

SUPPLEMENTARY MATERIAL

Antifungal siderophore conjugates for theranostic applications in invasive pulmonary aspergillosis using low molecular TAFC scaffolds

Joachim Pfister, Milos Petrik, Katerina Bendova, Barbara Matuszczak, Ulrike Binder, Matthias Misslinger, Alexander Kühbacher, Fabio Gsaller, Hubertus Haas and Clemens Decristoforo

MALDI-TOF MS: Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry was performed on a Bruker microflex™ bench-top MALDI-TOF MS (Bruker Daltonics, Bremen, Germany). Samples were prepared on a microscout target (MSP96 target ground steel BC, Bruker Daltonics) using dried-droplet method and α -cyano-4-hydroxycinnamic acid (HCCA, Sigma-Aldrich, Handels GmbH, Vienna, Austria) as matrix. All spectra were recorded by summarizing 600 laser shots per spot and Flex Analysis 2.4 software was used for data processing.

¹H-NMR Spectroscopy

¹H-NMR spectra were recorded on "Mars" 400 MHz Bruker Avance 4 Neo spectrometer in CDCl₃ or DMSO-d₆, respectively. Chemical shifts are expressed in ppm downfield relative to tetramethylsilane and the coupling constants (J) are reported in Hertz. Data for ¹H-NMR spectra are reported as follows: s = singlet, br s = broad singlet, d = doublet, dd = doublet of a doublet, t = triplet, q, quartet, m = multiplet.

Analytical RP-HPLC: Reversed-phase (RP) high-performance Liquid chromatography (HPLC) analysis was carried out using the following instrumentation: UltiMate 3000 RS UHPLC pump, UltiMate 3000 autosampler, UltiMate 3000 Variable Wavelength Detector; UV detection at λ = 220nm (Dionex, Germering, Germany) Radio-detector (Gabi Star, Raytest; Straubenhardt, Germany) using Jupiter 5 μ M C₁₈ 300 Å 150 x 4.6 mm (Phenomenex Ltd. Aschaffenburg, Germany) as column with acetonitrile (ACN)/H₂O/0.1% trifluoroacetic acid (TFA) as mobile phase; flow rate of 1 mL/min;

Gradient A: 0.0–3.0 min 10% ACN, 3.0–16.0 min 10–60 % ACN, 16.0–18.0 min 60% ACN, 18.0–18.1 min 60–10% ACN, 18.1–22.0 min 10% ACN.

Gradient B: 0.0–3.0 min 10 % ACN, 3.0–16.0 min 10–100 % ACN, 16.0–18.0 min. 100 % ACN, 18.0-18.1 min 100-10 % ACN, 18.1–23.0 min. 10 % ACN

Preparative RP-HPLC. Sample purification via RP-HPLC was carried out on a Gilson 322 Pump with a Gilson UV/VIS-155 detector (UV detection at λ = 220 nm) using a PrepFC™ automatic fraction collector (Gilson, Middleton, WI, USA), Eurosil Bioselect Vertex Plus 30 x 8 mm 5 μ m C₁₈A 300Å pre-column and Eurosil Bioselect Vertex Plus 300 x 8 mm 5 μ m C₁₈A 300Å column (Knauer, Berlin, Germany) and following ACN/H₂O/ 0.1 % TFA gradients with a flow rate of 2 mL/min:

Gradient 1: 0.0–1.0 min 0 % ACN, 1.0–35.0 min 0–50 % ACN, 35.0–36.0 min 50 % ACN, 36.0–36.1 min 50–0 % ACN, 36.1–43.0 min 0 % ACN

Gradient 2: 0.0–1.0 min 20 % ACN, 1.0–36.0 min 20–100 % ACN, 36.0–40.0 min 100 % ACN, 40.0–40.1 min. 100–20 % ACN, 40.1–47.0 min. 20 % ACN,

Gradient 3: 0.0–1.0 min 10% ACN, 1.0–36.0 min 10–80 % ACN, 36.0–37.0 min 80 % ACN, 37.0–37.1 min 80-10% ACN; 37.1–43.0 min 10% ACN

Gradient 4: 0.0–1.0 min 10 % ACN, 1.0–35.0 min 10–50 % ACN, 35.0–36.0 min 50 % ACN, 36.0–36.1 min 50–10 % ACN, 36.1–43.0 min 10 % ACN

Precursor preparation:

