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Novel lipophilic 7*H*-pyrido[1,2,3-*de*]-1,4-benzoxazine-6-carboxylic acid derivatives as potential antitumor agents: Improved synthesis and in vitro evaluation

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ABSTRACT

A convenient route for the synthesis of some acyloxymethyl esters and carboxamides of levofloxacin (LV) with modulated lipophilicity is described. The synthesized compounds were evaluated in vitro for their growth inhibitory effect in five human cancer cell lines. The most efficient LV derivatives (ester **2e** and amide **4d**) displayed IC_{50} values in the 0.2–2.2 μM range, while IC_{50} values for parent LV ranged between 70 and 622 μM depending on the cell line. The esters displayed no in vivo toxicity up to 80 mg/kg when administered intraperitoneally. This study thus shows that LV analogs displayed antitumor efficacy, at least in vitro, a feature that appeared to be independent from the lipophilicity of the grafted substituent.

1. Introduction

Fluoroquinolones (FQs) represent an important family of antibacterial agents that also display antiproliferative activity in some tumor cell types such as breast cancer cells,[1] bladder transitional cell carcinoma,[2–4] non-small cell lung carcinoma,[5] prostate carcinoma cells,[6,7] and colorectal carcinoma cells.[8] It has been demonstrated that ciprofloxacin displays antiproliferative and apoptosis-inducing activity both on prostate and bladder cancer cells.[9a,b] In recent years, 4-quinolone derivatives displaying 'non-classical' antitumor activity have been described.[10–12] In this context, the synthesis and cytotoxicity evaluation of 7-((4-substituted)piperazin-1-yl)quinolones (ciprofloxacin, norfloxacin and enoxacin) has been reported by Foroumadi et al.[13] and by our research group (ciprofloxacin).[14] The lead derivatives (Fig. 1) displayed in vitro antitumor activity with IC_{50} growth inhibitory values $\leqslant 5$ μM in various cancer cell lines.

In addition, various acyloxyalkyl ester prodrugs of butyric acid with anticancer activity were described in the literature.[15] The two lead anticancer prodrugs in this family are pivaloyloxymethyl butyrate[16] (Pivanex, AN-9, Fig. 1) and butyryloxymethyl-diethylphosphate[17] (AN-7, Fig. 1) that both release butyric acid together with formaldehyde upon hydrolytic degradation. AN-7 exhibited superior anticancer activity compared to AN-9 in various cancer models.[18] Formaldehyde-releasing prodrugs increase the level of reactive oxygen species (ROS) by forming *S*-formylglutathione adducts, thus diminishing the concentration of the ROS scavenger glutathione and thereby triggering signals for differentiation and death of cancer cells.[19]

The aim of the present study was therefore to potentiate the antitumor activity of a third generation FQ, levofloxacin (LV; (3*S*)-9-fluoro-2,3-dihydro-3-methyl-10-(4-methyl-1-piperazinyl)-7-oxo-7*H*-pyrido[1,2,3-*de*]-1,4-benzoxazine-6-carboxylic acid; **1**; Fig. 1). The in vitro IC_{50} growth inhibitory values found in various human cancer cell lines for lipophilic ciprofloxacin derivatives in our previous work[14] prompted us to investigate if the presence of a lipophilic moiety positioned at the C-6 of the 7*H*-pyrido[1,2,3-*de*]-1,4-benzoxazine skeleton of LV could also give rise

Abbreviations: FQ, fluoroquinolone; ROS, reactive oxygen species; LV, levofloxacin; MW, microwave; NA, nalidixic acid; TPP, triphenylphosphine; TCA, trichloroacetonitrile; TBAI, tetrabutylammonium iodide; DMPU, *N,N'*-dimethylpropyleneurea; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MTD, maximum tolerated dose; SRB, sulforhodamine B.

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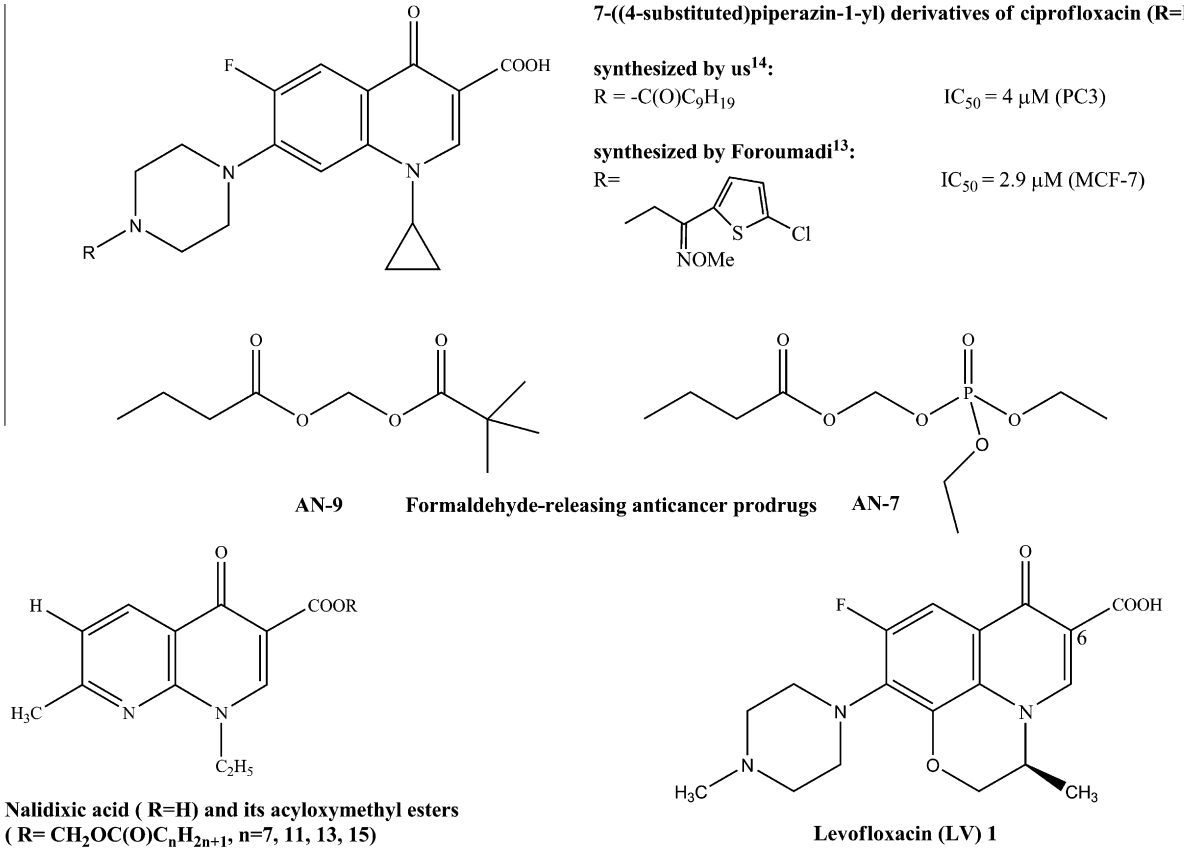


Figure 1. Structures of nalidixic acid and its acyloxymethylesters, 7-((4-substituted)piperazin-1-yl) derivatives of ciprofloxacin, levofloxacin, and anticancer acyloxymethyesters AN-7 and AN-9.

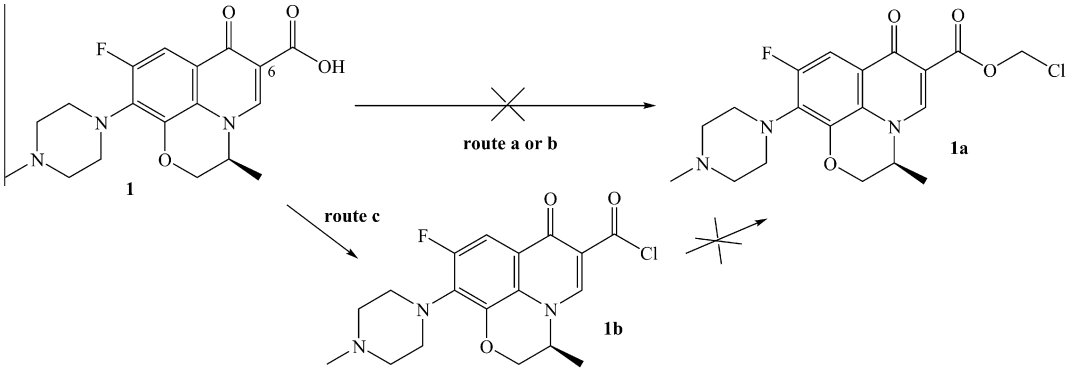
to antitumor compounds. In order to examine the contribution of formaldehyde to the anticancer activity, an efficient method for the synthesis of acyloxymethyl esters of LV is reported. The reactions were studied under microwave (MW) irradiation or by classical heating. Since the amide bond generally exhibits greater stability than ester linkages, we also synthesized an analogous series of amide derivatives of LV. The lipophilicity of all the compounds was calculated and their stability at pH 7.4 was determined. Their in vitro growth inhibitory activity was tested on a panel of five human cancer cell lines.

2. Results and discussion

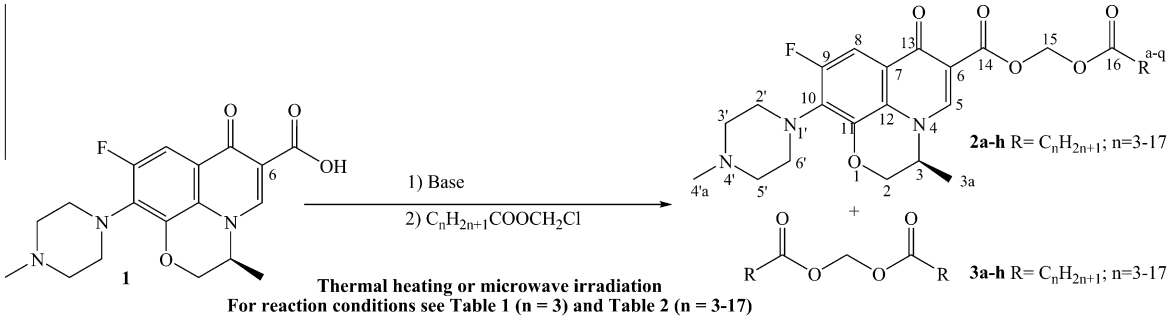
2.1. Chemistry

In a previous study, we have reported the synthesis of acyloxymethyl esters of nalidixic acid (NA),[20] the prototype quinolone (Fig. 1). We showed that the alkylation of NA potassium salt with 1 equiv of long chain alkanoyloxymethylchlorides gave the corresponding double esters in poor yields (11–39%). Acyloxymethyl esters of NA were thus conveniently synthesized by condensation of commercially available carboxylic acids with chloromethyl ester of NA obtained by chloromethylation with chloromethyl chorosulfate in phase transfer conditions.[20] This strategy was therefore applied to LV **1** (Scheme 1, route a). Unfortunately, the LV halomethylester **1a** could not be isolated by using the two main protocols (Scheme 1, routes a and b), described in the literature.[21,22] In good agreement with the true partition coefficient ('intrinsic lipophilicity' log *P*) described in literature,[23] we observed that LV (log *P* = 0.35) is more hydrophilic than NA (log *P* = 1.46), that could be a disadvantage for the phase transfer. Further experiments were carried out to evaluate the condensation of 1,3,5-trioxane with the acid chloride derived from LV in the presence of a Lewis acid (Scheme 1, route b). All the attempts by using oxalyl chloride (with or without catalytic amount of DMF) in anhydrous CH_2Cl_2 were unsuccessful. The formation of a dark-coloured reaction mixture and side-products were observed in all cases, suggesting a degradation of the quinolone substrate. An alternative method developed by Jang et al.[24] was then evaluated. For the first time we found that LV acyl chloride **1b** could be obtained under very mild conditions by simply using a combination of triphenylphosphine (TPP) and trichloroacetonitrile (TCA) in CH_2Cl_2 at room temperature (Scheme 1, route c). However, the subsequent conversion to the chloromethylester **1a** by using 1,3,5-trioxane and a Lewis acid ($ZnCl_2$, $AlCl_3$) failed whatever the solvent used.

Thus, an alternative strategy involving the alkylation of the drug carboxylate with various long-chain alkylcarbonyloxymethyl chlorides was evaluated for LV **1** (Scheme 2). The starting materials were prepared from recently developed in-house procedures.[20] The optimization of the reaction conditions was investigated with the commercially available propylcarbonyloxymethyl chloride as model compound. Several experimental conditions, including those described by Maeda et al.[25] (Table 1, entry 1), were evaluated: DMF or CH_3CN as solvent, temperature range from rt to 80 °C, reaction time (17 h, 4–7 days), with or without catalyst. Increasing both the temperature and the reaction time led generally to an improved yield of ester (Table 1, entries 1–6). The presence of a catalyst was of particular interest since the optimal conditions were refluxing LV potassium salt in acetonitrile with 1.2 equiv of propylcarbonyloxymethyl chloride and 0.1 equiv of tetrabutylammonium iodide (TBAI). This sequence (Table 1, entry 7) yielded 48% of the desired double ester **2a**; however, a long reaction time (7 days) was required.



