# Simple Carbohydrate Derivatives Diminish the Formation of Biofilm of Candida albicans

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# Abstract

The opportunistic human fungal pathogen Candida albicans rely on cell morphological transitions to develop biofilm and invade the host. In the current study, we developed new regulatory molecules, which inhibit the morphological transition of C. albicans from yeast-form cells to cells forming hyphae. These compounds, benzyl α-L-fucopyranoside and benzyl β-D-xylopyranoside, inhibit the morphological switching and adhesion of C. albicans to a polystyrene surface, resulting in a reduced biofilm formation. The addition of cAMP to cells treated with α-L-fucopyranoside restored the yeast-hyphae switch and the biofilm level to that of the untreated control. In the β-D-xylopyranoside treated cells, the biofilm level was only partially restored by the addition of cAMP, and these cells remained mainly as yeast-form cells.


# Introduction

A majority of human microbial infections involve the formation of biofilms, with serious implications for the use of medical devices such as prostheses, joint replacements, catheters, and pacemakers. Microbial biofilms are more resistant to antibiotics and antifungal drugs [1–7], and host immune responses [8, 9], and can promote further pathogen dissemination in the patient. Thus, it is important to develop new strategies for the prevention of biofilm formation, and to eliminate already formed biofilm.

*Candida albicans* is one of the most prevalent human opportunistic fungal pathogens. Although it is a common part of the human microbiome [10], this yeast can, under certain conditions, cause various diseases. Mucosal infections, such as vulvovaginal candidiasis, occur in up to 75% of all women [11]. Furthermore, immune-compromised individuals may develop candidiasis of the oral cavity, as well as systemic blood stream infections with high mortality [12].

The biofilms developed by *C. albicans* are made up of a dense matrix, composed of morphologically different cell types, i.e. blastospores (yeast-form cells), hyphae, and pseudo-hyphae cells. These cells form different biofilm layers, with a basal thin layer of yeast-form cells, and a thicker and more open hyphae layer [1, 13–15]. The hyphae development is an important virulence factor and facilitates the pathogen invasion through epithelial tissue of the host [16]. The cell walls of *C. albicans* hyphae and yeast-form cells differ in their composition, which affects its adhesion and also recognition by the host's immune system. For example, the hyphal glucan consists of a unique cyclic (1–3)-linked polymer backbone with long (1–6)-linked side chains [17]. Hyphae also have less mannan, compared to yeast-form cells [18]. The morphological transition from yeast-form cells to hyphae (i.e. switching) is a complex, tightly regulated process that is induced by several environmental conditions and quorum sensing molecules [16].

Carbohydrates have been explored for their possibilities to disturb or prevent adhesion of *C. albicans,*and its formation of biofilm and the extension of already existing biofilm [19–25]. In addition, some natural products of plant origin were recently shown to reduce the adhesion of *C. albicans* and its biofilm formation, by inhibiting the yeast-to-hyphae transition [26, 27].

In this study, we have synthesized and tested the effect of simple monosaccharides and glycosides (Chart 1) in relation to *C. albicans* biofilm development.

## Materials And Methods

# General methods, strains and growth conditions

Thin-layer chromatography was performed on precoated TLC glass plates with silica gel 60 $F_{254}$ 0.25 mm (Merck). Spots were visualized with UV light or by charring with an ethanolic anisaldehyde solution. Preparative chromatography was performed on Biotage Isolera One flash purification system using Biotage SNAP KP-Sil silica cartridges. NMR spectra were recorded at ambient temperatures on a Bruker Avance II at 400 MHz ($^{1}$H), and 100 MHz ($^{13}$C) and assigned using 2D methods (COSY, HMQC). Chemical shifts are reported in ppm, with reference to residual solvent peaks (δH $CHCl_3$ = 7.26 ppm and δC $CDCl_3$ = 77.0 ppm). Coupling constant values are given in Hz. $^{13}$C-NMR spectra are proton decoupled. High-resolution mass spectra (HRMS) were recorded on Waters QTOF XEVO-G2 (ESI+).