[Fe]Fusarinin C ([Fe]FsC)/[Ga]FsC: Fusarinin C was obtained by fungal culture according to Schrettl *et al* [1]. *Aspergillus fumigatus* mutant strain *AsidG* (which lacks the enzyme for acetylation of Fusarinine C) was grown (1×10^6 Spores/mL) in 200 mL iron depleted minimal medium, incubated for 28 h at 37°C and shaking at 200 rpm. After filtering of the culture supernatant, FeSO₄ was added to a final concentration of 10mM to get a red coloured solution. To produce [Ga]FsC, GaBr₃ was used instead of the FeSO₄, resulting in a colourless solution. The filtrate was subsequently loaded to a FlashPure cartridge (C18; 40 µm; 12g; column volume (CV) of 24 mL; BÜCHI Labortechnik AG, Flawil, Switzerland) by using a REGLO tubing pump (Type ISM795, Ismatec SA, Glattbrugg-Zürich, Switzerland) with a flow rate of 10 mL/min. Fixed [Fe]/[Ga]FsC on the cartridge was washed with 2 CV of water and then eluted with 5 CV of methanol. After evaporation of the organic solvent approximately 250 mg of [Fe]FsC could be obtained as a red-brown coloured solid with a purity of >90% confirmed by analytical RP-HPLC (gradient A t_R = 9.0 min) and the product was used for synthesis without further purification. MALDI-TOF-MS: m/z [M+H] = 780.68 [C₃₃H₅₁FeN₆O₁₂; exact mass: 779.63 (calculated)]. [Ga]FsC resulted in a yellow coloured solid (gradient A t_R = 8.7 min) MALDI-TOF-MS: m/z [M+H] = 793.68 [C₃₃H₅₁GaN₆O₁₂; exact mass: 792.28 (calculated)]

Acetylation of [Fe]/[Ga]FsC:

To acetylate FsC, 20-30 mg (38 µmol) dissolved in 500 µL water was rocked with 10-20 µL (0.2 µmol) of acetic anhydride for 2 min at room temperature and intense shaking. Resulting products mono-, di-, and triacetylfusarinine C were immediately purified via preparative RP-HPLC using gradient 1 to collect *N,N'*-diacetylfusarinine C ([Fe]/[Ga]DAFC, t_R = 17.8/18.1 min) in high purity (> 95%) followed by lyophilization. MALDI-TOF-MS: t_R ([Fe]DAFC= 10.9 min m/z [M+H] = 864.01 [C₃₇H₅₅FeN₆O₁₄; exact mass: 863.70 (calculated)] [2]; [Ga]DAFC was produced in the same way except of using [Ga]FsC as a starting material. Analytical data: t_R ([Ga]DAFC= 10.7 min m/z [M+H] = 877.37 [C₃₇H₅₅GaN₆O₁₄; exact mass: 876.30 (calculated)]

Modification of Fludioxonil

NaH (60% dispersion in mineral oil, 0.69 g, 17.2 mmol) were prewashed with pentane to get a white powdered dispersed in 30 mL DMF. Fludioxonil (2.01 g, 8.0 mmol) was dissolved in DMF (40 mL) and added dropwise to the NaH dispersion under argon atmosphere and stirred for 2h at room temperature. NaI (0.35 g, 2.3 mmol) and ethyl 4-bromobutyrate (2.5 g, 12.8 mmol) in DMF (30 mL) were added and stirred for additional 3 h. Hereafter the mixture was poured in ice cold water (250 mL) and extracted with ether. Combined organic layers were washed with brine and dried over Na₂SO₄. The solvent was removed under vacuum and the residue chromatographed with normal phase column, using CH₂Cl₂/ethyl acetate (1:2) to give the ester compound (3.04 g, 8.4 mmol: >100 % yield with some impurities) as white crystals. [3] ¹H-NMR (400 MHz, CDCl₃) δ (ppm): 7.72 (1H, d, J= 7.6 Hz), 7.26 (1H, br s), 7.16-7.12 (2H, m), 6.97 (1H, d, J= 7.6 Hz), 4.16 (2H, q, J= 6.7 Hz, OCH₂), 4.05 (2H, t, J= 6.6 Hz, NCH₂), 2.33 (2H, t, J= 6.2 Hz, CH₂COOEt), 2.19-2.12 (2H, m, CH₂), 1.27 (3H, t, J= 6.7 Hz, CH₃).

