Preparation of a Trp-BODIPY fluorogenic amino acid to label peptides for enhanced live-cell fluorescence imaging

A Trp-BODIPY fluorogenic amino acid to label peptides for enhanced live-cell fluorescence imaging

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Abstract

Fluorescent peptides are valuable tools for live-cell imaging due to the high specificity of peptide sequences for their biomolecular targets. When preparing fluorescent versions of peptides, labels need to be introduced at appropriate positions of the sequences to provide suitable reporters, while avoiding any impairment in the molecular recognition properties of the peptides. This protocol describes the preparation of the Trp-based fluorogenic amino acid Fmoc-Trp(C_2-BODIPY)-OH and its incorporation into peptides for live-cell fluorescence imaging. Fmoc-Trp(C_2-BODIPY)-OH contains a BODIPY (4,4-difluoro-4-bora-3a,4a-diaza-*s*-indacene) fluorogenic core attached to Trp via a spacer-free C–C linkage; it thus mimics the molecular interactions of Trp but includes an environmentally sensitive label that enables fluorescence detection and wash-free imaging. Fmoc-Trp(C_2-BODIPY)-OH can be prepared in 3–4 d before being employed for the solid-phase synthesis of fluorogenic peptides (6–7 d). We have used this technology to prepare the fluorogenic antimicrobial peptide **BODIPY-cPAF26** (3–4 d), which shows high chemical stability and minimal disruption of cellular activity when compared to the unlabeled sequence. We used **BODIPY-cPAF26** for wash-free imaging of fungal pathogens, including real-time visualization of *Aspergillus fumigatus* (5 d for culturing, 1-2 d for imaging). One major advantage of this procedure is the applicability to most peptide sequences, which enables the preparation of many peptide-based probes for enhanced fluorescence imaging. The procedure of this protocol covers the chemical synthesis of the fluorogenic amino acid Fmoc-Trp(C_2-BODIPY)-OH, the preparation of the labeled antimicrobial peptide **BODIPY-cPAF26** and their spectral and biological characterization as live-cell imaging probes for different fungal pathogens.

INTRODUCTION

Peptides are excellent molecular frameworks for the generation of imaging agents because they bind with high affinity and specificity to their targets. However, most peptides cannot be directly visualized using fluorescence imaging as they lack proper reporter groups and need to be modified with fluorophores or reactive tags for further derivatization.[1] A milestone in peptide and protein labeling has been the development of genetically encoded unnatural amino acids, which can be incorporated in response to nonsense or frameshift codons to embed suitable bioorthogonal groups at specific sites of peptides and proteins.[2] Recent advances that have driven a progress in the field of fluorescence imaging include the genetic encoding of norbornene amino acids, which can rapidly and specifically couple to tetrazine-containing fluorophores for protein labeling,[3] or the optimization of orthogonal variants of the inverse-electron-demand Diels-Alder cycloaddition reaction for dual color live-cell imaging and super-resolution microscopy.[4, 5]

In addition to synthetic biology strategies, many unnatural amino acids are available as building blocks to expand the chemical diversity within peptide sequences using solid-phase peptide synthesis (SPPS).[6, 7] These building blocks include unnatural amino acids with chemical groups amenable for two-step labeling processes (e.g. azides, alkynes, and alkenes). Once peptides containing these amino acids are synthesized, they are coupled to a suitable fluorophore (for instance, using 'click' chemistry), and the excess labeling agent is washed out prior to image acquisition. This approach has proven useful for many different applications,[8-11] although the need for washing steps might hamper its implementation in *in vivo* and real-time imaging. Recent progress has involved the preparation of the bioorthogonal fluorophores with bright fluorescence emission after the conjugation reaction, which leads to a reduction in fluorescence background and a minimization of the washing steps.[12-14] Moreover, further development of synthetic methodologies to diversify amino acids and peptides[15-17] will create opportunities for direct fluorescence labeling and *in situ* imaging studies.

Most commonly, peptides are readily labeled by attaching a fluorophore to a residue in the peptide sequence, yielding fluorescent conjugates that can be directly used for imaging. However, fluorophores have bulky structures that can impair the molecular recognition properties of the native peptide; it is, therefore, important to introduce fluorophores at appropriate positions within the amino acid sequence. Most of them are incorporated into peptides by reaction with terminal or side-chain chemical groups (e.g. amines, carboxylic acids, and thiols) from polar residues or through chemical spacers.[18-20] Since these modifications can disrupt the hydrogen bonding pattern with respect to the original peptide and have a detrimental effect on the biological properties of the fluorescent conjugate,[21] there is a demand for new technologies to incorporate fluorophores into peptides without affecting their inherent activity and, ideally, with suitable spectral properties for live-cell imaging applications.

Overview of the procedure

A schematic flowchart outlining the different sub-sections of the Procedure described in this protocol and their expected time requirement is reported in Figure 1. We have developed a new methodology to synthesize fluorogenic peptides that relies on a unique Fmoc-Trp(C_2-BODIPY)-OH amino acid (**1**),[22] which can be prepared by attaching a BODIPY fluorophore to the C_2 position of Trp *via* a spacer-free C–C linkage (Steps 1–35). Amino acid **1** mimics the molecular interactions of native Trp, and it possesses a fluorogenic label that can be used for live-cell imaging. Compound **1** shows high chemical stability, as we could prove by conducting the test described in Steps 36–37 (a quality control test that could be implemented before using a stock of compound **1** that has been stored as described in the Procedure), excitation and emission spectra in the visible range, and remarkable fluorescence enhancement in hydrophobic environments (i.e. when the peptide is binding to its target), which facilitates wash-free imaging experiments. We have validated the usefulness of **1** as a precursor of peptide-based imaging probes by derivatizing the antimicrobial peptide PAF26,[23] which

shows high affinity and selectivity for fungal cells. We detail in the Procedure the synthesis of the fluorogenic analogue of PAF26 (**BODIPY-cPAF26**) by conventional SPPS, simply using **1** instead of natural Trp (Steps 38–89). We have also recently employed the amino acid **1** for the preparation of cyclic peptides as fluorescent reagents for imaging apoptotic bodies.[24] **BODIPY-cPAF26** shows high resistance to proteolysis (which could be tested as described in Steps 90–94**)**, strong fluorogenic response in hydrophobic environments (see also procedure reported in Steps 95–106) and no impairment of the activity and selectivity of the native peptide in human and fungal cells (see tests described in Steps 107–131 and 132–162, respectively). All these tests represent a comprehensive panel of characterization experiments that can be readily implemented in other peptide sequences modified with the fluorogenic amino acid **1**. Finally, we describe the use of **BODIPY-cPAF26** for fluorescence live-cell imaging of several fungal pathogens (Steps 163–206) and real-time imaging of *Aspergillus fumigatus* (Steps 207–219), one of the most life-threatening human fungal pathogens. These imaging experiments should be considered as a template set of directions to be modified for the readers in their experiments of choice, which may vary depending on the instrumentation (e.g. confocal microscope) and cell systems used.

Figure 1.

Advantages and limitations of the procedure

A major advantage of this procedure is its general applicability, as it can be used to prepare fluorogenic analogues of most peptide sequences. Amino acid **1** is compatible with Fmoc-based SPPS protocols, tolerates standard deprotection and coupling conditions as well as mild acid-based deprotection strategy, without any observable degradation of the BODIPY fluorophore.[25, 26] Notably, careful design of the protecting group scheme is needed to ensure full orthogonality with the BODIPY structure; for instance, in the synthesis of **BODIPY-cPAF26**, we used Fmoc-Lys(Z)-OH

instead of Fmoc-Lys(Boc)-OH and Fmoc-Arg(NO_2)-OH instead of Fmoc-Arg(Pbf)-OH to avoid any acid-sensitive protecting groups.

Importantly, the exact impact of the Trp-BODIPY on the biomolecular properties of the labeled peptides needs to be examined on a case-by-case basis. Although most of the labeled bioactive peptides examined so far have shown similar activity patterns to their unlabeled, natural counterparts,[22] the introduction of Trp-BODIPY may prove detrimental to the activity of other peptides**,** and we recommend the readers to run proper tests (as those described in Steps 132-162) to compare the functional properties of labelled vs non-labelled peptide sequences.

Regarding spectral properties, **1** has both excitation and emission maximum wavelengths in the green region of the visible spectra ($\lambda_{exc.}$ ~500 nm, $\lambda_{em.}$ ~520 nm), which makes it ideally suited for conventional GFP (Green Fluorescent Protein)/ FITC (Fluorescein isothiocyanate) filters found in most spectrophotometers, flow cytometers, confocal microscopes and other imaging equipment. This characteristic is an important advantage with respect to naphthalene-, coumarin- and dansyl-based fluorescent amino acids that have been introduced as labels in peptides through SPPS.[27-30] Once inserted into peptides, these fluorogenic groups display shorter excitation and emission wavelengths than **1,** with relatively long Stokes shifts. Extensive work in this field has been performed by Imperiali and co-workers, who developed environmentally-sensitive fluorescent amino acids as reporters of protein–protein interactions.[31-34] These amino acids are mostly based on the naphthalimide scaffold and display remarkable fluorescence enhancements in hydrophobic environments, with excitation wavelengths around 400–450 nm and extinction coefficients around 10^3 to 10^4 $M^{-1}cm^{-1}$.[35, 36] Although the emission enhancement of **1** in hydrophobic environments might be slightly lower than that of naphthalimide-based amino acids, its high extinction coefficient (i.e. around one order of magnitude higher than its counterparts) results in overall stronger brightness.

Unlike the case of peptides containing unnatural amino acids with reactive orthogonal groups, the strong fluorogenic behavior of **1** makes it possible for the relevant labeled peptides to be used directly for live-cell imaging in wash-free conditions and without any additional labeling steps. This feature represents a major advantage in imaging experiments, where dynamic processes in living cells need to be monitored in real time.

The main limitation of the present protocol is that it can be implemented only to label the peptides or small proteins that are currently accessible via semi-synthetic approaches or by Fmoc-based SPPS.

Experimental design and crucial parameters

Fmoc-Trp(C_2-BODIPY)-OH: chemical synthesis, fluorescence properties and shelf life. We designed the synthesis of **1** so as to obtain the Fmoc-protected compound, which can be readily used for the synthesis of medium-sized peptides by conventional SPPS. The BODIPY fluorophore was selected because of its compact structure and excellent spectral photophysical properties.[37, 38] We decided to incorporate it into the C_2 position of Trp in a spacer-free C–C linkage to maintain the main recognition features of the labeled peptides (e.g. charge balance, overall polarity, and H-bond pattern). The retrosynthetic analysis of the amino acid **1** involves the arylation of the *m*-iodophenyl-BODIPY (**3**) on the unsubstituted C_2 position of Fmoc-Trp-OH (**2**) by Pd-catalyzed C–H activation[39-41], using an approach we recently developed (Figure 2).[16] The *m*-iodophenyl-BODIPY (**3**) can be prepared by acid-catalyzed condensation of the *m*-iodobenzaldehyde (**4**) with two equivalents of 2,4-dimethylpyrrole (**5**), followed by 2,3-dichloro-5,6-dicyano-*p*-benzoquinone oxidation and BF_3 complexation. Notably, all the starting materials needed for the synthesis of **1** (i.e. compounds **2**, **4** and **5**) are commercially available.

Figure 2.

Following similar reported procedures,[42] compounds **4** and **5** are mixed in conditions of trifluoroacetic acid (TFA) catalysis to yield the expected adduct with loss of a water molecule after stirring at room temperature for 22 h in anhydrous dichloromethane. Notably, in our hands, the synthetic yields did not significantly improve in the presence of dehydrating agents, such as 4Å molecular sieves. Next, 2,3-dichloro-5,6-dicyano-*p*-benzoquinone (DDQ) is added to the crude mixture with continuous stirring for 15 min, followed by addition of an excess of triethylamine and $BF_3 \cdot Et_2O$. The resulting dark green solution is stirred at room temperature for 4.5 h to produce compound **3** as the major product in a crude form. Liquid–liquid extraction followed by flash chromatography over silica gel affords compound **3** in ~30% yield (over the 3 steps). Of note, we observed slightly lower synthetic yields when this reaction was scaled up over 200 mg scale.

Next, the *m*-iodophenyl-BODIPY **3** is subjected to Pd-catalyzed arylation of Fmoc-Trp-OH (**2**) to afford the desired Fmoc-Trp(C_2-BODIPY)-OH (**1**) (Figure 3a).[43-45] The amino acid **2**, a slight excess of *m*-iodophenyl-BODIPY **3** (1.5 eq.), $AgBF_4$ (1.0 eq.), TFA (1.0 eq.) as the acid source, and catalytic $Pd(OAc)_2$ (0.05 eq.) are dissolved in *N*,*N*-dimethylformamide (DMF) and subjected to microwave irradiation for 20 min at 80 ºC. In our hands, **1** is isolated by normal-phase chromatography over silica gel in ~70% yield. Although our attempts to scale-up this reaction resulted in decreased yields, parallelization of the procedure is convenient and reproducible. Please note that conducting the reaction described above (Figure 3a) under conventional heating requires higher temperatures and longer reaction times than those mentioned for microwave-based synthesis, and it leads to the formation of very complex crude mixtures, due to the limited stability of the Fmoc and BODIPY groups in the mentioned conditions. These limitations make the preparation of **1** feasible only under microwave irradiation.

Figure 3.

Compound **1** displays excitation and emission maximum wavelengths in the green region of the visible spectrum ($\lambda_{exc.}$: 503 nm, $\lambda_{em.}$: 517 nm; Figure 3b). These spectral properties are optimal for its use in most spectrophotometers, flow cytometers and confocal microscopes, since these are generally equipped with excitation sources and emission filters matching the spectral properties of GFP or FITC ($\lambda_{exc.}$ ~ 490 nm, $\lambda_{em.}$ ~ 520 nm). Furthermore, we examined the chemical integrity of **1** in different conditions to determine the optimal storage conditions as well as its shelf life (Figure 3c and Supplementary Figures 1-2). Amino acid **1** is completely stable when stored in the dark as a solid, even at room temperature for up to 4 months (i.e. this was the longest time point measured at the time of submission). With respect to the common solvents used in peptide synthesis, we determined that **1** is fully stable in both dichloromethane and methanol solutions for up to 4 months, but loses the Fmoc group when it is stored in DMF for long periods of time at room temperature (13% degradation after 1 month, 45% after 2 months, and 97% after 4 months), in agreement with previous reports on the stability of Fmoc-protected amino acids.[46] Moreover, even though we did not observe any degradation of the BODIPY core upon exposure to daylight, we recommend storing **1** and its derivatives in the dark as a precautionary measure. Altogether, these results confirm that **1** can be shipped and employed as most Fmoc-protected amino acids, and even stored for several months in DMF at 4 ºC or –20 ºC.

Fluorogenic antimicrobial peptides: solid-phase synthesis, fluorogenic behavior and biological characterization. Given their selectivity for microbes over mammalian cells, fluorescent antimicrobial peptides are promising tools for imaging infectious pathogens.[47, 48] Our research group and others have studied the mechanism of action of Peptide AntiFungal 26 (PAF26), an antimicrobial hexapeptide with high affinity for fungal cells. PAF26 (H-RKKWFW-NH_2) has a short and conserved sequence with hydrophobic and cationic domains that are indispensable for its cellular activity.[49] We envisioned that fluorogenic PAF26 analogues, in which natural Trp is replaced by **1**,

would retain the activity and selectivity for fungal cells of the original sequence, while incorporating a suitable label for fluorescence imaging. Furthermore, since cyclic peptides show increased resistance to proteases[50] (such as those secreted by infective pathogens), we designed the synthesis of a cyclic fluorogenic peptide (**BODIPY-cPAF26**, Figures 4 and 5a) based on the sequence of PAF26. **BODIPY-cPAF26** is synthesized using 2-chlorotrityl polystyrene resin and Fmoc-based SPPS protocols (Figure 4).

Figure 4.

In the Procedure, as building blocks are used the amino acid **1** as well as Fmoc-Gly-OH, Fmoc-Phe-OH, Fmoc-Trp-OH, Fmoc-Lys(Z)-OH, and Fmoc-Arg(NO_2)-OH, which are fully mutually orthogonal in the mildly acidic cleavage conditions (TFA: DCM = 1: 99) and enable the isolation of the fully protected cyclic peptide without damaging the BODIPY core. In the synthesis of **BODIPY-cPAF26**, all non-labeled amino acids are incorporated at a concentration of 3 eq, using conventional *N*,*N*'-diisopropylcarbodiimide (DIC) and OxymaPure, whereas **1** is incorporated using a moderate excess (1.5 eq.), and (benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate, 1-hydroxybenzotriazole, and *N*,*N*-diisopropylethylamine (DIPEA) as coupling reagents. Acidic cleavage produces the protected linear peptide, which is subsequently cyclized by treatment with DIPEA and 1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate (HATU) in DMF in over 80% yield after precipitation in H_2O. Final hydrogenolysis and purification by semi-preparative HPLC affords the **BODIPY-cPAF26** peptide in very high purity (> 99%). Typically, the overall yield for the synthesis of the peptide **BODIPY-cPAF26** is 20–25% (~10 mg product per ~45 mg of resin).

