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INHIBITORS OF THE PULMONARY ANGIOTENSIN I-CONVERTING ENZYME

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SUMMARY

The peptides bradykinin, [Tyr^8]-bradykinin, Met–Lys–bradykinin, nonapeptide, bradykinin-potentiating peptide, bradykinin-potentiating peptide C, and the polypeptide thyroid-stimulating hormone have been shown to inhibit the angiotensin I-converting enzyme from rabbit lung. The venoms of several species of snakes, most notably *Crotalus*, have also been demonstrated to contain a converting enzyme inhibitor. Tranexamic acid (aminomethylcyclohexanecarboxylic acid), *N*-cyclohexyl-β-alanine, 3-cyclohexylamino-1-propylamine, *trans*-1,4-cyclohexanedicarboxylic acid, 2-cyclohexylaminoethanethiol, cyclohexylmethylamine, cyclohexanecarboxylic acid, cyclopentanecarboxylic acid, 2-pyrrolidone, and pyrrolidine also inhibited the enzyme; cyclohexane and *trans*-1,4-cyclohexanedimethanol had no effect on enzyme activity.

INTRODUCTION

A number of peptides, originally reported for their ability to potentiate the contractile response of the guinea-pig ileum to bradykinin, have since been observed to inhibit the angiotensin I-converting enzyme. Bradykinin-potentiating factor[1,2] has been well established as a pulmonary angiotensin I-converting enzyme inhibitor[3,4] by what may well be a competitive mechanism[3,5]. A nonapeptide (SQ 20881)[6,7] and the bradykinin-potentiating peptides B and C[8,9] have also been demonstrated to possess inhibitory activity in this system. Bradykinin-potentiating factor, nonapeptide, and five other peptides capable of inhibiting the converting enzyme have been isolated from the venom of the snake *Bothrops jararaca*[1,6,7,10,11]; bradykinin-potentiating peptides B and C were isolated from the venom of the snake *Agkistrodon halys blomhoffii*[9]. Isolation of these strong inhibitory peptides from snake venoms would suggest that other snake venoms may be the source of compounds which will inhibit angiotensin I-converting enzyme. With this end in mind, we have examined a wide variety of crude venoms in an effort to determine the possible existence of such inhibitory substances.

Previous reports from our laboratory have shown that several peptides, such as bradykinin, Met–Lys–bradykinin, and bradykinin-potentiating peptide C, are capable of inhibiting, by a competitive mechanism, the conversion of angiotensin I to angiotensin II[12]. We have now extended these inhibitor studies to non-peptide structures. Tranexamic acid (aminomethylcyclohexanecarboxylic acid), an experimental antifibrinolytic agent[13], is an effective angiotensin I-converting enzyme inhibitor. A number of related cyclohexane derivatives have been demonstrated to be even more active in this regard.

PROCEDURE

Materials

Angiotensin I and angiotensin II, bradykinin, Met–Lys–bradykinin, and bradykinin-potentiating factor were obtained from Schwarz/Mann, Orangeburg, N.Y. Samples of angiotensin I and bradykinin-potentiating peptide C were obtained from the Institute for Protein Research, Osaka, Japan. [Tyr^8]-Bradykinin was purchased from New England Nuclear Corp., Boston, Mass. The nonapeptide was furnished by Dr John Stewart, University of Colorado School of Medicine, Denver, Colo. The chemical formula for these compounds are shown in Fig. 1. *Laticauda semifasciata*

Asp–Arg–Val–Tyr–Ile–His–Pro–Phe–His–Leu
[Ile^5]-Angiotensin I

Arg–Pro–Pro–Gly–Phe–Ser–Pro–Phe–Arg
Bradykinin

Met–Lys–Arg–Pro–Pro–Gly–Phe–Ser–Pro–Phe–Arg
Met–Lys–Bradykinin

Arg–Pro–Pro–Gly–Phe–Ser–Pro–Tyr–Arg
[Tyr^8]-Bradykinin

Pyrrolidonecarboxylic acid–Gly–Leu–Pro–Pro–Gly–Pro–Pro–Ile–Pro–Pro
Bradykinin-potentiating peptide C

