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Three anthrones from *Rubus ulmifolius*

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Abstract

From the aerial parts of *Rubus ulmifolius* Schott three new anthrones, rubanthrone A, B and C, have been isolated. Their structures were established by spectral procedures including 1D and 2D NMR techniques and chemical derivatization. Rubanthrone A showed antimicrobial activity against *Staphylococcus aureus* at 4.5 mg/ml. © 2002 Elsevier Science Ltd. All rights reserved.

Keywords: *Rubus ulmifolius*; Rosaceae; Blackberry; Anthrones; Antimicrobial activity

1. Introduction

Rubus ulmifolius Schott, is a perennial shrub distributed in Italy from the sea level to 1100 m (Pignatti, 1982). Fresh bruised leaves are used in Italian folk medicine for abscesses, furuncles and ulcers; decoctions of leaves are used externally for redden eyes, vaginal lavages and aphta and internally for diarrhea, hemorrhoids, and intestinal inflammations (Uncini Manganelli and Tomei, 1999).

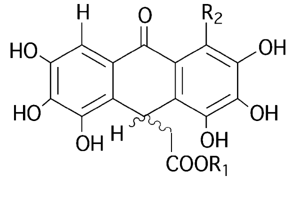
In Chilean folk medicine *R. ulmifolius* is used for its hypoglycaemic activity experimentally demonstrated on rats (Lemus et al., 1999).

Previous chemical studies of aerial parts of this species reported flavonoids such as quercetin and kaempferol; moreover caffeic and chlorogenic acids and saturated and unsatured fatty acids were isolated (Tzouwara-Karayanni and Philianos, 1981). Recently we have also identified, in the same plant, ursane and oleanane triterpenes and flavonoid glycosides and we have evaluated the antimicrobial activity of some pure compounds, isolated from active fractions, obtaining promising results on several microorganisms and particularly on *Staphylococcus aureus*, a multidrug resistant bacterium (Panizzi et al., 2002).

We now report the isolation and identification of three new anthrones: rubanthrones A, B and C and the antimicrobial activity against *Staphylococcus aureus* of rubanthrone A.

2. Results and discussion

Rubanthrone A (**1**) was obtained as a yellow precipitate. Its FABMS spectrum gave a quasimolecular $[M–H]^-$peak at 377 m/z corresponding to the molecular formula $C_{17}H_{14}O_{10}$, supported also by elemental analysis (see Experimental); another significant ion at 304 m/z showed the loss of a $-C_3H_5O_2$ fragment.



Rubanthrone A (**1**) : R_1=CH_3 R_2=OH
Rubanthrone B (**2**) : R_1=CH_3 R_2=H
Rubanthrone C (**3**) : R_1=H R_2=OH

The ^{1}H NMR spectrum showed a one proton singlet for the presence of an aromatic hydrogen at δ 7.29 (H-8), a second singlet, integrating for three protons, due to the presence of a methoxyl group at δ 3.61 (at position 2′) and signals of an AB_2 system (confirmed by COSY) at δ 2.42 (1H,*dd*, $J_{vic}=1.9$ Hz, $J_{gem}=19$ Hz, H-1′a), 2.97 (1H, *dd*, $J_{vic}=7$ Hz, $J_{gem}=19$ Hz, H-1′b), and 4.40 (1H, *dd*, $J_{vic}=1.9$ Hz, $J_{vic}=7$ Hz, H-10) respectively. The hydrogen bearing carbons C-8 at δ 108.2, C-10 at δ 40.9, C-1′ at δ 37.1 and the $-OCH_3$ group at δ 52.1 were assigned by HETCOR correlations.

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The position of the aromatic hydrogen was established by a combination of HETCOR, COLOC and NOESY experiments conducted on **1** and on the permethylated compound **1a** as shown in Figs. 1 and 2. A further confirmation came from literature data (El-Gamal et al., 1996; Ming-Tyan and Chiu-Ming, 1985).

The ^{13}C NMR spectrum displayed signals at δ 193.0 and δ 172.6 for the C-9 carbonyl group and the C-2′ methylester carbonyl group, the presence of a methyl ester function in this position was evidenced by COLOC correlations of the CH_3 to the carbonyl and the methylene group. The signal at δ 160.1 was attributed to the carbinolic group on C-7 on the basis of COLOC and NOESY experiments after methylation. The COLOC spectrum allowed assignments of the following carbons: the methine C-8 and the quaternary C-1a at δ 108.2, the methoxyl group at δ 52.1, the benzylic C-10 at δ 40.3, the aliphatic methylene C-1′ group at δ 37.0 and the other quaternary carbon C-8a at δ 115.0. The remaining quaternary carbons were attributed on the basis of literature data for similar compounds (El-Gamal et al., 1996; Habib et al., 1987).

