Mahmoud M. Sheha,
Nawal A. El-Koussi,
Hassan H. Farag

Medicinal Pharmaceutical Chemistry Department,
Faculty of Pharmacy,
Assiut University,
Assiut, Egypt

Brain Delivery of HIV Protease Inhibitors

To overcome the problems of peptidomimetic drug delivery to the specific organs, the use of dihydropyridine ↔ pyridinium chemical delivery systems to deliver peptides to the brain is considered in this work. An HIV protease inhibitor lead compound; KNI 279 was selected for the study. The *N*-alkylated dihydroisoquinoline derivatives of KNI-279 were synthesized and tested for their ability to be oxidized by brain homogenate and showed good results with reasonable half-life times specially for the *N*-alkoxycarbonyl-methyl derivative **8**. The *in-vivo* distribution of compound **8** proved the brain delivery and locked in property of HIV PR inhibitors in the brain. All the prepared compounds (both quaternary and dihydro derivatives) showed between 51 and 86 % HIV PR inhibitory activity compared to the parent compound.

Keywords: brain-specific delivery; HIV PR inhibitors; KNI; Dihydroisoquinoline

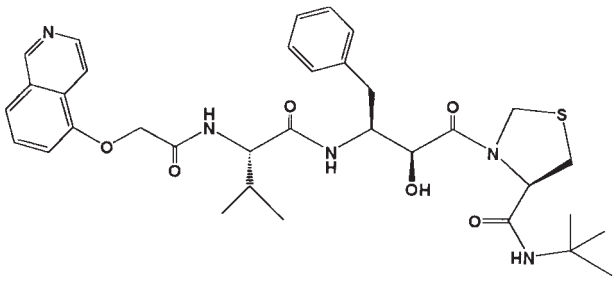
Received: June 5, 2002 [FP706]

Introduction

Human immunodeficiency virus (HIV), a member of the greater family of retroviruses, is the causative agent of the debilitating and fatal acquired immune deficiency syndrome (AIDS). During viral replication, the viral gag and gag/pol polyproteins undergo enzymatic cleavage by a viral encoded protease (PR) to generate the functional proteins of the mature virus. Inhibition of HIV PR results in the loss of proteolysis activity as well as production of non-infectious virons. Due to this critical role in viral maturation, HIV PR has become a prevalent target in AIDS therapy and the design of new PR inhibitors has escalated. As a consequence, a large number of specific HIV PR inhibitors with various chemical structures have been reported [1]. The most effective inhibitors are peptidomimetic compounds containing transition-state inserts in place of the amino acids occupying the P1P1′ position of the substrate peptide. However, the use of peptides as drugs is limited due to the poor brain bioavailability of the peptide derived drugs [2, 3].

The dihydropyridine ↔ pyridinium chemical delivery system (CDS) has been used successfully for brain-specific delivery of many drugs since the first publication by Bodor's group [4]. The drug carried on the dihydropyridine chemical delivery system (D-DHP) was shown to cross the blood-brain barrier (BBB) easily. Some of these dihydropyridines are oxidized in plasma but the major part crosses the BBB and is oxidized by oxidizing enzymes in the brain to the corresponding quaternary form (pyridinium) which is then retained (locked) in the brain and can release the drug. The main disadvantage of DHP-CDS, which retarded its development by the drug industry, is its instability during formulation and storage due to the hydration of the 5–6 double bond [5]. Certain 1,2-dihydroisoquinoline-CDS have been studied to overcome this problem and proven to be efficient in brain-specific delivery and shelf stable [6, 7]. Isoquinoline is present as a structural element of many HIV PR inhibitors [8]. Kynostatin compounds (KNI), potent HIV PR inhibitors, were selected as a model for a brain delivery study. In this case the active compound will be the dihydroisoquinoline and the quaternary isoquinolinium derivatives and no problem will be associated with the rate of release of the active drug from the carrier (the carrier is part of the active compound). KNI 279 (**1**) contains 5-isoquinolineoxyacetic acid at the P3 position. Derivatives of 1 were synthesized, and evaluated for brain delivery and HIV PR inhibitory activity.

Correspondence: Mahmoud M. Sheha, Medicinal Pharmaceutical Chemistry Department, Faculty of Pharmacy, Assiut University, Assiut 71526, Egypt. Phone: +20 12397 1632, fax: +20 8833 2776, e-mail: Sheha@acc.aun.edu.eg.



