J. Pharm. Pharmac., 1967, **19**, 95–101 Received June 27, 1966

An experimental approach to long-lasting hypotensive eledoisin-like peptides

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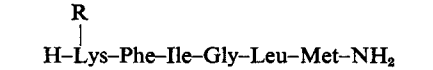
A new series of eledoisin-like peptides was synthesized with the object of obtaining long-lasting hypotensive drugs. Acyl residues were introduced into the molecule of the peptide



They were dissolved in some polar solvents (water, diacetin, triacetin, dimethylsulphoxide), and injected intramuscularly into either anaesthetized or unanaesthetized dogs. Comparison was made with eledoisin dissolved both in water and diacetin. Some of the peptides, mainly the butyryl and valeryl derivatives, in diacetin had a significantly longer-lasting action. The intensity of the hypotension could be also reduced, to avoid side-effects in unanaesthetized dogs, but it was not possible to make it gradual.

PREVIOUS reports (Bergamaschi & Glässer, 1963, 1964; Erspamer & Glässer, 1963; Fregnan & Glässer, 1966) have strongly supported the view that eledoisin exerts its hypotensive action in the dog by a direct vasodilating mechanism on the vascular smooth muscle. Clinical trials (Sicuteri, Fanciullacci, Franchi & Michelacci, 1963; Gersmeyer, Castenholz & Nicolay, 1965) were also successful in indicating that the endecapeptide is a powerful hypotensive vasodilator even in man. Unfortunately its action is short-lasting and abrupt when administered either intramuscularly or intravenously. Synthetic eledoisin-like peptides behaved similarly (Bernardi, Bosisio, Chillemi & others, 1964, 1965). Stürmer & Fanchamps (1965) were able to prolong the hypotension due to eledoisin by the use of a suitable vehicle (Depot-Präparat). We now report on the possibility of prolonging the hypotensive action of some eledoisin-like peptides and making it gradual in onset and disappearance. We felt that by suitable introduction of hydrophobic residues into the molecule of these peptides their distribution between hydrophylic and hydrophobic media could be varied and accordingly the absorption through the tissues could be modified.

For this purpose the hexapeptide Lys-Phe-Ile-Gly-Leu-Met-NH_2 was chosen because it was found to be almost as active as eledoisin and it had two free amine groups suitable for substitution. Lipophilic acyl substituents of different size were introduced into the ω-amino-group of lysine. The α-amino-group of lysine was kept free to provide a hydrophilic residue in the same molecule to influence, almost at will, the lipophilic-hydrophilic properties and solubility of these peptides.

The activity of the new peptides has been compared on a weight basis with that of eledoisin either in diacetin or water.

Experimental

CHEMICAL (de Castiglione)

The appropriate *N*α-t-butyloxycarbonyl-*N*ϵ-acyl-L-lysine was converted into the corresponding *p*-nitrophenyl ester and condensed with

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the pentapeptide L-phenylalanyl-L-isoleucyl-glycyl-L-leucyl-L-methioninamide (de Castiglione, 1965) to afford the protected *N*ε-acyl-hexapeptide. This was treated with hydrogen chloride in glacial acetic acid to eliminate the *N*α-t-butyloxycarbonyl group, and the free base was obtained by exchange with Amberlite IRA-410 (OH cycle).

N*α-t-Butyloxycarbonyl*-N*ε-acyl*-L-*lysine* (Table 1). To an ice-cold solution of *N*α-t-butyloxycarbonyl-L-lysine (Schwyzer, Costopanagiotis

TABLE 1. *N*α-t-BUTYLOXYCARBONYL-*N*ε-ACYL-L-LYSINE

Acyl group	Yield (%)	m.p. °C	$[\alpha]_D^{23}$	Formula	Found C	Found H	Found N	Required C	Required H	Required N
Butyryl ..	45	99–100	−16·4*	$C_{15}H_{28}N_2O_5$	56·7	8·9	8·9	56·9	8·9	8·8
Valeryl ..	40	86– 90	− 3·5†	$C_{16}H_{30}N_2O_5$	58·0	9·0	8·5	58·2	9·2	8·5
Caproyl ..	49	82– 84	−14·4*	$C_{17}H_{32}N_2O_5$	59·2	9·3	8·2	59·3	9·4	8·1
Cyclopentyl-propionyl ..	75	118–119	− 2·9†	$C_{19}H_{34}N_2O_5$	61·7	9·35	7·5	61·6	9·2	7·6
Cinnamyl ..	38	106–107	−12·6*	$C_{20}H_{28}N_2O_5$	63·7	7·4	7·7	63·8	7·5	7·4
Benzoyl ..	69	114–116	− 2·8†	$C_{19}H_{26}N_2O_5$	62·0	7·50	8·0	61·7	7·5	8·0
Nicotinyl ..	18	138–141	−15·5*	$C_{17}H_{25}N_3O_5$	57·9	7·45	11·8	58·1	7·2	12·0
1-Adamantane-carbonyl ..	71‡	187–188‡	+ 3*‡	$C_{22}H_{36}N_2O_5.C_{12}H_{23}N$	69·1	9·8	7·0	69·2	10·1	7·1

* *c*, 1 in *NN*-dimethylformamide.
† *c*, 1 in methanol.
‡ As dicyclohexylamine salt.