Scheme 1. Attempted synthetic routes to levofloxacin halomethylester **1a**. Reagents and conditions: Route a: (1) base (Na_2CO_3 or NaOH), (nBu_4)$NHSO_4$ cat.; (2) $ClCH_2OSO_2Cl$, solvent (CH_2Cl_2 or $CH_3COOC_2H_5$)/H_2O. Route b: (1) ClCOCOCl with or without DMF cat.; (2) $ZnCl_2$ + $CaCl_2$; (3) 1,3,5-trioxane, anhydrous CH_2Cl_2. Route c: (1) TPP (2.0 equiv), TCA (2.0 equiv), CH_2Cl_2, 0 °C to rt, 4 h.



Scheme 2. Synthetic route to the target alkylcarbonyloxymethyl esters **2a**–**h**.

Table 1
Optimisation runs for the synthesis of propylcarbonyloxymethyl ester **2a**

Entry	Base	Solvent	Catalyst (relative amounts catalyst/levofloxacin)	Thermal heating[a] (Δ)			Microwave method[b] (MW)		
				Temperature	Reaction time	Yield of **2a**[c] (%)	Temperature (°C)	Reaction time (min)	Yield of **2a**[c] (%)
1[d]	K_2CO_3	DMF		50 °C	17 h	Traces			
2	K_2CO_3	DMF		rt	4 days	23			
3	K_2CO_3	DMF		50 °C	7 days	28			
4	K_2CO_3	CH_3CN		rt	4 days	15			
5	K_2CO_3	CH_3CN		50 °C	7 days	36			
6	K_2CO_3	CH_3CN		Reflux	7 days	25			
7	K_2CO_3	CH_3CN	TBAI (0.1)	Reflux	7 days	**48**			
8	K_2CO_3	DMF	TBAI (0.2)				120	60	46
9	K_2CO_3	DMF	TBAI (0.2)				120	90	47
10	K_2CO_3	DMF	TBAI (0.2)				120	120	20
11	K_2CO_3	DMF	TBAI (0)				120	60	8
12	K_2CO_3	DMF	TBAI (1)				120	60	7
13	K_2CO_3	DMPU	TBAI (0.2)				120	60	16
14	K_2CO_3	CH_3CN	TBAI (0.2)				90	60	**56**
15	K_2CO_3	CH_3CN	TBAI (0.3)				90	60	52
16	K_2CO_3	CH_3CN	TBAI (0.1)				90	60	46
17	K_2CO_3	CH_3CN	TBAI (0.2)				90	90	32
18	Cs_2CO_3	CH_3CN	TBAI (0.2)				90	60	45
19	Cs_2CO_3	DMF	TBAI (0.2)				120	60	37

Bold values represent the best results in each experimental conditions.
[a] Classical heating conditions (Δ): (1) LV 3.03 mmol, K_2CO_3 (2 equiv), solvent (10 mL) reflux, 8 h. (2) $C_3H_7COOCH_2Cl$ (1.2 equiv) with or without TBAI.
[b] Microwave conditions (MW): (1) LV 0.276 mmol, base (2 equiv), solvent (2.5 mL), 20 min. (2) $C_3H_7COOCH_2Cl$ (1.5 equiv), TBAI.
[c] Isolated yields of analytically pure compound after purification with chromatography on silica gel.
[d] Experimental conditions described by Maeda et al.[25]

These conditions were then applied to long-chain alkylcarbonyloxymethyl chlorides bearing 11–17 methylene units. In all cases, the desired double esters **2e–h** were isolated in very poor yields (⩽10%) partially due to progressive decomposition of the alkylcarbonyloxymethyl chlorides during the long reaction time, and subsequent nucleophilic substitution leading to the formation of the corresponding diesters **3e–h** (Scheme 2), which were isolated in 14–35% yields.

To overcome these drawbacks, we decided to explore the use of MW energy.[26–28] Several optimisation runs were first carried out with propylcarbonyloxymethyl chloride within a single mode cavity synthesizer.[29] In an effort to ascertain the reaction parameters

governing the nucleophilic substitution, the reactive potassium salt was always prepared under MW in a discrete step using 20 min irradiation time. Three experiments were conducted to assess the effect of irradiation time on the substitution (Table 1, entries 8–10) and showed that increasing the reaction time from 60 to 120 min led to a loss of yield of the expected **2a**. In another set of experiments, we investigated the effect of the relative amounts of catalyst and LV on the isolated yields while keeping constant the irradiation time (60 min) (Table 1, entries 8, 11 and 12). The yield was significantly dependent on the amount of catalyst used. Three solvents were compared: DMF, *N*,*N*′-dimethylpropyleneurea (DMPU) and acetonitrile (Table 1, entries 8, 13 and 14). Since esterifications with chloroalkylesters were described as more effective when the reaction was conducted in DMPU,[30] we were surprised to find that the yield dropped to 16% when using this solvent. Finally, the yield was improved by using acetonitrile (Table 1, entries 14–17). Generally, when K_2CO_3 is replaced by Cs_2CO_3 the change in cation leads to an improved yield; however we did not observe such data (Table 1, entries 18 and 19). This preliminary study demonstrated that the best yield (56%) was obtained in acetonitrile with a molar ratio LV/propylcarbonyloxymethyl chloride/TBAI of 1:1.5:0.2 and 60 min of MW irradiation (Table 1, entry 14).

The application of these MW conditions to octadecanoyloxymethyl chloride (n = 17) gave ester **2h** with a slightly improved isolated yield (23% vs 5% in classical conditions) and very little formation of the corresponding diester **3h** (8% vs 28% in classical conditions). However, it should be pointed out that this MW procedure, when applied to a series of long chain derivatives (bearing 11–17 carbon atoms), was always plagued with difficulties during purification. Generally, several (2–3) silica gel columns (with different solvent systems) were necessary to isolate the lipophilic esters in pure form, without any residual trace of TBAI. Low isolated yields were thus obtained (10–23% depending on the n value).

To circumvent this problem, the reaction with octadecanoyloxymethyl chloride was further investigated using oil-bath heating and without TBAI. A mixture of LV and K_2CO_3 or Cs_2CO_3 (1 equiv) was heated in acetonitrile or DMF at 90 °C for 20 min. After adding 1 or 1.5 equiv of octadecanoyloxymethyl chloride, the reaction progress was checked every hour with LC-UV. Surprisingly, in contrast to the results obtained by Maeda et al.[25], the reaction proceeded rapidly. The best results (59–81% isolated yields of esters **2a–h**) were achieved when a mixture of LV and K_2CO_3 (1 equiv) was heated in DMF at 90 °C for 20 min and then 1 h more after addition of the various alkylcarbonyloxymethyl chlorides (1 equiv) (Table 2).

The potential instability of ester derivatives due to hydrolysis led us to synthesize amide derivatives of LV. The TCA/TPP system[24,31] was used for acylation (Scheme 1, route c). As outlined in Scheme 3, the treatment of LV **1** with TPP (2 equiv) and TCA (2 equiv) in CH_2Cl_2 at rt for 4 h, followed by elimination of triphenylphosphine oxide and reaction with amines containing 4–15 carbon linear chain lengths (1 equiv) in the presence of triethylamine (3 equiv) in CH_2Cl_2 (18 h, rt) gave the corresponding amides **4a–h**. The isolated yields were moderate, in the range 34–40% (Table 3).

The stability of eight LV derivatives of different lipophilicity (esters **2a**, **2c**, **2e**, **2g** and amides **4a**, **4b**, **4d**, **4g**) was determined by HPLC. As shown in Table 4, all these compounds were quite stable.

Table 2
Improved synthesis of alkylcarbonyloxymethyl esters **2a–h**

R = C_nH_{2n+1}		Yield[a] (%)
a	C_3H_7	59
b	C_5H_{11}	69
c	C_7H_{15}	75
d	C_9H_{19}	79
e	$C_{11}H_{23}$	79
f	$C_{13}H_{27}$	77
g	$C_{15}H_{31}$	79
h	$C_{17}H_{35}$	81

[a] Classical heating conditions (Δ): (1) LV 0.5 mmol, K_2CO_3 (1 equiv), DMF (20 mL), 90 °C, 20 min. (2) $C_nH_{2n+1}COOCH_2Cl$ (1 equiv), 90 °C, 1 h.

2.2. Pharmacology

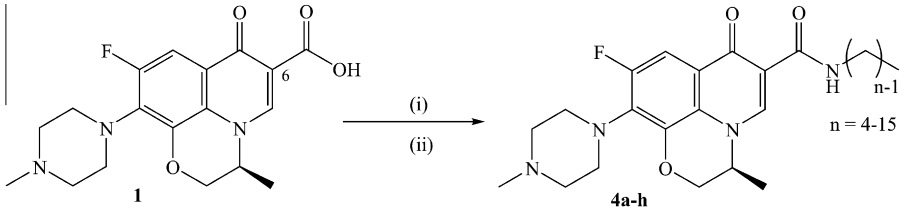
The newly synthesized LV derivatives were evaluated for their in vitro inhibition of human cancer cell line growth by means of the MTT colorimetric assay. Each of the seventeen compounds under study (including LV) were tested on two human apoptosis-resistant cancer cell lines (glioblastoma (U373-MG)[32a–c] and NSCLC (A549)[32d]) and three apoptosis-sensitive ones (prostate (PC-3),[32e] colorectal (LoVo)[32f] and breast (MCF-7)[32e]). The in vivo tolerance of compounds was also determined as the maximum tolerated dose (MTD) index.[33]

Although the ester derivatives of increasing lipophilicity (*C* log *P* ranging from 1.67 to 8.02) showed different antiproliferative effects depending on the substituent grafted on the carboxylic acid, there was no clear-cut relationship between in vitro antitumor activity and lipophilicity. Among the amide derivatives of increasing lipophilicity (ClogP ranging from 2.13 to 7.95), only the less lipophilic compound **4a** displayed IC_{50} values >10 μM; the introduction of a group with higher lipophilicity led to derivatives **4b–h** that exhibited IC_{50} values in the micromolar range. Amide **4d** (C_8H_{17}–N(H)– = R) with IC_{50} values 30- to 150-fold lower than that of **1**, in PC-3 and A549 NSCLC cell lines, turned out to be the most potent derivative in our series.

Only three esters (**2b**, **2c**, **2d**) possessed poor activity (IC_{50} >100 μM) against all cell lines (Table 4). As far as the A549 lung cell line is concerned, the esters (with the exception of **2a**) generally displayed poor growth inhibition or an effect comparable to that of parent LV; the amides were more potent. On the other hand, in the prostate, glioblastoma, colon and breast carcinoma cell lines, the LV derivatives showed in vitro antitumor activity generally greater than the parent FQ. Ester **2e** ($C_{11}H_{23}$–C(O)OCH_2O– = R) was the most active compound against U373-MG, LoVo and MCF-7 cell lines, with IC_{50} values below the micromolar range, that is, 220- to 2000-fold lower than that of **1**. At the moment we have no explanation about why compounds **2b**, **2c** and **2d** showed poor activity against all cell lines, whilst compounds **2a** and **2e** are significantly more active. Interestingly, even though esters analogs showed more potent in vitro growth inhibitory activity than LV on all studied cancer cell lines, their systemic in vivo toxicity was not simultaneously increased, because they displayed MTD indices >80 mg/kg.

In a separate set of experiments, we have compared the in vitro growth inhibitory effects of levofloxacin versus racemic ofloxacin and we have observed no major differences in the growth inhibitory activity induced by the two compounds on the five cancer cell lines under study (data not shown).

A difference in selectivity was observed between ester and amide series. For example, esters **2e–h** displayed a growth inhibitory effect against both the apoptosis-resistant U373-MG and apoptosis-sensitive LoVo and MCF-7 cancer cell lines, whereas they were not active against both apoptosis-sensitive PC-3 and apoptosis-resistant A549 cell lines. In contrast, amides **4b–h** produced similar effects irrespective of the cell lines. As shown in Figure 2, the various cell lines showed an heterogeneous profile of sensitivity to the lead ester **2e** in relation to apoptosis sensitivity versus resistance, while they all displayed similar sensitivity to the lead amide **4d**. In terms of antitumor activity, these data suggest that the amide derivatives are able to overcome the natural resistance of certain cancer cell types to apoptosis. The active derivatives



Scheme 3. Synthesis of *N*-alkylcarboxamides **4a–h**. Reagents and conditions: (i) TPP (2 equiv), TCA (2 equiv), CH_2Cl_2, 0 °C to rt, 4 h.; (ii) C_nH_{2n+1}–NH_2 (n = 4–15; 1 equiv), Et_3N (3 equiv), CH_2Cl_2, rt, 18 h.

