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Thielavins as Glucose-6-phosphatase (G6Pase) Inhibitors: Producing Strain, Fermentation, Isolation, Structural Elucidation and Biological Activities

SHINICHI SAKEMI*, HIDEO HIRAI, TOSHIO ICHIBA†, TAISUKE INAGAKI, YOSHINAO KATO, NAKAO KOJIMA††, HIROYUKI NISHIDA, JANICE C. PARKER[a], TOSHIYUKI SAITO, HIROKO TONAI-KACHI, MARIA A. VANVOLKENBURG[a], NOBUJI YOSHIKAWA and YASUHIRO KOJIMA

Exploratory Medicinal Sciences, Pfizer Global Research and Development (PGRD)-Nagoya Laboratories, Pfizer Inc., 5-2 Taketoyo, Aichi 470-2393, Japan
[a] Pfizer Global Research and Development (PGRD)-Groton Laboratories, Pfizer Inc., Eastern Point Road, Groton, CT 06340, U.S.A.

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High-throughput screening of microbial extracts using rat hepatic microsomal glucose-6-phosphatase (G6Pase) led us to find thielavin B as a G6Pase inhibitor with inhibition of glucose output from glucagon-stimulated hepatocytes. Further searching for more potent analogs identified 11 new thielavins F~P in addition to the known thielavins A and B from a fungus *Chaetomium carinthiacum* ATCC 46463. Thielavin G showed the strongest activity as a G6Pase inhibitor (IC_{50}=0.33 μM), while the IC_{50} of thielavin B was 5.5 μM. According to the structure-activity relationship, including authentic thielavins C, D and 3 partial hydrolysates from thielavins A and B, 3 benzoic acid-units and carboxylic acid functions are essential for G6Pase inhibition.

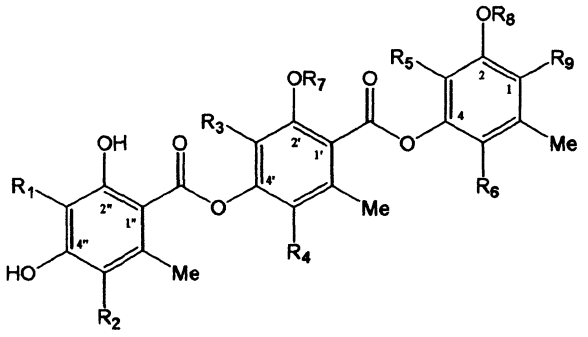
Diabetes mellitus is a major chronic disease and a cause of many debilitating and life-threatening complications. Because of such complications and its requirement of long term medical care, patient's "quality of life" has been disturbed. The medical costs associated with the treatment of diabetes mellitus are very large and continue to increase as the population ages and diabetes become more prevalent. Insulin has been very effective in the treatment of Type 1 diabetes mellitus and is used to treat Type 2 diabetic patients who fail to respond to existing oral therapies. However, there remains a need for additional, more effective oral anti-diabetic agents.

In the course of drug discovery efforts to identify anti-diabetic agents, we have focused on glucose-6-phosphatase (G6Pase). G6Pase has been recognized as a key enzyme in glucose homeostasis where G6Pase hydrolyzes glucose-6-phosphate (G6P) to glucose and releases it into the bloodstream. Inhibition of G6Pase activity should decrease hepatic glucose output from both glyconeogenesis and glycogenolysis, which would lead to the lowering of the concentration of plasma glucose in diabetes mellitus[1,2].

High throughput screening of our microbial extracts using the rat hepatic microsomal G6Pase enzyme was performed, which led us to identify thielavin B[3] as a G6Pase inhibitor with moderate inhibition of glucose output from glucagon (GGN)-stimulated hepatocytes. In the further searching for thielavin-analogs with more potent G6Pase inhibition, including the optimization of fermentation condition, we isolated 11 new thielavins F~P[§] in addition to the known thielavins A and B[3,4] from the fermentation broth of a fungus *Chaetomium carinthiacum* ATCC 46463 (Fig. 1). In this paper we describe producing

* Corresponding author: Shinichi.Sakemi@japan.pfizer.com
† Present address: Research & Development Division, Okinawa Industrial Technology Center (OITC), 12-2 Suzaki, Gushikawa, Okinawa 904-2234, Japan.
†† Present address: Faculty of Pharmacy, Meijo University, 150 Yagotoyama, Tenpaku-ku, Nagoya 468-8503, Japan.
§ New thielavins F~P in this paper have originally been coded in our laboratory as: F (CJ-19,586), G (CJ-19,587), H (CJ-19,588), I (CJ-19,589), J (CJ-20,089), K (CJ-20,090), L (CJ-20,092), M (CJ-20,093), N (CJ-20,094), O (CJ-20,095) and P (CJ-20,096).

Fig. 1. Structures of thielavin A~D and new thielavin analogs F~P (**1**~**11**).



	R_1	R_2	R_3	R_4	R_5	R_6	R_7	R_8	R_9
Thielavin A	Me	H	Me	Me	Me	Me	H	H	CO_2H
Thielavin B	Me	H	Me	Me	Me	Me	Me	Me	CO_2H
Thielavin C	Me	Me	Me	Me	Me	Me	Me	Me	CO_2H
Thielavin D	H	H	Me	Me	Me	Me	Me	Me	CO_2H
Thielavin F (1)	Me	H	Me	Me	H	Me	Me	Me	CO_2H
Thielavin G (2)	Me	H	H	Me	Me	Me	Me	Me	CO_2H
Thielavin H (3)	H	H	Me	Me	Me	Me	H	H	CO_2H
Thielavin I (4)	Me	H	Me	Me	Me	Me	H	H	H
Thielavin J (5)	Me	H	Me	Me	Me	H	Me	H	CO_2H
Thielavin K (6)	Me	H	Me	Me	Me	Me	Me	H	CO_2H
Thielavin L (7)	Me	H	Me	Me	Me	Me	Me	H	$CO_2CH_2CH(OH)CH_2OH$
Thielavin M (8)	H	H	Me	Me	Me	Me	Me	H	CO_2H
Thielavin N (9)	Me	H	Me	Me	H	Me	H	Me	CO_2H
Thielavin O (10)	Me	Me	Me	Me	Me	Me	Me	H	CO_2H
Thielavin P (11)	Me	Me	Me	Me	Me	Me	H	H	CO_2H

organisms, fermentation, isolation, structure elucidation and biological activity of a series of thielavins including 11 new analogs. Three hydrolysis products (CJ-20,556, CJ-20,557 and CJ-20,558), each of which has 2 benzoic acid-units, were also prepared from thielavins A and B. Their G6Pase-inhibitory activities were also measured and compared with those of the other thielavins including previously isolated authentic thielavins C and D[4,5].

