Semicochliodinol A and B: Inhibitors of HIV-1 Protease and EGF-R Protein Tyrosine Kinase Related to Asterriquinones Produced by the Fungus *Chrysosporium merdarium*

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The known bisalkylated 2,5-dihydroxybenzoquinones didemethylasterriquinone D and isocochliodinol as well as the new metabolites semicochliodinol A and B have been isolated as inhibitors of HIV-1 protease from the culture broth of the fungus *Chrysosporium merdarium* P-5656. The structures were elucidated by spectroscopic methods. The NMR spectra of two compounds were completely assigned. The metabolites inhibit HIV-1 protease with an IC_{50} value as low as 0.17 μM and epidermal growth factor receptor protein tyrosine kinase at 15 to 60 μM and are therefore valuable lead compounds for these targets. Molecular modelling of the HIV-1-protease-inhibitor complexes showed hydrogen bonding between the dihydroxybenzoquinone moiety of didemethylasterriquinone D and isocochliodinol to both active-site aspartic acids (Asp25/Asp25') of the protease and the indole parts of the inhibitors filling the P2 and P2' pockets of the protease.

Natural products are a continuous and proven source of new lead compounds to drug discovery programs. Recently we pursued a drug discovery program aimed at the identification of inhibitors of the HIV-1 protease. Inhibitors of this enzyme have been shown to be clinically useful agents for AIDS therapy[1]. In our search for inhibitors of the HIV-1 protease among secondary metabolites of microorganisms, inhibitors from the fungus *Chrysosporium merdarium* P-5656 were identified. The same compounds had been identified in our earlier work as inhibitors of epidermal growth factor receptor protein tyrosine kinase (EGF-R PTK). PTK's are a family of enzymes catalyzing phosphorylation of tyrosine residues of protein substrates. The deregulation of PTK activity in tumor cells is associated with malignant transformation[2]. Inhibitors of EGF-R PTK could have a therapeutic potential as anticancer agents[3]. In the present communication, the taxonomy of the strain P-5656 and the production, isolation, structure elucidation and enzyme-inhibitory properties of didemethylasterriquinone D (**1**), isocochliodinol (**2**) as well as of the new compounds named semicochliodinol A and B (**3** and **4**) are described.

Materials and Methods

Microorganism: Isolation and Preservation

The fungal strain P-5656 was isolated from a soil sample collected in a coconut grove near Tenacatita, Mexico and taxonomically classified as *Chrysosporium merdarium*. The microorganism was grown on LCSB agar (lactose 1.5%, cornsteep liquor 0.5%, peptone 0.5%, NaCl 0.4%, $MgSO_4 \cdot 7H_2O$ 0.05%, KH_2PO_4 0.06%, $FeCl_3 \cdot 6H_2O$ 0.0005%, $CuSO_4 \cdot 5H_2O$ 0.0002%, agar 3.0%). The pH was adjusted to 4.8 with 5 N H_2SO_4 or 5 N NaOH). The fungus was incubated at 28°C for ten days until complete sporulation. Working stocks are stored at 4°C, whereas long-term storage is at −80°C and −196°C, respectively.

Fermentation

A mature slant culture of the strain P-5656 was inoculated into a 500-ml Erlenmeyer flask with 1 baffle containing 100 ml of a medium with following composition: glucose 2.0%, Pharmamedia (Traders Protein, Southern Cotton Oil Company, Memphis, U.S.A.) 1.5%, $(NH_4)_2SO_4$ 0.3%, $ZnSO_4 \cdot 7H_2O$ 0.003%, $CaCO_3$ 0.4%. Before sterilization, the pH was adjusted to 7.0. The culture was shaken under aerobic conditions for 48 hours at 28°C and 250 rpm. 5 ml of the seed culture were transferred into 500 ml of the above medium in a 2000-ml Erlenmeyer flask with four baffles. The vegetative culture was incubated under the same conditions as already described. 5.0% of the second seed culture was transferred into a 3.5-liter fermenter (MBR Bioreactor AG, Wetzikon, Switzerland) filled with 2 liters of the following production medium: glucose 2.0%, potato starch 1.0%, soybean meal (defatted) 2.5%, yeast extract (Difco) 0.4%, meat extract (Difco) 0.1%, NaCl 0.25%. Prior to

