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Preliminary communication

Structural variations of piritrexim, a lipophilic inhibitor of human dihydrofolate reductase: synthesis, antitumor activity and molecular modeling investigations

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Abstract

Piritrexim (PTX) **(1)**, a lipophilic inhibitor of the human dihydrofolate reductase, has been evaluated as an anticancer agent. The synthesis of four structural variations **(2–5)** of PTX is reported. The PTX analogues ***2–5*** were obtained by reaction of suitable C_3-building blocks with pyrimidine-2,4,6-triamine **(14)** or with cyanacetamide **(7)** and guanidine **(10)**. The evaluation of ***2–4*** for antitumor activity against a panel of 60 human cancer cell lines showed inhibitory effects on the growth of the cell lines. These data are supported by molecular modeling and docking studies, which show that compounds ***2–4*** share the same binding mode within the DHFR active site. Moreover, the estimated ligand binding energies are in good agreement with the experimental activity data.

Keywords: Piritrexim; Pyrido[2,3-*d*]pyrimidines; Dihydrofolate reductase inhibitor; Antitumor activity; Molecular modeling; Docking studies

1. Introduction

Piritrexim (PTX) **(1),** first synthesized by Grivski et al. [1], is a lipophilic inhibitor of human dihydrofolate reductase with a pyrido[2,3-*d*]pyrimidine-2,4-diamine-structure. In contrast to methotrexate, PTX needs no active transport mechanism to reach into the cancer cell [2]. Piritrexim belongs to the lipophilic dihydrofolate reductase inhibitors of the second generation with lower affinity to histamin-*N*-methyl transferase resulting in less toxic side effects. [1,3,4]. In the NCI Cancer Test, PTX showed a significant activity against Walker 256 carcinoma, P 388 leukaemia, sarcoma 180 and the Ehrlich ascites carcinoma. In phase II studies activity against melanoma, lung cancer, colon cancer, sarcoma and head and neck cancer were reported [2].

One goal in our studies is the synthesis of new 5-deaza- and 8-deazapteridines as folate antagonists. Such a facile synthesis of alkyl and aryl substituted pyrido[2,3-*d*]pyrimidine-2,4-diamines has been reported [5]. In previous work, Iso-PTX with a methyl group in position 7 instead of position 5 and an alternative synthesis of PTX was described [6,7].

It is well known that the pyrimidine-2,4-diamine structure is essential for inhibition of the dihydrofolate reductase by PTX and that increase of the chain to the aromatic ring in position 5 reduces activity [8–10]. In order to prepare new PTX-analogues, derivatives with a missing methyl group in position 5, an additional methyl group in position 7, and with an elongated sidechain in position 6 were prepared. Also the 5,6,7,8-tetrahydro derivative of Nor-PTX **(3)** was synthesized as a new structure modification (Fig. 1).

2. Chemistry

In the course of our syntheses of new folate antagonists with a pyrido[2,3-*d*]pyrimidine structure, different C_3-building blocks, e.g., β-dicarbonyl derivatives or α,β-unsaturated aldehydes, were cyclocondensated with different

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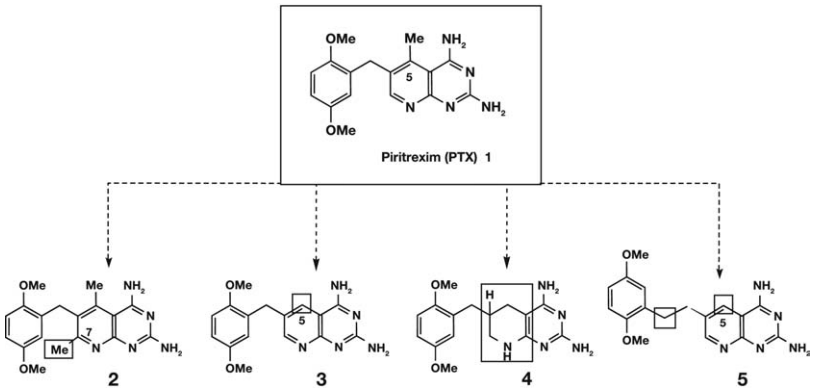

Fig. 1. Structural variation of PTX 1.

1,3-bisnucleophiles, e.g., cyanacetamide **(7)** or pyrimidine-2,4,6-triamine **(14)**.

For the preparation of PTX analogue **2**, we used the same route by refluxing 1,3-diketone **6** [11] with cyanacetamide **(7)** in ethanol in the presence of piperidine [12,13]. The resulting pyridone **8** was treated with dimethylformamide and thionylchloride to yield the 2-chloronicotinonitrile **9** [1] which was reacted with guanidine **(10)** to yield 7-methyl-PTX **(2)** [14] (Fig. 2).

Our synthesis of Nor-PTX **(3)** [15,16] started from the known malonate **11** [17], which was hydrolyzed to give the acid **12** [18]. **12** was than treated with dimethylformamide and phosphoroxychloride under mild conditions and further with *N*,*N*-dimethylammoniumperchlorate to yield the trimethinium perchlorate **13**, representing a reactive derivative of malondialdehyde [19]. **13** cyclocondensated in good yields with pyrimidine-2,4,6-triamine **(14)** giving Nor-PTX **(3)**. Recently an alternative preparation of Nor-PTX **(3)** has been published by condensation **14** and bromomalondialdehyde, followed by protection of amino groups and Pd-mediated coupling with 2,5-dimethoxybenzyl zinc chloride and final deprotection of the pivaloylated aminogroups [16] (Fig. 3).

An alternative C_3-building block for the synthesis of C-5 unsubstituted PTX derivatives **3** and **4** is the acrylaldehyde derivative **18**. The synthesis of **18** started from 3-(2,5-dimethoxyphenyl)propanol **(15)** [20] with a *Swern*-Oxidation with oxalylchloride and dimethylsulfoxide at –60 °C [21,22], followed by treatment with *N*,*N*-dimethyl-

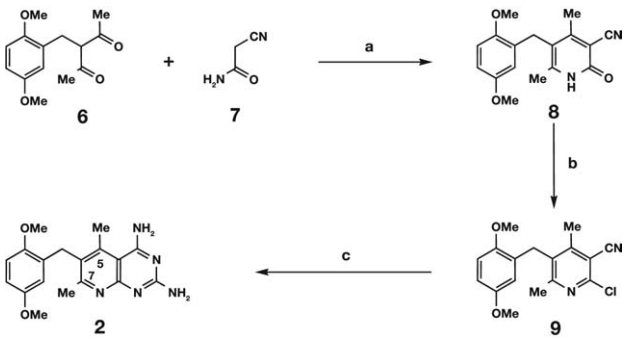

Fig. 2. Synthesis of 7-methyl-PTX reagents: (a) EtOH, piperidine, reflux; (b) $SOCl_2$, DMF, CH_2Cl_2, 80 °C; (c) pyridine, guanidine (10) reflux.

