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Enantioselective Synthesis and Teratogenicity of Propylisopropyl Acetamide, a CNS-Active Chiral Amide Analogue of Valproic Acid

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ABSTRACT Propylisopropyl acetamide (PID), an amide analogue of the major antiepileptic drug valproic acid (VPA), possesses favorable anticonvulsant and CNS properties. PID contains one chiral carbon atom and therefore exists in two enantiomeric forms. The purpose of this work was to synthesize the two PID enantiomers and evaluate their enantiospecific teratogenicity. Enantioselective synthesis of PID enantiomers was achieved by coupling valeroyl chloride with optically pure (4*S*)- and (4*R*)-benzyl-2-oxazolidinone chiral auxiliaries. The two oxazolidinone enolates were alkylated with isopropyl triflate, hydrolyzed, and amidated to yield (2*R*)- and (2*S*)-PID. These two PID enantiomers were obtained with excellent enantiomeric purity, exceeding 99.4%. Unlike VPA, both (2*R*)- and (2*S*)-PID failed to exert teratogenic effects in NMRI mice following a single 3 mmol/kg subcutaneous injection. From this study we can conclude that individual PID enantiomers do not demonstrate stereoselective teratogenicity in NMRI mice. Due to its better anticonvulsant activity than VPA and lack of teratogenicity, PID (in a stereospecific or racemic form) has the potential to become a new antiepileptic and CNS drug. *Chirality 11:645–650, 1999.*

KEY WORDS: antiepileptic drugs; isopropyl triflate; oxazolidinone chiral auxiliaries

INTRODUCTION

Valproic acid (VPA, Fig. 1), with carbamazepine, phenytoin, and phenobarbital, is one of the four major antiepileptic drugs (AEDs). Despite its lower potency compared to other major AEDs in classical animal models, VPA has a broad spectrum of antiepileptic activity and is clinically used in various types of epilepsy and other CNS disorders (migraine, bipolar disorders, and neuropathic pain).[1] Valpromide (VPD, Fig. 1), the primary amide of VPA is 3–15 times more potent than VPA in the above-mentioned models.[2–4] Propylisopropyl acetamide (PID) is a chiral isomer of VPD that contains one chiral carbon atom (Fig. 1), and has similar anticonvulsant potencies to VPD.[3,5]

VPA is a potent teratogen that causes several forms of congenital malformations to infants of pregnant women treated with it during early embryogenesis.[6] VPA therapy is mainly associated with an increased risk of developing neural tube defects (NTDs).[7,8] The incidence of VPA-induced spina bifida (a form of NTDs) is approximately 2%, which is 20-fold higher than in the general population.[9] VPA-induced NTDs are not exclusive to humans and can be observed in several laboratory animals.[10] Following a single 3 mmol/kg intraperitoneal injection of VPA to NMRI mice (a strain showing high sensitivity to VPA teratogenicity) on day 8 of gestation, exencephaly (the equivalent of NTDs in mice) was reproducibly observed in ~50% of all treated dams.[11,12] In a structure–teratogenicity study in mice of several VPA analogues and isomers, Nau and Hendrickx[10] found that VPD was not teratogenic and concluded that a VPA analogue must possess a carboxylic acid moiety in order to induce exencephaly. Subsequently, we recently demonstrated that the VPD isomer valnoctamide (Fig. 1) and its corresponding acid, valnoctic acid, are also not teratogenic.[11] To explore if amidation of a VPA isomer eliminates teratogenicity, we have investigated the teratogenicity of the racemate and pure enantiomers of the valproyl amide analogue PID.

