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Stereoselective Pharmacodynamic and Pharmacokinetic Analysis of *sec*-Butylpropylacetamide (SPD), a New CNS-Active Derivative of Valproic Acid with Unique Activity against Status Epilepticus

Naama Hen,[†,⊥] Tawfeeq Shekh-Ahmad,[†,⊥] Boris Yagen,[†,‡] John H. McDonough,[§] Richard H. Finnell,[∥] Bogdan Wlodarczyk,[∥] and Meir Bialer*[,†,‡]

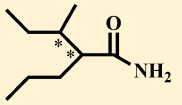
[†]Institute for Drug Research, Faculty of Medicine, School of Pharmacy, The Hebrew University of Jerusalem, Jerusalem 91120, Israel

[‡]David R. Bloom Center for Pharmacy, The Hebrew University of Jerusalem, Israel

[§]Pharmacology Branch, Research Division, U.S. Army Medical Research Institute of Chemical Defense, Aberdeen Proving Ground, Maryland, United States

[∥]Department of Nutritional Sciences, Dell Pediatric Research Institute, The University of Texas at Austin, Austin, Texas, United States

ABSTRACT: *sec*-Butylpropylacetamide (racemic-SPD) is a chiral CNS-active amide derivative of valproic acid (VPA). This study describes synthesis and stereospecific comparative pharmacodynamics (PD, anticonvulsant activity and teratogenicity) and pharmacokinetic (PK) analysis of four individual SPD stereoisomers. SPD stereoisomers' anticonvulsant activity was comparatively evaluated in several anticonvulsant animal models including the benzodiazepine-resistant status epilepticus (SE). SPD stereoisomers' PK−PD relationship was evaluated in rats. Teratogenicity of SPD stereoisomers was evaluated in SWV mice strain, susceptible to VPA-induced neural tube defect (NTD). SPD stereoisomers (141 or 283 mg/kg) did not cause NTD. SPD has stereoselective PK and PD. (2*R*,3*S*)-SPD and (2*S*,3*R*)-SPD higher clearance led to a 50% lower plasma exposure that may contribute to their relative lower activity in the pilocarpine-induced SE model. (2*S*,3*S*)-SPD, (2*R*,3*R*)-SPD, and racemic-SPD have similar anticonvulsant activity and a PK profile that are better than those of (2*R*,3*S*)-SPD and (2*S*,3*R*)-SPD, making them good candidates for development as new, potent antiepileptics with a potential in benzodiazepine-resistant SE.



INTRODUCTION

All 16 new antiepileptic drugs (AEDs) developed since 1990 for the symptomatic treatment of epilepsy have evolved from compounds receiving early evaluation in highly predictive animal seizure and epilepsy models.[1−3] *sec*-Butylpropylacetamide (SPD, Figure 1) is a one-carbon homologue of valnoctamide (VCD, Figure 1), a chiral constitutional isomer of VPA's corresponding amide valpromide (VPD) (Figure 1).[4−6] Similar to VCD, SPD possesses two stereogenic carbons in its structure.[7] Therefore, it exists as a racemic mixture of four stereoisomers in equal proportion as depicted in Figure 2.

SPD (racemate) was recently reported by us to possess a unique and broad-spectrum antiseizure profile superior to (lower ED_{50} values) valproic acid (VPA) and better than that of VCD.[8] In addition, SPD blocked behavioral and electrographic status epilepticus (SE) induced by pilocarpine (muscarinic agonist) and soman (organophosphate nerve gas) and afforded in vivo neuroprotection that was associated with cognitive sparing.[8,9] SPD's activity against SE is superior to that of diazepam in terms of rapid onset, potency, and ability to block SE when given 20−40 min after seizure onset. When administered 20 and 40 min after SE onset, SPD (100−174 mg/kg) produced long-lasting efficacy (e.g., 4−8 h) against soman-induced convulsive and electrographic SE in both rats and guinea pigs. SPD ED_{50} values in rats and guinea pigs were 71 and 92 mg/kg when administered 40 min after SE onset, respectively.[8]

Treatment of SE is aimed at rapid controlling of convulsive seizures before compensatory mechanisms fail and the patient enters into a "refractory" state. Benzodiazepines (lorazepam and diazepam), phenobarbital, and phenytoin are generally considered the first line drugs for the early treatment of SE. Second drugs of choice includes iv administration of VPA, levetiracetam, or lacosamide. SE can quickly become pharmacologically refractory when initial attempts to control the seizures fail despite adequate treatment. In addition to diazepam and lorazepam, other anesthetic drugs that might be considered for refractory SE include pentobarbital, thiopental, propofol, and ketamine.[8] A recent study showed that in contrast to diazepam or VPA, SPD (180 mg/kg) was as efficacious as propofol (100 mg/kg) and pentobarbital (30 mg/kg) in suppressing pilocarpine-induced electrographic SE (ESE) when administered 60 min after first seizure.[9] There is a clear need for more effective treatments for refractory SE that displays rapid onset and effective seizure control without producing dose-limiting sedation and respiratory depression. Further, the development of an effective therapy that attenuates refractory SE particularly when given 30−60 min after seizure onset and offers some neuroprotective potential would represent an important advance in the treatment of SE.

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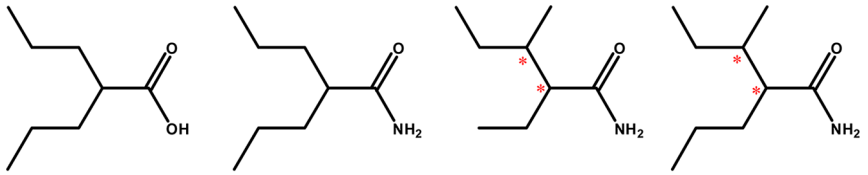


Figure 1. Chemical structures of valproic acid (VPA), valpromide (VPD), valnoctamide (VCD), and *sec*-butylpropylacetamide (SPD).

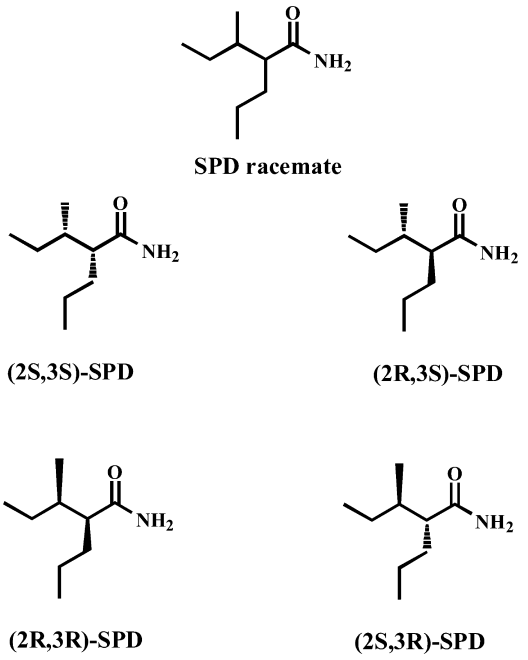


Figure 2. Chemical structures of the four SPD stereoisomers.

The current study was designed to evaluate stereospecifically the anticonvulsant activity, neurotoxicity, teratogenicity, and pharmacokinetics of SPD's four individual stereoisomers. The objectives of the current study were: (a) to synthesize SPD four individual stereoisomers, (2*S*,3*S*)-SPD, (2*S*,3*R*)-SPD, (2*R*,3*R*)-SPD, and (2*R*,3*S*)-SPD; (b) to comparatively evaluate their anticonvulsant activity, including two (pilocarpine- and soman-induced) benzodiazepine-resistant SE models, and teratogenicity in animal models in comparison to one another and to racemic-SPD and in the case of the teratogenicity studies, in comparison to VPA; (c) to comparatively analyze the pharmacokinetic profile of each SPD stereoisomer following ip administration to rats and to explore a possible pharmacokinetic–pharmacodynamic (PK–PD) correlation in regard to their anticonvulsant activity.

