and finally to a 5-hydroxypentyl group. Both IX and X are bound to the enzyme more strongly than the corresponding nonhydroxylated compounds (II and III), which is a demonstration of the importance of the OH group to the formation of an enzyme-inhibitor complex. That X is bound more tightly to the enzyme than IX is a consequence of the increase in hydrophobic bonds in X. Comparison of XI and IV reveals that these compounds are bound equally, whereas the index of inhibition of XII is higher than V. The lesser inhibition of adenosine deaminase by XII relative to V can be rationalized if it is assumed that the OH group on the terminal position of the pentyl group would be located near the nonpolar region of the enzyme, and thus, would interfere with the formation of hydrophobic bonds. Whereas it is true that the hydroxypentyl group of XII could undergo folding so that the hydroxyl group could reach the hydroxyl binding site utilized by IX and X, this folding would be energetically unfavorable; therefore, the energy that could be gained by OH binding at the site two carbons removed from the 9-position of the adenine nucleus is more than offset by the energy required for folding the pentyl chain and by the energy lost by repulsion to binding in the hydrophobic region of the enzyme. In the case of XI and IV, these two opposing forces are very nearly equal, *i.e.*, the energy lost by repulsive forces of the hydroxyl group in the nonpolar region of the enzyme and the energy required to fold the butyl chain of XI nearly equals the energy gained by OH binding to the site on the enzyme utilized by the hydroxyl group of IX and X.

Finally, on the basis of this work, it appears that the 9-alkyl group of the various adenine derivatives is binding to the enzyme outside of the active site. It may be that the adenine moiety of these inhibitors is binding to the same site on the enzyme which is normally utilized by the adenine portion of the substrate, adenosine. Because of the length and flexibility of the 9-alkyl group, it can bridge to a nonpolar region of the enzyme to which the normal 9-substituent (β-D-ribofuranosyl) of the substrate is neither attracted nor able to reach. We believe that it should be possible to synthesize inhibitors which take advantage of the polar and nonpolar regions of adenosine deaminase.

Glutamic Acid Analogs. The Synthesis of 3-Alkylglutamic Acids and 4-Alkylpyroglutamic Acids[1a,b]

YOON C. KIM AND GEORGE H. COCOLAS[1c]

School of Pharmacy, University of North Carolina, Chapel Hill, North Carolina

Received July 8, 1964
Revised Manuscript Received February 15, 1965

3-Alkylglutamic acids, designed as metabolic antagonists of glutamic acid, were prepared by the acid hydrolysis of their corresponding 4-alkyl-5,5-dicarbethoxy-2-pyrrolidinones. The pyrrolidinones were formed from the Michael condensation of ethyl β-alkyl-α,β-unsaturated esters with diethyl acetamidomalonate. The Michael condensation of diethyl alkylidenemalonates and diethyl acylaminomalonates in refluxing ethanol formed 4-alkyl-3,5,5-tricarbethoxy-2-pyrrolidinones. Acid hydrolysis of these pyrrolidinones and subsequent pyrolysis of the reaction residue gave 4-alkylpyroglutamic acids.

For some time we have been interested in the synthesis of analogs of glutamic acid with the idea that they might possess antimetabolic activity. Of particular interest are the glutamic acids which have aliphatic substituents on positions 2, 3, and 4. A review[2] of the literature on this type of glutamic acid analog reveals that only a limited number has been reported.

The mechanism of glutamic acid activation and glutamine synthesis as described by Meister, *et al.*,[3] depicts an enzyme site which makes use of at least two points of attack; the amino group and the α-carboxyl group. The possibility of a steric restriction by alkyl substituents on the carbon skeleton to inhibit enzyme action exists, since mapping of the enzyme site remains incomplete. The proximity of a β-substituent on glutamic acid to the functional groups which are actively involved in the metabolic reactions of glutamic acid may produce the nonpolar steric impedance desired.

The synthesis of glutamic acids with alkyl substituents on the carbon skeleton has been accomplished by several routes, many of which are not applicable to universal substitution of the skeletal carbons of glutamic acid. The preparation of 2-methylglutamic acid by the hydrolysis of γ-cyano-γ-valerolactam[4] requires a Strecker-type synthesis with ethyl levulinate to prepare the valerolactam. Another synthesis of 2-methylglutamic acid has been reported by Pfister, *et al.*,[5] by the hydrolysis of the hydantoin prepared from the corresponding methyl ketone. 2-, 3-, and 4-Methylglutamic acids were prepared by the Schmidt reaction.[6] Another synthesis of 4-methylglutamic acid[7] and of 3-

(1) (a) Presented at the 144th National Meeting of the American Chemical Society, Los Angeles, Calif., April 1963, Abstracts, p. 18L. (b) This work was supported by a grant (GM-08644) from the National Institutes of Health, Public Health Service. (c) To whom inquiries should be addressed.

