Antifertility Activity of *N*-Protected Glycine Activated Esters

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Abstract □ A series of *N*-protected glycine activated esters was found to have antifertility activity in mice when administered intravaginally. The *N*-carbobenzoxyglycine vinyl ester and *N*-carbobenzoxyglycine 1,2-dibromoethyl ester analogs possessed the best activity in inhibiting pregnancy but were much less active when administered intraperitoneally. The acrosin enzymatic activity of sperm also was inhibited by these *N*-protected glycine activated esters, as measured by *N*-α-benzoyl-L-arginine ethyl ester and azocasein hydrolysis. The ability to inhibit *N*-α-benzoyl-L-arginine ethyl ester hydrolysis, a trypsin-like activity, appeared to have a positive correlation with antifertility activity when the agents were administered intravaginally.

Keyphrases □ Antifertility activity—evaluation of *N*-protected glycine activated esters □ *N*-Protected glycine activated esters—evaluation for antifertility activity □ Glycine esters—*N*-protected analogs, evaluation for antifertility activity

A number of chemical (1) and endogenous (2) agents recently were shown to inhibit the proteolytic activity of the sperm's acrosomal enzyme, acrosin (EC 3.4.21.10) and thereby reduce sperm fertilization of the ovum. Acrosin enzymatic activity is similar to trypsin proteolytic activity and thus is inhibited by trypsin inhibitors. *N*-α-Tosyl-L-lysine chloromethyl ketone administered intravaginally in sterile lubricant before coitus inhibited fertilization in rabbits (3). A series of *N*-carbobenzoxyglycine, L-leucine, and L-proline esters administered intravaginally in mice demonstrated 100% inhibition at 10 mg/kg/day, as did *N*-α-tosyl-L-lysine chloromethyl ketone and tosyl-L-phenylalanine chloromethyl ketone (4). The glycine series has been expanded to include several types of esters as well as variations in the amino protecting group, and these results are reported here.

EXPERIMENTAL

Chemical Synthesis—The compounds evaluated in this study are listed in Table I. Several of the compounds were synthesized previously in this laboratory [*i.e.*, II and III (5, 6)] or elsewhere [*i.e.*, IV (7), V (8), XIV (9), and XV (10)]. Procedures for vinyl ester preparation using vinyl acetate and palladium chloride catalyst have been discussed in detail, as have those for cyanomethyl ester preparation (5, 6). The synthesis of new activated esters reported here (VI, VII, IX–XI, and XIII) was uncomplicated and involved reaction of *N*-carbobenzoxyglycine and active halogen compounds and triethylamine in ethyl acetate. In one case (VIII), the desired product was obtained using the alcohol, *N*-carbobenzoxyglycine, and *p*-toluenesulfonic acid in a standard esterification procedure. Since several compounds were difficult to purify, preparative details are included. All compounds appeared to have adequate stability when stored in a dry atmosphere, as judged by TLC and spectral methods[1].

Palladium chloride was obtained commercially[2]. *N*-Carbobenzoxyglycine *p*-nitrophenyl ester (XII), *N*-α-tosyl-L-lysine chloromethyl ketone (XVI), tosyl-L-phenylalanine chloromethyl ketone (XVII), *N*-carbobenzoxy-L-phenylalanine chloromethyl ketone (XVIII), 17-ethinyl estradiol (XIX), and diethylstilbestrol (XX) were purchased commercially[3]. The following materials were prepared as described previously: *N*-carbobenzoxyglycine vinyl ester (II) (6), *N*-carbobenzoxyglycine 1,2-dibromoethyl ester (III) (6), *N*-carbobenzoxyglycine ethyl ester (IV) (7), *N*-carbobenzoxyglycine cyanomethyl ester (V) (6), and *N*-trifluoroacetylglycine vinyl ester (XIV) (8).

N-*Carbobenzoxyglycine Acetonyl Ester (VI)*—A mixture of 1.5 g (7 mmoles) of *N*-carbobenzoxyglycine (6) and 0.8 g (8 mmoles) of triethylamine in 50 ml of tetrahydrofuran was heated to reflux. A solution of 1.0 g (7 mmoles) of bromoacetone (11) in 10 ml of tetrahydrofuran was added slowly dropwise over 1 hr. The reaction mixture then was stirred at reflux for 4 hr and cooled to room temperature, and the precipitate was collected. About 25 ml of chloroform was added to the filtrate, and this solution was washed with 20 ml of 5% HCl and dried (magnesium sulfate); the solvents were removed *in vacuo*.