Stereoselective teratogenicity has been demonstrated with several VPA analogues. Hauck and Nau[12,13] showed that *S*-(–)-4-ene VPA was four times more teratogenic and embryotoxic than *R*-(+)-4-ene VPA. A similar observation was demonstrated with *S*-(–)-4-yn VPA, being 7.5 times

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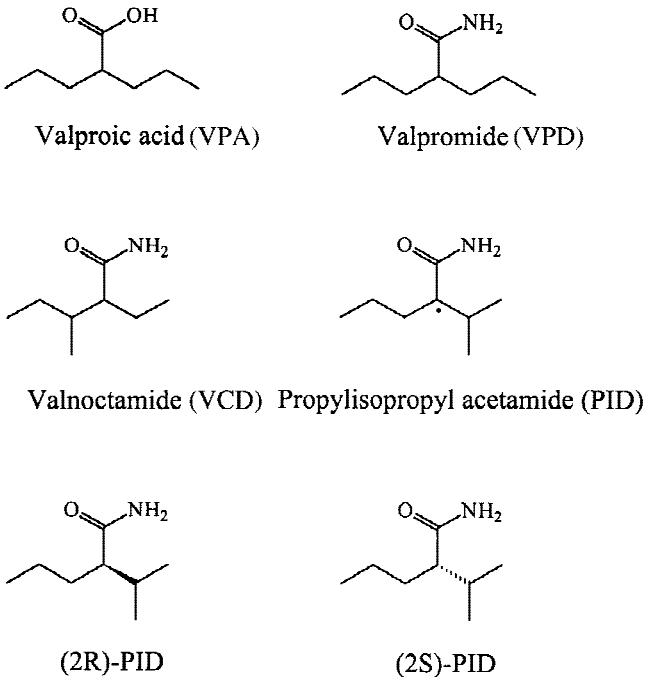


Fig. 1. Chemical structures of VPA, VPD, VCD, racemic PID, (2*S*)-PID, and (2*R*)-PID.

more teratogenic than its antipode.[12,14] The aim of the current study was to synthesize (2*R*)- and (2*S*)-PID, and to evaluate their stereospecific teratogenic potential in NMRI mice.

MATERIALS AND METHODS

Chemicals

(*S*)-(–)-Benzyl-(2)-oxazolidinone, (*R*)-(+)-benzyl-(2)-oxazolidinone, sodium sulfite, butyllitium, diisopropylamine, trifluoromethansulfonic anhydride, lithium hydroxide, 4A molecular sieves, and thionyl chloride were purchased from Aldrich Chemical Company Inc. (Milwaukee, WI). Tetrahydrofuran, sodium chloride, sodium hydrogen carbonate, dichloromethane, hydrogen peroxide, ammonium hydroxide, sodium hydroxide, diethyl ether, hydrochloric acid, ethyl acetate, and petroleum ether were purchased from Frutarom (Jerusalem, Israel). Ammonium chloride and pentane were purchased from J.T. Baker Chemical Co. (Philipsburg, NJ). Silica gel (silica gel 60 PF_{254} with gypsum) was purchased from Merck (Darmstadt, Germany).

Dry dichloromethane (DCM) and diisopropylamine were obtained by drying on CaH_2 and distillation. Dry THF was obtained by drying on sodium-benzophenome and distillation. Et_2O was dried on 4A molecular sieves.

Instruments

^1H and ^{13}C NMR were performed in $CDCl_3$ on a Varian VXR-300S spectrophotometer. All chemical shifts are reported in ppm relative to TMS. The gas chromatography–mass spectrometry (GC/MS) apparatus consisted of a Hewlett-Packard GCD plus 1800B series equipped with a HP-MS 5971 quadrupole mass analyzer and electron impact (EI) source operating at 70 eV and 250°C. The column was a Hewlett-Packard HP-5 fused silica capillary column (30 m × 0.25 mm, 0.25 μm) coated with a bonded stationary phase (5% phenyl silicone). Helium flow was 0.7 ml/min, injector 250°C, and column temperature 220°C for compounds **1**, **2**, **5**, and **6** and 110°C for compounds **3**, **4**, **7**, and **8.** Melting points (uncorrected) were measured on a Buchi model 530 apparatus (Buchi, Switzerland). Optical rotation was measured at 22°C with an Autopol III automatic polarimeter apparatus, Rudolph Research (Flanders, NJ). $[\alpha]_D$ values are reported as mean ± standard deviation.

