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Determination of aqueous stability and degradation products of series of coumarin dimers

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ABSTRACT

The stability in aqueous solution of five classes of coumarin dimers (**I–V**, compounds **1–29**) was studied by HPLC–MS/MS at various pH values. The relationship between chemical structure and stability is discussed. It was found that dimeric compounds with strong electron withdrawing groups (EWGs) on the α-carbon to the bridging C-atom are stable at all pH values, whereas other derivatives undergo retro-Michael addition at rates which are also affected by the substituents on the aromatic rings. In some cases formation of stable isomers or oxidation products was observed. In order to evaluate their developability and potential for progression to *in vivo* studies, representative compounds were tested in an *in vitro* microsomal stability assay.

1. Introduction

Coumarin derivatives have been found to have numerous therapeutic applications including photochemotherapy, antitumor and anti-HIV therapy [1], and are also active as antibacterials [2,3] anti-inflammatory agents [4], and anti-coagulants [5]. In addition, coumarins are known to be lipid lowering agents with moderate triglyceride lowering activity [6], whereas hydroxycoumarins are powerful chain-breaking antioxidants and can prevent free radical injury [7,8]. Recent discovery of coumarins having weak estrogenic activity resulted in the use of such derivatives as therapeutic agents in preventing the emergence of menopause related diseases, such as osteoporosis, increased risk for cardiovascular diseases and cognitive deficiencies [9].

The pharmaceutical use of coumarins therefore has excellent potential. A broad array of medicinal applications of coumarins has been summarized in recent reviews, especially concerning their antioxidant properties [10,11]. Application of coumarin derivatives in pharmacotherapy of breast cancer [12], progress of *Calophyllum* coumarins as potent anti-HIV agents [13,14], coumarins of *Corchorus* species as cardiac agents [15], chemistry and biological activity of natural and synthetic prenyloxycoumarins [16], in particular isopentenyloxycoumarin [17], and coumarins as versatile biodynamic agents [18] are also reviewed.

Stability in aqueous solution of coumarins of potential pharmacological importance has not been systematically studied up to date. An early report on the stability of coumarin acids, derived from coumarins by opening of the lactone ring, was based on isolation and structure determination (elemental analysis, mixed m.p.) of the products [19]. The first HPLC supported stability studies were performed qualitatively on some flavonoid and coumarin derivatives using a UV detector at two wavelengths [20]. A similar study involved the chemical and enzymatic stability of some coumarin-based cyclic prodrugs [21]. More recently, a set of coumarin-3-acyl derivatives was evaluated for their purity and stability by HPLC/photodiode array detector [22]. In order to determine absorption and fluorescence spectra of a water soluble coumarin, Arai and co-workers evaluated its stability at pH 7.2, in the dark, at room temperature, and did not observe any decomposition for up to a week [23]. The stability of four coumarins from biological origin during microwave-assisted extraction was

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Table 1
The mobile phase, solvent gradient condition and detection wavelengths for compounds **1**–**29**.

Compound	Solvent A	Solvent B	Linear gradient	Detection wavelength (nm)
1	0.1% HCOOH/H_2O	CH_3CN	10–90% Solvent B in 10 min	320
2	0.1% TEA/H_2O	CH_3CN	5–90% Solvent B in 10 min	320
3	0.1% TEA/H_2O	CH_3CN	10–30% Solvent B in 10 min	320
4	0.1% TEA/H_2O	CH_3CN	5–60% Solvent B in 10 min	320
5	0.1% TEA/H_2O	CH_3CN	5–30% Solvent B in 10 min	320
6	0.1% TEA/H_2O	CH_3CN	17–20% Solvent B in 10 min	320
7	0.1% TEA/H_2O	CH_3CN	10–30% Solvent B in 10 min	320
8	0.1% TEA/H_2O	CH_3CN	5–5% Solvent B in 10 min and to 15% Solvent B in 10 min	320
9	95% (0.1% TEA/H_2O) + 5% CH_3CN	CH_3CN	5–30% Solvent B in 10 min	320
10	95% (0.1% TEA/H_2O) + 5% CH_3CN	CH_3CN	5–60% Solvent B in 10 min	320
11	95% (0.1% TEA/H_2O) + 5% CH_3CN	CH_3CN	0–10% Solvent B in 5 min and to 90% Solvent B in 5 min	320
12	0.1% TEA/H_2O	CH_3CN	20–40% Solvent B in 10 min	320
13	95% (0.1% TEA/H_2O) + 5% CH_3CN	CH_3CN	5–60% Solvent B in 5 min	320
14	0.1% HCOOH/H_2O	0.1% HCOOH/CH_3CN	10–100% Solvent B in 10 min	320
15	0.1% TEA/H_2O	CH_3CN	5–40% Solvent B in 10 min	300
16	0.1% TEA/H_2O	CH_3CN	10–35% Solvent B in 10 min	290
17	0.1% HCOOH/H_2O	0.1% HCOOH/CH_3CN	10–90% Solvent B in 10 min	320
18	0.1% TEA/H_2O	CH_3CN	5–50% Solvent B in 10 min	270
19	0.1% TEA/H_2O	CH_3CN	20–80% Solvent B in 10 min	300
20	0.1% TEA/H_2O	CH_3CN	10–25% Solvent B in 10 min	320
21	95% (0.1% TEA/H_2O) + 5% CH_3CN	CH_3CN	10–90% Solvent B in 10 min	310
22	0.1% HCOOH/H_2O	0.1% HCOOH/CH_3CN	20–80% Solvent B in 10 min	240
23	0.1% TEA/H_2O	CH_3CN	10–45% Solvent B in 10 min	290
24	0.1% TEA/H_2O	CH_3CN	15–40% Solvent B in 10 min	320
25	0.1% TEA/H_2O	CH_3CN	10–50% Solvent B in 10 min	320
26	0.1% HCOOH/H_2O	0.1% HCOOH/CH_3CN	5–100% Solvent B in 10 min	320
27	0.1% TEA/H_2O	CH_3CN	5–85% Solvent B in 10 min	320
28	95% (0.1% TEA/H_2O) + 5% CH_3CN	CH_3CN	5–55% Solvent B in 10 min	320
29	0.1% TEA/H_2O	CH_3CN	5–30% Solvent B in 10 min	320

determined by HPLC, combining photodiode array and fluorescence detector and all compounds proved to be stable in methanol extracts up to 100 °C [24].

In this study, we investigated the stability of a series of coumarin dimers in aqueous solution at different pH values using HPLC-DAD detection, and the paths of the solvolytic and oxidative reactions were determined. The importance of *in vitro* chemical stability of drug candidates is well recognized [25–27]. Degradation products were proposed by means of HPLC–MS. Selected compounds were tested in an *in vitro* microsomal stability assay in order to evaluate their potential for further development. Where possible, potential metabolites were identified by HPLC–MS/MS.

2. Materials and methods

2.1. Materials

All studied compounds **1**–**29** were synthesized as described previously [28–31]. For HPLC-DAD and HPLC–MS assays the following reagents were used: acetonitrile (HPLC grade) and acetone (HPLC grade) obtained from Merck; triethylamine (TEA, p.a. eluent additive for HPLC–MS), ammonium hydroxide solution (p.a. ≥25%) and phosphate buffered saline obtained from Sigma–Aldrich; citric acid monohydrate (p.a.), formic acid (p.a.) and hydrochloric acid (p.a.) and magnesium chloride obtained from Kemika. Water used in this study was purified through a Millipore Milli-Q A10 system. Male CD-1 mouse liver microsomes were purchased from BD Gentest (USA). β-Nicotinamide adenine dinucleotide phosphate (NADP), D-glucose-6-phosphate and glucose-6-phosphate dehydrogenase were from Sigma–Aldrich.

