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

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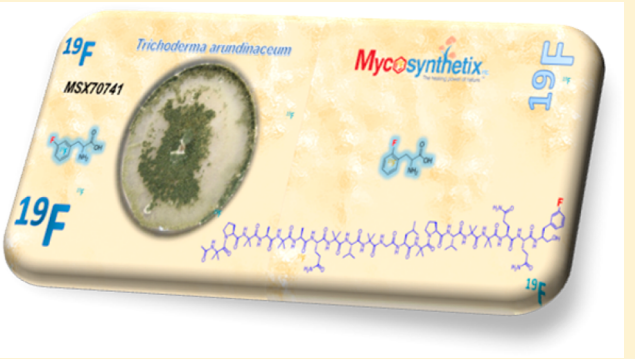
Supporting Information

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 ^{19}F) and spectrometric (HRESIMS/MS^n) 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).[1,2] Of these, about 4700 are halogenated,[3] and just 5 contain a fluorine atom,[4−7] 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.[8−11] Alternatively, drugs that include at least one fluorine atom (~274 up to 2009[12]) represent approximately 25−30% of pharmaceuticals,[13] including some of the top-selling drugs, such as sofosbuvir (Sovaldi; annual revenue of $9.4 billion in the U.S.), rosuvastatin (Crestor; annual revenue of $8.5 billion), and sitagliptin (Januvia; annual revenue of $5.0 billion).[13−15] However, these two classes of molecules do not often intersect, perhaps limiting the combination of privileged scaffolds in natural products[16−18] with the beneficial properties of fluorine atoms in medicinal chemistry.[13]

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,[19,20] such that, the benefits of incorporation in lead molecules or drugs could impact drastically on physicochemical properties, resulting in changes in absorption, distribution, metabolism, and molecular interactions *in vivo*[21] and *in vitro*.[22] 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.[13,23,24] 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.[25]

An alternative approach to modify natural product scaffolds is to employ precursor-directed biosynthesis,[26] using the biosynthetic machinery of microorganisms to incorporate fluorinated building blocks into natural products.[27−30] This technique has been widely used in the past, generating a vast number of microbial natural product analogues, with cyclo-

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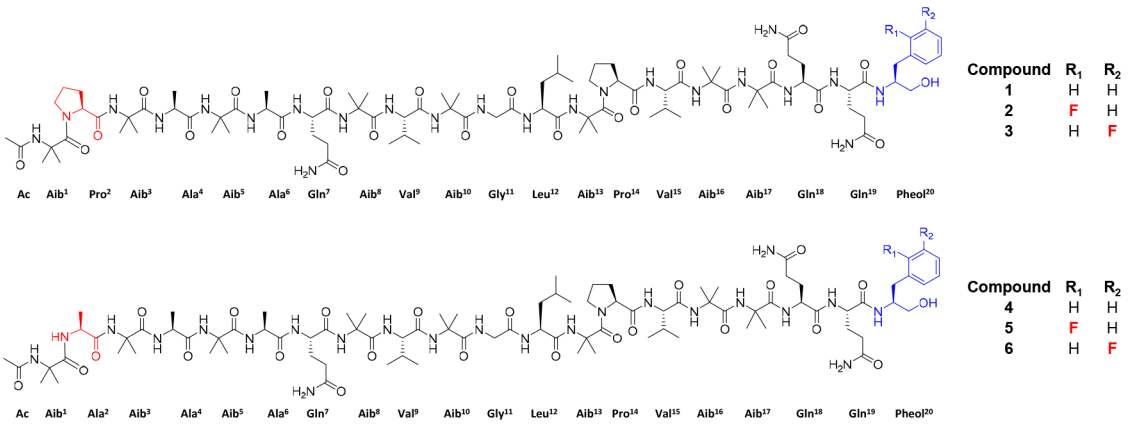

Figure 1. Structures of alamethicin F50 (**1**), *ortho*-F-Pheol alamethicin F50 (**2**), *meta*-F-Pheol alamethicin F50 (**3**), trichokonin VI (**4**), *ortho*-F-Pheol trichokonin VI (**5**), and *meta*-F-Pheol trichokonin VI (**6**). The amino acid residue targeted for modification is highlighted in blue, whereas the red residues highlight the difference between alamethicin F50 (**1**) and trichokonin VI (**4**).

