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REVIEW

Biosynthesis, total syntheses, and antitumor activity of tanshinones and their analogs as potential therapeutic agents

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Tanshinones are a series of abietane diterpenes, isolated exclusively from *Salvia miltiorrhiza* and related species. More than 40 tanshinones and their analogs have been isolated since the 1930s. Their biosynthetic pathway correlates with the MEP/DOXP pathway, and many key enzymes, such as SmCPS, are responsible for establishing their molecular scaffolds and stereospecificity. Because of their unique structural characteristics and promising biological activities, total syntheses of various tanshinones have attracted the interest of many synthetic chemists, including R. H. Thomson, H. Kakisawa, R. L. Danheiser, Y. Inouye and J. K. Snyder. Tanshinones and their analogs exhibit interesting and broad antitumor activity in various cell and animal models. Most recently, the tanshinone analog neo-tanshinlactone has shown potent and selective activity against breast cancer. This review will discuss the biosynthesis, total syntheses, and antitumor activities of tanshinones, especially neo-tanshinlactone and its analogs.

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1 Introduction

Tanshinones, which are abietane diterpenes, were first isolated by Nakao in 1930 from the roots of *Salvia miltiorrhiza* ('tanshen'), a well-known traditional Chinese medicine (TCM).[1,2] Tanshen has been used extensively for the treatment of coronary heart diseases, particularly angina pectoris and myocardial infarction,[3] as well as inflammatory diseases, including chronic hepatitis, arthritis, and endangitis.[4]

Over the past eight decades, natural product chemists have investigated the chemical constituents in tanshen, and so far more than 40 diterpenoids have been isolated from *S. miltiorrhiza*.[3] Tashinones are generally composed of four rings, including naphthalene or tetrahydronaphthalene rings A and B, an *ortho*- or *para*-quinone or lactone ring C, and a furan or dihydrofuran ring D. In the 1930s, Takiura[5–7] and Wessely[8,9] and their co-workers used spectroscopic data and degradative work to identify the structures of tanshinone I (**1**), tanshinone IIA (**2**), tanshinone IIB (**3**), and cryptotanshinone (**4**) (Fig. 1). These tanshinones are found exclusively in the *Salvia* genus. Structurally, all four compounds are characterized by an *ortho*-quinone

C-ring. Luo *et al.* reported the isolation and characterization of a tanshinone derivative, tanshinlactone (**5**), in 1986.[10] Recent studies by Lee and co-workers led to a new compound, neo-tanshinlactone (**6**), based on a bioassay-guided isolation approach.[11] Both **5** and **6** differ from the **1–4** by the presence of a lactone rather than an *ortho*-quinone C-ring. Compounds **5** and **6** are regio-isomers, differing in the positions of the lactone carbonyl and oxygen groups. In **5**, the lactone carbonyl is present at position-11 and oxygen at position-12 of the diterpenoid, while in **6**, the positions are reversed.

The biosynthetic pathways to the tanshinones have been explored for more than two decades, and work is still ongoing to determine the exact transformations and related enzymes. This research has provided various methods to increase the production of tanshinones in *S. miltiorrhiza*, such as by elicitation with a yeast elicitor and transformation of cells.[12–14]

Yizhou Dong

Yizhou Dong received his B.S. in pharmaceutical sciences from Peking University, Health Science Center (2002), his M.S. in organic chemistry from Shanghai Institute of Organic Chemistry (2005), and his Ph.D. degree from the University of North Carolina at Chapel Hill (UNC-CH) in 2009. His doctoral research, under Professor K.-H. Lee, focused on the design, synthesis, and biological evaluation of novel neo-tanshinlactone analogs as potent and selective anti-breast cancer agents. Following a one-year postdoctoral appointment in Professor K.-H. Lee's Natural Products Research Laboratories (UNC-CH), he is now a research fellow at Children's Hospital Boston, and also a research affiliate at the David H. Koch Institute for Integrative Cancer Research at MIT in the laboratory of Dr. Robert S. Langer.

Susan L. Morris-Natschke

Susan L. Morris-Natschke received her B.S. in chemistry from the University of Maryland at College Park (1975) and her Ph.D. in organic chemistry from the University of North Carolina at Chapel Hill (1982). She is currently Research Professor in the Division of Medicinal Chemistry and Natural Products, in the UNC Eshelman School of Pharmacy, where she has been on the faculty since 1983. Her interests include scientific writing/editing, as well as the synthesis and structure–activity relationships of bioactive natural products.

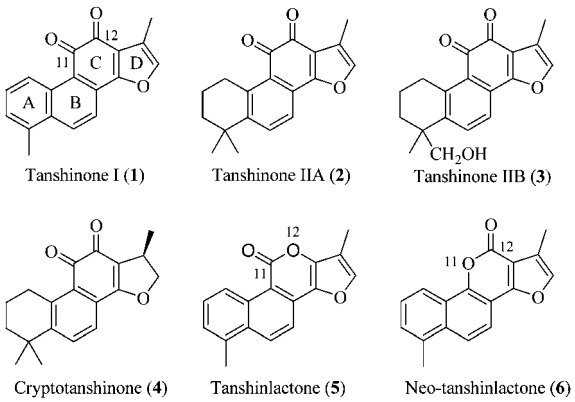


Fig. 1 Structures of tanshinones (**1–4**) and their analogs (**5–6**).

Extensive studies have demonstrated that tanshinones and their analogs exhibit various pharmacological activities,[15–20] including antibacterial, antioxidant, anti-inflammatory, and antineoplastic.[21–23] Recently, it was reported that tanshinones also display inhibitory activity on acetylcholinesterase (AChE), which suggested a potential application for the study and treatment of Alzheimer's disease.[24,25] Dihydrotanshinone and **4** inhibited AChE in a dose-dependent manner, with IC_{50} values of 1.0 and 7.0 μM, respectively.[25] Tanshinones and their analogs have shown antitumor activity in various cell lines and animal models. For instance, **6** was 10-fold more potent and 20-fold more selective than tamoxifen citrate (a widely used selective estrogen receptor modulator) against the MCF-7 breast cancer cell line *in vitro*.[11]

The unique structures, together with the potential therapeutic functions, of tanshinones have sparked much interest among synthetic chemists. This review will focus on the biosynthetic pathways, total syntheses, and antitumor activities of tanshinones (**1–4**) and their analogs (**5–6**).

Kuo-Hsiung Lee

Kuo-Hsiung Lee received his B.S. in pharmacy at Kaohsiung Medical University, Taiwan (1961), his M.S. in pharmaceutical chemistry at Kyoto University, Japan (1965), and his Ph.D. in medicinal chemistry at the University of Minnesota, Minneapolis (1968). Since 1970, he has been on the faculty of the UNC Eshelman School of Pharmacy, and is now Kenan Professor of Medicinal Chemistry and Director of the Natural Products Research Laboratories. In 2009, he was appointed as Honorable Director of the Chinese Medicine and Research Development Center, China Medical University and Hospital. He has published over 700 research articles, been granted over 70 patents, and received numerous awards, including most recently the Norman R. Farnsworth Research Achievement Award, American Society of Pharmacognosy (2009).

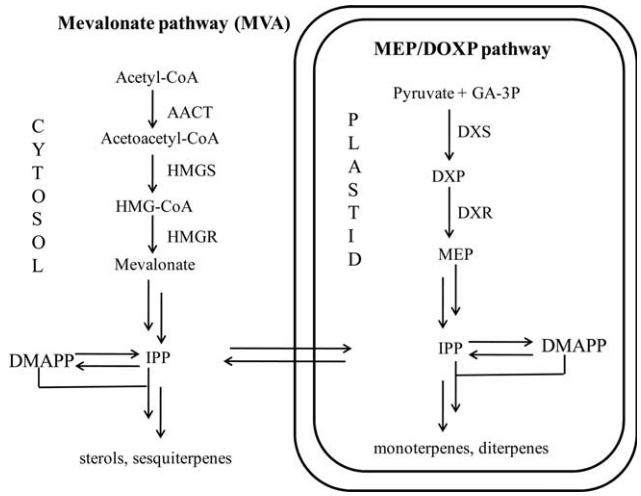
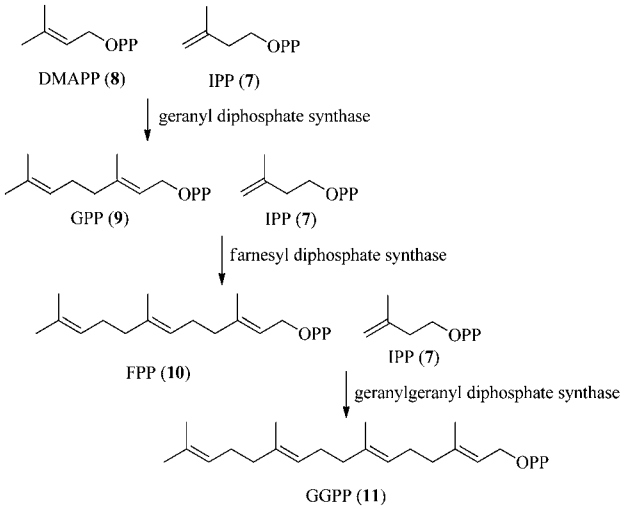