Results

Producing Strain and Fermentation

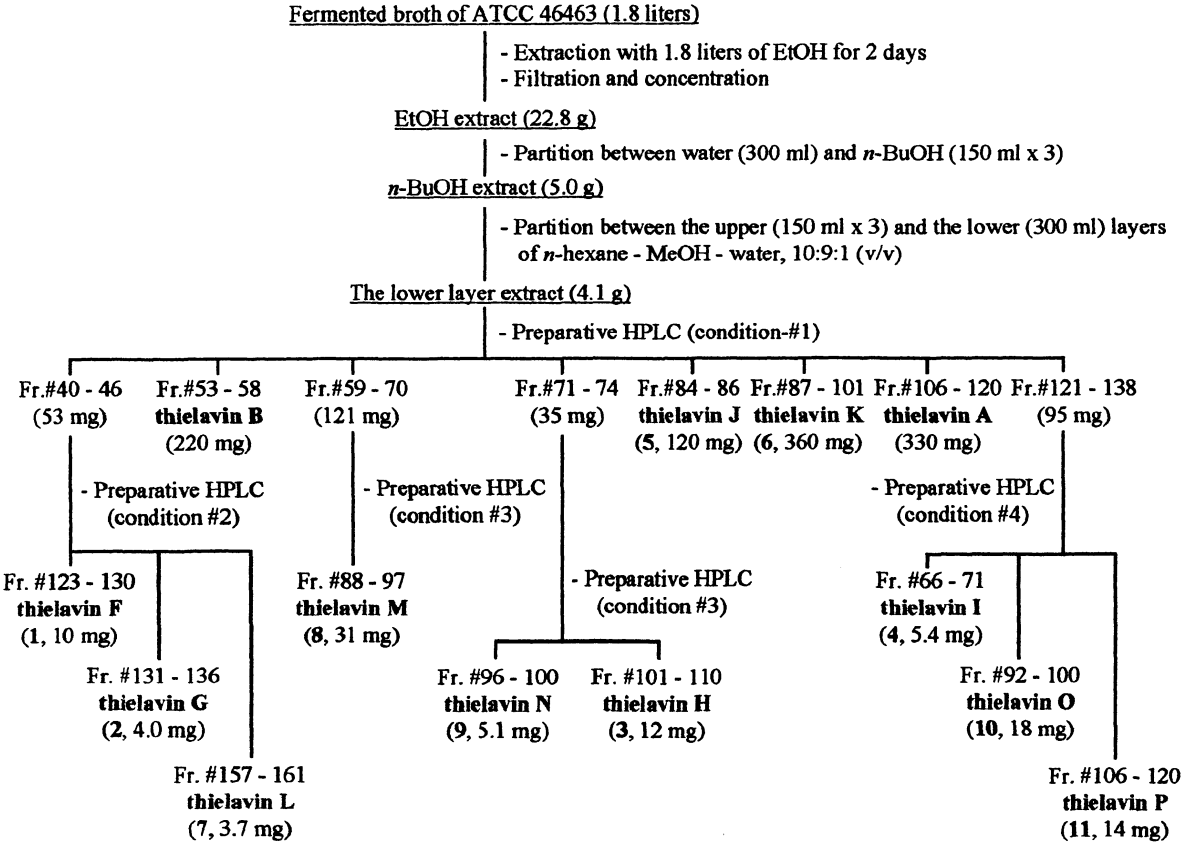
The producing strain, the fungus *Chaetomium carinthiacum* ATCC 46463 was obtained from the American Type Culture Collection.

ATCC 46463 was maintained on agar slants of potato dextrose agar medium. The cell suspension from the agar slant was used to inoculate 500 ml-Erlenmeyer's flasks containing 100 ml of seed medium (potato dextrose broth 2.4%, yeast extract 0.5% and agar 0.1%). After incubation at 27°C on a rotary shaker at 250 rpm for 4 days, 5 ml aliquots were inoculated into twenty 500 ml-Erlenmeyer's flasks containing 50 ml of production medium (glucose 1%, glycerol 6.6%, oil-less soybean meal 0.5%, NZ-amine® Type A [Wako] 0.5%, $(NH_4)_2SO_4$ 0.2%, tomato paste 0.5% and sodium citrate 0.2%, pH 6.5) and 25 g of buckwheat (Nikkoku Seihun). Stationary incubation was carried out at 27°C for 18 days.

Isolation of Thielavins

The fermented broth of ATCC 46463 (1.8 liters from 18 flasks) was extracted with 1.8 liters of ethanol for 2 days, and then filtered. The filtered broth (1.5 liters) was concentrated to dryness to yield 22.8 g of ethanol extract. The ethanol extract was suspended into *n*-butanol-saturated water (300 ml) and extracted with water-saturated *n*-butanol, three times (total, 150 ml×3). The dried *n*-butanol extract (5.0 g) was then suspended into the lower layer (300

Fig. 2. Isolation scheme of thielavins A, B and 11 new analogs F~P (**1**~**11**).



ml) of *n*-hexane - methanol - water, 10 : 9 : 1 (v/v) and washed with the upper layer of the same solvent mixture, three times (total, 150 ml×3). The resulting lower layer (4.1 g), which was confirmed to contains all thielavin analogs detected in the ethanol extract by HPLC analysis, was subsequently separated on preparative HPLC under an acetonitrile-gradient system (condition #1, see Experimental section), followed by methanol-gradient systems (conditions #2~4, see Experimental section) to afford thielavins A, B and 11 new analogs as shown in Fig. 2.

Physico-chemical Properties

Physico-chemical properties of new thielavin analogs **1**~**11** were summarized in Table 1. The compounds are soluble in most organic solvents but not in hexane and water. The UV spectra of **1**~**11** showed absorption maxima at 210~218, 267~276 and 308~318 nm, similar to those of thielavin A~D[3~5], suggesting the presence of a common benzoic acid skeleton for thielavins. The IR spectra of **1**~**11** showed the absorption at 3398~3423 and 1652~1743 cm^{-1}, suggesting the presence of hydroxy and carboxy groups, respectively. Retention times of these new thielavins under two different analytical HPLC conditions were also summarized in Table 1 (for the detailed conditions and the retention times of other thielavin analogs, see Experimental section).

Structural Elucidation

Thielavins A and B were identified by the direct comparisons with authentic thielavins A~D in our laboratory. The structures of the authentic thielavins had been elucidated by spectrometric analysis including 2D-NMR and by comparisons with previously reported data[3~5].