Rubanthrone B (**2**) appeared on TLC as a yellow spot after treatment with Naturstoffreagenz A-PEG.

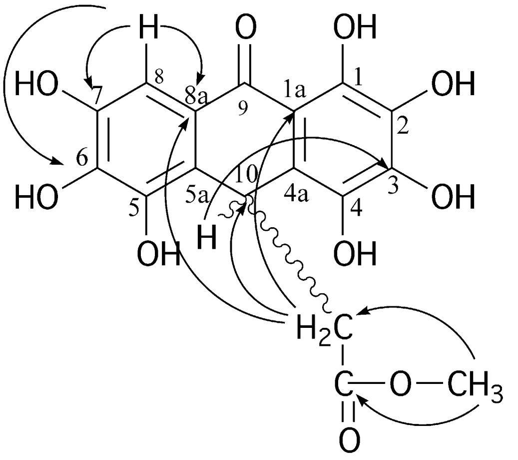

Fig. 1. COLOC experiment of rubanthrone A.

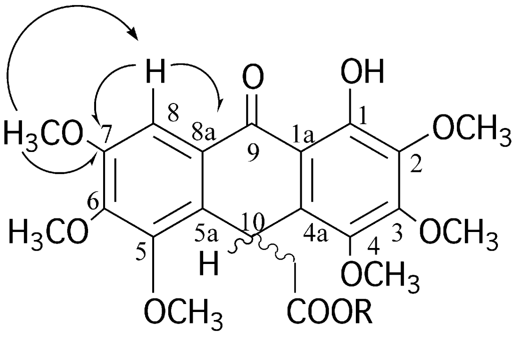

Fig. 2. Significant COLOC (⇀) and NOESY (→) correlations in permethylated rubanthrones A (**1a**) (R = OCH_3) and C (**3a**) (R = OH).

Its FABMS spectrum gave a quasimolecular $[M-H]^-$ peak at 361 *m/z* corresponding to the molecular formula $C_{17}H_{14}O_9$, supported also by elemental analysis (see Experimental). The ^1H NMR spectrum showed one two-protons singlet at δ 7.25 assignable to H-1 and H-8. The signals present in the DEPT spectrum confirmed the methine nature of this signal protons (δ 108.5) and showed again the signals (δ 41.6 and 51.4) due to the benzylic CH carbon and the $-OCH_3$ group respectively and one CH_2 signal (δ 37.3) for the aliphatic C-1′ carbon.

The NOESY experiment of the permethylated compound (**2a**) showed only an unique correlation between the most deshieleded $-OCH_3$ groups (linked to C-2/7 at δ 4.00) and the aromatic hydrogens (one two proton singlet at δ 7.25), so we could exclude the possibility for the aromatic hydrogens to be in position 7 or 6 (in this case correlations with two $-OCH_3$ groups should have been visible), moreover the presence in the COLOC experiment of a correlation between H-8/H-1 and C-8a/C-1a finally leaves out positions 5 and 4, confirming the presence of the hydrogens on positions 1 and 8.

COLOC experiments supported the structure of **2** which was analogous to that of **1** except for the presence of an aromatic hydrogen instead of an OH on the C-1 carbon.

This structure was supported also by the negative FABMS spectrum, where, besides the quasimolecular peak at 361 *m/z* $[M-H]^-$, the peak due to the loss of the $-CH_2COOCH_3$ side chain at 288 *m/z* $[M-73]^-$could be observed.

Rubanthrone C (**3**) appeared on TLC as a yellow spot after treatment with Naturstoffreagenz A-PEG.

Its FABMS spectrum gave a quasimolecular peak $[M-H]^-$at 363 *m/z* corresponding to the molecular formula $C_{16}H_{12}O_{10}$, supported also by elemental analysis (see Experimental).

The ^1H NMR spectrum showed a signal for an aromatic proton at δ 7.20, a signal for a benzylic proton at δ4.35 (1H, *br dd*, H-10), and one signal, integrating for two hydrogens, at δ 2.70 (2H, *m*, H-1′a and H-1′b).

Their carbons were assigned by HETCOR at δ 140.0, δ 41.6, δ 37.3 respectively.

