Oxytocin Analogues with Combined High Smooth Muscle and Negligible Antidiuretic Activities. Investigation of Position 7 in Neurohypophyseal Hormones[1]

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Received December 22, 1975

The proline residue in position 7 of oxytocin occupies one of the four corner positions in the two β turns proposed for the preferred conformation of the pituitary hormone. It has been suggested that synthetic modifications of the residues in these corner positions will yield analogues in which one or more of the biological activities of the parent hormone is highly accentuated in terms of potency relative to other activities. In a continued effort to test this hypothesis the following analogues of oxytocin were prepared: [7-glycine]oxytocin, [1-β-mercaptopropionic acid,7-glycine]oxytocin, [7-alanine]oxytocin, and [1-β-mercaptopropionic acid,7-alanine]oxytocin. These peptides were found to possess the following specific activities, respectively: rat uterotonic, 65 ± 2, 355 ± 3, 22 ± 1, 123 ± 4; avian vasodepressor, 5.3 ± 0.8, 17 ± 0.4, 4.8 ± 0.1, 9.8 ± 0.5; rat antidiuretic, <0.01, 0.062 ± 0.006, 0.081 ± 0.01, 0.17 ± 0.01; rat pressor, 0.3, 0.5, 0.4, 0.5 unit/mg. Thus the analogues retain high uterotonic activity but exhibit strongly diminished renal and vascular activities relative to oxytocin. Especially noteworthy is [1-β-mercaptopropionic acid,7-glycine]oxytocin with its high uterotonic activity but very low antidiuretic and pressor activities. The activity profile of this analogue combined with the fact that it is only slowly enzymatically degraded warrants further investigations of this peptide for clinical applications.

A comparison of the amino acid sequences of the nine naturally occurring neurohypophyseal nonapeptides characterized to date reveals an unusual evolutionary stability with substitutions occurring only in positions 3, 4, and 8.[3] The preferred conformation of oxytocin proposed by Urry and Walter places residues in these loci in the corner positions of the two β turns proposed for this hormone.[4] Position 7, which represents the fourth residue of these two β turns, is occupied by a proline residue in all the neurohypophyseal peptides isolated from animals thus far. From a conformational standpoint it was suggested that synthetic modifications or substitutions at these very four positions could yield hormone analogues in which one or more of the biological activities of the parent hormone is highly accentuated in terms of potency relative to the other specific activities. Peptides with such interesting activity profiles are potentially of great therapeutic value. Among the relatively few examples of neurohypophyseal hormone analogues with changes at position 7 studied to date,[5-12] [7-(thiazolidine-4-carboxylic acid)]oxytocin[12] is a dramatic case in point for the conformation–activity relationship discussed above. This analogue possesses an oxytocic activity twice that of oxytocin but a pressor activity less that of the natural principle. Other analogues of this series, although less potent than oxytocin, exhibit the same trend of enhanced oxytocic and milk-ejecting activities relative to the observed effects on the renal and vascular activities.

One of the 7-position analogues of oxytocin we became interested in some time ago was [7-glycine]oxytocin ([Gly^7]oxytocin), when we discovered that this compound showed significant resistance to enzymatic inactivation upon incubation with uterine tissue homogenate.[8,13] In contrast to oxytocin, which is predominantly hydrolyzed under these conditions at the Pro-Leu and the Leu-Gly peptide bonds, [Gly^7]oxytocin is resistant at these sites. The characteristics of slow enzymatic inactivation combined with the possibility of a more specific oxytocic activity and negligible antidiuretic activity made this analogue desirable for further investigation. [Gly^7]oxytocin had been prepared previously by two laboratories and one had reported an oxytocic activity of 330 U/mg,[6] while the other found an activity of 68 U/mg.[7] We resynthesized [Gly^7]oxytocin beginning from the partially protected nonapeptide, Z-Cys(Bzl)-Tyr-Ile-Gln-Asn-Cys(Bzl)-Gly-Leu-Gly-NH_2, generously supplied by Dr. M. Bodanszky, and we confirmed the lower value reported by Bespalova et al.[7] Since our interest is in obtaining analogues with high specific uterotonic activity and since it has been found that substitution of the N-terminal amino group by a hydrogen atom generally enhances the uterotonic activity, it was decided to synthesize [1-β-mercaptopropionic acid,7-glycine]oxytocin ([β-Mpr^1,Gly^7]oxytocin) and to investigate some of its biological properties. In addition, the preparation and biological activities of [7-alanine]-oxytocin ([Ala^7]oxytocin) and its deamino analogue, [1-β-mercaptopropionic acid,7-alanine]oxytocin ([β-Mpr^1,-Ala^7]oxytocin) are described.