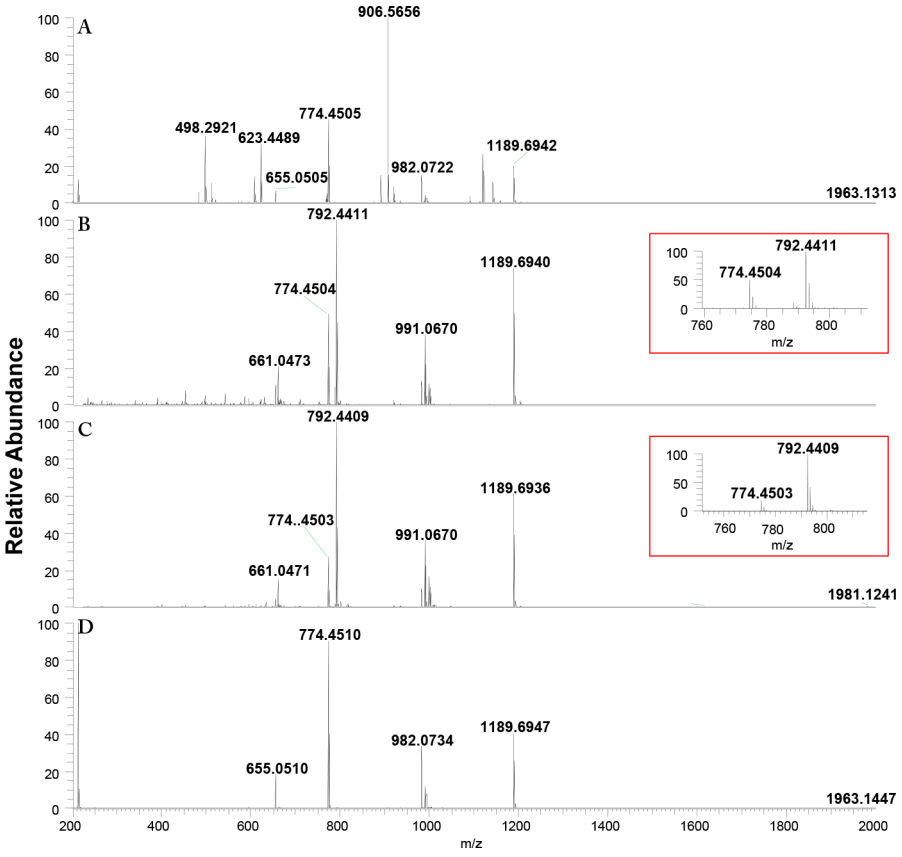

Figure 2. (A) Full-scan MS data of MSX70741 grown in PDA medium (control). (B) Full-scan MS data of MSX70741 grown in PDA supplemented with a racemic mixture of *ortho*-F-DL-Phe. (C) Full-scan MS data of MSX70741 grown in PDA supplemented with a racemic mixture of *meta*-F-DL-Phe. (D) Full-scan MS data of MSX70741 grown in PDA supplemented with a racemic mixture of *para*-F-DL-Phe. All cultures were sampled *in situ* using a droplet-liquid microjunction-surface sampling probe (droplet−LMJ−SSP) coupled to a hyphenated system (UPLC−PDA−HRMS−MS/MS). In panels B and C, the peaks corresponding to the fragment b_7^+ (*m*/*z* 792.4411 and 792.4409 for **2** and **3**, respectively), indicating the incorporation of fluorine (^{19}F), are boxed in red. Note, the peak at *m*/*z* 906.5656 in panel A is not associated with the targeted molecule.