Pyrrolidonecarboxylic acid–Trp–Pro–Arg–Pro–Gln–Ile–Pro–Pro
Nonapeptide

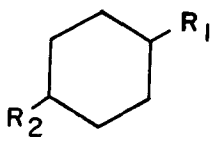
Pyrrolidonecarboxylic acid–Lys–Trp–Ala–Pro
Bradykinin-potentiating peptide

Fig. 1. The structures of the peptide angiotensin I-converting enzyme inhibitors and of angiotensin I.

venom was furnished by Dr Anthony Tu, Colorado State University, Fort Collins, Colo.; all other venoms and all polypeptide hormones were purchased from Sigma Chemical Co., St. Louis, Mo. Tranexamic acid was obtained from Lederle Labs, Pearl River, N.Y., and cyclohexanecarboxylic acid from Eastman Organic Chemicals, Rochester, N.Y.; all other cyclohexane derivatives (Fig. 2) were products of K and K Laboratories, Plainview, N.Y. Cyclopentane substitution analogues (Fig. 3) were products of Aldrich Chemical Co., Milwaukee, Wisc. Rabbits were obtained from the Tulane University Vivarium.

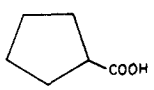
Preparation of converting enzyme

Young rabbits of either sex were sacrificed by a blow on the head and quickly



Name	R_1	R_2
N-Cyclohexyl-β-alanine	$NHCH_2CH_2COOH$	H
2-Cyclohexylaminoethanethiol	$NHCH_2CH_2SH$	H
3-Cyclohexylamino-1-propylamine	$NHCH_2CH_2CH_2NH_2$	H
Cyclohexylmethylamine	$NHCH_3$	H
trans-1,4-Cyclohexanedicarboxylic acid	COOH	COOH
Tranexamic acid (Aminomethylcyclohexanecarboxylic acid)	CH_2NH_2	COOH
Cyclohexanecarboxylic acid	COOH	H
trans-1,4-Cyclohexanedimethanol	CH_2OH	CH_2OH

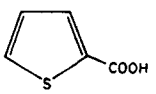
Fig. 2. The structures of the cyclohexane derivatives.



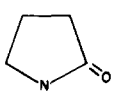
Cyclopentanecarboxylic acid

Thiazolidine

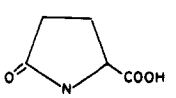
Pyrrolidine



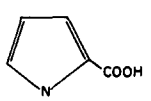
2-Thiophenecarboxylic acid



2-Pyrrolidone



L-2-Pyrrolidone-5-carboxylic acid



Pyrrole-2-carboxylic acid

Fig. 3. The structures of the cyclopentane substitution analogues.

exsanguinated. The lungs were rapidly removed and perfused with 0.9% saline until free of blood; all steps were carried out at 0–5 °C. The lung tissue was sliced, washed twice in 0.25 M sucrose containing 1 mM $MgSO_4$, and then homogenized in the sucrose–Mg^{2+} medium with a tri-R tissue homogenizer and teflon pestle, clearance 0.11–0.15

mm, at 2500 rev./min for 30 s (three strokes). The homogenate was filtered through cheesecloth, and the filtrate centrifuged at 1000 × *g* for 10 min (International Refrigerated Centrifuge); the pellet was resuspended and again centrifuged under similar conditions. The combined supernatant fluids were then centrifuged at 25 000 × *g* for 20 min (Spinco Model L, No. 30 rotor); the pellet was resuspended and again centrifuged. The resulting pellet which was resuspended in 0.25 M sucrose–1 mM $MgSO_4$ to a final concentration of 3–5 mg protein/ml has been previously demonstrated as that fraction with the highest angiotensin I-converting enzyme activity and to be devoid of angiotensinase activity[14]. This enzyme preparation was found to liberate only histidylleucine from angiotensin I during the incubation times (6 min) used in these studies.

