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Some Hydrazones of 2-Aroylamino-3-methylbutanohydrazide: Synthesis, Molecular Modeling Studies, and Identification as Stereoselective Inhibitors of HIV-1

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In accordance with our antiviral drug development attempt, acylhydrazone derivatives bearing amino acid side chains were synthesized for the evaluation of their antiviral activity against various types of viruses. Among these compounds, $\mathbf{8_S}$, $\mathbf{11_S}$, and $\mathbf{12_S}$ showed anti-HIV-1 activity with a 50% inhibitory concentration (IC_{50}) = 123.8 μM (selectivity index, SI > 3), IC_{50} = 12.1 μM (SI > 29), IC_{50} = 17.4 μM (SI > 19), respectively. Enantiomers $\mathbf{8_R}$, $\mathbf{11_R}$, and $\mathbf{12_R}$ were inactive against the HIV-1 strain III_B. Hydrazones $\mathbf{8_S}$, $\mathbf{11_S}$, and $\mathbf{12_S}$ which were active against HIV-1 wild type showed no inhibition against a double mutant NNRTI-resistant strain (K103N;Y181C). Molecular docking calculations of *R*- and *S*-enantiomers of **8**, **11**, and **12** were performed using the hydrazone-bound novel site of HIV-1 RT.

Keywords: Acylhydrazones / HIV-1 / Molecular modeling / Non-nucleoside reverse transcriptase inhibitors / Stereoselective activity

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Introduction

Infection with human immunodeficiency virus (HIV), the etiological agent of acquired immune deficiency syndrome (AIDS), is a significant global concern. Drug development efforts aimed at suppressing the viability, replication, and virulence of HIV have resulted in a number of active anti-HIV compounds, 26 of which have been FDA-approved to date. These clinically available compounds can be broadly classified into six groups according to their targets and mode of action in the HIV replicative cycle. These include the nucleoside reverse transcriptase inhibitors (NRTIs), nucleotide reverse transcriptase inhibitors (NtRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), protease inhibitors (PIs), cell entry inhibitors [fusion inhibitors (FIs) and co-receptor inhibitors (CRIs)], and integrase inhibitors (INIs) [1]. Despite these advances, there exists a pressing need to develop new treatment strategies for AIDS due to the emergence of drug-resistant HIV variants.

To infect its hosts, HIV uses three essential enzymes: HIV reverse transcriptase (HIV-RT), integrase (IN), and protease (PR) [2]. Among these, HIV-1 RT has been a major target for antiretroviral drug development and more than half of the currently approved drugs for the treatment of HIV-1 infection are RT inhibitors [3]. Conversion of the single stranded RNA genome into a double stranded DNA is the primordial function of HIV-1 RT. HIV-1 RT consists of a p66 and a p51 subunit and thus acts as a heterodimer. Despite the structural role of the p51 subunit as elucidated by means of conformational modifications, the NNRTI-binding pocket (NNRTI-BP), polymerase, and RNase H domains are located in the p66 subunit [4]. The NNRTI-BP with hydrophobic and hydrophilic residues

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is nearly 10 Å away from the catalytic site and the amino acid residues lysine at position 103 (K103N) and tyrosine at position 181 (Y181C) are prone to mutation.

There are five NNRTIs approved for clinical use: nevirapine, delavirdine, efavirenz, etravirine, and rilpivirine (Fig. 1). Crystallographic analysis of the HIV-1 RT–NNRTI complex of the first-generation NNRTIs such as nevirapine and delavirdine has revealed a common ''butterfly-like'' conformation despite the chemical diversity between these compounds [5, 6]. By contrast, diarylpyrimidine (DAPY) analogues such as etravirine and rilpivirine, belonging to the third generation NNRTIs, adopt different conformational modes popularly called ''giggle and wiggle'' through their torsional flexibility and ability to reposition within the NNRTI binding pocket [1, 7, 8].

The goal of this study was to identify and synthesize new NNRTIs which potentially prevent the emergence of resistant HIV-1 RT variants. Toward this end, we focused on the acylhydrazone scaffold as our antiviral development strategy [9]. Acylhydrazones have served as starting compounds for diverse heterocyclic ring structures and have been attributed with various biological activities [10] such as antituberculosis [11–18], antibacterial [19, 20], antiparasitic [21, 22], anticancer [23–26], analgesic, and anti-inflammatory [27–29]. Further, the anti-HIV activity of acylhydrazone scaffold compounds has been appraised in a limited number of studies [30–35] and analysis of their structures has revealed the presence of amino acid side chains (Fig. 2).

Keeping these structural parameters in view, we synthesized a series of 2-aroylamino-3-methylbutanohydrazide-hydrazones and evaluated them against diverse viruses. Compounds found to be active against the HIV-1(III_B) strain were further investigated for their mechanism of action. Finally, molecular modeling was employed to gain insight into the structural parameters for binding and stereoselectivity.

Results and discussion

Chemistry

Compounds $\mathbf{1}_S$ and $\mathbf{1}_R$ were prepared by benzoylation of L-valine or D-valine. Compounds $\mathbf{2}_S$ and $\mathbf{2}_R$ were obtained by esterification of compounds $\mathbf{1}_S$ and $\mathbf{1}_R$. Compounds $\mathbf{3}_S$ and $\mathbf{3}_R$ were obtained by heating compounds $\mathbf{2}_S$ and $\mathbf{2}_R$ with hydrazine hydrate. (2*S*)-2-(Benzoylamino)-3-methyl-*N*'-(arylmethylene)butanohydrazides (**4–7**, $\mathbf{8}_S$, **9–10**, $\mathbf{11}_S$, $\mathbf{12}_S$, **13–24**) were synthesized by refluxing compound $\mathbf{3}_S$ with various aromatic aldehydes (4-chlorobenzaldehyde, 2-chlorobenzaldehyde, 2,6-dichlorobenzaldehyde, 2,4–dichlorobenzaldehyde, 2-fluorobenzaldehyde, 3-fluorobenzaldehyde, 4-fluorobenzaldehyde, 2,6-difluorobenzaldehyde, 2-chloro-6-fluorobenzaldehyde, 4-(trifluoromethyl)benzaldehyde, 4-methoxybenzaldehyde,

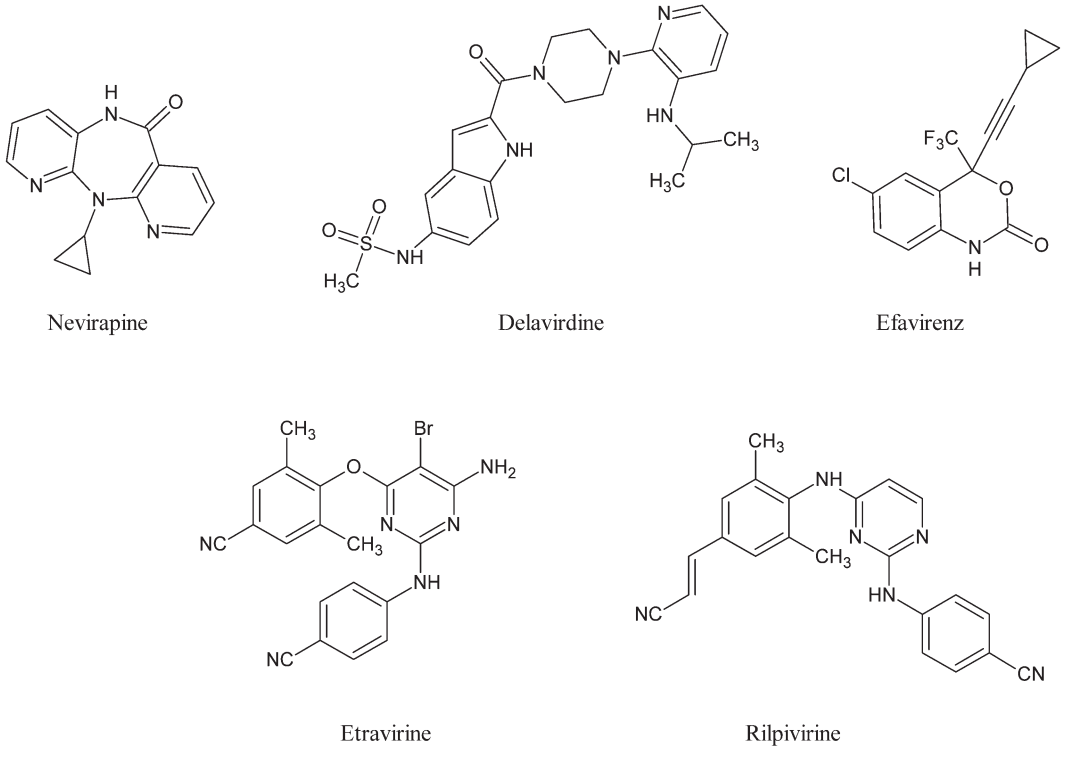


Figure 1. Structures of approved NNRTIs.

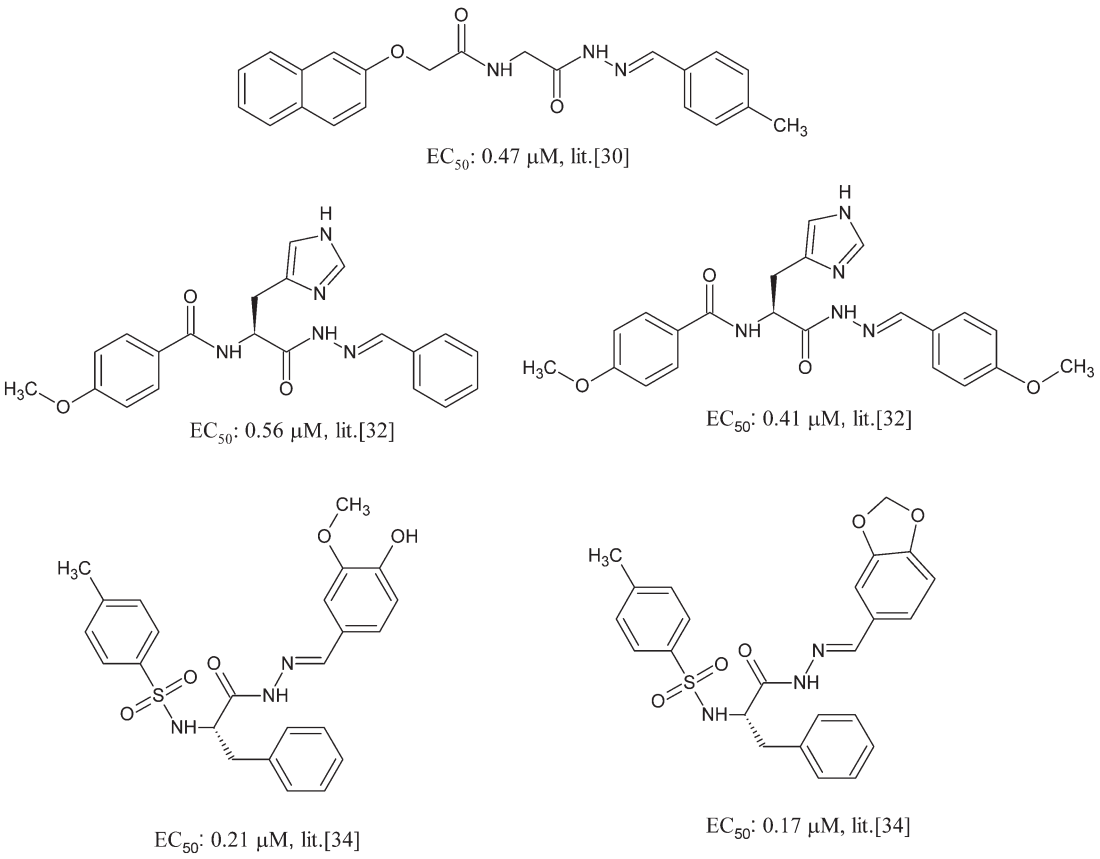


Figure 2. Chemical structures and anti-HIV activity of acylhydrazones with amino acid side chains.

