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Benzodioxole Derivatives as Negative Effectors of Plant Proteases

LUISA UGOLINI,[§] ISABELLA DELLA NOCE,[§,†] PAOLO TRINCIA,[‡] VALERIO BORZATTA,[‡] AND SANDRO PALMIERI*,[§]

CRA - ISCI, Research Institute for Industrial Crops, Via di Corticella 133, I-40128 Bologna, Italy, and Endura S.p.A., Via Baiona 107-111, I-48100 Ravenna, Italy

Previous work demonstrated that a commercial formulation of piperonyl butoxide (PBO) did inhibit the activity of some plant proteolytic enzymes. In this paper, the effect of pure PBO and nine pure PBO homologues (PBOH) appropriately synthesized toward bromelain and papain was studied in hydrocarbon solution using the bis(2-ethylhexyl)sodium sulfosuccinate (AOT) reverse micellar system. This study establishes that the majority of these compounds show, in vitro, interesting protease inhibition activities. The benzodioxole and dihydrobenzofuran structures, in particular, 5-[2-(2-butoxyethoxy)ethoxymethyl]-benzo[1,3]dioxole (EN 1-40) and 6-[2-(2-butoxyethoxy)ethoxymethyl]-5-propyl-2,3-dihydrobenzofuran (EN 16-5), respectively, appear to be responsible for protease inhibition. Measures of octanol/water partition coefficients on PBO and PBOH have demonstrated that water solubility plays a fundamental role in the expression of protease inhibition activity.

KEYWORDS: PBO; PBO homologues; proteases; inhibition; reverse micelles

INTRODUCTION

Piperonyl butoxide (PBO) belongs to a group of chemicals known as benzodioxole-derived compounds. Many of these chemicals are contained as natural safrole derivatives in various plant tissues and are used in synthetic form in several commercial formulations. Among these compounds, PBO is the most important because it is a well-known synergist of pyrethrin-, pyrethroid-, and carbamate-based insecticides, and it is considered to be the progenitor of benzodioxole polyoxyethylene compounds (**Figure 1**).

The low mammalian toxicity of PBO and its notable synergistic effect on so large a spectrum of insecticidal compounds make its wide use in agriculture possible (*1*, *2*). Although the mechanism of the synergistic effect of PBO has not been completely explained, there is some evidence that its action is directed toward some specific insects containing enzymes, namely, esterases, microsomal oxidases, and, in particular, cytochrome P-450 mono-oxygenases, that are responsible for catabolism or inactivation of insecticide molecules (*3*−*6*). The first discovery that PBO is an enzymatic inhibitor was reported in 1960 by Sun and Johnson (*7*), who observed an inhibition of the oxidative metabolism of some insecticides in house flies (*Musca domestica*). This finding was later confirmed by Casida in 1970 (*8*) and Hodgson and Philpot in 1974 (*9*), who found that PBO exerted a strong negative effect on cytochrome P450, which inhibited the microsomal oxidation of several insecticides and xenobiotics. Nevertheless, the effect of PBO and its derivatives on enzymes of plant origin, in particular proteases, has still not been described. Such enzymes are of great significance in important physiological and regulatory plant cell processes such as germination, storage, protein synthesis, protein mobilization, and protection against biotic stresses. Proteolytic activity is fundamental in the turn-over of all endogenous proteins, including those with natural insecticide or fungicide functions or with tissue repair functions, thus increasing and expanding the natural response of the plant cell toward possible parasitic aggression or different kinds of externally derived stress. In contrast with the proteases of insects and pathogenic microorganisms, the regulation of proteolytic enzymes in plants has a predominantly defensive role, for example, through the activation of specific inhibitors. In the case of injuries produced by mechanical or biological means, protease inhibitors are synthesized de novo, contributing to the plant protection strategy (*10*).

Whereas in a previous work we demonstrated that a PBO formulation (2% PBO w/v in water with appropriate excipients and emulsifier(s)) was able to inhibit the in vivo activity of plant protease(s) in cotton (*Gossypium* hirsutum) (*11*), the aim of this paper is to study the effect of pure PBO and some other PBO homologues (PBOH), appropriately synthesized, purified, and characterized, toward some commercial plant proteolytic enzymes. Given the high hydrophobicity of PBO and PBOH, it was impossible to carry out the enzyme assays in the aqueous solutions in which these enzymes normally work. Consequently,

* To whom correspondence should be addressed. Telephone and fax (+39)0516316851; fax institute (+39)374857; e-mail s.palmieri@isci.it.
[§] CRA − ISCI, Research Institute for Industrial Crops.
[†] Present address: Laboratory of Gastroenterology − Department of Internal Medicine, University of Modena and Reggio Emilia, L.go del Pozzo 71, 41100 Modena, Italy.
[‡] Endura S.p.A.

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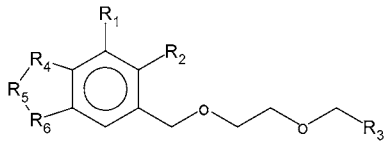


PBO R_1: -H; R_2: -$CH_2CH_2CH_3$; R_3: -$CH_2OCH_2CH_2CH_2CH_3$; R_4: -O; R_5: -CH_2; R_6: -O;

PBO homologues with modified side chain

EN 1-40 R_1: -H; R_2: -H; R_3: -$CH_2OCH_2CH_2CH_2CH_3$; R_4: -O; R_5: -CH_2; R_6: -O;

EN 1-42 R_1: -H; R_2: -$CH_2OCH_2CH_2OCH_2CH_2OCH_2CH_2CH_2CH_3$; R_3: -$CH_2OCH_2CH_2CH_2CH_3$; R_4: -O; R_5: -CH_2; R_6: -O;

EN 1-48 R_1: -H; R_2: -$CH_2CH_2CH_3$; R_3: -Ph ; R_4: -O; R_5: -CH_2; R_6: -O;

EN 1-14 R_1: -H; R_2: -$CH_2CH_2CH_2CH_3$; R_3: -$CH_2OCH_2CH_2CH_2CH_3$; R_4: -O; R_5: -CH_2; R_6: -O

EN 1-16 R_1: -H; R_2: -$CH_2CH_2CH_2CH_2CH_2CH_3$; R_3: -$CH_2OCH_2CH_2CH_2CH_3$; R_4: -O; R_5: -CH_2; R_6: -O;

PBO homologues with modified aromatic ring

EN 14-5 R_1: -H; R_2: -$CH_2CH_2CH_3$; R_3: -$CH_2OCH_2CH_2CH_2CH_3$; R_4: -CH_2; R_5: -CH_2; R_6: -CH_2;

EN 16-5 R_1: -H; R_2: -$CH_2CH_2CH_3$; R_3: - $CH_2OCH_2CH_2CH_2CH_3$; R_4: -O; R_5: -CH_2; R_6: -CH_2;

EN 16-6 R_1: -H; R_2: -H; R_3: - $CH_2OCH_2CH_2CH_2CH_3$; R_4: -O; R_5: -CH_2; R_6: -CH_2;

EN 18-5 R_1: -H; R_2: -$CH_2CH_2CH_3$; R_3: -$CH_2OCH_2CH_2CH_2CH_3$; R_4: -O; R_5: -CH_2CH_2 R_6: -O;

Figure 1. Chemical structures of PBO and the newly synthesized PBO homologues.

the effects of these compounds on some plant proteases were studied in a hydrocarbon solution, using the bis(2-ethylhexyl)-sodium sulfosuccinate (AOT) reverse micellar system.