sterilization, the pH was adjusted to 7.0. The fermentation was run for 128 hours at a temperature of 28°C, an aeration rate of 2 liters/minute and an agitation rate of 700 rpm. A typical fermentation profile of the strain *Chrysosporium merdarium* P-5656 is shown in Fig. 1.

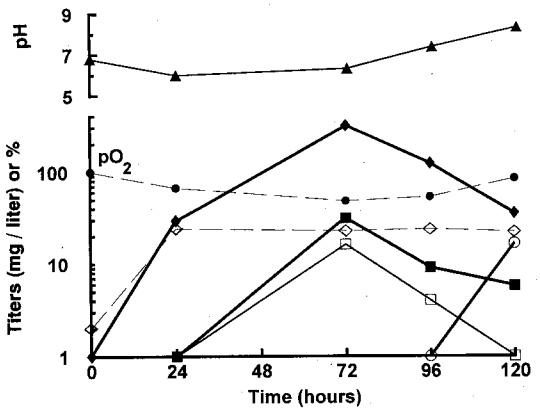
Isolation (Scheme 1)

General remarks: Melting points are uncorrected. Large scale liquid and preparative reversed phase chromatography was performed using a gradient mixer, a Besta-HD-200 pump, a Knaur UV detector (0.5 mm path) and a Büchi B-684 fraction collector. For semipreparative HPLC a Spectra Physics SP8800 delivery system with Shimadzu SPD-6AV UV/VIS detector with a preparative cell (path 0.5 mm) and integrator Merck-Hitachi D-2500 and a Nucleosil 100-5 C18 column (100 Å, 5 μm, 16 × 250 mm) attached was used.

The compounds were mainly bound to the mycelium. The whole broth (2 liters) was extracted three times with 2 liters of EtOAc to give 1.3 g of a black oil. Lipophilic impurities were removed by distribution between equal volumes of hexane and MeOH to give 480 mg of a solid which was insoluble in both solvents and not investigated further and 660 mg of a black solid in the MeOH phase after solvent removal. This material contained 2.2% of **1**, 21% of **2**, 2.0% of **3** and 1.2% of **4** according to HPLC analysis. Similar material obtained from a 200 liters fermentation was worked up to give the pure compounds.

Fig. 1. Production time course of the metabolites **1**, **2**, **3** and **4** in a 3.5-liter fermenter.

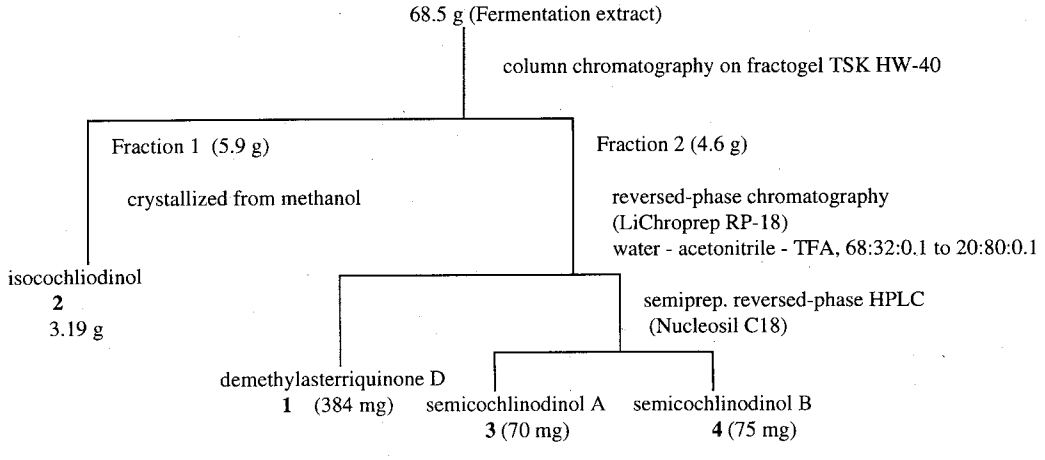
■ **1**, ◆ **2**, ○ **3**, □ **4**, ▲ pH, ◇ PMV, ● pO_2.