2.2. HPLC-DAD–MS analysis for samples from aqueous stability studies

HPLC-DAD–MS analysis was carried out on Agilent 1100 Series with DAD (diode array detector). Chromatographic separations were performed on a Waters Xterra C18 analytical column (50 mm × 2.1 mm, 3.5 μm), maintained at 30 °C, using gradient elution with appropriate mobile phases at a flow rate of 0.5 ml/min (Table 1). Total run time for a single injection was 17 min. The detection wavelength was selected at maximum absorption of each compound (Table 1).

2.3. Stability studies

Compound solutions were prepared at a concentration of 200 μg/ml by diluting a 1 mg/ml stock solution in acetone, CH_3CN or DMSO with citrate buffers of pH 4.0 and 5.0, and phosphate buffers of pH 7.4 and 9.0. Sampling from the solutions, maintained at 25 °C, was performed over 24 h at certain time intervals.

2.4. Mass spectrometry (MS) of degradation products

HPLC–MS analysis of degradation products was performed directly after DAD measurements using a Micromass Platform LCZ single quadruple mass spectrometer for compounds **14**, **17**, **19**, **22** and **26** and Agilent MSD single quadruple mass spectrometer for all other compounds.

The ESI-MS spectra on Micromass Platform LCZ were acquired in positive ion mode within a mass range of 100–800. Nitrogen was used as nebulizing gas. Drying gas flow rate was set at 730 l/h and its temperature was set at 300 °C. Source block temperature was set at 100 °C. The capillary voltage was 3000 V and cone voltage was 45 V.

The ESI-MS spectra on Agilent MSD single quadruple mass spectrometer were acquired in positive or negative ion mode within a mass range of 100–800. Nitrogen was used as nebulizing gas while its pressure was 25 psi. Drying gas flow rate was set at 10.0 l/min and its temperature set at 350 °C. The capillary voltage was 4000 V and fragmentor was set at 100.

2.5. Metabolic stability studies and HPLC–MS/MS analysis

Selected compounds (1 μM) were incubated at 37 °C for 60 min in 50 mM potassium phosphate buffer (pH 7.4) containing 0.5 mg microsomal protein/ml. The reaction was started by addition of co-

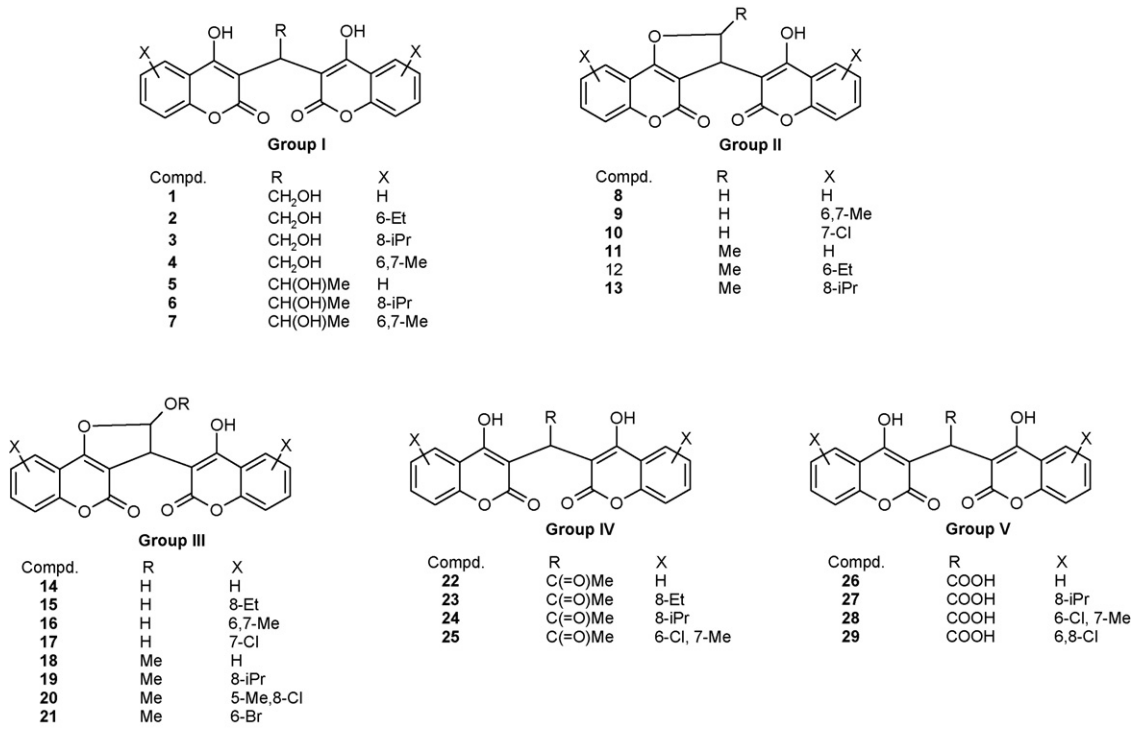


Fig. 1. Five sets of coumarin dimers selected for the stability study.

factor (2.0 mM NADP, 20 mM glucose-6-phosphate, 2.0 mM $MgCl_2$ and 4.0 U/ml glucose-6-phosphate dehydrogenase). At 0, 10, 20, 30, 45 and 60 min an aliquot (50 μl) was taken, quenched with acetonitrile/methanol (2:1, v/v) containing internal standard (warfarin, 1 ng/ml) and analysed by HPLC–MS/MS. Incubation without NADPH was used as a control.

Samples were analysed on an API 4000 Triple Quadrupole Mass Spectrometer (Applied Biosystems) coupled to a HPLC System (HP1200, Agilent) and a HTS PAL CTC Autosampler (CTC). Samples (5 μl) were injected onto an HPLC column (3.5 μm Waters Xterra MS C18, 2.1 mm × 50 mm) and eluted with a gradient at room temperature. The chromatographic conditions consisted of mobile phase A (10 mM NH_4HCO_3 in water pH 10) and mobile phase B (acetonitrile) that was run over a 1.5 min gradient at a flow rate of 0.8 ml/min. A negative ion mode with turbo spray, an ion source temperature of 550 °C and a dwell time of 150 ms were utilised for mass spectrometric detection. Quantitation of coumarin compounds (parent compound depletion) was performed using multiple reaction monitoring (MRM) and monitoring ion pairs of 365.2/160.9 (MH^-/product, compound **1**), 379.2/160.9 (compound **5**), 377.07/160.9 (compound **22**), 461.3/203 (compound **24**), 379.05/161 (compound **26**) and 463.14/203.1 (compound **27**).

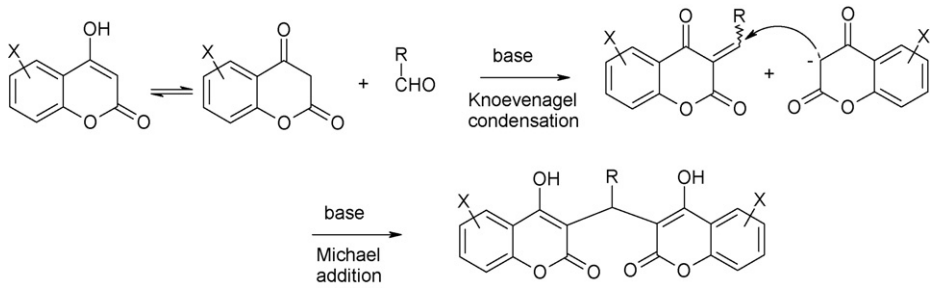
The intrinsic clearance (CLi) was determined from the first-order elimination constant by non-linear regression using Excel (Microsoft Excel 2002), corrected for the volume of the incubation and assuming 52.5 mg microsomal protein/g liver. Values for CLi were expressed as ml/min/g liver.