KNI-279 (1)

Investigations, results, and discussion

Synthesis of the dihydroisoquinoline derivative of KNI-279

KNI-279 (**1**) was prepared by a solution method for peptide synthesis using Boc-derivatives of thioproline (Thz), allophenylnorstatine (Apns), and valine (Val) and the ter-

Full Paper

 0365-6233/01/0047 $ 17.50+.50/0

minal isoquinolineoxyacetic acid (iQoa)[9]. Quaternization of the isoquinoline residue of **1** was performed by treating **1** with alkyl halide (methyl iodide, ethyl bromide, ethyl bromoacetate, or bromoacetic acid) at ambient temperature, and afforded the derivatives **2–5** as shown in Scheme1. Compound **5** was found as bromide salt, not as zwitterion (positive test for bromide ion after washing the crystalline product with mixture of anhydrous diethyl ether and methanol). Reduction of the quaternary isoquinolinium salts **2–4** with sodium dithionite in sodium bicarbonate under a stream of nitrogen afforded the dihydroisoquinoline derivatives **6–8**.

In vitro studies

Chemical oxidation

Silver nitrate and/or potassium ferricyanide were used as oxidizing agent for studying the kinetics of oxidation of dihydropyridine derivatives [10]. The oxidation of the prepared dihydro compounds **6–8** with silver nitrate solution was studied under pseudo first-order conditions. The rate constants of oxidation were determined by monitoring the disappearance of the dihydroisoquinoline derivatives, using HPLC, in alcoholic silver nitrate solution. The results (Table 1) reflect the susceptibility of the dihydroisoquinoline derivatives for oxidation to the corresponding quaternary isoquinolinium derivatives, which is essential for the brain-specific delivery process. Compound **8** is shown to undergo the fastest in oxidation ($t_{1/2}$ = 3.8 h), whereas compound **7** ungoes the slowest ($t_{1/2}$ = 14.5 h).

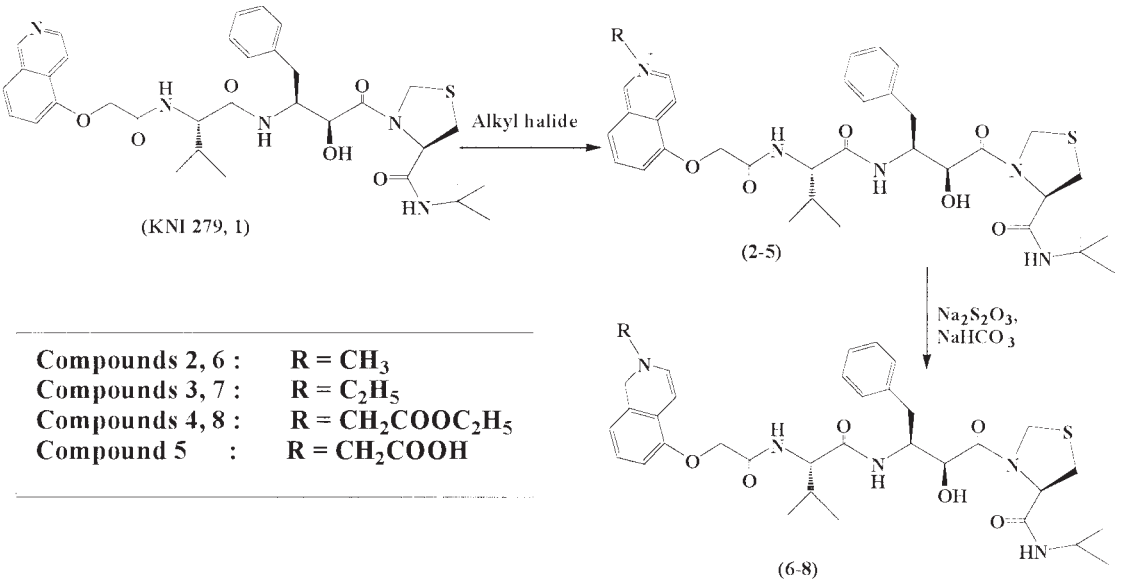
Table 1. Kinetics of oxidation of dihydroisoquinoline derivatives of KNI-279 (6–8) with silver nitrate.