The protected nonapeptides of [Ala^7]oxytocin as well as the protected key intermediates of [β-Mpr^1,Ala^7]-oxytocin and [β-Mpr^1,Gly^7]oxytocin were prepared by the general method of solid-phase peptide synthesis.[14] The peptide chain was elongated from the resin-bound glycine using nitrophenyl esters[15,16] of the appropriate *N*-butyloxycarbonyl (Boc) protected amino acids or using dicyclohexylcarbodiimide[17] (DCC) with Boc-protected free acids. The N-terminal *S*-benzylcysteine residue was introduced as the *N*-benzyloxycarbonyl (Z) derivative. The fully protected intermediates were liberated from the resin by ammonolysis[18] and the protecting groups were removed by treatment with sodium in liquid ammonia.[19] Formation of the disulfide ring was achieved by oxidation with potassium ferricyanide.[20] The resulting oxytocin analogues were purified by partition chromatography on Sephadex G-25[21] and in some instances also by gel filtration on Sephadex G-25 or LH-20.[22]

The compounds were tested for some of the biological activities characteristic of the neurohypophyseal hormones and the results are summarized in Table I. Immediately apparent is the relatively high ratio of uterotonic potency as well as milk-ejecting potency to all other activities tested for these analogues when they are compared to oxytocin or [β-Mpr^1]oxytocin (deamino-oxytocin). Also noteworthy is the consistent and high ratio of uterotonic potency to vasodepressor potency of these compounds which seems to be a peculiar and novel finding with these 7-position modified analogues, since usually uterotonic, milk-ejecting, and vasodepressor activities exhibit a similar trend. The behavior of these 7-position analogues in the pressor assay requires some further elaboration. The compounds possess

Table I. Comparison of Biological Activities of Oxytocin and Deamino-oxytocin with Analogues Possessing Amino Acid Substitutions in Position 7[a]

Peptide	Uterotonic (rat)	Milk-ejecting (rabbit)	Vasodepressor (fowl)	Antidiuretic (rat)	Pressor[b] (rat)
Oxytocin	546 ± 18[c]	440 ± 16[d]	507 ± 15[d]	2.7 ± 0.2[d]	3.1 ± 0.1[d]
[β-Mpr¹]oxytocin (deamino-oxytocin)	803 ± 36[e]	541 ± 13[e]	975 ± 24[e]	~19[e]	1.44 ± 0.06[e]
[Ala⁷]oxytocin	22 ± 1		4.8 ± 0.1	0.08 ± 0.01	0.4
[β-Mpr¹,Ala⁷]oxytocin	123 ± 4		9.8 ± 0.5	0.17 ± 0.01	0.5
[Gly⁷]oxytocin	65 ± 2 (68)[g]	224 ± 15[f] (235)[g]	5.3 ± 0.8	<0.01 (0.01)[g]	0.3
[β-Mpr¹,Gly⁷]oxytocin	355 ± 3		17 ± 0.4	0.062 ± 0.006	0.5

