dried to give 6.4 g (90%) of 3-(2-fluorobenzoylamino)-2-pyridone, mp 218 °C. The melting point was raised to 223 °C by crystallization from absolute ethanol. A solution of 5.4 g of the above crude amide in 55 mL of $POCl_3$ was heated under reflux for 5 h. After removing most of the solvent the residue was carefully treated with ice. The solid that separated was collected and dissolved in 50 mL of warm benzene. After adding 50 mL of Et_2O the mixture was filtered through Supercel and the filtrate concentrated. The addition of petroleum ether induced crystallization: yield 3.1 g (62%); mp 119–120 °C.

Method C. 2-(2-Cyanophenyl)oxazolo[5,4-*b*]pyridine (II-19). A mixture of 2 g (0.007 mol) of II-16, 1.6 g (0.18 mol) of CuCN, and 15 mL of 1-methyl-2-pyrrolidinone was purged with N_2 and heated in an oil bath until the bath temperature was 170 °C. After 3 h the mixture was cooled and diluted with 75 mL of 10% NH_4OH. The solid that separated was collected and weighed 2.3 g. This was extracted with 100 mL of boiling CH_2Cl_2 leaving 600 mg of dark-brown material. The CH_2Cl_2 solution was concentrated and diluted with petroleum ether to the cloud point and filtered through Supercel. Crystals separated in the filtrate: yield 1.1 g (68%); mp 146–147 °C.

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Potential Anticancer Agents. 16. Methotrexate Analogues with a Modified Peptide Side Chain

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Nine analogues of methotrexate, in which the side chain is modified by replacement of the terminal glutamyl moiety with other amino acids, were synthesized from 2,4-diamino-6-(chloromethyl)pteridine. None of these compounds exhibited significant activity against L1210.

The synthesis of new methotrexate (MTX) analogues, in order to find more effective anticancer agents and increase the number of compounds available for structure–activity analysis, has been one of the main goals of our laboratory since 1972.[2-4]

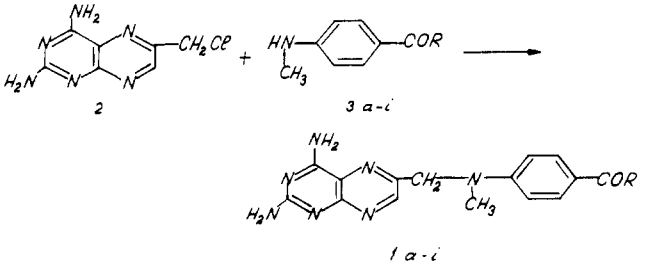
Both the position of attachment of the peptide side chain to the pteridine ring[3] and the influence of the optically active center[4,5] on the biological properties of these analogues were investigated previously. In this paper our attention is therefore focused on other changes in the peptide side chain.

Replacement of the terminal glutamic acid by other amino acids has been accomplished both in folic acid[6-8] and in aminopterin.[6,8,9] Surprisingly, only lysine, β-aminoglutaric acid, aspartic acid, α-aminoadipic acid, and α-aminopimelic acid congeners of MTX are known,[5,10-12] although a considerable number of alkyl ester and amide analogues have been prepared.[13-19] None of these is any more effective than MTX, although some of them are powerful inhibitors of dihydrofolate reductase (DHFR).[15,17] It has been demonstrated that rapid cleavage of the ester function occurs after in vivo administration.[15,16]

There is evidence that the glutamyl residue is involved in active transport of MTX across cell membranes.[20-23] This could explain the fact that attempts to replace it (except with α-aminoadipate and α-aminopimelate)[24] have not been successful, giving only inactive compounds. On the other hand, the role of the α- and γ-carboxyl groups is not clear.

