Kalanchosine Dimalate, an Anti-inflammatory Salt from *Kalanchoe brasiliensis*

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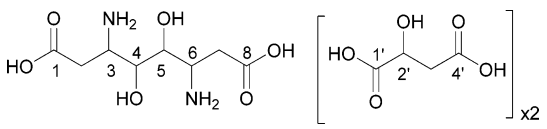
This report describes the isolation and characterization of kalanchosine dimalate (KMC), an anti-inflammatory salt from the fresh juice of the aerial parts of *Kalanchoe brasiliensis*. KMC comprises the new metabolite kalanchosine (**1**) and malic acid (**2**) in a 1:2 stoichiometric ratio. Kalanchosine (**1**), 3,6-diamino-4,5-dihydroxyoctanedioic acid, is the first naturally occurring dimeric bis(γ-hydroxy-β-amino acid) and is at least partially responsible for the anti-inflammatory properties of *K. brasiliensis*.

An interdisciplinary research program for the discovery of new bioactive molecules from Brazilian plants traditionally used in popular medicine against infectious diseases is currently under way in our laboratories. Among the plants studied, *Kalanchoe* species (Crassulaceae), which are renowned worldwide as ornamental plants and houseplants, are popularly used for their pharmacological properties.[1] We have a specific interest in *Kalanchoe brasiliensis* Camb., whose anti-inflammatory properties were first reported sixty years ago.[2] In continuation of our previous investigations on this species,[3] we are currently engaged in characterizing the substance(s) responsible for the in vivo anti-inflammatory effect recently demonstrated for the leaf juice.[4] An activity-directed fractionation process led to the unexpected isolation of an organic salt formed between a novel symmetrical bis(γ-hydroxy-β-amino acid)−kalanchosine−and malic acid, and we wish to report here the preliminary results on its chemical characterization as well as its anti-inflammatory properties.

Direct addition of EtOH to the juice from aerial parts of specimens of *K. brasiliensis* that had not yet bloomed and were collected at Rio de Janeiro State (Brazil) led to a partially water-soluble precipitate that was resuspended in H_2O and lyophilized to give an ashy white solid in a rather high yield (0.319% w/w fresh plant). This precipitate was shown by ^{1}H NMR to consist of a mixture of a new compound, called kalanchosine, and malic acid in a stoichiometric 1:2 ratio. Throughout this paper this compound is referred to as KMC (kalanchosine−malic acid complex salt), without attempting to specify the exact nature of the salt/coordination interactions between the constituent chemical entities.



Kalanchosine **1** Malic acid **2**

Similar 1:2 ratios have been found for kalanchosine−malic acid complexes isolated by EtOH precipitation from juices from several other *K. brasiliensis* specimens harvested in different places in Rio de Janeiro State, always during the nonblooming period. The successful isolation of KMC using this protocol was totally unexpected, since EtOH precipitation is generally employed to separate macromolecules such as proteins and polysaccharides from the fractions containing micromolecules soluble in H_2O.[5]

The main structural features of KMC were deduced from ^{1}H and ^{13}C NMR analysis and are summarized in Table 1.

^{1}H NMR data and ^{1}H−^{1}H COSY experiments (D_2O) for the kalanchosine constituent of KMC reveal a rather simple structure. Two *gem*-methylene protons at 2.56 and 2.73 ppm (H_2-2 or -7) appear as two sets of double doublets. The large geminal coupling constant (J = 17.2 Hz) indicates that they are the AB part of an ABX system, and their chemical shifts are in agreement with the presence of a vicinal carbonyl group (HO_2C-1 or -8). The X part was assigned to a nitrogen-bound methine appearing as a double doublet at 3.34 ppm (H-3 or -6), which in turn correlates with another methine doublet proton at 4.39 ppm (H-4 or -5).