Enantioselective Gas Chromatography (GC)

The GC apparatus used to separate PID enantiomers consisted of a Hewlett-Packard Model 5890 series 2 gas chromatograph equipped with a capillary split injector, FID detector and a Hewlett-Packard model 3396-A integrator. Separation was achieved on a capillary column (10 m, 0.25 mm, 0.25 μm) coated with Heptakis- (2,3-di-O-methyl-6-O-tert-butyldimethylsilyl)-β-cyclodextrin as the stationary phase.[15,16] The Carrier gas was nitrogen, column head pressure 50 KPa, split ratio 1:30, injector 250°C, detector 300°C and column temperature 120°C isothermal.

Synthesis

(4*S*)-3-(1′-Oxopentyl)-4-benzyl-2-oxazolidinone (1). To a cooled (–78°C) solution of (4*S*)-benzyl-2-oxazolidinone (23.2 g, 131 mmol) in dry THF (150 mL) was slowly added a solution of BuLi (86 mL, 1.6 M in hexane, 137 mmol). After stirring for 45 min, valeroyl chloride (17.1 ml, 144 mmol) was added dropwise via cannula. The reaction was slowly warmed to 0°C, stirred for 2.5 h, and quenched by saturated NH_4Cl solution. THF was evaporated and the reaction mixture extracted with DCM (3 × 150 ml). The combined organic phases were washed with water and saturated brine and dried with $MgSO_4$. White solid crystals of product **1** (29.5 g) were obtained by crystallization from 10% EtOAc in PE, yield, 75%. Melting point 45°C. ^1H NMR (300 MHz): δ 7.19–7.35 (m, 5H, aryl-H), 4.66 (m, 1H), 4.17 (m, 2H), 3.31 and 3.26 (dd, J = 3.3 Hz, 1H), 2.92 (m, 2H), 2.72–2.79 (dd, J = 10 Hz, 1H), 1.6–1.7 (m, 2H), 1.37–1.44 (m, 2H), 0.96 (t, J = 8 Hz, 3H). GC/MS (*m/z*): 261 (M+), 232, 219, 134, 118, 85, 57. Elemental analysis: found (calculated) C, 68.67% (68.94%); H, 7.47% (7.33%); N, 5.41% (5.36%). $[\alpha]_D$ 76.20 ± 0.14 (c = 1.85, DCM). Literature: $[\alpha]_D$ 53.2 (c = 2.4, $CHCl_3$).[14]

Isopropyl Trifluoromethane Sulfonate (Isopropyl Triflate). To a cooled (–17°C) solution of dry isopropyl alcohol (9.8 ml, 128 mmol) and dry TEA (18.6 mL, 134 mmol) in DCM (150 mL) was added dropwise a cooled (–17°C) solution of trifluoromethane sulfonic anhydride (37.0 g, 131 mmol). The reaction was stirred for 1 h and then washed with ice-cold 0.25 M HCl (2 × 250 mL) followed by ice-cold 0.5 M $NaHCO_3$ (2 × 125 mL). The organic phase was dried with $MgSO_4$ and evaporated without external heating (0°C bath). The yellowish liquid obtained was dissolved in cool (0°C) dry pentane (50 mL), vacuum-filtered through a short $MgSO_4$ plug, and concentrated. The product, a colorless liquid, was dissolved in 30 mL of

dry pentane and kept at –20°C (less than 24 h) until used. Yield 12 g, 48%. ^{1}H NMR (300 MHz): δ 5.2 (m, 1H), 1.5 (d, J = 2.7 Hz, 6H).

