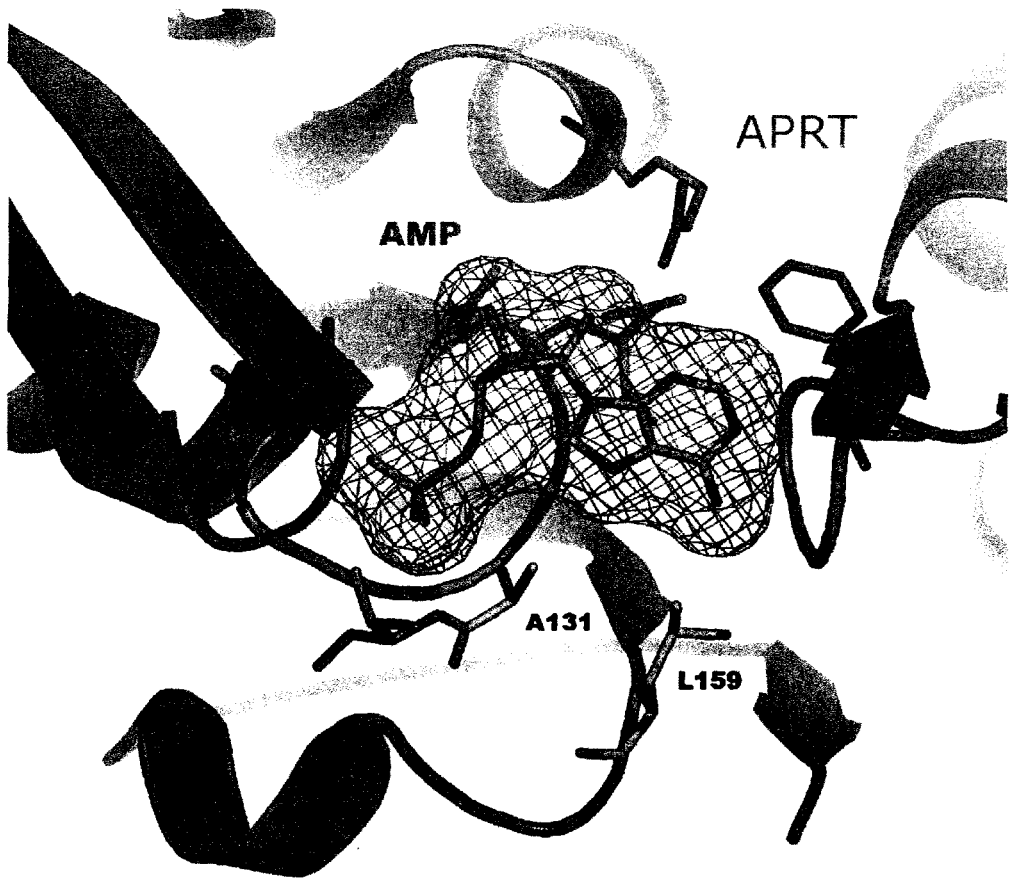
# Estudos moleculares e estruturais de enzimas de *Leishmania* visando o desenho racional de inibidores antiparasitários




Texto que sistematiza o trabalho científico do candidato para a obtenção do titulo de Livre Docência

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# Introdução

Esta tese trata de aspectos de biologia molecular e estrutural apresentados através de uma coletânea de artigos publicados ou submetidos para publicação, refletino nossa modesta contribuição na área da parasitologia molecular e estrutural. Nestes artigos as técnicas empregadas abrangem a bioquímica, biologia molecular, parasitologia molecular e cristalografia de proteínas.

A motivação que levou a escolha do tema desta tese focalizado nas vias de recuperação de purino nucleotídeos e na via glicolítica foram duas: Em primeiro lugar representa um conjunto de trabalhos realizados após meu doutoramento e assim caracteriza uma nova linha de pesquisa, tanto para mim quanto para o Grupo de Cristalografia de Proteínas e Biologia Estrutural do Instituto de Física de São Carlos; em segundo lugar permite a formulação de uma tese sobre uma tema coerente. Em contrapartida, os artigos aqui apresentados não representam a totalidade da minha produção científica ao longo destes anos.

Com a exceção do primeiro capítulo, cada capítulo traz uma breve introdução ao assunto a ser abordado e os artigos a ele relacionados.

O primeiro capitulo traz uma breve introducao aos temas desta tese, decrevendo a leishmaniose e ao desenho racional de inibidores viando futuro desenvolvimento de fármacos.

O segundo capitulo aborda a via de sintese de purinas. Apos uma breve introducao seguem-se quatro artigos que descrevem aspectos da bioquímica e estrutura das fosforibosil transferases (PRTases) de *Leishmania* e humana.

O terceiro capitulo aborda a via glicolítica. Apos uma breve introducao seguem-se cinco artigos. Os dois primeiros abordam a descrição da glucose-6-fosfato isomerase (PGI) humana e as observações decorrentes do estudo estrutural sobre esta enzima. Os dois artigos seguintes tratam da PGI de *Leishmania mexicana mexicana*, sua clonagem, caracterização, atividade de inibidores analogos ao substrato e sua estrutura cristalográfica. Finalmente o quinto artigo desreve os trabalhos realizados com a enolase de *Trypanosoma brucei*.

O quarto capitulo descreve trata da busca por inibidores de PRTases e *Leishmania* em extratos naturais, de plantas e animais marinhos. Compõe este capitulo sete artigos, os tres primeiros descrevem a estrutura cristalográfica de tres inibidores de uma Rutaceae *Adiscanthus fusciflorus* que possuem atividade inibitória sobre a APRT de *Leishmania*. O quarto artigo, aceito para publicação, descreve o isolamento e caracterização da atividade inibitória sobre o crescimento da cultura de *Leishmania major* do 7-geranyloxycoumarin, Aurapteno, uma cumarina isolada de *Esenbeckia febrifuga* (Rutaceae). O quinto artigo descreve os estudos bioquímicos e celulares de quatro inibidores isolados de *Adiscanthus fusciflorus*. Os dois artigos finais descrevem os avanços na identificação de compostos com atividade biológica de interesse a partir de extratos de animais marinhos.

# Capítulo I

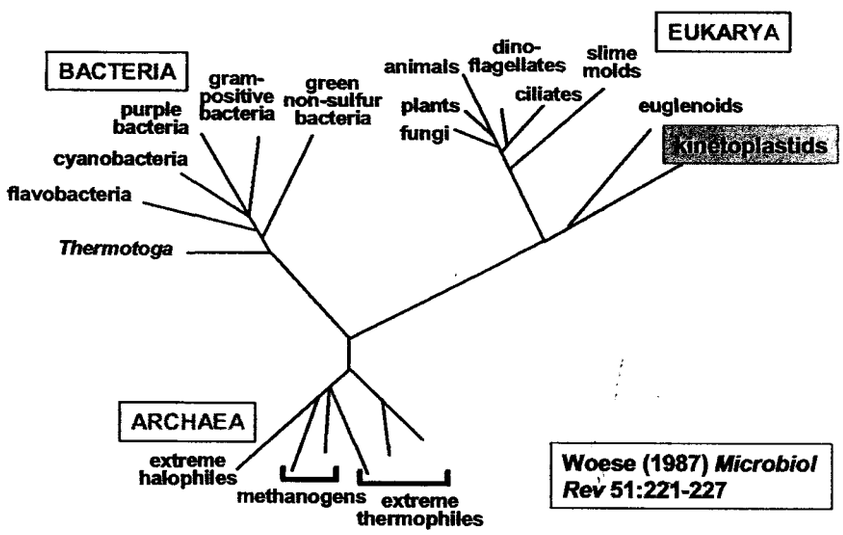
As décadas de 1950 e 1960 foram levadas por grande otimismo em assuntos referentes à saúde pública e agentes infecciosos. Em 1951, a Organização Mundial da Saúde (OMS) declarou que "... *malaria is no longer of major importance...*" e lançou-se uma ambiciosa campanha de erradicação da enfermidade [1]. Tal otimismo não se limitou a infecções por parasitos como a malária. Estendeu-se a todas as doenças infecciosas, bacterianas, virais, parasitarias, etc..., julgando-se que em poucos anos a humanidade estaria livre dos micróbios.

Apesar dos avanços da ciência nas ultimas décadas, muitos agentes etiológicos permanecem como uma ameaça à saúde publica e outros, considerados erradicados, de fácil tratamento ou mesmo sob controle, estão ressurgindo [2]. Isto se deve a fatores socioeconômicos e ambientais, fatores comportamentais de risco, especialmente em populações de refugiados, em emergências complexas, pela invasão de áreas silvestres ou pelo aparecimento de formas resistentes aos tratamentos disponíveis.

Dentre as patogenias para as quais nunca foi desenvolvido um tratamento adequado, seja por quimioterapia ou uma vacina eficaz, se destacam as doenças causadas por protozoários parasitas. Das etiologias causadas por espécies da ordem Kinetoplastida, destacamos as leishmanioses que estão colocadas na categoria I (doenças emergentes e não controladas) pela Organização Mundial da Saúde [3], junto com a doença do sono ou trypanosomíase africana e dengue,

apesar dos promissores resultados com o composto Miltefosina, em fase IV de testes clínicos.

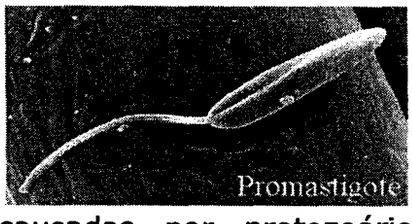
O interesse médico e de saúde pública despertado pelos Kinetoplastida se soma o interesse no sentido da pesquisa fundamental que os membros dessa ordem despertam. Esta ordem representa um ramo antigo dos eucariotos como descrito por Carl Woese e colaboradores em 1987 [4]. Em espécies de Kinetoplastida foram identificados fenômenos de grande interesse como a editoração do RNA mitocondrial por adição e deleção de uridinas. Este um fenômeno intrigante e ainda pouco resolvido. A falta de tRNAs codificados pelo genoma mitocondrial, apesar dessa organela possuir capacidade de tradução e síntese protéica, implica na importação de tRNAs do citoplasma para a matriz mitocondrial. O complexo genoma mitocondrial e sua incomum organização são alvos de intensos estudos desde sua descoberta. Transcrição policistrônica, uma característica de bactérias foi identificada em Kinetoplastida também, assim como a existência de um sistema de trans-splicing, ao invés do cis-splicing identificado em outros eucariotos [5].



Recentemente foi inclusive identificada a presença de genes de cloroplasto no genoma nuclear de *Trypanosoma* [6]. Esta descoberta implica em uma perda secundaria dessa organela e pode vir a ter interessantes desdobramentos.

As características peculiares citadas alem da importância a saúde publica humana, com as conseqüentes implicações sócio-econômicas resultantes de tornam os estudos desta ordem de grande interesse.

## 1.1- Leishmanioses




As leishmanioses são zoonoses classificadas em oito diferentes grupos segundo a sintomatologia e espécie de parasito envolvido (Tabela I). São causadas por protozoários de diversas espécies pertencentes ao gênero *Leishmania*, ordem Kinetoplastida. Estima-se que 400.000 novos casos ocorram anualmente nas regiões tropicais e subtropicais do globo, estando mais de 10 milhões de indivíduos contaminados e 400 milhões sob risco de infecção [7, 8]. Esses alarmantes números retratam um alto índice de mortalidade e morbidade das leishmanioses. Em humanos a doença tem manifestação crônica de apresentação cutânea ou visceral. As formas cutâneas do *Novo Mundo* se caracterizam por desenvolverem lesões metastáticas mutilantes nas várias mucosas. As formas viscerais, distribuídas em ambos os hemisférios, se caracterizam por infecções do sistema retículo-endotelial.

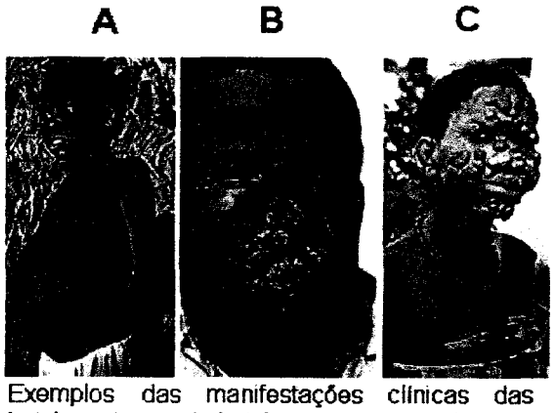
Tabela I. Classificação do gênero *Leishmania*.

| Species de *Leishmania* [a] | Condição clínica [b] | Epidemiologia [c] |
|---|---|---|
| A. Seção [d] Hypopylaria | | |
| Lagartos – Novo Mundo | | |
| *Leishmania agamae* | | |
| *Leishmania ceramodactyli* | | |
| B. Seção Peripylaria | | |
| Lagartos – Velho Mundo | | |
| *Leishmania adleri* | | |
| *Leishmania tarentolae* | | |
| Mamíferos – Novo Mundo | | |
| Complexo *Leishmania braziliensis* [i] (subgênero *Viannia* [j]) | | |
| *L. b. braziliensis* | C,M | ZVL |
| *L. b. guyanensis* | C | ZVL |
| *L. b. panamensis* | C, M | ZVL |
| *L. b. peruviana* | C | ZVL |
| *L. b. laisoni* [f] | C | |
| *L. b. colombiensis* | C | |
| *L. b. naiffi* [f] | | |
| *L. b. shawi* [f] | | |
| *L. b. equatorensis* | | |
| C. Seção Suprapylaria | | |
| Mamíferos – Novo Mundo | | |
| Complexo *Leishmania mexicana* [i] (subgênero *Leishmania*) | | |
| *L. m. mexicana* | C (D) | ZVL |
| *L. m. amazonensis* | C, D, V [h], P, M | ZVL |
| *L. m. pifanoi* | D (C) | |
| *L. m. aristidesi* | | |
| *L. m. venezuelensis* | C | |
| *L. m. garnhami* | C | |
| *L. m. enrietti* | | |
| *L. m. forattinii* | | |
| Complexo *Leishmania hertigi* (subgênero *Leishmania*) | | |
| *L. h. hertigi* | | |
| *L. h. deanei* | | |
| Mamíferos – Velho Mundo | | |
| Complexo *Leishmania donovani* (subgênero *Leishmania*) | | |
| *L. d. donovani* | V, P | AVL |
| *L. d. infantum* | V, C [h], A | ZVL |
| *L. d. chagasi* [e] | V, C [h], A | ZVL |
| *L. d. archibaldi* | V, C, O | |
| Complexo *Leishmania tropica* (subgênero *Leishmania*) | | |
| *L. t. tropica* | C, R, V [h], P, O | AVL |
| *L. t. killicki* | C | |
| *L. aethiopica* | C, D, M | ZVL |
| Complexo *Leishmania major* (subgênero *Leishmania*) | | |
| *L. m. major* | C, D, [V] | ZVL |
| *Leishmania gerbilli* [g] | | |
| *Leishmania turanica* n. sp. [g] | | |
| Lagartos – Velho Mundo | | |
| *Leishmania gymnodactyli* | | |
| *Leishmania hemidactyli* | | |
| *Leishmania hoogstraali* | | |
| *Leishmania zmeevi* | | |

[a] Adaptado de [10-16]. Esta tabela exclue a descrição "*Leishmania* sp." Para conter apenas aquelas espécies identificadas seguindo a proposta de Lainson. R. and Shaw. J. J.[10]. [b] Resumo das condições clínicas humanas [15,16]: A, leishmaniose asintomática; C, leishmaniose cutânea (CL); D, leishmaniose difusa cutânea (DCL, disseminada); M, leishmaniose mucocutânea (MCL); O, leishmaniose oronasal; P, leishmaniose post-kala-azar dermica (PKDL); R, leishmaniose recidiva (LR, forma de relapso); V, leishmaniose visceral (VL); ( ) existência incerta; [ ], baseada em um único caso [16]. [c] Características epidemiológicas de acordo com Desjeux, P. [15]: AVL, leishmaniose antroponótica visceral; ZVL, leishmaniose zoonótica visceral. [d] Seção é um termo taxonômico aceito e introduzido por Lainson R. e Shaw, J. J. in 1979 [12]. [e] *L. chagasi* foi incluído no complexo *Leishmania donovani* por Momen, H. et al. [17]. [f] *Leishmania naiffi*, *L. laisoni* e *L. shawi* foram classificadas por Desjeux, P. [15] como patogênicas a humanos apesar de apenas infecções em animais terem sido descritas [16]. [g] *L. gerbilli* e *L. turanica* foram isoladas de *Rhombomys opimus* e são endêmicas da Ásia Central [18] e Russia [19] respectivamente. Estas espécies são caracterizadas por desenvolvimento suprapylaria. Até o momento estas espécies não foram classificadas em um complexo específico. [h] Manifestação rara [15]. [i] A classificação de "complexo" foi introduzida por Lainson, R e Shaw, J. J. [11, 13]. [j] O subgênero *Viannia* foi proposto em 1989 por Lainson, R. et. al. 1989.

Célula de *Leishmania major* (amarelo) sendo fagocitada por um macrófago. Figura de microscopia eletronica de varredura colorida artificialmente.

As formas de tratamento têm se baseado no uso de antimônicos pentavelentes como o antimonato de N-methyl glucamina (Glucantine) e o estibogluconato de sódio (Pentostam) [20-22]. A duração da terapia e a resposta clínica variam de região a região e da interação patógeno-hospedeiro. Na prática, a aplicação de altas doses por prolongados períodos é comum. Isto se deve em parte a rápida eliminação destes compostos da corrente sangüínea. Em casos de recidiva e nas infecções por *L. aethiopica* ou leishmanioses viscerais, indica-se o emprego da anfotericina B (Fungizon) [22, 23]. A anfotericina B apresenta efeitos colaterais indesejados e até graves, portanto necessitando ser ministrada em hospitais, uma dificuldade adicional no tratamento, em se considerando as condições de saúde pública prevalentes nas regiões endêmicas. Esta terapia tem se mostrado pouco




Exemplos das manifestações clínicas das Leishmanioses. A- Leishmaniose visceral. Nota-se o abdomen distendido indicando splenomegalia e a severa atrofia muscular. B- a Leishmaniose mucocutânea. Nota-se o comprometimento da região da oral e nasal. C- Leishmaniose cutânea. Nota-se o aspecto pustular desta manifestação em particular. (de www.who.int/tdr)

eficiente em diversos casos. Em pacientes resistentes ao Glucantine e ao Fungizon não se identificam alternativas eficazes. Tende-se a repetir o tratamento, com doses nos limites da tolerância ou a utilizar o isotionato de pentamidina como uma última alternativa [20, 21]. Aliado a baixa eficácia de tratamento, se observam também diversas reações de toxicidade que vão desde náusea, vômitos e mialgia até, nos casos mais graves o choque, alterações eletrocardiográficas, hipersensibilidade e trombose venosa. Estas características indesejáveis, associadas ao aparecimento de formas resistentes de *Leishmania* [23], têm exacerbado a necessidade do desenvolvimento de drogas antileishmanióticas mais eficazes e menos tóxicas ao paciente. A toxicidade é particularmente severa no tratamento de crianças e idosos ou indivíduos portadores de infecções oportunistas secundárias.

## 1.2- Desenho e desenvolvimento de fármacos

A abordagem moderna para o desenvolvimento de drogas [24, 25] explora enzimas únicas ao metabolismo do parasito ou que possuam diferenças de especificidade catalítica entre a enzima do parasito e a do hospedeiro. Estas enzimas podem ser empregadas como alvo no desenvolvimento de inibidores da cadeia metabólica, sem com isso afetar o hospedeiro. Para tanto, torna se indispensável à determinação da estrutura tridimensional da enzima, a caracterização do mecanismo de catálise e identificação do sítio catalítico, bem como de domínios reguladores presentes na mesma [24]. Analises filogenéticas

mostram que os protozoários parasitos formam clades relativamente profundas tendo divergido do restante da linhagem eucariota em um ancestral primitivo [26, 27]. Esta observação é uma evidência adicional que mesmo enzimas homólogas entre o hospedeiro e os parasitos podem ter divergido suficientemente permitindo o seu uso como alvos em abordagens de quimioterapia. Um estudo dessa magnitude requer o desenvolvimento de proteínas mutantes, resistentes a inibidores ou com características cinéticas alteradas a fim de permitirem elucidar o mecanismo catalítico e os aminoácidos envolvidos no sítio catalítico. O primeiro composto desenvolvido desta forma foi o anti-hipertensivo Captopril, um inibidor da angiotensina convertase. O acúmulo de dados referentes a estruturas cristalográficas e cinética de diversas enzimas levou ao desenho de vários compostos adicionais para o emprego clínico (para uma revisão veja Kubinyi, H.[24]).