*Candida albicans* SC5314 [28] was grown at 37 °C in complete medium YPD (0.5% yeast extract, 1% peptone, 2% glucose) or minimal medium consisting of YNB (yeast nitrogen base without amino acids and ammonium sulphate, FORMEDIUM$^{TM}$, CYN0505) supplemented with 0.45% ammonium sulphate, 0.2% glucose and 100 mM L-proline. If needed 2% agar was used to solidify media.

The liquid minimal medium (YNB (yeast nitrogen base without amino acids and ammonium sulphate, FORMEDIUM$^{TM}$, CYN0505) supplemented with 0.45% ammonium sulphate, 0.2% glucose and 100 mM L-proline) *p*H 7.0 was used for biofilm assay (biofilm medium).

Different carbohydrates (Chart 1) were added to the biofilm medium at the final concentration 0.1–15 mg/mL. The cell permeable cAMP ($N^6$, 2'-*O*-Dibutyryladenosine 3',5'-cyclic monophosphate sodium salt, Sigma-Aldrich, D0260) was added to the biofilm medium at the final concentration of 10 mM to study if the sugars inhibition effect on the biofilm could be reversed.

# Biofilm assay

Prior the biofilm assay, yeast cultures were grown in liquid YPD medium for 24 hours until stationary phase ($OD_{600}$ 11–17), cells were then pelleted by centrifugation (1699 *g),,* washed with sterile water and cells were further inoculated into test biofilm medium (YNB (yeast nitrogen base without amino acids and ammonium sulphate) supplemented with 0.5% ammonium sulphate, 0.2% glucose and 100 mM L-proline*p*H 7.0)) at a final concentration of 0.2 $OD_{600}$/mL and incubated in 96-well flat-bottom polystyrene microtiter plates (Sigma-Aldrich, Corning® Costar® culture plates, CLS3596–50EA) for 24 or 48 h at 37 °C thermostat. At the defined time points, the biofilm was measured either by crystal violet staining as described [29, 30] or by improved XTT method, where the 200 mM glucose and XTT (2, 3-bis (2-methoxy–

4-nitro–5-sulfophenyl)–5-[(phenylamino)carbonyl]–2H-tetrazolium hydroxide, X4626, Sigma-Aldrich) were added to the reaction mixture [31]. The crystal violet (HT901–8FOZ, Sigma Aldrich) was added to the media at the final concentration of 0.05%. After 24 and/or 48 h of cells staining, plate wells were washed four times with 200 µL of water to remove planktonic cells, biofilms were then dried and dissolved in 200 µL of 96% ethanol. In XTT assay, the planktonic cells were removed by washing twice with PBS buffer. Total biomass and crystal violet biofilm staining measurements were performed at $OD_{560}$, the formazan formed during XTT biofilm assay was measured at $OD_{485}$ with FLUOstar OPTIMA plate reader, BMG LABTECH.

# Microfluidics and microscopy

Microfluidics plates (CellASIC® ONIX Y04D–02–5PK, Merck Millipore) were used with ONIX Microfluidic Perfusion System and were inoculated with yeast at 8 psi for 5 sec according to manufacturer recommendations, flowed at 1.5 psi with media tested (with/without carbohydrates, 15 mg/mL). The yeast growth and biofilm development were monitored over time on fully motorized and automated inverted widefield microscope Observer Z1 (Carl Zeiss) equipped with a sCMOS camera. The phase-contrast images were taken over time specified.