Fig. 2 Schematic representation of the mevalonate (MVA) pathway and the MVA-independent pathway (MEP/DOXP pathway).[28,29]



Scheme 1 Biosynthetic pathway of GGPP (**11**) from DMAPP (**8**) and IPP (**7**).[38]

2 Biosynthesis

It has long been known that terpenes or terpenoids are synthesized *via* at least two different pathways in plants, the mevalonate (MVA) pathway occurring in the cytosol, and the MVA-independent pathway (MEP/DOXP pathway) in the cell plastids (Fig. 2).[26–28] The former is responsible for the synthesis of sterols, certain sesquiterpenes, and the side chain of ubiquinone, and the latter is involved in production of monoterpenes, certain sesquiterpenes, diterpenes, carotenoids, and the side chains of chlorophylls and plastoquinone.[27] In the MVA pathway, mevalonate is synthesized from 3-hydroxy-3-methylglutaryl CoA through the catalysis of HMGR (3-hydroxy-3-methylglutaryl CoA reductase), which is an important step in the pathway.[29] In 2005, Ge *et al.* reported the accumulation of tanshinones in hairy root cultures of *S. miltiorrhiza* induced by a biotic elicitor, the carbohydrate fraction of yeast extract, and an abiotic elicitor, Ag^{+}.[28,30] Their results indicated that tanshinone production induced by the two elicitors was synthesized mainly *via* the MEP/DOXP pathway. However, this process could also depend on crosstalk between the MVA and MEP/DOXP pathways, as recent studies have proven that extensive crosstalk occurs between the two pathways.[27,31]

In the MEP/DOXP pathway (Fig. 2),[28,32] pyruvate and glyceraldehyde 3-phosphate (GA-3P) initially form 1-deoxy-D-xylulose-5-phosphate (DXP) through the action of DXP synthase (DXS). DXP is then converted to 2-*C*-methyl-D-erythritol 4-phosphate (MEP) by the action of DXP reductoisomerase (DXR). MEP then undergoes several steps catalyzed by a series of enzymes to afford isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). In the entire process, DXS plays an important role and is a rate-limiting enzyme for the synthesis of IPP and DMAPP.[33–35] In 2009, Wu *et al.* cloned and characterized a full-length cDNA encoding DXP from the hairy roots of *S. miltiorrhiza*.[36] Their study was the first to find a correlation between the elicitor-induced DXR transcription and the accumulation of tanshinone production.

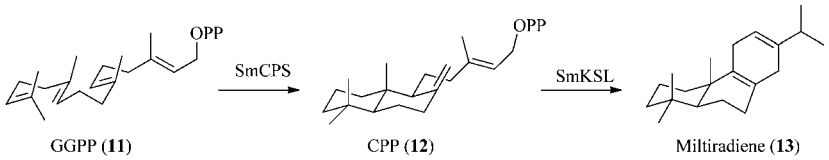
Reactions of DMAPP (**8**) and one or more IPP (**7**) residues lead to GPP (**9**), FPP (**10**), and GGPP (**11**) through the actions of geranyl diphosphate synthase (prenyltransferase), farnesyl diphosphate synthase, and geranylgeranyl diphosphate synthase (GGPPS) (Scheme 1).[37–40] GGPP is the universal precursor of diterpenoids.[41] In 2010, Kai *et al.* reported their results in characterization, expression profiling, and functional identification of a gene, *SmGGPPS*, encoding GGPPS from *S. miltiorrhiza*. They indicated that *SmGGPPS* showed extensive homology with other plant GGPPS, containing all five conserved domains and functional aspartate-rich motifs of the prenyltransferases.[42]

GGPP (**11**) is the precursor of labdadienyl/copalyl diphosphate (CPP, **12**), which has a fused bicyclic structure. The conversion of GGPP to CPP is catalyzed by CPP synthase (CPS),[43] a class II diterpene cyclase. Gao *et al.* applied a functional genomics approach and discovered that SmCPS can specifically transform GGPP to CPP with stereospecificity (Scheme 2).[44,45] Additional stereocenters are established by subsequent cyclization and rearrangement reactions catalyzed by CPP-specific class I diterpene synthase (often termed kaurene synthase-like, KSL). Gao *et al.* identified a CPP-specific class I diterpene synthase, SmKSL, from a cDNA library of *S. miltiorrhiza*.[44] Rapid amplification of the cDNA ends determined its full-length mRNA sequence. SmKSL produces miltiradiene (**13**) specifically from CPP as more than 95% of the total product output.[44] The structure of **13** was assigned based on HMBC, HMQC, and COSY data.

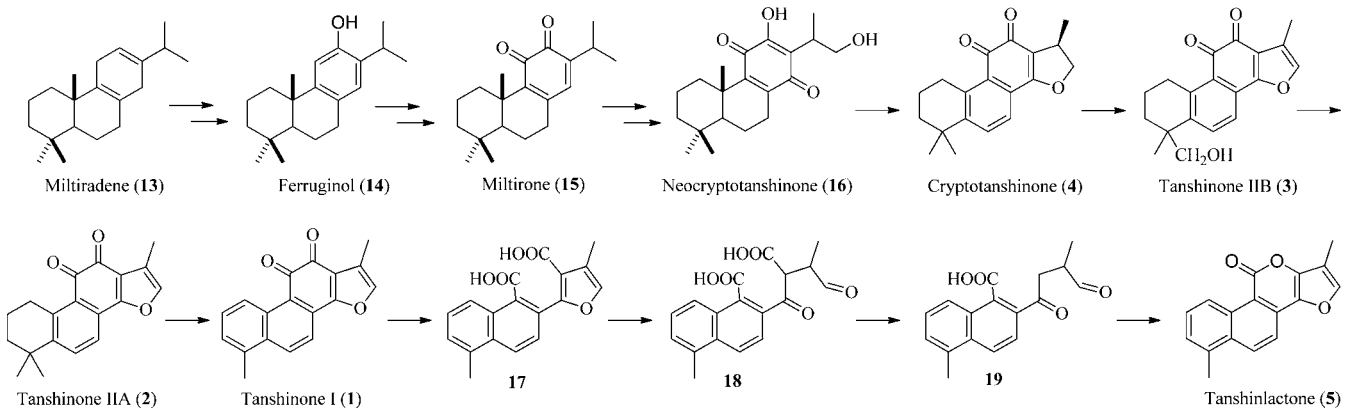
Because the cyclohexan-1,4-diene is relatively unstable, the authors proposed that miltiradiene (**13**) may undergo aromatization to ferruginol (**14**), followed by further installation of different groups to give miltirone (**15**) and neocryptotanshinone (**16**). Compound **16** can be converted stepwise to **4**, **3**, **2**, and **1** (Scheme 3).[44] In addition, Luo proposed that tanshinlactone (**5**) may be produced biologically from **1** through three intermediates, carboxylic acid **17**, β-keto carboxylic acid **18**, and ketone **19** (Scheme 3).[10]

3 Total syntheses

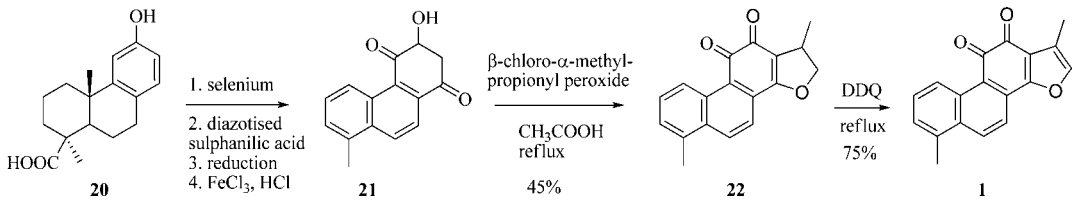
Total syntheses of tanshinones and their analogs have been attempted since the early 1960s,[46] and many synthetic strategies



Scheme 2 Biosynthesis of miltiradiene (**13**) from GGPP (**11**) with enzymes SmCPS and SmKSL.[44]



Scheme 3 Proposed biosynthetic pathway of tanshinones **1–4** and tanshinlactone **5**.[10,44]



Scheme 4 First total synthesis of tanshinone I (**1**) from podocarpic acid.

have been elucidated. This section will emphasize strategies for synthesis of the major tanshinones and their analogs.