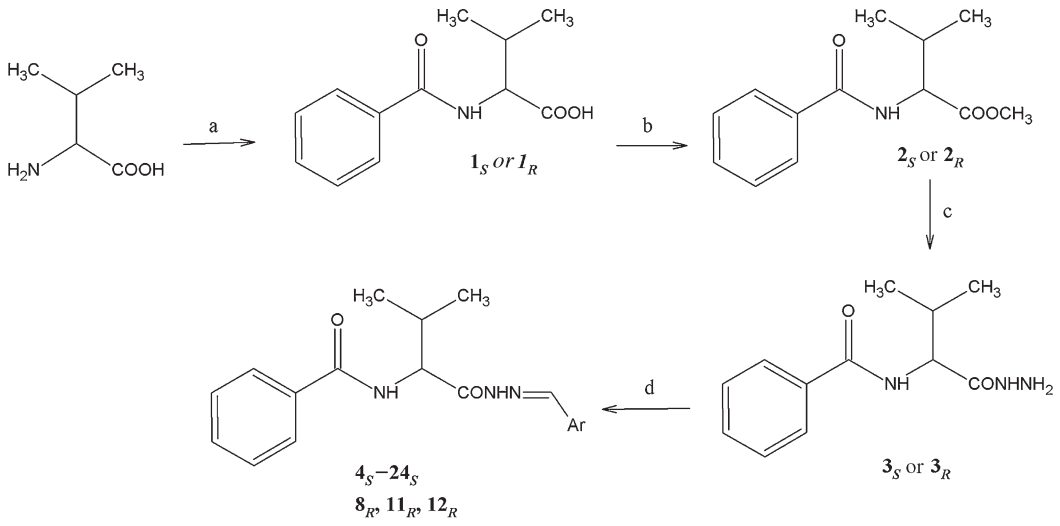
2,6-dimethoxybenzaldehyde, 4-methylbenzaldehyde, 2,6-dimethylbenzaldehyde, 2,4,6-trimethylbenzaldehyde, 4-hydroxybenzaldehyde, 4-hydroxy-3-methoxybenzaldehyde, 4-nitrobenzaldehyde, *N*,*N*-dimethyl-4-aminobenzaldehyde, cinnamaldehyde, 5-nitrofurfural) in ethanolic medium (Scheme 1). (2*R*)-2-(Benzoylamino)-3-methyl-*N*'-(arylmethylene)butanohydrazides (**8_R**, **11_R**, **12_R**) were synthesized by refluxing compound **3_R** with 2-fluorobenzaldehyde, 2,6-difluorobenzaldehyde and 2-chloro-6-fluorobenzaldehyde in ethanolic medium (Scheme 1).

While elemental analysis and HPLC data confirmed the purity of compounds **4–24** and **1–24**, respectively, their structures were identified by IR and ^{1}H NMR spectroscopy.

IR spectral data of compounds **4–24** were also confirmed on the basis of IR spectral data in the literature [30, 36]. The bands between 1676 and 1666 cm^{-1} were attributed to the C=O stretching bands and the bands between 1612 and 1577 were assigned to C=N stretching bands of acylhydrazones **4–24**.

The chemical shifts obtained from the ^{1}H NMR spectra of compounds **4–24** all supported the proposed structures of the compounds and they were in accordance with the literature [19, 37].

It is known that the hydrazones may exist as *E*/*Z* geometrical isomers due to the rotational restriction about the N=C double bond and *cis*/*trans* amide conformers [34, 38]. On the basis of literature, hydrazones comprising aldehyde and substituted hydrazide moieties prefer existing as *E* isomers in dimethyl-d_6 sulfoxide solution [34, 38, 39]. The chemical shift of the azomethine proton in the synthesized compounds was detected in the range of 8.01–8.69 ppm and two singlet peaks at 11.20–12.35 ppm were attributed to –NH– protons of the acylhydrazone moiety for compounds **4–24**. According to Easmon et al. [40] the N–H proton belonging to *E*-isomeric forms of acylhydrazones appears in the range 9–12 ppm; however, the same proton belonging to *Z*-isomeric forms of acylhydrazones appears in the range of 14–15 ppm in the ^{1}H NMR spectra. In the light of the foregoing, we propose that the compounds **4–24** exist as *E*-form. Two sets of signals belonging to *cis*/*trans* conformers of NH– protons of the acylhydrazone moiety were detected between 11.20–11.88 ppm and 11.36–12.35 ppm; the up-field signal of the mentioned proton was assigned to the *cis* conformer while the down-field



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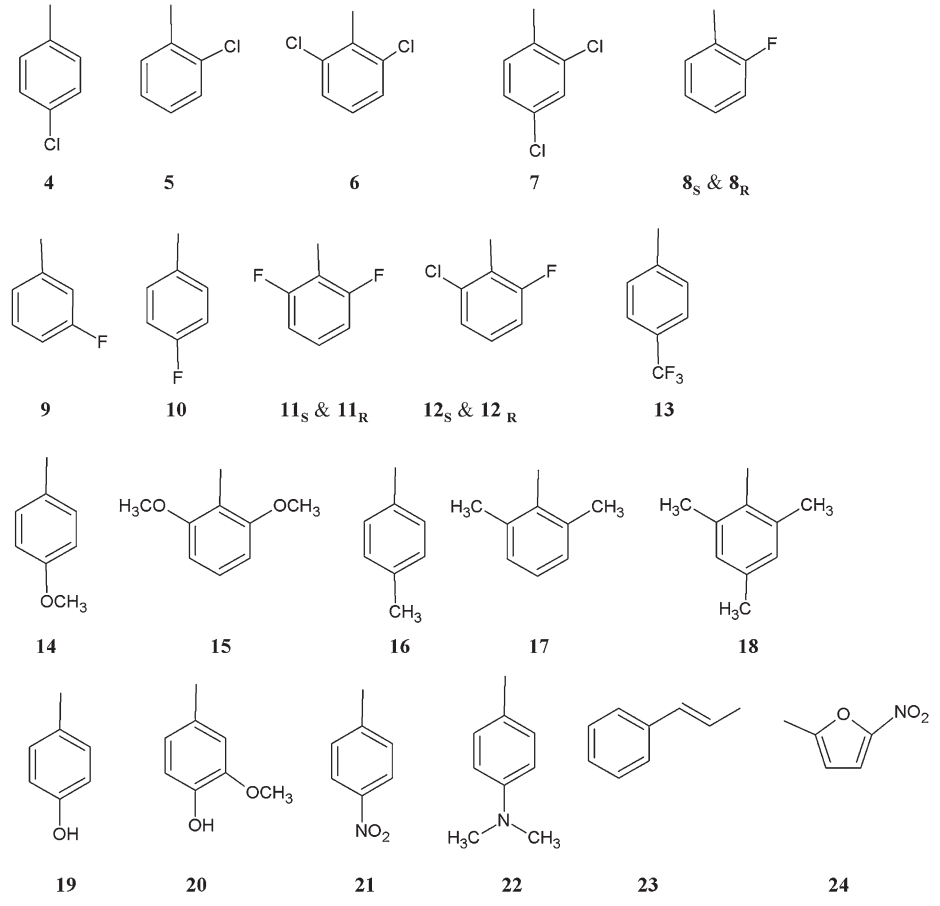


4 **5** **6** **7** **8_S & 8_R**

9 **10** **11_S & 11_R** **12_S & 12_R** **13**

14 **15** **16** **17** **18**

19 **20** **21** **22** **23** **24**

Scheme 1. Synthetic route to compounds **1_S**, **2_S**, **3_S**, **4–24**, **8_S**, **11_S**, **12_S**. *Reagents and conditions*: (a) C_6H_5COCl/NaOH; (b) MeOH/H_2SO_4; (c) $NH_2NH_2.H_2O$; (d) Ar–CHO/EtOH, reflux.

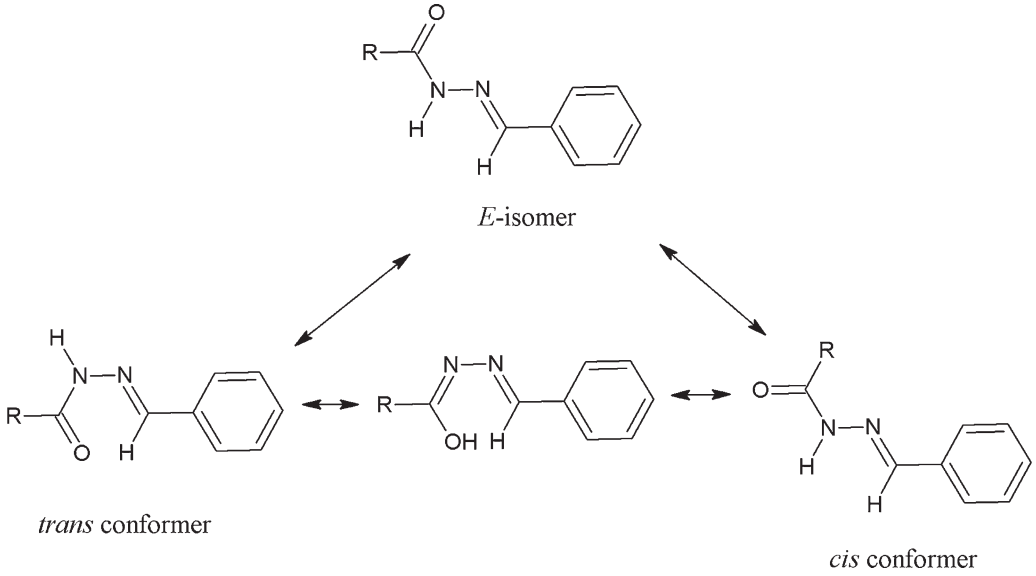
signal was assigned to the *trans* isomer (possible tautomeric forms of acylhydrazones **4**–**24** according to ^{1}H NMR data are depicted in Scheme 2). ^{13}C NMR spectrum of compound **11** was also recorded for further support. Detecting CH**C**H$(CH_3)_2$, **C**HCH$(CH_3)_2$ acylhydrazone **C**=O and some of the aromatic C-atoms as two peaks instead of one, thus, provided confirmatory evidence for the presence of both isomers. Specific rotation ($[\alpha]_D$) of all compounds was measured to judge optical purity. The specific optical rotation values of L-valine derived acylhydrazones **4**–**7**, **8**$_S$, **9**–**10**, **11**$_S$, **12**$_S$, **13**–**24** were determined and ranged between 243.311 and 122.387 while D-valine derived acylhydrazones **8**$_R$, **11**$_R$, and **12**$_R$ exhibited specific optical rotation values of −185.584, −154.594, and −151.258, respectively.

