Optically Active Antifungal Azoles. XII.[1] Synthesis and Antifungal Activity of the Water-Soluble Prodrugs of 1-[(1*R*,2*R*)-2-(2,4-Difluorophenyl)-2-hydroxy-1-methyl-3-(1*H*-1,2,4-triazol-1-yl)propyl]-3-[4-(1*H*-1-tetrazolyl)phenyl]-2-imidazolidinone

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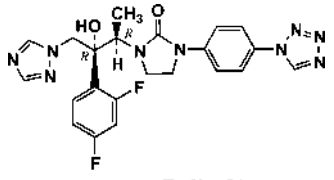
1-[(1*R*,2*R*)-2-(2,4-Difluorophenyl)-2-hydroxy-1-methyl-3-(1*H*-1,2,4-triazol-1-yl)propyl]-3-[4-(1*H*-1-tetrazolyl)phenyl]-2-imidazolidinone (1: TAK-456) was selected as a candidate for clinical trials, but since its water-solubility was insufficient for an injectable formulation, the quaternary triazolium salts 2 were designed as water-soluble prodrugs. Among the prodrugs prepared, 4-acetoxymethyl-1-[(2*R*,3*R*)-2-(2,4-difluorophenyl)-2-hydroxy-3-[2-oxo-3-[4-(1*H*-1-terazolyl)phenyl]-1-imidazolidinyl]butyl]-1*H*-1,2,4-triazolium chloride (2a: TAK-457) was selected as an injectable candidate for clinical trials based on the results of evaluations on solubility, stability, hemolytic effect and *in vivo* antifungal activities.

Key words water-soluble prodrug; triazolium salt; antifungal azole; TAK-457; TAK-456; 1,2,3-trisubstituted-2-butanol

In the course of our study on optically active azolone-based antifungal azoles, we selected 1-[(1*R*,2*R*)-2-(2,4-difluorophenyl)-2-hydroxy-1-methyl-3-(1*H*-1,2,4-triazol-1-yl)propyl]-3-[4-(1*H*-1-tetrazolyl)phenyl]-2-imidazolidinone (**1**: TAK-456) as a candidate for clinical trials (Chart 1). In previous reports, we described the stereocontrolled synthesis of TAK-456[1] and the antifungal activities *in vitro* and *in vivo*.[2] TAK-456 exhibited strong growth inhibitory activity against not only yeasts such as *Candida albicans* (*C. albicans*) and *Cryptococcus neoformans* but also against molds such as *Aspergillus fumigatus* (*A. fumigatus*), as well as highly potent protective effects against candidiasis and aspergillosis in mice *via* oral administration.[3]

Invasive aspergillosis still remains resistant to antifungal chemotherapy, although injectable amphotericin B has been used for the treatment of this disease in spite of the severe toxicity.[4] It is, therefore, considered that an injectable formulation of TAK-456 would have potential in the clinical treatment of this serious disease. We attempted to prepare an injectable solution of TAK-456 with sufficient concentration for experimental and clinical uses, but several trials by addition of pharmacologically acceptable cosolvents and/or excipients were unsuccessful because of the poor water-solubility (0.005 mg/ml) of TAK-456. Then, we turned our efforts to preparing a water-soluble prodrug of TAK-456. Bodor *et al.* has described the general concept of "soft drugs" or "prodrugs", for modifying the physicochemical properties, including water-solubility and pharmacokinetic profiles, of drugs containing a tertiary nitrogen atom by quarternizing them to the corresponding ammonium salts with a enzymatically and/or chemically labile group.[5] Thus, we designed the quaternary triazolium salts depicted by the general formula **2**. The prodrug strategy is represented by the scheme shown in Chart 2. Compounds **2**, which could be prepared by alkylation of TAK-456, might have enhanced water-solubility owing to their charged character and could be expected to be hydrolyzed enzymatically and/or non-enzymatically *in vivo* to generate the parent compound TAK-456. It was also expected that the water-solubility and the liability to hydrolysis would depend on the physicochemical properties of the substituent R. To search for a prodrug suitable for injectable formulation, we planned to prepare a series of compounds **2** with different physicochemical properties. We chose alkyl, alkoxy and substituted alkoxy groups (see Chart 2) for the substituent R and prepared the triazolium salts **2** (Table 1). In this paper, we describe the synthesis of **2** as well as the results of evaluations on their water-solubility, stability, hemolytic effect and antifungal activities.

Synthesis The water-soluble prodrugs (**2a**—**g**, **i**: Table 1) were prepared by alkylation of the parent compound **1** with halomethyl esters or halomethyl carbonates (**4**—**6**) in an aprotic polar solvent such as acetonitrile (MeCN) or acetone within a range of temperatures between 50 and 100 °C as shown in Chart 3. In the case of reaction of **1** with **5** or **6**, the resulting triazolium bromides or iodides were converted to the corresponding chlorides (**2a**, **d**, **e**, **g**, **i**) by anion-exchange with Dowex 1×8 Cl^- or mixing with aqueous saturated sodium chloride (NaCl) solution (brine). The hydroxypropoxy derivative **2h** was prepared from the benzyloxypropoxy derivative **2j** by catalytic hydrogenolysis with palladium on carbon (Pd–C). The compounds **2a**—**h**, except **2i** (amorphous), were obtained as crystals after purification



1 (TAK-456)

Chart 1

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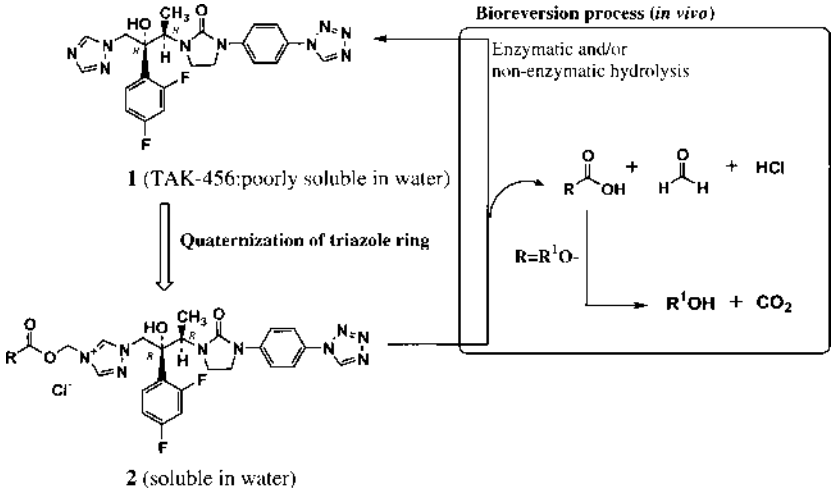


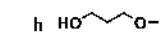
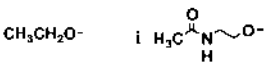
R	
a CH_3-	f $(CH_3)_2CHO$-
b $(CH_3)_2CH$-	g 
c $(CH_3)_3C$-	h 
d CH_3CH_2O-	i 
e $CH_3(CH_2)_2O$-	

