Aspartic protease inhibitory and nematocidal activity of phenyl-4-(2-phenylhydrazono)hexahydrofuro[3,2-c]pyridazin-7-ol (Percival dianhydroosazone)

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Abstract: We synthesized Phenyl-4-(2-phenylhydrazono) hexahydrofuro[3,2-*c*]pyridazin-7-ol (compound 3). The structure compound 3 was elucidated with IR, ^{1}H NMR, ^{13}C NMR and EIMS spectra. Compound 3 showed potent inhibitory activity against aspartic proteases, human cathepsin D and *Plasmodium falciparum* plasmepsin-II with $IC_{50} = 20$ μM. Enzyme-inhibitor complexes were predicted to stabilize by electrostatic and hydrophobic interactions between the side chains of amino acid residues at the active center and compound **3**. Moreover, compound **3** displayed good nematocidal activity against all developmental stages of *C. elegans*.

Keywords: Glucose phenyl osazone; Dianhdroosazone; Furopyridazine; Aspartic protease; Nematicidal; Cathepsin D; Malarial parasite *Plasmodium falciparum* plasmepsin-II; docking.

Introduction

Aspartic proteases play very important role in intracellular protein turnover. Several aspartic proteases are known to be involved in several diseases such as malaria, AIDS and neoplastic disorders. For instance, intracellular aspartic proteases plasmepsins[1], HIV protease[2], cathepsin D and cathepsin E[3,4] are involved in malaria, AIDS and breast cancer respectively. The cathepsin D is important for the intracellular degradation of proteins. Its increased expression is associated with a number of pathological conditions including neoplastic disorders and inflammatory diseases[1,5]. Over expression of human cathepsin D leads to uncontrolled protein degradation in neurodegenerative disorders[3,6].

A family of hemoglobin degrading aspartic proteases termed as plasmepsins is produced by malarial parasites i.e. Plasmodium species. These enzymes have been identified as potential anti-malarial drug targets [6], as inhibition of these enzymes could result in malarial parasites death[7,8]. The malarial parasite *Plasmodium falciparum* aspartic protease plasmepsin-II is involved in hemoglobin degradation during the intra-erythrocyte phase of infection. Plasmepsins are promising drug targets especially when combined with the

inhibitors of malarial parasite cysteine proteases facipains that are also involved in hemoglobin catabolism[9].

Numerous attempts have been made to design plasmepsin inhibitors to develop novel antimalarial drugs. A wide variety of synthetic and natural plasmepsin inhibitors have been reported[7-10]. We explored the biological activity of phenyl-4-(2-phenylhydrazono)hexahydrofuro[3,2-*c*]pyridazin-7-ol formulated as **3**, that is available from biomasses by conventional and microwave methods[11-14]. Herein, we report the aspartic protease inhibitory activities of compound **3** using human cathepsin D and malarial parasite *Plasmodium falciparum* plasmepsin-II. Ligand docking studies provided the basis of inhibition from structural stand point. Moreover, compound **3** displayed a potent nematicidal activity. Several reports describe the nematicidal activity of synthetic and natural compounds[15-17]. Our data pointed out compound **3** as a novel and potent nematicidal agent.

Results and Discussion

Chemistry

Acetylation of D-glucose phenylosazone **1** gave tetra-*O*-acetyl-D-arabinohexosulose-phenylosazone **2**. Deacetylation of 2 with 1.5 % aqueous sodium hydroxide gave (4a*S*,7*S*,7a*S*,*Z*)-1-phenyl-4-(2-phenylhydrazono)-1,4,4a,6,7,7a-hexahydrofuro[3,2*c*]pyridazin-7-ol **3**[13,14] (Figure 1). Better results were obtained by using microwave irradiation where higher yields and shorter reaction times were achieved[18].
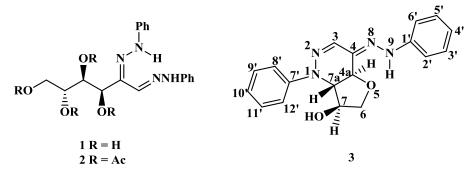

Figure 1. Structures of compounds **1-3**

The ^{1}H NMR spectrum of **3** showed a singlet at δ 4.73 assigned to the hydroxyl group. The protons H-6 and H-6a appeared as two doublet of doublets at δ 3.86 and 4.36 ppm, while H-7 resonated at δ 4.77 ppm, H-7a at δ 4.61 ppm and H-4a at δ 5.12 ppm.

