RSC Advances

Low-dose Paeonol Derivatives Alleviate Lipid Accumulation

Kuan-Chuan Pao,[a] Jin-Feng Zhao,[b] Tzong-Shyuan Lee,[b] Ying-Pei Huang,[a,c] Chien-Chung Han,[c] Lin-Chiang Sherlock Huang,[a,c] Kou-Hung Wu[a] and Ming-Hua Hsu*[a]

Representative fluorescent microscopy images of lipid accumulation after incubation with or without **Dil-oxLDL** in macrophages pretreated with paeonol or **7a**.
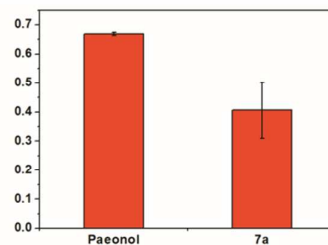
Fold changes of lipid accumulation in macrophages by fluorescent assay.

Here, we present a series of novel paeonol derivatives that prevent lipid accumulation at lower doses and exhibit improved water solubility.

ARTICLE TYPE

Low-dose Paeonol Derivatives Alleviate Lipid Accumulation

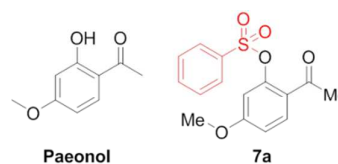Kuan-Chuan Pao,[a] Jin-Feng Zhao,[b] Tzong-Shyuan Lee,[b] Ying-Pei Huang,[a,c] Chien-Chung Han,[c] Lin-Chiang Sherlock Huang,[a,c] Kou-Hung Wu[a] and Ming-Hua Hsu*[a]

Here, we present a series of novel paeonol derivatives that prevent lipid accumulation at lower doses and exhibit improved water solubility. According to SAR analysis results, 1-(4-methoxy-2-(2-(piperidin-1-yl)ethoxy)phenyl)ethanone 10 (6a) and 4'-methoxy-2'-[(phenylsulfonyl)oxy]acetophenone (7a) demonstrate as good inhibition ability at low-dose (10 μ g/mL) as that of paeonol at high-dose (100 μ g/mL) treatment. In addition, compound 6a and 7a can be synthesized quantitatively with simple preparation methods. 15 Hence, we believe that compound 6a and 7a are potentially suitable antiatherogenic compounds for future drug development.

Paeonol 1-(2-hydroxy-4-methoxyphenyl)ethanone (1; Figure 1) is a phenolic compound isolated from the main active component of 20 Cortex Moutan, a traditional Chinese herbal medicine. Accompanying the considerable volume of scientific research on Chinese herbal medicine,[1–7] a growing body of evidence has indicated the benefits of using paeonol to treat various diseases. Paeonol is a widely used antiinflammatory agent for repairing 25 oxidative damage and enhancing immunity function *in vivo*.[8] In lipopolysaccharide-induced macrophages, paeonol can downregulate inflammation-associated genes.[9] At specific concentrations, paeonol can prevent oxidized low-density lipoprotein (ox-LDL)-induced monocyte adhesion to vascular 30 endothelial cells by attenuating the expression of vascular cell adhesion molecule-1 and phosphorylation of c-Jun N-terminal and extracellular signal-regulated kinases.[10] Moreover, paeonol represses the expression of micro-RNA-21 and release of tumor necrosis factor, which can protect rat vascular endothelial cells 35 from ox-LDL-induced injury.[11] Furthermore, paeonol can reduce the severity of liver fibrosis[12] and prevent ox-LDL-induced endothelial cell apoptosis.[13] Recent studies have indicated that paeonol is potentially suitable for treating various types of cancer. Paeonol enhances apoptosis in ovarian cancer cells by 40 downregulating survivin expression and activating caspase-3 expression.[14] Both *in vitro* and *in vivo* studies have indicated the radiosensitizing effect of paeonol on lung adenocarcinoma.[15] As a preventative Chinese herbal medicine, paeonol can facilitate preventing memory loss following ischemic stroke, and it can 45 improve oral glucose tolerance in patients receiving antidiabetic drugs.[16,17] Hence, paeonol is a potentially suitable candidate for further drug development.