[a] Results are expressed in USP U/mg ± SEM unless otherwise stated. At least three animals were used for each demonstration of oxytocin-like activity and five animals for vasopressin-like activity. [b] In this study pressor potencies were determined by using matches. See text for explanation. [c] Chan et al.[24] [d] Chan and du Vigneaud.[25] [e] Ferrier et al.[26] [f] Walter et al.[8] [g] Bespalova et al.[7]

low pressor activity, but a considerable attenuation (50–75% after 15 min) of the response to subsequent injections of USP posterior pituitary standard or oxytocin was observed lasting as long as 45 min after the administration of a dose of analogue. Therefore, it was necessary to estimate the pressor activity of these compounds using matches where the standard was always given prior to the analogue. Before proceeding with the next injection of the analogue, it was always ascertained that several doses of standard gave reproducible, unattenuated responses. In one case, [Gly⁷]oxytocin, a dose as high as 0.5 mg was injected, which actually produced a depressor response (~25 mmHg).

Of particular interest is [β-Mpr¹,Gly⁷]oxytocin with its high uterotonic activity accompanied by low pressor activity and, most importantly, a low antidiuretic activity. In contrast to the total resistance[23] to enzymatic degradation of [1,6-aminosuberic acid]oxytocin when incubated with rat uterine homogenate[8,13] and other tissue preparations, [β-Mpr¹,Gly⁷]oxytocin was inactivated at a slow rate of approximately 3% after 30 min and 30% after 60 min when incubated with uterine homogenate of rat under experimental conditions described in ref 13; this rate of inactivation is similar to that of [Gly⁷]oxytocin. The biological results obtained for [β-Mpr¹,Gly⁷]oxytocin make this analogue worthy of further investigations for clinical use during induction of labor and possibly for the regulation of fertility.

Experimental Section

All melting points were determined in open capillary tubes and are reported uncorrected. Thin-layer chromatography was carried out on Quantum Industries precoated silica gel G plates and the products were detected by chlorine–tolidine reagent.[27] Because of sparing solubility, the protected peptides did not move in any solvent system tried, and as such their purity could not be checked by TLC. The hormone analogues were shown to be homogeneous by TLC with loads to 50 μg on chromatographs 100–150 mm in length; R_f in solvent system 1-BuOH–AcOH–H_2O (4:1:5; upper phase) are [Ala⁷]oxytocin, 0.44; [β-Mpr¹,Ala⁷]oxytocin, 0.57; [Gly⁷]oxytocin, 0.21; [β-Mpr¹,Gly⁷]oxytocin, 0.53. Amino acid analyses[28] were performed on a Beckman Model 120C amino acid analyzer following hydrolysis in deareated 6 N HCl at 110°. Where elemental analyses are indicated only by the symbols for the elements, analytical results obtained were within ±0.4% of the theoretical values. Samples for analysis were dried at 100° at 0.01 Torr for 12 h.

Rat uterine assays were performed on isolated horns from virgin rats in natural estrus according to the method of Holton,[29] as modified by Munsick,[30] with the use of Mg^{2+}-free van Dyke–Hastings solution as bathing fluid. Avian vasodepressor assays were performed on conscious chickens by the method of Coon,[31] as described in the U.S. Pharmacopeia,[32] as modified by Munsick et al.[33] Pressor assays were carried out on anesthetized male rats as described in the U.S. Pharmacopeia.[34] Antidiuretic assays were performed on anesthetized male rats according to the method of Jeffers et al.,[35] as modified by Sawyer.[36] Either the four-point assay design of Schild[37] or matches were used to obtain specific activities as compared to U.S.P. posterior pituitary reference standard.