Reverse micelles are globe-shaped aggregates generated by surfactants in apolar solvents in which the polar heads of amphiphilic surfactant molecules are directed toward the inner part of the micelle, thus forming a polar core able to solubilize water, whereas the lipophilic chains of surfactant are exposed to the solvent (*12*). Reverse micelles are easily formed when certain surfactants and small amounts of water are added to an organic solvent. Under appropriate conditions, micellar solutions obtained by several surfactants are homogeneous, highly dynamic, thermodynamically stable systems and optically transparent. The dimension of micelles normally is on the order of nanometers and mostly depends on the ratio of water to surfactant concentration (Wo), which is expressed by the equation Wo = $[H_2O]$/[surfactant]. For example, when Wo increases from 5 to 40 with bis(2-ethylhexyl)sodium sulfosuccinate (AOT) as surfactant, the micelle diameter increases from about 4 to 15 nm (*13*).

From the early 1980s, numerous papers have demonstrated that reverse micelles are able to solubilize hydrophilic biopolymers such as enzymes and plasmids in hydrocarbon solvents. In addition, micellar enzyme kinetics (enzyme-catalyzed reactions where enzymes are entrapped in reversed micelles dispersed in organic solvents) were applied to determine the enzyme activity where hydrophobic substrates and enzyme effectors were used. In the case of lipase with glycerides, lipoxygenase with linoleic acid, and, as in our case, plant proteases with hydrophobic inhibitors, it is difficult to determine the enzyme activity in aqueous solutions with continuous direct spectrophotometric assays (*14*−*16*). Concerning the catalytic activity of already studied proteases in reverse micellar solutions, which were carried out mostly on trypsin and α-chymotrypsin, it was found that the hydrolytic activity for these enzymes was under optimal conditions (*17*−*19*). Starting from this finding, the kinetic study of some plant cysteine proteases in reverse micellar solution was interesting not only in view of their sensitivity toward PBO and some important PBOH but also for their behavior in a nonaqueous reaction medium. Given the established agricultural importance of PBO and the potential of some PBOH as new agrochemicals, the present study is dedicated to the comprehension of the negative effect that these compounds have shown toward some plant proteolytic enzymes. To this aim, reverse micelles are a good model to mimic plant cells (*20*) as well as provide an opportunity to gather information regarding the interaction efficiency between water-soluble enzyme(s) and a number of potential hydrophobic inhibitors. This is the first time that micellar enzyme kinetics has been applied to the study of hydrophobic molecules such as PBO and PBOH on plant proteases, and little is known about the behavior of these enzymes in a system such as AOT reverse micelles dispersed in hydrocarbon solutions.

MATERIALS AND METHODS

Protease and Inhibition Assays. *N*-benzoyloxycarbonyl-L-lysine *p*-nitrophenyl ester (CBZ) was used as a substrate, and its hydrolysis was followed and determined spectrophotometrically at 340 nm in 1 mL cuvettes with a path length of 1 cm at 30 °C using an $\epsilon_{340\ nm}$ = 6320 M^{-1} cm^{-1} (*21*). All assays were performed with a Cary model 2300 spectrophotometer equipped with a thermostated cell compartment, which was maintained at 30 °C in all enzymatic activity determinations.

Stock Solutions. Bromelain (4.51 U/mg solid) was dissolved in 10 mM sodium acetate buffer, pH 4.6, containing 1 mM cysteine to obtain a solution of 1 mg/mL (*21*). Papain (22.7 U/mg solid) was dissolved in 50 mM MES buffer, pH 6.0, containing 1 mM cysteine to obtain a solution of 1 mg/mL. The CBZ stock solution, used as substrate in the enzymatic assay of bromelain and papain, had a final concentration of 30 mM in acetonitrile containing 20% v/v water. The substrate stock solution was prepared by suspending the CBZ in acetonitrile (80 volumes); subsequently 20 volumes of water was added, and a clear solution was obtained. PBO and PBOH stock solutions had concentrations ranging from 0.1 to 1 M in isooctane. These compounds were protected from light during their preparation, use, and storage. Finally, AOT−isooctane stock solution was prepared by dissolving bis(2-ethylhexyl)sodium sulfosuccinate (AOT) in isooctane up to obtain a solution of 50 mM.

Protease Assays in Water. All assays were performed in 50 mM sodium acetate buffer, pH 4.6 (980 μL), to which enzyme stock solutions of bromelain or papain (10 μL) and CBZ stock solution (10 μL) were added directly in a quartz spectrophotometric cuvette. The self-hydrolysis of the substrate was previously determined in a blank solution.

Protease Assays in Reverse Micelles. All assays were performed in 50 mM AOT−isooctane solution (980 μL) to which enzyme solutions (10 μL) and CBZ stock solution (10 μL) were added directly in the

cuvette. Thus the final overall concentrations for papain, bromelain, and CBZ were 4.35×10^{-7}, 3.23×10^{-7}, and 2.99×10^{-4} M, respectively. By convention, overall concentration means that the solute concentration refers to the total volume of organic and water solution, whereas local concentration refers only to the water solution. The Wo value was maintained constant at 22.7 in all experiments. To find these conditions, a preliminary study to optimize the enzymatic assays in reverse micelles was carried out, where different AOT concentrations in isooctane and Wo's were tested. During these experiments, the overall concentration of the enzyme(s) and substrate were kept constant while the desired water content was obtained by an additional injection of the same buffer solution into the micellar solution. Every addition of aqueous solutions to the organic phase in the spectrophotometric cuvette was followed by vigorous stirring of the mixture to obtain a clear transparent solution. The enzyme activity was measured for 2 min following the absorbance increase at 340 nm. A unit of enzyme activity was defined as 1 μmol of substrate hydrolyzed per minute in the assay conditions. The specific activity is reported as the units per milligram of soluble protein.