Chart 2

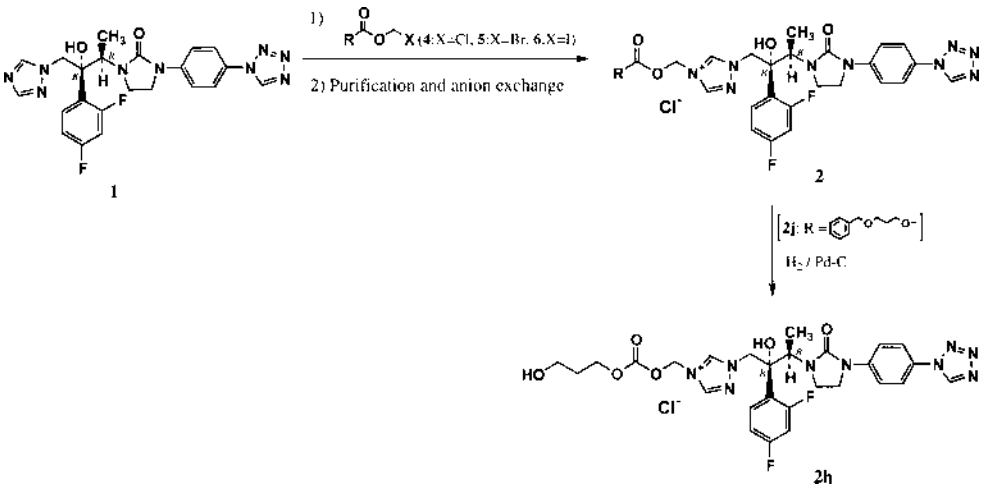


Chart 3

by chromatography [on silica gel or octadecyl silica (ODS)] and/or by crystallization from the following solvents; ethanol (EtOH), water, saline, ethyl acetate (AcOEt) or acetone. Among the crystalline quaternary triazolium salts, the acetoxymethyl derivative **2a** was obtained in two crystalline forms, *i.e.*, the unhydrated form crystallized from EtOH–acetone and the hydrated form crystallized from water.

The position of alkylation on the 1,2,4-triazole was assumed to be at the 4-nitrogen atom since Duffin *et al.* reported that quanternization of 1-substituted 1,2,4-triazoles generally occurs at the 4-position to give the corresponding 1,4-disubstituted triazolium salts.[6] The assignment of the 1,4-disubstituted triazolium structure was carried out using **2a** by NMR measurement, *i.e.*, the nuclear Overhauser and exchange spectroscopy (NOESY) and ^{1}H-detected multiple-bond heteronuclear multiple quantum coherrence (HMBC).[7] Thus, in NOESY of **2a**, a nuclear Overhauser effect (NOE) between the methylene protons of the acetoxymethyl group and the 3- and 5-protons on the triazole ring was observed. On the other hand, HMBC of **2a** showed C–H long range coupling between the methylene protons of the acetoxymethyl group and the 3- and 5-carbons on the triazole.

Water-Solubility and Stability The prodrugs for injectable use should have sufficient solubility and adequate stability in water to allow production of the injectable formulation. Therefore, the triazolium salts **2** were assessed for

Table 1. Physicochemical Properties of Prodrugs (**2**)

