Phenolic Compounds from *Miconia myriantha* Inhibiting *Candida* Aspartic Proteases

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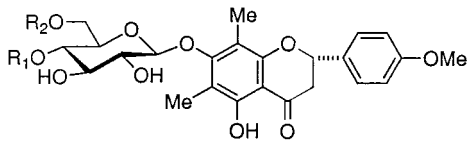
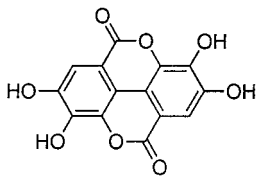
Assay-guided fractionation of the ethanol extract of the twigs and leaves of *Miconia myriantha* yielded two new compounds, mattucinol-7-*O*-[4″,6″-*O*-(*S*)-hexahydroxydiphenoyl]-*β*-D-glucopyranoside (**1**) and mattucinol-7-*O*-[4″,6″-di-*O*-galloyl]-*β*-D-glucopyranoside (**2**), along with mattucinol-7-*O*-*β*-D-glucopyranoside (**3**), ellagic acid (**4**), 3,3′-di-*O*-methyl ellagic acid-4-*O*-*β*-D-xylopyranoside, and gallic acid. Complete ^{1}H and ^{13}C NMR assignments of compound **1**, which possesses a hexahydroxydiphenoyl unit, were achieved using the HMBC technique optimized for small couplings to enhance the four-bond and two-bond H/C correlations. Compounds **1** and **4** showed inhibitory effects against *Candida albicans* secreted aspartic proteases, with IC_{50} of 8.4 and 10.5 μM, respectively.

The secreted aspartic proteases (SAP) of *Candida albicans* have been shown to be a major virulence factor in *Candida* infections.[1] Inhibition of SAP has been proposed as a new approach in the treatment of candidosis.[2] To date, there are no reports of investigating higher plants for inhibitors of secreted aspartic proteases.

In a search for *Candida* SAP inhibitors from higher plants, we have screened more than 300 plant specimens for inhibition of protease activity. *Miconia myriantha* Bentham (Melastomataceae), a small tree growing in Peru,[3] was selected for bioassay-guided fractionation on the basis of its potent in vitro activity against SAP. In this report, we present the isolation, structure elucidation, and SAP inhibitory effects of several phenolic compounds from this plant.



1 $R_1 + R_2$ = Hexahydroxydiphenoyl
2 $R_1 = R_2$ = Galloyl
3 $R_1 = R_2$ = H



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Results and Discussion

Guided by a primary screening assay adapted from a previously reported assay,[4] the ethanolic extract of the twigs and leaves of *M. myriantha* was fractionated by column chromatography on silica gel. The most active column fractions were further chromatographed on silica gel, reversed-phase silica gel, and Diaion HP-20 to yield two new compounds (**1** and **2**), along with four known compounds that were identified by their spectral data (NMR, MS, CD) as mattucinol-7-*O*-*β*-D-glucopyranoside (**3**),[5,6] ellagic acid (**4**),[7,8] 3,3′-di-*O*-methyl ellagic acid-4-*O*-*β*-D-xylopyranoside,[8,9] and gallic acid.[10]

Compound **1** was obtained as a yellow powder, $[\alpha]_D$ −38° (MeOH). Evidence of the polyhydroxy phenolic nature of **1** was derived from its strong dark blue color reaction with ferric chloride reagent on TLC visualization. The UV spectrum of **1** shows absorption maxima at 215, 276, and 362 nm. The ^{1}H NMR spectrum of **1** displays characteristic signals attributable to the skeleton of mattucinol-7-*O*-*β*-D-glucopyranoside (**3**)[5,6] with two additional singlets at δ 6.63 and 6.69 (Table 1). To facilitate comparison, the ^{1}H and ^{13}C NMR signals of **3** were completely assigned using contemporary 2D NMR techniques (COSY, HMQC, HMBC) and then are listed in Tables 1 and 2. The ^{13}C NMR spectrum of **1** (Table 2) supported the presence of **3** as a structural moiety in the molecule.[11] The additional two ester carbonyl signals (δ 168.4 and 168.5) and 12 aromatic carbon signals (see Table 2) suggested the presence of a hexahydroxydiphenoyl (HHDP) unit in **1**.[12] The high-resolution ESIMS established the molecular formula of **1** as $C_{38}H_{34}O_{18}$, consistent with **1** being comprised of **3** and one HHDP unit. Upon alkaline hydrolysis, **1** afforded **3** and **4**, which confirmed the structure of **1** as a mattucinol-7-*O*-*β*-D-glucopyranoside attached to an HHDP unit as shown. The linkage positions of the HHDP to **3** were determined by a series of 2D NMR experiments as follows. HMQC spectroscopy was first employed to correlate the protons with attached carbons. With the aid of COSY, the ^{1}H and ^{13}C NMR signals of the glucopyranosyl moiety were assigned (Tables 1 and 2). The remarkable downfield shifts of the signals for H-4″ (δ 4.92) and H-6″b (δ 5.14) relative to that of unsubstituted glucose indicated that the HHDP unit was linked to C-4″ and C-6″ of the glucose moiety. These linkages were further confirmed by HMBC (J_{CH}, 10 Hz), which revealed a correlation of H-4″ of the glucose with the ester carbonyl carbon at δ 168.4 (C-7‴), which also correlates with the aromatic proton H-5‴ (δ 6.69) of

