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Synthesis and evaluation of pyrido[1,2-*a*]pyrimidines as inhibitors of nitric oxide synthases

Ullvi Bluhm [a], Jean-Luc Boucher [b], Uwe Buss [a], Bernd Clement [a,*], Friederike Friedrich [a], Ulrich Girreser [a], Dieter Heber [a], Thanh Lam [a], Michel Lepoivre [c], Mojgan Rostaie-Gerylow [a], Ulrich Wolschendorf [a]

[a] *Pharmaceutical Institute, Department of Pharmaceutical Chemistry, Christian-Albrechts-University of Kiel, Gutenbergstraße 76, D-24118 Kiel, Germany*
[b] *Laboratoire de Chimie et Biochimie Pharmacologiques et Toxicologiques, UMR 8601, University Paris Descartes, 45 Rue des Saints-Pères, 75270 Paris Cedex 06, France*
[c] *CNRS URA 1116, University Paris Sud-Orsay, Bat 430, 91105 Orsay Cedex, France*

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ABSTRACT

A series of new 3-aroylpyrido[1,2-*a*]pyrimidines were synthesized from aryl methyl ketones in a simple two-step procedure and evaluated as nitric oxide synthases (NOS) inhibitors. In order to perform a structure–activity relationship study, different aroyl groups were introduced in 3-position and methyl groups were introduced at different positions of the pyrimidine heterocycle. Compounds with a biphenyloyl, benzyloxybenzoyl or naphthoyl group displayed the highest inhibitory effects which were further increased by introduction of a methyl group in position 8 of the pyrido[1,2-*a*]pyrimidine moiety. Some of the compounds exhibited promising inhibitory effects with selectivity toward the purified inducible NOS (iNOS) and were also active against iNOS expressed in stimulated RAW 264.7 cells.

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1. Introduction

Nitric oxide, NO, is a recently identified mediator acting as a regulator of the vascular tone, a neurotransmitter and a cytotoxic agent. NO also plays important roles in the pathogenesis of several diseases [1,2]. In particular, NO contributes to inflammatory and autoimmune diseases such as rheumatoid arthritis, osteoarthritis, inflammatory bowel disease and multiple sclerosis [3–7]. Therefore, nitric oxide synthases (NOS), the enzymes that produce NO by oxidation of L-arginine, are potential targets for new therapeutic agents. In mammals, three isoforms of NOS have been identified. The constitutive neuronal NOS (nNOS) and endothelial NOS (eNOS) are Ca^{++}-dependent enzymes that play key roles in the nervous and cardiovascular systems whereas the inducible nitric oxide synthases (iNOS) produce large quantities of NOS following immunological challenge [8–10]. NOS are homodimeric enzymes and each NOS subunit contains an NH_2-terminal oxygenase domain which bears binding sites for the substrate L-arginine, the heme prosthetic group and the cofactor (6*R*)-5,6,7,8-tetrahydro-L-biopterin (H_4B), and a CO_2H-terminal reductase domain which contains binding sites for the flavins, FMN and FAD, and the cofactor NADPH. These two domains are fused by a Ca^{++}-dependent calmodulin (CaM)-binding sequence. CaM-binding to NOS activates both intradomain as well as interdomain electron transfers and is required for maximal NO-forming activity [8–10].

There is much evidence that NOS inhibitors could be useful therapeutical agents in the treatment of diseases such as diabetes, congestive heart failure, atherosclerosis, migraine, asthma, cerebral ischaemia and Parkinson's disease [1,2,11–14]. However, they must be selective towards n- or iNOS to avoid interferences with the vital functions of eNOS involved in the control of vascular tone. Recent studies in the synthesis of new NOS inhibitors have thus focused on the development of either nNOS or iNOS selective inhibitors [15–18].

Several structurally diverse classes of NOS inhibitors have been investigated. Analogues of the natural substrate L-arginine, dipeptides and peptidomimetics derived from $N^{(\omega)}$-nitro-L-arginine have been studied, some of them exhibiting selectivities of up to three orders of magnitude over either eNOS or iNOS inhibition [19–24]. In the meantime, a great number of non-amino acid compounds have been tested as either nNOS or iNOS inhibitors. Substituted indazoles (with 7-nitroindazole, 7-NI, as the lead compound) or imidazoles, are potent NOS inhibitors but their selectivity remains low,

Abbreviations: n-NOS, neuronal nitric oxide synthase; i-NOS, inducible nitric oxide synthase; eNOS, endothelial nitric oxide synthase; $iNOS_{oxy}$, Oxygenase domain of the inducible NOS; 7-NI, 7-Nitro-1*H*-indazole; L-NNA, $N^{(\omega)}$-Nitro-L-arginine; L-NPA, $N^{(\omega)}$-Propyl-L-arginine; SEITU, *S*-Ethyl-*iso*-thiourea; 1400W, *N*-(3-(aminomethyl)benzyl)acetamidine.

* Corresponding author. Tel.: +49 0431 880 1126; fax: +49 0431 880 1352.
E-mail address: bclement@pharmazie.uni-kiel.de (B. Clement).

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at least *in vitro* [25,26]. Potent non-amino acid inhibitors with *iso*-thiourea and amidine functions have been reported [27–35]. 2-Aminopyridines have been described as NOS inhibitors, and selective nNOS inhibitors containing this scaffold were recently reported [36,37]. However, 2-amino-4-methylpyridine derivatives, which are reported to be highly potent inhibitors of all three isoforms, can exhibit high toxicity and should not be suitable for further development [36].

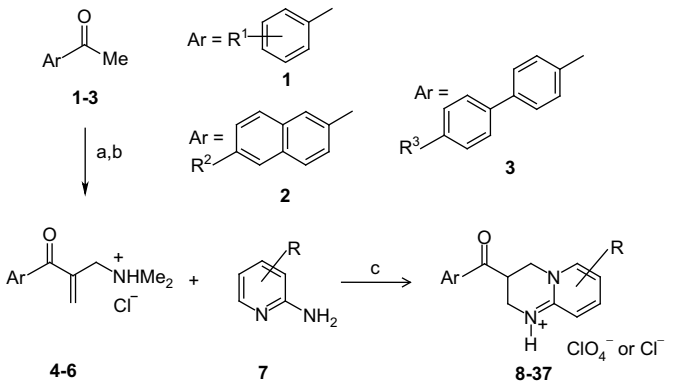
Pyrido[1,2-*a*]pyrimidines are heterocyclic compounds that include a 2-aminopyridine moiety and could thus appear as potential inhibitors of NOS. Therefore we have synthesized a series of new pyrido[1,2-*a*]pyrimidines bearing various aromatic substituents and one or two methyl groups at different positions of the pyrimidine ring.

These compounds were evaluated as inhibitors of the three purified NOS isoforms from various species and of an iNOS expressed in a murine macrophage cell line. Some of the compounds exhibited good inhibitory effects with some selectivity for one isoform of NOS thus opening the way to further SAR studies.

2. Chemistry

Pyrido[1,2-*a*]pyrimidines **8**–**37** were prepared by condensation of 2-aminopyridines **7** with enone Mannich bases **4**–**6**, which are readily accessible through the heating of aryl methyl ketones **1**–**3** and *N*,*N*′-dimethylmethyleniminium chloride in *N*,*N*-dimethylformamide [38] (see Scheme 1 for reactions). After the addition of the nucleophile to the enone structure, the dimethylamino group was eliminated followed by ring closure to form anellated heterocycles [39]. Isolating the resulting products as hydroperchlorates or as hydrochlorides showed that an addition–elimination mechanism had taken place. In the first step, the 2-aminopyridine adds to the double bond. Next a new electrophilic center is generated by the elimination of *N*,*N*′-dimethylammonium chloride to form a new enone moiety followed by a second addition to the ring closed heterocycle (see Scheme 2 for structures). The intermediates could not be isolated. The enones **4**–**6** were not characterized completely but immediately transformed to the heterocycles **8**–**37**. Details for the synthesis of some of these enone Mannich bases have been published previously [38].

The ring closure reaction was performed with 2-aminopyridine (**7a**), 2-amino-3-methylpyridine (**7b**), 2-amino-4-methylpyridine (**7c**), 2-amino-6-methylpyridine (**7d**), and 2-amino-4,6-dimethylpyridine (**7e**). Using phenyl, naphthyl, biphenyl, and benzyloxyphenyl substituted enone Mannich bases **4**–**6** for the ring closure reaction, the corresponding 3-aroylpyrido[1,2-*a*]pyrimidine derivatives **8**–**37** were obtained in moderate (30%) to good (70%) yields from commercially available aryl methyl ketones following simple procedures. Twenty six



Scheme 1. General method for the synthesis of pyrido[1,2-*a*]pyrimidines **8**–**37** from acetophenones **1**–**3** and 2-amino-pyridines **7a**–**e**. Reagents and conditions: (a) 2 $H_2C{=}NMe_2Cl$, DMF, 130 °C, 2 h; (b) EtOH, reflux; (c) $HClO_4$ or HCl, EtOH.

3-benzoyl derivatives **8**–**22**, five pyrido[1,2-*a*]pyrimidines **23**–**28** bearing a benzyloxybenzoyl substituent in position 3, nine naphthoyl (**28**–**30**) and ten biphenyloyl substituted heterocycles (**31**–**37**) were synthesized and evaluated as inhibitors of the three purified NOS isoforms.

3. Biological results

3.1. Screening of the inhibitory effects of compounds 7–37 on the activity of NOS using the Griess assay

In a first series of experiments, NOS inhibition data were obtained for all compounds introduced at 100 μM (final concentration) using recombinant NOS isoforms from different species and the classical colorimetric Griess assay for nitrite determination [40]. Experiments including the known inhibitors of NOS N^{ω}-nitro-L-arginine (L-NNA), N^{ω}-propyl-L-arginine (L-NPA), *S*-ethyl-*iso*-thiourea (SEITU), and *N*-(3-aminomethyl)benzylacetamidine (1400W) were performed for comparative purposes (see Fig. 1 for structures) [15–17]. As previously described, L-NNA and L-NPA more efficiently inhibited nitrite formation catalyzed by n- and eNOS whereas SEITU and 1400W were more efficient toward the inducible NOS than the constitutive isoforms [15–17]. Using this relatively insensitive assay, some of the tested pyrido[1,2-*a*]pyrimidines were found as active as the known inhibitors. However, no clear specificity for one isoform could be observed under our experimental conditions (Table 1).

3.2. Inhibitory effects of some pyrido[1,2-a]pyrimidines on the activity of NOS using a radioactive assay

Further experiments were performed to check the inhibitory effects of a more limited number of compounds using the conversion of [γ-^{14}C]L-arginine to [^{14}C]L-citrulline as a more sensitive assay to measure NOS activity [41]. This series of experiments was performed in the presence of 100 μM of each compound and 2.5 μM of substrate L-arginine, a value close to the K_m values of the various NOS isoforms [8–10]. The heterocyclic compound 7-NI was also introduced as a known heterocyclic inhibitor [15–17,25].

As a general observation, all the tested compounds more efficiently inhibited iNOS than nNOS or eNOS. Neuronal NOS was half-inhibited by 100 μM of compounds **18b**, **26c**, **34c** and **35c** whereas compounds **29e**, **34c** and **35c** (all at 100 μM) were the most potent inhibitors of eNOS. Interestingly, compounds **35c** and **26c** were found more potent than 7-NI against nNOS and eNOS, respectively. Pyrido[1,2-*a*]pyrimidines **26c**, **29c**, **29e**, **34c**, **35c**, and **37c** strongly inhibited the inducible NOS, with compounds **29c** and **35c** being more potent than L-NNA under those conditions. The IC_{50} values of the most promising compounds were determined and compared to those of L-NNA and 7-NI. The studied compounds displayed IC_{50} value in the 10–100 μM range and those of **35c** (with iNOS) and **26c** (with eNOS) were close to those of L-NNA for these enzymes (Table 2).

3.3. Mechanism of inhibition of iNOS by some pyrido[1,2-a] pyrimidines

Further experiments were performed in order to better investigate the mechanism of the inhibition of iNOS by some of the most promising pyrido[1,2-*a*]pyrimidines. In a first set of experiments, the inhibitory effects of compounds **29e**, **34c**, **35c** and **37c** (0, 10, 25 and 100 μM) were determined in the presence of different concentrations of L-arginine (0.25, 0.5, 1.0, 2.5, 5 and 25 μM) and [2,3,4-^{3}H]L-arginine.

