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Structure–activity relationships for naturally occurring coumarins as β-secretase inhibitor

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ABSTRACT

The present study was demonstrated to evaluate the effects of naturally occurring coumarins (NOCs) including simple coumarins, furanocoumarins, and pyranocoumarins on the inhibition of β-secretase (BACE1) activity. Of 41 NOCs examined, some furanocoumarins inhibited BACE1 activity, but simple coumarins and pyranocoumarins did not affect. The most potent inhibitor was 5-geranyloxy-8-methoxypsoralen (**31**), which has an IC_{50} value of 9.9 μM. Other furanocoumarin derivatives, for example, 8-geranyloxy-5-methoxypsoralen (**35**), 8-geranyloxypsoralen (**24**), and bergamottin (**18**) inhibited BACE1 activity, with the IC_{50} values <25.0 μM. Analyses of the inhibition mechanism by Dixon plots and Cornish-Bowden plots showed that compounds **18**, **31** and **35** were mixed-type inhibitor. The kinetics of inhibition of BACE1 by coumarins **24** was non-competitive inhibitors.

1. Introduction

Coumarins are widely distributed in nature and are found in all parts of plants.[1] These compounds are especially common in grasses, orchids, citrus fruits, and legumes.[1] Being so abundant in nature, coumarins make up an important part of the human diet. Based on chemical structure, they can be broadly classified as (a) simple coumarins (e.g., coumarin, **1**), (b) furanocoumarins of the linear (e.g., psoralen, **12**) or angular (e.g., angelicin, **38**) type, and (c) pyranocoumarins of the linear (e.g., xanthyletin, **40**) or angular (e.g., seselin, **45**) type.[1] Simple coumarins are very widely distributed in the plant kingdom.[1] Interestingly, citrus oils, in particular, contain abundant amounts of both simple as well as furanocoumarins.[2] Human are also exposed to furanocoumarins (e.g., bergapten, **14** and xanthotoxin, **20**) in umbelliferous vegetables such as parsnips, celery, and parsley in substantial amounts.[3] For example, parsnip root reportedly contains as much as 40 mg/kg of certain linear furanocoumarins such as psoralen (**12**), bergapten (**14**), and xanthotoxin (**20**), which are destroyed by normal cooking procedures (boiling or microwave).[3] In addition, in certain countries (e.g., China, India, and Mexico) and in certain geographical areas within the United States (e.g., the Southwest) fresh coriander leaves (also known as cilantro or Chinese parsley) are used extensively. The cilantro leaves are used in soups, chutneys, and sauces and flavoring curries and even wine. Recently, we reported the β-secretase (BACE1) inhibitory activities of several furanocoumarins isoimperatorin (**15**), oxypeucedanin (**16**), imperatorin (**21**), (+)-byakangelicol (**33**), and (+)-byakangelicine (**34**) from *Angelica dahurica*.[4]

Alzheimer's disease (AD) has become an increasingly severe medical and social problem, due to the rapid growth of aging people populations in industrialized countries, and even in some developing countries. The β-secretase (BACE1) has been recognized as a valuable target for the treatment of AD. The BACE1 inhibitors have potential to be developed as anti-dementia drugs. Nevertheless, all drugs considered for AD must be able to cross the plasma membrane, and most importantly the blood–brain barrier.[5] Enzyme inhibitors with therapeutic potential should preferably be smaller than 700 Da, making large peptide-based inhibitors not viable as drug candidates.[6] Thus, the secondary metabolites of plants, which have relatively low-molecular weights and high lipophilicity, may offer possibilities for drugs against AD.[6] There are only very few reports on natural products-based BACE inhibitors.[4,6–9]

In the present study, a series of 41 kinds of NOCs were investigated for their effects on inhibition of BACE1 activity, and we found that some NOCs are strongly inhibitors of BACE1.

