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Modified berberine and protoberberines from *Enantia chlorantha* as potential inhibitors of *Trypanosoma brucei*

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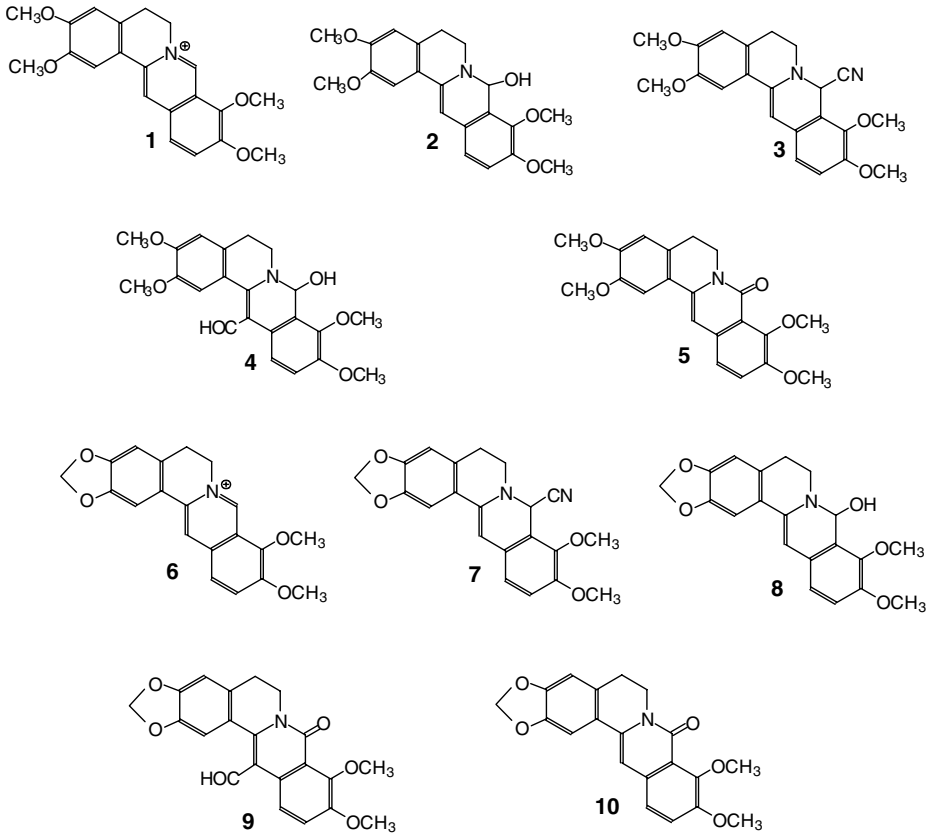
Phytochemical study of the stem bark of *Enantia chlorantha* resulted in the isolation of two protoberberines **1** and **2**. These alkaloids as well as commercially available berberine were modified chemically and tested *in vitro* against *Typanosoma brucei* proliferation as well as on three targeted glycolytic enzymes. The inhibitory activities observed were in the range of 20 μM (ED_{50} values).

1. Introduction

Sleeping sickness is a devastating disease in many regions of Subsaharan Africa. It is caused by *Trypanosoma brucei*, a protozoon belonging to the trypanosomatidae. More than 50 million people in some 34 African countries are at high risk of getting infected with this unicellular protozoan haemoflagellate [1]. If untreated, sleeping sickness invariably leads to death.