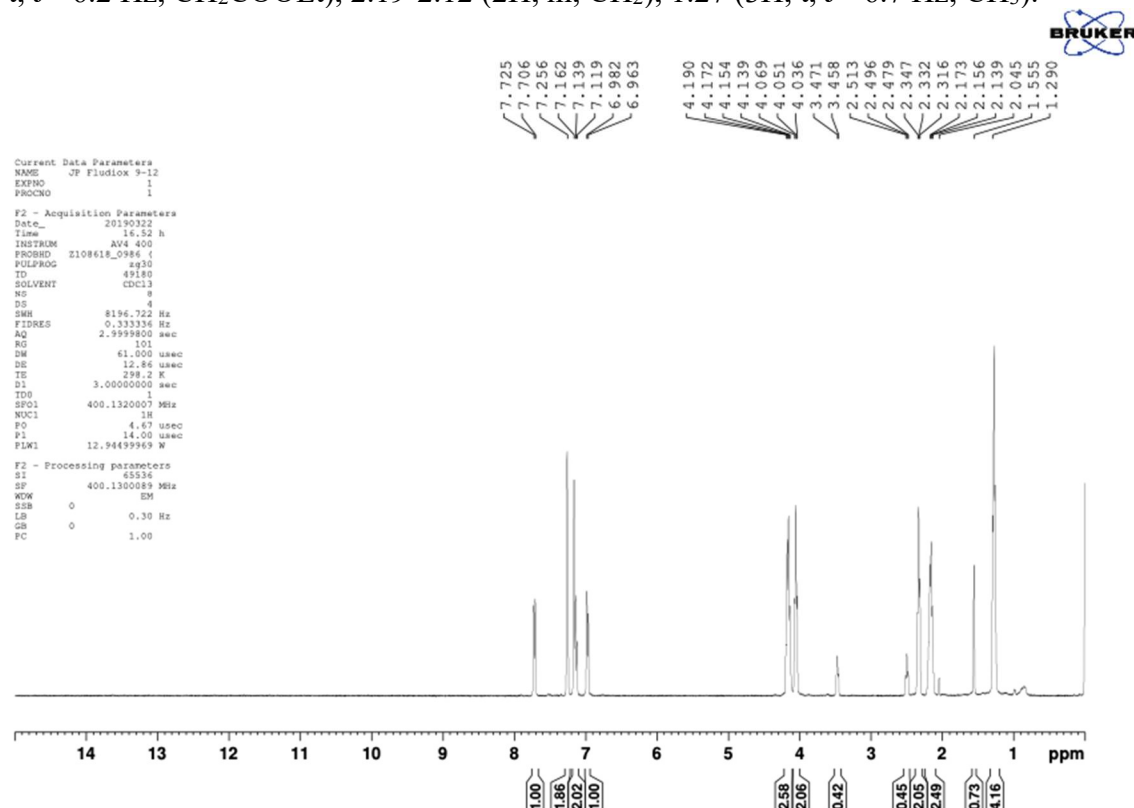


Figure S1. ¹H-NMR spectrum of ethyl fludioxonil-butyrate.

For cleavage of the ester group, 13.5 mL (16.7 μmol) of a 5% NaOH solution in water was added to the product dissolved in ethanol (40 mL) and the mixture was stirred for 2h at room temperature. Reaction solution was acidified in the ice bath with 2N HCl to adjust the pH to 1-2. Hereafter, the organic solvent was removed under vacuum and the precipitate filtered and dried to give the final product (1.156 g, 3.46 mmol, yield of 51 %) as a white powder. [4] NMR (400 MHz, DMSO) δ (ppm): 7.85 (1H, d, J=2.4 Hz), 7.52 (1H, dd, J=8.0 Hz, 1.6 Hz), 7.41 (1H, d, J= 2.4 Hz), 7.34-7.27 (2H, m), 4.04 (2H, t, J= 7.0 Hz, N-CH₂), 2.20 (2H, t, J= 7.4 Hz, CH₂-COOH), 2.01-1.94 (2H, m, CH₂).