The comparison of the ^{13}C NMR spectra of **1** and **3**, led us to the formulation of a structure identical to **1** but with a free carboxylic group instead of a methylester in the side chain.

The COLOC and NOESY experiments of the permethylated compound (**3a**) clearly established the substitution pattern of the aromatic hydrogen and the methoxy (OH) groups in **3a** (Fig. 2).

The side chain structure was confirmed by the negative FABMS spectrum, where besides the quasimolecular peak at 363 *m/z* $[M-H]^-$there was also the peak due to the loss of a $-CH_2COOH$ fragment at 304 *m/z* $[M-H-59]^-$.

In order to prove that rubanthrones A and B were not artifacts, rubanthrone C, the only one having a free carboxylic group in the side chain, was submitted again to an extraction/separation procedure analogous to that adopted during our plant investigation. It was macerated with methanol for two weeks and then subjected to a Sephadex LH-20 column chromatography eluting with methanol; its NMR spectra evidenced again the presence of the free carboxylic moiety leading to the conclusion that the methylated compounds were not artifacts.

The anthrones present in *Rubus ulmifolius* have never been previously isolated in nature, infact they are hexa or heptaoxygenated and have a methylencarboxylic side chain while all the known anthrones show three or four hydroxylic groups at most and can be linked to sugars and/or prenylic or geranylic side chains; moreover such molecules are commonly present in the families of Leguminosae, Rhamnaceae, Polygonaceae, Liliaceae, Simaroubaceae, Asphodelaceae, Guttiferae and Ochnaceae but they have never been found before in the Rosaceae family to which *Rubus ulmifolius* belongs.

2.1. Antimicrobial activity

Rubanthrone A, isolated in sufficient amount to be tested, was submitted to antimicrobial evaluation against *Staphylococcus aureus;* since the halo diameter of the inhibition zone surrounding the well was 17 mm we can say that it has a moderate activity. Its MIC value resulted 4.5 mg/ml (no reference compound was tested).

3. Experimental

3.1. General

FABMS were recorded in the negative mode with a VG ZAB instrument; ^{1}H and ^{13}C NMR spectra were obtained with a Bruker AC200 spectrometer in DMSO-d_6 using TMS as internal standard. All 1D and 2D NMR experiments were performed using the standard Bruker library of microprograms. The following adsorbents were used for purification: low-pressure chromatography, Merck Lobar Lichroprep RP-8 (31×2.5 cm); size-exclusion chromatography, Pharmacia Fine Chemicals Sephadex LH-20; analytical TLC, Merck Kieselgel 60 F_{254} precoated plates; chromatograms were visualized under UV light at 254 and 366 nm and/or sprayed with Naturstoffreagenz A-PEG.

3.2. Plant material

Leaves, branches and flowering tops were collected at the sea level in Asciano (Pisa, Italy) in July 1998. A voucher specimen is deposited in PI (Herbarium of the University of Pisa).

3.3. Extraction and isolation

The dried pulverized plant (2.00 kg) was successively extracted using a soxhlet apparatus with *n*-hexane, chloroform, chloroform/methanol 9:1 and then macerated with methanol at room temperature for 2 weeks.

The methanolic extract was successively partitioned with water saturated AcOEt and *n*-BuOH. Sephadex LH-20 column chromatography of the AcOEt residue, by elution with MeOH/H_2O (4:1), afforded anthrone A (80 mg), as a pale yellow powder. Sephadex LH-20 column chromatography of the *n*-BuOH fraction by elution with MeOH/H_2O (4:1), afforded 30 fractions. Fractions 18 and 19 were separately subjected to Lobar RP-8 column chromatography eluting with MeOH/H_2O (3.5:6.5) to give anthrones B (10 mg) and C (10 mg).

Rubanthrone A (**1**): $[\alpha]_D^{20}$ +6.1° (MeOH, *c* 0.09); UV λ_{max} MeOH nm: 278, 350 (*sh*), 365; IR KBr ν_{max} MeOH 1633 cm^{-1} (C=O), 1730 (ester CO), 3500 (OH) FABMS (negative mode) *m*/*z* 377 [M–H]$^-$, 304 [M–H-73]$^-$; elemental analysis: found: C 53.98%, H 3.73%, requires: C 54.12%, H 3.60%; ^{1}H NMR see Table 1, ^{13}C NMR see Table 2.

