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Synthesis and anti-inflammatory effect of lipophilic derivatives of threo-DL-phenylserine in rat experimental edema

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Novel N- and O-acyl derivatives of threo-DL-phenylserine containing lipophilic long chain carboxylic or aryl(sulfon)amide groups were synthesized. Anti-inflammatory activity in carrageenin-induced paw edema model in rats was studied. Compound **10** had the most expressed anti-inflammatory effect over 24 h.

Recently we have accomplished the synthesis of new derivatives of threo-DL-phenylserine, which revealed anti-inflammatory activity (Dirvianskyte et al. 2002; Straukas et al. 2002). In continuation of this project we performed the synthesis of novel N- and O-acyl derivatives containing lipophilic long chain carboxylic and/or aryl(sulfon) amide groups since recent reports demonstrated that the arylamide function (Caliendo et al. 2001) and long chain amino-alcohols (Magrioti et al. 2003) show activity on COX-1 and COX-2 in the carrageenin-induced rat paw edema model. The latter is a classic model of acute inflammation used in pharmacologic studies (see, for example, Ikeda et al. 2001; Lazzarini et al. 1996; Raymond et al. 1997; Wallace et al. 1998). This model is the most robust predictor of the clinical potency of NSAIDs and other inflammatory compounds (Murkherjee et al. 1996; Raymond et al. 1997) and is clearly the best predictor of human dose (Murkherjee et al. 1996). Seven new compounds **7–13** were screened for anti-inflammatory activities in the carrageenin-induced rat paw edema test.

Compounds **7–13** were synthesized from the corresponding amino acid derivatives **1–6**. A series of N-acyl and sulfonyl derivatives containing aromatic substituents **8–13** was obtained by acylation of phenylserine, its esters and O-acylated derivatives upon direct action with corresponding aryl and arylsulfonyl chlorides, respectively at 0–5 °C (Scheme). However, acylation with picolinic chloride proceeded only in the presence of dicyclohexylcarbodiimide (DCC) to give compound **7**. The reactions proceeded smoothly and resulted in high yield (65–75%), except for compound **11** (55%). Recrystallization from ethanol or a mixture of ethanol-ether, and for the lipophilic compounds **9, 11** and **12**, **13** from ether-hexane afforded pure compounds. IR and ^{1}H NMR spectral and elemental analysis data confirmed structures of the synthesized compounds.

The results of the anti-inflammatory investigation are summarized in the Table. All tested preparations reduced carrageenin-induced paw edema (Table), at all observation times and the obtained results prompted us to screen the compounds for their antiinflammatory action. Compared with control and acetylsalicylic acid and compound **10** had the most expressed statistically significant anti-inflammatory effect over a period of 24 h. The intensity of paw swelling was suppressed by 51.73 ($P < 0.02$) at the end of investigation. Compound **12** was also active and had a significant effect on the ascending (1 h) and descending (6 and 24 h) phases of the local inflammatory reaction and inhibited paw edema by 40.95 ($P < 0.01$) at the end of investigation (24 h). It should be noted that compounds **8, 9, 11** and **13** also showed significant anti-inflammatory effect at the same phases of investigation but the inhibition percent was lower (Table).

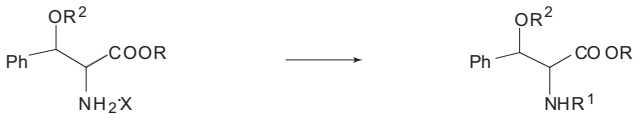
Thus, the current study using the inflammatory model of carrageenin paw edema demonstrates that compounds **10** and **12** showed the most expressed anti-inflammatory effect. These findings are valuable and compound **10** that exerts the most anti-inflammatory action might be of interest for further examination and designing new oral NSAIDs.

Experimental

1. Chemistry

M.p.'s were determined in open capillaries and are uncorrected. IR spectra were obtained in KBr on Perkin Elmer Spectrum BX spectrometer, ^{1}H NMR spectra – on a Hitachi R-22 spectrometer (90 MHz, Japan) using HMDS as an internal reference. Chemical shifts δ are reported in ppm, coupling constants (J) are given in Hz. Multiplicity of signals is expressed as s (singlet) or bs (broad singlet), d (doublet), dd (doublet of doublets), t (triplet), q (quadruplet), m (multiplet). Microanalyses of the synthesized compounds for C, H, N, and S were within ± 0.4% of the calculated values.