Antiviral evaluation

In view of the antiviral activity ascertained for similar acylhydrazones, the synthesized compounds were subjected to a preliminary screening for their antiviral effects against various types of viruses in HEL, HeLa, Vero, and CRFK (Crandell-Rees feline kidney) cell cultures. Compounds **4**–**24** were not found to be active against HSV−1(KOS), HSV-1(TK$^-$KOSACV^r), HSV-2(G), vaccinia virus, vesicular stomatitis virus, varicella zoster virus TK$^+$VZV, varicella zoster virus TK$^-$VZV, cytomegalovirus, vesicular stomatitis virus, respiratory syncytial virus, Coxsackie B4 virus, parainfluenza-3 virus, reovirus-1, Sindbis virus, and Punta Toro virus (*in vitro* antiviral evaluation and cytotoxicity data for compounds **4**–**24** in HEL, HeLa, and Vero cell cultures are available as Supporting Information Tables S1–S3). Compound **14** was found to have marginal activity against feline corona virus (EC_{50} = 26.8 μM) and feline herpes virus (EC_{50} = 60.6 μM, Supporting Information Table S4).

The anti-HCV activity of the compounds was investigated employing the *in vitro* HCV NS5BRdRp inhibition assay as described in the Experimental section. The compounds **4**–**5**, **7**, **8**$_S$, **9**, **12**$_S$, **14**–**18**, and **21**–**22** exhibited <20% inhibition of HCV NS5B RdRp activity at a compound concentration of 100 μM (Supporting Information Table S5), thus suggesting that these compounds are specific to HIV.

Anti-HIV and cytotoxicity data were obtained with the synthesized compounds using the strains HIV-1 (III_B) and HIV-2 (ROD) in an MT-4/MTT based assay [41] (see Supporting Information Table S6). The results of the anti-HIV study indicated that compounds **8**$_S$, **11**$_S$, and **12**$_S$ showed appreciable anti HIV-1 (III_B) activity with 50% inhibitory concentration (IC_{50}) of 123.8 μM and a selectivity index (SI) > 3, IC_{50} = 12.1 μM and SI > 29, IC_{50} = 17.4 μM and SI > 19, respectively (Table 1). Compounds **8**$_R$, **11**$_R$, and **12**$_R$ as enantiomers of three active hydrazones, were synthesized in order to evaluate their activity against the HIV-1 (III_B) strain in parallel with their *S*-enantiomers but none of them exhibited any significant activity. None of the evaluated compounds were found to be active against HIV-2 (strain ROD). Moreover, the hydrazones **8**$_s$, **11**$_s$, and **12**$_s$, which were active against HIV-1 wild type, showed no activity against a double mutant NNRTI resistant strain (K103N; Y181C; Table 1). It is noteworthy that the antiretroviral activity of compounds **8**, **11**, **12** is stereoselective and mainly imparted by their *S*-enantiomers. Although compound **8**$_S$ with 2-fluorophenyl moiety was found moderately active against HIV-1 (III_B), IC_{50} values of its analogous compounds **9** and **10** with 3-fluorophenyl



Scheme 2. Possible tautomeric forms of acylhydrazones **4**–**24** according to ^{1}H NMR data.

Table 1. Anti-HIV evaluation of selected compounds against wild-type HIV-1 (III_B), an HIV-1 NNRTI-resistant strain ($NNRTI^R$), and HIV-2 (ROD) in MT4 cells.

Compound	Strain	IC_{50} (μM)	CC_{50} (μM)	SI
$\mathbf{8_S}$	III_B	123.8 ± 116.5	>366	>3
	$NNRTI^R$	>366	>366	–
	ROD	>366	>366	–
$\mathbf{8_R}$	III_B	>366	>366	–
	ROD	>366	>366	–
$\mathbf{11_S}$	III_B	12.1 ± 6.3	>348	>29
	$NNRTI^R$	>348	>348	–
	ROD	>348	>348	–
$\mathbf{11_R}$	III_B	>348	>348	–
	ROD	>348	>348	–
$\mathbf{12_S}$	III_B	17.4 ± 11.2	>332	>19
	$NNRTI^R$	>332	>332	–
	ROD	>332	>332	–
$\mathbf{12_R}$	III_B	>332	>332	–
	ROD	>332	>332	–

and 4-fluorophenyl moieties were determined as >117.5 and >116.5 μg/mL, respectively. Among 2,6-halogenosubstituted acylhydrazones (compounds **6**, **11**, **12**); compounds $\mathbf{11_S}$ and $\mathbf{12_S}$ with 2,6-difluorophenyl and 2-chloro-6-fluorophenyl moieties showed satisfactory anti-HIV-1 (III_B) activity; however, compound **6** carrying the 2,6-chlorophenyl moiety was thoroughly inactive with an IC_{50} value of >113 μg/mL.

The compounds that displayed anti-HIV-1 activity in cell culture and their respective inactive enantiomer were evaluated for their inhibitory action on the RT polymerase activity in an enzymatic assay. In this assay the compounds $\mathbf{11_S}$ and $\mathbf{12_S}$ proved to be inhibitory with IC_{50} values of 162.2 ± 6.3 μM and 79.5 ± 9.7 μM, respectively, whereas the other compounds were completely devoid of activity even at concentrations of 3200 μM (Table 2).

The ultimate proof confirming the NNRTI mode of action on HIV-1 strain III_B replication in MT-4 cell culture was generated by performing a time-of-addition experiment with the most active compounds, $\mathbf{11_S}$ and $\mathbf{12_S}$. Dextran sulfate,

Table 2. Selected compounds assayed against wild-type HIV-1 RT polymerase activity.

Compound	IC_{50} (μM)[a)]
$\mathbf{11_R}$	>3200
$\mathbf{12_R}$	>3200
$\mathbf{11_S}$	162.2 ± 6.3
$\mathbf{12_S}$	79.5 ± 9.7
Nevirapine	12.3 ± 3.8
Efavirenz	0.17 ± 0.06

[a)] Compound concentration required to inhibit HIV-1 RT wild-type activity by 50%.

a polyanionic entry inhibitor, and clinically used RT inhibitors, AZT and nevirapine, were included as references. Both compounds, $\mathbf{11_S}$ and $\mathbf{12_S}$, completely block the replication of the virus in the same time frame as the established RT inhibitors, confirming their supposed NNRTI mode of action (Fig. 3).

Molecular docking studies

To evaluate the probable binding conformation of the representative chiral hydrazone derivatives, we performed docking study using Glide docking software v5.0 (Schrödinger, Inc., New York, NY, 2009). Since the hydrazone derivatives have been previously shown to inhibit HIV-1 RT through binding to a novel site located between the NNRTI-BP and the active site, the docking calculations of the chiral hydrazone derivatives were performed using the DHBNH (dihydroxy benzoyl naphthyl hydrazone)-HIV-1 RT co-crystal structure. It has been reported that, although DHBNH primarily inhibits the RNase H activity, it binds >50 Å away from the RNase H subdomain, at a site that partially overlaps the NNRTI-binding pocket [42]. As a preliminary step, we validated the accuracy of our docking approach by determining how closely the lowest energy binding conformation predicted by the Glidescore (Gscore) function resembles an experimental binding conformation as determined by X-ray crystallography. This validation resulted in a very good agreement between the localization of the inhibitor from docking and

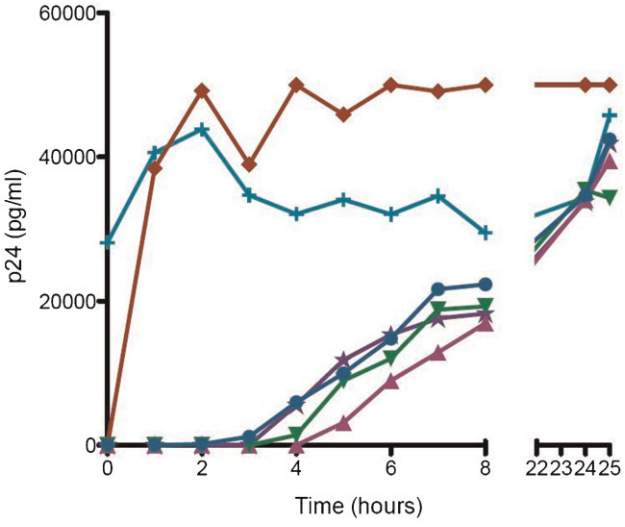


Figure 3. Time-of-addition experiment. MT-4 cells were infected with HIV-1, and the test compounds were added at different time points after infection. Virus production was determined by p24 Ag production in the supernatant at 31 h post infection. Crosses, control; diamonds, dextran sulfate 8000 (12.5 μM); stars, AZT (1.9 μM); triangles, nevirapine (7.5 μM); inverted triangles, $\mathbf{11_S}$ (95 μM); discs, $\mathbf{12_S}$ (125 μM). (This figure can be viewed in color mode in the online version.)

from the crystal structure as exemplified by a RMSD value of 0.5 Å between the predicted conformation and the observed X-ray crystallographic conformation of DHBNH. Analysis of the binding energy data for the docked conformations of representative chiral hydrazone derivatives (compounds **11$_R$** and **11$_S$**) at the novel site showed that the (*S*)-isomer (Gscore = −7.73 kcal/mol) binds more favorably than its (*R*)-counterpart (Gscore = −7.18 kcal/mol). Based on Glide docking scores, the *E*-isomer of compound **11$_S$** was found to bind more favorably than the *Z*-isomer.

The binding mode of compound **11$_S$** within the novel site of HIV-1 RT showed four key interactions (Fig. 4A). The phenyl ring of the benzoyl moiety is stabilized through the hydrophobic contacts with the methylene groups of Lys223 and the isopropyl group of Val108. The isopropyl group forms hydrophobic contacts with the side chains of Val108 and Leu234. The benzamide –NH and hydrazide –NH, respectively, forms hydrogen bonding interaction with the carboxylate group of the highly conserved Asp186. The highly electron-deficient 2,6-difluorophenyl ring forms a face-to-face pi–pi interaction with the electron-rich indole ring of the highly conserved primer grip residue Trp229, in addition to forming hydrophobic interactions with the side chains of Pro95, Leu100, and Tyr181.

The binding mode of compound **11$_R$** within the novel site of HIV-1 RT is shown in Fig. 4B. The binding conformation of compound **11$_R$** maintained all of the interactions of **11$_S$** except that the phenyl ring of the benzoyl moiety is fully solvent-exposed and does not participate in hydrophobic interaction with the methylene groups of the side chain of Lys223 and the isopropyl group of Val108.