The few drugs which are currently used to treat this disease are toxic [2] and require hospitalisation [3]. Therefore, there is an urgent need for new efficient and cost effective antitrypanosome agents. Such a need can 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 research programme devoted to the design of selective inhibitors of glycolysis [4–6] in species such as *Trypanosoma brucei*, the causative agent of African sleeping sickness [7], we also considered compounds of natural origin since the natural products literature mentions a wide variety of isolated substances showing activity against trypanosomes [8, 9]. Therefore, some protoberberines [10] isolated from the bark extracts from *Enantia chlorantha*, a plant used in African folk medicine against malaria and allied alkaloids have received special attention in this work. Given the fact that alkaloids of this type are very susceptible to attack by nucleophiles, a feature which is probably responsible for the biological properties of pentalenolactone isolated from *Streptomyces areae,* and which blocks glycolysis in both prokaryotic and eukaryotic species by selective inhibition of the enzyme glyceraldehyde phosphate dehydrogenase (GAPDH) [11–13], we were prompted to assay compound **1** and derivatives **2**–**10** on cultures of trypanosomes and on three glycolytic enzymes.



For these assays, the glycolytic enzymes purified and then overexpressed by Opperdoes's group [14, 15] were selected since they are potential targets for the inhibition of the glycolytic flux in the parasite [16]:

- glyceraldehyde phosphate dehydrogenase for the reason regarding pentanolactone but also because of structural similarities between compounds in the series and the cofactor NAD^+ of the GAPDH;
- phosphofructokinase (PFK) and phosphoglyceratekinase (PGK) are both ATP dependent enzymes acting in the hexose part of glycolysis and in the triose part, respectively. These two enzymes were considered on the basis of the structural analogies between compounds in the series and previously identified kinases inhibitors (tyrphostins and flavonoids) [17], which behave as competitive inhibitors for ATP and dehydrogenase inhibitors (pentalenolactone and koningic acid) [13–18].

Here, we describe the preparation of the protoberberines and the evaluation of their inhibitory activity on both the cultures of trypanosomes in *vitro* and on the three selected glycolytic enzymes.

2. Investigations, results and discussion

2.1. Chemistry

Palmatine (**1**) and its 8-hydroxydihydro-derivative **2** were isolated from the bark extract of *Enantia chlorantha* as previously described [10]. The synthetic derivatives were chosen to maximize structural diversity in order to establish a range-finding structure activity relationship with a modest series of compounds. 8-Cyanodihydropalmatine (**3**) and 8-cyanodihydroberberine (**7**) were obtained in high yields by treatment of a methanolic solution of palmatine (**1**) and berberine (**6**), respectively, with aqueous sodium cyanide following literature procedures [19]. Derivatives **4**, **5**, **8**, **9** and **10** were prepared according to a modified procedure of Reimer-Tiemann reaction. In this context, three distinct procedures depending on the composition of the reaction mixtures were used (Table 1):

1. When **1** was allowed to react with sodium hydroxide in a mixture of chloroform/ethanol, 8-hydroxydihydropalmatine (**2**) was obtained in 73% yield along with traces (less than 1%) of 13-formyl-8-hydroxydihydropalmatine (**4**) and 8-oxopalmatine (**5**);
2. In the absence of ethanol, **2**, **4** and **5** were obtained in 15, 71 and 9% yield, respectively;
3. The last procedure involving **1** without chloroform afforded the 8-substituted derivatives **2** (20%) and **5** (4%) only.

The reaction involving **2** according to the first procedure i.e. the reaction medium containing ethanol, afforded 20% of **1** and only traces of the 13-formylated derivative **4**. When the same reaction was repeated without alcohol, 13-formyl-8-hydroxydihydropalmatine **4** could be isolated in at least 50% yield.

Commercial berberine chloride (**6**), when submitted to sodium hydroxide/ethanol/chloroform gave the 13-formyl-8-oxoberberine (**9**) in 56% yield along with 8-hydroxydihydro- and 8-oxoberberine (**8**, **10**) in 5 and 9% respectively. In the absence of alcohol, the yield of compound **9** was maintained while that of **10** decreased to almost zero.