The crude, oily product was purified by column chromatography, with chloroform as the eluent, to give 0.8 g (45% yield) of a white powder, mp 71–72°; IR (mineral oil): 3320 (strong) (C═O, ester), 1720 (strong) (C═O, ketone), and 1685 (strong) (C═O, carbamate) cm^{-1}; NMR ($CDCl_3$): δ 2.17 (s, 3H, $COCH_3$) and 4.79 (s, 2H, OCH_2CO). Elemental analysis (carbon, hydrogen, and nitrogen) showed a formula of $C_{13}H_{15}NO_5$.

N-*Carbobenzoxyglycine Methoxymethyl Ester (VII)*—A mixture of 2.5 g (12 mmoles) of *N*-carbobenzoxyglycine and 1.3 g (12.8 mmoles) of triethylamine in 30 ml of ethyl acetate was heated to reflux. A solution of 1.0 g (12.4 mmoles) of chloromethyl methyl ether in 25 ml of ethyl acetate was added dropwise, with stirring, over 2 hr. The reaction mixture was stirred for 3 hr at reflux and cooled, and the precipitate was filtered. The filtrate was washed with 5% HCl followed by water and dried (magnesium sulfate), and the solvents were removed *in vacuo*.

The resulting oily product was purified by column chromatography, with chloroform as the eluent, to give 1.3 g (35% yield) of pure product as an oil; IR (CCl_4): 3380 (strong, broad) (NH) and 1710 (strong, broad) (2 C═O) cm^{-1}; NMR (acetone-d_6): δ 3.45 (s, 3H, OCH_3) and 5.35 (s, 2H, OCH_2O).

N-*Carbobenzoxyglycine Methoxyethyl Ester (VIII)*—A mixture of 2.5 g (12 mmoles) of *N*-carbobenzoxyglycine and 0.1 g (5 mole %) of *p*-toluenesulfonic acid monohydrate in 50 ml (0.65 mole) of 2-methoxyethanol was stirred at 70° for 24 hr (12). The reaction flask was fitted to a distillation apparatus, and the accumulated water was azeotropically distilled off while fresh 2-methoxyethanol was added to the reaction flask at about the same rate at which the distillate was collected. Approximately 10 ml of the distillate was removed over 103–110°. The reaction flask then was fitted with a reflux condenser, the reaction mixture was heated at 70° for 15 hr and cooled, and the solvent plus water was removed by azeotropic evaporation with toluene *in vacuo*.

This procedure resulted in 4.0 g of an oily product, which was partitioned between 15 ml of 5% sodium bicarbonate and 25 ml of chloroform. The organic layer was removed and dried (magnesium sulfate), and the solvent was removed *in vacuo* to give 2.9 g of a crude, oily product. It was purified by column chromatography, with chloroform as the eluent. Pure product, 1.6 g (50% yield), was obtained as a colorless oil; IR (CCl_4): 3380 (strong, broad) (NH) and 1720 (strong, broad) (2 C═O) cm^{-1}; NMR (acetone-d_6): δ 3.4 (s, 3H, OCH_3), 3.6 (m, 2H, $COOCH_2CH_2O$), and 4.3 (m, 2H, $COOCH_2CH_2O$). Elemental analysis showed a formula of $C_{13}H_{17}NO_5$.

N-*Carbobenzoxyglycine Carbethoxymethyl Ester (IX)*—Compound IX was prepared in the same manner as VII using ethyl bromoacetate. Without purification by column chromatography, pure product was

[1] Melting points were determined on a Thomas-Hoover melting-point apparatus and are uncorrected. IR spectra were determined with a Perkin-Elmer 257 grating spectrophotometer. NMR spectra were measured with a Jeolco C60 HL spectrometer, and chemical shifts are reported in δ (parts per million) units relative to the internal standard tetramethylsilane, with s = singlet, d = doublet, t = triplet, and m = multiplet; *J* values are in Hertz. Data were consistent with assigned structures for all intermediates and products. Silica gel for TLC refers to Merck silica gel G. Compounds were visualized by charring with sulfuric acid (50%). Silica gel used for column chromatography was silicAR cc-7, 200–325 mesh. Elemental analyses were performed by Atlantic Microlabs, Atlanta, Ga.

[2] Engelhart Industries.

