UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

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Síntese de *terc*-butil hidrazonas, avaliação da atividade antifúngica e seu modo de ação

Bruna Bento Casanova

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Trabalho de Conclusão de Curso apresentado como requisito parcial para a obtenção do título de Farmacêutico, pelo curso de Farmácia da Universidade Federal do Rio Grande do Sul.

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Dedico essa conquista a vocês!

Síntese de *terc*-butil hidrazonas, avaliação da atividade antifúngica e seu modo de ação

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Resumo

Leveduras emergentes estão entre as mais prevalentes infecções nosocomiais, com altas taxas de mortalidade e existe uma necessidade urgente de desenvolver agentes antifúngicos eficazes e não tóxicos com um espectro de atividade específica. Neste estudo 35 compostos, dentre eles aldeídos, hidrazonas e hidrazinas sintéticos foram obtidos e avaliados quanto a sua atividade antifúngica *in vitro*. A concentração inibitória mínima (CIM) dos compostos foi determinada em 20 isolados clínicos de *Candida parapsilosis* e *Tricosporon asahii*. Os compostos **13a** [*tert*-butyl (2Z)-2-(3,4,5-trihydroxybenzylidine)hydrazinecarboxylate] e **7b** [4-pyridin-2-ylbenzaldehyde] foram os mais ativos com valores de CIM na faixa de 8 a 16 ug/mL e 16 a 64 ug/mL, respectivamente. Utilizando 3 ensaios distintos, investigou-se o possível

modo de ação antifúngica dessas moléculas. Sugere-se ação dos compostos na membrana celular fúngica. Nossos resultados apontam para a descoberta de dois candidatos promissores para o desenvolvimento de novos agentes antifúngicos.

Palavras-chave: hidrazonas sintéticas, leveduras emergentes, antifúngico,

1. Introdução

Leveduras, especialmente espécies de cândida, surgiram ao longo das últimas duas décadas, como uma das principais causas de infecções humanas nosocomiais [1] [2]. O risco de infecções fúngicas tem aumentado, principalmente entre os indivíduos que são imunocomprometidos devido a neoplasias, SIDA e uso de quimioterapia intensiva e fármacos imunossupressores [3] [4].

Alguns anos atrás, *Candida albicans* representava 70-80% dos isolados clínicos do gênero *Candida* [5], mas nos últimos anos te m havido rápidas mudanças na epidemiologia. Em 2011, por exemplo, a prevalência de candidemia por de *Candida* não-albicans foi de 54,4% no mundo [6], e em 2013 um estudo revelou que nos maiores hospitais universitários do Brasil a prevalência dessas espécies foi de 56% [7]. As espécies de *Candida* não-albicans mais frequentes são *C. glabrata*, *C. parapsilosis* e *C. tropicalis* sendo a *C. parapsilosis,* a terceira espécie mais comum em isolados clínicos [6], [7], [8]. Dentre as leveduras que passaram a apresentar características oportunistas, destacam-se outros gêneros como *Tricosporon* sp. e *Rhodotórula* sp., que apesar de menos frequentes que as espécies de *Candida*, são o segundo e o terceiro gêneros causadores de infecções invasivas [6].

O que contribui para esta mudança é a utilização inadequada dos fármacos disponíveis na terapêutica, que provoca o aparecimento de espécies multirresistentes. E esse número de medicamentos utilizados é extremamente limitado, especialmente, quando comparado com o arsenal de antibacterianos, tornando a situação alarmante e despertando o interesse pela busca de novas alternativas seguras e eficazes [9].

Compostos que contém em suas estruturas as funções hidrazina e hidrazona são amplamente estudados por possuírem relatos, de atividades, tuberculostática [10], anticonvulsivante [11], analgésica [12], anti-inflamatória [12], antiplaquetária [13], antifúngica [14], antiviral [15], antitumoral [16] e antimalárica [17]. De acordo com uma revisão publicada em 2012 [18] as hidrazonas constituem uma classe importante de compostos para o desenvolvimento de novas entidades químicas (NCE) para tratar várias doenças de importância clínica, pois além de apresentar diversas aplicações biológicas, são facilmente sintetizadas e com bons rendimentos.