(2) (a) E. Borek and H. Waelsch, *J. Biol. Chem.*, **177**, 135 (1949); (b) C. E. Dent and D. I. Fowler, *Biochem. J.*, **56**, 54 (1954); (c) P. Ayengar and E. Roberts, *Proc. Soc. Exptl. Biol. Med.*, **79**, 476 (1952); (d) E. Roberts, *Federation Proc.*, **11**, 275 (1952).

(3) A. Meister, P. R. Krishnaswamy, and V. Pamiljans, *ibid.*, **21**, 1013 (1962).

(4) A. E. Gal, S. Avakian, and G. J. Martin, *J. Am. Chem. Soc.*, **76**, 4181 (1954).

(5) K. Pfister, W. J. Leanza, J. P. Conbere, H. J. Becker, A. R. Matzuk, and E. F. Rogers, *ibid.*, **77**, 697 (1955).

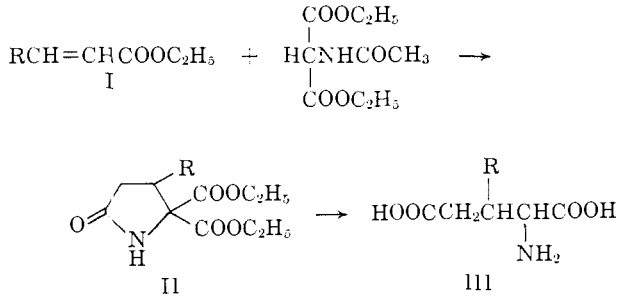
(6) J. Smrt and F. Sorm, *Collections Czech. Chem. Commun.*, **18**, 131 (1953); *Chem. Abstr.*, **48**, 3903 (1954).

(7) J. Fillman and N. Albertson, *J. Am. Chem. Soc.*, **74**, 4969 (1952).

methylglutamic acid[8] was reported by hydrolysis of adducts of a Michael reaction.

The Michael addition of amidomalonates to an appropriate derivative of acrylic acid has been a convenient route to the synthesis of glutamic acid[9] itself. By using appropriately substituted α,β-unsaturated esters, a 3-alkylglutamic acid could be obtained after hydrolysis of the intermediate, 4-alkyl-5,5-dicarbethoxy-2-pyrrolidinone (II). This is illustrated in Chart I.

CHART I



I II III

The α,β-unsaturated esters (I) were conveniently prepared through a modification of a procedure by Galat[10] by condensing monoethyl malonate with an aldehyde in the presence of a small amount of pyridine. The water, as it is formed, was removed by azeotropic distillation with the solvent of the reaction, toluene. The yields ranged from 53–79%.

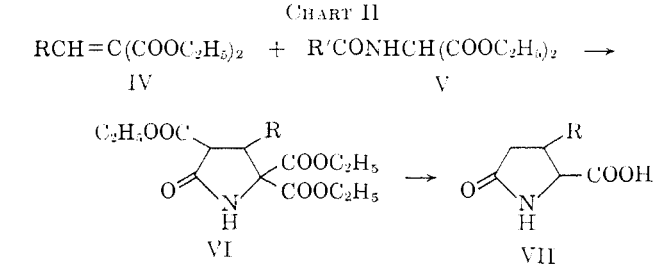
From previous studies[11] it was known that refluxing conditions in the reaction between diethyl acetamidomalonate and acrylic esters favored the formation of a cyclic intermediate instead of a normal addition product. The product obtained from the interaction of diethyl acetamidomalonate and the substituted α,β-unsaturated ester was therefore the corresponding 4-alkyl-5,5-dicarbethoxy-2-pyrrolidinone (II). It was also possible to obtain the cyclic product II by allowing the reactants to remain at room temperature for 14 days.

Hydrolysis of the addition–cyclization product II was routine. It was found more advantageous to use 49% fuming HBr to hydrolyze the ester–lactam rather than the more conventional HCl. The ammonium bromide formed on neutralization after hydrolysis was more soluble than ammonium chloride, in water or the water–ethanol mixture, from which the 3-alkylglutamic acids were precipitated. The yields ranged from 48–81%.