Accordingly, we directed our efforts toward the synthesis and biological evaluation of some MTX analogues, in which the glutamyl residue was replaced with monobasic

Scheme I



Scheme II

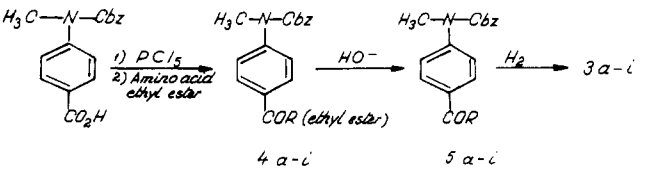


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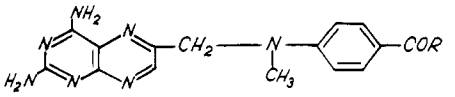
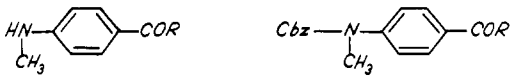
Table I. New Methotrexate Analogues



compd	R	formula[b]	mp, °C	UV data,[a] λ_{max}, nm (log ϵ)
1a	glycine	$C_{17}H_{18}N_8O_3$	225 dec	243 (4.22), 305 (4.27)
1b	DL-alanine	$C_{18}H_{20}N_8O_3 \cdot 2H_2O$	205 dec	244 (4.24), 307 (4.28)
1c	β-alanine	$C_{18}H_{20}N_8O_3 \cdot 2H_2O$	185	245 (4.19), 300 (4.20)
1d	sarcosine	$C_{18}H_{20}N_8O_3 \cdot 2H_2O$	195-200 dec	246 (4.35), 297 (4.20)
1e	DL-α-aminobutyric acid	$C_{19}H_{22}N_8O_3$	202 dec	245 (4.20), 308 (4.27)
1f	γ-aminobutyric acid	$C_{19}H_{22}N_8O_3 \cdot H_2O$	155	245 (4.12), 300 (4.09)
1g	DL-valine	$C_{20}H_{24}N_8O_3$	215-218	244 (4.24), 308 (4.17)
1h	L-leucine	$C_{21}H_{26}N_8O_3$	198 dec	245 (4.25), 308 (4.32)
1i	L-phenylalanine	$C_{24}H_{24}N_8O_3 \cdot H_2O$	193-195	245 (4.20), 308 (4.23)

[a] Determined in 0.1 N HCl solution. [b] All compounds were within ±0.4% of calculated values for C, H, and N.

Table II. Intermediate Peptides



3 α-i 4 α-i, 5 α-i

compd	R	formula[g]	mp,[a] °C	UV data,[b] λ_{max}, nm (log ϵ)
3a	$NHCH_2CO_2H$	$C_{10}H_{12}N_2O_3$	178-180	296 (4.22)
3b	$NHCH(CH_3)CO_2H$	$C_{11}H_{14}N_2O_3$	97-99	297 (4.07)[f]
3c	$NH(CH_2)_2CO_2H$	$C_{11}H_{14}N_2O_3 \cdot H_2O$	~66	294 (3.90)[f]
3d	$N(CH_3)CH_2CO_2H$	$C_{11}H_{14}N_2O_3$	133.5-135	287 (4.13)
3e	$NHCH(CH_2CH_3)CO_2H$	$C_{12}H_{16}N_2O_3 \cdot H_2O$	69-70	297 (4.21)[f]
3f	$NH(CH_2)_3CO_2H$	$C_{12}H_{16}N_2O_3$	42	295 (4.06)[f]
3g	$NHCH[CH(CH_3)_2]CO_2H$	$C_{13}H_{18}N_2O_3 \cdot H_2O$	~137	294 (3.98)[f]
3h	$NHCH[CH_2CH(CH_3)_2]CO_2H$	$C_{14}H_{20}N_2O_3$	87	297 (4.06)[f]
3i	$NHCH(CH_2C_6H_5)CO_2H$	$C_{17}H_{18}N_2O_3$	80-82	299 (4.10)
4a	$NHCH_2CO_2C_2H_5$	$C_{20}H_{22}N_2O_5$		270 (4.21)
4b	$NHCH(CH_3)CO_2C_2H_5$	$C_{21}H_{24}N_2O_5$	76-77	260 (4.19)
4c	$NH(CH_2)_2CO_2C_2H_5$	$C_{21}H_{24}N_2O_5$	44-45	260 (4.19)
4d	$N(CH_3)CH_2CO_2C_2H_5$	$C_{21}H_{24}N_2O_5$		250 (4.14)
4e	$NHCH(CH_2CH_3)CO_2C_2H_5$	$C_{22}H_{26}N_2O_5$	70-72	260 (4.08)[f]
4f	$NH(CH_2)_3CO_2C_2H_5$	$C_{22}H_{26}N_2O_5$	74-75	260 (4.10)[f]
4g	$NHCH[CH(CH_3)_2]CO_2C_2H_5$	$C_{23}H_{28}N_2O_5$		260 (4.26)
4h	$NHCH[CH_2CH(CH_3)_2]CO_2C_2H_5$	$C_{24}H_{30}N_2O_5$		260 (4.09)[f]
4i	$NHCH(CH_2C_6H_5)CO_2C_2H_5$	$C_{27}H_{28}N_2O_5$	60-62	260 (4.25)
5a	$NHCH_2CO_2H$	$C_{18}H_{18}N_2O_5$	134.5-135.5[c]	260 (4.25)
5b	$NHCH(CH_3)CO_2H$	$C_{19}H_{20}N_2O_5$	169.5-171.5[d]	262 (4.02)[f]
5c	$NH(CH_2)_3CO_2H$	$C_{19}H_{20}N_2O_5$	104.5-106	261 (4.22)
5d	$N(CH_3)CH_2CO_2H$	$C_{19}H_{20}N_2O_5$	137-139[c]	250 (4.29)
5e	$NHCH(CH_2CH_3)CO_2H$	$C_{20}H_{22}N_2O_5$	120-122	259 (4.12)[f]
5f	$NH(CH_2)_3CO_2H$	$C_{20}H_{22}N_2O_5$	117-118	258 (4.17)[f]
5g	$NHCH[CH(CH_3)_2]CO_2H$	$C_{21}H_{24}N_2O_5$	123-125	258 (4.13)[f]
5h	$NHCH[CH_2CH(CH_3)_2]CO_2H$	$C_{22}H_{26}N_2O_5$	129-130[e]	260 (4.28)
5i	$NHCH(CH_2C_6H_5)CO_2H$	$C_{25}H_{24}N_2O_5$	85-86[e]	260 (4.21)

