Design, synthesis and evaluation of hydrazine and acyl hydrazone derivatives of 5-pyrrolidin-2-one as antifungal agents

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ABSTRACT

Twenty-eight 5-pyrrolidine-2-ones decorated by hydrazine or acyl hydrazones groups have been designed, synthesized and evaluated as antifungal agents on a panel of twelve fungal strains and three *non albicans candida* yeasts species which have demonstrated reduced susceptibility to commonly used antifungal drugs. Half of the target compounds exhibited good to high antifungal activities on at least one strain with MIC_{50} lower than the control antifungal agent – hymexazol or ketoconazole. 5-Arylhydrazino-pyrrolidin-2-ones were found active and the –NH-NH- linker proved to be essential to maintain the antifungal potential. Compound **2a** is a broad-spectrum antifungal, active on 60% of the tested strains. Replacing the hydrazine linker by an acylhydrazone one narrowed the spectrum of activity but pyroglutamylaryl hydrazones, mainly aromatic ones, exhibited good activity, adequate "fungicide-like" properties and were devoted of cytotoxicity.

In the past decade, some molds and phytopathogenic fungal species *e.g.* from the Aspergillus, Fusarium or Alternaria genera, have proved to cause diseases in man, such as allergies, respiratory problems or infections. The effects of toxins produced by these pathogens have amplified and emerged as a common threat to both agricultural production, animal and human healths.[1,2,3,4] Indeed, this contamination by fungi and molds has an economic impact due to crops infestation, quality decrease of agricultural products and impact on health of humans and animals feed with contaminated food.[5,6] As a consequence, innovative antifungal agents targeting agricultural fungal strains and yeasts are needed. L-Pyroglutamic acid (PGA) **1** naturally occurred from the cyclisation of glutamine and glutamic acid issued from the beet root (*Beta vulgaris saccharifera*).[7,8] The structural features of **1** and other natural lactams[9-11] are wide and exhibit a large range of biological activities (Fig. 1).[12-14] On the other hand, hydrazones are frequently found in natural sources such as plants,[15] fungi[16] and marine organisms.[17] Natural and synthetic hydrazones are part of compounds displaying a large spectrum of biological properties including antimicrobial ones[18-20] while naturally occurring hydrazine derivatives are very scarce but also of biological importance.[21] Considering the importance of these building blocks, hydrazine or acylhydrazone functionalities were linked to a γ-lactam ring.

