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8-*O*-Azeloyl-14-benzoylaconine: A new alkaloid from the roots of *Aconitum karacolicum* Rapcs and its antiproliferative activities

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Abstract—A new alkaloid of *Aconitum karacolicum* Rapcs, from the Ranunculaceae family, collected in Kirghizstan, was isolated from the roots of this plant, using a purification scheme based upon its in vitro antiproliferative properties against three human tumour cell lines in culture. Structural identification was performed using high resolution MS–MS mass spectrometry and ^{1}H, ^{13}C, 2D NOESY NMR spectroscopy analysis. This compound consists of a 14-benzoylaconine moiety substituted on C-8 by an azeloyl chain. It presents in vitro cytotoxicity with an IC_{50} of about 10–20 μM, which warrants further investigation on its possible interest in cancer chemotherapy.

1. Introduction

Despite the wide variety of currently available anticancer drugs, the research and identification of new natural substances with antiproliferative activity remains a priority. Only a small number of available plants have been studied in this respect and natural biodiversity offers an unlimited field for the discovery of potential anticancer drugs. In addition, numerous plant extracts are currently used in traditional medicine and their active constituents have never been isolated and identified. The present report is based upon a preliminary screening of the medicinal plants growing in Kirghiz Republic. One of them, belonging to the genus *Aconitum*, fam. Ranunculaceae, has been selected for detailed investigation because of its traditional use against cancer in this country for many years.

Aconitum karakolicum Rapcs is a herbaceous perennial plant with a tall leafy stem (70–130 cm) bearing violet or blue zygomorphous flowers on long, packed racemes. The underground part of the plant is represented by cone-shaped tubercles measuring 2.0–2.5 × 0.7–1.0 cm, which grow by sticking to each other side by side. *Aconitum karakolicum* is endemic to Central Asia. All the parts of *Aconitum* species contain alkaloids of the diterpenoid group, the maximum content in alkaloids being found in tubercles after the period of vegetation (August–October).[1] The principal alkaloids identified in this plant are aconitine (0.8–1%), karakoline (0.05%), karakolidine (0.05%), zongorine (0.1%) and zongoramine (0.01%).[2] Napelline, aconifine, acetylnapelline and karakonitine have also been identified.[3] Since none of these compounds bear any antiproliferative activity, whereas plant extracts do, we undertook this work to isolate and to identify the compound(s) responsible for this activity. We propose the structure of 8-*O*-azeloyl-14-benzoylaconine as being responsible for the antiproliferative activity.

Keywords: Medicinal plants; *Aconitum* genus; Anticancer drugs; 8-*O*-Azeloyl-14-benzoylaconine.
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2. Results

2.1. Extraction, purification and cytotoxicity of the extracts

Figure 1 presents the purification procedure which was followed and Table 1 indicates the cytotoxicity of the fractions as determined on the three human tumour cell lines. From 1.5 kg of dried powdered tubercles, we obtained 14.7 g of crude extract. Since it was assumed that the possibly active substances in the extract were alkaloids, we only considered the residue obtained at pH 9–10. This crude ethanol/chloroform extract had an IC_{50} of about 95 μg/mL.

Five fractions were obtained by column chromatography. Only F2 presented a cytotoxic activity, with an IC_{50} of 30–50 μg/mL, while the IC_{50} values of the F1 fraction was >500 μg/mL and those of fractions F3, F4 and F5 were >200 μg/mL. Preparative HPLC of fraction F2 yielded nine subfractions, of which one, F2.9, presented an IC_{50} of 15–30 μg/mL while the others had no evaluable cytotoxicity (IC_{50} > 100 μg/mL). Several fractions prepared from F2.9 presented IC_{50} values of less than 20 μg/ml on all three cell lines (see Table 1). One of them, fraction F2.9.4, with IC_{50} values of 13–19 μg/mL, was further purified to yield a pure compound, as judged by HPLC, with IC_{50} values of 13, 15 and 8 μg/mL on the colon, lung and breast cancer cell