Compd.	Yield[a] (%)	mp (°C) (Solv.)[b]	Analysis (%) Calcd (Found) C	H	N	^{1}H-NMR (in DMSO-d_6) δ	IR (KBr) cm^{-1}	$[\alpha]_D$ {°C} (*c*) MeOH
2a	51[f,k,i]	204 (dec.) [ET–AC]	$C_{25}H_{26}ClF_2N_9O_4$ 50.89 (50.61)	4.44 (4.38)	21.37 (21.24) Cl: 6.01 (5.80)	0.98 (3H, d, *J*=7 Hz), 2.08 (3H, s), 3.60—3.66 (1H, m), 3.95—4.08 (3H, m), 4.70 (1H, q, *J*=7 Hz), 4.86 (1H, d, *J*=14.2 Hz), 5.07 (1H, d, *J*=14.2 Hz), 6.10 (1H, d, *J*=10.6 Hz), 6.19 (1H, d, *J*=10.6 Hz), 6.60 (1H, s), 6.96—7.06 (1H, m), 7.24—7.36 (2H, m), 7.90 (4H, s), 9.07 (1H, s), 10.06 (1H, s), 10.40 (1H, s)	3217, 2995, 1760, 1706, 1527, 1421, 1261	−39.7° {20} (0.52)
		195—196 (dec.) [W]	$C_{25}H_{26}ClF_2N_9O_4 \cdot H_2O$ 49.39 (49.56)	4.64 (4.64)	20.73 (20.85)	0.98 (3H, d, *J*=7 Hz), 2.08 (3H, s), 3.60—3.72 (1H, m), 3.96—4.10 (3H, m), 4.70 (1H, q, *J*=7 Hz), 4.87 (1H, d, *J*=14.4 Hz), 5.13 (1H, d, *J*=14.4 Hz), 6.11 (1H, d, *J*=10.8 Hz), 6.18 (1H, d, *J*=10.8 Hz), 6.70 (1H, s), 6.94—7.03 (1H, m), 7.21—7.38 (2H, m), 7.89 (4H, s), 9.06 (1H, s), 10.06 (1H, s), 10.50 (1H, s)	3226, 3120, 1754, 1706, 1525, 1415, 1261, 1230	−39.6° {20} (0.54)
2b	24[e,g]	217—219 (dec) [Saline]	$C_{27}H_{30}ClF_2N_9O_4 \cdot 1/2H_2O$ 51.72 (51.79)	4.98 (4.83)	20.10 (20.04)	0.97 (3H, d, *J*=7 Hz), 1.07 (6H, d, *J*=7 Hz), 2.59 (1H, quintet, *J*=7 Hz), 3.61—4.08 (4H, m), 4.65—4.75 (1H, m), 4.87 (1H, d, *J*=14 Hz), 5.11 (1H, d, *J*=14 Hz), 6.14—6.22 (2H, m), 6.69 (1H, s), 6.92—7.03 (1H, m), 7.22—7.37 (2H, m), 7.90 (4H, s), 9.09 (1H, s), 10.08 (1H, s), 10.48 (1H, s)	1753, 1694, 1524, 1501, 1481, 1422, 1269	—[c]
2c	48[f,h]	196—197 (dec.) [EA]	$C_{28}H_{32}ClF_2N_9O_4$ 53.21 (53.17)	5.10 (5.15)	19.94 (19.76)	0.98 (3H, d, *J*=7 Hz), 1.13 (9H, s), 3.61—3.66 (1H, m), 3.96—4.10 (3H, m), 4.71 (1H, q, *J*=7 Hz), 4.89 (1H, d, *J*=14.4 Hz), 5.15 (1H, d, *J*=14.4 Hz), 6.17 (2H, s), 6.75 (1H, s), 6.90—7.00 (1H, m), 7.23—7.34 (2H, m), 7.89 (4H, s), 9.10 (1H, s), 10.06 (1H, s), 10.55 (1H, s)	1707, 1522, 3181, 1736, 1481, 1420, 1262, 1167	−32.7° {20} (1.0)
2d	42[f,h,g,j,i]	203—206 (dec) [ET]	$C_{26}H_{28}ClF_2N_9O_5$ 50.37 (50.07)	4.55 (4.63)	20.33 (20.10)	0.97 (3H, d, *J*=7 Hz), 1.22 (3H, t, *J*=7 Hz), 3.60—4.08 (4H, m), 4.18 (2H, q, *J*=7 Hz), 4.63—4.73 (1H, m), 4.87 (1H, d, *J*=14 Hz), 5.10 (1H, d, *J*=14 Hz), 6.13 (1H, d, *J*=11 Hz), 6.21 (1H, d, *J*=11 Hz), 6.66 (1H, s), 6.96—7.04 (1H, m), 7.24—7.36 (2H, m), 7.90 (4H, s), 9.09 (1H, s), 10.07 (1H, s), 10.48 (1H, s)	3218,1752, 1709,1526, 1481, 1424, 1262	−38.7° {20} (0.55)
2e	22[f,h,g,k,i]	202—203 (dec.) [ET–AC]	$C_{27}H_{30}ClF_2N_9O_5 \cdot 1/2H_2O$ 50.43 (50.25)	4.86 (4.71)	19.60 (19.31) Cl: 5.51 (5.42)	0.88 (3H, t, *J*=7.2 Hz), 0.98 (3H, d, *J*=7 Hz), 1.62 (2H, tq, *J*=7.2 Hz), 3.62—3.67 (1H, m), 3.90—4.10 (3H, m), 4.09 (2H, t, *J*=7.2 Hz), 4.69 (1H, q, *J*=7 Hz), 4.87 (1H, d, *J*=14.2 Hz), 5.05 (1H, d, *J*=14.2 Hz), 6.16 (1H, d, *J*=11 Hz), 6.20 (1H, d, *J*=11 Hz), 6.53 (1H, s), 6.94—7.04 (1H, m), 7.23—7.35 (2H, m), 7.89 (4H, s), 9.09 (1H, s), 10.05 (1H, s), 10.38 (1H, s)	3221, 3056, 1752, 1709, 1524, 1422, 1264	−36.7° {20} (1.0)
2f	17[f,h,i]	220—222 (dec.) [ET–AC]	$C_{27}H_{30}ClF_2N_9O_5$ 51.15 (50.79)	4.77 (4.60)	19.88 (19.62)	0.98 (3H, d, *J*=7.4 Hz), 1.24 (6H, d, *J*=6.2 Hz), 3.62—3.66 (1H, m), 3.98—4.00 (3H, m), 4.69 (1H, q, *J*=7.4 Hz), 4.80 (1H, quintet, *J*=6.2 Hz), 4.87 (1H, d, *J*=14.4 Hz), 5.04 (1H, d, *J*=14.4 Hz), 6.11 (1H, d, *J*=11 Hz), 6.19 (1H, d, *J*=11 Hz), 6.51 (1H, s), 6.96—7.03 (1H, m), 7.22—7.38 (2H, m), 7.90 (4H, s), 9.10 (1H, s), 10.06 (1H, s), 10.36 (1H, s)	3181, 1746, 1709, 1524, 1426, 1267, 1098	−37.2° {20} (1.0)
2g	27[f,h,j]	218—220 (dec.) [ET]	$C_{28}H_{30}ClF_2N_9O_7$ 49.60 (49.60)	4.46 (4.46)	18.59 (18.40)	0.98 (3H, d, *J*=7 Hz), 3.61—3.66 (1H, m), 3.87—4.02 (7H, m), 4.52—4.60 (1H, m), 4.68—4.89 (3H, m), 4.89 (1H, d, *J*=13.8 Hz), 5.14 (1H, d, *J*=13.8 Hz), 6.18 (1H, d, *J*=11 Hz), 6.26 (1H, d, *J*=11 Hz), 6.70 (1H, s), 6.92—7.02 (1H, m), 7.22—7.38 (2H, m), 7.89 (4H, s), 9.11 (1H, s), 10.01 (1H, s), 10.56 (1H, s)	3252, 1765, 1703, 1524, 1422, 1260	−31.8° {20} (1.0)
2h	33[g,i]	181—185 (dec.) [ET]	$C_{27}H_{30}ClF_2N_9O_6$ 49.89 (49.48)	4.65 (4.75)	19.39 (19.18)	0.98 (3H, d, *J*=7 Hz), 1.74 (2H, quintet, *J*=6 Hz), 3.44 (2H, dt, *J*=6, 5 Hz), 3.60—4.10 (4H, m), 4.20 (2H, t, *J*=6 Hz), 4.59 (1H, t, *J*=5 Hz), 4.65—4.75 (1H, m), 4.87 (1H, d, *J*=14 Hz), 5.10 (1H, d, *J*=14 Hz), 6.13 (1H, d, *J*=11 Hz), 6.21 (1H, d, *J*=11 Hz), 6.63 (1H, s), 6.95—7.05 (1H, m), 7.25—7.37 (2H, m), 7.89 (4H, s), 9.09 (1H, s), 10.06 (1H, s), 10.48 (1H, s)	3216, 3127, 1755, 1705, 1524, 1481, 1424, 1265	−36.7° {20} (1.1)
2i	36[f,h,g,j]	A.P.[d]	$C_{28}H_{31}ClF_2N_{10}O_6 \cdot 2H_2O$ 47.16 (47.09)	4.95 (4.92)	19.64 (19.51)	0.97 (3H, d, *J*=7 Hz), 1.83 (2H, quintet, *J*=6 Hz), 3.20 (3H, s), 3.35 (2H, t, *J*=6 Hz), 3.55—3.70 (1H, m), 3.90—4.10 (3H, m), 4.18 (2H, t, *J*=6 Hz), 4.64—4.75 (1H, m), 4.87 (1H, d, *J*=14 Hz), 5.11 (1H, d, *J*=14 Hz), 6.14 (1H, d, *J*=11 Hz), 6.22 (1H, d, *J*=11 Hz), 6.66 (1H, s), 6.65—7.05 (1H, m), 7.25—7.39 (2H, m), 7.90 (4H, s), 9.10 (1H, s), 10.07 (1H, s), 10.48 (1H, s)	3237, 1763, 1690, 1661, 1524, 1483, 1424, 1267	−33.5° {20} (1.0)

a) **1a**—**g**, **i**: yields based on the parent compound (**2**); **1h**: yield of the deprotection reaction. *b*) Recrystallization solvent: EA, AcOEt; W, water; AC, acetone; ET, EtOH. *c*) Not measured. *d*) A.P.: Amorphous powder. *e*) The reaction was carried out in acetone. *f*) The reaction was carried out in MeCN. *g*) The product was purified by chromatography using a pre-packed column CPO-273LR (ODS). *h*) The product was purified by silica gel column chromatography. *i*) The product was purified by crystallization. *j*) Anion exchange was done with Dowex 1×8 Cl^-. *k*) Anion exchange was done by mixing with brine.

their solubility and stability in 5% glucose as an injectable carrier.[8] The solubilities (mg/ml) of **2b**, **d**—**i** were measured by the following simple method: 5% glucose was added portionwise (0.1 ml aliquots) to compound **2** (1 mg) with shaking followed by sonication until a clear solution was obtained. The solubility (mg/ml) was calculated based on the amount of 5% glucose added. In the case of **2a**, **c**, the solubilities were determined by another method: a mixture of **2** and 5% glucose was allowed to stand in a sonicator for 10 min at room temperature and the remaining solid (**2**) was

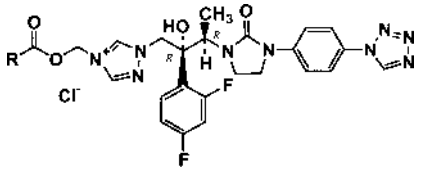
filtered off using a 0.2 μm membrane filter. The concentration (solubility: mg/ml) of **2** in the filtrate was determined by HPLC based on the peak area.[9] On the other hand, the stabilities were measured by the following method: a 1—5 mg/ml solution of **2** in 5% glucose was allowed to stand for 1 d at room temperature and the relative % content of **1** formed by hydrolysis of **2** was determined by HPLC.[9] This % value was regarded as the stability of the prodrugs **2**. The solubility and the stability results are shown in Table 2.