The 3-alkylglutamic acids gave characteristic positive ninhydrin tests. All the acids melted with decomposition, evolving a gas and subsequently solidifying and remelting at the temperature of the corresponding pyroglutamic acid. Ascending chromatographic techniques with a series of solvent systems[12] failed to show a separation of the two theoretically possible diastereoisomeric pairs.

In order to compare the 3-alkylglutamic acids insofar as purity and stability, the synthesis of their corresponding pyroglutamic acids was desired. These were best prepared according to the scheme shown in Chart II.

CHART II



IV V VI VII

The condensation between the diethyl alkylidenemalonates (IV) and diethyl acetamidomalonate (V, R′ = CH_3) at room temperature in the presence of 0.5 molar equiv. of $NaOC_2H_5$ produced crystalline products which were identified as 4-alkyl-3,5,5-tricarbethoxy-2-pyrrolidinones (VI). However, better yields were obtained when the condensation was carried out in refluxing ethanol.

Proof for the formation of the pyrrolidinone derivatives was made evident when the alkylidenemalonates were allowed to react with diethyl propionamidomalonate (V, R′ = CH_3CH_2) to give the identical 4-alkyl-3,5,5-tricarbethoxy-2-pyrrolidinones obtained from the reaction of diethyl acetamidomalonate and the same alkylidenemalonate. Additional proof for the products formed was given by agreeable results of elemental analysis and superimposed infrared spectra of the two compounds. All seven homologs of 4-alkyl-3,5,5-tricarbethoxy-2-pyrrolidinones are characterized by a sharp strong peak at 1750 cm.$^{-1}$.

The hydrolysis of 4-alkyl-3,5,5-tricarbethoxy-2-pyrrolidinones by refluxing in HCl appears to give a mixture of products. The crude residue gives a positive ninhydrin test giving evidence to the presence of the 3-alkylglutamic acid molecule. It is not unlikely that some partially hydrolyzed intermediates such as the pyroglutamic acids which may occur in partial decarboxylation and only partial hydrolysis are also present along with the 3-alkylglutamic acid hydrochloride.

Although it is not reported in the literature, it has been established in this laboratory that L-(−)-glutamic acid hydrochloride can be pyrolyzed to racemic pyroglutamic acid. In view of this, the residue so obtained from the hydrolysis of the triester–lactam VI containing the 3-alkylglutamic acid hydrochloride was fused at 200–220° in an oil bath to form a 4-alkylpyroglutamic acid (VII).

Microbiological studies on the effect of growth of three organisms, namely, *Staphylococcus aureus*, *Escherichia coli*, and *Proteus vulgaris*, in the presence of the substituted glutamic acids showed no apparent inhibition. None of the 3-substituted glutamic acid analogs had any significant effect on the rate of growth. Microscopic examination did not reveal any irregularity in the morphology of the organisms.

Experimental[13]

Monoethyl Malonate.—The procedure of Strube[14] was followed. The product was purified by distillation, b.p. 77–78° (0.15 mm.).

Anal. Calcd. C, 45.45; H, 6.10. Found: C, 45.21; H, 6.2 1.

(8) D. C. Morrison, *J. Am. Chem. Soc.*, **77**, 6072 (1955).

(9) (a) N. Albertson and S. Archer, *ibid.*, **67**, 2043 (1945); (b) H. R. Snyder, J. F. Shekleton, and C. D. Lewis, *ibid.*, **67**, 310 (1945); (c) C. S. Marvel and M. P. Stoddard, *J. Org. Chem.*, **3**, 198 (1938); (d) M. Dunn, B. Smart, C. Redemann, and K. Brown, *J. Biol. Chem.*, **94**, 599 (1931).

(10) A. Galat, *J. Am. Chem. Soc.*, **68**, 376 (1946).

(11) G. Cocolas and W. H. Hartung, *ibid.*, **79**, 5203 (1957).

(12) M. Gutcho and L. Laufer in "Glutathione," S. Colwick, *et al.*, Ed., Academic Press Inc., New York, N. Y., 1954, p. 79.

(13) Melting points were taken in open capillary tubes on a Mel-Temp apparatus (Laboratory Devices, Cambridge, Mass.) and are corrected.