For compound **37c**, plotting 1/activity as a function of 1/[L-arginine] led to straight lines that intercepted the *y*-axis at the

7	a	b	c	d	e
R	H	3-Me	4-Me	6-Me	4,6-Me_2

Ar = C_6H_5	R^1	H	4-Me	4-OH	4-F	4-CN	4-OMe	4-Cl	4-OEt	CO_2H
Compound		**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**[a]

Ar = C_6H_5	R^1	3-F,4-OMe	3,4-$(OMe)_2$	3,4-Cl_2	4-Br	cC_6H_{11}	3,4,5-$(OMe)_3$
Compound		**17**	**18**	**19**	**20**	**21**	**22**[a]

Ar = C_6H_5	R^1	OCH_2Ph	$OCH_2C_6H_4CN$	$OCH_2C_6H_4OMe$	$OCH_2C_6H_4Cl$	$OCH_2C_6H_4Br$
Compound		**23**	**24**	**25**	**26**	**27**[a]

Ar = naphthyl	R^2	H	Me	OMe	OMe
Compound		**28**	**29**	**30**	**30e**[a]

Ar = biphenyl	R^3	H	OH	F	CN	OMe	CO_2H	Br
Compound		**31**	**32** [a]	**33**	**34** [a]	**35** [a]	**36**[a]	**37**

Scheme 2. Structures of the compounds tested in this study. (a): Isolated as hydorchloride salts.

same point (constant V_m value), and the *x*-axis at different points which correspond to different 1/apparent K_m values (Fig. 2). These results suggested that compound **37c** inhibited iNOS in a competitive manner with L-arginine. Replot of the apparent K_m values as a function of the concentration of **37c** led to a K_i value of 14 μM (Insert of Fig. 2). Almost identical results were observed with compounds **29c** and **35c** which inhibited iNOS in a competitive manner and displayed K_i values of 23 and 15 μM, respectively (data not shown). However, a more complex mechanism for inhibition of iNOS by compound **34c** was observed and further studies are required to precisely determine its mode of action (data not shown). In another series of experiments, iNOS (2 μM) was preincubated for 15 min at 4 °C in the presence of 1 mM of **37c** and aliquots of this mixture were diluted 100 fold in order to perform the incubations under identical conditions as usual for the [γ-^{14}C]L-arginine assay. It was observed that the inhibition of iNOS activity by compound **37c** was abolished after dilution of the sample.

These data indicated that the inhibition of iNOS by some of the pyrimido[1,2-*a*]pyrimidines was competitive with the substrate L-arginine and reversible.

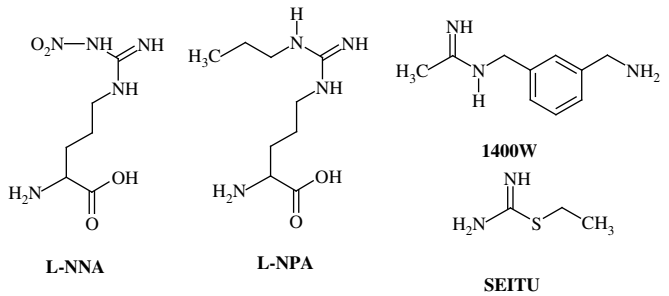


Fig. 1.

3.4. Effects of some pyrido[1,2-a]pyrimidines on the spectra of the heme

In order to check if pyrido[1,2-*a*]pyrimidines can interact with the heme active site of NOS, we have performed UV–visible studies on the oxygenase domain of iNOS ($iNOS_{oxy}$) which displays identical spectrophotometric properties than the full length enzyme. The UV–visible spectrum of native $iNOS_{oxy}$ in Hepes buffer pH 7.4 displayed a broad Soret peak with a maximum at 415 nm indicating that it exists as a mixture of hexacoordinated heme-Fe^{III}–H_2O (low spin) and pentacoordinated heme-Fe^{III} states (high spin), the former state being predominant, in agreement with literature data [42]. Addition of 1 mM imidazole (ImH) was done to completely convert $iNOS_{oxy}$ into a hexacoordinated heme-Fe^{III}–ImH complex absorbing at 430 nm. This method was very often used to more easily follow the binding of substrates to the NOS active site [43]. As already described, the addition of 100 μM L-NNA or 7-NI to the $iNOS_{oxy}$-Fe^{III}–ImH complex gave rise to difference spectra characterized by a trough at 425 nm and a peak at 396 nm showing that the binding of these compounds close to the heme shifted the $iNOS_{oxy}$ spin state equilibrium to the pentacoordinated heme-Fe^{III}-state corresponding to a displacement of bound ImH [43,44]. Under identical conditions, the addition of 100 μM of pyrido[1,2-*a*]pyrimidines **17c**, **19a**, **29c**, **29e**, **34c**, **35c** and **37c** led to difference spectra almost identical to that obtained with L-NNA (peak close to 396–400 nm, Table 2). All the other derivatives tested led to very weak, not significant, difference spectra after addition either to native $iNOS_{oxy}$ or to $iNOS_{oxy}$–ImH complex (Table 2).

3.5. Effects of compounds on the NADPH consumption by iNOS

In order to more deeply investigate the mechanism of inhibition and to get further informations on the effects of some pyrido[1,2-*a*]pyrimidines on the electron transfer in NOS, we have tested their

Table 1
Activity of various NOS isoforms incubated in the presence of 100 μM L-NNA, L-NPA, SEITU, 1400W, 2-aminopyridines **7**, and pyrido[1,2-*a*]pyrimidines **8**–**37**[a]

Compd	nNOS		iNOS		eNOS		Log $D_{7.4}$
	Human	Rat	Human	Murine	Human	Bovine	
L-NNA	13 ± 1		48 ± 4		25 ± 4		
L-NPA	53 ± 2	37 ± 8	95 ± 5	88 ± 2	96 ± 5	93 ± 8	
SEITU	16 ± 1	17 ± 3	6 ± 1	2 ± 1	37 ± 1	27 ± 12	
1400W	73 ± 3	43 ± 3	5 ± 1	3 ± 1	105 ± 7	107 ± 11	
7a	72 ± 2		95 ± 8		94 ± 8		
7c	21 ± 4		29 ± 8		50 ± 1		
7d	48 ± 1		47 ± 15		83 ± 2		
7e	10 ± 1		7 ± 3		12 ± 1		
8a		96 ± 1		102 ± 1		91 ± 18	1.4
8b		92 ± 2		103 ± 10		92 ± 2	
8c	78 ± 1[b]	57 ± 20	63 ± 6	16 ± 1	72 ± 1	40 ± 6	
8d		95 ± 1		104 ± 1		93 ± 6	1.5
9a		81 ± 1		86 ± 1		94 ± 1[b]	
9b		93 ± 1		100 ± 9		93 ± 3	1.6
9d		91 ± 3		99 ± 3		101 ± 6	1.6
10c	39 ± 10		30 ± 2		46 ± 8		
11a	83 ± 6		67 ± 15		79 ± 4		
11c	79 ± 3	51 ± 2	78 ± 3	78 ± 5	77 ± 5	37 ± 4	
11d	85 ± 5	79 ± 3	88 ± 3	83 ± 2	77 ± 3	41 ± 1	
12c	18 ± 5		54 ± 8		75 ± 6		
13a		90 ± 1		100 ± 8		90 ± 2	1.5
14c	2 ± 1[b]	2 ± 2	50 ± 6	28 ± 8	7 ± 1	20 ± 2	
15c		88 ± 7		97 ± 1		87 ± 11	
16c	69 ± 7		93 ± 6[b]		94 ± 2		
17c	89 ± 5		102 ± 17		84 ± 4		
18a		95 ± 1		123 ± 10		91 ± 6	1.4
18b		98 ± 1		102 ± 8		95 ± 1	
18d		92 ± 2		95 ± 2		90 ± 4	1.5
19a	49 ± 16	81 ± 1	105 ± 7	95 ± 2	89 ± 4	83 ± 4	1.8
20a	95 ± 6	88 ± 1	110 ± 5	92 ± 1	85 ± 2	86 ± 1	1.7
20c	65 ± 5	67 ± 2	69 ± 1	33 ± 1	73 ± 5	72 ± 4	
20d	89 ± 1	60 ± 4	93 ± 2	9 ± 3	86 ± 1	93 ± 5	
21c	42 ± 15		76 ± 1[b]		42 ± 1		
22a		89 ± 1[b]		105 ± 2		86 ± 3	
23c	81 ± 3		73 ± 8		75 ± 1		
24c	85 ± 14		105 ± 4		78 ± 4		
25c	90 ± 13		101 ± 13		46 ± 4		
26c	15 ± 4		94 ± 3		8 ± 2		
27c	39 ± 1[b]		83 ± 1[b]		16 ± 5		
28a	56 ± 7	16 ± 5	93 ± 5	97 ± 5	60 ± 7	80 ± 11	
28c	34 ± 1	15 ± 1	47 ± 2	28 ± 1	60 ± 4	50 ± 12	1.7
28d	61 ± 7	39 ± 10	89 ± 2	75 ± 3	75 ± 3	75 ± 2	
29c	24 ± 2		103 ± 8	90 ± 5	11 ± 1	49 ± 6	
29d	75 ± 17		88 ± 10		75 ± 8		2.1
29e	68 ± 1		83 ± 1		86 ± 7		2.2
30c	83 ± 6		88 ± 1	72 ± 13	98 ± 1	80 ± 8	
30d	72 ± 7		80 ± 14		6 ± 1		1.9
30e	69 ± 3		95 ± 10		121 ± 9		1.9
31c	4 ± 1	2 ± 1	17 ± 2	40 ± 15	8 ± 3	17 ± 2	
31d	4 ± 3		86 ± 4		20 ± 8		2.1
32c	79 ± 14		96 ± 7		86 ± 3		
33c	36 ± 5		85 ± 5		46 ± 3		
34c	70 ± 6		55 ± 6		55 ± 15		
35c	62 ± 3		49 ± 2		57 ± 2		
36c	70 ± 2		98 ± 2		91 ± 1		
37c	15 ± 1		120 ± 7		17 ± 3		2.7
37d	61 ± 8		106 ± 10		84 ± 3		2.8
37e	4 ± 3		89 ± 7		15 ± 10		

[a] Results are expressed as % of residual activities of the enzymes following incubations in the presence of 1% DMSO alone or 100 μM of the studied compounds. The activities were measured by the Griess assay for nitrite determination under the conditions described in the Experimental. Results are means ± S.D. from 5 experiments.
[b] Means ± S.D. from 3 experiments.

effects on the NADPH consumption catalyzed by iNOS. These data will give informations on their effects on the electron transfer from the reductase domain to the heme domain of iNOS and the possible generation of superoxide anion and hydrogen peroxide by iNOS. As previously described for this isoform, NADPH consumption by iNOS was very low in the absence of L-arginine [45]. It was increased about three times in the presence of L-arginine (100 μM) and completely abolished in the presence of L-NNA [45]. All the experiments were performed in the presence of 100 μM L-arginine and 100 μM (final concentrations) of pyrido[1,2-*a*]pyrimidines. Compounds **27c**, **34c**, **35c**, **37c** and **37e** led to a significant inhibition of NADPH consumption by iNOS (Table 2). For compounds **34c** and **37c**, IC_{50} values were determined to be 11 and 35 μM, respectively, close to the values obtained for the inhibition of citrulline formation. These data show that some of the pyrido[1,2-*a*]pyrimidines inhibit the NADPH consumption and could reduce the generation of reactive oxygen species such as hydrogen peroxide by iNOS.

3.6. Inhibitory effects of some compounds on the activity of iNOS expressed in RAW 264.7 cells

When activated by LPS and IFN-γ, this cell line expresses an inducible NOS [46]. We tested if some of our compounds could inhibit the induction of iNOS by introducing them at the same time as LPS + IFN-γ. In another set of experiments, we tested their inhibitory effects on the activity of this iNOS, 15 h after the addition of LPS + IFN-γ and washing of the cells. The effects of the compounds were measured using the Griess assay (nitrite formation). Identical results were obtained in both series of experiments, suggesting that the compounds did not affect the induction of iNOS in this cell line. Furthermore, it clearly appears that 100 μM of compounds **24c**, **26c**, **27c**, **35c**, **37c** and **37d** strongly inhibited nitrite accumulation in the supernatants of the cells (Table 3). Furthermore, the studied compounds were not toxic for these cells at this concentration (data not shown).

4. Discussion of structure–activity relationships

Our preliminary data obtained by screening using the Griess assay were reinforced by the experiments using the radioactive assay for NOS. For comparison, the discussion will focus on the inhibition data expressed as percentage of residual activity obtained at an inhibitor concentration of 100 μM in all test systems. These assays have shown that some of the studied pyrido[1,2-*a*]pyrimidines were inhibitors of NOSs with potencies close to those of compounds such as L-NNA, 1400W or 7-NI. No potent selectivity for one isoform of NOS from any species could be observed for this series of compounds. However, most of them appear to be more potent against purified iNOS than against nNOS and eNOS but the constitutive isoforms nNOS and eNOS were inhibited in almost similar manners. Interestingly, the studied compounds also inhibited iNOS expressed in intact cells without apparent toxicity.

