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The α-Chymotrypsin-catalyzed Hydrolysis of a Series of Hydrazides. II. Evaluation of the Kinetic Constants for Aqueous Systems at 25° and at the Optimum *p*H for Each Specific Substrate[1]

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The dependence of the α-chymotrypsin-catalyzed hydrolysis of L-tyrosinhydrazide and of six α-N-acylated-L-tyrosinhydrazides, in aqueous solutions at 25° and 0.02 *M* in the THAM component of a THAM–HCl buffer and at the optimum *p*H for each specific substrate, upon the initial specific substrate concentration has been determined. A comparison of the values of K_S and k_3 for α-N-formyl-, α-N-acetyl- and α-N-nicotinyl-L-tyrosinhydrazide with those for the corresponding acylated-L-tyrosinamides has shown that while the values of K_S for the members of any given amide-hydrazide pair may be approximately equal and the value of k_3 for the amide member may be greater than that for the hydrazide member of a particular pair, the ratio of the values of k_3 for each amide–hydrazide pair varies with the nature of the acyl moiety present in the three pairs.

It is known that L-tyrosinhydrazide, α-N-formyl-, α-N-acetyl-, α-N-trimethylacetyl-, α-N-dichloroacetyl-, α-N-benzoyl- and α-N-nicotinyl-L-tyrosinhydrazide are hydrolyzed in the presence of α-chymotrypsin and the optimum *p*H for the hydrolysis of each of the above specific substrates, in aqueous solutions at 25° and 0.02 *M* in the THAM[5] component of a THAM–HCl buffer, has been determined.[6] In this communication we shall be concerned with the determination of the dependence of the rate of the α-chymotrypsin-catalyzed hydrolysis of the above specific substrates upon the initial specific substrate concentration when such systems are examined in aqueous solutions at 25° and 0.02 *M* in the THAM component of a THAM–HCl buffer and in the immediate region of the *p*H optimum of each specific substrate.

It will become evident from the results of this study that insofar as dependency upon the initial specific substrate concentration is concerned, the initial stages of the α-chymotrypsin-catalyzed hydrolysis of the above specific substrates may be represented by equation 1 and that their respective

$$E_f + S_f \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E_f + P_{1f} + P_{2f} \qquad (1)$$

rates, during the initial stages of reaction, may be described, within the limits of experimental error, by equation 2, where $K_S = (k_2 + k_3)/k_1$. Therefore, in the evaluation of the constants K_S and k_3 the primary data, *i.e.*, extent of reaction *vs.* time, were first submitted to a first-order correction[7,8] in order to obtain linear relationships between extent of reaction and corrected time. From these linear relationships a least squares fit was employed to obtain values of v_0. Since it was observed that the values of v_0 and $[S]_0$ for each specific substrate gave apparent linear relationships in any one of the three plots based upon equation 2,[9,10] each set of values of v_0 and $[S]_0$ were fitted by the method of least squares to the line $1/v_0 = b/[S]_0 + a$, where $b = K_S/k_3\,[E]$, $a = 1/k_3\,[E]$ and $b/a = K_S$.[9] The values of a, b, k_3/K_S, K_S and k_3 so obtained are given in Table I along with the experimental parameters characteristic of each set of experiments, *e.g.*, *p*H, [E] and $[S]_0$. Since values of K_S and k_3 acquire significance only when the ratios $E'_S = [E]/K_S$ and $S'_S = [S]_0/K_S$ lie within certain limits,[11] values for these latter constants also are given in Table I.

$$v_0 = -d[S]/dt = k_3[E][S]/(K_s + [S]) \qquad (2)$$

If it is assumed as before[11] that the molecular weight of monomeric α-chymotrypsin is 22,000, its nitrogen content is 16.0%, the monomer possesses but one catalytically active site per molecule and that all of the enzyme is present in the reaction system as the monomer, it is clear that all values of E_S' given in Table I are well below the value of 0.6 associated with an experimental error of ±5%.[11] Therefore, insofar as values of E_S' are concerned, all values of K_S and k_3 were obtained under the conditions required if equation 2 is to have validity.[11]

If equation 2 is to be used for the evaluation of K_S and k_3 and the experimental error is ±5%, it is required that values of S'_S be between the limits of 0.05 and 20.[11] It will be seen from Table I that the values of S'_S for α-N-formyl- and α-N-dichloroacetyl-L-tyrosinhydrazide are well within the above limits. However, in all other cases the lower values of S'_S are either less than or close to the lower limit of 0.05 noted above.

