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Laboratory note

Synthesis and biological activities of diflunisal hydrazide–hydrazones

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Abstract

Several diflunisal hydrazide–hydrazone derivatives namely 2′,4′-difluoro-4-hydroxybiphenyl-3-carboxylic acid [(5-nitro-2-furyl/substitutedphenyl)methylene] hydrazide (**3a**–**o**) have been synthesised. Methyl 2′,4′-difluoro-4-hydroxybiphenyl-3-carboxylate (**1**) and 2′,4′-difluoro-4-hydroxybiphenyl-3-carboxylic acid hydrazide (**2**) were also synthesised and used as intermediate compounds. All synthesised compounds were screened for their antimycobacterial activity against *Mycobacterium tuberculosis* H37 Rv, antimicrobial activities against various bacteria, fungi and yeast species. Compound **3a** have shown activity against *Staphylococcus epidermis* HE-5 and *Staphylococcus aureus* HE-9 at 18.75 and 37.5 μg mL^{-1}, respectively. Compound **3o** have exhibited activity against *Acinetobacter calcoaceticus* IÖ-16 at a concentration of 37.5 μg mL^{-1}, whereas Cefepime, the drug used as standard, have been found less active against the microorganisms mentioned above. The synthesised compounds were found to provide 12–34% inhibition of mycobacterial growth of *M. tuberculosis* H37 Rv in the primary screen at 6.25 μg mL^{-1}. Anticonvulsant activity of the compounds were also determined by maximal electroshock (MES) and subcutaneous metrazole (scMET) tests in mice and rats following the procedures of antiepileptic drug development (ADD) program of the National Institutes of Health (NIH). Compound **3k** showed 25% protection against MES induced seizures in p.o. rat screening at a dose level of 30 mg kg^{-1} whereas **3n** and **3o** showed neurotoxicity after 4 and 0.5 h at a dose level of 100 and 300 mg kg^{-1}, respectively.

Keywords: Hydrazide–hydrazones; Diflunisal; Antibacterial activity; Anticonvulsant activity; HETCOR

1. Introduction

Hydrazide–hydrazones have been demonstrated to possess antibacterial [1–7], anticonvulsant [8–11] and antitubercular [12–16] activities. Compounds **A** [12], **B** [6] and **C** [7] have been reported as registered antitubercular and antibacterial agents, respectively. Nifuroxazide (**D**), which possesses a hydrazide–hydrazone structure, is used as intestinal antiseptic (Fig. 1). These observations led us to synthesize novel hydrazide–hydrazones and to investigate their possible antimicrobial, antimycobacterial and anticonvulsant activities.

It has been reported in the literature [17,18] that hydrazide–hydrazones can give corresponding hydrazide and aldehyde metabolites whereas the related hydrazides are known to yield carboxylic acids via hydrolytic route. Based on this knowledge, one can expect hydrazide–hydrazones, which were obtained from 2′,4′-difluoro-4-hydroxybiphenyl-3-carboxylic acid (diflunisal) (**E**), to metabolically decompose giving its parent drug. Diflunisal hydrazide–hydrazones (**F**) were also designed as possible dual acting antimicrobial/antituberculosis agents possesing antiinflammatory properties via its active metabolite, Diflunisal against pain and inflammatory events due to the cell damage arising from tuberculosis and accompanying infectious diseases. Structure of the synthesized compounds were confirmed using UV, IR, ^{1}H-NMR, ^{13}C-NMR, HETCOR and EI-mass spectral data besides elemental analysis.

The antimicrobial activities of these compounds against the laboratory strains belong to 56 bacterial species, isolates of six fungi and a yeast species were tested using disc-diffusion and microdilution assays.

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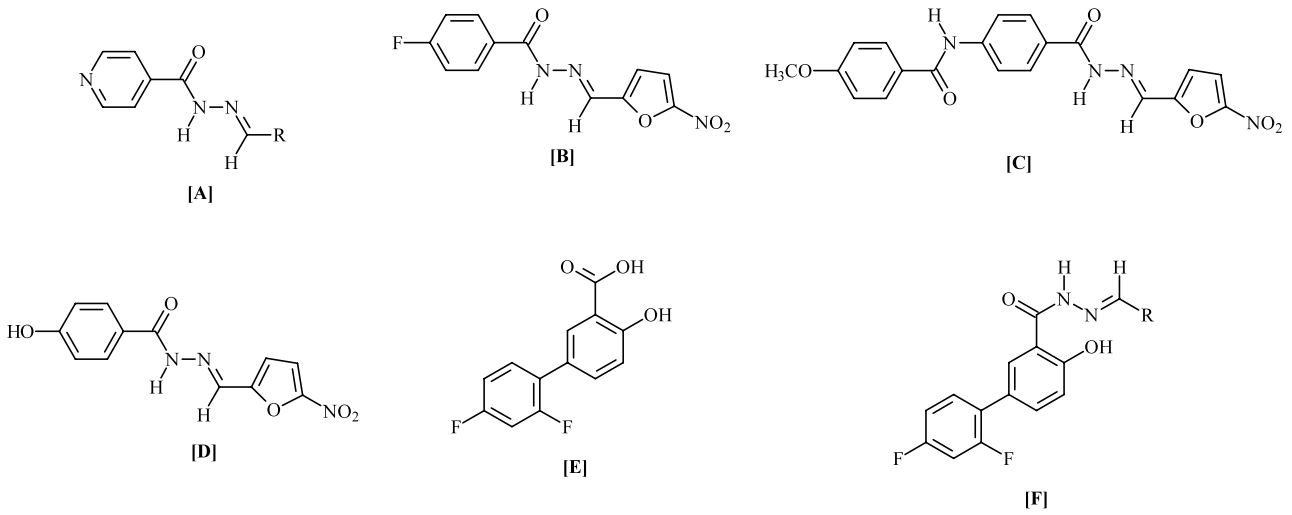

Fig. 1.

Antimycobacterial and anticonvulsant activities of synthesized compounds were also evaluated.