Z-Cys(Bzl)-Tyr(Bzl)-Ile-Gln-Asn-Cys(Bzl)-Ala-Leu-Gly-NH_2 (1). Boc-Gly-O-resin[38] (7.5 g), containing 0.38 mmol of glycine/g of substituted resin (polystyrene 2% cross-linked with divinylbenzene), was used with the following cycles of deprotection, neutralization and coupling for the introduction of each amino acid moiety (50-ml portions of solvent were used in washing steps): (1) three washings with AcOH; (2) Boc group removal with 1.25 N HCl in AcOH for 30 min (except that cleavage of Boc groups from Asn and Gln residues was carried out with TFA[39] for 15 min); (3) three washings with AcOH; (4) three washings with absolute EtOH; (5) three washings with DMF; (6) neutralization with 10% Et_3N in DMF for 10 min; (7) three washings with DMF; (8) addition of 8 mmol of the appropriate *tert*-butyloxycarbonylamino acid nitrophenyl ester in DMF (40 ml) followed by reaction overnight; (9) three washings with DMF; (10) three washings with absolute EtOH. Steps 5–10 were repeated for each residue incorporated[40] using 3 mmol of the nitrophenyl ester in step 8 to ensure complete reaction. Boc-Ala was coupled through reaction with DCC.[17] For DCC reactions step 8 was three washings with CH_2Cl_2 followed by (9) addition of 8 mmol of the appropriate protected residue in 25 ml of CH_2Cl_2 and mixing for 10 min; (10) addition of 8 mmol of DCC in 15 ml of CH_2Cl_2 and reaction overnight; (11) three washings with CH_2Cl_2; and (12) three washings with absolute EtOH. DCC couplings were also repeated; three washings with glacial AcOH were followed by steps 4–12 (using 3 mmol each of *tert*-butyloxycarbonylamino acid and DCC). After every coupling reaction had been repeated, the peptide resin was checked for unreacted amino groups by a ninhydrin test;[41] in each case coupling appeared to be complete. The coupling reactions were carried out with the following amino acid derivatives in succession: Boc-Leu-ONp, Boc-Ala-OH, Boc-Cys(Bzl)-ONp, Boc-Asn-ONp, Boc-Gln-ONp, Boc-Ile-ONp, Boc-Tyr(Bzl)-ONp, and Z-Cys(Bzl)-ONp. After the completion of synthesis the protected peptide resin was washed three times each with glacial AcOH and MeOH. The resin was washed out of the reaction vessel with MeOH, filtered, washed with Et_2O, and dried in vacuo: 10.25 g. The weight gain (2.75 g) indicated 83% incorporation of protected peptide based on the glycine content of the resin.

The protected peptide was removed from the peptide resin (10.2 g) by ammonolytic cleavage.[18] MeOH and NH_3 were removed in vacuo and the cleaved peptide was extracted with DMF followed by MeOH. The residue left after evaporation of solvents was triturated with MeOH and Et_2O, filtered, and dried in vacuo: 2.13 g. The compound was dissolved in DMF and precipitated with EtOH: 1.72 g (45% based on glycine); mp 267–269°; $[\alpha]^{24}D$ −43.4° (*c* 0.5, formic acid). Anal. ($C_{70}H_{90}N_{12}O_{14}S_2$) C, H, N. Amino acid analysis gave the following molar ratios: Asp, 1.0; Glu, 1.0; Gly, 1.0; Ala, 1.0; Ile, 1.0; Leu, 1.0; Tyr, 0.84; Cys(Bzl), 1.8; and NH_3, 3.1.

[7-Alanine]oxytocin (2). The protected peptide intermediate 1 (347 mg, 0.25 mmol) was dissolved in liquid NH_3 (350 ml, freshly distilled from Na) and reduced with Na at the boiling point of

