Discovery of an Angiotensin II Binding Inhibitor from a *Cytospora* sp. Using Semi-automated Screening Procedures

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Cytosporin A, B and C, three antagonists of [^{125}I]-angiotensin II binding to rat adrenal glands were discovered in fermentations of an endophytic *Cytospora* sp. during routine screening using semi-automated procedures. The most potent of these displayed an IC_{50} of $1.5 \sim 3\,\mu$M and was specific for angiotensin II AT_2.

The renin-angiotensin system (RAS) plays a central role in the regulation of normal blood pressure and appears to be critically involved in hypertension as well as in congestive heart failure, cirrhosis and nephrosis. Renin is synthesized by the kidneys and secreted into the circulation where it cleaves angiotensinogen to form the decapeptide, angiotensin I. Angiotensin I is converted to the octapeptide angiotensin II by angiotensin converting enzyme. Angiotensin II, the active hormone of the RAS, is a powerful arterial vasoconstrictor that exerts its action by interacting with specific receptors located on the cell membranes of various target organs[1,2].

Two subtypes of the angiotensin II binding sites have been characterized in rat adrenal glands and in other tissues. Work performed with two inhibitors, DuP 753 which blocks AT_1 and EXP 655 and other related compounds, which block AT_2, supports the theory that there are two distinct angiotensin II binding sites in rat adrenal glands and brain tissues[3~6].

In this paper, we describe the angiotensin II antagonistic activity of three novel hexahydrobenzopyran derivatives (one major component and two minor components), derived from fermentations of a *Cytospora* sp., designated cytosporin A, B and C, (Fig. 2) whose structure elucidation will be reported elsewhere[7]. The results indicate that these derivatives can inhibit the binding of angiotensin II at receptor sites in rat adrenal tissue. The technology used to perform an efficient and reproducible receptor binding assay for high volume screening for potential inhibitors of the angiotensin II receptor is also described.

Materials and Methods

Isolation and Maintenance of the Producing Organism

The producing organism, *Cytospora* sp. was recovered from living bark of *Betula alleghaniensis* (yellow birch) collected May, 1990, in Randolph County, West Virginia, U.S.A. using a specialized isolation method for endophytic bark fungi[8,9]. Cultures were maintained either as slants on malt-yeast extract agar (Difco Laboratories), as frozen vegetative mycelium (FVM) in 20% glycerol at −80°C, or as lyophilized mycelium and conidia in glass ampules. Cultures were accessioned to the Merck Microbial Resources Culture Collection as MF5658, and to the American Type Culture Collection as ATCC 74091. Descriptions of colony morphology are based on observations of 14-day cultures grown at 20°C, 12 hours photoperiod under cool-white fluorescent light. Capitalized color names in the descriptions are from Ridgway[10].

Fermentation Conditions

The three novel compounds were produced during the aerobic fermentation of aqueous nutrient media *via* inoculation with a lyophilized pellet of the organism. The seed fermentation was initiated in a 250 ml unbaffled flask containing 54 ml seed medium and then shaken at 220 rpm on a rotary shaker for 3 days at 28°C with 75% relative humidity in a room with constant fluorescent lighting. The seed medium contained corn steep liquor 5 g, tomato paste 40 g, oat flour 10 g, glucose 10 g and trace element mix 10 ml in 1 liter distilled water pH 6.8. The trace element mix contained $FeSO_4 \cdot 7H_2O$ 1 g,

$MnSO_4 \cdot 4H_2O$ 1 g, $CuCl_2 \cdot 2H_2O$ 25 mg, $CaCl_2$ 100 mg, H_3BO_3 56 mg, $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ 19 mg and $ZnSO_4 \cdot 7H_2O$ 200 mg in 1 liter of distilled water. Two ml portions of the 3 day culture growth were used to inoculate 45 ml portions of liquid production medium in 250 ml unbaffled flasks and shaken at 220 rpm on a rotary shaker at a constant temperature of 25°C and 50% relative humidity for up to 32 days. The production medium contained yellow corn meal 50 g, yeast extract 1 g and glucose 40 g in 1 liter distilled water.

