Bioorganic & Medicinal Chemistry Letters 19 (2009) 3502–3506

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

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

Semisynthesis and antiproliferative evaluation of a series of 3′-aminoflavones

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ARTICLE INFO

Article history:
Received 30 March 2009
Revised 30 April 2009
Accepted 3 May 2009
Available online 7 May 2009

Keywords:
Tangeretin
Flavones
Aminoflavones
Antiproliferative activity
Tubulin

ABSTRACT

A series of 3′-aminoflavones 5,6,7,8-tetra- or 5,7-dioxygenated on the A-ring was synthesized from tangeretin or naringin, two natural *Citrus* flavonoids. These flavones were evaluated for antiproliferative activity, activation of apoptosis, and inhibition of tubulin assembly. The most antiproliferative flavones exhibit a common 5-hydroxy-6,7,8-trimethoxy substitution pattern on the A-ring.

In 1998, Beutler et al. reported results of a comparative in vitro antitumor screening and subsequent tubulin polymerization studies carried out with a series of natural and synthetic flavones.[1] Most of the studied flavones contained only hydrogen, hydroxyl and methoxyl substituents. Some structure–activity relationships for cytotoxicity and associated inhibitory effects on tubulin polymerization were apparent from these results. Maximum potencies for cytotoxicity and tubulin interaction were found only with compounds bearing a hydroxyl group at C-5 on the A-ring, 3′-hydroxy-4′-methoxy groups on the B-ring and a methoxyl at C-3 on the C-ring. The substitution of the A-ring was apparently not critical for activity (except hydroxylation at C-5), but it could be observed that **1**,[2–4] the most potent of the studied flavones, was trimethoxylated at C-6, C-7 and C-8. We noticed that the required substitution pattern on the B-ring was the same as in combretastatin A4, **2**, a powerful inhibitor of tubulin assembly now under clinical investigation as a phosphate prodrug. As SARs within the combretastatins series reveal a slight increase in potency for the amino analog **3** compared to combretastatin A4,[5] we decided to prepare a series of 3′-amino-4′-methoxyflavones and to evaluate them for activities related to cancer (antiproliferative and proapoptotic activities and, inhibition of tubulin assembly). Owing to their substitution patterns, two *Citrus* flavonoids, tangeretin, **4**, a polymethoxyflavone (PMF) that occurs in high concentrations in the peel of various *Citrus* species such as sweet orange [*Citrus sinensis* (L.) Pers.] and mandarin (*Citrus reticulata* Blanco), and naringin, **5**, which are easily available from grapefruit (*Citrus* x *paradisi* Macfad), were chosen as starting materials for the semisynthesis.

Synthesis of 3′-aminoflavones with a 5,6,7,8-tetrasubstituted A-ring. Synthesis of these analogs was achieved from tangeretin (**4**) via the intermediate 3′-nitroflavones. First attempts of nitration were undertaken on tangeretin itself. As tangeretin has been described to yield by oxidation with excess nitric acid a red-coloured 5,8-flavoquinone structure,[6] we carried out nitration of **4** under various conditions: (a) with one equivalent of nitric acid in acetic acid at 60 °C (to dissolve **4**) or in trifluoroacetic acid at 0 °C; (b) with other reagents (nitric acid in acetic anhydride, NO_2BF_4 in acetonitrile, $(NH_4)_2Ce(NO_3)_6$ in trifluoroacetic acid, $Bi(NO_3)_3$ on Montmorillonite K10) already used with *para*-methoxybenzoic and cinnamic acids, or with *para*-methoxy aromatic ketones.[7–11] None of these reactions allowed isolation of the expected 3′-nitro derivative, but led either to red-coloured unstable products (at least two according to TLC), or to recovery of **4**. These results demonstrated that oxidation of the tetramethoxylated A-ring is favored over nitration of the B-ring at C-3′. So we decided to enhance reactivity of the B-ring by replacing the methoxyl group at C-4′ by the more strongly activating phenol group. Since the chemical regioselective demethylation of **4** at C-4′ seemed very unlikely to occur according to the major studies of Horie et al.,[12] semisynthesis of 4′-*O*-demethyltangeretin **6** from **4** was achieved in 70% yield by biotransformation using an *Aspergillus niger* strain as previously described in our laboratory.[13] When **6** was subjected to conditions of nitration used with tangeretin (1 equiv nitric acid, TFA, 0 °C), the expected 3′-nitro compound **7** was isolated in 45% yield, which confirms our hypothesis on the inversion of A and B-ring reactivities