& Sieber, 1963) (10 mmole) in N sodium hydroxide (10 mmole) and water (80 ml) were added dropwise and separately, with vigorous stirring, a solution of acyl chloride (10 mmole) in anhydrous tetrahydrofuran (10 ml) and N sodium hydroxide (10 ml) over 20 min. The stirring was continued for 20 min at room temperature. The reaction mixture was then saturated with sodium chloride, cooled below 0° and acidified at pH ~1 with 2N hydrochloric acid. The product was extracted with ethyl acetate and the combined extracts were washed with saturated sodium chloride solution, dried over anhydrous sodium sulphate, and the solvent evaporated *in vacuo* at 40°. Crystallization from ether-light petroleum (b.p. 40–60°) or acetone-light petroleum afforded the pure material.

For the nicotinyl derivative, a solution of *N*α-t-butyloxycarbonyl-L-lysine (15 mmole) in N sodium hydroxide (45 ml) was vigorously shaken with a mixture of crushed ice and nicotinyl chloride (15 mmole). After 1 hr at room temperature the reaction mixture was saturated with sodium chloride and treated with N hydrogen chloride (15 ml). The product was then worked up as before.

p-*Nitrophenyl* N*α-t-butyloxycarbonyl*-N*ε-acyl*-L-*lysinate* (Table 2). *NN*-Dicyclohexylcarbodiimide (5 mmole) was added to an ice-cold solution of *N*α-t-butyloxycarbonyl-*N*ε-acyl-L-lysine (5 mmole) and *p*-nitrophenol (5·5 mmole) in ethyl acetate (15 ml). The reaction mixture was allowed to stand 1 hr at 0° and 2 hr at room temperature. The dicyclohexylurea was filtered after cooling and washed with ethyl acetate. The filtrate was successively washed with N hydrochloric acid (at a temperature below 0°), 5% sodium bicarbonate and saturated sodium chloride solutions. After drying over anhydrous sodium sulphate the

TABLE 2. *p*-NITROPHENYL-*N*α-t-BUTYLOXYCARBONYL-*N*ε-ACYL-L-LYSINATE

Acyl group	Yield (%)	m.p. °C	$[\alpha]_D^{23}$	Formula	Found C	Found H	Found N	Required C	Required H	Required N
Butyryl ..	66	119–120	−29·8*	$C_{31}H_{31}N_5O_7$	57·6	7·1	9·6	57·6	7·1	9·6
Valeryl ..	80	119–120	−30·8†	$C_{32}H_{33}N_5O_7$	58·3	7·4	9·2	58·5	7·4	9·3
Caproyl ..	78	119–121	−26·8*	$C_{33}H_{35}N_5O_7$	58·9	7·6	9·0	59·3	7·6	9·0
Cyclopentyl-propionyl ..	77	150	−28†	$C_{35}H_{37}N_5O_7$	61·1	7·6	8·5	61·1	7·6	8·5
Cinnamyl ..	70	159–160	−22*	$C_{36}H_{31}N_5O_7$	62·4	6·5	8·5	62·8	6·3	8·4
Benzoyl ..	75	140–142	−27†	$C_{34}H_{33}N_5O_7$	61·3	6·3	8·8	61·1	6·2	8·9
Nicotinyl ..	41	109–111	−26·3*	$C_{33}H_{34}N_6O_7$	58·4	6·1	11·9	58·5	6·0	11·9

* *c*, 1 in *NN*-dimethylformamide.
† *c*, 1 in methanol.

solvent was evaporated *in vacuo* and the residue crystallized from acetone-light petroleum (b.p. 40–60°).

For the nicotinyl derivative, *NN*-dimethylformamide was used as solvent instead of ethyl acetate, and no acidic washings were made. The 1-adamantanecarbonyl derivative was isolated as an oil and used as such in the next reaction.