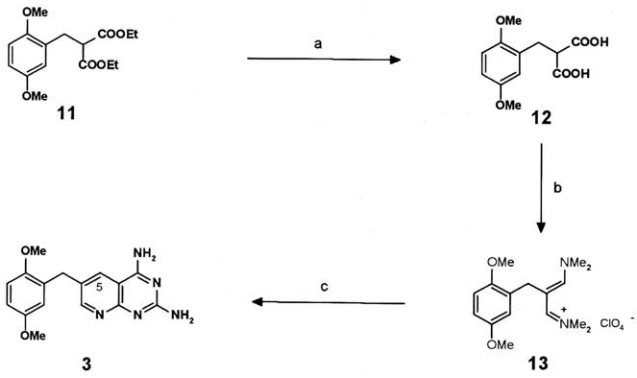

Fig. 3. Synthesis of Nor-PTX. Reagents: (a) NaOH 15% in H_2O:EtOH(1:1), 80 °C, HCl_{conc}; (b) dimethyl formamide, $POCl_3$, RT, $Me_2NH_2ClO_4$; (c) *n*-BuOH pyrimidine-2,4,6-triamine **(14)**, reflux.

methylenimmoniumchloride ("Böhme-Salz") **(16)** giving the Mannich base-hydrochloride **17** [23]. This Mannich base-HCl was unstable and eliminated dimethylamine and HCl giving the acrylaldehyde derivative **18** during purification. Reaction of **18** with pyrimidine-2,4,6-triamine **(14)** in a mixture of ethanol/acetic acid (1:1) at reflux yielded the dihydro compound **19**. Following oxidation with air in acetic acid under reflux gave Nor-PTX **(3)**. Reduction of **19** in trifluoroacetic acid with hydrogen and Pd/C gave rise to 5,6,7,8-tetrahydro derivative **4** (Fig. 4).

The synthesis of PTX analogue **5** with an elongated side chain is similar to our Nor-PTX procedure. Alkylation of diethylmalonate **(21)** with 2-(2,5-dimethoxyphenyl)ethyl-bromide **(20)** gave the substituted malonate **22** [24], which was hydrolyzed to 2-(2,5-dimethoxyphenyl)ethylmalonic acid **(23)** [25,26]. Treatment with dimethylformamide, phosphoroxychloride and crystallisation with *N*,*N*-dimethyl ammonium per chlorate gave trimethinium salt **24** [27], which cyclocondensated with pyrimidine-2,4,6-triamine **(14)** to give Homo-Nor-PTX **(5)** (Fig. 5).

3. Pharmacology

The enzyme dihydrofolate reductase is responsible for the reduction of dihydrofolic acid (DHFA) to tetrahydrofolic

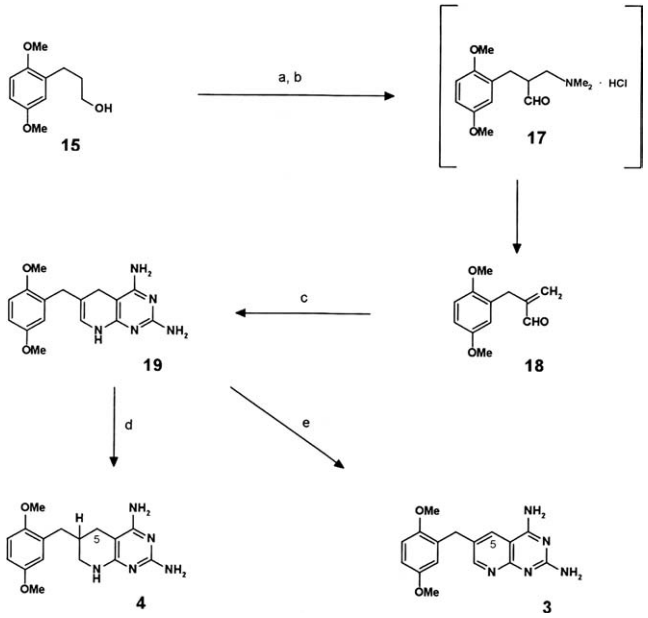

Fig. 4. Synthesis of Tetrahydro-Nor-PTX. Reagents: (a) oxaylchloride, dimethylsulfoxide, CH_2Cl_2, –60 °C; (b) Böhme salt **(16)** CH_2Cl_2, RT; (c) pyrimidine-2,4,6-triamine **(14)**, EtOH, HOAc, reflux; (d) H_2/Pd-Ac (10%), TFAA, RT; (e) air, HOAc, reflux.

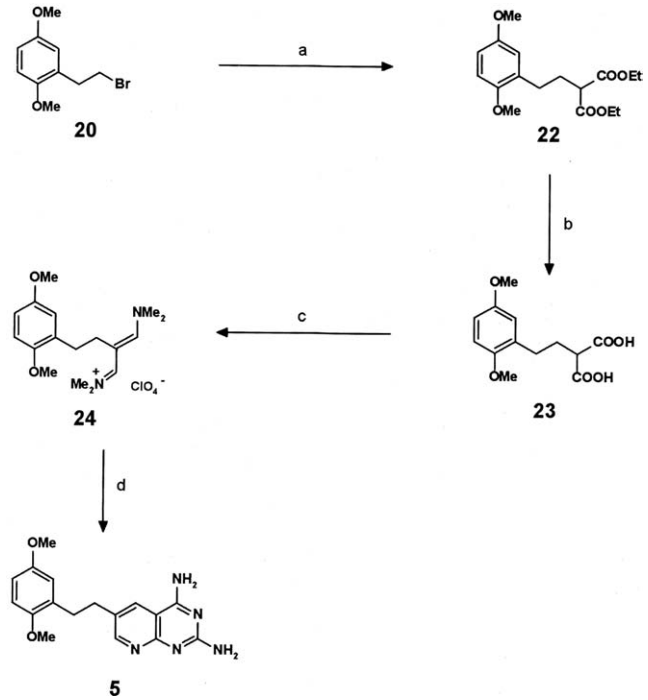

Fig. 5. Synthesis of PTX-analogue 5. Reagents: (a) diethylmalonate **(21)**, NaH, THF; (b) 15% aqueous NaOH, 80 °C; $HCl_{conc.}$ (c) dimethylformamide, $POCl_3$, 30 °C, $Me_2NH_2ClO_4$; (d) *n*-BuOH, pyrimidine-2,4,6-triamine (**14**), reflux.

acid (THFA), by using NADPH as cofactor. Tetrahydrofolic acid represents a C_1-building block for the cellular biosynthesis of purines, pyrimidines or amino acids [8,28,29]. Especially for the synthesis of thymidilate, a permanent supply of THFA out of DHFA is essential. Inhibition of dihydrofolate reductase results in lack of desoxythymidin-monophosphate and finally in a thymidinless cell-death [30,31].

