Bioorganic & Medicinal Chemistry Letters 19 (2009) 3973–3976

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Synthesis, configurational stability and stereochemical biological evaluations of (*S*)- and (*R*)-5-hydroxythalidomides

Takeshi Yamamoto [a], Norio Shibata [a,*], Daisuke Sukeguchi [a], Masayuki Takashima [a], Shuichi Nakamura [a], Takeshi Toru [a], Nozomu Matsunaga [b], Hideaki Hara [b], Motohiro Tanaka [c], Tohru Obata [c], Takuma Sasaki [c]

[a] Department of Frontier Materials, Graduate School of Engineering, Nagoya Institute of Technology, Gokiso, Showa-ku, Nagoya 466-8555, Japan
[b] Department of Biofunctional Evaluation, Molecular Pharmacology, Gifu Pharmaceutical University, Mitahora-higashi, Gifu 502-8585, Japan
[c] Department of Bioorganic Chemistry, School of Pharmacy, Aichi Gakuin University, 1-100 Kusumoto-cho, Chikusa-ku, Nagoya 464-8650, Japan

ARTICLE INFO

Article history:
Received 5 February 2009
Revised 25 February 2009
Accepted 26 February 2009
Available online 3 March 2009

Keywords:
Thalidomide
Asymmetric synthesis
Metabolites

ABSTRACT

The first asymmetric synthesis of (*S*)- and (*R*)-5-hydroxythalidomides, one of thalidomide's major metabolites, was achieved using HMDS/$ZnBr_2$-induced imidation as a key reaction. 5-Hydroxythalidomide was found to be configurationally more stable than thalidomide at physiological pH. Stereochemical biological effects of thalidomide and 5-hydroxythalidomide on anti-angiogenesis and antitumor activities were also investigated using racemic and pure enantiomers.

Thalidomide is one of the most notorious drugs that caused a tragic medical disaster in the late 1950s in Germany, England, Canada, Japan and more. It was prescribed as a sleeping drug/sedative and spread to pregnant women as a remedy for the relief of their nausea due to morning sickness. However, an epidemic of limb malformations broke out in these countries and years later it was ascertained that thalidomide was the cause of these terrible birth defects. Although thalidomide was banned worldwide in 1962, it has been licensed again for clinical use in several countries due to the discovery of thalidomide's unique multiple pharmacological actions against a number of intractable diseases, such as leprosy, human immunodeficiency virus replication in acquired immune deficiency syndrome, and cancer.[1]

Thalidomide possesses a single chiral center and was marketed as a racemate. In 1979, Blaschke et al found that the enantiomers of thalidomide have different biological properties.[2] While sedative and hypnotic effects are associated with the *R*-enantiomer, *S*-enantiomer of thalidomide is responsible for the teratogenic side effect. Nau and co-workers also supported the result using an enantiomerically pure thalidomide derivative, EM-12.[3] However, these reports are still a matter of debate because, under physiological conditions, optically active thalidomide rapidly undergoes racemization, making separation of its biological effects impossible.[4]

During recent studies on the structure–activity relationships of thalidomide and its derivatives, thalidomide metabolites have emerged as biologically significant targets.[5] Thalidomide is metabolically labile and several metabolites have been isolated.[6] Hence, the possibility should be considered that the teratogenic side effect or/and other multiple pharmacological activities might be due to the metabolites, including their enantiomers, rather than thalidomide itself. Two major metabolites of thalidomide are 5-hydroxythalidomide and 5′-hydroxythalidomide. Recently, we reported the synthesis and biological evaluation of optically active (*S*)- and (*R*)-5′-hydroxythalidomides.[7] As an extension of our work on thalidomide research,[8] we describe herein the asymmetric synthesis, configurational stability and stereochemical biological evaluation of (*S*)- and (*R*)-5-hydroxythalidomides (**2**) (Fig. 1).

Many research groups have actively studied the biological activity of racemic **2**.[5] For example, Hashimoto and co-workers revealed that **2** possesses moderate anti-angiogenic activity and tubulin-polymerization-inhibitory activity.[5c,d,f] Li and co-workers also reported that **2** is an active anti-angiogenic agent.[5b] On the other hand, to our knowledge, biological evaluation of optically active (*S*)- and (*R*)-**2** have never been examined, presumably due to both the possibility of racemization under physiological conditions and the lack of availability of enantiomerically pure (*S*)- and (*R*)-**2**. Previously, we reported a three-step synthesis of optically pure (*S*)- and (*R*)-**1**.[8b] This synthetic route is simple but fully determined for avoiding racemization during the sequence of its synthesis. We therefore examined the synthesis of optically pure (*S*)- and (*R*)-**2** by a strategy similar to that of optically active **1** (Scheme

* Corresponding author. Tel./fax: +81 52 735 7543.
E-mail address: nozshiba@nitech.ac.jp (N. Shibata).

