Pergamon

Bioorganic & Medicinal Chemistry Letters 11 (2001) 2637–2641

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

New Inhibitors of the Thioredoxin–Thioredoxin Reductase System Based on a Naphthoquinone Spiroketal Natural Product Lead

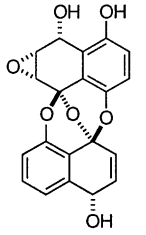
Peter Wipf,[a,*] Tamara D. Hopkins,[a] Jae-Kyu Jung,[a] Sonia Rodriguez,[a] Anne Birmingham,[b] Eileen C. Southwick,[c] John S. Lazo[c] and Garth Powis[b]

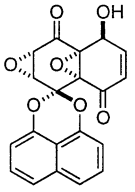
[a]*Department of Chemistry, University of Pittsburgh, Pittsburgh, PA 15260, USA*
[b]*Arizona Cancer Center, University of Arizona, 1515 North Campbell Avenue, Tucson, AZ 85724, USA*
[c]*Department of Pharmacology, University of Pittsburgh, Pittsburgh, PA 15261, USA*

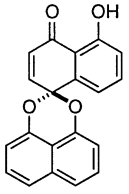
Received 24 May 2001; accepted 25 July 2001

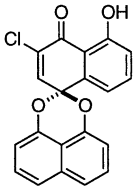
Abstract—Natural products of the naphthoquinone spiroketal structural type served as lead structures for the development of novel inhibitors of the thioredoxin–thioredoxin reductase redox system. The most potent compound in this series inhibited thioredoxin with an IC_{50} of 350 nM, and many derivatives showed low micromolar activities for growth inhibition against two breast cancer cell lines.

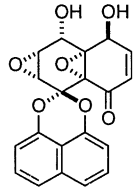
The preussomerin, palmarumycin, and diepoxin families of naphthoquinone spiroketal natural products have attracted considerable synthetic interest.[1] The known biological activities of these novel fungal metabolites span from antifungal antibiotic effects to FTPase, DNA gyrase, and phospholipase D inhibition.[1] Preliminary biological evaluation of 22 naphthoquinone spiroketals against two human breast cancer cell lines revealed several potent and selective growth inhibitors;[1a] in addition, analogue **SR-7** was found to arrest mammalian cells in the G2/M phase of the cell cycle.[2] We now report that several natural as well as synthetic members

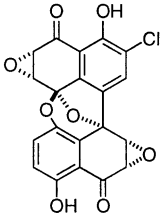

preussomerin A

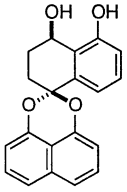

diepoxin σ (Sch 49209)

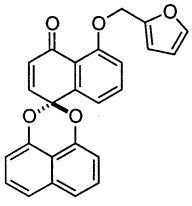

palmarumycin CP$_1$


palmarumycin C$_1$


diepoxin ζ (Sch 53514) (palmarumycin C$_{13}$) (cladospirone bisepoxide)

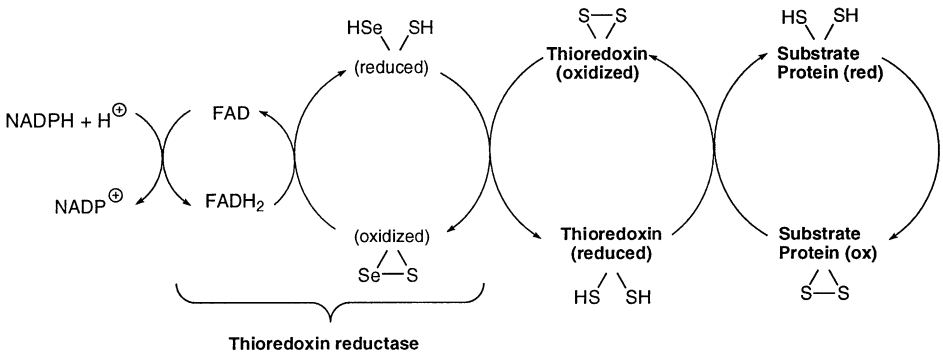

spiroxin A


CJ-12,371


SR-7

*Corresponding author. Tel.: +1-412-624-8606; fax: +1-412-624-0787; e-mail: pwipf@pitt.edu

0960-894X/01/$ - see front matter
PII: S0960-894X(01)00525-X



of the naphthoquinone spiroketal class are potent and selective inhibitors of the thioredoxin–thioredoxin reductase cellular redox system.