Table 1: Riemer-Tiemann reaction conditions and yields

Substrate	Reaction product	Yield (%)		
		Method 1	Method 2	Method 3
Palmatine (**1**)	**2**	73	15	20
	3	–	–	–
	4	traces	71	traces
	5	traces	9	4
8-hydroxydihydro-palmatine (**2**)	**1**	20	traces	20
	4	traces	50	traces
Berberine **6**	**7**	-	-	-
	8	5	16	10
	9	56	56	traces
	10	9	0	0

Method 1: $NaOH/CHCl_3/EtOH$. Method 2: $NaOH/CHCl_3$ and method 3: NaOH/EtOH

2.2. Biology

Compounds **1**–**4** and **7**–**9** were tested for their *Trypanosoma* and enzyme inhibitory effects. The results are presented in Table 2. Activities against *Trypanosoma brucei* are for the best case in the ten micromolar range, that is ten fold lower than the value for the reference compound, difluoromethyl ornithine ($ED_{50} = 100$ μM). IC_{50} values for the inhibition of the enzyme Tb. GAPDH obtained are at best in the 50 μM range which corresponds to the range of the Km values for the substrates NADH (20 μM) and NADH (450 μM) [7]. Compounds able to promote covalent bond formation with the enzyme showed no time dependent inhibition. The inhibition is not prevented by either substrate. IC_{50} values for inhibition of Tb PFK indicate inhibition effects in the range of the ATP cofactor Km value (60 μM). The results with the enzyme Tb. PGK indicates again for three compounds IC_{50} values lower than or similar to the Km values for ATP (100 μM).

From the first column of Table 2 regarding the activation on cultures of *Trypanosoma brucei*, it is evident that two of the studied compounds **3** and **9** exhibited inhibitory activities similar to that of DFMO. This observation suggests that these compounds deserve further chemical modifications.

Owing to the susceptibility of compounds **1**, **4** and **9** to attack by nucleophiles, possible covalent binding with the essential cysteine at GAPDH active site was considered. Indeed, such a covalent bonding has been observed with pentalenolactone [12, 13] and with other inhibitors previously developed [5]. No time dependent effect was observed with this enzyme and therefore no irreversible inactivation occurred. The observed inhibition was not protected by substrates indicating that it did occur out of the active site. However, affinities were significant for three compounds **1**, **3** and **7** among which compound **3** emerged as the most active on *T. brucei* cultures.

Table 2: Inhibition effects of the alkaloids on *T. brucei* and GAPDH, PGK and PFK enzymes

Compounds	ED_{50} (μM) *T. brucei* culture	IC_{50} (μM) GAPDH *T. b*	IC_{50} (μM) PGK *T. b*	IC_{50} (μM) PFK *T. b*
1	40	35	80	100
2	238	120	102	120
3	20	50	50	100
4	27	10	250	350
7	207	50	60	110
8	60	210	nt	nt
9	13	500	600	50

Inhibition effects on PFK and PGK were weak but still significant for the same compounds as those acting on GAPDH enzyme. However, no protection by ATP was observed suggesting, as already mentioned, that the inhibition did occur out of the active site.

In conclusion, although these compounds are active on *Trypanosoma* cultures, it is not certain that glycolysis represents their main target. Meanwhile, given their efficiency on cancer cells, other essential metabolisms must be considered such as action on tubulin, interference with polyamines metabolism and/or DNA intercalation. Further investigations in are in progress.

3. Experimental

3.1. Chemistry

M.p's. were determined on a Gallenkamp apparatus and are not corrected. TLC analyses were carried out on 0.25 mm thick precoated plates (Merck Fertigplatten Kieselgel 60 F_{254}). TLC plates were visualized under UV light and preferentially by 50% sulfuric acid spray and subsequent heating (black spots). CC was carried out on Merck Kieselgel 60 (70–230 mesh). NMR spectra were recorded on a JEOL JMN EX 400 spectrometer in ~5% solution at 25 °C. IR by a Mattson Polaris FTIR spectrometer in the solid state (KBr).

Compounds **1** and **2** were obtained and characterized as described previously [10]. Berberine was of analytical grade and was purchased from Fluka.