0960-894X/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2009.02.108

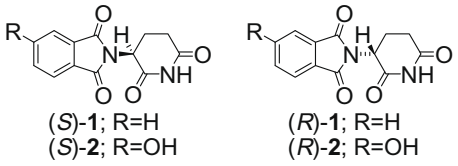
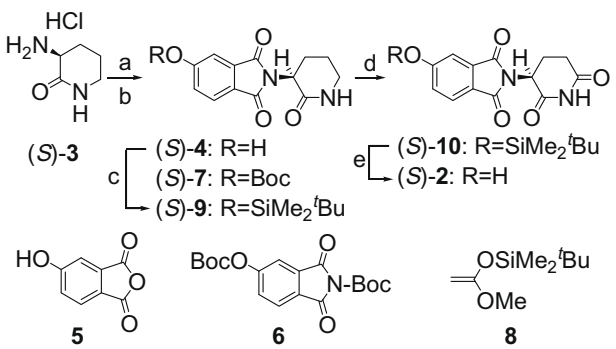


Figure 1. Structure of (*S*)- and (*R*)-thalidomides (**1**) and their metabolites, (*S*)- and (*R*)-5-hydroxythalidomides (**2**).

1). First, the synthesis of **4** was attempted by the reaction of (*S*)-**3** with 4-hydroxyphthalic anhydride (**5**) under heating condition, but **4** was obtained as a racemate. We next examined the reaction of **3** using *N,O-bis*-Boc-protected 4-hydroxyphthalimide **6** in the presence of Et_3N in CH_2Cl_2 at room temperature. Unfortunately, again, product **7** was obtained with a slight degree of racemization (91% ee). After careful optimization of the introduction of the 4-hydroxyphthaloyl group, a combination of hexamethyldisilazane (HMDS) and $ZnBr_2$[9] was found to be very effective for the condensation of **3** with **5** to produce (*S*)-**4** in high yield without any loss of enantiopurity. Namely, (*S*)-**3** was treated with **5** in the presence of Et_3N in 1,4-dioxane at room temperature for 4 h followed by the reaction with HMDS and $ZnBr_2$ in refluxing benzene for 6 h to afford (*S*)-**4** in high yield with >99% ee. The 5-hydroxyl group of **4** was protected by the use of *O*-silylated ketene acetal **8**[10] followed by oxidation using a catalytic amount of RuO_2 in the presence of excess $NaIO_4$ in a two-phase system to furnish silylated 5-hydroxythalidomide **10**. Finally, the treatment of **10** with concn HCl precipitated enantiomerically pure (*S*)-**2** in high yield with 99% ee. In a manner similar to that described for the preparation of (*S*)-**2**, (*R*)-**2** was obtained from (*R*)-**3** in five steps in good overall yield. The CD spectra of (*S*)-**2** and (*R*)-**2** indicated good agreement with those in the literature (Fig. 2).[11]

We next investigated the stabilities of **1** and **2** toward racemization and hydrolysis (decay). Optically pure (*S*)-**1** and (*S*)-**2** were incubated at 37 °C and varying pH values, and monitored by HPLC using a reported protocol.[4b,7] CHIRALCEL OJ-H with ethanol was used for the separation. Three-buffer systems, pH 6.24 (100 mM sodium phosphate monobasic, 100 mM sodium phosphate dibasic), pH 7.23 (100 mM sodium phosphate monobasic, 100 mM sodium phosphate dibasic) and pH 8.78 (40 mM tris base, 40 mM hydrochloric acid) were employed. The results are shown in Figs. 3 and 4. In the racemization study, (*S*)-**1** was racemized and decayed using all buffer solutions; the rate of racemization and hydrolysis was rather quick in neutral and alkaline buffer solutions, which was consistent with earlier studies by Hashimoto as well as by us.[4b,7] On the other hand, the stability of (*S*)-**2** to racemization and hydrolysis was higher than (*S*)-**1** under all the conditions. The half-life to racemization of (*S*)-**1** was estimated by a plot of the experimental data, $t_{0.5} = R/S = 0.5$. While the racemization half-lives of (*S*)-**1** were found to be 27.8 h at 6.24 pH, 5.1 h at 7.23, and 2.6 h at 8.78 in buffer solution at 37 °C, those of (*S*)-**2** were 53.2 h at 6.24 pH, 26 h at 7.23 and 13 h at 8.78.