Since its discovery in the early 1960s, the thioredoxin–thioredoxin reductase system has been the subject of intense pharmacological studies.[3] The two redox-active proteins have been isolated from many species, and their medical interest is based in part on their value as indicators of widespread diseases such as rheumatoid arthritis, AIDS, and cancer. The cytosolic 12 kDa thioredoxin-1 (Trx-1) is the major cellular protein disulfide reductase and its dithiol-disulfide active site cysteine pair (CXXC) serves as electron donor for enzymes such as ribonucleotide reductase, methionine sulfoxide reductase, and transcription factors including NF-κB and the Ref-1-dependent AP-1.[4] Therefore, thioredoxin-1 is critical for cellular redox regulation, signaling, and regulation of protein function as well as defense against oxidative stress and control of growth and apoptosis.[5] Thioredoxin-1 acts in concert with the glutathione–glutathione reductase system but with a rate of reaction orders of magnitude faster, and lack of cytosolic mammalian thioredoxin is embryonically lethal. Eukaryotic thioredoxin reductases (TrxR) are 112–130 kDa, selenium-dependent dimeric flavoproteins that also reduce substrates such as hydroperoxides or vitamin C.[6] They contain redox-active selenylsulfide-selenolthiol active sites and are inhibited by aurothioglucose and auranofin ($K_i = 4$ nM).[7] NADPH serves as reducing agent of Trx-1 via TrxR.

Pathophysiological effects of Trx-1/TrxR are indicated by Trx-1 overexpression in human tumors such as lung, colorectal, and cervical cancers and leukemia, and secreted Trx-1 stimulates cancer cell growth and

