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Optimized Synthesis of AMPA Receptor Antagonist ZK 187638 and Neurobehavioral Activity in a Mouse Model of Neuronal Ceroid Lipofuscinosis

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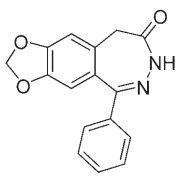
*Previous structure–activity relationship studies in the search for a potent, noncompetitive α-amino-3-(3-hydroxy-5-methyl-4-isoxazolyl)propionic acid (AMPA) receptor antagonist led to 2,3-dimethyl-6-phenyl-12*H*-[1,3]dioxolo[4,5-h]imidazo[1,2-c]-[2,3]benzodiazepine (ZK 187638). However, the first synthesis had some drawbacks regarding reagents, processes, and overall yield, which furthermore decreased when the synthesis was scaled up. Therefore, we now report a new synthetic route for this compound which requires fewer steps and is suited for large-scale production. This compound significantly relieved the symptoms of neuromuscular deficit in* mnd *mice, a model of neuronal ceroid lipofuscinosis with motor neuron dysfunction. After oral administration, the concentrations of the compound in the brain and spinal cord were about threefold higher than those in the plasma. In summary, this novel AMPA antagonist is accessible through an optimized synthetic route, has good neurobehavioral activity, oral bioavailability, and favorable brain penetration. This opens new possibilities for the treatment of devastating neurological diseases that are mediated by the AMPA receptor.*

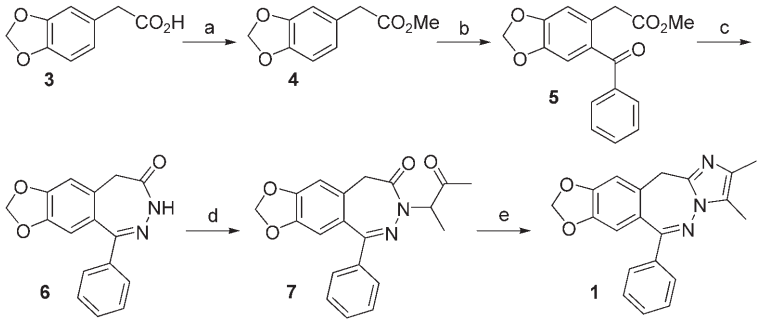
Introduction

The need for effective therapy of numerous neurological disorders is largely unmet. Evidence is accumulating that processes which are mediated by receptors of the AMPA type are a common feature in several of these diseases and are considered to represent cornerstones in the cascade of events leading to neuron death and dysfunction.[1] AMPA receptors are a subgroup of ionotropic glutamate receptors, which play a key role in synaptic transmission in the mammalian CNS on the one hand and which may cause excitotoxicity on the other if glutamate levels are excessively increased, which is the case for patients of stroke and amyotrophic lateral sclerosis (ALS), for example.[2] Furthermore, spinal motor neurons are particularly sensitive to glutamate excitotoxicity exerted through the AMPA receptor subtype in vitro.[3]

In the search for inhibitors of AMPA receptors, a novel 2,3-benzodiazepine (**1**, Scheme 1) has been found in studies of structure–activity relationships of derivatives of the selective and noncompetitive AMPA receptor antagonist GYKI 52466 (**2**, 1-(4-aminophenyl)-4-methyl-7,8-methylenedioxy-5*H*-2,3-benzodiazepine).[4] Compound **1** (2,3-dimethyl-6-phenyl-12*H*-[1,3]dioxolo-[4,5-h]imidazo[1,2-c][2,3]benzodiazepine, ZK 187638) blocks AMPA receptors very



2: GYKI 52466



Scheme 1. Synthesis of 2,3-dimethyl-6-phenyl-12*H*-[1,3]dioxolo[4,5-h]imidazo[1,2-c]-[2,3]benzodiazepine (**1**): a) amberlyst 15 (cat.), methanol, 100%; b) benzoyl chloride (1.5 equiv), tin tetrachloride (3 equiv), DMA (1.2 equiv), methylene chloride, 80%; c) hydrazine hydrate (2.7 equiv), ethanol, 62%; d) 3-bromobutan-2-one (5 equiv), KOH (5 equiv), tetrabutylammonium bromide (cat.), toluene, 72%; e) ammonium acetate (9 equiv), acetic acid, 88%.

