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Bioactive fluorinated derivative of amphotericin B

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Abstract—The first stably fluorinated derivative of amphotericin B (**2**) with a fluorine atom in the macrolide skeleton was synthesized using an electrophilic fluorination reagent, Selectfluor®. The derivative **2** showed hemolytic, K^+ permeable, and antifungal activities similar to those of AmB and thus was expected to be a powerful tool for NMR-based investigations on the mechanism of ion-channel formation by amphotericin B.

The incorporation of fluorine into a drug modulates its electronic, lipophilic, and steric parameters, which may critically influence the pharmacodynamic and pharmacokinetic properties. Therefore, derivatization to fluorinated analogues from current pharmaceuticals represents a potential avenue for the design of more potent drugs.[1] From the viewpoint of NMR studies for biological systems, the advantage of using a fluorine nucleus is evident; its relatively small size between hydrogen and oxygen, a nuclear spin of 1/2 with 100% natural abundance, high NMR sensitivity, and low background signals in cells make it an attractive nucleus for biological NMR studies.[2–4]

Polyene antibiotics, amphotericin B (AmB, **1**), is a standard drug for the treatment of deep-seated systemic fungal infections,[5] despite its severe side effects, including nephrotoxicity. Thus, design of less toxic derivatives of AmB has medical implications and fluorination is an option for better derivatives. We have been investigating the drug's mode of action in lipid bilayer membranes, especially the mechanism of ion-channel formation by AmB and sterol molecules, and the molecular recognitions for AmB–AmB and AmB–sterol.[6] In these experiments, ^{19}F-labeled compounds are also expected to be a versatile tool for examining the intermolecular interactions by NMR. Maclachlan reported the preparation of a fluorinated analogue of AmB in which 13-OH was substituted with fluorine.[7] The analogue, however, was easily hydrolyzed to AmB in aqueous conditions. In this paper, therefore, we report the preparation of the first stably fluorinated derivatives of AmB **2**, which is suitable for NMR measurements of an ion-channel complex in membranes. We also present its biological and ion-channel activities.

As illustrated in Scheme 1, treatment of Fmoc–AmB (**4**) with TMSOTf, followed by HF-pyridine, gave 13,14-anhydro derivative **5**, which was then reacted with an electrophilic fluorination reagent, Selectfluor®,[8] to afford **6**. Removal of the Fmoc group and purification with reverse-phase HPLC gave the objective compound **2**, which was found to be pure enough for further experiments based on ^{1}H NMR spectra.[9] The stereochemistry of C-14 was determined to be *S* on the basis of coupling constant data (Fig. 1): large coupling constants (9–10 Hz) for H14–H15, H15–H16, and H16–H17 indicate the chair conformation of the C13–C17 pyran ring with all protons in the axial position, consequently 14-F being equatorially oriented. It means that treatment of the glycal **5** with Selectfluor® almost exclusively gave an equatorially fluorinated product. We also prepared a readily available derivative **3** by reductive amination of AmB with *p*-fluorobenzaldehyde (Scheme 1) as a ^{19}F-labeled compound at the amino group for the use of NMR studies.[10]

We then evaluated the biological activity of these derivatives (Table 1). Compound **2** induced hemolysis against 1% human erythrocytes at concentrations comparable to those of AmB. The derivative also did show significant antifungal action against *Aspergillus nigar*. Despite the potent hemolytic activity comparable to that of AmB, **3** was virtually devoid of antifungal activity.

