Novel Spirodihydrobenzofuranlactams as Antagonists of Endothelin and as Inhibitors of HIV-1 Protease Produced by *Stachybotrys* sp.

I. Fermentation, Isolation and Biological Activity

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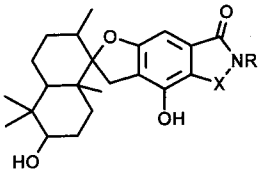
Six novel spirodihydrobenzofuranlactams I~VI (**1**~**6**) and a related spirodihydrobenzofuranalcohol, the previously described natural compound L-671,776 (**7**), were isolated from cultures of two different *Stachybotrys* species. These secondary metabolites showed antagonistic effects in the endothelin receptor binding assay and inhibited HIV-1 protease. Both biological activities are novel for L-671,776 (**7**). The pseudosymmetric spirodihydrobenzofuranlactam VI (**6**) is the most potent representative of this class of compounds exhibiting IC_{50} values of 1.5 μM in the ET-A receptor binding assay and 11 μM in the HIV-1 protease inhibition assay.

Endothelin-1 (ET-1), a 21 amino acid peptide, demonstrates potent vasoconstrictor, mitogenesis and bronchoconstrictor activity[1~3]. Previous studies have indicated that at least two distinct endothelin receptor subtypes exist, the ET-A and ET-B receptors[4,5]. Identification of novel compounds that inhibit the ability of ET-1 to bind to these receptors may lead to novel treatments for hypertension, congestive heart failure, asthma or atherosclerosis[6].

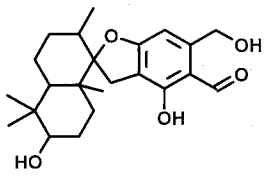
The protease of Human Immunodeficiency Virus (HIV) has been proven to be essential for correct processing of the viral *gag* and *gag-pol* precursor proteins[7]. Inhibition of this enzyme led to the formation of non infectious progeny virus *in vitro*. Therefore this enzyme has attracted the interest of the pharmaceutical research community aiming to identify enzyme inhibitors with antiviral activity, which would be valuable as anti-AIDS agents. Unfortunately most compounds identified as first generation inhibitors belonged to the class of peptidomimetics and displayed only low oral bioavailability[8]. Therefore programs to screen extracts from natural sources were initiated which were hoped to lead to the discovery of substances with improved oral bioavailability.

In the course of a screening program for antagonists of endothelin receptor binding and for inhibitors of

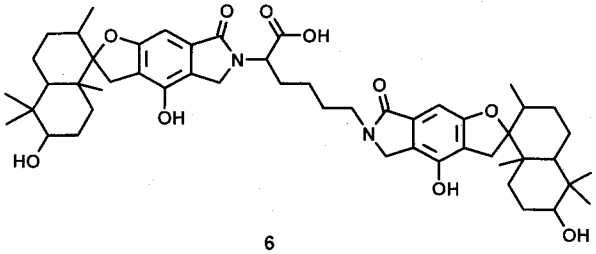
Scheme 1. Chemical structures of six new spirodihydrobenzofuranlactams I~VI (**1**~**6**) and of L-671,776 (**7**).



		X	R
1	I	CH_2	H
2	II	CH_2	$(CH_2)_2OH$
3	III	CH_2	$(CH_2)_4COOH$
4	IV	CH_2	$CH(COOH)(CH_2)_2COOH$
5	V	C=O	H



7
L-671,776



6
spirodihydrobenzofuranlactam VI

HIV-1 protease a *Stachybotrys chartarum* strain was found which produced the known spirodihydrobenzofuranalcohol L-671,776 (**7**)[9], and the spirodihydrobenzofuranlactams II , III, IV and VI (**2**, **3**, **4**, **6**) (Scheme 1). The spirodihydrobenzofuranlactam I (**1**) and its imide (**5**) were isolated from a different fungal strain *Stachybotrys atra* F11402. The lactams **1**~**4**, **6** and the imide **5** form a novel class of compounds of the spirodihydrobenzofuran family, while L-671,776 has already been patented as an inositol-phosphate phosphatase inhibitor[9] isolated from *Memnoniella echinata* and is structurally related to K-76[10] and to the recently described avian myeloblastosis virus protease (AMV) inhibitors Mer-NF5003B, E and F[11].

