washed with water and ether, and dried over phosphorus pentoxide yielding 0.206 g (84%).

The crude material (3 F, des-Lys[7] 1–20) was dissolved in anhydrous TFA (1.5 ml), and the solution was kept for 150 min at room temperature. Ice-cold ether was added, and after 30 min at −10° the precipitate was collected by centrifugation, washed with ether, and dried over potassium hydroxide pellets.

The residue, dissolved in 0.2 *M* sodium phosphate buffer, was purified by passing through an Amberlite CG 50 column, desalted by gel filtration on a Sephadex G-25 column, and lyophilized as described previously.

The product (3 G, des-Lys[7] 1–20) (0.085 g, 50% of the crude product) had $[\alpha]_D$ −75.0 ± 1° (*c* 0.104, water), single ninhydrin- and Pauly-positive component on paper electrophoresis at pH 1.9, 3.5, 6.4, and 9.5; amino acid ratios in acid hydrolysate: $Lys_{1.04}Orn_{1.05}His_{1.00}Glu_{2.95}Thr_{1.92}Ala_{4.85}Phe_{1.10}Met_{0.90}Asp_{0.92}Ser_{2.85}$; amino acid ratios in AP-M digest: $Lys_{1.05}Orn_{1.00}His_{0.95}Glu_{2.10}(Gln + Ser)_{8.78}Thr_{2.10}Ala_{4.95}Phe_{0.98}Met_{1.03}Asp_{1.00}$.

Lysylglutamylthreonylalanylalanylalanyllysylglutamylornithylglutaminylhistidylmethionylaspartylserylserylthreonylserylalanylalanine (3 G, des-Phe[8] 1–20). The condensation of 3 D, des-Phe[8] 1–12 (0.13 g, 0.074 mmol) with 3 E, 13–20[20] (0.132 g, 0.15 mmol as monoacetate trihydrate) by the azide procedure was carried out as described above for 3 G, des-Lys[7] 1–20 and gave the partially protected des-Phe[8]-[Orn[10]]-S-peptide (3 F, des-Phe[8] 1–20, 0.113 g, 61%).

Treatment with anhydrous TFA, purification on Amberlite CG-50 and on Sephadex G-25, followed by lyophilization, gave the pure nonadecapeptide 3 G, des-Phe[8] 1–20 (0.03 g, 33%), $[\alpha]^{20}_D$ −79.0 ± 1° (*c* 0.0994, water), single ninhydrin- and Pauly-positive component on paper electrophoresis at pH 1.9, 3.5, 6.4, and 9.5; amino acid ratios in acid hydrolysate: $Lys_{2.00}Orn_{1.02}His_{1.04}Glu_{2.93}Thr_{2.00}Ala_{4.80}Met_{1.05}Asp_{1.00}Ser_{3.07}$; amino acid ratios in AP-M digest: $Lys_{2.10}Orn_{0.95}His_{1.00}Glu_{2.10}Thr_{2.05}(Gln + Ser)_{8.70}Ala_{5.02}Met_{0.94}Asp_{1.00}$.

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The Structure of Batrachotoxin, a Steroidal Alkaloid from the Colombian Arrow Poison Frog, *Phyllobates aurotaenia*, and Partial Synthesis of Batrachotoxin and Its Analogs and Homologs

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Abstract: Four major toxic steroidal alkaloids are contained in skin extracts of the Colombian arrow poison frog *Phyllobates aurotaenia:* (i) batrachotoxinin A (LD_{50} 1 mg/kg mice), $C_{24}H_{35}NO_5$, 3α,9α-epoxy-14β,18β-(epoxyethano-N-methylimino)-5β-pregna-7,16-diene-3β,11α,20α-triol, the structure of which was elucidated by X-ray crystallography of the 20α-*p*-bromobenzoate; (ii) pseudobatrachotoxin, an extremely labile alkaloid of unknown composition which on standing spontaneously forms batrachotoxinin A; (iii) batrachotoxin, $C_{31}H_{42}N_2O_6$, the most toxic principle (LD_{50} 2 μg/kg mice), which has now been recognized as the 20α ester of batrachotoxinin A with 2,4-dimethylpyrrole-3-carboxylic acid, and (iv) homobatrachotoxin, the former "isobatrachotoxin," $C_{32}H_{44}N_2O_6$ (LD_{50} 3 μg/kg mice), now formulated as the 20α ester of batrachotoxinin A with 2-ethyl-4-methylpyrrole-3-carboxylic acid. A partial synthesis of batrachotoxin was achieved by the reaction of the anhydride of ethyl chloroformate and 2,4-dimethylpyrrole-3-carboxylic acid with batrachotoxinin A. The analogous esterification with the anhydride of the fully substituted 2,4,5-trimethylpyrrole-3-carboxylic acid gave a homolog of batrachotoxin which was *more stable* and *twice as active* (LD_{50} 1 μg/kg). The 20α ester of batrachotoxinin A with 1,2,4,5-tetramethylpyrrole-3-carboxylic acid was much less active than batrachotoxinin A itself. Reductive opening of the 3,9-hemiketal oxygen bridge of batrachotoxin with sodium borohydride leads to an acid-sensitive dihydrobatrachotoxin with $^1/_{250}$ of the activity of batrachotoxin.

After the elucidation of tetrodotoxin the many times more lethal venom of the Colombian arrow poison frog remained as one of the major challenges in the chemistry of natural products. Compared with the classical studies of Wieland on toad venoms[2] the difficulties were considerable. During the past 8 years four expeditions[3] had to be sent into the impervious jungle of the Choco region of western Colombia. The lability of the venom was a problem with which we learned to cope only after numerous failures. The paucity of the material forced us to carry out most operations on the level of micrograms. The advent of mass spectrometry, nuclear magnetic resonance, and, most important of all, the method of X-ray analysis of small asymmetric molecules without markers by heavy atoms[4] secured the final solution.

Four major steroidal alkaloids, batrachotoxin, "isobatrachotoxin," pseudobatrachotoxin, and batrachotoxinin A, have been isolated from extracts of the skin of the Colombian arrow poison frog.[5–7] Batrachotoxin and isobatrachotoxin[7] are extremely active

(1) Associate in the Visiting Program of the U. S. Public Health Service, on leave of absence from Osaka City University, 1965–1968.
(2) *Cf.* H. Michl and E. Kaiser, *Toxicon*, **1**, 186 (1963).
(3) M. Latham, *Natl. Geographic*, **129**, 683 (1966).
(4) J. Karle and I. L. Karle, *Acta Cryst.*, **21**, 841 (1966); J. Karle, *ibid.*, **B24**, 182 (1968). Although the *p*-bromobenzoate of batrachotoxinin A was used for X-ray analysis, the *heavy atom method*, which would have been quite unsuitable because of the special location of the bromine atom and the limitation of data, was *not* used.
(5) F. Märki and B. Witkop, *Experientia*, **19**, 239 (1963).
(6) J. W. Daly, B. Witkop, P. Bommer, and K. Biemann, *J. Am. Chem. Soc.*, **87**, 124 (1965).
(7) T. Tokuyama, J. Daly, B. Witkop, I. L. Karle, and J. Karle, *ibid.*, **90**, 1917 (1968).

