and embedded in Spurr resin. Ultrathin sections were stained with 8% uranyl acetate followed by 0.2% lead citrate and were examined under a Zeiss EM-10 transmission electron microscope.

Statistical Analysis. Fisher's test was used to determine the homogeneity of variance between groups. The Bonferroni test was applied to estimate the significance of the difference between means. The results were indicated as mean values ± SEM of the different groups at different time points of each experiment performed. All the experiments were repeated three times; $p \leq 0.001$ was considered to be extremely significant. GraphPad Instat v 3.05 software was used for the statistical testing.

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Supporting Information Available: Enzymatic activity of the ESP of treated tachyzoites with PMSF and **1**, zymogram of the ESP of treated tachyzoites with **1**, electron micrograph showing the cytopathic effects of maslinic acid in the extracellular and intracellular tachyzoites, and a table of inhibition of gliding motility. This material is available free of charge via the Internet at http://pubs.acs.org.

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