(14) R. E. Strube, *Org. Syn.*, **37**, 34 (1957).

TABLE I

β-SUBSTITUTED α,β-UNSATURATED ESTERS (I)

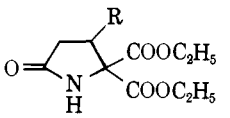
$RCH{=}CHCOOC_2H_5$

R	B.p., °C. (mm.)	% yield	Formula	% calcd. C	% calcd. H	% found C	% found H
CH_3[a]	...	..	...	...	...	...	...
C_2H_3	53 (13)	53	$C_7H_{12}O_2$	65.95	9.44	65.40	9.39
$(CH_3)_2$	78 (28)	70	$C_8H_{14}O_2$	67.57	9.92	67.69	9.85
$CH_3(CH_2)_2$	54 (8)	70	$C_8H_{14}O_2$	67.57	9.92	66.96	9.93
$CH_3(CH_2)_3$	81 (6)	79	$C_9H_{16}O_2$	69.19	10.33	69.66	10.22
$(CH_3)_2CHCH_2$	77 (15)	62	$C_9H_{16}O_2$	69.19	10.33	68.59	10.26
$CH_3(CH_2)_4$	92 (6)	73	$C_{10}H_{18}O_2$	70.55	10.66	69.96	10.55

[a] Ethyl crotonate, Sapon Labs, New York, N. Y.

TABLE II

4-ALKYL-5,5-DICARBETHOXY-2-PYRROLIDINONES (II)



R	M.p., °C.	% yield Reflux	% yield Room temp., 14 days	Formula	% calcd. C	% calcd. H	% calcd. N	% found C	% found H	% found N
CH_3	77–78	88	72	$C_{11}H_{17}NO_5$	54.31	7.04	5.76	54.39	6.94	5.75
C_2H_5	113–115	86	92	$C_{12}H_{19}NO_5$	56.02	7.44	5.44	56.03	7.50	5.32
$CH_3(CH_2)_2$	88–89	70	70	$C_{13}H_{21}NO_5$	57.55	7.80	5.16	57.78	7.94	4.99
$(CH_3)_2CH$	98–99	63	85	$C_{13}H_{21}NO_5$	57.55	7.80	5.16	57.85	7.77	5.18
$CH_3(CH_2)_3$	104–105	67	68	$C_{14}H_{23}NO_5$	58.93	8.13	4.91	58.61	8.19	4.98
$(CH_3)_2CHCH_2$	109	77	70	$C_{14}H_{23}NO_5$	58.93	8.13	4.91	58.96	8.17	4.75
$CH_3(CH_2)_4$	104–105	67	84	$C_{15}H_{25}NO_5$	60.18	8.42	4.68	60.41	8.41	4.46

TABLE III

3-ALKYLGLUTAMIC ACIDS (III)

$HOOCCH_2\underset{\displaystyle R}{\underset{|}{C}}H\underset{\displaystyle NH_2}{\underset{|}{C}}HCOOH$

R	M.p. dec., °C.	% yield	Formula	% calcd. C	% calcd. H	% calcd. N	% found C	% found H	% found N
CH_3	164	52	$C_6H_{11}NO_4$	44.74	6.88	8.69	44.31	7.01	8.39
C_2H_5	153	48	$C_7H_{13}NO_4$	47.99	7.48	8.00	47.96	7.55	8.04
$CH_3(CH_2)_2$	166	58	$C_8H_{15}NO_4$	50.78	7.99	7.40	50.88	8.05	7.46
$(CH_3)_2CH$	162	58	$C_8H_{15}NO_4$	50.78	7.99	7.40	50.67	7.96	7.40
$CH_3(CH_2)_3$	158	70	$C_9H_{17}NO_4$	53.19	8.43	6.89	53.32	8.19	6.95
$(CH_3)_2CHCH_2$	158	63	$C_9H_{17}NO_4$	53.19	8.43	6.89	53.43	8.26	6.84
$CH_3(CH_2)_4$	155	81	$C_{10}H_{19}NO_4$	55.28	8.82	6.45	55.47	8.95	6.50