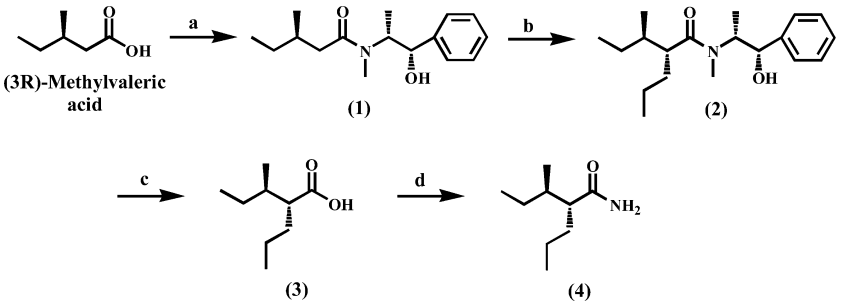
RESULTS

Chemistry. The general synthesis of the four individual SPD stereoisomers is depicted in Schemes 1 and 2 and detailed in the Experimental Section. The synthesized products were purified by crystallization. ^{1}H NMR spectra of the synthesized compounds were measured in DMSO using TMS as an internal standard. Elemental analyses were performed for all the synthesized compounds.

Pharmacokinetics of SPD Stereoisomers in Rats. The pharmacokinetics of SPD stereoisomers was studied following ip administration (60 mg/kg) to naive rats. The dose was chosen for the PK study as the intermediate dose among the various ED_{50} values of SPD stereoisomers. SPD has a low water solubility. Consequently, SPD was administered to rats in multisol, namely, a pure 5:1:4 solution of propylene glycol, alcohol, and water for injection, where SPD solubility was increased from 1.5 to 27 mg/mL. The plasma concentration–time plots of SPD individual stereoisomers in comparison to racemic-SPD are presented in Figure 3. The PK parameters of SPD and its four individual stereoisomers, calculated by noncompartmental analysis, are summarized in Table 1. The clearance (CL/F) of SPD stereoisomers ranged between 1.8 and 4.6 L h^{-1} kg^{-1} and their volume of distribution (V_z/F) ranged between 3.0 and 7.2 L/kg. (2*R*,3*S*)-SPD and (2*S*,3*R*)-SPD had 2-fold higher CL and V values compared to their other diastereoisomers. Consequently, the half-life ($t_{1/2}$) of SPD individual stereoisomers was similar and ranged between 1 and 1.5 h.

Time to Peak Effects (TPE) of SPD Stereoisomers and Determination of Their Median Effective (ED_{50}) or Behavioral Toxic Dose (TD_{50}). All quantitative in vivo

Scheme 1. Total Stereoselective Synthesis of (2*R*,3*R*)-SPD (4) and (2*S*,3*R*)-SPD[a]



[a]Reagents and conditions: (a) pivaloyl chloride, DCM, (2*R*,3*R*)-pseudoephedrine (chiral auxiliary), Et_3N; (b) LDA, LiCl, 1-propyliodide, THF; (c) H_2SO_4, 1,4-dioxane; (d) $SOCl_2$, CH_2Cl_2, 28–30% NH_4OH, ACN. For the synthesis of (2*S*,3*R*)-SPD, (2*S*,3*S*)-pseudoephedrine is the chiral auxiliary.

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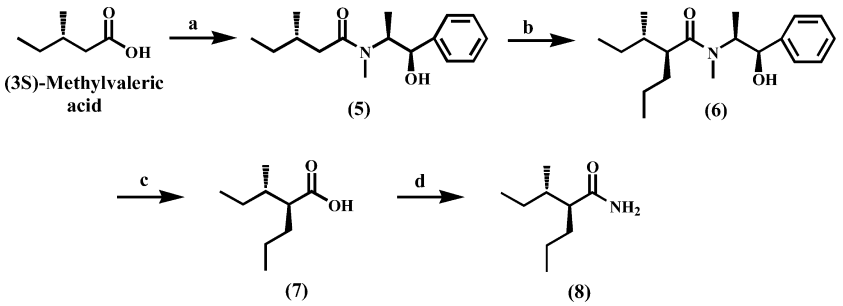
Scheme 2. Total Stereoselective Synthesis of (2*S*,3*S*)-SPD (8) and (2*R*,3*S*)-SPD[a]



[a]Reagents and conditions: (a) pivaloyl chloride, DCM, (2*S*,3*S*)-pseudoephedrine (chiral auxiliary), Et_3N; (b) LDA, LiCl, 1-propyliodide, THF; (c) H_2SO_4, 1,4-dioxane; (d) $SOCl_2$, CH_2Cl_2, 28−30% NH_4OH, ACN. For the synthesis of (2*R*,3*S*)-SPD, (2*R*,3*R*)-pseudoephedrine is the chiral auxiliary.

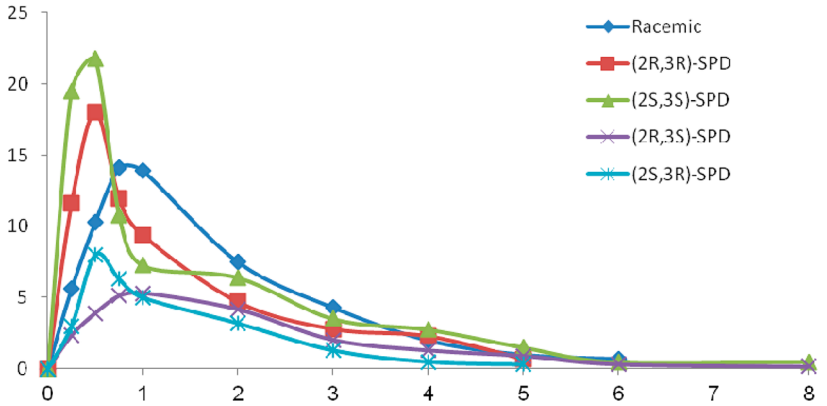


Figure 3. Plasma concentration−time plots of SPD (racemate), (2*R*,3*R*)-SPD, (2*S*,3*S*)-SPD, (2*R*,3*S*)-SPD, and (2*S*,3*R*)-SPD as obtained following ip administration of 60 mg/kg (of each compound) to rats.

Table 1. PK Parameters of SPD (Racemate), (2*S*,3*S*)-SPD, (2*R*,3*R*)-SPD, (2*R*,3*S*)-SPD, and (2*S*,3*R*)-SPD As Obtained after ip Administration of 60 mg/kg (of Each Compound) to Rats

PK parameter	racemic-SPD	(2*S*,3*S*)-SPD	(2*R*,3*R*)-SPD	(2*R*,3*S*)-SPD	(2*S*,3*R*)-SPD
$t_{1/2}$ (h)	1.1	1.5	1.1	1.3	0.95
CL/*F* (L h^{-1} kg^{-1})	1.8	1.8	2.2	3.9	4.6
V_z/F (L/kg)	3.0	3.9	3.6	7.2	6.3
AUC (mg L^{-1} h^{-1})	31.3	32.9	26.4	15.2	12.6
C_{max} (mg/L	14.1	21.7	18.0	5.3	8.0
t_{max} (h)	0.75	0.5	0.5	1.0	0.5
MRT (h)	1.8	1.8	1.5	2.2	1.5

Table 2. Anticonvulsant Activity and Neurotoxicity of SPD (Racemate) and Its Four Individual Stereoisomers after ip Administration to Mice

	ED_{50} (95% coinfidence interval) (mg/kg)				
anticonvulsant test	SPD (racemate)	(2*R*,3*S*)-SPD	(2*S*,3*S*)-SPD	(2*R*,3*R*)-SPD	(2*S*,3*R*)-SPD
maximal electroshock seizure (MES)	71 (55−90)	95 (80−118)	75 (58−91)	70 (57−81)	
metrazol-induced seizure (scMet)	62 (47−71)	93 (76−114)	69 (64−75)	53 (45−64)	
neurotoxicity (TD_{50})	88 (81−95)	109 (97−121)		94 (88−102)	
6 Hz, 32 mA	27 (24−30)	29 (26−34)	27 (24−29)	38 (27−48)	27 (19−33)
6 Hz, 44 mA	45 (40−49)	51 (29−73)	59 (48−74)	52 (43−68)	<100
neurotoxicity (TD_{50})	114 (100−134)	67 (56−79)	126 (103−160)	111 (100−130)	>75

anticonvulsant/toxicity studies were conducted at time to peak effect (TPE) previously determined in a qualitative analysis. The TPE of SPD strereoisomers was 0.25−0.5 and 0.5−1 h following ip and oral administration, respectively. Groups of four to eight mice or rats were tested with various doses of the candidate drug until at least two points were established between the limits of 100% protection or minimal toxicity and 0% protection or minimal toxicity. The dose of drug required to produce the desired end point in 50% of animals (ED_{50} or TD_{50}) in each test, the 95% confidence interval, the slope of the regression line, and the SEM of the slope were then calculated

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by a computer program based on the method described by Finney.[10]

Anticonvulsant Efficacy of SPD Stereoisomers in Seizure and Epilepsy Rodents Models. The anticonvulsant activity in various rat seizure and epilepsy models of SPD stereoisomers (in comparison to racemic-SPD) is depicted in Tables 2 and 3. Three SPD stereoisomers (2*R*,3*R*)-SPD, (2*S*,3*S*)-SPD, and (2*R*,3*S*)-SPD exhibited anticonvulsant activity similar to racemic-SPD, while (2*S*,3*R*)-SPD exhibited less potent anticonvulsant activity at the rat (po) MES model.

