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Structure-inspired design of a sphingolipid mimic sphingosine-1-phosphate receptor agonist from a naturally occurring sphingomyelin synthase inhibitor

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Ginkgolic acid obtained as sphingomyelin synthase inhibitor from plant extract library inspired the concept of sphingolipid mimic. Ginkgolic acid derived *N*-acyl anilines and ginkgolic acid 2-phosphate (GA2P) mimics ceramide and sphingosine 1-phosphate (S1P) respectively in structure and function. GA2P induced phosphorylation of ERK and internalization of S1P receptor 1 (S1P₁), indicated potent agonist activity. Docking study revealed that GA2P adopts similar binding pose to the bound ligand ML5, which is a strong antagonist of S1P₁.

Discovering enzyme inhibitors and agonists is a fundamental strategy to develop low molecular-weight drugs and biological tools, which significantly advances life science. Protein structure-based drug design *in silico* using structural information from solution-state NMR¹ and X-ray crystallography² often results in finding novel drug candidates that have a substantially different scaffold from the original substrate. However, in the case of membrane proteins, such as a membrane receptor and enzyme, synthetic inhibitors and agonists designed from original substrates are often more efficient because of difficulty with NMR and X-ray methods. This conventional method is not necessarily complimentary even though it is possible to discover partially active inhibitors. Therefore, we initially regarded naturally occurring inhibitors as mimics of the original substrate and developed novel chemical tools with new functions, such as agonist activity, by addition of chemical modifications. In this study, we focused on the membrane protein sphingomyelin synthase (SMS) whose substrate is ceramide and product is sphingomyelin.

Sphingomyelin is a major phospholipid component of the cell membrane and accounts for 85% of the total sphingolipid in humans. Additionally, the sphingomyelin-to-ceramide ratio has an important role in the maintenance of plasma membrane fluidity, regulation of micro-domain structure, and signal transduction. SMS is a membrane enzyme that catalyzes the synthesis of sphingomyelin from ceramide and has two isozymes, SMS1 and SMS2. SMS knockout mice have decreased levels of plasma inflammatory cytokines³ and are resistant to high-fat diet-induced obesity,⁴ insulin resistance,⁵ Alzheimer's disease,⁶ and tumorigenesis.⁷ Therefore, SMS inhibition is a novel therapeutic approach for these diseases. In this communication, we report isolation of ginkgolic acid (15:1) as the first naturally occurring SMS inhibitor from terrestrial plant extracts. Ginkgolic acid is an amphipathic compound composed of a hydrophilic head group from the salicylic acid part and a common hydrophobic tail group from the hydrocarbon chain and has similar physical properties to sphingosine (Figure 1). Because of its structure, we hypothesize that ginkgolic acid is a sphingolipid mimic and chemically modified its structure. As a sphingosine related compound, sphingosine-1-phosphate (S1P) is a mono-phosphorylated sphingosine and a well-known lipid mediator of sphingolipids that interacts with S1P receptor 1 (S1P₁) to regulate the vascular and immune systems.⁸ We also report the agonist activity of ginkgolic acid 2-phosphate (GA2P), which was chemically prepared by the phosphorylation of ginkgolic acid, was examined to understand chemical mimics and create novel chemical tools (Figure 1).

To discover sphingolipid mimics, we screened a natural product extracts library constructed from 650 plants grown wildy or cultivated in Hokkaido, including local medicinal plants, for cell-based inhibition of sphingomyelin synthase (SMS). A methanol extract of *Ginkgo biloba* had higher inhibition activity against SMS, and bioassay-guided fractionation of the *G. biloba* methanol extract resulted in isolation of ginkgolic acid (1, 15:1) as the active compound (Figure 2).