sporins likely being one of the most explored examples.[26,31,32] 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.[25,28−30,33−35]

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,[8,36,37] 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

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Table 1. NMR Data for Alamethicin F50 (1), *ortho*-F-Pheol Alamethicin F50 (2), and *meta*-F-Pheol Alamethicin F50 (3)[a]

		2			3		
residue	position	δ_C	type	δ_H, m (*J* in Hz)	δ_C	type	δ_H, m (*J* in Hz)
Ac	1	172.5	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		175.5	C	
	2	57.4	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[2]	1	175.6	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*
				2.34, *m*			2.34, *m*
	4	27.1[b]	CH_2	1.97, *m*	27.1[b]	CH_2	1.97, *m*
				2.08, *m*			2.08, *m*
	5	49.9	CH_2	3.48, *td*, (10.5, 6.3)	49.9	CH_2	3.49, *td*, (10.5, 6.3,)
				3.95, *m*			3.95, *m*
Aib[3]	1	178.5	C		178.5	C	
	2	57.4	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		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[5]	1	177.8	C		177.8	C	
	2	57.3	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[6]	1	178.1	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		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*
				2.30, *m*			2.30, *m*
	4	32.6	CH_2	2.34, *m*	32.5	CH_2	2.34, *m*
				2.54, *ddd*, (15.4, 9.8, 5.6)			2.54, *ddd*, (15.4, 9.8, 6.3)
	5	177.3	C		177.1	C	
	NH			8.00, *d*, (5.6)			7.99, *d*, (4.9)
	5-NH_2			6.77, *brs*			6.77, *brs*
				7.44, *brs*			7.45, *brs*
Aib[8]	1	178.2	C		178.2	C	
	2	57.6	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[9]	1	175.3	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[10]	1	179.0	C		179.0	C	
	2	57.6	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*

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Table 1. continued

		2			3		
residue	position	δ_C	type	δ_H, m (J in Hz)	δ_C	type	δ_H, m (J in Hz)
Gly[11]	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)
				3.94, *m*			3.95, m
	NH			8.34, *brt*, (5.6)			8.34, *brt*, (5.7)
Leu[12]	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
				1.96, *m*			1.96, *m*
	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*, (8.4)			8.11, *d*, (7.8)
Aib[13]	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*			8.40, *s*
Pro[14]	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*
				2.35, *m*			2.35, *m*
	4	26.9[b]	CH_2	1.99, *m*	26.9[b]	CH_2	1.99, *m*
				2.08, *m*			2.08, *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[15]	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*, overlapped			7.63, *d* (8.0)
Aib[16]	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*			7.59, *s*
Aib[17]	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*			7.81, *s*
Gln[18]	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*
				7.44, *brs*			7.45, *brs*
Gln[19]	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*
				2.34, *m*			2.34, *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*
				7.35, *brs*			7.35, *brs*

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Table 1. continued

		2			3		
residue	position	δ_C	type	δ_H, m (J in Hz)	δ_C	type	δ_H, m (J in Hz)
Pheol[20]/F-Pheol[20]	1	65.1	CH_2	3.65, *brt*	64.9	CH_2	3.63, *brt*
	2	52.9	CH	4.25, *m*	54.0	CH	4.16, *m*
	3	31.1	CH_2	2.70, *dd*, (14.0, 9.1)	37.7	CH_2	2.72, *dd*, (14.2, 9.7,)
				3.07, *dd*, (14.0, 4.9)			2.98, *dd*, (13.9, 4.8)
	4	126.5, *d*, (15.4)	C		142.6, *d*, (7.4)	C	
	5	162.6, *d*, (242.7)	CF		117.1, *d*, (21.0)	CH	7.06, *brd*, (10.2)
	6	115.5, *d*, (22.1)	CH	6.99, *t*, (9.1)	160.0, *d*, (242.4)	CF	
	7	129.1, *d*, (8.4)	CH	7.18, *dd*, (7.1, 7.0)	113.8, *d*, (21.0)	CH	6.88, *td*, (8.5, 2.3)
	8	124.9, *d*, (3.7)	CH	7.04, *t*, (7.7)	130.7, *d*, (8.2)	CH	7.24, *t*, (7.6)
	9	132.9, *d*, (4.4)	CH	7.37, *t*, (7.7)	126.3, *d*, (2.6)	CH	7.10, *d*, (7.6)
	NH			7.32, *d*, (9.1)			7.22, *d*, overlapped
	1-OH			5.23, *t*, (6.6)			5.27, *t*, (6.6)
	F			−119.7[c], *m*			−115.8[c], *m*