Compound	$k_{disapp.}$ [h^{-1}]	$t_{1/2}$ [h]	*R*
6	6.31×10^{-2}	11	0.986
7	4.75×10^{-2}	14.5	0.991
8	18.21×10^{-2}	3.8	0.990

k_{disapp} is the apparent pseudo first-order rate constant of disappearance of dihydroisoquinoline derivatives, $t_{1/2}$ is the half-time of the reaction, *R* is and regression coefficient.

Stability and oxidation in biological fluids

The rates of oxidation of the prepared dihydroisoquinoline derivatives **6–8** were investigated in 20 % rat brain homogenate (Table 2) and in fresh human plasma (Table 3). The rates of oxidation were found to follow pseudo first-order kinetics with correlation coefficient higher than 0.98. Compound **8** proved to undergo the fastest oxidation in 20 % brain homogenate ($t_{1/2}$ = 1.5 h) which is even faster than the rate of chemical oxidation by silver nitrate. The corresponding acid **5** was found to be oxidized in rat brain even faster than the ester **8**, to an extent that could not be monitored. Compound **7** was the slowest to be oxidized in brain homogenate yet also at a faster rate than by chemical oxidation. These fast rates of enzymatic oxidation in brain homogenate satisfy the requirements for efficient brain-specific delivery.



Scheme 1. Synthesis of the dihydroisoquinoline derivatives of KNI-279.

Table 2. Kinetics of stability of dihydroisoquinoline derivatives of KNI-279 (**6–8**) in rat brain homogenate.

Compound	$k_{disapp.}$ [h^{-1}]	$t_{1/2}$ [h]	*R*
6	14.46×10^{-2}	4.8	0.99
7	9.03×10^{-2}	7.7	0.98
8	45.92×10^{-2}	1.5	0.98

k_{disapp} is the apparent pseudo first-order rate constant of disappearance of dihydroisoquinoline derivatives, $t_{1/2}$ is the half-time of the reaction, *R* is and regression coefficient.

Table 3. Kinetics of stability of dihydroisoquinoline derivatives of KNI-279 (**6–8**) in fresh human plasma.

Compound	$k_{disapp.}$ [h^{-1}]	$t_{1/2}$ [h]	*R*
6	4.74×10^{-2}	14.6	0.99
7	3.71×10^{-2}	18.7	0.99
8	15.82×10^{-2}	4.4	0.98

k_{disapp} is the apparent pseudo first-order rate constant of disappearance of dihydroisoquinoline derivatives, $t_{1/2}$ is the half-time of the reaction, *R* is and regression coefficient.

In human plasma, the dihydroisoquinoline derivatives **6–8** proved to be more stable than in the brain homogenate used in our exiperiment. They follow the same trend as observed in the chemical oxidation and in the brain homogenate, with compound **8** being the least stable and compound **7** the more stable derivative.

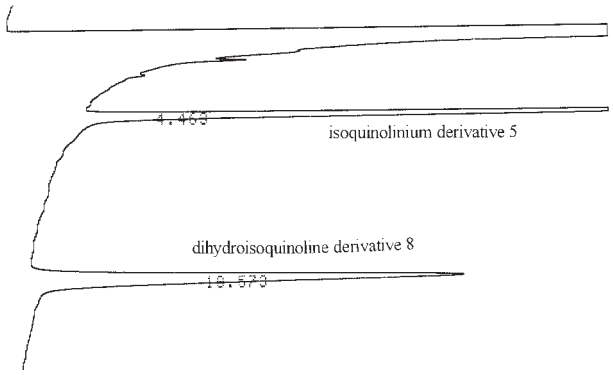


Figure 1. A representative HPLC chromatogram showing the presence of both the quaternary isoquinolinium derivative 5 (*Rt* = 4.46 min) and the dihydroisoquinoline derivative 8 (*Rt* = 10.57 min) in blood after 20 minutes from injection of compound **8** into rats.

Table 4. *In vivo* distribution (blood, and brain) of compound **8** in rats (*n* = 3).