In this article the cultivation of the producing microorganisms, as well as the isolation and biological activities of the natural products **1**~**7** are described.

Materials and Methods

Microorganisms: Isolation and Preservation

The fungal strain DSM 8767 was isolated from a soil sample collected at the Lake Kokonos, China. The fungus was taxonomically classified as *Stachybotrys chartarum* and has been deposited with the DSM (Deutsche Sammlung für Mikroorganismen und Zellkulturen, Braunschweig, Germany). The microorganism was grown on LCSB agar (Lactose 1.5%, cornsteep liquor 0.5%, peptone 0.5%, NaCl 0.4%, $MgSO_4 \times 7\ H_2O$ 0.05%, KH_2PO_4 0.06%, $FeCl_3 \times 6\ H_2O$ 0.0005%, $CuSO_4 \times 5\ H_2O$ 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 had occured. Working stocks are stored at 4°C, whereas long-term storage is at −80°C and −196°C, respectively.

The strain F11402 was isolated from a piece of wood collected in Sarno, Italy, and has been classified as a representative of the species *Stachybotrys atra*. This fungus was cultivated on slopes prepared of mycophil agar (BBL) at 25°C for ten days. The working stocks and the long-term preservation were carried out as described above.

The species names *Stachybotrys chartarum* and *Stachybotrys atra* are synonyms, named by S. HUGHES respectively Corda. The colony characteristics were growth on oatflake agar about 2 mm/day at 25°C, colorless mycelium, sparse aerial mycelium, conidial masses in blackish drops, reverse uncolored, no exudate, irregular and slow growth on malt extract and maximum growth temperature between 37°C and 40°C. The micromorphology showed conidiophores that were mostly simple, partly branched, 100 μm long and with a diameter of 3~6 μm, and were colorless at the base, but darkening and roughening to the apex. A cluster of ellipsoidal phialides was formed at the apex, the phialides were 10~12 μm long. Conidia aggregated into slimy masses, were ellipsoidal, had a dimension of $10 \times 6\ \mu$m and were smooth-walled to coarsely roughened.

Fermentation

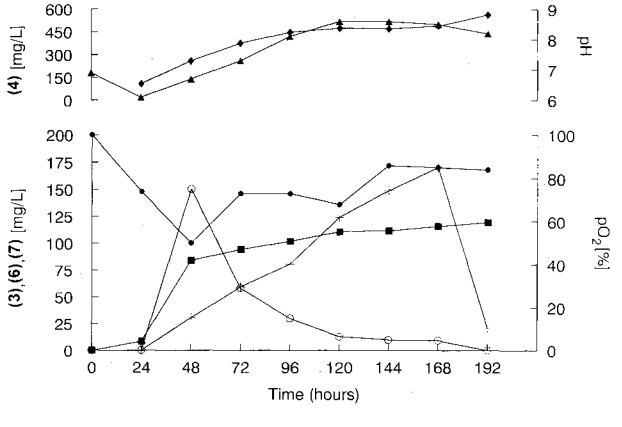
A mature slant culture of the strain DSM 8767 was inoculated into a 500 ml Erlenmeyer flask with 1 baffle containing 100 ml of a medium with the following composition: Glucose 2.0%, Pharmamedia 1.5%, $(NH_4)_2SO_4$ 0.3%, $ZnSO_4 \cdot 7H_2O$ 0.003%, $CaCO_3$ 0.4%. Before sterilization, the pH was adjusted 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 2,000 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 50 liter fermenter filled with 30 liters of the following production medium: Glucose 1.0%, potato starch 3.0%, casamino acids (Difco) 0.5%, LabLemco (Oxoid) 0.3%, yeast extract (Difco) 0.2%, N-Z Amine (Sheffield) 0.3%, KH_2PO_4 0.05%, $MgSO_4 \times 7H_2O$ 0.05%, KCl 0.03%, $CaCO_3$ 0.3%. Before sterilization, the pH was adjusted to 7.0. The aeration rate was 30 liters/minute and the agitation rate was adjusted to 600 rpm.

A typical fermentation profile of the strain *Stachybotrys chartarum* DSM 8767 is shown in Fig. 1. The cultivation was performed in a 3.5-liter fermenter (MBR) at 28°C for 192 hours with an aeration rate of 1 liter/minute and an agitation rate of 700 rpm. The preculture conditions have already been described above. The fermenter was filled with 1 liter of the previously mentioned production medium.