*Synthesis of Benzyl -L-fucopyranoside (4b)*

Acetyl chloride (0.43 mL, 6.1 mmol) was added to a stirred solution of benzyl alcohol (8.83 mL, 85.0 mmol) and L-fucose *4a* (1.0 g, 6.1 mmol). After 22 h at 50 C, the reaction mixture was allowed to reach rt before removal of benzyl alcohol by vacuum distillation (0.1 torr, 65 C). The dark brown crude residue was dissolved in pyridine (9.0 mL, 0.11 mol) and acetic anhydride (9.0 mL, 0.16 mol) and stirred at rt. After 15 h, toluene was added, and the solvent was removed under reduced pressure. Purification by column chromatography (Biotage, KP-Sil 50g, heptane:EtOAc 95:588:12) gave benzyl (2,3,4-*tri-O*-acetyl)L-fucopyranoside (986 mg, 56%, clear oil) and benzyl (2,3,4-*tri-O*-acetyl)L-fucopyranoside (247 mg, 14%, white amorphous solid). *Benzyl (2,3,4-tri-O-acetyl)-L-fucopyranoside:* $^{1}$H-NMR ($CDCl_3$) 7.37–7.28 (m, 5H) 5.42–5.38 (m, 1H) 5.30–5.29 (m, 1H) 5.15–5.12 (m, 2H) 4.71 ($d_{AB}$, 1H, $J_{AB}$ 12.4 Hz) 4.55 ($d_{AB}$, 1H, $J_{AB}$ 12.0 Hz) 4.17 (q, 1H, *J* 6.4 Hz) 2.16 (s, 3H) 2.03 (s, 3H) 1.98 (s, 3H) 1.11 (d, 3H, *J* 6.4 Hz); $^{13}$C-NMR ($CDCl_3$) 170.7, 170.5, 170.2, 137.3, 128.6, 128.1, 127.9, 95.6, 71.3, 70.0, 68.2, 68.2, 64.7, 20.9, 20.8, 20.8, 15.9; HRMS (ESI/QTOF) m/z [M + Na] calcd for $C_{19}H_{24}O_8Na$ 403.1363, found 403.1368. *Benzyl (2,3,4-tri-O-acetyl)-L-fucopyranoside:* $^{1}$H-NMR ($CDCl_3$) 7.37–7.28 (m, 5H) 5.28–5.22 (m, 2H) 4.98 (dd, 1H, *J* 3.6, 10.4 Hz) 4.92 ($d_{AB}$, 1H, $J_{AB}$ 12.4 Hz) 4.62 ($d_{AB}$, 1H, $J_{AB}$ 12.4 Hz) 2.18 (s, 3H) 2.00 (s, 3H) 1.98 (s, 3H) 1.25 (d, 3H, *J* 6.4 Hz); $^{13}$C-NMR ($CDCl_3$) 170.9, 170.4, 169.7, 137.2, 128.5, 128.0, 127.8, 99.8, 71.5, 70.7, 70.4, 69.3, 69.1, 20.9, 20.9, 20.8, 16.2; HRMS (ESI/QTOF) m/z [M + Na] calcd for $C_{19}H_{24}O_8Na$ 403.1363, found 403.1369. Benzyl (2,3,4-*tri-O*-acetyl)L-fucopyranoside (0.18 g, 0.63 mmol) was dissolved in MeOH (3 mL) and 1M NaOMe (0.15 mL, 0.15 mmol) was added. After 2 h, glacial acetic acid was added until neutral pH. The reaction mixture was co-evaporated with toluene several times. Purification by column

chromatography ($SiO_2$, $CH_2Cl_2$:MeOH 95:5) gave *4b* (90 mg, 57%) as white amorphous solid. Data analysis was according to published data [32].

## Results

# Carbohydrate derivatives

The unprotected carbohydrates (*1a, 2a, 3a,* and *4a)* and the methyl pyranosides (*1c, 2c,* and *3c)* were commercially available. The benzylated compounds *1b* [33], *2b* [34], and *3b* [35] have been described before. *4a* was benzylated at the anomeric position using benzyl alcohol and acetyl chloride [36] and deprotected to give compound *4b* in 32% yield (over two steps).

# Biofilm formation

To evaluate the effects of carbohydrate derivatives, the formation of biofilm was measured in liquid cultures, using *Candida albicans* SC5314 and the addition of 10 mg/mL of each compound. The formation of biomass/biofilm was measured after 24 h, using the crystal violet method [29]. The data are shown in Figure 1.

