termined from dose-response curves comprising four to five dose levels (number of determinations = 4) using the statistical program RS1 from BBN Research Systems.[22] Maximal decrease in DOPA was as follows: limbic 65%, striatum 80%, and cortex 50%. Maximal decrease in 5-HTP was as follows: limbic, striatum, and cortex 50%. Some of the compounds displayed partial agonist effects.

Motor Activity. The rats were injected with reserpine (5 mg/kg sc) 18 h before and test drug immediately before the activity session. Shown in Table I are the accumulated activity counts/30 min (mean ± SEM, n = 2-4). Reserpine controls were as follows: 3 ± 1 counts/30 min; n = 13. After the motility experiments the rats were injected with NSD 1015 (see above).

Inhibition of [^{3}H]Spiperone Binding. Inhibition of [^{3}H]-spiperone binding to D2 receptors in rat striatal membranes was determined as described by Hyttel.[23]

Inhibition of [^{3}H]N-0437 Binding. Male Wistar (Mol:Wist) rats (125-250 g) were sacrificed and their corpora striata were dissected and weighed. The tissue was homogenized (Ultra Turrax, 10-15 s) in 10 mL of ice-cold 50 nM Tris buffer, pH 7.5 (at 25 °C), containing 1 mM Na_2 EDTA, 5 mM KCl, and 2 mM $CaCl_2$. The homogenate was incubated for 15 min at 37 °C and then centrifuged twice at 20000g for 10 min at 4 °C, with rehomogenization of the pellet in 10 mL of ice-cold buffer. The final pellet was homogenized in 1600 vol (w/v) of ice-cold buffer. Incubation tubes kept on ice in triplicate received 100 μL of drug solution in water (or water for total binding) and 2000 μL of tissue suspension (final tissue content corresponds to 1.25 mg of original tissue). The binding experiment was initiated by addition of 100 μL of [^{3}H]N-0437 (from Amersham International plc., England, specific activity approximately 80 Ci/mmol, final concentration 0.2 nM) and by placing the tubes in a 25 °C water bath. After incubation for 90 min, the samples were filtered under vacuum (100-200 mbar) through Whatman GF/F filters (25 mm). The tubes were rinsed with 5 mL of ice-cold buffer which was then poured on the filters. Thereafter, the filters were washed with 5 mL of buffer. The filters were placed in counting vials, and 4 mL of appropriate scintillation fluid (e.g. Picofluor 15) was added. After shaking for 1 h and storage for 2 h in the dark, the content of radioactivity was determined by liquid-scintillation counting. Specific binding was obtained by substracting the nonspecific binding in the presence of 1 μM of 6,7-ADTN. For determination of the inhibition of binding, five concentrations of drugs covering 3 decades were used. The measured cpm's were plotted against drug concentration on semilogarithmic paper, and the best fitting s-shaped curve was drawn. The IC_{50} value was determined as the concentration at which the binding is 50% of total binding in control samples minus the nonspecific binding in the presence of 1 μM 6,7-ADTN.

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Synthesis of *N*-[*N*-(4-Deoxy-4-amino-10-methylpteroyl)-4-fluoroglutamyl]-γ-glutamate, an Unusual Substrate for Folylpoly-γ-glutamate Synthetase and γ-Glutamyl Hydrolase

Nicholas J. Licato,[†] James K. Coward,*[,†] Zenia Nimec,[‡] John Galivan,[‡] Wanda E. Bolanowska,[§] and John J. McGuire[§]

Departments of Medicinal Chemistry and Chemistry, The University of Michigan, Ann Arbor, Michigan 48109, Wadsworth Center for Laboratories and Research, New York State Department of Health, Albany, New York 12201, and Department of Experimental Therapeutics, The Grace Cancer Drug Center, Roswell Park Memorial Institute, Buffalo, New York 14263.
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N-[*N*-(4-Deoxy-4-amino-10-methylpteroyl)-4-fluoroglutamyl]-γ-glutamate has been synthesized and its ability to serve as a substrate for folylpolyglutamate synthetase and γ-glutamyl hydrolase has been investigated. It was anticipated that this compound would be a substrate for both of these enzymes. Although the title compound proved to be a good substrate for folylpolyglutamate synthetase, hydrolysis catalyzed by γ-glutamyl hydrolase was unexpectedly slow. These results suggest the use of fluoroglutamate-containing peptides as hydrolase-resistant folates or antifols in a variety of chemotherapeutic regimens.

Poly-γ-glutamate derivatives of folates have been known for many years.[1] Recently, the role of the naturally occurring folylpoly-γ-glutamates in folate-dependent one-carbon biochemistry[2] and the role of analogous derivatives of antifolates such as methotrexate (MTX) in the cytotoxic action of these drugs[3] have been the subject of extensive investigation. The enzyme which catalyzes the formation of poly-γ-glutamate derivatives of both the naturally occurring folates and the antifolates is folylpoly-γ-glutamate synthetase (FPGS, EC 6.3.2.17), an ATP-dependent enzyme which occurs in all species examined.[4] This enzyme specifically activates the γ-carboxyl group of the C-terminal glutamate in the growing polypeptide via formation of a γ-glutamyl phosphate intermediate.[5] Prior to the determination of the chemical structure and unambiguous chemical synthesis of the folylpoly-γ-glutamates,[6] they were referred to collectively as the folate "conjugates".

[†] The University of Michigan.
[‡] New York State Department of Health.
[§] Roswell Park Memorial Institute.