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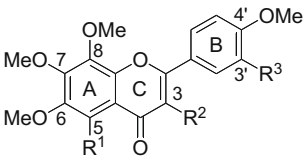
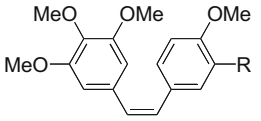
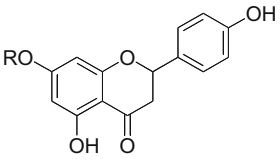
0960-894X/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2009.05.008



R^1	R^2	R^3	
OH	OMe	OH	**1**
OMe	H	H	**4**
OH	H	H	**23**
OMe	H	OH	**24**
OH	H	OH	**25**



R = OH **2**
R = NH_2 **3**



R = neohesperidosyl **5**

between **4** and **6**. In a following step, methylation of **7** (iodomethane, K_2CO_3, dimethylformamide) afforded 3′-nitrotangeretin **8** in a quantitative yield. Though this synthesis carried out on a small scale from **4** (0.15 mmol starting amount) was a very neat and effective method, we opted on a larger scale for the more classical chemical pathway that begins by the basic degradation of tangeretin to acetophenone **9**.[14] Preparation of **8** from **9** was then performed in two steps through the intermediate nitrochalcone **10** according to Scheme 1.

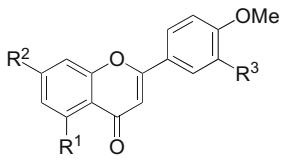
As pointed out on Scheme 2, 3′-aminotangeretin, **11**, was then obtained from **8** by catalytic hydrogenation over palladium, while preparation of **19** requires a regioselective 5-O-demethylation step with aluminium bromide into nitroflavone **18** prior to the reduction of the nitro group.[15] Access to C-3 oxygenated derivatives from **8** was anticipated, but the two attempted methods proved unsuccessful: Classical C-3 hydroxylation process with dimethyldioxirane provided only a complex mixture,[16,17] while hypervalent iodine oxidation with iodobenzene diacetate according to Moriarty et al. led to total recovery of **8**.[18,19] An alternative approach to C-3 oxygenated flavones from nitrochalcone **10** by means of the Algar–Flynn–Oyamada method (AFO method) was also unfruitful, since 2-hydroxy-3,4,5,6-tetramethoxybenzoic acid and 4-methoxy-3-nitrobenzoic acid were the major isolated compounds of the reaction.[20,21] As we could not access to C-3 oxygenated flavones, we then turned to synthesis of 3-chloro and bromo analogs, since such substituents effect on antiproliferative activity had previously been unexplored. 3-Chlorination and bromination of **8** that led to **12** and **15**, respectively, were carried out by *N*-chlorosuccinimide in a mixture dichloromethane-pyridine as previously reported in our laboratory,[22] and by *N*-bromosuccinimide according to the two-steps method of Bird et al. via a 2-methoxy-3-bromoflavanone intermediate.[23] Regioselective 5-O-demethylation of the nitroflavones **12** and **15** with aluminium bromide provided 5-hydroxynitroflavones **13** and **16**, then **14** and **17**, respectively, by a final reduction step of the nitro group by stannous chloride, dihydrate.

Synthesis of 3′-aminoflavones with a 5,7-disubstituted A-ring. Nitroflavone **20** (=3′-nitroacacetin) has been previously semisynthesized in four steps in our laboratory from naringin **5**.[24] Starting from **20**, 5,7-dioxygenated aminoflavones **21** and **22** were prepared by catalytic hydrogenation over palladium for **21** (82%), and a subsequent regioselective methylation (iodomethane, $KHCO_3$, dimethylformamide) for **22** (48% from **20**).

