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Synthesis of salicyl-peptides and their effect on human platelet aggregation *in vitro*

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Summary. A series of leucine dipeptide amides containing at their N-terminal amino group the salicyl-residue [(o)-RO-C_6H_4-CO-, where R=H or CH_3CO] have been synthesized by conventional solution techniques and tested for their inhibitory activity on human platelet aggregation *in vitro* induced by collagen, ADP or adrenaline. The salicyl-peptide (o)-HO-C_6H_4-CO-Leu-Asp(OBzl)-NH_2 was found to exert strong inhibitory activity on platelet aggregation induced by collagen with an IC_{50} value 4.5 mM. The corresponding dipetide H_2N-Leu-Asp(OBzl)-NH_2 was also examined and was found to be less active, indicating that the presence of the lipophilic β-benzyl ester group in combination with the salicyl group enhance the inhibitory activity. All the other salicyl-peptides examined either didn't show any inhibitory or aggregatory activity or a slight inhibition at the concentration of 9–10 mM.

Keywords: Amino acids – Collagen – Inhibitory activity – Platelet aggregation – Salicyl-peptides

Abbreviations: The abbreviations used are in accordance with the rules of IUPAC-IUB Joint Commission on Biochemical Nomenclature: Eur J Biochem (1984) 138: 9–37; J Biol Chem (1989) 264: 663–673. Additional abbreviations are: ADP, adenosine diphosphate; Boc, *tert*-butyloxycarbonyl; Bzl, benzyl; DIEA, N,N-diisopropylethylamine; DMSO, dimethyl sulfoxide; DMF, N,N-dimethylformamide; EtOAc, ethyl acetate; FCC, flash column chromatography; IC_{50}, molar concentration of salicyl-peptide for 50% inhibition of platelet aggregation; iso-BCF, isobutyl chloroformate; NMM, N-methylmorpholine; PyBOP, (benzotriazol-1-yloxy) tripyrrolidinophosphonium hexafluorophosphate; TLC, thin layer chromatography; TMS, tetramethylsilane

Introduction

Anticoagulants are effective agents in preventing blood clotting in the vein, while thrombolytic agents cause blood clots dissolution. On the arterial side during blood circulation clotting occurs by platelet aggregation. In general, the antithrombotic strategies are based on the inhibition of platelet aggregation and adhesion by blocking the primary stimulus caused by several agonists like thrombin, ADP, collagen etc. Inhibition of an agonist function by anticoagulant materials prevents the blood clotting and maintains its fluidity (Ojima et al., 1995; Das et al., 1995). The 65 amino acids peptide hirudin, secreted from the salivary gland of medicinal leech (Owen et al., 1988), as well as the synthetic 20 amino acids peptide hirulog-1 (Witting et al., 1992) are direct-acting potent inhibitors of thrombin. Smaller peptides, like the hirudin C-terminal fragments have been tested for inhibition of cell attachment (Owen et al., 1988; Krstenansky et al., 1987; Muramatsu et al., 1996). Progressively, studies on even smaller peptides led to the tetrapeptide Arg-Gly-Asp-Ser (RGDS), corresponding to the cell attachment site of fibronectin, which inhibits platelet aggregation stimulated by collagen and ADP (Pierschbacher et al., 1984; Gartner et al., 1985). Further investigation has shown that the amino acid sequence Arg-Gly-Asp (RGD) is the minimum requirement for cell attachment and cell recognition site of extracellular matrix and platelet adhesion proteins (Ruoslahti et al., 1987). The property of RGD-containing peptides to inhibit platelet aggregation and thrombus formation *in vitro* established them as antithrombotic materials. Extensive structure-activity and conformational studies on RGD-containing peptides have been performed on the pharmacophore group and have led to the development of cyclic analogues (Nishino et al., 1996) and non-peptidic mimetics of the RGD sequence (Callahan et al., 1992; Ku et al., 1993). In general a long-term therapy for prevention of myocardial infarction requires oral dosing of anticoagulant, so the trend is to search for small peptides or mimetics to be used as antithrombotic drugs. Furthermore, other non-peptidic molecules, like warfarin (a coumarin derivative) and aspirin are indirect-acting anticoagulants and they are currently been used for preventing myocardial infarction (Das et al., 1995; Weiss et al., 1967; Baenziger et al., 1977).