For the cultivation of *Stachybotrys atra* F11402, a piece of a well sporulated culture thereof was inoculated into a 500 ml Erlenmeyer flask with one baffle, filled with 100 ml of a medium which had the following composition:

Fig. 1. Time course of the production of the metabolites (**3**), (**4**), (**6**) and (**7**) in a 3.5-liter fermenter.

+ (**3**), ◆ (**4**), ■ (**6**), ○ (**7**), ▲ pH, ● pO_2.



Cerelose 2.0%, peptone (Difco) 2.0%, yeast extract (Difco) 1.0%. The pH was adjusted to 6.5 before sterilization. The incubation was carried out at 25°C and a rotation rate of 250 rpm. After 48 hours, 5.0% of the culture broth was used for inoculation of a second preculture with the same medium.

2,000 ml Erlenmeyer flasks with four baffles were filled with 500 ml of the described preculture medium. The cultivation was performed for 48 hours at 28°C with 125 rpm. For the production of the compounds **1** and **5**, 5.0% of the preculture were transferred into an 50 liter fermenter filled with the following production medium: Potato starch 6%, soybean meal (defatted) 2%, K_2HPO_4 0.03%. Before sterilization, the pH was adjusted to 6.4. The aeration rate was 30 liters/minute and the agitation rate was at 600 rpm.

Isolation

Chromatographic systems used: Preparative normal-phase liquid chromatography with silica gel (Lichroprep Si 60, 12~25 μm; Merck, Germany) as stationary phase was carried out using the following system: dual-pump gradient system (Gilson pumps type 303 and 306; Gilson, France), preparative glass column (49 × 460 mm; 870 ml; Büchi, Switzerland), a UV-detector equipped with a cell of 0.5 mm optical pathlength (Shimadzu type UV-120-02; Shimadzu, Japan) and a fraction collector (Büchi, type 684). Gravity was used to run gel filtration on Sephadex LH-20 (25~100 m; Pharmacia) in a glass column (50 × 500 mm; 1000 ml).

Preparative reversed phase liquid-chromatography was performed with a silica based C-18 modified stationary phase (Lichroprep RP-18, 15~25 μm; Merck, Germany), using the following system: preparative pump (Latek 500; Latek, Germany) in combination with a low-pressure gradient former (Labomat VS200; Labomatic, Switzerland), a conical glass column (26~49 × 230 mm; 260 ml; Büchi, Switzerland), a UV-detector equipped with a cell of 0.5 mm optical pathlength (Shimadzu type UV-120-02; Shimadzu, Japan) and a fraction collector (Büchi, type 684).

TLC-analysis of the fractions from silica gel chromatography was performed on HPTLC precoated plates (silica gel 60 F_{254}, with concentrating zone; Merck, Germany) using CH_2Cl_2 - CH_3OH 90 : 10 (v/v) as mobile phase and anisaldehyde spray-reagent (1% in a solution of 2% sulfuric acid (98%) in acetic acid) or UV-irradiation (254 nm, 316 nm) as detection methods.

The fractions collected from the preparative chromatography were analyzed using a standardized analytical reversed phase HPLC method. The instrumentation used consisted of an HPLC pump operated in the low-pressure gradient forming mode (Merck-Hitachi L-6200; Merck, Germany), a UV-detector (Merck-Hitachi L-4250) and an integrator (Merck-Hitachi D-2500). A spherical C-18 modified silica gel based stationary phase with a pore-size of 100 Å and a particle diameter of 5 μm was used (Lichrospher 100 RP-18, 5 μm, column size: 4 × 125 mm; Merck, Germany). Mobile phase A consisted of 2.2 mM KH_2PO_4 in H_2O adjusted to pH 3.0 with H_3PO_4 (about 0.3 mM). Mobile phase B was prepared by mixing 80% CH_3CN with 20% of mobile phase A (v/v). The wavelength for detection was set to 220 nm and 25 μl of each sample were injected for each run. A linear gradient was run from 5% to 100% mobile phase B in 25 minutes with a constant flow rate of 1.5 ml/minute.

