Improved Synthesis of Bradykinin

One of the major problems of peptide synthesis is protection of the guanido function of arginine. This has been accomplished by a variety of methods including even protonation (cf. SCHRÖDER and LÜBKE[1]). There has been no really adequate solution to the simultaneous requirements of introduction of the protecting group in high yield, inertness to the usual reagents and conditions of peptide synthesis, and removal in high yield under mild conditions.

Probably the most attractive guanido protecting group would be *p*-toluene-sulfonyl were it not that sodium-liquid ammonia reduction is required for its removal. We have now found that liquid hydrogen fluoride at 0° C removes the N^G-tosyl group rapidly and cleanly[2]. The synthesis of bradykinin provides an example.

Z-Arg(Tos)-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg(Tos)[3,4] was hydrogenated[6] in 90% acetic acid over palladium black at 4 atmospheres and room temperature. The crude product was purified by countercurrent distribution[7] in methanol-water-chloroform-carbon tetrachloride 37:10:26:27 for 200 transfers. Arg(Tos)[1]-Arg(Tos)[9]-bradykinin was obtained as an amorphous powder, giving a theoretical curve in the above countercurrent distribution, K = 2.33, and homogeneous on silica t.l.c. in *n*-butanol-acetic acid-water 7:1:2, Rf. 0.41. $[\alpha]_D^{24} - 56°$, $c = 1$, acetic acid. Anal.[9] Calcd. for $C_{64}H_{85}N_{15}O_{15}S_2 \cdot 3H_2O$: C, 54.03; H, 6.45; N, 14.77; S, 4.51. Found: C, 54.26; H, 6.58; N, 14.59; S, 4.53. Amino acid analysis[10]: Arg(Tos) 2.0; Pro 3.0; Gly 1.0; Phe 2.1; Ser 0.9. Duplicate bioassays[11] gave potencies of 2.3×10^{-5} and 4.1×10^{-5} bradykinin. Surprisingly, the dose-response curves for standard and test compound were parallel. No inhibition was observed.

A sample of Arg(Tos)[1]-Arg(Tos)[9]-bradykinin (0.500 g) was dissolved in 25 ml of liquid hydrogen fluoride and the solution stirred $^1/_2$ h at 0° C. The solution was placed in a 20° C bath and the hydrogen fluoride pumped off under vacuum over a period of about $^1/_2$ h[12]. The residue was dried in a vacuum desiccator over potassium hydroxide pellets and purified by gradient elution chromatography[7] on a weakly acidic ion exchange resin (IRC-50) using a linear gradient from 0.1 *N* acetic acid to glacial acetic acid.

The appropriate fractions were combined and lyophilized giving bradykinin as a white powder, 0.298 g (68% yield calculated as the triacetate). Amino acid analysis: Arg 2.1; Pro 2.8; Gly 1.0; Phe 2.1; Ser 0.9. Duplicate bioassays gave potencies of 1.35 and 1.25 bradykinin. We may have the purest bradykinin yet reported. The hydrogen fluoride method is now being used in the synthesis of potential competitive inhibitors of bradykinin.

Zusammenfassung. Eine neue Methode zur Entfernung der Schutzgruppe von N^G-Tos-Arg mittels wasserfreier Flußsäure und ihre Anwendung für die Synthese von Bradykinin wird beschrieben.

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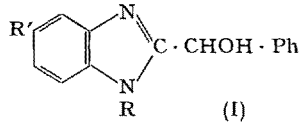
[11] Bioassays: J. H. SANNER. The effect on guinea-pig ileum was compared with standard bradykinin (Sandoz Pharmaceuticals, Hanover, New Jersey, BRS-640, Lot 64003).
[12] Under these conditions N^G-Tos-Arg was quantitatively converted to Arg while N^ϵ-Tos-Lys and Tos-Leu were each deprotected to the extent of about 0.1% as determined by t.l.c. comparison with solutions of known concentration.

D-5-Chloro-2-(α-hydroxybenzyl)benzimidazole and 1-Alkyl-5-chloro-2-(α-hydroxybenzyl)benzimidazoles as Inhibitors of Poliovirus Multiplication

As 5-chloro-2-(α-hydroxybenzyl)benzimidazole (5-chloro-HBB) (I; R = H, R′ = Cl) markedly inhibits the multiplication of type 2 poliovirus[1], it is important to decide if the activity is shared by both optical isomers and how it is influenced by N-alkylation.