Efficacy of SPD Stereoisomers in Slice Electrophysiology Studies. This model examined the ability of SPD (racemate) and its four inidividual stereoisomers to block spontaneous electrographic seizure-like activity in combined medial entorhinal cortex (mEC) hippocampal brain slices obtained from rats that have been treated with kainic acid (KA). Extracellular field potential recordings made on layer II of the mEC showed spontaneous bursting activity upon introduction of 6 mM KCl, and both the frequency (rate) and duration of these bursts are variously affected by AEDs.[11−13] As depicted in Table 4, the activity of racemic-SPD and its four individual stereoisomers was different in this in vitro model. Racemic-SPD significantly increased burst duration, while (2*R*,3*S*)-SPD and (2*S*,2*S*)-SPD significantly decreased burst duration. (2*R*,3*R*)-SPD did not affect burst duration but deceased the burst rate, while (2*S*,3*R*)-SPD did not change the burst duration or its rate.

SPD Stereoisomers Block Convulsive Seizures Induced by Cholinergic Agonist Pilocarpine. Administration of lithium pilocarpine induces SE characterized by convulsive and nonconvulsive seizures that can last for several hours. From a behavioral perspective, the number and severity of the observed convulsive seizures following pilocarpine administration were similar in the two treatment groups (pilocarpine alone and pilocarpine + SPD stereoisomer). The first convulsive stage 3 or greater seizure was observed 12 min after pilocarpine administration and lasted for approximately 60 s. Within the succeeding 30 min, rats were observed to have 4.9 ± 0.2 seizures with an interseizure interval of 3−5 min.[8] SPD stereoisomers, administered 30 min after the first observed stage 3 motor seizure (marking the SE onset), prevented the expression of further convulsive seizures in a dose-dependent fashion with ED_{50} values ranging between 95 and 135 mg/kg. (2*R*,3*R*)-SPD was the least potent SPD stereoisomer (ED_{50} > 130 mg/kg) (Table 3).

SPD Stereoisomers Block Electrographic and Convulsive Seizures Induced by Soman in Rats. In the rat nerve agent seizure model, SPD stereoisomers dissolved in multisol were administered at various doses along with the standard medical countermeasures at treatment delays of 20 min after the onset of soman-induced seizures to determine effective dose for termination of soman-induced electrographic seizures as described previously for racemic-SPD.[8] SPD stereoisomers were capable of stopping soman-induced seizures. The ED_{50} for seizure control was calculated by probit analysis and ranged between 40 and 70 mg/kg (Figure 4, Table 3). Following administration of SPD stereoisomers the average latency (s) for electrographic seizure termination at the 20 min treatment delay time (mean ± SEM) was the following: 550 ± 149, n = 16 [(2*R*,3*R*)-SPD]; 994 ± 280, n = 8 [(2*R*,3*S*)-SPD]; 719 ± 216, n = 15 [(2*S*,3*R*)-SPD]; 1589 ± 684, n = 13 [(2*S*,3*S*)-SPD] (Figure 5). All four individual SPD stereoisomers had a steep dose−response curve with Hill (shape)

Table 3. Anticonvulsant Activity and Neurotoxicity of SPD (Racemate) and Its Four Individual Stereoisomers after ip or Oral Administration to Rats

	ED_{50} (95% coinfidence interval) (mg/kg)				
anticonvulsant test	SPD (racemate)	(2*R*,3*S*)-SPD	(2*S*,3*S*)-SPD	(2*R*,3*R*)-SPD	(2*S*,3*R*)-SPD
ip Administration					
maximal electroshock seizure (MES)	20 (15−27)	33 (27−36)	31 (21−37)	36 (28−43)	
metrazol-induced seizure (scMet)	21 (15−27)	15 (9−20)	14 (8−19)	15 (7−20)	
pilocarpine-induced status (30 min)	84 (62−103)	135 (91−173)	94 (75−110)	98 (74−123)	>130
soman-induced status (20 min)	68 (61−76)	56 (54−59)	70 (68−71)	40.2 (39.7−40.8)	50 (48−53)
neurotoxicity (TD_{50})	49 (43−55)	41 (37−47)	34 (32−37)	34 (24−42)	
po Administration					
maximal electroshock seizure (MES)	29 (18−53)	72 (52−91)	48 (28−84)	79 (56−111)	106 (59−179)
metrazol-induced seizure (scMet)	(30 min) 18 (13−25), (60 min) 82 (42−98)	(60 min) 50 (37−65)	20 (14−26)	18 (11−26)	25 (18−35)
neurotoxicity (TD_{50})	(30 min) 131 (94−175), (60 min) 154 (124−192)	79 (72−87)	124 (105−141)	(30 min) 102 (87−116)	105 (54−150)

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Table 4. Effect of SPD (Racemate) and Its Four Individual Stereoisomers (100 μM) on Spontaneous Burst in in Vitro Slice Electrophysiology Studies (Rats)

	% control burst rate ± SEM	% control burst duration ± SEM
SPD (racemate)	76 ± 11	145 ± 11 ($p < 0.05$)
(2*R*,3*R*)-SPD	77 ± 5 ($p < 0.05$)	94 ± 10
(2*S*,3*S*)-SPD	109 ± 9	45 ± 7 ($p < 0.05$)
(2*R*,3*S*)-SPD	97 ± 9	78 ± 8 ($p < 0.05$)
(2*S*,3*R*)-SPD	91 ± 4	90 ± 5

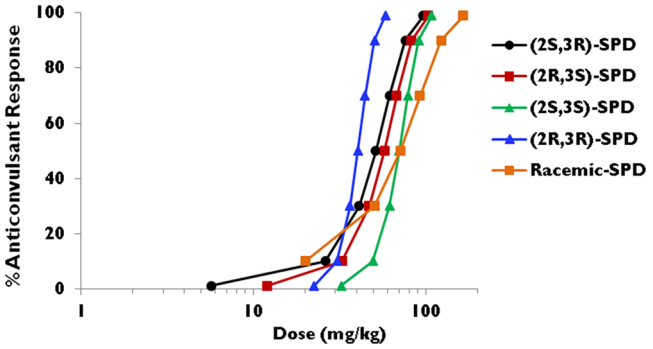

Figure 4. Anticonvulsant dose–response curve of SPD (racemate) and its four individual stereoisomers administered 20 min after onset of soman-induced (electrographic) status epilepticus (SE) in rats.

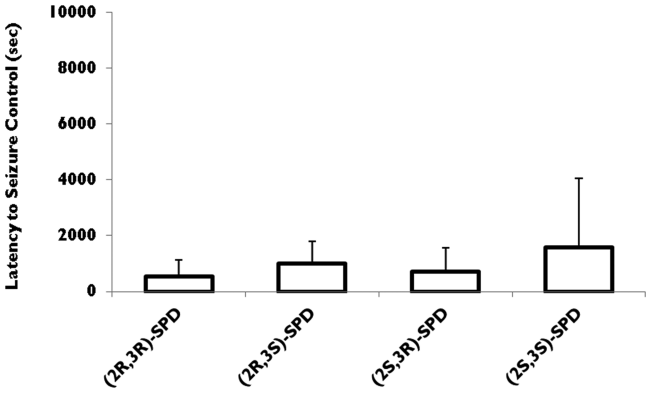

Figure 5. Latency (mean and SEM) for seizure control. The time from when SPD stereoisomers were administered to rats 20 min after seizure onset until the last epileptiform event could be detected on the EEG record.

coefficient of 4.5–8.9 (Figure 4) that led to a tight 95% confidence interval around their ED_{50} values. (2*R*,3*R*)-SPD was more potent than racemic-SPD as well as the three other individual SPD stereoisomers ($p < 0.05$) and had the shortest mean latency for seizure control (although it was not statistically significantly different because of the high inter-rat variability).