Time Intervals (minutes)	Blood*	Brain*
10	25.5 ± 6.2	NA
20	18.17 ± 2.4	2.23 ± 1.5
60	12.69 ± 3.3	6.05 ± 2.7
120	6.6 ± 1.2	10.6 ± 0.9
240	NQ	9.8 ± 1.8
300	ND	7.62 ± 2.1

NA, not available NQ; detected but cannot be quantified; ND; not detected.
* Total concentrations (± standard deviation) of compound **8** and its quaternary metabolite (compound **5**, without bromide) calculated in µg/g of rat brain or blood.

In vivo brain bioavailability

To validate the brain-specific delivery of the prepared dihydroisoquinoline derivatives, a brain delivery study for the most *in-vitro* promising compound **8** was performed on rats. A freshly prepared solution of compound **8** was given by intra-jugular injection in a dose of 20 mg/kg body weight of female Sprague Dawley rats. At specific time intervals the animals were killed, and blood samples and brains were collected. The samples were analyzed by HPLC to determine the total quantity of the dihydroisoquinoline derivative **8**, and its metabolites quaternary derivatives **4** and **5**. The results of these experiments are given in Table 4. Analysis of blood samples taken after 10 minutes proved the presence of compound **8** in high concentration in addition to its hydrolysis and oxidation products; compound 5 (**Figure 1**). At 1 and 2 hours only the quaternary acid **5** could be detected. Neither quaternary acid nor other oxidation or hydrolysis products could be quantified after **4** and **5** hours.

The picture looked different in the brain samples. The products detected after 20 minutes and 1hour are mainly the quaternary acid **5** and some of dihydroisoquinoline ester **8**. After 2 hours only the quaternary acid could be found. The concentration of HIV PR inhibitor was found to increase with time (for about 4 hours) and to be maintained for more than 5 hours.

HIV PR inhibitory activity

The prepared compounds **2–8** were evaluated for their HIV PR inhibitory activity against a synthetic enzyme using a reported technique [11]. The activities were com-

pared to that of the parent compound **1**. Both the dihydroisoquinoline derivatives **6–8** and the quaternary isoquinolinium derivatives **2–5** were found to be active as HIV PR inhibitor at a concentration on the 50 ng scale. The activities were found to be reduced (about 51–86 %) compared to that of the parent compound **1**, Table 5. The more lipophilic compounds **6–8** were more active as HIV PR inhibitors than the corresponding hydrophilic quaternary compounds **2–5**.

Acknowledgment

The authors acknowledge professor Yoshiaki Kiso and collaborators at Kyoto Pharmaceutical University, Japan, for conducting HIV PR inhibitory activity studies.

Experimental

Materials

Melting points were recorded (uncorrected) on a melting point /MS (Stuart Scientific, UK). Infrared spectra were recorded on a Shimadzu IR-470 Spectrometer (Shimadzu Corporation, Tokyo, Japan) and UV spectra on Shimadzu UV-120-02 Spectrophotometer (Shimadzu Corporation. Kyoto, Japan). Thin layer chromatography (TLC) was performed on precoated silica gel plates (Merck, 0.25 MM, 60 F254). Column chromatography was carried out on Merck silica gel 60 (Particle size 0.063–0.200 mm). ^{1}H-NMR spectra were recorded on a JEOL JNM-EX 270 MHz Spectrometer (Jeol, Tokyo, Japan); all chemical shifts are given in δ ppm, relative to tetramethylsilane (TMS) as internal standard. High resolution FAB MS analyses were recorded on JEOL JMS-SX 102 AQQ Hybrid Mass Spectrometer using thioglycerol as internal reference. All the obtained new compounds gave satisfactory high resolution mass spectra (error less than 3 ppm) and were fully characterized spectroscopically.

The HPLC system consisted of a Knauer model 64 solvent delivery module (Knauer, Germany), a Knauer variable wavelength UV detector a 20 µL sample loop, and a Shimadzu CR-6A chromatopac integrator (Shimadzu, Tokyo, Japan). The column used was a Knauer C18 (250 mm × 4.6 mm ID, 5 µm). The effluent monitored at 284 nm at flow rate of 1 mL/min. The mobile phase used consisted of 40 volumes of acetonitrile and 60 volumes of 0.1 % trifluoroacetic acid. Dimethylformamide (DMF) was purchased from El-Naser Chemical Industry Company (Cairo, Egypt), dried over phosphorus pentoxide and distilled under reduced pressure before use. All other chemicals and solvents were of analytical grade. The starting compound, KNI-279, was prepared in a pure crystalline form according to the reported method [9].

