Biosynthesis of Fluorinated Peptaibols Using a Site-Directed Building Block Incorporation Approach

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ABSTRACT: Synthetic biological approaches, such as site-directed biosynthesis, have contributed to the expansion of the chemical space of natural products, making possible the biosynthesis of unnatural metabolites that otherwise would be difficult to access. Such methods may allow the incorporation of fluorine, an atom rarely found in nature, into complex secondary metabolites. Organofluorine compounds and secondary metabolites have both played pivotal roles in the development of drugs; however, their discovery and development are often via nonintersecting tracks. In this context, we used the biosynthetic machinery of Trichoderma arundinaceum (strain MSX70741) to incorporate a fluorine atom into peptaibol-type molecules in a site-selective manner. Thus, fermentation of strain MSX70741 in media containing ortho- and meta-F-phenylalanine resulted in the biosynthesis of two new fluorine-containing alamethicin F50 derivatives. The fluorinated products were characterized using spectroscopic (1D and 2D NMR, including 19F) and spectrometric (HRESIMS/MSn) methods, and their absolute configurations were established by Marfey’s analysis. Fluorine-containing alamethicin F50 derivatives exhibited potency analogous to the nonfluorinated parent when evaluated against a panel of human cancer cell lines. Importantly, the biosynthesis of fluorinated alamethicin F50 derivatives by strain MSX70741 was monitored in situ using a droplet-liquid microjunction-surface sampling probe coupled to a hyphenated system.

Based on the literature and Dictionary of Natural Products, approximately 250,000 secondary metabolites have been isolated from plants, microorganisms, and other sources (particularly marine life). Of these, about 4700 are halogenated, and just 5 contain a fluorine atom, with no reports of natural fluorometabolites derived from fungi. Nature has been a fertile source for drug leads, particularly in the realms of anticancer and antimicrobial agents. Alternatively, drugs that include at least one fluorine atom (~274 up to 2009) represent approximately 25–30% of pharmaceuticals, including some of the top-selling drugs, such as sofosbuvir (Sovvaldi; annual revenue of $9.4 billion in the U.S.), rosuvastatin (Creostor; annual revenue of $8.5 billion), and sitagliptin (Januvia; annual revenue of $5.0 billion). However, these two classes of molecules do not often intersect, perhaps limiting the combination of privileged scaffolds in natural products with the beneficial properties of fluorine atoms in medicinal chemistry.

The high electronegativity, small atomic radius, and low polarizability of the C–F bond are some of the unique features that make fluorine so attractive from the point of view of medicinal chemistry, such that, the benefits of incorporating fluorine into lead molecules or drugs could impact drastically on physicochemical properties, resulting in changes in absorption, distribution, metabolism, and molecular interactions in vivo and in vitro. Due to the potential of fluorine-containing molecules in drug discovery, there has been a focus on the development of new synthetic and semisynthetic strategies to incorporate this atom into organic molecules, particularly in a site selective manner. However, the incorporation of a fluorine atom into structurally complex natural products remains a challenge, likely because of the perception that most of the fluorination reagents could degrade the parent molecule provided by nature.

An alternative approach to modify natural product scaffolds is to employ precursor-directed biosynthesis, using the biosynthetic machinery of microorganisms to incorporate fluorinated building blocks into natural products. This technique has been widely used in the past, generating a vast number of microbial natural product analogues, with cyclo-
There are challenges with this approach, because the building block selected for fluorine incorporation may not be able to compete with the natural moiety, or simply not be compatible with the enzymes involved in the biosynthesis of the desired product. Another challenge is that, in many of the cases, the organisms must be genetically modified in order to disrupt the biosynthetic pathway, so as to permit the incorporation of the fluorinated building blocks.

To probe the applicability of precursor-directed biosynthesis with fungal cultures, and in order to contribute to the expansion of the chemical space of natural products, a site-directed building block incorporation approach was applied to a wild type ascomycete using fluorinated amino acids as the source of fluorine. Previously, we reported the isolation of...
Table 1. NMR Data for Alamethicin F50 (1), ortho-F-Pheol Alamethicin F50 (2), and meta-F-Pheol Alamethicin F50 (3)\textsuperscript{a}