[a] Recrystallized from AcOEt-petroleum ether, if not otherwise noted. [b] Determined in EtOH when not otherwise noted. [c] AcOEt. [d] AcOEt-Me_2CO, 1:1. [e] AcOEt, extracted with $NaHCO_3$ aqueous solution. [f] In MeOH. [g] All compounds were within ±0.4% of calculated values for C, H, and N.

amino acids (that contain an α- or γ-carboxyl group).

Synthesis. The condensation of 2,4-diamino-6-(chloromethyl)pteridine (**2**)[2a,4] with the 4-(*N*-methylamino)-benzoylamino acids **3**, in aqueous media at pH 7.5, afforded compounds **1a–i** (Scheme I).

The crude products were purified by column chromatography on cellulose (compounds **1a,b,d,e,g–i**) or precipitation of the magnesium salts (compounds **1c,f**). The physical constants of the new MTX analogues are given in Table I.

The 4-(*N*-methylamino)benzoylamino acids **3** were obtained in good overall yield by the method of Fu[25] (Scheme II).

Reproducible, high yields of the amino acid ethyl esters were obtained via a new azeotropic esterification procedure using 4-toluenesulfonic acid as the catalyst. This procedure is particularly advantageous in cases where large amounts of such intermediates are needed.

Most of the *N*-carbobenzoxy esters **4** resulted as difficultly crystallizable oils (e.g., **4i** crystallized only after several months). Hydrolysis (1 N NaOH) of the esters **4**, followed by catalytic hydrogenolysis over 10% Pd/C at room temperature and atmospheric pressure, afforded the 4-(*N*-methylamino)benzoylamino acids **3**. The physical constants for the intermediate peptides **3–5** are summarized in Table II.

Biological Data. The new MTX analogues were tested against L1210 leukemia in the mouse and W 256 carcinosarcoma in the rat. The data are presented in Table III.

Replacement of the α- or γ-carboxyl group in the glutamyl moiety with a hydrogen atom (**1e** and **1f**) greatly affects the biological properties of the MTX analogues, resulting in a loss of anticancer activity. However, the α-CO_2H seems to be more important than the γ-CO_2H.