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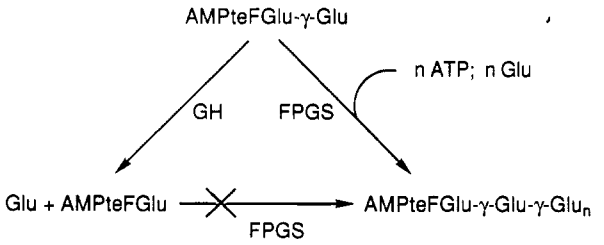
Certain microorganisms, e.g., *Lactobacillus casei*, are unable to grow on the conjugates but will grow if the conjugates are subjected to prior enzyme-catalyzed hydrolysis. The enzyme which catalyzes the hydrolysis reaction, formerly known simply as "conjugase", is now known to be a Zn^{2+}-dependent peptidase, γ-glutamyl hydrolase (GH, EC 3.4.22.12).[4] It is clear that the actions of these two enzymes could be responsible for the metabolic interconversion of the various polyglutamate forms.[7]

It would be of interest to be able to study the flux of folates and antifolates in and out of the polyglutamate pool. This has been done in the case of MTX metabolism by using pulse-chase techniques[7] and, in principle, also could be done with folates by using these techniques although the multiplicity of structures of the folate coenzymes and limitations of the analytical methods currently available make this much more difficult. In addition, the pulse-chase studies do not allow for an assessment of the relative importance of FPGS and GH in cellular folate homeostasis. As an approach to this problem, we have drawn on our previous work on the synthesis and biological activity of γ-fluoromethotrexate (FMTX), a MTX analogue in which the glutamate moiety of MTX has been replaced by 4-fluoroglutamate (FGlu).[8] Since the α-carboxyl groups of the poly-γ-glutamates are ionized at physiological pH and the folate transport proteins do not utilize the polyglutamate derivatives, efflux of the polyanions does not occur readily. This leads to an accumulation of the folate polyglutamates[9] or antifolate polyglutamates[10] in the cell. In FMTX, the simple change of fluorine for hydrogen results in a drug with very different biological properties compared to MTX due primarily to the markedly diminished ability of FMTX to form the polyglutamate derivatives. One consequence of the decreased glutamylation is that FMTX is rapidly lost from cells in the absence of extracellular drug.[8,11] On the basis of the literature data[4] and chemical reactivity considerations, we hypothesized that *N*-[*N*-(4-deoxy-4-amino-10-methylpteroyl)-4-fluoroglutamyl]-γ-glutamate (AMPteFGlu-γ-Glu; FMTX-γ-Glu; 1) would be a substrate for both of these enzymes. If this proved to be the case, the scheme shown in eq 1 would permit investigation of



the polyglutamate flux in cultured cells since hydrolysis of 1 to FMTX (AMPteFGlu) would short-circuit the cycle and prevent reformation of polyglutamate derivatives. All polyglutamates formed from 1 would have to be formed via the FPGS-mediated pathway. In order to test this hypothesis, we have synthesized 1 and have studied its ability to act as a substrate for FPGS and GH.

Scheme I

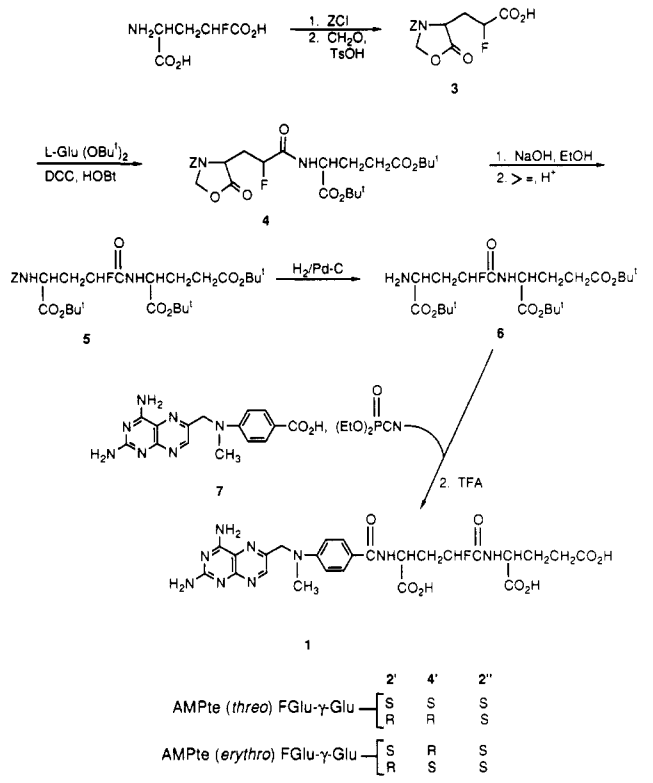


Table I. Kinetic Constants for FPGS-Catalyzed Reaction[a]

substrate	K_m, μM	rel V_{max}	rel V_{max}/K_m
MTX (AMPteGlu)	42 ± 14	1	0.024
AMPteGlu-γ-Glu	38 ± 22	0.45 ± 0.13	0.012
AMPteFGlu-γ-Glu (1)	100 ± 33	0.23 ± 0.05	0.0023

[a] Kinetic constants for all substrates were determined in the same experiment using four points in duplicate over a 20-fold concentration range. Reactions were allowed to incubate at 37 °C for 3 h during which time linearity was maintained. The experiment was repeated three times and values presented are average ± SD. Two different enzyme preparations were used and the absolute V_{max} values for each, using MTX as the substrate, were 1980 pmol/3 h and 3700 pmol/3 h; relative V_{max} values (rel V_{max}) were determined with the appropriate control.

