Alkylating Derivatives of Amino Acids and Peptides. Synthesis of *N*-Maleoylamino Acids,[1] [1-(*N*-Maleoylglycyl)cysteinyl]oxytocin, and [1-(*N*-Maleoyl-11-aminoundecanoyl)cysteinyl]oxytocin. Effects on Vasopressin-Stimulated Water Loss from Isolated Toad Bladder†

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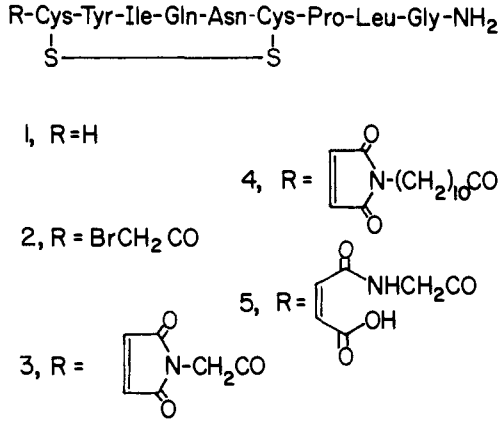
A method for the preparation of *N*-maleoylamino acids and esters is reported. These compounds were shown to inhibit both the oxytocin-induced smooth muscle contraction in the isolated rat uterus and the vasopressin-induced water loss from the isolated toad bladder. The inhibitory ability of the maleimides in the toad bladder assay was found to be related to their corresponding partition coefficients by the equation: $\log 1/C = -0.055 (\log P)^2 + 0.227 \log P + 3.96$. *N*-Maleoylamino acids can be coupled to peptides to form alkylating reagents which react rapidly with sulfhydryl groups. The synthesis of [1-(*N*-maleoylglycyl)cysteinyl]oxytocin (**3**) and [1-(*N*-maleoyl-11-aminoundecanoyl)cysteinyl]oxytocin (**4**) as potential affinity labeling reagents is described. These oxytocin analogs were shown to readily react with sulfhydryl-containing compounds; however, neither **3** nor **4** was seen to inhibit in the rat uterus assay at concentrations up to 3×10^{-5} *M*. When tested on the mucosal and serosal surfaces of the toad bladder, assay inhibition was seen only on the mucosal surface. These results are discussed with respect to the possible existence of sulfhydryl groups at neurohypophyseal receptors.

The binding of a hormone to its receptor initiates a complex sequence of events which ultimately produce a biological response. In the case of the neurohypophyseal hormones these responses are inhibited by the reagents *N*-ethylmaleimide (NEM)§ and *p*-chloromercuribenzoate (PCMB) which bind sulfhydryl groups.[2] These reagents inhibit the vasopressin-stimulated water loss from the isolated toad bladder and the oxytocin (**1**) induced contraction of the isolated rat uterus.[3] In rat uterus, the degree of inhibition produced by PCMB could be reduced by incubating the uterus with oxytocin prior to the addition of PCMB. Furthermore, the inhibition could be substantially reversed by treating the alkylated tissue with cysteine.[4] These results established that certain sulfhydryl groups are essential for the neurohypophyseal hormone induced responses and suggested that preincubation of tissue with hormone protected sulfhydryls from alkylation. Inhibition of metabolism did not appear to be important since this became evident only after periods of exposure to the reagent which were longer than those required for inhibition.[3]

The possibility has existed that the essential sulfhydryl groups, alkylated by NEM and PCMB, are at or near the oxytocic receptor as well as elsewhere in rat uterus. The finding that bromoacetyl-oxytocin (**2**), BrOxy, irreversibly inhibited the vasopressin receptor in toad bladder established that nucleophiles are present at or near this receptor.[5] Thus it was of interest to determine if these nucleophilic groups were sulfhydryl groups and to determine whether there are any essential sulfhydryl groups present on the serosal membrane surface of the toad bladder or on the surface of the rat uterus membrane.

To study the nature of these essential sulfhydryl groups we developed a method for preparing the maleimide derivatives of oxytocin: [1-(*N*-maleoylglycyl)cysteinyl]oxytocin (**3**), Mal-Gly-Oxy, and [1-(*N*-maleoyl-11-aminoundecanoyl)cysteinyl]oxytocin (**4**), Mal-AUA-Oxy. These maleimides, unlike NEM or PCMB, should possess a relatively high affinity for the receptors and, because the maleimide group reacts preferentially (although not exclusively) with sulfhydryl groups,[6] **3** and **4** should inhibit responses to oxytocin or vasopressin only if alkylatable sulfhydryl groups are present at the receptor. We report here the synthesis of the *N*-maleoylamino acids **6–14**, **20**, **21**, and **23**. Maleimides **6** and **12** were used to prepare the oxytocin derivatives **3** and **4**. The biological activities of the *N*-maleoylamino acids **6–14**, their esters **15–19**, and the oxytocin derivatives **3** and **4** on the oxytocin- and vasopressin-stimulated responses of the rat uterus and toad bladder are described. A correlation between biological activity in the toad bladder



assay and partition coefficient for compounds **6–19** is determined. The activities of all derivatives are evaluated in terms of the possible existence of sulfhydryl groups at the neurohypophyseal receptors.

Results

***N*-Maleoylamino Acids and Esters (Table I).** *N*-Maleoylamino acids **6–13** were prepared by refluxing a solution of the corresponding maleamic acids **24–31** (Table II) and triethylamine in toluene or benzene. The best yields were obtained using at least 2 equiv of the amine. It was possible to prepare optically active *N*-maleoylamino acids by this method (e.g., **20** and **21**). Maleamide **14** could not be prepared by this route and instead was prepared by a standard fusion technique.[7] No *N*-maleoylamino acids were formed when the maleamic acids **6–13** were subjected to several other standard cyclization procedures.[8] These results are consistent with other unsuccessful attempts to prepare compound **6**.[9]

The *N*-maleoylamino esters **15–19** were prepared by re-

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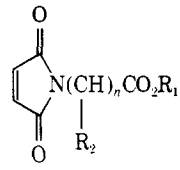
‡AFPE Edwin Leigh Newcomb Memorial Fellow, 1974–1975.

§Abbreviations used are NEM, *N*-ethylmaleimide; PCMB, *p*-chloromercuribenzoate; Mal-Gly, *N*-maleoylglycine; Mal-GlyOEt, *N*-maleoylglycine ethyl ester; Mal-AUA, *N*-maleoyl-11-aminoundecanoic acid; Mal-Gly-Oxy, [1-(*N*-maleoylglycyl)cysteinyl]oxytocin; Mal-AUA-Oxy, [1-(*N*-maleoyl-11-aminoundecanoyl)cysteinyl]oxytocin; BrOxy, bromoacetyl-oxytocin; DMF, dimethylformamide, DCC, dicyclohexylcarbodiimide; HOBt, 1-hydroxybenzotriazole; Me_2SO, dimethyl sulfoxide.