The COSY interactions (Figure 2) indicated that the doublet of doublet of H-6 has a ^3J (3.0 Hz) with H-7 and ^2J (9.9 Hz) with H-6a. The H-6a has a correlation with H-7 ^3J (5.7 Hz) besides the geminal coupling with H-6. While H-7 showed ^3J (8.7 Hz) coupling with H-7a in addition to its ^3J coupling correlations with H-6 and H-6a. H-7a showed ^3J (8.7 Hz) coupling correlation with H-4a and H-7. Moreover, H-4a has only one ^3J correlation with H-7a.

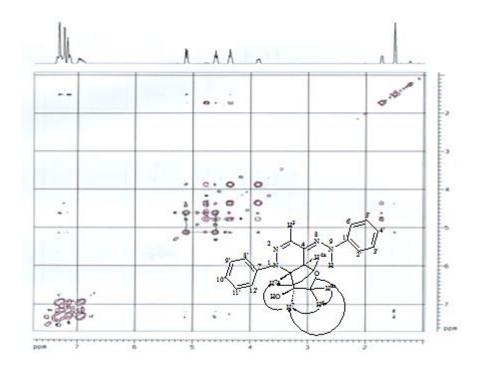**Figure 2.** COSY spectrum of **3** with a schematic representation of the observed correlations.

The correlation of the proton and carbon resonances in the HMQC spectra facilitated the assignment, see experimental part. HMBC interactions (Figure 3) showed that C-4a (δc 69.0 ppm) has a long range coupling correlation with H-3, H-6a and H-7a, respectively, while C-7 (δ 70.5 ppm) correlates with H-6 and H-4a. The C-6 (δ 73.7 ppm) has correlation with H-4a. C-4 (δ 146.0 ppm) showed a correlation to H-6a.
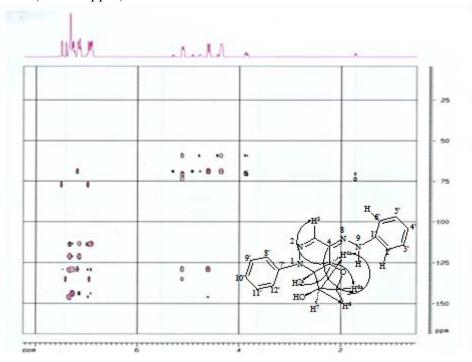

Figure 3. HMBC spectrum of **3** with a schematic representation of the observed correlations.

Biological assays

Inhibition of human cathepsin D and P. falciparum plasmepsin-II by dianhydro-hexosazone compound 3.

Compound **3** inhibited human cathepsin D and *P. falciparum* plasmepsin-II with IC_{50} value of 20 µM (Figure 4).
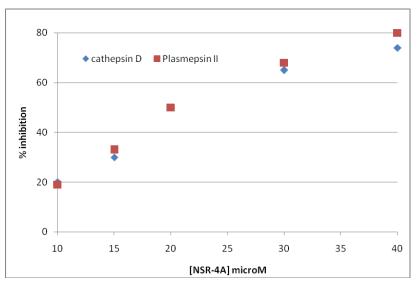

Figure 4. Human cathepsin D and *Plasmodium falciparum* plasmepsin-II inhibition plots as a function of compound **3** concentrations.

Docking of compound 3 in the substrate-binding site of P. falciparum plasmepsin-II.

The FlexX ligand docking program 11 predicted the binding of the compound **3** in the substrate-binding site of crystal structure of plasmepsin-II[15]. FlexX scoring function provided docking score for compound **3**.
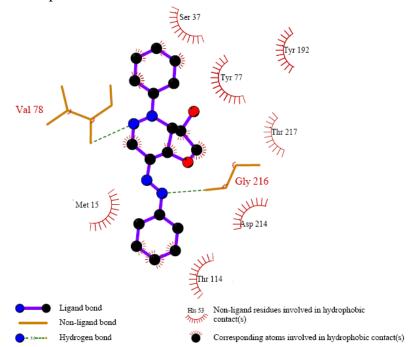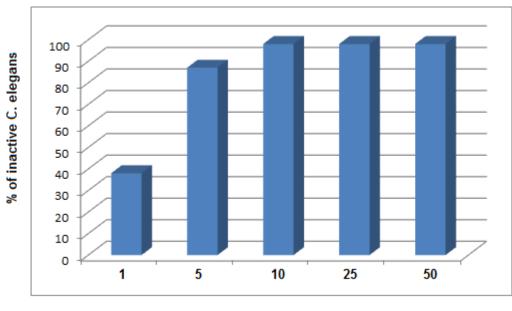

Figure 5. Ligplot [19] generated diagrammatic representation of the binding of compound **3** at the substrate binding site of Plasmepsin-II.