The APT ^{13}C NMR spectra exhibit signals that confirm the existence of a methylene group at 35.2 ppm and two methine groups at 47.7 and 73.1 ppm. Moreover, unexpectedly, three carboxyl groups (178.64, 178.57, 177.5 ppm) correlate with the methylene signal of kalanchosine (see below for comments). These data are consistent with the presence of a $-CH(OH)-CH(NH_2)-CH_2CO_2-$ sequence, which was later deduced to be duplicated in the dimeric-type structure of **1**.

The low-resolution Q-Star-ESI mass spectrum of KMC exhibits an ion at *m*/*z* 247 $[M + H - 2\ H_2O]^+$, in agreement with a quasi-molecular ion at 282, corresponding to $C_8H_{16}N_2O_6Na_2$ for **1**. High-resolution MALDI TOF-MS gives a significant ion at 275.0640, in agreement with $C_8H_{16}N_2O_6K$ (calc 275.0645). From these data, kalanchosine has been identified as 3,6-diamino-4,5-dihydroxyoctanedioic acid, a novel symmetrical dimeric molecule.

The rest of the ^{1}H NMR spectrum of KMC reveals a pair of signals not correlated with the above system and exhibiting double integral intensities in comparison with kalanchosine. An ABX methylene (2.70 and 2.79 ppm) and methine (4.46 ppm) signals indicate the existence of an independent $-CH(OH)-CH_2-$ subunit (^{13}C NMR peaks at 41.2 and 69.5 ppm). An unexpected supplementary set of three less intense carboxyl signals at 179.5, 179.3, and 177.2 ppm (see below for comments) correlates with the preceding two signals and allows the identification of a malic acid unit (**2**), a common carboxylic acid in the metabolism of the *Kalanchoe* (syn. *Bryophyllum*) genus.[6]

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Table 1. ^{1}H, ^{13}C, APT, and HETCOR NMR Data (D_2O, δ, ppm)[a] for KMC[b]

carbon number	δ ^{1}H, m	*J*, Hz	δ^{13}C	APT	HETCOR	HETCOR (long-range)
			Kalanchosine, **1** (ca. 1 equiv)			
1, 8			178.64[c]	CO		178.64, 178.57, 177.5−2.62,[d] 4.39
			178.57[c]			
			177.5			
2, 7	H_a: 2.56; dd	17.2; 5.5	35.2	CH_2	35.2−2.62[d]	35.2−3.34
	H_b: 2.73; dd	17.2; 9.0				
3, 6	3.34; dd	9.0; 5.5; 3.3	47.7	CH	47.7−3.34	47.7−3.34
4, 5	4.39; d	3.3	73.1	CH	73.1−4.39	73.1−2.62,[d] 4.39
			Malic Acid, **2** (ca. 2 equiv)			
1′			179.5	CO		179.3−2.74[d]
			179.3			
2′	H_a: 2.70; dd	17.4; 6.1	41.2	CH_2	41.2−2.74[d]	41.2−4.46
	H_b: 2.79; dd	17.4; 5.0				
3′	4.46; dd	6.1; 5.0	69.5	CH	69.5−4.46	69.5−2.74[d]
4′			177.2	CO		

[a] Traces of acid were added to the sample to improve the resolution. [b] In some lots, several signals attributed to the presence of minor amounts of malonic acid were present: ^{1}H, δ: H_a, 2.70 ppm, d, J = 16.1 Hz; H_b, 2.90, d, J = 16.1 Hz. ^{13}C, δ: 176.1 and 45.0 ppm. [c] Two decimal places are reported in order to differentiate between close signals. [d] Reported chemical shifts at 2.62 and 2.74 ppm in the HETCOR experiments correspond to the center of the multiplet signals at 2.56−2.73 ppm and 2.70−2.79 ppm, respectively.

