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Shahidine, a novel and highly labile oxazoline from *Aegle marmelos*: the parent compound of aegeline and related amides

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Dedicated to the fond memory of Professor Salimuzzaman Siddiqui FRS, (1897–1994) the founding director of HEJ Research Institute of Chemistry, University of Karachi, Karachi

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ABSTRACT

A rare alkaloid, shahidine (**1**), having an unstable oxazoline core has been isolated as a major constituent from the fresh leaves of *Aegle marmelos*. It is moisture-sensitive, and found to be the parent compound of aegeline and other amides, however, it is stable in dimethyl sulfoxide. Its structure was established by spectroscopic analysis. Biogenetically, oxazolines may be considered as the precursor of hydroxy amides and oxazoles found in plants. Shahidine (**1**) showed activity against a few Gram-positive bacteria.

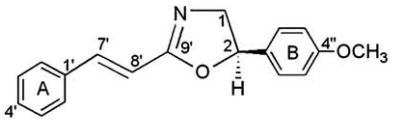
1. Introduction

Aegle marmelos (Linn.) Correa, commonly known as Bael, belongs to the family Rutaceae, and is distributed throughout tropical Asia and Africa.[1–4] From a medicinal point of view, the Bael tree is very important and every part of it is used in the Indian system of medicine;[1–4] indeed no drug has been longer or better known to the people of the Indo-Pakistan subcontinent than Bael.[4] The plant is of very high value in treating cardiac disorders, dysentery, diarrhea, diabetes, fever, inflammation, and pain.[2,5] The anticancer, antihyperglycemic, anti-inflammatory, antipyretic, analgesic and chemoprotective activities of the leaves of the plant have also been studied.[1–5]

In previous phytochemical investigations on the leaves, alkaloids, coumarins, flavonoids, steroids, triterpenes, and essential oils were isolated.[5–8] In our continuing search for bioactive compounds from local medicinal plants, two new compounds shahidine (**1**) and marmeline acetate (**2**) have been isolated from the leaves of *A. marmelos*. Compound **1** has a rare oxazoline ring and has been found to be the parent of aegeline (**3**)[6–9] and related amides (**4** and **5**)[7,10] (Figs. 1 and 2). Oxazoline (**1**) also possesses antibacterial activity.

2. Results and discussion

The petroleum ether extract of the fresh leaves of *A. marmelos*, on treatment with methanol afforded a methanol-soluble fraction, which contained one major compound identified as optically



2-(*Trans*-styryl)-5-(4-methoxyphenyl)- Δ^2-oxazoline: Shahidine (**1**)

Figure 1.

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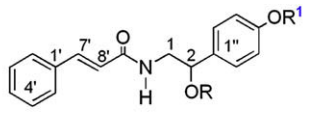


	R	R^1
2	$COCH_3$	$OCH_2CH=C(CH_3)_2$
3	H	CH_3
4	CH_3	CH_3
5	C_2H_5	CH_3
6	$COCH_3$	CH_3

Figure 2.

inactive amide (**4**).[7,10] A review of the literature disclosed it to be an artifact of the isolation procedure and aegeline (**3**) was suggested as its precursor,[7] which is not comprehensible. In fact methanol being a poor nucleophile cannot replace the hydroxy group of aegeline, which is a weak leaving group. This reaction would require strong acidic conditions while the process employed for isolation of **4** was mild.[7] To solve the discrepancy, the petroleum ether extract of the leaves of *A. marmelos* was reinvestigated. Thus TLC and co-TLC of **4** with the extract showed that the latter possessed no trace of amide **4**; instead it contained another major compound, having a very close R_f (0.18, silica gel, $CHCl_3$) value to that of **4** (0.16, silica gel, $CHCl_3$), which was separated through PTLC and isolated as a yellow amorphous powder and identified as shahidine (**1**). It was obtained in large amounts along with marmeline acetate (**2**)[9] and aegeline (**3**)[6] through flash column chromatography of the ethyl acetate soluble fraction of the petroleum ether extract. Oxazoline (**1**) was found to be highly labile and unstable as on contact with water, methanol, ethanol, and glacial acetic acid, it yielded aegeline (**3**),[6] amides **4**,[7,10] **5**,[7] and **6**,[6] respectively (Fig. 2).

Shahidine (**1**) $[\alpha]_D^{25}$ +98.33 (*c* 0.015, $CHCl_3$) has the molecular formula $C_{18}H_{17}NO_2$, as determined by HREIMS (*m*/*z* calcd: 279.1259; found: 279.1267) indicating eleven double bond equivalents. The UV spectrum of **1** displayed absorption maxima at 201, 278, and 340 nm, indicating the presence of a conjugated or aromatic system. As its molecular formula has a CH_3OH unit less than that of amide **4**, it was initially thought that it bears a double bond between C-1 and C-2, as is present in styrylamides isolated from *Piper guayranum* and *Amyris plumieri*.[11,12] However, the ^1H and ^{13}C NMR data did not support this hypothesis (Table 1) as no *trans* olefinic proton doublets at δ 7.65 and 6.22 were observed in the ^1H NMR spectrum of shahidine; instead, the ^1H NMR spectrum of **1** run in $CDCl_3$ (Table 1) showed a two-proton double doublet at δ 7.47 (J=1.2, 8.2) and a three-proton multiplet centered at δ 7.35, along with two doublets (J=16.3) each one-proton at δ 7.40 and 6.68 for the *trans* olefinic protons. These values, which resembled those for the cinnamide group of **4** (Table 2), have been assigned to H-2′,6′; H-3′,4′,5′; H-7′; and H-8′, respectively, of **1**. The HMQC plot has the interaction points for the correlation of H-2′,6′ with 127.6 (C-2′,6′), H-4′ with 129.6 (C-4′), H-3′,5′ with 128.8 (C-3′,5′), H-7′ with 140.5 (C-7′), and H-8′ with 115.0 (C-8′). In the ^{13}C NMR spectrum C-1′ and C-9′ resonated at 135.1 and 164.1, respectively. In this case, H-7′ and C-7′ resonated at low frequency while H-8′ appeared at high frequency and its corresponding carbon as well as C-9′ is shifted to low frequency as compared to that of **4** (Table 2). On the other hand, monosubstituted aromatic ring (A) protons and carbons have almost the same chemical shifts in both the compounds (Table 1, 2). This indicated that some changes occurred at C-9′, and shahidine has not exactly the same cinnamide moiety as that of **4**, instead it has the conjugated styryl group. Important HMBC interactions of C-9′ with H-7′ and H-8′; C-1′ with H-3′, H-5′ and H-8′; and C-7′ with H-2′ and H-6′ supported the observations discussed above (Fig. 3).