# Capítulo II

## 2.1- Síntese de purino-nucleotídeos

O metabolismo de purino-nucleotídeos em protozoários parasitos é significativamente diferente daquele de mamíferos. Em humanos os purino-nucleotídeos são sintetizados *de novo*, a partir de precursores não-nucleotídeo tais como aminoácidos, amônia e dióxido de carbono. Os purino-nucleotídeos podem também ser reciclados pela *via de recuperação* [28-31]. Os passos enzimáticos da via de síntese *de novo* são bem conservados ao longo de diversos taxa.

Os nucleotídeos podem também ser formados a partir de purinas e purino-nucleosídeos livres. Na *via de recuperação*, as purinas reagem com 5'-fosforibosil-1-pirofosfato (PRPP) formando os nucleosídeos-5'-monofosfatos correspondentes. Estas reações reversíveis são catalisadas por distintas fosforibosil-transferases (PRTases) substrato-específicas. As PRTases formam uma classe de aproximadamente 10 enzimas que catalisam reações semelhantes envolvendo PRPP e uma base nitrogenada [32]. Em mamíferos, essas reações são catalisadas por duas enzimas distintas: adenina-fosforibosil-transferase (APRT; AMP:pirofosfato fosforibosiltransferase; EC 2.4.2.7) e hipoxantina-guanina-fosforibosil-transferase (HGPRT; IMP:pirofosfato-fosforibosil-transferase; EC 2.4.2.8) nas reações reversíveis esquematizadas na figura 1. A HGPRT catalisa a remoção do grupo pirofosfato ($PP_i$) de PRPP e a adição de uma base purínica

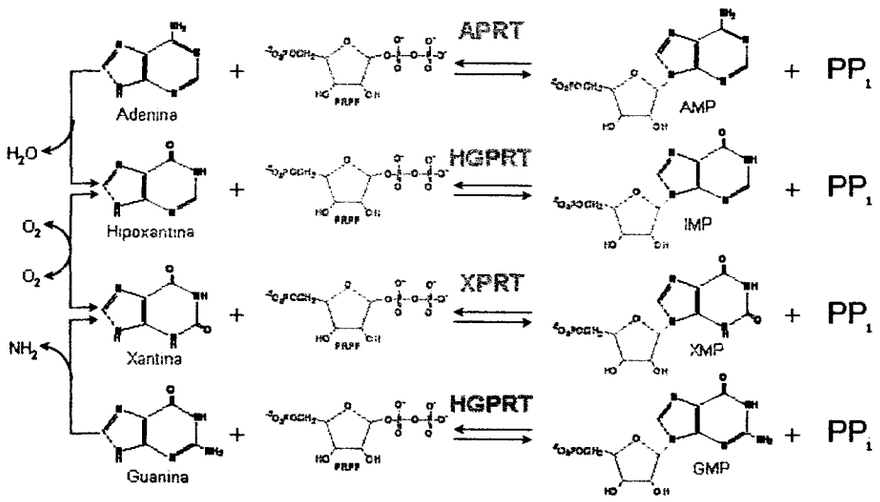



**Figura 1:** As diversas reações de transferência catalisadas pelas **PRTases** estão mostradas. **PRPP** 5'-fosforibosil-1-pirofosfato, **APRT** adenina-fosforibosil-transferase; **HGPRT** hipoxantina-guanina fosforibosil-transferase; **XPRT** xantina fosforibosil-transferase; **PPi** pirofosfato.

(hipoxantina ou guanina) em uma reação do tipo SN2 (ataque nucleofílico de segunda ordem) aparentemente. O mecanismo de reação da maioria das PRTases começa a ser elucidado. Mecanismo do tipo duplo-deslocamento (mecanismo "pingue-pongue") foi sugerido para a OPRT (orotato-fosforibosil-transferase, EC 2.4.2.10) [33] e UPRT (uridina-fosforibosil-transferase, EC 2.4.2.9) de levedura [34]. Giaconello A. e Salerno, C. [35] sugeriram que HGPRT de humanos segue o mecanismo seqüencial em um modelo aleatório e de rápido equilíbrio, onde os produtos se dissociam em seqüência ordenada, primeiro a base purínica seguida do complexo Mg-P-Rib-PP (magnésio-fosforibosil-pirofosfato). Entretanto, a análise de HGPRT de *Schistosoma mansoni* favorece o modelo de reação seqüencial ordenada, onde PRPP se liga a enzima primeiro e os produtos IMP ou GMP são liberados por último [36]. Este modelo difere do proposto para a HGPRT de humanos [35], e sugere a possibilidade de desenho de inibidores específicos para o complexo binário enzima-purina. O pirofosfato formado ($PP_i$) na reação de transferência é rapidamente hidrolisado favorecendo o deslocamento do equilíbrio de reação para a formação dos mononucleosídeos.

Esta cadeia de recuperação permite ao organismo a reutilização de purinas obtidas da degradação de ácidos nucleicos ou nucleotídeos (Figura 2). A *via de recuperação* está presente em todos os organismos estudados, incluindo protozoários parasitos [28, 31]. Em Kinetoplastida e diversos outros protozoários, a *via de recuperação* consiste na única forma de obtenção de purino-nucleotídeos [31, 37]. Como conseqüência cada gênero de parasito, auxotrófico para purino-nucleotídeos, desenvolveu um conjunto específico de enzimas da *via de recuperação* que lhes permite utilizar purinas pré-formadas (Figura 3). As implicações terapêuticas do metabolismo de purino-nucleotídeos em kinetoplastidas são substanciais [30, 31].

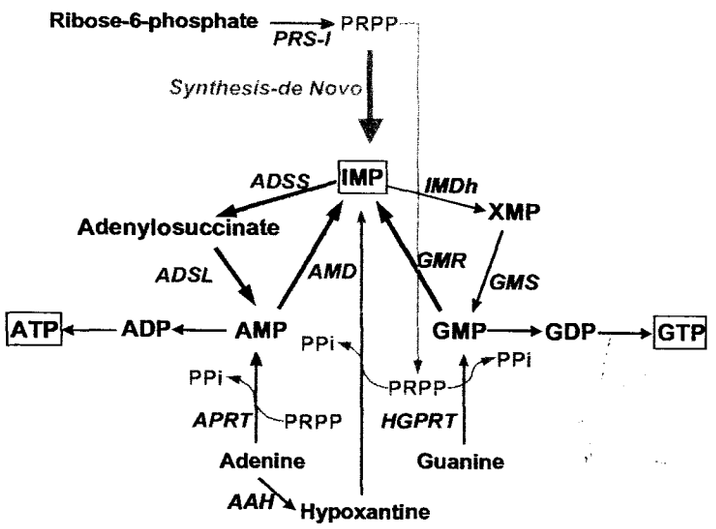



**Figura 2:** Via de recuperação e interconverção, em células de mamíferos. APRT- adenina-fosforibosil-transferase; HGPRT- hipoxantina-guanina-fosforibosil-transferase; PRPP- 5'-fosforibosil-1-pirofosfato.

Os Kinetoplastida se destacam pela suscetibilidade a análogos de purino-nucleotídeos [31, 37, 38, 39]. A HGPRT de *Leishmania* é relativamente inespecífica e pode utilizar pirazolopirimidinas como substrato. O mais estudado desses compostos é o análogo da hipoxantina, alopurinol (4-hidroxipirazol[3,4]pirimidina) (Figura 4). O alopurinol é metabolizado por HGPRT

aos vários análogos de IMP (Figura 4), um eficaz inibidor de succinil-AMP-sintase e GMP-redutase [40]. Conseqüentemente, alopurinol acarreta a inibição da biossíntese de purinas nesses organismos. Além do efeito inibitório descrito, o análogo de IMP é convertido a análogos de AMP que uma vez fosforilados a ATP são eficientemente incorporados na transcrição de RNA e levam a uma rápida degradação dos mesmos, acompanhado da inibição de síntese protéica [28].

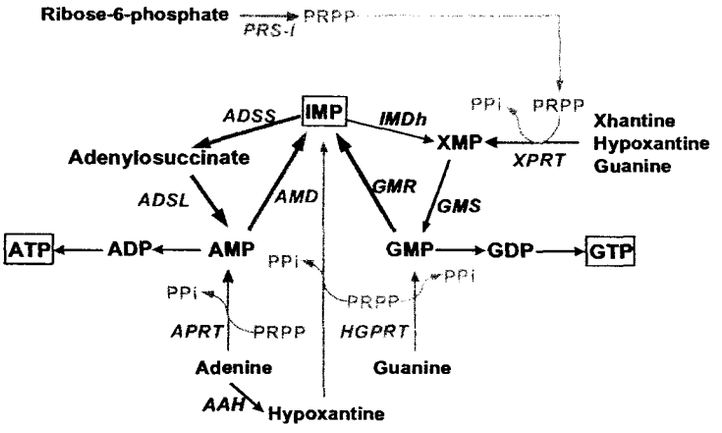



**Figura 3:** Via de recuperação e interconverção, em células de *Kinetoplastida*. APRT- adenina-fosforibosil-transferase; HGPRT- hipoxantina-guanina-fosforibosil-transferase; PRPP- 5'-fosforibosil-1-pirofosfato. XPRT- xantina-guanina-fosforibosil-transferase; AAH- adenina deaminase.

Alopurinol é utilizado extensivamente no tratamento de hiperuricemia (ácido úrico sangüíneo elevado) e artrite aguda (gota) [41]. Também tem sido explorado clinicamente no tratamento de leishmaniose cutânea [42] e Doença de Chagas crônica [43]. Isto se deve, em parte, pela incapacidade das PRTases humanas de metabolizarem este composto [31, 44]. Entretanto, reações adversas e a falha terapêutica foram observadas no uso de alopurinol [43, 42] alertando para a necessidade de aprimorar este composto. A *via de recuperação* de *Leishmania* e *Trypanosoma* se encontra esquematizada na figura 3. Esta via varia substancialmente entre os diversos protozoários parasitas e foi revisada em detalhe por Berens, R. L. *et. al.* (1995) [31]. A heterogeneidade nos componentes enzimáticos da *via de recuperação* dos

purino-nucleotídeos dentre os protozoários pode ser uma conseqüência da pressão seletiva sofrida em seus nichos ecológicos específicos. A maioria dos parasitos, incluindo *Plasmodium*, *Leishmania*, *Trypanosoma* e *Toxoplasma*, depende da atividade de fosforibosil-transferases para a recuperação de purino-nucleotídeos [31].

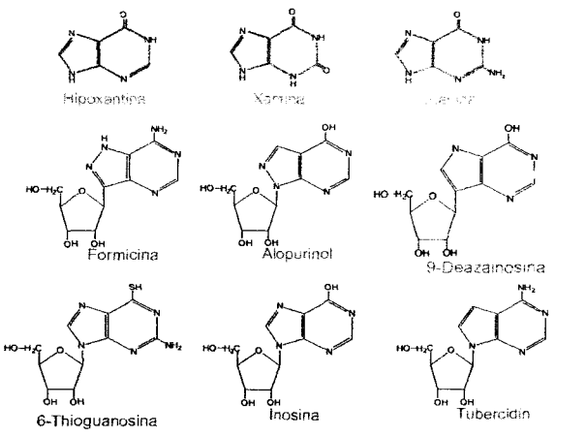



**Figura 4:** Bases nitrogenadas: Hipoxantina e Guanina, substratos das HGPRTases. Abaixo, alguns análogos de purino-nucleotídeos testados como inibidores em diversos parasitos protozoários.

Estas diferenças entre as espécies de *Leishmania* e os hospedeiros mamíferos tornam a resolução da estrutura tridimensional e o estudo de mutantes das PRTases muito atraentes como alvos para o desenho racional de drogas [29, 39, 40].

## 2.2- Trabalhos apresentados em seguida

Apresento em seguida quatro artigos relacionados com a familia das fosforibosil-transferases (PRTases). O primeiro descreve a estrutura cristalográfica da Adenina fosforibosil-transferase (APRT) de *Leishmania tarentolae* em complexo com o produto de reação AMP. Esta estrutura foi resolvida a 2.2Å e permitiu, baseado na sobreposição estrutural com outras PRTases e calculos de potenciais de interação molecular sugerir que o PRPP é o primeiro substrato a se ligar ao sítio ativo, enquanto o AMP é o ultimo substrato a deixar o sitio ativo.

O segundo artigo descreve a estrutura cristalográfica da APRT humana a uma resolucao de 2.1Å e contendo AMP ligado ao sítio ativo da enzima. A resolução dessa estrutura permitiu a identificação de importantes residuos envolvidos na especificidade ao substrato e discriminação de bases. A analise da estrutura da APRT humana com os dados clínicos de pacientes acometidos de urolitíase forneceu informações estruturais para os mecanismos pelos quais essas mutações levam a urolitiase.

O terceiro artigo descreve a clonagem, caracterização molecular e a cristalização da hipoxantina-guanina fosforibosil-transferase (HGPRT) de *Leishmania tarentolae*. Esta representa a primeira HGPRT de *Leishmania* cristalizada. Uma publicação descrevendo as analises decorrentes deste trabalho esta em fase de redação.

O quarto artigo descreve a clonagem e caracterização molecular da xantina fosforibosil-transferase (XPRT) de *Leishmania major*. A cristalização desta enima ainda não foi obtida, porem um modelo estrutural foi obtido baseado na estrutura de outras PRTases descritas anteriormente. Este artigo foi recentemente submetido para publicação.

1. Silva M, Silva C.H, Iulek J, Oliva G, and **Thiemann OH**. *Crystal structure of adenine phosphoribosyltransferase from Leishmania tarentolae: potential implications for APRT catalytic mechanism.* Biochim Biophys Acta. 2004;1696(1):31-9.

2. Silva M, Silva C.H, Iulek J, and **Thiemann O.H.** *Three-dimensional structure of human adenine phosphoribosyltransferase and its relation to DHA-urolithiasis*. Biochemistry. 2004;43(24):7663-71.

3. Monzani P.S, Alfonzo J.D, Simpson L, Oliva G, and **Thiemann O.H**. *Cloning, characterization and preliminary crystallographic analysis of Leishmania hypoxanthine-guanine phosphoribosyltransferase.* Biochim Biophys Acta. 2002;1598(1-2):3-9.

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SILVA, M.; SILVA, C. H. T. P.; IULEK, J.; OLIVA, G.; THIEMANN, O. H. Crystal structure of adenine phosphoribosyl transferase from Leishmania tarentolae : potential implications for APRT catalytic mechanism. **Biochimica et Biophysica Acta** : proteins and proteomics, Amsterdam, v. 1696, n. 1, p. 31-39, Jan. 2004.

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# Biochemical and structural characterization of *Leishmania major* Friedling xanthine phosphoribosyltransferase

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Protozoan parasites of the order Kinetoplastida, as well as several known protozoa, are strict purine nucleotide auxotrophs [1], lacking the entire *de novo* purine synthesis pathway and relying on the host and the recycling by the salvage pathway for the required purine nucleotides. In contrast, most organisms synthesize purine nucleotides by both the de *novo* and the salvage pathways. In the kinetoplastids three PRTase are known to be involved in the recycling of purine nucleotides; hypoxanthine–guanine PRTase (HGPRT) (EC 2.4.2.8), adenine PRTase (APRT) (EC 2.4.2.7) and xanthine PRTase (XPRT) (EC 2.4.2.22) [2]. XPRT is responsible for catalyzing the conversion of xanthine and α-D-5-phosphoribosyl 1-pyrophosphate (PRPP) into xanthine-5-monophosphate (XMP) and pyrophosphate (PPi) by the anomeric inversion of the ribofuranose ring [3]. Kinetic analysis of *Leishmania donovani* XPRT revealed that this enzyme preferentially phosphoribosylated xanthine but could also recognize hypoxanthine and guanine [4] as substrates. The structural explanation for such selectivity remains unclear, since the only structural information available comes from *E. coli* XGPRT crystallographic structure. In that structure, the substrate specific interaction with $Glu^{136}$ and $Asp^{140}$ residues through a water molecule, could be responsible for guanine > xanthine >> hipoxantine selectivity [5; 6].

In a broad project to design *Leishmania* specific inhibitors, we have cloned the *xprt* gene from *L. major* Friedling based on the sequence identified in the *Leishmania* genome effort. We overexpressed the recombinant enzyme to homogeneity in a heterologous expression system of *Escherichia coli.* The access to the recombinant protein allows the kinetic characterization of the recombinant enzyme and opens the possibility of its use in the search for novel PRTases inhibitors, as well as the molecular understanding of base selectivity of each PRTase. To advance our understanding on these essential enzymes a structural model based on homology principles was built. According to molecular dynamics protocols this model is stable and can correctly account for the XPRT topology.

The *L. major xprt* gene was amplified by PCR (Polymerase Chain Reaction) from genomic DNA with oligodeoxyribonucleotides primers specific for the 5' and 3' ends of the known *L. major xprt* gene. The primers for PCR amplification introduce a *Nde* I and a *Xho* I restriction site at the highlighted positions (5' TAGCTACATATGCTACCAACCCACATG - *Nde* I; 5'TCTCGAGTCAGAGCTTGGCAGGATAAC GGG 3' - *Xho* I). The *xprt* DNA fragment was cloned into the expression vector pET-29a (Novagen) at the *Nde* I/*Xho* I sites and transformed into *E. coli* BL21 (DE3) competent cells by standard protocols [7]

The amplified *xprt* DNA contained an open reading frame of 726 base pairs that encodes for a protein of 241 amino acids with sequence identity to other XPRTases. Alignment of *L. major* XPRT with other sequences highlights the conserved purine and PRPP binding domains (fig1) [8]. The predicted amino acid sequence of *L. major* XPRT shares 87% identity with the *L. donovani* XPRT [4] and sequence divergence is mostly concentrated at the N- and C- terminus regions.

The purification of recombinant XPRT was carried out at 4°C unless otherwise specified and using the following steps: *E. coli* cells were induced at an $O.D._{600}$ of 0.8 with 0.5mM IPTG for 15h at 20°C in LB media, and harvested by centrifugation (25000g for 15 min.). The cell pellet was dissolved in 100 mM Tris–HCl, 10 mM MgCl2, 1mM PMSF and 1mM leupeptin, pH 7.5 (buffer A) and cell lysis was achieved by ten 1-min cycles of sonication in an ice bath. The crude extract was clarified by centrifugation (25000g for 15 min.) and brought to 50% (w/v) ammonium sulfate for 25 min with slow stirring. The suspension was separated by centrifugation (25000g for 15 min.) and the pellet was dissolved in 5mL of buffer A and loaded in a 16-ml Phenyl-sepharose column, equilibrated in buffer A + 1M $(NH_4)_2SO_4$ at 0.8 ml/min flux. XPRT eluted at 0.7M $(NH_4)_2SO_4$. The protein fractions were pulled, desalted in a 15mL Hytrap desalting column and loaded into a 15mL Fast flow Q-sepharose. XPRT eluted in the column void with only trace contaminants as revealed by commansie stained SDS-PAGE (fig. 2).

