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ASYMMETRIC SYNTHESIS AND TERATOGENIC ACTIVITY OF (R)- AND (S)-2-ETHYLHEXANOIC ACID, A METABOLITE OF THE PLASTICIZER DI-(2-ETHYLHEXYL)PHTHALATE

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Summary

The stereoselectivity of the teratogenic activity of 2-ethylhexanoic acid (EHXA), a metabolite of the widely-used plasticizer di-(2-ethylhexyl)phthalate, was investigated. The enantiomers of EHXA were prepared via asymmetric synthesis with the aid of the chiral auxiliaries (R)- and (S)-1-amino-2-(methoxymethyl)pyrrolidine (RAMP, SAMP). The aqueous solutions of the sodium salts of (R)- and (S)-EHXA and the racemic EHXA ((±)-EHXA) were injected each morning and evening of day 7 and 8 of gestation in the NMRI mouse (500 mg/kg, i.p.), a period highly sensitive in regard to the production of neural tube defects (exencephaly) by branched-chain carboxylic acids. (S)-EHXA did not yield any teratogenic or embryotoxic response in this model, while (R)-EHXA was highly teratogenic (59% of living fetuses exhibited exencephaly) and embryotoxic (as indicated by embryolethality and fetal weight retardation); the exencephaly rate induced by (±)-EHXA was between those of the two enantiomeres (32%). It is therefore likely that stereoselective interactions of the enantiomers of EHXA with chiral molecules in the embryo are decisive in regard to the teratogenic response. This first example of the stereoselectivity of the teratological activity of an environmental pollutant suggests that the safety of man-made chemicals can be improved by the use of pure enantiomers instead of racemates.

Phthalic acid esters are used extensively as plasticizers, particularly for materials made of polyvinyl chloride. The annual production of phthalates in the USA alone is estimated to be about 1 billion pounds, world-wide production amounts to 3-4 billion pounds. The most commonly used phthalate is di-(2-ethylhexyl)phthalate (DEHP) which may constitute as much as 40% by weight of the plastic material (1,2). It has been shown that small quantities of phthalates may leak from plastic containers into milk, food, blood or various other materials (3-5); the presence of phthalates in human and animal blood and tissue is a reason for great concern with regard to possible toxic effects of these contaminants.

DEHP was shown to be teratogenic and embryotoxic at high dose levels in rats and mice (6,7); it is unknown whether the unintentional exposure to small amounts of DEHP derived from packaged food or from medical devices presents a potential reproductive hazard to pregnant women. DEHP is extensively metabolized in the mammalian organism via hydrolysis of one ester bond to mono-(2-ethylhexyl)phthalate (MEHP) and 2-ethylhexanol

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(EHXO), and most toxicological studies have focused so far on MEHP (8-11). The other hydrolysis product, EHXO, is apparently rapidly oxidized to 2-ethylhexanoic acid (EHXA) (12). On an equimolar basis, DEHP was found to be less teratogenic than MEHP, and MEHP less teratogenic than EHXA following single, high oral dosing in the rat. It was concluded that EHXA is the possible proximate teratogen of DEHP (13).

The α-carbon atom of EHXA is asymmetric and all teratological studies up to now have been performed with the racemic mixture ((±)-EHXA); the activities of the two enantiomers of EHXA are unknown. Previous studies with a number of drugs have indicated that the pharmacological and toxicological as well as teratological action (14) can strongly depend on the stereochemical configuration due to stereoselective interaction with proteins (enzymes, receptors) or other chiral cell constituents (15). We have therefore synthesized (R)- and (S)-EHXA using the chiral reagents (R)- and (S)-1-amino-2-(methoxymethyl)pyrrolidine (RAMP, SAMP). These enantiomers have previously been prepared via fractionated crystallization of the quinine and chinchonidine salts (16,17); the toxicological properties of these enantiomers were not investigated. We have studied the teratogenic activity of (±)-EHXA and (R)- and (S)-EHXA in a mouse model which has been established as a selective and specific assay for the teratogenic action of branched short-chain carboxylic acids (18). We demonstrate a profound stereoselectivity of the teratogenic action of EHXA a metabolite of the environmental pollutant DEHP.