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potently in a noncompetitive fashion and differs from the parent compound **2** essentially by an additional nitrogen-containing heterocycle attached to the positions 3 and 4 of the 2,3-benzodiazepine ring system. Initial studies on cerebral ischemia in rats and mice revealed that treatment with **1** decreased focal cerebral infarction, suggesting that **1** prevents acute neurodegeneration. However, scale-up of the synthesis to provide a larger quantity of compound **1** for further preclinical and clinical testing resulted in decreased yields of some steps (for instance, see reference [4]), thereby lowering the overall yield of this synthesis to less than 7.5%. Furthermore, some reagents and processes were not well suited for scale-up. Therefore, the aims of the present investigations were: 1) to find a new and scalable route for the chemical synthesis of **1** and 2) to extend the characterization of the compound with respect to demonstration of improved functional activity in the setting of a progressive, chronic neurological disease. The experiments were performed in *mnd* mice, which carry a spontaneous homozygous mutation in the coding region of the gene *Cln8*, which belongs to the family of neuronal ceroid lipofuscinosis-related genes (CLNs).[5] This mutation causes motor neuron dysfunction and late-life progressive paralysis of the hind legs. The role of AMPA receptor-mediated excitotoxicity is also well described in *mnd* mice. In fact, we previously found that *mnd* mice have altered expression of glutamate transporters[6] and AMPA receptor subtypes[7,8] in the spinal cord that may result in increased glutamatergic neurotransmission and play some role in the *mnd* pathology. Accordingly, treatment with the competitive AMPA receptor antagonist 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzol[f]quinoxaline (NBQX) reduces the loss of motor neuron function in *mnd* mice.[7] Because oral bioavailability and CNS uptake is a clear advantage in the therapy of chronic neurological disorders, the concentrations of **1** in plasma and at the target sites were also evaluated after repeated oral doses in this animal model.

Results and Discussion

Chemistry

The original synthetic route for compound **1** consisted of eight steps with an overall yield of approximately 7.5%, which decreased in scale-up as previously described.[4]

The new synthesis consists of only five chemical steps starting with the commercially available 3,4-(methylenedioxy)phenylacetic acid **3** (Scheme 1). The first transformation is an esterification of acid **3** to obtain the corresponding methylester **4**. This has been reported with 86% yield by using methanol/sulfuric acid.[9] We were able to optimize this reaction in terms of yield, which is now quantitative, and ease of workup by employing catalytic amounts of the acidic ion exchanger amberlyst 15 instead of sulfuric acid. The introduction of the benzoyl side chain on ester **4** can be envisioned by using Friedel–Crafts alkylation conditions. This has been reported repeatedly,[9–12] but in most cases, the cited yield of **5** is only moderate (31–48%),[9–11] and we obtained similar results by using standard reaction conditions. One report by De Sarro et al.[12] describes this Friedel–Crafts reaction to give 81% yield. Unfortunately, the general procedure given did not work as well in our hands. Presumably the unsatisfactory yield could be explained by complex formation between the product **5** and a metal species (such as tin). Also in our hands, the Lewis acid of choice for the starting material **4** is tin tetrachloride, owing to the fact that other Lewis acids such as aluminum trichloride are not compatible with the acetal moiety, or give no reaction at all, as is the case with bismuth(III)triflate.[13] We then came across the use of aluminum trichloride/*N*,*N*-dimethylacetamide (DMA) for Friedel–Crafts reactions.[14] The combination of the different procedures furnished compound **5** under optimized conditions (1.5 equiv benzoyl chloride, 3 equiv tin tetrachloride, 1.2 equiv DMA) to give an increased yield of 80% at larger scale (2.25 mol = 437 g starting material). This can be followed by the treatment of **5** with hydrazine to build up the seven-membered benzodiazepinone ring as described elsewhere,[9,11,12] but again, the reported yields were variable (29–74%). As stated earlier,[12] the use of hydrazine hydrate is feasible in this reaction, and in our hands gave an improved yield for **6** relative to the use of anhydrous hydrazine. An excess of hydrazine hydrate (2.7 equiv) was necessary, and this reagent had to be added into the reaction mixture in two portions—at the start of the reaction and after 20 h—to secure complete conversion. After workup and purification by crystallization, we obtained **6** in 63% yield with 50 g starting material. The best conditions for the N-alkylation of **6** were selected en route to our final product **1**. This kind of transformation is well precedented.[15–24] In our case an excess of base (KOH) and an excess of the reagent 3-bromobutan-2-one under phase-transfer conditions (cat. tetrabutylammonium bromide)[22,23] worked best for the generation of the substituted benzodiazepinone derivative **7**, which we obtained in 72% yield. In the final step the substituted imidazole ring has to be built, which can be carried out with ammonium acetate in acetic acid at elevated temperatures.[25–31] A large excess of ammonium acetate was needed for the synthesis of **1**. To reach complete conversion, it is essential to remove the solvent (acetic acid) partly during the reaction. Thereby, water, which is produced by this transformation, is also most probably removed. After workup and purification, the desired product **1** was obtained in 88% yield. In summary, our alternative synthesis makes **1** accessible in five synthetic steps and an overall yield of approximately 31%. In addition, reagents and procedures have been identified that are suitable for scale-up of the synthesis of **1.**

In vivo neuropharmacology

Neurological evaluation was done in *mnd* mice given **1** at oral doses of 70 and 140 mg kg^{-1} every second day from the age of 24 weeks (when neurological symptoms began) till 37 weeks (when motor behavior was greatly impaired). We used semi-quantitative tests, that is, scores for the loss of the hind-leg opening reflex and failure to grasp the cage rungs with the hind legs and quantitative tests, that is, rota-rod performance and the time required to walk up a 75-cm-long ramp raised 13 cm at one end (running time). Compound **1** did not appar-

ently modify behavior in mice of the nondiseased strain (C57BL6J mice, data not shown).