Results and Discussion

The synthesis of the title compound 1 is shown in Scheme I. The pK_a differences (α vs β/γ) observed in aspartic and glutamic acids have been exploited in the synthesis of aspartate β-esters and glutamate γ-esters. However, the presence of the 4-fluoro substituent in FGlu and the resultant decrease in the pK_a of the γ-COOH rendered the selective blocking of either the α- or γ-carboxyl group more difficult. Therefore, we used the substituted 5-oxo-4-oxazolidine 3 as the key intermediate in the synthesis of 1. This was prepared from FGlu and paraformaldehyde in the presence of catalytic TsOH, analogous to the method described by Itoh[12] for the synthesis of the corresponding oxazolidinones derived from aspartic and glutamic acids. Coupling of L-glutamic acid di-*tert*-butyl ester and 3 was accomplished with standard peptide coupling conditions (DCC, HOBt) to give dipeptide derivative 4. Hydrolytic ring opening of the oxazolidinone followed by reaction of the newly formed α-COOH with isobutylene led to fully blocked dipeptide 5.

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Table II. Product Analysis: FPGS-Catalyzed Glutamylation of AMPteGlu-γ-Glu vs AMPteFGlu-γ-Glu[a]

substrate	t, h	Glu_2[b]	Glu_3[b]	Glu_4[b]
AMPteGlu-γ-Glu	3	29, 22	424, 447	
	24	80, 83	2960, 3059	44, 50
AMPteFGlu-γ-Glu	3	22, 28	156, 178	
(1)	24	56, 79, 80	1186, 1220, 1241	19, 19, 21

[a] Product formation was monitored by incorporation of [^{3}H]-L-Glu into polyglutamate products and analyzed by HPLC as described in the Experimental Section. [b] Data presented in terms of pmol of [^{3}H]Glu added to the substrate (12.5 nmol) during duplicate or triplicate FPGS-catalyzed reactions.

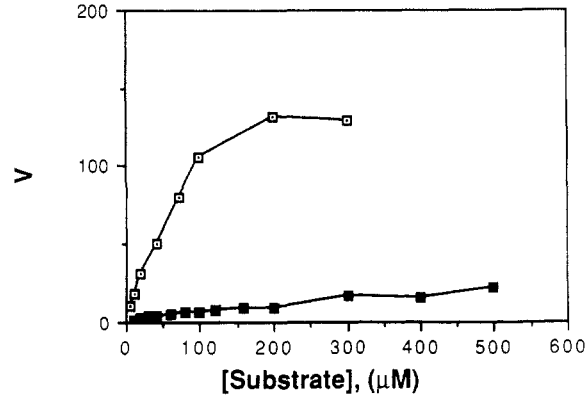

Figure 1. Hydrolysis of γ-glutamyl peptides of MTX (□, 2 h, 20 μg of protein) and FMTX (■, 3 h, 165 μg of protein) catalyzed by γ-glutamyl hydrolase isolated from porcine kidney. Velocity is in units of nmol h^{-1} (mg of protein)$^{-1}$.

Hydrogenolytic removal of the Cbz group gave **6**, which could be coupled to 4-amino-10-methylpteroic acid **7** previously activated in situ with diethyl phosphorocyanidate.[13] Removal of the *tert*-butyl esters with TFA gave the desired final product (**1**) after purification by chromatography on DEAE-cellulose. The FGlu used in this synthesis was racemic at both chiral centers (C2 and C4), whereas the L-glutamate used was stereochemically pure. Thus, the resulting final product (**1**) is a mixture of four diastereomers (Scheme I), which can be observed in the NMR spectra (^{1}H, ^{13}C, and ^{19}F) and in the HPLC data (vide infra).

The ability of **1** to act as a substrate for the FPGS-catalyzed addition of L-glutamate was investigated and, as can be seen in the data of Table I, the fluoro-substituted dipeptide derivative is a competent substrate but has kinetic parameters somewhat different from the protio dipeptide derived from MTX. The fact that the fluoro derivative has V_{max}/K_m = ca. 20% that of the protio dipeptide may result from the possibility that only one of the four diastereomers is a substrate for FPGS. Evidence for or against that hypothesis must await the stereospecific synthesis of the four FGlu isomers. Product analysis of the FPGS-catalyzed glutamylation of **1** was carried out, and the results are shown in Table II. Comparison of the protio dipeptide with fluoro dipeptide **1** shows that **1** is converted to the tripeptide to the extent of ca. 35–40% the amount observed with the protio dipeptide. In addition, a small but reproducible amount of a tetrapeptide product was formed from **1**.

GH from hog kidney was used to study the ability of **1** to undergo hydrolysis of the peptide bond adjacent to the fluoro substituent. As shown in Figure 1, the rate of GH-catalyzed hydrolysis of **1** is very low when compared to that of the protio dipeptide over a wide concentration range. In an independent experiment, all of the protio dipeptide was hydrolyzed during a 90-min incubation (165 μg of protein), whereas only 24% of the fluoro dipeptide was hydrolyzed using more enzyme (330 μg of protein) in 4 h. HPLC analysis of the reaction products (Figure 2) confirmed the slow rate of hydrolysis of **1** in that only after a 48-h incubation was nearly all of the substrate consumed to give a ca. 1:1 mixture of FMTX diastereomers (D,L-erythro- and D,L-threo). The products of the enzyme-catalyzed hydrolysis of **1** were shown to coelute with chemically synthesized FMTX[8] when coinjected for HPLC. The fact that **1** is such a poor substrate for GH is somewhat surprising. However, this low rate of enzyme-catalyzed hydrolysis of **1** is not due to its poor binding to the enzyme, as shown by the marked inhibition of hydrolysis of protio dipeptide AMPteGlu-γ-Glu by **1** (Figure 3). In the presence of 50 μM protio dipeptide substrate, a value of I_{50} = 10 μM was obtained.