[a]Data recorded in CD_3OH. ^1H (700 MHz), ^{13}C (175 MHz), and ^{19}F (470 MHz). [b]Signals may be exchangeable. [c]Recorded at 470 MHz.

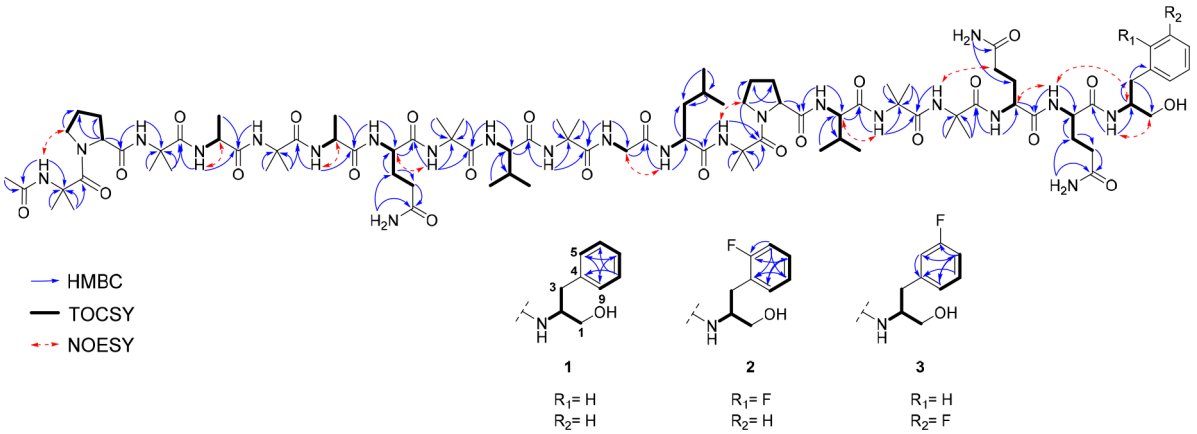

Figure 3. Key HMBC, TOCSY, and NOESY correlations for compounds **1**−**3**.

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-amino-isobutyric 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 Pheol[20] 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 modifiers and pore-forming antibiotics.[42−44] Therefore, in this communication we present the *in vivo* 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* 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.[45] 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 (b_{13}^+ fragment), 982.0722 ($[M + 2H]^{2+}$), 774.4505 (y_7^+ fragment), and 655.0505 ($[M + 3H]^{3+}$) (Figure 2).[38] 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 y_7^+= 792.4409), 9.00 amu ($[M + 2H]^{2+}$ = 991.0670), and 6.00 amu ($[M + 3H]^{3+}$ = 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-Pheol 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