Methotrexate, a classical folate antagonist, is well established in antitumor therapy [32]. Unfortunately the hydrophilic nature of the molecule limits transport into the cells of lung and CNS by diffusion [33]. Before methotrexate can act as an antitumor agent, an active transport mechanisms into the cell and polyglutamation by the enzyme folypolyglutamate synthase is necessary. A new class of dihydrofolate reductase inhibitors is represented by lipophilic molecules with a quinazoline or a pyrido[2,3-*d*]pyrimidine core, e.g., PTX **(1)**. These lipophilic inhibitors do not need an active transport mechanism for diffusion into the cell and no activation as they lack a glutamate sidechain. Piritrexim represents a non-classical lipophilic inhibitor of dihydrofolate reductase of the second generation as it has no affinity to histamin-*N*-methyltransferase giving less toxic side effects [34].

The idea behind the lipophilic dihydrofolate reductase inhibitors is to overcome drug resistances. In phase II studies activity of PTX **(1)** against malignant melanomas, lung cancer, colon cancer, sarcoma and head and neck cancer were reported [35]. In addition, PTX **(1)** is active as an antipsoriasis agent and as a drug against opportunistic infections with *Pneumocystis carinii* and *Toxoplasma gondii* [33]. Secondary infections with these organisms are the main cause of death for AIDS patients.

4. Molecular modeling studies

To understand the obtained pharmacological data on a structural basis, we analysed the DHFR inhibitors MTX, PTX, and the newly synthesized and tested compounds **2–4** by molecular modeling and docking techniques. There are several crystal structures of DHFR in complex with MTX and one in complex with PTX available through the RCSB Protein Data Bank [36]. Structural comparison of the human DHFR mutant L22Y-MTX complex (Protein Data Bank entry 4DLS) and the human DHFR mutant L22F-PTX complex (Protein Data Bank entry 1DLR) by fitting the protein C_α carbon atoms revealed that both inhibitors share a common interaction pattern with the protein, despite of their very different size. The pyrido[2,3-*d*]pyrimidine moiety of PTX is able to form the same hydrogen bond binding pattern with the protein as the corresponding substructure of MTX. Moreover, the *p*-dimethoxyphenyl subunit of PTX arranges in the DHFR binding site almost parallel to the 1,4-disubstituted phenyl group of MTX.

To investigate the influence of PTX structure modifications on the DHFR binding behaviour, we used the molecular docking package QXP (Quick eXPlore) to predict possible orientations of the various ligands within the DHFR binding site. The program allows the positioning of the fully flexible ligands within a partially flexible binding cleft by a Monte

Carlo procedure followed by energy minimization in combination with several scoring methods for binding affinity estimation [37]. To verify and check the docking procedure, we first applied the protocol to the already experimentally known MTX and PTX complexes named above. In both cases, QXP was able to predict the binding mode of the inhibitors within the DHFR active site correct (data not shown).

5. Results and discussion

Piritrexim analogues **2–4** were evaluated for antitumor activity in the NCI's in vitro disease-oriented antitumor screening against a panel of 60 human tumor cell lines derived from leukaemia, lung cancer, colon cancer, CNS cancer, melanoma, ovarian cancer, renal cancer, prostate cancer and breast cancer. The compounds were diluted to five concentrations, the highest being 10^{-4} mol/l, the others 10^{-5}, 10^{-6}, 10^{-7} and the lowest 10^{-8} mol/l. The growth inhibition power (GI_{50}) was evaluated and the results obtained are shown in Table 1.

The majority of tumors were inhibited by micromolar concentrations (10^{-6} mol/l) of the test compounds, in some cases even at concentrations of 10^{-7} or 10^{-8} mol/l. The 5,6,7,8-tetrahydro compound **4** showed the highest average activity against all the 60 cell lines with GI_{50} values between 0.024 and 89.1 μmol/l. It also was the most active compound against leukaemic cell lines with a GI_{50} concentration of 0.024 μmol/l against leukaemia K-562, followed by a GI_{50} concentration of 0.087 μmol/l against colon cancer HCT-116 and 0.10 μmol/l against non-small lung cancer NHI-H460.

The GI_{50} concentrations of Nor-PTX **(3)** were between 0.135 and >100 μmol/l. It mainly had a activity against leukaemia cell lines (K-562, 0.135 μmol/l; HL-60(TB), 0.138 μmol/l; MOLT-4, 0.174 μmol/l; SR, 0.417 μmol/l; CCRF, 0.832 μmol/l). It also inhibited the growth of non-small lung cancer cells A549/ATCC (GI_{50} = 0.295 μmol/l) and NCI-H460 (GI_{50} = 0.229 μmol/l). Finally, the colon cancer cell lines HCT-116 (GI_{50} = 0.209 μmol/l) and SW-620 (GI_{50} = 0.240 μmol/l) were sensitive against compound **3**.

The 7-methyl-PTX **(2)** had GI_{50} concentrations between 0.214 and 15.448 μmol/l in the antitumor test. **2** showed highest activity against leukaemia cell line K-562 with a GI_{50} concentration of 0.214 μmol/l and against colon cancer HCT-116 (GI_{50} = 0.389 μmol/l) (Table 1).

In order to compare the binding affinity of the newly synthesized PTX analogues, we docked the compounds **1–4** into the empty binding site of the experimentally known human DHFR mutant 1DLR. In these simulations, the ligand is fully flexible (free translation, rotation and rotation of functional groups about single bonds). Within the protein, the side chains Glu30 and Phe31, as well as the crystal water molecule 252, are also kept flexible to improve the interaction to the ligand. Fig. 6 shows as a representative example an overlay of the best docking solutions with the highest predicted binding affinity for PTX and compound **2**. Both compounds share the same binding mode, which is also the same for **3** and **4** (not visualized in Fig. 6). For the estimation of the binding affinity, the non-bonded Coulomb and van der Waals interactions between protein and ligand, as well as the ligand energy relative to the estimated global minimum of the free ligand, and the binding site energy relative to the local minimum for the empty site were taken into account. All new synthesized compounds show the same binding pattern as PTX within the experimentally resolved complex 1DLR. Moreover, the predicted binding affinities for the compounds **1–4** (**1**: –78.8; **2**: –48.3; **3**: –62.6; **4**: –60.1; all values in kJ mol^{-1}) are in good agreement with the experimentally determined activity data. Therefore, we are now able to rank new PTX homologues in terms of their expected affinity to the DHFR active site.

6. Conclusions

From literature and our own studies, we knew about the importance of the pyrimidine-2,4-diamine substructure for the inhibition of dihydrofolate reductase [8]. It was also reported that an increasing substituent in position 5 attenuates the potency of PTX derivatives [9]. In our studies, we focused on methyl-substituents in position 5 and 7 and hydrogenation of the pyridine ring of the molecule.

Test results showed that the activity of Iso-PTX, with a methyl group in position 7 instead of position 5 [6,7,15], is 20 times weaker than PTX **(1)**. **2** with an additional methyl group in position 7 of PTX is seven times lower in activity than PTX. Nor-PTX **(3)** is five times, the tetrahydro derivative **4** four times less active than PTX in average. The shift of the methyl group from position 5–7 lowers activity and also an additional group in position 7 gives no improvement. The experimental findings are in good agreement with predicted binding affinities obtained by molecular docking studies, which allow an at least qualitative ranking of new, not yet synthesized and tested PTX homologues.