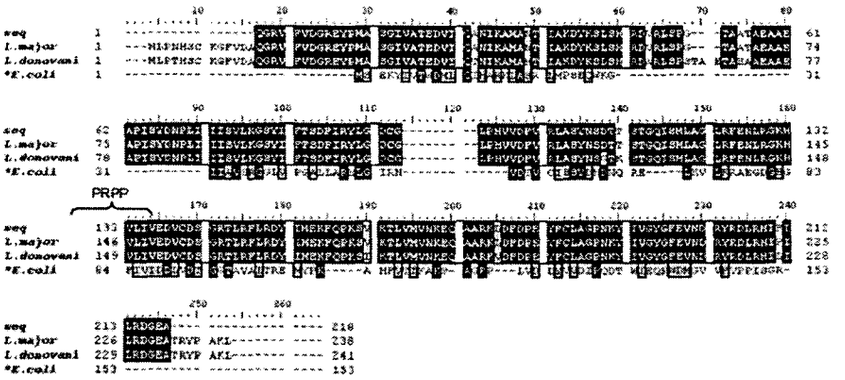
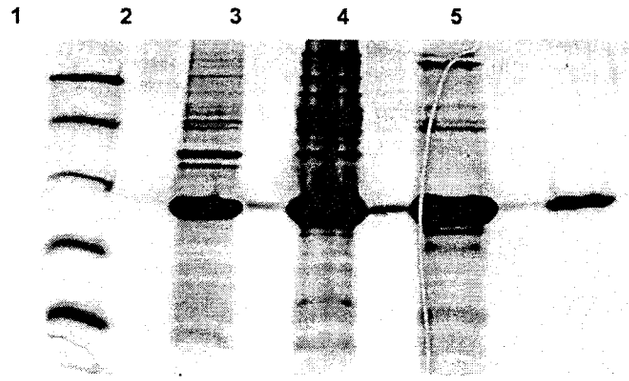



**Figure 1**- Multiple alignments of representative XPRT sequences. The amino acid sequences of several XPRT proteins are shown aligned with the *L. major* Friedling sequence. The amino acid positions are indicated at the sides of the alignment. The shaded boxes indicate conserved mutation (gray) and conserved residues (black) and the predicted PRPP binding site is highlighted in the picture. The sequence seq represents the *xprt* gene cloned in our laboratory. The sequences were aligned by the CLUSTALX program [9]




| Purification step | Total Protein (mg/ml) | Specific Activity (U) | Purification fold |
|---|---|---|---|
| Lysis | 217,8 | $8,01.10^{-4}$ | 0 |
| $(NH4)_2SO_4$ | 138,5 | $1,2.10^{-3}$ | 1,4 |
| Phenyl sepharose | 51,0 | $1,9.10^{-3}$ | 1,5 |
| Q sepharose | 15,5 | $3,6.10^{-3}$ | 4,5 |

Figure 2- (upper panel) Expression of *L. major* Friedling XPRT in *E. coli* and purification of the recombinant protein.. The proteins were separated in a 15% SDS-PAGE gel. Lanes: 1—molecular weigh marker, 2—clarified extracts (S20000 supernatant), 3—ammonium sulfate precipitate fraction, 4—pooled fractions from Phenyl sepharose HP, 5- pooled fractions from Q sapharose FF. (lower panel) Table of XPRT purification.

The kinetic parameters of *L. major* XPRT were determined spectrophotometricaly at 254 nm as described by Jardim et al [4] at 25°C over a 30 seconds reaction. The $K_m$ value for xanthine and guanine are similar to those reported for *L. donovani* XPRT 7 ± 2μM for xanthine and above 100 μM for guanine. The $K_m$ for hypoxanthine of 61 ± 26 μM for *L. major* XPRT differs by seven-fold to the *L donovani* XPRT 450 ± 97 μM for the same substrate. Such contrast in $K_m$ values can be due to small

structure differences between the two enzymes. Unfortunately, the structure of neither enzyme has been determined at atomic resolution. This fact prompted us to model *L. major* XPRT 3D structure using *T. cruzi* HGPRT, another Type I PRTase (PDB code 1TC2) [10], as a model (figure 3a). This task was performed with the Modeller program [11].

Despite low sequence identity among XPRTases, all type I enzymes show a conserved folding comprising 4-5 parallel β sheets flanked by 3-4 α helix [12] which should be conserved between the template (1TC2) and our homology based model. Ramachandran plots [13] and Verify-3D scores [14] were used to evaluate the quality of the best 3 models. All residues of our model are located in allowed regions. Verify-3D scores for individual residues, with a sliding window of 21 residues, were above 0.2.

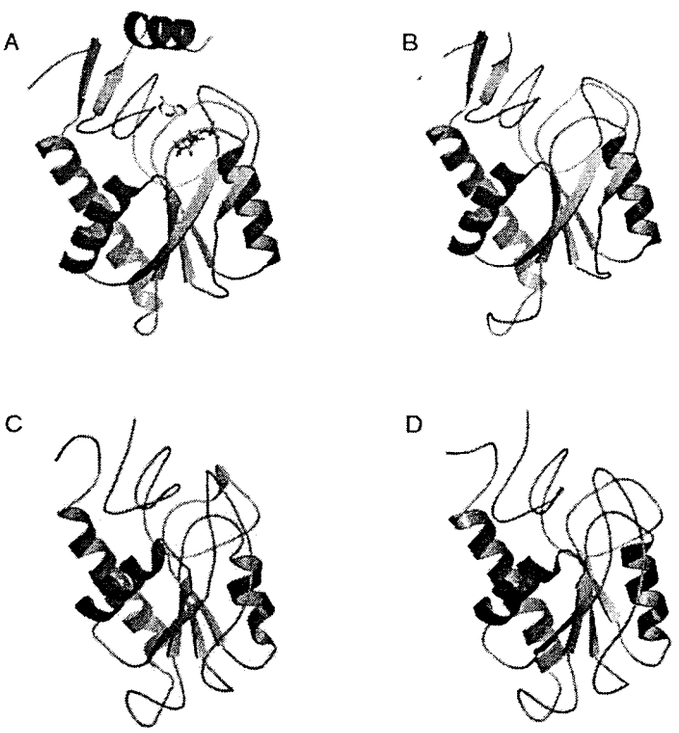



Figure 3- The panel shows 1TC2 crystallographic structure and the XPRT homology generated model in three different conformations. (a) 1TC2 secondary structure is presented as well as PRPP and alopurinol binding sites. (b) XPRT homology model generated by MODELLER 6.0 program shows high topological similarity to 1TC2, except for the first helix which has not been modeled. (c) XPRT energy minimized structure show that hood domain loses most secondary structure in the absence of ligands (during minimization and molecular dynamics studies only protein atoms were considered), although the PRPP binding domain is overall conserved. (d) XPRT model after 200 pico seconds shows that the overall topology is stable.

Molecular dynamic simulations, using the GROMACS [15] package, were used to evaluate the molecular stability of our model. The model was relaxed through energy minimization using the conjugate gradient algorithm, followed by a 100 pico seconds molecular dynamics at 300K ($27^0$C) in vacuum. Subsequently, 5260 water molecules were added to the system resulting in a rectangular box of 6.4 x 6.4 x 4.5 nm. An energy minimization was performed using the conjugate gradient algorithm and then a molecular dynamics simulation was carried on at 300K ($27^0$C) and 1 atm for 200 pico

seconds. A time step of 1 fento second and periodic boundary conditions were applied in all simulations. The long-range coulomb interactions were computed using the PME *(Particle Mesh Ewald)* [16] algorithm and for short-range coulomb and Van der Waals interactions a cut-off of 2.0 nm was used. For temperature and pressure coupling we used the Berendsen algorithms [17]. The overall three-dimensional structure of the model did not changed significantly through out the molecular dynamics simulation. For a matter of comparison, the initial and final structures are depicted in figure 3b.

From the superposition of 1TC2 on our homology model, it can be seen that when the purine substrate is located in the binding site it shall interact with residues $Val_{121}$, $Lys_{152}$, $Tyr_{174}$, $Ile_{175}$ and $Glu_{181}$. It is hypothesized that $Tyr_{174}$ and $Val_{121}$ encase the purine ring while the other residues are involved in hydrogen bond interactions.

In fact, XPRT purine binding site model differs from the template HGPRT only in residues $Val_{121}$, $Tyr_{174}$, $Phe_{180}$, $Glu_{181}$ ($Ile_{113}$, $Phe_{164}$, $Leu_{170}$, $Asp_{171}$ respectively). Therefore we hypothesize that these residues must be responsible for xanthine, over hypoxanthine selectivity. As previously described [18], several subtle structural differences contribute to the efficient use of xanthine over other substrates. Indeed greater xanthine selectivity may *not* be attributed to just one or a few particular amino acids, since substrate positioning in the active site may play an important role in this subject. For instance, in the human HGPRT [19,20], guanine binds through a hydrogen bond between $NH_2$ and the main chain oxygen of residue $Asp_{206}$, while xanthine binds through a hydrogen bond between carbonyl oxygen and $Asp_{206}$ main chain NH, revealing that selectivity is due to substrate shift inside the purine binding site.

Although an entire understanding of substrate selectivity would require a complete structural analysis, it is well established that some of the described mutations are related to xanthine selectivity. Just to name one, as described for *Tritrichomonas foetus* HGPRT [21], $Tyr_{174}$ hydrogen bonds to $Glu_{181}$, repositioning the main chain NH to interact with xanthine. It must be said though that there is some evidence that this interaction is not critical to xanthine use. First, human, *Trypanosoma cruzi*, and *Plasmodium falciparum* HGPRT have phenylalanine at this position, yet *P. falciparum* HGPRT *is* able to use xanthine effectively. Second, the $Trp_{199}Phe$ mutant of *T. gondii* HGPRT has robust xanthine PRTase activity [22].

Following the same superposition strategy, the PRPP binding site, whose residues are quite conserved in type I PRTases, can be easily identified in our model ($Lys_{59}$, $Gly_{60}$, $Tyr_{99}$, $Asp_{123}$, $Ser_{124}$, $Arg_{187}$). The *cis* geometry between $Leu_{58}$-$Lys_{59}$, also found in the structures of *T. cruzi* HGPRT [10 23], *T. foetus* HGPRT [21], and *E. coli* XGPRT [5,6] is observed in our construction. In this conformation, Cys NH would hydrogen bond to the pyrophosphate moiety and position $Gly_{60}$ to hydrogen bond to the

pyrophosphate (as observed in the other structures). These interactions would not be possible had $Leu_{58}$-$Lys_{59}$ a *trans* peptide bond.

Despite molecular dynamic simulations did not affect the XPRT model overall folding, the secondary structure from the hood domain was not conserved during all simulation interval. This result suggests that substrate binding might influence XPRT correct folding once simulation mimics an apo-enzyme. In fact, preliminary crystallographic studies show that upon concentration XPRT precipitates, unless in the presence of xanthine. Otherwise, the loss of secondary structure in the hood domain might be a reflex of great flexibility in this region.

The PRPP binding domain seems to be more stable in the absence of the substrate and was observed through out the molecular dynamics protocol.

In conclusion, the overall homology model represents a reasonable description of XPRT three-dimensional structure, which has already been used to rationalize the binding of XPRT inhibitors identified in our laboratory.

**Acknowledgment**

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# Capitulo III

## 3.1- Via glicolítica

O metabolismo celular para todos os seres vivos utiliza praticamente as mesmas enzimas distribuídas em algumas sequências de reações bem conhecidas denominadas vias metabólicas. Essas vias metabólicas são responsáveis pela manutenção da homeostase do organismo. A via glicolítica (Figura 5), por exemplo, é responsável pela transformação de glicose em compostos cada vez mais simples

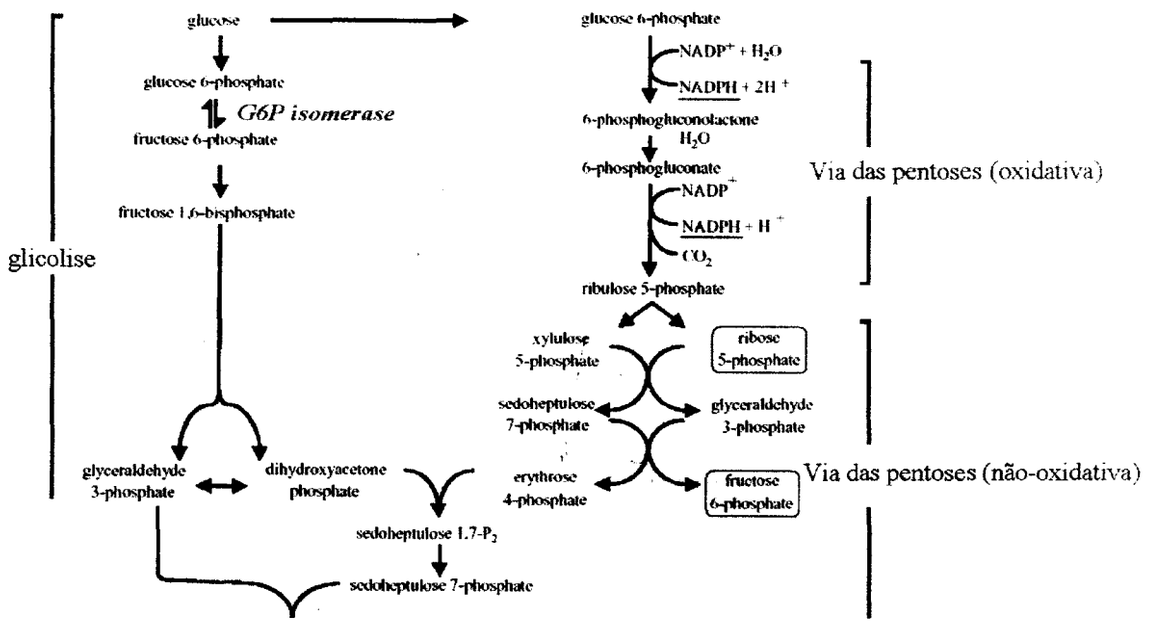


Figura 5: Esquema representativo da glicólise da via das pentoses em tripanosomatideos. A enzima glicose-6-fosfato isomerase está indicada em vermelho. A via das pentoses fornece o agente redutor NADPH e a ribose-5-fosfato, importantes no combate de agentes oxidantes e na biosintese de ácidos nucléicos (Figura adaptada de Hannaert et al, 2003; [47])

e a concomitente formação de moléculas armazenadoras de energia, conhecidas como ATP (adenosina-trifosfato) [45, 46]. Tais moleculas funcionam como verdadeiras "moedas de energia" e são consumidas por enzimas em outras vias metabólicas e demais processos fisiológicos, como por exemplo, a contração muscular.

A via glicolítica pode ser divida em duas etapas (figura 5). A primeira, conhecida como via de Embden-Meyerhof, é a fase anaeróbica na qual a glicose é capturada do meio extracelular e degradada até piruvato gerando como resultado líquido apenas duas moléculas de ATP; o piruvato (ou ácido pirúvico) pode ser convertido em ácido lático ou a alguns derivados alcóolicos. A segunda fase da glicólise ocorre nas mitocôndrias, depende de oxigênio (processo aeróbico) e é a responsável pela produção de 36 moléculas de ATP (por molécula de piruvato).

Outra via crucial no metabolismo é a via das pentoses (Figura 6). Esta é responsável pela produção da ribose-5-fosfato presente nos ácidos nucléicos e concomitante produção de $NADPH_2$ , coenzima necessária como agente redutor em muitas reações metabólicas. A via tem como ponto de partida a glicose-6-fosfato e produz por descarboxilação as pentoses, açurcares de 5 carbonos. O ciclo retorna a via Embden-Meyerhof pela produção de frutose-6-fosfato e gliceraldeído-3-fosfato.

Juntamente com as *Leishmania* ssp, o *Trypanosoma brucei* e *T. cruzi* são outros importantes protozoários parasitos humanos, transmitidos por insetos hematófagos e causadores da doença do sono e doença de Chagas, respectivamente. O *T. brucei* tem sido utilizado como modelo principal em

estudos de identificação e validação de potenciais alvos metabólicos para o desenvolvimento de novas drogas. *Trypanosoma* e *Leishmania* são dois gêneros da família Trypanosomatidea, ordem Kinetoplastida, são caracterizados pela compartimentalização de parte do metabolismo energético em organelas tipo peroxisomas, denominadas glicosomas [47].

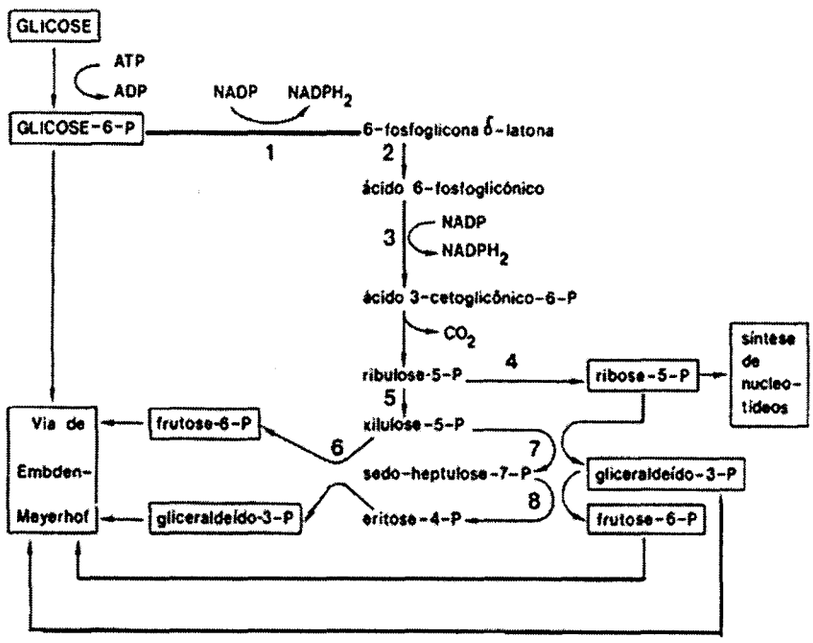


**Figura 6:** Via de formação da pentoses mostrando as enzimas participantes: (1) glicose-6-fosfato desidrogenase, (2) lactonase, (3)fosfogluconato desidrogenase, (4) fosforribose-isomerase, (5) fosfopentose isomerase, (6 e 7) trancetolase e (8) transaldolase.

A primeira etapa na pesquisa e desenvolvimento de novas drogas antiprotozoários é a identificação de enzimas essenciais para a sobrevivência ou desenvolvimento dos parasitos. A disponibilidade de seqüências gênicas obtidas pelos principais projetos genomas de parasitas permite a identificação de enzimas e vias metabólicas exclusivas dos parasitos ou bioquimicamente diferentes das de humanos [48]. A comparação bioquímica é a primeira evidência para incluir uma via metabólica e suas enzimas na lista de possíveis alvos de drogas antiprotozoários. Alguns exemplos de possíveis alvos identificados pela análise comparativa do metabolismo de trypanosomtideos incluem (1) a via

glicolítica e via das pentoses, ambas apresentam os primeiros passos de reação compartimentalizados no glicosoma, uma organela exclusiva dos parasitas; (2) o metabolismo do composto N1-N8-bisglutationil espermidina (ou tripanotiona) pela enzima tripanotiona redutase, o composto e enzima análogos em mamíferos são a glutationa e a glutationa redutase; e (3) metabolismo de folato pela enzima bifuncional dihidrofolato redutase-timidilato sintase que em mamíferos as funções redutase e sintase estão associadas a cadeias separadas [49,48].

A etapa seguinte à identificação das vias promissoras é a validação genética ou bioquímica das enzimas alvos. As duas principais ferramentas genéticas para validação de possíveis alvos são o silenciamento gênico (*gene knockout*) e mais recentemente o RNA de interferência (interference RNA, iRNA) [50]. Ambas as técnicas visam à anulação por silenciamento ou a redução por interferência dos níveis de expressão gênica [48]. A validação bioquímica requer o uso de compostos químicos que interagem de maneira especifica e seletiva bloqueando ou diminuindo a atividade das enzimas alvos. Esta é uma abordagem mais complexa, pois requer métodos experimentais para garantir a especificidade e seletividade in vivo dos compostos utilizados [48]. Somente após as etapas de identificação e validação é que se recomenda um maior investimento na busca de compostos líderes que atuem de maneira eficaz e seletiva no bloqueio da enzima alvo.