Scheme



threo-DL

	R	R^2	X
1	H	H	–
2	Et	H	HCl
3	$(CH_2)_7Ch_3$	H	p-$CH_3C_6H_4SO_3H$
4	$(CH_2)_{13}CH_3$	H	p-$CH_3C_6H_4SO_3H$
5	Et	$CO(CH_2)_{12}CH_3$	HCl
6	Et	$COCH_2C_6H_5$	HCl

	R	R^1	R^2
7	Et	COC_5H_4N	H
8	H	$SO_2C_{10}H_7$	H
9	$(CH_2)_7CH_3$	$SO_2C_{10}H_7$	H
10	Et	$SO_2C_6H_4NHCOCH_3$	H
11	$(CH_2)_{13}CH_3$	$SO_2C_6H_4NHCOCH_3$	H
12	Et	$COCH_2C_6H_5$	$CO(CH_2)_{12}CH_3$
13	Et	$CO(CH_2)_{12}CH_3$	$COCH_2C_6H_5$

Table: Antiinflammatory effects of compounds 7–13 on carrageenin-induced paw edema in rats

Compd.	n	Dose (mg/kg)	% inhibition of edema induced by carrageenin (deviation from control)					
			1 h post injection	2 h post injection	3 h post injection	4 h post injection	6 h post injection	24 h post injection
7	6	110	-54.93^{++}	-41.21^{++}	-34.07^{++}	-30.06^{*}	−22.80	-51.73^{++}
8	6	110	−38.53	−22.28	−7.18	−8.55	−7.71	−35.82
9	10	90	-16.83^{*}	−24.65	−20.00	-19.39^{++}	-21.39^{***}	-24.70^{**}
10	9	90	-24.85^{++}	−22.67	-28.67^{+}	-22.61^{**}	-21.96^{++}	-31.30^{++}
11	11	90	-18.94^{+}	−24.14	−13.47	−13.33	-19.31^{*}	-28.87^{*}
12	10	90	-25.68^{*}	−8.05	-18.70^{*}	−9.21	-25.68^{***}	-40.95^{**}
13	10	90	-37.03^{+}	−21.15	−14.56	−15.90	-19.89^{**}	-25.21^{**}
(ASA)	6	140	0	−28.84	−37.50	−27.69	−16.00	−18.50

Note: The values are significantly different from each control value:
$^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{+}P < 0.02$; $^{++}P < 0.002$.

threo-DL-Phenylserine (**1**) was purchased from Chemapol, Czech Republic, and esters **2–4** were synthesized by adding ethanol under reflux in the presence of gaseous HCl, and octanol or tetradecanol in presence of *p*-toluenesulfonic acid in benzene, respectively.

1.1. N-Picolinyl-threo-DL-phenylserine ethyl ester (**7**)

To a stirred solution of 10 mmol of threo-DL-phenylserine ethyl ester hydrochloride **2**, 11 mmol of picolinic acid in 10 ml of anh. pyridine was added 11 mmol of DCC at 0 °C. The reaction mixture was kept overnight in a refrigerator. The precipitate formed was filtered off and the solution poured into ice water. The product was filtered off, washed with water and recrystallized from ethanol. ($C_{17}H_{18}O_4N_2$): yield 2.1 g (68%), m.p. 118–120 °C); IR ν, cm^{-1}: 1660, 1740, 3310, 3380. ^{1}H NMR ($(CD_3)_2CO$) δ: 1.15 (3 H, t, J = 7.0, CH_3), 4.1 (2 H, q, J = 7.0, CH_2), 4.9 (1 H, dd, J = 4.0, α-CH); 5.2 (1 H, d, J = 5.0, OH), 5.4 (1 H, d, J = 3.5, β-CH); 7.0–8.0 (9 H, m, aromatic signals), 8.5 (1 H, d, J = 4, NH).