Table 3
Synthesis of *N*-alkylcarboxamides **4a–h**

$RNH_2R = C_nH_{2n+1}$		Yield[a] (%)
a	C_4H_9	34
b	C_6H_{13}	37
c	C_7H_{15}	36
d	C_8H_{17}	36
e	$C_{10}H_{21}$	35
f	$C_{12}H_{25}$	37
g	$C_{14}H_{29}$	39
h	$C_{15}H_{31}$	40

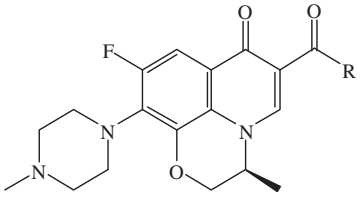
[a] Reagents and conditions: (i) LV 1 mmol, TPP (2 equiv), TCA (2 equiv) CH_2Cl_2, 0 °C to rt, 4 h; (ii) C_nH_{2n+1}–NH_2 (n = 4–15; 1 equiv), Et_3N (3 equiv), CH_2Cl_2 , rt, 18 h.

under study could exert their antitumor activity through activation of apoptotic or non-apoptotic cell death processes.

Glioblastomas are one of the most devastating forms of cancer arising from dramatic migration, and a number of publications reported that drugs able to reduce the levels of migration in glioblastoma cells are also likely to restore a certain level of sensitivity to apoptosis in these restricted-migration cells which thus can then be combated using conventional cytotoxic (pro-apoptotic) drugs.[32b,34a,b] Thus, novel types of antimigratory drugs are needed and we have already shown that nontoxic 2-quinolone derivatives have an antimigratory effect in vitro on cancer cells and are a cause of additive in vivo benefits when combined with etoposide or adriamycin in the case of the MXT mouse mammary adenocarcinoma.[35a] We thus decided to use computer-assisted phase-contrast microscopy[35b,c] in an attempt to quantitatively determine the effect of **2e** (0.1 μM) and **2h** (1 μM) on cell motility levels in A549 NSCLC and U373-MG glioblastoma cell lines. The study was conducted on U373-MG where **2e** and **2h** are the most effective compounds and on A549 where they have no effect (Table 4). The two esters did not show any antimigratory effect on tumor cell line over six days of observation (data not shown).

Mondal et al.[5] and Yamakuchi et al.[36] evaluated the antiproliferative and apoptotic activities of LV on human NSCLC and transitional bladder carcinoma cells in culture respectively. These investigators demonstrated that LV caused cellular growth inhibition in a time-dependent manner. We also investigated the time course of the increase on growth inhibition of the stable amide

Table 4
Levofloxacin and synthesized derivatives: structure, lipophilicity, stability, and IC_{50} in various cancer cell lines



R-	Compound	*C* log *P*[a]	Stability[b] (%)	IC_{50}[c] (μM)				
				U373-MG	A549	PC-3	LoVo	MCF-7
HO–	**1** (LV)	−0.51	–	188 ± 13	70 ± 6	238 ± 12	67 ± 5	622 ± 19
C_3H_7–C(O)OCH_2O–	**2a**	1.67	>92	40 ± 3	3 ± 1	31 ± 4	5 ± 1	35 ± 3
C_5H_{11}–C(O)OCH_2O–	**2b**	2.73	nd	199 ± 22	235 ± 16	207 ± 12	162 ± 15	183 ± 12
C_7H_{15}–C(O)OCH_2O–	**2c**	3.79	>90	243 ± 15	289 ± 12	230 ± 14	209 ± 12	238 ± 13
C_9H_{19}–C(O)OCH_2O–	**2d**	4.84	nd	>1000	>1000	>1000	>1000	>1000
$C_{11}H_{23}$–C(O)OCH_2O–	**2e**	5.90	>91	0.2 ± 0.1	65 ± 5	86 ± 6	0.3 ± 0.1	0.3 ± 0.1
$C_{13}H_{27}$–C(O)OCH_2O–	**2f**	6.96	nd	8 ± 2	617 ± 15	71 ± 7	21 ± 3	6 ± 1
$C_{15}H_{31}$–C(O)OCH_2O–	**2g**	8.02	>93	4 ± 1	46 ± 4	56 ± 5	6 ± 1	8 ± 1
$C_{17}H_{35}$–C(O)OCH_2O–	**2h**	nd	nd	0.9 ± 0.2	593 ± 34	100 ± 6	4 ± 1	12 ± 2
C_4H_9–N(H)–	**4a**	2.13	>97	113 ± 18	46 ± 1	30 ± 2	24 ± 3	55 ± 4
C_6H_{13}–N(H)–	**4b**	3.19	100	4 ± 1	6 ± 1	12 ± 2	5 ± 1	16 ± 2
C_7H_{15}–N(H)–	**4c**	3.72	nd	3 ± 1	3 ± 1	4 ± 1	1 ± 1	5 ± 1
C_8H_{17}–N(H)–	**4d**	4.25	100	2.3 ± 0.1	2.2 ± 0.1	1.5 ± 0.2	0.8 ± 0.4	2.1 ± 0.2
$C_{10}H_{21}$–N(H)–	**4e**	5.31	nd	3 ± 1	3 ± 1	3 ± 1	3 ± 1	5 ± 1
$C_{12}H_{25}$–N(H)–	**4f**	6.36	nd	5 ± 1	5 ± 1	5 ± 1	3 ± 1	10 ± 1
$C_{14}H_{29}$–N(H)–	**4g**	7.42	>98	3 ± 1	5 ± 1	3 ± 1	3 ± 1	5 ± 1
$C_{15}H_{31}$–N(H)–	**4h**	7.95	nd	7 ± 1	8 ± 1	5 ± 1	6 ± 1	15 ± 2

[a] Calculated *C* log *P* values with the ChemDraw Ultra 10.0 program.
[b] Stability studies were carried out using HPLC analysis (by monitoring both the loss of starting material and, if any, the formation of **1**) after incubation of each compound in phosphate buffer (pH 7.4) at 37 °C for 7 days.
[c] IC_{50} values were determined using the MTT colorimetric assay on five human cancer cell lines (U373-MG: glioblastoma, A549: lung cancer, PC-3: prostate cancer, LoVo: colon cancer, and MCF-7: breast cancer) treated for 5 days. The data are presented as mean ± SEM values calculated on hexaplicates.

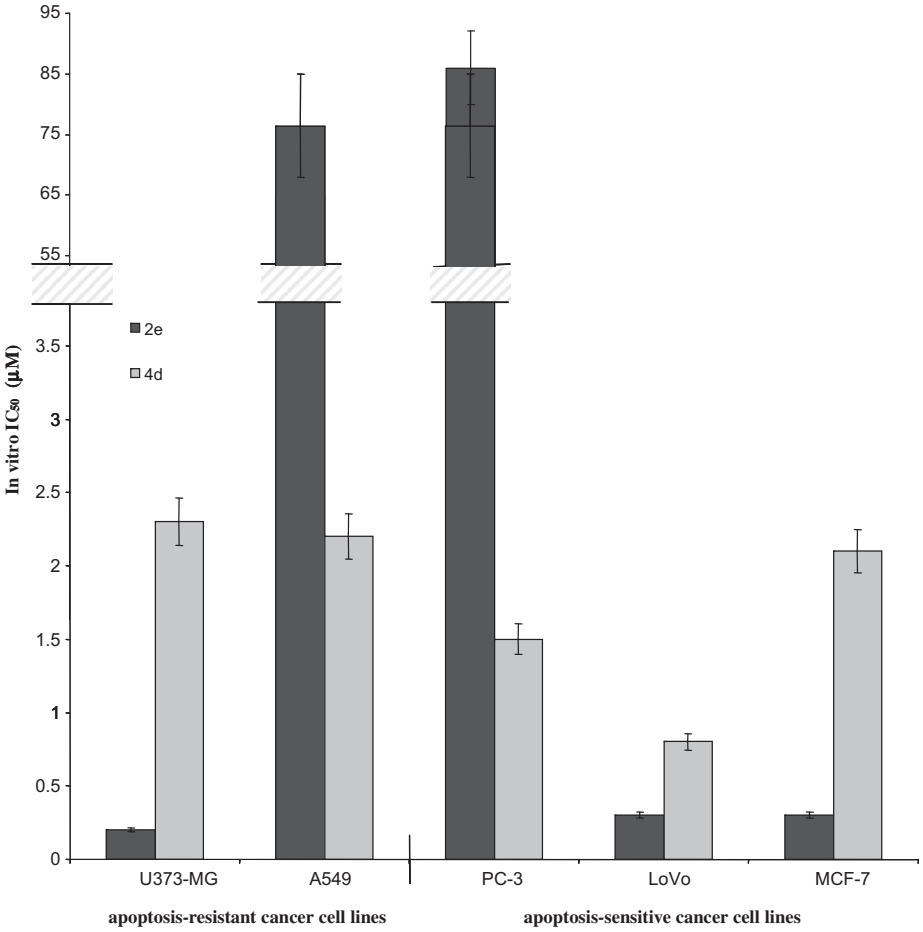


Figure 2. In vitro IC_{50} (±SEM) of compounds **2e** and **4d** with respect to apoptosis-resistant and sensitive cancer cell lines (using the colorimetric MTT assay) (PC-3: prostate cancer; U373-MG: glioblastoma; LoVo: colon cancer; A549: non-small-cell lung cancer; MCF-7: breast cancer).

derivatives that exhibited similar antitumor effect against all the cell lines under study. In agreement with previous reports,[5,36] we observed a time-dependent effect on IC_{50} growth inhibitory concentrations, on all tumor cell lines, when cancer cells were culture for 3 versus 5 days with compounds **1** and **4d**, these effects being particularly pronounced for compound **4d** (Fig. 3).

To our best knowledge, few reports in the literature described the synthesis of quinolones analogs by introducing new functionality at C-6 position of the 7*H*-pyrido[1,2,3-*de*]-1,4-benzoxazine skeleton.[37–42] You et al.[42] described the synthesis of C-6 substituted LV derivatives (compounds **A** and **B**; Table 5). They evaluated their in vitro growth inhibitory activity and showed that a moderate modification led to enhanced antitumor activities. The IC_{50} values for the LV derivative **B** bearing a nitrobenzothiazole group at the C-6 position were in the micromolar range[42] close to those of our lead compounds **2e** and **4d**. However, it must be kept in mind that the in vitro growth inhibitory effects observed with compounds currently used to treat cancer patients such as oxaliplatin, [33a] etoposide, [33a] temozolomide,[32b] lomustine[32b] and carmustine[32b] are associated with IC_{50} values >10 μM in vitro.

3. Conclusion

In summary, MW irradiation was successfully applied to the synthesis of butyryloxymethyl ester of LV with a short reaction time. The comparison of thermal and MW experimental conditions for the synthesis of long chain analogs is reported. Moreover, we showed that a synthetic approach involving the TCA/TPP system that utilizes easily available starting materials and simple procedures provided amide derivatives with 4–15 carbon atoms linear chain lengths in correct yields. We and another group had previously demonstrated that the in vitro growth inhibitory effects of several piperazinyl quinolones in various cancer cell lines can be positively modulated through the introduction of substituents on the 7-piperazinyl group of the quinolone skeleton.[14,13] In the present article, we demonstrated that a lipophilic moiety positioned at the C-6 of the 7*H*-pyrido[1,2,3-*de*]-1,4-benzoxazine framework can also give rise to antitumor compounds, at least in vitro.

The most efficient ester **2e** with $R = C_{11}H_{23}C(O)OCH_2O$ had a mean IC_{50} value of 0.3 μM on glioblastoma, colon and breast cancer cell lines with no in vivo toxicity while the most potent amide **4d** showed a mean IC_{50} value of 2 μM. Experiments are in progress to further characterize the mechanism(s) of action of these LV derivatives when exerting their anticancer effects. Indeed, at the moment we have not yet identified the major mechanism(s) of action through which these compounds exert anticancer activity, at least in vitro.