NH_3;[19] after 30 s the blue color was discharged with NH_4Cl. Ammonia was removed by lyophilization and the residue was dissolved in 0.05% aqueous TFA (500 ml). The pH of the solution was adjusted to 8.0 with 3% NH_4OH and 0.01 M $K_3Fe(CN)_6$ was added[20] with stirring until the yellow color persisted for 15 min. The pH of the solution was then adjusted to 6 with dilute TFA, resin suspension (AG3-X4, Cl^- form) was added, and stirring was continued for 15 min to remove ferro- and ferricyanide ions. The resin was separated by filtration and the filtrate was concentrated to a small volume. The product was purified by partition chromatography on a 3.10 × 96.8 cm column of Sephadex G-25[21] (fine) which had been equilibrated with the lower phase of the solvent system 1-BuOH–C_6H_6–3.5% AcOH (containing 1.5% pyridine) (12:1:13). The column was eluted with the upper phase of the solvent system at 23 ml/h and the peptide material was detected by the method of Lowry et al.[42] The product appeared as a major peak at R_f 0.20, well resolved from several minor peaks. The fractions corresponding to this peak were pooled and diluted with twice the volume of H_2O, and the solution was concentrated in vacuo and lyophilized: 166 mg. A portion (70 mg) was subjected to gel filtration[22] in 0.2 N AcOH on a 2.85 × 93.0 cm column of Sephadex G-25 (superfine). The compound emerged in a sharp symmetrical peak with maximum at 468 ml: 60 mg (55% as monoacetate based on the protected nonapeptide); $[\alpha]^{24}D$ –9.3° (*c* 0.5, 1 N AcOH). Anal. ($C_{41}H_{64}N_{12}O_{12}S_2 \cdot CH_3CO_2H$) C, H, N. Amino acid analysis gave the following molar ratios: Asp, 1.0; Glu, 1.0; Gly, 1.0; Ala, 1.0; $^1/_2$Cys, 1.9; Ile, 1.0; Leu, 1.0; Tyr, 0.89; and NH_3, 3.0.

β-Mpr(Bzl)-Tyr(Bzl)-Ile-Gln-Asn-Cys(Bzl)-Ala-Leu-Gly-NH_2 (3). This protected peptide intermediate was prepared on the same resin (7.5 g) as described for **1**, substituting β-Mpr(Bzl)-OH[20] for Z-Cys(Bzl)-ONp: weight gain 2.6 g (90% based on glycine). The product was cleaved from the resin by ammonolysis and reprecipitated from DMF–EtOH: 1.57 g (46% based on glycine); mp 261–263°; $[\alpha]^{24}D$ –38.6° (*c* 0.5, formic acid). Anal. ($C_{62}H_{83}N_{11}O_{12}S_2$) C, H, N. Amino acid analysis gave the following molar ratios: Asp, 1.0; Glu, 1.0; Gly, 0.93; Ala, 1.0; Ile, 1.0; Leu, 1.0; Tyr, 1.0; Cys(Bzl), 1.0; and NH_3, 3.0.

[1-β-Mercaptopropionic acid,7-alanine]oxytocin (4). The protected peptide amide (372 mg, 0.3 mmol) was dissolved in liquid NH_3 (350 ml), treated with Na, and oxidatively cyclized with $K_3Fe(CN)_6$ as described for **2**. The peptide material was purified by partition chromatography on the column described for **2** in the solvent system 1-BuOH–C_6H_6–3.5% AcOH (containing 1.5% pyridine) (1:1:2). The peptide material was detected by the method of Lowry et al. and the product emerged in a peak at R_f 0.23 which was isolated by lyophilization: 152 mg (52.4% based on protected peptide); $[\alpha]^{24}D$ –54.2° (*c* 0.5, 1 N AcOH). Anal. ($C_{41}H_{63}N_{11}O_{12}S_2$) C, H, N. Amino acid analysis gave the following molar ratios: Asp, 1.0; Glu, 1.1; Gly, 1.0; Ala, 1.0; $^1/_2$Cys, 0.45; mixed disulfide of Cys and β-Mpr, 0.39; Ile, 0.92; Leu, 1.0; Tyr, 1.0; and NH_3, 2.9.