7. Experimental protocols

7.1. Chemistry

Melting points (m.p.) were determined on a BÜCHI, Typ 310, capillary apparatus and are uncorrected. Boiling points were measured on a Kugelrohr distillation apparatus from BÜCHI (Typ GRK 50). IR spectra were determined with a PERKIN–ELMER Typ 1740 infrared Fourier-transformation spectraphotometer in KBr or $CHCl_3$. ^1H- and ^{13}C-NMR spectra were recorded in DMSO-d_6, if no other solvent is named (tetramethylsilane as internal standard), at 250.13 MHz (60.6 MHz) on a Bruker AC 250 or at 360.13 MHz (90.6 MHz) on a BRUKER AM 360. Mass

Table 1
Inhibition of in vitro tumor cell growth by PTX (1), methotrexate and PTX-derivatives **2**–**4**

Panel/cell line	Cytotoxicity GI (50) in μmol/1	([a]) ([b]) ([c]) GI (50) in μmol/1	GI (50) in μmol/1	GI (50) in μmol/1	GI (50) in μmol/1
	1	**2**	**3**	**4**	Methotrexate
Leukaemia					
CCRF-CEM	0.040	1.175	0.832	0.537	0.025
HL-60(TB)	N.D.	0.708	0.138	0.195	0.026
K-562	0.010	0.214	0.135	0.024	0.025
MOLT-4	0.030	0.776	0.174	0.692	0.029
RPMI-8226	0.468	6.166	3.467	9.550	0.151
SR	0.030	0.537	0.417	0.776	0.025
Non-small cell lung cancer					
A549/ATCC	0.010	0.646	0.295	0.219	0.028
EKVX	21.878	13.183	2.188	14.454	7.586
HOP-18	N.D.	N.D.	N.D.	N.D.	7.079
HOP-62	0.219	6.166	1.950	2.512	0.034
HOP-92	16.982	15.488	1.738	N.D.	12.882
NCI-H226	4.898	13.183	13.804	89.125	58.884
NCI-H23	0.224	0.759	0.525	0.245	0.035
NCI-H322M	0.355	8.710	9.333	1.585	0.501
NCI-H460	N.D.	0.741	0.229	0.100	0.025
NCI-H522	N.D.	4.898	N.D.	N.D.	0.151
LXPL 529	N.D.	N.D.	N.D.	N.D.	0.048
Small cell lung cancer					
DMS 114	N.D.	N.D.	N.D.	N.D.	0.026
DMS 273	N.D.	N.D.	N.D.	N.D.	0.039
Colon cancer					
COLO 205	14.125	10.715	7.943	7.079	1.905
DLD-1	N.D.	N.D.	N.D.	N.D.	0.052
HCC-2998	0.063	12.882	N.D.	4.571	0.240
HCT-116	0.013	0.389	0.209	0.087	0.025
HCT-15	0.015	1.622	1.413	N.D.	0.054
HT29	0.029	0.891	0.417	0.603	0.046
KM12	0.427	2.239	2.399	0.550	0.036
KM20L2	N.D.	N.D.	N.D.	N.D.	0.089
SW-620	0.055	1.622	0.240	0.479	0.036
CNS cancer					
SF-268	0.145	6.547	0.933	0.178	0.032
SF-259	0.355	0.813	0.603	0.479	0.042
SF-539	1.660	N.D.	1.122	1.122	0.062
SNB-19	N.D.	2.399	14.791	1.445	0.257
SNB-75	6.761	3.311	27.542	13.183	25.704
SNB-78	N.D.	N.D.	N.D.	N.D.	251.189
U251	5.129	1.413	2.591	0.776	0.141
XF498	N.D.	N.D.	N.D.	N.D.	158.489
Melanoma					
LOX IMVI	0.021	0.562	0.407	0.457	0.025
MALME-3M	N.D.	5.888	12.303	13.804	5.012
M14	0.043	0.955	0.759	0.468	0.028
M19-MEL	N.D.	N.D.	N.D.	N.D.	0.708
SK-MEL-2	0.072	15.488	1.318	40.738	239.883
SK-MEL-28	1.122	10.965	14.454	11.482	4.074
SK-MEL-2	0.098	0.759	0.676	0.741	0.058
UACC257	15.488	12.023	1.995	3.890	0.871
UACC-62	0.072	0.550	0.589	0.661	0.027
Ovarian cancer					
IGROV1	0.013	0.977	0.631	0.851	0.071
OVCAR-3	0.050	10.471	10.000	15.136	0.363
OVCAR-4	16.982	10.471	13.804	16.218	251.189

(continued on next page)

Table 1
(continued)

Panel/cell line	Cytotoxicity GI (50) in μmol/1	([a]) ([b]) ([c]) GI (50) in μmol/1	GI (50) in μmol/1	GI (50) in μmol/1	GI (50) in μmol/1
OVCAR-5	19.055	7.762	12.882	8.318	2.884
OVCAR-8	0.055	0.661	0.525	0.871	0.030
SK-OV-3	12.303	12.023	>100	30.903	128.825
Renal cancer					
786-0	0.263	0.646	0.589	0.427	0.027
A498	N.D.	10.715	2.399	18.197	2.399
ACHN	N.D.	2.455	1.072	1.202	0.046
CAKI-1	0.063	11.482	2.512	N.D.	0.030
RXF-393	2.239	15.136	1.585	13.183	32.359
RXF-631	N.D.	N.D.	N.D.	N.D.	0.059
SN12C	0.513	10.000	0.891	0.776	0.028
TK-10	12.023	11.220	11.220	5.754	93.325
UO-31	0.035	4.266	7.413	11.482	0.091
Prostata cancer					
PC-3	0.052	0.724	3.162	0.288	N.D.
DU-145	0.933	N.D.	2.344	3.162	N.D.
Breast cancer					
MCF7	12.023	3.981	1.820	2.344	N.D.
MCF/ADR-RES	4.467	9.333	63.093	9.120	N.D.
MDA-MB-231/ATCC	17.378	13.490	13.183	0.263	N.D.
HS578T	N.D.	12.023	19.055	8.318	N.D.
MDA-MB-435	0.204	0.794	0.617	0.398	N.D.
MDA-N	0.304	0.759	0.372	0.617	N.D.
BT-549	13.490	1.905	N.D.	10.715	N.D.
T-47D	15.136	N.D.	16.218	20.893	N.D.

[a] Data obtained from NCI's in vitro disease-oriented tumor cells screen.
[b] GI_{50} is the molar concentration causing 50% growth-inhibition of tumor cells. Compounds with GI50 > 100 μmol/l are considered inactive.
[c] N.D., not determined.

spectra were determined on a Finnigan TSQ 70 (electron impact, ionization energy 70 eV). Elemental analyses were measured on a Heraeus Typ CHN-Rapid. MPLC was performed with a Büchi pump Type B-688, Büchi glass columns and Macherey–Nagel silica gel 60, 0.04–0.063 mm, 230–400 mesh.