Individual flasks were extracted by shaking for an hour with an equal volume (45 ml) of methylethylketone (MEK). Components A, B and C were isolated from the extract. A peak titer of 200 μg/ml was observed at day 14 for the major component, component A.

Membrane Preparation

Adrenal glands were removed from rats, placed in cold buffer (50 mM Tris-HCL pH 7.7) and kept on ice. Fat was removed from the adrenal glands using tweezers (dry ice was packed under a beaker and the adrenal glands, placed on paper soaked in ice cold buffer, were placed on the top of the beaker). Whole, cleaned adrenals were placed in microcentrifuge tubes (20 glands per tube), frozen and stored in a liquid nitrogen tank. Membranes were then prepared as needed according to the following method. Homogenize one microcentrifuge of adrenal glands in 20 ml 50 mM Tris-HCL pH 7.7 and centrifuge at $48,000 \times g$ for 15 minutes at 4°C. Discard supernatant and resuspend pellet in buffer consisting of 120 mM NaCl, 10 mM Na_2HPO_4, 5 mM Na_2 EDTA and 0.1 mM PMSF and centrifuge at $48,000 \times g$ for 15 minutes at 4°C. Repeat this last step. Resuspend pellet in 2.5 ml of this same buffer, aliquot into microcentrifuge tubes and freeze.

Biochemical Assay Procedure

Membranes (the protein concentration was calculated to be 128 μg/ml in the assay) were incubated in the presence of $[^{125}I]$-Tyr^4-angiotensin II (final assay concentration = 40 pM). Assays were incubated at 37°C for 90 minutes in Marsh 1.2 ml tubestrips. Binding buffer (100 mM Tris-HCL pH 7.4 with 5 mM $MgCl_2$, 0.2% BSA and 0.2 mg/ml bacitracin) and sample were added *via* the Tecan pipetting station. Membrane and $[^{125}I]$-Tyr^4-angiotensin II were added using a 12 channel pipettor. Assays were filtered using a TomTek Mach II (format-6×16) 96 well cell harvester with GF/B filtermats. The filtermats were punched directly into minivials and counted using a 4-probe gamma counter. The results were collected on disk and calculated using a computer calculation program. Non-specific binding was defined as the number of counts found in the presence of 1 μg/ml cold angiotensin II.

Isolation

The methylethylketone extract was concentrated to dryness, reconstituted in methylene chloride and fractionated on a 200 ml column of silicagel (E. M. silicagel 60, 250~400 mesh, packed in methylene chloride) and eluted with methylene chloride containing increasing amounts of methanol. Fractions containing component A were pooled, concentrated to dryness and further fractionated on a 100 ml silicagel column (packed in methylene chloride - ethyl acetate (90 : 10)) and washed with the same solvent mixture, containing increasing concentrations of ethyl acetate. HPLC was used to examine the fractions from the second silicagel column and revealed the presence of component A in the early eluting volumes of methylene chloride - ethyl acetate (88 : 12). These fractions were concentrated to dryness, redissolved in methanol and further processed by gel filtration on a Sephadex LH-20 column (150 ml column, eluted with methanol) and preparative HPLC (Whatman Partisil-10 ODS-3 column (20 mm × 25 cm), maintained at room temperature and eluted at 8 ml/minute with 40% aqueous acetonitrile) resulting in a pure preparation of component A.

The presence of the minor analogs (components B and C) was detected when the major component (component A) was purified on a larger scale. The minor analogs were not present in large enough amounts to be picked up in the biochemical assay. They were recognized by certain similarities with component A.

Late eluting fractions from the second silica gel step, in particular, were processed by gel filtration on a Sephadex LH-20 column (as outlined for component A) and final purification was achieved by preparative HPLC (Whatman Partisil-10 ODS-3 column (20 mm × 25 cm) maintained at room temperature and eluted at 8 ml/minute with 20% aqueous acetonitrile for 60 minutes, then with a 40 minute gradient from 20% to 40% aqueous acetonitrile).