Materials and Methods

Instruments

^{1}H-NMR spectra were recorded with a Bruker WH270 spectrometer at 270 MHz. ^{1}H chemical shifts are reported in parts per million relative to internal tetramethylsilane. The gas chromatographic determination of the chemical purities were performed on a Hewlett-Packard 5700A using a SE 30 column (1.80 m x 2 mm; Applied Science Lab.) and flame ionization detection. The optical purities were determined on a Carlo Erba Fractovab 4160 gas chromatograph using a DB-210 capillary column (30 m x 0.25 mm; J & W Scientific) and nitrogen selective detection. The optical rotations were measured at 589 nm using a Perkin-Elmer 241 polarimeter.

Chemicals

(±)-2-Ethylhexanoic acid, ethyliodide, diisopropylamine, methyliodide, (S)-(-)-phenethylamine and hexanal were supplied by Aldrich (Steinheim, F.R.G.). (S)-(-)-1-amino-2-(methoxymethyl) pyrrolidine (SAMP), (R)-(+)-1-amino-2-(methoxymethyl)pyrrolidine (RAMP), butyllithium, silver nitrate, dry diethylether were obtained from Merck (Darmstadt, F.R.G.), N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) from Pierce (Rockford, Illinois, U.S.A.).

Synthesis of (R)- and (S)-2-ethylhexanoic acid

The synthetic pathways are summarized in FIG. 1. The enantioselective synthesis of the chiral 2-ethylhexanals **5** and **6** was accomplished according to the procedure described by Enders and Eichenauer (19). Thus we first prepared the hydrazones **1** and **2** using the auxiliaries (R)-1-amino-2-(methoxymethyl)pyrrolidine (RAMP) and (S)-1-amino-2-(methoxymethyl)pyrrolidine (SAMP) respectively, and hexanal. Metallation was performed through employment of a freshly prepared solution of lithium diisopropylamide (LDA) in dry diethylether (0° C, 6 h). The subsequent enantioselective alkylation using ethyliodide led to **3** and **4** (-110° C, 6 h). Cleavage of **3** and **4** according to the known methyliodide-method produced the 2'-chiral aldehyds **5** and **6**. Without further purification they were oxidized into (S)-(+)- and (R)-(-)-2-ethylhexanoic acid using a freshly prepared suspension of silver(I)-oxide. The products were purified by bulb-to-bulb distillation. Analysis by GC indicates a chemical purity of > 99% of the trimethylsilyl derivatives of both enantiomers, produced by reaction with MSTFA.