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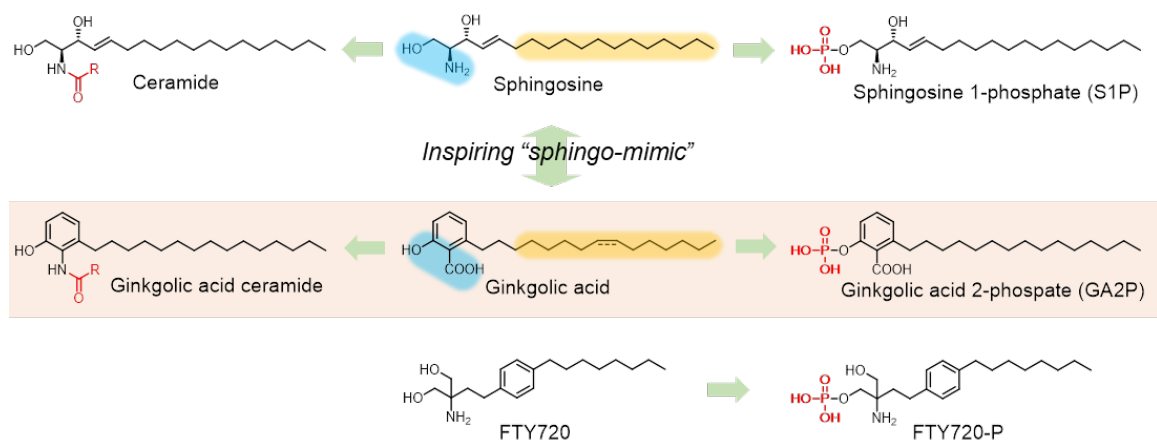


Figure 1. Ginkgolic acid, isolated from *Ginkgo biloba*, inspires sphingo-mimic and its derivatives behave as a lipid mediator.

Ginkgolic acid had relatively moderate inhibition of SMSs compared with that of previously synthesized inhibitors⁹ with IC_{50} of 1.5 μ M on both SMS1 and SMS2 (see supporting information (SI) for more details). Ginkgolic acid was reported to inhibit protein SUMOylation,¹⁰ suppress the development of pancreatic cancer,¹¹ protect against A β -induced synaptic dysfunction in the hippocampus,¹² and inhibit cell proliferation and migration of lung cancer cells.¹³ Ginkgolic acid is a natural salicylic acid with a long alkyl or alkenyl substituent at the ortho position to the carboxyl group, and sphingosine is an unsaturated 18-carbon amino alcohol. Both compounds have hydrophilic and hydrophobic parts in the molecule, and ginkgolic acid structurally resembles sphingosine (Figure 1).

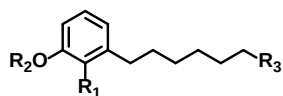
To elucidate the relationship between the structure of ginkgolic acid and its inhibition of SMS, we examined ginkgolic acid derivatives on SMSs in a SAR study. Ginkgolic acid derivatives (**2-5**) were prepared from 6-hydroxy salicylic acid starting material by modifying previously reported methods (Figure 2),¹⁴ and used for SMS1 and SMS2 inhibition assays (Figure 2, see SI). Ginkgolic acid (15:0) **2** with a saturated hydrophobic side chain did not have significantly different SMS inhibitory activity compared with that of **1**.

Compound **3**, which had a reduced chain length from C15 to C8, had less potent SMS inhibition. Methyl esters of ginkgolic acid (15:1), **4** and **5**, were inactive derivatives. Therefore, the carboxylic acid group and long hydrophobic chain of ginkgolic acid are essential moieties for inhibition of SMSs (Figure 2).

To extend the mimic function of this naturally occurring inhibitor, we chemically modified the structure of ginkgolic acid. In terms of structural similarity, the 1-hydroxy and 2-amino groups in sphingosine corresponded to the 2-hydroxy and 1-carboxyl groups respectively, of the salicylic acid portion in ginkgolic acid (Figure 1). Both molecules have long hydrophobic hydrocarbon chains near the hydrophilic parts with and without a double bond. Specifically, the carboxyl group in ginkgolic acid replaces the amino group in sphingosine. These aniline-type sphingosine mimics with C-15 (**6**) and C-8 (**7**) hydrocarbon chains were synthesized in three steps starting from commercially available 3-bromo-2-nitro-anisole (see SI).