3.1 First total synthesis of tanshinone I (1) from podocarpic acid

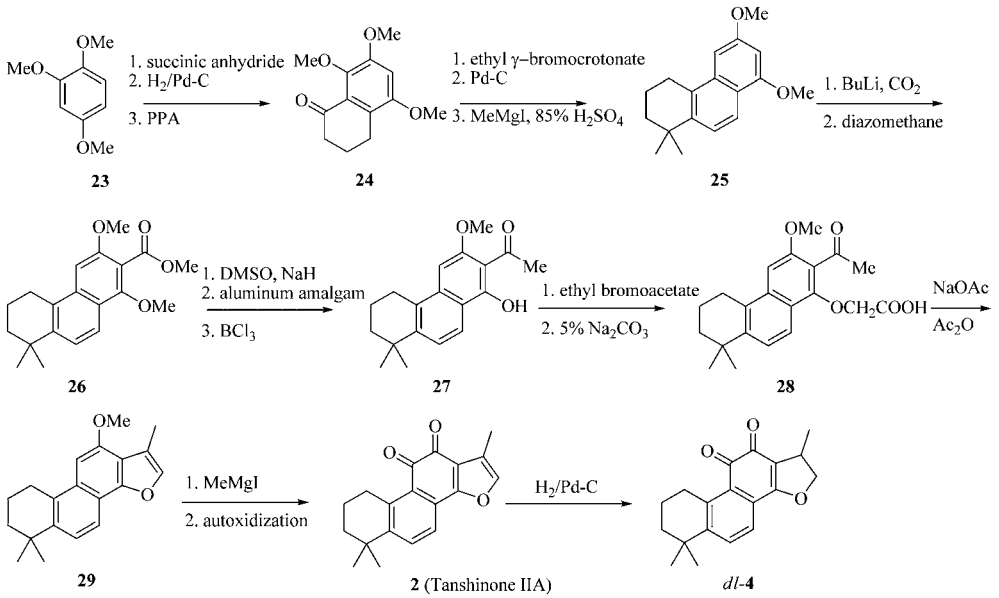
The first total synthesis of **1** was completed in 1968 by Baillie and Thomson starting from the natural product podocarpic acid (**20**) (Scheme 4).[47] Dehydrogenation of **20** with selenium powder gave 8-methyl-3-phenanthrol,[48,49] which was coupled with diazotized sulfanilic acid, then reduced to an amino-phenol, and oxidized to quinone **21** by Fieser's procedure.[50] Intermediate **21** was cyclized with β-chloro-α-methylpropionyl peroxide to afford quinone **22**, which was dehydrogenated to yield target compound **1**. This synthesis provided **1** in six steps and 34% overall yield (based on quinone **21**). A similar synthetic route was applied for the synthesis of (±)-**4** from 7-methoxy-1-tetralone.

3.2 Stepwise cyclization approach for the synthesis of (±)-cryptotanshinone (4)

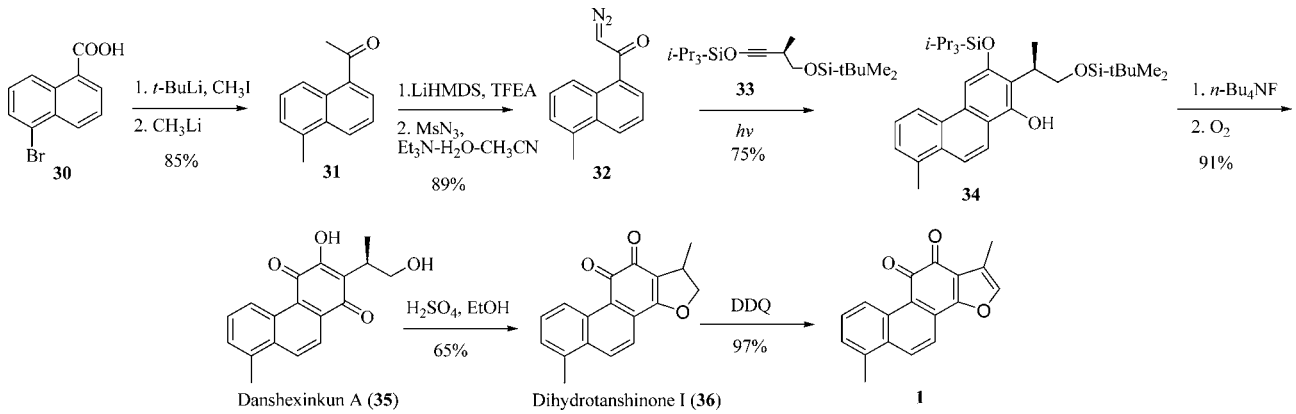
Kakisawa and co-workers reported the total syntheses of **2** and **4** through a stepwise cyclization approach from 1,2,4-trimethoxybenzene (**23**) (Scheme 5).[51,52] The bicyclic trimethoxy-α-tetralone **24** was prepared by Friedel–Crafts acylation of **23** with succinic anhydride, catalytic hydrogenation, and intramolecular Friedel–Crafts acylation with PPA. One OMe group was removed from the phenyl ring, and a third ring was installed by the sequence of Reformatsky reaction, hydrogenation, Grignard reaction, and subsequent cyclodehydration, producing the tetrahydrophenanthrene **25**. Treatment of **25** with butyllithium and carbon dioxide added a carboxylic acid, which was then esterified to give the methyl ester **26**. Compound **26** was converted by Corey's method (*via* the sulfoxide) to a methyl ketone intermediate,[53] and then one OMe group was selectively cleaved by boron trichloride to yield acetylmethoxyphenanthrol **27**. Reaction of **27** with ethyl bromoacetate followed by saponification provided the keto-carboxylic acid **28**. An intramolecular Perkin reaction provided furophenanthrene **29**, and then treatment with excess methylmagnesium iodide and autoxidization (oxidized with difficulty with Fremy's salt) afforded the desired **2** in a total of 13 steps. Compound **2** was easily transformed to (±)-**4** by catalytic hydrogenation.

3.3 Photochemical aromatic annulation strategy

Danheiser *et al.* developed a photochemical aromatic annulation strategy for the synthesis of polycyclic compounds, including **1**, **2**, and (−)-**4**.[54–56] The synthesis of **1** is shown in Scheme 6. 5-Bromo-1-naphthoic acid (**30**) was first converted to methyl ketone **31** through the Rubottom quenching procedure.[57] Next, the authors developed a two-step "detrifluoroacetylative" diazo



Scheme 5 Kakisawa's stepwise cyclization for synthesis of (±)-cryptotanshinone (**4**).



Scheme 6 Danheiser's photochemical aromatic annulations for synthesis of tanshinone I (**1**).

transfer method to synthesize the key diazo ketone **32** from **31**.[58] Using a low-pressure mercury lamp (254 nm), **32** and siloxyalkyne **33** were irradiated to accomplish the aromatic annulation step and produce the phenol **34**. Treatment of **34** with tetra-*n*-butylammonium fluoride in the presence of oxygen gave danshexinkun A (**35**), which was converted to dihydrotanshinone I (**36**) with concentrated sulfuric acid. Dehydrogenation of **36** with DDQ led to the final product **1**. This synthetic route provided **1** in six steps and 33% overall yield.

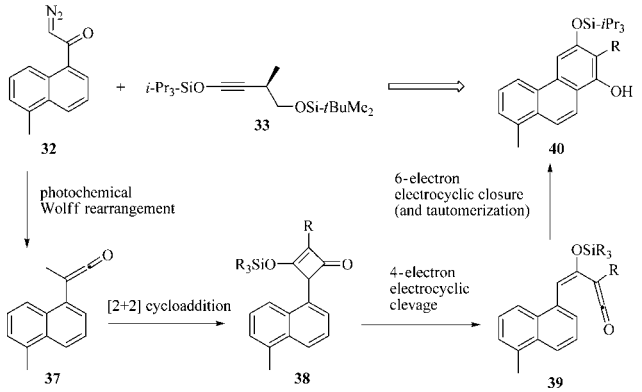
The intermediates in the photochemical aromatic annulations are shown in Scheme 7. Irradiation of the α-diazo ketone **32** initiated a photochemical Wolff rearrangement and produced the arylketene **37**, which reacted directly with siloxyalkyne **33** to afford the regiospecific [2 + 2] cycloaddition product **38**. Compound **38** underwent 4π electrocyclic opening and 6π electrocyclization to afford vinylketene **39** and then tricyclic phenol **40**, consecutively.

Danheiser *et al.* applied a similar strategy for the total syntheses of **2** and (−)-**4** from **33** and **41** through the key intermediates **42** and **43** (Scheme 8).[56] The intermediates and remaining synthetic steps are similar to those shown in Schemes 6 and 7.

3.4 Diels–Alder reaction strategy

Inouye and Kakisawa used a Diels–Alder reaction to build the tanshinone scaffold.[59] Their goals were to study the molecular orientation in the Diels–Alder reaction and establish a general synthetic route to all tanshinones (Scheme 9). *o*-Methylstyrene (**44**) and 3-methylbenzofuran-4,7-quinone (**45**) were heated without solvent to give the phenanthrene quinone **46** in less than 26% yield. Reduction of **46** with platinum oxide in acetic acid afforded the dihydro compound **47**, which was hydrolyzed with potassium hydroxide and then acidified with concentrated sulfuric acid to give **36**. Finally, **1** was obtained by treatment of **36** with DDQ. The synthetic route took four steps and resulted in approximately 4% overall yield.