Teratogenicity. The teratogenic potential of racemic-SPD and its four individual stereoisomers was assessed for their ability to induce gross morphological defects in the SWV/Fnn mice that are highly susceptible to VPA-induced exencephaly. VPA at a dose of 1.8 mmol/kg was embryotoxic and caused a greater than 2-fold increase in the resorption rate compared to the control groups (13.6% vs 6.3%, respectively). Two fetuses with exencephaly were observed in this VPA-treated group.

(2*R*,3*R*)-SPD and racemic-SPD were embryotoxic and induced resorptions in 22.9% and 13.4% of conceptions, respectively, when tested at the higher 1.8 mmol/kg dose. (2*R*,3*R*)-SPD was also teratogenic at the 1.8 mmol/kg dose, causing exencephaly in six fetuses. In contrast three of the SPD stereoisomers (2*S*,3*S*)-SPD, (2*R*,3*S*)-SPD, and (2*S*,3*R*)-SPD were neither embryotoxic nor teratogenic in our study of SWV mice (Table 5).

DISCUSSION

PK Analysis of SPD Stereoisomers. (2*R*,3*S*)-SPD and its enantiomer (2*S*,3*R*)-SPD have clearance values twice higher than racemic-SPD, (2*S*,3*S*)-SPD, and (2*R*,3*R*)-SPD (Table 1). This relatively high clearance led to lower plasma exposure (AUC < 50%) that may contribute to their lower anticonvulsant activity in the pilocarpine-induced benzodiazepine-resistant SE model, although the relative higher clearance of (2*R*,3*S*)-SPD did not affect its anticonvulsant activity [compared to racemic-SPD, (2*S*,3*S*)-SPD, or (2*R*,3*R*)-SPD] in the rat-MES and scMet models. (2*S*,3*S*)-SPD and (2*R*,3*R*)-SPD have similar plasma exposure and similar anticonvulsant activity.

Similar to its one-carbon homologue VCD, SPD is mainly eliminated by metabolism that presumably occurs primarily in the liver. Rat liver blood flow (Q) is 60–70 mL/min.[14] Assuming that SPD metabolism is mainly hepatic, then the liver extraction ratio ($E = CL/Q = CL_m/Q$) of the various SPD stereoisomers ranges between 0.12 [(2*R*,3*R*)-SPD and (2*S*,3*S*)-SPD] and 0.31 [(2*R*,3*S*)-SPD and (2*S*,3*R*)-SPD]. If these rat data can be extrapolated in humans, it may indicate that the less active SPD stereoisomers (in contrast to the active ones) might be slightly susceptible to hepatic first pass effect after oral dosing.

SPD Stereoisomers Activity in Anticonvulsant Animal Models. In the present study, we describe the anticonvulsant activity of SPD stereoisomers in a battery of traditional seizure and epilepsy models often employed in the search for novel AEDs. The results obtained from the study demonstrate that three SPD stereoisomers, (2*S*,3*S*)-SPD, (2*R*,3*R*)-SPD, and (2*R*,3*S*)-SPD, possess a broad-spectrum anticonvulsant profile in models of focal and generalized seizures and have similar activity like racemic-SPD. These three SPD stereoisomers demonstrated similar anticonvulsant activity (ED_{50}) as racemic-SPD at the MES, scMet, and 6 Hz tests and exhibited similar safety margin as expressed by their protective index ($PI = TD_{50}/ED_{50}$). In contrast (2*S*,3*R*)-SPD was found to be the least potent stereoisomer in rats. Racemic-SPD and three of its individual stereoisomers exhibited similar anticonvulsant activity in in vivo anticonvulsant rodent models (Tables 2 and 3). In contrast to in vivo (rodents) models, different activity was observed in in vitro slice electrophysiology studies that are resistant to phenytoin and VPA (Table 4). This in vitro model showed different effects of racemic-SPD and its four individual stereoisomers on burst duration and rate (Table 4). Nevertheless SPD anticonvulsant data should be mainly interpreted in light of the extensive in vivo studies, since in

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Table 5. Teratogenicity Data Effect of SPD and Its Four Individual Stereoisomers in Comparison to VPA after a Single ip Injection of the Tested Compounds to SWV Mice Dams on Day 8.5 of Gestation

compd	dose mg/kg (mmol/kg)	no. of litters	no. of implants	no. of resorptions (%)	no. of live fetuses (%)	no. of normal fetuses (%)	no. of fetuses with NTD[a] (%)
control	25% CEL	14	207	13 (6.3)	194 (93.7)	194 (100)	0
Na-VPA[d]	301 (1.8)	12	154	21 (13.6)[b]	133 (86.4)	131 (99)	2 (1.5)
Na-VPA[e]	181 (1.1)	12	156	12 (7.7)	144 (92.3)	144 (100)	0
SPD	283 (1.8)	12	179	24 (13.4)[b]	155 (86.6)	155 (87)	0
SPD	141 (0.9)	11	160	12 (7.5)	148 (92.5)	148 (93)	0
(2*S*,3*S*)-SPD	283 (1.8)	11	147	11 (7.5)	136 (92.5)	136 (100)	0
(2*S*,3*S*)-SPD	141 (0.9)	10	138	8 (5.8)	130 (94.2)	130 (100)	0
(2*R*,3*S*)-SPD	283 (1.8)	11	153	10 (6.5)	143 (93.5)	143 (100)	0
(2*R*,3*S*)-SPD	141 (0.9)	10	154	13 (8.4)	141 (91.6)	141 (100)	0
(2*S*,3*R*)-SPD	283 (1.8)	10	149	14 (9.4)	135 (90.6)	135 (100)	0
(2*S*,3*R*)-SPD	141 (0.9)	10	121	9 (7.4)	112 (92.6)	112 (100)	0
(2*R*,3*R*)-SPD	283 (1.8)	11	144	33 (22.9)[b,c]	111 (77.1)	105 (94.6)	6 (5.4)[b]
(2*R*,3*R*)-SPD	141 (0.9)	11	147	11 (7.5)	136 (92.5)	136 (100)	0

[a]Neural tube defects. [b]Significantly different when compared to the control group. [c]Significantly different when compared to group treated with similar dose of VPA. [d]Results from ref 28. [e]Results from ref 42.

vitro differences are not always manifested in intact animal models .

Although at present little can be said about the molecular mechanism through which SPD stereoisomers exert their acute antiseizure effects, the results from the anticonvulsant testing conducted thus far would support the conclusion that it exerts its effects through an ability to prevent seizure spread and elevate seizure threshold. This conclusion is based on the marked effect exerted by (2*R*,3*R*)-SPD, (2*S*,3*S*)-SPD, and (2*R*,3*S*)-SPD in the rat MES test (seizure spread) and its ability to elevate seizure threshold in the scMet seizure model. VPA is a major AED with multiple mechanisms of action (MOA), including histone deacetylase inhibition and others yet undiscovered.[15,16] As an amide derivative of VPA, it is likely that SPD will also have multiple MOA.

Lack of Teratogenicity of SPD and Its Individual Stereoisomers. Since most if not all commercially available AEDs are teratogenic, it is essential to develop new AEDs that are nonteratogenic.[17] SPD (racemate) and three of its individual stereoisomers were found to be nonembryotoxic and nonteratogenic, and they all failed to induce neural tube defects at doses 2−9 times [(2*R*,3*R*)-SPD] and 3−14 times [(2*S*,3*S*)-SPD] higher than their anticonvulsant ED_{50} values (Tables 2 and 3). SPD's one-carbon homologue valnoctamide (VCD, depicted in Figure 1) and two of its stereoisomers as well as its corresponding acid valnoctic acid (VCA) were nonteratogenic in SWV mice at similar dose as SPD (1.8 mmol/kg) and higher (2.7 mmol/kg). In contrast to VPA its constitutional isomer VCA was nonteratogenic and had a similar profile as VCD.[18] A similar nonteratogenic profile was observed with VCD's constitutional isomer propylisopropylacetamide (PID) and its two enantiomers.[19] Thus, VPA amide derivatives are superior to VPA not only by their more potent anticonvulsant activity but also by their lack of teratogenicity.