A glicólise é uma importante, e em alguns casos, a única via metabólica utilizada pelos tripamosomatideos para a obtenção de ATP. As primeiras sete, do total de dez, enzimas da glicólise estão compartimentalizadas no interior do

glicosoma [45]. Uma conseqüência desta compartimentalização é que as enzimas glicolíticas dos kinetoplastideos diferem em características cinéticas e estruturais quando comparadas com as enzimas homólogas de mamíferos e o fluxo metabólico da via é regulado de forma distinta. [45, 46]. Não apenas a glicólise é localizada no interior dos glicosomas, mas também um parte significativa da via das pentoses (Figura 6), que utiliza intermediários da glicólise para formação de D-ribose-5-fosfato e NADPH, utilizados em processos de biosintese de nucleotídeos e defesa contra stress oxidativo [46].

## 3.2- Glicose-6-fosfato isomerase

Glicose-6-fosfato isomerase (E.C. 5.3.1.9) - também conhecida como fosfoglicose isomerase - é uma enzima intracelular que catalisa a reação reversível entre a glicose-6-fosfato e a frutose-6-fosfato. Esta reação de isomerização é uma etapa comum a via glicolítica, gliconeogênica e a via das pentoses (Figura 5).

Em promastigotas de *L. mexicana*, 90% da atividade da PGI está localizada no citosol e o restante, menos de 10%, está associada aos glicosomas. Apesar desta dupla localização, apenas uma cópia do gene correspondente a PGI foi identificada e a atividade citosolica e glicosomal são bastante similares, descartando assim a possibilidade de se tratar de isoenzimas com diferentes localizações. A presença de enzimas glicolíticas tanto no citosol quanto no glicosoma varia entre diferentes espécies de parasitas tripanosomatideos e pode

ser detectada para a maioria das enzimas desta via [51]. A PGI de *T. brucei, T. cruzi* e *L. mexicana* apresentam menos de 50% de identidade com outras PGI's de mamíferos e todas as três apresentam um fragmento N-terminal de aproximadamente 50 aminoácidos que não apresenta identidade significativa com nenhuma outra seqüência (ou fragmento) pesquisados em bancos de dados públicos. A função deste fragmento é desconhecida e a hipótese que esteja envolvido na sinalização para o transporte pela membrana do glicosoma ainda precisa ser avaliada. Os resíduos catalíticos descritos nas estruturas de PGI de coelho, de porco, humana e de *Bacillus stearothermphilus* são conservados nas seqüências de trypanosomatídeos. Apesar da conservação dos resíduos catalíticos, diferenças nos parâmetros cinéticos entre a PGI de *L. mexicana* e a PGI humana foram observadas em condições experimentais idênticas levando a necessidade de uma análise estrutural mais detalha das PGIs de parasitas e humanos.

Do ponto de vista genético, experimentos de RNAi revelaram que a diminuição do nível de PGI na forma sanguínea de *T. brucei* resulta na inibição de crescimento de até 50% no primeiro dia e volta a normalidade daí em diante (Figura 7). Essa recuperação do fenótipo após os primeiros ciclos é um efeito comum na forma sanguínea do *T. brucei*.

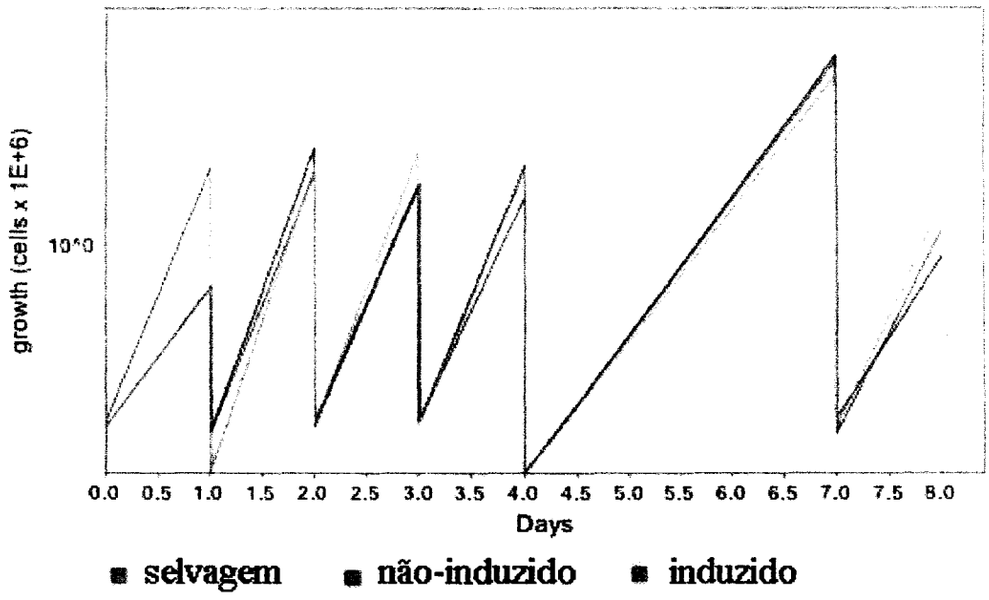


**Figura 7:** Representação gráfica do desenvolvimento de culturas da forma sanguínea de *T. brucei* (Figura adaptada do site http://www.trypanofan.org)

O resultado dos experimentos de RNAi em *T. brucei*, juntamente com as evidências bioquímicas, contribuem para a validação da PGI com potencial alvo para o desenho de drogas anti-tripanosomatideos.

## 3.3- Trabalhos apresentados em seguida

Apresento em seguida cinco artigos relacionados com as enzimas da via glicolítica, as glucose-6-fosfato isomerases (PGI) de humanos e *Leishmania mexicana mexicana* e a enolase de *Trypanosoma brucei*. Os dois primeiros artigos descrevem a clonagem, cristalização e a estrutura cristalográfica da PGI humana na sua forma livre de ligantes no sitio ativo. A estrutura foi resolvida a 2.1Å e

permitiu, pela comparação a outras PGIs de mamíferos permitiu propor um mecanismo para os primeiros passos catalíticos.

O terceiro e quarto artigos descrevem os trabalhos realizados com a PGI de L. mexicana em colaboração com o Prof. Dr. Paul A. M. Michels (Research Unit for Tropical Diseases and Laboratory of Biochemistry, Christian de Duve Institute of Cellular Pathology, Bruxelas, Belgica). O terceiro artigo relata a cristalização e os estudos com quatro inibidores fosforilados desenvolvidos pelo laboratório do Prof. Dr. Laurent Salmon (Laboratoire de Chimie Bioorganique et Bioinorganique, Orsay Cedex, França). O quarto artigo descreve a estrutura da PGI de *L. mexicana* e características de seu sítio ativo em comparação com a estrutura da PGI humana e de outros mamíferos.

O quinto artigo relata os resultados obtidos no estudo da enzima enolase de *Trypanosoma brucei* onde tive uma pequena participação. Este trabalho descreve a caracterização da enzima recombinante, sua cristalização e as observações decorrentes da modelagem molecular por homologia com outras enolases disponíveis.

1. Cordeiro A.T, Godoi P.H.C, Delboni L.F, Oliva G, **Thiemann O.H.** *Human phosphoglucose isomerase: expression, purification, crystallization and preliminary crystallographic analysis*. Acta Crystallogr D Biol Crystallogr. 2001; 57(Pt 4):592-5.

2. Cordeiro A.T., Godoi P.H.C., Silva C.H.T.P., Garratt R.C., Oliva G., **Thiemann O.H.** *Crystal structure of human phosphoglucose isomerase and analysis of the initial catalytic steps.* Biochim Biophys Acta. 2003, 1645 (2):117-22.

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# Capitulo IV

## 4.1- Rastreamento de inibidores a partir de extratos de plantas e animais marinhos

Durante séculos o homem utilizou-se de plantas e extratos derivados destas como unica fonte de agentes terapeuticos [52-54]. No inicio do século XIX as plantas passaram a representar a principal fonte de substancias para o desenvolvimento de medicamentos em decorrencia do desenvolvimento da química farmaceutica. Atualmente, apesar do grande desenvolvimento da sintese organica e de novos processos biotecnologicos, 25% dos medicamentos prescritos nos paises industrializados sao originarios de plantas e 40% de todas as novas drogas desenvolvidas possuem a participacao de produtos de origem natural [52]. Alguns exemplos importantes podem ser mencionados como a Aspirina® (Bayer) ou ácido acetilsalicílico. Este composto não é encontrado em plantas mas é o resultado de uma modificação estrutural símples, uma acetilação de compostos com conhecidas atividades antiinflamatórias e analgésicas como a salicina e a saligenina, isoladas de *Salix alba* L. Outro exemplo consiste na papoula (*Papaver somniferum* L.), de onde se obtem o ópio com conhecidas propriedades soporíficas e analgésicas.

A quimioterapia atualmente disponível para tratamento de infecções causadas por *Leishmania* esta distante de ser satisfatória. A resistência aos antimoniais

pentavalentes, que tem sido as drogas recomendadas para o tratamento da Leishmaniose visceral (VL) e cutânea (CL) por mais de cinqüenta anos, esta atualmente disseminada na Índia. Apesar de novos fármacos terem sido disponibilizados em anos recentes para o tratamento de VL (AmBisome®, Miltefosine) problemas de tratamento persistem [55-58]. A busca por novos fármacos persiste, com os bisfosfonados (residronato e pamidronato) e derivados de plantas como as licochalconas A e alcalóides quinolínicos tendo sido reportados como tendo atividade contra infecções experimentais em sistemas modelo. Diversos alvos em potencial para o desenvolvimento de fármacos têm sido identificados em estudos bioquímicos e moleculares e em alguns casos em sido validados. Os alvos moleculares, temas deste trabalho, envolvendo a via de recuperação de purino nucleotídeos permanece como um alvo em potencial de grande interesse [56-58].

Este aspecto revela a grande importancia que as plantas e outras fontes de produtos naturais têm frente ao desenvolvimento de novos fármacos. Nesta perpsectiva, vem sendo empregada a busca por composotos com atividade inibitória das enzimas alvo de *Leishmania*. Para o desenvolvimento dessa linha de investigação, sob a estreita e frutífera colaboração com os laboratórios de química e isolamento de produtos naturais do Prof. Dr. Paulo Cezar Vieira (Departamento de Química, Universidade Federal de São Carlos - UFSCar) e Prof. Dr. Roberto G. S. Berlinck (Instituto de Química de São Carlos, Universidade de São Paulo - USP) emprendemos o rastreamento de extratos de origem vegetal e de animais marinhos até a obtenção de compostos puros.

## 4.2- Trabalhos apresentados em seguida

Apresento em seguida sete artigos relacionados com o rastreamento de de extratos ea caractrização da atividade inibitória de diversos compostos.

Os primeiros tres artigos tratam do isolamento e caracterização estrutural de compostos isolados de *Adiscanthus fusciflorus* (Rutaceae), a isopimpinellina (5,8-dimethoxy-psoralen), skimmianine (7,8-dimethoxydictammine ou 4,7,8-trimethoxyfuro[2,3-b]quinoline), e o 3-(5,7-Dimethoxy-2,2-dimethyl-2H-benzo[b]- pyran-6-yl)propionic acid. Estes compostos foram isolados de extratos de *A. fusciflorus* em uma abordagem guiada pelos ensaios bioquímicos de inibição da APRT de *Leishmania*. O estudo estrutural por difração de raios-X de monocristais dos compostos se ostra importante do ponto de vista do desenho racional de novos compostos derivados por fornecer um maior conhecimento da molecula em questão.

O quarto artigo aceito para publicação se refre ao isolamento da cumarina Aurapteno (7-geranyloxycoumarin), isolada de *Esenbeckia febrifuga* (Rutaceae) por ensaios guiados pela inibição do crescimento de *L. major* em cultura. Esta abordagem foi possível graças a implantação da sala de cultivo de parasitas e células de mamíferos no Grupo de Cristalografia de Proteínas e Biologia Estrutural. Representa um avanço significativo por permitir o estudo bioquímico e celular dos compostos isolados como exemplificado nesta publicação e na seguinte.

O quinto artigo submetido para publicação se refere aos ensaios de inibição bioquímicos com as PRTases (APRT, HGPRT e XPRT) de *Leishmania* assim como aos estudos de inibição do crescimento do parasita e seletividade frente a células humanas.

Finalmene o sexto artigo relata, em uma revisão em colaboração com o Prof. Dr. Roberto G. S. Berlinck (Instituto de Química de São Carlos, Universidade de São Paulo - USP), os avanços e nossa modesta contribuição no isolamento de compostos derivados de animais marinhos e fungos marinhos.


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2. Napolitano H.B., Silva M., Ellena J., Rocha W.C., Vieira P.C., **Thiemann O.H.**, Oliva G. *Redetermination and comparative structural study of isopimpinellin: a new inhibitor against the Leishmania APRT enzyme*. Acta Crystallogr E Struc. Rep. 59: O1506-O1508 Part 10, 2003.

3. Silva M., Napolitano H.B., Ellena J., Rocha W.C., Vieira P.C., Oliva G., **Thiemann O.H.** *3-(5,7-Dimethoxy-2,2-dimethyl-2H-benzo[b]-pyran-6-yl)propionic acid: a potential inhibitor against Leishmania*. Acta Crystallogr E Struc. Rep. 59: O1575-O1577 Part 10, 2003.

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# Redetermination of skimmianine: a new inhibitor against the Leishmania APRT enzyme

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**Key indicators**

Single-crystal X-ray study
$T$ = 120 K
Mean $\sigma$(C–C) = 0.002 Å
$R$ factor = 0.038
$wR$ factor = 0.104
Data-to-parameter ratio = 12.9

For details of how these key indicators were automatically derived from the article, see http://journals.iucr.org/e.



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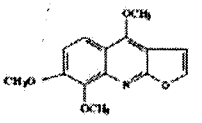
The title compound (alternative names 7,8-dimethoxydictamnine and 4,7,8-trimethoxyfuro[2,3-*b*]quinoline), $C_{14}H_{13}NO_4$, is a natural product extracted from *Adiscanthus fusciflorus* (*Rutaceae*). Our biochemical tests show that it has inhibitory activity against the enzyme adenine phosphoribosyltransferase (APRT) from Leishmania, a tropical parasite causing endemic disease in poor countries. It crystallizes in the centrosymmetric space group $P2_1/c$, with one molecule in the asymmetric unit, and has at least two C—H···O intermolecular interactions, leading to the formation of centrosymmetric dimers.

## Comment

Leishmaniasis is a disease caused by a protozoal parasite of the order Kinetoplastid. According to the World Heath Organization reports (WHO, 1998), 88 countries are affected, with 12 million infected people and approximately 350 million people at risk. The need for new drugs for the treatment of the leishmaniasis infections comes from a lack of safe drugs and the serious secondary effects observed in available chemotherapy (McGreevy & Marsden, 1986). The purine nucleotide salvage pathway in Kinetoplastid is a potential target for the development of new drugs, owing to its dependence on that biosynthetic pathway (Berens *et al.*, 1995). In Kinetoplastid, the phosphoribosyltransferase (PRTase) protein family is responsible for purine nucleotide salvage. Looking for new bioactive substances, potentially useful against leishmaniasis, we used the PRTase adenine phosphoribosyltransferase (APRT) from *L. tarentolae* as a model system to screen the inhibitory capacity of several small molecule compounds from Brazilian plants.



(I)

The screening was performed using the APRT inhibitory assay, either in the presence of extracts or with the purified compound, and was monitored spectrophotometrically (Tuttle & Krenitsky, 1980). The title compound, (I), was isolated from *A. fusciflorus* extracts and has been structurally investigated because of its inhibitory activity against APRT. Enzymatic tests of (I) at 50 µg/ml show an inhibition activity of 68%. Further investigations by molecular docking and dynamic simulations will be performed to study the interactions

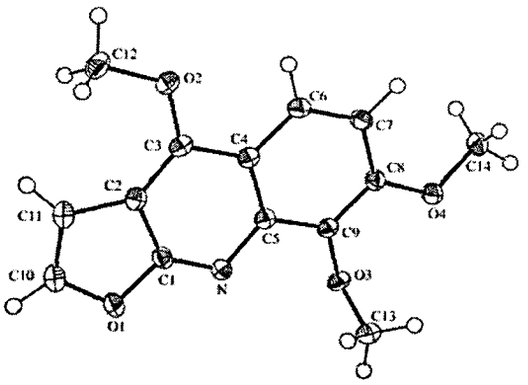



**Figure 1**
A view of the molecular structure of (I), showing the atom-labelling scheme. Displacement ellipsoids are drawn at the 50% probability level and H atoms are shown as spheres of arbitrary radii.

between this compound and the APRT active site. In light of this interest, structural characterization will give us important information with respect to the interaction mode of compound (I) with APRT, and allow the investigation of possible inhibition of that compound by other PRTases.

With the aim of obtaining more accurate structural data for comparison of (I) and other inhibitors against APRT enzyme, and in order to use this information in molecular docking and dynamic simulations, we undertook a structure determination at 120 K. An *ORTEP* view (Farrugia, 1997) of compound (I), together with the atom-labelling scheme, is shown in Fig. 1. All bond lengths and angles of this compound are close to normal values (Allen *et al.*, 1983). The crystallographic structure of (I), measured at room temperature, has been previously published (Cox *et al.*, 1989).

The crystal packing of (I) does not show any strong hydrogen bonds. Nevertheless, two weak intermolecular interactions of the type C—H···O (C10—H10···O3$^{i}$, C12—H12···O3$^{ii}$) and two of the type C—H···N (C10—H10···N$^{i}$ and C14—H14···N$^{iii}$) stabilize the three-dimensional structure (symmetry codes as in Table 1). The latter type links two neighbouring molecules in a centrosymmetric dimeric form, as shown in Fig. 2. The C10—H10···O3$^{i}$ interaction is responsible for the formation of infinite chains along the *b* axis, and C12—H12···O3$^{ii}$ for the formation of infinite chains along the *c* axis. All structural details of the intermolecular contacts for compound (I) were interpreted as hydrogen bonds on geometrical grounds (Ellena *et al.*, 2001).

## Experimental

The roots and leaves of *A. fusciflorus* were collected from the Manaus region of the Brazilian Amazon forest in December 2000. An authenticated specimen was deposited in the herbarium of the Instituto de Pesquisas da Amazonia–INPA (code 189859). The powdered parts (roots 2.380 kg and leaves 1.040 kg) were extracted successively with hexane (10 l) and methanol (8.5 l). The crude

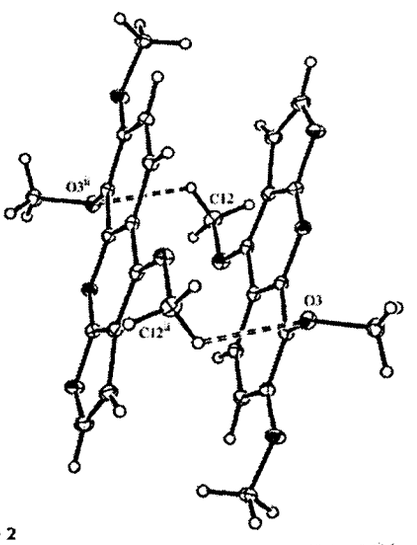



**Figure 2**
A view of (I), showing dimerization due to C12—H12···O3$^{ii}$ [symmetry code: (ii) 1 − *x*, 1 − *y*, −*z*] interactions.

hexane crude extract of the root (5.0 g) was extracted by chromatography on a silica gel column (Φ × *h* = 28 × 2 cm). Fractions 6 and 7 were combined and subjected to silica gel column chromatography, using the isocratic system (hexane/ethyl acetate 7:3). Fraction 8 was collected according to TLC analysis (normal phase) and fractions 6 and 7 were combined and subjected to CCDP using hexane/ethyl acetate 7:3 as the mobile phase. 10 mg of compound (I) were obtained; this crystallized by vapour diffusion as a prismatic, light yellow solid, using 1:1 hexane/dichloromethane as solvent.