To discover potent and selective inhibitors, ceramide mimics were synthesized by further acylation with variety of carboxylic acids. Selective acylation of the 1-amino group was problematic because of additional acylation of the phenolic hydroxyl group in **6**. Therefore, an *O*-methyl derivative of **6** was designated as a starting material and lead to *N*-acylation and *O*-demethylation. Twenty ceramide mimic ginkgolic acid derivatives were synthesized with two different hydrocarbon chain lengths by using various carboxylic acids. Table 1 shows the results of the SMS inhibition assay with these compounds (Table 1, see SI). Potent inhibition by these compounds for SMSs suggested that the modified molecules were ceramide mimics. Various acylation of the amino-modified mimic resulted in significant and selective inhibition of SMS2. Entry 7 and 10 in Table 1 had an IC_{50} of 5 μ M and 2 μ M, respectively, with 8–15 fold selectivity. The derivatives with a moderate hydrophobic alkyl chain might be required to accommodate the binding pocket of SMS1.

Mimic was applied to sphingosine-1-phosphate (S1P), which is the most significant lipid mediator of sphingolipids. Sphingosine-1-phosphate (S1P) receptors constitute a family of five related G protein-coupled receptors (GPCRs) named S1P₁-S1P₅, which are involved in pleiotropic physiological processes,



R = CO	= H,	R ₃ =		
1: R ₁ = CO ₂ H,	R ₂ = H,	R ₃ =	C ₆ H ₁₃	1.5 μ M (1), 1.5 μ M (2)
2: R ₁ = CO ₂ H,	R ₂ = H,	R ₃ =	C ₉ H ₁₉	2.0 μ M (1), 2.0 μ M (2)
3: R ₁ = CO ₂ H,	R ₂ = H,	R ₃ =	Et	20 μ M (1), 30 μ M (2)
4: R ₁ = CO ₂ Me,	R ₂ = Me,	R ₃ =	C ₆ H ₁₃	> 100 μ M (1 and 2)
5: R ₁ = NH ₂ Me,	R ₂ = H,	R ₃ =	C ₆ H ₁₃	> 100 μ M (1 and 2)
6: R ₁ = NH ₂ ,	R ₂ = H,	R ₃ =	C ₉ H ₁₉	50 μ M (1), 30 μ M (2)
7: R ₁ = NH ₂ ,	R ₂ = H,	R ₃ =	Et	50 μ M (1), 50 μ M (2)

Figure 2. Structure-activity relationship study on ginkgolic acid derivatives toward inhibition of SMS. Each concentration number indicates IC_{50} value for SMS1 as (1) and SMS2 as (2), respectively. The IC_{50} values were measured by cell-based assay system: SMS expressing cell lysates (20 mM Tris-buffer, 100 mL) and compounds were incubated for 3 hours at 37°C, then fluorescent lipids were extracted from lysates by the Bligh-Dyer method, and directly applied to HPLC (see SI).

Table 1. Inhibition activity of ceramide mimics toward sphingomyelin synthase 1 and 2. IC₅₀ values were measured by cell-based assay system: SMS expressing cell lysates (20 mM Tris-buffer, 100 mL) and compounds were incubated for 3 hours at 37°C, then fluorescent lipids were extracted from lysates by the Bligh-Dyer method, and directly applied to HPLC (see SI).

Entry	R ₄	R ₅	IC ₅₀ (μM): SMS1	IC ₅₀ (μM): SMS2
1	Me		30	>100
2	Me		15	5
3	Me		70	20
4	Me		7	10
5	Me		5	3
6	C ₈ H ₁₇		>100	>100
7	C ₈ H ₁₇		40	5
8	C ₈ H ₁₇		>100	35
9	C ₈ H ₁₇		15	7
10	C ₈ H ₁₇		30	2

and S1P₁ is critical in the vascular and immune systems.⁸ Fingolimod (FTY720), derived from naturally occurring myriocin produced by *Mycelia sterilia*, is a novel blockbuster drug used to treat relapsing-remitting multiple sclerosis.¹⁵ FTY720 is a prodrug whose active monophosphate (FTY720-P) is metabolized from FTY720 *in vivo* and acts as a functional antagonist of S1P receptors.¹⁶

To examine S1P mimic function of the ginkgolic acid skeleton, ginkgolic acid 2-phosphate (GA2P) was prepared by chemical phosphorylation of **2** as a mimic of S1P. We investigated the signalling activities of S1P₁ by measuring the downstream signalling phosphorylation of extracellular signal-regulated kinase (ERK) and internalization of S1P₁ using immunoblotting and indirect immunofluorescence microscopy.¹⁷ The phosphorylation levels of the ERK upon treatment with various concentrations of S1P, ginkgolic acid (15:0), and ginkgolic acid 2-phosphate were measured. Ginkgolic acid 2-phosphate induced ERK phosphorylation in a dose dependent manner,