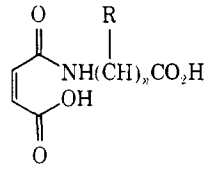
Table I. *N*-Maleoylamino Acids and Esters



Compd	*n*	R_1	R_2	Mp, °C	Purification method[a]	Yield, %	Formula[b]
6	1	H	H	113–113.5	A	46	$C_6H_5NO_4$
7	2	H	H	105–105.5	B	57	$C_7H_7NO_4$
8	3	H	H	90–92	B	40	$C_8H_9NO_4$
9	4	H	H	82–83	C	49	$C_9H_{11}NO_4$
10	5	H	H	79–80	C	45	$C_{10}H_{13}NO_4$
11	7	H	H	72–75	C	41	$C_{12}H_{17}NO_4$
12	10	H	H	84.5–86	C	33	$C_{15}H_{23}NO_4$
13	11	H	H	81–84	C	46	$C_{16}H_{25}NO_4$
14[c]				51.5–54	A	22	$C_{22}H_{37}NO_4$
15	1	C_2H_5	H	*d*	D	10	$C_8H_9NO_4$
16	2	C_2H_5	H	*d*	D	20	$C_9H_{11}NO_4$
17	3	C_2H_5	H	30.5–32	D	14	$C_{10}H_{13}NO_4$
18	5	C_2H_5	H	*d*	D	59	$C_{12}H_{17}NO_4$
19	10	C_2H_5	H	31.5–32	D	80	$C_{17}H_{27}NO_4$
20[e]	1	H	CH_3	97–98	C	57	$C_7H_7NO_4$
21[f]	1	H	$CH_2C_6H_5$	119–121	C	55	$C_{13}H_{11}NO_4$
22[g]	1	CH_3	$CH_2CH(CH_3)_2$	38–43	C	22	$C_{11}H_{15}NO_4$
23[h]	1	H	$CH_2CH_2SCH_3$	83–85	D	80	$C_9H_{11}NO_4S$

[a]Recrystallization solvents: A, $CHCl_3$; B, $CHCl_3$–*n*-hexane; C, EtOAc–*n*-hexane; D represents a molecular distillation. [b]Compound 14 was analyzed for C and H. All other compounds were analyzed for C, H, and N. All results were within ±0.3% of the calculated values. [c]Compound is *N*-maleoyl-12-aminooctadecanoic acid. [d]Oil. [e]$[\alpha]^{22}D$ −16.3° (*c* 1.10, EtOH0. [f]$[\alpha]^{22}D$ −108° (*c* 1.10, EtOH). [g]$[\alpha]^{22}D$ −24.2° (*c* 1.27, EtOH). [h]Synthesized from DL-methionine.

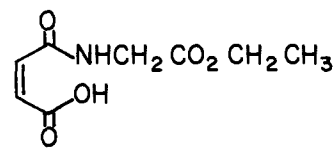
Table II. Maleamic Acids



Compd	*n*	R	Mp, °C	Recrystn solvent[a]	Yield, %	Formula[b]
24	1	H	187–188.5	A	96.5	$C_6H_7NO_5$
25	2	H	159.5–160	A	92.0	$C_7H_9NO_5$
26	3	H	161.5–162.5	A	87.1	$C_8H_{11}NO_5$
27	4	H	160–160.5	A	91.2	$C_9H_{13}NO_5$
28	5	H	159.5–161	B	93.6	$C_{10}H_{15}NO_5$
29	7	H	162–163	C	91.2	$C_{12}H_{19}NO_5$
30	10	H	152–153	C	97.1	$C_{15}H_{25}NO_5$
31	11	H	152–153	C	90.9	$C_{16}H_{27}NO_5$
32[c]	1	CH_3	142–143	D	100	$C_7H_9NO_5$
33[d]	1	$CH_2C_6H_5$	128–128.5	D	100	$C_{13}H_{13}NO_5$
34[e]	1	$CH_2CH_2SCH_3$	153–154.5	A	94.9	$C_9H_{13}NO_5S$

[a]Recrystallization solvents: A, H_2O; B, MeOH; C, EtOH; D, MeOH–Et_2O. [b]Compounds analyzed for C, H, and N. All results were within ±0.3% of the theoretical values. [c]Synthesized from L-alanine. [d]Synthesized from L-phenylalanine. [e]Synthesized from DL-methionine.

fluxing an acetic acid solution of the amino ester and maleic anhydride.[10] The optically active derivative of leucine (**22**) was prepared by this method. *N*-Maleoylamino acids could not be prepared from the esters **15–19**, as treatment of the *N*-maleoyl ester **15** with dilute ethanolic sodium hydroxide gave only the maleamic ester **37** due to preferential reaction of hydroxide with maleimides.[11] Ester **37** can also be prepared by reaction of maleic anhydride with glycine ethyl ester in chloroform.



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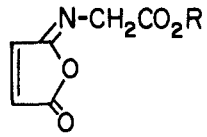
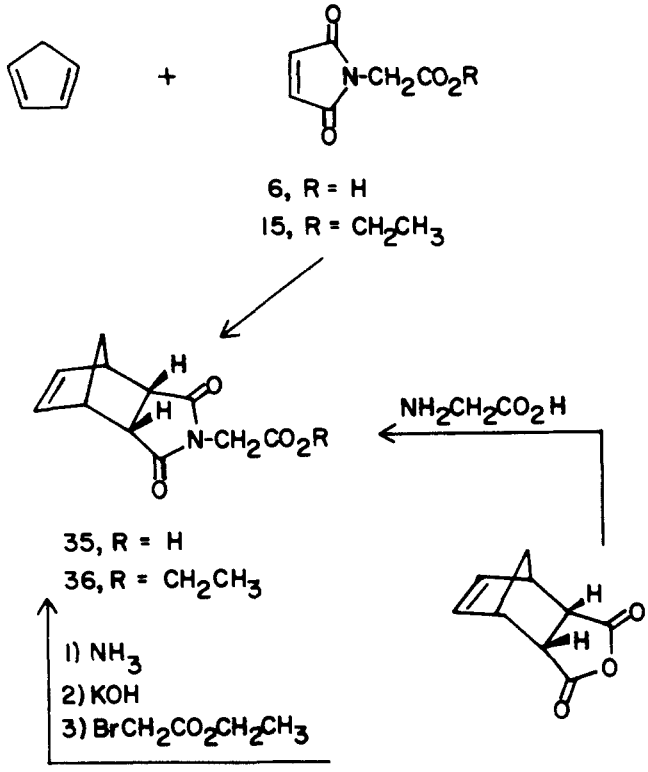
The structures of the cyclization products **6** and **15** were shown to be maleimides and not the kinetically favored isomalemides **38** and **39**.[12] The olefinic protons of both acids **6–14** and esters **15–19** appeared as sharp singlets in the NMR spectrum (at ca. 7 ppm) consistent with the symmet-



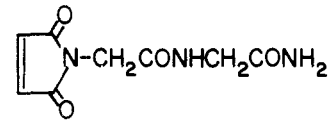
38, R = H
39, R = CH_2CH_3

rical maleimide structure.[13] Furthermore, both acid 6 and ester 15 reacted with cyclopentadiene to give adducts 35 and 36 which were identical with samples synthesized independently[14] (Scheme I).

