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THE SYNTHESIS AND EVALUATION OF NEW DERIVATIVES OF 2,4 DIAMINOPYRIMIDINES WITH MALE CONTRACEPTIVE ACTIVITY

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ABSTRACT

The discovery of the antifertility activity of gossypol led scientists and researchers to the development of compounds with antifertility activity that can be used as male contraceptives. These studies resulted in discovery of the antifertility activity of several classes of compounds which have been reported in the literature.

This article deals with the synthesis of four new analogues of pyrimethamine, the derivatives of 2, 4-diamino-5 (3,4-dichlorophenyl)-6-alkyloxymethyl pyrimidine (methyl, ethyl, n-propyl, iso-propyl), and the results of experimental antifertility evaluation of these compounds on male rats, which revealed a significant decrease in motility, viability, ESR and DSP indices in treated animals.

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INTRODUCTION

Reduction in the rates of demographic growth as well as maternal and child health care and mortality are directly linked to the habit of contraceptive use. The development and availability of safer, improved contraceptives, with a potential capability of being used by a larger group of individuals, will have a greater impact on population growth rate control, maternal and child health care, and mortality measures. These prospects are the fundamental basis for contraception research.

The discovery of the antifertility activity of gossypol, the active compound present in the seed and root of the cotton plant, inspired scientists and researchers to develop a compound with antifertility activity that can be used as a male contraceptive. With this goal in mind, the antifertility activity of different classes of compounds were evaluated, such as the antimalarial drug pyrimethamine and related 2,4-diaminopyrimidine compounds, as several reports exist on their antifertility activity.[1-3]

MATERIAL AND METHODS

In this report the synthesis of 4 new analogues of pyrimethamine with the general formula of (methoxy-ethoxy, n-propyloxy, and iso-propyloxy) derivatives of 2,4-diamino-5 (3, 4-dichlorophenyl)-6-alkyloxymethylpyrimidine (4a-d)], and their experimental antifertility activity has been evaluated. It is noteworthy to mention that the synthesis of 4a and 4b derivatives of this serial and their anticonvulsant and dihydrofolate reductase (DHFR) inhibitory activity has been reported previously.[4,5]

According to Fig. 1, the new compounds were prepared

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Table I. Ethyl-alkyloxy acetate (1a-d).

Substance	MW	b.pC° (20 mmHg)	Yields
1a	118	126-131	60%
1b	132	155-163	60%
1c	146	183-189	12%
1d	146	166-170	84%

Alkyloxy= methoxy, ethoxy, n-propyloxy, and iso-propyloxy

in 4 steps. Ethyl-alkyloxy acetate (1a-d) was prepared according to the method used by Raymond et al.[6] To a solution of sodium (2.78 atom gram) dissolved in the corresponding alkyl alcohol, halogenated (Cl or Br) ethyl acetate (2.4 mole) was added dropwise with stirring. The mixture was refluxed for 24 hours and then allowed to stand overnight. The solution was brought to a pH of 5-6 with concentrated HCl, filtered and distilled. The boiling points and the yields are reported in Table I.

α-Alkyloxy acetyl-3 , 4-dichlorophenyl acetonitrile[7] (2a-d)

To a solution of sodium (0.5 atom gram) in 250 mL absolute ethanol was added a mixture of 3,4 dichlorophenyl acetonitrile (0.5 mole) and the corresponding ethyl-alkyloxy acetate (0.5 mole). The mixture was refluxed on the steam-bath for four hours. After cooling, it was poured into water and extracted with ether, then the aqueous layer was acidified with N sulfuric acid and the separated oil extracted again with ether. The ethereal solution, after washing with sodium bicarbonate solution and again with water, was dried over anhydrous sodium sulfate. On removal of ether the residue crystallized, which was then recrystallized from ether petroleum ether. The melting points and yields are reported in Table II.

α-3, 4-Dichlorophenyl-β methoxy -β alkyloxymethyl acrylonitrile[7] (3a-d)

Alkyloxy acetyl-3, 4 dichlorophenyl acetonitrile (0.05 mole) was treated with 10g of diazomethane (0.24 mole) in cold dry ether (ice bath). The reaction subsided in a few seconds. After standing for five hours, the ether was evaporated, and the compound was used directly in the next step. The yields are reported in Table III.