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Table 1. ^{1}H NMR Data of Compounds **1**−**3** (δ, ppm; J value, Hz)

	1[a]	**2**[b]	**3**[b]
H-2	5.55 (dd, 12.3, 3.0)	5.50 (dd, 12.6, 3.0)	5.56 (dd, 12.2, 2.3)
3α	3.23 (dd, 17.2, 12.8)	3.30 m	3.34 m
3β	2.89 (dd, 17.2, 3.0)	2.80 (dd, 17.6, 3.0)	2.83 (17.1, 2.5)
2′,6′	7.51 (d, 8.6)	7.38 (d, 8.6)	7.45 (d, 8.6)
3′,5′	7.00 (d, 8.6)	6.96 (d, 8.6)	6.98 (d, 8.6)
Me-6	2.17 (s)	2.06 (s)	2.08 (s)
Me-8	2.16 (s)	2.00 (s)	2.06 (s)
MeO-4′	3.83 (s)	3.77 (s)	3.79 (s)
HO-5	12.2 (br s)	12.1 (br s)	12.1 (br s)
1″	4.89 (d, 7.0)	4.80 (d, 7.7)	4.59 (d, 7.3)
2″	3.76 m	3.58 (dd, 7.7, 9.2)	3.29 m
3″	3.80 m	3.65 (t, 9.2)	3.22 (dd, 8.8, 8.1)
4″	4.92 (t, 9.6)	5.00 (t, 9.2)	3.12 (dd, 8.1, 9.4)
5″	3.91 (dd, 9.6, 6.4)	3.80 m	3.11 m
6″a	3.67 (br d, 13.0)	4.13 (br d, 10.5)	3.62 (br d, 11.2)
6″b	5.14 (dd, 13.0, 6.4)	4.05 (dd, 12.2, 4.8)	3.43 m
5‴	6.63 (s)		
5⁗	6.69 (s)		
2‴, 6‴		6.89 (s)	
2⁗, 6⁗		6.95 (s)	

[a] Measured in acetone-d_6. [b] Measured in DMSO-d_6.

Table 2. ^{13}C NMR Data of Compounds **1**−**3** (δ, ppm)

	1[a]	**2**[b]	**3**[b]		**1**[a]	**2**[b]	**3**[b]
C-2	79.5	78.0	77.8	C-2″	76.2	74.3	74.1
3	43.7	42.4	42.2	3″	75.8	73.8	76.4
4	199.4	198.5	198.4	4″	72.8	70.7	69.9
5	159.7	157.9	157.7	5″	72.5	71.2	77.0
6	112.7	111.1	111.5	6″	64.0	62.1	61.0
7	162.3	160.8	161.4	1‴	116.0	119.2	
8	111.5	110.2	110.1	2‴	144.2	108.9	
9	158.8	157.4	157.2	3‴	136.5	145.5	
10	106.2	104.9	104.8	4‴	145.3	138.6	
1′	132.1	130.8	130.8	5‴	108.4	145.5	
2′	128.8	128.0	127.9	6‴	126.9	108.9	
3′	114.9	114.0	113.9	7‴	168.5	165.7	
4′	161.0	159.4	59.3	1⁗	116.2	119.1	
5′	114.9	114.0	113.9	2⁗	144.5	108.8	
6′	128.8	128.0	127.9	3⁗	136.7	145.4	
Me-6	9.2	8.6	8.6	4⁗	145.4	138.6	
Me-8	9.9	9.2	9.2	5⁗	108.3	145.4	
MeO-4′	55.8	55.2	55.2	6⁗	126.7	108.8	
1″	105.4	103.8	104.2	7⁗	168.4	165.1	