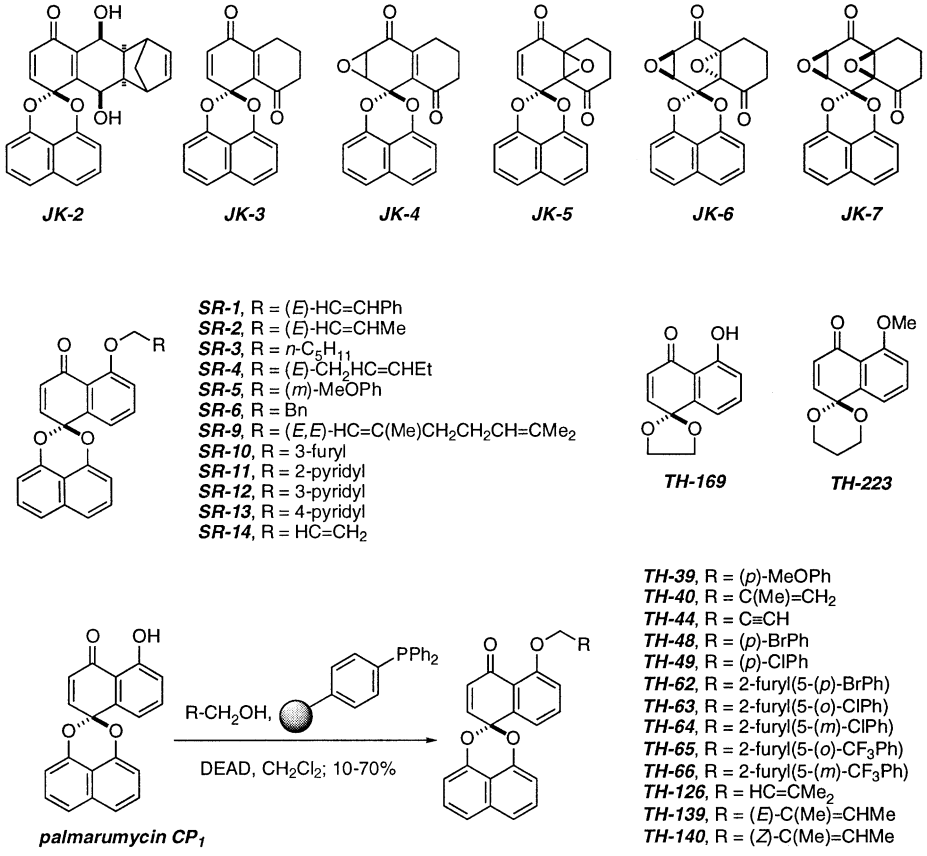


Figure 1. Structures of synthetic Trx-1/TrxR inhibitors.

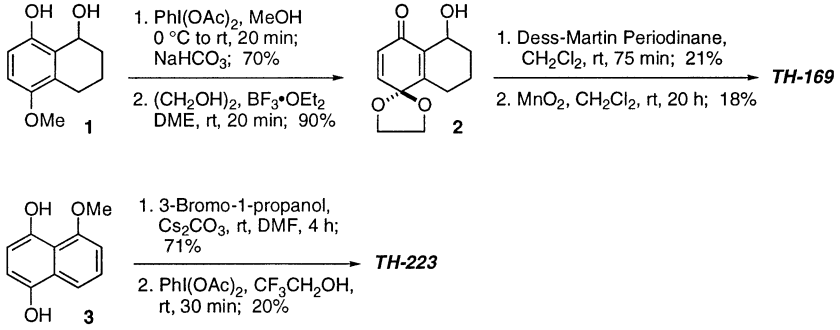
decreases sensitivity to induced apoptosis.[8] The Trx-1/TrxR system is therefore an important target for chemotherapeutic intervention. Alkyl 2-imidazolyl disulfides were found to be inhibitors of Trx-1/TrxR with IC_{50}s of 31/37 μM, respectively; they block MCF-7 human breast cancer cells in the G_2/M phase of the cell cycle and suppress the growth of several human primary tumors in the NCI 60 cancer cell line panel.[8,9] A COMPARE analysis revealed the most potent Trx-1/TrxR inhibitor known to date, the *para*-quinone NSC401005 a.k.a. the natural product pleurotin.[10] The IC_{50} of NSC401005 against Trx-1/TrxR was determined as 0.17 μM; however, the average IC_{50} of this compound for growth inhibition in the NCI 60 tumor cell line panel was only 21.5 μM. While inhibitors of TrxR such as auranofin and nitrosoureas are quite effective, the search for new, more specific, and less toxic compounds is well justified.

Syntheses and characterizations of palmarumycin CP_1, diepoxin σ, the **JK-**series and the **SR**-series of analogues have been reported elsewhere.[1] The **TH-39** through **TH-140** series of analogues was prepared in a similar fashion in 10–70% yield from synthetic palmarumycin CP_1 by Mitsunobu reactions with polystyrene-supported triphenylphosphine (Fig. 1).[11] **TH-169** was prepared by hypervalent iodine oxidation of diol **1**[1c] followed by transketalization with ethylene glycol and two-step aromatization (Scheme 1).[12] For the preparation of **TH-223**, the cesium salt of diol **3**[13] was alkylated and cyclized under oxidative conditions.[14]

Thioredoxin reductase was purified from human placenta as previously described[15] and recombinant human Trx-1 was prepared as previously described.[16] TrxR and Trx-1/TrxR activities were measured spectrophotometrically using previously published microtiter plate colorimetric assays, based on the increase in absorbance at 405 nm which occurs as dithionitrobenzoic acid (DTNB) is reduced by the enzyme-mediated transfer of reducing equivalents from NADPH.[16] Trx-1/TrxR-dependent insulin reducing activity was measured in an incubation with a final volume of 60 μL containing 100 mM HEPES buffer, pH 7.2, 5 mM EDTA (HE buffer), 1 mM NADPH, 1.0 μM TrxR, 0.8 μM Trx-1 and 2.5 mg/mL bovine insulin. Incubations were for 30 min at 37 °C in flat-bottom 96-well microtiter plates. The reaction was stopped by the addition of 100 μL of 6 M guanidine–HCl, 50 mM Tris, pH 8.0, and 10 mM DTNB, and the absorbance measured to 405 nm. TrxR activity was measured in a final incubation volume of 60 μL containing HE buffer, 10 mM DTNB, 1.0 μM TrxR and 1 mM NADPH. Compounds were diluted in HE buffer and added to the wells as 20 μL aliquots, and TrxR was then added, also as 20 μL aliquots in HE buffer. To start the reaction NADPH and DTNB were added as a 20 μL aliquot in HE buffer and the plate was moved to the plate reader which had been preheated to 37 °C. The optical density at 405 nm was measured every 10 s and initial reaction rates were measured.