Keywords: Amphotericin B; Selectfluor; Fluorination; Ion channel.
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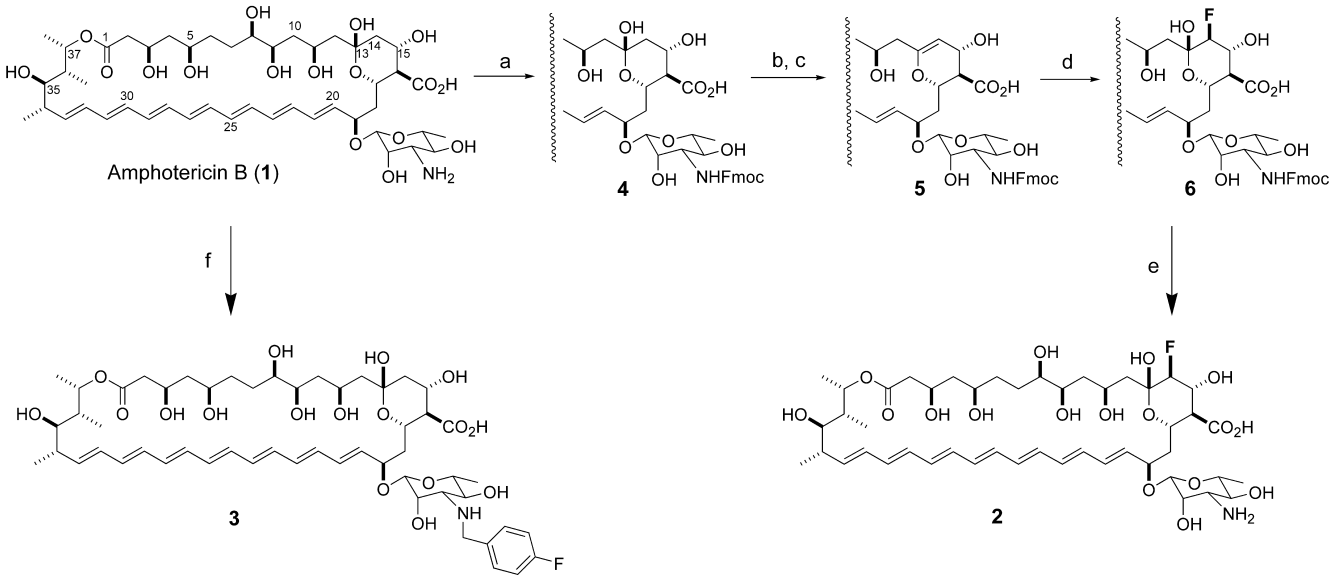

Scheme 1. Reagents and conditions: (a) FmocOSu, DMF, pyridine, rt, 18 h, quant; (b) TMSOTf, 2,6-lutidine, CH_2Cl_2, rt, 40 min; (c) HF-pyridine, THF, rt, 4 h, 50% (two steps); (d) Selectfluor, DMF-H_2O (3:1), rt, 1 h, 30%; (e) piperidine, DMSO–MeOH (4:1), rt, 1 h, 50% (isolation yield); (f) *p*-fluorobenzaldehyde, $NaBH_3CN$, DMF–MeOH (10:7), rt, 2 days, 32%.

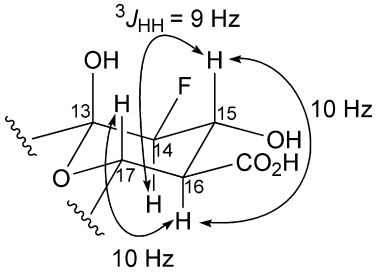

Figure 1. Stereochemistry at C14 of **2**. The equatorial orientation of 14-F was derived from axial orientations of H14–H17 that were determined by large $^3J_{HH}$ values (9–10 Hz).

Table 1. Hemolytic and antifungal assay results for AmB (**1**), **2**, and **3**

Compound	Hemolytic activity EC_{50} (μM)[a]	Antifungal activity (μg)[b]
1 (AmB)	1.4	10
2	1.3	10
3	2.6	>50

[a] Against 1% human erythrocytes.
[b] The minimal amount of samples on a paper disk that shows inhibitory zone on the culture of *Aspergillus niger*.

Then, we assessed the K^+- permeabilizing activity of the derivatives using artificial liposomes containing of egg phosphatidylcholine.[6b,d–f] The liposomes possess a higher external K^+ concentration and trans-membrane pH gradient, pH 5.5 inside and pH 7.5 outside. Once K^+ influx into the liposomes takes place through ion channels formed across the membrane, H^+ leaks out of the liposome in the presence of proton-transporter FCCP, leading to a pH increase in liposome lumen. This pH change was monitored by a chemical shift of phosphate anions in ^{31}P NMR; a downfield signal of phosphate ions at δ 3.1 was ascribed to permeabilized liposomes, while an up-field signal at δ 1.2 was due to intact liposomes. Signals of phosphate ions outside the liposomes were quenched by paramagnetic Mn^{2+}. Accordingly, the channel activities of drugs reduce the ^{31}P NMR peak at δ 1.2 and increase that at δ 3.1.