Mnd mice treated with vehicle progressively lost the hind-leg opening reflex, with a linear increase in "abnormal" responses from 27 to 37 weeks of age (Figure 1 A). The dose of **1** at 70 mg kg^{-1} significantly lowered the scores ($p < 0.01$), while the scores for the 140 mg kg^{-1} dose group were not different from the vehicle-treated group (Figure 1 A). From 27 weeks of age, *mnd* mice treated with vehicle increasingly failed to grasp the cage with the hind paws (Figure 1 B) which, at 36–37 weeks appeared to be locked in flexion. The 70 mg kg^{-1} dose significantly ($p < 0.01$) lowered the scores, while the scores for 140 mg kg^{-1} were not different from the vehicle group (Figure 1 B).

In the running time test, control mice of the nondiseased strain took about 3.5 s to walk up the ramp, independent of age and treatment. At the first time considered in this test (31 weeks of age), vehicle-treated *mnd* mice required a significantly longer time (5.5 s, $p < 0.01$) than healthy controls (3.6 s, not shown) to walk to the top (Figure 1 C). At this age, *mnd* mice treated with a dose of 70 mg kg^{-1} had a running time (3.5 s) similar to that of control mice, and significantly better than that of vehicle-treated *mnd* mice ($p < 0.05$, Tukey's test). The running time for *mnd* mice treated with 140 mg kg^{-1} was intermediate (4.4 s), but not significantly different from that of vehicle-treated *mnd* mice. From 31 to 37 weeks of age, the time required to reach the top of the ramp increased progressively in vehicle-treated *mnd* mice (Figure 1 C). Running time was significantly ($p < 0.01$) lowered at 70 mg kg^{-1}, whereas the effect of the 140 mg kg^{-1} treatment was not statistically significant.

Figure 1 D and Figure 1 E summarize the rota-rod test results for **1** in *mnd* mice. Animals of the healthy mouse strain did not fall off the rod up to the maximum time of 180 s, and **1** had no effect on their performance. Vehicle-treated *mnd* mice, starting from the age of 27–28 weeks, progressively lost the ability to balance on the rod, and by 34–37 weeks they were unable to perform. Figure 1 D shows a clear and significant effect of **1** (at 70 mg kg^{-1}) progressively delaying the decline in time on the rota-rod. For instance, at the age of 33 weeks, vehicle-treated *mnd* mice stayed on the rod for 39 ± 12 s, but those treated with 70 mg kg^{-1} stayed for 110 ± 63 s ($p < 0.01$). In the second experimental set, the mice treated with 140 mg kg^{-1} were statistically no different from those treated with vehicle (Figure 1 E).