2. Chemistry

Synthetic route to compounds **1**, **2** and **3a**–**o** are shown in Fig. 2. The physical and spectral data of hydrazide–hydrazones **3a**–**o** are given in Table 1. The compound 2′,4′-difluoro-4-hydroxybiphenyl-3-carboxylic acid (diflunisal) that possess analgesic and anti-inflammatory activity was chosen as starting compound to design several novel hydrazide-hydrazones. Methyl 2′,4′-difluoro-4-hydroxybiphenyl-3-carboxylate (**1**) was prepared by the reaction of diflunisal and methanol in the presence of a few drops of concentrated sulfuric acid. 2′,4′-Difluoro-4-hydroxybiphenyl-3-carboxylic acid hydrazide (**2**) was synthesised by heating hydrazine–hydrate and **1** in methanol. After condensing hydrazide with 5-nitro-2-furaldeyde or substituted benzaldeydes, 2′,4′-difluoro-4-hydroxybiphenyl-3-carboxylic acid [(5-nitro-2-furyl/substituted phenyl) methylene]hydrazide (**3a**–**o**) derivatives were obtained. Compounds **2** and **3a**–**o** are original compounds. The UV spectra of **3a**–**o** exhibited characteristic K bands arising from chromophoric C=N group at 319–395 nm [17]. In the IR spectra of compounds **3a**–**o**, the bands representing azomethine and carbonyl groups appeared at 1625–1600 and 1662–1639 cm^{-1}, respectively. The ^{1}H-NMR spectra of **3a**–**o** showed single signals corresponding to resonances of azomethine protons at 8.76–8.35 ppm. The phenolic and hydrazide–hydrazone N–H

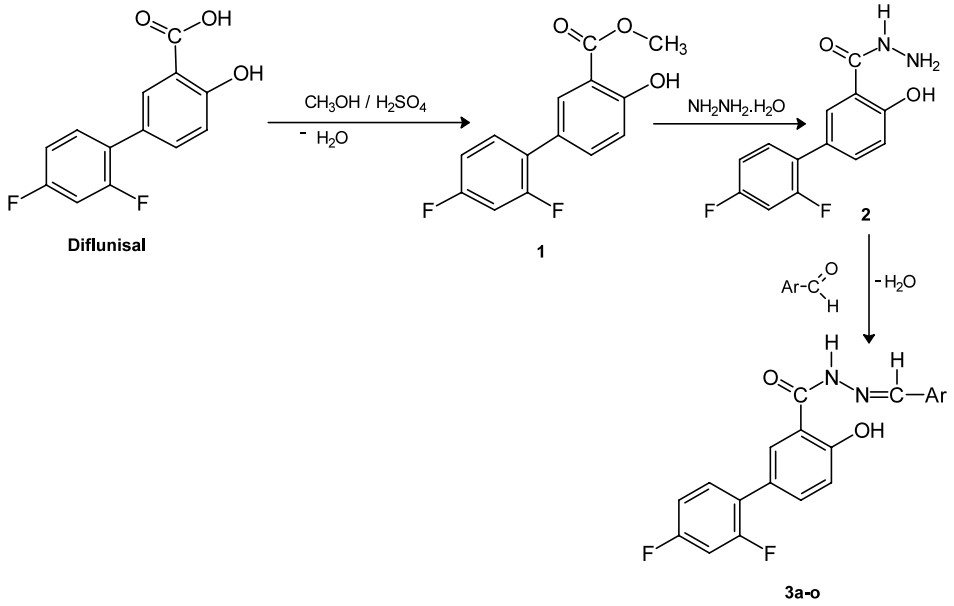

Fig. 2. Synthetic route of **1**, **2** and **3a**–**o**.

