A New Dimeric Dihydrochalcone and a New Prenylated Flavone from the Bud Covers of *Artocarpus altilis*: Potent Inhibitors of Cathepsin K

Ashok D. Patil,[†] Alan J. Freyer,*[,‡] Lew Killmer,[‡] Priscilla Offen,[‡] Paul B. Taylor,[‡] Bartholomew J. Votta,[‡] and Randall K. Johnson[‡]

Drug Discovery, Millennium Pharmaceuticals Inc., 75-5 Sidney Street, Cambridge, Massachusetts 02139, and Departments of Biomolecular Discovery, Analytical Sciences, and Cellular Biochemistry, GlaxoSmithKline Pharmaceuticals, 709 Swedeland, PO Box 1539, King of Prussia, Pennsylvania 19406-0939

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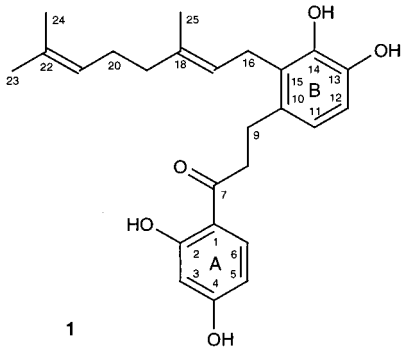
A MeOH/CH_2Cl_2 extract of the bud covers of *Artocarpus altilis* collected in Micronesia showed activity in a cathepsin K inhibition assay. In addition to the three known flavonoids isolated from the bud covers of this species, two new compounds have been identified whose structures were determined on the basis of spectral data. These compounds include a dimeric dihydrochalcone, cycloaltilisin 6 (**2**), and a new prenylated flavone, cycloaltilisin 7 (**3**). Novel compounds **2** and **3** have IC_{50} values of 98 and 840 nM, respectively, in cathepsin inhibition.

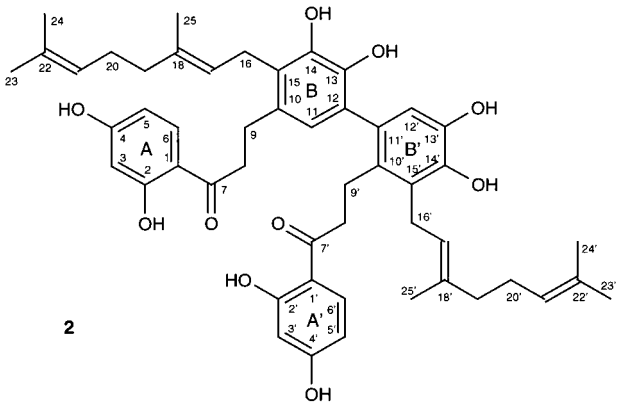
Cathepsin K is a novel cysteine protease that has been implicated in osteoporosis.[1,2] It has been established that, unlike serine protease inhibitors, cysteine protease inhibitors are very effective in preventing bone resorption.[3] The enzyme itself was identified from a cDNA library that was prepared from enriched osteoclasts present in osteoclastoma tissue.

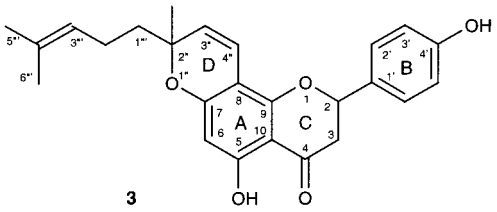
The buds of *Artocarpus altilis* (Moraceae) have been used traditionally in Taiwan for the treatment of liver cirrhosis and hypertension and has been reported to possess anti-inflammatory and detoxifying effects.[4] Flavonoids and triterpenoids have previously been isolated from various parts of this plant.[5−8] As part of our search for the biologically active compounds with utility against osteoporosis, we initiated a screen to evaluate natural product extracts for the inhibition of cathepsin K. An extract made from the bud covers of the plant *A. altilis* collected in Pohnpei showed cathepsin K inhibitory activity and was therefore selected for fractionation.

