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Semi-Synthetic Ecdysteroid 6-Oxime Derivatives of 20-Hydroxyecdysone Possess Anti-Cryptococcal Activity

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Abstract: Cryptococcosis, a life-threatening fungal infection, frequently occurs in patients suffering from AIDS. The treatment of the disease is hampered by the limited number of the effective drugs and the increasing resistance; therefore, to find new active substances is needed. As meningitis is the most serious infection affecting the AIDS patients, effective drugs have to be capable of entering to the central nervous system. Ecdysteroids are natural bioactive molecules with considerable anabolic activity and without toxic side effects on humans. The aim of this work was to study the anti-cryptococcal activity of a natural ecdysteroid, 20E, and its three semi-synthetic derivatives obtained by structural modification of the original molecule. We established the minimum inhibitory concentration of the compounds with microdilution method and demonstrated their fungicidal activity by flow cytometry and cultivation of the drug-treated cells. The interaction of the compounds with each other and efflux transporter inhibitors was assessed by checkerboard titration method. Two derivatives, 20E-EOx and 20E-ZOx, inhibited the growth of *Cryptococcus neoformans* with minimum inhibitory concentration 2 mg/mL and 1 mg/mL, respectively; both compounds possess fungicidal effect. A combination of the ecdysteroids with each other and verapamil resulted in additive interaction. This study confirmed that structural modification of an originally non-antimicrobial molecule can enhance its effectiveness.

Keywords: ecdysteroid; semi-synthetic derivatives; *Cryptococcus neoformans*; efflux transporter inhibitors

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1. Introduction

Cryptococcosis is a human disease caused by opportunistic pathogenic yeasts, *Cryptococcus neoformans* and *C. gattii* [1]. The infectious particles—basidiospores or blastoconidia—enter the human body via inhalation or, rarely, via skin lesions [2]. The infection usually remains latent in immunocompetent individuals, but in immunocompromised patients, the pathogen disseminates in the body and reaches the central nervous system, causing serious life-threatening cryptococcal meningitidis [3,4].

Today, three drugs are available for the treatment of cryptococcosis: amphotericin B, fluconazole and flucytosine; however, the high toxicity of amphotericin B and the increased resistance to fluconazole and flucytosine hamper the treatment of the disease [5,6]. Therefore, new alternative therapeutic strategies, preferably new anticryptococcal compounds, are needed to cure the infected patients [6].

Ecdysteroids represent the largest class of natural hydroxysteroids, occurring both in the flora and the fauna [7]. These compounds are generally known as the structural analogues of ecdysone and are responsible for regulating the molting and metamorphosis of arthropods [8]. However, ecdysteroids are bioactive in mammals too, and since their discovery in the 1950s, several beneficial biological effects had been described in their regard [9].

From these, their non-hormonal, seemingly adverse-effect-free anabolic activity is a subject of significant market interest. Although the number of human clinical studies available on ecdysteroids is still relatively low, the most abundant analogue, 20-hydroxyecdysone (20E), can be often met on the shelves of specialized shops as a food supplement ingredient [10–12]. Nevertheless, as a response to the indeed growing number of evidences on the physical performance enhancing effect of ecdysteroids, World Anti-Doping Agency (WADA) added 20E on the list of monitored substances in 2020 [https://www.wada-ama.org/sites/default/files/wada_2020_english_monitoring_program_.pdf] (accessed on 6 December 2022).

Ecdysteroids may have a potential use in the treatment of seriously ill COVID-19 patients. A clinical picture of the disease includes acute respiratory distress syndrome, resulting from the infection-related balance change in the renin-angiotensin system (RAS). 20E is assumed to restore the balance of the RAS through the activation of the Mas receptor (MasR), a key player in the system, promoting the survival and condition improvement of seriously ill patients [13,14]. In this regard, 20E is under clinical trials since 2020 [15].