Half of that material (68.5 g) was dissolved in 50 ml MeOH and applied on a fractogel TSK HW-40 (F) column (column size 1470 ml, flow 7 ml/minute) developed with MeOH yielding fraction 1 (3.2 liters to 4.9 liters, 5.90 g) and fraction 2 (4.9 liters to 6.5 liters, 4.63 g). Fraction 1 was recrystallized in 50 ml MeOH to give isocochliodinol **2** (3.19 g) as black crystals.

Fraction 2 was dissolved in 30 ml MeOH - acetone, (9 : 1) upon heating, added to LiChroprep RP-18 (10 g) and adsorbed by addition of H_2O (30 ml). A portion of this material was filled in a precolumn attached to a conically shaped Büchi column and was chromatographed in four runs on reversed phase (300 ml; LiChroprep RP-18, 15 ~ 25 μm, solvent A: water - TFA, (100 : 0.1); solvent B: acetonitrile - water - TFA, (80 : 20 : 0.07); gradient from 40% B to 100% B in 50 minutes; 30 ml/minute; 300 nm). Only the fraction containing **1** (22.7 ~ 28 minutes) or a mixture of **3** and **4** (35 ~ 42 minutes) were worked up by lyophilisation to give 384 mg of pure **1** and 468 mg of a mixture of **3** and **4**, both as brown solids. Pure **3** and **4** were obtained by semipreparative HPLC (solvent A: water - TFA, (100 : 0.1); solvent B: acetonitrile - water - TFA, (80 : 20 : 0.07); isocratic elution at 73% B; 8 ml/minute; 270 nm; 29 runs) to give **3** (70.1 mg; Rt 15.3 minutes) and **4** (75.5 mg; Rt

Scheme 1. Purification of **1** ~ **4** from *Chrysosporium merdarium*.



16.3 minutes) both as black solids after solvent removal.

HPLC Analysis (Fig. 2)

The following equipment was used: low pressure gradient delivery system SP8800 (Spectra-Physics), Rheodyne 7125 injection valve, UV-VIS Focus fast-scan detector (Spectra-Physics) with Hitachi Integrator D2500.

Assays for Kinase Inhibition and HIV-1 Protease Inhibition

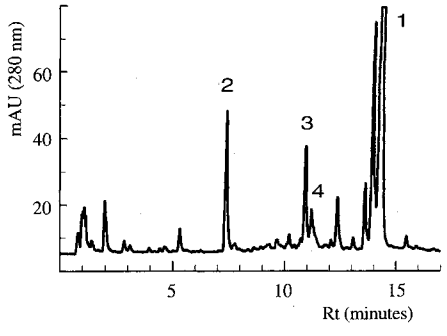
The compounds were tested for inhibition of the HIV-1 protease[4], human cathepsin D[4] and EGF-R PTK[5,6] as described previously.

Molecular Modelling into HIV-1 Protease

Molecular modelling of the HIV-1-protease-inhibitor complexes was performed using Monte Carlo docking combined with substructure minimizations using MacroModel software V4.0[7]. This procedure proved to yield geometries very close to the structures determined by X-ray for a variety of compounds[8,9].