Structural analysis of the predicted binding poses of compounds **3** revealed important information related to the basis of inhibition. The top binding poses of compound **3** were modeled into the active site of plasmepsin-II to examine interactions with protein residues. Compound **3** is placed at the center of binding cleft and interacted with aromatic amino acid residues of S1 and S1' sub sites i.e. Phe111, Tyr192, Tyr77, along with certain catalytic center residues i.e. Ser79, Gly216 and flap residues i.e. Val78. Analysis of FlexX docking solutions revealed that the enzyme-inhibitor complexes are stabilized by electrostatic as well as hydrophobic interactions between the side chains at the active center and compound **3** (Figure 5).

Nematicidal effect of compound 3

The *in vitro* nematicidal activity of compound **3** was tested using nematode *C. elegans.* The *C. elegans* were exposed to different concentrations i.e. 1, 5, 10, 25 and 50 µM of compound **3** and incubated for 24 hours. Compound **3** displayed good nematocidal activity against all developmental stage of *C. elegans* (Figure 6).

All the worms were found to be inactive at concentration ≥10 µM of compound **3**. Below this concentration, the nematicidal effect of compound **3** was observed to be concentration dependent. The LD_{50} value of compound **3** was estimated as 2 µM which indicated its effectiveness as a potent nematocidal agent.

Figure 6. Nematicidal activity of compound **3** against *C. elegans*.

Conclusion

Phenyl-4-(2-phenylhydrazono)hexahydrofuro[3,2-*c*]pyridazin-7-ol **3** was synthesized and its structure was confirmed using the spectroscopic analysis. This compound displayed inhibitory activity against aspartic proteases human cathepsin D and *Plasmodium falciparum* plasmepsin-II with IC_{50} = 20 µM. Compound **3** also exhibited considerable nematocidal activity against all developmental stage of *C. elegans*.

Experimental Section

Chemistry

All the reagents employed from Sigma (USA) or Aldrich (Germany) and were used without further purification. Melting points were measured in capillary tubes using Stuart melting point apparatus SMP10 and are corrected. Infrared (IR) spectra were measured on a Vector 22 infrared spectrophotometer (υ_{max} in cm^{-1}). Proton magnetic resonance (^{1}H NMR) spectra were recorded on Avenge AV 600 spectrometer (600 MHz). Chemical shifts are reported in δ values (parts per million, ppm) relative to tetramethylsilane (TMS) as internal standard and coupling constant values are given in Hz. Abbreviation used in NMR analysis are as follows: d = doublet, dd = doublet of doublets, m = multiplet, q = quartet, s = singlet, t = triplet. ^{1}H NMR spectral analysis was based on chemical-shift correlation (DQFCOSY) spectra. Assignments of the ^{13}C NMR spectra were based on carbon/proton shift-correlation spectra heteronuclear multiple quantum coherence (HMQC), heteronuclear multiple bond coherence (HMBC). Electron impact mass spectra (EI-MS) were recorded on a Finnigan MAT 312 mass spectrometer connected with a MASPEC Data System. Reactions were monitored by thin layer chromatography (TLC) on Merck silica gel 60 F$_{254}$ and the compounds were visualized by charring with H$_2$SO$_4$ in methanol and by UV light, using solvent 6:4 Ethyl acetate/Hexane.

(4aS,7S,7aS,Z)-1-phenyl-4-(2-phenylhydrazono)-1,4,4a,6,7,7a-hexahydrofuro[3,2-c]pyridazin-7-ol (3)

