Novel 2-Alkylamino-1,4-benzoxazine Derivatives as Potent Neuroprotective Agents: Structure−Activity Relationship Studies

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2-Alkylamino-substituted-1,4-benzoxazine derivatives, a new class of potential neuroprotective agents, were synthesized and examined for their intrinsic cytotoxicity and their capacity to inhibit oxidative stress-mediated neuronal degeneration in vitro. Through structure−activity relationship studies, the 3,3-diphenyl-substituted-1,4-benzoxazine derivative **3l** was identified as the optimal candidate, owing to its potent neuroprotective activity, without the manifestation of intrinsic cytotoxicity. Accordingly, **3l** proved to be effective in an animal model of excitotoxic lesions in newborn mice.

Introduction

Oxygen, though essential for aerobic metabolism, can be converted to toxic metabolites such as superoxide, hydrogen peroxide, and hydroxyl radicals, collectively known as reactive oxygen species (ROS). When ROS generation exceeds the capacity of endogenous enzymatic and nonenzymatic antioxidant defense systems, tissues become vulnerable to damage, as the result of a widely accepted phenomenon called oxidative stress.[1]

Compared to kidney or liver, the brain contains only low to moderate activities of superoxide dismutase, catalase, and glutathione peroxidase. In contrast, among the nonenzymatic endogenous defense systems, evidence is growing that glutathione is essential for the cellular detoxification of ROS in brain cells, and that alterations in brain glutathione metabolism might contribute to the occurrence of oxidative stress.[2] The latter has been associated with the loss of neurons in the course of the progression of many neurological diseases such as Alzheimer's and Parkinson's diseases, or ischemic stroke.[3] Consequently, supplementation with exogenous antioxidants could represent an important therapeutic potential to minimize central nervous system damage.[4] Hence, there is considerable interest in the discovery and development of efficient synthetic antioxidants.

In the course of our search for new neuroprotective agents, we have previously reported the synthesis of novel 8-alkylamino-substituted-1,4-benzoxazine derivatives **1**, as well as 3-alkylamino-2,4-dihydroxybenzophenones **2** (Chart 1). From their capacity to inhibit oxidative stress-mediated neuronal degeneration in vitro, these compounds were found to be potent neuroprotective agents, with activity close to that of standard α-tocopherol.[5] From the combined results of both intrinsic cytotoxicity and neuroprotection, substituted 1,4-benzoxazines were identified as the best candidates for therapeutic potential. On the basis of structure−activity relationship studies, two compounds, **1a** and **1b**, were selected for further in vivo biological evaluation. Both compounds were found to be effective in protecting against the lesions induced by *S*-bromo-willardiine injected into the cortex or white matter of 5-day old mice-pups.[5c] Although 3-alkylamino-2,4-dihydroxybenzophenones **2** could not be considered as promising compounds for therapeutic potential, due to their intrinsic cytotoxicity, they remained potent neuroprotective agents. We recently revealed a new mode of reactivity of these compounds in their oxidized 3,4-iminoquinone form, which led, through a cascade of transformation, to novel polyfunctionalized 1,4-benzoxazine derivatives **3a**−**n**.[6] Since these benzoxazines were topologically different from the 1,4-benzoxazine derivatives **1a** and **1b** reported earlier,[5a] we thought that it would be interesting to know if the new 1,4-benzoxazine derivatives **3a**−**n** also displayed neuroprotective activity.

Chart 1

1a 1b

2 3a-n

In this paper, we present the results of preliminary in vitro assays of variously substituted 1,4-benzoxazine derivatives **3a**−**n** (Table 1) aimed at exploring the structural requirements for efficient neuroprotective activity, without the manifestation of intrinsic cytotoxicity. Then, the neuroprotective effects of the most promising compound **3l** are tested in vivo, in a model

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Table 1. In Vitro Neuroprotective Activity of (2*H*-1,4-Benzoxazin-6-yl)(phenyl)methanones **3** and **4**