Modelling of **1** and **2** started with the X-ray structure of the CGP53437-HIV-1-protease complex[9]. The compounds were manually placed into the active site and preminimized in the rigid cavity for 1000 iterations. Then a Monte Carlo search (MCMM) was performed over 3000 steps, treating the chosen active site fragment of the protein (residues 8, 23, 25, 27~30, 32, 47~50, 76, 80~82, 84) with a constraint of 10 kcal/mol and using the AMBER forcefield, the PRCG minimization method and a distance dependent dielectric constance of 2.0 for a 1000 step minimization.

Fig. 2. Reversed phase HPLC chromatogram of a fermentation extract after 96 hours with methanol.



Experimental conditions: analytical reversed-phase cartridge (Nucleosil 100-5 C18, 5 μm; Macherey-Nagel, Düren, Germany; 4.0 × 125 mm); UV detection at 280 nm; mobile phase A: 0.1% aqueous TFA; mobile phase B: acetonitrile-TFA, (100:0.075); 1.5 ml/minute; gradient elution from 30% B to 80% B in 15 minutes; 10 μl of a solution 0.5 mg/ml in MeOH injected. The numbers indicate the assignment of individual peaks to the respective component. The solutions are sensitive to light.

Physico-chemical Data

The following instruments were used in this study: mass spectrometer VG 70-SE, Manchester; NMR Varian VXR-400 S, FT-IR spectrophotometer Bruker IFS-48. All ^{1}H NMR were recorded at 400 MHz and all ^{13}C NMR at 100 MHz except where stated.

Data of **1**: FAB-MS: 371 $(M+H)^+$, PD-MS (negative mode): 369 $(M-H)^-$, IR (KBr) cm^{-1} 3410, 3360, 2970, 2920, 2560, 1610, 1535, 1455, 1340, 1280, 1240, 1140, 1110, 1040, 985, 820, 760, 625.

Data of **2**: IR (KBr) cm^{-1} 3410, 3360, 2970, 2920, 2860, 1610, 1535, 1455, 1340, 1280, 1240, 1140, 1110, 1040, 985, 820, 760, 625.

Data of **3**: MP 195~197°C, HRFAB-MS Found: *m/z* 439.1672, Cacld for $C_{27}H_{23}N_2O_4$: 439.1658, IR (KBr) cm^{-1} 3400, 3330, 2920, 1630, 1530, 1460, 1425, 1340, 1320, 1280, 1240, 1100, 1040, 985, 800, 740.

Data of **4**: MP >240°C, HRFAB-MS Found: *m/z* 439.1654, Cacld for $C_{27}H_{23}N_2O_4$: 439.1658, IR (KBr) cm^{-1} 3420, 3360, 2920, 1610, 1530, 1460, 1340, 1320, 1280, 1240, 1130, 1105, 1040, 985, 805, 745.

Results and Discussion

Structure Elucidation

Structures of **1** and **2**

Compound **2** (Fig. 3) was identified as isocochliodinol by comparison of physico-chemical data, mainly ^{1}H NMR[10]. The position of the side chain was confirmed by NOE experiments: Irradiation on the methylene protons at 3.4 ppm gave rise to an NOE of 7% at the singlet at 7.2 ppm. Metabolite **1** (didemethylasterriquinone D) was identified as a more simple analogue by comparing ^{1}H NMR data and FAB-MS with **2**. For reference purposes the ^{13}C NMR were investigated (Table 1). The correlation of the shifts of the protonated ^{13}C atoms of **2** and **3** with those of the attached protons has been made with a PFG-HSQC (pulsed field gradient heteronuclear single quantum coherence) experiment[11]. Heteronuclear multiple-bond connectivities (HMBC) between protons and carbons[12] have been used to assign the quaternary carbon signals of **2** and **3**.

Fig. 3. Structure of didemethylasterriquinone D (**1**), isocochliodinol (**2**), semicochliodinol A (**3**) and B (**4**).