Because of the effectiveness of paeonol in treating various types of inflammatory disease, several studies[9,18,19] have reported 50 that paeonol can facilitate preventing cardiovascular disease and treating atherosclerosis. Moreover, a previous research[18] showed that paeonol effectively mitigated the development of atherosclerosis in animal models. Zhao et al.[20] reported that paeonol exhibits a novel effect on the formation of foam cells. 55 Furthermore, it positively attenuates cholesterol accumulation induced by ox-LDL in macrophages, and it can prevent foam cells from forming by increasing ATP-binding membrane cassette transport protein A1 (ABCA1)-dependent cholesterol efflux. Zhao et al. also reported that paeonol enhanced the 60 transcriptional regulation of ABCA1 via activating liver X receptor α (LXRα) activity. According to previous researches,[21] the accumulation of lipid-laden foam-cells is characterized by preatherosclerotic lesions, the potential mechanisms of which involve either the uncontrolled uptake of ox-LDL or impaired 65 cholesterol efflux in macrophages. Collectively, the findings of these studies indicate that paeonol is a highly suitable candidate for regulating cholesterol efflux and preventing atherosclerosis.

In a previous study, paeonol treatment markedly alleviated the exacerbation of atherosclerosis in animal models.[18] Similar 70 results have been observed in anticancer tests and diabetic prevention trials.[15,17] However, a typical dose of these assays is extraordinarily high; for example, radiosensitization of lung adenocarcinoma involves a combination of 1718 mg/kg of paeonol and 10 Gy of irradiation.[15] In addition, Xiao et al.[22] and 75 Xie et al.[23] have reported that the distribution of paeonol in the bloodstream ranges from 4 to 10 μg/mL after only one dose is orally delivered through rapid absorption.

Paeonol and its Schiff base derivatives (2, 3; Figure 1) can form complexes with copper ions, which exhibits high 80 antioxidant activity, moderate DNA-binding activity, and adequate tumor-cell cytotoxicity.[24] The nitrogen atom of the Schiff base contributes to the metal-chelating site and hydrogen bond acceptor in the biological system. Our lab had been study drug development for years, and we found that paeonol modified 85 with phenylsulfonyl groups in the side chain could acquire higher bioactivity.[25] This study presents a series of novel paeonol derivatives conjugated with the side chain of the Schiff base, O-alkylation heterocyclic rings and phenylsulfonyl groups, which prevent lipid accumulation at lower doses.
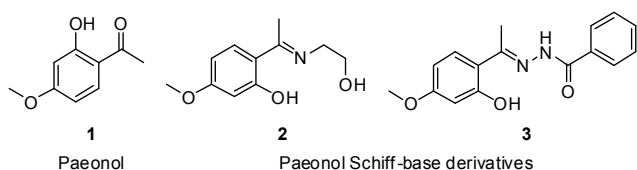
Figure 1 Structures of Paeonol, Paeonol Schiff-base derivatives.