(I)

We have determined (a) the maximum concentrations (of such compounds) tolerated by *ERK* cells (MTC's)[2], (b) the effectiveness of the compounds (at half MTC's) in delaying onset of cytopathic change in poliovirus-infected *ERK* cells[3], and (c) the concentrations of compound needed to produce various percentage reductions in poliovirus multiplication after 16 h incubation of *ERK* cells in the presence of both virus and compound[4].

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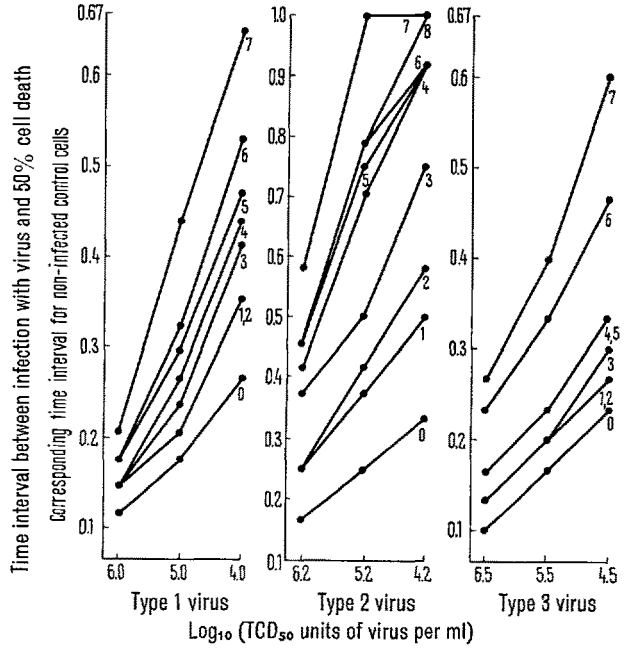
The effectiveness in protecting cells, simultaneously infected and treated with compound, is shown in the Figure. All compounds are more effective against type 2 than types 1 and 3 viruses (note the different scales of the ordinates in the Figure). Similar trends in activity are followed with all 3 viruses. In our cell-protection experiments, DL-5-chloro-HBB (I; R = H, R′ = Cl) is less effective, and its D-isomer is more effective, than HBB (I; R = R′ = H) in delaying onset of cytopathic change (Figure). The poliovirus-inhibitory concentrations in Table 1 show that the activities of the racemate reside entirely or almost entirely in the D-isomer. Selective activities of the D-isomer are approximately twice those of the racemate (Table I). Both D- and DL-isomers are also highly effective at inhibiting Coxsackie B 5 and A 9 and ECHO 6 viruses in monkey kidney cells and have some activity against herpes simplex and neurovaccinia viruses in *ERK* cells. They show no inhibiting influence on a selection of viruses from the other main virus groups[5].

The inhibiting actions on poliovirus multiplication of 1-alkyl derivatives of HBB (I; R = alkyl, R′ = H) increase to a maximum with the propyl (or butyl) derivative and then decline with further increase in length of the alkyl chain[3]. We have found a similar general trend with the 1-alkyl derivatives of 5-chloro-HBB. Very little increase in activity occurs with the introduction of the methyl group (usually too small to be apparent in the cell-protection experiments in the Figure). There is an

Table I. Maximum tolerated μmolarities (MTC), μmolarities giving 75% inhibition of poliovirus multiplication (VIC), activities relative to HBB (*A*)[2] and selectivities relative to HBB (S)[2] of 5-chloro-HBB and its 1 substituted derivatives (I; R′ = Cl)

	Virus type	Substituent R of the virus inhibitor present H(DL)	H(D)	Me	Et	Pr	Bu
MTC	–	100	100	100	140	100	60
VIC	1	160	85	160	140	20	25
	2	45	22.5	42.5	35	15	20
	3	170	85	165	160	27.5	30
A[a]	1	1.0	1.9	1.0	1.1	8.0	6.4
	2	0.78	1.6	0.81	1.0	2.3	1.8
	3	0.94	1.9	0.97	1.0	5.8	5.3
S	1	0.5	0.9	0.5	0.8	3.8	1.8
	2	0.4	0.7	0.4	0.7	1.1	0.5
	3	0.5	0.9	0.5	0.7	2.8	1.5

[a] *A* values quoted to 2 significant figures.