1.2. N-Arylsulfonyl-threo-DL-phenylserine esters (**8–11**).

To a stirred mixture of 10 mmol threo-DL-phenylserine (**1**) or ethyl ester hydrochloride (**2**), toluenesulfonates of threo-DL-phenylserine 1-octyl (**3**) and 1-tetradecyl (**4**) esters, respectively, and 20 mmol of 1 N KOH solution or 3.1 ml (22 mmol) of triethylamine in 30 ml of chloroform a solution of 10 mmol of the corresponding arylsulfonyl chloride in 10 ml of $CHCl_3$ was added dropwise over 10 min at 0–5 °C. The reaction mixture was refluxed for 2 h, and then cooled. Chloroform was added and the organic layer was separated and washed with water, 1 N HCl, and water, and dried over anh. $MgSO_4$. The solvent was evaporated *in vacuo* and the solid residue recrystallized to yield pure arylsulfonyl derivatives.
8 ($C_{19}H_{17}O_5NS$): yield 2.5 g (67%), m.p. 171 °C (ethanol-ether); IR ν, cm^{-1}: 1334, 1717, 3030, 3263. ^{1}H NMR (CD_3OD) δ: 4.1 (1 H, d, J = 3.5, α-CH); 4.75 (1 H, s, OH); 5.5 (1 H, d, J = 3.5, β-CH); 6.8–8.1 (12 H, m, aromatic signals).
9 ($C_{27}H_{33}O_5NS$): yield 3.38 g (70%), m.p. 98–99 °C (ether-hexane); IR ν, cm^{-1}: 1333, 1709, 3326, 3503. ^{1}H NMR ($(CD_3)_2CO$) δ: 0.95 (3 H, m, CH_3), 1.1–1.4 (12 H, m, $(CH_2)_6$), 3.6 (2 H, q, J = 7.0, CH_2O), 4.15 (1 H, d, J = 4.0, α-CH); 5.05 (1 H, dd, J = 4.0, β-CH); 6.9–8.2 (12 H, m, aromatic signals).
10 ($C_{19}H_{23}O_6N_2S$): yield 2.5 g (62%), m.p. 150–2 °C (ethanol-ether); IR ν, cm^{-1}: 1324, 1666, 1753, 3330, 3367. ^{1}H NMR ($(CD_3)_2CO$) δ: 1.1 (3 H, t, J = 7.0, CH_3); 2.25 (3 H, s, CH_3CO); 4.0 (2 H, q, J = 7.0, CH_2O); 4.2 (1 H, dd, J = 5.0, α-CH); 5.0 (1 H, d, J = 4.5, OH); 5.1 (1 H, d, J = 4.5, β-CH); 6.6 (1 H, d, J = 10.0, NH); 7.4–7.9 (9 H, m, aromatic signals); 9.5 (1 H, br s, NHCO).
11 ($C_{31}H_{46}O_6N_2S$): yield 3.15 g (55%), m.p. 91 °C; IR ν, cm^{-1}: 1373, 1690, 1763, 3337, 3450; ^{1}H NMR ($(CD_3)_2CO$) δ: 1.0 (3 H, m, CH_3); 1.3–1.5 (24 H, m, $(CH_2)_{12}$); 2.2 (3 H, s, CH_3), 4.1 (2 H, t, J = 4.0, CH_2,); 4.2 (1 H, dd, J = 4.0, α-CH); 5.2 (1 H, d, J = 5.0, β-CH); 7.6–7.9 (9 H, m, aromatic signals); 8.6 (1 H, br s, NH).

1.3. Synthesis of N,O-acyl threo-DL-phenylserine ethyl ester (**12**, **13**)

A mixture of 10 mmol of *O*-myristoyl or phenylacetyl-threo-DL-phenylserine ethyl ester hydrochloride **5**,**6** (Dirvianskyte et al. 2003) and 13 mmol of phenylacetyl or myristoyl chloride, respectively, in 20 ml of anh. 1,2-dichloroethane was heated until the compounds dissolved. The reaction mixture was cooled to room temperature and 23 mmol of triethylamine was added dropwise. The reaction mixture was refluxed for 2 h, then cooled, washed with water, 5% $NaHCO_3$ solution, and water. The organic layer was separated, dried over anh. $MgSO_4$ and the solvent was evaporated *in vacuo*.
12 ($C_{33}H_{47}O_5N$): yield 4.05 g (75%), m.p. 59–61 °C; IR ν, cm^{-1}: 1663, 1735, 3310; ^{1}H NMR (CF_3COOH) δ: 0.5 (3 H, m, CH_3); 0.8 (3 H, m, CH_3), 0.9–1.1 (22 H, m, $CH_2)_{11}$, 2.0 (2 H, t, J = 7, $COCH_2$); 3.3 (2 H, s, CH_2-Ar), 3.9 (2 H, q, J = 7.0, CH_2O), 4.8 (1 H, dd, J = 4.0, α-CH); 5.9 (1 H, d, J = 4.5, β-CH); 6.7–7.1 (11 H, m, aromatic signals and NH).
13 ($C_{33}H_{47}O_5N$): yield 3.85 g (72%), m.p. 67–69 °C; IR ν, cm^{-1}: 1660, 1735, 3310; ^{1}H NMR (CF_3COOH) δ: 0.5 (3 H, m, CH_3); 0.8 (3 H, m, CH_3), 0.9–1.0 (22 H, m, $CH_2)_{11}$, 2.0 (2 H, t, J = 7, $COCH_2$); 3.4 (2 H, s, CH_2-Ar), 3.8 (2 H, q, J = 7.0, CH_2O), 4.8 (1 H, dd, J = 4.0, α-CH); 6.0 (1 H, d, J = 4.5, β-CH); 6.7–7.0 (10 H, m, aromatic signals), 7.2 (1 H, d, J = 10, NH).