Table 1
Physical properties and spectral data of **3a–o**

Compound	R	Molecular formula	M.p. (°C) (yield %)	UV, λ_{max} (nm)	IR (ν, cm^{-1})	^{1}H-NMR (DMSO-d_6), (δ, ppm)
3a [a]	5-nitro-2-furyl	$C_{18}H_{11}F_2N_3O_5$	242–245 (83)	369, 280, 251, 205	3090 (Ar–OH, NH), 1662 (C=O), 1602 (C=N), 1144 (Ar–F).	7.09 (d, 1H, H_5, $J = 8.5$ Hz), 7.18 (t, 1H, H_9, $J = 8.4$ Hz), 7.25 (d, 1H, H_{14}, $J = 3.1$ Hz), 7.33 (t, 1H, H_{11}, $J = 8.1$ Hz), 7.56 (d, 1H, H_6 $J = 9$ Hz), 7.60 (d, 1H, H_{12} $J = 8.7$ Hz), 7.75 (d, 1H, H_{15}, $J = 3.7$ Hz), 7.95 (s, 1H, H_2), 8.42 (s, 1H, CH=N), 11.09–12.66 (2s, 2H, diflunisal OH and CONHN=CH).
3b	C_6H_5	$C_{20}H_{14}F_2N_2O_2$	250–254 (80)	331, 305, 296, 251, 221, 205	3259 (Ar–OH, NH), 1643 (C=O), 1612 (C=N), 1142 (Ar–F).	7.09 (d, 1H, H_5, $J = 8.6$ Hz), 7.21 (t, 1H, H_9, $J = 8.5$ Hz), 7.38 (t, 1H, H_{11}, $J = 8.8$ Hz), 7.47–7.77 (m, 7H,Ar–H), 8.04 (s, 1H, H_2), 8.48 (s, 1H, CH=N), 11.91 (s, 1H, diflunisal OH), 12.02 (b, 1H, CONHN=CH).
3c	$-C_6H_4-Br(4)$	$C_{20}H_{13}BrF_2N_2O_2$	275–276 (78)	332, 307, 296, 254, 226, 203, 193	3250 (Ar–OH, NH), 1645 (C=O), 1614 (C=N), 1140 (Ar–F), 1074 (Ar–Br).	7.09 (d, 1H, H_5, $J = 8.5$ Hz), 7.20 (t, 1H, H_9, $J = 8.4$ Hz), 7.23 (t, 1H, H_{11}, $J = 8.4$ Hz), 7.30–7.72 (m, 6H,Ar–H), 8.02 (s, 1H, H_2), 8.44 (s, 1H, CH=N), 11.95 (s, 2H, diflunisal OH and CONHN=CH)
3d [b]	$-C_6H_4-Cl(4)$	$C_{20}H_{13}ClF_2N_2O_2$	271–273 (77)	331,307, 297, 223, 202, 194.	3252 (Ar–OH, NH), 1645 (C=O), 1625 (C=N), 1142 (Ar–F), 1092 (Ar–Cl).	7.09 (d, 1H, H_5, $J = 8.6$ Hz), 7.20 (t, 1H, H_9, $J = 8.4$ Hz), 7.23 (t, 1H, H_{11}, $J = 8.4$ Hz), 7.54 (d, 2H, $H_{14,18}$, $J = 8.4$ Hz), 7.58 (d, 1H, H_6, $J = 8.3$ Hz), 7.62 (d, 1H, H_{12}, $J = 8.4$ Hz), 7.78 (d, 2H, $H_{15,17}$, $J = 8.4$ Hz), 8.03 (s, 1H, H_2), 8.46 (s, 1H, CH=N), 11.95 (s, 2H, diflunisal OH and CONHN=CH).
3e	$-C_6H_3-Cl_2(2,4)$	$C_{20}H_{12}Cl_2F_2N_2O_2$	255–257 (87)	386, 305, 234, 229, 213, 195	3250 (Ar–OH), 3125 (NH), 1652 (C=O), 1138 (Ar–F), 1099 (Ar–Cl).	7.09 (d, 1H, H_5, $J = 8.5$ Hz), 7.23 (t, 1H, H_9, $J = 8.5$ Hz), 7.39 (t, 1H, H_{11}, $J = 9$ Hz), 7.54–8.06 (m, 6H, Ar–H), 8.80 (s, 1H, CH=N), 11.87 (b, 1H, diflunisal OH), 12.15 (s, 1H, CONHN=CH).
3f	$-C_6H_4-F(2)$	$C_{20}H_{13}F_3N_2O_2$	260–262 (77)	329, 307, 294, 203	3260 (Ar–OH, NH), 1643 (C=O), 1614 (C=N), 1145 (Ar–F).	7.09 (d, 1H, H_5, $J = 8.6$ Hz), 7.20 (t, 1H, H_9, $J = 8$ Hz), 7.29–7.99 (m, 7H, Ar–H), 8.03 (s, 1H, H_2), 8.71 (s, 1H, CH=N), 12.00 (b, 2H, diflunisal OH and CONHN=CH)
3g	$-C_6H_4-F(3)$	$C_{20}H_{13}F_3N_2O_2$	259–260 (78)	331, 306, 292, 254, 203	3263 (Ar–OH, NH), 1641 (C=O), 1614 (C=N), 1140 (Ar–F).	7.09 (d, 1H, H_5, $J = 8.6$ Hz), 7.20 (t, 1H, H_9, $J = 8.4$ Hz), 7.22–7.68 (m, 7H, Ar–H), 8.03 (s, 1H, H_2), 8.47 (s, 1H, CH=N), 11.98–12.17 (s, 2H, diflunisal OH and CONHN=CH)
3h	$-C_6H_4-F(4)$	$C_{20}H_{13}F_3N_2O_2$	263–265 (73)	328, 304, 292, 219, 203	3600–3400 (Ar–OH), 3261(NH), 1641 (C=O), 1605 (C=N), 1149 (Ar–F).	7.09 (d, 1H, H_5, $J = 8.5$ Hz), 7.20 (t, 1H, H_9, $J = 8.5$ Hz), 7.30–7.84 (m, 7H, Ar–H), 8.03 (s, 1H, H_2), 8.46 (s, 1H, CH=N), 11.79–11.98 (b, 2H, diflunisal OH and CONHN=CH).
3i	$-C_6H_4-OH(2)$	$C_{20}H_{14}F_2N_2O_3$	290–291 (88)	367, 358, 328, 298, 287, 237, 202	3200–3175 (Ar–OH, NH), 1630 (C=O), 1616 (C=N), 1139 (Ar–F).	6.92–6.97 (m, 2H, Ar–H), 7.10 (d, 1H, H_5, $J = 8.6$ Hz), 7.22 (t, 1H, H_9, $J = 8.5$ Hz), 7.30–7.64 (m, 5H, Ar–H), 8.05 (s, 1H, H_2), 8.70 (s, 1H, CH=N), 11.16 (s, 1H, Ar–OH), 11.86–12.07 (b, 2H, diflunisal OH and CONHN=CH).
3j	$-C_6H_4-OH(4)$	$C_{20}H_{14}F_2N_2O_3$	268–271 (76)	326, 256, 229, 204	3323 (Ar–OH), 3150 (NH), 1647 (C=O), 1612 (C=N), 1142 (Ar–F).	6.86 (d, 2H, $H_{15,17}$, $J = 8.6$Hz), 7.07 (d, 1H, H_5, $J = 8.6$ Hz), 7.22 (t, 1H, H_9, $J = 8.4$ Hz), 7.38 (t, 1H, H_{11}, $J = 8.9$ Hz), 7.58–7.64 (m, 4H, H_6, H_{12} and $H_{14,18}$), 8.04 (s, 1H, H_2), 8.36 (s, 1H, CH=N), 10.00 (s, 1H, Ar–OH), 11.76 (s, 1H, diflunisal OH), 12.17 (b, 1H, CONHN=CH)
3k	$-C_6H_4-CH_3(4)$	$C_{21}H_{16}F_2N_2O_2$	266–268.5 (88)	371, 305, 227, 215, 202, 195	3248 (Ar–OH, NH), 1643 (C=O), 1612 (C=N), 1140 (Ar–F).	2.35 (s, 3H, CH_3), 7.08 (d, 1H, H_5, $J = 8.6$ Hz), 7.21 (t, 1H, H_9, $J = 8.4$ Hz), 7.28 (d, 2H, $H_{15,17}$, $J = 8$ Hz), 7.38 (t, 1H, H_{11}, $J = 8.9$ Hz), 7.57–7.62 (m, 2H, Ar–H), 7.64 (d, 2H, $H_{14,18}$, $J = 8$ Hz), 8.02 (s, 1H, H_2), 8.42 (s, 1H, CH=N), 11.83 (s, 1H, diflunisal OH), 11.88–12.18 (b, 1H, CONHN=CH)
3l	$-C_6H_4-OCH_3(4)$	$C_{21}H_{16}F_2N_2O_3$	242–245 (84)	366, 319, 307, 228, 200	3257(Ar–OH, NH), 1639 (C=O), 1608 (C=N), 1170 (Ar–F).	3.83 (s, 3H, OCH_3), 7.04 (d, 2H, $H_{15,17}$, $J = 8.6$ Hz), 7.08 (d, 1H, H_5, $J = 8.6$ Hz), 7.23 (t, 1H, H_9, $J = 8.5$ Hz), 7.38 (t, 1H, H_{11}, $J = 9$ Hz), 7.58–7.64 (m, 2H, H_6 and H_{12}), 7.70 (d, 2H, $H_{14,18}$, $J = 8.6$ Hz), 8.04 (s, 1H, H_2), 8.42 (s, 1H, CH=N), 11.08–12.97 (b,2H, diflunisal OH and CONHN=CH).