With L-tyrosinhydrazide the limited solubility, *i.e.*, *ca.* $5 \times 10^{-3} M$, coupled with a value of K_S = *ca.* $6 \times 10^{-3} M$ makes it unlikely that K_S and k_3 can be evaluated with greater precision than that attained in the present study unless the experimental error can be reduced to below ±1%. With α-N-benzoyl-L-tyrosinhydrazide the values of K_S and k_3 given in Table I are suspect for two reasons. First, they were obtained under conditions where the estimated values of S'_S were so low that the only constant of possible significance is the ratio k_3/K_S.[11] While it may appear that the situation could be improved to a modest degree by eliminat-

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(5) Tris-(hydroxymethyl)-aminomethane.

(6) R. Lutwack, H. F. Mower and C. Niemann, THIS JOURNAL, **79**, 2179 (1957).

(7) R. R. Jennings and C. Niemann, *ibid.*, **75**, 4687 (1953).

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TABLE I

SUMMARY OF VALUES OF KINETIC CONSTANTS[a]

–CH(CH₂C₆H₄OH)CONHNH₂	pH[b]	[E][c]	[S]₀[d]	a	b	k_3/K_S[e]	K_S[f]	k_3[g]	E'S[h]	S'S
H_2N–[i]	7.1	5.9	0.72–4.12[j]	151650 ± 56130	1500 ± 90	0.0032 ± 0.0002	6 ± 4	0.03 ± 0.02	0.6	0.07–0.4
HCONH–	7.8	4.1	3.71–13.47[k]	119640 ± 3960	1170 ± 20	.0059 ± .0001	9.8 ± 0.5	.058 ± .002	.4	.4 –1.4
CH_3CONH–	7.9	5.9	1.27–6.67[k]	7430 ± 1800	164 ± 4	.0294 ± .0007	22 ± 8	.7 ± .2	.3	.05–0.30
$(CH_3)_3CCONH$–	7.8	4.1	0.49–10.48[l]	17690 ± 7130	665 ± 6	.0104 ± .0001	45 ± 20	.4 ± .2	.1	.01– .23
$(CH_3)_3CCONH$–	7.8	4.1	2.99–10.48[l]	16780 ± 6280	690 ± 50	.0100 ± .0007	40 ± 20	.4 ± .2	.1	.07– .26
$Cl_2CHCONH$–	7.8	4.1	0.91–3.82[m]	10050 ± 1050	52.3 ± 1.7	.132 ± .004	5.2 ± 0.7	.70 ± .07	.8	.18– .73
C_6H_5CONH–	7.9	5.9	0.06–0.40[n]	9670 ± 2850	19.7 ± 0.3	.244 ± .004	2.2 ± 0.7[o]	.5 ± .2[p]	2.7	.03– .18
β-(C_5H_4N)CONH–	7.8	5.9	0.50–4.56[j]	.....	49 ± 3	.10 ± .06	9[p]	1[p]	0.7	.06– .51

[a] For systems involving α-chymotrypsin and the respective specific substrates in aqueous solutions at 25° and 0.02 *M* in the THAM component of a THAM–HCl buffer and at the indicated *p*H. [b] To within ±0.1 of a *p*H unit. [c] In units of 10^{-5} *M* and based upon an assumed molecular weight of 22,000 and a nitrogen content of 16.0%. [d] In units of 10^{-3} *M*. [e] In units of min./mg. protein-nitrogen per ml. [f] In units of 10^{-3} *M*. [g] In units of min./min./mg. protein-nitrogen per ml. [h] In units of 10^{-2}. [i] And its conjugate acid. [j] Maximum practical solubility <5 × 10^{-3} *M*. [k] Maximum practical solubility *ca.* 15 × 10^{-3} *M*. [l] Maximum practical solubility *ca.* 0.24 *M*. [m] Maximum practical solubility <4 × 10^{-3} *M*. [n] Maximum practical solubility *ca.* 0.4 × 10^{-3} *M*. [o] Value questionable, see text. [p] Based upon a subjective graphical evaluation, no estimate of variability can be given.