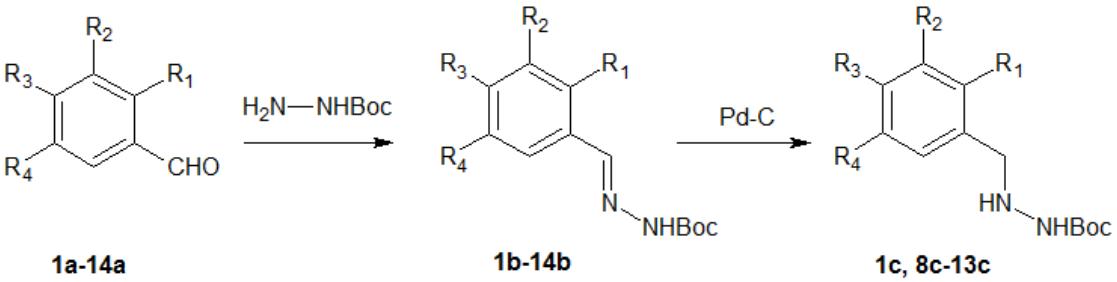
Considerando a importância clínica das infecções fúngicas e a ampla atividade biológica demostrada por hidrazinas e hidrazonas, realizou-se neste trabalho a síntese de uma série de compostos contendo essas funções químicas e avaliou-se sua atividade frente a isolados clínicos das espécies C. *parapsilosis* e T. *asahii.*

2. Resultados e Discussão

2.1. Síntese

Foram obtidos 14 hidrazonas e 7 hidrazinas através de uma rota sintética que emprega dois passos a partir de aldeídos comerciais ou modificados no laboratório (Tabela 1). A síntese química de compostos é apresentada de forma geral no Esquema1.

Esquema 1. Esquema geral de síntese dos derivados

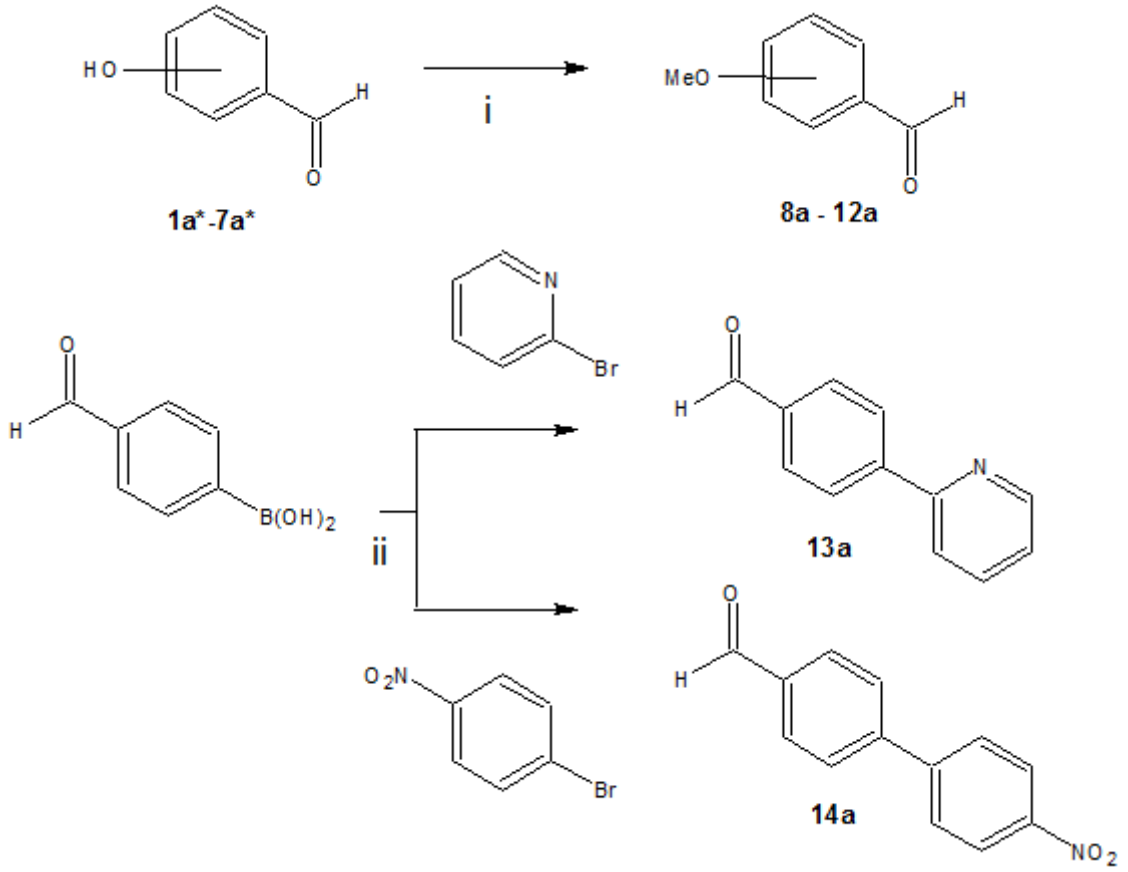


1a-14a **1b-14b** **1c, 8c-13c**

No Esquema 2 é apresentada a forma de obtenção dos aldeídos modificados. Sete aldeídos comerciais (**1a-7a**) foram utilizados como material