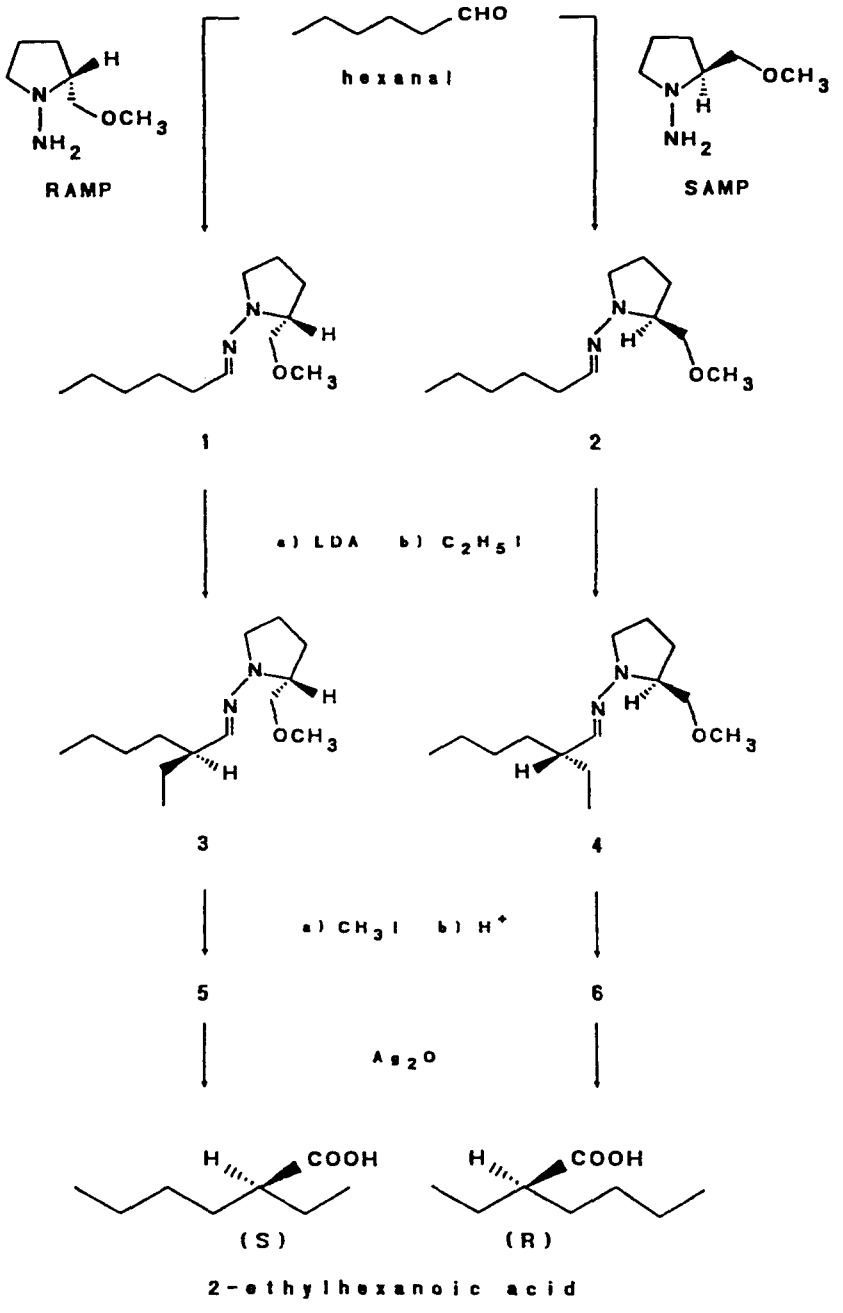

FIG. 1

Pathways for the enantioselective synthesis of (S)- and (R)-2-ethylhexanoic acid

(2R)-2-methoxymethyl-1-(hexylidenamino)pyrrolidine 1
^{1}H-NMR($CDCl_3$): δ = 0.90 (t, J = 7 Hz, 3H, 6'-H), 1.36 (mc, 6H, 3'-H, 4'-H, 5'-H), 1.88 (mc, 4H, 3-H, 4-H), 2.20 (m, 2H, 2'-H), 2.74 (m, 1H, 5-H_a), 3.32-3.50 (m, 3H, 5-H_b, OCH_2), 3.38 (s, 3H, OCH_3), 3.58 (m, 1H, 2-H), 6.66 (t, J = 7 Hz, 1H, 1'-H).

(2S)-2-methoxymethyl-1-(hexylidenamino)pyrrolidine 2
The ^{1}H-NMR($CDCl_3$) spectrum is identical with 1.

(2R,2'S)-2-methoxymethyl-1-(2-ethylhexylidenamino)pyrrolidine 3
^{1}H-NMR($CDCl_3$): δ = 0.90 (m, 6H, 6'-H, 2"-H), 1.36 (mc, 8H, 3'-H, 4'-H, 5'-H, 1"-H), 1.88 (mc, 4H, 3-H, 4-H), 2.08 (m, 1H, 2'-H), 2.74 (m, 1H, 5-H_a), 3.32-3.50 (m, 3H, 5-H_b, OCH_2), 3.38 (s, 3H, OCH_3), 3.58 (m, 1H, 2-H), 6.46 (d, J = 7 Hz, 1H, 1'-H).
$[\alpha]_D^{20} = +93.9^0$ (c = 0.88, C_6H_6)

(2S,2'R)-2-methoxymethyl-1-(2-ethylhexylidenamino)pyrrolidine 4
The ^{1}H-NMR($CDCl_3$) spectrum is identical with 3.
$[\alpha]_D^{20} = -93.8^0$ (c= 0.87, C_6H_6)

(S)-2-ethylhexanoic acid (16)
$[\alpha]_D^{22} = +7.2^0$ (c = 2.00, $CHCl_3$)

(R)-2-ethylhexanoic acid (16)
$[\alpha]_D^{22} = -7.4^0$ (c = 2.20, $CHCl_3$)

Determination of the optical purity

For determination of the enantiomeric purity, the chiral carboxylic acids were transformed into the diastereomeric (S)-(-)-1-phenethylamide derivatives, which were analysed by GC. The racemate served as standard:
(R)-(-)-2-ethylhexanoic acid: 86 ee [≈ 93 % (R)];
(S)-(+)-2-ethylhexanoic acid: 80 ee [≈ 90 % (S)].