The ^1H NMR spectrum also displayed a three-proton singlet at δ 3.80 for the aromatic methoxy group and two one-proton doublets (J=8.7) at δ 7.27 and 6.91 for H-2″,6″ and H-3″,5″ of the *p*-substituted phenyl ring (B), respectively. The HMQC spectrum showed the one-bond correlation of H-2″,6″ with C-2″,6″ (δ 127.5), and H-3″,5″ with C-3″,5″ (δ 114.2), while the HMBC spectrum showed the long-range correlation of 4″-OCH_3, 2″,6″, and 3″,5″ protons with C-4″at δ 159.8. In the broad band ^{13}C NMR spectrum C-1″ resonated at δ 132.7. These NMR data, which are consistent with the presence of a disubstituted aromatic ring with a *p*-methoxy group in the molecule, are almost identical with those of the corresponding protons and carbons for **4**. Ten out of the eleven double equivalents present in the molecule were thus accounted for.

The ^1H NMR spectrum also exhibited two double doublets at δ 3.91 (J=8.0, 15.2) and 4.35 (J=9.8, 15.2) ascribed to methylene protons at C-1, which has correlation with δ_C 62.4 in the HMQC plot. The high frequency shift of the ^1H and ^{13}C NMR δ values of this methylene unit as compared to those of **4** (Table 2) indicated its attachment to the nitrogen of an imino (C=N) moiety. This was supported by the absence of any NH resonance and the appearance of methylene protons as double doublets instead of doublet of double doublets as observed in the case of **4**. The ^1H NMR spectrum further displayed a resonance for the oxy-methine proton at δ 5.51 (dd, J=8.0, 9.8, H-2), correlated with δ_C 80.8 (C-2) in the HMQC spectrum. The C-1 and C-2 protons together formed the ABC pattern of the spin system. The ^1H–^1H COSY-45° measurements showed through-bond linkage of H-1_a with H-1_b; and H-2 with H-1_a and H-1_b (Fig. 3). The high frequency shift of H-2 as compared to that of **4**, absence of an aliphatic methoxy signal, and the large coupling constant of the methylene protons of C-1 as well as the molecular formula, suggested that C-1 and C-2 were part of a ring, which was likely to be an oxazoline located between the C-8′ and C-1″ positions, constructing the 2,5-disubstituted oxazoline nucleus[13,14] for the basic skeleton of **1**. In the case of **1**, oxazoline ring numbering is based on that of **4** for the sake of comparison and clarity. The linkage of C-1″ and C-2 was shown by the three-bond couplings of methylene protons with C-1″, H-2 with C-2″,6″ and H-2″,6″ with C-2 in the HMBC plot (Fig. 3). Another crucial long-range coupling was observed for C-9′ with methylene protons H-$1_{a,b}$ and H-2. The NOESY spectrum showed a through-space interaction of the methoxy group with the aromatic protons of ring B (Fig. 3). The absolute configuration of shahidine was proposed to be 5*S* on the basis of similarities in specific rotation (+98.33) with synthetic (+162.3)[13a] and natural (oxytriphine, +116.0)[13b] (+)-(5*S*)-2,5-diphenyl-2-oxazoline. On the basis of these facts, it was concluded

Table 1
^1H (400 MHz) and ^{13}C (100 MHz) NMR for shahidine* (**1**) in $CDCl_3$ (δ ppm; *J* Hz)

H/C	HMQC		HMBC	$^1H\times^1H$
	δ_C	δ_H (pattern, *J*)	C→H	COSY-45°
1_a	62.4	4.35 (dd, 9.8, 15.2)	—	H-1_b, H-2
1_b	—	3.91 (dd, 8.0, 15.2)	—	H-1_a, H-2
2	80.8	5.51 (dd, 8.0, 9.8)	H-2″, H-6″	H-$1_{a,b}$
1′	135.1	—	H-8′, H-3′, H-5′	—
2′,6′	127.6	7.47 (dd, 1.2, 8.2)	—	H-3′, H-5′
3′,5′	128.8	7.30–7.37 m	H-4′	H-2′, H-6′
4′	129.6	7.30–7.37 m	H-2′, H-6′	—
7′	140.5	7.40 (d, 16.3)	H-2′, H-6′	H-8′
8′	115.0	6.68 (d, 16.3)	—	H-7′
9′	164.1	—	H-$1_{a,b}$, H-7′, H-8′, H-2	—
1″	132.7	—	H-$1_{a,b}$, H-3″, H-5″	—
2″,6″	127.5	7.27 (d, 8.7)	H-2	H-3″, H-5″
3″,5″	114.2	6.91 (d, 8.7)	—	H-2″, H-6″
4″	159.8	—	H-2″, H-6″, H-3″, H-5″, –OCH_3	—
–OCH_3	55.2	3.80 s	—	—

*Numbering as that of **4** for the sake of comparison.

Table 2
NMR data for amides (**3**–**6**, δ ppm; J Hz)