3.1.1. Typical preparation for **3** *and* **7** [14]

To a solution of Berberine (**6**) or Palmatine (**1**) (5 mM) in 100 ml of hot MeOH, NaCN (50 mM) dissolved in 100 ml of H_2O was added. The resulting mixture was heated on a steam bath for 15 to 20 min following the formation of a homogenous solution. Upon cooling, **7** (1.60 g, 89% from berberine) or **3** (1.40 g, 74% from palmatine) was obtained as brown powder.

Compound **3**: M.p. 174–176 °C; IR (KBr): 2960, 2940, 2905, 2834, 2216 (CN), 1622, 1604, 1508 cm^{-1} ; ^1H NMR (400 MHz) δ 2.73–3.87 (4 H, m, 2H-5 and 2H-6); 3.89, 3.90, 3.95 and 3.96 (4 OCH_3, s); 5.60 (1 H, s, H-8); 6.60 (1 H, s, H-4); 6.64 (1 H, s, H-13); 6.90 (1 H, d, 8Hz, H-12); 7.01 (1 H, d, 8Hz, H-11) and 7.15 (1 H,s, H-1).

Compound **7**: M.p. 180–182 °C; ^1H NMR: 2.85–4.0 (4 H, m, 2H-5 and 2H-6); 3.87 and 3.97 (2 OCH_3, s); 5.75 (1 H, s, H-8); 5.96 (2 H, s, OCH_2O); 6.14 (1 H, s, H-1); 6.59 (1 H, s, H-13); 6.86 (2 H, s, H-4 and H-12) and 7.16 (1 H, s, H-11).

3.1.2. Method 1: preparation of **2** *and* **9**

Palmatine (**1**) or berberine (**6**) (0.5 mM) in a mixture of 30% aqueous NaOH (20 ml), EtOH (20 ml) and $CHCl_3$ (20 ml) was heated under reflux for 4 h after which the reaction mixture was cooled and neutralised with 10% aqueous HCl. The mixture was then extracted with $CHCl_3$ (3×10 ml). The organic phases were pooled and dried with $MgSO_4$ before the solvent was stripped off at reduced pressure to yield a viscous residue. Upon chromatography of such a residue over a silica gel column using $CHCl_3$/MeOH mixtures of increasing polarity, **2** (73%) or **9** (56%) were obtained as the main products along with traces of **4** and **5** or **8** and **10**.

Compound **9**: M.p. 176–178 °C; ^1H NMR: 2.70–4.10 (4 H, m, 2H-5 and 2H-6); 3.70 –3.90 (6 H, s, 2 OCH_3); 5.90 (2 H, s, OCH_2O); 6.70 (1 H, s, H-1); 6.90 (2 H, bs, H-4 and H-11) 7.95 (1 H, bs, H-12) and 9.20 (1 H, s, HCO-13).

Compound **8**: M.p. 160–162 °C; ^1H NMR: 2.85–4.0 (4 H, m, H-5,6); 3.8–4.0 (6 H, s, 2OCH_3); 5.00 (1 H, s, H-8); 5.90 (2 H, s, OCH_2O); 6.50 (1 H, s, H-13); 6.70 (1 H, s, H-4); 6.95 (1 H, d, 7.8 Hz, H-12); 7.20 (1 H, d, 7.8 Hz, H-11) and 7.25 (1 H, s, H-1).

Compound **10**: M.p. 188–189 °C; ^1H NMR: 3.3–4.1 (4 H, m, H-5,6); 4.10 (6 H, s, 2 OCH_3); 6.30 (2 H, s, OCH_2O); 6.40 (1 H, s, H-13); 6.90 (1 H,s, H-4); 7.30 (1 H, d, 8 Hz, H-11); 7.60 (1 H, s, H-1) and 7.90 (1 H, d, 8 Hz, H-12).

3.1.3. Method 2: preparation of **4**

Palmatine (**1**) (0.5 mM) was treated as above but in the absence of EtOH to afford **4** (71%) as the main reaction product after usual workup and purification.