Table I. Mass Spectra of Steroidal Alkaloids Isolated from *Phyllobates aurotaenia*[a]

Batrachotoxin		Homobatrachotoxin ("isobatrachotoxin")		Pseudobatrachotoxin		Batrachotoxinin A		Batrachotoxinin A *p*-brombenzoate	
m/e	Ion	*m/e*	Ion	*m/e*[d]	Ion	*m/e*	Ion	*m/e*	Ion
						417	$C_{24}H_{35}NO_5$	601, 599	$C_{31}H_{38}NO_6Br$
399[b]	$C_{24}H_{33}NO_4$	399[b]	$C_{24}H_{33}NO_4$	399[b]	$C_{24}H_{33}NO_4$	399[b]	$C_{24}H_{33}NO_4$	399[b]	$C_{24}H_{33}NO_4$
370[b]	$C_{23}H_{32}NO_3$	370[b]	$C_{23}H_{32}NO_3$	370[b]	$C_{23}H_{32}NO_3$	370[b]	$C_{23}H_{32}NO_3$	370[b]	$C_{23}H_{32}NO_3$
				(342)[b]	$(C_{22}H_{32}NO_2)$	330[b]	$C_{20}H_{26}O_4$		
312[b]	$C_{20}H_{24}O_3$	312[b]	$C_{20}H_{24}O_3$	312[b]	$C_{20}H_{24}O_3$	312[b]	$C_{20}H_{24}O_3$	312[b]	$C_{20}H_{24}O_3$
294[b]	$C_{20}H_{22}O_2$	294[b]	$C_{20}H_{22}O_2$	294[b]	$C_{20}H_{22}O_2$	294[b]	$C_{20}H_{22}O_2$	294[b]	$C_{20}H_{22}O_2$
286[b]	$C_{18}H_{22}O_3$	286[b]	$C_{18}H_{22}O_3$	286[b]	$C_{18}H_{22}O_3$	286[b]	$C_{18}H_{22}O_3$	286[b]	$C_{18}H_{22}O_3$
279	$C_{19}H_{19}O_2$	279	$C_{19}H_{19}O_2$	279	$C_{19}H_{19}O_2$	279	$C_{19}H_{19}O_2$	279	$C_{19}H_{19}O_2$
276	$C_{20}H_{20}O$	276	$C_{20}H_{20}O$	276	$C_{20}H_{20}O$	276	$C_{20}H_{20}O$	276	$C_{20}H_{20}O$
261	$C_{19}H_{17}O$	261	$C_{19}H_{17}O$	261	$C_{19}H_{17}O$	261	$C_{19}H_{17}O$	261	$C_{19}H_{17}O$
224	$C_{16}H_{16}O$	224	$C_{16}H_{16}O$	224	$C_{16}H_{16}O$	224	$C_{16}H_{16}O$	224	$C_{16}H_{16}O$
219	$C_{17}H_{15}$	219	$C_{17}H_{15}$	219	$C_{17}H_{15}$	219	$C_{17}H_{15}$	219	$C_{17}H_{15}$
				(206)[b]	?	202[b]	$C_{13}H_{14}O_2$	202, 200[b]	$C_7H_5O_2Br$
184[b]	$C_{13}H_{12}O$	184[b]	$C_{13}H_{12}O$	184[b]	$C_{13}H_{12}O$	184[b]	$C_{13}H_{12}O$	184[b]	$C_{13}H_{12}O$
		153[c]	$C_8H_{11}NO_2$	(166)	$C_{10}H_{16}NO$	158[b]	$C_{11}H_{10}O$	185, 183[b]	C_7H_4OBr
139[c]	$C_7H_9NO_2$	138[c]	$C_7H_8NO_2$					157, 155[b]	C_6H_4Br
122[c]	C_7H_8NO								
121[c]	C_7H_7NO	120[c]	C_7H_6NO						
		109[c]	$C_7H_{11}N$						
95[c]	C_6H_9N								
94[c]	C_6H_8N	94[c]	C_6H_8N						
88[b]	$C_4H_{10}NO$	88[b]	$C_4H_{10}NO$	88[b]	$C_4H_{10}NO$	88[b]	$C_4H_{10}NO$	88[b]	$C_4H_{10}NO$
71	C_4H_9N	71	C_4H_9N	71	C_4H_9N	71	C_4H_9N	71	C_4H_9N

[a] Fragments derived from the common steroidal moiety are presented in normal type while fragments derived from additional moiety are presented in bold-face type. [b] Intense signal. [c] Variable signal intensity due to thermal decomposition. [d] *m/e* values in parentheses may be due to impurities.

cardiotoxins and at the time appeared to be isomeric steroidal alkaloids ($C_{24}H_{33}NO_4$) on the basis of high-resolution mass spectra. They are easily differentiated from batrachotoxinin A and the extremely labile pseudobatrachotoxin by their characteristic ultraviolet

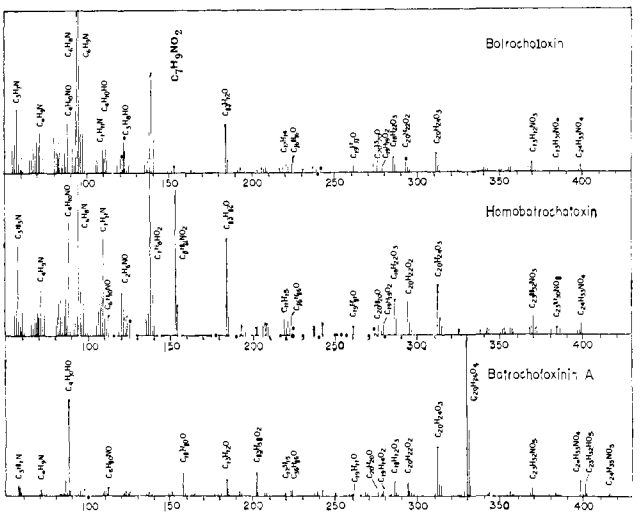


Figure 1. Fragmentation pattern of batrachotoxin, homobatrachotoxin (the former "isobatrachotoxin"), and batrachotoxinin A (AEI MS-9 mass spectrometer, direct inlet, 240°).

spectra (λ_{max} 234, 264 mμ), by an intense infrared absorption band at 1690 cm^{-1}, and by a strong positive reaction with Ehrlich's reagent. Pseudobatrachotoxin[7] appeared to be yet another isomer of the composition $C_{24}H_{33}NO_4$ according to mass spectra, but there is some reservation, because it could not be obtained in pure state as a result of its ready conversion to batrachotoxinin A ($C_{24}H_{34}NO_5$). This conversion, *prima facie*, appears to be the addition of the elements of water. The structure of batrachotoxinin A has been determined as Ia by X-ray diffraction analysis of a crystal of the 20-*p*-bromobenzoate derivative Ib.[7,8]