Paeonol is the extract of *Paeonia suffruticosa Andr.*, which has been used in traditional Chinese medicine for thousands of years; however, paeonol exhibits poor water solubility. The paeonol Schiff base derivatives **4a–4l** were prepared by condensing paeonol with a series of primary amines bearing heteroatom-substituted amine side chains to increase its solubility and bioactivity (Scheme 1). The nitrogen atom of the Schiff base can improve overall solubility in water molecules, not because nitrogen is hydrophilic but because it can function as a hydrogen bond acceptor. Dimeric paeonol derivatives were obtained by treating paeonol with hydrazine to produce the zero-length derivative **5a** or by using ethyldiamine to obtain a Schiff-base-conjugated double-head paeonol derivative **5b** (Scheme 2). Furthermore, we synthesized the O-alkylation products of paeonol (**6a–6c**) through the phenol group of the paeonol by using potassium carbonate as a base and acetone as a solvent under reflux conditions (Scheme 3). We also conjugated the phenylsulfonyl group with paeonol through the phenol group to obtain new series of paeonol derivatives **7a–7h** (Scheme 4).
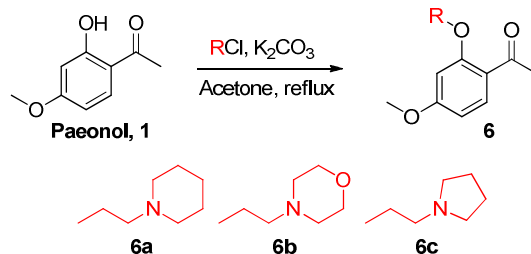
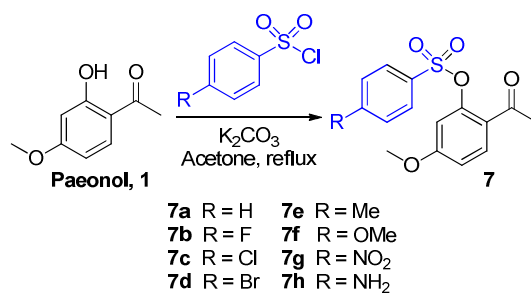Scheme 1 Synthesis of Paeonol Schiff-base derivatives.
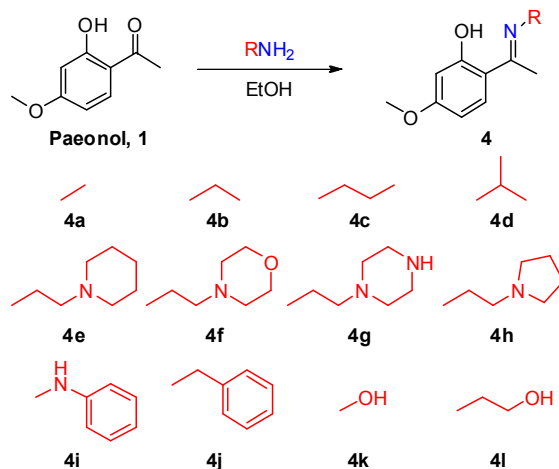
Scheme 2 Synthesis of Dimer Paeonol Schiff-base derivatives.
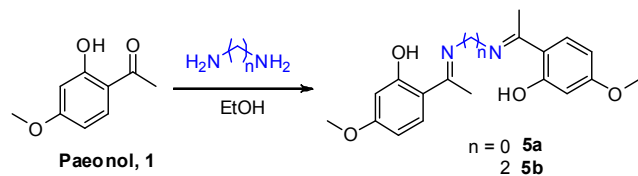
Scheme 3 Synthesis of O-alkylation Paeonol derivatives.

Scheme 4 Synthesis of Phenylsulfonyl Paeonol derivatives.

7a R = H   **7e** R = Me
7b R = F   **7f** R = OMe
7c R = Cl   **7g** R = NO_2
7d R = Br   **7h** R = NH_2

Various paeonol derivatives were prepared using a chemical synthesis technique to generate 14 Schiff-base-conjugated paeonol derivatives, including two double-head conjugated derivatives, 11 O-alkylation products, 3 of which with heterocycle ring and 8 of which bear phenylsulfonyl groups. Compared with natural paeonol, most of the synthesized compounds exhibited improved solubility in water because of the imine group in the core structure. Among these derivatives, the paeonol derivative **4g** bearing a piperazine ring (**4g**) exhibited a maximal solubility of 6.579 mg/mL (natural paeonol is 0.524 mg/mL; see Supporting Information Table S1). Improved water solubility is beneficial because it might improve pharmacokinetic research and lead to the development of novel drugs. However, some of the new synthesized compounds (**4i, 4j, 5,** and **7**) were more insoluble than natural paeonol, because these compounds contained a hydrophobic aromatic moiety that reduced the overall molecular hydrophilic affinity to the water.

The modified LDL (i.e., ox-LDL) disrupts the metabolism of lipids in macrophages, leading to foam cell formation and the induction of atherosclerosis. Therefore, we performed a fluorescent assay to confirm that the paeonol derivatives can prevent lipid accumulation in macrophages in ox-LDL treatment. Herein, we reported that the series of novel paeonol derivatives exhibited more favorable bioactivity in downregulating lipid accumulation than paeonol (Table 1). We found compounds **4i**, **5a**, **5b**, **7e**, and **7g** were precipitated in the media when treating with cell due to the poor solubility. Meanwhile, MTT results (see Supporting Information Figure S1) revealed that most of the phenylsulfonyl series compounds showed strong cytotoxicity, which caused the cell death. The results in Figure 2 indicated that some of these agents exerted the same effect as paeonol, but at lower doses (100-fold lower). Lee et al. reported that paeonol clearly influences lipid accumulation (Table 1). This study revealed that preincubation with novel paeonol derivatives exhibited exceptional bioactivity in regulating lipid content.