TABLE 3. *N*α-t-BUTYLOXYCARBONYL-*N*ε-ACYL-L-LYSYL-L-PHENYLALANYL-L-ISO-LEUCYL-GLYCYL-L-LEUCYL-L-METHIONINAMIDE

Acyl group	Yield (%)	m.p. °C	$[\alpha]^{23}_D$*	Formula	Found C	Found H	Found N	Found O	Required C	Required H	Required N	Required O
Butyryl ..	92	257–259	−29·5	$C_{48}H_{73}N_9O_9S.\frac{1}{2}H_2O$	58·5	8·2	12·5	17·3	58·3	8·3	12·6	17·1
Valeryl ..	85	~260	−29·3	$C_{44}H_{74}N_9O_9S$	59·4	8·5	12·3		59·4	8·3	12·5	
Caproyl ..	74	258–259	−28·7	$C_{45}H_{76}N_9O_9S$	59·5	8·6	12·3		59·6	8·5	12·4	
Cyclopentyl-propionyl ..	72	263–266	−27·2	$C_{47}H_{76}N_9O_9S$	60·9	8·6	12·0		60·6	8·4	12·0	
Cinnamyl ..	68	~260	−28·6	$C_{48}H_{72}N_9O_9S$	61·7	8·0	11·7		61·5	7·7	12·0	
Benzoyl ..	77	256–258	−29·3	$C_{46}H_{70}N_9O_9S$	60·9	8·1	11·9		60·6	7·7	12·3	
Nicotinyl ..	55	258–260	−28	$C_{45}H_{69}N_9O_9S.\frac{1}{2}H_2O$	58·7	7·8	13·8		58·7	7·7	13·7	
1-Adamantane-carbonyl ..	69	252–254	−25·5	$C_{50}H_{80}N_9O_9S.\frac{1}{2}H_2O$	61·6	8·3	11·3	15·8	61·4	8·4	11·4	15·5

* *c*, 1 in *NN*-dimethylformamide.

Nα-*t-Butyloxycarbonyl*-Nε-*acyl*-L-*lysyl*-L-*phenylalanyl*-L-*isoleucyl-glycyl*-L-*leucyl*-L-*methioninamide* (Table 3). A solution of *p*-nitrophenyl-*N*α t-butyloxycarbonyl-*N*ε-acyl-L-lysinate (1 mmole) and L-phenylalanyl-L-isoleucyl-glycyl-L-leucyl-L-methioninamide (1 mmole) in anhydrous *NN*-dimethylformamide (10 ml) was allowed to stand 5 days at 35°. The solution was then concentrated *in vacuo* and taken up with water. The product was filtered, dried, washed with ether and recrystallized from methanol.

Nε-*Acyl*-L-*lysyl*-L-*phenylalanyl*-L-*isoleucyl-glycyl*-L-*leucyl*-L-*methioninamide*. *N*α-t-Butyloxycarbonyl-*N*ε-acyl-L-lysyl-L-phenylalanyl-L-isoleucyl-L-glycyl-L-leucyl-L-methioninamide (0·4 mmole) was treated for 20 min at 25° with 1·33 N dry hydrogen chloride solution in glacial acetic acid (15 ml). The solvent was evaporated *in vacuo* and the residue was treated with anhydrous ether. The hydrochloride salt was dissolved in dioxane–water (1 : 1), and the solution was applied to an Amberlite IRA-410 column (OH cycle) which was eluted with dioxane–water

(1:1). The product, obtained after evaporation *in vacuo* at 40°, was dissolved in methanol and precipitated by addition of ether.

The physical constants of these products have already been reported (compare Bernardi & others, 1965). The new 1-adamantanecarbonyl derivative (95% yield) has m.p. 222–223°; $[\alpha]_D^{23}$–17° (*c* 1, in acetic acid 95%); $E_{1,9} = 0{\cdot}42$ Leu. Required for $C_{45}H_{72}N_8O_7S{\cdot}H_2O$: C, 61·2; H, 8·4; N, 12·4; O, 14·7. Found: C, 60·9; H, 8·4; N, 12·6; O, 14·4.

PHARMACOLOGICAL (Fregnan and Glässer)

Beagle dogs of either sex (10–15 kg), were anaesthetized intravenously with 35 mg/kg of pentobarbitone sodium. Anaesthesia was maintained with additional doses as needed. After endotracheal intubation the animals were maintained under artificial respiration by a Starling Ideal pump.

Arterial blood pressure was measured from a cannulated carotid artery and recorded on a kymograph either by a mercury manometer or by an Elema electromanometer connected to a galvanometer. The pressure variations were expressed in mm Hg as per cent variation over the control values.

A few experiments were also run on unanaesthetized dogs in which the blood pressure was measured through a cannula chronically implanted into the caudal artery.