The selectivity of this class of compounds seems to be modulated by the nature and position of the substituents on the aroyl group. In nearly all cases, the pyrido[1,2-*a*]pyrimidines bearing a methyl group in position 8 of the heterocyclic ring system (synthesized from 2-amino-4-methylpyridine **7c**), have a higher potency compared to any other substitution pattern, i.e. corresponding to structures **a**, **b**, **d** and **e**. In the structures under investigation, the higher inhibition was observed for the pairs of structures **8c**/**8d**, **11c**/**11d**, **20**c/**20d**, **28c**/**28d**, **29c**/**29d**, **31c**/**31d**, **37c**/**37d**, and only one pair, namely **30c**/**30d**, showed higher inhibition potency for the **d** isomer.

The lipophilicity of some compounds has been evaluated at pH 7.4 using an HPLC method [47] and the log $D_{7.4}$ values are given on Table 1. The lipophilicity is clearly linked to the type and size of the substituent at 3-position of the pyrido[1,2-*a*]pyrimidine. The position and the number of methyl substituent on the pyrimidine

Table 2
Effects of L-NNA, 7-NI and some pyrido[1,2-*a*]pyrimidines on the activities of three purified NOS, on the NADPH consumption by iNOS, and on the visible spectrum of $iNOS_{oxy}$.

Compd	Rat nNOS	% Activity[a]		% NADPH	Heme
		Murine iNOS	Bovine eNOS	Oxidation[b]	spectra shift[c]
L-NNA	2 ± 1	15 ± 5	5 ± 3	8	396
7-NI	36 ± 4 (27 ± 5)[d]	25 (40 ± 12)[d]	5 ± 3 (41 ± 15)[d]	n.d.[e]	398
9a	92 ± 8	100 ± 8	100 ± 12	91	n.d.
9d	76 ± 8	66 ± 8	84 ± 10	94	400
11d	83 ± 6	66 ± 10	71 ± 12	87	400
17c	69 ± 8	45 ± 8	59 ± 10	73	398
18b	48 ± 8	96 ± 10	94 ± 14	89	n.d.
18d	100 ± 5	91 ± 10	81 ± 14	92	n.d.
19a	87 ± 4	85 ± 8	83 ± 10	100	398
24c	60 ± 8	30 ± 6	44 ± 8	77	n.d.
26c	49 ± 10	25 ± 8	2 ± 2 (12 ± 5)[d]	64	n.d.
27c	62 ± 10	62 ± 13	77 ± 12	100	n.d.
29c	69 ± 5	13 ± 6 (29 ± 9)[d]	48 ± 12	70	398
29e	55 ± 5 (60 ± 15)[d]	28 ± 8 (35 ± 12)[d]	17 ± 8 (53 ± 12)[d]	72	400
34c	43 ± 6 (128 ± 15)[d]	18 ± 7 (50 ± 12)[d]	18 ± 8 (12 ± 5) [d]	40	396
35c	29 ± 6 (87 ± 14) [d]	13 ± 8 (18 ± 8)[d]	15 ± 5 (60 ± 15) [d]	40	396
37c	10 ± 4	19 ± 6 (28 ± 10)[d]	65 ± 10	64	400
37e	58 ± 4	35 ± 10 (120 ± 25)[d]	32 ± 8 (188 ± 25)[d]	72	n.d.

Data are expressed as % of the initial activities measured in the presence of 1% DMSO.
[a] Inhibition of purified recombinant n-, i- or eNOS was determined by the radioactive assay and are means ± S.D. from 3 experiments. Incubations contained 2.5 μM [γ-^{14}C]L-arginine, 1% DMSO alone or 100 μM of the studied compounds, and all the required cofactors as described in the Experimental.
[b] Incubations were performed in the presence of 100 μM L-arginine, 10 μM H_4B and 1% DMSO alone or 100 μM of the studied compounds, as described in the Experimental. Data are expressed as % of the NADPH oxidase activity of iNOS containing 1% DMSO alone (580 ± 110 nmol min^{-1} mg $prot^{-1}$).
[c] Position of the maximum appearing in the difference spectrum obtained after addition of 100 μM of the studied compounds to a solution of 0.8–1.2 μM $nNOS_{oxy}$ containing 1 mM ImH. This position is mentioned only for compounds leading to a significant difference spectrum. n.d.: no appearance of a significant difference spectrum.
[d] IC_{50} values (in μM) obtained from incubations performed in the presence of increasing concentrations of the studied compounds.
[e] Not determined.

heterocycle seem to have a small effect on the log $D_{7.4}$ values. However, increasing the size of the aromatic part of the 3-aroyl group increases the lipophilic character and the inhibition of NOS, as shown with the chlorophenyl derivative **14c**, the chloro substituted benzyl ether **26c**, and the 4-bromobiphenyl derivatives **37c** and **37e**. Considering the structures of molecules, compounds which have a simple phenyl group on the C=O group are almost inactive. Compounds **29c** and **29e** that contain a naphthyl group are less potent than those containing a biphenyl (**35c** and **34c**) or a benzyloxy group (**24c**, **26c**, **27c**). A methoxy or a bromo substituent on the biphenyl group reinforces the inhibitory effects. The more lipophilic structures led to the highest inhibitory effects and a slight preference for the inhibition of iNOS over n- and eNOS was observed.

For compounds **8a** and **20a**, a pK_a value of 12.4 was determined [48]. It can be assumed that substitution of the pyridine ring by one or two methyl groups influenced this value only marginally. So the basicity was not included as one of the factors of structure–activity relationship.

In this study, our primary aim was to check if the heterocyclic pyrido[1,2-*a*]pyrimidines could constitute a new series of inhibitors for NOS. The 2-aminopyridines **7a**–**e** used as starting materials are known NOS inhibitors, with the 4-methyl derivative described a potent but non-selective inhibitor [36]. Careful HPLC analysis showed no contamination of the tested pyridopyrimidines **8**–**37** with these aminopyridines, and no decomposition of **8**–**37** during testing was noticeable. Our experiments show that some of the pyrido[1,2-*a*]pyrimidines can efficiently inhibit NOS, sometimes more efficiently than the well-known inhibitors 7-NI or L-NNA. In our experiments, the high IC_{50} values determined for the known inhibitors could be linked to the high concentration of L-arginine used in the assays and to the fact that most of the studied compounds could act as competitive inhibitors with L-arginine, as observed with **29c**, **35c** and **37c**. Thus, it clearly appears that some of these pyrido[1,2-*a*]pyrimidines are as potent as 7-NI or 1400W when tested under identical conditions. Some of these compounds reduced NADPH consumption by iNOS suggesting that they could interfere with the electron transfer between the reductase domain and the heme domain and affect the generation of reactive oxygen species by NOS. Furthermore, our UV–visible and mechanistic studies suggest that some of the compounds could bind close to the heme in an identical manner than L-arginine and that they inhibit iNOS by competition with L-arginine binding at the active site. A more precise mode of binding of the compounds will be obtained with the help of crystallographic studies.

The synthetic approach to this class of heterocyclic compounds is straightforward and would allow easy modifications of the pyrido[1,2-*a*]pyrimidine backbone. Further SAR studies will help in the design of more potent and selective compounds bearing this scaffold.

5. Experimental

5.1. Chemistry

Melting points were determined with a Büchi 510 melting point apparatus and on a Thermowar microhotstage and are reported uncorrected. ^{1}H NMR spectra were obtained on a Bruker ARX 300 spectrometer at 300 K. All spectra were recorded in DMSO-d_6 as solvent. Chemical shifts (δ values) are reported in ppm using TMS as internal standard. All coupling constants (J values) are quoted in Hz. The following NMR abbreviations are used: br (broad), s (singlet), d (doublet), t (triplet), m (unresolved multiplet), Ar (aromatic proton). Elemental analyses and high resolution mass spectra were performed at the Institute of Organic Chemistry, University of Kiel. All reagents for the synthesis of the pyrido[1,2-*a*]pyrimidines were purchased from commercial sources and were used without further purification.

High performance liquid chromatography for the determination of purity of compounds was performed on a Waters System (Waters 600 controller with pump; Waters 2487 dual absorbance detector; Waters WISP 710B autosampler; EZ ChromTM Elite chromatography

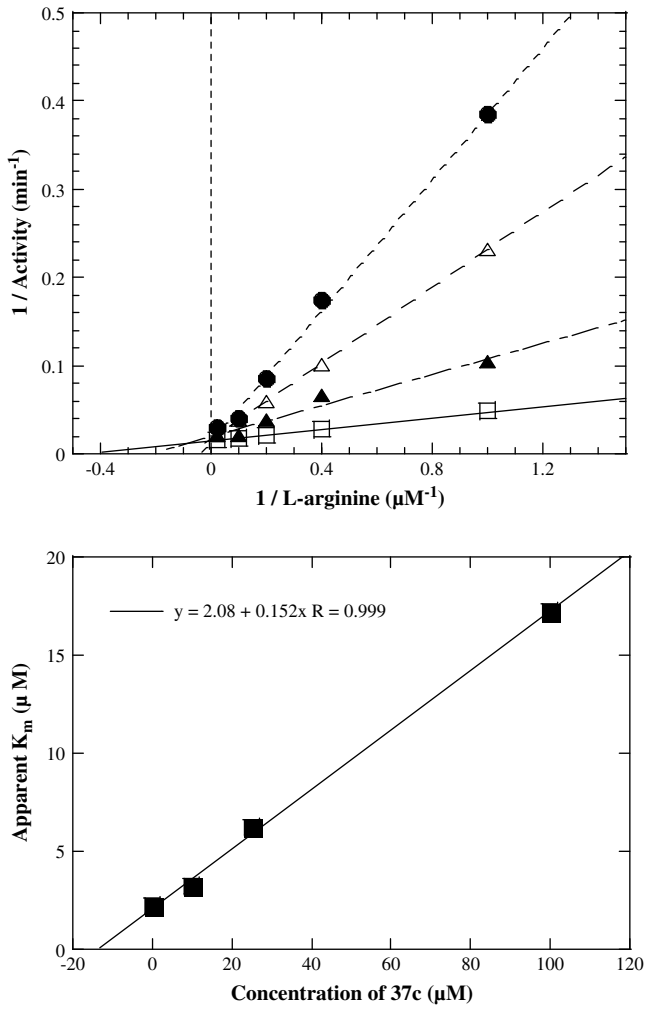


Fig. 2.

data system; LiChrospher® 100 RP-8 column; LiChroCART® 125 × 4 mm cartridge, 5 µm; guard column RP-Select B, 4 × 4 mm, 5 µm). The injection volume was 10 µl. "The mobile phase was changed from 100% solvent A (heptylsulfonate sodium salt 10 mM, pH 3/acetonitrile 85/15 v/v 10 mM tetramethylammoniumchloride) and 0% solvent B (heptylsulfonate sodium salt 10 mM, pH 3/acetonitrile 10/90 v/v 10 mM tetramethylammoniumchloride) within 10 min to 50% A, 50% B, hold for 15 min at 50% A, 50% B." Sample elution was detected by absorbance at 297 nm.

Table 3
Inhibition of iNOS expressed in stimulated RAW 264.7 cells by some pyrido[1,2-*a*]pyrimidines[a]

Compd	% Inhibition
9a	60 ± 8
9d	72 ± 4
11d	44 ± 4
17c	64 ± 3
18b	67 ± 3
18d	70 ± 2
19a	59 ± 5
24c	93 ± 2
26c	92 ± 2
27c	96 ± 2
29c	86 ± 2
29e	89 ± 2
34c	82 ± 2
35c	96 ± 4
37c	98 ± 2
37d	98 ± 2

[a] LPS and γ-interferon stimulated RAW 264.7 cells were incubated for 15 h at 37 °C in the presence of 1% DMSO alone, or 100 µM of the studied compounds. Nitrite determination in the supernatants was performed as described in the Experimental. Data are means ± S.D. from 6 to 8 determinations.

High performance liquid chromatography for the determination of log $D_{7.4}$ values of compounds [47] was performed on a Waters System – Waters 1525 binary pump; Waters 486 tunable absorbance detector; Waters 717 Plus autosampler; Waters Breeze software system; Kromasil 100 C18 5 µm, 60 – 4.6 mm column. The injection volume was 10 µl. The mobile phase was a gradient from 0% solvent A (acetonitrile) and 100% solvent B (a mixture of acetonitrile and Sörensen phosphate buffer 11 mM) over 30 min to 29.5% solvent A, 70.5% solvent B. Solvent B was a mixture of 30% acetonitrile and 70% buffer for compounds **8**–**27**, 37.5% acetonitrile and 62.5% buffer for compounds **28**–**36**, and 45% acetonitrile and 55% buffer for **37**. As reference compounds aniline, benzamide, benzonitrile, 4-ethylbenzaldehyde, 4-ethylbenzamide, 4-ethylbenzene, 4-ethylbenzonitrile, 4-methlybenzamide, *o*-cresol, phenol, *n*-propylbenzene, toluene, cinnamaldehyde and uracil were used. UV-detection was performed at 210 nm.

pK_a values were determined according to the procedure of Albert and Serjeant [48]. The pyrido[1,2-*a*]pyrimidines were dissolved in 10% of aqueous methanol. To 5 ml of these solutions were added 5 ml of NaOH (concentration range of 0.002–6 M) and 1 M HCl to make up a final concentration of 150 µM. The solutions were measured at a wavelength of 312 nm.

