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Article

New Pyrone and Quinoline Alkaloid from *Almeidea rubra* and their Trypanocidal Activity

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O estudo da fração acetato de etila do extrato metanólico das folhas de *Almeidea rubra* A. St.-Hil. (Rutaceae) permitiu o isolamento de duas substâncias inéditas: 4-metoxi-6-[2-(metilamino)fenil]-2*H*-piran-2-ona e acetato de *rel*-(7*R*,8*R*)-8-[(*E*)-3-hidroxi-3-metil-1-butenil]-4,8-dimetoxi-5,6,7,8-tetraidrofuro[2,3-*b*]quinolin-7-ila; e dos alcalóides arborinina, *N*-metil-1-hidroxi-3-metoxiacridona, esquimianina, cocusagina, isodutaduprina, isoesquimianina e isococusagina. Através da análise dos dados espectroscópicos foram estabelecidas as estruturas químicas das substâncias isoladas sendo que para os alcalóides inéditos tais dados são descritos pela primeira vez. Além disso, os ensaios bilógicos sobre as formas tripomastigotas do *Trypanosoma cruzi* das substâncias isoladas mostraram que elas possuem atividade tripanocida moderada.

The investigation of the ethyl acetate fraction of methanol extract from leaves of *Almeidea rubra* A. St.-Hil. (Rutaceae) afforded two new compounds 4-methoxy-6-[2-(methylamino)phenyl]-2*H*-pyran-2-one and *rel*-(7*R*,8*R*)-8-[(*E*)-3-hydroxy-3-methyl-1-butenyl]-4,8-dimethoxy-5,6,7,8-tetrahydrofuro[2,3-*b*]quinoline-7-yl acetate, along with the known compounds arborinine, *N*-methyl-1-hydroxy-3-methoxyacridone, skimmianine, kokusagine, isodutaduprine, isoskimmianine, and isokokusagine. Their structures were established based on their spectral data, and for the new compounds these data are described herein. Additionally, these compounds were assayed on the tripomastigote forms of *Trypanosoma cruzi* showing moderate trypanocidal activity.

Keywords: *Almeidea rubra*, Rutaceae, alkaloids, trypanocidal activity

Introduction

The genus *Almeidea* belongs to Rutaceae family and is widely distributed in Brazil. Data about the secondary metabolites of this genus are available. From *Almeidea guyanensis* was isolated *C*-arabinosyl flavones, 2- and 4-quinolones, and flavone *C*-glycosides.[1] We have previously reported the isolation of triterpenoids, acridone, 2-quinolone, and furoquinoline alkaloids, and a new tetrahydrofuroquinoline alkaloid from *A. coeruela* and *A. rubra*[2] besides steroids, one sesquiterpene, one cromone, and one flavone.[3]

Chagas' disease (American Trypanosomiasis) is caused by the flagellate protozoan *Trypanosoma cruzi*, affecting more than 18 million people in Latin America.[4] Its treatment is still a challenge and new less toxic and more effective drugs are necessary.

As a part of our search for new trypanocidal compounds and our studies on the chemistry of Rutaceae, a new specimen of *Almeidea rubra* A. St.-Hil. was investigated. Thus, here we report the isolation, identification and characterization of two new substances (**1** and **2a**) and seven known compounds (**3**-**9**) from methanol extract of leaves of this plant, in addition to their trypanocidal activities.

Results and Discussion

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methanol extract from leaves of *A. rubra* afforded two new compounds (**1** and **2a**) and known compounds (Figure 1) arborinine (**3**), *N*-methyl-1-hydroxy-3-methoxyacridone (**4**),[5] skimmianine (**5**),[6] kokusagine (**6**),[7] isodutaduprine (**7**),[8] isoskimmianine (**8**), and isokokusagine (**9**), which were identified through comparison of their NMR spectral data with the literature.