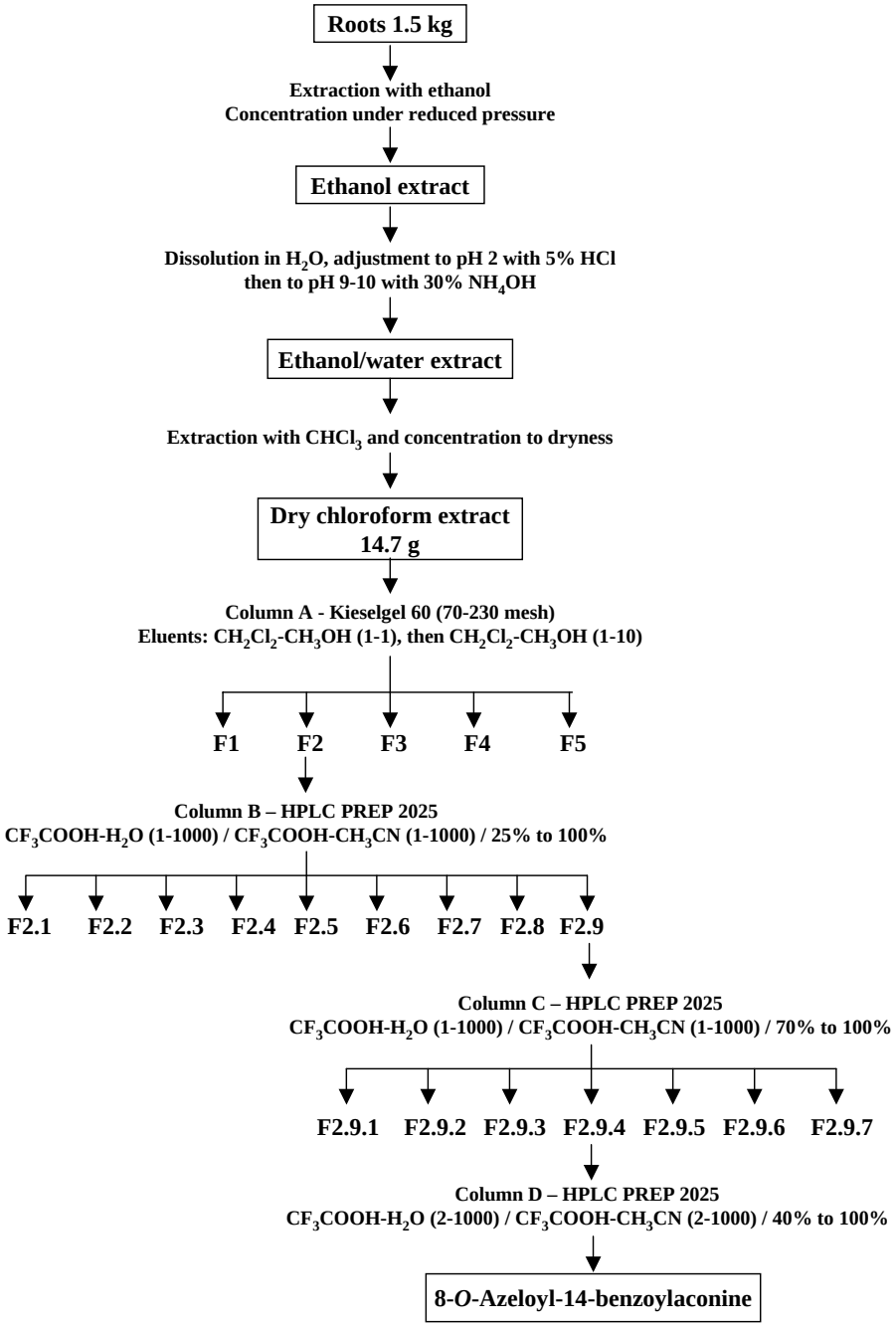

Figure 1. General scheme followed for extraction and purification of active substances from the roots of *Aconitum karakolicum*.

Table 1. The IC_{50} values of the fractions obtained after ethanol/chloroform extraction and column chromatography of *Aconitum karakolicum* tubercles

Fraction	Tumour cell line IC_{50} (μg/mL)		
	HCT-15 (colon)	A549 (lung)	MCF-7 (breast)
F1	>500	>500	>500
F2	**44**	**50**	**30**
F3–F5	>200	>200	>200
F2.1–F2.8	>100	>100	>100
F2.9	**25**	**30**	**15**
F2.9.1	25	29	16
F2.9.2	21	25	17
F2.9.3	19	23	12
F2.9.4	**16**	**19**	**13**
F2.9.5	16	19	12
F2.9.6	18	15	10
F2.9.7	14	18	13
8-*O*-Azeloyl-14-benzoylaconine	**13**	**15**	**8**

Cytotoxicity tests were performed as described in Section 4 on three human solid tumour cell lines.

Table 2. Comparison of the growth inhibitory activities of 8-*O*-Azeloyl-14-benzoylaconine to those of standard anticancer drugs

Cytotoxic agents	Tumour cell line IC_{50} (μM)		
	HCT-15 (colon)	A549 (lung)	MCF-7 (breast)
8-*O*-azeloyl-14-benzoylaconine	16.8	19.4	10.3
5-Fluorouracil	2.11	5.69	1.75
Cisplatin	3.08	7.21	3.01
Etoposide	3.61	17.6	5.73
Melphalan	28.7	39.4	11.1

lines, respectively. A comparison with the cytotoxicities of four major anticancer drugs, as they appear in the database of the Development Therapeutic Program of the National Cancer Institute on the three cell lines[4] is presented in Table 2. It shows that the molar concentrations of the active compound, which have been deduced from the structural identification presented below, is in the range of the active molar concentrations of usual anticancer drugs. The pure compound isolated and purified was then subjected to structural analysis by mass spectrometry and nuclear magnetic resonance spectroscopy.

2.2. Mass spectrometry

The ESI spectrum of the product isolated is shown in Figure 2. This spectrum exhibits an intense peak at *m*/*z* 774 and a minor one at *m*/*z* 586. The high intensity of the first peak is indicative of the molecular mass of the compound. Indeed, alkaloids are most of the time easily ionised and the detected mass corresponds to the protonated molecule (MH^+).[5]

To allow the elucidation of the structure, a study by multiple tandem mass spectrometry was performed to obtain structural information on the fragments of the molecular peak (Figs. 3 and 4). A fragmentation study of aconitine-type alkaloids by Wang et al. [6] enabled us to compare the fragmentation scheme of our unknown compound with those of known *Aconitum* alkaloids.