Conclusion

Among 21 newly designed and synthesized acylhydrazone compounds, **8$_S$**, **11$_S$**, and **12$_S$** were shown to possess anti-HIV-1 (III_B) activity with IC_{50} = 123.8 μM and SI > 3, IC_{50} = 12.1 μM and SI > 29, IC_{50} = 17.4 μM and SI > 19, respectively. The **8$_R$**, **11$_R$**, and **12$_R$** enantiomers of the three active hydrazones were synthesized and evaluated for their activity against the HIV-1 (III_B) strain, but none of them exhibited any significant antiviral activity. None of the compounds tested (**4**–**24**) were found to be active against the HIV-2 strain ROD. Moreover, the hydrazones **8$_S$**, **11$_S$**, and **12$_S$**, which were active against HIV-1 wild type, showed no activity against a double mutant NNRTI resistant strain (K103N;Y181C). The NNRTI activity of compounds **8**, **11**, and **12** is stereoselective, and is exclusively due to the activity of the *S*-enantiomer. It was clearly established, as could be expected, that the anti-HIV-1 activity results from an NNRTI mode of action.

Based on the results from anti-HIV screening and enzyme inhibition studies as well as molecular modeling findings, novel hydrazone derivatives targeting HIV-1 RT at lower concentrations with higher selectivity have been developed.

Experimental

Synthesis

All solvents and reagents were obtained from commercial sources and used without purification. All melting points (°C, uncorrected) were determined using a Kleinfeld SMP-II basic model melting point apparatus. Specific rotation was measured on a Autopol V Plus digital polarimeter (serial number 85123, manufactured by Rudolph Research Analytical) with a 2 dm optical

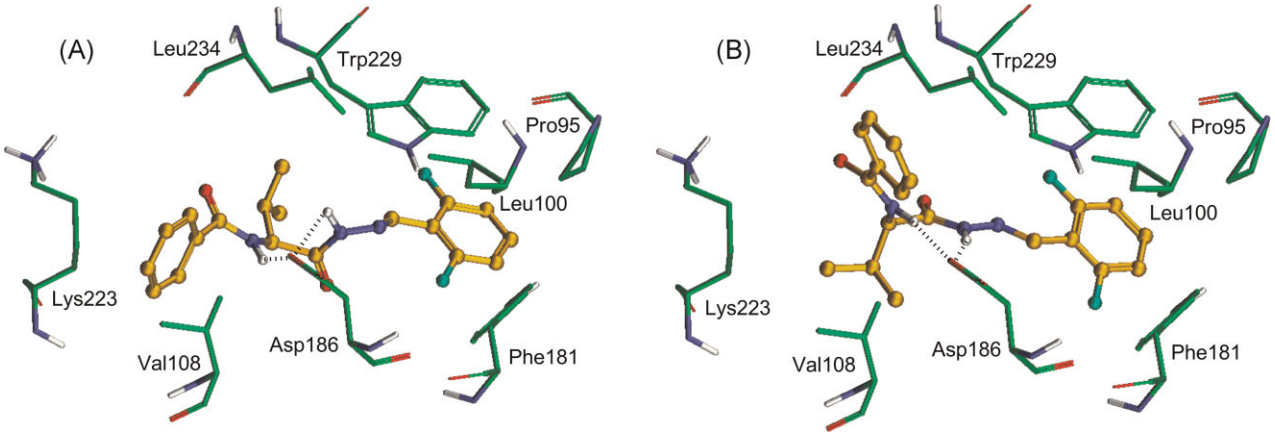


Figure 4. Glide-XP predicted binding model of compound (*S*)-**11** (A) and (*R*)-**11** (B) within the hydrazone binding site of HIV-1 RT. Amino acid residues are shown as stick model (please view this figure in color mode in the online version with the atoms colored as carbon – green, hydrogen – white, nitrogen – blue, and oxygen – red) whereas compounds are shown as ball and stick model with the same color scheme as above except that carbon atoms are represented in orange and the fluoro atoms are shown in green color. Dotted black line indicates hydrogen bonding interaction whereas dotted red line indicates electrostatic interactions.

length cell at 25.3°C. Elemental analyses were obtained using Leco CHNS-932 and are consistent with the assigned structures. Infrared spectra were recorded on a Shimadzu FTIR 8400S and data are expressed in wavenumber υ (cm^{-1}). NMR spectra were recorded on a Bruker AVANCE-DPX 400 at 400 MHz for ^1H NMR and 100 MHz for ^{13}C NMR (DEPT and decoupled), the chemical shifts were expressed in δ (ppm) downfield from tetramethylsilane (TMS) using DMSO-d_6 as solvent. The liquid chromatographic system consists of an Agilent Technologies 1100 series instrument equipped with a quaternary solvent delivery system and a model Agilent series G1315 A photodiode array detector. A Rheodyne syringe loading sample injector with a 50 μL sample loop was used for the injection of the analytes. Chromatographic data were collected and processed using Agilent Chemstation Plus software. The separation was performed at ambient temperature by using a reversed phase Nova-Pak C18 (3.9 mm × 150 mm, 5 μm particle size) column. All experiments were performed in isocratic mode. The mobile phase was prepared by mixing acetonitrile and bi-distilled water (40:60 v/v) and filtered through a 0.45 μm pore filter and subsequently degassed by ultrasonication, prior to use. Solvent delivery was employed at a flow rate of 1 mL/min. Detection of the analytes was carried out at 280 nm.

*(2S)-2-(Benzoylamino)-3-methylbutyric acid (**1_S**)*

(2*S*)-2-Amino-3-methylbutyric acid (L-valine, 1.17 g, 0.01 mol) was dissolved in sodium hydroxide solution (100 mL, 0.02 mol) and benzoyl chloride (1.40 g, 0.01 mol) was added to the reaction medium with stirring in an ice bath. The crude product was precipitated by addition of conc. HCl solution, filtered, dried, and washed with boiling petroleum ether. Yield 38%. m.p. 136–137°C [43]. $[\alpha]_D$ 31.859 (c: 1.0, DMF (dimethylformamide)). HPLC t_R (min.): 1.5. IR, υ (cm^{-1}): 3298 (amide NH str), 3228 (H-bonded OH str), 3066 (aromatic CH str), 2960 (alkyl CH str), 2874 (alkyl CH str), 1722 and 1695 (carboxylic acid C=O str), 1639 (amide C=O str).

*(2R)-2-(Benzoylamino)-3-methylbutyric acid (**1_R**)*

(2*R*)-2-Amino-3-methylbutyric acid (D-valine, 1.17 g, 0.01 mol) was dissolved in sodium hydroxide solution (100 mL, 0.02 mol) and benzoyl chloride (1.40 g, 0.01 mol) was added to the reaction medium with stirring in ice bath. The crude product was precipitated by addition of conc. HCl solution, filtered, dried, and washed with boiling petroleum ether. Yield 35%. m.p. 136–137°C [44]. $[\alpha]_D$ −27.785 (c: 1.0, DMF). HPLC t_R (min.): 1.5. IR, υ (cm^{-1}): 3298 (amide NH str), 3228 (H-bonded OH str), 3066 (aromatic CH str), 2960 (alkyl CH str), 2874 (alkyl CH str), 1722 and 1695 (carboxylic acid C=O str), 1639 (amide C=O str).

*(2S)-2-(Benzoylamino)-3-methylbutyric acid methyl ester (**2_S**)*

Compound **1_S** (2.21 g, 0.01 mol) was dissolved in 20 mL methanol and 1 mL conc. H_2SO_4 solution was added. The reaction mixture was heated under reflux for 3 h. The crude product was precipitated using $NaHCO_3$ solution (5% w/v), filtered, dried, and crystallized from petroleum ether. Yield 82%. m.p. 111–114°C [45]. $[\alpha]_D$ 14.176 (c: 1.0, DMF). HPLC t_R (min.): 3.54. IR, υ (cm^{-1}): 3074 (aromatic CH str), 2966 (alkyl CH str), 2874 (alkyl CH str), 1735 (ester C=O str), 1639 (amide C=O str), 1240 (C–O str).

*(2R)-2-(Benzoylamino)-3-methylbutyric acid methyl ester (**2_R**)*

Compound **1_R** (2.21 g, 0.01 mol) was dissolved in 20 mL methanol and 1 mL conc. H_2SO_4 solution was added. The reaction mixture was heated under reflux for 3 h. The crude product was precipitated by using $NaHCO_3$ solution (5% w/v), filtered, dried, and crystallized from petroleum ether. Yield 50%. m.p. 111°C [46]. $[\alpha]_D$ −15.954 (c: 1.0, DMF). HPLC t_R (min.): 3.54. IR, υ (cm^{-1}): 3074 (aromatic CH str), 2966 (alkyl CH str), 2874 (alkyl CH str), 1735 (ester C=O str), 1639 (amide C=O str), 1240 (C–O str).

*(2S)-2-(Benzoylamino)-3-methylbutyric acid hydrazide (**3_S**)*

Compound **2_S** (2.35 g, 0.01 mol) and hydrazine hydrate were heated under reflux for 1 h and 25 mL methanol was added to the reaction medium, subsequently the mixture was further heated under reflux for 1 h. The crude product was filtered and washed with boiling petroleum ether. Yield 77%. m.p. 210–211°C [47]. $[\alpha]_D$ 40.094 (c: 1.0, DMF). HPLC t_R (min.): 1.88. IR, υ (cm^{-1}): 3269 and 3184 (NH str), 1660 (hydrazide C=O str), 1624 (amide C=O str).

*(2R)-2-(Benzoylamino)-3-methylbutyric acid hydrazide (**3_R**)*

Compound **2_R** (2.35 g, 0.01 mol) and hydrazine hydrate was heated under reflux for 1 h and 25 mL methanol was added to the reaction medium, before further heating under reflux for 1 h. The crude product was filtered and washed with boiling petroleum ether. Yield 60%. m.p. 210–211°C [46]. $[\alpha]_D$ −38.269 (c: 1.0, DMF). HPLC t_R (min.): 1.88. IR, υ (cm^{-1}): 3269 and 3184 (NH str), 1660 (hydrazide C=O str), 1624 (amide C=O str).

*General procedure for the synthesis of (2S)-2-(benzoylamino)-3-methyl-N'-(arylmethylene)-butanohydrazides (**4–24**) and (2R)-2-(benzoylamino)-3-methyl-N'-(2-fluoro/2,6-difluoro/2-chloro-6-fluorobenzylidene)butanohydrazide (**8_R**, **11_R**, and **12_R**)*

Compound **3_S** was heated with various aromatic aldehydes under reflux for 2 h in ethanol. The crude products (**4–7**, **8_S**, **9–10**, **11_S**, **12_S**, **13–24**) were filtered and crystallized from appropriate solvents.

(2*R*)-2-(Benzoylamino)-3-methylbutyric acid hydrazide (**3_R**) was heated with 2-fluorobenzaldehyde, 2,6-difluorobenzaldehyde-2-chloro-6-fluorobenzaldehyde under reflux for 2 h in ethanol. The crude products (**8_R**, **11_R**, **12_R**) were filtered and crystallized from the appropriate solvents.