5.1.1. General method for the preparation of pyrido[1,2-a] pyrimidines

A solution of the 1-aryl-2-dimethylaminomethyl-2-propenone (4 mmol) and 2-aminopyridine (4 mmol) in ethanol (50 ml) was refluxed for 2–3 h. After evaporation of the solvent, the residue was dissolved in 2-propanol (20 ml) followed by cooling and stirring in an ice bath. Then perchloric acid (70% in water, 4 mmol) or concentrated hydrochloric acid was added. The precipitate was filtered, dried and recrystallised from methanol. The yields ranged from 10 to 30% [38,39].

*5.1.2. Preparation of compounds **8**–**37***

3-Benzoyl-3,4-dihydro-2*H*-pyrido[1,2-*a*]pyrimidine perchlorate (**8a**), 3-benzoyl-3,4-dihydro-9-methyl-2*H*-pyrido[1,2-*a*]pyrimidine perchlorate (**8b**), 3-benzoyl-3,4-dihydro-6-methyl-2*H*-pyrido[1,2-*a*] pyrimidine perchlorate (**8d**), 3,4-dihydro-3-(4′-methylbenzoyl)-2*H*-pyrido[1,2-*a*]pyrimidine perchlorate (**9a**), 3,4-dihydro-9-methyl-3-(4′-methylbenzoyl)-2*H*-pyrido[1,2-*a*]pyrimidine perchlorate (**9b**), 3,4-dihydro-6-methyl-3-(4′-methylbenzoyl)-2*H*-pyrido[1,2-*a*]pyrimidine perchlorate (**9d**), 3,4-dihydro-3-(4′-methoxybenzoyl)-2*H*-pyrido [1,2-*a*]pyrimidine perchlorate (**13a**), 3,4-dihydro-3-(3′,4′-dimethoxybenzoyl)-2*H*-pyrido[1,2-*a*]pyrimidine perchlorate (**18a**), 3,4-dihydro-3-(3′,4′-dimethoxybenzoyl)-9-methyl-2*H*-pyrido[1,2-*a*]pyrimidine perchlorate (**18b**), 3,4-dihydro-3-(3′,4′-dimethoxybenzoyl)-6-methyl-2*H*-pyrido[1,2-*a*]pyrimidine perchlorate (**18d**), 3-(3′,4′-dichlorobenzoyl)-3,4-dihydro-2*H*-pyrido[1,2-*a*]pyrimidine perchlorate (**19a**), and 3-(4′-bromobenzoyl)-3,4-dihydro-2*H*-pyrido[1,2-*a*]pyrimidine perchlorate (**20a**) were prepared following the general procedure and their physico-chemical properties have been previously reported [39].

3-Benzoyl-3,4-dihydro-8-methyl-2*H*-pyrido[1,2-*a*]pyrimidine perchlorate (**8c**) was prepared from 2-(dimethylaminomethyl)-1-phenylprop-2-en-1-one hydrochloride and 2-amino-4-methylpyridine **7c** according to the general method. Mp. > 290 °C. ^{1}H NMR: δ 2.29 (br s, 3H), 3.51 (m, 1H), 3.74 (d, 1H), 6.71 (dd, 1H, Ar-H, J = 6.8 Hz), 6.79 (s, 1H, Ar-H), 7.57 (m, 2H, Ar-H), 7.70 (t, 1H, Ar-H, J = 7.4 Hz), 7.90 (d, 1H, Ar-H, J = 6.8), 8.04 (d, 2H, J = 7.3 Hz), 9.38 (br

s, 1H, NH). Anal. ($C_{16}H_{17}N_2O_5Cl$) calc C: 52.90, H: 4.25, N: 5.37; found C: 53.20, H: 4.25, N: 5.31.

3,4-Dihydro-3-(4′-hydroxybenzoyl)-8-methyl-2*H*-pyrido[1,2-*a*] pyrimidine perchlorate (**10c**) was prepared from **24c** by ether cleavage using 3 equiv. aluminium chloride and 4 equiv. anisole in dichloromethane. The mixture was stirred for 40 min at room temperature. After treatment with acetic acid and water the aqueous solution was extracted with ethyl acetate and the organic layer washed with $NaHCO_3$-solution and brine. The solvent was evaporated and the residue was crystallised from methanol. Mp. 146 °C. ^{1}H NMR: δ 2.32 (s, 3H), 3.49 (m, 1H), 3.70–3.74 (m, 1H), 4.30–4.35 (m, 2H), 4.45–4.48 (m, 1H), 6.71 (d, 1H, $J = 6.1$ Hz), 6.80 (s, 1H), 6.91 (d, 2H, Ar-H, $J = 8.0$ Hz), 7.89–7.95 (m, 3H, Ar-H), 9,39 (br s, 1H), 10.66 (s, 1H). Anal. ($C_{16}H_{16}N_2O_2$) HR-MS: calc for $C_{16}H_{16}N_2O_2$ 268.1212; found 268.1212.

3-(4′-Fluorobenzoyl)-3,4-dihydro-2*H*-pyrido[1,2-*a*]pyrimidine perchlorate (**11a**) was prepared from 2-(dimethylaminomethyl)-1-(4-fluorophenyl)prop-2-en-1-one hydrochloride and 2-aminopyridine **7a** according to the general method. Mp. 149 °C. ^{1}H NMR: δ 3.54–3.61 (m, 1H), 3.79–3.85 (m, 1H), 4.41–4.60 (m, 3H), 7.06–7.10 (m, 1H, Ar-H), 7.44 (t, 2H, Ar-H, $J = 8.8$ Hz), 8.15–8.21 (m, 2H, Ar-H), 8.57 (dd, 1H, Ar-H, $J = 2.1$ Hz, $J = 6.6$ Hz), 8.82 (dd, 1H, Ar-H, $J = 2.1$ Hz, $J = 4.3$ Hz), 10.12 (br s, 1H, NH). Anal. ($C_{15}H_{14}N_2O_5ClF$) calc C: 50.50, H: 3.96, N: 7.85; found C: 50.87, H: 4.29, N: 7.52.

3,4-Dihydro-3-(4′-fluorobenzoyl)-8-methyl-2*H*-pyrido[1,2-*a*] pyrimidine perchlorate (**11c**) was prepared from 2-(dimethylaminomethyl)-1-(4-fluorophenyl)prop-2-en-1-one hydrochloride and **7c** according to the general method. Mp. 146 °C. ^{1}H NMR: δ 2.31 (s, 3H, CH_3), 3.50 (m, 1H, H-2/4), 3.73 (m, 1H, H-2/4), 4.28–4.52 (m, 3H, H-3, H-2/4), 6.72–6.76 (m, 2H, H-7/9), 7.40 (m, 2H, Ar-H), 7.90 (m, 1H, H-6), 8.18 (m, 2H, Ar-H), 9.22 (br s, 1H, NH). Anal. ($C_{16}H_{16}N_2O_5ClF$) calc C: 51.83, H: 4.35, N: 7.56; found C: 51.68, H: 4.49, N: 7.44.

3-(4′-Fluorobenzoyl)-3,4-dihydro-6-methyl-2*H*-pyrido[1,2-*a*] pyrimidine perchlorate (**11d**) was prepared from 2-(dimethylaminomethyl)-1-(4-fluorophenyl)prop-2-en-1-one hydrochloride and 2-amino-6-methyl-pyridine **7d** according to the general method. Mp. 149 °C. ^{1}H NMR: δ 2.52 (s, 3H), 3.43–3.53 (m, 1H), 3.77–3.81 (m, 1H), 4.26–4.48 (m, 3H), 6.81–6.89 (2d, 2H, Ar-H, $J = 7.1$ Hz, $J = 8.8$ Hz), 7.43 (t, 2H, Ar-H, $J = 8.8$ Hz), 7.71 (dd, 1H, Ar-H, $J = 7.2$ Hz, $J = 8.9$ Hz), 8.19 (dd, 2H, Ar-H, $J = 5.5$ Hz, $J = 8.9$ Hz), 9.31 (br s, 1H, NH). Anal. ($C_{16}H_{16}N_2O_5FCl$) calc C: 51.83, H: 4.35, N: 7.56; found C: 51.75, H: 4.29, N: 7.50.

3-(4′-Cyanobenzoyl)-3,4-dihydro-8-methyl-2*H*-pyrido[1,2-*a*] pyrimidine perchlorate (**12c**) was prepared from 1-(4-cyanophenyl)-2-(dimethylaminomethyl)prop-2-en-1-one hydrochloride and **7c** according to the general method. Mp. 149 °C. ^{1}H NMR: δ 2.32 (s, 3H), 3.49–3.55 (m, 1H), 3.74–3.78 (m, 1H), 4.39–4.54 (m, 3H), 6.74–6.76 (m, 2H), 7.92 (d, 1H, Ar-H, $J = 6.9$ Hz), 8.08 (d, 2H, Ar-H, $J = 8.4$ Hz), 8.21 (d, 2H, Ar-H, $J = 8.4$ Hz), 9.17 (br s, 1H). Anal. ($C_{17}H_{16}N_3O_5Cl$) calc C: 54.04, H: 4.28, N: 11.12; found C: 54.04, H: 4.32, N: 11.03.

3-(4′-Chlorobenzoyl)-3,4-dihydro-8-methyl-2*H*-pyrido[1,2-*a*] pyrimidine perchlorate (**14c**) was prepared from 1-(4-chlorophenyl)-2-(dimethylaminomethyl)prop-2-en-1-one hydrochloride and **7c** according to the general method. Mp. 166 °C. ^{1}H NMR: δ 2.31 (br s, 3H), 3.52 (m, 1H), 3.73–3.81 (m, 1H), 4.81 (m, 3H), 6.74 (d, 1H, Ar-H, $J = 6.9$ Hz), 6.78 (s, 1H, Ar-H), 7.67 (d, 2H, Ar-H, $J = 8.6$ Hz), 7.90 (d, 1H, Ar-H, $J = 6.9$ Hz), 8.08 (d, 2H, Ar-H, $J = 8.6$ Hz), 9.27 (br s, 1H, NH). Anal. ($C_{16}H_{16}N_2O_5Cl_2$) calc C: 49.63, H: 4.17, N: 7.23; found C: 49.77, H: 4.26, N: 7.11.

3-(4′-Ethoxybenzoyl)-3,4-dihydro-8-methyl-2*H*-pyrido[1,2-*a*] pyrimidine perchlorate (**15c**) was prepared from 1-(4-ethoxyphenyl)-2-(dimethylaminomethyl)prop-2-en-1-one hydrochloride and **7c** according to the general method. Mp. 131 °C. ^{1}H NMR: δ 1.36 (t, 3H, $J = 6.9$ Hz), 2.31 (s, 3H), 3.51 (m, 1H), 3.74 (d, 1H, $J = 13.3$ Hz), 4.18 (q, 2H, $J = 6.9$ Hz), 4.33–4.52 (m, 3H), 6.71–6.75 (m, 2H, Ar-H), 7.08 (d, 2H, $J = 8.9$ Hz), 7.90 (d, 1H, Ar-H, $J = 6.7$ Hz), 8.03 (d, 2H, Ar-H, $J = 8.9$ Hz), 9.15 (br s, 1H, NH). Anal. ($C_{18}H_{21}N_2O_6Cl$) calc C: 54.48, H: 5.33, N: 7.06; found C: 54.39, H: 5.26, N: 6.96.

3-(4′-Carboxybenzoyl)-3,4-dihydro-8-methyl-2*H*-pyrido[1,2-*a*] pyrimidine hydrochloride (**16c**) was prepared from **12c** by adding glacial acetic acid and water (1/1, v/v) and a few drops of ethanol until the substance is dissolved. The solution was refluxed 3 h and the solvent evaporated. The product was purified by crystallisation in methanol. Mp. 270 °C. ^{1}H NMR: δ 2.50 (s, 3H), 3.55 (m, 9H), 3.74–3.79 (m, 1H), 4.38–4.55 (m, 3H), 6.73 (d, 1H, $J = 6.7$ Hz), 6.84 (s, 1H), 7.93 (d, 1H. Ar-H, $J = 6.9$ Hz), 8.10 (d, 2H, Ar-H, $J = 8.4$ Hz), 8.16 (d, 2H, Ar-H, $J = 8.4$ Hz), 9.52 (br s, 1H), 13.00–14.00 (br s, 1H). Anal. ($C_{17}H_{17}N_2O_3Cl$) calc C: 61.35, H: 5.16, N: 8.42; found C: 60.98, H: 5.20, N: 8.30.