[3] Sigma Chemical Co.

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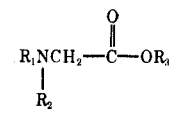
Table I—Physical Data for Glycine Esters



Compound	R_1	R_2	R_3	Yield, %	Melting Point	Crystallization Solvent	Formula	Analysis, %	Calc.	Found
II	Carbobenzoxy	H	$CH{=}CH_2$	50	Oil	—[a]	$C_{12}H_{13}NO_4$ (5, 6)		—	—
III	Carbobenzoxy	H	$CHBrCH_2Br$	15	Oil	—[a]	$C_{12}H_{13}Br_2NO_4$ (5, 6)		—	—
IV	Carbobenzoxy	H	CH_2CH_3	30	34–35°	Ethanol–water	$C_{12}H_{15}NO_4$		—	—
V	Carbobenzoxy	H	CH_2CN	70	69–70°	Chloroform–hexane	$C_{12}H_{12}N_2O_4$		—	—
VI	Carbobenzoxy	H	$CH_2{-}C(=O){-}CH_3$	45	71–72°	Chloroform	$C_{13}H_{15}NO_5$	C H N	58.86 5.70 5.28	58.88 5.71 5.27
VII	Carbobenzoxy	H	CH_2OCH_3	35	Oil	—[a]	$C_{12}H_{15}NO_5$	C H N	56.91 5.96 5.53	56.87 6.03 5.50
VIII	Carbobenzoxy	H	$CH_2CH_2OCH_3$	50	Oil	—[a]	$C_{13}H_{17}NO_5$	C H N	58.42 6.41 5.24	58.27 6.49 5.19
IX	Carbobenzoxy	H	$CH_2{-}C(=O){-}OCH_2CH_3$	80	42–43°	—[a]	$C_{14}H_{17}NO_6$	C H N	56.95 5.80 4.74	56.83 5.86 4.71
X	Carbobenzoxy	H	$CH(COCH_2CH_3)_2$	40	Oil	—[a]	$C_{17}H_{21}NO_8$	C H N	55.58 5.76 3.81	55.45 5.81 3.78
XI	Carbobenzoxy	H	$CH_2{-}C(=O){-}CH_3$	55	Oil	—[a]	$C_{13}H_{15}NO_6$	C H N	55.51 5.37 34.14	55.30 5.41 4.98
XIII	Tosyl	H	$CH{=}CH_2$	10	83.5–84.5°	Chloroform–hexane	$C_{11}H_{13}NO_4S$	C H N	51.75 5.13 5.49	51.66 5.16 5.45
XIV	Trifluoroacetyl	H	$CH{=}CH_2$	20	40.5–41.5°	Chloroform–hexane	$C_6H_6F_3NO_3$ (8)		—	—
XV	Phthaloyl		$CH{=}CH_2$	25	107–108°	Chloroform–hexane	$C_{12}H_9NO_4$ (9)		—	—

[a] Purified by column chromatography.

obtained in an 80% yield as a colorless oil. Upon drying in a desiccator for 2 weeks, the oil solidified and was used without recrystallization, mp 42–43°; IR (mineral oil): 3320 (strong) (NH) and 1730 (strong, broad) (3 C=O) cm^{-1}; NMR (acetone-d_6): δ 1.27 (t, 3H, J = 7.5, OCH_2CH_3), 4.24 (q, 2H, J = 7.5, OCH_2CH_3), and 4.77 (s, 2H, OCH_2CO). Elemental analysis showed a formula of $C_{14}H_{17}NO_6$.

N-Carbobenzoxyglycine Dicarbethoxymethyl Ester (X)—Compound X was prepared in the same manner as VII using diethyl bromomalonate. The crude product was purified by column chromatography, with chloroform as the eluent, to give a yellow oil. Attempts to crystallize the product from chloroform and hexane or ethanol and water failed. Final purification was accomplished by dissolving the product in chloroform and filtering the solution hot from activated charcoal. Removal of the chloroform *in vacuo* gave a colorless oil in a 40% yield; IR (CCl_4): 3420 (weak, broad) (NH) and 1740 (strong, broad) (4 C=O) cm^{-1}; NMR (acetone-d_6): δ 1.27 (t, 6H, J = 7.5, 2 OCH_2CH_3), 4.33 (q, 4H, J = 7.5, 2 OCH_2CH_3), and 5.69 [s, 1H, $OCH(COOC_2H_5)_2$]. Elemental analysis showed a formula of $C_{17}H_{21}NO_8$.