| residue | position | 2 | 3 |
|---------|----------|---|---|
| | | \(\delta_C\) | type | \(\delta_H, m (J \text{ in Hz})\) | \(\delta_C\) | type | \(\delta_H, m (J \text{ in Hz})\) |
| Ac | 1 | 172.5 | C | C | 172.5 | C |
| | 2 | 22.4 | CH\(_3\) | 2.05, s | 22.4 | CH\(_3\) | 2.05, s |
| Aib\(^1\) | 1 | 175.5 | C | C | 175.5 | C |
| | 2 | 57.4 | C | C | 57.4 | C |
| | 3 | 23.8 | CH\(_3\) | 1.46, s | 23.8 | CH\(_3\) | 1.46, s |
| | 4 | 26.6 | CH\(_3\) | 1.53, s | 26.6 | CH\(_3\) | 1.54, s |
| NH | | | | | 8.63, s | | 8.64, s |
| Pro\(^3\) | 1 | 175.6 | C | C | 175.6 | C |
| | 2 | 65.7 | CH | 4.25, t (8.4) | 65.7 | CH | 4.25, t (8.4) |
| | 3 | 29.7 | CH\(_2\) | 1.80, m | 29.7 | CH\(_2\) | 1.80, m |
| | 4 | 27.1\(^b\) | CH\(_2\) | 1.97, m | 27.1\(^b\) | CH\(_2\) | 1.97, m |
| | 5 | 49.9 | CH\(_2\) | 3.48, td (10.5, 6.3) | 49.9 | CH\(_2\) | 3.49, td (10.5, 6.3) |
| Aib\(^5\) | 1 | 178.5 | C | C | 178.5 | C |
| | 2 | 57.4 | C | C | 57.4 | C |
| | 3 | 23.1 | CH\(_3\) | 1.54, s | 23.1 | CH\(_3\) | 1.54, s |
| | 4 | 27.4 | CH\(_3\) | 1.56, s | 27.4 | CH\(_3\) | 1.56, s |
| NH | | | | | 7.62, s | | 7.62, s |
| Ala\(^4\) | 1 | 177.2 | C | C | 177.2 | C |
| | 2 | 54.1 | CH | 4.09, m | 54.1 | CH | 4.09, m |
| | 3 | 17.1 | CH\(_3\) | 1.48, d, (7.7) | 17.0 | CH\(_3\) | 1.48, d, (7.7) |
| NH | | | | | 7.56, d, (5.6) | | 7.56, d, (5.6) |
| Aib\(^6\) | 1 | 177.8 | C | C | 177.8 | C |
| | 2 | 57.3 | C | C | 57.3 | C |
| | 3 | 23.1 | CH\(_3\) | 1.54, s | 23.1 | CH\(_3\) | 1.54, s |
| | 4 | 27.1 | CH\(_3\) | 1.56, s | 27.1 | CH\(_3\) | 1.56, s |
| NH | | | | | 7.93, s | | 7.93, s |
| Ala\(^7\) | 1 | 178.1 | C | C | 178.1 | C |
| | 2 | 53.9 | CH | 4.02, m | 53.8 | CH | 4.01, m |
| | 3 | 16.9 | CH\(_3\) | 1.53, d, overlapped | 16.9 | CH\(_3\) | 1.53, d, overlapped |
| NH | | | | | 7.92, brs | | 7.91, brs |
| Gln\(^7\) | 1 | 175.8 | C | C | 175.8 | C |
| | 2 | 58.1 | CH | 3.94, m | 58.1 | CH | 3.94, m |
| | 3 | 27.1 | CH\(_2\) | 2.15, m | 27.3, | CH\(_2\) | 2.15, m |
| | 4 | 32.6 | CH\(_2\) | 2.30, m | 2.30, m | 2.30, m | 2.34, m |
| | 5 | 177.3 | C | C | 177.1 | C |
| 5-NH\(_2\) | | 8.00, d, (5.6) | 7.99, d, (4.9) | 7.99, d, (4.9) | 6.77, brs | 6.77, brs | 6.77, brs |
| | | 7.44, brs | 7.45, brs | 7.45, brs | |
| Aib\(^8\) | 1 | 178.2 | C | C | 178.2 | C |
| | 2 | 57.6 | C | C | 57.6 | C |
| | 3 | 23.3 | CH\(_3\) | 1.52, s | 23.3 | CH\(_3\) | 1.52, s |
| | 4 | 27.4 | CH\(_3\) | 1.55, s | 27.4 | CH\(_3\) | 1.55, s |
| NH | | | | | 8.09, s | | 8.08, s |
| Val\(^7\) | 1 | 175.3 | C | C | 175.3 | C |
| | 2 | 65.7 | CH | 3.58, dd (9.8, 3.5) | 65.7 | CH | 3.58, dd (9.3, 3.2) |
| | 3 | 30.4 | CH | 2.25, m | 30.6 | CH | 2.25, m |
| | 4 | 19.6 | CH\(_3\) | 1.00, d, (6.3) | 19.6 | CH\(_3\) | 1.00, d, (6.4) |
| | 5 | 20.8 | CH\(_3\) | 1.13, d, (6.3) | 20.8 | CH\(_3\) | 1.13, d, (6.3) |
| NH | | | | | 7.49, d, (4.9) | | 7.49, d, (4.9) |
| Aib\(^9\) | 1 | 179.0 | C | C | 179.0 | C |
| | 2 | 57.6 | C | C | 57.6 | C |
| | 3 | 26.8 | CH\(_3\) | 1.54, s | 26.8 | CH\(_3\) | 1.54, s |
| | 4 | 27.1 | CH\(_3\) | 1.56, s | 27.1 | CH\(_3\) | 1.56, s |
| NH | | | | | 8.22, s | | 8.22, s |
| residue | position | δ\(_C\) | type | δ\(_H\), m (J in Hz) | δ\(_C\) | type | δ\(_H\), m (J in Hz) |
|---------|----------|---------|------|---------------------|---------|------|---------------------|
| Gly\(^1\) | 1 | 173.0 | C | | 173.0 | C | |
| | 2 | 45.1 | CH\(_2\) | 3.67, m | 45.0 | CH\(_2\) | 3.67, dd (16.8, 5.6) |
| | NH | | | 8.34, brt (5.6) | | | |
| Leu\(^2\) | 1 | 175.8 | C | | 175.8 | C | |
| | 2 | 54.1 | CH | 4.46, m | 54.0 | CH | 4.45, m |
| | 3 | 41.5 | CH\(_2\) | 1.59, overlapped | 41.5 | CH\(_2\) | 1.59, overlapped |
| | 4 | 25.6 | CH | 1.91, m | 25.6 | CH | 1.91, m |