Table 1 (*Continued*)

Compound	R	Molecular formula	M.p. (°C) (yield %)	UV, λ_{max} (nm)	IR (ν, cm^{-1})	^{1}H-NMR (DMSO-d_6), (δ, ppm)
3m	$-C_6H_4-OCH_3$ OH (3.4)	$C_{21}H_{16}F_2N_2O_4$	253–257 (79)	351, 328, 300, 289, 231	3551 (Ar–OH), 3269 (NH), 1639 (C=O), 1143 (Ar–F).	3.84 (s, 3H, OCH_3), 6.86 (d, 1H, H_{17}, $J = 8$ Hz), 7.07; 7.11 (d;d, 2H, H_5 and H_{18}, $J = 8.5$ Hz and $J = 8.2$ Hz), 7.22 (t, 1H, H_9, $J = 8.5$ Hz), 7.34–7.64 (m, 4H, Ar–H), 8.04 (s, 1H, H_2), 8.35 (s, 1H, CH=N), 9.64 (s, 1H, Ar–OH), 11.78 (s, 1H, diflunisal OH), 11.90–12.46 (b, 1H, CONHN=CH)
3n	$-C_6H_4-NO_2$(4)	$C_{20}H_{13}F_2N_3O_4$	253–254 (86)	395, 336, 267, 200	3255 (Ar–OH, NH), 1643 (C=O), 1145 (Ar–F).	7.10 (d, 1H, H_5, $J = 8.6$ Hz), 7.20 (t, 1H, H_9, $J = 8.4$ Hz), 7.23 (t, 1H, H_{11}, $J = 8.5$ Hz), 7.57–7.63 (m, 2H, H_6 and H_{12}), 8.00 (d, 3H, $H_{14,18}$, $J = 8.6$ Hz and H_2), 8.31 (d, 2H, $H_{15,17}$ $J = 8.6$ Hz), 8.56 (s, 1H, CH=N), 11.85 (b, 1H, diflunisal OH), 12.11 (b, 1H, CONHN=CH)
3o	$-C_6H_2-CH_3$(2,4,6)	$C_{23}H_{20}F_2N_2O_2$	165–173 (61)	330, 303, 256, 230, 203	3250 (Ar–OH, NH), 1639 (C=O), 1610 (C=N), 1139 (Ar–F).	2.26 (s, 3H, CH_3), 2.45 (s, 6H, CH_3), 6.94 (s, 2H, $H_{15,17}$), 7.08 (d, 1H, H_5, $J = 8.6$ Hz), 7.22 (t, 1H, H_9, $J = 8.4$ Hz), 7.37 (t, 1H, H_{11}, $J = 9$ Hz), 7.60 (d, 1H, H_6, $J = 8.5$ Hz), 7.64 (d, 1H, H_{12}, $J = 8.8$ Hz), 8.05 (s, 1H, H_2), 8.76 (s, 1H, CH=N), 11.83 (s, 1H, diflunisal OH), 12.18 (s, 1H, CONHN=CH).

[a] EI-MS (70eV, *m/z*, %) 387 [M^+] (18), 249 (21), 233 (100), 206 (2), 205 (7), 178 (2), 177 (16), 155 (8), 154 (8).

[b] ^{13}C-NMR (DMSO-d_6) δ 105.30 (C11), 112.84 (C9), 117.24 (C3), 125.97 (C7), 129.75 (C14,C18, C15, C17, C2), 132.51 (C1), 132.60 (C6), 133.91 (C16), 134.97 (C12), 135.67 (C13), 148.45 (CH=N), 158.67 (C8), 159.49 (C4), 161.14 (C10), 165.27 (CONHN=CH). EI-MS (70eV, *m/z*, %) 386 [M^+] (33), 249 (4), 233 (100), 206 (2), 205 (5), 178 (3), 177 (15), 154 (10), 153 (2).

protons were observed at 11.08–12.97 ppm. ^{13}C-NMR spectra of **3d** exhibited resonances arising from azomethine (C=N) and hydrazide–hydrazone (C=O) carbons resonances at 148.45 and 165.27, respectively [19] (Fig. 3). HETCOR spectrum of **3d** also confirmed the identity of aromatic carbons by the use of proton data (Fig. 4). EI-mass spectra of selected compounds, **3a** and **3d** displayed molecular ions at m/z 387 and 386, respectively. The major fragmentation pathway appeared by the cleavage of CONHN=CH bonds of amide moiety.

3. Results and discussion

3.1. Antimicrobial activity

The antimicrobial activities of these compounds against laboratory strains belong to 56 bacterial species and isolates of six fungi and a yeast species were tested by using disc-diffusion [20] and microdilution assay [21]. Eleven out of 18 novel compounds tested in the present study were found to have no antimicrobial activity against the microorganisms listed in Table 2. Results of the remaining seven compounds, which were summarized in Tables 2 and 3, showed that they have some antibacterial activities, but no antifungal activities. 2′,4′-Difluoro-4-hydroxybiphenyl-3-carboxylic acid[(5-nitro-2-furyl)methylene]hydrazide **3a** have shown activity against *Staphylococcus epidermis* HE-5 and *Staphylococcus aureus* HE-9 at a concentration of 18.75 and 37.5 μg mL^{-1}, respectively. In addition, 2′,4′-difluoro-4-hydroxybiphenyl-3-carboxylic acid [(2,4,6-trimethylphenyl) methylene]hydrazide (**3o**) have exhibited activity against *Acinetobacter calcoaceticus* IÖ-16 at a concentration of 37.5 μg mL^{-1}, whereas cefepime, the drug used as standart, have been found less active against the microorganisms mentioned above.

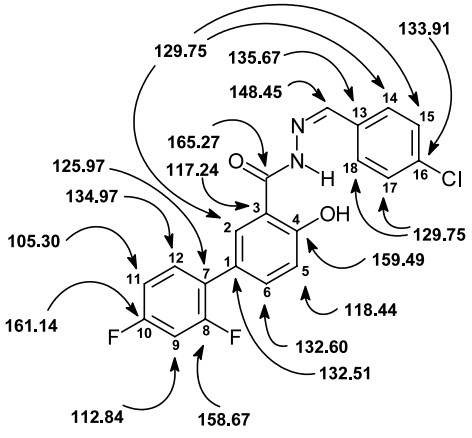

Fig. 3. ^{13}C-NMR spectral data of **3d**.