Scheme 1. Reagents, conditions and yields: (a) **6**, Et_3N, CH_2Cl_2, rt, 10 h, 89%, 91% ee; (b) (i) **5**, Et_3N, MS-3 Å, 1,4-dioxane, rt, 4 h; (ii) HMDS, $ZnBr_2$, benzene, reflux, 6 h, 85%, >99% ee; (c) **8**, MeCN. rt, 1 h; (d) RuO_2, 10% $NaIO_4$ aq AcOEt, CH_2Cl_2, 40 °C, 12 h, 78% (2 steps); (e) concn HCl aq, MeOH, rt, 1 h, 88%, >99% ee.

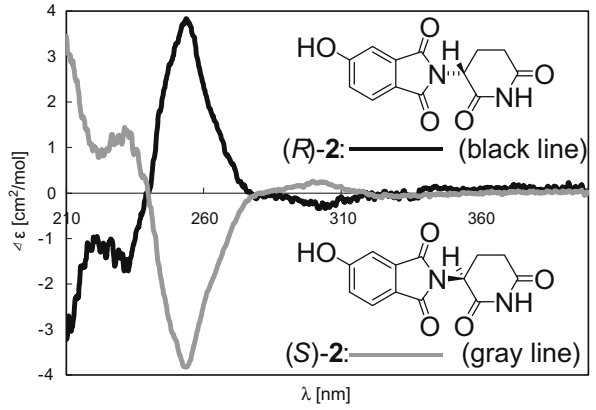


Figure 2. CD Spectra of (*S*)-**2** and (*R*)-**2**.

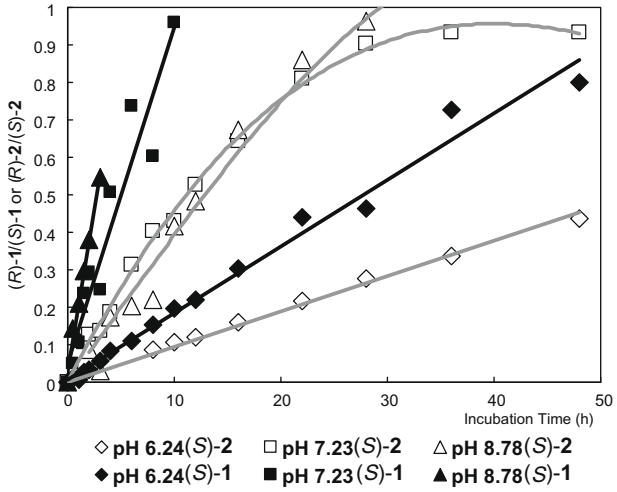


Figure 3. Racemization studies of (*S*)-**1** and (*S*)-**2**.

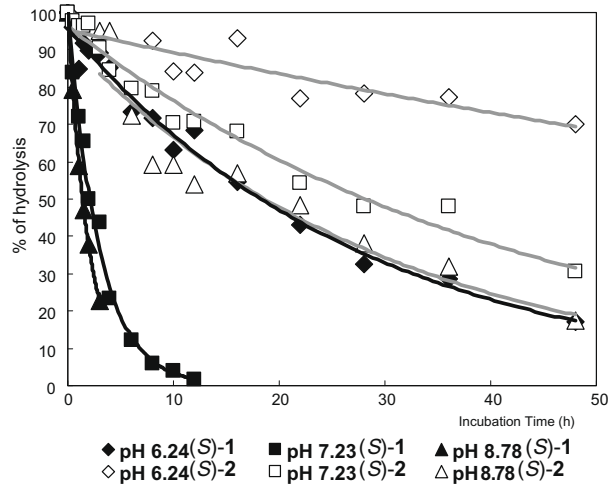


Figure 4. Hydrolysis (decay) studies of (*S*)-**1** and (*S*)-**2**.