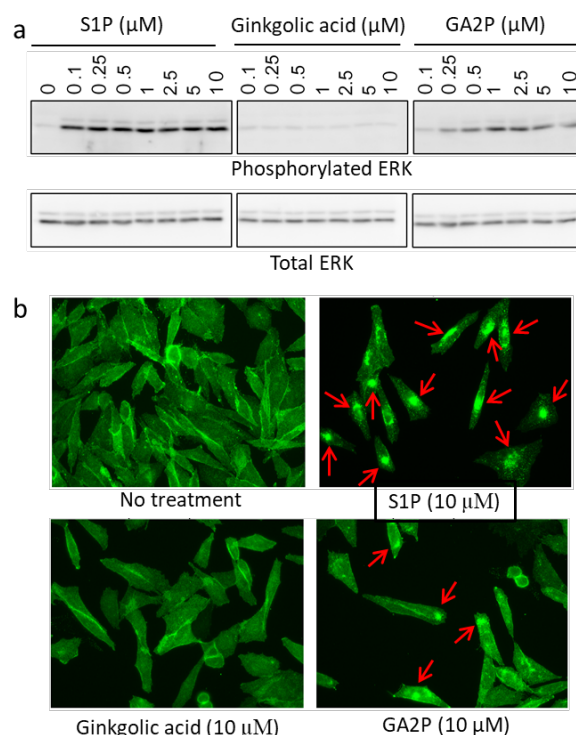


Figure 3 (a) S1P₁-CHO cells were incubated for 12 h with F-12 medium containing charcoal-treated FBS, and for another 12 h with F-12 medium. The cells were then stimulated with the indicated concentrations of S1P, ginkgolic acid (15:0) and GA2P respectively. Total cell lysates were separated by SDS-PAGE, followed by immunoblotting with anti-phosphorylated ERK (upper panel) and anti-ERK (lower panel) antibodies. (b) S1P₁-CHO cells were incubated for 12 h with F-12 medium containing charcoal treated FBS, and for another 12 h with F-12 medium. After a 1 h treatment with 10 μg/mL cycloheximide to inhibit protein synthesis, the cells were stimulated for 10 min with 10 μM S1P, ginkgolic acid (15:0) and GA2P respectively. After washing cells, Cells were stained with anti-FLAG M2 antibodies and Alexa Fluor 488 goat anti-mouse IgG (H+L) conjugated antibody (red arrows indicate the internalization of S1P₁).

but application of ginkgolic acid (15:0) did not result in ERK phosphorylation (Figure 3a). In addition, internalization of FLAG-tagged S1P₁ was observed by 10 μM ginkgolic acid 2-phosphate treatment (Figure 3b). These results suggest that ginkgolic acid 2-phosphate interacted with S1P₁ as an S1P agonist. Previously, Nizet *et al.* predicted that ginkgolic acid (15:0) stimulates S1P₁ using an *in silico* docking model.¹⁸ However, our results indicated that naked ginkgolic acid without the phosphate group did not stimulate S1P₁ (Figure 3). Furthermore, to confirm the direct interaction of GA2P to S1P₁, [³²P]S1P binding assay has been carried out, which proved GA2P was a competitive agonist (Figure S3).

The interaction between ginkgolic acid 2-phosphate and S1P₁ by docking simulations was confirmed by Autodock vina with flexible/fixed side chains. The docking result indicated that ginkgolic acid 2-phosphate adopts a similar binding manner to the bound ligand ML5, which was co-crystallized as a strong

antagonist of S1P₁.¹⁹ The phosphate group, which is crucial for the binding interaction, is close to that of ML5 and surrounded by hydrophilic side chains as shown in Figure 4. The hydrophobic tail part of ginkgolic acid