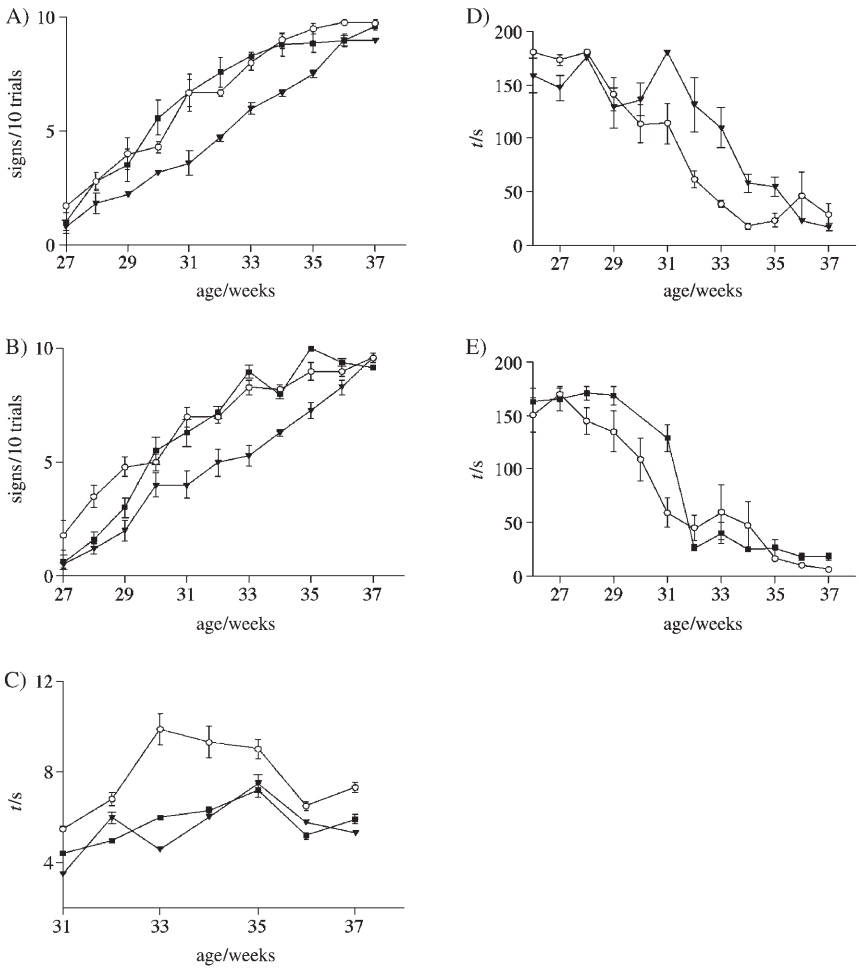

Figure 1. Effect of **1** on behavioral scores in *mnd* mice. A) Hind leg signs, B) grasping, and C) running time are reported for vehicle (○) and compound **1** at 70 mg kg^{-1} (▼) and 140 mg kg^{-1} (■). D), E) Rota-rod performance data in *mnd* mice are reported for vehicle (○) and compound **1** at 70 mg kg^{-1} (▼, panel D) or 140 mg kg^{-1} (■, panel E). Each data point represents the mean ± SD of 7–14 mice per group. Data were analyzed by ANOVA and Tukey's tests (see Results and Discussion section for statistical significance).

CNS distribution studies

Dose-related plasma concentrations of **1** were observed 3 h after the last dose of 70 and 140 mg kg^{-1} in *mnd* and C57BL6J mice (Table 1). Brain concentrations were about threefold higher than the plasma concentrations, and there was a linear relationship between brain and plasma concentrations regardless of the mouse strain (brain $= -0.15 +$ 3.24 plasma; $r^2 = 0.89$). Brain concentrations were also closely correlated with those in spinal cord (spinal cord $= 2.01 +$ 0.86 brain; $r^2 = 0.96$), further suggesting that the compound readily crosses the blood–brain barrier and diffuses within the CNS, as opposed to other AMPA antagonists such as RP119990 in mice.[32]

On correlating the efficacy of **1** on rota-rod performance with

Table 1. Plasma and CNS concentrations of compound **1** in *mnd* and C57BL6J mice.[a]

Mouse	Dose [mg kg^{-1}]	Compound **1** Plasma [μg mL^{-1}]	Brain [μg g^{-1}]	Spinal Cord [μg g^{-1}]
mnd	70	4.3 ± 2.0	12.6 ± 5.2	12.1 ± 6.5
	140	11.1 ± 4.5	41.8 ± 24.0	38.2 ± 19.1
C57BL6J	70	3.9 ± 6.0	9.7 ± 12.6	19.7 ± 14.5
	140	9.5 ± 8.0	29.7 ± 23.3	26.9 ± 20.5

[a] Values represent the mean ± SD of 5–6 mice.

brain levels in *mnd* mice, the peak concentrations reached in the brain and spinal cord were 12 μg g^{-1}, that is, around 30 μM, after oral administration of 70 mg kg^{-1}, which was effective against motor neuron dysfunction. At the higher dose (140 mg kg^{-1}), the compound possibly loses selectivity toward AMPA receptor blockade, reaching tissue concentrations high enough (≥20 μg g^{-1}) to activate other mechanisms that may counteract the main mechanism of action, thus explaining the lack of effect of this dose.

Our results of beneficial motor behavior function after pharmacological inhibition of AMPA receptor by **1** are in line with previous biochemical studies suggesting an important role of glutamate excitotoxicity in the *mnd* model of motor neuron disease[6–8] and further confirm the role of AMPA receptors on motor neuron activity. In fact, in vitro studies on cultured motor neurons have demonstrated inhibition of kainate-induced vulnerability of rat spinal motor neurons by both the competitive AMPA receptor antagonist NBQX[33] and GYKI 53655, a noncompetitive AMPA receptor antagonist.[34] The latter study also showed that AMPA receptor-mediated Ca^{2+} influx triggered by glutamate was 2.5-fold larger in motor neurons than in dorsal horn neurons, suggesting that a high density of functional AMPA receptors in motor neurons may explain their selective vulnerability.[34] Microglia, which are activated in the spinal cord of *mnd* mice,[35] also express AMPA receptors, the activation of which induces the release of tumor necrosis factor α (TNFα).[36] The expression of TNFα and its receptor p55 (TNF-R1) progressively increased in the spinal cord of *mnd* mice.[35,37] The continuous presence of TNFα may boost synaptic strength at excitatory synapses by increasing surface expression of the AMPA receptor,[38] and this might contribute to the potentiation of glutamate-mediated neural toxicity by this cytokine.[39] Findings that **1** is effective in an animal model of ALS, for which such mechanisms are also involved, have been reported.[40]