Table III. Anticancer Activity of Methotrexate Analogues

compd	LD_{50}[a] mg/kg	LD_{50}[a] mmol/kg	L1210[b] dose (mg/kg) × no. of admin[d]	L1210[b] ILS, %[e]	W 256[c] dose (mg/kg) × no. of admin[d]	W 256[c] TGI, %[f]
1a	250	0.65	23 × 7	0		
1b	349	0.81	33 × 6	0		
1c	250	0.58	25 × 6	19		
1d	400	0.92	50 × 6	0		
1e	925	2.25	78 × 9	16	60 × 14	47
1f	106	0.25	10 × 8	0	8 × 14	55
1g	200	0.47	20 × 7	0		
1h	710	0.42	90 × 9	24		
1i	500	1.02	45 × 10	14	40 × 9	58

[a] Single doses on Wistar rats: J. Cornfield and N. Mantel, *J. Am. Stat. Assoc.*, 45, 181 (1950). [b] In BDF_1 bearing mice: R. H. Adamson, S. T. Yancew, M. Ben, T. L. Loo, and D. P. Rall, *Arch. Int. Pharmacodyn. Ther.*, 153, 87 (1965). Treatment was begun 24 h after ip inoculation of 10^6 leukemic cells. [c] Walker 256 carcinosarcoma in rats; treatment was begun 7 days after tumor transplantation. [d] Daily administration in aqueous solution, pH 8.0–8.4. [e] % ILS = (T/C − 1) × 100. [f] % TGI = (1 − T/C) × 100.

This finding is also supported by recently published data on growth-inhibiting activity against human lymphoblastic leukemia exhibited by methotrexate α- and γ-glutamates.[24] An increase in the number of carbons in the alkyl chain (provided that the α-CO_2H group is still present) restores the antileukemic effectiveness to some extent (from 0% in **1a** to 24% in **1h**).

Experimental Section

Melting points were taken on a Boetius apparatus and are uncorrected. Ultraviolet spectra were determined with a Spekord UV–vis spectrophotometer, and infrared spectra were run on an IR 75 Carl Zeiss Jena spectrophotometer.

Amino Acid Ethyl Esters. General Procedure. A mixture of the desired amino acid hydrochloride (3 mol), EtOH (3300 mL), benzene (800 mL), and 4-toluenesulfonic acid (20 g) was introduced in the bottom of a rectification column (2.5 cm in diameter, 20 theoretical plates) equipped with a Dean-Stark trap to allow continuous water removal from the reaction. Completion of the esterification was monitored by TLC (Merck plates, Kieselgel F_{254}, 1:1:1:1 BuOH–AcOH–AcOEt–H_2O). When the reaction was completed, the reaction mixture was concentrated to dryness in vacuo and the residue crystallized by trituration with Et_2O. This procedure gave constant yields of 85–90%.[26,27] The following amino acid ethyl ester hydrochlorides were obtained (mp, °C): glycine (144–145), DL-alanine (80–82), β-alanine (56), sarcosine (122–124), DL-α-aminobutyric (148–150), γ-aminobutyric (63–65), DL-valine (98–100), L-leucine (126–128), L-phenylalanine (148–150), L-glutamic (107–109), D-glutamic (110.5–111.5), DL-glutamic (73.5–75), and DL-aspartic (87.5–89.5).

4-(*N*-Carbobenzoxy-*N*-methylamino)benzoylamino Acid Ethyl Esters 4a–i. General Procedure. A mixture of 4-(*N*-carbobenzoxy-*N*-methylamino)benzoic acid (0.1 mol),[4,25] PCl_5 (0.12 mol), and anhydrous Et_2O (400 mL) was stirred for 1 h at room temperature. The ether solution was washed with ice water and the organic layer was then added, with stirring at 0 °C, to a solution of the desired amino acid ethyl ester hydrochloride (0.12 mol)[26,27] and $NaHCO_3$ (66 g) in a mixture of H_2O (400 mL) and AcOEt (400 mL). The mixture was stirred continuously for 1 h at 0 °C and 2 h at room temperature. The organic layer was separated, washed successively with H_2O, 2 N HCl, and H_2O (200 mL each), dried (Na_2SO_4), and evaporated until a solid or an oil was obtained. Purification from AcOEt–petroleum ether (bp 45–60 °C) afforded products giving only a single spot on TLC (Merck plates, Kieselgel F_{254}, 9.5:0.5:0.5 $CHCl_3$–MeOH–AcOH). The yields were at least 80–85%.