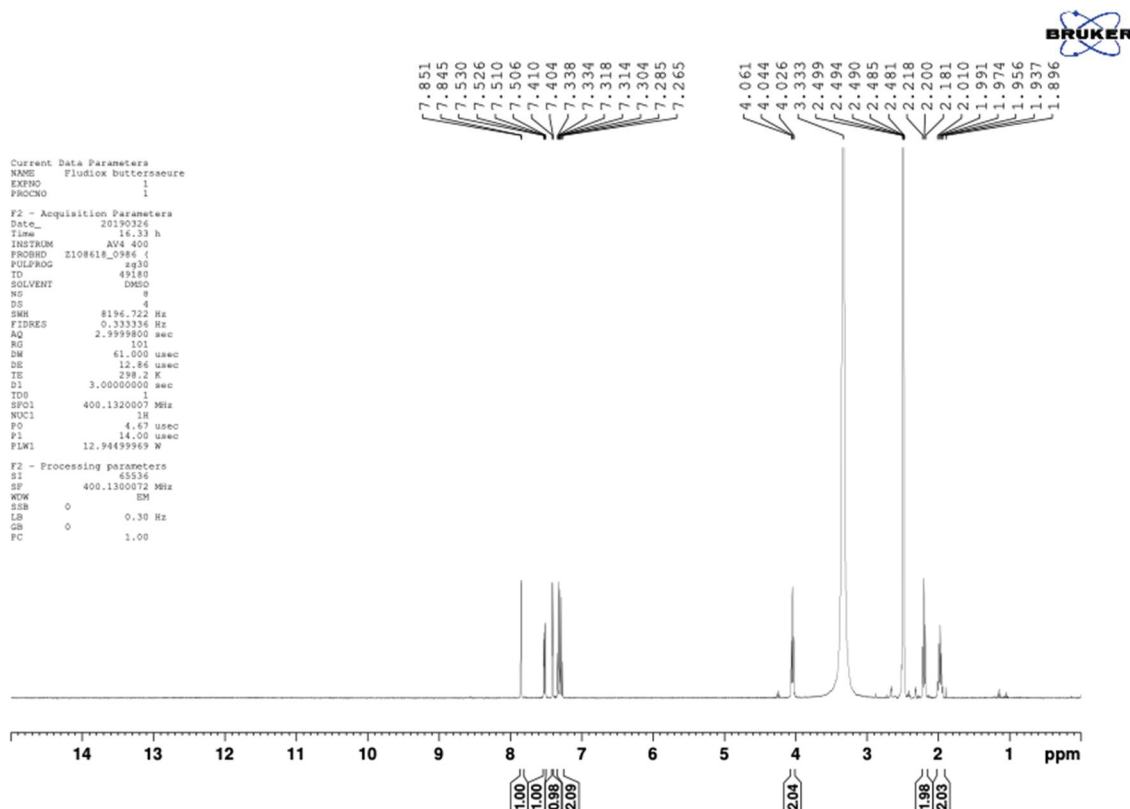


Figure S2. ^1H -NMR spectrum of fludioxonil-butiric acid

Conjugation of the antifungals:

Conjugation of Eflornithine

Eflornithine contains two primary amines that could interfere during the conjugation process and produce polymers. Therefore, as a first step, the free amine groups had to be protected with tert-butyloxycarbonyl protecting group (Boc). 100 mg (423 μmol) of eflornithine were dissolved in 750 μL water, mixed with 230 mg (1.05 mmol) of Boc-anhydride, dissolved in 500 μL dioxan, and left to react for 24h at room temperature. End of the reaction was confirmed by a Kaiser-Test and product was dried under vacuum and dissolved again in water. By adding 40 μL of NaOH (10 M), participation of $(\text{Boc})_2\text{-Eflornithine}$ occurred. This product was filtered and freeze dried. (32.6 mg; 85 μmol ; 20% yield) This Boc protected eflornithine was immediately used for conjugation to $[\text{Fe}]\text{DAFC}$ by dissolving 8.2 mg (21.4 μmol) of $(\text{Boc})_2\text{Eflornithine}$ in 500 μL dry DMF and after addition of 1.1 equiv. (8.9 mg, 23.5 μM) HATU, the mixture was left for 10 min at room temperature to activate the carboxylic acid. Subsequently, 0.8 equiv. of $[\text{Fe}]\text{DAFC}$ (14.2 mg, 16.4 μmol) were added and the mixture was stirred for further 30 min. The end of the reaction was confirmed by analytical RP-HPLC. After removing the organic solvent, product was purified by preparative RP-HPLC. (Gradient A: $t_R(+\text{Fe}) = 10.8/11.2$ min). Cleavage solution ($\text{TFA}:\text{H}_2\text{O}:\text{Triisopropylsilane}=95:2.5:2.5$) was added to dry product and followed by stirring for 45 min to remove the Boc-protecting groups. After evaporation of the TFA, the product could be isolated via preparative RP-HPLC (gradient 1: $t_R(-\text{Fe})= 21.0/21.7$ min; $t_R(+\text{Fe})= 20.7/21.1$ min) and freeze-drying. Analytical data: $[\text{Fe}]\text{DAFC-eflornithine}$ 1.27 mg [1.2 μmol ; 15% of theoretical yield] MALDI TOF-MS: m/z $[\text{M}+\text{H}] = 1028.62$ $[\text{C}_{43}\text{H}_{65}\text{F}_2\text{FeN}_8\text{O}_{15}$; exact mass: 1027.86 (calculated)]

[Ga]DAFC-eflornithine 2.4 mg [2.5 μ mol; 30% of theoretical yield] MALDI TOF-MS: m/z [M+H] = 1040.62 [C₄₃H₆₅F₂GaN₈O₁₅; exact mass: 1040.37 (calculated)]