ing the data obtained with values of $[S]_0$ from 0.06 to 0.12 × 10^{-3} *M* and basing an evaluation upon the data obtained with values of $[S]_0$ from 0.16 to 0.40 × 10^{-3} *M*,[12] it should be noted that in all cases the extent of reaction was between 53 and 83%. This latter situation coupled with the probability that the value of K_p, *i.e.*, the enzyme-product dissociation constant, may be of the order of 10^{-2} *M* creates the second uncertainty in that the use of equation 2 under these circumstances could lead to erroneous values of K_S and k_3. Since the need for observing the reaction under consideration for a longer than optimal period was a consequence of the very low solubility of the specific substrate, which could not be improved except by the undesirable practice of changing the solvent system, it was decided to make the best of a difficult situation by studying the hydrolysis of α-N-benzoyl-L-tyrosinhydrazide in competition with that of α-N-acetyl-L-tyrosinhydrazide.[13] The experimental data so obtained were subjected to a graphical analysis based upon consideration of all three plots derived from equation 2, and the following values were obtained: k_{3T}/K_{ST} = 0.10 min./mg. protein-nitrogen per ml.; k_{3T} = 0.15 × 10^{-3} *M*/min./mg. protein-nitrogen per ml. and K_{ST} = 1.5 × 10^{-3} *M*. From these values and the values of K_{S_2} = 22 × 10^{-3} *M*, k_{3_2}/K_{S_2} and k_{3_1}/K_{S_1} = 0.029 and 0.244 min./mg. protein-nitrogen per ml., respectively, it follows that K_{S_1} = 0.5 × 10^{-3} *M* and k_{3_1} = 0.12 × 10^{-3} *M*/min./mg. protein-nitrogen per ml. While no great reliability can be placed upon these latter values, it can be concluded that the value of K_S is of the order of 10^{-3} *M* and k_3 of the order of 10^{-4} *M*/min./mg. protein-nitrogen per ml. It is clear that the relationship between the probable values of K_S and k_3 and the solubilities of L-tyrosinhydrazide and α-N-benzoyl-L-tyrosinhydrazide are such as to preclude a precise evaluation of K_S and k_3 for these specific substrates.

The relatively low lower limits of *S'*s associated with the evaluation of K_S and k_3 for α-N-acetyl- and α-N-trimethylacetyl-L-tyrosinhydrazide suggests the desirability of a re-evaluation of the kinetic constants of these specific substrates at higher values of $[S]_0$ if constants of greater reliability are to be obtained. However, it is known that the values of K_S and k_3 given in Table I for α-N-acetyl-L-tyrosinhydrazide are in substantial agreement with values so determined.[14] Since it is possible to examine α-N-trimethylacetyl-L-tyrosinhydrazide under conditions where *S'*s can be varied between the limits of 0.05 and *ca.* 6.0,[15] in spite of the relatively high value of K_S, this specific substrate has been selected for an investigation

(12) Such an evaluation was found to give a value of k_3/K_S = 0.230 ± 0.006 min./mg. protein-nitrogen per ml.

(13) R. J. Foster and C. Niemann, THIS JOURNAL, **73**, 1552 (1951).

(14) R. J. Kerr, Ph.D. Thesis, California Institute of Technology, Pasadena, California, 1957.

(15) The association of the relatively high melting point, *i.e.*, 227–228°, with the limited solubility, *i.e.*, *ca.* 0.015 *M* in aqueous solutions at 25°, of α-N-acetyl-L-tyrosinhydrazide suggested that a more soluble neutral specific substrate derived from L-tyrosinhydrazide could be obtained if the lattice energy of the crystal could be decreased by the introduction of a bulky acyl moiety. α-N-Trimethylacetyl-L-tyrosinhydrazide was prepared and not only was its melting point, *i.e.*, 179–180°, approximately 50° lower than that of α-N-acetyl-L-tyrosinhydrazide[6] but its solubility in water at 25° was *ca.* 0.24 *M*.

of the nature of the rate equation for both the initial and extended reaction. These studies, which also should lead to more precise values of K_S and k_3, are now in progress.