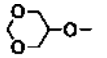
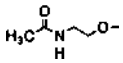
Compounds **2** generally had good water-solubility (1.5—$\geq$10 mg/ml). Notably, compounds **2h**, **i**, which have hydrophilic hydroxyl and acetoamino groups, respectively, revealed water-solubility higher than 10 mg/ml. In the case of **2a**, the solubilities of two crystalline forms were somewhat different; *i.e.*, 10 mg/ml for the unhydrated form and 4 mg/ml for the hydrated form.

The alkanoyl derivatives (**2a**—**c**) and the alkoxycarbonyl derivatives (**2d**—**f**) were considerably resistant to hydrolysis in 5% glucose (stability: 0.1—2.1%). Among these prodrugs (**2a**—**f**), the compounds having secondary and tertiary alkyl groups (**2b**, **c**, **f**: 0.3, 0.1, 0.3%, respectively) were found to be more stable than the compounds having unbranched alkyl groups (**2a**, **d**, **e**: 1.8, 2.1, 1.9%, respectively). On the other hand, **2g**—**i** with hydrophilic moieties were considerably unstable (6.4—25.6%). Especially, the hydroxyl compound **2h** showed the lowest stability (25.6%), which might be due to the neighboring group participation of the hydroxyl group accelerating the hydrolysis.

Biological Activity The *in vitro* antifungal activities of the triazolium salts **2** against *C. albicans* TA were compared with the parent compound **1** (TAK-456) and the non-prodrug quaternary salt **3** (Table 3). The *in vitro* activities are expressed as the minimum inhibitory concentrations (MIC, μg/ml), which were determined by an agar-dilution method on RPMI 1640 medium under 20% CO_2.[10] Compounds **2a**—

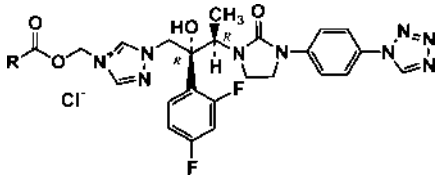
Table 2. Solubility and Stability of the Prodrugs

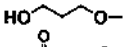
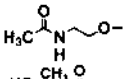
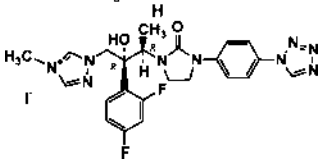


Compd. No.	R	Solubility in 5% glucose (mg/ml)	Stability Content (%) of **1** after 1 d in 5% glucose
2a	CH_3-	4,[a] 10[b]	1.8
2b	$(CH_3)_2CH$-	3	0.3
2c	$(CH_3)_3C$-	6	0.1
2d	CH_3CH_2O-	3	2.1
2e	$CH_3(CH_2)_2O$-	1.5	1.9
2f	$(CH_3)_2CHO$-	2	0.3
2g		5—8	6.4
2h		>10	25.6
2i		>10	6.4

a) Hydrated form of **2a**. *b*) Unhydrated form of **2a**.

Table 3. Antifungal Activities and Hemolytic Activities of the Prodrugs (**2**)



Compd. No.	R-	*In vitro* MIC (μg/ml) *C. albicans* TA	*In vivo* Candidiasis[c] *N*[d] (Day)[e]	Hemolysis (%)
2a	CH_3-	0.06[b]	3/5 (4.5)	1.8
2b	$(CH_3)_2CH$-	0.016[a]	—[f]	89
2c	$(CH_3)_3C$-	0.5[a]	1/5 (5.0)	100
2d	CH_3CH_2O-	0.13[b]	4/5 (6.0)	7.0
2e	$CH_3(CH_2)_2O$-	0.13[b]	—	64
2f	$(CH_3)_2CHO$-	0.25[b]	3/5 (5.5)	81
2g		0.06[b]	—	2.2
2h		0.06[b]	—	1.1
2i		0.06[b]	—	1.4
3		>16[a]	—	—
1 (TAK-456)		0.002,[a] 0.008[b]	2/5 (4.3)[g]	—

a) Determined under condition A. *b*) Determined under condition B. *c*) Prodrugs were administered interavenously to the mice at a dose of 1 μmol/kg immediately after infection. *d*) The number (*N*) of survivors per total number of mice (n=5) on day 7 after infection. *e*) The mean survival days (Day) of the mice which had died up to 7 d after infection. Control mice died up to 0.8 d. *f*) Not determined. *g*) Administered orally at a dose of 1 μmol/kg.

i (MICs, 0.016—0.5) were about ten times or more less potent than the parent compound **1** (MIC, 0.002—0.008). On the other hand, the *N*-methyl triazolium salt **3**, which is stable to hydrolysis, was substantially inactive (MIC, >16). Therefore, it is considered that the antifungal activity of compound **2** is inherently weak and the observed activity could be due to a small amount of the parent compound **1**, which is formed by hydrolysis in the assay medium during the experiment.

Four compounds **2a**, **c**, **d**, **f** were evaluated for *in vivo* antifungal activity using *C. albicans* TA infected mice (Table 3). The test compounds (1 μmol/kg) were administered intravenously once, immediately after infection. The activity is expressed as the number (*N*) of survivors per total number of mice ($n=5$) on day 7 after infection and as the mean survival days (Day) of the mice which had died up to 7 d after infection. All of the prodrugs tested were found to exert strong protective effects ($N=1/5$—$3/5$, Day=4.5—6.0) against candidiasis comparable to that of the parent compound **1** ($N=2/5$, Day=4.3). These results of antifungal activities *in vitro* and *in vivo* suggested that compounds **2** are hydrolyzed to the parent compound **1** in blood after intravenous administration.

In general, some amphiphilic compounds such as quaternary salts, which contain both hydrophobic and hydrophilic moieties, have been known to induce hemolysis. Thus, compounds **2** were assessed for hemolytic activity using rat blood. Table 3 shows the % of hemolysis caused by compounds **2**. Compounds **2g**—**i** with the hydrophilic moieties were found to have low hemolytic activity (1.1—2.2%). Among the alkanoyl derivatives (**2a**—**c**) and the alkoxycarbonyl derivatives (**2d**—**f**), compounds **2b**, **c**, **e**, **f**, possessing lipophilic alkyl or alkoxy groups showed high hemolytic activity (64—100%). On the other hand, the activity of compounds **2a**, **d** that have smaller alkyl or alkoxyl groups, proved to be low. Especially, compound **2a** bearing the acetoxymethyl group was substantially devoid of this effect (1.8%).

From the series of prodrugs, we selected **2a** as the most promising compound for further evaluation.