MS^2 fragmentation of *m*/*z* 774 produced a major fragmentation peak at *m*/*z* 586. This kind of fragmentation: $[M+H]^+ \rightarrow 586$ $[M+H-R]^+$ is well known as the

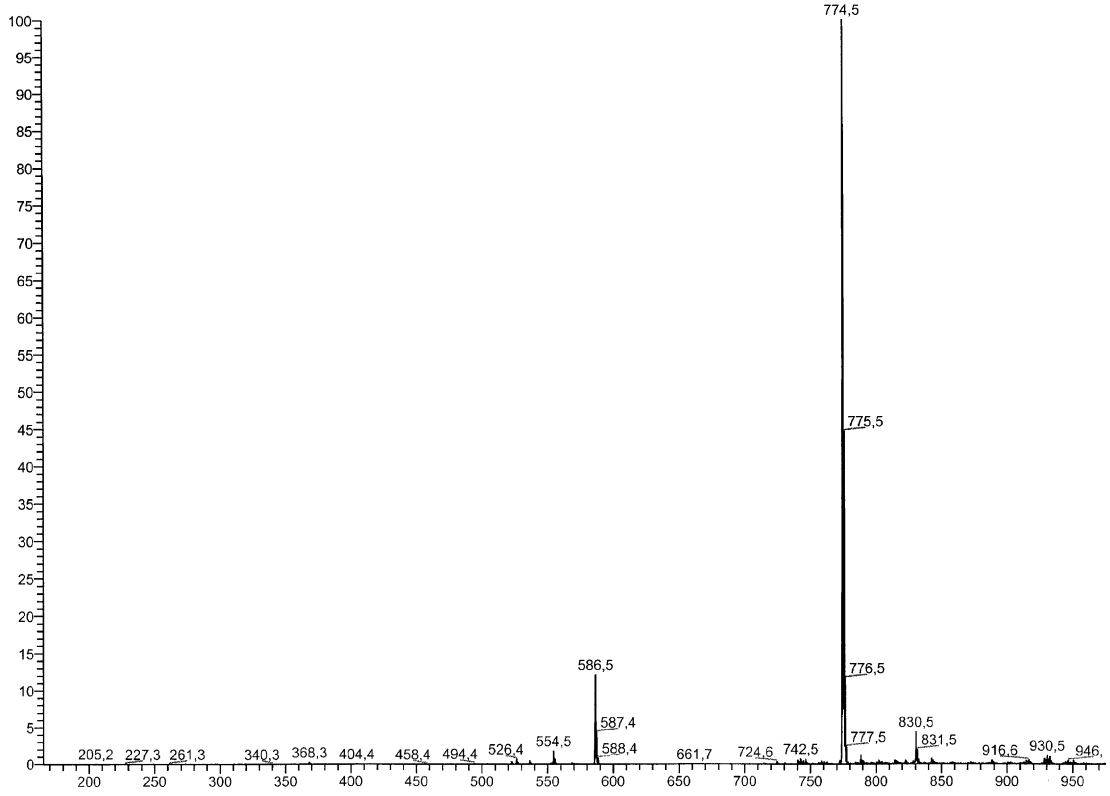

Figure 2. ESI-MS of fraction D3/#1.