Scheme I



Properties of the *N*-maleoylamino acids and esters are shown in Table I. *N*-Maleoylamino acids can be coupled with acylable peptides using DCC–HOBt[15] (e.g., **40**). The *N*-maleoyl derivatives were shown by NMR to alkylate the sulfhydryl compounds glutathione and benzylmercaptan.



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Mal-Gly-Oxy (3) and Mal-AUA-Oxy (4). Compounds 3 and 4 were prepared by acylation of highly purified synthetic oxytocin (1) with DCC, HOBt, Mal-Gly (6), and Mal-AUA (12), respectively. Both analogs were purified by gel filtration through Sephadex G-25. The use of partition chromatography over either G-25 or LH-20 for purification was not successful. The purified analogs 3 and 4 were shown to migrate as single spots well resolved from oxytocin itself in several solvent systems and were characterized by amino acid analysis, TLC, and NMR. The NMR data showed that compound 3 was a mixture of maleimide 3 and maleamic acid 5. The maleamic acid 5 is probably formed to some extent during the synthesis of 3 due to the known base lability of the maleimide ring system.[11] A sample of *N*-maleoylglycylglycinamide (**40**) was stable for at least 24 hr in the pH 7.4 buffer used during bioassays and therefore the maleimide rings of 3 and 4 were not hydrolyzed during the bioassay. No maleamic acid was formed during the synthesis of 4. Benzylmercaptan added to both maleoylamino acid derivatives 3 and 4 in a normal fashion as shown by the rapid disappearance of the maleimide olefinic protons in the NMR spectrum. BrOxy (2) was prepared by the method of Walter et al.[5]

Biological Results. The *N*-maleoyl acids 6–14 and esters 15–19 inhibited the vasopressin-induced water loss from the isolated toad bladder. Although most of the compounds are highly active at concentrations approximating 1 m*M*, the concentration at which NEM completely inhibits the system,[3a] acid 6 shows only weak activity at 10 m*M* and requires a concentration of greater than 40 m*M* to completely inhibit the system. A similar result was observed in

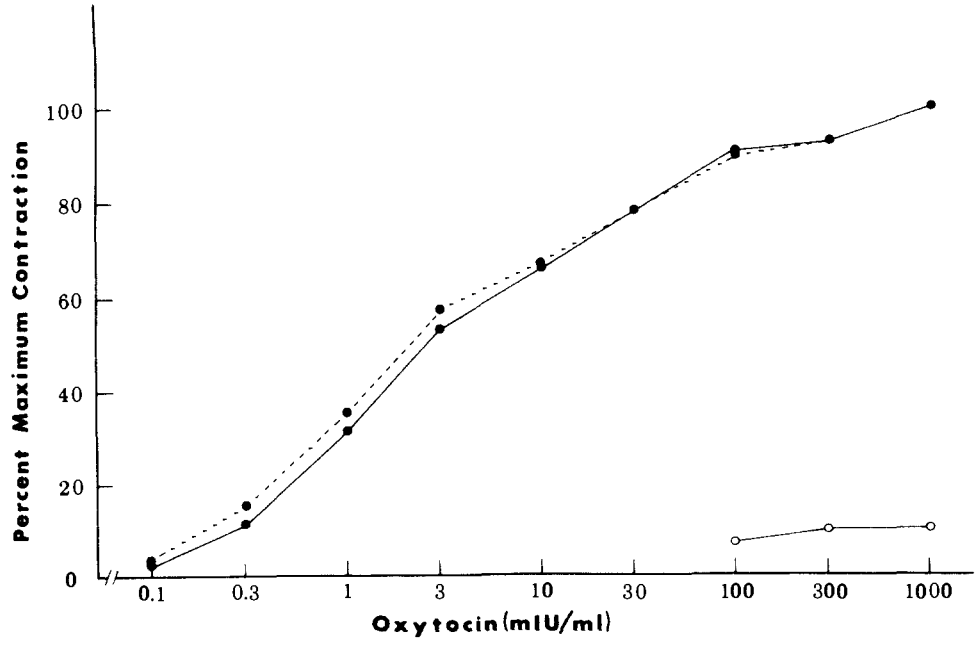

Figure 1. Dose–response curves (mean of three determinations) for oxytocin-induced contractions in isolated rat uterus: control (●—●), Mal-Gly (●- - - -●), and Mal-GlyOEt (○—○). Inhibitor concentrations, 3×10^{-4} *M*.

the inhibition of the oxytocin-induced contractions in the isolated rat uterus by Mal-Gly (**6**) and Mal-GlyOEt (**15**) (Figure 1). Preliminary analysis of the physical properties of compounds **6–19** suggested that the low activity of acid **6** was related to its lipophilicity. A quantitative study was undertaken to establish the nature of the relationship between lipophilicity and activity of all maleimides **6–19** and to determine if lipophilicity adequately accounted for the low potency of Mal-Gly (**6**). Several of the long-chain compounds were synthesized for this purpose only. Maleimides **6–19** were assayed to determine concentrations which would produce a standard inhibition (65%) of the vasopressin-induced water loss. Partition coefficients of maleoyl esters **15–19** were determined in an octanol–water system; partition coefficients of maleoyl acids **6–11** were determined in an octanol–water system buffered to the pK_a of the acid according to the method of Hansch[16] and converted to values prevailing at pH 7.4, the pH of the buffer used in the assay. These results are shown in Table III.