Extraction of the bud covers of *A. altilis* with MeOH/CH_2Cl_2 afforded a residue that was passed through a polyamide column to remove polyphenols and tannins. Bioassay-guided fractionation of this residue using column chromatography (Si gel) followed by PTLC (RP-18) and HPLC (Si gel) of the cathepsin K active fraction yielded novel compounds **2** and **3** plus the previously isolated[9] AC-3-1, AC-3-3, and AC-5-1 (**1**) from the dried flowers of the Indonesian plant *A. communis*.

Compound **1** was isolated as an amorphous powder, and the mass spectral data for this compound indicated that the molecular mass was 410 Da. The DCI mass spectrum using ND_3 produced a 5-Da increase in the molecular ion, signifying the presence of four exchangeable hydrogen atoms. HR-MS indicated a molecular formula of $C_{25}H_{30}O_5$. The ^{1}H and COSY NMR spectra in $CDCl_3$, taking into account the observed ^{13}C signals, revealed the presence of a sharp, hydrogen-bonded phenolic proton at δ 12.81 as well as an aromatic ABX and an AB spin system. Two olefinic triplets, five methylene pairs, and three methyl multiplets completed the ^{1}H NMR spectrum. These data strongly suggested the presence of a dihydrochalcone moiety with a geranyl side chain. The ^{13}C GASPE NMR spectrum of **1** displayed 10 quaternary carbons including a ketone carbonyl at δ 204.0, seven methines, five methylenes, and three methyl resonances. A database search







* To whom correspondence should be addressed. Tel: 610-270-6315. Fax: 610-270-6608. E-mail: Alan_J_Freyer@gsk.com.
[†] Millennium Pharmaceuticals Inc..
[‡] GlaxoSmithKline Pharmaceuticals.

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Table 1. ^{1}H and ^{13}C NMR Assignments for **2** and **3** in Acetone-d_6

	2		**2′**			**3**	
no.	δC	δH	δC	δH	no.	δC	δH
1	113.7		113.2		2	79.9	5.49 (1H,dd,3.0,13.0)
2	166.1		166.5		3	43.0	3.18 (1H,dd,13.0,17.0)
							2.76 (1H,dd,3.0,17.0)
3	103.5	6.26 (1H,d,2.4)	103.5	6.24 (1H,d,2.4)	4	197.4	
4	165.4		165.4		5	164.8	
5	108.7	6.31 (1H,dd,2.4,8.8)	108.8	6.24 (1H,dd,2.4,9.4)	6	97.3	5.87 (1H,s)
6	133.4	7.58 (1H,d,8.8)	133.8	6.88 (1H,d,9.4)	7	162.9	
7	204.9		205.7		8	102.2	
8	40.3	3.17 (2H,m)	40.7	2.83 (2H,m)	9	158.1	
9	27.8	2.97 (2H,m)	27.6	2.84 (1H,m)	10	103.6	
				2.76 (1H,m)			
10	132.1		131.1		1′	130.4	
11	122.8	6.58 (1H,s)	129.4		2′	128.9	7.40 (2H,d,8.6)
12	127.5		116.1	6.56 (1H,s)	3′	116.2	6.90 (2H,d,8.6)
13	140.7		143.2		4′	158.8	
14	144.6		144.0		2″	81.3	
15	126.1		127.7		3″	126.0	5.53 (1H,d,10.2)
16	26.0	3.62 (1H,dd,6.6,15.3)	26.2	3.49 (2H,d,6.6)	4″	116.7	6.54 (1H,d,10.2)
		3.49 (1H,dd,6.6,15.3)					
17	124.5	5.28 (1H,tm,6.6)	124.4	5.22 (1H,tm,6.6)	1‴	42.2	1.72 (1H,m)
							1.64 (1H,m)
18	135.1		135.1		2‴	23.3	2.07 (2H,m)
19	40.4	1.99 (2H,m)	40.5	1.99 (2H,m)	3‴	124.7	5.09 (1H,tm,7.2)
20	27.3	2.06 (2H,m)	27.3	2.06 (2H,m)	4‴	132.1	
21	125.1	5.05 (1H,m)	125.1	5.05 (1H,m)	5‴	25.7	1.63 (3H,dm,1.3)
22	131.6		131.6		6‴	17.6	1.55 (3H,dm,0.8)
23	25.8	1.59 (3H,dm,1.1)	25.8	1.57 (3H,dm,1.1)	2″Me	27.3	1.37 (3H,s)
24	17.7	1.53 (3H,dm,0.8)	17.7	1.52 (3H,dm,0.8)	OH		12.18 (1H,s,C_5-OH)
25	16.4	1.78 (3H,dm,1.1)	16.4	1.77 (3H,dm,1.1)			
OH		12.75 (1H,s,C_2-OH)		12.81 (1H,s,C_2′-OH)			