All drugs were dissolved, whenever possible, both in distilled water and diacetin, and injected into the medial group of the muscles of the thigh. Some drugs were also dissolved in triacetin and in dimethylsulphoxide (DMSO). Each dose was tested on at least 4 animals and always checked against eledoisin (10 μg i.m. in water).

Results

PHARMACOLOGICAL ASSAY

Table 4 summarizes the pharmacological results. Eledoisin dissolved either in water or in diacetin always caused an abrupt systemic hypotension with a peak effect proportional to the dose but with almost the same duration within the dose range studied. However, the solutions of eledoisin in diacetin were better than those in water, because they caused a weaker but somewhat longer-lasting hypotension in either anaesthetized or unanaesthetized dogs. The vascular effect due to the parent unacylated hexapeptide (soluble in water and not in diacetin) was identical in intensity to that of eledoisin. All the acyl derivatives (more soluble in diacetin than in water) caused a less marked but longer-lasting fall in blood pressure. The compounds containing the fatty acyl residues, mainly the butyryl and valeryl derivatives, were the best ones when injected into either anaesthetized or unanaesthetized dogs. As can be seen in Table 4 and in Figs 1–3, the two substances acted longer when dissolved in diacetin than in water. In fact, in diacetin the decrease in blood pressure was less marked (from −25 to −29 mm Hg against −60 mm Hg) but the hypotension lasted at least twice as long,

TABLE 4. PHARMACOLOGICAL RESULTS

Drugs	Vehicle	Dose µg/kg i.m.	Hypotension in dogs: Anaesthetized: % change mean arterial pressure: mmHg	Anaesthetized: % change mean arterial pressure: After min	Anaesthetized: Average duration min: 50% recovery	Anaesthetized: Average duration min: 100% recovery	Unanaesthetized: % change mean arterial pressure: mmHg	Unanaesthetized: % change mean arterial pressure: After min	Unanaesthetized: Average duration min: 50% recovery	Unanaesthetized: Average duration min: 100% recovery	Side-effects
Pyr-Pro-Ser-Lys-Asp(OH)-Ala-Phe-Ile-Gly-Leu-Met-NH_2 (Eledoisin)	Water	10	−36	3	32	70	−28	10	40	75	None
	"	30	−80	10	35	90	−60	15	60	110	Vomiting, diarrhoea, tachycardia
	Diacetin	10	−20	3	70	105					
	"	20	−55	12	50	100					
	"	30	−65	12	35	>90					
R | H–Lys–Phe–Ile–Gly–Leu–Met–NH_2											
R = hydrogen	Water*	10	−35	2	10	30					
R = butyryl	Water	1	−10	3	7	15					
	"	10	−60	2	30	80					
	Diacetin	30	−25	6	92	>200	−24	15	65	150	None
	"	60	−32	10	>90						
R = valeryl	Water	10	−60	2	27	70					
	Diacetin	10	− 9	3	30	55					
	"	30	−29	4	75	115†	−25	15	65	140	None
	Triacetin	30	−54	3	21	60					
	DMSO	30	−57	2	25	70					
R = caproyl	Diacetin	30	−16	10	33	75	−15	15	80	145	None
	"	60	−28	3	63	128					
R = cyclopentyl-propionyl	Diacetin	30	Inactive								
	"	60	−20	2	8	25	Inactive	120			None
	"	200	−40	2	23	45					
	DMSO	30	−24	5	44	100					
R = cinnamyl	Diacetin	30	−12	1	3	5					
	"	60	−20	4	63	100	Inactive	120			None
R = benzoyl	Diacetin	30	Inactive								
	"	60	−25	2	50	120					
R = nicotinyl	Diacetin	30	−37	5	12	60‡	−50	4	45	110	Vomiting, diarrhoea, tachycardia
R = 1-adamantane-carbonyl	Diacetin	30	−19	5	10	60					

* This compound was not soluble in diacetin at the concentrations used and it could not be tested in this solvent.
† The blood pressure did not completely recover in 2 dogs after 130 min when the recording was discontinued.
‡ The blood pressure did not completely recover in 1 dog after 230 min when the recording was discontinued.
The activity of the peptides is compared on a weight basis.

and no substantial side-effects were evoked in unanaesthetized dogs. By doubling the dose of the butyryl derivative the maximal fall in arterial pressure of anaesthetized dogs did not vary too much, being −32 mm Hg for 60 µg/kg against −25 mm Hg for 30µg/kg. Other solvents beside water and diacetin were also tried, e.g. the valeryl derivative was dissolved in triacetin and DMSO. In this instance, these two vehicles did

not behave differently from water. But the cyclopentylpropionyl derivative in DMSO seemed to be more suitable than in diacetin: while in diacetin the compound was almost inactive and short-lasting, in DMSO it caused a moderate fall in arterial pressure (−24 mm Hg) which lasted for about 100 min.