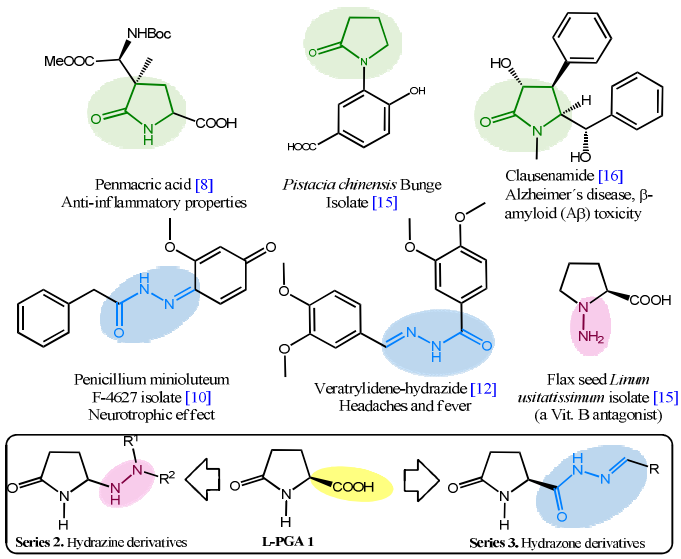
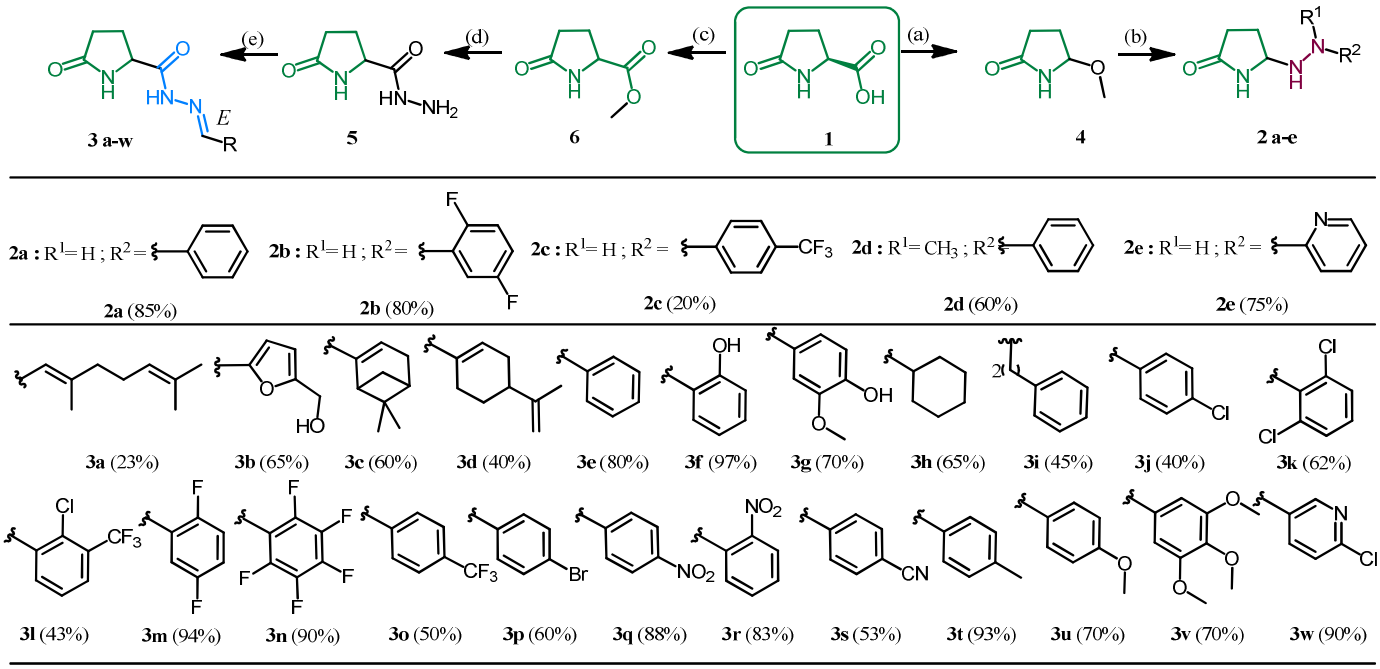


Fig. 1. Natural compounds containing lactam or hydrazine/hydrazone moieties.

Thus, two series of compounds (Series **2** and **3**, Fig. 1 and Scheme 1), issued from L-pyroglutamic acid were designed and evaluated for their antifungal properties on a large panel of fungi and molds.



Scheme 1. Reagents and conditions: (a) electrochemical decarboxylation, quant. yield;[22] (b) 1 eq of H_2N-N(R^1,R^2), CsF (5 mol%), solvent-less, 6-20 h, 80 °C;[23] c) CH_3SO_3H, MeOH/ $CHCl_3$, MS 3Å, reflux, quant. yield;[26] d) hydrazine monohydrate;[27] (e) aldehyde (1 equiv.), H_2O, rt, 1 h.