[a] Measured in acetone-d_6. [b] Measured in DMSO-d_6.

the HHDP. H-6″a and H-6″b displayed cross-peaks with the furthermost downfield ester carbonyl carbon at δ 168.5 (C-7‴), which also correlated to the aromatic proton H-5‴ (δ 6.63) of the HHDP. This not only established the direct linkages of the HHDP with the glucose but also facilitated differentiation of the two galloyl moieties (Gal-1‴ and Gal-1⁗) of the HHDP and assignment of the complicated quaternary carbon signals of the HHDP despite very close chemical shifts. With the HMBC experiment set at a delay time of 50 ms, corresponding to a J coupling of 10 Hz, the $^3J_{CH}$ correlations (such as H-5‴/C-1‴,-3‴,-7‴) and *ortho*-oxygenated $^2J_{CH}$ correlations (such as H-5‴/C-4‴, H-5⁗/C-4⁗) were readily obtained (Figure 1). To enhance the four- and two-bond H/C correlations, the HMBC experiment was set at the delay time of 400 ms, corresponding to a J coupling of 1.25 Hz.[8,13] Additional $^2J_{CH}$ (such as H-5‴/C-6‴) and $^4J_{CH}$ correlations (such as H-5‴/C-2‴) were observed (Figure 1, depicted with dashed line). The above two HMBC experiments allowed the complete assignment of carbon signals of the HHDP moiety. To the best of our knowledge, this is the first report of the complete ^{13}C NMR assignments of an HHDP moiety in a molecule. The NOESY experiment was also employed to provide further evidence for the structure of **1**. A key NOE correlation between the anomeric proton (H-1″) and Me-6 of the aglycone was observed. Such a selective NOE correlation

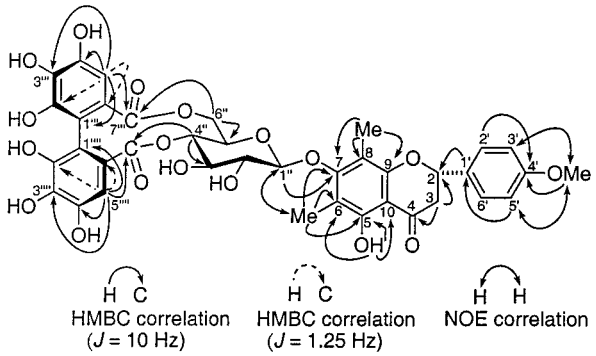

Figure 1. HMBC and NOE correlations of **1**.

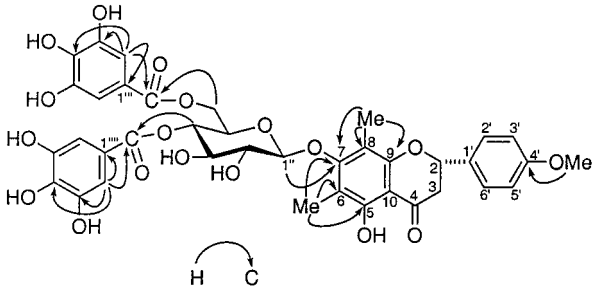

Figure 2. Key HMBC correlations of **2** (J = 6.25 Hz).

presumably reflects a buttressing effect of Me-8, leading to a preferred conformation about the C-7 → C-1″ bond which does not permit correlation between Me-8 and the anomeric proton. Other HMBC and NOE correlations are summarized in Figure 1.

In the CD spectrum of **1**, the strong positive Cotton effect at 240 nm, enhanced by the positive Cotton effect at ca. 250 nm of the aglycone (see Experimental section), indicates P-helicity and hence *S*-configuration[14] of the biphenyl chromophore of the HHDP structural moiety. In addition, in the longer wavelength region, the spectrum closely resembles that of **3**, indicating the *S* absolute configuration at C-2 of the aglycone.[15] On the basis of the above evidence, the structure of compound **1** was established as mattucinol-7-*O*-[4″,6″-*O*-(*S*)-hexahydroxydiphenoyl]-β-D-glucopyranoside.

