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Inhibition of both *Trypanosoma brucei* bloodstream form and related glycolytic enzymes by a new kolavic acid derivative isolated from *Entada abyssinica*

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A new kolavic acid derivative known from spectroscopic analyses as monomethyl ester-15-kolavic acid was isolated from the stem bark of *Entada abyssinica*, a plant traditionally used in West and East Africa for the management of sleeping sickness. The new derivative showed a strong and selective inhibitory activity on the GAPDH enzyme of *Trypanosoma brucei* with an IC_{50} value of 0.012 mM.

1. Introduction

Human African Trypanosomiasis (HAT), or sleeping sickness, is an old tropical disease transmitted through the bites of infected tsetse flies. The disease was largely controlled in the 1960s; however, a lack of human and financial resources and years of conflict in the most affected countries have hampered efforts to monitor and control the disease. Sleeping sickness, therefore, re-emerged in the 1980s (WHO 1998). Overall, 60 million people are exposed to HAT and 36,000 cases were reported to the WHO in 1998 (WHO 2001). However, only 3 to 4 million people are under surveillance, and, in fact, it is estimated that 300,000 people are infected with HAT. Countries most affected are Angola, the Democratic Republic of Congo, and Sudan. If untreated, sleeping sickness invariably leads to death.

The few drugs that are available to treat this disease are scarce, highly toxic, and encounter parasite resistance (Stanghellini 2001; WHO 2000; Legros 1999; Iten 1995; Pépin 1994; Kuzoe 1993; Van Nieuwenhove 1992). Thus, there is a great need for new efficient and cost-effective drugs to combat this disease. This challenge could be addressed in a rational manner by exploiting some unusual features exhibited by the trypanosomes such as the complete dependence of their bloodstream form on glycolysis to the stage of pyruvate as the sole source of energy supply. In this context, and as a continuation of a programme aimed at identifying lead compounds for the development of new and selective inhibitors of glycolysis (Nyasse 2002; Ladame 2001; Willson 1994) in species such as *Trypanosoma*, we also considered compounds from medicinal plants since the natural products literature mentions a wide variety of isolated substances showing activity against trypanosomes (Sepulveda-Boza 1996; Wright 1990). Thus, a new kolavane-type diterpenoid isolated from the bark extracts of *Entada abyssinica*, a plant used in African traditional medicine for the treatment of sleeping sickness (Freiburghaus et al. 1996), has received special attention in this work. Given the fact that the isolated compound contains a α-enone structure, a nucleophile reacting site, which is probably responsible for the biological properties of many naturally occurring compounds such a pentalenolactone obtained from *Streptomyces areae*, and which is known to block glycolysis in both prokaryotic and eukaryotic species by a selective inhibition of the enzyme glyceraldehyde-3-phosphate-dehydrogenases (GAPDH) (Cane 1994; Lambeir 1991; Cane 1989), we were prompted to assay compound **1** on culture of trypanosomes and three glycolytic enzymes.

In this contribution, we report on the first isolation of a new kolavic acid derivative and on its inhibitory effects on trypanosomes.

2. Investigations, results and discussion

2.1. Chemistry

Compound **1** was obtained as a white powder from the dichloromethane bark extract upon eluting the silica gel column chromatography with hexane/ethyl acetate mixtures of increasing polarity. The structure determination was mainly based on 1D (^{1}H, ^{13}C, ^{13}C-DEPT) and 2D NMR experiments (^{1}H-^{1}H-COSY, ^{1}H,^{13}C-HMBC, NOESY).

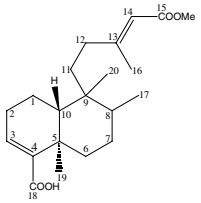

Table 1: ^{13}C and ^{1}H NMR spectral data for compound 1

Atom	Assignments		Observed correlations	
	C	H	HMBC (carbon number)	NOESY (proton shift)
1	17.45			
2	27.84	2.3, 2.15		2.15, 2.3
3	140.33	6.8	1, 2, 4, 6, 18	
4	142.06			
5	37.99			
6	38.06			
7	25.77	1.40		
8	35.20	1.6		0.93, 1.6
9	37.96			
10	45.50	1.45		1.45, 2.2
11	29.57	2.2		
12	34.80	2.08		
13	161.99			
14	115.20	5.68	12, 14	2.18, 3.68
15	167.62			
16	19.56	2.18 Me	12, 13, 14, 15	
17	15.12	0.93 Me	7	5.68
18	172.80			
19	20.69	0.95 Me	6, 8, 10	
20	21.70	1.3 Me	4, 6, 10, 11	
OMe	51.19	3.68 Me	14, 15	

The chemical shifts and correlations are given in Table 1.

