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Characterization of B-5354c, a New Sphingosine Kinase Inhibitor, Produced by a Marine Bacterium

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B-5354c is a new inhibitor of sphingosine kinase from a novel marine bacterium, SANK 71896. Kinetic study revealed that B-5354c inhibits sphingosine kinase with a *Ki* value of 12 μM. The inhibition is noncompetitive with respect to sphingosine. The compound also inhibits sphingosine-1-phosphate formation in human platelets. Experiments using synthetic derivatives of B-5354c indicate that all the three functional groups, *i.e.*, the long unsaturated aliphatic chain, 4-amino and 3-hydroxyl groups are necessary to inhibit sphingosine kinase.

The metabolic product of sphingosine (SPH) kinase, SPH-1-phosphate (SPP) is a bioactive sphingolipid metabolite which has diverse biological functions. It acts as a second messenger to regulate proliferation and survival intracellulary, and as a ligand for G protein-coupled receptors of the Edg-1 subfamily extracellularly[1,2].

SPP levels are mainly dependent on the activity of SPH kinase, but the precise regulation mechanisms of the enzyme remain unclear. Since low molecular weight inhibitors will be powerful tools to analyze the functions of the enzyme, we screened for inhibitors of SPH kinase in natural extracts and found new SPH kinase inhibitors; B-5354a, b and c (B-5354s, Fig. 1) in the culture broth of a novel marine bacterium, SANK 71896. In the preceding paper[3], we described the taxonomy of SANK 71896, and the fermentation, isolation, physico-chemical properties and structure determination of the B-5354s.

D,L-*Threo*-dihydro-SPH and *N,N*-dimethyl-SPH (DMS) were reported to inhibit SPH kinase in μM order[4,5], however they also have been reported to have several physiological functions besides the inhibition of SPH kinase probably on account of their structural similarity to SPH. For example, DMS acts as a potent inhibitor of PKC[6,7] and it induces apoptosis in a variety of human cell lines[8]. On the other hand, B-5354s, esters of 4-amino-3-hydroxybenzoic acid, are not structural analogs of SPH. Hence, the B-5354s are expected not to exhibit such unfavorable activities observed in SPH analogs. To shed light on the characteristics of B-5354s, we made some biological evaluations including kinetic analysis, enzyme inhibition in platelets and investigation of the inhibition specificity with focus on B-5354c that is the major component in the fermentation broth. In this paper, we report these results along with structure-activity relationships of the compound.

Materials and Methods

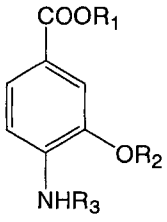
Materials

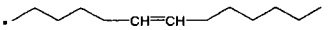
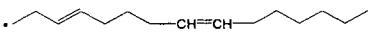
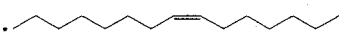
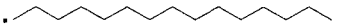
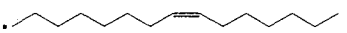
[3-^{3}H]D-*Erythro*-SPH (20 Ci/mmol) was purchased from Du Pont-New England Nuclear. SPH and SPH-1-phosphate (SPP) were from Matreya. *N,N*-DimethylSPH (DMS) was from Calbiochem. The 4-amino-3-hydroxybenzoic acid (**3** in Fig. 1) and di-*tert*-butyl dicarbonate were from Tokyo Chemical Industry. B-5354a, b and c were prepared as described in the preceding paper[3].

SPH Kinase Assay in a Cell-free System

As a source of SPH kinase, we used rat liver cytosol as previously described[9]. For the assay of SPH kinase activity in a cell-free system, we used the method of LOUIE *et al.*[10] with some modifications as previously described[9].

Fig. 1. Structures of the B-5354s and their synthetic derivatives.



Name	R_1	R_2	R_3
B-5354a		H	H
B-5354b		H	H
B-5354c		H	H
1		H	H
2		H	H
3	H	H	H
4		H	$COCH_3$
5		CH_3	H

[^{3}H]SPP Formation from [^{3}H]SPH in Platelets

[^{3}H]SPP formation from [^{3}H]SPH in washed human platelets was determined by the method of YATOMI *et al.*[5].

Other Enzyme Assays

Other enzyme assays were carried out as follows: Neutral sphingomyelinase (SMase) activity was evaluated by the method of NARA *et al.*[11] using rat brain microsomes as the enzyme source. Phosphatidylinositol (PI) 3-kinase activity was evaluated by the method of GOLD *et al.*[12] using Jurkat cell lysates as the enzyme source. Protein kinase C (PKC) activity was evaluated by a commercial PKC assay kit (Amersham Pharmacia Biotech) using partially purified rat brain PKC preparation (enriched in α, β and γ isoforms; Upstate Biotechnology).