Chemistry

Quaternization of KNI-279 with alkyl halides, general method

To a solution of KNI-279 (3.25 g, 5 mmol) in methanol (50 mL), alkyl halide (as indicated, 6 mmol) solution in methanol (20 mL) was added dropwise with stirring. The reaction mixture was stirred at about 50 °C for 2–5 hours, and concentrated under vacuum to one third of its volume. After cooling, the crystalline product was filtered and washed with anhydrous diethyl ether (3 × 50 mL) and dried in a desiccator over phosphorus pentoxide under vacuum. By the use of this procedure, the following compounds were prepared.

3-{3(S)-[N-(N-Methyl-5-isoquinolinyloxyacetyl)-l-valinyl]-amino-2(S)-hydroxy-4-phenylbutanoyl}-N-(tert-butyl)thiazolidine-4(R)-carboxamide iodide (**2**)

Methyl iodide (0.85 g), stirred for 2 hours to give **2** (3.5 g, 88.5 %): mp 124–126 °C, ^{1}H NMR (DMSO-d_6) δ 0.76 (d, 6 H), 1.26 (s, 9 H), 1.98 (m, 1 H), 2.67 (d, 2 H), 3.1 (m, 2 H), 4.28 (m, 2 H), 4.4 (s, 3 H), 4.66 (m, 2 H), 4.78 (m, 2 H), 4.92 (m, 3 H), 7.35–7.08 (m, 6 H), 7.53 (m, 1 H), 7.73 (s, 1 H), 7.93 (m, 1 H), 8.1 (m, 1 H), 8.25 (d, 1 H), 8.6 (d, 1 H), 8.69 (m, 1 H), 9.97 (s, 1 H); MS (HR FAB$^+$) *m/z* 664.3181 (+1.9 ppm) observed for $C_{35}H_{46}N_5O_6S$.

3-{3(S)-[N-(N-Ethyl-5-isoquinolinyloxyacetyl)-l-valinyl]amino-2(S)-hydroxy-4-phenylbutanoyl}-N-(tert-butyl)thiazolidine-4(R)-carboxamide iodide (**3**)

Ethyl bromide (1.0 g), stirred for 3 hours to give **3** (3.2 g, 80 %): mp 109–112 °C, ^{1}H NMR (DMSO-d_6) δ 0.78 (d, 6 H), 1.26 (s, 9 H), 1.78 (t, 3 H), 2.0 (m, 1 H), 2.67 (d, 2 H), 3.1 (m, 2 H), 4.29 (m, 2 H), 4.2 (q, 2 H), 4.58 (m, 2 H), 4.72 (m, 2 H), 4.92 (m, 3 H), 7.35–7.08 (m, 6 H), 7.5 (m, 1 H), 7.71 (s, 1 H), 7.81 (m, 1 H), 8.05 (m, 1 H), 8.25 (d, 1 H), 8.61 (d, 1 H), 8.66 (m, 1 H), 9.95 (s, 1 H); MS (HR FAB$^+$) *m/z* 678.3274 (+1.5 ppm) observed for $C_{36}H_{48}N_5O_6S$.

3-{3(S)-[N-(N-Ethoxycarbonylmethyl-5-isoquinolinyl-oxyacetyl)-l-valinyl]-amino-2(S)-hydroxy-4-phenylbutanoyl}-N-(tert-butyl)thiazolidine-4(R)-carboxamide bromide (**4**)

Ethyl bromoacetate (1.0 g), stirred for 5 hours at room temperature to give **4** (3.4 g, 85 %): mp 89–91 °C, ^{1}H NMR (DMSO-d_6) δ 0.76 (d, 6 H), 1.26 (s, 9 H), 1.32 (t, 3 H), 1.98 (m, 1 H), 2.67 (d, 2 H), 3.1 (m, 2 H), 4.25 (m, 4 H), 4.46 (m, 1 H), 4.67 (m, 2 H), 4.79 (m, 2 H), 4.92 (m, 4 H), 7.19–7.09 (m, 3 H), 7.37–7.31 (m, 2 H), 7.63 (m, 1 H), 7.70 (s, 1 H), 8.05 (m, 2 H), 8.31 (m, 2 H), 8.70 (d, 1 H), 8.82 (d, 1 H), 10.22 (s, 1 H); MS (HR FAB$^+$) *m/z* 736.3401 (+2.9 ppm) observed for $C_{38}H_{50}N_5O_8S$.