Figure 2 shows the results of the K^+ flux assays. As evident from the figure, derivatives **2** and **3** elicited an all-or-none type K^+-flux in ergosterol-containing liposomes, where two peaks were clearly seen at δ 1.2 and 3.1 resulting from intact and permeabilized liposomes, respectively. The all-or-none response reflects an instant pH increase in liposomes, thus implying the formation of ion channels with high conductance. Meanwhile, a graded behavior of ion flux, as observed for both

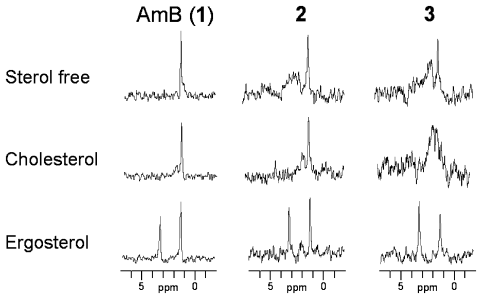

Figure 2. Membrane-permeabilizing activity of AmB (**1**) and fluorinated derivatives (**2** and **3**) using ^{31}P NMR spectra of liposome-entrapped phosphate. The drug was added to liposomes and incubated for 6 h. The lipid concentration of all the liposome suspensions was 12 mM. Liposomes were composed of egg-phosphatidylcholine (PC), 10% cholesterol-, or 10% ergosterol-containing PC. The peak around δ 1.2 corresponds to $H_2PO_4^-$ at pH 5.5 (intact liposomes) and that around δ 3.1 corresponds to HPO_4^{2-} at pH 7.5 (permeabilized liposomes). Signals between δ 1.2 and 3.1 are derived from liposomes with inside pH between 5.5 and 7.5. A molar ratio of drug/lipid was 1×10^{-4} for all the experiments.

compounds in cholesterol-containing and sterol-free membranes, is due to a gradual pH increase in liposomes and therefore indicates the formation of less conductive ion channels. Note that such spectral features of the derivatives agree well with those of AmB (Fig. 2), showing that **2** and **3** reproduce the ion-channel properties of AmB, particularly for ergosterol selectivity.

In conclusion, we synthesized the first stable AmB analogue with a fluorine atom in the backbone structure for use in solid-state NMR measurements, such as C-F REDOR.[11] We have just commenced NMR measurements using derivatives **2** and **3**, both of which showed functional similarity of ion-channels to those of AmB. Preliminary results obtained for **3** and ^{13}C-labeled AmB indicate any possible AmB–AmB intermolecular interactions, as suggested in the barrel-stave model[12] (details will be published elsewhere). These fluorinated derivatives should, therefore, serve as powerful tools in future NMR studies on ion-channel structure formed in membranes by AmB and lipid molecules.

Acknowledgments

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9. Compound **2**: ^{1}H NMR (500 MHz, DMSO-d_6) δ 4.24 (1H, m, H17), 3.99 (1H, m, H15), 3.78 (1H, dd, J = 51.5, 9 Hz, H14), 2.00 (1H, t, J = 10.0, H16), All other ^{1}H signals are identical to those of AmB;[13] ^{19}F NMR (470.40 MHz, DMSO-d_6) δ −199.86 (brd, J = 51.5 Hz); ESI MS calcd for $C_{47}H_{72}O_{17}NFNa$ ($M+Na^+$) 964.47. Found: 964.6.
10. Compound **3**: ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.42 (2H, dd, J = 5.0, 8.8 Hz, Ph), 7.11 (2H, dd, J = 8.0, 9.0 Hz, Ph), 4.32(1H, s, H1′), 3.81, 3.61 (2H, d, J = 13.2 Hz, Ph–CH_2), 3.73 (1H, d, J = 3.0 Hz, H2′), 3.10 (1H, m, H5′), 2.99 (1H, m, H4′), 2.36 (1H, dd, J = 3.0, 9.8 Hz, H3′). All other ^{1}H signals are identical with those of AmB;[13] ^{19}F NMR (470.40 MHz, DMSO-d_6) δ −117.8 (m); ESI MS calcd for $C_{54}H_{78}O_{17}NFNa$ ($M+Na^+$) 1054.52. Found: 1054.5.
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