de partida para a síntese dos demais derivados. Os aldeídos **8a-12a** foram sintetizados a partir da *O*-metilação dos aldeídos hidroxilados (**1a-7a**)**.** Através de acoplamento carbono-carbono entre o ácido borônico e as arilas bromadas obteve-se os compostos **13a** e **14a**.

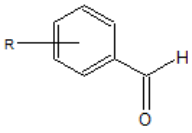
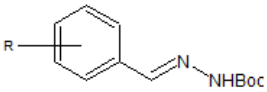
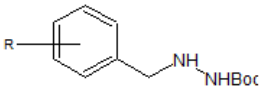
Esquema 2. Obtenção dos aldeídos precursores para a síntese das hidrazonas.



i. *O*-metilação dos aldeídos hidroxilados, **ii**. Acoplamento Suzuki Miyaura

Os aldeídos modificados (**8a-14a**) e os comerciais (**1a-7a**) foram submetidos a acoplamento com a amina simétrica protegida terc-butil carbazato formando as respectivas hidrazonas dos aldeídos utilizados (1**b-14b**). A etapa final foi a redução dessas hidrazonas, que levou a obtenção das hidrazinas **1c** e **8c**-**13c** (Esquema 1). As hidrazinas resultantes da redução das hidrazonas **2b-7b** e **14b** não puderam ser analisadas devidos a baixos rendimentos.

Tabela 1. Lista completa de derivados estudados.

Estrutura / Radical			
H	**1a***	**1b**	**1c**
3-OH	**2a***	**2b**	-
4-OH	**3a***	**3b**	-
4-Cl	**4a***	**4b**	-
2,4-OH	**5a***	**5b**	-
3,5-OH	**6a***	**6b**	-
2,3,4-OH	**7a***	**7b**	-
3-OMe	**8a**	**8b**	**8c**
4-OMe	**9a**	**9b**	**9c**
2,4-OMe	**10a**	**10b**	**10c**
3,5-OMe	**11a**	**11b**	**11c**
2,3,4-OMe	**12a**	**12b**	**12c**
4-(2)pyridinil)	**13a**	**13b**	**13c**
4-(4)NO_2benzil	**14a**	**14b**	-

2.2. Atividade Antifúngica

Inicialmente os todos compostos obtidos, descritos na Tabela 1, foram submetidos a um screening para avaliação da atividade antifúngica na concentração fixa de 500 µg/mL frente a 7 isolados clínicos de espécies causadoras de infecções invasivas, *C. albicans, C. krusei, C. parapsilosis, C. tropicalis, C. glabrata, C. lusitaneae e T. asahii*. Estes isolados estão depositados na coleção de culturas do Laboratório de Pesquisa Aplicada Micologia da Universidade Federal do Rio Grande do Sul, Porto Alegre, Brasil.

Os compostos que presentaram atividade de inibição para as espécies testadas na concentração de 500 µg/mL no screening estão apresentados na Tabela 2.

Tabela 2. Número de compostos de cada classe ativos na concentração de 500 µg/mL para as espécies testadas no screening.

	Compostos **1a**-**14a**	Compostos **1b**-**14b**	Compostos **1c, 8c-13c**
C. *glabrataa* [a]	5	2	1
C. *parapsilosis* [b]	7	6	2
C. *tropicalis* [c]	7	4	1
C. *krusei* [d]	5	3	1
C. *lusitaneae* [e]	5	5	1
C. *albicans* [f]	6	4	4
Tricosporon asahii [g]	6	6	4

Compostos que apresentaram atividade no teste para **a**: 2a, 5a, 7a, 13a, 14a, 7b, 12b, 12c; **b**: 2a, 6a, 7a, 8a, 10a, 11a, 13a, 2b, 5b, 6b, 7b, 11b, 13b, 10c, 13c; **c:** 2a, 5a, 8a, 10a, 12a, 13a, 14a, 7b, 8b, 11b, 14b, 13c; **d**: 2a, 5a, 12a, 13a, 14a, 7b, 9b, 12b, 8c; **e:** 2a, 4a, 6a, 13a, 14a, 2b, 6b, 7b, 9b, 11b, 13c; **f**: 2a, 5a, 7a, 10a, 11a, 13a, 7b, 10b, 11b, 13b, 1c, 9c, 10c, 13c; **g:** 1a, 2a, 5a, 6a, 8a, 13a, 3b, 5b, 6b, 7b, 11b, 13b, 9c, 10c, 12c, 13c,

