New Specific Angiotensin Antagonists. [8-Valine]-, [8-Isoleucine]-, and Chlorambucil-des-1-aspartic,8-valine-angiotensins I†

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[Val^8]-, [Ile^8]-, and Chl-des-Asp^1,Val^8-angiotensins I were synthesized by the solid-phase method and shown to be specific reversible antagonists of angiotensin I and II on the rat's blood pressure, the isolated guinea pig ileum, rat uterus, and rabbit aortic strip. The inhibition was of the competitive type, although lowering of the maximum response to the agonists was observed at high antagonist concentrations in the ileum and the uterus but not in the aorta preparations. pA_2 values were calculated and were the same for the two agonists in each of the smooth muscle organs. pA_2 values were of the same order of magnitude as pD_2 values for angiotensin II. The evidence favors the hypotheses that angiotensins I and II act on the same receptor and that the C-terminal residue of angiotensin II is important for intrinsic activity rather than for binding to the receptor.

After the discovery that [Ala^8]angiotensin II was a competitive antagonist of angiotensin II,[2] several other analogs, in which the phenylanine in position 8 of the peptide chain was replaced by other residues, were also shown to be angiotensin antagonists.[3-10] Since all of these have been octapeptides described as angiotensin II inhibitors, it was thought of interest to study 8-substituted decapeptide angiotensin I analogs as potential antagonists of angiotensin I and II. In this paper we report the syntheses and properties of [Ile^8]- and of [Val^8]-, as well as of Chl-des-Asp^1,Val^8-angiotensin I. The latter compound was prepared because Chl-[des-Asp^1]angiotensin II had been previously found to be a noncompetitive irreversible angiotensin II antagonist in the isolated guinea pig ileum.[11] The activities of the three analogs as antagonists of angiotensin I and II were studied in the rat blood pressure and in the isolated guinea pig ileum, rat uterus, and rabbit aortic strips.

Results

[Ile^8]-, [Val^8]-, and Chl-des-Asp^1,Val^8-angiotensins I did not show any agonistic angiotensin-like activity in assays that would have detected activities of 0.01% on the rat blood pressure, 0.005% on the isolated guinea pig ileum, 0.002% on the isolated rat uterus, and 0.02% on the rabbit aortic strip preparation.

Rat Blood Pressure. A marked inhibition of the pressor activities of both angiotensin I and II was observed during infusion of the rat with either [Val^8]-, [Ile^8]-, or Chl-des-Asp^1,Val^8-angiotensin I at a rate of 10 μg/(kg min). The antagonism was specific, no significant effect on the response to epinephrine being observed. The inhibition by the three analogs was reversible, the responses to angiotensins I and II returning to normal after the infusion was discontinued. There was no significant qualitative or quantitative difference between the inhibitions observed with [Ile^8]-, [Val^8]-, or Chl-des-Asp^1,Val^8-angiotensin I. A typical example of the observed antagonism is shown in Figure 1.

Smooth Muscle Preparations. The antagonism toward the angiotensins by different concentrations of [Val^8]-, [Ile^8]-, and Chl-des-Asp^1,Val^8-angiotensin I was studied in the guinea pig ileum, the rat uterus, and the rabbit aorta preparations. A very pronounced inhibition of the responses to both angiotensins I and II was observed with each of the three analogs studied. The antagonism was reversible, the response to the agonist returning to normal within 1 hr after removal of the antagonist from the medium. The inhibition was of the competitive type, with a parallel displacement of the agonists' log dose-response curves, even in the case of Chl-des-Asp^1,Val^8-angiotensin I. However, when large concentrations of any of the three inhibitors were present, a lowering of the maximum response was also observed in the guinea pig ileum and rat uterus. This was not seen in the rabbit aorta. These findings are exemplified by the experiments illustrated in Figures 2-4.

Employing antagonist concentrations for which parallel log dose-response curves were obtained, the pA_2 values[12] shown in Table I were calculated. None of the three analogs, at the concentrations tested, antagonized the activities of bradykinin or histamine on the guinea pig ileum; of bradykinin or oxytocin on the rat uterus; or of epinephrine on the rabbit aorta.