Fig. 6. Overlay of the best docking solutions of PTX (light grey) and the 7-methyl homologue **2** (dark grey) within the 1DLR-binding pocket. For clarity, the protein is visualized as a ribbon indicating the secondary structure elements without explicit sidechains.

*7.1.1. 6-(2,5-Dimethoxybenzyl)-5,7-dimethylpyrido[2,3-*d*]-pyrimidine-2,4-diamine (**2**)*

To a cold solution of sodium (506 mg, 22 mmol) in methanol (50 ml), guanidine hydrochloride (**10**-**HCl**) (1.84 g, 22 mmol) was added. The residue (NaCl) was separated and washed with methanol. The unified methanol phases were evaporated under reduced pressure. The resulting oil was diluted with pyridine (5 ml) and 2-chloro-5-(2,5-dimethoxybenzyl)-4,6-dimethylnicotinonitrile (**9**) (1040 mg, 3.3 mmol) was added. The mixture was refluxed for 5 h. Then water (25 ml) was added and the residue was isolated. Crystallisation from H_2O/dimethylformamide (20:1) provided 57% (630 mg) of a colourless powder; m.p. 310 °C (H_2O/DMF); IR: cm^{-1} 3339, 3167 (NH_2), 2999 (Ar–H), 1646 (C=N), 1575, 1545, 1498 (C=C); ^{1}H-NMR: δ 2.32 (3H, s, CH_3), 2.48 (3H, s, CH_3), 3.55 (3H, s, OCH_3), 3.82 (3H, s, OCH_3), 3.90 (2H, s, CH_2), 5.99 (1H, d, J = 3 Hz, 6′-H), 6.06 (2H, br, NH_2, D_2O exchangeable), 6.74 (1H, dd, J_1 = 9 Hz, J_2 = 3 Hz, 4′-H), 6.84 (2H, br, NH_2, D_2O exchangeable), 6.94 (1H, d, J = 9 Hz, 3′-H); ^{13}C-NMR:δ 17.37 (5-CH_3), 23.80 (7-CH_3), 27.96 (CH_2), 55.06 (OCH_3), 55.74 (OCH_3), 104.02 (C-4a), 110.19, 111.13, 114.28, 128.37 (C-1′, C-3′, C-4′, C-6′), 124.37 (C-6), 144.24 (C-5), 151.10 153.11 (C-2′, C-5′), 160.54, 161.65, 162.52, 163.74 (C-2, C-4, C-7, C-8a); MS: 339 (100) [M^+], 324 (22) [M^+ -CH_3], 309 (58) [M^+ -CH_3, -CH_3], 308 (40) [M^+ -OCH_3], 293 (19) [M^+ -CH_3,

-OCH_3]. Anal. calculated for $C_{18}H_{21}N_5O_2$ (339.40): C 63.7; H 6.24; N 20.6 (found: C 62.7; H 6.24; N 20.6).

*7.1.2. 6-(2,5-Dimethoxybenzyl)pyrido[2,3-d]pyrimidine-2,4-diamine (**3**) (Nor-PTX)*

Method 1: *N*-[(2*E*)-2-((2,5-dimethoxybenzyl)-3-dimethylamino)prop-2-en-1-ylidene]-*N*-methyl-methanium-perchlorate (**13**) (1.0 g, 2.7 mmol) was heated to reflux with pyrimidine-2,4,6-triamine (**14**) (0.33 g, 2.6 mmol) in *n*-butanol (10 ml) for 10 h. The precipitate was filtered off and crystallized from H_2O/dimethylformamide (20:1) to yield 75% (610 mg) **3** as a colourless powder.

Method 2: 6-(2,5-Dimethoxybenzyl)-5,8-dihydropyrido-[2,3-*d*]pyrimidine-2,4-diamine (**19**) (500 mg, 1.6 mmol) was solved in acetic acid (30 ml) and heated to reflux for 10 h while bubbling air through the solution. The solution was diluted with water (10 ml) and neutralized with 5N aqueous sodium hydroxide. The precipitate was filtered off and crystallized from H_2O/dimethylformamide (20:1) to yield 60% (300 mg) **3** as a colourless powder; m.p. 310 °C (H_2O/MF); IR: cm^{-1} 3429, 3354, 3157 (NH_2), 2996, 2937 (C-H), 2834 (OCH_3), 1675, 1651, (C=N), 1601, 1580, 1504 (C=C); ^{1}H-NMR:δ 3.69 (3H, s, OCH_3), 3.72 (3H, s, OCH_3), 3.91 (2H, s, CH_2), 6.75–6.92 (3H, m, 3′-H, 4′-H, 6′-H), 7.19 (2H, s, 4-NH_2, D_2O exchangeable), 8.28 (2H, s, 2-NH_2, D_2O exchangeable), 8.39 (1H, d, J = 2.5 Hz, 5-H), 8.59 (1H, d, J = 2.5 Hz, 7-H); ^{13}C-NMR:δ 33.20 (CH_2), 55.90 (OCH_3), 56.37 (OCH_3), 105.02 (C-4a), 112.41, 112.54, 117.05, 129.86 (C-1′, C-3′, C-4′, C-6′), 131.82, 133.75 (C-5, C-6), 151.61, 153.64 (C-2′, C-5′), 154.26 (C-4), 156.50 (C-7), 159.62 (C-8a), 163.70 (C-2); MS: 311 (100) [M^+], 296 (15) [M^+ -CH_3], 280 (29) [M^+ -(CH_3O)]. Anal. $C_{16}H_{17}N_5O_2$ (C,H,N).

*7.1.3. 6-(2,5-Dimethoxybenzyl)-5,6,7,8-tetrahydropyrido[2,3-d]pyrimidin-2,4-diamine (**4**)*

A mixture of 6-(2,5-dimethoxybenzyl)-5,8-dihydropyrido-[2,3-*d*]pyrimidine-2,4-diamine (**19**) (0.1 g, 0.032 mol) trifluoroacetic acid (5 ml) and palladium/carbon (10%) (25 mg) was hydrogenated at 400 kPa. The catalyst was separated over cellite and the filtrate concentrated under reduced pressure. The resulting oil was diluted with water (2 ml) and neutralized with concentrated aqueous sodium hydroxide. The precipitate was filtered off and crystallized from H_2O/dimethylformamide (20:1) to yield 60% **4** (60 mg) of a colourless powder; m.p. 268–269 °C (H_2O/DMF); IR: cm^{-1} 3447, 3341, 3181 (NH_2, NH), 2990, 2927 (C-H), 2834 (OCH_3), 1581 (C=N), 1500 (C=C); ^{1}H-NMR:δ 1.93–2.07 (2H, m, CH_2), 2.29–2.68 (3H, m, 5-H, 6-H), 2.72–3.05 (2H, m , 7-H), 3.69 (3H, s, OCH_3), 3.72 (3H, s, OCH_3), 5.10 (2H, s, NH_2, D_2O exchangeable), 5.38 (2H, s, NH_2, D_2O exchangeable), 5.91 (1H, s, NH, D_2O exchangeable), 6.70 (1H, d, J = 3 Hz, 6′-H), 6.74 (1H, dd, J_1 = 8.5 Hz, J_2 = 3 Hz, 4′-H), 6.88 (1H, d, J = 8.5 Hz, 3′-H); MS: 315 (28) [M^+], 284 (100) [M^+ -OCH_3]. Anal. $C_{16}H_{21}N_5O_2$ (C,H,N).