Compd	R^1	R^2	R^3	Toxicity[a]				Protection vs 2mM L-HCA[b]		Safety index	
				MTC(μM)		TC_{50}(μM)		PC_{50}(μM)		MTC/PC_{50}	
				MTT	LDH	MTT	LDH	MTT	LDH	MTT	LDH
1a				>250	>250	>250	>250	15.8	14.4	>15.8	>17.4
1b				>250	>250	>250	>250	2.5	4.0	>100	62.5
3a	Me	H	Bu^i	10	100	45	160	0.41	0.54	24.4	185.0
3b	Me	H	C_6H_{11}	10	100	29	120	2.40	2.10	4.2	47.6
3c	Me	H	$(CH_2)_2$-Ph	10	100	66	119	2.00	4.10	5.0	24.4
3d	Me	-$(CH_2)_5$-		10	100	72	120	3.20	4.10	3.1	24.4
3e	Me	-$(CH_2)_2O(CH_2)_2$-		10	100	75	190	1.70	1.50	5.9	66.7
3f	-$(CH_2)_4$-	H	CH_2-C_5H_9	5	25	27	53	1.39	0.82	3.6	30.5
3g	-$(CH_2)_5$-	H	CH_2-C_6H_{11}	5	100	16.5	>250	0.24	0.15	20.8	667.0
3h	-$(CH_2)_5$-	Me	H_2C–	5	25	25	>250	2.40	2.20	2.0	11.4
3i	-$(CH_2)_5$-	-$(CH_2)_5$-		100	>250	250	>250	12.3	18.5	8.1	>13.5
3j	-$(CH_2)_5$-	-$(CH_2)_2O(CH_2)_2$-		5	5	8.2	10.4	2.8	2.4	1.8	2.1
3k	Ph	H	CH_2-$CH(Ph)_2$	>250	>250	>250	>250	10.0	5.0	>25	>50
3l	Ph	Me	H_2C–	100	>250	>250	>250	0.5	1.0	200	>250
3m	Ph	H	CH_2-$CH(OMe)_2$	100	>250	>250	>250	5.0	2.5	20	100
3n	Ph	H	Bu^i	5	10	108	79	10.0	10.0	<1	1
4a	Me	-	-	10	100	48	>250	0.97	1.10	10.3	91.0
4g	-$(CH_2)_5$-	-	-	25	50	178	>250	4.0	3.0	6.2	16.6

[a] In vitro neurotoxicity monitored either by reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) or by quantification of cellular lysis (lactate dehydrogenase (LDH) assay). [b] In vitro neuroprotective activity estimated through their protective effects against L-homocysteic acid (L-HCA) cytotoxicity; MTC, maximum tolerated concentration; TC_{50}, concentration producing 50% toxicity; PC_{50}, concentration producing 50% protection.

Scheme 1

of brain damage mimicking the lesions underlying cerebral palsy.

Results and Discussion

Chemistry. Substituted 1,4-benzoxazine derivatives **3a–c**, **3f**, **3g**, **3k** were synthesized according to our recently reported multistep one-pot electrochemical cascade of transformation (Scheme 1).[6] Briefly, electrogenerated 3,4-azaquinone $\mathbf{2_{ox}}$ was used as an organic cofactor for the autorecycling oxidation of a $(R^1)_2CHCH_2$-NH_2 aliphatic primary amine, alone or in the presence of a second amine R^2NH_2. The catalytic cycle produced the reduced catalyst $\mathbf{2_{red}}$ and an alkylimine as the product of amine oxidation.[7] After a certain number of turnovers, the catalytic process ceased as the catalyst $\mathbf{2_{ox}}$ was trapped through [4 + 2] cycloaddition reaction with the tautomeric enamine form of the alkylimine extruded during the catalytic cycle. This reaction, which is an inverse-electron demand Diels–Alder reaction, wherein both cycloaddition partners are generated in situ, allowed the rapid construction of the expected 1,4-benzoxazine derivatives. For the synthesis of compounds **3d**, **3e**, **3h–j**, and **3l–n**, a variant, that uses a separately prepared secondary or tertiary enamine, replaced the aforementioned electrochemical procedure.[6b] Furthermore, treatment of 1,4-benzoxazines **3a** and **3g** under acidic workup conditions quantitatively yielded the corresponding hemiacetal derivatives **4a** and **4g** (Table 1).