The structural elucidation procedures of the new thielavin analogs **1**~**11** are essentially the same. Here, the structure elucidation of **2**, which is the most potent G6Pase

Table 1. Physico-chemical properties of new thielavin analogs **1**~**11**.

	1	**2**	**3**	**4**	**5**	**6**
Molecular formula	$C_{30}H_{32}O_{10}$	$C_{30}H_{32}O_{10}$	$C_{28}H_{28}O_{10}$	$C_{28}H_{30}O_{8}$	$C_{29}H_{30}O_{10}$	$C_{30}H_{32}O_{10}$
Molecular weight	552	552	524	494	538	552
HRFAB-MS (nega)	$C_{30}H_{31}O_{10}$	$C_{30}H_{31}O_{10}$	$C_{28}H_{27}O_{10}$	$C_{28}H_{29}O_{8}$	$C_{29}H_{29}O_{10}$	$C_{30}H_{31}O_{10}$
Calcd (*m/z*):	551.1917	551.1918	523.1604	493.1863	537.1760	551.1918
Found (*m/z*):	551.1928	551.1912	523.1601	493.1862	537.1750	551.1901
LRFAB-MS (posi, *m/z*):	553, 535, 389, 357, 343, 193, 179, 165, 151	553, 535, 461, 389, 343, 329, 193, 179, 165	525, 375, 329, 179, 151	495, 343, 331, 231, 179, 165	539, 375, 357, 343, 193, 165	553, 509, 389, 357, 193, 165
UV λ_{max} (MeOH) nm	210, 276, 310	210, 276, 310	215, 267, 308	218, 275, 310	214, 250, 275, 306	215, 250, 274, 307
IR ν_{max} (KBr) cm^{-1}	3400, 2937, 1743, 1652, 1604, 1461,	3400, 2941, 1743, 1651, 1419, 1269,	3400, 2929, 1660, 1446, 1315, 1255,	3398, 2931, 1660, 1622, 1417, 1307,	3400, 2933, 1654, 1622, 1456, 1265,	3400, 2935, 1726, 1620, 1417, 1265,
Rt/MeCN (minutes)[a]	15.9	15.9	17.3	19.4	18.5	18.5
Rt/MeOH (minutes)[b]	13.1	13.3	17.2	17.8	18.5	18.4

	7[c]	**8**	**9**	**10**	**11**
Formula	$C_{33}H_{38}O_{12}$	$C_{29}H_{30}O_{10}$	$C_{29}H_{30}O_{10}$	$C_{31}H_{34}O_{10}$	$C_{30}H_{32}O_{10}$
Molecular weight	626	538	538	566	552
HRFAB-MS (nega)	$C_{33}H_{37}O_{12}$	$C_{29}H_{29}O_{10}$	$C_{29}H_{29}O_{10}$	$C_{31}H_{33}O_{10}$	$C_{30}H_{31}O_{10}$
Calcd (*m/z*):	625.2285	537.1760	537.1761	565.2073	551.1918
Found (*m/z*):	625.2280	537.1766	537.1773	565.2078	551.1929
LRFAB-MS (posi, *m/z*):	627, 535, 463, 357, 193, 165	539, 389, 343, 193, 151	539, 461, 318, 179, 165	567, 553, 525, 193, 179, 165	553, 344, 193, 179
UV λ_{max} (MeOH) nm	214, 275, 310	214, 267, 304	214, 253, 275, 310	214, 252, 275, 318	216, 258, 273, 318
IR ν_{max} (KBr) cm^{-1}	3398, 2933, 1733, 1654, 1606, 1458,	3400, 2929, 1733, 1652, 1624, 1458,	3423, 2933, 1743, 1654, 1604, 1419,	3421, 2929, 1733, 1651, 1610, 1458,	3400, 2927, 1652, 1612, 1456, 1419,
Rt/MeCN (minutes)[a]	16.3	16.8	17.4	20.6	21.3
Rt/MeOH (minutes)[b]	15.2	16.3	16.7	20.6	21.6

[a] Retention time in acetonitrile condition (see Experimental section for the details).

[b] Retention time in methanol condition (see Experimental section for the details).

[c] $[\alpha]_D$ = +2.5°(*c* 0.2, MeOH).

inhibitor, was described as a representative. All of the ^{1}H- and ^{13}C-NMR assignments of the new analogs **1**~**11** were summarized in Tables 2-1 and 2-2.

The molecular formula of thielavin G (**2**) was determined to be $C_{30}H_{32}O_{10}$ by HRFAB-MS analysis (*m/z* found: 551.1912, calcd for $C_{30}H_{31}O_{10}$ $[M-H]^-$: 551.1918) and the number of hydrogens and carbons from NMR. The ^{1}H- and ^{13}C-NMR spectra (pyridine-d_5) showed 29 proton and 30 carbon signals. The carbon signals were classified into seven $-CH_3$, two $-O-CH_3$, two $-CH=$, ten $-C=$, six $-C(-O)=$ and three $-COO-$ carbonyls by analysis of their chemical shifts and the DEPT spectra. DEPT data also makes us count all 29 protons observed in ^{1}H-NMR, which suggests that the remaining 3 protons from the molecular formula are hydroxy groups. The degree of unsaturation from the molecular formula was fifteen: nine were assigned to double bonds (eighteen sp^2 carbons at δ 164.7, 163.0, 156.0, 153.9, 151.1, 149.1, 140.8, 137.4, 132.4, 130.9, 126.1, 122.6, 122.3, 122.2, 112.2, 110.2, 104.8 and 103.6), three to carbonyl groups (δ 171.1, 171.1 and 166.4) and the remainder to the three rings. In addition to the data comparisons to those of known thielavins, the structure of **2** was elucidated based on the results of HMBC experiment (Fig. 3) and the fragment analysis of the LRFAB-MS (Fig.

Table 2-1. ^{1}H- and ^{13}C-NMR chemical shifts of new thielavin analogs **1**~**6** in pyridine-d_5.