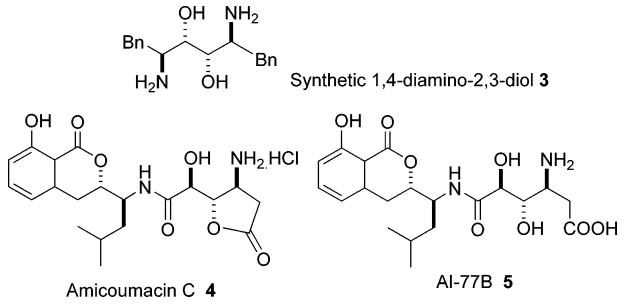
Additionally, the NMR spectra of some KMC samples exhibit several intercorrelated minor signals at 176.1 and 45.0 ppm (^{13}C) as well as an AB system at 2.70 and 2.90 ppm (H_a and H_b), d, J 16.1 Hz (^{1}H), consistent with the presence of a minor amount of malonic acid, which probably competes with malic acid in the salt formation.

The complexity of the carboxyl region of the ^{13}C NMR spectrum of KMC, where three unexpected carboxylic signals appear in the 176−179 ppm region, deserves additional comment. Although complexes of amino acids and malic acid (or other dicarboxylic acids) have not yet been isolated from a natural source, this type of complex has already been prepared and studied by X-ray crystallography.[7] These studies have shown that the exact stoichiometry in complexes of various amino acids and dicarboxylic acids (oxalic and succinic acids) depends on the nature of both partners, each of them being able to adopt different conformations and ionization states inside one crystal. In the case of KMC, the presence of several carboxyl signals for both kalanchosine (**1**) and malic acid (**2**) congeners probably occurs from different aggregation patterns where several ionization−coordination forms and/or conformations may be involved.

The infrared data obtained for KMC do not show any well-defined bands in the region expected for C═O vibrations of nonionized carboxylic acids (1720 cm^{-1} region). On the other hand, strong, broad bands are observed at 1603 cm^{-1} as well as at 1578 cm^{-1} together with a weaker band at 1409 cm^{-1}. These absorptions are characteristic of ionized carboxyl groups and could arise from the malate part of zwitterionic kalanchosine units.[8] The band at 1578 cm^{-1} may also arise from an NH_3^+ vibration.[9] All these data are in agreement with the presence of ionized malic acid and kalanchosine units in KMC but cannot be definitively attributed to the carboxyl versus carboxylate forms. The relative and absolute configurations of the four stereogenic centers present in kalanchosine (**1**) still remain to be established. The simple ^{1}H and ^{13}C NMR patterns of **1** clearly indicate a C_2-symmetrical structure of the molecule. The ^{1}H NMR pattern of chemical shifts and coupling constants as well as the ^{13}C data observed for **1** are reminiscent of those reported by Dondoni et al. for the synthetically designed dibenzyl C_2-symmetric 1,4-diamino-2,3-diol (**3**).[10]

Although no natural bis(γ-hydroxy-β-amino acid) has been described so far, the γ-hydroxy-β-amino acid moiety represents a critical substructure encountered in natural peptidic tuberactinomycin antibiotics[11] as well as in natural gastroprotective 3,4-dihydroisocoumarins such as amicoumacin C (**4**)[12] and AI-77B (**5**).[13]

Whether KMC represents an extraction artifact or a significant intermediate in the metabolism of the living plant remains to be established. However, the relatively high yield may be related to the so-called Crassulacean acid metabolism (CAM), a nocturnal uptake of CO_2 followed by its fixation in the form of organic acids, mainly malic acid, stored in vacuoles.[14]



With regard to structure/activity relationships, kalanchosine can be regarded as a β-amino acid, which represents a key substructure of an emerging class of promising compounds in medicinal chemistry.[15] It also resembles a 1,2-amino alcohol, a motif that is present in a variety of molecules endowed with an impressive array of biological activities that have stimulated considerable synthetic efforts over the past 15 years.[16] Anti-inflammatory properties, mainly protein kinase C inhibition, have been demonstrated for sphingosine,[17] some 3-amino-1,2-diols,[18] or even aroyl-2-amino alcohol derivatives.[19] 1,2-Amino alcohols have also shown potent semicarbazide-sensitive amino-oxidase (SSAO) inhibitor activity on human vascular adhesion protein, VAP-1.[20] 1,4-Diamino-2,3-dihydroxy compounds are isosteric inhibitors of natural HIV proteases.[21]