H/C	**3** $CDCl_3$ 500 MHz δ_H (J)	**4** $CDCl_3$ 125 MHz δ_C	**4** $CDCl_3$ 500 MHz δ_H (J)	**4** $(CD_3)_2SO$ 400 MHz δ_H (J)	**4** C_6D_6 400 MHz δ_H (J)	**5** $CDCl_3$ 400 MHz δ_H (J)	**6** $CDCl_3$ 500 MHz δ_H (J)
1_a	3.45 (ddd, 4.9, 7.9, 13.1)	46.0	3.33 (ddd, 4.0, 7.0, 13.5)	3.31–3.33 m	3.47 (ddd, 4.0, 8.5, 13.6)	3.43–3.46 m	3.76 (t, 6.0)
1_b	3.80 (ddd, 3.4, 9.4, 13.1)	—	3.81 (ddd, 4.0, 8.8, 13.5)	3.42 (ddd, 4.6, 5.9, 14.5)	4.07 (ddd, 4.0, 8.6, 13.6)	3.77–3.80 m	3.76 (t, 6.0)
2	4.85 (dd, 3.4, 7.9)	81.8	4.27 (dd, 4.0, 8.8)	4.23 (dd, 4.6, 3.2)	4.32 (dd, 4.0, 8.6)	4.37 (dd, 3.9, 8.8)	5.85 (t, 6.0)
1′	—	135.6	—	—	—	—	—
2′,6′	7.48 (dd, 2.0, 7.9)	127.8	7.49 (dd, 2.1, 7.8)	7.53 (dd, 1.9, 8.4)	7.32 (dd, 3.0, 7.9)	7.47–7.50 m	7.49 (dd, 2.0,7.8)
3′,5′	7.33–7.36 m	128.8	7.36–7.39 m	7.36–7.39 m	6.95–7.18 m	7.32–7.35 m	7.33–7.36 m
4′	7.33–7.36 m	129.6	7.36–7.39 m	7.36–7.39 m	6.95–7.18 m	7.32–7.35 m	7.33–7.36 m
7′	7.63 (d, 15.6)	141.1	7.62 (d, 15.6)	7.39 (d, 15.9)	8.03 (d, 15.5)	7.63 (d, 15.5)	7.61 (d, 15.6)
8′	6.38 (d, 15.6)	120.8	6.41 (d, 15.6)	6.69 (d, 15.9)	6.04 (d, 15.5)	6.38 (d, 15.5)	6.32 (d, 15.6)
9′	—	165.9	—	—	—	—	—
1″	—	131.1	—	—	—	—	—
2″,6″	7.29 (d, 8.7)	128.0	7.24 (d, 8.6)	7.23 (d, 8.6)	**	7.30 (d, 8.4)	7.30 (d, 8.6)
3″,5″	6.88 (d, 8.7)	114.21	6.91 (d, 8.6)	6.92 (d, 8.6)	6.91 (d, 8.6)	6.88 (d, 8.4)	6.89 (d, 8.6)
4″	—	159.7	—	—	—	—	—
4″–OCH_3	3.81 s	55.0	3.80 s	3.74 s	3.43 s	3.77 s	3.79 s
2-OCH_2-CH_3	—	—	—	—	—	3.38–3.41 m	—
2-OCH_2-CH_3	—	—	—	—	—	1.37 (t, 7.2)	—
2-OCH_3	—	56.8	3.24 s	3.11 s	3.16 s	—	—
2-OCOCH_3	—	—	—	—	—	—	2.08 s
N–H	6.12* (dd, 4.9, 9.4)	—	6.01* (dd, 4.0, 7.0)	8.16* (t, 5.9)	5.69* (br m)	6.05* (br s)	5.78* (t, 6.0)

*Exchangeable with D_2O.
**Signal obscured in the solvent peak.

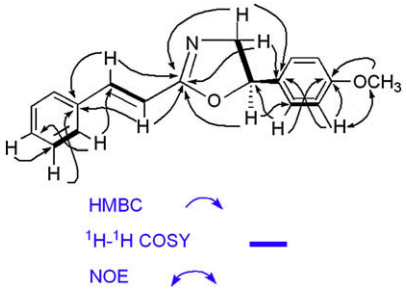
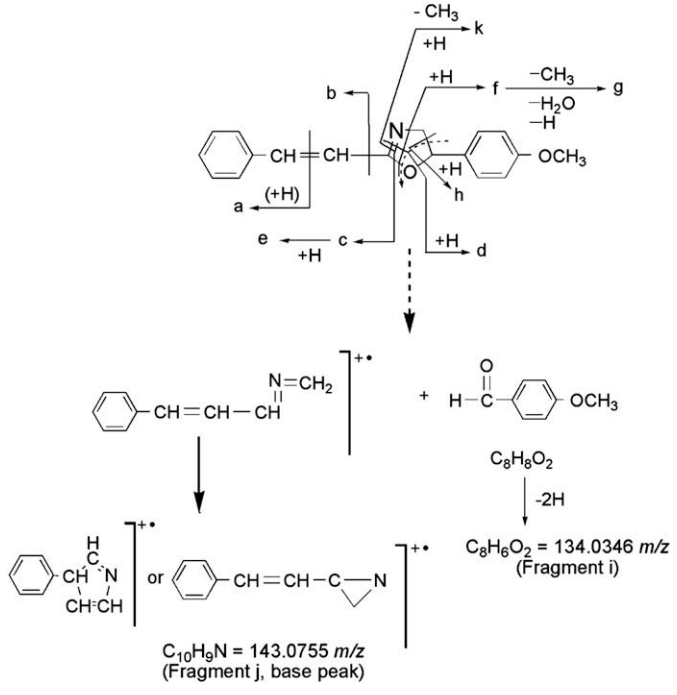


Figure 3.

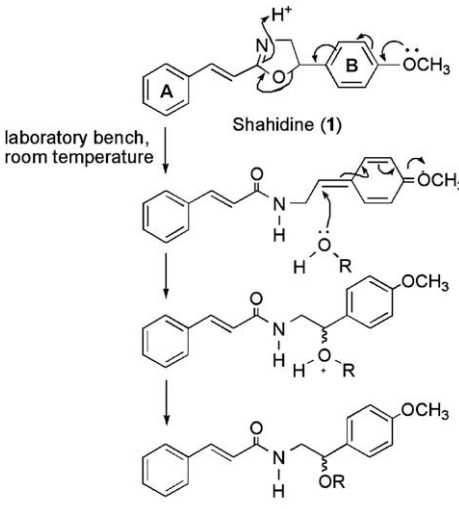
that shahidine has the structure (+)-(5*S*)-2(*trans*-styryl)-5-(4″-methoxyphenyl)-2-oxazoline (**1**), which was also corroborated by the diagnostic mass spectral fragments i and j observed at m/z 134.0346 ($C_8H_6O_2$) and 143.0755 ($C_{10}H_9N$). Other mass fragments appeared at m/z 91 (C_7H_7), 103.0542 (C_8H_7), 115.0535 (C_9H_7), 149.0798 ($C_9H_{11}NO$), 116 (C_9H_8), 151 ($C_9H_{11}O_2$), 117 (C_8H_5O), 121 (C_8H_9O), and 150.0592 ($C_8H_8NO_2$) (Scheme 1, fragments a–h, k), respectively, also substantiated the structure (**1**). The conjugated imino group in **1** may be responsible for the yellow color of **1** and that of the plant extracts containing it, like Schiff bases, which are usually yellow or orange in color.