***In Vivo* Antifungal Activity of 2a** The *in vivo* antifungal activity of **2a** against *C. albicans* TA and *A. fumigatus* 437 in mice was evaluated and compared with the parent compound **1** (TAK-456), fluconazole and itraconazole. *C. albicans* TA infected mice and *A. fumigatus* 437 infected neutropenic mice were used for the *in vivo* assay. In the *in vivo* assay against *C. albicans* TA, the test compounds were administered once, intravenously or orally, immediately after infection. On the other hand, in the case of the *in vivo* assay against *A. fumigatus* 437, the test compounds were administered intravenously or orally, once on the day of infection and twice daily on the following 2 d. The *in vivo* activity (Table 4) is expressed in terms of ED_{50} (mg/kg, the dose of the test compound which allows 50% survival of the infected mice). The activity (ED_{50}, 0.62 mg/kg) of **2a** against *C. albicans* TA infection was comparable to those of **1** (ED_{50}, 0.77 mg/kg) and fluconazole (ED_{50}, 0.22—0.39 mg/kg), and superior to that of itraconazole (ED_{50}, 5.19 mg/kg). Furthermore, in the therapeutic effect against aspergillosis in neutropenic mice, **2a** showed a low ED_{50} value (ED_{50}, 4.49 mg/kg), comparable to that of **1** (ED_{50}, 3.56 mg/kg), and remarkably superior to those of fluconazole (ED_{50}, 179 mg/kg) and itraconazole (ED_{50}, 19 mg/kg).

Table 4. *In Vivo* Antifungal Activity of Compound **2a**

Compd. No.	ED_{50} (mg/kg)[e]	
	C. albicans TA	*A. fumigatus* 437
2a[a]	0.62	4.49
1: TAK-456[b]	0.77	3.56
Fluconazole[c]	0.22—0.39	179
Itraconazole[d]	5.19	19

a) Administered intravenously with the solution in 5% glucose. *b*) Administered orally as a 0.5% carboxymethylcellulose (CMC) suspension. *c*) Administered orally as an aqueous solution. *d*) Administered orally as a 2-hydroxypropyl β-cyclodextrin solution. See ref. 11. *e*) The dose of the test compound which allows 50% survival of the infected mice.

Table 5. *In Vitro* Half-Lives ($T_{1/2}$) of **2a** (TAK-457, 12.25 μg/ml) in Plasma

$T_{1/2}$ (min)		
Mouse	Rat	Human
5.1	6.4	6.0

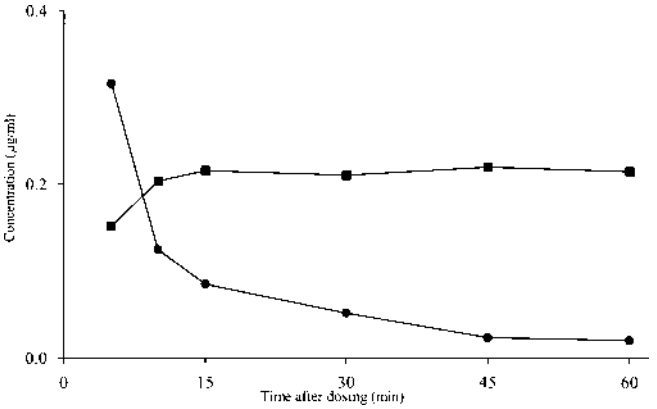


Fig. 1. Plasma Concentrations (Mean Values, $n=3$) of **2a** (●) and TAK-456 (**1**: ■) after a 1 mg/kg Intravenous Dose of [^{14}C]-**2a** in Rats

Pharmacokinetics of 2a The *in vitro* half-lives of **2a** were measured in the plasma of various animals, including mouse, rat and human. The results are shown in Table 5. Compound **2a** was hydrolyzed rapidly to form TAK-456 and the T-half-lives *in vitro* were about 5—6 min in the plasma of all the animals tested (mouse 5.1 min; rat 6.4 min; human 6.0 min). It is noteworthy that there was no species difference in hydrolysis.

This rapid conversion of **2a** to TAK-456 was also confirmed *in vivo* using rats. Figure 1 shows the time course of the plasma concentrations of **2a** and TAK-456 after intravenous administration of [^{14}C]-**2a** (1 mg/kg).[12] As seen in Fig. 1, **2a** was rapidly hydrolyzed with a T-half-life of 6 min and the parent compound TAK-456 appeared quickly. From the basis of the above *in vitro* and *in vivo* studies, **2a** is expected to be converted smoothly to TAK-456 in humans. Based on the results of the evaluations described above, compound **2a** (TAK-457) was designated as the injectable candidate for clinical trials.

In conclusion, we identified a new antifungal prodrug TAK-457 for injectable use. This biologically labile quaternary triazolium salt had sufficient solubility and adequate stability for an injectable formulation and exhibited excellent therapeutic effects against candidiasis as well as aspergillosis in mice *via* intravenous administration. Detailed studies on the therapeutic effects against aspergillosis using various experimental models will be described in a separate paper.[13)]

Experimental

Melting points were determined using a Yanagimoto melting point apparatus and are uncorrected. IR spectra were measured with a JASCO IR-810 spectrometer or a Shimazu FTIR-8200PC spectrometer. NMR spectra were recorded on Varian Gemini-200 and Bruker Avance DPX-300 spectrometers with tetramethylsilane as an internal standard. The following abbreviations are used: s=singlet, d=doublet, t=triplet, q=quartet, m=multiplet, br=broad. The optical rotations were recorded with a JASCO DIP-181 or DIP-370 digital polarimeter.

Reactions were followed by TLC on Silica-gel 60 F_{254} precoated TLC plates (E. Merck) or by HPLC using an ODS column (A-303, 4.6 mm i.d.×250 mm, YMC Co., Ltd.). Standard work-up procedures were as follows. The reaction mixture was partitioned between the indicated solvent and water. Organic extracts were combined and washed in the indicated order using the following aqueous solutions; water, 1 N aqueous sodium hydroxide solution (1 N NaOH), 5% aqueous $NaHCO_3$ solution (aqueous $NaHCO_3$), 1 N hydrochloric acid (HCl) and brine. Extracts were dried over anhydrous magnesium sulfate ($MgSO_4$), filtered and evaporated *in vacuo*.

Chromatographic separations were carried out on Silica gel 60 (0.063—0.200 mm, E. Merck) or ODS (CPO-273L^R, pre-packed column, 22 mm×300 mm, Kusano Kagaku Kikai Co.) using the indicated eluents.

Chloromethyl pivalate (**4c**), bromomethyl acetate (**5a**) were purchased from Aldrich Chemical Company, Inc.. Chloromethyl isobutylate (**4b**),[14)] chloromethyl propyl carbonate (**4e**), chloromethyl isopropyl carbonate (**4f**),[15)] ethyl iodomethyl carbonate (**6d**)[16)] were prepared according to the literature.

Chloromethyl (1,3-Dioxan-5-yl) Carbonate (4g) A solution of chloromethyl chloroformate (25 g) in diethyl ether (Et_2O, 50 ml) was added dropwise to a stirred solution of glycerol formal (14 g) and pyridine (15 g) in Et_2O (400 ml) over a period of 10 min at 0 °C. After stirring for 20 h, the precipitate was removed by filtration. The filtrate was worked up (Et_2O; brine). The residue was purified by silica gel flush column chromatography (AcOEt–hexane, 1 : 5→1 : 3, v/v) to give **4g** (1.7 g, 5%) as a colorless oil. ^{1}H-NMR ($CDCl_3$) δ: 4.05 (4H, d, J=3.2 Hz), 4.67 (1H, quintet, J=3.2 Hz), 4.82 (1H, d, J=6.2 Hz), 4.95 (1H, d, J=6.2 Hz), 5.75 (2H, s).