Ethyl β-Alkyl-α,β-unsaturated Esters (I).—The following is a general procedure for the preparation of substituted α,β-unsaturated esters. To 40 g. (0.3 mole) of monoethyl malonate and 0.4 mole of freshly distilled aldehyde was added 100 ml. of toluene and 2 ml. of pyridine. The mixture was refluxed vigorously for at least 4 hr., and the water was eliminated and collected in a moisture tube. It was possible to detect CO_2 from the effluent gases at the top of the condenser by directing them into a solution of lime water. Evolution of CO_2 was not determined quantitatively. The condenser was then set downward for distillation and the toluene was collected at atmospheric pressure. The distillation was continued *in vacuo*, and the product was collected over a 5° boiling range. The crude distillate was taken up in ether and washed first with dilute HCl, then 5% Na_2CO_3, and finally water until the washings were neutral. The ethereal solution was dried (Na_2SO_4) and then distilled through a 12-cm. Vigreux column and the ethyl β-alkyl-α,β-unsaturated ester was collected. The data are summarized in Table I.

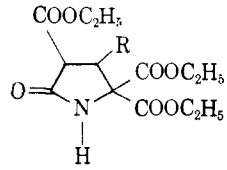
Diethyl-4-alkyl-5,5-dicarbethoxy-2-pyrrolidinone (II).—The following is a general procedure for the preparation of the 2-pyrrolidinone analogs listed in Table II. To 400 mg. (0.017 g.-atom) of sodium dissolved in 200 ml. of absolute ethanol was added 21.7 g. (0.1 mole) of diethyl acetamidomalonate and 0.125 mole of an ethyl β-alkyl-α,β-unsaturated ester. The reaction mixture was then refluxed overnight (10–12 hr.). After this period of time 2 ml. of glacial acetic acid was added, and the solvent and other volatile material were removed under reduced pressure with the aid of a water aspirator and a steam bath. On cooling, the residue solidified. The residue was dissolved in 50 ml. of toluene and to this was added about 20 ml. of petroleum ether (b.p. 30–60°). The product precipitated when the mixture was cooled. The crystals were collected and washed with water. A sample could be purified for analysis by recrystallization from toluene.

The procedure for the synthesis of the 4-alkyl-5,5-dicarbethoxy-2-pyrrolidinones at room temperature is identical with that described above except for the reaction conditions. The reagents were mixed and allowed to react for 14 days at room temperature before the $NaOC_2H_5$ was neutralized with glacial acetic acid.

3-Alkylglutamic Acids (III).—The following is a procedure which can be generally applied to the preparation of all the 3-alkylglutamic acids (Table III). Ten grams of a 3-alkyl-5,5-dicarbethoxy-2-pyrrolidinone was refluxed in 150 ml. of 49% fuming HBr for 4 hr. After this time, the contents were placed in an evaporator and the volatile constituents were removed *in vacuo* with the aid of a hot-water bath. The gummy residue was dissolved in 25 ml. of distilled water and the water was removed as before with the aid of the evaporator. This process was repeated once more. The residue was dissolved in 20 ml. of water, and

TABLE IV

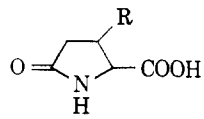
4-ALKYL-3,5,5-TRICARBETHOXY-2-PYRROLIDINONES (VI)



R	M.p., °C.	% yield	Formula	% calcd. C	H	N	% found C	H	N
CH_3	96–97	49	$C_{14}H_{21}NO_7$	53.32	6.71	4.44	53.26	6.66	4.44
C_2H_5	81–82	67	$C_{15}H_{23}NO_7$	54.70	7.04	4.25	54.85	6.97	4.19
$CH_3(CH_2)_2$	74–75	76	$C_{16}H_{23}NO_7$	55.97	7.34	4.08	55.80	7.31	3.98
$(CH_3)_2CH$	79–80	83	$C_{16}H_{26}NO_7$	55.97	7.34	4.08	56.31	7.43	4.28
$CH_3(CH_2)_3$	84–85	76	$C_{17}H_{27}NO_7$	57.13	7.62	3.92	57.04	7.87	4.06
$(CH_3)_2CHCH_2$	135	90	$C_{17}H_{27}NO_7$	57.13	7.62	3.92	57.49	7.39	3.98
$CH_3(CH_2)_4$	93–95	78	$C_{18}H_{29}NO_7$	58.21	7.87	3.77	58.75	7.74	3.76

TABLE V

4-ALKYLPYROGLUTAMIC ACIDS (VII)