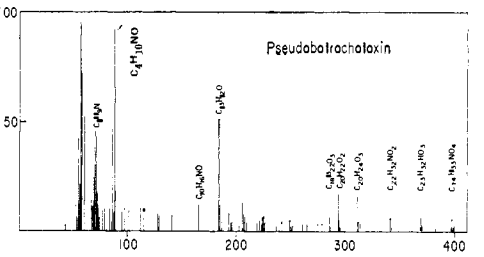
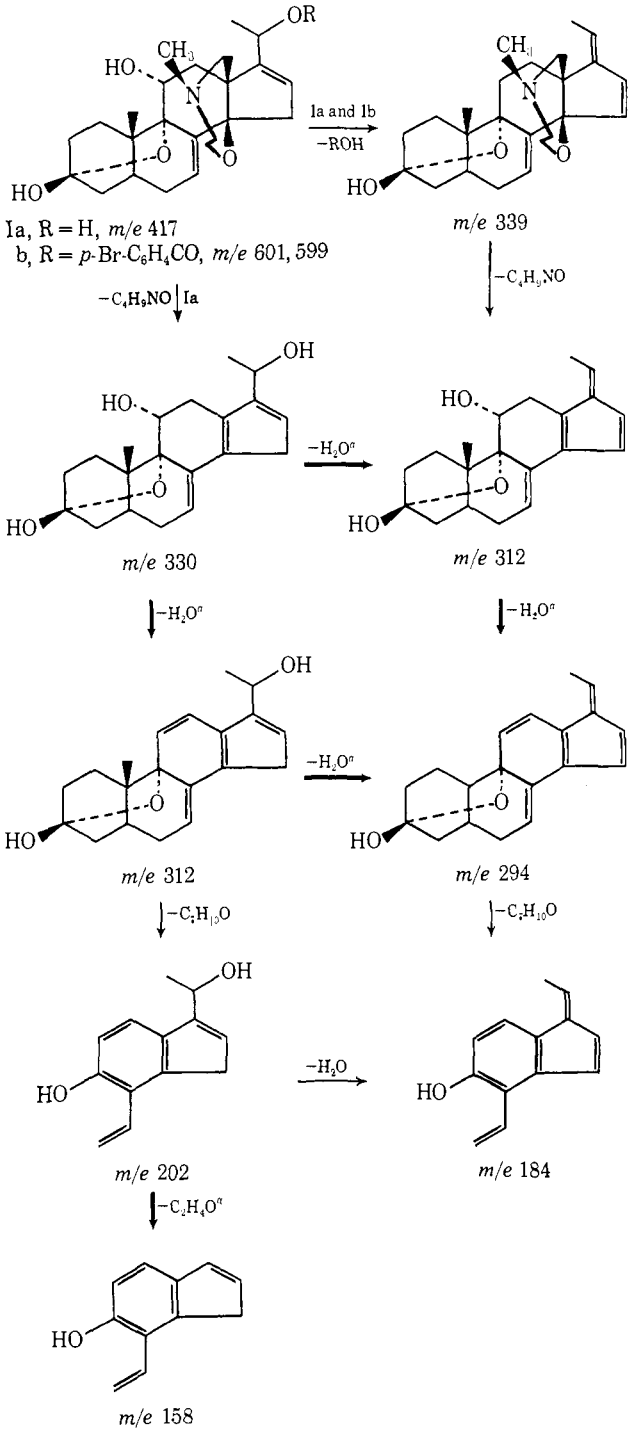


Figure 2. Fragmentation pattern of pseudobatrachotoxin which in common with batrachotoxin and homobatrachotoxin lacks the major peak at *m/e* 330 (see Chart I) characteristic of batrachotoxinin A.

Reinterpretation of Mass Spectra. The knowledge of the structure of batrachotoxinin A led to a reexamination of the spectral and chemical properties of batrachotoxin and isobatrachotoxin. The *m/e* 399 ion ($C_{24}H_{33}NO_4$) observed on mass spectrometry could no longer be the parent ion. Instead batrachotoxin and "isobatrachotoxin" presumably contain an additional moiety which probably accounts for the uv spectra, the positive Ehrlich reaction, and the presence of two additional methyl groups in batrachotoxin and one additional methyl and an ethyl group in isobatrachotoxin. The mass spectra of batrachotoxin, isobatrachotoxin, batrachotoxinin A, and the 20-*p*-bromobenzoate of batrachotoxinin A (molecular ion, *m/e* 599, 601) are presented in Table I with the peaks due to the steroid portion of batrachotoxinin A differentiated from the peaks marked in bold face due to the postulated additional moiety.

(8) I. L. Karle and J. Karle, *Acta Cryst.*, **B25**, 428 (1969).

Chart I. Mass Spectral Fragmentation of Batrachotoxin in A and Its 20-*p*-bromobenzoate



[a] Corresponding metastable peaks are observed.

The fragmentation of batrachotoxinin A and its *p*-bromobenzoate is interpreted in Chart I, while the mass spectra of the three steroidal alkaloids are presented graphically in Figure 1. The fragmentation of pseudobatrachotoxin, which was very difficult to obtain, is pictured in Figure 2.

The largest fragment coming from the postulated additional moiety in batrachotoxin appears as $C_7H_9NO_2$, *m/e* 139. Further fragments are formed by the loss of OH, H_2O, and CO_2. In isobatrachotoxin, the postulated moiety first appears as $C_8H_{11}NO_2$ and subsequently breaks down first by loss of methyl (metastable peaks) and then by loss of OH, H_2O, and

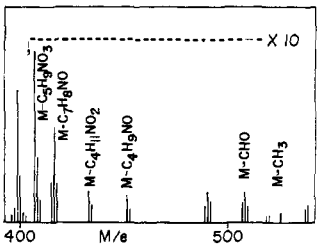


Figure 3. Fragmentation of batrachotoxin above 400. With special care, *i.e.*, rapid introduction of crystalline batrachotoxin carbonate into the vaporization chamber, the molecular ion at *m/e* 538 ($C_{31}H_{42}N_2O_6$) can be observed for a few seconds.

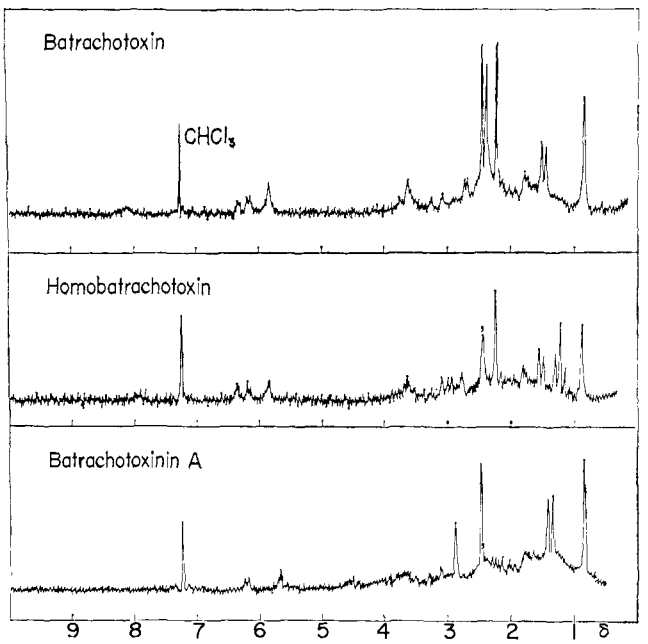


Figure 4. Nmr spectra of batrachotoxin, homobatrachotoxin, and batrachotoxinin A in $CDCl_3$.

CO_2. Since the $C_7H_9NO_2$ fragment of batrachotoxin must also account for the infrared absorption band at 1690 cm^{-1}, the ultraviolet chromophore, the two additional aryl methyl groups, and the positive Ehrlich reaction, it seemed likely that batrachotoxin was a dimethylpyrrolecarboxylate of batrachotoxinin A, while isobatrachotoxin was the corresponding ethylmethylpyrrolecarboxylate. Accordingly the name, *homobatrachotoxin*, should henceforth be used instead of the incorrect term, isobatrachotoxin. Finally, it was possible to demonstrate a true molecular ion for batrachotoxin, and an extremely weak peak was found at *m/e* 538, as predicted (Figure 3). It persisted for only a short period, probably due to thermal reactions. Fragmentation led to small peaks at *m/e* 513 (M − CH_3), 508 (M − CH_2O), 451 (M − C_4H_9NO), and 433 (451 − H_2O). A rearrangement ion at *m/e* 407 ($C_{26}H_{33}NO_3$) in batrachotoxin and 422 in homobatrachotoxin is probably due to loss of C_4H_9NO followed by explusion of the CO_2 of the carboxyl group.

Nuclear Magnetic Resonance Spectra. Comparison of nmr spectra of batrachotoxin in $CDCl_3$ and deuterioacetic acid provided evidence for the presence of the *tertiary* N-methyl group: δ^{DCCl_3} 2.40, $\delta^{D_3CCO_2D}$ 3.24.[9] A careful comparison of the nmr (Figure 4 and Table II)

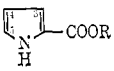
(9) J. C. N. Ma and E. W. Warnhoff, *Can. J. Chem.*, **43**, 1849 (1965).