No screening, os compostos demonstraram vasta e diversificada atividade. Dos 35 compostos testados, 30 apresentaram atividade de inibição em pelo menos uma espécie, os compostos **3a**, **9a**, **11b**, **4b** e **11c** não inibiram o crescimento em nenhuma das espécies, enquanto **2a**, **13a** e **7b** inibiram o crescimento de todas as espécies testadas. As espécies que demonstraram maior susceptibilidade foram T. *asahii*, C. *parapsilosis* e C. *albicans* que tiveram seu crescimento inibido por 16, 15 e 14 compostos, respectivamente. Destas, C. *parapsilosis* é descrita como a 3ª espécie de *Candida* não-albicans mais frequentes em isolados clínicos [6] [7] [8], e T. *asahii* como o segundo gênero mais importante [6]. Por essas razões os compostos que apresentaram atividade inicial para C*. parapsilosis* (**2a**, **6a**-**8a**, **10a**, **11a**, **13a**, **2b**, **5b**-**7b**, **11b**, **13b**, **10c** e **13b**) e T*. asahii* (**1a**, **2a**, **5a**, **6a**, **8a**, **13a**, **3b**, **5b**-**7b**, **11b**, **13b**, **9c**, **10c**, **12c** e **13c**), apresentados na Tabela 2, foram selecionados para o estudo da CIM. O ensaio de CIM foi realizado frente a 10 isolados clínicos de cada espécie. No mesmo experimento foi realizado o ensaio de CFM (Concentração Fungicida Mínima).

Dentre as moléculas testadas duas destacaram-se por apresentarem CIM inferior a 125 µg/mL para as duas espécies testadas, o aldeído **13a** e a hidrazona **7b**. Os resultados estão apresentados na Tabela 3. Os demais compostos testados apresentaram CIM entre 125 µg/mL e 500 µg/mL.

A variabilidade de resposta das leveduras aos compostos pode ser explicada pelo fato de tratarem-se de isolados clínicos, além de serem de espécies diferentes. O tratamento ao qual foi submetido cada paciente dos quais foram isoladas essas leveduras interfere nas suas respostas, podem ter adquirido fatores de resistência aos antifúngicos aos quais foram expostos.

Tabela 3. Concentração inibitória mínima (CIM) e Concentração fungicida mínima (CFM) dos compostos **13a** e **7b**, selecionados no screening inicial, frente a isolados clínicos.

	13a		**7b**		Fluc		**13a**		**7b**		Fluc
	CIM	**CFM**	**CIM**	**CFM**	**CIM**		**CIM**	**CFM**	**CIM**	**CFM**	**CIM**
T. asahii	µg/ml					***C. parapsilosis***	µg/ml				
TAH 05	125	>250	16	16	nd*	**RL 01**	32	125	16	>250	64
TAH 06	64	64	16	16	32	**RL 05**	32	>250	16	>250	≤1
TAH 07	64	>250	16	16	8	**RL 07**	32	125	16	>250	≤1
TAH 09	250	>250	8	>250	nd*	**RL 13**	64	250	16	>250	≤1
TAH 10	32	32	8	16	8	**RL 20**	16	>250	16	>250	4
TAH 11	64	>250	16	16	8	**RL 27**	32	>250	16	>250	≤1
TAH 12	64	64	16	16	nd*	**RL 32**	32	>250	16	>250	≤1
TAH 13	64	125	16	16	4	**RL 33**	32	>250	16	>250	2
TAH 14	32	125	16	16	4	**RL 36**	32	125	8	>250	2
TAH 15	32	64	16	16	4	**RL 38**	32	>250	8	>250	4

Fluc= Fluconazol, nd*= não determinado

As duas moléculas que apresentaram atividade, **13a** e **7b,** possuem estruturas e funções químicas distintas, (Tabela 1), enquanto **13a** é um aldeído e possui Nitrogênio como heteroátomo **7b** é uma hidrazona trihidroxilada. Ambos os compostos destacam-se pela elevada hidrofilia, Log P de 1,94 ± 0,2 e de 2,3 ± 0,59, respectivamente. A presença das hidroxilas na hidrazona **7b** confere a molécula maior possibilidade de realizar ligações de hidrogênio.