*Crystal data*

$C_{14}H_{13}NO_4$
$M_r$ = 259.25
Monoclinic, $P2_1/c$
$a$ = 7.2429 (1) Å
$b$ = 10.4418 (2) Å
$c$ = 15.4618 (3) Å
$\beta$ = 94.353 (1)°
$V$ = 1165.99 (4) Å$^3$
$Z$ = 4
$D_x$ = 1.477 Mg m$^{-3}$
Mo $K\alpha$ radiation
Cell parameters from 2808 reflections
$\theta$ = 3.4–27.5°
$\mu$ = 0.11 mm$^{-1}$
$T$ = 120 (2) K
Prism, light yellow
0.32 × 0.18 × 0.16 mm

*Data collection*

Nonius KappaCCD diffractometer
$\varphi$ and $\omega$ scans
Absorption correction: none
5117 measured reflections
2672 independent reflections
2301 reflections with $I > 2\sigma(I)$
$R_{int}$ = 0.013
$\theta_{max}$ = 27.5°
$h$ = −9 → 9
$k$ = −13 → 13
$l$ = −20 → 20

*Refinement*

Refinement on $F^2$
$R[F^2 > 2\sigma(F^2)]$ = 0.038
$wR(F^2)$ = 0.104
$S$ = 1.04
2672 reflections
207 parameters
H atoms treated by a mixture of independent and constrained refinement
$w = 1/[\sigma^2(F_o^2) + (0.0633P)^2 + 0.2061P]$ where $P = (F_o^2 + 2F_c^2)/3$
$(\Delta/\sigma)_{max} < 0.001$
$\Delta\rho_{max}$ = 0.29 e Å$^{-3}$
$\Delta\rho_{min}$ = −0.22 e Å$^{-3}$

**Table 1**
Hydrogen-bonding geometry (Å, °).

| $D$—H···$A$ | $D$—H | H···$A$ | $D$···$A$ | $D$—H···$A$ |
|---|---|---|---|---|
| C10—H10···O3$^{i}$ | 0.97 (1) | 2.47 (1) | 3.315 (1) | 144.8 (11) |
| C12—H12$A$···O3$^{ii}$ | 0.99 (1) | 2.54 (1) | 3.159 (1) | 120.3 (11) |
| C10—H10···$N^{i}$ | 0.97 (1) | 2.53 (1) | 3.290 (1) | 134.9 (11) |
| C14—H14$A$···$N^{iii}$ | 0.99 (1) | 2.69 (1) | 3.403 (1) | 128.0 (11) |

Symmetry codes: (i) $1-x, y-\frac{1}{2}, \frac{1}{2}-z$; (ii) $1-x, 1-y, -z$; (iii) $x, \frac{1}{2}-y, z-\frac{1}{2}$.

All of the H atoms, except those attached to the C atom C13, were found in a Fourier synthesis and subsequently refined freely. The H atoms attached to C13 were placed at calculated positions.

Data collection: *COLLECT* (Nonius, 1998); cell refinement: *HKL SCALEPACK* (Otwinowski & Minor, 1997); data reduction: *HKL DENZO* (Otwinowski & Minor, 1997) and *SCALEPACK*; program(s) used to solve structure: *SHELXS97* (Sheldrick, 1997); program(s) used to refine structure: *SHELXL97* (Sheldrick, 1997); molecular graphics: *ORTEP-3 for Windows* (Farrugia, 1997) and *PLATON* (Spek, 2002); software used to prepare material for publication: *WinGX* publication routines (Farrugia, 1999).

This work was supported by CNPq and FAPESP (São Paulo), Brazil, and by the WHO.

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# Redetermination and comparative structural study of isopimpinellin: a new inhibitor against the Leishmania APRT enzyme

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Key indicators
Single-crystal X-ray study
T = 120 K
Mean σ(C–C) = 0.002 Å
R factor = 0.044
wR factor = 0.124
Data-to-parameter ratio = 12.4

For details of how these key indicators were automatically derived from the article, see http://journals.iucr.org/e.



Received 28 July 2003
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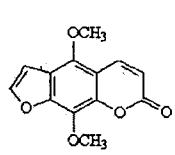
Online 18 September 2003

The title compound (alternative name 5,8-dimethoxypsoralen), $C_{13}H_{10}O_5$, is a natural product extracted from *Adiscanthus fusciflorus* (*Rutaceae*). Our biochemical tests show that this compound has inhibitory activity against the enzyme adenine phosphoribosyltransferase (APRT) from Leishmania, a tropical parasite causing endemic disease in poor countries. It crystallizes in the centrosymmetric space group $P2_1/c$, with one molecule in the asymmetric unit, and has at least two C—H···O intermolecular interactions, leading to the formation of centrosymmetric dimers.

## Comment

Leishmaniasis is a disease caused by a protozoal parasite of the order Kinetoplastid. According to the World Heath Organization reports (WHO, 1998), 88 countries are affected, with 12 million infected people and approximately 350 million people at risk. The need for new drugs for the treatment of leishmaniasis infections comes from a lack of safe drugs and the serious secondary effects observed in available chemotherapy (McGreevy & Marsden, 1986). Looking for new bioactive substances, potentially useful against leishmaniasis, we used the PRTase adenine phosphoribosyltransferase (APRT) from *L. tarentolae* as a model system to screen the inhibitory capacity of several small molecule compounds from Brazilian plants. The screening was performed using the APRT inhibitory assay, either in the presence of extracts or with the purified compound, and was monitored spectrophotometrically (Tuttle & Krenitsky, 1980). The title compound, (I), was isolated from *A. fusciflorus* extracts and has been structurally investigated because of its inhibitory activity against APRT. A comparative study between (I) and another inhibitor, skimmianine (II) (Napolitano *et al.*, 2003), against the APRT enzyme will be performed.




(I)

Enzymatic tests of compounds (I) and (II) at 50 μg/ml show inhibition activities of 50% and 68%, respectively. Further investigations by molecular docking and dynamic simulations will be performed to study the interactions between compounds (I) and (II) and the APRT active site. The new

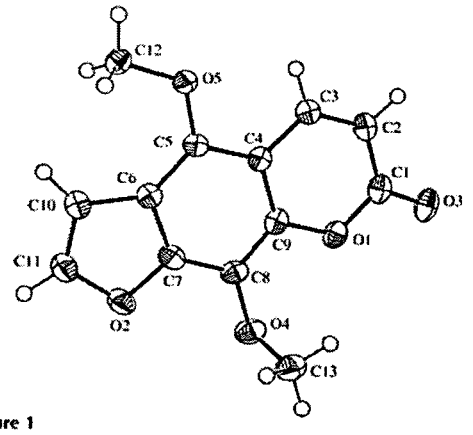



**Figure 1**
A view of the molecular structure of (I), showing the atom-labelling scheme. Displacement ellipsoids are drawn at the 50% probability level and H atoms are shown as spheres of arbitrary radii.

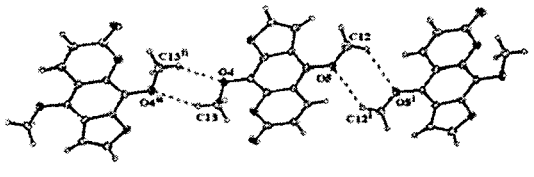



**Figure 2**
A view of (I), showing dimerization, viewed along the [111] direction. Dashed lines indicate intermolecular C—H···O hydrogen bonding.

information obtained will then be used to improve the inhibitory activity of these molecules. In light of this interest, a comparative structural characterization of the two compounds will give us important information with respect to the interaction modes of compounds (I) and (II) with APRT, and allow the investigation of possible inhibition of those compounds with other phosphoribosyltransferase (PRTases).

All bond lengths and angles of (I) are close to normal values (Allen *et al.*, 1983). The crystallographic structure of (I), measured at room temperature, has been previously published (Gopalakrishna *et al.*, 1977). With the aim of obtaining more accurate and comparable structural data of inhibitors (I) and (II), and in order to use this information in molecular docking and dynamic simulations, we determined the structure at 120 K. An *ORTEP* view (Farrugia, 1997) of (I), together with the atom-labelling scheme, is shown in Fig. 1.

Five significant intermolecular C—H···O contacts are found in the packing of compound (I), *viz.* C12—H12*B*···O5$^{i}$, C13—H13*B*···O4$^{ii}$, C12—H12*A*···O3$^{iii}$, C12—H12*C*···O3$^{iv}$ and C11—H11···O2$^{v}$ (symmetry codes as in Table 1). The first two interactions link neighbouring molecules in a centrosymmetric dimeric form, giving rise to an infinite chain of these dimers along the [111] direction, as seen in Fig. 2. The third and fourth interactions form an infinite chain along the *c* axis. The last interaction links two dimeric chains along the *b* axis. All structural details of the intermolecular contacts for compound (I) were interpreted as hydrogen bonds on geometrical grounds (Ellena *et al.*, 2001).

The molecular structures of (I) and (II) present small differences in overall planarity, except for carbon C13 in both compounds. The maximum deviation of their non-H atoms from the mean least-square plane through the planar part of the molecules are 0.162 (7) Å and 0.061 (1) Å, while the average deviations are 0.0597 (9) Å and 0.031 (1) Å for (I) and (II), respectively. The C13 methoxy group of (I) has the same orientation as the C5 methoxy group of (II), both being toward the respective C—H···O interaction, as described above. The dihedral angles between the plane formed by these methoxyl groups and the plane formed by the three rings of (I) and (II) are 1.2 (2)° and 2.3 (1)°, respectively. The torsion angles C4—C3—O2—C12 for (II) and C4—C5—O5—C12 for (I) are 0.1 (2)° and 0.2 (2)°, respectively. The orientation of the C8 methoxy group of (I) toward N$^{iii}$ allows this group to be positioned in the same ring plane. In (II), a carboxyl (instead of a methoxy) group is noted at the corresponding position.

The dihedral angles between the planes formed by the C9 methoxy group and the three rings of (I), and between the C8 methoxy group and the three rings of (I) are 89.09 (7)° and 77.95 (9)°, respectively. The torsion angles C5—C9—O3—C13 for (II) and C7—C8—O4—C13 for (I) are 93.4 (2)° and 76.8 (2)°, respectively. In both molecules, this functional group is attached to the benzene ring and in molecule (II) its orientation is driven by the intermolecular C—H···O interaction, as described above. These differences between compounds (I) and (II) seem to be consistent with the observed inhibitory activity differences against the Leishmania APRT enzyme.

## Experimental

The roots and leaves of *A. fusciflorus* were collected from the Manaus region of the Brazilian Amazon forest in December 2000. An authenticated specimen was deposited in the herbarium of the Instituto de Pesquisas da Amazonia–INPA (code 189859). The powdered parts (roots 2.380 kg and leaves 1.040 kg) were extracted successively with hexane (10 l) and methanol (8.5 l). The hexane extracte of the leaves (7.8 g), when chromatographed using a silica gel column with a hexane and ethyl acetate gradient followed by methanol, gave 33 fractions. Fractions 29 and 33 were chromatographed on a Sephadex LH-20 column using methanol as the mobile phase. Thirteen fractions were collected and fractions 6 and 7 resulted in 204.2 mg of compound (I), which crystallized by vapour diffusion from dichloromethane/methanol (1:1) as solvent, yielding light yellow prismatic needles.

*Crystal data*

$C_{13}H_{10}O_5$
$M_r$ = 246.21
Monoclinic, $P2_1/c$
$a$ = 16.9357 (5) Å
$b$ = 4.3669 (1) Å
$c$ = 16.2558 (4) Å
$\beta$ = 117.218 (1)°
$V$ = 1069.10 (5) Å$^3$
$Z$ = 4
$D_x$ = 1.53 Mg m$^{-3}$
Mo $K\alpha$ radiation
Cell parameters from 2772 reflections
$\theta$ = 1.0–27.5°
$\mu$ = 0.12 mm$^{-1}$
$T$ = 120 (2) K
Prism, light yellow
0.28 × 0.16 × 0.05 mm

*Data collection*

| | |
|---|---|
| Nonius KappaCCD diffractometer | $R_{int} = 0.023$ |
| $\varphi$ and $\omega$ scans | $\theta_{max} = 27.5°$ |
| Absorption correction: none | $h = -21 \rightarrow 22$ |
| 4114 measured reflections | $k = -4 \rightarrow 5$ |
| 2450 independent reflections | $l = -21 \rightarrow 20$ |
| 1841 reflections with $I > 2\sigma(I)$ | |

*Refinement*

| | |
|---|---|
| Refinement on $F^2$ | $w = 1/[\sigma^2(F_o^2) + (0.0713P)^2 + 0.1752P]$ where $P = (F_o^2 + 2F_c^2)/3$ |
| $R[F^2 > 2\sigma(F^2)] = 0.044$ | |
| $wR(F^2) = 0.124$ | |
| $S = 1.04$ | $(\Delta/\sigma)_{max} < 0.001$ |
| 2450 reflections | $\Delta\rho_{max} = 0.23$ e Å$^{-3}$ |
| 197 parameters | $\Delta\rho_{min} = -0.3$ e Å$^{-3}$ |
| All H-atom parameters refined | |

**Table 1**
Hydrogen-bonding geometry (Å, °).

| $D$—H···$A$ | $D$—H | H···$A$ | $D$···$A$ | $D$—H···$A$ |
|---|---|---|---|---|
| C12—H12$B$···O5$^{i}$ | 1.01 (2) | 2.64 (2) | 3.420 (1) | 134.2 (14) |
| C13—H13$B$···O4$^{ii}$ | 0.98 (2) | 2.58 (2) | 3.455 (2) | 149.4 (16) |
| C12—H12$A$···O3$^{iii}$ | 1.01 (2) | 2.61 (2) | 3.264 (1) | 141.3 (16) |
| C12—H12$C$···O3$^{iv}$ | 1.00 (2) | 2.67 (2) | 3.043 (1) | 102.0 (13) |
| C11—H11···O2$^{v}$ | 0.96 (1) | 2.45 (1) | 3.360 (1) | 156.7 (13) |

Symmetry codes: (i) $1-x, -2-y, 1-z$; (ii) $-x, -1-y, -z$; (iii) $x, -\frac{1}{2}-y, \frac{1}{2}+z$; (iv) $x, -\frac{1}{2}-y, \frac{1}{2}+z$; (v) $-x, y-\frac{1}{2}, \frac{1}{2}-z$.

All of the H atoms were found in a Fourier synthesis and were subsequently refined freely.

Data collection: *COLLECT* (Nonius, 1998); cell refinement: *HKL SCALEPACK* (Otwinowski & Minor, 1997); data reduction: *HKL DENZO* (Otwinowski & Minor, 1997) and *SCALEPACK*; program(s) used to solve structure: *SHELXS97* (Sheldrick, 1997); program(s) used to refine structure: *SHELXL97* (Sheldrick, 1997); molecular graphics: *ORTEP-3 for Windows* (Farrugia, 1997) and *PLATON* (Spek, 2002); software used to prepare material for publication: *WinGX* publication routines (Farrugia, 1999).

This work was supported by CNPq and FAPESP (São Paulo), Brazil, and by the WHO.

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# 3-(5,7-Dimethoxy-2,2-dimethyl-2*H*-benzo[*b*]-pyran-6-yl)propionic acid: a potential inhibitor against Leishmania

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**Key indicators**
Single-crystal X-ray study
$T$ = 120 K
Mean σ(C–C) = 0.002 Å
$R$ factor = 0.033
$wR$ factor = 0.090
Data-to-parameter ratio = 9.8

For details of how these key indicators were automatically derived from the article, see http://journals.iucr.org/e.



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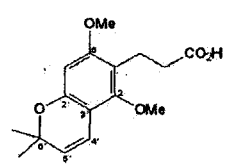
The title acid, $C_{16}H_{20}O_5$, was extracted from *Adiscanthus fusciflorus* (*Rutaceae*) and is shown to inhibit adenine phosphoribosyltransferase (APRT) enzyme activity. This compound crystallizes in the centrosymmetric space group *C*2/*c* with one molecule in the asymmetric unit. There is one strong hydrogen bond, with $O_D\cdots O_A$ = 2.6238 (12) Å and $O_D$—H$\cdots O_A$ = 171.1 (17)° involving the COOH group, forming a cyclic dimer about a center of symmetry. The packing of the molecules is additionally stabilized by one C—H$\cdots$O [$C_D\cdots O_A$ = 2.9820 (16) Å and $C_D$—H$\cdots O_A$ = 101.8 (10)°] and two C—H$\cdots\pi$ intermolecular hydrogen bonds.

## Comment

The title carboxylic acid, (I), has been investigated because of its interesting inhibitory activity against adenine phosphoribosyltransferase (APRT) from *Leishmania tarentolae* which is a member of the phosphoribosyltransferase (PRTase) family. The PRTases are responsible for the salvage of purine, pyridine and pyrimidine nucleotides, as well as aromatic amino acids. Most organisms synthesize adenine nucleotides by both the *de novo* and the salvage pathways. In contrast, protozoan parasites are strict purine nucleotide auxotrophs because of the absence of a purine *de novo* biosynthetic pathway (Berens *et al.*, 1995). Therefore, these microorganisms are absolutely dependent on scavenging pre-formed purine nucleotides from the host or the media (Ullman & Carter, 1997). To look for new potential anti-leishmania drugs, we used the APRT from *L. tarentolae* as a model system to investigate the inhibitory capacity of *A. fusciflorus* extracts. The screening was performed using a spectrophotometric assay (Tuttle & Krenitsky, 1980); the $IC_{50}$ of pure compound (I) is 147 μ*M*. In view of this interest, we have extracted the title compound, (I), and present here its crystal structure.




(I)

Compound (I) crystallizes in the centrosymmetric space group *C*2/*c* with one molecule in the asymmetric unit. The refined molecular structure, together with the atom-labeling scheme, are shown in Fig. 1 (Johnson, 1965). All the bond distances and angles are close to normal values (Allen *et al.*,

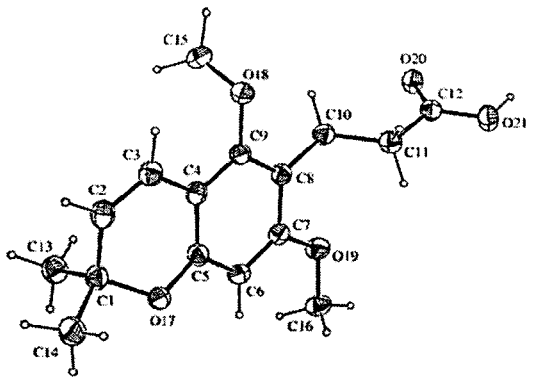



**Figure 1**
A view of the molecular structure of (I), showing the atom-labeling scheme. Displacement ellipsoids are drawn at the 50% probability level and H atoms are shown as spheres of arbitrary radii.

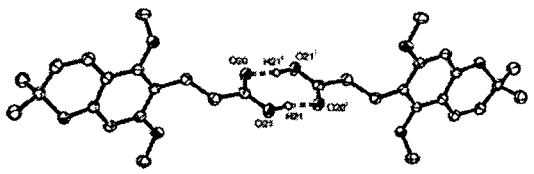


**Figure 2**
A view of (I), showing the dimerization due to O21—H21···O20$^{i}$ bonding [symmetry code: (i) $\frac{1}{2}-x, \frac{1}{2}-y, 1-z$].

1983). The benzene ring C4–C9 in the central part of the molecule is very nearly planar, the maximum deviation of any of its atoms from the least-squares plane describing them being 0.0051 (8) Å, while the average deviation is 0.0030 (9) Å. Atoms (C2, C3, C16, O17, O18 and O19) around the benzene ring are coplanar [r.m.s. deviation 0.0535 (10) Å]. Thus, the structure exhibits a planar central moiety, a typical structural feature observed in anti-leishmania inhibitors (Chan-Bacab & Peña-Rodrigues, 2001).

Several packing features may be noted (Spek, 1990). There are classical intermolecular hydrogen bonds [O21—H21···O20$^{i}$; symmetry code: (i) $\frac{1}{2}-x, \frac{1}{2}-y, 1-z$] between the COOH groups of neighbouring molecules, forming a centrosymmetric dimer (Fig. 2). O21···O20$^{i}$ is 2.6238 (12) Å and O21—H21···O20$^{i}$ is 171.1 (17)°.