In Vitro Biological Evaluation. The intrinsic neurotoxicity, as well as the neuroprotective activity of 2-alkylamino-1,4-benzoxazine derivatives **3**, was assessed in vitro on murine HT-22 hippocampal cell cultures. Compounds **1a** and **1b**, two established neuroprotective agents both in vitro and in vivo,[5a,c] were evaluated in the same test systems for comparison. The results are presented in Table 1.

All of the different classes of neuroprotective compounds tested to date are intrinsically cytotoxic but, at higher or lower concentrations, a neuroprotective effect can predominate. Antioxidants, and particularly alkylamino-1,4-benzoxazine derivatives,[5a,c] possess prooxidant activity, and consequently cytotoxic effects, probably via redox cycling mechanisms.[8] Accordingly, our initial objective was to determine the concentration range for cytotoxic effects of 2-alkylamino-1,4-benzoxazine derivatives **3**, to select a window of concentrations lacking intrinsic cytotoxicity aimed at testing the neuroprotective capacity of each compound in this range.

The intrinsic neurotoxic effects of each compound were evaluated following two different methods. Neurotoxicity was monitored, either by reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT reduction assay),[9] which allows an evaluation of the "redox state" of the cells and emphasizes oxidative stress, or by quantification of cellular lysis (death) after measurement of the lactate dehydrogenase activity released from damaged cells into the culture supernatant (LDH assay).[10] The maximum tolerated concentration tested lacking toxicity (MTC), and the concentration producing 50% toxicity (TC_{50}) was estimated for each tested compound using both MTT and LDH determination.

Neuroprotective properties of 1,4-benzoxazine derivatives were estimated through their protective effects against L-homocysteic acid (L-HCA) cytotoxicity. The critical role of the Xc^- cystine/glutamate antiporter in terms of cysteine supply for glutathione synthesis, is well documented.[11] L-HCA (or L-glutamate) toxicity in the neuronal cell line HT22 is a model for cell death by oxidative stress,[12] where an excess of extracellular L-HCA (or L-glutamate) results in a depletion of intracellular glutathione levels via competition for the cystine/glutamate antiporter Xc^-. The Xc^- antiporter system has been shown to be a particularly ubiquitous transporter in the central nervous system, present in both neurons (primary cortical neurons, HT 22 hippocampal cell line), oligodendrocytes, and glia cells (primary astrocytes, C6 glioma cells).[13] Hence, exposure of HT 22 hippocampal cell lines to L-HCA induces a depletion in intraneuronal glutathione which leads finally to the accumulation of ROS and the initiation of

programmed cell death by oxidative stress with features of necrosis and apoptosis.[14] Previous cell culture toxicity studies have demonstrated that these toxic effects could be attenuated with antioxidants.[3b,15] The concentration producing 50% protection (PC_{50}) was estimated for both MTT and LDH determinations.

As reported in Table 1, the new series of 2-alkylamino-1,4-benzoxazine derivatives **3a**–**n** showed generally significant in vitro neuroprotective activity, with PC_{50} (MTT) values between 0.24 and 12.30 μM, and PC_{50} (LDH) values between 0.15 and 18.50 μM. Interestingly, most of the compounds were found to be more active than **1a** and **1b**, considered as the most attractive derivatives of the previously reported 1,4-benzoxazine series **1**, with PC_{50} (MTT) values of 15.8 and 2.5 μM, respectively, and PC_{50} (LDH) values of 14.4 and 4.0 μM, respectively. Hemiacetal derivatives **4a** and **4g**, whose the neuroprotective effects were estimated as control experiments, showed activities different from those of their 2-alkylamino counterparts **3a**–**e** and **3g**–**j,** respectively. So, it could be concluded that, as expected, no hydrolysis of the 2-alkylamino chain occurred under the experimental conditions used.[6b]