Inhibition Assays in Reverse Micelles. The inhibition activity of PBO and each PBOH was determined with the addition of aliquots (from 1 to 20 μL) of PBO and PBOH stock solutions to the spectrophotometric cuvette containing the AOT−isooctane before the enzymes and CBZ solutions were added. For each inhibitor concentration, the enzyme activity was determined at least in triplicate. The inhibition curves were performed by plotting the percentage of protease inhibition versus the inhibitor concentration, and the slopes were used to calculate the concentration of inhibitor that reduces the protease activity by 50% (IC_{50}). The analyses were carried out without previous enzyme−inhibitor incubation; incubation times ranging from 15 to 45 min before analysis were ineffective. The enzyme activities were compared to those determined in blank solutions where isooctane was added instead of the inhibitor solution.

PBO and PBOH Preparation. Different homologues of PBO with modified aromatic rings and side chains (**Figure 1**) were synthesized to evaluate their biological activity in comparison with PBO, 5-[2-(2-butoxyethoxy)ethoxymethyl]-6-propyl-benzo[1,3]dioxole. The homologues with the modified side chains were 5-[2-(2-butoxyethoxy)-ethoxymethyl]-benzo[1,3]dioxole (EN 1-40), 5,6-bis-[2-(2-butoxy-ethoxy)ethoxymethyl]-benzo[1,3]dioxole (EN 1-42), 5-(2-benzyloxy-ethoxymethyl)-6-propyl-benzo[1,3]dioxole (EN 1-48), 5-[2-(2-butoxy-ethoxy)ethoxymethyl]-6-butyl-benzo[1.3]dioxole (EN 1-14), and 5-[2-(2-butoxyethoxy)ethoxymethyl]-6-hexyl-benzo[1,3]dioxole (EN 1-16), and the PBO homologues with the modified aromatic ring were 6-[2-(2-butoxyethoxy)ethoxymethyl]-5-propylindan (EN 14-5), 6-[2-(2-butoxyethoxy)ethoxymethyl]-5-propyl-2,3-dihydrobenzofuran (EN 16-5), 6-[2-(2-butoxyethoxy)ethoxymethyl]-2,3-dihydrobenzofuran (EN 16-6), and 6-[2-(2-butoxyethoxy)ethoxymethyl]-7-propyl-2,3-dihydro-benzo[1,4]dioxane (EN 18−5).

PBO and the homologues modified in the side chain were synthesized with a two-step procedure essentially based on the chloromethylation of benzodioxole, followed by an etherification. In particular, EN 1-48, EN 1-14, and EN 1-16 were produced starting from propyl, butyl, and hexyl benzodioxole. The homologues EN 14-5, EN 16-5, EN 16-6, and EN 18-5 were synthesized through a four-step synthesis, namely, acylation, reduction, chloromethylation, and etherification from commercially available indan, 2,3-dihydrobenzofuran, and 2,3-dihydrobenzo-[1,4]dioxane, respectively. The intermediates, when possible, were isolated and characterized by GC/MS and NMR, as well as the final product. Most of PBOHs were finally purified by distillation up to a minimum purity of 97% as confirmed by GC. Only EN 1-42 was purified by absorption column chromatography (silica gel 130−270 mesh), up to a minimum purity of 93%, using diisopropyl ether/methanol (99:1 v/v) as the mobile phase. The NMR and GC/MS analyses of PBOH confirm their structures. The NMR spectra were scanned in $CDCl_3$ containing tetramethylsilane as internal reference, using a Varian Gemini 300 spectrometer and Varian Gemini 200 spectrometer. The GC-MS analyses were carried out using a Varian instrument, model Saturn 2000, equipped with a capillary column (30 m, 0.25 mm i.d., 0.25 μm film thickness) packed with 5% diphenyl, 95% dimethyl polysiloxane. The carrier gas (He) flow rate was 1 mL min^{-1}. The experiments were carried out with electron impact ionization (EI) mode at an electron energy of 70 eV. The injector temperature was 250 °C. For GC analyses of PBO homologues with a modified aromatic ring, the column was held at 50 °C for 2 min, heated at 3.5 °C min^{-1} up to 71 °C, at 2.5 °C min^{-1} up to 200 °C, and at 3 °C min^{-1} up to 250 °C, and finally held at 250 °C for 10 min. For GC analyses of PBO homologues with a modified side chain, the column was held at 70 °C for 2 min, heated at 5 °C min^{-1} up to 285 °C, and then held at 285 °C for 30 min.

Partition Coefficient Determination. The partition coefficients were determined between *n*-octanol and water (P_{ow}), following the procedure reported by De Brujn et al. in 1988 (*22*) for hydrophobic organic chemicals. The presaturation of both phases was achieved at room temperature by stirring 980 mL of water and 20 mL of *n*-octanol in a suitable flask until equilibrium was reached. Pure compounds of PBO and EN 1-40 (2 g) were added to the two solvent systems, which were slowly stirred to avoid emulsion formation (*22*). Because the other PBOHs were available in smaller quantities, the P_{ow} determinations were carried out with 98 mL of water, 2 mL of *n*-octanol, and 0.2 g of each compound. The concentration of PBO and PBOH was then determined in the water phase on successive days until the steady-state condition was obtained.

Sampling and Analysis. Before sampling, the two solvent systems were left to settle in a separation funnel to allow the phases to separate. Three samples of the water phase were taken from the bottom of the separation funnel and centrifuged at $39\,800 \times g$ for 15 min at room temperature.

Spectrophotometric Method. The absorbance of the water phase was measured at 290 nm for determination of the PBO and PBOH concentrations, which were calculated using a calibration plot previously determined. The absorbance of PBO and PBOH in water solution was determined by reading the sample against a blank of aqueous solution presaturated with *n*-octanol. For the compound EN 14-5, the absorbance at 290 nm was not detectable, and the water phase concentration could not be determined by this method, so only a gas chromatographic analysis was carried out.

Gas Chromatographic Method. The water phase concentrations of the compounds were confirmed by gas chromatography−mass spectrometry (GC-MS). GC-MS analyses were carried out using a Hewlett-Packard GCD System model G1800A equipped with a 30 m × 0.25 mm capillary column HP-5. The flow rate of the carrier gas (He) was 1 mL min^{-1}. Aqueous samples were extracted with hexane, and 1 μL of the extract was injected. The column temperature was 160−180 °C at the start and 250 °C at the end with a rate of 8 °C min^{-1}; the temperatures of the injector and of the detector were 250 °C and 280 °C, respectively. As for the spectrophotometric method, the compound concentrations were calculated from a calibration plot previously determined. EN 1-16 was not analyzable by GC-MS, and therefore, its P_{ow} was determined only by UV spectrophotometry.

Calibration Plot. Water solutions of 0.1% PBO and PBOH (3.1 mM) were stirred for 2 days and then were diluted with distilled water until a clear transparent solution was produced. In this way, different concentrations of PBO and PBOH were obtained. The calibration curve was defined by plotting the absorbance at 290 nm of diluted solutions versus concentrations of PBO and PBOH. In the case of GC-MS analyses, the same diluted water solutions were extracted with hexane and analyzed by GC to obtain a calibration curve where the peak areas from the GC chromatogram of PBO and PBOH were plotted against their concentrations.