Similarly, **2** and (±)-**4** were synthesized from **45** and **48** (Scheme 10). The Diels–Alder reaction occurred in hot ethanol to provide **49**. Dehydrogenation of ring B, followed by hydrogenation of ring D with palladium-on-carbon rather than platinum oxide gave the dihydro compound **50**. Target tanshinones were obtained by established reaction sequences.



Scheme 7 Intermediates in photochemical aromatic annulations for synthesis of tanshinone I (**1**).

Lee and Snyder later reported an ultrasound-promoted Diels–Alder reaction to construct the tanshinone scaffold, which provided a simple and effective method to promote cycloaddition and produce tanshinones in high yield (Scheme 11).[60–62] For example, **2** was obtained from the Diels–Alder reaction of **48** and **51** (eight steps and 31% overall yield, including the three-step synthesis of **51**[63]). This strategy was also applied to the synthesis of nortanshinone, tanshindiol B, and similar compounds.

3.5 Base-induced photochemical cyclization

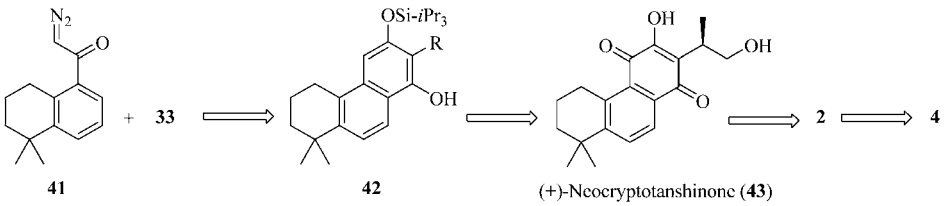
De Koning *et al.* developed a base-induced photochemical cyclization for the synthesis of substituted naphthalenes and phenanthrenes, which could be applied to produce the key intermediate (**56**) in Scheme 12.[64,65] Suzuki coupling of **53** with **54** afforded **55** (96% yield). Treatment of the biphenyl **55** with potassium *tert*-butoxide with simultaneous irradiation from a high-pressure mercury lamp gave the desired intermediate **56** in 70% yield. In 1974, Hout and Brassard demonstrated the conversion of **56** into **1** in six steps.[66] Demethylation and oxidation of **56** gave the quinone **57**, which was acetylated to triacetate **58**. Compound **59** was obtained by reaction with sodium methoxide, followed by addition of a methylallyl group to yield **60**. Acetylation and oxidative cleavage (Lemieux–Johnson oxidation) of the allyl group **60** provided aldehyde **61**. Basic hydrolysis followed by acid-promoted cyclization afforded **1**.

3.6 SmI_2-promoted radical cyclization

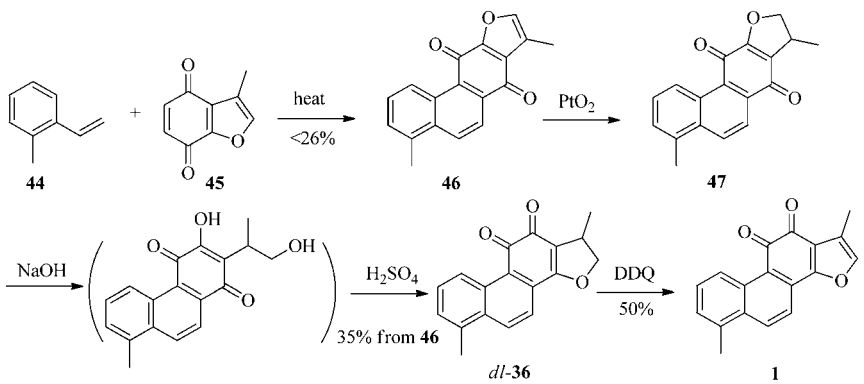
Jiang and co-workers have reported a radical cyclization process to make the furan ring of **4**, as shown in Scheme 13.[67] Monomethylation of 1,5-naphthalenediol (**62**) gave the phenol **63**. Treatment of **63** with sodium hydride and diethyl phosphorochloridate afforded aryl diethyl phosphate **64**. Cross-coupling of **64** with Grignard reagent followed by Friedel–Crafts alkylation provided tetrahydrophenanthrene **65**. Demethylation, bromination, and etherification provided bromide **66**. The key tetracyclic intermediate **67** was obtained *via* SmI_2-promoted intramolecular cyclization in 88% yield.[68] Aromatic nitration and hydrogenation produced the amine **68**. Oxidation of **68** led to (±)-**4** (11 steps and 7% overall yield from **62**), which was converted to **2** with DDQ, as described before.

3.7 Tandem esterification and intramolecular Friedel–Crafts acetylation

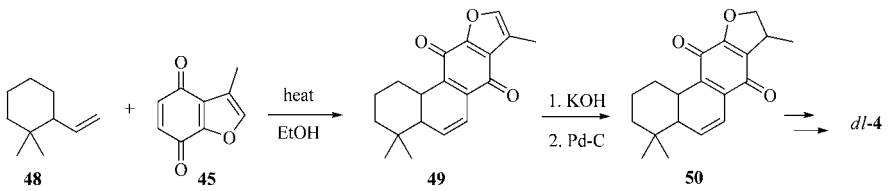
The total synthesis of neo-tanshinlactone (**6**) was achieved previously by Wang and Lee *et al.* (Scheme 14).[11] Tandem esterification/intramolecular Friedel–Crafts acetylation and tandem alkylation/intramolecular aldol reaction constructed rings C and D, respectively. Initially, 5-methoxy-1-tetralone (**69**) underwent Grignard, dehydration, aromatization, and demethylation reactions to provide 5-methyl-1-naphthol (**70**) in 70%



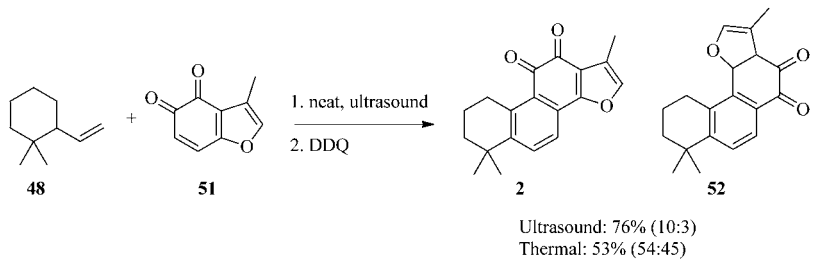
Scheme 8 Starting material and key intermediates in Daneheiser's synthesis of tanshinone IIA (**2**) and (−)-cryptotanshinone (**4**).



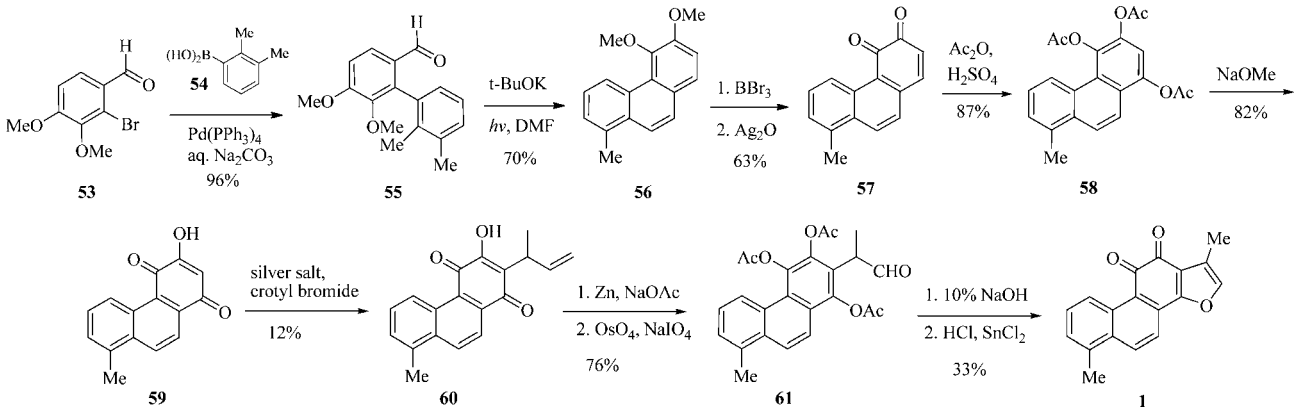
Scheme 9 Inouye's Diels–Alder reaction strategy for synthesis of tanshinone I (**1**).