Additionally, ecdysteroids are intensively studied in terms of various other bioactivities, such as antidiabetic [16,17], adaptogenic [18] or antitumor effects [19–21], but little is known regarding their antimicrobial potential. In a recent study, two phytoecdysteroids, ajugasterone C and 22-*epi*-ajugasterone C, isolated from *Serratula cichoracea*, were reported to exert antimicrobial properties against multiresistant strains such as *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae* and *Candida albicans* [22]. In an older study, 20E and its semi-synthetically prepared acyl derivatives were tested for their antimicrobial properties against microbes inducing inflammatory and purulent processes [23]. Interestingly, while the parental compound 20E did not exhibit antimicrobial effect with respect to most microbial strains studied, the introduction of 1, 3 or 4 acetyl groups to its structure resulted in active derivatives with enhanced antibacterial properties. This particular observation might underline the biological relevance of synthetic structural modifications in the case of ecdysteroids.

Based on this, the aim of our current study was to further explore the antimicrobial properties of semi-synthetic ecdysteroids, by preparing a series of nitrogen-containing 20E derivatives, and then test them against human pathogenic yeast *Cryptococcus neoformans*.

2. Materials and Methods

2.1. Strains and Cultivation Conditions

Cryptococcus neoformans strains (Table 1) were grown overnight in YPD medium (1% pepton, 1% dextrose, 0.5% yeast extract) at 30 °C. The cells were harvested by centrifugation (5 min, 3000 g) washed with sterile distilled water and suspended in RPMI 1640 medium (Biosera, Kampenhout, Belgium). The cell concentration of the suspensions was established by counting the cells in Bürker chamber then diluted to 1×10^5 cells/mL in RPMI 1640 medium.

Table 1. List of the tested strains.

Species	Strain Number
Cryptococcus neoformans	IFM 5844
Cryptococcus neoformans	IFO 410

IFM: Culture Collection of the Research Centre for Pathogenic Fungi and Microbial Toxicoses, Chiba University, Chiba, Japan. IFO: Institute for Fermentation, Osaka, Japan.

2.2. Solvents, Chemicals and Chromatographic Conditions

All solvents and reagents used for either chemical (e.g., organic synthetic) or microbiological purposes (e.g., indomethacin, verapamil, quinidine) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The obtained chemicals were used without any further purification.

The synthetic reactions were monitored by thin layer chromatography on Kieselgel $60F_{254}$ silica plates purchased from Merck (Merck KGaA, Darmstadt, Germany), and characteristic spots of products were examined under UV illumination at 254 and 366 nm. Flash chromatography was performed on a Combiflash Rf+ instrument (Teledyne ISCO, Lincoln, NE, USA) equipped with diode array and evaporative light scattering detection (DAD-ELSD). The apparatus was used with commercially available prefilled normal-phase "RediSep" columns (TELEDYNE Isco, Lincoln, NE, USA). Analytical-scale reversed-phase (RP) HPLC separations were carried out on a Jasco HPLC instrument (Jasco International Co., Ltd., Hachioji, Tokyo, Japan) equipped with an "MD-2010 Plus" PDA detector. The measurements were performed on a Kinetex XB-C18 250 mm × 4.6 mm column (Phenomenex Inc., Torrance, CA, USA) and compound purities were determined using the peak area % data of the PDA chromatogram recorded between 210 and 410 nm. During the analytical-scale HPLC measurements, the applied flow rate was a constant 1 mL/min. For preparative RP-HPLC purposes, an Armen Spot Prep II HPLC purification system (Gilson, Middleton, WI, USA) equipped with a dual-wavelength UV-VIS detector was utilized. The purifications were performed on a Kinetex XB-C18 250 mm × 21.2 mm column (Phenomenex Inc., Torrance, CA, USA) with adequately chosen eluents of methanol/water or acetonitrile/water.

2.3. Structure Elucidation

Structure elucidation was carried out by NMR spectroscopy and HR-MS. NMR spectra were recorded at 25 °C on a Bruker Avance DRX-500 NMR spectrometer (Bruker, Billerica, MA, USA) at 500 MHz (^{1}H) and 125 MHz (^{13}C). In general, 5–6 mg of the corresponding ecdysteroid was dissolved in DMSO-d_6 and transferred to NMR tubes for recording spectra. Chemical structures of the derivatives were determined by employing comprehensive one- and two-dimensional NMR methods [20]. Mass spectra were recorded on an Agilent 1100 LC-MS instrument (Agilent Technologies, Santa Clara, CA, USA) coupled with Thermo Q-Exactive Plus orbitrap analyzer (Thermo Fisher Scientific, Waltham, MA, USA) used in positive ionization mode.