The ^{13}C experiments (DEPT included) showed that compound **1** contains 15 carbon atoms distributed among five methyls, six methylenes, four methines and six unsubstituted carbon atoms. These findings coupled to the presence of two carboxylic functions (167.69 for $COOCH_3$ and 172.84 for COOH) and to that of two ethylenic protons at 6.80 ppm (1 H t, J = 3Hz) and 5.68 ppm (1 H, s) respectively led to the molecular formula $C_{21}H_{32}O_4$ which is suggestive of a bicyclic compound belonging to diterpenoids (Bohlmann 1980; Richomme 1991). Correlation experiments ($^{1}H,^{1}H$-COSY, HMQC and HMBC) exemplified by the links observed between the methine group (1 H 5.68 ppm and C 115.2 ppm) and its neighbours: an allylic methyl (3 H 2.18 and C 19.56 ppm), a methoxylcarbonyl (C 167.6 ppm) and the methylene group (2 H 2.08 and C 34.8 ppm) respectively showed that the side-chain of **1** (4-methoxycarbonyl-3-methyl-but-3-enyl) is identical to that of a known dehydropinifolic acid derivative (Richomme et al. 1991) and that compound **1** was a kolavane-type diterpene. Finally, a comparison between chemical shifts of **1** and those published (Miscra et al. 1964) on both kolavic acid and kolavenic acid methyl ester indicated that compound **1** differed from kolavic acid only by the additional methyl group attached to the carbon atom (C-15) in **1**. Thus, compound **1** was established as monomethyl ester-15-kolavic acid, a new and natural derivative of kolavic acid (Miscra et al. 1964).

2.2. Biology

Compound **1** exhibited an inhibitory activity on the growth of *T. brucei* (IC_{50} 1.7 μM) and *L. infantum* ($IC_{50} > 32\mu M$) with respect to the clinically used antitrypanosomal agents suramin (IC_{50} 0.044 μM) and melarsoprol (0.0068 μM) and was considered as a promising naturally occurring trypanocide. Since **1** contains two α-enone moieties and in an attempt to gain some insights into its mechanism of action against the growth of the trypanosomes, it was deemed necessary to evaluate its activities on GAPDH enzyme. In fact, the S–H group of the cysteine in position 149 of this glycolytic enzyme is known to give Michael addition products with such functionalities (α-enones). In the same context, the effects of **1** on other glycolytic enzymes namely phosphofructokinase (PFK), phosphoglycerate kinase (PGK) and pyruvate kinase (PyK) were also determined. The results obtained on the selected enzymes are displayed in Table 2.

Compound **1** appears as a selective inhibitor of GAPDH from parasites and its selectivity is at least 66 fold better

Table 2: Biological activities of compound 1

Inhibitory activities expressed in IC_{50} values (mM)

Enzyme	Rabbit muscle	*T. brucei*	*T. cruzi*	*L. mexicana*
GAPDH	0.80 ± 0.02	0.012 ± 0.02	0.25 ± 0.02	nd[a]
PFK	2.7 ± 0.1	1.20 ± 0.04	nd[a]	nd[a]
PGK	ni[b]	ni[b]	ni[b]	nd[a]
PyK	> 3	nd[a]	nd[a]	0.62 ± 0.04

[a] nd: not determined; [b] ni: no inhibition observed above 2 mM at which precipitation

on *T. brucei* GAPDH (IC_{50} 12 μM) than on its rabbit muscle homologue (IC_{50} 800 μM). The inhibitory effect of **1** on *T. cruzi* GAPDH (IC_{50} 250 μM) could also be considered as an interesting result since there is no efficient drug against this parasite, which is responsible for Chagas disease in South America.

Moreover, the lower inhibitory activity observed on the rabbit muscle GAPDH (IC_{50} 800 μM) as compared to the parasites may be interesting as well, since the enzyme GAPDH plays a central role in many topical human pathologies as apoptosis (Sunaga et al. 1995), cancer (Epner et al. 1993) and neuronal degeneration (Schulze et al. 1993).

Only a weak inhibitory effect was observed on PFK enzyme with again a better selectivity towards *Trypanosoma brucei* enzyme (IC_{50} 1200 μM). Such a weak activity could probably be due to a protein distorsion resulting from hydrophobic interactions generated by compound **1** near the active site of the protein since **1** is quite different from the enzyme substrate (fructose-6-phosphate) to reach the active site.

No inhibition was observed on PGK enzymes. Compound **1** has a structure quite different from that of the enzyme substrate and, since this enzyme is insensitive to allosteric distorsion it was logical not to observe even a weak activity as with PFK.

The modest activity observed on PyK enzyme of *Leishmania mexicana* (IC_{50} 620 μM) with compound **1** was probably due to hydrophobic interactions causing a distorsion of the allosteric site of this enzyme.