Antiproliferative activity was examined with estrogen receptor positive, p53 replete MCF-7 and estrogen receptor negative, p53 deficient MDA-MB-231 human breast cancer cells using a previously described colorimetric assay.[1a,2] Cells were seeded at 4000 cells/well in 96-well microtiter plates and allowed to attach overnight. Cells were treated with compounds (0.1–30 μM) or 0.1% DMSO (vehicle) for 72 h, after which the medium was replaced with serum-free medium containing 0.1% of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide. Plates were incubated for 3 h in the dark and the total cell number was calculated spectrophotometrically at 540 nm.

Table 1 summarizes Trx-1/TrxR assay data as well as growth inhibition values for selected compounds. The most active compounds inhibited Trx-1/TrxR with IC_{50} values from 0.35 to <15 micromolar. In particular, palmarumycin CP_1 rivaled the most active known inhibitor of the thioredoxin system, pleurotin, in activity (entry 1). Palmarumycin CP_1 also demonstrated considerable (>30-fold) selectivity for Trx-1 over TrxR. Alkylation at the phenol as shown in the **SR**-series of analogues mostly abolished activity, with the exception of **SR-10**, a 3-furylmethyl derivative (entry 17), and **SR-14**, an allylated phenol (entry 21), which were nonetheless >50-fold less active. For the most part, this trend is continued in the **TH**-series, but several derivatives show more significant affinity to the thioredoxin–thioredoxin reductase system. Specifically, **TH-40** (entry 23), **TH-44** (entry 24), and **TH-62** (entry 27) have IC_{50} values from 4.8 to 13.4 μM. The former two are closely related to **SR-14**, but the activity of **TH-62** is unexpected given the lack of activity of the closely related

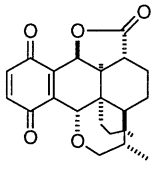


Scheme 1.

Table 1. IC_{50} values [μM] for TrxR, Trx-1/TrxR and cell growth inhibition[a]

Entry	Compound	TrxR	Trx-1/TrxR	MDA-MB-231	MCF-7
1	Palmarumycin CP_1	12.0	0.35	2.4	1
2	**JK-2**	nd[b]	8.0	2.1	1.3
3	**JK-3**	nd	2.1	6.4	3.8
4	**JK-4**	nd	12.2	23	4.6
5	**JK-5**	nd	44.0	3.4	1.3
6	**JK-6**	nd	>50	8.2	4.6
7	**JK-7**	nd	13.5	2.9	2.8
8	Diepoxin σ	13.5	4.5	2	1.5
9	**SR-1**	nd	>50	7.5	7.9
10	**SR-2**	nd	>50	2.9	1.3
11	**SR-3**	nd	>50	13.6	13.4
12	**SR-4**	nd	>50	9.2	>30
13	**SR-5**	nd	>50	2.7	2.3
14	**SR-6**	nd	>50	4.6	3.9
15	**SR-7**	nd	>50	2.5	1.1
16	**SR-9**	nd	>50	2	4.6
17	**SR-10**	>50	23.2	2	2
18	**SR-11**	>50	41.8	2.8	2
19	**SR-12**	>50	>50	1.4	1.5
20	**SR-13**	>50	>50	7.3	8
21	**SR-14**	>50	23.2	2.7	2
22	**TH-39**	>50	>50	2.2	0.7
23	**TH-40**	>50	4.8	8.2	7.8
24	**TH-44**	>50	13.4	4.7	4.3
25	**TH-48**	>50	>50	9.3	>10
26	**TH-49**	>50	>50	8	>10
27	**TH-62**	20.1	10.2	7.8	5.7
28	**TH-63**	>50	>50	>10	>10
29	**TH-64**	>50	>50	>10	>10
30	**TH-65**	>50	>50	4.9	5
31	**TH-66**	>50	42.4	5.2	5.5
32	**TH-126**	nd	>50	3.6	2
33	**TH-139**	nd	>50	5.3	4.3
34	**TH-140**	nd	>50	4.5	1.9
35	**TH-169**	8.8	3.4	4.3	4.2
36	**TH-223**	>50	40.2	5	4.4