Conjugation of Fludioxonil

For the conjugation of fludioxonil butyric acid, 3.8 mg (11.6 μ mol) was dissolved in 500 μ L anhydrous DMF and after addition of 2 equiv. (8.8 mg, 23.2 μ mol) HATU, the mixture was left for 10 min at room temperature to activate the carboxylic acid. Hereafter, 5.0 mg (5.8 μ mol) of [Fe]DAFC dissolved in 500 μ L DMF were added. The pH was adjusted to 8-9 with DIPEA and rock for 1 hour at room temperature. The end of the reaction was confirmed by analytical RP-HPLC. Crude product was purified by preparative RP-HPLC (gradient 3, t_R = 24.4 min) and freeze dried to give a red coloured solid. Analytical data: [Fe]DAFC fludioxonil butyric acid 3.15 mg [2.6 μ mol, 49% of theoretical yield] RP-HPLC gradient B, t_R = 12.5 min; MALDI TOF-MS: m/z [M+H] = 1180.32 [C₅₃H₆₅F₂FeN₈O₁₇; exact mass: 1179.96 (calculated)].

For [Ga]DAFC-fludioxonil, iron free DAFC-fludioxonil was labelled with stable gallium by adding a 10-fold molar excess of GaBr₃ solution at pH 4.5 and stirred for 10 min at room temperature. [Ga]DAFC-fludioxonil 390 μ g [2.6 μ mol, 35% of theoretical yield] RP-HPLC gradient B, t_R = 12.3 min; MALDI TOF-MS: m/z [M+H] = 1192.79 [C₅₃H₆₅F₂GaN₈O₁₇; exact mass: 1192.36 (calculated)].

Conjugation of Thiomersal

For the conjugation, thiomersal 3.04 mg (7.5 μ mol) was dissolved in 500 μ L dry DMF and after addition of 1.1 equiv. (3.1 mg; 8.2 μ mol) HATU, the mixture was left for 10 min at room temperature to activate the carboxylic acid. Subsequently, 5.06 mg (5.8 μ mol) of [Fe]DAFC were added and the reaction was stirred for further 30 min. End of the reaction was confirmed by RP-HPLC and immediately purified by preparative RP-HPLC (Gradient 3, t_R = 25,27 min) to give a red coloured solid after lyophilisation. Analytical data: [Fe]DAFC-thiomersal 3.26 mg [2.6 μ mol, 46 % of theoretical yield]; RP-HPLC gradient B, t_R = 17.51 min; MALDI-TOF-MS: m/z [M+H] = 1229.85 [C₄₆H₆₃FeHgN₆O₁₅S; exact mass: 1229.31 (calculated)];

For [Ga]DAFC-thiomersal, iron free DAFC-thiomersal was labelled with stable gallium by adding a 10-fold molar excess of GaBr₃ solution at pH 4.5 and the mixture was stirred for 10 min at room temperature. [Ga]DAFC-thiomersal 620 μ g [0.5 μ mol, 35 % of theoretical yield]; RP-HPLC gradient B, t_R = 17.18 min; MALDI-TOF-MS: m/z [M+H] = 1242.44 [C₄₆H₆₃GaHgN₆O₁₅S; exact mass: 1242.03 (calculated)]

Conjugation of 5-Fluoroorotic acid

For the conjugation of 5-Fluoroorotic acid, 3.78 mg (21.7 μ mol) was dissolved in 500 μ L dry DMF and after addition of 1.1 equiv. (9.0 mg, 23.8 μ M) HATU and 10 μ L DIPEA, the mixture was left for 10 min at room temperature to activate the carboxylic acid. Subsequently, 5.0 mg of [Fe]DAFC (5.7 μ mol) were added and the reaction was stirred for further 30 min. The end of the reaction was confirmed by analytical RP-HPLC. (Gradient A: t_R ([Fe]DAFC-FOA) = 12.65 min) Hereafter, the product was isolated via preparative RP-HPLC (Gradient 1: (t_R (+Fe)= 26.68 min) and freeze-dried as a white powder. Analytical data: [Fe]DAFC-FOA 1.74 mg [1.7 μ mol; 30% of theoretical yield]; MALDI TOF-MS: m/z [M+H] = 1020.24 [C₄₂H₅₆FeN₈O₁₇; exact mass: 1019.30 (calculated)]
[Ga]DAFC-FOA 2.96 mg [2.8 μ mol; 31% of theoretical yield]; MALDI TOF-MS: m/z [M+H] = 1033.38 [C₄₂H₅₆GaN₈O₁₇; exact mass: 1032.30 (calculated)]

Iron free conjugates:

For iron removal, half of the conjugate was stirred in 1 mL of 100 mM Na₂EDTA solution for 2 hours to produce the iron free compound, which was subsequently purified by preparative RP-HPLC and freeze-dried to get a white solid powder.