In our previous publications we have shown that several di- and tripeptide amides containing aspartic acid or asparagine in their sequence, inhibited platelet aggregation stimulated by collagen, ADP or adrenaline (Liakopoulou-Kyriakides et al., 1990; Liakopoulou-Kyriakides et al., 1992). Those observations prompted us to further investigate the inhibitory activity of such dipeptides coupled to salicylic acid. We therefore report here on the synthesis and biological activity as platelet aggregatory agents *in vitro* of some salicyl-peptides, that is, dipeptides which have been coupled with salicylic acid or acetylsalicylic acid at their N-terminal amino acid.

Materials and methods

All amino acids and derivatives used in this work are of the S-configuration and are purchased from MERCK KGaA (Germany), while all aggregating agents used including

collagen, ADP and adrenaline were purchased from Sigma Chem. Co (Germany). The solvents used were analytical grade. Elemental analyses were performed on a Perkin-Elmer 240 Elemental Analyser and they were within ±0.40% of the calculated values. Capillary melting points were determined on a Buchi 530 apparatus and are reported uncorrected. Optical rotations were measured with a Carl-Zeiss precision polarimeter (0.005°). IR spectra were recorded as KBr pellets on a Perkin-Elmer 16PC FT-IR spectrophotometer. ^{1}H NMR spectra were obtained at 400.13 MHz on a Bruker instrument using $CDCl_3$ or DMSO-d_6 as solvent and TMS as internal standard. Analysis by TLC was performed on precoated plates of silica gel 60 F_{254} (MERCK) with the following solvent systems (ratio by volume) (**1**), CH_2Cl_2/CH_3OH (9:1); (**2**), EtOAc/CH_3OH (8/2); (**3**), $CH_2Cl_2/CH_3OH/CH_3COOH$ (85/10/5). Spots on TLC plates were detected by UV light, with ninhydrin solution and/or by chlorination followed by a solution of 1% starch/1% KI (1:1 v/v).

Biological assays

Platelet aggregation was studied by a conventional photometric technique with a Dual Channel Coulter Electronic Aggregometer, at 37°C, with continuous recording of light transmission, according to the method of Born (Born, 1962). The aggregation agents used were at a final concentration of 60 μg/ml for collagen, 10 mM for ADP and 1 mM for adrenaline. Platelets were obtained from venous blood of healthy donors who had not taken any medication during the week preceding blood collection. The blood was immediately mixed at a 9:1 ratio with 3.8% sodium citrate solution and was centrifuged at 200 g for 5 min to yield platelet rich plasma.

Synthesis of Boc-Leu-Asp(OBzl)-NH_2

Boc-Asp(OBzl)-NH_2 (Karagiannis, 1993) (3.22 g, 10 mmol) was treated with CF_3COOH/anisole (10:1 v/v) for 1 h at room temperature. Subsequently the solvent was evaporated and the oily residue was solidified by addition of dry ether, filtered and washed on the filter with dry ether and dried over P_2O_5/KOH under *vacuo*. The CF_3COOH·H-Asp(OBzl)-NH_2 was dissolved in DMF (15 mL), chilled at 0°C and neutralized with NMM (1.23 mL, 11 mmol). To a solution of Boc-Leu-OH·H_2O (2.99 g, 12 mmol) in DMF (15 mL), cooled at −10°C, NMM (12 mmol) and *iso*-butyl chloroformate (12 mmol) were added. After 3 min the neutralized solution of H·Asp(OBzl)-NH_2 was added into the mixed anhydride of Boc-Leu-OH and the reaction mixture was stirred for 1.5 h at −10°C and for 1 h at room temperature. Subsequently the solvent was evaporated *in vacuo* and the residue was partitioned in EtOAc-H_2O. The organic layer was washed alternatively with 5% citric acid solution, water, 5% $NaHCO_3$ and water and dried (Na_2SO_4). Evaporation of the solvent and crystallization from EtOAc-hexane yielded 3.57 g (82%) of Boc-Leu-Asp(OBzl)-NH_2, m.p. 124–125°C, $[\alpha]_D^{25}$–40.4 (c1, DMF). Anal. ($C_{22}H_{33}O_6N_3$), C, H, N.