N-Carbobenzoxyglycine Acetoxymethyl Ester (XI)—Compound XI was prepared in the same manner as VII using bromomethyl acetate. The crude product showed several spots on TLC; purification by column chromatography, with chloroform as the eluent, gave a product that contained one small impurity. Further purification by column chromatography, with chloroform–ethyl acetate (1:1) as the eluent, gave a 20% yield of pure product and a 35% yield of a product with the impurity. The pure product was a colorless oil: IR (CCl_4): 3400 (strong, broad) (NH) and 1740 (strong, broad) (3 C=O) cm^{-1}; NMR ($CDCl_3$): δ 2.08 (s, 3H, $COCH_3$) and 5.74 (s, 2H, OCH_2O). Elemental analysis showed a formula of $C_{14}H_{15}NO_6$.

N-Tosylglycine Vinyl Ester (XIII)—*N*-Tosylglycine (3.0 g, 13 mmoles) was subjected to the usual vinylation procedure (5) twice. The reaction mixture was worked up to give an oily product, which was very impure on TLC. Purification by column chromatography, with chloroform as the eluent, gave 0.6 g of a brown solid that still was impure on TLC. This solid was purified further by column chromatography, with chloroform–methanol (97.5:2.5) as the eluent, to give a light-brown solid, which was recrystallized from chloroform and hexane.

Pure product (0.3 g, 10% yield) was obtained as white flakes, mp 83.5–84.5°; IR (mineral oil): 3250 (moderate) (NH), 1750 (strong) (C=O), and 1645 (weak) (C=C) cm^{-1}; NMR ($CDCl_3$): δ 2.42 (s, 3H, CH_3), 3.88 (broad, d, 2H, J = 4.9, NCH_2CO), 4.7 [ddd, 2H, J (*trans*) = 13.7, J (*cis*) = 6.3, J (*gem*) = 2.0, $=CH_2$], 7.1 [dd, 1H, J (*trans*) = 13.7, J (*cis*) = 6.3, OCH=], 7.29 (d, 2H, J = 8.3, aromatic), and 7.77 (d, 2H, J = 8.3, aromatic). Elemental analysis (carbon, hydrogen, and nitrogen) gave a formula of $C_{11}H_{13}NO_4S$.

Antifertility Screens—The newly synthesized agents were tested initially for antifertility activity in CF_1 female mice (~28 g) in much the same manner as steroidal contraceptives. For 28 days, virgin female mice, which had been isolated for 4 weeks, were administered test compounds at 10 mg/kg/day ip (0.2 ml). Test compounds were homogenized in 1% carboxymethylcellulose to obtain a fine suspension. On Day 10, female mice were exposed to male mice (two females per male) for the remainder of the experiment. Male mice were rotated once per week to eliminate the possibility of infertility.

On Days 17–21 of gestation, the females were sacrificed, and the numbers of viable fetuses, intrauterine deaths, and implantation sites were tabulated (4, 13). 17-Ethinyl estradiol and diethylstilbestrol were used as standards. Viable fetuses were examined for teratogenic effects of these drugs by the methods outlined by Wilson (14).

For intravaginal testing, the compounds were homogenized in 40% lactose or 5% carboxymethylcellulose and administered intravaginally (0.03–0.05 ml) at 10 mg/kg/day for 28 days. A tuberculin syringe without the needle or a low-dose (0.5-ml) insulin syringe with a 1–100-μl eppendorf pipet tip modified to fit the syringe was employed. On Day 10 and on all subsequent days, females were exposed to males for 1 hr. From this point, the protocol described earlier was followed (4). Compounds XVI–XVIII were used as standard proteolytic inhibitors.

For male antifertility activity, the method of Coppola (15) was followed. Male mice (~30 g) were administered II, III, and XVIII at 10 mg/kg/day ip for 8 weeks. At the 4-, 6-, and 8-week intervals, females were exposed to the treated males for 2 weeks and then removed. The number of females pregnant 4 weeks after exposure to the males was determined for each 2-week interval.