Discussion

[Val^8]-, [Ile^8]-, and Chl-des-Asp^1,Val^8-angiotensin I were found to be potent specific inhibitors of the pressor and myotropic activities of both angiotensins I and II. The inhibition was reversible and appeared to be of the competitive type, except for the lowering of the maximum responses to angiotensin at high inhibitor concentrations. We believe that this deviation from competitive antagonism behavior may be due to an enhancement of angiotensin tachyphylaxis by high concentrations of the inhibitors. This belief is based on the fact that the deviation from competitive antagonism was observed in the guinea pig ileum and the rat uterus, where tachyphylaxis occurs, but not in the rabbit aorta, where tachyphylaxis is not observed.[13] In spite of the deviations from competitive behavior at high inhibitor concentrations, the parallel log dose-response curves were used for estimation of pA_2 values to permit comparison with other antagonists for which pA_2 values have been reported.

The potencies of [Val^8]-, [Ile^8]-, and Chl-des-Asp^1,Val^8-angiotensin I as inhibitors of the myotropic activity of angiotensin II are comparable to those of 8-modified angiotensin II analogs.[2b,4,9] On the rat's blood pressure the analogs described here are better angiotensin inhibitors than [Ala^8]-[2b,4] and [Ile^8]angiotensin II,[9] since these analogs exert a pressor action of their own at the concentrations in which they are inhibitors. This, however, is not the case with [Sar^1,Ala^8]- and [Sar^1,Leu^8]angiotensins II, which

†This investigation was supported by Fundação de Ampara a Pesquisa do Estado de São Paulo (Projeto Bioq/FAPESP) and the Brazilian National Research Council (CNPq). Abbreviations used are: Chl = chlorambucil, *p*-[*N*,*N*-bis(2-chloroethyl)amino]phenylbutyryl; Boc = *tert*-butyloxycarbonyl. All optically active amino acids are of the L configuration and are abbreviated as recommended by the IUPAC Commission on nomenclature.[1]

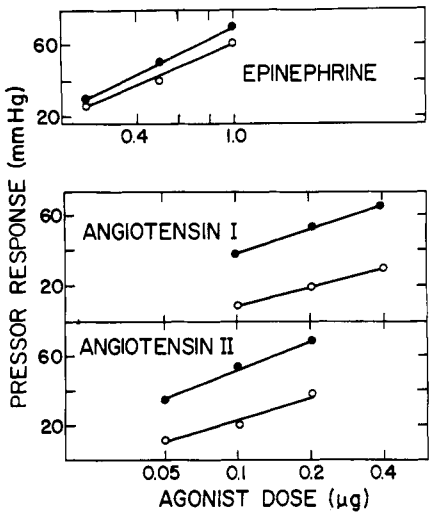

Figure 1. Log dose-response curves for epinephrine and angiotensins I and II on the rat blood pressure before (●–●) and during (○–○) infusion of [Ile^8]angiotensin I [10 μg/(kg min)].

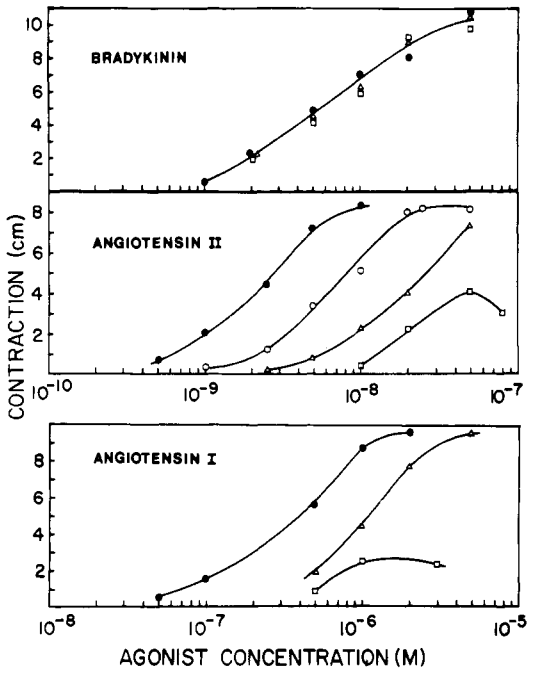

Figure 2. Log dose-response curves for bradykinin and angiotensins I and II on the isolated rat uterus in the presence of increasing concentrations of [Val^5]angiotensin I: (●–●), control; (○–○), 1 × 10^{-8} M; (△–△), 2 × 10^{-8} M; (□–□), 5 × 10^{-8} M.