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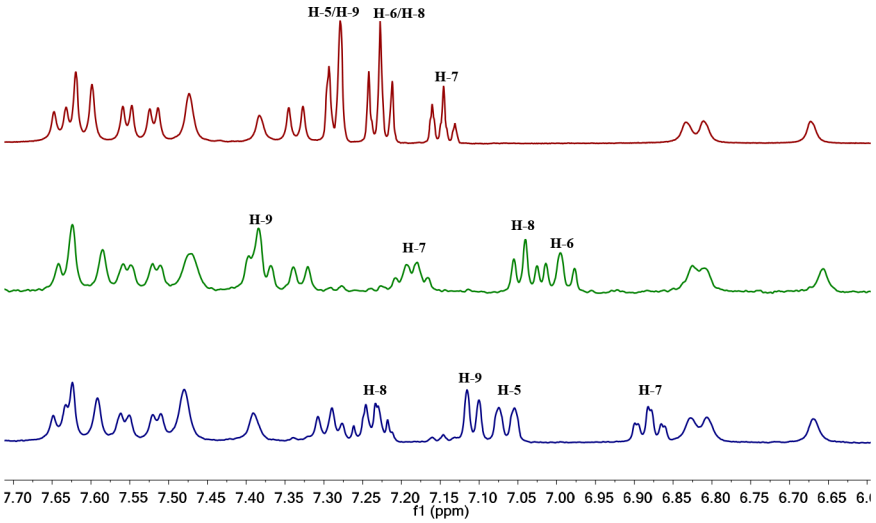

Figure 4. Comparison of the ^{1}H NMR spectra in the aromatic region (6.60−7.65 ppm) for alamethicin F50 (**1**; maroon), *ortho*-F-Pheol alamethicin F50 (**2**; green), and *meta*-F-Pheol alamethicin F50 (**3**; navy). All spectra were recorded in CD_3OH at 700 MHz. The spectra were identical from 0.75 to 6.50 ppm. For clarity, the signals belonging to the aromatic ring in each compound have been labeled and correspond to the data for the Pheol[20] residues shown in Tables S3, S4, and S5 for compounds **1**, **2**, and **3**, respectively.

(*ortho*/*meta*/*para*-F-DL-Phe), separately, following established procedures (Scheme S1).[49−52] HRESIMS analysis of the extracts obtained after 21 days of fermentation confirmed the presence of alamethicin F50 (**1**), as well as its *ortho* and *meta*-F-Pheol analogues (Figure S11), and support the hypothesis of the inability of the microorganism's biosynthetic machinery to incorporate fluorine by assimilation of *para*-F-Phe. Purification of the organic extract (1:1 MeCN-MeOH) of these cultures using a set of chromatographic procedures, and guided by MS analysis for the fluorinated signals, led to the isolation of two fluorinated peptaibols, namely, *ortho*-F-Pheol alamethicin F50 (**2**) and *meta*-F-Pheol alamethicin F50 (**3**), in ratios of 1:20 and 1:10 compared with the nonfluorinated parent (**1**).

Compounds **2** and **3** were isolated as white amorphous powders. Their molecular formulas were established as $C_{92}H_{150}FN_{23}O_{24}$ on the basis of the protonated molecule peaks $[M + H]^+$ at *m*/*z* 1981.1290 and 1981.1294 for **2** and **3**, respectively, in the HRESIMS spectrum (29 degrees of unsaturation). The incorporation of the fluorine atom into alamethicin F50 (**1**) was detected on the basis of the characteristic mass shifts of $[M + H]^+$, $[M + 2H]^{2+}$, $[M + 3H]^{3+}$, and y_7^+ ions (Figures S13, S21, and S31). The structures of **2** and **3** were confirmed by exhaustive interpretation of 1D and 2D NMR data, including ^{19}F-NMR spectra (Figures S30 and S40). The ^{1}H NMR (Table 1) spectra for **2** and **3** showed resonances for 18 NH groups (δ_H 6.50−8.70 ppm), three NH_2 (Gln^7, Gln^{18}, and Gln^{19}), four aromatic protons among δ_H 6.80−7.40 ppm, 13 αH ranging δ_H 3.30−4.50 ppm, one acetyl group at δ_H 2.05 ppm, and several signals in the shielded region (δ_H 0.70−2.50 ppm), including 16 methyl singlets (Aib) and eight methyl doublets, confirming **2** and **3** to be peptaibol derivatives.[38,53,54] Analysis of the 2D NMR data, in particular COSY, TOCSY, and HMBC experiments (Figure 3), permitted the assignment of the side chain for each amino acid (eight Aib, three Gln, two Ala, two Val, two Pro, one Leu, one Gly, and one F-substituted Pheol moiety). On the other hand, $^3J_{CH}$ and $^2J_{CH}$ HMBC correlations between NH protons and the αC and C═O signals, as well as the NOESY correlations among αH and NH protons of the neighboring amino acids, supported the structures of **2** and **3** as the *ortho*-F-Pheol and *meta*-F-Pheol analogues 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[20]) 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 (δ_C 115−163 ppm), instead of four singlet peaks observed for **1** (δ_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_{FC} values (Table 1). Importantly, the presence of a fluorine atom in **2** and **3** was verified by ^{19}F NMR spectroscopy with δ_F values of −119.7 and −115.8 ppm compared to δ_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−^{1}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_7^+ fragments, with the latter two generated from the cleavage between Aib^{13} and Pro^{14} (Figure S31).[38] 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 AcAib^1-Pro^2-Aib^3-Ala^4-Aib^5-Ala^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_7^+ permitted the assignment of the C-terminal fragment as Pro^{14}-Val^{15}-Aib^{16}-Aib^{17}-Gln^{18}-Gln^{19}-(*meta*-F)Pheol^{20}. Analogous mass spectrometric experiments were used to assign the amino acid sequence in **2** (Figures S21 and S22).