Characterization

Mass spectral data were obtained on Finnigan-MAT models MAT212 (EI mode at 90 eV) and TSQ70B (EI mode at 70 eV) mass spectrometers. NMR data were obtained on Varian XL-300 and Unity 400 NMR spectrometers.

Component A—The molecular weight was determined to be 308, corresponding to $C_{17}H_{24}O_5$. (calculated *m/z* 308.1624, found *m/z* 308.1634). ^{13}C NMR (CD_2Cl_2): δ 14.0, 15.9, 22.7, 24.4, 27.3, 30.4, 31.9, 35.0, 60.1, 61.2, 62.8, 73.5, 77.2, 139.6, 148.7, 191.6, 197.8 ppm.

Component B—The molecular weight was determined to be 346, corresponding to $C_{17}H_{25}ClO_4$. (calculated *m/z* 328.1441, found *m/z* 328.1435). ^{13}C NMR (~5% CD_3OD/CD_2Cl_2): δ 14.0, 22.7, 24.2, 26.6, 27.0, 30.3, 32.2, 37.6, 68.7, 71.6, 71.8, 76.9, 79.3, 83.9, 130.9, 163.0, 191.8 ppm

Component C—The molecular formula was determined to be 310, corresponding to $C_{17}H_{26}O_5$. (calculated *m/z* 310.1780, found *m/z* 310.1778). ^{13}C NMR ($CDCl_3$): δ 13.9, 16.0, 22.3, 27.8, 29.1, 29.9, 31.6, 35.5, 55.8, 57.8, 62.4, 68.9, 74.3, 77.2, 129.9, 157.7, 189.7 ppm. Structures are presented in Fig. 2.

Results and Discussion

Description of the Producing Organism

The isolate, MF5658, was assigned to genus *Cytospora* (Coelomycetes) on the basis of the following characteristics: tough stromatic conidiomata; the production of conidiophores in a well-defined palisade layer within a convoluted, multilocular chamber; highly branched conidiophores; enteroblastic conidiogenesis; small, hyaline, more-or-less allantoid conidia; and dark mycelial colors[11,12]. Members of the genus *Cytospora* typically inhabit stems of woody plants. *Cytospora* species are often the conidial states in the life cycles of *Valsa* and *Leucostoma* species (Ascomycetes, Diaporthales), but no sexual state of this strain was observed in culture. The stromatic tissues, conidiophores, and conidia of MF5658 are similar to many of the common *Cytospora* anamorphs of *Valsa* species described by SPIELMAN[12]. Because no workable identification system exists for these fungi in culture, and because our strain was derived from vegetative growth from living bark rather than from spores produced in an identifiable fruiting structure on the host, it is impossible to speciate with certainty this strain of *Cytospora*. Descriptions of its morphology on different mycological media and micromorphology of its conidiomata are presented below.

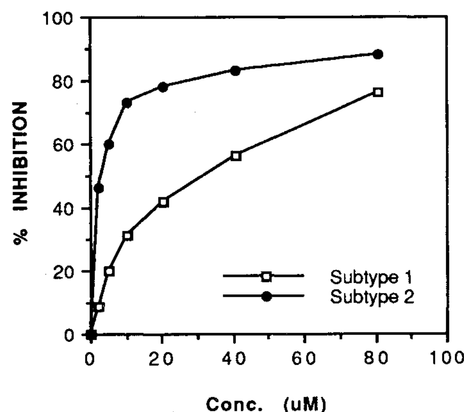
Colonies on oatmeal agar (Difco Laboratories) attaining 66~70 mm in diameter, with little development of aerial mycelium, appressed to submerged, pruinose or slightly floccose around inoculation point, dull, hyaline to pale olive when young, Buffy Olive, Light Yellowish Olive, soon dark olive to dark olivaceous black, Olive, Dark Greenish Olive, Deep Olive, Dark Grayish Olive, Dark Olive, with mottled pigment development over outer third of colony, with scattered stromatic conidiomatal pustules, pustules at first white, but soon pale grayish olive to olivaceous brown, margin submerged, irregular.