Compound **4**: M.p. 137–138 °C; IR (KBr): 3420 (OH), 1705 (C=O), 1650, 1590, 1550, 1490 cm^{-1}: ^1H NMR 2.7–4.15 (4 H, m, 2 H-5 and 2 H-6); 384–3.95 (12 H, s, 4 OCH_3); 5.80 (1 H, s, H-8); 6.70 (1 H, s, H-4); 7.00 (1 H, d, 8.4 Hz, H-11); 7.10 (1 H, s, H-1); 8.30 (1 H, d, 8.4 Hz, H-12) and 9.45 (1 H, s, HCO-13).

3.1.4 Alternative method for the preparation of **5** *and* **10** [20]

To 5 mM of palmatine (**1**) or berberine (**6**) dissolved in hot H_2O (50 ml) were dropped 15 g of KOH in 7 ml of H_2O. The mixture was stirred for 15 min and extracted with Et_2O (3×50 ml). The dark-brown precipitate was stirred with hot Et_2O for 20 min and filtered. The combined ether layers were washed with H_2O, dried (Na_2SO_4) and evaporated. Purification over column chromatogrphy on silica gel using EtOAc as solvent afforded **5** (42%) or **10** (46%).

Compound **5**: M.p. 183–185 °C; ^1H NMR: 2.90–4.5 (4 H, m, H-5,6); 3.8–4.0 (12 H, s, 4 OCH_3); 6.73 (1 H, s, H-4); 6.79 (1 H, s, H-1) and 7.23- 7.30 (3 H, m, H-11,12and 13).

3.2. Biology

The glycolytic enzymes from *Trypanosoma brucei* were prepared by overproducing in *Escherichia coli* and purified according to Hannaert et al. [14] for GAPDH and unpublished results for PGK and PFK. Commercialy available homologous enzymes: aldolase, triosephosphate isomerase, glycerol-3-phosphate deshydrogenase, pyruvate kinase were from rabbit muscle except lactate deshydrogenase (from beef heart) and 3-phosphoglycerate kinase (from yeast) as well as substrates, cofactors and linking enzymes were purchased from Boehringer Mannheim or from Sigma-Aldrich Company.

Activity was assayed spectrochemically at 340 nm with a Perkin Elmer spectrophotometer Lamda 15, at 25 °C, in a reaction volume of 1 ml. The assays were started by addition of the substrates in saturating concentration. Enzymes were used in quantities giving a maximal rate of about 30 $nmol \cdot min^{-1} \cdot l^{-1}$ and were incubated with varying concentrations of inhibitor for 5 min. The assay medium consisted of 0.1 M triethanolamine, HCl, pH = 7.6, ionic strength 0.15 adjusted with KCl.

The percentage of remaining activity was calculated by comparison with a control experiment in which the inhibitor was replaced by the same amount of solvent (DMSO). Possible effects of inhibitors on the absorbance of NADH were checked by reaction assays without enzyme.

The concentration of inhibitor required for 50% inhibition (IC_{50}) was calculated after at least five different concentrations of inhibitor.

Glyceraldehyde 3-phosphate dehydrogenase: 5 mM $MgSO_4$, 7 H_2O, 1 mM EDTA, 0.42 mM NADH, 1 mM $NaHCO_3$, 1 mM ATP disodium salt, 5.6 mM 3-phosphoglycerate, 50 μg 3-phosphoglycerate kinase from yeast.

3-Phosphoglycerate kinase: 10 mM $MgCl_2$, 6 H_2O, 0.50 mM KCl, 0.42 mM NADH disodium salt, 2.5 mM Phosphoenol pyruvate (PEP), 0.66 mM ATP disodium salt, 4.76 mM $NaHCO_3$, 5.6 mM 3-phosphoglycerate, 250 μg lactate deshydrogenase, 250 μg pyruvate kinase.

Phosphofructokinase: 2.5 mM $MgCl_2 \cdot 6H_2O$, 1.5 mM AMP, 0.6 mM NADH disodium salt, 2 mM F-6-P, 1 mM ATP disodium salt, 100 μg aldolase, 10 μg triosephosphate isomerase, 10 μg glycerol dehydrogenase.

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