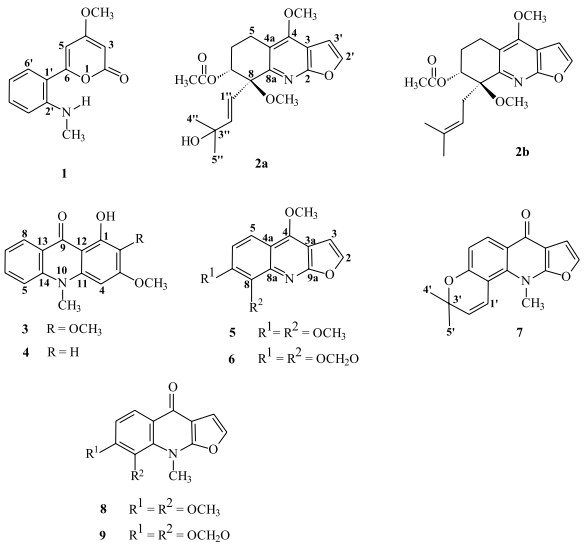

Figure 1. Compounds isolated from *Almeidea rubra*.

Compound **1** was isolated as a yellow powder and its structure was determined by analysis of NMR and LRMS.

The ^{1}H NMR spectrum of **1** showed aromatic protons at δ 7.33 (2H, m), and 6.70 (2H, m), assigned to H-4', H-6', and H-3', H5', respectively. Two olefinic protons (H-3 and H-5) appeared at δ 5.49, and 6.23 (d, *J* 2.2 Hz) assigned to an 2-pyrone by comparison with a styryl-2-pyrone described by Pizzolatti *et al*.,[9] besides broad *N*-H and *N*-CH_3 signals at δ 5.36, and 2.87. Furthermore, a singlet at δ 3.85 (3H) revealed the presence of the methoxyl group in the molecule.

The ^{13}C NMR spectrum of **1** showed 13 carb distinguished as 5 quaternaries (δ 171.7, 164.0, 16 147.6, 116.0), 4 aromatics (δ 132.3, 129.2, 116.4, 11 2 ofelinics (δ 100.1, 87.3), 1 methoxyl (δ 55.9), and methyl carbon (δ 30.4). The analysis of ^{13}C NMR HSQC spectrum allowed us to attribute undoubtedl chemical shifts to methyl, vinyl, and aromatic carb hydrogens of **1** (Table 1).

The assignments of quaternary carbons were poss from observed correlations in the HMBC spectrum. chemical shift of C-2' was deduced from ^3J correla between *N*-CH_3 hydrogens (δ 2.87) and one carbon 147.6. C-4 (δ 171.7) was assigned through ^3J correla between the lowerfield carbon and methoxyl hydro (δ 3.85). Furthermore, C-6 (δ 162.8) and C-1' (δ 11 were determined through ^2J-^3J correlation to H-5 (δ 6 Finally, through the ^2J correlation between H-3 (δ 5 and a carbonyl carbon at δ 164.0 was attributed C-2.

All these results allowed us to propose the structu methoxy-6-[2-(methylamino)phenyl]-2*H*-pyran-2-one compound **1**, which is described for the first time in literature and represents a new class of compounds for by the reaction of anthranilic acid and two acetyl/malo CoA units.

Single crystal X-ray diffraction establis undoubtedly the structure of **1** as a derivative of anthra acid (Figure 2).

The crystal structure of compound **1** shows the pres of two independent molecules per asymmetric unit (Fi 2). The main conformational difference between independent molecules is centered in the dihedral a between the least square planes that pass through aromatic rings in each molecule. The value of this ang molecule 1 is 33.58(8)° and in molecule 2 is 34.79 The crystal structure also shows the presence of intramolecular interaction in each molecule. Th interactions are slightly different due to the conforma

Table 1. NMR spectral data ($CDCl_3$, 9.8 T) of substance **1** isolated from *Almeidea rubra*