(4*S*,2′*R*)-3-(2′-Isopropyl-1′-oxopentyl)-4-benzyl-2-oxazolidinone (2). To a cooled (–78°C) solution of dry DIPA (11 mL, 78 mmol) in dry THF (40 mL) was added dropwise BuLi (49 mL, 1.6 M in hexane, 78 mmol). After stirring 30 min a cooled (–78°C) solution of **1** (18.5 g, 71 mmol in 70 mL of dry THF) was added slowly via cannula. After stirring for 1 h a cooled (–78°C) solution of isopropyl triflate (15 g, 78 mmol in 20 mL of dry pentane) was quickly added via cannula. The reaction was slowly warmed to –30°C, stirred 4 h, and quenched with saturated NH_4Cl. THF was evaporated, and the aqueous phase extracted with Et_2O (3 × 150 mL) and dried with $MgSO_4$. Purification of the crude product **2** (23 g of a viscous yellow oil) by column chromatography (silica gel, 0.5–3% EtOAc in PE) afforded a yellowish oil. Yield 8.8 g, 41%, ^{1}H NMR (300 MHz): δ 7.2–7.4 (m, 5H, aryl-H), 4.7 (m, 1H), 4.2 (m, 2H), 3.7 (m, 1H), 3.3–3.4 (dd, J = 3 Hz, 1H), 2.6–2.7 (dd, J = 6 Hz, 1H), 2.0 (m, 2H), 0.9–1.1 (m, 9H). GC/MS (*m/z*): 303 (M+), 261, 232, 178, 126, 57.

(2*R*)-Propylisopropyl acetic acid (3). To a cooled (0°C) solution of **2** (8.8 g, 29 mmol) in a 4:1 mixture of THFwater (550 mL) was added H_2O_2 (30%, 19.3 mL, 170 mmol) followed by LiOH (2.44 g, 58 mmol in 50 mL of water). The reaction was slowly warmed to room temperature and left to stir overnight. After 24 h, the reaction was cooled to 0°C and quenched by sodium sulfite solution (21 g, 170 mmol in 100 mL of water). THF was evaporated, and the basic aqueous phase (pH 11) was extracted with DCM (3 × 100 mL) to recover (4*S*)-benzyl-2-oxazolidinone, 80% yield. The aqueous phase was acidified with concentrated HCl (to pH 2) and extracted with EtOAc (3 × 100 ml). The combined organic phases were washed successively with dilute $NaHCO_3$, water and saturated brine and dried with $MgSO_4$. Evaporation of the solvent afforded product **3** as a colorless oil. Yield 3.49 g, 83%. The product was used without any further purification in the next step. GC/MS (m/z) 143, 115, 102, 87, 73, 69, 55.

(2*R*)-Propylisopropyl acetamide, ((2*R*)-PID, 4). To a cooled (0°C) solution of **3** (3.3 g, 23 mmol) dissolved in dry DCM (100 mL) was added dropwise a solution of thionyl chloride (6.78 g, 57 mmol). After stirring the reaction overnight at room temperature, the crude reaction mixture was slowly added via cannula to cold (–17°C) NH_4OH (20 mL, 25% solution in water, 140 mmol) and stirred for 1 h. The organic phase was washed with water, dilute HCl, and saturated brine, dried with $MgSO_4$, filtered, and concentrated. Product **4** was crystallized from 20% EtOAc in PE. Yield 2.14 g, 65%. Melting point 149–150°C. ^{1}H NMR (300 MHz): δ 5.6 (s, 1H, NH_a), 5.4 (s, 1H, NH_b), 1.7–1.8 (m, 2H), 1.4–1.5 (m, 3H), 1.2 (m, 1H), 0.88–0.96 (m, 9H, 3 × CH_3). ^{13}C NMR (75 MHz): δ 178.3, 54.3, 32.3, 30.7, 21.0, 20.8, 20.4, 14.2. Elemental analysis: found (calculated); C, 67.15% (67.1%): H, 11.92% (12.0%); N, 9.56% (9.80%). GC/MS (*m/z*): 128, 114, 101, 100, 86, 72, 57. $[\alpha]_D$ 9.37 ± 0.18 (*c* = 1.0, MeOH).