3-{3(S)-[N-(N-Carboxymethyl-5-isoquinolinyloxyacetyl)-l-valinyl]amino-2(S)-hydroxy-4-phenylbutanoyl}-N-(tert-butyl)thiazolidine-4(R)-carboxamide bromide (**5**)

Bromoacetic acid (0.85 g), stirred for 5 hours at room temperature to give **5** (2.9 g, 74 %): mp 65–68 °C, ^{1}H NMR (DMSO-d_6) δ

Table 5. Percent of HIV PR inhibition by the prepared compounds (**2–8**) and parent compound **1**, determined at concentration of 50 ng using peptidolytic assay.

Compound #	**2**	**3**	**4**	**5**	**6**	**7**	**8**	KNI-279 (**1**)
% HIV PR inhibitory activity	48	50	51	45	76	72	65	88

0.76 (d, 6 H), 1.26 (s, 9 H), 1.98 (m, 1 H), 2.67 (d, 2 H), 3.1 (m, 2 H), 4.25 (m, 2 H), 4.44 (m, 1 H), 4.67 (m, 2 H), 4.79 (m, 2 H), 4.88 (m, 4 H), 7.19–7.09 (m, 3 H), 7.37–7.31 (m, 2 H), 7.63 (m, 1 H), 7.70 (s, 1 H), 8.05 (m, 2 H), 8.31 (m, 2 H), 8.70 (d, 1 H), 8.82 (d, 1 H), 10.22 (s, 1 H), 11.65 (s, 1 H); MS (HR FAB$^+$) *m/z* 708.2175 (+2.6 ppm) observed for $C_{36}H_{46}N_5O_8S$.

Preparation of dihydroisoquinoline derivatives of KNI-279, general method

To a solution of quaternary KNI-279 derivative (2.5 mmol) in 50 mL of degassed water, sodium bicarbonate (0.25 g; 3 mmol) and 50 mL of dichloromethane were added. The mixture was stirred in an ice bath, and sodium dithionite (0.45 g; 2.57 mmol) was added portionwise over a period of 15 minutes, and stirring was continued for 3 hours under a nitrogen steam. The organic layer was then separated, washed with water, dried over anhydrous Na_2SO_4, and distilled under vacuum, to give the corresponding dihydro-derivative as an oily material, which was crystallized by trituration with hexane and preserved under nitrogen protected form light in refrigerator. By the use of this procedure, the following compounds were prepared.

3-{3(S)-[N-(N-Methyl-1,2-dihydroisoquinolin-5-yloxyacetyl)-l-valinyl]-amino-2(S)-hydroxy-4-phenylbutanoyl}-N-(tert-butyl)thiazolidine-4(R)-carboxamide (**6**)

Yield (1.3 g, 78 %): mp 74–76 °C, ^{1}H NMR (DMSO-d_6) δ 0.77 (d, 6 H), 1.26 (s, 9 H), 2.0 (m, 1 H), 2.39 (s, 3 H), 2.64 (d, 2 H), 3.13 (m, 2 H), 3.69 (s, 2 H), 4.28 (m, 1 H), 4.44 (m, 2 H), 4.62 (m, 1 H), 4.78 (m, 2 H), 4.92 (m, 3 H), 5.8 (d, 1 H), 6.20 (d, 2 H), 6.98–6.67 (m, 4 H), 7.23–7.05 (m, 6 H); MS (HR FAB$^+$) *m/z* 665.3079 (+2.7 ppm) observed for $C_{35}H_{47}N_5O_6S$.