2. Results and discussion

2.1. Inhibition of BACE1 activity by 41 NOCs

A total of 41 naturally occurring coumarins (NOCs) from several structural subclasses were investigated, including simple coumarins (**1**–**11**), linear-type furanocoumarins (**12**–**14**, **17**–**20**, **22**–**32**, **35**–**38**), angular-type furanocoumarins (**38** and **39**), linear-type pyranocoumarins (**40**–**44**), and angular-type pyranocoumarins (**45** and **46**) (Fig. 1). Inhibition of BACE1 activity of five

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furanocoumarins (**15**, **16**, **21**, **33** and **34**) was previously reported.[4] From the regression curves of coumarin concentration against percentage of control activity, IC_{50} values of each coumarin were estimated and presented in Table 1. Among the 41 compounds tested, member from furanocoumarin group were most active, with 5-geranyloxy-8-methoxypsoralen (**31**) being the most potent inhibitor at an IC_{50} value of 9.9 μM. Similarly, bergamottin (**18**), 8-geranyloxypsoralen (**24**), and 8-geranyloxy-5-methosypsoralen (**35**) exhibited IC_{50}s of 32.2, 20.4, and 11.1 μM, respectively. Several other compounds inhibited BACE1 to lesser extent, these included phellopterin (**32**) from furanocoumarin group (IC_{50} of 143 μM) as well as kinidilin (**30**) with IC_{50} value of 344 μM. All other coumarins, however, showed relatively less potent inhibitory effect of BACE1 activity with IC_{50} values of more than 500 μM.

2.2. Inhibition mechanism of BACE1 by coumarins

Further investigations were undertaken to explore the inhibition mechanism of several coumarins compounds exhibiting high inhibitory potency on BACE1 activity. Compounds for chosen for further study were bergamottin (**18**), 5-geranyloxy-8-methoxypsoralen (**31**), 8-geranyloxypsoralen (**24**), and 8-geranyloxy-5-methoxypsoralen (**35**). The inhibition constants were determined from Dixon plots,[10] in which the inverse of enzyme activity is represented as a function of inhibitor concentration for at least two different enzyme–substrate concentrations. For a more accurate determination of this constant, Dixon plots were constructed using two different substrate concentrations 1.5 and 0.75 μM. Following this methods, the extrapolated straight lines intersect in a single point from which the inhibition constants were determined to be K_i values of **18**, **24**, **31**, and **35** with 34.8, 61.6, 102.7, and 55.0 μM, respectively. The Dixon plots is very useful determining the inhibition parameters of coumarins to the activity of BACE1 activity but is not sufficient to fully elucidate the type of inhibition, because the Dixon plots for mixed and competitive inhibition is similar. The complementary plot, the Cornish-Bowden plots[11] in which the ratio of substrate concentration and enzyme activity is plotted versus inhibitor concentration, allows the distinction between these two types of inhibition. This plots, by itself, is also not sufficient to fully determine the type of inhibition because it