(4*R*)-3-(1′-Oxopentyl)-4-benzyl-2-oxazolidinone (5). Synthesized from valeroyl chloride and (4R)-benzyl-2-oxazolidinone by the same procedure as **1.** Yield 21.5 g, 76%. $[\alpha]_D$ –74.03 ± 0.19 (c=2.13, DCM). Elemental analysis: found (calculated): C, 68.72% (68.94%); H, 7.26% (7.33%); N, 5.05% (5.36%). GC/MS (*m/z*): 261 (M+), 232, 219, 117, 85, 57.

(4*R*,2′*S*)-3-(2′-Isopropyl-1′-oxopentyl)-4-benzyl-2-oxazolidinone (6). Synthesized from **5** and isopropyl triflate by the same procedure as **2.** Yield 5.5 g, 32%. GC/MS (*m/z*): 303 (M+), 261, 232, 212, 178, 127, 57.

(2*S*)-Propylisopropyl acetic acid (7). Obtained from **6** and lithium hydroperoxide by the same procedure as **3.** Yield 2.3 g, 89%. GC/MS (*m/z*): 143, 115, 102, 87, 73, 55.

(2*S*)-Propylisopropyl acetamide ((2*S*)-PID, 8). Synthesized from **7** and ammonium hydroxide by the same procedure as **4.** Yield 1.54 g, 67%. ^{1}H NMR (300 MHz): δ 5.6 (s, 1H, NH_a), 5.4 (s, 1H, NH_b), 1.77–1.82 (m, 2H), 1.4–1.5 (m, 3H), 1.2 (m, 1H), 0.88–0.96 (m, 9H, 3 × CH_3). ^{13}C NMR (75 MHz): δ 178.1, 54.3, 32.3, 30.7, 21.0, 20.8, 20.4, 14.2. Elemental analysis: found (calculated): C, 66.73% (67.1%); H, 11.62% (12.0%); N, 9.55% (9.80%). GC/MS (*m/z*): 128, 114, 101, 100, 86, 72, 57. $[\alpha]_D$ –9.28 ± 0.21 (*c* = 1.01, MeOH).

Animals

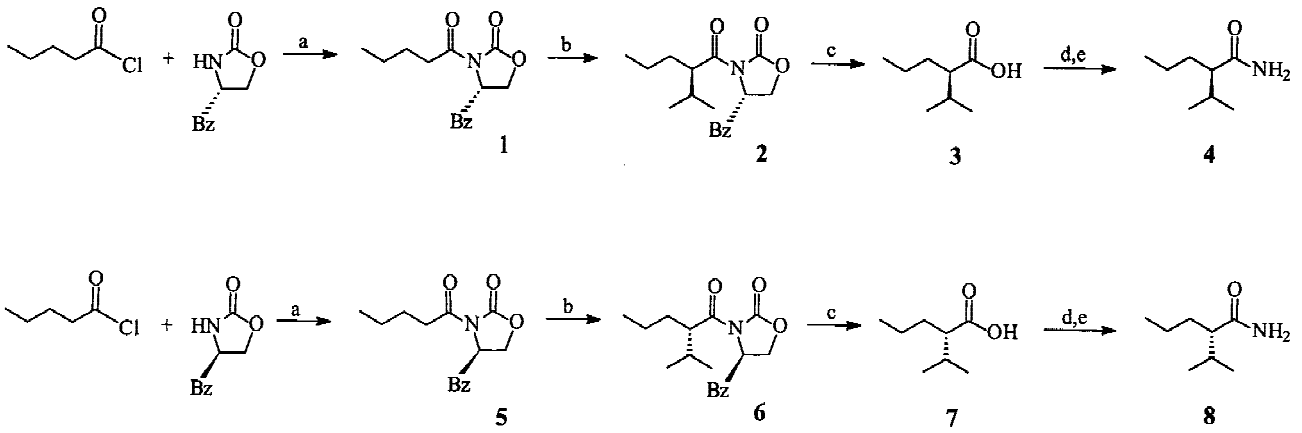
Mice of the NMRI strain (Harlan-Winkelmann GmbH, 33176 Borchen, Germany) were kept under controlled conditions: Room temperature (21 ± 1°C), relative humidity (50 ± 5%), and a 12 h light–dark cycle with the light period from 10 a.m. to 10 p.m. Females weighing 28–36 g were mated with males of the same strain for 3 h (from 6 a.m. to 9 a.m.). Females with vaginal plugs were separated, and the following 24-h period was designated as day 0 of pregnancy. The animals were given free access to food (Altromin 1324 diet, Lage, Germany) and tap water. Approval for the study was obtained from the Bezirksregierung, Hannover, Germany, approval number 97/956.