For the work up of cultures of the fungal strain *Stachybotrys chartarum* DSM 8767 (Scheme 2) the whole broth (120 liters) was mixed with EtOAc (240 liters). Subsequent filtration was performed using a filter aid (Celite; Celite Corp., U.S.A.). The aqueous and organic phases were separated, the aqueous phase was discarded, while the organic layer was washed with sodium chloride solution (20%, 9.8 liters) and concentrated *in vacuo*. The EtOAc-extract was partitioned between heptane (620 ml) and aqueous CH_3OH (10%, 770 ml). Evaporation of the CH_3OH phase yielded 44.8 g of crude extract.

The crude extract (44.8 g) was suspended in CH_2Cl_2-heptane 80 : 20 (200 ml) and adsorbed on silica gel (40 g). The adsorbed material forming a thick paste was applied on the column over a precolumn. This crude material was chromatographed on silica gel with stepwise elution of the following solvents (v/v): CH_2Cl_2 - heptane 80 : 20, 90 : 10; CH_2Cl_2; CH_2Cl_2 - isopropanol 99 : 1, 98 : 2, 95 : 5, 90 : 10, 75 : 25, 50 : 50; CH_2Cl_2 - CH_3OH 50 : 50 and CH_3OH. The flow rate of the mobile phase was 30 ml/minute, the detection wavelength was set to 254 nm. The fractions were combined based on TLC-analysis (see above), the pooled fractions were further analyzed by TLC and HPLC (see above) and in the bioassay. Crude L-671,776 (**7**) eluted from the column with the solvent CH_2Cl_2-isopropanol 75 : 25 (v/v) and a mixture of the spirodihydrobenzofuranlactams II, III, IV and VI (**2**, **3**, **4**, **6**) combined with undetermined components eluted with CH_2Cl_2 - CH_3OH 50 : 50 (v/v).

Crude L-671,776 (**7**) (21.8 g) was purified in portions (1~2 g) by reversed phase preparative HPLC with H_2O as mobile phase A and CH_3CN - H_2O 80 : 30 (v/v) as mobile phase B. The flow rate of the mobile phase was 30 ml/minute. A gradient was run from 50% to 100% phase B in 80 minutes with the detection wavelength set to 235 nm. The starting material (2.1 g) was suspended in 3 ml CH_3OH and applied to the above described reversed phase column. L-671,776 (**7**) (1.2 g) eluted from 68% to 100% mobile phase B.

The crude mixture of the spirodihydrobenzofuranlactams II, III, IV and VI (**2**, **3**, **4**, **6**) which eluted with CH_2Cl_2 - CH_3OH 50 : 50 (v/v) from the silica gel column, was further purified in portions (0.8~1.5 g) by reversed phase preparative HPLC with H_2O as mobile phase A and CH_3CN - H_2O 80 : 20 (v/v) as mobile phase B and a flow rate of 20 ml/minute. A gradient was run from 75% to 100% phase B in 80 minutes with the detection wavelength set to 235 nm. The starting material (0.86 g) was suspended in 3 ml CH_3OH and applied to the above described reversed phase column. Pure compound **2**

(21 mg) eluted with 82% mobile phase B, a mixture of **3** and **4** (83 mg) eluted from 83% to 84% phase B and pure metabolite **6** (105 mg) eluted from 87% to 92% mobile phase B.

A mixture of **3** and **4** (83 mg) was rechromatographed on reversed phase running a gradient from 10% to 80% phase B in 80 minutes. 83 mg of crude **3** and **4** were suspended in 0.3 ml CH_3OH and applied to the reversed phase column. Pure compound **4** (5 mg) eluted with 50% to 62% mobile phase B, while pure compound **3** eluted from 64% to 75% phase B.

For the work-up of *Stachybotrys atra* F11402 cultures (Scheme 3) the whole broth (90 liters) was lyophilized. The lyophilisate (1600 g) was mixed with EtOAc (30 liters) and filtrated. The filtered residue was suspended and extracted with CH_3OH (20 liters), filtered and the methanolic phase was concentrated *in vacuo*. The CH_3OH extract was suspended in H_2O, the pH value was adjusted to 2.5 with 5 N sulfuric acid and extracted with EtOAc (30 liters). The aqueous and organic phases were separated, the aqueous phase was discarded, while the organic layer was concentrated *in vacuo* yielding 188 g of crude extract.