DAFC-eflornithine: Analytical data DAFC-eflornithine 0.48 mg [0.4 μ mol, >98% purity] analytical RP-HPLC gradient A, t_R = 11.4/11.5 min; MALDI TOF-MS: m/z [M+H]⁺ = 975.36 [C₄₃H₆₈F₂N₈O₁₅; exact mass: 975.04 (calculated)].

DAFC-fludioxonil: Analytical data DAFC-fludioxonil 1.2 mg [1.1 μ mol, 99% purity] analytical RP-HPLC gradient B, t_R = 12.6 min; MALDI TOF-MS: m/z [M+H]⁺ = 1127.32 [C₅₃H₆₈F₂N₈O₁₇; exact mass: 1126.46 (calculated)].

DAFC-thiomersal: Analytical data DAFC-thiomersal 1.1 mg [0.9 μ mol, 96% purity] analytical RP-HPLC gradient A, t_R = 17.8 min; MALDI TOF-MS: m/z [M+H]⁺ = 1178.68 [C₄₆H₆₆HgN₆O₁₅S; exact mass: 1177.40 (calculated)].

DAFC-FOA: Analytical data DAFC-FOA 0.46 mg [0.47 μ mol, 98% purity] analytical RP-HPLC gradient A, t_R = 13.06 min; MALDI TOF-MS: m/z [M+H]⁺ = 967.24 [C₄₂H₅₉FN₈O₁₇; exact mass: 966.39 (calculated)].

Overview of all data

Table S1. Overview of Data included in this study except for biodistribution data which can be found in the main manuscript. Minimal inhibitory concentration (MIC), defined as no visible growth by the naked eye, were conducted in iron-sufficient and iron-depleted *Aspergillus* minimal medium (AMM).

(AMM).	Values		are		presented			as		±		SD.					
	Log D	Protein binding	Serum Stability	<i>in vivo</i> Stability		Uptake Assay			Competition Assay	MIC AMM (-)Fe [µg/mL]		MIC AMM (+)Fe [µg/mL]		MIC AMM (-)Fe [µM]		MIC AMM (+)Fe [µM]	
Compound	Value	Value	Value	Blood	Urine	Fe -	Fe - Block TAFC	Fe +	Fe -	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
[Fe]DAFC-Eflornithin	-3,45 ± 0,04	~7,9 %	>99 %	98%	99%	20.3 ± 1.2	16.8 ± 1.3	7.6 ± 3.2	32.3 ± 7.5	>256	>256	>256	>256	>249	>249	>249	>249
[Ga]DAFC-Eflornithin										>256	>256	256	>256	>246	>246	246	>246
Eflornithin										>256	>256	>256	>256	>1405	>1405	>1405	>1405
[Fe]DAFC-Fludioxonil	1,29 ± 0,02	~15.5 %	~95 %	40%	10%	145.5 ± 5.6	125.4 ± 9.3	65.9 ± 10.2	5.8 ± 0.2	128	256	128	>256	108	217	108	>217
[Ga]DAFC-Fludioxonil										16	64	>128	>128	13	54	>107	>107
Fludioxonil										32	64	16	32	96	191	48	2
Fludioxonil butyric acid										0.5	0.5	0.5	0.5	2	2	2	96
[Fe]DAFC-Thiomersal	0,24 ± 0,06	~70.0 %	~80 %	27%	31%	81.9 ± 9.8	48.7 ± 3.1	31.3 ± 6.0	11.1 ± 2.7	0.125	0.25	0.25	0.25	0.1	0.2	0.2	0.2
[Ga]DAFC-Thiomersal										0.5	1	0.5	1	0.4	0.8	0.4	0.8
Thiomersal										0.06	0.06	0.06	0.06	0.15	0.15	0.15	0.15
[Fe]DAFC-FOA	0,73 ± 0,01	~2.0 %	>99 %	>99 %	>99 %	65.7 ± 10.8	29.9 ± 14.4	25.7 ± 17.5	50.7 ± 12.9	256	>256	256	>256	251	>251	251	>251
[Ga]DAFC-FOA										16	64	>128	>128	15	62	>124	>124
Fluoroorotic acid										64	256	64	>256	368	1471	368	>1471
[Fe]DAFC-Cy5	1,03 ± 0,11	~13 %	99-97-96	>99%	5%	184.6 ± 52.9	185.3 ± 58.1	180.6 ± 61.9	11.8 ± 8.4	256	>256	256	>256	192	>192	192	>192
[Ga]DAFC-Cy5										16	32	256	>256	12	24	191	>191
Cy5 carboxylic acid										128	256	256	256	265	529	529	529
[Fe]TAFC	-2.08 ± 0.02	~3.0 %	>99 %	n.d.	n.d.	100.0 ± 10.0	25.3 ± 10.4	12.7 ± 23.0	25.3 ± 10.4	>256	>256	>256	>256	>283	>283	>283	>283
[Ga]TAFC										8	>256	>256	>256	9	>278	>278	>278