Protection given by $^1/_2$ MTC's of compounds to *ERK* cells infected with 3 dilutions of each poliovirus type. In the graphs, numbered lines join the 3 points for each compound as follows: line (0), controls with no test compound; (1), 5-chloro-HBB; (2), 5-chloro-1-methyl-HBB; (3), HBB; (4), 5-chloro-1-ethyl-HBB; (5), D-5-chloro-HBB; (6) 5-chloro-1-butyl-HBB; (7), 5-chloro-1-propyl-HBB; (8), 5-chloro-1-propyl-HBB (25 μM). Hydrochlorides were used in all cell culture experiments. Half the non-infected control cells survived 8.5 days in the type 1 virus experiment, 6.0 days in the type 2 virus experiment and 7.5 days in the type 3 virus experiment. The presence of $^1/_2$ MTC of any of the test compounds made no difference to these survival times for non-infected control cells.

Table II. 5-Chloro-2-(α-hydroxybenzyl)benzimidazole, its D-isomer and its 1-alkyl derivatives (I; R′ = Cl)

R =	H(DL)	H(D)[a]	Me	Et	Pr	Bu
m.p. (°)	192		189	168	172	117
Form[b]	Prisms		Needles	Plates	Plates	Prisms
Yield (%)	43.7	39.6	10.0	10.5	12.6	12.0
m.p. (°)[a]	211	220	176	183	174	204
Form[a,b]	Prisms	Needles	Prisms	Prisms	Prisms	Prisms
Formula[c]	Ø	Ø + HCl	Ø + CH_2	Ø + C_2H_4	Ø + C_3H_6	Ø + C_4H_8
C Reqd. (%)		57.0	66.1	67.0	67.9	68.7
Fd. (%)		57.0	66.2	66.7	68.2	68.4
H Reqd. (%)		4.11	4.82	5.28	5.71	6.10
Fd. (%)		4.07	4.84	5.43	5.90	5.88
N Reqd. (%)		9.5	10.3	9.8	9.3	8.9
Fd. (%)		9.4	10.4	10.0	9.3	8.9
Cl Reqd. (%)		24.1	13.0	12.4	11.8	11.3
Fd. (%)		23.6[d]	12.7	12.3	11.5	11.4

[a] Information refers to the hydrochloride. Hydrochlorides were crystallized from ethanol-ether. [b] Substances are all white crystals. [c] Ø = $C_{14}H_{11}ClN_2O$.

increase with the ethyl group, but the marked increase occurs on passing to the propyl derivative, followed by a decline to the butyl derivative. 5-Chloro-1-propyl-HBB (I; R = Pr, R′ = Cl) has high activity and selectivity (Table I), but is less active and usually less selective than PHBB (I; R = Pr, R′ = H)[4]. Each 1-alkyl compound is more active than its corresponding 5-chloro derivative. The latter compounds also have a disadvantage in being less soluble in tissue culture medium. However, prolonged warming will produce satisfactory dispersion even with the butyl derivative up to approximately 100 μmoles/l. Hydrochlorides of the 5-chloro compounds were used in our experiments, but the proportion of dissolved free base depends, of course, on the pH of the medium.