2.4. Availability and Semi-Synthesis of the Tested Compounds

20-hydroxyecdysone (20E) was purchased from Shaanxi KingSci Biotechnology (Xi'an, China) in a purity of 90%. By applying recrystallization from ethyl acetate–methanol (2:1, *v/v*), 20E was afforded in an RP-HPLC purity of 97.8%.

Ecdysteroid derivatives 20E-EOx and 20E-ZOx were prepared via semi-synthesis from 20E, following our previously described procedure [24]. Briefly, an aliquot of 1 g of hydroxylamine hydrochloride (14.39 mmol) was dissolved in 15 mL of freshly distilled ethanol. An ethyl alcoholic solution of potassium hydroxide (807.4 mg, 14.39 mmol) was added to the solution to liberate the hydroxylamine free base from its salt, and, subsequently, 1 g of 20E was added. The reaction mixture was stirred for 2 weeks at the boiling point. Following this, silica gel (4 g) was added to the solution, which was then evaporated to dryness under reduced pressure to prepare the sample for dry loading flash chromatographic purification. The normal-phase chromatographic separation was performed on a 24 g silica column (flow rate 35 mL/min, run time: 30 min) with a gradient of dichloromethane (A) and methanol (B), from 0% to 35% of solvent B in A. The eluted product mixture consisted of 20E-EOx and 20E-ZOx in an approximately 2:1 ratio. Subsequently, the ecdysteroid oxime isomers were separated using preparative RP-HPLC (16% aq. acetonitrile, flow rate: 15 mL/min), affording 20E-EOx in 49% yield (509 mg) and 20E-ZOx in 18% yield (181 mg).

20EL was synthesized from 20E-EOx, by slightly modifying our previously published procedure [20]. Briefly, a 300 mg aliquot of 20E-EOx (0.61 mmol) was dissolved in 20 mL of acetone. Subsequently, 83.3 mg of Na_2CO_3 (0.786 mmol, 3 equiv.) and 461.6 mg of *p*-toluenesulfonyl chloride (TsCl, 2.42 mmol, 4 equiv.) were added to the solution, and the mixture was stirred for 24 h at room temperature. Following this, silica gel (3 g) was added to the solution, which was then evaporated to dryness on a rotary evaporator to

prepare the sample for dry loading flash chromatographic purification. The product was purified on a 24 g silica column (flow rate 35 mL/min, run time: 30 min) with a gradient of dichloromethane (A) and methanol (B), from 0% to 35% of solvent B in A. In a second chromatographic step, the product's concentrated sample (approx. 230 mg) was further purified by preparative RP-HPLC (55% aq. methanol, flow rate: 15 mL/min), to afford ecdysteroid lactam 20EL in a yield of 61.3% (183.9 mg).