Shahidine (**1**), which is the main constituent of petroleum ether, dichloromethane, chloroform, dry acetone, and ethyl acetate extracts of the leaves, belongs to a rare class of 2-oxazoline natural product, which are rarely found in plants.[15,16a] Careful sifting of the literature revealed that oxytriphine was the first 2-oxazoline natural constituent isolated from the plant kingdom.[13b] On the other hand the 2-oxazoline ring is a component of numerous compounds isolated from microorganisms.[15,16a] It is important to mention that about 80% of plants live in close symbiotic relationship with symbiotic fungi (ectomycorrhiza, endophytes), which can produce the natural products isolated from plants.[16b] There are several natural cyclic hepta- and octapeptides, e.g., ascidiacyclamide bearing the 2-oxazoline ring, which impart conformational rigidity to these compounds.[16a] It is important to mention that while compounds bearing the oxazoline core are rare in Nature,[13,15] the oxazole ring is often found in natural products, and have also been discovered in the plant kingdom.[15,17,18]

Shahidine (**1**) bearing an oxazoline ring converted readily to aegeline (**3**) on keeping at room temperature. This indicated that **1** is the parent compound of aegeline (**3**). It may be conjectured that the oxazoline **1** transformed to its hydrated product (**3**) through the incorporation of a water molecule in the oxazoline core and simultaneous opening of the ring. The *p*-methoxy group on ring B is the driving force for the reaction (Scheme 2). Similarly methanol, ethanol, and acetic acid (glacial), added readily across the oxazoline ring of shahidine (**1**), furnishing amides **4**, **5**, and **6**, respectively. It is important to note in this context that shahidine (**1**), a masked amide, is inert toward aerial oxidation (auto-oxidation), and did not



Scheme 1. Mass fragmentation of shahidine (**1**).



R	Reagent	Compounds
-H	water	(3)
$-CH_3$	methanol	(4)
$-C_2H_5$	ethanol	(5)
$-COCH_3$	acetic acid	(6)

Scheme 2. Formation of amides (**3**–**6**) on addition of H_2O, CH_3OH, C_2H_5OH, and CH_3COOH, respectively, to compound (**1**).

produce oxidized products on exposure to light and oxygen. It is also stable in dimethyl sulfoxide (DMSO) solution as manifested by the periodic ^{1}H NMR spectra run in DMSO d_6; however, in chloroform it readily transformed into aegeline (**3**). Probably the slightly acidic nature of chloroform facilitates the attack of atmospheric water on the oxazoline ring of **1**. While kept in refrigerator **1** converted into **3**, within a few months. It seems it is sensitive to moisture, but, resistant to oxidation.

It is important to mention that in the literature, 2-methoxy amide (**4**) was reported to be formed from 2-hydroxy amide, aegeline (**3**).[7] However, the present investigation revealed that shahidine (**1**) is in fact the parent compound of amides **3**–**5** claimed as natural products.[6–10] This was strengthened by these observations: (a) Chatterjee et al., and Marcano et al., obtained aegeline from *A. marmelos* and *Zanthoxylum occumarense* as an optically inactive compound.[6,19] Moreover, amide (**4**) was found to be optically inactive in the present studies, as would be expected from the proposed mechanism depicted in Scheme 2. (b) When *A. marmelos* leaves were extracted separately with methanol and ethanol by Manandhar et al., amide **4** and amide **5** were obtained from the methanol and ethanol extracts, respectively.[7]

It is noteworthy that in the case of shahidine (**1**), hydrolysis of the C–N bond of the oxazoline ring did not take place, and therefore, no ester of the β-amino alcohol group was obtained. This may be due to two reasons: (a) hydrolysis of **1**, which has an oxazoline core requires acidic conditions.[20–22] (b) The methoxy group of B ring facilitated the cleavage of the C–O bond instead of fission of the C–N bond of the oxazoline ring in **1**, and it is the key factor.

It is concluded that due to the sensitivity and lability of the oxazoline ring, the isolation of natural products bearing this group is very difficult. This was evident by the fact that only two such compounds have been obtained from the plant kingdom, i.e., oxytriphine[13b] and shahidine (**1**). Reinvestigation of plants belonging to the genera, *Aeglopsis*,[18a] *Aegle*,[18a] *Amyris*,[18a] *Fagara*,[23] *Halfordia*,[17,18a] *Lolium*,[18a] *Micromelum*,[18a] *Oxytropis*,[13b] and *Zanthoxylum*,[19,24,25] which contained amides, hydroxy amides, and oxazoles and employing dry solvents, and careful rapid experimental procedures may lead to the isolation of parent compounds bearing an oxazoline core.

It is the first instance of isolation of an oxazoline-bearing compound from *A. marmelos*, which, contained both oxazoles and cinnamides as natural products.[6–10,26,27] Compounds such as oxazoline **1** can be considered the precursor of oxazole-type alkaloids, e.g., annuloline.[17] Crow and Hodgkin in 1963 suggested that the oxazole nucleus could well arise from a type of common precursor, which produced the amides aegeline (**3**) and *O*-methyl tyramine-*N*-methylcinnamide.[17]

Compound **2** (gum, $[\alpha]_D^{24}$ +52.71) was obtained from fraction sixteen of flash column chromatography. It has a composition $C_{24}H_{27}O_4N$ (MW 393) corresponding to twelve degrees of unsaturation as derived from the HREIMS spectrum, which showed a peak at *m*/*z* 334.1801, due to the loss of an acetyl group ($M^+-C_2H_3O_2$). The IR spectrum displayed peaks at 3465 (N–H), 1743 (acetoxy carbonyl), and 1661 (conjugated –C=C–) cm^{-1}, while its UV spectrum showed maxima at 220, 227, and 275 nm.