Conclusions

An optimized and scalable synthesis of the 2,3-benzodiazepine noncompetitive AMPA antagonist **1** was established with an increased overall yield of approximately 31%—a considerable improvement over 7.5% reported for our first approach of synthesis.[4] Compound **1** is orally bioavailable and concentrates in the target sites. The related plasma concentrations indicate free distribution across the blood–brain barrier. Compound **1** was well tolerated by mice for up to three months' treatment. Most importantly, this compound relieved the symptoms of neurobehavioral deficits in the *mnd* mouse model of motor neuron dysfunction and thus opens the way to new possibilities for therapeutic interventions in chronic neuromuscular disorders in which direct or glia-mediated excitotoxicity is involved, such as neuronal ceroid lipofuscinosis and ALS.

Experimental Section

General chemistry: All reagents and solvents were used as commercially received. ^{1}H NMR spectra were measured with a Bruker Avance 600 (600 MHz) or a Bruker Avance 400 (400 MHz), and ^{13}C NMR spectra were measured with a Bruker Avance 400 (100.54 MHz) unless stated otherwise. Chemical shifts are expressed in δ (ppm) and coupling constants (*J*) are in Hz. IR spectra were measured with a Nicolet 710 FTIR. MS and HRMS data were measured on a Fison AutoSpec EQ at 70 eV. Melting points were recorded on a Büchi SMP 20 and are not corrected.

Synthetic procedures: *3,4-(Methylenedioxy)phenylacetic acid methylester (**4**)*: Amberlyst 15 (50 g) was added to a stirred solution of 3,4-(methylenedioxy)phenylacetic acid (**3**, 500 g, 2.78 mol) in methanol (7.4 L). The reaction mixture was heated under reflux for 18 h and was then cooled to room temperature and filtered through celite (200 g). The filter cake was rinsed with methanol (3× 800 mL). The combined filtrates were evaporated to dryness to obtain 3,4-(methylenedioxy)phenylacetic acid methylester (**4**, 538 g, 100%) as a viscous oil (bp: 153–155 °C at 10 Torr); ^{1}H NMR (600 MHz, $CDCl_3$): δ = 3.53 (s, 2H), 3.69 (s, 3H), 5.93 (s, 2H), 6.705 (dd, *J* = 8.0 Hz, 1.0 Hz, 1H), 6.755 (d, *J* = 8.0 Hz, 1H), 6.78 ppm (d, *J* = 1.0 Hz, 1H); ^{13}C NMR (100.54 MHz, $CDCl_3$): δ = 40.69 (CH_2), 51.99 (CH_3), 100.96 (CH_2), 108.21 (CH), 109.64 (CH), 122.31 (CH), 127.45 (C), 146.64 (C), 147.68 (C), 172.06 ppm (C); IR (diamond cell/FTIR microscope): $\tilde{\nu}$ = 2953, 2894, 1738, 1504, 1492, 1446, 1249, 1164, 1040, 930, 811 cm^{-1}; MS: *m/z* (%) = 194 [M^+] (49), 181 (18), 169 (17), 135 (77), 131 (26), 119 (27), 77 (12), 69 (100); HRMS calcd for $C_{10}H_{10}O_4$ [M^+] 194.0579, found 194.0583.

*4,5-Methylenedioxy-2-benzoylphenylacetic acid methylester (**5**)*: A solution of *N*,*N*-dimethylacetamide (235 g, 250 mL, 2.70 mol) in methylene chloride (550 mL) was slowly (30 min) added at 0 °C to a solution of tin tetrachloride (1759 g, 789 mL, 6.75 mol) in methylene chloride (2.7 L) prepared at 0 °C. A white suspension was obtained, which was stirred for another 40 min. Benzoyl chloride (475 g, 392 mL, 3.38 mol) was introduced into the suspension at 0 °C within 20 min. A clear solution was formed. Finally 3,4-(methylenedioxy)phenylacetic acid methylester (**4**, 437 g, 2.25 mol), dissolved in methylene chloride (1.0 L), was added slowly (60 min) at 0 °C. The reaction mixture was stirred for 12 h and slowly warmed to room temperature. Thereby the colorless solution turned dark green. For workup the reaction mixture was cooled to −7 °C, and water (6.6 L) was added carefully over a period of 20 min (exothermic). The phases were separated, and the aqueous phase was extracted with methylene chloride (3×1.0 L). The combined organic phases were washed with an aqueous solution of NaOH (6 N, 2.0 L). After phase separation, the solvent was removed in vacuo and the resulting residue was purified by chromatography (matrex silica gel, methylene chloride) to yield 4,5-methylenedioxy-2-benzoylphenylacetic acid methylester (**5**, 537 g, 80%) as white crystals; mp: 74–75 °C; ^{1}H NMR (400 MHz, DMSO): δ = 3.48 (s, 3H), 3.75 (s, 2H), 6.13 (s, 2H), 6.88 (s, 1H), 7.05 (s, 1H), 7.52 (tt, *J* = 7.5 Hz,