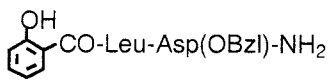
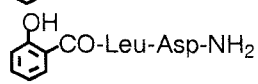
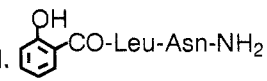
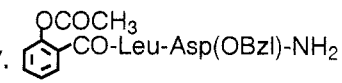
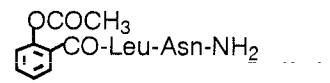
Synthesis of Boc-Leu-Asn-NH_2

The synthesis of the dipeptide Boc-Leu-Asn-NH_2 is described in Liakopoulou-Kyriakides (1992).

*Synthesis of HO-C_6H_4-CO-Leu-Asp(OBzl)-NH_2 (**I**)* (General procedure)

Boc-Leu-Asp(OBzl)-NH_2 (2.18 g, 5 mmol) was dissolved in 1.2 M HCl/CH_3COOH for 1 h at room temperature. The solvent was evaporated *in vacuo* and the residue was treated

Table 1. Yields and physical constants of salicyl-peptides

	Yield %	mp °C	$[\alpha]_D^{27}$ (c 0.5, DMF)	TLC			IR cm^{-1}
				Rf_1	Rf_2	Rf_3	
I. 	73	93–95	−22	0.46	0.51		3412 OH, 3318 CONH, 1731 COOR, 1693 CO
II. 	83	179–182	−33		0.10	0.71	3406 OH, 3265 CONH, 1740 COOH, 1682 CO
III. 	65	198–200	8.9	0.14	0.36		3408 OH, 3206 CONH, 1672 CO
IV. 	88	145–147	−27	0.50	0.40		3294 CONH, 1766 ArOCOR, 1,734 COOR
V. 	76	175–177	−24	0.16	0.30		3292 CONH, 1764 ArOCOR, 1660 CO

Rf_1 $CH_2Cl_2/CH_3OH(9:1)$; *Rf_2* $CH_3COOC_2H_5/CH_3OH$ (8:2); *Rf_3* $CH_2Cl_2/CH_3OH/CH_3COOH$ (85:10:5).

Table 2. ^{1}H NMR chemical sifts δ(ppm) for the compounds **I** and **IV**

Compound **I**	$N_\alpha H$	$C_\alpha H$	$C_\beta H$	$C_\gamma H$	$C_\delta H$	CH_2	C_6H_5	$CONH_2$	OH	C_6H_4
Asp	7.49 (J = 8.06 Hz)	4.81	3.00 2.77	–	–	5.09	7.32	6.61 5.80	–	–
Leu	7.22 (J = 6.00 Hz)	4.63	1.72	1.67	0.93	–	–	–	–	–
Salicyl	–	–	–	–	–	–	–	–	11.88	6.81–7.45
Compound **IV**	$N_\alpha H$	$C_\alpha H$	$C_\beta H$	$C_\gamma H$	$C_\delta H$	CH_2	C_6H_5	$CONH_2$	$COCH_3$	C_6H_4
Asp	7.36 (J = 7.96 Hz)	4.82	3.02 2.82	–	–	5.10	7.32	6.68 5.23	–	–
Leu	6.76 (J = 6.97 Hz)	4.55	1.77	1.58	0.97	–	–	–	–	–
Salicyl	–	–	–	–	–	–	–	–	2.33	7.13–7.79

with dry ether, filtered, washed on the filter with dry ether and dried over P_2O_5/KOH under *vacuo*. Subsequently the salt HCl·H-Leu-Asp(OBzl)-NH_2 was dissolved in DMF (10 mL) together with salicylic acid (0.69 g, 5 mmol), cooled at 0°C and PyBOP (2.60 g, 5 mmol) was added followed by the addition of DIEA (2.7 mL, 15.5 mmol). The reaction mixture was stirring for 30 min at 0°C and for 3 h at room temperature during which the pH was adjusted at 7.5–8.0 by addition of DIEA. The solvent was evaporated and the oily residue was directly chromatographed on FCC (silica gel 230–400 mesh, MERCK) using EtOAc/$PhCH_3$ (8:1, v/v) as eluent to afford pure product. Yield and other physical constants are reported in Table 1, ^{1}H NMR chemical shifts in Table 2. Anal. ($C_{24}H_{29}O_6N_3$), C, H, N.