Microscopy of antifungal susceptibility assays

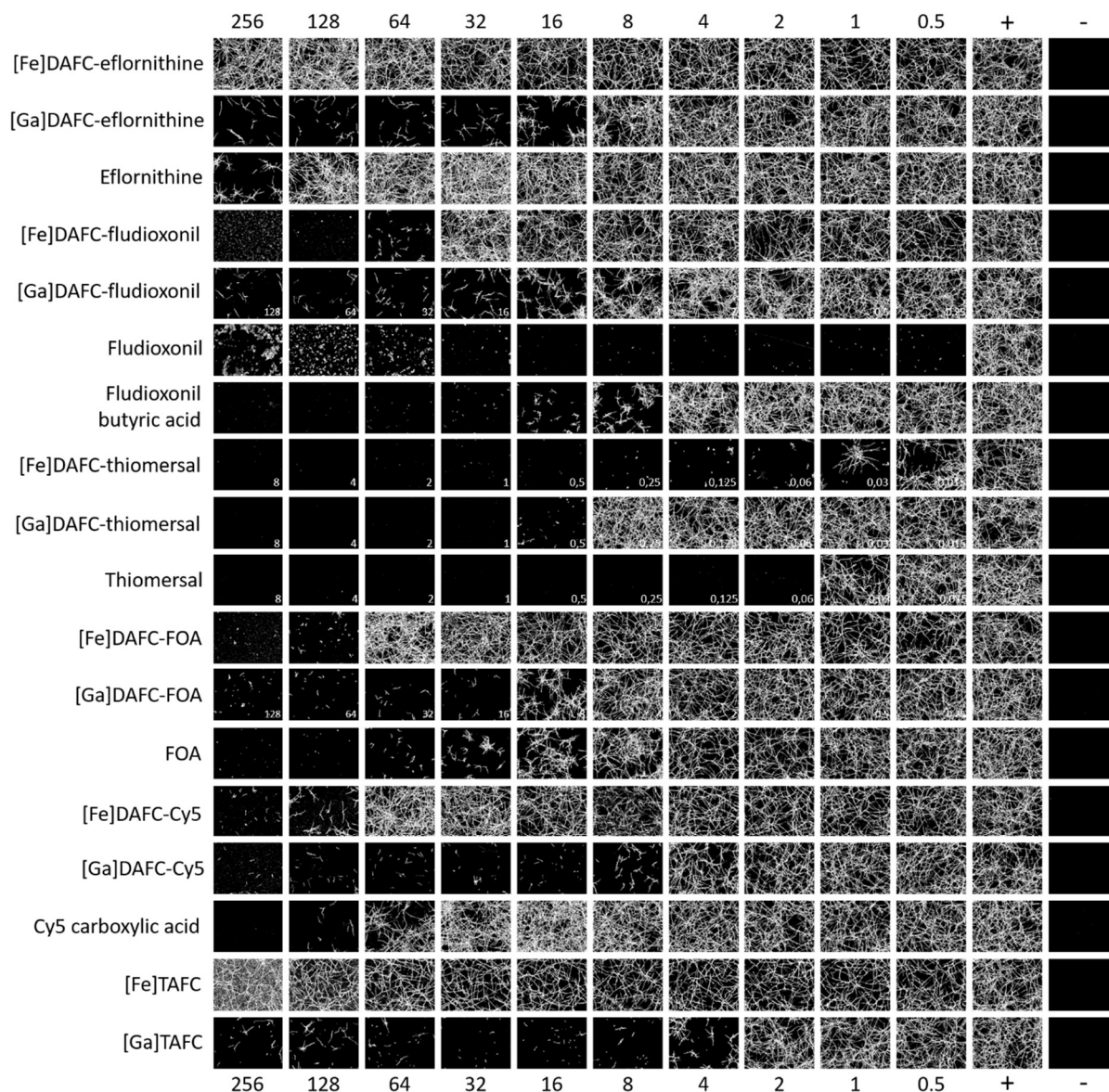


Figure S3. Microscopic images of *A. fumigatus* MIC assays with antifungal siderophore conjugates. Number on top and bottom display concentration of the antifungal in [$\mu\text{g/mL}$]. For practical reasons, different concentrations were chosen for some of the compounds as labelled in the lower right corner of the individual picture. Images were captured at 24 h incubated at 37°C in **iron depleted** *Aspergillus* minimal medium (AMM Fe(-)). Positive (+) (spores but no antifungal) and negative (-) (just medium) controls are shown in the two columns on the left. Artefacts at 64-256 $\mu\text{g/mL}$ wells of fludioxonil are unsolved crystals of the antifungal.