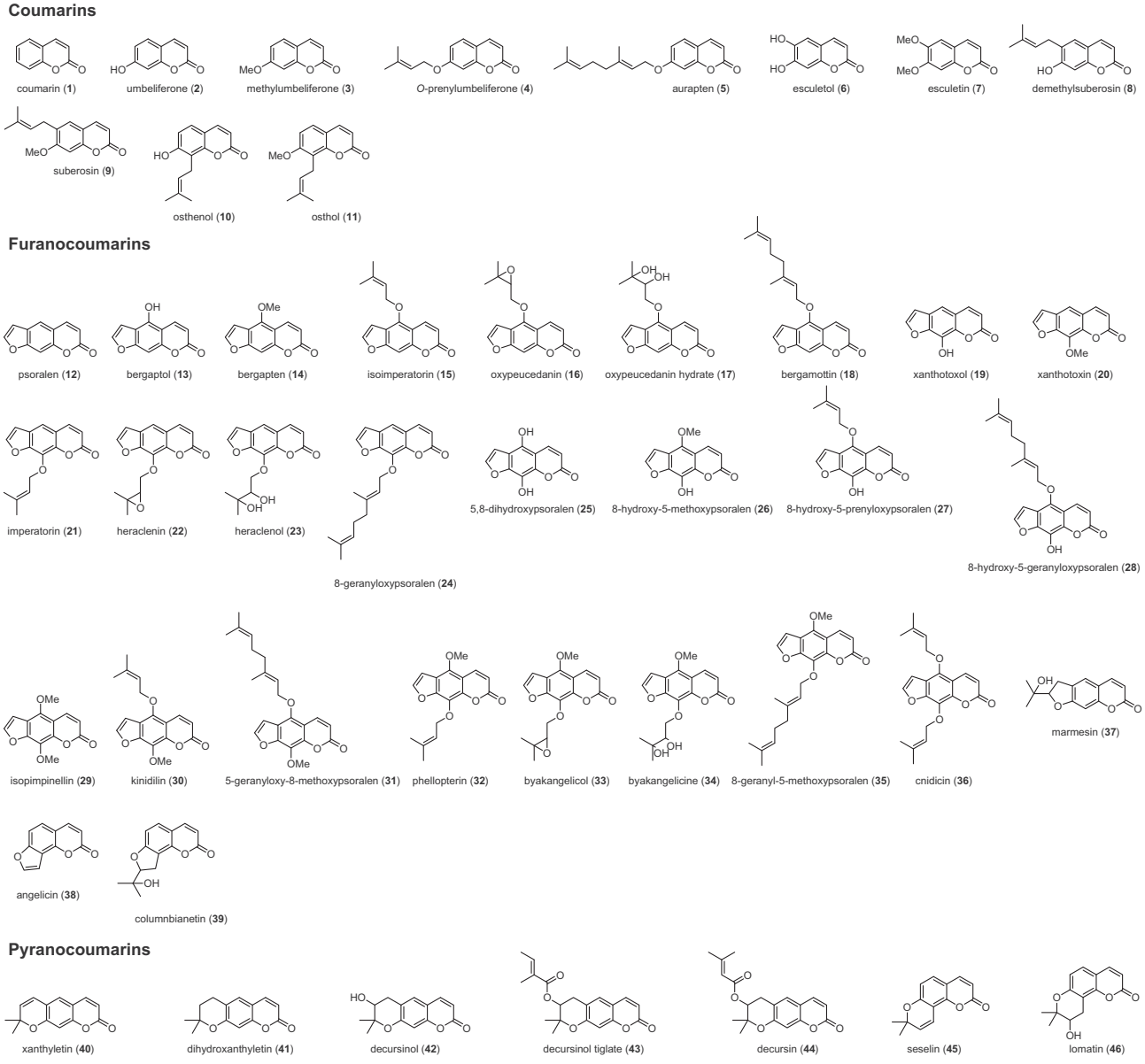

Figure 1. Structure of naturally occurring coumarins **1**–**46**.