Target compounds **2a-e** have been obtained by a two-steps route *via* pterolactam (5-methoxypyrrolidinone) **4** (Scheme 1). To the best of our knowledge, the general scaffold of hydrazines **2** was not reported in literature. Starting from L-pyroglutamic acid **1**, an electrochemical decarboxylative oxidation furnished racemic pterolactam **4** in quantitative yield.[22] We recently described a new procedure for the reaction of **4** with nucleophiles in non-acidic conditions.[23] By using this method, aromatic hydrazines and a catalytic amount of cesium fluoride were reacted under a moderate vacuum (30 mmHg), with lactam **4** at 80°C for 6 to 19 hours. In these conditions, racemic *N,N'*-aminals **2a,b** and **2d,e** were obtained in good 60-85% yields; however, because of purification difficulties, the *para*-trifluoromethyl analogue **2c** was isolated in a lower 20% amount. These lactams, in which the pyrrolidin-2-one ring is directly attached to an aryl hydrazine will be referred as type I derivatives. The general structure of hydrazones **3** can be found in literature, as hypertensive agents[24] or because of their central nervous system stimulating activity.[25] Target compounds **3a-w** were obtained in middle to very good yields (23-97%) by synthesizing firstly methyl pyroglutamate **6**[26] which was converted to the known pyroglutamyl hydrazide **5**[27] before reaction with the corresponding aldehydes in water at room temperature.[28] This series of compounds, in which a carbonyl group is inserted between the lactam ring and a hydrazone moiety, will be named type II. Derivatives type IIA (**3a,c,d,h,i**) distinguished from IIB (**3b**, **3e-g**, **3j-w**) by the presence of an aliphatic chain; for IIB, the hydrazone moiety is attached to an aromatic or heterocycle ring.

The *in vitro* antifungal activities of these original compounds were evaluated using the micro-dilution method, by using hymexazol, fluconazole and ketoconazole as the positive controls. A panel of twelve agricultural fungi (*Fusarium solani, Paecilomyces variotii, Penicillium ochrochloron*, *Aspergillus oryzae, Alternaria alternata*, *Cladosporium cladosporioides, Geotrichum candidum, Sclerotinia sclerotiorum* and *Botrytis cinerea*) known for causing allergies, asthma and mycosis, and also *non albicans Candida* yeasts species (*Candida pseudotropicalis*, *Candida tropicalis* and *Candida krusei*) which have demonstrated reduced susceptibility to commonly used antifungal drugs was selected.[29] The results of the first screening at a concentration of 100 µg/mL were listed in Table 1. To further evaluate the inhibitory potencies of the most promising synthesized compounds, the half maximal inhibitory concentration (MIC_{50}) values of products with high inhibition rate (> 70%) were determined.

In term of antifungal activity (Table 2), half of the newly synthesized compounds displayed antifungal activities against at least one strain. First, it is notable that all aliphatic hydrazone derivatives (type IIA) displayed poor antifungal activity (Table 2, entries **3a**, **3c**, **3d**, **3h**), and all active compounds exhibit an aromatic or heterocyclic ring. Noteworthy also, all products in this series displayed no activity against *B. cinerea*, *S. sclerotiorum* and *C. krusei.* Concerning hydrazine derivatives type I, all compounds but **2e**, bearing a 2-pyridyl moiety, displayed good antifungal properties, especially against *F. solani* and *P. variotii* (Table 2, **2a-d**). 5-(2-Phenylhydrazinyl)pyrrolidin-2-one **2a** showed a broad antifungal activity being able to fight against 8 strains *e.g. F. solani*, *P. variotii*, *P. ochrochloron*, *A. oryzae*, *A. alternata*, *S. sclerotiorum*, *C. pseudotropicalis* and *C. tropicalis*, with MIC_{50} values ranging from 0.6 to 58.5 µg/mL.

Changing R^1 from a hydrogen atom to a methyl group narrowed the spectrum of activity underlining the importance of the -NH-NH- linker to have a broad spectrum antifungal (Table 2, compound **2d**). Interestingly, replacing the phenyl group of **2a** by a 2-pyridinyl heterocycle led to a complete loss of the antifungal activity (Table 2, compound **2e**). Furthermore, substitution of the phenyl ring of **2a** led to more selective compounds targeting only two or three fungal strains. Thus, addition of two fluorine atoms in the 2- and 5-position in **2b** conserved the activity against *F. solani*, but this compound was 4-fold less active against *P. ochrochloron* and *A. alternata*, but still equivalent to that of the positive control Hymexazol, while the activity against *A. oryzae*, *C. pseudotropicalis* and *C. tropicalis* was lost. Compound **2c**, substituted by a *p*-trifluoromethyl group displayed a narrow impressive activity (2.0-11.7 µg/mL) specific for *F. solani*, *P. variotii* and *C. cladosporioides* but did not inhibit the growth of the other species. Moreover, **2c** is the only one towards which *C. cladosporioides* is sensitive, with MIC_{50} of 3.4 µg/mL, eight-time more active than the positive control hymexazol (Table 2, compound **2c**).