When the experimental data for α-N-nicotinyl-L-tyrosinhydrazide were evaluated, it was found that only the constants b and k_3/K_S could be obtained. The least squares treatment led to a negative value for a. However, a subjective graphical estimate, based upon uncorrected initial velocities, gave the values of K_S and k_3 presented in Table I. Although the k_3/K_S ratio, *i.e.*, 0.11 min./mg. protein-nitrogen per ml., derived from these values is in agreement with the value of k_3/K_S obtained by the least squares treatment, it is impossible to assign limits of variability to the values of K_S and k_3 given in Table I. However, they may be accepted as being substantially correct.[14]

Of the seven compounds considered in this study, it is clear that five, *i.e.*, α-N-formyl-, α-N-acetyl-, α-N-trimethylacetyl-, α-N-dichloroacetyl- and α-N-nicotinyl-L-tyrosinhydrazide are useful specific substrates of α-chymotrypsin. The ratio k_3/K_S is an index of over-all reactivity when $[S]_0$ loses significance with respect to K_S. The fact that values as low as 0.0032 and as high as 0.244 could be determined illustrates the exceptional sensitivity and flexibility of the analytical method that may be used for following the hydrolysis of the above specific substrates.

A comparison of the values of K_S and k_3 for α-N-formyl-, α-N-acetyl- and α-N-nicotinyl-L-tyrosinhydrazide with those for the corresponding amides which were determined under comparable conditions,[11] reveals that the values of K_S for a given amide–hydrazide pair are approximately equal for all three pairs and that the value of k_3 for the amide member of a given pair is greater than that for the hydrazide member of the pair, *cf.*, Table II. However, when the ratios of k_3 for a given pair are compared, it is seen that this ratio is *ca.* 8 for the α-N-formyl derivatives, *ca.* 3 for the α-N-acetyl derivatives and *ca.* 5 for the α-N-nicotinyl derivatives. Thus, even when the values of K_S are approximately the same for an amide–hydrazide pair, which differ only in one being an amide and the other a hydrazide, the differences in the values of k_3 for such pairs are still dependent upon the nature of the acyl moieties present in the different pairs. While a rigorous and quantitative explanation of this phenomenon cannot be given at this time, we believe that it may be a consequence of a situation where K_S is an index of the extent of combination of the enzyme and specific substrate in a variety of modes only some of which lead to orientations in the ES complex which permit the formation of a transition state and where k_3 is an index not only of the intrinsic energetics of the reaction involving the potentially hydrolyzable bond and its adjacent atoms but also of the probability of combination in a mode which can lead to a transition state.[16–18]

TABLE II

K_S AND k_3 VALUES OF AMIDE–HYDRAZIDE PAIRS[a]

Acyl moiety	K_S[b] Amide[d]	K_S[b] Hydrazide	k_3[c] Amide[d]	k_3[c] Hydrazide
α-N-Formyl	12 ± 3	9.8 ± 0.5	0.45 ± 0.05	0.058 ± 0.002
α-N-Acetyl	32 ± 4	28 ± 8	2.4 ± 0.3	0.7 ± 0.2
α-N-Nicotinyl	12 ± 3	9	5.0 ± 1.0	1

[a] For L-tyrosine derivatives in aqueous solutions at 25° and at the optimum *p*H. [b] In units of 10^{-3} M. [c] In units of 10^{-3} M/min./mg. protein-nitrogen per ml. [d] *Cf.*, ref. 11.

The values of K_S and k_3 obtained for α-N-dichloroacetyl-L-tyrosinhydrazide were unexpected since earlier studies with acetyl- and chloroacetyl-L-tyrosinamide,[11] conducted under comparable conditions, indicated that replacement of an acetyl group by a chloroacetyl group had little or no effect upon the values of K_S, *i.e.*, 32 ± 4 and 27 ± 2 × 10^{-3} M; but caused a marked increase in the value of k_3, *i.e.*, 2.4 ± 0.3 and 4.0 ± 0.2 × 10^{-3} M/min./mg. protein-nitrogen per ml. With the observation made in this study that replacement of an acetyl group by a dichloroacetyl group in the case of α-N-acylated-L-tyrosinhydrazides has the effect of causing no substantial change in the value of k_3, *i.e.*, 0.7 ± 0.2 and 0.70 ± 0.07 × 10^{-3} M/min./mg. protein-nitrogen per ml., but instead of causing a marked decrease in the value of K_S, *i.e.*, 22 ± 8 and 5.2 ± 0.7 × 10^{-3} M, it is clear that until the constants for the systems α-chymotrypsin–dichloroacetyl-L-tyrosinamide and α-chymotrypsin–α-N-chloroacetyl-L-tyrosinhydrazide are determined, it will remain unknown as whether the results noted in this study are a consequence of a unique behavior associated with the presence of the dichloroacetyl group or of the carbohydrazide group or of a combination of both.