20EL. White solid; Isolated yield: 61.3% (183.9 mg); RP-HPLC purity: 99.7%; HR-MS: $C_{27}H_{45}NO_7$, $[M + H]^+$ Calculated 496.32743, found: 496.32814. ^{1}H NMR (500 MHz, [D_6]DMSO) δ = 7.60 ppm (1H, d, J = 7.93 Hz), 5.48 ppm (1H, s), 4.45 ppm (1H, s), 4.37 ppm (1H, d, J = 5.95 Hz), 4.30 ppm (1H, d, J = 5.04 Hz), 4.22 ppm (1H, d, J = 2.75 Hz), 4.03 ppm (1H, s), 3.80 ppm (1H, m), 3.69 ppm (1H, m), 3.50 ppm (1H, s), 3.22 ppm (1H, m), 3.20 ppm (1H, m), 3.13 ppm (1H, m), 2.28 ppm (1H, m), 1.93 ppm (1H, m), 1.90 ppm (1H, m), 1.85 ppm (1H, m), 1.84 ppm (1H, m), 1.73 ppm (1H, dd, J = 13.12, 4.73), 1.67 ppm (1H, m), 1.65 ppm (1H, m), 1.60 ppm (1H, m), 1.56 ppm (1H, m), 1.52–45 ppm (3H, m), 1.41 ppm (1H, m), 1.29 ppm (1H, m), 1.26 ppm (1H, m), 1.12 ppm (1H, m), 1.08 ppm (3H, s), 1.06 ppm (3H, s), 1.05 ppm (3H, s), 0.85 ppm (3H, s), 0.78 ppm (3H, s); ^{13}C NMR (125 MHz, [D_6]DMSO) δ = 167.4 ppm, 153.5 ppm, 119.0 ppm, 85.8 ppm, 76.1 ppm, 75.6 ppm, 68.6 ppm, 68.4 ppm, 66.3 ppm, 53.3 ppm, 48.9 ppm, 47.8 ppm, 41.3 ppm, 40.8 ppm, 39.4 ppm, 38.4 ppm, 34.0 ppm, 32.3 ppm, 31.2 ppm, 29.7 ppm, 29.0 ppm, 26.0 ppm, 23.4 ppm, 23.0 ppm, 20.7 ppm, 20.2 ppm, 17.1 ppm. One- and two-dimensional NMR spectra of 20EL is provided as Supplementary Materials, Figures S1–S5, and its high-resolution mass spectrum as Figure S7.

Stock solutions for the bioactivity tests were prepared in methanol at 40 mg/mL concentration. Verapamil and quinidine were dissolved in DMSO at 5.0 (verapamil) and 2.5 (quinidine) mg/mL concentration, indomethacin stock solution was prepared in ethanol at 7.0 mg/mL concentration.

2.5. Antifungal Activity Assay

The inhibitory effect of the ecdysteroids was tested against *C. neoformans* strains. The minimum inhibitory concentration (MIC) was established with the micro-dilution method in 96-well microtiter plates. Briefly, 100 µL serially two-fold-diluted ecdysteroid compound solution was added to 100 µL of cell suspension. The final concentration of the ecdysteroids in the wells ranged from 2.00 to 0.125 mg/mL. After 72 h of incubation at 30 °C the optical density of the cultures was measured at 620 nm in SPECTROstar Nano plate reader (BMG LabTech, Offenburg, Germany). Minimum inhibitory concentration was defined as growth inhibition ≥90% compared to 100% growth of the untreated control. The experiments were carried out in three biological repeats always in triplicates.

Next, 5-µL samples were taken from the untreated and 20E-EOx- or 20E-ZOx-treated cultures and were added to 95 µL sterile distilled water and diluted to 10- and 100-fold. A volume of 5 µL from each dilution was placed on solid YPD medium and the growth of the strains was detected after 48-h incubation at 30 °C.

2.6. Yeast Viability Assay

The viability of *C. neoformans* IFM 5844 cells was examined by calcein-AM assay using flow cytometry. Briefly, 2×10^5 cells were exposed to 1.00 mg/mL 20E-ZOx in RPMI 1640 medium at 30 °C for 3 h. After the treatment, cells were washed twice with sterile distilled water, suspended in 100 µL RPMI 1640. Calcein-AM (Invitrogen, Waltham, MA, USA) was added in 10 µM concentration and the suspension was further incubated in the dark at 30 °C for 3 h. Cells were washed twice with sterile distilled water and the fluorescence intensity was measured by flow cytometer (FlowSight®, Amnis-EMD Millipore) using a 488-nm excitation laser. Non-treated cells stained with calcein-AM and cells stained with calcein-AM after exposure to 96% ethanol for 30 min were used as controls. Four independent experiments were carried out, the fluorescence intensity of 10,000 cells was detected in each sample during each experiment.

2.7. Combined Treatment of C. neoformans with 20E-EOx, 20E-ZOx and Transporter Inhibitors

The effect of the combinations of 20E-EOx and 20E-ZOx and efflux pump inhibitors (indomethacin, quinidine and verapamil) was determined by standard checkerboard titration method [25]. The inhibitors were tested in a concentration range from 200 to 12.50 µg/mL while the ecdysteroid concentration varied in concentration from 2.00 to 0.125 mg/mL. The initial cell concentration in each well was 1×10^5 cell/mL. After the incubation for 72 h at 30 °C, the optical density of the cultures was detected at 620 nm in SPECTROstar Nano plate reader (BMG LabTech, Offenburg, Germany). The inhibitory concentrations were determined for each compound alone and in combinations. The experiments were carried out at least three times.