In a preliminary metabolite screening, metabolites were analysed by HPLC–MS/MS method in MRM mode. MRM transitions of possible phase I metabolites were automatically generated by AB Analyst 1.4.1. script MakeMetIDMethods. Metabolites in mouse liver microsomes were detected by comparing samples at initial time (0 min) with 60-min incubation.

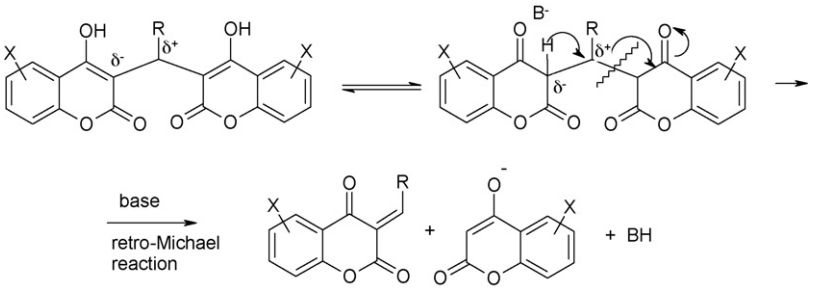
3. Results and discussion

In view of the known anti-inflammatory properties of some coumarin derivatives, several synthetic coumarin dimers **1**–**29** were synthesized as potential inhibitors of mast cell degranulation and inhibitors of leukotriene B_4 [28–31]. A significant number of these derivatives showed inhibition of mast cell degranulation or LTB_4 *in vitro* [28].

The synthesis of coumarin dimers **1**–**29**, Fig. 1, which are characterized by C_1 bridge between two identical units, is completed by the tandem Knoevenagel condensation/Michael addition reac-



Scheme 1. Tandem Knoevenagel condensation/Michael addition reaction in the synthesis of coumarin dimers **1**–**29**.



Scheme 2. Base-catalyzed retro-Michael reaction in coumarin dimers of the group **I**.

tion (Scheme 1). The yields of the compounds **1**–**29** in tandem reaction varied with the substituent pattern, type and number of substituents, amounted between 60% and 95% [28]. Due to the inherent reversibility of both steps, retrosynthetic dissolution of the central bond(s) can be expected as the main, pH-dependent process that determines the stability of all target structures.

Retro-Michael reaction of bis(coumarins) was first discussed by Appendino et al. in relation to the mechanism of their reductive fragmentation [32]. The authors suggested intermediacy of alkylidenchromandiones, for the structure see Scheme 2, in hydride reduction of bis(coumarins), and also suggested that acid and base catalysis are operative in the retro-Michael step. This proposal is amply confirmed in our study.

Assuming relative sensitivity of coumarin dimers to pH of the medium, which *in vivo* ranges from very acidic to basic depending on the biological compartment, we have undertaken a stability study of the representatives at three (or four) selected pH values.

3.1. pH-Rate profile of coumarin dimers

Five sets of coumarin dimers selected for this study are presented in Fig. 1.

They are representatives of approximately one hundred synthetic derivatives claimed [29–31] and tested for *in vitro* inhibition of mast cells degranulation or LTB_4 [28]. Compounds in each group have a common structural unit; the same oxidation level of the α-carbon to the bridging C-atom. Accordingly, these groups are characterized as follows:

- group **I**, compounds **1**–**7**, by *primary* or *secondary* hydroxyl group on α-carbon;
- group **II**, compounds **8**–**13**, by dihydrofuran ring, formed on dehydration of their precursors general formulae **I**;
- group **III**, compounds **14**–**21**, by an aldehyde function on α-carbon, present in the hemiacetal form (**14**–**17**) or, on *O*-methylation, in the acetal form (**18**–**21**);
- group **IV**, compounds **22**–**25**, by a ketone carbonyl group; and
- group **V**, compounds **26**–**29**, by a carboxylic group.

Various oxidation levels of α-C-atom are expected to give different stability properties to the five groups of coumarin dimers. On the other hand, similar behavior is expected for the members within each group, affected only by electronic effect of the substituents on the aromatic rings.

Table 2
Percentage of compounds **1–29** found in buffer solutions after 24 h.

Compound	Percentage of compound found in buffer solution after 24 h			
	pH 4.0	pH 5.0	pH 7.4	pH 9.0
1	92%	–[a]	76%	81%
2	Precipitation	87%	63%	61%
3	95%	–	62%	75%
4	85%	–	47%	33%
5	78%	–	72%	72%
6	83%	–	78%	78%
7	50%	–	37%	31%
8	Precipitation	100% (22 h)	100%	99%
9	Precipitation	Precipitation	100%	100%
10	Precipitation	Precipitation	100%	100%
11	Precipitation	100%	100%	100%
12	Precipitation	100%	99%	100%
13	Precipitation	100%	100%	100%
14	97%	–	36%	51%
15	99%	–	50% (21 h)	13% (21 h)
16	Precipitation	98%	8% (12 h)	11% (12 h)
17	Precipitation	97%	0%	0%
18	30%	–	90%	90%
19	40%	–	95%	95%
20	98%	–	100%	100%
21	51%	–	75%	75%
22	100%	–	93%	91%
23	100%	–	87%	86%
24	99%	–	95%	91%
25	98%	–	100%	100%
26	100%	–	99%	97%
27	99%	–	99%	100%
28	100%	–	99%	99%
29	100%	–	99%	97%

[a] Not analysed.