Colonies on EMERSON's YpSS (Difco Laboratories) attaining 35~45 mm in diameter, dull, aerial mycelium pruinose to downy, with scattered stromata, hyaline at margin but soon pale olive to olivaceous gray, Dark Olive-Buff, Citrine Drab, finally dark olive, Dark Greenish Olive, Dark Grayish Olive, margin minutely feathery or fimbriate. Colonies on malt-yeast extract agar (Difco Laboratories) attaining 70~75 mm in diameter, shiny to moist, aerial mycelium appressed or minutely floccose around inoculation point, hyaline to greenish yellow Yellowish Citrine, Serpentine Green, Dull Citrine, soon grayish olive to dark olive, Deep Grayish Olive, Dark Grayish Olive, Dark Olive, with scattered stromatic pustules in older portions, developing, long irregular plumose branches, with submerged, irregular margins.

Conidiomata scattered on colony surface, up to 1.5 mm wide, up to 2.5 mm tall, tough, stromatic, with base embedded in agar, pulvinate or hemispherical when young, becoming short cylindrical in age, with rounded apex, or sometimes with subapical constrictions, with surface minutely pubescent, dehiscing irregularly in age to yield a gelatinous spore mass or spore horns, with outer cortex a textura intricata, golden to dark brown, containing a irregularly convoluted conidial chamber, which overlies or surrounds an inner cortexwith inner cortex a textura intricata to texture angularis, dark brown to black. Conidiophores arranged in a dense palisade layer, lining the internal stromatic cavity, arising from broad, short cylindrical to subglobose hyphae, branching at 3~5 levels, acropleurogenous or not, thin-walled, hyaline, smooth. Conidiogenous cells enteroblastic, phialidic, cylindrical to awl-shaped, tapered to a narrow apex, $5\sim12\times1\sim3\,\mu$m, with minute pore at conidiogenous locus. Conidia $3\sim5\times1\sim1.5\,\mu$m, cylindrical, narrowly ellipsoidal, or allantoid, exuded from conidiomata in a golden brown to yellowish olive gelatinous mass or in spore horns. Hyphae septate, branching, hyaline to blackish brown, occasionally incrusted in age, up to 12 μm in diameter.

Fig. 1. Competition of [^{125}I]-Tyr^4-angiotensin II with component A in binding to rat adrenal glands.

□ AT_1, ● AT_2.



Tested in the *in vitro* assay for inhibition of angiotensin II binding to rat adrenal glands. The assay consists of 100 mM Tris-HCL pH 7.4, 5 mM $MgCl_2$, 0.2% BSA, 0.2 mg/ml bacitracin, 0.04 nM [^{125}I]-Tyr^4-angiotensin II and 128 ug/ml rat adrenal gland membranes. After a 90 minute incubation period at 37°C, the assay was filtered by cell harvester, punched into minivials and counted using a 4-probe gamma counter.

Fig. 2. Structure of Cytosporin A, B and C.

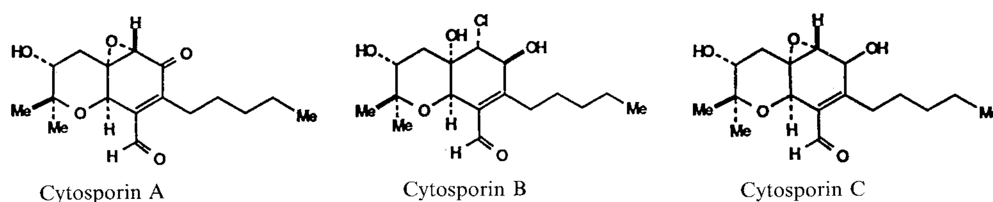


Table 1. L-921,301 Assay Results.