Assay of the converting enzyme

Incubation mixtures contained 10 μg antiogensin I (6 nmoles), 0.1 ml enzyme solution, and 0.2 M phosphate buffer (1% NaCl), pH 7.4, to a final volume of 1 ml. Enzyme solution and buffer were preincubated at 37 °C for 3 min, followed by the addition of angiotensin I to initiate the reaction and the incubation continued for 6 min at 37 °C. At this time, an aliquot was removed and assayed on the isolated rabbit aorta according to the methodology described by Huggins *et al.*[15]. When inhibitors were tested, the appropriate concentrations were preincubated with the enzyme solution. In those cases in which bradykinin and bradykinin analogues were used the inhibitor and angiotensin I were added simultaneously since these peptides are destroyed by the enzyme preparation[12]. The degree of inhibition is expressed as a percentage of inhibition which is calculated by comparing the tension generated during assay of an aliquot from an enzyme incubation in the presence of a specific inhibitor to that generated by similar aliquots from standard incubation solutions. In these studies the concentration of peptide, venom, cyclohexane derivative, cyclopentane analogue, or hormone used for inhibition studies was found to be without effect upon the contractile response of the rabbit aorta to angiotensin II. The crude snake venoms were heated to 60 °C for 60 min prior to their use as inhibitors to insure inactivation of any enzymes which may have been present.

RESULTS

Peptide inhibitors of the angiotensin I-converting enzyme

Nonapeptide and bradykinin-potentiating factor were potent angiotensin I-converting enzyme inhibitors, almost completely blocking the conversion of angiotensin I to angiotensin II at concentrations only slightly in excess of those of angiotensin I (Table I). L-Bradykinin and [Tyr^8]-bradykinin were somewhat less potent inhibitors, producing approximately 50% inhibition at equimolar concentrations; Met–Lys–bradykinin and bradykinin-potentiating factor required inhibitor:substrate ratios of 3:1 to obtain 50% inhibition.

Several polypeptide hormones were tested as possible converting enzyme inhibitors. Adrenocorticotropic hormone, somatotropic hormone, follicle-stimulating hormone, luteotropic hormone, and oxytocin (all at $4 \cdot 10^{-5}$ M), glucagon ($8.7 \cdot 10^{-5}$ M), and lysine vasopressin ($4.8 \cdot 10^{-5}$ M) failed to show any inhibitory activity. Thyroid-

TABLE I

INHIBITORS OF ANGIOTENSIN I-CONVERTING ENZYME: PEPTIDES

The inhibitor was preincubated with the enzyme for 3 min prior to the addition of angiotensin I, except in those cases marked by an (*), in which inhibitor and angiotensin I were added simultaneously. Incubation mixtures contained 6 nmoles angiotensin I ($6 \cdot 10^{-6}$ M).

Inhibitor	*No. of expts*	*Concentration (M)*	*Inhibition (%)*
L-Bradykinin	2	$7.9 \cdot 10^{-6}$	54*
		$1.6 \cdot 10^{-5}$	74*
Met–Lys–bradykinin	2	$1.7 \cdot 10^{-5}$	41*
		$3.8 \cdot 10^{-5}$	68*
[Tyr^8]-Bradykinin	2	$8.0 \cdot 10^{-6}$	70*
		$1.6 \cdot 10^{-5}$	75*
Nonapeptide	3	$8.9 \cdot 10^{-6}$	94
		$2.7 \cdot 10^{-5}$	100
Bradykinin-potentiating factor	3	$4.1 \cdot 10^{-7}$	25
		$3.3 \cdot 10^{-6}$	62
		$8.2 \cdot 10^{-6}$	80
Bradykinin-potentiating peptide C	2	$1.9 \cdot 10^{-5}$	47
		$3.8 \cdot 10^{-5}$	61

stimulating hormone, $2.4 \cdot 10^{-5}$ M, showed 34% inhibition of converting enzyme activity (three experiments).

Snake venom inhibitors

It has been reported from several laboratories that peptides obtained from the venom of *Bothrops jararaca* and *Agkistrodon halys blomhoffii* are capable of inhibiting angiotensin I-converting enzyme[3,4,6–11]. These reports have prompted us to investigate the venoms of several other species in order to ascertain their effectiveness as inhibitors of angiotensin I-converting enzyme activity. Table II shows the results from these studies. Two *Crotalus* species, *horridus horridus* (timber rattlesnake) and *adamanteus* (Eastern diamondback rattlesnake), contained potent inhibitors. *Bothrops jararaca* (South American pit viper), the source of nonapeptide and bradykinin-

TABLE II

INHIBITORS OF ANGIOTENSIN I-CONVERTING ENZYME: SNAKE VENOMS

The inhibitor was preincubated with the enzyme for 3 min prior to the addition of angiotensin I. Incubation mixtures contained 6 nmoles angiotensin I ($6 \cdot 10^{-6}$ M).