We have compared the antimicrobial activity of PAF26 to that of **BODIPY-cPAF26**, and determined their IC_{50} values for two different fungal species, *Neurospora crassa* and *Aspergillus fumigatus*. *N.*

crassa is an experimental model organism for filamentous fungi,[51] whereas *A. fumigatus* is a fungal pathogen causing multiple human diseases, the most lethal of which is invasive pulmonary aspergillosis (IPA), an infection that causes around 200,000 deaths per year worldwide.[52] Notably, **BODIPY-cPAF26** did not impair the antifungal activity of the original sequence, and it displayed slightly higher affinity for both fungal species than the unlabeled PAF26 peptide (Figure 5b and Supplementary Table 1). We also examined the fluorescence response of **BODIPY-cPAF26** in phospholipid liposomes, which mimic the microenvironment of cell membranes. Fluorescence spectra of **BODIPY-cPAF26** were recorded upon incubation with liposome suspensions of phosphatidylcholine:cholesterol (7:1) in phosphate buffer saline with increasing concentrations of phosphatidylcholine. As shown in Figure 5c, **BODIPY-cPAF26** displayed strong fluorogenic behavior, with quantum yields slightly over 30% after binding to phospholipid membranes (Supplementary Table 2). Finally, we evaluated the cytotoxicity of **BODIPY-cPAF26** in A549 lung epithelial cells as an indication of their poor affinity for human cells. We did not detect significant differences in the viability of A549 cells with or without incubation of **BODIPY-cPAF26** for several hours, even at relatively high concentrations (Figure 5d). Altogether, these results corroborate the suitability of the fluorogenic amino acid **1** as a Trp surrogate with minimal interference in the recognition properties of peptide sequences, which provides a suitable reporter to visualize molecular interactions using fluorescence-based techniques.

Figure 5.

Since imaging infection sites using peptide-based probes can be hindered by the presence of proteolytic enzymes, we examined the chemical integrity of **BODIPY-cPAF26** in proteolytic environments, and compared its degradation to the unlabeled PAF26. Both peptides were incubated at the same concentration in a protease cocktail for over 24 h, and time-course analysis was performed by HPLC-MS. Being a cyclic peptide, **BODIPY-cPAF26** showed high stability to

proteases, with marginal degradation after 24 h incubation (Supplementary Figures 3-4). In the same experimental conditions, the unlabeled linear peptide PAF26 was mostly degraded with only 20% of intact peptide left after 24 h.

Live-cell fluorescence imaging of fungal pathogens, including *A. fumigatus.* In view of the excellent properties of **BODIPY-cPAF26** as a highly stable and fluorogenic peptide to report the interaction with fungal cells, we employed it for live-cell imaging of different fungal species. In addition to *N. crassa* and *A. fumigatus*, we also examined the staining of **BODIPY-cPAF26** in other fungal pathogens, namely *Cryptococcus neoformans*, *Fusarium oxysporum* and *Candida albicans*. **BODIPY-cPAF26** showed bright staining in all the species, suggesting a potential common target for different fungi (Figure 6). Notably, the fluorogenic behavior of **BODIPY-cPAF26** enables live-cell imaging by simple incubation with the cells, in a single step and without the need for any washing protocols. This procedure represents an important advantage over imaging procedures with fluorescent peptides that are labeled using alternative procedures (e.g. 'click' fluorophores used in two-step labeling procedures or 'always-on' fluorophores that require washing).

Figure 6.

Since *A. fumigatus* is the fungal pathogen responsible for IPA, a life-threatening disease in immunocompromised patients,[52] we performed further studies to visualize the dynamic interaction between **BODIPY-cPAF26** and living cells of *A. fumigatus*. Germlings of *A. fumigatus* were pre-incubated with a red fluorescent probe that first stained the whole plasma membrane and then intracellular vacuoles. Before the red membrane stain was internalized, we added **BODIPY-cPAF26** to the cells and acquired time-course fluorescence images at high magnification to overlay the green signal from **BODIPY-cPAF26** and the red signal from the counterstain (Figure 7 and Supplementary Video 1). After 20 seconds of its addition, **BODIPY-cPAF26** stained the plasma membrane in the

apical region of the germling cells. In the following minutes, the green fluorescence signal moved towards the base of the germling, while the red counterstain started to accumulate in intracellular vacuolar organelles. These results validate our procedure using the amino acid Fmoc-Trp(C_2-BODIPY)-OH as an efficient chemical strategy to prepare fluorogenic peptides for enhanced fluorescence live-cell imaging without any washing steps. As a proof-of-concept, we have synthesized **BODIPY-cPAF26** as a stable, cyclic antimicrobial peptide for imaging dynamic processes in living fungal pathogens.

Figure 7.

MATERIALS

! CAUTION All organic solvents and bases are flammable, toxic and harmful by inhalation, ingestion or skin contact. Coupling reagents and additives are flammable and irritant. For these and other toxic, corrosive and/or irritant reagents, laboratory coat, gloves and safety goggles should be worn. All synthetic operations should be performed in a chemical fume hood.

REAGENTS

- Deionized water (dH_2O)
- Sterile water (Baxter, cat. no. UKF7114, stored at room temperature (r.t.)) (r.t.: 20–25 °C)
- Celite (Sigma-Aldrich, cat. no. 22138, stored at r.t.)
- Silica gel 60 Å (Carlo Erba, cat. no. P2000027, stored at r.t.)
- 2-Chlorotrityl chloride polystyrene resin (2-CTC-PS; Iris Biotech GMBH, cat. no. BR-1060, stored at r.t.)
- Dichloromethane (DCM; Scharlab, cat. no. CL0331, stored at r.t.)
- Anhydrous dichloromethane (anhydrous DCM; Sigma-Aldrich, cat. no. 494453, stored at r.t.)
- Hexane (Scharlab, cat. no. HE0228, stored at r.t.)
- Toluene (J.T. Baker, cat. no. 8078, stored at r.t.)
- Ethyl acetate (EtOAc; Scharlab, cat. no. AC0143, stored at r.t.)
- *N*,*N*-Dimethylformamide (DMF; Panreac Applichem, cat.no. 161785.1612, stored at r.t.)
- Anhydrous *N*,*N*-Dimethylformamide (anhydrous DMF; Sigma-Aldrich, cat.no. 227056, stored at r.t.)
- *N*,*N*-Diisopropylethylamine (DIPEA; Sigma-Aldrich, cat. no. 496218, stored at r.t)
- N_α-(9-Fluorenylmethyloxycarbonyl)-L-glycine (Fmoc-Gly-OH; Iris Biotech GMBH, cat. no. FAA1050, stored at 4 °C)
- N_α-(9-Fluorenylmethyloxycarbonyl)-L-tryptophan (Fmoc-Trp-OH; Sigma-Aldrich, cat. no. 47637, stored at 4 °C)
- N_α-(9-Fluorenylmethyloxycarbonyl)-L-phenylalanine (Fmoc-Phe-OH; Iris Biotech GMBH, cat. no. FAA1175, stored at 4 °C)
- N_α-(9-Fluorenylmethyloxycarbonyl)- N_ε-(benzyloxycarbonyl)-L-lysine (Fmoc-Lys(Z)-OH; Sigma-Aldrich, cat. no. 47577, stored at 4 °C)
- N_α-(9-Fluorenylmethyloxycarbonyl)- N_ω-(nitro)-L-arginine (Fmoc-Arg(NO_2)-OH; Sigma-Aldrich, cat. no. 47527, stored at 4 °C)
- Methanol (MeOH; Sigma-Aldrich, cat. no. 65542, stored at r.t.)
- Acetonitrile (ACN; Scharlab, cat. no. AC0333, stored at r.t.)
- Formic acid (Merck Millipore, cat. no. 100264, stored at r.t.)
- Trifluoroacetic acid (TFA; Fluorochem, cat. no. 001271, stored at r.t.) **! CAUTION** Toxic and corrosive
- Piperidine (Carlo Erba; cat. no. P0660216, stored at r.t.)
- Ethyl (hydroxyimino)cyanoacetate (OxymaPure; Luxembourg Bio Technologies, stored at r.t.)
- (Benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate (PyBOP; Novabiochem, cat. no. 8510090, stored at 4 °C)
- 1-Hydroxybenzotriazole (HOBt; Carbosynth, cat. no. FH02087, stored at 4 °C) **! CAUTION** Explosive, if dry. Always use as the monohydrate form and keep away from heat sources.
- (1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate) (HATU; Fluorochem, cat. no. 023926, stored at 4 °C) **! CAUTION** Irritant
- Hydrogen (H_2) **! CAUTION** It is extremely flammable; it can form explosive mixture with air and may react violently with oxidants.
- Palladium hydroxide on activated charcoal ($Pd(OH)_2$-C; Sigma-Aldrich, cat. no. 76063, stored at r.t.)
- 3-Iodobenzaldehyde (Apollo Scientific, cat. no. OR8221, stored at 4 °C) **! CAUTION** Irritant
- 2,4-Dimethylpyrrole (Sigma-Aldrich, cat. no. 390836, stored at 4 °C) **! CAUTION** Irritant
- 2,3-Dichloro-5,6-dicyano-*p*-benzoquinone (DDQ; Sigma-Aldrich, cat. no. D60400, stored at 4 °C) **! CAUTION** Toxic
- Triethylamine (TEA; Sigma-Aldrich, cat. no. T0886, stored at r.t.)
- Boron trifluoride diethyl etherate ($BF_3{\cdot}OEt_2$; Sigma-Aldrich, cat. no. 175501, stored at 4 °C) **! CAUTION** Flammable, toxic and corrosive
- Anhydrous sodium sulfate ($Na_2SO_{4\ anh}$; Panreac Applichem, cat.no. 191716.1211, stored at r.t.)
- Palladium acetate ($Pd(OAc)_2$; Sigma-Aldrich, cat. no. 205869, stored at r.t.) **! CAUTION** Irritant and causes skin corrosion
- Silver tetrafluoroborate ($AgBF_4$; Sigma-Aldrich, cat. no. 208361, stored at r.t.) **! CAUTION** Corrosive and harmful.
- Ethanol absolute (EtOH, VWR, cat. no. 20821.330, stored at r.t.)
- Sodium phosphate dibasic heptahydrate (Sigma Aldrich, cat. no. S9390, stored at r.t.)
- Citric acid monohydrate (Sigma Aldrich, cat. no. C-1909, stored at r.t.)
- Sodium hydroxide (Fisher, cat. no. S/4840/60, stored at r.t.)
- *N*,*N*'-Diisopropylcarbodiimide (DIC; Sigma-Aldrich, cat.no. D125407, stored at 4 °C)
- Fluorescein (Sigma-Aldrich, cat. no. 32615, stored at r.t.)
- Protease from *Streptomyces Griseus* type XIV (protease type XIV, Sigma Aldrich, cat. no. P5147, stored at -20 °C)
- PAF26 peptide (H-RKKWFW-NH_2, Genscript, lot no. P10271403, stored at 4 °C)
- Dimethyl sulfoxide for molecular biology (DMSO, Sigma Life Sciences, cat. no. D8418, stored at r.t.)
- Ca^{2+}-free Dulbecco's Phosphate Buffered Saline (Ca^{2+}-free DPBS; Gibco, Life Technologies, cat. no. 14040-091, stored at r.t.)
- MTT reagent (Trevigen, MTT Cell Proliferation assay kit, cat. no. 4890-25-01; stored at. 4 °C)
- Detergent reagent for MTT assay (Trevigen, MTT Cell Proliferation assay kit, cat. no. 4890-25-02, stored at r.t.)
- RPMI Cell Media 1640 (Gibco, Life Technologies, cat. no. 31870-025, stored at 4 °C)
- Phosphatidylcholine: cholesterol (7: 1) liposomes (Clodronate Liposomes, 10 mL PBS liposomes, stored at 4 °C)
- Heat inactivated Fetal Bovine Serum (FBS; Gibco Life Technologies, cat. no. 10500-064, stored at -20 °C)
- Penicillin-Streptomycin (Pen Strep; Gibco Life Technologies, cat. no. 15140-122, stored at -20 °C)
- L-Glutamine (L-Glu; Gibco Life Technologies, cat. no. 25030-024, stored at -20 °C)
- 0.05% (w/v) Trypsin-Ethylenediaminetetraacetic acid (EDTA) (Gibco Life Technologies, cat. no. 25300-054, stored at -20 °C)
- Sucrose (Sigma-Aldrich, cat. no. S0389, stored at r.t.)
- Oxoid™ Agar technical (Thermo Fisher Scientific, cat. no. LP0013B, stored at r.t.)
- Sodium citrate dihydrate (Sigma-Aldrich, cat. no. 71402, stored at r.t.)
- Potassium phosphate monobasic (KH_2PO_4; Sigma-Aldrich, cat. no. P5655, stored at r.t.)
- Ammonium nitrate (NH_4NO_3; Fluka, cat. no. A9642, stored at r.t.)
- Magnesium sulfate heptahydrate ($MgSO_4 \cdot 7\ H_2O$; Sigma-Aldrich, cat. no. M2773, stored at r.t.)

- Calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$; Sigma-Aldrich, cat. no. C3306, stored at r.t.)
- Citric acid (Sigma-Aldrich, cat. no. P5655, stored at r.t)
- Zinc sulfate heptahydrate ($ZnSO_4 \cdot 7\ H_2O$; Sigma-Aldrich, cat. no. Z0251, stored at r.t.)
- Ammonium iron (II) sulfate hexahydrate ($(NH_4)_2Fe(SO_4)_2 \cdot 6\ H_2O$; Sigma-Aldrich, cat. no. 09719, stored at r.t.)
- Copper (II) sulfate pentahydrate ($CuSO_4 \cdot 5\ H_2O$; Sigma-Aldrich, cat. no. C8072, stored at r.t.)
- Manganese (II) sulfate monohydrate ($MnSO_4 \cdot H_2O$; Sigma-Aldrich, cat. no. M7899, stored at r.t.)
- Boric acid (H_3BO_3; Sigma-Aldrich, cat. no. B6768, stored at r.t.)
- Sodium molybdate dihydrate ($Na_2MoO_4 \cdot 2\ H_2O$; Sigma-Aldrich, cat. no. M1651, stored at r.t.)
- D-biotin (SUPELCO, cat. no. 47868, stored at 4 °C)
- Glycerol (UltraPure™ Thermo Fisher Scientific, cat. no. 15514011, stored at r.t.)
- Oxoid™ Sabouraud (SAB) dextrose liquid medium (Thermo Fisher Scientific, cat. no. CM0147, stored at r.t.)
- Oxoid™ Sabouraud dextrose agar (SAB agar; Thermo Fisher Scientific, cat. no. CM0041, stored at r.t.)
- Oxoid™ Potato dextrose agar (PDA; Thermo Fisher Scientific, cat. no. CM0139, stored at r.t.)
- Potato dextrose broth (PDB; CONDA, cat. no. 1261, stored at r.t.)
- Tween80 (Sigma-Aldrich, cat. no. P4780, stored at r.t.)
- Tetramethylaminorhodamine-labelled PAF96 red counterstain (TAMRA-PAF96, TAMRA-AAAWFW-NH_2; Genscript, stored at 4 °C)
- A549 cells (ATCC, cat. no.: A549-ATCC CCL-185, stocks stored at -80 °C or -125 °C)
- *Neurospora crassa* (FGSC, cat. no: FGSC 2489, strain 74-OR23-1V A, stored at -80 °C)
- *Aspergillus fumigatus* (FGSC, cat. no.: FGSC A1163, strain CEA10, stored at -80 °C)
- *Cryptococcus neoformans* (FGSC, cat. no.: FGSC 9487, strain H99, stored at -80 °C)
- *Fusarium oxysporum* (FGSC, cat. no.: FGSC 9935, strain 4287, stored at -80 °C)
- *Candida albicans (*ATCC, cat. no.: ATCC MYA2876, strain SC5314, stored at -80 °C)