Reagents and Enzymes. Papain, bromelain and *N*-benzoyloxycarbonyl-L-lysine *p*-nitrophenyl esters were obtained from Sigma. Bis(2-ethylhexyl)sodium sulfosuccinate (AOT) was provided by Fluka. Isooctane and octanol for spectroscopy were obtained from Carlo Erba and Fluka, respectively. The other reagents were of analytical grade and were provided by Carlo Erba and by Merck.

RESULTS AND DISCUSSION

PBOH Characterization. At the end of preparation, PBOHs (**Figure 1**) were purified by distillation or by absorption column chromatography. The purity of these compounds, confirmed by

GC, ranged from 93% to 97% (see the Materials and Methods section). NMR and GC/MS analyses of PBOHs confirm their structures. Physical (boiling point, bp/mbar of vacuum) and spectrometric data for all compounds are reported.

EN 1-40, 5-[2−2(Butoxyethoxy)ethoxymethyl]-benzo[1,3]-dioxole. Colorless oil, bp 155−156 °C/0.3 mbar. ^{1}H NMR (300 MHz; $CDCl_3$) δ, ppm: 0.80 (*t*, 3H, $J = 6.4$ Hz, CH_3(19)); 1.28 (*m*, 2H, CH_2(18)); 1.48 (*m*, 2H, CH_2(17)); 3.38 (*t*, 2H, $J = 7.8$ Hz, O−CH_2(16)); 3.51 (*m*, 4H, 2O−CH_2); 3.57 (*m*, 4H, 2 O−CH_2); 4.38 (*s*, 2H, O−CH_2(8)); 5.85 (*s*, 2H, O−CH_2(2)); 6.68 (*m*, 2H, 2CH (4,5)); 6.80 (*m*, 1H, CH (7)). ^{13}C NMR (75 MHz; $CDCl_3$) δ, ppm: 13.80 (CH_3, C(19)); 19.15, 31.59 (CH_2, C(18,17)); 69.03, 69.97, 70.56, 71.089, 72.95 (6 O−CH_2, C(16,14,13,11,10,8)); 100.82 (O−CH_2, C(2)); 107.86, 108.38, 121.19 (CH, C(4,5,7)); 132.06 (C, C(6)); 146.95, 147.62 (2C, C(7a,3a)). MS *m*/*z* (relative abundance %): 41 (12), 45 (14), 51 (13),57 (13), 77 (23), 135 (100), 148 (15),150 (23),151 (46).

EN 1−42, 5,6-Bis-[2-(2-butoxyethoxy)ethoxymethyl]-benzo-[1,3]dioxole. Yellow oil purified by column chromatography. ^{1}H NMR (300 MHz; $CDCl_3$) δ, ppm: 0.91 (*t*, 6H, $J = 7.7$ Hz, 2CH_3(19,19′)); 1.36 (*m*, 4H, 2CH_2(18,18′)); 1.57 (*m*, 4H, 2CH_2-(17,17′)); 3.46 (*t*, 4H, $J = 6.8$ Hz, 2CH_2(16,16′)); 3.64 (*m*, 16H, 8 O−CH_2); 4.53 (*s*, 4H, 2 O−CH_2(8,8′)); 5.93 (*s*, 2H, O−CH_2-(2)); 6.90 (*s*, 2H, 2CH (4,7)). ^{13}C NMR (75 MHz; $CDCl_3$) δ, ppm: 14.07 (CH_3, C(19,19′)); 19.42, 31.86 (CH_2, C(17,17′,-18,18′)); 69.54, 70.25, 70.49, 70.80, 70.84, 71.32 (12 O−CH_2); 101.14 (O−CH_2, C(2)); 109.46 (2CH, C(4,7)); 130.49 (2C, C(5,6)); 147.10 (2C, C(7a,3a)). MS *m*/*z* (relative abundance %): 41 (20), 45 (24), 57 (20), 135 (10), 136 (13), 147 (11), 163 (23), 164 (21), 174 (30), 202 (25).

EN 1-48, 5-(2-Benzyloxyethoxymethyl)-6-propyl-benzo[1,3]-dioxole. Colorless oil, bp 150−152 °C/0.1 mbar. ^{1}H NMR (300 MHz; $CDCl_3$) δ, ppm: 0.94 (*t*, 3H, $J = 6.85$ Hz, CH_3(10′)); 1.56 (*m*, 2H, CH_2(9′)); 2.54 (*m*, 2H, CH_2(8′)); 3.65 (*s*, 4H, 2CH_2-(10,11)); 4.49 (*s*, 2H, CH_2(8)); 4.57 (*s*, 2H, CH_2(13)); 5.89 (*s*, 2H, CH_2(2)); 6.66 (*s*, 1H, CH(4)); 6.86 (*s*, 1H, CH(7)); 7.30 (*m*, 1H, CH−Ar). ^{13}C NMR (75 MHz; $CDCl_3$) δ, ppm: 14.68 (CH_3(10′)); 25.14 (CH_2(9′)); 35.00 (CH_2(8′)); 70.04, 70.28, 71.51, 73.91, 77 (O−CH_2, C(8,10,11,13)); 101.40 (O−CH_2, C(2)); 110.06, 110.13 (2CH, C(4,7)); 128.21, 128.34, 128.99 (5CH, C(14,15,16,17,18)); 129.56, 135.70, 138.94 (3C, C(5,6,-13)); 146.11, 147.64 (2C, C(7a,3a)). MS *m*/*z* (relative abundance %): 65 (14), 77 (13), 91 (46), 107 (12), 119 (14), 149 (18), 176 (100).

EN 1-14, 5-[2-(2-Butoxyethoxy)ethoxymethyl)]-6-butyl-benzo-[1.3]dioxole. Colorless oil, bp 190−191 °C/0.3 mbar. ^{1}H NMR (300 MHz; $CDCl_3$) δ, ppm: 0.91 (*t*, 6H, $J = 7.1$ Hz, 2CH_3-(19,19′)); 1.36 (*m*, 4H, 2CH_2(18,18′)); 1.57 (*m*, 4H, 2CH_2-(17,17′)); 3.48 (*t*, 4H, $J = 7.0$ Hz, 2 O−CH_2); 3.62 (*m*, 16H, 8 O−CH_2); 4.53 (*s*, 4H, 2 O−CH_2(8,8′)); 5.93 (*s*, 2H, O−CH_2-(2)); 6.90 (*s*, 2H, 2CH (4,7)). ^{13}C NMR (75 MHz; $CDCl_3$) δ, ppm: 14.10 (CH_3, C(19,19′)); 19.42, 31.86 (CH_2, C(17,17′,-18,18′)); 69.54, 70.25, 70.50, 70.80, 70.84, 71.32 (12 O−CH_2); 101.14 (O−CH_2, C(2)); 109.46 (2CH, C(4,7)); 130.49 (2C, C(5,6)); 147.10 (2C, C(7a,3a)). MS *m*/*z* (relative abundance %): 145 (11), 149 (42), 175 (14), 190 (100).