When the doses were identical (100 µg/mL) for the fluorescent assay, the compounds **4j, 6a,** and **7a** were substantially more effective in inhibiting lipid accumulation than natural paeonol (Figure 2). In particular, the compounds **6a** and **7a** downregulated
5 60%–65% of lipid accumulation, whereas paeonol downregulated only 30%. Moreover, at the high dose test of 100 µg/mL, the proposed derivatives clearly exhibited potential in reducing the dose requirements. To elucidate the low-dose effect of paeonol derivatives on lipid accumulation, we performed a dose-
10 dependent fluorescent assay (0.1–100 µg/mL), as shown in Figure 3. At a dose of 10 µg/mL, the compounds **4a, 4j, 6a, 6c,** and **7a** were as effective as a 100 µg/mL dose of natural paeonol. In Figure 4, the low-dose treatment of compound **7a** was more effective than natural paeonol administered in a high dose (40%
15 vs, 30%). At a dose of 1 µg/mL, the natural paeonol was ineffective in preventing lipid accumulation. By contrast, the compounds **4a, 4j, 6c,** and **7a** exhibited slight bioactivity. Furthermore, some of these compounds exhibited minor effects on lipid accumulation when the dose was reduced 1000-fold (0.1
20 µg/mL).

Table 1 Oxidized LDL-induced lipid accumulation in macrophage within Paeonol derivatives treatment.

Compounds[a]	Lipid Accumulation[b]	Compounds[a]	Lipid Accumulation[b]
Paeonol	0.669±0.006	**5a**	Not Resolve
4a	0.656±0.067	**5b**	Not Resolve
4b	0.785±0.007	**6a**	0.352±0.019
4c	0.509±0.07	**6b**	0.700±0.067
4d	0.836±0.242	**6c**	0.705±0.046
4e	0.727±0.033	**7a**	0.406±0.096
4f	0.931±0.175	**7b**	Cell Death
4g	0.782±0.107	**7c**	Cell Death
4h	0.584±0.087	**7d**	Cell Death
4i	Not Resolve	**7e**	Not Resolve
4j	0.464±0.054	**7f**	Cell Death
4k	Cell Death	**7g**	Not Resolve
4l	0.499±0.114	**7h**	Cell Death

[a] Paeonol derivatives in 100 µg/ml.
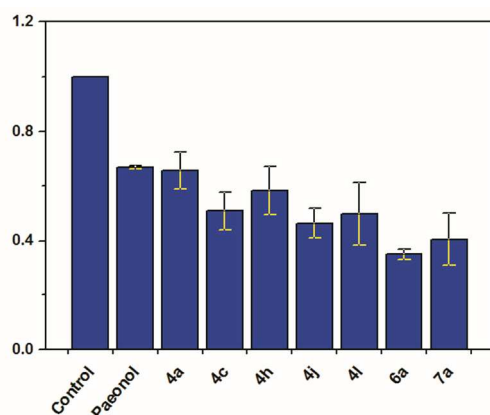25 [b] Lipid accumulation in macrophage by fluorescent assay.

Figure 2 Bio-activity of Paeonol derivatives for down-regulated lipid accumulation in macrophages. Data are mean ±S.E.M. from
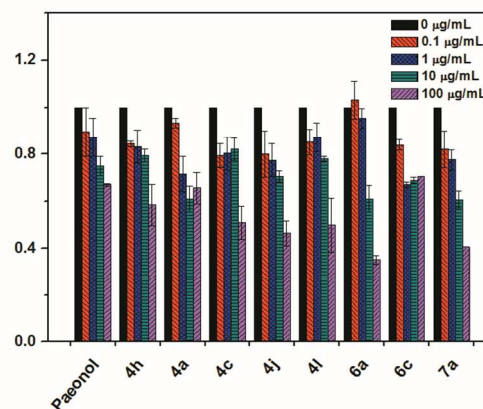30 three independent experiments. *P<0.05 versus Paeonol.