Teratology

VPA, racemic PID, (2*R*)-PID, and (2*S*)-PID were suspended in a 25% Cremophor EL aqueous solution. For another treatment group sodium valproate (VPA-Na) was dissolved in distilled water. The pregnant dams were injected subcutaneously (sc) with a single 3 mmol/kg dose (10 mL/kg) on the morning of day 8 of gestation. Mice of the control group were injected with the vehicle, 25% Cremophor EL solution (10 mL/kg). On day 18 of gestation the dams were sacrificed by cervical dislocation, the uteri removed, and the numbers of implants, resorptions, and dead fetuses recorded. Living fetuses were weighed individually and inspected for the presence of external malformations.

RESULTS AND DISCUSSION

Racemic PID is more potent than VPA in classical animal models for anticonvulsant screening.[3,5] Since biological macromolecules are chiral entities and PID is a chiral mol-



Scheme 1. Enantioselective synthesis of (2*R*)-PID **(4)** and (2*S*)-PID **(8).**[a]
[a](a) BuLi, THF, −78°C; (b) isopropyl triflate, LDA, THF, −30°C; (c) LiOH, H_2O_2, THF, H_2O; (d) $SOCl_2$, DCM; (e) NH_4OH, DCM.

ecule, we found it important to synthesize the individual PID enantiomers and investigate their potential to induce congenital malformations in NMRI mice.

The stereoselective synthesis of (2*R*)-PID and (2*S*)-PID is presented in Scheme 1. Preparation of imides **1** and **5** was achieved by coupling valeroyl chloride with (4*S*)- and (4*R*)-benzyl-2-oxazolidinones, respectively.[14] In our attempts to alkylate compounds **1** and **5,** we found that 2-bromo- and 2-iodopropane, which are conventionally used for alkylation, were completely unreactive even when the reaction was carried out at relatively high temperatures (0°C).[17] This observation is in agreement with reports of relative low nucleophilic reactivity of these oxazolidinone imides.[18] In order to overcome this problem, we synthesized isopropyl triflate and used it to alkylate imides **1** and **5.** Since many alkyl triflates are unstable at moderate temperatures,[19] the synthesis and isolation of isopropyl triflate was done at −17 to 0°C. Due to the ready decomposition of isopropyl triflate, it was stored in an inert solvent e.g. pentane for several days below −20°C.