Rubanthrone B (**2**): UV λ_{max} MeOH nm: 280, 353, 367; IR KBr ν_{max} MeOH 1654 cm^{-1} (C=O), 1742 (ester CO), 3500 (OH); FABMS (negative mode) *m*/*z* 361 [M–H]$^-$, 288 [M–H-73]$^-$; elemental analysis: found: C 56.36%, H 3.9%, requires: C 56.5%, H 3.77%.^{1}H NMR see Table 1, ^{13}C NMR see Table 2.

Rubanthrone C (**3**): $[\alpha]_D^{20}$ +2.0° (MeOH, *c* 0.05); UV λ_{max} MeOH nm: 281, 350, 360 (*sh*); IR KBr ν_{max} MeOH 1654 cm^{-1} (C=O), 1710 (carboxylic CO), 3500 (OH); FABMS (negative mode) *m*/*z* 363 [M–H]$^-$, 304 [M–H-59]$^-$; elemental analysis: found: C 57.76%, H 3.32%,

Table 1
^{1}H NMR data (200 MHz) of compounds **1**–**3** (DMSO-d_6)

	1	**2**	**3**
H-1	–	7.25 *s*	–
H-8	7.29 *s*	7.25 *s*	7.20 *s*
H-10	4.40 *dd* (1.9, 7)	4.34 *dd* (7.8, 1.8)	4.35 *br dd*
H-1′a	2.42 *dd* (1.9, 19)	Submerged by the solvent signal	2.70 *m*
H-1′b	2.97 *dd* (7, 19)	2.91 *dd* (7.8, 19)	2.70 *m*
−OCH3	3.61 *s*	3.73 *s*	–

Table 2
^{13}C NMR data (50 MHz) of compounds **1**–**3** (DMSO-d_6)

C	δ	δ	δ	Multipliciy DEPT*		
	1	**2**	**3**	**1**	**2**	**3**
1	138.4[c]	108.5	140.0[d]	C	CH	C
2	143.5[g]	160.4	144.2[h]	C	C	C
3	149.6	145.5	148.9[i]	C	C	C
4	138.4[c]	139.6	141.5[h]	C	C	C
5	145.9[g]	139.6	145.6[i]	C	C	C
6	140.2[g]	145.5	140.0[d]	C	C	C
7	160.1	160.4	160.6	C	C	C
8	108.2[a]	108.5	108.0[e]	CH	CH	CH
9	193.0	193.8	194.1	C	C	C
10	40.3	41.6	41.6	CH	CH	CH
1a	108.2[a]	115.2	108.0[e]	C	C	C
4a	113.1[b]	112.9	112.8[f]	C	C	C
5a	113.1[b]	112.9	112.8[f]	C	C	C
8a	115.0	115.2	115.3	C	C	C
1′	37.1	37.3	37.3	CH_2	CH_2	CH_2
$COOCH_3$	172.6	173.0	–	C	C	–
COOH	–	–	173.1	–	–	C
$-OCH_3$	52.1	51.5	–	CH_3	CH_3	–

[a] Overlapping between same letters.

[b] Overlapping between same letters.

[c] Overlapping between same letters.

[d] Overlapping between same letters.

[e] Overlapping between same letters.

[f] Overlapping between same letters.

[g] Interchangeable between same letters. *Multiplicity from DEPT ^{13}C NMR experiment.

[h] Interchangeable between same letters. *Multiplicity from DEPT ^{13}C NMR experiment.

[i] Interchangeable between same letters. *Multiplicity from DEPT ^{13}C NMR experiment.

requires: C 57.9%, H 3.19%. ^1H NMR see Table 1, ^{13}C NMR see Table 2.

3.4. Permethylation of compounds 1–3

Compounds **1**, **2** and **3** were separately dissolved in 2 ml THF (10 mg each), immerged in an ice-bath and stirred continuosly. An excess of ethereal diazomethane (approximately 3 ml of a 0.332 mol/l ethereal solution) was added to each solution and the reaction stirred for 12 h. After removal of the solvent under reduced pressure, NOESY and COLOC experiments were performed on each residue.

3.5. Antimicrobial screening

Qualitative antimicrobial screening was carried out using the agar-well diffusion assay (Clark et al., 1981) against *Staphylococcus aureus* ATCC 25923. The pure compound was dissolved 1:5 in DMSO, obtaining a solution whose concentration was 5 mg/ml, and 100 μl were placed into 6 mm wells (24 h incubation time at 37 °C). Results of the qualitative screening were recorded as the average diameter of the inhibition zone surrounding the wells containing the test solution. We also evaluated the MIC, without testing any reference compound, according to Eloff (1998).

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