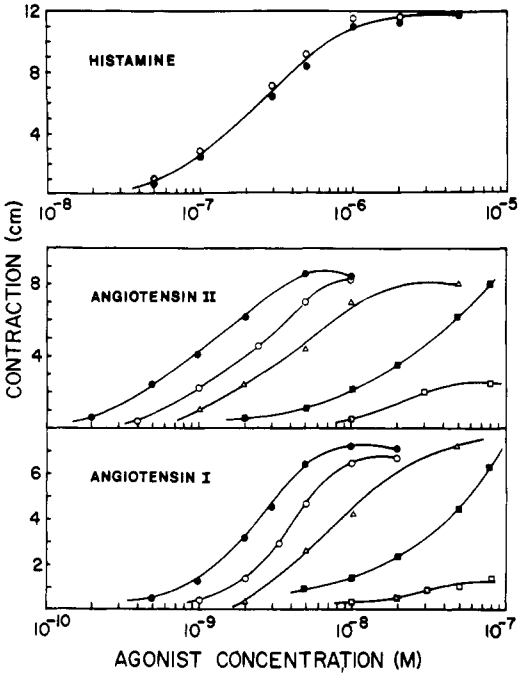

Figure 3. Log dose-response curves for histamine and angiotensins I and II on the guinea pig ileum in the presence of increasing concentrations of [Val^5]angiotensin I: (●–●), control; (○–○), 2 × 10^{-9} M; (△–△), 5 × 10^{-9} M; (■–■), 2 × 10^{-8} M; (□–□), 5 × 10^{-8} M.

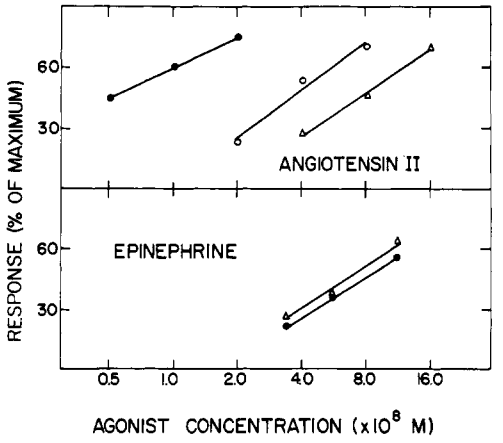

Figure 4. Log dose-response curves for epinephrine and angiotensin II on the isolated rabbit aortic strip in the presence of increasing concentrations of Chl-des-Asp^1,Val^8-angiotensin I: (●–●), control; (○–○), 5 × 10^{-8} M; (△–△), 1.5 × 10^{-7} M.

seem to be the best antagonists of angiotensin's pressor action yet described.[6,10]

Chl-des-Asp^1,Val^8-angiotensin I was a good inhibitor of the angiotensins' pressor and myotropic activity, but the inhibition was also reversible and competitive. This was contrary to expectation, since Chl-des-Asp^1-angiotensin II has been shown to be an irreversible noncompetitive angiotensin II antagonist, probably binding covalently to the receptor through alkylation by its nitrogen mustard moiety.[11] Although Chl-des-Asp^1,Val^8-angiotensin I had high affinity for the receptors, it appears that the drug-receptor interaction, in this case, does not allow the alkylating group to approach a suitable nucleophilic site on the receptor.

The activity of angiotensin I on different biological preparations depends to a different extent on its conversion to angiotensin II.[14] While most of its pressor activity is due to its conversion into angiotensin II,[15] this does not happen to a significant degree in the isolated rat uterus,[16] where a direct action of angiotensin I on the muscle probably occurs. The same considerations might apply to a possible conversion of [Val^8]-, [Ile^8]-, and Chl-des-Asp^1,Val^8-angiotensin I to their respective angiotensin II homologs. In the isolated rat uterus, the affinity of angiotensin I for its receptor is two orders of magnitude lower than that of angiotensin II, as judged by pD_2 values; the half-maximum responses to angiotensins I and II are obtained with concentrations of the order of 10^{-7}–10^{-9} M, respectively. This might be explained either by the existence of different receptors for the two peptides or by a difference in affinity of the same receptor for angiotensins I and II. The latter hypothesis is supported by the finding that, in the rat uterus, there was no significant difference in the affinity of each inhibitor for the receptors to angiotensins I and II, as inferred from pA_2 values (Table I). A similar reasoning may be valid also in