| | 5 | 21.3 | CH\(_3\) | 0.92, d (6.3) | 21.3 | CH\(_3\) | 0.92, d (6.3) |
| | 6 | 23.4 | CH\(_3\) | 0.94, d (6.3) | 23.4 | CH\(_3\) | 0.94, d (6.3) |
| NH | | | | | | 8.11, d (7.8) |
| Aib\(^3\) | 1 | 174.9 | C | | 174.9 | C | |
| | 2 | 58.1 | C | | 58.1 | C | |
| | 3 | 23.7 | CH\(_3\) | 1.61, s | 23.7 | CH\(_3\) | 1.61, s |
| | 4 | 26.7 | CH\(_3\) | 1.54, s | 26.6 | CH\(_3\) | 1.54, s |
| NH | | | | | | 8.40, s |
| Pro\(^4\) | 1 | 176.4 | C | | 176.4 | C | |
| | 2 | 64.6 | CH\(_2\) | 4.38, dd (9.1, 6.3) | 64.6 | CH\(_2\) | 4.39, dd (8.9, 6.5) |
| | 3 | 30.0 | CH\(_2\) | 1.80, m | 30.0 | CH\(_2\) | 1.80, m |
| | 4 | 26.9\(^b\) | CH\(_2\) | 1.99, m | 26.9\(^b\) | CH\(_2\) | 1.99, m |
| | 5 | 50.6 | CH\(_2\) | 3.73, m | 50.5 | CH\(_2\) | 3.73, m |
| | | | 3.88, dt (11.2, 6.3) | | | 3.88, dt (11.9, 6.2) |
| Val\(^5\) | 1 | 175.3 | C | | 175.3 | C | |
| | 2 | 64.3 | CH | 3.73, m | 64.3 | CH | 3.73, m |
| | 3 | 30.5 | CH | 2.34, m | 30.5 | CH | 2.34, m |
| | 4 | 19.4 | CH\(_3\) | 0.97, d (6.3) | 19.4 | CH\(_3\) | 0.98, d (6.5) |
| | 5 | 20.2 | CH\(_3\) | 1.07, d (6.3) | 20.2 | CH\(_3\) | 1.07, d (6.4) |
| NH | | | | | | 7.63, d (8.0) |
| Aib\(^6\) | 1 | 177.6 | C | | 177.6 | C | |
| | 2 | 57.6 | C | | 57.6 | C | |
| | 3 | 23.4 | CH\(_3\) | 1.54, s | 23.4 | CH\(_3\) | 1.54, s |
| | 4 | 27.4 | CH\(_3\) | 1.54, s | 27.4 | CH\(_3\) | 1.54, s |
| NH | | | | | | 7.58, s |
| Aib\(^7\) | 1 | 178.8 | C | | 178.8 | C | |
| | 2 | 57.7 | C | | 57.7 | C | |
| | 3 | 23.4 | CH\(_3\) | 1.53, s | 23.4 | CH\(_3\) | 1.53, s |
| | 4 | 27.4 | CH\(_3\) | 1.55, s | 27.4 | CH\(_3\) | 1.55, s |
| NH | | | | | | 7.81, s |
| Gln\(^8\) | 1 | 175.6 | C | | 175.5 | C | |
| | 2 | 57.0 | CH | 4.01, m | 57.0 | CH | 4.01, m |
| | 3 | 28.0 | CH\(_2\) | 2.25, m | 28.0 | CH\(_2\) | 2.25, m |
| | 4 | 33.2 | CH\(_2\) | 2.43, dt (15.4, 8.4) | 33.1 | CH\(_2\) | 2.43, dt (15.5, 8.6) |
| | | | 2.62, dt (15.4, 7.7) | | | 2.62, dt (15.2, 8.0) |
| | 5 | 177.4 | C | | 177.4 | C | |
| NH | | | | 7.78, d (5.6) | | 7.79, d (5.4) |
| 5-NH\(_2\) | | | | 6.78, brs | | 6.79, brs |
| 5-NH\(_2\) | | | | 7.44, brs | | 7.45, brs |
| Gln\(^9\) | 1 | 174.1 | C | | 174.0 | C | |
| | 2 | 55.7 | CH | 4.15, m | 55.6 | CH | 4.16, m |
| | 3 | 27.9 | CH\(_2\) | 1.99, m | 27.9 | CH\(_2\) | 2.01–2.05, m |
| | 4 | 32.9 | CH\(_2\) | 2.19, m | 32.9 | CH\(_2\) | 2.23, m |
| | 5 | 177.3 | C | | 177.3 | C | |
| NH | | | | 7.86, d (7.7) | | 7.87, d (7.5) |
| 5-NH\(_2\) | | | | 6.62, brs | | 6.63, brs |
| 5-NH\(_2\) | | | | 7.35, brs | | 7.35, brs |
alamethicin F50 (1; Figure 1) as the main constituent in an extract of fungal strain MSX70741 (Figure S1, Supporting Information), a Trichoderma isolate from the Mycosynthetix library.38 Compound 1 is a long-chain peptaibol (20 amino acid residues), which contains a high proportion of α-aminoisobutyric acid (Aib, 8 residues), includes an acyl substituted N-terminus, and has a C-terminal phenylalaninol (Pheol) moiety.39–41 Taking into account this structural information, we selected the Pheol20 building block as an attractive target for the introduction of a fluorine atom into the alamethicin F50 molecule (1) in a site-selective manner. We hypothesized that doing so would not drastically impact the biosynthesis of 1, and the resulting analogue should retain the α-helical conformation, which is a key feature for the biological activity of peptaibols as membrane modulators and pore-forming antibiotics.42 Therefore, in this communication we present the in vitro synthesis, as well as the isolation, structure elucidation, and biological evaluation of two new fluorinated-alamethicin F50 analogues, which were biosynthesized in wild type fungal species using the noncanonical amino acids ortho F-Pheol and meta F-substituted phenylalanine (Phe).