Table II. Correlation of Nmr Peaks of Batrachotoxin, Homobatrachotoxin, and Batrachotoxinin A[a]

Batrachotoxin		Homobatrachotoxin		Batrachotoxinin A	
Pyrrole NH	8.1 (br)	Pyrrole NH	8.1 (br)		
Pyrrole α-H	6.34 (s)	Pyrrole α-H	6.36 (s)		
H-16	6.18 (d) (5)	H-16	6.17 (d) (5)	H-16	6.24 (dd) (2.5, 5)
H-7	5.83 (s, br)	H-7	5.86 (s, br)	H-7	5.72 (t) (2)
H-20[b]	~5.86 (q)	H-20	~5.86 (q)	H-20	4.57 (q) (6.5)
H-11	3.23 (d) (17.5)	H-11	3.16 (d) (18)	H-11	3.22 (d) (18)
		Pyrrole α-CH_2CH_3	2.98 (dq) (7.5)		
H-18	2.85 (d) (14)	H-18	2.85 (d)	18-CH_2	2.89 (s)
H-18	2.70 (d) (14)	H-18	2.7 (d)		
Pyrrole α-CH_3	2.47 (s)				
N-CH_3	2.39 (s)	N-CH_3	2.43 (s)	N-CH_3	2.48 (s)
Pyrrole β′-CH_3	2.24 (s)	Pyrrole β′-CH_3	2.24 (s)		
21-CH_3	1.51 (d) (6.5)	21-CH_3	1.52 (d) (6)	21-CH_3	1.90 (d) (6.5)
		Pyrrole α-CH_2CH_3	1.22 (t) (7.5)		
19-CH_3	0.87 (s)	19-CH_3	0.87 (s)	19-CH_3	0.85 (s)

[a] Chemical shifts in δ, $CDCl_3$, TMS internal reference; s = singlet; d = doublet; t = triplet; q = quartet; figures in parentheses are coupling constants in cycles per second. [b] *Cf.* W. R. Benn, *J. Org. Chem.*, **28**, 3557 (1963).

Table III. Uv Spectra of Pyrrolecarboxylic Acids and Their Esters

	Substituents	R	Solvent	λ_{max}, mμ (log ε)	
(1) Pyrrole-2-carboxylates		Me	EtOH	234.5 (3.82)	261 (4.22)[a]
		H	EtOH	222 (s) (3.65)	258 (4.10)[b]
	N-Me	Et	EtOH	240 (3.78)	266 (4.18)[b]
	3,4-DiMe	Et	EtOH		273 (4.16)[c]
	3,5-DiMe	Et	EtOH	240 (3.70)	276 (4.29)[d]
	3-Me, 4-Et	H	EtOH	253 (s) (3.99)	270 (4.16)[d]
(2) Pyrrole-3-carboxylates		Me	EtOH		240 (3.82)[a]
		H	EtOH		245 (3.68)[e]
	2-Me	Et	EtOH	225 (3.9)	255 (3.8)[f]
	2,4-DiMe	Et	EtOH	232 (3.93)	258 (3.72)[c]
			EtOH	232 (3.93)	259 (3.70)[d]
	4,5-DiMe	Et	EtOH	231.5 (4.00)	263.5 (3.57)[d]
	2,5-DiMe	Et	0.1 *N* HCl–MeOH	225 (3.89)	267 (3.76)
	2,4,5-TriMe	Et	MeOH	232 (3.97)	271 (3.67)
	N-2,4,5-TetraMe	Et	MeOH	240 (3.97)	269 (3.64)
	2,4-DiMe-5-Et	Et	MeOH		270 (3.66)
(3) Batrachotoxin			0.1 *N* HCl–MeOH	234 (3.99)	262 (3.70)
(4) Homobatrachotoxin			0.1 *N* HCl–MeOH	233 (3.95)	264 (3.70)

[a] M. Scrocco and L. Caglioti, *Atti Accad. Nazl. Lincei*, **24**, 316 (1958). [b] R. Andrisano and G. Pappalardo, *Gazz. Chim. Ital.*, **85**, 1430 (1955). [c] U. Eisner and P. H. Gore, *J. Chem. Soc.*, 922 (1958). [d] G. H. Cookson, *J. Chem. Soc.*, 2789 (1953). [e] M. Scrocco and R. Nicolaus, *Atti Accad. Nazl. Lincei*, **22**, 500 (1957). [f] C. A. Grob and P. Ankli, *Helv. Chim. Acta*, **32**, 2023 (1949).

and the mass spectra led to the conclusion that batrachotoxin and homobatrachotoxin probably are both derived from the same steroidal skeleton of batrachotoxinin A. The C-20 hydrogen of batrachotoxinin A (δ 4.57, quartet) is missing in (homo)batrachotoxin, and additional peaks are present in the δ 5–7 region. One of these is probably the aromatic proton (δ ~6.35) of the dialkylpyrrolecarboxylate. It is possible that the new multiplet in this region (δ ~5.8) might be the C-20 hydrogen shifted downfield due to esterification of the 20α-hydroxyl group. A broad exchangeable hydrogen which could be due to a pyrrole NH was observed at δ 8.1 ($CDCl_3$). The infrared spectrum of batrachotoxin confirms this observation: a sharp absorption band at 3314 cm^{-1} is evidence for a pyrrole NH group. The additional (ethyl) methyl resonance peaks in (homo)batrachotoxin are discussed below.

It is noteworthy that batrachotoxin and homobatrachotoxin differ from batrachotoxinin A in lacking the fragmentation pathway leading from *m/e* 202 to 158 which corresponds to the loss of C_2H_4O (see Chart I). The 20α-*p*-bromobenzoate of batrachotoxinin A also lacks this fragmentation pathway. This observation is further evidence for C-20 as the site of esterification in (homo)batrachotoxin. In this way, batrachotoxin becomes batrachotoxinin A 20-dimethylpyrrolecarboxylate and homobatrachotoxin becomes batrachotoxinin A 20-ethylmethylpyrrolecarboxylate.

Ultraviolet Absorption Spectra. Table III and Figure 5 compare the uv spectra of esters of pyrrolecarboxylic acids with those of (homo)batrachotoxin. Batrachotoxin and homobatrachotoxin resemble esters of pyrrole-3-carboxylic rather than -2-carboxylic acids.[10]

The nmr spectrum of batrachotoxin in benzene and chloroform shows a characteristic shift: one aryl methyl resonance peak (δ^{DCCl_3} 2.48) exhibited a Δ ($\Delta = \delta^{DCCl_3} - \delta^{C_6D_6}$) of +0.10 while the other aryl methyl peak (δ^{DCCl_3} 2.24) showed a Δ of −0.11 ppm. Such solvent shifts can be used to differentiate 2(5)- from 3(4)-substituted pyrroles,[11,12] *i.e.*, 3(4) substituents show positive and 2(5) substituents negative Δ values. The 3-carboxylate group would probably have a smaller effect on the Δ of 2 substituents than on the

(10) Ryanodine is an example of a natural product containing a pyrrole-2-carboxylic ester: K. Wiesner, *Collection Czech. Chem. Commun.*, **33**, 2656 (1968).
(11) J. Ronayne and D. H. Williams, *J. Chem. Soc., B*, 805 (1967).
(12) J. Ronayne and D. H. Williams, *Chem. Commun.*, 712 (1966).