2,4- Diamino-5 (3,4 - dichlorophenyl) -6- alkyloxymethyl pyrimidine[7] (4a-d)

α (3, 4-dichlorophenyl-β methoxy-β alkyloxymethyl acrylonitrile (0.026 mole) was dissolved in 25 mL absolute ethanol. To this solution of guanidine base (0.026 mole) in 50 mL absolute ethanol, the result of the reaction between guanidine hydrochloride (0.026 mole) and sodium (0.026 atom gram) in absolute ethanol was added. The solution was heated on a steam bath for three hours. The ethanol was then removed and concentrated sodium hydroxide solution was added to the residue. The insoluble materials were filtered and dissolved in glacial acetic acid (10 mL) and diluted with water (30 mL). After treatment with active charcoal and filtration, the colorless solution was made alkaline with 2N sodium hydroxide. The white precipitate was filtered off and washed with water. Each precipitate was recrystallized in methanol and their melting points are shown in Table IV.

Antifertility activity evaluation[1,2]

Adult male Sprague-Dawley rats (approximately 200-250g) were obtained from the Institute of Biochemistry & Biophysics, University of Tehran, and were maintained on rat chow and water *ad libitum* with constant light-dark cycle and were housed in an animal room kept at 22°C.

Thirty rats were randomized to five groups. Each group consisting of six rats were injected subcutaneously on alternate days with 50 mg/kg/mL of compounds dissolved in propylene glycol for 60 days and the control group received 1 mL/kg propylene glycol during this period of time.

All rats were weighed in the course of the experiment to evaluate animal health. At the end of 50 days male rats were

TableII. α-Alkyloxy acetyl-3, 4 dichlorophenyl acetonitrile (2a-d).

Substance	MW	m.pC°	Yields	Recrystallization solvent
2a	258	100-105	69%	ether + petroleum ether
2b	227	95-98	73%	"
2c	286	102-107	94%	"
2d	286	70-82	78%	"