Basic exo-hydrolysis of **2** and **6** with lithium hydroperoxide was performed according to Evans et al.[20] and afforded (2*R*)- and (2*S*)-propylisopropyl acetic acids (compounds **3** and **7**). By using thionyl chloride, the acid chlorides of **3** and **7** were prepared. These acid chlorides were amidated with ammonium hydroxide to afford (2*R*)-PID (**4**) and (2*S*)-PID (**8**). Both **4** and **8** were obtained with enantiomeric purity exceeding 99.4%, as judged by GC (Fig. 2-A and 2-B), emphasizing that no racemization at the asymmetric carbon occurred at any stage of the asymmetric synthesis. Complete baseline separation of PID enantiomers (Fig. 2-C) is achieved by enantioselective GC with a chiral capillary column coated with a stationary phase made of derivatized β cyclodextrin.[16] Both 4-benzyl-2-oxazolidinone auxiliaries were recovered following the hydrolysis of **2** and **6** and could be reused.

The teratogenic potency of racemic PID and the individual enantiomers was evaluated in NMRI mice following a single 3 mmol/kg sc injection to pregnant dams at day 8 of gestation, as presented in Table 1. Racemic PID and the individual enantiomers failed to induce exencephaly in the developing mouse embryos, whereas VPA caused 37% and 73% exencephaly in living fetuses when administered in water and Cremophor EL suspension, respectively. Fetal deaths and early resorptions expressed as embryo lethality significantly ($P < 0.0001$) increased in VPA-treated animals. Mice treated with racemic PID or the individual PID enantiomers showed comparable embryolethality to controls. All PID and VPA treated animals (except those treated with racemic PID) showed significant ($P < 0.0001$) reductions in fetal weight as compared to controls.

Several studies have shown that VPD, the primary amide of VPA, exhibits a low teratogenic potential in mice.[4,11,21] It seems that the amidation of the carboxylic acid VPA can prevent most of its teratogenic effects. In order to test further the hypothesis that amidation of the carboxylic acid in VPA derivatives eliminates teratogenicity, we examined several other amide analogues of VPA. In a recent study racemic valvoctamide (VCD) was found to be nonteratogenic.[11] The present study clearly shows that PID, an isomer of VCD and VPD, is nonteratogenic as well.

Since PID is a chiral molecule with one asymmetric carbon atom, it is possible that the individual enantiomers induce teratogenic effects to different extents. One of the enantiomers might increase the teratogenic effects of its antipode or the teratogenicity of the latter may be decreased in the presence of its antipode. These potential phenomena might be caused by pharmacodynamic and/or pharmacokinetic interactions between the PID enantiomers.[22–24] Therefore, we synthesized (2*R*)- and (2*S*)-PID and tested their intrinsic ability to exert teratogenicity, in addition to testing racemic PID. As presented in Table 1 neither PID enantiomers, nor the racemic mixture that was administered at double the dose of the individual enantiomers, caused any significant teratogenic effects following a single sc injection. Unlike the unsaturated VPA analogues 4-ene-VPA and 4-yn-VPA which are markedly enantioselective teratogens,[13,14] both PID enantiomers were nonteratogenic in this well established mouse model for teratogenicity. This study shows that unlike unsaturated VPA analogues, the teratogenicity of the valproyl amide analogue PID is not stereoselective.