Table 1
Inhibition of BACE1 activity by NOCs

NOCs	IC_{50} (μM)	NOCs	IC_{50} (μM)
Simple Coumarins			
Coumarin (**1**)	>500 (8.1%)[a]	Esculetin (**7**)	>500 (6.2%)
Umbelliferone (**2**)	>500 (16.0%)	Demethylsuberosin (**8**)	412.9 ± 11.9
7-Methoxycoumarin (**3**)	>500 (1.1%)	Suberosin (**9**)	>500 (49.5%)
7-Prenyloxycoumarin (**4**)	>500 (33.1%)	Osthenol (**10**)	>500 (39.4%)
Aurapten (**5**)	345.1 ± 8.9	Osthol (**11**)	>500 (39.4%)
Esculetol (**6**)	>500 (9.8%)		
Furanocoumarins			
Psoralen (**12**)	>500 (19.3%)	8-Hydroxy-5-methoxypsoralen (**26**)	>500 (8.2%)
Bergaptol (**13**)	>500 (11.1%)	8-Hydroxy-5-prenyloxypsoralen (**27**)	>500 (42.1%)
Bergapten (**14**)	>500 (−6.0%)	5-Geranytoxy-8-hydroxypsoralen (**28**)	>500 (38.6%)
Isoimperatorin (**15**)	244.2 ± 8.2[b]	Isopimpnellin (**29**)	>500 (12.8%)
Oxypeucedanin (**16**)	359.2 ± 96[b]	knidillin (**30**)	3442 ± 49
Oxypeucedanin hydrate (**17**)	>500 (30.7%)	5-Geranytoxy-8-methoxypsoralen (**31**)	9.9 ± 1.3
Bergamottin (**18**)	32.2 ± 40	Phellopterin (**32**)	1431 ± 1.5
Xanthotoxol (**19**)	>500 (32.8%)	Byakangelicol (**33**)	104.9 ± 2.4[b]
Xanthotoxin (**20**)	>500 (−4.9%)	Byakangelicine (**34**)	219.7 ± 76[b]
Imperatorin (**21**)	91.8 ± 8.2b	8-Geranyloxy-5-methoxypsoralen (**35**)	11.1 ± 1.1
Heraclenin (**22**)	443.6 ± 20.1	Cnidicin(**36**)	>500 (1.4%)
Heraclenol (**23**)	>500 (17.2%)	Marmesin (**37**)	>500 (2.9%)
8-Geranyloxypsoralen (**24**)	20.4 ± 1.0	Angelicin (**38**)	>500 (9.5%)
5,8-Dihydroxypsoralen (**25**)	>500 (33.6%)	Columbianetin (**39**)	>500 (20.8%)
Pyranocoumarins			
Xanthyletin (**24**)	>500 (37.2%)	Decursin (**44**)	>500 (26.8%)
Dihydroxanthyletin (**41**)	>500 (13.5%)	Seselin (**45**)	>500 (14.0%)
Decursinol (**42**)	>500 (7.6%)	Lomatin (**46**)	>500 (12.8%)
Decursinol tiglate (**43**)	>500 (18.8%)	Reference[c]	0.2 ± 0.01

[a] Inhibition (%) at 500 μM.
[b] Ref. 4
[c] Lys-Thr-Glu-glu-Ile-Ser-Glu-Val-Asn-(statine)-Val-Ala-Glu-Phe-OH was used as the positive control.

has the disadvantage of not always distinguishing between mixed and uncompetitive inhibition. However, by analyzing the two plots together, all types of inhibition can be characterized. Figure 2 showed the Dixon plots and the Cornish-Bowden plots for furanocoumarins **18**, **24**, **31**, and **35** studied in this work using two different concentrations of substrate, 1.5 and 0.75 μM. For the compounds **18**, **31**, and **35** studied here, the plots showed mixed inhibition. Because $K_i > K_i'$ or $K_i < K_i'$ (where K_i, the inhibition constant, is the dissociation constant of the enzyme–inhibitor complex and K_i' is the dissociation constant of the enzyme–substrate–inhibitor complex), in both cases, the interaction is above the inhibitor concentration axis in the Cornish-Bowden plots and below the inhibitor axis in the Dixon plots. On the other hand, according to Dixon and Cornish-Bowden plots, the inhibition kinetics of BACE1 by **24** are typical non-competitive, where K_i value are equal to K_i' value and the interactions occurred on the **24** axis in both plots (Fig. 2 and Table 2).