The successful synthesis of **1** provides a useful drug for studying the intracellular flux of folate and antifolate polyglutamates in cells. We have shown that this fluoro dipeptide is a substrate for both the biosynthetic enzyme folylpolyglutamate synthetase and also the degradative enzyme γ-glutamyl hydrolase. The literature synthesis of FGlu[14] leads to a mixture of all four stereoisomers. As a

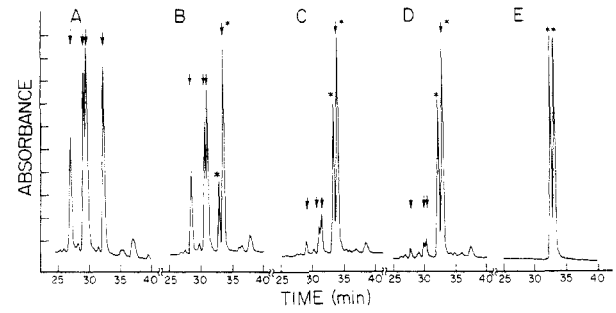

Figure 2. HPLC analysis of aliquots withdrawn from the reaction mixture during the enzyme-catalyzed hydrolysis (300 μg of protein) of (100 μM): (A) authentic **1**; (B–D) the reaction mixture after 4 h (B), 24 h (C), and 48 h (D); (E) authentic FMTX. Although the absolute retention times (t_R) differed slightly between runs, the relative t_R of the diastereomers of dipeptide **1** (arrows) and product FMTX (asterisks) remained constant. HPLC conditions are as described in the Experimental Section.

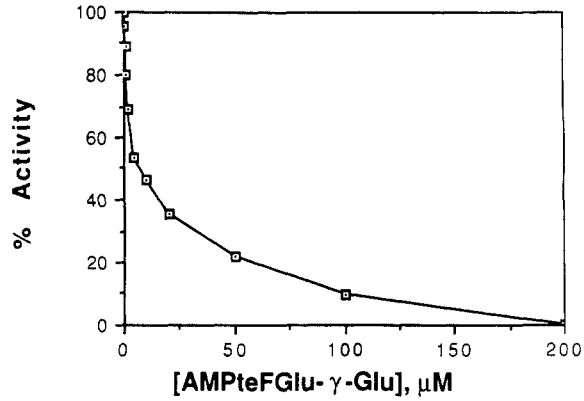

Figure 3. Inhibition of the hydrolysis (20 μg of protein) of the γ-glutamyl peptide of MTX (AMPteGlu-γ-Glu, 50 μM) by the γ-glutamyl peptide of FMTX (AMPteFGlu-γ-Glu, **1**); incubation time = 2 h.

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result, the coupling of FGlu to L-glutamate results in a final product (1) consisting of four diastereomers (Scheme I). This stereochemical heterogeneity makes a quantitative analysis of the enzyme data difficult since it is possible, even likely, that not all the isomers bind with equal affinity to the enzymes of interest in this work, namely FPGS and GH. Thus, the kinetic data obtained for the FPGS-mediated addition of L-glutamate to 1 are consistent with only one of the four diastereomers being a substrate. Resolution of this question must await the stereospecific synthesis of the FGlu isomers which is currently being investigated in our laboratory. Most recent studies on the mechanism of action of Zn^{2+}-dependent peptidases, e.g., carboxypeptidase A[15] suggest that the role of zinc is to assist in the nucleophilic attack by H_2O on the scissile amide bond via a zinc-bound hydroxide. Thus, the presence of an α-fluoro substituent would be expected to render the peptide bond more susceptible to basic hydrolysis.[16] However, the hydrolysis of 1 mediated by γ-glutamyl hydrolase (Figures 1 and 2) is unexpectedly slow. More work will be required to explain this unexpected finding. However, these results suggest a strategy for the inhibition of γ-glutamyl hydrolase mediated metabolism of folate and anti-folate polyglutamates. Specifically, it now seems feasible to design folate analogues containing fluoropeptides which would accumulate in cells and be less susceptible to metabolic degradation via hydrolysis than the corresponding protio derivative. Previous research has suggested that dipeptide derivatives of MTX are transported in L1210 leukemia cells.[17] In addition, recent studies from our laboratories have shown that 1 is taken up by H35 hepatoma cells in vitro (Galivan, J.; McGuire, J. J.; Coward, J. K., unpublished results).

Experimental Section

D,L-erythro,threo-4-fluoroglutamic acid (FGlu)[14] was prepared as previously described from 2-acetamidoacrylic acid[18] and diethyl fluoromalonate (Sigma). The mixture of two diastereomeric pairs (2*S*,4*R*:2*R*,4*S*,erythro; 2*S*,4*S*:2*R*,4*R*,threo) was used throughout the synthesis of the title compound. 4-Deoxy-4-amino-10-methylpteroic acid (7) was prepared by enzyme-catalyzed hydrolysis of methotrexate.[5,11] Nuclear magnetic resonance (NMR spectra were recorded on a Bruker WP-270SP, a Bruker AM-300, or a Bruker WM-360 instrument. Chemical shifts are reported in δ values (ppm) and are referenced to tetramethylsilane (TMS) for ^1H and ^{13}C NMR or to trifluoroacetic acid (TFA) for ^{19}F NMR. High-resolution mass spectra were determined on a VG 70-250-S mass spectrometer. Microanalyses were performed by Atlantic Microlab, Atlanta, GA. All TLC's were run on silica gel unless stated otherwise. Porcine kidney acetone powder was purchased from Sigma.