The data in Table III were subjected to a forward stepwise regression analysis. The first equation generated by the regression analysis was linear (eq 1); however, the final or best fit regression was parabolic (eq 2) and was significant at greater than the 99.9% level of significance as determined by the F statistic: $F_{2,11} = 389.08$, $F_{2,11;\alpha 0.001} = 13.81$. The statistical parameters are defined: n is the number of N-maleoyl derivatives assayed, r is the multiple correlation coefficient, and s is the standard error of estimate for the regression. The values in parentheses are the 95% confidence limits. Log P_0 is defined as the ideal lipophilic character and is generated from eq 2 in the usual fashion.[17]

Table III. Physical and Biological Data for N-Maleoylamino Acids and Esters

Compd	pK_a	Partition coeff P	Log $P_{7.4}$[b]	Log $1/C$[a] Obsd[c]	Log $1/C$[a] Calcd[d]	Δ log $1/C$
6	2.88	0.36[e]	−4.96	1.57	1.48	0.09
7	4.18	0.55[e]	−3.48	2.34	2.50	0.16
8	4.56	1.26[e]	−2.74	2.95	2.92	0.03
9	4.62	1.97[e]	−2.49	3.04	3.05	0.01
10	4.72	7.12[e]	−1.84	3.35	3.36	0.01
11	4.90[f]	49.0[e]	−0.81	3.73	3.74	0.01
12	4.90[f]		0.69[g]	4.00	4.09	0.09
13	4.90[f]		1.19[g]	4.06	4.15	0.09
14	4.90[f]		3.99[g]	4.00	3.99	0.01
15		3.12	0.49	4.00	4.06	0.06
16		2.58	0.41	4.21	4.05	0.16
17		4.64	0.67	4.27	4.09	0.18
18		31.4	1.50	4.17	4.18	0.01
19			4.00	3.96	3.99	0.03

[a] C is the molar concentration of drug required to produce a 65% inhibition of the vasopressin-stimulated water loss from the isolated toad bladder. For comparison purposes, NEM has a log P = 0.56 and a log $1/C$ = 3.43. [b] Log $P_{7.4}$ = log P − pH + pK_a. [c] The average weighted standard error of the log $1/C$ values is 0.095. [d] Calculated from eq 2. [e] Value for un-ionized species. [f] Estimated. [g] Calculated from additivity rules; see ref 16.

$$\log 1/C = 0.258\ (\pm 0.102) \log P + 3.609\ (\pm 0.261) \qquad n = 14,\ r = 0.846,\ s = 0.447 \qquad (1)$$

$$\log 1/C = -0.055\ (\pm 0.008)(\log P)^2 + 0.227\ (\pm 0.024) \log P + 3.961\ (\pm 0.081)\ \log P_0 = 2.06\ (1.61\text{–}2.67) \qquad n = 14,\ r = 0.993,\ s = 0.104 \qquad (2)$$

When Mal-Gly-Oxy (**3**) or Mal-AUA-Oxy (**4**) was preincubated with either the toad bladder (serosal side) or the rat uterus, no irreversible inhibition was detected at concentrations up to 3×10^{-5} M. Higher concentrations were not tested. Control experiments established that **3** weakly stimulated water loss from the toad bladder system (10 mU/μmol). Neither compound **3** nor **4** stimulated contractions in the rat uterus. Since the rat uterus is known to be much more sensitive to oxytocin than is the toad bladder,[18] the intrinsic activity of analog **3** cannot be caused by trace amounts of contaminating oxytocin.

BrOxy (**2**) (at 10^{-7} M) caused 93% inhibition of the vasopressin-stimulated water loss from toad bladder (lit.[5] 60%). BrOxy (**2**) did not inhibit oxytocin-stimulated contractions of rat uterus.

Mal-Gly (**6**) produced an 80% inhibition (average of three runs), when added to the solution bathing the serosal membrane of the toad bladder (3×10^{-2} M), but produced a 95% inhibition (average of three runs) when applied to the mucosal surface at the same concentration. Both Mal-Gly-Oxy (**3**) and Mal-AUA-Oxy (**4**) produced 13% inhibition of vasopressin-stimulated water loss from toad bladder when added to the mucosal bathing solution (10^{-4} M). Neither **3** nor **4** stimulated water loss when added to the mucosal surface.

Discussion

The synthesis of the N-maleoylamino acids reported here provides compounds that can be used to make sulfhydryl alkylating reagents which can be used in affinity labeling studies.[19] Acids **6–14**, **20**, **21**, and **23** react rapidly with sulfhydryl groups and can be coupled to peptides or other biologically active compounds which contain acylable functional groups. These compounds will be particularly useful for preparing maleimide derivatives of thermally and chemically labile peptides and other compounds which would be destroyed under the conditions normally used to prepare maleimides.

As expected, the N-maleoylamino acids inhibited vasopressin-stimulated water loss from toad bladder. The relationship between biological potency and partition behavior was studied carefully because preliminary experiments had shown that neither Mal-Gly (**6**) nor Mal-Gly-Oxy (**3**) inhibited either toad bladder or rat uterus. Thus it was necessary to establish that **6** could be an effective ligand for the affinity labeling studies. The partitioning study established that the low activity of **6** was due to its low lipophilicity and pK_a and was not caused by a low chemical reactivity such as might result from the proximity of the carboxylate anion in **6** to the maleimide ring. The partition studies also demonstrate that compound **6**, although not having special chemical properties, does possess useful distributional properties at physiological pH which could be used to study the accessibility of sulfhydryl groups in membrane systems, much as picric acid, which is a membrane impermeable uncoupler of oxidative phosphorylation, has been used to differentiate binding sites for different biological responses.[20] The ideal lipophilicity, log P_0 = 2.06, also establishes that NEM with a log P = 0.56 is not the optimal reagent for inhibition studies in the toad bladder system.

Having established that maleoyl acids **6–14** reacted with sulfhydryl groups and inhibited vasopressin-stimulated water loss and oxytocin-stimulated uterus contractions, two maleimide derivatives of oxytocin were prepared, purified, and tested. Neither maleoyl-oxytocin derivative **3** nor **4** produced irreversible inhibition in either rat uterus or

toad bladder whereas BrOxy (2), an analog which is modified at the same position in oxytocin and which is structurally very similar, rapidly and irreversibility inhibited the vasopressin-stimulated receptor. However, the maleimides 3 and 4, which both react rapidly with benzylmercaptan, can alkylate only sulfhydryl groups, whereas the bromoacetyl group in 2 is capable of alkylating a number of nucleophiles at physiological pH.[21] Analog 3 produced a small but measurable water loss in the toad bladder assay not caused by traces of oxytocin. This result suggests that maleimide 3 binds to the vasopressin receptor but does not alkylate it.

N-Acyl derivatives of oxytocin possess low biological activities;[5,18] *N*-acetyl-oxytocin possesses low oxytocic activity and low activity in the toad bladder assay.[18] This low activity is caused by a reduced affinity of the derivative for receptors.[5] BrOxy (2) would also be expected to possess a low affinity for the toad bladder receptor, prior to formation of the covalent bond, because the bromoacetyl group is of similar size and shape as the acetyl group. Since BrOxy is such a potent inhibitor of toad bladder, the low affinity of acyloxytocin analogs for the receptor does not prevent the derivative from alkylating the receptor if a nucleophile on the receptor is in proper position for the reaction to occur. Mal-Gly-Oxy (3) and Mal-AUA-Oxy (4) should have low intrinsic activities and affinities since they are *N*-acyl derivatives.