Enzymatic Studies—The acrosin enzyme was isolated from the epididymides collected from 10 male CF_1 mice (~30 g). The epididymides were sliced in 0.25 *M* sucrose–0.001 *M* disodium ethylenediaminetetraacetate (pH 6.5). The sperm suspension was filtered through two layers of sterile cheesecloth to remove debris, then centrifuged at 600×*g* for 15

Table II—Antifertility Activity of *N*-Protected Glycine Activated Esters in CF_1 Female Mice at 10 mg/kg/day

	Compound	Intraperitoneally				Intravaginally			
		n	Viable Fetuses per Litter	Intrauterine Deaths per Litter	Percent Pregnant	*n*	Viable Fetuses per Litter	Intrauterine Deaths per Litter	Percent Pregnant
I	*N*-Carbobenzoxyglycine	7	8.5	0.00	57	8	6.5	0.00	25
II	*N*-Carbobenzoxyglycine vinyl ester	8	11.0	0.00	12	8	0.0	0.00	0
III	*N*-Carbobenzoxyglycine 1,2-dibromoethyl ester	8	10.3	0.25	38	8	0.0	0.00	0
IV	*N*-Carbobenzoxyglycine ethyl ester	8	11.4	0.00	88	8	8.0	0.00	25
V	*N*-Carbobenzoxyglycine cyanomethyl ester	7	7.6	0.00	72	8	11.0	0.00	13
VI	*N*-Carbobenzoxyglycine acetonyl ester	8	9.3	0.83	75	8	7.7	0.00	38
VII	*N*-Carbobenzoxyglycine methoxymethyl ester	8	9.3	0.33	75	8	7.8	0.50	50
VIII	*N*-Carbobenzoxyglycine methoxyethyl ester	8	9.0	0.25	57	8	8.3	0.75	50
IX	*N*-Carbobenzoxyglycine carbethoxymethyl ester	8	10.7	0.67	38	8	1.0	0.00	13
X	*N*-Carbobenzoxyglycine dicarbethoxymethyl ester	8	6.7	2.50	75	8	8.4	0.20	63
XI	*N*-Carbobenzoxyglycine acetoxymethyl ester	8	8.8	1.80	50	8	10.0	1.30	50
XII	*N*-Carbobenzoxyglycine *p*-nitrophenyl ester	8	12.8	0.20	63	8	10.0	0.00	25
XIII	*N*-Tosylglycine vinyl ester	8	8.6	1.40	63	7	6.0	1.00	71
XIV	*N*-Trifluoroacetylglycine vinyl ester	8	9.2	1.20	63	8	7.7	0.70	38
XV	*N*-Phthaloylglycine vinyl ester	8	10.2	2.00	75	8	8.4	0.80	63
XVI	*N*-α-Tosyl-L-lysine chloromethyl ketone	7	8.6	0.20	71	7	0.0	0.00	0
XVII	Tosyl-L-phenylalaninechloromethyl ketone	7	4.5	0.50	57	8	0.0	0.00	0
XVIII	*N*-Carbobenzoxy-L-phenylalanine chloromethyl ketone	8	11.6	0.14	88	8	9.0	0.00	38
XIX	17-Ethinyl estradiol	8	0.0	0.00	0	—	—	—	—
XX	Diethylstilbestrol	8	0.0	0.00	0	—	—	—	—
	1% Carboxymethylcellulose	24	12.0	0.48	100	—	—	—	—
	40% Lactose	—	—	—	—	60	11.0	0.70	100
	5% Carboxymethylcellulose	—	—	—	—	34	9.6	0.64	74

min using a swinging bucket-type rotor head, and washed twice. The spermatozoa were resuspended in 20 ml of 0.25 *M* sucrose and frozen at −20° (9, 10).

After freeze–thawing, the tubes were agitated vigorously by vortexing for 30 sec and centrifuging at 1000×*g* for 15 min at 0°. The spermatozoa were resuspended, vortexed, and centrifuged again. The denuded spermatozoa were taken up in 5 ml of sucrose, and the pH was adjusted to 2.5 with hydrochloric acid. Acrosin was recovered in the supernate after centrifugation at 36,000×*g* for 15 min at 0° (16, 17).

Acrosin proteolytic enzymatic activity was determined by the method of Schleuning and Fritz (18). The incubation medium contained 0.7 ml of 0.1 *M* phosphate buffer (pH 7.6) and 0.3 ml of extracted crude rat acrosin, which were preincubated for 5 min at 30°. One milliliter of 2% azocasein and 1.5 μmoles of test compound in 0.2 ml of 40% lactose were added, and the mixture was incubated for 10 min at 30°. Tests were run in triplicate. The reaction was stopped with 5% trichloroacetic acid, and the mixture was centrifuged at 3500×*g* for 10 min. Absorption of the trichloroacetic acid-soluble peptides, which resulted due to the hydrolysis of azocasein, was measured at 366 nm. Enzymatic activity was calculated as the increase in absorbance per minute per milligram of protein (18, 19).