3-{3(S)-[N-(N-Ethyl-1,2-dihydroisoquinolin-5-yloxyacetyl)-l-valinyl]-amino-2(S)-hydroxy-4-phenylbutanoyl}-N-(tert-butyl)thiazolidine-4(R)-carboxamide (**7**)

Yield (1.25 g, 73 %): mp 80–82 °C, ^{1}H NMR (DMSO-d_6) δ 0.76 (d, 6 H), 1.25 (s, 9 H), 1.65 (t, 3 H), 1.98 (m, 1 H), 2.25 (m, 2 H), 2.64 (d, 2 H), 3.09 (m, 2 H), 3.71 (s, 2 H), 4.28 (m, 1 H), 4.44 (m, 2 H), 4.62 (m, 1 H), 4.78 (m, 2 H), 4.92 (m, 3 H), 5.8 (d, 1 H), 6.20 (d, 2 H), 6.98–6.67 (m, 4 H), 7.26–7.02 (m, 6 H); MS (HR FAB$^+$) *m/z* 679.3516 (+2.3 ppm) observed for $C_{36}H_{49}N_5O_6S$.

3-{3(S)-[N-(N-Ethoxycarbonylmethyl-1,2-dihydroisoquinolin-5-yloxy-acetyl)-l-valinyl]amino-2(S)-hydroxy-4-phenylbutanoyl}-N-(tert-butyl)-thiazolidine-4(R)-carboxamide (**8**)

Yield (1.5 g, 81 %) of pale yellow oil, ^{1}H NMR (DMSO-d_6) δ 0.76 (d, 6 H), 1.28 (s, 9 H) , 1.31 (t, 3 H), 2.01 (m, 1 H), 2.65 (m, 2 H), 2.931 (m, 2 H), 3.55 (s, 2 H), 3.79 (m, 3 H), 4.14 (m, 2 H), 2.36 (m, 3 H), 4.50 (m, 1 H), 4.67 (m, 1 H), 4.79 (m, 1 H), 4.92 (s, 2 H), 5.78 (d, 1 H), 6.16 (d, 1 H), 6.65 (m, 2 H), 6.89 (d, 1 H), 7.25–7.08 (m, 6 H), 7.45 (m, 2 H); MS (HR FAB$^+$) *m/z* 737.3475 (+2.4 ppm) observed for $C_{38}H_{51}N_5O_8S$.

Chemical oxidation of dihydro-derivatives with silver nitrate solution

In a series of tubes, 1 mL of a 0.2 mmol methanolic solution of the dihydro derivative (**6**, **7**, and 8) was added to 5 mL of 10 % methanolic silver nitrate solution and the mixture was agitated for 3 minutes. At the specific time intervals (5, 15, 30, 60, 120, 240, and 360 minutes) one tube is vortexed, centrifuged, and the supernatant was filtered on a 0.45 µm membrane filtration disc and analyzed by HPLC. The rate of disappearance of the dihydro-compounds and the appearance of the corresponding quaternaries were determined. The apparent pseudo first order rate constant of disappearance of dihydro derivatives (k_{disapp}, min^{-1}) were determined by linear regression of the ln of the mean peak area for 3 experiments against time in minutes.

Stability of dihydro-derivatives in biological fluids

In human plasma

Freshly collected heparinized human blood was centrifuged at 4000 rpm for 20 minutes; the supernatant (plasma) was then collected by a Pasteur pipette. To 5 mL of 80 % freshly collected plasma (diluted with phosphate buffer (0.11 M, pH 7.4), prewarmed in a water bath at 37 ± 1 °C for 5 minute, 300 µL of 0.2 mmol methanolic solution of freshly prepared dihydro compound was added. The mixture was kept at 37 ± 1 °C during the experiment. At time intervals (5, 15, 30, 60, 90, 120, 180 240, 360, and 480 minutes) 200 µL was withdrawn from the tested mixture, added immediately to 2 mL of ice cold methanol, vortexed, and kept frozen until analysis. When all the samples had been collected, they were centrifuged, and the supernatants were analyzed by HPLC for their content of the dihydro-compounds and corresponding quaternary derivatives. The apparent pseudo first-order rate constant of disappearance of dihydro compounds ($k_{disapp.}$, h^{-1}) were determined by linear regression of the ln of the AUC against time in minutes.