*(2S)-2-(Benzoylamino)-3-methyl-N'-(4-chlorobenzylidene)butanohydrazide (**4**)*

Yield 88%. m.p. 246–247°C (EtOH). $[\alpha]_D$ 200.721 (c: 1.2, DMF). HPLC t_R (min.): 7.90. IR, υ (cm^{-1}): 3281 and 3221 (NH str), 1674 (hydrazone C=O str), 1631 (amide C=O str), 1599 and 1579 (C=N, C=C str), 1091 (Ar–Cl str). ^1H NMR: δ (ppm) 0.94–1.02 (m, 6H, CHCH(**CH_3**)$_2$), 2.17–2.18 (m, 1H, CH**CH**$(CH_3)_2$), 4.30 and 5.34–5.38 (t, J = 8.5 Hz, J = 8.5 Hz and m, 1H, **CH**CH$(CH_3)_2$), 7.45–7.56 (m, 5H, ArH), 7.72 (d, J = 8.6 Hz, 2H, ArH), 7.88–7.92 (m, 2H, ArH), 8.01 and 8.25 (s and s, 1H, amide NH), 8.31 and 8.55 (d, J = 8.5 Hz and d, J = 8.2 Hz, 1H, –CH=N–), 11.55 and 11.75

(s and s, 1H, hydrazone NH). Anal. calcd. for $C_{19}H_{20}ClN_3O_2$ (357.83): C, 63.77; H, 5.63; N, 11.74%. Found C, 63.55; H, 4.26; N, 11.77%.

*(2S)-2-(Benzoylamino)-3-methyl-N'-(2-chlorobenzylidene)butanohydrazide (**5**)*

Yield 42%. m.p. 242°C (EtOH). $[\alpha]_D$ 159.331 (c: 0.96, DMF). HPLC t_R (min.): 8.03. IR, υ (cm^{-1}): 3276 and 3214 (NH str), 1668 (hydrazone C=O str), 1631 (amide C=O str), 1599 and 1579 (C=N, C=C str), 1091 (Ar–Cl str). ^{1}H NMR: δ (ppm) 0.96–1.03 (m, 6H, CHCH(**CH_3)$_2$**), 2.21–2.29 (m, 1H, CH**CH**$(CH_3)_2$), 4.28 and 5.36–5.40 (t, J = 8.5 Hz, J = 8.5 Hz and m, 1H, **CH**$CH(CH_3)_2$), 7.41–7.57 (m, 6H, ArH), 7.88–8.02 (m, 3H, ArH), 8.34 and 8.58 (d, J = 8.5 Hz and d, J = 8.2 Hz, 1H, –CH=N–), 8.41 and 8.67 (s and s, 1H, amide NH), 11.37 and 12.2 (s and s, 1H, hydrazone NH). Anal. calcd. for $C_{19}H_{20}ClN_3O_2$ (357.83): C, 63.77; H, 5.63; N, 11.74%. Found C, 63.49; H, 5.15; N, 11.70%.

*(2S)-2-(Benzoylamino)-3-methyl-N'-(2,6-dichlorobenzylidene)butanohydrazide (**6**)*

Yield 90%. m.p. 260–264°C (EtOH/DMF (90:10 v/v)). $[\alpha]_D$ 122.387 (c: 0.70, DMF). HPLC t_R (min.): 11.12 IR, υ (cm^{-1}): 3246 and 3198 (NH str), 1674 (hydrazone C=O str), 1626 (amide C=O str), 1602 and 1579 (C=N, C=C str). ^{1}H NMR: δ (ppm) 0.96–1.01 (m, 6H, CHCH(**CH_3)$_2$**), 2.28–2.33 (m, 1H, CH**CH**$(CH_3)_2$), 4.32 and 5.31–5.35 (t, J = 8.5 Hz, J = 8.5 Hz and m, 1H, **CH**$CH(CH_3)_2$), 7.42–7.60 (m, 6H, ArH), 7.86 and 7.92 (d, J = 7.1 Hz and d, J = 7.1 Hz, 2H, Ar–H), 8.32 and 8.47 (s and s, 1H, amide NH), 8.21 and 8.60 (d, J = 8.5 Hz and d, J = 8.3 Hz, 1H, –CH=N–), 11.72 and 11.96 (s and s, 1H, hydrazone NH). Anal. calcd. for $C_{19}H_{19}Cl_2N_3O_2$ (392.28): C, 58.17; H, 4.88; N, 10.71%. Found C, 58.03; H, 3.80; N, 10.81%.

*(2S)-2-(Benzoylamino)-3-methyl-N'-(2,4-dichlorobenzylidene)butanohydrazide (**7**)*

Yield 68%. m.p. 215–220°C (acetone). $[\alpha]_D$ 178.141 (c: 1.0, DMF). HPLC t_R (min.): 5.60. IR, υ (cm^{-1}): 3225 (NH str), 1672 (hydrazone C=O str), 1631 (amide C=O str). ^{1}H NMR: δ (ppm) 0.95–1.02 (m, 6H, CHCH(**CH_3)$_2$**), 2.19–2.29 (m, 1H, CH**CH**$(CH_3)_2$), 4.27 and 5.33–5.37 (t, J = 8.5 Hz, J = 8.5 Hz and m, 1H, **CH**$CH(CH_3)_2$), 7.44–7.57 (m, 5H, ArH), 7.72 (d, J = 2.1 Hz, 1H, ArH), 7.88–7.93 (m, 2H, ArH), 7.94, 7.96, 7.99, 8.01 (4s, 1H, amide NH), 8.36 and 8.60 (d, J = 8.2 Hz and d, J = 7.7 Hz, 1H, –CH=N–), 11.42 and 12.35 (s and s, 1H, hydrazone NH). Anal. calcd. for $C_{19}H_{19}Cl_2N_3O_2$ (392.28): C, 58.17; H, 4.88; N, 10.71%. Found C, 57.53; H, 3.89; N, 10.01%.

*(2S)-2-(Benzoylamino)-3-methyl-N'-(2-fluorobenzylidene)butanohydrazide (**8_S**)*

Yield 82%. m.p. 232–233°C (EtOH). $[\alpha]_D$ 198.465 (c: 1.2, DMF). HPLC t_R (min.): 5.46.IR, υ (cm^{-1}): 3244 and 3207 (NH str), 1670 (hydrazone C=O str), 1629 (amide C=O str), 1602 and 1579 (C=N, C=C str). ^{1}H NMR: δ (ppm) 0.95–1.02 (m, 6H, CHCH(**CH_3)$_2$**), 2.19–2.31 (m, 1H, CH**CH**$(CH_3)_2$), 4.28 and 5.35–5.39 (t, J = 8.5 Hz, J = 8.5 Hz and m, 1H, **CH**$CH(CH_3)_2$), 7.27–7.34 (m, 2H, ArH), 7.45–7.57 (m, 5H, ArH), 7.87–7.95 (m, 2H, ArH), 8.23 and 8.50 (s and s, 1H, amide NH), 8.33 and 8.59 (d, J = 8.5 Hz and d, J = 8.2 Hz, 1H, –CH=N–), 11.59 and 11.82 (s and s, 1H, hydrazone NH). Anal. calcd. for $C_{19}H_{20}FN_3O_2$ (341.38): C, 66.85; H, 5.91; N, 12.31%. Found C, 66.88; H, 4.67; N, 12.41%.

*(2R)-2-(Benzoylamino)-3-methyl-N'-(2-fluorobenzylidene)butanohydrazide (**8_R**)*

Yield 36%. m.p. 232°C (MeOH). $[\alpha]_D$ –185.584 (c: 1.0, DMF). HPLC t_R (min.): 5.40. IR, υ (cm^{-1}): 3246 and 3207 (NH str), 1672 (hydrazone C=O str), 1631 (amide C=O str), 1616 (C=N). ^{1}H NMR: δ (ppm) 0.95–1.03 (m, 6H, CHCH(**CH_3)$_2$**), 2.18–2.32 (m, 1H, CH**CH**$(CH_3)_2$), 4.28 and 5.35–5.40 (t, J = 8.5 Hz, J = 8.5 Hz and m, 1H, **CH**$CH(CH_3)_2$), 7.26–7.35 (m, 2H, ArH), 7.44–7.57 (m, 4H, ArH), 7.86–7.96 (m, 3H, ArH), 8.23 and 8.50 (s and s, 1H, amide NH), 8.32 and 8.57 (d, J = 8.4 Hz and d, J = 8.4 Hz, 1H, –CH=N–), 11.61 and 11.84 (s and s, 1H, hydrazone NH). Anal. calcd. for $C_{19}H_{20}FN_3O_2$ (341.38): C, 66.85; H, 5.91; N, 12.31%. Found C, 66.00; H, 5.75; N, 12.33%.

*(2S)-2-(Benzoylamino)-3-methyl-N'-(3-fluorobenzylidene)-butanohydrazide (**9**)*

Yield 69%. m.p. 242–243°C (EtOH/DMF (90:10 v/v)). $[\alpha]_D$ 197.284 (c: 1.2, DMF). HPLC t_R (min.): 5.54.IR, υ (cm^{-1}): 3265 and 3213 (NH str), 1670 (hydrazone C=O str), 1627 (amide C=O str), 1581 (C=N; C=C str), 1219 and 1141 (Ar–F str). ^{1}H NMR: δ (ppm) 0.95–1.02 (m, 6H, CHCH(**CH_3)$_2$**), 2.00–2.44 (m, 1H, CH**CH**$(CH_3)_2$), 4.30 and 5.36–5.40 (t, J = 8.5 Hz, J = 8.5 Hz and m, 1H, **CH**$CH(CH_3)_2$), 7.44–7.56 (m, 7H, ArH), 7.88–7.92 (m, 2H, ArH), 8.01 and 8.26 (s and s, 1H, amide NH), 8.35 and 8.58 (d, J = 8.2 Hz and d, J = 8.5 Hz, –CH=N– 11.60 and 11.79 (s and s, 1H, hydrazone NH). Anal. calcd. for $C_{19}H_{20}FN_3O_2$ (341.38): C, 66.85; H, 5.91; N, 12.31%. Found C, 66.76; H, 4.88; N, 12.36%.

*2-(Benzoylamino)-3-methyl-N'-(4-fluorobenzylidene)-butanohydrazide (**10**)*

Yield 86%. m.p. 233–235°C (EtOH/DMF (90:10 v/v)). $[\alpha]_D$ 197.520 (c: 1.0, DMF). HPLC t_R (min.): 5.13. IR, υ (cm^{-1}): 3255 and 3213 (NH str), 1670 (hydrazone C=O str), 1631 (amide C=O str), 1602 (C=N str), 1238, 1207, and 1153(Ar–F str). ^{1}H NMR: δ (ppm) 0.95–1.02 (m, 6H, CHCH(**CH_3)$_2$**), 2.19–2.27 (m, 1H, CH**CH**$(CH_3)_2$), 4.29 and 5.35–5.39 (t, J = 8.6 Hz, J = 8.6 Hz and m, 1H, **CH**$CH(CH_3)_2$), 7.26–7.33 (m, 2H, ArH), 7.44–7.56 (m, 3H, ArH), 7.74–7.78 (m, 2H, ArH), 7.88–7.92 (m, 2H, ArH), 8.28 and 8.56 (t, J = 8.6 Hz, J = 8.3 Hz and d, J = 8.3 Hz, amide NH), 8.01 and 8.69 (s and s, 1H, –CH=N–), 11.49 and 11.69 (s and s, 1H, hydrazone NH). Anal. calcd. for $C_{19}H_{20}FN_3O_2$ (341.38): C, 66.85; H, 5.91; N, 12.31%. Found C, 66.99; H, 5.13; N, 12.31%.