2.3. Structure–activity relationships of NOCs on BACE1 inhibitory activity

Coumarins have been reported to have several pharmacological activities, such as antitumor and antiacethylcholinesterase activities. Otherwise, in previous studies, furanocoumarins from medicinal plant *A. dahurica* showed inhibition effects of BACE1.[4] Therefore, in the present study, 41 kinds of naturally occurring coumarins (NOCs) were screened for their effects on BACE1 activity. All coumarins in this experiment were screened for BACE1 inhibitory activity at a concentration of 500 μM. Then a series of concentrations were used for an IC_{50} determination. Some structure–activity relationships of coumarins can be deduced. Of the simple coumarins in Table 1, coumarin (**1**), which is the fundamental skeleton lacking substitutions on the benzene ring, showed a no inhibitory effect (8.1%) on BACE1. The activity of aurapten (**5**), with a geranyloxy group at the C_7 position, exhibited a moderate inhibitory effect, whereas umbelliferone (**2**), 7-methoxycoumarin (**3**), and 7-prenyloxycoumarin (**4**) showed lower inhibitory effects. On the basis of this evidence, a geranyloxy group at the C_7 position of the simple coumarin skeleton seems to be very important for activity. In the series of furanocoumarins in Table 1, psoralen (**12**), having no additional substitutions on the benzene ring, was found to have significantly lower activity. The activities of isoimperatorin (**15**) and imperatorin (**21**), with a prenyloxy group at the C_5 or C_8 position, showed increased inhibitory effects, whereas in the latter, an additional methoxy group at the C_8 or C_5 position seems to have no significant influence. This evidence was comparing the structure and activity of **15**, **21**, knidilin (**30**), and phellopterin (**32**). In addition, bergapten (**14**) and xanthotoxin (**20**), with a methoxy group at the C_5 or C_8 position, showed no activity. Isoimpinellin (**29**), with the two methoxy groups at both the C_5 and C_8 positions, had no inhibitory activity to support of this inference. However, bergamottin (**18**) and 8-geranyloxypsoralen (**24**), with a geranyloxy group at the C_5 or C_8 position, were found to be significantly more active than were prenyloxy-groups (**15** and **21**), and an additional methoxy group at the C_8 or C_5 position seems to have significant influence. Based on results, mentioned above, functional-substitutions on the benzene ring selectively enhance or decrease inhibition of BACE1 activity, and a geranyloxy group seems to be very important for activity.

In conclusion, our data suggest that dietary NOCs, particularly geranyloxy appendage, effectively reach their target, BACE1, eliciting site-specific inhibition. The IC_{50} values of our inhibitors, although higher than peptide-derived transition state analog-based BACE1 inhibitors are none-the-less still in the low micromolar range. Considering the development of therapeutic for AD, chemical compounds must cross the blood–brain barrier (BBB) and the plasma membranes. On the other hand, NOCs are relatively