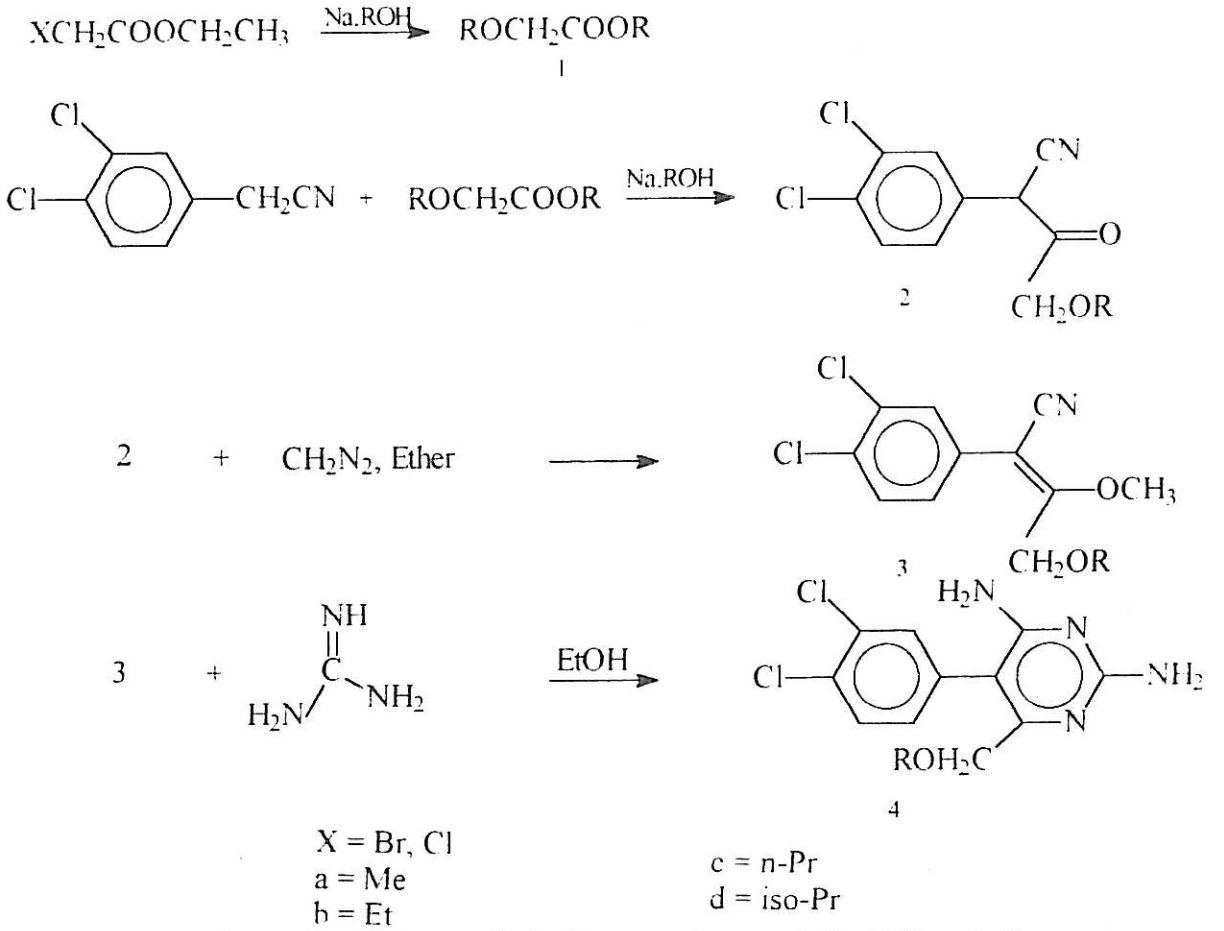

Fig.1. The steps involved in the synthesis of the proposed compounds (for details, see text).

Table III. α-3,4-Dichlorophenyl-β methoxy-β alkyloxymethyl acrylonitrile (3a-d).

Substance	MW	Form	Yields
3a	272	viscous liquid	95%
3b	286	"	96%
3c	300	"	99%
3d	300	"	88.5%

evaluated for fertility (mating test) and placed with 3 female rats for ten days. At the end of this period the male rats were euthanized by decapitation.

The trunk blood was collected and serum was used for testosterone determination by radioimmunoassay (RIA). The inferior portion of the abdomen was opened and the testis and epididymis (one side) were weighed and placed separately in physiologic solution (at 37°C). The number of advanced spermatids was determined in the removed testis.

DSP (daily sperm production) and ESR (epididymal sperm reserve) were determined by a homogenization method (Robb et al).[8]

Motility[9,10]

Motility is the progressive movement of the sperm, and is the ratio of motile sperm to non-motile forms, expressed in percentage. Sperm motility was determined according to the conventional method available in texbooks.[9,10]

Viability[10]

Viable sperm do not absorb dyes while nonviable forms do. Viability was determined according to the reported procedures, and expressed in percent.

ESR

The epididymal sperm reserve was determined according to the method of Robb et al.[8]

DSP

Daily sperm production was also determined according to the method of Robb et al.[8] DSP in rats is the ratio of the number of advanced spermatids over 6.3 days. This period is the time necessary for development of advanced spermatids in the rat seminiferous epithelium cycle.

Table IV. 2, 4-Diamino-5 (3, 4-dichlorophenyl)-6-alkyloxymethyl pyrimidine (4a-d).

Substance	MW	m.pC°	yields	Recrystallization solvent
4a	272	212-215	48%	methanol
4b	313	216-216	51%	"
4c	327	198-202	41%	"
4d	327	202-203	42%	"

GSI[12]

Gonado-somatic index is the ratio of the weight of the testes to body weight ×100.

Fertility[11]

Fertility was determined according to the reported procedures of Oberlander et al. and it is the ratio of implantation sites over the corpus luteum and expressed in percent.

Hormone measurement[2]

Serum testosterone (T) concentration was determined by a conventional RIA procedure, using an RIA kit obtained from Kavoshyar Company (Code 3900).

Statistical analysis

In all performed experiments, in each case and its control, the mean values have been calculated (mean±SEM), and Student's unpaired t-test was used to assess significant results. All results are shown in Table V.

RESULTS

Mouse weight

Statistical analysis of the recorded mouse weights during the experiment indicated that the result was significant only for group 4b ($p<0.05$).

Gonado-somatic index

Statistical analysis of the GSI studies indicated that the result was only significant for group 4c.

Epididymal weight

This factor was also determined and recorded in the course of the experiment. Statistical analysis of the studies indicated that the results were significant only for group 4a ($p<0.05$).

Motility

The motility study and its statistical analysis indicated that the results were significant for groups 4b, 4c and 4d ($p<0.01$).

Viability

Viability study and statistical analysis indicated that the results were significant for groups 4a, 4d and 4b, and their P values were (<0.05, <0.01, <0.001), respectively.

Epydidymal sperm reserve

Statistical analysis of ESR studies indicated that the

TableV. The effects of four synthesized compounds on physiologic and fertility indices together with their mean±SEM values.