Table 1. Antifungal activity of pyrrolidin-2-one derivatives at 100 µg/mL.

Compound	Average inhibition rate ± SD (%) (n = 3)											
	FS	***PV***	***PO***	***AO***	***BC***	***AA***	***CC***	***SS***	***GC***	***CK***	***CP***	***CT***
2a	100	78	90	71	62	82	44	76	0	35	96	89
2b	95	17	73	0	0	72	29	28	25	20	65	62
2c	100	82	35	37	39	2	81	40	22	51	38	51
2d	100	70	56	45	20	0	42	46	18	20	61	20
2e	64	11	21	23	0	0	0	0	32	0	0	8
3a	43	0	0	17	27	0	27	15	5	0	8	38
3b	72	45	28	0	0	42	0	0	88	66	28	38
3c	0	39	0	4	0	0	0	0	0	0	18	0
3d	0	31	45	0	13	0	56	0	39	18	0	0
3e	59	46	25	0	0	20	12	0	83	40	7	15
3f	40	14	30	7	0	38	0	21	70	38	18	7
3g	0	22	0	0	0	20	0	0	0	37	0	0
3h	0	36	23	8	8	8	1	0	62	0	30	0
3i	0	39	29	0	6	0	53	0	68	0	12	17
3j	61	27	92	0	34	20	98	58	92	26	50	47
3k	60	13	22	12	0	0	12	58	73	0	32	16
3l	0	19	44	4	0	12	36	0	62	47	18	0
3m	58	37	26	13	6	0	34	0	62	47	18	0
3n	40	0	21	4	17	0	11	5	36	0	0	0
3o	55	44	44	0	0	21	11	0	85	21	21	17
3p	100	0	77	43	40	0	72	37	100	0	2	92
3q	100	20	14	0	0	30	40	9	30	22	38	42
3r	100	3	13	32	27	0	0	21	50	0	35	19
3s	0	33	20	0	0	42	0	0	0	10	0	0
3t	94	35	0	11	0	0	5	0	28	28	32	33
3u	0	36	23	24	0	3	35	38	25	40	40	49
3v	0	26	32	0	0	29	0	0	0	50	18	0
3w	23	42	62	0	0	0	33	0	72	2	10	0

FS – Fusarium solani, PV - Paecilomyces variotii, PO – Penicillium ochrochloron, AO – Aspergillus oryzae, BC – Botrytis cinerea, AA – Alternaria alternata, CC – Cladosporium cladosporioides, SS – Sclerotinia sclerotiorum, GC – Geotrichum candidum, CK – Candida krusei, CP – Candida pseudotropicalis, CT – Candida tropicalis.

Nine on sixteen acylhydrazones from type IIB, obtained from aromatic aldehydes, presented interesting activities, but with a narrower spectrum of antifungal potency. All compounds showed no activity against *P. variotii, A. oryzae, A. alternata, C. pseudotropicalis* or *C. tropicalis.* Interestingly, *para* substitution of the phenyl ring by a chloride or bromine atom led to compounds **3j** and **3p** respectively, with a broader spectrum, and activities better than hymexazol against *F. solani* and *C. cladosporioides*. The electrodonating OCH_3 substituent in the *para* position (**3u**, **3v**) abolished the global antifungal activity (Table 2, **3u**, **3v**). Exchanging the *para*-Br of **3p** for a CF_3, CH_3 or NO_2 group (**3o**, **3t**, **3q**) reduced its activity against *F. solani*. Interestingly, moving the *para*-NO_2 group of **3q** to the *ortho* position led to **3r** which maintained its activity against the same fungi. In this series, poly-substitution generally dramatically decreased the activities, except for **3k** which presented the same good activity as **3j** (~ 1.8 µg/mL) against *G. candidum,* being three times more efficient than the *para*-bromo compound **3p**. *G. candidum* proved to be rather sensitive to the aryl hydrazones of the series IIB because six on nine of these products inhibited its growth, three of this series being as potent as the controls (Table 2, compounds **3j**, **3k**, **3p**). Even the *ortho*-OH and *para*-CF_3 phenyl (**3f**, **3o**) as well as the chloro-pyridyl (**3w**) compounds which were inactive against all the other strains, led to a middle inhibition of its growth at 38-64 µg/mL (positive control ketoconazole: 1.6 µg/mL). It is also notable that for compounds **3b** and **3e**, the sequential dilution for inhibition of *G. candidum* growth led to a dramatic decrease in activity (less than 10% inhibition at 50 µg/mL).