Its ^{1}H NMR spectrum ($CDCl_3$, Table 3) displayed the same cinnamoyl moiety as that of compound **4** (Table 2), the main difference between the benzylic methine proton in compounds **2** and **4** was the presence of a high frequency chemical shift value (δ 5.87) of H-2 in the case of **2**, which is comparable to that for amide **6** (Table 2). The HMBC spectrum confirmed the placement of an acetyl group at C-2 showing important long-range coupling of H-2 and N–H with the carbonyl carbon of the acetyl group at (δ 173.5). Furthermore, the ^{1}H NMR spectrum of **2** exhibited a two-proton doublet at δ 4.48 (J=6.5, H-1‴), one-proton triplet at δ 5.45 (J=6.5, H-2‴) and two closely spaced three-proton singlets at δ 1.77 and 1.71 due to the *gem* dimethyl groups indicating the presence of a prenyloxy chain. The HMQC plot illustrated the correlation of H-1‴ with δ_C 64.8 (C-1‴) and H-2‴ with δ_C 119.5. That the prenyloxy chain was linked at C-4″ was confirmed with the important ^3J correlation of H-1‴ in the HMBC spectrum. It was also supported by the NOESY plot, which showed a through-space interaction of H-1‴ with H-3″,5″. In light of above experimental details the structure of **2** was determined to be *N*-[2-acetoxy-2-[4″-(3‴,3‴-dimethylallyloxy)-phenyl]ethylcinnamide (marmeline acetate), which was substantiated by the diagnostic fragment ions in the mass spectrum particularly at *m*/*z* 334.1801 ($M^+-C_2H_3O_2$) and 324.1231 ($C_{19}H_{18}O_4N$). It is important to note that compound **2**, which was

Table 3
^{1}H (400 MHz) and ^{13}C (100 MHz) NMR for marmeline acetate (**2**) in $CDCl_3$ (δ ppm; *J* Hz)

H/C	HMQC		HMBC	^{1}H×^{1}H
	δ_C	δ_H (pattern, *J*)	C→H	COSY-45°
1	44.6	3.75–3.82 m	H-2, N–*H*	H-2, N–*H*
2	74.0	5.87 (t, 6.5)	H-2″, H-6″, H-1	H-1
1′	134.7	—	H-8′	—
2′,6′	128.1	7.44–7.48 m	—	H-3′, H-5′
3′,5′	130.5	7.30–7.34 m	—	H-2′, H-6′
4′	131.9	7.30–7.34 m	—	—
7′	141.6	7.59 (d, 15.6)	H-8′	H-8′
8′	120.1	6.23 (d, 15.6)	—	H-7′
9′	166.0	—	H-8′, H-1	—
1″	128.2	—	H-2, N–*H*	—
2″,6″	128.8	7.26 (d, 8.5)	H-3″, H-5″, H-2	H-3″, H-5″
3″,5″	114.7	6.87 (d, 8.5)	—	H-2″, H-6″
4″	159.0	—	H-1‴, H-3″, H-5″, H-2″, H-6″	—
1‴	64.8	4.48 (d, 6.5)	—	H-2‴
2‴	119. 5	5.45 (t, 6.5)	H1‴, 5‴-CH_3	H-1‴
3‴	128.2	—	H-1‴, 5‴-CH_3	
4‴-CH_3	18.1	1.71 br s	H-2‴, 5‴-CH_3	—
5‴-CH_3	25.7	1.77 br s	H-2‴, 4‴-CH_3	—
OCOCH_3	18.1	2.15 s	—	—
O$COCH_3$	173.5	—	H-2, N–*H*	—
–NH	—	5.85* (dd, 3.5,6.5)	—	H-1

*Exchangeable with D_2O.

previously known as a synthetic compound,[9] is now found in Nature for the first time. Its ^{13}C NMR data, which were not reported earlier are included in Table 3.

A plausible biogenetic pathway for the formation of **1** and **2** is illustrated in Scheme 3. Enzymatic condensation of cinnamic acid with tyramine may provide the key intermediate (**7**), β-hydroxylation of which can lead to the formation of the hydroxy amide (**8**). Internal cyclization of **8** followed by methylation can produce shahidine (**1**). The formation of dihydroxy amide intermediate (**8**) was supported by the isolation of marmeline acetate (**2**) from the plant. On the other hand alkylation of amide **7** followed by β-hydroxylation can give marmeline (**10**), which can also be biosynthesized by alkylation of amide **8**. Enzymatic acetylation of **10** can ultimately yield marmeline acetate (**2**). The intermediates **7** and **8**–**10** have been isolated from *Piper steerni*[28] and *A. marmelos*,[8,9] respectively.

The versatility of oxazolines[29] to serve as precursors to a variety of functional groups amplifies the importance of shahidine (**1**), which is present abundantly in the fresh leaves of *A. marmelos*. Moreover, compounds bearing an oxazoline core are also very important as they are used as chiral auxiliaries in asymmetric synthesis.[30] To examine the antibacterial activity of the leaves, its various extracts and pure compounds were evaluated against Gram-positive and Gram-negative organisms. In preliminary experiments, all the extracts and compounds **1**, **3**, and **4** showed good activity against Gram-positive bacteria, while aegeline (**3**) also inhibited the growth of a few Gram-negative organisms (Table 4). Amide **4** was also evaluated for any anticancer activity in NCI, NIH, Bethesda (USA). It was tested against three NCI cancer cell lines,[31] MCF7 (breast), NCI-h460 (lung), and SF-268 (CNS) and found to be inactive as an anticancer agent.