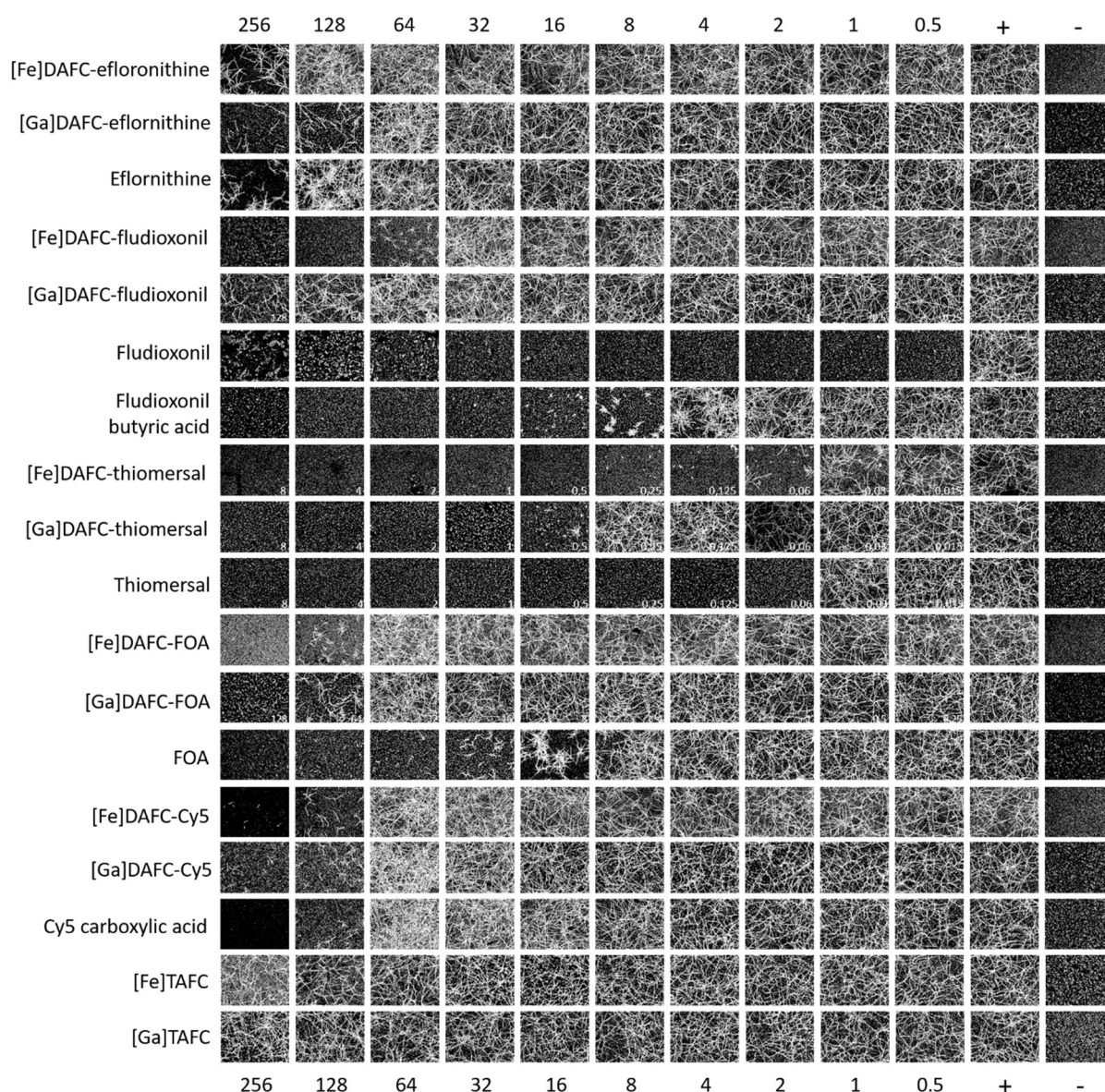


Figure S4. Microscopic images of *A. fumigatus* MIC assays with antifungal siderophore conjugates. Number on top and bottom display concentration of the antifungal in [$\mu\text{g/mL}$]. For practical reasons, different concentrations were chosen for some of the compounds as labelled in the lower right corner of the individual picture. Images were captured at 24 h incubated at 37°C in **iron sufficient** *Aspergillus* minimal medium (AMM Fe(+)). Positive (+) (spores but no antifungal) and negative (-) (just medium) controls are shown in the two columns on the left. Diffuse punctual background signal origins from the AMM Fe(+) medium.

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