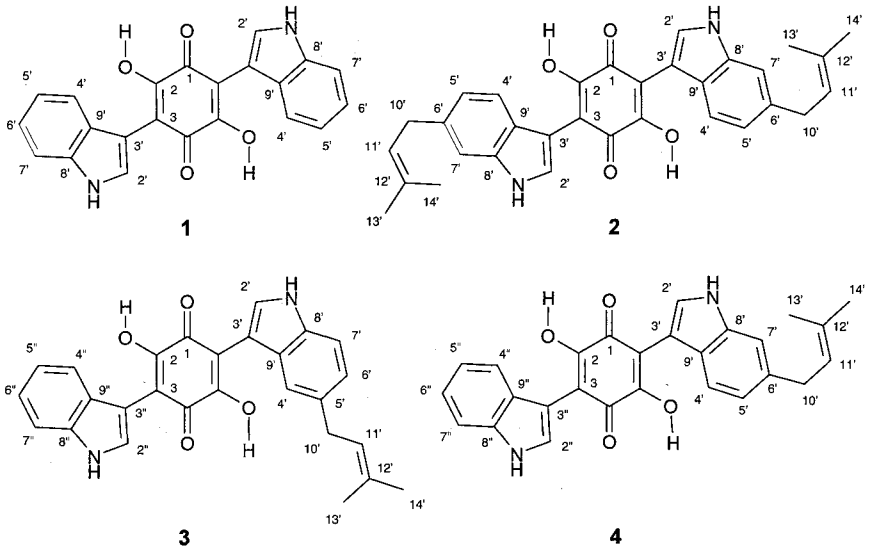


Table 1. ^{1}H NMR and ^{13}C NMR chemical shifts (in ppm).

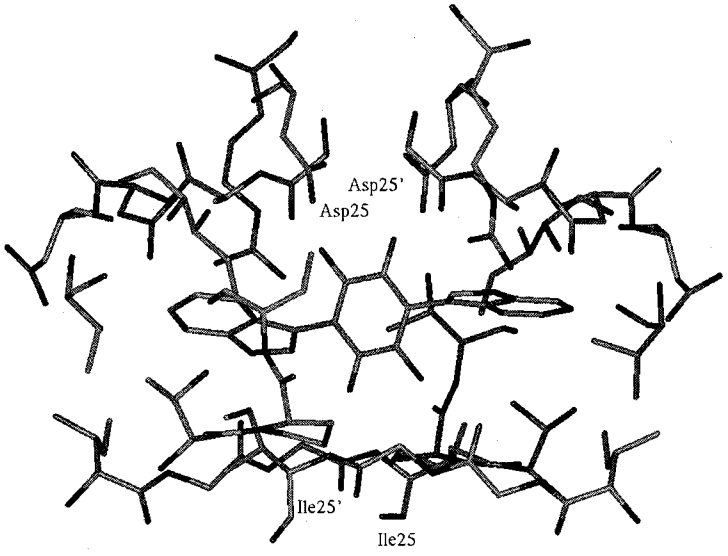
	^{1}H NMR		^{13}C NMR			
	3	**4**	**1**[a]	**2**	**3**	**4**
1, 4			n.d.	184.0[b] s	n.d.	n.d.
2, 5			n.d.	152.9[b] s	n.d.	n.d.
3, 6			111.1 s	112.0 s	111.9 s	112.7 s
					112.0 s	
1′	10.55* s br	10.46* s br				
1″	10.61* s br	10.60* s br				
2′	7.63 m	7.64* m	127.4 d	128.0 d	128.5 d	129.1 d
2″	7.68 d	7.68* d			128.4 d	128.7 d
3′			104.3 s	105.4 s	105.2 s	106.1* s
3″					105.6 s	106.2* s
4′	7.45 s	7.55 d	121.5* d	122.7 d	122.0 d	123.4* d
4″	7.64 m	7.64 m			122.8 d	122.9* d
5′		6.91 d	120.9* d	121.1 d	133.1 s	123.5* d
5″	7.06 t	7.06 t			119.9 d	120.6 d
6′	6.98 d		118.6 d	135.9 s	123.2 d	138.2* s
6″	7.14 t	7.14 t			122.2 d	121.8 d
7′	7.37 d	7.27 s	111.3 d	111.2 d	111.9 d	112.0 d
7″	7.46 d	7.46 d			112.1 d	112.8 d
8′			126.4 s	125.8 s	135.7 s	136.6* s
8″					137.1 s	137.8* s
9′			135.6 s	137.5 s	127.7* s	126.5* s
9″					127.9* s	128.3* s
10′	3.42 d	3.44 d		35.0 t	35.4 t	35.8 t
11′	5.38 t	5.39 t		125.2 d	125.8 d	125.9 d
12′				131.9 s	131.4 s	132.6 s
13′	1.72 s	1.77* s		17.8 q	17.9 q	18.5 q
14′	1.75 s	1.76* s		25.9 q	25.9 q	26.6 q