Table I. pA_2 Values[a] for the Inhibition of Angiotensins I and II by [Ile^8]-, [Val^8]-, and Chl-des-Asp^1,Val^8-angiotensins I

Antagonist[b]	Agonist[b]	Guinea pig ileum	Rat uterus	Rabbit aorta
[Ile^8]AI	AI	8.0 ± 0.2	7.0 ± 0.5	8.3 ± 0.4
	AII	7.9 ± 0.2	7.3 ± 0.5	8.0 ± 0.5
[Val^8]AI	AI	9.1 ± 0.3	8.1 ± 0.4	8.2 ± 0.5
	AII	7.9 ± 0.2	7.3 ± 0.5	8.0 ± 0.5
Chl-des-Asp^1,Val^8-AI	AI	8.0 ± 0.3	7.9 ± 0.4	7.9 ± 0.3
	AII	7.5 ± 0.4	7.4 ± 0.6	7.6 ± 0.5

[a] pA_2 values were calculated from the x intercept of the equation $\log (x - 1) = pA_2 - pA_x$ (see Experimental Section) and are shown with ±1 standard deviation. [b] AI = angiotensin I; AII = angiotensin II.

the case of the rabbit aorta and the guinea pig ileum, where a significant portion of angiotensin I activity may be ascribed to a direct action of the decapeptide.[17]

The behavior of the antagonists described in this paper is considered evidence that angiotensins I and II exert their direct myotropic actions by interacting with the same receptor. The pA_2 values calculated for the antagonists (Table I) were of the same order of magnitude as the pD_2 values for angiotensin II and comparable to pA_2 values reported for several 8-modified angiotensin II analogs.[2b,4,6,9] This indicates that the affinity of these inhibitors for the angiotensin receptor is similar to that of angiotensin II itself. It is concluded that the importance of the C-terminal residue of angiotensin II for biological activities resides on its participation in the triggering of the response (intrinsic activity) rather than on the binding of the peptide to its receptor (affinity).

Experimental Section

Syntheses. The peptides were synthesized by the solid-phase method.[18,19] The C-terminal Boc-amino acid was esterified to 2% cross-linked chloromethyl polymer, and chain elongation was performed on an automatic peptide synthesizer.[20] $CHCl_3$ was used as the solvent for all reagents and Boc-amino acids, with the exception of Boc-Arg(NO_2), which was dissolved in $CHCl_3$–DMF (2:1). The Boc groups were removed with 25% (v/v) CF_3COOH in $CHCl_3$ for 30 min. The coupling steps were carried out with 2.5 equiv of Boc-amino acid and DCI. The amino acids with reactive side chains were used in the form of the following derivatives: Boc-Asp(Bzl), Boc-Arg(NO_2), Boc-Tyr(Bzl), and Boc-His(Tos).[21,22] After the last coupling, the peptide was cleaved from the resin and deprotected by stirring for 60 min at 0° in anhydrous HF containing 5% (v/v) anisole. After removal of HF and anisole, by vacuum distillation and washing with EtOAc, the peptides were extracted with glacial AcOH and lyophilized.

Tlc was performed on silica gel with fluorescent indicator (Eastman) with the following solvent systems: A, *n*-BuOH–AcOH–H_2O (4:1:1); B, *n*-BuOH–AcOH–H_2O–pyridine (15:3:12:10); C, $CHCl_3$–MeOH–AcOH–H_2O (65:30:4:1); D, $CHCl_3$–Me_2CO (5:1); and on cellulose (Merck) with the solvent system *n*-PrOH–H_2O (2:1) (E).