The fact that maleimides 3 and 4 do not inhibit suggests that the nucleophiles in the vasopressin receptor of toad bladder alkylated by BrOxy (2) were not sulfhydryl groups *or* that any sulfhydryl groups present in these receptors can not react with the maleimides 3 and 4 but can react with the bromoacetyl group of 2. Steric hinderance at the receptor could cause this selectivity.

The biological activity of Mal-Gly (6) depends on whether it is added to the serosal or mucosal side of the bladder. When added to the serosal side, 6 inhibits completely only at high concentrations ($>4 \times 10^{-2}$ *M*). It is apparent from eq 2 and from the data in Table III that the unusually low activity of 6 is caused by the low pK_a and lipophilicity of the molecule which prevent it from either binding to or penetrating the serosal membrane. However, 6 does completely inhibit the vasopressin-induced water loss at a lower concentration (3×10^{-2} *M*) when added to the mucosal side. Similar results have been reported by Rasmussen et al.[3a] with NEM. Thus there are either fewer essential sulfhydryl groups accessible from the mucosal side or they are more sensitive than those accessible from the serosal side. These results suggest that at least some of the sulfhydryl groups essential for toad bladder response to vasopressin are not on the serosal surface.

Further support for this conclusion is derived from the observation that 3 and 4 irreversibly inhibited water loss when added to the mucosal surface. Limited supplies of the analogs prevented testing them at high concentrations to see if complete inhibition could be achieved. The potency of 3 and 4 to inhibit water loss is approximately intermediate to that of NEM and 6 at the same concentration.

The results obtained with Mal-Gly (6), Mal-Gly-Oxy (3), and Mal-AUA-Oxy (4) neither prove nor disprove the proposal that essential sulfhydryl groups are at or near the oxytocin or vasopressin receptor. No irreversible inhibition was detected in the normal assays but since the maleimide group is not isosteric with the bromoacetyl group alkylation may have been prevented for steric reasons. The nature of the essential sulfhydryl groups in the oxytocin and vasopressin systems remains to be identified.

Experimental Section

Melting points were determined with a Thomas-Hoover Uni-Melt apparatus and are uncorrected. NMR spectra were determined in $CDCl_3$, Me_2SO-d_6, or MeOH-d_4 on a Varian EM-360, Varian A-60A, or a Bruker HX 90-E spectrometer, interfaced with a Nicolet 1080 data system for use with Fourier transform, with Me_4Si as an internal standard. Uv spectra were determined in absolute EtOH on a Cary 15 recording spectrophotometer and a Gilford 240 spectrophotometer. Ir spectra (KBr) were determined on a Beckman IR-5A spectrophotometer. Elemental analyses were performed by Galbraith Laboratories, Inc., Knoxville, Tenn. The pK_a of compounds 6–10 were determined on a Sargent Model 7 pH meter. Optical rotations were determined on a Bendix series 1100 automatic polarimeter (0.1-dm cell). Solid-phase synthesis of oxytocin was performed on a Beckman 990 peptide synthesizer. Amino acid analyses were determined on a Beckman Spinco 120B amino acid analyzer. No correction was made for decomposition of Tyr. All gel permeation chromatographies of oxytocin (1) or oxytocin analogs 2, 3, and 4 were done at 4°. Thin-layer chromatography was carried out on compounds 1, 3, and 4 using glass plates coated with silica gel (E. Merck). The following solvent systems were used: (A) *n*-BuOH–EtOAc–HOAc–H_2O (1:1:1:1) or (B) *n*-BuOH–HOAc–H_2O (4:1:5, upper phase). The peptides were located with chlorine spray.[22]

The following commercially available reagents and solvents were purified prior to use as follows. Triethylamine (Et_3N) was distilled from phthalic anhydride followed by redistillation from sodium ribbon; toluene (benzene) was dried over KOH pellets and filtered immediately before use; 1-octanol was washed with 4 *N* H_2SO_4, 0.5 *N* NaOH, and deionized H_2O and fractionally distilled in vacuo; bromoacetyl bromide was distilled in vacuo; dimethylformamide (DMF) was purified by the method of Stewart and Young;[23] dicyclohexylcarbodiimide (DCC) was distilled in vacuo; 1-hydroxybenzotriazole (HOBt) was recrystallized from deionized water; 1-butanol was distilled from magnesium butylate; and acetic acid (HOAc) was distilled from triacetyl borate.

Arginine-vasopressin and oxytocin used in the bioassays were purchased from Sigma Chemical Co.

Protected amino acids were purchased from Cyclo Chemical Co., Sigma Chemical Co., and Beckman. The 1% chloromethyl resin was purchased from Bio-Rad.

Determination of Partition Coefficients. Partition coefficients were determined according to the method of Hansch[16] in a 1-octanol–water system in the case of compounds 15–18 and in a 1-octanol–0.1 *M* citrate, 0.2 *M* phosphate buffer[24] system in the case of compounds 6–11 with the pH of the buffer adjusted to the pK_a of the corresponding *N*-maleoylamino acid. In this latter case the experimentally derived partition coefficients were converted first to that expected for the completely un-ionized species and then converted to the values expected at pH 7.4,[16] which is the pH of the bioassays. The partition coefficients for compounds 12–14 and 19 were not determined experimentally due to low aqueous solubilities coupled with the low extinction coefficients utilized in the determination but rather were calculated by utilization of accepted additivity principles.[16] The partitioning was carried out in scintillation vials and phase volumes were adjusted to give approximately equal ratios of solute in each phase. Pipets used to transfer the 1-octanol were allowed to drain for 5 min due to this solvent's high viscosity. Each vial was inverted 100 times by hand in 2 min and then swirled for 2 hr at room temperature on a mechanical shaker. The separated layers were centrifuged. The maleimides were found to deviate from linearity with respect to Beer's law when the absorbance was above 0.4. The organic or aqueous layers were therefore diluted with enough absolute EtOH to give a final absorbance between 0.2 and 0.4. All partition coefficients were checked for a concentration dependence by running each compound at three or more distinct concentrations. The final partition coefficient for each compound represents the mean of from three to nine determinations.

Biological Methods. Rat uteri, prepared from albino, female rats (Holtzmann Co., Madison, Wis.), were assayed following the procedure of Holton.[25] Mechanical movements of the uterine strips were recorded on a Grass Model 5 polygraph via force-displacement transducers (FT-03). Each strip was maintained at a resting tension of 1 g during a 1-hr equilibration period before addition of any drugs. Dose–response effects of oxytocin were obtained by adding threefold increasing concentrations to the bath. After the peak contraction was obtained, the tissue was washed each minute for 5 min and allowed to equilibrate for 5 min following the last wash before the next concentration was added. This procedure was continued until the contraction produced by oxytocin did not further increase. After obtaining the control dose–re-

sponse curve to oxytocin, a single concentration of antagonist was added to the bath and allowed to interact with the tissue for varying periods of time. The tissue was then washed seven times for 15 min and allowed to equilibrate for 15 min after the last wash before beginning the second dose–response curve to oxytocin.