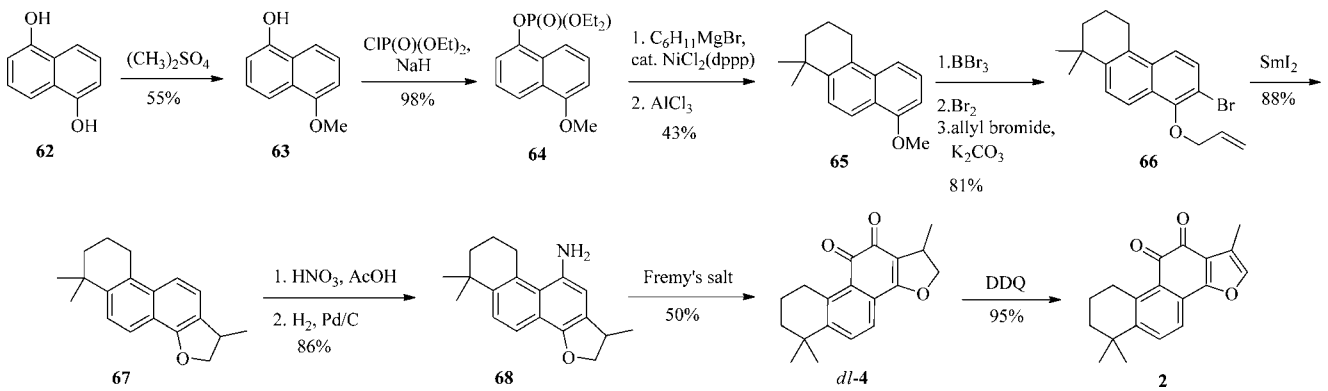
Scheme 10 Inouye's synthetic pathway to (±)-cryptotanshinone (**4**).



Scheme 11 Snyder's ultrasound-promoted Diels–Alder reaction strategy for synthesis of tanshinone IIA (**2**).



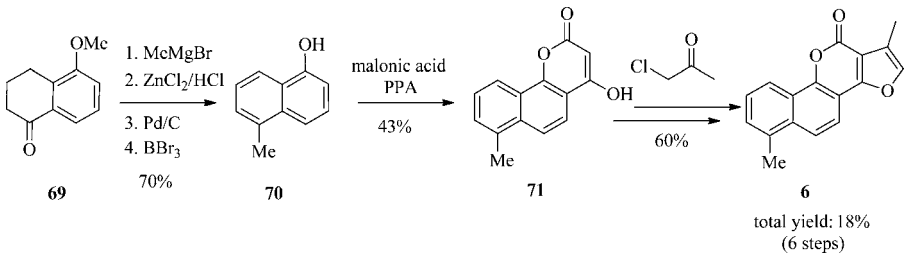
Scheme 12 Base-induced photochemical cyclization approach for synthesis of tanshinone I (**1**).



Scheme 13 SmI_2-promoted radical cyclization approach for synthesis of (±)-cryptotanshinone (**4**) and tanshinone IIA (**2**).

yield. Treatment of **70** with malonic acid in the presence of PPA yielded 4-hydroxybenzochromenone **71** through a tandem esterification and intramolecular Friedel–Crafts acetylation.[69] Neo-tanshinlactone (**6**) was subsequently obtained *via* a tandem alkylation/intramolecular aldol reaction.[70] The synthesis took six steps and resulted in an 18% overall yield.

Chemical modifications of **6** for structure–activity relationship (SAR) studies by Lee led to the preparation of 4-ethyl neo-tanshinlactone **72** (Fig. 3), a synthetic congener of **6**.[71] The synthesis of **72** was optimized to five steps and an overall yield of 18%,[72] compared with seven steps and 3% yield reported initially.[69] Structural simplification of natural products is a powerful and



Scheme 14 Tandem esterification and intramolecular Friedel–Crafts acetylation for synthesis of neo-tanshinlactone (**6**).

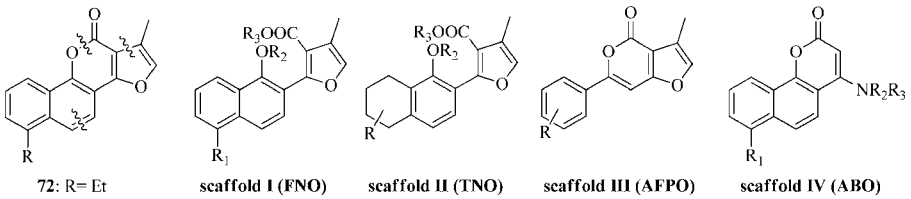
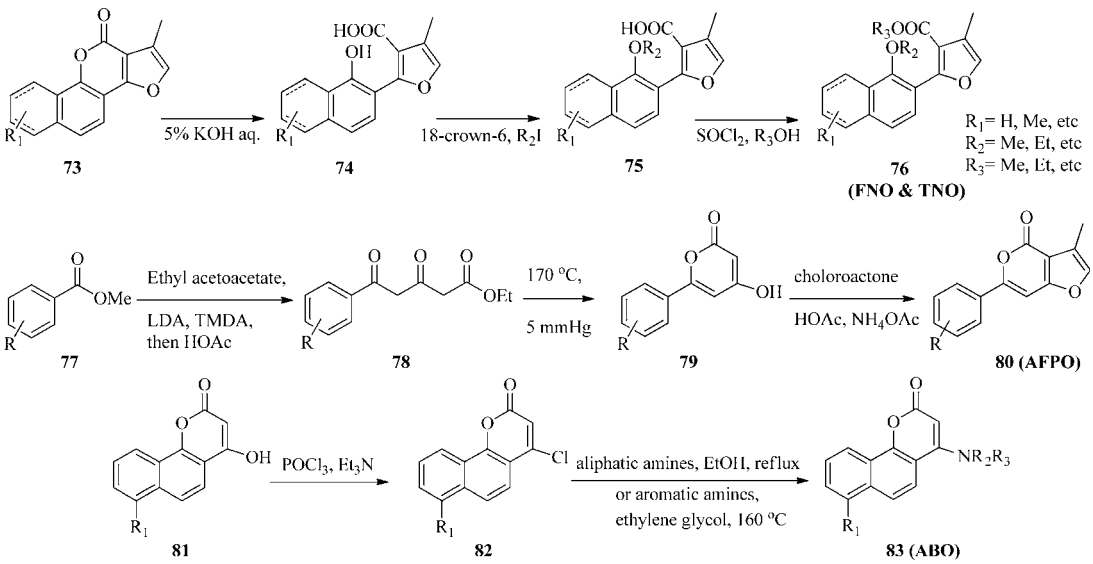


Fig. 3 4-Ethyl neo-tanshinlactone (**72**) and newly designed scaffolds I–IV.

highly productive tool for lead development and analogue design. Based on this strategy, four scaffolds (Fig. 3), including 2-(furan-2-yl)naphthalen-1-ol (FNO),[73] tetrahydronaphthalene-1-ol (TNO),[74] 6-phenyl-4*H*-furo[3,2-*c*]pyran-4-one (AFPO),[75] and 4-amino-2*H*-benzo[*h*]chromen-2-one (ABO),[76] were designed and developed as novel analogs with promising anticancer activity.

Syntheses of these scaffolds are shown in Scheme 15. Neo-tanshinlactone-analogs (**73**) were hydrolyzed to cleave the lactone C-ring and derive the related hydroxy-carboxylic acids **74**.[77] An alkyl iodide and 18-crown-6 ether were added directly to the crude mixture, without work up, to provide selective alkylation of the hydroxy group of **74**.[78] The resulting ethers **75** were converted to esters **76** with thionyl chloride and the appropriate alcohol at room temperature to afford FNO and TNO analogs.[79] For the AFPO scaffold, various substituted benzoate esters (**77**) were reacted with a dianion intermediate to give substituted phenyl triketoesters **78** as tautomeric mixtures. 6-Phenyl-pyrones **79** were prepared by heating **78** under reduced pressure.[80] AFPO analogs **80** were obtained *via* a tandem alkylation/intramolecular aldol reaction of **79**.[11,70] In addition, ABO analogs (**83**) were synthesized from 4-hydroxybenzochromenones (**81**).[11] Treatment of **81** with $POCl_3$ afforded the related chlorides (**82**).[81] The target analogs (**83**) were prepared by substitution reaction of the chloro groups in **82** with various amines.[81,82]

In summary, extensive studies have been carried out toward the total synthesis of tanshinones and their analogs. Early work provided pioneering concepts in the synthetic strategies, which are still being applied today. However, these studies were generally performed on a small scale and resulted in low yields. Danheiser *et al.* achieved the first total synthesis of optically pure (−)-cryptotanshinone (**4**) in seven steps and 18% overall yield.[54–56] Many other analogs can also be synthesized by using similar methodology. Lee and Snyder's synthetic pathway to tanshinone IIA (**2**) gave the highest overall yield (31%) compared with other methods; however, the regioselectivity was moderately low (10 : 3).[60–63] Lee and coworkers have reported



Scheme 15 Synthesis of scaffolds I–IV.

the only synthetic route to neo-tanshinlactone (**6**), which is efficient for gram-scale synthesis and further structural optimization.[11]

4 Antitumor activity and mechanisms of action

Many natural tanshinones and their synthetic analogs have been evaluated for cytotoxic activity against different human tumor cell lines.[3] Various studies and several brief reviews have also discussed or proposed possible mechanisms of action for different tanshinones.[19,83,84] This section will systematically describe the antitumor activity and mechanisms of action of tanshinones and their analogs.