[Val^8]angiotensin I was purified by 400 transfers of countercurrent distribution in *n*-BuOH–AcOH–H_2O (4:1:5). A homogeneous peak was obtained with a distribution coefficient $K = 0.08$. After concentration by distilling *in vacuo*, followed by lyophilization, a white powder was obtained in a final yield of 28%, based on the amount of Boc-Leu initially esterified to the resin. The product gave only one Pauly- and ninhydrin-positive spot on high voltage electrophoresis at pH 2.8 (1 *M* AcOH) and at pH 4.9 (pyridine acetate), with the expected mobilities, and was also homogeneous on tlc with the following R_f values: 0.27 (A), 0.40 (B), 0.12 (E). Amino acid analyses (Beckman Mod. 120C amino acid analyzer) yielded the following molar ratios: Asp, 1.01; Arg, 0.95; Val, 2.16; Tyr, 0.94; Ile, 1.07; His, 1.92; Pro, 1.06; Leu, 1.10.

[Ile^8]angiotensin I was purified in the same way as [Val^8]angiotensin I: $K = 0.124$, yield, 25%. The product was homogeneous to high voltage paper electrophoresis at pH 2.8 and 4.9, with the expected mobilities, and to tlc with R_f values 0.20 (A), 0.35 (B), 0.14 (E). Amino acid ratios found were: Asp, 0.95; Arg, 0.98; Val, 1.05; Tyr, 0.85; Ile, 1.90; His, 2.02; Pro, 1.06; Leu, 1.09.

Chl-des-Asp^1,Val^8-angiotensin I appeared as only one major uv- and Pauly-positive and ninhydrin-negative spot, on tlc, with R_f values 0.67 (B), 0.65 (C), 0.12 (D). Amino acid ratios were: Arg, 0.96; Val, 2.08; Tyr, 1.05; Ile, 1.10; His, 1.94; Pro, 0.95; Leu, 0.92. The presence of the chlorambucil group in the peptide was demonstrated by following the kinetics of its hydrolysis at pH 7.4 and 38°, with a Radiometer TTT-11 pH-stat. The expected release of H^+, with $t_{1/2} = 103$ min, was observed and confirmed by Volhard Cl^- titration. Due to its short life in aqueous solution, this peptide was dissolved in propylene glycol for its biological assays, and the concentration of its solutions was checked by diluting in the physiological media, filtering, and determining the uv absorbance at 280 nm.

Bioassays. The isolated guinea pig ileum,[23] rat uterus,[24] and rabbit aorta[25] preparations were done as described earlier. The bath volume was 5 ml, the media were aerated with a stream of air, and the isotonic contractions were recorded under a 1-g load with six-fold magnification. The pA_2 values were estimated from the regression equation relating $\log (x - 1)$ to $-\log$ (inhibitor concentration) (pA_x), where x is the ratio of the agonist's concentrations that produced the same response in the presence and in the absence of the inhibitor.[12] The regression equations were calculated by the method of least squares, from at least eight data points obtained from at least four different preparations. The standard error of the pA_2 values was estimated from the standard error of the estimate and of the slope of the regression equation. The rat blood pressure assays were performed on urethane anesthetized animals. The blood pressure was recorded from the carotid artery and the two femoral veins were canulated to permit simultaneous infusion and single injections.