Venom	*No. of expts*	*Concentration (μg)*	*Inhibition (%)*
Crotalus horridus horridus	4	4	69
		10	100
Crotalus adamanteus	5	40	71
Agkistrodon contortrix contortrix	4	40	28
Agkistrodon piscivorus piscivorus	4	40	38
Acanthropis antarticus	3	40	28
Bothrops jararaca	5	40	68
Bothrops atrox	4	40	21
Bufo marinus	4	40	0
Laticauda semifasciata	1	200	0

potentiating factor, inhibited as expected. *Agkistrodon contortrix contortrix* (broad-banded copperhead), *Agkistrodon piscivorus piscivorus* (Eastern cottonmouth moccasin), *Acanthropis antarticus* (common death adder), and *Bothrops atrox* (fer de lance) also contained factors capable of inhibiting angiotensin I-converting enzyme. The venoms of the South American marine toad *Bufo marinus* and the sea snake *Laticauda semifasciata* were inactive in this regard.

Cyclohexane derivatives and cyclopentane structural analogues

Tranexamic acid (aminomethylcyclohexanecarboxylic acid) has been shown to be an effective antifibrinolytic agent, presumably by inhibiting fibrinolytic activity[13]. More recently Park and Regoli[16] has shown that a synthetic analogue of angiotensin II containing a cyclohexane derivative, 1-aminocyclopentanecarboxylic acid, in position 8 was an effective physiological antagonist of angiotensin II activity. Such observations prompted us to study the effects of various cyclohexane derivatives (Fig. 2) on angiotensin I-converting enzyme. Both cyclohexane and *trans*-1,4-cyclohexanedimethanol, at substrate:inhibitor ratios far in excess of those employed for any of the other cyclohexane derivatives do not inhibit the conversion of angiotensin I to angiotensin II.

TABLE III

INHIBITORS OF ANGIOTENSIN I-CONVERTING ENZYME: CYCLOHEXANE DERIVATIVES AND CYCLOPENTANE SUBSTITUTION ANALOGUES

The inhibitors were preincubated with the enzyme for 3 min prior to the addition of angiotensin I. Incubation mixtures contained 6 nmoles angiotensin I ($6 \cdot 10^{-6}$ M).

Inhibitor	*No. of expts*	*Concentration (M)*	*Inhibition (%)*
Cyclohexane	2	$9.3 \cdot 10^{-2}$	0
trans-1,4-Cyclohexanedimethanol	2	$6.4 \cdot 10^{-2}$	0
		$9.6 \cdot 10^{-2}$	0
Tranexamic acid	2	$6.4 \cdot 10^{-2}$	100
		$6.4 \cdot 10^{-3}$	31
N-Cyclohexyl-β-alanine	3	$6.4 \cdot 10^{-2}$	78
		$6.4 \cdot 10^{-3}$	22
3-Cyclohexylamino-1-propylamine	2	$6.4 \cdot 10^{-2}$	100
		$3.2 \cdot 10^{-2}$	47
trans-1,4-Cyclohexanedicarboxylic acid	2	$6.4 \cdot 10^{-2}$	100
		$6.4 \cdot 10^{-3}$	50
2-Cyclohexylaminoethanethiol	3	$6.4 \cdot 10^{-3}$	100
		$3.2 \cdot 10^{-3}$	71
Cyclohexylmethylamine	2	$7.5 \cdot 10^{-2}$	100
		$6.4 \cdot 10^{-3}$	21
Cyclohexanecarboxylic acid	2	$6.4 \cdot 10^{-2}$	100
		$3.2 \cdot 10^{-2}$	93
		$6.4 \cdot 10^{-3}$	21
Cyclopentanecarboxylic acid	2	$6.4 \cdot 10^{-2}$	100
2-Pyrrolidone	2	$6.4 \cdot 10^{-2}$	26
Pyrrolidine	2	$6.4 \cdot 10^{-2}$	93