LABWARE AND EQUIPMENT

- Set of micropipettes (200-1000, 20-200 and 2-20 µL, Gilson)
- Pipette disposable universal tips (5-200 and 100-1000 µL, Daslab)
- Teflon stick
- 6 mL polystyrene syringes cartridge with polyethylene porous disc (Agilent technologies)
- Spatula
- Timer
- 50 mL polypropylene centrifuge tubes with screwcaps (VWR)
- 15 mL polypropylene conical tubes (Corning)
- 75 cm^2 polystyrene cell culture flasks with canted neck (Corning, cat. no. 430720U)
- 50 mL polystyrene disposable pipette basins (Fisherbrand, cat. no. 13-681-502)
- Pipet aid PIPETBOY acu 2 (Integra, cat. no. 155000)
- 96-well tissue culture plates, black, flat bottom with lid (Falcon, cat. no. 353376)
- 96-well cell culture plates, flat bottom with low-evaporation lid, polystyrene (Costar, cat. no. 3595)
- Plastic syringes (Terumo or BD Plastipak, hypodermic Syringes without needle)
- 1-mL glass syringes (Thomas Scientific)
- Needles (BD Microlance, 21 G)
- 1.5 mL microcentrifuge tubes (VWR)
- Plastic (Agilent technologies) and Teflon (Biotage) stopcocks
- Glass vials (7 x 50 mm, VWR)
- Filters (0.45 µm, PVDF hydrophilic) (Millipore)
- Latex (VWR) or nitrile gloves (GEN-X, Kisher Biotech)
- Plastic septa (Φ 14.9 mm and Φ 30.7 mm, Saint-Gobain Performance Plastics)
- Parafilm
- Round-bottom flasks
- Plastic funnels
- Separatory funnels
- Conical flasks
- Glass Pasteur pipettes (VWR)
- Plastic Pasteur pipettes (VWR)
- Cotton
- Stirring bars
- Aluminium TLC plates (Silica gel 60 F_{254} 20 × 20 cm, Merck KGaA)
- Fritted glass funnels (Φ 55 mm, grade 4, Duran)
- Adapter flask funnels
- 10 mL capped reaction vessels (CEM, cat. no. 19.909050)
- CEM Discover SP microwave synthesizer with Synergy software (CEM)
- Redi*Sep* R_f frits for Redi*Sep* 25 g cartridges (Teledyne ISCO, cat. no. 60-5237-053)
- Syringe filters (25 mm polypropylene 0.45 µm, Phenex, cat. no. AF7-1101-12)
- Columns for ISCO flash chromatography: Silica HP Redi*Sep* R_f 40 g flash columns, cat. no. 69-2203-347; Silica HP Redi*Sep* R_f 30 g flash column, cat. no. 69-2203-345 (Teledyne ISCO)
- 60 mL polystyrene syringe cartridge with polyethylene porous disc for flash chromatography (Agilent technologies)
- CombiFlash purification system (Teledyne ISCO)
- Rotary evaporator (Laborota 4003, Heidolph)
- Analytical balance (AB204-S, Mettler Toledo)
- Orbital platform shaker (Unimax 1010, Heidolph)
- Magnetic stirrer (IKA RCT Basic S1 Werke Safety Control Magnetic Hotplate Stirrer)

- UBD heating block (Grant)
- Hand-held UV lamp (6W, UVP)
- pH-meter (CyberScan pH 510, Eutech)
- Termoblock shaker (Eppendorf Thermomixer)
- Vortex mixer (BV1000, Benchmark Scientific)
- Multichannel electronic pipette (Rainin E4 XLS 12-channel, 20-200 µL LTS, Mettler-Toledo)
- Multichannel electronic pipette tips (Rainin Tips LTS, 200 µL, GP-L200F, Mettler-Toledo)
- Cell counter (ChemoMetec A/S, Nucleocounter)
- Lyophilizer (Virtis Freezemobile)
- Centrifuge for peptide synthesis (Beckman Allegra 21R)
- Centrifuge for cell culture (Sanyo Mistral 3000i)
- Sonicator (Clifton Ultrasonic bath)
- Water bath (Aqualine AL18, Lauda)
- Vacuum pump
- High-performance liquid chromatography system (HPLC): Waters 2695 separations module, Waters 2996 photodiode array detector
- HPLC system coupled to mass spectrometer (HPLC-MS): Waters 2695 separations module, Waters 2996 photodiode array detector. The MS spectrometer contains an electrospray ionization source (Micromass ZQ) and the MassLynx 4.1 software.
- Semi-preparative HPLC: Waters 2707 autosampler, Waters 2489 detector, Waters fraction collector III and software ChromeScope.
- HPLC vials (300 µL polypropylene plastic, Waters)
- Nuclear Magnetic Resonance spectrometers (NMR): Varian Mercury 400 MHz or Bruker Avance III 600 MHz
- High-resolution mass spectrometer (HRMS): LTQ-FT Ultra (Thermo Scientific)
- Class-II microbiological safety cabinet (Biomat2)
- Synergy H1 hybrid spectrophotometer (Gen5 2.01 analysis software, Biotek).
- Cell incubator (Panasonic Incusafe)
- Inverse microscope (Olympus CK2)
- Cell culture flasks (TC Flask T25 stand, Vent. Cap, SARSTEDT, cat. no. 83.3910.002)
- Inoculation loop (10 µL, blue, sterile, Greiner bio-one, cat. no. 731171)
- Glass flat bottom flasks (250 mL, PYREX®)
- Foam plug for flask (50 × 38 mm, King Scientific, cat. no. FS5038)
- 8-well chamber (µ-Slide, ibiTreat, V2.0, sterilized, ibidi GmbH, cat. no. 80826)
- Miracloth (Merck Calbiochem, cat. no. 475855)
- Sterile disposable Petri dishes (9 cm, Thermo Scientific, cat. no.101IRR)
- Haemocytometer (Profondeur, 0.200 mm, 0.0625 mm^2, Fuchs-Rosenthal, SUPERIOR MARIENFELD)
- Lens cleaning tissue (Whatman105, 100 × 150 mm, GE Healthcare, cat. no. 2105-841)
- Aluminum foil (45 cm, Terinex)
- 3M Micropore surgical-tape (Micropore)
- TriStar LB941 multimode microplate reader (BertholdTech)
- Inverted confocal fluorescent microscope (Leica SP8) with temperature control unit (550W, the Cube E5CN-H) and environmental control box
- Autoclave (AL02-01, Advantage-Lab)
- Refrigerated incubator (Model MIR-153, 126 L, Sanyo)
- Shaking incubator (Innova 4400, New Brunswick)

REAGENT SETUP

▲ **CRITICAL** Autoclave all media on the same day they are prepared.

! CAUTION Do not place materials that are not autoclave-safe in autoclave (non-heat resistant plastics and glass), as they will melt or shatter during sterilization cycle. Fill the container up to 2/3 of the maximum volume and always loosen up the lid of the bottles before autoclaving.

Piperidine solution. Prepare a minimum of 110 mL of a piperidine: DMF (1:4) solution. This solution should be prepared fresh before use.

! CAUTION Piperidine is toxic by inhalation. It must be handled with care inside a fume hood.

Sample preparation for analytical HPLC and HPLC-MS. Dissolve an aliquot of the crude or the final products in a ACN: H_2O (1:1) mixed solvent or in MeOH and then transfer into an HPLC vial. Aliquots containing other organic solvents must be first evaporated and redissolved in MeOH. Filter, when appropriate, through a 0.45 µm filter to remove any insoluble material. Typically, 5-50 µL volume injections are used for HPLC analysis.

5 mM DMSO solutions of BODIPY-cPAF26 and PAF26 peptides. Dissolve 1.0 mg of each peptide in DMSO (152 µL for **BODIPY-cPAF26**, 210 µL for PAF26) and store at -20 °C. In order to avoid several freeze-thaw cycles, DMSO stocks should be ideally stored in small multiple aliquots, which can be kept at -20 °C for at least 6 months without observing any degradation.

! CAUTION DMSO is highly toxic. Gloves must be worn at all times to avoid contact with skin.

1 µM fluorescein in 0.1 N NaOH in EtOH. Prepare a 10 mM solution of fluorescein in DMSO by weighing 3.3 mg and dissolving them in 1 mL of DMSO. Weigh 40 mg of NaOH in a 15 mL conical tube and dissolve in 10 mL EtOH. Dilute the 10 mM fluorescein solution in 0.1 N NaOH in EtOH to a final concentration of 1 µM. This solution can be stored at r.t. in the dark for several days.

! CAUTION NaOH is very corrosive; hence protective equipment (goggles, gloves, and lab coat) should be worn when handling this chemical.

Citric acid/phosphate buffer (pH 7.0-7.1). Weigh 1 g of citric acid monohydrate in a 50 mL centrifuge tube and dissolve the solid in 50 mL of sterile H_2O. On a separate tube, weigh 2.7 g dibasic sodium phosphate heptahydrate and dissolve in 50 mL sterile H_2O. Mix

21.9 mL of phosphate solution with 3.1 mL of citric acid solution and dilute to a total of 50 mL with sterile H_2O. Check pH with the help of a pH meter (pH = 7.0-7.1). This buffer solution can be stored at 4 ˚C for several months.
Protease cocktail. Weigh 1.5 mg protease type XIV in a 1.5 mL microcentrifuge tube and dissolve in 1.5 mL citric acid/phosphate buffer (pH 7.1) to obtain a 1 mg mL^{-1} stock solution. Dilute the stock solution in the same buffer to a final concentration of 1 µg mL^{-1}. The protease cocktail should be prepared fresh before use.
Cell culture medium. Mix 500 mL RPMI cell media 1640, 50 mL FBS, 5 mL Pen Strep and 5 mL L-Glu in a sterile environment. The resulting medium can be filtered using disposable bottle top filters with PES membranes and stored at 4 °C for 2–3 months.
▲ **CRITICAL** Cell culture experiments must be performed in sterile conditions inside a class-II microbiological safety cabinet.
Vogel's sucrose minimal medium. Prepare solid Vogel's sucrose minimal agar medium according to **Tables 1-3**, adding 2% (w/v) agar before autoclaving. To prepare liquid Vogel's sucrose minimal medium, follow the same recipe excluding agar addition (autoclave and store at r.t.). This medium can be safely stored under sterile conditions at 4 ºC for up to 12 months.
SAB dextrose liquid medium. Dissolve 30 g of SAB liquid medium in 1 L dH_2O (autoclave and store at r.t.). This medium can be safely stored under sterile conditions at 4 ºC for up to 12 months.
SAB dextrose agar medium. Dissolve 65 g of SAB agar in 1 L dH_2O (autoclave and store at r.t.). This medium can be safely stored under sterile conditions at 4 ºC for up to 12 months.
PDA medium. Dissolve 40 g of PDA agar in 1 L dH_2O (autoclave and store at r.t.). This medium can be safely stored under sterile conditions at 4 ºC for up to 12 months.
PDB medium. Dissolve 26.5 g of PDB medium in 1 L dH_2O. Mix well and dissolve by heating with frequent agitation until complete dissolution. Boil for 1 min if needed (autoclave and store at r.t.). This medium can be safely stored under sterile conditions at 4 ºC for up to 12 months.

Table 1.

Table 2.

Table 3.

Preparation of agar flasks for culturing fungal cells. Heat up and melt sterile agar medium using a conventional microwave oven and pour 10 – 15 mL of it into sterile T25 cell culture flasks. Tighten the lids, lay the flasks down and let the agar cool down at r.t. Store the flasks at 4 ºC in a sealed plastic bag for up to 6 months**.**
Preparation of stock suspension aliquots of *A. fumigatus*. Streak *A. fumigatus* spores (purchased from FGSC) using a sterile inoculation loop onto the surface of solid Vogel's sucrose minimal agar medium within a T25 cell culture flask, and incubate the flask at 37 ˚C in the dark for 3 days. Add 1 mL of sterile dH_2O to the flask, shake gently and transfer the suspension containing fungal spores (conidia) with a P1000 micropipette to a 2 mL microcentrifuge tube. Add 0.5 mL glycerol to the tube, shake gently and prepare stock suspension aliquots of 100 µL into sterile 1.5 mL microcentrifuge tubes. Seal the tubes with parafilm and store them at – 80 ºC for up to 12 months.
Preparation of stock suspension aliquots of *C. neoformans*. Streak *C. neoformans* (purchased from FGSC) using a sterile inoculation loop onto 15 mL SAB dextrose agar medium within a 9 cm Petri dish. Transfer a single colony with a sterile pipette tip into a sterile glass flat-bottom flask containing 10 mL of liquid SAB dextrose liquid medium. Plug the flask with a sterile foam plug, seal the flask with aluminum foil and incubate the flask on a shaking incubator at 37 ºC (170 rpm) for 20 h. Filter the suspension from the flask through folded Miracloth (4 layers) into a polypropylene conical tube, centrifuge for 5 min at r.t. (4,000 rpm, 1,755 g) and remove the supernatant. Resuspend the pellet in 2 mL glycerol: dH_2O (3:7) and prepare stock aliquots of 100 µL into 1.5 mL microcentrifuge tubes. Seal the microcentrifuge tubes with parafilm and store them at – 80 ºC for up to 12 months.
Preparation of stock suspension aliquots of *F. oxysporum*. Add *F. oxysporum* microconidia spores (purchased from FGSC) using a sterile inoculation loop into a sterile glass flat-bottom flask with 20 mL of PDB medium. Plug the flask with a sterile foam plug, seal the flask with aluminum foil and incubate the flask on a shaking incubator at 28 ºC (170 rpm) for 5 days. Filter the suspension from the flask through folded Miracloth (4 layers) into a polypropylene conical tube. Centrifuge for 10 min at r.t. (4,000 rpm, 1,755 g), discard the supernatant and resuspend the pellet in 1.5 mL of sterile dH_2O (these steps must be repeated twice). Add 0.5 mL glycerol into the tube, shake the suspension and prepare stock aliquots of 100 µL into 1.5 mL microcentrifuge tubes. Seal the microcentrifuge tubes with parafilm and store them at – 80 ºC for up to 12 months.
Preparation of stock suspension aliquots of *C. albicans*. Transfer a single colony of *C. albicans* (purchased from ATCC) using a sterile inoculation loop into a sterile glass flat-bottom flask with 15 mL of PDB medium. Plug the flask with a sterile foam plug, seal the flask with foil and incubate the flask on a shaking incubator at 30 ºC (170 rpm) for 12 h. Filter the suspension from the flask through folded Miracloth (4 layers) into a polypropylene conical tube, centrifuge for 5 min at r.t. (4,000 rpm, 1,755 g) and remove the supernatant. Resuspend the pellet in 2 mL glycerol: dH_2O (3:7) and prepare stock aliquots of 100 µL into 1.5 mL microcentrifuge tubes. Seal the microcentrifuge tubes with parafilm and store them at – 80 ºC for up to 12 months.
▲ **CRITICAL** All these preparations must be performed in sterile conditions inside a class-II microbiological safety cabinet.

EQUIPMENT SETUP
Manual peptide-synthesis manifold. Place a vacuum manifold in the fume hood and connect it to the vacuum line including a trap. Use polystyrene syringe cartridges with polyethylene porous discs to retain the resins, and hold them on the manifold with two-way stopcocks. Cap all unused inlets with plastic septa. Use a Teflon stick for manual stirring.

Thermoblock shaker. Before incubating samples for protease degradation assays, the thermoblock shaker must be equilibrated at 37 ˚C. Once equilibrated, microcentrifuge tubes containing samples are covered in aluminum foil and shaken at 37 ˚C for the indicated times.
Spectrophotometer. Settings for fluorescence readings: monochromator-based, excitation: 450 nm, emission: 480-600 nm with 1 nm resolution, gain: 90-100. Settings for absorbance readings: 420-550 nm with 1 nm resolution or single endpoint measurements.
HPLC-based analysis and purification. See **Tables 4-6.**

Table 4.

Table 5.

Table 6.

Autoclave. Autoclave all samples at 121 ^{o}C for 18 min without any drying cycles. Autoclave any lab ware/equipment (pipette tips etc.) at 121 ^{o}C for 15 min with a 15 min drying cycle and fast exhaust.
! CAUTION Do not place materials that are not autoclave safe in autoclave (non-heat resistant plastics and glass) as they will melt or shatter during sterilization cycle. Fill the container up to 2/3 of the maximum volume and always loosen up the lid of the bottles before autoclaving.