[a]IC_{50} values were calculated from five concentrations (0.1–50 μM) for both the Trx-1/TrxR and TrxR assays.
[b]nd, not determined.



pleurotin

Figure 2. Structure of pleurotin.

TH-63–66. The beneficial effects of the free phenol group in the palmarumycin pharmacophore for Trx-1/TrxR inhibition are most strikingly demonstrated in the comparison of **TH-169** and **TH-223**. Only the free phenol **TH-169** maintains significant activity (entry 35) while the methyl ether **TH-223** is practically inactive. The comparison between palmarumycin CP_1 and **TH-169** also demonstrates the contribution to activity by the naphthalenediol ketal; a replacement with the 1,3-dioxolane group decreases activity ca. 10-fold and, most significantly, reduces the Trx-1 selectivity from >30- to ca. 2-fold. The presence of the conjugated enone is not crucial, as comparisons in the **JK**-series demonstrate. The diepoxydiketones **JK-7** and, in particular, diepoxin σ, maintain respectable levels of inhibition of Trx-1/TrxR (entries 7 and 8), even though successive replacements of the double bonds in **JK-4**, **JK-5**, and **JK-6** with epoxide rings lead to noticeable decreases in activity vs. the parent dienone **JK-3**.

It is intriguing to compare the structures of the most active palmarumycin analogues with pleurotin, a 0.17 μM inhibitor (Fig. 2). The polycyclic perhydroanthracene core of pleurotin is most closely related to **JK-2**, but the latter has considerably lower Trx-1/TrxR activity (entry 2). This difference is likely due to the presence of a *para*-quinone in pleurotin, which is a significantly more electrophilic moiety than the cyclohexadienone in **JK-2**. It is therefore noteworthy that the IC_{50} of palmarumycin CP_1 almost matches that of pleurotin, since the presence of the phenol ring attached to the cyclohexenone further reduces its electrophilicity and intrinsic reactivity toward thiols. Both **JK-2** and palmarumycin CP_1 demonstrate 10–20 times greater cell growth inhibition against MCF-7 and MDA-MB-231 cancer cell lines compared with pleurotin. Interestingly, the parent natural products as well as >80% of the synthetic analogues show IC_{50} values <10 μM against estrogen receptor positive and negative breast adenocarcinoma cells. Thus, this series is quite enriched for cytotoxic compounds directed toward human malignancies. All six compounds that inhibit Trx-1 in vitro with an IC_{50} <10 μM, namely palmarumycin CP_1, **JK-2** (entry 2), **JK-3** (entry 3), diepoxin σ, **TH-40** (entry 23), and **TH-169** (entry 35), have an IC_{50} for growth inhibition with the two human breast cancer cell lines of <10 μM. Several of the compounds that lacked TrxR or Trx-1/TrxR inhibitory activity, such as **TH-48** (entry 25), **TH-49** (entry 26), **TH-63** (entry 28), and **TH-64** (entry 29), have IC_{50} values for growth inhibition in excess of 10 μM. Nonetheless, at this time we do not have any direct evidence that the cytotoxicity was caused by inhibition of the Trx-1/TrxR system. This correlation will require additional investigation. In general, however, there is no obvious direct relationship between cell growth inhibitory activity and Trx-1/TrxR inhibition in the **JK**-series. This could reflect undesirable pharmacological attributes of some of the good in vitro inhibitors of Trx-1, such as poor cellular uptake, rapid cellular efflux, metabolism, or binding to multiple macromolecular targets, which limit their cellular toxicity. The substantial cytotoxicity of compounds lacking obvious in vitro activity against the Trx-1/TrxR system is most likely due to interactions with other, as yet undefined, molecular targets.