In 20 % rat brain homogenate

About four grams of rat brain was taken, washed with ice-cold saline solution, and homogenized in a tissue homogenizer with about 20 mL of aqueous ice cold isotonic phosphate buffer (0.2 M, pH 7.4), while keeping the homogenizer tube in an ice bath. To 5 mL of the freshly prepared brain homogenate, previously equilibrated at 37 ± 1 °C in a water bath for 5 minutes, 300 µL of 0.2 mmol methanolic solution of freshly prepared dihydro compounds was added. The mixture was kept at 37 ± 1 °C during the experiment. Samples of 0.5 mL were withdrawn from the tested mixture at different time intervals (0, 10, 20, 30, 60, 90, 120, 240, and 300 minutes) and immediately added to 2 mL ice cold methanol, vortexed, and placed in the deep freezer (–20 °C). When all the samples have been collected, they were centrifuged, and the supernatants were analyzed by HPLC for their content of the dihydro-compounds and corresponding quaternary derivative. The apparent pseudo first-order rate constants of disappearance of dihydro-derivative ($k_{disapp.}$, h^{-1}) were determined by linear regression of the ln of the AUC against time in minutes.

In-vivo distribution studies

Six groups, each of three Sprague Dawley female rats of average weight of 120–140 g were anesthetized with urethane. The freshly prepared solution of compound **8** at a concentration of 25 mg/mL in dimethyl sulfoxide (DMSO) was injected through the external jugular vein at a dose level of 20 mg/kg of body weight. At appropriate time intervals (10, 20, 60, 120, 240, and 360 minutes), 1 mL of blood was withdrawn from the eye and added immediately to a centrifuge tube containing 4 mL of acetonitrile, which was afterwards weighed to determine the amount of blood added. The animal was then decapitated, and the brain was collected, weighed, and kept frozen together with the blood samples. The whole brain was homogenized in 1 mL of water and mixed with 4 mL of 5 % DMSO in acetonitrile. The mixture was homogenized again and centrifuged at 4000 rpm for 10 minutes.

The blood samples were also centrifuged at 4000 rpm for 10 minutes and the supernatants from both brain and blood samples were analyzed by HPLC. Control tests were performed on six (one for every test time) Sprague Dawley female rats injected with DMSO at a dose of 1 mL/kg animal weight.

HIV-1 PR inhibitory activity [11]

Inhibition of HIV-1 PR was quantified using a peptidolytic assay with a synthetic nonapeptide substrate Ac-Arg-Ala-Ser-Gln-Asn-Tyr-Pro-Val-Val-NH_2. The inhibitors were dissolved in DMSO and assayed at 50 nM concentration.

References

[1] J. J. Eron, *Clin. Infect. Dis.* **2000**, June 30, Suppl 2, S 160–170.

[2] K. A. Witt, T. J. Gillespie, J. D. Huber, R. D. Egleton, T. P. Davis, *Peptides* **2001**, *22*, 2329–2343.

[3] C. Adessi, C. Soto, *Curr. Med. Chem.* **2002**, *9*, 963–978.

[4] N. Bodor, H. H. Farag, M. E. Brewster, *Science* **1981**, *214*, 1370–1372.

[5] N. Bodor, H. H. Farag, M. D. C. Barros, W.-M. Wu, P. Buchwald, *J. Drug Targeting* **2002**, *10*, 63–71.

[6] S. Mahmoud, *Master Thesis,* Assiut University, Egypt, **2002.**

[7] M. Abu-Elnile, *Master Thesis,* Assiut University, Egypt, **1997**.

[8] M. M. Sheha, S. Nakata, H. Enomoto, T. Kimura, T. Mimoto, N. Hattori, N. M. Mahfouz, H. Y. Hassan, A. F. Youssef, K. Akaji, Y. Kiso, in *Peptide Chemistry* (Ed.: M. Ohno), Protein Research Foundation, Osaka, **1995**, pp. 349–352.

[9] M. M. Sheha, N. M. Mahfouz, H. Y. Hassan, A. F. Youssef, T. Mimoto, Y. Kiso, *Eur. J. Med. Chem.* **2000**, *35*, 887–894.

[10] M. E. Brewster, J. J. Kaminski, Z. Gabanyi, K. Czako, A. Simay, N. Bodor, *Tetrahedron* **1989**, *45*, 4395.

[11] T. Mimoto, R. Kato, H. Takaku, S. Nojima, K. Terashima, S. Misawa, T. Fukazawa, T. Ueno, H. Sato, M. Shintani, Y. Kiso, H. Hayashi, *J. Med. Chem.* **1999**, *42*, 1789–1802.