The crude extract (188 g) was purified by gel filtration on Sephadex LH-20 using CH_3OH - CH_2Cl_2 50 : 50 (v/v) as mobile phase. Enriched compound **5** (8.7 g) eluted after 4.7 liters mobile phase and crude **1** (10 g) after 5 liters CH_3OH - CH_2Cl_2 50 : 50 (v/v).

Enriched **1** was further purified on silica gel using CH_2Cl_2 - isopropanol as mobile phase. Compound **1** (10 g), dissolved in CH_2Cl_2, was applied to the column and eluted with CH_2Cl_2 - isopropanol 90 : 10 (v/v). Recrystallisation from EtOAc (150 ml) yielded pure **1** (5.9 g).

In analogy to the separation of **1** crude compound **5** was further purified by silica gel chromatography using CH_2Cl_2 - CH_3OH as mobile phase. Enriched metabolite **2** (8.7 g) was dissolved in CH_2Cl_2 and eluted with CH_2Cl_2 - CH_3OH 94 : 6 (v/v). Recrystallisation from EtOAc (200 ml) yielded pure compound **5** (6.8 g).

Biological Properties

Endothelin receptor binding assay methodologies: Normal rat kidney (NRK) cells, which were used as the source of ET-A receptors, were scraped and transferred to a conical centrifuge bottle (250 ml) on ice. The cells were rinsed with 10 ml TE8 buffer (10 mM Tris-NaOH, 1 mM EDTA, pH 8.0) with 1 mM DTT and then homogenized using a Polytron for 10 seconds at setting 6. PMSF (0.1 M, 10 μl/ml) was added and the cells were centrifuged at 1000 × *g* for 10 minutes. The supernatant was centrifuged at 48000 × *g* for 10 minutes. The pellets were resuspended in 5 ml HBSS (Ca^{++}/Mg^{++} free) per flask of cells. Cells were stored in 1 ml aliquots at −80°C until assay.

On the day of the experiment, membrane preparations (18.5 μg/ml protein for NRK cells) were thawed and suspended in 20 mM Hepes buffer, pH 7.4, containing 145 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.1% bovine serum albumin and 0.02% bacitracin. Binding experiments were performed by incubating 100 μl of the suspension, 50 μl of either 10 pM [^{125}I] ET-1 (NEN-Du Pont, Boston, MA; specific activity 2200 Ci/mmol) and 50 μl of drug or buffer at a final volume of 0.5 ml for 90 minutes (NRK cells) at 37°C. Non-specific binding was determined in the presence of 100 nM unlabeled ET-1. Bound and free radioactivity were separated by vacuum filtration through Whatman GF/C glass fiber filters presoaked in 2% powdered milk. Filters were washed three times in 4 ml of ice-cold buffer and counted in a Genesys gamma counter (Schaumburg, IL).

HIV protease assay: During the purification procedure the anti-HIV-1 protease activity was monitored using the scintillation proximity assay[12] (HIV Proteinase ^{125}I SPA Enzyme Assay System (IMK 8939); Amersham International Plc., Cardiff, UK). All fractions and pure compounds were solubilized in DMSO (final concentration < 5 mM). The IC_{50} values for compound **1** to **7** were determined by a peptide-based enzyme assay: The cleavage of the icosapeptide RRSNQVSQNY*PIVQNIQGRR (164 μM) in 20 mM MES (morpholinoethanesulfonic acid; pH 6.0) was monitored. The reaction was started by the addition of *E. coli* expressed and purified HIV-1 protease[13]. After 60 minutes incubation at 37°C the reaction was stopped by adding $HClO_4$ to a final concentration of 0.03 μM and product formation was analyzed by RP-HPLC[14].

Results and Discussion

The time course for the production of the three novel spirodihydrobenzofuranlactams III, IV and VI (**3**, **4**, **6**) and the related spirodihydrobenzofuran alcohol L-671,776 (**7**) was determined by experiments in a 3.5 liter fermenter and was monitored by HPLC-analysis (Fig. 1). It could be shown that L-671,776 (**7**) is a biosynthetic precursor of the spirodihydrobenzofuranlactams **3**, **4** and **6**. After 48 hours of fermentation the highest titer of L-671,776 (**7**) was achieved. In parallel to the titer decrease of L-671,776 (**7**) the production of the spirodihydrobenzofuranlactams increased as could be monitored by HPLC-analysis of the culture broth after different incubation times (Fig. 1).