based on the molecular mass and the molecular formula suggested the identity of **1** to be AC-5-1, and this proposal was confirmed by heteronuclear correlations.[8−10]

Cycloaltilisin 6 (**2**) was obtained as a white solid, and its DCI-MS (NH_3) data indicated that the molecular mass was 818 Da. The DCI mass spectrum using ND_3 produced a 9-Da increase in the molecular ion, signifying the presence of eight exchangeable hydrogen atoms. HR-MS indicated a molecular formula of $C_{50}H_{58}O_{10}$, two hydrogens less than double the molecular formula of **1**. Genesis of an intense fragment ion at *m/z* 409 indicated that **2** readily fragmented into two identical halves, and this observation suggested that cycloaltilisin 6 (**2**) was a dimer of **1**. The ^{1}H and COSY NMR spectra of **2** (see Table 1) in acetone-d_6 exhibited two hydrogen-bonded phenolic protons at δ 12.81 and 12.75 as well as two aromatic ABX spin systems and two aromatic singlets. This evidence suggested that **2** consisted of two monomers of compound **1** connected through their B rings, thus accounting for the presence of the ring B aromatic singlets rather than doublets. The ^{1}H and COSY NMR spectra, taking into account the observed ^{13}C signals, also revealed the presence of four olefinic triplets, 10 methylene pairs, and six methyl multiplets. If the two monomers were symmetrically bridged (via C-11 to C-11′ or C-12 to C-12′), only half the number of NMR signals would be observed. The number of unique NMR resonances indicated that the bridge in cycloaltilisin 6 (**2**) must be asymmetric, i.e., C-12 to C-11′. As expected, the ^{13}C GASPE NMR spectrum of **2** contained 50 unique carbon resonances, double the number of carbon resonances in **1**. These carbon resonances included two ketone resonances at δ 205.7 and 204.9 and 12 downfield methine signals rather than 14 (double the seven found for **1**), confirming the ring B bridge, which was further substantiated by HMQC and HMBC correlation data.

The chemical shifts, coupling constants, integrals, multiplicities, and correlations between the ^{1}H and ^{13}C signals in cycloaltilisin 6 (**2**) were totally analogous to their counterparts in dihydrochalcone **1**. The resonances of the two individual dihydrochalcone moieties making up the dimeric structure were, however, readily distinguishable because there existed a center of bifurcation between the OH-2′ proton and the oxygen atoms of both ketone carbonyls. Because the phenolic OH-2′ proton was more tightly hydrogen-bound than that of OH-2, the OH-2′ NMR signal appeared noticeably sharper and slightly further downfield. The geranyl side chain resonances were more distant from the center of bifurcation, and as result, it was more difficult to differentiate between their chemical shifts.