3-(3′-Fluoro-4′-methoxybenzoyl)-3,4-dihydro-8-methyl-2*H*-pyrido [1,2-*a*]pyrimidine perchlorate (**17c**) was prepared from 1-(3-fluoro-4-methoxyphenyl)-2-(dimethylaminomethyl)prop-2-en-1-one hydrochloride and **7c** according to the general method. Mp. 143 °C. ^{1}H NMR: δ 2.32 (s, 3H), 3.41–3.55 (m, 1H), 3.72–3.77 (m, 1H), 3.96 (s, 3H), 4.33–4.53 (m, 3H), 6.72–6.76 (m, 2H, Ar-H), 7.36 (t, 1H, Ar-H, $J = 8.5$ Hz), 7.87–7.97 (m, 3H, Ar-H), 9.16 (br s, 1H, NH). Anal. ($C_{17}H_{18}N_2O_6FCl$) calc C: 50.95, H: 4.53, N: 6.99; found C: 51.08, H: 4.53, N: 6.98.

3-(4′-Bromobenzoyl)-3,4-dihydro-8-methyl-2*H*-pyrido[1,2-*a*] pyrimidine perchlorate (**20c**) was prepared from 1-(4-bromophenyl)-2-(dimethylaminomethyl)prop-2-en-1-one hydrochloride and **7c** according to the general method. Mp. 160 °C. ^{1}H NMR: δ 2.31 (s, 3H, CH_3), 3.51 (m, 1H, H-2/4), 3.75 (d, 1H, H-2/4, $J = 12.6$ Hz), 4.35–4.51 (m, 3H, H-3, H-2/4), 6.72–6.76 (m, 2H, H-7/9), 7.81 (d, 2H, H-3′/5′, $J = 8.67$ Hz), 7.91 (d, 1H, H-6, $J = 6.8$ Hz), 7.98 (d, 2H, H-2′/6′, $J = 8.7$ Hz), 9.20 (br s, 1H, NH). Anal. ($C_{16}H_{16}N_2O_5BrCl$) calc C: 44.52, H: 3.74, N: 6.49; found C: 44.48, H: 3.83, N: 6.39.

3-(4′-Bromobenzoyl)-3,4-dihydro-6-methyl-2*H*-pyrido[1,2-*a*] pyrimidine perchlorate (**20d**) was prepared from 1-(4-bromophenyl)-2-(dimethylaminomethyl)prop-2-en-1-one hydrochloride and **7d** according to the general method. Mp. 174 °C. ^{1}H NMR: δ 2.50 (br s, 3H), 3.48 (m, 1H), 3.76 (d, 1H, $J = 13.3$ Hz), 4.29–4.50 (m, 3H), 6.81 (d, 1H, Ar-H, $J = 7.2$ Hz), 6.87 (d, 1H, Ar-H, $J = 8.9$ Hz), 7.70 (m, 1H, Ar-H), 7.82 (d, 2H, $J = 8.5$ Hz), 8.02 (d, 2H, $J = 8.4$), 9.39 (br s, 1H, NH). Anal. ($C_{16}H_{16}N_2O_5BrCl$) calc C: 44.52, H: 3.74, N: 6.49; found C: 44.37, H: 3.83, N: 6.39.

3-(4′-Cyclohexylbenzoyl)-3,4-dihydro-8-methyl-2*H*-pyrido[1,2-*a*] pyrimidine perchlorate (**21c**) was prepared from 1-(4′-cyclohexylphenyl)-2-(dimethylaminomethyl)prop-2-en-1-one hydrochloride and **7c** according to the general method. Mp. 189 °C. ^{1}H NMR: δ 1.23–1.47 (m, 5H), 1.70–1.81 (m, 5H), 2.31 (s, 3H), 2.50–2.62 (m, 1H), 3.50–3.55 (m, 1H), 3.73–3.77 (m, 1H), 4.36–4.54 (m, 3H), 6.72–6.75 (m, 1H), 6.79 (s, 1H), 7.41 (d, 2H, Ar-H, $J = 8.2$ Hz), 7.75 (d, 2H, Ar-H, $J = 8,2$ Hz), 10.87 (br s, 1H). Anal. ($C_{22}H_{27}N_2O_5Cl$) calc C: 60.76, H: 6.26, N: 6.44; found C: 60.98, H: 6.35, N: 6.43.

3,4-Dihydro-3-(3′,4′,5′-trimethoxybenzoyl)-2*H*-pyrido[1,2-*a*] pyrimidine hydrochloride (**22a**) was prepared from 2-(dimethylaminomethyl)-1-(2,3,4-trimethoxyphenyl)prop-2-en-1-one hydrochloride and **7a** according to the general method. Mp. 207 °C. ^{1}H NMR: δ 3.52 (m, 1H), 3.77 (s, 3H), 3.83 (m, 1H), 3.87 (s, 6H), 4.42 (m, 1H), 4.55–4.58 (m, 2H), 6.85 (t, 1H, Ar-H, $J = 6.4$ Hz), 7.14 (d, 1H, Ar-H, $J = 8.4$ Hz), 7.36 (s, 2H, Ar-H), 7.80 (t, 1H, Ar-H, $J = 7.8$ Hz), 8.04 (d, 1H, Ar-H, $J = 6.6$ Hz), 9.90 (br s, 1H, NH). Anal. ($C_{18}H_{21}N_2O_4Cl$) calc C: 59.26, H: 5.80, N: 7.68; found C: 59.18, H: 5.94, N: 7.52.

3-(4′-Benzyloxybenzoyl)-3,4-dihydro-8-methyl-2*H*-pyrido[1,2-*a*] pyrimidine perchlorate (**23c**) was prepared from 1-(4′-benzyloxybenzoyl)-2-(dimethylaminomethyl)prop-2-en-1-one hydrochloride

and **7c** according to the general method. Mp. 139 °C. ^{1}H NMR: δ 2.37 (s, 3H), 3.32–3.54 (m, 1H), 3.72–3.77 (m, 1H), 4.35–4.39 (m, 2H), 4.45–4.53 (m, 1H), 5.25 (s, 2H), 6.72–6.76 (m, 2H, Ar-H), 7.19 (d, 2H, Ar-H, $J = 8.9$ Hz), 7.33–7.49 (m, 5H), 7.91 (d, 1H, Ar-H, $J = 6.8$ Hz), 8.05 (d, 2H, Ar-H, $J = 8.9$ Hz), 9.15 (br s, 1H). Anal. ($C_{23}H_{23}N_2O_6Cl$) calc C: 60.20, H: 5.05, N: 6.10; found C: 60.19, H: 5.15, N: 6.17.

5.1.3. 3-[4′-(4″-Cyanobenzyloxy)benzoyl]-3,4-dihydro-8-methyl-2H-pyrido[1,2-a]pyrimidine

Perchlorate (**24c**) was prepared from 1-[4′-(4″-cyanobenzyloxyphenyl)]-2-(dimethylaminomethyl)prop-2-en-1-one hydrochloride and **7c** according to the general method. Mp. 285 °C. ^{1}H NMR: δ 2.33 (s, 3H), 3.52–3.58 (m, 1H), 3.75–3.83 (m, 4H), 4.38–4.55 (m, 3H), 6.74–6.77 (m, 2H), 7.08 (d, 2H, Ar-H, $J = 8.8$ Hz), 7.75 (d, 2H, Ar-H, $J = 8.8$ Hz), 7.86 (d, 2H, Ar-H, $J = 8.4$ Hz), 7.93 (d, 1H, Ar-H, $J = 6.8$ Hz), 8.11 (d, 2H, Ar-H, $J = 8.4$ Hz), 9.17 (br s, 1H). Anal. ($C_{24}H_{22}N_3O_6Cl$) calc C: 59.57, H: 4.58, N: 8.68; found C: 59.18, H: 4.68, N: 8.67.

3,4-Dihydro-3-[4′-(4″-methoxy)benzyloxybenzoyl]-8-methyl-2*H*-pyrido[1,2-*a*]pyrimidine perchlorate (**25c**) was prepared from 2-(dimethylaminomethyl)-1-[4′-(4″-methoxybenzyloxyphenyl)]-prop-2-en-1-one hydrochloride and **7c** according to the general method. Mp. 201 °C. ^{1}H NMR: δ 2.33 (s, 3H), 3.52–3.58 (m, 1H), 3.75–3.83 (m, 4H), 4.38–4.55 (m, 3H), 6.74–6.77 (m, 2H), 7.08 (d, 2H, Ar-H, $J = 8.8$ Hz), 7.75 (d, 2H, Ar-H, $J = 8.8$ Hz), 7.86 (d, 2H, Ar-H, $J = 8.4$ Hz), 7.93 (d, 1H, Ar-H, $J = 6.8$ Hz), 8.11 (d, 2H, Ar-H, $J = 8.4$ Hz), 9.17 (br s, 1H). Anal. ($C_{24}H_{25}N_2O_7Cl$) calc C: 58.95, H: 5.15, N: 5.73; found C: 59.25, H: 5.48, N: 6.26.

3-[4′-(4″-Chlorobenzyloxy)benzoyl]-3,4-dihydro-8-methyl-2*H*-pyrido[1,2-*a*]pyrimidine perchlorate (**26c**) was prepared from 1-[4′-(4″-chlorobenzyloxyphenyl)]-2-(dimethylaminomethyl)prop-2-en-1-one hydrochloride and **7c** according to the general method. Mp. 178 °C. ^{1}H NMR: δ 2.33 (s, 3H), 3.52–3.58 (m, 1H), 3.75–3.83 (m, 4H), 4.38–4.55 (m, 3H), 6.74–6.77 (m, 2H), 7.08 (d, 2H, Ar-H, $J = 8.8$ Hz), 7.75 (d, 2H, Ar-H, $J = 8.8$ Hz), 7.86 (d, 2H, Ar-H, $J = 8.4$ Hz), 7.93 (d, 1H, Ar-H, $J = 6.8$ Hz), 8.11 (d, 2H, Ar-H, $J = 8.4$ Hz), 9.17 (br s, 1H). Anal. ($C_{23}H_{22}N_2O_6Cl_2$) calc C: 55.99, H: 4.49, N: 5.68; found C: 55.96, H: 4.59, N: 5.69.

3-[4′-(4″-Bromobenzyloxy)benzoyl]-3,4-dihydro-8-methyl-2*H*-pyrido[1,2-*a*]pyrimidine hydrochloride (**27c**) was prepared from 1-[4′-(4″-bromobenzyloxyphenyl)]-2-(dimethylaminomethyl)prop-2-en-1-one hydrochloride and **7c** according to the general method. Mp. 110 °C. ^{1}H NMR: δ 2.33 (s, 3H), 3.52–3.58 (m, 1H), 3.75–3.83 (m, 4H), 4.38–4.55 (m, 3H), 6.74–6.77 (m, 2H), 7.08 (d, 2H, Ar-H, $J = 8.8$ Hz), 7.75 (d, 2H, Ar-H, $J = 8.8$ Hz), 7.86 (d, 2H, Ar-H, $J = 8.4$ Hz), 7.93 (d, 1H, Ar-H, $J = 6.8$ Hz), 8.11 (d, 2H, Ar-H, $J = 8.4$ Hz), 9.17 (br s, 1H). Anal. ($C_{23}H_{22}N_2O_2BrCl$) calc C: 58.31, H: 4.68, N: 5.91; found C: 57.92, H: 4.95, N: 5.80.

3,4-Dihydro-3-(2-naphthoyl)-2*H*-pyrido[1,2-*a*]pyrimidine perchlorate (**28a**) was prepared from 2-(dimethylaminomethyl)-1-(2-naphthyl)prop-2-en-1-one hydrochloride and **7a** according to the general method. Mp. 211 °C. ^{1}H NMR: δ 3.62 (m, 1H), 3.86 (d, 1H, $J = 13.4$ Hz), 4.46–4.67 (m, 3H, H-3), 6.83 (t, 1H, Ar-H, $J = 6.7$ Hz), 7.05 (d, 1H, Ar-H, $J = 8.9$ Hz), 7.70 (m, 2H, Ar-H), 7.82 (t, 1H, Ar-H, $J = 7.2$ Hz), 8.00–8.16 (m, 5H, Ar-H), 8.88 (s, 1H, Ar-H), 9.49 (br s, 1H). Anal. ($C_{19}H_{17}N_2O_5Cl$) calc C: 58.69, H: 4.41, N: 7.20; found C: 58.47, H: 4.72, N: 7.11.