Cultures of the *Stachybotrys chartarum* DSM 8767 strain were worked up after 120 hours of fermentation time. The extraction of the whole broth of the *Stachybotrys chartarum* DSM 8767 by EtOAc and the partitioning of the organic phase between heptane and 10% aqueous CH_3OH yielded crude extract containing L-671,776 (**7**) and the spirodihydrobenzofuranlactams II, III, IV, and VI (**2**, **3**, **4**, **6**). The spirodihydrobenzofuranlactams (**2**, **3**, **4**, **6**) could be separated from L-

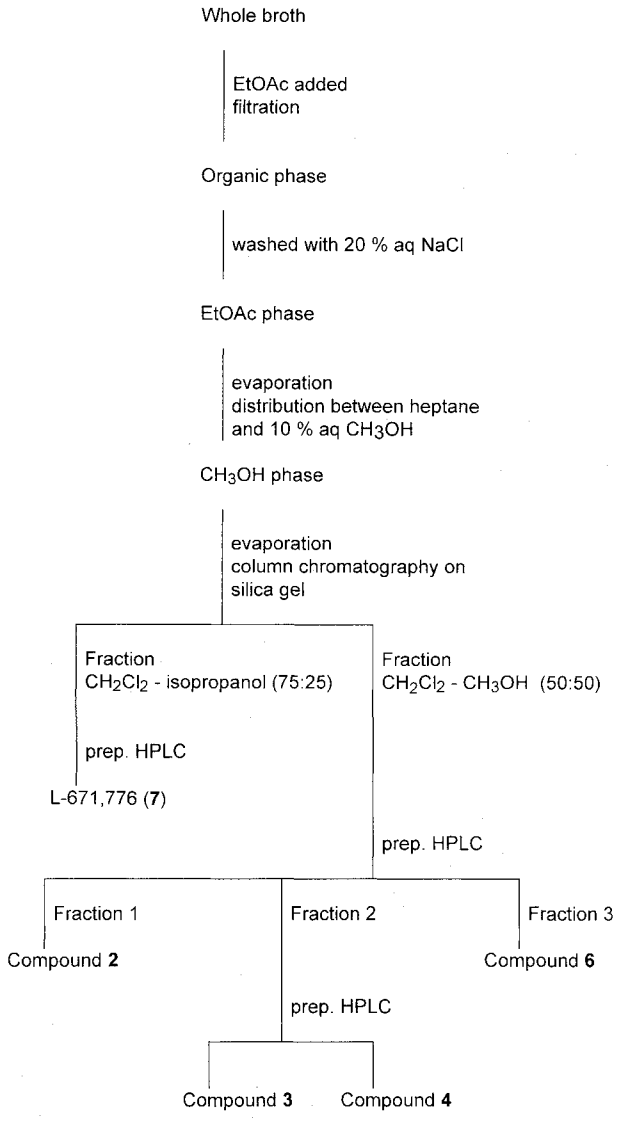
671,776 (**7**) by chromatography on silica gel (Scheme 2). Crude L-671,776 was further purified by reversed phase (C_{18}) chromatography. The different spirodihydrobenzofuranlactams (**2**, **3**, **4**, **6**) were separated by multiple chromatographic steps on reversed phase (C_{18}) using different gradient systems.

The fermentation time for cultures of *Stachybotrys atra* F11402 was 120 hours. Separation of the spirodihydrobenzofuranlactam I (**1**) and its imide **5** was achieved by gel filtration on Sephadex LH-20 using CH_3OH-CH_2Cl_2 50:50 (v/v) as mobile phase. The metabolites **1** and **5** were further purified by additional chromatography on silica gel.

The structure elucidation of the six novel spirodihydrobenzofuranlactams I~VI (**1**~**6**) and the physicochemical data of these compounds will be described elsewhere[15]. The FAB-MS, the ^1H NMR and ^{13}C NMR spectra of L-671,776 (**7**) are well in accordance with the reported data in the literature[9].

The six novel spirodihydrobenzofuranlactams I~VI (**1**~**6**) and L-671,776 (**7**) have been identified as receptor binding antagonists of endothelin and as inhibitors of HIV-1 protease. The biological activities for all the compounds are summarized in Table 1. The spirodihydrobenzofuranlactam VI (**6**) containing two spirodihydrobenzofuranlactam rings linked *via* a lysine moiety

Scheme 2. Isolation procedure for the spirodihydrobenzofuranlactams II, III, IV, VI (**2**, **3**, **4**, **6**) and L-671,776 (**7**) from *Stachybotrys chartarum*.