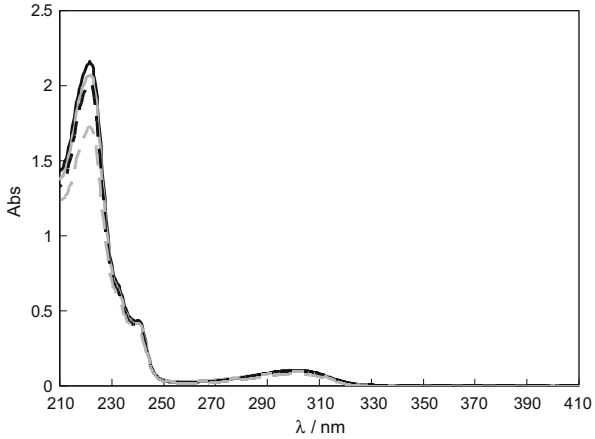


Figure 5. UV–vis spectra of **1** in buffer solution: pH 6 (black line), pH 7 (gray line), pH 8 (black broken line), pH 9 (gray broken line).

It is interesting to note that the stabilization effect is markedly high in basic conditions; we next investigated the UV–vis spectra of (*S*)-**1** and (*S*)-**2** in different buffer solutions. The spectra are shown in Fig. 5 and 6. While (*S*)-**1** showed similar UV–vis spectra in any pH conditions (Fig. 5), the UV–vis spectra of (*S*)-**2** were pronouncedly red-shifted in basic solution (Fig. 6). These results indicate that the contribution of phenolphthalein-like resonance structure in phthalimide moiety is responsible for the higher stability of (*S*)-**2** (Fig. 7).

Biological evaluation of enantiomers of **1** and **2** were investigated next. Among the diverse biological activities of thalidomide that have been suggested, first, we were interested in anti-angiogenesis activity of thalidomide. It is hypothesized that the anti-angiogenic activity is correlated with teratogenicity but not with the sedative or the mild immunosuppressive properties of thalidomide.[12] The disruption of blood vessel formation in the fetal limb bud might be responsible for the teratogenicity of thalidomide.

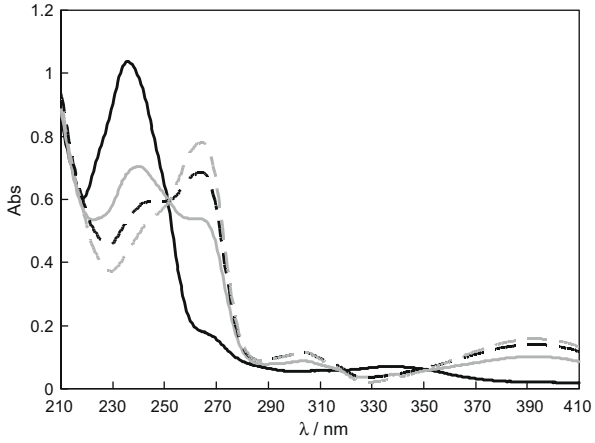


Figure 6. UV–vis spectra of **2** in buffer solution: pH 6 (black line), pH 7 (gray line), pH 8 (black broken line), pH 9 (gray broken line).

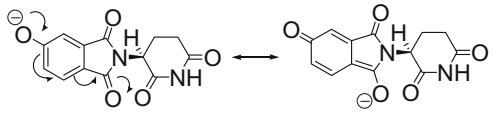


Figure 7. Resonance of **2**.

The tube formation assay, one of the trusted assay systems, was examined using an angiogenesis assay kit according to the manufacturer's instructions. Human umbilical vein endothelial cells (HUVECs) co-cultured with fibroblasts were cultivated in the presence or absence of various concentrations of test drugs plus Vascular endothelial growth factor-A (VEGF-A, 10 ng/ml).[7,13] After 11 days, cells were fixed in 70% ethanol. The cells were incubated with diluted primary antibody (mouse anti-human CD31, 1:4000) for 1 h at 37 °C, and with the secondary antibody (goat anti-mouse IgG alkaline phosphatase-conjugated antibody, 1:500) for 1 h at 37 °C, and visualization was achieved using 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT). Images were obtained from five different fields (5.5 mm^2 per field) for each well, and tube area was quantified using an angiogenesis image analyzer. The results are shown in Figure 8. While racemic **1** and (*S*)-**1** blocked vascular sprout formation in high concentration (tube area; <60%), (*R*)-**1**, racemic **2**, (*R*)-**2** and (*S*)-**2** showed weak inhibitory activity (tube area; >70%). Although all the activities are weak, (*R*)-**2** is slightly more potent than (*S*)-**2**.