	HSQC		HMBC	
Carbon	δ_C	δ_H ($^1J_{C\text{-}H}$)	$^2J_{C\text{-}H}$	$^3J_{C\text{-}H}$
2	164.0			
3	87.3	5.49 d (*J* 2.2 Hz)	171.7, 164.0	100.1
4	171.7			
5	100.1	6.23 d (*J* 2.2 Hz)	171.7, 162.8	116.0, 87.3
6	162.8			
1'	116.0			
2'	147.6			
3' and 5'	116.4, 111.2	6.70 m	147.6, 132.3, 129.2	116.0
4' and 6'	132.3, 129.2	7.33 m		162.8, 147.6
4-OCH_3	55.9	3.85 s		171.7
2'-*N*CH_3	30.4	2.87 s		147.6
2'-*NH*		5.36 br s		

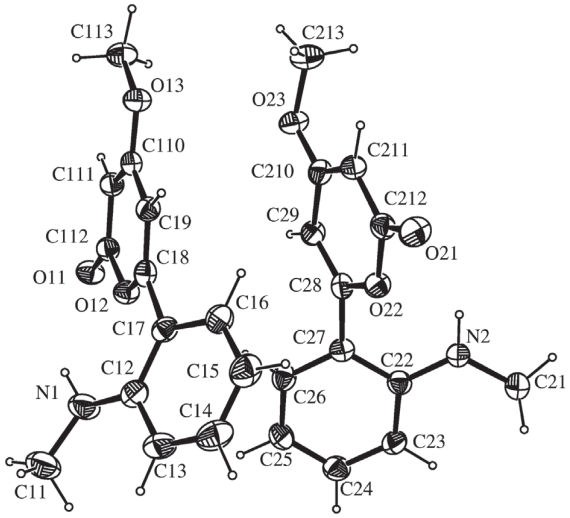

Figure 2. ORTEP-3 diagram of compound **1**.

changes. The analyses of the crystal packing show the presence of four intermolecular interactions of the type C-H…O that give rise to the formation of chains along the (101) direction. These chains are connected with the chain just above and below it through three intermolecular interactions of the N-H…O type.

The quinoline and acridine alkaloids, which are widely distributed in Rutaceae, arise from combination of anthranilic acid and acetate/malonate.[10] In these cases, the ciclization takes place by nucleophilic attack of nitrogen atom to the carboxyl carbon. However, compound **1** should be formed by the nucleophilic attack of the oxygen atom to the carboxyl carbon, giving rise an α-pyrone.

We have previously reported the isolation of the new tetrahydrofuroquinoline alkaloid **2b** (Figure 1) from *A. coerulea*.[2] Reinvestigation of *A. rubra* afforded an alkaloid **2a** whose spectral data seem to be very similar to those of **2b**. This alkaloid was isolated as a white powder and identified by NMR and LRMS. The molecular formula $C_{20}H_{25}NO_6$ of **2a** was defined by elemental analysis.

In the ^{1}H NMR spectrum of **2a** were observed a pair of doublets (J 2.6 Hz) at δ 7.58 and 6.97, and a singlet at δ 4.30, which indicate that **2a** was a tetrahydrofuroquinoline alkaloid. The signals at δ 2.78, 2.20, and 2.01, assigned to H-5, and H-6, respectively, confirmed that **2a** had a reduced furoquinoline ring.

^{1}H NMR spectrum of **2a** also showed two singlets at δ 3.14, and 2.03, and a group of signals at δ 6.06 (d, J 16.1 Hz), 5.69 (d, J 16.1 Hz), 1.34 (s), and 1.31 (s), which indicated the presence of the 3-hydroxy-2-isopentenyl substituent. Through the spectral data comparison, it was established that **2a** distinguished from **2b** by the presence of 3-hydroxy-2-isopentenyl substituent instead of γ,γ-dimethylallyl moiety.

Analysis of HSQC correlations and literature data[2] allowed us to attribute undoubtedly chemical shifts to methyl, methylene, vinyl, furanyl hydrogen/carbon of **2a** (Table 2).