Compound **2** was obtained as a pale yellow powder, $[\alpha]_D$ −25° (MeOH). It showed a similar color reaction with ferric chloride and UV absorptions as those of **1**. Its molecular formula was determined as $C_{38}H_{36}O_{18}$ by ESIMS and its ^{13}C NMR spectrum. The ^{1}H NMR spectrum of **2** closely resembles that of **3**, with the exception of two additional singlets at δ 6.89 and 6.95, each integrating for two protons. The ^{13}C NMR spectrum displayed two sets of signals characteristic of two galloyl units (Table 2). These data suggested that the structure of **2** is a mattucinol-7-*O*-β-D-glucopyranoside attached to two galloyl units. In a manner similar to that for **1**, 2D NMR techniques (COSY, HMQC, HMBC) were employed to assign the proton and carbon signals and hence to confirm the structure. The significant downfield shifts of H-4″ and H-6″ of the glucopyranosyl unit when compared with those of compound **3** indicated the linkage positions of the two galloyl units are at C-4 and C-6 of the glucose, which was further supported by the long-range H/C correlations observed in the HMBC experiment performed with the delay time of 80 ms (corresponing to a J coupling of 6.25 Hz) (Figure 2). In addition, this HMBC experiment permitted differentiation of the two sets of nearly overlapping galloyl carbon signals via correlations of the two ester carbonyl carbons (Figure 2). Thus, all the ^{1}H and ^{13}C NMR signals of **2** were assigned

(Tables 1 and 2). The CD spectrum of **2** was similar to that of **3**, confirming the *S* absolute configuration of C-2 of the flavanone alycone.[15] Therefore, the structure of **2** is mattucinol-7-*O*-[4″, 6″-di-*O*-galloyl]-*β*-D-glucopyranoside.

Purified compounds, **1**, **3**, **4**, 3,3′-di-*O*-methyl ellagic acid-4-*O*-*β*-D-xylopyranoside, and gallic acid, were evaluated for inhibitory effects against *Candida albicans* secreted aspartic proteases. Only compounds **1** and **4** are active, with IC_{50} of 8.4 and 10.5 μM, respectively. Pepstatin A was used as a positive control and shows an IC_{50} of 0.016 μM. To determine if the inhibitory effects of **1** and **4** were selective for *Candida* SAP, their activities against a general aspartic protease, pepsin, were determined. The IC_{50} of **1** and **4** against pepsin were 66.6 and 37.1 μM, respectively, while pepstatin A had an IC_{50} of 0.31 μM. These data indicate that the two compounds selectively inhibit *Candida* SAP. Although the potency of these two compounds is weak relative to pepstatin A, this is the first reported *Candida* SAP inhibitory activity of compounds from plants.

Experimental Section

General Experimental Procedures. Optical rotations were determined on a JASCO DIP-370 digital polarimeter. CD spectra were recorded on a JASCO J-715 spectrometer. UV spectra were measured on a Hewlett-Packard 8453 spectrometer. IR spectra were run on an ATI Mattson Genesis Series FTIR spectrometer. NMR spectra were recorded on either a Bruker Avance DRX-400 spectrometer operating at 400 MHz for ^{1}H and 100 MHz for ^{13}C NMR or a Bruker Avance DPX-300 spectrometer operating at 300 MHz for ^{1}H and 75 MHz for ^{13}C NMR. Chemical shifts (δ, ppm) are relative to internal TMS. COSY, HMQC, HMBC (J = 10 Hz, 6.25 Hz, 1.25 Hz), and NOESY (mixing time 800 ms) were measured with standard pulse programs. ESI-FTMS were measured on a Bruker-Magnex BioAPEX 30es ion cyclotron high-resolution HPLC-FT spectrometer by direct injection into an electrospray interface. Column chromatography was performed on silica gel (40 μm, J. T. Baker), reversed-phase silica gel (RP-18, 40 μm, J. T. Baker), and Diaion HP-20 (Supelco). TLC was performed on silica gel sheets (Alugram Sil G/UV_{254}, Macherey-Nagel, Germany) and reversed-phase plates (RP-18 F_{254S}, Merck, Germany). Sabouraud dextrose broth (prepared as labeled and filter sterilized) and dextrose were purchased from Difco Labs (Detroit, MI). The fluorescent renin substrate was purchased from Molecular Probes (#R-2931, Eugene, OR). All other chemicals (yeast extract, bovine serum albumin, pepsin, and pepstatin A) were obtained from Sigma (St. Louis, MO). Fluorescence was measured on a Cytofluor 2350 fluorescence measurement system using CytoCalc software (version 2.00.06, PerSeptive Biosystems, Inc., Framingham, MA).