1.5 Hz, 2H), 7.62–7.69 ppm (m, 3H); ^{13}C NMR (100.54 MHz, DMSO): δ = 38.04 (CH_2), 51.59 (CH_3), 102.11 (CH_2), 109.93 (CH), 112.28 (CH), 128.59 (CH), 129.60 (C), 129.84 (CH), 131.35 (C), 133.02 (CH), 137.81 (C), 145.74 (C), 149.34 (C), 171.22 (C), 196.20 ppm (C); IR (KBr): $\tilde{\nu}$ = 3441, 3029, 2947, 2916, 1742, 1655, 1502, 1376, 1340, 1283, 1153, 1035, 926, 877, 718 cm^{-1}; MS: *m/z* (%) = 298 [M^+] (35), 267 (9), 239 (67), 238 (100), 193 (7), 181 (14), 152 (28), 105 (14), 77 (21), 51 (5); HRMS calcd for $C_{17}H_{14}O_5$ [M^+] 298.0841, found 298.0836.

5-Phenyl-7H-1,3-dioxolo[4,5-h][2,3]benzodiazepine-8(9H)-one (***6***): Hydrazine monohydrate (14.3 g, 13.9 mL, 286.3 mmol) was added slowly to a solution of 4,5-methylenedioxy-2-benzoylphenylacetic acid methylester (**5**, 50 g, 168 mmol) in ethanol (500 mL), and the reaction mixture was heated under reflux for 20 h. A second portion of hydrazine monohydrate (8.3 g, 8.0 mL, 164.7 mmol) was then added, and the reaction mixture was heated under reflux for another 10 h. The reaction was cooled to room temperature, aqueous HCl (2 N, 2500 mL) was added, and stirring was continued for 1 h. The precipitated product **6** was filtered and washed with water (2×50 mL). The wet product was dissolved in ethanol (160 mL), heated under reflux for 1 h, and then cooled to room temperature. The crystallized product **6** was filtered off and dried to obtain 5-phenyl-7*H*-1,3-dioxolo[4,5-h][2,3]benzodiazepine-8(9*H*)-one (**6**, 29.2 g, 62%) as an ochre solid; mp: 181.5–182.5 °C, Lit: 182–184 °C,[17] Lit: 182.9 °C;[4] ^{1}H NMR (400 MHz, DMSO): δ = 3.38 (s, 2H), 6.10 (s, 2H), 6.56 (s, 1H), 7.08 (s, 1H), 7.41–7.56 (m, 5H), 10.92 ppm (br s, 1H); ^{13}C NMR (100.54 MHz, DMSO): δ = 41.34 (CH_2), 102.08 (CH_2), 107.80 (CH), 108.07 (CH), 124.52 (C), 128.45 (CH), 129.00 (CH), 129.73 (CH), 131.73 (C), 138.26 (C), 146.10 (C), 150.40 (C), 159.60(C), 169.88 ppm (C); IR (KBr): $\tilde{\nu}$ = 3245, 2907, 1665, 1486, 1391, 1327, 1256, 1039, 765, 696 cm^{-1}; MS: *m/z* (%) = 280 [M^+] (100), 251 (67), 238 (14), 223 (15), 193 (4), 165 (18), 139 (5), 126 (5), 97 (6), 77 (8), 51 (5); HRMS calcd for $C_{16}H_{12}N_2O_3$ [M^+] 280.0848, found 280.0848.