	Animal weight (g)	Gonado-somatic index (×10³)	Epididymal weight (g)	Motility (%)	Viability (%)	Epididymal sperm reserve (ESR) (×10⁶)	Daily sperm production (DSP) (×10⁶)	Fertility (%)	Serum testosterone (ng/mL)
Control	297.88 ±6.48	0.92±0.06	0.57±0.01	59.55±4.55	72.96±3.48	253.87±21.62	18.46±0.8	83.10±8.73	3.42±0.52
4a	295.33±6.09 N/S	1.07±0.09 N/S	0.28±0.01 *	52.5±2.53 N/S	61.4±4.03 *	98.46±19.71 ***	22.06±0.07 *	63.16±9.88 N/S	4±0.5 N/S
4b	280.83± 3.67 *	0.87±0.5 N/S	0.49±0.17 N/S	39.45±2.2 **	43.12±2.90 ***	100.05±7.05 ***	8.12±0.6 **	22.2±0 ***	0.61±0.54 *
4c	284.33±18.16 N/S	1.05±0.08 *	0.29±0.01 N/S	42.75±3.69 **	62.43±5.7 N/S	69.96±12.77 ***	12.51±1.12 *	87.32±2.52 N/S	3.3±0.33 N/S
4d	288.67±9.37 N/S	0.86±0.53 N/S	0.48±0.01 N/S	38.5±1.72 **	50.13±2.05 **	100.43±2.22 ***	13.68±0.67 *	59.03±2.86 *	3.43±1.04 N/S

$p<0.05$, *** $p<0.001$, N/S= not significant.

results were significant for all the groups with $p<0.001$ (4a, 4b, 4c, 4d).

Daily sperm production

The statistical analysis of DSP studies indicated that the results for groups 4c ($p<0.05$), 4d ($p<0.05$), and 4b ($p<0.01$) were significant.

quantitative spermatogenesis at the level of pachytene spermatocytes and spermatids. This consequently led to reductions in the number of testicular advanced spermatids and epididymal sperm content.

The latter conclusion together with the result of reduction in folinic acid bioavailability may be the fundamental basis in reduction of advanced spermatids and epididymal sperm reserve. Concerning Sertoli cell function in the testis, Awoniyi et al. concluded further that the primary function of these nursing cells is to provide the essential factors and create the proper environment for the development of germ cells. Part, if not all, of the control of spermatogenesis by gonadotropin and testosterone (T) seems to be mediated by the actions of these hormones on Sertoli cells. However, the reduction in the number of advanced spermatids in this study can not be explained by serum gonadotropin and T levels, because the levels of these hormones did not change following PYR treatment.

A possible explanation for the decrease in the number of spermatocytes and round spermatids could, however, be explained by the direct cytotoxic effect of PYR on the Sertoli cell and/or spermatocytes and spermatids themselves. The damaging effects of PYR on Sertoli cells may be due to a decrease in the production of certain essential proteins secreted by these cells.

Reduction in the secretion of Sertoli cell-specific proteins

Fertility

The statistical analysis of fertility studies indicated that the results for 4d ($p<0.05$) and 4b ($p<0.001$) were significant.

Serum testosterone

The statistical analysis of serum testosterone studies indicated that the result was significant for group 4b only.

DISCUSSION

Trimethoprim (TRIM) and pyrimethamine (PYR) are two members of the 2,4 diaminopyrimidine class of compounds with antibacterial and antimalarial activity, respectively. These compounds are DHFR inhibitors. Trimethoprim is active against bacterial DHFR, and pyrimethamine against malarial DHFR.

Consentino et al.[1] evaluated the possibility of DHFR inhibitory mechanism involvement in the antifertility effects of PYR and concluded that the concomitant administration of activated intermediate folinic acid caused reversal of the antifertility effects of PYR in male mice.

Awoniyi et al.[2] concluded that the antifertility effect of PYR is the consequence of direct cytotoxic effects on the seminiferous epithelium.

They also made important further observations that the administered dose of PYR led to profound inhibition of such as transferrin, sulfated glycoprotein-2 and inhibin could thereby lead to possible reduction in sperm production.

One caveat in regard to the lack of reduction of testosterone concentration in the seminiferous tubule fluid and its consequent effects on spermatogenic cells, is the fact that the production of androgen receptors and androgen binding protein levels are under the control of Sertoli cells. Damage to the functions of Sertoli cells may result in decreased androgen receptors and androgen binding proteins and thus interfere with T uptake and utilization by germ cells. Therefore, the antifertility effects could simply reflect decreased Sertoli cell ability to respond to the androgen present. Thus the significance of the damaging effect of PYR on Sertoli cell function may only be expressed in the form of an assumption, and further studies are necessary to elucidate the possible mechanisms.

In our report we have evaluated different parameters. The weight index is the sign of the animals general health in the course of experiments. GSI and epididymal weight indices are signs of the animal's reproductive organ development in the course of experiments. The motility, viability, fertility and serum T levels are the factors attributed to sperm capability in the animal's general process of reproduction. ESR and DSP are two signs that are consistent with the conclusion of previous reports and show that reduction in DSP and ESR is either the result of DHFR inhibition and/or the result of detrimental effects of 2,4 diaminopyrimidine compounds on the process of spermatogenesis.

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