*(2S)-2-(Benzoylamino)-3-methyl-N'-(2,6-difluorobenzylidene)butanohydrazide (**11_S**)*

Yield 98%. m.p. 246–247°C (EtOH/DMF (90:10 v/v)). $[\alpha]_D$ 178.826 (c: 1.0, DMF). HPLC t_R (min.): 6.11. IR, υ (cm^{-1}): 3250 and 3211 (NH str), 1670 (hydrazone C=O str), 1631 (amide C=O str), 1602 (C=N str), 1236, 1207, and 1153 (Ar–F str). ^{1}H NMR: δ (ppm) 0.95–1.01 (m, 6H, CHCH(**CH_3)$_2$**), 2.28–2.41 (m, 1H, CH**CH**$(CH_3)_2$), 4.27 and 5.29–5.33 (t, J = 8.5 Hz, J = 8.5 Hz and m, 1H, **CH**$CH(CH_3)_2$), 7.17–7.24 (q, 2H, ArH), 7.45–7.56 (m, 4H, ArH), 7.87–7.92 (m, 2H, ArH), 8.16 and 8.43 (s and s, 1H, amide NH), 8.18 and 8.59 (d and d, J = 8.5 Hz, J = 8.2 Hz 1H, –CH=N–), 11.62 and 11.83 (s and s, 1H, hydrazone NH). ^{13}C NMR-DEPT: δ (ppm) 19.71 and 19.77 (CH_3), 30.8 and 30.21 (CH), 55.46 and 59.22 (CH), 111.88 (Ar–C_q), 112.65, 112.75, 112.89, 112.99 (Ar–CH), 128.02 and 128.11 (Ar–CH), 128.65 (Ar–CH), 131.70 and 131.82 (Ar–CH), 134.46 and 134.70 (Ar–C_q), 137.88 (CONHN=**CH**–), 159.58 and 161.95 (Ar–C_q), 167.23 and 168.52 (CONHN=), 173.83 (Ar–CONH).

Anal. calcd. for $C_{19}H_{20}F_2N_3O_2$ (359.37): C, 63.50; H, 5.33; N, 11.69%. Found C, 63.40; H, 5.15; N, 11.73%.

*(2R)-2-(Benzoylamino)-3-methyl-N'-(2,6-difluorobenzylidene)butanohydrazide (**11$_R$**)*

Yield 32%. m.p. 248°C (MeOH). $[\alpha]_D$ −154.594 (c: 0.9, DMF). HPLC t_R (min.): 6.08. IR, υ (cm^{-1}): 3257 and 3211 (NH str), 1672 (hydrazone C=O str), 1627 (amide C=O str), 1232, 1209 (Ar–F str). ^{1}H NMR: δ (ppm) 0.95–1.01 (m, 6H, CHCH(**CH_3)$_2$**), 2.18–2.33 (m, 1H, CH**CH**$(CH_3)_2$), 4.27 and 5.28–5.34 (t, J = 8.5 Hz, J = 8.5 Hz and m, 1H, **CH**$CH(CH_3)_2$), 7.16–7.25 (q, 2H, ArH), 7.44–7.57 (m, 4H, ArH), 7.87–7.92 (m, 2H, ArH), 8.16 and 8.42 (s and s, 1H, amide NH), 8.19 and 8.58 (d and d, J = 8.4 Hz, J = 8.1 Hz 1H, –CH=N– 11.64 and 11.83 (s and s, 1H, hydrazone NH). Anal. calcd. for $C_{19}H_{20}F_2N_3O_2$ (359.37): C, 63.50; H, 5.33; N, 11.69%. Found C, 62.62; H, 5.21; N, 11.67%.

*(2S)-2-(Benzoylamino)-3-methyl-N'-(2-chloro-6-fluorobenzylidene)butanohydrazide (**12$_S$**)*

Yield 84%. m.p. 248–249°C (EtOH/DMF (90:10 v/v)). $[\alpha]_D$ 163.498 (c: 0.98, DMF). HPLC t_R (min.): 8.33. IR, υ (cm^{-1}): 3248 and 3201 (NH str), 1676 (hydrazone C=O str), 1626 (amide C=O str), 1602 and 1577 (C=N; C=C str). ^{1}H NMR: δ (ppm) 0.96–1.01 (m, 6H, CHCH(**CH_3)$_2$**), 2.11–2.41 (m, 1H, CH**CH**$(CH_3)_2$), 4.29 and 5.29–5.34 (t, J = 8.5 Hz, J = 8.5 Hz and m, 1H, **CH**$CH(CH_3)_2$), 7.33–7.55 (m, 6H, ArH), 7.87–7.93 (m, 2H, ArH), 8.22 and 8.60 (d, J = 8.4 Hz, and d, J = 8.2 Hz, 1H, CH=N–), 8.28 and 8.51 (s and s, 1H, amide NH), 11.68 and 11.92 (s and s, 1H, hydrazone NH). Anal. calcd. for $C_{19}H_{19}ClFN_3O_2$ (375.82): C, 60.72; H, 5.10; N, 11.18%. Found C, 60.99; H, 4.94; N, 11.29%.

*(2R)-2-(Benzoylamino)-3-methyl-N'-(2-chloro-6-fluorobenzylidene)butanohydrazide (**12$_R$**)*

Yield 56%. m.p. 252–253°C (MeOH). $[\alpha]_D$ −151.258 (c: 0.92, DMF). HPLC t_R (min.): 8.30. IR, υ (cm^{-1}): 3248 and 3200 (NH str), 1676 (hydrazone C=O str), 1626 (amide C=O str), 1600 (C=N; C=C str). ^{1}H NMR: δ (ppm) 0.96–1.01 (m, 6H, CHCH(**CH_3)$_2$**), 2.18–2.33 (m, 1H, CH**CH**$(CH_3)_2$), 4.29 and 5.29–5.34 (t, J = 8.5 Hz, J = 8.5 Hz and m, 1H, **CH**$CH(CH_3)_2$), 7.33–7.57 (m, 6H, ArH), 7.87–7.93 (m, 2H, ArH), 8.21 and 8.60 (d, J = 8.4 Hz and d, J = 8.1 Hz, 1H, –CH=N– 8.28 and 8.51 (s and s, 1H, amide NH), 11.70 and 11.93 (s and s, 1H, hydrazone NH). Anal. calcd. for $C_{19}H_{19}ClFN_3O_2$ (375.82): C, 60.72; H, 5.10; N, 11.18%. Found C, 60.04; H, 4.96; N, 11.17%.

*2-(Benzoylamino)-3-methyl-N'-(4-trifluoromethylbenzylidene)butanohydrazide (**13**)*

Yield 57%. m.p. 243–244°C (EtOH/DMF (90:10 v/v)). $[\alpha]_D$ 177.633 (c:1.0, DMF). HPLC t_R (min.): 11.87. IR, υ (cm^{-1}): 3244 and 3213 (NH str), 1672 (hydrazone C=O str), 1633 (amide C=O str), 1602 (C=N str). ^{1}H NMR: δ (ppm) 0.95–1.03 (m, 6H, CHCH(**CH_3)$_2$**), 2.19–2.29 (m, 1H, CH**CH**$(CH_3)_2$), 4.32 and 5.35–5.38 (t, J = 8.5 Hz, J = 8.5 Hz and m, 1H, **CH**$CH(CH_3)_2$), 7.45–7.57 (m, 4H, ArH), 7.81–7.87 (m, 2H, ArH), 7.88–7.93 (m, 4H, ArH), 8.35 (s, 1H, amide NH), 8.09 and 8.61 (s and d, J = 8.2 Hz 1H, –CH=N–), 11.68 and 11.89 (s and s, 1H, hydrazone NH). Anal. calcd. for $C_{20}H_{20}F_3N_3O_2$ (391.38): C, 61.38; H, 5.15; N, 10.74%. Found C, 61.77; H, 5.69; N, 10.74%.

*(2S)-2-(Benzoylamino)-3-methyl-N'-(4-methoxybenzylidene)butanohydrazide (**14**)*

Yield 93%. m.p. 255–256°C (EtOH/DMF (90:10 v/v)). $[\alpha]_D$ 145.682 (c:1.0, DMF). HPLC t_R (min.): 4.53. IR, υ (cm^{-1}): 3275 and 3232 (NH str), 1668 (hydrazone C=O str), 1631 (amide C=O str), 1608 (C=N str). ^{1}H NMR: δ (ppm) 0.94–1.01 (m, 6H, CHCH(**CH_3)$_2$**), 2.06–2.38 (m, 1H, CH**CH**$(CH_3)_2$), 3.80 (d, J = 9.5 Hz, 3H, –**OCH_3**), 4.28 and 5.35–5.37 (t, J = 8.6 Hz, J = 8.6 Hz and m, 1H, **CH**$CH(CH_3)_2$), 6.99–7.04 (m, 2H, ArH), 7.44–7.49 (m, 2H, ArH), 7.52–7.55 (m, 1H, ArH), 7.65 (d, 2H, J = 8.8 Hz, 2H, ArH), 7.88–7.92 (m, 2H, ArH), 7.95 and 8.19 (s and s, 1H, amide NH), 8.25 and 8.54 (d, J = 8.6 Hz and d, J = 8.3 Hz 1H, –CH=N–), 11.50 and 11.58 (s and s, 1H, hydrazone NH). Anal. calcd. for $C_{20}H_{23}N_3O_3$ (353.42): C, 67.97; H, 6.56; N, 11.89%. Found C, 67.75; H, 6.01; N, 11.92%.

*(2S)-2-(Benzoylamino)-3-methyl-N'-(2,6-dimethoxybenzylidene)butanohydrazide (**15**)*

Yield 60%. m.p. 248–249°C (EtOH/DMF (90:10 v/v)). $[\alpha]_D$ 180.901 (c:1.2, DMF). HPLC t_R (min.): 5.75. IR, υ (cm^{-1}): 3275 and 3232 (NH str), 1668 (hydrazone C=O str), 1631 (amide C=O str), 1608 (C=N str). ^{1}H NMR: δ (ppm) 0.95–1.01 (m, 6H, CHCH(**CH_3)$_2$**), 2.06–2.27 (m, 1H, CH**CH**$(CH_3)_2$), 3.79 (d, J = 6.5 Hz, 6H, –**OCH_3**), 4.28 and 5.21–5.25 (t, J = 8.6 Hz, J = 8.6 Hz and m, 1H, **CH**$CH(CH_3)_2$), 6.70–7.75 (m, 2H, ArH), 7.32–7.37 (m, 1H, ArH), 7.43–7.55 (m, 3H, ArH), 7.84 and 7.90 (d, J = 7.1 Hz and d, J = 7.1 Hz 1H, ArH), 8.15 and 8.44 (d, J = 9.1 Hz and d, J = 9.1 Hz 1H, –CH=N–), 8.25 and 8.41 (s and s, 1H, amide NH), 11.27 and 11.44 (s and s, 1H, hydrazone NH). Anal. calcd. for $C_{21}H_{25}N_3O_4$ (383.44): C, 65.78; H, 6.57; N, 10.96%. Found C, 65.32; H, 5.97; N, 10.90%.