DL- and D-5-chloro-2-(α-hydroxybenzyl)benzimidazoles (I; R = H, R′ = Cl) and the DL-1-alkyl-5-chloro derivatives (I; R = alkyl, R′ = Cl) were obtained by heating under reflux, the appropriate 4-chloro-*o*-phenylenediamine (1 mole) with DL- or D-mandelic acid (1 mole) in 2*M* hydrochloric acid (2.5 moles) for 6 h (for the butyl derivative, 4*M* hydrochloric acid and 12 h were necessary). Those hydrochlorides that separated were removed, and the bases were obtained on neutralizing either the reaction mixtures or aqueous solutions of the hydrochlorides with 3*M* potassium carbonate. Repeated crystallization from aqueous methanol with charcoal treatment gave the benzimidazoles (Table II). D-5-chloro-2-(α-hydroxybenzyl)benzimidazole hydrochloride crystallized from ethanol-ether mixture and had $[\alpha]_D^{17}$ of −78.6° (*c* 0.81 in ethanol), but its base (m.p. 85–100°) was not obtained in a pure state. Our yield of the parent DL-isomer was better than that previously obtained by fusing the diamine with mandelic acid[6]. N-Alkyl-4-chloro-2-nitroanilines were prepared from the alkylamine and 2,5-dichloro-1-nitrobenzene[7]. 4-Chloro-2-nitro-N-propylamine was obtained, in 84% yield, as orange prisms from ethanol, m.p. 49–50° (Anal.-Found: C, 50.4; H, 5.12; N, 13.1; Cl, 16.5. $C_9H_{11}ClN_2O_2$ requires C, 50.4; H, 5.13; N, 13.1; Cl, 16.6%). These nitroanilines were hydrogenated to give the diamines[3]. Hydrogen uptake continued until an excess of up to 21% had reacted. Partial dechlorination may explain the low yields of pure benzimidazoles (Table II).

As both relative selectivities (for poliovirus in *ERK* cells) and solubilities in water of the 5-chloro derivatives are generally inferior to those of the corresponding non-halogenated compounds the 5-chloro derivatives are, at present, potentially of less value in relation to polioviruses than the non-halogenated compounds, in spite of their quite high activities. We are testing other halogen derivatives of HBB, of its 1-alkyl derivatives, and of its optical isomers in order to elucidate the overall structure-activity pattern. Results will be reported elsewhere[8].

Zusammenfassung. Die D-isomere Verbindung ist verantwortlich für die Poliovirus-hemmende Aktivität von DL-5-Chlor-2-(α-oxy-benzyl)-benzimidazol. DL-1-Propyl-5-chlor- und DL-1-Butyl-5-chlor-Derivate sind ausserordentlich wirksam zur Verhinderung der Vermehrung der Typen 1, 2 und 3 des Poliovirus; andere 1-Alkyl-5-chlor-Derivate sind ebenfalls aktiv. Die 1-Alkyl-Grundverbindungen sind jedoch wirksamer als deren gleichartige 1-Alkyl-5-chlor-Derivate.

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Courtauld Institute of Biochemistry, Middlesex Hospital Medical School, London, W.1. (England), 4 December 1967.

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Glutamate Dehydrogenase Activity in Normal and Tumoural Skin in Humans

In previous studies, the authors demonstrated that a coenzyme-independent lactate dehydrogenase is active in Ehrlich ascites tumour cells[1,2]. They also found an increased activity of glutamate dehydrogenase (GLDH) in the liver of Guérin-epithelioma rats[3].

In the present work, some of the results concerning GLDH activity in normal skin and skin epithelioma will be discussed.

Materials and methods. Samples of skin and tumoural tissue (spindlecell epitheliomas and basaliomas) were homogenized in 0.9% NaCl with $7 \times 10^{-4}M$ EDTA. The supernatant resulting from a 10-min centrifugation (1800 *g*) at low temperature, was used for testing GLDH activity in 340 nm[4] with a VSU 1 K Zeiss Jena spectrophotometer.

Results and discussion. As seen in the Table, GLDH activity is higher in skin epithelioma than in normal skin in man. The enzyme is inhibited by Zn^{++} ions and O-phenantroline both in normal skin and in skin epithelioma. While in normal skin there is a total Zn^{++} inhibition with $1.9 \times 10^{-4}M$ $ZnSO_4$, in skin epithelioma the activity is found to be more than 50% of the original enzyme activity in tumours, and also exceeds that of normal skin. Total inhibition was only noted in epithelioma at a concentration of $2.5 \times 10^{-4}M$ $ZnSO_4$. While in normal skin $1 \times 10^{-2}M$ O-phenantroline totally inactivates the enzyme, in epithelioma with the same concentration of O-phenantroline, one fifth of the original activity is still found. It should be mentioned that in one instance of epithelioma, inactivation was not obtained even when Zn^{++} and O-phenantroline concentrations were 100 times

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