Preparation of Derivatives of B-5354c

Dihydro-B-5354c (**1**): A solution of B-5354c (13 mg) in 5 ml of MeOH was subjected to hydrogenation over 10% palladium carbon (5 mg) for 2 hours under atomospheric pressure at room temperature. The catalyst was filtered off and the solvent was evaporated. The residue was purified by preparative HPLC (column: Nacalai Tesque COSMOSIL 5C18-AR 20 i.d.×250 mm, flow rate: 6 ml/minute, mobile phase: 85% aqueous acetonitrile, detection: UV absorption at 210 nm, retention time: 24 minutes) to give 7 mg (54%) of **1**. **1**: white powder. Molecular formula: $C_{21}H_{35}NO_3$. ^{1}H NMR (360 MHz, $CDCl_3$): δ 7.61 (1H, d, J=1.7 Hz), 7.50 (1H, dd, J=8, 1.7 Hz), 6.68 (1H, d, J=8 Hz), 4.27 (2H, t, J=6.7 Hz), 1.74 (2H, tt, J=7.3, 6.7 Hz), 1.42 (2H, m), 1.40~1.20 (20H, brs), 0.89 (3H, t, J=6.7 Hz).

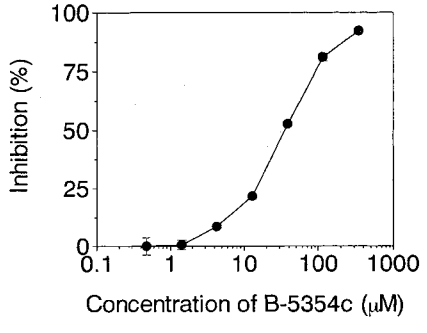
n-Butyl analogue (**2**): To a solution of 4-amino-3-hydroxybenzoic acid (1 g) in 5 ml of CH_2Cl_2, 3.6 g of di-*tert*-butyl dicarbonate and 5 ml of pyridine were added. The reaction mixture was stirred for 1 hour at room temperature. After aqueous workup and extraction with EtOAc, 1.5 g of *N*-Boc-4-amino-3-hydroxybenzoic acid was obtained. To a solution of *N*-Boc-4-amino-3-hydroxybenzoic acid (25 mg) and 4-dimethyl-amino-pyridine (8 mg) in 2 ml of *n*-BuOH, 40 mg of dicyclohexylcarbodimide dissolved in 1 ml of *n*-BuOH was added. The reaction mixture was stirred for 2 hours at room temperature, and then 5 ml of H_2O was added. After removing *n*-BuOH under *vacuo*, the resulting aqueous solution was extracted with EtOAc and the extract was washed with brine, dried over Na_2SO_4 and concentrated *in*

vacuo. The extract was purified by a silica gel column equilibrated with hexane - EtOAc 8 : 1. The major product was concentrated and deprotected by TFA at 0°C. After purification by a silica gel column equilibrated and developed with hexane - EtOAc 4 : 1, 17 mg (82%) of **2** was obtained. **2**: white powder. Molecular formula: $C_{11}H_{15}NO_3$. ^{1}H NMR (360 MHz, $CDCl_3$): δ 7.61 (1H, d, J=1.7 Hz), 7.50 (1H, dd, J=8, 1.7 Hz), 6.67 (1H, d, J=8 Hz), 4.27 (2H, t, J=6.7 Hz), 1.72 (2H, tt, J=7.3, 6.7 Hz), 1.46 (2H, tq, J=7, 6.7 Hz), 0.97 (3H, t, J=7 Hz).

N-Acetyl-B-5354c (**4**): **4** was prepared by *N*-acetylation of B-5354c as described in a previous paper[3].

O-Methyl-B-5354c (**5**): To a solution of B-5354c (11 mg) in 2 ml of CH_2Cl_2, excess amount of diazomethane dissolved in ethylether was added. The mixture was stirred for 16 hours at room temperature. After concentration *in vacuo* and purification by HPLC (column: Nacalai Tesque COSMOSIL 5C18-AR 20 i.d.×250 mm, flow rate: 6 ml/minute, mobile phase: 85% aqueous acetonitrile, detection: UV absorption at 210 nm, retention time: 51 minutes) to give 2 mg (17%) of **5**. **5**: white powder. Molecular formula: $C_{22}H_{35}NO_3$ (HR-FABMS ($M^{+\cdot}$), *m/z* 361.2644, Δ +2.7 mmu). ^{1}H NMR (360 MHz, $CDCl_3$): δ 7.55 (1H, dd, J=8, 1.6 Hz), 7.46 (1H, d, J=8, 1.6 Hz), 6.66 (1H, d, J=8 Hz), 5.40~5.30 (2H, m), 4.26 (2H, t, J=6.7 Hz), 3.90 (3H, s), 2.0 (4H, m), 1.75 (2H, m), 1.49~1.21 (14H, m), 0.88 (3H, t, J=7 Hz). ^{13}C NMR (90 MHz, $CDCl_3$): δ 166.9 (s), 146.2 (s), 141.0 (s), 130.1 (d), 129.7 (d), 124.0 (d), 120.0 (s), 113.1 (d), 111.3 (d), 64.6 (t), 55.6 (q), 31.8 (t), 29.8 (t), 29.7 (t), 29.0 (t), 29.0 (t), 28.9 (t), 27.3 (t), 27.1 (t), 26.0 (t), 22.7 (t), 14.1 (q).