*Synthesis of HO-C_6H_4-CO-Leu-Asn-NH_2 (**III**)*

Following the same procedure HO-C_6H_4-CO-Leu-Asn-NH_2 (III) was prepared. The product was also purified on a FCC using as eluent CH_2Cl_2/CH_3OH (6:1 v/v). Physical data also appear in Table 1.

*Synthesis of $CH_3COOC_6H_4$-CO-Leu-Asp(OBzl)-NH_2 (**IV**)*

To a solution of compound I (0.23 g, 0.5 mmol) in CH_2Cl_2 (5 mL), NMM (1 mmol) was added followed by $(CH_3CO)_2O$ (0.5 mmol) under vigorous stirring. The reaction progress was monitored by TLC and a new portion of NMM and $(CH_3CO)_2O$ were added to complete the reaction. The reaction mixture was diluted with CH_2Cl_2 and washed with water, 5% $NaHCO_3$ solution, water and dried (Na_2SO_4). Filtration through a folded filter paper and evaporation of the filtrate under reduced pressure provided an oily product which was crystallized from EtOAc-hexane. For physical constants see Table 1 and for ^{1}H NMR chemical shifts see Table 2. Anal. ($C_{26}H_{31}O_7N_3$), C, H, N.

*Synthesis of $CH_3COOC_6H_4$-CO-Leu-Asn-NH_2 (**V**)*

Similar procedure was followed for the synthesis of $CH_3COOC_6H_4$-CO-Leu-Asn-NH_2. For date see Table 1.

*Synthesis of HO-C_6H_4-CO-Leu-Asp-NH_2 (**II**)*

Compound I (0.28 g, 0.5 mmol) was dissolved in methanol and hydrogenated over 10% Pd/C. When the reaction was completed (TLC) the catalyst was removed by filtration, the solvent was evaporated under reduced pressure and the residue was crystallized from CH_3OH-ether. For data see Table 1.

Results and discussion

A series of Leu-Asp(or Asn)-NH_2 dipeptides have previously been examined for their inhibitory activity on human platelet aggregation *in vitro* against collagen, ADP or adrenaline (Liakopoulou-Kyriakides et al., 1990). These peptides were further coupled at their N-terminal amino group with salicyl- or an acetylsalicyl-moiety in an attempt to synthesize more potent inhibitors of platelet aggregation. The idea derives from the concept that enhanced activity may be obtained by combining in the same molecule active compounds with different "backbones", like salicylic acid and a peptide.

The synthesis of the desired compounds was carried out in solution applying techniques of peptide chemistry. The dipeptides were synthesized using the carboxylic-carbonic anhydride method. The same method was found insufficient when applied for incorporation of acetylsalicyl group at the N-terminal amino group of dipeptides. Especially during the incorporation of acetylsalicyl-moiety a side reaction takes place leading to an O → N acetyl group shift, which afforded a mixture of acetyl-dipeptides and salicyl-dipeptides. Thus the N^{α}-acetyl dipeptides were the main products of this undesirable reaction. The O → N acetyl migration was also established by reacting acetylsalicylic acid with leucine methyl ester. The acetyl-leucine methyl ester was isolated in 55% yield. The same product was obtained by treatment of leucine methyl ester with acetic anhydride and identified by TLC, FT-IR and ^{1}H NMR. Using PyBOP (Coste et al., 1990) as the condensing reagent a series of salicyl-peptides was synthesized in very good yields. Examining further the coupling reaction between salicylic acid and peptides it was found that the more efficient reaction route in synthesizing acetylsalicyl-peptides is to precede the synthesis of salicyl-peptides followed by conversion into acetyl-derivatives using acetic anhydride as acylating reagent. Figure 1 shows the synthetic route of (o)-HO-C_6H_4-CO-Leu-Asp(OBzl)-NH_2. Similar synthetic routes were followed for the other salicyl-peptides described here.