7-[(R,S)-1-Methyl-2-oxopropyl]-5-phenyl-7H-1,3-dioxolo[4,5-h]-[2,3]benzodiazepine-8(9H)-one (***7***): 3-Bromobutan-2-one (4.75 g, 3.18 mL, 31.4 mmol) was added to a solution of 5-phenyl-7*H*-1,3-dioxolo[4,5-h][2,3]benzodiazepine-8(9*H*)-one (**6**, 2.2 g, 7.85 mmol), KOH (1.76 g, 31.4 mmol), and tetrabutylammonium bromide (0.51 g, 1.57 mmol) in toluene (22 mL), and the mixture was heated under reflux for 6 h. Then second portions of KOH (0.44 g, 7.85 mmol) and 3-bromobutan-2-one (1.19 g, 0.84 mL, 7.85 mmol) were added, and the reaction mixture was heated under reflux for another 6 h. The reaction mixture was cooled to room temperature, ethyl acetate (100 mL) was added, and the mixture was washed with brine (100 mL). The phases were separated, and the organic phase was washed again with brine (100 mL). The combined organic phases were evaporated to dryness under reduced pressure. The resulting residue was purified by chromatography (matrex silica gel, hexane/acetone 1:0→4:1→3:2) to afford 7-[(*R*,*S*)-1-methyl-2-oxopropyl]-5-phenyl-7*H*-1,3-dioxolo[4,5-h]-[2,3]benzodiazepin-8(9*H*)-one (**7**, 1.97 g, 72%) as a light-yellow solid; mp: 146–149 °C; ^{1}H NMR (400 MHz, DMSO): δ = 1.22 (br s, 0.5×3H), 1.41 (br s, 0.5×3H), 1.74 (br s, 0.5×3H), 2.09 (br s, 0.5×3H), 3.43 (br s, 1H), 3.63 (br s, 1H), 5.10 (q, *J* = 6.8 Hz, 1H), 6.13 (s, 2H), 6.63 (s, 1H), 7.17 (s, 1H), 7.41–7.56 ppm (m, 5H); ^{13}C NMR (100.54 MHz, DMSO): δ = 26.46 (CH_3), 40.99 (CH_2), 61.79 (CH), 102.27 (CH_2), 107.85 (CH), 108.14 (CH), 123.90 (C), 128.58 (CH), 129.27 (CH), 130.42 (CH), 132.08 (C), 137.60 (C), 146.36 (C), 150.91 (C), 162.51 (C), 167.84 (C), 205.13 ppm (C); IR (KBr): $\tilde{\nu}$ = 3418, 2986, 1720, 1665, 1483, 1391, 1327, 1253, 1036, 948, 703 cm^{-1}; MS: *m/z* (%) = 350 [M^+] (10), 307 (100), 279 (28), 265 (13), 237 (14), 160 (7), 104 (6), 77 (6), 51 (2); HRMS calcd for $C_{20}H_{18}N_2O_4$ [M^+] 350.1267, found 350.1271.

5-Phenyl-8,9-dimethyl-11H-1,3-dioxolo[4,5-h]imidazo[1,2-c][2,3]benzodiazepine (***1***): A solution of 7-[(*R*,*S*)-1-methyl-2-oxopropyl]-5-phenyl-7*H*-1,3-dioxolo[4,5-h][2,3]benzodiazepine-8(9*H*)-one (**7**, 1.80 g, 5.14 mmol) and ammonium acetate (3.60 g, 46.7 mmol) in acetic acid (38 g, 36 mL) was heated under reflux for 3 h. The solvent was partly removed (18 mL) under reduced pressure (5 kPa) at the same temperature. The mixture was cooled to room temperature, water (72 mL) and ethyl acetate (50 mL) were added. Stirring was continued for 30 min, and the phases were separated. The aqueous phase was extracted with ethyl acetate (50 mL). The combined organic phases were evaporated to dryness under reduced pressure. The resulting residue was purified by chromatography (matrex silica gel, hexane/acetone 1:0→4:1→1:1) to afford 5-phenyl-8,9-dimethyl-11*H*-1,3-dioxolo[4,5-h]imidazo[1,2-c] [2,3]benzodiazepine (**1**, 1.50 g, 88%) as cream-white solid. An analytic sample can be obtained by recrystallization from ethanol/water (10:30); mp: 174.5–175 °C, Lit: 176.6 °C;[4] ^{1}H NMR (400 MHz, DMSO): δ = 2.02 (s, 3H), 2.20 (s, 3H), 3.82 (s, 2H), 6.08 (s, 2H), 6.54 (s, 1H), 7.14 (s, 1H), 7.48–7.57 (m, 3H), 7.67–7.72 ppm (m, 2H); ^{13}C NMR (100.54 MHz, DMSO): δ = 8.16 (CH_3), 12.75 (CH_3), 32.45 (CH_2), 102.20 (CH_2), 107.92 (CH), 109.77 (CH), 128.56 (CH), 129.64 (CH), 130.48 (CH), 131.02 (C), 135.76 (C), 138.41 (C), 140.89 (C), 145.80 (C), 150.82 (C), 162.37 ppm (C); IR (KBr): $\tilde{\nu}$ = 3421, 2918, 1620, 1533, 1485, 1385, 1294, 1273, 1256, 1232, 1037, 699 cm^{-1}; MS: *m/z* (%) = 331 [M^+] (100), 316 (14), 289 (4), 254 (6), 249 (7), 190 (6), 166 (4), 96 (6), 77 (8), 51 (4); HRMS calcd for $C_{20}H_{17}N_3O_2$ [M^+] 331.1321, found 331.1315.