Thus, the inhibition caused by those particular derivatives listed in Table III may be regarded as displaying some degree of specificity and not simply due to the high concentration of material present. The following cyclohexane derivatives, listed in descending order of potency, were found to inhibit the angiotensin I-converting

enzyme: 2-cyclohexylaminoethanethiol, *trans*-1,4-cyclohexanedicarboxylic acid, tranexamic acid, cyclohexanecarboxylic acid, cyclohexylmethylamine, 3-cyclohexylamino-1-propylamine, and *N*-cyclohexyl-β-alanine. With the exception of *N*-cyclohexyl-β-alanine, these agents, as well as cyclopentanecarboxylic acid, completely inhibited enzymatic activity at inhibitor:substrate molar ratios of 11 000:1 or less. The heterocyclic cyclopentane substitution analogues (Fig. 3) 2-pyrrolidone and pyrrolidine also blocked the converting enzyme. However, pyrrole-2-carboxylic acid, L-2-pyrrolidone-5-carboxylic acid, thiazolidine, and 2-thiophenecarboxylic acid failed to significantly alter converting enzyme activity.

DISCUSSION

It has been observed that a number of peptides will inhibit the enzymatic conversion of angiotensin I to angiotensin II by the pulmonary angiotensin I-converting enzyme[3–12]. Bradykinin, Met–Lys–bradykinin, bradykinin-potentiating peptide C, and bradykinin-potentiating factor have been reported to competitively inhibit this conversion. Both non-competitive and competitive mechanisms have been suggested for inhibition by the nonapeptide[18,19]. There has, however, been little attempt to determine the structural features of these inhibitory peptides which enable them to compete with angiotensin I for the active site of the angiotensin I-converting enzyme. The structures of these peptides are illustrated in Fig. 1. The nonapeptide is the most potent of these inhibitors, followed by, in decreasing order of potency, bradykinin-potentiating factor, [Tyr^8]-bradykinin, L-bradykinin, Met–Lys–bradykinin, and bradykinin-potentiating peptide C. Certain general observations may be made concerning the correlations between the structures and their relative activities as inhibitors. The two most potent inhibitors—the nonapeptide and bradykinin-potentiating factor (both isolated from *Bothrops jararaca* venom)—contain N-terminal pyrrolidonecarboxylic acid and C-terminal proline residues. However, bradykinin-potentiating peptide C, which also contains an N-terminal pyrrolidonecarboxylic acid residue and a C-terminal proline residue is far less potent. This fact, further supported by the failure of pyrrolidonecarboxylic acid to inhibit the enzyme, suggests that the N-terminal residue may not be of much significance in determining potency of inhibition. Both bradykinin-potentiating factor and nonapeptide are basic peptides, whereas bradykinin-potentiating peptide C is not a basic molecule. This may be of significance since the natural substrate angiotensin I and the hydrolysis products histidylleucine and angiotensin II are all basic. Furthermore, bradykinin, [Tyr^8]-bradykinin, and Met–Lys–bradykinin, also strong inhibitors, are highly basic peptides. However, as will be discussed later, acidic cyclohexane derivatives tend to be stronger inhibitors than their basic counterparts, and thus the effect of the basic groups in the peptides must at present remain uncertain. The weaker inhibitory capability of the undecapeptide, Met–Lys–bradykinin, as compared to that of [Tyr^8]-bradykinin or bradykinin itself may be due to the N-terminal Met–Lys sequence. Although this residue increases the basicity of the peptide, it may also increase the size of the peptide sufficiently to hinder it from binding at the angiotensin I-converting enzyme active site. Bradykinin-potentiating peptide C is also an undecapeptide and thus size could be another factor in its lower efficacy as an inhibitor.