In summary, we have discovered a new, nanomolar lead structure for the development of inhibitors of the thioredoxin–thioredoxin reductase system. Palmarumycin CP_1 rivals the enzyme inhibitory activity reported for the structurally much more complex and more electrophilic quinone pleurotin and shows 50% cancer cell growth inhibition at 10–20 times lower concentrations. Our SAR studies on synthetic derivatives demonstrate that both the phenol group and the enone moiety in palmarumycin CP_1 are important for maximizing enzymatic activity, and that the presence of the naphthalenediol ketal enhances Trx-1 over TrxR selectivity. It is

possible that the antifungal and antibacterial effects found for palmarumycins[17] are a consequence of in vivo inhibition of the cellular redox state; certainly, the palmarumycins represent an attractive new opportunity for the development of chemotherapeutic agents that target the Trx-1/TrxR system.

Acknowledgements

This work was supported in part by grants from the National Science Foundation (CHE-9453461), the Department of Defense (DAMD 17-00-1-0412), and by the National Institutes of Health (U19CA-52995) and Merck & Co. We thank Professor Billy Day (University of Pittsburgh) for stimulating discussions and Ms. Angela Wang for technical support.

References and Notes

1. (a) Wipf, P.; Jung, J.-K.; Rodriguez, S.; Lazo, J. S. *Tetrahedron* **2001**, *57*, 283. (b) Coutts, I. G. C.; Allcock, R. W.; Scheeren, H. W. *Tetrahedron Lett*. **2000**, *41*, 9105. (c) Wipf, P.; Jung, J.-K. *J. Org. Chem*. **2000**, *65*, 6319 and references cited therein.
2. Lazo, J. S.; Tamura, K.; Vogt, A.; Jung, J.-K.; Rodriguez, S.; Balachandran, R.; Day, B. W.; Wipf, P. *J. Pharm. Exp. Ther*. **2001**, *296*, 364.
3. Williams, C. H. *Eur. J. Biochem*. **2000**, *267*, 6101.
4. Arnér, E. S. J.; Holmgren, A. *Eur. J. Biochem*. **2000**, *267*, 6102.
5. Davis, W.; Ronai, Z.; Tew, K. D. *J. Pharm. Exp. Ther*. **2001**, *296*, 1.
6. Williams, C. H.; Arscott, L. D.; Müller, S.; Lennon, B. W.; Ludwig, M. L.; Wang, P.-F.; Veine, D. M.; Becker, K.; Schirmer, R. H. *Eur. J. Biochem*. **2000**, *267*, 6110.
7. Becker, K.; Gromer, S.; Schirmer, R. H.; Müller, S. *Eur. J. Biochem*. **2000**, *267*, 6118.
8. Powis, G.; Kirkpatrick, D. L.; Angulo, M.; Baker, A. *Chem.-Biol. Interact*. **1998**, *111*, 23.
9. Vogt, A.; Tamura, K.; Watson, S.; Lazo, J. S. *J. Pharm. Exp. Ther*. **2000**, *294*, 1070.
10. Kunkel, M. W.; Kirkpatrick, D. L.; Johnson, J. I.; Powis, G. *Anti-Cancer Drug Des*. **1997**, *12*, 659.