Compound	IC_{50} (μM)	
	AT_1	AT_2
Component A, major	25～30	1.5～3.0
Component B, minor	>290	55～70
Component C, minor	>320	30～40

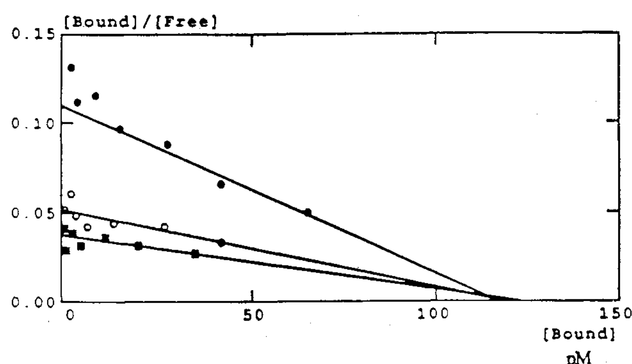
Characterization of Angiotensin II Inhibitors

In the process of screening microbial extracts, three novel compounds were found as specific inhibitors of angiotensin II binding to receptors of AT_2. The dose response competition of [^{125}I]-Tyr^4-angiotensin II and component A in binding to rat adrenal glands is depicted in Fig. 1. The IC_{50} of component A in the biochemical assay was found to be approximately 25～30 μM (8～10 μg/ml) in AT_1 and 1.5～3 μM (0.5～1 μg/ml) in AT_2 (Table 1). These results were obtained by blocking one of the two angiotensin II binding sites (AT_1 or AT_2) in rat adrenal glands as described in our previous report[4].

Scatchard analysis of [^{125}I]-Tyr^4-angiotensin II binding to AT_2 sites of rat adrenal glands in the absence of the inhibitor and in the presence of the inhibitor at 2 and 4 μM (Fig. 3) suggests reversible inhibition by this compound and thus it appears to be a competitive inhibitor.

Fig. 3. Scatchard analysis of [^{125}I]-Tyr^4-angiotensin II binding to rat adrenal glands in the absence of the inhibitor and in the presence of the inhibitor at 2 and 4 μM.

● Control, ○ 2 μM component A, ■ 4 μM component A.



New Technology

Since the evaluation of these compounds, the angiotensin II receptor binding assay has been further automated to provide for more efficient and accurate results. The Tecan Megaflex 4-tip pipetting station and the Tomtek Quadra 96 are used for sample addition and the LKB beta plate counter and Packard Topcount are used for counting in either a 6×16 well format or an 8×12 well format, respectively. More recently, Amersham International has developed a scintillation proximity assay[13] for use with angiotensin II, as well as other receptors and enzymes, that simplifies and increases the speed of performing the assays. With this new technique, there is no need to separate bound from free ligand and the assay can be performed and counted in 96 well (8×12) plates without the use of filtermats or counting tubes.

Other Angiotensin II Antagonists from Natural Products

In recent years, several other agents from natural products have been shown to exhibit anti-angiotensin II activity. TAN-1446, an angiotensin II receptor antagonist was prepared from the culture of *Penicillium restrictum* and is useful as an agent for preventing and/or treating hypertension or congestive cardiac insufficiency[14]. Halistanol trisulfate, a sulfated steroid derivative, was isolated from the extracts of two different marine sponges of the genus, Topsentia. Through kinetic studies, halistanol trisulfate was found to be a competitive inhibitor of the peptide substrate, [val5]-angiotensin II[15]. A tetrazole derivative prepared by the fermentation of a Streptomyces species MA6966 (ATCC 55293) has been found useful in the treatment of hypertension and congestive heart failure[16]. Several novel angiotensin II antagonists were manufactured using a novel Strepto-

myces species[17]. The agents discussed above display functions similar to those displayed by Cytosporin A, B and C described in our paper.

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