4. Experimental

4.1. Chemistry

All reagents were of commercial quality, reagent grade, and were used as supplied without further purification. LV (>98%) was purchased from Fluka; chloromethyl alcanoates were prepared as previoulsly described.[20] Merck silica gel 60 F_{254} plates were used

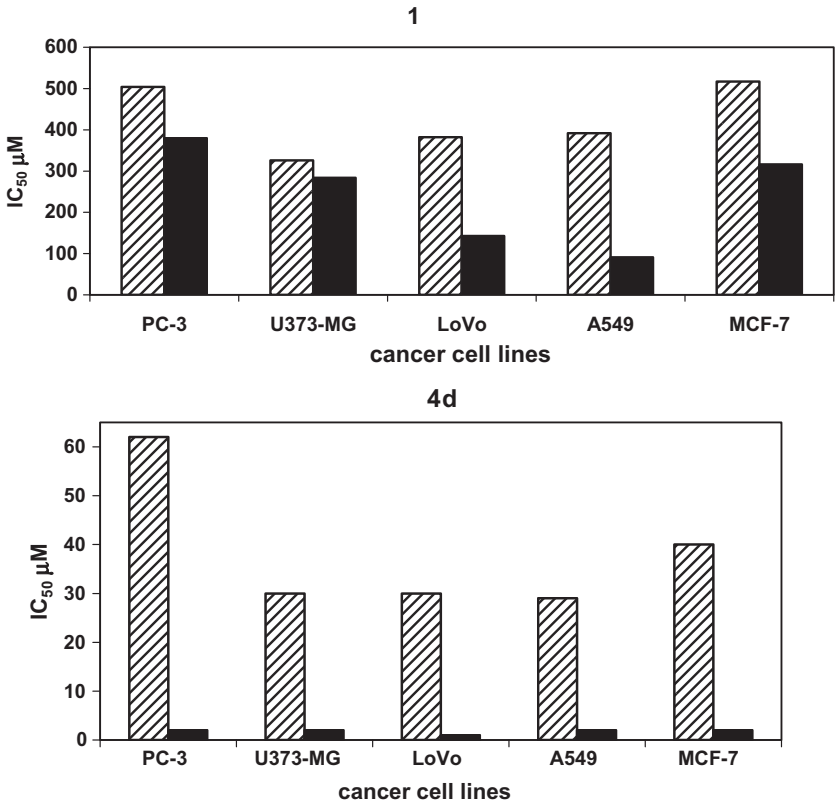
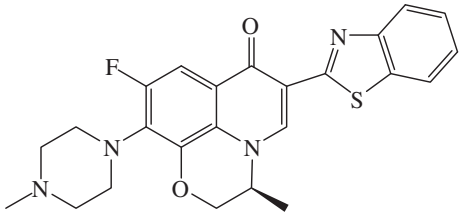
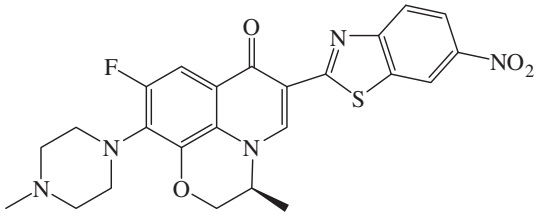


Figure 3. Time-dependent growth inhibition observed with levofloxacin **1** and amide **4d** in five human tumor cell lines (PC-3: prostate cancer; U373-MG: glioblastoma; LoVo: colon cancer; A549: non-small-cell lung cancer; MCF-7: breast cancer). The cells were cultured for 3 (hatched bars) or 5 (black bars) days with compound **1** or **4d**.

Table 5
Structure and in vitro assay results against selected tumor cell lines for LV derivatives (data from literature[42])

Compound	IC_{50}[a] (μM)					
	KB	A2780	Bel7402	PC-3	A549	MCF-7
	60.2 ± 11[b]	26.6 ± 2.2[b]	92.2 ± 21.1[b]	nd	nd	nd
	0.6 ± 0.2[b]	1.2 ± 0.2[b]	2 ± 0.4[b]	9.9[c]	>10[c]	>10[c]

[a] IC_{50} values on selected cell lines (KB: oral epidermal carcinoma, A2780: ovarian carcinoma, Bel7402: hepatocellular carcinoma, PC-3: prostate cancer, A549: lung cancer and MCF-7: breast cancer).
[b] In vitro MTT assay.
[c] In vitro sulforhodamine B (SRB) assay.

for analytical TLC with UV light detection (λ = 254 nm). Preparative column chromatography purifications were performed on silica 60 Å (Merck) finer than 70 μm by means of the solvent systems indicated. Melting points (mp) were determined on a Kofler hot stage apparatus and are uncorrected. ^{1}H NMR spectra were recorded on a Bruker AC-300 spectrometer. Data are reported in the following order: chemical shift δ in ppm, signal multiplicity, value(s) of coupling constant(s), number of protons, and assignment. ^{13}C NMR spectra and ^{19}F NMR spectra were recorded on a Bruker Avance-300 spectrometer. NMR spectra were recorded in deuteriochloroform unless indicated otherwise. The chemical shifts δ are expressed relative to TMS or $CFCl_3$. The attributions in the quinolone moiety are reported according to the atom numbering indicated in Scheme 2, and those of the methylene groups of alkyl chains in alphabetical order starting

from CH_3. Mass spectra were recorded on a Perkin Elmer SCIEX API 365 operating in electrospray mode. Elemental analyses were carried out by the 'Service Inter-universitaire de l'ENSIACET' in Toulouse, on an EA 1110 Thermo.

4.1.1. General procedure for the synthesis of 2a–e under thermal conditions (Δ)

A mixture of LV (0.180 g, 5.0×10^{-4} mol) and grinded K_2CO_3 (0.069 g, 5.0×10^{-4} mol) in dry DMF (20 mL) was heated at 90 °C and left stirring at this temperature for 20 min. Chloromethyl alcanoate (5.0×10^{-4} mol) was added and the reaction mixture was vigorously stirred at 90 °C for 1 h. The mixture was cooled, diluted to 50 mL with CH_2Cl_2, washed with a 5% aqueous solution of sodium chloride (5×100 ml). The organic layer was dried over anhydrous $MgSO_4$ and concentrated in vacuo to provide after flash chromatography on silica gel the desired compound.

4.1.2. Procedure for the synthesis of 2a under microwave activation (MW)

A mixture of LV (0.100 g, 2.76×10^{-4} mol) and K_2CO_3 (0.076 g, 5.52×10^{-4} mol) in acetonitrile (2.5 mL) was heated to 300 W/90 °C and maintained at this temperature for 20 min. After cooling, (nBu)$_4$NI (0.020 g, 5.52×10^{-5} mol) and chloromethylpropanoate (52 μL, 4.14×10^{-4} mol) were added. The reaction mixture was vigorously stirred at 300 W/90 °C for 1 h. The mixture was cooled and concentrated in vacuo. Water (50 mL) and CH_2Cl_2 (50 mL) were then added to the residue. The organic layer was washed with water (2×50 mL). This aqueous layer was extracted with CH_2Cl_2 (30 mL). The organic layers were separated, washed with water (3×30 mL), dried over anhydrous Na_2SO_4 and evaporated. The remaining residue was purified by flash chromatography on silica gel.

4.1.3. (3*S*)-6-[(Butanoyloxy)methyl]9-fluoro-2,3-dihydro-3-methyl-10-(4-methyl-1-piperazinyl)-7-oxo-7*H*-pyrido[1,2,3-*de*]-1,4-benzoxazine-6-carboxylate 2a

The procedure described above (Δ) applied to $C_3H_7COOCH_2Cl$ (62 μL, 5.0×10^{-4} mol) gave after chromatography on silica gel with NEt_3/acetone (2:100) and subsequent acetone wash (10 mL) **2a** (0.143 g, 59% yield) as a white solid. R_f 0.19 (90:10 CH_2Cl_2/MeOH). Mp 210 °C (dec).

^{1}H NMR (300 MHz): 0.96 (t, $^3J_{H\text{-}H}$ = 7.2 Hz, 3H, C_a–H); 1.52 (d, $^3J_{H\text{-}H}$ = 6.8 Hz, 3H, C_{3a}–H); 1.68 (sext, $^3J_{H\text{-}H}$ = 7.4 Hz, 2H, C_b–H); 2.37 (s, 3H, $C_{4'a}$–H); 2.39 (t, $^3J_{H\text{-}H}$ = 7.4 Hz, 2H, C_c–H); 2.55 (m, 4H, $C_{3'}$-H and $C_{5'}$-H); 3.35 (m, 4H, $C_{2'}$-H and $C_{6'}$-H); 4.28–4.61 (m, 3H, C_2–H and C_3–H); 5.95 (AB syst, $^2J_{H\text{-}H}$ = 5.6 Hz, 2H, C_{15}–H); 7.38 (d, $^3J_{H\text{-}F}$ = 12.5 Hz, 1H, C_8–H); 8.17 (s, 1H, C_5–H). ^{13}C NMR (75 MHz): 13.5 (C_a); 18.1 (C_b); 18.3 (C_{3a}); 35.8 (C_c); 46.4 ($C_{4'a}$); 50.5 (d, $^4J_{C\text{-}F}$ = 4.2 Hz, $C_{2'}$ and $C_{6'}$); 54.9 (C_3); 55.7 ($C_{3'}$ and $C_{5'}$); 68.1 (C_2); 79.5 (C_{15}); 105.2 (d, $^2J_{C\text{-}F}$ = 24.1 Hz, C_8); 107.9 (C_6); 122.9 (d, $^3J_{C\text{-}F}$ = 8.5 Hz, C_7); 123.5 (d, $^4J_{C\text{-}F}$ = 1.4 Hz, C_{12}); 131.9 (d, $^2J_{C\text{-}F}$ = 14.4 Hz, C_{10}); 139.6 (d, $^3J_{C\text{-}F}$ = 6.8 Hz, C_{11}); 145.6 (C_5); 155.7 (d, $^1J_{C\text{-}F}$ = 245.9 Hz, C_9); 163.4 (C_{14}); 172.5 (d, $^4J_{C\text{-}F}$ = 2.7 Hz, C_{13}); 172.6 (C_{16}). ^{19}F NMR (282 MHz): -120.9 (d, $^3J_{H\text{-}F}$ = 12.4 Hz). MS (ESI, positive mode): 462.5 [M+H]$^+$. Elemental Anal. Calcd for $C_{23}H_{28}FN_3O_6$: C, 59.86; H, 6.12; N, 9.11. Found: C, 59.57; H, 5.88; N, 9.02.

4.1.4. (3*S*)-6-[(Hexanoyloxy)methyl]9-fluoro-2,3-dihydro-3-methyl-10-(4-methyl-1-piperazinyl)-7-oxo-7*H*-pyrido[1,2,3-*de*]-1,4-benzoxazine-6-carboxylate 2b

The procedure described above (Δ) applied to $C_5H_{11}COOCH_2Cl$ (0.082 g, 5.0×10^{-4} mol) gave after flash chromatography on silica gel with NEt_3/acetone (1:100) **2b** (0.171 g, 69% yield) as a white solid. R_f 0.22 (90:10 CH_2Cl_2/MeOH). Mp 190 °C (dec).

^{1}H NMR (300 MHz): 0.89 (t, $^3J_{H\text{-}H}$ = 6.9 Hz, 3H, C_a–H); 1.22–1.37 (m, 4H, C_b–H and C_c–H); 1.53 (d, $^3J_{H\text{-}H}$ = 6.8 Hz, 3H, C_{3a}–H); 1.66 (m, 2H, C_d–H); 2.38 (s, 3H, $C_{4'a}$–H); 2.40 (t, $^3J_{H\text{-}H}$ = 7.4 Hz, 2H, C_e–H); 2.57 (m, 4H, $C_{3'}$-H and $C_{5'}$-H); 3.36 (m, 4H, $C_{2'}$-H and $C_{6'}$-H); 4.32–4.55 (m, 3H, C_2–H and C_3–H); 5.95 (AB syst, $^2J_{H\text{-}H}$ = 5.6 Hz., 2H, C_{15}–H); 7.44 (d, $^3J_{H\text{-}F}$ = 12.6 Hz, 1H, C_8–H); 8.21 (s, 1H, C_5–H). ^{13}C NMR (75 MHz): 13.9 (C_a); 18.3 (C_{3a}); 22.3 (C_b); 24.3 (C_c); 31.2 (C_d); 34.0 (C_e); 46.4 ($C_{4'a}$); 50.5 (d, $^4J_{C\text{-}F}$ = 4.2 Hz, $C_{2'}$ and $C_{6'}$); 54.9 (C_3); 55.7 ($C_{3'}$ and $C_{5'}$); 68.1 (C_2); 79.5 (C_{15}); 105.4 (d, $^2J_{C\text{-}F}$ = 23.7 Hz, C_8); 108.0 (C_6); 123.0 (d, $^3J_{C\text{-}F}$ = 7.8 Hz, C_7); 123.5 (d, $^4J_{C\text{-}F}$ = 1.4 Hz, C_{12}); 131.9 (d, $^2J_{C\text{-}F}$ = 14.2 Hz, C_{10}); 139.6 (d, $^3J_{C\text{-}F}$ = 6.7 Hz, C_{11}); 145.6 (C_5); 155.7 (d, $^1J_{C\text{-}F}$ = 245.9 Hz, C_9); 163.4 (C_{14}); 172.5 (d, $^4J_{C\text{-}F}$ = 2.3 Hz, C_{13}); 172.8 (C_{16}). ^{19}F NMR (282 MHz, $CDCl_3$): −121.0 (d, $^3J_{H\text{-}F}$ = 11.4 Hz). MS (ESI, positive mode): 490.8 [M+H]$^+$. Elemental Anal. Calcd for $C_{25}H_{32}FN_3O_6$: C, 61.34; H, 6.59; N, 8.58. Found: C, 61.52; H, 6.48; N, 8.49.

4.1.5. (3*S*)-6-[(Octanoyloxy)methyl]9-fluoro-2,3-dihydro-3-methyl-10-(4-methyl-1-piperazinyl)-7-oxo-7*H*-pyrido[1,2,3-*de*]-1,4-benzoxazine-6-carboxylate 2c

The procedure described above (Δ) applied to C_7H_{15}-$COOCH_2Cl$ (0.096 g, 5.0×10^{-4} mol) gave after flash chromatography on silica gel with NEt_3/acetone (1:100) **2c** (0.192 g, 75% yield) as a white solid. R_f 0.23 (90:10 CH_2Cl_2/MeOH). Mp 189 °C (dec).