*Figure 1.* The biomass and biofilm formation of *C. albicans* in the minimal medium supplemented with different carbohydrate compounds at 10 mg/mL (crystal violet staining). Unfilled columns indicate biomass. Filled columns indicate biofilm. All experiments were performed in duplicates. The data was normalized by untreated control, which was set to 100%.

During the biofilm experiments, the *C. albicans* biomass substantially increased only on mannose (*1a)*,, but not on the other carbohydrates. None of the tested compounds inhibited biomass except for *1c,* that reduced the biomass formation by ~40% (Figure 1). The unprotected monosaccharides *1a-4a* and the methyl glycosides *1c-3c* did not show any significant effect on the formation of biofilm. In contrary, the benzyl-glycosides *3b* and *4b* reduced the formation of biofilm. After 24 hours of incubation, 7.1 and 4.3 times less biofilm was formed, respectively, compared to the untreated control (Figure 1).

The viability of biofilms of *C. albicans* after treatment with *3b* and *4b* at different concentrations was evaluated by staining the adherent cells with XTT, a colorimetric assay for quantification of cellular viability and cytotoxicity [31]. Compounds *3b* and *4b* each displayed a dose-dependent inhibitory effect on biofilm viability (Figure 2a).

The addition of cAMP to the media with carbohydrates affected the biofilm development, rescued the inhibition by *4b* and restored the biofilm level to that of the untreated control (Figure 2b). On the other hand, the inhibitory effect of *3b* was only partially restored.

*Figure 2.* The biofilm formation of *C. albicans* on polystyrene in liquid culture measured by XTT assay. a) The biofilm growth after 24 h at different concentrations (1 - 15 mg/mL) of compounds *3b* (filled

columns) and *4b* (unfilled columns). b) The biofilm growth after 24 h supplemented with 10 mg of compounds *3b* and *4b,* without (filled columns) or with addition of cAMP (unfilled columns). Samples compared to control (set to 100%), i.e. YNB supplemented with 100 mM L-proline and 0.2% glucose, *p*H 7.0. All experiments were performed in duplicates.

## *Yeast-hyphae transition*

The formation of hyphae by *C. albicans* is important for biofilm formation since hyphae are more adherent than yeast cells. After the formation of hyphae, the adhesins, which are expressed mostly on hyphae, play an important role in the further adhesion [9]. To monitor the *C. albicans* cell morphology, we studied the biofilm development also using microscopy and microfluidics.

After the yeast cells were inoculated, hyphae started to form within the first hour of incubation in the biofilm medium (YNB supplemented with 100 mM L-proline and 0.2% glucose, *p*H 7.0, see Figure 3a). The addition of benzyl β-D-xylopyranoside (*3b)* or benzyl α-L-fucopyranoside (*4b)* resulted in a substantial decrease of hyphae formation (Figure 3b and c), and the majority of the cells remained in yeast-form during the incubation period. High-resolution pictures after 10 hours and 18.5 hours of incubation can be found in the Supporting Information.

*Figure 3.* The effect of compounds *3b (*panel b), and *4b (*panel c), on the morphological transition of *C. albicans.*The images were taken at 10 h of incubation at 37 °C. Untreated, UT (panel a) - YNB supplemented with 100 mM L-proline and 0.2% glucose, *p*H 7.0; the different carbohydrates were tested at the concentration of 15 mg/mL. The scale bar is 32 □m.

The branching of hyphae and cell size could be affected by vacuole inheritance [37, 38]. However, the benzylated compounds did not exhibit any effect on vacuole morphology. Furthermore, the addition of cAMP to the media released the hyphae formation inhibitory effect caused by benzyl α-L-fucopyranoside (*4b)* (Supporting Information) and the switching of yeast-form cells to hyphae occurred effectively. On the contrary, the cAMP did not rescue the benzyl β-D-xylopyranoside (*3b)* inhibition effect, and most cells remained in yeast-form after 24 hours of the treatment (Supporting Information).