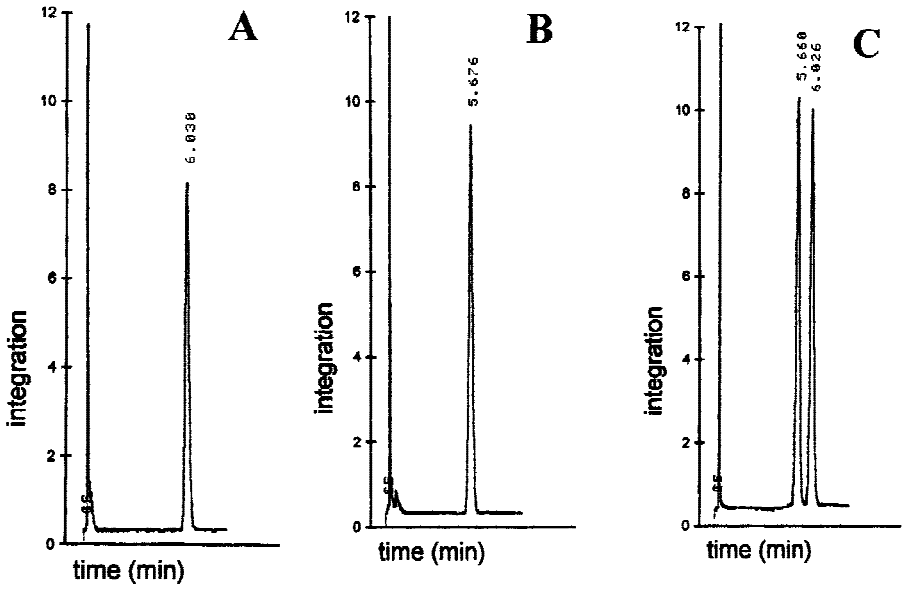


Fig. 2. GC Chromatograms of PID. (A) (2*R*)-PID (4); (B) (2*S*)-PID (8); (C) racemic PID.

In humans, VPD is a prodrug of VPA,[25,26] so that VPD has no clinical advantage (better antiepileptic potency or lack of teratogenicity) over VPA. In dogs[27,28] and rats,[29] 30–55% of a VPD dose is metabolized to VPA. In contrast to humans, dogs and rats, in NMRI mice, only 8% of an oral VPD dose is biotransformed to VPA but causes 6% exencephaly.[11] These findings were also observed when the vehicle was composed of dimethyl sulfoxide: Tween 20: water in a 1:2:7 ratio. In other experimental conditions where the vehicle was made of a propylene glycol mixture, VPD was not teratogenic.[4,21] Unlike VPD, PID is completely stable to metabolic hydrolysis in dogs to the corresponding acid, propylisopropyl acetic acid (PIA).[5] This stability of PID amide-acid biotransformation may have contributed to its lack of teratogenicity, since racemic PIA induces significant embryolethality in 18% of developing embryos.[30]

In conclusion, we found that (4*R*)- and (4*S*)-benzyl-2-oxazolidinone chiral auxiliaries and isopropyl triflate are suitable for stereospecific synthesis of (2*R*)- and (2*S*)-PID with excellent enantiomeric excess. Neither (2*R*)- nor (2*S*)-PID, in addition to racemic PID, were teratogenic to NMRI mice, in contrast to VPA, which exerted pronounced teratogenic effects in this common animal model for teratogenicity.

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TABLE 1. Teratogenicity of racemic PID, (2*R*)-PID, and (2*S*)-PID as compared to VPA in NMRI mice at day 18 of gestation.

Compound	Dose mmol/kg	Litters *n*	Total implants *n*	Live Fetuses *n*	Fetal weight[a] g	Embryo lethality[b] *n* (%)	Exencephaly[c] *n* (%)
(2*R*)-PID[d]	3	9	92	78	1.16 ± 0.08*	14 (15.2)	0 (0)
(2*S*)-PID[d]	3	5	64	58	1.15 ± 0.09*	6 (9.4)	0 (0)
Racemic PID[d]	3	8	96	87	1.21 ± 0.07	9 (9.4)	0 (0)
VPA-Na[d]	3	7	86	41	0.96 ± 0.08*	45 (52.3)**	30 (73.2)**
VPA-Na[e]	3	8	92	63	1.09 ± 0.11*	29 (31.5)**	23 (36.5)**
Controls[f]	–	20	258	238	1.23 ± 0.09	20 (7.8)	3 (1.3)

[a]Mean ± standard deviation.
[b]Percent of total implants.
[c]Percent of live fetuses.
[d]Administered subcutaneously in the vehicle: 25% Cremophor EL.
[e]Administered intraperitonealy dissolved in distilled water.
[f]Controls received the vehicle: 25% Cremophor EL.
*Significantly different from controls ($p < 0.0001$, Fisher exact test).
**Significantly different from controls ($p < 0.0001$, student's *t*-test).

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