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Experimental

Specific Substrate Stock Solutions.—The specific substrates and their stock solutions were prepared as described previously.[6]

Buffer and Enzyme Stock Solutions.—The solutions were prepared with the same precautions as before[6] from THAM and crystalline bovine α-chymotrypsin, Armour preparations nos. 10705 and 00592.

Analysis of Reaction Systems.—The procedure described by Lutwack, Mower and Niemann[6] was used without modification.

Reaction Systems.—The general procedure has been described previously.[6] All experiments were conducted in aqueous solutions at 25° and 0.02 M in the THAM component of a THAM–HCl buffer. The reaction conditions employed with each specific substrate are summarized below.

L-Tyrosinhydrazide.—Eleven experiments with values of $[S]_0$ from 0.72 to 4.12 × 10^{-3} M, [E] = 0.208 mg. protein-nitrogen per ml. of Armour preparation no. 10705, total reaction time of 45 to 195 min., total extent of reaction of 3.0 to 12.7% gave 11 corrected values of v_0 of 0.441 to 1.904 × 10^{-6} M/min. with each value being determined by a least squares fit of 6 to 9 points corresponding to the same number of observations of the corrected extent of reaction[7,8] *vs.* time within the above total reaction times.

α-N-Formyl-L-tyrosinhydrazide.—Nine experiments with values of $[S]_0$ from 3.71 to 13.47 × 10^{-3} M, [E] = 0.1444 mg. protein-nitrogen per ml. of Armour preparation no. 00592, total reaction time of 32 to 38 min., total extent of reaction of 1.2 to 2.7% gave 9 corrected values of v_0 of

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(17) G. S. Eadie and F. Bernheim, *Bull. Math. Biophys.*, **15**, 33 (1953).

(18) S. Levine, *Enzymologia*, **16**, 256, 265 (1953–1954).

2.330 to 4.940 × 10^{-6} M/min. with each value being determined by a least squares fit of 8 to 9 points.

α-N-Acetyl-L-tyrosinhydrazide.—Fifteen experiments with values of $[S]_0$ from 1.27 to 6.67 × 10^{-3} M, [E] = 0.208 mg. protein-nitrogen per ml. of Armour preparation no. 10705, total reaction time of 13 to 55 min., total extent of reaction of 5.8 to 28.5% gave 15 corrected values of v_0 of 0.735 to 3.117 × 10^{-5} M/min. with each value being determined by a least squares fit of 7 to 8 points.

α-N-Trimethylacetyl-L-tyrosinhydrazide.—Two sets of six experiments with values of $[S]_0$ from 0.494 to 1.08 × 10^{-3} M and 2.99 to 10.48 × 10^{-3} M, [E] = 0.1444 mg. protein-nitrogen per ml. of Armour preparation no. 00592, total reaction time of 32 to 60 min. and 16 to 36 min., total extent of reaction of 6.2 to 8.6% and 1.9 to 4.4% gave 12 corrected values of v_0 of 0.071 to 1.380 × 10^{-5} M/min. with each value being determined by a least squares fit of 8 to 9 points.

α-N-Dichloroacetyl-L-tyrosinhydrazide.—Ten experiments with values of $[S]_0$ from 0.909 to 3.82 × 10^{-3} M, [E] = 0.1444 mg. protein-nitrogen per ml. of Armour preparation no. 00592, total reaction time of 9 to 10 min., total extent of reaction of 10.0 to 15.0% gave 10 corrected values of v_0 of 0.145 to 0.420 × 10^{-4} M/min. with each value being determined by a least squares fit of 7 to 9 points.

α-N-Benzoyl-L-tyrosinhydrazide.—Two sets of experiments, one with 8 values of $[S]_0$ from 0.06 to 0.12 × 10^{-3} M and the other with 10 values of $[S]_0$ from 0.16 to 0.402 × 10^{-3} M, [E] = 0.208 mg. protein-nitrogen per ml. of Armour preparation no. 10705, total reaction time of 15 to 27 min. and 15 to 39 min., total extent of reaction of 57.1 to 80.1% and 53.4 to 82.7% gave 18 corrected values of v_0 of 0.290 to 1.862 × 10^{-5} M/min. with each value being determined by a least squares fit of 8 to 9 points. The conditions used in the competitive hydrolysis of α-N-acetyl- and α-N-benzoyl-L-tyrosinhydrazide are summarized in Table III.