# Discussion

In the current study we developed carbohydrates derivatives which diminish the biofilm formation of the opportunistic pathogen *Candida albicans.* Carbohydrates are abundant in nature and are part of building blocks of living cells. Xylose and fucose are found in fungal extracellular biofilm matrix [39]. Xylose comprises around 12% of total carbohydrates of *C. albicans* biofilm matrix [39]. Furthermore, L-fucose is a component of glucan surface structures of host (mammalian cells). *C. albicans* Als1 protein specifically recognizes fucose-containing sugars of glucan of the host [40]. The addition of these carbohydrates can possibly perturb the pathogen interaction with the host. Thus, these carbohydrates have been investigated for their ability to inhibit adhesion to human cells.

In the early 1980s, the effect of both fucose and xylose was studied on *C. albicans* adhesion to human cells. Sobel et al., showed that both D- and L-fucose inhibited *C. albicans* adhesion to vaginal epithelial cells (~30%) [19]. Xylose inhibited the adhesion to buccal cells by 15% [20].

In our study, the addition of L-fucose and D-xylose to the biofilm media (containing the hyphae inducer L-proline) slightly decreased the biofilm formation on polystyrene. On the other hand, the benzyl-derivatives of both fucose and xylose had significantly higher inhibitory effect on *C. albicans* biofilm formation. We found that they affect the switching of yeast-form cells to hyphae. Upon addition of either benzyl α-L-fucopyranoside (*4b)* or benzyl β-D-xylopyranoside (*3b)*,, the cells continued to grow in yeast-like form and produced only few hyphae. On the other hand, methyl glycosides did not affect the morphological transition indicating that the inhibitory effect is linked to the benzyl group. Interestingly, aromatic compounds have been reported to influence the adhesion of both *Saccharomyces cerevisiae* and *C. albicans* by inducing the expression of adhesins genes [41, 42]. For *S. cerevisiae,* tryptophol and phenyl ethanol were reported to be a part of quorum sensing activating the *FLO11* gene expression via PKA pathway [41, 43]. *C. albicans* was found to respond to the aromatic alcohol tyrosol [44], while tryptophol and phenylethanol did not induce phenotypic changes in *C. albicans* [43]. Farnesol is a negative regulator of quorum sensing, through the cAMP pathway, in both *C. albicans* and *S. cerevisiae,* [42, 45] as it was found to inhibit *C. albicans* adhesion [46] and inhibit cell growth of *S. cerevisiae [*47].

The addition of cAMP to the benzyl-glycosides containing biofilm medium reversed the hyphae formation inhibitory effect of benzyl α-L-fucopyranoside (*4b)* but not of benzyl β-D-xylopyranoside (*3b)*.. This suggests that the effects of these two compounds are mediated by different mechanisms.

## Conclusions

We have shown that benzyl β-D-xylopyranoside (*3b)* and benzyl α-L-fucopyranoside (*4b)* each inhibit the morphological transition of *C. albicans* from yeast-form cells to hyphae, diminishing the biofilm. Neither the unprotected monosaccharides, nor the corresponding methyl-glycosides showed any significant effects.

The cAMP treatment of α-L-fucopyranoside treated cells restored yeast-hyphae switching and biofilm level to that of untreated control. While the biofilm level was partially restored by the addition of the cAMP, the benzyl β-D-xylopyranoside treated cells remained mainly in yeast-like form. We propose that the effects shown by these compounds are mediated by different mechanisms.

## Declarations

# Availability of data and materials

All data generated or analysed during this study are included in this published article and its supplementary information files.

# Competing interest statement

OS, UE and SM are shareholders of Gedea Biotech AB, a company with relationship to the present work.

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# Author’s contributions

UE and SM designed and coordinated the study. OI performed the experiments and analyzed the data. All authors interpreted and reviewed the results. All authors were active in the writing of the manuscript.