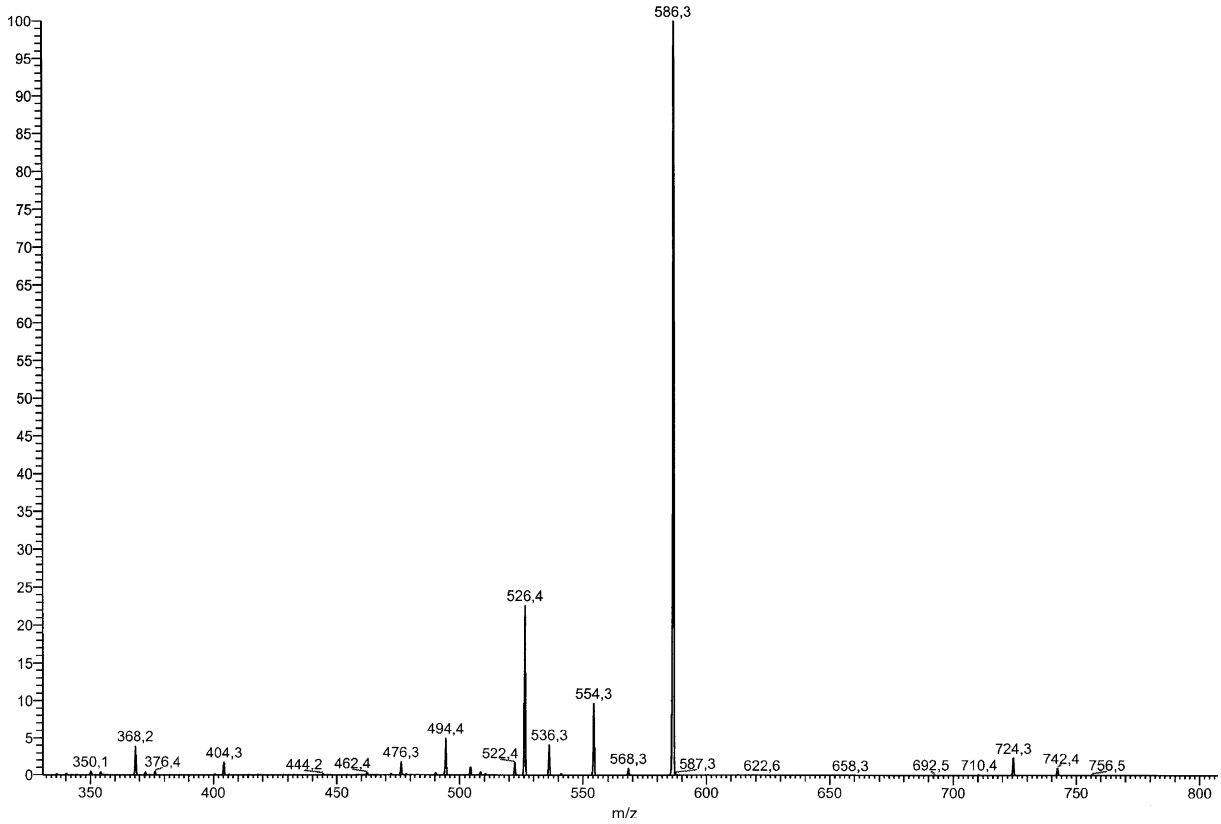

Figure 3. MS^2 spectrum of MH^+ (*m*/*z* 774).

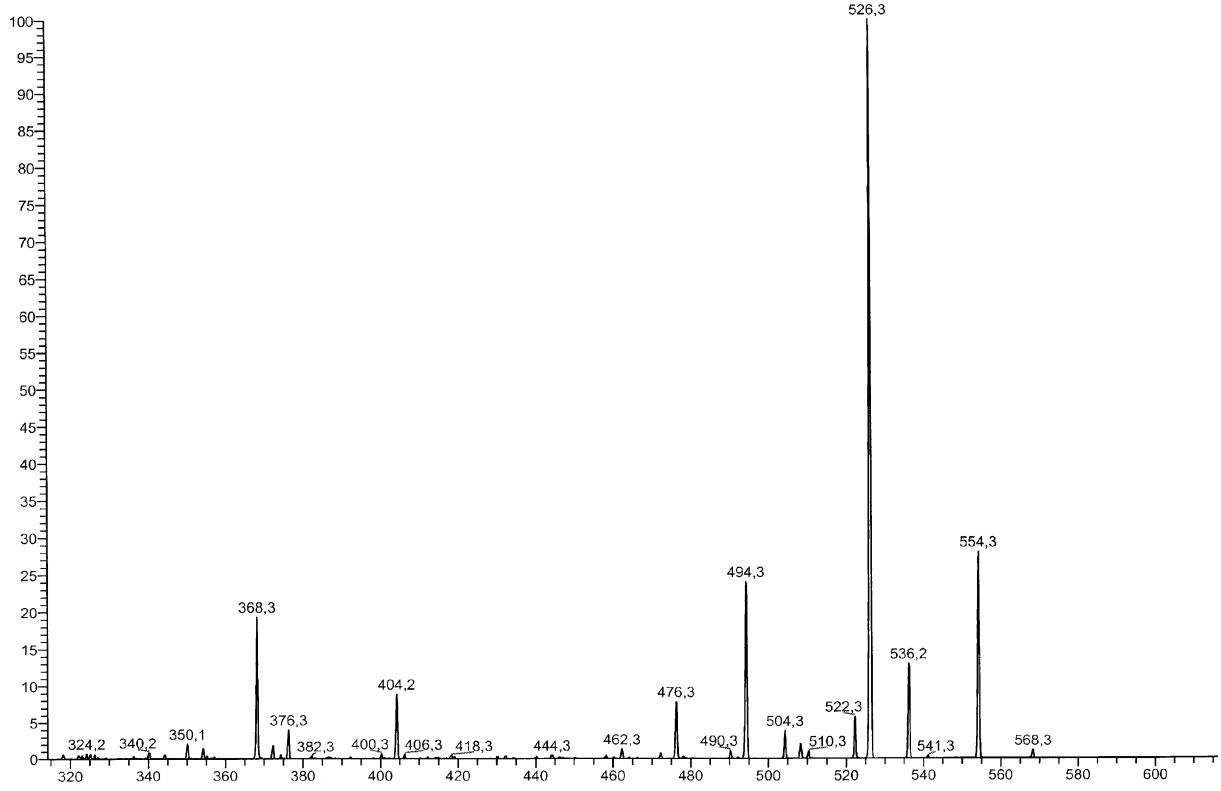

Figure 4. MS^3 spectrum of *m*/*z* 586.

elimination of an acyl moiety R on the C8 position of an aconitine-type alkaloid.[7] In the literature, R is usually an acetyl group or a fatty acyl group, but in our case the molecular mass of this leaving group (188) did not correspond to any simple fatty acid.[8,9] High resolution mass spectrometry (HRMS) was used for this

identification. MS^3 fragmentation (Fig. 4) was similar to the one obtained for aconitine, where R is CH_3COOH. Tables 3 and 4 summarise the attributions of the fragments.

HRMS measurements were performed simultaneously by LSIMS high resolution. HRMS of *m/z* 586 confirmed the nature of the aconitine-type alkaloid (experimental mass 586.3012, theoretical mass for $C_{32}H_{44}NO_9$ 586.3016). HRMS of *m/z* 774 allowed us to determine the molecular mass of our compound: experimental mass 774.4062, theoretical mass for $C_{41}H_{60}NO_{13}$ 774.4065 MH^+. These two measurements gave the mass of the $C_9H_{16}O_4$ group at the C8 position, which could correspond to an azelaic acid moiety.