The role of KMC in the already demonstrated anti-inflammatory properties of the aqueous extract of *K. brasiliensis* has been investigated using the zymosan-induced inflammation test in mice.[22] As shown in Figure 1, treatment with 240 mg/kg KMC-precipitate impaired the increase in the popliteal lymph nodes of mice in a manner similar to treatment with 480 mg/kg juice, indicating that KMC is at least 2-fold more active than *K. brasiliensis* juice. In contrast, the filtrate demonstrates only a residual activity, probably due to unprecipitated KMC material.

In conclusion, a 1:2 organic salt (KMC) between kalanchosine (**1**) and malic acid (**2**) has been isolated in rather high yield from *K. brasiliensis*. The kalanchosine component of KMC is the first natural compound showing the symmetrical −CH(NH_2)CH(OH)-CH(OH)CH(NH_2)− core, and current efforts are devoted to the definitive elucidation of the configuration of this dimeric γ-hydroxy β-amino acid. Very recent results from our laboratories have led to the characterization of KMC from different *Kalanchoe* species

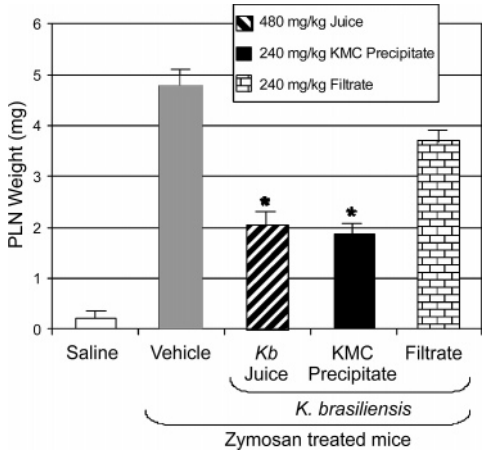


Figure 1. Popliteal lymph node (PLN) assays for anti-inflammatory activity against zimosan in mice, using crude juice of *K. brasiliensis* (*Kb*) or KMC fraction obtained after precipitation with ethanol and the corresponding filtrate. The results are expressed as mean ± SEM of 3 experiments with 5 mice per group/experiment. *p < 0.01 in relation to vehicle data.

as well as from other Crassulaceae, thus suggesting a rather ubiquitous occurrence of kalanchosine and a possible role of this metabolite in CAM processes involving malic acid turnover. The importance of KMC in the renowned anti-inflammatory properties of *K. brasiliensis* is demonstrated.

Experimental Section

General Experimental Procedures. Optical rotations were measured with a Perkin-Elmer 243B polarimeter, and melting points with a Kofler apparatus (not corrected). The IR spectrum was obtained on a Nicolet 210 spectrometer. ^{1}H and ^{13}C NMR spectra were recorded with a Varian Gemini 200 (^{1}H, 200 MHz; ^{13}C, 50 MHz) spectrometer. Q-Star (electrospray mode) low-resolution MS was obtained with a Voyager-DE PRO spectrometer model (Applied Biosystems). High-resolution MALDI TOF-MS was performed on a Biflex III (Bruker Daltonics, Billerica, MA) in a reflectron mode (IS1: 19 kV, IS2: 16.5 kV, reflector: 20 kV), and α-cyano-4-hydroxycinnamic acid (HCCA, Aldrich, Milwaukee, WI) was used as matrix. A saturated 0.5 μL sample solution in 50:50 MeOH/H_2O−1% HCO_2H was prepared and spotted on the target, followed by 0.5 μL of matrix. Mixing was accomplished by pipetting up and down, and the solution was allowed to dry at room temperature. MALDI spectra were internally calibrated using matrix peaks. HRMS was obtained with an accuracy of 2 ppm.