Fig. 2. Inhibitory effect of B-5354c on SPH kinase activity in a cell-free system.

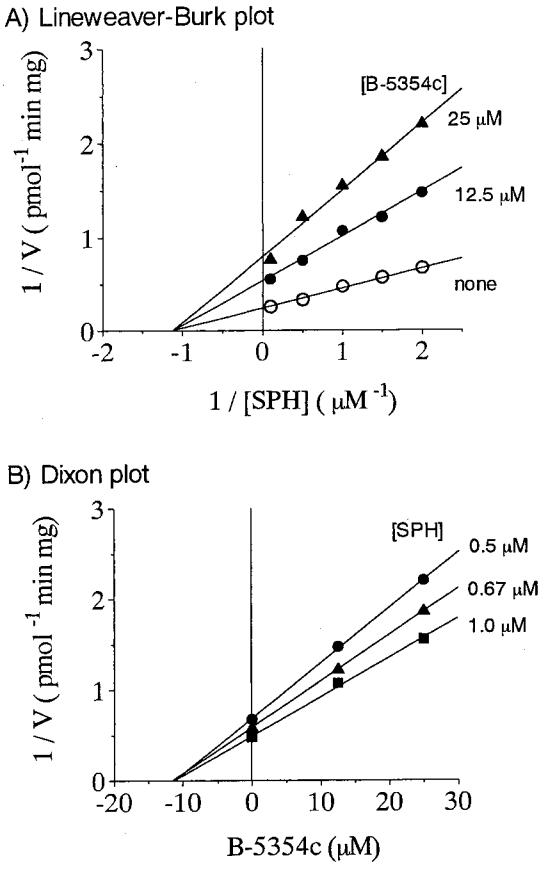


Results

Kinetic Studies

B-5354c inhibited rat liver SPH kinase in a dose-dependent manner with an IC_{50} value of 38 μM as shown in Fig. 2. To characterize the further inhibitory properties of B-5354c, we analyzed the kinetics using classical Michaelis-Menten methods. Lineweaver-Burk plot analysis, as shown in Fig. 3A, indicated that B-5354c decreased the apparent Vmax values in a dose-dependent manner while the *Km* value for SPH remained unaltered. Thus, B-5354c clearly showed noncompetitive-type inhibition with respect to SPH, suggesting that B-5354c and SPH do not share a common binding site on the SPH kinase. Further evidence for a noncompetitive-type inhibition was provided by the Dixon plot analysis in Fig. 3B. The *Ki* value was calculated 12 μM by both plots.

Fig. 3. Kinetic analyses of SPH kinase inhibition.



A) The *Km* value for SPH is unchanged at 1.0 μM. Vmax values for control, 12.5 μM B-5354c and 25 μM B-5354c are 4.3, 1.9 and 1.3 pmol/minute/mg, respectively.

Table 1. Activities of the B-5354s and their synthetic derivatives.

Compounds	Inhibition of SPK *	Inhibition of SPP formation in platelets **
Natural products		
B-5354a	21	93
B-5354b	58	65
B-5354c	38	91
Synthetic derivatives		
1	> 500	11
2	> 500	0
3	> 500	6
4	> 500	17
5	> 500	8

* IC_{50} value (μM) in a cell-free assay system using rat liver cytosol as the enzyme source.

** SPP/SPH ratio (% inhibition at 30 μg/ml).

SPH Kinase Inhibition in Human Platelets

We evaluated the effect of B-5354c on [^{3}H]SPP formation from [^{3}H]SPH in human platelets. As reported previously by YATOMI *et al.*[5], platelets are good tools for analyzing SPH kinase activity in intact cells because they lack the SPP degradation pathway. Therefore, the radioactivity added as [^{3}H]SPH is rapidly converted to [^{3}H]SPP by SPH kinase and remains unchanged as [^{3}H]SPP in the platelets. As shown in Fig. 4, B-5354c inhibited the SPP formation in a dose-dependent manner even when added extracellularly, suggesting that B-5354c permeates the plasma membrane and inhibits SPH kinase in intact cells. In a control study, DMS also inhibited the SPP formation.