Position	1			2			3			4			5			6		
No.	^{13}C (ppm)		^{1}H (ppm)	^{13}C (ppm)		^{1}H (ppm)	^{13}C (ppm)		^{1}H (ppm)	^{13}C (ppm)		^{1}H (ppm)	^{13}C (ppm)		^{1}H (ppm)	^{13}C (ppm)		^{1}H (ppm)
1	125.9	s		130.9	s		115.2	s		115.2	d	6.86 (1H, s)	113	s		115	s	
2	154.7	s		153.9	s		159.9	s		155.3	s		164.3	s		159.7	s	
3	103.5	d	6.88 (1H, s)	122.6	s		118.3	s		115.7	s		117	s		116.6	s	
4	149.7	s		149.1	s		152.1	s		149.7	s		153.1	s		151.8	s	
5	121.8	s		126.1	s		120.8	s		119.4	s		116	d	6.83 (1H, s)	120.7	s	
6	132.9	s		132.4	s		138.3	s		135.6	s		141	s		138.1	s	
1'	127	s		122.2	s		116.7	s		117.5	s		127.6	s		126.4	s	
2'	154.2	s		156	s		156.3	s		156.5	s		154.7	s		154.9	s	
3'	122.2	s		104.8	d	6.95 (1H, s)	117.8	s		117.8	s		122.7	s		122.6	s	
4'	149.2	s		151.1	s		151.6	s		151.4	s		150.2	s		150.2	s	
5'	125.9	s		122.6	s		122.1	s		121.8	s		126.4	s		127.2	s	
6'	134.4	s		137.4	s		135.8	s		135.6	s		133.4	s		133.9	s	
1"	102.7	s		103.6	s		105	s		103.2	s		103.2	s		103	s	
2"	164.6	s		164.7	s		166.4	s		165	s		165.1	s		165	s	
3"	109.9	s		110.2	s		102.3	d	6.71 (1H, d)[a]	110.3	s		110.4	s		110.2	s	
4"	162.8	s		163	s		165.2	s		163.1	s		163.3	s		163.1	s	
5"	112	d	6.66 (1H, s)	112.2	d	6.62 (1H, s)	113.4	d	6.64 (1H, d)[a]	112.4	d	6.64 (1H, s)	112.6	d	6.64 (1H, s)	112.4	d	6.64 (1H, s)
6"	140.5	s		140.8	s		144.3	s		140.9	s		141.1	s		140.9	s	
1-carbonyl	*170.3	s		*171.1	s		*175.8	s					*176.2	s		*175.6	s	
2-OMe	55.5	q	3.55 (3H, s)	62.2	q	3.82 (3H, s)												
3-Me				10.6	q	2.29 (3H, s)	10.7	q	2.41 (3H, s)	18.4	q	2.44 (3H, s)	9.8	q	2.38 (3H, s)	10.7	q	2.43 (3H, s)
5-Me	11.9	q	2.12 (3H, s)	13.2	q	2.13 (3H, s)	13.7	q	2.19 (3H, s)	12.9	q	2.12 (3H, s)				13.4	q	2.23 (3H, s)
6-Me	16.9	q	2.30 (3H, s)	17.1	q	2.25 (3H, s)	18.9	q	2.60 (3H, s)	20	q	2.01 (3H, s)	24.2	q	2.68 (3H, s)	18.7	q	2.63 (3H, s)
1'-carbonyl	*166.7	s		*166.4	s		*168.9	s		*169.2	s		*166.9	s		*166.5	s	
2'-OMe	62	q	3.76 (3H, s)	56	q	3.68 (3H, s)		q					62.6	q	3.75 (3H, s)	62.3	q	3.71 (3H, s)
3'-Me	9.9	q	2.08 (3H, s)				10.8	q	2.24 (3H, s)	19.7	q	2.19 (3H, s)	10.4	q	2.07 (3H, s)	10.4	q	2.08 (3H, s)
5'-Me	12.5	q	1.92 (3H, s)	12.5	q	1.93 (3H, s)	13.3	q	2.03 (3H, s)	13.1	q	1.98 (3H, s)	13.1	q	1.92 (3H, s)	13	q	1.94 (3H, s)
6'-Me	16.5	q	2.19 (3H, s)	17.5	q	2.28 (3H, s)	18.4	q	2.41 (3H, s)	10.5	q	2.44 (3H, s)	17.1	q	2.23 (3H, s)	17.3	q	2.24 (3H, s)
1"-carbonyl	*170.3	s		*171.1	s		*169.9	s		*170.8	s		*170.9	s		*170.7	s	
3"-Me	8.3	q	2.37 (3H, s)	8.7	q	2.38 (3H, s)				8.7	q	2.37 (3H, s)	8.9	q	2.36 (3H, s)	8.7	q	2.36 (3H, s)
5"-Me																		
6"-Me	24.6	q	2.57 (3H, s)	24.7	q	2.55 (3H, s)	24.6	q	2.62 (3H, s)	25	q	2.59 (3H, s)	25.1	q	2.57 (3H, s)	25	q	2.58 (3H, s)

[a] J = 2.3 Hz.

*Assignment is ambiguous because of no HMBC correlation.

4). The HMBC experiment revealed three benzene rings (**A**~**C**). The long-range couplings from three methyl protons [3-CH_3 (δ 2.29) to C-2 (δ 153.9), C-3 (δ 122.6) and C-4 (δ 149.1); 5-CH_3 (δ 2.13) to C-4, C-5 (δ 126.1) and C-6 (δ 132.4); 6-CH_3 (δ 2.25) to C-5, C-6 and C-1 (δ 130.9)] indicated the presence of 2,4-dioxygenated-3,5,6-trimethylbenzene ring (**A**). The long-range coupling from 2-OCH_3 (δ 3.82) to C-2 indicated the attachment of the methoxy group to the 2 position of the benzene ring (**A**). The second benzene ring (**B**) was proven by the long-range couplings from two methyl protons [5'-CH_3 (δ 1.93) to C-4' (δ 151.1), C-5' (δ 122.6) and C-6' (δ 137.4); 6'-CH_3 (δ 2.28) to C-5', C-6' and C-1' (δ 122.2)] and an olefinic proton [H-3' (δ 6.95) to C-2' (δ 156.0), C-4' and C-5']. The long-range coupling from 2'-OCH_3 (δ 3.68) to C-2' also indicated the attachment of the methoxy group to the 2' position of the benzene ring (**B**). From its chemical shift, C-4' also bears oxygen. The third benzene ring (**C**) was proved by the long-range couplings from two methyl protons [3"-CH_3 (δ 2.38) to C-2" (δ 164.7), C-3" (δ 110.2) and C-4" (δ 163.0); 6"-CH_3 (δ 2.55) to C-5" (δ 112.2), C-6" (δ 140.8) and C-1" (δ 103.6)] and an olefinic proton [H-5" (δ 6.62) to C-1" and C-3"]. Again, from their chemical shifts, C-2" and C-4" bear oxygen. From the UV spectra and chemical shifts of C-1, C-1' and C-1", three carbonyl groups (δ 171.1, 171.1 and 166.4) should be attached to the C-1, C-1' and C-1" like other known thielavins, although the assignment of these carbonyl groups might be interchangeable because of no HMBC correlation. The connection of these three benzoyl groups (**A**, **B** and **C**) and the residual hydroxy groups was established by the analysis of the positive FAB-MS fragmentation as shown in Fig. 4. The positive FAB-MS of **2** gave the fragment ion peaks at *m/z* 389 (weak), 343 (weak), 179 (weak) and 165 (strong). Two fragment ion peaks at *m/z* 343 and 165 indicated the successive elimination of **A** and **B** from **2**, respectively. The fragment ion peak at *m/z* 389 also suggested the presence of an **A**-**B** unit. All of the other sequential possibilities of units **A**, **B** and **C** cannot make the fragment ions described above. As the connection of the three benzoyl groups was

Table 2-2. ^{1}H- and ^{13}C-NMR chemical shifts of new thielavin analogs **7**~**11** in pyridine-d_5.