The hydrolysis of *N*-α-benzoyl-L-arginine ethyl ester, a trypsin substrate, was followed as described previously (19). Two milliliters of tris(hydroxymethyl)aminomethane, 0.3 ml of substrate, 0.1 ml of crude mouse acrosin, and 1.5 μmoles of test sample in 0.2 ml of 40% lactose were mixed, and the increase in absorbance at 253 nm was determined for 30 min. The absorbance (*A*) of the control equaled 0.320/min (18, 19). Compounds XVI–XVIII were used as proteolytic inhibitor standards.

RESULTS AND DISCUSSION

Previous screening showed that the 1,2-dibromoethyl esters of *N*-carbobenzoxyglycine, leucine, phenylalanine, and proline afforded 100% inhibition of fertility when administered intravaginally (4). However, only *N*-carbobenzoxyglycine vinyl ester demonstrated 100% inhibition compared to leucine, phenylalanine, and proline vinyl esters. Since the vinyl esters were far less toxic (*i.e.*, $LD_{50} \geq 500$ mg/kg) than the 1,2-dibromoethyl esters, it was decided to examine other esters in the glycine series and other amino-protecting groups of the vinyl esters.

With the newly reported compounds (IV–XV), intravaginal administration compared to intraperitoneal administration at 10 mg/kg/day resulted in the best antifertility activity in mice (Table II). However, the vinyl ester analog (II) and the 1,2-dibromoethyl ester (III) of *N*-carbobenzoxyglycine still were the most active, giving 100% inhibition (Table II). The carbethoxymethyl ester (IX) and the cyanomethyl ester (V) resulted in 87% inhibition. The free acid (I), the ethyl ester (IV), and the *p*-nitrophenyl ester (XII) resulted in 75% inhibition. Administration of the methoxymethyl ester (VII) and the methoxyethyl ester (VIII) resulted in only 50% inhibition of fertility, as did the acetoxymethyl ester (XI). Examination of other amino-protecting groups of glycine vinyl esters again showed that the *N*-carbobenzoxy derivative (II) possessed the best inhibitory activity (*i.e.*, 100%) when administered intravaginally, with the *N*-trifluoroacetyl analog (XIV) having the next best inhibitory effect at 62%. The *N*-tosyl (XIII) and *N*-phthaloylglycine vinyl esters (XV) were essentially ineffective when administered intravaginally in mice.

Intraperitoneal administration of II, III, and XVII in males at 10 mg/kg/day for 8 weeks demonstrated no antifertility activity. It is believed that the enzymes of the acrosomal layer of the sperm become activated in the vaginal tract (17, 20) and are not normally active (*i.e.*, enzymes are complexed with inhibitors) in the male reproductive tract as well as in ejaculated sperm. This may account for the absence of an antifertility effect of these agents when administered to male mice, or there may be a lack of distribution of drugs to the semen and sperm after intraperitoneal administration.

Mouse acrosin enzymatic studies showed that inhibition of *N*-α-ben-

Table III—*In Vitro* Effects of *N*-Protected Glycine Activated Esters (1.5 μmoles) on Acrosin Proteolytic Activity

Compound	Inhibition, %	
	N-α-Benzoyl-L-arginine Ethyl Ester Hydrolysis	Azocasein Hydrolysis
I	58 ± 1.2	63 ± 5.3
II	91 ± 6.7	83 ± 3.1
III	84 ± 0.3	100 ± 4.2
IV	64 ± 6.5	55 ± 5.1
VI	62 ± 5.5	64 ± 11.7
VII	48 ± 10.3	56 ± 9.0
VIII	45 ± 11.7	37 ± 16.6
IX	76 ± 8.2	76 ± 11.2
X	42 ± 8.5	51 ± 15.4
XI	48 ± 14.0	47 ± 11.7
XII	64 ± 10.3	66 ± 18.8
XIII	33 ± 2.3	62 ± 3.9
XIV	59 ± 5.0	96 ± 5.4
XV	52 ± 7.1	67 ± 28.9
XVI	91 ± 1.2	100 ± 4.3
XVII	18 ± 0.6	100 ± 3.2
XVIII	35 ± 2.3	100 ± 3.8
1% Carboxymethyl cellulose	0 ± 4	0 ± 6