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References

(1) *J. Biol. Chem.*, **247**, 977 (1972).
(2) (a) P. A. Khairallah, A. Toth, and F. M. Bumpus, *J. Med. Chem.*, **13**, 181 (1970); (b) R. K. Turker, M. Yamamoto, P. A. Khairallah, and F. M. Bumpus, *Eur. J. Pharmacol.*, **15**, 285 (1971).
(3) G. R. Marshall, W. Vine, and P. Needleman, *Proc. Nat. Acad. Sci. U. S.*, **67**, 1624 (1970).
(4) D. T. Pals, F. D. Masucci, F. Sipos, and G. S. Denning, *Circ. Res.*, **29**, 664 (1971).
(5) D. Regoli, W. K. Park, F. Rioux, and C. S. Chan, *Rev. Can. Biol.*, **30**, 319 (1971).
(6) D. T. Pals, F. D. Masucci, G. S. Denning, F. Sipos, and D. C. Fessler, *Circ. Res.*, **29**, 673 (1971).
(7) D. Regoli and W. K. Park, *Can. J. Physiol. Pharmacol.*, **50**, 99 (1972).
(8) M. C. Khosla, S. Kumar, R. R. Smeby, and F. M. Bumpus, *J. Med. Chem.*, **15**, 627 (1972).
(9) M. Yamamoto, R. K. Turker, P. A. Khairallah, and F. M. Bumpus, *Eur. J. Pharmacol.*, **18**, 316 (1972).
(10) D. Regoli, F. Rioux, and W. K. Park, *Rev. Can. Biol.*, **31**, 73 (1972).
(11) T. B. Paiva, A. C. M. Paiva, R. J. Freer, and J. M. Stewart, *J. Med. Chem.*, **15**, 6 (1972).
(12) H. O. Schild, *Brit. J. Pharmacol. Chemother.*, **4**, 277 (1949).
(13) P. A. Khairallah, I. H. Page, F. M. Bumpus, and R. K. Turker, *Circ. Res.*, **19**, 247 (1966).
(14) E. A. Carlini, Z. P. Picarelli, and J. L. Prado, *Bull. Soc. Chim. Biol.*, **40**, 1825 (1958).
(15) K. K. F. Ng and J. R. Vane, *Nature (London)*, **225**, 1142 (1970).
(16) I. H. Page and F. M. Bumpus, *Physiol. Rev.*, **41**, 331 (1951).
(17) J. W. Aiken and J. R. Vane, *Nature (London)*, **228**, 30 (1970).
(18) R. B. Merrifield, *J. Amer. Chem. Soc.*, **85**, 2149 (1963).
(19) J. M. Stewart and J. Young, "Solid Phase Peptide Synthesis," W. H. Freeman, San Francisco, Calif., 1969.
(20) R. B. Merrifield, J. M. Stewart, and N. Jernberg, *Anal. Chem.*, **38**, 1905 (1966).
(21) S. Sakakibara and T. Fujii, *Bull. Chem. Soc. Jap.*, **42**, 1466 (1969).

(22) J. M. Stewart, M. Knight, A. C. M. Paiva, and T. B. Paiva in "Progress in Peptide Research," S. Lande, Ed., Gordon and Breech, New York, N. Y., 1972.
(23) T. B. Paiva and A. C. M. Paiva, *Brit. J. Pharmacol. Chemother.*, **15**, 557 (1960).
(24) T. B. Paiva and A. C. M. Paiva, *J. Pharmacol. Exp. Ther.*, **130**, 177 (1960).
(25) R. F. Furchgott and S. Bhadrakom, *ibid.*, **108**, 129 (1953).

Novel Analgetics and Molecular Rearrangements in the Morphine–Thebaine Group. 28.[1] Derivatives of 6,14-*endo*-Etheno-7-oxo-6,7,8,14-tetrahydrothebaine and 6,14-*endo*-Etheno-6,7,8,14-tetrahydrothebaine

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6,14-*endo*-Ethenotetrahydrothebaine (**1a**) obtained *via* the 7-oxo derivative **3a** is equipotent with morphine as an analgetic; the derived oripavine **1b** is 40 times more potent. The *N*-cyclopropylmethyl derivatives **1e** and **2e** are morphine antagonists more potent than nalorphine.

The chemistry of derivatives of 6,14-*endo*-ethenotetrahydrothebaine has been extensively studied as has the relationship between structure and analgetic activity in this series.[2] However, all the derivatives so far described have had a C-7 substituent. We here report the synthesis of the parent 6,14-*endo*-ethenotetrahydrothebaine (**1a**), the corresponding oripavine **1b**, and some related compounds.

Chemistry. The oripavine **1b** was obtained by Huang-Minlon reduction of 6,14-*endo*-etheno-7-oxotetrahydrothebaine (**3a**) which was prepared from either the thebaine-2-chloroacrylonitrile adduct **5a**[3] or the thebaine-ethyl 2-acetoxyacrylate adduct **5b**.[3]

The C-7 epimeric chloronitriles **5a** undergo a variety of reactions with basic reagents.[4] Both epimers in boiling aqueous alcoholic NaOH gave the 7-oxo derivative **3a**. The ketone was also obtained from **5b** either by reduction with $LiAlH_4$ to give diol **5c** followed by HIO_4 cleavage or by Curtius degradation *via* **5d** and **5e**.