EN 1-16, 5-[2-(2-Butoxyethoxy)ethoxymethyl]-6-hexyl-benzo-[1.3]dioxane. Colorless oil, bp 192−196 °C/0.2 mbar. ^{1}H NMR (300 MHz; $CDCl_3$) δ, ppm: 0.85 (*m*, 6H, 2CH_3(19,13′)); 1.26 (*m*, 8H, 4CH_2); 1.50 (*m*, 4H, 2CH_2); 2.48 (*t*, 2H, $J = 7.5$ Hz, CH_2); 3.39 (*t*, 2H, $J = 7.5$ Hz, CH_2); 3.57 (*m*, 8H, 4CH_2-(10,11,13,14)); 4.41 (*s*, 2H, O−CH_2(8)); 5.83 (*s*, 2H, O−CH_2-(2)); 6.59, 6.78 (*s*, 2H, 2CH (4,7)). ^{13}C NMR (75 MHz; $CDCl_3$) δ, ppm: 13.83, 14.02 (CH_3, C(19,13′)); 19.19, 22.55 (CH_2, C(17,18)); 29.19, 31.37, 31.63, 31.70, 32.26 (CH_2, C(12′,11′,-10′,9′,8′)); 69.26, 70.02, 70.62, 70.74, 71.16 (O−CH_2, C(8,-10,11,13,14,16)); 100.66 (O−CH_2, C(2)); 109.34 (2CH, C(4,7)); 128.74, 135.23 (2C, C(5,6)); 145.34, 146.92 (2C, C(7a,3a)). MS *m*/*z* (relative abundance %): 149 (48), 175 (20), 218 (100).

EN 14-5, 6-[2-(2-Butoxyethoxy)ethoxymethyl]-5-propylindan. Colorless oil, bp 135 °C/0.3 mbar. ^{1}H NMR (200 MHz; $CDCl_3$) δ, ppm: 0.95 (*m*, 6H, 2CH_3 (10, 21)); 1.35 (*m*, 2H, CH_2 (20)); 1.55 (*m*, 4H, 2CH_2 (19, 9)); 2.05 (*quint*, 2H, CH_2 (2)); 2.60 (*m*, 2H, CH_2); 2.85 (*t*, 4H, $J = 7.0$ Hz, 2CH_2); 3.45 (*t*, 2H, $J = 6.3$ Hz, CH_2); 3.85 (*m*, 8H, 4CH_2); 4.60 (*s*, 2H, CH_2 (11)); 7.04 (*s*, 1H, CH (7)); 7.19 (*s*, 1H, CH (4)). ^{13}C NMR (50 MHz; $CDCl_3$) δ, ppm: 14.47, 14.83 (CH_3, C(10), C(21)); 19.84, 25.16, 26.06, 32.19, 33.03, 35.06 (CH_2); 69.90, 70.67, 71.22, 71.28, 71.75, 72.01 (O−CH_2); 125.80 (CH, C(4) and C(7)); 137.01, 139.80, 141.01, 142.07 (C, C(5), C(7a), C(6), C(3a)). MS *m*/*z* (relative abundance %): 41 (13), 45 (11), 57 (12), 115 (11), 128 (15), 131 (22), 144 (14), 145 (38).

EN 16-5, 6-[2-(2-Butoxyethoxy)ethoxymethyl]-5-propyl-2,3-dihydrobenzofuran. Colorless oil, bp 180−185 °C/0.1 mbar. ^{1}H NMR (200 MHz; $CDCl_3$) δ, ppm: 0.95 (*m*, 6H, 2CH_3 (10, 21)); 1.35 (*m*, 2H, CH_2 (20)); 1.55 (*m*, 4H, 2CH_2 (19, 9)); 2.50 (*t*, 2H, $J = 7.0$ Hz, CH_2); 3.45 (*t*, 2H, $J = 6.3$ Hz, CH_2); 3.65 (*m*, 8H, 4CH_2); 4.55 (*m*, 4H, 2CH_2); 6.98 (*s*, 2H, CH (4,7)). ^{13}C NMR (50 MHz; $CDCl_3$) δ, ppm: 14.42, 14.51 (CH_3, C(10), C(21)); 19.87, 25.62, 30.45, 32.32, 38.10 (CH_2); 68.51, 70.08, 70.70, 70.78, 71.18, 71.23, 71.79 (O−CH_2); 124.82, 128.52 (CH, C(4) and C(7)); 119.81, 127.32, 135.31, 156.79 (C, C(5), C(7a), C(6), C(3a)). MS *m*/*z* (relative abundance %): 41 (11), 105 (11), 174 (30), 175 (100), 191 (36).

EN 16-6, 6-[2-(2-Butoxyethoxy)ethoxymethyl]-2,3-dihydrobenzofuran. Colorless oil, bp 168−170 °C/0.2 mbar. ^{1}H NMR (300 MHz; $CDCl_3$) δ, ppm: 0.90 (*t*, 3H, $J = 7.27$ Hz, CH_3 (19)); 1.35 (*m*, 2H, CH_2 (18)); 1.56 (*quint*, 2H, $J = 7.60$ Hz, CH_2 (17)); 3.15 (*t*, 2H, $J = 8.77$ Hz, CH_2 (1)); 3.56 (*t*, 2H, $J = 7.6$ Hz, CH_2 (16)); 3.67−3.56 (*m*, 8H, 4CH_2 (10,11,13,14)); 4.46 (*s*, 2H, CH_2 (8)); 4.54 (*t*, 2H, $J = 8.77$ Hz, CH_2 (2)); 6.71 (d, 1H, $J = 8.1$ Hz, CH (5)); 7.03 (d, 1H, $J = 8.1$ Hz, CH (4)); 7.18 (s, 1H, CH (7)). ^{13}C NMR (75 MHz; $CDCl_3$) δ, ppm: 12.87 (CH_3, C(19)); 19.20, 29.57 (CH_2, C(18), C(17)); 31.65 (CH_2, C(1)); 68.98 (O−CH_2, C(2)); 70.02, 70.58, 70.62, 71.14, 71.22, 73.17 (O−CH2, C(16), C(14), C(13), C(11), C(10), C(8)); 108.81, 124.95, 128.11 (CH, C(4), C(5), C(7)); 127.08, 130.19, 159.67 (C, C(7a), C(6), C(3a)). MS *m*/*z* (relative abundance %): 41 (7), 77 (5), 105 (8), 133 (100), 149 (14).