2.8. Data Analysis

The mode of the interaction was evaluated by Abott formula [25] using Microsoft Excel Software version 16.0.

Ie is the expected inhibition for a given interaction calculated as $I_e = X + Y - (XY/100)$. X and Y are inhibition expressed as percent given for each compound when used alone. $I_{0:}$ is the observed inhibition. Interaction ratio (IR) = I_0/I_e. When IR is between 0.5 and 1.5, the interaction is additive, IR > 1.5 means synergistic interaction, while IR < 0.5 refers to antagonism.

3. Results

3.1. Semi-Synthetic Modification of 20E and Its Derivatives

A total of three semi-synthetic ecdysteroid derivatives, including one new compound (20EL) was prepared through the structural modification of 20E (Figure 1). At first, the 6-carbonyl moiety of 20E was converted to an oxime following previously published procedures, which resulted a mixture of two geometrically isomeric oxime analogues [20,26]. Subsequently, 20-hydroxyecdysone (6E)-oxime (20E-EOx) and 20-hydroxyecdysone (6Z)-oxime (20E-ZOx) had been succesfully separated by RP-HPLC.

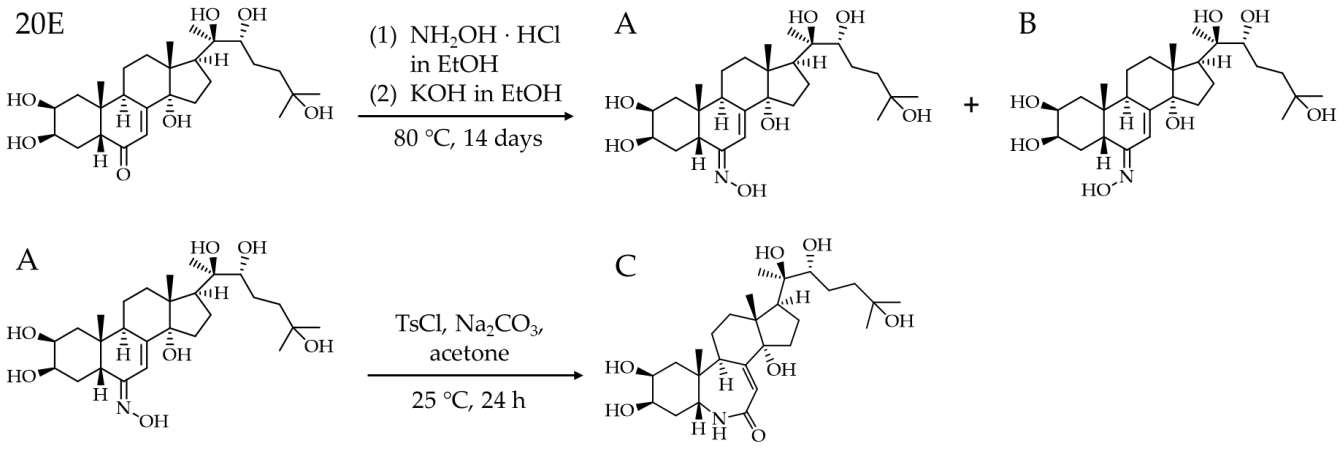

Figure 1. Semi-synthetic preparation of the tested ecdysteroids. (**A**): 20E-EOx; (**B**): 20E-ZOx; (**C**): 20EL.

In a second synthetic step, the B-ring of the ecdysteroid oximes was attempted to be transformed to a seven-membered lactam ring. According to previous literature data, the Beckmann-rearrangement of an oxime to a lactam is a stereospecific transformation involving the migration of the chemical moiety located in anti-position with respect to the oxime's hydroxyl. In case of 20E-ZOx, the group in anti-position is C^7H with an sp^2-hybridized carbon atom whose migration is chemically inhibited, hindering the rearrangement of the subtrate to a lactam [20,27]. Therefore, the transformation was carried out solely from 20E-EOx, which resulted the corresponding lactam derivative (20EL) in fair yield. The

synthetic scheme, including the chemical structure of the ecdysteroid derivatives is shown in Figure 1.