Anticonvulsant Effects of SPD in Animal Models of SE. SE is initially treated with a benzodiazepine such as diazepam or lorazepam. Both are extremely effective when given early in SE; however, the benzodiazepines lose their efficacy when given after 20 min of spontaneous self-sustaining seizures; e.g., animals that experience prolonged SE quickly develop pharmacoresistant SE if treatment is not initiated within a short period from seizure onset.[20]

Two SPD stereoisomers, (2*S*,3*S*)-SPD and (2*R*,3*R*)-SPD, were found to be a highly effective antiseizure drugs in the lithium pilocarpine induced SE model. The results obtained demonstrate that (2*S*,3*S*)-SPD and (2*R*,3*R*)-SPD as well as racemic-SPD have a unique capability not shared by other AEDs to arrest ongoing behavioral seizure activity when administered 30 min after seizure onset, although it is possible that the tested rats were exhibiting nonconvulsive SE.

In addition, all SPD strereoisomers exhibited activity in the soman-induced SE models when given 20 min after onset of electrographic seizures in rats (Figures 4 and 5). This unique activity in ceasing electrographic seizures is not shared by benzodiazepines or other AEDs. (2*R*,3*R*)-SPD was more potent in this SE model than racemic-SPD as well as three other individual SPD stereoisomers and had the shortest latency for seizure control.

Stereoselective Pharmacokinetics (PK) and Pharmacodyanmics (PD). Interaction of a racemic drug, with a receptor or an enzyme, may result in a distinguished pharmacological response of the individual stereoisomers that may also display distinguished PK behavior that could lead to PD stereoselectivity.[21−24] Consequently, consideration of chirality should be implemented into PK and PD studies.

Therefore, the FDA's 1992 policy "Statement for Development of the New Stereoisomeric Drugs" triggered the development of single individual stereoisomers and not the use of racemic mixtures in the development of drugs containing stereogenic carbon atoms in their structures.[25] This policy coupled with marketing incentives of further profitability as a "line extension" has encouraged companies to look for chiral switches of established chiral drugs that were first introduced to the market as racemic mixtures.[21,26]

Racemic-VCD was equipotent to SPD when given at SE onset but in contrast to SPD, VCD (80 mg/kg) lost its activity in behavioral SE when administered 30 min after the pilocarpine-induced SE onset, while SPD had an ED_{50} value of 84 mg/kg.[8] At electrographic SE (ESE) VCD was found to be less potent than SPD.[9] VCD (180 mg/kg) stopped ECE when given 30 min after seizure onset, while an identical dose of SPD stopped ESE at 60 min. At 30 min SPD stopped ESE at a lower dose of 130 mg/kg.[9] In that regard SPD and its more potent stereoisomers (2*R*,3*R*)-SPD and (2*S*,3*S*)-SPD top not

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only VCD and VPA but also diazepam and other benzodiazepines.

Similar to the current SPD study, stereoselectivity was demonstrated in a recent study with two of VCD stereoisomers: (2*R*,3*S*)-VCD and (2*S*,3*S*)-VCD. In the MES test (2*R*,3*S*)-VCD had a more potent ED_{50} value (34 mg/kg) than its diastereoisomer (2*S*,3*S*)-VCD (64 mg/kg).[18]

CONCLUSIONS

SPD exhibits strereoselective PK and PD in rats with a similar time to peak plasma concentration (t_{max}) and time to peak effect (TPE). The higher clearance (2*R*,3*S*)-SPD and (2*S*,3*R*)-SPD led to a 50% lower plasma exposure (AUC) and contributed to their lower activity (compared to racemic-SPD and its other two stereoisomers) in the benzodiazepine-resistant SE model. SPD and its individual stereoisomers (141 or 283 mg/kg) did not cause NTD. These doses are 2−9 times higher than their anticonvulsant ED_{50} values. (2*S*,3*S*)-SPD was found to be significantly less embryotoxic than racemic-SPD and its enantiomer (2*R*,3*R*)-SPD. (2*R*,3*R*)-SPD was the most active compound in the soman-induced SE model.

(2*S*,3*S*)-SPD and (2*R*,3*R*)-SPD and racemic-SPD have similar anticonvulsant activity and a PK profile that is better than that of (2*R*,3*S*)-SPD and (2*S*,3*R*)-SPD. If SPD would exert its broad-spectrum anticonvulsant activity due to a single MOA it is likely that it would exhibit stereoselective PD. The fact that there was no significant difference between (2*R*,3*S*)-SPD and (2*S*,3*R*)-SPD and racemic-SPD in the various anticonvulsant rodent models (except the soman-induced SE) may indicate that SPD anticonvulsant activity is due to multiple MOA. The choice for further drug development between racemic-SPD, (2*S*,3*S*)-SPD, or (2*R*,3*R*)-SPD will be based on comparative toxicological analysis and additional anticonvulsant testing that will discriminate between these three CNS-active compounds.

EXPERIMENTAL SECTION

Chemicals. All the solvents were of analytical grade or HPLC grade and were purchased from J.T. Baker (The Netherlands). Pivaloyl chloride, (1*S*,2*S*)-pseudoephedrine, (1*R*,2*R*)-pseudoephedrine, lithium chloride, anhydrous triethylamine, *n*-butyllithium 1.6 M in hexane, anhydrous diisopropylamine, 1-iodopropane, methyl *tert*-butyl ether (MTBE), anhydrous 1,4-dioxane, *n*-heptane, sulfuric acid, thionyl chloride, ammonium hydroxide 28−30% in water, and 4 Å molecular sieves were purchased from Sigma-Aldrich Chemical Company Inc. (Rehovot, Israel). (3*S*)-Methylvaleric acid was purchased from Asta Tech Product Inc. (U.S.). (3*R*)-Methylvaleric acid was purchased from Avonyx Laboratories LLC (U.S.). Tetrahydrofuran (THF), dichloromethane (DCM), petroleum ether, acetonitrile (ACN), and ethyl acetate were purchased from Frutarom (Israel). Dry tetrahydrofuran, dichloromethane, and acetonitrile were obtained by drying over calcium hydride and distillation.

Materials and Methods. Product formation follow-up was performed by means of GC−MS and TLC. TLC analyses were performed on precoated silica gel on aluminum sheets (Kieselgal 60 F_{254}, Merck). A gas chromatography−mass spectrometry assay was performed on a HP5890 series II gas chromatograph equipped with a Hewlett-Packard MS engine (HP5989A) single quadrupole mass spectrometer, HP7673 autosampler, HP MS-DOS Chemstation, and HP-5MS capillary column (0.25 μm × 15 m × 0.25 mm). The temperature program was as follows: injector temperature, 180 °C; initial temperature, 40 °C for 6 min; gradient of 20 °C/min until 140 °C; gradient of 10 °C/min until 200 °C; hold time, 3 min. The MS parameters were set as follows: source temperature, 180 °C; transfer line, 280 °C; positive ion monitoring; EI-MS (70 eV). The molecular ion and the five most-pronounced ions are provided.

^{1}H NMR spectra, in DMSO using TMS as internal standard, were recorded on a Varian Mercury series NMR 300 spectrometer. Chemical shifts (δ scale) are reported in parts per million (ppm) relative to residual TMS. Multiplicity is indicated as follows: s (singlet), d (doublet), t (triplet), m (multiplet), dd (doublet of doublet), br m (broad multiplet). Coupling constants (J) are given in (Hz).

Chemical structures of the newly synthesized compounds were assessed by ^{1}H NMR and elemental analyses. Melting point was determined on a Buchi 530 capillary melting point apparatus. Elemental analyses were performed on a 2400-2 Perkin-Elmer C, H, N analyzer. C, H, N analyses of all newly synthesized compounds were within ±0.4 of theoretical values and thus were considered satisfactory.

SPD (racemate) was synthesized according to previously described procedures.[27] SPD stereoisomers [(2*S*,3*R*)-SPD, (2*R*,3*R*)-SPD, (2*S*,3*S*)-SPD, and (2*R*,3*S*)-SPD] were synthesized according to the methods outlined hereunder.