Table IV. Comparison of the Effect of Solvent on the Chemical Shift of Methyl Resonance Peaks in Batrachotoxin, Homobatrachotoxin, and Pyrrolecarboxylates

Pyrrolecarboxylate derivatives	Chemical shift[f] 2 substituent	4 substituent	5 substituent	Other
Ethyl 2,4-dimethylpyrrole-3-carboxylate[a]	CH_3 2.45 (+0.07)	CH_3 2.22 (−0.24)	H 6.36 (+0.24)	
Ethyl 2,5-dimethylpyrrole-3-carboxylate[a]	CH_3 2.45 (+0.06)	H 6.13 (−0.28)	CH_3 2.17 (+0.29)	
Ethyl 2,4,5-trimethylpyrrole-3-carboxylate[b]	CH_3 2.45 (+0.08)	CH_3 2.15 (−0.23)	CH_3 2.10 (+0.30)	
Ethyl N^2,4,5-trimethylpyrrole-3-carboxylate[c]	CH_3 2.50 (+0.09)	CH_3 2.19 (−0.22)	CH_2 2.12 (+0.37)	N-CH_3 3.38 (+0.83)
Ethyl 2,4-dimethyl-5-ethylpyrrole-3-carboxylate[d]	CH_3 2.48 (+0.07)	CH_3 2.18 (−0.21)	CH_2 2.54 (+0.26)	
Ethyl 2-ethyl-4-methylpyrrole-3-carboxylate[e]	CH_2 2.95 (+0.04)	CH_3 2.27 (−0.18)	H 6.38 (+0.21)	
Ethyl 2,4-dimethyl-5-acetylpyrrole-3-carboxylate[a]	CH_3 2.50 (+0.40)	CH_3 2.55 (+0.18)		
Batrachotoxin	CH_3 2.48 (+0.10)	CH_3 2.24 (−0.11)	H 6.34 (?)	
Homobatrachotoxin	CH_2 2.98 (+0.02)	CH_3 2.24 (−0.12)	H 6.36 (?)	

[a] W. Küster, W. Weber, H. Maurer, P. Schlack, W. Niemann, K. Willig, and R. Schlayerbach, *Z. Physiol. Chem.*, **121**, 135 (1922). [b] L. Knorr and K. Hess, *Ber.*, **44**, 2762 (1911). [c] Prepared from ethyl 2,4,5-trimethylpyrrole-3-carboxylate by methylation: A. H. Corwin and W. M. Quattlebaum, Jr., *J. Am. Chem. Soc.*, **58**, 1081 (1936). [d] The ester, mp 107–108°, was prepared by catalytic reduction (10% Pd-C in glacial acetic acid) of ethyl 5-acetyl-2,4-dimethylpyrrole-3-carboxylate. [e] The ester was prepared from α-nitrosoacetone and ethyl propionylacetate following Küster's method.[a] [f] δ, $CDCl_3$, TMS; the solvent shift is in parentheses ($\delta^{CDCl_3} - \delta^{C_6D_6}$).

Δ of 4 substituents, in analogy to results with vinylcarboxylates.[13] Table IV compares solvent shifts of methyl groups in batrachotoxin, homobatrachotoxin, and various substituted ethyl pyrrolecarboxylates. This comparison of nmr spectra indicates that batrachotoxin is an ester of 2,4-dimethylpyrrole-3-carboxylic acid and that homobatrachotoxin is derived from 2-ethyl-4-methyl-3-pyrrolecarboxylic acid.

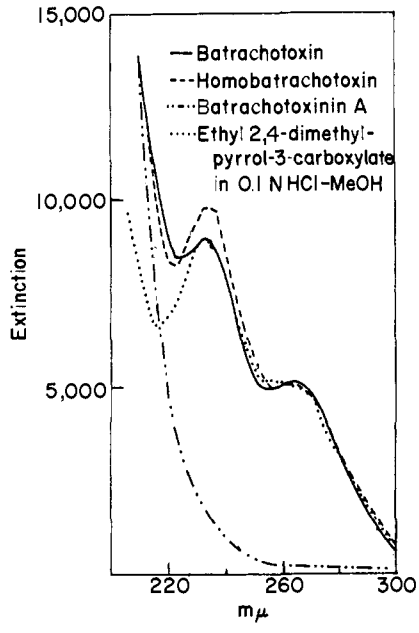


Figure 5. Comparison of the uv spectra of the three major ingredients of kokoi venom with ethyl 2,4-dimethyl-3-carboxylate. All spectra were recorded in 0.1 *N* methanolic hydrogen chloride solution.

Supporting evidence was obtained from the color reaction of batrachotoxin and ethyl 2,4-dimethylpyrrole-3-carboxylate with modified Ehrlich's reagent (*p*-dimethylaminocinnamaldehyde). The spectra of the colored solutions were identical in both cases: $\lambda_{max}^{CH_3OH}$ 420 mμ (shoulder) and 640 mμ (Figure 6).

Hydrolysis of Batrachotoxin to Batrachotoxinin A. Hydrolysis of batrachotoxin with base proved difficult. The steric hindrance of the two methyl groups was finally overcome by treatment with 2.0 *N* sodium hydroxide at 60° for 16 hr which led to partial hydrolysis and to a material identical with batrachotoxinin A by tlc and color reactions.

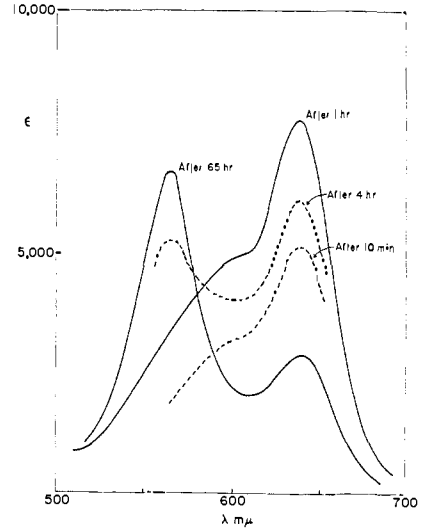
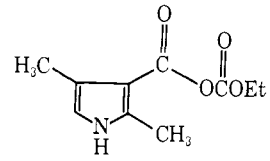


Figure 6. Uv spectra of the solutions of batrachotoxin with *p*-dimethylaminocinnamaldehyde in 0.1 *N* HCl in methanol as a function of time. The spectra were identical with those obtained with ethyl 2,4-dimethyl-3-carboxylate.

Partial Synthesis of Batrachotoxin from Batrachotoxinin A. The anhydride II of 2,4-dimethylpyrrole-3-carboxylic acid with ethyl chloroformate was prepared as described in the Experimental Section. This anhydride reacted with batrachotoxinin A (Ia) under Schotten–Bauman conditions to give a 35% (or 70% corrected) yield of a basic ester identical with batrachotoxin VI by the criteria of thin-layer chromatography, color reaction, toxicity (Table V), nmr ($DCCl_3$ and C_6D_6), and mass spectrometry.



II

(13) J. Ronayne and D. H. Williams, *J. Chem. Soc., C*, 2642 (1967).