Observamos assim que a hidrofilia e a possibilidade de ligação de hidrogênio parece ser importante para atividade e sugerem indícios do modo com estas moléculas possam agir sobre as células fúngicas.

Outro indicio do modo de ação das moléculas pode ser observado quando se avaliou a atividade dos compostos sobre os isolados T. *asahii* TAH06 e C. *parapsilosis* RL01. Ambos os isolados são considerados resistentes ao Fluconazol segundo os parâmetros estabelecidos pelo CLSI (Clinical and Laboratory Standards Institute). Que determina que isolados que apresentem CIM para o Fluconazol maior que 8 ug/mL sejam considerados resistentes a este fármaco. O composto **7b** apresentou CIM de 16 ug/mL para ambos os isolados resistentes ao Fluconazol, e essa atividade pode indicar diferença no mecanismo de ação do fármaco e dos compostos testados, bem como diferença entres mecanismos de ação dos compostos em teste já que o isolado resistente a um foi sensível ao outro.

Os dois compostos apresentaram ainda diferença no tipo de atividade, enquanto **13a** apresentou padrão predominantemente fungistático (CFM > CIM) para as duas espécies, **7b** foi fungistático para C. *parapsilosis* e fungicida (CFM=CIM) para T. *asahii.* Esses podem ser indícios também de diferença nos mecanismos de ação dessas duas moléculas.

A partir dos resultados da CIM e das observações de prováveis mecanismos de ação distintos e diversos do Fluconazol nossos esforços foram focados na investigação do modo de ação dos compostos ativos.

2.3. Modo de ação

Para os compostos avaliados, foram realizados 3 testes a fim de investigar seus modos de ação conforme descrito a seguir.

a) Avaliação da Ação sobre a Estabilidade da Parede Celular: Teste de Proteção do Sorbitol:

A parede celular fúngica serve como uma barreira de proteção, impede rebentamento osmótico das células e confere forma. A parede celular é, portanto, essencial para o crescimento e viabilidade de fungos num ambiente hipotônico. A parede celular é dispensável se os fungos são protegidos com um

suporte osmótico em condições específicas, então mesmo que as células tenham sua parede celular lesada com moléculas que inibam sua síntese elas continuarão a crescer. Sorbitol foi utilizado neste experimento com protetor osmótico.

A nova CIM dessas moléculas foi determinada com meio YNB suplementado com glicose em experimentos paralelos com e sem a adição de sorbitol 0,8M. Os fungos tratados com Anidulafungina, antifúngico que atua na síntese da parede celular voltaram a crescer após a incubação por 7 dias. Como mostrado na Tabela 4, os derivados **13a** e **7b** não apresentaram atividade na parede celular dos isolados, pois o MIC permaneceu o mesmo nas leituras em 2 e 7 dias e nos meios com e sem sorbitol.

Tabela 4. CIM em µg/mL dos compostos **13a** e **7b**, na presença e ausência de 0,8M de sorbitol, em *C. parapsilosis* RL 33 e *T. asahii* TAH 10

Compostos	2 dias		7 dias	
	s/sorbitol	c/sorbitol	s/sorbitol	c/sorbitol
Anidulafungina	<1,0	<1,0	<1,0	>125
7b	16	16	16	16
13a	32	32	32	32

b) Avaliação da Ação sobre a Estabilidade da Membrana Celular: Fuga Celular

Quando ocorrem danos a membrana celular fúngica, componentes celulares como os nucleotídeos, extravasam das células. Esses compostos exibem forte absorvância a 260 nm permitindo assim sua quantificação, e por meio desses dados é possível avaliar a extensão do dano a membrana do fungo como mostra a Figura 1.