2.3. Nuclear magnetic resonance

A complete assignment of the proton and carbon signals was obtained by analysis and study of correlations of 2D spectra (COSY, HMQC, HMBC, NOESY) (Tables 5–8). The study of the J_1 ^1H–^{13}C coupling correlations by HMQC allowed us to assign to each non-quaternary carbon the chemical shifts of its corresponding protons.

Table 3. Comparison between the MS^2 fragmentations of aconitine[5,6] and 8-*O*-azeloyl-14-benzoylaconine

	AC R = CH_3COOH CID (28%)	Product R = 188 CID (20%)
MH^+	646 (42)	774 (54)
MH^+-18	628 (1)	756 (<1)
MH^+-32	614 (3)	742 (2)
$MH^+-32-18$	596 (2)	724 (1)
MH^+-R	586 (100)	586 (100)
MH^+-R-18	568 (1)	568 (<1)
MH^+-R-32	554 (4)	554 (2)
$MH^+-R-32-18$	536 (2)	536 (<1)
$MH^+-R-32-28$	526 (3)	526 (4)
$MH^+-R-32-32$	522 (<1)	522 (<1)

Table 4. Comparison between the MS^3 fragmentations of aconitine[5,6] and 8-*O*-azeloyl-14-benzoylaconine $[F1+H]^+ = [M+H-R]^+$

	AC R = CH_3COOH CID MS1 28% CID MS2 27%	Product R = 188 CID MS1 30% CID MS2 20%
$F1H^+$	586 (30)	586
$F1H^+-18$	568 (4)	568 (1)
$F1H^+-32$	554 (50)	554 (28)
$F1H^+-32-18$	536 (16)	536 (13)
$F1H^+-32-28$	526 (100)	526 (100)
$F1H^+-32-32$	522 (3)	522 (6)
$F1H^+-32-28-18$	508 (<1)	508 (2)
$F1H^+-32-32-18$	504 (2)	504 (4)
$F1H^+-32-32-28$	494 (5)	494 (24)
$F1H^+-32-32-32$	490 (<1)	490 (1)
$F1H^+-32-32-28-18$	476 (2)	476 (8)
$F1H^+-32-32-32-18$	472 (<1)	472 (<1)
$F1H^+-32-32-32-28$	462 (<1)	462 (1)
$F1H^+-32-122-28$	404 (1)	404 (9)
$F1H^+-32*3-122$	368 (18)	368 (19)
$F1H^+-32*3-122-18$	350 (<2)	350 (2)

Table 5. ^1H NMR shifts and assignments

H	δ (ppm)	*J* (Hz)
1	3.49	m
2a[a]	1.41	br d
2b	2.40	d (11.3)
3	4.34	br s
5	2.91	s
6	4.15	d (6.0)
7	2.60	d (6.0)
9	2.96	t (5.8)
10	2.36	m
12a	1.94	d (9.6)
12b[a]	2.36	br d
14	4.91	d (5.2)
15	4.51	d (5.2)
16	3.26	m
17	3.32	s
18a	3.49	br d
18b[a]	3.27	br d
19a	3.14	d (11.4)
19b	3.94	d (11.4)
N-CH_2CH_3	3.27	m
N-CH_2CH_3	1.44	t (7.6)
1-OCH_3	3.38	s
6-OCH_3	3.24	s
16-OCH_3	3.79	s
18-OCH_3	3.29	s
H-2′/H-6′	7.99	d (7.2)
H-3′/H-5′	7.47	t (7.2)
H-4′	7.61	t (7.2)
$-NH^+$	8.26	s br
8-CO_2-(CH_2)1″a	1.58	m
8-CO_2-(CH_2)1″b	1.82	m
8-CO_2-(CH_2)2″	1.58	m
8-CO_2-(CH_2)3″a	0.87	m
8-CO_2-(CH_2)3″b	1.26	m
8-CO_2-(CH_2)4″	0.99	q (7.4)
8-CO_2-(CH_2)5″	1.12	m
8-CO_2-(CH_2)6″	1.58	m
8-CO_2-(CH_2)7″	2.28	t (7.7)

[a] Superposition with other protons.

Moreover, the ^1H and ^{13}C NMR chemical shifts of this compound were based on the unambiguous assignments of the previously described 14-benzoylaconine-8-palmitate and 14-benzoylaconine-8-stearate.[8,9]

The ^1H NMR spectrum (400.13 MHz, $CDCl_3$) (Table 5) showed four singlets at 3.38, 3.24, 3.79 and 3.29 ppm, attributed to the various methoxy groups, that is, 1-OCH_3, 6-OCH_3, 16-OCH_3 and 18-OCH_3, respectively. The protons H-2′/H-6′ (7.99 ppm, d, $J = 7.2$ Hz), H-3′/H-5′ (7.47 ppm, t, $J = 7.2$ Hz) and H-4′ (7.61 ppm, t, $J = 7.20$ Hz) were easily assigned to the benzoyl group.[10] The doublet observed at 4.91 ppm, ($J = 4.8$ Hz), and attributed to H-14β, indicated the presence of an ester group at C-14.[11]

The ^{13}C NMR spectrum (100.6 Mz, $CDCl_3$) (Table 7) showed 41 signals among which 32 could be attributed to the benzoylaconine skeleton,[12] and nine to the azeloyl group. A DEPT experiment defined eight quaternary carbons of which two could be assigned to the azelaic acid moiety, that is, the 8-COO^- and COOH functions were noted at 175.87 and 176.90 ppm, respectively.