R	M.p., °C.	% yield	Formula	% calcd. C	H	N	Neut. equiv.	% found C	H	N	Neut. equiv.
H	183–185	50	$C_5H_7NO_3$	46.51	5.46	10.85	129.1	46.15	5.62	10.94	130.2
CH_3	149–150[a]	53	$C_6H_9NO_3$	50.34	6.34	9.79	143.1	50.33	6.35	9.76	143.0
C_2H_5	138–139	31	$C_7H_{11}NO_3$	53.49	7.05	8.91	157.1	53.51	7.05	8.95	156.5
$CH_3(CH_2)_2$	143–145	30	$C_8H_{13}NO_3$	56.13	7.65	8.18	171.2	56.28	7.84	8.25	170.8
$(CH_3)_2CH$	189–190	88	$C_8H_{13}NO_3$	56.13	7.65	8.18	171.2	56.06	7.85	8.29	171.1
$CH_3(CH_2)_3$	150–151	39	$C_9H_{15}NO_3$	58.36	8.16	7.56	185.2	58.32	8.23	7.55	186.2
$(CH_3)_2CHCH_2$	169–171	70	$C_9H_{15}NO_3$	58.36	8.16	7.56	185.2	57.71	8.40	7.55	185.3
$CH_3(CH_2)_4$	153–155	74	$C_{10}H_{17}NO_3$	60.28	8.60	7.03	199.3	60.36	8.60	6.94	198.0

[a] Lit.[11] m.p. 148–152°.

the pH of the solution was adjusted to 3.2 with concentrated NH_3 solution. At this point the chain length of the individual 3-alkylglutamic acids altered the solubility so that those whose side chains were larger precipitated with ease from solution. Precipitation of the alkylglutamic acids with smaller substituents (methyl, ethyl, and propyl) could be encouraged by cooling on an ice bath or by diluting the aqueous solution with 100 ml. of absolute ethanol. Precipitation from the water–alcohol mixture is complete in 48 hr. Care must be taken to add the ethanol slowly to prevent the precipitation of an amorphous solid which is not characteristic of the desired 3-alkylglutamic acids. Samples of the amino acids were purified for analysis by recrystallizing from a water–ethanol mixture. All melted with decomposition. Melting points of the decomposed 3-alkylglutamic acids corresponded with those of their pyroglutamic acids.

4-Alkyl-3,5,5-tricarbethoxy-2-pyrrolidinone (VI). A. From Diethyl Acetamidomalonate.—A solution of 25 g. (0.115 mole) of diethyl acetamidomalonate in 200 ml. of absolute ethanol was added to 50 ml. of absolute ethanol in which was dissolved 1.3 g. (0.057 g.-atom) of sodium. When the two solutions were mixed 0.13 mole of diethyl alkylidenemalonate was added and the mixture refluxed for 10 hr. The reaction mixture was then neutralized with glacial acetic acid, and the solvent was removed under reduced pressure. The residue which remained was treated with 100 ml. of water, and the crystals which formed were filtered and recrystallized from 95% ethanol (see Table IV).

B. From Diethyl Propionamidomalonate.—A solution of 10 g. (0.043 mole) of diethyl propionamidomalonate was dissolved in 100 ml. of ethanol containing 0.50 g. (0.022 g.-atom) of sodium. To this mixture was added 0.05 mole of the diethyl alkylidenemalonate and the mixture refluxed for 10 hr. The reaction mixture was treated in the same manner as described above to obtain the product.

4-Alkylpyroglutamic Acids (VII).—Ten grams of the 4-alkyl-3,5,5-tricarbethoxy-2-pyrrolidinone was dissolved in 80 ml. of concentrated HCl and the mixture refluxed for 3 hr. The solvent was then removed *in vacuo* with the aid of a water bath until the residue remaining was a viscous mass. The residue was transferred to a porcelain evaporating dish and heated on a steam bath. The residue, on cooling overnight, became a plastic mass. The dish was then placed in an oil bath, and the temperature was maintained at 200–220° for 10 min. during which time vigorous bubbling occurred and diminished. The fused mass was dissolved in 30 ml. of hot water, adding ethanol when necessary to complete the solution, and then filtered. The filtrate was decolorized with 0.5 g. of charcoal, concentrated to at least half of its original volume and cooled to precipitate the 4-alkylpyroglutamic acids. The product was recrystallized twice from water or a 3:1 water–ethanol mixture for purification for analysis (see Table V).