There is also a weak C16—H16*B*···O21$^{ii}$ [symmetry code: (ii) $x, -y, z-\frac{1}{2}$] intermolecular hydrogen bond that is responsible for stabilization of the infinite parallel chains (Fig. 3). Furthermore, two intermolecular C—H···$\pi$ interactions involve atoms C15 and C16 and the $\pi$ cloud of the benzene ring. The former is between atom H15*A* and the $\pi$-ring of a molecule at $(-x, 1-y, -z)$ and the second between atom H16*A* and the molecule at $(-x, -y, -z)$. These are characterized by the distances C15···*CgBz* and C16···*CgBz* of 3.5534 (15) and 3.6568 (15) Å, respectively, and by the angles C15—H15*A*···*CgBz* and C16—H16*A*···*CgBz* of

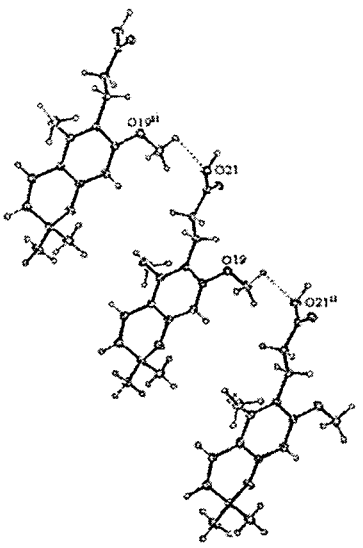



**Figure 3**
The crystal structure of (I). Dashed lines indicate intermolecular C16—H16*B*···O21$^{ii}$ hydrogen bonding [symmetry code: (ii) $x, -y, z-\frac{1}{2}$].

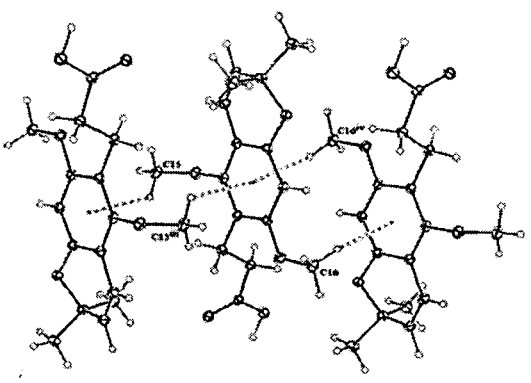



**Figure 4**
The C—H···$\pi$ interactions in the structure of (I).

129.4 (11) and 152.2 (11)°, respectively (*CgBz* denotes the centroid of the benzene ring). These interactions link infinite parallel chains, as shown in Fig. 4. All geometrical details of the intermolecular contacts were interpreted as hydrogen bonds on geometrical grounds (Ellena *et al.*, 2001); Table 2 reports the relevant geometrical parameters.

## Experimental

The roots and leaves of *A. fusciflorus* were collected in Manaus-AM/Brazil in December 2000. An authenticated specimen was deposited in the herbarium of the Instituto de Pesquisas da Amazonia, INPA/Brazil, reference code 189859. The powdered parts of the dihydrocinnamic acid title compound, isolated by extraction (roots 2.380 kg

and leaves 1.040 kg), were then extracted successively with hexane (10 l) and methanol (8.5 l). The hexane extract of the root (5.0 g) was chromatographed on an silica gel column ($\Phi \times h = 28 \times 2$ cm) using a hexane/EtOAc gradient to fractionate the extract. Nine fractions were collected. Fraction 5 (hexane–ethyl acetate 7:3) was chromatographed on an silica gel column ($\Phi \times h = 50 \times 1.5$ cm) using a gradient system of hexane, ethyl acetate and methylene chloride. 42 fractions were collected and, based on normal phase thin-layer chromatography (TLC), seven fractions were pooled. Fraction 3 (hexane/ethyl acetate/methylene chloride 7:2:1) produced an amorphous white solid (25 mg) that was washed successively with hexane and crystallized by vapor diffusion at room temperature from hexane/methylene chloride (1:1). The purity of the compound was checked by TLC (silica gel, Merck PF 254, 0.25 mm thickness).

*Crystal data*

$C_{18}H_{20}O_3$
$M_r = 292.32$
Monoclinic, $C2/c$
$a = 23.3022$ (3) Å
$b = 10.2248$ (2) Å
$c = 15.0785$ (3) Å
$\beta = 122.273$ (1)°
$V = 3037.60$ (9) Å$^3$
$Z = 8$
$D_x = 1.278$ Mg m$^{-3}$
Mo $K\alpha$ radiation
Cell parameters from 3662 reflections
$\theta = 1.0$–$27.5$°
$\mu = 0.10$ mm$^{-1}$
$T = 120$ (2) K
Prism, colorless
0.16 × 0.14 × 0.10 mm

*Data collection*

Nonius KappaCCD diffractometer
$\varphi$ and $\omega$ scans
5170 measured reflections
2666 independent reflections
2207 reflections with $I > 2\sigma(I)$
$R_{int} = 0.016$
$\theta_{max} = 25$°
$h = -27 \rightarrow 27$
$k = -12 \rightarrow 12$
$l = -17 \rightarrow 17$

*Refinement*

Refinement on $F^2$
$R[F^2 > 2\sigma(F^2)] = 0.033$
$wR(F^2) = 0.090$
$S = 1.05$
2666 reflections
271 parameters
H atoms treated by a mixture of independent and constrained refinement
$w = 1/[\sigma^2(F_o^2) + (0.0472P)^2 + 0.8048P]$ where $P = (F_o^2 + 2F_c^2)/3$
$(\Delta/\sigma)_{max} < 0.001$
$\Delta\rho_{max} = 0.19$ e Å$^{-3}$
$\Delta\rho_{min} = -0.16$ e Å$^{-3}$
Extinction correction: *SHELXL97*
Extinction coefficient: 0.0043 (7)

**Table 1**
Selected geometric parameters (Å, °).

| | | | |
|---|---|---|---|
| O20—C12 | 1.2252 (15) | C12—O21 | 1.3188 (15) |
| O20—C12—O21 | 122.90 (11) | O21—C12—C11 | 113.66 (11) |
| O20—C12—C11 | 123.42 (11) | | |

**Table 2**
Hydrogen-bonding geometry (Å, °).

| $D$—H···$A$ | $D$—H | H···$A$ | $D$···$A$ | $D$—H···$A$ |
|---|---|---|---|---|
| O21—H21···O20$^{i}$ | 1.04 (2) | 1.59 (2) | 2.6238 (12) | 171.1 (17) |
| C16—H16$B$···O21$^{ii}$ | 1.011 (17) | 2.607 (15) | 2.9820 (16) | 101.8 (10) |
| C15—H15$A$···$Cg$B$z^{iii}$ | 1.024 (16) | 2.814 (16) | 3.5534 (15) | 129.4 (11) |
| C16—H16$A$···$Cg$B$z^{iv}$ | 0.985 (15) | 2.756 (15) | 3.6568 (15) | 152.2 (11) |

Symmetry codes: (i) $\frac{1}{2} - x, \frac{1}{2} - y, 1 - z$; (ii) $x, -y, z - \frac{1}{2}$; (iii) $-x, 1 - y, -z$; (iv) $-x, -y, -z$.

Atoms H15$A$, H16$A$, H16$B$ and H21 were found in a Fourier synthesis and were freely refined. The other H atoms were placed at calculated positions.

Data collection: *COLLECT* (Nonius, 1997–2002); cell refinement: *HKL SCALEPACK* (Otwinowski & Minor, 1997); data reduction: *HKL SCALEPACK* and *DENZO* (Otwinowski & Minor, 1997); program(s) used to solve structure: *SHELXS97* (Sheldrick, 1997); program(s) used to refine structure: *SHELXL97* (Sheldrick, 1997); molecular graphics: *ORTEP-3 for Windows* (Farrugia, 1997); software used to prepare material for publication: *WinGX* (Farrugia, 1999).

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**Aurapten, a novel coumarin with growth inhibition against *Leishmania major* promastigotes**

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**Abstract**

Several natural compounds have been identified for the treatment of leishmaniasis. Among them are some alkaloids, chalcones, lactones, tetralones and saponins. The new compound reported here, 7-geranyloxycoumarin, called aurapten, belongs to the chemical class of the coumarins and has a molecular mass of 298.37 g/mol. The compund was extracted from the Rutaceae species *Esenbeckia febrifuga*. The compound was purified from an hexane extract starting from 407,7g of dried leaves and followed by four silica gel chromatographic fractionation steps using different solvents as the mobile phase. The resulting 47mg of compound shows significant growth inhibition with an $LD_{50}$ of 30μM against the tropical parasite *Leishmania major*, which causes severe clinical manifestations in humans and is endemic in the tropical and subtropical regions. In the present study we investigated the atomic structure of aurapten in order to determine the

existence of common structural motifs that might be related to other coumarins and potentially to other identified inhibitors of *Leishmania* growth and viability. This compound has a comparable inhibitory activity of other isolated molecules. The aurapten is a planar molecule constituted of an aromatic system with electron delocalization. A hydrophobic side chain consisting of ten carbon atoms with two double bonds and negative density has been identified and may be relevant for further compound synthesis.

Key words: Coumarin, Aurapten (7-geranyloxycoumarin), X-rays, *Leishmania*, Inhibitor.

## Introduction

Leishmaniasis is a disease caused by a protozoan parasite of the order Kinetoplastida. According to the World Health Organization (1), 12 million infected people exist in 88 countries, with approximately 350 million people being at risk. In view of the lack of safe drugs and the serious secondary effects caused by available chemotherapy (2), there is a need for new drugs for the treatment of leishmaniasis infections. Therefore, the discovery of novel classes of inhibitors can be an important step which contributes to overcoming the drug resistance of *Leishmania* (3).

The use of phytotherapy to treat human diseases has its roots in pre-historical times and most of the effective drugs currently available are obtained from plants (4). The biodiversity existing in Brazilian is a potential source of many new bioactive molecules to be studied and explored (5).

In a search for new bioactive substances potentially useful against leishmaniasis, we used *L. major* Friedlin (WHO MHOM/IL/80/Friedlin) cells as a model system to screen small molecule compounds obtained from Brazilian plants. The screening was done using an *in vivo* culture assay in the presence or absence of the purified compound and the parasite growth rate was monitored microscopically. This approach permitted us to identify a new compound which was subsequently shown to be 7-geranyloxycoumarin (aurapten). The compound belongs to the class of coumarins and shows significant growth inhibition of *L. major* promastigotes at concentrations of micrograms per ml.

## Material and Methods

### Extraction of the plant inhibitor

The leaves of *Esenbeckia febrifuga* (Rutaceae) were collected in Piracicaba, SP, Brazil, by Prof. Ricardo Ribeiro Rodrigues (Escola Superior de Agricultura Luiz de Queiroz, ESALQ, code 84295) in February 2000 and extracted according to the procedure illustrated in Figure 1 and described below.

Ground leaves (407.7 g) were submitted to a first extraction step with 4.5 L of hexane, resulting in 9.5 g of extract after the solvent was removed. The extract was fractionated by silica gel chromatography through a 30 cm high column measuring 5 cm in diameter using a hexane/ethyl acetate (100:0, 9:1, 8:2, 7:3, 1:1, 0:100) and an ethyl acetate/methanol (100:0, 9:1, 7.5:2.5, 6:4, 1:1, 0:100) gradient system. From this purification 30 fractions of 250 mL each were collected. Fraction 14 (hexane:ethyl acetate 8:2) was further fractionated on a 3 x 26 cm silica gel column using a gradient system with hexane, methylene chloride and methanol (hexane 100%; hexane/methylene chloride 100:0, 90:10, 80:20, 70:30, 1:1; methylene chloride/methanol 100:0, 90:10, 70:30, 1:1, 0:100). The 79 fractions (25 mL each) were collected and, based on thin-layer chromatography (TLC) on silica gel (0.25-mm thick silica gel, Merck PF 254, hexane/methylene chloride/aceton 3:1:0.5), and detected with, Vaniline Acid, UV light 254 and 360 nm and Dragendorff reagent, eight fractions were pooled. Fraction four was submitted to chromatographic separation on a 2 x 31 cm silica gel column diluted isocratically with 3:1:0.5 hexane, methylene chloride and acetone. This step resulted in 87 fractions (25 mL each), twelve of which were pooled after TLC analysis as above. Fraction two was submitted to chromatographic separation on a silica gel column on a 1 x 20 cm silica gel column using methylene chloride as the mobile phase resulted in 24 fractions (25 mL each), four of which were pooled after TLC analysis. Fraction four resulted in an amorphous yellow solid (47 mg) which is 7-geranyloxycoumarin (aurapten) according to NMR $^{1}H$, $^{13}C$ spectra and GC/MS, that was crystallized by vapor diffusion, at room temperature, from hexane/methylene chloride 2:1. The purity of the compound was confirmed by TLC (0.25-mm thick silica gel, Merck PF 254, Darmstadt, Germany), NMR and GC/MS.

**Parasite assays**

Stock solutions of aurapten were prepared in dimethyl sulfoxide (DMSO) at 5.0 mg/ml. Further dilutions of aurapten were made directly in the *L. major* culture medium immediately before use. In all experiments the final concentration of DMSO less than 0.5% (v/v), a concentration that does not affect parasite growth rate, mobility or morphology (6).

*L. major* Friedlin promastigotes were grown to a concentration of 1.5 x $10^4$ cells/ml in M199 (Gibco Laboratories, New York, NY, USA) supplemented with 10% fetal bovine serum (FBS), 10 mM adenine, 10 mg/ml penicillin, 10 mg/ml streptomycin, 0.25% hemin chloride, 0.1% biotin, and 1.0 M HEPES, pH 7.5. The parasite cultures were prepared with or without aurapten at final concentrations of 2.0, 5.0, 7.0, 10.0 and 15.0 µg/ml. The effect of aurapten on parasite growth was monitored by counting in triplicate in a Nebauer chamber after 48-h incubation at 26ºC.

**Crystallographic characterization of aurapten**

Aurapten was crystallized from 2:1 hexane/methylene chloride by vapor diffusion at room temperature. The typical crystal was yellow and was needle shaped. X-ray diffraction was carried out using a Nonius KappaCCD diffractometer at room temperature. The structure was solved by direct methods using the SHELXL97 software (7) and was refined anisotropically with full-matrix least-squares on $F^2$ using SHELXL97 (7). The hydrogen atoms were placed at the calculated position, except for those involved in H-bonds, found on difference maps and further refined. Final indices were: $R_1(F_o)$ = 0.0895 and $wR_2(F^2)$ = 0.2723 for 165 refined parameters. The crystallographic parameters for $C_{19}H_{22}O_3$ are: $M_w$ = 298.37, Orthorhombic, space group Pnma [No. 62], Z = 4, $a$ = 6.557 (1) Å, $b$ = 7.117 (2) Å, c = 35.650 (2) Å, V = 1664.0 (9) Å$^3$, $d_c$ = 1.191 Mg.m$^{-3}$, λ (Mo Kα) = 0.71073 Å, µ = 0.104 mm$^{-1}$, 3205 measured reflections, 1616 unique ($R_{int}$ = 0.0301) 1579 of which were considered for calculation purposes as observed with an I ≥2σ (I). The crystallographic data were deposited in the Cambridge Crystallographic Data Centre (deposit@ccdc.camac.uk) under accession number 232201.

**Results and Discussion**

We investigated and characterized the antiproliferative activity of the coumarin (7-geranyloxycoumarin), or aurapten, with a molecular mass of 298,37 g/mol against *L. major* Friedlin promastigotes in axenic cultures. As shown in Figure 2, *L. major* promastigote growth was significantly inhibited by aurapten, with an $LD_{50}$ of 30 µM. This value represents a significant inhibitory activity against *L. major* and is similar to those obtained for other inhibitors isolated from other plants (8). The negative control, corresponding to the parasite in the presence of DMSO, the aurapten solvent, showed no detectable inhibition of growth, alteration in cell morphology or motility at the concentrations tested.

Most studies directed at the detection of plant secondary metabolites with leishmanicidal activity have been done using the promastigote form of the parasite because of its easier maintenance under *in vitro* conditions. However, since the promastigote is not the infective form of the parasite in vertebrate hosts, these evaluations have only a suggestive value of the possible leishmanicidal activity of the compound tested.

There are several active compounds obtained from medicinal plants traditionally used worldwide for the treatment of leishmaniasis. Among these active molecules, described in the recent literature are quinoline alkaloids, isoquinoline alkaloids, indole alkaloids, terpenes, acetogenins, and lignans (9). Other natural compounds with antileishmanial activity are coumarins, chalcones, lactones, tetralones, and saponins (9). The compound described here belongs to the class of the coumarins and its activity against *Leishmania* promastigotes is reported here for the first time.

To unambiguously assign the structure of this inhibitor and to gain insight into common structural motifs related to other coumarins and other leishmania inhibitors, its crystal structure was determined, and several structural features emerged. This new information will be valuable for the development of a new anti-*Leishmania* drug since resistance to the currently available drugs such as antimonials and amphotericin B has emerged (3).

The refined molecular structure together with the atom-numbering scheme is shown in Figure 3. The molecule is a derivative of cinnamic acid that contains an aromatic system with electron delocalization. There is also a hydrophobic side chain consisting of ten carbon atoms with two double bonds with negative density. All bond distances and angles are close to normal values (10,11) and the molecule is planar, as can be seen in Figure 3. Three double weak intermolecular interactions of type C–H...O support the molecular packing (12) shown in Figure 4. They are the intermolecular C8—H8...O1$^{i}$ and C6—H6...O2$^{i}$ [symmetry code: i) 1+x, y, z] hydrogen bonds between two neighbor molecules. The distance and dihedral angles between donor and acceptor are 3.38 (1) Å, 180 (5)° and 3.27 (1) Å, 172 (5)°, respectively. The last of the intermolecular hydrogen bonds C5—H5...O2$^{ii}$ [symmetry code: ii) 0.5+x, y, 1,5-z] linking these two molecules shows 3.34 (1) Å of distance and 163 (3)° of dihedral angle between donor and acceptor. The planarity shown in the molecular configuration and the intermolecular interactions are consistent with typical structural features observed in anti-*Leishmania* inhibitors (13-15).

Figure 5 shows the *L. major* culture in the presence and absence of the inhibitor. Forty-eight h after inoculation, a significant decrease in surviving promastigotes was observed in the presence of aurapten (Figure 5b) at a final concentration of 30 μM. The surviving parasites showed abnormal morphology with the presence of large vacuoles and low flagellum motility.

This preliminary evaluation of aurapten's activity using promastigotes yielded interesting results indicating that the compound should be investigated further as a potential anti-*Leishmania* compound.

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**Figures**

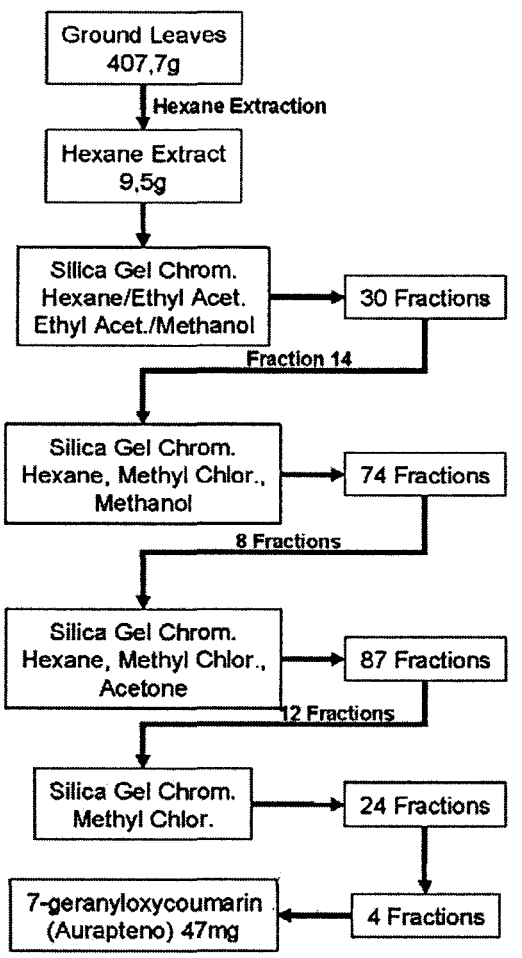


Figure 1. Flow diagram of the aurapten isolation procedure. Individual steps are described in Material and Methods. The solvents used for elution of the chromatography column are listed in the boxes on the left side. Acet = acetate; chrom. = chromatography; chlor. = chloride. Silica gel column effluents were monitored by TLC to select fractions for further purification.