Table V. Toxicity (LD_{50}) of Batrachotoxin and Related Compounds[a]

	LD_{50}, μg/kg
Batrachotoxin	2
Homobatrachotoxin	3
Batrachotoxinin A	1000
Batrachotoxinin A 20-(2,4-dimethylpyrrole-3-carboxylate)	2
Batrachotoxinin A 20-(2,5-dimethylpyrrole-3-carboxylate)	2.5
Batrachotoxinin A 20-(pyrrole-2-carboxylate)	>1000
Batrachotoxinin A 20-(2,4,5-trimethylpyrrole-3-carboxylate)	1
Batrachotoxinin A 20-(2,4-dimethyl-5-ethylpyrrole-3-carboxylate)	8
Batrachotoxinin A 20-(2,4-dimethyl-5-acetylpyrrole-3-carboxylate)	280
Batrachotoxinin A 20-(N,2,4,5-tetramethylpyrrole-3-carboxylate)	>1000
Batrachotoxinin A 20-*p*-bromobenzoate	>1000
Dihydrobatrachotoxin	250
4-Pregnen-3-one 20β-(pyrrole-2-carboxylate)	>10000

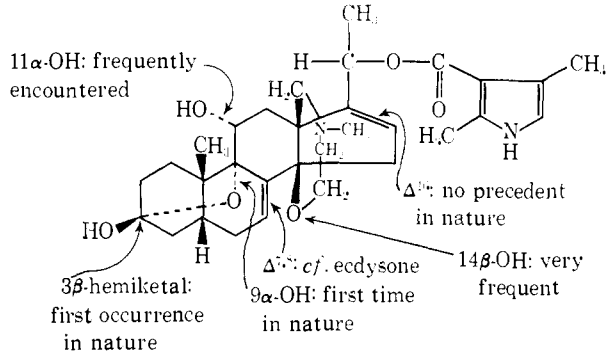
[a] Established by subcutaneous injection into 20-g NIH general purpose mice.

A variety of other anhydrides between pyrrolecarboxylic and ethylcarbonic acid reacted with batrachotoxinin A to afford analogs and homologs of batrachotoxin. Certain of these analogs were as toxic or of greater toxicity than batrachotoxin (Table V). The pyrrole-2-carboxylate of batrachotoxinin A was virtually inactive. The analogs were synthesized on a micro scale, purified by column chromatography, and identified by mass spectrometry.

The lability of batrachotoxin is largely caused by the free α position of the pyrrole moiety which is easily subject to autoxidation and deterioration by the action of acid. Accordingly, the ester of batrachotoxinin A with 2,4,5-trimethylpyrrole-3-carboxylic acid was synthesized. The new homolog of batrachotoxin had twice the activity, presumably because the much more stable derivative survives in the organism longer and more of it reaches the site of action. Substitution by methyl groups in the 1,2,4,5-tetramethyl analog essentially abolishes activity. The free pyrrole NH group may be required for activity, possibly as a site for chelation with an important ion.

Reduction of batrachotoxin or homobatrachotoxin with sodium borohydride in absolute ethanol leads to dihydro compounds which have higher R_f values on tlc (silica gel) than the original venoms. Each dihydro compound on treatment with acid is isomerized to a new dihydro derivative with a much lower R_f value. Likewise batrachotoxinin A gives two dihydro products on borohydride reduction, indicative of the facile isomerization of the newly generated alcohol. Presumably these dihydro products are formed by reduction of the hemiketal IV → V, as shown in Chart II. The dihydro derivative V of batrachotoxin was found to retain only $^1/_{200}$ of the original toxicity. Apparently the intact 3β-hydroxy-3α,9α-oxido arrangement is important for the biological activity of batrachotoxin.

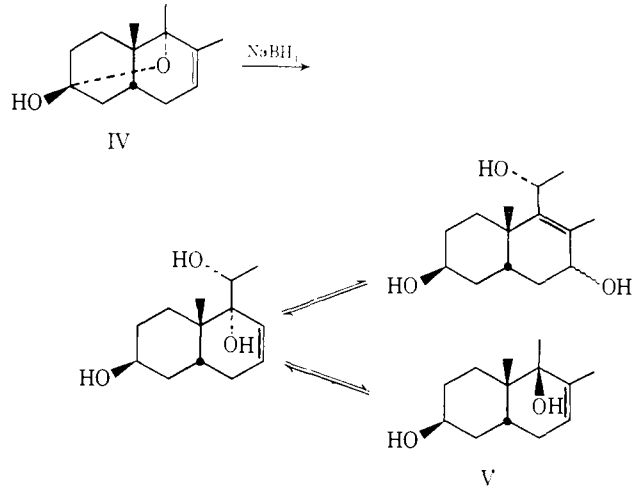
Comparative Structural Features. Certain features of the structure of batrachotoxin (VI) have never been



VI

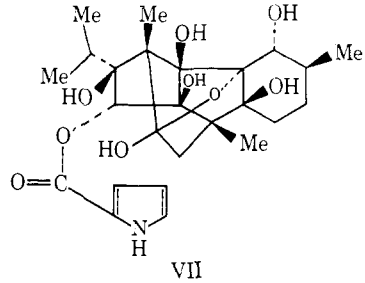
observed before in a naturally occurring pregnane derivative: (i) the 9α-OH, (ii) the 3α,9α-oxide, (iii) the 3β-hemiketal, (iv) the seven-membered 14β,18β-heterocyclic ring encompassing N-methylaminoethanol, a precursor of choline, (v) the Δ^{16} unsaturation, and (vi) the comparable pyrrole ester attached to a 20α-OH.

Chart II

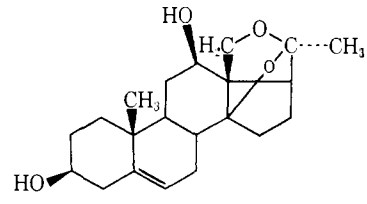


IV

V



VII



VIII

The only other naturally occurring pyrrole ester is ryanodine (VII),[10,14] which has some pharmacological properties in common with batrachotoxin.[15]

The general stereochemistry of the rings, A/B-*cis*, C/D-*cis* junctions, and of the side chain is in keeping with the cardenolide glycosides[16] and bufodienolides.[2] A connection between 14β-OH and 18β-CH_2 has been postulated for stapelogenin VIII.[17]

Biosynthetic Considerations. Colombian poison arrow frogs have been maintained in terrariums for over 3 years, and a number of young have been raised. The amount of poison in these frogs in markedly reduced from that found in the natural state. Initial biosynthetic studies have been unsuccessful, perhaps due to the slow rate of synthesis of batrachotoxin. Radioactive serine, a possible precursor of the methylaminoethanol, was not incorporated to a measurable extent during a period of 2 weeks. Long-term administration of radioactive precursors will probably be required. In this way radioactive cholesterol was recently demonstrated as a precursor of the salamander alkaloids.[18]

Pharmacology. The chemistry and structural features, unusual as they may be, are eclipsed by the pharmacology of batrachotoxin, which appears to be a most valuable tool for the study of ion equilibria across membranes and for a better understanding of several fundamental processes in neurophysiology.[19]

Phyllogenetic Considerations. The presence of batrachotoxin and congeners may be a characteristic of frogs of the genus *Phyllobates*.[20] Such compounds have now been identified from *Phyllobates aurotaenia* (Choco, Colombia) and *P. vittatus* (southwestern Costa Rica), and, tentatively, from *P. lugubris* (Bocas del Toro, Panama). Batrachotoxin has not been detected in frogs of the related genera, *Dendrobates* or *Colostethus* (*Prostherapis*).[21,22] Instead, frogs of the genus *Dendrobates* contain another class of alkaloids[21-23] which appear to be substituted octa- and decahydroquinolines.[24]

Experimental Section

Mass spectral data were obtained on an AEI MS-9 or a Hitachi RMU-6D mass spectrometer. Nmr data were measured with a Varian A-60 or HA-100 instrument.