All compounds have been purified either by crystallization or by flash column chromatography (FCC). The yields and physical constants of the synthesized salicyl-peptide are summarized in Table 1. ^{1}H NMR data for compounds **I** and **IV** appear in Table 2.

The effect of the salicyl-peptides listed in Table 1 on platelet aggregation was examined in platelet rich plasma collected from venous blood. Compounds **I**, **II** and **IV** were found to inhibit platelet aggregation *in vitro*. Figure 2 shows the inhibition of platelet aggregation by these compounds at

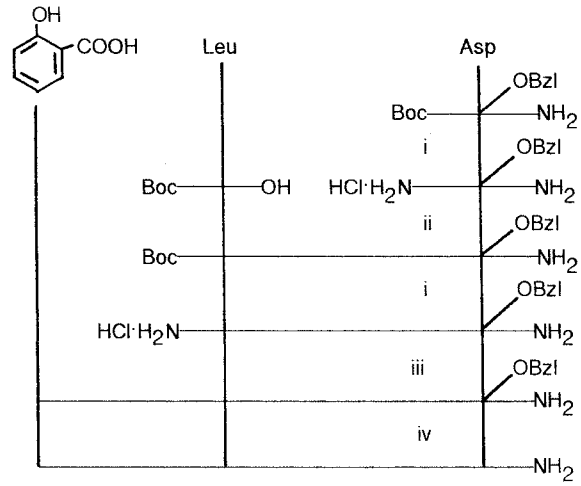


Fig. 1. Synthetic route of salicyl-peptides: **i** 1.2 M HCl/CH_3COOH; **ii** NMM, iso-BCF; **iii** DIEA, PyBOP; **iv** H_2/10%Pd-C

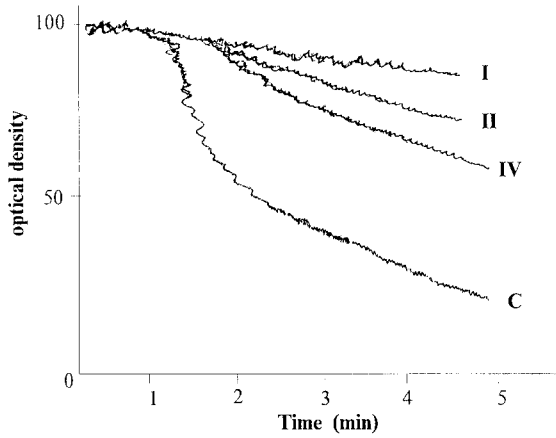


Fig. 2. Effect of salicyl-peptides **I**, **II** and **IV**, at the concentration of 9 mM, on platelet aggregation induced by collagen. *C* control (normal serum + collagen)

the concentration of 9 mM. Compounds **III** and **V** didn't show any inhibition or aggregation at least at the concentrations tested here (9–10 mM). Interesting enough, the derivatives **I** and **II** which do not carry the acetyl group on their salicyl-residue proved to be the more potent inhibitors than derivative **IV** which possesses the acetyl group. Furthermore the presence of the benzyl ester group on the side chain of aspartic acid seems to have considerable contribution to the inhibitory activity, as the compound **II**, which does not possess the benzyl ester is less active than **I**. Compound **IV** which brings both acetyl and benzyl ester groups showed lower inhibition activity than the other salicyl-peptides having aspartic acid at their C-terminal position. Salicyl-peptides incorporating asparagine at the same position are less active (curves not shown) than the derivatives with aspartic acid and in accord with the observations mentioned above compound **V**, having an acetyl group on the salicyl-residue showed the lower activity. The results show that introduction of hydrophilic groups at the side chain of C-terminal amino acid together with the acetyl protection of the hydroxyl group of salicyl-moiety reduces the inhibitory activity. The inhibitory activity of the most potent compound (o)-HO-C_6H_4-CO-Leu-Asp(OBzl)-NH_2 was studied further. Figure 3(a) shows inhibition curves obtained with compound **I** at the concentration of 9 mM(1) and 4.5 mM(2) respectively, against platelet aggregation stimulated by collagen (60 μg/ml). Figures 3(b) and 3(c) show the inhibition curves of compound **I** against platelet aggregation induced by ADP and adrenaline respectively, at the concentration of 9 mM. It is worth mentioning that none of the compounds showed aggregatory activity at least at the tested concentration (10 mM).