[2-(*N*-Acetylamino)ethyl] chloromethyl carbonate (**4i**) and 3-benzyloxypropoxy chloromethyl carbonate (**4j**) were prepared from the corresponding alcohols (*N*-acethylethanolamine, 3-benzyloxypropanol[17)]) by the same method as described above. **4i** (85%): ^{1}H-NMR ($CDCl_3$) δ: 2.01 (3H, s), 3.58 (2H, q, J=6 Hz), 4.32 (2H, t, J=6 Hz), 5.75 (2H, s), 5.87 (1H, br). **4j** (89%): ^{1}H-NMR ($CDCl_3$) δ: 2.01 (2H, quintet, J=6.2 Hz), 3.57 (2H, t, J=6.0 Hz), 4.36 (2H, t, J=6.4 Hz), 4.51 (2H, s), 5.71 (2H, s), 7.33 (5H, s).

(1,3-Dioxan-5-yl) Iodomethyl Carbonate (6g) A mixture of **4g** (1.7 g), sodium iodide (5.1 g) and MeCN (40 ml) was stirred at 60 °C for 2 h. The resulting mixture was concentrated *in vacuo*. The residue was worked up (Et_2O; 5% aqueous sodium thiosulfate solution, water, brine) to give **6g** (3.2 g, 100%) as a pale yellow oil. ^{1}H-NMR ($CDCl_3$) δ: 4.04 (4H, d, J=3.0 Hz), 4.66 (1 H, quintet, J=3.0 Hz), 4.81 (1H, d, J=6.2 Hz), 4.95 (1H, d, J=6.2 Hz), 5.97 (2H, s).

Iodomethyl propyl carbonate (**6e**), [2-(*N*-acetylamino)ethyl] iodomethyl carbonate (**6i**) and 3-benzyloxypropoxy iodomethyl carbonate (**6j**) were prepared from the corresponding chloromethyl carbonates **4e**, **i**, **j** by the same method as described above. **6e** (88%): ^{1}H-NMR ($CDCl_3$) δ: 0.97 (3H, t, J=7.0 Hz), 1.73 (2H, m), 4.19 (2H, t, J=7.0 Hz), 5.96 (2H, s). **6i** (48%): ^{1}H-NMR ($CDCl_3$) δ: 2.00 (3H, s), 3.54 (2H, q, J=6 Hz), 4.28 (2H, t, J=6 Hz), 5.93 (2H, s), 6.12 (1H, br). **6j** (93%): ^{1}H-NMR ($CDCl_3$) δ: 2.00 (2H, quintet, J=6.2 Hz), 3.57 (2H, t, J=6.0 Hz), 4.36 (2H, t, J=6.4 Hz), 4.51 (2H, s), 5.94 (2H, s), 7.34 (5H, s).

4-Acetoxymethyl-1-[(2*R*,3*R*)-2-(2,4-difluorophenyl)-2-hydroxy-3-[2-oxo-3-[4-(1*H*-1-terazolyl)phenyl]-1-imidazolidinyl]butyl]-1*H*-1,2,4-triazolium Chloride (2a: Table 1) A solution of **1** (200 mg) and **5a** (77.1 mg) in MeCN (2 ml) was stirred for 7 h at 80 °C under an argon atmosphere. The reaction mixture was diluted with MeCN (11 ml), and the resulting mixture was stirred at 80 °C until the clear solution was obtained. After having been cooled to room temperature (r.t.), silica gel (400 mg) was added, and the mixture was stirred for 10 min at r.t. The silica gel was filtered off and washed with MeCN–tetrahydrofuran (THF) (1 : 1, v/v, 2 ml×3). The mother liquor and the washings were combined, and brine (10 ml) was added. The resulting mixture was stirred for 30 min at r.t., and the organic layer was separated. The same operations as above, the addition of brine (10 ml) to the organic layer followed by the stirring for 30 min at r.t. and the subsequent separation of the organic layer, were performed further three times. The organic layer was dried over $MgSO_4$, and the solvent was distilled off under reduced pressure. The residue was dissolved in THF (3 ml), and the solution was allowed to stand for 5 h at r.t. The precipitate (179 mg) was collected by filtration and dissolved in methanol (MeOH)–THF (1 : 1, v/v, 4 ml). The solution was concentrated *in vacuo* and the residue was dissolved in THF (2 ml). The solution was allowed to stand for 5 h at r.t. The precipitate was collected by filtration and dissolved in ethanol (EtOH)–acetone (12 : 1, v/v, 6.5 ml). The resulting solution was concentrated *in vacuo* to a volume of *ca*. 2 ml and allowed to stand for 3 h at r.t. The precipitate was collected by filtration to give the unhydrated form of **2a** (126 mg, 51%) as a white crystalline powder. Compound **2a** (0.63 g) obtained above was recrystallized from water (10 ml) to give the hydrate form (0.61 g) as a white crystalline powder.

1-[(2*R*,3*R*)-2-(2,4-Difluorophenyl)-2-hydroxy-3-[2-oxo-3-[4-(1*H*-1-terazolyl)phenyl]-1-imidazolidinyl]butyl]-4-[(2-methylpropanoyloxy)methyl]-1*H*-1,2,4-triazolium Chloride (2b: Table 1) A mixture of **1** (0.5 g), **4b** (1.37 g) and acetone (20 ml) was refluxed for 50 h. The resulting mixture was concentrated *in vacuo* and diisopropylether (isoPr$_2$O, 10 ml) was added to the residue. The precipitate was collected by filtration and purified by ODS column chromatography (MeOH–H_2O, 3 : 2, v/v) to give **2b** (0.15 g, 24%) as a white amorphous powder. This powder (50 mg) was crystallized from saline (1 ml) to obtain **2b** (41 mg) as a white crystalline powder.

1-[(2*R*,3*R*)-2-(2,4-Difluorophenyl)-2-hydroxy-3-[2-oxo-3-[4-(1*H*-1-terazolyl)phenyl]-1-imidazolidinyl]butyl]-4-[(2,2-dimethylpropanoyloxy)-methyl]-1*H*-1,2,4-triazolium Chloride (2c: Table 1) A mixture of **1** (0.5 g, 1.04 mmol), **4c** (15.7 g, 104.2 mmol) and MeCN (3 ml) was stirred at 100 °C for 6.5 h. The resulting mixture was concentrated *in vacuo* and isoPr$_2$O was added to the residue. The precipitate was collected by filtration and purified by silica gel flush column chromatography (AcOEt→acetone→acetone–EtOH, 10 : 1→5 : 1, v/v) to give **2c** (0.32 g, 48%) as a white amorphous powder. This powder (0.4 g) was crystallized from AcOEt to obtain **2c** (0.3 g) as a white crystalline powder.