## RESULTS AND DISCUSSION

Strain MSX70741 was identified as Trichoderma arundinaceum based on morphological and molecular characterization (Figures S2–S4). Cultures of this strain grown in potato dextrose agar (PDA) or PDA supplemented with a racemic mixture of either ortho-F-DL-Phe, meta-F-DL-Phe or para-F-DL-Phe (500 ppm; see Figure S1 for photographs of the cultures) were monitored in situ using a droplet—liquid microjunction—surface sampling probe (droplet—LMJ—SSP) coupled to a UPLC—PDA—HRMS—MS/MS system.43 All of these cultures showed characteristic in-source ion peaks for alamethicin F50 (1), such as m/z 1963.1313 ([M + H]+; monoisotopic precursor ion), 1189.6942 ([M + H]+ fragment), 982.0722 ([M + 2H]+, 774.4505 (y7 fragment), and 655.0505 ([M + 3H]+) (Figure 2).43 Moreover, in the culture supplemented with meta-F-DL-Phe, the mass spectrum also showed a set of peaks shifted by 17.99 amu ([M + H]+ = 1981.1241, and y7 = 792.4409, 9.00 amu ([M + 2H]+ = 991.0670), and 6.00 amu ([M + 3H]+ = 661.0471), indicating the incorporation of a fluorine atom (F = 18.9984 amu, exact mass) into alamethicin F50 (1) (Figures 1 and 2). Similar results were observed in the MS spectrum of cultures supplemented with ortho-F-DL-Phe (Figure 2). Importantly, incubating the microorganism with para-F-DL-Phe did not result in the biosynthesis of the para-F-Phe alamethicin F50 analogue (Figure 2), as previously reported for beauvericin,46 pseurotin and synerazol analogues,47 giving insights into the specificity in building block recognition by nonribosomal peptide synthetases (NRPS).48 To obtain enough material for structural characterization of the putative fluorinated alamethicin F50 analogues, T. arundinaceum strain MSX70741 was grown on rice and rice supplemented with each of the fluorinated building blocks