D-Glucose phenylosazone (20 g) was suspended in a mixture of pyridine (80 mL) and acetic anhydride (60 mL) and shaked occasionally. It was left for overnight, and then crushed ice was added. The resulting sticky material of the tetra-*O*-acetyl-D-glucosazone **2** was washed with water several times. It was dissolved in ethanol and precipitated with water to give yellow crystals, R$_f$ 0.5 (H/E, 2:1), yield 86%, m.p 121-124 °C (Lit.[13] 115-117 °C). A solution of the acetate (20 g) was dissolved in acetone (1 L) and a solution of 1.5 % sodium hydroxide in water (1.3 L) was added at room temperature. After few hours pale yellow crystals were appeared. After 24 hours, they were filtered and recrystallized from ethanol as yellow needles in 89 % yield, m.p. 238 °C [lit.[14] 238°C]. IR: υ_{max} (KBr/cm^{-1}): 3423 (OH), 3323 (N-H), 2924 (C-H), 1599 (C=N). ^{1}H NMR (CDCl$_3$, 600 MHz): δ 9.66 (s, 1 H, NH, disappear upon deuteration), 7.36-7.26 (m, 6 H, H-Ar), 7.18 (s, 1 H, H-3), 7.14 (d, 2 H, J = 7.8 Hz, H-2',6'), 6.98-6.91 (m, 2 H, H-4', H-10'), 5.12 (d, 1 H, J = 8.7 Hz, H-4a), 4.77 (m, 1 H, H-7), 4.73 (s, 1 H, O*H*, D$_2$O *exchangeable*), 4.61 (dd, 1 H, $J_{7a,4a}$ = 5.7 Hz, $J_{7a,7}$ = 8.7 Hz, H-7a), 4.36 (dd, 1 H, $J_{6a,7}$ = 5.7 Hz, $J_{6a,6}$ = 9.9 Hz, H-6a), 3.86 (dd, 1 H, $J_{6,7}$ = 3.0 Hz, $J_{6,6a}$ = 9.9 Hz, H-6); ^{13}C NMR (CDCl$_3$, 150 MHz): δc 146.0 (C-4), 143.8 (C-1'), 135.1 (C-3), 129.5 (C-7'), 129.3 and 129.1 (C-3',5' and C-9',11'), 121.3 and 121.2 (C-10', C-4'), 113.8 and 113.4 (C-2',6' and C-8',12'), 73.7 (C-6), 70.5 (C-7), 69.0 (C-4a), 59.2 (C-7a). EI-MS: (m/z %) 322.4 (80) [M]$^+$, 277.4 (7), 261.3 (100), 233.3 (2), 200.2 (1), 185.2 (4), 171.2(6), 157.2 (13), 144.2 (3), 131.2 (17), 116.2 (3), 104.1 (13), 93.1 (11), 77.1 (37), 65.1 (9), 51.1 (6); HREIMS Calcd for C$_{18}$H$_{18}$O$_2$N$_4$ [M$^+$] 322.1430, found: [M$^+$] 322.1435.

Biological assays

Plasmepsin-II and cathepsin D inhibition assays

The enzyme activities of plasmepsin-II and cathepsin D were measured as described earlier [10] using a fluorescence energy transfer (FRET) based assay with the fluorogenic

substrate Dabcyl-Glu-Arg-Nle- Phe-Leu-Ser-Phe-Pro-EDANS (malarial FRET-1; AnaSpec Inc., USA) with an excitation and emission wavelengths of 336 and 490 nm, respectively. The assay was performed in 0.1 M sodium acetate buffer pH 5.0, containing 10% glycerol and 0.01% tween 20. The compound 3 (dissolved in 5% final concentration of DMSO) were added in the reaction mixture before the addition of substrate. The enzyme assays using 'standard inhibitor' pepstatin-A (Sigma Inc, USA) was performed in the same experimental manner as for compound 3. The concentration-response curves were plotted for IC50 determination by plotting the data as percentage inhibition as a function of inhibitor concentration. The IC50 defined by the midpoint (50% inhibition) of the inhibitor titration.

Docking of Compound 3 to the substrate-binding site of plasmepsin-II

Molecular docking was carried out by FlexX ligand docking program [10] using the crystal structural coordinates of *Plasmodium falciparum* plasmepsin-II (PDB ids; 1M43)[14]. 3D models of compound **3** were generated in SYBYL mol2 format and utilized for docking to the active site of plasmpsin-II.   FlexX method of ligand docking involves incremental construction of ligands from smaller fragments in the cavity of a receptor. The ranking of the generated docking solutions is performed using a scoring function similar to that developed by Bohm[20] which estimates the free binding energy (ΔG) of the protein-ligand complex. After the docking run, top ranking docking solutions were saved and considered for detailed analysis.

Nematicidal assays of Compound 3

The nematicidal assay of compound **3** was conducted using *Caenorhabditis elegans* as a model nematode. The *C. elegans* strain N2 and *E. coli* strain OP50 were received from Caenorhabditis Genetics Center, University of Minnesota, USA.

The nematode *C. elegans* were cultured at 20 ℃ on nematode growth medium (NGM) seeded with *E. coli* [21]. The nematocidal assay was carried out in sterile 96 well plates (Corning, New York, USA). Nematodes (n= 20-50) of different developmental stages (i.e. larvae-adult males and hermaphrodites) were placed in 200 µl of S medium in wells of microtiter plate[22], containing 10 µl of 5X concentrated overnight culture of *E. coli*. The test compound **3** were dissolved in DMSO and added to wells with different concentrations ranging from 1-100 µM.

The final concentration of DMSO in all wells was $\leq 1.0\%$. The plates were incubated at 20 °C. The LC_{50} (lethal concentration causing 50% mortality) was assessed under inverted microscope after 24 hours of incubation by counting the percentage of motile and dead worms. The experiments were conducted in triplicate and mean percentage mortality was calculated.

Acknowledgement

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