Finally in vitro antitumor activities of enantiomers of **1** and **2** (100 μg/ml) were evaluated against KB cells from oral cancer, MCF-7 from breast cancer, and multiple myeloma (MM) cell lines (IM-9, and Hs Sultan). Tumor cell suspension (5 × 103 cells/mL) in RPMI-1640 medium was prepared before treatment. Each sample was dissolved in DMSO at the concentration of 10 mg/mL. Two microliters of sample solution and 198 μl of cell suspension were plated into a 96 well microplate and incubated for 72 h at 37 °C in a humidified atmosphere of 5% CO_2. The surviving cells were determined by the MTT assay.[14] As shown in Table 1 showed weak inhibitory activity in MCF-7, IM-9, and Hs-Sultan cell lines. On the

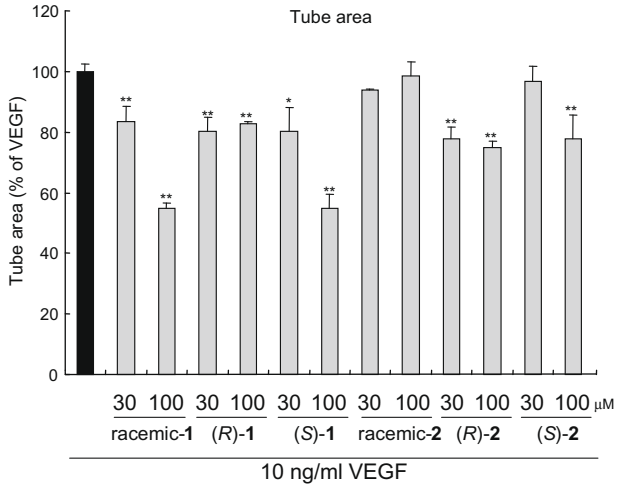


Figure 8. Effects of **1** and **2** on tube area. Comparison of angiogenesis induced by VEGF: mean ± S.E.M (VEGF: $n = 9$, VEGF + compounds: $n = 3$). *; $P < 0.05$, **; $P < 0.01$ versus VEGF (Dunnett's multiple comparison test).

Table 1
Antitumor activities of **1** and **2**.

Compds	Cell viability (%) at 100 μg/ml			
	KB	MCF-7	IM-9	Hs-Sultan
Racemic-**1**	92.2	86.5	107.0	85.9
(*S*)-**1**	95.6	81.3	93.5	88.6
(*R*)-**1**	96.9	89.8	87.1	75.6
Racemic-**2**	118.1	122.1	48.2	55.6
(*S*)-**2**	121.0	199.7	67.2	35.0
(*R*)-**2**	151.0	204.6	57.9	46.4

other hand, growth of the MM cell lines was markedly inhibited by **2**. It is also interesting to note that metabolite **2** possesses a proliferating effect on KB and MCF-7 cells. No significant stereospecific effect was observed for the antitumor activities between enantiomers of **1** and **2**.

In summary, we have reported the first asymmetric synthesis of (*S*)- and (*R*)-5-hydroxythalidomides (**2**). Racemization was effectively avoided by the use of HMDS/$ZnBr_2$-induced imidation method. Incubation experiments at physiological pH revealed that metabolite **2** is configurationally more stable than thalidomide itself due to the contribution of phenolphthalein-like resonance structures. A preliminary tube formation assay to assess the ability to inhibit angiogenesis suggests that (*S*)-**1** is the most active and its enantiomer (*R*)-**1**, metabolites (*R*)-**2** and (*S*)-**2** are rather weak compared to (*S*)-**1**. On antitumor activities, **2** exhibited proliferating effects in KB and MCF-7 cells while **1** has very weak antitumor activities. Despite the higher configurational stability of **2**, none of the significant stereochemical effects in the antitumor assays was observed for enantiomers of **2**. It should be noted that the inhibitory activity against MM cell lines of **2** is much stronger than that of **1**. All the results indicate that the multiple myeloma inhibitory activity of thalidomide may be responsible for its metabolite **2**, while anti-angiogenesis is responsible for (*S*)-**1**. Although it is reported that hydroxylation of **1** results in increased teratogenicity in experiments using a developing chick embryo,[5a] our results appear to indicate that metabolite **2** is slightly safer for the treatment of multiple myeloma due to its weaker anti-angiogenesis property. Further detailed studies to investigate the influence of stereochemistry and metabolites on biological activities of thalidomide would give useful information to develop safer non-teratogenic drugs based on thalidomide's structure.