(2*R*,3*R*)-*sec*-Butylpropylacetamide [(2*R*,3*R*)-SPD (4), Scheme 1] and (2*S*,3*R*)-*sec*-Butylpropylacetamide [(2*S*,3*R*)-SPD]. (3*R*)-Methylpentanoic Acid, ((1′*R*,2′*R*)-2′-Hydroxy-1′-methyl-2′-phenylethyl)methylamide (1). Anhydrous DCM (60 mL) was added to a round-bottomed flask cooled to −5 °C, containing (3*R*)-methylvaleric acid (10 g, 0.08 mol) under nitrogen (N_2), followed by addition of Et_3N (8.5 g, 0.084 mol, 1.05equiv) and pivaloyl chloride (9.64 g, 0.08 mol, 1.00 equiv), while maintaining an internal temperature of <0 °C. The reaction mixture was stirred for 1 h, and then Et_3N (8.5 g, 0.084 mol, 1.05 equiv) was added. (1*R*,2*R*)-Pseudoephedrine (13.21 g, 0.08 mol, 1.00 equiv), for the synthesis of (2*R*,3*R*)-SPD, or (1*S*,2*S*)-pseudoephedrine (13.21 g, 0.08 mol, 1.00 equiv) for the synthesis of (2*R*,3*R*)-SPD was added as a solid in portions and allowed to stir for an additional 1 h below 5 °C, and then water (60 mL) was added. The organic layer was isolated, washed with 1 M HCl (60 mL), 1 M NaOH (60 mL), and water (60 mL), dried over $MgSO_4$, filtered, and evaporated to yield 97% oily product. The residue (oily product) was used in the next step without any further purification or treatment.

(2*R*)-Propyl-(3*R*)-methylpentanoic Acid, ((1′*R*,2′*R*)-2′-Hydroxy-1′-methyl-2′-phenylethyl)methylamide (2). To a solution of anhydrous LiCl (6.8 g, 0.16 mol, 2.00 equiv) in dry THF (80 mL) and diisopropylamine (18.6 g, 0.184 mol, 2.3 equiv)) under N_2 atmosphere at −20 °C, *n*- BuLi (1.6 M in cyclohexane, 0.184 mol, 2.3 equiv) was added slowly while maintaining an internal temperature of less than −10 °C. After 30 min, a solution of amide **1** (0.08 mol) in THF (40 mL) was slowly added while maintaining an internal temperature of less than −10 °C. After 30 min, 1-iodopropane (27.2gr, 0.16 mol, 2.00 equiv) was slowly added while maintaining an internal temperature of less than −10 °C. After 2 h, the reaction mixture was warmed to 20 °C and stirred overnight. Saturated NH_4Cl (82 mL) was added to quench the reaction. The organic layer was separated and washed with water (82 mL). The combined aqueous layers were extracted with MTBE (82 mL). The layers were separated, and the MTBE layer was washed with water (82 mL). The organic layer was dried over $MgSO_4$, filtered, and evaporated to yield 96% oily product. The residue (oily product) was used in the next step without any further purification or treatment.

(2*R*,3*R*)-*sec*-Butylpropylcarboxylic Acid (SPA) (3). 1,4-Dioxane (50 mL) was added to a round-bottomed flask containing amide **2** (0.08 mol) followed by the addition of 3 M H_2SO_4 (40 mL, 0.12 mol, 1.5 equiv). The reaction mixture was refluxed (~90 °C) for 6 h and then cooled to ~30 °C. Concentrated H_2SO_4 (27 mL, 0.48 mol, 6.00 equiv) was added, and the mixture was refluxed for 16 h followed by cooling to 20 °C. The black mixture was extracted with *n*-heptane (60 mL × 3). The combined organic layers were washed with water (40 mL). The organic layer was extracted with 4 M NaOH (40 mL × 3). The combined basic aqueous extracts were washed with *n*-heptane (40 mL). Concentrated HCl (30 mL) was carefully added to the combined basic aqueous extracts to obtain an acidic solution (pH < 2) and then was extracted with *n*-heptane (30 mL × 3). The combined organic

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layer was washed with water (30 mL). The organic layer was dried over $MgSO_4$, filtered, and evaporated to yield 50% oily product. The residue (oily product) was used in the next step without any further purification or treatment.

(2*R*,3*R*)-*sec*-Butylpropylacetamide [(2*R*,3*R*)-SPD] (4). To acid **3** solution (6 g, 0.038 mol) in dry DCM (50 mL) under N_2 atmosphere at 0 °C, a 1:1 solution of thionyl chloride (3.32 mL, 0.045 mol, 1.2 equiv) dissolved in dry DCM (7 mL) was added dropwise, and the mixture was allowed to stir overnight followed by distillation of the solvents under normal pressure. The oily product was dissolved in dry ACN (20 mL) and was added dropwise to a 50 mL ammonium hydroxide solution (28−30%) at 0 °C and left to stir for 2 h. Reaction mixture was extracted with ethyl acetate (3 × 30 mL). The organic phase was washed with 2 N NaOH, dried over $MgSO_4$, and evaporated, and the oily product was recrystallized with ethyl acetate petroleum ether (3:1) to obtain the product as a white powder. Purity was assessed using melting point, GC−MS, ^{1}H NMR, and elemental analysis.

(2*S*,3*S*)-*sec*-Butylpropylacetamide [(2*S*,3*S*)-SPD (8), Scheme 2] and (2*R*,3*S*)-*sec*-Butylpropylacetamide [(2*R*,3*S*)-SPD]. (3*S*)-Methylpentanoic Acid, ((1′*S*,2′*S*)-2′-Hydroxy-1′-methyl-2′-phenylethyl)methylamide (5). Anhydrous DCM (60 mL) was added to a round-bottomed flask cooled to −5 °C, containing (3*S*)-methylvaleric acid (10 g, 0.08 mol) under nitrogen (N_2), followed by addition of Et_3N (8.5 g, 0.084 mol, 1.05equiv) and pivaloyl chloride (9.64 g, 0.08 mol, 1.00 equiv), while maintaining an internal temperature of <0 °C. The reaction mixture was stirred for 1 h, and then Et_3N (8.5 g, 0.084 mol, 1.05 equiv) was added. (1*S*, 2*S*)-Pseudoephedrine (13.21 g, 0.08 mol, 1.00 equiv) for the synthesis of (2*S*,3*S*)-SPD or (1*R*, 2*R*)-pseudoephedrine (13.21 g, 0.08 mol, 1.00 equiv) for the synthesis of (2*R*,3*S*)-SPD was added as a solid in portions and allowed to stir for an additional 1 h below 5 °C, and then water (60 mL) was added. The organic layer was isolated, washed with 1 M HCl (60 mL), 1 M NaOH (60 mL), and water (60 mL), dried over $MgSO_4$, filtered, and evaporated to yield 97% oily product. The residue (oily product) was used in the next step without any further purification or treatment.

(2*S*)-Propyl-(3*S*)-methylpentanoic Acid, ((1′*S*,2′*S*)-2′-Hydroxy-1′-methyl-2′-phenylethyl)methylamide (6). To a solution of anhydrous LiCl (6.8 g, 0.16 mol, 2.00 equiv) in dry THF (80 mL) and diisopropylamine (18.6 g, 0.184 mol, 2.3 equiv) under N_2 atmosphere at −20 °C was added *n*- BuLi (1.6 M in cyclohexane, 0.184 mol, 2.3 equiv) slowly while maintaining an internal temperature of less than −10 °C. After 30 min, a solution of amide **5** (0.08 mol) in THF (40 mL) was slowly added while maintaining an internal temperature of less than −10 °C. After 30 min, 1-iodopropane (27.2 g, 0.16 mol, 2.00 equiv) was slowly added while maintaining an internal temperature of less than −10 °C. After 2 h, the reaction mixture was warmed to 20 °C and stirred overnight. Saturated NH_4Cl (82 mL) was added to quench the reaction. The organic layer was separated and washed with water (82 mL). The combined aqueous layers were extracted with MTBE (82 mL). The layers were separated, and the MTBE layer was washed with water (82 mL). The organic layer dried over $MgSO_4$, filtered, and evaporated to yield 96% oily product. The residue (oily product) used in the next step without any further purification or treatment.