***N*-Cbz-D,L-*erythro,threo*-4-fluoroglutamic Acid (2).** To a stirred solution of $NaHCO_3$ (336 mg, 4 mmol) in 20 mL of H_2O at 4 °C (ice bath) was added 300 mg (1.82 mmol) of FGlu. CbzCl (414 μL, 2.91 mmol) was then added in 3 portions at 3-min intervals. After 5 min, a second addition of 336 mg (4 mmol) $NaHCO_3$ was made in small portions and the ice bath was removed. After rapid stirring for 5 h, the absence of FGlu was established by TLC analysis, and the solution was extracted with 2 × 20 mL of $CHCl_3$ to remove excess CbzCl and benzyl alcohol. The aqueous solution was adjusted to pH 1 with 3 N HCl at 4 °C and immediately extracted with 3 × 30 mL of EtOAc. Between each extraction the pH of the aqueous solution was checked and readjusted to pH 1 with 3 N HCl as needed. The EtOAc extracts were combined and dried over $MgSO_4$. A water/brine wash of the organic extract was avoided due to the slight solubility of *N*-CbzFGlu in such washes. The dried EtOAc solution was filtered and the solvent was evaporated in vacuo. After placing the gummy, solid residue under pentane overnight, a semicrystalline solid was obtained, which was dried under vacuum to yield 470 mg (86%) of the desired product: TLC, CH_3CN/H_2O (4:1) R_f = 0.35; mp 116–117 °C; mass spectrum (FAB), m/z 300 (MH^+); 256 (– CO_2); ^1H NMR ($CDCl_3$) δ 7.78 and 7.72 (1 H, 2 d, NH), 7.35 (5 H, s, aryl CH), 5.12 and 4.94 (1 H, 2 octets partially obscured by the benzylic CH_2, $^2J_{H,F}$ = 48.8 Hz, $^3J_{H,H}$ = 8.1 Hz, $^3J_{H,H}$ = 4.2 Hz, CHF), 5.03 and 5.04 (2 H, benzylic CH_2), 4.3–4.1 (1 H, m, α-CH), 2.4–2.0 (2H, m, CH_2); ^{19}F NMR (DMSO-d_6): δ -113.7, -115.5 (2 septets). Anal. ($C_{13}H_{14}FNO_6$) C, H, N.

4-(*N*-Cbz-D,L-*erythro,threo*-5-oxo-4-oxazolidinyl)-2-fluoropropionic Acid (3). *N*-CbzFGlu (2; 400 mg, 1.35 mmol) and paraformaldehyde (130 mg, 4.64 equiv of CH_2O) were suspended in 25 mL of dry benzene together with 15 mg (catalytic) of TsOH and the mixture was heated at reflux temperature for 7 h, the last 6.5 h with a Dean–Stark trap in place. At the end of this time, the reaction solution was cooled and then washed with H_2O (2 × 20 mL) and 5% aqueous $NaHCO_3$ (3 × 10 mL). The pooled aqueous $NaHCO_3$ washes were then cooled (ice bath), adjusted to pH 1.5 with 6 N HCl, and extracted with EtOAc (3 × 15 mL). The combined EtOAc solution was dried over $MgSO_4$ and concentrated in vacuo to give a clear, gummy residue: 273 mg (65%); TLC, CH_3CN/H_2O (4:1), R_f = 0.69; mass spectrum (EI), m/z 311 (M^+); exact mass calcd for $C_{14}H_{14}FNO_6$ M^+ 311.0795, obsd 311.0805; ^1H NMR ($CDCl_3$) δ 8.63 (1 H, s, γ-CO_2H), 7.33 (5 H, s, aryl CH), 5.50 (m, partial CHF), 5.47 and 5.18 (2 H, 2 d, J = 4.2 Hz, oxazolidine CH_2), 5.23 and 5.13 (2 H, 2 s, benzylic CH_2), 5.2–5.0 (m, partial CHF, other signals due to CHF are obscured by the benzylic CH_2 and oxazolidine CH_2), 4.46 (b s, α-CH), 2.9–2.5 (m, β-CH_2); ^{13}C NMR ($CDCl_3$) δ 172.65 and 172.35 (2 d, $^2J_{CF}$ = 35.73, 24.13 Hz, γ-C=O), 171.49 and 171.42 (α-C=O), 153.19 and 153.09 (Cbz C=O), 134.98 and 134.87 (aryl quaternary), 128.74 and 128.34 (aryl CH), 87.35 and 86.95 (2 d, $^1J_{CF}$ = 186.71, 184.85 Hz, CHF), 78.01 (oxazolidine CH_2), 68.60 (benzylic CH_2), 51.27 (α-CH), 32.68 and 32.42 (β-CH_2); ^{19}F NMR ($CDCl_3$) δ -120.8, -121.8 (2 m). Anal. ($C_{14}H_{14}FNO_6$) C, H, N.

***N*-[4-(*N*-Cbz-D,L-*erythro,threo*-5-oxo-4-oxazolidinyl)-2-fluoropropionyl]-L-glutamic Acid, Di-*tert*-butyl Ester (4).** Fluoropropionic acid 3 (273 mg, 0.88 mmol) was dissolved with stirring in 20 mL of CH_2Cl_2 in a flask fitted with a drying tube and cooled in an ice bath. DDC (254 mg, 1.23 mmol) and HOBt (237 mg, 1.75 mmol) were then added, followed by di-*tert*-butyl glutamate (249 mg, 0.96 mmol). After 5 min the flask was removed from the ice bath, and the reaction solution was stirred for 3 days at ambient temperature. Filtration of the cold CH_2Cl_2 solution removed DCU (142 mg) and concentration of the filtrate in vacuo gave 859 mg of an oily residue. Filtering column chromatography[19] (1:1 EtOAc/hexanes) gave the pure product as a viscous oil after evaporation of the solvent in vacuo: yield, 243 mg (50%); TLC, CH_2Cl_2/EtOAc (4:1) with 1% Et_3N, R_f = 0.7 vs 3 R_f = 0.38; HPLC, Waters Z-module C18 μ-bondapak, CH_3OH/H_2O (7:3), flow rate = 1.5 mL min^{-1}, t_R = 23.3 min, the peak is a broad doublet due to the diastereomeric product mixture; mass spectrum (CI), m/z 553 (MH^+); exact mass calcd for $C_{27}H_{39}FN_2O_9$ MH^+ 553.2561, obsd 553.2564; ^{19}F NMR ($CDCl_3$) δ ca. -119.5 (m).