Our initial objective was to design targets that effectively protected neuronal cell lines in vitro from the toxicity induced by L-HCA, without causing any intrinsic toxic effects to the neurons. A safety index was then estimated through the relative difference between the toxic effect of the compound and its neuroprotective effect, to select candidates with the widest safety index in vitro. This was calculated as the MTC/PC_{50} ratio. As shown in Table 1, the MTC determined from the MTT reduction assay appeared to be the most sensitive parameter for all evaluated compounds. This was not surprising because antioxidants may initially interfere with the cellular redox state, before provoking any significant cellular lysis. With regard to this parameter, all targeted 3,3-dimethyl-substituted-1,4-benzoxazine derivatives **3a**–**e**, as well as hemiacetal **4a**, with the same MTC values (10 μM), were found to be at least 25-fold more toxic than benzoxazines **1a** and **1b** (MTC > 250 μM). So, although exhibiting significant PC_{50} values ranging from 0.41 μM to 3.20 μM slightly depending on the 2-substitution, benzoxazines **3a**–**e** could not be considered as promising compounds for therapeutic potential since the safety index values did not exceed 24.4 (compound **3a**). Similarly, within the subseries of 3-cyclopentyl- or 3-cyclohexyl-substituted-1,4-benzoxazine derivatives **3f**–**j** and **4g**, the results gave no evidence that the intrinsic cytotoxicity and the neuroprotective activity could be dissociable from one another, since the rank order of the compounds was closely the same for both toxicity and activity: the less toxic benzoxazine **3i** (MTC = 100 μM), that bore a 2-piperidyl substituent, was also the less active compound (PC_{50} = 12.3 μM), whereas the very cytotoxic benzoxazine **3g** (MTC = 5 μM), possessing a 2-cyclohexylmethylamino chain, was also very active (PC_{50} = 0.24 μM).

Except for compound **3n** which was cytotoxic (MTC = 5 μM), introduction of phenyl groups at the 3-position of the 1,4-benzoxazine ring resulted in a large decrease in in vitro cytotoxicity (at least 10 times). The most striking example was given by the comparison of compounds **3h** (MTC = 5 μM) and **3l** (MTC = 100 μM), both bearing a 2-(*N*-methyldioxolanyl)methylamino substituent. Furthermore, within the most attractive subseries of 3,3-diphenyl-substituted-1,4-benzoxazine derivatives **3k**–**n**, replacement, on the 2-alkylamino chain, of a methyl substituent by a phenyl group, reduced at least 50-fold the cytotoxicity: compare **3n** (MTC = 5 μM) with **3k** (MTC > 250 μM); to a lesser extent, the replacement of a methoxy substituent by a phenyl group also induced a decrease in cytotoxicity (2.5 times): compare **3m** (MTC = 100 μM) with **3k**. However, a lot of phenyl groups concomitantly reduced the neuroprotective activity: compare **3l** (PC_{50} = 0.5 μM) with **3k** (PC_{50} = 10.0 μM). Finally, it appeared that benzoxazine **3l**, with a safety index of 200, could be considered as the most attractive compound from our preliminary in vitro evaluation.

At this point, it could be noted that these results obtained from neuronal cell lines do not necessarily reflect the in vivo situation, where the different types of brain cells are in close contact to each other. Thus, evidence is growing that an intensive metabolic exchange occurs, especially between astrocytes and neurons.[2,3e] So, the potential therapeutic interest as neuroprotective agent of the optimal candidate **3l** had to be further corroborated in vivo.

In Vivo Biological Evaluation. For several risk factors of cerebral palsy, it has been established that excess release of excitatory amino acids could represent a common final pathway leading to neuronal cell damage and death. Excessive release of glutamate will lead to excessive production of ROS. Consequently, antioxidant molecules could have therapeutic benefits in brain insults underlying cerebral palsy. A well-characterized animal model of excitotoxic lesions in newborn mice has been used.[16] In this model, brain damage is induced with intracerebral administration of *S*-bromo-willardiine, a glutamatergic agonist acting on α-3-amino-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate receptors. *S*-Bromo-willardiine injection induces marked toxic effects in both cortex and white matter, where the cortical lesion is an accepted model of neonatal hypoxic-ischemic brain lesion and the white matter lesion is a model of periventricular leukomalacia, two brain lesions found in patients with cerebral palsy. In this model, the neuroprotective effects of 2-alkylamino-1,4-benzoxazine **3l** were assessed and compared with those of reference compounds **1a** and **1b**.