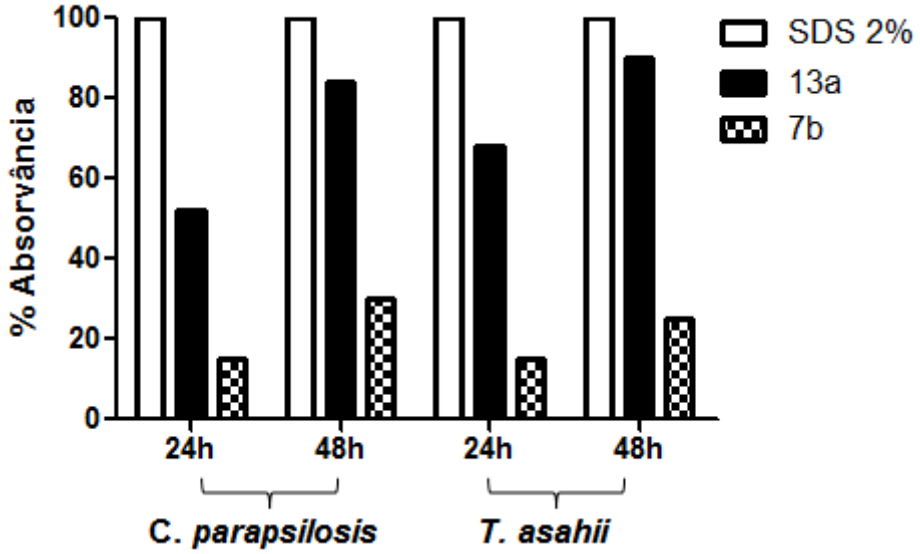


Figura 1. Liberação de componentes intracelulares de *C. parapsilosis* RL 33 e *T. asahii* TAH 10 tratados com os compostos **13a** e **7b**.

Tomando a leitura da absorvância do SDS 2% (Dodecil Sulfato de Sódio) como padrão de 100% de lise celular comparamos os resultados dos compostos testados. O composto **13a** em 48h chegou a uma absorvância de 90% do padrão, levando a conclusão de que causa forte dano a estabilidade da membrana celular para ambos os isolados. No entanto, o composto **7b** parece causar dano a membrana, porém de forma moderada quando comparado com o padrão e com o anterior.

c) Avaliação da Ação sobre o Ergosterol de Membrana: Efeito do Ergosterol

Para determinar se o dano a membrana celular ocorre por ligação aos esteróis de membrana, a CIM destes compostos foi determinada novamente com e sem a adição do ergosterol. Neste teste pode-se avaliar se a atividade dos derivados se dá devido a ligação ao ergosterol de membrana, uma vez que ergosterol exógeno é adicionado e este impede a ligação dos compostos ao ergosterol endógeno. Como consequência, nos casos positivos a CIM aumenta na presença de ergosterol exógeno. O padrão para este teste foi a Anfotericina B, fármaco que atua através deste mecanismo. Conforme observado na Figura 2, nenhum dos derivados apresentou aumento significativo da CIM, o que nos

leva a crer que estes possuem um mecanismo de ação que não envolve esta via.

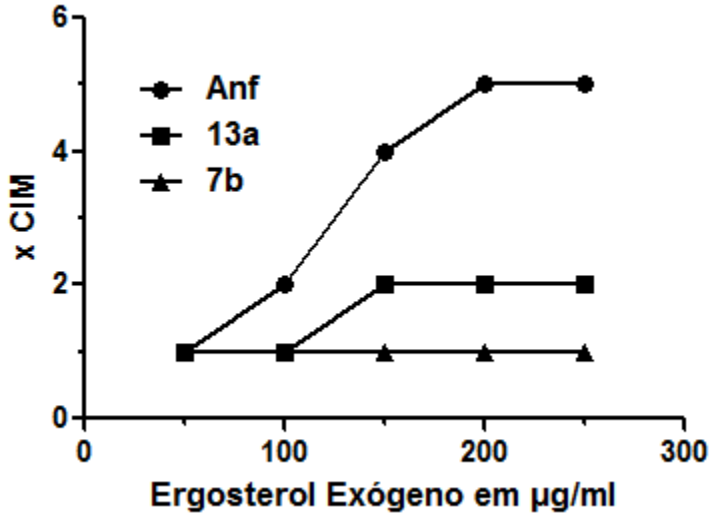


Figura 2. Efeito do ergosterol exógeno (50–250 µg/mL) na CIM dos compostos **13a**, **7b** e Anfotericina B contra *C. parasilosis* RL33 e *T. asahii* TAH10.

3. Conclusão

Neste estudo trinta e cinco compostos foram estudados, dentre eles 28 foram sintetizados com bons rendimentos e submetidos aos testes de atividade antifúngica *in vitro* contra 7 espécies de leveduras. Duas moléculas,**13a** e **7b**, destacaram-se pela CIM demostrada nos ensaios realizados, entre 8 e 16 µg/mL, frente a 20 isolados clínicos de *C. parapsilosis* e *T. asahii* que são espécies de grande importância epidemiológica e de grande severidade na infecção.

Nos testes de investigação do modo de ação desses compostos nas células fúngicas, demostraram ter grande efeito na estabilidade da membrana celular sem interação com ergosterol.

Demostrou-se, assim, que o grupo de moléculas aqui testadas apresenta promissora atividade contra leveduras emergentes, e que pode ser de grande importância no desenvolvimento de alternativas para o tratamento dessas infecções.