*(2S)-2-(Benzoylamino)-3-methyl-N'-(4-methylbenzylidene)butanohydrazide (**16**)*

Yield 92%. m.p. 251–253°C (EtOH/DMF (90:10 v/v)). $[\alpha]_D$ 185.166 (c: 1.0, DMF). HPLC t_R (min.): 6.59.IR, υ (cm^{-1}): 3276 and 3230 (NH str), 1670 (hydrazone C=O str), 1635 (amide C=O str), 1612 (C=N str). ^{1}H NMR: δ (ppm) 0.95–1.02 (m, 6H, CHCH(**CH_3)$_2$**), 2.19–2.21 (m, 1H, CH**CH**$(CH_3)_2$), 2.34 (s, 3H, Ar–**CH_3**), 4.29 and 5.35–5.40 (t, J = 8.6 Hz, J = 8.6 Hz and m, 1H, **CH**$CH(CH_3)_2$), 7.25–7.28 (m, 2H, ArH), 7.44–7.60 (m, 5H, ArH), 7.88–7.92 (m, 2H, ArH), 7.98 and 8.22 (s and s, 1H, amide NH), 8.25 and 8.54 (d, J = 8.5 Hz and d, J = 8.3 Hz 1H, –CH=N–), 11.42 and 11.61 (s and s, 1H, hydrazone NH). Anal. calcd. for $C_{20}H_{23}N_3O_2$ (337.42): C, 71.19; H, 6.87; N, 12.45%. Found C, 71.11; H, 7.27; N, 12.49%.

*(2S)-2-(Benzoylamino)-3-methyl-N'-(2,6-dimethylbenzylidene)butanohydrazide (**17**)*

Yield 41%. m.p. 242°C (MeOH). $[\alpha]_D$ 206.099 (c: 1.1, DMF). HPLC t_R (min.): 11.98. IR, υ (cm^{-1}): 3273 and 3229 (NH str), 1672 (hydrazone C=O str), 1631 (amide C=O str), 1609 (C=N str). ^{1}H NMR: δ (ppm) 0.96–1.01 (m, 6H, CHCH(**CH_3)$_2$**), 2.19–2.21 (m, 1H, CH**CH**$(CH_3)_2$), 2.41 and 2.47 (s and s, 6H, Ar–**CH_3**), 4.31 and 5.36–5.44 (t, J = 8.6 Hz, J = 8.6 Hz and m, 1H, **CH**$CH(CH_3)_2$), 7.08–7.19 (m, 3H, ArH), 7.45–7.55 (m, 3H, ArH), 7.88 and 7.92 (dd, J = 7.2 Hz, J = 7.2 Hz, 2H, ArH), 8.20 and 8.48 (d, J = 8.5 Hz and d, J = 8.3 Hz, 1H, –CH=N–), 8.43 and 8.58 (s and s, 1H, amide NH), 11.35 and 11.61 (s and s, 1H, hydrazone NH). Anal. calcd. for $C_{21}H_{25}N_3O_2$ (351.44): C, 71.77; H, 7.17; N, 11.96%. Found C, 71.09; H, 7.09; N, 11.88%.

*(2S)-2-(Benzoylamino)-3-methyl-N′-(2,4,6-trimethylbenzylidene)butanohydrazide (**18**)*

Yield 56%. m.p. 248–253°C (EtOH/DMF (90:10 v/v)). $[\alpha]_D$ 149.004 (c: 0.82, DMF). HPLC t_R (min.): 20.15. IR, υ (cm^{-1}): 3254 and 3205 (NH str), 1668 (hydrazone C=O str), 1629 (amide C=O str). ^{1}H NMR: δ (ppm) 0.96–1.00 (m, 6H, CHCH(**CH_3**)$_2$), 2.24–2.31 (q, 4H, CH**CH**$(CH_3)_2$ and Ar–**CH_3**), 2.38 and 2.44 (s and s, 6H, Ar–**CH_3**), 4.29 and 5.35–5.37 (t, J = 8.5 Hz, J = 8.5 Hz and m, 1H, **CH**$CH(CH_3)_2$), 6.91 and 6.94 (s and s, 2H, ArH), 7.45–7.56 (m, 3H, ArH), 7.87–7.93 (m, 2H, ArH), 8.20 and 8.40 (d, J = 8.6 Hz and s, 1H, amide NH), 8.54 (s, 1H, –CH=N– 11.28 and 11.53 (s and s, 1H, hydrazone NH). Anal. calcd. for $C_{22}H_{27}N_3O_2$ (365.46): C, 72.30; H, 7.45; N, 11.50%. Found C, 72.20; H, 7.11; N, 11.38%.

*(2S)-2-(Benzoylamino)-3-methyl-N′-(4-hydroxybenzylidene)butanohydrazide (**19**)*

Yield 83%. m.p. 216–217 °C (EtOH). $[\alpha]_D$ 153.713 (c: 0.97, DMF). HPLC t_R (min.): 2.39. IR, υ (cm^{-1}): 3410 (OH str), 3254 and 3205 (NH str), 1670 (hydrazone C=O str), 1630 (amide C=O str). ^{1}H NMR: δ (ppm) 0.94–1.01 (m, 6H, CHCH(**CH_3**)$_2$), 2.16–2.28 (m, 1H, CH**CH**$(CH_3)_2$), 4.28 and 5.35–5.38 (t, J = 8.6 Hz, J = 8.6 Hz and m, 1H, **CH**$CH(CH_3)_2$), 6.83 (t, J = 8.9 Hz, J = 8.9 Hz, 2H, ArH), 7.44–7.56 (m, 5H, ArH), 7.88–7.92 (m, 2H, ArH), 8.14 (s, 1H, amide NH), 8.20 and 8.51 (d, J = 8.7 Hz and d, J = 8.4 Hz 1H, –CH=N–), 9.91 (s, 1H, Ar–O**H**), 11.28 and 11.46 (s and s, 1H, hydrazone NH). Anal. calcd. for $C_{19}H_{21}N_3O_3$ (339.38): C, 67.24; H, 6.24; N, 12.38%. Found C, 66.61; H, 5.89; N, 12.03%.

*(2S)-2-(Benzoylamino)-3-methyl-N′-(4-hydroxy-3-methoxybenzylidene)butanohydrazide (**20**)*

Yield 46%. m.p. 204–206°C (EtOH/acetone (90:10 v/v)). $[\alpha]_D$ 196.947 (c: 1.0, DMF). HPLC t_R (min.): 2.56. IR, υ (cm^{-1}): 3413 (OH str), 3250 and 3210 (NH str), 1672 (hydrazone C=O str), 1629 (amide C=O str). ^{1}H NMR: δ (ppm) 0.94–1,01 (m, 6H, CHCH(**CH_3**)$_2$), 2.09–2.31 (m, 1H, CH**CH**$(CH_3)_2$), 3.82 (s, 3H, –**OCH_3**), 4.27 and 5.34–5.37 (t, J = 8.6 Hz, J = 8.6 Hz and m, 1H, **CH**$CH(CH_3)_2$), 6.82 and 6.84 (dd, J = 2.5 Hz, J = 2.5 Hz, 1H, ArH), 7.05–7.09 (m, 1H, ArH), 7.26 and 7.30 (dd, J = 1.8 Hz, J = 1.8 Hz, 1H, ArH), 7.44–7.56 (m, 3H, ArH), 7.87–7.92 (m, 2H, ArH), 8.13 (s, 1H, amide NH), 8.22 and 8.52 (d, J = 8.7 Hz and d, J = 8.3 Hz 1H, –CH=N–), 9.52 (s, 1H, Ar–O**H**), 11.32 and 11.48 (s and s, 1H, hydrazone NH). Anal. calcd. for $C_{20}H_{23}N_3O_4$ (369.41): C, 65.03; H, 6.28; N, 11.37%. Found C, 64.48; H, 6.09; N, 11.20%.

*(2S)-2-(Benzoylamino)-3-methyl-N′-(4-nitrobenzylidene)-butanohydrazide (**21**)*

Yield 45%. m.p. 207–208°C (EtOH). $[\alpha]_D$ 243.311 (c: 1.0, DMF). HPLC t_R (min.): 5.62. IR, υ (cm^{-1}): 3254 and 3205 (NH str), 1670 (hydrazone C=O str), 1635 (amide C=O str), 1520 (NO_2 str), 1350 (NO_2 str). ^{1}H NMR: δ (ppm) 0.96–1.23 (m, 6H, CHCH(**CH_3**)$_2$), 2.08–2.29 (m, 1H, CH**CH**$(CH_3)_2$), 4.32 and 5.36–5.40 (t, J = 8.5 Hz, J = 8.5 Hz and m, 1H, **CH**$CH(CH_3)_2$), 7.45–7.57 (m, 3H, ArH), 7.88–7.98 (m, 4H, ArH), 8.12 and 8.61 (s and d, J = 8.1 Hz, 1H, ArH), 8.28 and 8.31 (dd, J = 5.7 Hz, J = 5.7 Hz, 1H, amide NH), 8.37 (d, J = 8.7 Hz, 1H, –CH=N–), 11.78 and 11.98 (s and s, 1H, hydrazone NH). Anal. calcd. for $C_{19}H_{20}N_4O_4$ (368.38): C, 61.95; H, 5.47; N, 15.21%. Found C, 62.02; H, 4.89; N, 15.16%.

*(2S)-2-(Benzoylamino)-3-methyl-N′-(4-dimethylaminobenzylidene)butanohydrazide (**22**)*

Yield 46%. m.p. 289–292°C (EtOH/acetone (90:10 v/v)). $[\alpha]_D$ 155.281 (c: 1.0, DMF). HPLC t_R (min.): 7.14. IR, υ (cm^{-1}): 3253 and 3211 (NH str), 1666 (hydrazone C=O str), 1633 (amide C=O str). ^{1}H NMR: δ (ppm) 0.94–1.00 (m, 6H, CHCH(**CH_3**)$_2$), 2.16–2.29 (m, 1H, CH**CH**$(CH_3)_2$), 2.94 and 2.97 (s and s, 6H, –N(**CH_3**)$_2$), 4.37 and 5.35–5.39 (t, J = 8.6 Hz, J = 8.6 Hz and m, 1H, **CH**$CH(CH_3)_2$), 6.72–6.80 (m, 2H, ArH), 7.45–7.56 (m, 5H, ArH), 7.88–7.89 (*m*, 2H, ArH), 7.92 and 8.09 (s and s, 1H, amide NH), 8.16 and 8.49 (d, J = 8.7 Hz and d, J = 8.4 Hz 1H, –CH=N–), 11.20 and 11.36 (s and s, 1H, hydrazone NH). Anal. calcd. for $C_{21}H_{26}N_4O_2$ (366.45): C, 68.83; H, 7.15; N, 15.29%. Found C, 68.30; H, 7.99; N, 14.89%.