Figure 3 Fold changes of lipid accumulation within Paeonol
35 derivatives treatment in indicated concentrations. Data are mean ±S.E.M. from three independent experiments.
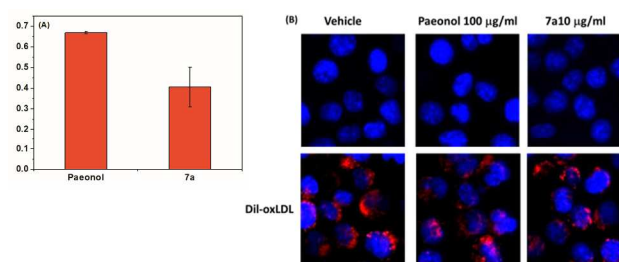

Figure 4 **7a** in low-dose treatment exhibited good activity as
40 paoenol in high-dose. A. Fold changes of lipid accumulation in macrophages by fluorescent assay. B. Representative fluorescent microscopy images of lipid accumulation after incubation with or without Dil-oxLDL in macrophages pretreated with paeonol or **7a**. Data are mean±S.E.M. from three independent experiments.
45

The structure–activity relationship (SAR) of the compounds was evaluated based on our results. Among the series of imine-conjugated paeonol derivatives, the compound **4c** bearing a propyl group was the most efficacious, compared with those
50 bearing methyl, ethyl, and isopropyl groups. The heterocyclic compounds bearing piperidine, pyrrolidine, morpholine, and piperazine rings (**4e–4h**, respectively) exhibited increased solubility in water; however, this had no effect on downregulated lipid accumulation in macrophages. The double-head conjugated
55 paeonol derivatives **5a** and **5b** were insoluble in water and precipitated in media during cell-culture treatment. Compounds **4i** and **4j** were similar structures bearing phenyl rings with poor water solubility. Although **4j** exhibited adequate regulation of lipid accumulation at low doses, **4i** caused cell death. Similar
60 phenomena were observed for compounds **4k** (cell death) and **4l** (adequate regulation). Among the O-alkylation paeonol derivatives, compound **6a** was the most potent compound in inhibiting lipid accumulation. The piperidine-modified derivative **6a** was more active than either morpholine **6b** or pyrrolidine **6c**.
65 Furthermore, we found most of the phenylsulfonyl paeonol derivatives cause cell death; however, the **7a** exhibited high

activity. The SAR analysis suggested that phenylsulfonyl moieties bearing halide (**7b**, **7c**, and **7d**), OMe (**7f**), and NH$_2$ (**7h**) cause cell death. Meanwhile, the **7c** with CH$_3$ group and **7d** with NO$_2$ group were too hydrophobic to precipitate in the media. The low-dose treatment of compound **7a** achieved the same effect as paeonol administered in 100 µg/mL (Figure 4). The results indicated that substituting the phenol group with a phenylsulfonyl side chain is a new strategy for achieving further chemical modification.

Conclusions

In summary, we synthesized 14 paeonol derivatives containing Schiff-base conjugates and 11 O-alkylation derivatives. The water solubility of the compounds was evaluated, and the results revealed that the compounds bearing a heterocyclic ring exhibited increased solubility in water, although such increase exerted no effect on downregulated lipid accumulation in macrophages. The SAR analysis results indicated that compounds **6a** and **7a** are potent inhibitors of lipid accumulation at low doses. In accordance with Zhao et al.[20], we hypothesize that this novel series of paeonol derivatives follows the paeonol pathway to regulate lipid accumulation by upregulating the nuclear translocation of LXRα, which enhances the mRNA and protein expression of ABCA1, thereby reducing the accumulation of cholesterol. The results indicated that compounds **6a** and **7a** are potentially suitable antiatherogenic compounds for future drug development.

Notes and references

[a] *Nuclear Science & Technology Development Center, National Tsing Hua University, Hsinchu 30013, Taiwan. Tel: +886-3-573-1180 ; Fax: +886-3-572-5974. E-mail: mhhsu@mx.nthu.edu.tw*

[b] *Department of Physiology, National Yang-Ming University, Taipei 11221, Taiwan.*

[c] *Department of Chemistry, National Tsing Hua University, Hsinchu 30013, Taiwan.*

‡ Kuan-Chuan Pao and Jin-Feng Zhao contributed equally to this work.

1   S. Akhondzadeh and S. H. Abbasi, *Chin. J. Integr. Med.,* 2006, **21**, 113-118.

2   G. G. Yue, K. M. Chan, M. H. To, L. Cheng, K. P. Fung, P. C Leung and C. B. Lau, *J. Nat. Prod.,* 2014, **77**, 1074-1077.