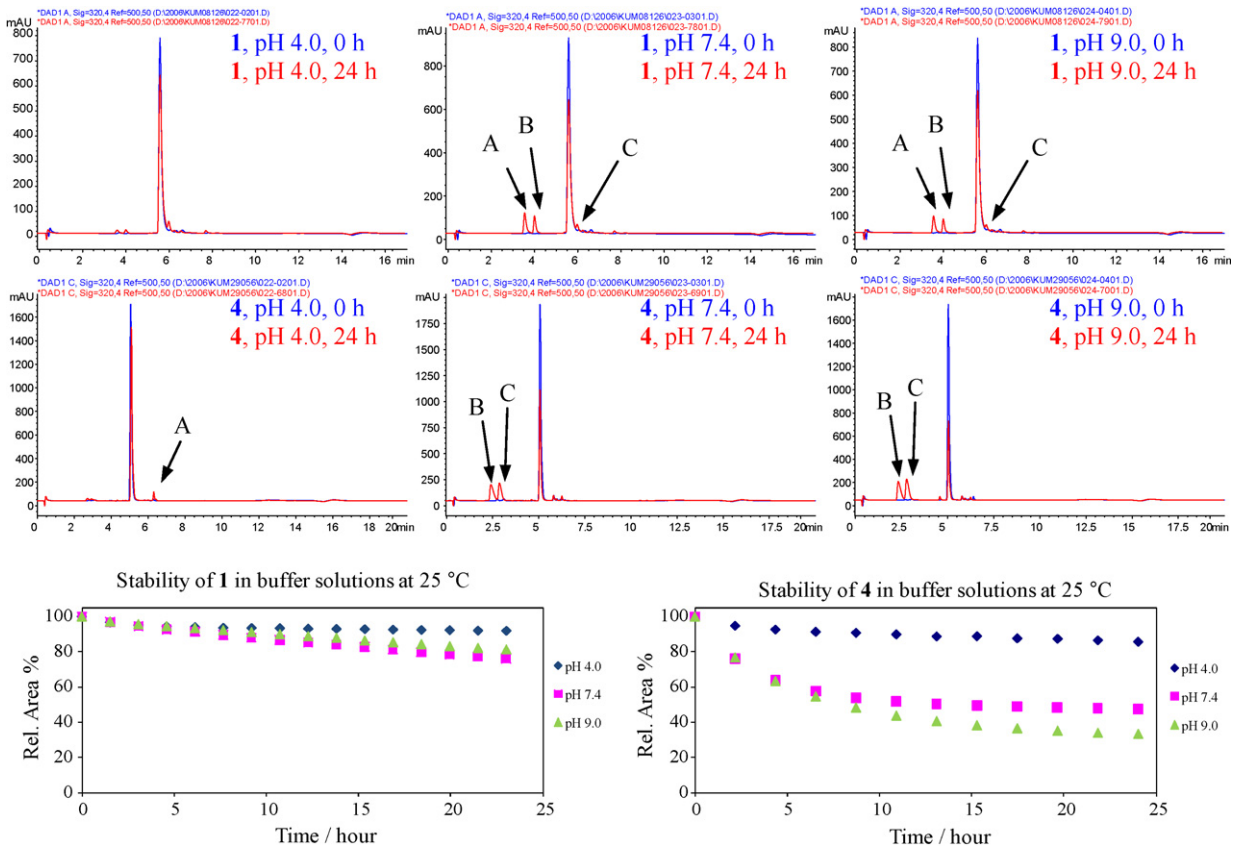


Fig. 2. HPLC-DAD chromatograms after 0 and 24 h, and progress curves for decomposition of **1** and **4** in solution at pH 4.0, 7.4 and 9.0.

pH-Dependent stability data, expressed as % of starting compound **1**–**29** after 24 h, are collected in Table 2.

Data from Table 2 reveal that the final percentage of starting dimers varied between 0% and 100%, depending on the group present on the α-C-atom. Dimers **2**, **8–13**, **16** and **17** precipitate at pH 4.0, but their solubility at pH 5.0 was high enough to monitor their stability (except dimers **9** and **10** that precipitated at pH 5.0 as well).

Progress curves were obtained by sampling of the reaction solution at regular time intervals by HPLC/DAD and determination of the composition of the reaction mixture by HPLC/MS.

Figs. 2 and 3 summarize chromatograms and progress curves at three pH values (4.0, 7.4 and 9.0) for the compounds **1** and **4**, then **5** and **7**, representatives of group **I** with a *primary* and *secondary* OH group α-carbon to the bridging C-atom. All compounds in this group exhibited limited stability and kinetic data for their first-order decomposition reaction are given in Table 3.

Mass spectra and structures of the degradation products are discussed in Section 3.3.

Compounds **1**–**7** showed a rather different stability at various pH values. There is an evident effect of the four methyl groups in **4** and **7** on the decomposition rate. A faster retro-Michael reaction can be explained by stabilization of the positive charge on the bridging carbon in the retro-step, resulting with 1 mol of starting coumarin and 1 mol of 3-alkylidenechromandione, according to Scheme 2. Compound **5**, instead, was completely stable toward retro-Michael, only equilibration with its structural isomer was observed; see Scheme 5 and related discussion.

Compounds within group **II**, characterized by the presence of an annelated dihydrofuran ring, proved to be stable at all three pH values. No formation of degradation products was observed after 24 h with 99–100% of starting compound remaining. This difference in stability, in comparison to their open-chain analogs, can be explained by the fixed enolic form in one coumarin ring of these compounds. Hence, deprotonation of the second ring in its 1,3-dicarbonylic form cannot trigger splitting of the central C–C bond in the αβ-position to the merging carbanionic center.

Compounds of group **III** are characterized by an aldehyde function on the α-carbon, present in the hemiacetal form in **14**–**17** or in the acetal form in **18**–**21**. Easy ring opening of cyclic hemiacetals in basic medium is well known [33], whereas acid-catalyzed ring opening of acetals is the preferred process [34]. This general behavior of hemiacetals and acetals is reflected in pH-dependent decomposition rates of the two sets of compounds within group **III**. Whereas all hemiacetal derivatives **14**–**17** proved stable over 24 h at acidic pH (4.0 or 5.0), but exhibited rather fast decomposition rates at pH 7.4 and pH 9.0, acetals 18 and 19 were much more stable in the neutral and basic media and decomposed relatively fast at acidic pH. The stability of hemiacetals in acidic medium and fast de-dimerization under neutral and basic conditions can be explained by the presence of a ring-chain prototropic equilibrium (Scheme 3).

Chromatograms and pH-dependent progress curve for hemiacetal **16** are shown in Fig. 4. Since this equilibrium is shifted to the left under acidic conditions, high stability of hemiacetals **14**–**17** is regularly observed under these conditions.

Pronounced dependence of the decomposition rate at neutral and basic pH on the substitution pattern in compounds **14**–**17** is another important observation (Table 4). Whereas the rates for **14** and **15** are similar and in the range of $3.1–6.6 \times 10^{-2}\ h^{-1}$, the other two congeners, **16** and **17**, decompose at notably higher rates, $3.5–9.2 \times 10^{-1}\ h^{-1}$. The ethyl group in the *meta* position to the C–C bond to the benzene ring in dimer **15** has a minor effect on the rate of retro-Michael reaction, whereas this rate is significantly enhanced by the accumulated effect of four methyl

Table 3
Pseudo-first-order kinetics and half-lives for the decomposition of the selected representatives of coumarin dimers **I**.

Compound	pH 4.0 (or pH 5.0)		pH 7.4		pH 9.0	
	k (h^{-1})	$t_{1/2}$ (h)	k (h^{-1})	$t_{1/2}$ (h)	k (h^{-1})	$t_{1/2}$ (h)
1	ND	ND	4.34×10^{-2}	16.0	3.22×10^{-2}	21.5
2	4.09×10^{-1}	1.7	6.45×10^{-2}	10.7	5.42×10^{-2}	12.8
3	ND	ND	4.87×10^{-2}	14.2	5.06×10^{-2}	13.7
4	1.53×10^{-1}	4.5	2.74×10^{-1}	2.5	1.77×10^{-1}	3.9
5	7.36×10^{-2}	9.4	7.35×10^{-2}	9.4	1.33×10^{-1}	5.2
6	7.28×10^{-2}	9.5	5.70×10^{-2}	12.2	5.95×10^{-2}	11.6
7	1.08×10^{-1}	6.5	1.13×10^{-1}	6.1	1.18×10^{-1}	5.9

ND = not determined.