Confocal microscopy. We used a Leica TCS SP8 laser scanning confocal microscope with a tunable white light laser (WLL, 450-750 nm), photomultiplier tubes (PMT), hybrid GaAsP (HyD) detectors and a 63× water immersion objective (HC PL APO UVIS CS2, 1.4 N.A.) for fluorescence live-cell imaging. For live-cell imaging of different fungal species (steps 199-206), we used one excitation wavelength (496 nm, laser intensity: 20%) from the WLL source (power level: 70%) and the emission was detected between 505 and 550 nm. A transmitted light detector was used for brightfield image acquisition (gain 355, offset 0%). For time-lapse high resolution imaging (steps 207-219), we used two different excitation wavelengths (i.e. 496 nm (laser intensity: 25%) and 570 nm (laser intensity: 10 %) from the WLL (power level: 70%) and two emission bands (i.e. 505-550 nm (green) and 585-650 nm (red)). The transmitted light detector was used with the same settings as above. Images for time-lapse movies were acquired at 5 sec intervals. All images were acquired in a 1024 × 1024 pixel format and a scanning speed of 400 Hz. The pixel size of all images was 39.95 × 39.95 nm^2.
If live-cell images are taken with a different microscope, excitation should be performed within the 450-500 nm (green) and the 550-600 nm ranges (red), and fluorescence emission should be collected using either band-pass or long-pass filters covering 500-550 nm (green) or 590-650 nm (red) ranges. Water and oil immersion objectives can be both used for image acquisition. The settings for image format and scanning speed should be adjusted depending on the requirements of the experiment. However, we recommend setting high pixel format and slow scanning speeds whenever possible.

PROCEDURE

▲ **CRITICAL** Cultures of the five different fungal species — *N. crassa*, *A. fumigatus*, *C. neoformans*, *F. oxysporum*, and *C. albicans* — (see steps 132–151) must be performed under sterile conditions using a class-II microbiological safety cabinet. All reagents and pipette tips must be sterile. Cultures of the various fungal species can be performed in parallel to maximize time efficiency.

▲ **CRITICAL** Preparation of fungal cells for fluorescent live-cell imaging (see steps 163–189) must be performed under sterile conditions using a class-II microbiological safety cabinet. All reagents and pipette tips must be sterile. Preparation of the multiple species can be performed in parallel to maximize time efficiency.

Synthesis of 4,4-difluoro-8-(3-iodophenyl)-1,3,5,7-tetramethyl-4-bora-*3a*,*4a*-diaza-*s*-indacene (*m*-iodophenyl-BODIPY (3)) ● **TIMING 32 h**

▲ **CRITICAL** As a visual aid, in Figure 8 are reported photos of various stages of the present sub-section of the Procedure.

1| Weigh 500 mg 3-iodobenzaldehyde (1.0 eq., 2.2 mmol) in a 250 mL round-bottom flask containing a magnetic stir bar (egg-shaped, 25 × 10 mm).

2| Cap the flask with a rubber septum (Φ 30.7 mm), place it on a magnetic stirrer and add to the flask 50 mL of anhydrous DCM using a 60 mL syringe under N_2 stream while stirring the contents of the flask (stirring mot.: 70 % of full power).

3| Add to the flask 500 µL 2,4-dimethylpyrrole (2.2 eq., 4.7 mmol) with a 1 mL syringe under N_2 stream and constant stirring.

4| Add to the flask four drops (~15 µL) of TFA with a 1-mL syringe under N_2 stream. The solution turns orange.

! CAUTION We recommend using a glass needle when working with TFA to avoid any potential contamination due to degradation of plastic syringes.

5| Stir the reaction mixture under N_2 stream at r.t. for 30 min (stirring mot.: 70 % of full power).

6| Suspend the N_2 flow and stir the reaction mixture under N_2 atmosphere at r.t. for 22 h (stirring mot.: 70 % of full power). The solution turns dark red.

7| Monitor the consumption of 3-iodobenzaldehyde and the formation of a new product by sampling an aliquot from the reaction mixture and performing analysis by TLC (DCM: hexane (1: 1), Rf ≈ 0.4-0.5) or HPLC (Gradient_2, **Table 4**).

8| Weigh 490 mg DDQ (1.0 eq., 2.2 mmol) in a 50 mL conical flask and add to it 30 mL of anhydrous DCM.

9| Shake and sonicate the resulting yellow suspension until it is almost completely dissolved.

10| Add dropwise (for ~ 10 min) the solution prepared in step 9 to the reaction mixture with a syringe under N_2 stream (stirring mot.: 80 % of full power).

11| Rinse the conical flask with 2 mL of anhydrous DCM and transfer the solution dropwise to the reaction mixture (for ~ 5 min).

12| Stir the reaction mixture under N_2 for 5 min.

13| Suspend the N_2 flow and stir the reaction mixture under N_2 atmosphere for 10 min.

14| Add to the reaction mixture dropwise 4 mL of TEA (45.0 eq., 97.0 mmol) with a syringe and under N_2 stream. The solution turns dark green.

15| Add to the reaction mixture dropwise (for ~ 3 min) 4 mL $BF_3{\cdot}OEt_2$ (30.0 equivalents, 64.7 mmol) with a syringe and under a N_2 stream.

16| Stir the reaction mixture under a N_2 stream for 3 min; then, suspend the N_2 flow and stir the reaction mixture under N_2 atmosphere for 4 h 30 min.

17| Monitor the formation of the new product by sampling the reaction mixture and performing analysis by TLC (DCM: hexane (1: 1), Rf ~ 0.2-0.3) or HPLC (t_R = 8.0 min; Gradient_2, **Table 4**).

18| Remove the septum and transfer the reaction mixture into a 250 mL separatory funnel containing 50 mL of dH_2O. Rinse the reaction flask with 20 mL of DCM.

19| Place a stopper on the separatory funnel and shake vigorously 10 times. Remove the stopper, transfer the organic layer into a 250 mL conical flask and discard the aqueous layer into another 250 mL container. Transfer the organic layer back to the separatory funnel and repeat the step after adding another 50 mL of dH_2O.

20| Combine the aqueous layers and back-extract the resulting solution with 20 mL of DCM. Combine the organic layers, transfer them into a 250 mL conical flask and add to it the necessary amount of anhydrous Na_2SO_4.

21| Filter the DCM solution into a 250 mL round-bottom flask through a funnel containing a small piece of glass wool at the bottom. Rinse the reaction flask with 20 mL DCM and add the resulting solution to the filtrate.

22| Remove the organic solvent under reduced pressure (water bath max. T: 40 °C) using a rotary evaporator.

■ **PAUSE POINT** For storage, cap the flask and store the crude mixture at 4 °C. The crude mixture can be safely stored for at least 24 h.

23| Purify compound **3** (DCM: hexane (1: 1), Rf ~ 0.2) by flash column chromatography (Silica RediSep R_f 40 g flash column CV 53.9 mL - 40 mL/min), using a DCM: hexane linear gradient (increment rate: 1% DCM min^{-1}) and dual-wavelength detection (254 and 360 nm). When a compound is detected, change to isocratic mode until the compound is completely eluted. For loading, dissolve the crude mixture in 10 mL of DCM in a round-bottom flask and add to it two spatula tips of silica. Subsequently, remove the solvent under reduced pressure (water bath max. T: 30 °C) using a rotary evaporator. Transfer the silica-adsorbed crude mixture into a 60 mL polystyrene syringe cartridge and load into a CombiFlash purification system. Run the flash column chromatography with DCM: hexane at 40 mL min^{-1} and collect 12 mL fractions. Typically, compound **3** elutes when the DCM: hexane volume ratio is 88: 12. The compound **3** might crystallize in some of the collection tubes.

? TROUBLESHOOTING

24| Identify the fractions containing compound **3** by sampling aliquots from the collected tubes and analyzing them by HPLC (t_R = 8.0 min; Gradient_2, **Table 4**).

25| Combine the pure fractions into a 100 mL round-bottom flask and remove the solvent under reduced pressure (water bath max. T: 40 °C) using a rotary evaporator to obtain a red solid. The solid can be analyzed by HPLC-MS to check the purity and exact mass (tR: 4.5 min; HPLC-MS (2), **Table 5**).

? TROUBLESHOOTING

PAUSE POINT For storage, cap the flask and store compound **3** under N_2 at 4 °C. Compound **3** can be safely stored for at least 9 months.

Figure 8.

Synthesis of Fmoc-Trp(C_2-BODIPY)-OH (1) ● TIMING 5 h

CRITICAL As a visual aid, in Figure 9 are reported photos of various stages of the present sub-section of the Procedure.

26| Weigh 3.3 mg $Pd(OAc)_2$ (0.05 eq., 0.02 mmol), 57.1 mg $AgBF_4$ (1.0 eq., 0.29 mmol) and 125 mg Fmoc-Trp-OH (1.0 eq., 0.29 mmol) and transfer them as solids into a 10 mL microwave reaction vessel.

27| Add to the microwave reaction vessel a magnetic stir bar (cylindrical stirring bar 10 × 3 mm).

28| Place 198 mg of compound **3** (1.5 eq., 0.44 mmol) in a 5 mL round-bottom flask and transfer the compound to the microwave reaction vessel with anhydrous DMF (1.2 mL in 100 µL portions) using a micropipette. Shake the resulting solution.

29| Add to the microwave reaction vessel 22 µL (0.29 mmol) of TFA using a micropipette and close the reaction vessel with a microwave cap.

! CAUTION Due to the volatility and corrosiveness of TFA, we recommend using a glass syringe or pipette.

30| Sonicate the solution for 10 seconds.

31| Place the reaction mixture under microwave irradiation (250 W) at 80 °C for 20 min in a CEM Discover microwave synthesizer. Monitor the formation of compound **1** by sampling an aliquot from the crude mixture and performing analysis by HPLC (t_R = 7.0 min; Gradient_2, **Table 4**).

? TROUBLESHOOTING

32| Pack a pad of Celite into a fritted glass funnel and wash it twice with 25 mL EtOAc.

33| Load the crude reaction mixture with a glass pipette.

34| Rinse the reaction vessel with 25 mL of EtOAc and add the resulting solution to a flask fitted with a filter. Filter the solution containing the reaction mixture through Celite using vacuum and collect the filtrates in a 100 mL round-bottom flask. Wash the Celite pad twice with 17 mL of EtOAc each time. Evaporate the solvent under reduced pressure at 43 °C using a rotary evaporator (add portions of toluene to enhance DMF removal).

PAUSE POINT For storage, cap the flask and store the crude mixture at 4 °C The crude mixture can be safely stored for at least 24 h.

35| Purify compound **1** (EtOAc, Rf ~ 0.3) by flash column chromatography (Silica RediSep R_f 12 g flash column CV 16.8 mL - 30 mL min^{-1}) using a EtOAC: hexane linear gradient (increment rate: 2% EtOAc min^{-1}) and dual-wavelength detection (254 and 306 nm). When a compound is detected, change to isocratic mode until the compound is completely eluted. For loading, dissolve the crude mixture in 15 mL of EtOAc in a round-bottom flask and add to the resulting solution two spatula tips of silica. Subsequently, remove the solvent under reduced pressure (water bath max. T: 40 °C) using a rotary evaporator. Transfer the silica-adsorbed crude mixture into a 60 mL polystyrene syringe cartridge and load into a CombiFlash purification system. Run the flash column chromatography with EtOAc: hexane at 30 mL min^{-1} and collect 12 mL fractions. Typically, compound **1** elutes when the EtOAc: hexane volume ratio is 3: 7. When 40 g columns are used, the product elutes when the EtOAc: hexane volume ratio is 1: 1. Compound **1** is isolated

as a red amorphous solid. The solid can be analyzed by HPLC-MS to check the purity and exact mass (tR: 9.6 min; HPLC-MS (1), **Table 5**).

■ **PAUSE POINT** Compound **1** can be safely stored for at least 4 months at 4 °C under N_2.

Figure 9.

Analysis of samples of the amino acid 1 stored for up to 4 months ● **TIMING 1 h**

36| If compound **1**, as obtained after completing step 35 above, is not used immediately after purification and is stored instead, its chemical purity needs to be reconfirmed before use. To test the compound stability as a solid, weigh 5 mg of the amino acid **1** in a 2 mL glass vial and wrap in aluminum foil. To test the compound stability in solution, weigh 2.2 mg of the amino acid **1** in a 50 mL centrifuge tube and dissolve it in 30 mL of DCM, MeOH, or DMF to obtain 0.1 M solution. Aliquot the solution into three separate 15 mL conical tubes (10 mL each) and wrap them in aluminum foil.

37| Store the three solution and one solid samples at three different temperatures: r.t., 4 ˚C, and -20 ˚C. At the indicated time points (i.e. 1 month, 2 month and 4 months), sample aliquots (spatula tip for solid, 50 µL for solutions), dilute in MeOH (50 µL) and analyze by HPLC-MS (tR: 9.6 min; HPLC-MS (1), **Table 5**).

? TROUBLESHOOTING

Solid-phase peptide synthesis of BODIPY-cPAF26 ● **TIMING 36 h**

▲ **CRITICAL** As a visual aid, in Figure 10 are reported photos of various stages of the present sub-section of the Procedure.

38| Weigh 46 mg of 2-CTC-PS resin (loading capacity: 1.6 mmol g^{-1}) into a 6 mL polystyrene syringe with a polyethylene porous disc. Place it in the vacuum manifold using a two-way stopcock.

39| Swell the resin with DCM with gentle agitation using a Teflon stick (2 × 4 mL × 15 s).

40| Calculate the amount of the first amino acid (Fmoc-Gly-OH) to achieve a loading capacity around 1 mmol g^{-1} (i.e. mass of resin (g) × 1 mmol g^{-1} × amino acid molecular weight (g mol^{-1}), see **Table 7**).

41| Weigh 14 mg of Fmoc-Gly-OH in a 1.5 mL microcentrifuge tube. Add to the tube 0.5 mL of DCM and 24 µL of DIPEA. Shake for a few seconds until the solid dissolves completely and add the solution to the syringe followed by manual stirring using a Teflon stick. Wash the microcentrifuge tube with 0.5 mL DCM and add the washing liquid to the syringe followed by manual stirring using a Teflon stick.

42| Shake the syringe in an orbital shaker for 10 min at 200 rpm (4 g).

43| Add to the syringe 55 µL of DIPEA without filtering and shake the syringe in an orbital shaker for 40 min at 200 rpm (4 g).

44| Add 36 µL of MeOH (0.8 µL MeOH per mg resin) to the resin without filtering to cap unreacted groups. Shake the syringe in an orbital shaker for 10 min at 200 rpm (4 g).

45| Put the syringe in the vacuum manifold and drain the resin.

46| Wash the resin thoroughly with DCM (4 × 3 mL × 10 s) and DMF (4 × 3 mL × 10 s), and apply vacuum to drain the resin after each wash.

47| Add to the syringe a piperidine: DMF (1: 4) solution to remove the Fmoc protecting group (1 × 5 mL × 1 min + 2 × 5 mL × 5 min). For each wash, gently shake the reaction mixture with the Teflon stick and then drain the resin under vacuum.

48| Calculate the amount of the second amino acid and the coupling reagents (Fmoc-Trp-OH, DIC and OxymaPure) needed on the basis of the amount of resin and functionalization (i.e. mass of resin (g) × 1 mmol g^{-1} × 3 eq. × reagent molecular weight (g mol^{-1}) see **Table 7**).

49| Weigh the amino acid and OxymaPure as calculated in step 48 in a 1.5 mL microcentrifuge tube.

50| Wash the resin thoroughly with DCM (4 × 3 mL × 10 s) and DMF (4 × 3 mL × 10 s), and apply vacuum to drain the resin after each wash.

51| Dissolve the reagents from step 49 in 0.5 mL of DMF, add 21 µL of DIC, and shake the resulting solution for 5 min.

52| Add the resulting activated amino acid solution to the resin and gently shake the resulting reaction mixture with the Teflon stick for 4 min.

53| Shake the syringe in an orbital shaker for 56 min at 200 rpm (4 g) and then drain the resin. Wash the resin as in step 50.

54| Run a Kaiser test[53] to assess the extent of the peptide coupling.

? TROUBLESHOOTING

55| Repeat steps 48–54 for coupling all following amino acid (i.e. Fmoc-Phe-OH) and steps 46–47 for Fmoc removal.

■ **PAUSE POINT** Peptide elongation can be paused at any point after the incorporation of the third amino acid. Pausing after the incorporation of the second amino acid is not recommended due to the potential formation of diketopiperazines.[54] Storing the peptide as Fmoc-protected is not recommended, because partial Fmoc removal can lead to re-incorporation of the fluorenylmethyl moiety to the free amine group giving the corresponding secondary amine. For storage, wash the resin 3 times with DCM or DMF (5 mL each), wrap the syringe in aluminium foil and store at r.t. The resin can be safely stored at 4 °C for at least 24 h.