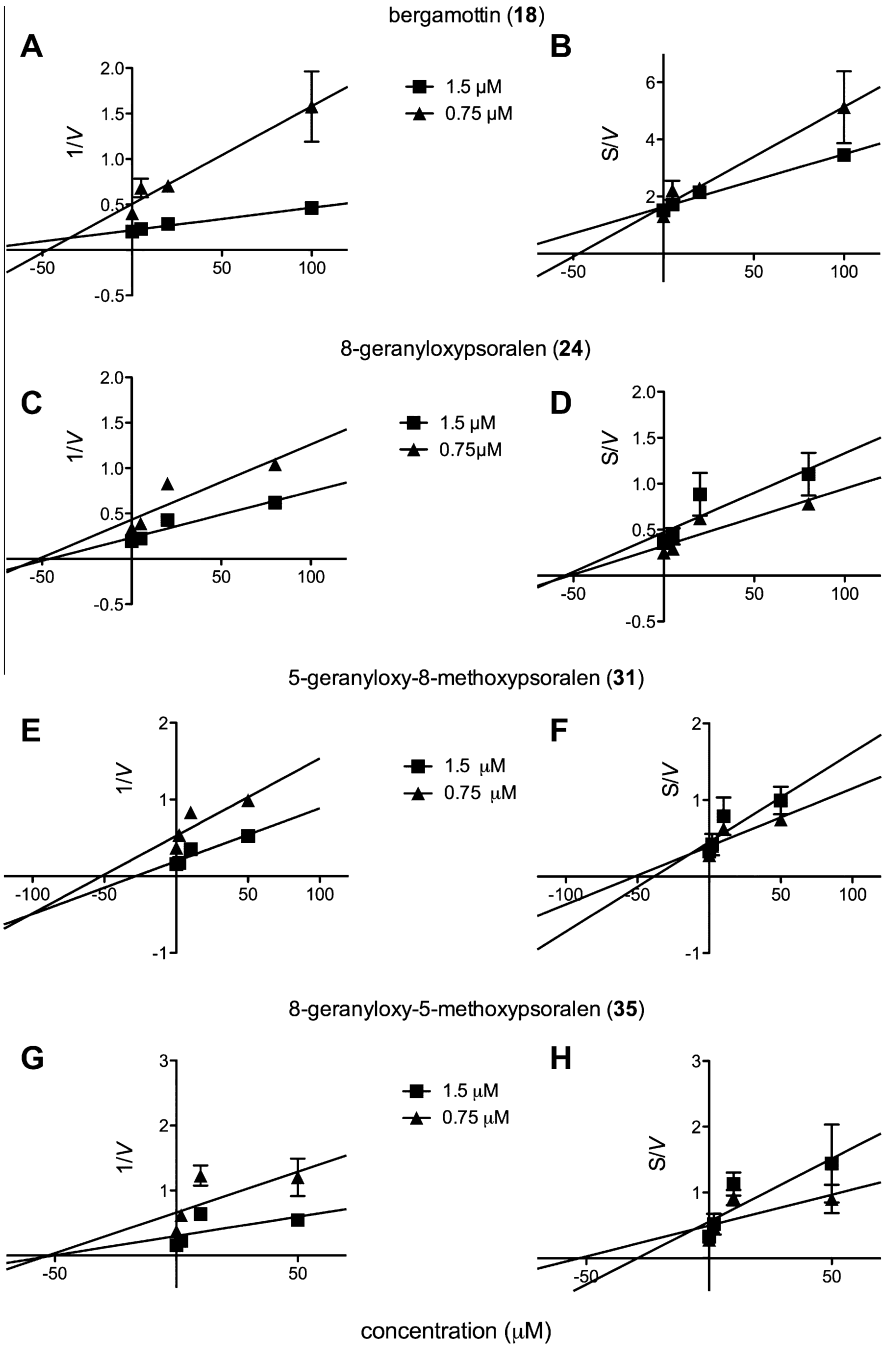

Figure 2. Dixon plots (panels A, C, E, and G) and Cornish-Bowden plots (panels B, D, F, and H) for inhibition of NOCs **18**, **24**, **31**, and **35** on BACE1 activity. The BACE1 substrate concentrations used were 1.5 μM (■) and 0.75 μM (▲). The results are presented as means ± S.E.M. of triplicate experiments.

Table 2
Kinetics parameters for inhibition of BACE1 activity by compounds **18**, **24**, **31**, and **34**

Compounds	K_i (μM)	K_i' (μM)	Type of inhibition
18	34.8	0.7	Mixed
24	61.6	61.9	Non-competitive
31	102.7	13.7	Mixed
34	55.0	6.2	Mixed

low-molecular-weight and high lipophilicity. Zhang et al. reported that imperatorin (**21**) could pass through the BBB easily in rat.[12] Given their enhanced lipophilic potency in cell, we believe that our compounds **18**, **24**, **31**, and **35** may be a possibility to prevent Alzheimer's disease.

3. Experimental procedure

3.1. Chemicals

A total of 46 coumarin derivatives were used for the inhibition studies of BACE1 activity (Table 1). Coumarins; coumarin (**1**), umbelliferone (7-hydroxycoumarin, **2**), 7-methoxycoumarin (**3**), 7-prenyloxycoumarin (**4**), aurapten (7-geranyloxycoumarin, **5**), esculetol (6,7-dihydroxycoumarin, **6**), esculetin (6,7-dimethoxycoumarin, **7**), demethylsuberosin (7-hydroxy-6-prenylcoumarin, **8**), suberosin (7-methoxy-6-prenylcoumarin, **9**), osthenol (7-hydroxy-8-prenylcoumarin, **10**), osthol (7-methoxy-8-prenylcoumarin, **11**), furanocoumarins; psoralen (**12**), bergaptol (5-hydroxypsoralen,