4-(*N*-Carbobenzoxy-*N*-methylamino)benzoylamino Acids 5a–i. General Procedure. To a solution of esters **4a–i** (0.1 mol) in MeOH (200 mL), cooled at 0 °C, was added 1 N NaOH (110 mL) with stirring and cooling to less than 6 °C. The reaction was continued for 1 h more at this temperature and then completed at room temperature. The end of the reaction could be determined by TLC (Merck plates, Kieselgel F_{254}, 9.5:0.5:0.5 $CHCl_3$–MeOH–AcOH). The reaction mixture was treated with charcoal, filtered, diluted with H_2O, and adjusted to pH 2 with 6 N HCl. The 4-(*N*-carbobenzoxy-*N*-methylamino)benzoylamino acids, which crystallized after standing several hours or days at room temperature, were dissolved in AcOEt and extracted with a $NaHCO_3$ solution. The organic layer was discarded and the free acid precipitated from the aqueous solution by adding 6 N HCl. Filtration, drying, and recrystallization (AcOEt–petroleum ether) yielded a pure product according to TLC (Merck plates, Kieselgel F_{254}, 4:1:1 BuOH–AcOH–H_2O). The yields were ca. 90%.

4-(*N*-Methylamino)benzoylamino Acids 3a–i. General Procedure. A 10% (w/v) solution of 4-(*N*-carbobenzoxy-*N*-methylamino)benzoylamino acid in MeOH was subjected to catalytic hydrogenolysis over 10% Pd/C, at room temperature and atmospheric pressure. After the completion of the reaction, the catalyst was filtered and the solution evaporated to dryness in vacuo. The resultant solid was of high purity according to TLC (4:1:1 BuOH–AcOH–H_2O).

4-[*N*-[(2,4-Diamino-6-pteridinyl)methyl]-*N*-methylamino]benzoylamino Acids 1a–i. General Procedure. The pH of an aqueous solution containing the desired 4-(*N*-methylamino)benzoylamino acid (0.1 mol) was adjusted to 7.5 with solid $NaHCO_3$ (or $KHCO_3$ for compounds **3c,f**), and finely powdered 2,4-diamino-6-(chloromethyl)pteridine (0.12 mol) was added at 45 °C during 1 h. The mixture was stirred at 45 °C for 24 h, the pH being maintained at 7.5 with occasional addition of $NaHCO_3$ or $KHCO_3$, and the hot solution was then filtered. The filtrate was adjusted to pH 3.5–4 with AcOH and kept overnight at 4 °C. The deposited solid was filtered, washed successively with H_2O, Me_2CO, and Et_2O, and dried. The crude product was subjected to further purification as described below.

Purification of MTX Analogues. Purification of the products from the condensation of 2,4-diamino-6-(chloromethyl)pteridine with 4-(*N*-methylamino)benzoylamino acids was achieved as follows: (1) compounds **1c,f** were separated as Mg salts; (2) compounds **1a,b,d,e,g–i** were chromatographed on a cellulose column (50:1) using as eluant a 0.1 M Na_2PO_4 solution adjusted to pH 7.0 with HCl. The collected fraction was checked by paper chromatography [Whatman No. 1, descending, Na_2HPO_4 buffer (pH 7.0)] and acidified to pH 3.5–4 in order to precipitate the product. High-purity compounds were generally obtained with an overall yield of 5–10%.

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Potential Anticancer Agents. 17. Analogues of Methotrexate with a Tripeptide Side Chain

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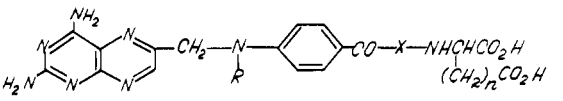
Nine tripeptide analogues of methotrexate were synthesized from 2,4-diamino-6-(chloromethyl)pteridine. Only *N*-[*N*-[4-[(2,4-diamino-6-pteridinyl)methyl]amino]benzoyl]glycyl-DL-aspartic acid (**1a**) showed moderate activity against L1210 murine leukemia (ILS = 69%) and W 256 carcinosarcoma (TGI = 55%).