The ^1H NMR ($CDCl_3$) spectrum was similar to the spectrum reported for 3 except that the multiplets at δ ca. 4.5 (α-CH) and δ 2.8–1.8 (CH_2) were more complex, reflecting the presence of the glutamate moiety, and no peak was observed for the free COOH.

In CD_3OD, one can readily distinguish the FGlu α-CH (δ ca. 4.5) from the Glu α-CH (δ ca. 4.35), as well as the FGlu β-CH_2 (δ ca. 2.8–2.4) from the Glu β-CH_2 (δ ca. 2.2–2.0 and 2.0–1.8), although the spectra are extremely complex.

The ^{13}C NMR ($CDCl_3$) showed a peak at ca. 87.25 (2 d, $^1J_{CF}$ = 184.5 Hz, CHF). Otherwise, the spectrum is similar to that reported for 3 except for the presence of a multiplet at δ ca. 168 due to two C=O of the Glu *tert*-butyl esters. In addition, the presence of another chiral center in L-Glu leads to the increased complexity of the entire spectrum.

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***N*-(*N*-Cbz-D,L-*erythro,threo*-4-fluoroglutamyl)-γ-glutamic Acid, Tri-*tert*-butyl Ester (5). Saponification.** Oxazolidinone fluoro dipeptide 4 (230 mg, 0.42 mmol) was dissolved in 4 mL of EtOH, and 1 mL of 1 N NaOH was then added dropwise. The solution immediately went from colorless to light yellow upon addition of base. After 1 h, TLC analysis (acetone/H_2O (4:1)) showed that the reaction was complete. The pH of the solution was then adjusted to 5 with 6 N HCl at 4 °C, after which EtOH was removed in vacuo and the resulting slurry was partitioned between H_2O and EtOAc (2 × 15 mL). The combined EtOAc extracts were washed in the cold with 0.1 N HCl (2 × 30 mL) and saturated NaCl (1 × 30 mL), then dried over $MgSO_4$, and finally evaporated in vacuo to give 183 mg (81%) of a gummy solid. NMR (^1H, ^{13}C) and chromatographic analysis indicated that the acid product was sufficiently pure for conversion to the *tert*-butyl ester.

Esterification. A portion of the saponification product (123 mg, 0.228 mmol) was dissolved in 5 mL of dry $CHCl_3$ and placed in a pressure bottle together with a magnetic stirring bar. The bottle was chilled to −30 °C in a dry ice bath, and 26 μL of concentrated H_2SO_4 was added. Immediately, approximately 3 mL of isobutylene gas was condensed within the bottle which was then quickly closed, the dry ice bath was removed, and the reaction was allowed to stir for 2 days at ambient temperature. The vessel was then chilled to −30 °C prior to being opened and 0.5 mL of aqueous 20% K_2CO_3 was added to neutralize the acid. The contents of the vessel were transferred to a round-bottom flask, after which $CHCl_3$ was evaporated in vacuo. The residue was partitioned between H_2O and EtOAc (3 × 10 mL). The EtOAc solution was then extracted with 25 mL of 5% aqueous $NaHCO_3$ and 25 mL of saturated NaCl. The EtOAc solution was dried over $MgSO_4$ and evaporated in vacuo to give 124 mg (89%) of an oil: TLC, $CHCl_3$/MeOH (9:1) R_f = 0.73; EtOAc/hexanes (2:1), R_f = 0.25; ^1H NMR (CD_3OD) δ 7.32 (5 H, m, aryl CH), 5.1 (3 H, m, benzyl CH_2, CHF), 4.3 (2 H, m, α-CH), 2.6–1.8 (6 H, m, CH_2), 1.46–1.44 (27 H, 3 s, $C(CH_3)_3$); ^{13}C NMR (CD_3OD) δ 173.6, 172.1, 171.8, 171.5 (ester, amide C=O), 158.5, 158.2 (Cbz C=O), 138.1 (aryl quaternary), 129.4, 129.0, 128.8 (aryl CH), 91.0, 90.5, 88.6, 88.1 (4 d, $^1J_{CF}$ = 186 Hz, CHF), 83.2, 83.1, 81.9 ($C(CH_3)_3$), 67.7 (Cbz CH_2), 53.5 (Glu α-CH), 52.7, 52.4 (FGlu α-CH), 35.9, 35.8, 35.6, 35.5 (FGlu β-CH_2), 32.5 (Glu γ-CH_2), 28.4, 28.25, 28.2 ($C(CH_3)_3$), 27.4 (Glu β-CH_2).

***N*-(D,L-*erythro,threo*-4-Fluoroglutamyl)-γ-glutamic Acid, Tri-*tert*-butyl Ester (6).** The *N*-blocked fluoro dipeptide triester 5 (123 mg, 0.20 mmol) was dissolved in 10 mL of MeOH and a solution of 50 mg of Pd/C (10%) in 2 mL of EtOH was added. This solution was subjected to hydrogenation under 42 psi of H_2 with shaking on a Parr apparatus for 9 h. After removal of the catalyst by filtration, the solvent was removed by rotary evaporation to yield 83 mg (86%) of the amine product as an oil; TLC, EtOAc, R_f = 0.29, 0.33 (diastereomers). This product was sufficiently pure for use in the coupling step described below.