There were some significant differences observed between the aromatic ring B and B′ resonances in dimer **2**. The H-11 aromatic singlet (δ 6.58) shared HMBC correlations with C-13 (δ 140.7) and C-15 (δ 126.1), with the benzylic C-9 (δ 27.8) in the propiophenone moiety, and with the C-11′ (δ 129.4) quaternary aromatic signal in the adjoining ring B′. The correlation with C-9 established the position of the δ 6.58 aromatic singlet in ring B at C-11, two carbons removed from the propiophenone moiety. The correlations with C-15 (to which the geranyl side chain was attached) and the phenolic C-13 were also consistent with the proposed dimeric structure. Conversely, the H-12′ aromatic singlet at δ 6.56 shared HMBC correlations with C-10′ (δ 131.1) and phenolic C-14′ (δ 144.0) in ring B′ as well as with the quaternary C-12 in ring B. No correlation was observed between H-12′ and C-9′, indicating that H-12′ was not adjacent to the second propiophenone moiety. These observations collectively established the structure of **2** as a dimer consisting of two monomers of **1** connected through an asymmetric bridge between C-12 and C-11′.

Cycloaltilisin 7 (**3**) was obtained as a pale yellow solid whose DCI-MS (NH_3) data indicated a molecular mass of 406 Da with two exchangeable hydrogens. HR-MS indicated a molecular formula of $C_{25}H_{26}O_5$, four hydrogens less than were present in dihydrochalcone **1**. The IR spectrum

of **3** showed bands at 3565 (OH) and 1709 (C=O) cm^{-1}, and the UV absorptions at 272, 296, and 360 nm were suggestive of a flavone skeleton. The DCI-MS fragment ions at *m/z* 287 and 120, arising from a retro Diels−Alder cleavage of **3** followed by a hydrogen transfer, indicated only one hydroxyl group was present on ring B. The proposal of a *p*-substituted phenolic B ring was supported by the ^{1}H NMR spectrum (see Table 1) of **3** in acetone-d_6, which contained an aromatic A_2B_2 spin system at δ 7.40 and 6.90. Upon inspection of the ^{1}H and ^{13}C GASPE NMR spectra data for **3**, the presence of three methyl multiplets (δ_C 27.3, 17.6, and 25.7), three methylene signals (δ_C 43.0, 42.2, and 23.3), one oxygenated quaternary aliphatic carbon (δ_C 81.3), and two double bonds (δ_C 116.7, 126.0 and 124.7, 132.1) were revealed. The presence of two olefinic proton doublets at δ 5.53 and 6.54, sharing a *cis* coupling (J = 10.2 Hz), in the ^{1}H NMR spectrum of **3** suggested that the oxygen atom at the C-7 position had cyclized onto C-2″ of one of the prenyl groups, forming an additional pyran ring as shown in the **3**. This proposal was supported by the observation of a fragment ion at *m/z* 323 that evolved from loss of the C_6H_{11} prenyl side chain from the molecular ion. The ^{1}H and ^{13}C NMR assignments were confirmed by HMBC correlations. In addition to the correlations observed for the flavone nucleus, the following important correlations were noticed. H-4″ (δ 6.54) shared HMBC correlations with C-7 (δ 162.9) and C-9 (δ 158.1) of the flavone moiety, as well as with the oxygen-bearing quaternary C-2″ (δ 81.2). The CH_3-2″ (δ 1.37) protons correlated to C-2″, as did olefinic H-4″ and methylenes H-1‴ (δ 1.72, 1.64) and H-2‴ (δ 2.07). The COSY and NOE difference data showed that the H-2‴ methylene protons were part of a prenyl side chain directly attached to the H-1‴ methylene group. The stereochemistry at chiral centers C-2 and C-2″ was not determined.

All the compounds isolated from the *A. altilis* were shown to be potent inhibitors of cathepsin K. Cycloaltilisin 6 (**2**) was found to be the most potent inhibitor with an IC_{50} of 98 nM followed by AC-5-1 (**1**) with an IC_{50} of 170 nM and cycloaltilisin 7 (**3**) with an IC_{50} of 840 nM. AC-3-1 and AC-3-3 were relatively weak inhibitors of cathepsin K with IC_{50} values of 1 and 4 μM, respectively.