Table 1. continued

| residue   | position | δ_C  | type | δ_H m (J in Hz) | δ_C  | type | δ_H m (J in Hz) |
|-----------|----------|------|------|----------------|------|------|----------------|
| Pheol20/F-Pheol20 | 1 65.1 | CH₂ | CH₂ | 3.65, brt | 64.9 | CH₂ | 3.63, brt |
|           | 2 52.9 | CH  | CH  | 4.25, m  | 54.0 | CH  | 4.16, m  |
|           | 3 31.1 | CH₂ | CH₂ | 2.70, dd, 14.0, 9.1 | 37.7 | CH₂ | 2.72, dd, 14.2, 9.7, 4.9 |
|           | 4 125.6, d, (15.4) | C | C | 142.6, d, (7.4) | 117.1, d, (21.0) | CH | 7.06, brd, (10.2) |
|           | 5 162.6, d, (242.7) | CF | CF | 160.0, d, (242.4) | 137.8, d, (8.5, 2.3) | CH | 6.88, td, (8.5, 2.3) |
|           | 6 115.5, d, (22.1) | CH | CH | 113.8, d, (21.0) | 130.7, d, (8.2) | CH | 7.24, t, (7.6) |
|           | 7 129.1, d, (8.4) | CH | CH | 126.3, d, (2.6) | 7.10, d, (7.6) |
|           | 8 124.9, d, (3.7) | CH | CH | 7.04, t, (7.7) |
|           | 9 132.9, d, (4.4) | CH | CH | 7.37, t, (7.7) |
| NH        | 7.32, d, (9.1) | C | C | 7.22, d, overlapped |
| 1-OH      | 5.23, t, (6.6) | CH | CH | 5.27, t, (6.6) |
| F         | −119.7, m | CH | CH | −115.8, m |

aData recorded in CD₃OH. ¹H (700 MHz), ¹³C (175 MHz), and ¹⁹F (470 MHz). bSignals may be exchangeable. cRecorded at 470 MHz.

Figure 3. Key HMBC, TOCSY, and NOESY correlations for compounds 1–3.
analogue of alamethicin F50 (1), respectively (Figure 3). In general, the $^1$H and $^{13}$C NMR spectra for compounds 2 and 3 were almost identical to those recorded for 1 (Table 1; Figure 4), the main differences being in the chemical shifts and splitting of signals attributed to an ortho and meta F-substituted phenyl (2 and 3, respectively), equivalent to the phenylalaninol (Pheol)$^{50}$ moiety in 1 (Figure 4, Table 1). Thus, in the $^{13}$C NMR spectrum for 2 and 3, a set of six doublets were displayed in the aromatic region ($\delta_{C} 115-163$ ppm), instead of four singlet peaks observed for 1 ($\delta_{C} 127-140$ ppm; Table 1). The observation of splitting in a proton-decoupled $^{13}$C NMR experiment confirmed the incorporation of the fluorinated building blocks into the products, as noted by the prominent $J_{CH}$ values (Table 1). Importantly, the presence of a fluorine atom in 2 and 3 was verified by $^{19}$F NMR spectroscopy with $\delta_{F}$ values of $-119.7$ and $-115.8$ ppm compared to $\delta_{F}$ values of $-119.7$ and $-114.8$ ppm for the racemic mixtures of amino acids ortho-F-DL-Phe and meta-F-DL-Phe, respectively (Tables 1 and S2). The $^{19}$F-NMR signals for products 2 and 3 had similar $^{19}$F-$\text{H}$ coupling patterns to those observed for their corresponding building blocks, confirming their incorporation into alamethicin F50 (1) (Table S2).

The amino acid sequences in 2 and 3 were also examined by HRESIMS/MS (Figures S22 and S32). In the case of compound 3, the full scan HRESIMS spectra exhibited several common in-source ions, specifically $[M + H]^+$, $[M + 2H]^{2+}$, $[M + 3H]^{3+}$, $b_{13}^+$ and $y_{2}^+$ fragments, with the latter two generated from the cleavage between Aib$^{13}$ and Pro$^{14}$ (Figure S31).$^{58}$ In compound 3, for example, fragmentation of the ion $b_{13}^{+}$ at m/z 1189.69 gave peaks at m/z 934.5372, 849.4816, 750.4141, 665.3605, 537.3030, 466.2657, 381.2129, 310.1757, and 225.1231 indicating the successive losses of Aib$^{13}$-Leu$^{12}$-Gly$^{11}$, Aib$^{10}$, Val$^{9}$, Aib$^{8}$, Gln$^{7}$, Ala$^{6}$, Aib$^{5}$, Ala$^{4}$, and Aib$^{3}$ and supporting the sequence AcaAib$^1$-Pro$^2$-Aib$^4$-Aib$^5$-Aib$^6$-Gln$^7$-Aib$^8$-Val$^{9}$-Aib$^{10}$-Gly$^{11}$-Leu$^{12}$-Aib$^{13}$. Similar fragmentation of the $[M + 2H]^{2+}$ ion, in particular the fragment $y_{2}^+$ permitted the assignment of the C-terminal fragment as Pro$^{14}$-Val$^{13}$-Aib$^{12}$, Aib$^{11}$-Gln$^{10}$-Gln$^{9}$-(meta-F)Pheol$^{20}$. Analogous mass spectrometric experiments were used to assign the amino acid sequence in 2 (Figures S21 and S22).
The absolute configuration of each amino acid in 2 and 3 was confirmed by acid hydrolysis, Marfey’s derivatization under alkaline conditions, and analysis of the derivatives using a 10 min UPLC protocol.38 For this, the appropriate standards for the D and L enantiomers of the ortho and meta F-Pheol building blocks were prepared (Supporting Information).38 As expected, the absolute configuration of all amino acids in 2 and 3 was L, as previously reported for alamethicin F50 (1) by Ayers et al.38 Importantly, these results indicated that T. arundinaceum strain MSX70741 incorporated only the L-enantiomer of the fluorinated building blocks into the alamethicin F50 analogues (2,3) (Figures S9 and S10). In previous studies, the absolute configurations of the fluorinated analogues of beavericin, psuerotin, and synerazol were presumed to be the same as in the parent compounds, based on the detection of one diastereomer and specific rotation data.46,47