Scheme 3. Isolation procedure for the spirodihydrobenzofuranlactams I and V (**1** and **5**) from *Staphybotrys atra* F11402.

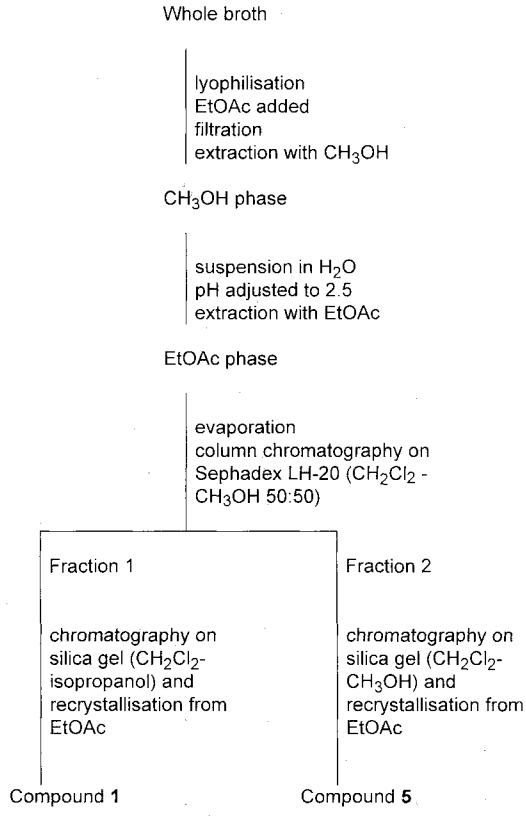


Table 1. Antagonistic effects in the endothelin receptor binding assay (ET-A) and inhibition of HIV-1 protease by the spirodihydrobenzofuranlactams I~VI (**1**~**6**) and L-671,777 (**7**).

	1	**2**	**3**	**4**	**5**	**6**	**7**
ET-A receptor binding IC_{50} (μM)	$\gg$10	$\gg$10	—	—	—	1.5	>100
HIV-1 protease assay IC_{50}* (μM)	161	115	39	288	375	11	115

*=mean value from three independent determinations.

showed IC_{50} values of 1.5 μM in the ET-A receptor binding assay and of 11 μM in the HIV-1 protease assay. Interestingly this pseudosymmetric compound **6** is 4 to 30 times more active against the HIV protease and over 40 times more potent in the endothelin receptor binding assay than the metabolites **1**~**5** and **7**. Recently three inhibitors of avian myeloblastosis virus (AMV) protease Mer-NF5003B, E and F[11] have been described. These compounds are structurally related to L-671,776 (**7**) and showed inhibitory activity against AMV protease in the range of 10 to 20 μM. Both enzymes AMV protease and HIV-1 protease are aspartic proteases of retroviral origin. While L-671,776 (**7**) inhibits HIV-1 protease only weakly (IC_{50} value of 115 μM), the related pseudosymmetric spirodihydrobenzofuranlactam VI (**6**) inhibits HIV-1 protease to an extent comparable to that of Mer-NF5003B, E and F against AMV protease. The only remote antiviral activity of compound **6** did not justify the determination of pharmacokinetic parameters in animals.

The spirodihydrobenzofuranalcohol L-671,776 (**7**) is known as an inositol-phosphate phosphatase inhibitor[9]. However, its weak endothelin activity and the moderate inhibiting activity against HIV-1 protease are new biological properties of this compound, which have not been reported previously. In addition it could be shown that L-671,776 (**7**) is an important precursor of the most interesting representative of the spirodihydrobenzofuran family, the metabolite **6**.

In conclusion the six spirodihydrobenzofuranlactams I~VI (**1**~**6**) represent a novel class of biologically active natural products. Hexapeptides[16] and pentapeptides[17] have been reported as naturally occurring antagonists of endothelin. Recently the sesquiterpene S-50-235[18] has been isolated as another natural antagonist from the plant *Myrica cerifera*, but the spirodihydrobenzofuranlactams are structurally unique antagonists of endothelin.

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