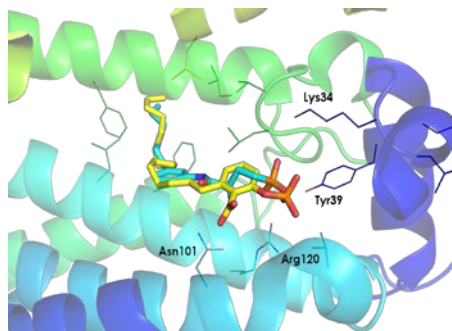


Figure 4. Docking result of GA2P. The protein structure of S1P₁ (PDB code 3V2Y) is shown in ribbon model and the residues surrounding the binding site are shown in line model. GA2P and ML5 (bound ligand in 3V2Y) are represented in stick model. Carbon atoms are highlighted in yellow (GA2P) and cyan (ML5). Oxygen, nitrogen and phosphate atoms are shown in red, blue and orange, respectively. GA2P binds to the binding pocket with binding affinity of -8.1 kcal/mol. The aliphatic C-15 tail fills the hydrophobic pocket, and the head group interacts with S1P₁ residues Lys34, Tyr39, Asn101 and Arg120 in hydrogen-bond distance.

2-phosphate adopts a similar binding conformation as ML5 and is located at the hydrophobic region of the binding site. In the ML5-S1P₁, Lys34 forms a hydrogen bond with the phosphate group but remains far away from that of ginkgolic acid 2-phosphate.

In conclusion, we identified ginkgolic acid (15:1) as a novel naturally occurring inhibitor of SMSs by screening a plant extracts library. There was structural similarity between this naturally occurring SMS inhibitor and sphingolipid, which suggests that the inhibitor was a mimic of the original molecule. Modified ceramide mimic derivatives were prepared to selectively inhibit SMS2, which is valuable to understand SMS biology. Analysis of S1P ginkgolic acid 2-phosphate mimic activity on the S1P receptor supported this “sphingo-mimic” concept. Several natural products that have a hydrophilic core and hydrophobic hydrocarbon side chain have been characterized such as cardols, urushiols, and gentsides.²⁰ Our study implied that the physiological activities of these compounds work through lipid-related systems.

Conflicts of interest

There are no conflicts to declare.

Notes and references

- 1 a) D. Zheng, Y. Huang, H. Moseley, R. Xiao, J. Aramini, G. Swapna, G. Montelione, *Protein Sci.*, 2003, **12**, 1232; b) N. Oezguen, L. Adamian, Y. Xu, K. Rajarathnam, W. Braun, *J.*