Acknowledgments

Support was provided by JSPS KAKENHI (19390029, 19020024) and Nagai Foundation Tokyo (Research Grant).

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.02.108.

References and notes

1. Hashimoto, Y. *Arch. Pharm.* **2008**, *341*, 536.
2. Blaschke, G.; Kraft, H. P.; Fickentscher, K.; Kohler, F. *Arzneim.-Forsch.* **1979**, *29*, 1640.
3. Schmahl, H. J.; Nau, H.; Neubert, D. *Arch. Toxicol.* **1988**, *62*, 200.
4. (a) Knoche, B.; Blaschke, G. *J. Chromatogr., A* **1994**, *666*, 235; (b) Nishimura, K.; Hashimoto, Y.; Iwasaki, S. *Chem. Pharm. Bull.* **1994**, *42*, 1157.
5. (a) Boylen, J. B.; Horne, H. H.; Johnson, W. J. *Can. J. Biochem. Physiol.* **1964**, *42*, 35; (b) Marks, M. G.; Shi, J.; Fry, M. O.; Xiao, Z.; Trzyna, M.; Pokala, V.; Ihnat, M. A.; Li, P.-K. *Biol. Pharm. Bull.* **2002**, *25*, 597; (c) Inatsuki, S.; Noguchi, T.; Miyachi, H.; Oda, S.; Iguchi, T.; Kizaki, M.; Hashimoto, Y.; Kobayashi, H. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 321; (d) Noguchi, T.; Shinji, C.; Kobayashi, H.; Makishima, M.; Miyachi, H.; Hashimoto, Y. *Biol. Pharm. Bull.* **2005**, *28*, 563; (e) Noguchi, T.; Miyachi, H.; Katayama, R.; Naito, M.; Hashimoto, Y. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 3212; (f) Noguchi, T.; Fujimoto, H.; Sano, H.; Miyajima, A.; Miyachi, H.; Hashimoto, Y. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 5509; (g) Nakamura, T.; Noguchi, T.; Kobayashi, H.; Miyachi, H.; Hashimoto, Y. *Chem. Pharm. Bull.* **2006**, *54*, 1709.
6. Lepper, E. R.; Smith, N. F.; Cox, M. C.; Scripture, C. D.; Figg, W. D. *Curr. Drug Metab.* **2006**, *7*, 677.
7. Yamamoto, T.; Shibata, N.; Takashima, M.; Nakamura, S.; Toru, T.; Matsunaga, N.; Hara, H. *Org. Biomol. Chem.* **2008**, *6*, 1540.
8. (a) Takeuchi, Y.; Shiragami, T.; Kimura, K.; Suzuki, E.; Shibata, N. *Org. Lett.* **1999**, *1*, 1571; (b) Suzuki, E.; Shibata, N. *Enantiomer* **2001**, *6*, 275; (c) Shibata, N.; Yamamoto, T.; Toru, T. In *Top. Heterocycl. Chem.*; Eguchi, S., Ed.; Springer: Berlin, Heidelberg, 2007; Vol. 8, pp 73–97.
9. Reddy, P. Y.; Kondo, S.; Toru, T.; Ueno, Y. *J. Org. Chem.* **1997**, *62*, 2652.
10. Kita, Y.; Haruta, J.; Fujii, T.; Segawa, J.; Tamura, Y. *Synthesis* **1981**, 451.
11. Meyring, M.; Müehlbacher, J.; Messer, K.; Kastner-Pustet, N.; Bringmann, G.; Mannschreck, A.; Blaschke, G. *Anal. Chem.* **2002**, *74*, 3726.
12. (a) Stephens, T. D. *Teratology* **1988**, *38*, 229; (b) D'Amato, R. J.; Loughnan, M. S.; Flynn, E.; Folkman, J. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 4082; (c) Parman, T.; Wiley, M. J.; Wells, P. G. *Nat. Med.* **1999**, *5*, 582.
13. (a) Izuta, H.; Chikaraishi, Y.; Shimazawa, M.; Mishima, S.; Hara, H. *Evid. Based Complement. Altern. Med.* **2007**. doi:10.1093/ecam/nem152; (b) Chikaraishi, Y.; Shimazawa, M.; Hara, H. *Exp. Eye Res.* **2007**, *84*, 529.
14. Carmichael, J.; DeGraff, W. G.; Gazdar, A. F.; Minna, J. D.; Mitchell, J. B. *Cancer Res.* **1987**, *47*, 943.