To assess the bioactivities of compounds 2 and 3, their IC50 values were determined against a panel of cancer cell lines [MDA-MB-435 (melanoma), MDA-MB-231 (adenocarcinoma), and OVCAR3 (ovarian cancer)].51,52,56-58 In these cytotoxicity assays, compounds 2 and 3 were equipotent to their nonfluorinated parent, with IC50 values ranging from 4.8 to 6.4 μM (Table 2). These data suggested that the incorporation of fluorine into the alamethicin F50 (1) molecule did not drastically impact the cytotoxicity of the compounds.

| compd | IC50 (μM)* |
|-------|------------|
|       | MDA-MB-435 | MDA-MB-231 | OVCAR3 |
| 1     | 4.9        | 5.6        | 1.5     |
| 2     | 6.4        | 5.9        | 4.8     |
| 3     | 4.8        | 5.9        | 6.3     |
| 4     | 4.8        | 5.1        | 5.1     |
| 5     | 4.7        | 5.0        | 5.0     |
| 6     | 2.8        | 4.3        | 4.4     |
| Paclitaxel (taxol) | 0.0005 | 0.009 | 0.002 |

*IC50 values were determined as the concentration required to reduce cellular proliferation by 50% relative to the untreated controls following 72 h of continuous exposure.

In an attempt to stimulate the fungus to increase the biosynthesis of the fluorinated analogues of alamethicin F50, an experiment was designed as follows: T. arundinaceum was cultured on PDA for 3 days. Subsequently, an agar plug with mycelium from the leading edge of the colony was used to inoculate 10 mL of liquid medium containing 2% of soy peptone, 2% dextrose, and 1% yeast extract (YESD). After 3 days of growth, the liquid media was used to inoculate either autoclaved rice (10 g of rice and 20 mL of H2O control) or autoclaved rice containing either (1) 130 mg of powder of ortho-F-DL-Phe; (2) 37.5 mg of ortho-F-DL-Phe in 5.0 mL of H2O (final concentration: 1250 ppm); (3) 15 mg of ortho-F-DL-Phe in 2.0 mL of H2O (final concentration: 500 ppm); or (4) 15 mg of meta-F-1-Phe in 2.0 mL of H2O (final concentration: 500 ppm) (Scheme S2). All four cultures were incubated for 21 days and then extracted and analyzed by UPLC-PDA-MS. The fluorinated analogues were detected in all cultures. Subsequent isolation of compound 2 by HPLC-PDA-MS following the protocol described in the experimental section indicated that (1) supplementing the microorganism with 130 mg of ortho-F-DL-Phe increased the ratio of compounds 2/1 to 1:1, although the overall yield of product declined significantly (1 mg), and (2) the optimum way to obtain the ortho fluorinated analogues of alamethicin F50 was supplementing the media with 500 ppm of ortho-F-DL-Phe, yielding 11.4 mg of a 2:5 ratio of 2/1.

Finally, to validate the protocol used for biosynthesis of peptoids fluorinated in the Pheol moiety at the C-terminal, we selected Trichoderma albolutescens strain MSX57715 (details about the strain identification are provided in the Supporting Information, Figure S5), which biosynthesizes the peptaibol trichokin VI (4).36 Cultivation of this strain under the same conditions used for strain MSX70741 led to the isolation of the ortho and meta-F-Pheol analogues of trichokin VI (4–6, Figure 1). As observed with MSX70741, MSX57715 incorporated only the ortho and meta-F-1-Phe building blocks into trichokin VI (4), and not the para substituted analogue. From the point of view of bioactivity, compounds 5 and 6 displayed potency similar to the nonfluorinated parent when evaluated against the same panel of cancer cell lines, with IC50 values in the lower μM range (Table 2).