EN 18-5, 6-[2-(2-Butoxyethoxy)ethoxymethyl]-7-propyl-2,3-dihydrobenzo[1,4]dioxane. Colorless oil, bp 195−196 °C/0.4 mbar. ^{1}H NMR (200 MHz; $CDCl_3$) δ, ppm: 0.95 (*m*, 6H, 2CH_3-(11, 23)); 1.35 (*m*, 2H, CH_2); 1.53 (*m*, 4H, 2CH_2); 2.55 (*t*, 2H, $J = 7.7$ Hz, CH_2(9)); 3.45 (*t*, 2H, $J = 6.8$ Hz, CH_2(20)); 3.62 (*m*, 8H, 4CH_2); 4.20 (*m*, 4H, 2CH_2), 4.45 (*s*, 2H, CH_2(12)); 6.72 (*s*, 1H, CH(5)); 6.84 (*s*, 1H, CH(8)). ^{13}C NMR (50 MHz; $CDCl_3$) δ, ppm: 14.45, 14.62 (CH_3, C(11), C(23)); 19.76, 24.79, 32.25, 34.23 (CH_2); 64.83, 64.98, 69.77, 70.62, 71.10, 71.22, 71.70 (O−CH_2); 118.27, 118.62 (CH, C(5) and C(8)); 122.27, 129.40, 135.16, 141.65 (C, C(6), C(8a), C(7), C(4a)). MS *m*/*z* (relative abundance %): 91 (14), 163 (63), 175 (11), 190 (100), 191 (33).

Protease Activity Assay in Reverse Micelles. Whereas papain was studied in reverse micelles to establish its stability and activity in comparison to water solutions (*23*), to the best of our knowledge bromelain activity has not been studied in a micellar hydrocarbon solution before. Therefore, no specific references were available to set up an optimal protease assay

Table 1. Dependence of Bromelain Activity on the Water Content and AOT Concentration of Reverse Micelles at Constant Wo (22.7) at 30 °C in 0.01 M Sodium Acetate, pH 4.6, and 1 mM Cysteine

[AOT] (mM)	% H_2O v/v	[bromelain] (μM) overall	[bromelain] (μM) local	[CBZ] (mM) overall	[CBZ] (mM) local	activity (U/mL)
25	1	0.323	32.3	0.3	30.0	0.377
50	2	0.323	16.1	0.3	15.0	0.209
100	4	0.323	8.07	0.3	7.5	0.082

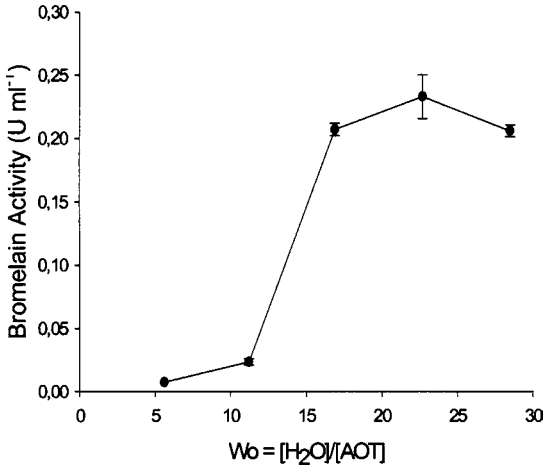


Figure 2. Velocity of bromelain-catalyzed hydrolysis on *N*-benzoyloxycarbonyl-L-lysine *p*-nitrophenyl ester in 50 mM AOT/isooctane reverse micelles as a function of Wo at 30 °C. $[CBZ]_{ov}$ = constant = 0.3 mM; $[bromelain]_{ov}$ = constant = 0.323 μM (0.01 M sodium acetate, pH 4.6, 1 mM cysteine). 1 U = 1 μmol of CBZ hydrolyzed per minute.

for this enzyme in these conditions. Before other results are discussed, it is of interest to comment on the behavior of bromelain in reverse micelles, which is preliminary to the work made to find the optimal enzyme assay conditions.

When Wo and the overall concentrations of substrate and enzyme are kept constant, the velocity of the bromelain-catalyzed reaction depends on water content in the whole system because the enzyme and substrate concentrations change in the water pool of micelles (**Table 1**). In fact, as was observed with other enzymes in reverse micelles, the reaction velocity increases when the water concentration decreases in the micellar system (*18*). The determination of the optimal Wo has been another important preliminary study carried out for optimizing the enzymatic assays used in this work. Wo is an important parameter that affects most of the physicochemical properties of reverse micelles dispersed in hydrocarbon solutions, including their dimension. **Figure 2** reports the reaction rate as a function of Wo at pH 4.6 (optimal pH for bromelain when CBZ is used as substrate). This experiment indicates the range of Wo values in which bromelain activity can be measured in an enzyme reverse micelle assay with 50 mM AOT in isooctane: at values below 5.6, bromelain activity was not detectable, whereas values above 33 were impractical because homogeneous and transparent micellar solutions cannot be achieved. Furthermore, the experiment of **Figure 2** shows that although the local concentration of enzyme and substrate decreases due to the increase of water concentration and micelle dimension, the reaction rate increases up to Wo 22.7. This optimal value was kept constant in all experiments. Finally, this trial shows that the relationship between Wo and bromelain activity describes a bell-shaped curve, similar to what was reported for several other enzymes (*24*, *25*).

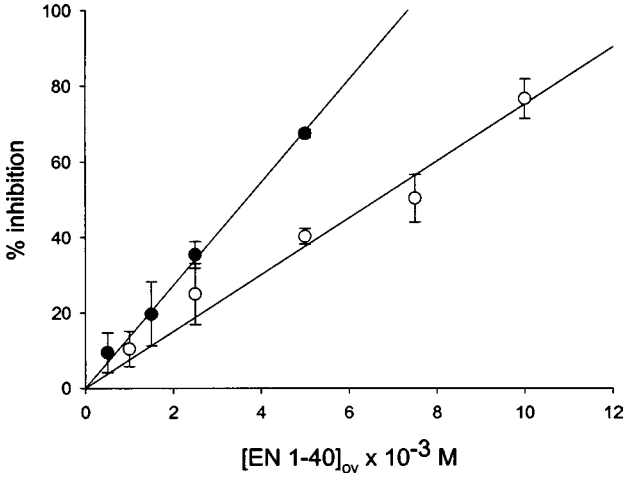


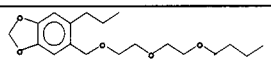
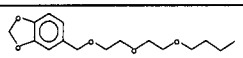
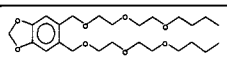
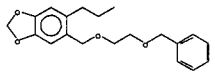
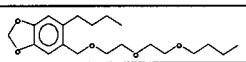
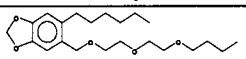
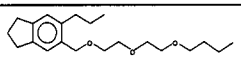
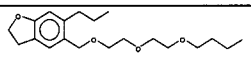
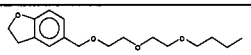
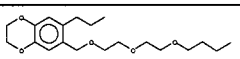
Figure 3. Percent inhibition of bromelain and papain ativity in 50 mM AOT/isooctane reverse micelles at different overall concentrations of EN 1-40 (0.5–10 mM). Wo = 22.7; $[CBZ]_{ov}$ = 0.3 mM; $[bromelain]_{ov}$ = 0.323 μM (0.01 M sodium acetate, pH 4.6, 1 mM cysteine) (●); $[papain]_{ov}$ = 0.435 μM (0.05 M MES, pH 6.0, 1 mM cysteine) (○).