*7.1.4. 6-[2-(2,5-Dimethoxyphenyl)ethyl]pyrido[2,3-d]pyrimidine-2,4-diamine (**5**)*

N-[(2*E*)-2-[2-(2,5-Dimethoxyphenyl)ethyl]-3-(dimethylamino)prop-2-en-1-ylidene]-*N*-methylmethanaminium-perchlorate (**24**) (0.8 g, 2.1 mmol) and pyrimidine-2,4,6-triamine (**14**) (0.24 g, 1.9 mmol) in *n*-butanol (10 ml) were heated to reflux for 10 h. The precipitate was filtered off and crystallized from H_2O/dimethylformamide (20:1) to yield 80% **5** (0.5 g) of a colourless powder; m.p. 303–305 °C (H_2O/DMF); IR: cm^{-1} 3460, 3344, 3352 (NH_2), 2992, 2937 (C–H), 2834 (OCH_3), 1695, 1654, 1619 (C=N), 1598, 1580, 1553, 1500 (C=C); ^{1}H-NMR: Δ 2.89 (4H, s, 1′-H, 2′-H), 3.66 (3H, s, OCH_3), 3.71 (3H, s, OCH_3), 6.70–6.90 (3H, m, 3″-H, 4″-H, 6″-H), 7.11 (2H, br, 4-NH_2, D_2O exchangeable), 8.24 (2H, br, 2-NH_2, D_2O exchangeable), 8.40 (1H, d, J = 2.5 Hz, 5-H), 8.53 (1H, d, J = 2.5 Hz, 7-H); ^{13}C-NMR:δ 31.57 (CH_2CH_2), 32.52 (CH_2CH_2), 55.93 (OCH_3), 56.42 (OCH_3), 104.96 (C-4a), 112.18, 112.43, 116.82 (C-3′, C-4′, C-6′), 130.34 (C-1′), 132.73, 133.40 (C-5, C-6), 151.90, 153.52 (C-2′, C-5′), 154.24, 156.50, 159.63, 163.81 (C-2, C-4, C-7, C-8a); MS: 325 (6) [M^+], 310 (1) [M^+ -CH_3], 174 (100) [M^+ -(CH_3O)$_2$ $C_6H_2CH_2$]. Anal $C_{17}H_{19}N_5O_2$ (C, H, N).

*7.1.5. 5-(2,5-Dimethoxybenzyl)-4,6-dimethyl-2-oxo-1,2-dihydropyridine-3-carbonitrile (**8**)*

3-(2,5-Dimethoxybenzyl)pentan-2,4-dione (**6**) (1.6 g, 6.4 mmol) was heated to reflux with cyanacetamide (**7**) (530 mg, 6.3 mmol) in ethanol (5 ml) with 10 drops of piperidine for 5 h. After cooling, the residue was filtered off and crystallized from ethanol and gave (**8**) with 58% (1.11 g) yield as colourless crystals, m.p. 151 °C (ethanol); IR: cm^{-1} 3311, 3150 (NH), 2998, 2937 (C-H), 2842 (OCH_3), 2224 (CN), 1652 (C=N); ^{1}H-NMR:δ 2.19 (3H, s, CH_3), 2.20 (3H, s, CH_3), 3.62 (3H, s, OCH_3), 3.67 (2H, s, CH_2), 3.77 (3H, s, OCH_3), 6.22 (1H, d, J = 3 Hz, 6′-H), 6.76 (1H, dd, J_1 = 9 Hz, J_2 = 3 Hz, 4′-H), 6.93 (1H, d, J = 9 Hz, 3′-H). 12.33 (1H, br, N-H, D_2O exchangeable); MS: 297 (76) [M^+], 42 (100). Anal. $C_{17}H_{18}N_2O_3$ (C, H, N).

*7.1.6. 2-Chloro-5-(2,5-dimethoxybenzyl)-4,6-dimethylnicotinonitrile (**9**)*

To a solution of *N*,*N*-dimethylformamide (7.3 ml, 0.095 mo1) in absolute chloroform (50 ml), cooled in an ice bath, thionylchloride (11.9 g, 0.1 mol) in absolute chloroform (20 ml) was added drop wise at a maximum temperature of 5 °C. At the end of the exothermic reaction, 5-(2,5-dimethoxybenzyl)-4,6-dimethyl-2-oxo-1,2-dihydropyridine-3-carbonitrile (**8**) (3.0 g, 0.01 mol) was added within 10–15 min in small portions. The mixture was allowed to warm at room temperature and heated to reflux for 3 h. After cooling, an ethanolic solution of potassium hydroxide was added slowly, then the mixture was diluted with water and the organic layer was separated. The aqueous phase was extracted with chloroform for three times. The unified organic phases were washed three times with water (10 ml), dried over Na_2SO_4 and concentrated under reduced pressure. The

residue was purified by MPLC (cyclohexane/acetic acid (8:2)) and following crystallization from ethanol to yield 79% (2.6 g) of **9** as colourless crystals, m.p. 121–122 °C (ethanol); IR: cm^{-1} 3435 (NH), 3076, 3027 (Ar-H), 2997, 2950 (C-H), 2834 (OCH_3), 2230 (CN), 1721 (C=N), 1611, 1592, 1563, 1545, 1499 (C=C); ^{1}H-NMR:δ 2.40 (3H, s, CH_3), 2.42 (3H, s, CH_3), 3.60 (3H, s, OCH_3), 3.78 (3H, s, OCH_3), 3.96 (2H, s, CH_2), 6.14 (1H, d, J = 3 Hz, 6′-H), 6.79 (1H, dd, J_1 = 9 Hz, J_2 = 3 Hz, 4′-H), 6.95 (1H, d, J = 9 Hz, 3′-H); MS: 316/318 (100/33) [M^+], 301/303 (25/8) [M^+ -CH_3], 285/287 (25/9) [M^+ -OCH_3]. Anal. $C_{17}H_{17}N_2O_2Cl$ (C, H, N).