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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).[55] 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 beauvericin, pseurotin, 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 IC_{50} 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 IC_{50} 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.

Table 2. Bioactivity Data of Compounds 1−6

	IC_{50} (μM)[a]		
compd	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

[a]IC_{50} 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 H_2O; control), or autoclaved rice containing either: (1) 130 mg (powder) of *ortho*-F-DL-Phe; (2) 37.5 mg of *ortho*-F-DL-Phe in 5.0 mL of H_2O (final concentration: 1250 ppm); (3) 15 mg of *ortho*-F-DL-Phe in 2.0 mL of H_2O (final concentration: 500 ppm); or (4) 15 mg of *ortho*-F-L-Phe in 2.0 mL of H_2O (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 peptaibols 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 trichokonin VI (**4**).[38] 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 trichokonin VI (**4**−**6**, Figure 1). As observed with MSX70741, MSX57715 incorporated only the *ortho* and *meta*-F-L-Phe building blocks into trichokonin 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 IC_{50} 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,[60] such as legal access to biodiversity, identification of biological activity, and most recently, intellectual property associated with composition of matter patents,[60,61] which many would consider the most desirable of "Orange Book" patents.[62] 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,[8] may lead to the generation of valuable compounds.

In summary, we report the biosynthesis of fluorine containing analogues of the peptaibols alamethicin F50 (**2**,**3**) and trichokonin 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 peptaibol 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 CD_3OH with presaturation of the OH peak at δ_H 4.92 ppm (wet experiment). NMR instrumentation was a JEOL ECA-500 NMR spectrometer operating at 500 MHz for ^1H, 470 MHz for ^{19}F, and 125 MHz for ^{13}C, or an Agilent 700 MHz NMR spectrometer equipped with a cryoprobe, operating at 700 MHz for ^1H and 175 MHz for ^{13}C. All chemical shifts were referenced to the residual solvent peaks (δ_H 3.31 and δ_C 49.0). HRESIMS data were obtained using a Thermo QExactive Plus mass spectrometer (ThermoFisher

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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 Acquity ultraperformance liquid chromatography (UPLC) system (Waters Corp.) to a Thermo QExactive Plus via procedures described previously by Sica et al.[45] Briefly, extractions were performed using Fisher Optima LC/MS grade solvents consisting of 50:50 MeOH-H_2O. 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-H_2O (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 C_{12} column (21 × 250 mm) at a flow rate of 20.0 mL/min. Flash column chromatography was carried out with 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.[51,58,63,64] All solvents were obtained from Fisher Scientific and used without further purification. The *o*/*m*/*p*-F-DL-Phe, *o*/*m*-F-D-Phe, and *o*/*m*-F-L-Phe were purchased from Acros Organics. The standards of *o*/*m*-F-D-Pheol and *o*/*m*-F-L-Pheol were prepared as detailed in the Supporting Information.