3.2. Anti-Cryptococcal Activity of 20-Hydroxyecdysone and Its Derivatives

The effect of 20-hydroxyecdysone (20E) and its three semi-synthetic derivatives, 20E-EOx, 20E-ZOx and 20EL on the growth of an opportunistic human pathogen yeast *C. neoformans* was tested in microdilution assay. Two-fold serial dilutions of the compounds in concentration range 2.00–0.125 mg/mL was used to determine the minimal inhibitory concentrations. All of the compounds showed inhibitory activity against both *C. neoformans* strains. However, minimal inhibitory concentration could be determined only for 20E-EOX and 20E-ZOx as 20E and 20EL had only moderate effect on the growth of the strains. The minimal inhibitory concentration of 20E-EOx was 2.00 mg/mL while 20E-ZOx proved more effective with MIC 1.00 mg/mL (Table 2).

Table 2. Minimal inhibitory concentration of the tested ecdysteroids.

Strains	Minimum Inhibitory Concentration (mg/mL)			
	20E	**20EL**	**20E-EOx**	**20E-ZOx**
IFM 5844	>2.00	>2.00	2.00	1.00
IFO 410	>2.00	>2.00	2.00	1.00

To determine if the inhibitory effect of 20E-EOx and 20E-ZOx arises from fungistatic or fungicidal mode of action, the cells of *C. neoformans* IFM 5844 strain after the treatment with 20E-EOx and 20E-ZOx was inoculated onto the surface of solid YPD medium. The results showed that both compounds have fungicidal action in their minimum inhibitory concentration (Figure 2).

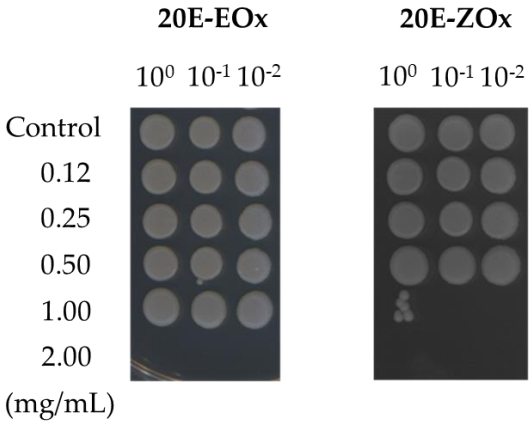

Figure 2. Growth of *C. neoformans* IFM 5844 after 20E-EOx or 20E-ZOx treatment. (10^0, 10^{-1} and 10^{-2} represent the dilution factor).

3.3. 20E-ZOx Reduces the Viability of C. neoformans IFM 5844 Cells

The inhibitory activity of 20E-ZOx on the viability of C. neoformans IFM 5844 cells was tested by calcein-AM assay in flow cytometry. The cells treated with 20E-ZOx at MIC value for 3 h showed reduced fluorescence comparing to the stained untreated control (Figure 3) indicating that 20E-ZOx is effectively inhibits the viability of the cells even during short incubation time.