Table 1
Antiproliferative, proapoptotic and antitubulin activities of synthesized flavones

Compd	Cytotoxicity on KB cells[a] IC_{50} (μM)	Activation of apoptosis in HL60[b]	ITA activity[d]
3′-Aminoflavones			
14	86% IC_{50} = 0.14	1 μM (×4.5)	15% inhibition
19	83% IC_{50} = 0.16	1 μM (×6.0)	27% inhibition
17	55%	1 μM (×3.6)	
22	30%	No activation at 100 μM	
21	7%	100 μM (×4.0)	
11	6%	No activation at 100 μM	
3′-Nitroflavones			
16	23%	n.d.	
8	3%	n.d.[c]	
18	2%	n.d.	
3′-Unsubstituted flavones			
23	28%	100 μM (×3.8)	
4	12%	No activation at 100 μM	
3′-Hydroxyflavones			
1	92% IC_{50} = 0.02	0.1 μM (×5.7)	IC_{50} = 10 μM 3[e]
25	IC_{50} = 0.08	1 μM (×3.8) 10 μM (×5.2)	IC_{50} = 86 μM 27[e]

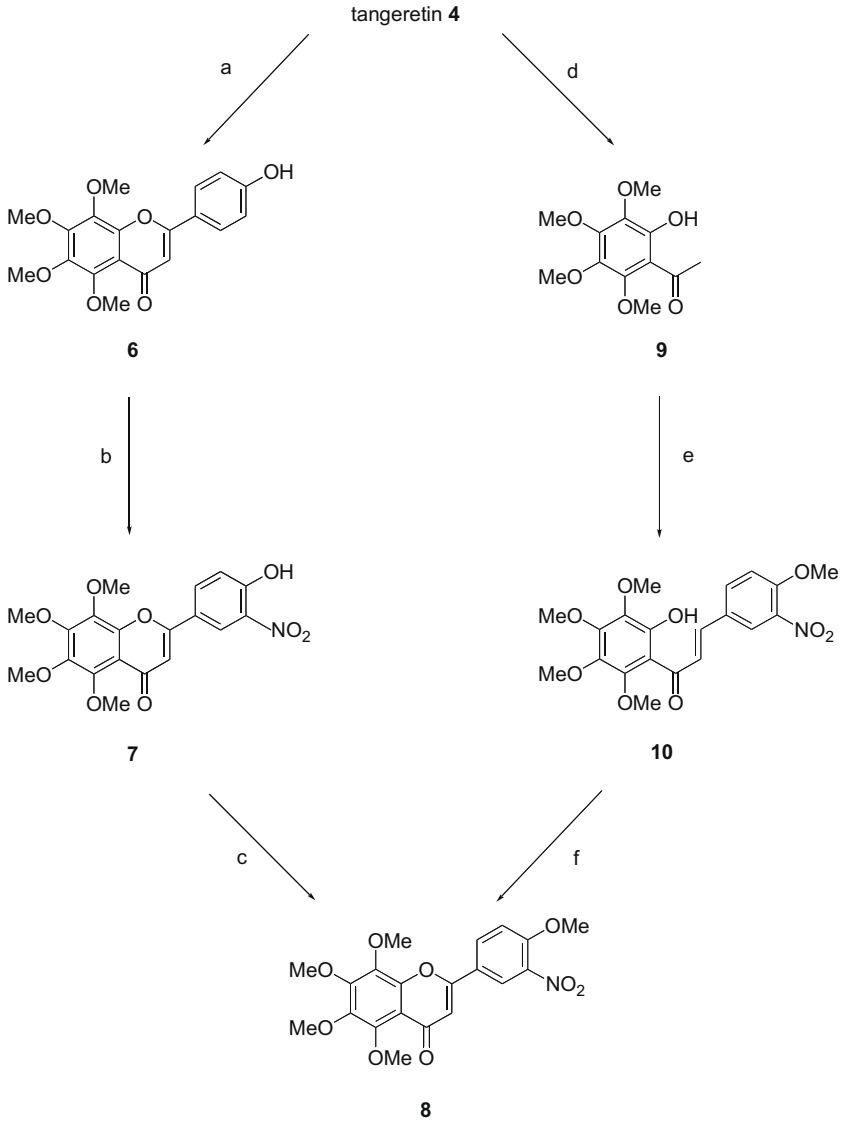
[a] As measured by the MTS assay after 72 h incubation of cells with drug: results are expressed as the percentage of inhibition of cell growth with 10^{-6} M flavone concentration, or as IC_{50}, calculated only for the most active compounds.
[b] Activation of caspases 3/7 activity: optimal concentration of compound and fold activation.
[c] Not determined.
[d] Results are expressed as the percentage of ITA at 6.6×10^{-5} M, or as IC_{50}, calculated only for **1** and **25**.
[e] IC_{50} **1** or **25**/IC_{50} deoxypodophyllotoxin.