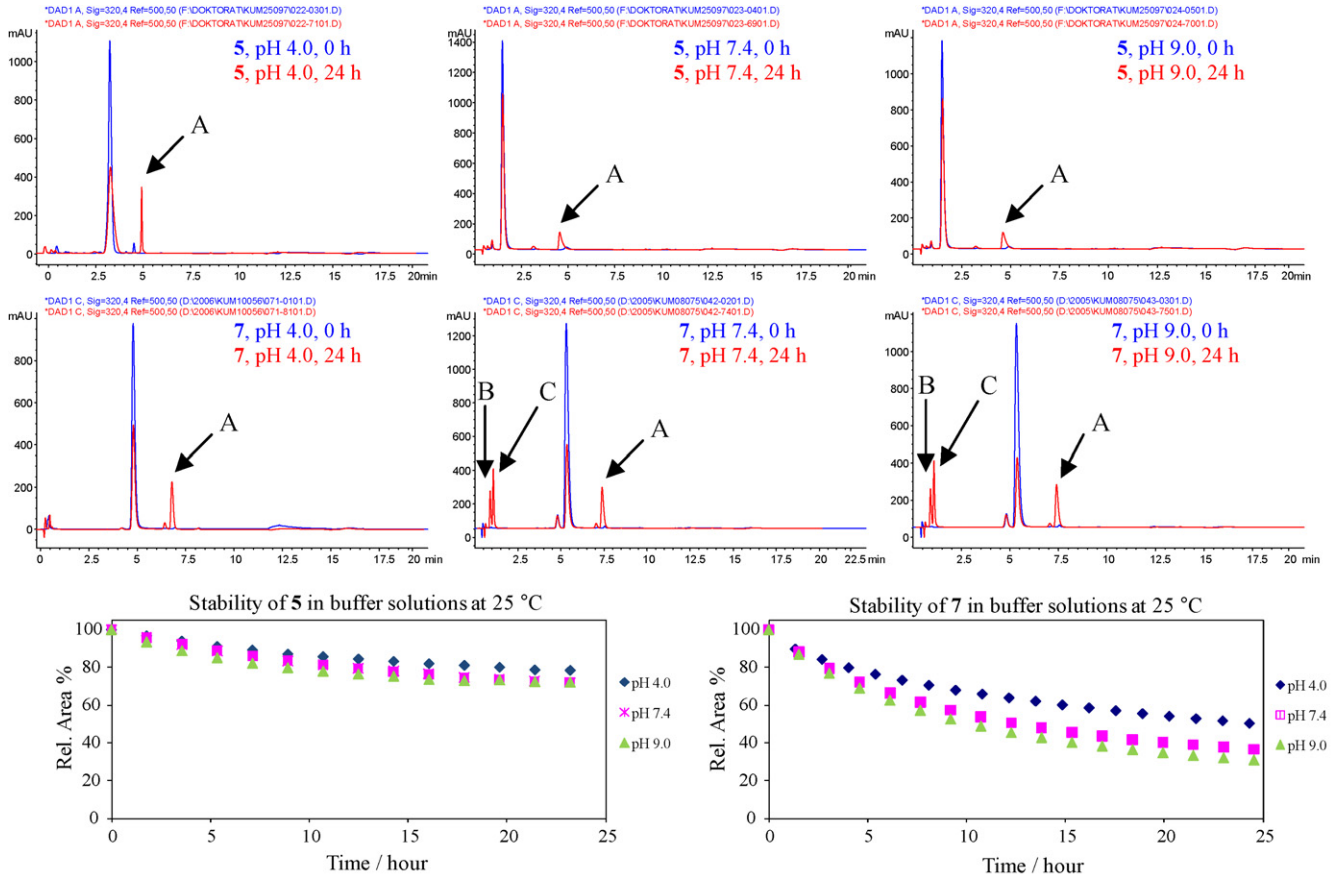
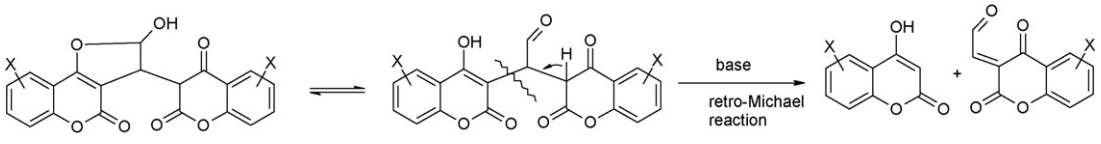


Fig. 3. HPLC-DAD chromatograms after 0 and 24 h, and progress curves for decomposition of **5** and **7** in solution at pH 4.0, 7.4 and 9.0.

groups in compound **16** and *para*-Cl atoms in compound **17**. In compound **16**, the hyperconjugative effect of the *para*-methyl groups function to stabilize the positive charge on the central C-atom. Chlorine atoms in the *para* position are expected to exert an inductive, electron withdrawing effect, thereby destabilizing the positive charge in **17** and reducing base-promoted retro-Michael reaction. The small enhancement of the rate is presumably a consequence of its week resonance contribution from this position [35]. However, decomposition rate of **17** slower in comparison to **16** but faster in comparison to **14** and **15** is presumably a consequence of its resonance contribution from this position.

Chromatograms and decomposition progress curve for coumarin dimer **18**, with acetal unit at α-C-atom, are presented in Fig. 4. There is a surprising inversion of the pH-dependent rate for compound **18** as compared to **16**. Faster decomposition is observed for the former in acidic, than in neutral and basic medium. This can be explained by the acid-promoted opening



Scheme 3. Prototropic equilibrium proceeds retro-Michael reaction in compounds of group **III**.

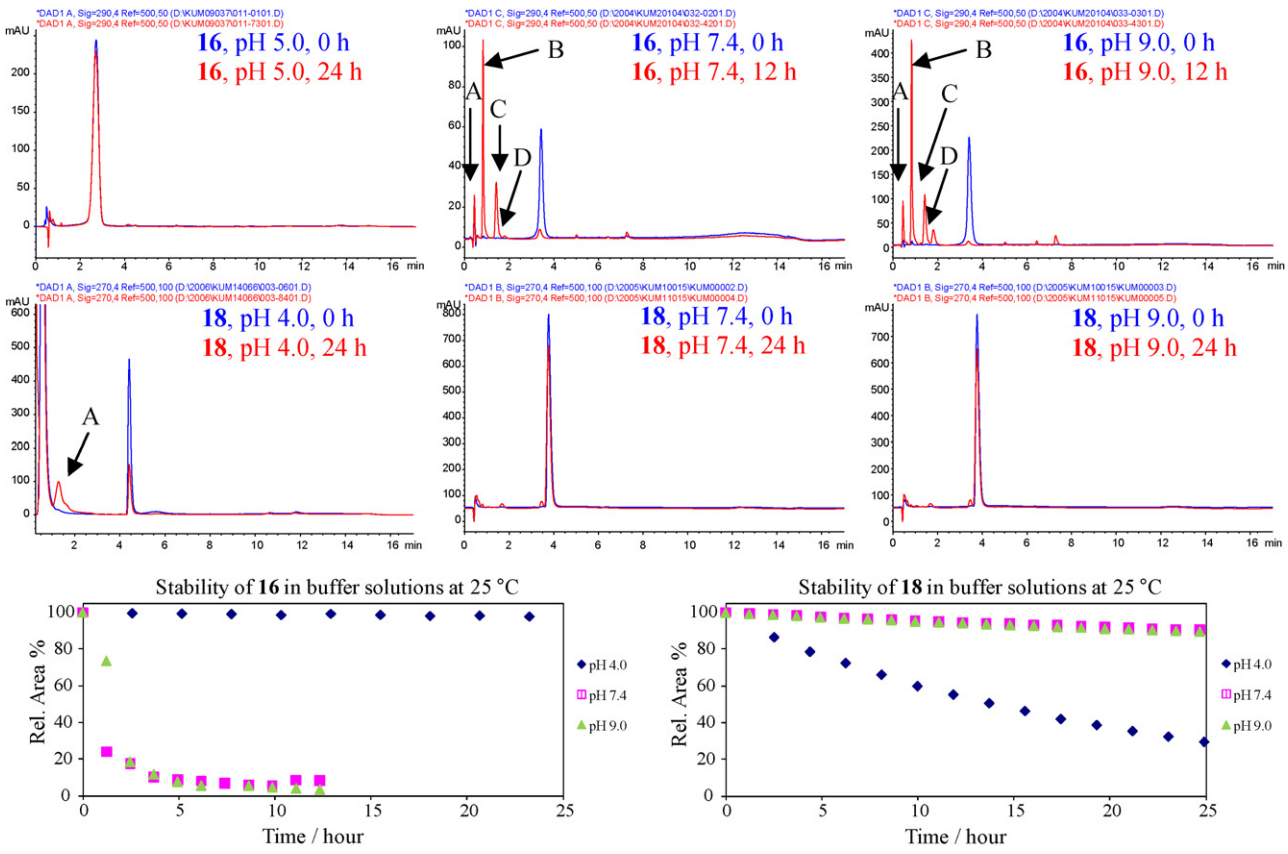
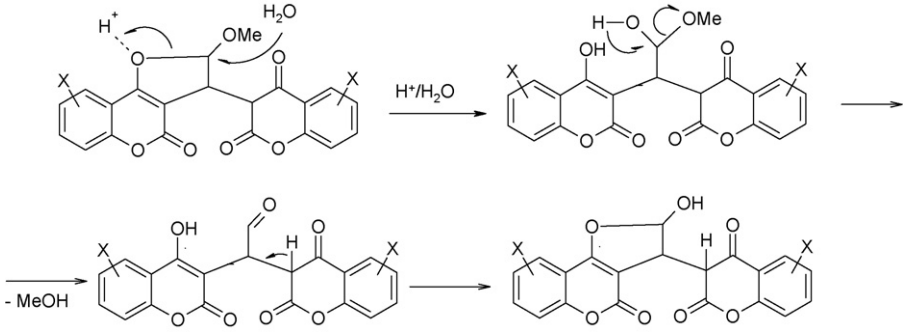