1-[(2*R*,3*R*)-2-(2,4-Difluorophenyl)-2-hydroxy-3-[2-oxo-3-[4-(1*H*-1-terazolyl)phenyl]-1-imidazolidinyl]butyl]-4-ethoxycarbonyloxymethyl-1*H*-1,2,4-triazolium Chloride (2d: Table 1) A mixture of **1** (1.31 g, 2.72 mmol), **6d** (1.25 g, 5.43 mmol) and MeCN (15 ml) was stirred at 60 °C for 14 h under an argon atmosphere. The resulting mixture was concentrated *in vacuo*. The residue was purified by silica gel flush column chromatography (AcOEt→acetone→acetone–EtOH, 4 : 1, v/v) followed by ODS column chromatography (MeOH–H_2O, 3 : 2, v/v) to give the iodide salt (1.1 g) as a pale yellow amorphous powder. The iodide salt (1.1 g) was then ion-exchanged using Dowex 1×8 Cl^- (water) followed by crystallization from EtOH to give **2d** (0.7 g, 42%) as a white crystalline powder.

1-[(2*R*,3*R*)-2-(2,4-Difluorophenyl)-2-hydroxy-3-[2-oxo-3-[4-(1*H*-1-terazolyl)phenyl]-1-imidazolidinyl]butyl]-4-propoxycarbonyloxymethyl-1*H*-1,2,4-triazolium Chloride (2e: Table 1) Compound **1** (1.0 g) was allowed to react with **6e** (0.9 g) in a manner similar to that described for the synthesis of **2d** to obtain the iodide (1.0 g) as a pale yellow amorphous powder. This iodide (0.3 g) was dissolved in THF–AcOEt (3/1, 100 ml) and ion-exchanged by mixing with brine (50 ml×4). The organic layer was dried over $MgSO_4$, and concentrated *in vacuo* followed by crystallization from EtOH–acetone to give **2e** (0.09 g, 22% from **1**) as a white crystalline powder.

1-[(2*R*,3*R*)-2-(2,4-Difluorophenyl)-2-hydroxy-3-[2-oxo-3-[4-(1*H*-1-terazolyl)phenyl]-1-imidazolidinyl]butyl]-4-isopropoxycarbonyloxymethyl-1*H*-1,2,4-triazolium Chloride (2f: Table 1) The reaction of **1** (0.5 g) with **4f** (3.17 g) was carried out in a manner similar to that described for the synthesis of **2c**. Compound **2f** (110 mg, 17%) was obtained as a white crystalline powder (recrystallized from AcOEt–acetone).

1-[(2*R*,3*R*)-2-(2,4-Difluorophenyl)-2-hydroxy-3-[2-oxo-3-[4-(1*H*-1-terazolyl)phenyl]-1-imidazolidinyl]butyl]-4-[(1,3-dioxan-5-yl)oxycarbonyloxymethyl-1*H*-1,2,4-triazolium Chloride (2g: Table 1) The reaction of **1** (1.0 g) with **6g** (0.9 g) was carried out in a manner similar to that described for the synthesis of **2d** to obtain the iodide (4.22 g) as a pale yellow amorphous powder. This iodide salt (1 g) was subjected to column chromatography on Dowex 1×8 Cl^- (water). The eluate was lyophilized to obtain **2g** (0.23 g, 27%) as a white amorphous powder. This powder (0.15 g) was crys-

tallized from EtOH (20 ml) to give **2g** (0.14 g) as a white crystalline powder.

1-[(2*R*,3*R*)-2-(2,4-Difluorophenyl)-2-hydroxy-3-[2-oxo-3-[4-(1*H*-1-terazolyl)phenyl]-1-imidazolidinyl]butyl]-4-[(3-hydroxypropyl)oxycarbonyloxymethyl]-1*H*-1,2,4-triazolium Chloride (2h: Table 1) The reaction of **1** (2.0 g) with **6j** (2.9 g) was carried out in a manner similar to that described for the synthesis of **2d**. The product was ion-exchanged by Dowex 1×8 Cl^- (H_2O) to obtain 4-[(3-benzyloxypropoxy)carbonyloxymethyl]-1-[(2*R*,3*R*)-2-(2,4-difluorophenyl)-2-hydroxy-3-[2-oxo-3-[4-(1*H*-terazol-1-yl)phenyl]-1-imidazolidinyl]butyl]-1*H*-1,2,4-triazolium chloride (**2j**, 0.79 g, 26%) as a white amorphous powder. *Anal.* Calcd for $C_{34}H_{36}ClF_2N_9O_6 \cdot 1/2H_2O$: C, 54.51; H, 4.98; N, 16.83. Found: C, 54.37; H, 5.10; N, 16.87. ^{1}H-NMR ($CDCl_3$) δ: 0.97 (3H, d, J=7.2 Hz), 1.89 (2H, quintet, J=6.4 Hz), 3.47 (2H, t, J=6.2 Hz), 3.60—4.09 (4H, m), 4.22 (2H, t, J=6.6 Hz), 4.44 (2H, s), 4.65—4.75 (1H, m), 4.86 (1H, d, J=14 Hz), 5.09 (1H, d, J=14 Hz), 6.12 (1H, d, J=11 Hz), 6.20 (1H, d, J=11 Hz), 6.62 (1H, s), 6.94—7.04 (1H, m), 7.24—7.36 (7H, m), 7.90 (4H, s), 9.09 (1H, s), 10.07 (1H, s), 10.45 (1H, s) IR (KBr): 3125, 1759, 1696, 1524, 1481, 1422, 1267 cm^{-1}.

A solution of **2j** (0.66 g) and 1 N HCl (0.89 ml) in MeOH (25 ml) was hydrogenated over 10% Pd–C (50% wet, 0.33 g) at r.t. under atmospheric pressure for 1.5 h. The catalyst was removed by filtration, and the filtrate was concentrated *in vacuo*. The residue was purified by ODS column chromatography (MeOH–H_2O, 3 : 2, v/v) followed by crystallization from EtOH to give **2h** (0.19 g, 33%) as a colorless crystalline powder.

1-[(2*R*,3*R*)-2-(2,4-Difluorophenyl)-2-hydroxy-3-[2-oxo-3-[4-(1*H*-1-terazolyl)phenyl]-1-imidazolidinyl]butyl]-4-[(2-*N*-acetylaminoethoxy)carbonyloxymethyl]-1*H*-1,2,4-triazolium Chloride (2i: Table 1) The reaction of **1** (2.0 g) with **6i** (2.9 g) was carried out in a manner similar to that described for the synthesis of **2d**. The iodide was ion-exchanged by Dowex 1×8 Cl^- (water) to obtain **2i** (0.5 g, 36%) as a white amorphous powder.

1-[(2*R*,3*R*)-2-(2,4-Difluorophenyl)-2-hydroxy-3-[2-oxo-3-[4-(1*H*-1-terazolyl)phenyl]-1-imidazolidinyl]butyl]-4-methyl-1*H*-1,2,4-triazolium Iodide (3) A mixture of methyliodide (0.36 ml, 6 mmol), **1** (48 mg, 0.1 mmol) and acetone (2 ml) was refluxed for 64 h. The whole was concentrated *in vacuo*. The residue was purified by ODS column chromatography (MeOH–H_2O, 3 : 2, v/v) to give **3** (47 mg, 75%) as a white amorphous powder. ^{1}H-NMR (DMSO-d_6) δ: 0.98 (3H, d, J=7 Hz), 3.50—4.05 (4H, m), 3.84 (3H, s), 4.60—4.80 (1H, m), 4.86 (2H, br), 6.32 (1H, br), 7.00—7.10 (1H, m), 7.20—7.50 (2H, m), 7.90 (4H, s), 8.83 (1H, s), 9.96 (1H, s), 10.06 (1H, s). IR (KBr): 1688, 1524 1499, 1483, 1424, 1269 cm^{-1}. *Anal.* Calcd for $C_{23}H_{24}F_6IN_9O_2 \cdot H_2O$: C, 43.07; H, 4.09; N, 19.65. Found: C, 43.20; H, 4.22; N, 19.17.