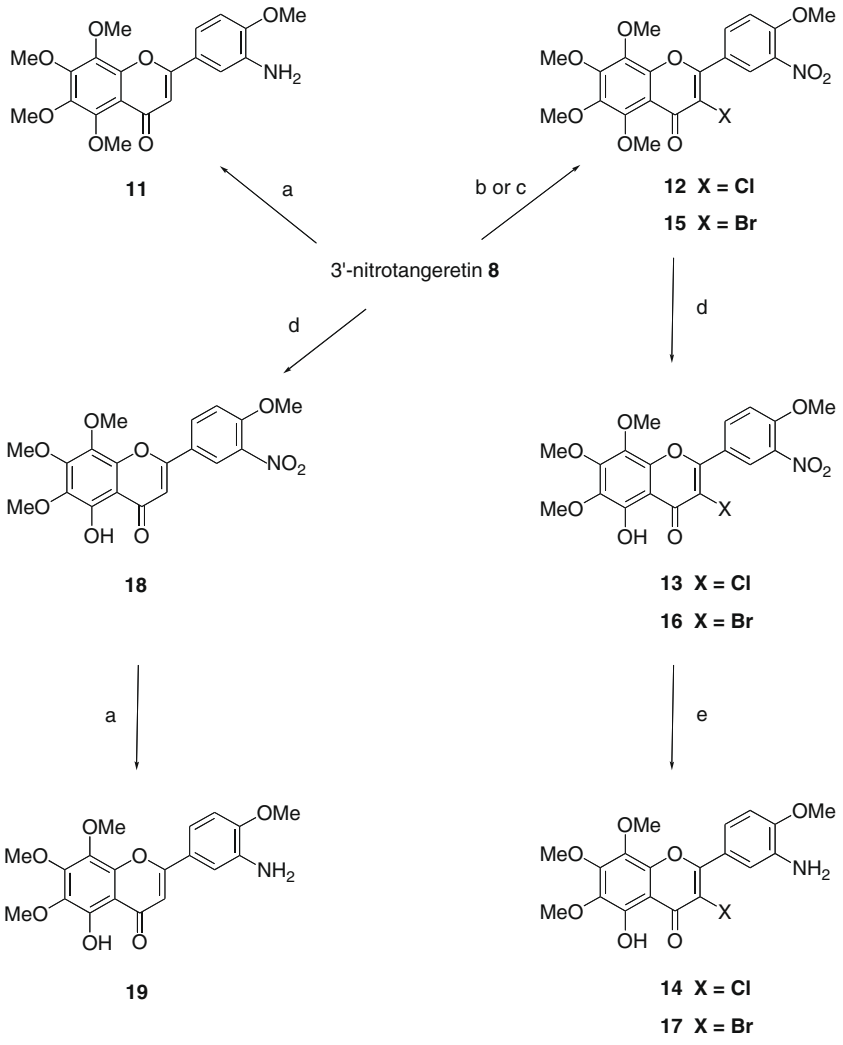
R^1	R^2	R^3	
OH	OH	NO_2	**20**
OH	OH	NH_2	**21**
OH	OMe	NH_2	**22**

Lastly, in order to evaluate SARs within this series, three analogs without nitrogen on the B-ring, 5-hydroxy-6,7,8,4′-tetramethoxyflavone (=5-*O*-demethyltangeretin = gardenin B) **23**, 3′-hydroxy-5,6,7,8,4′-pentamethoxyflavone **24** (=3′-hydroxytangeretin), and its 5-*O*-demethyl derivative **25** (=gardenin D) were also prepared.[25] Synthesis of **24** from tangeretin **4** gave 11% yield by the same classical three-steps pathway (steps d–f) as described for **8** in the Scheme 1, but with isovanillin instead of 3-nitro-4-methoxybenzaldehyde at the step e. The 5-O-demethylation step (**4**·**23** and **24**·**25**) was carried out as mentioned in the Scheme 2 (step d).