The assignments of quaternary carbons of **2a** were made from observed correlations in the HMBC spectrum. The chemical shift of C-4 was deduced from ^3J correlation between methoxyl hydrogens at C-4 (δ 4.30) and one carbon at δ 158.2. The correlation of the other methoxyl hydrogens (δ 3.14) and one carbon at δ 79.7 determined

Table 2. NMR spectral data ($CDCl_3$, 9.8 T) of alkaloid **2a** isolated from *Almeidea rubra*

		HSQC	HMBC	
Carbon	δ_C	δ_H ($^1J_{C\text{-}H}$)	$^2J_{C\text{-}H}$	$^3J_{C\text{-}H}$
2	162.7			
3	105.2			
4	158.2			
4a	117.6			
5	19.5	2.78 m	117.6, 23.5	158.2, 150.0, 72.6
6	23.5	2.20, 2.01 m	72.6, 19.5	117.6, 79.7
7	72.6	5.43 dd (J 8.5, 2.9 Hz)	79.7	170.3, 126.9, 19.5
8	79.7			
8a	150.0			
2'	142.9	7.58 d (J 2.6 Hz)		162.7, 105.2
3'	104.6	6.97 d (J 2.6 Hz)	142.9, 105.2	162.7
1"	126.9	6.06 d (J 16.1 Hz)		70.8
2"	141.1	5.69 d (J 16.1 Hz)	70.8	79.7, 29.6, 29.5
3"	70.8			
4" and 5"	29.6, 29.5	1.34, 1.31 s	70.8	141.1
4-OCH_3	58.5	4.30 s		158.2
8-OCH_3	51.3	3.14 s		79.7
-$OO\underline{C}CH_3$	170.3			
-$OOCCH_3$	21.2	2.03 s		170.3

C-8. C-4a was assigned through correlation between the carbon at δ 117.6 and H-5, and H-6 (δ 2.78, and 2.01). H-5 also correlated to C-8a (δ 150.0). Furthermore, methyl hydrogens at δ 1.34, and 1.31, correlated with an oxygenated carbon at δ 70.8, establishing C-3". Finally, the correlation between furanyl hydrogens (δ 7.58, and 6.97) and quaternary carbons at δ 162.7, and 105.2 determined C-2, and C-3, respectively.

The close similarity between the chemical shifts observed for carbons of reduced furoquinoline ring when compared with the model compound 7-*O*-acetylhaplophyllidine (**2b**)[2] led us to suggest a *trans* relationship between the 7-acetoxy and 8-methoxy groups. Alkaloid **2a**, *rel*-(7*R*,8*R*)-8-[(*E*)-3-hydroxy-3-methyl-1-butenyl]-4,8-dimethoxy-5,6,7,8-tetrahydrofuro [2,3-*b*]quinoline-7-yl acetate, is described for the first time in the literature, and the occurrence of tetrahydrofuroquinoline alkaloid such as **2a** and **2b** seems to be restricted to the genera *Almeidea* and *Haplophyllum* of Rutaceae family.[2,11]

In order to improve the therapy of Chagas disease, we have been studying species of the order Rutales (Rutaceae, Meliaceae, Simaroubaceae, Burseraceae, and Cneoraceae) aiming to isolate hit compounds that could be used in the development of new antichagasic drugs.

Compounds described in this paper were isolated from an active fraction of *A. rubra* (62.9% lysis – percent reduction of the parasite number) on the tripomastigote forms of *T. cruzi*.[12] Their *in vitro* trypanocidal actions are shown on Table 3. Three of them (**7**-**9**) could not be assayed due to their instability.

Table 3. Trypanocidal activity of compounds isolated from *Almeidea rubra*

Compound	Concentration (μg/mL) x Lysis %			IC_{50}
	500	250	100	(mmol/L)
1	74.10	45.08	27.23	1.271
2a	58.93	44.20	28.12	0.977
3	57.99	42.39	24.00	1.231
4	45.60	43.20	31.20	2.600
5	65.17	30.35	25.44	1.455
6	58.38	57.13	46.58	0.5596

The results showed that all of compounds had moderate trypanocidal actions and kokusagine (**6**) was the most active one. Probably, they are responsible by the activity of the *A. rubra* fraction.[12] However, they could not be considered good prototype for the development of new antichagasic drugs, mainly when their activities are compared with crystal violet and some lignans also isolated from other Rutaceae.[13]

Experimental

General experimental procedures

The ^{1}H NMR, ^{13}C NMR and 2D correlation spectra w
obtained in $CDCl_3$ using either Bruker DRX-200 and A
400 NMR spectrometers, and using tetramethylsi
(TMS) as internal standard.