Pyrolysis of Glutamic Acid Hydrochloride.—In a porcelain evaporating dish 50 g. of L-glutamic acid hydrochloride was mixed with 20 ml. of distilled water, and the dish was placed on an oil bath and supported thereon by wire mesh. The oil was heated to 200–220°, and the dish was allowed to remain in the oil until the effervescence ceased and the residue became a brown mass. The fused material was cooled and dissolved in a minimum quantity of hot distilled water. The solution was filtered and cooled to precipitate 13 g. of racemic pyroglutamic acid, m.p. 183–185°. Concentration of the mother liquor yielded more pyroglutamic acid.

Inhibition Studies.—The effect of the 3-alkyl-substituted DL-glutamic acid analogs on the growth and morphology of *S. aureus* M-250, *E. coli* J M-206, and *P. vulgaris* M-240 was evaluated by a turbidimetric method at concentrations of 10, 100, and 1000 γ/ml. The organisms were grown in tryptose–phosphate broth and the glutamic acid analog was added either at the beginning of the growth curve or at a later time during the period of exponential growth. Growth rate was compared in a Klett–Summerson colorimeter using a 540 filter. The cells exposed to the glutamic analogs were also examined microscopically for the presence of aberrant forms.

Acknowledgment.—The authors are indebted to Dr. R. R. Mohan, Mr. R. S. Pianotti, and Mr. B. S. Schwartz, Department of Microbiology of the Warner-Lambert Research Institute, research affiliate of the Warner-Chilcott Laboratories, for carrying out the inhibition studies.

The Metabolite of 3′,5′-Dichloro-4-amino-4-deoxy-N^{10}-methylpteroylglutamic Acid (Dichloromethotrexate)

TI LI LOO AND RICHARD H. ADAMSON

Laboratory of Chemical Pharmacology, National Cancer Institute, National Institutes of Health, Bethesda, Maryland

Received February 1, 1965

Dichloromethotrexate (DCM) is partly metabolized by man and three rodent species. This metabolite is also obtained by incubating DCM with rat liver homogenate. Based on degradation experiments, the metabolite is shown to be 3′,5′-dichloro-4-amino-4-deoxy-7-hydroxy-N^{10}-methylpteroylglutamic acid.

In studies of the physiological disposition[1a-c] of dichloromethotrexate[2a-c] (abbreviated DCM in this paper), a pteroylglutamic acid antagonist of clinical interest[6] in the treatment of leukemia, the presence of a metabolite of DCM was recognized in the urine of animals that had received the drug by different routes. Employing Cl^{36}-labeled DCM, it was observed that almost all the radioactivity was recoverable from the urine and feces of man,[1c] dog, and three rodent species[1b] treated with this drug. In addition to unaltered DCM, one and only one metabolite, amounting to almost one-third of the dose of DCM administered, was detected. The dog, however, excreted DCM unchanged.

An identical metabolite was isolated from the *in vitro* incubation of DCM with rat liver homogenate.[7] As in the case of the *in vivo* experiments, dog liver homogenate was ineffective in metabolizing DCM.

Because of the minute quantity of pure DCM metabolite available for study and the inherent difficulties involved in pteridine chemistry, classical techniques are not always useful in structural elucidation.[8] To establish identity most of the following criteria were relied upon: elementary analysis if possible, ultraviolet absorption spectroscopy,[9] electrophoretic mobility,[10] and position of elution from a diethylaminoethylcellulose column.[3] Taken alone, none of these criteria would be sufficient to warrant a conclusive structural proof.

The fact that there is only one metabolite which is not identical with any simple pteridine related to DCM excludes the possibility that it is a cleavage product of DCM. Also, the metabolite is distinctly different from 3′,5′-dichloro-N^{10}-methylpteroylglutamic acid[5] in spectra and electrophoretic mobility and hence cannot result from DCM by simple hydrolysis of the 4-amino group.

Analytical data of DCM and its magnesium salt can best be expressed by the respective empirical formulas, $C_{20}H_{20}Cl_2N_8O_6 \cdot H_2O$ and $C_{20}H_{18}Cl_2MgN_8O_6 \cdot 5H_2O$.[11] In other words, tentatively, the metabolite seems to differ from its parent compound only in an oxygen atom. Existing knowledge of drug metabolism suggests that the extra oxygen atom is located in either the pteridine or the benzene ring.