Position	7[a]			8			9			10			11		
No.	^{13}C (ppm)		^{1}H (ppm)	^{13}C (ppm)		^{1}H (ppm)	^{13}C (ppm)		^{1}H (ppm)	^{13}C (ppm)		^{1}H (ppm)	^{13}C (ppm)		^{1}H (ppm)
1	118.7	s		115.1	s		122.9	s		115.3	s		115.1	s	
2	155.1	s		159.7	s		156.2	s		159.9	s		156.2	s	
3	117.5	s		116.6	s		104.9	d	6.94 (1H, s)	116.9	s		118.2	s	
4	151.2	s		151.9	s		149.9	s		152.1	s		151.7	s	
5	121.5	s		120.7	s		122.5	s		120.9	s		120.6	s	
6	135.3	s		138.1	s		137.6	s		138.3	s		138.1	s	
1'	127.1	s		127.2	s		116.2	s		127.4	s		117.6	s	
2'	154.9	s		154.9	s		160.1	s		155.1	s		159.8	s	
3'	122.7	s		122.7	s		116.7	s		122.9	s		116.5	s	
4'	150.7	s		149.4	s		151.2	s		150.7	s		151.9	s	
5'	126.4	s		126.5	s		120.8	s		126.7	s		122	s	
6'	133.9	s		133.9	s		138.3	s		134.1	s		135.7	s	
1"	103.1	s		104.9	s		103.8	s		106	s		105.8	s	
2"	165	s		166.1	s		164.9	s		161.2	s		161.1	s	
3"	110.3	s		102.2	d	6.71 (1H, d)[b]	110.3	s		110.4	s		110.2	s	
4"	163.2	s		165	s		163.1	s		160.8	s		160.6	s	
5"	112.4	d	6.64 (1H, s)	113.2	d	6.64 (1H, d)[b]	112.4	d	6.62 (1H, d)	118.2	s		118	s	
6"	140.9	s		144	s		140.9	s		137.7	s		137.6	s	
1-carbonyl	170.3	s		*175.6	s		*171.2	s		*175.8	s		*175.6	s	
2-OMe							56.1	s	3.67 (3H, s)						
3-Me	10.7	q	2.39 (3H, s)	10.4	q	2.44 (3H, s)				10.7	q	2.45 (3H, s)	10.8	q	2.41 (3H, s)
5-Me	13.1	q	2.12 (3H, s)	13.4	q	2.23 (3H, s)	12.6	q	1.93 (3H, s)	13.6	q	2.24 (3H, s)	13.3	q	2.20 (3H, s)
6-Me	17.9	q	2.26 (3H, s)	18.7	q	2.62 (3H, s)	17.7	q	2.29 (3H, s)	18.9	q	2.63 (3H, s)	19.4	q	2.60 (3H, s)
1'-carbonyl	*166.5	s		*166.5	s		*166.5	s		*166.7	s		*168.7	s	
2'-OMe	62.3	q	3.70 (3H, s)	62.3	q	3.71 (3H, s)				62.5	q	3.72 (3H, s)			
3'-Me	10.3	q	2.09 (3H, s)	10.4	q	2.14 (3H, s)	10.7	q	2.45 (3H, s)	10.6	q	2.17 (3H, s)	10.6	q	2.27 (3H, s)
5'-Me	13.1	q	1.94 (3H, s)	13.1	q	2.00 (3H, s)	13.6	q	2.22 (3H, s)	13.4	q	2.02 (3H, s)	13.1	q	2.06 (3H, s)
6'-Me	17.3	q	2.23 (3H, s)	17.3	q	2.25 (3H, s)	18.9	q	2.65 (3H, s)	17.5	q	2.26 (3H, s)	18.3	q	2.42 (3H, s)
1"-carbonyl	*170.7	s		*169.6	s		*171.2	s		*170.9	s		*170.8	s	
3"-Me	8.7	q	2.38 (3H, s)				8.9	q	2.37 (3H, s)	10.1	q	2.42 (3H, s)	9.9	q	2.41 (3H, s)
5"-Me										13.2	q	2.30 (3H, s)	13.5	q	2.30 (3H, s)
6"-Me	25	q	2.58 (3H, s)	24.4	q	2.61 (3H, s)	24.9	q	2.55 (3H, s)	19.6	q	2.61 (3H, s)	18.7	q	2.61 (3H, s)

[a] The glycerol part of 7: 4.83 (1H, dd, J = 11.1 and 4.3 Hz), 4.72 (1H, J = 11.1 and 5.9 Hz), 4.40 (1H, m) and 4.03 (2H, d, J = 5.9 Hz).
[b] J = 2.3Hz.
*Assignment is ambiguous because of no HMBC correlation.

established, the position of the three hydroxy groups were settled to the remaining three open bonds. Thus, the structure of **2** was determined to be 4-[4'-(2",4"-dihydroxy-3",6"-dimethylbenzoyloxy)-5',6'-dimethyl-2'-methoxybenzoyloxy]-2-methoxy-3,5,6-trimethylbenzoic acid as shown in Fig. 1.

Structures of thielavin I (**4**) and thielavin L (**7**) were also determined by the same procedure described above. During the analysis, it became clear that **4** has a hydrogen at C-1 and **7** has a carboxylic glyceride at C-1 by HMBC spectra and their molecular formula.

Biological Properties

G6Pase-inhibitory activity of all new thielavins isolated, known thielavins A~D and three partial hydrolysates (**12**~**14**, see Fig. 5 and Experimental section) of thielavins A and B were examined (G6Pase Inh. in Table 3).

The most potent compound showing G6Pase inhibition was thielavin G (**2**, IC_{50}=0.33 μM). The activity was 15-fold stronger than that of thielavin B. Thielavins A, B, C, D, **1**, **3**, **5**, **8** and **9** showed moderate activities (IC_{50}=1.3~7.4 μM). These compounds showed inhibitory activities of glucose output from glucagon-stimulated hepatocytes (about 20~60% inhibition: Glc. output Inh. in Table 3) except for **9**. Thielavins **6**, **7**, **10** and **11** had lower activities (IC_{50}=16.8~36.2 μM). Thielavin I (**4**, no carboxylic acid), thielavin L (**7**, glycerol ester) and three partial hydrolysates (**12**~**14**) showed almost no activity for G6Pase inhibition.