Fig. 4. HPLC-DAD chromatograms after 0 and 24 h, and progress curve for decomposition of **16** in solution at pH 5.0, 7.4 and 9.0 and of **18** in solution at pH 4.0, 7.4 and 9.0.



Scheme 4. Protonation and opening of cyclic acetals **18**–**21**.

of cyclic acetal followed by formation of the open-chain aldehyde, in equilibrium with cyclic hemiacetal form, as presented in Scheme 4. In basic medium, the retro-Michael reaction is largely suppressed in all compounds from this group, with the exception of compound **21** where ca. 25% of retro-adduct was observed.

Coumarin dimers in group **IV** (**22**–**25**) and **V** (**26**–**29**) are characterized by the ketone and carboxylic group, respectively, on the

Table 4
Pseudo-first-order kinetics and half-lives for the decomposition of the selected representatives of coumarin dimers **III**.

Compound	pH 4.0 (or pH 5.0)		pH 7.4		pH 9.0	
	k (h^{-1})	$t_{1/2}$ (h)	k (h^{-1})	$t_{1/2}$ (h)	k (h^{-1})	$t_{1/2}$ (h)
14	ND	ND	6.60×10^{-2}	10.5	3.46×10^{-2}	20.0
15	ND	ND	3.10×10^{-2}	22.4	1.10×10^{-1}	6.3
16	ND	ND	9.20×10^{-1}	0.8	4.57×10^{-1}	1.5
17	ND	ND	3.50×10^{-1}	2.0	2.98×10^{-1}	2.3
18	4.96×10^{-2}	14.0	ND	ND	ND	ND
19	3.81×10^{-2}	18.2	ND	ND	ND	ND
20	ND	ND	ND	ND	ND	ND
21	2.72×10^{-2}	25.4	1.16×10^{-2}	59.5	1.17×10^{-2}	59.4

ND = not determined.

α-carbon to the bridge. Both groups stabilize the dimeric structures toward hydrolytic retro-Michael reaction nearly completely at all three selected pH values. Very slow decomposition was observed only for compounds **22**–**24** at pH 7.4 and 9.0. Good stability of compounds from groups **IV** and **V** in comparison to compounds from groups **I**–**III** can be explained by destabilization of the carbocationic center on either of the two C-atoms on the bridging C–C bond(s). On the contrary, both 1,4-related carbonyl groups in **IV** and **V** stabilize the carbanion on the vicinal C-atom. This is known "mismatch" relation of the two electron withdrawing groups (EWGs) which makes formation of the central C–C bond energetically unfavorable process [36–38].

3.2. Detection and structure determination of degradation products

A tentative assignment of the degradation products was made using the quasi-molecular ion $[M+H]^+$ for compounds **14**, **17**, **22** and **26** and $[M-H]^-$ ion for all other compounds obtained from HPLC–MS experiments.

HPLC analysis at pH 7.4 and 9.0 revealed that compounds from group **I** produce two degradation peaks whose mass spectra, Fig. 5a and b correspond to retro-Michael reaction products. In addition, HPLC–MS chromatograms of compound **1** show an additional peak, Fig. 5c with the same molecular ion as compound analysed.

The most interesting structural aspect of coumarin dimers is the presence of more tautomers in equilibrium. For the compounds from the groups **I**–**V** three tautomers A–C, and two isomers according to the position of the C=C bond, A and D, are conceivable (Scheme 5).

While tautomeric equilibria A–C include migration of the proton from O to C atom, isomer D results from a 1,3-proton shift between two C-atoms. All these species characterize a high degree of conjugation of C=O and C=C bonds. As discussed with Scheme 2, form C can participate in retro-Michael reaction of the compounds from group **I**, whereas in the polycyclic structures **II** and **III** tautomer B is locked and therefore stable. Isomers D are stable species for the group **I** but can be expected as fluctuating structures in the compounds from groups **IV** and **V** due to high C–H acidity of the bridging C-atom in tautomer A, flanked by the three unsaturated bonds. Compounds from these two groups are therefore stabilized against retro-Michael reaction by high double-bond character of the bonds toward the bridging carbon.

It is known that tautomeric equilibria are fast processes, but the final ratio of tautomers is sometimes reached over a considerable time and under forced conditions. An illustrative example is 1,4-dihydroxynaphthalene which isomerizes into naphthalene 1,4-dione in benzene at 150 °C (in a sealed tube), with first-order kinetics, and equilibrium was reached after 3 days (1:1 ratio) [39]. In trifluoroacetic acid the equilibrium is reached after 30 min at room temperature and ratio of *ca.* 1:9 is retained. The dione is kinetically stable at room temperature but rapidly converts into the phenolic