Fungal Strain Isolation and Identification. Mycosynthetix 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.[38] Both strains were used previously for the isolation of peptaibols.[38] A description of the procedures used to identify these strains was outlined recently,[65] 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. albolutescens*. The sequence data for both strains were deposited in GenBank (accession numbers: ITS: KY630171, *tef1*: KY630169, KY630170, *RPB2*: KY630166 for strain MSX70741 and accession numbers: *tef1*: KY630167, KY630168, *RPB2*: KY630164, KY630165 for strain MSX57715).

Fermentation, Extraction, and Isolation. Fungal strains MSX70741 and MSX57715 were each grown on a malt extract agar, and subsequently, a small piece from the leading edge of the colony was transferred into YESD 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 prepared by adding a vitamin solution and twice the volume of rice with H_2O. These flasks were incubated at 22 °C until the culture showed good growth. In the case of media supplemented with a racemic mixture of *ortho*-F-DL-Phe (E2), *meta*-F-DL-Phe (E3), or *para*-F-DL-Phe (E4), 100 mg of the amino acid were added to the culture after a week of growth (Scheme S1).

To each solid fermentation culture of MSX70741 (EC, E2-E4; see Scheme S1), 60 mL of 1:1 MeOH−$CHCl_3$ were added, and the resulting slurry was shaken for 16 h on an orbital shaker. These mixtures were filtered under vacuum. To each filtrate 90 mL of $CHCl_3$ and 150 mL of H_2O were added, and the mixtures were stirred for 30 min and then transferred into a separatory funnel. The organic layer was drawn off and dried *in vacuo*. This dried organic extract was defatted by reconstituting in a mixture of 100 mL of 1:1 MeOH−MeCN and 100 mL of hexane, and then partitioned in a separatory funnel. The MeOH−MeCN layer was collected and concentrated *in vacuo*. The resulting MeOH−MeCN extracts were then adsorbed on Celite 545 (Acros Organics) and fractionated via flash chromatography on a 4 g RediSep Rf Gold Si-gel column using a gradient solvent system of hexane-$CHCl_3$- MeOH at a flow rate of 18 mL/min over 90 column volumes (CV) for a duration of 24.0 min. Fractions were collected every 9.0 mL and pooled according to the UV and ELSD profiles, which resulted in four combined fractions in total (F_I-F_{IV}). MS-directed resolution of fraction F_{IV} from E2 and E3 (eluted with 100% MeOH, 92.4 and 136.0 mg, respectively) via reversed-phase HPLC (Synergi column), using a linear gradient from 40% to 100% MeCN in H_2O (0.1% formic acid) at a flow rate of 20.0 mL/min over 30 min afforded seven subfractions ($F_{IV\text{-}1}$−$F_{IV\text{-}7}$). Fractions $F_{IV\text{-}1}$ (t_R 18.5−19.5 min) from E2 and E3 were further characterized as alamethicin F50 (**1**, 28.5 and 42.9 mg, respectively). Purification of fractions $F_{IV\text{-}2}$ from E2 and E3 by MS-directed semipreparative HPLC using the above-mentioned conditions at 4.60 mL/min led to the isolation of compounds **2** (1.4 mg) and **3** (4.5 mg). Analogous procedures were used to isolate compounds **4** (24.0 mg), **5** (3.4 mg), and **6** (6.2 mg).

*Alamethicin F50 (**1**).* White powder; $[\alpha]_D^{26}$ −6.5 (*c* 0.2,MeOH); UV (MeOH) λ_{max} (log ε) 204 (4.52) nm; ^{1}H NMR (CD_3OH, 700 MHz) and ^{13}C NMR (CD_3OH, 175 MHz), see Table S3; HRESIMS *m*/*z* 1963.1388 $[M + H]^+$ (calcd for $C_{92}H_{152}N_{23}O_{24}$, *m*/*z* 1963.1375).