Table 6. ^{1}H–^{1}H *J* correlation COSY

H	COSY
1	H-2a
2a	H-1, H-3, H-2b
2b	H-2a
3	H-2a
5	H-6
6	H-7, H-5
7	H-6, H-17
9	14, H-10
10	H-12a, H-9
12a	12b, H-10
12b	H-12a
14	H-9
15	H-16
16	H-15
17	H-7
18a	H-18b
19a	H-19b
N-CH_2CH_3	CH_2CH_3
H-1″a	H-1″b, H-2″
H-1″b	H-1″a
H-2″	H-3″b, H-1″a
H-3″a	H-3″b
H-3″b	H-3″a, H-4″, H-2″
H-4″	H-3″b, H-5″
H-5″	H-4″, H-6″
H-6″	H-5″, H-7″
H-7″	H-6″

Table 7. ^{13}C NMR shifts and assignments, HMBC *J* correlation

Carbon	δ (ppm)	HMBC
1	80.19	1-OCH_3
2	29.87	
3	70.33	
4	43.70	5, 6, 7, 18b
5	45.39	6, 7
6	82.93	6-OCH_3, 12
7	41.97	17
8	90.22	5, 6, 9, 10, 12, 14, 17, 15
9	43.97	5, 7, 14
10	40.50	7, 9, 17
11	51.05	5, 7, 12a, 17,
12	35.36	9, 10, 16,
13	74.40	14, 16,
14	78.71	16
15	79.11	
16	90.30	15, 16-OCH_3
17	63.51	5, 7, 10
18	76.05	18-OCH_3
19	50.77	
N-CH_2CH_3	50.70	N-CH_2CH_3
N-CH_2CH_3	11.34	
1-OCH3	55.63	N-CH_2CH_3
6-OCH3	59.53	
6-OCH3	62.13	
18-OCH3	59.60	
1′	129.73	3′, 5′
2′/6′	129.39	
3′/5′	130.19	
4′	134.30	2′, 6′
7′	166.31	2′, 6′, 14
8-COO	175.87	1″a,
1″	34.76	
2″	24.79	1″a,
3″	30.27	
4″	28.52	5″, 7″
5″	28.84	7″
6″	24.39	7″
7″	34.33	
COOH	176.90	7″

The chemical shift of the methoxy protons (δ_H 3.79 ppm) at C-16 suggested the presence of hydroxyl groups at the C-13 and C-15 positions. Moreover, the comparison of the ^{13}C chemical shift of C-15, observed at 79.1 ppm, with those of C-15 of various aconitine alkaloids bearing an OH group in the α position ($\delta \sim$78.5–79.0 ppm) and in the β position ($\delta \sim$68 ppm) led us to attribute to the hydroxyl group an α configuration.[3]

In Table 8, the proton–proton correlations resulting from the most significant space interactions, obtained by a NOESY experiment are collected. We observed a H-1 interaction with H-10 and H-12 as well as CH_3CH_2N with the 1-OCH_3 and H-17. We could also observe an interaction between the protons H-18 and H-19a. These correlations seem to imply a chair conformation of the cyclohexyl C1–C2–C3–C4–C5–C11 ring, and thus an equatorial orientation for the substituent in C4 position. Specifically, the H-18a proton has a nuclear Overhauser effect (NOE) with the H-3 proton, which implies an axial orientation of the H-3 proton, and an equatorial orientation of the hydroxyl group in the C3 position.[13]

We also observed a space correlation between protons H-3″b of the azelaic chain and the H-6 proton. In addition, the H-5″ and H-4″ protons present a NOE with the 6-OCH_3 protons. These correlations show that the azeloyl chain is directed toward the amino function, thus the proton of the acid function directly interacts with the nitrogen atom. In ^{13}C NMR, the COOH at 176.90 ppm is particularly broadened, because of the quadripolar effect of the nitrogen atom. In ^{1}H NMR, the proton related to nitrogen was noticed at 8.26 ppm, which is not compatible with a free acid proton ($\delta \geqslant 10$ ppm). These observations were in favour of a quaternization of the nitrogen atom with the azelaic acid chain. This zwitterionic structure has been confirmed by IR spectrometry. The IR spectrum indicated the presence of a carboxylate COO^- band and a sharp ammonium NH^+ band, respectively, observed at 1645 and 2240 cm^{-1}.

Based on all these spectroscopic data, we proposed the structure of this new alkaloid, identified in the F2.9.4 fraction, as being 8-*O*-azeloyl-14-benzoylaconine (Fig. 5).