^{1}H NMR (300 MHz): 0.86 (t, $^3J_{H\text{-}H}$ = 6.8 Hz, 3H, C_a–H); 1.21–1.36 (m, 8H, $C_{b\text{-}e}$–H); 1.53 (d, $^3J_{H\text{-}H}$ = 6.6 Hz, 3H, C_{3a}–H); 1.60–1.70 (m, 2H, C_f–H); 2.38 (s, 3H, $C_{4'a}$–H); 2.40 (t, $^3J_{H\text{-}H}$ = 7.2 Hz, 2H, C_g–H); 2.56 (m, 4H, $C_{3'}$-H and $C_{5'}$-H); 3.36 (m, 4H, $C_{2'}$-H and $C_{6'}$-H); 4.33–4.54 (m, 3H, C_2–H and C_3–H); 5.96 (AB syst, $^2J_{H\text{-}H}$ = 5.7 Hz, 2H, C_{15}–H); 7.41 (d, $^3J_{H\text{-}F}$ = 12.0 Hz, 1H, C_8–H); 8.19 (s, 1H, C_5–H). ^{13}C NMR (75 MHz): 14.0 (C_a); 18.3 (C_{3a}); 22.6 (C_b); 24.6 (C_c); 28.9 (C_d); 29.0 (C_e); 31.6 (C_f); 34.0 (C_g); 46.4 ($C_{4'a}$); 50.5 (d, $^4J_{C\text{-}F}$ = 4.1 Hz, $C_{2'}$ and $C_{6'}$); 54.9 (C_3); 55.7 ($C_{3'}$ and $C_{5'}$); 68.1 (C_2); 79.5 (C_{15}); 105.3 (d, $^2J_{C\text{-}F}$ = 24.0 Hz, C_8); 107.9 (C_6); 123.0 (d, $^3J_{C\text{-}F}$ = 8.5 Hz, C_7); 123.5 (d, $^4J_{C\text{-}F}$ = 1.4 Hz, C_{12}); 131.9 (d, $^2J_{C\text{-}F}$ = 14.2 Hz, C_{10}); 139.6 (d, $^3J_{C\text{-}F}$ = 6.8 Hz, C_{11}); 145.6 (C_5); 155.7 (d, $^1J_{C\text{-}F}$ = 245.9 Hz, C_9); 163.4 (C_{14}); 172.5 (d, $^4J_{C\text{-}F}$ = 2.6 Hz, C_{13}); 172.8 (C_{16}). ^{19}F NMR (282 MHz): −120.9 (d, $^3J_{H\text{-}F}$ = 11.3 Hz). MS (ESI, positive mode): 518.8 [M+H]$^+$. Elemental Anal. Calcd for $C_{27}H_{36}FN_3O_6$: C, 62.65; H, 7.01; N, 8.12. Found: C, 62.67; H, 6.86; N, 8.12.

4.1.6. (3*S*)-6-[(Decanoyloxy)methyl]9-fluoro-2,3-dihydro-3-methyl-10-(4-methyl-1-piperazinyl)-7-oxo-7*H*-pyrido[1,2,3-*de*]-1,4-benzoxazine-6-carboxylate 2d

The procedure described above (Δ) applied to $C_9H_{19}COOCH_2Cl$ (0.110 g, 5.0×10^{-4} mol) gave after flash chromatography on silica gel with NEt_3/acetone 1/100 **2d** (0.215 g, 79% yield) as a white solid. R_f 0.24 (90:10 CH_2Cl_2/MeOH). Mp 189 °C (dec).

^{1}H NMR (300 MHz): 0.86 (t, $^3J_{H\text{-}H}$ = 6.8 Hz, 3H, C_a–H); 1.19–1.36 (m, 12H, $C_{b\text{-}g}$–H); 1.53 (d, $^3J_{H\text{-}H}$ = 6.9 Hz, 3H, C_{3a}–H); 1.64 (m, 2H, C_h–H); 2.38 (s, 3H, $C_{4'a}$–H); 2.40 (t, $^3J_{H\text{-}H}$ = 7.5 Hz, 2H, C_i–H); 2.57 (m, 4H, $C_{3'}$-H and $C_{5'}$-H); 3.36 (m, 4H, $C_{2'}$-H and $C_{6'}$-H); 4.32–4.55 (m, 3H, C_2–H and C_3–H); 5.95 (AB syst, $^2J_{H\text{-}H}$ = 5.7 Hz, 2H, C_{15}–H); 7.41 (d, $^3J_{H\text{-}F}$ = 12.6 Hz, 1H, C_8–H); 8.20 (s, 1H, C_5–H). ^{13}C NMR (75 MHz): 14.1 (C_a); 18.3 (C_{3a}); 22.6 (C_b); 24.6 (C_c); 29.0 (C_d); 29.2 (C_e); 29.2 (C_f); 29.4 (C_g); 31.8 (C_h); 34.0 (C_i); 46.4 ($C_{4'a}$); 50.5 (d, $^4J_{C\text{-}F}$ = 4.1 Hz, $C_{2'}$ and $C_{6'}$); 54.9 (C_3); 55.7 ($C_{3'}$ and $C_{5'}$); 68.1 (C_2); 79.5 (C_{15}); 105.3 (d, $^2J_{C\text{-}F}$ = 24.1 Hz, C_8); 107.9 (C_6); 123.0 (d, $^3J_{C\text{-}F}$ = 8.5 Hz, C_7); 123.5 (d, $^4J_{C\text{-}F}$ = 1.4 Hz, C_{12}); 131.9 (d, $^2J_{C\text{-}F}$ = 14.2 Hz, C_{10}); 139.6 (d, $^3J_{C\text{-}F}$ = 6.8 Hz, C_{11}); 145.6 (C_5); 155.7 (d, $^1J_{C\text{-}F}$ = 245.9 Hz, C_9); 163.4 (C_{14}); 172.5 (d, $^4J_{C\text{-}F}$ = 2.7 Hz, C_{13}); 172.8 (C_{16}). ^{19}F NMR (282 MHz): −121.0 (d, $^3J_{H\text{-}F}$ = 11.3 Hz). MS (ESI, positive mode): 546.5 [M+H]$^+$. Elemental Anal. Calcd for $C_{29}H_{40}FN_3O_6 \cdot 1.2H_2O$: C, 61.40, H, 7.53; N, 7.41. Found: C, 61.33; H, 7.00; N, 7.39.

4.1.7. (3*S*)-6-[(Dodecanoyloxy)methyl]9-fluoro-2,3-dihydro-3-methyl-10-(4-methyl-1-piperazinyl)-7-oxo-7*H*-pyrido[1,2,3-*de*]-1,4-benzoxazine-6-carboxylate 2e

The procedure described above (Δ) applied to $C_{11}H_{23}COOCH_2Cl$ (0.124 g, 5.0×10^{-4} mol) gave after flash chromatography on silica gel with NEt_3/acetone (1:100) **2e** (0.233 g, 79% yield) as a white solid. R_f 0.25 (99:1 CH_2Cl_2/MeOH). Mp 189 °C (dec).

^{1}H NMR (300 MHz): 0.88 (t, $^3J_{H\text{-}H}$ = 6.6 Hz, 3H, C_a–H); 1.21–1.35 (m, 16H, $C_{b\text{-}i}$–H); 1.53 (d, $^3J_{H\text{-}H}$ = 6.9 Hz, 3H, C_{3a}–H); 1.65 (m, 2H, C_j); 2.37 (s, 3H, $C_{4'a}$–H); 2.40 (t, $^3J_{H\text{-}H}$ = 7.5 Hz, 2H, C_k–H); 2.56 (m, 4H, $C_{3'}$-H and $C_{5''}$-H); 3.36 (m, 4H, $C_{2'}$-H and $C_{6'}$-H); 4.32–4.54 (m, 3H, C_2–H and C_3–H); 5.96 (AB syst, $^2J_{H\text{-}H}$ = 5.4 Hz., 2H, C_{15}–H); 7.44 (d, $^3J_{H\text{-}F}$ = 12.6 Hz, 1H, C_8–H); 8.20 (s, 1H, C_5–H). ^{13}C NMR (75 MHz): 14.1 (C_a); 18.3 (C_{3a}); 22.7 (C_b); 24.6 (C_c); 29.1, 29.3, 29.3, 29.5 and 29.6 ($C_{d\text{-}i}$); 31.9 (C_j); 34.0 (C_k); 46.4 ($C_{4'a}$); 50.6 (d, $^4J_{C\text{-}F}$ = 4.1 Hz, $C_{2'}$ and $C_{6'}$); 54.9 (C_3); 55.7 ($C_{3'}$ and $C_{5'}$); 68.1 (C_2); 79.5 (C_{15}); 105.4 (d, $^2J_{C\text{-}F}$ = 24.0 Hz, C_8); 108.0 (C_6); 123.0 (d, $^3J_{C\text{-}F}$ = 8.5 Hz, C_7); 123.5 (d, $^4J_{C\text{-}F}$ = 1.4 Hz, C_{12}); 131.9 (d, $^2J_{C\text{-}F}$ = 14.2 Hz, C_{10}); 139.6 (d, $^3J_{C\text{-}F}$ = 6.8 Hz, C_{11}); 145.6 (C_5); 155.7 (d, $^1J_{C\text{-}F}$ = 245.9 Hz, C_9); 163.4 (C_{14}); 172.5 (d, $^4J_{C\text{-}F}$ = 2.6 Hz, C_{13}); 172.8 (C_{16}). ^{19}F NMR (282 MHz): −120.9 (d, $^3J_{H\text{-}F}$ = 11.3 Hz). MS (ESI, positive mode): 574.7 $[M+H]^+$. Elemental Anal. Calcd for $C_{31}H_{44}FN_3O_6{\cdot}0.4H_2O$: C, 64.10; H, 7.77; N, 7.23. Found: C, 64.01; H, 7.52; N, 7.20.

4.1.8. (3*S*)-6-[(Tetradecanoyloxy)methyl]9-fluoro-2,3-dihydro-3-methyl-10-(4-methyl-1-piperazinyl)-7-oxo-7*H*-pyrido[1,2,3-*de*]-1,4-benzoxazine-6-carboxylate 2f

The procedure described above (Δ) applied to $C_{13}H_{27}COOCH_2Cl$ (0.137 g, 5.0×10^{-4} mol) gave after flash chromatography on silica gel with MeOH/NEt_3/CH_2Cl_2 (7.5:1:100) and subsequent acetone wash (10 mL) **2f** (0.234 g, 77% yield) as a white solid. R_f 0.27 (90:10 CH_2Cl_2/MeOH). Mp 184 °C (dec).

^{1}H NMR (300 MHz): 0.88 (t, $^3J_{H\text{-}H}$ = 6.8 Hz, 3H, C_a–H); 1.20–1.37 (m, 20H, $C_{b\text{-}k}$–H); 1.53 (d, $^3J_{H\text{-}H}$ = 6.6 Hz, 3H, C_{3a}–H); 1.65 (m, 2H, C_l–H); 2.38 (s, 3H, $C_{4'a}$–H); 2.40 (t, $^3J_{H\text{-}H}$ = 7.5 Hz, 2H, C_m–H); 2.57 (m, 4H, $C_{3'}$-H and $C_{5'}$-H); 3.36 (m, 4H, $C_{2'}$-H and $C_{6'}$-H); 4.33–4.55 (m, 3H, C_2–H, C_3–H); 5.95 (AB syst, $^2J_{H\text{-}H}$ = 5.7 Hz, 2H, C_{15}–H); 7.42 (d, $^3J_{H\text{-}F}$ = 12.6 Hz, 1H, C_8–H); 8.20 (s, 1H, C_5–H). ^{13}C NMR NMR (75 MHz): 14.1 (C_a); 18.3 (C_{3a}); 22.7 (C_b); 24.6 (C_c); 29.1, 29.3, 29.3, 29.5, 29.6, 29.6 and 29.7 ($C_{d\text{-}k}$); 31.9 (C_l); 34.0 (C_m); 46.4 ($C_{4'a}$); 50.5 (d, $^4J_{C\text{-}F}$ = 4.1 Hz, $C_{2'}$ and $C_{6'}$); 54.9 (C_3); 55.7 ($C_{3'}$ and $C_{5'}$); 68.1 (C_2); 79.5 (C_{15}); 105.4 (d, $^2J_{C\text{-}F}$ = 24.0 Hz, C_8); 108.0 (C_6); 123.0 (d, $^3J_{C\text{-}F}$ = 8.4 Hz, C_7); 123.5 (d, $^4J_{C\text{-}F}$ = 1.4 Hz, C_{12}); 131.9 (d, $^2J_{C\text{-}F}$ = 14.3 Hz, C_{10}); 139.6 (d, $^3J_{C\text{-}F}$ = 6.8 Hz, C_{11}); 145.6 (C_5); 155.7 (d, $^1J_{C\text{-}F}$ = 245.9 Hz, C_9); 163.4 (C_{14}); 172.5 (d, $^4J_{C\text{-}F}$ = 2.6 Hz, C_{13}); 172.8 (C_{16}). ^{19}F NMR (282 MHz): −121.0 (d, $^3J_{H\text{-}F}$ = 11.3 Hz). MS (ESI, positive mode): 602.5 $[M+H]^+$. Elemental Anal. Calcd for $C_{33}H_{48}FN_3O_6$: C, 65.87; H, 8.04; N, 6.98. Found: C, 65.65; H, 7.79; N, 6.94.