The antiproliferative effect of flavones was assayed on KB human buccal carcinoma cells and the activation of apoptosis with DEVD-AMC as substrate in HL60 human leukemia cells. Inhibition of tubulin assembly (ITA) was determined according to Zavala and Guenard's method[26] for the most antiproliferative compounds only. Results were given as the percentage of ITA at 6.6×10^{-5} M or as IC_{50}, calculated and also expressed in relation to deoxypodophyllotoxin (DPPT) in terms of the $IC_{50}/IC_{50\ DPPT}$ ratio. As depicted in Table 1, the three most antiproliferative and proapoptotic 3′-aminoflavones, **14**, **19** and **17**, have in common a persubstituted A-ring with a phenol function at C-5 and three methoxyl groups at C-6, C-7 and C-8. When one of these structural requirements is lacking (5-methoxylated flavone **11** vs **19**; 5,7-dioxygenated flavones **21** and **22** vs **19**), the biological response is very weak. Loss of antiproliferative activity by methylation of the 5-phenol group confirms the



Scheme 1. Reagents and conditions: (a) *Aspergillus niger*, 70%; (b) 1 equiv HNO_3, TFA, 0 °C, 0.5 h, 45%; (c) iodomethane, K_2CO_3, DMF, rt, 1 h, quantitative yield; (d) EtOH–40% aq KOH, reflux, 5 h, 56%; (e) MeOH–50% aq KOH 10:1, 3-nitro-4-methoxybenzaldehyde, rt, 15 h, 60%; (f) I_2, pyridine, 120 °C, 10 h, 52%.



Scheme 2. Reagents and conditions: (a) H_2, Pd–C 10%, DMF, rt, 3 h, 93%, (**11**), 95% (**19**) ; (b) NCS, CH_2Cl_2–pyridine 4:1, rt, 48 h, 95% ; (c) NBS, CH_2Cl_2–MeOH 2:1, rt, 5 h; extraction then THF–NaOH N 1:1, rt, 0.25 h, 95%; (d) $AlBr_3$, acetonitrile, 0 °C, then HCl 1 N, 50 °C, 0.33 h, 60% (**13**), 64% (**16**), 69% (**18**); (e) $SnCl_2$, $2H_2O$, MeOH, 60 °C, 14 h, 53% (**14**), 41% (**17**).

previously reported importance of the 5-hydroxyl group.[1,27] Contribution of the 3′-amino substituent to the activity is obvious from observed results with **19** in regard to **23** and **18**, the 3′-unsubstituted or nitro analogs, respectively. As we could not prepare the 3′-amino analog of **1**, the compared effect of C-3′ substituent (amino vs hydroxyl) was carried out with analogs **19** and **25**: Antiproliferative activity and most ITA are better for **25**, while activation of apoptosis pathway is more effective with **19**. Lastly, the superior antiproliferative, proapoptotic and ITA activities of the reference flavone **1** confirms the crucial role of the C-3 methoxyl group.

Though failing in its initial goal of access to the amino analog of the potent natural flavone **1**, this study is noteworthy from a pharmacomodulation point of view. Influence on the cytotoxicity of substitution patterns at C-3′ (nitro and amino vs hydroxyl), and C-3 (bromo and chloro substituents) are previously unpublished to our knowledge. Preparation of this lacking amino analog is in progress by an alternative synthetic process and its biological evaluation will be described subsequently.

Acknowledgments

We thank Jean-Christophe Jullian for NMR measurements, Dr. Françoise Gueritte for a sample of **1**, and Kevin Spelman for English corrections of the Letter.

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