*o-F-Pheol-Alamethicin F50 (**2**).* White powder; $[\alpha]_D^{26}$ −7.0 (*c* 0.2, MeOH); UV (MeOH) λ_{max} (log ε) 204 (4.54) nm; ^{1}H NMR (CD_3OH, 700 MHz) and ^{13}C NMR (CD_3OH, 175 MHz), see Table 1; HRESIMS *m*/*z* 1981.1290 $[M + H]^+$ (calcd for $C_{92}H_{151}FN_{23}O_{24}$, 1981.1280).

*m-F-Pheol-Alamethicin F50 (**3**).* White powder; $[\alpha]_D^{27}$ −1.0 (*c* 0.03, MeOH); UV (MeOH) λ_{max} (log ε) 204 (4.52) nm; ^{1}H NMR (CD_3OH, 700 MHz) and ^{13}C NMR (CD_3OH, 175 MHz), see Table 1; HRESIMS *m*/*z* 1981.1294 $[M + H]^+$ (calcd for $C_{92}H_{151}FN_{23}O_{24}$, 1981.1280).

*Trichokonin VI (**4**).* White powder; $[\alpha]_D^{24}$ −10.0 (*c* 0.2, MeOH); UV (MeOH) λ_{max} (log ε) 204 (4.57) nm; HRESIMS *m*/*z* 1937.1232 $[M + H]^+$ (calcd for $C_{90}H_{150}N_{23}O_{24}$, 1937.1218).

*o-F-Pheol-Trichokonin VI (**5**).* White powder; $[\alpha]_D^{25}$ −8.0 (*c* 0.3, MeOH) ; UV (MeOH) λ_{max} (log ε) 204 (4.54) nm; HRESIMS *m*/*z* 1955.1147 $[M + H]^+$ (calcd for $C_{90}H_{149}FN_{23}O_{24}$, 1955.1124).

*m-F-Pheol-Trichokonin VI (**6**).* White powder; $[\alpha]_D^{25}$ −5.0 (*c* 0.2, MeOH) ; UV (MeOH) λ_{max} (log ε) 204 (4.48) nm; HRESIMS *m*/*z* 1955.1149 $[M + H]^+$ (calcd for $C_{90}H_{149}FN_{23}O_{24}$, 1955.1124).

Cytotoxicity Assay. Human melanoma cancer cells MDA-MB-435, human breast cancer cells MDA-MB-231, and human ovarian cancer cells OVCAR3 were purchased from the American Type Culture Collection (Manassas, VA, U.S.A.). The cell lines were propagated at 37 °C in 5% CO_2 in RPMI 1640 medium, supplemented with 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% CO_2). 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 AQ_{ueous} One Solution Cell Proliferation Assay, Promega Corp, Madison) that measured viable cells. IC_{50} 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 YESD followed by incubation 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 H_2O. These flasks were supplemented with 130 mg of *ortho*-F-DL-Phe powder (Condition 1), 2.0 mL of a stock solution 7500 ppm of *ortho*-

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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 H_2O, 20 μL of 1 M $NaHCO_3$, and 100 μL 1% Marfey's reagent (*Nα*-(2,4-dinitro-5-fluorophenyl)-L-alaninamide) 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. Also, aliquots of all of 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 H_2O over 10 min on a BEH column, and the eluent was monitored at 340 nm.

To generate the digested and derivatized peptaibols, 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 24 h, at which time they were evaporated under a stream of nitrogen. To each hydrolysis product was then added 25 μL of H_2O, 10 μL of 1 M $NaHCO_3$, and 50 μL of 1% Marfey's reagent in acetone. 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**−**3** (PDF)

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Notes

The authors declare no competing financial interest.

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