In newborn mice, there were no deaths within the experimental groups. In most of the pups, a 15 μg *S*-bromo-willardiine injection into the cortex or white matter of 5-day old mice pups induced tonic and tonicoclonic seizures. These epileptic manifestations were observed during the first 8 h following intracerebral injection and were not modified in terms of frequency, severity, or phenotype by ip treatments. Histological lesions induced by *S*-bromo-willardiine affected both the cortical plate and the white matter (Figure 1). Cotreatment with **1a**, **1b**, or **3l** protected in a dose-dependent manner both the white matter and the cortical plate against the insult (Figures 1 and 2). Both doses (1 and 10 mg/kg) of **1a** and **3l** were neuroprotective, while only the highest dose (10 mg/kg) of **1b** yielded a significant protection in this model (Figure 2). Thus, in newborn

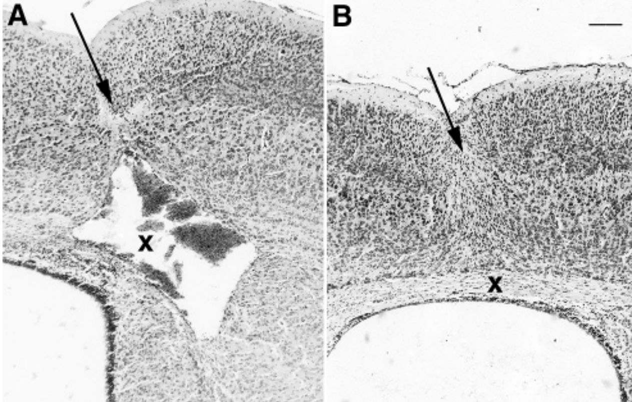

Figure 1. Compound **3l** prevents *S*-bromo-willardiine-induced neuronal death and white matter cysts. Cresyl violet-stained sections showing typical brain lesions induced by *S*-bromo-willardiine, injected at postnatal day 5 and studied at the age of postnatal day 10. A. Brain injected with *S*-bromo-willardiine alone, showing the typical neuronal loss in layers II–VI (arrow) and the white matter cystic lesion (X). B. Brain cotreated with *S*-bromo-willardiine and compound **3l**; note the absence of detectable white cystic matter lesion (X) and the limited neuronal death in the cortical plate (arrow). Bar: 70 μm.

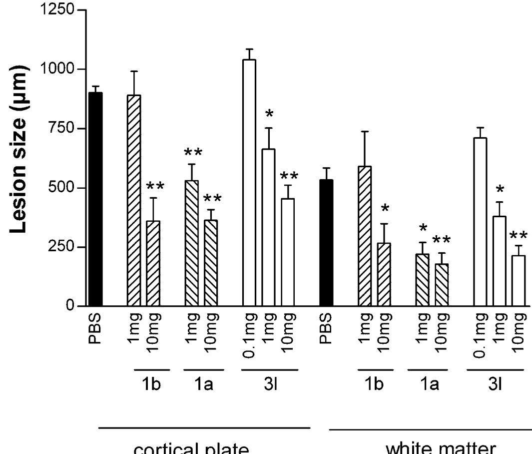

Figure 2. The histograms represent the mean length of the neocortical lesion in the sagittal fronto-occipital axis ± SEM. Asterisks indicate difference from control (* $P < 0.05$, ** $P < 0.01$ in ANOVA with Dunnet's multiple comparison test). PBS: control animals injected with *S*-bromo-willardiine into the cortex and white matter and ip with physiological saline; all the other experimental groups were cotreated with *S*-bromo-willardiine and the indicated drug at the doses indicated in mg/kg, ip.

mice, 2-alkylamino-1,4-benzoxazine **3l** showed a neuroprotective activity close to that of **1a**, considered as the most promising compound of the previously reported benzoxazine series.[5c] Furthermore, as glutamatergic lesions in white and gray matter involve both neurons and glia, the effectiveness of 2-alkylamino-1,4-benzoxazine **3l** confirms the protective effects observed in neuronal cell lines and also suggests that **3l** could be protective for glial cells.

However, the present in vivo model did not permit accurate evaluation of the capacity of compound **3l** to cross the blood–brain barrier, since this barrier is likely not fully competent at this developmental stage.[17] Partition or distribution coefficient has been the most frequently used physicochemical parameter to rapidly estimate the permeability of druglike molecules through biological membranes. The calculated log distribution coefficient for compound **3l** (calculated using SciLogP ULTRA) was found to be 5.57, a value which was very close to those calculated for reference benzoxazines **1a** (log P = 5.11) and **1b** (log P = 5.28). Therefore, it appeared that compound **3l** was rather lipophilic and would be expected to penetrate the blood–brain barrier.