We recently reported the synthesis of new methotrexate analogues, in which the terminal glutamic acid moiety was replaced by other amino acids.[2a] There is considerable evidence in the literature which points to the importance of the terminal glutamyl residue for biological activity.[2b-6] It is known, also, that the triglutamyl derivative of folic acid exhibits some antitumor activity, whereas folic acid itself is completely ineffective.[7] For these reasons, it was of interest to prepare peptide analogues of methotrexate (MTX), in which the terminal glutamyl moiety remains intact. Since most of the tested homo- and heteropolymeric analogues of MTX have been shown to be ineffective against L1210 murine leukemia, as well as against microorganisms[8-10] (except MTX–immunoglobulin and MTX–albumin covalent complexes[11-13]), we chose to limit ourselves to the synthesis of tripeptide analogues of general structure **1** (see Table I), in which X is a supplementary amino acid.

Synthesis. Condensation of 2,4-diamino-6-(chloromethyl)pteridine (**2**)[14,15] with tripeptides **3**, in water at pH 7.5, afforded methotrexate analogues **1a–i** (Scheme I). Purification was accomplished readily by column chromatography on cellulose or Sephadex G-10. The physical constants for these new derivatives are given in Table II. The synthesis of tripeptides **3a–i** was achieved as shown in Scheme II. The 4-(*N*-carbobenzoxy-*N*-methylamino)benzoylamino acids **4** were prepared as previously described,[2a] purified carefully, and condensed with diethyl glutamate or diethyl aspartate[2a,16,17] in the presence of *N,N'*-dicyclohexylcarbodiimide (DCC) in order to obtain the protected tripeptides **5a–i**. Saponification of the ester groups yielded the free acids **6a–i** which, on catalytic hydrogenolysis, gave the tripeptides **3a–i**. The physical constants for the new intermediates **3**, **5**, and **6** are listed in Table III.

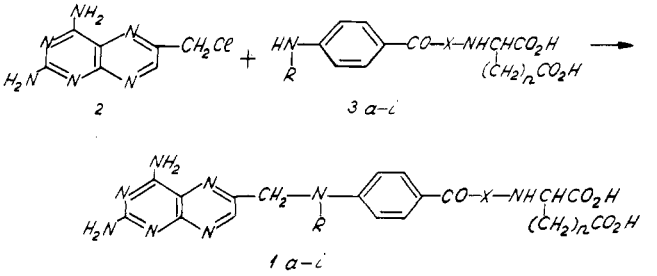
Biological Data. The antitumor effectiveness of the new methotrexate analogues **1a–i** was evaluated against L1210 mouse leukemia and W 256 rat carcinosarcoma. The data given in Table IV indicate that the insertion of an extra amino acid between the aminobenzoyl and glutamic acid moieties may lead to compounds with borderline activity against the L1210 tumor. One compound, the glycylaspartate analogue **1a**, exhibited moderate activity against both tumors.

Table I. New Tripeptide Analogues of Methotrexate



compd	R	X	*n*
1a	H	Gly	1
1b	H	Gly	2
1c	CH_3	Gly	1
1d	CH_3	Gly	2
1e	CH_3	DL-Ala	2
1f	CH_3	Sar	2
1g	CH_3	L-Leu	2
1h	CH_3	L-Phe	1
1i	CH_3	L-Phe	2

Scheme I



Experimental Section

Melting points were taken on a Boetius apparatus and are uncorrected. Ultraviolet spectra were determined with CF_4 Optica Milano and Spekord UV–vis Carl Zeiss Jena spectrophotometers, and infrared spectra were run on an UR-10 Carl Zeiss Jena spectrophotometer.

Ethyl 4-(*N*-Carbobenzoxyamino)- and 4-(*N*-Carbobenzoxy-*N*-methylamino)benzoyl Dipeptides 5a–i. Diethyl glu-

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