3. Experimental

3.1. General experimental procedures

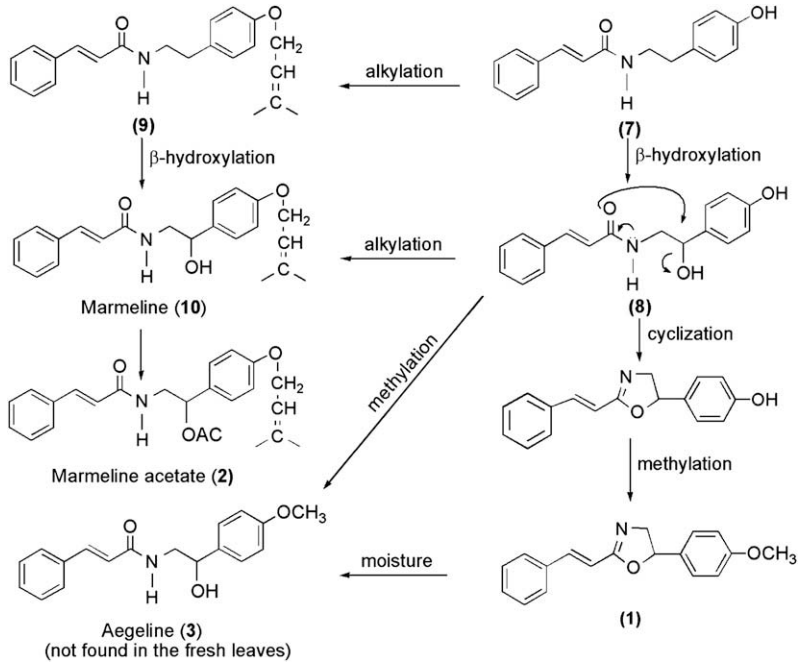
IR (in $CHCl_3$) and UV (in MeOH) spectra were measured on JASCO-A-302 and Hitachi-U-3200 spectrophotometers, respectively, while optical rotation was measured with a Schmidt and Haansch Polartronic-D instrument. EI and HREI mass spectra were recorded on Finnigan MAT-112 and JMS HX-110 spectrometers. ^1H NMR spectra were measured using $CDCl_3$, $(CD_3)_2SO$ or C_6D_6 on a Bruker Aspect AM-400 and a -500 spectrometer at proton frequencies of 400 and 500 MHz. Standard Bruker acquisition and processing software was used for the 1D (DEPT) and the 2D-COSY experiments. Assignments of proton chemical shifts are based on COSY-45°, NOESY and HMQC spectroscopy. Chemical shifts are in parts per million (δ) and coupling constants (J) are in hertz. ^{13}C NMR spectra (Broad Band decoupled and DEPT) were recorded at (100 and 125 MHz) in $CDCl_3$, spectral assignments of which have been made partly through DEPT, HMQC, and HMBC spectra and partly through comparison with the reported values of similar compounds. E. Merck Kieselgel 60 GF_{254} precoated cards (0.2 mm thickness) and glass plates were used for analytical (thin layer) and preparative (thick layer) chromatography, respectively, while for flash column chromatography (Model Aldrich) silica gel 9385 (E. Merck) was used.

3.2. Plant material

The leaves of *A. marmelos* were collected from the Karachi University Campus in January 1999 and October 2005. The plant was authenticated by Dr. Sherwali at the Department of Botany, University of Karachi, and the voucher specimen (No. 68509 KUH) was deposited in the same department.

3.3. Extraction and isolation

Fresh, undried, and uncrushed leaves (1.00 kg) of *A. marmelos* were cut into small pieces and kept in petroleum ether (2×5 l) for two days. The extracts were filtered and combined together and the two layers formed in the extract due to the presence of plant water were separated. The upper layer was evaporated under vacuum to give a dark and gummy residue, which was treated with methanol, affording a greenish brown methanol-soluble fraction and an insoluble white powder. The former fraction was dried with anhydrous Na_2SO_4, treated with charcoal and filtered, and the filtrate (37.5 mg) was subjected to preparative thin layer chromatography (PTLC) (silica gel, $CHCl_3$), affording six bands of which band three showed a single UV and iodine-active spot on TLC (silica gel, $CHCl_3$, R_f=0.16). It was



Scheme 3. Proposed biogenetic pathway for the formation of (**1**) and (**2**) in the fresh leaves of *A. marmelos*.

Table 4
Preliminary antibacterial activity of different extracts and pure compounds from *A. marmelos* leaves

Samples (20 mg/ml)	Shahidine (**1**)	Aegeline (**3**)	Compound (**4**)	AGLP (pet. ether extract)	AGLDC (dichloromethane extract)	AGLC (chloroform extract)	AGLM (methanol extract)
Gram-positive bacteria							
Staphylococcus aureus ATCC	8	7	9	7	12	8	9
Staphylococcus aureus AB188	9	7	9	—	8	9	7
Staphylococcus epidermidis	7	8	9	7	11	9	10
Staphylococcus saprophyticus	8	ND	ND	ND	15	12	9
Staphylococcus fecalis	9	—	9	—	—	—	—
MRSA-1	8	9	9	7	—	—	ND
MRSA-2	9	ND	ND	ND	11	—	—
MRSA-3	9	ND	ND	ND	9	—	7
Corynebacterium xerosis	11	7	8	—	9	10	9
C. hoffmanii	10	8	7	7	8	9	7
C. diphtheriae	9	8	—	—	9	11	7
Bacillus cereus	8	8	9	7	10	8	8
Bacillus subtilis ATCC	9	7	9	8	11	10	—
B. thuringiensis	9	8	8	—	14	8	8
Micrococcus luteus ATCC	9	—	8	7	10	9	9
Micrococcus luteus WT	9	8	—	—	9	9	9
Gram-negative bacteria							
Proteus mirabilis	—	16	—	—	12	—	20
Proteus vulgaris	—	8	—	—	15	—	20
Salmonella paratyphi ATCC	ND	8	—	—	—	—	—
Salmonella paratyphi A	—	—	—	—	—	—	—
Salmonella paratyphi B	—	7	—	—	—	—	—
Esherichia coli MDR	—	8	9	8	—	—	—
Esherichia coli WT	—	7	—	—	—	—	—
Pseudomonas aeruginosa PAO286	—	ND	ND	ND	—	—	—
Pseudomonas aeruginosa	—	7	11	12	—	—	—
Shigella flexneri	—	7	9	—	—	—	—
Shigella boydii	—	—	—	—	—	—	—
Shigella dysenteriae	—	8	9	9	12	—	—
Klebsiella pneumoniae	—	7	10	—	—	—	—

—=No zone of inhibition; ND=not done.

identified as *N*-[2-methoxy-2-(4-methoxyphenyl)ethyl]cinnamide[7] (**4**, 28.0 mg) through spectroscopic studies.