3   Q. Zhou, W. Z. Qin, S. B. Liu, J. SW. Kwong, J. Zhou and J. Chen, *Cochrane Database. Syst. Rev.,* 2014, **4**.

4   J. L. Ru, P. Li, J. N. Wang, W. Zhou, B. H. Li, C. Huang, P. D. Li, Z. H. Guo, W. Y. Tao, Y. F. Yang, X. Xu, Y. Li, Y. H. Wang and L. Yang, *J. Cheminform.*, 2014, **6**:13.

5   Y. Ueda, K. Mori, S. Satoh, H. Dansako, M. Ikeda and N. Kato, *Biochem. Biophys. Res. Commun.*, 2014, **447**, 341-345.

6   Y. C. Chen, F. P. Chen, T. J. Chen, L. F. Chou and S. J. Hwang, *Hepatogastroenterology.*, 2008, **55**, 467-470.

7   S. Wang, S. Penchala, S. Prabhu, J. Wang and Y. Huang, *Curr. Drug. Discov. Technol.*, 2010, **7**, 67–75.

8   B. Chen, M. L. Ning and G. S. Yang, *Molecules.*, 2012, **17**, 4672-4683.

9   H. Huang, E. J. Chang, Y. Lee, J. S. Kim, S. S. Kang and H. H. Kim, *Inflamm. Res.*, 2008, **57**, 189–198.

10   Y. Q. Wang, M. Dai, J. C. Zhong and D. K. Yin, *Biol. Pharm. Bull.*, 2012, **35**, 767–772.

11   Y. R. Liu, J. J. Chen and M. Dai, *Acta. Pharmacol. Sin.*, 2014, **35**, 483-488.

12   D. Kong, F. Zhang, D. Hua. Wei, X. J. Zhu, X. P. Zhang, L. Chen, Y. Lu and S. Zheng, *J. Gastroenterol. Hepatolo.,* 2013, **28**, 1223–1233.

13   M. H. Bao, Y. W. Zhang and H. H. Zhou, *J. Ethnopharmacol.*, 2013, **146**, 543–551.

14   J. Yin, N. S. Wu, F. Q. Zeng, C. Cheng, K. Kang and H. Yang, *Acta Histochem.*, 2013, **115**, 835–839.

15   Y. Lei, H. X. Li, W. S. Jin, W. R. Peng, C. J. Zhang, L. J. Bu, Y. Y. Du, T. Ma and G. P. Sun, *Int. J. Radiat. Biol.*, 2013, **89**, 1079–1086.

16   S. Yu. Su, C. Yi. Cheng, T. H. Tsai and C. L. Hsieh, *Evid. Based Complememt. Alternat. Med.,* 2012*,* vol. **2012**, Article ID 932823, 11 pages.

17   C. H. Lau, C. M. Chan, Y. W. Chan, K. M. Lau, T. W. Lau, F. C. Lam, W. T. Law, C. T. Che, P. C. Leung, K. P. Fung, Y. Y. Ho and C. B. S. Lau, *Phytomedicine*, 2007, **14**, 778–784.

18   H. Li, M. Dai, W. Jia, *Planta. Med.*, 2009, **75**, 7–11.

19   Y. J. Li, J. X. Bao, J. W. Xu, F. Murad and K. Bian, *Vasc. Pharmacol.*, 2010, **53**, 169–176.

20   J. F. Zhao, S. J. J. Leu, S. K. Shyue, K. H. Su, J. Wei and T. S. Lee, *Am. J. Chinese. Med.*, 2013, **41**, 1079–1096.

21   Andrew C. Li and Christopher K. Glass, *Nat. Med.*, 2002, **8**, 1235–1242.

22   Y. Xiao, Y. H. Zhang, Y. X. Sheng, J. L. Zhang, *Biomed. Chromatogr.*, 2008, **22**, 527–534.

23   Y. Xie, H. Zhou, Y. F. Wong, H. X. Xu, Z. H. Jiang, L. Liu, *J. Pharm. Biomed. Anal.*, 2008, **46**, 748–756.

24   D.-D. Qin, Z.-Y. Yang, F.-H. Zhang, B. Du, P. Wang, T.-R. Li, Inorg. Chem. Commun. 2010, **13**, 727–729.

25   T.-J. Huang, H. Chuang, J.-C. Horng, Y.-C. Kuo, C.-W. Chen, F.-Y. Tsai, Y.-C. Liang, S.-C. Yen, S.-C. Chou, M.-H. Hsu, *Eur. J. Med. Chem.* 2015, **90**, 428–435.