3,4-Dihydro-8-methyl-3-(2-naphthoyl)-2*H*-pyrido[1,2-*a*]pyrimidine perchlorate (**28c**) was prepared from 2-(dimethylaminomethyl)-1-(2-naphthyl)prop-2-en-1-one hydrochloride and **7c** according to the general method. Mp. 142 °C. ^{1}H NMR: δ 2.32 (br s, 3H), 3.62 (m, 1H), 3.84 (d, 1H, $J = 13.9$ Hz), 4.41–4.61 (m, 3H), 6.74 (d, 1H, Ar-H, $J = 6.9$ Hz), 6.81 (s, 1H, Ar-H), 7.69 (m, 2H, Ar-H), 7.92 (d, 1H, Ar-H, $J = 6.9$ Hz), 7.99–8.15 (m, 5H, Ar-H), 8.86 (s, 1H, Ar-H), 9.31 (br s, 1H). Anal. ($C_{20}H_{19}N_2O_5Cl$) calc C: 59.63, H: 4.75, N: 6.96; found C: 59.61, H: 4.85, N: 6.90.

3,4-Dihydro-6-methyl-3-(2-naphthoyl)-2*H*-pyrido[1,2-*a*]pyrimidine perchlorate (**28d**) was prepared from 2-(dimethylaminomethyl)-1-(2-naphthyl)-prop-2-en-1-one hydrochloride and **7d** according to the general method. Mp. 128 °C. ^{1}H NMR: δ 2.52 (br s, 3H), 3.56 (m, 1H), 3.85 (d, 1H, $J = 13.9$ Hz), 4.38–4.62 (m, 3H), 6.82 (d, 1H, Ar-H, $J = 7.0$ Hz), 6.96 (d, 1H, Ar-H, $J = 8.8$ Hz), 7.69 (m, 2H, Ar-H), 7.98–8.16 (m, 5H, Ar-H), 8.90 (s, 1H, Ar-H), 9.59 (br s, 1H). Anal. ($C_{20}H_{19}N_2O_5Cl$) calc C: 59.63, H: 4.75, N: 6.96; found C: 59.55, H: 4.75, N: 6.84.

3,4-Dihydro-8-methyl-3-[2-(6-methylnaphthoyl)]-2*H*-pyrido[1,2-*a*]pyrimidine perchlorate (**29c**) was prepared from 2-(dimethylaminomethyl)-1-[2-(6-methylnaphthyl)]prop-2-en-1-one hydrochloride and **7c** according to the general method. Mp. 128 °C. ^{1}H NMR: δ 2.33 (s, 3H), 2.52 (s, 3H), 3.57–3.63 (m, 1H), 3.81–3.86 (m, 1H), 4.40–4.59 (m, 3H), 6.74–6.79 (m, 2H, Ar-H), 7.52 (d, 1H, Ar-H, $J = 8.4$ Hz), 7.81 (s, 1H, Ar-H), 7.95–7.97 (m, 3H, Ar-H), 8.04 (d, 1H, Ar-H, $J = 8.4$ Hz), 9.21 (br s, 1H). Anal. ($C_{21}H_{21}N_2O_5Cl$) calc C: 60.51, H: 5.08, N: 6.72; found C: 60.17, H: 5.10, N: 6.88.

3,4-Dihydro-6-methyl-3-[2-(6-methylnaphthoyl)]-2*H*-pyrido[1,2-*a*]pyrimidine perchlorate (**29d**) was prepared from 2-(dimethylaminomethyl)-1-[2-(6-methylnaphthyl)]prop-2-en-1-one hydrochloride and **7d** according to the general method. Mp. 198 °C. ^{1}H NMR: δ 2.53 (s, 6H), 3.56–3.59 (m, 1H), 3.83–3.87 (m, 1H), 4.35–4.56 (m, 3H), 6.83 (d, 1H, Ar-H, $J = 7.2$ Hz), 6.90 (s, 1H, Ar-H, $J = 8.7$ Hz), 7.51 (dd, 1H, Ar-H, $J = 1.4$ Hz, $J = 8.4$ Hz), 7.70–7.78 (m, 1H, Ar-H), 7.81 (s, 1H, Ar-H), 7.95–8.11 (m, 3H, Ar-H), 8.89 (s, 1H, Ar-H), 9.36 (br s, 1H). Anal. ($C_{21}H_{21}N_2O_5Cl$) calc C: 60.51, H: 5.08, N: 6.72; found C: 61.07, H: 5.12, N: 6.49.

3,4-Dihydro-6,8-dimethyl-3-[2-(6-methylnaphthoyl)]-2*H*-pyrido[1,2-*a*]pyrimidine perchlorate (**29e**) was prepared from 2-(dimethylaminomethyl)-1-[2-(6-methylnaphthyl)]prop-2-en-1-one hydrochloride and 2-amino-4,6-dimethyl-pyridine **7e** according to the general method. Mp. 160 °C. ^{1}H NMR: δ 2.29 (s, 3H), 2.49 (s, 3H), 2.53 (s, 3H), 3.51–3.58 (m, 1H), 3.80–3.85 (m, 1H), 4.31–4.53 (m, 3H), 6.67 (s, 1H, Ar-H), 6.71 (s, 1H, Ar-H), 7.52 (dd, 1H, Ar-H, $J = 1.5$ Hz, $J = 8.4$ Hz), 7.81 (s, 1H, Ar-H), 7.95–8.06 (m, 3H, Ar-H), 8.69 (s, 1H, Ar-H), 9.18 (br s, 1H). Anal. ($C_{22}H_{23}N_2O_5Cl$) calc C: 61.33, H: 5.38, N: 6.50; found C: 61.57, H: 5.58, N: 6.78.

3,4-Dihydro-3-[2-(6-methoxynaphthoyl)]-8-methyl-2*H*-pyrido[1,2-*a*]pyrimidine perchlorate (**30c**) was prepared from 2-(dimethylaminomethyl)-1-[2-(6-methoxynaphthoyl)]prop-2-en-1-one hydrochloride and **7c** according to the general method. Mp. 198 °C. ^{1}H NMR: δ 2.33 (s, 3H), 3.56–3.85 (m, 1H), 3.93 (s, 3H), 4.40–4.60 (m, 3H), 6.74–6.79 (m, 2H, Ar-H), 7.30 (dd, 1H, Ar-H, $J = 2.5$ Hz, $J = 8.9$ Hz), 7.45 (d, 1H, Ar-H, $J = 2.4$ Hz), 7.79–8.06 (m, 4H, Ar-H), 8.77 (s, 1H, Ar-H), 9.22 (s, 1H). Anal. ($C_{21}H_{21}N_2O_6Cl$) calc C: 58.27, H: 4.89, N: 6.47; found C: 58.14, H: 5.02, N: 6.43.

3,4-Dihydro-3-[2-(6-methoxynaphthoyl)]-6-methyl-2*H*-pyrido[1,2-*a*]pyrimidine perchlorate (**30d**) was prepared from 2-(dimethylaminomethyl)-1-[2-(6-methoxynaphthyl)]prop-2-en-1-one hydrochloride **7d** according to the general method. Mp. 190 °C. ^{1}H NMR: δ 2.53 (s, 3H), 3.52–3.58 (m, 1H), 3.93 (s, 3H), 4.33–4.55 (m, 2H), 6.82 (d, 1H, Ar-H, $J = 7.1$ Hz), 6.90 (d, 1H, Ar-H, $J = 8.8$ Hz), 7.31 (dd, 1H, Ar-H, $J = 2.5$ Hz, $J = 9.0$ Hz), 7.45 (d, 1H, Ar-H, $J = 2.5$ Hz), 7.69–7.74 (m, 1H, Ar-H), 7.91–8.06 (m, 3H, Ar-H), 9.37 (br s, 1H). Anal. ($C_{21}H_{20}N_2O_2$) HR-MS: calc for $C_{21}H_{20}N_2O_2$ 332.1525; found 332.1525.

3,4-Dihydro-3-[2-(6-methoxynaphthoyl)]-6,8-dimethyl-2H-pyrido[1,2-*a*]pyrimidine hydrochloride (**30e**) was prepared from 2-(dimethylaminomethyl)-1-[2-(6-methoxynaphthyl)]prop-2-en-1-one hydrochloride and **7e** according to the general method. Mp. 264 °C. ^{1}H NMR: δ 2.28 (s, 3H), 2.49 (s, 3H), 3.50–3.56 (m, 1H), 3.80–3.83 (m, 1H), 3.93 (s, 3H), 4.30–4.49 (m, 3H), 6.73 (s, 2H, Ar-H), 7.30 (dd, 1H, Ar-H, $J = 2.5$ Hz, $J = 9.0$ Hz), 7.45 (d, 1H, Ar-H, $J = 2.4$ Hz), 7.93–8.07 (m, 3H, Ar-H), 8.81 (s, 1H, Ar-H), 9.62 (br s, 1H). Anal.

($C_{22}H_{23}N_2O_2Cl$) calc C: 69.01, H: 6.05, N: 7.32; found C: 68.99, H: 6.14, N: 7.23.

3,4-Dihydro-8-methyl-3-(4′-phenylbenzoyl)-2*H*-pyrido[1,2-*a*] pyrimidine perchlorate (**31c**) was prepared from 1-(biphenylyl)-2-(dimethylaminomethyl)prop-2-en-1-one hydrochloride and **7c** according to the general method. Mp. 111 °C. ^{1}H NMR: δ 2.33 (br s), 2.84–2.88 (m, 1H), 3.59 (m, 1H), 4.39–4.56 (m, 3H), 6.67–6.78 (m, 2H, Ar-H), 7.43–7.56 (m, 3H, Ar-H), 7.78 (d, 2H, Ar-H, $J = 7.0$ Hz), 7.92 (d, 2H, Ar-H, $J = 8.5$ Hz), 7.94 (d, 1H, Ar-H, $J = 6.8$), 8.16 (d, 2H, Ar-H, $J = 8.5$ Hz), 9.20 (br s, 1H, NH). Anal. ($C_{22}H_{21}N_2O_5Cl$) calc C: 61.61, H: 4.93, N: 6.53; found C: 61.73, H: 5.11, N: 6.52.

3,4-Dihydro-6-methyl-3-(4′-phenylbenzoyl)-2*H*-pyrido[1,2-*a*] pyrimidine perchlorate (**31d**) was prepared from 1-(biphenylyl)-2-(dimethylaminomethyl)prop-2-en-1-one hydrochloride and **7d** according to the general method. Mp. 98 °C. ^{1}H NMR: δ 2.5 (br s, 3H), 3.51 (m, 1H), 3.79 (m, 1H), 4.35–4.55 (m, 3H), 6.81 (d, 2H, Ar-H, $J = 7.0$ Hz), 6.95 (d, 1H, Ar-H, $J = 9.0$ Hz), 7.42–7.55 (m., 3H, Ar-H), 7.70 (m, 1H, Ar-H), 7.79 (d, 2H, Ar-H, $J = 7.0$ Hz), 7.90 (d, 1H, Ar-H, $J = 8.4$ Hz), 8.18 (d, 2H, Ar-H, $J = 8.4$ Hz), 9.60 (br s, 1H, NH). Anal. ($C_{22}H_{21}N_2O_5Cl$) calc C: 61.61, H: 4.93, N: 6.53; found C: 61.82, H: 5.02, N: 6.38.

3,4-Dihydro-3-[4′-(4″-hydroxyphenyl)benzoyl]-8-methyl-2*H*-pyrido [1,2-*a*]pyrimidine hydrochloride (**32c**) was prepared from 2-(dimethylaminomethyl)-1-(4-hydroxybiphenylyl)prop-2-en-1-one hydrochloride and **7c** according to the general method. Mp. 295 °C. ^{1}H NMR: δ 2.32 (s, 3H), 3.53–3.56 (m, 1H), 3.75–3.79 (m, 1H), 4.34–4.54 (m, 3H), 6.73 (d, 1H), 6.85 (s, 1H), 6.91 (d, 2H, Ar-H, $J = 8.5$ Hz), 7.63 (d, 2H, Ar-H, $J = 8.6$ Hz), 7.81 (d, 2H, Ar-H, $J = 7.9$ Hz), 7.94 (d, 1H, Ar-H, $J = 6.5$ Hz), 8.09 (d, 2H, Ar-H, $J = 8.2$ Hz), 9.57 (s, 1H), 9.85 (br s, 1H). Anal. ($C_{22}H_{21}N_2O_2Cl$) calc C: 69.38, H: 5.56, N: 7.36; found C: 69.81, H: 5.64, N: 7.32.