[7-Glycine]oxytocin (5). This was prepared from Z-Cys(Bzl)-Tyr-Ile-Gln-Asn-Cys(Bzl)-Gly-Leu-Gly-NH_2 (250 mg, 0.19 mmol)[6] in the same manner as described for **2**. The crude product was subjected to partition chromatography on a 2.85 × 63 cm column of Sephadex G-25 (fine) in the system BuOH–H_2O (1:1, the aqueous phase 1.5% pyridine and 3.5% AcOH). The product emerged as a major peak at R_f 0.36. Fractions comprising the peak area were pooled with 2 vol of H_2O and the product was isolated by evaporation and lyophilization: 110 mg (60% from protected peptide). The entire product was subjected to gel filtration chromatography on a 0.9 × 112 cm column of Sephadex LH-20 in the upper phase of the solvent system BuOH–AcOH–H_2O (4:1:5). The product emerged as a sharp peak at the void volume of the column. Fractions comprising the peak area were pooled with H_2O and evaporated to a small volume, and the product was recovered quantatively by lyophilization: $[\alpha]^{24}D$ –41° (*c* 0.5, 1 N AcOH). Anal. ($C_{40}H_{62}N_{12}O_{12}S_2 \cdot CH_3CO_2H \cdot 4H_2O$) C, H, N. Amino acid analysis gave the following molar ratios: Asp, 1.1; Glu, 1.1; Gly, 1.9; $^1/_2$Cys, 1.8; Ile, 1.0; Leu, 1.0; Tyr, 0.86; and NH_3, 2.8.

β-Mpr(Bzl)-Tyr(Bzl)-Ile-Gln-Asn-Cys(Bzl)-Gly-Leu-Gly-NH_2 (6). This protected nonapeptide was prepared in the same manner and from the same Boc-Gly-O-resin (7.0 g) as described for 1, substituting Boc-Gly-OH for Boc-Ala-OH and β-Mpr(Bzl)-OH for Z-Cys(Bzl)-ONp: weight gain 2.55 g (92% based on glycine). The product was cleaved from the resin (9.5 g) by ammonolysis and precipitated from DMF–H_2O followed by reprecipitation from DMF–EtOH: 1.40 g (43.1% based on glycine resin); mp 253.5–254.5°, $[\alpha]^{24}D$ –31.5° (*c* 0.5, DMF). Anal. ($C_{61}H_{81}N_{11}O_{12}S_2$) C, H, N. Amino acid analysis gave the following molar ratios: Asp, 1.0; Glu, 1.0; Gly, 1.9; Ile, 1.0; Leu, 1.0; Tyr, 1.0; Cys(Bzl), 0.92; and NH_3, 3.1.

[1-β-Mercaptopropionic acid,7-glycine]oxytoxcin (7). The protected peptide amide (306 mg, 0.25 mmol) was dissolved in liquid NH_3 (350 ml), treated with Na, and oxidatively cyclized with $K_3Fe(CN)_6$ as described for **2**. The peptide material was purified by partition chromatography on the column described for **5** which had been equilibrated with the lower phase of the solvent system 1-BuOH–C_6H_6–3.5% AcOH (containing 1.5% pyridine) (1:1:2). The column was eluted with the upper phase of the solvent system at 27 ml/h, peptide material was detected by the method of Lowry et al., and the product emerged as a single peak at R_f 0.24. The fractions corresponding to the peak area were pooled, evaporated, and lyophilized: 139 mg (58.4% based on the protected peptide); $[\alpha]^{24}D$ –54.9° (*c* 0.5, 1 N AcOH). Anal. ($C_{40}H_{61}N_{11}O_{12}S_2$) C, H, N. Amino acid analysis gave the following molar ratios: Asp, 1.0; Glu, 1.0; Gly, 2.0; $^1/_2$Cys, 0.54; mixed disulfide of Cys and β-Mpr, 0.45; Ile, 1.0; Leu, 1.0; Tyr, 0.94; and NH_3, 3.2.