11. General procedure for Mitsunobu reactions. 8-(4-Methoxy-benzyloxy)-1-oxo-1,4-dihydronaphthalene-4-spiro-2′-naphtho[1″,8″-de][1′,3′]dioxin (TH-39). A solution of palmarumycin CP_1 (20.1 mg, 0.0635 mmol), diphenylphosphino-polystyrene (230 mg, 1.41 mmol/g, 0.230 mmol) and 4-methoxybenzyl alcohol (39.6 μL, 0.318 mmol) in dry CH_2Cl_2 (2 mL) was stirred for 45 min at room temperature and cooled to 0 °C. Then, DEAD (50.0 μL, 0.318 mmol) was added dropwise to the reaction mixture at 0 °C. The solution was warmed to room temperature, stirred for 35 h, diluted with additional CH_2Cl_2, and washed with 5% aq KOH solution followed by 5% HCl. The organic extracts were filtered. The resin was washed further with CH_2Cl_2 and the combined extracts were concentrated in vacuo. Chromatography on SiO_2 (hexanes/EtOAc, 25:1→10:1→4:1) gave 6.1 mg (69%) of **TH-39**: ^{1}H NMR ($CDCl_3$) δ 7.70–7.45 (m, 8 H), 7.21 (dd, 1 H, J = 8.1, 0.8 Hz), 6.98 (t, 4 H, J = 8.2 Hz), 6.87 (d, 1 H, J = 10.5 Hz), 6.31 (d, 1 H, J = 10.5 Hz), 5.26 (s, 2 H), 3.84 (s, 3 H); ^{13}C NMR ($CDCl_3$) δ 182.7, 159.2, 158.8, 147.4, 141.0, 135.1, 134.7, 134.1, 132.2, 128.4, 128.3, 127.6, 121.2, 120.4, 115.9, 114.1, 109.8, 93.4, 70.7, 55.3; HR-MS (EI) calcd for $C_{28}H_{20}O_5$ 436.1311, found 436.1323.
12. Spectroscopic data for **TH-169**: mp 96.2–100.5 °C; IR (neat) 2956, 2919, 2852, 1662, 1617, 1460, 1393, 1344, 1296, 1240, 1157, 1083, 967, 843, 806, 746 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 12.16 (s, 1 H), 7.54 (t, 1 H, J = 8.7 Hz), 7.12 (d, 1 H, J = 7.6 Hz), 7.01 (d, 1 H, J = 8.3 Hz), 6.85 (d, 1 H, J = 10.3 Hz), 6.33 (d, 1 H, J = 10.3 Hz), 4.4–4.2 (m, 4 H); ^{13}C NMR ($CDCl_3$) δ 189.6, 161.8, 144.1, 141.4, 136.2, 128.3, 118.9, 118.0, 114.6, 99.9, 65.9; HR-MS (EI) calcd for $C_{12}H_{10}O_4$ 218.0579, found 218.0571.
13. Contant, P.; Haess, M.; Riegl, J.; Scalone, M.; Visnick, M. *Synthesis* **1999**, 821.
14. Spectroscopic data for **TH-223**: mp 147.5–152.1 °C; IR (neat) 2960, 2919, 2840, 1670, 1636, 1595, 1475, 1322, 1281, 1258, 1094, 1060 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 7.69–7.55 (m, 3 H), 7.03 (dd, 1 H, J = 7.8, 1.5 Hz), 6.37 (d, 1 H, J = 10.8 Hz), 4.33 (td, 2 H, J = 12.6, 2.5 Hz), 4.09 (dd, 2 H, J = 7.2, 4.6 Hz), 3.95 (s, 3 H), 2.5–2.2 (m, 1 H), 1.65–1.60 (m, 1 H); ^{13}C NMR ($CDCl_3$) δ 183.4, 159.4, 145.0, 134.6, 134.5, 130.9, 119.3, 118.6, 112.5, 90.8, 61.3, 56.2, 25.1; HR-MS (EI) calcd for $C_{14}H_{14}O_4$ 246.0892, found 246.0896.
15. Oblong, J. E.; Gasdaska, P. Y.; Sherrill, K.; Powis, G. *Biochemistry* **1993**, *32*, 7271.
16. Gasdaska, P. Y.; Oblong, J. E.; Cotgreave, I. A.; Powis, G. *Biochim. Biophys. Acta* **1994**, *1218*, 292.
17. Krohn, K.; Michel, A.; Flörke, U.; Aust, H.-J.; Draeger, S.; Schulz, B. *Liebigs Ann. Chem*. **1994**, 1093.