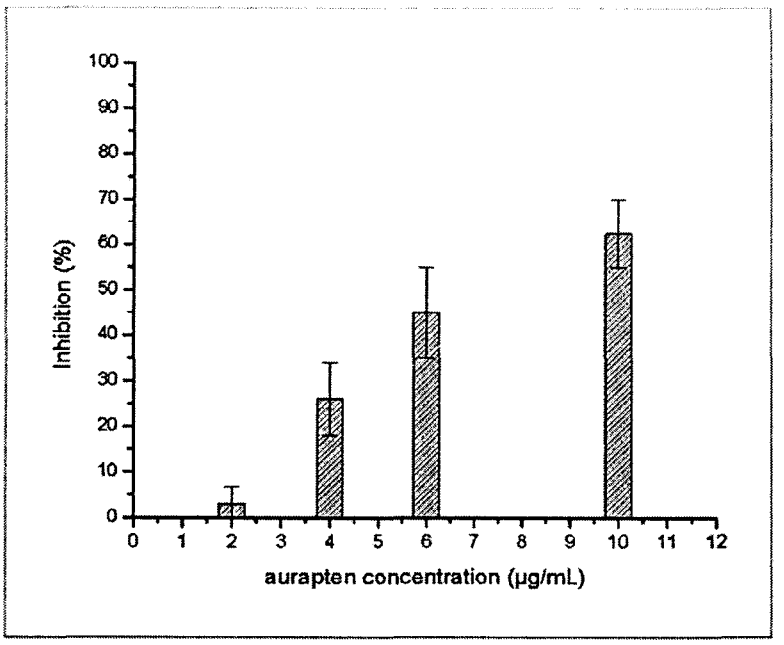


Figure 2. Antileishmanial activity of aurapten against *Leishmania major* Friedlin promastigotes. Cells were cultivated in the presence of different concentrations of aurapten and counted after 48 h. The height of the bars indicates de percentage of growth inhibition at each concentration compared to the control experiment containing only the aurapten solvent DMSO. The experiments were performed three times independently and each counting was performed in triplicate. Data are reported as means ± SD.

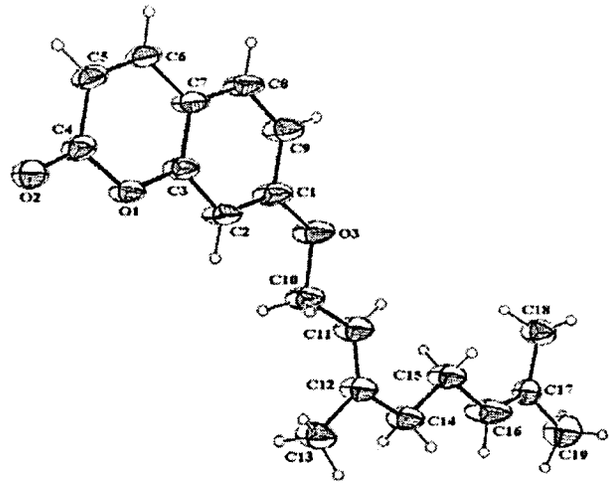


Figure 3. X-ray crystallographic structure of the aurapten. Displacement ellipsoids are shown at the 30% probability level. Carbon and oxygen atoms are labeled for clarity.

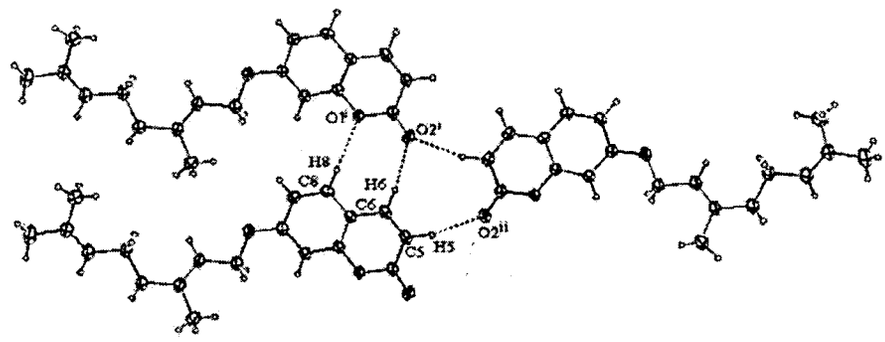


Figure 4. A view of aurapten's packing showing C—H...O interactions. Symmetry codes (i) and (ii) correspond to [1+x, y, z] and [0.5+x, y, 1.5-z], respectively.

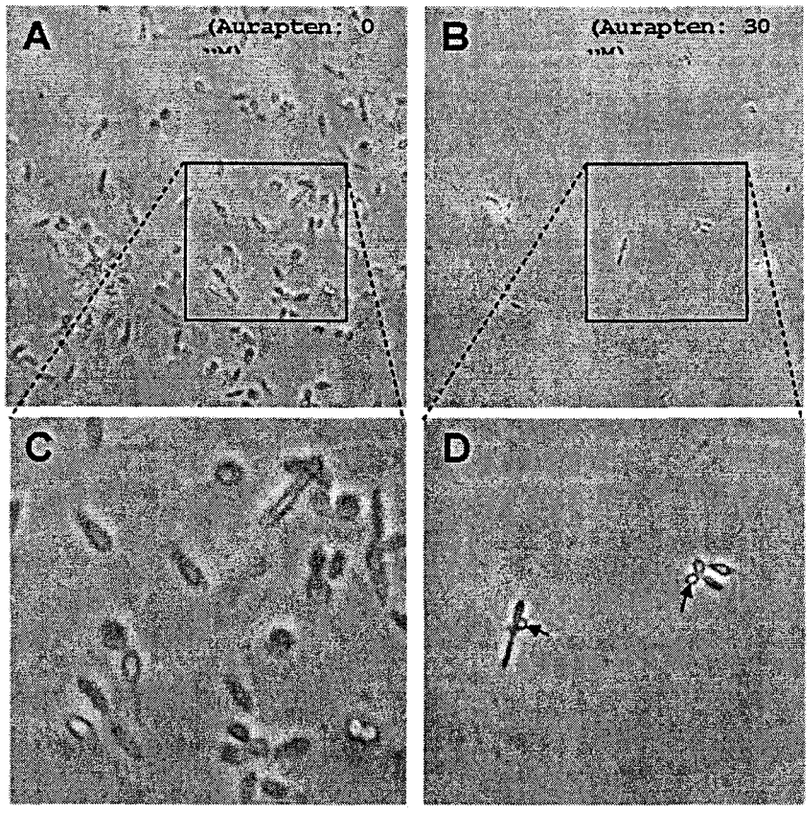


Figure 5. *Leishmania major* promastigote culture. Promastigotes were cultured in M199 medium supplemented with 10% fetal bovine serum at 26°C and cell viability was estimated by counting in a Neubawer chamber after 48 h of incubation in the presence or absence of the inhibitor. *A*, Negative control without aurapten in the presence of 0.5% DMSO. *B*, Condition containing 30 µM aurapten and 0.5% DMSO. *C* and *D* are expansions of Figures *A* and *B*, respectively (indicated by the squares in A and B) showing the different morphological characteristics of the parasite in the presence of aurapten. Vacuoles are indicated by the arrow.

# In-vivo and in-vitro effect of four compounds of *Adiscanthus fusciflorus*: Inhibition of phosphoribosyl transferases activity and antileishmanial effect.

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**Objectives: The aim of this study was the screening of novel *Leishmania* inhibitors.**

**Methods: Plant extracts of were fractionated guided by APRT enzyme inhibition. The inhibitors obtained were further tested in XPRT and HGPRT enzymes. The antileishmanial properties of the selected molecules were analyzed on *in vitro L. major* promastigotes culture *in vivo*. The interactions between inhibitors and the enzymes were analyzed by computational molecular docking.**

**Results: Four selected alkaloids and cumarines present inhibitory properties in both systems used, the biochemical PRTase assay and the in vitro *Leishmania* inhibition.**

**Conclusions: The results reported here concerning the inhibition of PRTase enzymes and *L. major* promastigote parasites indicate that the four natural molecules selected can be further exploited as lead compounds in a rational drug design approach of compound optimization against human leishmaniasis.**

**Abbreviations:** 5'-phospho-α-d-ribosyl-1'-pyrophosphate - PRPP;

Keywords: *Leishmania*, phosphoribosyltransferase, natural product, alkaloids, cumarine, *Adiscanhus fusciflorus*.

## Introduction

In countries of Africa, Asia, Middle East and Latin America [1] leishmaniais has been identified as a serious public health problem by the World Health Organization (WHO) and the disease is also endemic in the southern countries of Europe. In the last two decades the appearance of cases of co-infection of AIDS and *Leishmania* have added a new dimension

41 to the problem [2,3]. The peopling of new habitats and the increased human mobility has
42 taken leishmaniasis to regions previously unaffected, such as the North American region [4].
43 The estimated prevalence of 12 million infected and 350 million lives at eminent risk of
44 infection reflects the global dimension to leishmaniasis as public health concern [5].
45 To oppose this scenario, new drugs and new formulations of old drugs are either approved
46 or on clinical trial, rendering the current leishmaniasis chemotherapy more promising than
47 it has been for several years [6]. In spite of the progress, antileishmanial drugs in use are
48 generally toxic, expensive and demanding long-term treatment with hospitalization. Those
49 are characteristics attributable to low target specificity [7] and contribute to a general
50 inefficacy of the current treatment approaches. The new drugs and formulations may
51 alleviate, but is doubtfully alter significantly, the current global scenario [8]. Therefore, there
52 is a great and urgent need for the development of new, effective and safe drugs for the
53 treatment of leishmaniasis.
54 The institution of a rational therapeutic regimen for the treatment of parasitic
55 diseases depends on the exploitation of fundamental biochemical disparities between
56 parasite and host. One of the most striking metabolic discrepancies between parasites and
57 human is the purine pathway [9-11]. Whereas mammalian cells can synthesize the purine
58 heterocycle *de novo*, all Kinetoplastida studied are auxotrophic for purines [12,13]. In these
59 organisms the enzymes that enable the organism to scavenge host purines are the
60 hypoxanthine-guanine phosphoribosyltransferase (HGPRT; EC 2.4.2.8), adenine
61 phosphoribosyltransferase (APRT; EC 2.4.2.7) and xanthine phosphoribosyltransferase
62 (XPRT; EC 2.4.2.22) [12,13]. Unique features of the purine salvage pathway of *Leishmania*
63 and *Trypanosoma* constitute the basis for the susceptibility of these genera to several
64 pyrazolopyrimidine analogs of naturally occurring purine bases and nucleosides [11,13-15].
65 A rich source of novel lead compounds for the drug discovery process has been the
66 screening of natural compound sources such as plants, animals and microorganisms. Brazil
67 is recognized as a repository of a large, and mostly unexplored, biodiversity with a vast
68 potential to yield novel therapy alternatives.
69 In the present study we used the *Leishmania* PRTase enzymes as targets to screen
70 the inhibitory capacity of several small molecules compounds from Brazilian plants. We
71 describe in this communication four new PRTase inhibitors and their respective

antileishmanial activities. The selectivity of the compounds was tested against human and *Leishmania* cells and the molecular basis for the compound inhibition was investigated by molecular docking techniques with each PRTase. Such systematic approach of investigation identified the alkaloids and a cumarine as lead compounds and are used in their further improvement.

## Materials and methods

*Purification of enzymes and inhibitors.* *L. tarentolae* APRT, HGPRT and *L. major* XPRT enzymes were purified from *Escherichia coli* in recombinant expression systems as described previously [16,17].

The skimmianine, isopimpinellin, γ-fagarine and dictamine inhibitors were purified from roots and leaves of *Adiscanthus fusciflorus* (Rutaceae) collected from the region of Manaus in the Amazon State of Brazil. The purification protocol includes successive extractions with hexane and methanol as described previously [18,19]. The structure of the tested compounds is shown in Figure 1.

*Enzymatic assay and inhibitor Screening.* The APRT, HGPRT and XPRT activity and inhibition assay were performed as described previously in a spectrophotometric assay at the wavelengths of 259, 255 and 254 respectively [20]. The principle of the assay permits to explore the rate of change in absorbance resulting from the conversion of the enzyme specific substrates to the resulting nucleotide. The assays were carried out at 25°C in a reaction mixture of 500 µL containing the following reagents: 100 mM Tris-HCl pH 7.4, 100 µM PRPP and 5 mM $MgCl_2$. The APRT assay contained 50 µM Adenine, HGPRT 40 µM guanine, and the XPRT assay 50 µM xanthine and no $MgCl_2$ was added. All PRTase reactions were initiated by the addiction of enzyme to a final concentration of 2µg/mL and monitored during 1 min for APRT and HGPRT and 30 seconds for XPRT assay.

The screening of natural products with inhibitory activity against the *Leishmania* PRTases was performed in triplicate using the standard activity assay described. The inhibitors stock solutions were prepared in dimethyl sulfoxide (DMSO) at 1 mg/mL. Further dilutions were performed in the assay reaction buffer immediately prior to use. The

final concentration of DMSO in the enzymatic reaction did not exceed 5% (v/v), a concentration at which the solvent did not affect the activities of the PRTases tested.

*Cell culture and inhibition assays. L. major* promastigotes were grown in M199 (Gibco Laboratories) supplemented with 10% heat inactivated fetal bovine serum (FBS), 10 mM adenine, 10 mg/mL penicillin, 10 mg/mL streptomycin, 0.25% haemin chloride, 0.1% biotin and 1.0 M Hepes pH 7.5 at 26°C. The inhibitory effect of each compound was tested in promastigote cultures at $1.5x10^4$ cells/mL with and without inhibitors in 12 well plates, 2 mL/well. The concentrations of inhibitor tested were 2.0, 5.0, 7.0, 10.0 and 15.0 µg/mL. Cells were counted in a Newbawer chamber in six independent experiments after 48h incubation at 26°C. In all experiments the final concentration of DMSO was kept lower than 0.5% (v/v), a concentration that doesn't show evidence of affecting the parasite growth rate, mobility or morphology [21].

Human U937 promielocytes cells were used to test the selectivity of the inhibitors. The cells were cultured in RPMI 1640 media at 37 °C in a 5% $CO_2$ atmosphere. A concentration of $1.5x10^5$ cells/mL were incubated in the presence or absence of inhibitors and DMSO in 12 well plates, 2 mL/well, and counted in a Newbawer chamber after a period of 48h.

Growth inhibition protection experiments were carried out as described for *L. major* promastigote assays. In each culture, in triplicate, were added 50µM and 500µM of each purine (adenine, guanine, xanthine and hypoxanthine) as well as the inhibitor (skimmianine, dictamine, γ-fagarine and isopimpinellin) at the $LD_{50}$ concentration of each compound. Controls without inhibitors with added purines and with inhibitors in the absence of purines were performed in parallel.

*Docking procedure.* Docking simulations were performed with the GOLD 2.1 software [22], which does flexible docking using a genetic algorithm. The parameters used in this algorithm were originally optimized from a set of 305 complex structures with coordinates deposited in the Protein Data Bank (PDB). Among the parameters available in the program, we used a population equivalent 100000 operations, 95 mutations and 95 crossovers. Docking calculations were done in the interior of spheres of 15 Å radius with origins at: Ala80 (Cβ atom) of the APRT structure; Asp126 (Oδ1 atom) of the HGPRT

structure and Val121 (Cγ1 atom) of the XPRT model. The ten orientations of highest score for each compound were then selected using the score function denominated GoldScore. On the basis of this function, the program classifies the orientations of the molecules in a decreased order of affinity (score) with the ligand site of the receptor.

Before the docking calculations and after removal of ligands and the crystallographic waters from the active sites of the APRT and HGPRT structures, as well as the XPRT model, we added and oriented hydrogens of the side chains of the residues of the respective active sites. Charges and atomic potentials were assigned to the protein structures and model using the Insight II program [23].

## Results

*Effect of isolated compounds on the inhibition of Leishmania PRTases.* The recombinant APRT enzyme from *Leishmania tarentolae* was used to guide the purification of novel PRTase inhibitors. In this approach we screened the inhibitory activity of 1.126 extracts from Brazilian plants, the best four inhibitors selected from *Adiscanthus fusciflorus* (Rutaceae) are shown in figure 1. The inhibitors selected using the *Leishmania* APRT were then tested for their inhibitory capacity in HGPRT and XPRT enzymatic assays since it will be interesting from the standpoint of a lead compound to be able to inhibit all three enzymes simultaneously. The inhibitory activity of each selected compound (Figure 1) on the three PRTases and on the *L. major* promastigotes are shown in table 1.

The effect of each compound isolated from *A. fusciflorus* on the *Leishmania* PRTases is shown in Figure 2. All 4 inhibitors exhibited a clear linear concentration dependent effect. In comparison with the control experiments with solvent (DMSO) alone the compounds showed a significant effect on the PRTases activity ($P<0.05$).

A correlation of dictamine, γ-fagarine and isopimpinellin inhibitory effect over each PRTases is observed. Skimmianine however showed a distinct selectivity for both XPRT and HGPRT, whereas it inhibited APRT activity at a similar level as the other three compounds.

The $IC_{50}$ calculated for each inhibitor against the PRTases was calculated and is shown in Table1. The inhibitory effect of skimmianine on HGPRT and XPRT activity is 100 fold

higher as its activity with APRT. All other compounds show a similar inhibitory activity over each PRTase, reflecting a low selectivity of those inhibitors for the enzymes.

*Effect of isolated compounds on the in-vitro growth of Leishmania major promastigotes and human promyelocite cells.* Figure 3 shows the effect of the alkaloids and the cumarine on the in-vitro growth of *L. major* promastigotes. All four compounds exhibited a clear concentration-dependent inhibitory effect. In comparison with the control, the compounds showed a significant inhibitory effect on the in-vitro growth of *L. major* promastigotes at concentration of 20 μg/mL and above ($P<0.05$).

Table 1 shows the $LD_{50}$ values of the four compounds on the in-vitro growth of *L. major* promastigotes and human promielocyte cells. The $LD_{50}$ values on the promastigote and human cells are equal, reflecting a lack of cellular selectivity. A small difference is though observed on the effect of skimmianine, that shows a reduced effect on *L. major* promastigotes ($LD_{50}$=98μM) wen compared to the human cells (245μM).

Control experiments have been performed to evaluate the effect of DMSO on the growth of the *L. major* promastigotes and human promielocyte cells. No significant level of growth inhibition was detected at the concentrations used in the tests as well as no morphological alterations were observed by phase contrast microscopy (Data not shown).

*Effect of the addition of purines to the Leishmania culture.* The observed biochemical and cell culture effects of the copounds is not obviously a linked phenomenon. To initially identify any correlation we used the addition of purines (adenine, guanine, hypoxanthine and xanthine) to the culture media in an attempt to identify a possible *protection* or *survival rescue* that could be suggestive of a correlation between the biochemical and cellular observations.

Table 2 shows the inhibitory effect (in % growth inhibition) of each alakaloid and cumarine in the presence of two different concentrations of purines, 50μM and 500μM each. Except for dictamine, the other compounds (skimmianine, isopimpinellin and γ-fagarine) affected less severely the growth of the parasites. The data is preliminary, but reveals an interesting possibility of correlation.