Plant Material. The twigs and leaves of *Miconia myriantha* were collected in Peru in July 1990. A voucher specimen of this plant is deposited at the National Center for Natural Products Research (Voucher # IBE 9732).

Extraction and Isolation. The dried powdered twigs and leaves (170 g) were extracted with 95% EtOH (1.2 L × 3) at 37 °C for 5 h. Removal of the solvent in vacuo at 45 °C yielded an EtOH extract (10.5 g), of which 5.0 g was chromatographed on normal silica gel using $CHCl_3$−MeOH as the gradient eluting solvent system (0% → 100% MeOH in $CHCl_3$, 7.5 L), followed by MeOH (1.5 L) to give pooled fractions A−M.

The most active fractions, K and L (1.1 g, 53% SAP inhibition at 50 μg/mL), were crystallized from MeOH to yield **3** (22.6 mg). The mother liquid was concentrated and subjected to column chromatography on normal silica gel with $CHCl_3$−MeOH−H_2O (70:10:1), followed by reversed-phase silica gel (C-18) with MeOH−H_2O (0% → 100%) to give **1** (10.5 mg), **2** (4.2 mg), 3,3′-di-*O*-methyl ellagic acid-4-*O*-*β*-D-xylopyranoside (6.9 mg), and gallic acid (10.1 mg). Column fraction M (2.16 g, 30% SAP inhibition at 50 μg/mL) was chromatographed on Diaion HP-20 eluting with MeOH−H_2O (60% → 100%) to afford **4** (21.7 mg) and **1** (50.0 mg).

Mattucinol-7-*O*-[4″,6″-*O*-(*S*)-hexahydroxydiphenoyl]-*β*-D-glucopyranoside (1): yellow powder, $[\alpha]_D^{25}$ −38° (*c* 0.70, MeOH); UV (MeOH) λ_{max} (log ϵ) 215 (4.69), 276 (4.38), 362 (3.54) nm; IR (KBr) ν_{max} 3410 (br), 1731, 1623, 1516. 1448, 1347, 1234, 1046 cm^{-1}; ^{1}H NMR (DMSO-d_6, 400 MHz) δ 12.1 (1H, br s, HO-5), 7.47 (2H, d, J = 8.4 Hz, H-2′,6′), 6.99 (2H, d, J = 8.4 Hz, H-3′,5′), 6.53 and 6.40 (1H each, s, H-5‴/H-5⁗), 5.59 (1H, dd, J = 12.6, 2.6 Hz, H-2), 4.93 (1H, dd, J = 13.2, 6.2 Hz, H-6″b), 4.75 (1H, d, J = 7.3 Hz, H-1″), 4.62 (1H, t, J = 9.6 Hz, H-4″), 3.48 (1H, m, H-2″), 3.53 (1H, m, H-3″), 3.85 (1H, m, H-5″), 3.56 (1H, m, H-6″a), 3.79 (3H, s, MeO-4′), 3.30 (1H, overlapping with H_2O signal, H-3α), 2.88 (1H, br d, 16.7 Hz, H-3*β*), 2.12 and 2.10 (3H each, s, Me-6, Me-8) (sugar signals' assignments were based on a COSY spectrum in DMSO-d_6); ^{1}H and ^{13}C NMR data (acetone-d_6, 400 MHz), see Tables 1 and 2; ESIMS m/z 801.1678 {calcd for [M($C_{38}H_{34}O_{18}$) + Na]$^+$, 801.1643}; FABMS (neg.) m/z 777 [M − H]$^-$, 475 [777 − HHDP]$^-$; CD (c 2.57 × 10^{-4} M, MeOH) λ ([θ]) 240 (+111 543), 268 (−46 465), 282 (−39 001), 290 (−38 221), 352 (+8346) nm.