As demonstrated in this study, a site-directed building block incorporation approach can be a powerful tool for studying, and perhaps expanding upon, the chemical diversity available through nature. Primarily, this approach facilitates the incorporation of fragments that are rarely found in nature into complex secondary metabolites. Second, these unnatural metabolites, which may be otherwise difficult to obtain, contribute to the expansion of chemical space around privileged scaffolds.59 Moreover, these new biosynthetic products may address some perceived challenges to the screening of natural products, such as legal access to biodiversity, identification of biological activity, and most recently, intellectual property associated with composition of matter patents, which many would consider the most desirable of “Orange Book” patents.52 In short, this approach imparts another way to translate natural products discoveries into further development. In particular, this methodology opens up new avenues for targeting the biosynthesis of bioactive compounds (i.e., privileged scaffolds) with potentially improved physicochemical and pharmacological properties. This technique, in combination with appropriate genomic approaches, may lead to the generation of valuable compounds.

In summary, we report the biosynthesis of fluorine containing analogues of the peptoids alamethicin F50 (2,3) and trichokin VI (5,6) using a site-directed building block incorporation approach. Importantly, the biosynthesis of these products was carried out using wild type Trichoderma strains. Biosynthesis of products 2, 3, 5, and 6 represent the first report of the application of a site-directed building block incorporation approach targeting the incorporation of a fluorine atom into peptoid type molecules. Notably, examination of the ability of Trichoderma species to incorporate the fluorinated building blocks was monitored in situ, facilitating the identification of the products in an early stage of the study, before the scaling up of the cultures.

### EXPERIMENTAL SECTION

#### General Experimental Procedures

NMR experiments were conducted in CD3OD with presaturation of the OH peak at δH 4.92 ppm (water experiment). NMR instrumentation was a JEOL ECA-500 NMR spectrometer operating at 500 MHz for 1H, 700 MHz for 13C, and 125 MHz for 19F, equipped with a cryoprobe, operating at 700 MHz for 1H and 175 MHz for 13C. All chemical shifts were referenced to the residual solvent peaks (δH 3.31 and δC 49.0). HRESIMS data were obtained using a Thermo QExactive Plus mass spectrometer (ThermoFisher...
Scientific) paired with an electrospray ionization source. Monitoring the biosynthesis of secondary metabolites in fungal cultures *in situ* was performed using the droplet-LMJ-SSP coupled with a Waters Acuity ultraperformance liquid chromatography (UPLC) system (Waters Corp.) to a Thermo QExactive Plus via procedures previously by Sica et al. Briefly, extractions were performed using Fisher Optima LC/MS grade solvents consisting of 50% MeOH/H2O. An initial 5 μL of solvent was drawn into the syringe. Droplets of 4 μL were dispensed onto the surface of the sample at a rate of 2 μL/s, held on the surface for 2 s, and withdrawn back into the syringe at the same rate. This extraction process was repeated in triplicate for a single spot prior to injection into the UPLC–MS system. The higher-energy collisional dissociation (HCD) used a normalized energy of 35 for all the compounds to obtain MS/MS data. The UPLC separations were performed using an Acquity BEH C18 column (50 mm × 2.1 mm, internal diameter, 1.7 μm) equilibrated at 40 °C and a flow rate set at 0.3 mL/min. The mobile phase consisted of a linear MeCN/H2O (acidified with 0.1% formic acid) gradient starting at 15% MeCN to 100% MeCN over 8 min. The mobile phase was held for another 1.5 min at 100% MeCN before returning to the starting conditions. The HPLC separations were performed using a Varian ProStar HPLC system connected to a ProStar 335 photodiode array detector (PDA) with UV detection set at 195 and 210 nm. Preparative HPLC purifications of isolated compounds were performed on a Phenomenex Synergi 4 μm particle size C18 column (21 × 250 mm) at a flow rate of 20 mL/min. Fractions from column chromatography were carried onto a Teledyne ISCO Combiflash RF connected to ELSD and PDA detectors, with the latter having UV detection set at 200–400 nm, all according to established protocols. All solvents were obtained from Fisher Scientific and used without further purification. The orF-D-L-Phe, orF-Phe- and orF-F-DL-Phe were purchased from Acros Organics. The standards of orF-P-D-L-Pheol and orF-F-D-L-Pheol were prepared as detailed in the Supporting Information.

**Fungal Strain Isolation and Identification.** Mycosynthetic fungal strain MSX70741 was isolated from wood collected in a humid mountain forest (April 1993), whereas strain MSX57715 was isolated from leaf litter in a predominately oak forest (October 1991) both by Dr. Barry Katz. Both strains were used previously for the isolation of peptaibolks. A description of the procedures used to identify these strains was outlined recently, and the specific details are also provided in the Supporting Information (Table S1, Figures S2–S5). MSX70741 was identified as *Trichoderma arundinaceum*, whereas strain MSX57715 was identified as *T. atrovicans*. The sequence data for both strains were deposited in GenBank (accession numbers: ITS: KY301717, tefl: KY630169, KY630170, RPBI: KY630166 for strain MSX70741 and accession numbers: tefl: KY3630167, KY630168, RPBI: KY630164, KY630165 for strain MSX77715).