(2*S*,3*S*)-*sec*-Butylpropylcarboxylic Acid (SPA) (7). 1,4-Dioxane (50 mL) was added to a round-bottomed flask containing amide **6** (0.08 mol) followed by addition of 3 M H_2SO_4 (40 mL, 0.12 mol, 1.5 equiv). The reaction mixture was refluxed (∼90 °C) for 6 h and then cooled to ∼30 °C. Concentrated H_2SO_4 (27 mL, 0.48 mol, 6.00 equiv) was added, and the mixture was refluxed for 16 h followed by cooling to 20 °C. The black mixture was extracted with *n*-heptane (60 mL × 3). The combined organic layers were washed with water (40 mL). The organic layer was extracted with 4 M NaOH (40 mL × 3). The combined basic aqueous extracts were washed with *n*-heptane (40 mL). Concentrated HCl (30 mL) was carefully added to combined basic aqueous to provide an acidic solution (pH < 2) and then was extracted with *n*-heptane (30 mL × 3). The combined organic layer was washed with water (30 mL). The organic layer was dried over $MgSO_4$, filtered, and evaporated to yield 58% oily product. The residue (oily product) was used in the next step without any further purification or treatment.

(2*S*,3*S*)-*sec*-Butylpropylacetamide (SPD) (8). To acid **7** solution (6 g, 0.038 mol) in dry DCM (50 mL) under N_2 atmosphere at 0 °C, a 1:1 solution of thionyl chloride (3.32 mL, 0.045 mol, 1.2 equiv) dissolved in dry DCM (7 mL) was added dropwise, and the mixture was allowed to stir overnight followed by distillation of the solvents under normal pressure. The oily product was dissolved in dry ACN (20 mL) and was added dropwise to a 50 mL ammonium hydroxide solution (28−30%) at 0 °C and left to stir for 2 h. Reaction mixture was extracted with ethyl acetate (3 × 30 mL). The organic phase was washed with 2 N NaOH, dried over $MgSO_4$, and evaporated, and the oily product was recrystallized with ethyl acetate petroleum ether (3:1) to obtain the product as a white powder. Purity was assessed using melting point, GC−MS, ^{1}H NMR, and elemental analysis.

(2*R*,3*R*)-*sec*-Butylpropylacetamide [(2*R*,3*R*)-8]. White powder, 60% yield, mp 129−132 °C. MS-EI, *m*/*z* (%): 142 (M^+ − 15, 0.3), 101 (20), 86 (25), 72 (100), 55 (34). ^{1}H NMR (300 MHz, DMSO, δ TMS): 0.78− 0.88 (m, 9H), 0.98−1.22 (br m, 7H), 1.88− 1.98 (m, 1H), 6.6− 6.7 (s, 1H), 6.15−6.25 (s, 1H). Anal. ($C_9H_{19}NO$) C, H, N. $[\alpha]_D$ +14.8 ± 0.4 (*c* 1, MeOH).

(2*S*,3*R*)-*sec*-Butylpropylacetamide [(2*S*,3*R*)-8]. White powder, 62% yield, mp 151−154 °C. MS-EI, *m*/*z* (%): 142 (M^+ − 15, 0.3), 101 (33), 86 (31), 72 (100), 55 (18). ^{1}H NMR (300 MHz, DMSO, δ TMS): 0.78− 0.88 (m, 9H), 0.98−1.22 (br m, 7H), 1.88−1.98 (m, 1H), 6.6−6.7 (s, 1H), 6.15−6.25 (s, 1H). Anal. ($C_9H_{19}NO$) C, H, N. $[\alpha]_D$ +12.0 ± 0.5 (*c* 1, MeOH).

(2*S*,3*S*)-*sec*-Butylpropylacetamide [(2*S*,3*S*)-8]. White powder, 72% yield, mp 131−134 °C. MS-EI, *m*/*z* (%): 142 (M^+ − 15, 0.3), 101 (33), 86 (32), 72 (100), 55 (20). ^{1}H NMR (300 MHz, DMSO, δ TMS): 0.78− 0.88 (m, 9H), 0.98−1.22 (br m, 7H), 1.88−1.98 (m, 1H), 6.6−6.7 (s, 1H), 6.15−6.25 (s, 1H). Anal. ($C_9H_{19}NO$) C, H, N. $[\alpha]_D$ −16.5 ± 0.6 (*c* 1, MeOH).

(2*R*,3*S*)-*sec*-Butylpropylacetamide [(2*R*,3*S*)-8]. White needles, 79% yield, mp 153−156 °C. MS-EI, *m*/*z* (%): 142 (M^+ − 15, 0.4), 101 (34), 86 (35), 72 (100), 55 (19). ^{1}H NMR (300 MHz, DMSO, δ TMS): 0.78− 0.88 (m, 9H), 0.98−1.22 (br m, 7H), 1.88−1.98 (m, 1H), 6.6−6.7 (s, 1H), 6.15−6.25 (s, 1H). Anal. ($C_9H_{19}NO$) C, H, N. $[\alpha]_D$ −10.8 ± 0.4 (*c* 1, MeOH).

Biological Testing/Anticonvulsant Activity. Pharmacokinetic Studies. Analysis of SPD Stereoisomers in Plasma. Plasma concentrations of each SPD stereoisomer were quantified by a gas chromatography−mass spectrometry (GC−MS) assay.[8] The GC−MS analysis was performed on a Hewlett-Packard (HP) 5890 series II GC apparatus (Hewlett-Packard, Palo Alto, CA, U.S.) equipped with an HP5989A single quadruple mass spectrometer operating in electron impact (EI) mode, an HP7673 autosampler, an HP MS-DOS Chemstation, and an HP-5MS capillary column (0.25 μm × 15 m × 0.25 mm).

Plasma (200 μL) was added to the test tubes followed by 25 μL of methanol and 25 μL of internal standard solution (α-F-TMCD 250 μg/mL in methanol),[28] and the tubes were vortexed thoroughly. Chloroform (2 mL) was used for the extraction of the compounds. The dry residues obtained after evaporation of 1.8 mL of chloroform were reconstituted with 60 μL of methanol, of which 1 μL was injected into the GC−MS apparatus. The temperature program was as follows: injector temperature, 200 °C; initial temperature, 50 °C for 6 min; gradient of 20 °C/min until 140 °C; gradient of 10 °C until 200 °C; hold time, 3 min. The MS parameters were set as follows: source temperature, 200 °C; transfer line, 280 °C; positive ion monitoring, EI-MS (70 eV). The pressure of the carrier gas, helium, was set at 5 psi. For EI analysis, the ionization energy was 70 eV with a source pressure of 10^{-6} Torr. Retention times of SPD and internal standard were 9.9 and 7.9 min, respectively. Calibration curves were constructed for each analytical run and were linear in the concentration range between 0.5 and 50 μg/mL.

Calculation of Pharmacokinetic (PK) Parameters. The PK parameters of each SPD stereoisomer was calculated by non-compartmental analysis based on statistical moment theory using PK

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software Phoenix Winnonlin Tripos L.P. (Pharsight Co., Mountain View, CA).[29] The terminal half-life was calculated as $0.693/\lambda_z$ where λ_z is the linear terminal slope of the log of the drug plasma concentration (C) vs time (t) curve. The plasma exposure or area under the C vs t curve (AUC) from zero to infinity was calculated by using the trapezoidal rule with extrapolation to infinity. The mean residence time (MRT) was calculated from the quotient AUMC/AUC, where AUMC is the area under the $C \times t$ product vs t (first moment-time) curve from zero to infinity. The apparent (total) clearance (CL/F) was calculated from the quotient of Dose/AUC, with F being the absolute bioavailability of the drug after ip administration. The apparent volume of distribution (V/F) was calculated from the quotient of CL and λ_z. The peak plasma concentration (C_{max}) and the time to reach C_{max} (t_{max}) were determined by visual inspection.

Animals and Test Substances Used for Seizure Testing. Male and female albino CF1 mice (18−25 g, Charles River, Portage, MI) and male albino Sprague−Dawley rats[30] (100−150 g, Charles River, Wilmington, MA) were used as experimental animals. Animals were housed in an Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) accredited temperature and humidity controlled facility and maintained on a standard 12 h/12h light−dark (lights on at 06:00) cycle with free access to standard laboratory chow Prolab RMH 3000) and water ad libitum. All animal experiments were performed in accordance with the guidelines set by National Institutes of Health and the University of Utah Institutional Animal Care and Use Committee (IACUC). All animals were allowed free access to both food and water except when they were removed from their cages for the experimental procedure. Except for the kindling studies, animals were used once. All animals were euthanized in accordance with the Institute of Laboratory Resources policies on the humane care of laboratory animals.