***N*-[*N*-(4-Amino-4-deoxy-10-methylpteroyl)-D,L-*erythro,threo*-4-fluoroglutamyl]-γ-glutamic Acid (1).** In a 15-mL round-bottom flask fitted with a drying tube was placed 2.5 mL of DMF, 23 μL of Et_3N (0.16 mmol), and 25 μL (0.16 mmol) of diethyl phosphorocyanidate. Diaminopteroate 7[5,11] (52 mg, 0.16 mmol) was then added and the reaction solution was stirred at ambient temperature. After 3 h, the solution remained slightly cloudy, an indication that unreacted pteroate remained. An additional 5 μL (0.04 mmol) of diethyl phosphorocyanidate was added and within 15 min the solution became clear. TLC analysis ($CHCl_3/CH_3OH$ (4:1)) of the solution indicated only a small amount of unreacted pteroate and after an additional 2 h, TLC analysis indicated that the activation reaction was complete. Peptide 6 (76 mg, 0.16 mmol) was dissolved in 1 mL of DMF and was added to the reaction flask and stirring continued for 72 h at ambient temperature. Solvent DMF was removed in vacuo, the residue was dissolved in 35 mL of $CHCl_3$ and washed with 1% NH_4OH (2 × 20 mL) to remove unreacted 7. The organic layer was washed with 20 mL of H_2O, then dried over Na_2SO_4 and evaporated in vacuo to provide 155 mg of crude blocked 1; TLC, $CHCl_3/CH_3OH$ (4:1), R_f = 0.6, 0.7 (diastereomers). This coupled material was dissolved in neat TFA (3 mL) and progress of the deesterification reaction was monitored by TLC ($CHCl_3/CH_3OH$ (4:1), product R_f = 0 vs triester 6 R_f = 0.6, 0.7). After 24 h, the solvent TFA was removed by rotary evaporation and the residue was dried in vacuo. A total of 166 mg of crude material was recovered.

The crude product was dissolved in 20 mL of H_2O, the pH was adjusted to 8 with dilute NH_4OH, and the sample volume brought to 160 mL in order to obtain sufficiently low conductance prior to loading on a DEAE-cellulose (Whatman DE-52) column (30 × 1 cm). The column was washed with 100 mL of water and the desired pteroyl dipeptide 1 was eluted from the column with a linear gradient formed from 175 mL of 15 mM NH_4HCO_3 and 175 mL of 500 mM NH_4HCO_3. Fractions were collected and analyzed by TLC and UV spectroscopy: yield of pure 1, 34 mg (35% overall from 6); TLC on cellulose, 5% NH_4HCO_3, R_f = 0.83; UV, λ_{max} (pH 1) = 244, 307 nm; λ_{max} (pH 7) = 257, 304, 365 nm; λ_{max} (pH 13) = 257, 302, 370 nm; mass spectrum (FAB, thioglycerol) positive ion, m/z 602 ($M + H^+$); negative ion, m/z 600 ($M - H^+$); HPLC, see Figure 2A; ^1H NMR (D_2O/DCl, pD = 1) δ 8.50, 8.47 (1 H, 2 s, C7-H), 7.61, 7.30 (4 H, 2 m, aryl CH), 5.2–4.9 (3 H, m, C9-CH_2, CHF), 4.59 (1 H, m, Glu α-CH), 4.3–3.7 (1 H, 4 q, FGlu α-CH), 3.27 (3 H, s, NCH_3), 2.6–1.5 (6 H, b m, FGlu/Glu CH_2); ^{19}F NMR (^1H-decoupled) (DMSO-d_6) δ -111.7, -112.0, -113.8, -114.0.

Enzymology. Rat liver FPGS was partially purified as previously described[20] and assayed by monitoring the incorporation of [^3H]glutamate into folylpolyglutamate products.[20,21]

GH was isolated from porcine kidney on the basis of properties reported in the literature.[4] Porcine kidney acetone powder (10 g) was dissolved in 200 mL of KH_2PO_4 (20 mM, pH 6.0) buffer with stirring at 0 °C for 1 h. The solution was brown with a fair amount of undissolved tissue. The solution was brought to 50% $(NH_4)_2SO_4$ saturation by adding 62.6 g (4 °C) over a period of 0.5 h and then stirring was continued for an additional 0.5 h. The mixture was transferred to six centrifuge tubes and centrifuged at 15000 rpm (27000*g*) in a refrigerated Sorvall (4 °C) centrifuge to give a pellet and a floating solid in the buffer. The supernatant was pipetted off and filtered from the floating solid residue to give 140 mL of a clear brown solution (2.49 mg/mL). The solution was brought to 80% $(NH_4)_2SO_4$ saturation (4 °C) by adding 42.8 g over a 20-min period followed by stirring for 0.5 h. Following centrifugation at 15000 rpm as described above, the supernatant was removed and the pellet was resuspended in 20 mL of KH_2PO_4 buffer. The resulting suspension was dialyzed against 2 L of KH_2PO_4 buffer for 24 h (4 °C), during which time some precipitate formed. The solution was transferred to two culture tubes, heated at 60 °C for 0.5 h, and then centrifuged (Sorvall GLC-1; 1600*g*) for 5 min. The clear, light brown supernatant (18.5 mL; [protein] = 3.3 mg/mL by Lowry protein assay[22]) was decanted off and frozen at −80 °C in aliquots.

A typical GH assay mixture consisted of the following components in a final volume of 0.3 mL: 30 μL of 0.5 M NaOAc, pH 4.5; 6 μL of 1 M β-mercaptoethanol, enzyme solution (6 μL with AMPte[^3H]Glu-γ-Glu as substrate, 50 μL or 100 μL with 1 as substrate), and varying amounts of substrate as indicated in Figures 1–3. Incubations were carried out at 37 °C. The amount of product formed in each experiment was quantitated by HPLC analysis;[23] a linear gradient of 0 to 11% acetonitrile was used. The enzyme-catalyzed reaction was linear with respect to time and protein concentration under the conditions specified with AMPte[^3H]Glu-γ-Glu as substrate.