4.1.9. (3*S*)-6-[(Hexadecanoyloxy)methyl]9-fluoro-3,7-dihydro-3-methyl-10-(4-methylpiperazin-1-yl)-7-oxo-2*H*-[1,4]oxazino-[2,3,4-ij]quinoline-6-carboxylate 2g

The procedure described above (Δ) applied to $C_{15}H_{31}COOCH_2Cl$ (0.152 g, 5.0×10^{-4} mol) gave after flash chromatography on silica gel with MeOH/NEt_3/CH_2Cl_2 (7.5:1:100) and subsequent acetone wash (10 mL) **2g** (0.252 g, 79% yield) as a white solid. R_f 0.28 (90:10 CH_2Cl_2/MeOH). Mp 182 °C (dec).

^{1}H NMR (300 MHz): 0.88 (t, $^3J_{H\text{-}H}$ = 6.8 Hz, 3H, C_a–H); 1.21–1.36 (m, 24H, $C_{b\text{-}m}$–H); 1.53 (d, $^3J_{H\text{-}H}$ = 6.9 Hz, 3H, C_{3a}–H); 1.65 (m, 2H, C_n–H); 2.38 (s, 3H, $C_{4'a}$–H); 2.40 (t, $^3J_{H\text{-}H}$ = 7.2 Hz, 2H, C_o–H); 2.56 (m, 4H, $C_{3'}$-H and $C_{5'}$-H); 3.36 (m, 4H, $C_{2'}$-H and $C_{6'}$-H); 4.33–4.55 (m, 3H, C_5–H and C_6–H); 5.96 (AB syst, $^2J_{H\text{-}H}$ = 5.6 Hz, 2H, C_{15}–H); 7.42 (d, $^3J_{H\text{-}F}$ = 12.3 Hz, 1H, C_8–H); 8.20 (s, 1H, C_5–H). ^{13}C NMR (75 MHz): 14.1 (C_a); 18.3 (C_{3a}); 22.7 (C_b); 24.6 (C_c); 29.1, 29.3, 29.4, 29.5, 29.6, 29.6 29.7 and 29.7 ($C_{d\text{-}m}$); 31.9 (C_n); 34.0 (C_o); 46.4 ($C_{4'a}$); 50.5 (d, $^4J_{C\text{-}F}$ = 4.1 Hz, $C_{2'}$ and $C_{6'}$); 54.9 (C_3); 55.7 ($C_{3'}$ and $C_{5'}$); 68.1 (C_2); 79.5 (C_{15}); 105.4 (d, $^2J_{C\text{-}F}$ = 24.0 Hz, C_8); 108.0 (C_6); 123.0 (d, $^3J_{C\text{-}F}$ = 8.4 Hz, C_7); 123.5 (d, $^4J_{C\text{-}F}$ = 1.5 Hz, C_{12}); 131.9 (d, $^2J_{C\text{-}F}$ = 14.3 Hz, C_{10}); 139.6 (d, $^3J_{C\text{-}F}$ = 6.8 Hz, C_{11}); 145.6 (C_5); 155.7 (d, $^1J_{C\text{-}F}$ = 245.9 Hz, C_9); 163.4 (C_{14}); 172.5 (d, $^4J_{C\text{-}F}$ = 2.6 Hz, C_{13}); 172.8 (C_{16}). ^{19}F NMR (282 MHz): −120.9 (d, $^3J_{H\text{-}F}$ = 11.3 Hz). MS (ESI, positive mode): 630.7 $[M+H]^+$. Elemental Anal. Calcd for $C_{35}H_{52}FN_3O_6{\cdot}0.2H_2O$: C, 66.37; H, 8.34; N, 6.63. Found: C, 66.29; H, 8.03; N, 6.60.

4.1.10. (3*S*)-6-[(Octadecanoyloxy)methyl]9-fluoro-2,3-dihydro-3-methyl-10-(4-methyl-1-piperazinyl)-7-oxo-7*H*-pyrido[1,2,3-*de*]-1,4-benzoxazine-6-carboxylate 2h

The procedure described above (Δ) applied to $C_{17}H_{35}COOCH_2Cl$ (0.166 g, 5.0×10^{-4} mol) gave after flash chromatography on silica gel with MeOH/NEt_3/CH_2Cl_2 (5:1:100) and subsequent acetone wash (10 mL) **2h** (0.266 g, 81% yield) as a white solid. R_f 0.29 (90:10 CH_2Cl_2/MeOH). Mp 180 °C (dec).

^{1}H NMR (300 MHz): 0.88 (t, $^3J_{H\text{-}H}$ = 6.8 Hz, 3H, C_a–H); 1.20–1.36 (m, 28H, $C_{b\text{-}o}$–H); 1.52 (d, $^3J_{H\text{-}H}$ = 6.6 Hz, 3H, C_{3a}–H); 1.64 (m, 2H, C_p–H); 2.39 (s, 3H, $C_{4'a}$–H); 2.40 (t, $^3J_{H\text{-}H}$ = 7.5 Hz, 2H, C_q–H); 2.58 (m, 4H, $C_{3'}$-H and $C_{5'}$-H); 3.37 (m, 4H, $C_{2'}$-H and $C_{6'}$-H), 4.33–4.57 (m, 3H, C_2–H and C_3–H); 5.95 (AB syst, $^2J_{H\text{-}H}$ = 5.7 Hz., 2H, C_{15}–H); 7.39 (d, $^3J_{H\text{-}F}$ = 12.6 Hz, 1H, C_8–H); 8.18 (s, 1H, C_5–H). ^{13}C NMR (75 MHz): 14.1 (C_a); 18.3 (C_{3a}); 22.7 (C_b); 24.6 (C_c); 29.1, 29.3, 29.4, 29.5, 29.6, 29.6, 29.6, 29.7 and 29.7 ($C_{d\text{-}o}$); 31.9 (C_p); 34.0 (C_q); 46.3 ($C_{4'a}$); 50.4 (d, $^4J_{C\text{-}F}$ = 4.1 Hz, $C_{2'}$ and $C_{6'}$); 54.9 (C_3); 55.7 ($C_{3'}$ and $C_{5'}$); 68.1 (C_2); 79.5 (C_{15}); 105.3 (d, $^2J_{C\text{-}F}$ = 24.0 Hz, C_8); 107.9 (C_6); 123.0 (d, $^3J_{C\text{-}F}$ = 8.4 Hz, C_7); 123.5 (d, $^4J_{C\text{-}F}$ = 1.4 Hz, C_{12}); 131.8 (d, $^2J_{C\text{-}F}$ = 14.3 Hz, C_{10}); 139.7 (d, $^3J_{C\text{-}F}$ = 6.8 Hz, C_{11}); 145.6 (C_5); 155.7 (d, $^1J_{C\text{-}F}$ = 245.8 Hz, C_9); 163.4 (C_{14}); 172.5 (d, $^4J_{C\text{-}F}$ = 2.7 Hz, C_{13}); 172.8 (C_{16}). ^{19}F NMR (282 MHz): −121.0 (d, $^3J_{H\text{-}F}$ = 11.3 Hz). MS (ESI, positive mode): 658.5 $[M+H]^+$. Elemental Anal. Calcd for $C_{37}H_{56}FN_3O_6{\cdot}0.5H_2O$: C, 66.64; H, 8.62; N, 6.30. Found: C, 66.53; H, 8.22; N, 6.22.

4.1.11. General procedure for the synthesis of amides 4a–h

To a cooled (0 °C) and stirred mixture of LV (0.361 g, 10^{-3} mol) and TCA (0.2 mL, 2×10^{-3} mol) in 5 mL of dry CH_2Cl_2 was added dropwise, under argon, a solution of TPP (0.525 g, 2×10^{-3} mol) in 1 mL of dry CH_2Cl_2. The reaction mixture was stirred for 4 h at rt to give a yellow residue which was centrifuged, washed with CH_2Cl_2 (5 mL) and used without further purification. A suspension of this acyl chloride in 1 mL of dry CH_2Cl_2 was then treated with alkylamine (10^{-3} mol) followed by triethylamine (0.303 g, 3×10^{-3} mol). The reaction mixture was allowed to react for 18 h at rt; it was diluted to 25 mL with CH_2Cl_2 and washed with brine (3×50 mL). The organic layer was dried over anhydrous $MgSO_4$, concentrated under reduced pressure to provide after flash chromatography on silica gel the desired compound.

4.1.12. (3*S*)-*N*-Butyl-9-fluoro-2,3-dihydro-3-methyl-10-(4-methyl-1-piperazinyl)-7-oxo-7*H*-pyrido[1,2,3-*de*]-1,4-benzoxazine-6-carboxamide 4a

With $C_4H_9NH_2$ (0.059 g, 10^{-3} mol) the procedure described above provided after flash chromatography on silica gel with CH_2Cl_2/MeOH 100:7.5 the desired compound **4a** (0.143 g, 34% yield) as yellowish solid. R_f 0.14 (90:10 CH_2Cl_2/MeOH). Mp 160 °C.

^{1}H NMR (300 MHz): 0.95 (t, $^3J_{H\text{-}H}$ = 7.4 Hz, 3H, C_a–H), 1.37–1.51 (m, 2H, C_b–H), 1.57 (d, $^3J_{H\text{-}H}$ = 6.6 Hz, 3H, C_{3a}–H), 1.58 (m, 2H, C_c–H), 2.36 (s, 3H, $C_{4'a}$–H), 2.56 (m, 4H, $C_{3'}$–H and $C_{5'}$-H), 3.28–3.51 (m, 6H, C_d–H, $C_{2'}$–H and $C_{6'}$–H), 4.26–4.47 (m, 3H, C_2–H and C_3–H), 7.69 (d, $^3J_{H\text{-}F}$ = 12.6 Hz, 1H, C_8–H), 8.63 (s, 1H, C_5–H), 9.97 (t, $^3J_{H\text{-}H}$ = 5.6 Hz, 1H, N–H). ^{13}C NMR (75 MHz): 13.8 (C_a), 18.2 (C_{3a}), 20.3, 31.7, 38.9 ($C_{b\text{-}d}$), 46.4 ($C_{4'a}$), 50.6

(d, $^4J_{C\text{-}F}$ = 4.1 Hz, $C_{2'}$ and $C_{6'}$), 54.8 (C_3), 55.7 ($C_{3'}$ and $C_{5'}$), 68.2 (C_2), 105.1 (d, $^2J_{C\text{-}F}$ = 23.9 Hz, C_8), 111.3 (C_6), 122.5 (d, $^3J_{C\text{-}F}$ = 8.7 Hz, C_7), 124.3 (d, $^4J_{C\text{-}F}$ = 1.3 Hz, C_{12}), 131.8 (d, $^2J_{C\text{-}F}$ = 14.6 Hz, C_{10}), 139.4 (d, $^3J_{C\text{-}F}$ = 6.7 Hz, C_{11}), 143.8 (C_5), 155.8 (d, $^1J_{C\text{-}F}$ = 245.8 Hz, C_9), 164.8 (C_{14}), 175.4 (d, $^4J_{C\text{-}F}$ = 3.0 Hz, C_{13}). ^{19}F NMR (282 MHz): −121.2 (d, $^3J_{H\text{-}F}$ = 12.4 Hz). MS (ESI, positive mode): 417.3 $[M+H]^+$. Elemental Anal. Calcd for $C_{22}H_{29}FN_4O_3{\cdot}0.3H_2O$: C, 62.63; H, 7.07; N, 13.28. Found: C, 62.59; H, 6.99; N, 13.09.

4.1.13. (3*S*)-*N*-Hexyl-9-fluoro-2,3-dihydro-3-methyl-10-(4-methyl-1-piperazinyl)-7-oxo-7*H*-pyrido[1,2,3-*de*]-1,4-benzoxazine-6-carboxamide 4b

With $C_6H_{13}NH_2$ (0.101 g, 10^{-3} mol) the procedure described above provided after flash chromatography on silica gel with CH_2Cl_2/MeOH 100:7.5 the desired compound **4b** (0.166 g, 37% yield) as yellowish solid. R_f 0.15 (90:10 CH_2Cl_2/MeOH). Mp 113 °C.