Specificity of B-5354c

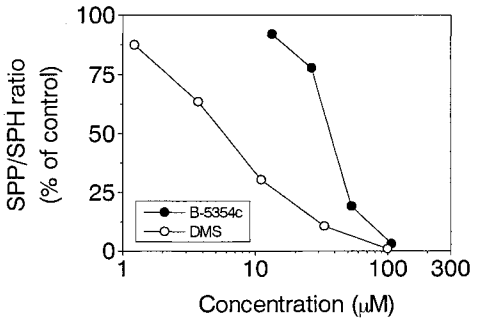
In order to assess the specificity of B-5354c, we evaluated the effects of the compound on other sphingolipid metabolic enzyme, neutral SMase and on other kinases, PI 3-kinase and PKC. B-5354c virtually showed no inhibitory activity towards these enzymes at 500 μM. These results indicate that B-5354c is a specific inhibitor for SPH kinase.

Structure-activity Relationships

B-5354c is a di-substituted benzoic acid ester with unsaturated alcohol. To access which functionality may contribute to the inhibitory activity, derivatives of B-5354c, **1**~**5**, were prepared (Fig. 1). The activities of these compounds along with the natural products were summarized in Table 1. The amino group and the phenolic hydroxyl group were essential for the activity since *N*-acetate (**4**) and methyl ester (**5**) were devoid of the activity. It is noteworthy that unsaturation in the aliphatic side chain was necessary for enzyme inhibition since the derivative bearing a saturated side chain, **1** was inactive at 500 μM. Derivatives with a short side chain (**2**) and without an aliphatic chain (**3**) were also inactive. Among the natural products, slight deviation in inhibitory activity was observed maybe reflecting length and number of unsaturated bonds in the aliphatic chain.

In summary, this study indicates that polar head group with an amino and a hydroxyl group as well as an unsaturated aliphatic chain is required for the enzyme inhibition of B-5354c.

Fig. 4. Inhibitory effect of B-5354c on the SPP formation in human platelets.



Discussion

In the present study, we have confirmed that B-5354c is a specific and noncompetitive inhibitor of SPH kinase. These results suggest that B-5354c may interact with a domain distinct from the SPH binding site and regulate the enzyme activity. There are many reports that SPH kinase activities are regulated by growth factors or cytokines, such as platelet-derived growth factor[13], nerve growth factor[14] and tumor necrosis factor-α[15]. Since SPH kinases are activated transiently within a short time after those stimuli[13~15], the kinase activities might be regulated by post-translational mechanisms such as protein-protein interaction or phosphorylation. The precise regulation mechanisms of SPH kinase remain unclear, hence B-5354c may be a useful tool to study the mechanisms.

Concerning the noncompetitive regulation of SPH kinase, it was reported that phosphatidylserine (PS) enhances SPH kinase activity in a noncompetitive manner[16]. Interestingly, PS also needs double bonds in its aliphatic chain to regulate SPH kinase activity just like B-5354c, which we have reported here. Therefore, the double bond in the aliphatic chain of B-5354c may be necessary to interact with SPH kinase or its substrate, SPH.

B-5354c reduces SPP formation in platelets with a dose response similar to that in the cell-free system (Fig. 2, 4). The results implied that B-5354c permeates the plasma membrane and inhibits SPH kinase with high efficiency in intact cells. From these facts, we may use this inhibitor to study the functions of SPH kinase in intact cells.

SPH analogs, especially DMS, have been used as a potent inhibitor of SPH kinase. In practice, as shown in Fig. 4, inhibitory potency of DMS exceeds that of B-5354c. However, DMS has been reported to have several physiological functions besides the inhibition of SPH kinase. For example, DMS acts as a potent inhibitor of PKC[6,7] and it induces apoptosis in a variety of human cell lines[8]. Furthermore, it is reported that DMS is a catabolite resulting from ceramide breakdown under physiological conditions[17] and it can be phosphorylated in activated platelets[18]. This evidence confirmed the need for a more specific inhibitor of SPH kinase. B-5354c may fit our requirement for this, because its inhibition is specific for SPH kinase as far as examined.

In summary, B-5354c is a new, specific and membrane-permeable inhibitor of SPH kinase. The inhibition is noncompetitive with respect to SPH. It may be a useful tool for studying the biological functions and the regulation mechanisms of SPH kinase in intact cells.

Acknowledgment

We wish to thank Dr. F. NARA for measurement of the neutral SMase activity.

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