Temp. ambient, acetone-d_6 except
[a] DMSO-d_6. [b] Visible only at −60°C.

Structures of **3** and **4**

Both compounds had a molecular composition of $C_{27}H_{23}N_2O_4$ as determined by HR-MS suggesting a C_5H_8 unit in addition to structure **1**. The ^{1}H NMR of **3** and **4** (Table 1) showed the signals of compound **1**, but the signals of the symmetric indole rings in **1** were split into pairs and one proton was missing. In addition the typical signals of a dimethylallyl side chain were

Fig. 4. Molecular modelling of didemethylasterriquinone D (**1**) into the active site of HIV-1- protease.
Only the amino acid residues that are spatially close to the inhibitor are shown.

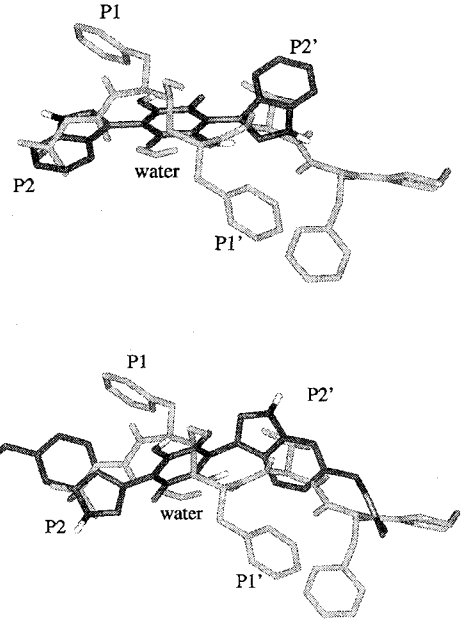


visible. These findings suggested that **3** and **4** had the same core structure as didemethylasterriquinone D (**1**) and were substituted on two different positions on one of the two indole rings of **1** with a dimethylallyl side chain. In the ^{1}H NMR of both compounds the substituted indole ring shows a singlet and two doublet signals suggesting substitution at 5′ and 6′, respectively. Analysis of ^{1}H NMR and ^{13}C NMR data reveal that semicochliodinol B has the same substitution as isocochliodinol (**2**) and must have structure **4**. Therefore semicochliodinol A must have structure **3** which was confirmed by analysis of the ^{13}C NMR and ^{1}H NMR by HSQC and HMBC.

Fermentation and Isolation

Didemethylasterriquinone D (**1**) has been described as a semisynthetic derivative of asterriquinone D[13], a metabolite of *Aspergillus terreus*[14] with *in vivo* anitumor activity[15], but not as a microbial metabolite before. Isocochliodinol **2** has been isolated from *Chaetomium murorum*[10]. Both symmetric compounds seem to be quite common as fungal metabolites: From 3000 fungal strains screened in the assay for EGF-R PTK five were shown to produce **1** and **2** belonging to various genua *Gilmaniella humicola*, *Trichosporiella hyalina*, *Chaetomium* sp., *Botryotrichum piluliferum* and *Humicola grisea*. Semicochliodinol A and B are produced only in small amounts. Analysis of the time course of product formation by HPLC (Fig. 1) showed that **3** was produced at a later stage of the fermentation.