13), bergapten (5-methoxypsoralen, **14**), isoimperatorin (5-prenyloxypsoralen, **15**), oxypeucedanin (5-epoxyisopentenyloxypsoralen, **16**), oxypeucedanin hydrate (5-(2,3-dihydroxy-3-methylbutoxy)psoralen, **17**), bergamottin (5-geranyloxypsoralen, **18**), xanthotoxol (8-hydroxypsoralen, **19**), xanthotoxin (8-methoxypsoralen, **20**), imperatorin (8-prenyloxypsoralen, **21**), heraclenin (8-epoxyisopentenyloxypsoralen, **22**), heraclenol (8-(2,3-dihydroxy-3-methylbutoxy)psoralen, **23**), 8-geranyloxy psoralen (**24**), 5,8-dihydroxypsoralen (**25**), 8-hydrpxy-5-methoxy psoralen (**26**), 8-hydroxy-5-prenyloxypsoralen (**27**), 5-geranyloxy-8-methoxypsoraren (**28**), isopimpinellin (5,8-dimethoxypsoralen, **29**), knidilin (8-methoxy-5-prenyloxy psoralen, **30**), 5-geranyloxy-8-methoxypsoralen (**31**), phellopterin (5-methoxy-8-prenyloxypsoralen, **32**), byakangelicol (5-epoxyisopentenyloxy-8-methoxypsoralen, **33**), byakangelicine (5-methoxy-8-(2,3-dihydroxy-3-methylbutoxy) psoralene, **34**), 8-geranyloxy-5-methoxypsoralen (**35**), cnidicin (5,8-diprenyloxypsoralen, **36**), marmesin (**37**), angelicin (**38**), columbianetin (**39**), pyranocoumarins; xanthyletin (**40**), dihydroxanthyletin (**41**), decursinol (**42**), decursinol tiglate (**43**), decursin (**44**), seselin (**45**), lomatin (**46**). coumarins **1**–**3**, **6**, **7**, **12**, **14**, **20**, and **38** were obtained from Sigma Chemical Co. (St. Louis, MO), Wako Pure Chemical (Osaka), or Tokyo Chemical Industry (Tokyo). All other chemicals and reagents were of the highest purity commercially available. Coumarins **11**, **15**, **16**, **21**, **33**, and **34** were isolated from *A. dahurica* and *Cnidium monnieri*.[4,13] Other NOCs were synthesized as described previously in Supplementary data. [14–22] A BACE1 (recombinant human BACE1) assay kit was purchased from the PanVera Co. (United States).

3.2. β-Secretase (BACE1) enzyme assay

The β-secretase (BACE1) assay was carried out according to the supplied manual with modifications.[4] Briefly, a mixture of 10 μl of assay buffer (50 mM sodium acetate, pH 4.5), 10 μl of BACE1 (1.0 U/ml), 10 μl of the substrate (750 nM Rh-EVNLDAEFK-Quencher in 50 mM ammonium bicarbonate), and 10 μl of sample dissolved in 30% DMSO was incubated for 60 min at room temperature in the dark. The mixture was irradiated at 550 nm and the emission intensity at 590 nm was recorded. The inhibition ratio was obtained by the following equation:

$$\text{Inhibition } (\%) = [1 - \{(S - S_0)/(C - C_0)\}] \times 100 \quad (1)$$

Where C was the fluorescence of the control (enzyme, buffer, and substrate) after 60 min of incubation, C_0 was the fluorescence of control at zero time, S was the fluorescence of the tested samples (enzyme, sample solution, and substrate) after incubation, and S_0 was the fluorescence of the tested samples at zero time. To allow for the quenching effect of the samples, the sample solution was added to the reaction mixture C, and any reduction in fluorescence by the sample was then investigated. All data are the mean of three experiments.

Supplementary data

Supplementary data (these data include synthesis and spectroscopic data of compounds **4**, **5**, **8**–**10**, **13**, **18**, **19**, **22**–**32**, **35**–**37**, **39**, and **42**–**46**) associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.12.002.

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