Conclusion

We have synthesized and evaluated a series of new 2-alkylamino-1,4-benzoxazine derivatives **3a–n** as neuroprotective agents in vitro. Interestingly, the cytotoxicity of these compounds could be structurally modulated by varying the substitution at the 3-position, without markedly affecting the neuroprotective activity. In this respect, introduction of 3-phenyl substituents appeared to constitute a method of detoxification. This result was different from that obtained with the previously reported 1,4-benzoxazine series **1**, for which 3-phenyl substituents abolished both the cytotoxicity and the neuroprotective activity, while the presence of phenyl groups on the 8-alkylamino chain constituted a prerequisite for minimizing the neurotoxic effects.[5a] Finally, 3,3-diphenyl-substituted-1,4-benzoxazine derivative **3l** was found to be an efficient neuroprotective agent, in an animal model of excitotoxic lesions in newborn mice, suggesting that it may be considered as a potential candidate for the treatment and prevention of cerebral palsy, and might be active in other neurodegenerative diseases.

Experimental Section

All compounds **3a–h, 3j–n**, **4a**, and **4g** were prepared according to our published procedures.[6] Spectral data for these compounds are reported in ref 6b.

[(*R*,*S*)-5-Hydroxy-2-piperidino-3-spiro-1′-cyclohexyl-3,4-dihydro-*2H*-1,4-benzoxazin-6-yl](phenyl)methanone (3i). Freshly distilled enamine (269 mg, 1.5 mmol) was dissolved in methanol (250 mL) that contained tetraethylammonium perchlorate (TEAP) (1.15 g, 5 mmol), along with piperidine (50 μL, 0.5 mmol). The addition of the latter was necessary to produce the monoanionic species of $\mathbf{2_{red}}$, which was the sole form that can be oxidized to 3,4-azaquinone $\mathbf{2_{ox}}$. Then, 3,4-aminophenol $\mathbf{2_{red}}$ (114.5 mg, 0.5 mmol) was added by small portions (22.9 mg, 0.1 mmol) to the solution which was oxidized at a mercury pool whose potential was fixed at + 50 mV vs SCE, under nitrogen, at room temperature. After exhaustive electrolysis (4 h, 2 Faraday), the solvent was removed under reduced pressure. The brown oil residue was then poured into diethyl ether (20 mL). Insoluble TEAP was filtered off, and the filtrate was evaporated under reduced pressure, at 30 °C. Flash chromatography of the residue on silica gel, with toluene as the eluent, afforded the expected 1,4-benzoxazine **3i** in 25% yield (66 mg, 0.12 mmol) as a yellow solid which was recrystallized from ether: mp 138–140 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.2–1.9 (m, 16H), 2.5 (m, 2H), 3.3 (m, 2H), 4.30 (s, 1H), 4.65 (s, 1H), 6.35 (d, J = 9 Hz, 1H), 6.95 (d, J = 9 Hz, 1H), 7.50 (m, 3H), 7.70 (d, J = 8 Hz, 2H), 12.75 (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 21.4, 21.6, 24.5, 25.4, 26.3, 34.2, 35.2, 49.7, 52.1, 97.7, 106.9, 112.3, 120.3, 123.9, 128.1, 128.8, 131.1, 138.6, 150.6, 152.2, 200.4; MS DCI *m/z* 407 (MH^+). Anal. ($C_{25}H_{30}N_2O_3$) C, H, N: calcd, 73.89, 7.34, 6.89; found, 73.60, 7.39, 6.86.

Partition Coefficient Determination. Log P values were calculated using SciLogP ULTRA which is a unique scientific software tool for such a determination. The new SciLogP ULTRA prediction formula is generated on the basis of 8905 compounds training set in molecular weight range from 30 to

900, using the Neutral Network engine. When tested, it gave R2 = 0.95 and mean error of 0.3 log *P* units on 3800 new and diverse compounds outside the training and test sets.

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Supporting Information Available: Experimental procedures for biological evaluation; microanalytical data. This material is available free of charge via the Internet at http://pubs.acs.org.

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