Table 2. MIC_{50} values of the active compounds **2** and **3** against 10 fungal strains.[a]

Compounds	MIC_{50} (µg/mL)										
	FS	PV	PO	AO	AA	CC	SS	GC	CP	CT	Spectrum[b]
2a	18.5	7.8	4.4	43.4	3.6	/	23.0	/	0.6	58.5	8
2b	24.5	/	21.1	/	18.6	/	/	/	/	/	3
2c	11.7	2.0	/	/	/	3.4	/	/	/	/	3
2d	31.0	14.8	/	/	/	/	/	/	/	/	2
3b	>75	/	/	/	/	/	/	>75	/	/	0
3e	/	/	/	/	/	/	/	>75	/	/	0
3f	/	/	/	/	/	/	/	62.0	/	/	1
3j	/	/	15.4	/	/	23.9	/	1.8	/	/	3
3k	/	/	/	/	/	/	/	1.8	/	/	1
3o	/	/	/	/	/	/	/	63.7	/	/	1
3p	6.5	/	5.3	/	/	53.0	/	5.9	/	>75	4
3q	59.1	/	/	/	/	/	/	/	/	/	1
3r	51.6	/	/	/	/	/	/	/	/	/	1
3t	101.5	/	/	/	/	/	/	/	/	/	1
3w	/	/	/	/	/	/	/	38.4	/	/	1
Ketoconazole[c]	/	/	/	/	/	/	/	1.5	9.6	15.9	-
Hymexazol	15.6	57.2	62.2	45.7	38.3	28.9	26.5	>50	/	/	-
Fluconazole[d]	/	/	/	/	/	/	/	1.6	/	/	-

FS – Fusarium solani, PV - Paecilomyces variotii, PO – Penicillium ochrochloron, AO – Aspergillus oryzae, AA – Alternaria alternata, CC – Cladosporium cladosporioides, GC – Geotrichum candidum, CP – Candida pseudotropicalis, CT – Candida tropicalis.

[a] Table 2 only summarized the compounds which exhibited antifungal activities against at least one strain among the tested panel of strains.

[b] Spectrum refers to the number of strains killed by the compound.

[c] Ketoconazole was used as positive control for *G. candidum*, *C. pseudotropicalis* and *C. tropicalis* bioassay.

[d] Fluconazole was used as positive control for *G. candidum* and *C. krusei* bioassay.

To further evaluate their potential for candidate lead fungicide, some of the molecular physicochemical properties [octanol/water partition coefficient (LogP), topology polar surface area (TPSA), number of hydrogen bond donor sites (NDS), number of hydrogen bond acceptors sites (NAS) and molecular weight (MW)] were calculated (Table 3),[30] with MarvinSketch 18.24, a ChemAxon software. All these compounds met the Lipinski "Rule of five" criteria[31] with Log P < 1.8, NDS < 4, NAS < 9 and MW < 310, and the Briggs "Ground rules of three" suggesting that for a fungicidal to be available, it should have a Log P < 3.[31] It is also interesting to note that, as indicated by Tice,[32] the TPSA did not offer any advantage over the Lipinski Rule. However, while no obvious correlation can be found between the antifungal activity over a particular fungal species and one of these parameters, it can be interesting to underline that the number of hydrogen bond acceptors sites (NAS) is lower than 5 for all the products displaying some activity on many strains.