3-[4′-(4″-Fluorophenyl)benzoyl]-3,4-dihydro-8-methyl-2*H*-pyrido [1,2-*a*]pyrimidine perchlorate (**33c**) was prepared from 2-(dimethylaminomethyl)-1-(4-fluorobiphenylyl)prop-2-en-1-one hydrochloride and **7c** according to the general method. Mp. 144 °C. ^{1}H NMR: δ 2.33 (s, 3H), 3.55–3.59 (m, 1H), 3.76–3.81 (m, 1H), 4.39–4.55 (m, 3H), 6.74–6.78 (m, 2H), 7.36 (t, 2H, Ar-H, $J = 8.8$ Hz), 7.81–7.95 (m, 5H, Ar-H), 8.14 (d, 2H, Ar-H, $J = 8.4$ Hz), 9.21 (br s, 1H). Anal. ($C_{22}H_{20}N_2O_5FCl$) calc C: 59.13, H: 4.51, N: 6.27; found C: 59.18, H: 4.70, N: 6.33.

3-[4′-(4″-Cyanophenyl)benzoyl]-3,4-dihydro-8-methyl-2*H*-pyrido [1,2-*a*]pyrimidine hydrochloride (**34c**) was prepared from 1-(4-cyanobiphenylyl)-2-(dimethylaminomethyl)-prop-2-en-1-one hydrochloride and **7c** according to the general method. Mp. 285 °C. ^{1}H NMR: δ 2.31 (s, 3H), 3.52–3.56 (m, 1H), 3.77–3.81 (m, 1H), 4.41–4.57 (m, 3H), 6.72 (d, 1H, $J = 6.1$ Hz), 6.93 (s, 1H), 7.94–7.99 (m, 7H, Ar-H), 8.19 (d, 2H, Ar-H, $J = 8.3$ Hz), 9.93 (br s, 1H). Anal. ($C_{23}H_{20}N_3OCl$) calc C: 70.85, H: 5.17, N: 10.78; found C: 70.57, H: 5.31, N: 10.68.

3-[4′-(4″-Methoxyphenyl)benzoyl]-3,4-dihydro-8-methyl-2*H*-pyrido[1,2-*a*]pyrimidine hydrochloride (**35c**) was prepared from 1-(4′-methoxybiphenyl)-2-(dimethylaminomethyl)prop-2-en-1-one hydrochloride and **7c** according to the general method. Mp. 201 °C. ^{1}H NMR: δ 2.33 (s, 3H), 3.52–3.58 (m, 1H), 3.75 (s, 3 H), 3.75–3.81 (m, 1H), 4.38–4.55 (m, 3H), 6.74–6.77 (m, 3H), 7.08/7.75 (2×d, 2 × 2H, $J = 8.8$ Hz), 7.86/8.11 (2×d, 2 × 2H, $J = 8.4$ Hz), 7.93 (d, 1H $J = 6.8$ Hz), 9.17 (bs 1H). Anal. ($C_{23}H_{22}N_2O_2$) HR-MS: calc for $C_{23}H_{22}N_2O_2$ 358.1681; found 358.1680.

3-[4′-(4″-Carboxyphenyl)benzoyl]-3,4-dihydro-8-methyl-2*H*-pyrido[1,2-*a*]pyrimidine hydrochloride (**36c**) was prepared from **34c** by adding 50% acetic acid in water and a few drops of ethanol until the substance was dissolved. The solution was refluxed 3 h and the solvent evaporated. The product was purified by crystallisation from methanol. Mp. > 245 °C. ^{1}H NMR: δ 2.33 (s, 3H), 3.52–3.58 (m, 1H), 3.75–3.83 (m, 4H), 4.38–4.55 (m, 3H), 6.74–6.77 (m, 2H), 7.08 (d, 2H, Ar-H, $J = 8.8$ Hz), 7.75 (d, 2H, Ar-H, $J = 8.8$ Hz), 7.86 (d, 2H, Ar-H, $J = 8.4$ Hz), 7.93 (d, 1H, Ar-H, $J = 6.8$ Hz), 8.11 (d, 2H, Ar-H, $J = 8.4$ Hz), 9.17 (br s, 1H). Anal. ($C_{23}H_{21}N_2O_3Cl$) calc C: 67.56, H: 5.18, N: 6.85; found C: 67.65, H: 5.26, N: 6.74.

3-[4′-(4″-Bromophenyl)benzoyl]-3,4-dihydro-8-methyl-2*H*-pyrido [1,2-*a*]pyrimidine perchlorate (**37c**) was prepared from 1-(4-bromobiphenylyl)-2-(dimethylaminomethyl)-prop-2-en-1-one hydrochloride and **7c** according to the general method. Mp. 182 °C. ^{1}H NMR: $\delta = 2.33$ (s, 3H), 3.52–3.59 (m, 1H), 3.77–3.81 (m, 1H), 4.39–4.55 (m, 3H), 6.73–6.77 (m, 2H, Ar-H), 7.69–7.77 (m, 4H, Ar-H), 7.92 (t, 3H, Ar-H, $J = 7.8$ Hz), 8.15 (d, 2H, Ar-H, $J = 8.4$ Hz), 9.18 (br s, 1H, NH). Anal. ($C_{22}H_{20}N_2O_5BrCl$) calc C: 52.04, H: 3.97, N: 5.52; found C: 52.03, H: 4.38, N: 5.70.

3-[4′-(4″-Bromophenyl)benzoyl]-3,4-dihydro-6-methyl-2*H*-pyrido [1,2-*a*]pyrimidine perchlorate (**37d**) was prepared from 1-(4-bromobiphenylyl)-2-(dimethylaminomethyl)prop-2-en-1-one hydrochloride and **7d** according to the general method. Mp. 235 °C. ^{1}H NMR: δ 2.53 (s, 3H), 3.29 (s, 2H), 3.49–3.55 (m, 1H), 3.79–3.83 (m, 1H), 4.33–4.50 (m, 3H), 6.82 (d, 1H, Ar-H, $J = 7.1$ Hz), 6.92 (d, 1H, Ar-H, $J = 9.0$ Hz), 7.68–7.78 (m, 5H, Ar-H), 7.91 (d, 2H, Ar-H, $J = 8.5$ Hz), 8.18 (d, 2H, Ar-H, $J = 8.5$ Hz), 9.32 (s, 1H, NH). Anal. ($C_{22}H_{20}N_2O_5BrCl$) calc C: 52.04, H: 3.97, N: 5.52; found C: 52.37, H: 4.05, N: 5.50.

3-[4′-(4″-Bromophenyl)benzoyl]-3,4-dihydro-6,8-dimethyl-2*H*-pyrido[1,2-*a*]pyrimidine perchlorate (**37e**) was prepared from 1-(4′-bromobiphenyl)-2-(dimethylaminomethyl)prop-2-en-1-one hydrochloride and **7e** according to the general method. Mp. 302 °C. ^{1}H NMR: δ 2.28 (s, 3H), 2.49 (s, 3H), 3.49–3.54 (m, 1H), 3.77–3.81 (m, 1H), 4.30–4.45 (m, 3H), 6.66 (s, 1H, Ar-H), 6.71 (s, 1H, Ar-H), 7.70–7.78 (m, 4H, Ar-H), 7.91 (d, 2H, Ar-H, $J = 8.5$ Hz), 8.17 (d, 2H, Ar-H, $J = 8.5$ Hz), 9.12 (br s, 1H). Anal. ($C_{23}H_{21}N_2OBr$) HR-MS: calc for $C_{23}H_{21}N_2O^{79}Br$ 420.0837; found 420.0837, calc for $C_{23}H_{21}N_2O^{81}Br$ 422.0817; found 422.0819.

5.2. Biological assays

5.2.1. Reagents and materials

N-(3-Aminomethyl)benzylacetamidine, N^{ω}-nitro-L-arginine, N^{ω}-propyl-L-arginine, and tetrahydrobiopterine were purchased from Alexis Biochemicals. Calcium chloride hexahydrate, NADPH, DMSO, EDTA, sulfanilamide and 1-naphthylethylenediamine dihydrochloride were obtained from Merck. Tetramethylammonium chloride and L-arginine were purchased from Fluka and Calmoduline from pig brain from Roche Diagnostics. Acetonitrile was obtained from LGC Promochem. All other chemicals were obtained from Sigma-Aldrich.

5.2.2. Origin of proteins

Isoforms of NOS used in the screening tests (Griess assay) were rat brain nNOS (Alexis, recombinant from Sf9 insect cells, specific activity 700 nmol/min × mg prot); human nNOS (B. Mayer, University of Graz, from *Pichia pastoris*, specific activity 800 nmol/min × mg prot); murine iNOS (Cayman Chemical, from Sf9 insect cells, specific activity 6 nmol/min × mg prot); human iNOS (Alexis, from Sf9 insect cells, specific activity 1 nmol/min × mg prot); bovine eNOS (Cayman Chemical, from Sf9 cells, specific activity 3 nmol/min × mg prot); and human eNOS (B. Mayer, University of Graz, from *Pichia pastoris*, specific activity 184 nmol/min × mg prot).

Isoforms of NOS used in the radioactive experiments were as follows: recombinant rat brain nNOS purified from the yeast *Saccharomyces cerevisiae* (specific activity 290 nmol nmol/min × mg prot); murine iNOS purified from *Escherichia coli* (specific activity 340 nmol/min × mg prot); bovine eNOS from *E. coli* (specific activity 40 nmol/min × mg prot), and $iNOS_{oxy}$ from *E. coli* were gifts from D.J. Stuehr (Lerner Research Fundation, Cleveland, USA).

Protein concentrations were determined by the method of Bradford using bovine serum albumin as a standard and the Bradford reagent from Biorad [49]. They were estimated to be more than 95% pure by SDS-PAGE electrophoresis. Their heme concentrations were determined optically from the [Fe^{II}–CO] – [Fe^{II}] difference spectrum using a $\Delta\epsilon_{445-480\ nm}$ of 74 $mM^{-1} \times cm^{-1}$ [50].

5.2.3. Nitrite determination (Griess assay)

The formation of nitrite (stable end-product of NO) was followed by the reaction with sulphanilamide and 1-naphthylethylenediamine hydrochloride in hydrochloric acid. The absorption of the resulting azo dye was measured at 543 nm on a Beckmann DU7500 UV/vis spectrophotometer. Since NADPH can affect low values by reducing the diazonium cation as well as the azo dye, it was necessary to remove NADPH from the reaction using an additional incubation with lactate dehydrogenase and sodium pyruvate [40].

Buffers contained 50 mM triethanolamine (iNOS and eNOS, pH = 7.5, nNOS, pH = 7.0), 1 mM CHAPS, 10 mM 2-mercaptoethanol and 0.5 mM EDTA. Typical assay mixtures contained 0.5 mM Ca^{2+} (nNOS and eNOS), 1 mM Mg^{2+} (iNOS), 5 μM flavine adenine dinucleotide (FAD), 5 μM flavine mononucleotide (FMN), 0.5 mM NADPH, 10 μM H_4B, 10 μg/ml CaM, 0.5 mM (nNOS) or 1 mM L-arginine (iNOS and eNOS) in a total volume of 80 μl. These mixtures were incubated at 37 °C (nNOS 30 min, iNOS and eNOS 20 min) after the addition of proteins. Then a solution of 1.6 mM pyruvic acid, sodium salt, and 20 U/ml L-lactic dehydrogenase (20 μl) was added. These preparations were incubated for 20 min at 37 °C and the reactions were stopped by the addition of 50 μl ice cold acetonitrile and centrifugation (15,000 rpm, 4 °C). Then 12 μl each of a solution of 5.8 mM naphthylethylendiamine dihydrochloride and 52 mM sulfanilamide were added to 120 μl of the supernatants. Absorbances at 543 nm were measured after 5 min at 20 °C. All data were referred to 100% control experiments containing 1% DMSO but without inhibitor. All experimental data are means ± of 3–5 determinations.

5.2.4. Radioactive assays

NOS-dependent oxidation of L-arginine to L-citrulline was determined according to a previously described protocol [41]. Briefly, enzymatic reactions were conducted at 37 °C for 5 min in 50 mM Hepes, pH 7.4, containing 5 mM DTT, 1 mM NADPH, 1 mM $CaCl_2$, 10 μg/ml CaM, 10 μM H_4B, 2 μM FAD, 2 μM FMN, about 200,000 cpm [γ-^{14}C]-L-arginine (Perkin–Elmer, specific activity 50 mCi/mmol, 2.5 μM L-arginine), and the tested compounds. The pyrido[1,2-*a*]pyrimidines were added to the incubation mixtures as 1 μl portions dissolved in DMSO. Final incubation volumes were 100 μl. Control incubations contained similar amounts (1%) of DMSO without inhibitors and values are expressed relative to the DMSO controls. The reactions were started by the addition of protein and terminated by the addition of 500 μl cold stop buffer (20 mM sodium acetate, pH 5.5, 1 mM L-citrulline, 2 mM EDTA, and 0.2 mM EGTA). Samples (500 μl) were applied to small glass columns containing 800 μl of Dowex AG 50 W-X8 (Na^+ form, prepared from the H^+ form), pre-equilibrated with stop buffer and a total of 1.5 ml of stop buffer was added to eluate [^{14}C]L-citrulline. Aliquots were then mixed with 1.5 ml Pico-Fluor 40 (Packard) and counted on a Packard Tri-Carb 2300 liquid scintillation spectrometer. Control sample without NOS are included for background determinations. Incubations in the presence of iNOS were performed similarly but $CaCl_2$ and CaM were omitted. When experiments had to be done with lower concentrations of L-arginine, [^{3}H-2,3,4]L-arginine (Perkin–Elmer, specific activity 50 Ci/mmol) was used and various concentrations of unlabelled L-arginine were added.