3. Discussion

We have identified the most active substance present in ethanol/chloroform extracts of *A. karakolicum* roots. The molecular formula $C_{41}H_{59}NO_{13}$ was assigned to 8-*O*-azeloyl-14-benzoylaconine by combined NMR analysis and high- resolution EI mass spectroscopy. This compound results in fact from the replacement of the acetyl group by an azelaidic acyl moiety on carbon 8

Table 8. Significant NOEs from NOESY spectrum

Observed H	Show NOEs to
1	H-2a, H-12a, H-10
2a	H-1, H-2β
2b	H-2a, 1-OCH_3
3	H-18a
5	6-OCH_3
6	H-7, 6-OCH_3, H-9
7	H-6, 6-OCH_3, H-10, H-1″a
9	H-6, H-14, H-10
10	H-14, H-7, H-1, H-9
12a	H-1, H-12b, H-16
12b	H-12a
14	H-10, H-9
15	H-16, 16-OCH_3
16	H-12a, H-15, 16-OCH_3
17	N-CH_2CH_3, H-12
18a	H-18b, H-5, H-19a, H-3
18b	H-18a, H-19a
19a	H-19b
19b	H-19a, H-18a
N-CH_2CH_3	CH_2CH_3, 1-OCH3, H-17
N-CH_2CH_3	N-CH_2CH_3
H-1″a	H-1″b
H-1″b	H-1″a, H-2″, H-5″, H-3″b
H-2″	H-4″, H-1″b
H-3″a	H-3″b
H-3″b	H-3″a, H-4″, H-5″, H-6, H-1″b
H-4″	H-2″, H-3″b, H-6″, 6-OCH_3
H-5″	H-6″, H-3″b, 6-OCH_3, H-1″b
H-6″	H-4″, H-5″, H-7″
H-7″	H-6″

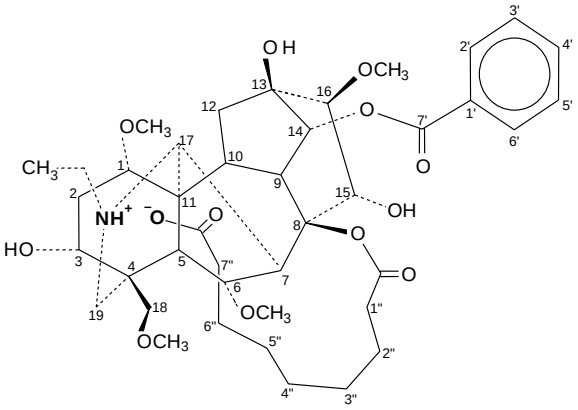

Figure 5. Structure of 8-*O*-azeloyl-14-benzoylaconine.

of the aconitine skeleton. The compound thus identified has never been described in the chemical literature, although several fatty acyl esters of aconine were previously described.[10] The novel feature of this newly identified alkaloid compound is its zwitterionic structure between the negative charge of the carboxylate function at the extremity of the azelaic acid chain and the positive charge of the quaternary ammonium formed on the nitrogen atom of the heterocycle. Given the length of the azelaidic chain, an internal ionic bond between these two moieties could be postulated.

Aconitine itself is devoid of antiproliferative activity. In the in vitro test on the 60 human tumour cell lines of the NCI, the IC_{50} of aconitine (NSC 56464) is below 100 μM in two cell lines only[14]; according to the molecular weight assigned to 8-*O*-azeloyl-14-benzoylaconine (774), the IC_{50} of this compound was about 10–18 μM in three cell lines out of three, which warrants further investigations.

Concerning azelaic acid, it has been shown to be an inhibitor of mitochondrial oxidoreductases in tumour cells.[15] As a consequence, it was proposed as a general antitumour agent.[16] However, its antiproliferative activity in vitro was in the millimolar range, far beyond the IC_{50} observed for the new compound present in fraction F2.9.4. Azelaic acid (NSC 19493) has never been tested in the in vitro screening panel of the NCI but some experiments have been performed on mouse tumours in vivo,[17] which concluded in the total absence of antitumour activity. The antiproliferative activity observed for 8-*O*-azeloyl-14-benzoylaconine cannot therefore be attributed to the activity of its individual constituents.

It is remarkable that the antiproliferative activity of 8-*O*-azeloyl-14-benzoylaconine in terms of molar concentrations is in line with the activity of major anticancer drugs belonging to several classes (antimetabolites, alkylating agents, platinum compounds, topoisomerase inhibitors). This appears encouraging for further analysis of the anticancer potential of this molecule. The proof of the cytotoxic activity of this new substituted aconine would be firmly assessed upon synthesis and evaluation in the whole NCI panel. This will be the next step of our investigations.

4. Materials and methods

4.1. Plant material

Tubercles of *A. karakolicum* Raspc were collected in the eastern mountainous area of Issyk-Kul region of Kirghizstan in August 2003, and identified at the Kirghiz Agricultural Institute. The voucher specimen is deposited at the herbarium of the Department of Botany of Kirghiz National University.

4.2. Extraction, fractionation and purification

Dried and powdered tubercles (1.5 kg) were extracted with ethanol. The solvent was then evaporated under reduced pressure. The residue was dissolved in water acidified to pH 2 with 5% HCl; the solution was then brought to pH 9–10 by addition of 30% ammonia and re-extracted with chloroform. After evaporation of chloroform, 14.7 g of residue was obtained.