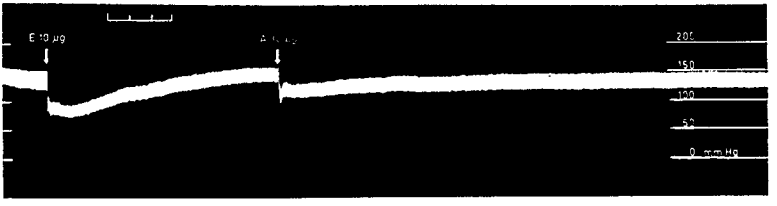

FIG. 1. Carotid blood pressure in an anaesthetized dog measured by a mercury manometer. Hypotension following intramuscular injection of eledoisin in water (E: 10 μg/kg) and butyryl derivative in diacetin (A: 30 μg/kg). Time intervals = 10 min.

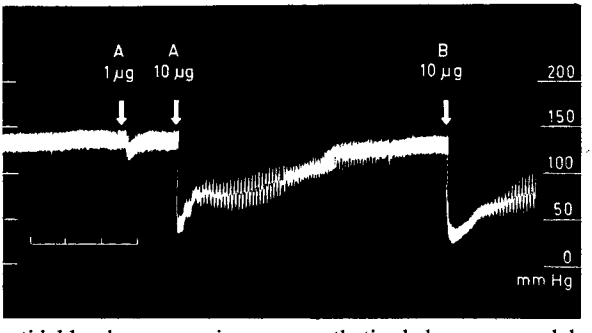

FIG. 2. Carotid blood pressure in an anaesthetized dog measured by a mercury manometer. Hypotension following intramuscular injections of the butyryl (A) and valeryl (B) derivatives in water. Doses in μg/kg. Time intervals = 10 min.

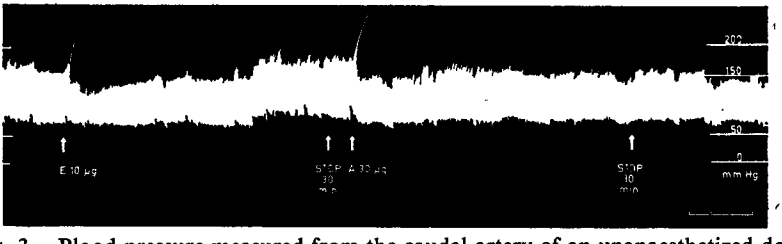

FIG. 3. Blood pressure measured from the caudal artery of an unanaesthetized dog by an electromanometer connected to a galvanometer. Hypotension following intramuscular injections of eledoisin (E) and butyryl derivative (A). Doses in μg/kg. Time intervals = 10 min.

Discussion

Eledoisin and all the eledoisin-like peptides so far known cause a sudden fall in arterial pressure which is short-lasting, possibly because of their rapid metabolism. On the other hand, the period of hypotension

cannot be prolonged to make it useful clinically, by simply increasing the dose. At higher doses the fall in blood pressure becomes more and more marked while the duration of this effect is not always proportional to the dose. In addition, abrupt and strong hypotensions stimulate the reflex mechanisms to restore normal conditions, and they might also evoke numerous side-effects (vomiting, diarrhoea, and tachycardia) particularly evident in unanaesthetized animals.

The problem is how to make the hypotension caused by eledoisin and eledoisin-like peptides gradual in onset and of sufficient duration without evoking side-effects.

Sturmer & Fanchamps (1965) claimed to have prolonged the depressor effect of eledoisin (Depot-Präparat) and to have made it gradual. We also found that it was possible to reduce the intensity of the hypotension and to prolong it, both by suitable changes in the molecule of the peptides and by the use of an appropriate solvent. Among the compounds tested the butyryl and valeryl derivatives dissolved in diacetin gave the best performance. The choice of an appropriate solvent for a given peptide seemed also to be important. The valeryl derivative was dissolved in water, diacetin, triacetin, and DMSO to study the influence of the vehicle. The results showed that diacetin was the best solvent for this drug; the blood pressure did not fall too much and the hypotension lasted for several hours. Triacetin and DMSO were no better than water. Diacetin also prolonged the action of eledoisin but did not significantly reduce the intensity of the hypotension to make it useful for therapeutic trials. In contrast, diacetin was useless for the cyclopentylpropionyl derivatives from a pharmacological point of view. In this instance, DMSO was more suitable.

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