^{1}H NMR (300 MHz): 0.88 (t, $^3J_{H\text{-}H}$ = 6.9 Hz, 3H, C_a–H), 1.25–1.47 (m, 6H, $C_{b\text{-}d}$–H), 1.57 (d, $^3J_{H\text{-}H}$ = 6.6 Hz, 3H, C_{3a}–H), 1.62 (m, 2H, C_e–H), 2.36 (s, 3H, $C_{4'a}$–H), 2.55 (m, 4H, $C_{3'}$–H and $C_{5'}$–H), 3.28–3.49 (m, 6H, C_f–H, $C_{2'}$–H and $C_{6'}$–H), 4.27–4.48 (m, 3H, C_2–H and C_3–H), 7.68 (d, $^3J_{H\text{-}F}$ = 12.6 Hz, 1H, C_8–H), 8.64 (s, 1H, C_5–H), 9.96 (t, $^3J_{H\text{-}H}$ = 5.4 Hz, 1H, N–H). ^{13}C NMR (75 MHz): 14.1 (C_a), 18.3 (C_{3a}), 22.6, 26.8, 29.6, 31.6, 39.3 ($C_{b\text{-}f}$), 46.4 ($C_{4'a}$), 50.6 (d, $^4J_{C\text{-}F}$ = 3.8 Hz, $C_{2'}$ and $C_{6'}$), 54.8 (C_3), 55.7 ($C_{3'}$ and $C_{5'}$), 68.2 (C_2), 105.1 (d, $^2J_{C\text{-}F}$ = 23.3 Hz, C_8), 111.3 (C_6), 122.4 (d, $^3J_{C\text{-}F}$ = 8.3 Hz, C_7), 124.3 (d, $^4J_{C\text{-}F}$ = 1.5 Hz, C_{12}), 131.8 (d, $^2J_{C\text{-}F}$ = 14.3 Hz, C_{10}), 139.4 (d, $^3J_{C\text{-}F}$ = 6.8 Hz, C_{11}), 143.8 (C_5), 155.8 (d, $^1J_{C\text{-}F}$ = 246.0 Hz, C_9), 164.8 (C_{14}), 175.4 (d, $^4J_{C\text{-}F}$ = 3.0 Hz, C_{13}). ^{19}F NMR (282 MHz, $CDCl_3$): -121.2 (d, $^3J_{H\text{-}F}$ = 12.4 Hz). MS (ESI, positive mode): 445.5 $[M+H]^+$. Elemental Anal. Calcd for $C_{24}H_{33}FN_4O_3$: C, 64.84; H, 7.48; N, 12.60. Found: C, 64.83; H, 7.35; N, 12.57.

4.1.14. (3*S*)-*N*-Heptyl-9-fluoro-2,3-dihydro-3-methyl-10-(4-methyl-1-piperazinyl)-7-oxo-7*H*-pyrido[1,2,3-*de*]-1,4-benzoxazine-6-carboxamide 4c

With $C_7H_{15}NH_2$ (0.115 g, 10^{-3} mol) the procedure described above provided after flash chromatography on silica gel with CH_2Cl_2/MeOH 100:7.5 the desired compound **4c** (0.184 g, 36% yield) as yellowish solid. R_f 0.16 (90:10 CH_2Cl_2/MeOH). Mp 115 °C.

^{1}H NMR (300 MHz): 0.87 (t, $^3J_{H\text{-}H}$ = 6.8 Hz, 3H, C_a–H), 1.20–1.45 (m, 8H, $C_{b\text{-}e}$–H), 1.57 (d, $^3J_{H\text{-}H}$ = 6.7 Hz, 3H, C_{3a}–H), 1.63 (m, 2H, C_f–H), 2.36 (s, 3H, $C_{4'a}$–H), 2.54 (m, 4H, $C_{3'}$–H and $C_{5'}$–H), 3.28–3.50 (m, 6H, C_g–H, $C_{2'}$–H and $C_{6'}$–H), 4.27–4.48 (m, 3H, C_2–H and C_3–H), 7.68 (d, $^3J_{H\text{-}F}$ = 12.5 Hz, 1H, C_8–H), 8.64 (s, 1H, C_5–H), 9.98 (t, $^3J_{H\text{-}H}$ = 5.4 Hz, 1H, N–H). ^{13}C NMR (75 MHz): 14.1 (C_a), 18.2 (C_{3a}), 22.6, 27.1, 29.0, 29.6, 31.7, 39.3 ($C_{b\text{-}g}$), 46.4 ($C_{4'a}$), 50.6 (d, $^4J_{C\text{-}F}$ = 3.8 Hz, $C_{2'}$ and $C_{6'}$), 54.8 (C_3), 55.7 ($C_{3'}$ and $C_{5'}$), 68.2 (C_2), 105.1 (d, $^2J_{C\text{-}F}$ = 24.0 Hz, C_8), 111.3 (C_6), 122.4 (d, $^3J_{C\text{-}F}$ = 9.0 Hz, C_7), 124.3 (d, $^4J_{C\text{-}F}$ = 1.5 Hz, C_{12}), 131.8 (d, $^2J_{C\text{-}F}$ = 14.3 Hz, C_{10}), 139.4 (d, $^3J_{C\text{-}F}$ = 6.8 Hz, C_{11}), 143.8 (C_5), 155.8 (d, $^1J_{C\text{-}F}$ = 246.0 Hz, C_9), 164.8 (C_{14}), 175.4 (d, $^4J_{C\text{-}F}$ = 3.0 Hz, C_{13}). ^{19}F NMR (282 MHz, $CDCl_3$): -121.2 (d, $^3J_{H\text{-}F}$ = 12.5 Hz). MS (ESI, positive mode): 459.1 $[M+H]^+$. Elemental Anal. Calcd for $C_{25}H_{35}FN_4O_3$: C, 65.48; H, 7.69; N, 12.22. Found: C, 65.41; H, 7.47; N, 12.22.

4.1.15. (3*S*)-*N*-Octyl-9-fluoro-2,3-dihydro-3-methyl-10-(4-methyl-1-piperazinyl)-7-oxo-7*H*-pyrido[1,2,3-*de*]-1,4-benzoxazine-6-carboxamide 4d

With $C_8H_{17}NH_2$ (0.129 g, 10^{-3} mol) the procedure described above provided after flash chromatography on silica gel with CH_2Cl_2/MeOH 100:7.5 the desired compound **4d** (0.168 g, 36% yield) as yellowish solid. R_f 0.17 (90:10 CH_2Cl_2/MeOH). Mp 122 °C.

^{1}H NMR (300 MHz): 0.88 (t, $^3J_{H\text{-}H}$ = 6.8 Hz, 3H, C_a–H), 1.21–1.47 (m, 10H, $C_{b\text{-}f}$–H), 1.58 (d, $^3J_{H\text{-}H}$ = 6.9 Hz, 3H, C_{3a}–H), 1.63 (m, 2H, C_g–H), 2.37 (s, 3H, $C_{4'a}$–H), 2.56 (m, 4H, $C_{3'}$–H and $C_{5'}$–H), 3.29–3.51 (m, 6H, C_h–H, $C_{2'}$–H and $C_{6'}$–H), 4.26–4.47 (m, 3H, C_2–H and C_3–H), 7.71 (d, $^3J_{H\text{-}F}$ = 12.3 Hz, 1H, C_8–H), 8.64 (s, 1H, C_5–H), 9.98 (t, $^3J_{H\text{-}H}$ = 5.6 Hz, 1H, N–H). ^{13}C NMR (75 MHz): 14.1 (C_a), 18.2 (C_{3a}), 22.7, 27.2, 29.2, 29.3, 29.7, 31.8, 39.3 ($C_{b\text{-}h}$), 46.4 ($C_{4'a}$), 50.6 (d, $^4J_{C\text{-}F}$ = 4.1 Hz, $C_{2'}$ and $C_{6'}$), 54.8 (C_3), 55.7 ($C_{3'}$ and $C_{5'}$), 68.2 (C_2), 105.1 (d, $^2J_{C\text{-}F}$ = 24.0 Hz, C_8), 111.3 (C_6), 122.5 (d, $^3J_{C\text{-}F}$ = 8.7 Hz, C_7), 124.3 (d, $^4J_{C\text{-}F}$ = 1.3 Hz, C_{12}), 131.8 (d, $^2J_{C\text{-}F}$ = 14.6 Hz, C_{10}), 139.4 (d, $^3J_{C\text{-}F}$ = 6.7 Hz, C_{11}), 143.8 (C_5), 155.8 (d, $^1J_{C\text{-}F}$ = 245.9 Hz, C_9), 164.8 (C_{14}), 175.4 (d, $^4J_{C\text{-}F}$ = 2.9 Hz, C_{13}). ^{19}F NMR (282 MHz): -121.2 (d, $^3J_{H\text{-}F}$ = 11.3 Hz). MS (ESI, positive mode): 473.2 $[M+H]^+$. Elemental Anal. Calcd for $C_{26}H_{37}FN_4O_3{\cdot}0.5H_2O$: C, 64.84; H, 7.95; N, 11.63. Found: C, 64.87; H, 7.72; N, 11.67.

4.1.16. (3*S*)-*N*-Decyl-9-fluoro-2,3-dihydro-3-methyl-10-(4-methyl-1-piperazinyl)-7-oxo-7*H*-pyrido[1,2,3-*de*]-1,4-benzoxazine-6-carboxamide 4e

With $C_{10}H_{21}NH_2$ (0.157 g, 10^{-3} mol) the procedure described above provided after flash chromatography on silica gel with CH_2Cl_2/MeOH 100:7.5 the desired compound **4e** (0.176 g, 35% yield) as yellowish solid. R_f 0.17 (90:10 CH_2Cl_2/MeOH). Mp 133 °C.

^{1}H NMR (300 MHz): 0.88 (t, $^3J_{H\text{-}H}$ = 6.6 Hz, 3H, C_a–H), 1.19–1.47 (m, 14H, $C_{b\text{-}h}$–H), 1.58 (d, $^3J_{H\text{-}H}$ = 6.6 Hz, 3H, C_{3a}–H), 1.64 (m, 2H, C_i–H), 2.37 (s, 3H, $C_{4'a}$–H), 2.56 (m, 4H, $C_{3'}$–H and $C_{5'}$–H), 3.29–3.50 (m, 6H, C_j–H, $C_{2'}$–H and $C_{6'}$–H), 4.27–4.46 (m, 3H, C_2–H and C_3–H), 7.71 (d, $^3J_{H\text{-}F}$ = 12.6 Hz, 1H, C_8–H), 8.64 (s, 1H, C_5–H), 9.98 (t, $^3J_{H\text{-}H}$ = 5.6 Hz, 1H, N–H). ^{13}C NMR (75 MHz): 14.1 (C_a), 18.2 (C_{3a}), 22.7, 27.2, 29.3, 29.4, 29.5, 29.6, 29.6, 31.9, 39.3 ($C_{b\text{-}j}$), 46.4 ($C_{4'a}$), 50.6 (d, $^4J_{C\text{-}F}$ = 4.1 Hz, $C_{2'}$ and $C_{6'}$), 54.8 (C_3), 55.7 ($C_{3'}$ and $C_{5'}$), 68.2 (C_2), 105.1 (d, $^2J_{C\text{-}F}$ = 24.0 Hz, C_8), 111.3 (C_6), 122.5 (d, $^3J_{C\text{-}F}$ = 8.7 Hz, C_7), 124.3 (d, $^4J_{C\text{-}F}$ = 1.3 Hz, C_{12}), 131.8 (d, $^2J_{C\text{-}F}$ = 14.5 Hz, C_{10}), 139.4 (d, $^3J_{C\text{-}F}$ = 6.7 Hz, C_{11}), 143.8 (C_5), 155.8 (d, $^1J_{C\text{-}F}$ = 245.9 Hz, C_9), 164.8 (C_{14}), 175.4 (d, $^4J_{C\text{-}F}$ = 2.9 Hz, C_{13}). ^{19}F NMR (282 MHz): −121.2 (d, $^3J_{H\text{-}F}$ = 11.3 Hz). MS (ESI, positive mode): 501.5 $[M+H]^+$. Elemental Anal. Calcd for $C_{28}H_{41}FN_4O_3{\cdot}0.3H_2O$: C, 66.46; H, 8.29; N, 11.07. Found: C, 66.35; H, 7.97; N, 11.07.

4.1.17. (3*S*)-*N*-Dodecyl-9-fluoro-2,3-dihydro-3-methyl-10-(4-methyl-1-piperazinyl)-7-oxo-7*H*-pyrido[1,2,3-*de*]-1,4-benzoxazine-6-carboxamide 4f

With $C_{12}H_{25}NH_2$ (0.185 g, 10^{-3} mol) the procedure described above provided after flash chromatography on silica gel with CH_2Cl_2/MeOH 100:7.5 the desired compound **4f** (0.197 g, 37% yield) as yellowish solid. R_f 0.19 (90:10 CH_2Cl_2/MeOH). Mp 101 °C.