*7.1.7. 2,5-Dimethoxybenzylmalonic acid (**12**)*

Diethyl 2,5-dimethoxybenzylmalonate (**11**) (3.9 g, 0.019 mol) and 15 g NaOH in 100 ml H_2O/EtOH (1:1) were heated to 80 °C for 2 h. After cooling, water (100 ml) was added; the solution was acidified with HCl_{conc} and extracted with ether (20 ml) for three times. The organic layer was washed with water (20 ml) three times, dried with Na_2SO_4, concentrated under reduced pressure and crystallized from toluol to yield 43% (1.37 g) of colourless crystals, m.p. 155–157 °C (toluol), IR: cm^{-1} 3200–2500 (COOH), 3060 (Ar-H), 2956, 2911 (C–H), 2836 (OCH_3), 1718, 1713 (C=O), 1506 (C=C) 1293 (O–H); ^{1}H-NMR:δ 2.96 (2H, d, J = 7.5 Hz, CH_2), 3.55 (1H, t, J = 7.5 Hz , H-2), 3.66 (3H, s, OCH_3), 3.73 (3H, s, OCH_3), 6.69 (1H, d, J = 3 Hz, 6′-H), 6.75 (1H dd, J = 9 Hz, J = 3 Hz, 4′-H) 6.86 (1H, d, J = 9 Hz, 3′-H), 12.64 (2H, br, OH, D_2O exchangeable); ^{13}C-NMR:δ 29.76 (CH_2), 55.66 (OCH_3), 56.20 (OCH_3), 111.96, 112.15, 117.08, 127.49 (C-l′, C-3′, C-4′, C-6′), 151.77 (C-5′), 153.14 (C-2′), 170.69 (COOH); MS: 254 (34) [M^+], 210 (100) [M^+ -CO_2]; Anal $C_{12}H_{14}O_6$ (C, H).

7.1.8. N-*[(2E)-2-(2,5-dimethoxybenzyl)-3-dimethylamino) prop-2-en-1-ylidene]*-N-*methyl-methanium-perchlorate (**13**)*

N,*N*-Dimethylformamide (2.1 g, 0.029 mol) was stirred and cooled in an ice bath. Phosphoroxychloride (2.09 g, 0.014 mol) was added drop wise. To this mixture, 2,5-dimethoxybenzyl-malonic acid (**12**) (2.55 g, 0.01 mol) was added in small portions and stirred for 24 h at room temperature, then 30 min under reduced pressure. After cooling in an ice bath, ethanol (4 ml) and a solution of *N*,*N*-dimethyl ammoniumperchlorate (1.45 g, 0.01 mol) in ethanol (4 ml) were added. The precipitate was separated and crystallized from ethanol to yield **13,** 32% (1.2 g) of colourless crystals, m.p. 154 °C (ethanol); IR: cm^{-1} 3018 (Ar-H), 2970, 2943 (C-H), 2841 (OCH_3), 1597 (C=N), 1495 (C=C); ^{1}H-NMR: Δ 3.01 (6H, br, NCH_3), 3.23 (6H, br, NCH_3), 3.70 (3H, s, OCH_3), 3.72 (2H, s, CH_2), 3.78 (3H, s, OCH_3), 6.52 (1H, d, J = 2.2 Hz, 6′-H), 6.84 (1H, dd, J_1 = 6.0 Hz, J_2 = 2.2 Hz, 4′-H), 6.97 (1H, d, J = 6.0 Hz, 3′-H), 7.67 (2H, s, 1-H, 3-H); ^{13}C-NMR:δ 24.63 (CH_2), 48.66 (CH_3), 55.24 (OCH_3), 55.74 (OCH_3), 98.36 (C-2), 110.90, 111.44, 115.98, 129.65 (C1′, C-3′, C-4′, C-6′), 150.21, 153.40 (C-2′, C-5′), 166.40 (C-1, C-3); MS: 277 (8) [M^+ -ClO_4^-], 232 (16) [M^+ -ClO_4^- -$(CH_3)_2NH$], 188 (100). Anal. $C_{16}H_{25}ClN_2O_6$ (C, H, N).

*7.1.9. 2-(2,5-Dimethoxybenzyl)acrylaldehyde (**18**)*

To a stirred solution of oxalylchloride (3.1 g, 0.024 mol) in 50 ml dry methylene chloride, that was cooled to –70 °C, dimethylsulfoxide (3.72 ml, 0.048 mol) in 20 ml dry methylene chloride was added drop wise. After 10 min, 3-(2,5-dimethoxy)phenylpropanol (**15**) (4.28 g, 0.022 mol) was added in small portions at –60 °C within 5 min. The solution was stirred for further 15 min. Then triethylamine (12.4 ml, 0.090 mol) was added cautiously. After the solution had warmed at room temperature *N*,*N*-dimethyl(methylene)-immoniumchloride ("Böhme-Salz") (**16**) (4.12 g, 0.044 mol) was added and the solution was stirred for 15 h. After the addition of methylenechloride (30 ml), the organic layer was washed three times with a saturated aqueous solutions of $NaHCO_3$ and NaCl, then washed with water (20 ml), dried with Na_2SO_4 and concentrated under reduced pressure giving 2.83 g of a yellow oil (1**8**) in 63% yield, that was purified by vacuum distillation, b.p. 190–210 °C (0.4 kPa), IR: cm^{-1} 3017 (Ar-H), 2955, 2911 (C-H), 2836 (OCH_3), 1693 (CHO), 1503 (C=C), ^{1}H-NMR: ($CDCl_3$) δ3.53 (2H, dd J_1 = 0.5 Hz, J_2 = 0.5 Hz, CH_2), 3.75 (6H, s, OCH_3), 6.01 (1H, dt, J_1 = 1 Hz, J_2 = 0.5 Hz, 3-H), 6.03 (1H, dt, J_1 = 1 Hz, J_2 = 0.5 Hz, 3-H), 6.69–6.82 (3H, m, 3′-H, 4′-H, 6′-H), 9.61 (1H, s, CHO); ^{13}C-NMR: Δ 32.67 (CH_2), 55.22 (OCH_3), 55.68 (OCH_3), 147.93 (C-2), 135.71 (C-3), 111.67, 111.72, 116.51, 127.25 (C-l′, C-3′, C-4′, C-6′), 151.21 (C-2′), 152.96 (C-5′), 194.68 (C-1); MS: 206 (90) [M^+], 175 (100) [M^+ -OCH_3]. Anal. $C_{12}H_{14}O_3$ (C, H).