4.1 Tanshinone I (1)

Kim *et al.* reported that **1** inhibited cell growth dose-dependently at concentrations from 0.5–25 μM and induced apoptosis in activated T-HSC/C1-6 hepatic stellate cells.[85] Typical morphological changes and DNA ladders were observed by treatment of **1** at 5 and 10 μM. Flow cytometric analysis showed increased apoptotic sub-G1 cells dose-dependently. In these studies, **1** increased caspase 3 activation *via* cytochrome c release and loss of mitochondrial membrane potential. Later in 2008, Nizamutdinova *et al.* studied the effect of **1** at 1–50 μM on induction of apoptosis in MCF-7 and MDA-MB-231human breast cancer cell lines.[86] At 50 μM, **1** increased the apoptotic cell population by 14, 26 and 27% at 24, 48 and 72 h, respectively in MCF-7 cells, as shown through FACS analysis. TUNEL assays displayed similar results. The authors concluded that **1** induced apoptosis in the above two cell lines by the activation of caspase 3, down-regulation of the anti-apoptotic protein Bcl-2, and up-regulation of the pro-apoptotic protein Bax. Similarly, Su and co-workers explored the induction of apoptosis by **1** at 1–10 μg mL^{-1} in human colon cancer Colo 205 cells.[87] They observed increased activation of caspase 3, bax, p53, and p21. Their results also showed that treatment with **1** at 0, 1, 2.5, 5 and 10 μg mL^{-1} for 72 h increased the percentage of cells in sub-G1 phase from 3.83% to 7.22%, 8.68%, 14.4% and 32.98%, respectively. They speculated that **1** induced apoptosis in Colo 205 cells through both mitochondrial-mediated intrinsic cell-death pathways and p21-mediated G0/G1 cell cycle arrest.[87]

Nizamutdinova and co-workers also reported that, in a concentration range from 1–50 μM, **1** regulated adhesion molecules in human MDA-MB-231 breast cancer cells.[88] Their study showed that, at 5–50 μM, **1** completely inhibited the expression of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) in tumor necrosis factor-α (TNF-α) stimulated human umbilical vein endothelial cells (HUVECs). Compound **1** also reduced adhesion of monocyte U937 and MDA-MB-231 cells to HUVECs. Moreover, **1** displayed inhibitory activity against TNF-α-induced production of vascular endothelial growth factor (VEGF) and VEGF-mediated tube formation in HUVECs. At 10 and 50 μM, **1** inhibited more than 80% invasion of cancer cells. In addition, in nude mice treated with **1**, both tumor mass volume and metastasis of MDA-MB-231 breast cancer cells to lung tissue decreased.[88]

Lee *et al.* investigated the mechanism of action of **1** in human non-small-cell lung cancer.[89] Their results indicated that **1** can significantly reduce metastasis and tumorigenesis by inhibiting NF-κB and AP-1, and then suppressing the expression of the angiogenic factor IL-8. Compared with dexamethasone (positive control at 5 μg mL^{-1}), compounds **2**, **4**, and **1** at 10 μg mL^{-1} significantly decreased migration *in vitro* in CL1-5, a highly invasive human lung adenocarcinoma cell line. Zymographic analysis revealed that **1**, **2**, and **4** greatly reduced gelatinase activity in CL1-5 cells (treated with conditioned medium). In addition, treatment with **1** (0.3 mg kg^{-1} d^{-1} for 12 days) decreased tumor size by 85% compared with the control group in macrophage-conditioned medium-stimulated CL1-5-bearing severe combined immunodeficient mice. Moreover, no metastatic nodules were observed by fluorescence microscopy in the lungs of mice treated with **1**, which suggested that **1** significantly reduced tumor metastases. However, **2** showed no significant effect on the number of tumor metastases in the lung. The authors suggested that the mechanisms of action of **1** may be associated with the Ras-mitogen-activated protein kinase and Rac1 signaling pathways, such as inhibiting PDGF-β, IL-8 and Ras, based on microarray and pathway analysis.[89]

Park *et al.* evaluated the inhibitory effects of **1** and **2** against the jun-fos–DNA complex formation, which is capable of binding 5′-TGAGTCA-3′ DNA elements (AP-1 binding site) and is critical to gene expression.[90] Their results suggested that both **1** and **2** suppressed AP-1 function and cell proliferation; however, both compounds were much less active than doxorubicin in MTT assays. Moreover, **1** and **2** reduced the interaction of jun-fos and DNA with IC_{50} values of 0.15 and 0.22 mM, respectively in TPA-induced NIH 3T3 cells.

Most recently, Liu and coworkers reported the effect of **1** at dosages from 10–50 μg mL^{-1} in three different monocytic leukemia cells (U937, THP-1 and SHI 1).[91] Compound **1** reduced the expression of hTERT mRNA, as well as activity of telomerase, and down-regulated survivin levels. Caspase-3 activity also increased dramatically in a time-dependent manner *via* a colorimetric assay. The results indicated that **1**-induced apoptosis in monocytic leukemia U937 THP-1 and SHI 1 cells correlated with activation of caspase-3 and reduction of hTERT mRNA expression and telomerase activity, as well as down-regulation of survivin expression.[91]

4.2 Tanshinone IIA (2)

Tanshinone IIA (**2**), one of the major constituents in *Salvia miltiorrhiza*, has been studied biologically at length. Major studies will be described briefly below. Many cells, tissues, and animal models have been used in the studies, and many different mechanisms have been proposed. It is very likely that **2** may interact with more than one target. In addition, several studies also explored combination chemotherapy of **2** with other anticancer agents including, cisplatin,[92] and 5-FU,[93] which will not be covered in this review.

4.2.1 Inducing apoptosis. Treatment with **2** can induce apoptosis in several different cancer cell lines, including HeLa, leukemia, liver, breast, and gliomas.[94–101] However, various mechanisms of action have been reported regarding how apoptosis is triggered. Several studies reported that **2** can activate caspase-3, down-regulate the anti-apoptotic protein bcl-2, and

up-regulate the pro-apoptotic protein bax in different human cancer cell lines, including THP-1 leukemia, MDA-MB-231 breast cancer, HL60 promyelocytic leukemia, K562 erythroleukemia, and hepatocellular cancer (HCC).[94,96,99,102,103] Wang *et al.* examined the effect of **2** at 25–100 ng mL^{-1} in human glioma cells, and found that apoptotic cells increased significantly.[97] Cells in the G0/G1 phase also increased and those in the S phase decreased. The mRNA expressions of ADP-ribosyltransferase 1 (ADPRTL1), CYP1A1 and GFAP increased 1–2-fold, while that of nestin decreased dramatically. These results are consistent with the findings of Yoon and Wang in 1999 and 2005, respectively.[96,104] Oligonucleotide microarray analysis by Wang *et al.* showed that 41 genes (1.22%) were up-regulated and 24 genes (0.71%) were down-regulated by treatment of MCF-7 cells with **2** at 0.25 μg mL^{-1}, from which they proposed that ADPRTL1 might be the main target.[104] The relationships of the above-mentioned factors in cancer and apoptosis are as follows. The ADPRT gene encodes a zinc-finger DNA-binding protein, poly(ADP-ribose) polymerase-1 (PARP-1), whose alteration has been associated with response to oxidative damage and the cellular response.[105,106] An increase of ADPRT can induce significant apoptosis of cancer cells.[107,108] CYP1A1 encodes cytochrome P450, subfamily 1.[109] Its activation by anticancer agents results in cytotoxicity through DNA damage-induced apoptosis.[110] GFAP is a glial-specific intracyptoplasmic filamentous protein, whose expression levels have been correlated positively with differentiation grade and reversibly with malignant phenotype of glioma cells.[111,112] Nestin, a type VI intermediate filament protein, is abundantly expressed in nerve cells.[113,114] It is also found in gliomas/glioblastomas, and has been used as a marker for undifferentiated astrocytic precursors. Glioblastomas express higher nestin levels compared with malignant gliomas.[115,116] Furthermore, the *in vivo* (s.c. injection at 30 mg kg^{-1} three times a week for 10 weeks) studies in human breast IDC-xenografted nude mice showed that tumor size in treated mice was 1.82 times lower than that in control groups, indicating that there was significant inhibition of tumor growth in mice treated with **2**.[104]

Using time-lapse imaging measurement and cell phase analysis, Zhou *et al.*[98] found that **2** (5 μg mL^{-1}) disrupted the mitotic spindle of HeLa cells in mitosis, but its effects were different from those of paclitaxel and vincristine used as controls. Cells were arrested first in the G2/M phase, before apoptosis was induced through a mitochondrial-dependent pathway. The results also indicated that **2** did not destroy the microtubule structure in interface cells. In more recent studies, Pan *et al.* examined the microtubule organization in HeLa cells by immunofluorescence staining of α- and β-tubulin and suggested that **2** (0.5 μg mL^{-1}) interfered with microtubule assembly and then induced apoptosis.[117] On the basis of flow cytometry analysis, Pan and Dong separately reported that apoptosis may occur in the G2/M phase in HeLa cells and MKN-45 cells, respectively.[117,118] Pan *et al.* also provided a binding model of the **2**–microtubule complex, which speculated that **2** may locate in the pocket of the microtubule β-subunit.