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# Figures

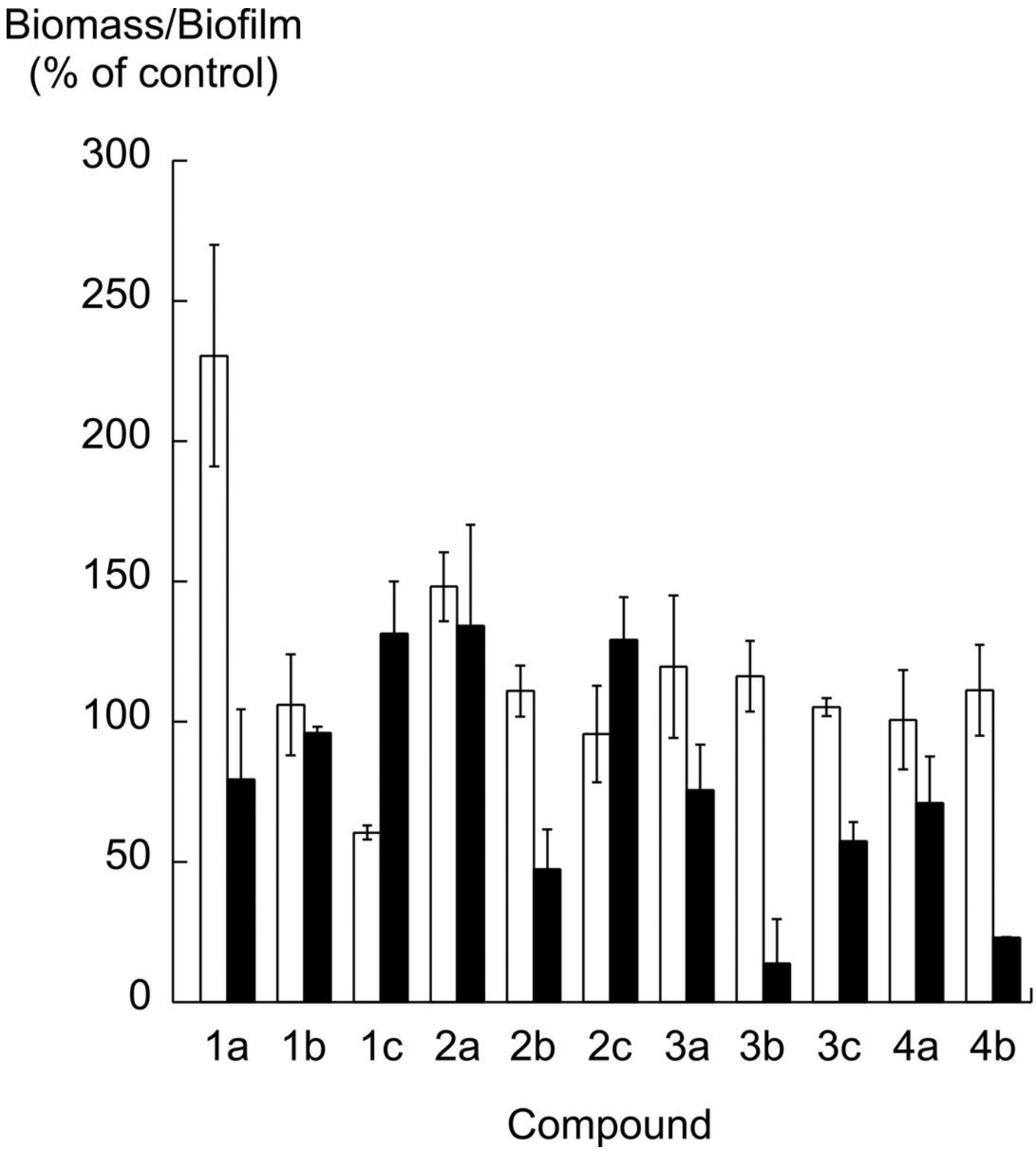
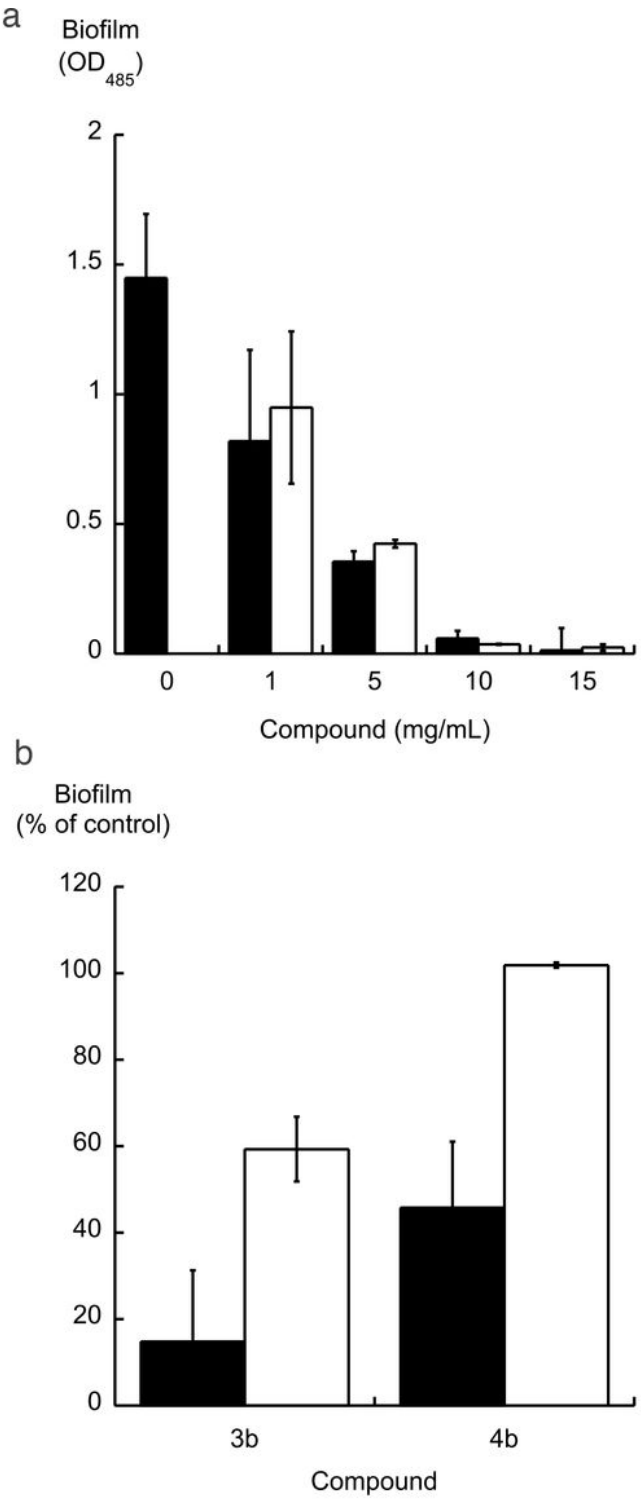