Fig. 5. Superimposition of the model of **1** (above) or **2** (below) with X-ray data of CGP 53437[9] in the HIV-1 protease and indication of the pockets of the protease occupied.



Molecular Modelling into HIV-1 Protease

The HIV-1 protease is a homodimer with symmetric pockets P1/P1′; P2/P2′ *etc.* for the side chains of the peptide/protein substrate. The dihydroxybenzoquinone moiety of compounds **1** and **2** allows hydrogen bonding to both active-site aspartic acids (Asp25/Asp25′) and the two flap isoleucines (Ile50/Ile50′) (Fig. 4). This means

Table 2. Inhibition of HIV-1 protease, cathepsin D and EGF-R PTK by compounds **1**~**4**.

		IC_{50} (μM)		
		HIV-1 prt[a]	CD	EGF-R PTK[a]
Didemethylasterriquinone D	**1**	0.24	4.2	15
Isocochliodinol	**2**	0.18	4.1	20
Semicochliodinol A	**3**	0.37	2.5	20
Semicochliodinol B	**4**	>0.5	4.9	60

[a] Means from triplicates.
HIV-1 prt: HIV-1 protease; CD: human cathepsin D, EGF-R PTK: epidermal growth factor receptor protein tyrosine kinase.

that the critical water molecule, which is present in other HIV-1 protease inhibitor complexes and forms hydrogen bonds with the flaps as well as with the inhibitor atoms, is replaced by part of the inhibitor. This water replacement is similar to that observed with molecules such as 7-membered cyclic ureas[16] or 4-hydroxypyrone derivatives[17,18], which were specifically designed and later proved by X-ray to offer this feature.

The indole parts of the molecules fill the P2 and P2′ pockets of the protease (Fig. 5). This was achieved differently in case of **1** than for the substituted compound **2**. This because the 6′-dimethylallyl chain in **2** appears to be in an unfavorable position of the indole ring. When part of the substituted molecule is accommodated in the binding site, the rest of the molecule is shifted away from the most favorable complex geometry. The different binding modes proposed by the molecular modelling were not reflected by differences in antienzymatic activities of compounds **1** and **2**. Observation of the binding mode would be important for further chemical modifications of this class of compounds. According to this model, the P1/P1′ and P3/P3′ pockets are not filled at all. This allows interesting speculations, of how P1 might be reached from the 2′ or 4′ position of the indole ring (*e.g.* by attaching a benzyl in one of these positions).

The model obtained from the Monte Carlo docking studies can be used for subsequent structure-based drug design efforts.

Biological Properties

The compounds are not light stable in dilute solutions. Their enzyme-inhibitory activity is shown in Table 2. Compounds **1** to **3** inhibited the HIV-1 protease with IC_{50} values in the submicromolar range. Only compound **4** is a weaker inhibitor of HIV-1 protease. Human cathepsin D is a related aspartic protease with pleiotropic activity. The IC_{50} values against human cathepsin D are approximately 10 fold higher for these compounds.

The nakijiquinones C and D with a similar central hydroxybenzoquinone ring have recently been described as inhibitors of c-ErbB-2 kinase[19]. All compounds **1** to **4** inhibited EGF-R PTK around 20 μM, suggesting that the dimethylallyl side chains are not important for the binding to the enzyme. Superimposition of **1** with known inhibitors of EGF-R PTK like staurosporine[6] or DAHP 1 (CGP52411)[20] suggests that new derivatives of **1** with meta-substitution at the benzoquinone ring could be more potent as inhibitors of this enzyme. In conclusion, **1** and its derivatives are attractive lead compounds for inhibitors of HIV-1 protease and EGF-R PTK. However the molecules clearly require optimization to become clinically useful agents.

Acknowledgments

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