4. Experimental

4.1. Procedimentos gerais de síntese dos grupos de compostos

Todos os solventes utilizados possuíam grau analítico e foram destilados com pressão positiva de nitrogênio. Todas as reações que exigiam atmosfera inerte foram realizadas em aparato previamente seco e sob atmosfera de nitrogênio. As reações foram monitoradas através de cromatografia em camada delgada (CCD), as quais foram realizadas em placas de gel de sílica 60 F254 Merck. A visualização foi obtida através de uma lâmpada de luz ultravioleta no comprimento de onda de 254nm.

4.1.2. Derivados 8a-12a

A *O*-metilação foi feita pela reação dos aldeídos com 4eq de CH_3I por hidroxila a ser protegida e 3 eq de K_2CO_3 por hidroxila em acetona com aquecimento de 80 ºC em frasco schlenk por 12h.

4.1.3. Derivados 13a e 14a

Em um tubo de Schlenk foram adicionados os radicais bromados (2-bromopiridina ou 1-bromo-4-nitrobenzeno) e 1,1 equivalentes do ácido 4-formilfenil borônico. Em seguida adicionou-se os catalisadores $Pd(OAc)_2$ a 2 mol% e PPh_3 a 4 mol%. Então sob atmosfera inerte foi adicionada a mistura metanol/THF 1:1 e a base hidróxido de potássio 2 eq. O sistema é agitado por 24h na temperatura de 60°C, e então analisado por cromatografia em camada delgada. [19]

4.1.4. Derivados 1b a 14b

O aldeídos **1a**–**14a** foram adicionados a 1 eq de *terc*-butilcarbazato em um tubo de Schlenk sob atmosfera inerte em uma mistura tolueno/isopropanol 1:1. O sistema foi agitado por 2h sob a temperatura de 85ºC e então mantêm-se a

agitação por mais 14h em temperatura ambiente. O precipitado formado foi purificado por sucessivas lavagens com ciclohexano [20].

4.1.5. Derivados 1c, 8c-14c

As hidrazonas foram submetidos a uma redução catalítica com Pd/C 10% 0,2 eq, formiato de sódio 1,8 eq, dissolvidos em uma mistura de etanol/água 5:1. A mistura foi agitada por 1,5h na temperatura de 60°C e após, resfria-se à temperatura de 40°C e segue agitação por 12h [20].

4.2. Dados de caracterização para os novos compostos

4.2.1. 4'-nitrobiphenyl-4-carbaldehyde (14a)

^{1}H NMR (400 MHz, $cdcl_3$) δ 10.10 (d, *J* = 3.8 Hz, 1H), 8.36 – 8.31 (m, 2H), 8.05 – 7.98 (m, 2H), 7.82 – 7.76 (m, 4H). ^{13}C NMR (101 MHz, cd_3od) δ 192.1, 148,8, 147.15, 146.92, 136.8, 126.36, 123,95, 123,23, 123,13.

4.2.2. *tert*-butyl(2Z)-2(3,5-dihydroxybenzylidene)hydrazinecarboxylate (6b)

^{1}H NMR (400 MHz, cd_3od) δ 7.72 (s, 1H), 6.61 (d, *J* = 2.2 Hz, 2H), 6.27 (t, *J* = 2.2 Hz, 1H), 1.52 (s, 9H). ^{13}C NMR (101 MHz, cd_3od) δ 158.41, 154.07 , 144.27, 136.27, 105.08, 103.83, 80.65, 80.46, 27.33.

4.2.3. *tert*-butyl(2Z)-2-[(4'nitrobiphenyl-4-l)methylene]hydrazinecarboxylate (14b)

^{1}H NMR (400 MHz, cd_3od) δ 8.6 (s, 1H), 8.617 (d, *J* = 2.2 Hz, 2H), 7,82 (t, *J* = 2.2 Hz, 1H),7.46 (s, 2H), 7.24 (s, 2H) 1.61 (s, 9H). ^{13}C NMR (101 MHz, cd_3od) δ 155.5, 148.14, 147.15, 146.92, 145.03, 128.81, 127.45, 123.95, 81.03, 28.4.

4.2.4 .*tert*-butyl2-(2,4-dimethoxybenzyl)hydrazinecarboxylate (10c)

^{1}H NMR (400 MHz, cd_3od) δ 7.21 (s, 1H), 6.62 (d, *J* = 2.2 Hz, 2H), 4,14 (s, 2H), 3.75 (s, 3H), 3.4 (s, 3H) 1.42 (s, 9H).). ^{13}C NMR (101 MHz, cd_3od) δ 162.55, 158.65, 154.5, 132.04, 119.26, 104.8, 98.84, 83.93, 55.26, 55.0, 45.47, 28.15.