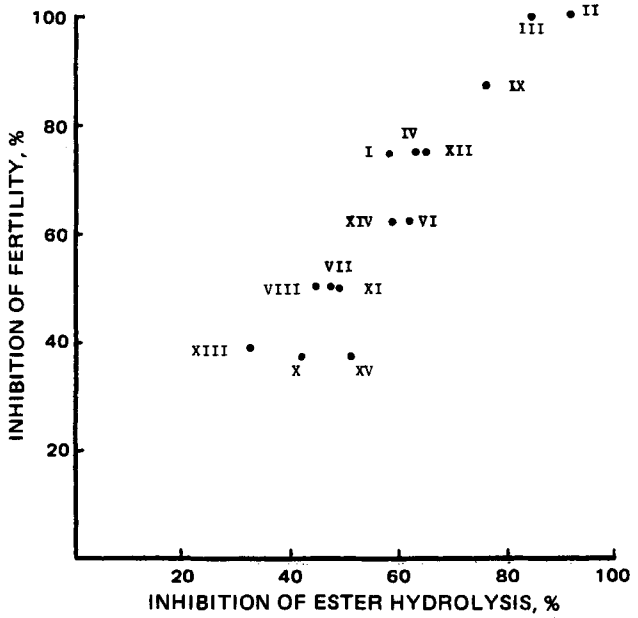


Figure 1—*Correlation between intravaginal antifertility activity at 10 mg/kg/day and percent inhibition of α-benzoyl-L-arginine ethyl ester hydrolysis at 1.5 μmoles.*

zoyl-L-arginine ethyl ester hydrolysis was related more directly to antifertility activity than to inhibition of azocasein hydrolytic activity (Table III). *N*-α-Benzoyl-L-arginine ethyl ester is a substrate that is readily hydrolyzed by trypsin due to its proteolytic or hydrolytic activity, whereas azocasein is a nonspecific substrate that can be hydrolyzed by several proteolytic- and esterase-type enzymes. Whereas azocasein hydrolysis was inhibited significantly by certain compounds (*i.e.*, XIV and XVIII), inhibition of azocasein hydrolysis did not appear to be positively correlated with antifertility activity. This does not preclude the possibility that these agents may inhibit other enzymatic activity of the sperm, which may contribute to the antifertility activity of these agents.

Inhibition of *N*-α-benzoyl-L-arginine ethyl ester hydrolysis by mouse acrosin preparations demonstrated a positive correlation with antifertility activity when the drug was administered intravaginally. Table II shows that those compounds that promoted >80% inhibition of *N*-α-benzoyl-L-arginine ethyl ester hydrolysis by mouse acrosin were potent fertility inhibitors, *e.g.*, I and II. Compound IX, the carbethoxymethyl ester, demonstrated 76% inhibition of *N*-α-benzoyl-L-arginine ethyl ester hydrolysis and 87% inhibition of fertility. Compounds that showed 50% inhibition of pregnancy demonstrated ~45–48% inhibition of *N*-α-benzoyl-L-arginine ethyl ester hydrolysis. Linear regression analysis showed a correlation coefficient of 0.95 between antifertility activity when administered intravaginally and reduction of *in vitro* mouse *N*-α-benzoyl-L-arginine ethyl ester hydrolysis at $p = 0.001$ (Fig. 1). When the intravaginal antifertility activity was compared to azocasein hydrolysis by mouse acrosin, a correlation coefficient of 0.68 was obtained at $p = 0.4$.

These compounds may inhibit *N*-α-benzoyl-L-arginine ethyl ester hydrolysis by conventional enzymatic inhibition mechanisms, by enzyme acylation, or by acting as a competitive substrate for the enzyme. Upon hydrolysis, II and III generate acetaldehyde or bromoacetaldehyde, which have the capability of nonreversible alkylation of the active site of the enzyme (21), thereby inhibiting its activity. Acrosin, an endopeptidase that cleaves the carboxyl bond of arginine and lysine derivatives, can be inhibited by known trypsin inhibitors. Proteolytic hydrolytic enzymatic activity is involved in sperm capacitation labilization of the external membrane of the sperm and penetration of the ovum's zona pellucida layer for fertilization. Acrosin is thought to play a vital role in the latter physiological process (4).

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