Experimental Section

General Experimental Procedures. IR spectra were recorded on a Nicolet Model 20 DXB FTIR spectrophotometer. All homo- and heteronuclear one- and two-dimensional NMR data were recorded on a Bruker AMX-400 spectrometer in either $CDCl_3$ or acetone-d_6. DCI-MS data were obtained using a Finnigan Model 4610 quadrupole mass spectrometer using CH_4, NH_3, and ND_3 reagent gases. The HR-DCI-MS data were acquired on a VG-70SE with CH_4 and with NH_3 reagent gases. Analytical and preparative TLC were carried out on precoated Si gel G (Kiesel gel G254) and reversed-phase (Whatman KC18F) plates. A Rainin HPXL solvent delivery system equipped with a refractive index detector, Model 156, was used for HPLC separations employing a Lichrosorb Si 60 or a Whatman Magnum-9 ODS-3 column. Optical rotations were recorded on a Perkin-Elmer 241 MC polarimeter. Reagent grade chemicals (Fisher and Baker) were used throughout.

Biological Assay. The experimental procedure was modeled after a previously described method for the proteolytic cleavage of small peptide fluorogenic substrates.[11] All screening was performed using an Orca robotic arm that facilitated the addition of components to a total volume of 100 μL per well. The final buffer composition in the assay was 100 mM sodium acetate, 5 mM EDTA, and 20 mM L-cysteine, pH 5.5. The L-cysteine was added to the buffer on the same day that the assay was performed. The substrate used was the cleavable fluorogenic peptide conjugate ZFR-AMC (phenylalanine-arginine-aminomethylcoumarin, Bachem). With exception of the inhibitor source plates, all assay components were kept at 4 °C until they were added to the test plates that were left at room temperature for 1 h. It was unnecessary to quench the cleavage reaction because the assay duration could be accurately constrained by robot scheduling. The final assay composition was 160 ng/mL (6 nM) cathepsin K, 20 μM ZFR-AMC, and 100 μg/mL (or lower if necessary) inhibitor. The final assay mix contained 2% DMSO, which was the summation of the substrate and inhibitors each contributing 1%. After substrate cleavage by cathepsin K, the AMC fluorescence signal was recorded by a Fluostar fluorimeter (Tecan) which had custom filters installed (Omega Optical) possessing excitation and emission wavelengths of 360 nm (15 nm bandwidth) and 440 nm (20 nm bandwidth), respectively.

Plant Material. The plant material was collected in Pohnpei by Dr. Austin Bowden-Kirby in June 1989, and voucher specimens have been deposited at the University of Guam.

Extraction and Isolation. Exhaustive extraction of the dried, ground bud covers (323 g) of *A. altilis* with MeOH/CH_2Cl_2 (1:1, 3 × 1 L) afforded a brown residue (47 g). A portion of this extract (2.5 g) was subjected to a polyamide column and eluted with MeOH. The eluent obtained from the polyamide column was evaporated under reduced pressure and temperature (37 °C) to yield a cathepsin K inhibitory-active, pale yellow residue (1.77 g), which was applied to a Si gel column. Elution with MeOH/CH_2Cl_2 (5:95) and then with increasing percentages of MeOH in CH_2Cl_2 afforded several fractions that were collected and monitored by TLC. Like fractions were combined to yield a total of 10 fractions (A−J). The Si gel PTLC of the Cathepsin K inhibitory fraction C (0.216 g) in acetone/hexane (30:70) followed by RP-18 HPLC (20:80 H_2O/CH_3CN; flow rate 3 mL/min; RI detection) yielded AC-3-1 (78 mg) and AC-3-3 (37 mg). The PTLC (RP-18) of the active fraction D (0.158 g) employing H_2O/CH_3CN (30:70) followed by Si gel HPLC using *i*-PrOH/CH_2Cl_2 (9:91) yielded cycloaltilisin 7 (**3**, 16 mg) and AC-5-1 (**1**, 85 mg). The RP-18 PTLC of the F fraction (0.084 g) using H_2O/CH_3CN (30:70) followed by Si gel PTLC (5:95 MeOH/CH_2Cl_2) afforded the most potent compound, cycloaltilisin 6 (**2**, 46 mg).