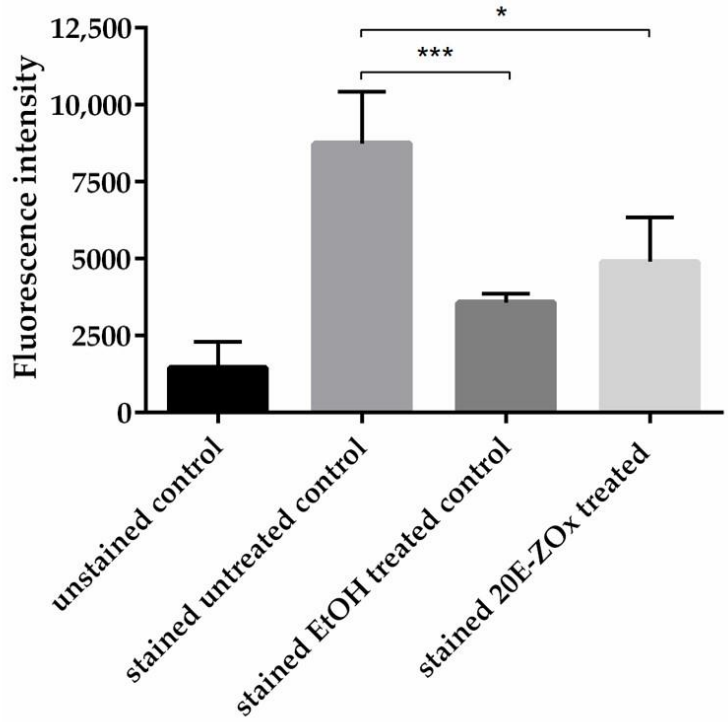

Figure 3. The effect of 20E-ZOx on the viability of *C. neoformans* IFM 5844 cells. The values represent the mean ± standard deviation calculated from four independent experiments (*, $p \leq 0.05$, ***, $p \leq 0.001$, unpaired *t* test).

3.4. In Vitro Interaction between Ecdysteroids on C. neoformans IFM 5844 Cells

The studied *C. neoformans* strains were slightly sensitive to 20E and 20EL. Combined treatment of strain IFM 5844 with 20E and 20EL did not show interaction between them. Combination of 20E-ZOx, with 20E, 20EL and 20E-EOx derived additive interaction (Table 3). Additive interaction was observed when 20E-EOx was combined with 20E and 20EL.

Table 3. In vitro interaction between 20E-ZOx (1.00 mg/mL) and 20E.

20E (mg/mL)	I_0 (%)	I_e (%)	IR	Type of Interaction	Growth Inhibition Rate (%)
2.00	37	92	0.80	Additive	100
1.00	28	91	0.88	Additive	100
0.50	26	30	0.89	Additive	100
0.25	4	27	1.11	Additive	100

3.5. Combined Treatment of C. neoformans IFM 5844 Cells with 20E-ZOx and Efflux Pump Inhibitors

The application of efflux pump inhibitors can increase the effect of certain drugs. Therefore, we combined 20E-EOx and 20E-ZOx with efflux pump inhibitors, indomethacin, quinidine or verapamil, respectively, to check if they can enhance the activity of the studied ecdysteroid compounds. Neither of the efflux pump inhibitors affected the growth of *C. neoformans* alone in the applied concentration range. The interaction ratio (IR) calculated by Abbott formula indicated additive interaction between verapamil and 20E-EOx similarly verapamil and 20E-ZOx (Table 4). No interaction was observed when indomethacin or quinidine was combined with 20E-EOx or 20E-ZOx.

Table 4. Combined effect of 20E-EOx and 20E-ZOx with verapamil on C. neoformans IFM 5844.

20E-EOx (mg/mL)	I_0 (%)	I_e (%)	IR	Type of Interaction	Growth Inhibition Rate (%)
2.00	98	30	0.77	Additive	98
1.00	13	30	0.56	Additive	24
0.50	10	30	0.50	Additive	20
0.25	9	30	0.45	Additive	17
20E-ZOx (mg/mL)	**I_0 (%)**	**I_e (%)**	**IR**	**Type of Interaction**	**Growth Inhibition Rate (%)**
2.00	89	30	0.83	Additive	99
1.00	79	30	0.88	Additive	96
0.50	40	30	0.71	Additive	49
0.25	36	30	0.68	Additive	45

4. Discussion

Along with the worldwide spreading of antibiotic resistance and frequent occurrence of hardly curable microbial infections, the demand to find new compounds or application of present drugs against resistant microbes has augmented [28]. Recently, numerous natural substances and bioactive compounds used in human therapy for other purposes turned out to have antimicrobial activity. These molecules can be used in the treatment of microbe-caused diseases or can be the basis of the development of new antimicrobials.