Separation and Purification of Steroidal Alkaloids from *Phyllobates aurotaenia*. Fresh skins from 5000 adult *Phyllobates aurotaenia* were extracted three times with methanol (total volume 20 l.). The methanol extracts were concentrated cautiously *in vacuo* at 30° to approximately 5 l. This extract was chilled to 5°. All subsequent partitions were carried out at this temperature. After dilution with three volumes of water the aqueous methanol solution was extracted with chloroform. The chloroform solution was extracted with cold 0.2 *N* hydrochloric acid. The acidic extract was made alkaline (pH ~10) with 1.0 *N* ammonium hydroxide and again extracted with chloroform. Concentration of the chloroform extract *in vacuo* yielded 157 mg of crude bases. Two-dimensional tlc of this fraction gave Ehrlich-positive (marked with +) and Ehrlich-negative spots as shown in Figure 7. Chromatography on a silica gel column (35 cm × 28 mm) with cyclohexane, chloroform, triethylamine, and methanol (16:4:1:1) gave four major fractions consisting of pooled individual fractions of 4 ml each. Fraction A (11–20) contained 40 mg of nontoxic material which was not further investigated. Fraction B (29–38) consisted of 28 mg of fairly pure homobatrachotoxin. Fraction C (40–57) consisted of 43 mg of batrachotoxin, pseudobatrachotoxin, and trace amounts of batrachotoxinin A. Fraction D (58–85) consisted of 39 mg of almost pure batrachotoxinin A which after rechromatography in chloroform–methanol (9:1) yielded 34 mg of the crystalline carbonate, mp 222–224° (from acetone).

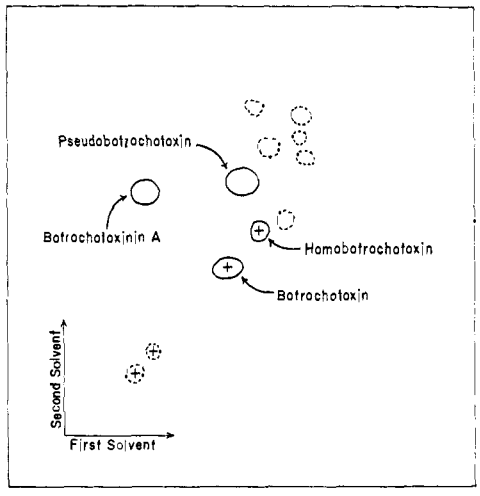


Figure 7. Two-dimensional tlc of the crude venom obtained from the original methanol extract of the frog skins by fractionation with chloroform. The spots marked + were Ehrlich positive. First solvent system: cyclohexane–chloroform–triethylamine–methanol, 5:4:1:1. Second solvent system: chloroform–methanol, 7:1.

Fraction B, purified by chromatography on a silica gel column (0.7 g) with chloroform containing 4% methanol, yielded 16 mg of pure homobatrachotoxin.

Fraction C was purified twice by column chromatography, first in chloroform containing 6% methanol and then in cyclohexane–chloroform–triethylamine–methanol (20:4:1:1) to yield 11 mg of batrachotoxin and 8 mg of a mixture of pseudobatrachotoxin and batrachotoxinin A. The latter fraction was rechromatographed using chloroform containing 6% methanol to yield 1 mg of pure pseudobatrachotoxin and 7–8 mg of batrachotoxinin A. The low recovery of pseudobatrachotoxin is due to the ease with which it converts to batrachotoxinin A during purification.

20α-*p*-Bromobenzoate of Batrachotoxinin A. To the solution of 5 mg of batrachotoxinin A and 20 mg of *p*-bromobenzoic anhydride in 10 ml of chloroform was added 1 ml of 0.4 *N* aqueous sodium hydroxide solution, and the mixture was vigorously shaken at room temperature for 3 hr under nitrogen. The basic product was extracted from the chloroform solution with dilute hydrochloric acid. The acid extract was made alkaline with aqueous ammonia and reextracted into chloroform. The basic product (~7 mg) was chromatographed on a column of silica gel (0.75 g) with chloroform containing 3% methanol. The first fractions contained 55 mg of crystalline batrachotoxinin A 20α-*p*-bromobenzoate which was recrystallized three times from acetone–cyclohexane to give colorless needles, mp 213°. From these needles was selected the single crystal for complete X-ray crystallography.[7]

Sodium Borohydride Reduction of Batrachotoxin. To a solution of 0.2 mg of batrachotoxin in 0.5 ml of absolute ethanol was added a trace amount of solid sodium borohydride, and the mixture was kept for 30 hr at room temperature. There was no change in the uv spectrum during this time. The residue was then removed by a stream of nitrogen dissolved in water and extracted into chloroform. Tlc of the extract showed the quantitative conversion to

(14) K. Wiesner, Z. Valenta, and J. A. Findlay, *Tetrahedron Letters*, 221 (1967).
(15) E. X. Albuquerque, personal communication.
(16) T. Reichstein, *Naturwissenschaften*, **54**, 53 (1967).
(17) U. Eppenberger, W. Vetter, and T. Reichstein, *Helv. Chim. Acta*, **49**, 1505 (1966).
(18) G. Habermehl and A. Haaf, *Ber.*, **101**, 198 (1968).
(19) E. X. Albuquerque, J. Warnick, J. Daly, and B. Witkop, to be presented at the Fourth International Congress of Pharmacology, Basel, July 14–18, 1969.
(20) C. W. Myers and J. W. Daly, in preparation.
(21) J. W. Daly and B. Witkop, *Mem. Inst. Butantan* (Sao Paulo), 33 (2), 425 (1966).
(22) J. W. Daly and B. Witkop, "Venomous Animals and Their Venoms," Vol. III, W. Bücherl, E. E. Buckley, and V. Deulofeu, Ed., Academic Press, New York, N. Y., in press.
(23) J. W. Daly and C. W. Myers, *Science*, **156**, 970 (1967).
(24) J. W. Daly, T. Tokuyama, G. Habermehl, I. L. Karle, and B. Witkop, in preparation.

dihydrobatrachotoxin, R_f 0.55 in chloroform–methanol (7:1) on silica gel. Dihydrobatrachotoxin on treatment with dilute hydrochloric acid at room temperature easily changed to an equilibrium mixture containing a new isomer, R_f 0.39. The mass spectrum of isodihydrobatrachotoxin showed peaks at *m/e* 401, 314, 312, 296, 278, 263, 245, 235, 184, 139, 94, and 71. The mass spectra of dihydrobatrachotoxin showed peaks at 401, 386, 353, 341, 288, 285, 239, 95, and 71.

Borohydride Reduction of Homobatrachotoxin and Batrachotoxinin A. Analogously homobatrachotoxin and batrachotoxinin A were reduced to the corresponding dihydro derivatives. The R_f values of these products were as follows: homobatrachotoxin, 0.52; dihydrohomobatrachotoxin, 0.57; isodihydrohomobatrachotoxin, 0.45; batrachotoxinin A, 0.40; dihydrobatrachotoxinin A, 0.51; isodihydrobatrachotoxinin A, 0.26.

Hydrolysis of Batrachotoxin to Batrachotoxinin A. A solution of 0.5 mg of batrachotoxin was dissolved in 0.5 ml of 4.0 *N* aqueous methanolic (1:1) potassium hydroxide and heated to 60° in a sealed tube under a nitrogen atmosphere for 16 hr. Methanol was removed by a stream of nitrogen. The residue was extracted into chloroform. The chloroform solution was extracted with dilute hydrochloric acid. The acid solution was made alkaline by the addition of ammonia solution and extracted with chloroform.

The presence of batrachotoxinin A was confirmed by tlc (silica gel) in chloroform–methanol (7:1).

Anhydride II of 2,4,5-Trimethylpyrrole-3-carboxylic Acid and Ethyl Chloroformate. 2,4,5-Trimethylpyrrole-3-carboxylic acid (910 mg, 6×10^{-3} mol) and 0.60 g (6×10^{-3} mol) of triethylamine were dissolved in 10 ml of dry tetrahydrofuran. When the reaction mixture was kept below 0° a solution of 0.65 g (6×10^{-3} mol) of ethyl chloroformate in 3 ml of tetrahydrofuran was added dropwise and the reaction mixture was allowed to stand for 5 hr. After evaporation of the solvent *in vacuo*, the residue was extracted into benzene and washed with dilute hydrochloric acid and sodium bicarbonate. The solvent was removed by distillation and the residue was recrystallized from benzene–cyclohexane to give 1.15 g of the anhydride, mp 93°; ir spectrum in chloroform solution, ν (cm^{-1}) 1795, 1724 (CO); nmr spectrum (δ, $CDCl_3$, TMS), 2-CH_3, 2.44; 4-CH_3, 2.12; 5-CH_3, 2.10; ethoxyl group, 2.51 and 1.13.