In order to check the mammalian cell toxicity of the tested compounds presented herein, selected products were screened against human embryonic kidney cells (HEK293).[34] All arylhydrazines **2a-e** and *N*-acyl arylhydazones **3** showed a slight to no toxicity in viable kidney cells, the viability of cells dropping down around 10% for the most toxic compounds at a concentration of 100 µM. On the other side, some selected compounds were submitted to the National Cancer Institute (NCI) for screening on a large panel of cancer cells and exhibited slight to no cytotoxicity (see data in SI).

The inhibition activity of 28 new compounds mixing the structure of hydrazine with a pyrrolidinone ring were evaluated against a large panel of fungal strains, and their structure-activity relationships were analyzed: 1) 5-arylhydrazino pyrrolidin-2-ones **2** were found active; 2) the –NH-NH- linker proved to be essential to maintain the antifungal potential; 3) pyroglutamylaryl hydrazones **3**, mainly aromatic ones, exhibited good activity, adequate "fungicide-like" properties and were devoted of cytotoxicity. In particular, these series of compounds proved to be novel fungicide candidates to fight *F. solani* and *G. candidum*, two strains which are known to pose health risks related to major barrier breaks among people with weak immune systems.

Table 3. Predicted molecular properties of the new compounds[33]

Compound	Log *P*[a]	TPSA[b]	NDS[c]	NAS[d]	MW[e]	Spectrum
2a	0.93	53.16	3	4	191.23	7
2b	1.22	53.16	3	4	227.21	3
2c	1.81	53.16	3	4	259.23	3
2d	0.98	44.37	2	4	205.26	2
3f	0.14	90.79	3	7	247.25	1
3j	1.05	70.56	2	5	265.70	3
3k	1.65	70.56	2	5	300.14	1
3o	1.32	70.56	2	5	299.25	1
3p	1.21	70.56	2	5	310.15	4
3q	0.38	116.38	2	9	276.25	1
3r	0.38	116.38	2	9	276.25	1
3t	0.96	70.56	2	5	245.28	1
3w	0.17	83.45	2	6	266.68	1

[a] Log P = octanol/water partition coefficient; [b] TPSA = topology polar surface area; [c] NDS = number of hydrogen bond donor sites; [d] NAS = number of hydrogen bond acceptors sites; [e] MW = molecular weight (g/mol).

Conflict of interest

The authors declare no conflict of interest.

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28. General procedure: *N*-acylhydrazones were obtained from the condensation reaction of 5-oxopyrrolidine-2-carbohydrazide **5**, which was preliminary obtained in a large quantity through the hydrazinolysis of methyl-pyroglutamate **6**, with the corresponding aldehyde. The reaction was conducted at room temperature for one hour, in green solvents such as water or ethanol to afford the wanted *N*-acylhydrazones. No side products were observed for this type of reaction.
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33. The herein calculations were performed with MarvinSketch 18.24, a ChemAxon software.
34. The human embryonic kidney 293 cell line (HEK293) was cultured in Dulbecco's modified Eagle medium (DMEM) (Gibco, Waltham, MA) supplemented with 2 mM L-glutamine, 100 IU/ml penicillin/streptomycin, non-essential amino acid solution (1/100) and 5% (v/v) heat-inactivated foetal bovine serum (Sigma-Aldrich, Saint-Louis, MO), and grown at 37 °C in a humidified incubator with 5% CO_2. Cells were seeded at 3000 cells per well onto 96-well plates in DMEM medium. Cells were incubated in a culture medium that contained 100 µM of the different test compounds and 2 µM of the references, each dissolved in less than 1% DMSO. After 72 h of incubation, cell viability (in proliferation and cytotoxicity) was estimated by the colorimetric MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium) assay.

Graphical Abstract

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Design, synthesis and evaluation of hydrazine and acyl hydrazone derivatives of 5-pyrrolidin-2-one as antifungal agents

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