**Figure 1**

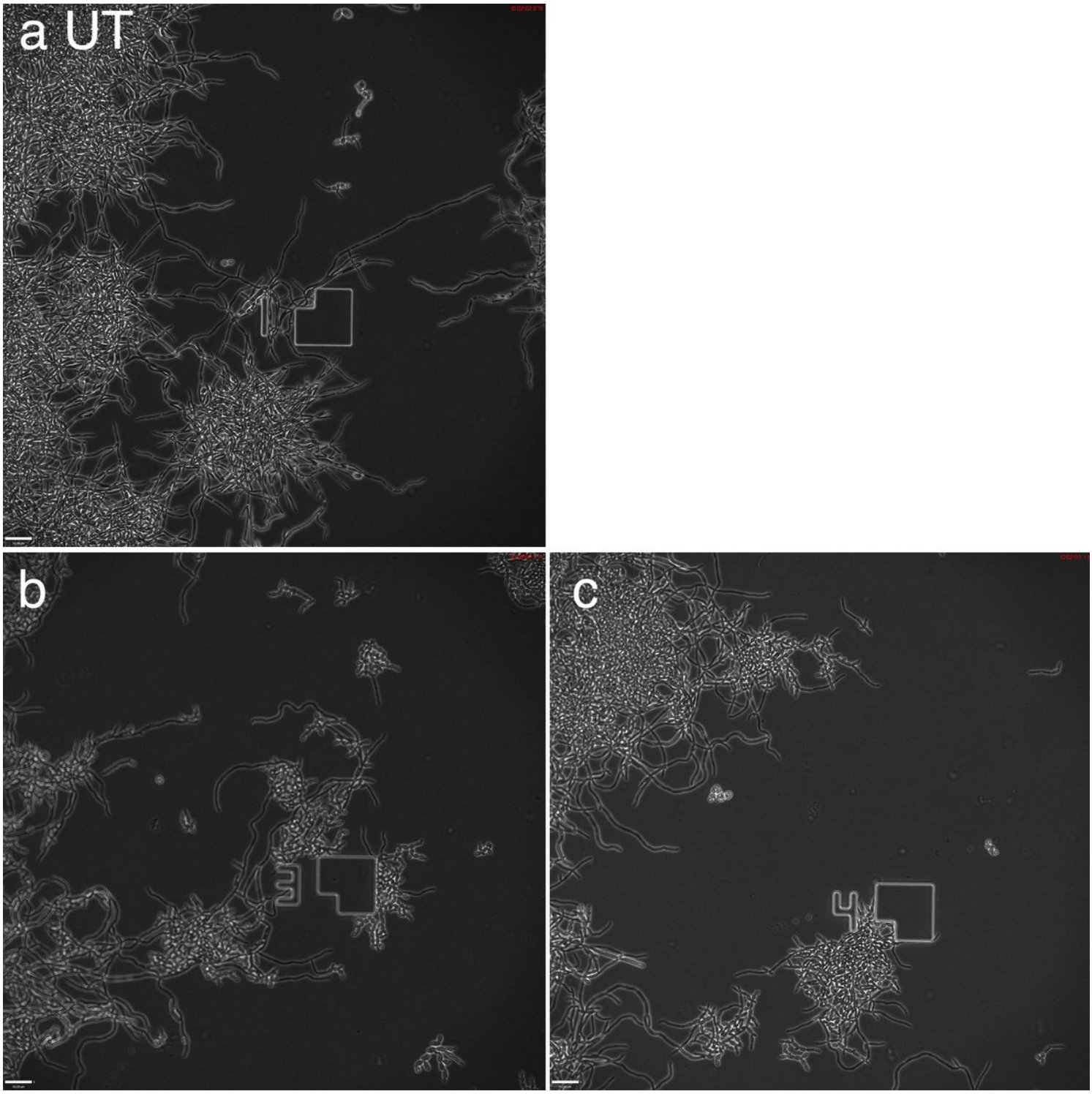
The biomass and biofilm formation of C. albicans in the minimal medium supplemented with different carbohydrate compounds at 10 mg/mL (crystal violet staining). Unfilled columns indicate biomass. Filled columns indicate biofilm. All experiments were performed in duplicates. The data was normalized by untreated control, which was set to 100%.




## Figure 2

The biofilm formation of C. albicans on polystyrene in liquid culture measured by XTT assay. a) The biofilm growth after 24 h at different concentrations (1 - 15 mg/mL) of compounds 3b (filled columns) and 4b (unfilled columns). b) The biofilm growth after 24 h supplemented with 10 mg of compounds 3b and 4b, without (filled columns) or with addition of cAMP (unfilled columns). Samples compared to

control (set to 100%), i.e. YNB supplemented with 100 mM L-proline and 0.2% glucose, pH 7.0. All experiments were performed in duplicates.




## Figure 3

The effect of compounds 3b (panel b), and 4b (panel c), on the morphological transition of C. albicans. The images were taken at 10 h of incubation at 37 °C. Untreated, UT (panel a) - YNB supplemented with 100 mM L-proline and 0.2% glucose, pH 7.0; the different carbohydrates were tested at the concentration of 15 mg/mL. The scale bar is 32 □m.

# Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- IshchukSuppInfor.pdf