56| For the coupling of the amino acid **1**, calculate the amount of amino acid and coupling reagents (**1**, HOBt, PyBOP and DIPEA) needed on the basis of the amount of resin and functionalization (i.e. mass of resin (g) × 1 mmol g^{-1} × 1.5 or 2 eq. × reagent molecular weight (g mol^{-1}), see **Table 7**).
57| Weigh the amino acid and HOBt as calculated in step 56 in a 1.5 mL microcentrifuge tube. Separately, weigh PyBOP in a separate 1.5 mL microcentrifuge tube.
58| Wash the resin thoroughly with DCM (4 × 3 mL × 10 s) and DMF (4 × 3 mL × 10 s), and apply vacuum to drain the resin after each wash.
59| Dissolve PyBOP from step 57 in 0.1 mL of DMF.
60| Dissolve the reagents from step 57 in 0.4 mL of DMF. Add to the resulting solution 16 µL DIPEA (2 eq.) and the PyBOP solution from step 59. Shake the resulting solution for 2 s, add it to the resin and gently shake the resulting reaction mixture with the Teflon stick for 4 min.
61| Shake the syringe in an orbital shaker for 56 min at 200 rpm (4 g) and then drain the resin. Wash the resin as in step 58.
62| Run a Kaiser test[53] to assess the extent of the coupling.
? TROUBLESHOOTING

■ **PAUSE POINT** For storage, wash and drain the resin three times with DCM (5 mL of DCM per wash-drain cycle), wrap the syringe in aluminum foil and store the resin at r.t. for 24 h.

Table 7.

63| Wash the resin thoroughly with DCM (4 × 3 mL × 10 s) and DMF (4 × 3 mL × 10 s), and apply vacuum to drain the resin after each wash.
! CAUTION Always use a Teflon two-way stopcock when working with TFA (see next step).
64| Add 3 mL of a freshly prepared TFA: DCM (1: 99) solution to the resin. Shake gently with a Teflon stick for 1 min. Collect the filtrates in a 100 mL round-bottom flask with 30 mL DCM. Keep constant stirring of the solution in a magnetic stirrer (egg-shaped stirring bar 2 cm × 1 cm, stirring mot.: 60% of full power). Wash the resin with DCM (3 mL × 5 s) and collect the filtrates in the same flask.
65| Repeat step 64 another four times with an additional final wash with DCM (3 mL × 5 s), collecting all the filtrates in the same flask.
66| Remove the solvent under reduced pressure (water bath max. T: 40 °C) using a rotary evaporator.
▲ **CRITICAL** Low acidic conditions and short reaction times are critical to preserve the integrity of the BODIPY fluorophore.
67| Dissolve the crude reaction mixture obtained in step 66 in 14 mL of ACN: H_2O (1:1), adding first ACN and then H_2O in a 50 mL polypropylene centrifuge tube.
▲ **CRITICAL** The ACN volume should be reduced to the minimum in order to avoid melting the crude peptide solution during freeze-drying.
68| Freeze the crude peptide solution using liquid N_2. Lyophilize for 24 h, wrapping the glass container in aluminum foil.
69| Sample a small aliquot of the peptide crude, dissolve in ACN: H_2O (1: 1) and analyze by HPLC-MS to check the crude purity and the exact mass (tR: 3.1 min; HPLC-MS (2), **Table 5**).

Figure 10.

Cyclization of the protected peptide ● TIMING 3 h
70| Weigh 14 mg of HATU (1.0 eq., 0.04 mmol) in the 50 mL centrifuge tube from step 68, which should contain 61 mg of linear protected peptide (0.04 mmol). The amount of HATU should be stoichiometrically determined according to the amount of peptide obtained.
71| Dissolve the compounds in 0.9 mL anhydrous DMF and add 16 µL DIPEA (2.5 eq., 0.09 mmol).

72| Stir the solution for 2 h at r.t. on a magnetic stirrer (cylindrical stirring bar 8 × 3 mm, stirring mot.: 80% of full power). Sample a small aliquot of the peptide crude, dissolve in ACN: H_2O (1: 1) and analyze by HPLC-MS to confirm the reaction is completed (tR: 4.1 min; HPLC-MS (2), **Table 5**).
73| Add to the 50 ml centrifuge tube dropwise 12 mL of cold (5-10 °C) H_2O to precipitate the protected peptide.
74| Vortex the mixture for 1 min, centrifuge for 5 min at 4,000 rpm (1,610 g) and carefully decant H_2O.
75| Repeat steps 73-74 two additional times.

Lyophilization of the cyclic protected peptide ● TIMING 24 h
76| Re-dissolve the protected peptide in 8 mL of ACN: H_2O (1: 1). Freeze the crude peptide solution using liquid N_2. Lyophilize for 24 h, wrapping the glass container in aluminum foil.
77| Sample a small aliquot of the peptide crude, dissolve in ACN: H_2O (1: 1) and analyze by HPLC to check the purity (tR: 4.1 min; HPLC-MS (2), **Table 5**).
■ **PAUSE POINT** For storage, wrap the centrifuge tube in aluminum foil and store at 4 °C. The peptide can be safely stored at 4 °C for at least 7 days.

Hydrogenation reaction ● TIMING 49 h
▲ **CRITICAL** As a visual aid, in Figure 11 are reported photos of various stages of the present sub-section of the Procedure.
78| Weigh 45 mg of protected **BODIPY-cPAF26** peptide from step 76 (1.0 eq., 0.03 mmol) and 23 mg of $Pd(OH)_2$-C in a 25 mL round-bottom flask containing a magnetic stir bar (cylindrical stirring bar 10 × 3 mm).
79| Cap the flask with a rubber septum (Φ 14.9 mm). Evacuate and backfill the flask with Ar or N_2.
80| Add 4.5 mL of freshly prepared MeOH: formic acid (95: 5) with a syringe and purge for 5 min.
81| Connect a balloon filled with hydrogen (H_2) to the system through a needle and purge the flask with H_2.
82| Stir the reaction mixture under H_2 atmosphere on a magnetic stirrer at r.t. for 48 h (stirring mot.: 80% of full power). Refill the H_2 balloon periodically along with re-addition of $Pd(OH)_2$-C (3 times overall). Sample a small aliquot of the peptide crude, dissolve it in 0.5 mL ACN: H_2O (1: 1) and analyze by HPLC-MS to confirm that the reaction is completed (tR: 5.4 min for **BODIPY-cPAF26**; HPLC-MS (1), **Table 5**).
! CAUTION H_2 is a highly flammable gas. H_2 gas must be always handled inside a ventilated fume hood.
! CAUTION Once the reaction is complete and before the work-up, the H_2 atmosphere should be replaced by N_2 or Ar before filtering in order to avoid ignition of the charcoal.
? TROUBLESHOOTING
83| Filter the solution through a glass Pasteur pipette containing Celite and a tiny piece of cotton at the bottom. Wash the filter with 3 mL MeOH to ensure complete elution of the peptide.
84| Remove the solvent under reduced pressure (water bath max. T: 40 °C) using a rotary evaporator.

Purification of BODIPY-cPAF26 ● TIMING 51 h
85| Dissolve 25 mg of the crude **BODIPY-cPAF26** peptide from step 84 in 1.5 mL ACN: H_2O (1: 1). Heating for a few minutes in a water bath at 40 °C or sonicating help to dissolve the sample**.**
86| Filter the solution through a 0.45 µm syringe filter into a 3 mL vial for semi-preparative HPLC purification (see **Table 6**). Rinse the round-bottom flask with 0.5 mL ACN: H_2O (1: 1), filter and transfer to the same vial.
87| Inject 500 µL of the solution just prepared to the semi-preparative HPLC. **BODIPY-cPAF26** elutes around t_R: 12-14 min (ACN: H_2O (4:6)). Collect the pure **BODIPY-cPAF26** peptide in a 50 mL centrifuge tube (~ 30 mL per injection).
88| Repeat step 87 three more times**.**
89| Combine all the pure fractions. Freeze them using liquid N_2 and lyophilize for 48 h, wrapping the glass container in aluminum foil. Sample a small aliquot of the peptide, dissolve in ACN: H_2O (1: 1) and analyze by HPLC-MS to confirm the purity of the peptide (tR: 5.4 min for **BODIPY-cPAF26**; HPLC-MS (1), **Table 5**).
■ **PAUSE POINT** For storage, wrap the centrifuge tubes in aluminum foil and store them at 4 °C under N_2 atmosphere. The peptide can be safely stored at 4 °C for at least 6 months.

Figure 11.

Proteolytic stability of BODIPY-cPAF26 and PAF26 peptides ● TIMING 25 h
90| Aliquot 20 µL of 5 mM DMSO solutions of **BODIPY-cPAF26** and PAF26 peptides into two separate 1.5 mL microcentrifuge tubes.

91| Dilute both peptides in 480 µL of protease cocktail (1 µg mL^{-1}, pH 7.1) to reach 200 µM final concentration.
92| Vortex the two solutions. Aliquot samples (62 µL) for the first time point (t = 0), precipitate the proteins by microcentrifugation (4 ˚C, 13,000 rpm**, 18,530 g**), and analyze the supernatants by HPLC-MS (tR: 4.2 min for PAF26, 5.4 min for **BODIPY-cPAF26**; HPLC-MS (1), **Table 5**).
93| Place the solutions in the pre-heated thermoblock shaker at 37 ˚C (see Equipment setup). Shake the tubes at 950 rpm and take aliquots (62 µL) at the indicated time points (i.e. 0.25 h, 1 h, 4 h, 8 h, and 24 h).
94| Precipitate the proteins by microcentrifugation (4 ˚C, 13,000 rpm, 18,530 g), and analyze the supernatants by HPLC-MS (tR: 5.4 min for **BODIPY-cPAF26**; HPLC-MS (1), **Table 5**).
? TROUBLESHOOTING

Test of BODIPY-cPAF26 fluorogenic behavior of in phospholipid membranes ● TIMING 1.5 h
95| Dilute 40 µL of the 5 mM DMSO solution of **BODIPY-cPAF26** with 40 µL of DMSO to reach 2.5 mM final concentration.
96| Vortex the phosphatidylcholine (PC): cholesterol (7: 1) liposome suspension in PBS (7.5 mg mL^{-1} PC) for 10 seconds.
▲ **CRITICAL** Liposome suspensions must be used at r.t. and stored at 4 ˚C, but cannot be frozen.
97| Dilute the 7.5 mg mL^{-1} PC liposome suspension in 2-fold serial dilutions (495 µL in 1.5 mL microcentrifuge tubes) using DPBS. Prepare 10 serial dilutions, ranging from 3.75 mg mL^{-1} PC (2-fold dilution) to 0.007 mg mL^{-1} PC (1024-fold dilution). Prepare a blank sample with only DPBS (495 µL in a 1.5 mL microcentrifuge tube).
98| Add 5 µL of 2.5 mM **BODIPY-cPAF26** solution to all liposome suspensions and also to the DPBS blank to reach 25 µM final concentrations.
99| Vortex all the liposome suspensions and the DPBS blank for 10 seconds.
100| Transfer 150 µL (× 3) from every suspension onto separate wells of a 96-well black plate with flat bottom and lid. Include 3 wells (150 µL each) of 1 µM fluorescein in 0.1 N basic EtOH as a reference for the quantum yield measurements.
101| Incubate the plate for 15 min at r.t. in the dark.
▲ **CRITICAL** Do not incubate **BODIPY-cPAF26** with liposomes for longer than 30 min as sedimentation of the peptide can lead to inaccurate measurements.
102| Measure fluorescence emission spectra for the 96-well black plate using a Synergy H1 spectrophotometer. Excitation wavelength: 450 nm, emission range: 480-600 nm (every 1 nm).
103| Transfer samples to a 96-well polystyrene transparent plate using a multichannel pipette. As a reference for background measurements, make sure to fill three wells with 150 µL of DPBS each.
104| Measure absorbance spectra of the 96-well transparent plate using a Synergy H1 spectrophotometer. Absorbance range: 420-550 nm (every 1 nm).
105| Transfer **BODIPY-cPAF26** suspensions with high liposome content, low liposome content, DPBS blank as well as fluorescein in basic EtOH into 2 mL glass vials.
106| Irradiate the glass vials with 365 nm light using a hand-held UV-lamp. Differences in fluorescence intensity of **BODIPY-cPAF26** can be detected by naked eye (see Figure 12).
! CAUTION Exposure to UV radiation can cause skin or eye burns; hence protective equipment (goggles, gloves and lab coat) should be worn.
? TROUBLESHOOTING

Figure 12.

Testing BODIPY-cPAF26 cytotoxicity in A549 human lung epithelial cells ● TIMING 42 h
▲ **CRITICAL** Cell culture experiments must be performed in sterile conditions using a class-II microbiological safety cabinet. All reagents (media, DPBS, Trypsin-EDTA) and pipette tips must be sterile.
107| Use a running culture of A549 cells, typically one T-75 cell culture flask with 25 mL cell culture medium (see Reagent setup) incubated at 37 ˚C in 5% CO_2.
108| Check confluency and morphology of the cells under a microscope.
▲ **CRITICAL** Live A549 cells should be adhering to the flask. If most cells are floating or show signs of contamination (e.g. presence of bacteria or a cloudy medium), discard the culture and use a new batch.
109| Remove and discard the medium with a 25 mL plastic pipette.
110| Gently add 10 mL of Ca^{2+}-free DPBS to wash the cells then remove and discard the washing liquid with a 10 mL plastic pipette.
111| Add 1 mL of Trypsin-EDTA to the flask, rock gently to cover the entire surface of the flask and incubate for 3 min at 37 ˚C in 5% CO_2.

? TROUBLESHOOTING

112| Take the flask out of the incubator, tap gently to detach cells, add 10 mL of cell culture medium to the flask and transfer the cell suspension to a 50 mL centrifuge tube.

113| Collect a 50 µL aliquot of the cell suspension for cell counting.

114| Determine the number of cells using a cell counter. In one fully confluent flask of A549 cells, counts are typically ~ 0.5–2 × 10^6 cells mL^{-1}, depending on their manipulation.

115| Centrifuge the cell suspension (5 min, 20 ˚C, 350 rpm, 189 g).

116| Discard the supernatant and resuspend the cell pellet in 1 mL of cell culture medium. Calculate the amount of cells needed for the cytotoxicity assay depending on the wells to be used (25,000 cells/well). In a standard assay with six different conditions and four replicates (total of 24 wells), around 6 × 10^5 cells are needed.

117| Dilute the cell suspension in cell culture medium to achieve a final concentration of 2.5 × 10^5 cells mL^{-1}, and place the cell suspension in a 50 mL pipette basin.

118| Plate cells (100 µL/well) in sterile 96-well flat bottom polystyrene clear plates with lid using a multichannel pipette. Discard leftover cells following biological waste disposal regulations.

119| Incubate the plate(s) for 16 h at 37 ˚C in 5% CO_2.

120| Check confluency and morphology of the cells under a microscope.

? TROUBLESHOOTING

121| Transfer 4 µL of a 5 mM **BODIPY-cPAF26** solution to a 1.5 mL microcentrifuge tube and dilute the solution to 1 mM final concentration adding to it 16 µL of DMSO.

122| Prepare three more dilutions from 1 mM **BODIPY-cPAF26** in DMSO (2-fold serial dilutions) in separate 1.5 mL microcentrifuge tubes to obtain 0.5 mM, 0.25 mM and 0.125 mM DMSO solutions.

123| Dilute the four DMSO solutions in cell culture medium (9 µL of DMSO solutions plus 90 µL medium) to obtain 90 µM, 45 µM, 22.5 µM, and 11.25 µM solutions of **BODIPY-cPAF26**. Prepare an additional blank solution with 9 µL of DPBS plus 90 µL of cell culture medium.

124| Add 12.4 µL of the solutions to the cells in the 96-well plate to reach a final concentration of **BODIPY-cPAF26** of 10 µM, 5 µM, 2.5 µM, and 1.25 µM. As negative controls, add 12.4 µL of DPBS solution, and as positive controls, remove medium from the wells and add 112 µL DMSO to the cells.

125| Rock the plate(s) gently and incubate the cells for 4 h at 37 ˚C in 5% CO_2.

126| Dilute an appropriate volume of MTT reagent with cell culture medium (2.3-fold dilution, a minimum of 30 µL solution/well is required), and place the solution in a 50 mL pipette basin.

127| Add 30 µL of the MTT solution in cell culture medium just prepared to each well using a multichannel pipette.

128| Rock the plate(s) gently and incubate the cells for 4 h at 37 ˚C in 5% CO_2.