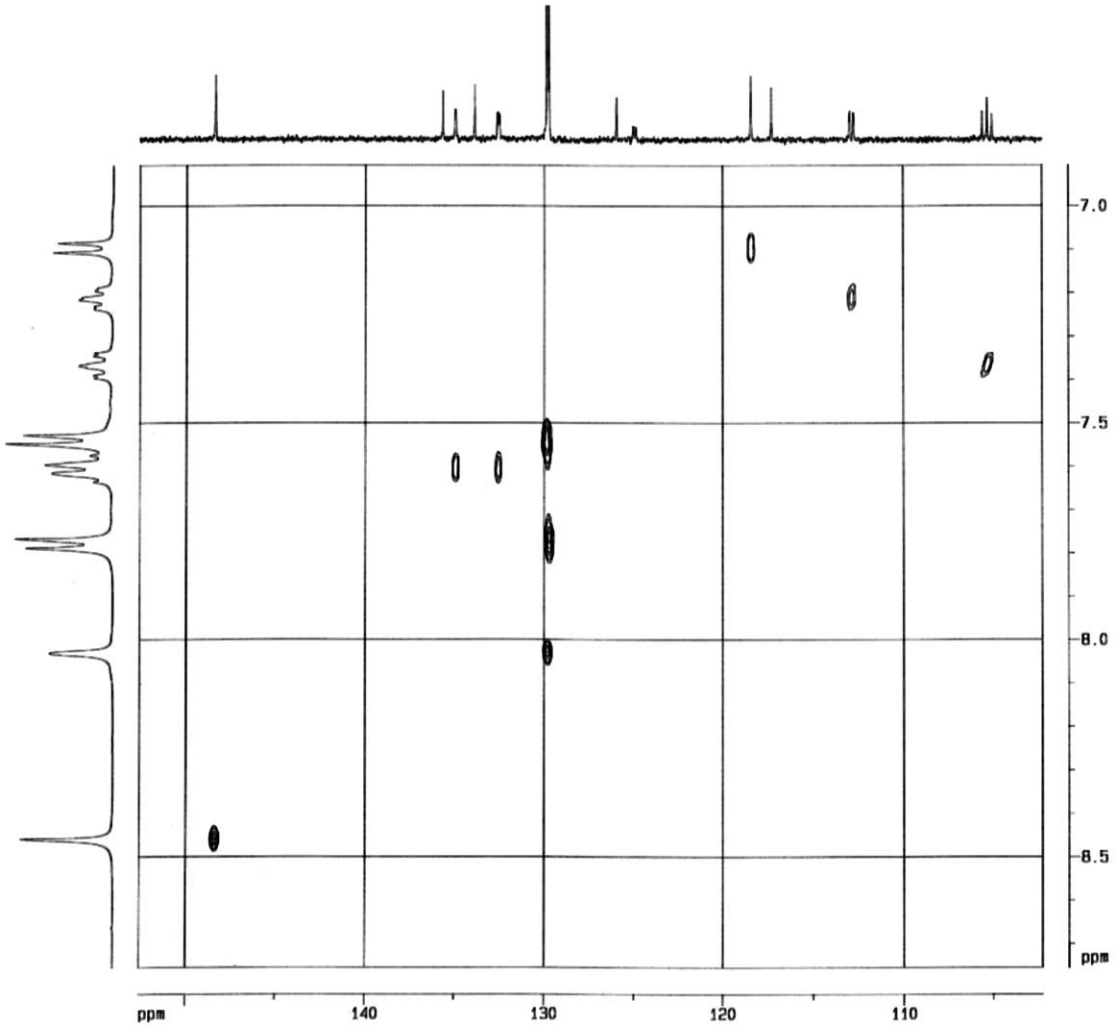

Fig. 4. The HETCOR spectrum of compound **3d**.

3.2. Antimycobacterial activity

The synthesized compounds were tested in vitro for their antimycobacterial activity. Primary screen was conducted at 6.25 μg mL^{-1} against *Mycobacterium tuberculosis* H37 Rv in BACTEC 12B medium using a broth microdilution assay, the Microplate Alamar Blue Assay (MABA) [22]. Compound **3b** exhibiting fluorescence were tested in the BACTEC 460 radiometric system [22]. Rifampin was used as the standard in the tests. None of the compounds were considered for further evaluation as they had mycobacterial inhibitions less than 90% at 6.25 μg mL^{-1}. The results of antimycobacterial activity studies are presented in Table 4.

3.3. Anticonvulsant activity

The compounds were each tested in male albino mice (weight 20–25 g, CF no. 1 from Charles River in Wilmington, MA) in the maximal electroshock (MES), subcutaneous metrazol (ScMET) and neurotoxicity (Tox) models of epilepsy [23]. Substances were administrated via the i.p route at doses of 30, 100 and 300 mg kg^{-1} at two time points (1/2 and 4 h). Including both time periods and both anticonvulsant models (MES & scM screens) a total of 16 mice were evaluated. All 16 were also evaluated for neurotoxicity effects.

Compounds **1**, **2**, **3b**–**3h**, **3l**, **3i**, **3m**, **3n** and **3o** were not active in the models used. Compound **3j** showed trace signs of anticonvulsant protection in the primary model screens. This compound was chosen for reevaluation screening in the 6 Hz screen which uses a threshold stimulus (32 mA for a 3-s duration) vs. the maximal electroshock supra threshold stimulation. The 6 Hz results were performed at 100 mg kg^{-1} over five time points, but compound **3j** did not exhibit any protection in this model. Compounds **3a** and **3k** were also evaluated using qualitative oral rat screening. In this experiment, male albino rats (Sprague–Dawley, 150–185 g) were used as experimental animals. Compound **3k** protected only one-quarter of the rats at a dose level

Table 2
Antimicrobial activities of seven newly synthesized compounds against the tested bacterial strains and fungal isolates using disc diffusion method (300 μg mL^{-1}) [a]