*7.1.10. 5,8-Dihydro-6-(2,5-dimethoxybenzyl)pyrido[2,3-*d*] pyrimidine-2,4-diamine (**19**)*

2-(2,5-Dimethoxybenzyl)acrylaldehyde (**18**) (911 mg, 4.4 mmol) was heated under reflux with pyrimidine-2,4,6-triamine (**14**) (428 mg, 3.4 mmol) in a mixture of ethanol (5 ml) and acetic acid (5 ml) for 5 h. After cooling, the residue was filtered off and gave **19** as a colourless powder, yield 30% (320 mg), m.p. 262–263 °C; IR: cm^{-1} 3322, 3161 (NH_2, NH), 3058 (Ar-H), 2944 (C-H), 2833 (OCH_3), 1700, 1658 (CHO), 1584, 1528, 1498 (C=C); ^{1}H-NMR:δ 2.90 (2H, s, CH_2), 3.09 (2H, s, 5-H), 3.68 (3H, s, OCH_3), 3.72 (3H, s, OCH_3), 5.33 (2H, s, NH_2, D_2O exchangeable), 5.61 (2H, s, NH_2, D_2O exchangeable), 5.67 (1H, d, J = 5 Hz, 7-H), 6.73 (1H, d, J = 3.5 Hz, 6′-H), 6.74 (1H, dd, J_1 = 9 Hz, J_2 = 3.5 Hz, 4′-H), 6.88 (1H, d, J = 9 Hz, 3′-H), 7.51 (1H, d, J = 5 Hz, 8-NH, D_2O exchangeable); MS: 313 (59) [M^+], 312 (59) [M^+ -1], 298 (5) [M^+ -CH_3], 282 (76) [M^+ -OCH_3], 162 (100). Anal. $C_{16}H_{19}N_5O_2$ (C, H, N).

*7.1.11. Diethyl [2-(2,5-dimethoxyphenyl)ethyl]malonate (**22**)*

Diethylmalonate (**21**) (2.72 g, 0.017 mol) was dissolved in dry tetrahydrofuran (25 ml). To this solution, a 60% dispersion of sodium hydride in petroleum (0.72 g, 0.018 mol)

and 2-(2,5-dimethoxyphenyl)ethylbromide **(20)** (4.13 g, 0.017 mol) were added at room temperature. Then the mixture was heated to reflux for 12 h. After the addition of water (20 ml), the aqueous phase was extracted with ether (30 ml) for three times. The unified ether fractions were washed with water (20 ml) three times, dried over Na_2SO_4 and concentrated under reduced pressure. The residue was purified by MPLC (cyclohexane/acetic acid (8:2)) to yield 3.3 g (60%) of a colourless oil (**22**), b.p. 230–250 °C (0.2–0.4 kPa); IR: cm^{-1} 3029 (Ar–H), 2986, 2941, 2909 (C–H), 2874, 2836 (OCH_3), 1746, 1729 (COOEt), 1610, 1591, 1504, 1500 (C=C); ^{1}H-NMR: Δ 1.18 (6H, t, J = 7 Hz, CH_3), 2.01 (2H, m, CH_2), 2.53 (2H, m, CH_2), 3.35 (1H, t, J = 7.5 Hz, CH), 3.68 (3H, s, OCH_3), 3.70 (3H, s, OCH_3), 4.11 (4H, q, J = 7 Hz, OCH_2), 6.69 (1H, d, J = 3 Hz, 6′-H), 6.74 (1H, dd, J_1 = 9 Hz, J_2 = 3 Hz, 4′-H), 6.87 (1H, d, J = 9 Hz, 3′-H); ^{13}C-NMR:δ 13.73 (OCH_2CH_3), 27.12 (CH_2CH_2), 28.45 (CH_2CH_2), 50.60 (C-2), 50.81 (OCH_2), 55.20 (OCH_3), 55.66 (OCH_3), 111.49, 111.60, 115.92 (C-3′, C-4′, C-6′), 129.52 (C-1′), 151.15, 152.96 (C-2′, C-5′), 168.76 (COOR); MS: 324 (4) [M^+], 164 (100). Anal. $C_{17}H_{24}O_6$ (C, H).

*7.1.12. [2-(2,5-Dimethoxyphenyl)ethyl]malonic acid (**23**)*

Diethyl [2-(2,5-dimethoxyphenyl)ethyl]malonate (**22**) (3.24 g, 0.010 mol) was added to a solution of 50 ml 15% aqueous sodium hydroxide and heated to 80 °C for several hours. After addition of conc. hydrochloric acid to pH 4–5 and extraction with 50 ml ethylacetate for three times, the unified organic phases were dried over Na_2SO_4 and concentrated under reduced pressure. The resulting residue was crystallized from toluol yielding 85% of **23** (2.27 g) as colourless crystals, m.p. 111–112 °C (toluol); IR: cm^{-1} 3079, 3014 (Ar–H), 2950, 2927 (C–H), 2874, 2836 (OCH_3), 1708 (COOH), 1607, 1507 (C=C); ^{1}H-NMR:δ 1.90–2.02 (2H, m, CH_2), 2.49–2.58 (2H, m, CH_2), 3.14 (1H, t, J = 7.5 Hz, CH), 3.68 (3H, s, OCH_3), 3.71(3H, s, OCH_3), 6.68 (1H, d, J = 3 Hz, 6′-H), 6.74 (1H, dd, J_1 = 9 Hz, J_2 = 3 Hz, 4′-H), 6.87 (1H, d, J = 9 Hz, 3′-H), 10.50 (2H, br, COOH); MS: 268 (7) [M^+] 224 (66) [M^+ -CO_2], 152 (100). Anal. $C_{13}H_{16}O_6$ (C, H).

7.1.13. N-*[(2E)-2-[2-(2,5-Dimethoxyphenyl)ethyl]-3-(dimethylamino)prop-2-en-1-ylidene]-N-methylmethanaminium-perchlorate (**24**)*

N,*N*-dimethylformamide (1.4 g, 19.2 mmol) was stirred and cooled in an ice bath. Phosphoroxychloride (1.39 g, 9.1 mmol) was added drop wise. This mixture was stirred for 30 min at room temperature. Then [2-(2,5-dimethoxyphenyl)ethyl]malonic acid (**23**) (1.0 g, 3.7 mmol) was added in small portions and stirred for 12 h at 30 °C; finally 30 min under reduced pressure. After cooling on ice, ethanol (8 ml) and a solution of *N*,*N*-dimethylammoniumperchlorate (725 mg, 5.0 mmol) in ethanol (4 ml) were added. The precipitate was separated and crystallized from ethanol to yield **24**, 69% (1.0 g) of colourless crystals, m.p. 161–162 °C (ethanol); IR: cm^{-1} 3001 (Ar-H), 2931 (C-H), 2836 (OCH), 1588 (C=N), 1505 (C=C); ^{1}H-NMR:δ 2.59–2.71 (4H, m, CH_2), 3.32 (12H, s, NCH_3), 3.69 (3H, s, OCH), 3.72 (3H, s, OCH_3), 6.74–6.93 (3H, m, 3′-H, 4′-H, 6′-H), 7.44 (2H, s, 1-H, 3-H); ^{13}C-NMR:δ 33.03 (CH_2CH_2), 33.34 (CH_2CH_2), 48.66 (NCH_3), 55.29 (OCH_3), 55.51 (OCH_3), 102.97 (C-2), 111.39, 111.47, 116.34 (C-3′, C-4′, C-6′), 129.25 (C-1′), 151.02, 153.07 (C-2′, C-5′), 165.50 (C-1, C-3); MS: 291 (4) [M^+ -ClO_4^-], 246 (7) [M^+ -ClO_4^- -$(CH_3)_2NH$], 202 (100). Anal. $C_{17}H_{27}ClN_2O_6$ (C, H, N).

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