Anal. Calcd for $C_{12}H_{15}NO_5$: C, 58.66; H, 6.71; N, 6.22. Found: C, 58.85; H, 6.46; N, 6.14.

The following acid anhydrides were prepared by the method described above: mixed anhydride of ethyl chlorocarbonate with (mp) 2,4-dimethylpyrrole-3-carboxylic acid (88–90°), 2,5-dimethylpyrrole-3-carboxylic acid (75–76.5°), 1,2,4,5-tetramethylpyrrole-3-carboxylic acid (oil), 2,4-dimethyl-5-ethylpyrrole-3-carboxylic acid (58°), and 2,4-dimethyl-5-acetylpyrrole-3-carboxylic acid (96–97°).

Acylations of Batrachotoxinin A. About 0.8 mg of batrachotoxinin A was used for each acylation experiment. Eight milligrams of the anhydride of the respective pyrrole-3-carboxylic acid (see footnotes to Tables III and IV) and ethyl chloroformate was added to a chloroform solution (1 ml) of batrachotoxinin A and, after the addition of 1 ml of 0.2 *N* sodium hydroxide solution, the mixture was stirred vigorously for 4 hr at room temperature. The chloroform layer was separated and washed with 0.3 *N* hydrochloric acid. The acid solution was made basic with ammonia and the reaction product was extracted into chloroform. The reaction products were purified by chromatography on a column (height 13 cm) containing 1.5 g of silica gel and eluted by a mixture of chloroform and methanol, 9:1. Fractions were collected after every 0.6 ml (60 drops). Batrachotoxinin A 20α esters (0.1–0.3 mg, 35%) appeared generally in fractions 11–13, while recovered batrachotoxinin A (~55%) showed up in fractions 16–23. The batrachotoxinin A 20α esters were identified by mass spectrometry.

With the anhydrides of 2,4,5-trimethyl-, 1,2,4,5-tetramethyl-, and 2,4-dimethyl-5-ethylpyrrole-3-carboxylic acids the yields in the esterification of batrachotoxinin A were quantitative. Esterification with the acid chloride of pyrrole-2-carboxylic acid gave about a 30% yield. The preparation of acid chlorides of 2,4- and 2,5-dimethylpyrrolecarboxylic acids, from a benzene solution of the acids with thionyl chloride, phosphorus oxychloride, and oxalyl chloride in the presence of triethylamine, failed. The symmetric anhydride of 2,5-dimethylpyrrole-3-carboxylic acid was prepared from the anhydride with ethyl chloroformate and excess free acid by heating in dioxane overnight with triethylamine present. The oily symmetric anhydride [ν (cm^{-1}) 3472 (NH), 1751, 1718 (CO); nmr (δ, $CDCl_3$), 2.13, 2.44, no signals for OCH_2CH_3] failed to esterify batrachotoxinin A in chloroform solution under Schotten–Baumann conditions.

Synthesis of Batrachotoxin. Eighteen milligrams of batrachotoxinin A and 170 mg of anhydride II (8.2 molar excess) were dissolved in 3 ml of chloroform and stirred vigorously with 3.0 ml of 0.2 *N* potassium hydroxide solution for 5 hr at room temperature. The chloroform layer was separated and extracted with 0.3 *N* hydrochloric acid. The aqueous phase was made basic with ammonia and was extracted with chloroform. The chloroform was removed *in vacuo* and the 16 mg of basic products was purified over a column containing 14 g of silica gel (height 18 cm). The eluting solvent consisted of a mixture of chloroform and methanol, 9:1. Each fraction consisted of 1.1 ml. Synthetic batrachotoxin (5 mg) appeared in fractions 21–24 and unreacted batrachotoxinin A (8 mg) was isolated after fraction 50. The synthetic batrachotoxin was identified by tlc, mass spectrometry, and nmr in deuteriochloroform and deuteriobenzene and by its unique toxicity, LD_{50} 2 μg/kg in mice.

Communications to the Editor

Electron Spin Resonance of Organosilyl Radicals in Solution

Sir:

Organosilicon chemistry encompasses a wide range of reactions and mechanisms.[1] Silicon-centered free radicals play important roles in a number of these processes,[2] particularly those involving the hydrosilation of unsaturated systems.[3] Except for silyl radicals[4] (which have only been observed in an argon or krypton matrix at 4.2°K), no direct evidence is available for these

(1) L. H. Sommer, "Stereochemistry, Mechanism and Silicon," McGraw-Hill Book Co., New York, N. Y., 1965.

(2) C. Eaborn, "Organosilicon Compounds," Butterworths Scientific Publications, London, 1960, pp 45 ff, 116; V. Bazant, J. Joklik, and J. Rathousky, *Angew. Chem.*, **80**, 133 (1968); J. J. Zuckermann, *Advan. Inorg. Chem. Radiochem.*, 6, 383 (1964); D. Seyferth and R. B. King, *Ann. Survey Organometal. Chem.*, **2**, 110 (1966); J. Curtice, H. Gilman, and G. S. Hammond, *J. Am. Chem. Soc.*, **79**, 4754 (1957); H. Sakurai, A. Hosomi, and M. Kumada, *Bull. Chem. Soc. Japan*, **40**, 1551(1967); L. E. Nelson, N. C. Agelotti, and D. R. Weyenberg, *J. Am. Chem. Soc.*, **85**, 2663 (1963); C. Eaborn, R. A. Jackson, and R. W. Walsingham, *Chem. Commun.*, 300 (1965); 920 (1967), *J. Organometal. Chem.*, **5**, 297 (1966); M. A. Nay, G. N. C. Woodall, O. P. Strausz, and H. E. Gunning, *J. Am. Chem. Soc.*, **87**, 179 (1965); N. J. Friswell and B. G. Gowenlock, *Advan. Free Radical Chem.*, **2**, 43 (1967); Y. Nagai, K. Yamazaki, *et al.*, *J. Chem. Soc., Japan, Pure Chem. Sect.*, **88**, 79 (1967); H. Sakurai, M. Murakami, and K. Kumada, *J. Am. Chem. Soc.*, **91**, 520 (1969).

(3) E. Y. Lukevits and M. G. Voronkov, "Organic Insertion Reactions of Group IV Elements," Consultants Bureau, New York, N. Y., 1966; T. G. Selin and R. West, *J. Am. Chem. Soc.*, **84**, 1860 (1962).

(4) (a) F. J. Adrian, E. L. Cochran, and V. A. Bowers, "Free Radicals in Inorganic Chemistry," Advances in Chemistry Series, No. 36, American Chemical Society, Washington, D. C., 1962 p 50 ff; (b) R. L. Morehouse, J. J. Christiansen, and W. Gordy, *J. Chem. Phys.*, **45**, 1751 (1966); (c) the esr of stable radical ions containing Si has been reviewed recently: G. Urry, "Radical Ions," T. T. Kaiser and L. Kevans, Ed., Interscience Publishers, Inc., New York, N. Y., 1968, p 275 ff; (d) A. G. Brook and J. H. Duff, *J. Am. Chem. Soc.*, **91**, 2119 (1969); (e) H. Sakurai, M. Murakami, and K. Kumada, *ibid.*, **91**, 519 (1969).