By using circular dichroism spectra with distamysin A as a positive control, Zhang's group determined that **2** bound to the DNA minor groove and subsequently caused DNA damage, but did not act as an intercalator or produce free radicals.[119,120] The DNA binding consequently led to RNAPII phosphorylation and degradation, resulting in p53 activation and apoptosis. The results were confirmed *in vitro* (in the hydroperitoneum hepatoma cells-H22 cell line with a 0.2–4 μM dose of **2**) and *in vivo* (in mice bearing ascitic-type H22, i.p. injection every other day with various concentrations of **2** for 10 days). A molecular modeling study suggested that the importance of the furan oxygen is related to hydrogen bond formation within the DNA minor groove. Other tanshinones, including **4**, were also examined in the study. However, **4** did not induce any significant variation of RNAPII level at concentrations from 2–24 μM.

4.2.2 Inhibiting invasion and metastasis. In 2006, Liu *et al.* reported that **2** may reduce cell adhesion to and invasion through the extracellular matrix (ECM) in acute promyelocytic leukemia (APL) NB4 cells *in vitro*.[99] They performed *in vitro* adhesion and invasion assays with **2** at 40 μg mL^{-1} by use of laminin-, fibronectin-, collagen- and vitronectin-coated plates and Matrigel matrix-coated Boyden chambers. The above inhibitions occurred in a dose-dependent manner. Later in 2009, Shan *et al.* found that **2** significantly inhibited tumor invasion and metastasis in human colon carcinoma (CRC) cell lines HT29 and SW480[121] both *in vitro* (0.5–2.5 mg mL^{-1}) and *in vivo* (0–80 mg kg^{-1} d^{-1} for four weeks). Levels of urokinase plasminogen activator (uPA) and matrix metalloproteinases (MMP)-2 and MMP-9 were decreased, and levels of tissue inhibitor of matrix metalloproteinase protein (TIMP)-1 and TIMP-2 were increased. In addition, Shan *et al.* also observed a significant decrease of p65 protein, which is a major phosphorylated subunit of nuclear factor-kappaB (NF-κB). Because MMP and TIMP proteins are regulated *via* the activation of the NF-κB transcription factor,[122] the results suggested that **2** may suppress the NF-κB signal transduction pathway, consistent with a discovery by Jang *et al.* in RAW 264.7 cells.[123] Similarly, Xu and coworkers reported that **2** inhibited the invasion and metastasis of HCC both *in vitro* (0–2 μg mL^{-1}) and *in vivo* (0–13.5 g kg^{-1} d^{-1} for 5 consecutive weeks in male athymic BALB/c nu/nu mice).[124] Compound **2** down-regulated the protein expression of MMP-2 and MMP-9, which was consistent with Shan's results. Inhibition of invasion and metastasis of HCC cells by **2** was at least partly due to this down-regulation. Ye and Yang *et al.* also reported that **2** reduced expression of MMP-2 and MMP-7 in the human MKN-45 gastric carcinoma cell line.[125,126]

4.2.3 Inhibiting angiogenesis. Yang and coworkers proved that **2** can elicit human endothelial cell death independent of oxidative stress, which is dependent on quinone oxidoreductase (NQO1) activity.[127] Treatment of human endothelial EAhy926 cells with **2** at 25 μM caused at least 75% cell apoptosis. Compound **2** increased intracellular calcium and consequently induced the release of cytochrome c, which led to loss of the mitochondrial membrane potential and activated caspases. They also proposed a model for the mechanism of action of **2**-induced human endothelial EAhy926 cell apoptosis. In the presence of NQO1, **2** can be reduced from the quinone form to the hydroquinone form, which is unstable and auto-oxidizes to its original parent form. Such a futile cycle may lead to rapid and severe loss of cellular NADP(H) and induce the sequestration of calcium

from the endoplasmic reticulum, resulting in a rise in cytosolic calcium levels. The change of the calcium pools can cause cytochrome c release and mitochondrial membrane depolarization. Consequently, the process would increase the activation of caspases, and then induce apoptosis. This year, Chiu *et al.* also reported that, at 5 μg mL^{-1}, **2** decreased mitochondrial membrane potential and induced apoptosis with a higher ratio of Bax/Bcl-2 in human A549 lung cancer cells.[128] They observed an increase of calcium and reactive oxygen species (ROS) in A549 cells by flow cytometry.

Fu *et al.* studied the effects of **2** on apoptosis and the expression of vascular endothelial growth factor (VEGF) in the SMMC-7721 HCC cell line.[129] Their results suggested that **2** not only induced apoptosis, but also reduced the expression of VEGF. *In vivo* studies in s.c. colorectal cancer xenografts by Zhou *et al.* showed that treatment with **2** at a dose of 1 and 2 mg kg^{-1} d^{-1} greatly reduced microvessel density.[130] VEGF levels also decreased significantly by 15.8%, 34.2%, and 74.8% in the low-, mid-, and high-dose (0.5, 1, 2 mg kg^{-1} d^{-1}, respectively) **2**-treated groups compared with the control group.

In 2009, Xu and Xi reported that **2** caused anti-angiogenic, anti-proliferation, and anti-migration effects. They performed various assays including MTT, 2-D/3-D migration, tube formation, PCR, and chick embryo chorioallantoic membrane (CAM).[131] Their results indicated that **2** inhibited the proliferation of the human MDA-MB-435 breast cancer cell line with an IC_{50} of 21 nM, and prevented breast cancer cell migration at 5 and 6 nM in a wound-healing assay and a transwell migration assay, respectively. Moreover, **2** inhibited the tube formation of newborn cattle aortic endothelial cells (NCAECs) after 2 h co-incubation with MDA-MB-435. In addition, **2** inhibited mRNA expression of vascular endothelial growth factor (VEGF) and two transcription factors (HIF-1α/c-Myc) in a dose-dependent manner and also inhibited angiogenesis *in vivo* in the CAM assay.

Similarly, Feng and Zong reported the down-regulation of HIF-1α expression in SGC-7901 gastric cancer cells treated with **2**.[132,133] Feng and Du reported that the expression of c-Myc protein was inhibited by **2** in SGC-7901 gastric cancer and MDA-MB-231 breast carcinoma cells, respectively.[132,134] These results suggested that **2** may exert its anti-proliferative, anti-migratory, and anti-angiogenic effects through multi-target interactions.

4.2.4 Down-regulating epidermal growth factor receptors. In 2008, Su *et al.* reported that **2** down-regulated the expression of ErbB-2 and up-regulated TNF-α in colon cancers both *in vitro* (1–5 μg mL^{-1}) and *in vivo* (20 mg kg^{-1} d^{-1} for 30 days, male SCID mice xenografted with Colo 205 cells).[135] However, a recent study by Lu and coworker showed that **2** decreased the expression of p53 and bcl-2, but did not affect the expression of ErbB-2 in breast cancers (30 mg kg^{-1}, four times per week for four weeks, female nude mice xenografted with human MCF-7 or MDA-MB-231 breast cancer cells).[136] Compound **2** displayed around 10-fold greater inhibitory activity than tamoxifen *in vitro*, a positive control. Later, Zhai and coworkers investigated the effect of **2** on the expression of epidermal growth factor receptor (EGFR) in the human SMMC-7721 hepatocellular carcinoma cell line.[137] Compound **2** significantly reduced the expression rate of EGFR, which may be related to the induced apoptosis in SMMC-7721.