In addition to these preliminary studies, the best experimental conditions to determine protease activities and their inhibition in the presence of PBO and PBOHs were found using different substrates (CBZ and BAEE), substrate (CBZ) concentrations (from 7.5 to 60 mM), cysteine concentrations (from 1 to 25 mM), pH (from 2.6 to 6.6), and ionic strength (10, 50, and 100 mM) with acetate and MES buffers with the aim to set up suitable assays for permitting a better comparison among enzymes and effectors tested and, at the same time, to improve the assay sensitivity, linearity, and reproducibility. To better feature the inhibitory effect, the enzyme assays were carried out in pre-steady-state conditions, as was also done by Heinrikson and Kézdy in water solution (*21*). At the end of a series of trials, the performances of the assays for both the enzymes were acceptable, even though enzyme activities in reverse micelles were from 5 to 10 times lower than those in water solution.

Inhibition Curves. The relationship between bromelain and papain inhibition and concentration of EN 1-40 is shown in **Figure 3**. The results indicate that there is a linear increase of inhibition at a wide range of inhibitor concentrations, although with different slopes according to the nature of the enzyme tested, thus showing specific sensitivity toward these PBOHs. Similar curves were determined for PBO and the other PBOHs (not shown), which were necessary to calculate the values reported in **Table 2**.

Protease Inhibition by PBO and Different PBOHs. The majority of the studied compounds inhibited esterase activity in the two plant proteases considered. **Table 2** shows the enzymatic inhibition data of PBO and PBOHs and the concentration of inhibitor calculated to reduce 50% of the protease activity (IC_{50}). Considering these data, it is easy to assess that PBO and PBOHs appear to be medium-low negative effectors of plant proteases, especially if they are compared with typical macromolecular inhibitors of serine and cysteine proteases such as the Bowman Birk inhibitor(s) and different kinds of cystatins, respectively. Nevertheless, the inhibition values of **Table 2** are not so far from those determined in water solution for other important synthetic inhibitors, such as benzamidine and fluorine derivatives normally considered strong generic protease inhibitors, or for phenyl-methane-sulfonyl fluoride, diisopropyl-fluorophosphate, and phenylmethylsulfonylfluoride for serine

Table 2. Inhibition of Bromelain and Papain by PBO and PBOH[a]

Inhibotors	IC_{50} Bromelain $[inhibitor]_{overall}$ ($\times 10^{-2}$ M)	IC_{50} Papain $[inhibitor]_{overall}$ ($\times 10^{-2}$ M)
PBO 	1.32 ± 0.09	4.35 ± 0.35
EN 1-40 	0.37 ± 0.02	0.66 ± 0.07
EN 1-42 	1.24 ± 0.22	1.81 ± 0.18
EN 1-48 	2.53 ± 0.46	2.76 ± 0.45
EN 1-14 	1.44 ± 0.08	1.67 ± 0.69
EN 1-16 	1.44 ± 0.22	2.64 ± 0.32
EN 14-5 	6.56 ± 1.68	3.11 ± 0.7
EN 16-5 	0.78 ± 0.08	0.90 ± 0.04
EN 16-6 	0.41 ± 0.04	0.44 ± 0.02
EN 18-5 	7.37 ±1.27	6.91 ±1.52

[a] IC_{50}'s of PBO and PBOHs were calculated using the calibration plot: % of inhibition of enzyme vs inhibitor concentration.

proteases, cysteine proteases, and cotton carboxypeptidase, respectively (*26*−*28*). In addition, if one considers the water solubility of PBO and PBOH, which ranges between 10^{-6} and 10^{-4} M (see **Table 3**) and the micellar system as a simple two-phase system, it is reasonable to think that the local concentration of these molecules in the water pool of reverse micelles that interacts with the proteases is at least of 2 orders of magnitude lower than the overall concentration used to calculate the data reported in **Table 2**. In this view, the inhibitory potential of these molecules is much more valuable. Nevertheless, a precise determination of the IC_{50} referring to the local concentration of each inhibitor cannot be calculated because the whole reaction inhibition rate is the addition of inhibition rates occurring in each domain of the micellar system: free water inside of reverse micelles, bound water in the interphase, and the hydrocarbon solution (*29*).

Considering the IC_{50} data in **Table 2**, EN 1-40 was the strongest inhibitor of the two enzymes considered, showing a level of inhibition at least three times higher than the other benzodioxole derivatives of PBO, EN 1-14, EN 1-16, and EN 1-42. These compounds showed similar inhibition activities toward bromelain, which was the most inhibited enzyme by PBOHs containing the benzodioxole structure. EN 18-5 and EN 14-5, which do not contain a benzodioxole ring, even if they were more effective with papain, were very weak inhibitors toward bromelain. Nevertheless, EN 16-5 and EN 16-6, which show a dihydrobenzofuran (or benzo-mono-oxygen) ring instead of the benzodioxole are able to exert an important inhibition activity on cysteine proteases.

Although it is difficult to make reliable conclusions about the relationship between chemical structure and inhibition activity, it is important to remark that the benzodioxole and benzo-mono-oxygen structures appeared to be necessary to produce a certain protease inhibitory activity. In this regard, it is interesting to remark that the inhibition effect appeared to be strongly modulated by water solubility of PBO and its homologues. Thus, to have more elements to establish a realistic relationship between chemical structure and inhibition activity, it is important also to evaluate the data of **Table 2** in light of the P_{ow} of PBO and each PBOH reported in **Table 3**.

Partition Coefficients (Octanol/Water) of Inhibitors and Their Effect on Protease Inhibition. The partition coefficient between octanol and water is a widely used parameter for organic compounds to describe physicochemical properties such as hydrophobicity and water solubility (*21*).