Discussion

There are some compounds, such as 2-hydroxy-5-nitrobenzaldehyde and chlorogenic acid[6], that have been identified as weak G6Pase inhibitors in the past, while synthetic derivatives of chlorogenic acid were recognized as more potent inhibitors of glucose-6-phosphate translo-

Fig. 3. Assignments of ^{1}H- and ^{13}C-NMR and HMBC correlations on thielavin G (**2**).

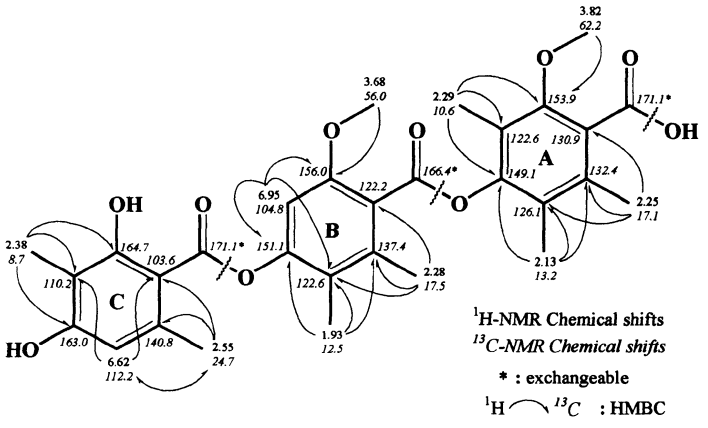
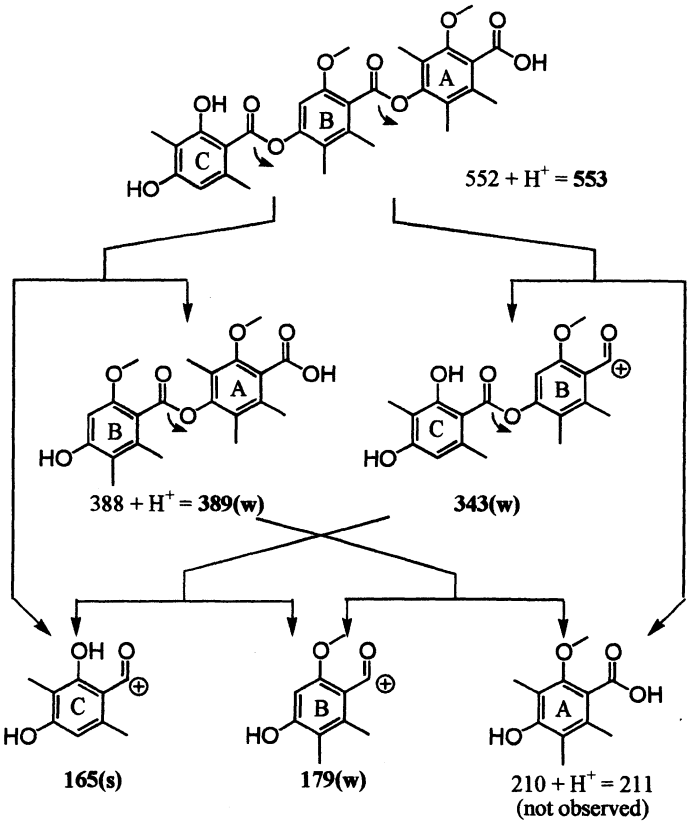


Fig. 4. Positive FAB-MS fragmentation of thielavin G (**2**).



case[7,8]. Here, we presented 11 new thielavin analogs together with 4 known thielavins A~D as a new structural type of G6Pase inhibitors.

Considering the structure-activity relationship of thielavin analogs, 3 benzoic acid-units and carboxylic acid at the end are essential for G6Pase-inhibitory activity. Further, the hydroxy group and hydrogen on the benzene rings are preferable to methoxy and methyl groups,

Fig. 5. Structures of CJ-20,556 (**12**), CJ-20,557 (**13**) and CJ-20,558 (**14**).

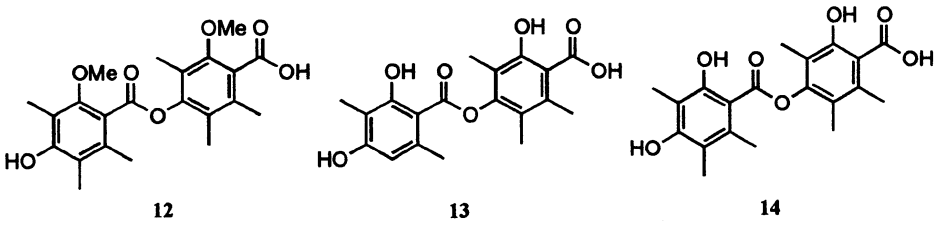


Table 3. G6Pase inhibitory activity and glucose output inhibitory activity of thielavin analogs.

Sample ID	G6Pase Inh. IC_{50} (μM)[a]	Glc. output Inh. (%)[b]
Thielavin A	4.6	19
Thielavin B	5.5	33
Thielavin C[c]	7.2	43
Thielavin D[c]	7.4	39
Thielavin F (**1**)	1.4	26
Thielavin G (**2**)	0.33	30
Thielavin H (**3**)	1.3	57
Thielavin I (**4**)	>202	NT
Thielavin J (**5**)	7.2	67[d]
Thielavin K (**6**)	36.2	NT
Thielavin L (**7**)	20.8	NT
Thielavin M (**8**)	5.6	33
Thielavin N (**9**)	5.6	0
Thielavin O (**10**)	16.8	NT
Thielavin P (**11**)	18.1	NT
CJ-20,556 (**12**)	249	NT
CJ-20,557 (**13**)	183	NT
CJ-20,558 (**14**)	88.2	NT

NT: not tested.
[a] G6Pase inhibitory activity (IC_{50}, μM).
[b] Inhibition (%) of glucose output from GGN stimulated hepatocytes, assayed at x30 higher concentration of G6Pase-inhibitory activity (IC_{50}), at 100 μM (thielavins B, C and D) and at 97.5 μg/ml (CJ-20,089).
[c] Authentic sample previously identified from a different fungus in our laboratory.
[d] The result is ambiguous due to the toxicity.

respectively, for stronger G6Pase inhibitory activity.