AC-5-1 (1): amorphous powder; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 12.81 (1H, s, C_2-OH), 7.60 (1H, d, J = 8.7 Hz, H-6), 6.73 (1H, d, J = 8.2 Hz, H-12), 6.68 (1H, d, J = 8.2 Hz, H-11), 6.38 (1H, d, J = 2.5 Hz, H-3), 6.35 (1H, dd, J = 2.5, 8.7 Hz, H-5), 5.19 (1H, tm, J = 6.7 Hz, H-17), 5.04 (1H, tm, J = 6.7 Hz, H-21), 3.42 (2H, d, J = 6.7 Hz, H-16), 3.12 (2H, m, H-8), 2.99 (2H, m, H-9), 2.08 (2H, m, H-20), 2.07 (2H, m, H-19), 1.80 (3H, dm, J = 1.1 Hz, Me-25), 1.67 (3H, dm, J = 1.1 Hz, Me-23), 1.59 (3H, dm, J = 0.8 Hz, Me-24); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 204.0 (s, C-7), 165.2 (s, C-2), 162.7 (s, C-4), 142.8 (s, C-13), 142.4 (s, C-14), 138.9 (s, C-18), 132.3 (d, C-6), 132.2 (s, C-22), 131.1 (s, C-10), 126.0 (s, C-15), 123.7 (d, C-21), 121.7 (d, C-17), 121.4 (d, C-11), 113.7 (s, C-1), 112.9 (d, C-12), 107.8 (d, C-5), 103.6 (d, C-3), 39.7 (t, C-8), 39.6 (t, C-19), 27.8 (t, C-9), 26.3 (t, C-20), 25.9 (t, C-16), 25.7 (q, C-23), 17.7 (q, C-24), 16.3 (q, C-25); DCI-MS (NH_3) *m/z* 428 (11%) [M + NH_4], *m/z* 411 (59%) [M + H]$^+$, *m/z* 287 (3%), *m/z* 259 (2.9%), *m/z* 123 (2.5%).

Cycloaltilisin 6 (2): white solid; IR (KBr) ν_{max} 3380, 3100−3000, 1700, 1632, 1603, 1512, 1445, 1291, 1265, 850, 804 cm^{-1}; UV (MeOH) λ_{max} (log ϵ) 234 (3.44), 278 (3.02), 315 (3.67), and 375 (3.48) nm; for ^{1}H and ^{13}C NMR data, see Table 1; DCI-MS (NH_3) *m/z* 836 (22%) [M + NH_4]$^+$, *m/z* 819 (21.3%) [M + H]$^+$, *m/z* 428 (25.3%), *m/z* 409 (32.9%), *m/z* 165 (100%), *m/z* 144 (92.1%), *m/z* 137 (42.5%); HR-DCI-MS 819.4112 [M + H]$^+$, calcd for $C_{50}H_{59}O_{10}$, 819.4108.

Cycloaltilisin 7 (3): pale yellow solid; $[\alpha]_D$ −23.1° (*c* 0.19, MeOH); IR (KBr) ν_{max} 3565, 3400, 3100−3000, 1709, 1641, 1616, 1590, 1519, 1476, 1449, 1374, 1271, 832 cm^{-1}; UV (MeOH) λ_{max} (log ϵ) 213 (3.13), 227 (3.30), 272 (3.12), 296 (3.69), and 360 (3.42) nm; for ^{1}H and ^{13}C NMR data, see Table 1; DCI-MS (NH_3) *m/z* 407 (100%) [M + H]$^+$, *m/z* 323 (2.2%), *m/z* 287 (8.7%); HR-DCI-MS *m/z* 407.1845 (M + H)$^+$, calcd for $C_{25}H_{27}O_5$, 407.1858.

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