The present findings indicate that the combination of independent active compounds in one molecule afforded a potent inhibitor of platelet aggregation induced by collagen, and other compounds with minor antiplatelet activity. The inhibition curves and the IC_{50} value show that the best inhibitory activity is obtained when the hydroxyl group on salicyl-moiety is unprotected

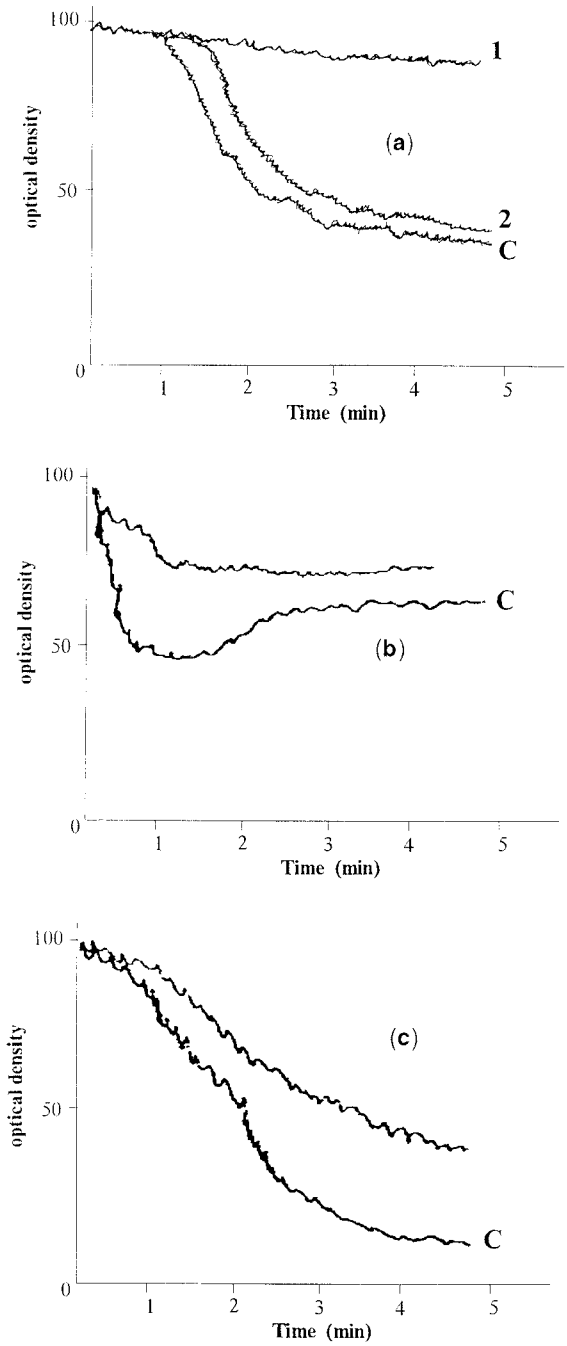


Fig. 3. Effect of salicyl-peptide **I** on platelet aggregation induced by collagen (**a**), ADP (**b**) and adrenaline (**c**). *C* control (normal serum + collagen, ADP or adrenaline). **a***1* Salicyl-peptide **I** at concentration of 9 mM. **a***2*; Salicyl-peptide **I** at concentration of 4.5 mM

and the side chain of the C-terminal amino acid contains an ester group. Further investigation on the amino acid(s) side chain influence as well as the salicylic acid derivatives would bring more information about the biological effect of the salicyl-peptides. These results in addition to the ones reported previously (Liakopoulou-Kyriakides et al., 1990; Liakopoulou-Kyriakides et al., 1992) may be the start for the synthesis of a new class of platelet aggregation inhibitors and we continue to work on this project.

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