Microorganisms/code/source [b]	Inhibition zone in diameter (mm) around test disc							Standard ant. discs [c]
	Diflunisal	**2**	**3a**	**3e**	**3f**	**3j**	**3o**	
Acinetobacter baumanii IÖ-15 (1)	9	–	–	–	–	–	–	15 (OFX)
Acinetobacter calcoaceticus IÖ-16 (1)	6	7	–	–	7	–	8	16 (OFX)
Alcaligenes pacificus RK-105 (1)	–	–	17	6	–	–	–	32 (OFX)
Alcaligenes xylosoxydans A1C (1)	-	–	8	–	–	–	–	12 (SCF)
Bacillus amyloliquefaciens 142 (2)	–	18	22	–	–	7	7	27 (SCF)
Bacillus antracsis AO-2000 (1)	–	–	6	–	–	–	–	21 (SCF)
Bacillus cereus RK-75 (2)	–	8	14	7	–	8	7	30 (OFX)
Bacillus licheniformis RK-8 (2)	–	9	15	–	–	9	9	29 (SCF)
Bacillus macerans M-58 (2)	–	14	13	7	–	9	7	26 (SCF)
Bacillus sphaericus M-9 (2)	–	12	20	–	–	8	–	18 (OFX)
Bacillus substilis M-19 (2)	–	7	9	–	–	–	–	28 (OFX)
Escherichia coli E59 (1)	12	9	21	–	–	–	–	– (OFX)
Flavobacterium blastinum HH299 (2)	–	7	–	–	–	–	–	32 (OFX)
Klebsiella pneumoniae RK-27 (1)	–	11	13	–	–	7	6	12 (OFX)
Pseudomonas syringae pvs. *tomato* RK-14 (3)	–	14	18	–	–	9	11	24 (OFX)
Ralstonia pickettii RK-212 (3)	–	–	–	–	–	–	–	25 (OFX)
Salmonella typhimurium RK-22 (1)	–	11	16	9	–	8	–	27 (SCF)
Staphylococcus aureus HE-9 (1)	–	14	15	–	–	11	8	22 (SCF)
Staphylococcus epidermis HE-5 (1)	–	22	25	–	–	–	13	NT
Serratia spp. RK-150 (2)	–	–	–	–	–	14	9	27 (SCF)

[a] The strains which were not susceptible to none of the compounds tested were as follows: bacteria: *Acinetobacter lwoffii* RK-25 (1), *Acinetobacter johnsonii* RK-62 (1), *Agrobacterium radiobacter* RK-190 (2), *Bacillus megaterium* M-3 (2), *Bacillus pumilus* RK201 (2), *Brevundimonas diminuta* RK-66 (2), *Brucella abortus* H-46 (1), *Brucella melitensis* H-71 (1), *Burkholdria cepacia* PR-1217 (1), *Burkholdria gladioli* B-7 (2), *Citrobacter freundii* RK26 (1), *Clavibacter michiganense* Cmm783 (3), *Curtobacterium flaccumfaciens* RK-250 (3), *Enterobacter aerogenes* H-IK (1), *Enterobacter cloacae* RK-210 (1), *Enterobacter intermedius* RK-81 (1), *Enterobacter pyrinus* H52 (1), *Erwinia amylovora* RK-5 (3), *Erwinia carotovora* RK-20 (3), *Erwinia chrysanthemi* RK-67, *Klebsiella trevisanii* RK-245 (1), *Kocuria varians* M-8 (1), *Leclercia adecarboxylata* RK-186 (2), *Micrococcus luteus* AO-H (4), *Neisseria* spp RK-231 (1), *Pantoea agglomerans* RK-79 (2), *Plesiomonas shigelloides* RK-159 (1), *Proteus vulgaris* H-IK-2 (1), *Pseudomonas aeruginosa* E-47 (1), *Pseudomonas fluorescens* RK-242 (2), *Pseudomonas huttiensis* RK-200 (3), *Pseudomonas putida* B-8 (2), *Streptococcus pneumoniae* H-IK-3 (1), *Streptococcus pyogenes* H-IK-2957 (1), *Xanthomonas axonopodis* pv. *vesicatoria* Xcv-110c (3), *Yersinia enterocolitica* RK-13 (1). Yeast: *Candida albicans* 1432 (1). Fungi: *Alternaria alternata* AA-3 (3), *Aspergillus flavus* Hak-23 (1), *Fusarium oxysporum* Hak-F15 (3), *Penicillium* spp. Hak-P21 (1), *Rhizopus* spp. Hak-R1 (2) *Trichophyton rubrum* Hak-Tr8 (1), *Microsporum Canis* EH-2001 (1).

[b] Source of microorganisms: 1 = clinic human pathogenic organism, 2 = Env: microorganisms isolated from environmental samples (soil, water or plant associated), 3 = plant pathogenic, 4 = food pathogenic.

[c] OFX: ofloxacin (10 μg/disc); SCF: sulbactam (30 μg)+sefoperazone (75 μg) (105 μg/disc); NET: netilmicin (30 μg/disc) were used as positive reference standarts antibiotic discs (oxoid); NT: not tested.

of 30 mg kg^{-1}. In addition, compounds **3n** and **3o** showed neurotoxicity at 4 and 0.5 h at doses of 100 and 300 mg kg^{-1}, respectively.

4. Experimental

All chemical compounds were purchased from Fluka. Diflunisal was generously provided by Sanovel Pharmaceuticals. Melting points were taken on Buchi-530 apparatus and are uncorrected. Elemental analyses were performed on a Leco CHNS-932 instrument. UV Spectra were recorded on a Beckman DU 530 spectrophotometer (1 mg/100 mL in EtOH). IR spectra were run on Schimadzu FTIR-830 spectrophotometer. ^{1}H-NMR and ^{13}C-NMR spectra were obtained on a Bruker AVANC-DPX 400 instrument. EI-mass spectra were performed using a Micro MS Zabspec Double Focussing Magnetic sector. The purities of the synthesized compounds were checked by thin layer chromatography on silica gel plates using chloroform: methanol (90:10, v/v) as mobile phase (λ = UV 254 nm, t = 20 °C).

4.1. Chemistry

4.1.1. Methyl-2′,4′-difluoro-4-hydroxybiphenyl-3-carboxylate (*1*)

Diflunisal (0.01 mol) and methanol (20 mL) refluxed for 3 h in a few drops of concentrated sulfuric acid. The obtained solid was washed with $NaHCO_3$ solution (%5), dried and recrystallised twice from methanol.