This residue was purified by column chromatography (column A) using Kieselgel 60 (70–230 mesh) with dichloromethane/methanol, first in 10:1, and then in 1:1, as eluents. Fractionation was controlled by TLC on silica gel plates (Kieselgel 60 F 254, Merck), the spots being visualised with iodine vapours. Five different fractions (F1–F5) were collected according to the TLC spots. After the determination of the cytotoxicity of

these fractions, the active fraction F2 was subjected to further purification. After evaporation to dryness, the sample was dissolved in methanol/water (1:1) and filtered at 0.45 mm. Three steps of HPLC purification were then performed, all using an Ultrasep ES 10 RP18 6.0 μm reversed phase C18 column, 250 × 20 mm (Bischoff, Germany), protected by a guard column. Absorbance was monitored at 234 nm. For the first step (column B), the solvents were: A, trifluoroacetic acid/water, 1:1000; B, trifluoroacetic acid/cyanhydric acid, 1:1000 and we used the following elution conditions: injection volume, 5 mL; gradient from 25% B to 100% in 60 min; flow time, 70 min; flow rate, 8 mL/min. Nine different fractions (F2.1–F2.9), according to the peaks detected by UV, were separated and collected, and each of them was tested on the human tumour cell lines. The most active fraction, F2.9, underwent one more purification by preparative HPLC (column C) with a 70–100% gradient of solvent B, and seven different subfractions, from F2.9.1 to F2.9.7, were collected. After testing these fractions on tumour cell lines, the F2.9.4 subfraction was subjected to further HPLC purification (column D). The solvents were: A, trifluoroacetic acid/water, 2:1000; and B, trifluoroacetic acid/cyanhydric acid, 2:1000. Elution was performed with a linear gradient of solvent B (40–100%). A pure alkaloid was thus obtained and tested on tumour cell lines.

4.3. Cell culture and cytotoxicity evaluation

The human tumour cell lines HCT-15 (colon cancer), A549 (lung cancer) and MCF-7 (breast cancer) were obtained from the Developmental Therapeutics Program of the National Cancer Institute (Rockville, MD, USA). Cells were routinely grown with RPMI 1640 medium supplemented with 10% foetal calf serum, both obtained from Biochrom AG (Berlin, Germany). They were grown on Petri dishes (Nunc, Denmark) at 37 °C in a humidified atmosphere containing 5% CO_2. Cells were replicated every 4 days and the medium changed once in-between.

Cytotoxicity was evaluated on exponentially growing cells grown for 24 h in the presence of the extracts to be tested. Briefly, 4000 cells were seeded in 96-well plates with 200 μL of complete medium; 24 h later, the medium was supplemented with a series of definite amounts of crude extracts or purified fractions and the contact maintained for 24 h. The dry extracts and fractions were dissolved in pure water adjusted to pH 2.5 with 1% HCl, the same solvent being used as a control. All samples were first sterilised using polycarbonate membrane filters of 0.22 μm (Millipore, Molsheim, France). The cells were then allowed to re-grow for 48 h; viable and metabolically active cells were quantitatively estimated by coloration with the MTT dye (1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan, Sigma-Aldrich Chimie, Saint-Quentin-Fallavier, France). The amount of extract or fraction allowing a 50% decrease in cell numbers is indicative of the cytotoxicity and represents the IC_{50} of the extract. This technique allows a rapid, sensitive and reproducible screening of various extracts and substances.[18]

4.4. Mass spectroscopy

ESI mass measurements were performed on a Finnigan LCQ mass spectrometer equipped with an electrospray source in positive mode. The spraying voltage was 5 kV. The capillary temperature was set at 220 °C. Sample solutions were infused at 3 μL/min with a syringe pump. The collision energies (%) for the MS^n analyses ranged from 20 to 30%, depending on the mass of the parent or fragment ions.

Accurate mass determination was carried out using an AutoSpec mass spectrometer arranged in an EBE geometry (Micromass, Manchester, UK). The instrument was operated at 8 kV accelerating voltage in positive mode. The caesium gun was set to 35 keV energy and 1 μL of sample was mixed in the tip of the probe with a nitrobenzylalcohol matrix.

4.5. Nuclear magnetic resonance (NMR)

The 1D and 2D NMR experiments were performed on a Bruker DPX400 spectrometer at 400.13 and 100.6 MHz for ^1H and ^{13}C experiments, respectively, equipped with a 5 mm broadband probe and Bo gradients. All spectra were recorded using 9 mg of substance dissolved in 0.7 mL of $CDCl_3$, Chemical shifts in ppm are given relative to TMS. The ^1H–^1H shift correlated two dimensional (COSY)[19] spectra obtained using the COSY 90 pulse sequence. The type of carbon is defined by 2 experiment 1D (DEPT 90 and 135). The one-bond ^1H–^{13}C chemical shift correlation (HMQC) spectra had been obtained according to the Bax sequence[20] using Bo gradient pulses for the selection of ^1H coupled to ^{13}C carbons. The ^1H detected heteronuclear multiple bond correlation (HMBC) spectra were recorded using the pulse sequence proposed by Bax and Summers[21] involving a low pass Jn filter (3.8 ms) and a delay to observe the long-range coupling (60 ms) as in the HMQC experiment, Bo gradient pulses were applied to select ^1H coupled to ^{13}C nuclei. Gradient selected NOESY spectra were acquired with the NOESYGPPH Bruker pulse program.

4.6. Infrared spectroscopy (IR)

IR spectrum was recorded on a BRUKER IFS-25 spectrophotometer.

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