The data we present here does not demonstrate a clear correlation between the G6Pase inhibition and the inhibition of glucose output from glucagon-stimulated rat-hepatocytes. However, since each measurement of glucose output was made at a single concentration of compound, rather than over a range of concentrations that would permit an estimate of the EC_{50}, this incomparable correlation may not be significant at this point. On the other hand, this might be because of compound's properties such as low solubility, affinity and permeability to membrane, easiness to be metabolized, and toxicity to the rat-liver cells, *etc.* We need further study to clarify them.

Thielavins were originally isolated as inhibitors of prostaglandin biosynthesis[3,4], and later they were found to be PLA2 inhibitors with thielocins which have dimeric structures of thielavins[5,9~12]. More recently, thielavins have been claimed as telomerase inhibitors[13], cell wall trans-glycosylation inhibitors[14], teststerone 5a-reductase inhibitors[15], PLC inhibitors[16] and antibiotics[17]. Here, we report the novel use of thielavins as G6Pase inhibitors. Recently, a new thielavin-type compound that consists of 4 benzoic acid-units has been reported as a G6Pase inhibitor[18]. It is our interest to study the G6Pase-inhibitory activity of other thielavins, especially thielocins with dimeric structure, to identify and develop compounds suitable for the treatment of diabetes.

Experimental

General

Spectral and physico-chemical data were obtained by the following instruments: UV, JASCO Ubest-30; IR, Shimadzu IR-470; NMR, JEOL LAMBDA270; FAB-MS, JEOL JMS-700; Optical rotations, JASCO DIP-370 with a 5 cm cell. All NMR spectra were measured in pyridine-d_5 unless otherwise indicated and peak positions are expressed in parts per million (ppm) based on the reference of a pyridine peak at δ 8.55 ppm for ^{1}H-NMR and δ 149.8 ppm

for ^{13}C-NMR. The peak shapes are denoted as follows: s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), br (broad) and sh (shoulder). All FAB-MS spectra were measured using glycerol as a matrix.

Analytical HPLC of Thielavin Analogs

For the retention times in Table 1, following conditions were applied to the analytical HPLC: column=YMC ODS-AM (4.6×50 mm, 3 μ), solvent A=acetonitrile with 0.05% TFA (v/v), solvent B=MeOH with 0.05% TFA (v/v), solvent C=H_2O with 0.05% TFA (v/v), flow rate=0.9 ml/minute, temperature=40°C.

Liner gradient system with acetonitrile (for the Rt/MeCN in Table 1): start with 5%-A/C, go up to 30% at 3.0 minutes, increase to 70% at 23.0 minutes, to 100% at 26.0 minutes and stay with 100%-A till 28.5 minutes. On this acetonitrile-condition, known thielavins A, B, C, D and partial hydrolysates (**12**~**14**) showed their Rt (minutes) at 19.0, 16.9, 18.9, 15.2, 9.8, 11.5 and 13.5, respectively.

Liner gradient system with methanol (for Rt/MeOH in Table 1): start with 5%-B/C, immediately go up to 55% at 0.5 minutes, keep increasing to 85% at 25.0 minutes, to 100% at 26.0 minutes and stay 100%-B till 28.5 minutes. On this methanol-condition, known thielavins A, B, C, D and partial hydrolysates (**12**~**14**) showed their Rt (minutes) at 19.4, 15.0, 17.3, 12.8, 5.4, 8.3 and 10.8, respectively.

Preparative HPLC

For the isolation of thielavin analogs, following conditions were applied to the preparative HPLC: column=YMC ODS-AM (5 μ, 120 Å, 20×250 mm with 20×50 mm guard column), solvent A=MeCN with 0.1% TFA (v/v), solvent B=MeOH with 0.1% TFA (v/v), solvent C=H_2O with 0.1% TFA (v/v), at room temperature, UV monitoring=at 220 nm.

Condition #1: flow rate=20 ml/minute, gradient system=stay at 55%-A/C in the first 55 minutes and linear gradient to 80%-A/C in the next 25 minutes, sample injection=*ca.* 300 mg in 1.0 ml MeOH solution, fractionation=void first 80 ml (4 minutes) and then collected every 10 ml (0.5 minutes). The separation was repeated 14 times and the fractions were combined.

Condition #2: flow rate=10 ml/minute, linear gradient from 65%-B/C to 70%-B/C in the first 120 minutes and to 80%-B/C in the next 60 minutes, fractionation=void first 50 ml (5 minutes) and then collected every 10 ml (1 minute).

Condition #3: flow rate=10 ml/minute, linear gradient from 70%-B/C to 80%-B/C in the first 120 minutes and to 90%-B/C in the next 60 minutes, fractionation=void first 50 ml (5 minutes) and then collected every 10 ml (1 minute).

Condition #4: flow rate=10 ml/minute, linear gradient from 75%-B/C to 85%-B/C in the first 120 minutes and to 100%-B in the next 60 minutes, fractionation=void first 50 ml (5 minutes) and then collected every 10 ml (1 minute).

Partial Hydrolysis of Thielavin B

Thielavin B (20.0 mg) was stirred with a mixture of 2.0 ml of 2 N NaOH and 2.0 ml of dioxane, at 50°C for 24 hours. To the reaction mixture, 5 ml of 1 N HCl and 40 ml of H_2O were added. The solution was extracted with 50 ml of EtOAc, twice. The EtOAc solution was passed through anhydrous Na_2SO_4 column (1.5×7 cm), and concentrated to pure CJ-20,556[11,19] (**12**, 19.3 mg).

CJ-20,556 (**12**): Colorless glass; HRFAB-MS $(M-H)^-$= *m/z* 401.1596 calcd for $C_{22}H_{25}O_7$ (Δ −0.4 mmu); UV λ_{max} 254 nm; IR ν_{max} (KBr) 3501, 2941, 1711, 1580, 1462, 1410, 1323, 1292, 1221, 1175, 1094, 1080, 1057, 1040, 974, 770, 708, 664 and 509 cm^{-1}; LRFAB-MS (positive) *m/z* 403 and 193; LRFAB-MS (negative) *m/z* 401, 209 and 165; ^{1}H-NMR (pyridine-d_5) δ 9.11 (2H, br), 3.81 (3H, s), 3.74 (3H, s), 2.31 (9H, s), 2.24 (3H, s), 2.22 (3H, s) and 2.16 (3H, s).