Mattucinol-7-*O*-[4″,6″-di-*O*-galloyl]-*β*-D-glucopyranoside (2): pale yellow powder, $[\alpha]_D^{25}$ −25° (*c* 0.28, MeOH); UV (MeOH) λ_{max} (log ϵ) 216 (4.67), 280 (4.36), 362 (3.63) nm; IR (KBr) ν_{max} 3420 (br), 1705, 1623, 1516. 1451, 1348, 1244, 1213 cm^{-1}; ^{1}H and ^{13}C NMR data (DMSO-d_6, 400 MHz), see Tables 1 and 2; ESIMS m/z 819 [M($C_{38}H_{36}O_{18}$) + K]$^+$; FABMS (neg.) m/z 779 [M − H]$^-$, 475 [779 − Gal × 2]$^-$; CD (c 6.4 × 10^{-4} M, MeOH) λ ([θ]) 245 (+ 8 399), 277 (−18 967), 284 (−17 401), 352 (+12 679) nm.

Mattucinol-7-*O*-*β*-D-glucopyranoside (3): granular crystal, ^{1}H NMR (pyridine-d_5, 300 MHz) δ 12.6 (1H, s), 7.54 (2H, d, J = 8.6 Hz), 7.05 (2H, d, J = 8.6 Hz), 5.50 (1H, d, J = 6.9 Hz), 5.42 (1H, dd, J = 13, 3 Hz), 4.6−3.8 (m), 3.70 (3H, s), 3.20 (1H, dd, J = 17, 13 Hz), 2.93 (1H, dd, J = 17, 3 Hz), 2.65 and 2.56 (3H each, s), identical to the literature data;[6] ^{1}H and ^{13}C NMR data (DMSO-d_6, 400 MHz), see Tables 1 and 2; FABMS (neg.) m/z 475 [M − H]$^-$; CD (c 1.1 × 10^{-3} M, MeOH) λ ([θ]) 250 (+9722), 287 (−39 348), 350 (+12 243) nm.

Alkaline Hydrolysis of 1. A solution of **1** (1.5 mg) in 10% Et_3N−MeOH (0.2 mL) was kept at room temperature overnight. The reaction mixture was concentrated under a stream of N_2 and then subjected to TLC analysis using benzene−EtOAc−HCOOH (3:6:1) as a developing system. Spots were visualized under UV lamp. Compounds **3** and **4** were detected with R_f values of 0.62 and 0.71, respectively.

Induction of SAP Production. The procedure of Capobianco et al.[4] was used to prepare the SAP-rich extract. Briefly, *Candida albicans* ATCC 10231 was grown overnight at 30 °C in Sabouraud dextrose broth. The cells were washed once with 10 mM phosphate-buffered saline (pH 7.0), resuspended in 15 mL inducing medium (0.2% yeast extract, 0.2% bovine serum albumin, 2.0% dextrose), and incubated for 8 h at 30 °C. The SAP-rich supernatant was collected, sterilized by filtration, aliquoted, and stored at −80 °C until needed.

SAP Inhibition Assay. Using 50 mM sodium citrate buffer (pH 4.5) as the diluent, the optimum substrate and SAP extract concentrations were determined to be 63 μM and a 1:71 dilution of the SAP-rich supernatant, respectively. The final volume of the reaction mixture (sample, diluted SAP extract, substrate) was 100 μL. Blanks that contained ≤4% v/v DMSO were also included. Immediately after adding 63 μM substrate the fluorescence at 360ex/530em was measured. The plate was incubated for 1 h at 37 °C, and then fluorescence was measured again. The increase in fluorescence over the incubation time was determined and expressed as % fluorescence compared to a positive control, which contained no sample but the same DMSO concentration.

Crude extracts were initially tested at one concentration of 200 μg/mL to identify active extracts (≥80% inhibition). Active extracts were confirmed by retesting at three concentrations (50, 10, and 2 μg/mL). Solvent partitions and chromatographic fractions were tested at three concentrations to guide isolation. Isolated compounds were tested at eight concentrations, and % fluorescence was plotted versus concentration. Prism soft-

ware from GraphPad (San Diego, CA) was used to fit the resulting data to sigmoidal curves and determine the concentration that inhibited 50% of activity (IC_{50}). The aspartic protease inhibitor pepstatin A was used as a positive control in these assays.

Pepsin Inhibition Assay. Optimum final pepsin and substrate concentrations were determined to be 10 units/mL and 30 μM, respectively. The assay was performed as above except that pepsin was substituted for the SAP extract and a 50 mM sodium citrate buffer (pH 4.0) was used as the diluent.

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