Tranexamic acid (aminomethylcyclohexanecarboxylic acid) has been used as an

experimental antifibrinolytic agent, apparently acting by inhibition of fibrinolytic peptidases[13]. We have found that tranexamic acid and a number of other cyclohexane derivatives are angiotensin I-converting enzyme inhibitors. 2-Cyclohexylaminoethanethiol was the most potent in this series of inhibitors, completely blocking activity at an inhibitor substrate ratio of 1100:1. Following 2-cyclohexylaminoethanethiol in decreasing order of potency are *trans*-1,4-cyclohexanedicarboxylic acid, tranexamic acid, cyclohexylmethylamine, 3-cyclohexylamino-1-propylamine, and cyclohexanecarboxylic acid. The latter three molecules are of approximately the same inhibitory strength. *N*-Cyclohexyl-β-alanine is the least potent in this series. As was the case with the peptide inhibitors, there is no definitive correlation between structure and potency of inhibition. The fact that neither cyclohexane nor cyclohexanedimethanol inhibit the enzyme strongly suggests that the ability to inhibit the enzyme must be derived from those specific groups attached to the cyclohexane nucleus. 2-Cyclohexylaminoethanethiol, *N*-cyclohexyl-β-alanine, and 3-cyclohexylamino-1-propylamine have similar structures, differing mainly in the functional groups terminating the side chains, and yet 2-cyclohexylaminoethanethiol is a much more potent inhibitor than either of the others. The sulfhydryl group is the most probable cause of the potency of 2-cyclohexylaminoethanethiol; this group may well act not only by virtue of its reducing potential, but by its metal chelating potential as well. Bakhle and Reynard have demonstrated inhibition of the converting enzyme from dog lung by 2,3-dimercaptopropanol, and have speculated that it inhibits the dog lung enzyme by virtue of its metal chelating properties and not merely because of the presence of sulfhydryl groups[20].

trans-1,4-Cyclohexanedicarboxylic acid and tranexamic acid follow 2-cyclohexylaminoethanethiol in potency. These two molecules are similar in that they both possess functional groups on the cyclohexane ring in the *trans*-1,4 configuration, with the more acidic dicarboxylic acid displaying the greater affinity for the enzyme. The monofunctional cyclohexane derivatives cyclohexylmethylamine and cyclohexanecarboxylic acid are less effective enzyme inhibitors.

In the cyclopentane substitution analogue series, the monosubstituted cyclopentanecarboxylic acid was a potent inhibitor, as was pyrrolidone. There is as yet insufficient data to warrant any structure–activity correlations with this group of inhibitors.

Ferreira first observed the presence of a peptide fraction, bradykinin-potentiating fraction, from the venom of *Bothrops jararaca* which potentiated the contractile response of bradykinin on the isolated guinea pig ileum[1,2]. A pentapeptide (subsequently termed bradykinin-potentiating fraction) isolated from this fraction was observed to inhibit angiotensin I-converting enzyme[3]. Subsequently six additional peptides also capable of inhibiting converting enzyme have been isolated from the venom of this snake[6,7]. Five bradykinin-potentiating peptides, A through E, have been found in the venom of the Japanese snake *Agkistrodon halys blomhoffii*. Two of these, B and C, have been reported to inhibit angiotensin I-converting enzyme[9]. In order to determine whether or not any other snake venoms contained material capable of inhibiting converting enzyme, we tested a number of crude venoms as possible inhibitors. Two species of *Crotalus*, namely, *horridus horridus* and *adamanteus*, showed strong inhibition. On a crude weight basis *Crotalus horridus horridus* produced much greater inhibition than did *Bothrops jararaca*. However, since no attempt was made

to purify this venom, it is possible that it merely contained a greater concentration of inhibitory material rather than stronger inhibitors. That the inhibition was indeed due to inhibitors and not due to alteration of the converting enzyme by venom enzymes was established by heating the venoms at 60 °C for 60 min; horridus venom was heated for 10 additional minutes at 100 °C. Such heating would be expected to inactivate whatever proteolytic enzyme may have been present. The venoms of *Agkistrodon contortrix contortrix*, *Agkistrodon piscivorus piscivorus*, *Acanthropis antarticus*, and *Bothrops atrox* also contained inhibitory material. Neither the venom of the sea snake *Laticauda semifasciata* nor that of the horned toad *Bufo marinus* showed any inhibition. This raises the possibility that inhibitory peptides may be peculiar to the venoms of land snakes.

These studies illustrate the great diversity of molecules which possess the ability to inhibit the pulmonary angiotensin I-converting enzyme. Of particular interest are the non-peptide inhibitors. Such molecules may well provide an experimental and pharmacological tool for elucidating the biochemical and physiological role of converting enzyme. It seems possible by further modification of the cyclohexane nucleus to design an agent capable of chemically labeling the active site of the enzyme. It may further be possible to design drugs with sufficient potency for use in determining the possible role of angiotensin II formation in those hypertensive conditions in which renin levels are increased[21].

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