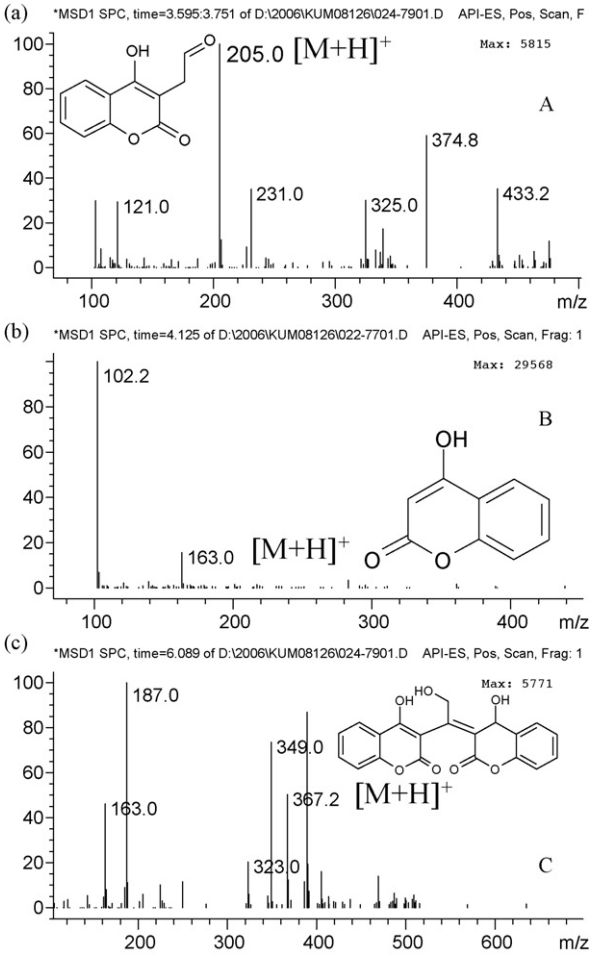
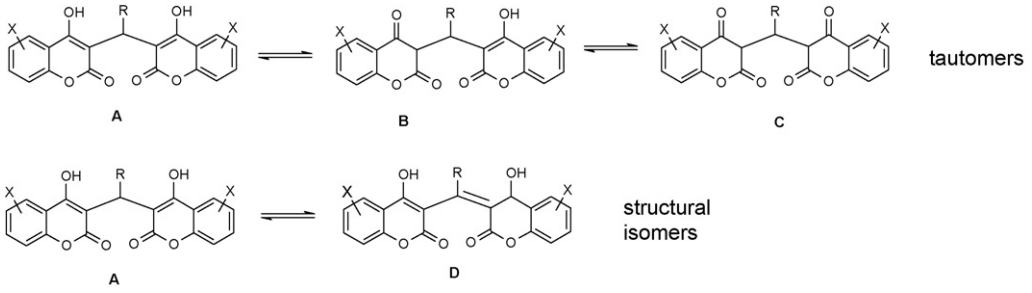


Fig. 5. Proposed structures and mass spectra of degradants A (a), B (b) and isomer C (c) of compound **1**.

form in the presence of a base. Coumarins, hydroxyl coumarins and other derivatives are present in various tautomeric forms [40,41], and in some cases large difference in the stability of tautomers are observed [42].

Data from the literature support our observation that most compounds from group **I**, beside retro-Michael reaction, approach equilibrium where the kinetically stable isomer D accumulates. This is exemplified by compounds **1**, **4**, **5** and **7** in progress curves shown in Figs. 1 and 2, and by mass spectra of degradants A (a), B (b) and isomer C (c) of compound **1** in Fig. 5. Progress curves asymptotically approach certain values of the starting compounds, whereas mass spectra reveal formation of a new peak with the same M^+ value, beside retro-Michael products, for which the structure of the isomeric form **D** is proposed.



Scheme 5. Conceivable tautomers and isomers for coumarin dimers **I**–**V**.

For compounds **14, 15** and **17** formation of products with 2 Da less than the parent compounds was observed under neutral and basic conditions, which is a result of dehydrogenation (Fig. 7c). The structure of these products is not assigned with confidence, and two isomers, E and F, can be assumed (Fig. 6).

HPLC-MS chromatograms after 24 h for compound **17**, from group **III**, show two new peaks, observed under basic conditions. Plausible structures of degradants A (a) and B (b) in Fig. 7 are the results of retro-Michael reaction, as outlined in Scheme 4.

Mass spectra from coumarin dimer **21**, presented in Fig. 8a indicate that degradant result from the open aldehyde form.

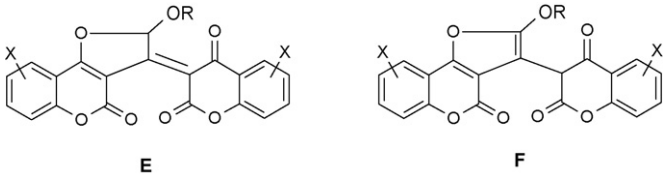


Fig. 6. Tentative structures of the dehydrogenation products from the compounds in the group **III**.

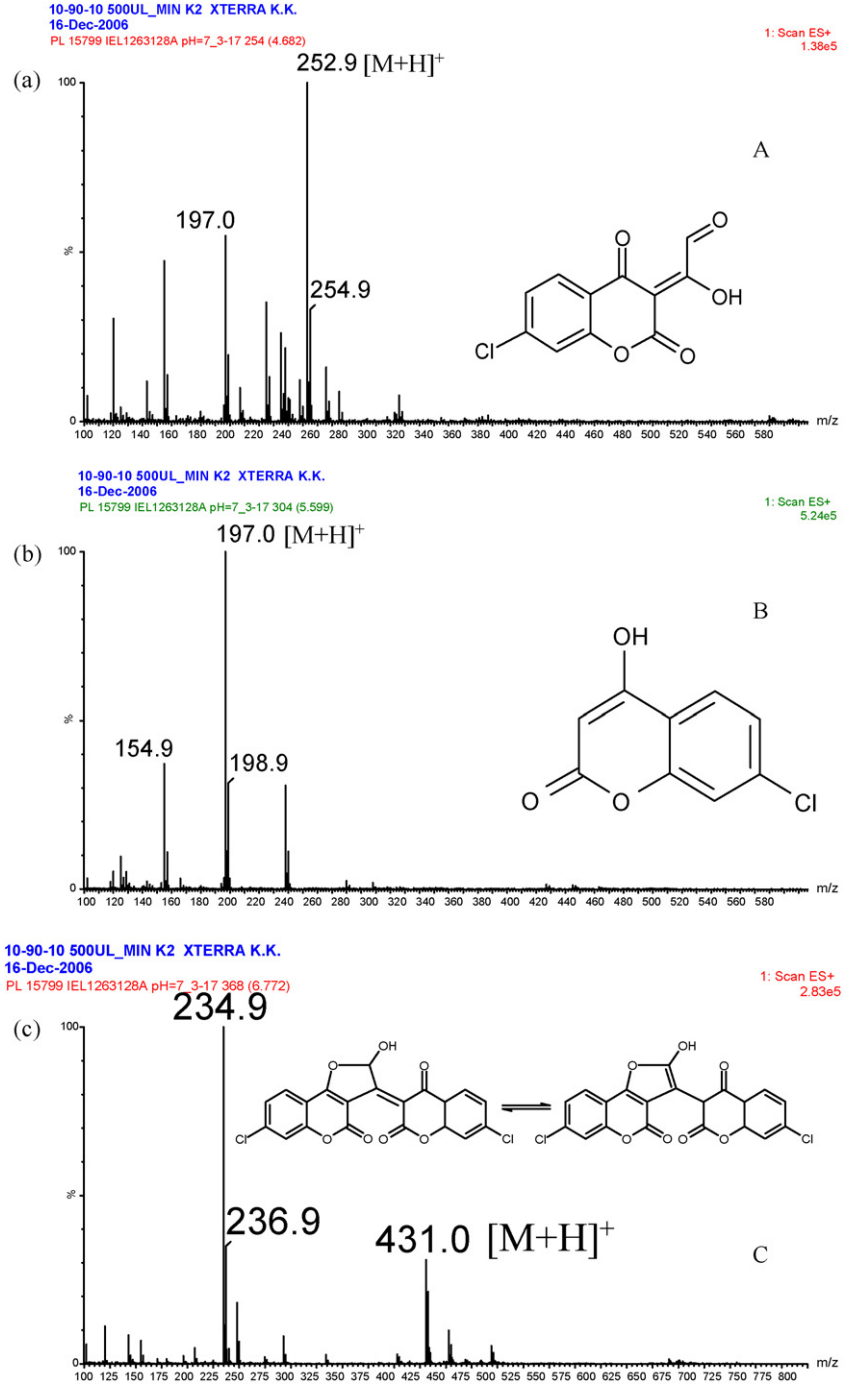


Fig. 7. Proposed structures and mass spectra of degradants A (a), B (b) and C (c) of compound **17**.