Animal Experiments

Female mice (Han: NMRI) were mated between 6.00 and 9.00 a.m. The first 24 hours after conception were day 0 of gestation. The mice were fed an Altromin 1324 diet and given access to water ad libitum. Controlled conditions were maintained (room temperature 21 ± 1°C, air moisture 50 ± 5%), a 12 hour light-dark cycle was employed with light from 10.00 a.m. - 10.00 p.m. and darkness from 10.00 p.m. - 10.00 a.m. The substances were injected 4 x i.p. as their sodium salts each morning and evening of day 7 and 8 between 7.00 and 9.00 a.m., and between 7.00 and 9.00 p.m. Also (±)-EHXA was injected 1 x i.p. as the sodium salt in the morning of day 8. Solutions of 50 mg/ml (of the acid) were administered as the sodium salt at 10 ml/kg for a dosage of 500 mg/kg body weight (3.0 mmol/kg). On day 18 of gestation the number of implantations as well as the embryolethality (resorptions and dead fetuses) were determined, every living fetus was weighed individually and examined for exencephaly.

Results and Discussion

We have previously established a mouse model, where single injections of carboxylic acids or analogs were administered as the sodium salt during the morning of day 8 of gestation. The neural tube defect exencephaly was a highly sensitive indicator for the teratogenic action of this class of compounds administered during this early organogenesis period

(14,18). In this teratologic assay the administration of 500 mg/kg of the (±)-EHXA produced an exencephaly rate of only 5% which was increased to 32% using a multiple administration regimen (TAB. I).

TABLE I

Teratogenicity of the enantiomers and the racemic mixture of EHXA in mice

Substance	Dose[a] [mg/kg]	Number of litters	Number of live fetuses	Embryo-[b] lethality [%]	Exencephaly[c] [%]	Fetal[d] weight [g]
(R)-EHXA	4 x 500	17	172	11	59[e]	1.00 ± 0.05[f]
(S)-EHXA	4 x 500	9	100	1	1	1.16 ± 0.10
(±)-EHXA	4 x 500	20	212	10	32	1.01 ± 0.08
(±)-EHXA	1 x 500	14	157	7	5	1.17 ± 0.09
Control[g]		10	126	6	0	1.14 ± 0.05

[a] Single i.p. injection of the sodium salt per kg body weight on the morning of day 8 of gestation or four i.p. injections of the sodium salts per kg body weight on the mornings and evenings of days 7 and 8 of gestation
[b] resorptions and dead fetuses in % of total implants
[c] percentage of live fetuses
[d] mean ± s.d.
[e] significantly different from (S)-EHXA-induced exencephaly rate ($p < 0.005$; χ^2 - test)
[f] significantly different from (S)-EHXA-induced fetal weight retardation ($p < 0.01$; t - test)
[g] 3.0 mmol NaCl/kg

We have studied the stereoselectivity of the teratogenic potency of the two EHXA enantiomers with this multiple dosing regimen. The compounds were injected as the sodium salts during the mornings and evenings of day 7 and 8. (S)-EHXA did not elicit any teratogenic or embryotoxic response above control rates: the fetal weights and embryolethality were normal, and only 1 out of 100 living fetuses exhibited exencephaly (which was within the background incidence of 0-1.5% in our strain of mice). In sharp contrast, (R)-EHXA induced a potent teratogenic response with 59% of the living fetuses exhibiting exencephaly; there was also a reduction of fetal weight; the exencephaly rate induced by (±)-EHXA was between those of the two enantiomers (TAB. I). Because of the high stereoselectivity of the teratogenicity of EHXA it is likely that the interaction of the enantiomers with chiral molecules (e.g. proteins) in the embryo may play a decisive role; also, possible differences in the pharmacokinetics of the two enantiomers may be important. These questions are presently under active investigation.

In conclusion we have found a strong enantioselectivity for the teratogenic action of EHXA: (R)-EHXA was a potent teratogen, while (S)-EHXA did not show any activity when administered with the same dosing regimen. To our knowledge, this is the first demonstration of high stereoselectivity of a teratological response induced by an environmental compound. We suggest that the safety of man-made chemicals can be improved by the synthesis and use of the less toxic enantiomer.

Acknowledgements

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