2. Pharmacology

2.1. Animals

Experiments were performed on 90 male Wistar rats, initially weighing 200–250 g. The animals were purchased from "Bioreglament" (Vilnius, Lithuania) and kept under standardized conditions. Food and water were provided *ad libitum*. After a resting period of one week, animals were subjected to the experimental protocol. All experimental and control groups consisted of 6 to 11 animals.
Approval of the respective institutional ethic committee for Laboratory Animal Use was obtained prior to commencement of the experiment.

2.2. Induction of carrageenin inflammation

A 1% solution of carrageenin (Sigma Chemical Co) prepared in sterile saline was used to induce the paw edema (Lazzarini et al. 1996). The rats were anesthetized with Apaurin® (KRKA) and carrageenin was injected subplantarly (0.1 ml/rat) into the left hind paw. The volume of induced edema was mesured immediately before and 1, 2, 3, 4, 6 and 24 h after carrageenin injection to determine the differences in paw volumes.

2.3. Test compounds and investigation procedures

Compounds **7–13** were used throughout the study. All preparations were prepared as fine homogenized suspension in 1% starch gel and administered orally in doses 110 mg/kg **7**, **10** and 90 mg/kg **8**, **9**, **11–13** in 1 ml volume to the experimental animals 2 h before the induction of carrageenin edema. Each experimental protocol included an equivalent number of control animals that received the same volume of starch gel. The effect of compounds was also compared with the action of acetylsalicylic acid (ASA). The antiedematous effects of the compounds were estimated in terms of percent inhibition from control groups.

2.4. Statistical analysis

The results were expressed as mean values ± S.E.M. Differences between control and tested groups were statistically analyzed by Student's t test with $P < 0.05$ considered as significant. The percentage of deviation from the control group was derived by the following formula: $(T\text{-}C)/C \times 100$, where T is the data on the tested group and C is the data on the control.

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Identification and determination of invertase secreted by tomato cells

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A simple and rapid procedure for the identification and determination of extracellular invertase from a culture medium of tomato cell suspension cultures is described. Sucrose was used as substrate for the determination of the extracellular and intracellular activities of the enzyme. The culture medium (without cells) was used for identification and determination of extracellular enzyme activity. Intracellular activity was estimated from the cell suspension.

Glycosidases are involved in several important biological processes such as digestion, biosynthesis of glycoproteins, and catabolism of glycoconjugates.

Invertase (β-D-fructofuranosidase EC 3.2.11.26, saccharase called also sucrase) catalyses the hydrolysis of sucrose to glucose and fructose under concentrations lower than 10% (wt./vol.) and has transfructosylating activity under sucrose concentrations higher than 10% (wt./vol.) (Rubio et al. 2002). The enzyme is an important industrial product with applications in the production of non-crystalizable invert sugars and soft-centered chocolates (Wiseman 1979). Invertase has been widely studied especially in yeast and fungi (Costalioli et al. 1997; Romero-Gómez et al. 2000).

The development of a new method for identification and determination of biocatalysts is highly connected with progress of biotechnological processes. Although invertase is generally present in plants, this source has not used previously.

It is the aim of this paper to report on our investigation concerning the hydrolysis of sucrose by intra- and extracelullar invertase from tomato cells. Sucrose was used for the study of intracelullar and extracelullar invertase activity.

Homogenized cell suspension cultures and culture medium alone after 7 days cultivation were used for assaying the activity of intracellular and extracellular invertase. The distribution of intracellular and extracellular enzyme activity is shown in the Table.

The data indicate an 90% intracellular and 10% extracellular distribution of the enzyme activity tested. The intracellular specific enzyme activity is 2.5 times higher.

The production of extracellular galactosidases as well as proteolytic enzymes (Mulinami and Devendra 1999; Stano