Ecdysteroids are natural substrates that commonly present in wide range of living organisms. Based on their favorable effect on the metabolism in mammals they are frequently used as dietary supplement at low amounts (up to 1 mg day), but bodybuilders use higher doses (up to 1000 mg per day) [11,29]. Previous studies on the antimicrobial property of natural ecdysteroids, e.g., 20-hydroxyecdysone could not demonstrate any activity against bacteria of different genus, but recent study revealed antibacterial and antifungal activity of two phytoecdysteroids [22,23]. In this study, we investigated the antimicrobial effect of the natural ecdysteroid 20E, and its three semi-synthetic derivatives. The semi-synthetic derivatives were prepared by structural modification of the original molecule, 20E. Despite extensive survey against different species (*Arthrobacter globiformis, Agrobacterium tumefaciens, Bacillus subtilis, Bacillus cereus, Escherichia coli, Klebsiella pneumoniae, Micrococcus luteus, Pseudomonas aeruginosa, Serratia marcescens, Staphylococcus aureus, Xanthomonas campestris*), none of the compounds showed antibacterial activity in concentration range 2.00 mg/mL–0.125 mg/mL (data not shown). However, inhibitory effect was detected against an opportunistic pathogenic yeast species *C. neoformans*. 20E had only moderate activity against *C. neoformans* strains as no MIC could be established in the tested concentration range. The modification of the molecule enhanced the activity and the two derivatives 20E-EOx and 20E-ZOx effectively inhibited the growth of the studied strains. There was some difference between the two derivatives as 20E-ZOx was more effective than 20E-EOx. The third derivative, 20EL, proved less potent than 20E-EOx and 20E-ZOx. Our results demonstrated that the two active compounds killed the cells of the tested strains.

The treatment of cryptococcosis is difficult due to the limited number of the available drugs. The most effective therapy is the combination of amphotericin B and flucytosine, but the long-term therapy can have serious side effects [6]. Fluconazole is also frequently suggested for the treatment of cryptococcosis, but the increasing resistance against this drug hampers its application [30]. The function of efflux transporters can be one reason of the resistance [30]. Therefore, the combination of transporter inhibitors with antimicrobial drugs can be beneficial as it can decrease the effective drug dose during the therapy. We combined 20E-EOx and 20E-ZOx with efflux transporter inhibitors verapamil, indomethacin and quinidine [31–33]. The combination of 20E-EOx or 20E-ZOx with verapamil led to additive interaction, while the combination with indomethacin or quinidine did not have any reaction, suggesting that 20E-EOx and 20E-ZOx are not substrates of the transporters inhibited by these drugs.

Our results confirmed that the synthetic modification of a natural molecule can increase its biological activity and it might aid in the potential therapeutic application.

5. Conclusions

Cryptococcus neoformans can cause life-threatening infections in humans, especially in immunocompromised individuals. The treatment of the disease is complicated; therefore, the search for new effective drugs is compelling. Natural ecdysteroid, 20EL showed moderate inhibitory activity against this pathogen but the activity of its semi-synthetic derivatives was increased. Our results demonstrated that synthetic modification of a natural compound, 20-hydroxyecdysone, can improve its anti-cryptococcal activity.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/microbiolres13040071/s1, Figure S1: ^{1}H NMR spectrum of 20EL.; Figure S2: ^{13}C (JMOD) spectrum of 20EL.; Figure S3: Two-dimensional HSQC spectrum of 20EL.; Figure S4: Two-dimensional COSY spectrum of 20EL.; Figure S5: Two-dimensional HMBC spectrum of 20EL. Figure S6: Chemical structure of 20EL with ^{1}H and ^{13}C NMR signal assignments.; Figure S7: High resolution mass spectrum of 20EL recorded in positive ionization mode.

Author Contributions: Conceptualization, I.P., C.V. and A.H.; methodology, B.S., K.B., M.V. (Mónika Vörös), M.B.H. and M.V. (Máté Vágvölgyi); software, B.S. and M.V. (Máté Vágvölgyi); validation, I.P., C.V. and A.H.; formal analysis, C.V. and A.H.; investigation, B.S., K.B., M.V. (Mónika Vörös), M.B.H. and M.V. (Máté Vágvölgyi); data curation, I.P. and M.V. (Máté Vágvölgyi); writing—original draft preparation, I.P. and M.V. (Máté Vágvölgyi); writing—review and editing, C.V. and A.H.; supervision, I.P., C.V. and A.H. All authors have read and agreed to the published version of the manuscript.

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