**Fermentation, Extraction, and Isolation.** Fungal strains MSX70741 and MSX77715 were each grown on a malt extract agar, and subsequently, a small piece from the leading edge of the colony was transferred into YES media (followed by incubation for 7 days at 22 °C with agitation at 125 rpm). The seed cultures were transferred into 250 mL Erlenmeyer flasks containing 50 mL of rice, which was diluted and added to the appropriate wells (several concentrations; 0.2, 0.5%, DMSO: 0.5%). The cells were incubated in the presence of test substance for 72 h at 37 °C and then harvested by trypsinization followed by two washes to remove all cell debris. This dried organic extract was transferred into 250 mL Erlenmeyer flasks containing 30 mL of rice medium, prepared using 10 g of rice and twice the volume of rice with 10% fetal bovine serum (10%), penicillin (100 units/mL), and streptomycin (100 μg/mL). Cells in log phase of growth were harvested by trypsinization followed by two washes to remove all traces of enzyme. A total of 5000 cells were seeded per well of a 96-well clear, flat-bottom plate (Microtest 96, Falcon) and incubated overnight (37 °C in 5% CO2). Samples dissolved in DMSO were then diluted and added to the appropriate wells (several concentrations; total volume: 100 μL; DMSO: 0.5%). The cells were incubated in the presence of test substance for 72 h at 37 °C and evaluated for viability with a commercial absorbance assay (CellTiter 96 AQueous One Solution Cell Proliferation Assay, Promega Corp, Madison) that measured viable cells. IC50 values were determined as the concentration required to reduce cellular proliferation by 50% relative to the untreated controls following 72 h of continuous exposure. Paclitaxel (taxol) was used as a positive control.

**Optimization of Biosynthesis of Fluorinated Analogues.** For each different condition (Scheme S2) a seed of the fungal strain MSX70741 was grown on liquid YES media incubated for 3 days at 22 °C with agitation at 125 rpm. The seed culture was transferred into 250 mL Erlenmeyer flasks containing 30 mL of rice medium, prepared using 10 g of rice and twice the volume of rice with H2O. These flasks were supplemented with 130 mg of orF-F-DL-Phe powder (Condition 1), 2.0 mL of a stock solution 7500 ppm of orF-
F-DL-Phe (500 ppm, Condition 2), 5.0 mL of a stock solution 7500 ppm of ortho-F-DL-Phe (1250 ppm, Condition 3), or 2.0 mL of a stock solution 7500 ppm of ortho-F-L-Phe (500 ppm, Condition 4) (Scheme S2). The flasks were incubated at 22 °C until they showed good growth and then extracted according to the procedure mentioned in the fermentation, extraction, and isolation section.

Marfey's Analysis. Approximately 0.2 mg of each amino acid standard was weighed into separate glass 2 mL reaction vials. To each standard was added 50 μL of H2O, 20 μL of 1 M NaHCO3, and 100 μL 1% Marfey's reagent (Nε-(2,4-dinitro-5-fluorophenyl)-L-alanina-mide) in acetone. The reaction mixtures were agitated at 40 °C for 1 h. The reactions were halted by the addition of 10 μL of 2 N HCl. The product of the reactions was dried under a stream of nitrogen and dissolved in ~1.7 mL of MeOH. Each derivatized standard was injected individually (0.7 μL) onto the UPLC column, and the derivatized standards were combined to give a mixed standard, which was injected too. UPLC conditions were 10–70% MeOH in 0.1% of formic acid in H2O over 10 min on a BEH column, and the eluent was monitored at 340 nm.

To generate the digested and derivatized peptaiobals, approximately 0.2–0.3 mg of compounds 1–3 were weighed separately into 2 mL reaction vials, to which was added 0.5 mL of 6 N HCl. The compounds were hydrolyzed at 90 °C for 10 min on a BEH column, and the reaction mixtures were agitated at 40 °C for 1 h. The reactions were halted by the addition of 5 μL of 2 N HCl. The mixtures were dried under a stream of nitrogen and brought up in ~200 μL of MeOH and injected onto the UPLC using the same conditions as for the standards (Figures S7–S10).

### ASSOCIATED CONTENT

#### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnatprod.7b00189.

Molecular phylogenetic trees of T. arundinaceum and T. albolutescens; culture conditions of strains MSX70741 and MSX57715; experimental procedures; 1D and 2D NMR spectra and tabulated data of compounds 1–3; HRESIMS spectra of compounds 1–3; and Marfey's analysis of standards and compounds 1–5 (PDF)

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**Notes**

The authors declare no competing financial interest.

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