^{1}H NMR (300 MHz): 0.88 (t, $^3J_{H\text{-}H}$ = 6.8 Hz, 3H, C_a–H), 1.19–1.47 (m, 18H, $C_{b\text{-}j}$–H), 1.58 (d, $^3J_{H\text{-}H}$ = 6.6 Hz, 3H, C_{3a}–H), 1.63 (m, 2H, C_k–H), 2.37 (s, 3H, $C_{4'a}$–H), 2.55 (m, 4H, $C_{3'}$–H and $C_{5'}$–H), 3.28–3.49 (m, 6H, C_l–H, $C_{2'}$–H and $C_{6'}$–H), 4.26–4.46 (m, 3H, C_2–H and C_3–H), 7.71 (d, $^3J_{H\text{-}F}$ = 12.6 Hz, 1H, C_8–H), 8.64 (s, 1H, C_5–H), 9.98 (t, $^3J_{H\text{-}H}$ = 5.6 Hz, 1H, N–H). ^{13}C NMR (75 MHz): 14.1 (C_a), 18.2 (C_{3a}), 22.7, 27.2, 29.3, 29.4, 29.5, 29.6, 29.6, 29.7, 31.9, 39.3 ($C_{b\text{-}l}$), 46.4 ($C_{4'a}$), 50.6 (d, $^4J_{C\text{-}F}$ = 4.1 Hz, $C_{2'}$ and $C_{6'}$), 54.8 (C_3), 55.7 ($C_{3'}$ and $C_{5'}$), 68.2 (C_2), 105.1 (d, $^2J_{C\text{-}F}$ = 24.0 Hz, C_8), 111.3 (C_6), 122.5 (d, $^3J_{C\text{-}F}$ = 8.8 Hz, C_7), 124.3 (d, $^4J_{C\text{-}F}$ = 1.2 Hz, C_{12}), 131.8 (d, $^2J_{C\text{-}F}$ = 14.6 Hz, C_{10}), 139.4 (d, $^3J_{C\text{-}F}$ = 6.7 Hz, C_{11}), 143.8 (C_5), 155.8 (d, $^1J_{C\text{-}F}$ = 245.9 Hz, C_9), 164.8 (C_{14}), 175.4 (d, $^4J_{C\text{-}F}$ = 2.9 Hz, C_{13}). ^{19}F NMR (282 MHz): −121.2 (d, $^3J_{H\text{-}F}$ = 12.4 Hz). MS (ESI, positive mode): 529.6 $[M+H]^+$. Elemental Anal. Calcd for $C_{30}H_{45}FN_4O_3$: C, 68.15; H, 8.58; N, 10.60. Found: C, 68.29; H, 8.36; N, 10.70.

4.1.18. (3*S*)-*N*-Tetradecyl-9-fluoro-2,3-dihydro-3-methyl-10-(4-methyl-1-piperazinyl)-7-oxo-7*H*-pyrido[1,2,3-*de*]-1,4-benzoxazine-6-carboxamide 4g

With $C_{14}H_{29}NH_2$ (0.213 g, 10^{-3} mol) the procedure described above provided after flash chromatography on silica gel with

CH_2Cl_2/MeOH 100:7.5 the desired compound **4g** (0.216 g, 39% yield) as yellowish solid. R_f 0.20 (90:10 CH_2Cl_2/MeOH). Mp 90 °C.

^{1}H NMR (300 MHz): 0.89 (t, $^3J_{H\text{-}H}$ = 6.8 Hz, 3H, C_a–H), 1.20–1.47 (m, 22H, $C_{b\text{-}l}$–H), 1.58 (d, $^3J_{H\text{-}H}$ = 6.6 Hz, 3H, C_{3a}–H), 1.64 (m, 2H, C_m–H), 2.38 (s, 3H, $C_{4'a}$–H), 2.57 (m, 4H, $C_{3'}$–H and $C_{5'}$–H), 3.30–3.50 (m, 6H, C_n–H, $C_{2'}$–H and $C_{6'}$–H), 4.27–4.47 (m, 3H, C_2–H and C_3–H), 7.72 (d, $^3J_{H\text{-}F}$ = 12.6 Hz, 1H, C_8–H), 8.64 (s, 1H, C_5–H), 9.98 (t, $^3J_{H\text{-}H}$ = 5.6 Hz, 1H, N–H). ^{13}C NMR (75 MHz): 14.1 (C_a), 18.2 (C_{3a}), 22.7, 27.2, 29.4, 29.4, 29.5, 29.6, 29.7, 29.7, 29.7, 29.7, 31.9, 39.3 ($C_{b\text{-}n}$), 46.4 ($C_{4'a}$), 50.6 (d, $^4J_{C\text{-}F}$ = 4.1 Hz, $C_{2'}$ and $C_{6'}$), 54.8 (C_3), 55.7 ($C_{3'}$ and $C_{5'}$), 68.2 (C_2), 105.1 (d, $^2J_{C\text{-}F}$ = 24.0 Hz, C_8), 111.3 (C_6), 122.5 (d, $^3J_{C\text{-}F}$ = 8.8 Hz, C_7), 124.3 (d, $^4J_{C\text{-}F}$ = 1.3 Hz, C_{12}), 131.8 (d, $^2J_{C\text{-}F}$ = 14.6 Hz, C_{10}), 139.4 (d, $^3J_{C\text{-}F}$ = 6.6 Hz, C_{11}), 143.8 (C_5), 155.8 (d, $^1J_{C\text{-}F}$ = 245.9 Hz, C_9), 164.8 (C_{14}), 175.4 (d, $^4J_{C\text{-}F}$ = 2.9 Hz, C_{13}). ^{19}F NMR (282 MHz): -121.2 (d, $^3J_{H\text{-}F}$ = 14.1 Hz). MS (ESI, positive mode): 557.6 $[M+H]^+$. Elemental Anal. Calcd for $C_{32}H_{49}FN_4O_3$: C, 69.03; H, 8.87; N, 10.06. Found: C, 68.78; H, 8.67; N, 9.93.

4.1.19. (3*S*)-*N*-Pentadecyl-9-fluoro-2,3-dihydro-3-methyl-10-(4-methyl-1-piperazinyl)-7-oxo-7*H*-pyrido[1,2,3-*de*]-1,4-benzoxazine-6-carboxamide 4h

With $C_{15}H_{31}NH_2$ (0.227 g, 10^{-3} mol) the procedure described above provided after flash chromatography on silica gel with CH_2Cl_2/MeOH 100:7.5 the desired compound **4h** (0.231 g, 40% yield) as yellowish solid. R_f 0.20 (90:10 CH_2Cl_2/MeOH). Mp 87 °C.

^{1}H NMR (300 MHz): 0.90 (t, $^3J_{H\text{-}H}$ = 6.6 Hz, 3H, C_a–H), 1.21–1.49 (m, 24H, $C_{b\text{-}m}$–H), 1.59 (d, $^3J_{H\text{-}H}$ = 6.6 Hz, 3H, C_{3a}–H), 1.65 (m, 2H, C_n–H), 2.38 (s, 3H, $C_{4'a}$–H), 2.57 (m, 4H, $C_{3'}$–H and $C_{5'}$–H), 3.29–3.52 (m, 6H, C_o–H, $C_{2'}$–H and $C_{6'}$–H), 4.28–4.49 (m, 3H, C_2–H and C_3–H), 7.72 (d, $^3J_{H\text{-}F}$ = 12.6 Hz, 1H, C_8–H), 8.65 (s, 1H, C_5–H), 10.00 (t, $^3J_{H\text{-}H}$ = 5.7 Hz, 1H, N–H). ^{13}C NMR (75 MHz): 14.1 (C_a), 18.3 (C_{3a}), 22.7, 27.2, 29.4, 29.4, 29.6, 29.7, 29.7, 29.7, 29.7, 29.7, 31.9, 39.3 ($C_{b\text{-}o}$), 46.4 ($C_{4'a}$), 50.6 (d, $^4J_{C\text{-}F}$ = 4.1 Hz, $C_{2'}$ and $C_{6'}$), 54.9 (C_3), 55.7 ($C_{3'}$ and $C_{5'}$), 68.2 (C_2), 105.1 (d, $^2J_{C\text{-}F}$ = 24.0 Hz, C_8), 111.3 (C_6), 122.5 (d, $^3J_{C\text{-}F}$ = 8.7 Hz, C_7), 124.3 (d, $^4J_{C\text{-}F}$ = 1.3 Hz, C_{12}), 131.9 (d, $^2J_{C\text{-}F}$ = 14.6 Hz, C_{10}), 139.4 (d, $^3J_{C\text{-}F}$ = 6.8 Hz, C_{11}), 143.8 (C_5), 155.8 (d, $^1J_{C\text{-}F}$ = 245.8 Hz, C_9), 164.8 (C_{14}), 175.4 (d, $^4J_{C\text{-}F}$ = 2.9 Hz, C_{13}). ^{19}F NMR (282 MHz): −121.2 (d, $^3J_{H\text{-}F}$ = 12.4 Hz). MS (ESI, positive mode): 571.6 $[M+H]^+$. Elemental Anal. Calcd for $C_{33}H_{51}FN_4O_3{\cdot}0.6H_2O$: C, 69.44; H, 9.01; N, 9.82. Found: C, 69.10; H, 8.93; N, 9.75.

4.2. Stability tests

HPLC analysis was performed on a Waters system (Waters Associates Inc., Milford, MA, USA) consisting of a 600 controller pump, a PDA996 diode array detector, a 717 plus autosampler, and a Lisa 30 Ecrosas (ICS) oven at 30 °C. The instrument was controlled by the Empower software. The experiments were carried out on a C18 Luna (Phenomenex) reverse-phase column (length 100 × 3 mm i.d., 3 μm particle size) eluted with a mobile phase consisting of various linear gradients of Solvent A (MeOH + 0.1% TFA)/Solvent B (water + 0.1% TFA) at a flow rate of 0.6 mL/min. The eluting solvents were prepared daily and degassed with helium during analyses. The detection was set at 292 nm. All injections (10 μL) were made in duplicate.

In a typical experiment, 100 μL of a 1 mM solution of tested compound in DMSO was diluted with acetonitrile/phosphate buffer (pH 7.4) (50:50) to a final volume of 1 mL. An aliquot was discarded and kept at 4 °C (t_0). The remaining solution placed in a sealed tube was heated in a water bath at 37 °C for 7 days (t_{7d}). To evaluate the rate of degradation of the tested compounds, LV (degradation product expected for esters **2a–i** and amides **4a–h**) was injected. Under these conditions, all derivatives were totally separated with respective retention times at 2.4 min (LV **1**), 7.1–15.0 min (esters **2a**,**c**,**e**,**g**) and 11.7–15.4 min (amides **4a**,**b**,**d**,**g**). For compounds whose stability was less than 100%, the following ratio was calculated: stability(%) = [mean measured concentration (n = 2) at 7d/mean measured concentration (n = 2) at t_0] × 100.

4.3. Pharmacology

4.3.1. Evaluation of in vitro cell proliferation by means of the MTT colorimetric assay

The assessment of cell population growth is based on the capability of living cells to reduce the yellow product MTT (3-[4,5-dimethylthiazol-2yl]-diphenyl tetrazolium bromide; Sigma, Bornem, Belgium) to a blue product, formazan, by a reduction reaction occurring in the mitochondria.[32a,b] The five cell lines were incubated for 24 h in 96-microwell plates (at a concentration of 10^4 to 3.10^4 cells/mL culture medium depending on the cell type) to ensure adequate plating prior to cell growth determination. The number of living cells after 120 h of culture in the presence or absence (control) of the various drugs is directly proportional to the intensity of the blue color, measured by spectrophotometry using a 680XR microplate reader (Bio-Rad Laboratories Inc, Hercules, CA) at a wavelength of 570 nm (with a reference at 630 nm). Each experiment was carried out in hexaplicate. Nine concentrations ranging from 10^{-3} to 10^{-7} M (with semi-log decrease in concentration) were tested for each of the compounds under study. The compounds were dissolved in DMSO at a final concentration of 0.1% DMSO in the cell culture media for the highest concentrations analyzed for each compound. These DMSO concentrations have no detectable effects at both cell proliferation[32a,b] and cell migration[35b,c] features of various normal and cancer cell lines.

4.3.2. In vivo testing: maximum tolerated dose[33]

The maximum tolerated dose (MTD) of a drug is defined as the maximum dose which can be administered acutely to healthy animals (i.e., not grafted with tumors). The acute MTD was determined following single ip administration of increasing drug doses to groups of three healthy B6D2F1 female mice (Charles River, Brussels, Belgium). Five dose levels (5, 10, 20, 40 and 80 mg/kg) of drug were evaluated. The MTD was defined as the dose just below the lowest dose level that killed at least one mouse in a treatment group after a maximum of 28 days.

4.3.3. Cell migration assay

The effects of compounds **2e** and **2h** on cell motility levels in A549 and U373-MG human cancer cell lines were analyzed. The cancer cell migration levels were characterized quantitatively on a computer-assisted device, as detailed elsewhere.[34a,35b,c] This device enables the trajectories of culture maintained living cells to be quantified. The greatest linear distance migrated by each cell was calculated from these trajectories. This distance is in fact the maximum relative distance from the point of origin, that is, the MRDO quantitative variable. The experiments were all performed over 72 h, and one image was recorded every 4 min.[34a,35b,c] Since the analyses were carried out in triplicate, a minimum of 121 and a maximum of 184 cells were analyzed in each experimental condition. The influence of compounds **2e** and **2h** on human cancer cell migration was analyzed at 0.1 and 1 μM, respectively.

Supplementary data

Supplementary data (HPLC analytical conditions for stability tests and retention times for compounds **1**, **2a**,**c**,**e**,**g** and **4a**,**b**,**d**,**g**) associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.10.039.

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