196 *Molecular fitness of the isolated compounds in the PRTase active site.* To obtain a
197 molecular understanding of the type of interactions occurring between the alkaloid and
198 cumarine compounds and the targeted enzymes, molecular docking experiments were
199 performed. Those experiments used as target molecules the molecular structures of the
200 *Leishmania* APRT and HGPRT and the molecular model of the *Leishmania* XPRT
201 determined in our group [16,17]. In general, the docking results obtained with GOLD 2.1
202 (Table 1) for the compounds here studied agree with the enzymatic inhibition experiments
203 shown in Table 1.
204 Clear interactions of each inhibitor with amino acid side chains are observed from
205 this analysis. The top scoring interactions are shown in figure 4a-c.
206
207 **Discussion**
208
209 Scientific evolution of medicinal plants has, in the past, provided the basis of
210 modern medicine. Several compounds from higher plants are currently being evaluated in
211 various laboratories as potential antiparasitic agents for malaria, trypanosomiasis and
212 leismaniasis [24]. The search for bioactive molecules normally begins with the screening of
213 several plant extracts, isolation of bioactive fractions and identification of the active
214 components when feasible [25]. Most of the studies directed toward the detection of plant
215 secondary metabolites with leishmanicidal activity have been done using the promastigote
216 form of the parasite due to the facility to maintain under *in vitro* conditions [26]. In spite of
217 that one of the critical points of the leishmaniasis phytotherapeutical research is the lack of
218 good and rapid screening methods to find potential plants and to evaluate them [27]. For a
219 rational drug design approach it is paramount to understand the molecular target enzyme
220 whose inhibition is intended. The enzymes of the purine salvage pathway have been
221 considered as appropriate targets by different laboratories [9-12]. In this context, the inhibition
222 of the three phosphoribosyl transferases (PRTases) of the pathway; adenine, guanine-
223 hypoxanthine and xanthine PRTases, is necessary. We present in this paper four new *L.*
224 *major* promastigotes inhibitors with bioactivity similar at Pentostam, a commercial
225 leishmanicida [21]. Although of moderate inhibition capacity [26], their selection process
226 represents a novel approach. This strategy permitted us the selection and isolation of

alkaloids and a cumarine, which are the most important group of natural compounds with antileishmanial activity [24]. In recent years a few attempts for screening medicinal plants used for the treatment of leishmaniasis have been carried out in Spain, Sudan and Guinea-Bissau. These studies have confirmed the importance of many plant species as potential source for the isolation of novel compounds with leishmanicidal activity [26].

All four compounds (Figure 1) selected from our screening approach showed an inhibitory activity against the *Leishmania* PRTases in a concentration dependent manner (Figure 2). The inhibition effect on each individual PRTase is very similar for dictamine, isopimpinellin and γ-fagarine and varies from an $IC_{50}$ of 260μM for dictamine against APRT to 80μM for γ-fagarine against HGPRT (Table 1). The inhibitor skimmianine however, presented a marked selectivity for XPRT and HGPRT (1.1 μM and 1.8 μM, respectively) over APRT inhibition (142 μM).

This selectivity is also revealed, although to a lesser extent, in the in-vitro *Leishmania* promastigote and human promyelocite culture assays. From the four inhibitors selected, only skimmianine has shown a small selectivity (2.5 fold) against *Leishmania* cells ($LD_{50}$ of 98 μM) over human promyelocite cells ($LD_{50}$ of 245 μM) as shown in table 1. This result doesn't mean that the compounds are acting specifically on the PRTases in-vivo, but it represents a first indication that they may be good starting compounds. In an attempt to understand if there is a correlation between the biochemical inhibition of the PRTases and the growth inhibition of promastigotes several experiments must be performed in the future. An initial experiment trying to protect the promastigote cells from the purified compounds using a mixture of purine nucleotides in the culture media revealed that with the exception of dictamine, the other alkaloids (skimmianine, and γ-fagarine) and the cumarine isopimpinellin, affected less severely the growth of the parasites (Table 2). The data is preliminary, but reveals an interesting possibility of correlation.

All three alkaloids and the cumarine represent lead compounds that can be interesting starting points in a rational based drug design approach. For this aim, we used the molecular docking technique to address the interaction of each inhibitor with each PRTase. In general, the docking results obtained with GOLD 2.1 for the compounds here studied agree with the enzymatic inhibition experiments shown in Table 1.

Concerning APRT, the experimental affinity order for the active site is the following: skimmianine > isopimpinellin > γ-fagarine >> dictamine. In other words, skimmianine and isopimpinellin have similar $IC_{50}$ values and they are above the values obtained for γ-fagarine and dictamine. This last compound has the lowest and far $IC_{50}$ value amongst all the inhibitors. From the Table 1, the GoldScore of the compounds concerning the APRT structure is very similar to the order obtained in the experimental work. The fitness of both the skimmianine (46.11) and isopimpinellin (45.97) with the enzyme is very similar and they are higher than the scores obtained for γ-fagarine (44.28) and dictamine (42.42).

Analyzing the top-ranked solutions obtained with GOLD for these 4 compounds inside the APRT active site, dictamine is the only molecule that does not perform any strong hydrogen bond with residues of APRT, theoretically. Skimmianine is hydrogen bound to both the conserved Lys102 and Lys105 residues. Similarly, isopimpinellin has strong hydrogen bonds with the side chain of Arg316 as well as the side chain of Lys102. On the other hand, the GOLD result obtained for γ-fagarine suggests that this molecule interact by hydrogen bond with the Lys102 side chain. Top-ranked solution of skimmianine inside the APRT active site is shown in Figure 4a.

Concerning HGPRT, the experimental $IC_{50}$ values (Table 1) reveal skimmianine and dictamine as the best and the worse inhibitors, respectively. Our theoretical results (Table 1) agree with the enzymatic inhibition experiments, suggesting skimmianine as the ligand with highest GoldScore (37.91). On the other hand, dictamine has the lowest score (34.93) and could be theoretically the worse inhibitor. Analyzing the top-ranked solutions obtained with GOLD for these 2 alkaloids inside the HGPRT active site, skimmianine is strongly hydrogen bound to the nitrogen atom of the ASP129 main chain (Figure 4b).

Concerning XPRT, our docking results (Table 1) suggest skimmianine as the best inhibitor (GoldScore = 49.95). In fact, the experimental $IC_{50}$ value obtained from the XPRT inhibition with this alkaloid is the lowest (1.1 μM) amongst all the 4 inhibitors (Table 1). The GOLD top-ranked solutions for skimmianine inside the XPRT active site suggests a strong hydrogen bond between the nitrogen atom of the ASP123 main chain of the enzyme and the oxygen atom of the furane ring of the inhibitor, which are separated by a distance of 2.3 A (Figure 4c). The results obtained with GOLD for XPRT must be analyzed with a

certain caution because we have used a homology model and not a structure obtained by single crystal X-ray diffraction techniques.

In conclusion, three alkaloids and a cumarine have been selected by biochemical assays directed towards the *Leishmania* PRTases and further characterized by their ability to inhibit the growth of *L. major* promastigotes in culture. A parallel experiment testing their selectivity was performed with human promyelocite cells.

The correlation of the biochemical data with the culture data has been preliminary obtained by demonstrating a protecting effect of a mixture of purines in the presence of the inhibitors.

Furthermore, a molecular docking analysis has been performed to access the structural elements involved in the binding of each inhibitor to the active site of each PRTase, indicating the structural elements involved in selectivity and binding for further exploration in a rational drug enhancement program.

Those compounds isolated from a Brazilian plant show promising results as lead compounds for future studies toward the development of an effective antileishmanial compound.

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409
410 FIGURES
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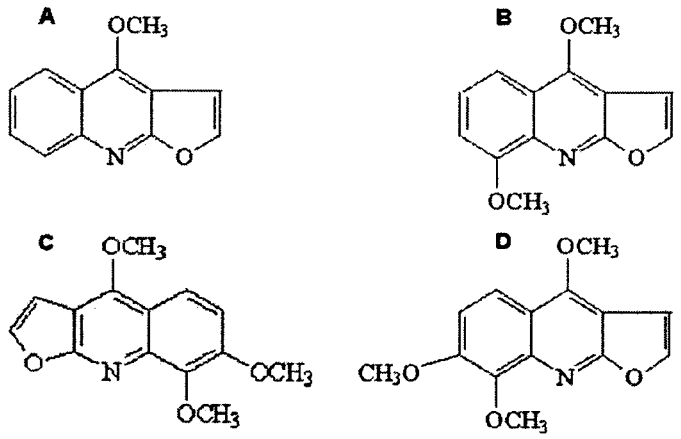


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415 **Figure 1.** Chemical structure of isolated compounds. (A) Dictamine (B) γ-fagarine (C)
416 isopimpinellin (D) skimmianine.

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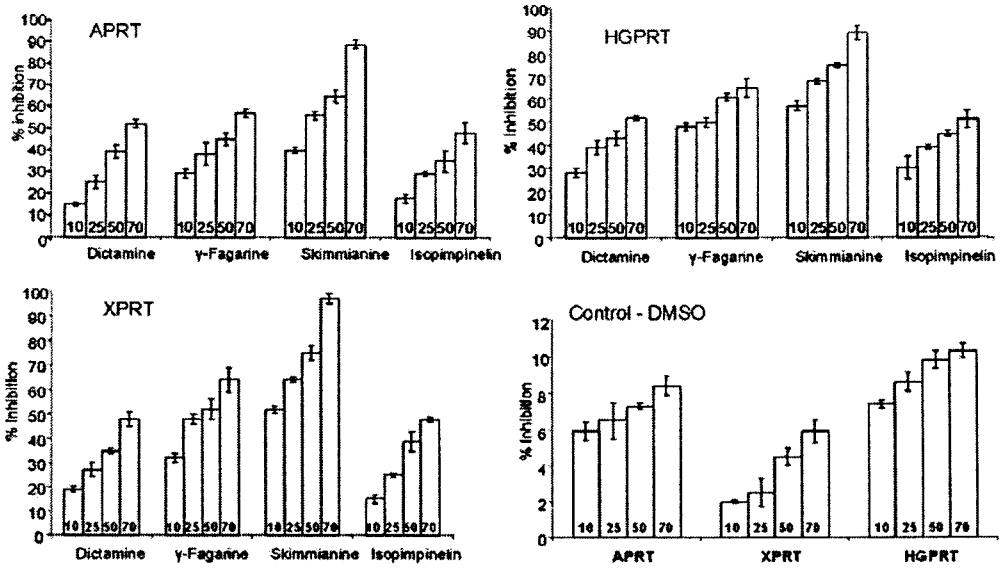


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419 **Figure 2.** Effect of the four compounds on the *Leishmania* PRTases. The results are from
420 six experiments in triplicate and are given as percentage of inhibition. The vertical lines
421 represent the amplitude of the standard deviation from the statistical analyses of the
422 obtained mean. DMSO-Control: Control experiment of inhibitory effect of DMSO on the
423 enzyme activity, showing a much reduced effect even at the higher concentration of 70 μM.
424 The numbers in each bar indicate the concentration of the compound tested, 10 μM, 25 μM,
425 50 μM and 70 μM.

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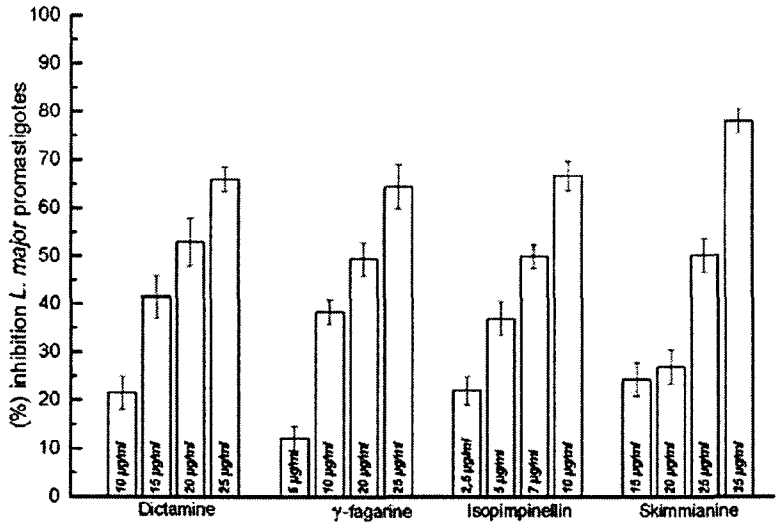


427

428 **Figure 3.** Effect of the four isolated compounds on the *in-vitro* growth of *L. major*
429 promastigotes. Promastigotes (1.5 x $10^4$ cells/mL) were incubated in the presence of
430 compounds for 48 h. The results are from six experiments in triplicate and are given as
431 percentage of inhibition. The vertical lines represent the amplitude of the standard deviation
432 from the statistical analyses of the obtained mean.

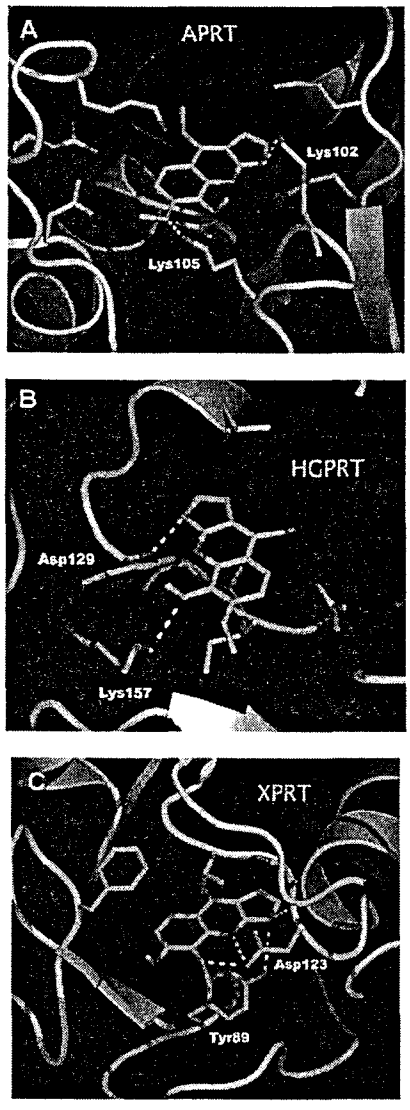


**Figure 4.** Top-ranked solution obtained with GOLD for (A) skimmianine-APRT complex. Selected residues of the active site are shown, with the hydrogen bonds represented by dashed lines in yellow with the Lys102 and Lys105 residues. (B) skimmianine-HGPRT complex. The hydrogen bonds are represented by dashed lines in yellow with the Asp129 and Lys157 residues and (C) skimmianine-XPRT complex. Hydrogen bonds represented by dashed lines in yellow with the Tyr89 and Asp123 residues.

446 TABLES
447
448 **Table 1.** Effect of 4 compounds on the enzymatic activity of PRTases, *L. major*
449 promastigotes, human cell and top-ranked solutions obtained with GOLD 2.1 interactions.
450
451 [a] The results of the enzymes inhibition are from three independent experiments in triplicate
452 and measured by spectrophotometric method at the wavelength 259, 255 and 254

| Compounds | [a] Enzymes $IC_{50}$ (µM) | | | Enzymes Gold-Score | | | Cell cultures $LD_{50}$ (µM) | |
|---|---|---|---|---|---|---|---|---|
| | APRT | XPRT | HGPRT | APRT | XPRT | HGPRT | [b] *H. sapiens* | [c] *L. major* |
| Dictamine | 262 | 199 | 250 | 42.42 | 42.61 | 34.93 | 90 | 90 |
| Isopimpinellin | 142 | 196 | 142 | 45.97 | 46.76 | 35.64 | 25 | 20 |
| Skimmianine | 142 | 1 | 2 | 46.11 | 49.95 | 37.91 | 245 | 98 |
| γ-fagarine | 151 | 120 | 82 | 44.28 | 43.18 | 35.02 | 70 | 69 |

453 respectively .
454 [b] The results of the in-vitro inhibition of mammal cells are from two independent
455 experiments in triplicate and measured by Newbauer chamber.
456 [c] The results of the in-vitro inhibition of *L. major* promastigotes are from six independent
457 experiments in triplicate and measured by Newbauer chamber.
458
459
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468 **Table 2.** Effect of the addition of purines (adenine, guanine, hypoxanthine and xanthine) on
469 the protection of *L. major* promastigotes against the four selected compounds.
470

| Purine concentration | Skimmianine | Isopimpinellin | γ-Fagarine | Dictamine |
|---|---|---|---|---|
| 0µM | 0 | 0 | 0 | 0 |
| 50µM | -8 | -55 | -220 | 7.4 |
| 500µM | -650 | -122 | -80 | 0 |

471 The results are presented in % inhibition of growth compared to the no purines added
472 experiment. The negative numbers represent a stimulation of growth.
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BERLINCK, R. G. S.; HAJDU, E.; ROCHA, R. M.; OLIVEIRA, J. H. H. L.; HERNÁNDEZ, I. L. C.; SELEGHIM, M. H. R.; GRANATO, A. C.; ALMEIDA, E. V. R.; NUÑEZ, C. V.; MURICY, G.; PEIXINHO, S.; PESSOA, C.; MORAES, M. O.; CAVALCANTI, B. C.; NASCIMENTO, G. G. F.; THIEMANN, O. H.; SILVA, M.; SOUZA, A.O.; SILVA, C. L.; MINARINI, P. R. R. Challenges and rewards of research in marine products chemistry in Brazil. **Journal Natural Products**,Pittsburgh, v. 67, p. 510-522, 2004.

# Capitulo V

## Conclusões

Os trabalhos apresentados nesta tese são referentes a uma das linhas de pesquisa que eu coordeno. Esta linha de pesquisa visa o emprego da estrutura molecular e da busca por inibidores na biodiversidade brasileira. Essa busca tem como objetivo obter novos inibidores das enzimas alvo que possam ser estudados por processos de *Docking* e técnicas de química medicinal.

Os trabalhos se iniciaram com a clonagem e caracterização das três PRTases de Leishmania de interesse para o projeto, a APRT, HGPRT e XPRT. Foi iniciada em paralelo a clonagem e caracterização da APRT humana, visando não somente obter o conhecimento de sua estrutura, mas também para ser utilizada em testes comparativos de inibição com a APRT de *Leishmania major*.

Desse esforço resultou a publicação da estrutura cristalográfica das APRTs de *Leishmania* e Humana, a obtenção e resolução da estrutura da HGPRT e a modelagem molecular da estrutura da XPRT de *Leishmania*. A analise da estrutura da HGPRT de *L. tarentolae* se encontra em fase de redação do artigo para submissão.

Essas enzimas forma empregadas em testes de rastreamento por inibidores em ensaios bioquímicos empregando extratos de plantas, animais marinhos e da flora microbiana. Uma serie de compostos foram obtidos desses ensaios e diversos

puderam ser cristalizados e tiveram sua estrutura resolvida em colaboração com o Prof. Dr. Javier Ellena (IFSC - USP). Desse trabalho resultaram três publicações. Os trabalhos de ensaios com inibidores foram estendidos, visando testar a sua eficácia sobre o parasito e as células de mamíferos. Com isso foi implantada e se encontra em funcionamento, uma sala de cultivo de parasitas e células de mamíferos. Os testes celulares foram realizados com diversos inibidores e resultaram na identificação da atividade celular de quatro compostos. Este trabalho foi recentemente submetido para publicação.

Esta abordagem representa uma linha inovadora implantada no laboratório e com potencial de ser aplicada a outras enzimas e inibidores, aprofundando a investigação e caracterização de compostos de interesse.

Uma linha de investigação paralela levou aos estudos com a enzima glucose-6-fosfato isomerase (PGI) de *Leishmania*. Essa enzima foi clonada e caracterizada. Assim como foi feito para a APRT de *Leishmania*, também iniciamos a investigação da PGI humana.

Obtivemos com sucesso a resolução da estrutura da PGI humana que resultou na publicação de dois trabalhos. Estes trabalhos contribuíram com o conhecimento geral do mecanismo catalítico das PGIs.

A PGI de *Leishmania mexicana* foi clonada a partir de dois fragmentos genicos cedidos por nosso colaborador, Prof. Dr. Paul A. M. Michels (*Research Unit for Tropical Diseases and Laboratory of Biochemistry, Christian de Duve Institute of Cellular Pathology*, Bruxelas, Belgica). Com a clonagem dessa enzima e a obtenção da proteína recombinante iniciamos ensaios de inibição com quatro

compostos desenvolvidos pelo Prof. Dr. Laurent Salmon (*Laboratoire de Chimie Bioorganique et Bioinorganique, Orsay Cedex*, França) e ensaios de cristalização. Resultou deste trabalho a publicação de dois artigos, descrevendo a clonagem, cristalização e atividade inibitória dos compostos testados. O segundo artigo descreve a estrutura cristalográfica da PGI de *Leishmania* e características peculiares do sitio ativo da enzima.

Estas linhas de pesquisa continuam ativas e representam uma modesta contribuição a área de Parasitologia Molecular e Estrutural

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