Low resolution EIMS were recorded on GC-1
GCMS-QP5000 Shimadzu.

Plant material

The leaves of *Almeidea rubra* A. St.-Hil. were colle
in May 2000 in Espírito Santo State, southeast of Br
The plant material was identified by Dr. José Rubens P
from the Department of Botany, University of São P
(Brazil) and the voucher herbarium specimen (Pirani *e*
4746) was deposited at the Herbarium of that Departm

Extraction and isolation

Dried leaves (313.5 g) were extracted first with hex
and then with MeOH at room temp. Part of the meth
extract (15.9 g) was submitted to VLC on silica gel 60
230 mesh) using a hexane-CH_2Cl_2-EtOAc-MeOH grad
to yield the corresponding fractions (ALFMH, ALF
ALFMA, ALFMM). The fraction ALFMA (3.3208 g)
submitted to column chromatography over silica (s
gel 230-400 mesh, 410 x 43 mm i.d., stepwise wi
hexane-EtOAc-MeOH gradient) to give eleven fracti

The second fraction (ALFMA2; 0.6302 g)
chromatographed on silica gel column (230-400 m
358 x 31 mm i.d.) and eluted with solvents of increa
polarity (hexane:AcOEt 8:2→MeOH) to afford
fractions. Through column chromatography over sepha
LH-20 (492 x 32 mm i.d., eluted with CH_2Cl_2:MeOH
from the fraction five (ALFMA2,5; 103.6 mg) was obta
four fractions. Then, the second fraction (ALFMA2
68.0 mg) was subjected to column chromatography
silica gel (230-400 mesh, 394 x 32 mm i.d., stepwise
a CH_2Cl_2→MeOH gradient) to give four fractions. The
fraction (ALFMA2,5,2,3; 36.0 mg) was chromatograp
on sephadex LH-20 column (492 x 32 mm i.d.) and el
with CH_2Cl_2:MeOH (1:1) to afford six fractions. Thro
column chromatography over silica gel (230-400 m
394 x 32 mm i.d., stepwise with a CH_2Cl_2→MeOH grad
of the fraction five (ALFMA2,5,2,3,5; 14.3 mg) was isol
the alkaloid **2a** (2.6 mg).

Fraction four (ALFMA4; 178.1 mg) was chro
tographed on sephadex LH-20 column (492 x 32 mm

and eluted with MeOH to afford five fractions. Through column chromatography over sephadex LH-20 (492 x 32 mm i.d., eluted with CH_2Cl_2:MeOH 1:1) of the third fraction (ALFMA4,3; 59.9 mg) was obtained the alkaloid **5** (ALFMA4,3,7; 2.5 mg) and eight fractions. Then, the fourth fraction (ALFMA4,3,4; 2.3 mg) was subjected to column chromatography over sephadex LH-20 (332 x 25 mm i.d., eluted with CH_2Cl_2:MeOH 1:1) to give three fractions. Further purification of the fraction two (ALFMA4,3,4,2; 2.0 mg) on preparative planar silica plate with hexane:EtOAc (8:2) afforded alkaloid **6** (1.6 mg). Through column chromatography over sephadex LH-20 (492 x 32 mm i.d., eluted with CH_2Cl_2:MeOH) of the fraction five (ALFMA4,3,5; 21.5 mg) was isolated the compound **1** (14.7 mg).

The alkaloids arborinine (**3**) (43.2 mg), isodutaduprine (**7**) (25.6 mg), and isoskimmianine (**8**) (28.8 mg) were crystallized in MeOH from fraction six (ALFMA6; 104.5 mg), seven (ALFMA7; 317.3 mg) and nine (ALFMA9; 120.5 mg), respectively.

The other alkaloid isokokusagine (**9**) (32.1 mg) was purified on sephadex LH-20 (492 x 32 mm i.d., eluted with MeOH) from fraction eight (ALFMA8; 90.3 mg), and the alkaloid **4** (3.2 mg) was obtained from fraction three (148.8 mg).