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Registry No. (2′*S*,4′*S*,2″*S*)-1, 124716-15-0; (2′*R*,4′*R*,2″*S*)-1,

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124716-16-1; (2′S,4′R,2″S)-1, 124716-17-2; (2′R,4′S,2″S)-1, 124716-18-3; (DL-erythro)-2, 124688-42-2; DL-threo)-2, 124688-43-3; (DL-erythro)-3, 124688-44-4; (DL-threo)-3, 124688-45-5; (2′S,4′S,2″S)-4, 124688-46-6; (2′R,4′R,2″S)-4, 124815-52-7; (2′S,4′R,2″S)-4, 124815-53-8; (2′R,4′S,2″S)-4, 124815-54-9; (2′S,4′S,2″S)-5, 124688-47-7; (2′R,4′R,2″S)-5, 124688-48-8; (2′S,4′R,2″S)-5, 124688-49-9; (2′R,4′S,2″S)-5, 124688-50-2; ('S,4′S,2″S)-6, 124688-51-3; (2′R,4′R,2″S)-6, 124688-52-4; (2′S,4′R,2″S)-6, 124688-53-5; (2′R,4′S,2″S)-6, 124688-54-6; 7, 19741-14-1; FPGS, 63363-84-8; GH, 9074-87-7; (DL-erythro)-FGlu, 91383-48-1; (DL-threo)-FGlu, 91383-47-0; H-Glu(OBu-t)-OBu-t, 16874-06-9.

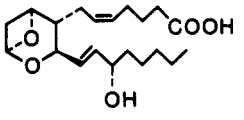
Synthesis and in Vitro Activity of Stereoisomers of a Novel Thromboxane Receptor Antagonist, (±)-(5*Z*)-7-[3-*endo*-[(Phenylsulfonyl)amino]bicyclo[2.2.1]hept-2-*exo*-yl]heptenoic Acid

Mitsuaki Ohtani* and Masayuki Narisada

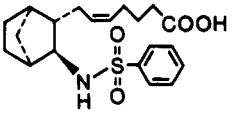
Shionogi Research Laboratories, Shionogi & Co., Ltd., Fukushima-ku, Osaka 553, Japan. *Received July 18, 1988*

Three stereoisomers of S-145 (1) with variations at the side-chain junctions were synthesized. Endo-cis isomer **10** and *N*-exo-trans isomer **18** were obtained via the common intermediate **5** having an endo-fused ring structure. Exo-cis isomer **28** was prepared via exo-fused azetidino compound **21**. Inhibitory concentrations (IC_{50}) of the sodium salts newly obtained for platelet aggregation were measured using washed rat platelets (WP) and human platelet-rich plasma (PRP). The IC_{50} values of these compounds for contraction of the rat aorta were also measured. Compound **1** of *N*-endo-trans structure and *N*-exo-trans isomer **18** exhibited more potent inhibitory activity than cis-isomers **10** and **28** against responses induced by TXA_2-related substances for rat WP and rat thoracic aorta. However, these compounds exhibited almost comparable inhibitory activity for human PRP.

Thromboxane A_2 (TXA_2) is a very potent inducer of human platelet aggregation and vasoconstriction of bronchial and vascular smooth muscles. Therefore, TXA_2 has been considered to be an important mediator in a variety of circulatory diseases.[1] In the previous paper,[2] we described the synthesis and in vitro activity of a novel TXA_2 receptor antagonist, S-145 [(±)-(5*Z*)-7-[3-*endo*-[(phenylsulfonyl)amino]bicyclo[2.2.1]hept-2-*exo*-yl]heptenoic acid, **1**], and emphasized the importance of the aromatic sulfonylamino group as a side chain for exhibiting the potent inhibitory activity against responses induced by TXA_2-related substances. The effect of stereochemical variation in the attached ring system on the biological activity of the TXA_2 receptor antagonist is not yet understood completely, and one stereoisomer frequently shows a different character from another in biological examinations.[3] We were very interested in finding the dependence of the biological activity upon the variation in the stereochemical structure and directed our research toward the synthesis and evaluation of the in vitro activity of the racemic three stereoisomers of compound **1**.



TXA_2



1 (S-145)

Chemistry

Isomers having different stereochemistry from that of compound **1** at the junctions of the side chains were synthesized as depicted in Scheme I. Endo-cis isomer **10** and *N*-exo-trans isomer **18** were derived from a key intermediate (**5**), which was prepared from dicyclopentadiene (**2**). Catalytic hydrogenation of dicyclopentadiene (**2**) with a catalytic amount of nickel boride occurred regioselectively at the norbornene skeleton and gave **3** in 84.5% yield.[4] Ozonolysis of **3** followed by Zn–acetic acid treatment and subsequent Jones' oxidation afforded dicarboxylic acid **4** in 41.7% yield. Treatment of **4** with acetic anhydride gave cyclic anhydride which, without purification, was treated with methanol under reflux, regioselectively giving acid ester **5** in 93.5% yield. Curtius rearrangement of **5** and subsequent alcoholysis with benzyl alcohol afforded benzyl carbamate **6** in 59.1% yield. Diisobutylaluminum hydride (DIBAL) reduction of **6** gave an unstable product (**7**),[5] which was treated with Wittig reagent prepared from (4-carboxybutyl)triphenylphosphonium bromide and dimsyl sodium in DMSO, giving a crude acid. Esterification of the acid with diazomethane gave methyl ester **8** in 34.3% yield from **6**. Removal of the carbobenzoxy group[2] in **8** followed by phenylsulfonylation afforded sulfonylamino ester **9** in 80.4% yield. Hydrolysis of **9** with KOH in aqueous methanol gave **10**, which was transformed into its sodium salt **11** for biological assay in vitro by neutralizing with sodium methoxide in methanol in 88.8% yield. The

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(5) Cyclization occurred and gave 2-hydroxyoctahydroindole derivative **7**, which was unstable and used for the next reaction without purification. See the Experimental Section.

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