In another working, the original yellow-colored petroleum ether extract (32.5 mg) was subjected directly to preparative thin layer chromatography, (silica gel, $CHCl_3$) affording three bands. Band two showed a single spot on TLC (silica gel, chloroform, R_f=0.18), and was characterized as a new Δ^2-oxazoline alkaloid (shahidine, **1**, 24.1 mg, 0.519%, dry wt basis) through spectroscopic studies (UV, IR, Mass, 1D, and 2D NMR). Shahidine (**1**), on treatment with water, methanol, ethanol and glacial acetic acid, afforded compounds **3**,[6] **4**,[7] **5**,[7] and **6**,[6] respectively.

To obtain **1** in large quantities, fresh, undried, and uncrushed leaves (2.00 kg) of *A. marmelos* were also percolated in petroleum ether (2×11 l) for 2 days. The golden yellow extract on evaporation yielded a residue, which was taken up in ethyl acetate (300 ml). In the ethyl acetate fraction, white powder deposited, which was filtered and the filtrate was treated with Na_2SO_4 anhydrous and charcoal and filtered. The filtrate thus obtained was evaporated under reduced pressure furnishing a residue (4.93 g), which showed one major and a few minor spots on TLC (silica gel, $CHCl_3$). This residue was subjected to flash column chromatography [Aldrich model; column size: 1000 ml, silica gel (180 g); petroleum ether and ethyl acetate, in order of increasing polarity by 5%] affording 46 fractions. The volume eluted with each system was 0.25 l. Fraction sixteen (P.E/E.A, 7.5:2.5) exhibiting a single UV active spot on TLC (silica gel, $CHCl_3$, R_f=0.21) was identified by spectral studies as a new natural product, marmeline acetate[9] (**2**, 115 mg). Eluate 17 (P.E/E.A, 7.0:3.0) and 25 (P.E/E.A, 1:1) showed single spots on TLC (silica gel, $CHCl_3$, R_f=0.18) and (silica gel, $CHCl_3$, R_f=0.09), respectively, and characterized as shahidine (**1**, 365 mg) and aegeline (**3**, 207 mg), respectively, through spectroscopic studies (UV, IR, EIMS, and ^{1}H NMR).

3.4. Characterization of compounds

*3.4.1. 2-(trans-Styryl)-5-(4-methoxyphenyl)-Δ^2-oxazoline: shahidine (**1**)*

Yellow and amorphous; $[\alpha]_D^{24}$ +98.33 (*c* 0.015, $CHCl_3$); UV (MeOH) λ_{max} nm (ε): 201 (83,700), 278 (11,559), 340 (3236); IR ($CHCl_3$) ν_{max}: 2881 (m) (aliphatic –CH), 1661 (s) (C=N stretching in conjugation with C=C), 1613 (s) (>C=C< conjugated with aromatic ring), 1513 (m), 1611 (s) (benzene stretchings), 1170 (s) (C–O stretching), 825 (m) (*para*-disubstituted benzene) cm^{-1}; FAB (+ve): 280 *m/z* [M$^+$+1] ($C_{18}H_{18}NO_2$); FAB (−ve): 278 *m/z* [M$^+$−1] ($C_{18}H_{16}NO_2$); HREIMS *m/z*: 279.1267 [M$^+$] (calculated for $C_{18}H_{17}NO_2$, 279.1259), 150.0592 ($C_8H_8NO_2$, fragment k), 149.0798 ($C_9H_{11}NO$, fragment d), 143.0755 ($C_{10}H_9N$, fragment j), 134.0346 ($C_8H_6O_2$, fragment i), 121.0668 (C_8H_9O, fragment h), 115.0535 (C_9H_7, fragment c), 103.0542 (C_8H_7, fragment b); EIMS *m/z* (relative intensity, %): 279 (M$^+$, $C_{18}H_{17}NO_2$, 38.06), 151 ($C_9H_{11}O_2$, fragment f, 69.88), 149 ($C_9H_{11}NO$, fragment d, 37.65), 143 ($C_{10}H_9N$, fragment j, 100), 134 ($C_8H_6O_2$, fragment i, 55.83), 121 (C_8H_9O, fragment h, 57.09), 117 (C_8H_5O, fragment g, 15.99), 116 (C_9H_8, fragment e, 51.79), 115 (C_9H_7, fragment c, 87.76), 103 (C_8H_7, fragment b, 61.55), 91 (C_7H_7, fragment a, 55.78); ^{1}H and ^{13}C NMR data, see Table 1.

*3.4.2. N-[2-Acetoxy-2-[4′-(3″,3″-dimethylallyloxy)]phenyl]-ethylcinnamide: marmeline acetate (**2**)*

Colorless gum; $[\alpha]_D^{24}$ +52.71 (*c* 0.019, $CHCl_3$); UV (MeOH) λ_{max} nm (ε): 220 (17,779), 227 (17,370), 275 (16,988); IR ($CHCl_3$) ν_{max}: 3465 (m) (N–H), 1661 (s) (C=O), 1545 (m) cm^{-1}; HREIMS *m/z*: 334.1801 (calculated for M$^+$−$C_2H_3O_2$, $C_{22}H_{24}O_2N$, 334.1806), 324.1231 ($C_{19}H_{18}$ NO_4); EIMS *m/z*: 334 (M$^+$−$C_2H_3O_2$), 324 (M$^+$−C_5H_9), 308 (M$^+$−C_5H_9O), 289, 278, 262, 247, 232 ($C_{13}H_{14}O_3N$); ^{1}H and ^{13}C NMR data, see Table 3.