Hemibladders from doubly pithed toads (*Bufo marinus*, Mogul-ED, Oshkosh, Wis.) were attached to glass tubes with the mucosa on the inside of the sac.[26] The bladders were placed in 30 ml of a physiological salt solution aerated with 5% CO_2 in O_2 and the inside of the sac was filled with 2 ml of a 1:5 dilution of the physiological salt solution.[27] The inside and outside solutions were changed twice during a 1-hr equilibration period before any drugs were added. Flow of water out of the sac during 40-min incubation periods was determined gravimetrically[26] and basal water flow was obtained during the first 40-min period after equilibration. A single concentration of a maleimide dissolved in 95% EtOH was added to one bladder and an equal volume of vehicle (0.1–0.3 ml) was added to the paired control bladder and allowed to remain in contact with the tissue for 30 min. The inside and outside solutions were then replaced twice with drug-free salt solution during a 20-min period and the bladders were incubated for 40 min in the presence of arginine-vasopressin (Sigma Chemical Co.), 1 μg/ml. All drugs were added to the solution bathing the serosal surface of the bladder unless otherwise indicated. Vasopressin-stimulated water loss from the maleimide-treated bladder was calculated as a percent of the stimulated water loss from the control bladder after correction for basal flow. Effects of the maleimide derivatives are expressed as percentage inhibition of vasopressin-stimulated water flow. Each maleimide (**6–19**) was tested at varying concentrations and a linear regression performed on the concentration vs. percent inhibition data. A value was then computed from the generated equation for each compound for the concentration required to give a 65% inhibition.

Regression Analyses. The experimentally determined values for the partition coefficient (P) and the molar concentration (C) of maleimide required to produce a 65% inhibition of the vasopressin-stimulated water loss from the isolated toad bladder were converted to log P and log $1/C$ values and subjected to a forward stepwise linear regression analyses program generated at the Academic Computing Center, University of Wisconsin, Madison, Wis., and stored in a Univac 1110 computer. The 99.9% level of significance was used as criterion for rejection or acceptance of each successive term in log P.[28] See Figure 2.

***N*-Maleoylamino Acids (6–13, 20, 21, and 23).** The general procedure may be illustrated by the following example. A solution of maleic anhydride (41.7 g, 0.425 mol) in HOAc (175 ml) was added to a solution of glycine (31.9 g, 0.425 mol) in AcOH (510 ml), and the mixture was stirred at room temperature for 3 hr. The white precipitate was filtered, washed with cold H_2O (50 ml), and dried (71.0 g, 0.410 mol, 96.5%). Crystallization from H_2O afforded analytically pure maleamic acid **24**.[9a] See Table II.

Maleamic acid **24** (2.91 g, 16.8 mmol) was suspended in dry toluene (500 ml) and treated with Et_3N (3.55 g, 35.1 mmol). This solution was refluxed with vigorous overhead stirring for 1 hr with concomitant removal of formed H_2O via a Dean-Stark apparatus. The toluene solution was decanted away from an orange oil. The toluene was removed by evaporation to give the triethylammonium salt of **6** as a hygroscopic solid. The solid was acidified to pH 2 with HCl, extracted with EtOAc, and dried ($MgSO_4$). The EtOAc was removed in vacuo to give **6** (1.21 g, 7.8 mmol, 46.4%). A work-up of the orange oil by similar methods gave another 10% of **6**. See Table I.

***N*-Maleoylamino Esters (15–19 and 22).** The general procedure[10] may be illustrated by the following example. Maleic anhydride (1.39 g, 14.2 mmol) was added to a solution of ethyl 11-aminoundecanoate (3.26 g, 14.2 mmol) in HOAc (20 ml), and the solution was heated at reflux for 5 hr. The HOAc was removed in vacuo, saturated $NaHCO_3$ solution was added to pH 8, the solution was extracted with EtOAc and dried ($MgSO_4$), and the EtOAc was removed in vacuo to give **19** (2.60 g, 11.3 mmol, 79.6%) as a slightly yellow oil. Compound **19** was further purified by distillation in a Kügelrohr apparatus to give an analytical sample of a clear colorless liquid which solidified on standing. Yields were not optimized. See Table I.

Oxytocin (1). The protected nonapeptide Z-Cys(SBzl)-Tyr-(OBzl)-Ile-Gln-Asn-Cys(SBzl)-Pro-Leu-Gly was synthesized by standard solid-phase peptide synthetic techniques on a 1% resin.[23,29] After removal of the peptide material from the resin by ammonolysis it was recrystallized from DMF–MeOH. This purified protected nonapeptide amide was deprotected and oxidized to the disulfide.[30] The crude oxytocin was desalted and partially separated from dimers by the method of Manning et al.[31] Highly purified oxytocin was obtained by partition chromatography on LH-20 using freshly prepared *n*-BuOH–HOAc–H_2O (2:1:10, lower phase). Purity of the oxytocin was established by amino acid analysis, NMR spectroscopy, thin-layer chromatography (R_{fA} 0.51, R_{fB} 0.22), bioassay, and comparison with authentic oxytocin.

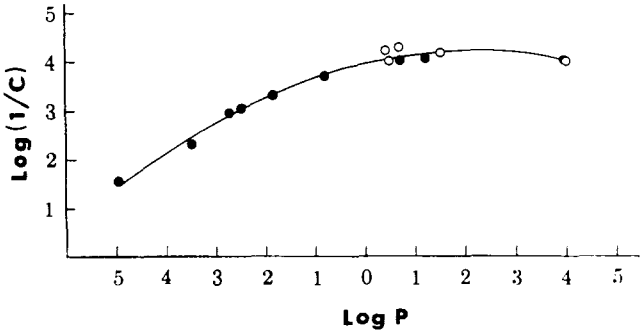

Figure 2. Relationship between biological activity and partition coefficient of *N*-maleoylamino acid derivatives: acids, ●; esters, ○.

Bromoacetyl-oxytocin (2). Bromoacetyl-oxytocin was prepared by the method of Walter et al.[5] and purified by gel filtration over G-15 (50% HOAc) followed by partition chromatography over LH-20; *n*-BuOH–HOAc–H_2O (2:1:10, lower phase).