▲ **CRITICAL** Under the microscope, check the progression of the MTT reaction by observing the precipitation of a dark solid within the cells.

? TROUBLESHOOTING

129| Place enough detergent reagent in a 50 mL pipette basin (a minimum of 100 µL detergent/well is required).

130| Add 100 µL detergent to each well using a multichannel pipette. Rock gently the plate, wrap it in aluminum foil and keep at r.t. for a minimum of 2 h.

■ **PAUSE POINT** At this point, the experiment can be paused and the plate can be kept at r.t. overnight.

▲ **CRITICAL** Typically, 2-4 h incubation with detergent are enough to obtain the needed data, but overnight incubation is recommended to ensure full solubilization of the MTT reagent.

131| Measure absorbance at 570 nm and 650 nm (background signal) of the 96-well transparent plate using a Synergy H1 spectrophotometer. Wells with high cell viability values (DPBS-containing negative control) are dark colored, whereas wells with low cell viability (full DMSO positive control) are yellow colored (see Figure 13). Absorbance values should be between 0.5 and 1.5 AU.

? TROUBLESHOOTING

Figure 13.

Culture of *N. crassa* ● TIMING 121 h

132| Streak *N. crassa* spores from a stock culture (purchased from FGSC) using a sterile inoculation loop onto the surface of solid Vogel's sucrose minimal agar medium (10-15 mL) within a T25 cell culture flask.

133| Incubate the flask at 25 ˚C in the refrigerated incubator under 15 W 'warm white' light for 5 days.

? TROUBLESHOOTING

134| Add 1.5 mL of sterile dH_2O to the flask, shake gently and recover the suspension containing fungal cells with a P1000 micropipette. Filter the suspension through folded Miracloth (4 layers) into a sterile 1.5 mL microcentrifuge tube.

135| Remove a 4-µL aliquot of the cell suspension and transfer it to a hemocytometer to count the spores.

■ **PAUSE POINT** The *N. crassa* conidial suspension in sterile dH_2O can be stored for up to 4 days at 4 ˚C.

▲ **CRITICAL** Past the 4-days storage mark, *N. crassa* conidia must be discarded following biological waste disposal regulations. All inoculations should be performed from original stock cultures and not from sub-cultures to reduce the possibility of cultures acquiring new mutations.

Culture of *A. fumigatus* ● TIMING 73 h

136| Thaw and vortex one stock suspension aliquot of *A. fumigatus* to produce a homogeneous cell suspension.

137| Add 50 µL of the homogeneous cell suspension onto the surface of solid Vogel's sucrose minimal agar medium within a T25 cell culture flasks and spread the conidia over the agar surface with a sterile inoculation loop.

138| Incubate the flask at 37 ˚C in the dark for 3 days.

? TROUBLESHOOTING

139| Prepare 0.05 % (v/v) Tween 80 solution in sterile dH_2O and add 1.5 mL of it to the flask. Shake gently the resulting mixture and recover the suspension with a P1000 micropipette.

140| Remove a 4 µL-aliquot of the cell suspension and transfer to a hemocytometer to count the spores.

■ **PAUSE POINT** *A. fumigatus* conidial suspension in sterile dH_2O can be stored for up to one month at 4 ˚C.

▲ **CRITICAL** Past the one-month storage mark, *A. fumigatus* conidia must be discarded following biological waste disposal regulations. All inoculations should be performed from original stock cultures and not from sub-cultures to reduce the possibility of cultures acquiring new mutations.

Culture of *C. neoformans* ● TIMING 73 h

141| Thaw and vortex one stock suspension aliquot of *C. neoformans* and gently streak onto 15 mL SAB dextrose agar medium within a 9 cm Petri dish.

142| Seal the plate with Micropore tape, place it inverted in an incubator and keep it there at 30 ˚C for 3 days.

? TROUBLESHOOTING

■ **PAUSE POINT** Petri dishes containing *C. neoformans* colonies can be stored for up to one month at 4 ˚C.

▲ **CRITICAL** Past the one-month storage mark, *C. neoformans* colonies must be discarded following biological waste disposal regulations. All inoculations should be performed from original stock cultures and not from sub-cultures to reduce the possibility of cultures acquiring new mutations.

Culture of *F. oxysporum* ● TIMING 121 h

143| Thaw and vortex one stock suspension aliquot of *F. oxysporum* to produce a homogeneous cell suspension.

144| Add 50 µL of the homogeneous cell suspension to a sterile glass flat-bottom flask with 20 mL of PDB medium.

145| Plug the flask with a sterile foam plug and seal the flask with aluminum foil. Incubate the flask on a shaking incubator at 28 ˚C for 5 days.

? TROUBLESHOOTING

146| Filter the cell suspension through folded Miracloth (4 layers) and transfer it into a sterile 50 mL centrifuge tube.

147| Centrifuge the suspension for 10 min at r.t. (4,000 rpm, 1,755 g) and discard the supernatant.

148| Re-suspend the pellet in 2 mL of sterile dH_2O and transfer the resulting suspension with a P1000 micropipette into a 2 mL microcentrifuge tube.

149| Remove a 4 µL-aliquot of the cell suspension and transfer it to a hemocytometer to count the spores.

■ **PAUSE POINT** *F. oxysporum* conidia suspension in sterile dH_2O can be stored for up to one month at 4 ˚C.

▲ **CRITICAL** Past the one-month storage mark, *F. oxysporum* conidia must be discarded following biological waste disposal regulations. All inoculations should be performed from original stock cultures and not from sub-cultures to reduce the possibility of cultures acquiring new mutations.

Culture of *C. albicans* ● TIMING 73 h

150| Thaw and vortex one stock suspension aliquot of *C. albicans* and gently streak onto 15 mL PDA medium within a 9 cm Petri dish.

151| Seal the plate, place it inverted in an incubator, and keep it there at 30 ˚C for 3 days.

? TROUBLESHOOTING

■ **PAUSE POINT** Petri dishes containing *C. albicans* colonies can be stored for up to one month at 4 ˚C.

▲ **CRITICAL** Past the one-month storage mark, *C. albicans* colonies must be discarded following biological waste disposal regulations. All inoculations should be performed from original stock cultures and not from sub-cultures to reduce the possibility of cultures acquiring new mutations.

Antifungal activity measurements of BODIPY-cPAF26 and PAF26 peptides in *N. crassa* and *A. fumigatus* ● TIMING 26 h

152| In a 1.5 mL microcentrifuge tube, dilute 20 µL of a 5 mM **BODIPY-cPAF26** or **PAF26** solution with 80 µL of DMSO to obtain a 1 mM solution.

153| Prepare another two dilutions of the 1 mM **BODIPY-cPAF26** or **PAF26** solution in DMSO in separate 1.5 mL microcentrifuge tubes to obtain 30 µM (30 µL of 1 mM peptide and 970 µL dH_2O) and 25 µM (25 µL of 1 mM peptide and 975 µL dH_2O) solutions.

154| Add 100 µL of the 30 µM peptide solution into each of the wells (A1-G1) of a transparent U-bottom 96-well plate (Figure 14a).

155| Add 100 µL of the 25 µM peptide solution into each of the wells (A2-G2) of a transparent U-bottom 96-well plate (Figure 14a).

156| Add 100 µL of sterile dH_2O into each of wells D1, H1, D2, and H2, and 50 µL of sterile dH_2O into each of the rest of the wells.

157| Perform 2-fold serial dilutions of the 30 µM peptide solution prepared in step 153 using a multichannel electronic pipette by transferring 50 µL from column 1 into column 3 and then 5 and so on. Discard the 50 µL taken from column 11 (Figure 14b).

158| Perform 2-fold serial dilutions of the 25 µM peptide solution prepared in step 153 using a multichannel electronic pipette by transferring 50 µL from column 2 into column 4 and then 6 and so on. Discard the 50 µL taken from column 10. (Figure 14c).

159| Dilute the conidial suspension from step 134 (*N. crassa*) or step 139 (*A. fumigatus*) with 5 mL solution of liquid Vogel's sucrose minimal medium in dH_2O (1: 4) to reach a final concentration of 10^6 cells mL^{-1}. Vortex the suspension and place it in a 50 mL pipette basin.

160| Add 50 µL of the conidial suspension just obtained into each well using a multichannel pipette to achieve a final volume of 100 µL/well and a concentration of 5×10^5 cells mL^{-1}, excluding wells that will act as blanks in rows D and H. To the blank wells in rows D and H, add 50 µL of a solution of liquid Vogel's sucrose minimal medium in dH_2O (1: 4) to achieve a final volume of 100 µL/well (Figure 14d).

▲ **CRITICAL STEP** The addition of the conidia to the wells of the 96-well plate must be performed quickly to avoid any of the conidia settling in the pipette basin.

161| Put the lid on the plate and wrap it in aluminum foil. Incubate for 24 h at 37 ˚C in the case of *A. fumigatus* or at 25˚C in the case of *N. crassa*.

Figure 14.

162| Measure the optical density of the contents of the plate at 610 nm using a spectrophotometer. Optical density values for the control group (conidia alone) should be ~ 0.1-0.2 AU. IC_{50} values are determined by four-parameter logistic regression analysis.

? TROUBLESHOOTING

Preparation of *N. crassa* conidial germlings for live-cell fluorescence imaging ● TIMING 4.5 h

163| Vortex the cell suspension of *N. crassa* conidia from step 134.

164| Dilute *N. crassa* conidia to 10^5 cells mL^{-1} with a 2 mL solution of liquid Vogel's sucrose minimal medium in dH_2O (1: 4).

165| Vortex the diluted suspension and transfer it to an 8-well chamber (200 µL/well) with a P200 micropipette.

166| Incubate the 8-well chamber in a refrigerated incubator for 4 h at 25 ˚C in the dark.

▲ **CRITICAL** A dilution of liquid Vogel's sucrose minimal medium in dH_2O (1: 4) must be used instead of 100% liquid Vogel's sucrose minimal medium to produce *N. crassa* as conidial germlings and avoid the formation of mature hyphae, which are not suitable for single-cell imaging.

Preparation of *A. fumigatus* conidial germlings for live-cell fluorescence imaging● TIMING 25 h

167| Vortex the cell suspension of *A. fumigatus* conidia from step 139.

168| Dilute *A. fumigatus* conidia to 10^5 cells mL^{-1} with a 2 mL solution of liquid Vogel's sucrose minimal medium in dH_2O (1: 4).

169| Vortex the diluted suspension and transfer it to an 8-well chamber (200 µL/well) with a P200 micropipette.
170| Incubate the 8-well chamber in a refrigerated incubator for 24 h at 25 ˚C in the dark.
▲ **CRITICAL** A dilution of liquid Vogel's sucrose minimal medium in dH_2O (1: 4) must be used instead of 100% liquid Vogel's sucrose minimal medium to produce *A. fumigatus* as conidial germlings and avoid the formation of mature hyphae, which are not suitable for single cell imaging.

Preparation of *C. neoformans* cells for live-cell fluorescence imaging ● TIMING 21.5 h
171| Transfer 3-4 colonies of *C. neoformans* from step 142 into a sterile glass flat-bottom flask containing 10 mL of liquid SAB medium.
172| Plug the flask with a sterile foam plug and seal the flask with aluminum foil. Incubate the flask on a shaking incubator at 37 ˚C for 20 h (170 rpm).
173| Filter the cell suspension through folded Miracloth (4 layers) then transfer it into a sterile 50 mL centrifuge tube.
174| Centrifuge the suspension for 5 min at r.t. (4,000 rpm, 1,755 g)) and discard the supernatant.
175| Re-suspend the pellet in 2 mL of sterile dH_2O and transfer the resulting suspension to a 2 mL microcentrifuge tube.
176| Remove a 4 µL-aliquot of the cell suspension and transfer it to a hemocytometer to count the spores.
177| Dilute *C. neoformans* conidia to 5×10^5 cells mL^{-1} in sterile dH_2O.
178| Vortex the diluted suspension and transfer it to an 8-well chamber (200 µL/well) with a P200 micropipette.
179| Incubate the 8-well chamber at 37 ˚C for 30 min.
▲ **CRITICAL** The chamber containing *C. neoformans* cells must be handled carefully as they will not adhere to the bottom of the 8-well chamber as well as the other fungal species.
? TROUBLESHOOTING

Preparation of *F. oxysporum* cells for live-cell fluorescence imaging ● TIMING 9 h
180| Dilute *F. oxysporum* microconidial suspension from step 148 to 5×10^5 cells mL^{-1} with 2 mL of a solution of PDB medium: dH2O (1:99).
181| Vortex the diluted cell suspension and transfer it to an 8-well chamber (200 µL/well) with a P200 micropipette.
182| Incubate the 8-well chamber in a refrigerated incubator at 25 ˚C for 8 h.

Preparation of *C. albicans* cells for live-cell fluorescence imaging ● TIMING 13.5 h
183| Transfer one single colony of *C. albicans* from step 151 into a sterile glass flat-bottom flask containing 15 mL PDB medium.
184| Plug the flask with a sterile foam plug and seal the flask with aluminum foil. Incubate the flask on a shaking incubator at 30 ˚C for 12 h (170 rpm).
185| Filter the suspension through folded Miracloth (4 layers) and transfer the filtrate into a sterile 50 mL centrifuge tube.
186| Remove a 4 µL-aliquot of the cell suspension and transfer it to a hemocytometer to count the yeast cells.
187| Dilute the *C. albicans* cells to 5×10^5 cells mL^{-1} in PDB medium.
188| Vortex the diluted cell suspension and transfer it to an 8-well chamber (200 µL/well) with a P200 micropipette
189| Incubate the 8-well chamber at 30 ˚C for 30 min.
▲ **CRITICAL** The chamber containing *C. albicans* cells must be handled carefully as they will not adhere to the bottom of the 8-well chamber as well as the other fungal species.
? TROUBLESHOOTING
General setup of the fluorescence confocal microscope for live-cell imaging ● TIMING 30 min
190| Switch on the laser(s) and set the stage temperature: 25 ˚C for *N. crassa*; 28 ˚C for *F. oxysporum*; 30 ˚C for *C. neoformans* and *C. albicans*; 37 ˚C for *A. fumigatus*.
191| Adjust the WLL laser power to 70 %.
192| Clean the 63× water immersion objective with lens cleaning tissue.
193| Set the excitation beam wavelength(s), laser intensity(ies) and emission wavelength(s) as described in the Equipment setup section.
▲ **CRITICAL** Due to the high sensitivity of HyD detectors, it is important to set the laser intensity at a low level (~10 %) to avoid causing damage to the detectors.
194| During imaging experiments, place the 8-well chamber on the microscope stage, focus the sample using the eyepieces and select the region of interest to image.
? TROUBLESHOOTING
195| Adjust the Z-position as well as gain and offset for each channel.

196| At this point, individual images can be acquired or successive images can be recorded in a time course with an interval time of 10 seconds. For time-lapse experiments extending over 30 min, we recommend interval times of at least 20 s.
197| Save the experimental data to appropriate folders and export images as .tif files.
198| Remove the sample and clean the objective. Cool down the laser or lasers and switch it or them off.

Live-cell fluorescence imaging of fungal cells using BODIPY-cPAF26 ● TIMING 45 min
199| Prepare samples as described in steps 163–166 (*N. crassa*), 167–170 (*A. fumigatus*), 171–179 (*C. neoformans*), 180–182 (*F. oxysporum*), and 183–189 (*C. albicans*).
200| In a 1.5 mL microcentrifuge tube, dilute 2 µL of 5 mM **BODIPY-cPAF26** DMSO stock solution with 998 µL of sterile dH_2O to obtain a 10 µM **BODIPY-cPAF26** solution.
201| Using a P200 micropipette, carefully remove 40 µL of the 200 µL medium from one well of the 8-well chamber, which contains the fungal cells.
202| Add 40 µL of the solution prepared in step 200 to the same well mentioned in the previous step to obtain a 2 µM solution of **BODIPY-cPAF26**.
203| Incubate the cells with **BODIPY-cPAF26** for 10 min at r.t.
▲ **CRITICAL** Cells must be handled carefully to minimize cell detachment from the bottom of the well.
204| Acquire images as required. Live-cell imaging can be performed for up to 60 min without causing obvious deleterious effects on the cells. However, immediate imaging following **BODIPY-cPAF26** treatment is highly recommended.
205| After acquisition, save the files to an appropriate location and subsequently back up the files at an appropriate time to prevent any data loss.
206| Discard cells following biological waste disposal regulations.
■ **PAUSE POINT** After acquisition, images can be analyzed at any time.