Acknowledgment. The authors wish to thank Dr. R. T. Havran for his synthetic help and Ms. Dasha Surovec for her assistance in the bioassays. We also wish to thank Dr. M. Bodanszky for his interest in this work and for generously supplying us with a sample of protected nonapeptide of [Gly^7]oxytocin from his laboratory. This work was supported in part by the Population Council (Contract No. M73.1381/ICER/C3) and by U.S. Public Health Service Grant AM-18399.

References and Notes

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Folate Analogues Altered in the C^9–N^{10} Bridge Region. 10-Oxafolic Acid and 10-Oxaaminopterin[1a]

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The unambiguous synthesis of two folate analogues, in which the 10-amino group of folic acid was replaced with oxygen, is described. The synthetic sequence employed commercially available methyl *p*-hydroxybenzoate and *N*-(2,3-epoxypropyl)phthalimide as starting materials. The use of cesium bicarbonate as a coreactant in the nucleophilic displacement reaction between bromo ketone **3** and the nucleophile **4** was found to be unique in character. The aminoacetonyl oxime **7** obtained by the hydrazinolysis of **6** was used as a common intermediate for the synthesis of both compounds. The generality of the use of the TFA–HCl mixture to deprotect the carbonyl group of both **10** and **12** and reductions involving sodium hydrosulfite in aqueous DMF were further substantiated by conversions of **11** and **13** to **14** and **15** quickly and efficiently without employing catalytic hydrogenations. Subsequent cyclizations, oxidations, and hydrolysis of these reduction products to the pteroate analogues **17** and **19** were carried out efficiently as described for the synthesis of the sulfur analogues. Activation of the carboxyl group of **19** by way of the mixed anhydride **22** and subsequent coupling to glutamic acid was carried out using the solid-phase coupling procedure. However, compound **17** required trifluoroacetylation to **20** prior to the coupling reaction due to solubility problems. Both 10-oxafolic acid (**1**) and 10-oxaaminopterin (**2**) showed potent antifolate activity when tested against two folate-requiring organisms. Compound **2** was a very powerful inhibitor of DCM-resistant *Lactobacillus casei* dihydrofolate reductase. The activity was comparable to that of methotrexate while the 4-hydroxy analogue did not show inhibition. 7,8-Dihydro-10-oxafolic acid failed to show any substrate activity to this enzyme and did not inhibit the enzymatic reaction when used with an equimolar concentration of the natural substrate.

The use of folate analogues as chemotherapeutic agents for the treatment of neoplastic diseases is well known.[1b-4] A concept of in vivo lethal synthesis[5,6] has led to the search for new folate analogues which are substrates of the enzyme dihydrofolate reductase (E.C. 1.5.1.3) and the reduction products thus formed capable of showing antifolate activity either by interference with folate uptake[7] or by inhibiting enzymes that are involved in DNA biosynthesis.[8] As part of a continuing program aimed at developing compounds which may meet these requirements, we have reported the design and synthesis of several folate analogues which are altered in the C^9–N^{10} bridge region.[9-13] In our continuing search for new and better substrates to this enzyme it appeared interesting to investigate the mode of interaction of the 10-oxa analogues toward dihydrofolate reductase and also their antifolate activities. This paper describes the chemical synthesis and characterization of the 10-oxa analogues of folic acid and aminopterin[14] and their preliminary antifolate activities.

More than 20 years ago Fairburn[15,16] and co-workers reported the synthesis of 10-oxafolic acid (**1**) and 10-oxaaminopterin (**2**) (Chart I). In this connection, these authors reported the characterization of their respective pteroate analogues as well. All four compounds were evaluated for their antifolate activity by the method of Wooley[17] and all of these showed varying degrees of antifolate activity when tested against *Streptococcus faecalis* R. Significant among these results is the fact that none of the analogues showed any response when a 10:1 ratio of the respective analogue to folic acid was employed. At a ratio of 100:1, 10-oxaaminopterin (**2**) showed very little antifolate response. Furthermore, the 4-amino-4-deoxy pteroate analogue was remarkably more responsive to this organism than the respective folate analogue. The validity