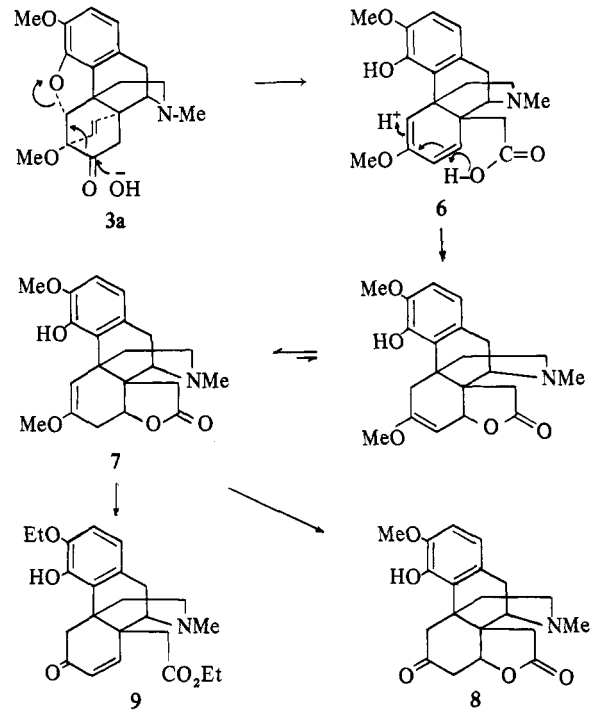
The chloronitrile hydrolysis product contained a C-4 phenolic γ lactone **7** in addition to ketone **3a**. This phenol was also obtained from **3a** by reaction with NaOH in boiling aqueous 2-ethoxyethanol. Its formation involves a benzilic acid type rearrangement of **3a** to **6** followed by lactonization when the reaction mixture is neutralized (Scheme I). Treatment of **7** with cold HCl gave **8** while with boiling EtOH–HCl, **7** gave keto ester **9** in which transetherification of the 3-*O*-methyl group had occurred in addition to opening of the lactone.

Demethylation of the 3-*O*-methyl group of **3a** was achieved by treatment of the dimethyl ketal with NaOH in diethylene glycol at 210°. The ethanotetrahydrooripavine **4c** was similarly obtained from the hydrogenated 7-oxo compound **4a**.

N-Demethylation of **3a** by the azodicarboxylate route[5] followed by reaction of the nor ketone **3b** with appropriate halides afforded the *N*-allyl **3d**, *N*-propargyl **3e**, and *N*-2-methylallyl **3f** analogs. Similar procedures applied to the ethano ketone **4a** gave **4d-f** (Table I). The *N*-cyclopropylmethyl etheno ketone **3g** was obtained directly from the *N*-cyclopropylmethylchloronitrile **5a** (*N*-CPM replaces *N*-Me) by reaction with NaOH–EtOH; the *N*-CPM lactone **7** (*N*-CPM replaces *N*-Me) was also isolated from the reaction mixture.

Huang-Minlon reduction of **3a** gave 6,14-*endo*-ethenotetrahydrooripavine (**1b**) which was converted to the corresponding tetrahydrothebaine **1a** with methyl iodide. A similar reduction of the ethano ketone **4a** produced 6,14-*endo*-ethanotetrahydrooripavine (**2b**); the corresponding tetrahydrothebaine **2a** was in this case made by hydrogenation of 6,14-*endo*-ethenotetrahydrothebaine (**1a**). The

Scheme I



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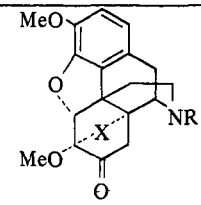

No.	R	X	Mp, °C	Formula[a]
3d	$CH_2CH=CH_2$	C_2H_2	152–155	$C_{23}H_{25}NO_4$
3e	$CH_2C\equiv CH$	C_2H_2	197–198	$C_{23}H_{23}NO_4$[b]
3f	$CH_2C(Me)=CH_2$	C_2H_2	158–161	$C_{24}H_{27}NO_4$
4d	$CH_2CH=CH_2$	C_2H_4	110–111	$C_{23}H_{27}NO_4$
4e	$CH_2C\equiv CH$	C_2H_4	145–147	$C_{23}H_{25}NO_4$
4f	$CH_2C(Me)=CH_2$	C_2H_4	171–173	$C_{24}H_{29}NO_4$

[a] All compounds were analyzed for C, H, N and are within ±0.4% of the theoretical values except where indicated. [b] C: calcd, 73.19; found, 72.60.