*4-methoxy-6-[2-(methylamino)phenyl]-2H-pyran-2-one (**1**)*

Yellow powder; elemental analysis (Found: C, 68.0432; H, 5.565904; N, 6.11885. Calc. for $C_{13}H_{13}NO_3$: C, 67.53; H, 5.63; N, 6.06%); UV λ_{max}/nm (CH_2Cl_2): 240, 250, 283, 303, 332, 371; IR ν_{max}/cm^{-1}: 2920, 2855, 1719, 1630, 1557, 1401, 1010 (film); ^{1}H NMR ($CDCl_3$, 400 MHz): Table 1; ^{13}C NMR ($CDCl_3$, 100 MHz): Table 1; EIMS *m/z*: 231, 200, 199, 104, 77.

*Rel-(7R,8R)-8-[(E)-3-hydroxy-3-methyl-1-butenyl]-4,8-dimethoxy-5,6,7,8-tetrahydrofuro[2,3-b]quinoline-7-yl acetate (**2a**)*

White powder; $[\alpha]_D^{25}$ = - 8.7° (*c* 0.15, CH_2Cl_2); elemental analysis (Found: C, 61.76958; H, 7.457999; N, 4.316954. Calc. for $C_{20}H_{25}NO_6$: C, 64.00; H, 6.67; N, 3.73%); IR ν_{max}/cm^{-1}: 2922, 2855, 1741, 1579, 1371, 1327, 1244, 1093, 1017 (film); ^{1}H NMR ($CDCl_3$, 400 MHz): Table 2; ^{13}C NMR ($CDCl_3$, 100 MHz): Table 2; EIMS *m/z*: 226, 266, 326.

Single crystal X-ray analysis

Low temperature X-ray diffraction data collection was performed at 120(2) K, on an Enraf-Nonius Kappa-CCD diffractometer equipped with an Oxford Cryosystem liquid N_2 device, using graphite-monochromated MoKα radiation (0.71073 Å). Data were collected up to 50° in 2θ, with a redundancy of 4. The final unit cell parameters were based on all reflections. Data collections were made using the COLLECT program;[14] integration and scaling of the reflections were performed with the HKL Denzo-Scalepack system of programs.[15] No absorption corrections were applied.

The structure was solved by direct methods with SHELXS-97.[16] The model was refined by full-matrix least squares on F^2 with SHELXL-97.[17] All the hydrogen atoms were set isotropic and freely refined. The programs SHELXL-97,[17] and ORTEP-3[18] were used within WinGX[19] to prepare materials for publication. Atomic coordinates, bond lengths and angles, and thermal parameters have been deposited at the Cambridge Crystallographic Data Centre.

The supplementary crystallographic data have been sent in electronic format to the Cambridge Crystallographic Data Centre, as CIF file No. CCDC 242119. These can be obtained obtained free of charge by contacting the CCDC (12 Union Road, Cambridge CB2 1EZ, UK; fax: +44 1223 336033; www.ccdc.cam.ac.uk/conts/retrieving.html; deposit@ccdc.cam.ac.uk).

Trypanocidal activity in vitro

As described by Ambrozin *et al.*,[12] the bioassays were carried out using blood of infected Swiss albino mice, which was collected by cardiac puncture at the peak of parasitemic infection (7th day of infection for Y strain of *T. cruzi*). The infected blood was diluted with healthy mice blood to achieve a concentration of 2.10^6 forms/mL. Solutions of the compounds were prepared by dissolving in dimethylsulfoxide (DMSO). The activities of substances were evaluated at 500, 250, and 100 μg/mL.

The bioassays were performed in triplicate on titration microplates (96 wells) which contained 400 μL mixture/well. The plates were incubated at 4 °C, and the number of parasites counted after 24 h, following the method described by Brener.[20]

Infected blood with the same volume of DMSO was used as control, and gentian violet to a concentration of 250 μg/mL was used as positive control.

The activity is expressed as percent reduction of the parasite number (lysis) and IC_{50} values were calculated using the program GraphPad Prims v.3.0.

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