Compound **1**: yield 65%, m.p. 102–104 °C (lit. [24] 95–97 °C) ^{1}H-NMR (DMSO-d_6) δ 3.90 (s, 3H, $COOCH_3$), 6.84–7.91 (m, 6H, Ar–H), 10.75 (s, 1H, Ar–OH). IR (KBr, cm^{-1}) 3132 (Ar–OH), 1679 (C=O), 1139 (Ar–F). UV (EtOH, λ_{max}) 316, 256, 229. Anal.

Table 3
The in vitro antimicrobial activity results of the selected compounds against the tested bacterial strains and fungal isolates based on microdilution method (MIC in μg mL^{-1})

Microorganisms/Code	MIC values in μg mL^{-1}							
	Diflunisal	**2**	**3a**	**3e**	**3f**	**3j**	**3o**	Maxipime
Acinetobacter baumanii IÖ-15	150	–	–	–	–	–	–	250
Acinetobacter calcoaceticus IÖ-16	150	150	–	–	> 300	–	37.5	125
Acinetobacter lwoffii RK-25	–	–	–	–	–	–	–	125
Alcaligenes pacificus RK-105	–	–	150	300	–	–	–	NT
Alcaligenes xylosoxydans A1C	–	–	150	–	–	–	–	NT
Bacillus amyloliquefaciens 142	–	150	75	–	–	150	150	NT
Bacillus antracsis AO-2000	–	–	150	–	–	–	–	NT
Bacillus cereus RK-75	–	300	75	150	–	75	150	62.5
Bacillus licheniformis RK-8	–	300	75	–	–	75	150	NT
Bacillus macerans M-58	–	150	75	150	–	75	150	NT
Bacillus sphaericus M-9	–	300	75	–	–	150	–	NT
Bacillus substilis M-19	–	150	150	–	–	–	–	NT
Escherichia coli E59	75	150	75	–	–	–	–	250
Flavobacterium blastinum HH299	–	75	–	–	–	–	–	31.25
Klebsiella pneumoniae RK-27	–	150	150	–	–	150	150	125
Pseudomonas syringae pvs. *tomato* RK-14	–	150	75	–	–	150	75	62.5
Salmonella typhimurium RK-22	–	150	75	150	–	150	–	125
Staphylococcus aureus HE-9	–	150	37.5	–	–	75	150	125
Staphylococcus epidermis HE-5	–	37.5	18.75	–	–	–	37.5	31.25
Serratia spp. RK-150	–	–	–	–	–	75	75	62.5

Table 4
Antimycobacterial activity of the synthesized compounds

Compound no.	R	MIC (μg mL^{-1})	Method	% Inhibition
1	–	> 6.25	Alamar	30
2	–	> 6.25	Alamar	21
3a	5-nitro-2-furyl	> 6.25	Alamar	34
3b	C_6H_5	> 6.25	BACTEC	0
3c	C_6H_4Br (4)	> 6.25	Alamar	16
3d	C_6H_4Cl (4)	> 6.25	Alamar	15
3e	$C_6H_3Cl_2$ (2,4)	> 6.25	Alamar	21
3f	C_6H_4F (2)	> 6.25	Alamar	17
3g	C_6H_4F (3)	> 6.25	Alamar	23
3h	C_6H_4F (4)	> 6.25	Alamar	19
3i	C_6H_4OH (2)	> 6.25	Alamar	17
3j	C_6H_4OH (4)	> 6.25	Alamar	12
3k	$C_6H_4CH_3$(4)	> 6.25	Alamar	15
3l	$C_6H_4OCH_3$ (4)	> 6.25	Alamar	17
3m	$C_6H_3OCH_3OH$ (3,4)	> 6.25	Alamar	27
3n	$C_6H_4NO_2$ (4)	> 6.25	Alamar	23
3o	$C_6H_2CH_3$ (2,4,6)	> 6.25	Alamar	31

Calc. for $C_{14}H_{10}F_2O_3$: C, 63.64; H, 3.81. Found: C, 63.87; H, 4.32.

4.1.2. 2′,4′-Difluoro-4-hydroxybiphenyl-3-carboxylic acid hydrazide (**2**)

To methanolic solution of **1** (25 mL, 0.01 mol) was added hydrazine-hydrate (99%) (4 mL) and refluxed for 2 h. The reaction mixture was then cooled, diluted with water and allowed to stand overnight. The solid precipitated was washed with water, dried and recrystallised twice from methanol.

Compound **2**: yield 65%, $C_{13}H_{10}F_2N_2O_2$, m.p. 200–202 °C ^{1}H-NMR (DMSO-d_6) δ 4.15–5.74 (b, 2H, $CONHNH_2$), 7.20 (d, 1H, H_5, $J = 8.5$ Hz), 7.40 (t, 1H, H_9, $J = 8.5$ Hz), 7.55 (t, 1H, H_{11}, $J = 9$ Hz), 7.74–7.83 (m, 2H, H_6 and H_{12}), 8.19 (s, 1H, H_2), 9.83–10.95 (b, 1H, Ar–OH), 11.77–13.66 (b, 1H, $CONHNH_2$). IR (KBr, cm^{-1}) 3365 (Ar–OH, N–H), 1641 (C=O), 1136 (Ar–F). UV (EtOH, λ_{max}) 307, 256, 208.

4.1.3. General procedure for the synthesis of 2′,4′-Difluoro-4-hydroxybiphenyl-3-carboxylic acid [(5-nitro-2-furyl/substitutedphenyl)methylene]hydrazide (**3a–o**)

A solution of 0.01 mol of **2** and equimolar amount of appropriate aldehyde in 60 mL of EtOH was heated under reflux for 1–2 h. The precipitate obtained was filtered off, washed with water and cleaned twice with boiling EtOH. The physical and spectral data of hydrazide–hydrazones **3a–o** are given in Table 1.

4.2. Biology

4.2.1. Antimicrobial activity

Representative strains of 56 bacteria, one yeast and six fungi species, were used in this study. Microorganisms were provided by Department of Clinical Microbiology, Faculty of Medicine; and Plant Diagnostic Laboratory, Faculty of Agriculture at Ataturk University, Erzurum, Turkey. Identity of the microorganisms

used in this study was confirmed by Microbial Identification System in Biotechnology Application and Research Center at Ataturk University. The test cultures of bacteria, yeast and fungi were maintained and assayed on/in nutrient agar (NA), sabouraud dextrose agar (SDA), and potato dextrose agar (PDA) medium/broth (DIFCO, Detroit, MI), respectively.