Considering the log P_{ow} data reported in **Table 3**, it is possible to split PBO and the PBOHs into two groups, depending on their water solubility. The first group is the most numerous, and it is represented by a mixture of benzodioxole and non-benzodioxole compounds (PBO, EN 1-48, EN 18-5, EN 14-5, and EN 1-14) with a low water solubility evidenced by a log P_{ow} ranging from 4.03 to 4.46. The second group is constituted by benzodioxole compounds that, compared to PBO, have modified side chains: EN 1-40 (log $P_{ow} = 2.63$) provides a similar structure to PBO but without the aliphatic tail, whereas EN 1-42 (log $P_{ow} = 2.61$) contains two 2-(2-butoxyethoxy)-ethoxymethyl chains. EN 16-5, EN 16-6, and EN 1-16 deserve special consideration. Taking into account their log P_{ow}, these compounds cannot be placed either in the first group or in the second. In particular, the presence of the aliphatic side chain was much more effective on water solubility of benzodioxole compounds (see PBO and EN 1-40) rather than of those with benzo-mono-oxygen configuration such as EN 16-5 and EN 16-6. In addition, the aliphatic side chain extension produced a dramatic decrease of water solubility in the benzodioxole molecules such as PBO and EN 1-16.

The relationship between bromelain inhibition and the hydrophobicity of different inhibitors shows that the benzodioxole compounds are well placed around a correlation curve, although they have different water solubility and inhibition activity (**Figure 4**). In contrast, the two non-benzodioxoles, EN 18-5 and EN 14-5, are notably far from this curve even if they show similar hydrophobicity to some other compounds. This means that in the case of the benzodioxole compounds, the

Table 3. Inhibitor Concentrations in the Water Phase of the Two-Solvent System Octanol/Water and Partition Coefficients (Octanol/Water) of PBO and PBOH

Inhibitors	Molecular weights	Concentrations in water phase ($\times 10^{-4}$ M)	Log Pow
PBO	338	0.29±0.04	4.06±0.07
EN 1-40	296	7.22±0.54	2.63±0.04
EN 1-42	470	4.69±0.33	2.61±0.04
EN 1-48	328	0.38±0.11	4.08±0.01
EN 1-14	352	0.127±0.009	4.35±0.03
EN 1-16	380	0.010±0.002	5.45±0.11
EN 14-5	334	0.09±0.04	4.46±0.09
EN 16-5	336	1.92±0.27	3.18±0.06
EN 16-6	294	3.43±0.05	2.98±0.005
EN 18-5	352	0.26±0.00	4.03±0.00

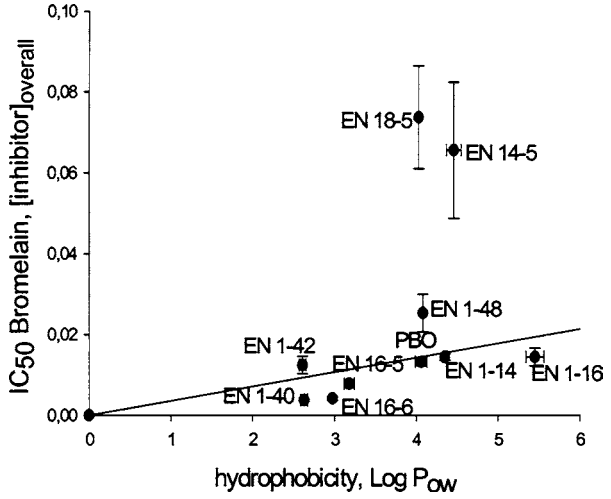


Figure 4. Relationship of bromelain inhibition, IC_{50}, to inhibitor hydrophobicity, log P_{ow}. IC_{50} = $[inhibitor]_{overall}$ calculated to reduce 50% of the protease activity.

inhibition potential is due to a combination of two factors: chemical structure and water solubility. For instance, among PBOHs with similar benzodioxole structure, EN 1-40 and EN 1-42 are the most active inhibitors because they show the highest water solubility. In this circumstance, the partition equilibrium between water and isooctane plays an important role in enzyme inhibition because it is shifted toward the water pool of micelles. In principle, this means that a higher concentration of these inhibitors in the water pool makes a more efficient interaction with the enzyme possible. On the other hand, despite the moderate water solubility of EN 14-5 and EN 18-5, their structures do not allow similar inhibitory effects. Consequently, the observed inhibition activity of PBO and some PBOHs appears to be due to the benzodioxole and benzo-mono-oxygen configuration of these compounds. Water solubility, modulated by chemical structure, can indirectly increase the inhibition activity. This is an additional value in the potential inhibition of proteases.

Analogous findings were obtained with papain (not shown). In this case, the behavior of EN 14-5 is similar to that observed with the other benzodioxole and benzo-mono-oxygen PBOHs. A hypothetical explanation could be found in consideration of the different natures of the enzymes, although both are cysteine proteases, and the singular chemical structure of EN 14-5.

Concluding Remarks. The hydrocarbon reverse micellar system is an original and useful system for isolating different types of proteins and enzymes (*19*, *30*, *31*), for carrying out specific enzymatic analyses (*16*, *32*), and for immobilizing enzymes (*12*), microorganisms (*33*), and plant cells (*34*). In our case, the use of hydrated reverse micelles dispersed in an organic solvent was the only viable way to study the protease inhibition potential of PBO and of a series of new PBOHs, which showed a variable hydrophobicity due to the different side chain structures and modifications of their basic structure. The main objective of this study was to evaluate the inhibition potential of PBO and PBOHs on some plant proteases using pure effectors and pure enzymes in reverse micelles, which can simulate plant cells in vivo (*20*).

The results of this study indicate that benzodioxole compounds show interesting protease inhibition activities. In particular, EN 1-40 shows the maximum inhibition activity toward all the proteases considered, whereas the non-benzodioxole compounds, namely, EN 18-5 and EN 14-5, were very weak inhibitors. The benzo-mono-oxygen compounds, EN 16-5 and EN 16-6, deserve special consideration due to the negative effect exerted on cysteine proteases that can be associated with their relatively good water solubility and their special chemical structure. It is interesting to note that the latter, despite their modified structure, succeed in cysteine protease inhibition with comparable IC_{50} values to the best benzodioxole compounds.

This finding indicates not only that the benzodioxole and benzo-mono-oxygen structure is the necessary precondition to induce a negative effect on protease activity but also that the oxidation level of the indane prosthetic group is not a crucial feature.

In conclusion, this study suggests the following: (i) the benzodioxole and benzo-mono-oxygen structures appear to be responsible for in vitro plant protease inhibition; (ii) water solubility, which is modulated by chemical structure, plays a fundamental role in the expression of the inhibition activity of PBO and PBOH toward proteases; finally, on the basis of this last consideration, (iii) PBO is in vitro an important cysteine protease inhibitor.

ABBREVIATIONS USED

PBO, piperonyl butoxide; PBOHs, PBO homologues; P_{ow}, octanol−water partition coefficient; AOT, bis(2-ethylhexyl)-sodium sulfosuccinate; CBZ, *N*-benzoyloxycarbonyl-L-lysine *p*-nitrophenyl ester.

ACKNOWLEDGMENT

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