Real-time fluorescence imaging of *A. fumigatus* using BODIPY-cPAF26 ● TIMING 1 h
▲ **CRITICAL** To maximize the localization of **BODIPY-cPAF26** at the cell membrane and the quality of the fluorescence images, we recommend performing steps 207-217 one well at a time.
207| Prepare samples as described in steps 167–170.
208| Prepare a 5 mM DMSO stock solution of the red counterstain (TAMRA-PAF96) using a 1.5 mL microcentrifuge tube (4.8 mg in 1 mL DMSO).
209| In a 1.5 mL microcentrifuge tube, dilute 4 µL of the 5 mM red counterstain DMSO stock solution with 996 µL of sterile dH_2O to obtain a 20 µM solution of the counterstain.
210| Using a P200 micropipette, carefully remove 40 µL of the 200 µL medium from a well of the 8-well chamber containing the fungal cells.
211| Add 40 µL of the solution prepared in step 208 to the well referred to in the previous step to obtain a 5 µM final concentration of the red counterstain.
212| Incubate the cells with 5 µM red counterstain for 10 min at r.t.
213| Using a P200 micropipette, carefully remove 160 µL of the 200 µL medium from the well of the 8-well chamber and add 160 µL of a fresh solution of liquid Vogel's sucrose minimal medium in dH_2O (1: 4).
▲ **CRITICAL** Cells must be handled carefully to minimize any cell detachment from the bottom of the well.
214| Using a P200 micropipette, carefully remove 40 µL of the 200 µL medium from the well of the 8-well chamber and place the chamber on the stage of the confocal microscope.
215| Identify a region of interest containing one or more red-stained cells. Re-adjust the Z-position for optimal focus.
216| Using a P200 micropipette, gently add 40 µL of the solution prepared in step 200 to obtain a 2 µM solution of **BODIPY-cPAF26**.
217| Acquire a series of images in a time course (recommended interval time between images: 5 sec).
? TROUBLESHOOTING
218| After acquisition, back up the files to prevent any data loss.
219| Discard cells following biological waste disposal regulations.
■ **PAUSE POINT** After acquisition, images can be analyzed at any time.

? TROUBLESHOOTING
Troubleshooting advice can be found in **Table 8**.

Table 8.

● **TIMING**

Synthesis of *m*-iodophenyl-BODIPY (3): Steps 1-25
Steps 1-7, Condensation of 3-iodobenzaldehyde and 2,4-dimethylpyrrole: 23 h
Steps 8-13, Oxidation: 1 h
Steps 14-17, $BF_3{\cdot}Et_2O$ coordination: 5 h
Steps 18-22, Work-up: 1 h
Steps 23-25, Purification: 2 h
Synthesis of Fmoc-Trp(C_2-BODIPY)-OH (1): Steps 26-35
Steps 26-31, Fmoc-Trp-OH arylation with compound **3**: 1 h
Steps 32-34, Work-up: 1 h
Steps 35, Purification: 3 h
Stability analysis of the amino acid 1: Steps 36-37
Steps 36-37, HPLC analysis: 1 h
Solid-phase peptide synthesis of BODIPY-cPAF26: Steps 38-69
Steps 38-39, Resin preparation: 15 min
Steps 40-45, Coupling of the first amino acid to the resin: 1.25 h
Steps 46-47, Fmoc removal: 15 min
Steps 48-55, Coupling of second amino acid and peptide elongation: 9 h
Steps 56-62, Coupling of amino acid **1**: 1.25 h
Steps 63-67, Cleavage of the linear protected peptide from the resin: 1 h
Steps 68-69, Solvent removal from the crude peptide: 24 h
Peptide cyclization: Steps 70-77
Steps 70-75, Cyclization of the protected peptide: 3 h
Steps 76-77, Lyophilization of the cyclic protected peptide: 24 h
Hydrogenation reaction: Steps 78-84
Steps 78-82, Hydrogenation reaction: 48 h
Steps 83-84, Work-up: 1 h
Purification of the BODIPY-cPAF26 peptide: Steps 85-89
Steps 85-88, HPLC purification: 3 h
Step 89, Solvent removal and isolation of **BODIPY-cPAF26**: 48 h
Proteolytic stability of BODIPY-cPAF26 and PAF26 peptides: Steps 90-94
Steps 90-92, Sample preparation: 1 h
Steps 93-94, Incubation and HPLC analysis: 24 h
Fluorogenic response of BODIPY-cPAF26 in phospholipid membranes: Steps 95-106
Steps 95-101, Sample preparation: 1 h
Steps 102-106, Spectroscopic measurements: 30 min
Cytotoxicity of BODIPY-cPAF26 in A549 human lung epithelial cells: Steps 107-131
Steps 107-118, Cell counting and plating: 1 h
Steps 119-120, Overnight cell incubation: 16 h
Steps 121-123, Preparation of **BODIPY-cPAF26** and control solutions: 1 h
Steps 124-125, Incubation of cells with compounds: 4 h
Steps 126-131, MTT cell viability assay: 20 h
Culture of fungal species: Steps 132-151 (can be performed in parallel)
Steps 132-135, Culture of *N. crassa*: 121 h
Steps 136-140, Culture of *A. fumigatus*: 73 h
Steps 141-142, Culture of *C. neoformans*: 73 h
Steps 143-149, Culture of *F. oxysporum*: 121 h
Steps 150-151, Culture of *C. albicans*: 73 h
Antifungal activity measurements of BODIPY-cPAF26 and PAF26 peptides in *N. crassa* and *A. fumigatus*: Steps 152-162
Steps 152-153, Peptide dilutions: 30 min
Steps 154-160, Plate preparation: 1 h
Steps 161-162, Overnight incubation and measurements: 24.5 h
Preparation of fungal cells for live-cell fluorescence imaging: Steps 163-189 (can be performed in parallel)

Steps 163-166, Preparation of *N. crassa* cells: 4.5 h
Steps 167-170, Preparation of *A. fumigatus* cells: 25 h
Steps 171-179, Preparation of *C. neoformans* cells: 21.5 h
Steps 180-182, Preparation of *F. oxysporum* cells: 9 h
Steps 183-189, Preparation of *C. albicans* cells: 13.5 h
General setup of the fluorescence confocal microscope for live-cell imaging: Steps 190-198
Steps 190-198, Microscope set-up: 30 min
Live-cell fluorescence imaging of fungal cells using BODIPY-cPAF26: Steps 199-206
Steps 199-202, Sample preparation: 15 min
Steps 203-206, Incubation of **BODIPY-cPAF26** and image acquisition: 30 min
Real-time fluorescence imaging of *A. fumigatus* using BODIPY-cPAF26: Steps 207-219
Steps 207-213, Sample preparation and incubation of counterstain: 30 min
Steps 214-219, Incubation of **BODIPY-cPAF26** and image acquisition: 30 min

ANTICIPATED RESULTS

Chemical properties of amino acid 1 and peptide BODIPY-cPAF26. Both **1** and the **BODIPY-cPAF26** display excitation and emission maximum wavelengths in the green region of the visible spectra ($\lambda_{exc.}$ ~ 500 nm, $\lambda_{em.}$ ~ 520 nm), which makes the use of these compounds compatible with GFP/FITC filters in most equipments (**Figure 3**). **BODIPY-cPAF26** displays strong fluorogenic behavior, with quantum yields over 30 % in phospholipid membranes and negligible fluorescence in aqueous media, as proven by experiments conducted by our group (see Figure 12). **BODIPY-cPAF26** is also expected to display a high affinity for fungal cells (see **Figures 5b** and **5c**), high proteolytic stability (see **Supplementary Figure 3**) but its presence should not affect the viability of, for instance, human cells (Figure 5d).

Fluorescence live-cell imaging of fungal cells and real-time imaging of *A. fumigatus* using the peptide BODIPY-cPAF26. We successfully employed the peptide **BODIPY-cPAF26** for confocal fluorescence imaging of several fungal pathogens, including *N. crassa*, *A. fumigatus*, *C. neoformans*, *F. oxysporum* and *C. albicans*. The fluorogenic behavior of **BODIPY-cPAF26** is expected to enable direct live-cell imaging by simple incubation of the peptide with the fungal cells, in a single step and without the need for any washing (**Figure 6**). We also performed real-time imaging experiments using **BODIPY-cPAF26** in *A. fumigatus* (**Figure 7**). Time-course fluorescence imaging at high magnification show staining of the cells a few seconds after the addition of **BODIPY-cPAF26** to the media, and its preferential localization in the fungal cell membranes.

Analytical data
***m*-iodophenyl-BODIPY (3).**
Red powder, 234 mg (98 % purity, 24 % yield). HPLC t_R = 8.0 min (Gradient_2, **Table 4**).
^{1}H NMR ($CDCl_3$, 400 MHz): *δ* 7.76 (dt, *J* = 7.7, 1.5 Hz, 1H), 7.62 (t, *J* = 1.6 Hz, 1H), 7.24 – 7.20 (m, 1H), 7.19 – 7.14 (m, 1H), 5.92 (s, 2H), 2.48 (d, *J* = 1.3 Hz, 6H), 1.36 (s, 6H).
^{13}C NMR ($CDCl_3$, 100 MHz): δ 155.9, 142.9, 139.3, 138.0, 137.1, 136.8, 130.7, 127.3, 121.5, 94.3, 14.7, 14.6 (one quaternary carbon signal not seen).
HRMS (m/z): $[M+H]^+$ calcd. for $C_{19}H_{18}BF_2IN_2$, 451.0654; found: 451.0651.

Fmoc-Trp(C_2-BODIPY)-OH (1).
Red powder, 122 mg (> 99 % purity, 56% yield). HPLC t_R = 7.0 min (Gradient_2, **Table 4**).
^{1}H NMR ($CDCl_3$, 400 MHz): *δ* 8.12 (s, 1H), 7.69 – 7.56 (m, 4H), 7.48 (t, *J* = 7.7 Hz, 1H), 7.41 (t, *J* = 1.7 Hz, 1H), 7.37 (d, *J* = 4.9 Hz, 2H), 7.30 (t, *J* = 8.0 Hz, 3H), 7.25 – 7.21 (m, 1H), 7.20 – 7.13 (m, 3H), 7.07 (ddd, *J* = 8.0, 7.0, 1.1 Hz, 1H), 5.90 (s, 1H), 5.87 (s, 1H), 5.09 (d, *J* = 8.0 Hz, 1H), 4.55 (d, *J* = 7.5 Hz, 1H), 4.17 (q, *J* = 10.3, 9.4 Hz, 2H), 4.01 (s, 1H), 3.44 – 3.37 (m, 1H), 3.37 – 3.28 (m, 1H), 2.47 (s, 3H), 2.46 (s, 3H), 1.38 (s, 3H), 1.35 (s, 3H).
^{13}C NMR ($CDCl_3$, 100 MHz): δ 174.8, 163.1, 156.0, 155.9, 143.9, 143.2, 141.4, 140.7, 136.1, 136.0, 135.1, 133.9, 131.5, 130.1, 129.1, 128.8, 127.9, 127.8, 127.2, 125.2, 123.2, 121.6, 120.5, 120.1, 119.3, 111.2, 108.2, 67.2, 47.2, 36.9, 28.0, 14.8, 14.7.
HRMS (m/z): $[M+Na]^+$ calcd. for $C_{45}H_{39}BF_2N_4O_4$, 771.2930; found: 771.2925.

H-Arg(NO_2)-Lys(Z)-Lys(Z)-Trp(C_2-BODIPY)-Phe-Trp-Gly-OH.
Red powder, 61 mg (90 % purity, 75 % yield).
HPLC-MS (m/z): $[M+H]^+$ calcd for $C_{86}H_{98}BF_2N_{17}O_{14}$, 1642.6, found 1643.2.

Cyclo(Arg(NO_2)-Lys(Z)-Lys(Z)-Trp(C_2-BODIPY)-Phe-Trp-Gly).
Red powder, 53 mg (88 % purity, 77 % yield).
HPLC-MS (m/z): $[M+H]^+$ calcd for $C_{86}H_{96}BF_2N_{17}O_{13}$, 1624.6, found 1626.2.

BODIPY-cPAF26 peptide.
Crude form: red powder, 25 mg (85 % purity, 58 % yield)
Pure form: red powder, 9.2 mg (> 99 % purity, 25 % yield). HPLC t_R = 5.8 min (Gradient_1, **Table 4**).
^{1}H NMR (CD_3OD, 600 MHz): δ 8.55 (s, 3H), 7.89 – 7.84 (m, 1H), 7.71 (t, *J* = 7.7 Hz, 1H), 7.65 (d, *J* = 7.8 Hz, 1H), 7.60 – 7.55 (m, 2H), 7.42 (dt, *J* = 8.1, 0.9 Hz, 1H), 7.40 – 7.36 (m, 2H), 7.18 – 7.12 (m, 5H), 7.11 (s, 1H), 7.06 (m, 3H), 6.94 (m, 1H), 6.10 (s, 1H), 6.06 (s, 1H), 4.45 (t, *J* = 7.4 Hz, 1H), 4.32 (d, *J* = 10.5 Hz, 1H), 4.25 (m, 1H), 4.17 – 4.07 (m, 2H), 4.04 (d, *J* = 16.5 Hz, 1H), 4.00 (m, 1H), 3.63 (dd, *J* = 14.9, 9.5 Hz, 1H), 3.51 – 3.45 (m, 1H), 3.38 (m, 1H), 3.30 (1H), 3.27 – 3.23 (m, 2H), 3.15 (dd, *J* = 14.2, 7.5 Hz, 1H), 3.10 (dd, *J* = 14.0, 5.9 Hz, 1H), 2.90 (td, *J* = 8.6, 4.2 Hz, 2H), 2.72 (t, *J* = 7.7 Hz, 2H), 2.63 (dd, *J* = 14.2, 8.5 Hz, 1H), 2.51 (s, 3H), 2.50 (s, 3H), 2.09 – 1.88 (m, 4H), 1.76 (m, 1H), 1.67 (m, 3H), 1.52 (m, 7H), 1.48 – 1.41 (m, 4H), 1.37 (m, 2H), 1.26 – 1.14 (m, 2H).
HRMS (m/z): $[M+H]^+$ calcd for $C_{70}H_{85}BF_2N_{16}O_7$, 1311.6926, found 1311.6864.

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Author contributions

L. M.-T. performed all compound syntheses and chemical characterization; R. S.-F. performed in vitro spectral and biological characterization; C. Z. and N. D. R. designed and performed the experiments with fungal cells; F. A., R. L. and M. V. designed the chemical syntheses; R. L. and M. V. supervised the project; M. V. analyzed the data and wrote the paper. All authors discussed the results and commented on the manuscript.

Competing financial interests

The authors declare competing financial interests (see HTML version of this article for details).

Figure Legends

Figure 1. Flowchart outlining all the experimental procedures described in this protocol, which includes chemical synthesis (yellow), in vitro characterization (blue), and fluorescence imaging assays (red).

Figure 2. Retrosynthetic analysis of the amino acid Fmoc-Trp(C_2-BODIPY)-OH (**1**).

Figure 3. Synthesis and chemical characterization of amino acid Fmoc-Trp(C_2-BODIPY)-OH (1). a) Preparation of the amino acid **1** from Fmoc-Trp-OH (**2**) and *m*-iodophenyl-BODIPY (**3**). b) Spectral properties of the amino acid **1**. c) Long-term stability (as determined by HPLC analysis) of the amino acid **1** as solid and in solution (0.1 M) at different temperatures. *: determined after redissolvement due to solvent evaporation.

Figure 4. Synthetic scheme for the preparation of **BODIPY-cPAF26** on solid-phase.

Figure 5. BODIPY-cPAF26, a fluorogenic peptide with high affinity for fungal cells. a) Chemical structure of **BODIPY-cPAF26**. b) Cell viability plots and non-linear regressions for **BODIPY-cPAF26** and unlabeled PAF26 in *N. crassa*. Both peptides were incubated at the same concentrations with *N. crassa* conidia, and after 24 h at 25 ºC fungal growth was determined by measuring the optical density at 610 nm. Data represented as means ± s.d. (n=3). c) Fluorescence spectra of **BODIPY-cPAF26** after incubation with liposome suspensions of PC: cholesterol (7:1) in PBS ranging from 3.75 mg mL^{-1} to 0.007 mg mL^{-1} of PC in 2-fold serial dilutions, $\lambda_{exc.}$: 450 nm. d) Viability of human lung A549 epithelial cells after incubation with different concentrations of **BODIPY-cPAF26**. Individual data points represented together with means ± s.d. (n=4). Non-significant (n.s.) differences ($p > 0.05$) were found between untreated cells and any of the treatments.