*(2S)-2-(Benzoylamino)-3-methyl-N′-[3-[4-(dimethylamino)phenyl]prop-2-en-1-ylidene]butano-hydrazide (**23**)*

Yield 67%. m.p. 289°C (EtOH/acetone (90:10 v/v)). $[\alpha]_D$ 159.811 (c: 1.0, DMF). HPLC t_R (min.): 13.44. IR, υ (cm^{-1}): 3250 and 3207 (NH str), 1665 (hydrazone C=O str), 1633 (amide C=O str). ^{1}H NMR: δ (ppm) 0.93–0.98 (m, 6H, CHCH(**CH_3**)$_2$), 2.16–2.38 (m, 1H, CH**CH**$(CH_3)_2$), 2.95 (s, 6H, –N(**CH_3**)$_2$), 2.97–2.99 (m, 1H, Ar–CH=**CH**–CH=N–), 3.31 (s, 1H, Ar–**CH**=CH–CH=N–), 4.28 and 5.33–5.37 (t, J = 8.6 Hz, J = 8.6 Hz and m, 1H, **CH**$CH(CH_3)_2$), 6.68–6.77 (m, 3H, ArH), 6.84, 6.88, 6.92 (s, s, s, 1H, ArH), 7.41–7.49 (m, 3H, ArH), 7.52–7.56 (m, 1H, ArH), 7.77 and 7.94 (d, J = 9.2 Hz and d, J = 9.3 Hz, 1H, ArH), 7.88–7.91 (m, 1H, amide NH), 8.12 and 8.48 (d, J = 8.8 Hz and d, J = 8.5 Hz 1H, –CH=N–), 11.20 and 11.39 (s and s, 1H, hydrazone NH). Anal. calcd. for $C_{23}H_{28}N_4O_2$ (392.49): C, 70.38; H, 7.19; N, 14.27%. Found C, 69.87; H, 7.11; N, 14.16%.

*(2S)-2-(Benzoylamino)-3-methyl-N′-(5-nitrofurfurylidene)butanohydrazide (**24**)*

Yield 94%. m.p. 188°C (EtOH/DMF (90:10 v/v)). $[\alpha]_D$ 188.737 (c: 1.0, DMF). HPLC t_R (min.): 3.86. IR, υ (cm^{-1}): 3254 and 3205 (NH str), 1672 (hydrazone C=O str), 1635 (amide C=O str), 1540 (NO_2 str), 1365 (NO_2 str). ^{1}H NMR: δ (ppm) 0.94–1.02 (m, 6H, CHCH(**CH_3**)), 2.18–2.27 (m, 1H, CH**CH**$(CH_3)_2$), 4.29 and 5.25–5.29 (t, J = 8.4 Hz, J = 8.4 Hz and m, 1H, **CH**$CH(CH_3)_2$), 7.24 (t, J = 3.9 Hz, J = 3.9 Hz, 1H, ArH), 7.46–7.57 (m, 3H, ArH), 7.79 (t, J = 4.1 Hz, J = 4.1 Hz, 1H, ArH), 7.96 and 8.23 (s and s, 1H, amide NH), 8.32 and 8.64 (d, J = 8.3 Hz and d, J = 8.1 Hz, 1H, –CH=N–), 11.88 and 12.11 (s and s, 1H, hydrazone NH). Anal. calcd. for $C_{17}H_{18}N_4O_5$ (358.35): C, 56.98; H, 5.06; N, 15.63%. Found C, 56.74; H, 4.88; N, 15.43%.

In vitro antiviral assays

Inhibition of HIV-induced cytopathicity in MT-4 cells

Evaluation of the antiviral activity of the compounds against HIV-1 strain III_B and HIV-2 strain ROD in MT-4 cells was performed using the MTT assay as previously described [41, 48]. Stock solutions (10× final concentration) of test compounds were added in 25 μL volumes to two series of triplicate wells so as to allow simultaneous evaluation of their effects on mock- and HIV-infected cells at the beginning of each experiment. Serial fivefold dilutions of test compounds were made directly in flat-bottomed 96-well microtiter trays using a Biomek 3000 robot (Beckman Instruments, Fullerton, CA). Untreated control HIV- and mock-infected cell samples were included for each sample.

HIV-1(III_B) [49] or HIV-2 (ROD) [50] stock (50 μL) at 100–300 $CCID_{50}$ (cell culture infectious dose) or culture medium was added to either the infected or mock-infected wells of the microtiter tray. Mock-infected cells were used to evaluate the effect of test compound on uninfected cells in order to assess the cytotoxicity of the test compound. Exponentially growing MT-4 cells [51] were centrifuged for 5 min at 220 g and the supernatant was discarded. The MT-4 cells were resuspended at 6×10^5 cells/mL, and 50-μL volumes were transferred to the microtiter tray wells. Five days after infection, the viability of mock- and HIV-infected cells was examined spectrophotometrically by the MTT assay.

The MTT assay is based on the reduction of yellow colored 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Acros Organics, Geel, Belgium) by mitochondrial dehydrogenase of metabolically active cells to a blue-purple formazan that can be measured spectrophotometrically. The absorbances were read in an eight-channel computer-controlled photometer (Safire2, Tecan, Mechelen, Belgium), at two wavelenghths (540 and 690 nm). All data were calculated using the median OD (optical density) value of three wells. The 50% cytotoxic concentration (CC_{50}) was defined as the concentration of the test compound that reduced the absorbance (OD_{540}) of the mock-infected control sample by 50%. The concentration achieving 50% protection from the cytopathic effect of the virus in infected cells was defined as the 50% effective concentration (EC_{50}).

Time-of-addition experiments

Time-of-addition experiments were adapted from previously reported methods [52, 53]. Briefly, MT-4 cells were infected with HIV-1(III_B) at an m.o.i. of 0.5. Following a 1 h adsorption period cells were distributed in a 96-well tray at 45 000 cells/well and incubated at 37°C. Test compounds were added at different times (0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 24, and 25 h) after infection. HIV-1 production was determined at 31 h post infection via a p24 enzyme-linked immunosorbentassay (Perkin Elmer, Brussels, Belgium). Dextran sulfate was used at 12.5 μM, AZT at 1.9 μM, nevirapine at 7.5 μM, compound $\mathbf{11}_{\boldsymbol{S}}$ at 95 μM, and compound $\mathbf{12}_{\boldsymbol{S}}$ at 125 μM.

Reverse transcriptase assay

Recombinant wild type p66/p51 HIV-1 RT was expressed and purified as described previously [54]. The RT assay is performed with the EnzCheck Reverse Transcriptase Assay kit (Molecular Probes, Invitrogen), as described by the manufacturer. The assay is based on the dsDNA quantitation reagent PicoGreen. This reagent shows a pronounced increase in fluorescence signal upon binding to dsDNA or RNA–DNA heteroduplexes. Single-stranded nucleic acids generate only minor fluorescence signal enhancement when a sufficiently high dye/base pair ratio is applied [55]. This condition is met in the assay.

A poly(rA) template of approximately 350 bases long, and an oligo(dT) 16 primer, are annealed in a molar ratio of 1:1.2 (60 min at room temperature). Fifty-two nanograms of the RNA/DNA is brought into each well of a 96-well plate in a volume of 20 μL polymerization buffer (60 mM Tris–HCl, 60 mM KCl, 8 mM $MgCl_2$, 13 mM DTT, 100 μM dTTP, pH 8.1). Five microliters of RT enzyme solution, diluted to a suitable concentration in enzyme dilution buffer (50 mM Tris–HCl, 20% glycerol, 2 mM DTT, pH 7.6), is added. The reactions are incubated at 25°C for 40 min and then stopped by the addition of EDTA (15 mM fc). Heteroduplexes are then detected by addition of PicoGreen. Signals are read using an excitation wavelength of 490 nm and emission detection at 523 nm using a spectrofluorometer (Safire2, Tecan). To test the activity of compounds against RT, 1 μL of compound in DMSO is added to each well before the addition of RT enzyme solution. Control wells without compound contain the same amount of DMSO. Results are expressed as relative fluorescence, i.e., the fluorescence signal of the reaction mix with compound divided by the signal of the same reaction mix without compound.

NS5B inhibition assay

The biological activity of the compounds against NS5B polymerase were evaluated in a reaction buffer containing 20 mM Tris–HCl (pH 7.0), 100 mM NaCl, 100 mM sodium glutamate, 0.1 mM DTT, 0.01% BSA, 0.01% Tween-20, 5% glycerol, 20 U/mL of RNase Out, 0.25 μM of polyrA/U_{12}, 25 μM UTP, 2 μCi [alpha-^{32}P]UTP, 300 ng of NS5BCΔ21and 1.0 mM $MnCl_2$ with or without inhibitors (100 μM) in a total volume of 25 μL for 1 h at 30°C as previously described [56, 57].

Reactions were terminated by the addition of ice-cold 5% v/v trichloroacetic acid (TCA) containing 0.5 mM pyrophosphate. Reaction products were precipitated on GF-B filters and quantified on a liquid scintillation counter. NS5B activity in the presence of DMSO control was set at 100% and that in the presence of the compounds was determined relative to this control.

Molecular modeling

Ligand structure preparation

Representative chiral hydrazone derivatives were built using the fragment dictionary of Maestro 9.0 and energy minimized by Macromodel program v9.7 (Schrödinger, Inc., New York, NY, 2009) using the OPLSAA force field with the steepest descent followed by truncated Newton conjugate gradient protocol. The low-energy 3D structures of the hydrazone derivatives were generated with the following parameters present in LigPrep v2.3: different protonation states at physiological pH, all possible tautomers, ring conformations and stereoisomers. The output obtained from the LigPrep run was further used for generating 100 ligand conformations using default parameters of the conformational search panel of the Macromodel submenu which uses a combination of mixed torsional/low-mode sampling function. Minimized structures were filtered with a maximum relative energy difference of 5 kcal/mol to exclude redundant conformers. The output conformational search file (CSearch file) containing at most 100 unique conformers of the hydrazone derivatives was used as input for docking simulations.

Protein structure preparation

The X-ray co-crystal structure of HIV-1 RT-dihydroxy benzoyl naphthyl hydrazone (DHBNH) (PDB ID: 2I5J) [42] obtained from the RCSB Protein Data Bank was used for docking into the novel non-nucleoside inhibitor binding site located between the NNRTIBP and the active site of HIV-1 RT. The initial structure thus obtained was refined by means of default parameters mentioned in the protein preparation tool implemented in Maestro v9.0 and Impact program v5.5 (Schrödinger, Inc., New York, NY, 2009), in which the protonation states of residues were adjusted to the dominant ionic forms at pH 7.4. Refined HIV-1 RT model was further used to generate the grid by selecting the bound inhibitor (DHBNH).

Docking protocol

The conformational library of the chiral hydrazone derivatives was docked at the hydrazone binding novel site of HIV-1 RT using the ''Extra Precision'' (XP) Glide docking program v5.0 (Schrödinger, Inc., New York, NY, 2009) and the default parameters. The top scoring pose of each hydrazone derivative within the novel site was then subjected to energy minimization using Macromodel program v9.7 and used for graphical analysis. All computations were carried out on a Dell Precision 470n dual processor with the Linux OS (Red Hat Enterprise WS 4.0).

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