Os demais compostos foram caracterizados conforme literatura existente.

4.3. Atividade antifúngica

4.3.1. Isolados

O conjunto dos isolados das espécies *Candida albicans*, *Candida krusei*, *Candida glabrata*, *Candida tropicalis* e *Candida lusitaneae*, *Candida parapsilosis* e *Tricosporon asahii* foram obtidos a partir das coleções de culturas do Laboratório de Pesquisa Aplicada Micologia da Universidade Federal do Rio Grande do Sul, Porto Alegre, Brasil.

4.3.2 Screening da atividade antifúngica

As leveduras foram inoculadas em Sabouraud Dextrose Agar (Oxoid) e normalizadas para a turbidez de um padrão Mc Farland de 0,5 e dilui-se a 1:50 em soro fisiológico seguido por diluição 1:20 em meio Sabouraud Dextrose Agar. Em placa de 96 poços foram adicionadas às amostras em uma concentração final de 500 ug/mL incubadas a 35°C por 48h. As moléculas foram consideradas ativas quando não houve crescimento aparente do fungo.

4.3.3 Concentração Inibitória Mínima

Inóculos foram preparados de acordo com as determinações do CLSI [21]. As leveduras foram cultivadas em Sabouraud Dextrose Agar (Oxoid) durante 48 h a 35°C e normalizado para a turbidez de um padrão McFarland de 0,5 e dilui-se a 1:50 em soro fisiológico seguido por diluição 1:20 em meio RPMI-MOPS.

Os valores de concentração inibitória mínima (CIM) foram determinadas por microdiluição do caldo utilizando o método de diluição dupla de acordo com as orientações do CLSI com meio RPMI-MOPS [21]. As concentrações das amostras testadas variou 0,5-256 ug/mL. A CIM foi definida como a menor concentração de amostra à qual o microrganismo testado não demonstrar o crescimento visível.

4.3.4 Concentração Fungicida Mínima

A concentração fungicida mínima (CFM) foi determinada por sub-cultura de alíquotas de 10 ul de todos os poços sem crescimento visível de SDA com cloranfenicol e incubando a 35 °C durante 48 h. CFM foi definida como a menor concentração produzindo subculturas negativos.

4.4. Mecanismo de ação

4.4.1 Ensaio do Sorbitol

Os valores de CIM foram determinados pelo procedimento de microdiluição padrão CLSI. As células foram inoculadas a uma concentração final de $2x10^3$ UFC/mL e cultivadas em Yeast Nitrogen Base (Difco), com 0,5% de glicose e incubados a 30 ° C. Duplicata das placas contendo as amostras de teste foram preparadas e em uma delas 0.8M sorbitol é adicionado no meio como suporte osmótico. As placas foram lidas em 2 e 7 dias [22], [23], [24]

4.4.2 Fuga Celular

Células são cultivadas em tampão MOPS, pH 6 a 10^7 UFC/mL e transferidas para tubos. Os compostos foram adicionados a uma concentração final = CIM. SDS (2%) foi utilizado como composto de referência, que produz 100% de perda celular. Durante a incubação a 30 ° C foram retiradas alíquotas em intervalos de tempo (24 e 48 h), para a análise de absorvância a 260 nm em espectrofotômetro Beckman DU-600 [22], [23].

4.4.3 Ensaio do Ergosterol

Realizado através microdiluição em caldo padrão CLSI em duplicata, na ausência e na presença de diferentes concentrações (50-250 µg/mL) de ergosterol (Sigma Chemical Co.) adicionado ao meio. A anfotericina B foi usada como um controle para este mecanismo. A CIM foi determinada após 24 h de incubação [22], [23].

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Books without editor:

3. Doe, J. S.; Smith, J. J. Bioorganic Chemistry; Pergamon: Oxford, 1990, Chapter 6.

Theses:

4. Doe, J. S. Ph.D. Thesis, University of California at San Diego, January 2000.

Patent/Chem. Abstract:

5. Lyle, F. R. U.S. Patent 6,973,257, 1995; Chem. Abstr. 1995, 123, 2870.
Abstract of meeting papers:
6. Doe, J. S. Abstract of Papers, 195th National Meeting of the American Chemical Society, Anaheim, CA; American Chemical Society: Washington, DC, 1995; Abstract 3028.

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