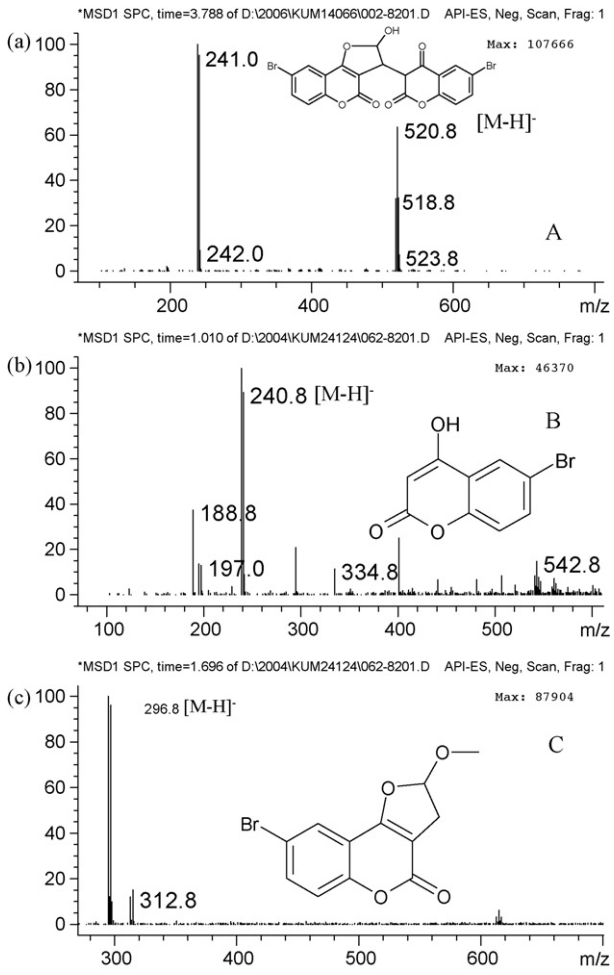


Fig. 8. Proposed structures and mass spectra of degradants A (a), B (b) and C (c) of compound **21**.

3.3. *In vitro* metabolic stability of selected coumarin dimers

In order to gain a better understanding of the relationship between the chemical structure and biological fate of tested coumarins, as well as their potential to progress into further *in vivo* pharmacokinetic studies, the hepatic metabolic stability was investigated. Representative compounds from groups **I**, **IV** and **V** were selected for metabolic stability assessment in mouse liver microsomes and their respective *in vitro* intrinsic clearances are summarized in Table 5.

Generally, all coumarin dimers tested have a low intrinsic clearance in mouse liver microsomes (CLi < 0.6 ml/min/g liver), suggesting a potential for low clearance *in vivo*. Compound **24**, which showed a moderate intrinsic clearance of 2.56 ml/min/g, was the least stable. When comparing corresponding analogs within these groups, some differences in microsomal stability are observed. Namely, the depletion of compound **1**, containing the primary hydroxyl group, was more rapid in comparison to compound **5**, containing the secondary hydroxyl group. Within groups **IV** and **V**, isopropyl substituents resulted in a lower *in vitro* stability in comparison to their corresponding analogs, likely due to their increased lipophilicity.

Compound **24**, from group **IV**, resulted in the lowest *in vitro* stability with 80% of parent compound being eliminated after a 60 min of incubation and was screened for potential *in vitro* oxidative metabolites. In order to detect phase I metabolites, preset transitions were monitored during this HPLC–MS/MS analysis. Qualitative analysis of microsomal incubations of compound **24**, indicates oxidative metabolism (M+16, M+32, M−2) with different retention times (presented in Fig. 9). Although the exact position of the hydroxyl group could not be discerned from the MS data, hydroxylation of the isopropyl moiety, as observed in [43], is likely.

Table 5
In vitro hepatic microsomal intrinsic clearances.

Group	Compound	CLi (ml/min/g)
I	**1**	0.41
	5	0.1
IV	**22**	0.34
	24	2.56
V	**26**	0.01
	27	0.36

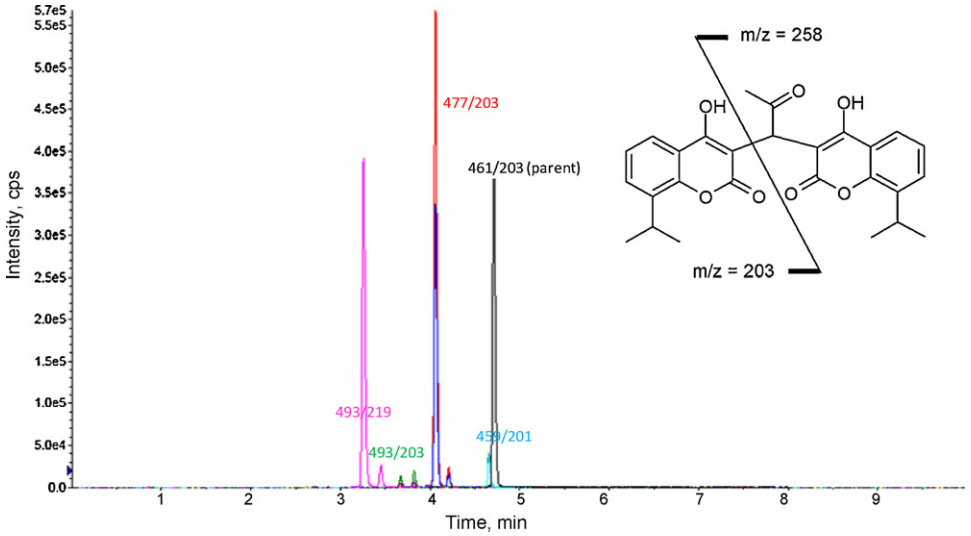


Fig. 9. Chromatogram of 1 μM compound **24** incubation with mouse liver microsomes (0.5 mg/ml) for 60 min.

4. Conclusions

Coumarin dimers **1**–**29**, classified into groups **I**–**V** according to the functionality on the α-atom to the bridging carbon, exhibited pH-dependent aqueous stability. The principal hydrolytic, de-dimerization step includes the retro-Michael reaction.

For the compounds in group **I**, with *primary* or *secondary* OH groups on the α-carbon, decompositions are accompanied by formation of isomer D. Compounds with an annelated dihydrofuran, group **II**, proved stable toward decomposition and oxidation.

For the hemiacetals and acetals in group **III** ring opening was observed under acidic conditions and oxidation (dehydrogenation) under neutral and basic conditions, leading to tentative isomeric structures **E** and **F**.

Compounds in groups **IV** and **V**, with EWGs at the α-carbon proved stable at all pH values due to a "mismatch" effect of 1,4-dicarbonylic system. This precludes a heterolytic split of the central C–C bond and enables possible formations of the stable structural isomer D.

Minor effects of substituents on the decomposition rate can be explained by stabilization or destabilization of partial positive charge on the bridging C-atom.

Overall, the coumarin derivatives tested show a good *in vitro* stability in mouse liver microsomes, with the exception of compound **24**, and are suitable for further screening *in vivo*.

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