***N*-Maleoylglycyl-oxytocin (3).** Et_3N (1.07 mg, 10 μmol) in DMF (0.104 g) was added to the acetate salt of oxytocin (**1**) (10.5 mg, 9.8 μmol). The solution was stirred at 0° for 0.5 hr. HOBt (36.7 mg, 220 μmol), Mal-Gly (**6**) (15.1 mg, 97 μmol), and DCC (21.9 mg, 106 μmol) in DMF (1.1 ml) were added. The solution was stirred at 0° for 2 hr and further for 22 hr at room temperature. The reaction mixture was filtered, the precipitate was washed with DMF, and the filtrates were evaporated to dryness in vacuo. The crude product was dissolved in 0.2 *N* HOAc and the solution filtered to remove insolubles. The filtrate was lyophilized. The dried powder was applied to a column of Sephadex G-25 in 0.2 *N* HOAc and the column eluted with this solvent. Maleimide **2** eluted at 1.56 void volume (2.3 mg, 20%), oxytocin at 1.92, Mal-Gly (**6**) at 1.94, and HOBt at 2.10 void volumes. Amino acid analysis gave $Asp_{0.88}Pro_{1.08}Glu_{1.07}Gly_{2.00}Cys_{2.14}Ile_{0.86}Leu_{0.91}Tyr_{0.71}(NH_3)_{3.15}$; TLC R_{fB} 0.39. The NMR (CD_3OD) of compound **2** showed the presence of the maleimide protons as a sharp singlet at 6.92 ppm (90%) and an AB quartet (J = 12.0 Hz, ν_A = 6.26 ppm, ν_B = 6.65 ppm) which was assigned to the maleamic acid protons **5** (10% of sample).

***N*-Maleoyl-11-aminoundecanoyl-oxytocin (4).** This compound was synthesized in the same manner as **3** and purified by dissolving in 50% HOAc, filtering to remove insolubles, and chromatographing on G-25 using 50% HOAc as solvent. From 12.0 mg (11.2 μmol) of **1** and 31.6 mg (112 μmol) of **12**, 4.1 mg (3.23 μmol, 28.8%) of pure **4** was obtained which eluted at 1.13 void volumes: R_{fB} 0.45. The NMR (CD_3OD) of compound **4** showed the presence of the maleimide protons as a sharp singlet at 6.78 ppm. Amino acid analysis gave $Asp_{0.96}Pro_{1.03}Glu_{1.08}Gly_{1.00}Cys_{2.08}Ile_{1.00}Leu_{1.04}Tyr_{0.78}(NH_3)_{2.89}$.

***N*-Ethylaceto-5-norbornene-2,3-dicarboximide (36). (A)** To 5-norbornene-2,3-dicarboximide (0.595 g, 3.65 mmol), prepared by the method of Tawney et al.,[32] in EtOAc (35 ml) was added 1.15 ml of an alcoholic KOH solution (3.05 *M* in 95% EtOH). A white precipitate formed immediately. The mixture was stirred for 0.4 hr, filtered, and triturated with boiling benzene to yield the potassium salt (0.54 g, 2.68 mmol, 73%) which was used without further purification. Ethyl bromoacetate (0.202 g, 1.21 mmol) in DMF (1.0 ml) was added to the potassium salt of 5-norbornene-2,3-dicarboximide (0.303 g, 1.51 mmol) in DMF (2.0 ml). The resultant milky white suspension was stirred for 0.5 hr. Crude **36** was chromatographed on silica gel eluting with a benzene–EtOAc gradient. The ester was isolated as a solid (0.250 g, 1.00 mmol, 83%): mp 56.5–58° (lit.[32] 53°).

(B) Compound **15** was allowed to react with freshly distilled cyclopentadiene in the usual manner.[33] The product of this reaction was shown to be identical with compound **36** prepared by method A. See Scheme I.

***N*-Carboxymethyl-5-norbornene-2,3-dicarboximide (35). (A)** Glycine (5.00 g, 66.6 mmol) and 5-norbornene-2,3-dicarboxylic anhydride (10.81 g, 65.8 mmol) were suspended in toluene (100 ml). Et_3N (0.624 g, 6.17 mmol) was added and the solution was refluxed for 20 hr while H_2O was removed via a Dean-Stark appara-

tus. The solvent was evaporated, the solid residue triturated with 0.12 *N* HCl, filtered, dissolved in saturated $NaHCO_3$ solution, and extracted with EtOAc, the basic aqueous fraction acidified to pH 2 with concentrated HCl, extracted with $CHCl_3$, and dried ($MgSO_4$), and the $CHCl_3$ removed in vacuo to yield **35** (4.08 g, 18.4 mmol, 28.0%): mp 150–152°.

(**B**) Compound **6** was allowed to react with freshly distilled cyclopentadiene in the usual manner.[33] The product of this reaction was shown to be identical with compound **35** prepared by method A. See Scheme I.

***N*-Maleoylglycylglycinamide (40).** HOBt (2.34 g, 15.3 mmol) and **6** (0.778 g, 5.02 mmol) were added to a solution of glycinamide hydrochloride (0.555 g, 5.02 mmol) and Et_3N (0.505 g, 4.99 mmol) in DMF (10 ml). The reaction mixture was cooled to 0° for 0.5 hr. A solution of DCC (1.05 g, 5.09 mmol) in DMF (6 ml) was added and the mixture stirred for 1 hr at 0° and room temperature for 2.5 hr. The reaction mixture was filtered, the DMF was removed in vacuo, and after recrystallization from MeOH the peptide **40** was isolated as a white solid (0.804 g, 3.81 mmol, 76.4%): mp 189.5–192° dec. Anal. ($C_8H_9N_3O_4$) C, H, N.

***N*-Maleoyl-12-aminooctadecanoic Acid (14).** A suspension of 12-aminooctadecanamide (5.77 g, 19.3 mmol) in 6 *N* HCl (105 ml) was refluxed for 22 hr. The product was extracted from the reaction mixture with EtOAc and precipitated in the cold to yield 12-aminoundecanoic acid hydrochloride (5.51 g, 16.4 mmol, 85.0%): mp 91–93°. The amino acid hydrochloride was dissolved in 50% EtOH (100 ml) and the pH was adjusted to 7.6 with 2 *N* NH_4OH. The free amino acid which precipitated out (4.39 g, 14.7 mmol) was added to maleic anhydride (1.40 g, 14.3 mmol) dissolved in HOAc (24 ml). The mixture was stirred for 17 hr at room temperature. HOAc was removed in vacuo, and the maleamic acid was obtained as a pale yellow liquid (5.69 g, 14.3 mmol, 100%). The maleamic acid (0.91 g, 2.29 mmol) was heated at 185° for 2 hr in a round-bottom flask. A saturated bicarbonate solution was added to the brown oil and the solution washed with EtOAc. Compound 14 was isolated as a white solid from the EtOAc washings (0.19 g, 0.50 mmol, 21.8%). See Table I.