For the bioassays, C57B110 male mice, weighing 22−25 g (Fiocruz, Rio de Janeiro, Brazil), were housed in a temperature-controlled room and received water and food ad libitum. All experiments were carried out in accordance with Brazilian guidelines for animal use in laboratory experiments.

Plant Material. The aerial parts of cultivated, nonblooming specimens of *K. brasiliensis* were collected in the morning in Itaguaí at Rio de Janeiro State (Brazil). A voucher specimen (304627) was deposited at the Botanical Garden in the city of Rio de Janeiro, Brazil.

KMC Isolation. The fresh aerial parts of *K. brasiliensis* (24 kg) were washed with aqueous NaOCl at 100 ppm and rinsed and dried at room temperature. The fresh plant material was triturated 24 h later in an industrial food processor to afford a green juice (19 L), which was clarified by decantation at 5 °C for 24 h. The resulting yellow supernatant (17.3 L) was collected via siphon and mixed with EtOH (18 L). The mixture was shaken and kept at 5 °C for 5 days, forming a white-flake precipitate that was separated by paper filtration (changing the filter when necessary to maintain an acceptable flow). The filters were washed with Milli-Q H_2O (1.0 L) and the washings combined with the precipitate to give a slurry, to which was added supplementary EtOH (1.5 L). After filtration through paper, the collected solid material, exhibiting the consistency of fresh cheese, was partially dried in a ventilated oven at 40 °C, dissolved in Milli-Q H_2O, frozen, and lyophilized. This precipitate (76.3 g, 0.319% yield w/w from the fresh plant) was further shown by ^{1}H NMR analysis to be the pure KMC.

KMC: 3,6-diamino-4,5-dihydroxyoctanedioic acid (1, kalanchosine)−malic acid, 1:2: ashy white amorphous solid; decomposition without fusion at 300 °C; $[\alpha]_D^{25}$ −14 (*c* 1.0, water); IR (KBr, cm^{-1}) 3385 (s, br), 1603 (s), 1578 (s), 1409 (s), 1322 (m), 1286 (m), 1112 (m), 1081, 1035 (w), 876 (w), 784 (w), 676 (m), 534 (m); Q-Star *m*/*z* 247.089 [M of **1** − 2 H_2O + H]$^+$; HRMS *m*/*z* [M of **1** + K]$^+$, 275.0640, $C_8H_{16}N_2O_6K$ (calc 275.0645); ^{1}H and ^{13}C NMR, see Table 1 and spectra in the Supporting Information.

Anti-inflammatory Bioassay. Zymosan-induced inflammation: a group of 5 mice received a subcutaneous injection (10 μL) of zymosan (150 μg in sterile saline) in the left hind footpad. The control group received saline. After 7 days, the weight of popliteal lymph nodes (PLN) from both legs, after dissecting and removing the attached adipose tissue, was evaluated. Treatment with Kb juice and fractions: groups of 5 mice were treated intraperitoneally with daily injections of lyophilized juice of Kb (480 mg/kg), KMC precipitate (240 mg/kg), and filtrate (240 mg/kg) resuspended in sterile deionized H_2O to a final volume of 200 μL. Indomethacin (3 mg/kg) was used as a standard. The sample solutions were filtered through a 0.22 μm Millipore membrane before use. Control mice received sterile deionized H_2O. In all cases, mice were treated during 5 days, starting 2 days after zymosan injection. At the end of the treatment (7 days after zymosan injection), PLN weights were evaluated. Data were subsequently analyzed using the Statsworks program. Differences between means were evaluated by use of the Student's unpaired *t*-test and considered to be statistically significant at p < 0.05. Results were expressed as means ± SEM.

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Supporting Information Available: Copy of original spectra files. This material is available free of charge via the Internet at http://pubs.acs.org.

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