Hemolytic Activity Distilled water and 5% glucose (Otsuka Co.) was used for positive control and blank, respectively. Compounds were dissolved in 5% glucose at a concentration of 2 mg/ml and pre-warmed at 37 °C. The solution (1 ml) and rat heparinaized whole blood (0.1 ml) were mixed and incubated at 37 °C for 30 min. The mixture was centrifuged at 1870 ***g*** for 10 min and the hemoglobin level in the supernatant was determined using HEMOGLOBIN TEST WAKO (Wako Pure Chemical Co.), according to manufacturer's manual. The % of hemolysis was calculated by the following formula: hemolysis (%)=OD_{540} of sample supernatant/OD_{540} of control supernatant ×100.

Antifungal Activity *in Vitro* MICs against *C. albicans* TA were determined by an agar dilution method using RPMI-1640 medium (Gibco BRL, Grand Island, N.Y.). A double concentration of RPMI-1640 medium was prepared with 0.3 M morpholinepropanesulfonic acid (MOPS; Dojindo, Tokyo, Japan) buffer (pH 7.0), sterilized by filtration through a membrane filter (pore size, 0.45 μm), and mixed with an equal volume of 2.0% agar (condition A) or 3.0% agar (condition B) (Wako, Osaka, Japan) which had been autoclaved at 121 °C for 15 min and kept at 55 °C. The agar medium (9.9 ml) was then poured into petri dishes containing 0.1 ml of serial dilutions of antifungal agents dissolved in dimethylsulfoxide (DMSO; Wako) and allowed to solidify. About 10^3 CFU of fungal cells suspended in saline was inoculated with a multiple inoculator (Sakuma, Tokyo, Japan) onto the agar plates prepared as described above. The plates were then incubated in a CO_2 incubator at 35 °C for 20 h. The MIC was defined as the lowest concentration of antifungal agent giving no visible growth or causing almost complete inhibition of growth.

Antifungal Activity *in Vivo* *In vivo* antifungal activity against *C. albicans* TA was measured by the following method: Six-week-old CDF_1 female mice were infected intravenously with 2×10^6 CFU of *C. albicans* TA per mouse. The compounds were administered intravenously or orally immediately after infection. The efficacy of the compound is expressed as *N* and Day (Table 3) or ED_{50} values (Table 4). ED_{50} values were calculated by the method of Reed and Muench[18] from survival rates on day 7 after infection.

ED_{50} values of the compounds against aspergillosis were determined by the following method: Six-week-old CDF_1 female mice were infected intravenously with 6×10^4 CFU of *A. fumigatus* 437 per mouse 4 d after the intraperitoneal administration of 200 mg/kg of cyclophosphamide. The test compounds were administered intravenously or orally 2 h after infection and twice daily on the following 2 d. ED_{50} values (Table 4) were calculated by the method of Reed and Muench from survival rates on day 10 after infection.

In Vitro* Half-Lives of 2a (Table 5)** The standard solution (10 μl) of **2a** (1.225 mg/ml) was added to the animal [SD(IGS) rat or ICR mouse] or human plasma (990 μl), which had been pre-incubated for 5 min at 37 °C. The mixtures were incubated at 37 °C. An aliquot (100 μl) was taken at 5, 10, 15, 30, 60 and 120 min and deproteinized with MeCN–acetic acid (AcOH) (4 : 1, v/v, 100 μl). After centrifugation at 19000 ***g for 10 min, the supernatant (150 μl) was added to 150 μl of 0.01 mol/l aqueous ammonium acetate solution adjusted to pH 6.0 with AcOH. An aliquot (20 μl) of the solution was injected to an HPLC. HPLC conditions: The HPLC system consisted of an LC-10AD pump, a SIL-10A autosampler, a CTO-10AC column oven, a SPD10AV UV-detector, and a CLASS-LC10 workstation (all from Shimadzu Co., Kyoto, Japan). Column: L-Column ODS (5 μm particle size, 4.6 mm i.d.×150 mm; Chemicals Evaluation and Research Institute, Tokyo, Japan). Mobile phase: A mixture of 0.01 mol/l aqueous ammonium acetate solution adjusted to pH 6.0 with AcOH and CH_3CN (67 : 33, v/v). Column temperature: 40 °C. Flow rate: 1.0 ml/min. UV detection: 274 nm.

Concentration of 2a and TAK-456 in Rat Plasma (Fig. 1) Animals used in this study were male Crj:CD(SD)IGS rats (weight, 281 to 283 g; Charles River Japan Inc., Yokohama, Japan). They were fed laboratory chow (CR-LPF; Oriental Yeast Co., Ltd., Tokyo, Japan), had free access to water, and were housed in temperature- and humidity-controlled rooms (20 to 26 °C, 40 to 75%) with 12-h light–dark cycles for more than a week before use. [^{14}C]-**2a** was dissolved in 5 w/v % glucose solution for intravenous injection at a dose of 1 mg/ml/kg. The test compound was injected to fed animals. After dosing, blood samples were taken from the tail vein, and centrifuged at 1870 ***g*** for 10 min at 4 °C to obtain plasma samples. The sampling times were as follows: 5, 10, 15, 30, 45, 60 min. The samples, after determination of the radioactivity, were kept frozen at −20 °C until analyses.

Compound **2a** and TAK-456 (**1**) in rat plasma were quantified by HPLC. Prior to the HPLC analysis, to a portion of plasma was added a half volume of phosphate buffered saline in the ice-cooled bath, and to the supernatant obtained by the centrifugation at 1870 ***g*** and 4 °C for 10 min was added 2 volumes of a mixture of MeCN and AcOH (1 : 9, v/v). The supernatant obtained by the same manner described above was subjected to HPLC. The HPLC separations were achieved at 40 °C with a Shield RP8 column (4.6 mm i.d.×150 mm, Waters Assoc., Milford Massachusetts, U.S.A.). A mixture of 0.01 mol/l ammonium acetate, MeCN and AcOH (75 : 25 : 0.2, v/v/v) and a mixture of 0.01 mol/l aqueous ammonium acetate solution, MeCN and AcOH (60 : 40 : 0.2, v/v/v) were used for the mobile phase A and B [MP(A) and MP(B)], respectively. The flow rate was 1 ml/min. The time program for the gradient elution was as follows: the concentration of MP(B) was held at 0 vol% for 15 min, linearly increased from 0 to 100 vol% over a period of 15 min and cycled back to the initial condition (0 vol%). The effluent was collected into 1 ml size of fractions, followed by the counting of radioactivity. Data Processing: Data were expressed as the mean values or the mean values with standard deviations (S.D.) for the results from three to four animals, unless otherwise indicated. Half-life ($T_{1/2}$) in plasma was calculated by the linear regression analysis and trapezoidal rule.

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