3.4.3. N-[2-Hydroxy-2-(4-methoxyphenyl)ethyl]-3-phenyl-2-propenamide: aegeline (**3**)

White crystalline solid; $[\alpha]_D^{24}$ 0 (*c* 0.010, $CHCl_3$); UV (MeOH) λ_{max} nm (ε): 201 (53,994), 217 (49,401), 222 (50,094), 275 (51,866); IR ($CHCl_3$) ν_{max}: 3405 (m) (N–H), 3355 (m) (O–H br), 2910 (w) (aliphatic), 2852 (w) (aryl ether), 1665 (s) (conjugated C=C), 1692 (m) (conjugated C=O), 1625 (s) (trans conjugation), 1512 (m) (amide II; N–H bending), 1460 (m), 1575 (w) (benzene stretching), 1507 (w) (C=C aryl C–H vibration), 1577 (m) (aryl C–H vibrations), 980 (m) (CH=CH trans), 818 (m) (*para*-disubstituted benzene) cm^{-1}; HREIMS *m/z*: 297.1365 [M^+] (calcd for $C_{18}H_{19}NO_3$, 297.1360), 280.1285, ($C_{18}H_{18}NO_2$, M^+-OH), 279.1283 ($C_{18}H_{17}NO_2$, M^+-H_2O), 160.0777 ($C_{10}H_{10}NO$), 150.0677 ($C_9H_{10}O_2$), 137.0617 ($C_8H_9O_2$), 131.0532 (C_9H_7O), 103.0556 (C_8H_7); EIMS *m/z*: 297 (M^+, $C_{18}H_{19}NO_3$), 220 ($C_{12}H_{14}NO_3$), 207 ($C_{11}H_{13}NO_3$), 194 ($C_{10}H_{12}NO_3$), 190 ($C_{11}H_{12}NO_2$), 166 ($C_9H_{12}NO_2$), 151 ($C_9H_{11}O_2$), 150, 148, 146 (C_9H_8NO), 136, 135, 107 (C_7H_7O), 103 (C_8H_7), 90 (C_7H_6), 77 (C_6H_5); δ_C (125 MHz, $CDCl_3$) 167.0, 159.3, 141.7, 134.6, 133.8, 129.8, 128.8, 127.8, 127.1, 120.0, 114.0, 73.4, 55.3, 47.6; ^1H NMR data, see Table 2.

3.4.4. N-[2-Methoxy-2-(4-methoxyphenyl)ethyl]-3-phenyl-2-propenamide (**4**)

Colorless and gummy; $[\alpha]_D^{24}$ 0 (*c* 0.012, $CHCl_3$); UV (MeOH) λ_{max} nm (ε): 217 (19,965), 222 (22,915), 274 (24,559); IR ($CHCl_3$) ν_{max}: 3405 (m) (N–H), 2952 (m) (aliphatic-CH_2), 1663 (s) (amide I; C=O stretching), 1626 (s) (>C=C< conjugated with aromatic ring), 1630 (s) (*trans* olefinic bond), 1502 (m) (C=C), 1512 (m) (amide II; N–H bending), 1443 (w), 1500 (m) (benzene stretchings), 1112 (m) (C–O), 825 (m) (*para*-disubstituted benzene), 981 (s) (–HC=CH–) cm^{-1}; Peak matching *m/z*: 311.1517 (M^+, calcd for $C_{19}H_{21}NO_3$, 311.1521), 164.0792 ($M^+-C_9H_9NO$), 135.0382 ($M^+-C_{10}H_{10}NO_2$); EIMS *m/z*: 311 (M^+), 280 (M^+-OCH_3), 164 ($C_{10}H_{12}O_2$), 163 ($C_{10}H_{11}O_2$), 152 ($C_9H_{12}O_2$), 151 ($M^+-C_{10}H_{10}NO$), 135 ($C_9H_{11}O$), 103 (C_8H_7), 77 (C_6H_5); ^1H and ^{13}C NMR data, see Table 2.

3.4.5. N-[2-Ethoxy-2-(4-methoxyphenyl)ethyl]-3-phenyl-2-propenamide (**5**)

White powder; UV (MeOH) λ_{max} nm (ε): 215 (12,028), 224 (12,030), 272 (12,581); IR ($CHCl_3$) ν_{max}: 3401 (s) (N–H), 2950 (m) (aliphatic-CH_2), 1664 (m) (amide I; C=O stretching), 1619 (m) (>C=C< conjugated with aromatic ring), 1510 (w) (amide II; N–H bending), 1463 (m), 1614 (m) (benzene stretchings) cm^{-1}; EIMS *m/z*: 325 (M^+, $C_{20}H_{23}NO_3$), 280 ($M^+-OC_2H_5$), 179 ($C_{11}H_{15}O_2$), 165 ($C_{10}H_{13}O_2$), 103 (C_8H_7), 77 (C_6H_5); δ_C (125 MHz, $CDCl_3$) 165.7, 159.4, 141.1, 134.8, 131.9, 129.6, 128.7, 128.0, 127.8, 120.7, 114.0, 79.9, 64.1, 55.2, 45.6, 27.9; ^1H NMR data, see Table 2.

3.4.6. N-[2-Acetoxy-2-(4-methoxyphenyl)ethyl]-3-phenyl-2-propenamide (**6**)

White powder; UV (MeOH) λ_{max} nm (ε): 222 (10,181), 273 (10,324), 280 (9806); IR ($CHCl_3$) ν_{max}: 3345 (s) (N–H), 1742 (s) (O–C=O), 1641 (s) (NH–C=O) cm^{-1}; EIMS *m/z*: 339 (M^+, $C_{20}H_{21}NO_4$), 265 ($M^+-C_2H_3O_2-CH_3$), 192 ($C_{11}H_{12}O_3$), 160 ($M^+-C_{10}H_{11}O_3$), 149 ($C_9H_9O_2$), 137 ($C_8H_9O_2$), 131 (C_9H_7O), 103 (C_8H_7), 77 (C_6H_5); δ_C (100 MHz, $CDCl_3$) 170.5, 165.9, 159.7, 141.5, 134.7, 129.8, 129.7, 128.8, 127.9, 127.8, 120.2, 114.0, 74.3, 55.2, 44.4, 21.2; ^1H NMR data, see Table 2.

4. Antimicrobial activity

4.1. Antibacterial activity

Antibacterial activity was determined by the disc diffusion method of Baur et al.[32] using Nutrient Agar Medium. For this purpose, sterile discs of 6 mm diameter filter paper were prepared. From a stock solution of 10 mg/ml; 10 μl, i.e., 100 μg of the sample was applied to a disc. The discs were air dried and then placed on a lawn of the test organism. The plates were incubated at 37 °C for 24 h and then zones of inhibition were observed around each discs, measured, and recorded (Table 4).

5. Anticancer activity

Compound (**4**) was tested for anticancer activity against three NCI cancer cell lines, MCF7 (breast), NCI-H460 (lung), and SF-268 (CNS), in National Cancer Institute, National Institute of Health, Bethesda, USA.[31]

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