TABLE III

α-CHYMOTRYPSIN-CATALYZED COMPETITIVE HYDROLYSIS OF α-N-ACETYL- AND α-N-BENZOYL-L-TYROSINHYDRAZIDE[a]

$[S_1]_0$ × 10^3 M	$[S_2]_0$ × 10^3 M	$[S_T]_0$ × 10^3 M	t_{max} (min.)	v_0[b] × 10^5 M/min.
0.140	0.268	0.408	14	0.696
.140	.358	.498	14	.762
.140	.447	.487	11	.918[c]

[a] In aqueous solutions at 25° and pH 7.9 and 0.02 M in the THAM component of a THAM–HCl buffer with [E] = 0.208 mg. protein-nitrogen per ml. of Armour preparation no. 10705. [b] From a least squares fit of 7 points unless otherwise noted. [c] 8 points.

α-N-Nicotinyl-L-tyrosinhydrazide.—Thirteen experiments with values of $[S]_0$ from 0.50 to 4.56 × 10^{-3} M, [E] = 0.208 mg. protein-nitrogen per ml. of Armour preparation no. 10705, total reaction time of 7 to 30 min., total extent of reaction of 13.1 to 47.8% gave 13 corrected values of v_0 of 0.102 to 0.808 × 10^{-4} M/min. with each value being determined by a least squares fit of 7 to 9 points.

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[CONTRIBUTION FROM THE CLAYTON FOUNDATION FOR RESEARCH, THE BIOCHEMICAL INSTITUTE AND THE DEPARTMENT OF CHEMISTRY, THE UNIVERSITY OF TEXAS]

DL-4-Oxalysine, an Inhibitory Analog of Lysine

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2-Amino-3-(β-aminoethoxy)-propionic acid (4-oxalysine) has been prepared through an acetamidomalonic ester condensation using 2-chloroethyl chloromethyl ether, followed by ammonolysis and hydrolysis of the intermediate, to give a mixture of products. The reaction mixture was separated into several pure components by chromatography on an alumina column. 4-Oxalysine inhibits the growth of a number of microörganisms. The reversal of this toxicity by lysine has been most extensively studied with *Leuconostoc dextranicum* 8086 and was found to be competitively reversed over a 300-fold range of concentrations with an inhibition index from 1 to 3.

The introduction of an oxygen atom in place of a methylene group in the carbon skeleton of a naturally occurring amino acid has resulted in the formation of an inhibitory analog.[1-3] In the present study, it was desired to prepare an analog of lysine by the introduction of an oxygen atom in place of the methylene group at the 4-position. Such an analog has the structural similarity and similar distance between the amino groups which would be necessary for antagonism of lysine. For example, 2,6-diaminoheptanoic acid (ε-C-methyllysine) has been reported to be an effective lysine inhibitor, but other compounds of similar structure but having amino groups different in distance apart from that of lysine are ineffective as inhibitors.[4] Accordingly, 2-amino-3-(β-aminoethoxy)-propionic acid (4-oxalysine) was prepared and found to be an effective inhibitory analog of lysine for several microorganisms.

In all of the synthetic approaches attempted, which resulted in the formation of 4-oxalysine, a mixture of products was obtained which proved difficult to separate. Further, the yield of by-products in every case exceeded that of the desired compound. The most satisfactory technique found was through an acetamidomalonic ester condensation with 2-chloroethyl chloromethyl ether. The desired product of this condensation, 2 - acetamido - 2 - (β - chloroethoxymethyl) - malonate, was always contaminated with unreacted starting material and only a 38% conversion[5] was obtained. Several attempts to improve this yield by using inverse addition, altering the temperature and/or time of addition, using other condensing agents[6] and by direct fusion of the reactants at elevated temperatures[7] failed. The substituted malonic ester derivative was treated with concentrated ammonium hydroxide at room temperature for several days to replace the terminal chlorine by an amino- group and then acid hydrolyzed; or

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