Figure 6. Confocal live-cell images (*top*: fluorescence, *bottom*: bright field) of different fungal species after incubation with **BODIPY-cPAF26** (2 μM for a-d, 10 μM for e,) for 10 min, without any washing steps. a) *N. crassa*, b) *A. fumigatus*, c) *C. neoformans*, d) *F. oxysporum* and e) *C. albicans*. $\lambda_{exc.}$: 496 nm, $\lambda_{em.}$: 505-550 nm. Scale bar: 10 μm.

Figure 7. Time-course fluorescence imaging of *A. fumigatus* with BODIPY-cPAF26. High-resolution fluorescence confocal images of the fungal pathogen *A. fumigatus* after incubation with a cell membrane counterstain (red) and following direct addition of **BODIPY-cPAF26** (2 μM, green) without any washing steps. **BODIPY-cPAF26** initially interacts rapidly with the apical plasma membrane of *A. fumigatus*, its staining moves then towards the base of the germling cell within a few minutes (see Supplementary Video 1). $\lambda_{exc.}$: 496 nm, $\lambda_{em.}$: 505-550 nm (green); $\lambda_{exc.}$: 570 nm, $\lambda_{em.}$: 585-650 nm (red). Scale bar: 5 μm.

Figure 8. **Time-course illustration of the reaction set-up for the synthesis of compound 3 under N_2 atmosphere**. a) Addition of anhydrous DCM. b) Addition of 2,4-dimethylpyrrole. c) Solution resulting after addition of TFA. d) Slow addition of DDQ. e) Addition of $BF_3{\cdot}OEt_2$ to the TEA-containing mixture. f) Isolated pure compound **3**.

Figure 9. **Time-course illustration of the reaction set-up for the synthesis of the amino acid 1**. a) Reaction vessel after microwave irradiation. b) Filtration of the crude reaction through Celite under vacuum. c) Crude mixture prior to purification. d) Isolated pure amino acid **1**.

Figure 10. **Setup for the solid-phase synthesis of BODIPY-cPAF26**. a) Manifold for manual SPPS. b) Experimental set-up for resin cleavage. c) Crude protected peptide after lyophilization.

Figure 11. **Experimental setup for the hydrogenation of the cyclic protected peptide**. a) Purge under N_2 stream. b) Hydrogenation reaction under H_2 at atmospheric pressure. c) Filtration of crude

solution of the reaction mixture through a glass Pasteur pipette. d) Pure **BODIPY-cPAF26** after semi-preparative HPLC purification.

Figure 12. Pictograms of BODIPY-cPAF26 and fluorescein under excitation with 365 nm light using a hand-held UV lamp. a) **BODIPY-cPAF26** (10 μM) in high concentration of liposomes (1.8 mg mL^{-1}). b) **BODIPY-cPAF26** (10 μM) in low concentration of liposomes (0.03 mg mL^{-1}). c) **BODIPY-cPAF26** (10 μM) in DPBS. d) Fluorescein (4 μM) in basic EtOH.

Figure 13. **Pictogram of A549 cells in a 96-well plate after detergent incubation**. Cells were incubated in four replicates with different concentrations of **BODIPY-cPAF26** (rows B-E) as well as full DMSO (row F) and DPBS (row G) for positive and negative controls, respectively.

Figure 14. **Plate layout for the antifungal activity assays.** a) Steps 154–156. b) Step 157. c) Step 158. d) Step 160. Conidia are incubated in six replicates with different concentrations of peptide (rows A-C and E-G) and controls without peptide (column 12). In addition, 'blank' wells contain Vogel's sucrose minimal medium only (rows D and H).

Tables

Table 1. Composition of Vogel's trace element solution (store at 4 °C for up to 12 months)

Citric acid · H_2O	5 g
$ZnSO_4$ · 7 H_2O	5 g
$(NH_4)_2Fe(SO_4)_2$ · 6 H_2O	1 g
$CuSO_4$ · 5 H_2O	250 mg
$MnSO_4$ · H_2O	50 mg
H_3BO_3	50 mg
Na_2MoO_4 · $2H_2O$	50 mg
dH_2O	To a total volume of 1 L

Table 2| Composition of Vogel's salts 50X stock solution (store at 4 °C for up to 12 months).

Sodium citrate · 2 H_2O	127 g
KH_2PO_4	250 g
NH_4NO_3	100 g
$MgSO_4$ · 7 H_2O	10 g
$CaCl_2$ · 2 H_2O	5 g
Vogel's trace element solution (see Table 1)	5 mL
Biotin solution (0.5 % (w/v) of d-biotin in dH_2O)	5 mL
dH_2O	To a total volume of 1 L

Table 3| Composition of solid Vogel's sucrose minimal agar medium (store at 4 ^{o}C for up to 12 months).

Vogel's salts 50X stock solution (see Table 2)	20 mL
Sucrose	20 g
Agar	20 g
dH_2O	To a total volume of 1 L

Table 4| HPLC conditions for the analysis of crude mixtures and purified compounds.

Equipment	HPLC
Column	XBridge BEH 130 C18 reverse-phase column 4.6 × 100 mm (3.5 µm)
Solvents	A: H_2O (0.045 % TFA (v/v)) B: ACN (0.036 % TFA (v/v))
Flow rate	1 mL min^{-1}
Gradient_1	5-100% (v/v) B over 8 min; 100% (v/v) B over 3 min; 100-5% (v/v) B over 0.5 min; 5% (v/v) B over 3 min
Gradient_2	50-100% (v/v) B over 8 min; 100% (v/v) (v/v) B over 3 min; 100–50% (v/v) B over 0.5 min; 50% (v/v) B over 3 min
Injection volume	2-15 µL
Detection wavelengths	210-400 nm

Table 5| HPLC-MS conditions for the analysis of crude mixtures and purified compounds.

Equipment	HPLC-MS (1)	HPLC-MS (2)
Column	Phenomenex Gemini C18 110A reverse-phase column, 4.6 x 50 mm (particle size 3.5 µm)	XSelect CSH C18 reverse-phase column, 4.6 x 50 mm (particle size 3.5 µm)
Solvents	A: H_2O (0.1 % FA (v/v)) B: ACN (0.1 % FA (v/v))	A: H_2O (0.1 % FA (v/v)) B: ACN (0.07 % FA (v/v))
Flow rate	1 mL min^{-1}	2 mL min^{-1}
Gradient	0-100% (v/v) B over 8 min; 100 %(v/v) over 3 min; 100–0% (v/v) B over 0.5 min; 0% (v/v) B over 2.5 min	5-100% (v/v) B over 4.5 min; 100 % over 1.5 min; 100–5% (v/v) B over 0.1 min; 5% (v/v) B over 0.4 min
Injection volume	10-50 µL	2-15 µL
Detection wavelengths	210-600 nm	210-400 nm

Table 6| Semi-preparative HPLC conditions for the purification of compounds.

Column	Jupiter C12 reverse-phase column 21.2 × 100 mm (10 µm), Proteo 90 Å, Ax (Phenomenex)
Solvents	A: H_2O (0.1 % TFA (v/v)) B: ACN (0.05 % TFA (v/v))
Flow rate	16 mL min^{-1}
Gradient	0-35% (v/v) B over 5 min; 35-55% (v/v) B over 18 min; 100–0% (v/v) B over 1 min; 0% (v/v) B over 1 min
Injection volume	500 µL
Detection wavelengths	220 and 310 nm

Table 7| Reagents needed for the solid-phase peptide synthesis of **BODIPY-cPAF26**.

Resin weight: 45.0 mg	**Loading capacity:** 1 mmol g^{-1}			
Reagent	**Equivalents**	**Molecular weight**	**Density**	**Quantity**
Fmoc-Gly-OH	1.0	297.3 g mol^{-1}	-	13 mg
DIPEA	3.0	129.2 g mol^{-1}	0.742 g mL^{-1}	24 µL
DIPEA	7.0	129.2 g mol^{-1}	0.742 g mL^{-1}	55 µL
Fmoc-Trp-OH	3.0	426.5 g mol^{-1}	-	57 mg
Fmoc-Phe-OH	3.0	387.4 g mol^{-1}	-	52 mg
Fmoc-Lys(Z)-OH	3.0	502.6 g mol^{-1}	-	68 mg
Fmoc-Arg(NO_2)-OH	3.0	441.4 g mol^{-1}	-	60 mg
DIC	3.0	126.2 g mol^{-1}	0.815 g mL^{-1}	21 µL
OxymaPure	3.0	142.1 g mol^{-1}	-	19 mg
Fmoc-Trp(C_2-BODIPY)-OH	1.5	748.6 g mol^{-1}	-	51 mg
PyBOP	1.5	520.4 g mol^{-1}	-	35 mg
HOBt (hydrate)	1.5	153.1 g mol^{-1}	-	10 mg
DIPEA	2.0	129.2 g mol^{-1}	0.742 g mL^{-1}	16 µL

Table 8 | Troubleshooting.

Step(s)	Problem	Possible reason	Possible solution
23	Compound **3** is eluted with only hexane during column chromatography	Incorrect equilibration of the purification system or traces of DCM remaining after evaporation	Combine all fractions in a round-bottom flask to remove the solvent and repeat the purification steps
25	Low recovery yield after column chromatography	Compound **3** co-elutes with some byproducts	Combine impure fractions in a round-bottom flask, remove the solvent and crystallize compound **3** in DCM: hexane (1: 6)
31	Low conversion in the formation of compound **1**	Crude impurities or large scale may decrease the synthetic yield	Add more $Pd(OAc)_2$ and $AgBF_4$ and perform an additional microwave irradiation cycle
37	Samples of amino acid **1** in solution dry out	Low boiling point of the solvent used	Re-dissolve the dry sample in MeOH and analyze by HPLC-MS
54	Positive Kaiser test	Partial amino acid incorporation	Repeat the coupling. Alternatively, employ longer reaction times (16 h) or employ the reaction conditions from steps 48-52**.**
54, 62	Unclear result in Kaiser test	Partial amino acid incorporation or too many resin beads used for the test	Repeat the test with fewer resin beads. If still unclear, cleave a small portion of the resin and analyze it by HPLC. If the incorporation of the amino acid is partial, repeat the coupling
62	Positive Kaiser test	Partial amino acid incorporation	Repeat the coupling using 0.5 eq. of amino acid **1**, HOBt and PyBOP and 1 eq. DIPEA
62	Abnormal color in Kaiser test	The BODIPY fluorophore gives intense yellow color	Repeat the test with fewer resin beads
82	**BODIPY-cPAF26** peptide not formed and/or formation of byproducts	Low catalyst efficiency	Repeat the hydrogenation reaction with a different source/batch of $Pd(OH)_2$-C
94	Minor degradation observed for PAF26	Low protease concentration	Increase the pH of the buffer to 7.5-7.8 with sodium dibasic phosphate to speed up the degradation

106	No differences in fluorescence between different liposome suspensions	Wrong concentration of **BODIPY-cPAF26** or liposome suspensions	Repeat the preparation of the liposome dilutions in DPBS, ensuring all samples are properly vortexed
106	Fluorescein shows little fluorescence emission	pH of the solution is not adequate	Increase the solution pH with basic EtOH (0.1 N NaOH) or repeat the sample preparation
111	A549 cells do not detach from cell culture flask	The proteolytic activity of trypsin is reduced	Incubate for longer time (5 min). Alternatively, wash the cells again with 10 mL Ca^{2+}-free DPBS and use a fresh batch of Trypsin-EDTA
120	A549 cells do not adhere to the bottom of the plate(s) or there are signs of contamination	Inadequate manipulation of the cells	Discard plate(s) and use a fresh batch of cells and reagents (i.e. media, DPBS, Trypsin-EDTA)
128	No dark precipitate is observed in the cells	The MTT reaction is slow	Incubate the cells for longer time, typically another 2 h
131	Absorbance values are too low	Cell number per cell is too low or incubation times are too short	Repeat the experiment with more cells per well or increase the MTT reagent and detergent incubation times
131	Absorbance values are too high	Cell number per well is too high	Decrease cell density when plating
131	High standard deviation between replicates	Inaccurate plating or pipetting	Increase accuracy of cell plating and check the performance of pipettes
133, 138, 142, 145, 151	Fungal cells do not grow properly or there are signs of contamination	Fungi in the stock culture are dead or mutated. Poor microbiological technique implemented or the recipe of the growth medium was not followed correctly	Discard flasks. Prepare new cultures using a new batch of medium. Inoculate with a fresh stock culture. Check that the temperature of incubation is correct
162	High variability in absorbance values between replicates	Inaccurate preparation of the 96 well-plate	Confirm the multichannel pipette is working well for all the channels
162	Absorbance values are too low	Not enough cells have been transferred to the 96 well-plate	Gently homogenize the cell suspension during plating to avoid any cell settling in the pipette basin

179,189	*C. neorformans* or *C. albicans* detach from the bottom of the chamber	Sudden movement of the chamber	Discard cells if they have been treated with peptide. If untreated, leave them in the incubator for further 30 min and avoid sudden movements
194	Fluorescence is not detected during image acquisition	Laser intensity is too low or laser is switched off	Check the laser is switched on and adjust its intensity appropriately
217	Red-stained cell(s) have disappeared from the field of view after the addition of **BODIPY-cPAF26**	Cells have detached during the addition of **BODIPY-cPAF26**	Move to another well and, when gently adding **BODIPY-cPAF26**, place the pipette tip close to the surface of the medium to minimize any movement generated by impact of the droplet

Additional information

Supplementary Information is available in the online version of the paper.

Supplementary Figure 1.

Supplementary Figure Legend 1. Analysis of the long-term stability of the amino acid 1 when stored as a solid at different temperatures. HPLC-MS traces of the amino acid **1** after being stored in the dark for 4 months at r.t., 4 ºC, and -20 ºC. UV detection: 500 nm.

Supplementary Figure 2.

Supplementary Figure Legend 2. Analysis of the long-term stability of the amino acid 1 when dissolved in organic solvents at different temperatures. HPLC-MS traces of the amino acid **1** after being stored in the dark for 4 months in: a) DCM at -20 ºC, b) MeOH at 4 ºC, c) DMF at r.t. In c), the green arrow points at the remaining amino acid **1** and the main peaks correspond to Fmoc-removed side products. UV detection: 500 nm.

Supplementary Table 1. Cellular activity of BODIPY-cPAF26 and PAF26 in different fungal species. Cell viability was measured after 16 h incubation with different concentrations of **BODIPY-cPAF26** or PAF26 peptides at 25 ˚C for *N. crassa* or 37 ˚C for *A. fumigatus*. IC_{50} values (µM) are represented as means ± s.d. (n=3).

Supplementary Table 2. Fluorescence quantum yields of BODIPY-cPAF26 in PC: cholesterol (7: 1) liposome suspensions in PBS with increasing hydrophobicity. PBS alone was used as a negative control. Quantum yields were determined by comparing the integrated emission area of the fluorescence spectra to the emission area of fluorescein in basic EtOH (QY: 0.97), λ_{exc}.: 450 nm. Data represented as means ± s.d. (n=3).

Supplementary Figure 3.

Supplementary Figure Legend 3. Time-course analysis of the chemical integrity of BODIPY-cPAF26 and unlabeled linear PAF26 in proteolytic environments. HPLC traces of **BODIPY-cPAF26** (a) and unlabeled PAF26 (b) before incubation (*top*) and after incubation (*bottom*) at a

concentration of 200 µM in a protease cocktail (1 mg L^{-1}). Green arrows point at the peaks of intact **BODIPY-cPAF26** and red arrows point at intact PAF26. UV detection: 280 nm. Purities were determined by integration of the peak areas in respective HPLC chromatograms at 280 nm.

Supplementary Figure 4.

Supplementary Figure Legend 4. Electrospray analysis of BODIPY-cPAF26 and unlabeled PAF26 after 24 h incubation in a protease cocktail. Both peptides (200 µM) were incubated in 1 mg L^{-1} of the protease cocktail, and their respective mass spectra were recorded on a Waters Micromass ZQ mass spectrometer (ESI positive mode). a) MS analysis of **BODIPY-cPAF26** (exact mass: 1311 Da). b) MS analysis of unlabeled PAF26 (exact mass: 949 Da).

Supplementary Video 1.

Supplementary Video Legend 1. Time-course high-resolution imaging of *A. fumigatus* upon treatment with BODIPY-cPAF26. *A. fumigatus* were pre-treated with a cell membrane counterstain (*red*) and imaged under the confocal microscope. Cells were then treated with **BODIPY-cPAF26** (2 µM, *green*) and further imaged without any washing steps. The movie shows the rapid fluorogenic response of **BODIPY-cPAF26** upon interaction with the cell membrane of *A. fumigatus*. Scale bar: 5 µm.

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