Each of the SPD stereoisomers was administered (ip or po) in 0.5% methylcellulose in a volume of 0.04 mL/10 g body weight in rats.

Anticonvulsant Tests. In vivo anticonvulsant activity was established by both electrical and chemoconvulsant seizure tests which have been described previously.[30−32] The electrical tests used were the maximal electroshock (MES) seizure test, and the 6 Hz psychomotor seizure test. The chemical test was the sc metrazol.

MES Test and 6 Hz Test. For the MES and 6 Hz tests, a drop of anesthetic/electrolyte solution (0.5% tetracaine hydrochloride in 0.9% saline) was applied to the eyes of each animal prior to placement of the corneal electrodes. The electrical stimulus in the MES test was 50 mA, 60 Hz for mice and 150 mA, 60 Hz for rats delivered for 0.2 s by an apparatus similar to that originally described by Woodbury and Davenport.[33] Abolition of the hind leg tonic extensor component of the seizure was used as the end point.

The ability of the test substance to prevent seizures induced by 6 Hz corneal stimulation (32 and 44 mA, 3 s duration) in mice was evaluated at a convulsive current that evokes a seizure in 97% of the population tested (CC_{97}). The 6 Hz seizures are characterized by a minimal clonic phase that is followed by stereotyped, automatistic behaviors described originally as being similar to the aura of human patients with partial seizures.[34,35] Animals not displaying this behavior were considered protected.

Minimal Behavioral Toxicity Tests. Minimal toxicity was identified in rats as minimal motor impairment (MMI) as determined by overt evidence of ataxia, abnormal gait, and stance.

Effect of SPD Stereoisomers on Lithium Pilocarpine Induced SE. Seizures were induced by systemic administration of pilocarpine hydrochloride (50 mg/kg, ip), a muscarinic cholinergic agonist. Administration of LiCl (20 mg/kg, ip) 24 h prior to pilocarpine reduces the dose of pilocarpine needed to induce status epilepticus (SE). Pilocarpine induces behavioral seizures within a few minutes, and those animals showing no seizures after 45 min of pilocarpine were removed from the study. At the time of the first stage 3 (Racine scale) seizure or higher, rats were randomized into two groups, pilocarpine alone and pilocarpine + an individual SPD stereoisomer. The latter group received the test compound (130 mg/kg, ip, SPD stereoisomer) 30 min after the first stage 3 seizure. Animals were observed and scored for seizure severity for 1.5 h before being returned to their home cages. All animals were given 1 mL of 0.9% saline (oral) to compensate for the fluid loss induced by excessive cholinergic activation. The median effective dose (ED_{50}) for anticonvulsant activity for the various SPD stereoisomers was determined by probit analysis.[36]

Effect of SPD Stereoisomers on in Vitro Electrophysiology Testing on Rat Hippoacamapal Brain Sliced from Kainic Acid Treated Rats. Male Sprague Dawley rats were injected with either saline or kainic acid (KA) per hour to the onset of stage 4/5 seizure on the Racine scale.[12] Extracellular recording from combined medial entorhinal cortex (mEC) hippocamppal (HC) horizontal brain slices (400 μm) was performed 1 week later. Baseline electrographic responses from layer II medial EC were recorded in normal oxygenated Ringer solution (3 mM KCl). The extraceleulr solution was then switched to one containing 6 mM KCl and 50 μM picrotoxin to compare differences in spontaneous burst (SB) rate and SB duration in slices from control and KA-treated rats. Further, the ability of SPD and its individual stereoisomers (100 μM) to block SBs was compared in slices from KA- and saline-treated rats. All experiments were recorded at 31 ± 1 °C.[12]

Effect of SPD Stereoisomers on Soman-Induced SE. An established rodent model of nerve agent-induced SE was used: a rat HI-6 pretreatment model.[8,37−39] The model utilizes a pretreatment and adjunctive drug that counters the acute immediate lethal effects of the nerve agent but that does not inhibit the development of SE. In this model the challenge dose of soman is sufficient to elicit SE in all animals 5−8 min following soman challenge. The animals were typically tested in squads of eight and were randomized among treatment groups each test day. The animals were weighed, placed in individual recording chambers, and connected to the recording apparatus. EEG signals were recorded using CDE 1902 amplifiers and displayed on a computer running Spike2 software (Cambridge Electronic Design, Ltd., Cambridge, U.K.). Baseline EEG was recorded for at least 20 min. The animals were then pretreated with 125 mg/kg, ip, of the oxime HI-6 to prevent the rapid lethal effects of the soman challenge. At 30 min after pretreatment the rats were challenged with 180 μg/kg, sc, soman ($1.6 \times LD_{50}$) and 1 min later treated with 2.0 mg/kg, im, atropine methyl nitrate to inhibit peripheral secretions. The rats were then closely monitored both visually and on the EEG for seizure onset. Seizure onset was operationally defined as the appearance of >10 s of continuous rhythmic high amplitude spikes or sharp waves that were at least twice the baseline amplitude accompanied by a rhythmic bilateral flicking of the ears, facial clonus, and possibly forepaw clonus. The rats received standard medical countermeasures [0.1 mg/kg atropine sulfate + 25 mg/kg 2-PAM Cl admixed to deliver 0.5 mL/kg, im, and 0.4 mg/kg, im, diazepam] at 20 min after seizure onset and then were immediately given a dose (10−165 mg/kg), ip, of SPD (racemate or an individual stereoisomer) dissolved in mutisol (a 5:1:4 solution of propylene glycol, alcohol, and water for injection). These standard medical countermeasures (atropine, 2-PAM, and in the rat model, diazepam), at the doses and times used, are insufficient by themselves to terminate soman-induced seizures in either model. The rats were monitored for at least 5 h after exposure and then returned to the animal housing room. At 24 h after the exposure, surviving animals were weighed and the EEG was again recorded for at least 30 min.

Teratogenicity. For this study, the highly inbred SWV/Fnn mouse strain with a known susceptibility to AED-induced NTDs[40] was used according to the previously published procedure.[41] Dams were allowed to mate overnight with male mice and were examined on the following morning for the presence of vaginal plugs. The onset of gestation was set at 1 a.m. of the previous night. On gestational day 8.5, pregnant females received a single ip injection (10 μL per gram body weight) of sodium valproate at doses 1.8 or 1.1 mmol/kg or SPD (racemate or its individual stereoisomers) at doses 1.8 or 0.9 mmol/kg. A 25% Cremophor EL water solution was injected ip to dams constituting the control group. On gestation day 18.5, the dams were euthanized by CO_2 asphyxiation, the abdomen opened and the gravid uteri removed. The locations of all viable, dead, and resorbed fetuses were recorded,

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and the fetuses were grossly examined for the presence of gross malformation.

AUTHOR INFORMATION

Corresponding Author

*Phone: 972-2-6758610. Fax: 972-2-6757246. E-mail: bialer@md.huji.ac.il.

Author Contributions

⊥N.H. and T.S.-A. contributed equally.

Notes

Dr. Meir Bialer has received in the past 3 years speakers or consultancy fees from Bial, CTS Chemicals, Desitin, Janssen-Cilag, Johnson & Johnson, Medgenics, Rekah, Sepracor, Teva, UCB Pharma, and Upsher-Smith. Dr. Bialer has been involved in the design and development of new antiepileptics and CNS drugs as well as new formulations of existing drugs. None of the other authors have any conflict of interest to disclose. We, the authors, confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines. The authors declare no competing financial interest.

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ABBREVIATIONS USED

CNS, central nervous system; AED, antiepileptic drug; MES, maximal electroshock seizure; scMet, subcutaneous metrazol; SE, status epilepticus; PI, protective index; VPA, valproic acid; LDA, lithium diisopropylamide; DCM, dichloromethane; THF, tetrahydrofuran; NMR, nuclear magnetic resonance; GC–MS, gas chromatography–mass spectrometry; TLC, thin-layer chromatography

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