4.2.5 Inhibiting Stat3. The seven proteins in the signal transducers and activators of transcription (Stat) family are Stat1, Stat2, Stat3, Stat4, Stat5A, Stat5B, and Stat6. Several studies have demonstrated that constitutively activated Stat3 is involved in proliferation, angiogenesis, and immune evasion in various human solid tumors. Studies by Tang *et al.* suggested that, at concentrations of 1–8 μg mL^{-1}, **2** inhibited the constitutive Stat3 activation in rat C6 glioma cells.[138] At 8 μg mL^{-1}, **2** almost completely blocked the Stat3 binding activity. In contrast, Shin *et al.* reported that, compared with **4** at 7 μM (see section 4.4), **2** did not inhibit Stat3 activity in DU145 prostate cancer cells.[139]

4.3 Neo-tanshinlactone (6) and its analogs

Lee's group has proved that **6** showed significant and selective inhibition of two ER+ human breast cancer cell lines, and was 10-fold more potent and 20-fold more selective than tamoxifen citrate, a widely used selective estrogen receptor modulator.[11] Further structural modification established the SAR and led to 4-ethyl neo-tanshinlactone (**72**), which was about twice as active as **6** against MCF-7 and SK-BR-3 cell lines.[71,72] In addition, **72** showed potent *in vivo* activity against a ZR-75-1 xenograft model, but not PC-3 and MDA-MB-231 xenografts. Furthermore, **72** was tested independently against cell lines derived from normal breast tissue (MCF10A and 184A1) *versus* SK-BR-3 as a positive breast cancer cell line control, which showed that **72** was selective for a subset of breast cancer-derived cell lines and was significantly less active against normal breast-derived tissue. Kinase assays indicated that **72** significantly suppressed several important protein kinases, including CK2R1, ABL, and AKT1. As mentioned before, Lee *et al.* also designed and developed four series of neo-tanshinlactone analogs (Fig. 3): FNO,[73] TNO,[74] AFPO,[75] ABO.[76] Some analogs of FNO (**84**), TNO (**85**), and AFPO (**86–87**) exhibited high anti-breast cancer selectivity, for example, being approximately 100–250-fold more potent against SK-BR-3 than other tested human tumor cell lines (Fig. 4; Table 1).[71–73] In contrast, lead compounds (**88** and **89**) with the ABO scaffold generally displayed potent antitumor activity against a broad range of cancer cell lines, with ED_{50} values of 0.01–0.76 μM.[76] The 4′-methoxyphenyl derivative (**88**) showed the most potent *in vitro* antitumor activity, with ED_{50} values of 0.01–0.17 μM. More importantly, these lead compounds **88–89** were seven-fold more potent than paclitaxel against KB-VIN, a vincristine-resistant MDR KB subline. Further mechanism-of-action studies are ongoing for the lead compounds. Overall, these novel analogs displayed significant antitumor activity, high selectivity compared with normal cell lines, and different tumor-tissue-type selectivity. Thus, they show significant promise for development as clinical trials candidates.

4.4 Other tanshinones and their analogs

Shin *et al.* have studied the interaction of tanshinones **2** and **4** with Stat3 in DU145 prostate cancer cells.[139] They proved that, at a concentration range of 0.2 to 50 μM for 24 h, **4** inhibited Stat3 in a dose-dependent manner, with an IC_{50} value of 4.6 μM. However, **2** did not inhibit Stat3 at the same concentration range in Stat3-dependent dual-luciferase assays. Their results showed

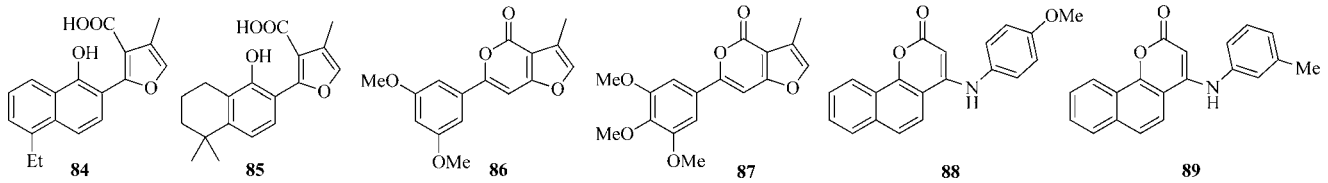


Fig. 4 Structures of lead compounds **84–89**.

Table 1 Cytotoxicity of **1–6**, **72**, **84–89** against the Human Tumor Cell Line Panel (in μM)[3,a]

Compd	KB	KB-VIN	A549	DU145	SKBR-3	ZR-75-1	MDA-MB-231
1	10.14	N/A	15.58	N/A	N/A	N/A	N/A
2	13.95	N/A	3.06	N/A	N/A	N/A	2.55
3	3.55	N/A	16.77	N/A	N/A	N/A	2.26
4	6.76	N/A	2.36	N/A	N/A	N/A	0.54
5	>15	N/A	>15	N/A	N/A	N/A	N/A
6	>30	>30	>30	>30	0.95	0.95	>30
72	>30	>30	>30	>30	0.36	0.36	>30
84	>30	23.6	>30	29.4	3.4	1.0	>30
85	4.7	4.7	3.3	3.0	0.7	1.7	24.7
86	>30	>30	>30	>30	0.28	>30	>30
87	>30	>30	>30	>30	0.44	29.1	>30
88	0.11	0.13	0.17	0.11	0.13	0.01	0.02
89	0.14	0.14	0.15	0.14	0.15	0.08	0.76
Paclitaxel[b]	5.92	>1000	6.52	4.92	5.47	3.07	6.91

[a] Mean ED_{50} (μM), from 2 or more independent tests. [b] Mean ED_{50} (nM).

that **4** inhibited Stat3 Tyr705 phosphorylation through a JAK2-independent mechanism in DU145 prostate cancer cells, resulting in decreased expression of Stat3 downstream target proteins, such as cyclin D1, survivin, and Bcl-xL. Compound **4** may bind to the SH2 domain of Stat3 and inhibit the formation of Stat3 dimers. Shin *et al.* also performed computational modeling of **4** binding to Stat3 using the AutoDock 1.0 program. According to the refined model, **4** may bind at the specific site where the Tyr705 residue interacts within the SH2 domain, and it can also form a number of hydrogen bonds with nearby amino acid residues, including Arg609 and Ile634. In addition, studies by Don *et al.* reported that, at 1–30 μM, **4** displayed anti-inflammatory activity by inhibiting PI3K activation and consequently reducing the phosphorylation of Akt and ERK1/2.[140]

Lee and co-workers studied the effect of four tanshinones (**1**, **2**, **4**, and **36**, at 0.39–100 μM) on reactive oxygen species (ROS) and p-38 mitogen-activated protein kinases (MARK) in HepG2 cells.[141] With an ED_{50} value of 2.52 μM, **36** was more potent than the three controls (cisplatin, doxorubicin, and retinoic acid) against this cell line. The results also showed that all four tanshinones induced ROS generation, while only **36** activated p38 MAPK. Thus, **36**-induced apoptosis in HepG2 cells could be related to ROS-mediated p38 MAPK activation. More recently, Lee also reported the anti-hepatocellular carcinoma potential of the same four tanshinones.[142] The study suggested that **4** suppressed P-glycoprotein (Pgp)-mediated doxorubicin efflux in a Pgp-overexpressed HepG2 subclone (R-HepG2 cells). Compound **2** displayed the best synergistic effect with doxorubicin among the four tanshinones.

Wong found that 15,16-dihydrotanshinone I (**36**) inhibited endothelial cell proliferation dose-dependently (50 μg mL^{-1} with 84.4% of endothelial cell growth inhibition).[143] Compound **36** also inhibited the proliferation of several cancer cell lines *in vitro*, including SW480 (colon cancer) and HTB 72 (melanoma).

In summary, current studies have suggested that tanshinone IIA (**2**) can interact with various biological targets and inhibit tumor growth through different mechanisms of action.[94–99,102–104,117–139] This work could be extended to investigate the cross-talk between different pathways and develop new analogs with optimal pharmaceutical properties. Although tanshinone I (**1**)[85–91] and cryptotanshinone (**4**)[139–142] also exhibited potent antitumor activity, structural modifications on these structures have not provided any promising results in the literature. Other current studies on **1–4** have focused on the therapeutic effects of tanshinones in cardiovascular disease.[3] In addition, neo-tanshinlactone (**6**) and its analogs exhibited potent and selective activity against breast cancer both *in vitro* and *in vivo*.[71–76] This activity might be related to unique mechanism(s) of action, and merit further exploration. Moreover, some analogs showed highly potent antitumor activity, comparable to that of paclitaxel.[76] The results indicate that lead compounds derived from **6** are promising candidates for future development as chemotherapy drugs.

5 Conclusions

Numerous tanshinones and their analogs have been isolated and characterized from *Salvia miltiorrhiza*, which has been used in Chinese TCM for multiple therapeutic indications. Ongoing research on biosynthetic pathways continues to uncover specific reaction sequences and related enzymes. Various strategies from total syntheses have provided powerful tools to explore potential therapeutic applications and mechanisms of action, as well as establish SAR based on modified synthetic tanshinones, identify

novel bioactive lead compounds, and develop new structurally related chemical entities. Many tanshinones and their analogs, including neo-tanshinlactone analogs, have been identified as potent and selective antitumor agents, which merit further investigation as clinical trial candidates.

6 Acknowledgements

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