5.2.5. UV/visible interaction studies of NOS_{oxy} domain with pyrido[1,2-a]pyrimidines

Studies were carried out at room temperature in 1-cm pathlength cuvettes containing 0.8–1.0 μM $iNOS_{oxy}$ in 50 mM Hepes, pH 7.4. Assayed compounds were dissolved in DMSO, and equivalent amounts of DMSO were added to the reference cuvette. At the beginning, these experiments were done without the addition of imidazole but no clear effect could be observed. This comes from the fact that native $iNOS_{oxy}$ is an equilibrium of low- and high spin heme-Fe^{III} complexes, and that compounds modify only a small part of the equilibrium. We then saturated the enzyme with an excess of imidazole, a compound that binds to the iron of the heme and completely forms a low spin complex absorbing at 425–430 nm. Further additions of compounds that bind in an identical manner than L-arginine can displace imidazole from the iron and restore the high spin heme Fe^{III} complex absorbing at 395–400 nm [43]. Solutions of 0.8–1 μM $iNOS_{oxy}$ in 50 mM Hepes buffer containing 1 mM imidazole were transferred to each cuvette (150 μl total volume) and a baseline between 380 and 480 nm was recorded. Then, successive additions of 1 μl of the compounds (1 mM or 10 mM in DMSO) were done and the same volumes of DMSO were added to the reference cuvette. After each addition, the spectra were recorded.

5.2.6. Effects of pyrido[1,2-a]pyrimidines on NADPH consumption by iNOS

The initial rates of NADPH oxidation by iNOS were quantitated spectrophotometrically at 340 nm using an extinction coefficient of 6.2 $mM^{-1} \times cm^{-1}$. Cuvettes contained 150 μl of 50 mM Hepes, pH 7.4, 5 mM DTT, 100 μM $CaCl_2$, 200 μM NADPH, 10 μM H_4B, L-arginine (100 μM) and the tested agents. After preincubating the cofactor mixture and the inhibitor for 2 min at 37 °C, the iNOS sample (final concentration 15–50 nM) was added and the absorption at 340 nm was followed for 3 min. Control incubations were run in the presence of 1% DMSO, in the absence of NADPH, or in the presence of 1 mM L-NNA, a known inhibitor of NADPH consumption by NOS [45].

5.2.7. Effects of pyrido[1,2-a]pyrimidines on NOS expressed in RAW 264.7 cells

The murine macrophage RAW 264.7 cells were plated at 100,000 cells/well in 96-well plates in 200 μl of RPMI 1640 culture medium supplemented with 5% FCS and antibiotics, and incubated for 24 h at 37 °C under a 5% CO_2 atmosphere. Fresh medium and various concentrations of compounds were added 1 h before the addition of 40 U/ml γ-IFN and 50 ng/ml LPS. After incubation for 20 h at 37 °C, 100 μl of the supernatants were analysed for nitrite formation using the Griess' reagent and absorbance at 550 nm was measured on a Victor plate reader. Quantitation of nitrite formation was performed with calibration curves using known amounts of $NaNO_2$ treated under the same conditions.

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References

[1] J.F. Kerwin, J.R. Lancaster, P.F. Feldman, J. Med. Chem. 38 (1995) 4343–4362.
[2] S. Pfeiffer, B. Mayer, B. Hemmens, Angew. Chem. Int. Ed. 38 (1999) 1714–1731.
[3] S. Moncada, R.M. Palmer, E.A. Higgs, Pharmacol. Rev. 43 (1991) 109–142.
[4] J.F. Kerwin, M. Heller, Med. Res. Rev. 14 (1994) 23–74.
[5] J.L. Dinerman, C.J. Lowenstein, S.H. Snyder, Circ. Res. 73 (1993) 217–222.

[6] K.L. Davis, E. Martin, I.V. Turko, F. Murad, Annu. Rev. Pharmacol. Toxicol. 41 (2001) 203–236.
[7] C. Nathan, J. Clin. Invest. 100 (1997) 2417–2423.
[8] W.K. Alderton, C.E. Cooper, R.G. Knowles, Biochem. J. 357 (2001) 593–615.
[9] B.S.S. Masters, K. McMillan, E.A. Sheta, J.S. Nishimura, L.J. Roman, P. Martasek, FASEB J. 10 (1996) 552–558.
[10] H. Li, T.L. Poulos, J. Inorg. Biochem. 99 (2005) 293–305.
[11] R.J. Esper, J.O. Vilariño, R.A. Machado, A. Paragano, Adv. Cardiol. 45 (2008) 17–43.
[12] H. Li, T. Wallerath, T. Münzel, U. Förstermann, Nitric Oxide 7 (2002) 149–164.
[13] L.L. Thomsen, J. Olesen, Clin. Neurosci. 5 (1998) 28–33.
[14] K.K. Chung, T.M. Dawson, V.L. Dawson, Cell. Mol. Biol. 51 (2005) 247–254.
[15] B.R. Babu, O.W. Griffith, Curr. Opin. Chem. Biol. 2 (1998) 491–500.
[16] L. Salerno, V. Sorrenti, C. Di Giacomo, G. Romeo, M.A. Siracusa, Curr. Pharm. Des. 8 (2002) 177–200.
[17] A.C. Tinker, A.V. Wallace, Curr. Top. Med. Chem. 6 (2006) 23–74.
[18] E.P. Erdal, E.A. Litzinger, J. Seo, Y. Zhu, H. Li, R.B. Silverman, Curr. Top. Med. Chem. 5 (2005) 603–624.
[19] N.M. Olken, M.A. Marletta, Biochemistry 32 (1993) 9677–9685.
[20] C. Frey, K. Narayanan, K. McMillan, L. Spack, S.S. Gross, B.S. Masters, O.W. Griffith, J. Biol. Chem. 269 (1994) 26083–26091.
[21] H.Q. Zhang, W. Fast, M.A. Marletta, P. Martasek, R.B. Silverman, J. Med. Chem. 40 (1997) 3869–3870.
[22] M. Flinspach, H. Li, J. Jamal, W. Yang, H. Huang, B.R. Silverman, T.L. Poulos, Biochemistry 43 (2004) 5181–5187.
[23] E.P. Garvey, J.A. Oplinger, G.J. Tanoury, P.A. Sherman, M. Fowler, S.C. Wang, D.K. Nakayama, R.L. Simmons, S. Snyder, T.R. Billiar, J. Biol. Chem. 269 (1994) 26669–26676.
[24] R. Ijuin, N. Umezawa, S.-I. Nagai, T. Higuchi, Bioorg. Med. Chem. Lett. 15 (2005) 2881–2885.
[25] R.C. Babbedge, P.A. Bland-Ward, S.L. Hart, P.K. Moore, Br. J. Pharmacol. 110 (1993) 225–228.
[26] D.J. Wolff, B.A. Lubeskie, S. Umansky, Arch. Biochem. Biophys. 314 (1994) 360–366.
[27] J.L. Collins, B.G. Shearer, J.A. Oplinger, S. Lee, E.P. Garvey, M. Salter, C. Duffy, T.C. Burnette, E.S. Furfine, J. Med. Chem. 41 (1998) 2858–2871.
[28] B.G. Shearer, S. Lee, J.A. Oplinger, L.W. Frick, E.P. Garvey, E.S. Furfine, J. Med. Chem. 40 (1997) 1901–1905.
[29] P.E. Chabrier, M. Auguet, B. Spinnewyn, S. Auvin, S. Cornet, C. Demerlé-Pallardy, C. Guilmard-Favre, J.G. Marin, B. Pignol, V. Gillard-Roubert, C. Roussillot-Charnet, J. Schulz, I. Viossat, D. Bigg, S. Moncada, Proc. Natl. Acad. Sci. USA 96 (1999) 10824–10829.
[30] E.P. Garvey, J.A. Oplinger, E.S. Furfine, R.J. Kiff, F. Laszlo, B.J. Whittle, R.G. Knowles, J. Biol. Chem. 272 (1997) 4959–4963.
[31] G.J. Southan, C. Szabó, M.P. Connor, A.L. Salzman, C. Thiemermann, Eur. J. Pharmacol. 291 (1995) 311–318.
[32] W.M. Moore, R.K. Webber, K.F. Fok, G.M. Jerome, J.R. Connor, P.T. Manning, P.S. Wyatt, T.P. Misko, F.S. Tjoeng, M.G. Currie, J. Med. Chem. 39 (1996) 669–672.
[33] T.J. Hagen, A.A. Bergmanis, S.M. Kramer, K.F. Fok, A.E. Schmelzer, B.S. Pitzele, L. Swenton, G.M. Jerome, C.M. Kornmeier, W.M. Moore, L.F. Branson, J.R. Connor, P.T. Manning, M.G. Currie, E.A. Hallinan, J. Med. Chem. 41 (1998) 3675–3683.
[34] R.K. Webber, S. Metz, W.M. Moore, J.R. Connor, M.G. Currie, K.F. Fok, T.J. Hagen, D.W. Hansen, G.M. Jerome, P.T. Manning, B.S. Pitzele, M.V. Toth, M. Trivedi, M.E. Zupec, F.S. Tjoeng, J. Med. Chem. 41 (1998) 96–101.
[35] D.W. Hansen, K.B. Peterson, M. Trivedi, S.W. Kramer, R.K. Webber, F.S. Tjoeng, W.M. Moore, G.M. Jerome, C.M. Kornmeier, P.T. Manning, J.R. Connor, T.P. Misko, M.G. Currie, B.S. Pitzele, J. Med. Chem. 41 (1998) 1361–1366.
[36] S. Connolly, A. Aberg, A. Arvai, H.G. Beaton, D.R. Cheshire, A.R. Cook, S. Cooper, D. Cox, P. Hamley, P. Mallinder, I. Millichip, D.J. Nicholls, R.J. Rosenfeld, S.A. St-Gallay, J. Tainer, A.C. Tinker, A.V. Wallace, J. Med. Chem. 47 (2004) 3320–3323.
[37] J.A. Lowe, W. Qian, S.E. Drozda, R.A. Volkmann, D. Nason, R.B. Nelson, C. Nolan, D. Liston, K. Ward, S. Faraci, K. Verdries, P. Seymour, M. Majchrzak, A. Villalobos, W.F. White, J. Med. Chem. 47 (2004) 1575–1586.
[38] U. Girreser, D. Heber, M. Schütt, Synthesis (1998) 715–717.
[39] U. Girreser, D. Heber, M. Schütt, Synlett (1998) 263–264.
[40] D.J. Stuehr, N.S. Kwon, S.S. Gross, B.A. Thiel, R. Levi, C.F. Nathan, Biochem. Biophys. Res. Commun. 161 (1989) 420–426.
[41] D.S. Bredt, H.H.W. Schmidt, in: M. Feelisch, J.S. Stamler (Eds.), Methods in Nitric Oxide Research, Wiley, 1996, pp. 249–254.
[42] A. Presta, U. Siddhanta, C. Wu, N. Sennequier, L. Huang, H.M. Abu-Soud, S. Erzurum, D.J. Stuehr, Biochemistry 37 (1998) 298–310.
[43] K. McMillan, B.S.S. Masters, Biochemistry 32 (1993) 9875–9880.
[44] A. Tuynman, C. Perollier, Y. Frapart, P. Schumann-Bard, V. Collot, S. Rault, J.L. Boucher, Nitric Oxide 9 (2003) 86–94.
[45] N. Sennequier, D.J. Stuehr, Biochemistry 35 (1996) 5883–5892.
[46] M. Lepoivre, J.M. Flaman, Y. Henry, J. Biol. Chem. 267 (1992) 22994–23000.
[47] J.D. Krass, B. Jastorff, H.G. Genieser, Anal. Chem. 69 (1997) 2575–2581.
[48] A. Albert, E.P. Serjeant (Eds.), The Determinaton of Ionization Constants, Chapman and Hall, New York, 1984.
[49] M. Bradford, Anal. Biochem. 72 (1976) 248–252.
[50] D.J. Stuehr, M. Ikeda-Saito, J. Biol. Chem. 267 (1992) 20547–20550.