Measurement of neurobehavioral activity: Procedures involving animals and their care were conducted in conformity with the institutional guidelines that are in compliance with national (D.L. no. 116, G.U. suppl. 40, February 18, 1992, Circolare No. 8, G.U., 14 luglio 1994) and international laws and policies (EEC Council Directive 86/609, OJ L 358, 1 December 12, 1987; NIH Guide for the Care and Use of Laboratory Animals, U.S. National Research Council, 1996). Male *mnd* mice (B6 KB2) were imported from Jackson Laboratories (Bar Harbor, ME, USA) at the age of 6–8 weeks. Controls of the same strain (C57BL6J) and age were obtained from IFFA Credo (Lyon, France). On arrival, the animals were housed five to a cage (Tecniplast Gazzada S.R.L., Buguggiate, VA, Italy) and acclimatized for at least three months. The animals were housed under standard conditions (22±1 °C, 60% relative humidity, 12 h light/dark schedule) with free access to food (Altromin-MT, Rieper, Bolzano, Italy) and water.

Mnd mice were treated by gavage starting from 24 weeks of age as follows: 13 *mnd* and 14 controls received 70 mg kg^{-1} of compound **1**; 7 *mnd* and 7 controls received an initial dose of 100 mg kg^{-1} then of 140 mg kg^{-1}; 12 *mnd* and 13 controls received vehicle alone. The compound was dissolved in 10% Tween 80 and administered orally in a volume of 15 mL kg^{-1} every second day. Treatment lasted three months (until the age of 37 weeks), and mice were killed 3 h after the last dose. Body weight was recorded during treatment.

The *mnd* mice were observed for neurological scores once a week, by the same operator, blinded to the treatments. Hind-leg signs and cage-rung grasping with hind paws were determined as described elsewhere.[7] Quantitative assessment of the motor behavioral deficit involved measurement of running time on an inclined plane and rota-rod performance. For running time, *mnd* mice were trained to walk up a 75-cm ramp raised 13 cm at one end. The aversive stimulus was gently tapping their backs. The time (in sec-

onds) to run from the base to the top was recorded. For rota-rod testing, mice were trained to remain on the rod and habituated to the handling involved for at least a week before testing. On the day of testing, mice were transferred to the rota-rod room at least 15 min before the test. Strategies to overcome deficits (gripping the rod and rotating with it, falling and jumping back, etc.) were noted but not considered for the test. Performance was evaluated as the time spent on the rotating rod (12 rpm) without falling off (Rota-Rod treadmill for mice, Ugo Basile, Comerio, VA, Italy). The test was stopped after 3 min. Mice that fell off before the third minute were tested three times, with at least 5 min between tests.

For statistical analysis of behavior in *mnd* mice, a repeated-measures test (ANOVA) followed by post-hoc Tukey's test was used. All the statistical analyses were done using GraphPad Prism 2.0a for Power Macintosh (GraphPad Software Inc., San Diego, CA, USA), designed by Dr. Harvey J. Motulsky (1994–1995).

Assessment of brain availability of compound 1: At the end of the chronic studies of neuromuscular function, groups of *mnd* mice and their respective controls were killed by decapitation 3 h after the last drug dose, and their blood, spinal cord, and brain were rapidly dissected and frozen in dry ice. Plasma and CNS tissue were processed by HPLC, after a liquid–liquid extraction procedure. Briefly, 0.05 mL of a methanolic solution of flutoprazepam (100 $\mu g\,mL^{-1}$) were added to 0.05 mL of plasma. The samples were then shaken twice with 1 mL diethyl ether. After centrifugation, the organic phase was separated and evaporated to dryness. The residue was dissolved in the mobile phase and analyzed by HPLC with UV detection ($\lambda = 315$ nm). CNS tissue was homogenized (20 $mL\,g^{-1}$) in KH_2PO_4 (0.01 M, pH 7.4), and 0.5 mL containing approximately 25 mg of tissue was extracted as described for plasma. Separation was carried out on a Discovery C18 column (15 cm × 4.6 mm i.d., 5 μm particle size) (Supelco, Bellefonte, USA), protected by a Lichrospher RP-select B 5-μm precolumn (Merck, Germany) at room temperature. The mobile phase was 0.01 M KH_2PO_4/CH_3OH/CH_3CN (39:58:3 *v/v*) adjusted to pH 5.0 with H_3PO_4, at a flow rate of 1 $mL\,min^{-1}$. The retention times were approximately 10.6 min for **1** and 14.6 min for the internal standard. The assay was both precise and accurate with intra- and inter-assay coefficients of variation (CV) and relative error (RE) within the usual acceptance criteria.

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