- Biomol. NMR*, 2002, **22**, 249; c) C. Bailey-Kellogg, A. Widge, J. J. Kelley, M. J. Berardi, J. H. Bushweller, B. R. Donald, *J. Comput. Biol.*, 2000, **7**, 537; d) K. Pervushin, R. Riek, G. Wider, K. Wutrich, *Proc. Natl. Acad. Sci. USA*, 1997, **94**, 12366.
- 2 J. Erickson, D. J. Neidhart, J. VanDrie, D. J. Kempf, X. C. Wang, D. W. Norbeck, J. J. Plattner, J. W. Rittenhouse, M. Turon, N. Wideburg, et al. *Science*, 1990, **249**, 527.
- 3 Y. Fan, F. Shi, J. Liu, J. Dong, H. H. Bui, D. A. Peake, M. S. Kuo, G. Cao, X.-C. Jiang, *Arterioscler. Thromb. Vasc. Biol.*, 2010, **30**, 2114.
- 4 S. Mitsutake, K. Zama, H. Yokota, T. Yoshida, M. Tanaka, M. Mit-sui, M. Ikawa, M. Okabe, Y. Tanaka, T. Yamashita, H. Takemoto, T. Okazaki, K. Watanabe, Y. Igarashi, *J. Biol. Chem.*, 2011, **286**, 28544.
- 5 Z. Li, H. Zhang, J. Liu, C.-P. Liang, Y. Li, G. Teitelman, T. Beyer, H. H. Bui, D. A. Peake, Y. Zhang, P. E. Sanders, M.-S. Kuo, T.-S. Park, G. Cao, X.-C. Jiang, *Mol. Cell. Biol.*, 2011, **31**, 4205.
- 6 K. Yuyama, S. Mitsutake, Y. Igarashi, *Biochim. Biophys. Acta*, 2014, **1841**, 793.
- 7 T. Ohnishi, C. Hashizume, M. Taniguchi, H. Furumoto, J. Han, R. Gao, S. Kinami, T. Kosaka, T. Okazaki, *FASEB J.*, 2017, **31**, 3816.
- 8 a) A. Kihara, S. Mitsutake, Y. Mizutani, Y. Igarashi, *Prog. Lipid Res.*, 2007, **46**, 126; b) J. Rivera, R. L. Proia, A. Olivera, *Nat. Rev. Immunol.*, 2008, **10**, 753.
- 9 a) M. Kato, M. A. S. Hammam, T. Taniguchi, Y. Suga, K. Monde, *Org. Lett.*, 2016, **18**, 768; b) X. Deng, F. Lin, Y. Zhang, Y. Li, L. Zhou, B. Lou, Y. Li, J. Dong, T. Ding, X. Jiang, R. Wang, D. Yea, *Eur. J. Med. Chem.*, 2014, **73**, 1; c) R. Adachi, K. Ogawa, S.-I. Matsumoto, T. Satou, Y. Tanaka, J. Sakamoto, T. Nakahata, R. Okamoto, M. Kamaura, T. Kawamoto, *Eur. J. Med. Chem.*, 2017, **136**, 28.
- 10 I. Fukuda, A. Ito, G. Hirai, S. Nishimura, H. Kawasaki, H. Saitoh, K. Kimura, M. Sodeoka, M. Yoshida, *Chem. Biol.*, 2009, **16**, 133.
- 11 J. Ma, W. Duan, S. Han, J. Lei, Q. Xu, X. Chen, Z. Jiang, L. Nan, J. Li, K. Chen, L. Han, Z. Wang, X. Li, E. Wu, X. Huo, *Oncotarget*, 2015, **6**, 20993.
- 12 D. Mango, F. Weisz, R. Nisticò, *Front. Pharmacol.*, 2016, **7**, 1.
- 13 S. H. Baek, J. Ko, J. H. Lee, C. Kim, H. Lee, D. Nam, J. LEE, S. Lee, W. M. Yang, J. Um, G. Sethi, K. S. Ahn, *J. Cell. Physiol.*, 2017, **232**, 346.
- 14 a) N. Satoh, T. Takeuchi, T. Nishimura, T. Ohta, S. Tobinaga, *Chem. Pharm. Bull.*, 2001, **49**, 18-22; b) I. R. Green, F. E. Tocoli, *Synt. Commun.*, 2002, **32**, 947-957.
- 15 S. Park, D. Im, *Biomol. Ther.*, 2017, **25**, 80.
- 16 A. Billich, F. Bornancin, P. De'vay, D. Mechtcheriakova, N. Urtz, T. Baumruker, *T. J. Biol. Chem.*, 2003, **278**, 47408.
- 17 Y. Ohno, A. Ito, R. Ogata, Y. Hiraga, Y. Igarashi, A. Kihara, *Genes Cells*, 2009, **14**, 911.
- 18 A. Hollands, R. Corriden, G. Gysler, S. Dahesh, J. Olson, S. R. Ali, M. T. Kunkel, A. E. Lin, S. Forli, A. C. Newton, G. B. Kumar, B. G. Nair, J. J. P. Perry, V. J. Nizet, *J. Biol. Chem.*, 2016, **291**, 13964.
- 19 a) M. G. Sanna, S. Wang, P. J. Gonzalez-cabrera, A. Don, D. Mar-solais, M. P. Matheu, S. H. Wei, I. Parker, E. Jo, W. -C. Cheng, M. D. Cahalan, C. -H. Wong, H. Rosen, *Nat. Chem. Biol.*, 2006, **2**, 434; b) M. A. Hanson, C. B. Roth, E. Jo, M. T. Griffith, F. L. Scott, G. Reinhart, H. Desale, B. Clemons, S. M. Cahalan, S. C. Schuerer, G. Sanna, G. W. Han, P. Kuhn, H. Rosen, R. C. Stevens, *Science*, 2012, **335**, 851.
- 20 a) M. T. S. Trevisan, B. Pfundstein, R. Haubner, G. Wuertele, B. Spiegelhalter, H. Bartsch, R. W. Owen, *Food Chem. Toxicol.*, 2006, **44**, 188; b) W. F. Symes, C. R. Dawson, *Nature*, 1953, **171**, 841; c) L. Gao, J. Li, J. Qi, *Bioorg. Med. Chem.*, 2010, **18**, 2131.