3. Experimental

3.1. General experimental procedures

M.p's were determined on a Buchi Melting point apparatus B-540 and are not corrected. TLC analyses were carried out on 0.25 mm thick precoated silica plates (Merck Fertigplatten Kieselgel 60 F_{254}) with the mobile phase hexane/ethyl acetate 9/1. TLC spots were visualized under UV light and preferentially either by iodine vapour spray or by 50% sulfuric acid and subsequent heating (black spot). CC was carried out on Merck Kieselgel 60 (70–230 mesh). NMR spectra were recorded on a 500 MHz Brucker spectrometer at 25 °C. IR by a Mattson Polaris FTIR spectrometer in the solid state (KBr). GAPDH from Rabbit muscle, auxiliary enzymes and substrates were purchased from Boehringer Mannheim and Sigma-Aldrich Chemical Company.

3.2. Plant material

The bark samples of *Entada abyssinica* were collected in Dschang (West Province, Cameroon) in December 2001 and identified by one of us (B. Sonké) by comparison with the material available at the National Herbarium, Yaoundé (YA). A voucher specimen of this plant has been deposited in the National Herbarium, Yaoundé.

3.3. Extraction and Isolation

Dried bark pieces (5 kg) of *E. abyssinica* were powdered and macerated at room temperature with methylene chloride for six days. The residue obtained upon evaporation of the solvent to dryness was then dissolved in hexane. The hexane soluble part was evaporated to dryness affording 73 g of the hexane extract. This extract was successful chromatographed on a silica gel pad and yielded pure compound **1** (330 mg) under elution with hexane/ethyl acetate 4 : 1. This material was recrystallized in a hexane-ethyl acetate mixture (4 : 1) to give a colourless powder (200 mg).
Compound **1** (colourless powder): m.p. 162–163 °C; For ^{1}H and ^{13}C-data see Table 1.
Primary screening on *Trypanosoma brucei brucei, T. cruzi* and *Leishamania parasites* was carried through the WHO/TDR/DDR supported network of screening and biological evaluation laboratories.

3.4. Inactivation studies on GAPDH, PFK and PGK

Inhibitory effects of compound **1** on glycolytic enzymes were determined by spectrophotometry following published procedures by Claustre et al. (2002) for PFK, Willson et al. (1994) for GAPDH and Misset et al. (1984) for PGK.

3.4.1. Source of enzymes, substrates and cofactors

GAPDH, PFK, PGK, PyK from rabbit muscle, LDH from beef heart, all substrates and cofactors were purchased from Sigma-Aldrich Chemicals Co. The glycolytic enzymes from *Trypanosoma brucei* were prepared by overproducing in *Escherichia coli* and purified according to Hannaert et al. (1995). *Trypanosoma cruzi* GAPDH was prepared by overproducing according to Souza et al. (1998) and provided by G. Oliva from the University of Sao Paulo (Brazil).

3.4.2. Inhibition studies

The inhibitor concentration required for 50% inhibition (IC_{50}) was calculated for each enzyme at substrate and cofactor saturating concentrations. In each case there was a preincubation of the enzyme with different concentrations of compound **1** for 5 min followed by addition of the reaction mixture to trigger off the reaction. The percentage of remaining activity was calculated by comparison with an inhibitor-free control experiment.
Activity was followed spectrochemically at 25 °C by absorbance of NADH at 340 nm ($\varepsilon_{340} = 6.22\ mM^{-1}.cm^{-1}$) with a spectrophometer SAFAS.
The composition of the reaction mixture (1mL) was different for each enzyme:
– GAPDH: Buffer (Triethanolamine hydrochloride: TEA 0.1 M, pH 7.6), 1 mM EDTA, 2 mM NAD^+, 0.8 mM D-glyceraldehyde-3-phosphate (GAP), 0.1 M KCl and 10 mM potassium phosphate.
– PyK: Buffer (TEA 0.5 M, pH 7.2), 6 mM MgSO4, 5 mM $KHCO_3$, 50 mM KCl, 0.42 mM NADH, 2.2 mM PEP, 2 mM ADP and 6.2 μg LDH.
– PFK: Buffer (TEA 0.1 M, pH 7.6), 1 mM EDTA, 0.6 mM NADH, 2.2 mM PEP, 0.5 mM ATP, 2.5 mM $MgCl_2$, 1 mM $NaHCO_3$, 1.5 mM AMP, 2.5 μL PyK/LDH (1.1 unit).
– PGK: Buffer (TEA 0.3 M, pH 7.5), 10 mM $MgCl_2$, 0.5 mM KCl, 0.42 mM ATP, 2.5 mM phosphoenolpyruvate (PEP), 0.66 mM ATP, 4.8 mM $NaHCO_3$, 5.6 mM 3-phosphoglycerate, 2.5 μL PyK/LDH (1.1 unit).

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