4.2.1.1. Disc diffusion assay. The synthesized and lyophilized compounds were dissolved in dimethylsulfoxide (DMSO) to a final concentration of 20 mg mL^{-1} and sterilized by filtration by 0.45 μm millipore filters. Antimicrobial tests were then carried out by disc diffusion method [20] using 100 μL of suspension containing 10^8 CFU mL^{-1} of bacteria, 10^6 CFU mL^{-1} of yeast and 10^4 spore mL^{-1} of fungi spread on nutrient agar (NA), sabourand dextrose agar (SDA), and potato dextrose agar (PDA) medium, respectively. The discs (6 mm in diameter) were impregnated with 15 μL of each compounds (300 μg/disc) at the concentration of 20 mg mL^{-1} and placed on the inoculated agar. DMSO impregnated discs were used as negative controls. Ofloxacin (10 μg/disc), sulbactam (30 μg)+cefoperazone (75 μg) (105 μg/disc) and/or netilmicin (30 μg/disc) were used as positive reference standards to determine the sensitivity of one strain/isolate in each microbial species tested. The inoculated plates were incubated at 37 °C for 24 h for bacterial strains, 48 h for yeast and 72 h for fungi isolates. Plant associated microorganisms were incubated at 27 °C. Antimicrobial activity in disc diffusion assay was evaluated by measuring the zone of inhibition against the test organisms. Each assay in this experiment was repeated twice.

4.2.1.2. Microdilution assays. The minimal inhibition concentration (MIC) values were also studied for the microorganisms, except one, sensitive to at least one of the seven compounds determined in disc diffusion assay. The inocula of microorganisms were prepared from 12 h broth cultures and suspensions were adjusted to 0.5 McFarland standard turbidity. The test compounds dissolved in dimethylsulfoxide (DMSO) were first diluted to the highest concentration (600 μg mL^{-1}) to be tested, and then serial twofold dilutions were made in a concentration range from 9.37 to 600 μg mL^{-1} in 10 mL sterile test tubes containing nutrient broth. MIC values of each compound against bacterial strains were determined based on a micro-well dilution method [21] with some modifications.

The 96-well plates were prepared by dispensing into each well 95 μL of nutrient broth and 5 μL of the inoculum. A 100 μL from each of the test compounds initially prepared at the concentration of 600 μg mL^{-1} was added into the first wells. Then, 100 μL from their serial dilutions was transferred into six consecutive wells. The last well containing 195 μL of nutrient broth without compound and 5 μL of the inoculum on each strip was used as negative control. The final volume in each well was 200 μL. Maxipime (Bristol–Myers Squibb) at the concentration range of 500–7.8 μg mL^{-1} was prepared in nutrient broth and used as standard drug for positive control. The plate was covered with a sterile plate sealer. Contents of each well were mixed on plate shaker at 300 rpm for 20 s and then incubated at appropriate temperatures for 24 h. Microbial growth was determined by absorbance at 600 nm using the ELx 800 universal microplate reader (Biotek Instrument inc, Highland Park, Vermont, USA). All of the compounds tested in this study were screened two times against each microorganism. The MIC was defined as the lowest concentration of the compounds to inhibit the growth of microorganisms.

4.2.2. In vitro evaluation of antimycobacterial activity against M. tuberculosis H37Rv

Primary screen was conducted at 6.25 μg mL^{-1} against *M. tuberculosis* H37Rv in BACTEC 12B medium using the BACTEC 460 radiometric system [22]. Compounds effecting $<90\%$ inhibition in the primary screen (MIC >6.25 μg mL^{-1}) are not considered for further evaluation. Compounds demonstrating at least 90% inhibition in the primary screen are re-tested at lower concentration (MIC) in a broth microdilution assay alamar Blue. The MIC is defined as the lowest concentration inhibiting 99% of the inoculum.

4.2.2.1. BACTEC radiometric method of susceptibility testing. Inocula for susceptibility testing were either from a positive BACTEC isolation vial with a growth index (GI) of 500 or more, or a suspension of organisms isolated earlier on a conventional medium.

The culture was well mixed with a syringe and 0.1 ml of a positive BACTEC culture was added to each of the vials containing the test drugs. The drug vials contained rifampin (0.25 μg mL^{-1}). A control vial was inoculated with a 1:100 dilution of the culture. A suspension equivalent to a Mc Farland no. 1 standard was prepared in the same manner as a BACTEC positive vial, when growth from a solid medium was used.

Each vial was tested immediately on a BACTEC instrument to provide CO_2 in the headspace. The vials were incubated at 37 °C and tested daily with a BACTEC instrument. When the GI in the control reads at least 30, the increase in GI (ΔGI) from the previous day in the control was compared with that in the drug vial. The following formula was used to interpret results:

ΔGI control $>$ ΔGI drug = susceptible

ΔGI control $<$ ΔGI drug = resistant

If a clear susceptibility pattern (the difference of ΔGI of

control and the drug bottle) was not seen at the time the control GI is 30 the vials were read for 1 or 2 additional days to establish a definite pattern of ΔGI differences.

4.2.3. Anticonvulsant activity

The anticonvulsant evaluation were undertaken by the National Institue of Neurological Disorders and Stroke (NIH). Male albino rats (18–25 g) and male albino rats (Sprague–Dawley, 150–185 g) were used as experimental animals. The compounds were suspended in 0.5% methylcellulose–water mixture. All the compounds were administered i.p in a volume of 0.01 mL g^{-1} body weight for mice and 0.004 mL g^{-1} body weight for rats at doses of 30, 100 and 300 mg kg^{-1} to one to four animals. Activity was established using the MES, scMET and neurotoxicity. The MES screen utilizes an electric stimulation delivered for 0.2 sec via the corneal membrane of 50 mA at 60 Hz for mice and 150 mA, 60 Hz for rats. Animals are provided an anaesthetic to the eyes before placement of electrodes.

The scMET screen uses a s.c. dose of the chemoconvulsant metrazol dissolved in 0.9% saline and injected at a vol of 0.01 mL g^{-1} body weight in mice. A dose of 85 mg kg^{-1} to elicit seizure activity for mice and 70 mg kg^{-1} for rats. The volume for rats was 0.2 mL/10 g. Endpoint for protection is absence of a 3-s clonic episode.

Some selected compounds described in this study were examined for oral activity in the MES screen and for 6 Hz screen which uses a threshold stimulus vs. the maximal electroshock suprathreshold stimulation.

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