A comparison of the ultraviolet absorption spectrum of DCM in dilute alkali with that of its metabolite discloses that the absorption maximum at 370 mμ for DCM has been displaced 26 mμ to 344 for the metabolite. Such a hypsochromic shift is strongly reminiscent of a similar shift of 26 mμ in dilute alkali from 364 for 2,4-diamino-6-methylpteridine[4] to 338 mμ for 2,4-diamino-7-hydroxy-6-methylpteridine.[12] As a whole, the general pattern of change in the spectra in both acid and alkali matches closely for these two pairs of compounds. Nevertheless, in view of the corresponding

(1) (a) V. T. Oliverio and T. L. Loo, *Proc. Am. Assoc. Cancer Res.*, **3**, 140 (1960); (b) V. T. Oliverio and J. D. Davidson, *J. Pharmacol. Exptl. Therap.*, **137**, 76 (1962); (c) J. D. Davidson and V. T. Oliverio, *Clin. Pharmacol. Exptl. Therap.*, in press.

(2) (a) Dichloromethotrexate is the 3′,5′-dichloro derivative of methotrexate. Methotrexate or amethopterin are generic names for 4-amino-4-deoxy-N^{10}-methylpteroylglutamic acid or N-(*p*-{[(2,4-diamino-6-pteridinyl)-methyl]methylamino}benzoyl)glutamic acid. (b) The DCM was supplied by Lederle Laboratories Division of American Cyanamid Co. through the courtesy of Cancer Chemotherapy National Service Center of the National Cancer Institute. The drug used in the rabbit experiments was estimated to be over 90% pure by chromatography.[3] For incubation with rat liver homogenate, DCM was purified by column chromatography on DEAE cellulose.[3] (c) The synthesis of methotrexate was reported in ref. 4, and that of DCM in ref. 5, and also by R. Angier and W. V. Curran, *J. Am. Chem. Soc.*, **81**, 2814 (1959).

(3) V. T. Oliverio, *Anal. Chem.*, **33**, 263 (1961).

(4) D. R. Seeger, D. B. Cosulich, J. M. Smith, Jr., and M. E. Hultquist [*J. Am. Chem. Soc.*, **71**, 1753 (1949)] reported absorption maximum at 369 mμ in alkali.

(5) D. B. Cosulich, D. R. Seeger, M. J. Fahrenbach, B. Roth, J. H. Mowat, J. M. Smith, Jr., and M. E. Hultquist, *ibid.*, **73**, 2554 (1951).

(6) E. Frei, III, C. L. Spurr, C. O. Brindley, O. Selawry, J. F. Holland, D. P. Rall, L. R. Wasserman, B. Hoogstraten, B. I. Shnider, O. R. McIntyre, L. B. Matthews, Jr., and S. P. Miller, *Clin. Pharmacol. Exptl. Therap.*, **6**, 160 (1965).

(7) T. L. Loo and R. H. Adamson, *Biochem. Pharmacol.*, **11**, 170 (1962). In this paper the structure proposed for the metabolite 3′,5′-dichloro-7-hydroxy-N^{10}-methylpteroylglutamic acid or 4-deamino-4,7-dihydroxy-DCM was later shown to be erroneous. See text.

(8) For an excellent discussion on this point, see A. Albert, *Quart. Rev.* (London), **6**, 197 (1952).

(9) Infrared spectroscopy, on the other hand, proves to be of little application to our problem owing to overlap of hydrogen-bonded OH and NH stretching frequencies.

(10) See J. D. Davidson, *J. Natl. Cancer Inst.*, **29**, 792 (1962), for a description of method and equipment.

(11) In pteridine chemistry the interpretation of analytical data must proceed with extreme caution.[8] In the present case, ultimate analysis provides no basis for discrimination between the proposed empirical formula $C_{20}H_{20}Cl_2N_8O_6 \cdot H_2O$ and $C_{20}H_{20}Cl_2N_8O_6 \cdot 2H_2O$ (DCM dihydrate) or $C_{20}H_{18}Cl_2MgN_8O_6 \cdot 5H_2O$ and $C_{20}H_{18}Cl_2MgN_8O_6 \cdot 6H_2O$ (hexahydrated magnesium salt of DCM). Determination of water content by the Karl Fisher reagent gave inconclusive results in our hands probably because of addition of water across double bonds in some of these pteridines.[8]

(12) G. B. Elion, G. H. Hitchings, and P. B. Russell [*J. Am. Chem. Soc.*, **72**, 78 (1950)] reported absorption maximum in alkali at 340 mμ.