Reaction of *N*-Maleoyl Derivatives with Thiols. Various *N*-maleoylamino acids, esters, and amides were mixed with equimolar amounts of glutathione or benzylmercaptan in D_2O, Me_2SO-d_6, or MeOH-d_4 and NMR spectra obtained immediately. In all cases the singlet for the olefinic protons of the maleimide had disappeared within 10 min of addition of the thiol.

Spectral Data of *N*-Maleoyl Derivatives. The *N*-maleoyl derivatives exhibited the following general spectral behavior: NMR 6.67–6.78 ($CDCl_3$), 6.78–6.92 (MeOH-d_4), 7.00–7.13 ppm (Me_2SO-d_6), singlet for olefinic protons; uv λ max (EtOH) 289–298 nm (log ε 2.64–2.79); ir 1720 cm^{-1} broad (KBr), imide and acid carbonyls.

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References and Notes

(1) While this manuscript was in preparation, an alternate synthesis of *N*-maleoylamino acids was reported: O. Keller and J. Rudinger, *Helv. Chim. Acta*, **58**, 531 (1975).
(2) (a) C. F. Fox and E. P. Kennedy, *Proc. Natl. Acad. Sci. U.S.A.*, **57**, 698 (1965); (b) J. R. Carter, Jr., *Biochemistry*, **12**, 171 (1973).
(3) (a) H. Rasmussen, I. L. Schwartz, M. A. Schoessler, and G. Hochster, *Proc. Natl. Acad. Sci. U.S.A.*, **46**, 1278 (1960); (b) P. J. Bentley, *J. Endocrinol.*, **30**, 103 (1964).
(4) Y. Ishida, H. Moritoki, and K. Hara, *Yakugaku Zasshi*, 81, 1713 (1961); *Chem. Abstr.*, **56**, 14866*g* (1962).
(5) R. Walter, I. L. Schwartz, O. Hechter, T. Dousa, and P. L. Hoffman, *Endocrinology*, **91**, 39 (1972).
(6) (a) D. G. Smyth, O. O. Blumenfeld, and W. Konigsberg, *Biochem. J.*, **91**, 589 (1964); (b) C. F. Brewer and J. P. Riehm, *Anal. Biochem.*, 18, 248 (1967).
(7) J. R. Heitz, C. D. Anderson, and B. M. Anderson, *Arch. Biochem. Biophys.*, **127**, 627 (1968).
(8) (a) D. H. Marrian, *J. Chem. Soc.*, 1515 (1949); (b) K. Tsou, R. J. Barrnett, and A. M. Seligman, *J. Am. Chem. Soc.*, **77**, 4613 (1955); (c) L. E. Coleman, Jr., J. F. Bork, and H. Dunn, Jr., *J. Org. Chem.*, **24**, 135 (1959); (d) C. K. Sauers, *ibid.*, **34**, 2275 (1969).
(9) (a) F. E. King, J. W. Clarke-Lewis, R. Wade, and W. A. Swindin, *J. Chem. Soc.*, 873 (1957); (b) B. Helferich and W. Wesemann, *Chem. Ber.*, **100**, 421 (1967).
(10) S. L. Shapiro, L. Freedman, and M. J. Karten, U.S. Patent 3,129,225 (1964); *Chem. Abstr.*, **61**, 7029*a* (1965).
(11) J. D. Gregory, *J. Am. Chem. Soc.*, **77**, 3922 (1955).
(12) T. M. Pyriadi and H. J. Harwood, *J. Org. Chem.*, **36**, 821 (1971).
(13) (a) H. Rubinstein and J. E. Skarbek, *J. Org. Chem.*, **36**, 3372 (1971); (b) H. Rubinstein, M. Parnarouskis, and H. Feuer, *ibid.*, **38**, 2166 (1973).
(14) M. Furdik and V. Sutoris, *Chem. Zvesti*, **17**, 31 (1963); *Chem. Abstr.*, **60**, 7929*h* (1964).
(15) W. Konig and R. Geiger, *Chem. Ber.*, **103**, 788 (1970).
(16) (a) W. P. Purcell, G. W. Bass, and J. M. Clayton, Ed., "Strategy of Drug Design: A Guide to Biological Activity", Wiley, New York, N.Y., 1973, p 126; (b) C. Hansch and J. M. Clayton, *J. Pharm. Sci.*, **62**, 1 (1973).
(17) C. Hansch and W. R. Glave, *Mol. Pharmacol.*, **7**, 337 (1970).
(18) E. Schroder and K. Lubke, "The Peptides", Academic Press, New York, N.Y., 1966.
(19) (a) B. R. Baker, "Design of Active-Site-Directed Irreversible Enzyme Inhibitors", Wiley, New York, N.Y., 1967; (b) S. J. Singer, *Adv. Protein Chem.*, 1–54 (1967).
(20) W. G. Hanstein and Y. Hatefi, *Proc. Natl. Acad. Sci. U.S.A.*, **71**, 288 (1974).
(21) G. E. Means and R. E. Feeney, "Chemical Modification of Proteins", Holden-Day, San Francisco, Calif., 1971.
(22) D. E. Nitecki and J. W. Goodman, *Biochemistry*, **5**, 665 (1966).
(23) J. M. Stewart and J. Young, "Solid Phase Peptide Synthesis", W. H. Freeman, San Francisco, Calif., 1969, pp 31–32.
(24) T. C. McIluaine, *J. Biol. Chem.*, **49**, 183 (1921).
(25) P. A. Holton, *Br. J. Pharmacol. Chemother.*, **3**, 328 (1948).
(26) (a) P. J. Bentley, *J. Endocrinol.*, **17**, 201 (1958); (b) I. L. Schwartz and R. Walter, *Am. J. Med.*, **42**, 769 (1967).
(27) M. Sugita, S. Urakabe, J. S. Handler, and J. Orloff, *Comp. Biochem. Physiol. A*, **45**, 157 (1973).
(28) N. R. Draper and H. Smith, "Applied Regression Analysis", Wiley, New York, N.Y., 1966.
(29) (a) R. B. Merrifield, *J. Am. Chem. Soc.*, **85**, 2149 (1963); (b) W. S. Hancock, D. J. Prescott, P. R. Vagelos, and G. R. Marshall, *J. Org. Chem.*, **38**, 774 (1973); (c) D. Yamashiro and C. H. Li, *J. Am. Chem. Soc.*, **95**, 1310 (1973).
(30) M. Walti and D. B. Hope, *Experientia*, **15**, 389 (1973).
(31) M. Manning, T. Wuu, and J. W. M. Baxter, *J. Chromatogr.*, **38**, 396 (1968).
(32) P. O. Tawney, R. H. Snyder, R. P. Conger, K. A. Lei, B. Brand, C. H. Stiteler, and A. R. Williams, *J. Org. Chem.*, **26**, 15 (1961).
(33) J. G. Martin and R. K. Hill, *Chem. Rev.*, **61**, 537 (1961).