Partial Hydrolysis of Thielavin A

Thielavin A (51.5 mg) was stirred with a mixture of 5.0 ml of 2 N NaOH and 5.0 ml of dioxane, at 50°C for 32 hours. To the reaction mixture, 40 ml of 1 N HCl and 50 ml of H_2O were added. The solution was extracted with 100 ml of EtOAc, twice. The EtOAc solution was passed through anhydrous Na_2SO_4 column (1.5×7 cm), and concentrated to the dryness (52.5 mg). Pure CJ-20,557 (**13**, 2.7 mg), CJ-20,558 (**14**, 4.1 mg) and non-reacted thielavin A (7.3 mg) were obtained after HPLC separation: same condition as Preparative HPLC described above with flow rate=10 ml/minute, linear gradient system=5%-A/C for first 10 minutes, then increase to 70% at 140 minutes, and to 100% at 150 minutes; Rt=118, 128 and 149 minutes, respectively.

CJ-20,557 (**13**): Colorless glass; HRFAB-MS $(M-H)^-$=*m/z* 359.1141 calcd for $C_{19}H_{19}O_7$ (Δ +1.1 mmu); UV λ_{max} 216, 274 and 308 nm; IR ν_{max} (KBr) 3408 (br), 3126 (br), 2932, 1653, 1622, 1501, 1440 (sh), 1416, 1385, 1308, 1267, 1204, 1150, 1098, 1074, 1030, 841, 800, 725, 708, 623, 600, 583 and 471 cm^{-1}; LRFAB-MS (positive) *m/z* 361, 193, 179, 165 and 157; LRFAB-MS (negative) *m/z* 359, 195, 181, 177 and 163; ^{1}H-NMR (pyridine-d_5) δ 6.61 (1H, s), 2.62 (3H, br s), 2.56 (3H, s), 2.36 (3H, s), 2.18 (3H, s) and 1.96 (3H, s).

CJ-20,558 (**14**): Colorless glass; HRFAB-MS $(M-H)^-=m/z$ 373.1287 calcd for $C_{20}H_{21}O_7$ (Δ 0.0 mmu); UV λ_{max} 216, 276 and 317 nm; IR ν_{max} (KBr) 3499, 3130 (br), 2932, 1651, 1614, 1589 (sh), 1450 (sh), 1425, 1385, 1321, 1283, 1269 (sh), 1210 (sh), 1177, 1124, 1097, 1074, 1028, 837, 806, 795, 772, 725, 696, 681, 596 and 519 cm^{-1}; LRFAB-MS (positive) *m/z* 375, 179 and 157; LRFAB-MS (negative) *m/z* 373, 195 and 177; ^{1}H-NMR (pyridine-d_5) δ 2.60 (3H, br s), 2.59 (3H, s), 2.40 (3H, s), 2.28 (3H, s), 2.25 (3H, s) and 2.03 (3H, s).

Preparation of Rat-liver Microsomes

Microsomes were prepared from the livers of fasted Sprague-Dawley rats. Livers were removed from the rats anesthetized by ether, and immediately washed with saline. Liver weights were recorded and the livers chopped and homogenized for 30 seconds (Polytron, level 6) with homogenization buffer (250 mM sucrose, 25 mM HEPES, 2.5 mM EDTA and 0.1 mM phenylmethylsulfonyl fluoride, pH 7.4). After centrifugation for 10 minutes at 4°C (12,000 *g*, 11,000 rpm), the resulting supernatant was further centrifuged for 60 minutes at 4°C (100,000 *g*, 29,500 rpm). The resulting microsome pellet was re-suspended in homogenization buffer at a concentration equivalent to 1 g original wet liver per ml, and stored in aliquots in a freezer at −80°C. For the G6Pase-inhibition assay described below, this microsome suspension was further diluted 60 times with assay buffer.

Determination of G6Pase-inhibitory Activity

The activity of the microsomal G6Pase was measured based on the colorimetric reaction of inorganic phosphate. The enzyme reaction was initiated by the addition of 20 μl of microsome suspension (see above) to the reaction mixture, containing 70 μl of assay buffer (50 mM HEPES, 100 mM KCl, 2.5 mM EGTA, 2.5 mM $MgCl_2$ 1 mM DTT and 1.43 mM G6P, pH 7.2 by KOH) and 10 μl of the test compound in 10% (v/v) DMSO. After a 15 minutes incubation at room temperature, 150 μl of the colorimetric reagent (mixture of 1 part of 34 mM ammonium molybdate in 4 N HCl and 3 parts of 1.3 mM malachite green in water) was added, and the optical absorbance at 690 nm (A_{690}) was measured. Percent inhibition of G6Pase activity was calculated by the formula below: in which A_{690} (background) is the absorbance when we used S 4048 as a test compound (final concentration=1.65 μM), since S 4048 is known to inhibit G6P transport into microsomes[8,20].

Inhibition of Glucose Output from Glucagon (GGN)-stimulated Rat-hepatocytes

Hepatocytes were prepared from the livers of fed male Sprague-Dawley rats by collagenase digestion[21] and incubated as described previously[8]. The cells were suspended into Krebs-Ringer bicarbonate (KRB) buffer, pH 7.4, containing 2.5 mM $CaCl_2$ and 1% (w/v) gelatin, at a final concentration of about 6.0×10^6 cells/ml. The following incubations were run in 25 ml-flask at 37°C (in a shaking water bath) with continuous gas-flow of 95% O_2 and 5% CO_2. The cells suspension was divided into four test conditions, each run in quadruplicate. Two sets of flasks received a test compound dissolved in DMSO (0.1% final v/v) and the other two sets received the DMSO vehicle (0.1% final v/v) alone. All flasks were pre-incubated for 5 minutes, and then, aliquots of the cell suspension were taken for measurement of glucose in the medium. GGN (20 μl, final concentration 25 nM) was added to one of the two sets of flasks with a test compound, and to one of the sets of vehicle flasks. The remaining flasks received 20 μl of the KRB buffer as a control. All flasks of hepatocyte were incubated for 30 minutes, after which aliquots of cell suspension were taken for the measurement of glucose. After centrifugation of each aliquot of cell suspension collected, the glucose concentration in the supernatant was measured using a CCX Spectrum Systems Autoanalyzer (Abbott, North Chicago, IL). The difference of glucose concentration (ΔGlc) between the 5 minutes- and the 30 minutes-incubations for each flask was calculated. Percent inhibition of glucose output was calculated as follows:

$$\text{Inhibition (\%)}=\left[\frac{\Delta\text{Glc (total)}-\Delta\text{Glc (sample)}}{\Delta\text{Glc (total)}-\Delta\text{Glc (basal)}}\right]\times100$$

where ΔGlc (total) was glucose output by cells treated with glucagon but without test compound, ΔGlc (sample) was glucose output by cells treated with both glucagon and test compound, and ΔGlc (basal) was glucose output by untreated cells. ΔGlc with test compound but without GGN was monitored to check for inhibition of basal glucose output by the test compound.

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